27th INTERNATIONAL SYMPOSIUM ON ESSENTIAL OILS

(27. Internationales Symposium über Ätherische Öle)

September 8-11, 1996 - Wien/Vienna, Austria

BOOK OF ABSTRACTS

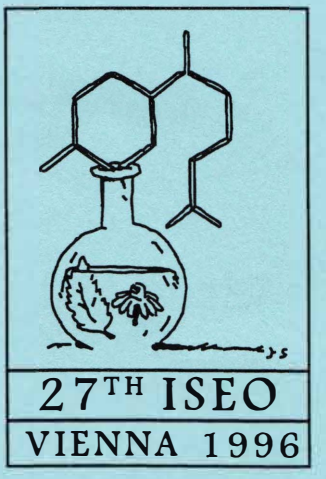

TABLE OF CONTENTS:

LECTURES / ORAL PRESENTATIONS

O 1 - 1

A PSYCHOLOGIST EXAMINES THE USE OF ESSENTIAL OILS IN AROMA-THERAPY TECHNIQUES

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"The main use of aromatherapy within conventional health care is as a means of relieving stress and anxiety. Despite the publication of three clinical trials in the first six months of 1995, there remains a dearth of clinical research on this intervention. Most are of low methodological quality and the majority fail to find aromatherapy to be statistically superior to massage with plain carrier oil" (Vickers, 1996. p 127). As this quotation so succinctly highlights, there are many loose and unvalidated statements made about the success of aromatherapy techniques and most findings reported from within the aromatherapy community can be explained by placebo effects. The lecture will centre on the limited number of controlled studies that have been carried out and the conclusions that can be drawn from them.

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O 1 - 2

ESSENTIAL OILS AND HUMAN VIGILANCE

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In studying the effects of possibly psychoactive substances like essential oils on human attention (on which most other cognitive functions are based), several forms of attention have to be discriminated. The most basic level of attention is alertness, which may be described as the ability of the organism to react to simple stimuli in terms of speed. One very important aspect of attention is its extension in time, i.e. the sustaining of attention. The process of sustaining attention over periods of time (minutes to hours), especially if critical stimuli (to which there must be reacted) occur infrequently and unpredictably, is called vigilance. In the present study, we examined the influence of an essential oil on measures of vigilance.

Vigilance was measured by means of a computer-based test. In one session four trials took place. In the first trial (10 minutes), subjects just had to perform the task. In the second trial (10 minutes), subjects were wearing a surgical mask with no substance applied during performing the task. At the beginning of the third trial (15 minutes), a substance was applied to the mask; the substance was water in the control group and 1,8-cineol (20 microliters) in the experimental group. This procedure was repeated at the beginning of the forth trial (15 minutes). At the beginning of trials 2 and 3 and at the end of trials 2 and 4 subjects had to rate the odours on the dimensions of hedonics, intensity, effect and degree of relaxation on analogue scales.

Between-group comparisons (water vs essential oils) showed no significant general effects due to the inhalation if cineol. Intra-group analysis, however, revealed complex interactions between subjectively experienced effects of inhaling cineol and objective measures of vigilance. It is concluded that relatively low doses of cineol do not have a generally stimulating effect with respect to human vigilance but that influences on vigilance mediated by subjective experience can be shown.

O 1 - 3

ANALYSIS OF THE CHIRAL FRAGRANCE COMPOUNDS (+)/(-)-CARVONE IN BIOLOGICAL FLUIDS AND TISSUES

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The chiral fragrance compounds (+)- and (-)-carvone were studied, which are commercially available in high enantiomeric purity. With concentrations ranging from 55 to 80 % (-)-carvone is the main constituent of the essential oil of *Mentha spicata var. crispa* (Lamiaceae), being responsible for the typical spearmint odour of the oil. In contrast (+)-carvone, found in the essential oil of *Carum carvi* (Apiaceae) in concentrations between 50 and 85 %, exerts an herbal odour of caraway seeds. Referring to the literature (-)-carvone acts as a mild sedative agent, whereas (+)-carvone shows stimulating effects. Since (+)- and (-)-carvone are used as flavoring compounds in a number of foodstuffs as well as in mouth care products and cosmetics, the aim of this study was to investigate the pharmacokinetics of (+)- and (-)-carvone after peroral administration using an animal model.

Enantiomeric pure (+)- and (-)-carvone are well absorbed and reach the maximum concentration in blood almost equally fast (17.1 min and 16.8 min, respectively). In contrast to (+)-carvone the area under the blood concentration time curve for (-)-carvone only showed a value of 29.8 %, indicating a higher rate of metabolism for (-)-carvone. To investigate this hypothesis, carveol, the main metabolite, was analysed in liver samples from animals pretreated with (+)- and (-)-carvone, respectively. After administration of (-)-carvone the content of carveol in the liver samples was indeed about 9 times higher than that for (+)-carvone.

Based on these results we further investigated the pharmacokinetics of (+)- and (-)-carvone in human subjects during a massage treatment. Again, (+)-carvone exhibited significantly higher blood levels than (-)-carvone, although the dose of both compounds was the same.

The difference in the pharmacokinetics of (+)- and (-)-carvone should therefore always be considered when using one of these fragrance compounds in cosmetical or medical treatment.

O 1 - 4

CORRELATION OF THE CHEMICAL PROFILES OF ESSENTIAL OIL MIXES WITH THEIR RELAXANT OR STIMULANT PROPERTIES IN MAN AND SMOOTH MUSCLE PREPARATIONS IN VITRO.

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Essential oils have been categorised by aromatherapists as been either relaxant (sedative) or stimulant. It is not clear whether this refers to the action on the brain or to some or all of the muscles, as many essential oils are also classified as anti-spasmodic. Aromatherapists use mixtures of three or more essential oils to massage their clients and reports of beneficial effects on stress-related symptoms abound. Stress is mediated through the sympathetic branch of the autonomic nervous system and also mediated by hormones and is not under conscious control. The small intestine has a dual innervation : sympathetic and parasympathetic and it also has an additional plexus of nerves in the walls (the enteric nervous system) which involves several neurotransmitters. Essential oil mixtures were tested on guinea-pig ileum in vitro. All the mixtures were analysed by gas chromatography and their components were segregated according to their Retention Times: these were then correlated with their action on the smooth muscle. The range of mixtures was also assessed by aromatherapists as to their predicted sedative or stimulant effect on their clients. It was found that there was a remarkably high correlation between the three results. The results suggest that the small intestine, even in vitro, has an ability to mimic the human psyche. The results also indicate that the stimulant (or spasmogenic) effect of essential oil mixtures was directly correlated with the total monoterpene concentration, with very few exceptions.

O 1 - 5

The Chemistry and Pharmacology of *Origanum (Kekik)* Water

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Origanum onites L. (*kekik*) is a widely used herb as a substitute of thyme since antiquity. Today it has also gained considerable commercial importance besides its widespread ethnomedical use. Recently, the use of its aqueous distillate which accumulates under the essential oil (***Kekik water***) has been popular for various therapeutic purposes in Turkey.

In order to evaluate the pharmacological effects of kekik water, *in vivo* and *in vitro* experiments were carried on experimental animals namely; acute and chronic toxicity, antibacterial and antifungal tests, effects on general behaviour and hippocratic screening, analgesia test, barbiturate sleeping time and bile flow tests, *in vivo* blood pressure, bronchospasm, general performance (swimming), anti-obesity tests; microscopic investigations of liver, pancreas and spleen, biochemical tests for blood glucose, triglyceride and cholesterol, and isolated organ bath experiments of ileum, aorta and vas deferens. *Kekik water* was observed to be active on gastrointestinal and cardiovascular systems in terms of having inhibitory and stimulatory effects, respectively. The gastrointestinal inhibitory action was found to be in the n-hexane fraction, while the cardiovascular stimulant actions were found to reside in the chloroform fraction of *kekik water*. The fractions were identified by bioassay guided fractionation and the chemical constituents were characterized by GC and GC/MS.

O 1 - 6

ANTIVIRUS ACTIVITY OF THE ESSENTIAL OILS OF SOME *HERACLEUM L.* SPECIES

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At present we know that essential oils from a lot of higher plants have a bacteriostatic and bacteriocidic effect on Gram-positive and Gram-negative bacteria, and also fungistatic effect on some funghi. These species of plants are usually used in scientific and folk medicine as sanitary substances, as a prophylactic (preventive) for acute inflammation of respiration (breathing). The essential oils of hogweeds *Heracleum L.* species have antibacterial and fungitoxic effects.

We have investigated antivirus effects of essential oils from fruits and roots of some *Heracleum* species (*Apiaceae* = *Umbelliferae*), which were cultivated at the scientific station of the Komarov Botanical Insitute (St. Petersburg District).

The essential oils were obtained by water distillation in all-glass Ginzberg's apparatus as described in the USSR Pharmacopoeia from air dried fruits and fresh roots. The results of chemical investigation of these essential oils were published earlier.

In all experiments 0,2 ml solution (1 part of essential oil and 4 parts of sunflower oil) per mouse (albino mice, 10-12 g) were used (intranasal and per oral), and also as spray - only pure essential oil - 0,01 - 0,05 ml per 10 l^3. Two types of viruses, type A (A/Bethesda) and B (B/Lee) were used in the experiments.

As final results: all of the essential oils from *Heracleum* species have antivirus activity. The essential oils from roots are more active. But in general antivirus activity of hogweed oils is less than such drugs as: Remantadin and Adapromin. Hence , oils of *Heracleum* are more interesting as source for new drugs and ways for using essential oils.

O 2 - 1

BIOCHEMICAL AND MOLECULAR GENETIC ASPECTS OF MONOTERPENE FORMATION

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Most monoterpenes are derived by allylic oxygenation of a parent olefin that is formed by enzymatic cyclization of the ten-carbon isoprenoid precursor geranyl diphosphate. The commercial mint (*Mentha*) species produce either C3-oxygenated or C6-oxygenated *p*-menthane monoterpenes (e.g., menthol or carvone) as the major essential oil components. The biosynthetic origin of both of these monoterpene families from the common olefin precursor 4*S*-(-)-limonene is reviewed as a general model for this type of metabolic pathway. The properties of the limonene synthase, and of the two regiospecific cytochrome P450 limonene hydroxylases catalyzing oxygen insertion at C3 or C6 of the cyclic skeleton, are reported. The isolation of the cDNA species encoding these enzymes, the first of monoterpene metabolism to be cloned, is described and the results of sequence comparison with other terpenoid cyclases and hydroxylases are noted. The organization and regulation of monoterpene metabolism in *Mentha* are delineated and the roles of the cyclase and hydroxylases in the control of essential oil yield and composition are defined. Prospects for genetic engineering of monoterpene production in plants and microorganisms are discussed.

hydrox - RE
dhydrog - Cy
Synt/Cy - Post

O 2 - 2

LIMONENE SYNTHASE FROM *Perilla frutescens*

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Perilla frutescens Britton (Labiatae), which is used as a Chinese drug in Asian countries, shows chemical variation with regard to the essential oil components in the leaves. Genetic analyses of various chemotypes demonstrated that several genes control the biosynthesis of monoterpenoids including the major component perillaldehyde, which is accumulated in the presence of two dominant genes *G* and *H*. It has also been shown that limonene synthase activity, which plays a primary role in synthesizing *l*-limonene, the precursor of perillaldehyde, was detectable only in the seedlings of the *GH* genotype. To study the regulatory mechanism of the expression of the limonene synthase in relation to the function of genes *G* and *H*, we have cloned a cDNA encoding limonene synthase from the seedlings of *P. frutescens*.

A cDNA library constructed from *Perilla* seedlings (strain No. 9, *GGHH*) was screened with the limonene synthase cDNA (LC5.2) from *Mentha spicata* as a hybridization probe, to isolate 10 independent positive clones from *ca.* 30,000 plaques. A representative clone PFLC1 was 2031 bp in length, containing an open reading frame of 603 amino acids. The deduced amino acid sequence of PFLC1 exhibited appreciable sequence identity with those of spearmint limonene synthase(65%), tobacco 5-*epi*-aristolochene synthase(35%), and castor bean casbene synthase(30%). Two cDNA clones of the *Perilla* limonene synthase were functionally expressed in *Escherichia coli*, yielding enzymes catalytically active in generating 4(*S*)-limonene from geranyldiphosphate *in vitro*. Genomic Southern blot analyses of various genotypes (*GGHH*, *GGhh*, *ggHH*, and *gghh*) of *P. frutescens* suggested that at least two copies of the PFLC1 DNA exists in strains having the *HH* genotype. In contrast, no PFLC1 DNA sequences were detectable in the genomes of strains with the *hh* genotype that are incapable of producing cyclohexanoid monoterpenes for lack of limonene synthase activity. The present study has clearly demonstrated that *Perilla* strains of the genotype *hh* completely lack PFLC1, suggesting that the dominant gene *H* must be either PFLC1, the limonene synthase structural gene itself, or a gene locus containing PFLC1 as a part of its structure. Northern blot analyses, using a PFLC1 3'-flanking region as a hybridization probe, showed that PFLC1 mRNA accumulated in all the aerial parts of the *GGHH* plants, particularly in the leaves. In the *ggHH* plants, on the other hand, only low levels of the PFLC1 mRNA could be detected in the stem and calyx, consistent with the minute amount of perillaldehyde detected in those tissues.

GERMACRENE BIOSYNTHESES IN CARAWAY (*CARUM CARVI*) AND CHICORY (*CICHORIUM INTYBUS L.*)

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Chicory is known for its bitter taste which is associated with the presence of sesquiterpene lactones in the roots and leaves. Cyclisation of farnesyl pyrophosphate (FPP) towards a germacrene is thought to be the first step in their biosynthesis[1] and recently we have demonstrated that chicory roots contain a germacrene A synthesising sesquiterpene cyclase. However, we were not able to detect the notoriously unstable germacrene A itself, but only its degradation products[2] α-selinene, β-selinene and β-elemene.
In order to detect the formation of a true (stable) germacrene we have become interested in caraway (*Carum carvi L.*). In contrast to the oil produced from its seeds, which contains mainly monoterpenes, the essential herb oil of caraway is rich in sesquiterpenes. The main component of caraway herb oil is germacrene D (76%). Other important components of this oil are *trans*-caryophyllene (6.2%), β-elemene (4.6%) and δ-cadinene (3.8 %). The herb oil of caraway seedlings, especially that of the roots, is known to be rich in germacrene B (64%)[3]. Incubation with [^{3}H]FPP of a 100.000 *g* supernatant prepared from etiolated caraway seedlings showed on radio-GC the production of germacrene B. Production of farnesol was also observed, obviously due to the abundant phosphatase activity in crude plant extracts. However, no degradation products of germacrene B or any other labelled products were detected. The same experiments undertaken with leaves of full-grown caraway showed the enzymatic synthesis of germacrene D, some other sesquiterpenes and farnesol.
The germacrene producing sesquiterpene cyclases represent an important step in the biosynthesis of many sesquiterpenes, but have not been described in detail yet. In order to characterise these germacrene synthases we are now trying to purify them. In a first purification step using anion exchange chromatography we managed to omit almost all interfering phosphatase activity.

[1]Piet D.P. *et al.*, Tetrahedron, vol. 5, p. 6303-6324 (1995).
[2]Teissere P.J., Chemistry of fragrant substances, New York (1994).
[3]Wichtman E.V., Ph.D.-thesis, University of Hamburg (1988).

VARIATION AND INHERITANCE OF MONOTERPENES IN *LARIX* SPECIES

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Branches of three species of larch and six hybrids were collected at Ruotsinkylä Field Station (Forest Research Institute), 20 km north of Helsinki, in June 1994 and August 1995. The leaves were crushed and hydrodistilled. The essential oils were analysed by GC-MS on a heptakis-β-cyclodextrin column.

The identification was based on GC retention times of authentic samples, GC-MS spectra and retention data obtained on β-cyclodextrin columns.

The studied species/hybrids and the components characteristic for each of them were as follows:

Larix sibirica (Münch.)Ledeb. was a high 3-carene type. 3-Carene is usually followed by γ-terpinene and terpinolene. Of the α-pinenes (-)-α-pinene was dominating.

L. leptolepis (Sieb. et Zucc.) was a low 3-carene type with a high content of myrcene and (+)-α-pinene higher than (-)-α-pinene.

L. decidua Mill. showed almost equal amounts of (-)- and (+)-α-pinene and a quite high content of 3-carene.

L. sibirica x *leptolepis* and *L. leptolepis* x *sibirica* controlled crossings gave a progeny of which about 50 % were high 3-carene types and 50 % low 3-carene types.

L. decidua x *leptolepis* and *L. leptolepis* x *decidua* open pollinated crossings produced a progeny which inherited either a low 3-carene from *L. leptolepis* or a high 3-carene from *L. decidua*.

L. sibirica x *decidua* showed a high 3-carene content (typical for *L. sibirica*), and as an influence from *L. decidua* a higher (+)-α-pinene content than in *L. sibirica*.

L. laricina x *decidua* crossings were high 3-carene types. (+)-α-Pinene dominated over (-)-α-pinene and the content of (-)-β-pinene was higher than in any other of the studied species or crossings.

O 2 - 5

MULTIVARIATE STATISTICAL ANALYSIS AS A TOOL FOR THE DEFINITION OF CHEMOTYPES IN ESSENTIAL OIL PLANTS - POTENTIALS AND LIMITATIONS

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Chemical polymorphism is widespread in the plant kingdom, and many species of the Lamiaceae are well-known for this phenomenon[1], especially the genus Thymus[2]. It can only be discovered by analysing the essential oil compositions of a large number of individual plants which results in an immense amount of quantitative data. The computer-based multivariate statistical analysis (MSA), namely the cluster analysis in combination with factor analysis and discriminant analysis, is suited for comparing all these data simultaneously. Through this it is possible to discover a hidden grouping of oil types which is not necessarily evident only on checking through the tables and gas chromatograms. As a pre-requisit for a correct result, a guideline, three times more cases (= individual plants) than variables (= individual essential oil constituents) should be analysed. However, the precision of MSA depends on how exact the chromatographical and integrated data are, which may be affected with inaccuracies caused by the distillation parameters (e.g. formation of artifacts), by a varying detector response, by overlapping peaks, mode of peak integration, injection mistakes etc.. Therefore the significance of the MSA must not be overestimated. All of the problems connected with MSA will be discussed using as an example the classification of the *Thymus* plants of the Alpine region of Austria (*Thymus praecox* ssp. *polytrichus*) on the bases of a data set calculated from 141 individual plants and 60 constituents[3]. This population turned out to be extremely polymorphous, bordering on the limits of what it is possible to process.

[1] Lawrence B.M. (1980) The existence of infraspecific differences in specific genera in the *Labiatae* family. In: Annales techniques, VIIIe Congrès International des Huiles Essentielles, Cannes-Grasse, 118-131.

[2] Stahl-Biskup, E. (1991) The chemical composition of *Thymus* Oils: A review of the Literature 1960-1991. J. Ess. Oil Res. 3, 61-82

[3] Holthuijzen, J. and Stahl-Biskup, E. (1994) Multivariate analysis of the essential oil data for the classification of *Thymus* plants of the Austrian Alpine region. Poster on the 25th International Symposium on Essential Oils, Grasse.

O 2 - 5

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O 2 - 6

SELECTION FOR SEED YIELD AND EARLINESS IN FENNEL (*Foeniculum vulgare* Mill.) AND CORRELATED RESPONSE IN SEED-OIL YIELD.

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Fennel is an important medicinal plant which also is edible and has uses in food processing. Current cultivated variety in Egypt is highly heterogeneous in plant growth characteristics, and seed yield and quality. Limited information, is available on the major genetic parameter needed for breeding in this crop plant. The present study was undertaken in 1994, 1995 and 1996, to estimate associations and heritability of seed yield and some of its components. Selection was also conducted based on the information provided by these genetic parameters. The results indicated significant positive correlations between total seed yield and both of plant height and number of branches developed on the main stem per plant. High yielding plants tended to develop greater numbers of both umbels containing full seeds and umbels with shrink seeds. High yielding individuals also tended to produce high amounts of both fully developed seeds and shrink seeds. Narrow-sense heritability (h^2) for total seed yield was 0.16 in 1995 and 0.11 in 1996. The estimates of h^2 in 1995 and 1996, respectively, were 0.33 and 0.38 for plant height, 0.42 and 0.46 for number of branches, 0.31 and 0.28 for number of umbels per plant. Individual plants were screened in 1995 growing season simultaneously and independently based on greater number of umbels containing fully developed seeds but with reduced number of umbels containing shrink seeds and early in seed maturity. The population composed of bulk progenies form the selected plants, relative to the original population, exhibited 15.2% increase in yield of fully developed seeds and 17.2% reduction in weight of shrink seeds. The ratios for the weight of full developed seeds : weight of shrink seeds were 10:1 and 7.2:1 in the improved and original populations, respectively. The improved population contained 26% of their plants, in contrast to 7.5% in the original population, developed mature seeds in the 1st, 2nd and 3rd order umbels 3 weeks earlier than the remaining plants. Changes in seed-oil yield was detected.

PHYSIOLOGICAL ASPECTS OF ESSENTIAL OIL PRODUCTION

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Apart from the evolution and from genetic factors that influence the compostion of essential oils from plants, several physiological factors are known to influence the yield and the composition of such oils, Table 1.

Table 1: Factors that influence the yield and composition of essential oils.

Pollinator and pollination	Climate
Type of plant material	Soil properties
Type of secretory structure	Hydric stress and method of irrigation
Development of organs	Mechanic or chemical injuries
Time of harvest	Addition of herbicides and/or fertilizers
Storage	Catabolism
Method and time of propagation	*In vitro* production

Some of these factors have been studied to some extent, in particular for commercially important crops, in order to determine the optimum conditions of cultivation and time of harvest, for attaining higher yields and a better quality of the oils.

Since essential oils can be isolated from many plant taxa, turning them into ideal characters for systematic studies, they have been used to support the identification of species, to document natural hybrids, to define genetic differences, to work out centers of origin and spread of populations, and to study variations within taxa. Chemotaxonomy is, therefore, another field where the influence of the growth conditions of plants on the composition of their essential oils is of great importance.

O 3 - 2

VARIABILITY OF ESSENTIAL OIL ACCUMULATION IN FENNEL AFFECTED BY ECOLOGICAL CONDITIONS AND DEVELOPMENT

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Foeniculum vulgare is one of the most important species of Apiaceae family. Both the seed and the essential oil (Aetheroleum foeniculi) are utilised in phytoterapy as well as in food industry. All over the word people are looking for populations of high productivity accumulating appropriate quality of the essential oils at the same time. For selecting the most suitable populations the regularity of essential oil accumulation has to be identified. In the present work the influence of the ecological conditions and the development stages on essential oil accumulation are discussed.

The plant material drown into the investigation consisted of 13 populations of Foeniculum vulgare Mill. var. vulgare, which were classified by our earlier investigations (Bernáth et al. 1994, 1996) into three different chemovarieties and four chemoforms of the lower rank. The populations were investigated through three vegetation cycles in the research field of UHFI University (Soroksar) and in the Botanical Garden of JATE University (South part of Hungary) parallely. The changes of the phenological and morphological characters (including structural modification of the seed tissue), the accumulation of the essential oils and their interaction were measured.

It was proved that the highest values of the essential oil occur in seeds being in "green seed" development stage. It seems to be an universal phenomenon and hardly depend either on the type of the population or position of the branches, where the seeds come from. The green seed of cultivar `Soroksári` (SO) contains 16.47 % essential oil, and there are three more populations which accumulate about 10 % at this stage. The high essential oil content in the green seeds can be explained by the structural changes of seed tissue going in the course of the seed development.

The accumulation level of the essential oil in the seed is effected by both the number of the vegetation cycle, and ecological conditions. However the compositional character of the oils seems to be rather stable.

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O 3 - 3

ESSENTIAL OIL STORAGE AND SECRETION IN GLANDS OF SOME LAMIACEAE (AN ULTRASTRUCTURAL STUDY).

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Essential oils in Lamiaceae plants are produced in glandular trichomes which occures mainly on the leaf surface. Glandular trichomes in *Agastache rugosa* (Fisch. et Mey) O.Kuntze, *Dracocephalum moldavica* L., *Nepeta cataria* L., *Nepeta cataria* L. var. *citriodora* Balb., *Nepeta cyanea* Stev. and *Scutellaria baicalensis* Georgi were studied by Transmission and Scanning Electron microscopes. Secretory cells of oil-producing trichomes of all investigated samples have abundant endoplasmic reticulum (ER), and well-developed plastidome which is presented by leucoplasts with invaginations (in *Nepeta cataria*, *Nepeta cataria* var. *citriodora*, *Scutellaria baicalensis*) or without ones (in *Agastache rugosa*, *Dracocephalum moldavica*, *Nepeta cyanea*). The essential oils (terpenes) in the glands examined are visualised as the electron dense droplets. They usually occur in storage vacuoles (which are supposed to be derivatives of ER), periplasmic and subcuticular space. The electron dense droplets in *Agastache rugosa* and *Dracocephalum moldavica* are also found in the intermembrane space of mitochondria and plastids. Terpenes are excreted into periplasmic space by exocytosis and passes through the cell wall into subcuticular space. The amount of terpenes in storage vacuoles is nearly the same as it is in subcuticular space. The cuticule which covers glandular trichomes has the thickness comparable to that of the cell wall. Some amount of essential oils may probably pass through the cuticule via dendrites which occures in it.

O 3 - 4

INULIN AS CARBON SOURCE IN THE CULTURE OF EDELWEISS HAIRY ROOTS

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The roots of many perennial members of the Compositae (eg. *Inula*) contain the fructan inulin as reserve carbohydrate. Although *Leontopodium alpinum* was recently reclassified into the tribe/subtribe Gnaphalieae/Gnaphaliinae [Anderberg 1994] its original classification with *Inula* into the Inuloideae suggests the involvement of the fructan in the metabolism of Edelweiss.
We have previously reported on the presence of an essential oil in normal Edelweiss roots [Comey et al 1992] and "hairy" roots, developed by genetic transformation with *Agrobacterium rhizogenes* (Strain 9402) [Hook 1994]. Although sucrose is the most commonly used carbon source in the culture of hairy roots, it was of interest to determine how its partial or complete replacement by inulin would affect hairy root growth, biomass production, essential oil yields and composition.
Roots were grown for six weeks as batch cultures in a phytohormone-free medium. A series of media basal salts (Murashige & Skoog, Gresshoff & Doy, Litvay's, White's) were formulated with sucrose (3%), inulin (3%) or sucrose (1.5%) + inulin (1.5%). Cultures were grown in both the presence and absence of light.
Results obtained indicated that basal salt formulations and illumination during the culture period had highly significant effects on biomass growth, essential oil content and composition. In contrast, replacement of sucrose by inulin in the various media formulations led to only small reductions in biomass production and insignificant changes in % essential oil yields. Inulin can therefore be considered as an alternative, though not superior, carbon source to sucrose.

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O 4 - 1

Fragrance Analysis Using Semiautomatic Spectra (MS, IR) Interpretation and Olfactoric Data

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In the first part of this presentation the analysis of various perfume creations is discussed. In such perfume samples a complex system of components exhibiting distinguished olfactory properties is involved, however the resulting scent impression can be a priori hardly predicted. The components used may constitute either low price synthetic products (e.g. monoterpenic compounds like limonene or linalool) or in turn, again consist of high value complex natural systems (e.g. essential oils). In this respect the aim of presented study was to find out, if there is a possible prediction or correlation of resulting olfactoric perfume characterization with attributes of particular components used.

As starting material a primary basic set consisting of 22 commercially available odor composition (as basic materials for fragrance creations) was selected and analyzed with respect to components involved by means of GC-coupled methods (GC/FID and GC/FTIR/MS) and with respect to the most important olfactorially active compounds using the "GC-sniffing technique". The spectra interpretation was performed by substructure based MS-spectra library (NIST/EPA/NIH), PBM Bench Top spectra library, own spectra collections and semiautomatic preselection of spectral data recorded. In this way, a list of approx. 50 odor intense components together with more than fifty further compounds with restricted olfactory activity was compiled. All 22 systems investigated were evaluated by totally six perfume trained persons in order to determine the sample specific odor attributes. Already at this stage of the study a surprising result was a high percentage of "so-called" high price samples investigated consisting of low price (especially synthetic) odor components and solvatizing agents mainly.

In the same way and by additional use of the principal component analysis (PCA) as well as the multivariate data anaylsis (MVDA) further more than 100 perfume creations (partly also components of basic set stored at different conditions) were investigated. Also these results confirmed the tendency of the use of low prized constituents accompanied by moderate alteration only.

In the second part of this presentation the effectivity of substructure orientated spectra libraries in the aroma research is discussed. The investigation of more than 25 complex fragrance systems (e.g. essential oils and headspace samples) with GC-FID, GC-FTIR-MS and GC-sniffing technique resulted in the identification of totally more than 350 odor active compounds. By means of substructure orientated spectra libraries and multivariate methods (PCA and MVDA) for data analysis it was possible to get a significant correlation odor attribute (like floral, green, fresh, etc.) with the variation of single substance groups (like ketones, aldehydes or alcohols). This allows the principally prediction of odor attributes in complex systems only by the use of multivariate analyses of concentration variety of volatiles.

NEW APPROACHES IN ESSENTIAL OIL ANALYSIS USING POLYMER-COATED SILICA FIBERS

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Polymer-coated silica fibers are mainly used for solventless microextraction of analytes from aqueous solutions of pollutants. Sample preparation is based on the absorption of analytes from an aqueous sample onto a coated fused silica fiber, which is mounted in a GC syringe. After absorption the analytes are recovered from the fiber by thermal desorption in a conventional GC injection port.

Since the development of solid-phase microextraction (SPME) in 1989 (1) and the marketing of a commercially available device (2) the field of its application has grown enormously.

We have investigated the applicability of this new and efficient technique in the field of essential oil analysis and will demonstrate some new and very efficient applications:

1) The rapid and simple gc-investigation of volatile components in highly diluted aqueous solutions such as tea infusions and distillation waters.
2) Head space analysis of crude drugs from aromatic plants.
3) GC analysis of very small samples of aromatic plants after heating.
4) Solventless sampling of essential oil from individual oil glands.
5) Transfer of a capillary gc fraction onto a second gc-capillary column.

(1) C.L. Arthur and J. Pawliszyn: Anal. Chem. 62, 2145 (1990).

(2) Supelco, Inc. (Bellefonte, USA).

O 4 - 3

SUPERCRITICAL FLUID EXTRACTION (SFE) AS A FRACTIONATION TECHNIQUE FOR VEGEBLE MATRICES

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Supercritical fluid extraction (SFE) is now a well-established extraction technique; in particular it has been successfully applied to the extraction of the volatile fraction of plants and/or of parts of them. The solvating power of a supercritical fluid can be varied physically, through its density, and/or chemically, by adding a modifier miscible with it.

Preliminary results will be presented concerning the possibility of extracting specific fractions from vegetable matrices selectively, using supercritical fluids. Some examples concerning extractions of volatiles and antioxidants from sage and rosemary, and the characterizing components from saffron will be reported, together with examples of essential oil fractionation.

O 4 - 4

CARBON-13 NMR AS A TOOL FOR ENANTIOMERIC DIFFERENTIATION OF TERPENES IN ESSENTIAL OILS

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The enantiomeric excess determination of terpenes present in essential oils is carried out usually following two possible ways : i) pre-fractionation of the oil by a chromatographic technique leading to pure compounds, followed by the separation of both enantiomers using GC on chiral columns ; ii) Multi Dimensional Gas Chromatography which allows direct injection of the sample into the chromatograph ; a first column, achiral, brings to a pre-separation of the compounds which are transferred into the second column, chiral, using the heart-cutting technique.

In the laboratory, we developed an original method which allows the identification of the individual components of essential oils, using Carbon-13 NMR Spectroscopy without previous separation (up to 25 compounds, minimum ratio 0.5%). This technique is well suited for stereoisomers identification as well as chemical polymorphism studies.

The aim of this work was to evaluate the potentiality of Carbon-13 NMR to differentiate enantiomers of natural terpenes, in order to be able, using the same technique, in a first step, to study the chemical composition of the essential oil and, in a second step, to determine directly the enantiomeric excess of the main constituent(s).

NMR chiral determination can be carried out by several techniques. We used chiral lanthanide shift reagents (LSR). The Lewis acidic-site of the LSR coordinates with the Lewis-basic site of the substrate (for instance, an oxygenated function). Ytterbium being the most suitable metal for Carbon-13 NMR Spectroscopy, we choose $Yb(hfc)_3$ as the LSR (tris-(3-heptafluoropropyl-hydroxymethylene-(+)camphoratoytterbium III).

We will describe our results relative, in the first step, to the differentiation of pure oxygenated terpenes in a racemic form : alcohols, acetates or ketones bearing a linear, cyclic or bicyclic skeleton, and in a second step, to the determination of the enantiomeric excess of some of them, which are the major components of essential oils (for instance, menthone in *Calamintha nepeta* oil).

O 4 - 5

New method for the determination of organochlorine pesticides in essential oils and pesticide residues data from 110 essential oil samples

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The presence of organochlorine pesticides in essential oils is discussed since a long time because the similar properties of volatile and lipophilic pesticides let expect them in essential oils. Due to the analytical problems only few results were published till now.

With a new cleanup-procedure it is possible to determine the most used 21 organochlorine pesticides by gaschromatography in quantities of > 10 ng/g. Gelchromatography on a Sephadex LH-20 column with ethanol (isopropanol) is the first cleanup-step followed in most cases by a second one with conc. sulphuric acid. The analysis will be done on a gaschromatograph system using two different capillary-columns (DB 5 and Rtx-1701) and ECD.
Out of 110 samples of 34 different essential oils we found organochlorine pesticides in 72 samples and higher amounts in 64 samples. Especially in the essential oil samples from *Mentha arvensis* var. *piperascens* (mint oil) we found α-HCH, γ-HCH (lindane), α-Endosulfane, *p,p'*-DDT, *p,p'*-DDE and HCB.
Our results show, that it is important to analyze also pesticides which are banned in many countries (e.g. DDT and technical HCH). But it is also remarkable, that about 35 percent of all tested samples contained no pesticides > 10 ng/g. We propose own maximum limits for essential oils with regard to the toxicological relevance.

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O 5 - 1

CHEMICAL EXPLORATION OF BRAZILIAN AROMATIC SPECIES BELONGING TO THE MYRTACEAE FAMILY

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As part of our research program on the essential oils of brazilian aromatic plants, we present the results of our chemical investigations on thirty species belonging to the Myrtaceae family. Eleven genera have been investigated : *Calyptranthes, Eugenia, Gomidesia, Hexachlamys, Marlierea, Myrceugenia, Myrcia, Myrciaria, Paramyrciaria, Plinia and Psidium.*

The analytical methodology is described and discussed ; the chemical compositions of the volatile extracts obtained by hydrodistillation of plant leaves are presented taking into account the major metabolic pathway of their constituents.

Most of the essential oils examined are complex sesquiterpene mixtures containing a majority of cyclic structures which have been regrouped according to their mode of cyclisation. A chemical classification of these samples are proposed on the grounds of the statistical analysis of the data using the Principal Component Analysis (PCA) method.

Among the thirty samples we have analyzed, four of them are interesting for their essential oil chemical composition from a scientific aspect and/or for potential further exploitation.

Eugenia stigmatosa DC. is characterized by a high content of an unusual component, cis-tetradec-4-enoic acid, which has already been found, in minor amounts, in fatty oil of seeds of Lauraceae.

The leaf essential oil of *Calyptranthes concinna* DC. contains about 80 % of elemicin whereas that of *Calyptranthes tricona* Legrand is interesting for two major new chromene derivatives likely to present biological activity.

Finally, *Myrcia fallax* Richard provided 0.35 % of an essential oil containing 84 % of (-) α-bisabolol ; on account of its chemical composition, this species is worth being submitted to further investigations.

VOLATILE COMPONENTS OF SELECTED LIVERWORTS

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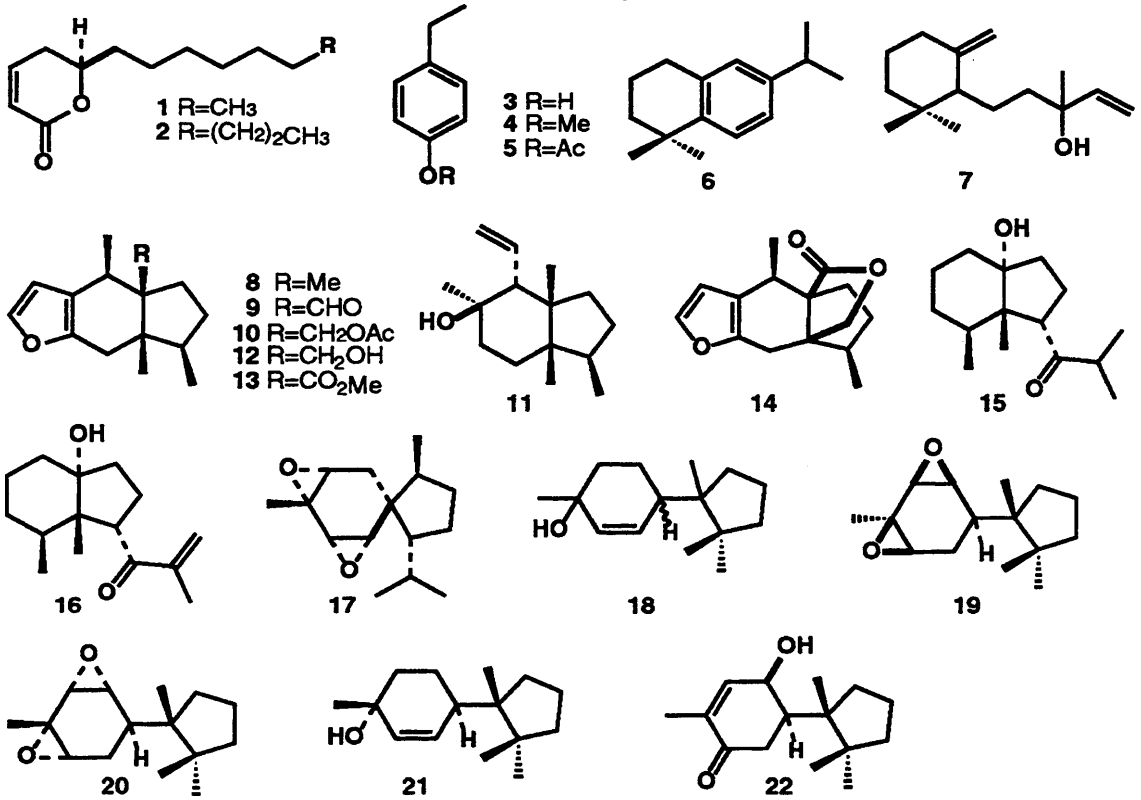
Oil bodies of liverworts are rich sources of terpenoids and lipophilic aromatic compounds, several of which show strong mossy odour [1,2]. Recently we studied the chemical constituents of 7 selected liverworts and isolated the following compounds. (*R*)-dodec-2-en-1,5-olide (**1**), (*R*)-tetradec-2-en-1,5-olide (**2**) from *Cheilolejeunea imbricata*, three ethyl benzene derivatives (**3-5**) from *Leptolejeunea elliptica*, olivacene (**6**) and β-monocyclonerolidol (**7**) from *Archilejeunea olivacea*, three pinguisane- sesquiterpenes (**8-10**), one neopinguisane-type sesquiterpenes (**11**) from *Dicranolejeunea yoshinagana*, four pinguisanes (**9**, **12-14**) from *Lopholejeunea nigricans*, two chiloscyphanes (**15,16**) from *Jungermannia vulcanicola* and six cuparene-type sesquiterpenes (**17-22**) from *J. hattoriana*. Compounds (**1**, **2**) which are chiroptically pure are responsible for the strong milky smell of *C. imbricata*. Powerful mossy smell of *L. elliptica* is due to the simple 4-ethylphenol and its derivatives (**3-5**). Compounds (**1,6,9,10,11,12,13,14,17-20**) were newly isolated from natural sources.



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O 5 - 3

IDENTIFICATION OF NEW SESQUITERPENES IN LIVERWORTS

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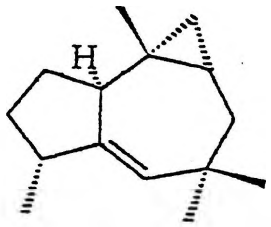
In contrast to other mosses liverworts (Hepaticae) form a large variety of terpenoid compounds which are contained in special oil bodies. Reports in the literature [1] showed that many of the sesquiterpenes from liverworts have the *unusual* configuration. We were primarily interested in the separation of enantiomeric sesquiterpenes by enantioselective gas chromatography. The presence of sesquiterpene hydrocarbons and alcohols with a configuration opposite to that of the same constituents in higher plants could be confirmed. In the course of our investigations several new compounds were isolated by preparative GC and identified by spectroscopic methods and by conversion into known compounds. Beside 2-dimensional GC- and NMR techniques the investigation of derivatives of the unknown structures obtained by oxydation, hydrogenation and rearrangement reactions were essential tools for deriving the correct skeleton and stereochemical details.

These techniques will be illustrated in the case of some new africanane (**1**, **2**) derivatives from *Pellia epiphylla*, a furanoeudesmatrienol (**3**) from *Lophocolea heterophylla* and a spirovetivenol (**4**) from *Lepidozia reptans*.



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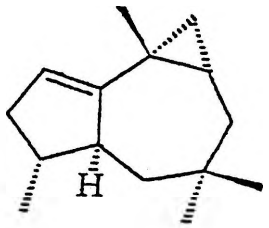

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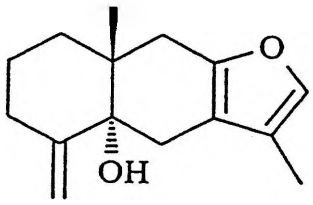

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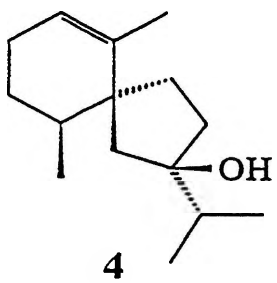

4

O 5 - 4

Constituents of the Haitian Vetiver Oil

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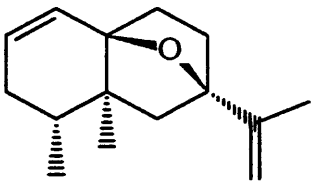
The commercial Vetiver oil, obtained by steam distillation of the dried roots of *Vetiveria zizanioides* (L.) Nash, consists of a complex mixture of more than 300 sesquiterpenoids and some *nor*-derivatives. Although the similar chemical and physical properties of its constituents complicate their separation and structure elucidation, we started an analysis of the Haitian Vetiver oil.

The isolation of several new sesquiterpene ethers, e.g. the epoxyeremophiladiene **1** and epoxyspirovetivadiene **2**, was achieved by combination of flash chromatography and distillation. A complete structure elucidation of some compounds by extensive ^{1}H- and ^{13}C-NMR spectroscopic measurements will be presented .



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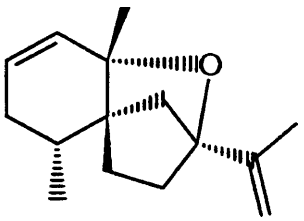

2

O 5 - 5

THE ESSENTIAL OILS OF *SATUREJA* OCCURING IN TURKEY

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The genus *Satureja* (*Labiatae*) is represented in the flora of Turkey by fourteen species of which five are endemic. *Satureja* species are used as a substitute of thyme in Turkey. Water distilled essential oils from eleven *Satureja* species were analysed by GC and GC/MS. The results will be presented in a comparative manner.

PRODUCTION OF ESSENTIAL OILS

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A broadly illustrated and practically oriented lecture on the production of essential oils will be given.
Main aspects of the production of essential oils are the preparation of the plantmaterial, the isolation methods, the yields and economics and the quality control.

The preparation of the plantmaterial may concern e.g.: harvesting, threshing, drying, grinding, hydrolysis and fermentative conversion.

Citrus oils can be produced by expression, such as the Italian pellatrici and sfumatrice methods, the American Brown oil extractor, and the FMC apparatus.
For the production of leaf, seed and flower oils steamdistillation, hydrodistillation and hydrodiffusion are applied.
Other natural isolates, such as oakmoss absolute are obtained by solvent extraction, and for instance flower oils are produced by supercritical fluid carbondioxide extraction.
Modern continuous distillation and extraction processes, as carried out by Biolandes Technologies and Texarome, are practiced today.

Ranges and anomalies in the yields of natural isolates are noticed.
Scope and limitations of the economics, such as raw material, capital, energy and labour costs will be commented.

The physical standards for many essential oils have been published.
The analysis of essential oils by modern spectroscopic techniques will be discussed. The quality assessment in essential oil studies is critically reviewed.

The chemical composition of essential oils from the same plant species and various origins are compared with an up-to-date computerized database.

Anis st-dist yield 2.1-2.3

adv st-dist disv
comm ap possi hidrol
low capit cst high energ cost
quality well def

Biolandes Spain

O 6 - 2

INFLUENCE OF STORAGE ON QUANTITY AND QUALITY OF ESSENTIAL OIL YIELD FROM TWENTY HERB SPECIES

Svoboda, KP[1], Galambosi, B[2] and Hampson, J[1]

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[2] Research Station for Ecological Agriculture, SF-50600, Mikkeli, Finland

In order to maintain the quality and quantity of the oil yield from dried herbs, it is important to provide proper storage conditions; dry, but not over hot, and protected from light, especially sunlight. In this small scale experiment, we wanted to monitor a range of dried herbs stored in conditions available to everyone. To this effect, they were stored in white paper 'sugar bags', in drawers, and at 'living room' temperature (14-20°C). Samples of 20 herb species were harvested, varying from leaves, seeds and roots, then dried carefully at 35°C. The initial distillation was done to assess the actual oil yield as an indicator of how much material would have to be used to extract a reasonably measurable amount of oil. The samples for future distillation were then weighed out, put into separate bags and stored. The root material was stored 'whole' and not broken up until just before distillation. Prior to distillation, the weight was re-checked so that an accurate oil yield could be obtained. The samples were distilled for 2 hours using the BP apparatus, the yield measured, then the oil was analysed by GC. This gives us a check on both yield and quality of the oil, allowing us to monitor any changes occurring during storage. The distillations were done at intervals of 6 months. With herbs whose yield deteriorated markedly over time, further experiments could be carried out to try to find more suitable conditions in which to store them i.e. lower temperatures/freezing. The following list gives main results in oil yield, (%v/w) monitored in November 1993 and April 1996 respectively: wormwood (0.6; 0.4); tarragon (1.0; 0.3); fennel (0.4; 0.1); hyssop (1.0; 0.6); lovage (1.0; 0.4); spearmint (1.0; 0.6); catmint (0.8; 0.2); basil (0.5; 0.5); oregano (3.3; 2.4); Greek oregano (3.5; 2.4); sweet marjoram (1.4; 1.4); rosemary (1.6; 1.4); sage (1.7; 1.4); summer savory (1.0; 0.6); wild thyme (0.6; 0.4); thyme (1.8; 1.0); dill seed (3.9; 3.0); coriander seed (0.9; 0.6); angelica root (1.1; 0.5); lovage root (0.4; 0.1). The existing species will be held in storage for another year. Essential oil composition was markedly changed in certain species (catmint, coriander seed, dill seed, fennel leaf, hyssop, lovage root, lovage leaf, summer savory, tarragon, wormwood), whilst other species did not show any deterioration or only very slight changes (angelica root, basil, greek oregano, oregano, rosemary, sage, spearmint, sweet marjoram, thyme, wild thyme).

O 6 - 3

DEVELOPMENT OF ESSENTIAL OIL INDUSTRIES IN DEVELOPING COUNTRIES : PROSPECTS AND CONSTRAINTS

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Many developing countries are endowed with vast resources of aromatic plants. Apart from the traditional uses of these plants, many are exported to industrialized countries as spices, primary processed products and mainly as raw materials for the production of fragrances and flavours. With a few exceptions, harvesting of aromatic plants for export has been from wild resources, resulting in the depletion of natural flora. This type of indiscriminate exploitation has created biodiversity conservation problems, and even endangered valuable species.

The lack of information on the social and economic benefits to be derived from the industrial utilization of aromatic plants has been a major factor impeding the development of the essential oils industry in the developing countries. Except for the use of these plants for domestic purposes, not much information has been available on their market potential and trading possibilities. As a result, the real potential of these plants is not known to the governments or the entrepreneurs. Furthermore inventories on the types of useful plants and their abundance are not available for assessing the possibilities for their industrial utilization.

Today the development of plant based products has gained momentum due to green consumerism and the current resurgence on the use of "Naturals" in developed countries. The demand for eco-friendly organic products and the free market economy bringing in expanding markets have given a fresh impetus to the development of plant based products. Furthermore the requirement of essential oils for use in aromatherapy is increasing, creating a demand for organically produced exotic oils.

Poor propagation and agricultural practices leading to low quality raw materials, poor post harvest treatment, inefficient processing techniques leading to low yields and poor quality products, lack of quality control, high energy losses during processing, poor facilities for R & D on product and process development, lack of trained manpower and difficulties in marketing are some of the problems associated with the essential oil industries in developing countries.

The development of the essential oils industries is therefore important to many developing countries which have rich resources of raw materials or the climatic conditions for the initiation of cropwise cultivation programmes. There is therefore an urgent need to assist the developing countries in the proper and useful utilization of their aromatic plant resources so they can obtain the maximum economic benefits and equally importantly, conserve their environment and biodiversity.

O 6 - 4

PIPER BETLE LEAF OIL: FACTORS AFFECTING PRODUCTION AND COMPOSITION

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Betel leaf plant cultivated abundantly in eastern and southeastern India is an important cash crop. The use of betel leaves is a part of conventional hospitability, a habit and an innocent aftermeal breath-sweeting practice, involving over one fourth of human race of Asia, Africa and Gulf countries. Betel leaf is reliever of thirst, cerebral congestion and a stimulant. The oil is antiseptic an has marked activity against a number of gram positive and gram negative bacteria.

Chemical composition of essential oil from betel leaf widely differs from one another explaining marked differences in flavour of the leaves amongst different varieties. However common presence in most of the varieties of chavicol, chavibetol cadiene and allyl-catechol is notable. High eugenol content in some of the varieties is likely to make the oil attractive to aroma and pharmaceutical industry.

Content of oil in betel leaf is dependent on seasons, maturity of leaves, different agronomical regimes and generally ranges from 0,1 to 1,0 percent. Betel plant is best cultivated in „baroj" (conservatory) and thrives best under 18-30 C, high humidity, very diffuse light , precipitation 150-175 cm/year and on upland soil having pH slightly acidic to neutral. The growth is responsive to N:P:K fertilisation and both oil and its constituents are appreciably stimulated by application of herbal origin n-triacontanol formulation, a saturated fatty alcohol. The oil and its constitutents vary from variety to variety and from region to region of cultivation.

For effective distillation of oil, hydrodistillation in Clevengers-type still with automatic feed back system is desirable. For maximum yield of oil optimum ratio (10:2:5) of the capacity of still (lit) quantity of plant material (kg) and volume of water (lit) is recommended. Optimum duration of distillation being three hours. Distillate water after oil recovery and non voltile solid fraction also merit commercial exploitation because of presence of aroma traces and betel oleoresins.

O 6 - 5

DESIGN AND CONSTRUCTION OF FIELD DISTILLATION EQUIPMENT IN EL SALVADOR

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Essential–oil–producing plant species are of common use in the Primary Health Care Systems of tropical areas. Most of the tropics belong to the developing countries where the essential oils industry thrives as a cottage–scale industry employing quite basic still designs.
For many developing countries, regional and international trade in non–traditional agricultural products (such as medicinal and aromatic plant extracts and especially essential oils) is an important source of foreign exchange, and is thus vital to the economic development of those countries. The rural sector plays a key role in the production of essential oils, and thus enhancement of trade will benefit the most needy sectors of the population.
However, the lack of technical know–how often means to many small and medium–sized enterprises that controlled wild collection and cultivation of herbal raw material cannot be followed by the subsequent on–site processing of essential oils to achieve the desired value added in the country of origin itself.

Not only for the use in projects the Protrade manufacturing and plant con–struction handbook "The Distillation of Essential Oils" has been well received, but also for private enterprises and research institutions in partner countries, USA and inside Europe.

The construction of distillation equipment in El Salvador uses an open access to technology options and information.

Protrade firma que comerc dest (liv)

O 7 - 1

- Efeitos dos óleos
- intensification of impulses (taste
- " secretion
- " activ digest-enz
- " nutrient absorption
- inhibition of bact growth
  fung.
  mould-growth
- " oxidat proces

Essential Oils as Phytogenic Feed Additives

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In the Plant Kingdom 24 Families are reported to contain more than 1, and further 40 Families only 1 essential oil producing genera (Protzen, 1993).

The biological activity of essential oil plants has been known and utilized since ancient times (e.g. food seasoning, ethnomedicine, etc.)

Changes in the nutritional habits of the human population, the increased concern for the environment have brought about an upsurge of interest towards the consumption and production of natural foods. To achieve this goal, similarly to organic agriculture, the food producing 'animal industry' will also have to reduce the application of synthetic chemicals and turn towards the more healthy natural ways and means of production.

Nutritional products used as feed additives/supplements to bulk feedstuffs (e.g. grains, oilseeds, forage, etc.) are meant to improve performance, or in certain cases to cure nutritional deficiency and/or metabolic disorders. To date, mainly synthetics have been used to this end.

Owing to the versatility of their biological activity (antimicrobial-, antifungal-activity, activity against insects, etc.), essential oil plants/essential oils can be regarded as potential substitutes for the synthetic feed additives. In this sense, they could find favorable utilization not only in animal keeping (e.g. insect repellents or insecticides), but as feed additives also in animal feeding.

In discussing the possible positive role of essential oils in animal feeding (e.g. increased feed uptake, improved feed-utilization, improved acceptance of feed with unpleasant taste, the reduction of chemoterapeutical application, etc.), special attention will be paid to the antioxidant-activity of essential oils that can influence both feed and meet quality, as well as shelf-life.

Further favorable applications of essential oils (plants) in animal keeping (e.g. improving barn climate, etc.) will also be discussed with the ultimate goal to call attention both to the physiological/economic importance of *phytogenic feed-additives* and to the need for revealing the scientific basis of their application.

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O 7 - 2

FEEDING HERBAL INGREDIENTS PRODUCES A PERFORMANCE ENHANCEMENT IN FATTENING SWINE

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The effects of a herbal feed supplement on several parameters of swine production were studied on a commercial UK unit over a 15 month period.
The unit sells on average approximately 2000 finished pigs per month at a body weight of 92 kgs. They enter the unit from breeding establishment at approximately 37 kgs live weight. The pigs are fed a balanced diet as a liquid ration delivered by pipeline. For 6 months of the 15 months study period pigs were fed a ration containing a herbal supplement. This was in two separate periods of 2 and 4 months. For the remainder of the time pigs were fed an unsupplemented ration.

At the end of the trial the performance of the unit was evaluated on a monthly basis for following parameters:

- Number of pigs entering the unit
- Number of pigs sold fat
- Feed usage
- Mortality
- Feed conversion ratio
- Daily liveweight gain

Average figures for the months when the herbal supplement was fed were:

- Daily Live Weight Gain 825 g/day
- FCR 2,40
- Mortality 2,82 %

This compares to the period when a normal ration was fed of

Daily Live Weight Gain 627 g/day	(24 % lower)
FCR 2,53	(5 % higher)
Mortality 3,35 %	(19% higher)

The position of the UK market for pigs and pigmeat puts a conservative value on the effects of the herbal supplement of over £ 7000.00 per month. This shows a cost-effectivness ratio in favour of the use of the herbal feed supplement of greater than 8:1.

POSTERS

P 1 - 1

EVALUATION OF INHIBITORY DATA OF ESSENTIAL OIL CONSTITUENTS OBTAINED WITH DIFFERENT MICROBIOLOGICAL TESTING METHODS

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In the past a number of different testing methods have been elaborated in order to investigate the antimicrobial properties of essential oils and their constituents. However, the gained results obtained with different testing set-ups are limited, because they are strongly dependent from the applied experimental conditions and cannot be compared in most cases.

To demonstrate the data variety, inhibitory data of eugenol - one of the best examined compound found in essential oils - from own investigations and from literature against *Escherichia coli* were chosen as an example and evaluated after compilation.

As a result from the critical discussion methodical parameters are outlined for testing the antimicrobial activity of essential oils and their constituents in order to improve the comparability of the obtained results.

P 1 - 2

ESSENTIAL OILS AND ANTIBACTERIAL ACTIVITY AGAINST *HELICOBACTER PYLORI*

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Helicobacter pylori is an organism which has been proved to play a major role in the etiology of human antral gastritis and an essential factor on peptic ulcer disease (1). Moreover the evidence that *H. pylori* is one of the important independent risk factor for the non cardial gastric cancer is growing (2). The object of the study reported here was to evaluate the ability of essential oils to inhibit the *in vitro* growth of *H. pylori*. The essential oils tested for the anti *H. pylori* activity were basil, boldo, cinnamon, cypress, French tarragon, bitter fennel, sweet fennel, clove, geranium, juniper, lavender "Abrialis", lavender "grosso", lavender "Super A", peppermint, oregano, Spanish oregano, rosemary, sage, winter savory and thyme all obtained by steam distillation. Minimal inhibitory concentration (MICs) were determined by broth dilution method. Oils were prepared as 10% (vol/vol) solution in ethanol and added in the range 200-2000 ppm to BHI with yeast extract (0.1%) and horse serum (10%). One hundred µl of this solution was distributed in each well of a Corning cell wells plate and inoculated with 10 µl of two days old culture by "Stepper™ repetitive pipette". Inoculated plates were incubated for 3-4 days in microaerophily. The growth was evaluated through the amount of cells on the bottom of the microwell. In all repeated tests *H. pilory* showed a surprising resistance to the highest concentration of all the essential oils tested with the only exceptions of cinnamon oil that is active at MIC of 800 ppm and clove oil, active at MIC of 1800 ppm. Geranium, juniper, oregano, Spanish oregano, winter savory, thyme oils are only able to delay the growth. Recent evidence that consumption of onion reduces the risk of stomach carcinoma has directed our attention to onion, leek and garlic essential oils hoping to find some experimental evidence supporting the case control studies by Dorant *et al* (3).

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P 1 - 3

GROWTH INHIBITION ACTIVITY OF ESSENTIAL OILS AND OTHER ANTIMICROBIAL AGENTS TOWARDS BIFIDOBACTERIA FROM DENTAL CARIES

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Bifidobacteria are typically found in the alimentary tracts of humans and animals. In 1974 Scardovi and Crociani (1) described *Bifidobacterium dentium,* a species isolated from dental caries and in 1996 other two species *B. inopinatum* and *B. denticolens* were found in the same habitat and proposed as new species (2). Essential oils are widespread used in oral hygiene and dentistry but only recently, data about their effects on fastidiously and facultatively anaerobic oral bacteria have been published. In this communication the effect of twenty essential oils from aromatic plants typical of the Italian Mediterranean flora as well as from species originated in tropical countries were surveyed on 16 strains of bifidobacteria from dental caries. In addition the antimicrobial susceptibility of the same strains to 13 antibiotics was tested. The essential oils were obtained by steam distillation of plant material in Cleavenger type apparatus. Microtitre test plates were used to determine MICs (minimal inhibitory concentrations) in TPY as growth medium. Inoculated plates were incubated for 3-4 days in anaerobic condition. Clindamycin, erythromycin, lincomycin, penicillin G and vancomycin were the most inhibitory antibiotics. Gentamicin, kanamycin, metronidazole and neomycin showed a low activity. Bacitracin, chloramphenicol, streptomycin and tetracycline possess antibacterial properties with different degrees of effectiveness. In general the antibiotics are more effective against the strains of the two new species *B. inopinatum* and *B. denticolens* than against the strains of *B. dentium* with the only exceptions of erythromycin and metronidazole. *B. inopinatum* and *B. dentium* have the same behavior when tested for resistance to essential oils. Ten of the twenty oils tested were more active against *B. denticolens* than to the other two species. Cinnamon oil was the most effective with MICs of 200-400 ppm. Basil, geranium, winter savory, oregano and Spanish oregano have shown an inhibitory effect at low concentrations against most of the strains tested while boldo, cinnamon, cypress, French tarragon, bitter fennel, sweet fennel, clove, juniper, lavender "Abrialis", lavender "grosso", lavender "Super A", peppermint, rosemary, sage, and thyme possess antibacterial properties with different degrees of effectiveness.

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P 1 - 4

ESSENTIAL OILS AS EFFECTIVE SANATORS OF HOSPITAL INFECTIONS

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Hospital purulent septic infections (HPSI) are at first place in modern classification of microbial infections. The economic damage as a result of HPSI is a considerable one.
We are conducting works on development of new effective, ecologically pure and harmless means for air sanation from HPSI.
Essential oil from *Origanum vulgare* was used for sanation of air in the rooms. Allergenicity of the essential oil has not been discovered.
A dressing room in a purulent surgical department of a hospital was used for examination of the method.
Preliminary bacteriological analyses of air in the examined object has been conducted. The dynamic of the next observations has demonstrated a decrease in quantitative composition of bacterial flora 5-7 times as compared to the standard, determined by the Russian Ministry of Health. The sanative effect of *Origanum vulgare* essential oil has continued for 18 hours.

P 1 - 5

ANTIMICROBIAL PROPERTIES OF THE ESSENTIAL OIL OF *ARTEMISIA MOLINIERI* QUEZEL.

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Artemisia molinieri Quezel cultivated in the Medical Plants Garden, Institute of Biology and Botany, Medical Academy in Wrocław, Poland, yields 0.75% of the essential oil. Its composition differs from those observed for the oil obtained from *A. molinieri* from natural origin (1). The essential oil was separated by vacuum distillation into the following fractions:

1. 75.6% p-cymene, 12.8% α-terpinene,
2. 66.4% ascaridole,
3. residue, 33.1% and 35.6% - two isomeric bisabololoxide A acetate.

The antimicrobial properties of the total oil and its three fractions were studied against three fungi (*Candida albicans, Rhodotorula rubra* and *Aspergillus fumigatus*) by using well test and against four bacteria (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*) and *C.albicans* by using suspension test (2).

The results of suspension test showed that Gram - bacteria were more resistant (MIC 4-5 µl/ml) than Gram + bacteria (MIC 2-3 µl/ml), fungi were most sensitive (MIC 1-2 µl/ml).

In well test *C.albicans* was more resistant than other fungi : inhibition zone of 0.4 ml of 1.1% essential oil's solution was 35 mm, while for two others no growing was observed.

Small differences in activity of the oil and its fractions against bacteria and greater ones against fungi occur : the second fraction showed the highest activity.

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This study was supported by State Committee of Sciences No. 5 PO6B 103 08.

P 1 - 6

CHEMICAL AND BIOLOGICAL STUDIES ON THE ESSENTIAL OILS OF FOUR *HELICHRYSUM* SPECIES GROWING IN GREECE

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The genus *Helichrysum* belongs to the family of Asteraceae. The 4 studied species: *Helichrysum amorginum*, *H. italicum ssp. italicum*, *H. stoechas ssp. barrelieri* and *H. taenari* are shrubs with yellow flowers, growing in dry places. *H. amorginum* is endemic in the island of Amorgos and *H. taenari* is endemic in South Peloponesus.

We report in this study the chemical constituents of the essential oils obtained from the aerial parts of the above mentioned species of *Helichrysum*.

GC and coupled GC-MS analyses of the oils led to the identification of their components. The major components out of the 24 constituents (82.06%) of the oil of *H. italicum* were: geraniol (35.59%), geranyl acetate (14.69%) and nerolidol (11.86%); 25 constituents, were identified from *H. amorginum* (89.98%) with major ones: geraniol (32.11%), geranyl acetate (20.76%) and neryl acetate (17.54%). The oil obtained from *H. stoechas* showed the occurrence of 24 constituents (73.87%) with main components β-caryophyllene (15.56%), β-elemene (13.11%) and benzyl benzoate (5.69%); while the oil isolated from *H. taenari* had a high percentage of geraniol (49.99%), camphene (18.63%) and α-pinene (8.91%) out of the 17 identified constituents (87.41%).

The bacteriostatic activities of the oils were determined, using the dilution technique, by measuring their MIC values against six Gram (±) bacteria *S. epidermidis*, *E. coli*, *E. cloacae*, *K. pneumoniae* and *P. aeruginosa*, strains of ATCC. The oil of *H. amorginum* and *H. taenari* exhibited the strongest activity against the six test bacteria while the two other oils showed a weaker one. The bacteriostatic properties of the oils is suspected to be associated with the significant contribution of geraniol, geranyl acetate, neryl acetate and camphene, which are known to possess strong antibacterial activity.

P 1 - 7

CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF TAGETES ERECTA AND TAGETES PATULA ESSENTIAL OILS

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Many species of the genus *Tagetes* (marigold) members of the *Compositae* family have been used to obtain essential oils. Numerous reports describing their applications as natural insecticides, especially for the control of mosquitoes, can be found in the literature. In this study we report the chemical composition of 4 essential oils obtained by hydro distillation of flowers and leaves from *Tagetes erecta* and *T. patula* and their growth inhibition activity towards 15 species of the genera *Lactobacillus* and *Clostridium.*

Chromatographic profiling, of the marigold oils, was performed on a Carlo Erba HRGC gas chromatograph at conditions reported by Marotti and Piccaglia (1). The main components detected were limonene, trans-ocimene, terpinolene, piperitone and β-caryophyllene. Relevant quantitative differences were found between the two species and between the oils from leaves and flowers. Microtitre test plates were used to determine MICs (minimal inhibitory concentrations) in TPY (2) as growth medium. Incubation, of inoculated plates, was performed in anaerobic conditions for 48 hours. The growth was evaluated through the cells density, the reduction of the pH and the color variation of bromocreosol indicator. Despite the differences of the chemical composition, the antimicrobial activity of the four oils tested was uniform. Surprising, at low concentrations the oils showed inhibitory effects against all the *Clostridium* species tested while the *Lactobacillus* species showed resistance to the higher concentrations tested.

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THE CHEMICAL COMPOSITION AND ANTIBACTERIAL PROPERTIES OF *THYMUS LONGICAULIS* SUBSP. *CHAUBARDII* OILS: Three Chemotypes in the Same Population.

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The infraspecific variability of the essential oils in the genus *Thymus* (Labiatae) has been the subject of several studies reviewed by Stahl-Biskup[1].

The genus *Thymus* is represented in Greece by 32 species (23 of which are endemic) [2]. In continuation of our studies on Greek aromatic plants we recently came across a chemically diverse population of *Thymus longicaulis* C. Presl. subsp. *chaubardii* Jalas. This subspecies is a wild growing endemic plant spread in Yugoslavia, Albania, Greece and Asiatic Turkey. It is noteworthy that even though the individual plants of the population are almost morphologically identical, they can easily be separated by their characteristic odors.

Aerial parts of the specimens with distinct odors were collected at full flowering stage from the same population (growing in an area of 1 m^2) at Mt. Parnis (altitude 1200 m) in Attiki.

GC and GC-MS analyses of the essential oils obtained by hydrodistillation of the plant material, led to the identification of 35 components. The GC analyses confirmed the existence of three types of oils as these were originally recognized by their odor: thymol (19.44%) and limonene (18.74%) were the main components of the thyme-odor type (chemotype I); geraniol (56,78%) and geranyl acetate (7.62%) were the main components of the rose-odor type (chemotype II); linalool (63.12%) and α-terpinyl acetate (20.38%) were the main components of the lavender-odor type (chemotype III).

The results of the present study show the chemical polymorphism of *T. longicaulis* a phenomenon that has also been observed in other *Thymus* species.

The antibacterial activity of the essential oils was tested against six Gram (±) bacteria. Even though all oils exhibited significant antibiotic properties Chemotype II showed the strongest activity against all tested bacterial strains.

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P 1 - 9

ANTIMUTAGENIC ACTIVITY OF (+)-β-EUDESMOL AND PAEONOL FROM *DIOSCOREA JAPONICA*

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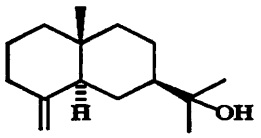
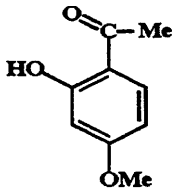
In our search for new naturally occurring antimutagenic compounds in plants, which have a history of safe use as Chinese crude drugs (1-3), we found that the methanol extract of *D. japonica* (Sanyaku in Japanese) exhibited suppression of the SOS-inducing activity of furylfuramide. In this presentation, we report the isolation and identification of the antimutagenic compounds contained in *D. japonica* (4).

A methanol extract from *Dioscorea japonica* showed a suppressive effect on *umu* gene expression of the SOS response in *Salmonella typhimurium* TA1535/pSK1002 against the mutagen 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (furylfuramide). The suppressive compounds in the methanol extract were isolated by SiO_2 column chromatography, and identified as (+)-β-eudesmol (**1**) and 2-hydroxy-4-methoxy-acetophenone (paeonol, **2**). The antimutagenic activities of these compounds against furylfuramide and Trp-P-1 were tested by an Ames test using *Salmonella typhimurium* TA100, which indicated that these compounds showed antimutagenic activity.



Compound 1



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Anti-inflammatory, analgesic and antipyretic activities of essential oil of Ageratum Conyzoïdes

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ABSTRACT: Ageratum conyzoïdes (A.C.) is an asteracea widely used in african and asiatic traditional medicines. Among these indications are mentionned enteralgia, cephalgia, fever and inflammatory diseases. In this study, we investigated the oral activity of essential oil of A.C. on cotton pellet granuloma (anti-inflammatory activity), tail-flick and acetic acid methods (analgesic effect) and brewer's yeast injection (antipyretic activity) on mice and rat.

Doses of 3 and 4 ml/kg were found to have a significant anti-inflammatory activity. At 3ml/kg, the antipyretic effect was comparable with that of reference substance (acetylsalicylate lysine 100mg/kg os) whereas the analgesic activity was shown at 2,3 and 4ml/kg. The dasly oral administration for 7 days failed to show gastric toxicity .

These results supports the folk use of A.C.

key words : Ageratum conyzoïdes Analgesic, anti inflammatory and antipyretic effects.

P 1 - 11

THE EFFECT OF ESSENTIAL OILS ON THE UTERUS COMPARED TO THAT ON OTHER MUSCLES.

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Essential oils are been used with increasing frequency during childbirth as well as during pregnancy. Their effect on the foetus is not known and very litle scientific data exists on their effect on the uterus. Although there is a substantial amount of data as to the effects of essential oils on smooth muscle, there is also considerable variation in results between authors. The extrapolation of data on essential oil effects on one tissue should be viewed with caution as different tissues can react in opposing ways to the same chemicals.

The possible variability of effects of essential oils on different tissues was studied using essential oils singly and in mixtures of two to four on preparations of the rat uterus, stomach, coecum and guinea-pig ileum in vitro. The results suggest that there is a wide variation in effect from one tissue to another. There was also a variability in the effect of the same essential oil or mixture on uterus preparations from rats at different stages in the oestrus cycle. There is also a wide variation in the effects of the same essential oil samples obtained from different commercial sources. This poses a serious question over the safety of widespread usage of essential oils during the very delicate stages of foetal development and parturition without further research.

P 1 - 12

3-D Studies on Odour Molecules

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Investigations on the molecular similarity are of high importance for structure avtivity relationship studies. Molecular shape and electrostatic potentials are features which are widely used for the description of the molecular stereochemistry and related electronic effects. Threedimensional comparisons can be performed on a set of structurally different molecules with same biological effect in order to obtain concrete information about the structural subunits, which might be common for all these molecules. Also the comparison with compounds of similar structure or electrostatic properties without the investigated biological effect leads to information about important differences between the molecules of various biological effects.

Molecular similarity investigations on Sandalwood odour molecules were done extensively recently [1-3] and models for osmophoric regions on the molecular surfaces were developed e. g. by Active Analog Approach studies [4]. As examples differences in fragrance impression of various isocamphanyl-cyclohexanols are explained by an analysis of the molecular surfaces, where deviations in these parts of the surfaces, which are most important for an interaction with the receptor site, are responsible for an increase or decrease or even loss of the Sandelwood fragrance.

That differences of electrostatic potentials are also very important for a selective odour impression, is documented in a study of the comparison between β-santalol and its 7-oxa- derivative. For both compounds the molecular shape is rather similar, but the complete different electrostatic potential caused by the oxygen bridge in the second compound leads to the total loss of Sandelwood scent.

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P 1 - 13

STRUCTURE-ODOUR RELATIONSHIPS OF SANDALWOOD ODORANTS: SYNTHESIS OF DOUBLEBOND-MODIFIED SANTALOLES

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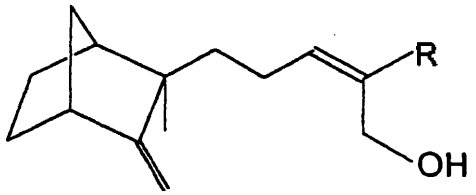
ß-Santalol (**1**), one of the main constituents of East Indian Sandalwood oil, is responsible for its warm and woody odour.
In connection with our studies on structure-activity relationships of fragrance compounds emitting the sandalwood odour, we have already shown that a modification of the methylene bridge of the norbornane skeleton does not change the fragrance of this sesquiterpene alcohol to a great extent [1,2,3]. By replacing the $-CH_2-$ unit of the methylene bridge with an ether bridge it could be shown that this molecule was odourless [4].

Continuing our studies on structure modifications of Santalol analogues and guided by results which have been obtained by means of computer aided fragrance design, it seemed interesting and worthwhile to replace the olefinic methyl group in the sidechain with other substituents (**2**,**3**).
In six steps each of the desired molecules could be obtained [1,5,6]. The olfactory evaluation was made for the Z- and E-Isomers. Thus it could be shown that the methyl group is necessary for this odour impression.



1. R=CH_3
2. R=H
3. R=C_2H_5

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P 1 - 14

Studies on the odor - structure relationship of some terpene substituted doixanes and dioxolanes.

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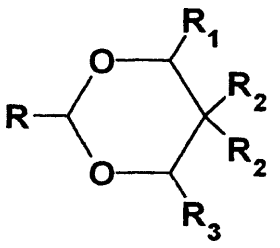
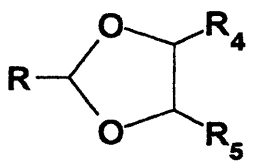
Odor characteristics of some substituted dioxanes (**1**) and dioxolanes (**2**) are given

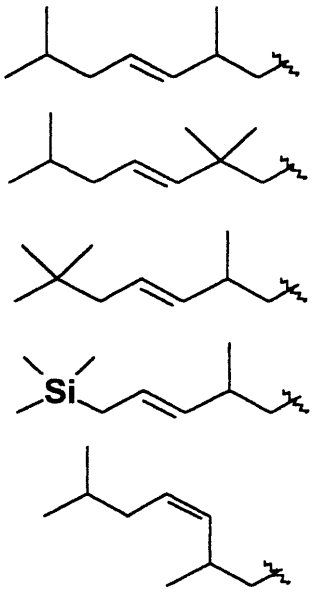


1



2

R =



R_1 = H, $-CH_3$

R_2 = H, $-CH_3$

R_3 = H, $-CH_3$

R_4 = H, $-CH_3$

$-CH_2CH_3$

$-C(CH_3)_3$

R_5 = H, $-CH_3$

The results of studies on structure - odor relationship carried out on the group of twenty six dioxanes and dioxolanes are presented.

MONOTERPENES FROM CUBAN PINES AND THEIR POSSIBLE ROLE IN THE HOST-PLANT RECOGNITION BY *DIORYCTRIA HORNEANA*

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Monoterpene hydrocarbons constitute the basic volatile components of oleoresins produced by pines. Monoterpenes from the loblolly pine were reported by Hanula et al. (1985) to act as oviposition stimulants for *Dioryctria amatella* (Pyralidae). A related pyralid *Dioryctria horneana*, a Cuban endemite, often attacks pines of the species *Pinus caribaea* and *P. cubensis* in Cuba. We have studied the monoterpene composition of oleoresins of Cuban pines to evaluate their degree of attractiveness for the insect pest. Both unattacked and attacked trees of *Pinus caribaea*, *P. tropicalis*, and *P. cubensis* were used for the study. Groups of trees growing in the same locality (a potential subject of insects' choice) were statistically evaluated according to the monoterpene composition of their oleoresins. Chirality of monoterpenes was taken into account, too. A multivariate data analysis (program CODEX®) of our analytical results showed significant differences between the different species, while only slight differences in the oleoresin composition were found between the unattacked and attacked trees of the same species (*Pinus caribaea* resp. *P. cubensis*). The presence of higher proportions of (-)-β-pinene and (+)-limonene was significant for the groups of attacked trees compared to unattacked ones.

A series of monoterpenic standards was tested electrophysiologically. Females of *D. horneana* were more sensitive to the tested monoterpenes than the corresponding males.

Literature:

Hanula J.L., Berisford C. W., DeBarr G.L. (1985). Monoterpene oviposition stimulants of *Dioryctria amatella* in volatiles from fusiform rust galls and second-year loblolly pine cones. *J. Chem. Ecol.* **11**, 943-952.

REGULATION OF ESSENTIAL OIL FORMATION IN CARAWAY AND POSSIBILITIES FOR MODIFICATION

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Essential oils are an important natural product and manipulation of content and composition has been the objective of many studies. However, although there are some positive exceptions it generally proved to be difficult to achieve an improvement in content or composition. In order to enable manipulation of essential oils, a study of the *in planta* regulation of content and composition is required. Although caraway may not be the economically most important essential oil crop, it is a suitable model system as it contains a rather simple essential oil with only two main compounds, limonene and carvone, of which one (carvone) is more attractive than the other. Therefore, our research is focused at increasing carvone yield of caraway.

Physiological research showed that formation of limonene and carvone was stimulated by high light conditions, likely as a result of increased carbohydrate availability from photosynthesis. Moreover, under high light conditions the ratio between carvone and limonene changed in favour of carvone. A breeding strategy aimed at improving sucrose availability during essential oil formation may lead to a caraway variety containing a higher carvone content.

Because of the strongly increased knowledge of biochemical and genetic regulation of essential oil formation, now the time seems ripe for a more direct approach, *i.e.* engineering the activity of enzymes involved in essential oil biosynthesis, and therefore the enzymatic regulation of the formation of limonene and carvone in caraway was studied. The activities of the three enzymes involved, (+)-limonene cyclase (+)-limonene hydroxylase and (+)-*trans*-carveol dehydrogenase, are developmentally regulated and the conversion of limonene to *trans*-carveol which is catalysed by a cytochrome P-450 enzyme, is the rate limiting step in the formation of carvone. An increase in the activity of this enzyme could possibly lead to a higher carvone content.

The strategy to use this knowledge to create a caraway variety rich in carvone and the possibilities to translate this study to other essential oil crops will be discussed.

P 2 - 2

STEREOCHEMISTRY OF FOUR 1,2-EPOXYMENTHYL ACETATES ISOLATED FROM S_1 OILS OF *MENTHA ROTUNDIFOLIA*

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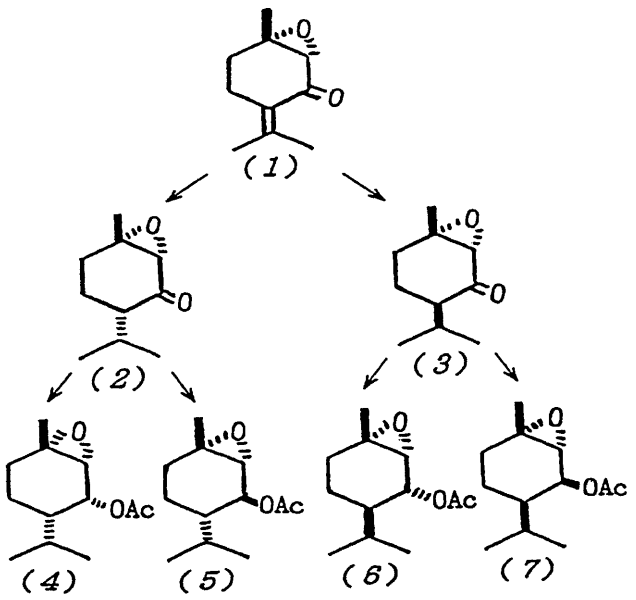
Present report deals with the conformation and the absolute configuration of compound *6* and *7*, which were supposed as stereoisomers of 1,2-epoxymenthyl acetate, isolated from the essential oil of the self-pollinated S_1 plants of *Mentha rotundifolia* with (1S:2S)-(+)-piperitenone oxide(*1*) as a main component. The collected S_1-17 oil in November consisted mainly of (1S:2S:4S)-(-)-*trans*-piperitone oxide(*2*), (1S:2S:4R)-(-)-*cis*-piperitone oxide(*3*), (1S:2S:3R:4S)-(+)-1,2-epoxyneomenthyl acetate(*4*) and (1S:2S:3S:4S)-(+)-1,2-epoxymenthyl acetate (*5*). On the other hand, the S_1-3 oil in November showed to contain *2*, *3*, *6* and *7* as major components. Both *6* and *7* were converted to *3* by deacetylation, followed by oxidation with MnO_2. The stereochemistry of compound *6* and *7* were assigned to be 1S:2S:4R both of them, and the absolute configuration of C-3 position have to be R for *6* and S for *7* by comparing the coupling constant observed with those of *4* and *5*. These conformations were discussed from H^1NMR data as follows: the coupling constant $J_{C2-H,\,C3-H}$=2.7Hz and $J_{C3-H,\,C4-H}$=2.7Hz could be determined as compound *6* due to (3R)-(-)-1,2-epoxyneoisomenthyl acetate, and $J_{C2-H,\,C3-H}$=9.9Hz and $J_{C3-H,\,C4-H}$=9.9 Hz could be determined as compound *7* due to (3S)-(-)-1,2-epoxyisomenthyl acetate.

The biosynthetic characteristics were discribed from the stereochemistry of 1,2-epoxymenthyl acetates between S_1-17 and S_1-3. It seems that compound *2* and *3* derived from *1* is stereospecific produced to give *4* and *5* in the S_1-17 plant, whereas to afford *6* and *7* in the S_1-3 plant.



P 2 - 3

CLASSIFICATION OF 92 STRAWBERRY GENOTYPES BASED ON THEIR LEAF ESSENTIAL OIL COMPOSITION

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Quebec, Canada J3B 3E6.

A great deal of work has been done on the inherent resistance of genotypes to two-spotted spider mite (TSSM), and some specific diseases like leaf spot, leaf scorch, and red stele. These pest and diseases are the most important pests and diseases of strawberry in Quebec. Chemical composition of leaves is among the most important factor that may affect plant susceptibility or resistant to pests and diseases. The purpose of our research was to compare the natural concentration of leaf essential oils (EOs) of 92 strawberry genotypes and construct a of pests and diseases susceptibility and plant and fruit characteristics in a attempt to correlate them to the concentration of leaf EOs composition. The cultivars and selections used in our study were chosen from a wide range of breeding lines and programs including Belgium, Canada, Denmark, England, France, Italy, New Zealand, Scotland, The Netherlands and USA and four wild species. Four major clusters were produced based on the leaf EOs composition. Two *Fragaria chiloensis* (Accession number 590 & 859) and *F. viridis* created their own cluster and being resistant to spider mites and leaf diseases. *Fragaria virginiana* created its own group being completely different from the rest.

P 2 - 4

COMPARISON OF THE CONTENT AND CHEMICAL COMPOSITION OF ESSENTIAL OILS OF TWO DILL CULTIVARS FROM POLAND

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Two cultivars of the dill : *Ambrozja* and *Lukulus* are cultivated nowadays in Poland. The fresh and dried herb before the bud formation are used in the "kitchen". The herb at the flowering stage and unripe umbells are used as a spice for pickled cucumber.

The objective of present study is to compare the content and chemical composition of the essential oil obtained from two cultivars of the dill (*Anethum graveolens L.*): *Ambrozja* and *Lukulus* being at different vegetative stages of the plant.

The oils are analysed by gas chromatography (GC) and by GC/MS. Oil characteristics are presented in the table below.

Cultivare	Yield essential Stage of growth	Characteristics Essential Oils of Dill at Various Growth Stages																			
		oil in dry weight %				α–felandrene				limonene				dill ether				carvone			
		H	U	L	S	H	U	L	S	H	U	L	S	H	U	L	S	H	U	L	S
Ambrozja	initial flowering	1,8	8,1	1,9	0,2	57,6	47,6	73,6	40,4	14,6	19,4	3,4	3,8	14,3	21,9	7,1	13,8	tr	tr	-	tr
Lukulus		1,8	6,9	1,7	0,5	56,5	50,4	63,1	tr	12,9	27,6	3,4	4,5	15,8	9,8	15,4	13,5	0,8	2,4	tr	0,3
Ambrozja	flowering stage	2,1	7,7	2,3	0,4	22,7	14,1	70,7	61,4	44,9	43,8	3,9	3,5	2,8	4,7	6,6	18,3	22,6	31,7	1,7	0,8
Lukulus		3,2	6,1	2,7	0,9	23,6	11,5	68,5	60,7	37,1	59,3	3,9	3,3	6,6	1,4	8,8	18,5	25,9	22,9	0,5	0,6
Ambrozja	ripe umbells	2,1	3,0	1,5	0,4	18,6	12,0	58,8	62,6	25,4	25,7	6,3	3,9	3,7	2,6	2,1	6,7	46,3	55,2	1,2	0,2
Lukulus		2,5	4,6	0,6	0,3	17,0	10,6	77,4	63,9	27,7	28,8	4,7	3,6	3,8	2,6	tr	7,6	46,0	53,2	tr	tr
Ambrozja	fruits	3,9				0,4				42,4				0,05				54,2			
Lukulus		4,7				0,2				44,7				0,06				51,7			

H - herb, U - umbells, L - leafs, S - stalks

ESSENTIAL OIL YIELD FROM DIFFERENT PLANT ORGAN AND DIFFERENT ACCESSIONS OF CORIANDER

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Abstract: Essential oil from different plant organs and different accessions of Coriandrum sativum L. were determined by using clevenger apparatus. The maximum quantity of oil per hectare was obtained from aerial part followed by the leaf with the exception of accession 223114, which has been followed by seed oil. The relative oil yield is dependent on the oil content and the biomass of that particular accession. In all accessions the relative oil content has been the highest in the flower except the accession 223066 where the essential oil in the seed exceeded the flower. Furthermore, the composition of the oils were analyzed by GC-MS. The qualitative and quantitative composition of the oils from the leaf, stem, flower and aerial has been found to be different from seed oil.

CUTTING TIME, YIELD AND ESSENTIAL OIL COMPOSITION IN THREE CULTIVAR OF PARSLEY.

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Parsley [*Petroselinum crispum* (Miller) Nyman ex A.W.Hill] biennal plant native of Mediterranean areas, is cultivated in Europe and North America for its aromatic and attractive leaves used, fresh or dried, as a condiment, garnish and flavoring ingredient. The essential oil is utilised as a spices or fragance in perfumer's product. The aim of this study was to evaluate the influence of cutting time on yield and oil composition in different cultivars.
The trial was carried out in Bari, in southern Italy; three cultivars, different for size and form of leaves: normal (NL), giant (GL) and curled (CL) were directly seeded in the field in single rows 20 cm apart using 1g of seed m^{-2} and cut 3 times:135-215 and 260 days after sowing.
Plots of 12 m^2 with 4 replications in randomized block design were arranged. Standard cultural and pest control system were used. Total yield, height, number, weight and dry matter of leaves were collected at each cut. Data were processed by analysis of variance.
The essential oil was obtained by steam distillation for 60 minutes of 1 kg of fresh leaves and petioles and the composition was determined by GC/FID and GC/MS spectroscopy analysis.
Total yield was of 40, 32 and 22 t ha^{-1} respectively in the cultvars GL, NL and CL; the 1st cut gave about 50% of total yield in all cultivars. Dry matter in the 1st cut was, on average, 9.3% and 14.8% in the 3rd cut.
Essential oil content increased from 0.06% of fresh weight in 1st and 2nd cuts to 0.1% in the last cut. The highest oil production, about 22 l ha^{-1}, was obtained by cultivar GL, the lower, 15 l ha^{-1}, in CL.
Generally the major components of essential oil were: *p*-1,3,8-menthatriene, 1-methyl-4-isopropenylbenzene, myristicin, apiol, thymol, anethole. In the 3rd cut myristicin, and apiol content was higher than in the others. The cultivar CL was richer in myristicin, while NL and GL in apiol.

P 2 - 7

INVESTIGATION ON THE COMPOSITION OF ESSENTIAL OILS OF *HYSSOPUS OFFICINALIS* L. POPULATIONS

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[2]Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót

Pluhár, Zs[3]., Németh, É[3]., Bernáth, J[3].,
[3]University of Horticulture and Food Industry, Department of Medicinal Plant Production, Budapest, Hungary

Hyssopus officinalis L. (*Lamiaceae*) is a well-known spice and medicinal plant, the flowering shoots and the essential oil of which are used.

The cultivated populations of hyssop can be characterised by a significant heterogeneity. In the course of its breeding the uniformity of flower colour (e.g. blue form), an increase in the oil content are the main achievable purposes. As our knowledge on (the variation or genetic fixation of) oil composition is incomplete and the international preferences on it is less determined, we decided to study this question. The variation of the chemical composition of oils is studied in various stages of hyssop breeding, when the traditional breeding purposes of getting high oil content, high yield, dark blue coloured flowers, etc. were regarded as achievable ends.

The different populations of *Hyssopus officinalis* grown from seeds and the offsprigs of the individually selected breeds gained from them served as starting material for our investigations. The original seeds had been obtained from abroad, from various Botanical Gardens.

The essential oils were gained with Water Steam Distillation (WSD) and Supercritical Fluid (SCF) Extraction with CO_2. The oils were analysed by GC, GC-MS techniques.

The main observations are as follows:

- In the case of applying SCF extraction the oil yield is higher and the composition of oils is more uniform comparing to WSD oils. The changes in oil composition, when WSD was applied, were mainly due to the formation of artefacts.
- The heterogeneity in the oil composition of the various populations was independent of the seed supplying botanical gardens (origin).
- Chemical heterogeneity in oil composition could be found among the offsprings of a plant with a particular chemical composition. If only the main four components (a: β-pinene, b: limonene, c: pinocamphone, d: isopinocamphone) are regarded, among the offsprings clear and mixed lines alike could be found independently of original composition.

The above-mentioned observations justify the necessity and usefulness of comparative chemical studies in hyssop selection which is going on.

P 2 - 8

STUDY ON THE CONTENT OF SOME TERPENE COMPOUNDS IN THE ESSENTIAL OIL OF SOME BULGARIAN VARIETIES POPULATIONS AND PERSPECTIVE CLONES FROM PEPPERMINT (*MENTHA PIPERITA* HUDS.)

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[1]Research Institute for Roses, Essential Oil and Medicinal Plants, 6100 Kazanlak, Bulgaria
[2]Higher Institute of Agriculture in Plovdiv, 12 Mendeleev str, 4000 Plovdiv, Bulgaria

Abstract

The investigation was carried out in 1994 -1995 in the Research Institute for Roses, Essential oil and Medicinal Plants in Kazanlak, Bulgaria. In this paper results are given from GC analyses of the essential oil from varieties Sofia, Tundja, Zefir and Kliment, some populations with different origins as well as some perspective clones obtained via the methods of induced mutation and individual selection of native forms from peppermint (*Mentha piperita* Huds.). Discussion is focused on the variation of chemical compounds α- pinene, β- pinene, limonene and β- caryophyllene in the essential oil of the tested varieties and clones. Results from this investigation could be used in the process of selection of new varieties of peppermint in Bulgaria.

P 2 - 9

SEED PRODUCTION IN MALE STERILE PLANTS IN *SALVIA SCLAREA* L.(*LAMIACEAE*)

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Seed production in male sterile and fertile plants of *Salvia sclarea* was studied to determinate the influence of male sterility on its quantity. In the literature data about female generative organs in male sterile plants are very scarce.

Normally 4 loculed pistils were presented in the flowers of *S. sclarea*. In every loculus of the pistil a tenuinuncellate anatropous unitegmic ovule was formed.

The formation of the embryo sac was of Polygonum-type, essentially uniform in *Lamiaceae*. After a porogamous fertilization 4-oneseeded nutlets were formed. These seed were without endosperm and investing - type of embryo.

The important changes of stamens, calyx and corolla in the male sterile plants were associated with increased number of pistil loculi. Their number varied in the different plants from four to ten, but the greater part remained empty.

Due to these anomalies in the male sterile plants 1-oneseeded and 2-oneseeded nutlets and even no nutlets were formed.

All these anomalies decreased the seed production of male sterile plants on the average by 20-30% compared to this of the fertile ones and have to be provided by the heterosis selection of specific cultivars.

OBSERVATIONS ON GLANDULAR TRICHOMES AND ESSENTIAL OILS IN *ROSMARINUS OFFICINALIS* L.

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Within a series of researches on the secretory structures in *Labiatae* and related volatile compounds (Bini Maleci, Pinetti, Servettaz, 1992 in Harley & Reynolds (eds): Advances in *Labiate* Sciences, Kew, 349-355; Servettaz, Bini Maleci, Pinetti, 1992, Plant Syst. Evol. 179:129-139; Servettaz, Pinetti, Bellesia, Bini Maleci, 1994, Bot. Acta 107: 369-472; Bini Maleci, Pinetti, Servettaz, 1995, Flora 190:237-242;) we intend to clear up the problem of better characterizing the kind of metabolites elaborated in the different trichomes.

For this research, we used *Rosmarinus officinalis* leaves, on which two kinds of glandular hairs are present: peltate and capitate with only one or two stalk cells. Peltate trichomes have many secretory cells and a large subcuticular space; capitate ones have only a secretory cell with a small subcuticular space. In both the trichome types secretion is accumulated in the subcuticular space.

The contents of these secreting trichomes have been analized both by means of histochemical tecniques (autofluorescence, Sudan black B, Nile Red, Nadi) and by using GC - MS. For the latter technique, the two kinds of trichomes have been isolated separately from the leaf tissues, using the tecnique of microneedle shuttle analysis (Tirillini, Stoppini, 1994, J. Essent. oil Res. 6: 249-252; Tirillini, Stoppini, 1995, J. Chrom. Science 33: 139-142).

Both histochemical and GC-MS analyses are in full agreement, and clerly indicate that the essential oils are chiefly contained in the small capitate hairs; on the contrary they are almost absent in the peltate trichomes.

The histochemical analyses carried out in order to identify what kind of substances are present in the large subcuticular space of the peltate trichomes, indicate la presence of a variable amount of flavonoids ($AlCl_3$ tecnique).

P 3 - 2

Histological Approach for the Assessment of Essential Oils' Potential Yield in *Origanum vulgare* ssp. *hirtum*

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After characterizing histologically the secretion tissue of oregano, in which unicellular-head capitate hairs and peltate glandular hairs were identified, the relations between the essential oils' content and the number of peltate glandular hairs on the leaf blade were shown.
The histologic study is part of a comprehensive research on oregano, aimed at providing the general view of the plant, with ist physiological, histological and agronomic aspects being also analysed with special emphasis on the production quality.
Estabilishing a relation between the tissue analysis and the esssential oils' content provides a helpful additional factor to handle in plant breeding.

P 3 - 3

EVALUATION OF LIGNIN SYNTHESIS IN RELATION TO ESSENTIAL OIL AND EUGENOL CONTENTS IN CLOCIMUM (*OCIMUM GRATISSIMUM L.*)

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Experiments purposing to evaluate lignin formation at different growing stages of the plant were conducted in selected source genotypes of „Clocimum" (Ocimum gratissimum) rich in eugenol. The analysis of growth-wise leaf and stem samples started from 4 weeks old plants and continued at regular intervals till dormancy during first and second year of plant growth. The isolated lignin was of the type of Klason lignin following Tappi's method of analysis. Each lignin analysis coincided with the distillation of respective leaf samples for measurement of essential oil and eugenol contents. The eugenol percentages showed gradual decline with corresponding increase in the lignin percentages once the plant growth crossed the optimum level and the stem growth became woody. The analytical results offered ample proof to obviate the conversion process from eugenol to lignin bearing direct relationship, which was inversely proportional. It corroborated the experimental evidences of Siegel (1955), Higuchi (1957) and Stafford (1960) based on phenylpropanoid amino acid pathway.

P 3 - 4

Effect of salicylic acid on the yield and quality of essential oil in aromatic crops

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Abstract:

Essential oils are a mixture of a large number of secondary metabolites of plants. Defence against stresses including pathogens in plants is atleast partly due to secondary metabolites. Salicylic acid (SA) is known to directly or via certain other elicitors induce synthesis of secondary metabolites involved in defence against stresses especially diseases caused by viruses, bacteria and fungi. We have asked the question do aromatic crop plants accumulate more essential oils after application of SA than the normal plants. The idea underlying this question is that if SA would accumulate relatively more essential oil, and possibly of a different quality. To answer this question growing populations of Japanese- and pepper-mints and palmarosa, citronella and lemongrass were sprayed with varying amounts of SA, once or twice. The sprayed and comparable nonsprayed plants were in plots arranged in randomised block design. The plants were harvested after two to four weeks of SA spray and analysed for herbage and essential oil yields and quality of essential oil. It was observed that SA application did not affect the herbage and essential oil yields as well as the quality of oil in the aromatic plants examined. It is concluded that the accumulation of essential oil in mints and aromatic grasses is not affected by exogenous SA. It is hypothesised that either the synthesis of essential oil constituents in aromatic plants occurs constitutively, without the intervention of SA or SA amounts required for the induction of synthesis of essential oil constituents are already available in the aromatic plants.

P 3 - 5

ESSENTIAL OILS AT DIFFERENT DEVELOPMENT STAGES OF ETHIOPIAN TAGETES MINUTA L.

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Abstract: Tagetes minuta L. is a common weed, widely spread in tropical and subtropical countries originated from South America. As its essential oil is already an established perfumery raw material attempts are made to introduce its production also in Ethiopia.

The reports of Lawrence (1) and Thappa et al (2) indicate that the chemical composition of the oil varies according to seed origin and stage of plant development, enabling to produce oil with the desired composition.

Based on this, attempts were made to quantify and characterize the oil at different stage of maturity grown under Wondo Genet, Ethiopian condition.

P 3 - 6

EFFECT OF HEAVY METAL POLLUTED SOILS ON SOME QUALITATIVE AND QUANTITATIVE CHARACTERS OF MINT AND CORNMINT

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In 1991-1993 in pot experiments we studied the effect of highly heavy metal polluted soils on some quantitative and qualitative characters of *M. piperita L.* (cv Tundja and Clone No 1) and *M. arvensis var piperascens Malinv.* (cv Mentolna-14). Soils were taken at distances of 0.5, 3, 6 and 10 km from Non-Ferrous Metals Combine (NFMC) near Plovdiv.

The following was estimated: fresh herbage yield, content of essential oil in the fresh herbage, yield of essential oil, heavy metal concentration in soils, plant parts (roots, stems, leaves and flowers) and in the essential oils, GC analyses of the oils. Plants, grown on the first soil contained excessive amounts of Cd, Pb, Zn and Cu, and the yield of essential oil was up to 17 % lower than from the control soil. High heavy metal concentration in the plants did not affect the essential oil content, but decreased menthol content in the oils. Despite high heavy metal content in the plants, (above the critical levels for Cd, Pb, Cu and Zn in the plants), oils as final product were not contaminated. Although heavy metal concentration in the other soils was relatively high, yields and quality of essential oil were not significantly affected.

Advances in the Authenticity Assessment of Citrus Oils

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Abstract

Chirality evaluation is proved to be an efficient tool for the authenticity control of neroli, petitgrain and bergamot oils by enantioselective multidimensional gas chromatography (enantio-MDGC). The simultaneous stereochemical analysis of the main compounds linalool, linalyl acetate, α-terpineol using heptakis-(2,3-di-O-acetyl-6-O-tert.-butyldimethyl-silyl)-β-cyclodextrin as the chiral main column is described. α-Pinene, β-pinene, limonene, terpinen-4-ol and Z-/E-nerolidol are simultaneously stereoanalyzed with heptakis-(2,3-di-O-methyl-6-O-tert.-butyldimethylsilyl)-β-cyclodextrin.

Characteristic authenticity profiles of neroli, petitgrain, bergamot and other citrus oils are deduced by enantioselective cGC as well as *i*sotope *r*atio *m*ass *s*pectrometry (IRMS) online coupled with capillary gas chromatography. Enantiomeric ratios, isotopic data, as well as quantification of bergamot oil compounds are evaluated integrally. Scope and limitations of the techniques are discussed.

Key words

Chirality evaluation, enantio-MDGC analysis, isotope ratio mass spectrometry (IRMS), genuineness of essential oils, authenticity profiles

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3-Butylphthalide: Chirospecific Analysis, Structure and Properties of the Enantiomers

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Abstract. Using enantioselective gas chromatography (enantio-GC) the direct analysis of 3-butylphthalide enantiomers was achieved. Investigations relating to their sensory characteristics and odour thresholds were done via enantio-GC / olfactometry. In order to elucidate stereochemical features the 3-butylphthalides were converted with (R)-2-phenylpropionic acid into diastereomeric ester derivatives. After isolation and separation via HPLC, absolute configurations were derived from ^{1}H-NMR studies. Ester cleavage and recyclisation yielded the corresponding 3-butylphthalide enantiomers. Investigations of celery seed oil show enantiomeric distributions in the range of 95 : 5 in favour of the (3 S) - enantiomer, which presents a significantly lower GC odour threshold value than the (3 R) - enantiomer.

Key words: Enantioselective gas chromatography, 3-butylphthalide, celery aroma compounds, natural enantiomeric distribution

Literature

D. Bartschat, B. Maas, S. Smietana, A. Mosandl , Phytochem. Anal. **7** (1996), 131-135

GC-ANALYSIS OF ESSENTIAL OILS ON CHIRAL COLUMNS - RELEVANT FOR THE PHARMACOPOEIA

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The separation of enantiomers by GC on chiral columns - based on different cyclodextrin phases - is a well-established method (1). In recent years it has been demonstrated that this method is very reliable for the documentation of the purity and identity of essential oils.
Monographs for certain essential oils of the European Pharmacopoeia give an exact definition on their origins. For the analytical control of these essential oils GC-analysis on chiral columns is not in use.
For the assessment of the quality of an essential oil this method seems to be useful and state of art.
One prerequisite for all pharmacopoeia methods is a general applicability with the following parameters: Maximal two-step temperature gradients, preferentially seperation of all important components in a single run and use of commercially available chiral columns and standards.
With respect to these conditions the seperation specificities of a broad range of compounds derived from different essential oils, e.g. peppermint oil, was examined on commercially available columns in dependence of temperature and column length.
The results are presented in comparison with an achiral column - based on polyethylenglycol - which is used in some monographs of the European Pharmacopoeia.

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Acknowledgement: This investigation was supported by a grant from the Bundesinstitut für Arzneimittel und Medizinprodukte, Berlin.

P 4 - 4

Comparaison between heptakis (6-*O*-thexyldimethylsilyl)-2,3-di-*O*-acetyl-β-Cyclodextrin and heptakis (6-*O*-*tert.*-butyldimethylsilyl)-2,3-di-*O*-acetyl-β-cyclodextrin as Chiral Stationnary Phase in Gas Chromatography.

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Heptakis (6-*O*-*tert.*-butyldimethylsilyl)-2,3-di-*O*-acetyl-β-cyclodextrin is a chiral stationnary phase introduced by *Mosandl and al.* [1] for the resolution of many racemic mixtures.
For the synthesis of this product, *tert.*-butyl-dimethylsilyl chlorid (TBDMSCl) was used as starting material. It presents the double disadvantage to be expensive and difficult to handle (solid and moisty sensitive).

Here, we report the preparation of a new chiral stationnary phase, the heptakis (6-*O*-thexyldimethylsilyl)-2,3-di-*O*-acetyl-β-cyclodextrin from the thexyl-dimethylsilyl chloride (TDSCl). This starting material is easy to manipulate, inexpensive and gives TDS-ethers which are about 2 to 3 times more stable to acidic hydrolysis than the corresponding TBDMS-ethers.

We also report the chiral analysis and an interesting comparative study of the new phase and the known one heptakis (6-*O*-*tert.*-butyldimethylsilyl)-2,3-di-*O*-acetyl-β-cyclodextrin.

Examples of separations of essential oils constituents will be presented.

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SFC/MS INVESTIGATION OF SPICE EXTRACTS

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Essential oils and spice extracts are used as an alternative to spices and mixtures of spices in food industry. The main advantage is the microbiological stability of these products. Common extraction methods are solvent extraction, supercritical fluid extraction (SFE) and the recently developed accelerated solvent extraction by means of ASE 200™ (Dionex).

Spice extracts are composed of volatile and less or non volatile compounds. The investigation of spice extracts by GC is only confined to volatile compounds. A new method to investigate the total extracts is the capillary supercritical fluid chromatography (SFC). This method is usable for volatile and non volatile compounds.

The SFE extracts and ASE extracts and in addition the respective essential oils of black pepper and other spices were investigated by GC and SFC/MS (1;2). SFC-MS analyses were performed on a Dionex, Series 600, supercritical fluid chromatograph equipped with a Rheodyne 7256 pneumatic controlled loop injector, a Mplus SFC/MS interface and a Finnigan 4500 mass spectrometer (1;2).

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SUPERCRITICAL FLUID EXTRACTION OF CLARY SAGE AND STUDY OF SCLAREOL AND ELEMENTS CONTENT IN PARTS OF PLANT

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Supercritical Fluid Extraction (SFE) is a relatively new procedure to give better quality and cleaner product than the traditional organic solvent extraction. The natural materials can be extracted by SFE without their components changing.
SFE of clary sage (*Salvia sclarea* L.) was investigated in this paper. Carbon dioxide was used as a supercritical solvent. The extracts were precipitated in two separators series. The oily, waxy products were collected in the 1st separator, and the essential oil rich product was recovered in the 2nd separator. In all experiments the extract quantities and the mass flow of solvent were measured and the influence of extraction and separation parameters on the amount of the extracts was examined.
The composition of the SFE extract containing the essential oil were compared with that of the essential oil obtained by steam distillation. The major components of clary sage oil were linalool and linalyl acetate, further minor components were identified such as α-pinene, geranyl acetate, neryl-acetate, p-cymol, myrcene, linalool, nerol and borneol.
The oily, waxy fractions were examined by TLC. We tried to identify the components appearing in these extracts with the help of available standards.
Quantitative estimation was given concerning the yield of sclareol from the clary sage in two steps. SFE and the steam distillation were used in the first step, and than clary sage was extracted in alcohol in the second step.
The sclareol content of the plant was investigated during the vegetation period, too.
Supercritical Fluid Extraction can be a suitable way of manufacturing of herb extracts for use in the food industry, in the medicine and in the perfumery. Nevertheless, the analysis of these extracts is not simple, further experiments and analytical measurements are needed to determine the optimum parameters for SFE.

(This work was supported by OTKA under grant numbers TO07693 and TO16880)

P 4 - 7

EVALUATION OF ESSENTIAL OILS ON THE BASIS OF CHROMATOGRAPHIC DATA USING PRINCIPAL COMPONENT ANALYSIS

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Capillary gas chromatography with flame ionisation detection was applied to the separation of terpenes in essential oil samples. Twenty seven samples from 6 different species were investigated. Eight components found in those samples were used as variables for the further characterisation by a chemometric procedure. Principal component analysis was applied to the differentiation of samples from different species and sources.

Our results indicate that principal component method of chromatographic data handling seems to be a fruitful approach to compare chromatograms obtained from such a complex mixtures essential oils, particularly because it is difficult to detect even major differences by direct inspection from char paper or tabular data.

It was found that this classification is connected more with the chemical composition of an essential oil than with botanic systematic. On the other hand,
however, in some of the cases it was even possible to check the origin of the plant material from which the essential oils were derived. Therefore an intensive
work is needed in order to resolve the complex relationships between the quality of the final industrial product.

P 4 - 8

INVESTIGATIONS OF TEA INFUSIONS AND DISTILLATION WATERS USING SOLID PHASE MICROEXTRACTION (SPME) TECHNIQUE

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In order to prepare gc ready samples of volatiles from highly diluted aqueous solutions usually liquid-liquid extraction or solid phase extraction using sorbent packings and successive solvent elution has been applied. Both methods are time consuming and labour intensive. Furthermore, liquid-liquid extraction requires a relatively large volume of an appropriate solvent, which has to be concentrated prior to gc analysis.

In contrast, the recently developed (1) solid-phase microextraction technique (SPME), which has been mainly applied to aqueous samples of pollutants, offers the advantage of very simple, solventless extraction of analytes and subsequent thermal desorption in the injection port of a gaschromatograph.

We have investigated its applicability in the field of analyzing tea infusions, aromatic waters, and distillation waters from aromatic plants. In a first step an aqueous solution of several terpenoids with different polarities and molecular weights was analyzed in order to optimize methodical parameters such as extraction time, desorption time and temperature, etc.. Using the optimized experimental conditions tea infusions, aromatic waters and distillation waters from Peppermint and Sage were analyzed. The obtained results are discussed and compared with results gained by conventional extraction methods.

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P 4 - 9

Evaluation of a Fennel Collection by Classical Extraction and SPME - Headspace - Analysis

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The fennel seed collection of the genebank Gatersleben was investigated applying solid-phase microextraction headspace-analysis. The collection contained 41 samples of different chemotypes.
The SPME-results were compared with those obtained by classical solvent extraction. The reproducibility of the headspace method applied at the determination of the main components trans-anethole, estragole and fenchone has been found to be comparable with the extraction method.
Furthermore the direct comparison of the two methods is also possible. The absolute values of the essential oil components are similiar. This is surprising, because we investigated in the first case the hexane extracts of fennel samples, in the second case the headspaces of aqueous fennel suspensions.

ANALYSIS OF ESSENTIAL OIL COMPOUNDS USED IN FRAGRANCE LAMPS

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There is, in the United States, a fast growing field of natural healing, which is well known in Europe and has been widely practised, mainly in the countries of England and France. This application, called Aromatherapy, is included in some health professionals practice and extensive research is under way on this subject. In the last years the use of essential oils in Fragrance Lamps has became very popular in Austria and other European countries. Objective of these investigations was to find out whether the essential oil compounds in this form of application submit any form of reactions or changes.

In the first part of this work 16 commercially available essential oils, 7 of them bought in Austria and 9 from Italy were selected and analysed by means of GC, GC-FTIR-MS and GC-sniffing-technique. In the next step the volatiles, under room temperature conditions and under heated conditions (the fragrance compounds were trapped by full tank as well as after 2h of running the lamp) were adsorbed on Dräger „Niosh Tubs" by using the „Closed Loop Trapping-Method", eluted with 400 µl CH_2Cl_2 and analysed as mentioned above. All essential oils and the corresponding headspace-samples were evaluated by 3 professional perfumers in order to determine the specific odour attributes.

The study shows that in all investigated systems no pyrolytic products were detectable by gaschromatographic-spectroscopic methods, but a clear change of the composition of the fragrance compounds from lower boiling to higher boiling ones was shown. Indeed, under heated conditions the fragrance compounds were multiply emitted in the air, so that they can easily be inhaled.

P 4 - 11

ANALYSIS OF ESSENTIAL OIL MIXES USED IN AROMATHERAPY

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Essential oils are used to treat many health conditions. They can be applied to the skin by various methods, and they can also be ingested or inhaled. For clinical use it is important that the oils are of high quality and that once mixed they remain chemically stable throughout the treatment period. Before the start of the experiment, the 18 oils to be used in the mixtures were checked for purity and chemical constituents (oils supplied by S Price Aromatherapy Ltd). Immediately after the mixes were prepared, they were checked by GC analysis (Day 0), then after 1 week of storage, and thereafter at 1, 2, 3, 6 and 12 months respectively. Testing will continue for another year. The oil mixes have been stored in glass vials in the dark, and the original oils in their original brown glass bottles, all at room temperature. Caddy Diagrams and Caddy Classic Profiles were used for comparison of chemical stability of individual oils and their mixes during that time. A Caddy Diagram is a coloured pie chart generated by clustering chemical constituents into chemical families (esters, aliphatic aldehydes, ketones, sesquiterpenes, lactocoumarins, oxides, aromatic aldehydes, monoterpenes, alcohols, phenols and phenolic esters). By using these diagrams, it is possible to compare quickly the oil quality against a classic profile of a natural unadulterated oil. Of the 11 mixes under investigation, this report deals in detail with two: 1) mix used for head tension - 2:4:4:1 lavender, sweet marjoram, eucalyptus and peppermint and 2) mix used for sinuses - 1:1:1:1 lavandin, basil, eucalyptus and peppermint. The standards of all oils used were of a high quality, and their profiles have not changed at all during one year storage. Both mixes also remained stable over that period and very little chemical reaction appears to have taken place on mixing the oils. This is shown by the expected Caddy Diagram for the mixes matching very closely to the actual GC analyses of the mixes. Caddy Diagrams, full chemical analysis of the oils (basil, bergamot, black pepper, chamomile, eucalyptus, juniper, lavandin, lavender, lemon, lemon grass, myrrh, oregano, peppermint, rosemary, sandalwood, sweet marjoram, tea tree) and the results of other mixes, are available on demand.

THE ESSENTIAL OIL COMPOSITION OF THREE *ABIES* SPECIES GROWING IN SOUTH BALKANS.

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The genus *Abies* belongs to the *Pinaceae* family and is represented by more than 50 species, found mainly in northern temperate regions. In South Europe and more specifically in the Balkan Peninsula only 2 recognized species are forming extensive natural forests: The common Silver Fir (*Abies alba* Miller), found in Spain, Poland and the Balkans and the Greek Fir (*Abies cephalonica* Loudon), native to the high mountains of Greece. Besides these two distinct species in the same area a third morphotype is found (*Abies borisii-regis* Mattf.) which is probably of a hybrid origin between *A.alba* and *A.cephalonica*. *A.borisii-regis* shows intermediate morphoanatomic characteristics between the putative parental species.

In order to check the phylogenetic relationship, as it is represented by their secondary metabolites, the leaf essential oil of a statistically representative sample of sympatric trees from the 3 taxa was obtained. The composition of the volatile metabolites was investigated by GC and GC-MS analyses.

Forty metabolites were identified and quantified on the basis of their retention indices and their mass spectra characteristics. The majority of the oils was found to be composed of monoterpenes: *A. alba* (81,43%); *A. cephalonica* (74,04%); *A. borisii-regis* (66,93%).

The major metabolites were the same for all studied species but their contribution varied, thus allowing the assignment of characteristic chemotypes based on their contribution rank.

The five major components were found to be a = β-pinene, b= camphene, c = fenchyl acetate, d = α-pinene and e = limonene.

Chemotype A (*A. alba*): a > b >c > d > e

Chemotype B (*A. cephalonica*): a > d > e > b > c

Chemotype C (*A. borisii-regis*): e >> a > b >d > c

Careful analysis of the overall terpenoid composition of the 3 taxa shows that they can be easily distinguished on the basis of their major volatile metabolites and that the composition of the *A. borisii-regis* is intermediate to that of the two parental species. This conforms the hybrid origin of *A. borisii-regis*.

P 5 - 2

THE ENANTIOMERIC COMPOSITION OF THE MONOTERPENE HYDROCARBONS OCCURRING IN THE NEEDLES OF JUNIPERUS COMMUNIS L. VAR. SAXATILIS PALL. (NORWEGIAN MOUNTAIN JUNIPER)

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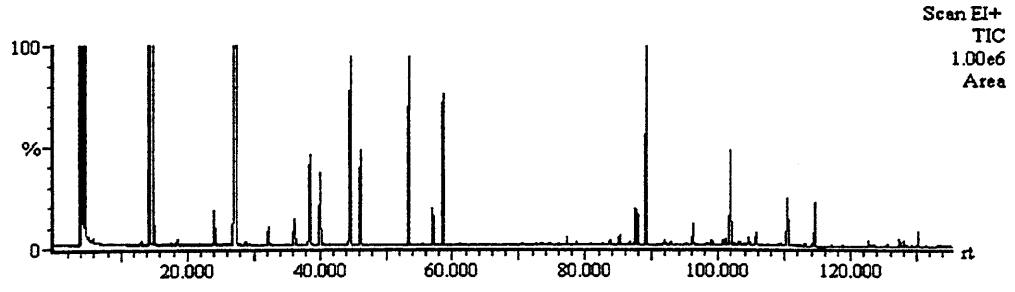
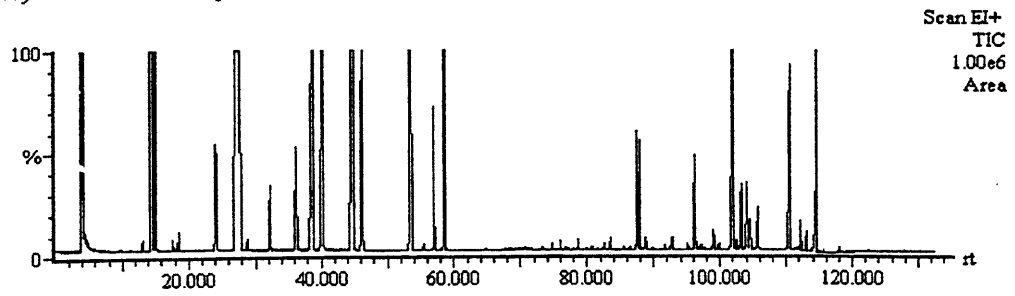
The essential oil of Juniperus communis L. has been the object of our studies on essential oil components for several years. The juniper oil is of interest due to the high concentration of volatile hydrocarbons in the plant material. By capillary gas chromatography we could indicate more than 300 components in the oil of which 55 in the fraction of the monoterpene hydrocarbons. However, the identity of the trace components is not easy to verify and we have been looking at the more common monoterpene hydrocarbons using partly capillary columns described by König et al. , partly coupled columns where one column contained an optical active stationary phase. The identity has been verified by several GC/MS systems,Kovats indices and retention indices. The plant material was collected in the Valdres area which is a typical district for mountain juniper in Norway. All samples were collected at 1200 m above sea level. The analysis reported here is of a typical oil sample obtained by hydrodistillation of needles. The main constituent is (-)-Sabinene which may account for more than 60% of the total oil. The enantiomeric separation showed the presence of 21 (23) monoterpene hydrocarbons. Some samples contained no (+/-)-alpha-Phellandrene. The variation throughout the year indicated little change in the monoterpene composition.p-Cymene was found in the freshly distilled oil and may be formed during prolonged distillation of the plant material. The essential oil of Juniper communis L. could be used as a standard mixture for hydrocarbon analysis.

Essential oil of Juniperus communis L.



Hydrocarbons of Juniperus communis L.



P 5 - 3

ENANTIOMERIC COMPOSITION OF MONOTERPENIC HYDROCARBONS IN VARIOUS *JUNIPERUS COMMUNIS* SPECIMENS

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Aside from our preliminary investigations concerning the chromatographic separation and direct chiral recognition of terpenic hydrocarbons in commercially available juniper oils (1), to our knowledge, more detailed studies have not yet appeared in the literature. It has been made clear that differences in the chiral composition of monoterpenes depend on the origin of raw material.

The present study concerns essential oils from *Juniperus communis* L. carefully harvested in learning if, and what extent, the place of growing, the season of harvesting and the morphological part of one plant species influences the composition of monoterpenes.

The samples of plant material were harvested and prepared under similar, controlled conditions. Surprisingly large variations in the monoterpenic composition were observed.

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P 5 - 4

COMPOSITION OF THE ESSENTIAL OIL OF BERRIES OF JUNIPERUS COMMUNIS L. OF CROATIAN ORIGIN.

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Using a combination of hydrodistillation of crushed berries (orgin Perusic, Lika, Croatia) and solid-liquid chromatography (Baker spe Silica columns, 3 ml), the terpene hydrocarbons (pentane) and the oxygen containing compounds (diethyl ether/pentane) were separated. Gas chromatography of the total oil shows ± 200 peaks. Based on gas chromatography on fused silica capillary columns: Supelcowax 10, 60 m, 0.25 mm ID, film 0,25 µm and DB·5, 30 m, 0.25 mm ID, film 0.25 µm; Kovats indices and GC/MS, monoterpene hydrocarbons, the oxygen containing monoterpenes and the sesquiterpene hydrocarbons were identified.

IDENTIFIED COMPOUNDS IN *JUNIPERUS COMMUNIS* L. (CROATIA)

1	TRICYCLENE	26	γ-ELEMENE
2	α-THUJENE	27	β-HUMULENE
3	α-PINENE	28	SANTALENE (EPI-α)
4	α-FENCHENE	29	α-HUMULENE
5	CAMPHENE	30	GERMACRENE D
6	THUJA-2,4(10)-DIENE	31	β-SELINENE
7	VERBENENE	32	(CIS-β)-GUAIENE
8	β-PINENE	33	VIRIDIFLORENE
9	MYRCENE	34	GERMACRENE A
10	δ·2-CARENE	35	γ-CADINENE
11	α-PHELLANDRENE	36	(TRANS) CALAMENE
12	δ-3-CARENE	37	α-CADINENE
13	α-TERPINENE	38	GERMACRENE B
14	*p*-CYMENE	39	CIS SABINENE HYDRATE
15	LIMONENE	40	TRANS SABINENE HYDRATE
16	β-PHELLANDRENE	41	(CIS-PARA) MENTH-2-EN-1-OL
17	(Z)-β-OCIMENE	42	(TRANS -PARA) MENTH-2-EN-1-OL
18	(E)-β-OCIMENE	43	BORNEOL
19	γ-TERPINENE	44	TERPINENE-4-OL
20	TERPINOLENE	45	(PARA) CYMEN-8-OL
21	MENTHATRIENE (1,3,8-PARA)	46	α-TERPINEOL
22	CYCLOSATIVENE	47	CIS PIPERITOL
23	α-COPAENE	48	TRANS PIPERITOL
24	β-BOURBONENE	49	(TRANS) CHRYSANTHENYL ACETATE
25	CYPERENE	50	METHYL CITRONELLATE
		51	BORNYL ACETATE

P 5 - 5

COMPARATIVE STUDY ON THE NEEDLE ESSENTIAL OILS OF *JUNIPERUS COMMUNIS* L. AND *J. COMMUNIS VAR. NANA* WILD. IN BULGARIA

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Juniperus communis L. is distributed in the mountain regions of Bulgaria from 500 to 1700 m above sea level (a.s.l.) and *J. communis var. nana* Wild. above 1700 to 2500 m a.s.l.

Young common juniper twigs were collected from different sites at 1200 m a.s.l. in Plana mountain and from *J. communis var. nana* at 1800 m a.s.l. Vitocha mountain. The main content of α and β-pinene, Δ^3 - carene, limonene, terpenene 4-ol and bornylacetate was assessed by gas and liquid chromatography (Perkin-Elmer 8200). The main components in both types of oils are α and β pinene. α - pinene prevailing in the oil of J. *communis var. nana* - 32,74 % (28,38 - 44,21 %) and in *J. communis* - 20,10 % (10,98-30,78 %). β -pinene displayed a reverse pattern - higher amounts in *J.communis* - 15, 12 % and lower in *J. communis var. nana* - 3,77 %. The quantities of Δ^3 carene, terpinene 4-ol and bornylacetate in *J. communis var. nana* are greater that in *J. communis* and the amount of limonene is higher in *J. communis*.

Juniper oils have the characteristic scent of a plant raw material, with a wellsensed fragrance of an oil terpene. With its specific aroma, juniper oil is of interest, as a substance to be used singly, to the parfume-and-cosmetics and pharmaceutic industries.

P 5 - 6

COMPOSITION OF THE ESSENTIAL OIL OF *JUNIPERUS PHOENICEA* L.

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Juniperus phoenicea L. grows spontaneously in Portugal, near the maritime zone, over sands and dunes or over carbonic and calcarean rocks.
The essential oils of collective and individual samples isolated by hydrodistillation from the twigs were obtained in the yield of 0.35 % to 0.9 %. The compositions evaluated by GC and GC/MS were dominated by the monoterpenic hydrocabons (60%-93%). The main constituents are α-pinene, β-felandrene, limonene and α-terpenyl acetate. Nevertheless in one sample of twenty six samples analysed the α-terpenyl acetate occurred in trace amounts. The variableness of the concentrations of the main compounds and the presence of particular constituents in some samples, namely the α-cedrol, indiciate chemovariability for this species.

P 5 - 7

THE ESSENTIAL OIL OF *AEGOPODIUM PODAGRARIA* L.*

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Aegopodium podagraria L. (Apiaceae), a perennial herb with subterranean runners is used in traditional European medicine against gout, rheumatism, abscesses and hemorrhoids. Apart from hot water extracts of the herb the application of a poultice of the squashed fresh herb is known. (1, 2)

Hitherto concerning the essential oil composition of *Aegopodium podagraria* a head space analysis of the volatiles released from the flowers has been reported (3). As part of a phytochemical survey of this medicinal plant we now present the results of our investigation of the essential oil which was obtained by hydrodistillation of the flowering herb.

By GC/MS analysis on different stationary phases 40 compounds could be identified using comparison with reference compounds, retention data references (4) and mass spectra library search. Aliphatic aldehyds and ketones were present in minor amounts (up to 0.69 %). In the monoterpene fraction limonene (9.4 %), γ-terpinene (6.08 %) and ß-myrcene (5.91 %) represented the main compounds. The essential oil of *Aegopodium podagraria* proved to be rich in sesquiterpenes (54 % of the total oil obtained by hydrodistillation of the fresh herb). *trans*-ß-Farnesene (3.05 %), ß-elemene (2.96 %), ß-caryophyllene (1.90 %), α-humulene (1.86 %) and spathulenol (1.68 %) could be identified among the main constituents of the sesquiterpene fraction. Due to pharmacological as well as GC/MS investigations of fractions of an aqueous extract the essential oil of *Aegopodium podagraria* does not seem to contribute to the antiphlogistic activity of this herb (5).

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* Dedicated to Univ.Prof.Dr. Th. Kartnig on occasion of his 65th birthday.

P 5 - 8

MONOTERPENE ENANTIOMERS OF ANGELICA ROOT AND SEED OIL

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Roots (2 samples) and seeds (32 samples) of *Angelica archangelica* L., Apiaceae, collected in north Finland from different localities, were extracted with hexane and analysed by GC-MS using a chiral stationary phase (heptakis (6-O-methyl-2,3-di-O-pentyl)-β-cyclodextrin; 70 % in OV 1701 w/w, 25 m x 0.25 mm i.d.). For comparison a root oil was hydrodistilled from a plant grown in Karila, south east Finland. The following enantiomeric pairs [(+)- and (-)-forms] were found: α-thujene, α-pinene, camphene, β-pinene, sabinene, 3-carene, limonene and β-phellandrene. The α-phellandrene enantiomers were not very well separated.

The root oil was characterized by (-)-α-pinene (19-42 %), (+)-sabinene (5-28 %), (+)-3-carene (tr-22 %) and (+)-β-phellandrene (tr-22 %). The studied root oils were all different from each other; the hydrodistilled oil was a (-)-α-pinene/(+)-β-phellandrene type, the other two were (+)-sabinene/(+)-3-carene and (-)-α-pinene/(+)-sabinene types.

The seed oils were clearly divided into two main groups according to the content of (+)-β-phellandrene. The first group had (+)-β-phellandrene as a main component (> 75 %) and small proportions of other components. The other group had smaller amounts of (+)-β-phellandrene, but the main component was either (+)-α-pinene, (+)-sabinene or myrcene. Two samples of 32 showed a higher content of (-)-α-pinene than of (+)-α-pinene. The (+)-limonene/(-)-limonene ratio was usually 1/1, except for samples from the most northern part of Finland, in which the (+)-form was a little more abundant.

It seems that the (+)-enantiomers are more common in the seed oil, while the root oil was dominated by (-)-enantiomers. (+)-3-Carene was not detected in the seed oil, although it was abundant in the root oil.

COMPOSITION OF THE ESSENTIAL OIL OF *CHAEROPHYLLUM AZORICUM* TREL., AN ENDEMIC SPECIES OF THE AZORES

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Chaerophyllum azoricum Trel. (Apiaceae) is an endemic species of the Azores that occurs, generally above an altitude of 700 m, on the islands S. Miguel, S. Jorge, Pico and Flores.[1,2] This species grows in wet and sheltered places, in very few localities, most of these on Flores.[2]

C. azoricum is highly threatened by extinction due to the cutting of protecting trees and shrubs.[2] The present work is part of a screening programme on the endemic aromatic flora of the Azores.

The essential oil was isolated, by hydrodistillation and distillation-extraction, from plants collected during the vegetative phase at Pico Morro Pelado, S. Jorge. The oil, obtained in a yield of 0.1% (v/w), was analysed by GC and GC-MS.

Twenty-seven components were identified amounting to 99% of the total oil. The monoterpene fraction (86.2%) was dominant, terpinolene (62.2%) and γ-terpinene (13.0%) being the main oil components. The oxygen-containing monoterpenes represented only 0.6% of the oil.

The sesquiterpene fraction, consisting only of hydrocarbons, occurred in low amount (7.0%), zingiberene (4.3%) being the main component. In addition to monoterpenes and sesquiterpenes, the oil contained a significant amount of myristicin (5.4%).

Acknowledgement - This study was partially granted by the Instituto de Biotecnologia e Química Fina (IBQF) - Centro de Biotecnologia Vegetal, Lisbon, and by the Junta Nacional de Investigação Científica e Tecnológica (JNICT) under research contract no PBIC/C/BIA/2070/95.

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P 5 - 10

ANALYSIS OF ESSENTIAL OIL OF *ACHILLEA SETACEA WALDST. ET KIT.*

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Many *Achillea* species growing in Turkey are used in folk medicine as diaphoretic, diuretic, emmenagogue and carminative. Essential oil was obtained from aerial parts of *A. setacea* and analysed by GC and GC/MS techniques. Major components in the oil were identified as camphor (10,2%), myrtenol (9,7%), 1,8-cineol (7,8%) and α-bisabolol (7,5%). Ist physicochemical characteristics were also determined.

P 5 - 11

A STUDY OF THE ESSENTIAL OIL OF *ARTEMISIA annua* L ADAPTED TO BRAZILIAN CLIMATE.

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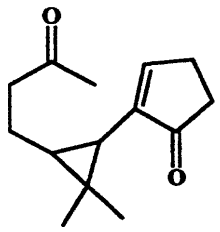
The Asteracea *Artemisia annua* L. native of the Asian continent is well known for the production of a potent antimalarial compound, artemisinine. The purpose of this work was to study some chemical aspects of the essential oil of the plant adapted to Brazilian climate.

The identification of the essential oil of *Artemisia annua* L. cultivated in Brazil was studied using GC/MS (HP 5890 /HP 5970). The essential oil was prepared by hydrodistillation of freshly picked leaves and flowers. The constituents were determined by association of retention time data and co-injection of authentic standarts. Owing to extensive work undertaken by CPQBA-UNICAMP acess to pure samples of qinghaosu acid was possible. From this compound Qinghaosu alkane, Qinghaosu epoxide, and Qinghaosu ketone were obtained by functional group interconversion. These sesquiterpenens were used to analyse the minor components of the essential oil of the plant adapted to Brazilian climate. Forty nine constituents were identified in the essential oil. A new carbon skeleton was suggested from our GC/MS data. The compound 2-cyclopenten-1-one, 2-[2.2-dimethyl-3-(3-butanone)cyclopropyl] **1** was obtained by Pauson-Khand synthesis which made it possible to confirm the presence of the *cis* and *trans* isomers of this compound in our oil.

The gas chromatography profile of *Artemisia annua* L. essential oil produced in Brazil compared with those of other origins revealed significant variation of their composition. The seasonal behavior of artemisinine content versus essential oil production was observed during one harvest period. The plant produced maximine of artemisinine content prior to flowering.The essential oil obtained from leaves had a major content of sesquiterpenes. After full bloom the plant produced an essential oil rich in monoterpenenes. The main constituent observed after flowering was camphor.



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P 5 - 12

PRELIMINARLY ANALYSIS OF ESSENTIAL OIL FROM SARDINIAN *HELICHRYSUM ITALICUM* G. DON SUBSPECIES *MICROPHYLLUM.*

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Several *Helichrysum* species (*Compositae*) are widely represented in the Mediterranean flora. The first data about *Helichrysum* oils were cited by Gildemeister (1), other notices were pubblished more recently (2), however, few data are available about the composition of the oil, because the published data are not complete and the influence of the cultivation was not investigated. Thus, we aimed to study the variation between the essential oil of *Helichrysum italicum* G. Dom ssp. *microphyllum* growing wild in Sardinia and the essential oil of the same plant cultivated in a site near to the wild station. Both stations were located in the north Sardinia. The cultivated samples were obtained from cuttings of the wild plants. Cultivation consisted of irrigating and and pruning. The volatile compounds from dryed inflorescences were extracted by hydrodistillation in a Clevinger apparatus using distilled water and a small amount of pentane. The isolated oil, containing a little amount of water, was extracted with ethyl ether and dried over anhydrous sodium sulphate. The yield in essential oil was comparable in the two considerated stations and reach 0.47% w/w that represent about the double with respect to the literature data (3). Analytical GC was carried out on a Hewlett Packard 5890 Series II Gas-Chromatograph connected with a Mass Detector HP 5971 A using a 30 m x 0.2 mm i.d. DB5MS capillary column. 21 constituents of total volatiles of *H. italicum* G. Dom ssp. *microphyllum* essential oil have been identified and quantified in a single GC capillary run. The quantitation was performed using the method of internal standard (2,6-dimethylphenol) and identification was performed by comparation with pure samples and by GC-mass spectrometry. The chromatograms of the essential oils of *H. italicum* G. Dom ssp. *microphyllum* wild showed a different composition in the major contituentes with respect to the essential oil obtained from the cultivated plant. In particularly differences in content of limonene, linalol, α-terpineol, nerol and neril acetate were observed. For instance, the percentage of nerol was significantly higher in the wild plants than in the cultivated one. On the contrary the cultived plant was reacher in neril acetate content.

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GERMACRENE D - A SOURCE OF RARE SESQUITERPENE HYDROCARBONES

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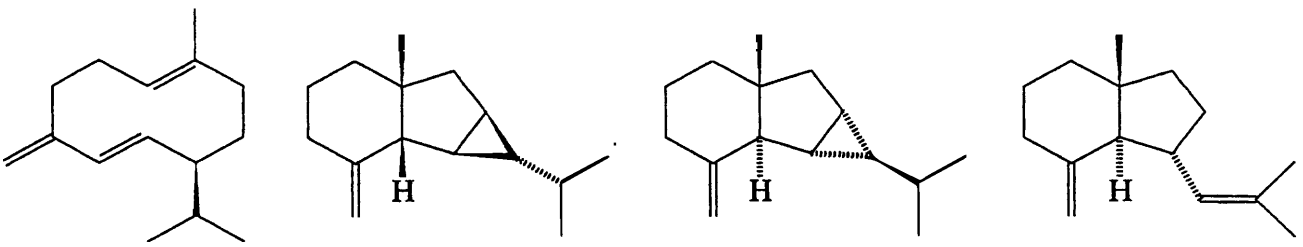
The ten-membered ring sesquiterpene germacrene D occurs in many plants. The more common (-)-germacrene D **1** was first isolated by Yoshihara *et al.* from *Pseudotsuga japonica* S. (1). Some sources of the (+)-enantiomer were mentioned (2) and both enantiomers were found in *Solidago altissima* L. (3).

To obtain germacrene D in a preparative scale we examined several plants of *Solidago canadensis* L. and *S. gigantea* L., collected at different locations in Hamburg and Schleswig-Holstein (northern Germany) in the summer of 1995. The steam distillates contained germacrene D as the main component in both enantiomeric forms while the enantiomeric composition differed. It is remarkable that in some samples only **(-)-1**, but in no sample enantiomerically pure **(+)-1** was present.

Germacrene D is known to isomerize by treatment with silica gel and UV-light to yield several hydrocarbons (1). We wanted to use germacrene D as a precursor for the preparation of various rare sesquiterpene reference compounds. The aim of our work was to rearrange germacrene D of known enantiomeric composition under acidic and thermal conditions and in presence of UV-light and to perform a detailed investigation of the products. After isomerization of germacrene D (purified by preparative gas chromatography) the resulting products were isolated by silica gel chromatography and subsequent preparative gas chromatography. Beside the expected rearrangement products new compounds (**2**, **3**, **4**) were identified by 2D-NMR techniques.



1 **2** **3** **4**

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P 5 - 14

The Essential Oil of *Tanacetum fruticulosum* Ledeb.

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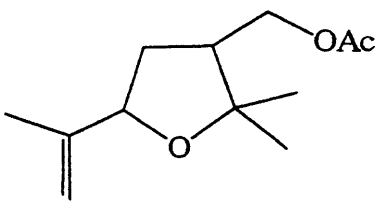
Tanacetum fruticulosum (Compositae) is a shrub growing in the hills and plains of several parts of Middle East and Central Asia. The essential oil of this plant, not examined previously, was prepared from air dried material collected near Hamedan, Iran.

The oil was separated by distillation and repeated flash chromatography. The isolated constituents were analyzed by a combination of RRI, GC-MS, ^{1}H- and ^{13}C-NMR spectra.

94 components representing about 95% of the oil could be identified. Typical for this oil are oxidized derivatives of lavandulol and nerolidol, mostly unknown, such as **1** and **2**.



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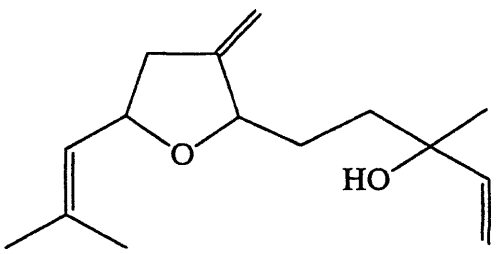

2

P 5 - 15

ESSENTIAL OIL FROM SARDINIAN *MELISSA OFFICINALIS* L. AND *MELISSA ROMANA* MILL.

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The essential oil of *Melissa officinalis* L. (*Labiatae*) leaves is of interest for pharmacology due to its sedative, antispasmodic, bacteriostatic, and antiviral activity (1). This perennial herb is commonly known as lemon balsam, owing to its citrus aroma due principally to the presence of geranial and neral (2). There are, however, other forms of this variable species, *Melissa romana* Mill. o ssp. *altissima* and ssp. *inodora*, as well as intermediate forms. *Melissa romana* Mill. is wild growing in Sardinia and *Melissa officinalis* L. has been naturalised and cultivated; in the present paper, we studied the steam-volatile leaf oil of these two species to compare their composition. Specimens of *M. officinalis* L. and *M. romana* Mill. were pick up in June 1995 in different stations located in the north Sardinia very close each other to minimise the climatic influences. The volatile compounds from fresh leaves harvested from the flowering plant were extracted by hydrodistillation in a Clevinger apparatus using distilled water and a small amount of pentane. The isolated oil, containing a little amount of water, was extracted with ethyl ether and dried over anhydrous sodium sulphate. As far as yield in essential oil for *M. romana* Mill. is concerned it was 0.035% and 0.012% (w/w) in two different wild stations and 0.044% in the cultivated one. The yield in essential oil for *M. officinalis* L. was 0.051% and 0.040% in the two naturalised examined stations. Analytical GC was carried out on a Perkin Elmer 4200 Gas-Chromatograph using a 30 m x 0.2 mm i.d. DB5 capillary column. The GC-MS analyses were carried out using an Ion-Trap instrument, operating in EI mode. Spectra were acquired each 0.5 s. The chromatograms of the essential oils of *M. officinalis* L. showed the prevalence of isomeric terpenes with molecular weight 152 and 154 and a low content in compounds with higher m.w. The more represented components were neral and geranial. On the contrary in the essential oils of *M. romana* Mill. we found a high concentration of terpenes with m.w. 204 having the typical mass spectra of cariophyllene, muurulen and cadinene. No evidence of neral and geranial was found in this essential oil. Since sometimes the plant smell of lemon such as *M. officinalis* L., work is in progress to investigate the composition of the essential oil during the year.

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P 5 - 16

COMPOSITION OF THE ESSENTIAL OILS OF SOME TURKISH NEPETA SPECIES

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Nepeta is a large genus of the family Labiatae, it comprises about 250 species. In Turkey, 33 *Nepeta* species are growing. Some species are important medicinal plants and used in folk medicine due to diuretic, bacteriostatic activities and to cure skin disorders of the eczema type.

In a continuation of our studies on *Nepeta* species, we have now studied four species growing in southearn Turkey, *Nepeta nuda* L.ssp. *albiflora* (Boiss) Gams, *N. italica* L. *N. cilicia* Boiss and *N. sulfuriflora* P.H. Davis by capillary GC and GC-MS analyses.

The essential oil of *N.nuda ssp. albiflora* is rich of nepetalactones consisting of 4aα,7α,7aα-nepetalactone (35.5 %) and 4aα,7α,7aß-nepetalactone (37.6 %) while the essential oil of *N. italica* and *N. sulfuriflora* carries 1,8 cineol as the main component, 80.8 % and 61.5, respectively. The main constituent of *N. cilicia* is formed of sesquiterpenes, mainly ß-caryophyllene (15.7%) and caryophyllene oxide (40%).

Since nepetalactones are responsible for the feline attractant or insect repellant properties, nepetalactones and the essential oils are being investigated for biological activities.

P 5 - 17

THE ESSENTIAL OIL COMPOSITION OF SOME *NEPETA* SPECIES OF TURKEY

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Water distilled essential oils from thirteen species of *Nepeta* growing in Turkey were analysed by GC and GC/MS. The following compounds were identified as major components in the essential oils of the following *Nepeta* species:
1,8-cineol: *N. sulfuriflora, N. nuda ssp. albiflora, N. nuda ssp. nuda, N. italica,*
geijerene: *N. nuda ssp. nuda,*
caryophyllene oxyde: *N. conferta, N. isaurica, N. cilicia, N, nuda ssp. glandulifera,*
ß-pinene: *N. phyllochlamys,*
α-terpineol: *N. viscida,*
linalool: *N. flavida,*
4aα, 7a, 7aα-nepetalactone: *N. cadmea, N. caesarea.*

P 5 - 18

The Essential Oils of Three New Labiatae Taxa from Turkey: *Origanum husnucan-baserii*, *Sideritis gülendamii* and *Salvia aytachii*

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Water-distilled essential oils from the aerial parts of three new endemic species of Turkey were analysed by GC and GC/MS. Seventy five components were characterized with borneol (20.23 %), α-terpineol (11.46 %) and *trans*-sabinene hydrate (10.97 %) as major constituents in the essential oil of *Origanum husnucan-baserii* H. Duman, Z. Aytaç et A. Duran (1). The essential oil of *Sideritis gülendamii* H. Duman et F. A. Karavelioğulları (1) were found to contain β-pinene (34.32 %) and α-pinene (13.21 %) as major constituents. Camphor (30.78 %) and 1,8-cineole (27.28 %) were identified as major constituents among fifty eight compounds characterized, in the essential oil of *Salvia aytachii* M. Vural et N. Adıgüzel (2).

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P 5 - 19

CHEMICAL ANALYSIS OF *SALVIA OFFICINALIS L.* PLANT AND EXTRACTS

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Garden sage (*Salvia officinalis L.*) of family *Labiatae L.* (*Lamiaceae Lindl.*) is a very important medicinal plant. Within this species there is a great variability of morphological features and chemical composition. Leaves and tops of sprouts are used in pharmacy as a drug. The subject of researches were dry leaves of plants cultivated in collection of Department of Medicinal Plants of Warsaw Agricultural University (Poland). The aim of this work was to identify essential oil composition in the leaves. The flavonoids and the tannins were identified in methanol, 70% ethanol and trichlorofluoromethane extracts from leaves and in leaves themselves. By gas chromatography were also analysed the main chemical compounds of essential oil and extracts, that represent from 50 up to 60% of the sum of all components.
On the ground of researches it was found that there is relatively similar content (quantitative and qualitative) of volatile compounds in essential oil and in examined 70% C_2H_5OH, CH_3OH and CCl_3F extracts. Carried in relation to stock, flavonoids and tannins balance in 70% C_2H_5OH, CH_3OH and CCl_3F extracts and remainders after extraction showed that these remainders are a good, complementary source of flavonoids and tannins. Remainders after distillation are also a good source of flavonoids and tannins - compounds of an important pharmacological activity.

P 5 - 20

STUDY ON THE ESSENTIAL OIL CONTENT AND COMPONENTS OF *SALVIA OFFICINALIS* L.

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In the period 1995-1996 in the Research Institute for Roses, Essential Oil and Medicinal Plants in Kazanlak, Bulgaria, an investigation on essential oil content and composition of *Salvia officinalis* L. was conducted. As experimental material we used different forms from *S. officinalis* population, characterised with differences in occurrence of blooming and differences in the colour of flowers.

Gas chromatographic analyses were performed on the essential oils obtained from different forms. There were established the content of α- thujene, β- pinene, cineole, β- caryophyllene, camphene, camphor and others. There was no great variation in the essential oil components in the oils from different forms. However, content of the main compound β- thujene was higher in the later blooming forms and in the forms with lilac colour of the flowers. Forms with white flowers had higher content of β- caryophyllene and β- pinene, while β- thujene was higher in the forms with pink flowers.

P 5 - 21

COMPARATIVE ANALYSIS OF *SALVIA OFFICINALIS* AND *S. TOMENTOSA* ESSENTIAL OILS

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The constituents of *Salvia officinalis L.* essential oil have been widely investigated during the last two
decades. However, there are only few records can be found about the ssential oil of *S. tomentosa Mill.* [1,2] as this species has a limited distribution in native flora mainly of East Europe and South-west Asia [3]. As a continuation of our earlier work [4], we have performed recently a GC-MS analysis of *S. officinalis* (I.) and *S. tomentosa* (II.) essential oils grown in Hungary and a *S. tomentosa* oil (III.) of Bulgarian origin. The results indicated a significant difference in ratio of mono- and sesquiterpene fractions with the three oils. I. and II. were found to be rich in monoterpenes, while III. contained a considerable amount of sesquiterpenes. Among the main components of I., II. and III. occur b-pinene (2.3%, 6.7%, 14.85%), 1,8-cineole (3.15%, 10.9%, 1.95%), campor (9.7%, 12.7%, 7.8%), borneol (8.7%, 8.9%, 9.7%), bornyl acetate (5.0%, 3.2%, 2.5%), b-caryophyllene (0.18%, 0.70%, 5.6%) and a-humulene (4.3%, 6.4%, 2.9%). The a-, b-thujone of the oils acceded 35.9% / 9.7% in I., 7.1% / 3.2% in II., and 0.5% / 046% in oil III. respectively. Although, the very low thujone values of III. measured are in good agreement with the data reported by Bayrak et al (1). Tsankova et al (2) reported the absence of these components. The relatively high thujone content of S. tomentosa grown in Hungary (II.) arouses questions about responsible factors,

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P 5 - 22

COMPARATIVE STUDIES OF THE ESSENTIAL OILS OF SOME SPECIES OF SECT. *SALVIA*

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Some European species of *Section Salvia* (*Lamiaceae*) like *Salvia officinalis L., S. lavandulifolia Vahl., S. tomentosa Mill., S. fruticosa Mill., S. candelabrum Boiss., S. ringens Sibth. et Sm.* have been grown in Hungary. As these species being Mediterranean ones, their cultivation under Hungarian climatic conditions, out of their native growing areas may be interesting from many respects, among others, of studying what kind of differences there are in their essential oil contents and composition, how these species can be cultivated in temperate belt, etc. This type of our comparative studies has been done on *Salvia* species for a long time- [2,3].

The plants were grown from seeds, available from various European botanical gardens in open air conditions at Vácrátót. The essential oils of the freshly harvested plants were obtained by traditional water steam distillation and the oil composition was analysed by GC, GC-MS instruments. The quantity of some non-volatile components such as rosmarinic, caffeic, ursolic/oleanolic acids was measured by densitometry.

Unlikely to the majority of *Salvia* species all the species provided essential oils in measurable quantity. Some of their main components identified and being present in all the oils are as follows: β-pinene (a), 1,8-cineole (b), α-, β-thujone (c/d), camphor (e), borneol (f), bornyl acetate (g), β-caryophyllene (h). They could be found in the following ratios (%):

Species	a	b	c / d	e	f	g	h
S. officinalis	2.3	3.45í	35.9 / 9.7	8.7	5.0	0.7	4.3
S. ringens	2.9	16.5	9.7 / 1.8	16.0	2.0	1.4	2.3
S. candelabrum	7.5	50.6	1.6 / 0.5	9.0	4.5	0.9	0.18
S. tomentosa	6.7	10.9	7.1 / 3.2	9.7	8.9	3.2	6.03
S. lavandulifolia	6.9	47.3	0.15 / 0.03	12.7	2.2	0.6	4.8
S. fruticosa	1.75	15.0	21.5 / 3.7	25.9	1.04	1.0	1.4

As the plants were gathered at the same time and from the same field the differences in the oil composition are seemingly genetically fixed ones. The thujon content shows the greatest differences in accordance with others observations.
The contents of non-volatile components do not differ significantly from the figures gained for other *Salvia* species.

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P 5 - 23

ANALYSIS OF THE ESSENTIAL OIL OF *SALVIA CARDIOPHYLLA*

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The essential oil of the leaves of *Salvia cardiophylla* from Paraguay was obtained by hydrodistillation using a Clevenger-type apparatus, giving a yield of 0.03% (v/w).

Analysis of the oil were carried out by GC and GC-MS using two fused silica capillary columns of different stationary phases (Supelcowax 10™ and methylsilicone SE-30). The identification of its constituents was performed by comparison of their retention indices in both stationary phases and their mass spectra with those of known compounds and those reported in the literature (1-3).

More than 85% of the total oil was identified. It was characterized by being mainly constituted by sesquiterpenes, which reached a percentage higher than 75%. Among them, ß-caryophyllene (23%), germacrene-D (14%), ß-caryophyllene oxide (10%), bicyclogermacrene (6%) and spathulenol (7%) were the major constituents. Monoterpenes were scarcely present in the essential oil of the leaves of *S. cardiophylla*, showing a percentage of *ca.* 1.5%. Palmitic acid was found in a percentage of 6%. No previous reports on the composition of the volatile oil of this species were found in the literature.

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P 5 - 24

COMPOSITION OF THE ESSENTIAL OIL OF *TEUCRIUM HAENSELERI* BOISS.

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The essential oil of *Teucrium haenseleri* was isolated by hydrodistillation and distillation-extration from the aerial parts collected during the vegetative phase and flowering period of the plant and analysed by GC and GC-MS.

The essential oil samples showed an orange-yellowish colour and possessed a strong odour. The oil isolated from the leaves collected during the vegetative phase was obtained in a higher yield (0.8%) than that isolated from the flowers (0.5%) and leaves (0.3%) collected during the flowering period.

The oil isolated from the flowers was a complex mixture, from which 46 compounds were identified, representing 92% of the total oil. In the oils isolated from the leaves collected during the flowering period and vegetative phase of the plant, 36 and 45 components were identified amounting to 94% and 90%, of the total oil respectively.

All the oils isolated from *T. haenseleri* consisted mainly of monoterpenes (83%-84%), where α-pinene (18%-23%) and β-pinene (24%-31%) were the major constituents. The sesquiterpene fraction was always lower than 12% of the total oil, and was dominated by δ-cadinene (3%-5%).

Conversely to what has been found with the essential oils of other *Teucrium* species, usually dominated by the sesquiterpene fraction,[1-7] the essential oils of *T. haenseleri* were dominated by the monoterpene fraction, α-pinene and β-pinene being the main components. The above compounds were found only in trace amounts in the essential oils of other *T.* species,[1-6] although in *T. heterophyllum* α-pinene was found as an important component (16%).[7]

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P 5 - 25

QUANTITY AND QUALITY OF ESSENTIAL OIL FROM *THYMUS SIBTHORPII* CV. KRESNA

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Thyme is a well-known and broadly applied medicinal and essential oil-bearing plant.

Essential oil from a new thyme cultivar "Kresna" bred in 1985 in the experimental field of the Institute of Botany has been investigated. Wild population of *Thymus sibthorpii* Benth. originating from the floristic region of the Strouma River Valley was used as initial material.

Preliminary studies on essential oil from "Kresna" were carried out only at full flowering stage. In the process of cultivation we have observed that the stage of full flowering does not commence simultaneously in all plants.

The aim of the present study was to trace the changes in quality and quantity of essential oil at different stages of flowering (budding, initial flowering, full flowering, end of flowering) and to set the most appropriate time for harvesting.

Quality and quantity of essential oil extracted from the above-ground mass harvested at different stages of flowering were studied from 1993 to 1994. The essential oil was obtained by hydrodistillation for 1 h using Clevenger-type apparatus. It varies from 1.67% (budding) to 2.08% (full flowering) (averaged data for the survey period). Seventy-one compounds were established and 15 of them were identified by GC. The content of the main component, carvacrol, varies from 63.87% (budding) to 74.81% (end of flowering).

The most appropriate time for harvesting is when the plants at full flowering stage account for more than 50% and those at the end of flowering for about 15%. At this time the yield of the above-ground mass is high which ensures higher yield of essential oil.

The author gratefully acknowledges financial support from NFRI.

P 5 - 26

COMPOSITION AND INFRASPECIFIC VARIABILITY OF THE ESSENTIAL OIL OF *THYMUS VILLOSUS* SUBSP. *LUSITANICUS*

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Thymus villosus L. subsp. *lusitanicus* (Boiss) Coutinho is an Iberian endemic taxon, which belongs to the section *Pseudothymbra* of the genus *Thymus* (Lamiaceae).
The composition of the essential oils of four populations of different localities from Portugal was investigated by GC and GC-MS using two fused silica capillary columns of different stationary phases.
In total 86 compounds were identified, meaning a percentage of the essential oils ranging from 95% to 97%. Important quantitative differences on the major constituents of the populations were found, indicating the existence of infraspecific variability.
In order to investigate the chemical polymorphism, the essential oil of individual plants of *Thymus villosus* subsp. *lusitanicus* was analyzed mainly by GC, and, when necessary, by GC-MS. The results were submitted to a multivariate analysis (Principal Component Analysis and Cluster Analysis). Two main types of essential oils were found: linalool and geraniol/geranyl acetate, although some subgroups in both types can be appreciated.

P 5 - 27

THE ESSENTIAL OIL COMPOSITION OF THYMUS SPECIES FROM ETHIOPIA.

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The essential oils of two Thymus species growing in Ethiopia were investigated. Using a combination of hydrodistillation and solid-liquid chromatography (Baker spe Silica columns, 3 ml), the terpene hydrocarbons (pentane) and the oxygen containing compounds (diethyl ether/pentane) were separated. Based on gas chromatography on fused silica capillary columns: Supelcowax 10, 60 m, 0.25 mm ID, films 0,25 µm and DB · 5, 30 m, 0.25 mm ID, film 0.25 µm, Kovats indices and GC/MS, a number of the compounds present were identified. Of special interest were the quantitative differences of thymol/carvacrol in the species - also dependent on the habitat (T. schimperi).

		thymol	carvacrol
Thymus schimperi	(Debra Berhan)	+++	+
Thymus schimperi	(Bale)	+	+++
Thymus serrulatus	(Tigray)	++	+

P 5 - 28

VARIABILITY OF THE ESSENTIAL OILS OF *LIPPIA GRAVEOLENS* HBK FROM GUATEMALA

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Several species are used as Oregano, of which the most important ones are
„Greek Oregano" : *Origanum vulgare* ssp. hirtum (*Labiatae*)
„Turkish Oregano" : *Origanum onites* (*Labiatae*)
„Spanish Oregano" : *Thymus capitatus* (*Labiatae*)
„Mexican Oregano": *Lippia graveolens* (*Verbenaceae*).
Strong variations have been observed in the concentracion and composition of the essential oil not only between but also within the different species dependent on their origin and genotype. Main components are, however, thymol and carvacrol.

In the frame of a domestication project of indigenous medicinal and aromatic plants of Guatemala *Lippia graveolens* was successfully introduced into cultivation. Wild plants of different areas of the country have been collected as starting material. Mostly homogeneous cuttings were grown in same conditions at a experimental station of ICTA (Instituto de Ciencia y Tecnología Agricola). The essential oil obtained from the dried leaves was analysed to find the differences in the yield and the content of the main chemical compounds as well as the minor compounds. The content of the essential oil varied between 1,4%-3,9%(m/m). Significant differences existed also in the composition of the oils. It could be distinguished between a carvacrol type with a content of 40,7% carvacrol, a thymol type with 56,6%, 66,5% and 80,3% thymol and a mixed type without a main component.

P 5 - 29

COMPOSITION OF THE ESSENTIAL OILS OF SOME *PIPER* USED IN TRADITIONAL MEDICINE IN S.TOMÉ AND PRÍNCIPE

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The Republic of S. Tomé and Príncipe, located in West Africa's Gulf of Guinea some 180 miles off the coast of Gabon, is one of the smallest states in the world.

In S. Tomé, like in many other developing countries, there is a treasure of traditional medicine and traditions concerning naturally occurring drugs, based on the empirical knowledge of medicinal and toxic plants, gained by the ancestors and passed on from generation to generation by oral tradition.

Data on about 350 folk medicinal plants were collected from different localities of S. Tomé and Príncipe, during an ethnobotanical survey that was conducted among 1993 - 1995. The information on medicinal uses of plants is based on first hand information collected by personnel contact with traditional healers.

The essential oil-bearing plants are among the plants that are widely used in this country. They are utilized in different forms, such as whole herbs, powders, extracts and vapours, for a variety of purposes.

The aim of the present paper is to present some of the results already achieved in the study of the aromatic medicinal plants of S. Tomé. The essential oils of some Piperaceae - *Piper capense* L., *Piper umbellatum* L. and *Piper nigrum* L. - were obtained by hydrodistillation of the aerial parts (*P. capense* and *P. umbellatum*) and fruits (*P. nigrum*). They were analysed by GC and GC-MS, using two fused silica capillary columns of different stationary phases, and by ^{13}C NMR.

Monoterpene hydrocarbons were the main group of constituents in all samples. ß-pinene (31%) and trans-caryophyllene (12.9%) were the major compounds in the volatile oil of *Piper capense*. The essential oil of *Piper umbellatum* was characterised by its high ß-pinene (26.2%), α-pinene (17.6%) and trans-nerolidol (11.9%) content. The most important constituents in the essential oil of *Piper nigrum* were limonene (18.9%), *trans*- caryophyllene (16.5%), sabinene (15.8%) and ß-pinene (10.3%).

THE ESSENTIAL OIL COMPOSITION OF LEAVES AND FRUITS OF *HEDYOSMUM MEXICANUM*

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As a part of a program of research on the chemical composition of the essential oils of aromatic flora from Costa Rica, we report here the results obtained in the analysis of the volatile oils of leaves and fruits from *Hedyosmum mexicanum* Cordemoy (Chlorantaceae). To our knowledge, no information on the composition of the essential oils from *Hedyosmum* sp. has been previously reported in the literature.
Both essential oils of leaves and fruits were obtained by hydrodistillation and were analyzed by GC and GC-MS using two fused silica capillary columns of different stationary phases (Supelcowax 10™ and SE-30). Identification of the components was carried out in the basis of their retention indices on the two stationary phases and their mass spectra (1,2,3). GC-IR was performed to improve the identification.
In total, 36 constituents were identified, meaning more than 75 % of the total oil in both samples. The essential oils from leaves and fruits showed a high amount of monoterpene hydrocarbons, especially sabinene (27,97 % and 24,62 %, respectively). Among sesquiterpenes, a furane derivative, which was tentatively identified from its IR and MS spectra, was found in a high percentage in both samples. This kind of compound had been previously reported in the Chlorantaceae (4). The co-occurrence of germacrane derivatives (isomers B and D) in both oils was also detected.

Acknowledgements: Financial support from the Ministerio de Educación y Ciencia (Spain) inside the Iberoamerican Cooperation Program is gratefully acknowledged.

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Indole derivatives in bark essential oil of Anonidium manni (Oliv.) Engl. and Diels (Annonaceae) from Gabon

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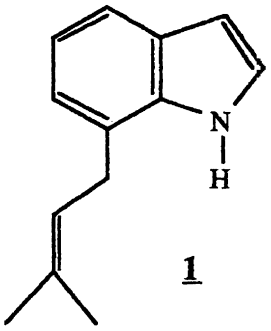
The barks of *Anonidium mannii* give essential oil mainly constituted by sesquiterpens and volatile alkaloids.

Two samples of different geographic origins (Bitam and Libreville) have been obtained in 0.10 and 0.13 % yields respectively. The analyses (GC and GC/MS) show that the same constituents are present in both oils, but in widely different amounts.

Two alkaloids (M=185) have been isolated by column chromatography and identified by ^{1}H and ^{13}C NMR, as 7- and 3-isopentenylindole (compounds 1 and 2 below)



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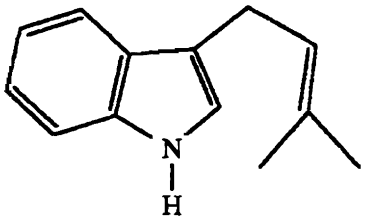

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1 has already been found by Achenbach and Renner (1) in the alcoolic extracts of the bark of *Anonidium mannii*; 2 in those of *Monodora tenuifolia* (2).

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P 5 - 32

Monodora myristica seeds - Analytical and sensory investigation of the essential oil of calabash nutmeg of different origin

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Calabash nutmegs are the seeds of the tree *Monodora myristica (Gaertn.)* Dun. of the *Annonaceae* family, originating from and cultivated in West-Africa. The bean-like seeds, 10-30 mm long, 8-15 mm wide, 6-12 mm thick, have a glossy, smooth to slightly wrinkled, light to strongly brown surface.

The round seeds are used as savory food additive, but also for the preparation of stimulants, drugs (e.g. as a stomatic or to relieve constipation) and disinfectants. Further, rosaries and necklaces are made from the seeds.

Smell and taste of the seeds and the essential oil are stated differently. They are described as pleasant, savory, slightly citric or thymol-like, on the one hand, or as nutmeg-like, on the other hand, which explains the terms "African nutmeg", "Calabash nutmeg" and "False nutmeg".

In literature, the content of essential oil is given within the range from 3 % to 6 %. The content of fatty oils amounts to approx. 25 %. The essential oil consists of more than 75 % monoterpene hydrocarbons. The major constituents are stated differently which led Onyenekwe *et al.* [1993] to suppose not only habitat-related differences, but also the existence of more than one variety of the plant, in Nigeria. Ekundaye *et al.* [1988] have described the components p-cymene and linalool as major constituents, while Onyenekwe has analyzed the monoterpene hydrocarbons α-phellandrene (50 %), p-cymene (8.5 %) and α-pinene (5 %) as major constituents. Lamaty *et* al. [1987] report a content of 48 % α-phellandrene. Also a few sesquiterpene hydrocarbons and oxygenated compounds, such as germacrene-D-4-ol and cardinenes, are further constituents.

Sabinene and α-pinene, the major constituents of the essential oil of nutmeg (*Myristica fragrans),* are not contained in the essential oil of calabash nutmeg, nor safrole and eugenol.
The hallucinogen myristicine, too, is detected only in *Myristica fragrans.*

The essential oils of *Monodora myristica* seeds of four different habitats were obtained by steam distillation with yields between 3.4 % and 4.8 %. The composition of the oil was analyzed by gas chromatography/mass spectrometry. The contents of α-phellandrene/p-cymene amounted between < 1 %/57 % and 45 %/20 %.

The ground seeds and essential oils also were evaluated sensorily. The samples had an intensive, savory and strongly terpenic flavour.

The conception that by application of *Monodora myristica* seeds the typical nutmeg flavour will be created, is not supported.

P 5 - 33

VOLATILES OF THE ESSENTIAL OILS OF THE LEAVES AND ROOT BARK OF *UVARIA NARUM* WALL. (ANNONACEAE)

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The essential oil of *Uvaria narum* Wall. (Annonaceae) leaves was analyzed by GC, GC/MS, GC/FTIR/MS and GC-sniffing techniques. The sample found to be rich in beta-caryophyllene (9.99%), benzoic acid (9.75%), benzyl benzoate (6.23%), alpha-bulnesene (5.74%), beta-elemene (5.61%), alpha-copaene (5.39%), 3-hexenoic acid (4.46%) and germacrene-D (4.26%). Further more sixty compounds, mainly sesquiterpenes (ca. 60% incl. main compounds), aromatic components (ca. 20% incl. main compounds), hexane derivatives (ca. 10% incl. main compounds) and monoterpenes (ca. 8% incl. main compounds), were also identified to be of olfactoric importance for the characteristic dry-fruity, damascone and osmanthus-like odour of this natural product.

In the same way the essential oil of the *Uvaria narum* boot bark was analyzed. Beside dominating well-known mono- (e.g. borneol and ist derivatives) and sesquiterpenes (e.g. patchoulenone and elemenes) further more than 70 compounds, partly unknown as genuine constitutents of this folk medicine used root bark oil, were identified. The compounds and their olfactoric impressions will be also discussed in this presentation.

P 5 - 34

VOLATILE COMPONENTS OF CHINESE CRUDE DRUGS, *DIOSCOREA JAPONICA*

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Batatatis Rhizoma ("Sanyaku" in japanese) is the root of *Dioscorea japonica* (Dioscoreaceae) and has been traditionally used in medicine for treatment of diarrhea, asthma, polyuria, and diabetes. In our previous reports, we found (+)-β-eudesmol and paeonol as antimutagenic compounds from *D. japonica* (1). The volatile components of several chinese crude drugs have been investigated in our flavor chemical research (2-13). However, no study of the components of the volatile oils, and odour has yet been reported for this chinese crude drug. In this study, the composition of the volatile oil from the root of *D. japonica* was investigated by GC and GC-MS. The volatile constituents of *D. japonica* were mainly contained fatty acids (90.2%). These fatty acids were palmitic acid (41.00%), linoleic acid (28.76%), pentadecanoic acid (4.90%), 9-hexadecenoic acid (4.47%), linolenic acid (3.02%), oleic acid (3.03%), tetradecanoic acid (0.48%), and dodecanoic acid (0.11%). Terpenoids were 1.3%, which were hinesol (0.49%), farnesol (0.22%), β-eudesmol (0.16%), ar-curcumene (0.08%), menthol (0.07%), β-bisabolene (0.05%), elemol (0.03%), endo-borneol (0.03%), and δ-cadinene (0.03%).

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P 5 - 35

FLAVOUR COMPONENTS OF JAPANESE TRADITIONAL FOOD, NEWLY TREATED CURL "NORI"(*PORHYLA YEZOENSIS* F. *NARAWAENSIS*).

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The volatile oil from roasted curl "Nori"(*Porhyla yezoensis* f.*narawaensis*) was obtained by steam distllation. More than 70 kind of flavor components from the oil were identified by capillary GC, GC-MS. The main consistuts were palmitic acid, phytol, (1Z,3Z)-cyclooctaziene, heptadecene, tetradecanoic acid, β-ionone, 6-undecanone, α-ionone, dihydroactinidiolide and (9Z, 12Z)-octadecadienoic acid. These components were compared to dried and roasted sheet "Nori".(1, 2)

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The Chemical Constituents of Chinese Traditional Medicine *Chrysanthemum morifolium* Ramat

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The flower of *Chrysanthemum morifolium* Ramat(Compositae), known as "Hang Bai Ju", are used as antipyretic, antidote, antibacteria, and improvement of visual efficiency in Chinese traditional medicine(1). Its decoction is also used for treatment of coronary heart disease. The chrysanthemum health drink is very popular in China and said to be beneficial for hypertension and atherosclerosis.

In this study, we isolated 16β,22α-dihydroxypseudotaraxasterol-3β-O-palmitate **1**, lup-16β-hydroxypseudotaraxasterol-3β-O-palmitate **2**, 16β-hydroxypseudotaraxasterol-3β-O-palmitate **3**, pseudotaraxasterol **4**, taraxasterol **5**, β-sitosterol **6**, octacosanol **7**, hexacosanic acid **8**, palmitic acid **9**, pratentsein **10**, apigenin **11**, luteolin **12**, acacetin **13**, dacosterol **14**, apigenin-7-O-β-D-glucopyranoside **15**, luteolin-7-O-β-D-glucopyranoside **16**, pratentsein-7-O-β-D-glucopyranosyl(6→1)-α-L-rhamnopyranoside **17**, acacetin-7-O-β-D-glucopyranosyl(6→1)-α-L-rhamnopyranoside **18**, chlorogenic acid **19**, quinic acid 4-O-caffeate **20**, quinic acid 3,4-di-O-caffeate **21**, quinic acid 3,5-di-O-caffeate **22**, butyl caffeate **23**, ethyl caffeate **24**, n-pentyl-β-D-fructofuranoside **25**, β-dictyopterol **26**, chrysanthediol A **27**, diacetyl chrysanthediol B **28**, and diacetyl chrysanthediol C **29**. The structures of new compounds, **1**, **2**, **25**, **27**, **28**, and **29** were elucidated by chemical and spectroscopicmethods, in particular 2D NMR techniques. The ethy, butyl caffeate, and other caffeate derivatives may be the active principles of 5-lipoxygenase antagonists.

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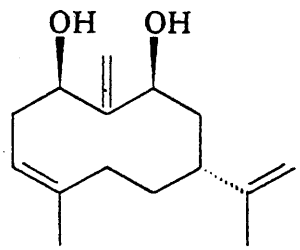

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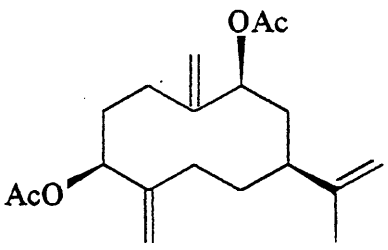

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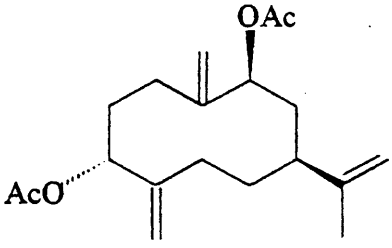

29

P 5 - 37

Diversity of Plants Producing Essential Oils in Thailand

by

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Abstract

Thailand is enriched with a diversity of tropical plant species, many of which produce essential oils. The plant structures bearing such oils range from roots, stems, barks, leaves and reproductive structures such as flowers and fruits. This paper will report a diversity of these aromatic plants distributed among many plant families throughout the country.

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1. This study is supported by aresearch grant from KURDI (K.I.P.1.2.35)
2. To be presented at the 27th International Symposium of Essential oils held during September 8-11, 1996 at the Centre of Pharmacy, University of Vienna, Austria

Distillation of Essential Oils - A Kinetic Study

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The merits and demerits of hydro- and steam-distillation system have been discussed. The biomass and water ration in the hydro-distillation system being an important and critical parameter has been optimised and found to be 1:7. A mathematical model representing the kinetics of the process has been proposed and verified on a few aromatic biomass samples. The experimental results obtained fully corroborate the validity of model proposed.

$$I = I_o * [1 - e^{-kt}]$$

The testing and analysis of the oils has also been carried out as per Indian Standards and results compared with that of commercially produced oils.

SORPTION PROCESSES AT THE EXTRACTION OF *LAVANDULA VERA D. C.*

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It was proved that the extraction of lavender is accompanied by a sorption of aromatic substances from miscella to the stems of the raw material. It is due to the uneven distribution of the extractable substances in the different parts of the lavender racemes.

The extraction of lavender carried out by a closed periodical process should be repeated 2 or 3 times beginning with a short first extraction to cause the process to run at high rate and diffusion coefficients and to reduce the influence of the sorption phenomena.

ON THE EXTRACTION OF *ROSA DAMASCENA Miller*

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The equilibrium state of the extraction of fresh rose flowers was studied. The continuance of the single extraction was from 5 min, to 24 hours. Hexan was used as a solvent.

It was found out that the basic amount of concrete was obtained during the first 5 min - more than 50% as compared to the yield for the 24 - hour extraction. The equilibrium state of this process was not reached even after 24 hours.

The yield of concrete influenced by the duration and number of single extraction was also studied. Hexan and miscella were used as solvents.

Biotransformation of Terpenoids and Related Compounds by Microorganisms - Production of Biologically Active Substances -

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We are continuing to investigate the microbiological introduction of oxygen functional groups for terpenoids and related compounds because many biologically active compounds possess hydroxyl, carbonyl, carboxylic and epoxide groups.

We have obtained carvone-8,9-epoxides(**1**), dihydrobottrospicatol(**2**), bottrospicatols(**3**), *p*-menthane-3,8-diols(**4**), 5-hydroxycarvone, 5-hydroxy-(+)-neodihydrocarveol, 5-hydroxydihydrocarvone, 8 kinds of 8-hydroxydihydrocarveols, 10-hydroxydihydrocarveol and 8,9-dihydroxydihydrocarveol, dihydrocarvone-8,9-epoxide and dihydrocarveol-8,9-epoxide, 2α-, 3α- and 3β-hydroxy-1,8-cineoles(**5**) and isopiperitenone (**6**) in the biotransformation of carvone, 1,8-cineole(**7**), menthol and related compounds. Compounds **1-4**, **11**, **12**, dihydro-4-oxoisophorone obtained from 4-oxoisophorone, β-resorcyl aldehyde and 3-nitrosalicyl aldehyde exhibited the strong inhibitory activity for the germination of lettuce seeds at the concentration of 200 ppm. The formation of compounds **5** and **6** from **7**(*Eucalyptus*) and limonene(*Citrus*) as a biomass, respectively, is very important on the view point of the formation of the precurser of **4**, which is known as mosquito repellents and allelochemicals. Aromatic aldehydes such as vanillin and ethylvanillin are easily convertible to the coressponding alcohols by various microorganims. However, vanillin, ethylvanillin, genticin aldehyde and protocatechu aldehyde showed the strong inhibitory activity for the root's elongation of lettuce and rice at the concentration of 200ppm. The metabolites such as from isopterocarpolone(**8**, 2-oxo-α-(-)-eudesmol, a constituent of *Pterocarpus santalinus* heartwood) from α-(-)-eudesmol(*Porella stephamina*) by *Asp.niger* and *Asp. cellulosae M-77* and 12-hydroxy-(-)-cyclocolorenone(**9**) and 6β-hydroxy-4,11-guaiadien-3-one(**10**) from (-)- (**11**, prepared from*Solidago altissima*)and (+)-cyclocolorenone(**12**, prepared from*Plagiochila sciophila*), respectively, are expected to have more biologically activity.

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P 6 - 5

ENANTIOSELECTIVE CYCLIZATION OF (±)-LAVANDUOL TO (-)-(2*S*,4*S*)-1,5-EPOXY-5-METHYL-2-(1-METHYLETHENYL)-4-HEXANOL BY *GLOMERELLA CINGULATA*

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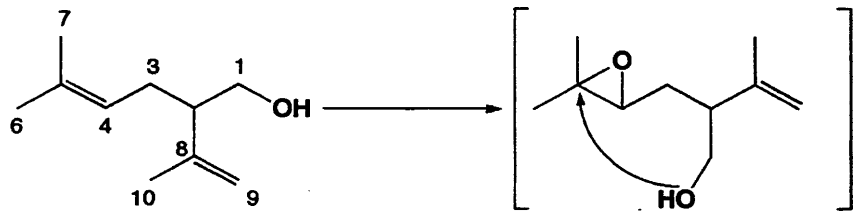
Lavandulol [5-methyl-2-(1-methylethenyl)-4-hexen-1-ol] (**1**), an acyclic monoterpene alcohol, occurs in lavender oil. Its acetate is widely used in fragrances. So far, the biotrans-formation of **1** has not been reported. As part of our continuing program to investigate the microbial transformations of acyclic terpenes (1-4).

The microbial transformation of (±)-lavandulol using the plant patogenic fungus, *Glomerella cingulata* as a biocatalyst was investigated. One of the metabolites is determined to be (-)-(2*S*,4*S*)-1,5-epoxy-5-methyl-2-(1-methylethenyl)-4-hexanol (**2**), which is enantiomeric pure (100% e.e.).



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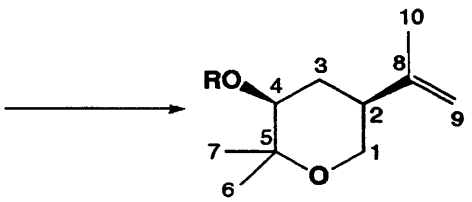

R=H; **2**

R=Ac; **3**

Possible metabolic pathway of (±)-lavandulol (**1**) to (-)-(2*S*,4*S*)-1,5-epoxy-5-mehtyl-2-(1-methylethenyl)-4-hexanol (**2**) by *G. cingulata*.

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P 6 - 6

STORAGE STABILITY OF HERBAL DRUGS CONTAINING ESSENTIAL OIL

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According to the German Law every drug has to be effective, safe and of good pharmaceutical quality. Judgement of the quality of herbal drugs containing essential oil presents special problems due to the fact of uncertain storage stability.

Investigations were carried out in order to define shelflife times for some of the most common herbal drugs contatining essential oils.

Following plant materials (DAB 10 quality) were included into the test panel:
Absinhii herba, Anisi fructus, Carvi fructus, Foeniculi fructus, Juniperi fructus, Salviae folium, Valerianae radix, Curcumae xanthorrhizae rhizoma.

In relation to the specific characteristics of each material, various cut sizes of each drug were prepared. These materials were stored under conditions according to the guideline 'Stability Testing of New Drug Substances and Products'[1]. Three alternative packaging materials commonly used in pharmaceutical practice were chosen. At defined time intervals, samples were taken and examined. Essential oil content of the samples was determined by German Pharmacopoeia 10 steam distillation method. The extracted essential oils were examined by gas liquid chromatography. As a result considerable changings of quantitative and qualitative composition (fingerprint!) were observed.

As a consequence, we suggest drug specific defined indications for shelflife in dependence on the cut size. These should be considered as the basis for further practical utilization, i.e. Pharmacopoeiae and 'Standardzulassungen'.

1. Feiden, Arzneimittelprüfrichtlinien (5.Erg.lfg. 1994)

Acknowledgement: This investigation was supported by a grant from PhytoLab GmbH & Co.KG, Vestenbergsreuth and BfArM, Berlin.

P 6 - 7

QUALITATIVE-QUANTITATIVE CHARACTERISTICS OF THE AUTOCHTONOUS POPULATIONS OF CHAMOMILE ON THE EAST-SLOVAKIAN LOWLAND

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Chamomile, *Chamomilla recutita* (L.) Rauschert, belongs to the most popular medicinal plants. Composition of its essential oil influences on its medical quality.

This contribution is aimed at study of essential oil content and its composition variability of autochtonous chamomile populations on the East-Slovakian Lowland.

Plant material (chamomile anthodia) was harvested from the natural locations. The main components of chamomile essential oil were determined by gas chromatography. A gas chromatograph HEWLETT-PACKARD 5890 Series II was used for these analyses.

Chamomile, *Chamomilla recutita* (L.) Rauschert, is occured mainly in the secondary plant communities on the East-Slovakian Lowland such as trodden societes of arid and mois soil, weed societes and dump societes.

Chamomile stands are cultivated as the diploid or tetraploid varieties in large-scale production in Slovakia, too.

The results confirmed that there is a bisabololoxide chemotype of chamomile in the region of the East-Slovakian Lowland.

Chamomile flowers from the natural societes are harvested by hand by Gypsy people. Unfortunately, harvested flowers have poor quality with the high content of bisabololoxides, uncomparable with a world standard. Not enough equipped and educated drug distributors sell poor quality chamomile dry material on the domestic market. It is unsaleable on the markets abroad.

P 7 - 1

ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF PLANT ESSENTIAL OILS.

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The antimicrobial activity of essential oils, complex mixtures of organic volatile compound, has been known for more than fifty years. The increase of their use in food, cosmetic and pharmaceutical industries requires wider studies on their antimicrobial and antioxidant properties. We investigated the properties of twenty essential oils some from aromatic plants typical of the Italian and Mediterranean flora and some from species originated in tropical countries. The study evaluated the MICs (minimal inhibitory concentrations) against 47 strains as well as the antioxidant properties. The bacteria were chosen for their diversity and belong to different species of the genera *Bacillus*, *Streptococcus*, *Lactobacillus*, *Clostridium*, *Bifidobacterium*, *Erwinia*, *Xanthomonas*, *Pseudomonas* and *Agrobacterium*. In addition seven genera of yeast were tested. The oils were obtained by steam distillation of plant material in Clevenger-type apparatus. MICs were defined in the range 200 ppm - 2000 ppm with intervals of 200 ppm. Antioxidant analysis were performed in agar plate added with linoleic acid and ß-carotene. Filters (6mm) soaked with essential oils (18 µl) were put on the agar plate and after 4h at 45°C the zone of color retention was measured.
Oils from cinnamon, clove, oregano and Spanish oregano, winter savory, thyme and geranium were the most inhibitory to the growth of all bacteria. Basil, French tarragon, peppermint, sage, juniper, boldo and rosemary oils possess antibacterial properties with different degrees of effectiveness: oils from cypress, bitter and sweet fennel were not inhibitory to all the tested bacteria. All the oils were inhibitory to at least one yeast. Cinnamon, Spanish oregano, juniper, winter savory, thyme and geranium oils were the most active while sweet fennel was the least effective. Spanish oregano oil has the greatest antioxidant activity. Oils from clove, thyme and oregano were also very active. On the other hand basil, rosemary, French tarragon, sweet and bitter fennel, cypress, lavender "Abrialis" and "Grosso" did not have antioxidant activity.

P 7 - 2

VOLATILE COMPONENTS AND ANTIOXIDANT PROPERTIES OF *MYRISTICA FRAGRANS* H.

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The chemical compositions and antioxidant properties of volatile extracts from nutmeg (the grayish-brown kernel of *Myristica fragrans* Houtt) by various isolation methods, including steam-distillation (SD), simultaneous steam distillation-solvent extraction (SDSE), headspace adsorption (HSA) and supercritical fluid extraction (SFE) using carbon dioxide under different pressure conditions, were investigated in this study.

Twenty-six major components of the volatile extracts were identified by GC and GC/MS. The yield of the major components by different isolation methods was with the order of SFE4000psi > SDSE > SFE3000psi > SD > SFE2000psi > HSA. The percentage yield of myristicin which is one of the major components responsible for the nutmeg flavor was 45.8%, 39.8%, 36.5%, 17.2%, 4.8% and 1.3% for SFE2000psi, SFE4000psi, SFE3000psi, SD, SDSE and HSA, respectively. The antioxidant properties of the volatile extracts, including SFE and SD extracts, were analyzed by the ferric thiocyanate method and compared with butylated hydroxyanisole (BHA) and α-tocopherol. Relative antioxidant activity of these extracts was BHA > α-tocopherol > SFE3000psi > SD > SFE4000psi > SFE2000psi. The content of the phenolic components which are the main antioxidant components in the volatile extracts of nutmeg was in the order of SFE2000psi > SFE4000psi > SFE3000psi > SD. It seemed that the antioxidant effect of volatile extracts was not only from the phenolic content.

THE IMPACT OF *LEVISTICUM OFFICINALIS* L. (LOVAGE) VOLATILE OIL ON MAMMALIAN LIPID METABOLISM.

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Lovage [*Levisticum officinalis* L.] volatile oil has been shown to have pronounced antioxidant activity [1] which suggests the possibility of using the oil as an alternative source of raw materials for the prevention of lipid peroxidation, as synthetic antioxidants previously used have been linked to incidence of cancer in animal feeding trials [2].

Lovage is a member of the family Umbelliferae and exhibits a strong celery-like odour. Lovage grows wild in the hills of southern Iran and south west Europe [3], but was shown to grow and over-winter well in trials carried out at SAC Auchincruive. As all parts of the plant are strongly aromatic, lovage is cultivated for its leaves, roots and seeds.

Having identified the strong antioxidant properties exhibited by the lovage plant, rat feeding trials were initiated using the volatile oil extracted from dried lovage leaves by steam distillation. The feeding trials were set up to determine if dietary administration of lovage volatile oil to pregnant rats would result in a protective, beneficial effect in the fatty acid profiles of the brain and liver tissues of both the mother rats and their progeny. The results of these trials showed the volatile oils to have beneficial effects in the mother rats which were in turn passed across the placenta to the rat pups [4]. The protective effect appeared more significant in the case of the liver tissue than in the brain tissue. The effect was also most pronounced in the offspring compared with the mothers. Administration of lovage oil resulted in an increase in specific fatty acid levels in the liver fractions from the mother rats in eight individual cases and resulted in an increase in specific fatty acid levels in the fractions of liver tissue from rat pups in eleven individual cases.

The beneficial effects of certain plant volatile oils on the polyunsaturated fatty acid [PUFA] levels in specific animal tissues has already been proven [5] and even though the mechanism by which these oils apparently prevent oxidation is unknown, the presence of this antioxidant capacity must not be ignored or under-valued.

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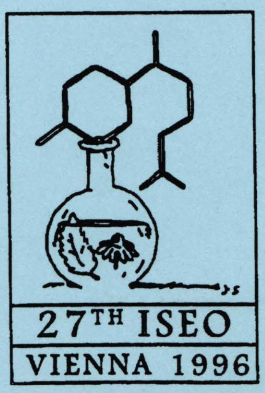
Ch. Franz, Á. Máthé, G. Buchbauer (editors)

Essential Oils:

Basic and Applied Research



Proceedings of the 27th International Symposium on Essential Oils

Allured Publishing Corporation

Essential Oils:

Basic and Applied Research

Proceedings of the 27th International Symposium on Essential Oils
September 8 - 11, 1996. - Vienna, Austria

Edited by

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1997
Allured Publishing Corporation, Carol Stream, IL

Distributor:
Allured Publishing Corporation, 362 S. Schmale Rd., Carol Stream, IL 60188, USA

Essential Oil Symposium Proceedings - 1996

[27th International Symposium on Essential Oils (1996 : Vienna, Austria)]
Essential Oils.
Includes Index.
1. Essential Oils -- Congresses. I. Franz, Chlodwig. II. Máthé, Ákos.
III. Buchbauer, Gerhard. IV. Title.
ISBN 0-931710-59-6

Professor Dr. Gerhard Franz

President of the „Gesellschaft für Arzneipflanzenforschung“

dedicated

to

his 60th Birthday

Table of Contents

Biological Activity

Genetic Aspects and Breeding

Physiological Aspects

Analytics - Technical Aspects

Analytics - Chemical Aspects

Production and Processing of Essential Oils

Phytogenic Feed Additives

Technical Communications

Index of Authors

Preface

Over the last almost 30 years the scientific interest in essential oils has remarkably intensified. The enormous diversity of compounds and compositions, attracted the attention of phytochemists, analysts, flavourists and perfumers, and has stimulated work in systematic botany, pharmacognosy and pharmacology. However, in order to make full use of these resources, it is not sufficient to document the variation by increasingly sophisticated analytical methods only. It is also interesting to understand the biochemistry, physiology and genetics of their biosynthesis, as well as the biological activity of single compounds and entire mixtures. Based on these results, new applications are under discussion, e.g. essential oils as food preservants or feed additives. Finally, techniques to produce these renewable natural products in required quantities and qualities are under development.

The whole range of the mentioned topics was covered by the contributions to the 27th International Symposium on Essential Oils, Vienna 1996. It was attended by more than 200 participants coming from some 40 countries. 33 oral contributions and almost 90 posters have been delivered of which a selected peer-reviewed number is compiled in the present proceedings in order to give an insight into the recent progress.

The scientific program was enriched by an introductory lecture given by Prof. W. Kubelka, Vienna and a critical summary by. Dr. B.M. Lawrence, Winston-Salem, two cavalcades impossible to catch in written form. Nevertheless, we do hope that the contributions to this book highlight the entire field, find numerous readers and stimulate farther discussions.

The Organizers of the symposium, as well as the Editors of the proceedings wish to express their sincere thanks to all, who either as Authors, Referees, Participants or Sponsors have contributed to the success of this event.

Vienna, 1997

Ch. M. Franz

Biological Activity

A PSYCHOLOGIST EXAMINES THE USE OF ESSENTIAL OILS IN AROMATHERAPY TECHNIQUES

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Numerous books have been written about aromatherapy and the use of essential oils in massage. Most are little more than 'cookbooks', having a short historical introduction followed by a series of recipes based on the supposed properties of certain odours and the reactions they are claimed to produce. Perhaps the most succinct overview and basic underlying philosophy of the discipline has been given by Tisserand (1989). In this short review I will not deal in detail with the confusion perpetuated by many of the practitioners over the concept of the 'vital nature' of essential oils; this has previously been adequately dealt with by Dodd (1988) and King (1994). Aromatherapy is a metaphysical rather than a scientific discipline in that it consists of a collection of facts and knowledge about the effects or supposed effects of essential oils on human behaviour and physiology. Most of the collected knowledge is based upon individual cases and, as a consequence, aromatherapists tend to generalise from particular cases. In recent years aromatherapy has tended to fragment into various areas ranging from aesthetic aromatherapy to the more extreme fringe who claim medical cures based on the smell alone (Kusmirek,1992; Tisserand, 1994). In my experience there are few cases where knowledge about the effects of aromatherapy treatment is based upon principles of scientific experimentation (King, 1994). However many aromatherapists incorrectly use scientific ideas and concepts to promote their cause. This view is borne out by a recent book in which the author states:

"The main use of aromatherapy within conventional health care is as a means of relieving stress and anxiety. Despite the publication of three clinical trials in the first six months of 1995, there remains a dearth of clinical research on this intervention. Most are of low methodological quality and the majority fail to find aromatherapy to be statistically superior to massage with plain carrier oil" (Vickers, 1996. p 127).

Thus it seems that most of the claims espoused by the aromatherapists can be simply explained as placebo effects.

Scientific knowledge concerning the effects of fragrances

In the last decade there has been a suddenly increase in the amount of scientific research devoted to the sense of smell. An aim of the First International Conference on the Psychology of Perfumery, held at the University of Warwick in 1986, was to bring together members of the aromatherapy community with the fragrance industry and researchers undertaking scientific studies about the effects of smells. This highly successful meeting was subsequently published as an edited book (Van Toller and Dodd, 1988). A Second International Conference on the Psychology of Perfumery was held in 1990 and the proceedings were again published as a book (Van Toller and Dodd, 1992). One essential question arises in our understanding of the sense of smell as to whether or not reactions to smells are learnt or innate. In the terminology of computers are reactions to smells software (learnt) or hardware(innate). Engen (1982 and1988) claims that all associations to odours are learnt. Kirk-Smith, Van Toller and Dodd (1983) in a pioneering study carried out by the Warwick Human Olfaction Research Group (WORG) showed that odours could be associated with a stressful task without the subjects being aware of the smell. Later the odours were evaluated to see if they evoked stress related reactions. Finally subjects were presented with the smell and asked if they had previously encountered the it. Thirty of the thirty two subjects run in the study claimed not to have encountered the smell previously. Kirk-Smith and Booth (1987)

reviewed the literature and again concluded that human reactions to smells were learned.

What are the known effects of odour on human behaviour ? Baron (1990) reported that subjects working in the presence of a pleasant smelling air-freshner set higher goals and were likely to use more efficient strategies. Earlier Rotten (1983) had found that the converse was the case when subjects were placed in a malodorous environment. The Shiseido Co Ltd, Tokyo, have designed a computer controlled system to dispense odorised air throughout the Kajima Corporation Building. Clerical workers in this building are refreshed in the morning and after the lunch-break by citrus smells. During mid morning, floral scents are circulated to "help improve concentration". In the lunch break and towards the end of the afternoon, woodland scents are circulated throughout the building "to relax the workers". Increased productivity has been claimed for this process (see Anonymous, 1990). In a complex laboratory study, Ludvigson and Rottman (1989), using the essential oils lavender and cloves, were disappointingly unable to show positive effects on three cognitive skills: a) multiple-choice vocabulary task; b) an analogy task; c) a test of arithmetic reasoning. The authors of this study also had their subjects (21 male and 51 female) complete three personality tests. In addition, the authors measured mood and affective reactions of their subjects. Their study involved three sessions with the odours being given during the second and third sessions. A twice yearly publication of the New York, Olfaction Research Fund, is concerned with reporting the fragrance research, called aromachology, funded by the Fund. In the Summer,1991 edition there is an account of a study carried out at the Sloan-Kettering Cancer Centre. The report concerns the use of fragrances to reduce stress in patients undergoing magnetic resonance imaging (MRI). The MRI procedure requires patients to lie motionless for periods of up to one hour in the small noisy claustrophobic tunnel surrounded by massive magnetic coils. Patient anxiety was said to result in ten per cent of the patients prematurely terminating the process. Redd and Manne reported that the use of heliotropin (a sweet vanilla-like odour) in a group of patients (N=40) resulted in approximately 63 per cent less anxiety compared to a control group (N=45) in which no odour was used. The anxiety levels of the patients were said 'to be determined by a battery of psychological, physiological, and behavioural measures'. Five fragrances were said to have been tested but, with the exception of heliotropin, they were not named.

Warm and Dember (1990 and 1991) have studied the effects of peppermint and muguet (lily-of-the-valley) on a demanding vigilance task. Both of the odours were said by the authors to have gained high 'pleasantness' scores in mood rating studies carried out by researchers working for International Flavours and Fragrances (IFF). However, whereas peppermint was reported to be stimulating and alerting, muguet was reported as being relaxing. In their study thirty six subjects monitored a video screen for 40 minutes looking for the occasional change of a visual pattern. The task demanded sustained attention on the part of the subjects because the signals to be detected were interspersed among a larger number of rapidly occurring signals. The 'to be detected non signals' were 10 mm distance from a central dot, whereas the 'normal signals' were 12 mm distance from a central dot. Warm, Dember and Parasuraman claimed that their laboratory task was equivalent to long distance driving.

In common with most vigilance tasks they found that the efficiency of subjects declined over time. The two odours plus non-odorised air, used as a control condition, was administered through a face mask for a thirty second period every 5 minutes during the 40 minute period. Warm, Dember and Parasuraman collected various measures of performance, these included:
the percentage of correctly detected patterns, so-called 'hits'; the percentage of errors made when a 'hit' was falsely scored, this is 'false alarms'. In addition, measures of subjects' levels of drowsiness, irritability and perceived work-load were recorded. The results shown that when the delivery of the odour was under the control of the experimenter both peppermint and the muguet improved vigilance. In a second experiment, using 16 college students, odourless air interspersed with peppermint odour was compared with a control group in which subjects carried out the task without a mask or the use of peppermint. The results replicated the first study with the group receiving the peppermint odour being better than the control group. It was argued by the authors that in both experiments both odours produced increased

sensitivity to the signals to be detected. Importantly, they did not affect the response bias. Response bias is a measure of the willingness of a subject to report an error. For example, a subject may freely report errors or be conservative in their willingness to report errors. It was claimed that the improvements obtained were not merely due to increased responding on the part of the subjects in the odour groups. A third experiment, involving *ad libitum* self-application of the odour, was carried out. This produced an ambiguous result in which males failed to show the improvements found in female subjects.

The results of these sensory stimulation studies are interesting in that, despite prior claims that peppermint was stimulating and muguet relaxing, both odours produced positive results in term of improved performances in a demanding vigilance task. However, there was no significant effect in terms of the self-reported stress. The reason for this is not clear, perhaps the task was not sufficiently demanding overall, or perhaps subjective 'stress' in this experimental study equals subjective 'boredom'; the latter was not measured. How might with our existing level of knowledge about the effects of odours on behaviour explain this vigilance finding ? One of the important effects of an odour is to produce a change of state arising from the sensory input (Van Toller, 1988), this could serve to increase the concentration of a person. A common example might be a driver sucking peppermint flavoured sweets as an aid to concentration. However, an increase in concentration may not actually require an odour because Knasko and Gilbert (1990) have shown that a positive effect can be obtained in a situation where the subject merely believes that a performance enhancing 'odour' is present. Using three groups and appropriate cover stories Knasko and Gilbert told their subjects that they would be in the presence of an odour with a very low detection rate which would improve their performance levels. In fact, Knasko and Gilbert sprayed pure odourless water into the experimental environment. The suggestion that the odour had performance enhancing properties resulted in improvements in the clerical coding task carried out by the subjects.

The findings from these experimentally controlled studies indicate that odours can be effective in overcoming fatigue and stress. The effectiveness of specific odours has not, to date, been shown. It seems that what has been shown is a general sensory arousal property of odours. An alternative explanation may be that it is a classic example of a placebo effect where the desired effect is obtained after the subjects are told what that effect will be.

Psychophysiology and odours

During the last few years the WORG research team have examined a number of psychophysiological techniques in an attempt to develop objective methods of studying sensory responses to odours. To this end we have undertaken a number of psychophysiological studies (Van Toller,1991). The author together with John King, a psychiatrist interested in stress relaxation, attempted to measure the levels of muscle relaxation produced by an odour (King, 1988). This research was carried out in a laboratory setting where we were able to measure all the components of a complex relaxation therapy procedure. The technique developed by King involves the use of sound, light, warmth and smell to simulate as accurately as possible a seaside scene. He found that a seaside scene was a popular, happy and relaxing memory for many anxious patients. By measuring the frontalis muscle in the forehead (this is a difficult muscle to relax consciously) we were able to show that the seaside odour developed by King, produced an additional 15 percent increase in relaxation of the frontalis muscle. The other components (sound, warmth etc) failed to produce similar effects. The relaxation was shown to be specific to the seaside smell and did not generalise to other smells.

EEG and olfaction

The basic information units of the brain are the billions of nerve cells which collect and pass on information via electrochemical activity. The electrical activity over the surface of the cerebral cortex can measured and recorded from the scalp as an electroencephalogram (EEG). EEG can be measured by a number of techniques. A common and early method was to fix electrodes onto the scalp and record the fluctuating electrical signals as analogue ink traces. The fluctuating traces of EEG represents a spatial summation of all the intracellular, extracellular and evoked membrane potentials. They show waxing and waning of activity as the

electrochemical inhibitory and excitatory components interact. In recent years we have used brain electrical activity maps (BEAM) which involve producing the EEG activity as coloured maps. Other authors have reported different techniques used to measure the effects of odour stimulation on brain activity, see Van Toller and Dodd (1988 and 1992) for details.

The Warwick EEG experiment protocol

We always attempt to test our subjects in an as naturalistic a setting as the experimental conditions allow. The first requirement in our current experimental situation is that our subjects wear a tightly fitting fabric cap. This looks like an Australian life-saver's swimming cap. The cap contains 28 electrodes which are seated firmly on the head by the use of chest straps to pull and hold the cap down onto the head. It is not uncomfortable and before it is placed on the head the experimenter clips two reference electrodes to either earlobe. Each electrode is then filled with an electrolyte gel in order to ensure good electrical contact between the scalp and the electrodes. When this procedure is completed subjects are taken into a specially constructed low odour chamber (LOC) and seated in a comfortable armchair. We then explain the procedure and tell the subjects that we will blindfold them and play a tolerable level of white noise through the headphones they are asked to wear. Subjects are told that this procedure is required to help them to detect any odours they might receive. We then instruct our subjects in a technique of breathing in through the nose and out through the mouth. This is to prevent retronasal stimulation of the olfactory receptors from the mouth, throat or lungs during odour presentations. Our experimental method involves periods when an odour may be presented and during these periods subjects are asked to adopt the 'nose in and mouth out' breathing pattern. They are asked to sit very still and to not to make overt efforts to think about any smells they may detect. During inter-trial periods, subjects are allowed to breathe in any manner they like and are permitted to move. During the odour presentation periods, the experimenter determines when an odour is actually presented but subjects feel in control because the odours are presented in synchrony with their breathing. Clearly, subjects differ in the way they respond to the experimental situation but all, during the debriefing phase, report that the experiment was a restful and pleasant experience. Clearly, cognitive as well as sensory components are involved in our experimental situation but it is relatively easy for our subjects to relax and just 'float along' in the experimental situation. Note that our subjects are unlikely to have had previously experience of many of the odours presented to them.

Each randomly presented odour or control trial is divided into three distinct phases: a) an initial 'baseline' EEG recording; b) a period of EEG recording when an odour or control material is presented beneath both nostrils; c) a final post-odour EEG phase when the odour or control material has been removed. Following each complete set of EEG recordings we ask the subjects to smell the odour again and to try to describe the odours used in the study. They are also asked to scale each odour using three bipolar psychometric scales: familiar unfamiliar; strong - weak; pleasant - unpleasant. During this phase of the experiment, subjects are allowed to smell the odours as many times as they like. The use of psychometric techniques allow us to later correlate EEG activity with the value judgements made by the subjects (Van Toller, 1991).

The Brain Electrical Activity Maps (BEAM)

A major problem with EEG is the sheer amount of data generated from even a simple study. Analogue EEG traces are not easy to read and summarise without considerable experience. The introduction of EEG techniques involving computer generated topologies overcomes problems of summarising EEG data. BEAM techniques have been reviewed by Duffy (1986) and a good introduction can be found in Wong (1991). The BEAM method is, therefore, an extension of the traditional methods and it involves transformation of the EEG data using Fast Fourier Transformations (FFT) which are used to generate coloured topographic maps. The collection interval for individual EEG maps is 2.56 sec which means that the display is virtually real-time brain activity. EEG Data is collected for each of the conventional EEG bands i.e., delta, (0-3.5 Hz); theta (4- 7 Hz); alpha (8-14 Hz); beta 1 (13-30 Hz); beta 2 (31+ Hz).

The Neuroscience series III brain imager used in our studies stores the original EEG waveforms on a large capacity optical disc which allows the data to be replayed later. Details of the methods

and procedures are to be found in Kendal-Reed (1990). As mentioned above, after recording the EG subjects are asked to produce psychometric ratings of the odours. This means we are able to look at the maps to see if we can determine how a subject rated an odour. From our early studies we found that we were able to 'read' the topographical maps and predict fairly accurately how the subject psychometrically rated the odours. In order to check our results we had other observers perform judgements and we found that they were also able to do this. This finding was in marked contrast to the periods of time required earlier to confidently interpret the analogue ink EEG traces. Klemm, Lutes, Hendrix and Warrenburg (1992) have also shown cortical topologies and reported similar qualitative EEG data correlated with psychometric evaluations. Their findings confirm our earlier reports (Van Toller and Kendal-Reed, 1989) of positive effects on EEG from stimulation using odours.

An infant EEG research study made in the WORG laboratories has centred around trying to understand how infants respond in a preferential manner to common food odours. To our knowledge, this is the first EEG study of its kind that has been attempted in this age-group.

The aim of this study was to elicit and catalogue responses in young infants using the BEAM. technique. Healthy 12 week old babies were tested whilst awake, usually within an hour of their last feed. The technical details and full details of this work are reported by Kendal-Reed, 1990. The evidence suggests that the twelve week old subjects respond differentially in terms of EEG responses to the various odours. It is still not clear how very young human infants respond to food odour in terms of preference. It could be an affective (emotional) response to the odour, such as liking or disliking. Such responses may derived from intrinsic/innate circuitry in the brain. This is the position taken by Steiner (1979) to explain facial reactions of neonates to various tastes. In addition, Schmidt (1992) has shown adult-like affective responses to odours in nine-month old babies. This raises the intriguing possibility that odour preferences are not learned over the first few years of life, which is the traditional view (Peto, 1935), but rather that some odours are intrinsically pleasant or aversive. It is likely that such odours would have biological significance. See Van Toller and Kendal-Reed (1995) for further discussion of this point.

Analysis and interpretation of BEAM data

Notwithstanding the valuable clues and findings obtained from topological maps, our earliest attempts were directed at going beyond qualitative representation to allow statistical evaluation of the EEG data. Accordingly, we developed techniques to download the EEG data in a form we could used for quantitative analyses. Analysing EEG activity can be likened to the proverbial problem of looking for a needle in a haystack. The background electrical 'noise' of the brain is very large in relation to the signals we are measuring. In addition, there is considerable inter- and intra-subject variability. Notwithstanding these problems we decided to set up an experiment to see if there was agreement in terms of cortical activity across different subjects responding to a range of odours. Additionally we were also interested to see if we could confirm the correlation's between a subject's pyschometric rating and their cortical EEG activity during the presentation of that odour. EEG responses from the visual, auditory, motor, somatosensory and taste areas of the cerebral cortex have been plotted in some detail but the olfactory system has, until recently, been ignored. Kolb and Whishaw (1990) have commented on this lack of interest in the olfactory system. We were, therefore, interested to see if we could identify cortical areas that responded to olfactory stimuli in a systematic way. It is generally accepted that EEG activity anterior to the central sulcus of the cerebral cortex is motor, and EEG activity posterior is somatosensory in character(Kolb and Wishaw, 1990).

Following discussions with our colleagues and collaborators at Quest International we set up an experiment to test out these ideas. Full technical details and results can be found in Van Toller, Behan, Howells, Kendal-Reed and Richardson (1992). The proposal was to test subjects using our normal procedures using a range of odours. The odours, diluted to approximate isointense concentrations, as judged by the researchers and a small group of post-graduates working in other areas, were:

1) blank control strip i.e., with no odour absorbed onto it

2) 5-alpha-androstan-3-one, reported as smelling (i) 'urinous', (ii) 'sandalwoody' (iii) odourless

3) Chandanol, a sandalwood odour, Quest International

4) White Sapphire, a green floral fine fragrance, Quest International

5) linalyl acetate, a component of lavender and bergamot oils

6) indole, a 'faecal' odour

7) McKenzies' smelling salts, eucalyptus oil and smelling salts.

Smelling salts was used because of its qualities as a combined olfactory and trigeminal stimulus. The trigeminal sense is mediated by the 5th cranial nerve with extensive innervation to the facial and head regions. The trigeminal nerve is found mixed in with the olfactory tissue and in the nasal passageways of the nose. It is essentially concerned with the tactile sense and pain. For example, it is experienced when peeling onions because trigeminal nerves on the cornea of the eye are stimulated. The olfactory receptors are innervated by the 1st cranial nerve. The experimental procedure, as outlined above , involved partial perceptual isolation of the subjects. It was decided that the analysis would centre on the classical alpha EEG(8-14 Hz) band because this was the dominant frequency expected from a subject placed in our experimental situation. Other frequency bands were also examined and analysed but were not found to contain significant activity and they will not be reported further. As explained previously, the BEAM technique collects data over a 2.56 second time period; therefore, it was decided that the analysis would be concerned with the first frame after presentation of the odour. It would be during this initial reception of the odour that we expected to find maximum emotional, novelty or orienting responses to the odour. Our subjects were recruited from Open University students who were attending a Summer School at Warwick. They consisted of 9 males and 5 females and covered a wide age range. They all had limited previous experience of olfaction and certainly no experience in the manner with which we presented and required evaluation of the odours. Before entering the experiment subjects were screened for nasal patency.

The statistical method used for our analysis was multi-dimensional scaling (MDS), (Schiffman, Reynolds and Young, 1981). MDS is a descriptive statistical technique used widely in the perfume and fragrance industry in sensory evaluations which usually have a large number of variables requiring categorising. In our particular study, we wanted to know which electrodes showed statistically significantly activity during the odour presentations. As mentioned above each mean electrode amplitude value was calculated for the first 2.56 sec following odour presentation. These values were then used as the input data for the MDS calculations. The MDS result gave a plot with three clusters. The positioning of the main cluster lay in the somatosensory part of the cortex behind the central sulcus.

Following recording of the EEG data, the odour qualities were obtained by getting the subjects to use bipolar psychometric rating scales to record their judgements. A detailed account of psychometric scales and their used in olfactory studies can be found in Van Toller, Dodd and Billing (1985). Sensory olfactory evaluation measurements can be classified under three main headings. The first heading is purely sensory and refers to characteristics of an odour with examples, being the 'lemoness' of a lemon or the 'rosiness' of a rose. The second heading consists of hedonic characteristics relating to subjective perception of the attractiveness of an odour, this scale ranges from unpleasant to pleasant. The third heading refers to comparative data learned by association with the sensory data; e.g., smells 'clean', 'fresh' etc. As stated previously, the experimental design allow us to examine correlations between psychometric ratings and EEG activity. Mean psychometric scores were used to construct an MDS configuration and the electrodes that were found to have significant activity lay as a band of electrodes anterior to the central sulcus across both hemispheres in a motor part of the cerebral cortex.

This study involved naive subjects with no previous detailed information about smells and odours and the results indicate that we had identified certain groups of electrodes which were responding to the odours. The first group, frontal to the central sulcus, appeared to be

evaluative in nature, corresponding to the learned attributes of an odour. The electrodes showing significant effects were located in the motor and premotor parts of the cortex. The second group of electrodes showing significant activity lay in an area of the cortex posterior to the central sulcus. This area corresponds to the sensory part of the cerebral cortex. The fact that the data were averages derived from a number of subjects of both sexes implies that the results have generality. Despite the fact that the olfactory signals have passed through subcortical processing levels they were eliciting similar EEG responses across subjects. However, we are not in a position, nor do we wish at this stage, to make claims about how and where the EEG signals arrive at the surface.

It is important to realise that the results were obtained from a single shot approach and that the cortical results arise from a series of complex interactions between emotion and cognition. Were we to have taken an averaging approach, or indeed have tried to reproduce these responses later, it is likely that we may have been unable to do so. From our early psychophysiological studies (Van Toller et al, 1983) we found that the brain rapidly ceased to record input from different odours, presumably because the different odours we were using were treated as belonging to a similar category e.g., 'smells'. The approach we have taken in our experiments impinges on many areas of neuropsychology and we are only at the stage of beginning to appreciate what questions we might sensibly ask rather than obtain answers to questions. Inferential statistical techniques impose restrictions in the analysis of EEG and most statistical techniques require large numbers of repetitions of the stimulus in order to help reduce variance. If, as in our method, you are attempting to examine the initial emotional reactions of a subject to an odour, the signal will, as suggested above, be rapidly lost over time. Subjects are easily bored in experimental situations. For example, suppose a particular fragrance initially elicited recall of an emotional and vividly charged episodic memory of an early romance. By the 30th or 40th presentation the memory network elicited may have extended to recalling negative aspects. More likely subjects will be bored by the repetitions not to mention the habituation that will occur to the odour. It is the first ephemeral emotional/cognitive responses that we are attempting to capture in our investigations.

As suggested above inferential statistics assume clear-cut situations and normal distributions which probably rarely exist. This being the case we have begun to look for alternative ways to analyse EEG data. Increasingly, psychologists are turning to other analytical methods that permit reflection of the nature of the data rather than attempting to force the data into moulds that are perhaps not entirely suitable. An example is the use of fuzzy sets (Zetenyi,1988) to analyse data. Fuzzy logic recognises that the information we take from our subjects is complex, incomplete and does not fit into neat exclusive, non-overlapping categories. An alternative method is to analyse the EEG activity using Chaos techniques (Freeman, 1991) . At the present time these techniques are complex and require additional refinements but they hold great potential hope for future investigations into neurobiological effects from olfactory stimulation by allowing us to examine different aspects of brain electrical activity.

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ESSENTIAL OILS AND HUMAN VIGILANCE

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Introduction

One basic problem in studies investigating the effect of fragrances on higher cognitive functioning is the possibility that resulting changes in cognition (e.g. improvement of memory) may be confounded with changes at a more general level of cortical activity, i.e. attention. Cognitive functions like perception, language and memory depend on the integrity and and the level of processing effectivity of the attentional systems. If someone performs a memory test being drowsy after having not enough sleep, memory scores will be low - but not because this person's memory is bad per se but because his attention was impaired during the test.

The term "attention" clearly has several different meanings; in neuropsychology, alertness, divided and selective attention and vigilance are differentiated. In this distinction, alertness means the general level of arousal of the organism which may be measured as the speed of information processing. Divided attention refers to the capability to attend to several aspects of an environment simultaneously, while selective attention is the ability to focus on one aspect of a situation while ignoring other aspects. Vigilance refers to the sustainment of attention over longer periods of time, especially if critical stimuli to which there has to be a reaction occur only rarely in time. Vigilance thus can be seen as a counterforce against increasing fatigue in boring situations, as given for example for pilots during long distance flights.

In this study we investigated the possibly activating effect of 1,8-cineol on humans in a vigilance task.

Method

Both the control and the experimental group consisted of 30 healthy subjects. The mean age in the control group was 32.6 years (17 to 56 years), in the experimental group it was 36.7 years (18 to 64 years).

Vigilance was measured by means of a computer based test. Subjects sat in front of a monitor on which two squares, one above the other, were visible. A pattern appeared in one of the squares which oscillated between the squares. If the pattern appeared in one square twice in immediate succession, the subjects should react to this critical stimulus by pressing a button as quickly as possible. The critical stimulus appeared at interstimulus intervals from 8 to 40 seconds. In one individual session four trials took place. In the first trial (10 minutes), subjects just had to perform the vigilance task. In the second trial (10 minutes), subjects were wearing a surgical mask with no substance applied during performing the task. At the beginning of the third trial (15 minutes), a substance was applied to the mask. The substance was water in the control-group and 20 microliters of 1,8-cineol in the experimental group. In the fourth trial (15 minutes) this procedure was repeated. At the beginning of trials 2 and 3 and at the end of trials 2 and 4 subjects were asked to rate the odours on the dimensions of hedonics, intensity, effect and degree of relaxation on visual analogue scales.

Results

In the evaluation of the results, differences between the individual reaction times in the four trials and between the individual values on the four rating scales were calculated for each group.

Comparison of the subjective ratings of the control group (water) with those of the experimental group (1,8-cineol) showed significant differences for all of the four dimensions. 1,8-cineol was rated more pleasant ($p = 0.005$), more intnsive ($p = 0.000$) and more activating than water ($p = 0.000$), but it was rated less relaxing than water ($p = 0.030$).

Visual inspection of the individual reaction time differences between trials 2 and 3 of the control and the experimental group (Fig. 1) showed a negative difference (i. e. slowing) in the control group but a very small positive difference in the experimental group. This difference, however, failed to reach statistical significance.

More detailed analysis of the data revealed that this positive difference was due to an acceleration in the second interval (min. 5 to 10) of trial 3, which, however, also not significant.

In order to see whether reaction time depended on the subjective experience of the inhaled fragrance, multivariate models were built and stepwise regression was performed. A significant nonlinear (quadratic) relation ($p = 0.040$) between the individual reaction time differences in the second interval of trial 2 and trial 3 and the difference of the ratings of the effect of 1,8-cineol at the end of trial 2 and at the beginning of trial 3 was found (Fig. 2). This means that subjects reacted more quickly if they rated 1,8-cineol as activating.

Discussion

Inhalation of 1,8-cineol did not change performance of healthy subjects in a vigilance task in terms of group effects between control and experimental group. Within-group analysis, however, revealed that performance of the experimental group depended on the subjective rating of the inhaled fragrance: the more activating the fragrance was thought to be, the faster the reaction was when compared with the baseline condition. This effect was not found immediately at the beginning of inhalation, but only in minutes 5 to 10 after beginning of inhalation. Jäger et al. (1996) in a pharmacokinetic study showed in prolonged inhalation of 1,8-cineol, the peak plasma concentration is reached after about 18 minutes. Thus, there seems to be an interaction between psychological and pharmacological factors which may explain the described results.

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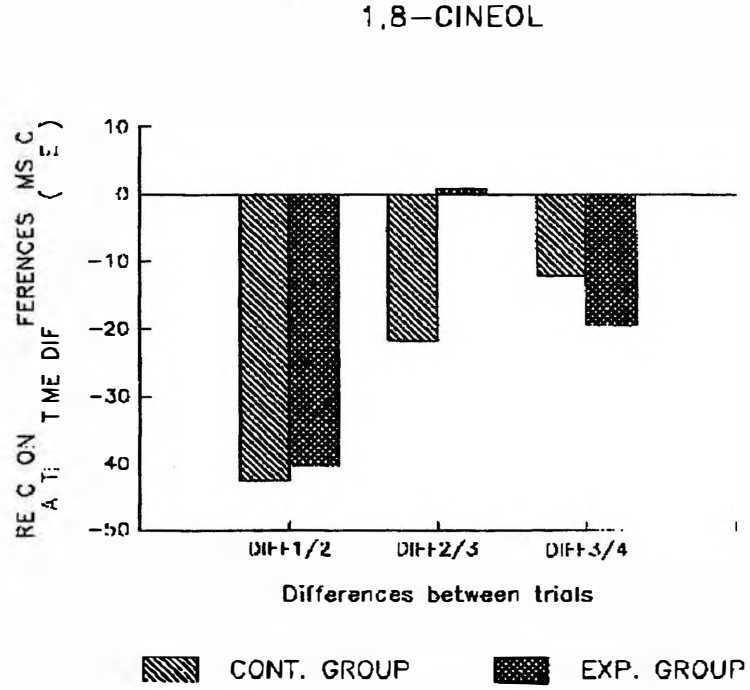

Figure 1. Mean values of the individual reaction time differences between trials of the control and the experimental group

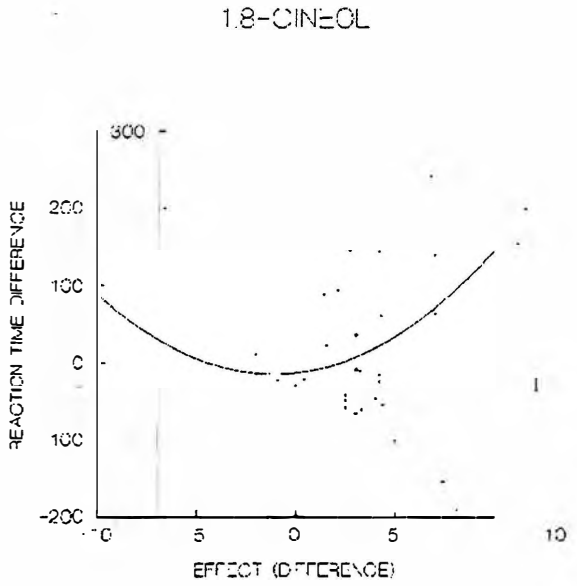

Figure 2. Relation between the individual reaction time differences in the second interval of trial 2 and 3 and the individual differences of the ratings of the effect at the end of trial 2 and the beginning of trial 3

ANALYSIS OF THE CHIRAL FRAGRANCE COMPOUNDS (+)-/(-)-CARVONE IN BIOLOGICAL FLUIDS AND TISSUES

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For this study the chiral fragrance compounds (+)-/(-)-carvone were chosen, which are commercially available in high enantiomeric quality. With concentrations ranging from 55 to 80 % (-)-carvone is the main constituent of the essential oil of *Mentha spicata var. crispa* (Lamiaceae). The drug consists of the spearmint leaves and has a spicy taste, which in contrast to peppermint is characterized as not cooling.
The leaves contain 1 - 3 % essential oil, flavonoids, tannins and bitter substances. Beside (-)-carvone, which is the main constituent of the essential oil, other monoterpenes like dihydrocuminalcohol, carveol, 6-hydroxycarvone and 1,8-cineole have been identified. In contrast to peppermint leaves menthol is absent in spearmint leaves.
Spearmint leaves are frequently used as a stomachic and carminative. The essential oil of the drug is recommended for catarrhal complaints, mainly for inhalation. Large amounts of the oil are incorporated into mouth washes and toothpastes, as well as into chewing gums and liqueurs. Moreover it is also cosmetically used for dry and irritated as well as for oily skin.

In contrast, (+)-carvone can particularly be found in the essential oil of *Carum carvi* (Apiaceae) in concentrations between 50 and 85 %. The drug consists of the caraway seeds, which exert a spicy, aromatic taste.
The main constituents of the fruits include the essential oil in concentrations ranging from 3 to 7 %, further protein, carbohydrate and flavonoids. As mentioned above, (+)-carvone is the main constituent of the essential oil. Besides, (+)-limonene and other terpenes including the isomers of dihydrocarvone, carveol and dihydrocarveol could be identified.
The caraway seeds enjoy a widespread use as a stomachic, since the essential oil promotes gastric secretion and stimulates the appetite. Because of its good spasmolytic activity it is applied as a carminative and as a cholagogue. In folk medicine caraway is also employed as a galactagogue. The essential oil is included in mouth washes for gargling and in skin frictions to bring about hyperaemia. But the greatest amount of caraway is used as a spice and taste enhancer and for the preparation of liqueurs. Besides caraway oil has been shown to have marked fungicidal activity.

(+)- and (-)-carvone were given the GRAS status by FEMA in 1965, they are approved by the FDA for food use and were listed with an ADI of 1,25 mg/kg by the Council of Europe. The acute oral LD_{50} was reported to be 1640 mg/kg in rats. Tested on human subjects at a concentration of 2 % in petrolatum no irritation of the skin could be detected after a 48h closed patch test. A maximization test showed no sensitization reactions either.

Concerning the organoleptic properties (+)-/(-)-carvone show huge differences: Whereas (-)-carvone is characterized by a spicy-minty odor, (+)-carvone possesses an herbal odor reminiscent of caraway seeds. Regarded from the stereochemical point of view, (+)-/(-)-carvone belong to the group of optical isomers. These isomers often differ widely as far as their biological efficacy is concerned. In most cases they show great differences in their quantitative behaviour, but sometimes even qualitative distinctions have been investigated. As a consequence one may even find completely different pharmacokinetics as well as different metabolism fates between two optical isomers.
Referring to the literature (-)-carvone acts as a mild sedative agent, whereas (+)-carvone shows stimulating effects. Moreover it has been investigated in the last few years that monoterpenes like carvone or limonene show interesting cytostatic properties in mice and thus may lead to a strong regression of the carcinogenesis mainly of breast cancer. This was another strong argument for us to investigate the pharmacokinetics of (+)-/(-)-carvone, using an animal model.

Oil/water emulsions were prepared seperately with 50 mg of (+)- or (-)-carvone and 10 % tween 80 in water in order to create a stable emulsion. Female *Sprague Dawley* rats perorally received a single, 0,3 ml dose of one of the fragrance compounds in 10 % tween 80 by means of an oral administration. Afterwards the plasma was centrifuged immediately. Having added piperitone as internal standard and methanol to the plasma, the protein precipitation was centrifuged and the clear solution was analysed with the help of GC measurements.

It was especially important for the preparations of the blood samples to work on ice in order to decrease the high volatility of the fragrance compounds.
Referring to our investigations, (+)-/(-)-carvone were well absorbed and reached the maximum concentration in blood almost equally fast after approximately 20 minutes. In the case of (-)-carvone we could not find any traces of the terpene after one hour, whereas we were able to detect (+)-carvone even three hours after the peroral administration. In contrast to (+)-carvone the area under the blood concentration time curve for (-)-carvone showed a value of 17.3 %, indicating a higher rate of metabolism for (-)-carvone.

As liver is the main organ for metabolism, our next aim was to examine the liver samples of the animals. After the homogenization and centrifugation piperitone was added to the clear solution, which was extracted by using a Bond-Elut-C18 column. Afterwards the samples were immediately analysed with GC and possible metabolites identified with the help of GC/MS measurements. Besides a cyclodextrine column was applied on the same GC-apparatus in order to examine the chiral properties of carvone and possible metabolites.
The experiments showed, that most of (-)-carvone was quickly metabolised to (-)-carveol, whereas only little (+)-carveol was built out of (+)-carvone. We were able to detect traces of (-)-carveol even three hours after the peroral administration of (-)-carvone. In contrast (+)-carveol, the main metabolite of (+)-carvone was only detectable for approximately 1 hour. After administration of (-)-carvone the content of carveol in the liver samples was indeed about 9 times higher than that for (+)-carvone.

These results stress the hypothesis, that the much higher blood levels of (+)-carvone are due to its poor metabolism rate and consequently due to an elimination taking place more slowly than that of (-)-carvone.

Having finished the investigations on the animal model, we wanted to study the behaviour of (+)- and (-)-carvone in human blood. In order to get the fragrance compounds gently into the human body, we decided to apply a massage treatment. The massage oil was prepared with (+)- or (-)-carvone in concentrations of 20% in peanut oil. Our aim was to study a possible absorption of the two volatile compounds and to obtain quantitative results, showing the pharmacokinetic behaviour of both isomers in defined intervals in human blood.
Before starting the massage on a female subject with a body weight of 50 kg, the voluntary was advised to put on a breathing mask, in order to prevent any kind of inhalation of the applied fragrance compound. Then two and one-half grams of the massage oil were spread on a defined skin area of the stomach of the female subject. For ten minutes the oil was gently massaged into the skin and afterwards residues of the oil were completely removed. Blood samples were drawn from the cubital vein and after their centrifugation the internal standard piperitone was added to the plasma. Again the further preparation of the samples was done on ice in order to keep the volatility of the fragrance compounds as low as possible. For the extractions a Bond-Elut-C18 column was chosen and the analysis was performed with the help of GC measurements.
(+)-/(-)-carvone were well absorbed and reached the maximum concentration in blood after approximately 32.5 minutes.

Again (+)-carvone exhibited significantly higher blood levels than (-)-carvone. In contrast to (+)-carvone the area under the blood concentration time curve for (-)-carvone this time only showed a value of 29.8 %.
These results confirm the investigations on the animal model and may once again be based on a completely different metabolism fate of the two enantiomeric fragrance compounds.

Summing up, our analysis showed important differences in the pharmacokinetic behaviour of (+)-/(-)-carvone not only in the animal model, but also in the human being with the help of a massage treatment. In case of cosmetical or medical treatments one should always keep in mind these results, especially when considering the possible dosage of the two terpenes. Since (+)-carvone reaches higher concentrations in blood than (-)-carvone and seems to undergo a poor metabolism fate, one should always distinguish between the two fragrance compounds and use them carefully in order to gain an optimum of efficacy and a minimum of side effects.

Acknowledgement: The support of the Austrian Science Fund (project: 11 606 - MED) and of Dragoco, Vienna is gratefully acknowledged.

3-D STUDIES ON ODOUR MOLECULES

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Investigations on the molecular similarity are widely used for structure acvtivity relationship studies. Molecular shape, properties of the molecular surface, electrostatic potentials of the molecules are features used for the comparison of a set of structurally different molecules with the same biological effect in order to obtain concrete information about those structural subunits, which might be in common for all these molecules. It can be assumed that the geometry of these subunits and their properties are responsible for a concrete biological effect by the interaction with a convenient receptor site. Also the comparison with compounds of similar structural or electrostatic properties without the investigated biological effect leads to information about important differences between the molecules of various biological properties.

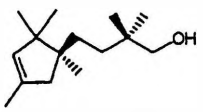
One of the pronounced problems in molecular similarity studies is the large conformational flexibility of molecules with three or more single bonds, a property, which leads to a great number of local conformational minima. The population of the minima is controlled thermodynamically and can be estimated with the help of the Boltzmann statistics. At room temperature conformations with energies higher than 5 kcal/mol are generally excluded, because their population is too low to contribute to a thermodynamic equilibrium at physiological temperature.

In the comparison of e.g. molecular surfaces of a set of flexible molecules the conformational space, consisting of all energetically possible conformations, has to be considered, because for association processes at a convenient receptor site not always the lowest energy conformation is responsible. In many cases conformations with higher steric energy may interact more suitable and so therefore the common structural subunit exists only in these conformations of slightly enhanced energy content..

Molecular similarity investigations on Sandalwood odour molecules were done extensively recently [1-3] and a model for osmophoric regions on the molecular surfaces was developed by Active Analog Approach studies [4]. Some examples of the comparison of flexible Sandalwood odour molecules with structurally very similar but odourless molecules are given here.

The compounds used for investigations are from the group of cyclopentenes. These substances have become more important in the last years. Relatively easy synthetic access and excellent fragrance properties make these products interesting for commercial applications [5, 6]. The main starting component in this group of substances is α-campholene aldehyd ((2′,2′,3′-trimethylcyclo-pent-3′-en-1′-yl)-acetaldehyd). Via aldolconden-sation followed by reduction, different alcohols with a woody scent can be synthesized [7]. One of the most important compounds is 4-(2′,2′,3′-trimethylcyclopent-3′-en-1′-yl)-2-methyl-but-2-en-1-ol, which is known as Sandacore®, Sandalmysore® or Madrol® and 4-(2′,2′,3′-trimethylcyclopent-3′-en-1′-yl)-2-methyl-butan-1-ol known as trade mark Brahmanol® (**1**) [8]. This saturated alcohol is more stable than the unsaturated analogues and is therefore better suitable for commercial applications.

1



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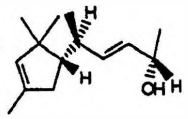

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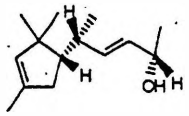

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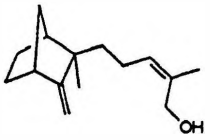

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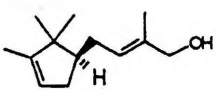


7

All these compounds have one or more chiral centers, which leads to different configurations. Only in few cases some information about the biological effects of the enantiomers is available. In the class of Sandalwood odour compounds it has been proven even optical isomers have different fragrance impressions, a fact, which was studied extensively for ß-santalol **5** [9], tert.-butyl-bicyclodecan-1-ol **6** [10, 11] and Madrol **7** [12]. It has to be assumed that the association pattern of these isomers at the chiral receptor site is different. Molecules **3** and **4** are enantiomers due to the different configuration of the carbon atom in the α-position to the cyclopentene ring. The different fragrance of these compounds is therefore also a consequence of the different molecular shape.

The other point of discussion is that also small structurally differences can lead to different biological effects, for instance in the case of Brahmanol (**1**), a compound with a campholene skeleton, showing strong sandalwood odour and the analogous compound with a fencholene skeleton (**2**), which is odourless.

In the following diagrams the energies of the various conformations of **1** (fig. 1) and **2** (fig. 2) are plotted in dependence on the relevant dihedral angles. These local minima are connected by solid lines in increasing order of energy.

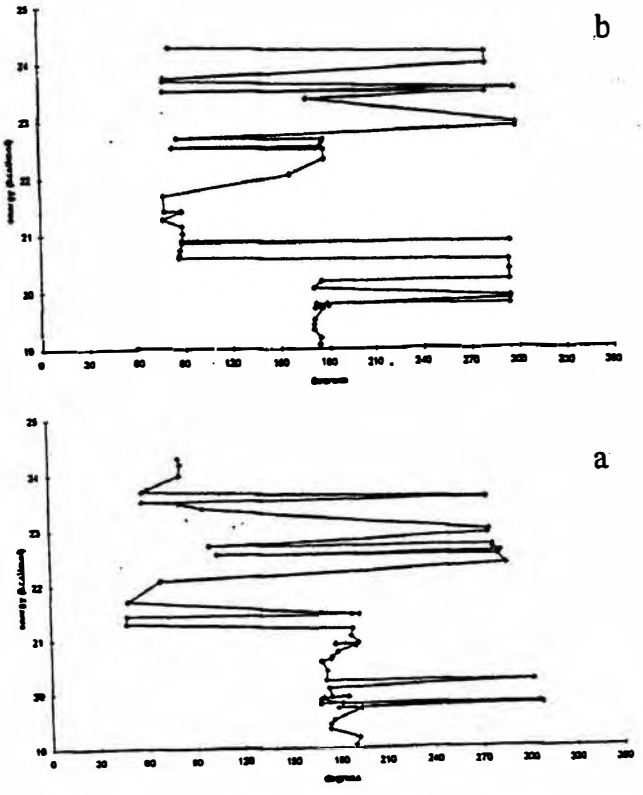


Figure 1. Energies of the different conformation of **1** in dependence on the dihedral angle α (a) and β (b).

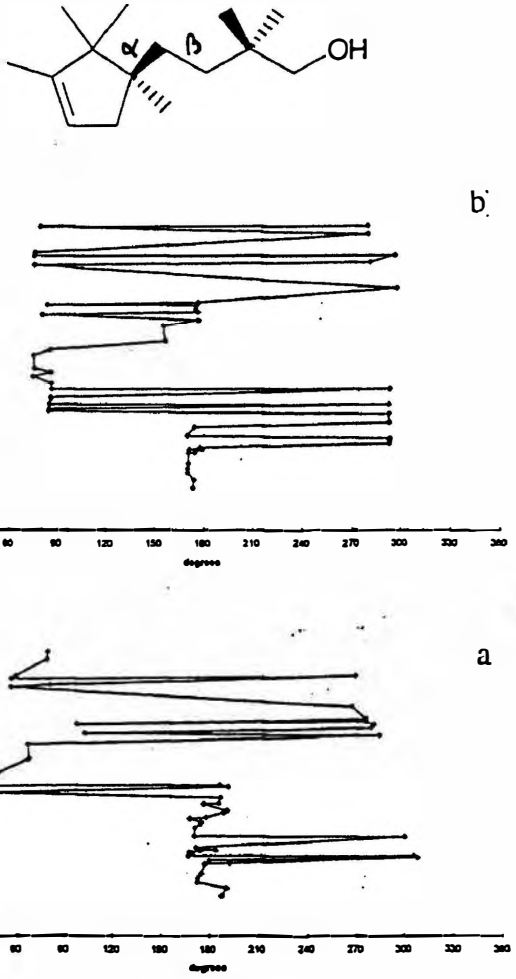


Figure 2. Energies of the different conformation of **2** in dependence on the dihedral angle α (a) and β (b).

For the dihedral angle α the absolute energy minimum was observed round 170 degrees. The next conformations with slightly increased energy possess the same values for α and β, but with different (oscillating) values for the other dihedral angles. For higher energies angle ranges were detected for α between 285 and 310 degrees and between 70 and 95 degrees, whereas the corresponding values for β appear between 170 and 205 degrees, between 280 and 310 degrees and between 40 and 100 degrees, respectively. Conformations with energies higher than 5 kcal/mol from the absolute energy minimum are not shown in the diagrams.

For this first pair of substances, it can be seen that the conformational spaces for every dihedral angle are very similar. The correlation between the energy of each conformation and the dihedral angle leads to a very similar pattern although in one region the two compounds differ in the position of a methyl group.

Following the procedure of the molecular surface comparisons based on force field calculations and

the construction of comparable moleclar surfaces [13, 14], the agreement of the molecular surfaces of the selected conformations of **1** and **2** is close to 100% in large parts of their surfaces. In one octant the agreement is only 47,7%. Following the association model developed by Active Analog Approach investigations [4] osmophoric points were found in both the hydrophilic and the hydrophobic part of the molecule. As the side chain of both molecules is more or less identical and their orientations is not influenced by the substitution at the cyclopenten ring, the osmophoric points found close to the hydroxyl group (hydrophobic molecule part) are also identical. But the differences at the third osmophoric point, located at the octant of the molecule in which the methyl group of the fencholen skeleton leads to an additional surface bulk. This causes a steric repulsion at the receptor site, which diminishes the association force to such an extent, that no Sandalwood fragrance can be detected anymore.

In figure 3 the bulk at the molecular surface of **2** is shown (dotted black points) in comparison to the molecular surface of Brahmanol (**1**) (solid grey surface). The deviation of compound **2** occurs in one of the three osmophoric points.

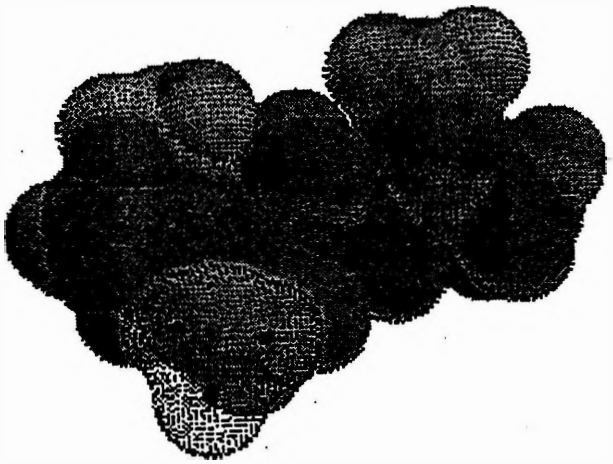


Figure 3. Superpositioning of compound **1** (grey solid surface) and **2** (dotted black surface).

In a molecular surface comparison (using the model of van der Waals surfaces) with (+)-tert.-butyl-bicyclodecan-1-ol (**5**), a rather rigid compound with strong and clean sandalwood odour used as calculation standard, both substances show similar results in most parts of the molecular surface. All conformations, which differ less than 5 kcal/mol from the absolute energy minimum have been compared with this reference molecule (5) to find an explanation for the different biological effect in spite of the high structural similarity. The conformations indicated with a circle in figure 1 were found to have the best agreement with the standard compound and they were used for a further comparison.

As second example of compounds with a flexible side chain the enantiomers **3** and **4** were studied. In the first example the compound with the fencholene skeleton was found to be odourless. The aldolcondensation of (optical pure) fencholene aldehyde followed by reduction leads to the alcohol, which is a mixture of 4 enantiomers. This mixture is described to possess a woody freagrance with cedric tonality [15]. From comparison of the conformational space as explained for the first example it can be shown that caused by steric interactions the energy differences of the various conformations are much larger, which is demonstrated in figure 4 for compound **3** and in figure 5 for the isomeric substance **4**. In contrary to the high flexibility of the side chain, the number of enegetically possible conformations is small. Additionally the differences between the various enantiomers are tremendous and conformations which may agree with the Sandalwood odour standard molecule **5** are not populated in thermodynamic equlilibrium.

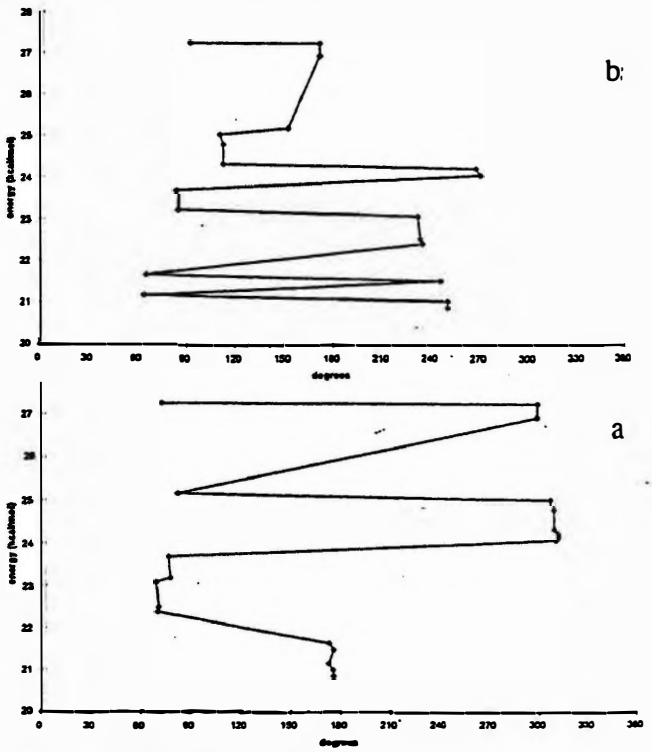
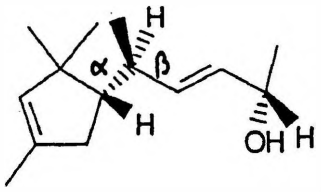


Figure 4. Energies of the different conformation of **3** in dependence on the dihedral angle α (a) and β (b).



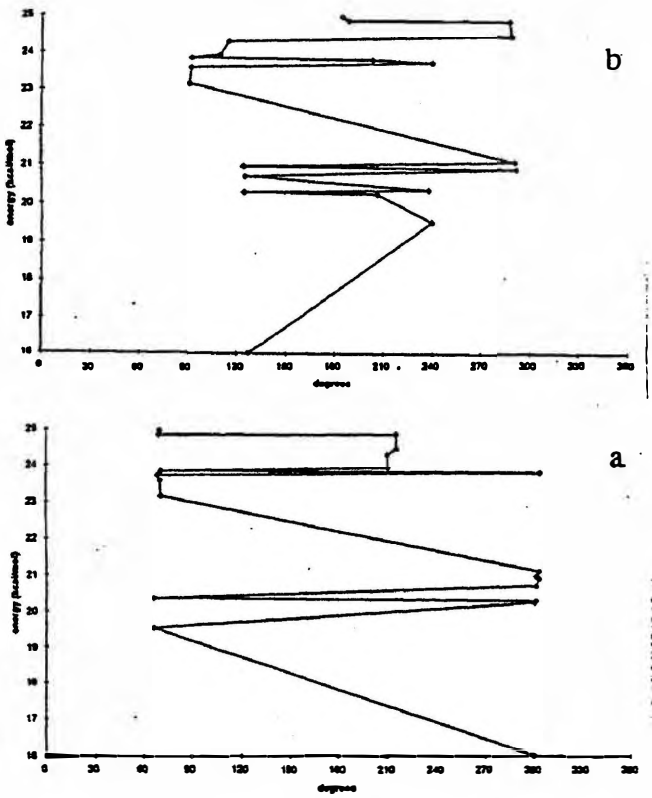


Figure 5. Energies of the different conformation of 4 in dependence on the dihedral angle α (a) and β (b).

The conformational space of flexible molecules plays an important role in molecular similarity investigations. The search for the active conformations as well as the estimation of the relative energies, which determine the population of the relevant conformation in the thermodynamic equilibrium are main topics of structure activity studies.

Acknowledgements: The authors wish to thank the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich (Project 11349 CHE) for financial support and Mrs. E. Liedl for technical assistance.

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STUDIES ON THE ODOR-STRUCTURE RELATIONSHIP OF SOME TERPENE SUBSTITUTED DIOXANES AND DIOXOLANES

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Introduction

The structure-odor relationship studies are one of the fascinating part of research on odoriferous compounds. Some results of these type of the studies have been excellently presented by Ohloff in his book[1]. Our contribution in the research on odoriferous compounds is concerned with synthesis of some acyclic[2], monocyclic[3] and bicyclic[4] terpenoids.

Here we present the synthesis and odor characteristics of some terpene substituted dioxanes and dioxolanes as well as the approach to correlation of odor with some structural factors of the molecule.

Materials and Methods

Dioxanes **5-16** and dioxolanes **17-28** were obtained as products of the reaction of corresponding aldehydes **4a-c** with the 1,3- or 1,2-diols respectively. (Scheme 1)

Aldehydes **4a-c** were synthesized in four steps synthesis from appropriate organo-magnesium halide (-Br or -Cl) and crotonaldehyde or 3-methyl-2-butenal. Allyl alcohols **1a-c** were converted into corresponding esters **2a-c** by Claisen rearrangement (orthoacetate modification). Esters were reduced with lithium aluminium hydride to the alcohols **3a-c** which in the last step were oxidized to the aldehydes **4a-c**. Z-isomer of the aldehyde **4a** was obtained via isomerization of the alcohol **3a** according to a procedure described by Vedejs and Fuchs[5].

The structure of all compounds obtained was confirmed by IR and ^{1}H NMR spectra. Physical and spectral data of dioxanes and dioxolanes are given in Polish Patent Applications[6]. Odor characteristics of compounds synthesized are given in the Table I. Compounds, for which the odor was evaluated were above 97% purity (GC).

Results and Discussion

Correlation between structural factors of the compounds studied and their odors were carried out by MS Exel program. The odor was characterized by intensivity (I), fragrance (II), note (III) and additional description (IV) eg. fresh, sweet as listed in the Table II.

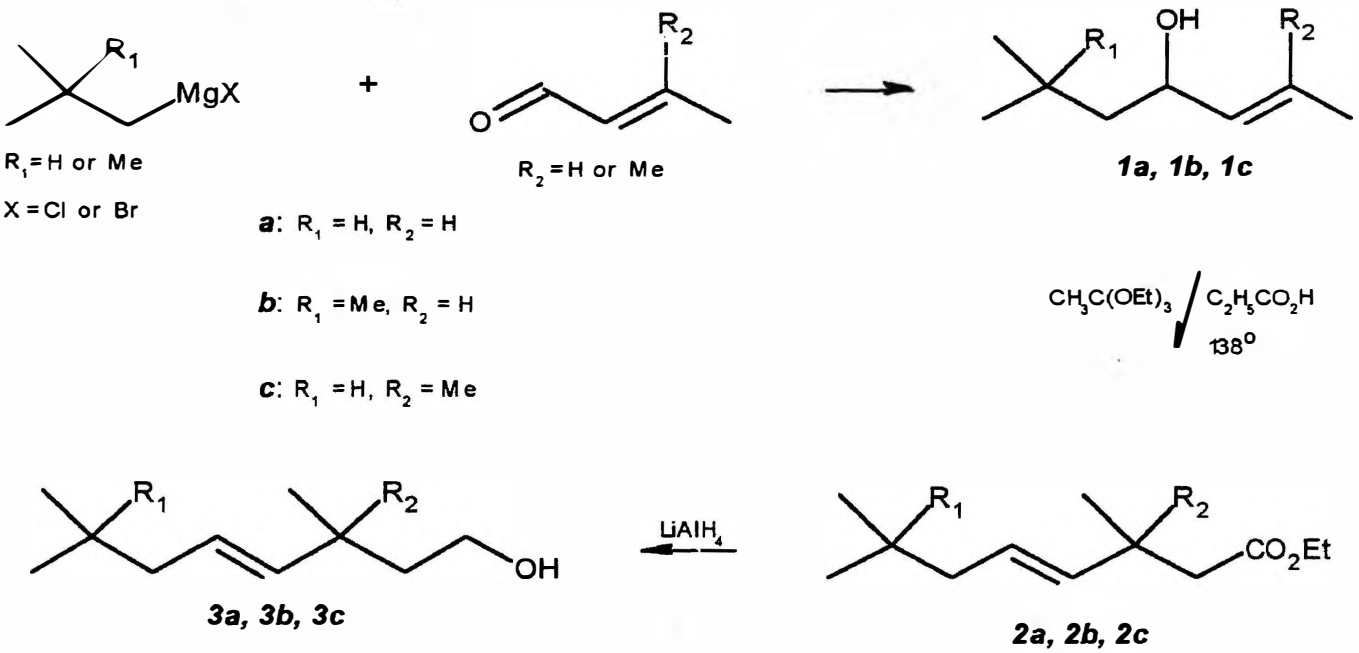
Table II

Determination of odor factors (OF) used in the correlation

Intensivity	OF	**Fragrance**	OF
inodorous, weak	-1	floral, floral-fruity	-1
medium-intensive	0	fruity-floral, fruity, fruity-vegetable	0
intensive, strong intensive	1	vegetable-fruity, vegetable, woody, humus-woody	1

Note	OF	**Description**	OF
floral,	-1	agreeable, sweet	-1
fruity,	0	undetermined	0
vegetable, woody,	1	fresh, penetrating	1

Scheme 1.

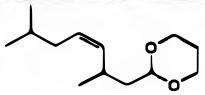
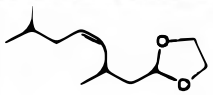
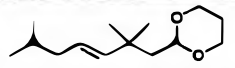
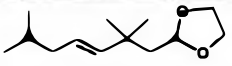
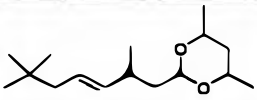
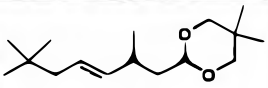
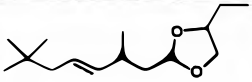
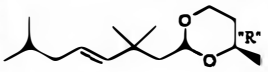
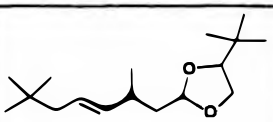
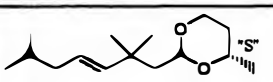
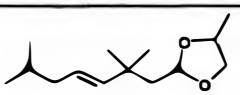
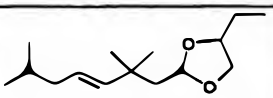
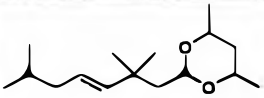
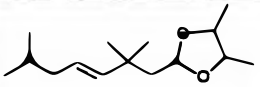


		R1	R2	R3	R4	R5	R6
	5	H	H	H	H	H	H
(Z)-	**6**	H	H	H	H	H	H
	7	Me	H	H	H	H	H
	8	H	Me	H	H	H	H
	9	Me	H	H	H	H	Me
	10	Me	H	Me	H	H	Me
	11	Me	H	H	Me	Me	H
"R"	**12**	H	Me	H	H	H	Me
"S"	**13**	H	Me	H	H	H	Me
	14	H	Me	H	H	H	Me
	15	H	Me	Me	H	H	Me
	16	H	Me	H	Me	Me	H

		R1	R2	R7	R8
	17	H	H	H	H
(Z)-	**18**	H	H	H	H
	19	Me	H	H	H
	20	H	Me	H	H
	21	Me	H	Me	H
	22	Me	H	Me	Me
	23	Me	H	Et	H
	24	Me	H	tBu	H
	25	H	Me	Me	H
	26	H	Me	Et	H
	27	H	Me	Me	Me
	28	H	Me	tBu	H

Table I

Odor characteristics of dioxanes and dioxolanes

 **5**	medium-intensive, fresh, floral, with ambergris note	 **17**	medium-intensive, floral, with fruity note
 **6**	medium-intensive, fruity- floral, with fresh note	 **18**	medium-intensive, agreeable, floral, with marked humus-woody note
 **7**	medium-intensive, agreeable, fruity-floral	 **19**	intensive, agreeable, fruity-floral, with woody note
 **8**	medium-intensive, floral-fruity with quincefruit note	 **20**	medium-intensive, sweet, floral-fruity, with honey note
 **9**	medium-intensive, agreeable, fruity-floral, with apricot note	 **21**	intensive, vegetable with marked floral note
 **10**	medium-intensive, agreeable, fruity-floral, with bindweed flower note	 **22**	intensive, vegetable with floral note
 **11**	medium-intensive, agreeable, fruity-floral, with apricot note	 **23**	medium intensive, agreeable, fruity-floral,
 **12**	weak, vegetable, with carrot note less marked then 13	 **24**	weak, almost inodorous with weak floral note
 **13**	medium-intensive, vegetable, with note of carrot	 **25**	medium-intensive, agreeable, floral, with sweet pea note
 **14**	medium-intensive, vegetable, with floral-fruity note	 **26**	medium-inten-sive, agreeable, floral-fruity, with lime flower note
 **15**	weak, fruity-floral	 **27**	weak, floral, with lily flower note (*lilium candidum*)
 **16**	medium-intensive, agreeable, fruity-floral, with apricot note	 **28**	Weak, almost inodorous

The structural factors were categorized in terms (0,1) as follow:
(A) = additional Me group at C-6,
(B) = additional Me group at C-2,
(C) = additional Me group at C-6 in the ring,
(D) = additional Me group at C-5 in the ring,
(E) = additional Me group at C-4 in the ring,
(F) = five membered ring. The combinations of structural factors were also considered:
(G) = A + B, (H) = A + C, (I) = B + C, (J) = A + B + C, (K) = D + E, (L) = D + F, (M) = A + B + D, (N) = E + F, (O) = A + B + E, (P) = A + B + F, (R) = A + B + C + D + E, (S) = C + D + E, (T), = total number of carbon atoms in additional alkyl groups, both in terpenoid chain and in the ring.

Table III

Correlation coefficients of odor - structure relationship

	I	II	III	IV
A	0.1530	0.2083	-0.1037	-0.1880
B	-0.2897	0.0471	-0.2391	-0.1389
C	-0.2928	-0.2870	-0.4049	-0.0213
D	0.00	0.0675	0.0326	-0.2761
E	0.00	**0.5516**	-0.0844	0.1433
F	0.00	**-0.5666**	0.0346	-0.1170
G	-0.1777	0.2971	-0.4071	-0.4138
H	-0.1000	-0.0556	-0.3358	-0.1412
I	-0.3980	-0.1665	-0.4509	-0.1089
J	-0.3067	-0.0250	**-0.5213**	-0.2480
K	0.00	0.4350	-0.0265	-0.1623
L	0.00	-0.3294	0.0503	-0.3115
M	-0.0914	0.2026	-0.1837	-0.4162
N	0.00	-0.0930	-0.0450	0.0127
O	-0.1068	**0.5285**	-0.2970	-0.1434
P	-01181	-0.2650	-0.2487	-0.3454
Q	0.00	**0.5666**	-0.0346	0.1170
R	-0.2131	0.2554	-0.3681	-0.2813
S	0.00	0.00	0.00	-0.2933
T	**-0.5041**	0.0197	-0.3748	-0.1295

The full correlation matrix was then calculated. Only some coefficients were in order of 0.5-0.6. The others were very small (Table III).

Data obtained from the correlation matrix allowed us to express some odor - structure relationship conclusions.

Additional methyl group at C-2 in the isoprenoid chain changes olfactory properties from floral odor (**5** and **17**) to floral-fruity (**8** and **20**) and to fruity-floral when the additional Me group is at C-6 atom (**7** and **19**).

Presence of alkyl group in the dioxolane ring with the 2,2,6-trimethyl-4-heptenyl unit makes change of the odor from floral-fruity (**20**) to floral (**25**, **26** and **27**) whereas in dioxolane with the 2,6,6-trimethyl-4-heptenyl unit from fruity-floral (**19**) to vegetable (**21** and **22**).

In the case of dioxane derivatives additional methyl group in the ring with the 2,6,6-trimethyl-4-heptenyl unit does not make change of fruity-floral odor (**9**, **10** and **11**) whereas with 2,2,6-trimethyl-4-heptenyl unit changes the odor from floral-fruity (**8**) to vegetable (**12**, **13** and **14**). Additional two methyl groups in the dioxane ring cause change of odor to fruity-floral (**15**, **16**).

Odor intensivity decreases with an increase of number of additional alkyl groups in terpenoid chain and in the ring. Dioxolanes with the *t*-butyl group (**24** and **28**) are almost inodorous.

The configuration of chiral carbon atom in the dioxane ring with the methyl group does not affect the odor. Both isomers (**13** and **14**) are characterized by vegetable odor with carrot note.

Comparing odors of *E/Z* pairs of dioxanes (**5** and **6**) as well as dioxolanes (**17** and **18**) one can see small difference in note only.

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CORRELATION OF THE CHEMICAL PROFILES OF ESSENTIAL OIL MIXES WITH THEIR RELAXANT OR STIMULANT PROPERTIES IN MAN AND SMOOTH MUSCLE PREPARATIONS *IN VITRO*

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INTRODUCTION

Essential oils have always been categorised by aromatherapists as been either relaxant (sedative) or stimulant. It is not clear whether this refers to the action on the brain or to some or all of the muscles, as many essential oils are also classified as antispasmodic. Most essential oils are used by aromatherapists in mixes of 3 or more, diluted by 95% with almond or other carrier oil and then massaged into the skin. The overall effect of this aromatherapy massage is doubtless a major factor in relieving stress and in so doing may also "cure" or at least alleviate many of the stress-related conditions like eczema, stomach ache, back ache, headache etc. Some of the beneficial effect may also arise from counselling the client or at least listening to the problem and acknowledging its existence.

Changes in the body which occur outside of the brain, as a result of stress are not under conscious control but are mediated by the sympathetic branch of the autonomic nervous system. The activity of most organs of the body is controlled by the autonomic nervous system and as a general rule the sympathetic system may be considered to be activated in times of flight or fight which will include stress. Stress-related changes in the body will also be mediated by hormones, such as those released from the adrenal gland. Stimulation of the sympathetic system, and adrenaline released from the adrenal gland, will increase heart rate and stroke volume and by dilating and contracting different blood vessels will cause blood to be distributed to those organs such as skeletal muscle, heart and lungs which are involved in exercise. Smooth muscle will also be either contracted or relaxed such that the body is prepared for exercise, thus bronchial muscle relaxes and sphincters of the gastro-intestinal system contract. If one considers the fight response in animals, smooth muscle contracts to give dilated pupils and make hair stand on end. In both man and other animals, stimulation of the sympathetic system will cause metabolic changes which favour activity, such as an increase in blood glucose.

The nerves of the sympathetic system which innervate smooth, cardiac and vascular smooth muscle all release noradrenaline as their neurotransmitter and the differential response, either contraction or relaxation, is brought about by the presence of different adrenoceptors on the innervated tissue.

In general, alpha adrenoceptors mediate contraction and betaadrenoceptors relaxation, but of course there are exceptions to this rule. Further differentiation and control of the system is obtained by the presence of sub-types of alpha and beta adrenoceptors. Occupation of a receptor by an appropriate agonist results in a change in cell activity (such as contraction or relaxation) which is mediated via a secondary messenger within the cell. Alpha-2 adrenoceptors mediate their actions via a fall in cyclic AMP (cAMP), whilst beta-adrenoceptor activation is associated with a rise in cAMP. Alpha-adrenoceptors are linked to the phosphoinositide pathway. In general, contraction is associated with an increase in the concentration of calcium ions within the muscle fibre whilst relaxation involves either a removal of calcium, the blocking of calcium channels or the opening of potassium channels.

Many tissue of the body receive a dual innervation from the two branches of the autonomic nervous system (sympathetic associated with activity and parasympathetic with feeding and the restoration of energy). In the gastro-intestinal tract we have this dual innervation plus an additional plexus of nerves in the wall of the intestine, often called the enteric nervous system, which involves several other neutotransmitters. It is on account of this rich

innervation of the intestine that we have studied the action of essential oils on the smooth muscle of the guinea-pig ileum *in vitro*.

The preparation will remain viable for several hours after removal from the animal and will respond to electrical field stimulation with reproducible contractions which are due to the stimulation of the parasympathetic nerve with the release of acetylcholine.

Essential oils which stimulate smooth muscle contraction can be recognised immediately whilst the site of action of those which reduce the size of the electrically-induced contraction can be determined. Possible sites of action include inhibition of the release of acetylcholine, or relaxation of the tissue via stimulation of adrenoceptors, action on secondary messengers or on calcium or potassium channels. This preparation thus allows us to recognise spasmogenic and spasmolytic activity, to determine whether or not the activity is dose-related, to measure duration of action and also attempt to determine the mechanism of action.

The question arises whether the knowledge of the activity of essential oils on smooth muscle gives us any clues about the likely actions of these compounds if and when they enter the central nervous system (CNS). A famous English pharmacologist suggested that the intestine could be considered a paradigm of the CNS but it still remained almost impossible to infer action in the CNS from activity on isolated smooth muscle. The reason for this is simply the complexity of the CNS, with the interaction between excitatory and inhibitory fibres being such that reduced activity in one neurone can lead either to sedation or excitation. Thus alcohol appears to stimulate some behaviour although it is a CNS depressant, the explanation being that the inhibition of inhibitory pathways removes a normal break and behaviour therefore changes.

Another aspect of the complexity of the CNS is that any one particular behaviour is controlled by several neurotransmitters, each of which is likely to be able to bind to different sub-groups of receptors. If one for example considers pain, this involves neurotransmitters in the afferent pathway such as Substance P, glutamate and nitric oxide and this afferent pathway can be modulated by neuronal pathways releasing a range of neurotransmitters including opioid peptides, acetyl choline, histamine, 5-hydroxytryptamine and cholecystokinin. With so many neurotransmitters involved in the pain pathway it is not surprising that the experience of pain can be influenced by many different compounds. For example, monoterpenes like menthone and alphaterpineol (administered by the subcutaneous route) showed activity similar to that of accepted analgesics eg. indomethacin and naproxen in reducing the behavioural activity of the mouse to a noxious stimulus (Hart et al., 1994). In experiments studying the motor activity of mice after exposure to the aroma of various essential oils (Buchbauer, 1991, 1992; Jäger et al., 1992) rosemary, jasmine and ylang ylang increased activity whilst neroli, lime-blossom, passiflora, lavender, citronellol and linalool decrease motor activity. The presence of components in the blood when applied by inhalation has also been demonstrated (Jäger et al. 1992). The effect on the motor activity has been shown to be similar to that when the essential oil was injected. It has been assumed that changes in motor activity are a central effect but the possible action on neuromuscular transmission has not been investigated. However, recent experiments on the motor-nerve skeletal muscle preparation (rat phrenic-nerve diaphragm) by the authors has shown that eg. lavender and tea tree oils cause a reduction in the size of the twitch of the skeletal muscle in response to electrical stimulation of the motor nerve.

Linalool, which was shown to reduce motor activity in the mouse has also been shown to have an action within the brain itself: using membranes from rat cerebral cortex, linalool exhibited a dose-related inhibition of the binding of glutamate, a main excitatory neurotransmitter of the CNS (Elisabetsky et al., 1995).

The effect of essential oils in man has been studied in several different ways including measuring the alertness and reaction times (Manley, 1993) and human brain activity (Torii et al., 1988; Kubota et al. 1992) using Contingent Negative Variation (CNV). The latter is the brain potential which occurs between a warning stimulus and an imperative stimulus i.e. when the subject is expecting something to happen. The CNV amplitude is increased by caffeine, jasmine and peppermint and decreased by chlorpromazine, lavender and marjoram. There is some discrepancy between results from different groups regarding many oils.

The present experiments were designed to see whether the effect of stress on man could be mimicked using isolated segments of small intestine and by monitoring their spasmogenic or spasmolytic effects, it could be possible to assess their relaxant or stimulant nature.

MATERIALS AND METHODS

Essential oils were obtained from various commercial sources and each oil was analysed by GC using a Shimadzu GC8A with a 50 m x 0.32 mm OV101 column; the temperature program was set at 4 °C min-1 from 100 to 230 °C. The percentage of all the components was calculated in each selected Retention Time interval of under 10 min, 11-15,16-20, 21-30 and 30+ min. The main components present in each RT interval was also determined.

The essential oils were diluted in methanol x 1,000 and 0.1 to 0.2 ml was applied to the tissue preparations in the organ bath giving a final dilution of x 200,000 to x 400,000 (i.e. a concentration of 2.5 x 10-6 to 5 x 106). Solvent activity was nil at the highest volume used.

Pharmacological studies were carried out on guinea-pig ileum and the smooth muscle preparations were mounted in 25 ml organ baths containing Krebs bicarbonate buffer at 34 ^{O}C, gassed with 95 % oxygen in carbon dioxide. Field stimulation was applied, when needed, by two parallel platinum electrodes placed either side of the tissue; these were attached to a stimulator (0.5ms pulse, 0.1 HZ, 70v). Changes in tension were recorded with an isometric transducer attached to a pen recorder.

Aromatherapist's prediction of the effect of essential oils on the patient (alone or as mixtures) was determined by a practising Aromatherapist with many years of experience and confirmed by many others.

RESULTS

Previous comparisons of the pharmacological activity of many components and essential oils suggested that monoterpenes were responsible for contractions in the guinea-pig ileum *in vitro (* Lis-Balchin et al 1 996a, b). This was best illustrated by work on two New Zealand essential oils Manuka and Kanuka. The former was largely composed of sesquiterpenes and produced a relaxation in the gut, whilst the latter was composed largely of monoterpenes and produced a contraction (Lis-Balchin et al. 1996a).

The results therefore suggested that it was simply the actual percentage composition of the monoterpenes which determined whether the effect on the smooth intestinal muscle would be contractile or relaxant. This hypothesis was put to the test, using essential oils alone or in mixes, by calculating the total percent of components in different RT intervals (Table 1) and predicting what the effect on the smooth muscle would be. It was noted that monoterpenes were in the RT >10 interval, with the exception of 1,8cineole which was also found here, whilst alcohols, ketones and aldehydes occurred in the 11-15 min interval, esters and phenols in the 15-20 min interval and sesquiterpenes thereafter.

Table 1. The predicted effect on guinea-pig ileum based on the percentage of components at different Retention Time Intervals

RT	>10	11 - 15	16 - 20	21 - 30	30+	Effect
1.	---	85	2	---	7	R
2.	41	5	49	---	---	S/R
3.	85	1	---	6	---	S
4.	78	22	1	14	---	S/r
5.	86	2	2	1	---	S
6.	44	45	---	5	---	S/R
7.	37	47	8	4	---	S/R
8.	4	90	---	2	---	R
9.	9	56	12	15	---	s/R
10.	---	---	---	58	37	R
11.	98	---	---	---	---	S
12.	99	---	---	---	---	S
13.	---	---	---	71	19	R
14.	22	69	---	---	---	S/R
15.	10	26	4	41	6	s/R
16.	10	39	36	9	---	s/R
17.	78	9	3	5	---	S

Whenever there was a considerable percentage of components in the >10 min interval, this would be associated with a small to large contraction of the ileal muscle (depending on the actual percentage).

Predictions of pharmacological activity could therefore be easily made based on the chemical composition, with the exception of essential oils containing 1 ,8-cineole e.g. *Eucalyptus globulus* (Table 2). The chemical predictions were largely similar to both the actual observed effect on the smooth muscle and also similar to the Aromatherapist's prediction on the patient. The latter effect was either a relaxant effect or a stimulant effect on the patient; the stimulant effect could be directly related to a contraction on the isolated muscle.

Table 2. Comparison of the actual effect of Essential oils on Guinea-pig ileum and the predicted effects using chemical composition and Aromatherapist's prediction

		Actual effect on tissue	Chem. prediction	Aromather. prediction
a.	Te tree	R & s/R	s/R	s/r
b.	Neroli	R	s/R	S/r
c.	Camomile German	R	R	R
d.	Frankincense	S	S	R
e.	Camphor	S	S	S
f.	Black Pepper	S/r	S/r	S/r
g.	Rosemary	S/r	S/r	S
h.	Lemongrass	R	R	S/r
i.	Juniper	R	S	S
j.	Lavender	R & s/R	R	R
k.	Bergamot	s/R	S/R	S/R
l.	Ylang Ylang	R	R	r
m.	Sandalwood	R	R	r
n.	Vetivert	R	R	R
o.	Petitgrain	S/r	R	S/R
p.	Rosewood	R	R	R
q.	Geranium Bourbon	R	R	R
r.	Eucalyptus globulus	R	S	S
s.	Clary Sage	S & S/r	R	r
t.	Ginger	R	s/R	s/r
u.	Dillweed	S/r	s/R	r
v.	Nutmeg	S	S	r
w.	Manuka	R	R	R
x.	Spikenard	R	R	r
y.	Camomile Roman	S/R & s/R	S/R	R
z.	Valerian	R	R	R

Mixtures of two or more essential oils also showed the same trend, some of which are shown in Table 3. This proves that contractions of smooth muscle are largely as a result of a high monoterpene concentration, regardless of the actual monoterpene component.

As before, the correlation broke down if 1,8-cineole was involved e.g. in mixtures with rosemary or *Eucalyptus globulus*. There is no easy explanation for this discrepancy. It is also of interest that if *Eucalyptus globulus*, containing 95% of 1,8-cineole is presented to the smooth muscle preparation it will cause a relaxation, whereas if 1,8-cineole alone is presented it causes a contraction.

Table 3. Comparison of the predicted effect of essential oil blends on clients by an Aromatherapist and by the chemical composition with their actual effect on Guinea-pig ileum

	Blend	Effects Predicted		Actual
1.	Orange 2: Nutmeg 1: Dill 1	S	S	s/R
2.	Lemongrass 1: Juniper 1: Rosemary 2	S	S	R
3.	Frankincense 1: Rose Abs. 1: Clary Sage 2	R/S	R/S	s/R
4.	Eucalyptus glob. 1: Black Pepper 1: Ginger 1	S	S	s/R
5.	Ginger 1: Tea Tree 1: Rosemary 2	S	S/R	S/R
6.	Frankincense 2: Ylang Ylang 1: Geranium 1	R/S	S/R	R
7.	Frankincense 1: Geranium 1: Bergamot 2	S/R	S/R	S/R
8.	Camomile Roman 1: Lavender 1: Geranium 1	R/S	s/R	S/R
9.	Frankincense 1: Mandarin 2: Scotch Pine 1	S/R	S	S/R
10.	Camomile Roman 1: Valerian 1: Rose Abs. 1	R/S	s/R	R
11.	Ylang Ylang 1: Marjoram 1: Thyme Red 1	R	s/R	R
12.	Petitgrain 1: Melissa 1: Sage Dalmatian 1	s/r	R	R
13.	Kanuka 1: Lavender 1: Frankincense 1	?R	S/r	R
14.	Manuka 1: Lavender 1: Frankincense 1	?S	s/R	R
15.	Fennel 1: Orange 1: Bergamot 1	S/r	S/R	S/R
16.	Basil 1: Bergamot 1: Clary Sage 1: Jasmine 1	S	s/R	S/R

CONCLUSIONS

The present results indicate that there is a very close correlation between the pharmacological activity of essential oils on the isolated smooth ileal muscle of the guinea-pig and the predicted effect on the human psyche. This "holistic" effect would probably have originated from the direct effect of essential oils on the CNS with the concomitant effect of the massage and of course counselling. The actual effect on the isolated smooth muscle is less complex and probably involves various adrenoceptors, but there could also be a simple direct action of components on the membrane with all monoterpenes initiating a rise in calcium levels which cause contraction of the muscle.

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THE EFFECT OF ESSENTIAL OILS ON THE UTERUS COMPARED TO THAT ON OTHER MUSCLES.

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INTRODUCTION

Aromatherapy is increasingly more popular and is being used indiscriminately at every possible occasion - including pregnancy and childbirth. Very few studies exist concerning the effect of essential oils on human pregnancy (apart from the toxic and supposedly abortifacient effects of a few selected examples taken in large doses eg. camphor, pennyroyal, parsley and juniper). No studies exist on the effect on babies. There is however a growing blase attitude towards the use of essential oils during pregnancy and parturition, simply because there has so far been no reported fatality or abnormality associated with the use of essential oils!

The main dangers lie in the first few months of pregnancy (during organogenesis) and during parturition whereby the essential oils may concentrate in the baby's brain and cause an anaesthetic effect resulting in the inability of the baby to breathe on delivery. There is another danger concerning the actual contractile effect on the uterine musculature itself. A recent Aromatherapy book (Price & Price, 1996) lists "uterotonic oils which facilitate delivery" giving no scientific references. These include fennel, bay, aniseed, clove bud and nutmeg which are all very strong and reactive oils and not the ideal ones to rub into the skin or bathe in. Many essential oils cause contact dermatitis (Rudzki et al.,1976) even when airborne (Schaller & Korting,1995). The safety to mother and child must therefore be considered further.

Clinical studies on the use of aromatherapy in childbirth (Burns & Blaney, 1994) were not conclusive, mainly because a large number of different essential oils were used at different times and in different ways and there was a bias towards the use of just a few oils eg. lavender and clary sage, the latter having a probably undeserved (and unproven) status of being oestrogenic. Essential oils are used during pregnancy for counteracting stretchmarks, healing cracked nipples and stretching the vulva, all of which could be probably be done equally effectively with just the carrier oil used in the massage mixture.

There is now scientific evidence for the effect of inhaled essential oils on the motility of mice (Buchbauer et. al., (1992), also the Cognitive Negative Variation ie. a direct effect on brain waves (Kubota et al. 1992; Torii et al., 1993) and mood (Warren & Warrenberg. 1993). However does this indicate that there is some beneficial use during delivery of a baby, other than relaxation?

Unlike other smooth muscle, the response of the uterus to both endogenous and exogenous compounds is affected by oestrogen and progesterone. The plasma levels of these two hormones change during the menstrual cycle and during pregnancy. Other hormones like prostaglandins and oxytocin have a direct effect on the uterus at childbirth. These are natural contractants and they may be influenced by many even simple chemicals eg. aspirin. The effect of essential oils on these hormones is unknown.

The effect of essential oils on smooth muscle has been studied by many authors, with often differing results (Reiter & Brandt, 1985; Lis-Balchin,1995; Lis-Balchin et al., 1996). As there has been no recent work on the uterus, the present study was initiated to show the effect of essential oils on the uterus compared with that on other muscles in vitro using guinea-pig and rat tissues.

MATERIALS AND METHODS

Pharmacological studies were carried out on guinea-pig ileum and rat ileum, caecum, vas deferens and uterus. Smooth muscle preparations were mounted in 25ml organ baths containing Krebs bicarbonate buffer at 34oC, gassed with 95% oxygen in carbon dioxide. Field stimulation was applied, when needed, by two parallel platinum electrodes placed either side of the tissue; these were attached to a stimulator (0.5ms

pulse, 0.1 HZ, 70v). Changes in tension were recorded with an isometric transducer attached to a pen recorder. Rat phrenic nerve-diaphragm preparations were suspended in 150ml organ baths.

Essential oils were obtained from various commercial sources and each oil was analysed by GC for future reference. The essential oils were diluted in methanol x 1,000 and 0.1 to 0.2 ml was applied to the tissue preparations in the organ bath giving a final dilution of x 200,000 to x 400,000 (ie. a concentration of 2.5 x 10-6 to 5 x 10-6). Solvent activity was nil at the highest volume used.

RESULTS

Table 1 shows the effect of components on the guinea-pig ileum and rat uterus applied at the same concentration. The effect on the uterus was constantly a reduction in spontaneous contractions, whilst some components showed a contractile effect on the ileum.

Table 1. Effect of components on rat uterus compared with guinea-pig ileum

Component	Effect	
	rat uterus	guinea-pig ileum
2-carene	R	R/S
(-) carvone	R	R
(+) carvone	R	R
1,8-cineole	R	S
p-cymene	R	S
farnesol	R	R
fenchone	R	R
geraniol	R	R
limonene	R	S
linalool	R	R
linalyl acetate	R	R
menthol	R	R
myrcene	R	S
nerol	R	R
a-pinene	R	S
sabinene	R	S
terpinen-4-ol	R	R
a-terpineol	R	R
a-terpinene	R	S
g-terpinene	R	S/R

R (uterus) = reduction in size (force) of pontaneous contraction; S (ileum) = spasmogenic action; R/r (ileum) = spasmolytic action ie. reduction in response of tissue to Electrical Stimulation.
0.1ml of solution applied in each case, diluted x 1000 with methanol

All essential oils studied alone at the same concentration had a constant reduction effect on spontaneous contractions in the uterus (Table 2),

Table 2. Effect of selected essential oils on rat uterus and guinea-pig ileum

Essential oil	Effect	
	uterus	ileum
Angelica	R	S
Camphor	R	S
Chamomile, Roman	R	S/R
Chamomile, German	R	R
Celery	R	S
Dill	R	S/r
Fennel	R	S
Frankinsence	R	S
Geranium	R	R
Kanuka	R	S/R
Lavender, French	R	s/R
Lemon	R	S
Manuka	R	R
Nutmeg	R	S
Orange	R	S
Parsley	R	S/R
Peppermint	R	R
Pineneedle	R	S/r
Rosemary	R	S/r
Thyme	R	R
Valerian	R	R

R (uterus) = reduction in size (force) of spontaneous contraction; S (ileum) = spasmogenic action; R/r (ileum) = spasmolytic action ie. reduction in response of tissue to Electrical Stimulation; r=smaller reduction

but often a different effect on the ileum. The trace for frankinsence (Fig.1) illustrates the contractile effect on guinea-pig ileum compared to the reduction in force of the rat uterus in both oestrus and pregnancy.

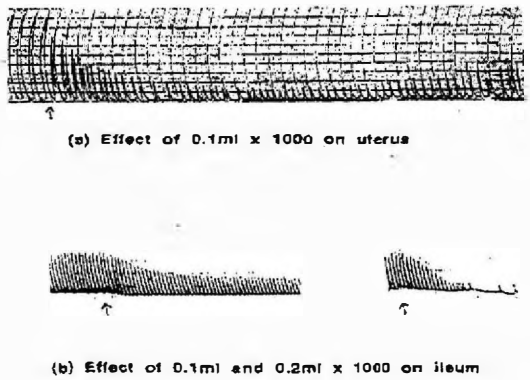

Figure 1. Effect of farnesol on rat uterus and guinea-pig ileum

Many essential oils eg. lavender showed diverse effects on different preparations of uterus at different stages of oestrus and preoestrus (not illustrated). Mixtures of two or more essential oils produced different effects in the two tissues (Table 3).

Table 3. Effects of blends of essential oils on rat uterus and guinea-pig ileum

Blend	Effect	
	Uterus	G-P ileum
Angelica 1: Nutmeg 1 : Roman camomile 1	R + S**	R/S
Angelica 1: Nutmeg 1 : Dill 1	R + S*	S/R
Basil 1: Bergamot 1: Clary Sage 1: Jasmine1	R	S/R
Clove bud 1: Caraway 1: Celery 1	R	S/R
Caraway : Aniseed 1: Cassia 1	R	S/R
Cinnamon 1: Coriander 1: Rosewood1	R	S/R
Camomile Roman 1: Dill 1: Angelica 1	R + S**	S/R
Dill 1: Aniseed 1: Rosemary 1: Angelica 1	R + S*	S/R
Dill 1: Orange 1: Kanuka 1	R	S/r
Frankincense 1: Geranium 1: Bergamot 2	R	S/R
Frankincense 2: Ylang Ylang 1: Geranium 1	R	R
Frankinsence 1: Rose 1: Clary Sage 1	R	S/R
Kanuka 1: Lavender 1: Frankincense 1	R	R
Marjoram 1: Jasmine 1: Palmarosa 1	R	R
Orange 2: Nutmeg 1: Dill 1	R	S/R
Rose Abs. 1: Geranium 2: Lavender	R	R/S
Valerian 1: Dill 1: Rosemary 1	R	R
Ylang ylang 1: Marjoram 1: Thyme 1	R	R

R (uterus) = reduction in size (force) of spontaneous contraction S (ileum) = spasmogenic action; R/r (ileum) spasmolytic action ie. reduction in response of tissue to Electrical Stimulation; S = Increase in rate/force of spontaneous contraction after wash-out; S** = Increase in rate/force of spontaneous contractions, even without wash-out, following initial spasmolytic action*

There was often a different effect according to the dose and the action of a single component was often different in a mixture eg. angelica root alone and in a mixture with nutmeg and dill (Fig.2).

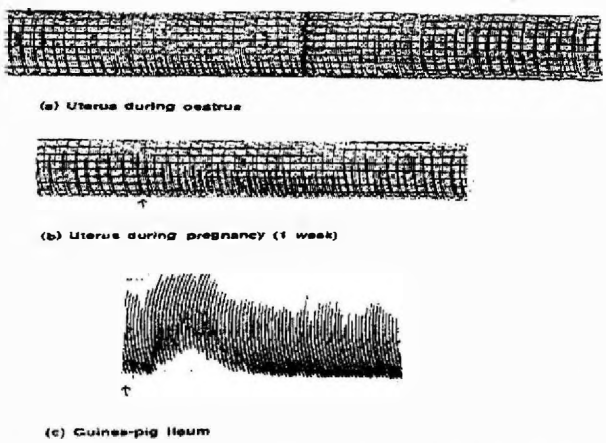

Figure 2. Effect of Frankinsence on rat uterus and guinea-pig ileum

On the other hand, angelica included in a slightly different mix showed a similar effect on the uterus and on the ileum, as contractions were produced in the latter and an increase in spontaneous contractions in the uterus (Fig.3).

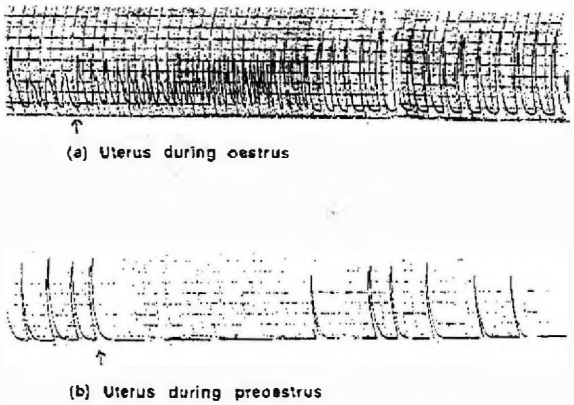

Figure 3. Effect of French Lavender on rat uterus

There is a profound difference in the pharmacological activity of essential oils on different tissues in different species (Table 4) and even different smooth muscle preparations in the same animal.

Table 4. Pharmacological action of essential oils on guinea - pig ileum, rat stomach, rat caecum and rat vas deferens *in vitro*

Essential oil	Ileum G-P	rat	Stomach rat	Caecum rat	Vas def. rat
Angelica root	***	!!	NA	!!!	! +
Aniseed	**!	!	!	!!! or *	! +
Camphor	***	!	!	!!!	NA
Celery	***	!	!	!!!	! +
Chamomile, blue	!!	!!!	!!!	!!	!!!
Clary Sage (a)	***	NA	!	!!!	!
Clary Sage (b)	**!	NA	!!	!	!!
Dill	++!	NA	!!	!!	! +
Fennel	***	!	!!!	!!	!!!
Frankinsence	***	!!!	!!!	!!!	NA
Geranium	!!	NA	!	!!!	!!
Jasmine abs.	!!	NA	!!!	NA	!
Kanuka	**!	!	!!!	!!!	**
Lavender French	!!	NA	!	NA	!! +
Lemon	***	!	nil+	!!!	nil+
Manuka	!!!	NA	NA	!!!	!
Nutmeg	***	!!!	!!	!!!	!!
Orange	***	!!!	!!	!!!	! +
Petitgrain	**	!!!	NA	!	NA
Parsley	**!	NA	!	!!!	!
Peppermint	!!!	NA	NA	!!	NA
Rose abs.	!	NA	!!	NA	!!!
Rosemary	***	!!!	!	!	!
Thyme	!	NA	!!!	!	NA

All diluted x 1000 except when marked + where diluted x 100

DISCUSSION AND CONCLUSIONS

The effect of essential oils, singly and as mixtures, and their components is almost always different on the uterus compared with the most sensitive smooth muscle preparation ie. guinea-pig ileum. There is a difference in the effect of the same essential oils during different phases of the rat oestrus cycle (which lasts only 3 days) and during pregnancy. There is also a difference in the effect of a single essential oil when presented alone or in a mixture.

The results suggest that there is no way of assessing in advance the effect of essential oils on the female uterus during pregnancy and parturition, due to the different effects shown on different tissues and the lack of information on extrapolation from *in vitro* animal studies to the *in vivo* effects in humans. Further research is urgently required before the usage of essential oils during pregnancy and parturition can be considered safe.

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EVALUATION OF INHIBITORY DATA OF ESSENTIAL OIL CONSTITUENTS OBTAINED WITH DIFFERENT MICROBIOLOGICAL TESTING METHODS

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INTRODUCTION: In vitro examinations of essential oils and of their constituents on their growth inhibiting properties against microorganism have been done in the past by use of different testing methods. Results obtained by these methods strongly depend on the applied experimental conditions, and therefore, their comparability is impaired in most of the cases. An evaluation of the antimicrobial efficiency of an examined compound becomes more or less complicated by these influences. To demonstrate the data variety, inhibitory data of eugenol – one on the best examined constituent found in essential oils – against *Eschericia coli* resulting from different test methods were chosen and compiled together including results from own investigations.

RESULTS: Eugenol inhibited growth of *E. coli* in all test systems used. With none of the different types of test methods strictly constant inhibitory data are reported.

Inhibitory zone formation is observed either with the paper disk technique (*Table I, Figure I*) or hole technique (*Table II, Figure II*) in agar diffusion test with eugenol. The size of the inhibitory zones – often taken as a measure for the efficiency of a compound – ranged from 12 to 26 mm. In comparison to neomycin, eugenol was more active by this method.

Table I. Inhibitory data of eugenol obtained in the agar diffusion paper disk test

Strain	Inhibition zone	Agar	Compound dose	Incubation time/T	Solubility enhancer	Reference compound	Reference
unspecified	12 mm	blood agar	moistened disk ?	18 h/37°C	none	none	Suresh et al., 1992
unspecified	13 mm	malat agar	9 mm disk, 20 µl	18 to 24 h/30°C	none	none	Weigand, 1986
unspecified	20 mm	pennassay base agar, seed agar	moistened disk ?	24 to 72 h/37°C	EtOH, acetone	none	Nadal et al., 1973
unspecified	21 mm	tryptone-yeast-glucose agar	9. 1 mm disk, 2 µl	18 to 24 h/37°C	EtOH	none	Morris et al., 1979
unspecified	25 mm	nutrient, potato-dextrose agar	10 mm disk, moistened ?	cited	Tween	none	Megalla et al., 1980

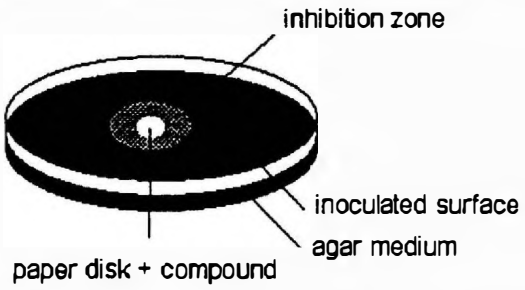

Figure I. Model of the agar diffusion paper disk test

Table II. inhibitory data of eugenol obtained in the agar diffusion hole test

Strain	inhibition zone	Agar	Compound dose	Incubation time/T	Solubility enhancer	Reference compound	Reference
NCIB 8879	18.5 mm	Iso-sensitest agar	4 mm hole, 10 µl	48 h/25°C	none	none	Deans et al., 1989
unspecified	26 mm	nutrient agar	10 mm hole, 100 µl	72 h/30°C	none	none	Dey, 1984
unspecified	1.2-fold rel. to neomycin	Diagnostic sensitivity test agar	7 mm hole, ? µl	24 h/°C27	Tween 20, DMSO	neomycin	Laekeman et al., 1990

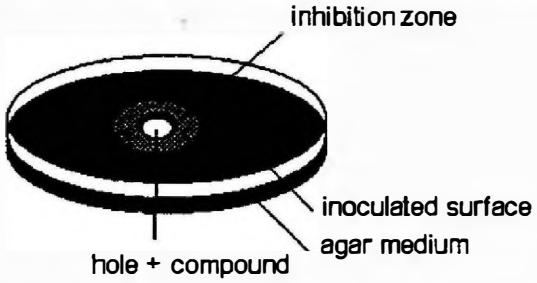

Figure II. Model of the agar diffusion hole test

Results obtained with the agar dilution technique revealed growth inhibition by eugenol from 10 to 1000 mg/l (*Table III, Figure III*).

Table III. Inhibitory data of eugenol obtained in the agar dilution test

Strain	MIC in mg/l	Agar	Inoculum size	Incubation time/T	Solubility enhancer	Reference compound	Reference
UPR	10	nutrient agar	not given	24 to 72 h/37°C	EtOH, acetone	none	Nadal et al., 1973
unspecified	500	tryptic soy agar	10 E5/ml	cited	Tween 80	none	Prudent et al., 1993
unspecified	500	cited	cited	cited	cited	cited	Katayama et al., 1960
Migula, 1895	250 - 500 66% inhibition	meat extract, peptone agar	loopful	24h/37°C	acetone, glycol	phenol, hexa-chlorophen	Weuffen et al., 1970
Migula, 1895	1000 ("MMC")	meat extract, peptone agar	loopful	24h/37°C	acetone, glycol	phenol, hexa-chlorophen	Weuffen et al., 1970

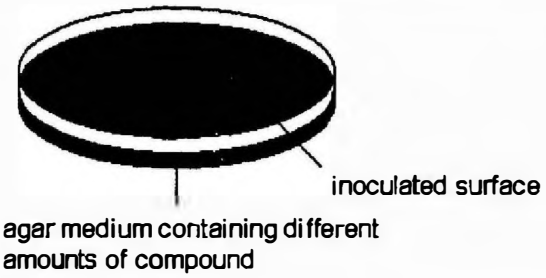

Figure III. Model of the agar dilution test

MIC data obtained with eugenol in serial dilution tests ranged from 63 to 600 mg/l (*Table IV, Figure IV*), and MMC data from 500 to 3000 mg/l (*Table V, Figure IV*). Comparison of the relative efficiency of eugenol has been done with phenol and naladixinic acid.

Table IV. Minimal inhibitory concentrations of eugenol obtained in the serial dilution test

Strain	MIC in mg/l	Broth	Inoculum size	Incubation time/T	Solubility enhancer	Reference compound	Reference
unspecified	63	nutrient	10 E5/ml	48 h/30°C	DMSO	none	Jay et al., 1984
ATCC 23716	296 (ED_{50})	meat extract, casein peptone	adjusted by optical density	4 h/37°C	without ?	phenol	Barelmann, 1994
NCTC 363	390	nutrient	microbial suspension	48 h/37°C	Tween 20	none	Yousef et al., 1979
ATCC 9673	400	nutrient, yeast extract, glucose	adjusted by optical density	48 h/37°C	DMF	none	Kubo et al., 1991
unspecified	500	tryptone-yeast-glucose	300000 viable organisms	18 to 24 h/ 37°C	EtOH	none	Morris et al., 1979
ATCC 11229	500	nutrient	microbial suspension	24 h/37°C	none	none	Maruzzella et al., 1961
ATCC 25922 mutant IV	550	meat extract, casein peptone	100 - 300 CFU /ml	18 h/37°C	Tween 20	naladixinic acid, phenol	Pauli, 1994
ATCC 25922	550	meat extract, casein peptone	100 - 300 CFU /ml	18 h/37°C	Tween 20	phenol	Pauli, 1994
clinical isolate	600	meat extract, casein peptone	100 - 300 CFU /ml	18 h/°7°C	Tween 20	phenol	Pauli, 1994
ATCC 25922	4000 (survivors)	63% sucrose, bovine serum	10 E7/ml	2 min./37°C	PEG 400	none	Briozzo et al., 1989

Table V. Minimal microbicidal concentrations of eugenol obtained in the serial dilution test

Strain	MMC in mg/l	Broth	Inoculum size	Incubation time/T	Solubility enhancer	Reference compound	Reference
ATCC 23716	500 (approx.)	meat extract, casein peptone	adjusted by optical density	4 h/37°C	without ?	phenol	Barelmann, 1994
ATCC 25922	700	meat extract, casein peptone	100 - 300 CFU /ml	18 h/37°C	Tween 20	phenol	Pauli, 1994
ATCC 25922 mutant IV	750	meat extract, casein peptone	100 - 300 CFU /ml	18 h/37°C	Tween 20	naladixinic acid, phenol	Pauli, 1994
NCTC 363	780	nutrient	microbial suspension	48 h/37°C	Tween 20	none	Yousef et al., 1979
clinical isolate	800	meat extract, casein peptone	100 - 300 CFU /ml	18 h/37°C	Tween 20	phenol	Pauli, 1994
unspecified	1000	Merck, Standard 1-Nährbouillon	microbial suspension	24 h/37°C	ultrasonic	phenol	Münzing et al., 1980
CCM 180	3000	mercapto acetate	10 cells/ml	cited	DMSO	none	Zemek et al., 1979

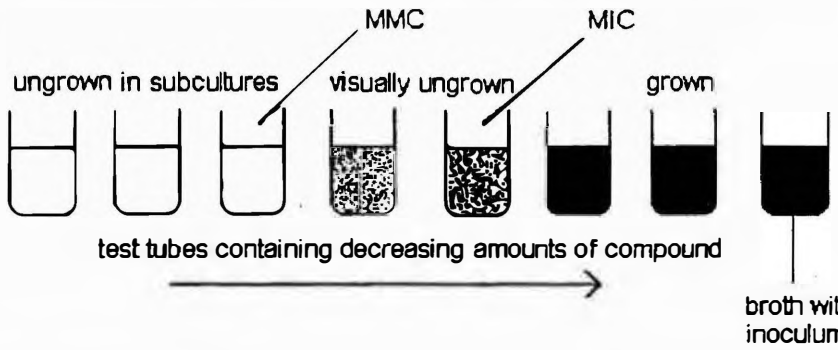

Figure IV. Model of the serial dilution test

Eugenol vapors are capable to inhibit growth of *E. coli* (*Table VI*).

Table VI. Inhibitory data of eugenol obtained in vapor phase activity tests

Strain	Inhibition	Agar, inoculation	Compound dose	Incubation time/T	Solubility enhancer	Reference compound	Reference
unspecified	+++	dextrose, *E. coli* on surface	4 drops, inverted plate	24 h/37°C	none	none	Kellner et al., 1955
ATCC 11229	25 mm	nutrient, *E. coli* on surface	0.5 ml, inverted plate	48 h/37°C	5% glycerol	none	Maruzzella et al., 1961
IAM 1239	surface: no growth	agar included *E. coli* cells	20 mg, chamber	24 h/37°C	none	none	Gocho, 1991

DISCUSSION: Results obtained with the agar diffusion test depend — beside the inhibitory activity of a test compound — on the ability of the test compound to move through the agar medium. Diffusion rates of essential oil constituents in the agar medium are generally unknown, and therefore, quantitative comparison of inhibitory zones, as usually done with antibiotics, is critical (see *Table I*). Inhibitory data of eugenol varied markedly, which can be explained through the influence of various factors (7), e.g., disk size, amount of compound applicated to disk, agar type, agar content, pH, volume of agar, and type of microbial strains used.

The agar dilution test requires 75 mg of a compound for four dilutions steps (2000, 1000, 500, 250 mg/l) by use of agar layers with 20 ml volume. In contrast, the need of compound at the 2000 mg/l dose is 100-times lower in the serial dilution test with 12 dilution steps as it was used in the own examinations. In addition, the serial dilution test allows to compare inhibitory data with well-known antimicrobials, e.g., phenol or naladixinic acid, an actual antibiotic compound. The deviations of MIC and MMC data of eugenol against *E. coli* were tolerable: in the mean MIC values ranged from 250 to 600 mg/l and MMC from 500 and 1000 mg/l, which seem to be a consequence of different test modifications used in the listed examinations. Therefore, it appears to be promising to consider a standardization of this method for testing essential oils and their constituents.

CONCLUSION: Among the methods used to examine the in vitro antimicrobial activity of essential oil components, the serial dilution technique appears to be the most promising one. This method yields two types of inhibitory data, the MIC and MMC from subcultures. Additionally, this method allows to work with very low amounts of test substances and enables comparison with clinically relevant antibiotics. A standardized serial dilution test should reflect the following factors:

1) Registered microbial strains
2) Controlled counts of inoculated microorganisms
3) Appropriate culture media for each type of microorganisms
4) Defined inoculation time and inoculation temperature
5) Appropriate solubility enhancer, e.g. Tween 20
6) Evaluation and control of inhibitory data by use of antibiotics
7) Testing of short-time inhibitory activity
8) Recording of ED_{50} inhibitory data

EXPERIMENTAL: Microbial strains used were *Eschericia coli* ATCC 25922, *E. coli* ATCC 25922 mutant IV and one clinical isolate.

Compound dilution: 10% stock solution of eugenol was prepared with Tween 20 (1/4 v/v). Microtiter plates containing 8 series of 12 tubes were taken. A defined volume of the stock solution was given to the first test tube of a series and Mueller-Hinton Bouillon (MHB) was added to give 250 µl liquid volume. 200 µl thereof were transferred to the second tube and MHB was added to 250 µl. This procedure was repeated till the last tube.

Inoculation: *E. coli* strains were grown overnight at 37°C in MHB. The microbial suspension was adjusted to 0.5 McFarland turbidity standard and was then diluted five times 1:10 containing at least 100 to 300 colony forming units (CFU) per ml. 25 µl of this suspension were added to each test tube. The plates were incubated for 16 to 18 hours at 37°C.

MIC and MMC determination: the minimal inhibitory concentration (MIC) was taken from the lowest dosed test tube showing visually no growth. A 10 µl portion of each visually ungrown test tube was transferred to Mueller-Hinton agar medium. These subcultures were incubated overnight for controlling presence or absence of growth. The minimal microbicidal concentration (MMC) was taken from the lowest dosed test tube showing no growth in subcultures.

Control of inhibitory data: the experimental conditions, such as test medium and the number of bacteria determined by CFU/ml, were appropriate to reproduce inhibitory data of a known antibiotic (naladixinic acid). The antibiotic was used as reference compound in each test series.

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ESSENTIAL OILS AND ANTIBACTERIAL ACTIVITY AGAINST *HELICOBACTER PYLORI*

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INTRODUCTION

Since the beginning of the 1980s, there has been a deep revision on in the understanding of gastroduodenal pathology, thanks to the discovery of *Helicobacter pylori* (1). It is a Gram-negative bacterium able to colonize the human stomach even in conditions of normal acidity. This is due to its capacity to penetrate under the mucus layer and adhere to the epithelial surface.

Its prolonged contact with the gastric mucosa induces the frequent development of chronic active gastritis that, over the years, can became atrophic (2,3). This is a condition which leads to high risk of gastric cancer *(4)*. Furthermore, *H. pylori* infection is directly correlated with the development of duodenal ulcer and lymphoma-type Malt of the stomach (5). H. *pylori* infection occurs early in life and can persist for a lifetime if the subject is not treated with target therapy (6).

The current therapy against *H. pylori* consists only of antibiotics (i. e. ampicillin, metronidazole, claritromicine) or antacid drugs (omeprazole, lansoprazole) (7). However, it is not excluded that some natural substances can have antibacterial activity against *H. pylori,* e.g. derivatives of certain aromatic plants which have bacteriostatic activity towards other bacterial species.

The object of the study reported here is to evaluate the ability of essential oils to inhibit the *in vitro* growth of *H. pylori.*

EXPERIMENTAL

Bacterial isolates

Two strains of *H. pylori* freshly isolated from a patient with a duodenal ulcer were utilized. Two antral gastric bioptic specimens were homogenized with Ultraturrax (20000 rpm) in cold water, cultivated in selective medium (Columbia Agar added with 10 % horse serum, 0.01 % hemine, 10 mg/l amphoteicin B and 6 mg/l of vancomycin) and incubated at 37 °C in an anaerobic jar with Campy-PAK plus (BBL, Microbiology System Lockeysville, HD) for 6-7 days. The colonies were transferred in liquid medium (BHI added with 0.1 % yeast extract and 10 % horse serum). *H. pylori* was identified by morphology and biochemical tests (urease and oxidase positive).

Assay of bacterial growth inhibition

Minimal inhibitory concentration (MICs) were determined by broth dilution method. Oils were prepared as a 10 % (v/v) solution in ethanol and added in the range 200-2000 ppm to BHI with yeast extract (0.1 %) and horse serum (10 %). One hundred microlitres of this solution were distributed in each well of a Corning cell wells plate and inoculated with 10 µl of 2-day-old culture by "Stepper™ Repetitive Pipette". Inoculated plates were incubated for 3-4 days in microaerophily. Growth was evaluated by the amount of cells on the bottom of the microwell.

Oil isolation and analyses

The essential oils were obtained by steam distillation of plant material in a Clevenger-type apparatus. The percentage range of the main components of some essential oils tested are reported in Table 1. The oils were stored in a dark glass bottle at 4 °C. Chromatographic profiling of the oils was performed on a Carlo Erba HRGC gas chromatograph under the conditions reported by Marotti and Piccaglia (8). The identification of compounds was performed by comparison of their retention times with those of pure substances and with those reported by Adams (9), by peak enrichment with standards and by mass spectrometric data.

RESULTS AND DISCUSSION

In all repeated tests *H. pylori* showed a resistance to the highest concentrations of all the essential oils tested with the only exceptions being cinnamon and clove oils, the two oils with a high percentage of eugenol (Table 1). These data agree somewhat with previous studies which reported a low activity of

essential oils against Gram-negative as opposed to Gram-positive strains (10). In our experience, only cinnamon and clove oils showed activity against *H. pylori* with MICs of 800 and 1800 ppm, respectively. Geranium, juniper, oregano, Spanish oregano, winter savory and thyme oils are only able to delay the growth. In general cinnamon exhibits high antibacterial activity at 400 ppm with an inhibition of 80 % of 60 strains belonging to *Bacillus, Clostridium, Lactobacillus, Bifidobacterium* and other genera (11). Similar results were obtained when clove oil was used but only at higher concentrations. The active component against *H. pylori is* still unknown although eugenol may play an important role as is the case with other bacteria (12) Today, antibiotic therapy against *H. pylori is* effective only in 80 % of treated patients and this is due to the fact that MIC of the antibiotics used (ampicillin, erythrocin, ciprofloxacin) changes in accordance with gastric pH. In the different parts of the stomach there is an uneven distribution and dissociation of the antibiotics themselves due to pH changes. Unfortunately, there is evidence that *H. pylori is* developing higher resistance to the nitroimidazoyl antibiotics, which are not affected by variation of intragastric pH. For this reason the essential oils, which are active against *H. pylori,* could play an important role in future therapy. Moreover, some epidemiological aspects of *H. pylori* infection seem to suggest a correlation with the dietary use of some officinal plants (13).

There is evidence of oral transmission of *H. pylori* through water or food, although no direct studies have been focused on the presence of the bacterium in food products. On the contrary, the use of some aromatic plant might prevent the initial steps of infection. In fact, populations with a high onion consumption show a great reduction of gastric cancer (13). Since *H. pylori* infection is correlated with risk of gastric cancer, it can be hypothesized that the anticancer action of onion can, in part, be related to its bactericidal activity.

Table 1. Main components of some essential oils tested (*range % w/v*)

Clove		**Geranium**		**Sage**		**Sweet fennel**	
eugenol	82-85	linalool	10-14	1,8-cineole	5-13	limonene	2-6
caryophyllene	12-14	citronellol	24-26	α-thujone	36-46	fenchone	1-2
		geraniol	11-13	β-thujone	4-10	*trans*-anethole	86-91
Oregano		citronellyl formate	7-9	camphor	16-21		
p-cymene	7-9	geranyl formate	3-7			**Bitter fennel**	
y-terpinene	14-16			**Rosemary**		limonene	2-7
carvacrol	54-58	**French tarragon**		α-pinene	9-30	fenchone	5-10
		α-pinene	1-2	borneol	1-8	*trans*-anthole	82-90
Winter savory		*cis*-ocimene	5-9	1,8-cineole	14-50		
p-cymene	11-16	*trans*-ocimene	5-9	camphor	7-22	**Cinnamon**	
y-terpinene	1-6	methyl chavicol	73-82			eugenol	70-90
carvacrol	26-41			**Lavender**		cinnamaldehyde	1-2
		Peppermint		1,8-cineole	2-9	benzyl benzoate	3-5
Thyme		1,8-cineole	5-6	linalool	28-30	caryophyllene	2-5
p-cymene	19-25	menthone	25-28	camphor	4-8		
y-terpinene	12-13	menthofuran	3-5	linalyl acetate	21-30		
thymol	17-38	menthol	45-47				
		pulegone	3-4				

ACKNOWLEDGMENT

The excellent technical assistance of M. Albeltoni is gratefully acknowledged. This research, supported by a grant of the National Research Council, is part of a project on *"Biological activity of vegetable substances"*.

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GROWTH INHIBITION OF ESSENTIAL OILS AND OTHER ANTIMICROBIAL AGENTS TOWARDS BIFIDOBACTERIA FROM DENTAL CARIES

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INTRODUCTION

Three species of bifidobacteria have so far been found in the human dental caries. *Bifidobacterium dentium* was described in 1974 (1); *B. inopinatum* and *B. denticolens* were only recently isolated and characterized (2). *B. dentium* is also present in the colon of infants and adults and in the human vagina while there is no evidence of the presence of the other two species in other anatomic sites.
The pioneering work on the antimicrobial sensitivity of the genus *Bifidobacterium* was done on a limited number of species (3–5). The first study of the antimicrobial susceptibility of 34 strains related to seven species of *Bifidobacterium* was conducted by Miller and Finegold (6). In 1983, a wide study was reported on the *in vitro* effect of 16 antimicrobial agents against 459 strains of bifidobacteria, which referred to 15 species of human and animal origin (7). All the species tested show high sensitivity to penicillin, erythromycin, clindamycin and vancomycin, but resistance to metronidazole which normally is active against the obligate anaerobes. Essential oils have widespread use in oral hygiene and dentistry but data about their effects on fastidiously and facultatively anaerobic oral bacteria, have only recently been published (8).

The purpose of this study was to investigate the antimicrobial susceptibility of the bifidobacteria from dental caries for their further characterization and to acquire some knowledge for a possible application in dental pathology.

EXPERIMENTAL

Assessment of inhibition properties of antibiotics

A total of 16 bacterial strains, out of 48 isolated from human dental caries, were studied for their susceptibility to 16 antimicrobial substances: 8 strains of *B. inopinatum* and 8 of *B. denticolens,* selected from different samples of dental caries. Seven strains of *B. dentium* were also studied for vancomycin, clindamycin, gentamicin, lincomycin and metronidazole, which were not considered in the previous study on the antimicrobial susceptibility of the genus *Bifidobacterium* (7).

The minimal inhibitory concentrations (MICs) were determined by means of a dilution micromethod in TPY liquid medium (9), using microtitre plates (96 wells, with round bottoms, diameter 6.4 mm; Corning, Laboratory Sciences Company, New York), supplied with a lid. The 16 laboratory-standard powders tested were: penicillin G, tetracycline, chloramphenicol, erythromycin, bacitracin, neomycin, streptomycin, kanamycin, polymyxin B, gentamicin, vancomycin, lincomycin, clindamycin, metronidazole, nitrofurantoin and nalidixic acid, all purchased from Sigma (St. Louis, MO, USA).

The antimicrobial agents were weighed, dissolved, diluted in appropriate diluents and filtered (Millipore 0.22 µm), and then added to TPY medium, which was adjusted to pH 7.0–7.2, and were dispensed into wells of microtitre plates (0.2 ml/well) by a Multipette 4780 Eppendorf equipped with an 8-channel dispenser (Eppendorf, USA). For each strain, control wells without the antibiotic were considered as a positive test of growth. A 5.0 µl sample of a 24 h anaerobic culture in TPY

(10^6 cells/ml), diluted 1:10, was used as inoculum. Microtitre plates incubated anaerobically in Gas-Pak jars (BBL) for 48 h at 37°C were set up in duplicate. Growth was determined by the amount of cell sediment, visibly evaluated, and by the final pH values.

The MIC was the lowest concentration of compound which prevented visible growth and did not lower the pH. The MICs were subjected to correlation and linear regression analysis. The MIC_{50} and MIC_{90}values were the lowest concentrations of antibiotic which inhibited the growth of at least 50% and at least 90% of the strains tested, respectively.

Assessment of inhibition properties of essential oils

The effect of 20 essential oils (Table 1) from aromatic plants typical of the Italian Mediterranean flora as well as from species which originated in tropical countries were surveyed on 16 strains of bifidobacteria from dental caries. The essential oils were obtained by steam distillation of plant material in a Clevenger-type apparatus. The methods used to test the inhibitory activity of the oils were the same as used for antibiotics with some adaptation due to the different nature of the components. Oil samples were prepared as 10% (v/v) solutions in 95° ethanol due to the insolubility of the oils and were stored at 4°C. Oil concentration ranged from 200 to 2000 ppm with intervals of 200 ppm. Microtitre test plates were used to determine MICs in TPY growth medium. Inoculated plates were incubated for 3–4 days in anaerobic conditions.

RESULTS AND DISCUSSION

The sensitivities to antibiotics of the bifidobacteria tested are shown in Table 2. Among the six antibiotics with a Gram-positive spectrum, clindamycin, erythromycin, lincomycin, penicillin G and vancomycin were the most effective antimicrobials, while bacitracin was the least effective of the group. *B. dentium* seems to be almost always less sensitive, except to bacitracin.

Within the broad-spectrum antimicrobial agents, chloramphenicol and tetracycline were similarly effective against the three species with MIC_{90} ranging between 4.7 and 8.9; gentamicin was active at these values but only towards *B. inopinatum*, whereas it was less effective against *B. denticolens* and *B. dentium*. The strains of the two new species studied were quite resistant to kanamycin, neomycin and nitrofurantoin with MIC_{90} 25.6–169 μg/ml, while *B. dentium* was more resistant to kanamycin and neomycin. The comparison between the three species showed a wide range of MIC_{90} values for gentamicin, kanamycin and neomycin. The compounds with a Gram-negative spectrum, such as nalidixic acid and polymyxin B, showed low activity against the three species. Nalidixic acid inhibited 90% of all strains between 364 and 821 μg/ml; polymyxin B presented a wide diversity of MIC_{90} values between *B. inopinatum* (271 μg/ml) on the one hand and *B. denticolens* and *B. dentium* (>1000 μg/ml) on the other. Metronidazole showed very low activity towards *B. inopinatum* (MIC_{90} 848 μg/ml); in contrast *B. denticolens*, and in particular *B. dentium*, appeared to be more sensitive (MIC_{90} 229 and 17 μg/ml, respectively). The two new species were quite sensitive to streptomycin (MIC_{90} about 4 μg/ml) while *B. dentium* showed more resistance (MIC_{90} 146 μg/ml).

The results of our study on the antimicrobial susceptibility of *B. inopinatum* and *B. denticolens*, the two new species isolated from human dental caries, demonstrated that clindamycin, penicillin G, erythromycin, lincomycin, vancomycin, bacitracin and streptomycin were the most active antimicrobial agents. In addition chloramphenicol, tetracycline and, to a lesser degree, kanamycin and nitrofurantoin showed high activity. Gentamicin and neomycin were active only against *B. inopinatum*.

The two new species showed a similar behaviour towards clindamycin, penicillin G, vancomycin and kanamycin. *B. inopinatum* was more resistant to erythromycin, lincomycin and metronidazole and more sensitive to the remaining nine antimicrobials, when compared with *B. denticolens*.

In contrast to the two new species, *B. dentium* was more sensitive to bacitracin, chloramphenicol, tetracycline and metronidazole; it showed a similar response to

vancomycin and, alternatively against either species, to gentamicin, nitrofurantoin, polymyxin B and nalidixic acid. Furthermore, *B. dentium* showed more resistance to the other seven antimicrobiotics tested. The minimal resistance of *B. dentium* against metronidazole was noteworthy; in contrast it had very low levels of activity against *B. inopinatum* and *B. denticolens* and the five species of bifidobacteria of human origin previously tested (7). Therefore, we suggest that metronidazole cannot always be used for identification of the genus as indicated by Essers (10) and confirmed by Matteuzzi et al. (7).

Our results on the susceptibility of *B. inopinatum* and *B. denticolens* agree with those obtained previously (7) for the species of human origin, for seven of the antibiotics studied, whereas for the remaining nine the two new species are more sensitive.
B. inopinatum and *B. dentium* exhibit the same behaviour when tested for resistance to essential oils. Ten of the 20 oils tested were more active against *B. denticolens* than the other two species (Table 1). Cinnamon oil was the most effective with MICs of 200–400 ppm. Basil, clove, geranium, winter savory, oregano and Spanish oregano have shown an inhibitory effect at low concentrations against most of the strains tested while boldo, cypress, French tarragon, bitter fennel, sweet fennel, juniper, lavender "Abrialis", lavender "grosso", lavender "Super A", peppermint, rosemary, sage, and thyme possess antibacterial properties with different degrees of effectiveness.

The role of bifidobacteria in the formation of dental caries is still a subject of discussion. Their presence in a very high percentage of the samples examined (unpublished data) is an index of the importance of this genus in the development and/or progression of dental disease.

Table 1. Antibacterial activity of 20 plant essential oils to three species of bifidobacteria from dental caries.

ESSENTIAL OILS	*B.INOPINATUM* (6)[a]	*B.DENTICOLENS* (6)	*B.DENTIUM* (4)
BASIL	1000 - >2000[b]	400 - 600	1400 - >2000
BOLDO	>2000	1600 - 2000	>2000
CINNAMON	<200 - 400	<200 - 400	400
CYPRESS	>2000	>2000	>2000
FRENCH TARRAGON	1800 - >2000	600 - 1200	>2000
BITTER FENNEL	>2000	>2000	N.T.[c]
SWEET FENNEL	>2000	N.T.	>2000
CLOVE	800 - 1400	600	1000 - 1400
GERANIUM	400 - 1400	400	1000 - 1400
JUNIPER	>2000	>2000	>2000
LAVANDER "ABRIALIS"	>2000	>2000	>2000
LAVANDER "GROSSO"	>2000	600 - 1000	>2000
LAVANDER "SUPER A"	>2000	N.T.	>2000
PEPPERMINT	>2000	600 -1000	1600 - >2000
OREGANO	600 - 1200	400 - 600	800 - 1000
SPANISH OREGANO	600 - 800	400 - 600	800
ROSEMARY	>2000	>2000	>2000
SAGE	>2000	>2000	>2000
WINTER SAVORY	400 - 1000	400	600 - 1000
THYME	1000 - 2000	600 - 800	400 - >2000

[a]In brackets the number of strains tested. [b]Range of MICs (minimal inhibithory concentrations) in ppm. [c]N.T. Not tested.

Table 2. Comparative *in vitro* activities of 16 antimicrobial agents against three species of the bifidobacteria isolated from dental caries

Species	No. of strains tested	MIC range	MIC_{50}[a]	MIC_{90}[a]	No. of strains tested	MIC range	MIC_{50}	MIC_{90}
	Bacitracin[b]				Clindamicin			
B. inopinatum	8	2.5–5[c]	3.3	4.5	8	0.05	0.05	0.05
B. denticolens	8	6	6	6	8	<0.01–0.07	0.03	0.06
B. dentium	7	0.8–1.5	<0.8	1.3	7	0.03–0.6	0.18	0.45
	Lincomycin				Penicillin G[b]			
B. inopinatum	8	0.3–0.5	<0.3	0.42	8	0.05	0.05	0.05
B. denticolens	8	<0.01–0.15	0.04	0.12	8	<0.01–0.05	0.023	0.046
B. dentium	7	0.1–0.7	0.2	0.54	8	0.3	0.3	0.3
	Chloramphenicol[b]				Gentamicin			
B. inopinatum	8	5–7	5.9	6.8	8	4–6	4.4	5.7
B. denticolens	8	5–9	6.5	8.5	8	80–120	93.3	111
B. dentium	7	5–6	5.1	5.8	7	45–100	71.9	97
	Neomycin[b]				Nitrofurantoin[b]			
B. inopinatum	8	20–80	20.1	61	8	5–30	10.4	25.6
B. denticolens	8	70–>150	122	169	8	5–50	22	46
B. dentium	8	150–500	187	413	8	15–60	22	44
	Nalidixic acid[b]				Polymyxin B[b]			
B. inopinatum	8	200–400	273	364	8	50–350	113	271
B. denticolens	8	600–850	691	821	8	400–1100	756	1103
B. dentium	7	400	400	400	6	>1000	>1000	>1000
	Metronidazole				Streptomycin[b]			
B. inopinatum	8	700–900	720	848	8	2–3.5	2.6	3.2
B. denticolens	8	150–250	<150	229	8	3–5	3.7	4.7
B. dentium	7	10–20	10	17	8	100–300	<100	146
	Erythromycin[b]				Kanamycin[b]			
B. inopinatum	8	0.05–0.2	0.08	0.16	8	30–60	30.9	53
B. denticolens	8	<0.01–0.05	0.03	0.034	8	20–70	27	60
B. dentium	8	0.1–0.5	0.21	0.43	8	200–>1500	261	501
	Vancomycin				Tetracycline[b]			
B. inopinatum	8	0.9–1	0.9	0.98	8	2–7	3.8	5.9
B. denticolens	8	0.7–1	0.87	1.02	8	4–6	4.6	8.9
B. dentium	7	0.6–0.8	0.67	0.78	7	3–5	3.6	4.7

[a]MIC of 50% and 90% of strains studied, respectively. [b]*B. dentium* data from Matteuzzi et al. (7).[c]Concentrations in μg/ml.

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CHEMICAL AND BIOLOGICAL STUDIES OF THE ESSENTIAL OILS OF FOUR *HELICHRYSUM* SPECIES OF GREEK ORIGIN

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INTRODUCTION

As a part of a systematic research on the chemical composition of Greek *Helichrysum* species, we present in this study the chemical constituents of the essential oils obtained from the aerial parts of four *Helichrysum* species. The genus *Helichrysum* comprises 16 species belonging to the family of *Asteraceae* (Clapham et al. 1976) the 7 of which are naturalised in Greece. The 4 studied species are: *Helichrysum amorginum Boiss. et Orph. in Boiss.*, *H. italicum (Roth) G. Don fil. in Loudon ssp. italicum*, H. *stoechas (L.) Moench ssp. barrelieri (Ten.) Nyman* and *H. taenari Rothm.* All of them are dwarf shrubs with yellow flowers, growing in dry places. The first two species (*H. amorginum* and *H. italicum*), were collected in the Greek island of Aegean Amorgos, where the first one is endemic, *H. stoechas* was collected in the region of Attiki (Mount Imittos) and *H. taenari* was collected from south Peloponesus (cape Taenaron) where is endemic.

Species of the genus *Helichrysum* have been used in folk medicine as antimicrobial, anti-inflammatory, digestive and choleretic agents (Rios et al. 1991, Recio et al. 1990).

EXPERIMENTAL

Plant Material

All four plants were collected during their flowering period (June-July). *Helichrysum amorginum Boiss. et Orph. in Boiss.* and *H. italicum (Roth) G. Don fil. in Loudon ssp. italicum* were collected from the Aegean Island of Amorgos, where the latest is endemic, *Helichrysum stoechas (L.) Moench ssp. barrelieri* was collected from the rocky open area of the mountain Imittos (region of Attiki). *H. taenari Rothm.* was collected on the limestone rocks of Mani Peninsula, in South Greece (Peloponesus, Cape Taenaron) where is endemic taxon. Voucher specimens of all species have been deposited in the Herbarium of the Laboratory of Pharmacognosy, University of Athens

Methods and Analyses

The plant material was cut in small pieces and hydrodistilled for 2 hr with a water cooled receiver, to reduce hydrodistillation overheating artifacts. The essential oils were analyzed by GC and GC-MS. The identification of the chemical constituents was based on NBS/NIST and with comparison of their R_ts and mass spectra with those obtained from authentic samples and/or Wiley libraries spectra.

The bacteriostatic activities of the oils were determined, using the dilution technique (Janssen et al. 1987), by measuring the minimal inhibition concentration (MIC) of the oils against the above shown Gram (±) bacteria. Standard antibiotics (netilmicine, and amoxicilline with clavulanique acid) were used in order to control the sensitivity of the test organisms.

RESULTS AND DISCUSSION

GC and coupled GC-MS analyses of the oils led to the identification of the investigated Helichrysum species components (Table 1).

The major contributors out of the 24 constituents (82.06%) of the oil of *H. italicum* were: geraniol (35.59%), geranyl acetate (14.69%) and nerolidol (11.86%). 25 constituents, were identified in the oil of *H. amorginum* (89.98%). The main ones were: geraniol (32.11%), geranyl acetate (20.76%) and neryl acetate (17.54%) (Chinou et al. 1996).The essential oil obtained from *H. stoechas* showed the occurrence of 24 constituents which were identified and quantified representing 73.87% of the total oil. The major metabolites were: α-caryophyllene (15.56%), β-elemene (13.11%) and benzyl benzoate (5.69%). The essential oil isolated from *H. taenari* had a high percentage of geraniol (49.99%), camphene (18.63%) and α-pinene (8.91%) out of its 17 constituents (87.41%).

The essential oils of *H. amorginum*, *H. italicum* and *H. taenari* were found to be dominated by oxygenated monoterpenes whereas the main contributors in the oil of *H. stoechas* were oxygenated and non-oxygenated sesquiterpenes. Through the antibacterial screening, (Table 2) the oil of *H. amorginum* and *H. taenari* exhibited the strongest activity against all the six tested bacteria, while the oil of the two other taxa *(H. italicum ssp. italicum, H.stoechas ssp. barrelieri)* showed weaker ones.

The bacteriostatic properties of *H. amorginum*, *H. italicum* and *H. taenari*, is suspected to be associated with the significant contribution of the oxygenated monoterpenes geraniol, geranyl and neryl acetates, which are known to possess strong antibacterial activity (Knobloch et al. 1989). The strong activity of *H. stoechas* especially against the two *Staphylococcus* strains is probably due to the presence of camphene, which has been found, from antibacterial screenings, to exhibit a similar activity against Gram positive bacteria (Knobloch et al. 1989, Hinou et al. 1989).

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Acknowledgements

The authors wish to thank Dr. Kit Tan of the University of Copenhagen Denmark, for the collection of Helichrysum taenari, as well as for the taxonomic identification of the specimen.

Table 1. Chemical composition of the four investigated *Helichrysum* species

Compounds	*H. amorginum*	*H. italicum*	*H. stoechas*	*H. taenari*
Bicyclo[2.2.1]hept-2-ene, 2,7,-trimethyl	-	-	-	1.47
6-Methyl-5-hepten-2-one	0.09	0.01	-	-
β-Myrcene	0.11	0.29	-	-
Tricyclene	-	-	-	2.36
α-Pinene	-	-	1.01	8.91
Camphene	-	-	-	18.63
β-Pinene	-	-	-	0.34
Myrcene	-	-	-	0.24
o-Cymene	-	-	-	0.10
Limonene	0.07	0.10	-	0.49
β-Phellandrene	-	-	-	0.16
E-β-Ocimene	0.03	0.05	-	-
Terpinolene	-	0.23	-	-
Linalool	-	-	0.10	-
E-Linalool oxide (furanoid)	0.04	0.04	-	-
Decanol	0.04	0.87		
α-Campholenal	-	-	-	0.62
cis-Verbenol	-	-	-	1.49
Myrtenal	-	-	-	0.19
α-Terpineol	0.01	-		
Verbenone	-	-	-	1.54
Decanal	-	-	0.19	-
Nerol	1.33	1.55		
Citronellol	0.27	0.43		
z-Citral	0.32	0.47	-	0.55
Geraniol	32.11	35.59	-	49.99
Geranyl formate	0.22	0.08	-	-
Phenol,5-methyl-2-(1-methylethyl)	0.21	0.06	-	-
Citronellyl acetate	6.60	3.15	-	-
Neryl acetate	17.54	7.25	-	-
Decanoic acid	-	-	2.20	-
Geranyl acetate	20.76	14.69	-	-
α-Copaene	-	-	0.90	-
β-Elemene	0.44	0.39	13.11	0.39
α-Gurjunene	-	-	1.39	-
β-Caryophyllene	0.20	0.45	15.56	-

(Table 1. - Contnd)

Compounds	*H. amorginum*	*H. italicum*	*H. stoechas*	*H. taenari*
β-Selinene	0.30	0.53	1.05	-
α-Humulene	-	-	-	3.38
Aromadendrene	-	-	4.68	-
Alloaromadendrene	-	-	1.23	-
α-Muurolene	0.47	0.81	-	-
γ-Cadinene	1.18	1.94	-	-
E-Nerolidol	6.85	11.86	0.54	-
Palustrol	-	-	0.79	-
Spathulenol	-	-	2.15	-
Globulol	-	-	1.53	-
1H-Cyclopropane azulene-4-ol	-	-	5.12	-
Torreyol	-	-	1.57	-
δ-Cadinol	0.31	0.67	3.00	-
Viridiflorol	0.32	0.60	3.60	-
9-Octodecenoic acid	0.15	0.18	-	-
Benzyl benzoate	-	-	5.69	-
6,10,14Trimethyl pentadecan-2-one	-	-	0.73	-
Total	90.17	82.29	66.14	90.85

Table 2. Bacteriostatic activity of the essential oils and the main components of the four*Helichrysum* species studied

Essential Oils	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*E. cloacae*	*K. pneumoniae*	*E. coli*
H. amorginum	0.75	0.75	1.25	1.25	1.25	7.50
H. italicum	3.25	3.25	3.75	3.50	3.50	-
H. stoechas	1.25	1.25	7.50	3.50	3.75	2.50
H. taenari	1.25	1.25	-	-	7.50	3.50
Geraniol	0.13	0.13	0.38	0.38	0.38	0.25
Geranyl acetate	0.25	0.25	-	0.75	0.75	-
Neryl acetate	0.25	0.25	0.38	0.25	0.75	-
Camphene	0.13	0.13	-	-	1.25	0.90

CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF *TAGETES ERECTA* AND *TAGETES PATULA* ESSENTIAL OILS

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INTRODUCTION

The genus *Tagetes* (marigold), belonging to the Asteraceae family, includes numerous species e. g. *patula, erecta, minuta, tenuifolia, lucida* and *riojana*. It is native to Middle- America and widespread and well known all over the world as ornamental plant. The marigold essential oils are strong smelling and have been used in a wide variety of applications such as folk medicine, perfumes, insect repellents and as antimicrobial and antifungal agents (1-4). The field of utilization of the oils depends on their composition which changes from species to species and is affected by the provenance, the vegetative period and by the part of the plant used to extract the oils (5-7).

The bacteria tested belong to the genus *Lactobacillus* and *Clostridium*. The species of genus *Lactobacillus* are Gram-positive strictly fermentative aerotolerant or anaerobic, aciduric or acidophilic and have complex nutritional requirements. They grow wherever high levels of soluble carbohydrate, protein breakdown products, vitamins and a low oxygen tension occur and thus in a variety of habitats such as human and animal oral cavities, intestines and vagina, plants and materials of plant origin, soil sewage, manure silage and man-made habitats such as fermenting or spoiling food. Recently the genus was reviewed by Hames and Vogel (8). The genus *Clostridium* represents one of the largest genera of the prokaryotes with more then 100 species having ubiquitous distribution . They are characterized by the formation of endospores, an anaerobic metabolism, the inability to carry out dissimilatory sulfate reduction and are Gram-positive. If the parameters for growth are favorable, spores will germinate and a population will be established.The four nutritional groups of clostridia are: saccharolytic, proteolytic, a combination of both, or specialized on one or a few substrates (9).

The aim of our work was to characterize the essential oils from flowers and leaves of *T.patula* and *T. erecta* and to evaluate their growth inhibition activity towards 15 species of the genera *Clostridium* and *Lactobacillus*.

EXPERIMENTAL

Plant Material

Seedlings of *T. patula* and *T. erecta*, grown in a greenhouse, were transplanted in spring in an experimental field in Northern Italy and harvested at full flowering stage in summer. A representative sample of each species, made up of numerous plants, was utilized to separate flowers and leaves which were air-dried and stored until oil distillation.

Oil Isolation

The air-dried materials were left to macerate in water for 12 h and then hydrodistilled for 5 h in a Clevenger-type apparatus. The oils were stored in a dark glass bottle at 4°C until analysis.

GC and GC/MS Analyses

The analyses were performed with a Carlo Erba HRGC 5160 Mega gas chromatograph equipped with a FID and a Hitachi D-2000 chromato-integrator. The separation of compounds was achieved using a fused silica column SPB5 (Supelco), 30 m x 0.32 mm, 0.25 μm film thickness, operating from 70 to 200°C at 5°C/min, holding the initial temperature for 18 min. The carrier gas was helium at a flow rate of 1ml/min, the detector temperature was 250°C. Quantitative data were expressed as relative percentages of the peak areas. A Finnigan Mat ion trap detector model 800 set at 70 eV and equipped with software, Relase 3.0, was employed for mass spectral analyses under the GC conditions reported above. The identification of compounds was performed by comparison of their retention times with those of pure substances and with those reported by Adams (10), by peak enrichment with standards and by mass spectrometric data

Bacterial strains and assessment of inhibition

The surveyed *Clostridium* and *Lactobacillus* strains were taken from the culture collection of the Institute of Agricultural Microbiology of Bologna University, Italy. A list of the species examined appears in Table 2. Microtitre test plates were used to determine minimal inhibitory concentrations (MICs) in TPY (11) growth medium. Oil samples were prepared as 10% (v/v) solutions in 95° ethanol, due to the insolubility of oils, and stored at 4°C. Oil concentrations ranged from 200 to 2000 ppm with intervals of 200 ppm. Plates with wells containing 100 µl of solution were inoculated with 10 µl of diluted 1-to 4- day-old culture by "Stepper TM Repetitive Pipette". Incubation of inoculated plates was performed in anaerobic conditions for 48 hours. Growth was evaluated through the cell density, the reduction of the pH and the color variation of bromocreosol indicator.

RESULTS AND DISCUSSION

The quali-quantitative composition of the oils along with the yields are reported in Table 1. *T. patula* produced, on average, a greater amount of oil than *T. erecta* and the leavse were richer in oil than the flowers. Twenty-six compounds were identified, the main ones being limonene, (E)-b-ocimene, terpinolene, piperitone and b-caryophyllene. Generally oils from *T. erecta* were richer in (E)-b-ocimene and piperitone than those from *T.patula* whereas the latter were characterized by higher amounts of limonene, dihydrotagetone and terpinolene. The oils from the flowers of both species showed higher percentages of (E)-b-ocimene and b-caryophyllene than those from leaves. This trend was particularly evident for b-caryophyllene which was found at 16.6% in the flowers and at 2.0% in the leaves. The leaves produced oils with high percentages of limonene, terpinolene, (Z)-ocimenone and piperitone. All the oils examined were characterized by the presence of components known to have potential biological activity.

This is the case of dihydrotagetone, (E)-ocimenone and piperitone which, as compounds belonging to the ketons, are considered of great importance in the control of microorganisms (2). Also (Z)-b-ocimene and limonene have been reported to be biologically active substances (12).

Table 1. Relative percentages of compounds and yields (%) of *Tagetes erecta* and *Tagetes patula* essential oils.

	T. erecta		*T. patula*	
Compound	flowers	leaves	flowers	leaves
a-pinene	tr.	0.39	0.38	1.05
camphene	tr.	0.05	tr.	tr.
sabinene	tr.	0.45	0.35	0.61
b-pinene	tr.	0.10	tr.	tr.
myrcene	0.79	0.91	0.63	tr.
a-terpinene	tr.	0.10	tr.	tr.
p-cymene	tr.	0.20	tr.	0.86
limonene	6.65	10.91	9.08	26.78
(Z)-b-ocimene	0.34	1.17	10.99	tr.
(E)-b-ocimene	15.58	11.53	4.78	tr.
dihydrotagetone	tr.	tr.	0.68	3.91
g-terpinene	tr.	0.37	0.22	0.39
terpinolene	9.08	11.34	15.73	25.58
linalool	3.21	0.80	0.66	0.67
p-mentha-1,3,8 triene	tr.	0.52	tr.	tr.
terpinen-4-ol	11.6	1.39	1.45	2.13
(Z)-ocimenone	0.99	3.63	1.78	5.92
(E)-ocimenone	1.55	0.62	0.75	1.31
piperitone	22.60	32.05	3.85	11.53
b-caryophyllene	15.85	3.56	17.43	0.50
a-humulene	0.34	0.15	tr.	tr.
germacrene D	tr.	tr.	tr.	tr.
germacrene B	0.25	0.22	tr.	tr.
trans- nerolidol	0.96	0.80	0.62	0.37
spatulenol	2.26	2.68	2.60	2.12
caryophyl. oxide	2.70	2.47	7.33	1.92
yield*	0.04	0.14	0.13	0.36

** expressed on air-dried matter*
tr. = trace (< 0.04).

Despite the differences in chemical composition, the antimicrobial activity of the four oils tested was rather uniform. Surprisingly, at low concentrations the oils showed inihibitory effects against almost all the *Clostridium* species while the *Lactobacillus* species showed resistance to the higer concentrations (Table 2). This encouraging result

might allow the use of oils to be tested as preservatives in foods where the presence of lactobacilli is desiderable, or even essential, while the growth of clostridia is harmful.

Table 2. Antibacterial activity of *T. erecta* and *T. patula* essential oils to *Lactobacillus* and *Clostridium* species.

	T.erecta (flowers)	*T.patula* (flowers)	*T.erecta* (leaves)	*T.patula* (leaves)
Cacetobutylicum	NT[a]	NT	200[b]	200
C. beijerinckii	200	200	200	200
C. bifermentans	NT	800	2000	1400
C. butyricum	200	200	400	1000
C. pasteurianum	1400	400	600	400
C. putrefaciens	200	200	1600	1200
C. sporogenes	200	200	200	200
C. tyrobutyricum	1200	800	2000	2000
L. acidophilus	>2000	>2000	>2000	>2000
L. brevis	1800	>2000	>2000	>2000
L. casei	>2000	>2000	>2000	>2000
L. delbrueckii[c]	>2000	1600	1400	>2000
L. delbrueckii[d]	>2000	>2000	>2000	>2000
L. helveticus	>2000	>2000	>2000	>2000
L. reuteri	>2000	>2000	>2000	>2000

a Not tested;
b MICs (minimal inhibitory concentrations) in ppm;
c sp.*bulgaricus;*
d subsp.*delbrueckii.*

Acknowledgment

This research, supported by a grant of the National Research Council, is a part of a project on "Biological activity of vegetable substances".

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THE CHEMISTRY AND PHARMACOLOGY OF *ORIGANUM (KEKIK)* WATER

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INTRODUCTION

Kekik is a collective term given in Turkey to plants containing carvacrol or thymol, hence smelling like thyme or oregano such as *Thymus, Origanum, Thymbra, Coridothymus* and *Satureja*. Such plants are used as condiment or as herbal tea taken as a pleasant hot drink or to cure various disorders. These plants are also used to distil **kekik oil**. Turkey is the major exporter of dried kekik, each year exporting around 5000 tonnes for a return of about 13 million dollars (1).

Kekik is a well known herb since antiquity in Turkish folk medicine (2-4) being used as stimulant, antidiarrheal, for gingivitis (5), analgesia, common colds, as antitussive and expectorant (5,6), diuretic and against kidney stones (7), sedative (8), cardiovascular stimulant (1), antiparasitic and antihelminthic (4,5), and as an antidote for many centuries (9). Mostly it is observed to be used for gastrointestinal complaints and in liver disorders as choleretic (2-5,8,10). It was popular against tumours in the medieval ages (11) (Table 1,2).

Kekik water has, in recent years, become a commercial commodity. It is the aromatic water obtained after removing essential oil from the distillate. Although kekik water has been known and produced in western and southern parts of Turkey in villages for use as a household remedy, rising demand for it especially in urban areas has forced commercial essential oil distillers to market it in cities. Reliable statistics are hard to come by on the local comsumption of kekik water in Turkey, but one supplier informed us that he had sold 70 tonnes in 1994.

For the preparation of kekik water in villages, a cauldron is placed over a fireplace. Plant material is placed in the cauldron and water is added to cover the plant line. At the inner centre of the cauldron a pot is placed over the plant material. The lid of the cauldron is turned upside down on top and the shallow part is filled with cold water. During distillation, aromatic vapour condenses on the cold surface of the lid and collects in the pot. Distillation is terminated when the pot is filled with the distillate. Essential oil floating on the distillate is scooped out with a spoon and transferred to a bottle or discarded. The aromatic water so prepared is cooled and consumed.

A more sophisticated distillation still which is also used for the production of essential oil, aromatic water and alcoholic drinks especially in mountain villages consists of two copper or tin vessels fitting on top of the other. The bottom vessel (ca.9 L) contains plant material and water. The smaller capacity (3 L) top vessel has a conical shape inside. Outside the cone cooling water is run through the inlet and outlet pipes. Bottom circle of the cone is lined with a trough in which the distillate accumulates and is run out through a pipe.

Since kekik water can be obtained from several species called kekik, we have decided to work with only one species in order to make sure of the identity of the plant material. We have chosen *Origanum onites* which is the main source of kekik water in Western Anatolia. Dried *Origanum onites* herb was distilled in our laboratories to obtain kekik water. This water was used throughout the experiments. From now on it will be called **Origanum water (OW)**.

The aim of this study was to study the chemistry and to screen the pharmacological activities of OW which have not previously been done.

MATERIALS AND METHODS

Animals and microorganisms

Adult albino mice (*Swiss*; 25-35 g.) rats (*Sprague-Dawley* and *Wistar* strain; 200-350 g.) and guinea pigs of either sex are used in this study. They were housed in well ventilated rooms with suitable room temperature and light cycle. Mice and rats were were fed with standard diet (Yem fabrikalari, Eskisehir), guinea pigs with

green vegetables obtained from local bazaar and water *ad libitum.*

Each test group consisted at least 5 experimental animals and care being taken to compare the test results against chemicals used as control (KCl and Noradrenaline for aorta and vas deferens, KCl and Acetylcholine for ileum, 0.9 % NaCl solution for *in vivo* tests. All the animals were handled with care and light ether anesthesia was applied prior to any surgical operation. Suitable anesthetic agent (Ketamine or Urethane) was used for *in vivo* operations. The chemicals used for pharmacological experiments were purchased from Sigma and Merck, except test materials which were produced in our laboratories (origanum water and its n-hexane and chloroform fractions (OW, OW_h and OW_c respectively)).

The following organisms were used for microbiological tests which were obtained from the Microbiology Laboratory of Medical School (Osmangazi University, Eskisehir): *Candida albicans, Penicillium* sp. *Aspergillus* sp. *Salmonella typhimurium, Pseudomonas aeruginosa, Escherichia coli, Klebsiella* sp. *Bacillus subtilis* and *Branhamella catarrhalis.*

Pharmacological Tests

Antimicrobial test, acute and chronic toxicity tests, general behaviour, general performance (swimming), analgesia (tail-flick), barbiturate sleeping time, bile flow tests and chromatographic analysis of bile, measurement of blood pressure, bronchospasm test, tests on isolated ileum, aorta and vas deferens, histological investigations of liver, spleen and pancreas, biochemical tests of blood sugar, triglyceride and cholesterol analyses were carried on KW and its fractions (fractionations by n-hexane and chloroform) and statistical analysis were performed according to standard methods described previously elsewhere (13-39). Sprague-Dawley rats ware injected OW as test substance and 0.9 % NaCl for control (2.0 mL/kg, i.p) and weighed every day for the anti-obesity test (Table 3).

Plant Materials and Chemical Analysis

In initial pharmacological screening experiments, Origanum water was used as such. For further tests, OW was subjected to exhaustive liquid-liquid extraction first with n-hexane and then with chloroform to yield 0.1 % and 0.02 % oily extracts, respectively. The chloroform extract was applied on a TLC plate and developed with chloroform. The major band at Rf7 was scraped and eluted with chloroform. The extracts were analysed by capillary GC/MS using Hewlett-Packard GCD system. HP-Innovax FSC column (60 m x 0.25 mm id) was used with helium as carrier gas. GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept at 220°C for 10 min then programmed to 240°C at a rate of 1°C/min. Split ratio was 50:1. Injector temperature was 250°C and MS temperature was at 165°C. MS were taken at 70 eV. Mass range was from m/z 35 to 425. Library search was carried out using Wiley GC/MS library and TBAM Library of Essential Oil Constituents. The identity of the compounds was confirmed by ^{1}H-NMR using a JEOL JNM-EX90A FT-NMR system in $CDCl_3$.

Solid-Phase Micro Extraction (SPME) of OW was also carried out. The adsorptive tip of the SPME syringe was exposed to OW in a vial placed on a magnetic stirrer for 20 minutes. Thermal desorption of the extractive components in the injection port of the gas chromatograph through the stream of helium gas led them into the capillary column.

Pharmacological Studies with OW

First set of pharmacological tests were performed using Origanum water as such. In these experiments, 0.1 mL of OW was applied in the organ bath. Its final concentration in the bath was 0.001 mL. In in vivo tests, 3.3 mL/kg (i.p.) on mice, 2 mL/kg (i.p.) on rats and 0.2 mL/kg (i.v.) on Guinea-pigs were used. In acute toxicity tests an unusually high amount of 21 mL/kg (i.p.) on mice were used and no toxicity was observed.

The n-hexane and chloroform extracts were tested only on isolated organs. The hexane extract (OWh) was dissolved in DMSO (10^{-3} g/mL stock) and applied to make the final concentration in the bath as 10^{-5} g/mL. The chloroform extract (OWc) was dissolved in DMSO ($5x10^{-3}$ g/mL stock) and the final concentration in the bath was $5x10^{-5}$ g/mL).

The Rf7 fraction isolated from the chloroform extract was tested *in vivo* on rats (i.v., 0.3 mL/kg) for blood pressure, after dissolving it in DMSO (10^{-5} g/mL).

Pure carvacrol (99.2% purity) (10^{-6} g/mL) was tested on isolated rat ileum and on mice for analgesic activity (1 mg/kg).

RESULTS AND DISCUSSION

The GC analysis of the Solid-Phase Micro Extraction (SPME) product of OW gave the following composition: Carvacrol (63.8 %), thymol (9.6 %), cis-p-menth-4-ene-1,2-diol (8.7 %) and linalool (5.6 %) as major constituents among the 27 components identified (Table 7 and Fig 2).

GC and GC/MS analysis of the n-hexane and chloroform extracts showed the following compositons: Carvacrol (73 %) was the main component of the n-hexane extract. The other major components comprised thymol (6 %), alpha-terpineol (4.7 %), borneol (2.7 %) and linalool (3.4 %). However, the chloroform extract gave a different composition with carvacrol (26.2 %) still as the major component, but compounds such as 3,7-dimethyl-1-octen-3,7-diol (21 %), cis-p-menth-4-ene-1,2-diol (15.6 %), cis-p-menthan-1,8-diol (6.2 %) which were either not present or present in trace amounts in the n-hexane extract (Fig.2 and Table 7)

The occurrence of two p-menthendiols in the active fraction (Rf7) of the chloroform extract was significant. These compounds are rarely found in nature and cis-p-menth-4-ene-1,2-diol and cis-p-menth-3-ene-1,2-diol are reported here for the first time from the family Labiatae. They comprised 75.8 % and 8.5% of the Rf7 fraction. Their identities were established by GC/MS and NMR studies.

These two menthendiols were previously isolated and identified from the essential oil of *Ferula jaeschkeana* (39). Cis-p-menth-4-ene-1,2-diol was also identified in Juniper Berry Oil (40) and Nutmeg Oil (41), It was obtained by microbial transformation of gamma-terpinene with *Diplodia gossypina* (42) and was detected during quantitative analysis of Chenopodium Oil as a reaction product of ascaridole (43-45). Trans-p-menth-4-ene-1,2-diol was also isolated from the oil of *Ferula jaeschkeana* and was obtained by microbial transformation with *Diplodia gossypina* (46,47).

p-Menth-3-ene-1,2-diol was obtained by microbial transformation of gamma-terpinene with *Diplodia* and *Corynespora* species (47-49).

The aim of this study was to perform a pharmacological screening of Kekik Water which has not been carried out previously. As kekik water, *Origanum onites* L. water was selected for pharmacological screening due to its widespread use among other species which are also known as *kekik*. Therefore, negative (inactive) test results are also reported here and attention is payed, naturally, on positive activities which resulted in unexpected activities and active chemicals. A series of pharmacological activities were performed to screen the OW. No activity was observed in most of the tests (namely; antimicrobial, acute and chronic toxicity, general behaviour, general performance, analgesia, bronchoconstriction, anti-obesity, histological appearance of liver, spleen and pancreas, serum glucose, triglyceride and cholesterol levels). Activities were observed in gastrointestinal and cardiovascular systems. OW inhibited gastrointestinal contractions but stimulated the cardiovascular system, in other words elevated blood pressure.

The results of pharmacological tests performed with intact OW are shown in Tables 4 - 6.

The positive results obtained are the following: Bile flow (Fig. 1e), barbiturate sleeping time, isolated ileum and aorta experiments and lack of any toxic effect of OW.

Two main extracts obtained with n-hexane (OW_h) and chloroform (OW_c) from Origanum water (OW) yielded different pharmacological activities and different chemical compositions. OW_h was responsible for the gastrointestinal inhibitory actions, whereas OW_c was responsible for the cardiovascular stimulant actions (Fig. 1a-c).

The Rf7 fraction showed a positive blood pressure elevating (hypertensive) effect on rats. When cis-p-menth-4-ene-1,2-diol, the major component of this fraction, obtained as a microbial transformation product, was tested under the same conditions no activity was observed. This may be due to the use of incorrect enantiomer, or to the possible effect of the 3-ene-isomer.

Pure carvacrol (99.2 % purity) was also tested on rat ileum and was found to possess strong antispasmodic activity (Fig. 1d). It was also shown to have analgesic activity in tail-flick test on mice (5). Although carvacrol is the major component, no analgesic activity was observed with OW but there was a marked analgesia with

carvacrol (1 mg/kg i.p.) (Fig. 1f). We have recently reported the analgesic activity of *Origanum onites* oil (50).

Acute and chronic toxicity of OW were tested on mice and rats, respectively. LD_{50} was found to be more than 21.9 mL/kg i.p. on mice indicating the safe use of Origanum Water. The results are summarised in Table 5.

As a last note for the mechanism of action of these simple but active compounds, it can be suggested that a possible specific adrenergic agonistic action on adrenergic receptors may be expected since adrenergic mechanisms are known to be involved in gastrointestinal inhibitory, cardiovascular stimulant and also analgesic actions (51) which awaits further investigations.

Acknowledgment:

The authors cordially thank Dr. Roman Kaiser of the Givaudan-Roure company in Zurich, Switzerland for his kind advice and help in the identification of menthendiols.

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Table 1. Ethnopharmacological uses of Kekik*

(* Origanum, Thymus, Coridothymus, Thymbra, Satureja)

- antiseptic	(oil)
- stimulant	(herb)
- gastrointestinal disorders, diarrhea	(herb, water)
- liver disorders, choleretic	(herb, water)
- gingivitis	(herb)
- common cold, antitussive, expectorant	(herb, oil)
- sedative	(herb)
- analgesic	(oil)
- diuretic	(herb)
- kidney stones	(herb)
- cardiovascular stimulant	(herb)
- antiparasitic and antihelminthic	(herb, oil)
- antitumor	(herb)
- antidote for poisons and venoms	(herb)

Table 2. Ethnopharmacological uses of Kekik Water

- gastrointestinal disorders
- nausea
- liver disorders, choleretic
- common cold
- endocrine diseases (diabetes mellitus)

Table 3. Pharmacological tests carried out on Origanum Water (OW)

Test	Animal	Result
* Antimicrobial	(antibacterial and antifungal)	Negative
* Toxicity tests		
* -- Acute toxicity	(mouse; in vivo)	Nontoxic
* -- Chronic toxicity	(rat; in vivo)	Nontoxic
* General behaviour	(mouse; in vivo)	inactive
* Swimming performance	(mouse; in vivo)	inactive
* Analgesic	(mouse; in vivo)	inactive
* Bronchospasm	(guinea pig; in vivo)	inactive
* Barbiturate sleeping time	(mouse; in vivo)	Active
* Bile flow	(rat; in vivo)	Active
* Blood pressure	(rat; in vivo)	Active
* Isolated organ bath experiments		
* -- isolated ileum	(rat and guinea pig; in vitro)	Active
* -- isolated aorta	(rat and guinea pig; in vitro)	Active
* -- isolated vas deferens	(rat and guinea pig; in vitro)	Active
* Histological investigations		
* -- liver	(rat and mouse)	inactive
* -- pancreas	(rat and mouse)	inactive
* -- spleen	(rat and mouse)	inactive
* Biochemical tests (in serum)		
* -- glucose	(rat)	inactive
* -- triglyceride	(rat)	inactive
* -- cholesterol	(rat)	inactive

Table 4. Pharmacological Tests, Animals and Doses (Only active tests included)

Test	Animal	Method	Material	Doses
Toxicity				
- acute	mouse	in vivo	OW	20 mL/kg (max.)
- chronic	rat	in vivo	OW	2.0 mL/kg
Bile flow	rat	in vivo	OW	2.0 mL/kg

(Table 4. Pharmacological Tests, Animals and Doses (cont'd))

Test	Animal	Method	Material	Doses
Barbiturate Sleeping Time	mouse	in vivo	OW	3.3 mL/kg
Analgesia	mouse	in vivo	Carvacrol	1mg/kg
Blood pressure	Rat & G.pig	in vivo	Rf7	10^{-5} g/mL
Isolated organ bath				
- isolated aorta	Rat & G.pig	in vitro	OW	0.001 mL (*)
- isolated aorta	Rat & G.pig	in vitro	OWh	10^{-5} g/mL (*)
- isolated aorta	Rat & G.pig	in vitro	OWc	$5x10^{-5}$ g/mL (*)
- isolated ileum	Rat & G.pig	in vitro	OW	0.001 mL (*)
- isolated ileum	Rat & G.pig	in vitro	OWh	10^{-6} g/mL(*)
- isolated ileum	Rat & G.pig	in vitro	Carvacrol	10^{-5} g/mL(*)

(*) final organ bath concentration

Table 5. Results of Toxicity Tests of Origanum Water (OW)

Test	Animal	Time	Doses	Result (Mortality)
Acute toxicity	Mice	48 hours	3.3 ml/kg i.p.	0/3
			13.3 ml/kg i.p.	0/3
			21.9 ml/kg i.p.	0/3
			LD_{50} >21.9 ml/kg	
Chronic toxicity	Rat	43 days	2.0 ml/kg ip.	0/5

Table 6. Activities and Active Fractions

		OW	OWh	OWc	Rf7	Carvacrol
Gastrointestinal inhibition	active	strong	-	-	strong	
Cardiovascular stimulation		active	-	strong	strong	
Isolated aorta		-	inhibition contraction			

Table 7. Major components of OW and its extracts

Compound	Hexane	Chloroform	SPME
Linalool	3.35	0.55	5.55
Terpinen-4-ol	1.83	0.26	1.45
alpha-terpineol	4.65	0.90	0.59
borneol	2.66	0.48	1.61
(Z)-3-hexenyl nonanoate	-	2.41	-
3,7-dimethyl-1-octen-3,7-diol	0.34	**21.03**	0.15
cis-p-menthan-1,8-diol	<0.01	6.22	-
3,7-dimethyl-1,7-octadien-3,6-diol*	-	1.61	0.10
cis-p-menth-4-ene-1,2-diol	1.15	**15.59**	8.71
cis-p-menth-3-ene-1,2-diol	-	1.57	0.78
thymol	6.10	1.47	9.56
carvacrol	**73.01**	**26.24**	**63.76**

(*) tentative identification

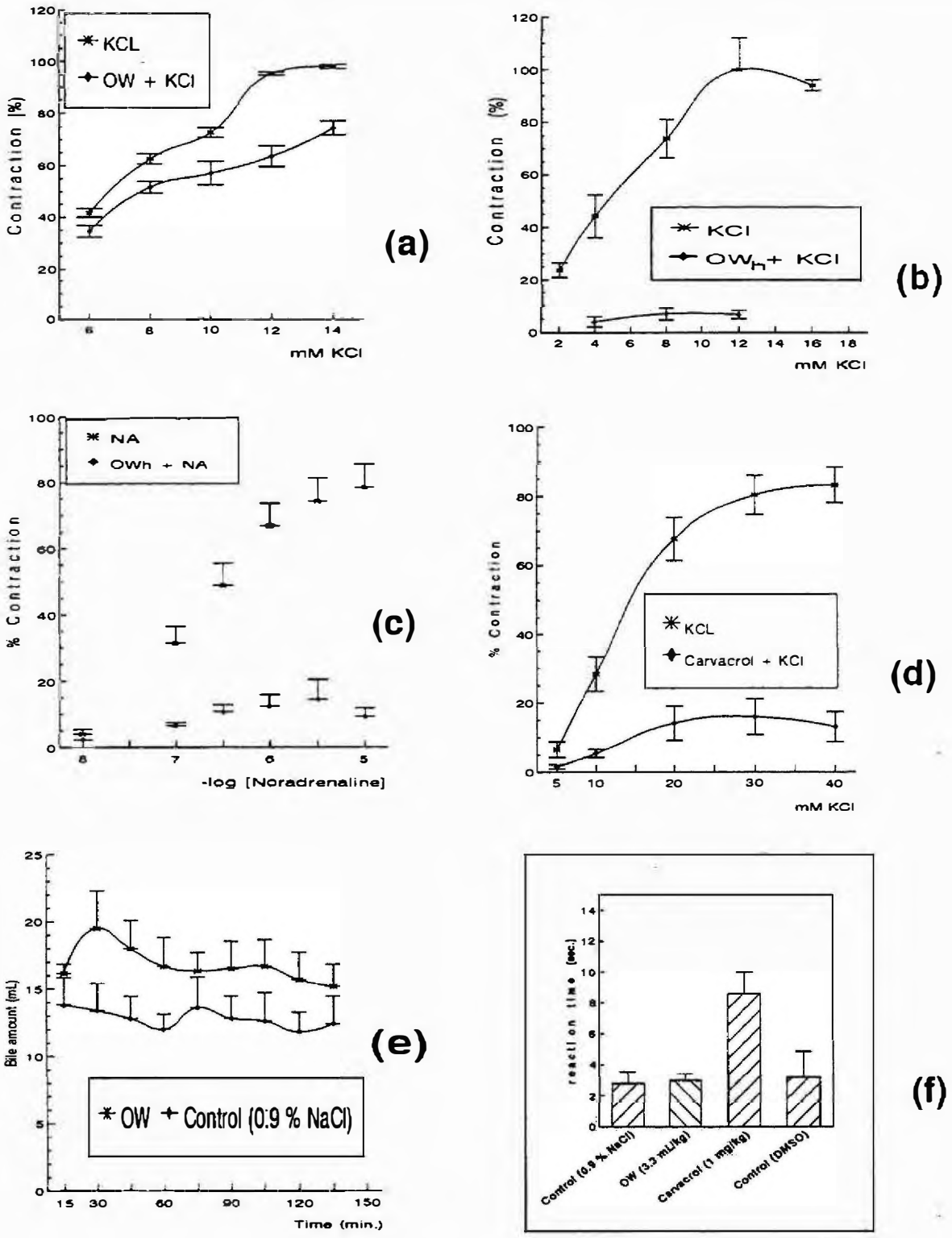

Fig. 1 Effects a) of OW, b) OWh and d) Carvacrol on isolated rat ileum and c) of OWh on rat aorta; e) of OW on bile flow and f) analgesic actions of carvacrol.

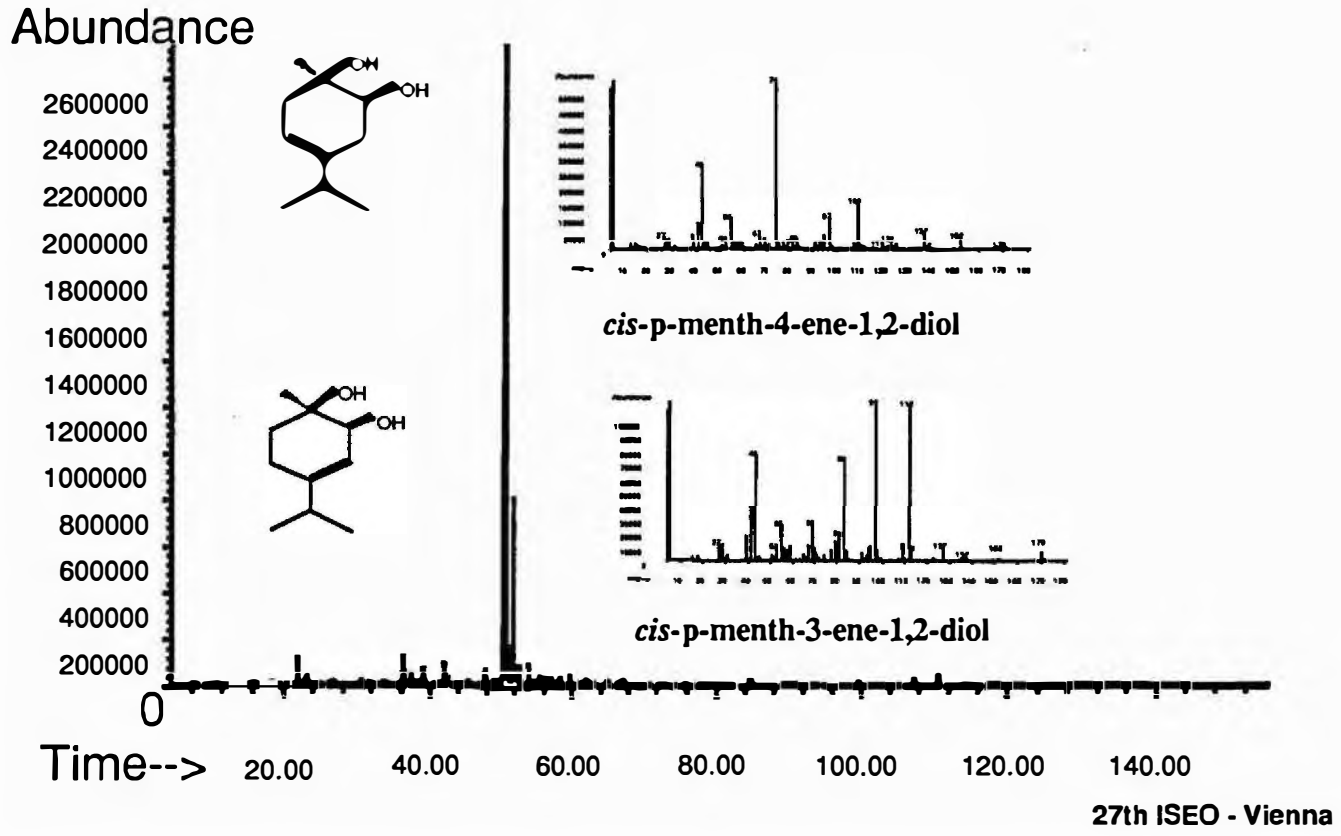

Fig. 2 Major components of the Rf7 fraction

THE CHEMICAL COMPOSITION AND ANTIBACTERIAL PROPERTIES OF *THYMUS LONGICAULIS* SUBSP. *CHAOUBARDII* OILS: THREE CHEMOTYPES IN THE SAME POPULATION

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INTRODUCTION

The genus *Thymus* (Labiatae) has been credited with a long list of pharmacological properties (diuretic, cholagogic, spasmolytic, antiseptic etc.). In Greece it is represented by more than 30 species, 5 of which are endemic (Greuter et al. 1986). The infraspecific variability of the essential oils in the genus *Thymus* has been the subject of several studies reviewed by Stahl-Biskup (1991).

In pursuit of our studies on Greek aromatic plants we recently came across a chemically diverse population of *7hymus longicaulis* C. Presl subsp. *chaubardii* (Reichenb. fil.) Jalas. This subspecies belongs to sectio *Serpyllum* Bentham and is a wildgrowing plant, spread in former Yugoslavia, Albania, Greece and Asiatic Turkey (Greuter et al. 1986, Davis 1982). The chemical polymorphism of *Thymus longicaulis* subsp. *chaubardii* has earlier been reported from Asiatic specimens (Baser et al. 1994). In the present study it is noteworthy, that even though the individual plants of the examined population are morphologically almost identical, they were easily recognizable by their characteristic odors.

EXPERIMENTAL

Plant Material and Distillation - Aerial parts, of the plants with distinct odors, harvested at full flowering stage, were collected from the same population (growing in an area of 1 m^2) in mountain Pamis, Attiki, at an altitude of 1200 m, in June 1995. Voucher specimens were deposited in the Herbarium of the Laboratory of Pharmacognosy, University of Athens.

Prior to the chemical analysis the specimens were separated in three classes: thyme-odor class; rose-odor class; lavender-odor class. Air-dried plant material was cut in small pieces and hydrodistilled for 3 hrs, in a modified Clevenger-type apparatus with a water cooled receiver, to reduce hydrodistillation overheating artifacts and afforded light yellow oils (0.6 -0.8% yield). The oils were dried over anhydrous sodium sulphate and were kept at -4°C until they were analyzed. The composition of the volatile constituents was established by GC and GC/MS.

Screening For Bacteriostatic Activity
The bacteriostatic properties of the essential oils from the three *Thymus* chemotypes were examined using the dilution method in agar (Janssen et al. 1987). The bacteriostatic activities were determined by measuring the minimal inhibitor concentration (MIC) against the following Gram (+) bacteria: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC *25922),* *Enterobacter cloacae* (ATCC 13047), *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 227853). Standard antibiotica (netilmicin, ciprofloxacin, imipenem, ceftazidim and amoxicillin with clavulanic acid, Sanofi Diagnostics Pasteur) were used in order to control the sensitivity of the test organisms.

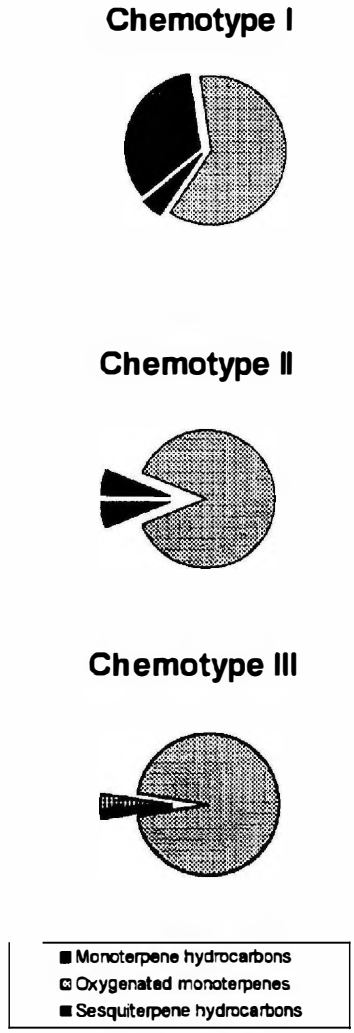
RESULTS AND DISCUSSION

Thirty five metabolites representing the majority (91.9-97.8%) of the three odor classes, were identified and quantified.

The results of the chemical analyses revealed that the three odor classes of *T. longicaulis* subsp. *chaubardii,* are

characterized by different and distinct chemical profiles. On the basis of the major constituents, characteristic chemotypes were assigned to the studied "odor-classes". Limonene (18.74 %) and thymol (19.44 %) are the main components of the thyme-odor class (chemotype I.); geraniol (56.78 %) and geranyl acetate (7.62 %) are the major components of the rose-odor class (chemotype II.); linalool (63.12 %) and a-terpinyl acetate (20.38 %) are the predominant components of the lavender-odor class (chemotype III.).

The biological studies showed that chemotype II. and III. possess the strongest antibacterial activity, probably due to the high content of non-phenolic alcohols such as geraniol and linalool (Hinou et al. 1989). Chemotype I. showed a weaker activity against all the tested bacteria, probably due to the low concentration of the phenolic alcohols thymol and carvacrol that are some of the most active investigated terpenes (Knobloch et al. 1989).



Acknowledgment

We thank Mr. Th. Constandinidis (Institute of Systematic Botany, University of Athens) for the identification of the plant material.

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ANTIMUTAGENIC ACTIVITY OF (+)-B-EUDESMOL AND PAEONOL FROM *DIOSCOREA JAPONICA*

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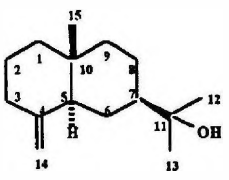
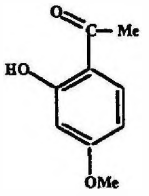
INTRODUCTION

Chinese yam (Dioscoreaceae) occurs naturally in Japan where it is cultivated and its storage rhizome is used mainly for treatment of diarrhea, asthma, polyuria, and diabetes. Several phenanthrenes, dihydrophenanthrenes, and bibenzyls have been isolated from *Dioscorea* species (Takasugi et al., 1987; Sunder et al., 1978), and antifungal compounds were isolated from *D. batatas* (Takasugi et al., 1987). Hikino et al. (1986) reported on the isolation of hypoglycemic active compounds (dioscorans A, B, C, D, E, and F) from *D. japonica*.

In evaluating the carcinogenicity or mutagenicity of environmental chemicals, it is quite important to determine factors present in our environment that may affect these activities. With the development of techniques for detecting possible environmental carcinogens and mutagens (Ames et al., 1975), it has been shown that ordinary diets contain many kinds of mutagens and antimutagens, and as one example Kakinuma et al. (1984a) reported the identification of an antimutagen (cinnamaldehyde) from *Cinnamomum cassia* using *Escherichia coli* WP2 B/r *uvr*A⁻*trp*E⁻.



Compound 1



Compound 2

The *umu* test system was developed to evaluate the genotoxic activities of a wide variety of enviromental carcinogens and mutagens, using the expression of one of the SOS genes to detectDNA-damaging agents (Oda et al., 1985; Nakamura et al., 1987).

In our search for new naturally occurring antimutagenic compounds in plants, which have a history of safe use as Chinese crude drugs (Miyazawa et al., 1995a, b, c), we found that the methanol extract of *D. japonica* (Sanyaku in Japanese) exhibited suppression of the SOS-inducing activity of furylfuramide. In this paper, we report the isolation and identification of the antimutagenic compounds contained in *D. japonica*.

Umu Test

The *umu* test for detecting the SOS-inducing activity of chemicals was carried out essentially as described by Oda et al. (1985) using *S. typhimurium* TA1535/pSK1002 whose plasmid pSK1002 carries a *umu*C'-*lac*Z' fused gene. The SOS-inducing potency is estimated by the measurement of the level of *umu* operon expression in terms of cellular b-galactosidase activity.

Ames Test

The mutation test was carried out by the preincubation method (Yahagi et al., 1977), which is a modification of Ames method (Ames et al., 1975).

The antimutagenic compounds in *D. japonica* were clearly identified as **1** and **2**. These compounds had a suppressive effect on *umu* gene expression of the SOS response in *S. typhimurium* TA1535/pSK1002 against furylfuramide and Trp-P-1. As shown in Table 2, **1** suppressed the SOS-inducing activity of both mutagen at lower concentrations than **2**. In the

Table 2. Suppressive Effect of 1 and 2 on Furylfuramide[a] and Trp-P-1[b] Using *S. typhimurium* TA1535/pSK1002.

Chemical	furylfuramide	Trp-P-1	Control[c]	Dose response[d] 0.18	0.11	0.07	0.04(μmol/mL)
1	930.1		252.9	382.9	451.7	645.5	787.7
		567.6	210.0	395.9	411.0	448.7	477.8

Chemical	furylfuramide	Trp-P-1	Control	Dose response 1.20	0.60	0.24(μmol/mL)
2	930.1		252.9	529.2	668.0	782.5
		567.6	210.0	295.1	346.6	420.3

[a] Furylfuramide (1μg/mL in DMSO) was added at 60μL. [b] Trp-P-1 (40μg/mL in DMSO) was added at 50μL. [c] Control was treatment without mutagens. [d] β-galactosidase activity (units).

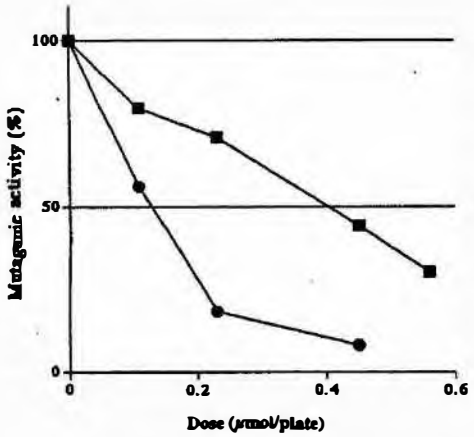


Figure 2. Effect of 1 on the mutagenicity of furylfuramide and Trp-P-1 in *S. typhimurium* TA100: (l) effect of 1 on the mutagenicity of furylfuramide; (n) effect of 1 on the mutagenicityof Trp-P-1. Furylfuramide (0.5 μg/mL in DMSO) was added at 20 μL/plate. Trp-P-1 (40 μg/mL in DMSO) was added at 50 μL/mL. In case of the effect of 1 on the mutagenicity of furylfuramide, 1 was toxic under the 0.56 μmol/plate.

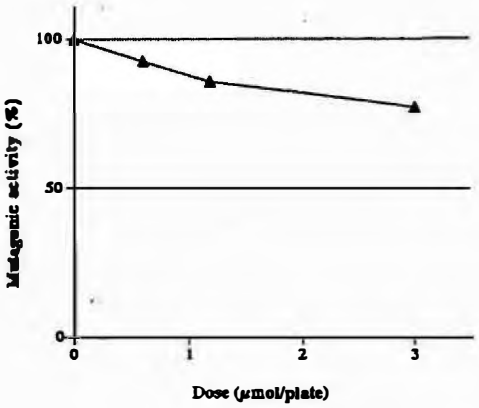


Figure 3. Suppressive effect of 2 on the furylfuramide using *S. typhimurium* TA100. Furylfuramide (0.5 μg/mL in DMSO) was added at 20 μL/plate.

Ames test using *S. typhimurium* TA100, **1** similarly inhibited the mutagenicity of furylfuramide and Trp-P-1, and **2** showed a low suppressive effect of the mutagenicity against furylfuramide (Figure 2 and 3).

Compounds **1A** and **1D** did not show any suppressive effects on the SOS-inducing activity of furylfuramide (data not shown). In this effect indicated that a hydroxyl group at C-11 is important for the suppressive effect. Compound **1** has other biological activities, for example it has a preventive activity against experimental ulcerations (Nogami et al., 1986) and antianoxic activity (Yamahara et al., 1990). Further, **1** has an inhibitory activity on Epstein-Barr virus early antigen (EVB-EA) activation . EVB-EA activation is an assay to detect a tumor promoters, so it indicated that **1** might be an antitumor promoter (Konoshima, et al., 1991). This seems interesting in relation to their antimutagenic activities shown in the present study.

Compound **1** also showed toxicity for *S. typhimurium* TA1535/pSK1002 at more than 0.18 μmol/mL with each mutagen (furylfuramide and Trp-P-1). On the other hand, it showed toxicity for TA100 at more than 0.45 μmol/plate with furylfuramide and at more than 0.56 μmol/plate with Trp-P-1. For similarly example, Kakinuma et al. (1984b) reported antimutagenic diterpenoids (Enmein, Nodosin, and Oridonin) from *Isodon japonicus* using *E. coli* B/r WP2 *trp*$^-$. Oridorin showed bactericidal activity at a higher dose. In this matter, it is necessary to test the antimutagens for their toxicity. Previously, Fukuhara et al. (1987) reported on a bio-antimutagen (paeonol) from *Paeonia suffruticosa* using *E. coli* B/r WP2 *trp*$^-$, *uvr*A$^-$, and paeonol has a bio-antimutagenic effect on 4NQO-induced mutation.

Compound **1** is one of the components of the essential oils of *Chenopodium botrys* (El-Sayed et al., 1989) and *Humulus lupulus* (Hop) (Tressl et al., 1983). Compound **2** is a component of the essential oils of *Paean mouton* and *P. lactiflora* (Miyazawa et al., 1983 and 1984). Osawa et al. (1986) reported on the desmutagenic action of food compounds (ascorbic acid, cysteine) on mutagens formed by the sorbic acid/nitrite reaction, and yam juice exhibited a desmutagenic action. In this paper, **1** and **2**

were principal components of antimutagenic activity in *D. japonica*, though these were minor components in this crude drug.

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MONOTERPENES FROM CUBAN PINES AND THEIR POSSIBLE ROLE IN THE HOST-PLANT RECOGNITION BY *DIORYCTRIA HORNEANA*

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INTRODUCTION

Monoterpene hydrocarbons constitute the basic volatile components of oleoresins produced by pines. Monoterpenes from the loblolly pine were reported by Hanula et al. (1985) to act as oviposition stimulants for *Dioryctria amatella* (Pyralidae). A related pyralid *Dioryctria horneana*, a Cuban endemite, is a moth ovipositing in the resin exuded at bark injuries (Valdes et al, 1985). *D. horneana* often attacks pines of the species *Pinus caribaea* in Western Cuba and *P. cubensis* in Eastern Cuba. *P. tropicalis*, on the other hand, is not attacked by this pest in its natural habitat. *Pinus caribaea* growing in Eastern Cuba is never attacked by this insect. One reason could be a different attractiveness of the pine species due to different compositions of the volatiles released from the resin. We have studied the monoterpene composition of oleoresins of Cuban pines to evaluate their degree of attractiveness for the insect pest. Both unattacked and attacked trees of *Pinus caribaea* and *P. cubensis*, as well as the unattacked *P. tropicalis* trees were used for the study. A large study of the enantiomeric composition of monoterpene hydrocarbons in Cuban pines was published recently (Valterová et al., 1995). In the present work, a new approach to the evaluation of chemical data was tested. Groups of trees growing in the same locality (a potential subject of insects' choice) were statistically evaluated according to the monoterpene composition of their oleoresins. Chirality of monoterpenes was taken into account, too. The electroantennographic responses of *Dioryctria horneana* males and females to a series of monoterpene hydrocarbons were tested, including dose-response curves of the most active compounds.

MATERIAL AND METHODS

Insects. Dyorictria horneana used in the study originated from Cuba (Mayarí). The individuals were collected as larvae. Larvae were allowed to pupate and as pupae, they were transported to the laboratory. There pupae were sexed and males were kept separately from the females under 16:8 light:darkness regime. Two to three days old males and females were used for electroantennographic (EAG) experiments.

Electroantennography. The compounds were dissolved in hexane forming a series of dilution from 1 ng to 1 μg per microliter. 5 μl aliquots were pipetted on a filter paper disc which was loaded into the Pasteur pipette. After overnight equilibration, the cartridges were used for antennal stimulation. The following series of monoterpenic standards was tested: (–)-limonene, (+)-limonene, (–)-α-pinene, (+)-α-pinene, (–)-β-pinene, (+)-sabinene, (+)-3-carene, *p*-cymene, and myrcene.

Stimulus delivery system. Stimuli were delivered onto the antennal preparation by air pulses blown through the odour cartridge. The cartridge outlet was positioned in a distance 1.5 cm from the antenna. Stimulus duration was 1 second, the air flow rate 1 l/min. Intervals between two successive stimuli were used at a range from 1 - 20 min. depending on the type and intensity of the stimuli. During these periods, the antennal preparation was blown by a continual stream of clean and humidified air.

EAG recording. The whole animal preparation was used. Male in a disposable pipette tip was fixed in place by small droplets of melted wax. The head and one antenna were exposed to the air. Tip of one antenna was cut off and connected with the recording electrode. Ground electrode was positioned into the head capsule. Glass Ag/AgCl microelectrodes filled with insect hemolymph saline (Kaissling, 1974) were used for EAG recordings. Antennal responses were amplified, displayed on oscilloscope (Tektronix 5113) and recorded by a pen recorder. The EAG amplitude was evaluated statistically using the Student's test.

Plant material. The collection of the oleo-resin samples from *Pinus tropicalis*, *P. caribaea*, and *P. cubensis* was described earlier (Valterová et al., 1995). The samples originated from two localities - Pinar del Río (Western Cuba) and Mayarí (Eastern Cuba). The following groups of trees were included in the study: Locality Pinar del Río (Western Cuba): *Pinus tropicalis* - unattacked (group A); *Pinus caribaea* - unattacked (group B) and attacked by *D. horneana* (group C); Locality Mayarí (Eastern Cuba): *Pinus caribaea* - unattacked (group D); *Pinus cubensis* - unattacked (group E) and attacked by *D. horneana* (group F).

Analytical and statistical methods. The gas chromatographic separation with a multidimensional GC system was described earlier (Borg-Karlson et al., 1993; Valterová et al., 1995). The obtained analytical data were subjected to a multivariate data analysis (Wold et al., 1989; Sjödin et al., 1989). Projection to latent structures - discriminant analysis (PLS-DA; program CODEX®) was used for the evaluation of differences between selected groups of trees. (+)- And (–)-enantiomers of the chiral monoterpenes were treated as separate compounds. The trees growing in the two localities were treated separately: groups A, B, and C in one run and groups D, E, and F in the second run, in order to determine if there are differences between groups and, if so, to find out which monoterpenes make up the differences.

RESULTS AND DISCUSSION

EAG results showed that all compounds tested elicited responses in both males and females of *Dyorictria horneana* (Fig. 1). Except for (–)-limonene, females were significantly more sensitive to host terpenes than males (95% significance level). Sabinene, (+)-3-carene and (+)-α-pinene were the most active compounds followed by (–)-α-pinene, (–)-β-pinene, (+)- and (–)-limonene. *p*-Cymene and myrcene elicited only weak responses. (+)-α-Pinene was significantly more active than (–)-α-pinene and (+)-β-pinene. EAG responses to (–)-α-pinene and (–)-β-pinene were not significantly different. Also EAG responses to (+)-limonene were higher in both sexes than to (–)-isomer.

The responses of both females and males to (+)-α-pinene, (+)-sabinene, and (+)-3-carene were dose-dependent (Fig 2). This fact means that these compounds might be of importance for the insect. On the other hand, (–)-α-pinene did not show any dependence on the tested dose.

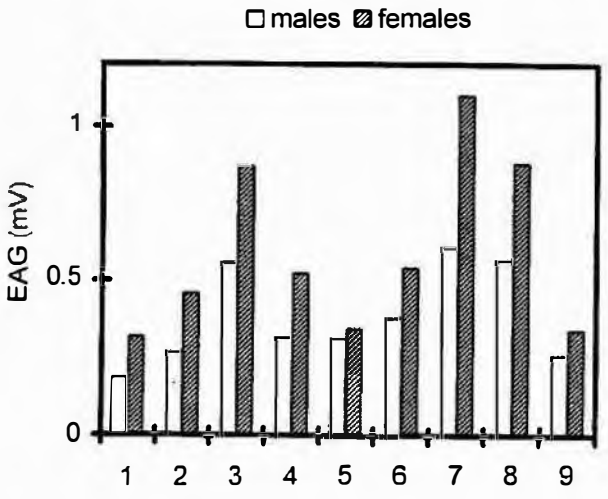

Fig. 1. EAG Responses of *D. horneana* to monoterpenes. 1, *p*-cymene; 2, (–)-α-pinene; 3, (+)-α-pinene; 4, (–)-β-pinene; 5, (–)-limonene; 6, (+)-limonene; 7, (+)-sabinene; 8, (+)-3-carene; 9, myrcene.

EAG results showed that both sexes are able to perceive the host odour, however, females are much more sensitive. Myrcene and *p*-cymene probably don't play a key role in host discrimination, as the EAG responses were very weak. These compounds, however, cannot be excluded from further tests, because they can modulate the effect of key compounds. (–)-α-Pinene elicited a higher response, but it is not expected to belong to the key compounds as the response was not dose-dependent (Fig 2). On the other hand, (+)-α-pinene, (+)-sabinene and (+)-3-carene might be much more important.

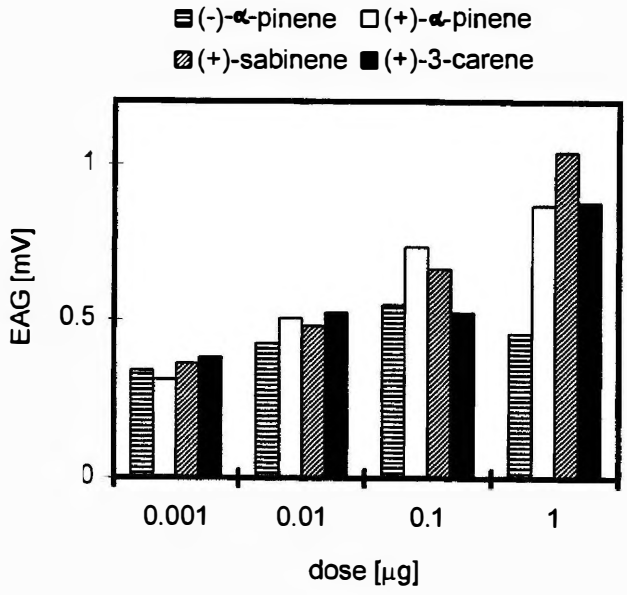

Fig. 2. EAG Responses of *D. horneana* females to monoterpenes; A dose-response diagram.

Table I.

Average proportions (in %) of the main components in the studied groups of pines

Compounds	*P. tro.* (A)	*P. car.* (B)	*P. car.* (C)	*P. car.* (D)	*P. cub.* (E+F)
(–)-α-pinene	10.6	22.0	45.0	16.0	4.2
(+)-α-pinene	79.7	33.3	21.4	27.0	81.3
(–)-β-pinene	1.3	1.4	3.1	1.2	3.8
(+)-β-pinene	0.6	0.4	0.7	0.4	0.5
myrcene	1.0	2.4	2.1	3.1	2.2
(–)-limonene	0.5	1.0	1.0	1.1	3.7
(+)-limonene	0.6	0.5	0.7	0.4	0.5
(–)-β-phellandrene	3.0	35.5	22.8	47.4	1.2
(+)-β-phellandrene	0.03	0.3	0.02	0.4	0.1

(+)-sabinene, (+)-3-carene, and *p*-cymene formed trace components

The chemical analyses showed that the main components of the oleoresins were (+)- and (–)-α-pinene (all pine species), and (–)-β-phellandrene (*P. caribaea*). The mean values of the proportions of main monoterpenes in all species in this study are summarised in Table I. A detailed information is given in our recently published paper (Valterová et al., 1995).The statistical analysis of all trees collected in Cuba did not show any separation into groups of attacked *versus* unattacked trees. In *P. cubensis*, no significant difference between unattacked and attacked trees was found, while in *P. caribaea*, a significant difference was found between unattacked and attacked trees (Valterová et al., 1995). Both enantiomers of β-pinene as well as (–)-α-pinene and (–)-camphene were important for the group of attacked *P. caribaea* trees.

When pines of different species within one locality were evaluated, a clear distinction between *P. caribaea* (attacked) and *P. tropicalis* (unattacked; both from locality Pinar del Río), respectively *P. caribaea* (unattacked) and *P cubensis* (attacked, both from locality Mayarí) was found (Figs 3, 4).

In the locality Pinar del Río, the trees of *P. tropicalis* (unattacked, A) and *P. caribaea* (both unattacked, B, and attacked, C) showed the tendency to separate into three groups. (Fig. 3). The important components for the individual groups were (+)-α-pinene, (+)-camphene, (+)-β-phellandrene for *P. tropicalis*, α- and (–)-β-phellandrene for unattacked *P. caribaea*, and finally (–)-α-pinene, (–)-camphene, (–)-β-pinene for attacked *P. caribaea* trees. (–)-Limonene, myrcene, *p*-cymene and (–)-sabinene were also of some importance for the group of unattacked *P. caribaea* trees.

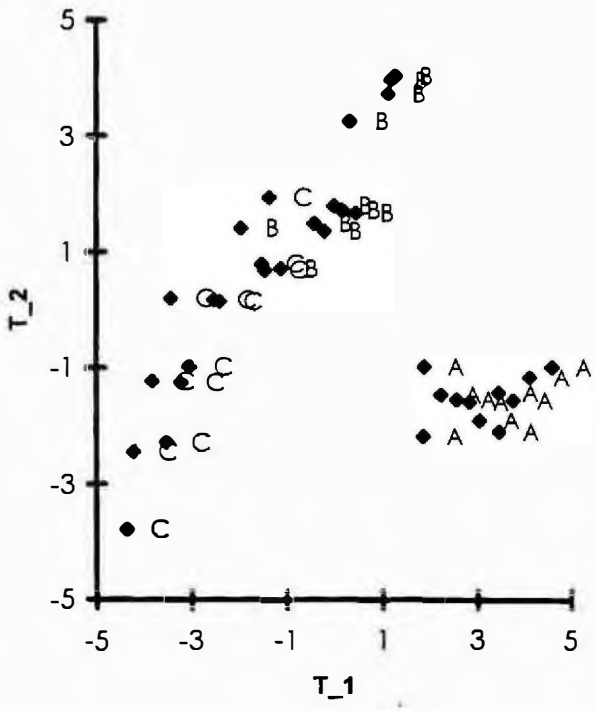

Fig. 3. PLS-DA of pines growing in Pinar del Río; *P. tropicalis* (unattacked, A), *P. caribaea* (unattacked, B, and attacked, C).

In the Mayarí locality, forming of two groups was obvious (Fig. 4). The significant components for the unattacked *P. caribaea* (D) trees were (–)-β-phellandrene, α-phellandrene, and (–)-α-pinene. For *Pinus cubensis* (E+F), (+)-α-pinene, (+)-camphene, and tricyclene were the most important compounds.

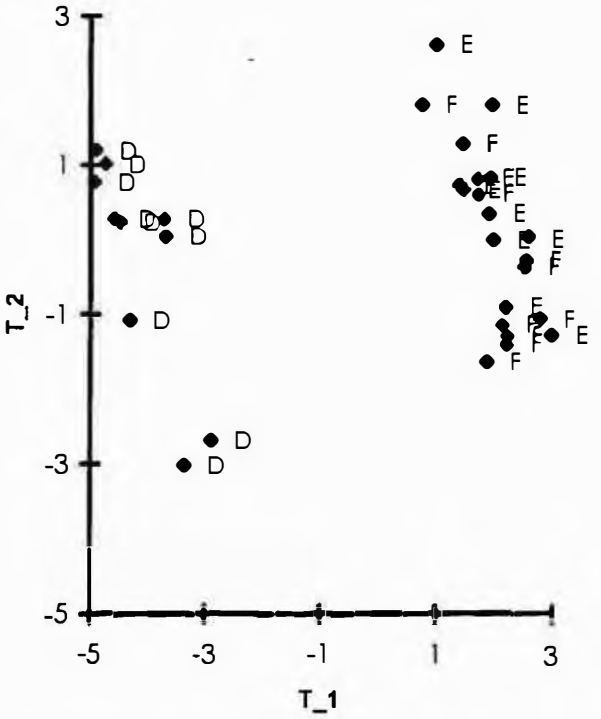

Fig. 3. PLS-DA of pines growing in Mayarí; *P. caribaea* (unattacked, D), *P. cubensis* (unattacked, E, and attacked, F).

Statistical results showed that the separation of trees into groups is mostly due to the different pine species rather than due to the insect attack. No clear correlation between the chemical and electrophysiological results was found.

Our EAG data demonstrate that the antennae of both sexes are broadly tuned to more than one compound. It means that *Dyorictria horneana* antennae are not specialised to a single specific host compound. Perhaps a specific mixture of volatiles rather than a single compound might be important to guide moths to their host. Question arises about the significance of host discrimination for males. There is a possibility that host volatiles may help males to find mating partner at the vicinity of place suitable for oviposition and thus enhance the chances for survival of offspring.

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Genetic Aspects and Breeding

BIOCHEMICAL AND MOLECULAR GENETIC ASPECTS OF MONOTERPENE FORMATION

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INTRODUCTION

Several hundred naturally occurring monoterpenes are known and they constitute the most common odor-bearing components of the essential oils (1). Essentially all monoterpenes are biosynthesized from geranyl diphosphate (see Figs. 1 and 2), the ubiquitous C_{10} intermediate of the isoprenoid pathway (2). Monoterpene synthases, often referred to as "cyclases", catalyze the reactions by which geranyl diphosphate is cyclized to the various monoterpene carbon skeletons. These enzymes have received considerable recent attention because the cyclization process determines the basic structural character of the monoterpene end-products and because the cyclization mechanism is quite complex, involving multiple steps in which many of the carbon atoms of the substrate undergo alterations in bonding, hybridization and configuration (2). Research on monoterpene synthases has also been stimulated by the possible regulatory importance of these enzymes that function at a branch point in isoprenoid metabolism (3).

Most of the cyclic parent compounds formed from geranyl diphosphate by the action of the monoterpene synthases are olefins (4). The subsequent metabolism of these key intermediates to oxygenated derivatives is of great interest since such oxygenated derivatives are largely responsible for the characteristic odors and flavors of the essential oils (1). In the monoterpene series, cytochrome P450 hydroxylases are generally responsible for establishing the oxygenation pattern of the various skeletal types (5,6). Thus, cytochrome P450 catalyzed allylic hydroxylation of the parent olefins sets in motion a series of enzymatic reactions involving subsequent oxidation to the corresponding conjugated carbonyl compound, reduction of the now-activated α,β-double bond and, ultimately, reduction of the carbonyl function to give a broad spectrum of regiochemically related products (7-9). The origin of most of the naturally occurring monoterpenes can be rationalized by variations in the cyclization reaction coupled to the "allylic oxidation-conjugate reduction" sequence, with the regiospecific cytochrome P450 hydroxylases establishing the basic oxygenation pattern on a given structural theme generated upon cyclization of the geranyl substrate (2,5).

Model Systems

Much of our recent research has been focused on the commercial mint species, peppermint (*Mentha piperita* L.) and spearmint (*Mentha spicata* L.), in part because the physiology and genetics of essential oil formation in this family of plants is better understood than any other (3,10,11). The principal monoterpene constituents of the essential oils of *Mentha* (family Lamiaceae) are members of the *p*-menthane (1-methyl-4-isopropylcyclohexane) family and are distinguished by the position of oxygenation on the *p*-menthane ring (10,11). Thus, peppermint and related species produce almost exclusively monoterpenes bearing an oxygen function at C3 such as menthol, whereas spearmint types produce almost exclusively monoterpenes bearing an oxygen function at C6, typified by carvone (Fig. 1).

The biosynthesis of both families of *p*-menthane monoterpenes in *Mentha* species proceeds from geranyl diphosphate via the cyclic olefin (-)-4*S*-limonene (12). The transformation of geranyl diphosphate to limonene is seemingly the least complicated terpenoid cyclization (13) in having ample precedent in solvolytic model studies (14), and the responsible enzyme has become a prototype for the terpenoid cyclization reaction (15).

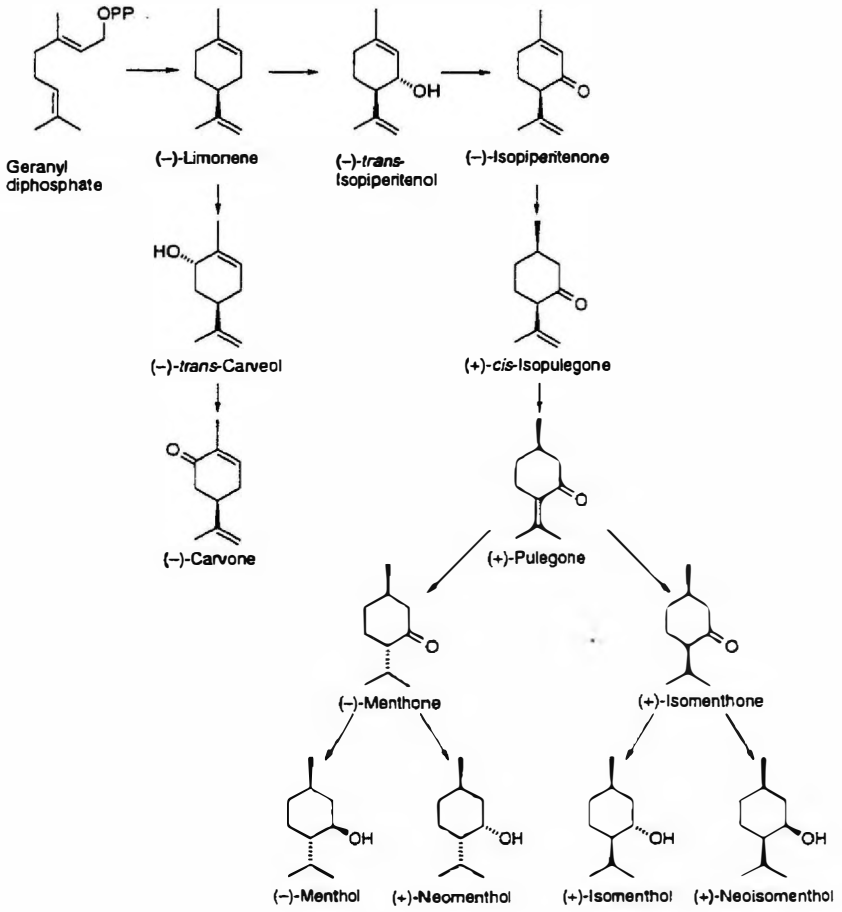

Figure 1. Pathways for the conversion of geranyl diphosphate to *p*-menthane monoterpenes of peppermint and spearmint.

Previous investigations have demonstrated that the regiospecificity of oxygenation is established very early in the monoterpene biosynthetic sequence in *Mentha* at which the common olefinic precursor (-)-limonene (12) is hydroxylated at C3 to yield (-)-*trans*-isopiperitenol (in peppermint-type species) or at C6 to afford (-)-*trans*-carveol (in spearmint-type species) (16). These regiospecific, and species specific, hydroxylases are cytochrome P450 catalysts (16). The remaining enzymatic machinery responsible for the subsequent redox transformations of isopiperitenol to menthol is present in both peppermint and spearmint species; however, carveol is a poor substrate for these reactions, with the result that only the dehydrogenation product, carvone, accumulates in spearmint types (17). *Mentha* species provide a good example of the deployment of the allylic oxidation-conjugate reduction pathway to elaborate a simple olefinic cyclization product into a series of oxygenated derivatives, and further illustrate how selectivity in the hydroxylation and subsequent redox steps determine oil composition.

The monoterpenes of *Mentha* and other members of the Lamiaceae are produced and stored in glandular trichomes, highly specialized secretory structures found on the surfaces of the leaves (18,19). The secretory cells of these glandular structures are not photosynthetic and contain only leucoplasts (20). Thus, these cells must rely in the import of carbon from the underlying tissue to support terpenoid synthesis that leads to essential oil accumulation. Secretory cells of these glandular trichomes can be mechanically isolated in high yield

as a disc of eight cells each, reflecting the original anatomy of the gland (21). The isolated cells are capable of the *de novo* synthesis of monoterpenes from basic precursors such as [^{14}C]sucrose (22), indicating that they contain all of the enzymatic machinery necessary for the synthesis of the essential oil terpenoids. This feature, in addition to the non-specific permeability of the cells towards low-molecular weight water soluble compounds, permits a broad range of metabolic studies (22,23) and, as importantly, provides a highly enriched source of both the enzymes that carry out monoterpene biosynthesis and the mRNA species that encode these catalysts.

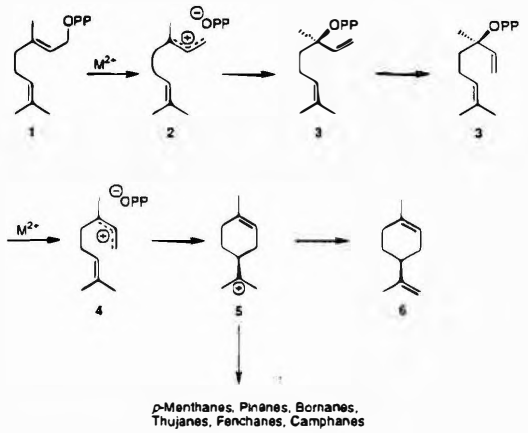

Figure 2. Mechanism of cyclization of geranyl diphosphate (1), via 3*S*-linalyl diphosphate (3) and the 4*S*-α-terpinyl cation (5), to (-)-4*S*-limonene (6) catalyzed by limonene synthase.

(-)-Limonene Synthase

The enzyme that produces (-)-4*S*-limonene (geranyl diphosphate:(-)-4*S*-limonene cyclase or, simply, (-)-4*S*-limonene synthase) has been purified from peppermint and spearmint oil glands (24), and highly specific antibodies directed against this enzyme have been prepared (25). In general properties (molecular weight, divalent metal ion requirement, hydrophobicity, pI, etc.), (-)-4*S*-limonene synthase is typical of the cyclase class of enzymes, and it seemingly catalyzes a slow, possibly rate-limiting, step of monoterpene biosynthesis in *Mentha* (26, 27). The mechanism of cyclization is illustrative of catalysts responsible for the construction of cyclohexanoid monoterpenes (26) (Fig. 2). Thus, metal ion dependent ionization of the geranyl substrate **1** leads to the charge-delocalized carbocation:pyrophosphate anion pair **2** that collapses to the enzyme-bound tertiary allylic isomer linalyl diphosphate **3** (in this instance, stereochemical considerations dictate the formation of the 3*S*-enantiomer). Rotation about the C2-C3 bond axis of the linalyl intermediate, from transoid to cisoid form, overcomes the original geometric impediment to cyclization of the geranyl precursor, and a second ionization step (to **4**) promotes C6-C1 ring closure from the *anti*, *endo*-form to yield the corresponding 4*S*-α-terpinyl cation **5**, an intermediate common to the formation of all cyclohexanoid monoterpenes (2). Subsequent internal addition of the cationic center to the remaining double and/or hydride shifts or other rearrangements lead to the pinane, bornane, thujane, camphane, fenchane and other skeletal types; however, in the case of limonene synthase, simple deprotonation from the adjacent methyl group of the α-terpinyl cation yields the monocyclic olefin **6** directly. A more thorough discussion of the mechanism of the monoterpene cyclase reaction can be found elsewhere (2,28).

A detailed understanding of the control of monoterpene biosynthesis and of the cyclization reaction mechanism required the relevant cDNA clone as a tool for evaluating patterns of developmental and environmental regulation and for examining active site structure-function relationships. Internal amino acid sequences of the purified limonene synthase from spearmint oil glands were utilized to design three oligonucleotide probes that were subsequently employed to screen a spearmint oil gland cDNA library (15). Several full-length clones were isolated and were functionally expressed in *Escherichia coli*, yielding a protein that was immunologically recognized by polyclonal antibodies directed against the native enzyme (25) and that was catalytically active in generating from geranyl diphosphate a product distribution identical to that of the native enzyme (principally limonene with small amounts of the coproducts α- and β-pinene and myrcene) (15). The longest open reading frame is 1800 nucleotides and the deduced amino acid sequence contains a putative plastidial transit peptide of about 6 kDa. Surprisingly, the targeting sequence does not appear to compromise catalytic activity as the recombinant preprotein expressed in *E. coli* is essentially as

active as the mature (truncated) native form. Sequence comparisons with other terpenoid cyclases of plant origin demonstrated a significant degree of similarity which led to the development of a general homology-based polymerase chain reaction (PCR) strategy for cloning of this enzyme type (29). The cDNA encoding limonene synthase from spearmint has been utilized to isolate the corresponding cDNA encoding the limonene synthase from *Perilla frutescens* (30) (see chapter by A. Yuba *et al.* in these Proceedings).

(-)-Limonene Hydroxylases

Microsomal preparations from the epidermal oil glands of peppermint and spearmint catalyze the NADPH- and O_2-dependent allylic hydroxylation of (-)-limonene, and the responsible enzymes meet the established criteria for cytochrome P450-dependent mixed function oxidases (16,17). The reactions catalyzed are completely regiospecific. In spearmint, the cytochrome P450 hydroxylase gives rise exclusively to (-)-*trans*-carveol. (-)-*trans*-Isopiperitenol cannot be detected as a product, indicating the presence of only (-)-limonene-6-hydroxylase (16) (Fig. 1). Conversely, microsomes obtained from peppermint glands produce only (-)-*trans*-isopiperitenol when incubated with (-)-limonene; (-)-*trans*-carveol is not detected as a product, indicating the exclusive presence of (-)-limonene-3-hydroxylase (16). These results are entirely consistent with the accumulation of C6-oxygenated *p*-menthane derivatives in spearmint and of C3-oxygenated *p*-menthane compounds in peppermint, and they established these cytochrome P450 hydroxylases as the key enzymes that determine the type of monoterpenes formed in most *Mentha* species.

All of the *Mentha* hydroxylases thus far examined appear to be highly specific for limonene as substrate, although they exhibit only a modest degree of enantioselectivity. Thus, (+)-4*R*-limonene is utilized as a hydroxylation substrate at 25-50% of the rate of the (-)-4*S*-enantiomer indicating that the orientation of the C4-isopropenyl substituent with respect to the cyclohexenyl system is of minor significance (16). By contrast, of numerous other monoterpene olefins tested, including most positional isomers of limonene, only the 8,9-dihydro analog serves as an alternate substrate for ring (C3 or C6) hydroxylation. In addition to the strict regiochemistry of the *Mentha* C3- and C6-hydroxylases, these enzymes are also readily distinguishable based on differential inhibition by substituted azoles (16); in all other measured properties, they are very similar.

The limonene-6-hydroxylase from spearmint microsomes was the first to be solubilized and purified by hydrophobic interaction chromatography. Partially amino acid sequencing allowed development of a PCR-based strategy for isolation of the corresponding clone from a spearmint gland cDNA library, and sequence information from the full-length spearmint hydroxylase was utilized to construct a selective hybridization probe for the isolation of the corresponding (-)-limonene-3-hydroxylase from peppermint. Both clones were confirmed by functional expression of the appropriate hydroxylase activity using the baculovirus-*Spodoptera* system, and both cDNAs expressed hydroxylase proteins of molecular weight 57,000 corresponding to that previously determined for the native enzymes. As might be expected, the two sequences were very similar in showing over 70% identity at the amino acid level.

Regulation of Metabolism

Limonene synthase catalyzes the first committed step of monoterpene biosynthesis in *Mentha* and, therefore, is an obvious target of regulatory studies. When limonene synthase activity in oil gland extracts and total monoterpene biosynthetic rate (as determined by $^{14}CO_2$ incorporation ***in vivo***) are measured as a function of peppermint leaf development, an essentially coincidental pattern is observed. Both synthase activity and total biosynthetic rate rise rapidly from leaf emergence, peak at or about the first week, and then decrease to essentially zero by the second week. Western immunoblotting, using anti-limonene synthase polyclonal antibodies (25) to quantify synthase protein, indicated that enzyme activity parallels enzyme protein. This observation suggests that the limonene synthase activity level directly reflects the amount of synthase present at any given time and that the activity of the enzyme *per se* is unlikely to be regulated by allosterism, covalent modification or other means. Finally, mRNA blot hybridization studies (northern analysis) demonstrated the time course of appearance and

disappearance of limonene synthase transcripts to slightly precede the appearance and subsequent disappearance of the enzyme itself, as might be expected if newly transcribed message was immediately translated to the encoded protein and was followed by turnover of both. The summation of these observations suggests that control of monoterpene biosynthesis (as evidenced by the regulation of limonene synthase) resides at the level of gene expression, and indicates that the oil glands are active in essential oil biosynthesis only during the first two weeks of leaf development, a period much shorter than previously appreciated.

Organization of Metabolism

Spearmint has been utilized as the primary model for studies on the organization of monoterpene metabolism because the biosynthetic pathway in this species is simpler than that of peppermint (Fig. 1). Nevertheless, there are several lines of evidence that indicate that many organelles within the glandular trichome secretory cells participate in monoterpene metabolism and that metabolite trafficking, even in the abbreviated pathway to (-)-carvone, is quite complex. The efficiency of transfer to and utilization of pathway intermediates in the various cellular compartments has clear implications relating to the composition of the essential oil that is ultimately sequestered in the gland subcuticular space.

Studies with isolated mint glands indicate that gland cell plastids (leucoplasts) are capable of synthesizing isopentenyl diphosphate, the fundamental precursor of terpenoids, from basic carbon substrates (23), and several lines of evidence indicate that limonene also originates at this site. Thus, the deduced amino acid sequence of the cDNA encoding (-)-limonene synthase contains a putative amino-terminal plastidial transit peptide (15), and the *in vitro* translated preprotein of about 70 kDa is proteolytically processed to a size corresponding to the mature form of about 64 kDa in isolated pea chloroplasts. Finally, immunogold cytochemical techniques have localized limonene synthase specifically in the lumen of the secretory cell leucoplasts.

By contrast, the deduced amino acid sequence of the cDNA encoding the limonene-6-hydroxylase yields the mature protein in size (57 kDa) and does not appear to contain a transit peptide, but rather specifies a typical amino-terminal membrane insertion sequence. The latter is typical of endoplasmic reticulum proteins such as cytochrome P450, and is consistent with the location of the hydroxylase in the microsomal (light membrane) fraction of gland cell homogenates (16). The carveol dehydrogenase responsible for carvone formation is an operationally soluble enzyme (17), and is unlikely to originate in either plastids or endoplasmic reticulum thus suggesting the participation in metabolism of yet another compartment. Whether the enzyme is cytosolic, or perhaps associated with the vacuolar structures thought to be involved in essential oil secretion (see chapter by M.R. Kolatite in these Proceedings), is uncertain.

It is notable that spearmint oil contains 10-20% limonene, depending on leaf age, implying some inefficiency in either transfer of the olefin to the endoplasmic reticulum or in the conversion to carveol at this site. Carveol, on the other hand, does not appreciably accumulate in spearmint oil, indicating that this intermediate is efficiently transformed to carvone prior to oil secretion to the subcuticular storage cavity. This situation contrasts markedly to that of peppermint in which the more extensive pathway operates in menthol biosynthesis. In this instance, neither limonene nor isopiperitenol accumulate in the oil (<1%), although other downstream intermediates, such as pulegone and menthone, are present in readily measurable amounts. The precise composition of an oil can be seen to depend upon the efficiencies of coupling of the various biosynthetic steps but whether this relates to differences in metabolite trafficking from one site to the next, or to transformation kinetics at each site, or both, is not yet clear, even for the case of spearmint where only three enzymatic reactions are involved.

Prospects

With success in the cDNA cloning of limonene synthase and limonene hydroxylases, and the development of general cloning strategies, it seems likely that other sequences for monoterpene synthases and hydroxylases will soon be available. With such genes in hand, the molecular genetic manipulation of monoterpenoid composition and yield in essential oil plants can be contemplated. It is also conceivable that the monoterpene pathway

can be engineered into "non-essential oil" plants, such as fruits and vegetables, and even ornamental species, to impart desirable odor properties; the transformation of microbes for the fermentative production of selected monoterpenoid compounds can be readily realized based on existing technologies. Given the ecological roles of the monoterpenes (31,32), their use in the genetic engineering of chemical defenses in field crops against insect pests and pathogens also seems inevitable. However, the availability of the structural genes encoding key biosynthetic pathway steps is not in itself sufficient to insure adequate production of the target metabolites at the appropriate location in a transgenic organism. It is clear that successful genetic engineering of monoterpene production will ultimately require a more detailed understanding of the organization and regulation of metabolism, especially as findings related to precursor supply, metabolite trafficking, and the secretion process can be applied to the biosynthesis of monoterpenes in transgenic species that normally do not produce appreciable quantities of these compounds.

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LIMONENE SYNTHASE FROM *PERILLA FRUTESCENS*

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Introduction

Perilla frutescens Britton (Labiatae), which is used as a food, Chinese crude drug, natural pigment, and spice in Asian countries, shows chemical variation with regard to the essential oil components of the leaves (1). Figure 1 illustrates six defined *Perilla* chemotypes; one that accumulates cyclohexanoid monoterpenoids (PA type), three that produce different types of furanoid monoterpenes (EK, PK and PL types), one that contains only acyclic monoterpenes (C type), and one that does not produce any leaf monoterpenoids but, accumulates phenylpropanoids (PP type). Genetic analyses of various chemotypes demonstrated that several genes control the biosynthesis of monoterpenoids including the major component perillaldehyde, which is accumulated in the presence of two dominant genes *G* and *H*. Furthermore, it has been shown that limonene synthase activity, which plays a primary role in synthesizing *l*-limonene, the precursor of perillaldehyde, was detectable only in the seedlings of the *GH* genotype (2). To study the regulation mechanism of the limonene synthase activity in relation to the function of genes *G* and *H*, we have cloned a cDNA encoding limonene synthase from the seedlings of *P. frutescens*.

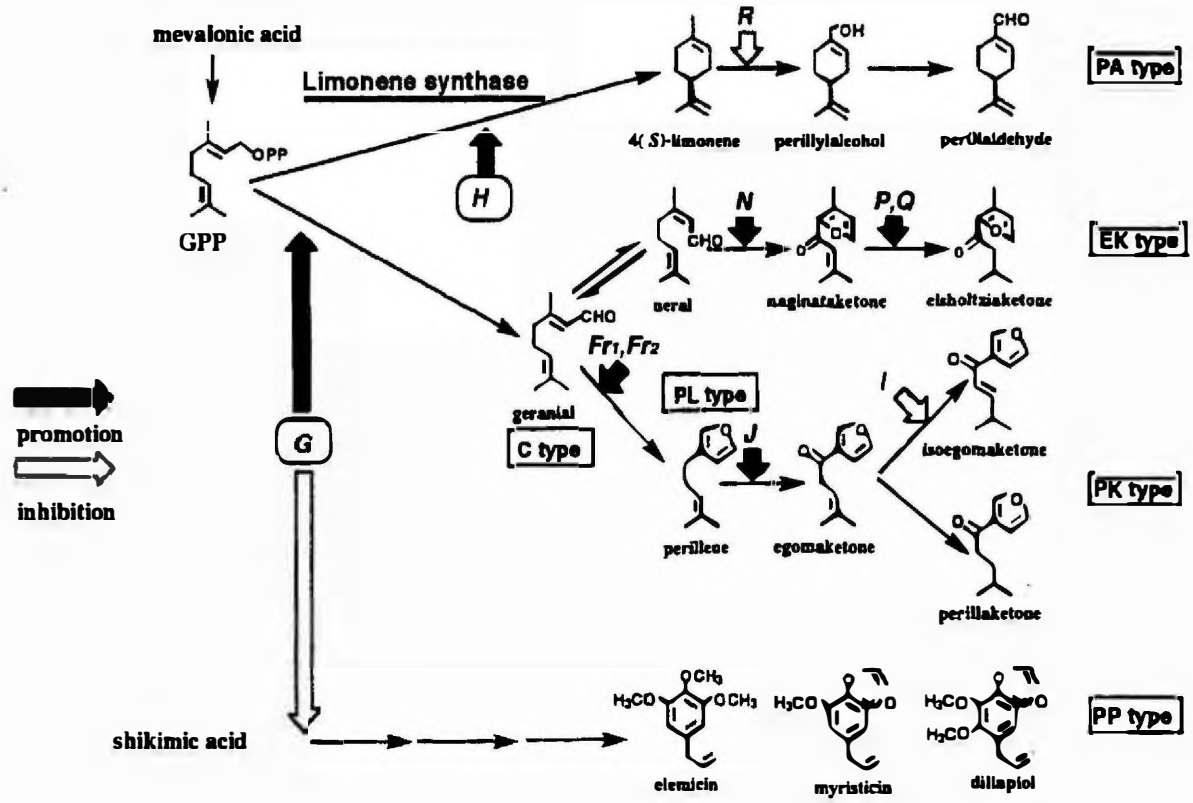

Figure 1. Hypothetical biosynthetic pathways for essential oil constituents of *Perilla frutescens* and genes controlling the reaction steps. Black arrows indicate promotion, and white arrows indicate inhibition, of the indicated biosynthetic steps

cDNA Library Construction and Cloning

A l ZAPII cDNA library constructed from *Perilla* seedlings (strain No. 9, *GGHH*) was screened with the limonene synthase cDNA (LC5.2) from *Mentha spicata* (3) as a probe to isolate 10 independent positive clones from *ca*. 30,000 plaques. A representative clone PFLC1 (accession No. D49368) was 2031 bp in length, containing an open reading frame of 603 amino acids (4). Its identity to the open reading frames of spearmint limonene synthase, tobacco 5-*epi*-aristolochene synthase, and castor bean casbene synthase were 65%, 35% and 30%, respectively (5, 6). All of four terpene cyclase amino acid sequences contain the well-known DDXXD consensus motif which is thought to represent a binding site for the substrate prenyldiphosphate/metal ion-complex (7). This conserved motif is also present in FPP (farnesyldiphosphate) synthase of various species (8), and the important role of the conserved aspartates for enzymatic activity has been demonstrated by directed mutagenesis experiments.

Expression of PFLC1 in *E. coli*

One of these cDNA clones was functionally expressed in *Escherichia coli,* yielding enzyme which was catalytically active in generating 4(*S*)-limonene from geranyldiphosphate. The open reading frame of a cDNA clone, pPFLC9 is in frame with the b-galactosidase that is driven by the *lacZ* promoter of the vector. This translational fusion construct was used to test functional expression in *E. coli* XL1-Blue. For comparison, pPFLCl and pBluescriptII SK(+) were employed as controls. Transformed bacteria were induced with IPTG, and the cultured cells were harvested, homogenized, and assayed for limonene synthase activity. The enzymatic reaction product gave the identical mass fragmentation pattern on GC-MS with authentic specimen, 4(*S*)-limonene. Preparations from the culture containing pPFLC9 afforded measurable levels of limonene synthase activity (0.1-1.0 nmol / incubation), whereas no other olefin product was detected. SDS-PAGE analysis of the corresponding protein fractions showed an extra band at *ca.* 70 kDa compared to non-induced controls.

Genomic Southern Analyses of PFLC1

Genomic Southern blot analyses of various genotypes (*GGHH*, *GGhh*, *ggHH*, and *gghh*) of *P. frutescens* suggested that at least two copies of the PFLC1 encoding limonene synthase exist in strains having the *HH* genotype. In contrast, no PFLC1 DNA sequences were found in the genomes of strains with the *hh* genotype (Table 1). The *hh* genotype strains, accumulating non-cyclohexanoid-type monoterpenoids such as perillaketone or geranial (Fig. 1), can not produce

Table 1. Genotypes and Chemotypes of *Perilla frutescens* used in the present study

Strain No	9	32	5539	3	6	8	11	63	79	1828	1841
Genotype	*GGHH*	*GGHH*	*GGHH*	*GGhh*	*GGhh*	*GGhh*	*GGhh*	*GGhh*	*GGhh*	*GGhh*	*GGhh*
Chemotype	PA	PA	PA	EK	PK	PK	PK	PK	EK	PK	EK

Strain No.	**1847**	**1863**	**1864**	**1866**	**1**	**10**	**16**	**25**	**12**	**5316**
Genotype	*GGhh*	*GGhh*	*GGhh*	*GGhh*	*ggHH*	*ggHH*	*ggHH*	*ggHH*	*gghh*	*gghh*
Chemotype	EK	PK	PL	PK	PP	PP	PP	PP	PP	PP

Note: Chemotypes were classified according to the main constituents: PA type, perillaldehyde and l-limonene; EK type, elsholtziaketone and naginataketone; PK type, isoegomaketone and perillaketone; PL type, perillene; and PP type, elemicin or myristicin or dillapiol

the essential biosynthetic intermediate 4(*S*)-limonene because they lack limonene synthase. Classical genetic analyses showed that the dominant gene *H* is completely epistatic to other genes such as *Fr1*, *Fr2* and *N* which are involved in the biosynthesis of non-cyclohexanoid-type monoterpenes (1). Non-cyclohexanoid-type monoterpenes are produced only in the absence of *H*, suggesting that a sequential expression of the *N*, *Fr1* and *Fr2* genes might exist in the *Perilla* genome (1).

Organ Specific Expression of PFLC1

Organ-specific expression analysis demonstrated that the PFLC1 mRNA accumulates in the aerial parts of *GGHH* plants, and showed the highest mRNA level in leaves. In *ggHH* plants, only minute amounts of perillaldehyde can be detected in the stem and calyx, consistent with the low levels of the PFLC1 mRNA detected in these tissues. In the *ggHH* genotype, it is known that a gene *G'* (necessary for the expression of *H*), is expressed exclusively in the calyx (9); a *G'*-like gene controlling the stem-specific biosynthesis of monoterpenes has not yet been discovered.

Summary

The present study has clearly demonstrated that *Perilla* strains of the genotype *hh* completely lack PFLC1, indicating that the dominant gene *H* must be either PFLC1, the limonene synthase structural gene itself, or a gene locus containing PFLC1 as a part of its structure.

It is still not known how the expression of PFLC1 is regulated in the presence of the gene *G*, whose exact function needs to be clarified in connection with the expression of *H*. Analysis of the promoter region of the *H* (PFLC1) gene should help to clarify the regulatory mechanism.

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SELECTION FOR SEED YIELD AND EARLINESS IN FENNEL (*FOENICULUM VULGARE* MILL.) AND CORRELATED RESPONSE IN SEED-OIL YIELD

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INTRODUCTION

Fennel (*Foeniculum vulgare* Mill.) is considered as an important medicinal plant. It is also edible as vegetable crop and has utilization in food processing and bakery products. A local cultivar raised from commercial seeds is currently used in some areas of Egypt. The cultivar has a wide phenotypic variations in plant growth characteristics, earliness and seed yield. Vigorous plant growth is not necessarily associated with high seed yield (Mamedova and Aliev, 1985). Characteristics of a successful variety in fennel includes earliness in maturity with high seed yield, limited growth height for suitability to combine harvest, high oil contents of the seeds, and low estragol content in the oil (Reichardt and Pank, 1993). There is a need to improve fennel seed production from this local cultivar. Towards this goal, an elite version derived from this adapted cultivar should be developed. The new version may be proven successful as an improved variety or it may be crossed subsequently with one or more exotic varieties to incorporate new traits if needed.

The main objectives of the present study were: 1) to conduct selection for increasing fully developed seed yield and enhanced earliness in maturity, and 2) to study correlated responses in essential oil and its components. We have found no information available on the major genetic parameters to aid breeding in this fennel population for growth and developmental characteristics, and seed yield under South-Egyptian conditions. Therefore, initial studies were undertaken to get estimations for narrow-sense heritabilities and phenotypic associations of some growth and developmental characteristics and seed yield.

MATERIALS AND METHODS

Source of seeds and general experimental procedures

Local Egyptian cultivar of fennel (*Foeniculum vulgare* Mill) was used in the present study. Commercial seeds of this cultivar were obtained from local seed retailers at Assiut district. All experiments in this study were conducted at the Ornamental Research Station, Assiut University, Assiut, Egypt.

Common in all experiments, triple supper-phosphate was added, at rate of 150 kg/fedd., during soil preparation. Two to three seeds were planted during the first week of Oct. in hills 30 cm apart on the northern side of 70 cm wide and 120 cm long rows. Fifteen to twenty days after planting, seedlings were thinned to a single plant per hill. Plants were fertilized with ammonium-nitrate at rate of 200 kg/fedd. Half of the ammonium-nitrate amount was added 2 weeks after thinning. The other half was added in addition to potassium-sulphate (at rate of 50 kg/fedd.) at the time of blooming of the first order umbel. Growing plants were irrigated as needed. The following traits were recorded on the individual mature plants at the harvest time: 1) plant height, (cm) 2) number of primary branches, 3) total number of umbels, 4) number of umbels containing fully developed (FD) seeds, 5) number of umbels with shrink partially developed (PD) seeds, 6) total seed yield (g), 7) weight (g) of fully developed (FD) seeds, and 8) weight (g) of shrink (PD) seeds.

Selection procedures

Initial studies were undertaken to decide the appropriate selection procedure based on information about phenotypic associations and narrow-sense heritabilities of some growth and developmental characteristics, and seed yield. A random sample of 250 individual plants from the cultivar grown in 1994 and 1995 were used to get estimations for correlation coefficients (r). Open-pollinated seeds of each of these plants were harvested and stored separate. The total linear correlation coefficients were estimated as $r = \text{cov. } xy / (\text{var. } x * \text{var. } y)^{0.5}$, where x and y are two random variables (Gomez and Gomez, 1984).

Seeds from thirty individual plants were selected at random from the population of the cultivar grown in 1994. Progenies of these 30 plants were grown in 1995 and 1996. A randomized complete-block

experiment with four replicates was used to evaluate these progenies. Nine plants were grown from each progeny per replicate. Narrow-sense heritability was estimated as $h^2 = 2*b_{op}$, in which b_{op} is the coefficient estimated by regression of offspring on parental values (Falconer, 1981).

Plant selection started in 1995 based on the information about r estimates (in 1994 and 1995) and h^2 estimates (in 1995), the following selection procedure was applied to the data file of the individual plants of the cultivar grown in 1995: 1) the top 40 plants (16% of the population) for the number of umbels containing FD seeds were marked, and 2) these plants were then screened based on the reduced number of umbels with PD seeds, and greater weight of FD seeds. The selected plants were seventeen (about 7% of the original population). Seven of the 17 plants were among plants labelled in the field in 1995 for being 3 to 4 weeks earlier in maturity (developed mature dry seeds at first week of May) than other plants in the population.

Evaluation of the selection materials

A randomized complete-block experiment with four replicate was used to evaluate the following treatments in 1996 growing season: 1) bulked seeds from progenies of all the 17 selected plants (C_1 population), 2) seeds represent the original cultivar [base population (C_0), control treatment), and 3) seeds from each of 3 early and high yielding plants among the 7 early selections (E_2, E_4, and E_5). Each of the first two treatments was represented by 60 plants per replicate. The third treatment was 20 plants from each progeny per replicate. In addition to data on the traits mentioned elsewhere in this text, the essential oil and its components in the seeds were also determined. All data were subjected to the analysis of variance and treatment means were compared using the orthogonal contrast procedure (Gomez and Gomez, 1984).

Essential oil and separation of monoterpenes

Samples of 5 g dry seeds were used for oil determination by hydrodistillation. For oil constituents, 1 mL of the oil was diluted in 2 mL acetone then 1 mL of this solution was injected into a Hewlett Packard Gas Chromatograph (GC) Model 5890 Series II using a Flame Ionization Detector (FID) temperature of 280°C and an injection temperature of 250 °C. The injection was made onto a 25m OV225 capillary column (Macherey-Nagel) with 0.25 mm ID. The temperature program was 120 °C, 2 min; 12-200 °C, 25 °C/min; 200 °C/min. The carrier gas was H_2, 1 ml/min constant flow. Six main components were identified in the oil: μ-pinene, myrcene, limonene, fenchone, estragole, and trans-anethole (Fig. 1). Identity of the peaks was confirmed through the use of standards and data were reported in area percentages.

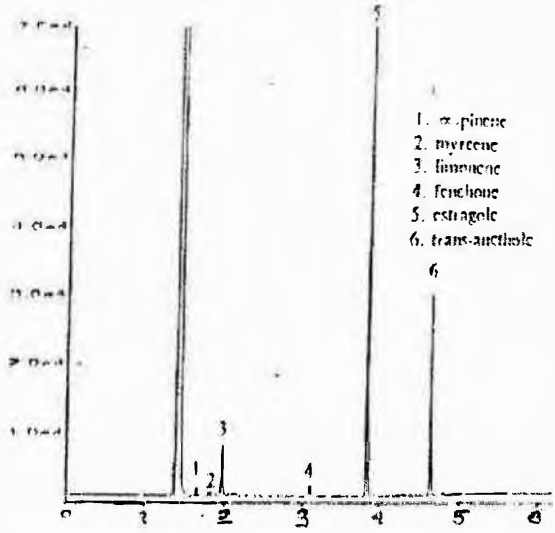

Figure 1. Gas chromatogram of essential oil of a local Egyptian fennel cultivar

RESULTS AND DISCUSSION

Estimates presented in Table (1) and in Table (2) verify the use of umbel counting and classifications as primary selection criterion to indirectly screen for improving seed yield. Seed yield had low heritability coefficients. Number of umbels containing FD seeds exhibited relatively high h^2, in addition to high r values with seed yield criterions. Due to the positive association between number of umbels containing FD seeds and those with PD seeds, screening was suggested against the latter to reduce its frequency within those individuals having great number of umbels containing FD seeds.

Table 1. Phenotypic correlation coefficients (r) among some growth and developmental characteristics, and seed yield in population of Egyptian local cultivar of fennel, 1994 (top-right diagonal) and 1995 (bottom-left diagonal).

Trait[a]	H	B	TU	UFD	UPD	TY	YFD	YPD
H		0.24**	0.13	0.02	0.21**	0.32**	0.27**	0.17*
B	0.27**		0.13	0.00	0.12	0.23**	0.15*	0.14
U	0.12	0.11		0.88**	0.83**	0.71**	0.68**	0.53**
UFD	0.14*	0.13	0.82**		0.56**	0.63**	0.65**	0.41**
UPD	0.17*	0.09	0.83**	0.45**		0.59**	0.53**	0.52**
TY	0.28**	0.21**	0.71**	0.66**	0.59**		0.98**	0.64**
YFD	0.27**	0.23**	0.66**	0.64**	0.53**	0.96**		0.46**
YPD	0.16*	0.11	0.53**	0.40**	0.48**	0.64**	0.43**	

[a] *H=height, B=branches, TU=total number of umbels, UFD= umbels of fully developed seeds, UPD=umbels with partly developed seeds, TY=total yield(g/plant), YFD=yield of fully developed seeds, YPD=yield of partly developed seeds.*

* *and* ** *are significant at 0.05 and 0.01 levels of probability, respectively.*

Table 2. Regression heritability estimates (h^2) for seed yield, and some growth and developmental characteristics in population of Egyptian local cultivar of fennel.

Trait	1995	1996
Plant height	0.32 (0.13)*	0.38 (0.10)
Primary branches	0.41 (0.09)	0.47 (0.08)
Umbels (no.)		
total number	0.31 (0.14)	0.29 (0.10)
with fully developed seeds	0.41 (0.15)	0.43 (0.16)
with shrink partly developed seeds	0.25 (0.14)	0.24 (0.16)
Seed yield (g/Plant)		
total yield	0.16 (0.11)	0.11 (0.08)
fully developed seeds	0.14 (0.16)	0.13 (0.12)
partly developed seeds	0.15 (0.17)	0.08 (0.13)

* *Data between parenthesis are the standard errors*

The bulked seeds from the progenies of the 17 selected plants (C_1 population) produced greater number of umbels containing FD seeds and less number of umbels with PD seeds than the original cv. (C_0 population) (Table 3). Higher yield of FD seeds causing an increase in total seed yield were also produced in the C_1 than in the C_0 population. The increases were 15.2 % and 11.8 % in weight of FD seeds and total seed yield, respectively. The weight of PD seeds was 13.5 % less in the C_1 population than in the C_0 population. The ratios for the weights of FD seeds : PD seeds were 10:1 and 7.5:1 in the C_1 and in the C_0 populations, respectively. The progenies tested for the three early selections were similar to each other and to C_0 population in weight of FD seeds, PD seeds, and total seed yield.

Table 3 . Performance of bulked progenies from 17 selected plants (C_1), separately tested progenies of 3 individuals of them (E_2, E_4 and E_5), and base population (C_0) of Egyptian local cultivar of fennel for some growth and developmental characteristics[a], 1996 growing season

Entry	TU	UFD	UPD	TY (*g/plant*)	YFD (*g/plant*)	YPD (*g/plant*)
Progeny E_2	43.2	27.8	15.4	88.4	79.1	9.3
Progeny E_4	51.6	36.7	14.9	94.6	86.1	8.5
Progeny E_5	47.9	32.6	15.3	92.9	84.1	8.7
C_1 population	52.6	35.8	16.8	98.7	89.7	9.0
C_0 population	53.5	31.1	22.4	88.3	77.9	10.4
Contrasts[b]						
E_4 vs E_2 & E_5	**	**	ns	ns	ns	ns
E_2 vs E_5	**	**	ns	ns	ns	ns
E_2 &E_4 &E_5 vs C_0	**	ns	**	ns	ns	ns
C_1 vs C_0	ns	**	**	**	*	ns

[a] *TU=total number of umbels, UFD=umbels of fully developed seeds, UPD=umbels with partially developed seeds, TY=Total seed yield, YFD= yield of fully developed seeds, YPD=yield of partially developed seeds.*

[b] *ns, *, and ** are not significant and significant at 0.05 or 0.01 level of probability, respectively.*

Twenty-six per cent of the plants in C_1 population and 38% of the plants in the individually tested progenies, in contrast to 7.5% in the C_0 population, developed mature dry seeds 3 weeks earlier than the remaining plants in these populations. Significant decrease in plant height and increase in number of primary branches occurred (Table 4) in C_1 and the 3 progeny populations compared to C_0 population.

These results indicate that selection based on number of umbels was effective in improving seed yield in this cultivar of fennel. Also, enhanced earliness in maturity and decrease in plant height can be achieved without affecting seed yield. Similarly, selection within the local Ukrainian population of fennel (Zhurbenko, 1977) was effective in developing forms which were 20 - 60% higher in seed yield and 14 to 17 days earlier in maturity. The use of umbels as primary selection criteria has the following technical

advantages: 1) number of umbels can be observed several weeks before seed harvest, and 2) number of umbels needs less labour than seed yield, which should be collected dry and weighted, to determine.

Essential oil contents and its components except trans-anethole were not changed in C_1 population as result of selection for increasing seed yield and enhanced earliness in maturity (Table 4).

The progenies of the three early selections as a bulk showed no differences in essential oil and its estragole component compared to C_0 population. Limonene, fenchone, and trans-anethole were higher in the population of these progenies than C_0 population. Differences in essential oil and its components existed among these progenies. Seeds of E_4 had higher essential oil contents compared to E_2 and E_5. The highest limonene and trans-anethole levels were also found in E_4. Seeds of E_2 contained the highest level of fenchone. Estragole was the major component of essential oil in the three tested progenies, C_1 and C_0 populations. Cavaleiro et al., (1993) proposed an existence of different chemotypes in fennel. Genotypic variation exists in fennel of different origin for essential oil and its components (Kapelev, 1980; Aralsan et al., 1989). Recently released breeding strain producing essential oil yield of 7.0 ml/100g dry seeds which contains above 30% fenchone and less than 5% estragole was reported by Reichardt and Pank, 1993.

Our results suggest that essential oil and its components should be used as direct selection criterion to improve the traits related to seed-oil contents in this cv. of fennel. Inbreeding and progeny test would be helpful to differentiate promising individuals and accelerate the improvement for essential oil and its components. Concerning the reduction of the estragol, in this cultivar, incorporation of exotic germplasm would be useful.

Table 4. Plant height (H), number of primary branches (B), essential oil yield and its constituents in bulked progenies of 17 selected individuals (C_1 population), separately tested progenies of 3 individuals of them (E_2, E_4 and E_5), and base population (C_0) from Egyptian local cultivar of fennel,1996 growing season.

Entry	H	B	% Oil yield	% Oil constituents			
				Limonene	Fenchone	Estragole	Trans-anethole
Progeny E_2	108.1	6.0	1.45	9.1	7.5	78.9	0.18
Progeny E_4	114.4	7.7	2.11	21.2	1.3	69.2	5.80
Progeny E_5	113.6	6.8	1.12	8.3	1.7	83.2	0.22
C_1 population	105.6	6.7	1.76	11.4	2.6	78.5	2.69
C_0 population	116.1	6.2	1.56	9.4	2.4	84.2	0.78
Contrasts[a]							
E_4 vs E_2 & E_5	*	**	**	**	**	*	*
E_2 vs E_5	**	**	*	ns	**	ns	ns
E_2&E_4&E_5 vs C_0	**	*	ns	*	**	ns	**
C_0 vs C_1	*	*	ns	ns	ns	ns	**

[a] *ns, *, and ** are not significant and significant at 0.05 or 0.01 level of probability, respectively.*

Acknowledgement

The first author would like to thank the staff members of the Institute für Qualitätsanalytik at Quedlinburg, Germany for the lab facilities, equipments and sincere help they offered to analyze the fennel samples during her visit to their institute.

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SEED PRODUCTION IN MALE STERILE PLANTS IN *SALVIA SCLAREA* L. (LAMIACEAE)

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INTRODUCTION

Salvia sclarea is a valuable aromatic and medicinal plant with a great application in perfumery and folk medicine. It is wild growing on limestone places throughout Bulgaria. From air dried leaves 1.5 - 2.5 % essential oil has been obtained.

In Bulgaria there are several cultivars: "Iskra", "Trakiika" and "Boyana", but they can not completely satisfy the need for this plant. The heterosis selection of new cultivars based on male sterile aromatic plants is of great importance and will help to solve this problem.

Recently at the Institute of Botany male sterile plants of *S. sclarea* were found in the available collection of freely pollinated lines and the "Boyana" cultivar. Cytoembryological investigations were carried out on microsporo-, microgametogenesis and development of male gametophyte with the purpose to determine the genetic nature of male sterility (1).

The present study is aimed at finding out how male sterility affects female gametophyte and the quantity and quality of seed production, literary date about which are scarce (2). To the best of our knowledge, which is based on the available literature, *S. sclarea* is investigated for the first time in such aspect.

EXPERIMENTAL

Material and Methods - Flower buds and flowers of *S. sclarea*, collected from 1989 to 1990 of freely pollinated lines and "Boyana" cultivar at the experimental field of the Institute of Botany (Bulgarian Academy of Sciences) located at the village of Gorni Lozen were used as material.

They were fixed in Navashin's mixture and treated according to standard paraffin methods. Sections (6-10 μm thick) were prepared using "Minot" rotary microtome. Heidenhein's hematoxylin was used as a colouring agent. The study was carried out with "Amplival" light microscope.

RESULTS

The important changes in stamens, calyx and corolla in the male sterile plants were associated with sterilization processes in the female generative organs of some flowers.

The early stages of pistil organogenesis in fertile and male sterile plants were identical. Further on differences in pistil formation became visible in formation of more than 4 loculi per one pistil (5-8) in some male sterile plants. The greater part of these loculi remained empty.

Often in such flowers more than one ovule in every loculus was formed. The greater part of these ovules degenerated later, at the stage of mature embryo sacs.

Normally, a tenuinucellate, anatropous, unitegmic ovule is formed in each of the four loculi of the pistil of *S. sclarea*. A hypodermal archesporial cell functioned directly as a megaspore mother cell and epidermis remained as a single layer.

A linear megaspore tetrad was formed, the chalazal cell of which developed into a *Polygonum*-type (8-nucleate) embryo sac, essentially uniform in Lamiaceae (3).

Parallel to the normal running embryonal processes degeneration of some ovules was observed starting at 2-celled embryo sacs and reaching its maximum at the stage of mature embryo sacs.

Fertilization was porogamous. Endosperm formation was ab initio cellular. The primary endosperm cell divided transversely in two chambers. Only the primary micropylar chamber divided by incomplete wall and the chalazal ones developed directly into a 2-nucleate haustorium. So endosperm development in *S. sclarea* can be referred to the most distributed in *Lamiaceae Prunella* - Typus (3).

The embryogenesis was of *Onagrad*-type like all other studied taxa of *Lamiaceae* (4). It followed the entire formation of cellular endosperm. After fertilization the zygote enlarged considerably and its first division was by transversal wall.

The mature seed was almost without endosperm and with a straight embryo belonging to the evolutionary most advanced in *Lamiaceae* "Investing"-type (5). The embryo possessed thick formed cotyledons, overlapping and encasing the radicle and the hypocotyl for at least half its length, like appendices.

In some seeds of male sterile plants we observed degeneration of the embryo at the Torpedo- and Heart-stages. This caused later the formation of embryoless seeds only with cellular endosperm.

In other studied mature seeds of *S. sclarea* the degeneration of the embryos was caused by the degeneration of the cellular endosperm. In this way empty seeds covered only by testa were formed. The correlation between normal, embryoless and empty seeds was summarized in Table1.

Further we studied the mature seeded nutlets development in fertile and sterile plants with the purpose of determining the influence of these anomalies on its quantity. In fertile plants the fruits were normally of four 1 - seeded nutlets, but rarely also of 2 - or 3 - 1 - seeded nutlets.

In male sterile plants there were more often formed only one to two 1 - seeded nutlets. In some cases even no nutlets were formed. As a result the number of the mature seeds has increased considerably.

DISCUSSION

On the 700 species distributed all through temperate and tropical regions male sterility is known only in *S. nemorosa* and *S. officinalis* (6). Recently male sterile forms have been found also in *S. sclarea* (1). To establish the nature of male sterility the microsporo - and microgametogenesis of these plants have been studied (1). Data about female generative organs in male sterile plants are very scarce (6).

The need for new specific cultivars of S. sclarea based on heterosis selection of male sterile plants determine a detailed cytoembryological study of the quantity and quality of seed production.

It has been found that male sterility increased the quantity of seed production compared with these of the fertile plants by the average of 20 %, due to sterilization processes in different stages of megagameto - and embryogenesis. Our results are similar with these for male sterile plants in S. officinalis, where lower seed production was obtained (6).

Further we find out that male sterility has an influence also on the quality of the seed production. Among mature seeds parallel with the normal embryoless and empty ones covered only by testa were presented. The last two types are visible externally like normal seeds.

All these sterilization processes during the megagameto - and embryogenesis affected the seed production of male sterile plants by the average of 20-30 % compared with these of fertile ones. These peculiarities have to be provided by the heterosis selection of specific cultivars.

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Table 1. Heterogeneity of seed production in male sterile plants of *S. sclarea*

Number of plants	Kinds of seeds (% per plant)		
	normal	embryoless	empty
1	91	5	4
2	85	9	6
3	79	10	11
4	87	8	5
5	82	11	7

LEAF ESSENTIAL OIL COMPOSITION OF A SELECTED STRAWBERRY POPULATION IN QUEBEC

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INTRODUCTION

The two-spotted spider mite (TSSM), *Tetranychus urticae* Koch., and some specific diseases like leaf spot (*Mycosphaerella fragariae* Tul.), leaf scorch, (*Diplocarpon earlina* Ell. and Ev.) and red stele (*Phytophthora fragariae* Hickman) are among the most important pest and diseases of strawberry in eastern central Canada.

In the last 10 years many crosses have been made and evaluated in our breeding program in an attempt to breed a new hardy strawberry cultivars resistant to specific pests and diseases adapted to Quebec Climate. Unfortunately most selections which were resistant to these disease and pests did not produce a good crop or their fruit did not have a good agronomic value. However some of the selections which produced a good commercial crop were susceptible to one or a combination of two-spotted-spider mite (TSSM), tarnish plant bug, clipper weevil, leaf spot, leaf scorch, leaf blight, verticellium wilt, mildew or botrytis fruit rot.

Among many methods that we use to improve the pest and disease susceptibility we also look at the essential oil (EO) composition of a randomly selected strawberry genotype in an attempt to correlate the degree of pest and disease susceptibility with leaf EOs composition. Our previous work (Khanizadeh and Bélanger, 1996) showed some relationship between EOs compositions and degree of mite susceptibility but it was not conclusive since we did not have a large population.

The first part of this research is to compare leaf EOs concentrations of a 92 strawberry genotypes and then construct a of pests and diseases susceptibility table based on our field and greenhouse observation and literature, and to determine the similarity of the genotypes based on their leaf EOs and pests and disease susceptibility.

MATERIALS AND METHODS

Ninety two strawberry lines and commercial cultivars with different degrees of susceptibility or resistant to two-spotted spider mite (TSSM), verticellium wilt, leaf spot, leaf scorch, mildew, anthracnose, botrytis fruit rot, etc. were used in our study. The 88 cultivars and selections used in our study were chosen from a wide range of breeding lines and programs from Belgium, Canada, Denmark, England, France, Italy, New Zealand, Scotland, The Netherlands and USA. Four wild genotypes which were reported to be pest and disease resistant were also added to the population for comparison. Two of these wild genotypes were *F. Chiloensis* were donated by Dr. H. Daubeny to Smithfield Canadian Germplasm repository (Accession number 590 & 859). There were collected from Vancouver and known to be resistant to leaf diseases and mite (Personal communication with M. Lufmman, the curator). The other two leaf disease resistant wild genotypes were *F. viridis* and *F. virginiana.*

All of these commercial genotypes and selected breeding lines and wild species are maintained in our tissue culture bank. They are used in our breeding program and their plant and fruit characteristics were kept in our data base program (Pedigree, 1990). In 1993, plants produced from tissue culture materials were transplanted in the field. Approximately 0.5 kg of leaves of each cultivar were collected at the end of the summer of 1994 for leaf EO analysis. The leaf analysis, steam-distillation and identification of EOs were performed as previously described (Khanizadeh and Bélanger, 1993).

A cluster analysis which hierarchically clusters all the observations was used for grouping the

genotypes based on their complete leaf essential oil composition similar to our previous investigation (Khanizadeh and Bélanger, 1996).

RESULTS

Ninety compounds were detected from 92 strawberry genotypes. Thirty four compounds were identified (Table 1), and the rest are under investigation for identification. The cluster analysis were performed on all the compounds and also on the ones that we could identified, however the results (Fig. 1) is based on the identified compounds find in the EOs.

Four major clusters were produced based on the leaf EO composition. Two *Fragaria chiloensis* (Accession number 590 & 859) and *F. viridis* created their own cluster and being resistant to spider mites and leaf diseases. *Fragaria virginiana* created its own group being completely different from the rest (Fig. 1). In our preliminary comparison, we averaged the EOs of genotypes which were grouped together based on their leaf essential oil composition (Table 1). No Linaloloxide or Cis-Linaloloxide were detected in *F. virginiana*, *F. viridis* and the two *F. Chiloensis* (Accession # 590 & 859). The concentration of Linalool, Damascenone, α-Copaene, were very low or absent in *F. chiloensis* and *F. viridis*. No trace of Docosane was detected for *F. virginiana* (Table 1). The absence of these chemical might be due to their low concentration in the leaf which make it difficult to be detected by our instrument. A large differences were observed on certain leaf EO composition of wild species and cultivated genotypes. Farnesol, α-Pinene, Nonanal, 1-Octanol, β-Caryophyllene, Heptanal, Phenylacetaldehyde, Hexadecanoic acid, Phytol, Hexahydrofarnesyl Acetone, Heptane 2-4-Dimethyl, β-Pinene, β-Ionone and (Z)-3-Hexenylacetate were observed in all wild species compared to commercial lines and cultivars. The concentration of certain Linalool, Decanal, 1-Octanol, Farnesol, Thymol and Damascenone were very high in *F. virginiana*. Similar high concentration of Farnesol, α-Pinene and Nonanal were observed in *F. viridis* and *F. chiloensis* (Table 1). However it is possible that the composition of leaf EOs varies due to the interaction of a specific cultivar with environmental factors like temperature, humidity, light or other plant characteristics or cultural practices. Much more work is needed to study the pest and disease resistance of strawberry plants and their interaction with other plant and environmental factors. Our next step is to test individual genotype with one or a combination of selected EOs in comparison to wild species and construct a table of pests and diseases using the information which is already available in our data base (Pedigree, 1990) in an attempt to reveal any possible relationship between EOs and degree of pest and disease susceptibility. This technique might be also useful in identification of resistant lines early in the breeding program to eliminate susceptible seedlings.

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3. Pedigree®, Version 3.0 © Copyright 1990. Registration No. 403061, December 1990, File number 316275, Consumer and Corporate Affairs Canada, Copyright Office.

Table 1. Comparison of concentration leaf essential oil composition of cultivated strawberry cultivars and selections vs three wild species (µg/g dry matter of the leaf).

Essential Oil	Commercial Genotypes[z]	*F. chiloensis* & *F. viridis*	*F. virginiana*
hexanal	8794	12391	19767
heptane 2-4-dimethyl	9749	20604	48398
2-propylcyclohexane	38808	33083	92011
(Z)-3-hexen-1-ol	29330	21450	45720
(E)-2- hexen-1-ol	4069	2366	13297
oxirane	5289	7239	25097
heptanal	1908	7823	10023
α-Pinene	728	8532	8970
β-Pinene	3844	7117	12090
2-heptenal	4755	5133	5918
(Z)-3-hexenylacetate	14446	23683	39757
phenylacetaldehyde	5855	22914	17340
1-octanol	1568	7316	88388
linaloloxide	2889	5147	6502
cis-linaloloxide	617	0	0
nonanal	38927	219809	444968
linalool	6223	0	393377
α-Terpinol	15587	14040	58447
decanal	5604	4264	226274
β-cyclocitral	22528	22844	83089
thymol	4987	1739	64140
$C_9H_{10}O_2$	5553	11464	6721
damascenone	978	0	11616
α-copaene	14074	0	11847
β-caryophyllene	1521	6777	8474
β-ionone	3515	7655	10425
α-muurolene	28689	13005	20500
α-farnesene	3540	1412	8666
farnesol	981	15557	5313
benzyl salicylate	6608	6673	18072
hexahydrofarnesyl acetone	3546	6417	23841
hexadecanoic acid	17209	44512	66231
phytol	14216	38040	26279
docosane	1979	2549	0

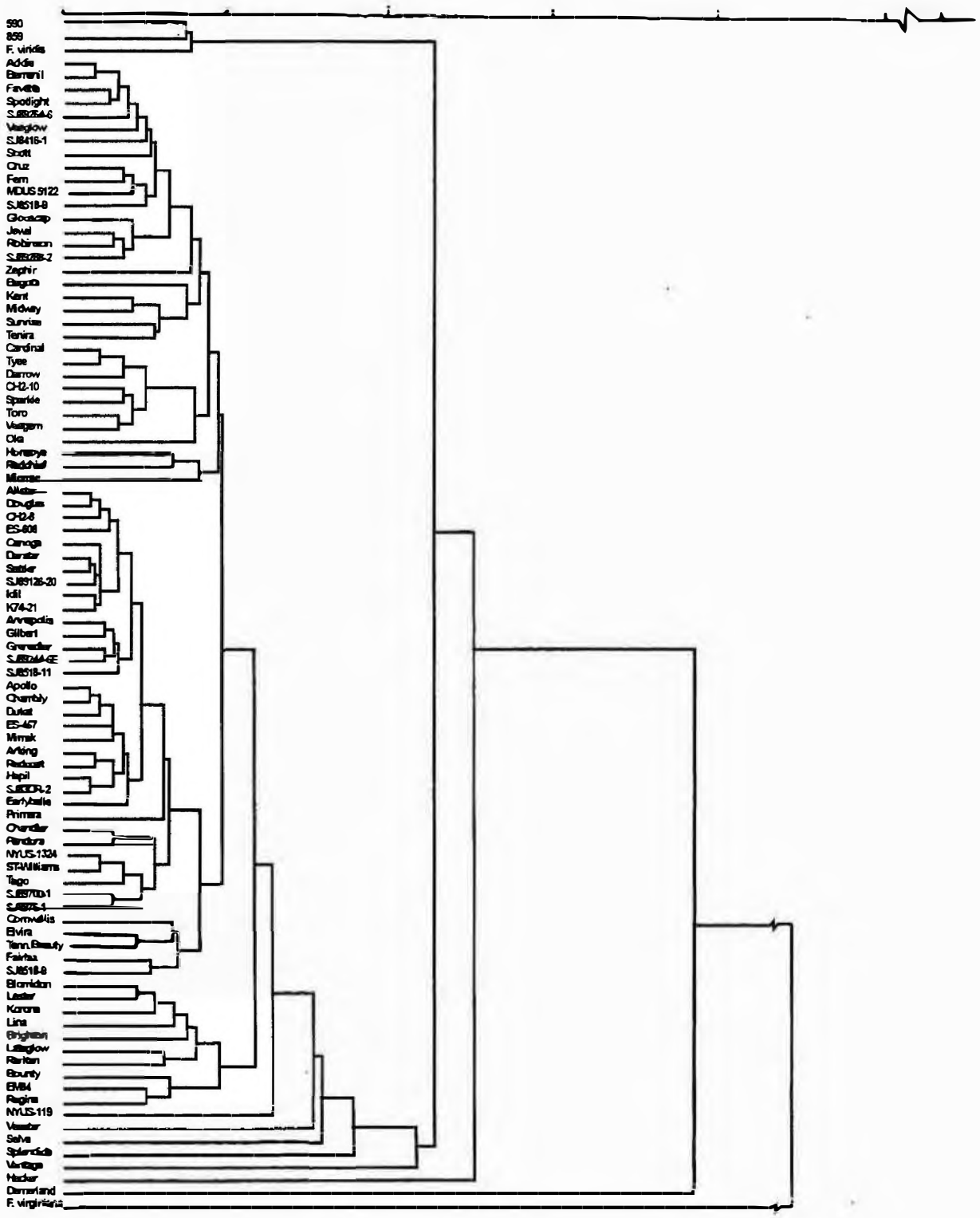

Fig 1. Ward's minimum cluster analysis of selected strawberry cultivars based on known leaf essential oil composition.

Physiological Aspects

PHYSIOLOGICAL ASPECTS OF ESSENTIAL OIL PRODUCTION

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INTRODUCTION: Apart from the evolution and from genetic factors that influence the composition of essential oils from plants, several physiological factors are known to influence the yield and composition of such oils (Table 1).

Some of these factors have been studied to some extent, in particular for commercially important crops, in order to determine the optimum conditions of cultivation and time of harvest, for attaining higher yields and a better quality of the oils.

Since essential oils can be isolated from many plant taxa, turning them into ideal characters for systematic studies, they have been used to support the identification of species, to document natural hybrids, to define genetic differences, to work out centers of origin and spread of populations, and to study variations within taxa. Chemotaxonomy is, therefore, another field where the influence of the growth conditions of plants on the composition of their essential oils is of great importance. Nevertheless, a direct comparison of the essential oil content and composition among the different existing studies is hampered because of different methods of isolation and qualitative and quantitative analysis, differences in geographical origin and environmental conditions, different plant parts and developmental stages when harvested, and variations within the varieties evaluated.

The simple fact of picking up the flowers or fruits from the mother plant is known to induce chemical changes in the aroma profile; some components present in a living plant are not found in the picked plant material, as it has been shown for jasmine, freesia, lilac, Easter lily, peach, and strawberries (Mookherjee *et al.*, 1988). Thus, the essential oil yield and composition can be affected in several ways since its formation to its isolation.

POLLINATOR AND POLLINATION: The scents of plants are, in most cases, related to the attraction of pollinators. As such the emission of volatiles attains its maximum at the time of nectar availability or of pollen maturation, that is when the flower is ready for pollination (Harborne, 1982; Jakobsen and Olsen, 1994).

Apart from the monthly and annual fluctuations, or the changes associated with the vegetative or flowering period of the plant, there are also diurnal fluctuations that seem to be related to the activity of the pollinator. Indeed, in plants with diurnal pollination, the emission of volatiles attains its maximum during the day, while the contrary is observed for those plants having night pollinators, such as bats, mice or nocturnal moths (Repčák *et al.*, 1980b; Harborne, 1982; Ecroyd *et al.*, 1995). The honeysuckle (*Lonicera japonica*) is well known to smell strongly, similar to jasmine and orange flowers, at night. Ikeda *et al.* (1994) have shown changes in the volatile components emitted from the living flowers of honeysuckle throughout the whole day, and the strongest odour was found to be emitted from 7.30 p.m. to 7.30 a.m., with its maximum between 11.30 p.m. to 3.30 a.m.

Other examples on the diurnal or nocturnal rhythmic emission of volatiles of several species can be found in the literature: *Hoya carnosa*, *Stephanotis floribunda*, *Odontoglossum constrictum*, *Citrus medica* (Altenburger and Matile, 1990), *Melaleuca alternifolia* (Murtagh and Etherington, 1990), *Nicotiana sylvestris* (Loughrin *et al.*, 1990), *N. suavolens* (Loughrin *et al.*, 1991), *Trifolium repens* (Jakobsen and Olsen, 1994). For *Trifolium repens* a special device was developed to analyse *in situ* the flower volatiles emission (Jakobsen and Olsen, 1994). Although the composition of the fragrance remained fairly constant during a 24 h period,

Table 1: Factors that influence the yield and composition of essential oils.

Pollinator and pollination	Climate
Type of plant material	Soil properties
Type of secretory structure	Hydric stress and method of irrigation
Development of organs	Mechanic or chemical injuries
Time of harvest	Addition of herbicides and/or fertilizers
Storage	Catabolism
Method and time of propagation	*In vitro* production

Table 2: Plants with differences in the essential oil composition between distinct plant organs.

Plant	Plant organs	Reference
Achillea ligustica	Leaves and flowers	Tzakou *et al.* (1995)
Achillea ptarmica	Green parts, roots and flowers	Kuropka *et al.* (1991)
Aframomum pruinosum	Seeds and leaves	Menut *et al.* (1994)
Aloysia gratissima	Leaves and flowers	Soler *et al.* (1986)
Alpinia breviligulata	Fruit peel and seed	Dũng *et al.* (1994)
Amomum villosum	Leaves and fruits	Ji *et al.* (1988)
Annona muricata	Leaves, peel and fruit pulp	Pélissier *et al.* (1994)
Calendula officinalis	Flowers and whole plant	Chalchat *et al.* (1991)
Cananga odorata	Whole flowers, petals, ovaries	Stashenko *et al.* (1993)
Canella winteriana	Leaves and stem bark	Abaul *et al.* (1995)
Carum carvi	Umbels, leaves and stems	Fleisher and Fleisher (1988)
Catimbium latilabre	Seed and fruit peel	Leclercq *et al.* (1994)
Cinnamomum glaucescens	Whole fruit, pericarp and seed only	Adhikary *et al.* (1992)
Cinnamomum parthenoxylon	Root bark and wood	Dũng *et al.* (1995)
Cinnamomum spp. (7 species)	Leaves, bark and wood	Jantan and Goh (1992)
Cinnamomum zeylanicum	Leaves and stem bark	Nath *et al.* (1996a)
Citrus aurantium	Flowers, leaves and peel	Boelens and Sindreu (1988)
Citrus limon	Fruit peels and leaves	Ayedoun *et al.* (1996a)
Citrus volkamericana, *C. reticulata* and *C. sinensis*	Fruit peels and leaves	Tirado *et al.* (1995)
Clausena heptaphylla	Leaves and fruit	Nath *et al.* (1996b)
Croton aff. nepetifolius	Leaves, stems, bark, wood	Craveiro *et al.* (1980)
Curcuma longa	Leaves and fresh rhizomes	McCarron *et al.* (1995)
Dactylanthus taylorii	Female and male inflorescence nectar	Ecroyd *et al.* (1995)
Foeniculum vulgare	Flowers, stem, leaves, bud, waxy and ripe seed	Bernáth *et al.* (1996)
Foeniculum vulgare	Flowers, leaves, fruits and stems	Venskutonis *et al.* (1996a)
Heterotropa sp.	Leaves and roots	Komae *et al.* (1988)
Heterotropa takoi	Leaves, petiole, subterranean stem, root, calyx and shoot	Hayashi *et al.* (1988)
Hyssopus officinalis	Flowers, stems, leaves and roots	Schulz and Stahl-Biskup (1991)
Juniperus oxycedrus	Berries and leaves	Stassi *et al.* (1995)
Lavandula pinnata	Flowers and leaves	Figueiredo *et al.* (1995a)
Levisticum officinale	Root and seed	Toulemonde and Noleau (1988)
Ligusticum mutellina	Fruits, herb and root	Brandt and Schultze (1995)
Lindera neesiana	Leaves and stems	Singh *et al.* (1995a)
Melissa officinalis	Calyx and leaves	Schultze *et al.* (1992)
Monarda citriodora	Leaves and flowers	Collins *et al.* (1994)
Monarda didyma	Flowers and leaves	Carnat *et al.* (1991)
Myrtus communis	Leaves, flowers, unripe and ripe fruits	Boelens and Jimenez (1992)
Nelumbo nucifera	Stamens and petals	Omata *et al.* (1991)
Ocimum basilicum	Whole plant and flower spikes	Bonnardeaux (1992)
Paederia foetida	Flowers, leaves and stems	Wong and Tan (1994)
Petroselinum sativum	Herb and seed	Shaath *et al.* (1988)
Picea abies	Root, trunk, branch and twig xylem and phloem, needles, male and female flowers	Persson *et al.* (1993)
Pilocarpus trachyllophus	Leaves, root and trunk bark, root and trunk heartwood	Neto *et al.* (1995)
Pinus nigra	Leaves and branches	Vidrich *et al.* (1996)
Pistacia lenticus	Gum, leaves, ripe and unripe fruits	Boelens and Jimenez (1991)
Protium heptaphyllyum	Leaves and stems	Zoghbi *et al.* (1995)
Pseudotsuga menziesii	Needles and twigs	Buchbauer *et al.* (1994)
Rhus coriaria	Leaves, fruit, pericarp and branch/bark	Kurucu *et al.* (1993)
Ridolfia segetum	Flowers and leaves	Fleisher and Fleisher (1996)
Rosa sp.	Flowers and pollen	Dobson *et al.* (1987)
Sassafras albidum	Leaves and twigs	Tucker *et al.* (1994)
Schinus molle	Berries and leaves	Maffei and Chialva (1990)
Sideritis mugronensis	Flowers and leaves	Máñez *et al.* (1991)
Tagetes minuta	Leaves, flowers and whole plant	Chalchat *et al.* (1995)
Thymus lotocephalus	Flowers and leaves	Figueiredo *et al.* (1993)
Vitex agnus-castus	Leaves, flowers and fruits	Kustrak *et al.* (1994)
Xylopia aethiopica	Leaves and fruits	Ayedoun *et al.* (1996b)
Xylopia pynaertii	Stem bark and root	Fournier *et al.* (1994)
Xylopia sericea	Leaves, fruits, roots and trunk	Câmara *et al.* (1996)

the emission was two to three times higher in the middle of the day, compared with the dark period. Moreover, the time of maximum emission coincided with flight activity of *Apis* sp. and *Bombus* sp., which are the primary pollinators of *T. repens.*

TYPE OF PLANT MATERIAL: The oil content and composition depend also on the type of plant organ analysed: flowers (calyx, corolla, nectary, ovaries, pollen or stamens), green parts (leaves and stems), bark, wood, whole fruits, pericarp or seed only, or roots (Table 2). This is

particularly evident in entomophilous flowers, whose flower volatiles are used as orientation clues; the fragrance emitted by the flowers, or flower parts, is quite distinct from that of other plant parts.

In several cases, floral oils contain a higher proportion of monoterpenes, such as α-phellandrene, limonene and fenchone, which are, according to some authors, directly related to pollinators attraction (Máñez *et al.*, 1991). The differences found between the essential oils of different plant organs can be partly explained by the existence of different secretory structures that are heterogeneously distributed over the plant body.

Nevertheless, there are also examples of plant species from which the oil obtained from each plant organ is similar (Table 3).

TYPE OF SECRETORY STRUCTURE: Volatile oils are synthesized, stored and released by a variety of secretory structures which are in general characteristic of a taxonomic group (Table 4). In some species, however, different types of the same secretory structure can exist, heterogeneously distributed over the plant body. There is some accumulating evidence suggesting that these structures may not develop synchronously, that they do not always secrete the same type of compounds and that they can have different secretion processes.

In *Leonotis leonurus* (Ascensão *et al.*, 1995), capitate and peltate trichomes differ in their secretion process: in the peltate trichomes the secretion seems to remain accumulated in the subcuticular space, unless an external factor disrupts the cuticle, whereas in the capitate trichomes the secretion is probably released by micropores. It is noteworthy that in this species, peltate trichomes are abundant both on the leaves and flowers, while capitate trichomes are only numerous on the leaves, and are rare or absent on the flowers.

Whereas in *Salvia officinalis* the direct analysis of glandular contents indicated that both stalked and sessile glands produced a very similar oil (Venkatachalam *et al.*, 1984), in *Poncirus trifoliata*, Heinrich *et al.* (1980) found two types of glands, one type in the exocarp and the other in the endocarp, producing essential oils of different composition with regard to the mono- and sesqui-terpene percentages. Either by trichome microsampling coupled to HPLC analysis or by ultrastructural/histochemical tests, secretion differences could also be found for the capitate and non-capitate trichomes of *Helianthus annuus* (Spring *et al.*, 1992) and for the glandular uniseriate trichomes of Type I, II and III of *Geranium robertianum* (Pedro *et al.*, 1990). Brun *et al.* (1991) found that on the leaves of *Mentha x piperita*, capitate trichomes appear before the peltate ones. Monoterpene production was only detected if the leaf bears peltate trichomes, limonene being the first compound accumulated.

Also in *Plecthrantus madagascariensis* there is an heterogeneous distribution of capitate and peltate trichomes over the vegetative and reproductive organs. Restricted to the calyx, there is an unusual type of capitate trichomes (Figueiredo *et al.* 1996). Characteristic peltate trichomes, with an orange to reddish colour, occur particularly concentrated at the leaf intervein areas, while all the other trichomes have no colour. The main component of the oils of *P. madagascariensis* was 6,7-dehydroroyleanone, a diterpene, which was isolated as orange to reddish crystals.

Table 3: Plants with similar essential oil composition observed for different plant organs.

Plant	Plant organs	Reference
Actinodium cunninghamii	Flowers and leaves	Brophy and Goldsack (1994)
Agastache rugosa	Flowers and leaves	Dũng *et al.* (1996)
Ageratum conyzoides	Flowers and leaves plus stem	Riaz *et al.* (1995)
Alpinia speciosa	Leaves, stem and rhizome	De Pooter *et al.* (1995)
Amomum schmidtii	Leaves, stem and root	Dũng *et al.* (1992)
Artemisia argentea	Flowers and leaves from flowering and vegetative phase	Figueiredo *et al.* (1994)
Artemisia judaica	Flowers, leaves and branches	Putievsky *et al.* (1992)
Chaerophyllum coloratum	Ripe fruits and umbels	Vajs *et al.* (1995)
Doryphora sassafras	Leaves and bark	Brophy *et al.* (1993)
Geleznowia verrucosa	Flowers and leaves	Brophy and Goldsack (1995)
Lippia adoensis	Flowers and leaves	Abegaz *et al.* (1993)
Michelia alba	Flowers and leaves	Ueyama *et al.* (1992)
Ocimum gratissimum	Flowers and leaves	Pino *et al.* (1996)
Ocimum gratissimum	Flowers, leaves and stems	Charles and Simon (1992)
Spondias cytherea	Green and ripe fruit	Wong and Lai (1995)
Thymus capitellatus	Flowers and leaves	Figueiredo *et al.* (1993)
Vitex agnus-castus	Inflorescences, leaves and fruit	Senatore *et al.* (1996)

Table 4: Different types of secretory structures occurring in some plant families (adapted from Fahn, 1988).

Secretory structures	Families
External secretory structures	
Trichomes	Asteraceae, Lamiaceae, Rutaceae, Geraniaceae, Solanaceae and Cannabinaceae
Osmophores	Piperaceae, Orchidaceae and Araceae
Internal secretory structures	
Idioblasts	Lauraceae, Magnoliaceae, Piperaceae, Araceae, Aristolochiaceae, Calycanthaceae and Saururaceae
Cavities	Rutaceae, Myrtaceae, Myoporaceae, Hypericaceae and Leguminosae
Ducts	Apiaceae, Asteraceae, Pinaceae, Myrtaceae, Hypericaceae, Leguminosae and Anacardiaceae

DEVELOPMENT OF ORGANS: A large number of examples can be found in the literature, even previous to 1949 (Repčák *et al.*, 1980a), on the influence of the developmental stage of plant organs on the yield and composition of the essential oils (Table 5).

In most cases, there is an increase of essential oil yield from flower bud to full flowering. Simultaneously, the essential oil composition undergoes important changes, some components varying their concentration from traces-10% in the initial stages, to 50-70% in the full flowering stage. Several authors have interpreted this increase of some components as a contribution to pollinators attraction.

In many aromatic plants (peppermint, sage, rose scented-geranium, Japanese mint and lemongrass), most of the essential oil is accumulated much before the leaves are fully expanded. The lower yield of essential oil in

Table 5: Plant species with differences in the essential oil yield and composition throughout developmental stages.

Plant	Plant organs	Reference
Achillea collina	Flower buds and open flowerheads	Cernaj *et al.* (1983)
Achillea millefolium	Flowers ontogeny	Figueiredo *et al.* (1992b)
Achillea millefolium	Leaves in flowering and vegetative phases	Figueiredo *et al.* (1992a)
Ananas comosus	Green and ripened fruits	Umano *et al.* (1992)
Anethum graveolens	Flowering, lower than 50% fruit formation, 50% and complete fruit formation	Pino *et al.* (1995)
Artemisia annua	Budding stage to peak of flowering	Chalchat *et al.* (1994)
Astronium fraxinifolium	Young and mature leaves from young and adult plants	Alencar *et al.* (1996)
Cananga odorata	Small undeveloped green flowers, intermediate and fully matured flowers	Stashenko *et al.* (1995)
Carum carvi	Development of the roots	Stahl-Biskup and Wichtmann (1991)
Carum carvi	Whole plant and umbels	Fleisher and Fleisher (1988)
Carum carvi	Development of the seeds	Bouwmeester *et al.* (1995)
Chrysanthemum balsamita	Young and fully developed flowerheads	Strobel *et al.* (1987)
Citrus volkamericana	Green fruits, intermediate maturity and fully yellow-orange ripe fruits	Combariza *et al.* (1994)
Citrus volkamericana, *C. reticulata* and *C. sinensis*	Completely green fruits, intermediate maturity and fully ripe fruits	Tirado *et al.* (1995)
Cymbopogon winterianus	Leaf ontogeny	Luthra *et al.* (1991)
Eucalyptus spp. (12 species)	Juvenile and adult leaves	Li *et al.* (1995)
Foeniculum vulgare	Development of the roots	Stahl-Biskup and Wichtmann (1991)
Foeniculum vulgare	Early, late and ripe seed	Marotti *et al.* (1994b)
Heterotropa takoi	Maturation of the leaves	Hayashi *et al.* (1988)
Matricaria chamomilla	Flowers ontogeny	Franz *et al.* (1978), Franz (1980)
Melaleuca alternifolia	Leaf ontogeny	Southwell and Stiff (1989)
Mentha arvensis	Leaf ontogeny	Duriyaprapan and Britten (1982)
Mentha citrata	Aerial parts at full- and post-flowering	Malizia *et al.* (1996)
Mentha x piperita	Juvenile to senescent plants	Murray *et al.* (1988)
Micromeria varia	Aerial parts in flowering and vegetative phases	Pedro *et al.* (1995)
Myrtus communis	Ripening of fruits	Boelens and Jimenez (1992)
Nelumbo nucifera	Flowers before and after anthesis	Omata *et al.* (1991)
Ocimum sanctum	Maturation of the leaves	Dey and Choudhuri (1983)
Ocimum spp. (7 species)	Eight phenophases	Gupta (1996)
Olea europea	Under-ripe, ripe and over-ripe fruits	Morales *et al.* (1996)
Origanum majorana	Different leaf nodes	Circella *et al.* (1995)
Pastinaca sativa	Development of the roots	Stahl-Biskup and Wichtmann (1991)
Pelargonium sp.	First leaf (at the apical bud) and the twelfth leaf	Rao *et al.* (1993)
Satureja montana	Start to full bloom	Bilia *et al.* (1992)
Sideritis mugronensis	Flower and leaf development	Máñez *et al.* (1991)
Tagetes minuta	Flower, leaves, fruit and seeds	Thappa *et al.* (1993)
Tagetes minuta	Flowers and leaves from the start to the end of flowering	Chalchat *et al.* (1995)

mature leaves has been attributed to catabolism of the terpenes, and also, for plants with external secretory structures, to the fact that in fully expanded leaves the secretory structures have matured and may have released the secretion by cuticle disruption. In plants with internal secretory structures such as *Pinus* (Simić *et al.*, 1996) or *Eucalyptus* (Li *et al.*, 1995), the oil yield remains more or less constant or is higher in juvenile than in adult material.

The decrease in the amount of the phenylpropanoids eugenol and methyleugenol, with the development of the leaves, has been explained by the use of these components in the synthesis of lignin and/or to higher oxidation of phenol compounds catalysed by the increase of the activity of polyphenoloxidase and peroxidase (Dey and Choudhuri, 1983). According to Máñez *et al.* (1991) the changes in the composition of an essential oil associated with the maturation of the organ, are directly related to higher rates of cyclization and dehydration of the components of the oil.

No major changes could be found in essential oil composition through development of *Otacanthus coeruleus* from two months old entire plants, young shoots and after bloom (De Pooter *et al.*, 1989).

TIME OF HARVEST: The harvesting season can also affect the essential oil content and composition, but this effect varies from species to species. In some cases, the decrease in the essential oil content and/or changes in oil composition were correlated with weather parameters (daylength, temperature and humidity) and to the attack of fungal pathogens, particularly during the months of rainfall. In all cases, the best harvesting time needs to be determined, according to the commercially most interesting component and yield of the oil.

In *Crithmum maritimum*, Barroso *et al.* (1992) observed that during the flowering period the dominant component was sabinene, while γ-terpinene was the major component during the rest of the year. The effect of different harvesting seasons was also noticeable on the oil content and composition of several other species (Table 6). Another example is the choice of the right time to harvest *Vanilla* beans: if the beans are harvested to early, they are susceptible to mould and the vanillin content is lower, whereas overripening produces split ends beans of lower quality and lower price (Moyler, 1994; Ranadive, 1994). However, no major differences were recorded for the oils of *Diplolophium africanum* (Koumaglo *et al.*, 1994), *Melaleuca quinquenervia* (Ramanoelina *et al.*, 1994) or of *Jasminum grandiflorum* over a period of four months (Verghese and Sunny, 1992).

It is interesting to mention that in one case, *Chrysothamnus nauseosus* (Halls *et al.*, 1994), the seasonal changes in the volatiles composition were correlated with herbivory. Although this

Table 6: Plant species where the time of harvest affected the essential oil yield and composition.

Plant	Plant organs	Reference
Aristolochia asclepiadifolia	Roots	Sagrero-Nieves *et al.* (1993)
Artemisia judaica	Leaves	Putievsky *et al.* (1992)
Calamintha nepeta	Aerial parts	De Pooter *et al.* (1986)
Chrysanthemum balsamita	Flowers and leaves	Strobel *et al.* (1987)
Chrysothamnus nauseosus	Leaves	Halls *et al.* (1994)
Citrus bergamia	Fruit peel	Dugo *et al.* (1991)
Citrus kinokuni	Fruit peel	Shiota and Itoo (1991)
Citrus sinensis	Fruit	Dugo *et al.* (1994), Verzera *et al.* (1996)
Crithmum maritimum	Leaves	Barroso *et al.* (1992)
Cymbopogon spp. (3 species)	Leaves	Singh *et al.* (1994)
Dracocephalum moldavica	Aerial parts	Holm *et al.* (1988)
Eucalyptus camaldulensis	Leaves	Doran *et al.* (1995)
Eucalyptus spp. (3 of the 5 species)	Adult leaves	Zrira and Benjilali (1996)
Ledum groenlandicum	Aerial parts	Belleau and Collin (1993)
Matricaria chamomilla	Flowers	Franz *et al.* (1978)
Matricaria recutita	Aerial parts	Aslaksen *et al.* (1996)
Mentha x piperita	Aerial parts	Murray *et al.* (1988)
Olearia phlogopappa (4 clones)	Leaves	Dragar and Menary (1992)
Origanum vulgare	Leaves	Putievsky *et al.* (1985)
Pelargonium sp.	Aerial parts	Prakasa Rao *et al.* (1995)
Salvia coccinea	Aerial parts	Onayade *et al.* (1991)
Salvia officinalis	Leaves	Pitarević *et al.* (1985)
Satureja hortensis	Aerial parts	Hay (1993)
Satureja obovata	Aerial parts	Arrebola *et al.* (1994)
Thymus capitatus	Leaves and flowers	Arras and Grella (1988)
Thymus vulgaris	Aerial parts	McGimpsey *et al.* (1994)
Vanilla planifolia	Beans	Moyler (1994), Ranadive (1994)

species is heavily browsed in winter, it is only slightly so in summer. Despite the fact that a lack of forage in winter could result in an increased rabbitbrush browsing, it seems that the higher concentration of volatiles in summer could act as feeding deterrent to deer and other browsers.

STORAGE: Air-drying, either with or without heat, and freeze-drying are two common procedures for storage. The safe limit of herb storage varies depending on the plant species and the storage conditions. The rapid metabolic transformations that take place as the plant material starts to dry, do not only cause morphological modifications, but may also cause drastic changes in the aroma profile. Nevertheless, in some cases the drying process has no remarkable effect. Several authors consider oxidation, evaporation and resinification responsible for these changes, although contaminations, the age of the plant material, and the light and temperature/humidity conditions can also affect the process. Again here, the presence of internal secretory structures may determine that the plant species is less vulnerable to oil losses or transformations. External secretory trichomes can naturally release their secretion by cuticle disruption and are more exposed to mechanical damages (handwork, transport, crushing in piles).

Different responses to hay storage were recorded for the oil content of three *Cymbopogon* spp. (Singh *et al.*, 1994), being advantageous to *C. martinii* and *C. winterianus*, but not to *C. flexuosus*.

Stashenko *et al.* (1993) have shown that the essential oil obtained from fresh flowers of ylang-ylang contained from 1.5 to 3 times more monoterpenes and light oxygen-containing compounds than oils distilled 24h after the flowers had been collected. The oil from the fresh flowers was considered as a so-called Extra Grade. Also for *Ocimum basilicum* (Baritaux *et al.*, 1992; Venskutonis *et al.*, 1996b), *Sassafras albidum* (Tucker *et al.*, 1994), *Carum carvi* (Fleisher and Fleisher, 1988), *Zingiber officinale* (Ekundayo *et al.*, 1988), *Anethum graveolens* (Huopalahti and Kesälahti, 1985), *Chamomila recutita* (Letchamo, 1993), *Xylopia aethiopica* (Ayedoun *et al.*, 1996b) and *Thymus vulgaris* (Venskutonis *et al.*, 1996c) the oil yield and/or quantitatively important volatile compounds were found to be reduced during storage and/or drying. Conversely, air-drying of *Melissa officinalis* prior to distillation did not change qualitatively the composition of the oil, but the relative amounts of the constituents were affected (Shalaby *et al.*, 1995). Air-drying of *Eucalyptus camaldulensis* (Zrira and Benjilali, 1991) or of *Artemisia afra* (Worku and Rubiolo, 1996) did not affect the essential oil composition. Storage of *Melaleuca alternifolia* under several unheated conditions has shown no oil loss up to two weeks after harvesting (Murtagh and Curtis, 1991). Recent studies on the same species (Whish and Williams, 1996) have shown that there was no detrimental effect of drying tea tree leaves prior to distillation, whereas drying of tea tree leaf on stem increased the oil yield when compared to the immediately distilled leaf-stripped material. According to the authors this may correspond to an active form of postharvest uptake or production.

The effect of microwave oven and warm-air drying (40-100°C) on the microflora and volatile oil profile of ten herbs was assayed by Deans *et al.* (1991). Both microwave treatment and warm-air drying at temperatures >60°C induced a loss of volatiles and the formation of new components.

In some cases curing after harvest is even needed to develop the aroma, as is the case for *Vanilla planifolia* beans, which have no aroma at the time of harvest. This develops only during curing, that should start soon after picking (one week to ten days) to prevent beans from splitting (Moyler, 1994; Ranadive, 1994).

The preparation for storage may also be important: sacks of peppercorns (*Piper nigrum*) which are stored under damp conditions, without free circulation of air, develop a "musty" note which remains in the oil even after extraction (Moyler, 1994). For *Chamomila recutita* no major differences were found with the use of different packing materials (Letchamo, 1993).

Irradiation has been shown to be effective for decontaminating stored spices. Some authors have studied the effect of several irradiation doses on the essential oil profile of several species (majoram, cardamom, spice mixtures, black pepper, basil, thyme), and, in general, there was no effect, or only slight changes in the oil composition were observed with irradiation doses between 10-50kGy (Venskutonis *et al.*, 1996b, c).

METHOD AND TIME OF PROPAGATION: Galambosi *et al.* (1989) observed that the propagation method (sowing and planting) did not change the oil yield of *Dracocephalum moldavica*, although the composition was quite different. Nevertheless, for *Mentha x piperita* and *M. arvensis* cultivars the essential oil yields and the menthol content varied based on the method

of propagation: autumn planted rooted cuttings, or autumn and summer planted rhizomes (Zheljazkov *et al.*, 1996a). Similar results were reported by Marotti *et al.* (1994a) for *Mentha x piperita.*

Likewise, the time of the year for propagation (spring or autumn) induced changes in the oil yield (Gasic *et al.*, 1991): most of the seventeen cultivars of chamomile showed a significantly higher oil yield if the plant was spring sown rather than autumn sown. For *Mentha spicata* (Singh *et al.*, 1995b), from the eight planting times from November to March, the maximum biomass and oil yield was obtained from crop planted at the end of December.

CLIMATE: Essential oil production is highly dependent on climatic conditions (Letchamo *et al.*, 1980; Spring *et al.*, 1985; Hornok, 1988; Murray *et al.* 1988; Maffei, 1988; Gasic *et al.*, 1992; Singh *et al.*, 1995a). According to Cabo *et al.* (1987), during the months of lowest temperature and fewest hours of sunlight there is an obvious decrease in essential oil production. The yield and composition of the essential oils of *Mentha x piperita* (Murray *et al.*, 1988), *Thymus vulgaris* (Letchamo *et al.*, 1994), *Anethum graveolens* (Hälva *et al.*, 1993), *Trifolium repens* (Jakobsen and Olsen, 1994), chamomile and peppermint (Schütte, 1976) have been shown to be temperature and light dependent, higher oil yields being obtained at increased temperatures and high irradiances. According to Jakobsen and Olsen (1994), the positive influence of irradiation may be in part a temperature effect. The increased light regime can trigger the enzymes for the biosynthesis of terpenes and other essential oil compounds, together with other lipophilic substances. Letchamo *et al.* (1994) have shown that *Thymus vulgaris* grown under natural light released a typical thyme aroma without any mechanical pressure, while the plant grown under supplemental light did not release any aroma until some pressure was applied. This may be due to differences in epicuticular wax deposition on the leaf surface.

In *Pinus elliotii*, monoterpene emission rates, particularly those of α- and β-pinene, myrcene, limonene and β-phellandrene, also increased exponentially with the increase of temperature, between 20 °C and 46 °C (Tingey *et al.*, 1980). Nevertheless, according to the same authors, light had no influence on the emission rates of these components. According to Craveiro and Lemos (1980), there was no conversion of *trans*- into *cis*-anethole when plants of *Croton aff. zehntneri* were exposed to sunlight during 80 days. However, this conversion did occur when the extracted oil was submitted to the same conditions. According to Badoc and Lamarti (1991), a tropical climate favours the formation of oxidized components in the oils.

Daylength considerably influenced flowering of *Origanum majorana*, but had no direct influence on the essential oil formation (Circella *et al.*, 1995).

SOIL PROPERTIES: The type and composition of the soil has been considered, by several authors, as one of the determinant factors in the composition of essential oils, and also as one of the explanations for the differences found in oils of the same species (Gouyon *et al.*, 1986; Cabo *et al.*, 1987; Murray *et al.*, 1988; Morgan, 1989; Misra, 1995).

Soil factors closely correlated with pH are also important both for growth and essential oil production of plants producing volatiles. For instance, high rainfall leads to leaching of calcium and formation of acidic soils, which limits plant growth simply because H^+ is much more toxic to the roots in the absence of calcium. The pH also strongly influences the solubility of certain elements in the soil, and the rate at which they are absorbed by plants. Iron, zinc, copper and manganese are less soluble in alkaline than in acidic soils because they precipitate as hydroxides at high pH values. Moreover, the critical level of one element determined for one soil, may be different for another soil, especially when considering crops that are grown over a wide range of geographical regions.

Row spacing can also play an important role in crop development, since closer row spacing can be detrimental due to allelopathic factors or by mutual leaf-shading.

Zheljazkov and Nielsen (1996) have shown that soil and air pollution with heavy metals did not affect the phenological stages and essential oil yield of *Lavandula angustifolia.* Although the lavender inflorescences accumulated different amounts of heavy metals, the essential oil was not contaminated. However, recent studies of Schilcher and Habenicht (1996) revealed the presence of organochlorine pesticides in 72 out of 110 samples of 34 different essential oils.

HYDRIC STRESS AND METHOD OF IRRIGATION: Water stress induces biochemical, physiological and developmental changes in plants, but this seems to be only beneficial to the essential oil yield of some species such as *Anethum graveolens*, *Artemisia dracunculus*,

Satureja douglasii, *Mentha x piperita* and *Ocimum basilicum*. In other aromatic species, such as *Artemisia annua*, *Coriandrum sativum* and *Thymus vulgaris*, a yield increase was only reached with normal or higher irrigation (Hornok, 1988; Harborne, 1990; Simon *et al.*, 1992; Chalchat *et al.*, 1994; Letchamo *et al.*, 1994, 1995).

According to Harborne (1990), in the Mediterranean climates, where the plants are usually under hydric stress, about 38% of the plants are essential oil producers, while in the temperate climates this number decreases to 11%. Simon *et al.* (1992) verified that an increase of hydric stress was coupled with a corresponding increase, and change of composition, of the essential oil of *Ocimum basilicum*. According to the same authors the yield increase can be partly related to a higher trichome density. With the increase of hydric stress, linalol, eugenol and the sesquiterpene concentration decreased, while, inversely, the methylchavicol concentration raised (Simon *et al.*, 1992).

Severe drought altered the metabolism in leaves of apple tree (*Malus domestica*) and induced an increase of some volatile compounds, probably due to an increased lipoxygenase activity (Ebel *et al.*, 1995).

The method of irrigation as well as the period of development of the plant in which it is more abundant, are also important for the essential oil yield (Hornok, 1988; Nedkov and Georgiev, 1991). Of the four irrigation systems assayed (Sprinkler, surface drip, subsurface at 35cm depth, and subsurface irrigation with microporous hose at 15cm depth) the highest yield of *Mentha x piperita* oil was attained by surface drip irrigation or subsurface irrigation with microporous hose at 15cm depth (Nedkov and Georgiev, 1991). In *Satureja montana*, the waterings did not influence the quality and quantity of the oil produced, but only contributed to higher yields of extractable material (Bilia *et al.*, 1992).

MECHANICAL OR CHEMICAL INJURIES

The result of mechanical injuries such as wounding, or infestation by predators is particularly important in oleoresin-producing plants, which accumulate the resin in internal secretory structures such as ducts or cavities. In *Pinus pinaster* it was shown that after perforation there was a two and half-fold increase in oil yield, while inoculation with mycelium of *Verticicladiella* sp. led to a 60-fold enrichment in oil yield (Cheniclet, 1987). Furthermore, the infection with the mycelium had a longer lasting effect on the terpenes production than the wounding. Both the wounding and the infection with mycelium did not induce significant changes in the relative percentages of the main components (Cheniclet, 1987).

Steele *et al.* (1995) have shown that monoterpene and diterpene biosynthesis was induced in *Abies* wounded stems. The activity of monoterpene and diterpene cyclases was 25- to 500-fold higher than in non-wounded control stems. According to these authors, the induced resin response mechanism is not only species dependent, but it also varies according to the seasonal development, water stress and light regime.

Wounding is known to induce the activity of a number of proteins, some of these having an unknown function. Lipoxygenase has an increased activity in wounded tissues, and it is possible that some of its reaction products or derivatives (hexenal, nonenal, β-ionone) act as signals to produce antipest metabolites (Hildebrand *et al.*, 1989).

ADDITION OF HERBICIDES AND/OR FERTILIZERS: According to Hornok (1988), the supplementation of the soil with three of the most important nutrients (N, P and K) has generally produced an increase in the yield of the oil, but the separate addition of the same nutrients gave different results in the yield and composition of the same oils. Other authors have shown that inversely to what happened with the yield, the composition of the essential oil was not influenced by the concentration of nitrogen in the soil (Galambosi *et al.*, 1989; Prakasa Rao and Singh, 1991). Similar results were reported by Court *et al.* (1993), who conducted field experiments with *Mentha x piperita*, using different rates of nitrogen (0, 60, 120, 180, 240 and 300kg/ha). Oil yields increased quadratically with N fertilization up to 180kg/ha, but higher concentrations had no influence on the yield. Menthol, the major component of peppermint oil, and many of the other oil constituents were not influenced by the N fertilization. The differences seen for some other components seemed to be related to a delay in plant maturity with increased N fertilization. Similar results were reported by Kothari and Singh (1995) for *Mentha gracilis* using 0, 100, 200 and 300kgN/ha. The green herbage and oil yields increased up to 200kgN/ha, higher amounts of N and larger plant-spacing not increasing oil yield. For *Mentha x piperita*, Marotti *et al.* (1994a) found that mineral

fertilization increased the menthol content, and decreased that of menthone and β-caryophyllene. Deficiencies of N, P and S drastically reduced herbage and oil yields of *Tagetes minuta*, and markedly changed the oil composition (Graven *et al.*, 1991).

Dragar and Menary (1995) assayed the effect of various N, P, Ca and K concentrations on the essential oil yield and composition of *Olearia phlogopappa*. According to these authors, oil yield increased as P (0.8µmol) and N (12µmol) became limiting. Nevertheless, overlowering the amount of N can be detrimental since the plants become smaller, thus producing less oil in total. No significant differences were observed for the Ca or K treatments. With regard to the oil composition it was seen that low P increased the amount of sesquiterpenes, namely kessane and liguloxide.

In field trials with *Rosa damascena*, the foliar application of kinetin (5-20mg/l) affected the essential oil composition and increased the number of flowers per plant and the oil yield (Farooqui *et al.*, 1993). Similarly, the application of commercial formulations of the plant growth regulators triacontanol and mixtalol, affected both the yield and the composition of the essential oil of *Pelargonium* sp. (Bhattacharya and Rao, 1996).

The foliar application of different herbicides and growth regulators on *Salvia officinalis* showed marked changes in the composition and yield of the essential oil, although it was impossible to establish a correlation with the results obtained (El-Keltawi and Croteau, 1987). Conversely, Vaverkova *et al.* (1995) found that the use of Afalon 50WP did not affect the oil yield or composition of *Salvia officinalis*.

Stahl and Wollensah (1986) studied the effect of eleven herbicides on the development of the trichomes and azulene production in *Achillea millefolium*. Several of these herbicides delayed the glandular development and caused giant trichomes, with two to three times the number of cells. The production of azulene was also reduced, particularly when 2,4-D, Dicamba, Triazin, Chlorpropham and Brompyrazon were applied.

Unrestricted weed growth caused a 58% to 78% reduction in oil yield of *Mentha arvensis* (Kothari and Singh, 1994). The application of either Terbacil, Pendimethalin, Oxadiazon or Oxyfluorfen did not affect the oil yield, when compared with the weed-free control. According to Zheljazkov *et al.* (1996b), the highest essential oil yield for *Mentha xpiperita* and *M. arvensis* var. *piperascens* was obtained when mechanical or combined mechanical and chemical weed control was used. In both species, the oil composition was not significantly affected by the method of weed control. For *Coriandrum sativum* some differences could be seen, both in oil yield and composition, after the use of herbicides independently or in combination, or after one or two years of treatment (Zheljazkov and Zhalnov, 1995).

Mildew, rust and black spot are some known devastating diseases of essential oil bearing plants such as *Coriandrum sativum* (Kalra *et al.*, 1995) or *Rosa damascena* (Margina and Zheljazkov, 1995). Several fungicides are very effective in controlling the diseases without affecting the quality and the yield of the oil.

CATABOLISM: The diurnal fluctuations in the composition and/or the yield of an essential oil, as well as the changes in the oil composition with the development of the organ, or of the plant, have been considered as a normal metabolic terpene turnover (Croteau, 1987, 1988). While in the case of the diurnal fluctuations the terpene turnover depends on the photosynthesis and on the utilization of photosynthates, in the changes related to the development, the content in monoterpenes decreases as a result of the turnover of the stored material (Croteau, 1987). The study of two model systems (*Mentha x piperita* and *Salvia officinalis*) has shown that the catabolism of terpenes leads to the formation of glycoside derivatives that are transported to the roots where they are used either as energy source or in the synthesis of lipids (Croteau, 1987).

Nevertheless, the studies of Stahl-Biskup and co-workers (Stahl-Biskup, 1987; Stengele and Stahl-Biskup, 1994; Stahl-Biskup and Holthuijzen, 1995) have shown that glycosidically bound volatiles are not obligatorily involved in essential oil biosynthesis or metabolism. From their studies on *Thymus x citriodorus*, *Mentha x piperita*, *M. pulegium* and *Lamium album*, it is assumed that glycosylation may function, in some species, as a protective mechanism.

***IN VITRO* PRODUCTION:** *In vitro* essential oil production is only known to occur in a number of systems. In general, the cultures are unable to produce the same type of compounds as found in the essential oil of the *in vivo* plant, and/or the production is rather low (Figueiredo *et al.*, 1995b).

Several procedures have been assayed to increase the essential oil production by plant cell

cultures, from which hairy roots obtention is, nowadays, receiving increased attention (Deans and Svoboda, 1993; Kennedy *et al.*, 1993; Santos *et al.*, 1996). The essential oils from *Pimpinella anisum* hairy roots grown in different culture media were qualitatively very similar, but showed major quantitative differences regarding the main components. In the cultures grown under dark conditions, the major component was always *trans*-epoxy-pseudoisoeugenyl 2-methylbutyrate, whereas in those grown under continuous light the major component was either this same compound, geigerene and pregeigerene or pregeigerene and β-bisabolene. The highest essential oil yield from the hairy roots (0.05-0.1%) was attained with cultures growing in SH medium (Santos *et al.*, 1996). The qualitative composition of the oil obtained from the hairy roots was similar to that from the roots of the mother plant.

In conclusion, when chemosystematic studies are involved, the plant material conditions, namely plant age, plant part, time of harvest, type of storage, among others, should be specified, in order to minimize the problems when comparing data between different studies.

Although some of the results here reported were based on experiments that were conducted with plants grown in greenhouses or under other controlled conditions, and may thus not apply directly to field grown plants, where other environmental factors interact to influence the plant status, it is clear that the plant growing conditions may alter the essential oil yield and composition. For each particular interesting species, the growing conditions may be manipulated to preserve a unique character of an oil, or different cultivars with distinct aroma profiles can be developed, under specific ecological conditions and with a proper time of harvest, in accordance with market demands (e.g. food, pharmaceutical or cosmetic industries).

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ULTRASTRUCTURE OF GLANDULAR TRICHOMES ON LEAVES OF FIVE LAMIACEAE REPRESENTATIVES

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INTRODUCTION

Many species of the Lamiaceae are aromatic because of essential oils that they produce. Buds and young leaves are the most aromatic ones. Essential oils are produced in glandular trichomes that cover the surface of leaves, calyx and stems. The present study deals with the ultrastructure of the glandular trichomes of *Agastache rugosa* (Fish. et Mey) O. Kuntze, *Dracocephalum moldavica* L., *Nepeta cataria* L., *Nepeta cataria* L. var. *citriodora* Balb. and *Scutellaria baicalensis* Georgi .

MATERIAL AND METHODS

The plants for investigation were grown in the scientific station of Komarov Botanical Institute in St. Petersburg district. Buds, young and mature leaves were used for study by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The plants have been investigated at different developmental stages. For TEM small pieces of leaves were prefixed for 4 hr with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.2M phosphate buffer (pH 7.2) and postfixed with 2% OsO_4 in the same buffer, or fixed only in 2% OsO_4. After dehydration in alcohol series the material was embedded in an Epon-Araldite mixture. Ultrathin sections were stained with lead citrate and observed in Tesla-BS-500 and Hitachi-600 electron microscopes. For SEM, material was fixed in 2.5% glutaraldehyde in 0.2M phosphate buffer (pH 7.2), dehydrated with alcohol, critical-point dried, coated with gold and examined in JSM-35 scanning electron microscope.

RESULTS AND DISCUSSION

Secretory trichomes appear on very young leaf buds. The indumentum of young leaves of the investigated species is so dense that one can hardly see the leaf surface beneath trichomes. Leaves are covered with non-glandular and glandular trichomes. Glandular trichomes are of two types: capitate and peltate glands (or scales). The capitate trichomes were observed on both faces of the leaf. Peltate glands were found mostly on the abaxial face. They do not occur on the veins (Fig. 1).

Mature glandular trichomes consist of a single basal cell, a stalk and a secretory head. Capitate trichomes of *Agastache rugosa*, *Dracocephalum moldavica*, *Scutellaria baicalensis* and *Nepeta cataria* have unicellular stalk, those of *Nepeta cataria* var. *citriodora* have unicellular or bicellular stalk. Secretory cells of capitate trichomes of all investigated species consisted of 4 cells. Ultrathin sections of mature head cells of capitate trichomes showed well developed rough endoplasmic reticulum and numerous dictiosomes. Such ultrastructure is characteristic for the cells that secrete not lipophylic substances, but slime (Schnepf, 1972; Heinrich, 1977).

Peltate trichomes of *Agastache rugosa*, *Dracocephalum moldavica*, *Scutellaria baicalensis* and *Nepeta cataria* have unicellular stalk and a head consisting of 4-6 central and 12-18 periferal cells. *Nepeta cataria* var. *citriodora* have scales with unicellular or bicellular stalk with the head of 4 central and 12-16 periferal cells and scales with unicellular or bicellular stalk and 6-celled head (fig. 2).

The secretetion is produced in the head and is accumulated between head cells and the cuticle, in subcuticular space. The secreting glands with elevated cuticle are larger than the developing glands and glands at early stages of secretion, that have no secretion under the cuticle (fig. 3).

The ultrastructure of secretory cells of peltate trichomes is basically similar for all investigated species. At the stage of secretion two organelles predominate in these cells: plastids (leucoplasts) and endoplasmic reticulum (ER). These cell compartments were

found in secretory cells of different plants that produce terpenes (Fahn, 1988) and are supposed to be the site of terpene biosynthesis (Carde, Bernard-Dagan, 1982).

Dracocephalum moldavica has numerous plastids of irregular shape which are not associated with ER. They do not contain any lamellae and have very few plastid reticulum. Rough ER cisternae are combined in stacks. There are dense inclusions between the membranes of plastidal and mitochondrial envelope, in ER (fig. 4). These inclusions has similar osmiophilia to that in subcuticular space. They disappear after fixation with osmium tetroxide only, which is known to be a specific fixative which does not fixate terpenes (Vassilyev, 1977). Osmiophilic droplets are also found in vacuoles of different size. Some of these vacuoles are very small and are formed as associations of smooth tubular ER. On later stage vacuoles are found in the apical part of the head cells. The material is excreted to extracellular space by exocitosis.

Plastids in secretory cells of *Agastache rugosa* have irregular shape. They have contain a network of smooth tubular elements which contain osmiophilic material (fig. 5). Plastids are associated with elements of smooth ER. Osmiophylic droplets are found between the membranes of plastidal envelope and within the lumen of periplastidal smooth ER.(fig. 5, arrows).

Secretory cells of *Nepeta cataria*, *Nepeta cataria* var. *citriodora* and *Scutellaria baicalensis* have plastids of irregilar shape which look on the ultrathin sections as a cross section of a cup stack (fig. 6). They have few plastoglobuli and a network of smooth tubular elements. Plastids are associated with tubular smooth ER (fig. 6, arrows). Osmiophylic material was not found neither within plastids, nor in periplastidal ER. Secretory droplets accumulate only in vacuoles. The vacuoles are located in apical part of head cells.

Secretion is excreted by exocytosis to extraplasmic space and then pass through the cell wall into subcuticular cavity. In all investigated species a portion of secretion remains inside the secretory cells in storage vacuoles. The secretion appear in subcuticular space as small droplets adjacent to the cell wall. On later stages of development large droplets of excreted material fill subcuticular cavity.

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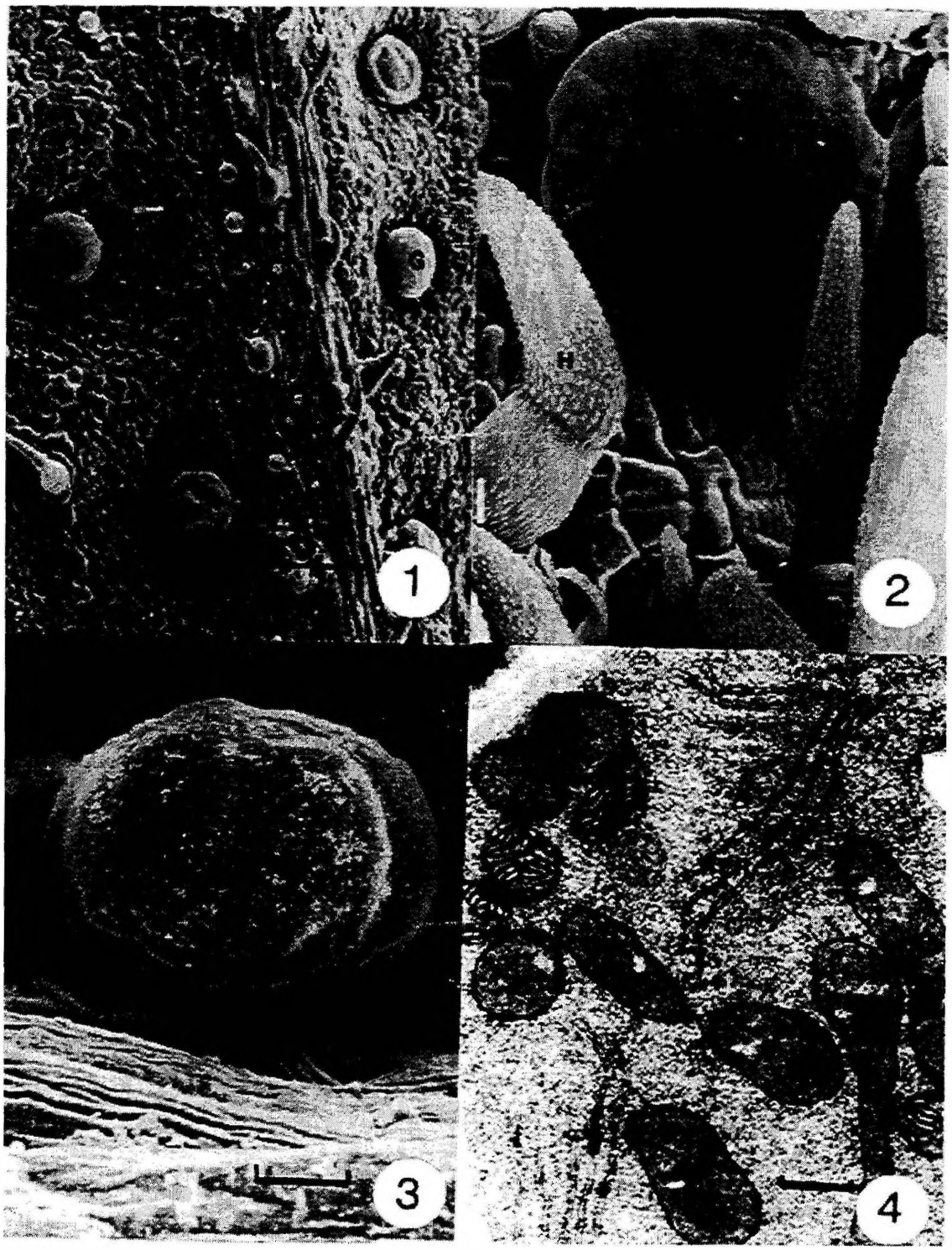

Figure 1. ***Agastache rugosa*** **abaxial leaf surface. G peltate gland; arrows - capitate trichomes. Bar - 100 μm**

Figure 2. ***Dracocephalum moldavica.*** **peltate glands and non glandular hairs. H - non glandular hair; G - peltate gland; GE - peltate gland with elevated cuticle. Bar - 20μm**

Figure 3. Peltate glandular trichome of ***Nepeta cataria*** **var.** ***citriodora*** **Bar - 10μm**

Figure 4. Plastids (P) and endoplasmic reticulum (ER) in secretory cell of ***Dracocephalum moldavica.*** **Bar - 0.4 μm**

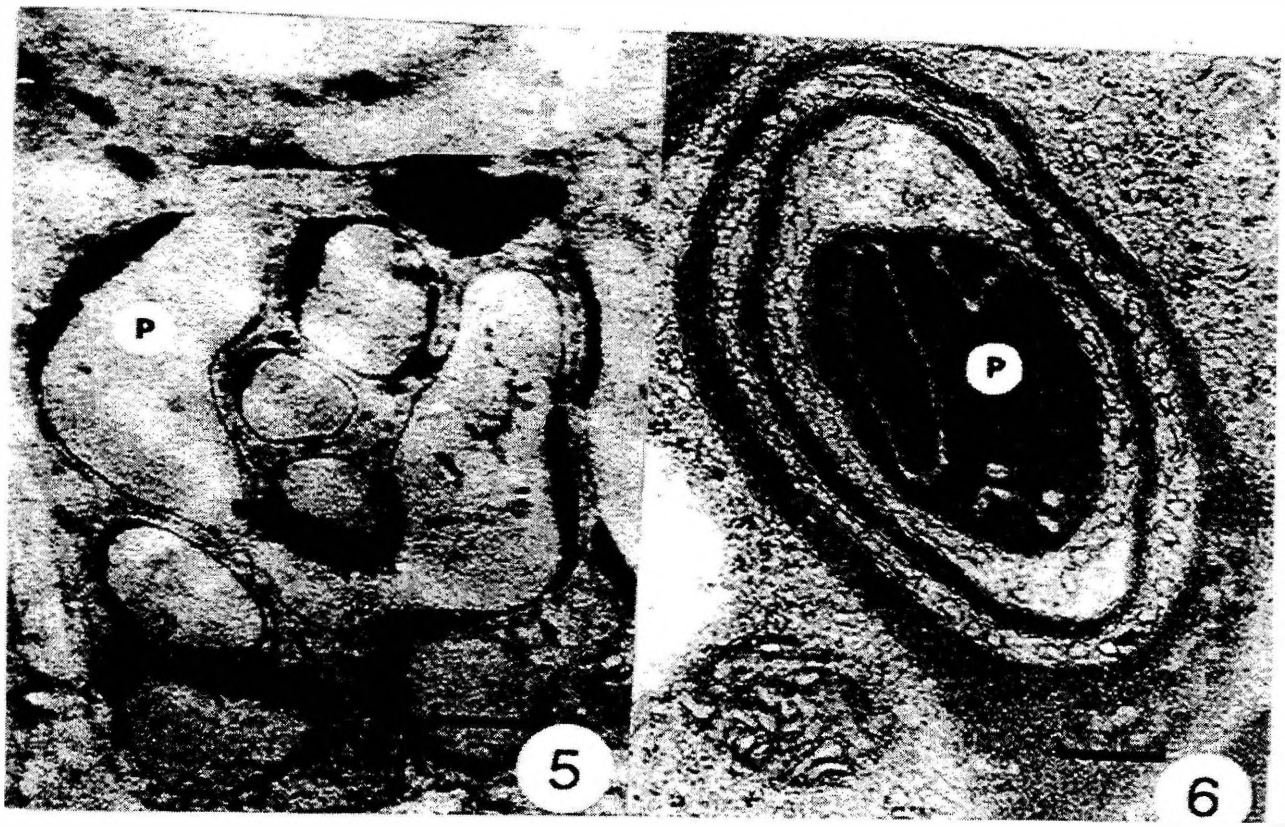

Figure 5. Plastids (P) in secretory cell of *Agastache rugosa*. Arrows - elements of smooth ER, associated with plastids. Bar - 0.4 μm

Figure 6. Cross section of a plastid (P) in secretory cell of *Nepeta cataria*. Arrows tubes of endoplasmic reticulum. Bar - 0.2 μm

OBSERVATIONS ON GLANDULAR TRICHOMES AND ESSENTIAL OILS IN *ROSMARINUS OFFICINALIS* L.

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INTRODUCTION

Within a series of researches on the secretory structures in *Labiatae* and the related volatile compounds (Bini Maleci *et al.* 1992; Servettaz *et al.* 1992; Servettaz *et al.* 1994; Bini Maleci *et al.* 1995), the problem arose of better characterizing the kind of metabolites elaborated in the different trichomes. In almost all aromatic species at least two kinds of secreting trichomes are described (peltate and capitate), which differ in structure and mode of secretion; it is generally accepted that both kind of trichomes secrete essential oils, but also proteins and glucides can be found (Werker, 1993).

In this research we intend to better define the contents of these trichomes using *R. officinalis* leaves, where both above cited kinds of glandular hairs are present. According to Werker *et al.* (1985), a third kind of trichome, capitate with 2-5 elongated stalk cells, can be found in *R. officinalis*. Owing to the scarcity of this kind of trichome, we here didn't consider this one. The trichomes were first histochemically analyzed on leaf sections, while for chemical analysis (GC-MS) the two kind of trichomes have been separately collected in order to evidentiate the possible differences.

METHODS

SEM analysis. Small pieces of material were fixed by 2.5% glutaraldehide in 0.1 phosphate buffer at pH 7.4 and postfixed in 0.2% OsO_4. The material has been dehydrated with acetone, followed by critical point drying and carbon gold coated. An electron microscope Philips 515 was utilized.

Histochemical methods. Fresh material was sectioned and preliminarly observed with an epifluorescence microscope with UV light to evidentiate the autofluorescence. Fresh sections were then stained with the following methods: Nadi (David and Carde, 1964), Sudan Black B, Sudan III-IV, Calcofluor, Acriflavine (O'Brien and McCully 1981), Nile Red (Greenspan *et al.*, 1985), Nile Blue (Werker and Fahn, 1981), $AlCl_3$ (Guerin *et al.*, 1971), and Aniline Blue-Black (Fisher, 1968) - (table 1).

Sampling of the contents of the glandular hairs. The secretion sampling was done using cilindrical type (20± μm i.d.) microneedles (MNs) (Tirillini and Stoppini, 1994; Tirillini and Stoppini, 1995).

The glass MNs were inserted in a fused silica needle (the MN protruded about 0.5 mm), the glands were perforated and the liquid secretion filled the MN due to a light suction.A piston in the fused needles allowed the MN to be transferred into a glass tube (about 1 mm diameter x 50 mm); the glass tube was sealed by end fusion. The sampling of the secretory contents of the peltate trichomes was done touching the trichomes with the MN tip. In order to avoid contamination, the sampling of the secretory contents of the capitate trichomes was done cutting the leaf and sampling the capitate trichomes that protrude, well isolated from the edge.

GC-MS analysis. The glass tubes were introduced into a glass U-shaped. The tube was thermostated at 80°C and flushed with helium (high grade purity, 20 ml/min) for 40 min; the volatile compounds were absorbed in a Chrompack Carbotrap 300 trap and transferred by means of a Chromopack Purge and Trap Injector into a Hewlett Packard CG-MS, model 5989 A MS Engine, equipped with a SE 54 column (25m x 0.2 mm, film thickness 0.5μm) and He 0.8 ml/min. The column was held at 35°C for 2 minuts and then programmed to 250°C at 5°C/min. Compounds were identified by comparison with their retention time and their mass spectra with

those of authentic samples or/and with data reported in literature.

Table 1 - Histochemical tests. Results: - absent; ± scarce; + intense; ++ very intense.

Stain	Stained compound	Colour	Peltate trichomes	Short capitate trichomes
Aniline Blue-Black	Proteins	Black	–	–
Nadi	Essential oils	Blue	±	++
Sudan Black B	Fats	Black	±	++
Sudan III-IV	Neutral fats	Red	–	+
Nile Red	Neutral fats	Yellow	–	++
Nile Blue	Terpenoids Fats	Pink Blue	– +	++ +
Al Cl_3	Flavonoids	Green - -Yellow	+	–
Calcofluor	Polysacchari -des	Blue	–	–
Acriflavine	Polysacchari -des	Yellow - - Orange	–	–

RESULTS AND DISCUSSION

Owing to the economical importance of the species, *R. officinalis* has been the object of innumerable researches (Werker *et al.*, 1985; Vokou and Margaris, 1986; Catalano *et al.*, 1993) however up to now no study was known on separated secretory trichomes. As illustrated in Fig. 1, these secreting structures are: peltate trichomes with many secretory head cells and a large subcuticular space; short capitate trichomes with only one secretory head cell with a small subcuticular space. In both the trichome types, secretion is accumulated in the subcuticular space. This one is very large in the peltate trichomes and small in the capitate ones.

The histochemical stain with Nadi demonstrates that the subcuticular space in the short capitate trichomes is full of secretion (Fig. 2), while only few small drops of secretion are evidentiated by this stain in the subcuticular space of peltate trichomes (Fig. 3). Sudan black B reaction for fats is more intense in short capitate trichomes (Fig. 4). The autofluorescence (Fig. 5) and the Nile red stain evidence terpenoids only in capitate trichomes (Figs. 6 and 7). The remaining of the secretion is not well known; until now we could only demonstrate the presence of flavonoids in the peltate trichomes, using $AlCl_3$ stain, which produces a yellowish-green fluorescence in their presence.

Until now, our method of analysis could not give the absolute amount of essential oils in the different kind of hairs, also because of the presence of a lot of water in the peltate trichomes.

In Table 2 the percentage composition of volatile compounds in both kinds of trichomes is reported. 2-Methyl-2-pentenal is present, in big amount, only in the peltate trichomes. This compound was known as a natural component of essential oils of *Allium victorialis* (Nishimura and Ariga, 1994), *A. cepa* (Dembele and Dubois, 1973), *A. porrum* (Schreyen *et al.*, 1976), as well as of *Ferula asafoetida* (Noleau *et al.*, 1991). To our knowledge, the finding in *R. officinalis* is new, notwithstanding the huge amount of researches on this species. This fact might depend on the volatily of the compound, which would thus escape the vapor distillation, still the most utilized method of essential oil production.

Table 2: Composition (%) of *R. officinalis* L. essential oils of peltate and short capitate trichomes.

Compounds	Composition (%)	
	Peltate Trichomes	Short Capitate Trichomes.
2-methyl-2-pentenal	36.84	-
α-pinene	18.77	20.00
camphene	7.82	3.52
β-pinene	0.61	1.32
β-myrcene	0.69	1.61
limonene	0.61	0.29
1-8-cineole	6.17	15.80
linalool	4.00	16.00
camphor	0.52	11.70
borneol	3.04	8.00
α-thujone	0.87	1.94
α-terpineol	9.64	1.37
bornyl acetate	9.50	12.80
eucarvone	0.52	1.04
α-humulene	0.43	4.80

Apart this difference, the qualitative composition is practically identical in the two kinds of trichomes, and is more or less the same until now founded in the species (Manunta, 1985, Werker *et al.*, 1985, Rosua and Garcia-Granados, 1987, Vokou and Margaris, 1986; Catalano *et al.*, 1993,).

In the peltate trichomes apart from 2-methyl-2-pentenal the main compounds are α-pinene (18.77%), α-terpineol (9.64%), bornyl acetate (9.5%) and camphene (7.82%), while in the short capitate trichomes main compounds are α-pinene (20%), linalool (16%), 1-8-cineole (15.8%), bornyl acetate (12.80%) and camphor (11.70%). Very interesting is the presence of a good amount of camphor only in the short capitate trichomes, while the same compound is very scarce in the

peltate ones. The amount of camphor in *R. officinalis* may be very variable, is scarce on acidic soils and abundant on calcareous ones (Manunta, 1985), it increases in specimens transplanted in more basic soil (Servettaz, unpublished data), and it is very important as determing the peculiarity of the essential oils.

The major interest of this work is the possibility of a separate analysis of the two kind of trichomes in *R. officinalis* leaves. In this way the chemical analysis can confirm the histochemical data, also in a very small structure, as capitate hairs are.

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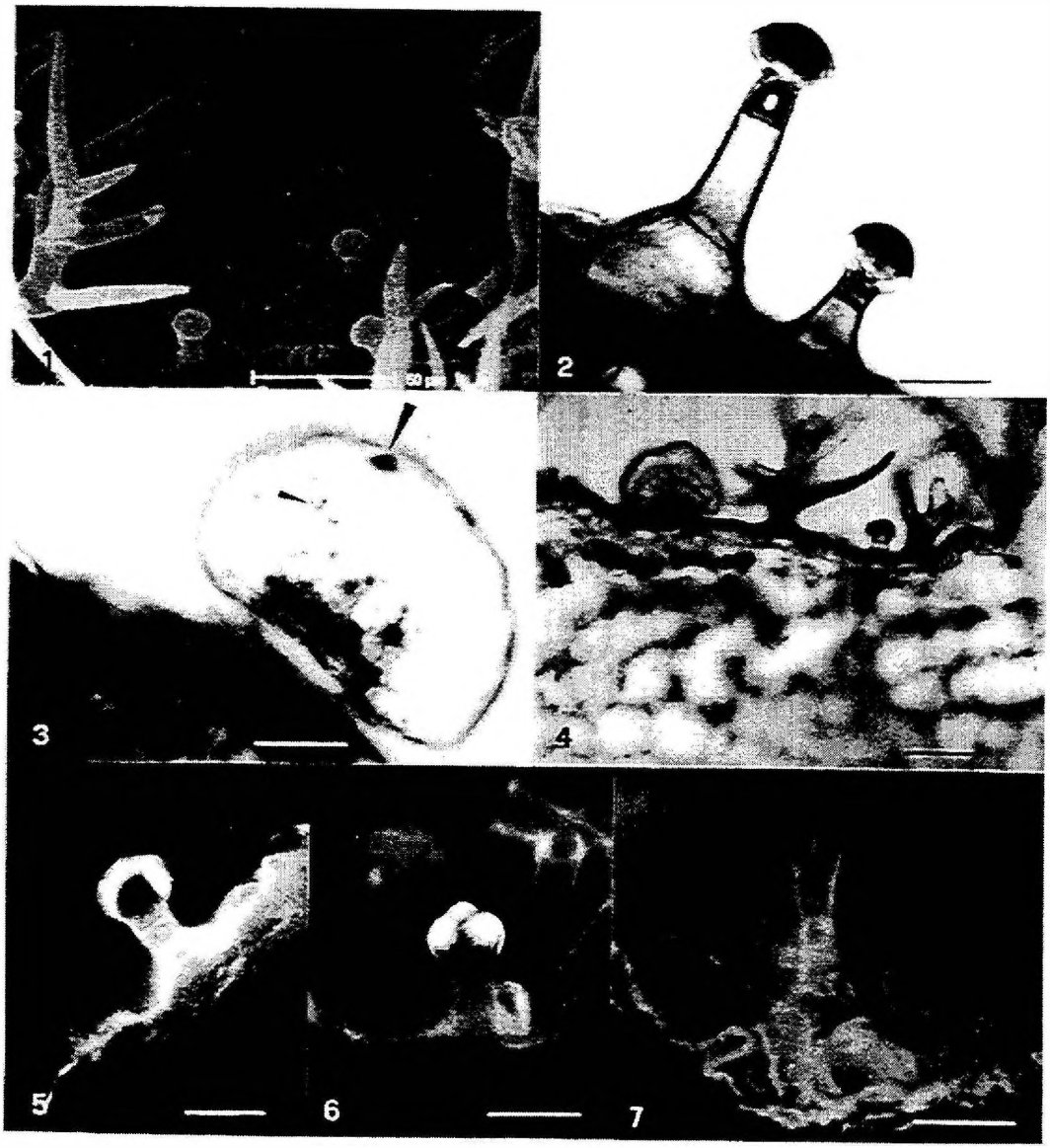

Figure 1. Scanning electron micrograph, leaves of *R. officinalis* with peltate and short capitate trichomes (bar 50μm).

Figures 2-7: Histochemical characterization of glandular trichomes secretion.

***Fig. 2**. Short capitate trichomes appear intensely stained with NADI (bar 20μm). **Fig. 3**. Peltate trichome shows only small Nadi stained drops [arrows] (bar 20μm). **Fig. 4**. Only the cuticle of peltate trichomes and cuticle and secretion of the short capitate trichomes appear stained with Sudan Black B. (bar 50μm). **Fig. 5.** Short capitate trichomes, observed in UV light, show a light-yellow autofluorescence. (bar 20μm). **Fig. 6**. Short capitate trichomes reveale the presence of terpenoids when stained whit. Nile Red stain. (Blue light; bar 20μm). **Fig. 7.** The peltate trichome secretion appears negative to Nile Red stain. (Blue light; bar 50μm).*

Fig.1 X 830 / Fig.2 X 600 / Fig.3 X 600 / Fig.4 X 180 / Fig .5 X 580 / Fig.6 X 600 / Fig.7 X 260 /

INFLUENCE OF INULIN, CULTURE MEDIUM AND LIGHT ON THE GROWTH AND ESSENTIAL OIL CONTENT OF EDELWEISS HAIRY ROOTS

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INTRODUCTION

Hairy roots developed from dicotyledonous plants have been found to produce the same secondary metabolites as roots of uninfected parent plants (1). Due to a scarcity of natural plant material, 'hairy' roots were developed from intact plants of Edelweiss (*Leontopodium alpinum* Cass.) after infection and genetic transformation with *Agrobacterium rhizogenes* (Strain 9402) (2). These were found to yield an essential oil which was qualitatively similar to that produced by roots of intact, soil-grown plants (3). Edelweiss is a member of the Compositae, a family where the ubiquitous reserve carbohydrates are considered to be fructans (4). In this family the fructans are synthesised as an homologous series of unbranched ß-2,1-linked polymers called inulins (5). The suggested function of fructans is in maintaining growth during periods of limited water-availabilty or seasonally low soil-water temperatures (6). Fructans are water-soluble and occur in the vacuoles of leafy vegetables as well as "fleshy" roots, several of which have been used traditionally as souces of inulin [eg. *Helianthus tuberosus* (Jerusalem artichoke) and *Inula helenium* (Elecampane)]. Although *Leontopodium* was classified with *Inula* into the tribe Inuleae and resembles it in producing an essential oil, it differs from it in not having fleshy roots.

Plant secondary metabolites, including essential oils, are known to be produced by hairy root cultures (7, 8, 9). Since their formation can be influenced by culture conditions and nutrients (10), it was of interest to examine how changes in carbon source, culture medium and light influenced yield and composition of the essential oil isolated from Edelweiss hairy roots.

EXPERIMENTAL

Hairy roots of Edelweiss were established as indicated in ref (3). All cultures were grown for 6 weeks as batch cultures, at 25°, on an orbital shaker (90rpm), and either under fluorescent lights (Super Gro 65-80W) at a photoperiod of 18hr light-6hr dark or in the absence of light when flasks were surrounded by aluminium foil. Roots [Line a] were maintained in a phytohormone-free medium, containing Murashige & Skoog [M+S] basal salts (11), thiamine HCl 0.4mg l^{-1}, mesoinositol 100 mg l^{-1}, and sucrose 30 g l^{-1}. Subcultures of roots [inoculum weight ca. 0.7g] were made from the maintenance medium into media [150 ml in 250 ml conical flasks] formulated as above, except with the basal salts of Murashige & Skoog [M+S] being replaced by those of Gresshoff & Doy [G+D] (12), Litvay [L] (13) or White [W] (14) and sucrose 30 g l^{-1}, being replaced by inulin 30g l^{-1} or sucrose plus inulin each at 15 g l^{-1}. All experiments were replicated [x8].

Oil Isolation - Freshly harvested roots were dried (<40°), crushed and subjected to hydrodistillation for 5h using an Apparatus for the Determination of Essential Oils in Vegetable Drugs (15). The volume was recorded and the separated oil used for chromatographic analysis.

Oil Analysis - Samples were analysed on a Perkin-Elmer (8700) FID instrument fitted with a 2m x 3mm stainless steel, glass-lined column packed with 10% Carbowax 20M on Gas Chrom. W(AW) 100-120 mesh. The temperature programme used was 100 °C for 5minutes, rising to 150° at 2°/ min, held for 5 minutes and then rising to 200° at 3°/min. The injector and detector temperatures were 220 ° and 200 °C respectively. The flow rate of the carrier gas (N_2) was 30 mL/min. Percentage relative abundances of individual compounds were calculated by internal normalisation protocols using integrator-generated data. Each analysis was duplicated.

Statistical Analyses Analyses were performed using Instat ^{R} statistical software (Graphpad, San Diego, USA). Data were analysed by one-way Analysis of Variance [ANOVA]. Additional analyses of data were performed using the Tukey-Kramer multiple comparison post test.

RESULTS AND DISCUSSION

Effects on Growth and Biomass production

Increase in biomass over the culture period, expressed as Growth Index [fresh weight at harvest ÷ fresh weight of inoculum] was most pronounced in media supplemented with Litvay's basal salts and poorest with White's, especially when grown in the light [Fig. 1].

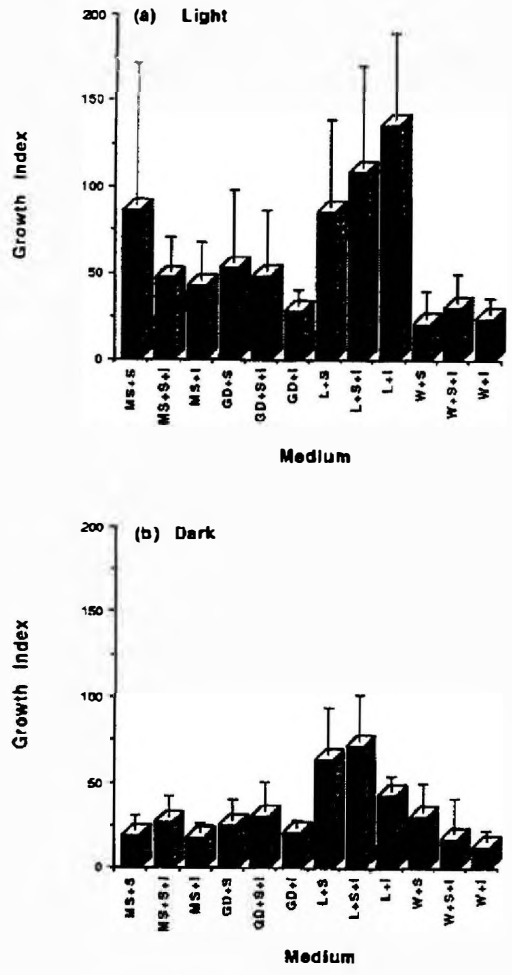

Figure 1 Biomass production [Growth Index] of *Leontopodium alpinum* hairy roots (line a) cultured under [a] light and [b] dark conditions; in different basal salts (Murashige & Skoog [MS]; Gresshoff & Doy [GD]; Litvay [L]; White[W]); in the presence of sucrose [S] or inulin [I]. Cultures were grown in 150mL medium, in 250 mL flasks for 6 weeks. All determinations were replicated (x8).

Biomass, recorded as dry weight per flask, was maximum in the M+S medium supplemented with 3% sucrose and cultured in the light where a yield of 2.04 g [s.d. 0.095] was obtained. Of the basal salt formulations examined White's gave the lowest yields ($P<0.001$). Partial or total replacement of sucrose by inulin in the four basal salt formulations did not result in statistically significant differences in biomass yields. Culture in the absence of light reduced growth in all media, with the most significant reductions in biomass being recorded with the M+S salts, where, in comparison to light culture, reductions of 33% were found in the presence of 3% sucrose ($P<0.001$), 42% with sucrose plus inulin ($P<0.001$) and 51% with 3% inulin ($P<0.001$).

Essential oil content

Edelweiss hairy roots when cultured in a phytohormone-free modified M+S medium supplemented with 3% sucrose and grown in light always contains between 0.4 - 0.6% (v/w) of an essential oil. Culture in the absence of light increases this oil yield [Fig. 2].

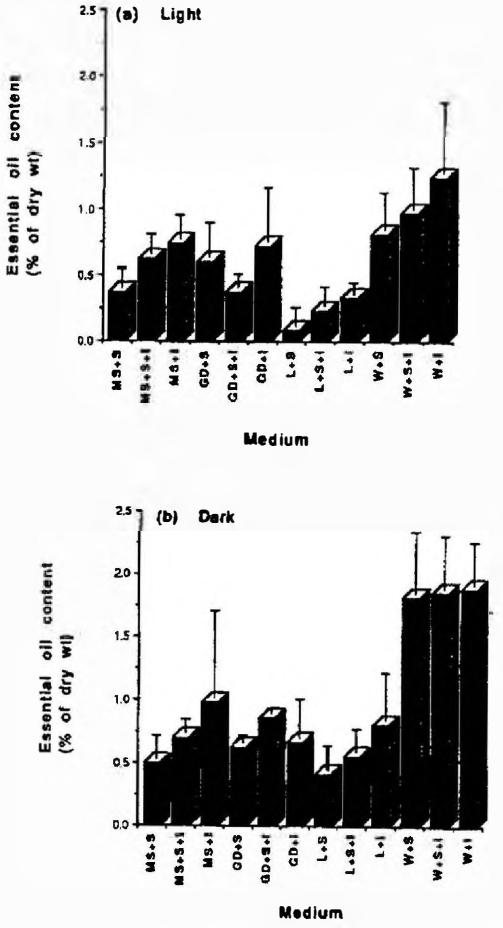

Figure 2 Essential oil content (%v/w) of *Leontopodium alpinum* hairy roots (line a) cultured under [a] light and [b] dark conditions; in different basal salts (Murashige & Skoog [MS]; Gresshoff & Doy [GD]; Litvay [L]; White[W]); in the presence of sucrose [S] or inulin [I]. Cultures were grown in 150mL medium, in 250 mL flasks for 6 weeks. All determinations were replicated (x4).

Comparison of the three basal salt formulations with the M+S based-medium showed roots cultured in White's medium to contain significantly higher amounts of

essential oil (P<0.001), an effect more obviously seen when culture was in the dark. Replacing sucrose with inulin as carbon source did not significantly affect oil contents.

Essential oil composition

The essential oil produced by Edelweiss roots, both natural and hairy, is a complex mixture of sesquiterpenes (16). Two constituents, a hydrocarbon [Sq HC] and a structurally related acetate [Sq Ac], are always present in major concentrations, together representing more than 50% of the oil. Changing the basal salt formulation from M+S resulted in significant reductions in the % content of the sesquiterpene hydrocarbon, especially when grown in light (Fig.3).

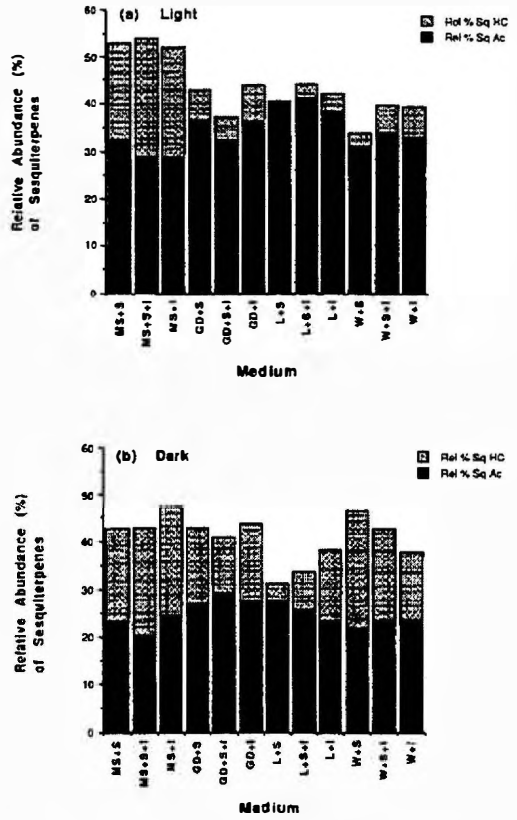

Figure 3 Relative abundance of essential oil constituents isolated from hairy roots (line a) of *Leontopodium alpinum* cultured under [a] light and [b] dark conditions; in different basal salts (Murashige & Skoog [MS]; Gresshoff & Doy [GD]; Litvay [L]; White[W]); in the presence of sucrose [S] or inulin [I]. Cultures were grown in 150mL medium, in 250 mL flasks for 6 weeks. All determinations were replicated (x4)

Replacement of sucrose with inulin produced variable results, though enhanced contents of the sesquiterpene acetate were especially obvious in the oil isolated from roots cultured with inulin in the presence of Litvay's and Gresshoff+Doy's salts. However the most significant effects on essential oil composition were produced by the presence or absence of light during the culture period, with the hydrocarbon content increasing in the dark, at the expense of the acetate, and vise versa in the light.

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BIOTRANSFORMATION OF TERPENOIDS AND RELATED COMPOUNDS BY MICROORGANISMS - PRODUCTION OF BIOLOGICALLY ACTIVE SUBSTANCES

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INTRODUCTION

We are continuing to investigate the microbiological transformation in order to clarify the metabolism of terpenoids and the utilization of the metabolites [1-3]. The microbiological introduction of oxygen functional groups for terpenoids and related compounds is interesting because many biologically active compounds possess hydroxyl, carbonyl, carboxylic and epoxide groups. Here, we report the production of biologically active substances in the bio-transformation of terpenoids and related compounds.

EXPERIMENTAL

Culture *and Biotransformation - Aspergillus* spp. such as *A. niger* and *A. cellulosae* were cultivated under the rotatory condition (100 rpm) at 30 °C for 3 days in the following medium (200 ml) in the 500 ml *Erlenmeyer* flask: glucose (15g), sucrose (l5g), polypepton (5g), KH_2PO_4 (1 g), $MgSO_4$ 7 H_2O (0.5 g), KCI (0.5 g), $FeSO_4$ 7 H_2O (0.01 g) (pH 7.0) [1] Actinomycetes such as *Streptomyces bottropensis* and S. *ikutamanensis* were cultivated statically at 30 °C for 3 days in the same medium (40 ml) used for fungi in the 100 ml *Erlenmeyer* flask. Yeasts such as *Hansenula anomala* were cultivated under the rotatory condition (100 rpm) at 30 °C for 3 days in the following medium (200 ml) in the 500 ml *Erlenmeyer* flask: glucose (10 g), dried extract of yeast (0.5g), L-asparagin (2g), KH_2PO_4 (1g), $MgSO_4$ 7 H_2O (1 g), NaCl (0.5 g), $CaCl_2$ (0.5 g) (pH *5.5). Euglena gracilis Z* was cultivated photoheterotrophically (light illumination at *ca* 2000-3000 lux) and statically at 25 °C for 7-10 days in the following medium in the 100 ml *Erlenmeyer* flask: KH_2PO_4 (0.4 g), $(NH_4)_2HPO_4$ (0.2 g), $MgSO_4$ 7 H_2O (0.5g), $CaC0_3$ (0.2 g), DL-malate (2 g), Na-glutamate (5 g), EDTA 2Na (50 mg), $ZnSO_4$ $7H_20$ (22 mg), $MnSO_4$ H_20 (5.8 mg), $FeSO_4$ (NH_4) 2 SO_4 6 H_20 (5.7 mg), Na_2MoO_4 H_20 (1.5 mg), $CuSO_4$ 5 H_20 (1.6 mg), $CoSO_4$ 7 H_2O (1.5 mg), H_3BO_4 (11.4 mg), vitamin B_1 (2.5 mg) and vitamin B_{12} (0.02 mg) in 1 liter distilled water (pH 3.3, adjusted with HCI) [4]. *Dunaliella tertiolecta* was cultivated photoautotrophically at 25 °C for 7 days in the same manner in case of *Euglena* in the following medium: $MgCl_2$ 6 H_2O (1.5 g), $MgSO_4$ 7 H_2O (0.5 g), Kcl (0.2 g), $CaCl_2$ (0.2 g), $KN0_3$ (1.0 g), $NaHCO_3$ (0.045 g), Tris (2.45 g), K_2HPO_4 (0.045 g), Fe-EDTA (3.64 mg), EDTA 2Na (1.89 mg), $ZnSO_4$ 7 H_20 (0.087 mg), H_3BO_3 (0.61 mg), $CoCl_2$ H_20 (0.015 mg), $CuSO_4$ 5 H_20 (0.06 mg), $MnCl_2$ (0.23 mg), $(NH_4)6Mo_7O_{24}$ 4 H_2O (0.38 mg) and NaCl (29 g) in 1 liter distilled H_2O (pH 8.0) [5]. *Chlorella ellipsoidea* IAM C-27 was cultivated under the rotatory condition(100 rpm) in 100 ml of the same medium used for *Dunaliella* without NaCl at 25 °C for 7-10 days.

On obtaining full growth of microorganims, each substrate was added into the cultured mediurm and the biotransformation was carried out under the sarne conditions as described above. After the finish of the biotransformation, each cultured broth was extracted with ether. The ether extract was analyzed by GC and GC-MS and the metabolites were separated and purified by a combination of column chromatography on silica gel and prep. GC. The products were identified by GC, IR, ^{1}H- and ^{13}C-NMR and mass spectra.

RESULTS

In the course of the investigation for the biotransformation of monoterpenoids and related compounds by using *A. niger, A. cellulosae, S. bottropensis, S. ikutamanensis, Euglena, Dunaliella, Chlorella* and other microorganisms, we have clarified the metabolic pathways of monoterpenoids such as carvone and related compounds. Further, we have obtained *l*-carvone-8,9-epoxide (**1**), 5β-hydroxy-*d*-neodihydrocarveol (**2**), *d*-bottrospicatol (**3**), *l*-

iso-bottrospicatol (**4**), isodihydrobottrospicatol (6), *p*-menthane-3,8-*cis*-diol (**7**), *p*-menthane-3,8-*trans*-diol (**8**), 5β-hydroxy-*l*-carvone, 5β-hydroxydihydrocarvone, 8 kinds of 8-hydroxydihydrocarveols, 10-hydroxydihydrocarveol, 8,9-dihydroxydihydrocarveol, dihydrocarvone-8,9-epoxide, dihydrocarveol-8,9-epoxide, 2α-, 3α- and 3β-hydroxy-1,8-cineoles (**9**), isopiperitenone (**l0**), 3β-hydroxy-1-methylcyclohexene (**11**), dihydro-4-oxoisophorone (**12**) as the biologically active substances or their precursors. Compounds **1, 3, 4, 6-8, 12,** 3-nitrosalicyl aldehyde (**15**) and β-resorcyl aldehyde (**18**) exhibited the strong inhibitory activity for the germination of lettuce seeds at the concentration of 200 ppm (Table 1. and Figure 1.). Furthermore, compound **5** synthesized from **3** showed the strongest inhibitory activity among the analogues such as **6**, methoxylated **3** and esterified **3** [9]. The formation of compound **9** from 1,8-cineole (*Eucalyptus*) and **10** from *d*-limonene (*Citrus*) as a biomass is very important and interesting from the view point of the formation of the precursor of **7** and **8**, which are known as mosquito repellents [7] and allelochemicals in *Eucalyptus citriodora [6, 8-9].* Compound **11** is known as insect pheromone [2]. Aromatic aldehydes such as vanillin (**13**) and ethylvanillin (**l4**) are easily convertible to the corresponding alcohols by *Euglena [3], Dunaliella [2]* and *Chlorella.* However, compounds **13**, **14**, genticin aldehyde (**16**) and protocatechualdehyde (**17**) showed the strong inhibitory activity for the root's elongation of lettuce and rice after the germination at the concentration of 200 ppm. Benzalacetophenone was also easily biotransformed by *Euglena, Dunaliella, Aspergillus* spp., *Hansenula* and *S.ikutamanensisto* 4-phenyl-2-butanone, which is known as allelochemicals in *Eucalyptus pauciflora.* Isopterocarpolone (**20**) from α-(-)-eudesmol (**19**, *Porella stephamina)* and 12-hydroxy-(-)-cyclocolorenone (**22**) and 6β-hydroxy-4,11-guaiadien-3-one (**24**) from (-)-(**21**, *Solidago altissima)* and (+)-cyclocolorenone (**23**, *Plagiochila sciophila),* respectively, as metabolites of sesquiterpenoids by *A. niger* and *A. cellulosae* are expected to have more biologically activity than the starting materials.

Acknowlegements

The authors would like to thank Prof. H. Nishimura, Hokkaido Tokai University, who was a co-worker to study the formation of bottrospicatol and p-menthane3,8-diols, Dr. Toru Katsuragi, University of Osaka Prefecture, for supplying microorganisms, Dr. Toshihiro Hashirnoto, Tokushima Bunri University, for his useful discussion concerning the metabolites of α-eudesmol and cyclocolorenone.

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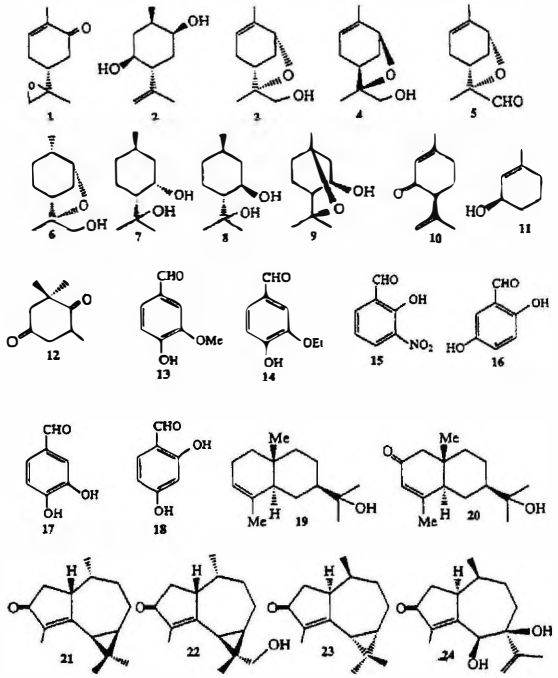

Figure 1. Compounds obtained in the course of biotransformations

TABLE 1. Production of Biologically Active Substances in the Biotransformation of Terpenoids and Related Compounds by Microorganisms -

Substrates	Microorganisms	Products	Biologically activity	
l-Carvone	*S. bottropensis*	*l*-Carvone-8,9-epoxides(**1**)		A
		5β-Hydroxycarvone		
l-Carvone	*S.ikutananensis*	5β-Hydroxy-(+)-neodihydrocarveol(**2**)		B
		5-Hydroxydihydrocarvone		
l- & *d*-Carvone & dihydrocarveol	*A.niger, Euglena*	8 kinds of 8-Hydroxydihydrocarveols		
Carvone & dihydrocarveol	*A.niger*	10-Hydroxydihydrocarveol		
l-Carvone	*A.niger*	8,9-Dihydroxydihydrocarveol		
l- & *d* -Carvone	*S.bottropensis*	Dihydrocarvone-8,9-epoxides		
Dihydrocarvone & dihydrocarveol		Dihydrocarveol-8,9-epoxide		
l-cis-Carveol	*S.bottropensis*	*d*-Bottrospicatols(**3**)		A
d-cis-Carveol	*S.bottropensis*	*l*-Isobottrospicatols(**4**)		A
3	CrO_3/Py[9]	*d*-Bottrospicatal(**5**)		A
Dihydrocarveols	*S.bottropensis*	Isodihydrobottrospicatol(**6**)		A
3	1atmH_2.PtO_2[9]	**6**		A
Menthols	*A.niger, Aspergillus* spp.	*p*-Menthane-3,8-*cis*-diols(**7**)		A B
Neomenthol	*A.niger, Aspergillus* spp.	*p*-Menthane-3,8-*trans*-diols(**8**)		A B
1,8-Cineole	*A.niger, S.ikutananensis* and *S.bottropensis*	2α-Hydroxy-1,8-cineole		
	A.niger, S.ikutananensis and *S.bottropensis*	3α-Hydroxy-1,8-cineole		
	S.ikutananensis	3β-Hydroxy-1,8-cineoles(**9**)	pro-A	pro-B
d-Limonene	*A.cellulosae*	Isopiperitenone(**10**)	pro-A	pro-B
1-Methyl-1- cyclohexene	*A.cellulosae*	2-Methyl-2-cyclohexen-1-ol(**11**)		D
		2-Methyl-2-cyclohexen-1-one		
4-Oxoisophorone	*A.niger*	Dihydro-4-oxoisophorone(**12**)		A
Vanillin(**13**)	*Euglena , Dunaliella and Chlorella*	Vanillyl alcohol		C E
Ethylvanillin(**14**)	*Euglena , Dunaliella*	Ethylvanillyl alcohol		C

	and Chlorella		
3-Nitrosalicylaldehyde(**15**)	*Euglena ,Dunaliella and Chlorella*	3-Nitrosalicyl alcohol	A
Genticin aldehyde(**16**)			A
Protocatechu aldehyde(**17**)			A
β-Resorcylaldehyde(**18**)			A
Benzalacetophenone -	*Euglena , Dunaliella, Aspergillus spp. Hansenula, S. ikutananensis*	4-Phenyl-2-butanone	A
α-(-)-Eudesmol(**19**)	*A.niger , A.cellulosae*	Isopterocarpolone(**20**)	A
(-)-Cyclocolorenone(**21**)	*A.niger*	12-hydroxy-(-)-cyclocolo none(**22**)	A
(+)-Cyclocolore none(**23**)	*A.niger*	6β-hydroxy-4,11-guaiadien3-one(**24**)	A

A. The inhibition of the germination of green foxtail, radish, garden cress, wheat and lettuce seeds at the concentration of 50-200 ppm.

B. The mosquito repellents and allelochemicals

C.The strong inhibitory activity for the root's elongation of lettuce and rice at the concentration of 200ppm.

D. Sex pheromone

E. Bio-antimutagen

ENANTIOSELECTIVE CYCLIZATION OF (±)-LAVANDULOL TO (-)-(2*S*,4*S*)-1,5-EPOXY-5-METHYL-2-(1-METHYLETHENYL)-4-HEXANOL BY *GLOMERELLA CINGULATA*

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INTRODUCTION

Lavandulol [5-methyl-2-(1-methylethenyl)-4-hexen-1-ol] (1), an acyclic monoterpene alcohol, occurs in lavender oil [1]. Its acetate is widely used in fragrances [2]. So far, the biotransformation of **1** has not been reported. As part of our continuing program to investigate the microbial transformations of acyclic terpenes [3-6], we report the enantioselective microbial cyclization of (±)-**1** using plant pathogenic fungus *Glomerella cingulata* as a biocatalyst.

EXPERIMENTAL

Spores of *G. cingulata* [the strain stored in Gifu University (Japan)] which have been preserved on potato dextrose agar at 4°C were inoculated into 200 mL of sterilized culture medium (1.5% saccharose, 1.5% glucose, 0.5% polypepton, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.1% K_2HPO_4 and 0.001% $FeSO_4 \cdot 7H_2O$ in distilled water) in a 500 ml shaking flask, and the flask was shaken (reciprocating shaker, 100 rpm) at 27°C for 3 days.

Precultured *G. cingulata* (5 mL) was transplanted into a stirred fermentor (3.5 L) containing medium (3 L). Cultivation was carried out at 27 °C and stirring under aeration for 4 days. After the growth of *G. cingulata*, 1 (1.5 g) was added into the medium and cultivated 6 more days.

After the fermentation, culture medium and mycelia were separated by filtration and both of them extracted with dichloromethane. The solvent was evaporated and the dichloromethane extract was separated on Si-60 columns with a hexane/ethyl acetate gradient to give compound **2** (100 mg).

Table 1. ^{1}H-NMR spectral data of compounds **1-3** (270.05MHz, $CDCl_3$, TMS as int. standard).

H	1	2	3
1_{ax}	3.50 *dd* (8,10.5)	3.37 *t* (11.5)	3.41 *t* (11.5)
1_{eq}	3.57 *dd* (5.5,10.5)	3.65 *ddd* (2,4.5,11.5)	3.67 *ddd* (2,4.5,11.5)
2	1.96-2.34 *m*	2.33 *m*	2.40 *m*
3_{ax}	1.96-2.34 *m*	1.56 *ddd* (11.5,11.5,12.5)	1.56 *ddd* (11.5,11.5,12.5)
3_{eq}	1.96-2.34 *m*	1.91 *dddd* (2,3.5,4.5,12.5)	2.00 *dddd* (2,3.5,4.5,12.5)
4	5.08 *m*	3.50 *dd* (4.5,11.5)	4.70 *dd* (4.5,11.5)
6	1.61 *s*	1.17 *s*	1.19 *s*
7	1.70 *m*	1.29 *s*	1.23 *s*
9	4.82 *m*	4.73 *m*	4.71 *m*
9'	4.93 *m*	4.81 *m*	4.80 *m*
10	1.70 *m*	1.73 *s*	1.72 *s*
CH_3CO	-	-	2.06 *s*

Chemical shifts in ppm. Coupling constants in Hz.

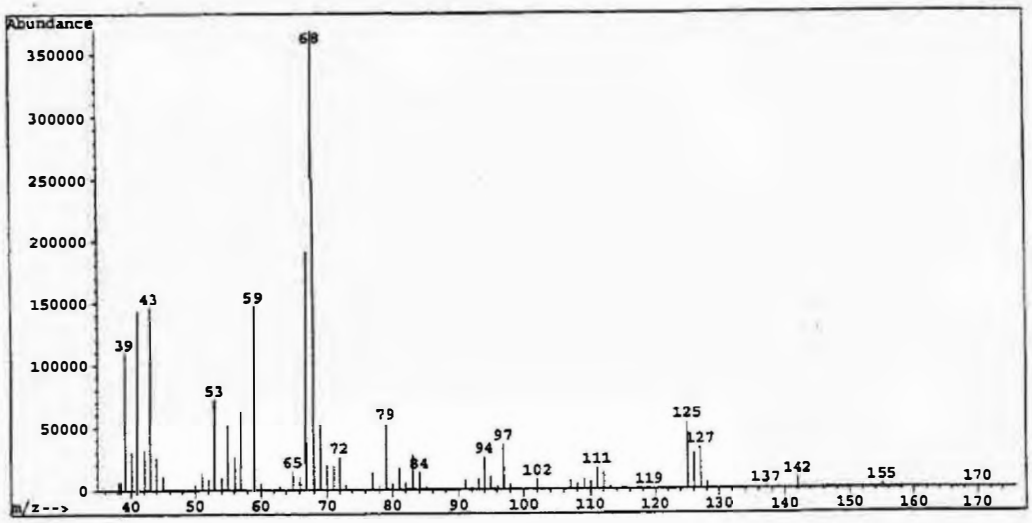

Figure 1. EI-mass spectrum of **2**.

Table 2. ^{13}C-NMR spectral data of compounds **1**-**3** (67.80MHz, $CDCl_3$, residual $CHCl_3$ used as int. ref. δ=77.00).

C	1	2	3
1	63.6 (CH_2)	64.5 (CH_2)	64.7 (CH_2)
2	49.9 (CH)	43.7 (CH)	43.2 (CH)
3	28.4 (CH_2)	33.3 (CH_2)	30.0 (CH_2)
4	122.0 (CH)	74.1 (CH)	75.5 (CH)
5	132.8 (C)	74.8 (C)	73.0 (C)
6	25.7 (CH_3)	27.8 (CH_3)	27.8 (CH_3)
7	17.8 (CH_3)	15.8 (CH_3)	16.9 (CH_3)
8	145.4 (C)	144.7 (C)	144.4 (C)
9	113.1 (CH_2)	110.5 (CH_2)	110.7 (CH_2)
10	19.5 (CH_3)	21.4 (CH_3)	21.5 (CH_3)
$\underline{C}H_3CO$	-	-	21.2 (CH_3)
$CH_3\underline{C}O$	-	-	170.2 (C)

Chemical shifts in ppm. Multiplicities were determined by the DEPT pulse sequence.

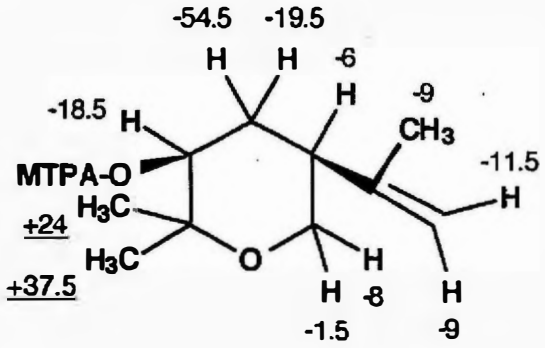

Fig. 2. δΔ Values obtained for the MTPA esters of **2**. δΔ Values are expressed in hertz (500.00 MHz).

RESULTS AND DISCUSSION

The structure of **2** [colorless oil. $[\alpha]^{D}_{20}$ -8.7° (*c* 0.95 in $CHCl_3$)] was determined by spectral data of **2** and **3** (acetate of **2**). The EI-MS (Fig. 1)[7] indicated **2** had a molecular formula $C_{10}H_{18}O_2$. ^{1}H and ^{13}C NMR signals (Tables 1 and 2) and IR spectrum [7] indicated **3** had one acetyl group [δH 2.06; δC 170.2 (C=O); ν_{max} 1746 cm^{-1}] but no hydroxyl group. That is, **2** had only one hydroxyl group. Compound **2** had a secondary hydroxyl group [δH 3.50; δC 74.1 (CH)], an ether linkage [δH 3.37, 3.65; δC 64.5 (CH_2), 74.8 (C)], an isopropenyl group but no trisubstituted double bond. From the spectral data, **2** has a tetrahydropyran ring and the planer structure of **2** was clarified. H-2 and H-4 were shown to be axial from their coupling constants. ^{1}H NMR signals of **2** and **3** showed the long-range coupling (*W*-type) from H-1_{eq} to H-3_{eq}. The structure of **2** elucidated to be 1,5-epoxy-5-methyl-2β-(1-methylethenyl)-4β-hexanol.

To clarify the enantiomeric excess, **2** was converted to (*R*)-MTPA ester. ^{1}H NMR showed the ester is not mixture of diastereomers, consequently **2** is enantiomerically pure (100% e.e.). To determine the absolute configuration of the secondary alcohol (C-4 position), the (*S*)-MTPA ester of **2** was also obtained and (*S*)- and (*R*)-MTPA esters applied to Mosher's method [8,9]. As shown in Fig. 2, δΔ values ($\delta_{(S)\text{-MTPA}}$-$\delta_{(R)\text{-MTPA}}$) were assigned and C-4 determined to be *S*-configuration. Compound **2** was therefore assigned (2*S*,4*S*)-form.

From the above result, two possible metabolic

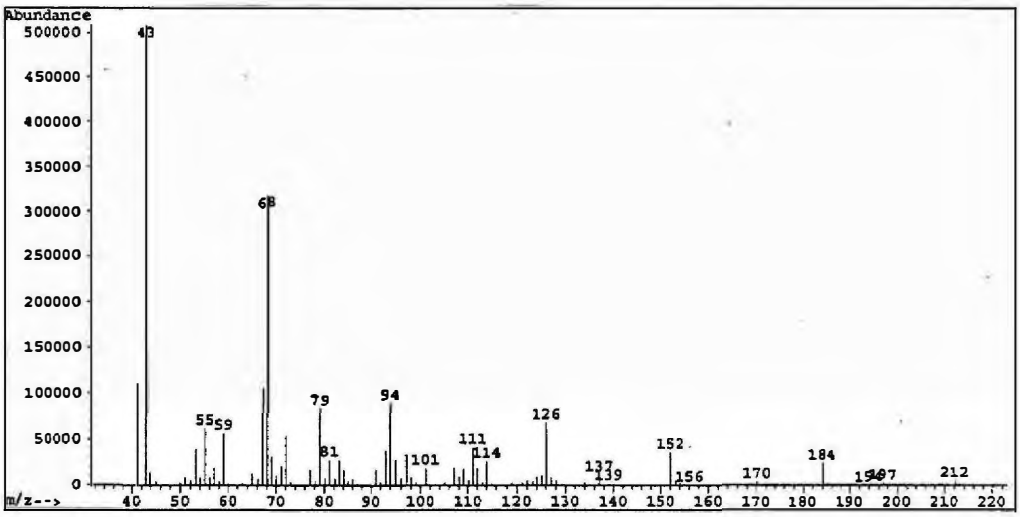

Figure 3. EI-mass spectrum of **3**.

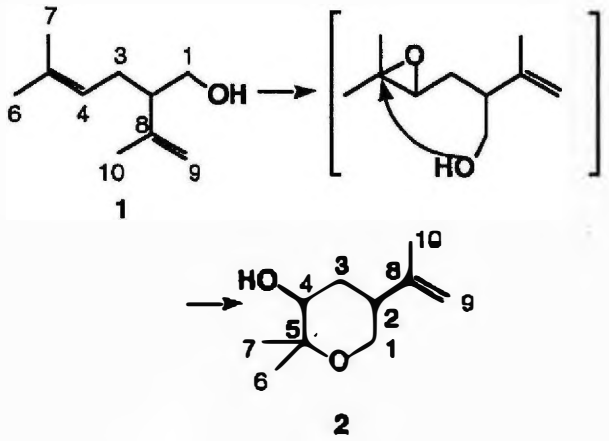

Fig. 4. Possible metabolic pathway of (±)-lavandulol (**1**) to (-)-(2*S*,4*S*)-1,5-epoxy-5-methyl-2-(1-methylethenyl)-4-hexanol (**2**) by *G. cingulata*.

pathways were considered; 1) only (2*S*)-**1** was transformed to **2** enantioselectively or 2) (±)-**2** was produced and then (2*R*,4*R*)-**2** was further transformed to other metabolite enantioselectively.

Similar to our previous study[3-6], the oxidation at the remote double bond is the main metabolic pathway in the biotransformation of **1** by *G. cingulata*. As shown in Fig. 4, **2** was possibly formed by way of the epoxidation at the remote double bond and subsequent attack of the lone pair of the hydroxyl group to C-5 position, although the intermediate epoxide could not be detected.

Work is now in progress to clarify the metabolic pathway and to identified the structures of other metabolites, we now further investigated the microbial transformation of (±)-**1** by *G. cingulata*.

ACKNOWLEDGMENT

This work was supported by the Grant-in-Aid for Scientific Research on General Areas No. 07640793 from the Ministry of Education, Science and Culture, Japan.

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7. Compound **2**; IR ν_{max} cm^{-1}: 3461, 2978, 1646, 1455, 1375, 1153, 1095, 1054, 895, 831. EI-MS m/z (rel. int.): 170(0.5), 155(1), 152(0.4), 142(2), 125(14), 111(4), 97(9), 79(14), 68(100), 59(40), 53(20), 43(40), 41(40).
   Compound **3**; IR ν_{max} cm^{-1}: 2984, 1746, 1646, 1456, 1371, 1237, 1163, 1081, 1040, 895, 839. EI-MS m/z (rel. int.): 212(1), 197(0.5), 184(5), 170(0.7), 152(6), 137(2), 126(12), 111(7), 94(16), 79(16), 68(57), 55(12), 43(100).
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EFFECT OF HEAVY METAL POLLUTED SOILS ON SOME QUALITATIVE AND QUANTITATIVE CHARACTERS OF MINT AND CORNMINT

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INTRODUCTION

Industrial pollution of agricultural soils is among the most serious ecological problems in Bulgaria. Of all chemical elements and compounds which are regarded as environmental pollutants, heavy metals (Cd, Pb, Zn, Cu, Mn, Hg and some others) are the most widely spread out and found in agricultural soils especially around big smelters. According to Jorgensen (1), in a world scale, these metals cause the highest environmental stress, much higher than pesticides for instance.

In Bulgaria, there are some industrial regions where soils contain excessive amounts of these elements (2, 3, 4). Production of edible crops in these regions often results in contaminated produce. According to our early investigation (4, 5), instead of growing edible crops, it is possible to grow some aromatic and medicinal species which final product is not used for human consumption or as forage for animals. Besides, in the new economic conditions in Bulgaria, growing of aromatic crops is economically more sound, these crops are much more attractive for numerous small farmers and landowners. The objective of our study is to explore possibilities for growing of three cultivars from peppermint and cornmint on soils with different concentration of heavy metals and eventual use of *M. piperita* and *M. arvensis* for phytoremediation of metal polluted soils.

EXPERIMENTAL

In 1991-1993 we conducted pot experiments to study the effect of highly heavy metal polluted soils on productivity and quality of *Mentha piperita L.* (cv Tundja and Clone No 1) and *Mentha arvensis var piperascens Malinv.* (cv Mentolna-14) from fam. *Lamiaceae* (*Labiatae*). Soils were taken at distances of 0.5, 3, 6 and 10 km from Non-Ferrous Metals Combine (NFMC) near Plovdiv. Four standard seedlings were planted in every pot which contained similar amount of soil (12 kg). The experiment was set up every year, (three times) i.e. we grew mint plants as one-year crop. Every variant was in four replications. During the vegetative period we kept the necessary soil humidity (80 % of Field Water Capacity). Plants from all variants were harvested in the stage of full blossoming. Essential oil content was measured in fresh herbage by using Clevenger type apparatus. Oil samples, necessary for GC and heavy metal analyses were obtained via steam distillation of the herbage in small containers. For measurement of Cd, Pb, Cu, Mn and Zn in soils, plant parts and in the oils, we used Atomic Absorption Spectrophotometer Perkin-Elmer 5000 with a background correction. Oil samples were analysed on Perkin-Elmer GFAA model 5100. The method for preparing of sample solutions from plants, soils and oils was as described by us earlier (4, 5, 6).

RESULTS AND DISCUSSION

Heavy metal concentration in the four soils was as published by us earlier (6), the concentration of Pb, Cd, Cu and Zn being much higher than their critical concentration in soils. Heavy metal concentration of soil 1 decreased the yield of fresh material and essential oil by 14-17 % within different cultivars and years compared to the yields from the control soil (taken from unpolluted region) (Figure 1). However, essential oil content in the herbage of the three cultivars was not significantly affected by the heavy metal concentration in soils. Yields of herbage and essential oil obtained from plants grown on soil 2 and soil 3 (taken at 3 and 6 km from the smelter), were not significantly decreased compared to the control yields, i.e. the tested cultivars could be grown at this distance from the smelter.

Cadmium concentration in plants strongly depends on its soil concentration (7, 8, 9), (Figure 2). Plants, grown on soil 1 accumulate significantly more of this element than plants grown on the control soil. Excessive amounts of Cd are found in the roots from soil 1, twice as much as the value for critical Cd concentration in plants (the dashed line -8, 9). Cd concentration in the other plant parts from soil 1 is between 2 and 3 mg/kg. Within plants from all variants, most Cd is found in the roots, then in other plant parts, which is due to its specific translocation in plants (3, 4, 5, 8, 16). Plants from the three cultivars contain similar amounts of the element, i.e. there is no cultivar response to Cd, a matter discussed in other publications (4, 10).

As with Cd, lead concentration in plants also depends on its soil concentration. Again, most of the element is found in roots which supports the general understanding for the mechanism of uptake and translocation of this element within plants. No differences between cultivars in respect to Pb accumulation are found.

Copper concentration in plants "copies" to a great extend its soil concentration. There are significant differences between plant parts in respect to Cu accumulation i.e. roots > leaves > flowers > stems. No differences between cultivars are found, supporting our findings from the field experiments, conducted with the same cultivars (4).

Zinc is one of the major pollutants in the area. Measured Zn concentration in plants correlate to Zn concentration in soils. Higher concentrations are accumulated in plant roots and leaves and less in flowers and stems. Most of the other authors also found the highest concentration in plant roots. No differences between three cultivars in respect to Zn content are found, although in other experiments some authors reported such differences (11).

We did not observe any toxicity symptoms on plants which, although the concentration of Cd, Pb, and Zn in soils and plants was much higher than their critical soil and plant concentration, a matter discussed widely in some papers (3, 9, 12, 13, 14).

In all oil samples we did not detect Cd (by using GFAAS), while the concentration of Pb was negligible. The concentration of the other measured heavy metals are much under the maximum acceptable concentration for respective elements in cooking oils (although essential oils can be hardly used as widely as the cooking oils). Surely, heavy metals do not pass over to the essential oils during the process of steam distillation, oils are free of metals.

Gas-chromatographic analyses of the oils show no significant variation in the content of most constituents as menthone, menthylacetate, menthofurane, α-pinene, limonene, cineole and others, due to elevated concentrations of heavy metals in soils and plants. However, there is slight decrease in menthol content in the oils obtained from plants, grown on soil 1. This is in some respects in conformity with the results, reported by Misra (15) and with our results from the field experiments. Nevertheless, the quality of the oils remained within the Bulgarian State Standard for the oils from respective cultivars.

CONCLUSIONS

Plants, grown on soil 1 (taken at 500 m from the smelter), accumulated excessive amounts of Cd, Pb, Zn and Cu, and the yield of essential oil was up to 17 % lower than from the control soil, without visible toxicity symptoms. High heavy metal concentration in the plants grown on soil 1 did not affect the essential oil content, but slightly decreased menthol content in the oils. Oil quality as a whole remain within typical one for the respective cultivars. Despite high heavy metal content in the plants, (above the critical levels for Cd, Pb, Cu and Zn in the plants), oils as final product were not contaminated.

Although heavy metal concentration in soil 2 and soil 3 (taken at distances of 3 and 6 km from the smelter), was relatively high, yields and quality of essential oil were not significantly affected.

Bulgarian cultivars of *M. piperita* (Tundja and Clone 1) and *M. arvensis* (Mentolna 14) can be successfully grown on heavy metal polluted soils and may be used for their phytoremediation.

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Figure 1. Yield of herbage (g/pot) and oil yields (% to the control) from the three cultivars, as depending on the heavy metal concentration of soils

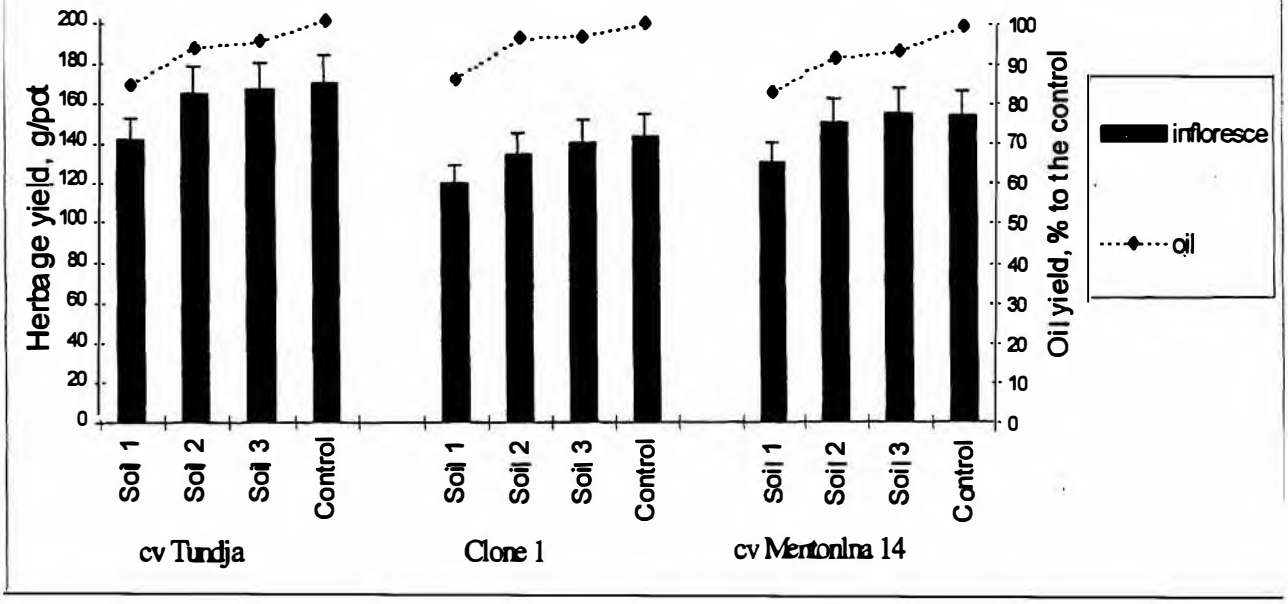


Figure 2. Cd concentration in plant parts, mg/kg DW

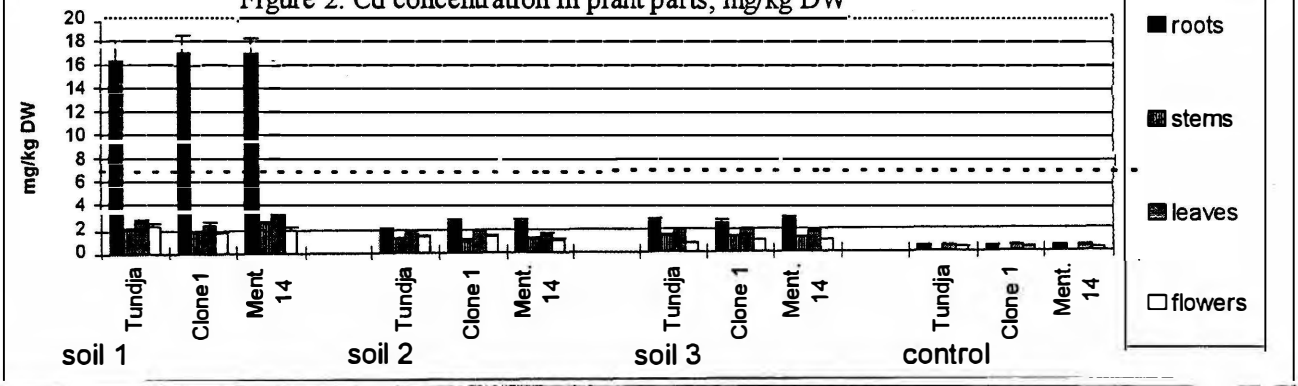


Figure 3. Pb concentration in plant parts, mg/kg DW

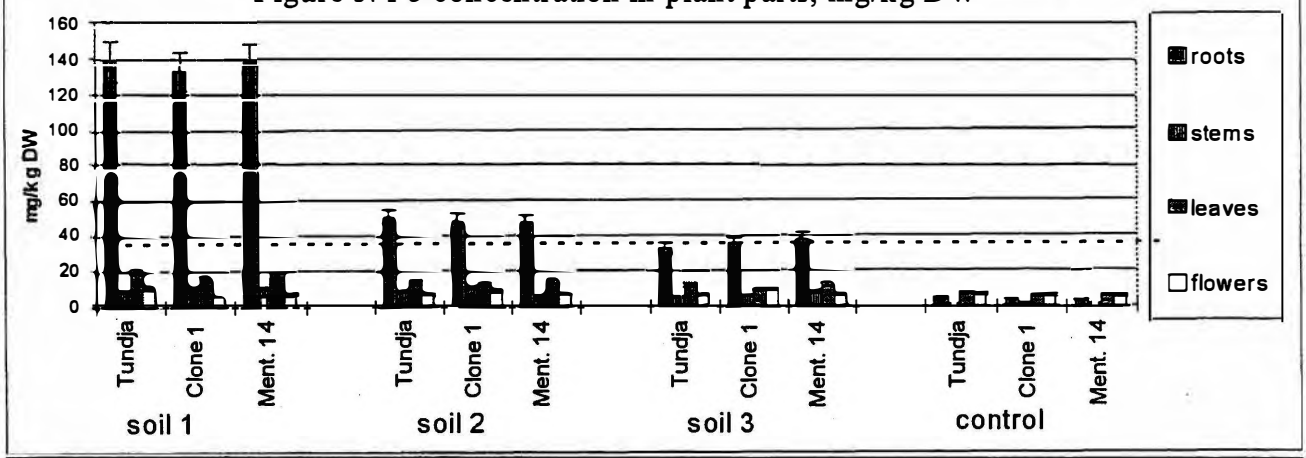


Figure 4. Cu concentration in plant parts, mg/kg DW

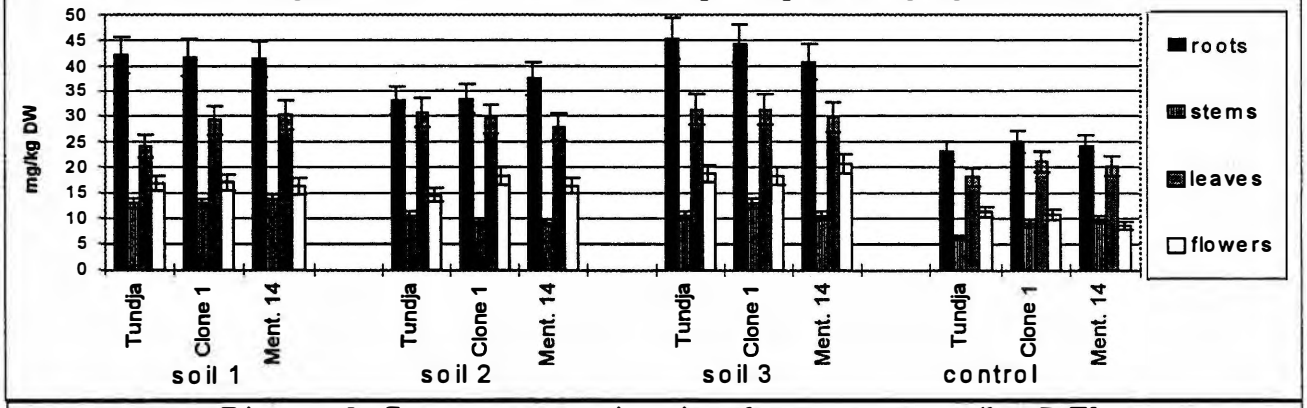
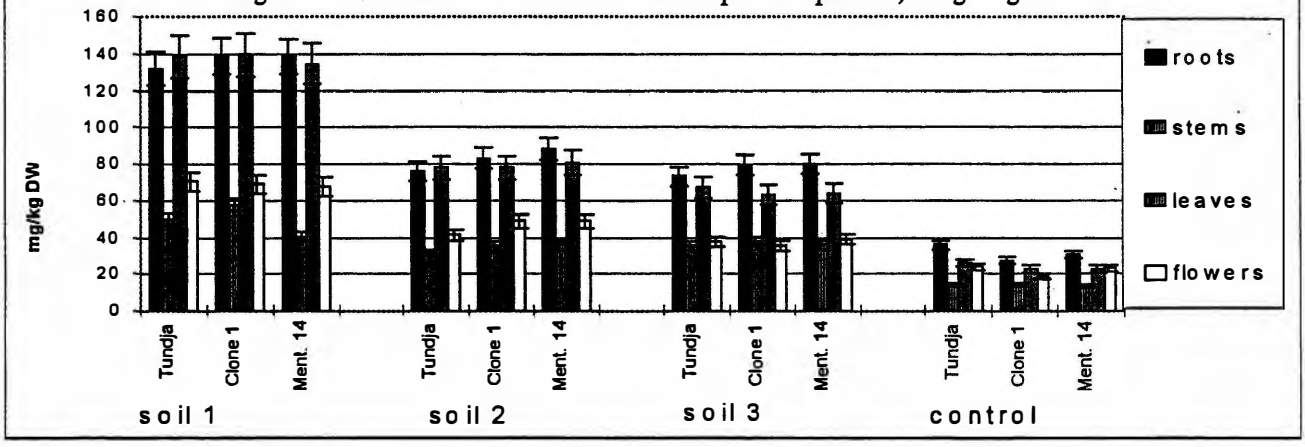


Figure 5. Zn concentration in plant parts, mg/kg DW



Analytics - Technical Aspects

Fragrance creations analysis using semiautomatic spectra (MS,IR) interpretation and olfactoric data

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Perfume creation involves a complex system of components exhibiting distinguished olfactory properties and the resulting scent impression is a priori hard to predict. The components used in turn, may be either low price synthetic products (e.g. monoterpenic compounds such as limonene or linalool) or consist of high value complex natural systems (e.g. essential oils). In this respect, the aim of this study was not only to present a short survey of basic underlying principles involved in instrumental and olfactory analysis of fragrance systems - along with the basic computer supported semiautomatic interpretation, but also to show by selected examples possible prediction or correlation of resulting perfume attributes on the basis of instrumental analysis data alone.

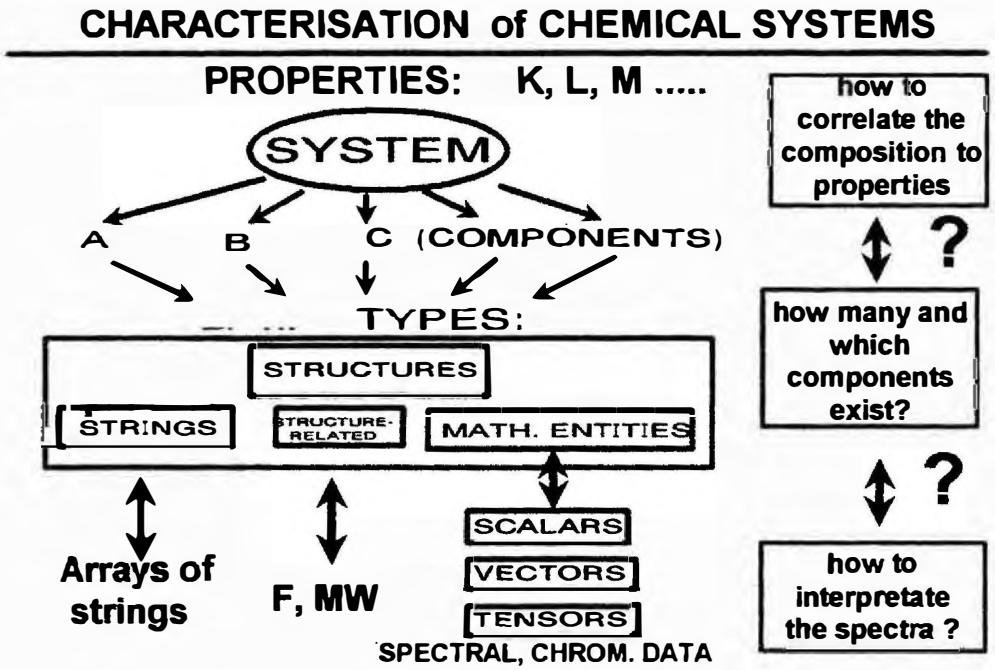


Fig. 1: Data types most frequently involved in characterization of chemical systems.

Fig.1 displays the three data types involved most in the characterization of chemical systems. This includes string, structural and mathematical data, the latter stemming from instrumental analyses (numbers, spectra or spectra arrays) together with the problems encountered most often in chemical analysis. In the analysis of fragrance mixtures, therefore, the question of how many and which components constitute the sample, as well as the problem of data interpretation are secondary to the main question, as to which components contribute to the olfactory properties of the system. In solutions of practical problems the profiling and sample comparison based on complex full GC-MS data sets carried out via semiautomatic procedures has become increasingly important. Nevertheless, the first part of the flavor chemist's work must be the interpretation of spectral data, mostly GC-MS, provided by instrumental analysis. Some examples of the tools stemming from PC desktop applications which may prove helpful

in performing this task are given in fig.2. They comprise either knowledge based programmes with expert rules, such as SpecTools of Chemical concepts Weinheim, Analytical Expert from

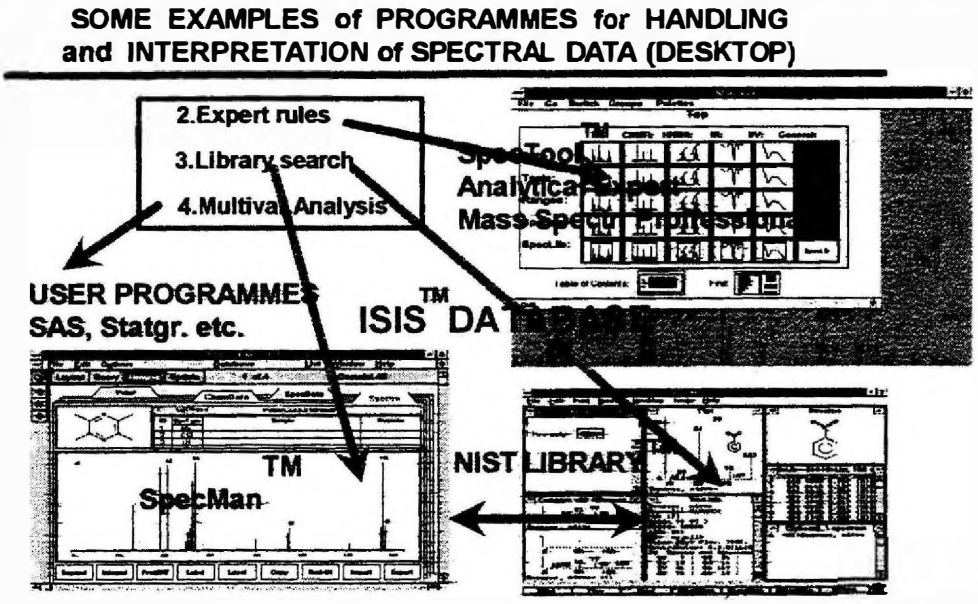


Fig.2: Examples of tools for spectral data-handling.

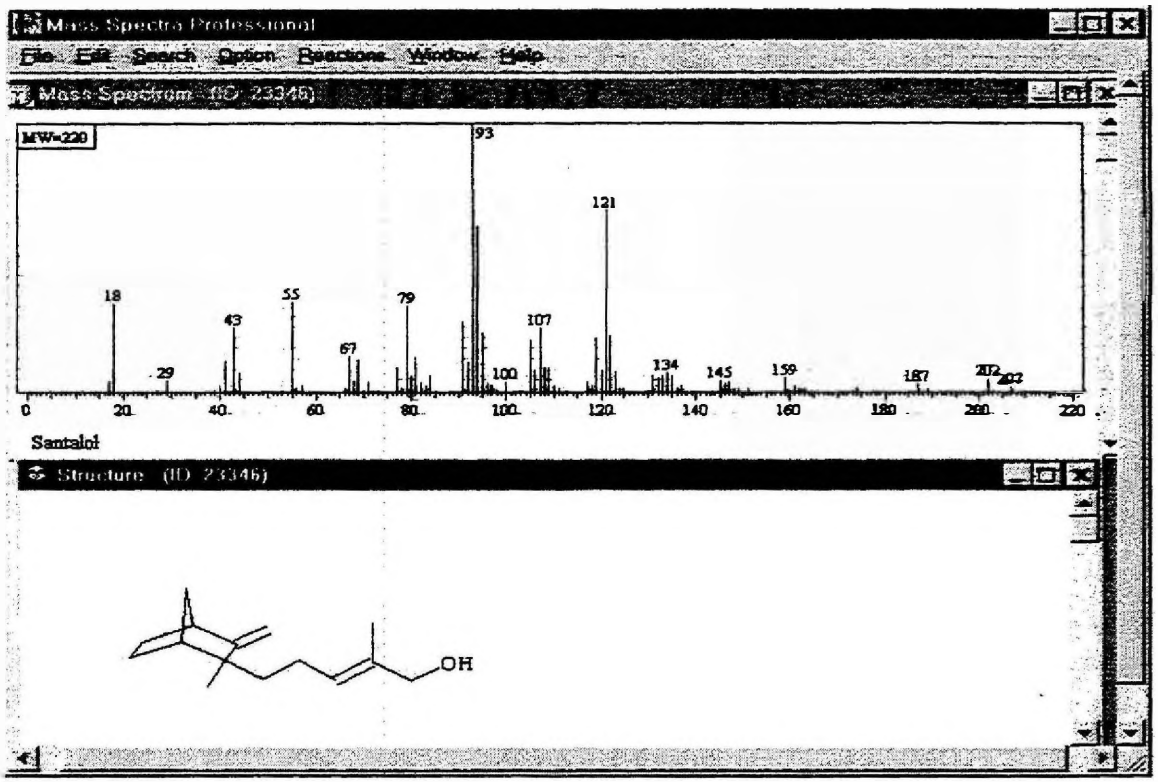


Fig.3 Applications of Mass Spectra Professional to the structure and MS of Santalol (for red marked signals fragmentation reaction-proposals are made)

Shimadzu or Mass spectra Professional created by R.Mistrik from NIST, or spectral libraries -

preferably including substructure search options or tools from the multivariate-statistical data-evaluation kit. Application examples of such tools are given in fig. 3 and 4. Thus, fig. 3 shows the spectrum of ß-santalol (MW 220), red lines marking the ions for which fragmentation pathways based on standard fragmentation reactions like McLafferty rearrangement, multiple H-migration or alfa-cleavage are proposed. Quality control also make good use of these tools (see the missing explanation for the M-13 ion at m/z 207 in fig.3 !). The underlying structure is supplied as an .MOL file. Fig. 4 shows another example, where the resulting benchmarks achieved for substructure-based mass spectra query using a combination of ISIS BASE (MDL Information Systems) and ISIS BASE, NIST structure files provided by Chemical Concepts are shown. The following shows an application of this to two complex examples for the corre-

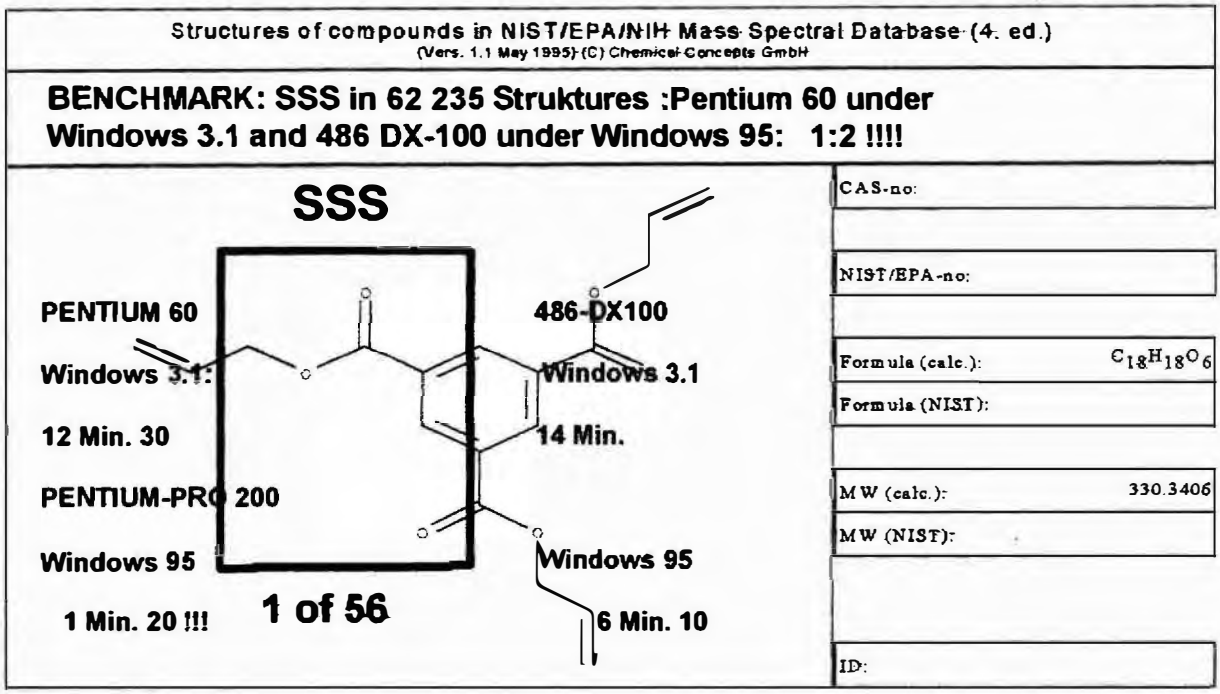


Fig.4 Substructure search for meta-phthalic acid derivatives with benchmarks values.

lation of olfactoric systems.

In the first example, taking samples of perfumes - no interpretation of recorded GC-MS data was undertaken at all. The composition of investigated samples remained essentially unknown and the projections of full GC-MS data sets for the samples, each represented by a point, were compared, followed by correlation with olfactory data. In the second example, the procedures discussed were used in order to elucidate the components in the particular samples investigated and used in turn to generate concentration profiles of the 350 compounds found. These were used for sample comparison and the ensuing correlation of olfactoric attributes.

The basic principle of general correlation procedure used in both examples is given in fig. 5. This shows that an instrumental data set and a set of attributes is allocated to every particular sample. Data for similar samples are expected to exhibit similarities when data and attributes for a given set are projected on separate planes i.e. the data of similar samples should show clustering of instrumental data. The same applies for the projection of attributes on a plane. Again, if similar samples display similar sets of attributes, a clustering of these samples' attributes for samples should be visible.

The point is that if the instrumental data somehow also represent instrumentally undetectable properties of samples - such as olfactory attributes - then a coincidence of clustering of both, the data and the attributes should be detectable.

In the first example the multivariate correlation of full extend GC-MS data sets of 30 commercial perfume samples (of 3rd order data set) was carried out (every full GC-MS data set was projected as point in a plane). The resulting clusters were correlated with the clustering of the corresponding olfactory profiles (established on the basis of 30 attributes and PCA) given in fig.6. From 30 samples 5 samples according to olfactory attributes can not be assigned, 17 samples can be considered as floral, citrus like or fresh and 8 samples appear to be balsamic, oriental and musty. The instrumental GC-MS ER data without any further interpretation indicate that from the group of floral citrus like and fresh attributes the prediction of 12-13 (clusters red or green) on basis of instrumental data can be done and therefore at least principally the prediction of olfactory attributes considered is possible. This seems to be also indicated by results of second example where more exact and extensive work was done.

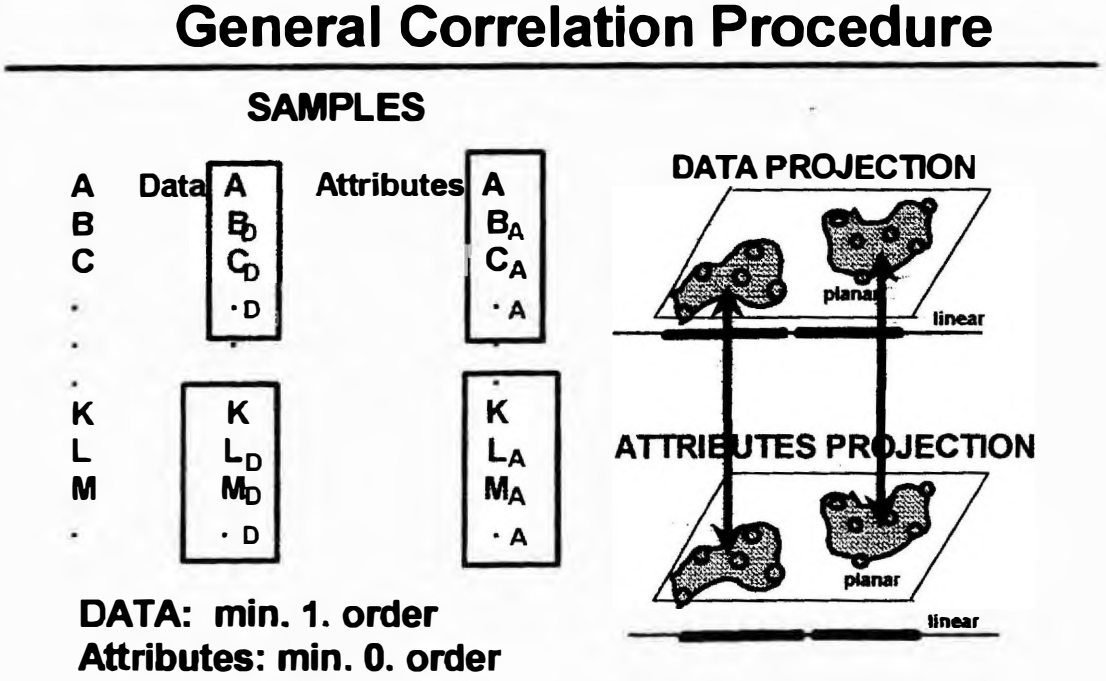
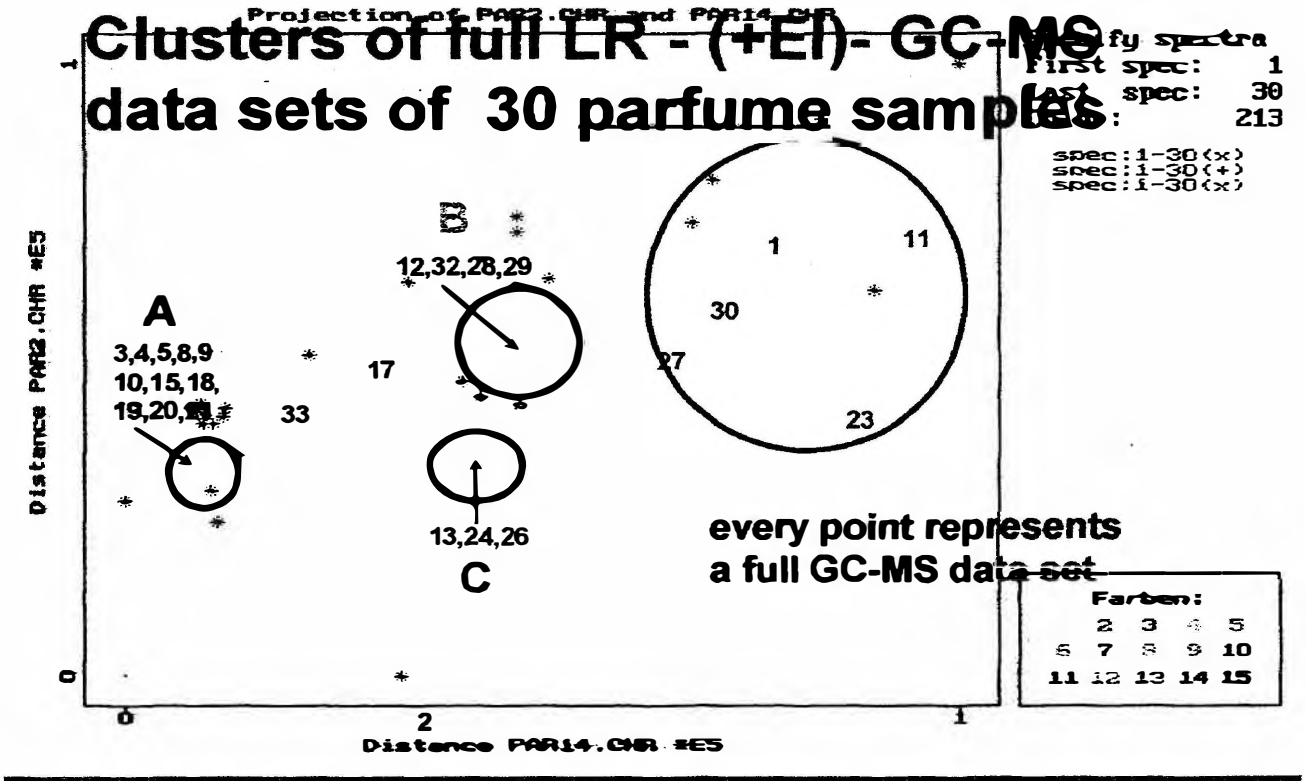


Fig. 5. Basic principle for coincidence in clustering of instrumental and of attribute data.

In second example the instrumental data of 25 different systems which were classified by professional perfumers with simple attributes and where the GC-sniffing did not indicate the overwhelming odor impression were first interpreted. This resulted in a table of totally 350 different compounds. For each particular sample a concentration profile was established and the principle component analysis of the set of this concentration profiles was carried out. The projection of each of such concentration was correlated with dominating odor impression as seen in fig.7. In this case the results are much more pronounced.

Acknowledgement: The authors acknowledge the assistence of chief perfumers Mr.Höpner and Mr.Haussmann from Dragoco Comp. Vienna in this work.



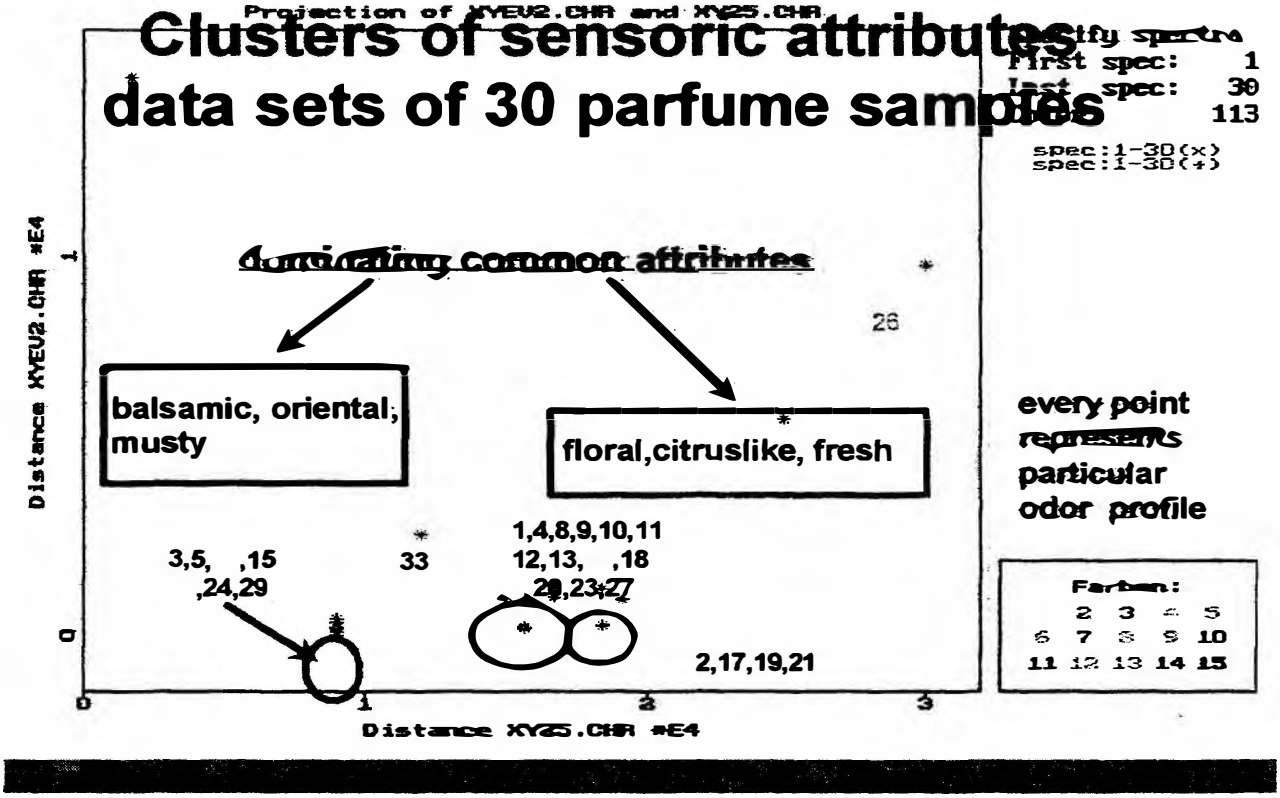


Fig.6. Clusters of 30 perfume samples according full GC-MS data (upper part) and according profiles containing 30 olfactory attributes (lower part)

ANALYSED ODOR SYSTEMS

Flowers: apple, grape, Exacum affine,
Mushrooms: Boletus edulis, Cantharellus cibarius, Amillaria mellea, Lepiota procera, Agaricus campestris and Agaricus bisporus
Plant parts: diff. parts of Aesculus hippocastanum, leaves of Plecthranthus coleoides, sunflower stems, Juglans nigra peels, Chenopodium botrys plant, SCF-extract of shellac, needles and twigs of Douglas Fir
Other systems: old books, diff. fragrance candles

MULTIVARIATE EVALUATION of 25 ODOR SYSTEMS

(Projection of Terpenes, Ketones, Lactones, Esters)
DATA MATRIX 350 x 25 (Components x Odor systems)

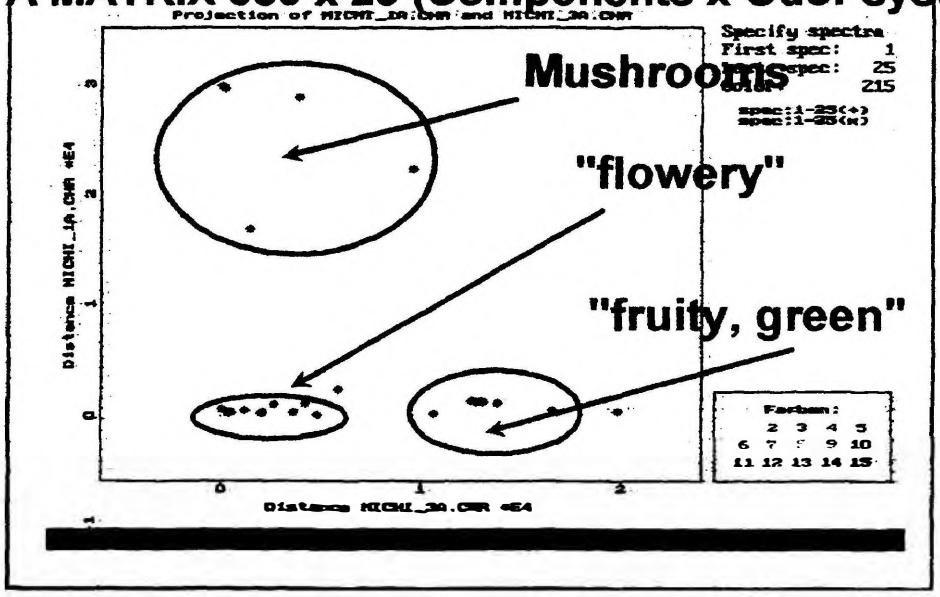


Fig.7: Projection of concentration profiles on the basis of 350 possible components for 25 system classifiable in olfactoric terms as „flowery“,“fruity“,“green“ and „mushroom like“

NEW APPROACHES IN ESSENTIAL OIL ANALYSIS USING POLYMER-COATED SILICA FIBERS

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The investigation of essential oils from plant material or from plant preparations typically requires several distinct steps, each of which can be critical for obtaining accurate and reproducible results. Sample preparation to isolate the components of interest from the raw material usually is achieved by hydro-distillation, by solvent extraction and by adsorption techniques. Especially sample preparation based on adsorption has been extensively used to separate analytes from complex matrices and to concentrate trace components.

Recently, a new adsorption technique has been developed by Pawliszyn and co-workers (1), called solid-phase-microextraction (SPME) using polymer-coated fused silica fibers, which has several advantages in comparison with conventional techniques. Sample preparation is based on the adsorption of analytes from a sample onto a coated fused silica fiber which is mounted in a modified GC syringe. After introducing the coated fiber into a liquid or gaseous sample the compounds to be analyzed are enriched according to their distribution coefficients and can be subsequently thermally desorbed from the coating after introducing the fiber into the hot injector of a gaschromatograph.

The commercially available SPME device (2) consists of a 1 cm length of fused silica fiber of ca. 100 µm diameter coated on the outer surface with a stationary phase fixed to a stainless steel plunger and a holder that looks like a modified microliter syringe (Figure 1). The fiber can be drawn into the syringe needle to prevent damage. To use the device the needle is pierced through the septum that seals the sample vial. Then, the plunger is depressed lowering the coated fiber into the liquid sample or the headspace above the sample. After sample adsorption, which takes some minutes, the fiber has to be drawn back into the needle and withdrawn from the sample vial. By the same procedure the fiber can be introduced into the gas chromatograph injector where the adsorbed substances are thermally desorbed and flushed by the carrier gas into the capillary GC column.

The selectivity and capacity of the fiber coating can be adjusted by changing the phase type or thickness of the coating on the fiber according to the properties of the compounds to be analyzed. Commercially available are at present coatings of 7, 30 and 100 µm of polydimethylsiloxane, a 85 µm polyacrylate and a 65 µm Carbowax®/divinylbenzene coating for polar components. We have only used the polydimethylsiloxane coatings of 7 and 100 µm.

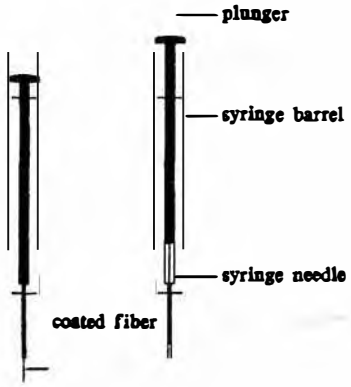

Figure 1. SPME-device (schematically)

Investigation of a tea infusion

The first type of application we have investigated concerns the investigation of aqueous solutions of essential oils as for example tea infusions from medicinal aromatic plant drugs. Analyses dealing with the composition of tea infusions are found relatively seldom in literature. Therefore, we wish to report in the following on the composition of tea infusions of medicinal plant drugs containing essential oil. For our experiments we have selected a commercial tea-bag preparation of fennel fruits. As a further example the investigation of peppermint-tea is given in a seperate article (3).

The fennel tea infusion was prepared by pouring 150 ml boiling water over one tea bag containing 1 g crushed plant material and leaving to stand 5 minutes. During this time the tea bag was moved 10 times up and down.

After the tea infusion reached room temperature, the length of a 100 µm polydimethylsiloxane coated fiber was immersed into the aqueous solution (Figure 2) where the dissolved oil components partition between the polymer coating and the water. Previous investigations have shown, that extraction time can be shortened substantially by agitation of the solution. We have, therefore, applied vigorous agitation during the extraction, using a magnetic stirrer. Thus, the extraction length could be limited to 5 minutes. After this time, the fiber containing the adsorbed compounds was removed from the tea

infusion and transferred to the heated (220°C) injector of a gas chromatograph for 1 minute desorption and successive analysis.

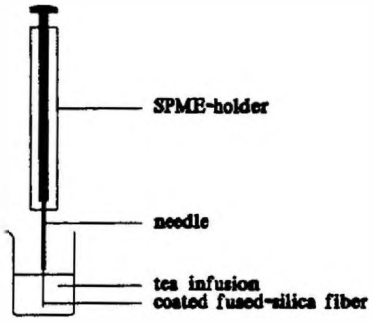

Figure 2. SPME-extraction procedure from a tea infusion

The obtained gas chromatogram on a DB-5 column is shown in Figure 3 exhibiting well separated peaks.

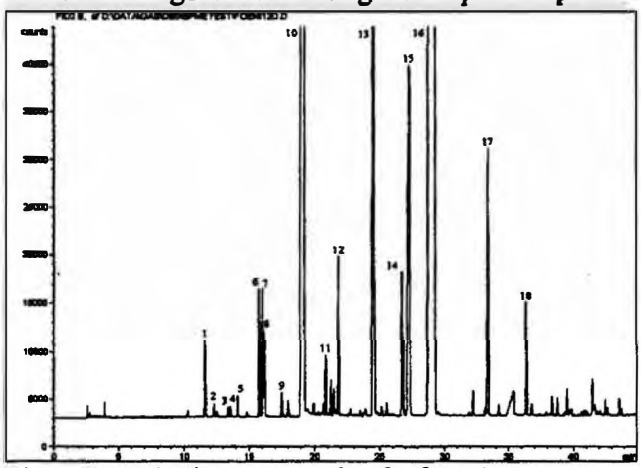

Figure 3. Volatile compounds of a fennel-tea infusion extracted by SPME

Main constituents are trans-anethole (Peak No. 16) and Fenchone (No. 10) and lower amounts of monoterpene hydrocarbons, anisaldehyde and some other oxidation products from anethole.

The percentages of the individual components are listed in Table 1 and compared with the respective essential oil obtained by hydrodistillation. As can be taken from this table, the percentual proportions of both analyses are in an unexpected good agreement. Only the proportions of the monoterpene hydrocarbons are significantly lowered in the tea infusion, due to their lower solubility in the aqueous phase. This effect can also be observed in an aromatic water preparation from fennel oil. However, in this oil-saturated aqueous preparation the anethole/fenchone ratio was considerably changed. Solid phase extraction (SPE) of the tea infusion finally yielded proportions of the individual components which exhibited higher differences to the oil composition.

Table 1. SPME of a fennel tea infusion

No.	Compound	SPME	SPE	Oil	Arom. water
1	α-Pinene	0.30	trace	2.99	0.46
2	Camphene	0.05	-	0.31	0.07
3	Sabinene	0.04	-	0.14	0.04
4	β-Pinene	0.04	-	0.22	0.05
5	Myrcene	0.08	-	0.38	0.10
6	p-Cymene	0.49	0.07	0.76	0.34
7	Limonene/β-Phellandrene	0.51	trace	1.71	0.54
8	1,8-Cineole	0.33	0.42	0.33	0.89
9	γ-Terpinene	0.09	-	0.25	0.08
10	Fenchone	28.99	38.76	26.36	61.47
11	Campholenealdehyde	0.22	0.24	0.26	0.33
12	Camphor	0.61	1.14	0.76	1.45
13	Estragole	5.61	2.67	3.68	2.80
14	Carvone	0.90	1.58	0.08	0.26
15	Anisaldehyde/cis-Anethole	3.56	11.85	3.68	2.84
16	trans-Anethole	53.90	30.35	55.15	25.39
17	p-Methoxy phenylacetone	1.25	3.77	0.22	0.10
18	p-Methoxy propiophenone	0.46	1.08	0.37	0.44

Headspace analysis

The next series of investigations using coated fibers and SPME, respectively, refer to head space analysis. The used arrangement is given in Figure 4. As an example we have analyzed the head space of a 250 mg sample of crushed fennel fruits, which were placed in a 10 ml crimp-cap glass vial with a teflon-lined rubber septum. After piercing the septum with the needle of the SPME-device, the 100 µm polydimethylsiloxane coated fiber we used was exposed to the vapour over the sample, the head-space, for 1 minute. This time has been shown to be sufficient, since the diffusion coefficients controlling the process of enrichment on the fiber coating are about 4 orders of magnitude higher than those of a liquid phase.

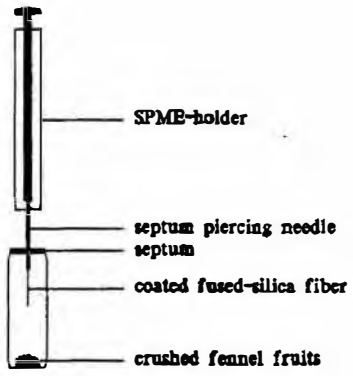

Figure 4. Arrangement for head-space sampling by SPME

After withdrawing the SPME-device from the sample vial and injection into the heated gc split-

injector - the split ratio was adjusted to 1:20 - the following gas chromatogram was obtained (Figure 5). As a result of the applied inlet liner of 0.75 mm i.D. with a small volume, which is recommended by the SPME-device manufacturer, an excellent peak shape without any peak-broadening is obtained. Comparison with the gas chromatogram of the respective hydrodistillate indicated, that all constituents of the fennel oil up to the higher boiling oxidation products of anethole could be found.

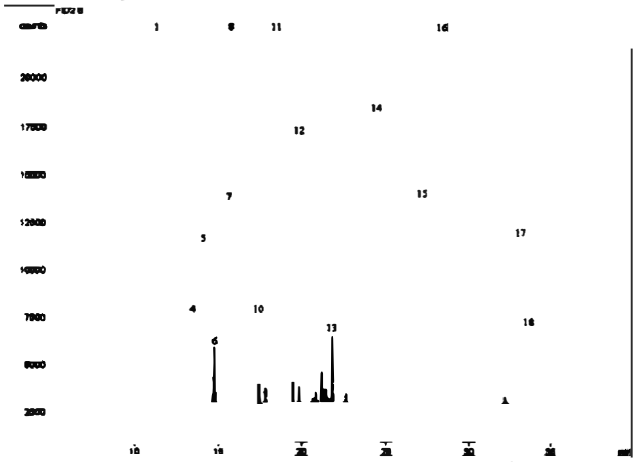

Figure 5. Head-space volatiles of fennel fruits extracted by SPME

However, the percentual proportions of the individual components differ significantly from the essential oil composition as can be taken from Table 2 showing the composition of the head space analysis versus the results obtained from the oil.

Table 2. Head space analysis of *Foeniculum vulgare* fruits

No.	Compound	Headspace	Oil
1	α-Pinene	11.69	2.99
2	Camphene	1.31	0.31
3	Sabinene	0.53	0.14
4	β-Pinene	0.82	0.22
5	Myrcene	1.39	0.38
6	α-Phellandrene	0.53	0.15
7	p-Cymene	1.83	0.76
8	Limonene / β-Phellandrene	5.82	1.71
9	1,8-Cineole	1.10	0.33
10	γ-Terpinene	0.82	0.25
11	Fenchone	44.35	26.36
12	n-Nonanal (?)	2.49	0.04
13	Camphor	0.69	0.76
14	Estragole	2.91	3.68
15	Anise aldehyde / cis-Anethole	2.16	3.68
16	trans-Anethole	17.43	55.15
17	Anethole epoxide (?)	1.73	0.05
18	p-Methoxy phenylacetone	0.81	0.22

This is easy to understand since the concentration of a compound in the head space depends on its vapour pressure at a given temperature. Therefore, the relatively low boiling monoterpene hydrocarbons are enriched at the expense of the higher boiling constituents, such as anethole. Consequently, head space analysis using a SPME-device with an unpolar coated fiber is well suited for qualitative analysis of aromatic plants but it has to be taken into account, that the quantitative proportions may differ significantly from the respective oil composition.

Thermo-extraction

In order to overcome those limitation we took advantage of thermo-extraction. The optimal arrangement would be a kind of micro-hydro-distillation combined with recovering the vapour on the coating of a fused silica fiber. We tested an arrangement consisting of an oven block with a drilled hole into which a special glass cartridge can be inserted (Figure 6).

Figure 6. Device for thermo-extraction of volatile compounds from crude drugs
a = septum; b = glass cartridge; c = sample; d = heating block; e = coated fused-silica fiber; f = septum piercing needle; g = SPME-holder

After heating the oven block (d) to 200°C, the glass cartridge (b) was introduced, containing the sample (c), in our investigation 16 mg fennel fruits, cutted in halves, and 50 mg silica gel with a water content of 20 %. In advance the cartridge was sealed at one end by a silcon rubber septum (a) and in order to reduce oxidation of labile oil constituents a continuous flow of nitrogen was admitted.

During heating of the sample the water of the silica gel and the sample is vaporized, improving the release and transport of the essential oil to the capillary end of the cartridge into which the coated fiber (e) of the SPME-device has been inserted. The process can be regarded as a rapid micro steam distillation. After 1 minute of heating and adsorption of the volatiles at the exposed fiber, coated with a 100 µm layer of polydimethylsiloxane, the fiber was drawn back into the piercing needle and injected into a split injector of a gas chromatograph. In the obtained gas chromatogram (Figure 7) a high amount of trans-anethole (Peak No. 16) can be observed, which is characteristic for the respective essential oil.

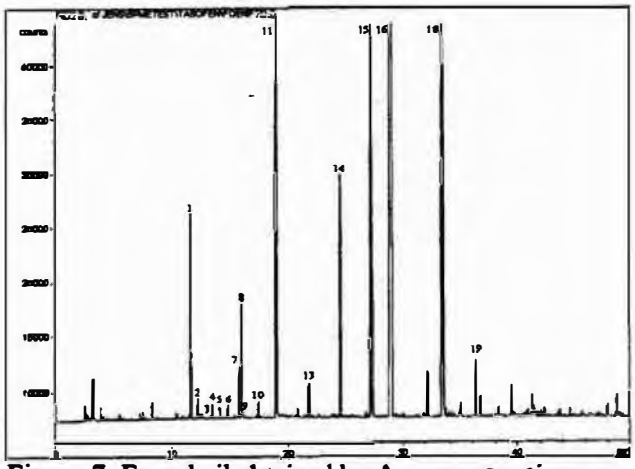

Figure 7. Fennel oil obtained by thermo-extraction and SPME sampling

Comparison of the quantitative results (Table 3) obtained from fennel fruits by thermo-extraction and conventional hydrodistillation indicates a considerable better correlation of the individual percentages than with data obtained by headspace analysis. The significant higher amounts of oxidation and rearrangement products from anethole in the thermo-extract, e.g. anisaldehyde, cis-anethole, and p-methoxy phenylacetone, the so-called "anisketone", are results of the applied high temperature. They can be minimized by choosing a lower temperature for thermo-extraction of the plant sample with the need of a longer extraction time.

Under those conditions the analytical results obtained after thermo-extraction are a fairly good approach to data obtained by conventional hydrodistillation if the possibility of thermally induced rearrangements is taken into account.

Table 3. Thermo-extraction of *Foeniculum vulgare* fruits

No. Compound	Th.extr.	Headspace	Oil
1 α-Pinene	1.90	11.69	2.99
2 Camphene	0.21	1.31	0.31
3 Sabinene	0.05	0.53	0.14
4 β-Pinene	0.14	0.82	0.22
5 Myrcene	0.10	1.39	0.38
6 α-Phellandrene	0.15	0.53	0.15
7 p-Cymene	0.50	1.83	0.76
8 Limonene / β-Phellandrene	1.10	5.82	1.71
9 1,8-Cineole	0.05	1.10	0.33
10 γ-Terpinene	0.15	0.82	0.25
11 Fenchone	16.38	44.35	26.36
12 n-Nonanal (?)	-	2.49	0.04
13 Camphor	0.36	0.69	0.76
14 Estragole	2.56	2.91	3.68
15 Anise aldehyde / cis-Anethole	7.82	2.16	3.68
16 trans-Anethole	49.62	17.43	55.15
17 Anethole epoxide (?)	-	1.73	0.05
18 p-Methoxy phenylacetone	11.06	0.81	0.22
19 p-Methoxy propiophenone	0.65	0.03	0.37

Picking oil glands

In order to avoid thermally induced and hydrolytic processes etc. in course of the separation of an essential oil from plant material, extraction using different solvents has been recommended. However, the main disadvantage of solvent extraction is the presence of non-volatile lipophilic compounds such as fatty oil, waxy material, plant pigments etc. in the extract, which may cause difficulties during subsequent gc investigation of the sample. As an alternative the picking of oil glands using a coated fused silica fiber and successive introduction of the adsorbed oil into a gas chromatograph seemed to be feasible. This technique should provide valuable information on the genuine composition of an essential oil. As a further advantage the possibility to investigate the oil composition of different areas of a single plant organ can be quoted. Finally, the solvent-free sampling and transfer into an analytical device seemed to be favourable.

For our experiments at first the fruit of the rue *Ruta graveolens* was chosen. The small green fruits, which have a diameter of approximately 8 mm, show under the surface numerous oil cavities as brownish-green dots. The essential oil was obtained by piercing the coated fiber of the SPME-device into an oil cavity. In this experiment we used a fiber with 7 µm coating of chemical bonded polydimethylsiloxane to avoid disintegration of the coating and overloading of the gc capillary after introduction into the gas chromatographic split-injector (split ratio 1:20). The obtained gas chromatogram is given in Figure 8, showing several strong peaks, which represent the homologue 2-alkanones with chain lengths from 9 to 13 carbon atoms. The main constituent is 2-undecanone (Peak No. 5).

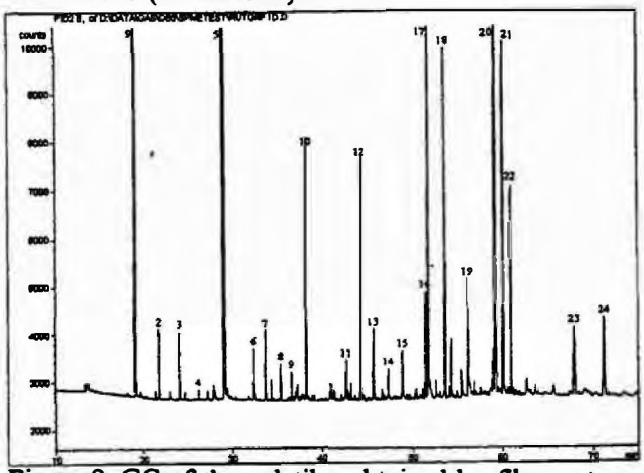

Figure 8. GC of the volatiles obtained by fiber extraction from an oil cavity of the green fruit of *Ruta graveolens*

The more detailed analytical results are given in Table 4 and compared with results obtained from an extract and a respective hydrodistillate.

Remarkable is the fairly good correlation of the percentual portions in all the three preparations. A further aspect to be mentioned is the occurrence of furano-coumarins such as psoralen, xanthotoxin and bergapten in the fiber- and solvent extract and their lacking in the hydrodistillate. The occurrence of the coumarins in the fiber extract confirms the suggestion, that these substances are located in the oil glands, an information which cannot be obtained by an other technique.

Table 4. Fiber extract from an oil gland of *Ruta graveolens* (green fruit)

No.	Compound	Fiber	Extr.	Dist.
1	Nonan-2-one	6.17	8.88	9.62
2	Geijerene	0.41	trace	0.11
3	Decan-2-one	0.39	0.62	0.83
4	Non-2-yl acetate	0.06	0.07	0.11
5	Undecan-2-one	62.33	63.79	82.53
6	10-Methylundecan-2-one	0.32	0.45	0.52
7	Dodecan-2-one	0.44	0.58	0.74
8	Undec-2-yl acetate	0.23	0.21	0.27
9	11-Methyldodecan-2-one	0.16	0.18	0.24
10	Tridecan-2-one	1.54	1.72	2.13
11	KI = 1602	0.28	0.42	-
12	KI = 1642	1.62	-	-
13	KI = 1678	0.61	-	0.22
14	KI = 1720	0.22	-	-
15	KI = 1761	0.48	0.08	-
16	Moskachan B	0.72	0.64	0.13
17	Psoralen	4.18	4.82	-
18	KI = 1888	2.31	-	-
19	KI = 1962	0.85	0.42	0.26
20	Xanthotoxin	6.16	4.66	-
21	Bergapten	3.01	3.18	-
22	KI = 2097	1.62	-	-
23	KI = 2244	0.77	0.69	-
24	KI = 2303	1.12	-	-

Similar investigations have been performed with lime fruits yielding comparable results regarding the similarity of the oil composition from the peel, if the oils were obtained by fiber extraction and cold pressing, respectively.

Figure 9 shows the gc of fiber-extracted oil from an oil cavity of the lime peel. Main constituent of this oil, which consists of more than 90 % hydrocarbons, is limonene, but the sensorial more important constituents are oxygenated monoterpenoids as for example neral, geranial as well as neryl and geranyl acetate. The quantitative results are given in Table 5, which are contrasted to analytical results from the cold pressed oil, a commercial product used in flavor industry. Comparison of the percentual proportions shows that the data obtained by the new fiber-picking method are in good agreement with the commercial product.

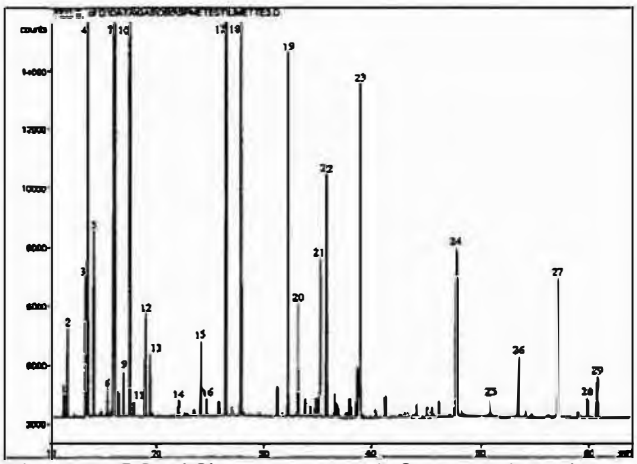

Figure 9. GC of fiber-extracted oil from an oil cavity of the peel of lime *Citrus aurantifolia*

Table 5. Composition of a fiber-extracted lime oil versus a commercial cold pressed oil

No.	Compound	Fiber	c.p.
1	α-Thujene	0.13	0.43
2	α-Pinene	0.52	1.59
3	Sabinene	0.91	1,54
4	β-Pinene	4.74	8.66
5	Myrcene	1.19	1.43
6	α-Terpinene	0.20	0.28
7	Limonene	52.17	55.74
8	cis-β-Ocimene	0.16	0.07
9	trans-β-Ocimene	0.30	0.14
10	γ-Terpinene	12.33	11.99
11	n-Octanol	0.10	0.09
12	Terpinolene	0.69	0.58
13	Linalool	0.43	0.34
14	Citronellal	0.12	0.06
15	α-Terpineol	0.56	0.39
16	n-Decanal	0.12	0.07
17	Neral	3.29	1.42
18	Geranial	5.16	2.33
19	Neryl acetate	2.81	1.12
20	Geranyl acetate	0.83	0.32
21	β-Caryophyllene	1.30	0.60
22	trans-α-Bergamotene	2.01	1.01
23	β-Bisabolene	2.72	1.46
24	KI = 1731	1.53	0.64
25	KI = 1816	0.15	0.12
26	KI = 1885	0.49	-
27	Limettin	1.25	0.67
28	Bergaptene	0.20	0.27
29	KI = 2093	0.38	-

Heracleum

In order to demonstrate the ability to investigate the individual oil glands of a single plant organ with the aid of a coated fused silica fiber, investigations of fruits from the umbellifer *Heracleum mantegazzianum*, the Giant Hogweed have been per-

formed. The more than 2 meters reaching plants native of S.W. Asia are expanding their growing area in Northern Europe rapidly and become more and more to be pest. The flat fruits, consisting of 2 parts (mericarps), have a length of up to 12 mm. During ripening 4 oblong oil ducts are visible on the dorsal side (Figure 10a) and 2 of them on the commissural side (Figure 10b), that means the inner surface. The cross-section of the fruit (Figure 10c) shows the eliptical oil ducts very clear, which have a diameter of ca. 200 x 900 µm, thus making the introduction of a coated fiber feasible.

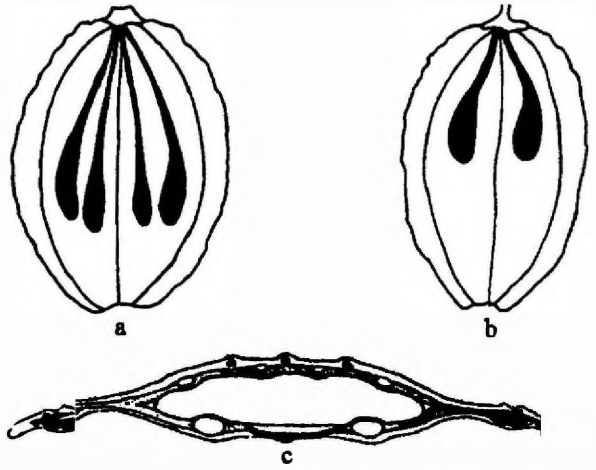

Figure 10. *Heracleum mantegazzianum* fruit
a = dorsal view of the mericarp with 4 oil ducts; b = commissural view of the mericarp with 2 oil ducts; c = cross-section of the mericarp

We took therefore samples from both sides of the half-fruit by dipping a 7 µm polydimethylsiloxane coated fiber into an oil duct and analyzed the respective oils. The gas chromatogram of the dorsal oil duct is represented in Figure 11, showing well resolved peaks of numerous aliphatic esters which are characteristic for this oil. In addition, high amounts of furano-coumarins can be recognized at higher retention times which cause severe inflammation after contact with the skin.

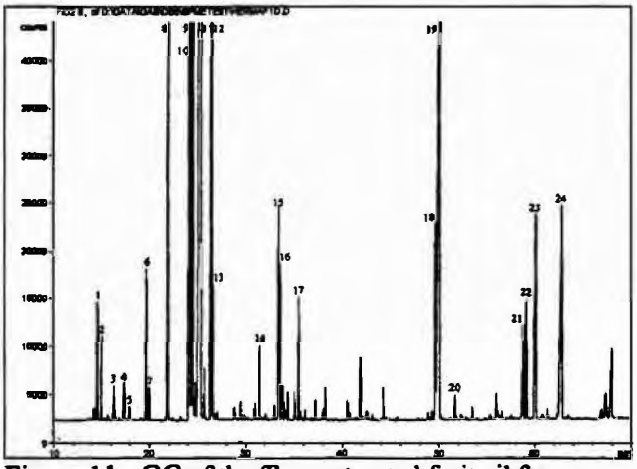

Figure 11. GC of the fiber-extracted fruit oil from a dorsal oil duct of *Heracleum mantegazzianum*

Comparison of the analytical results (Table 6) exhibits a similar composition of both oils from the dorsal and the commissural ducts. In addition, the fluorescence microscopical observation, that the furano-coumarins of Heracleum species are located within the oil ducts can be confirmed by our investigation.

Table 6. Composition of fiber-extracted fruit oils from *Heracleum mantegazzianum*

No.	Compound	Fiber dorsal	Fiber commiss.	Dist. oil
1	Octanal	0.55	0.82	2.46
2	Hexylacetate	0.40	0.13	0.94
3	n-Butyl 2-methylbutanoate	0.17	0.18	0.80
4	KI = 1060	0.19	0.13	0.23
5	Octanol	0.08	trace	0.37
6	KI = 1106	0.82	0.20	1.34
7	KI = 1112	0.16	0.05	0.22
8	n-Hexyl isobutanoate	3.38	2.07	6.21
9	n-Hexyl butanoate	11.05	9.68	14.83
10	n-Octenyl acetate	3.36	3.96	5.62
11	n-Octyl acetate	42.29	36.85	47.50
12	n-Hexyl 2-methylbutanoate	6.35	3.61	6.98
13	n-Hexyl 3-methylbutanoate	0.71	0.74	0.88
14	n-Octyl isobutanoate	0.41	0.31	0.84
15	KI = 1387	1.29	1.28	0.45
16	n-Octylbutanoate	0.89	0.89	1.37
17	n-Octyl 2-methylbutanoate	0.70	0.47	0.75
18	KI = 1781	1.17	0.97	-
19	Angelicin (?)	9.23	16.97	-
20	Psoralen	0.15	2.28	-
21	KI = 2040	1.00	0.70	-
22	Xanthotoxin	1.18	2.14	-
23	Bergapten + unknown	2.89	3.95	-
24	KI = 2142	3.26	2.28	-

Peak transfer

One of the most interesting applications of polymer coated fused silica fibers which we have investigated is the new approach of transferring fractions of a capillary gas chromatographic run onto a second capillary column, that means to perform a kind of column-switching or 2-dimensional gas chromatography in a very simple manner. The idea which is the basis of our approach has been: If highly diluted organic vapours in the head space of a sample can be adsorbed at the coating of a fiber and admitted to capillar gas chromatographic separation after thermal desorption in a heated injector, why not adsorb the highly diluted organic vapour of a gc fraction eluting from a gc-capillary in the carrier gas flow on a coated fiber and introduce it to a second capillary?

As can be demonstrated no modification of the gas chromatograph has to be performed in order to realize the idea. Figure 12 shows the FID cross

section of a HP 5890 Series II gas chromatograph, we have used, where the eluting fractions are sampled after shutting the valves of the air, of hydrogen and the make-up gas if applied.

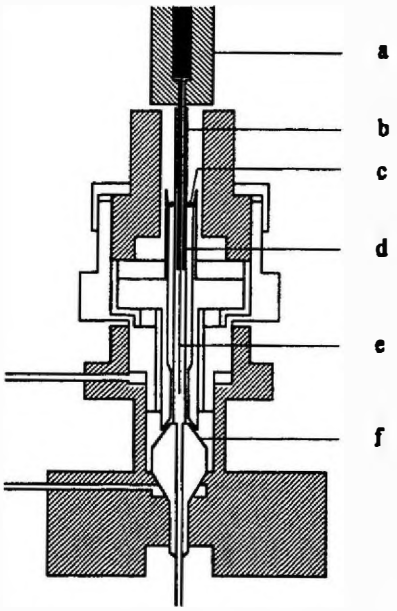

Figure 12. Cross-section of the FID of a HP 5890 Series II gas chromatograph with the inserted SPME fiber
a = SPME-holder; b = inserted glass tubing; c = O-ring; d = septum piercing needle; e = FID-jet

This has to be done after a first normal gc-run yielding the exact information on retention times of the peaks to be transferred. In order to minimize the volume of the detector to avoid dilution of the eluting fraction and to direct the gas flow to the fiber surface, we inserted a capillary glass tubing of 1.5 mm iD which was fixed and tightened by a O-ring.

At the beginning of peak-elution controlled only by time, a 100 µm Polydimethylsiloxane coated fiber was introduced into the mounted glass capillary tubing (Figure 12) and withdrawn at the end of peak elution. Afterwards, the fiber within the needle could be introduced into the injector of a second capillary column, e.g. with a different polar stationary phase. The given example (Figure 13) shows the cutting of a peak from a gc-run of distilled lime oil on a DB-Wax column. We suggested an unknown peak to be a mixture of two components. The injection of the peak-cut on a DB-5 column confirmed our suggestion. After adsorbing the respective gc-fraction at the coated fiber and transferring to a DB-5 capillary 2 components could be seperated: 1,4-cineole and α-terpinene, which are not separated on the polar DB-Wax capillary. The transfer of chiral components from a non-chiral capillary onto a enantio-selective capillary using our new technique will be published in more detail elsewhere (4).

Acknowledgement - The author is grateful to I. Hoffmann and J.-A. Protzen for excellent technical assistance.

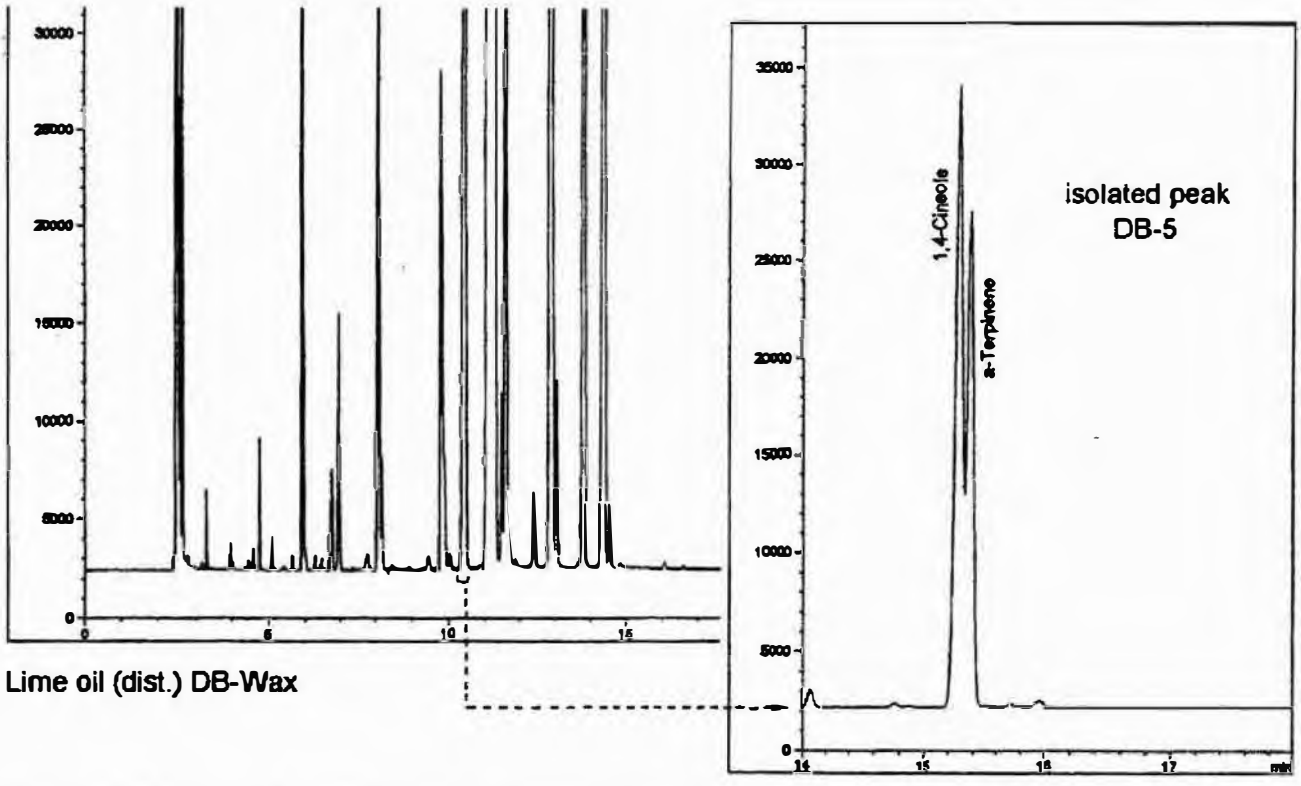

Figure 13. GC-separation of lime oil on a DB-Wax capillary and peak transfer to a DB-5 coloumn

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GC-ANALYSIS OF ESSENTIAL OILS ON CHIRAL COLUMNS - RELEVANT FOR THE PHARMACOPOEIA

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Introduction

General practicability is a prerequisite for all pharmacopoeia methods. For essential oil analysis the following experimental design is considered as being important for reliable and reproducible results: maximal two-step temperature gradients, preferentially separation of all important components in a single run and use of commercially available columns and standards.

In recent years chiral separation of enantiomers on modified cyclodextrins became a well-established method to document purity and identity of essential oils (Werkhoff et al. 1993) in the European Pharmacopoeia, however, this method is not utilized.

The relevant monograph for peppermint oil contains a GC profile based on a polyethyleneglycol 20000 bonded phase capillary column which enables good separations of the investigated components. The detection of racemic adulterations which have been discovered in peppermint oil, e.g. menthylacetate, is not possible by this method (Faber et al. 1994).

With respect to the above requirements for an analytic method in the pharmacopoeia we studied the separation of chiral compounds related to peppermint oil on a modified β-cyclodextrin phase capillary column. The separation behaviour of these components was examined under isothermal conditions as documented in this paper.

Experimental Part

Sample preparation:

Test mixture:
(+)-Menthone 120 µl (Fluka No.: 63675) (3), (-)-Menthone 280 µl (Fluka No.: 63678) (2), (+)-Menthofuran 50 µl (Fluka No.: 63661) (1), (+)-Isomenthone 50 µl (Roth No.: 6458) (4), (+)-Menthylacetate 120 µl (Fluka No.: 45987) (8), (-)-Menthylacetate 280 µl (Fluka No.: 46985) (7), (+)-Menthol 150 µl (Fluka No.: 63658) (5), (-)-Menthol 300 µl (Fluka No.: 63660) (6), (+)-Pulegone 140 µl (Fluka No.: 82569) (9), (-)-Pulegone 60 µl (Fluka No.: 82579) (10), (+)-Carvone 30 µl (Fluka No.: 22070) (11), (-)-Carvone 70 µl (Fluka No.: 22060) (12), n-Hexane 14,85 ml (Fluka No.: 52765) (Fig. 2, 3)

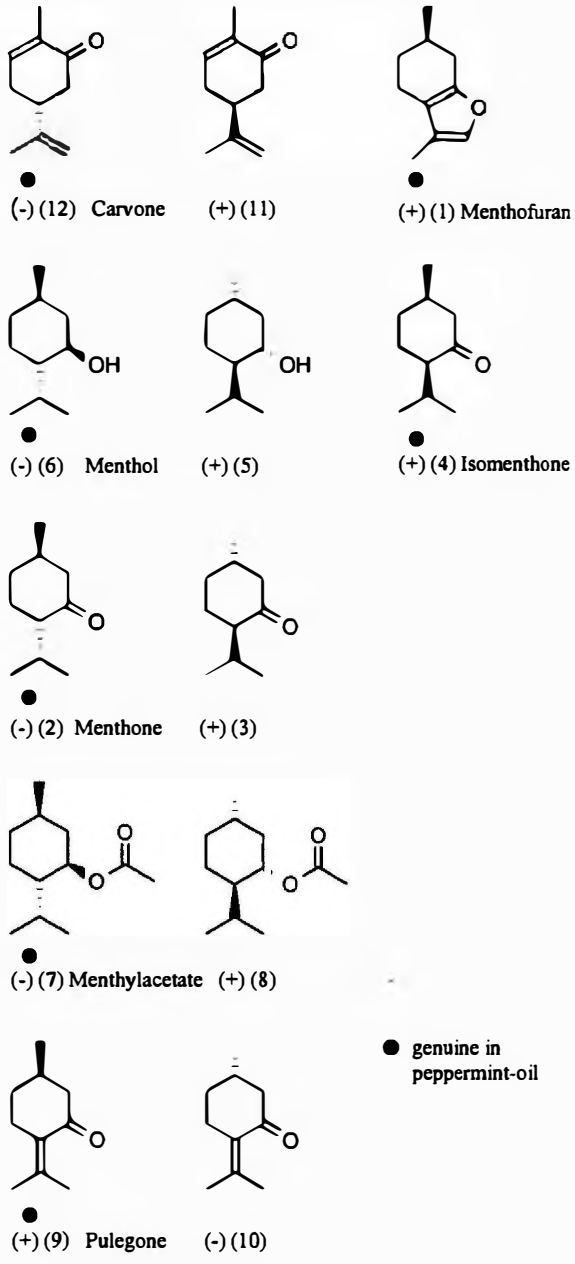

Figure 1. Investigated Components

Peak identification has been performed by coelution with authentic standards.

Peppermint oil (100 µl) was diluted with n-Hexane (900 µl) (Fig. 4)

Column:
FS-Hydrodex β-3P, 50 m x 0,25 mm ID, Column-No.:20314-6, Macherey & Nagel (Düren / Germany) Cat. No.: 723359, Heptakis-(2,6-di-O-methyl-3-O-pentyl)-β-cyclodextrin / OV 1701

GC-System:
GC: Packard 437 A, injector: 180°C, FID-detector: 260°C,carrier gas: 1,1 bar N_2, split: 1:100, injection-volume: 1µl

Temperature:

isothermal (°C):
94, 102, 110, 118, 126, 134, 142, 150, 158, 166, 174, 182, 190, 198 (Fig. 2, 3)

gradient:
80°C / 10 min isothermal > 2°C / min > 140°C / 30 min (Fig. 4)

Conclusions

The investigated components of the peppermint oil show a specific temperature related behavior of the respective elution sequence under isothermal conditions (Fig. 2, 3). (+)-Isomenthone intersects the unseparated menthones and (+)-menthofuran in the tested range of temperatures; (-)- and (+)-menthol are crossing the peaks of the menthylacetates, pulegones and carvons. No change of the elution order of the enantiomeres could be observed.
In some cases the separation of enantiomeres is drastically improved e.g. menthylacetate.
In a temperature region with peak crossing the development of a temperature gradient becomes difficult due to the possible change of the elution sequence.
The different isotherms are characteristic for the various chiral columns (data not shown).

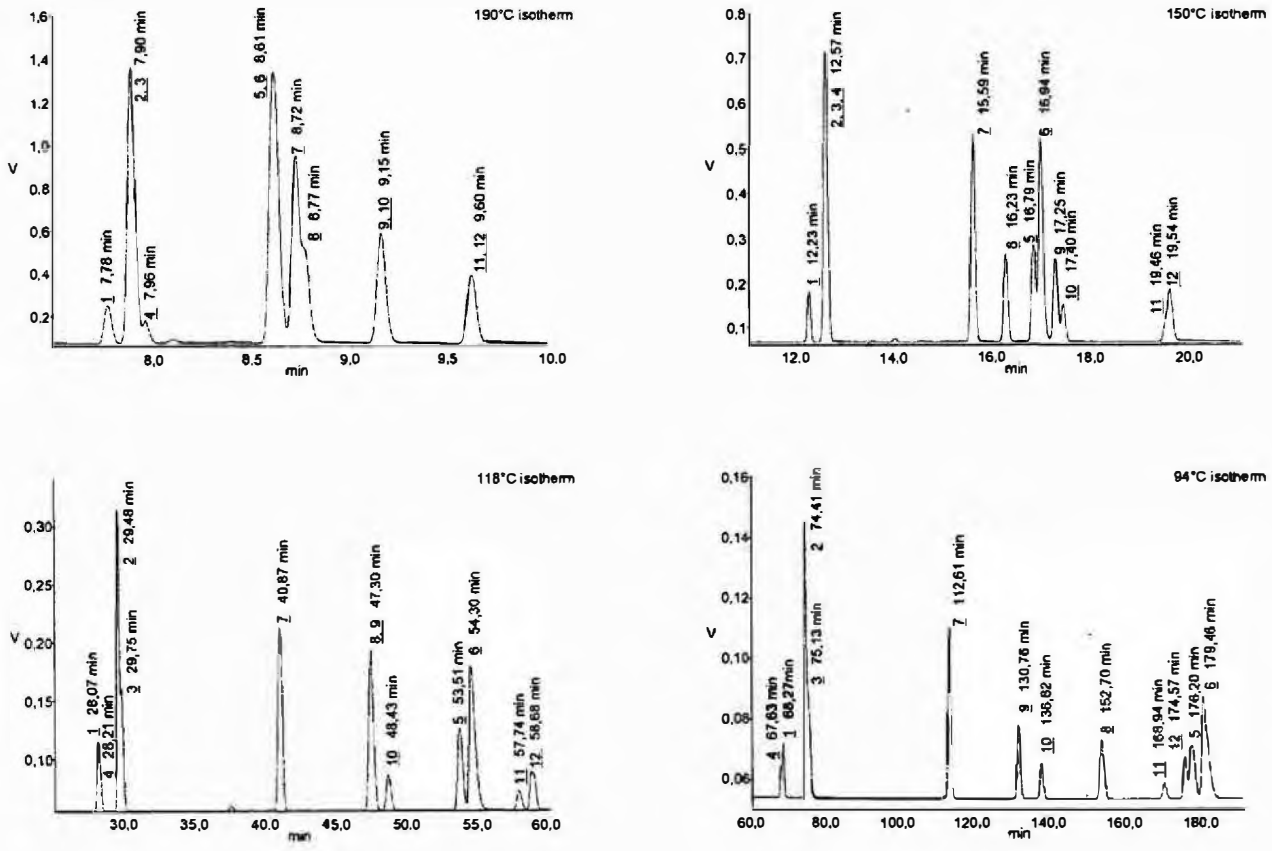

Figure 2. Elution sequence of test mixture depending on different isothermal column temperatures (examples), assignment of underlined numbers see Fig. 1

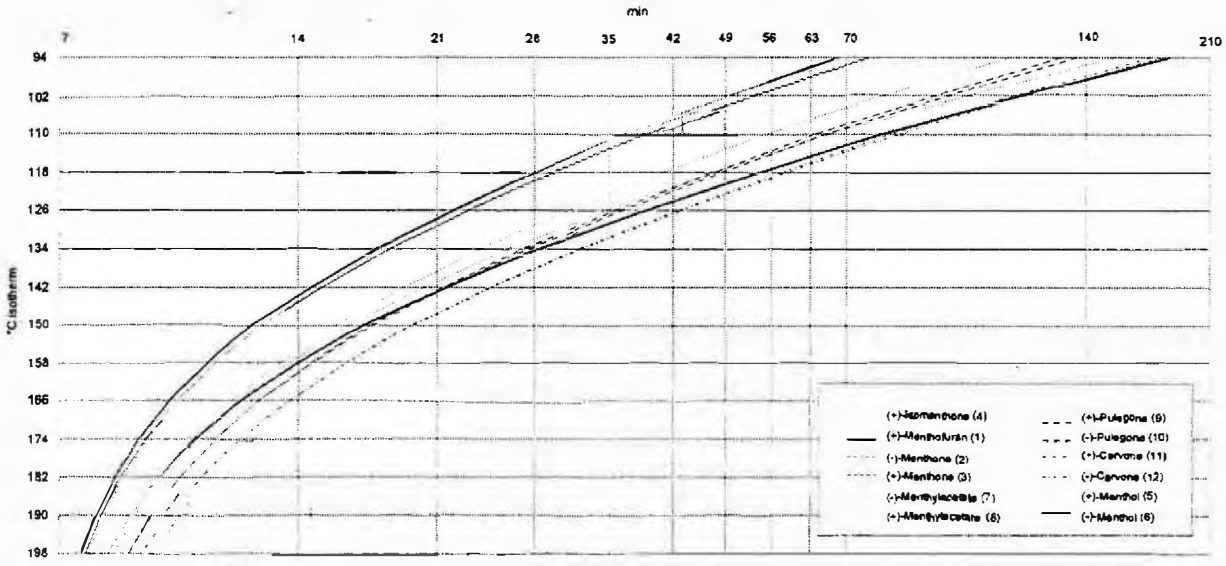

Figure 3. Elution sequence depending on different column temperatures (isothermal conditions)

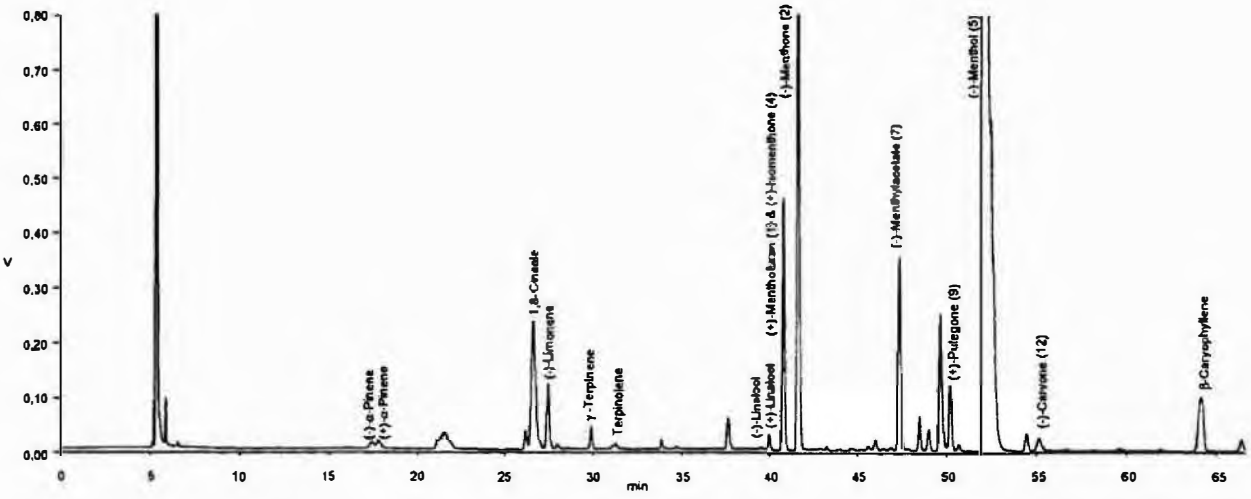

Figure 4. GC of peppermint oil on FS-Hydrodex ß-3P

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Acknowledgement: These investigations were supported by a grant from the Bundesinstitut für Arzneimittel und Medizinprodukte, Berlin, Germany

SFC/MS INVESTIGATION OF SPICE EXTRACTS

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INTRODUCTION

Essential oils and spice extracts are used as an alternative to spices and mixtures of spices in food industry. The main advantage is the microbiological stability of these products. Common extraction methods are solvent extraction, supercritical fluid extraction (SFE) and the recently developed accelerated solvent extraction (ASE) by means of ASE 200™ (Dionex).
Spice extracts are composed of volatile and non-volatile compounds. The investigation of spice extracts by GC is only confined to volatile compounds.
A new method to investigate the total extracts is the capillary supercritical fluid chromatography (SFC). This method is usable for volatile and non volatile compounds.
The SFE extracts and ASE extracts and in addition the respective essential oils of black pepper and other spices were investigated by GC and SFC/MS. Capillary supercritical fluid chromatography - mass spectrometry has been used successfully to identify components of commercial supercritical fluid extracts (Fa. Raps) and ASE extracts of black pepper.

EXPERIMENTAL

Freshly milled black pepper was filled in 33 mL extraction cells and extracted (*table 1*) by the ASE 200™ system (Dionex).

Table 1:
ASE conditions

solvent	ethanol
temperature	100°C
pressure	140 bar
heating up time	5 min
static extraction time	5 min
static cycles	1
wash out (ethanol)	60%
wash out (nitrogene)	200 s

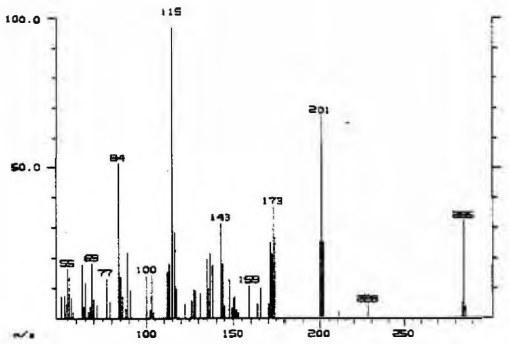
The SFC/MS analyses were performed on a Dionex, Series 600, supercritical fluid chromatograph equipped with a Rheodyne 7256 pneumatic controlled loop injector with 0,2 µl loop size, a SFC column (Dionex SB-Biphenyl-30 10 m × 50 µm ID, 0.25 µm film), a Mplus SFC-MS interface (70°C; restrictor 250°C) and a Finnigan 4500 mass spectrometer. The applied pressure programm at 70°C oven temperature was:

- 100 bar, 1 min isobar
- 10 bar/min to 200 bar; 2 min isobar
- 20 bar/min to 450 bar; 5 min isobar

RESULTS AND DISCUSSION

The extraction of black pepper with SFE (CO_2) produced comparable results to the extraction with ASE (ethanol). The extracts were composed of about 10% volatile components (mono- and sesquiterpenes) and about 40% low, respectively non-volatile hot tasted components (piperidides, pyrrolidides and isobutyl amides). In each case piperine (*figure 1*) was the main component.
The terpenes were well separated of the piperidides. The hot tasting components are separated at low temperature (70°C), so that column bleeding remains negligible.

Figure 1:
SFC/EI-MS: piperine



The SFC/MS EI-mass spectra of the volatile components (e.g. *figure 2*) were comparable to GC/MS EI-mass spectra.

Figure 2:
SFC/EI-MS: α-humulene

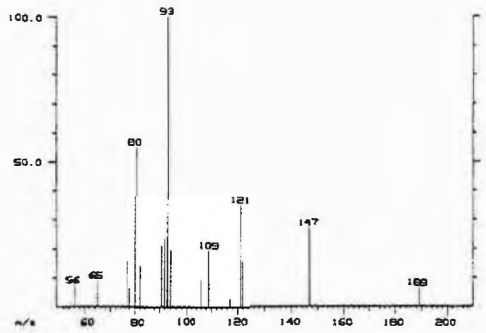


Figure 3:
SFC/EI-MS: piperettine

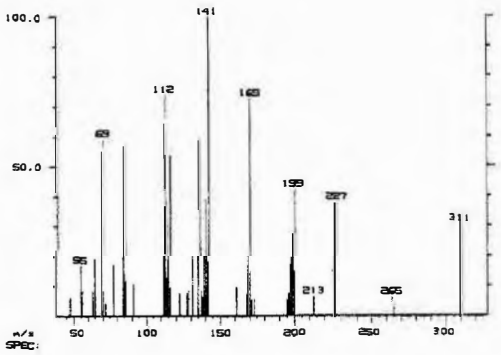
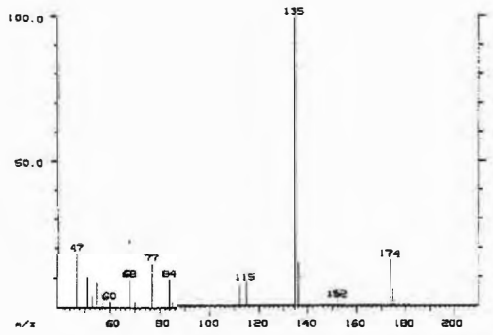


Figure 4:
SFC/EI-MS: piperanine



However, the SFC/MS EI-mass spectra of the hot tasting components (e.g. *figure 1, 3-4*) were of higher quality.

CONCLUSION

SFE and ASE are well-suited extraction methods for black pepper. The applied SFC/MS method proved to be useful especially for investigation of non-volatile constituents of spice extracts.

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SUPERCRITICAL FLUID EXTRACTION OF CLARY SAGE AND STUDY OF SCLAREOL AND ELEMENT CONTENT IN PARTS OF PLANT

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INTRODUCTION

SFE of clary sage (*Salvia sclarea* L.) cultivated in Vácrátót was investigated in our work. Supercritical Fluid Extraction (SFE) is a relatively new procedure to give better quality and cleaner product than the traditional organic solvent extraction. The natural materials can be extracted by SFE without their components changing [1]. Carbon dioxide was used as a supercritical solvent. The extracts were precipitated in two separators in series. The oily, waxy products were collected in the 1st separator, and the essential oil rich products were recovered in the 2nd separator. In the experiments the extract quantities and the mass flow of solvent were measured, and the influence of extraction and separation parameters on the amount on the extracts was examined.

The composition of the SFE extract containing the essential oil were compared with that of the essential oil obtained by steam distillation. The latter one obtained from the different plant parts (stem, leaves, bracteolas, petals) was examined for the distibution of element content and other metal components by atomic absorption and ICP methods.

The oily, waxy fractions were examined by TLC. We tried to identify the components appearing in these extracts with the help of avaible standards.

Quantitative estimation was given concerning the yield of sclareol from the clary sage in two steps. SFE and the steam distillation were used in the first step, and than clary sage was extracted in alcohol in the second step.

Salvia sclarea is a biennial plant in Central Europe. Sporadically it flowers in the first year, but usually only in the second year. The first flowering period is in June -July and the second one in September. The lynalil acetate content, which characterizes the fragrance of clary sage oil is highest during the first flowering period in the second year. The esther value, calculated on lynalil acetate and used for standardization of the oil may be as high as 70 %. The free linalool, 1,8–cineole and limonene contents are also characteristic factors with their maximum in the second year as well.

The composition of the essential oil in leaves differs from that of the essential oil in flowers. Main constituents of leaf oil are β–thujone, 1,8–cineole, terpenic acid and bornyl acetate. Studying the essential oil composition in *Salvia sclarea* during the second flowering period we found that the contents of β–thujone and borneol are higher in the full-flowering stage than in the bud stage. The composition of the oil changes during the harvest and the distillation as follows: the lynalil acetate content decreases, α–pinene and β–pinene often dissapear, 1,8–cineole and myrcene decresase, influencing the scent of the oil. In the first period of the harvest a substantial higher lynalil acetate content was observed [2,3].

Micro and macro elements from natural sources take a prominent part in phytotherapy for the prevention and the treatment of some illness. The human and animal organisms need magnesium and other elements for their vital biochemical processes. Especially magnesium needs for producing energy, the electrolyte ballance, keeping pregnancy, in chronic diarrhoea, against developing of diabetes, etc. The magnesium can be supplemented on several ways, e.g. with enriched drinking water, kitchen salt, food, tablets, herbal teas [4,5].

METHODS AND MATERIALS

Soxhlet hexane extraction

Ground dried plant materials (stem, leaf, bracteola, petal) were measured and then 200 cm^3 hexane was added to a 250 cm^3 bulb. The solvent was recycled until the extract became colorless. After extracting the sample was concentrated by vacuum ecaporator at 40 °C.

Water distillation

The steam distillation apparatus described in the VII. Hungarian Pharmacopoeia was used. After weighing the required amount of ground dried plant material the required amount of water was added to a 2 dm^3 distill flask. The distillation was carried out under the boiling condition.

Supercritical CO_2 fluid extraction

A schematic flow diagram of the extraction apparatus, made in Hungary, is shown in Figure 1. Liquid CO_2 is supplied from a gas cylinder and released into the inner storage vessel. The desired temperature and pressure were adjusted, and after loading the extraction vessel with the plant material the CO_2 feed was started. The volatile compounds

and other lipophilic substances were extracted during extraction. Fractionation of the extracts was conducted by releasing the pressure of carbon dioxide at two stages using two separators in series. The pasty SFE product was collected in the first separation vessel.

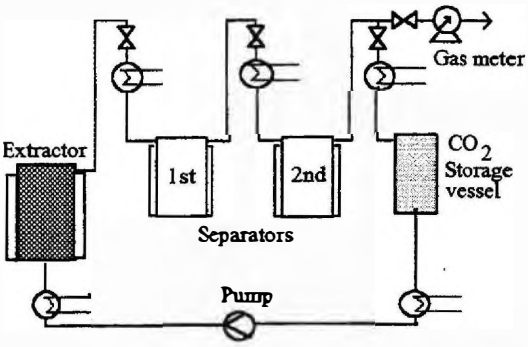

Figure 2. Schematic flow diagram of the extraction apparatus

The product containing the volatile compounds was recovered in the second separator. The total volume of CO_2 consumed is measured by the volumetric gas meter. The accumulated product samples were collected and weighed at certain time intervals.

The CO_2 used in this study was 95-96 % (w/w) pure and supplied by Messer Griesheim Hungaria.

Measurements of the elements contents

1. Atom absorption spectrometric method [6,7]
   Apparatus: Perkin Elmer 2380 Atom Absorption Spectrometer
   Wavelenght: 285.2 nm
   Solution: 50 % sulfuric acid and 5 % calciumchloride

2. ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometer)
   Apparatus: Atom Scan 25 (Thermo Jarell Ash), a sequential plasma emission spectrometer.
   Emission source: an inductively coupled argon plasma (2 kW crystal controlled radio frequency generator induced at 27.17 MHz)
   Optical system: an evacuated Czerny-Turner monochromator existed including two photomultiplier detector (R 477 solar blind, R 899 IR enhanced) and a unique grating ruled at two different line densities (2400 and 1200 lines/mm) to provide both high resolution (0.008 mm) and high sensity over the complete wavelenght range (from 160 to 850 mm).
   Sampling: 0.5 g dry milled matter of the samples were digested with the mixture of 5 cm^3 HNO_3 and 3 cm^3 H_2O_2 in teflon vessels. After digestion the samples were diluted to 25 cm^3. From the solution of the samples (three parallel) quantities of 15 elements were measured as follows: Al, Ca, Cr, Cu, Fe, K, Li, Mg, Mn, Na, P, V, Zn.

Botanical examination

Apparatus: Hitachi Sceanning Electron Microscope S-246 on Video processor P67 E (Mitsubischi), Zeiss Axoskop.

GC analysis parameters

Essential oil
Apparatus: Buck Applications Lab
Carrier Gas: N_2
Injector: 200 °C
Column: capillar (Mxt-1, 30 m x 0.53 mm)

TLC

Kieselgel 60 F_{254}, Toluene-ethylacetate (93:7), benzene, dichloromethane, benzene-ethanol (95:5), Simadzu Dual-Wavelenght Flying-Spot Scanner CS-9000, UV (365 nm)

Plant materials

Flowering tops of the plant, leaf, stem, calyx-leaf, bracteol, petal

RESULTS AND DISCUSSION

Supercritical Fluid Extraction

The supercritical fluid extraction experiments were carried out using feshly ground dried flowering tops of clary sage. However, the quantity of plant sample was not enough to examine quantitative effects of pressure and temperature on the extraction yields.

The temperature was kept at 40 °C in the extractor vessel to prevent the changing of the thermolabile components during the extraction. A typical extraction curve is shown in Figure 2, where the total extraction yields (mass of extract/mass of dried plant) are plotted against the specific solvent mass passed through the extraction vessel.

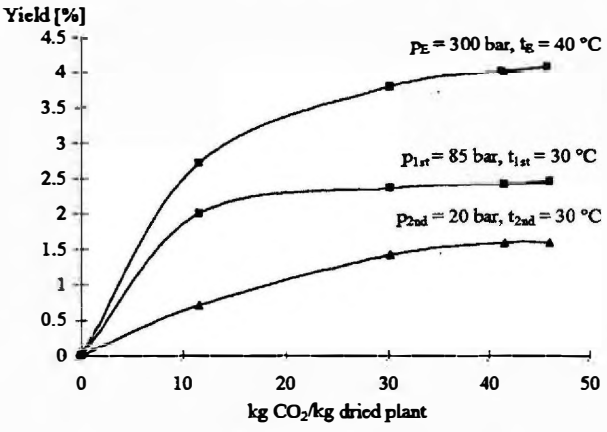

Figure 2. Extraction curve of clary sage

Water, which is present in relatively dry plant material is also extracted. It can be physically separated from the products and is not included in the extraction yields. The maximum yield was 4.06 %. The colour of the subsequent samples changed gradually from yellow through brownish yellow to dark brown, indicating the extraction of pigments at higher extraction times.

The essential oil of the flowering tops was 0.15 ml/100 g dried plant material. SFE yield (mass of extract · 100 % / mass of dried lowering tops) on the volatile compounds rich product was 1.61 %. The oil obtained by supercritical fluid extraction and water distillation were analyzed by capillary GC. The chemical percentage composition of these oils is given in Table 1. Flowering tops of clary sage was used for SFE. The SFE products marked 1, 1*, 2, 3 were collected at certain time intervals in the 2nd separator (1 sample was brownish yellow oil, 1* sample was brownish yellow oil and yellow powder).

Table 1. Chemical percentage composition of clary sage essential oil and SFE oil (GC %)

Compounds	Water distn.	SFE (2nd separator) 1	1*	2	3
α–Pinene	0.78	5.19	9.15	24.04	15.34
Myrcene	2.66	-	-	-	0.38
Borneol	0.08	0.34	0.35	-	-
Linalool	24.99	3.95	3.11	1.50	2.02
Lynalil acetate	15.98	23.64	37.35	3.04	9.72
Geranil acetate	10.18	4.41	10.13	2.00	6.16

Linalool and lynalil acetate as the main components in clary sage essential oil were found in each samples. The volatile components rich SFE extracts (1 and 1*) involved much higher level of linalool and lynalil acetate than the distilled oil. The succesively samples collected in the 2nd separator could be characterized by increasing α–pinene content, and the percentage level of α–pinene was higher than that of α–pinene in the distilled oil.

The ratio of lynalil acetate and linalool in the essential oil of flowering tops obtained by water distillation and SFE were analysed by GC-MS and the results are shown in Table 2.

Table 2. The ratio of lynalil acetate and linalool (GC area %)

Samples	Linalool	Lynalil acetate	Lynalil acetate : linalool
SFE	3.79	23.85	~ 6.3
Water distn.	24.21	16.06	~ 1.5

Comparing the clary sage extracts containing the essential oils to the distilled oil by GC-MS demonstrated the hydrolysis of esters (e.g. linalyl acetate) to the corresponding alcohols during the water distillation. The lynalil acetate : linalool ratio was much higher (~ 6.3) in the SFE product than in the distilled oil (~ 1.5).

To examine the sclareol content in the clary sage SFE extracts from flowering tops of plant was used GC-MS, TLC and densitometry. The extracts containing non-volatile compounds (4, 5 samples) could be characterized significant sclareol content (9.87 %, 6.22 %, respectively), which was appr. 5-7 times higher than in the distilled oil (1.37 %). Sclareol content was the highest in the 1* marked product (10.89 %).

The plant material, which was used for SFE and water distillation in the first step, was extracted with ethyl alcohol for 5-7 days in the second step. Sclareol content of the plant residue after water distillation was appr. one and a half times higher (1.148 mg/g) than that of the plant residue after SFE (0.689 mg/g).

Study of elements contents in plant parts

The amounts of the important elements (Li, Ca, Mg, Zn, Cr) were significantly different in the examined plant parts extracts produced by hexane extraction (Table 3). The magnesium content of the leaf was 3769 mg/kg. The essential oil content of the leaf was 0.019 %.

Table 3. Element contents of the plant parts of *Salvia sclarea* L. (mg/kg)

Element	Leaf	Bracteol	Petal	Calyx-leaf	Fruit
Al	36.7	120.9	180.9	21.4	9.4
B	33.7	50	35	36	17
Ca	29838	48051	29047	12483	48001
Cu	18.1	29.6	34	27.2	15
Fe	85	220	314	93	43
K	6265	20301	32070	10940	12030
Li	15.9	102	73.9	2.7	3.9
Mg	3769	6088	4454	3468	310
Mn	28	42	34	41	30
Na	515.1	1553	485.9	321.0	262
P	963.7	2053	2714	7439	12071
Zn	32.7	62	51.1	68.7	40

The magnesium content of this essential oil was relatively low (66.5 mg/kg) (Table 4).

Table 4. Element contents of the extracts produced from clary sage leafs. (mg/kg)

Element	Essential oil
Al	25.7
B	15.5
Ca	515
Cu	109.1
Fe	25.7
K	39.2
Mg	66.5
P	26.9
Zn	210.8

The maximum magnesium content was found in the bracteol (6088), however relatively high magnesium compositions were detected in petal and calyx-leaf too. Besides the well-known chlorophyll magnesium complex, the magnesium is located in many enzymes and takes part in the forming of volatile and fatty oils as well.

Botanical examination

The green leaf and the calyx-leaf of clary sage with very much unicellular glanduar hairs is shown in Figure 3 and Figure 4, respectively. The unicellular glanduar hairs with long handles are the place of the essential oil localization.

The bracteol and petal were violete colour. Only the Labiatate type glanduar hairs was found on them. The essential oil amount of the bracteol was 0.02 % and that of the petal was 0.01 %.

Calyx-leaf is the place, where the volatile oil is synthetised in the secondary metabolism. The essential oil content of the calyx-leaf was much more higher than that of the other parts of the plant (0.06 %). In this

Figure 3. Glanduar hairs on the leaf

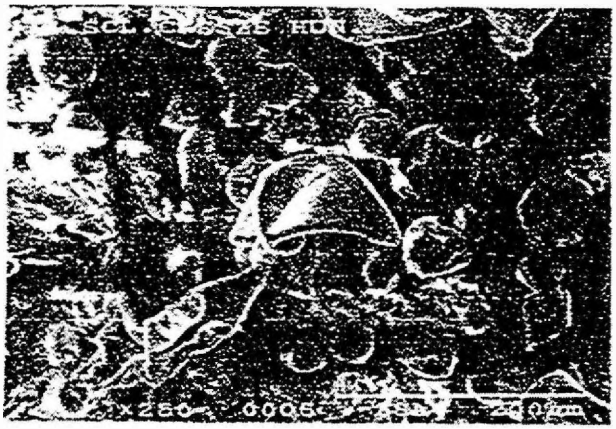

Figure 4. Glanduar hairs on the calyx-leaf

parts of the plant there are very numerous glanduar Labiatae type hairs. On the surface of the glanduar hairs gumlike substances can be observed The most of the glanduar hairs can be found near the veins. Clotted hairs are present as well.

The chemical percentage composition of the volatile components in the extracts of the different plant parts is shown in Table 5.

Table 5. Chemical percentage composition of clary sage oils obtained hexane extraction (GC %)

Compounds	Bracteol	Calyx-leaf	Petal
α–Pinene	0.5	0.7	-
β–Pinene	0.2	0.5	0.2
Limonene	0.4	0.7	0.7
Myrcene	0.2	0.3	0.1
Borneol	3.6	7.1	2.1
Linalool	10.5	15.9	20.1
Lynalil acetate	16.0	37.0	29.0
Geranyl acetate	3.5	5.0	17.0

SUMMARY

Clary sage was extracted by SFE, water distillation and hexane extraction. The extracts were analyzed by GC, TLC and GC-MS. The SFE products containing non-volatile compounds were characterized higher sclareol content than the distilled oil. Clary sage was tested as a possible element source e.g. magnesium by ICP-AES. High magnesium content was found in the different plant parts.

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This work was supported by OTKA under grant numbers T007693 and T016880.

INVESTIGATIONS OF TEA INFUSIONS AND DISTILLATION WATERS USING SOLID PHASE MICROEXTRACTION (SPME) TECHNIQUE

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INTRODUCTION

To prepare gc ready samples of volatiles from highly diluted aqueous solutions usually a liquid-liquid extraction or a solid phase extraction using sorbent packings and successive solvent elution has been applied. Both methods are labour intensive and time consuming. Furthermore, liquid-liquid extraction requires a relatively large volume of an appropriate solvent, which subsequently has to be concentrated prior to gc analysis.

In contrast, the recently developed solid-phase microextraction technique (SPME) (1), so far mainly applied to aqueous samples of pollutants, offers the advantage of a very simple, solventless extraction of analytes and subequent thermal desorption in the injection port of a gaschromatograph.

In this investigation the SPME technique is applied to the aromatic water, the distillation water and the tea infusion of *Mentha piperita* (L.) and the results are compared to conventional isolation and extraction techniques.

MATERIAL AND METHODS

Solid-phase microextraction (SPME): 3 ml of an aqueous solution stored in a 4 ml vial were extracted with a 100 µm polydimethylsiloxane coated glass fiber (Supelco Cat-No. 5-7300) for 15 min whilst stirring.

Solid Phase Extraction (SPE): 25 ml of a solution were pulled through a BondElut LRC C18 catridge (Varian 12111-3027) using a vacuum pump. The analytes were eluted with 2 ml of n-pentane and concentrated to 100 µl under nitrogen and reduced pressure.

Solvent extraction: 25 ml of an aqueous solution were extracted three times with 10 ml of n-pentane; the combined extracts were concentrated to 100 µl under nitrogen and reduced pressure.

Distillation: 25 ml of the aqueous solution were destilled for 2 h with cohobation. The volatiles were collected in 1 ml n-pentane. This solution was concentrated to 100 µl under nitrogen and reduced pressure.

Testmixture: Approximately equal amounts of the 8 compounds and 2 isomer mixtures (see Results; 100 µl for liquids, 100 mg for solids) were mixed together. A stock solution was prepared using 50 µl of the mixture and diluted to 1.0 ml with absolute ethanol. A working solution was made by taking 100 µl of the stock solution and diluting to 100 ml with deionized water.

Essential Oil: The plant material of *Mentha piperita* (L.) was grown in the Willamette Valley (Oregon, USA), and distilled in August, 1996. The essential oil was taken directly out of the separator ("Florentine Flask") and used without further clean up except filtration prior to gc analysis.

Distillation Water: The distillation water was taken at the same time and from the same distillation run as the essential oil used in this investigation.

Aromatic Water: For this investigation 100 µl of the peppermint oil were shaken in 100 ml of deionized water and filtered after 24 h of standing.

Tea infusion: 150 ml boiling water was poured over a commercial peppermint tea bag (Kneipp) containing 1.5 g crushed plant material and left to stand 10 min. During this time the tea bag was moved 10 times up and down.

Gaschromatography: HP 5890 Series II GC with FID, 30 m DB-5 (J&W) fused silica capillary (0.25 mm i.d.; 0.25 µm film, 46 °C to 220 °C, 3 °C/min; injector and detector: 220 °C; Peak area percentage calculation: HP 3365 Series II ChemStation software (Hewlett Packard).

RESULTS Before starting our investigations we tested and evaluated the feasibility of SPME technique for our special requirements.

At first an aqueous solution of 12 compounds (see below) with different polarities and molecular weights was analysed in order to optimise methodical parameters.

cis-3-hexenol	1,8-cineole	menthylacetate
α-pinene	menthone	β-caryophyllene
β-pinene	isomenthone	cis-nerolidol
p-cymene	menthol	trans-nerolidol

As a result we obtained a set of reasonable parameters for our further studies as stated below

extraction time: 15 min (with stirring)

desorption time: min (at 220 °C inj.temp.)

Essential Oil The Essential Oil should not be considered as a typical "whole essential oil", but a cut, as it was sampled during a distillation process (after 30 min). Because of this fact, the composition may differ from other oils of this origin (table 1).

Aromatic Water In the beginning of this century aromatic waters were very common and described in many pharmacopoeia. However, nowadays they are rarely used. Today, instead of genuine distillation waters (see below) as they were used in former times similar products are prepared by mixing the essential oil with deionized water by the optional aid of solubilizers like ethanol or talcum.

Table 1 gives an impression of the discriminating behaviour of the used fiber coating, which has a high affinity to menthone and menthylacetate, whereas the content of menthol is considerable lower compared to the other extraction techniques.

Due to its preparation method mentioned above, the aromatic water resembles the essential oil with respect to the qualitative composition.

Distillation water The water which is separated from the essential oil in the separator ("Florentine Flask") during the distillation process is called distillation water. It was taken at the same time and from the same distillation run as the essential oil used in this investigation. Therefore the chromatographic results have to be considered with the same reservations as those for the oil, as the composition of the distillation water changes with distillation time (2).

Tea Infusion Eventhough the tea infusion is different from the aromatic water and the distillation water, it is integrated in this investigation, because the tea infusion completes the trio of essential oil containing aqueous solutions.

The main differences are due to different plant material used, and the preparation method. Therefore the tea infusion also contains non steam volatile components like dyes and tannins. These substances may have matrix effects and influence the extraction behaviour of the solution. This could be the reason for the different analytical results for the solid-phase extraction techniques.

DISCUSSION

Our findings are in accordance to the expectation (3) that the composition of the investigated aqueous solutions can be considered as a result of a partitioning process of the volatiles of the oil and the water phase with an obvious shift to oxygenated compounds when compared to the essential oil.

With exception of the mentioned problems with the tea infusion, all applied conventional isolation techniques, namely

- solvent extraction
- SPE
- distillation

yielded fairly good comparable results.

The results obtained using the SPME technique are different from those because of the selectivity of the fiber coating. Especially polar and slightly water soluble compounds like menthol and cis-3-hexenol seemed to have a too high polarity for a proportional adsorption at the unpolar coating, resulting in a percentual underrepresentation (Table 1).

SUMMARY Keeping in mind the discriminating behaviour of the fiber coating (100 µm polydimethylsiloxane), the SPME technique is very useful in the analysis of aqueous solutions.

Both for economic as well as ecologic reasons the SPME technique is a state of the art technique as it has the following advantages:

low sample consumption,
no solvent use,
high sensitivity,

and

rapid sample preparation.

It is well qualified as a screening method prior to further investigations of distillation waters, which will be a subject of further studies.

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Table I. Comperative percentage composition of the isolation experiments

Compound	Essential Oil	Aromatic Water				Distillation Water				Tea Infusion			
		SPME	SPE	Extraktion	Distillation	SPME	SPE	Extraktion	Distillation	SPME	SPE	Extraktion	Distillation
trans-2-hexenal	0,05	t	0,08	0,04	0,04	0,08	0,78	0,72	0,63	-	0,13	t	t
cis-3-hexenol	0,04	t	t	t	t	t	1,19	0,96	0,28	-	t	t	t
α-pinene	0,79	0,29	0,03	0,06	0,06	0,05	-	-	-	0,06	-	-	-
sabinene	0,61	0,37	-	0,06	0,07	0,04	-	-	-	0,07	-	-	-
β-pinene	1,36	0,68	0,38	0,38	0,39	0,36	0,85	0,88	0,91	0,20	t	t	0,17
myrcene	0,31	0,18	t	t	0,04	t	-	-	-	0,04	-	-	-
3-octanol	0,33	0,14	0,55	0,46	0,51	0,38	0,85	0,89	0,96	0,07	0,27	0,30	0,50
α-terpinene	0,38	0,28	t	0,05	0,07	0,29	-	-	-	0,05	-	-	-
limonene	1,96	1,22	0,09	0,27	0,24	t	-	-	-	0,44	t	-	-
1,8-cineol	6,17	5,30	10,76	8,40	9,21	12,95	13,27	13,70	15,09	3,15	5,44	3,34	3,73
cis-ocimene	0,51	0,32	0,04	0,07	0,08	t	-	-	-	t	-	-	-
unknown	-	-	-	-	-	t	1,05	0,89	0,32	-	-	-	-
γ-terpinene	0,61	0,49	0,06	0,10	0,13	0,69	t	t	t	0,10	t	-	-
trans-sabinene hydrat	1,10	0,31	1,42	1,53	1,43	t	t	0,07	t	0,33	0,98	1,83	1,70
linalool	0,45	0,26	0,63	0,72	0,71	0,36	0,63	0,78	0,77	0,16	0,42	0,88	0,91
menthone	19,76	36,50	21,54	21,00	21,93	30,12	11,58	11,37	13,33	53,78	38,04	26,11	26,40
isomenthone / menthofurane / neomenthol	9,60	11,04	9,67	9,45	9,56	7,48	6,01	6,13	6,69	10,55	15,16	10,95	12,40
menthol	36,35	27,32	45,83	47,77	45,99	30,61	36,35	37,60	39,23	14,24	23,58	32,31	36,32
terpinen-4-ol	0,78	0,33	1,96	1,74	1,66	1,20	4,13	4,11	4,31	0,25	1,21	1,05	1,34
isomenthol	0,79	0,88	0,98	0,91	0,90	0,75	0,52	0,53	0,57	0,24	0,38	0,30	0,31
pulegone	1,20	1,16	1,86	1,82	1,73	1,59	1,85	1,82	1,84	2,05	5,66	4,77	4,40
piperitone	0,51	0,22	0,77	0,90	0,77	1,70	5,40	5,42	4,73	2,85	1,79	3,10	3,46
menthylacetate	6,08	6,89	1,10	1,64	1,62	3,91	0,38	0,31	0,38	3,47	0,45	0,38	0,20
β-caryophyllene	2,33	1,13	0,32	0,42	0,46	0,08	-	-	t	t	-	-	-
germacrene D	2,70	1,30	0,37	0,49	0,52	0,05	-	-	-	0,14	t	t	0,41
viridiflorol	0,27	0,18	0,07	0,08	0,06	0,94	0,24	0,26	0.22	0,45	-	-	-

306

EVALUATION OF A FENNEL COLLECTION BY CLASSICAL EXTRACTION AND SOLID PHASE MICROEXTRACTION HEADSPACE ANALYSIS

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INTRODUCTION

Plant breeding requires the investigation of single plants or parts of plants. Moreover, the analysis of small amounts of plantmaterial is important for the characterization of living plants, for physiological studies and for chemotaxonomy. In order to analyze very small samples of essential oil plants micromethods have been described, which perform the separation of volatile substances by means of distillation [1; 2]. Our analytical approach to analyze those samples was, to condense and to concentrate the volatile substances at solid phase cartridges [3; 4]. In this context solid phase microextraction (SPME) delivers new aspects.

MATERIAL AND METHODS

The fennel collection (41 samples) received from the genebank Gatersleben has been investigated applying solid phase microextraction headspace analysis (figure 1). The results have been compared with those received by the classical hexane extraction method (table 1). The sample material consisted of chemotypes which presented extreme differences in regard to their main components fenchone, estragole and trans-anethole.
At screening experiments of the fennel assortment SPME-headspace analysis has been prefered, since the direct contact with the liquid fennel suspension may lead to a fast inhibition of the solid phase by non-volatile ingredients (e.g. sugar, organic acids, fat components).

SPME-Headspace-conditions

50mg of milled fennel seeds were stirred in 5ml of water at 30°C for 15 minutes while the SPME-fiber (100µm polydimethylsiloxane, Supelco) is located in the headspace of the suspension.
GC: Hewlett Packard 5890 series II, injector temperature: 250°C, desorption time: 2 min.

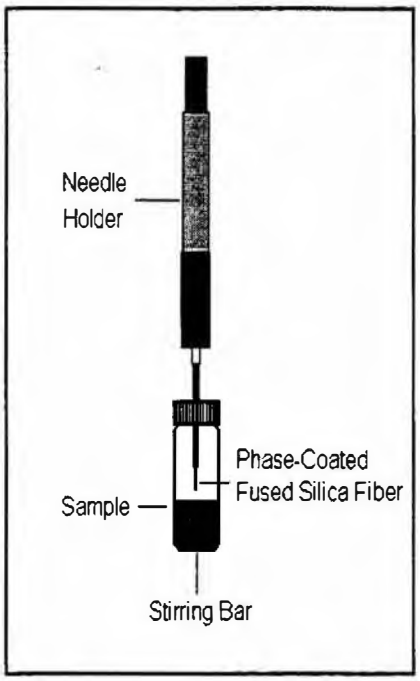

Figure 1. Solid Phase Microextraction Headspace Analysis

RESULTS

The reproducibility of the headspace method applied at the determination of the main essential oil components has been found to be comparable with that obtained at the extraction method. But also the absolute values of those components received at both procedures are similiar (table 1, figures 2-4). This is surprising, because we investigated in the first case the hexane extracts of fennel samples, in the second case the headspaces of aqueous fennel suspensions.

Table 1. Results of the determination of α-pinene, myrcene, limonene, fenchone, estragole and trans-anethole applying SPME-headspace analysis and hexane extraction, respectively

Sample-Nr.	Method	Oil composition in %					
		a-Pinene	Myrcene	Limonene	Fenchone	Estragole	Anethole
FOE 7/76	Extraction	5,53	2,51	4,27	27,50	2,01	55,0
	SPME	6,56	3,26	7,67	28,38	2,66	44,8
FOE 9/83	Extraction	4,62	1,42	1,55	11,10	2,99	76,5
	SPME	6,77	1,86	3,35	9,82	4,23	68,9
FOE 10/83	Extraction	5,25	2,08	1,56	12,20	57,00	19,0
	SPME	5,58	1,56	2,30	9,48	62,98	12,9
FOE 11/75	Extraction	5,70	2,38	3,08	24,67	2,26	59,0
	SPME	4,44	2,09	4,40	25,13	3,59	56,1
FOE 12/75	Extraction	3,12	1,83	2,59	21,40	2,52	65,4
	SPME	5,07	2,71	5,20	22,85	3,46	54,3
FOE 14/78	Extraction	6,53	1,94	1,97	15,50	2,56	69,1
	SPME	8,69	2,08	3,70	16,18	3,59	60,4
FOE 15/78	Extraction	4,19	1,27	2,31	21,25	2,42	65,9
	SPME	6,25	1,27	4,66	18,13	3,42	60,7
FOE 16/95	Extraction	6,22	1,42	2,23	18,75	2,41	65,9
	SPME	4,67	0,93	2,87	17,70	3,77	64,8
FOE 17/81	Extraction	1,69	1,45	7,48	8,90	2,87	76,6
	SPME	2,26	1,85	15,93	6,37	3,61	66,5
FOE 18/83	Extraction	1,58	0,98	10,90	2,14	3,02	77,7
	SPME	1,33	0,36	16,90	2,06	4,00	67,4
FOE 19/82	Extraction	2,07	1,37	7,65	11,10	2,75	74,1
	SPME	2,08	1,50	12,45	8,24	3,63	69,7
FOE 20/94	Extraction	1,89	1,41	10,35	9,59	2,74	72,6
	SPME	1,51	1,31	16,33	7,90	3,73	66,5
FOE 21/81	Extraction	4,89	1,99	1,98	16,20	2,51	69,2
	SPME	4,97	1,87	3,11	16,78	3,73	63,8
FOE 22/95	Extraction	25,87	4,06	14,90	17,93	33,67	0,0
	SPME	20,93	1,51	19,35	11,13	38,90	0,4
FOE 23/91 o	Extraction	2,56	1,66	12,65	12,35	2,48	66,3
	SPME	2,06	1,45	20,73	7,05	3,40	61,8
FOE 24/92	Extraction	6,26	2,49	1,93	13,40	57,45	15,4
	SPME	4,27	1,47	1,98	10,26	64,40	13,4
FOE 27/88	Extraction	1,81	1,26	7,88	9,84	71,15	5,6
	SPME	1,79	0,82	10,22	6,88	72,93	3,8
FOE 28/95	Extraction	10,12	2,41	2,90	14,60	25,40	42,8
	SPME	10,13	1,64	3,93	14,33	31,28	34,9
FOE 29/95	Extraction	1,59	1,24	11,70	6,53	2,85	75,0
	SPME	2,22	1,72	23,85	5,05	3,38	60,7
FOE 30/89	Extraction	1,56	1,40	13,25	9,72	2,64	70,1
	SPME	1,88	1,78	22,63	7,71	3,41	59,5
FOE 31/89	Extraction	1,18	1,03	4,88	4,51	3,67	82,7
	SPME	0,87	0,40	5,40	4,72	6,26	79,6
FOE 32/89	Extraction	1,05	0,94	5,50	7,26	3,42	80,9
	SPME	1,49	1,20	10,93	7,38	4,47	70,1
FOE 33/87	Extraction	1,43	1,68	2,10	17,20	2,55	70,0
	SPME	1,71	2,00	3,54	13,08	3,68	67,1
FOE 34/89	Extraction	4,29	1,32	1,55	7,50	3,08	80,4
	SPME	6,00	1,29	2,48	7,59	4,41	72,2
FOE 35/89	Extraction	2,52	2,16	2,87	24,80	62,97	0,2
	SPME	1,84	1,51	3,54	15,80	71,00	0,6
FOE 36/89	Extraction	5,18	1,60	1,69	10,32	2,82	75,7
	SPME	7,61	1,74	3,15	9,24	4,04	66,6
FOE 37/89	Extraction	1,83	1,04	3,94	11,95	2,68	74,3
	SPME	2,38	0,78	7,49	11,88	3,70	64,2

(Table 1 Contnd)

Sample-Nr.	Method	Oilcomposition in % a-Pinene	Myrcene	Limonene	Fenchone	Estragole	Anethole
FOE 38/91 x	Extraction	2,03	1,55	7,41	6,37	2,84	77,1
	SPME	2,94	1,69	12,08	6,07	3,99	66,5
FOE 39/89	Extraction	6,21	2,02	4,59	11,65	56,35	15,3
	SPME	6,36	1,33	6,66	8,40	56,03	13,6
FOE 40/91 o	Extraction	8,89	1,61	2,35	22,35	2,63	56,3
	SPME	4,46	1,12	2,88	20,35	4,50	63,4
FOE 41/95	Extraction	1,38	1,51	6,94	10,75	3,05	74,7
	SPME	1,85	1,43	11,83	10,29	4,08	66,3
FOE 42/89	Extraction	1,55	1,23	2,16	11,05	2,73	80,3
	SPME	2,01	1,81	4,58	10,63	4,36	73,7
FOE 43/94	Extraction	2,29	1,69	2,29	17,20	30,95	40,1
	SPME	1,61	1,70	2,85	14,95	57,18	16,0
FOE 44/90 x	Extraction	1,05	0,84	5,91	2,59	4,34	82,7
	SPME	1,77	0,60	15,48	3,50	5,34	68,5
FOE 45/93	Extraction	2,95	2,98	3,19	29,70	46,10	9,3
	SPME	2,66	2,42	3,83	20,10	57,18	6,4
FOE 46/95	Extraction	5,71	2,27	3,40	27,85	2,00	54,7
	SPME	9,34	3,08	7,31	21,25	2,75	47,6
FOE 47/95	Extraction	2,26	0,80	2,53	20,75	2,75	63,9
	SPME	2,77	0,72	4,29	17,20	4,55	60,1
FOE 48/95	Extraction	7,68	3,35	3,70	32,70	46,05	0,0
	SPME	5,39	2,50	4,53	28,28	50,45	0,4
FOE 49/95	Extraction	2,59	0,80	2,96	21,20	2,90	62,5
	SPME	3,06	0,64	4,09	18,43	4,50	59,4
FOE 51/95	Extraction	1,21	0,73	11,95	6,37	2,82	76,0
	SPME	1,58	0,94	21,00	5,84	3,88	64,7
FOE 52/95	Extraction	3,11	1,47	16,05	3,60	73,15	0,36*
	SPME	2,00	0,70	14,95	3,59	71,48	3,5

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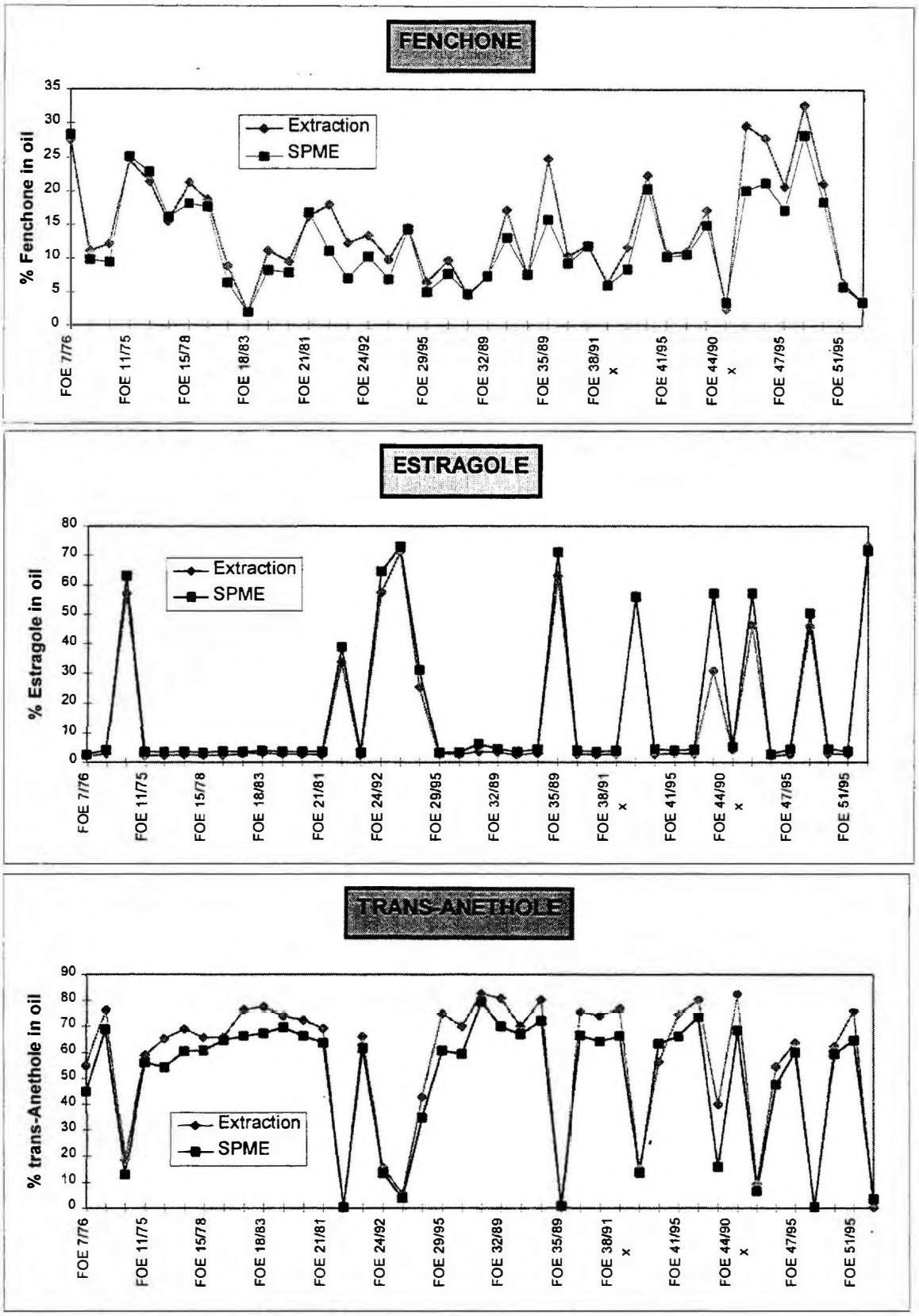

Figure 2 - 4. Comparison of the classical extraction method and SPME characterized by the individual amounts of fenchone, estragole and trans-anethole

A NEW METHOD FOR THE DETERMINATION OF ORGANOCHLORINE PESTICIDES IN ESSENTIAL OILS WITH RESULTS OF ANALYSIS OF 110 ESSENTIAL OIL SAMPLES AND OF 10 MEDICINAL PRODUCTS

5th report: Exposure to crude drugs and crude-drug preparations

Prof. Heinz Schilcher* and Dr. Malte Habenicht

1. INTRODUCTION

For many years it has been a more or less open secret that for various reasons the presence of organochlorine pesticides (here: OPs) must be expected in essential oils.

One reason for this is the wide distribution of OPs throughout the world. Despite their relatively low volatility at normal soil temperatures, OPs evaporate from plant surfaces and also from the ground and thus find their way into the atmosphere. From there they are distributed ubiquitously. Precipitation distributes them over the earth's surface. They can be identified, for example, in polar surface ice, in dwarf pine needles, and at an altitude of 2000 m and an air distance of up to 40 km from the nearest agricultural land.

Another reason is the high degree of stability of OPs to meteorologic and metabolic influences. For example, the half-life of DDT in the soil can be more than 10 years. Though this longevity can be desirable and useful for agricultural and public health purposes, it inevitably results in accumulation of these substances in the environment. Another reason worthy of mention is the substantial increase in the concentration of OPs that occurs during the preparation of essential oils either by steam distillation or by lipophilic extraction.

The paucity of reports of the presence of OPs in essential oils in the published scientific literature is due exclusively to the fact that detection is difficult because the physical and chemical characteristics of OPs are very similar to those of essential oils. It is therefore scarcely surprising that few data are available on levels of OP residues in essential oils, especially as the concentrations concerned may be as low as, or even less than, 1 to 10 ng of OP per gram of essential oil.

1.1 Objective of the present study

1. To examine all analytical methods that are described in the literature and currently used in practice for the detection of pesticides, and in particular organochlorine pesticides, in biological matrices so as to assess their suitability for the detection of organochlorine pesticides in essential oils.
2. To perform a quantitative determination of 21 / 18 important OPs with special reference to *DDT* and related substances such as methoxychlor and dicofol, *hexachlorocyclohexanes* such as lindane and technical HCH mixture, *cyclodienes* such as heptachlor, endosulfan, aldrin, dieldrin, and endrin, and *chlorinated benzenes* such as hexachlorobenzene and quintozene.
3. To validate the analytical method for use with economically and pharmaceutically significant essential oils.
4. To investigate the greatest possible number of pharmaceutically and economically interesting essential oils of the most varied origins in order to compile a "contamination trend analysis".
5. To determine the uptake rates of some OPs into essential oils with special reference to steam distillation.
6. To determine the concentrations of OPs in essential oil-containing medicinal products.
7. To propose maximum allowable concentrations of OPs bearing in mind toxicologic considerations, the ADI values proposed by WHO and FAO, and the limits permitted by the European Pharmacopeia.

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2. MATERIAL AND METHODS

2.1 Experimental studies

In order to satisfy the following four basic criteria:

1. detection of important contaminants in a single working cycle;
2. high sensitivity of detection;
3. high degree of accuracy in terms of the qualitative and, above all, quantitative determination of the individual components; and
4. apparatus and work expenditure that permit routine testing;

the proposed analytical method must achieve the following:

firstly, as near as possible to 100% extraction of OPs from the biological matrix; *secondly*, removal of interfering substances, in other words the most selective possible cleanup method; and *thirdly*, precise and valid identification and accurate quantitative determination with a high recovery rate (at least 70%) and a relative standard deviation of less than 20%.

The experimental studies were commenced with optimization of the qualitative and quantitative determination of *OP pure substances* by means of *gas chromatography* and various detectors (electron capture detector, mass-selective detector, and flame ionization detector). The following analytical parameters were taken into account: possible stationary phases (nonpolar, moderately polar, and polar); sample feed, whether as unsplit feed, split feed in front of the stationary phase, or cool-on-column feed directly onto the column; the geometry of the vaporization tube, and optimization of the injector temperature.

A great deal of time was spent on the experimental studies on the various possible *cleanup steps*. The following cleanup methods were tested: liquid-liquid partitioning; cleanup via a Florisil or silica gel column; separation of interfering substances by means of calcium silicate (Calflo E); cleanup by sweep co-distillation; cleanup by freezing out; cleanup by treatment with sulfuric acid; and cleanup by gel chromatography using Bio Beads SX-3 (polystyrene gel) or Sephadex LH-20.

For the *extraction*, the following solvents were tested: n-hexane, petroleum benzin, acetone / dichloromethane (2/1, v/v), acetonitrile and acetonitrile-water mixture, and solid-phase extraction on RP materials. Extraction was performed by maceration, percolation, or by accelerated maceration with an Ultra-Turrax apparatus.

2.2 Results of the method development studies

After an initial cleanup step on a Sephadex LH 20 column and a subsequent cleanup step with sulfuric acid, the analytical method described below succeeded in quantitatively determining 17 organochlorine pesticides in 34 different essential oils with an adequate recovery rate of between 76 and 100% and a relative standard deviation of 2.9 to 8.3%. Where the cleanup step with concentrated sulfuric acid can be dispensed with, for example in the case of mint and peppermint oils, the OPs aldrin, endrin, dieldrin, and methoxychlor can also be quantitatively determined. In this case the recovery rates were between 70 and 105% and the relative standard deviation between 1.8 and 10.2%.

2.3 Working specification

Exactly 3.0 g of essential oil are placed in a 5-ml volumetric flask and isopropanol is added up to the mark. After mixing, 2 ml of the sample solution are transferred to the prepared, i.e. preswollen, Sephadex LH 20 column by means of a transfer pipette. After absorption into the column, extraction is performed with isopropanol at an elution rate of 35 ml/h. The first 200 ml of eluate, which contains 95% of the components of the essential oils, are discarded. The subsequent eluate (201 to 450 ml) is used for determination of pesticides.

The 250 ml of "pesticide eluate" are diluted with 350 ml of distilled water in a 1000-ml separating funnel and shaken out twice with 50 ml of *n-hexane* in each case. After washing of the n-hexane phases twice with 50 ml of distilled water in each case, the hexane solution is carefully dried with 5 g of sodium sulfate.

After filtration into a 250-ml round-bottomed flask and addition of 0.5 ml of isooctane ("as a keeper"), the solution is concentrated to 2–3 ml by means of a rotary vacuum evaporator equipped with a vacuum control, the water bath temperature being 20°C. With the aid of a gentle stream of nitrogen (approx. 100 ml/min) the "pesticide concentrate" is now concentrated to 1 ml and quantitatively transferred into a 2-ml volumetric flask with rinsing of the round-bottomed flask. The volumetric flask is filled exactly to the mark with n-hexane and used for the GC assay.

The residue content of the sample is calculated by means of the following equation:

$$\text{Pesticide content y} = \frac{\text{Response factor y} \times \text{peak height} \times 5}{\text{Sample weight}}$$

where:

Pesticide content y	=	Residue content of pesticide y in essential oil [µg/g]
Response factor y	=	Relationship between peak height y and amount of pesticide y, as determined by calibration of the system [mV/µg]
Peak height y	=	Measured peak height of pesticide y in the sample [mV]
Sample weight	=	Weight of sample of essential oil [g]

Where the second cleanup step with concentrated sulfuric acid is required, the approximately 100 ml of dried hexane extract are transferred into a 100-ml glass jar *before* being concentrated in the rotary vacuum evaporator. The glass jar is then filled to exactly 100 ml with n-hexane. The contents of the glass jar are transferred into a 200-ml separating funnel and 10 ml of concentrated sulfuric acid are added slowly. After shaking for some time the lower phase generally darkens. After the initial separation of the lower, dark phase, shaking is repeated with another 10 ml of concentrated sulfuric acid. A calibrated transfer pipette is then used to withdraw 50.0 ml of the upper, hexane phase. This hexane solution is concentrated to 1 ml as described above and is then applied to the above equation and used for the GC assay.

The qualitative and quantitative gas-chromatographic determination of organochlorine pesticides is performed in accordance with the specifications given in Tables 1 to 4.

Table 1.

GC system 1	
Equipment:	GC Autosystem, Perkin Elmer
Separating column:	DB-5 capillary column 30 m x 0.25 mm i.d., 0.25 µm film thickness, J & W
Temperature program:	Initial temperature 50°C (hold for 1 min), then 30°C/min to 160°C (hold for 2 min), then 2°C/min to 250°C (hold for 10 min)
Injector:	Split-splitless injector, split ratio 1:35, temperature 240°C
Purge-off time:	1 min
Carrier gas:	Helium, initial pressure 140 kPa
Detector:	ECD, ^{63}Ni 560 MBq, temperature 320°C
Make-up gas:	Argon/methane 95/5, 60 ml/min
Sample feed:	1 µl
Analytical unit:	PE Nelson 1020, Perkin Elmer

Table 2.

GC system 2	
Equipment:	GC Autosystem, Perkin Elmer
Separating column:	DB-5 capillary column 30 m x 0.25 mm i.d., 0.25 µm film thickness, J & W
Temperature program:	Initial temperature 150°C (hold for 0 min), then 2°C/min to 220°C (hold for 5 min)
Injector:	Split-splitless injector, split ratio 1:35, temperature 240°C
Purge-off time:	0 min
Carrier gas:	Helium, initial pressure 140 kPa
Detector:	ECD, ^{63}Ni 560 MBq, temperature 320°C
Make-up gas:	Argon/methane 95/5, 60 ml/min
Sample feed:	1 µl
Analytical unit:	PE Nelson 1020, Perkin Elmer

Table 3.

GC system 3	
Equipment:	GC Sigma 300, Perkin Elmer
Separating column:	Rtx-1701 capillary column 15 m x 0.25 mm i.d., 0.25 µm film thickness, Restek
Temperature program:	Initial temperature 50°C (hold for 1 min), then 30°C/min to 160°C (hold for 2 min), then 2°C/min to 250°C (hold for 10 min)
Injector:	Split-splitless injector, split ratio 1:35, temperature 240°C
Purge-off time:	1 min
Carrier gas:	Helium, initial pressure 70 kPa
Detector:	ECD, ^{63}Ni 370–560 MBq, temperature 320°C
Make-up gas:	Argon/methane 95/5, 60 ml/min
Sample feed:	1 µl via Autosampler AS 300
Analytical unit:	LCI 100, Perkin Elmer

The following GC system was used for analysis of the organochlorine pesticides with the mass-selective detector.

Table 4.

GC system 4	
Gas chromatograph:	MFC 500, Carlo Erba
Mass spectrometer:	Kratos 25 Rf
Ionization:	EI, 80 eV
Separating column:	DB-1 capillary column 30 m x 0.25 mm i.d., 0.25 µm film thickness, J & W
Temperature program:	Initial temperature 50°C (hold for 1 min), then 30°C/min to 160°C (hold for 2 min), then 2°C/min to 250°C (hold for 10 min)
Injector:	Split-splitless injector, split ratio 1:35, temperature 240°C
Purge-off time:	1 min
Carrier gas:	Helium, initial pressure 140 kPa
Sample feed:	1 µl

The *qualitative* analysis can be performed either by comparing the retention times on at least two different stationary phases with those of authentic pesticide standards (see Table 5), or else by means of GC-MS analysis.

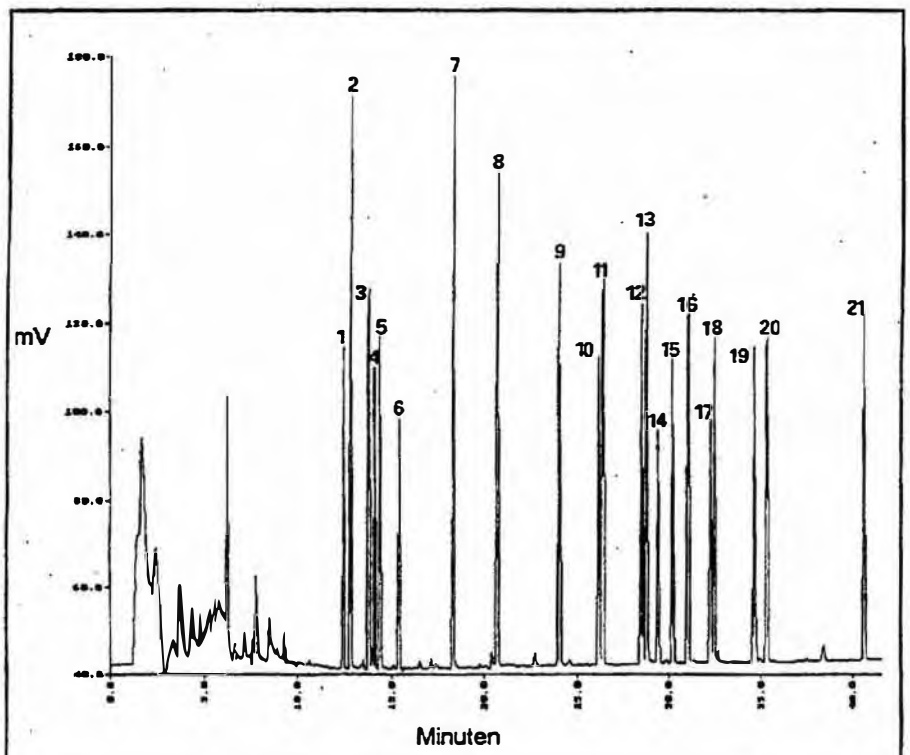
For the *quantitative* analysis, the apparatus used must first be calibrated with pure reference substances. Analysis can be performed via peak height or peak area. We found calculation via peak height to give more accurate results, especially when the peak of the substance to be analyzed was not completely separated from interfering substances.

Table 5. Retention times (R_t) and relative retention times (RR_t) calculated therefrom obtained using GC systems 1 and 2.

	GC-System 1		GC-System 2	
	$R_{t\ [min]}$	RR_t	$R_{t\ [min]}$	RR_t
α-Hexachlorcyclohexan	12.73	0.60	9.94	0.53
Hexachlorbenzol	13.17	0.62	10.36	0.56
β-Hexachlorcyclohexan	14.12	0.67	11.34	0.61
Lindan	14.32	0.68	11.70	0.63
Quintozen	14.74	0.70	12.00	0.64
δ-Hexachlorcyclohexan	15.80	0.75	13.09	0.70
Heptachlor	18.81	0.89	16.21	0.87
Aldrin	**21.16**	**1.00**	**18.62**	**1.00**
Heptachlorepoxid	24.46	1.16	22.02	1.18
*o,p'*DDE	26.57	1.26	24.45	1.30
α-Endosulfan	26.85	1.27	24.45	1.31
Dieldrin	28.96	1.37	26.60	1.43
*p,p'*DDE	29.16	1.38	26.84	1.44
o,p'*DDD*	29.78	1.41	27.46	1.47
Endrin	30.64	1.45	28.29	1.52
β-Endosulfan	31.44	1.49	29.10	1.56
*p,p'*DDD	32.57	1.54	30.29	1.63
*o,p'*DDT	32.80	1.55	30.52	1.64
Endosulfansulfat	35.06	1.66	32.77	1.76
*p,p'*DDT	35.72	1.69	33.46	1.80
Methoxychlor	41.00	1.94	38.78	2.08

After calculation of the peak heights of all 21 organochlorine pesticides (see Table 5) at four concentration levels, the correlation coefficients of the corresponding calibration lines were calculated. Figure 1 shows the thus-calculated linear regions of the various organochlorine pesticides.

Figure 1.



1. α-HCH, 2. HCB, 3. β-HCH, 4. γ-HCH, 5. Quintozen, 6. δ-HCH, 7. Heptachlor, 8. Aldrin, 9. Heptachlorepoxid, 10. *o,p`*DDE, 11. α-Endosulfan, 12. Dieldrin, 13. *p,p`*DDE, 14. *o,p`*DDD, 15. Endrin, 16. β-Endosulfan, 17. *p,p`*DDD, 18. *o,p`*DDT, 19. Endosulfansulfat, 20. *p,p`*DDT, 21. Methoxychlor

The method was validated by means of the add-on method (added concentration of between 10 and 40 ng/g of organochlorine pesticide).

Additional experimental studies and data, such as the determination of the uptake rates of individual OPs into essential oils after steam distillation from added raw drug material, are described in [1].

3. RESULTS

3.1 Results of analysis of 110 essential oil samples and 10 essential oil-containing medicinal products

In 1994 and 1995 we used the analytical method developed by us to analyze 110 samples of 32 different plants (see Table 6), plus a number of medicinal products whose active constituents are essential oils.

Table 6. The essential oils analyzed

1. Menthae piperitae aetheroleum	2. Menthae arvensis aetheroleum
3. Anisi aetheroleum	4. Foeniculi aetheroleum
5. Lavandulae aetheroleum	6. Caryophylli aetheroleum
7. Eucalypti aetheroleum	8. Rosmarini aetheroleum
9. Carvi aetheroleum	10. Cinnamomi aetheroleum
11. Pini pumilionis aetheroleum	12. Piceae aetheroleum
13. Juniperi aetheroleum	14. Matricariae aetheroleum
15. Melissae aetheroleum	16. Melaleuca alternifolia (Teebaumöl)
17. Aurantii pericarpium aetheroleum	18. Citri aetheroleum
19. Bergamottae aetheroleum	20. Petitgrainöl (C. aurantium ssp. aurantium)
21. Grapefruitöl (Citrus paradisi)	22. Mandarinae aeth. (Citrus madurensis)
23. Aurantii dulcis aeth. (Citrus sinensis)	24. Petroselini aetheroleum ex herba
25. Anethum aetheroleum ex herba	26. Levistici aetheroleum ex herba
27. Citronellae aetheroleum	28. Cinnamomi Cassicae aetheroleum
29. Cajaputi aetheroleum	30. Annonae aetheroleum
31. Anibae roseodora aetheroleum	32. Santali lignum aetheroleum

Organochlorine pesticides were unequivocally demonstrated in 72 samples, i.e. around two thirds of all the samples analyzed.

In 64 of these the levels were considerably greater than those permitted by the German Residue Limits Ordinance (RHmV 1994) [2]. By contrast, the limits proposed in Pharmeuropa 1993 [3] were exceeded in only 31 of the samples. Using an initial sample weight of 3.0 g, approximately 35% of the samples analyzed showed no OPs.

In 62 samples more than one OP was found, α-hexachlorocyclohexane, lindane, hexachlorobenzene, α-endosulfan, and p,p'DDE being the most common. Especially noteworthy is the presence of technical hexachlorocyclohexane in 66 essential oils, the permitted limit of 0.02 mg/kg being exceeded in 56 cases, while one sample contained α-HCH at a concentration of 17 310 ng/g, some 50 times the permitted limit for this substance.

Another substance found to be responsible for a major residue problem was the fungicide *hexachlorobenzene* (HCB). This was identified in 27 of the 72 contaminated oils. Also relatively common was contamination with endosulfan / endosulfan sulfate, the level of 0.1 mg/kg of this permitted by the German Residue Limits Ordinance (RHmV 1994) [2] being exceeded in 23 samples.

Another highly significant form of contamination is that with DDT. Despite the fact that its use has been prohibited in the Federal Republic of Germany since as long ago as 1972, this insecticide was found to be present in 21 samples.

Further details relating to individual OPs are given in [1].

Analysis by geographical origin was rendered difficult by the fact that no information was available on the origin of around a third of the samples. Nevertheless, there was a tendency to higher concentrations of OPs, and in particular to contamination with technical HCH mixture and DDT — two long-since prohibited products — in essential oil samples derived from developing countries and also from Eastern Europe.

Also of interest is the geographically determined relationship between *mint oil* and *peppermint oil*. Whereas the limits specified by RHmV 1994 [2] were exceeded in all but two of the 24 analyzed samples of mint oil (derived from the plant

Mentha arvensis var. *piperascens*), these limits were satisfied in 55% of the samples of peppermint oil (derived from the plant *Mentha x piperita*), and the limits proposed in Pharmeuropa 1993 [3] were exceeded in only two of the samples of peppermint oil.

The limits specified by RHmV 1994 [2] were exceeded in only one of the ten analyzed samples of ethanolic-aqueous medicinal products. The presence of α-HCH, lindane, α-endosulfan, and hexachlorobenzene was detected, the concentrations of these being in the range 1 ng/g to 55 ng/g. These levels of contamination are considerably below those that were found in the samples of the various essential oils from which the same medicinal products were derived.

3.2 Consequences in terms of toxicology and drug legislation

The limits for organochlorine pesticides in food specified by the German Residue Limits Ordinance (RHmV 1994) were exceeded in over 50% of the analyzed samples of essential oils. From the point of view of adherence to this ordinance, this is certainly an unwelcome result. On the other hand, it may be asked whether the limits for essential oils specified by this ordinance are justified. In order to answer this question, the following model calculation is proposed. According to this formula, a 60-kg person with a daily intake of 1.5 g of essential oil containing lindane at the relatively high concentration of 1 µg/g would thereby be ingesting only 0.3% of his ADI of lindane:

$$\frac{1\ \mu g/g \times 1.5\ g \times 100}{60\ kg \times 8\ \mu g/g} = 0.3\%\ \text{of ADI of lindane (8 µg/kg body weight)}$$

Thus, though it may be undesirable, ingestion of a typical daily amount of this relatively highly contaminated essential oil represents no toxicologic risk to the consumer. We therefore recommend special maximum limits for organochlorine pesticides in essential oils. For example, the recommendations of Pharmeuropa 1993 [3] could be adopted not only for medications but also for foods, especially bearing in mind the small amounts of essential oils present in foods and cosmetics.

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Analytics - Chemical Aspects

CHEMICAL EXPLORATION OF BRAZILIAN AROMATIC SPECIES BELONGING TO THE MYRTACEAE FAMILY

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INTRODUCTION

Brazil, which occupies half the surface area of the Southern American continent, is astonishing for its richness and variety, for the great diversity of climates and soils resulting in very different phytogeographic formations according to the region.
This richness is also found in the aromatic flora, in which a large number of species have still to be discovered and studied.
A scientific program related to aromatic plants from Southern Brazil was initiated in 1990 by the Faculty of Pharmacy of Porto Alegre (Rio Grande do Sul) in collaboration with our laboratory (Faculty of Science, Montpellier II University).
Southern Brazil, which includes the states of Paraná, Santa Catarina and Rio Grande do Sul benefits from a temperate climate and is named "European Brazil" with however a rich diversity of vegetation.
It is traditionally known for its large grassy plains where cattle raising is practiced and for the araucarias forests found in higher altitudes. It is also an area of fertile ground, especially in Paraná, where coffee and soya plantations have been developped.
Our research program concerns the aromatic species found in the Atlantic rain forest, a plant formation which extends along the east coast of Brazil.

Three families have been selected to start this study : Lamiaceae, Piperaceae and Myrtaceae, but the majority of the results already obtained concerns the latter family.

Scott *et al.* [1] have underlined the ecological importance of the Myrtaceae in Eastern Brazil and indicated that this family is predominant in terms of the total number of species represented and the surface area covered.
This family is so much more interesting, that despite the important number of its representative species in Brazil, very few have been the object of chemical studies up to now. Even so, the use of aromatic plants in herbal medicine is traditional in Brazil.
G. Buchbauer has clearly discussed the biological effects and possible therapeutic applications of essential oils.[2] Furthermore, possible uses of plants or essential oils as safe natural herbicides, growth boosters and other agents in agriculture is presently a field of increased interest.[3] It is for this reason that our colleagues of the University of Porto Alegre have directed their studies towards plants traditionally known for their therapeutic uses or their insect repellent properties.

The program is centred on two principal axes of research :

- botanical study of plant material and screening of the biological properties of their volatile extracts (part performed at Porto Alegre)
- chemical analyses of these volatile constituents for a chemotaxonomic classification purpose and to complete the biological evaluation (part performed at Montpellier).

Only the results obtained from the chemical analyses will be presented and discussed in the context of this conference.

PLANT MATERIAL

The Myrtaceae family covers about 144 genera divided in two sub-families : Leptospermoideae and Myrtoideae, widely spread in Tropical America, South East Asia, East Australia and Pacific Islands.[4]
Our study includes 30 species distributed into 12 genera of which the systematic classification is illustrated in Fig. 1.

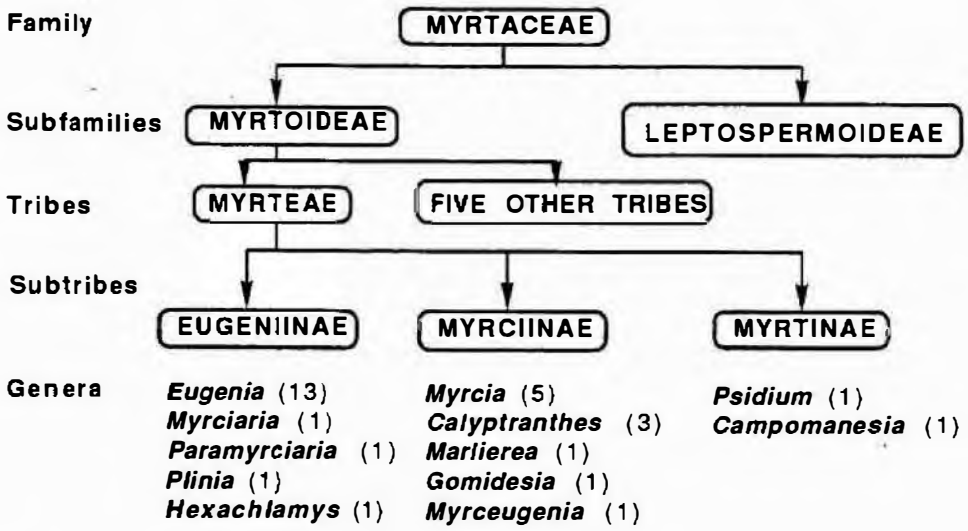
These results represent a part of a PhD work presented by P. VERIN in July, 1996 at the University of Montpellier II

Table 1

Collecting sites of the studied species and yields of the essential oils obtained by hydrodistillation of their leaves

Botanical Name	Yield (mL/100g)	Locality*
Calyptranthes concinna DC.	0.24	Tenente Portela, RS
Calyptranthes grandifolia Berg	0.12	Ilhéus, BA
Calyptranthes tricona Legrand	0.18	Tenente Portela, RS
Campomanesia xanthocarpa Berg	0.35	Morro Santana, Porto Alegre, RS
Eugenia beaurepaireana (Kiaerskov) Legrand	0.12	Morrinhos do Sul, RS
Eugenia brasiliensis Lam.	0.56	Blumenau, SC
Eugenia cuprea (Berg) Niedenzu	0.13	Peruibe, SP
Eugenia involucrata DC.	0.28	Porto Alegre, RS
Eugenia plicato-costata Berg	0.09	Una, BA
Eugenia prismatica Legrand	0.88	Sao Mateus do Sul, PA
Eugenia ramboi Legrand	0.15	Morrinhos do Sul, RS
Eugenia rostrifolia Legrand	0.21	Porto Alegre, RS
Eugenia schuechiana Berg	0.14	Porto Alegre, RS
Eugenia stigmatosa DC.	0.09	Peruibe, SP
Eugenia tinguyensis Cambess.	0.04	Ilhéus, BA
Eugenia uniflora L.	0.54	Porto Alegre, RS
Eugenia xiriricana Mattos	0.12	Peruibe, SP
Gomidesia spectabilis (DC.) Berg	0.18	Garuva, SC
Hexachlamys itatiaiae Mattos	0.09	Morrinhos do Sul, RS
Marlierea eugeniopsoides (Kausel & Legrand) Legrand	0.34	Morrinhos do Sul, RS
Myrceugenia euosma (Berg) Legrand	0.33	Osório, RS
Myrcia acuminatissima Berg	0.12	Garuva, SC
Myrcia bombycina (Berg) Niedenzu	0.95	Esmeralda, RS
Myrcia fallax (Richard) DC.	0.35	Garuva, SC
Myrcia glabra (Berg) Legrand	0.10	Morro Santana, Porto Alegre, RS
Myrcia multiflora (Lam.) DC.	0.20	Taquari, RS
Myrciaria cuspidata Berg	0.38	Morro Santana, Porto Alegre, RS
Paramyrciaria delicatula (Kausel) DC.	0.14	Osorio, RS
Plinia brachybotrya (Legrand) Sobral	0.09	Morrinhos do Sul, RS
Psidium cattleianum Sabine	0.22	Porto Alegre, RS

*BA : Bahia, SP : Sao Paulo, SC : Santa Catarina, RS : Rio Grande do Sul, PA : Parana



( ) Number of species which have been examined, in each genus.

Figure 1: Botanical classification of the twelve examined genera

RESULTS AND DISCUSSION

Most of the species examined have been collected in Southern Brazil (Paraná, Santa Catarina and principally Rio Grande do Sul), three of them originating from Sao Paulo state and three others from Bahia (Table 1). The yields of the essential oils obtained by hydrodistillation of leaves of the selected species have been found to be generally low [0.1% to 1.0%] and a survey of their gas chromatograms indicates that the samples are, for the most part, rather complex and constituted by a majority of sesquiterpenes.
Among the species studied, very few had already been the object of a chemical study of their volatile constituents. [5-9]

The analytical techniques applicable to essential oils are more and more sophisticated and a very complete presentation of these modern analysis methodologies, in particular the recent so-called *hyphenated* techniques, has been published by D. Joulain[10] who gives some advice about their utilization :

➪ risk of " qualitative abuses "
- by misuse of GC/MS semi-automatic or automatic data processing, specially in the case of sesquiterpenoid compounds which do not display sufficiently specific mass spectral data patterns,
- by referring to GC retention data from different sources (especially in the case of highly polar components that generate unreliable indices with aging columns),

➪ risk of " quantitative abuses "
- by excessive confidence in integrator calculations in the absence of prior standardization,

and D. Joulain concludes that, in the case of non-isolated constituents, the use of his / her own databases developed by the analyst himself / herself from genuine samples (obtained by isolation or synthesis) should avoid any mistaken identification.
Nevertheless, a complete description (MS, GC, FTIR, NMR) of identified components is recommended.

But, in the context of an exploration of a botanical family, what is the best way to efficiently conduct the chemical exploration of many new esssential oils ?

➪ should we purify each constituent (even those present in minor amounts) then initiate its complete spectroscopic analysis ? According to such a process, a lot of time will be necessary to unmask all the secrets of a mixture which may be finally uninteresting !

➪ should we perform hasty analyses by GC/MS ... with probably some hazardous identifications ?

➪ we have chosen a middle ground with "careful examination" by GC and GC/MS of prefractionated samples, accurate identification by co-injection with authentic samples when available and / or the isolation of some key-components for a complete spectroscopic analysis and formal identification (Fig. 2).

All our samples have been analysed in this way which has enabled us to identify 140 components in total, of which 104 are sesquiterpenes.

B. Lawrence[11] noted that the essential oils obtained in a weak yield were often high in sesquiterpenes content. This observation seems to also apply to our samples of which the largest part is rich in sesquiterpenes.

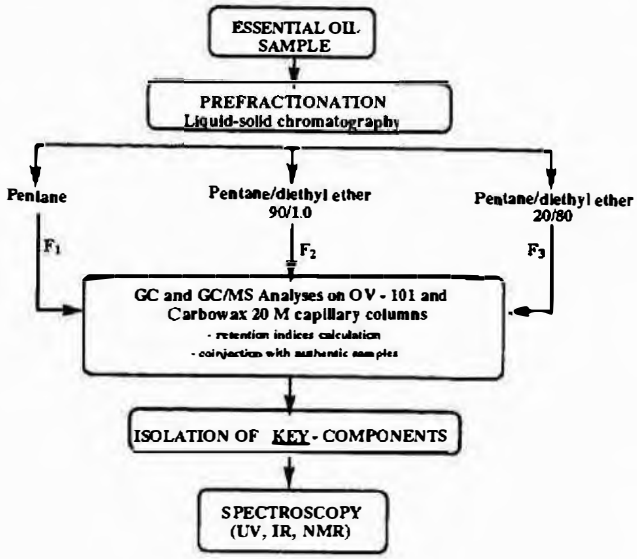


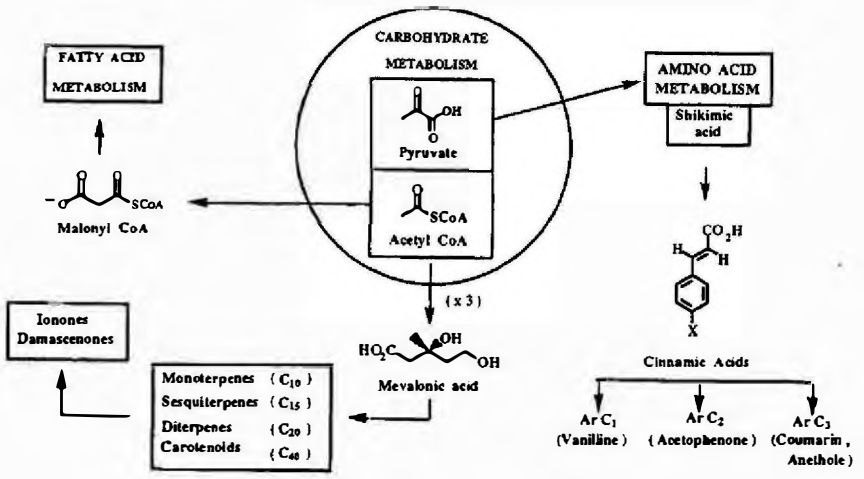
Figure 2 : Analytical procedure

Having taken account of their complexity and of the possible presence of artefacts due to their method of obtention or storage (conversion of germacrene B into γ-elemene by Cope rearrangement [5,12], oxidations...), we have classified our samples on the basis of the biosynthetic pathway taken by the majority of their constituents. A diagram of the three principal biosynthetic pathways of the volatile constituents is shown in Fig. 3.

The pathway of the mevalonic acid is by far the best represented; only two species (*Calyptranthes concinna* and *Calyptranthes tricona*) are characterized by a majority of compounds originating from the shikimic acid and one species (*Eugenia stigmatosa*) provides an essential oil almost exclusively composed of an olefinic fatty acid (Fig. 4).

No clear distribution results from this first classification as the three sub-tribes have been found in the "mevalonic acid pathway group" in which, nevertheless, the sesquiterpenic samples plainly predominate. So, before attempting another mode of chemical classification of these samples, we will deal with the three most typical species, characterized by the presence of chemical structures rarely found in essential oils.

➪ *Eugenia stigmatosa* DC. (vernacular name : "guamirim") is a tree 6m high that grows in coastal forests from Bahia to Santa Catarina; the essential oil, obtained by hydrodistillation of leaves, is extremely simple, containing more than 90% of (*Z*) - tetradec-5-enoic acid (physeteric acid, **1**). This olefinic fatty acid[13] has already been found in lipids of several marine organisms: identified for the first time in whale and dolphin oils, it was subsequently isolated from sardines and prawns.[14] It has also been found in minor amounts in the plant kingdom : in rape seed oils [15], *Pinus* and *Picea* resins [16], shellac [17] and *Thumbergia alata* seed oil.[18]



R. Tressi and coll. " Biogenesis of Volatiles in fruits and vegetables " in : Aroma Research (H. Maarse and P.J. Groenen, eds.), Pudoc, Wageningen (1975).

Figure 3 : Principal biogenetic pathways of natural volatiles

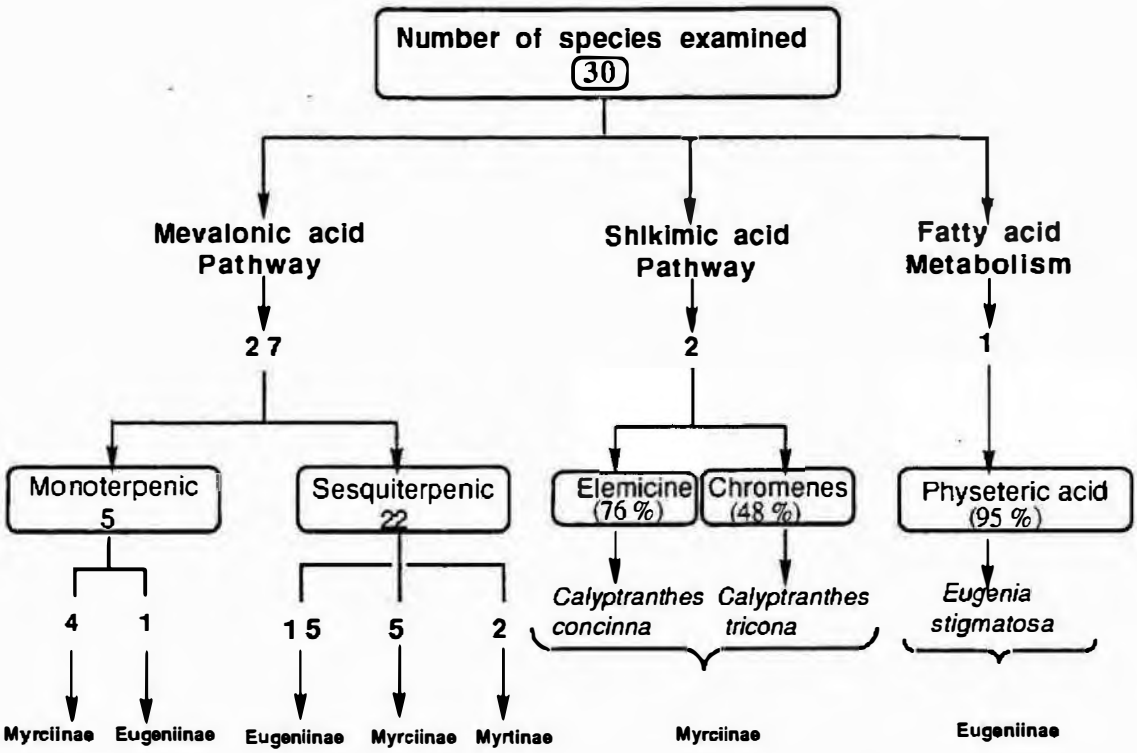


Figure 4 : Metabolic pathways occurring in the examined species

To the best of our knowledge, this compound has been identified for the first time in the Myrtaceae family.

Spectral data of compound **1** :

- MS : 226 (M^+, 5), 208 (22), 166 (21), 138 (20), 124 (31), 110 (41), 96 (71), 81 (92), 69 (100), 55 (95).

- ^{13}C NMR : d 179.8 (C-1), 131.3 (C-6), 128.1 (C-5), 33.4 (C-2), 31.8 (C-12), 29.5 (C-8), 29.5 (C-10), 29.3 (C-9), 29.3 (C-11), 27.2 (C-7), 26.4 (C-4), 24.6 (C-3), 22.6 (C-13), 14.0 (C-14).

- ^1H NMR : d 5.40 (dt, 1H, $J_{5,6}$ 10.8 Hz, $J_{6,7}$ 7.2 Hz, H-5), 5.30 (dt, 1H, $J_{3,4}$ 7.2 Hz, H-6), 2.35 (t, 2H, $J_{2,3}$ 7.4 Hz, H-2), 2.02 (m, 2H, H-4), 1.98 (m, 2H, H-7), 1.67 (m, 2H, H-3), 1.30 (m, 2H, H-8), 1.25 (m, 10H, H-[9-13]), 0.82 (t, 3H, J_{13-14} 6.2 Hz).

➪ *Calyptranthes concinna* DC.(vernacular name : "guamirim ferro") is a small tree 3-6m high that grows in araucarias forests and in other vegetation formations in Southern Brazil, in Argentina, Paraguay and Uruguay. The leaf essential oil, obtained in 0.24% yield, presents a sweet-woody olfactive note; it contains about 76% of elemicine (**2**), the three other major components being *trans*-isoelemicin **3** (2.5%), α-cadinol (2.3%) and β-caryophyllene (2.2%).
Another species of Myrtaceae family, *Backousia myrtifolia* Hook & Harvey (Leptospermoideae) has been shown to exist as an elemicin form [19] along with three other chemotypes : isoelemicin, methyleugenol and methylisoeugenol. It is noteworthy that our sample does not contain any trace of these two last components; this result means probably that the corresponding chemotypes will not be found in *Calyptranthes concinna*.

➪ *Calyptranthes tricona* Legrand (vernacular name: "guaburiti") is a tree 5 to 10m high which grows in the continental forests of Rio Grande do Sul and Santa Catarina. The fruits are edible. The leaf essential oil, obtained in 0.18% yield, is characterized by the presence of three major components. One is an acyclic sesquiterpene, (Z)-β-farnesene, which represents 26.6% of the mixture. The other two constituents - M = 206 (19.1%) and M = 220 (28.1%) - present very different retention indices on an apolar column (1664 and 1729 respectively) but are of a very close polarity as they co-elute on a capillary column coated with Carbowax 20M.

The spectral analysis of the components isolated by column chromatography on silica gel 60 (Merck, 70-230 mesh ASTM) eluted with pentane : diethyl ether / 90 : 10, indicates that both are chromene derivatives and differ from one another in the presence of a methyl group on the aromatic nucleus : the structures, corresponding to 5,7-dimethoxy-2-methyl-2H-benzopyranne **4** and 5,7-dimethoxy-2,8-dimethyl-2H-benzopyranne **5**, were proposed on the basis of their ^{1}H and ^{13}C NMR data.
Other components of the chromene series have already been found in different species of the Asteraceae family [20] and in minor amounts in other families as Rutaceae, Liliaceae and Cyperaceae.
Some of them are known for their biological activity : precocenes I and II (**6**, **7**), which are characteristic of the *Ageratum* genus[21], have been found to possess "insect-control" properties as anti-juvenile hormones.[22] Encecaline **8** is known for its fungicidal and insecticidal properties.[23]

The two structures identified in the essential oil of *Calyptranthes tricona* differ from the other more common chromenes by the respective positions of methyl and methoxy groups on the aromatic nucleus. These have been, to our knowledge, identified and described for the first time in the plant kingdom.

Spectral data :

4.- MS : 206 (M^+, 22), 192 (15), 191 (100), 176 (18), 147 (8), 77 (10), 69 (12).

- ^{13}NMR : d 159.0 (C-7), 156.6 (C-5), 154.1 (C-9), 122.2 (C-3), 119.1 (C-4), 105.2 (C-10), 94.1 (C-8), 92.1 (C-6), 71.9 (C-2), 55.9 (C-12), 55.7 (C-13), 21.4 (C-11).

- ^{1}NMR : d 6.7 (ddd, 1H, $J_{3,4}$ 9.8 Hz, $J_{2,4}$ 1.8 Hz, $J_{4,8}$ 0.5 Hz, H-4), 6.05 (dd, 1H, $J_{6,8}$ 2.3Hz, H-8), 6.02 (d, 1H, H-6), 5.43 (dd, 1H, $J_{2,3}$ 3.1 Hz, H-3), 4.91 (m, 1H, H-2), 3.78 (s, 3H, H-13), 3.75 (s, 3H, H-12), 1.42 (d, 3H, $J_{2\text{-}11}$ 6.6 Hz, H-11).

5.- MS : 220 (M^+, 25), 206 (16), 205 (100), 190 (20), 175 (15), 91 (12), 77 (11).

- ^{13}NMR : d 161.4 (C-7), 155.7 (C-9), 153.0 (C-5), 122.6 (C-3), 119.5 (C-4), 106.7 (C-8), 105.2 (C-10), 88.4 (C-6), 71.6 (C-2), 56.1 (C-12), 56.0 (C-13), 21.4 (C-11), 8.0 (C-14).

- ^{1}NMR : d 6.6 (dd, 1H, $J_{3,4}$ 9.9 Hz, $J_{2,4}$ 1.8 Hz, H-4), 6.04 (s, 1H, H-6), 5.47 (dd, 1H, $J_{2,3}$ 3.2 Hz, H-3), 4.90 (m, 1H, H-2), 3.84 (s, 3H, H-13), 3.80 (s, 3H, H-12), 1.99 (s, 3H, H-14), 1.41 (d, 3H, $J_{2,11}$ 6.6 Hz, H-11).

➪ Among the 27 species favouring the biosynthetic pathway derivated from the mevalonic acid, only five contain a majority of classical monoterpenes.

◆ Four of them belong to the sub-tribe of Myrcinae :

- *Calyptranthes grandifolia* Berg (vernacular name : "guamirim-chorao") is a tree 5-10m high that grows in the southern brazilian coastal forests. The leaf essential oil, obtained in 0.12% yield, was found to possess the following main components :

α-pinene (24.4%)
viridiflorene (2.5%)
β-pinene (31.5%)
bicyclogermacrene (2.3%)
β-caryophyllene (10.5%)
cis-calamenene (1.6%)
α-humulene (1.5%)
δ-cadinene (2.6%)
germacrene D (3.9%)
spathulenol (1.7%)

- *Marlierea eugeniopsoides* (Kaus. & Legrand) Legrand (vernacular name "guamirim-branco") is a tree 4-12m high that grows in the coastal forests of Paraná, Santa Catarina and Rio grande do Sul. The fruits are edible. The leaf essential oil, which was produced in 0.34% yield, was determined to contain the following compounds :

α-pinene (18.2%)
limonene (5.9%)
sabinene (6.2%)
1,8-cineole (5.3%)
β-pinene (6.9%)
γ-terpinene (6.0%)
myrcene (8.4%)
terpinolene (10.7%)
α-phellandrene (8.8%)
terpinen-4-ol (8.4%)

- *Myrcia acuminatissima* Berg (vernacular name "guamirim-ferro", syn. *Myrcia racemosa*, *Aulomyrcia acuminatissima*), is a tree 5-8m high that can be found in the southern brazilian coastal

forests. Chemical analysis of its leaf essential oil, obtained in 0.12%, revealed the presence of the following constituents :

α-pinene (4.1%)
α-terpineol (4.7%)
β-pinene (5.0%)
β-caryophyllene (8.1%)
1,8-cineole (2.9%)
spathulenol (7.5%)
linalol (22.3%)
caryophyllene oxide (5.5%)
terpinen-4-ol (5.2%)
caryophylladienol (2.2%)

- *Myrcia bombycina* (Berg) Niedenzu (vernacular name : "guamirim do campo") is a tree 3-8m high which grows in the southern brazilian araucarias forests, in Paraguay and Argentina. It was found to possess an oil yield of 0.95% and a chemical composition that can be summarized as follows :

α-pinene (23.9%)
β-phellandrene (2.2%)
β-pinene (12.4%)
α-terpineol (2.3%)
myrcene (3.2%)
elemol (2.2%)
α-phellandrene (3.1%)
γ-eudesmol (7.8%)
limonene (7.0%)
β-eudesmol (4.3%)
1,8-cineole (2.5%)
α-eudesmol (4.1%)

◆ The fifth species belongs to the Eugeniinae sub-tribe : *Eugenia prismatica* Legrand (vernacular name : "guamirim") is a treelet 3-8m high occurring in araucarias forets of Paraná and Santa Catarina. The oil content of the leaves was found to be 0.88%. The analysis of its volatile constituents revealed the following composition

α-pinene (13.4%)
viridiflorene (3.9%)
limonene (35.5%)
bicyclogermacrene (11.5%)
β-caryophyllene (2.7%)
spathulenol (2.2%)
aromadendrene (2.5%)
globulol (3.5%)
allo-aromadendrene (1.8%)
epiglobulol (2.1%)

The chemical composition of the essential oil of *Eugenia prismatica* is very different to that obtained for the other *Eugenia* species studied in the context of this work which are characterized by sesquiterpenic essential oils, with the exception of *Eugenia stigmatosa* whose leaves contain physeteric acid. It is worth mentioning at this point that *E. prismatica* is in the process of being reexamined by L. R. Landrum and will be reclassified soon in the Myrtinae sub-tribe under the name of *Mosiera prismatica*. It appears that this interesting example illustrates a certain agreement between the botanical and chemical data in the systematic classification of plants.

➪ The species characterized by a majority of sesquiterpenes are distributed into the three sub-tribes

◆ In the Eugeniinae, we counted 11 *Eugenia*, as well as the species *Hexachlamys itatiaiae*, *Myrciaria cuspidata*, *Paramyrciaria delicatula* and *Plinia brachybotrya*.

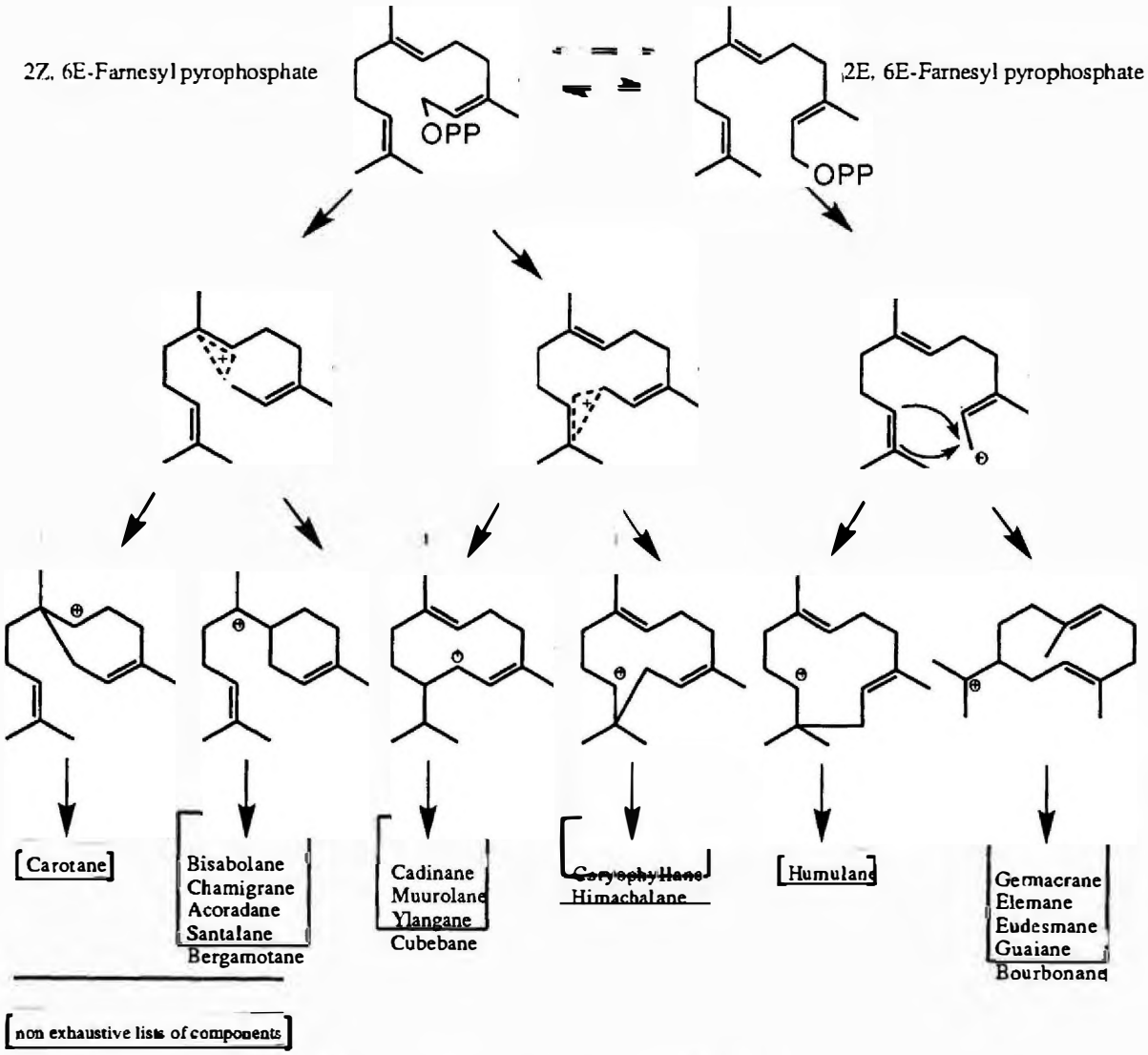
◆ In the group of Myrciinae, we noted three species of *Myrcia* : *Myrcia fallax*, *M. glabra* and *M. multiflora* as also *Gomidesia spectabilis* and *Myrceugenia euosma*.

◆ Finally, *Psidium cattleianum* and *Campomanesia xanthocarpa*, of which the essential oils were also rich in sesquiterpenes, represented the sub-tribe of Myrtinae.

In each case, the cyclic structures predominate over the acyclic structures in the sesquiterpenes group.

The chemotaxonomic importance of the cyclic sesquiterpenes has been noticed by Mann[24] : "often, different skeletal types co-occur in several species of a particular genus; and in some instances, a particular species has been assigned to a genus because it produces metabolites with skeletons characteristic of that genus".

Considering the high content of these metabolites in most of our samples, we had considered the possibility of proceeding to a chemotaxonomic classification according to their skeletal type. Each of these structures can be assumed to derive from the (2E, 6E)-farnesyl pyrophosphate or from the (2Z, 6E)-isomer, *via* intermediates which contain incipient cationic sites, according to pathways shown in Figure 5. The figure is somewhat simplified - especially in the cases of the four routes emanating from the (2Z, 6E)-FFP which proceed, probably, by nerolidyl



* D. E. Cane, C. Chang, R. Croteau, A. Saito and J. Shaskus, *J. Amer. Chem. Soc.*, 1984, 106, 1142
J. Mann, Secondary Metabolism, 2nd Ed., Clarendon Press, Oxford (1987).

Figure 5 : Main pathways involved in cyclic sesquiterpenes formation

pyrophosphate[25] and because each cationic species can of course undergo rearrangements, hydride shifts, so that, in some cases, more than one route is possible - but it does demonstrate the way in which the origins of the various skeletal types can be rationalized, at least on paper.

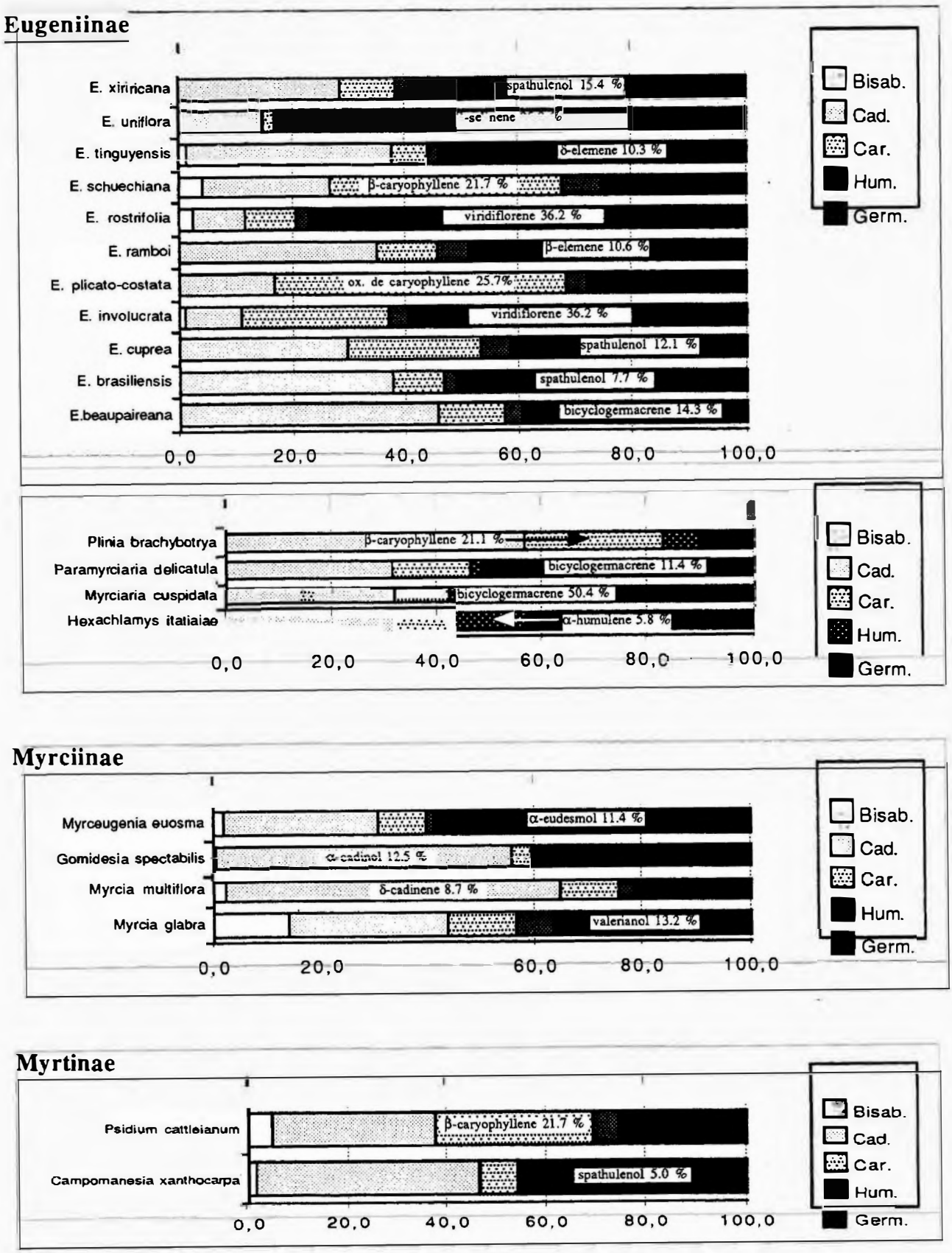
Among the six groups defined in that way, only the route to carotane skeleton is not represented; the identified components are distributed among the five other groups in diverse proportions.

For the convenience of this presentation, one sample has been separated from the others because of its very different chemical compostion, characterized by a high content of structures emanating from γ-bisabolene : it is the essential oil obtained in 0.35% yield from the leaves of *Myrcia fallax* (Richard) DC., a treelet widespread in the neotropics, ranging from eastern Mexico to southeastern brazilian coastal forests.

The chemical composition of this essential oil can be summarized as follows

6-methylhept-5-en-2-one (0.6%)
trans-γ-bisabolene **9** (1.9%)
trans-α-bergamotene (1.8%)
β-bisabolol (0.7%)
trans-β-bergamotene (0.6%)
bisabolol oxide A **10** (1.2%)
cis-β-farnesene (1.8%)
bisabolol oxide B **11** (0.8%)
β-bisabolene (1.1%)
α-bisabolol **12** (83.8%)

Figure 6 : Distribution of the different classes of cyclic sesquiterpenes
(only the species rich in sesquiterpenes are being considered)



After purification by column chromatography and determination of the optical rotation, the major constituent was found to be the levogyre enantiomer of the α-bisabolol : $[\alpha]_D$ - 65° ($CHCl_3$). The anti-inflammatory properties of this compound are well known; C. Franz has presented the results of breeding of different chemotypes of *Matricaria recutita* (Asteraceae) selected for their (-)-α-bisabolol, chamazulene or bisabolol oxides contents allowing the selection of a chamomile cultivar with high percentages of the two former constituents.[26] A high content of (-)-α-bisabolol has equally been noticed in the bark essential oil of another Asteraceae, *Vanillosmopsis arborea* Baker, collected in Brazil.[27]

- With regard to the 21 other samples, the relative percentages of each type of cyclic sesquiterpenes have been calculated and the result of this calculation is shown in Figure 6 (for each species, the major sesquiterpene is indicated, with its percentage in the essential oil, in the graph for the corresponding group).

The two principal cyclisation pathways are those giving germacrane (and the related components) and cadinane (and the related components) skeletons, while the humulane skeleton pathway is always in the minority. Furthermore,we can notice, globally, a predominance of the constituents originating from the (2E, 6E)-farnesyl pyrophosphate (in dark in Figure 6) in the Eugeniinae samples (10/15) whereas the sesquiterpenes derived from the (2Z, 6E)-isomer predominate in the two other sub-tribes.

These data were analysed statistically by the Principal Component Analysis method. Five variables, corresponding to the five groups of cyclic sesquiterpenes which have been previously defined, were used for this analysis and the first and second principal components accounted for 68% of the total variance of the data set. The projection of the factor scores *versus* the first and second principal components are plotted in Figure 7. As expected on the basis of our previous observation, the main contributors to the first and second factors were found to be related to "germacrane" and "cadinane" variables respectively.
A hierarchical ascendant classification performed with the variables obtained by the PCA method resulted in four groups demarcated in Figure 7 : the species representative of the Eugeniinae sub-tribe (Δ) are distributed in groups 1 and 4; the second group is constituted by only one sample *(Myrcia gomidesia)* belonging to the Myrciinae sub-tribe (●) whereas the third group is constituted by two other Myrciinae species along with *Campomanesia xanthocarpa* (❐), Myrtinae sub-tribe). Therefore, the four groups defined on this chemical basis, do not agree completely with the botanical classification.
We can observe quite a large distribution of samples, as the different skeleton types co-occur in several species of a particular genus and even in several genera, which did not allow the establishment of a new chemical classification. A more important sampling would perhaps give more interesting results; on the other hand, we must keep in mind that the biosynthetic pathways adopted in our classification must be regarded as hypothetical.

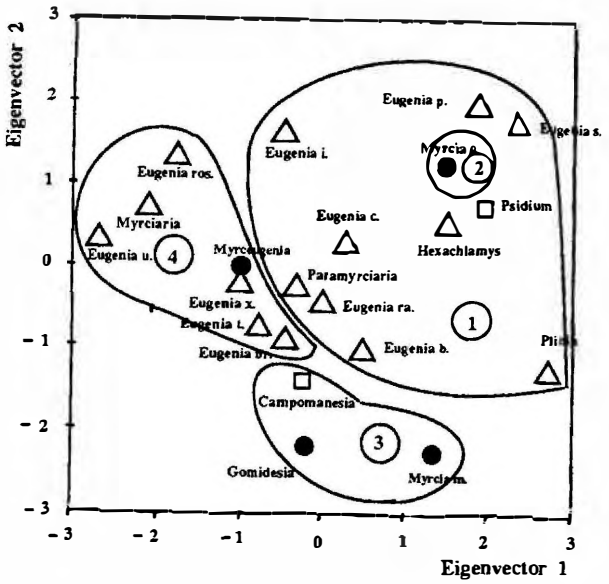


Figure 7 : Eigenvector 1 in function of eigenvector 2 referred to sesquiterpenic samples

Δ species belonging to the Eugeniinae sub-tribe
● species belonging to the Myrciinae sub-tribe
❐ species belonging to the Myrtinae sub-tribe

CONCLUSION

Out of the thirty species presented, only three of which had previously been studied for their volatile constituents, four are noteworthy for the chemical composition of their essential oil

- the volatile extract of *Eugenia stigmatosa* is almost exclusively composed of an olefinic fatty acid : physeteric acid, identified for the first time in the Myrtaceae family.

- the essential oil of *Calyptranthes tricona* is characterized by a large percentage of two new chemical structures, derived from the chromene nucleus. As other related components have been shown to have a strong biological activity, we can only hope for similar effects with our samples: their biological evaluation will begin soon.

- finally, two other species could be exploited for essential oil production and certainly deserve a more in depth investigation : *Myrcia fallax*, on account of its high content of α-bisabolol which is much appreciated by the cosmetic industry and *Calyptranthes concinna* for its elemicin.

The result is therefore globally positive with more than 10% success in the research of new sources of natural products.

D. Joulain, at the 13th International Congress of Flavour, Fragrances and Essential Oils in Istanbul, gave a number of good reasons for the exploration of new aromatic species, while warning scientists of the risk in conducting a research which would be too academic.
His speech addressed in particular university scientists involved in this subject & I quote : "when one inquires about the motivations for conducting research on essential oil by university research groups, rarely is the rather crucial consideration of economical interest taken into account".
It is fair to say that our educational mission does not train us to take profitability into account. But, is it not true, that research free from the constraints associated with the anguish of results is often more destined for success?
Universities and Industry have to work hand in hand; their fate is linked! In supporting us in the direction and the development of our research topics, the industrialists will gain from a more harmonious and fruitful approach in preparing for our childrens' future.

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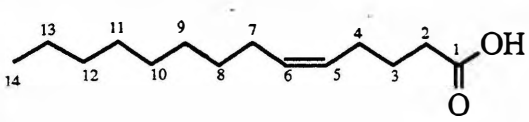

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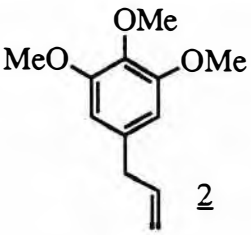

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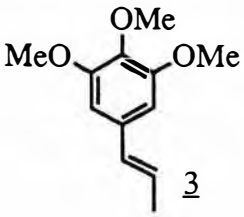

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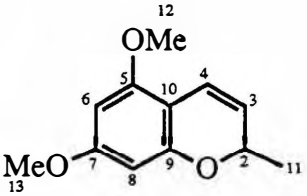

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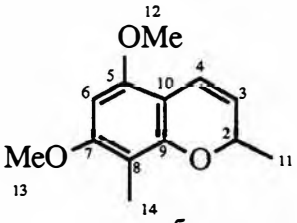

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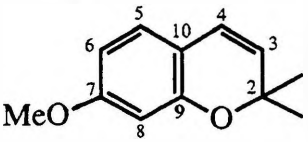

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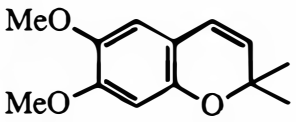

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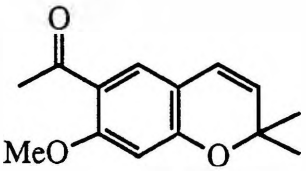

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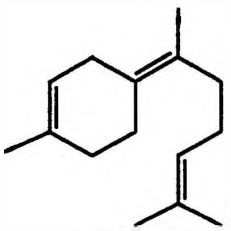

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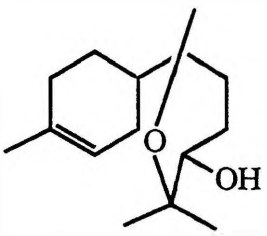

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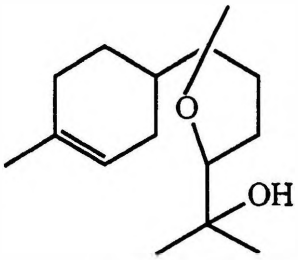

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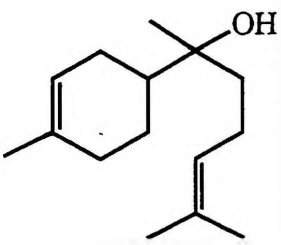

12

VOLATILE COMPONENTS OF SELECTED LIVERWORTS

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INTRODUCTION

Oil bodies of liverworts are rich sources of terpenoids and lipophilic aromatic compounds, several of which show strong mossy odour and interesting biological activity (1, 2). Recently we studied volatile components of 7 selected liverworts using TLC, GC-MS, column chromatography (silica gel and Sephadex LH-20), prep. TLC and prep. HPLC and isolated 17 sesquiterpenoids and two δ-lactones and detected three ethyl benzene derivatives.

EXPERIMENTAL

Plant Material - The liverworts were collected in the following locations. *Archilejeunea olivacea* (Hook. & Tayl.) Schiffn: Tom Bowrin Bay, New Zealand, December 1993, *Cheilolejeunea imbricata* (Nees) Hatt.: Sanagouchi-son, Myoudo-gun, Tokushima, Japan, *Leptolejeunea elliptica* (Lehm. et Lindenb.) Schiffn: Aioicho, Naka-gun, Tokushima, Japan, in Jan. 1995, *Dicranolejeunea yoshinagana*: Kamigun, Beppu valley, Kochi, Japan, in July 1994.

Lopholejeunea nigricans:Taniguchi, Kamikatsu-cho, Katsuura-gun, Tokushima, Japan, in March 1995, *J. vulcanicola* (Schiffn.) Steph.: Okayama prefecture, Japan, in May 1995, *J. hattoriana* (Amak.) Amak.: Niigata prefecture, Japan in July 1995.

TLC: TLC was carried out on silica gel precoated glass plates with ***n***-hexane-EtOAc (1:1 and 4:1). Detection was with Godin' s reagent.

GC/MS: GC/MS analysis performed from 50 °C isothermal for 3 min, then 50-250 °C at 5 °C/min. A fused silica column coated with DB-17 (30 m x 0.25 mm i.d., film thickness 0.25mm) was used. The temperature programming of the GC-MS analysis for the examination of enatiomerical purity was performed from 50 °C for 3 min, then 50-230 °C at 3 °C/min, and finally isothermal at 230 °C for 5 min. A fused-silica column coated with β-Dex 120 (30 m x 0.25 mm i.d., film thickness 0.25 mm) was used.

Spectral Data: NMR spectra were recorded at 150, 100 or 50 MHz for ^{13}C and 600, 400 or 200 MHz for ^{1}H. EIMS were measured at 70 eV.

EXTRACTION AND ISOLATION

The air-dried liverworts (*A. olivacea*, *C. imbricata*, *D. yoshinagana*, *L. nigricans*, *J. vulcanicola* and *J. hattoriana*) were extracted with ether for 1 week. Filtration and solvent evaporation gave a green oil which was chromatographed on silica gel using n-hexane and EtOAc gradient. The fractions obtained were further purified by column chromatography on Sephadex LH-20 using MeOH-CH_2Cl_2 (1:1), prep. TLC or prep. HPLC. *L. elliptica* was gently washed with water and plant material ground in a mortar with ether and then filtered through a short column packed with celite. Concentration of the filtrate was carried out under a stream on N_2 gas. The green oil was analyzed by GC/MS.

The following compounds were isolated: (*R*)-dodec-2-en- 1,5-olide [**1**] (34 mg), (*R*)-tetradec-2-en- 1,5-olide [**2**] (3.1 mg) from *C. imbricata* (4.0 g dried material), three ethyl benzene derivatives, 1-ethyl-4-hydroxybenzene [**3**] (3.6% in GC), 1-ethyl 4-methoxybenzene [**4**] (71.8 %) and 1-ethyl 4-acetoxybenzene [**5**] (24.5 %), from *L elliptica* (100 mg). The structures of compounds **3 - 5** were confirmed by the comparison of retention times and mass spectra with those of authentic samples. Olivacene [**6**] (6.3 mg) and β-monocyclonerolidol [7] (23 mg) from *A. olivacea* (16.0 g), three pinguisane-type sesquiterpenes, deoxopinguisone |**8**| (6.0 mg), 14-oxodeoxopinguisone |**9**| (2.0 mg), 14-

acetoxydeoxopinguisone [**10**] (6.8 mg) and one neopinguisane type sesquiterpene, 5-neopinguisenol [**11**] (136.8 mg) from *D. yoshinagana* (31.0 g), four pinguisanes, [**8**] (6.0 mg), [**9**] (2.0 mg), [**11**] (24 mg), 14-hydroxydeoxopinguisone [**12**] (3.0 mg), methyl deoxopinguisone-14-ate [**13**] (3.0 mg) and deoxopinguisone-14,12-olide [**14**] (14.2 mg) from *L. nigricans* (15.0 g), two chiloscyphane-type sesquiterpenoids, dihydrochiloscypholone [**15**] (4.6 mg) and chiloscypholone [**16**] (33.8 mg) (2) from *J. vulcanicola* (14.2 g), and one acorane-type, acoradiepoxide [**17**] (3.6 mg) and six cuparene-type sesquiterpenoids, cuparene (684 mg), neocuprenenol [**18**] (5.6 mg), cuparadiepoxide [**19**] (22.6 mg), epi-cuparadiepoxide [**20**] (18.5 mg), cuprenenol [**21**] (36.0 mg) (1), and rosulantol [**22**] (32.0 mg) (1) from *J. hattoriana* (1.14 Kg).

RESULTS AND DISCUSSION

Compounds [**1, 6, 9-14 15** and **17-20**] were newly isolated from natural sources and their absolute structures established by a combination of spectral data, X-ray crystallographic analysis, chemical degradation and modified Mosher's method. *C. imbricata* and *L. elliptica* are very important species among the *Hepaticae* from the view point of fragrance chemistry. Compounds [**1, 2**] which are chiroptically pure (more than 99 % *ee* estimated by GC-MS analysis using chiral capillary column) are responsible for the strong milky odour (3) of *C. imbricata.* Powerful phenol-like mossy odour of *L. elliptica is* due to the mixture of simple 4-ethylphenol [**3**] and its derivatives [**4, 5**].

Olivacene [**6**] is the first naturally occurring sesquiterpene hydrocarbon, although it has been reported as the synthetic product (4). Matsuo et al. (5) reported the presence of 4 and three monoterpene hydrocarbons, α-pinene, β-pinene and camphene in *L. elliptica.* The present species, however, does not contain any terpenoids.

The isolated compounds from the present species are also significant for the chemosystematics of the Jungemanniales liverworts. There are no chemical affinity between *A. olivacea*, *C. imbricata* and *L. elliptica* which belong to the Lejeuneaceae. *D. yoshinagana* and *L. nigricans* (Lejeuneaceae) are closely related chemically because these species produce characteristic pinguisane-type sesquiterpenoids. *J. vulcanicola* is chemically similar to *Chiloschyphus pallescens* since both species elaborate the same chiloscyphane-type sesquiterpenoids (2). *J. hattoriana* chemically resembles *J. rosulans* (1) since both species elaborate cuparane-type sesquiterpenoids whereas there is no chemical affinity between *J. hattoriana* and *J. vulcanicola* since the latter species produces chiloscyphane-type sesquiterpenoids. The structural elucidation of the new compounds will be reported elsewhere.

Acknowledgments

The authors are grateful to Dr. J. Braggins (Univ. of Auckland, New Zealand) for the collection and identification of the liverwort, *A. olivacea.* This work was supported in part by a Grant in Aid for General Research (08459026 YA) from the Ministry of Education, Science and Culture, Japan.

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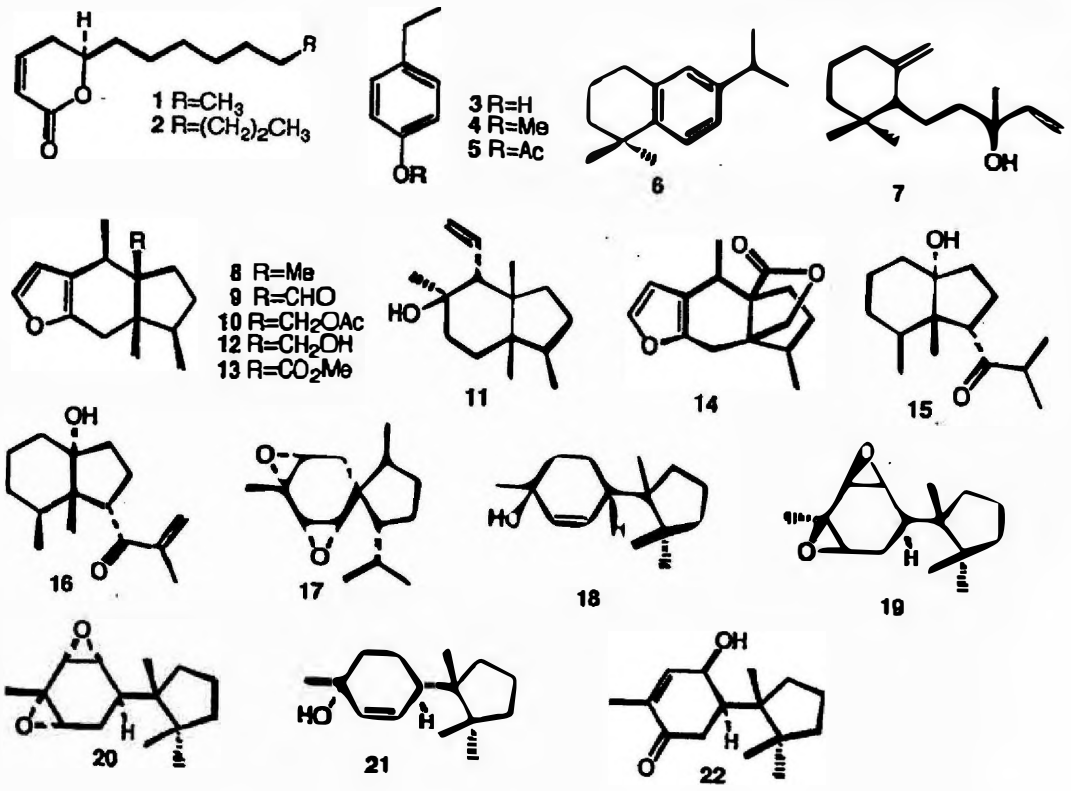

Figure 1. Isolated compounds from selected liverworts

THE ESSENTIAL OIL COMPOSITIONS OF THREE *ABIES* SPECIES GROWING IN SOUTH BALKANS

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INTRODUCTION

The genus *Abies* belongs to the *Pinaceae* family and is represented by more than 50 species, found mainly in northern temperate regions. The chemical composition of the volatile metabolites, produced by several species of the genus as well as their ecological importance, in the host-pest relationships, have been the subject of numerous studies (Ross et al. 1996, Regimbal et al. 1994, Wagner et al. 1990, Russell et al. 1976). In South Europe and more specifically in the Balkan Peninsula only two recognized species are forming extensive natural forests: the common Silver Fir (*Abies alba* Miller), found in Spain, Poland and the Balkans and the Greek Fir (*Abies cephalonica* Loudon), native to the high mountains of Greece. Besides these two distinct species in the same area a third morphotype is found (*Abies borisii-regis* Mattf.) which is probably of a hybrid origin between *A. alba* and *A. cephalonica*. *A. borisii-regis* shows intermediate morphoanatomic characteristics between the putative parental species (Chater 1993).

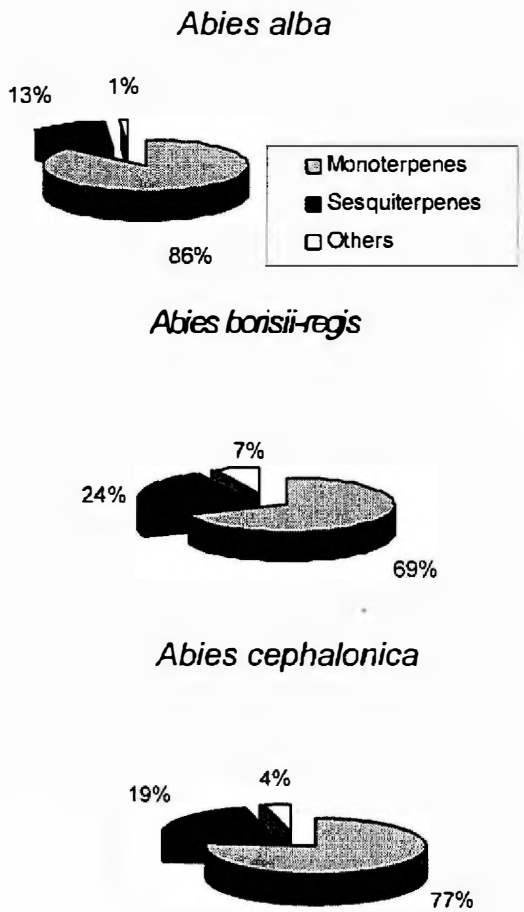
In order to check the phylogenetic relationship, as it is represented by their secondary metabolites, the leaf essential oil of a statistically representative sample of sympatric trees from the 3 taxa was obtained. The composition of the volatile metabolites was investigated by GC and GC-MS analyses.

MATERIAL AND METHODS

The study area is the Southern Balkan tip of Europe and the plant material was collected in natural forests. Based on their morphoanatomical characteristics, a total of 23 trees were chosen as representatives of the 3 *Abies* species.

Needles were gathered in a random manner from various parts of the tree crowns to reduce plant plasticity effects. The needles were cut to small pieces and hydrodistilled for 2 hr with a water cooled receiver, to reduce hydrodistillation overheating artifacts.

The essential oils were analyzed by GC (DB-1 and DB-5 columns) and GC-MS. The identification of the chemical constituents was based on comparison of their R_ts and mass spectra with those obtained from authentic samples and/or libraries spectra. The composition data of all detected constituents were submitted to cluster analysis. The main constituents of the essential oils of the three studied *Abies* species can be seen in Table 1 .



RESULTS AND DISCUSSION

The hypothesis of Panetsos (1975) that *A. borisii-regis* is a hybrid between *A. alba* and *A. cephalonica* is supported. Moreover, it is chemotypically closer to the southern parental species (*A. cephalonica*) than the northern one (*A. alba*).

A. alba is differentiated from the other two species by having a higher percentage of fenchyl acetate. The variation within the *A. alba* population is declared by the relative concentrations of α-pinene, limonene and the almost equal concentrations of camphene and fenchol. The same terpenes were found by Lang (1994) who investigated provenance differentiation in terms of the terpene content of the cortex resin of twigs. Lang also found α-phellandrene to be an important component of variation in European populations of silver fir.

The results obtained in this study could be helpful in reforestation projects where they indicate that local genetic diversity which is manifested at the level of the secondary metabolites must be maintained in *A. cephalonica* and *A. borisii-regis* populations. Some unique features of these two species such as the wider climatic tolerance of the first and the ability of the second to withstand competition by deciduous trees such as *Fagus sylvatica* must be maintained well represented in the Balkan gene pool.

Table 1. Major volatile metabolites of the three studied *Abies* species

	***Abies cephalonica* (%)**	***Abies borisii-regis* (%)**	***Abies alba* (%)**
α-pinene	16.02 (3.94)	8.17 (3.03)	10.87 (1.53)
camphene	9.26 (1.59)	10.19 (3.46)	15.32 (1.20)
ß-pinene	21.26 (3.97)	11.38 (9.14)	19.78 (3.96)
limonene	14.22 (6.77)	22.64 (9.81)	11.04 (4.12)
fenchyl acetate	1.81 (2.35)	5.41 (3.32)	14.15 (3.06)
globulol	7.22 (2.24)	7.76 (3.43)	1.49 (0.83)

Standard deviations are shown in parentheses

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ENANTIOMERIC COMPOSITION OF THE MONOTERPENE HYDROCARBONS OCCURRING IN THE NEEDLES OF *JUNIPERUS COMMUNIS* L. VAR. SAXATILIS PALL. (NORWEGIAN MOUNTAIN JUNIPER)*

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INTRODUCTION AND RESULTS

The essential oil of *Juniperus communis* L. var. saxatilis Pall. (Norwegian mountain juniper) has been the object of our studies on essential oil components for several years. The juniper oil is of interest due to the high concentration and the variety of the volatile hydrocarbons in the plant material. By capillary GC we could indicate more than 300 separate components, 55 of these in the fraction of the monoterpene hydrocarbons.

The plant material was collected at various times during January - May 1996 in Valdres,Norway at about 1200 m above sea-level. The plant material was analyzed on its total content of essential oil as well as on the variation of individual components during a growing season. We were interested in the main constituents as well as the distribution of the enantiomeric monoterpene hydrocarbons as this group represents the main constituents of the oil. The plant material was subjected to different modes of volatile oil extraction such as Clevenger, Likens - Nickerson and Supercritical Fluid Extraction to verify the occurrence of the main constituents. To identify the separated compounds we applied retention indices on two different stationary phases (DB-5 and CP-WAX52B), and 60 m capillary Gccolumns. Total oil and separate fractions were analyzed by GC. Two coupled GC - MS instruments were used to verify the mass spectra obtained and the library given by R.P. Adams used for the tentative identification. We have focussed our attention on the main constituents where the mass spectra obtained showed a very close resemblance with the published data and where therefore the tentative identification seemed reasonably sure when the retention indices was added. The monoterpene hydrocarbon fraction was subjected to further analysis by applying stationary phases used for enantiomeric separation. We also coupled Carbowax columns in tandem to the enantiomeric columns to obtain a better separation. Especially Königs special column material for terpene hydrocarbons was important for the separation of optical isomers. A total separation of the enantiomeric monoterpene hydrocarbons could not be achieved on one single column.

The main constituent in the Norwegian juniper oil is (-)-Sabinene which account for more than 60% of the total oil depending upon the harvesting time of the plant material. P-Cymene was found in the freshly distilled oil and could be formed by prolonged distillation. The essential oil of juniper contains a very large number of hydrocarbons and could be used as a terpene hydrocarbon standard mixture. Figure 1 shows the chromatogram of the total juniper oil as well as the hydrocarbon fraction. The oil was fractionated on Bakerbond-Silica columns using different solvents as eluent. A description of the procedure will be found in earlier publications by our group. Figure 2 shows the main tentatively identified constituents using retention indices, GC-MS spectra on different columns and terpene library search for the best fit of spectra.

* Studies on Terpenes and Terpene Products. LXXVII

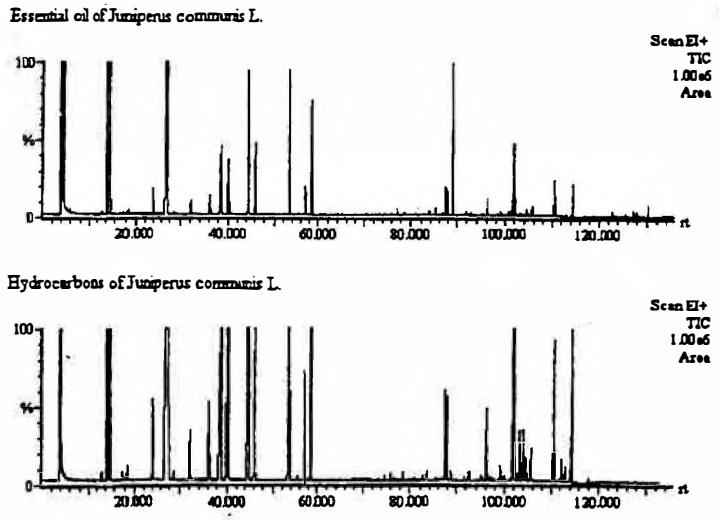

Figure 1. Chromatogram of the essential oil of *Juniperus communis* L.

A chromatogram of the total essential oil as well as that of the hydrocarbon fraction of *Juniperus communis* L. var. saxatillis is shown. Such a run is used for screening purposes to indicate the main group of constituents.

	TRICYCLENE	29	CIS MUUROLA-4(14),5-DIENE
	α-THUJENE	30	γ-MUUROLENE
	α-PINENE	31	GERMACRENE D
	α-FENCHENE	32	β-BISABOLENE
	CAMPHENE	33	β-SELINENE
	β-PINENE	34	α-SELINENE
	SABINENE	35	α-MUUROLENE
8	δ-2-CARENE	36	α-BULNESENE
9	δ-3-CARENE	37	γ-CADINENE
10	MYRCENE	38	SELINENE <7-EPI-α>
11	α-PHELLANDRENE	39	δ-CADINENE
12	α-TERPINENE	40	CADINA-1,4-DIENE
13	LIMONENE	41	α-CADINENE
14	β-PHELLANDRENE	42	SELINA-3-7(11)-DIENE
15	γ-TERPINENE	43	GERMACRENE B
16	TRANS-β-OCIMENE	44	TRANS SABINENE HYDRATE
17	p-CYMENE	45	LINALOOL
18	TERPINOLENE	46	CIS SABINENE HYDRATE
19	α-CUBEBENE	47	MENTH-2-EN-1-OL <CIS PARA>
20	ISOLEDENE	48	MENTH-2-EN-1-OL <TRANS PARA>
21	α-COPAENE	49	BORNYL ACETATE
22	β-BOURBONENE	50	METHYL ETHER THYMOL
23	β-ELEMENE	51	TERPINENE-4-OL
24	CYPERENE	52	TRANS PULEGOL
24	β-CARYOPHYLLENE	53	TRANS PIPERITOL
26	α-GUAIENE	54	CIS PIPERITOL
27	α-HUMULENE	55	α-TERPINEOL
28	ISO GERMACRENE D	56	PERRILLA KETONE

Figure 2. Identified compounds in *Juniperus communis* L. subsp. saxatillis Pall.

The enantiomeric composition of the juniper oil achieved through GC-analysis on combined stationary phases is as follows:

1. Tricyclene
2. (-)- alpha-Fenchene
3. (+)-beta-Pinene
4. (-)-beta-Pinene
5. (+)-Camphene
6. (-)-Camphene
7. (+)-alpha-Pinene
8. (-)-alpha-Pinene
9. (-)-Sabinene
10. delta-2-Carene
11. delta-3-Carene
12. (+)-Limonene
13. (-)-Limonene
14. (+)-alpha-Phellandrene
15. (-)-alpha-Phellandrene
16. Myrcene
17. Terpinolene
18. alpha-Terpinene
19. gamma-Terpinene
20. p-Cymene
21. trans-beta-Ocimene

CONCLUSIONS

The complex composition of essential oils requires very strict control of the chromatographic conditions to obtain optimum separation power of the analytical system. Careful monitoring of the temperature of the

chromatographic column is necessary and temperature variation of the Kovats indices on Carbowax stationary phases is often overlooked. The different extraction/isolation procedures of essential oils from plant material will result in a quantitative variation of the constituents present. Reported quantitative data with several decimals is of little value. Due to variation in the mass spectra reported in libraries and uncertainties in exact identification through GC - MS analysis and library search there is a strong need for standard mixtures of terpenes. Selected essential oils showing small variation in composition could be a solution to this problem.

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COMPOSITION OF THE ESSENTIAL OIL OF BERRIES OF *JUNIPERUS COMMUNIS* L. OF CROATIAN ORIGIN

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INTRODUCTION AND RESULTS

The plant material was collected at Perusic,Lika in Croatia. The dried plant material was subjected to Clevenger distillation,Likens-Nickerson extraction and Supercritical Fluid Extraction to verify the presence of the main constituents of the essential oil. The isolated oil was separated into chemical groups on Bakerbond Silica columns. Total essential oil and oil fractions were subjected to capillary gas chromatography on column with different polarity of stationary phases.Mainly DB-5 and CP-WAX 52 CB were used as many retention indices of terpenes have been reported on these stationary phases. The main constituents were tentatively identified by GC-MS analysis and library search on the ADAMS terpene library for spectral fitting.

As can be seen in Figure 1 , the main group of compounds in this oil is hydrocarbons. A closer analysis of the monoterpene hydrocarbons reveals the same constituents as in the Norwegian mountain juniper, however the quantitative distribution is different. In our sample we could not detect sabinene among the monoterpene hydrocarbons.

As was observed when the oils isolated by different techniques was analyzed by GC there were large quantitative differences between the individual constituents. The main constituents,however, remained the same. These compounds,tentatively identified, are listed in Table 1. Figure 1 shows chromatograms of the total oil and of the hydrocarbon fraction of the oil.

CONCLUSIONS

The essential oil from berries of Juniperus communis L. of Croatian origin contains as main constituents hydrocarbons.The main monoterpene hydrocarbons are alphaPinene and beta-Pinene. The main sesquiterpene hydrocarbons are the germacrenes.The quantitative composition of the

essential oil varies according to the isolation procedure.Distillation time and storage also greatly influences the quantitative composition

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Table 1. Identified compounds in *Juniperus communis* l. (Croatia)

1. TRICYCLENE
2. α-THWENE
3. α-PINENE
4. α-FENCHENE
5. CAMPHENE
6. THUJA-2,4(10)-DIENE
7. VERBENENE
8. β-PINENE
9. MYRCENE
10. δ-2-CARENE
11. α-PHELLANDRENE
12. δ-3-CARENE
13. α-TERPINENE
14. p-CYMENE
15. LIMONENE
16. β-PHELLANDRENE
17. -β-OCIMENE
18. -β-OCIMENE
19. y-TERPINENE
20. γ-TERPINOLENE
21. MENTHATRIENE (1,3,8-PARA)
22. CYCLOSATIVENE
23. α-COPAENE
24. β-BOURBONENE
25. CYPERENE
26. γ-ELEMENE
27. β-HUMULENE
28. SANTALENE (EPI-α)
29. β-HUMULENE
30. GERMACRENE D
31. β-SELINENE
32. (CIS-β-GUAIENE
33. VIRIDIFLORENE
34. GERMACRENE A
35. γ-CADINENE
36. (TRANS) CALAMENE
37. α-CADINENE
38. GERMACRENE β
39. CIS SABINENE HYDRATE
40. TRANS SABINENE HYDRATE
41. (CIS-PARA) MENTH-2-EN-I-OL
42. (TRANS -PARA)MENTH-2-EN-I-OL
43. BORNEOL
44. TERPINENE-4-OL
45. (PARA) CYMEN-8-OL
46. α-TERPINEOL
47. CIS PIPERITOL
48. TRANS PIPERITOL
49. (TRANS) CHRYSANTHENYL ACETATE
50. METHYL CITRONELLATE
51. BORNYL ACETATE

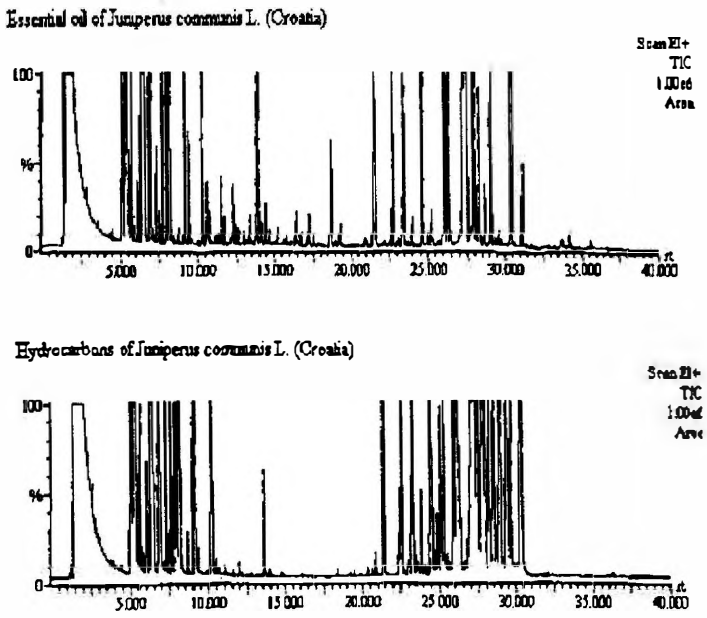

Figure 1. Chromatogram of the total oil and hydrocarbon fraction of *Juniperus communis* L. oil

COMPARATIVE STUDY ON THE NEEDLE ESSENTIAL OILS OF *JUNIPERUS COMMUNIS* L. AND *J. COMMUNIS VAR. NANA* WILD. IN BULGARIA

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INTRODUCTION

The common juniper (*Juniperus communis* L.) is a representative for the group of medicinal plants that contain essential oils with antiseptic, antiinflammatory and disinfective main activity (Stoyanov, 1973). All the organs of this species contain essential oil but it is obtained from the twigs and foliage as well as from the cones.

The genus *Juniperus* (fam. *Cupressaceae*) in Bulgaria is represented by 4 naturally occurring and some cultivated species. The most widely spread among them is the common juniper. *Juniperus communis* L. This is a very variable species with several geographical varieties and some garden forms, as well. It is spread in the lower parts of the medium mountain belt, between 500 and 1700 m a.s.l. whereas in the higher parts of the mountains *Juniperus communis* var. *nana* Wild. is to be found. This is a typical high-mountainous form adapted excellently to the tree line (1700-2000-2200 m a.s.l.).

The purpose of this work is to make a comparative study on essential oils from the needles of *Juniperus communis* L. and *Juniperus communis* var. *nana* Wild.

MATERIALS AND METHODS

The plant material has been collected from natural habitats of *J. communis* L. at 1200 m a.s.l. in the Plana planina mountain and of *J. communis* var. *nana* Wild. at 1800 m a.s.l. in the Vitosha mountain.

From each sample plot, 6 and 5 model shrubs have been investigated, respectively. The juniper twig samples (young branches with a width up to 0.8 cm) were collected during the winter period from the southern central part of the crown to avoid the influence of the biochemical processes during the growing period both on the biosynthesis of essential oil and on its contents (Von Rudloff, 1972).

The essential oil was extracted by a Clevenger microapparatus with a triple reiteration of each sample.

The analysis of the essential oils was made in accordance with the Bulgarian State Standard 9200-80 "Method for analysing essential oils". Gas chromatography on an apparatus Perkin Elmer 8200 was carried out under the following working conditions:

- Supercowax column with a length of the wave 25 m;
- inner diameter 0.25 mm;

- heating rate 5 °C/min.;
- final temperature 210 °C for 15 min.;
- linear velocity of the carrier gas - 14 m/s.;
- splitter 1:100;
- a temperature of the injector and detector 210 °C.

RESULTS

The identified components of the oils from *Juniperus communis* L. and *Juniperus communis* var. *nana* Wild. are presented in Table 1.

The obtained results show that the investigated oils have identical quality (composition) in which the contents of terpene hydrocarbons dominates.

The basic macrocomponents in the essential oil-composition in both species are α- and β- pinene. The difference is only quantitative - in *Juniperus communis* L. the average content of α pinene is 20.1 % average, in *J. communis* var. *nana* Wild. it is 32.74 %. Svendsen *et al.* (1985) determined a higher quantity of α pinene in *Juniperus communis* L. (57 %) and lower value in *J. communis* var. *nana* Wild. (20 %), in Norway. Regarding β pinene the correlation is the opposite - 15.1 % and 3.8 %, respectively.

Δ^3 carene can be found in smaller quantities and in *Juniperus communis* L. it is almost 5 times less (0.44 %) than in *J. communis* var. *nana* Wild. (2.18 %).

The limonene quantity had similar values in both species - 10.49 % and 9.02 %, respectively.

The rate of oxygen-containing compounds, such as terpinene 4-ol, in *Juniperus communis* L. is 0.71 % and in *J. communis* var. *nana* Wild. - 1,4 7%, respectively. The bornilacetate content of *J. communis* var. *nana* Wild. is characterized by almost double high values (2.45 %) and in *Juniperus communis* L. - 1.39 %.

CONCLUSION

The studied essential oils from the needles from *Juniperus communis* L. and *J. communis* var. *nana* Wild. belong to the essential oils with clearly determined terpene pattern. The fragrance is a typical, richly balsamic, and reminding of pine fragrance that can be of a particular interest for the perfumery, cosmetic and pharmaceutical industries.

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Table 1. Components of the chemical composition of needle essential oils

Juniperus		α-Pinene	β-Pinene	Δ^3-Carene	Limonene	Terpinene-4-ol	Bornil-acetate
communis	1	17.50	33.20	0.13	4.55	0.57	1.15
	2	10.98	4.12	0.02	6.34	0.88	2.56
	3	30.78	8.35	0.22	13.05	1.05	1.18
	4	15.58	24.55	0.54	13.92	0.62	1.23
	5	20.02	10.43	0.81	11.54	0.71	1.13
	6	25.80	10.12	0.93	13.54	0.71	1.13
	M ± m	20.1 ± 2.66	15.12 ± 4.18	0.44 ± 0.14	10.49 ± 1.5	0.71 ± 0.07	1.39 ± 0.21
Juniperus	1	34.15	1.05	2.15	8.12	1.19	2.39
communis	2	42.11	4.12	2.18	8.62	1.53	3.04
var. nana	3	28.28	2.23	2.05	10.05	1.54	2.28
	4	29.13	5.62	2.24	8.32	1.22	2.11
	5	30.05	5.84	2.30	10.05	1.85	2.43
	M ± m	32.74 ± 2.24	3.77 ± 0.84	2.18 ± 0.04	9.02 ± 0.37	1.47 ± 0.11	2.45 ± 0.14

344

THE ESSENTIAL OIL OF *AEGOPODIUM PODAGRARIA* L.

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ABSTRACT: The essential oil obtained by hydrodistillation from the aerial parts of *Aegopodium podagraria* L. (Apiaceae) was subjected to GC/MS analysis on different stationary phases. 40 compounds representing 70.2 % of the essential oil could be identified. Non terpenoid aldehyds, ketones, alcohols and acetats were present in minor amounts (up to 0.7 %). Limonene (9.4 %), ß-myrcene (5.9 %) and γ-terpinene (6.1 %) represented the main compounds in the monoterpene fraction. Germacrene D (11.0 %), α- and ß-farnesene (4.6 % and 3.1 %, respectively) and ß-elemene (3.0 %) were major constituents of the sesquiterpene fraction. Due to pharmacological as well as GC/MS investigations of fractions of an aqueous extract of the herb of *Aegopodium podagraria* the essential oil does not seem to contribute to the antiphlogistic activity of this extract.

KEY WORD INDEX: *Aegopodium podagraria* L., Apiaceae, GC/MS, essential oil composition, antiphlogistic activity.

INTRODUCTION: *Aegopodium podagraria* L. (Apiaceae) (*Ger.* Giersch, Geißfuß; *Engl.* Ashweed, Goutwort) is growing as a creeping perennial with typically umbelliferous flowers and 1 - 2 trefoil leaves. It is known as a troublesome weed in Europe. Nevertheless, in traditional European medicine it is used against gout, rheumatism, abscesses and hemorrhoids. Apart from hot water extracts of the herb the application of a poultice of the squashed fresh herb is known (1-3).
Hitherto concerning the essential oil composition of *Aegopodium podagraria* a head space analysis of the volatiles released from the flowers (4) as well as a TLC investigation (5) have been reported. As part of a phytochemical survey of this medicinal plant we now present the results of our investigation of the essential oil which was obtained by hydrodistillation of the flowering herb.
Furthermore fractions of an aqueous extract which showed inhibitory activity in the carrageenin induced rat paw oedema model (6) were analysed for essential oil constituents by GC/MS.

EXPERIMENTAL: Plant Material - The flowering aerial parts were used. Wild plants were collected near Graz, Austria, in July 1995 and 1996. Voucher specimen are deposited at the herbarium of the Institute of Pharmacognosy, University of Graz.

Isolation of the Essential Oil - The oil was obtained by hydrodistillation from the fresh plant material (3 h, apparatus according to the Austrian Pharmacopoea). The oil (0.04 % yield on a fresh weight basis) was light green and of a peculiar, unpleasant aroma.

Qualitative and Quantitative Analysis - GC/MS analyses were carried out on a Hewlett-Packard 5890 Series II Plus gas chromatograph interfaced to a Hewlett-Packard 5989B mass spectrometer. Separations were performed on HP5MS (30 m x 0.25 mm I.D., film 0.25 µm), Ultra 1 (50 m x 0.20 mm I.D., film 0.11 µm) and J&W DB-Wax (60 m x 0.25 mm I.D., film 0.25 µm) capillary columns. Helium was used as carrier gas (0.9 ml/min constant flow). The oven temperature was programmed as: 70 to 230 °C with 2 °C/min. Injector and interface temperatures

were 230 °C and 250 °C, respectively. EI mass spectra were recorded at 70 eV ionization voltage over the mass range 40-400 u. Samples (0.5 µl of oil solutions 1:10 in hexane) were injected by split injection (1:50). Temperature programmed retention indices of the compounds were determined relatively to the retention times of a series of n-alkanes. The constitutents of the oil were identified by GC/MS comparison with reference compounds, matching their mass spectra with reference libraries (Wiley 138 K and laboratory-own data base) and comparison of the retention indices with those reported in literature (7-9). Quantification of the relative amounts of the individual components was done according to the Area Percent Method without consideration of calibration factors.

Antiphlogistic activity - For investigation of the antiphlogistic activity the air-dried aerial parts of *Aegopodium podagraria* were used. Preparation of the aqueous extract as well as pharmacological testing in the carrageenin-induced rat paw oedema model are described elsewhere (10). Separation of the aqueous extract was performed on Sephadex LH-20/MeOH and Sephadex G-25/EtOH-H_2O (1:1, v/v). Combined fractions were submitted to pharmacological testing. The pharmacologically active fractions were analysed by GC/MS for essential oil constituents as described above.

RESULTS AND DISCUSSION: The essential oil of *Aegopodium podagraria* which was obtained by hydrodistillation of the fresh herb was investigated. By GC/MS analysis on different stationary phases (100 % methyl silicone, 5% phenyl-95 % methyl silicone, polyethylene glycole) 40 compounds could be identified. Table I gives the identified compounds listed in order of their elution from the DB-Wax column. A total ion chromatogramm of a separation on the DB-Wax column is shown in Fig. 1.
Non terpenoid aldehyds, ketones, alcohols and acetates were present in minor amounts (up to 0.7 %). In the monoterpene fraction limonene (9.4 %), ß-myrcene (5.9 %), γ-terpinene (6.1 %), *cis*-ß-ocimene (3.0 %), ß-pinene (2.9 %) and p-cymene (2.4 %) represented the main compounds. Oxidized monoterpenes were detected in amounts less than 1.0 %.
The essential oil of *Aegopodium poda-*

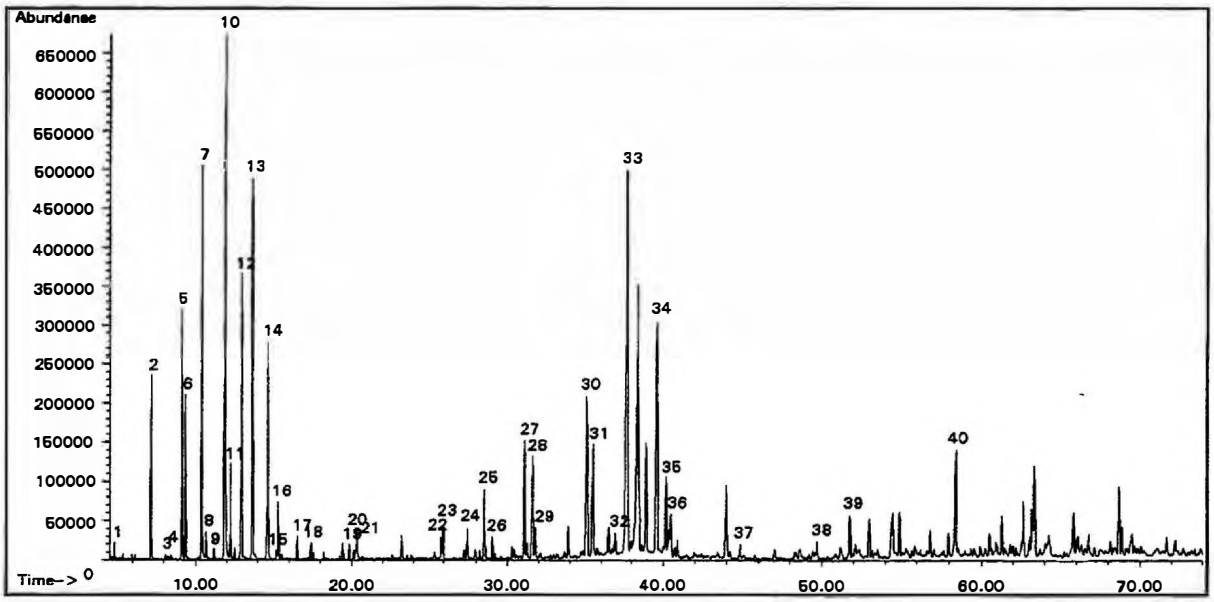

Fig. 1. Total ion chromatogramm of the essential oil obtained by hydrodistillation of the fresh herb of *Aegopodium podagraria* L.
(J&W DB-Wax, 60 m x 0.25 mm I.D., 0.25 µm, 70 - 230 °C, 2 °C/min)

graria proved to be rich in sesquiterpenes (>1 % calculated on basis of peak area %). Germacrene D (11.0 %), α- and ß-farnesene (4.6 and 3.1 %, respectively; the isomers were identified tentatively according to their retention indices as well as reference mass spectra as *trans*, *trans*-α- and *trans*-ß-), α-humulene (2.2 %) and ß-elemene (3.0 %) were identified as major constituents of the sesquiterpene fraction. Though, 19 sesquiterpenes (including oxidized compounds) remained unidentified. Furthermore, dihydroedulan I and II could be detected in each sample of *Aegopodium podagraria* which was analysed, hence these more uncommon compounds contribute to the characterization of this essential oil.

An aqueous extract of the herb of *Aegopodium podagraria* exhibited an antiphlogistic activity in the carrageenin induced rat paw oedema model (35 % inhibition at a dose of 330 mg/kg, p.o., based on dry herb; in comparison: indomethacin showed an inhibition of 39 % at a dose of 2 mg/kg) (6). After preseparation on Sephadex LH-20/MeOH and Sephadex G-25/EtOH-H_2O (1:1, v/v) in the active fractions no essential oil constituents could be identified by GC/MS. Hence the essential oil does not seem to contribute to the antiphlogistic activity of the aqueous extract obtained from the herb of *Aegopodium podagraria*.

ACKNOWLEDGEMENT: The authors wish to thank Prof.Dr. H. Juan and Dr. W. Sametz, Zentrale Tierbiologische Einrichtung, Graz, for carrying out pharmacological testing.

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Table I. GC/MS analysis of the essential oil of the herb of *Aegopodium podagraria* L.[1]

No.	Compound	RIp[2]	RInp[3]	Area %	Identification[4]
1	nonane*	900	900	< 0.1	MS, Co-GC
2	α-pinene*	1040	934	1.8	MS, Co-GC
3	camphene*	1084	946	< 0.1	MS, Co-GC
4	hexanal	1090	776	< 0.1	MS, Co-GC
5	β-pinene*	1126	972	2.9	MS, Co-GC
6	sabinene*	1135	968	1.7	MS, RI
7	β-myrcene*	1169	983	5.9	MS, Co-GC
8	α-phellandrene	1176	996	0.2	MS, Co-GC
9	heptanal	1190	879	< 0.1	MS, Co-GC
10	limonene*	1211	1023	9.4	MS, Co-GC
11	β-phellandrene*	1220	1020	1.1	MS, Co-GC
12	*cis*-β-ocimene*	1240	1028	3.1	MS, Co-GC, RI
13	γ-terpinene*	1258	1049	6.1	MS, Co-GC
14	p-cymene*	1282	1012	2.4	MS, Co-GC
15	α-terpinolene*	1294	1077	0.1	MS, Co-GC
16	octanal	1296	981	0.7	MS, Co-GC
17	3-hexen-1-yl acetate	1324	987	0.3	MS, RI
18	3-octanyl acetate	1343	1108	0.2	MS, RI
19	3-hexen-1-ol	1389	839	0.2	MS, RI
20	2-nonanone	1394	1071	< 0.1	MS, RI
21	nonanal	1397	1082	0.4	MS, Co-GC
22	α-copaene*	1510	1368	0.3	MS, Co-GC
23	dihydroedulan II	1512	1280	0.5	MS, RI
24	dihydroedulan I	1538	1284	0.4	MS, RI
25	linalool	1556	1084	0.9	MS, Co-GC
26	linalyl acetate	1564	1245	0.3	MS, Co-GC
27	β-elemene*	1595	1381	3.0	MS, RI
28	β-caryophyllene*	1603	1400	1.9	MS, Co-GC
29	terpinen-4-ol	1607	1158	0.5	MS, Co-GC
30	*trans*-β-farnesene*	1675	1451	3.1	MS, RI
31	α-humulene	1683	1438	1.9	MS, Co-GC
32	α-terpineol	1710	1168	0.5	MS, Co-GC
33	germacrene D	1724	1469	11.0	MS, RI
34	*trans,trans*-α-farnesene	1736	1502	4.6	MS, RI
35	δ-cadinene	1767	1511	1.3	MS, RI
36	γ-cadinene*	1770	1506	0.4	MS, RI
37	geraniol	1856	1238	0.2	MS, Co-GC
38	*cis*-jasmone	1953	1363	0.3	MS, RI
39	caryophyllene oxide	1990	1554	0.8	MS, Co-GC
40	spathulenol	2150	1552	1.7	MS, RI
			Total	70.2	

[1] Compounds sorted by elution order on J&W DB-Wax capillary column; [2] retention index on DB-Wax (J&W Scientific); [3] retention index on Ultra 1 (Hewlett-Packard); [4] methods of identification: MS = mass spectra library matching (Wiley 138K and laboratory-own spectral data base); Co-GC = GC/MS-comparison with reference compounds; RI = comparison of retention indices with those reported in literature (7-9); * also identified by (4) as volatiles released from the flowers of *Aegopodium podagraria*.

ANALYSIS OF *ACHILLEA SETACEA* WALDST.& KIT. ESSENTIAL OIL

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INTRODUCTION

Achillea setacea is a perennial herb belonging to Asteraceae family.. It attains a height of 10 - 80 cm. with stems longitudinally striped, ± seriecous-woolly. It grows in steppe, stony slopes, meadows, fallow fields in Inner and North Anatolia. It is also described from South Central and East Europe and Caucasia. Since *Achillea* species have been used in folk medicine as antiinflammatory and haemostatic agents, the essential oils of some *Achillea* species have already been analyzed (2-6).

EXPERIMENTAL

Plant Material - Aerial parts of *A. setacea* Waldst. & Kit. were collected from a wild population along roadside from Ankara to Cerkes in July 1993. The aerial parts were subjected to water distillation in a Clevenger - type apparatus to produce oil 0.17 % yield.

Analysis Physical and chemical characteristics of the oil, refractive index, density and optical rotation were determined by standard methods. The essential oil was analysed by GC/MS. Analyses were performed using a Hewlett-Packard GCD system. Innowax FSC column (60m x 0.25mm) was used with helium as carrier gas. GC oven temparature was kept 60 ^{0}C for 10 min. and programmed to 220 ^{0}C at rate of 4 ^{0}C / min. and then kept constant at 220 ^{0}C for 10 min. programmed to 240 ^{0}C at rate of 1^{0}C / min. Split flow was adjuted at 50 ml/ min. The injector and detector temparatures were at 250 ^{0}C. MS were taken at 70 eV. Mass range was from m/z 10 to 425. Library search was carried out using Wiley GC / MS Library and TBAM Library of Essential Oil Constituents (9-17).

RESULT AND DISCUSSION

Our previous work on the essential oils of Turkish Achillea species include *A. wilhelmsii, A.biebersteinii* and *A. nobilis* subsp. *neilreichii*(2-4). Tanker and Küsmenoglu detected borneol (13 %), α- terpineol (26 %) as major constituents in the essential oil of *A.wilhelmsii,* while Brunke reported camphor (36 %), and 1,8 cineole (21 %) as main components (2, 5). We had detected 1,8 cineole (46 %) and camphor (18 %) as main constituents in the oil of *A. biebersteinii* (3). Chialva et al. reported the same constituents as major components (46 % and 18 %, respectively)(6). In the oil of *A. nobilis* subsp. *neilreichii* , piperitone (10.3 %), viridiflorol (9.06 %) and 1,8 cineole (5,6 %) were detected as main constituents by our group (4). Maffei et al. reported that germacrene D (46 %) was the major component of the essential oil of *A.nobilis* in Italy (7).

During the present study, 105 constituents were characterized comprising 85.3 % of the oil of *A.setacea.* The main components detected in the oil were camphor (10.2 %), myrtenol (8.6 %), 1,8-cineole (7.8 %), α-bisabolol (7.5 %) , caryophyllene oxide (5.1 %), lavandulyl acetate (4.6 %), α-terpineol (4.5 %), piperitone (3.3 %) which cosstitute 51.6 % of the oil (table 1). Camphor and 1,8 cineole have been reported as major constituents in the several *Achillea* species (4-11). Authors reported that camphor, 1,8 cineol, borneol as main oxiganeted compounds of *A.setacea* growing in Middle Europe. In this study, we detected myrtenol, α-terpineol, lavandulyl acetate, piperitone as major constituents not mentioned by Kastner et al. (11). In an another work, the main

oxygen containing compounds were identified as 1,.8 cineole and borneol in the oil of *A.setacea* (12). They also found farnesen, bisabolen as sesquiterpenes while we detected large amount of caryophyllene oxide and α-bisabolol. This could be a differentiating character. Caryophyllene oxide was found in essential oils of *A.asiatica* and *A.millefolium* (7,10). If we compare of the qualitative composition of the essential oil of *A.setacea* with the literature data, a higher number of identified components in the present study.

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Table 1. Physicochemical Constants and Chemical Composition of the Oil of *A. setacea* W&K.

(oil yield (v/w): 0.17 %, d^{20}_{20} : 0.9592; $[\alpha]_D^{20}$: -5.21°; n_D^{20}: 1.4750)

Compound	%	Compound	%	Compound	%	Compound	%
α-pinene	0.18	cis- sabinene hydrate	0.44	cis- piperitol	0.20	decanoic acide	0.20
camphene	0.18	octanol	0.08	cis- chrysanthenol	0.56	α-caryophylladienol*	0.43
hexanal	0.05	1-methyl 4-acethylcyctohex-1en	0.05	ar-curcumene	0.10	β-caryophylladienol*	1.30
β-pinene	0.15	trans- p-menth-2en-1-ol	0.37	cis- sabinol	0.04	caryophyllenol II	0.75
sabinene	0.28	trans- chrysanthenylacetate	0.28	cuminaldehyde	0.11	α-bisabololoxide A	0.52
α-terpinene	0.04	pinocarvone	0.24	myrtenol	8.65	pentacosane	0.17
dehydro 1,8-cineole	0.07	bornylacetate	0.72	p-mentha-1,5 dien-7-ol	0.30	γ-costol	0.09
limonene	0.08	6-methyl 3,5-heptadiene-2-one	0.07	grandisol	0.97	isobutyl phthalate	0.07
1,8-cineole	7.79	β-caryophyllene	0.17	trans-carveol	0.18	9-hexacosene	0.04
(E)- 2-hexenal	0.05	lavandulyl acetate	4.59	p-cymen-8-ol	tr.	heptacosane	0.04
amylfuran	0.04	hotrienol	tr.	ascaridole	0.20	buthylphthalate	0.23
γ-terpinene	0.17	cis- p-menth-2en-1-ol	0.37	benzylisovalerate	0.03	hexadecanoic acid	1.52
p-cymene	0.53	thuj-3-en-10-al	0.06	cis-jasmone	0.30		
terpinolene	0.04	myrtenal	1.55	caryophyllene oxide	5.14	* *tentative*	
2-methylbutylisovalerate	0.05	sabinaketone	0.18	perillyl alcohol	0.08		
6-methyl-5-hepten-2-one	0.04	1-nonanol	0.04	pentadecanal	0.11		
hexanol	0.07	cis- verbenol	0.12	(E)-nerolidol	0.20		
nonanal	0.07	trans- pinocarveol	0.27	13-tetradecanolide	0.56		
yomogi alcohol	0.07	p-mentha-1,5-dien-8-ol	0.08	humuleneepoxide-II	0.44		
α-thujone	0.07	γ-terpineol	0.40	p-mentha-1,4-dien-7-ol	0.59		
(E)-2-octenal	tr.	lavandulol	1.22	cuminalcohol	0.22		
β-thujone	0.50	trans- verbenol	1.17	hexahydrofarnesyl acetone	0.35		
1-octen-3-ol	0.17	myrtenyl acetate	0.49	spathulenol	0.53		
trans- sabinenehydrate	0.41	α-terpineol	4.49	α-bisabololoxide B	1.30		
α-campholeraaldehyde	0.12	borneol	3.56	bisabololoxide	1.50		
(E,E)-2,4 heptadienal	0.08	trans- sabinol	0.40	γ-eudesmol	0.13		
artemisia alcohol	0.18	thujol	0.07	eugenol	0.41		
chrysanthenone	0.73	verbenone	0.24	thymol	0.43		
camphor	10.15	nerylacetate	0.37	α-bisabolol	7.50		
benzaldehyde	0.16	trans- p-menth-2-en 1,8 diol	0.24	carvacrol	0.40		
linalool	0.19	piperitone	3.33	β-eudesmol	0.81		

351

ESSENTIAL OIL OF *ARTEMISIA ANNUA* L. ADAPTED TO BRAZILIAN CLIMATE

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INTRODUCTION

The Chinese have used *Artemisia annua* L. (Asteraceae) for more than fifteen centuries to treat malaria fever. In the 1970's various groups studied the genera *Artemisia* to certify antimalarial activity of the plants extracts. The active compound was isolated in 1972 and identified by Chinese researchers in 1979 [1]. Further work proved that only *A. annua* L. and *A. apicea* had antimalarial activity. This was attributed to the endoperoxide sesquiterpene, artemisinine **1**, only found in these two species.

The volatile components of *A. annua* L. have been studied by Woerdenbag *et al.* [2] and Lawerence [3]. The essential oil has been reported to possess antimicrobial activity, providing an additional market for this plant [3].

The World Health Organization (WHO) estimate that acute and chronic malaria are responsible for approximately 230 million cases annually, of which 2 million lead to death. The world-wide cases of malaria are superior to that observed for AIDS (acquired immuno deficient syndrome) [4]. The scope of our work was to study some chemical aspects of the plant adapted to Brazilian climate and ascertain its economical viability.

RESULTS AND DISCUSSION

We determined the oils composition weekly detecting significant oil content variation during the whole plant cycle. According to Figure 1 and 2 we determined the essential oil content, artemisinine content and the ratio of monoterpenes/sesqui and oxygenated terpenes (% R) weekly

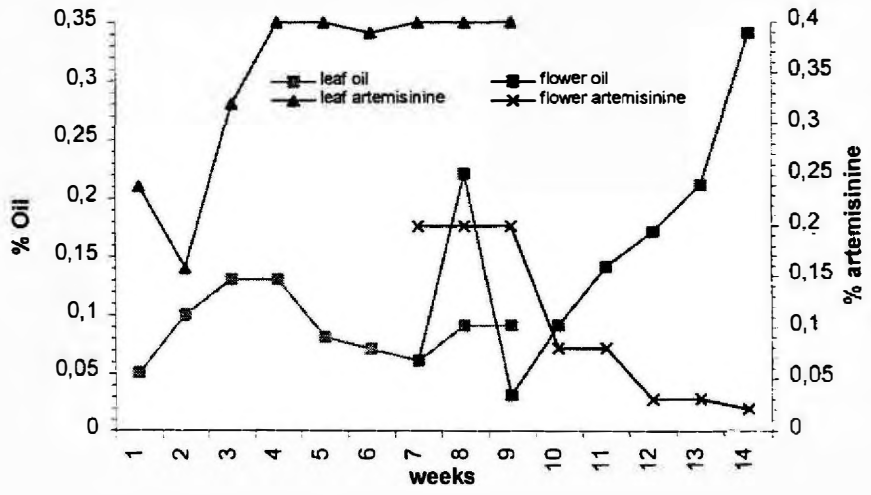

Figure 1. Oil and artemisinine content behavior throughout a harvest period

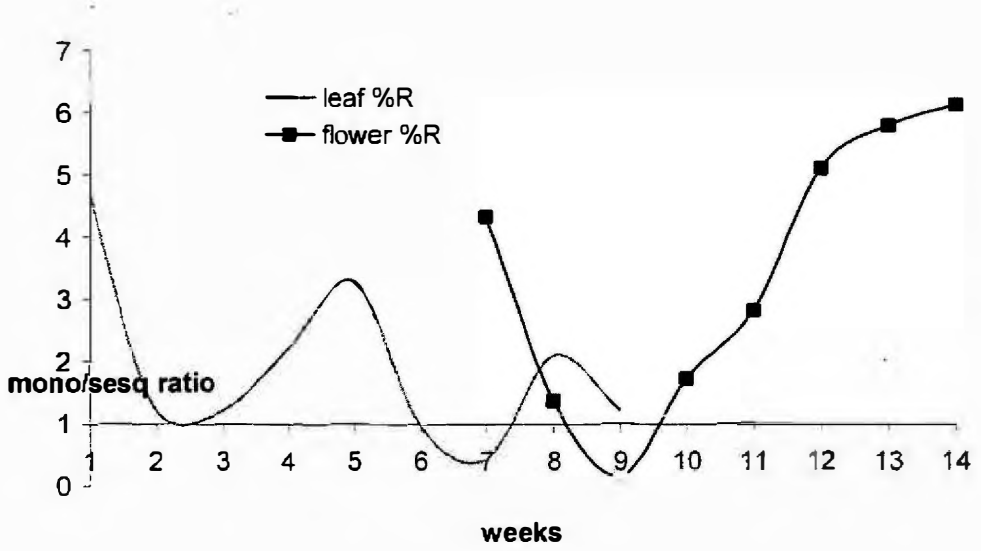

Figure 2. Ratio of mono/ hydrocarbon and oxygenated sesquiterpenes seasonal behavior

This data was plotted illustrating the parameter's variations. The maximum oil content were obtained on the 8th and 14th week from the flowers both rich in monoterpenes. The higher R ratio observed on the 14th week conferred a stronger monoterpene odour to the essential oil. Whereas the 8th week oil had a weaker scent of monoterpenes. The oils obtained from leaves had low R values with rather an unpleasant smell.

Simultaneously the artemisinine **1** content was determined showing that the highest amounts were found prior to flowering as observed in Figure 1.

The identification of the essential oil was studied using GC/MS (HP 5890 / HP 5970). The samples were prepared by hydrodistillation of freshly picked leaves and flowers. The composition was determined by association of retention time data and co-injection of authentic standards .Owing to extensive work undertaken by CPQBA-UNICAMP access to pure samples of qinghaosu acid **2**, **3**, **4**, **5**, **6**, **7**, **8**,and **9** were possible [5].

Forty nine components were identified in the essential oil (Table 1 and Figure 3). Compounds **1**, **2**, **5**, and **9** were found in trace amounts. A new carbon skeleton 2 - cyclopenten - 1 - one, 2 - [2,2 - dimethyl - 3 - (3-butanone)cyclopropyl] **10** was suggested from our GC/MS data. The compound was obtained by Pauson-Khand synthesis [6] enabling to confirm the existence of the *cis* and *trans* isomers in our oil.

From Figure 1 we concluded that the plant leaves favour artemisinine **1** production whereas the essential oil obtained from the flowers is rich in monoterpenes. The resulting data permitted to ascertain the plants economical viability throughout its harvest period.

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Table 1. Compounds identified in the essential oil

Compound	RI	Compound	RI	Compound	RI
α-pinene	945.0	estragole	1224.2	humulene	1447.4
camphene	962.7	l-terpineol	1232.4	aromadendrene	1458.1
β-pinene	972.3	citral	1242.6	β-ionone	1464.4
sabinene	1007.3	carvone	1252.3	alloaroma-dendrene	1476.7
myrcene	1010.4	citral	1266.3	guaiol	1481.3
2-carene	1018.6	isobornyl acetate	1300.8	ledene	1503.3
phelandrene	1021.6	eugenol	1312.9	nerolidol (Z)	1519.1
1,8-cineol	1031.6	α- cubebene	1338.1	nerolidol (E)	1525.2
artemisia ketone	1055.3	α-copaene	1353.2	epiglobulol	1551.7
fenchone	1073.4	longicyclene	1389.8	caryophelene oxide	1567.1
isopulegol	1091.5	α-gurjunene	1401.1	globulol	1573.4
camphore	1174.8	β-cedrene	1417.6	viriflorol	1579.7
citronelal	1183.7	longifolene	1426.5	farnesol	1610.0
borneol	1189.0	calarene	1429.6	nor-tailorine (Z)	1716.0
isoborneol	1214.3	α-neoclovene	1442.3	nor-tailorine (E)	1732.8

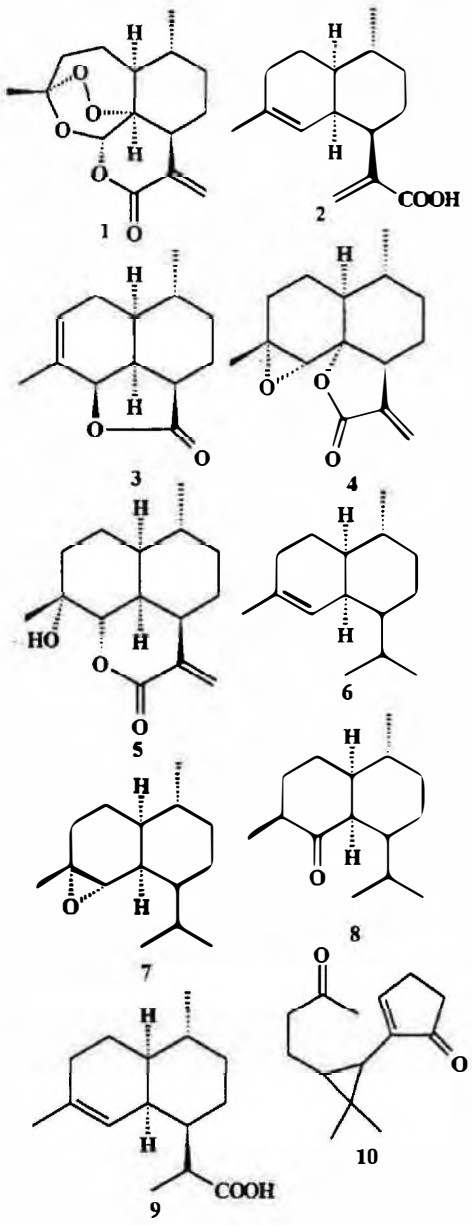

Figure 3. Sesquiterpene derivatives used as standards

PRELIMINARY ANALYSIS OF ESSENTIAL OIL FROM SARDINIAN *HELICHRYSUM ITALICUM* (ROTH) G. DON SSP. *MICROPHYLLUM* (WILLD.) NYMAN

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INTRODUCTION

The genus *Helichrysum* (*Compositae*) is represented by herbs or dwarf shrubs, often lanate or tomentose. Capitula small to medium, florets yellow, all tubular (1). Several *Helichrysum* species are widely represented in the Mediterranean flora. The first data about *Helichrysum* oils were cited by Gildemeister (2), other notices, about the oil of this genus, were published more recently (3). The species *Helichrysum italicum* (Roth) G. Don ssp. *microphyllum* (Willd.) Nyman is widely diffused in all Sardinia island and it is beginning to be cultivated for its aromas. The composition of this essential oil, until now, is still less known and the influences of cultivation have not been investigated. In the present paper, we aimed to study the variation between the major constituents of the essential oil of *H. italicum* growing wild in Sardinia and that from the same plant cultivated in sites near to the wild station. The studied stations were located in northern Sardinia.

EXPERIMENTAL

Specimens of wild and cultivated *Helichrysum italicum* (Roth) G. Don ssp. *microphyllum* (Willd.) Nyman were collected in June 1995 in stations located in the North of Sardinia (Gallura). The cultivated samples were obtained from seeds and the plants were irrigated and pruned. The volatile compounds from dried inflorescence were extracted using a Clevenger apparatus.

GC-MS- Four replicates of each sample were analysed by using a Hewlett-Packard model 5890 Series II GC fitted with a 30m x 0.25mm DB5MS fused silica capillary column connected with a 0.5m x 0.53 mm i.d. fused silica pre column coated with deactivated methyl siloxane. Temperature was for injection port 63°C and for detector 280°C. The column temperature was held at 60 °C for 6 min and than programmed to 130°C at 3°C/min, to 200°C at 10°C/min. The samples (using 2,6-dimethylphenol as internal standard) were injected using a split/splitless automatic injector HP 7673. The GC was connected to a Mass Selective Detector HP 5971A. The identification of constituents was performed by comparison of Rt and/or by comparison of mass spectra of the analysed samples with mass spectra of pure standards or by comparison with published data (4-6). The quantitative analysis was performed using the internal standard method.

RESULTS AND DISCUSSION

The yield in essential oil was comparable in the two stations considered and reach 0.47% w/w. The chromatogram of the essential oil of *H. italicum* wild showed a different composition in the major constituents with respect to the essential oil obtained from the cultivated plant. Particularly, differences in content of limonene, linalool, α-terpineol, nerol and neryl acetate were observed. The quantitative data for each oil component found in the three studied stations are reported in the table. The oil components were divided into five groups: monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxides, esters and alcohols. As far as the sesquiterpene hydrocarbons is concerned, it is of interest to note the 9.93% and 8.49% reached by *H. italicum* collected in the cultivation C and in the wild station B, while the plants collected in the cultivation A reached only a 2.6%. This different behaviour is to impute to curcumene that in the station A is present only in traces while in B and C reached the 6%. The oil obtained from the wild plants (B) contained the minimum (38.60%) amount of esters; they are essentially represented by neryl acetate that in the plants deriving from the two cultivation (total esters are 49.00% and 48.20%) reached ca. 43%.

In the wild plants its quantity (33%) is the 10% less than in the cultivated one. Limonene content affect the total of monoterpenes because the other singular constituents are not so variables. The alcohols assembled the major number of the identified compounds in the studied oil of *H. italicum* and some of them are present in relevant quantity. The most important constituent of the alcohol group is nerol that represent 11.1% in the inflorescence from the wild plants and 4.4% in the oil from cultivation C while in the other cultivated station the percentage of nerol is 9%. Finally all groups of components showed significant variations and interesting similarities in the cultivated and in the wild plants.

Acknowledgements

The work was financially supported by M.U.R.S.T. (60% and 40%).

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Table 1. Major constituents (%) of essential oils obtained from wild and cultivated plants of *Helichrysum italicum* (Roth) G. Don ssp. *microphyllum* (Willd.) Nyman.

		% (w/w)		
	Compounds[a]	A	B	C
MH*	α-pinene	0.11	0.42	0.44
MH	camphene	0.21	0.27	0.25
MH	β-pinene	0.15	0.19	0.12
MH	p-cymene	0.13	0.17	0.14
MH	limonene	0.62	2.03	1.47
MH	γ-terpinene	0.16	0.13	0.11
ALC	eucalyptol	2.21	0.14	0.13
ALC	linalool	3.45	4.91	7.13
ALC	α-terpineol	2.83	1.25	0.66
ALC	nerol	9.11	11.12	4.42
ALC	tymol	0.19	0.14	0.14
ALC	carvacrol	0.12	0.17	0.11
EST	neryl acetate	43.08	33.53	42.46
EST	geranyl acetate	0.11	0.12	0.15
SH	β-cariophyllene	0.131	0.55	0.63
EST	neryl propanoate	5.87	5.03	5.63
SH	γ-curcumene	2.19	1.91	2.05
SH	curcumene	0.11	6.03	6.55
ALC	guaiol	0.13	0.71	0.61
ALC	β-eudesmol	2.29	2.04	2.48
ALC	α-eudesmol	0.89	1.31	0.99

[a]Peak identifications are based on MS comparisons with file spectra and relative retention time; [b]total identified components of *Helichrysum italicum* G. Dom ssp. *microphyllum* oil; absolute weight percent (wt%) using internal standard method; average of four GC runs. A and C = cultivated plants; B = wild plants.*MH = Monoterpene hydrocarbons; ALC = Alcohols; EST = Esters; SH = Sesquiterpene Hydrocarbons.

Germacrene D - A Source of Rare Sesquiterpene Hydrocarbons

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Introduction

In 1969 Yoshihara *et al.* reported the first isolation of germacrene D and mentioned the facility to isomerize this sesquiterpene by treatment with acids (e.g. silica), heat or UV-light resulting in hydrocarbons of the cadinene group and bourbonenes [1]. Further rearrangements with germacrene D were performed by Lawrence *et al.* [2] and Nishimura *et al.* [3].

(-)-Germacrene D is widespread in higher plants. The less common (+)-enantiomer was found in liverworts [4], some other sources were mentioned by Lorimer and Weavers [5]. Although a synthesis of racemic germacrene D was reported [6], it is more convenient to obtain both enantiomers from natural sources [7].

We wanted to use germacrene D of known enantiomeric composition as a precursor for the preparation of various rare sesquiterpenes as reference compounds for the analysis of essential oils. To get both enantiomers of germacrene D in a preparative scale we examined specimens of *Solidago canadensis* L., *S. gigantea* Ait. and *S. virgaurea* ssp. *praecox* L. (golden rod) growing ubiquitously in central Europe.

Results and Discussion

Germacrene D was found to be the main component in the steam distillates of all *Solidago* species. Both enantiomeric forms were present with variable enantiomeric compositions depending on the collection site of the plants. The enantiomeric ratio is also dependent on the part of the plant (blossoms, stems, leaves). It is remarkable that in some samples only (-)-germacrene D, but only in one sample of *Solidago virgaurea* ssp. *praecox* L. the pure (+)-

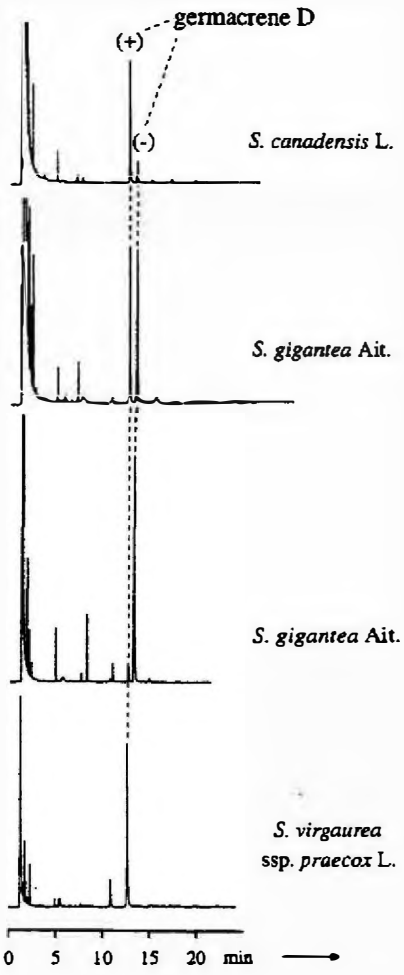
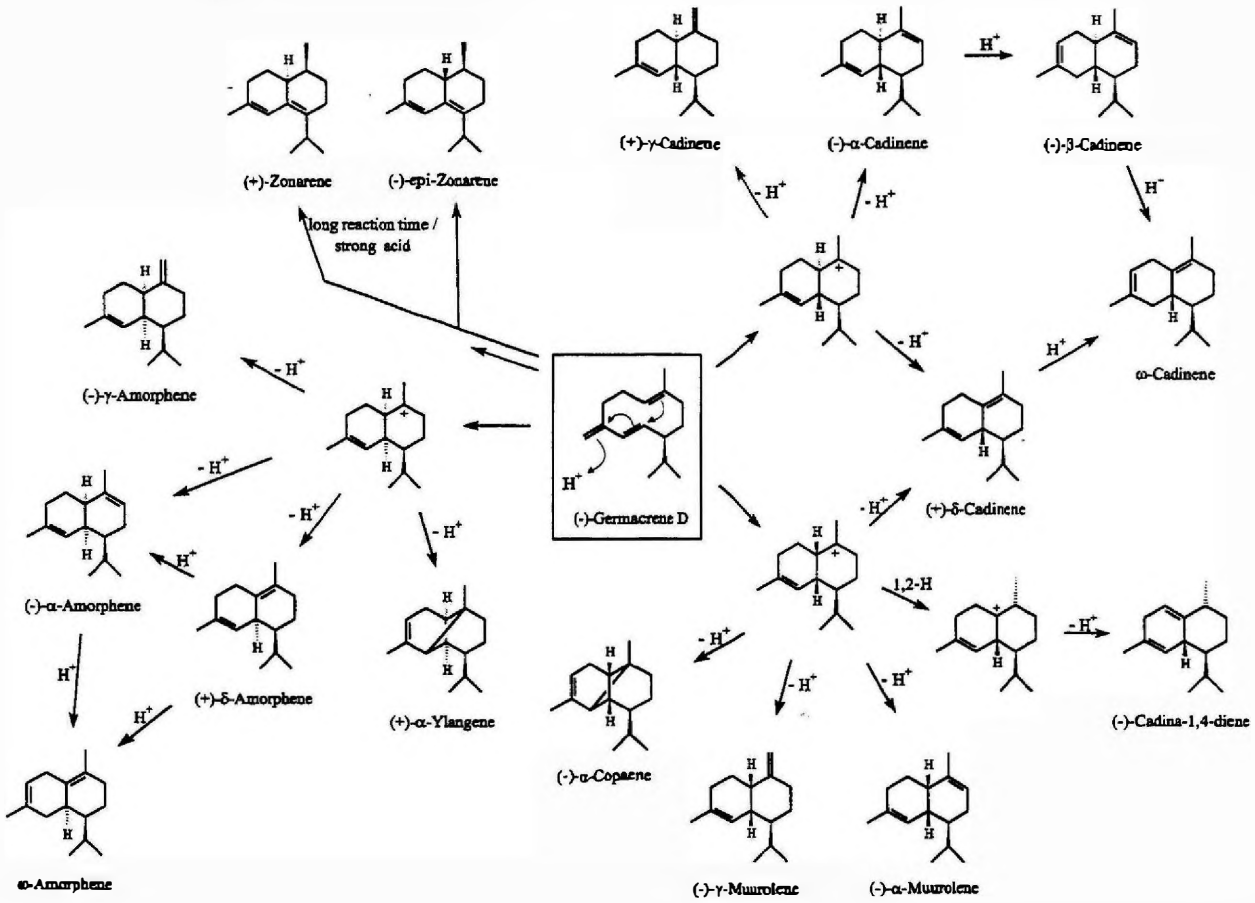


Figure 1
Investigation of the enantiomeric composition of samples of *Solidago canadensis* L., *S. gigantea* Ait. and *S. virgaurea* ssp. *praecox* L. on heptakis(2,3-di-*O*-methyl-6-*O*-TBDMS)-β-cyclodextrin/OV 1701 (50%, 25 m), 115°C isothermal

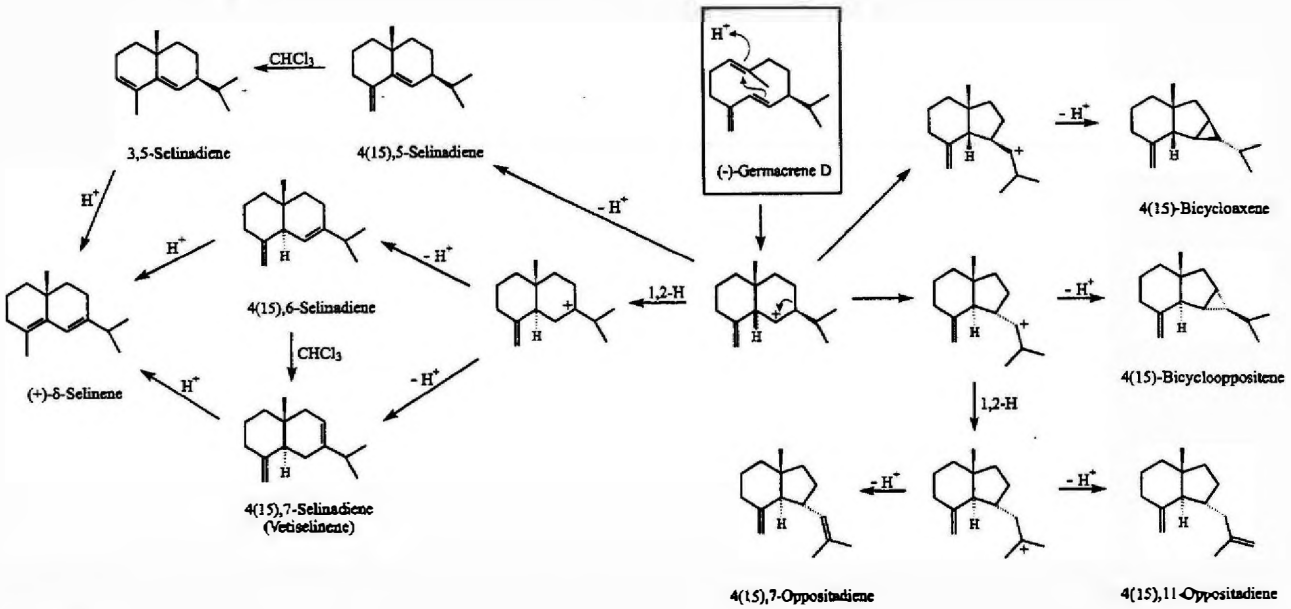


Scheme 1
Acid catalyzed reaction of (-)-germacrene D resulting in cadinenes, muurolenes and amorphenes.

enantiomer was present. Some examples are given in **Figure 1**.

The acid catalized rearrangement resulted in a complex mixture of components as shown in **Schemes 1**, **2** and **3**. As main compounds cadinenes, muurolenes and amorphenes were obtained. As an example for the utility of these standards we were able to identify both enantiomers of δ-amorphene as natural compounds in liverworts and in higher plants [8]. Except for a smaller number of selinenes some members of the rare oppositene and axene sesquiterpenes [9] were formed, among them fused ring systems bearing cyclopropyl groups. The formation of substituted oppositene derivatives from epoxygermacrene D as a precursor was reported by Yamamura *et al.* [10]. Additionally, we obtained isodaucenes, which are known as isomerization products of α-humulene [11] and were also formed in substituted form from epoxygermacrene D [12].

The main products of the thermal rearrangement are shown in **Scheme 4**. Isogermacrene D with a *cis*-substituted 5-6-double bond was identified by comparison of the coupling constant $J_{5\text{-}6}$ = 11,4 Hz instead of $J_{5\text{-}6}$ = 15,8 Hz as found in germacrene D. The configuration of the 1-10-double bond is under investigation. The formation of the ε-compounds can be explained by an *ene*-reaction. ε-Amorphene was subsequently identified as a trace component in the essential oil of *Mentha piperita* L. of Bulgarian origin. β-Ylangene and β-copaene may be formed thermally and by irradiation with UV-light in a similar way (**Scheme 5**). The minor products are principally identical to those formed in the presence of acids. Additionally, we identified α-/β-cubebene and cadina-3,5-diene by GC-MS,

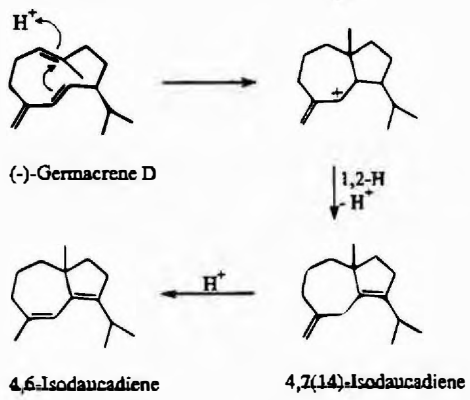


Scheme 2
Acid catalyzed reaction of (-)-germacrene D resulting in selinenes, oppositenes and axenes.

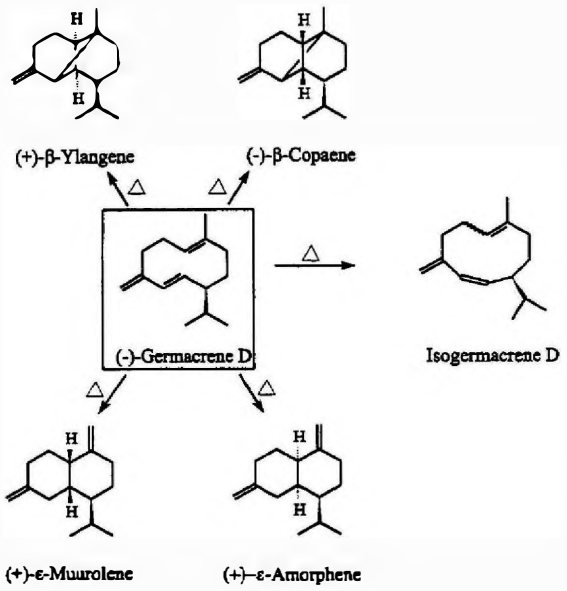
which was also subsequently identified as a natural product in several plants [8].

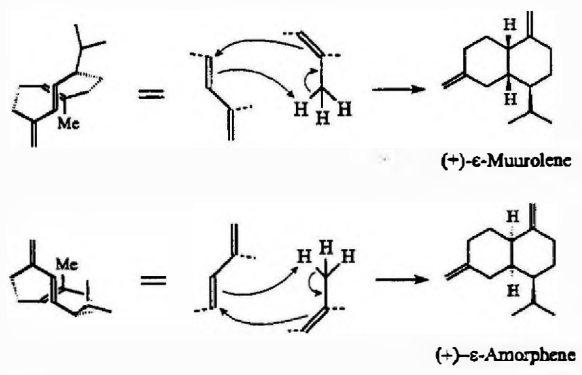
The photochemically induced isomerization yielded [2+2]-cycloaddition products, which are shown in **Scheme 5**. Only a trace of the α-isomer was formed from the main product β-bourbonene at elevated temperature during irradiation. The formation of both β-copaene and β-ylangene can be explained by the conformations in the excited state as shown in **Scheme 5**.

The results demonstrate that germacrene D is a valuable precursor for the preparation of a

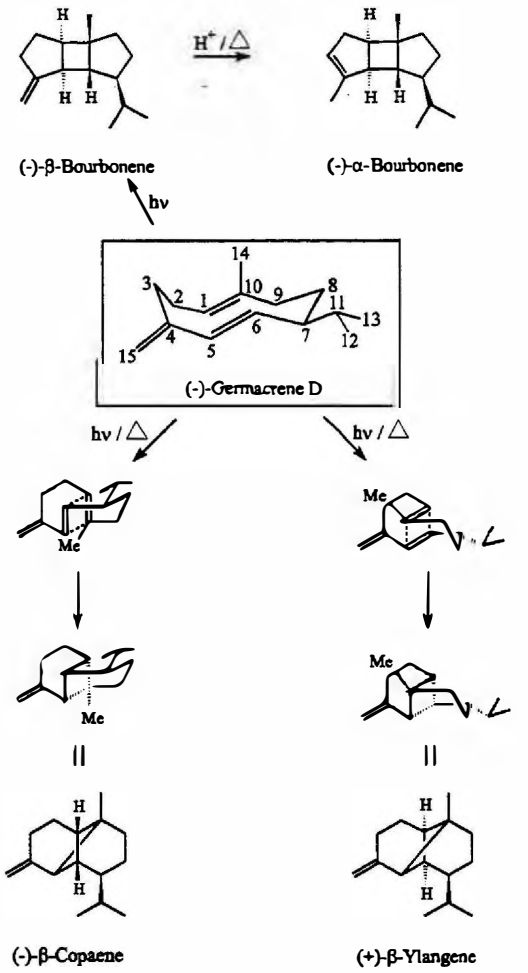


Scheme 3
Acid catalyzed reaction of (-)-germacrene D resulting in isodaucenes.





Scheme 4
Thermal rearrangement of (-)-germacrene D. The formation of ε-muurolene and ε-amorphene is proposed to involve an *ene*-reaction.



Scheme 5
UV-light induced [2+2]-cycloadditions of (-)-germacrene D. The mechanism of the formation of β-ylangene and β-copaene is illustrated and applies also to the thermal pathway.

variety of sesquiterpenes and, furthermore, support the importance of this compound or its cationic biogenetic precursor as a key intermediate in the biosynthesis of several other sesquiterpenes [1, 3].

Experimental

Several plants of *Solidago canadensis* L. and *S. gigantea* Ait. were collected in and near Hamburg (northern Germany) in 1995 and 1996; *S. virgaurea* ssp. *praecox* L. was provided by the Botanischer Garten in Hamburg. The essential oil was prepared by steam distillation (1,5 h) of aq. homogeates of fresh plants using n-hexane as collection solvent. Germacrene D was isolated by preparative GC, identified by GC-MS and NMR spectroscopic methods and investigated by enantioselective GC [13].

Germacrene D was isomerized with various acidic catalysts (p-TsOH/$CHCl_3$; Amberlyst® 15 ion exchange resin/n-hexane; BF_3/Et_2O/0°C; $AlCl_3/CH_2CL_2$), under thermal conditions (400°C/injector of the prep. GC; 200°C/n-hexane/sealed glass tube) and in the presence of UV-light (254 nm/n-hexane solution/3 hours). The resulting products were isolated by silica gel chromatography and/or subsequent preparative GC and identified by means of GC-MS and 2D-NMR techniques. The detailed reaction conditions and isolation methods and the spectroscopic data of the obtained new compounds will be published elsewhere.

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THE ESSENTIAL OIL OF *TANACETUM FRUTICULOSUM* LEDEB. FROM IRAN

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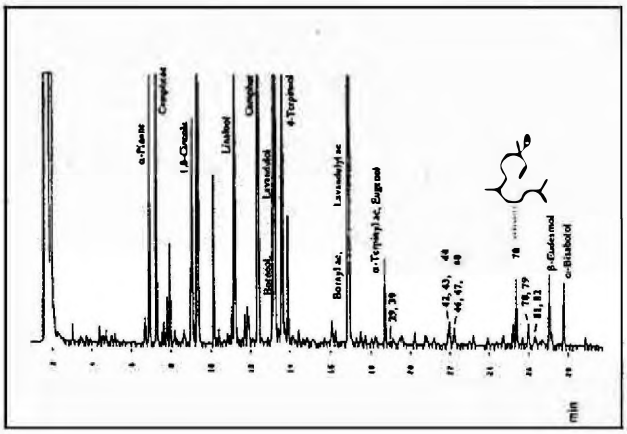
Tanacetum fruticulosum syn. *Chrysanthemum fruticulosum* (Compositae) is a shrub growing wildly in the hills and plains of several parts of Central Asia and the Middle East.

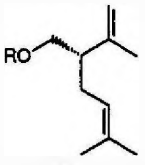
The *Tanacetum* oil was prepared by hydrodistillation of air dried aerial parts of the plants which were collected at the end of the flowering-period at the beginning of September 1994 40 km south of Hamedan, **Iran**. It smells fresh-terpenoid, sweet-herbal and slightly mugwort-like. 30 g of the essential oil were separated by distillation and the residue was submitted to repeated flash chromatography. The isolated constituents were analysed by a combination of GC-MS, RRI, ^{1}H- and ^{13}C-NMR spectra.

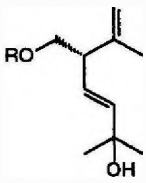
About 90 components were identified representing about 95% of the oil. The main constituents are 1,8-cineole (17%), camphor (13%), lavandulol (11%), and lavandulyl acetate (9%). Typical for the *Tanacetum* oil are photooxidation products of lavandulol and nerolidol.



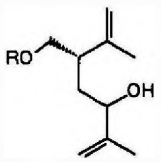
GC of the *Tanacetum fruticulosum* oil from Iran (CP Sil CB-column, 25 m x 0.25 mm i.d., temperature program: 60°C, 5°C/min to 220°C)

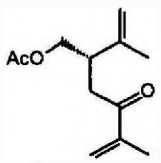
Lavandulol Derivatives





12 R = H	11%	**31** R = Ac	0.1%
24 R = Ac	9%	**53** R = COiPr	trace
32 R = COEt	0.2%	**72** R = COCH(Me)Et	trace
37 R = COiPr	0.1%	**73** R = COiBu	trace
57 R = COCH(Me)Et	trace		
58 R = COiBu	trace		

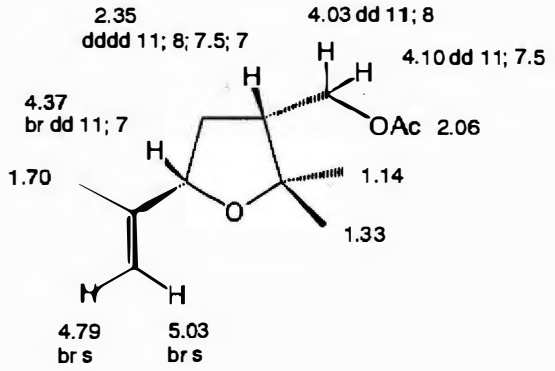




35, 36 R = Ac	0.2%	**34**	0.2%
64 R = COiPr	0.1%		
84 R = COCH(Me)Et	0.1%		

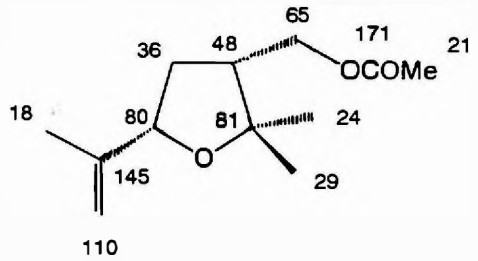
Most of them were also isolated from Lavender oil[1]

Two compounds **29** and **30** were isolated from the medium polar fraction. HR-MS (212.1412) gave the molecular formula $C_{12}H_{20}O_3$. Typical values in the NMR spectra (^{1}H: s 2.06, ^{13}C: s 171, q 21) provide the structure of an acetate of a C_{10} alcohol. Two signals in the ^{13}C-NMR spectrum (d 80, s 81) indicate an ether. The compound possesses an isopropenyl group at the ether bridge. Two downfield shifted methyl singlets must be located at the quaternary C atom of the ether group. Combination of all data gave formula **29** and **30**. The stereochemistry of the two tetrahydrofuran isomers was confirmed by NOED spectra. Their formation by cyclisation of **35** and **36** is logical.

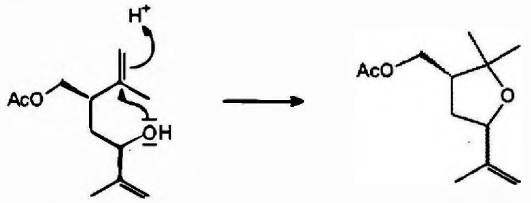


29 (^{1}H-NMR)

Odor: strong sweet, fruity (apricot), woody

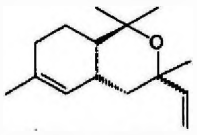


29 (^{13}C-NMR)

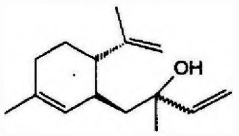


35, 36 **29, 30**

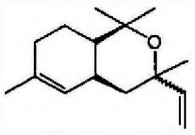
Nerolidol Derivatives



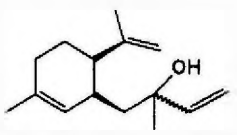
40, 45 A, C trace



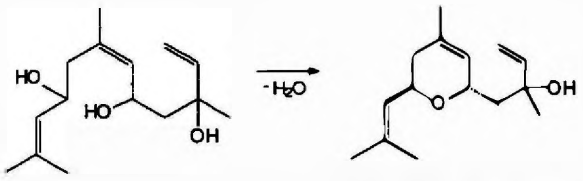
55, 56 A, C trace



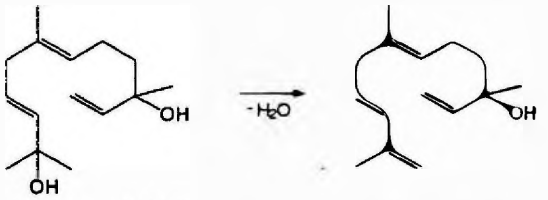
44, 50 B, D trace
Cabreuva Oxides



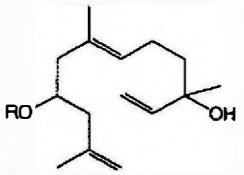
60, 61 B, D 0.2%
Isohumbertiols



67, 76 A, B trace
trans-Bejarols

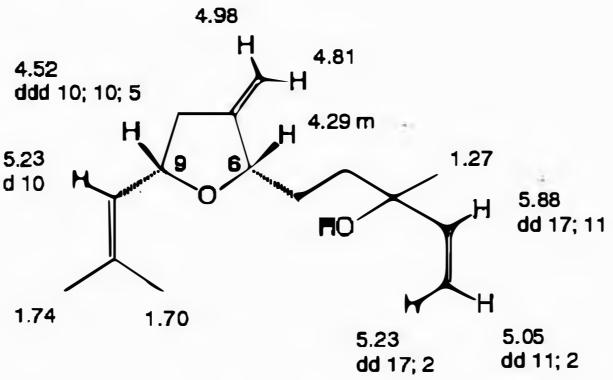


86 0.2% **70** 0.8%

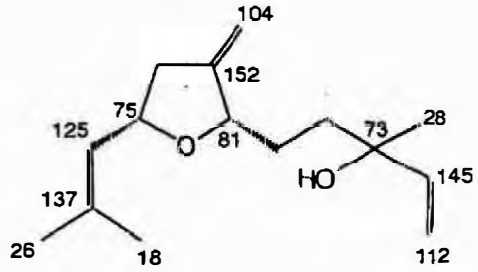


93 R = COiPr trace
96 R = CO CH(Me)Et trace

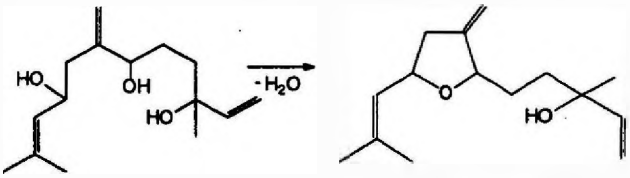
Four epimers (**78, 79, 81, 82**) were isolated from the polar fraction with a MW of 236. These epimers possess a tertiary hydroxy group (^{13}C: s 73). Furthermore, there are two doubletts at δ = 75 and 81 in the ^{13}C-NMR spectrum indicating an ether, and six lowfield shifted signals for one trisubstituted double bond, one methylene and one vinyl group. The ether proton at δ_H = 4.52 couples with the olefinic proton of the trisubstituted double bond, and with an allylic CH_2 group. These protons couple with the methylene protons, one of them shows a NOE to a second ether proton. This proton (δ_H = 4.29) couples with a CH_2 group of a CH_2CH_2 chain. The vinyl group is located at a quaternary C atom bearing the hydroxy and the methyl group. The stereochemistry of this stereocenter remained unknown.



78 (^{1}H-NMR)
NOE between 6-H and 9-H

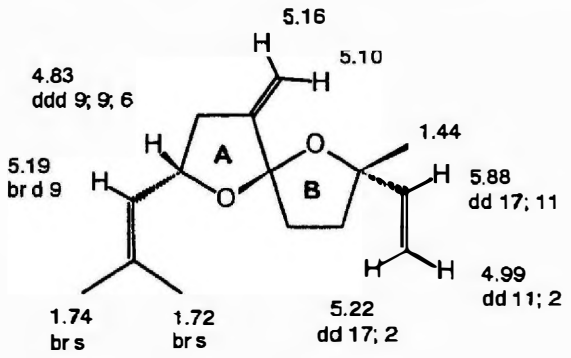


78 (^{13}C-NMR)



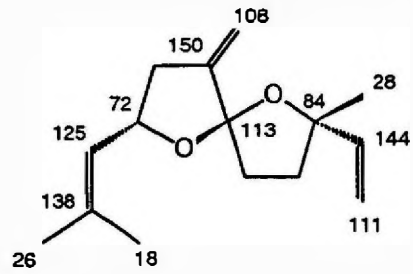
4 Epimers
(78, 79, 81, 82)

In the less polar fraction we found four epimers (**42, 43, 46, 47**): $C_{15}H_{22}O_2$ (HR-MS: 234.1620). Typical for all these epimers is a singlet at δ_C = 113. Compound **42** shows two signals at δ = 84 and 72 in the ^{13}C-NMR spectrum. Hence, the component proves to be an acetal. The molecule has one trisubstituted double bond, one methylene and one vinyl group, therefore the compound must be bicyclic. In the ring **A**, there is an ether proton at δ = 4.83 coupling with a proton of the isopropylidene groups, and with a CH_2 group. The protons of the CH_2 group couple with the methylene protons. In the ring **B** a downfield shifted methyl group (δ = 1.44) must be situated at the ether C atom. At this position there is also a vinyl group which shows a strong NOE to the methyl group. The stereochemistry of the four isomers was partially confirmed by NOED spectra.

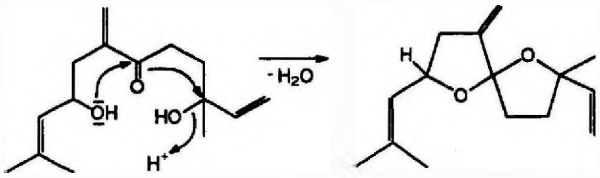


42 (^{1}H-NMR)

Odor: strong woody, sweet, fruity

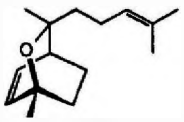


42 (^{13}C-NMR)

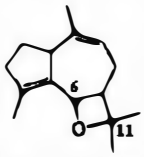


4 Epimers
(42, 43, 46, 47)

Some other interesting constituents were identified by their ^{1}H-NMR spectra:

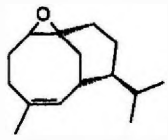


48 0.1%
Dehydrosesquicineole

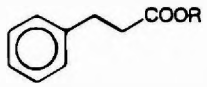


65 0.1%
6,11-Epoxyguaia-4,9-diene

68 trace
Dendrolasine



90 trace
Salsolene Oxide



19	R = Me	0.1%
26	R = Et	0.1%
73	R = CH(Me)Pr	trace
83	R = $(CH_2)_4$Me	trace
89	R = (Z) CH_2CH_2CH=CHEt	0.1%

Reference:
[1] R. Kaiser, D. Lamparsky; *Tetrahedron Lett.* 1977, **33**, 665

INVESTIGATION OF THE COMPOSITION OF ESSENTIAL OILS OF *HYSSOPUS OFFICINALIS L.* POPULATIONS

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INTRODUCTION

Hyssopus officinalis L. (*Lamiaceae*) is a well-known spice, the flowering shoots and essential oil being used. It is also an excellent plant for attracting bees and is a decorative garden ornamental. The plant native to the Mediterranean has long been cultivated in Central Europe. In many countries, including Hungary, this species is used as a folk medicine against certain respiratory diseases, e.g. bronchitis due to its spasmolytic activity. However, little is known as concerns the variations in its oil content and composition under Central European, climatic conditions.

The cultivated populations of hyssop are characterised by a significant heterogeneity. The main aims in its breeding are flower colour uniformity (e.g. the blue form) and an increased oil content. As knowledge on the variation or genetic fixation of the oil composition is incomplete, we decided to study this question.

This paper deals with the study of the essential oils of *Hysssopus officinalis* of different origins, grown under the same climatic conditions in Hungary.

We studied how the oil composition depends upon the origin and also the time when the samples were harvested. The compositions of essential oil samples obtained by Supercritical Fluid Extraction (SFE) with CO_2 and by Water Steam Distillation (WSD) were compared. The oils were analysed by GC and GC/MS techniques.

MATERIAL AND METHODS

Different populations of *Hyssopus officinalis* grown from seeds and the offspring of the plants individually selected according to the colour of the flowers obtained from them served as starting material for our investigations. The original seeds had been acquired from various botanical gardens abroad.

Table 1. Identity and origin of populations of hyssop grown in Vácrátót

Samples	Origin of seed
I*	St Gallen
II*	Zürich
III*	Frankfurt
IV*	Latvia
V*	Halle
VI*	Montreal
VII**	Antverpen
VIII**	Salaspils
IX**	Wroclaw

* Date of sowing 10. 04. 1991
** Date of sowing 11. 11. 1993

The essential oils were obtained by means of WSD and SFE in a small-scale CO_2 extractor.

The fresh plant material was subjected to WSD for 2 h in a Hungarian Pharmacopoeia distillation apparatus [1].

SFE was performed on air-dried herbs, using an Isco SFX 2-10 instrument at 40 °C and at 50 °C and at increasing pressures (80 to 500 bar). The extraction time was 30 min. and the flow rate was 0.6 ml/min.

The oils were analysed by GC and GC/MS techniques. Analytical GC was carried out on a HP 5890 SERIES II gas chromatograph (FID), using a 30 m HP-5 fused silica capillary column which was programmed from 60 °C (2 min. hold)

to 220 °C (2 min. hold) at 5 °C/min. Other important parameters were injector temp.: 250 °C; carrier gas: N_2; split sample introduction. GC/MS was performed on a FINNIGAN GCQ mass spectrometer. All conditions were as above, except that the carrier gas was He.

RESULTS AND DISCUSSION

The main observations were as follows:

The GC study of the oils demonstrated three main components in the oil: β-pinene (**a**), pinocamphone (**c**) and isopinocamphone (**d**) (Fig. 1). In addition to these substances and in contrast with literature data [2-5] limonene (**b**) was one of the main components (about 38 %) in some samples (Fig. 2). Such a chemotype has not been described previously. Accordingly, the inves-tigation of the proportions of the four compounds was considered interesting.

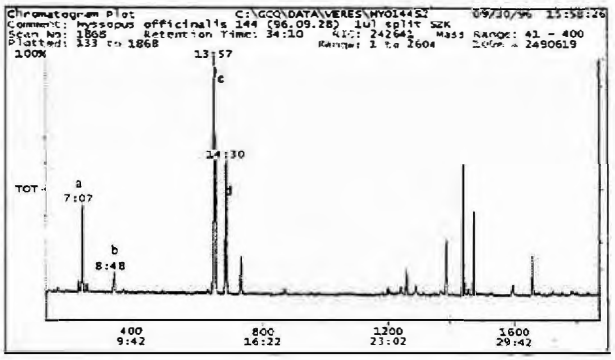


Fig. 1. Total ion chromatogram of the essential oil of *Hyssopus officinalis* L.

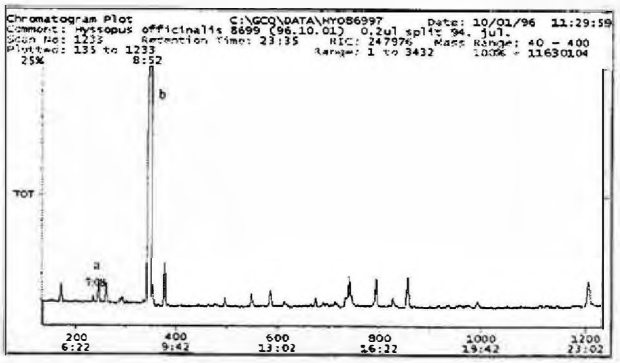


Fig. 2. Total ion chromatogram of the essential oil of *Hyssopus officinalis* L.

The heterogeneity in the oil composition of the various populations was found to be independent of the botanical gardens supplying the seed (Fig. 3).

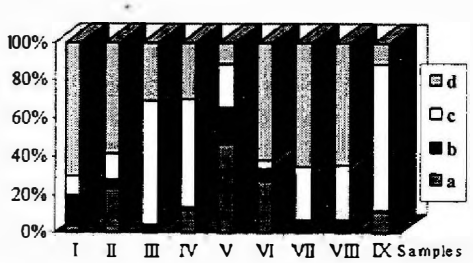


Fig. 3. Proportions of main components in the volatile oil of *Hyssopus officinalis* L. of different origins (samples harvested in October, 1994)

On SFE extraction, the oil yields were in general higher that those obtained by WSD (Fig. 4). In the CO_2 extractor, the parameters 100 bar, 40 °C and 30 min. were considered optimal.

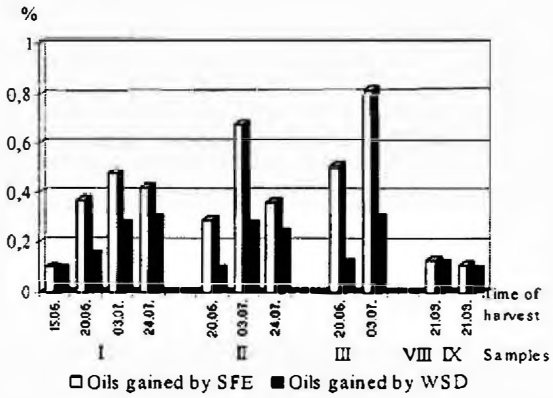


Fig. 4. Yields of essential oils obtained by different extraction methods

The variation in the oil composition of three populations (of various origins) was followed during the vegetation period. No changes in the proportions of the four main components were found in one population (Fig. 5), but the other two exhibited opposite changes (Figs. 6 and 7). Further studies are necessary to explain these differences.

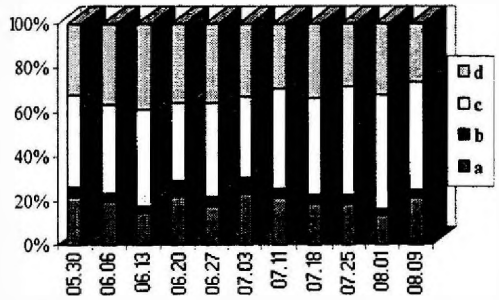


Fig. 5. Proportions of the main components in the volatile oil of *Hyssopus officinalis* L. (sample II)

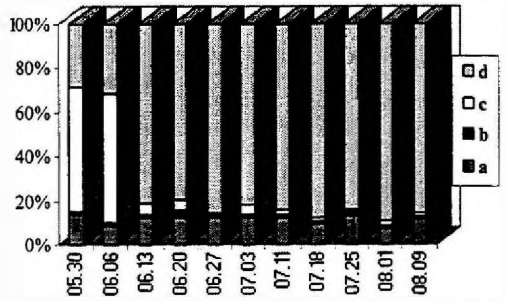


Fig. 6. Proportions of the main components in the volatile oil of *Hyssopus officinalis* L. (sample I)

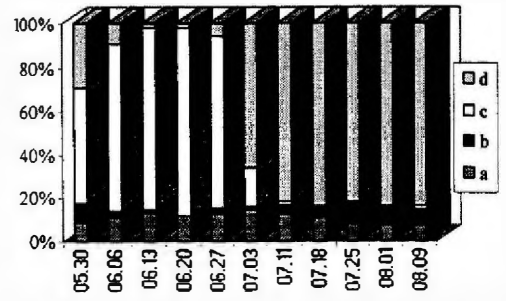
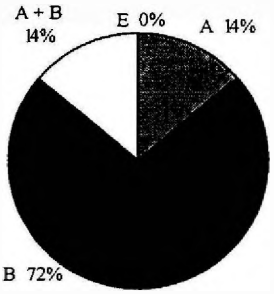
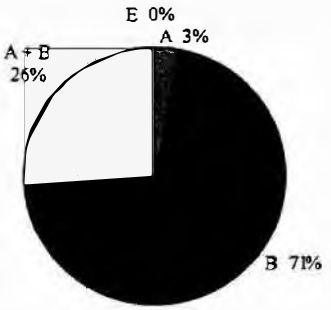


Fig. 7. Proportions of the main components in the volatile oil of *Hyssopus officinalis* L. (sample III)

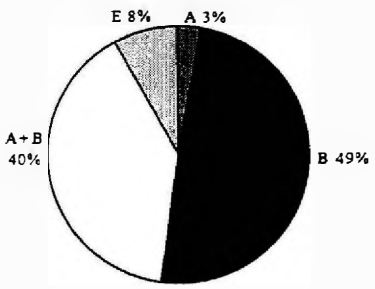
Our observations indicated that selection on the basis of the flower colour does not guarantee the homogeneity of the oil composition. It was therefore worth differentiating between populations as concerns the main components of the oils, e.g. pinocamphone, isopinocamphone, pinocamphone + isopinocamphone, limonene and oils with miscellaneous composition (Fig. 8).



Distribution of the individuals in strain marked 7.



Distribution of the individuals in strain marked 9.



Distribution of the individuals in strain marked 10.

A: isopinocamphone is the main component of the oil (more than 50% of the oil)
B: pinocamphone is the main component of the oil (more than 50% of the oil)
A+B: pinocamphone and isophinocamphone are together the main components (20-50%)
E: another compound is the main component of the oil (more than 30% of the oil)

Fig. 8. Distribution of the main components in the volatile oil of the offspring of *Hyssopus officinalis* L.

Chemical heterogeneity in oil composition was observed among the offspring of a plant with a particular chemical composition. When only the main four components (a: β-pinene, b: limonene, c: pinocamphone, d: isopinocamphone) were considered, clear and mixed lines alike could be found among the offspring independently of the original composition.

The above observations justify the necessity and usefulness of comparative chemical studies in hyssop selection, and these are currently ongoing.

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ESSENTIAL OIL FROM SARDINIAN *MELISSA OFFICINALIS* L. AND *MELISSA ROMANA* MILL.

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ABSTRACT

Analyses of the essential oils of *M. officinalis* L. showed the prevalence of isomeric terpenes with molecular weight 152 and 154 and a low content in compounds with higher m.w. The more represented components were neral and geranial. On the contrary in the essential oils of *M. romana* Mill. we found a high concentration of terpenes with m.w. 204 having the typical mass spectra of caryophyllene, muurolene and cadinene; no evidence of neral and geranial was found in this essential oil.

INTRODUCTION

The essential oil of *Melissa officinalis* L. (*Labiatae*) leaves is known for its properties as: sedative, antispasmodic, bacteriostatic, and antiviral activity (1). This perennial herb is commonly known as lemon balsam, owing to its citrus aroma due principally to the presence of geranial and neral (2). In Sardinia are present two taxa of *Melissa* genus: *Melissa officinalis* L. str. s. (= *Melissa officinalis* L. subsp. *officinalis*) and *Melissa romana* Mill. (= *M. officinalis* L. subsp. *altissima* (Sm.) Arcangeli). *M. romana* is widely diffused almost in all Sardinia island, but it is rare in the North-East of island. It is a Mediterranean autochthon entity, constituents of the wild vegetation of the semi-shaded hedges, of the riparian flora, of the edge of the wood, etc. *M. officinalis* is sporadically founded in few individuals among entity of *M. romana* or in distinct stations (more frequently in the submountain regions of the island). This second entity, more continental than Mediterranean, requires more fresh and humid environment and in the island it seems to be authocthon or naturalised rather than cultivated and spontaneized. In the present paper, we studied the steam-volatile leaf oil of these two species, wild and cultivated, to compare their composition.

EXPERIMENTAL

Specimens of cultivated *M. officinalis* L. were picked in two different stations located in: (a) S. Giovanni (Sassari) and in (b) Sassari city. Specimens of cultivated *M. romana* Mill. were picked in an experimental station of the University of Sassari located in (e) Ottava (Sassari); the wild plants were collected in Florinas (Sassari) in two different stations: the first one (c) located near at a fountain, the second one (d) in a semiarid uncultivated land growing among other wild species of plants. The volatile compounds from fresh leaves harvested from plants collected at the end of June 1995 were extracted by hydrodistillation in a Clavenger apparatus. Three replicates of each sample were analysed by using a Hewlett-Packard model 5890A GC fitted with a 50 m x 0.20 mm NS-54 fused silica capillary column (Supelco). Injection port and detector temperature were 240°C. The column temperature starts the run at 50 °C and than programmed to 130°C at 3°C/min (1min), to 205°C at 5°C/min (20 min), 20°C/min up 240°C and held for 15 min. The quantitation of each compound was expressed as absolute percentage using internal standard and response factors. The detector response factors (RF_S) were determined for key components relative to tetradecane (3). GC-MS analyses were carried out with a Perkin Elmer ITD (Ion Trap Detector) coupled with a Perkin Elmer model 4200 gas-chromatograph, equipped with a fused capillary column DB5 30m x 0.20 mm (ID) which was connected to the ion source of the mass spectrometer. Mass spectral matches were made by comparison of mass spectra with published data (4, 5).

RESULTS AND DISCUSSION

The quantitative data for each oil component found in the leaves of the two studied species and coming from different stations are reported in Table 1.

The oil components of leaves were divided into five groups: aldehydes, terpenes, alcohols, esters, ketones and oxides. In *M. romana* Mill. the aldehydes are present only in traces while in *M. officinalis* L. the aldehydes are well represented by citral (neral around 27% and geranial about 40%) and citronellal (6%).

With regard to terpenes it is worthy to note that in *M. officinalis* L. their percentage is around 11% and in *M. romana* Mill they reached the 71% and 89 % of total of constituents. This increase, in *M. romana*, is due to the high percentage of β-caryophyllene and α-muurolene that alone represent between 38.38% and 76.16% of the total amount of terpenes. In the alcohols group the highest level (7.07%) was found in all examined *M. officinalis* L. The content in alcohols in *M. romana* Mill. collected in the semiarid land was 5.85% while in the others we found a 2.66% and a minimum of 0.09% in the cultivated one. The variation in esters was from 1.14 % in *M. officinalis* L. collected in S. Giovanni and 4.13% in *M. romana* Mill. cultivated and in the wild plants the values were never higher than 3.58%. Ketones and oxides are represented by a maximum of the 7.93% of (-)-caryophyllene oxide in *M. romana* Mill. growing in the semiarid station. This compound is less than half in the others two considered *M. romana* Mill.; in *M. officinalis* L. it is never more than 0.57% because in this species the more represented compound is piperiton with a maximum of 3.60% in the plants collected in the station in Sassari city. In conclusion *Melissa officinalis* L. and *M. romana* Mill showed a clear difference in the qualitative and qualitative composition of the leaves essential oil. In *M. officinalis* the major constituent is citral (69%) while in *M. romana* the more important constituents are α-muurolene and β-caryophyllene. *M. romana* Mill. cultivated showed an increase in the yield of essence of leaves reaching the yield of *M. officinalis* L.; also in *M. officinalis* L. cultivated the yield was major. We can suppose that the cultivation supports the increase of the yield of essence.

ACKNOWLEDGEMENTS

The work was financially supported by M.U.R.S.T. (60% and 40%). The authors gratefully acknowledge Mr. Gianuario Manca and Mr. Nino Pittalis for technical assistance.

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Table 1. Chemical composition (%) of balm-mint oils obtained from cultivated plants of *Melissa officinalis* L. and from wild and cultivated plants of *Melissa romana* Mill.

Compounds[a]	*M. officinalis* L. *(a)	*M. officinalis* L. (b)	*M. romana* Mill. (c)	*M. romana* Mill. (d)	*M. romana* Mill. (e)
cis-hexen-3-enol	< 0.01	< 0.01	-	-	-
benzaldeide	< 0.01	< 0.01	-	-	-
6-methyl-5-hepten-2-en	0.11	0.07	-	-	-
octan-3-en	0.13	0.09	-	-	-
6-methyl-5-hepten-2-ol	0.09	0.05	-	-	-
linalool oxide anidrous	< 0.01	< 0.01	-	-	-
linalool oxide anidrous	< 0.01	< 0.01	-	-	-
limonene	0.05	0.07	-	-	-
cis-β-ocimene	0.15	0.12	0.62	-	< 0.01
trans-β-ocimene	0.52	0.44	0.91	-	< 0.01
cis-linalool-oxide	< 0.01	< 0.01	-	-	-
trans-linalool-oxide	< 0.01	< 0.01	-	-	-
linalool	0.73	0.25	-	-	-
pulegolo	0.15	0.18	-	-	-
citronellal	6.48	6.83	0.98	-	0.09
nerol oxide	1.13	0.75	-	-	-
isopulegol	2.00	1.24	-	-	-
safranal	< 0.01	< 0.01	-	-	-
δ-terpineol	< 0.01	< 0.01	-	-	-
β-citronellol	0.08	0.09	-	-	-
citronellil propionate	-	-	0.87	-	0.08
nerol	1.85	2.37	0.10	0.59	< 0.01
neral	28.76	28.14	-	-	-
geraniol	1.99	2.50	0.13	0.72	< 0.01
piperiton	2.30	3.60	-	-	-
geranial	40.62	41.11	-	-	-
methylgeraniato	0.24	0.25	0.32	0.33	0.11
nerilacetate	< 0.01	< 0.01	-	0.78	0.10
geranylacid	-	-	0.60	-	0.40
geranylacetate	0.90	1.27	2.39	0.94	3.84
α-copaene	0.22	0.19	3.32	1.79	< 0.01
β-copaene	0.13	0.14	3.40	1.68	< 0.01
β-elemene	0.17	0.12	-	-	-
methyleugenolo	0.18	0.20	-	-	-
β-caryophyllene	5.87	5.59	28.99	20.53	31.36
germancrene-D	0.12	0.09	2.53	1.40	2.47
α-bergamotene	0.12	0.08	1.60	0.77	< 0.01
humulene	0.34	0.40	2.72	1.78	2.90
α-muurolene	2.11	2.30	37.85	17.57	44.80
β-bisabolene	0.17	0.17	2.33	2.46	2.21
γ-cadinene	0.85	0.30	1.65	5.70	< 0.01
calamene	0.05	0.06	0.42	0.37	0.12
δ-cadinene	0.46	0.30	2.07	1.70	1.99
elemene	0.14	0.11	0.71	6.50	0.21
(–)-caryophyllene oxide	0.57	0.42	2.79	7.93	3.35
(-)-α-cubebene	-	-	-	9.48	-
α-terpineol	-	-	-	0.53	-
T-cadinol	-	-	-	0.74	-
α-cadinol	-	-	2.43	3.27	-

** **(a)** = Melissa officinalis L., cultivated, collected in S. Giovanni; **(b)** = Melissa officinalis L., cultivated, collected in Sassari; **(c)** = Melissa romana Mill., wild, collected in Florinas, Station A; **(d)** = Melissa romana Mill., wild, collected in Florinas, Station B; **(e)** = Melissa romana Mill., cultivated, collected in Ottava. [a]Peak identifications are based on MS comparisons with file spectra and relative retention time; [b]total identified components of balm-mint oil; absolute weight percent (wt%) using response factors; average of three GC runs.*

THE ESSENTIAL OIL COMPOSITION OF SOME *NEPETA* SPECIES FROM TURKEY

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INTRODUCTION

The genus *Nepeta* is represented in Turkey by 33 species and 38 taxa of which 17 are endemic (1). As part of our continuing research into essential oils of Turkish Labiatae, we have analysed the essential oils of 13 *Nepeta* taxa occurring in Turkey. The study materials, their collection sites and oil yields are listed in Table 1.

MATERIAL AND METHOD

Aerial parts of 13 *Nepeta* species were studied. The plant materials were collected from different regions of Turkey. Voucher specimens are kept at the Herbarium of the Faculty of Pharmacy, Anadolu University in Eskisehir, Turkey (**ESSE**). The air dried aerial parts of the plants were subjected to hydrodistillation for 3 h using a Clevenger apparatus to produce essential oils ranging from trace to 3.7 % yield. The percentage yields of the oils were calculated on moisture free basis (Table 1).

The essential oils were analysed by GC/MS using a Hewlett-Packard GCD system. An Innowax FSC column (60 m x 0.25 mm) was used with Helium as carrier gas. GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4°C/min and then kept constant at 220 °C for 10 min. Split ratio was adjusted at 50:1. The injector temperature was 250 °C. MS were taken at 70 eV. Mass range was from m/z 10 to 425. Library search was carried out using Wiley GC/MS Library and TBAM Library of Essential Oil Constituents (2-11). Relative percentage amounts of the separated compounds were calculated from Total Ion Chromatograms by the computerized integrator.

RESULTS AND DISCUSSION

The results have enabled us to group the species studied into several groups according to main components in their oils and their GC/MS results are expressed in separate list (see Table 2). Please note that only the compounds which are present in the oils over 1 % are listed.

The following compounds were identified as major components in the essential oils of the following *Nepeta* species. **1,8-cineole:** *N. sulfuriflora*, *N. nuda* subsp. *albiflora*, *N. nuda* subsp. *nuda*, *N. italica*; **geijerene:** *N. nuda* subsp. *nuda*; **caryophyllene oxide:** *N. conferta*, *N. isaurica*, *N. cilicia*, *N. nuda* subsp. *glandulifera*; **β-pinene:** *N. phyllochlamys*; **α-terpineol:** *N. viscida*; **linalool:** *N. flavida*; **4aα, 7α, 7aα-nepetalactone:** *N. cadmea*, *N. caesarea*.

***Nepeta sulfuriflora* P. H. Davis**: This endemic species has not previously been investigated. We have studied the essential oil composition of the two materials collected from two different regions. One of the samples was collected at fruiting, the other one at flowering stages. The flowering specimen yielded 0.44 % oil. GC/MS analysis resulted in the characterization of 92 compounds presenting 93% of the oil, with **1,8-cineole** (46.4 %) as the main constituent. 1,8-cineole content was lower (24.2 %) in the oil of the fruiting specimen which yielded 0.33 % essential oil. 86 components representing 94 % of the oil were identified (12,13).

***Nepeta italica* L.:** Herbal parts of *N. italica* yielded 3.7 % oil in which 62 components were characterized representing 95.52 % of the oil. Main constituents were **1,8-cineole** (51.61 %). To the best of our knowledge this is the first report on the essential oil composition of *N. italica*.

***Nepeta nuda* L. subsp. *albiflora* (Boiss.) Gams:** *Nepeta nuda* has four subspecies in Turkey. The oil of subsp. *albiflora* obtained in 0.1 % yield from the herbal parts was also rich in **1,8-cineole** (10.63 %). 128 components were identified representing 82.09 % of the oil. The composition of this oil has not previously been reported .

***Nepeta nuda* L. subsp. *nuda*:** This is the more common subspecies of *N. nuda*. It has been reported to have several chemotypes (14). 1,8-cineole-rich chemotypes have also been reported

(13). One specimen of *N. nuda* subsp. *nuda* collected in Kütahya yielded 0.2 % oil, rich in **1,8-cineole** (14.94 %). Nepetalactone-rich *N. nuda* is also quite common. However, one sample of *N. nuda* subsp. *nuda* yielded an oil (1.5 %) rich in **geijerene** (23.31 %). Geijerene is a rare compound found in the oils of *Geijera parviflora* (15,16), *Pimpinella* subsp. (17,18), *Ruta graveolens* (19, 20), *Ruta angustifolia* (21, 22), *Ruta chalepensis* (23), *Chromolaena odorata* (24,25), *Boenninghausenia albiflora* (26) and *Wiedemannia orientalis* (27).

***Nepeta phyllochlamys* P. H. Davis:** This is the first report on the chemistry of this endemic species. The herbal parts of this species yielded 1.1 % oil rich in **β-pinene** (16.26 %) 110 components were identified representing 94.13 % of the oil.

***Nepeta viscida* Boiss.:** All parts of this endemic plant are quite viscid, hence the name. It yielded an oil in which the main component was **α-terpineol** (31.6 %) (28). The oil yield from the aerial parts was 0.4 %. GC/MS analysis resulted in the characterization of 113 components representing 92.25 % of the oil. The main component was **α-terpineol** (18.73 %).

***Nepeta flavida* Hub.-Mor.:** Aerial parts of *N. flavida* yielded 1.4 % oil. Such a high yield is quite rare in *Nepeta* species which are generally oil poor. 84 compounds were identified representing 92.9 % of the oil with **linalool** (37.7 %) and **1,8-cineole** (22.7 %) as main constituents (12).

***N. cadmea* Boiss.:** This endemic species yielded 0.5 % oil in which the main constituent was **4aα,7α,7aα-nepetalactone** (74.96 %). It was among the 66 compounds identified representing 94.42 % of the oil.

***N. caesarea* Boiss.:** This also an endemic species. 0.6 % oil was distilled from its herbal parts. The main constituent of its oil was **4aα,7α,7aα-nepetalactone** (90.55 %). GC/MS analysis resulted in the characterization of 23 constituents representing 97.54 % of the oil (29).

Caryophyllene oxide was the main constituent in the ***Nepeta*** oils listed in Table 3.

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Table 1. The percentage yields of the oils calculated on moisture free basis

Code	Name	Material	Collection Place	Date	ESSE	Oil Yield (%)
NS1	*Nepeta sulfuriflora*	Flowering Aerial Parts	Antalya: Anamur	1995	11623	0.4
NS2	*N. sulfuriflora*	Fruiting Aerial Parts	Antalya: Gazipasa to Anamur	1995	11640	0.3
NNA	*N. nuda* ssp. *albiflora*	Aerial Parts	Rize	1993	10055	0.1
NNN	*N. nuda* ssp. *nuda*	Aerial Parts	Kütahya: Radar	1995	9199	0.2
NI	*N. italica*	Aerial Parts	Mu¤la: Fethiye	1995	11342	3.7
NNN2	*N. nuda* ssp. *nuda*	Aerial Parts	Bursa: Uluda¤	1992	10265	1.5
NC	*N. conferta*	Aerial Parts	Antalya: Elmal›	1995	11331	0.1
NIs	*N. isaurica*	Aerial Parts	Antalya: Alanya	1995	11509	0.5
NCi	*N. cilicia*	Aerial Parts	Antalya: Alanya	1995	11520	tr
NNG	*N. nuda* ssp. *glandulifera*	Aerial Parts	Antalya: Anamur	1995	11622	0.1
NP	*N. phyllochlamys*	Aerial Parts	Antalya: Kemer	1995	11296	1.1
NV	*N. viscida*	Aerial Parts	Mugla: Fethiye	1995	11350	0.4
NF	*N. flavida*	Aerial Parts	Adana: Düziçi	1995	11618	1.4
NCa	*N. cadmea*	Aerial Parts	Antalya: Kemer	1995	11294	0.5
NCs	*N. caesarea*	Aerial Parts	Adana: Yarpuz	1995	11530	0.6

Table 3. *Nepeta* oils with caryophyllene oxide as main constituent

Species	Endemic	A	B	C
N. conferta Hedge et Lamond	+	15.77	99	78.32
N. isaurica Boiss. et Heldr.	+	15.47	86	86.39
N. cilicia Boiss.	-	36.44	28	87.70
N. nuda subsp. *glandulifera* Hub.-Mor. et Davis	+	30.71	60	65.43

A: Caryophyllene oxide (%)
B: Number of components identified
C: Percentage of total components identified

Table 2. The composition of *Nepeta* oils analysed. *(Only the components >1% are listed. The blanks are either <1% or 0)*

	NS1	NS2	NI	NNN	NNA	NC	NIs	NCi	NNG	NCa	NCs	NF	NP	NNN1	NV
a-pinene	2.72	1.80	3.78												1.55
a-thujene		1.07											1.49		
b-pinene	4.03	2.33	3.75									1.87	16.26		8.94
sabinene	6.38	12.79	2.88									1.70	6.56		4.32
myrcene															1.11
a-terpinene		1.01													
limonene								2.36							1.14
1,8-cineole	46.35	24.23	51.61	14.94	10.63	1.00	3.90					22.67	5.91	7.99	9.41
b-phellandrene					2.07										
g-terpinene		1.97													
(E)-b-ocimene					1.53								1.28		
p-cymene						1.43		1.93					1.19		
geijerene														23.31	1.02
cis-linalool oxide (furanoid)						3.47							1.05		
trans-sabinene hydrate	1.11	1.39	1.25				1.96								
trans-linalool oxide (furanoid)						2.50									
octyl acetate					1.23										
a-copaene	1.53			1.95		1.33		2.58							
campholenal							1.18								
b-bourbonene				1.10	2.15		1.11								
linalool	2.13	4.65				11.41	2.52			3.42	1.11	37.65	7.64		1.17
b-cubebene								2.67							
pinocarvone							2.92						1.20		
pregeijerene														7.36	
terpinen-4-ol	3.02	5.76	1.92	1.30		1.93	1.06			2.53			8.37		
b-caryophyllene				7.82	4.68			2.57	5.34			4.74			4.56
myrtenal			1.07				3.52						1.35		
aromadendrene				1.76					2.40						
(Z)-b-farnesene														2.48	
trans-pinocarveol	1.02					1.65	3.95						2.21		
(E)-b-farnesene					3.29										1.82
d-terpineol	1.28		2.88												
a-humulene				1.80	1.84										
trans-verbenol	1.55	2.08	1.65			3.55	4.36						2.13		
g-muulolene					1.23										
a-terpineol	2.94	2.74	7.14	1.53			4.74					2.21			18.73
germacrene-D	1.88	1.47	1.35	4.82				1.18		1.37		1.90			1.78
b-bisabolene				1.10	4.01				1.47					3.10	1.80
bicyclogermacrene															2.61
carvone				3.65			1.31								
d-cadinene	1.35	1.47		1.80				1.08							
b-sesquiphellandrene														2.21	1.28
myrtenol							3.43						1.08		
trans-carveol							1.32								
calamenene												1.03			
epicubebol								1.00							
neoisodihydrocarveol								2.22							
1,11-oxidocalamenene*									2.81						
a-calacorene-I						1.00									
palustrol							2.01		3.48						
cubebol								5.49							
isocaryophyllene oxide						1.09	3.62	6.36							
caryophyllene oxide	2.55	4.64	1.33	6.25	10.47	15.77	15.47	36.44	30.71			2.40	7.78		2.90
4aa,7a,7aa-nepetalactone										74.96	90.55				
(E)-nerolidol										2.72		1.03			
ledol							7.24		1.55			1.52			1.03

4ab,7a,7aa-nepetalactone														3.68	
4aa,7a,7ab-nepetalactone														7.73	
germacrene D-4-ol				1.15											
humulene epoxide-II					1.73	1.00	1.00	4.01	2.53						
4ab,7a,7ab-nepetalactone														13.47	
elemol						2.65							1.96		7.14
spathulenol				1.00	1.53	2.37	4.58	5.85	4.80						1.81
(Z)-3-hexenylbenzoate						1.51			1.00						
g-eudesmol															1.28
T-cadinol			3.21												
eugenol		2.79													
thymol				2.24	2.47										
cembrene															1.15
carvacrol		2.39		7.13	3.32		1.60	4.48							
a-eudesmol															1.26
cadalin (=cadalene)						2.03									
a-cadinol		8.05		1.00								1.04			1.89†
oxo-a-ylangene								1.84							
a caryophylladienol*									1.01						
14-nor-cadin-5-en-4-one						2.38									
caryophyllenol-II				1.21	1.79	1.07	1.16	1.72	2.13						
phytol					1.91										

** tentative identification by GC/MS data alone , † mixed with b-eudesmol*

THE ESSENTIAL OILS OF THREE NEW LABIATAE TAXA FROM TURKEY: *ORIGANUM HUSNUCAN-BASERII, SIDERITIS GULENDAMII* AND *SALVIA AYTACHII*

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INTRODUCTION

The family Labiatae (Lamiaceae) is represented in Turkey by 556 species and altogether 741 taxa. Turkey is also considered a gene centre for several Labiatae genera such as *Origanum*, *Sideritis* and *Salvia*. The genus *Origanum* is represented by 24 species and 27 taxa in Turkey 15 of which are endemic. *Sideritis* is represented by 45 species and 52 taxa, 37 taxa being endemic. The genus *Salvia* is represented by 88 species and 93 taxa in Turkey. The ratio of endemism in *Salvia* is 51 %.

Recently, three new species belonging to the above genera have been described from Turkey. These are *Origanum husnucan-baserii* H. Duman, Z. Aytaç et A. Duran, *Sideritis gulendamii* H.Duman et F.A.Karaveliogullari[1]. and *Salvia aytachii* M.Vural et N.Adigüzel [2].

Here we report on the essential oil compositions of these newly described endemic species of the flora of Turkey.

MATERIAL AND METHOD

Plant Materials

Plant materials were collected during flowering stage from the following sites: *O.husnucan-baserii*, Antalya: Alanya; *S. gulendamii*, Eskisehir: Sivrihisar and Mihaliççik (two collections); *S.aytachii*, Ankara: Beypazari to Nallihan road.

Voucher specimens are kept at the Herbarium of the Anadolu University Faculty of Pharmacy in Eskisehir, Turkey (ESSE).

Distillation

Plant materials were hydro distilled for 3 h using a Clevenger-type apparatus. The percentage yields of the oils calculated on moisture free basis were as follows: *O. husnucan-baserii* (0.13 %), *S. gulendamii* (0.07 % and 0.14 %) and *S. aytachii* (0.90 %).

GC/MS

The essential oils were analysed by GC and two GC/MS systems. GC analysis was carried out using a Shimadzu GC-15A with C-R4A integrator. HP-5 FSC column (30 m x 0.32 mm) was used with nitrogen as carrier gas. Oven temperature was programmed from 60 °C to 240 °C at a rate of 3 °C/min, then kept at 240 °C for 10 min. The following GC/MS systems were used: **A**= A Shimadzu GC/MS QP2000A system. The same column and operational conditions as in GC were applied. Carrier gas was helium. **B**= A Hewlett-Packard GCD system. Innowax FSC column (60 m x 0.25 mm) was used with helium as carrier gas. GC oven temperature was kept at 60°C for 10 min and programmed to 220 °C at a rate of 4 °C/min, and then kept constant at 220 °C for 10 min. Split ratio was adjusted at 50:1. The injector temperature was at 250 °C. MS were taken at 70 eV. Mass range was from *m/z* 10 to 425. Library search was carried out using Wiley GC/MS Library and TBAM Library of essential oils constituents. The MS were also compared with those of reference compounds and confirmed with the aid of retention indices from published sources[3-10] Relative percentage amounts of the separated compounds were calculated from total ion chromatograms by the computerized integrator.

RESULTS AND DISCUSSION

The yields and chemical compositions of oils obtained by hydrodistillation from the aerial parts of three new endemic species of Turkey were analysed by GC/MS.

Origanum husnucan-baserii

Turkey is considered as the centre of origin of the genus *Origanum*. We have studied the essential oil composition of 20 out of 26 *Origanum* taxa existing in Turkey.

These studies led us to categorize the *Origanum* species studied into three groups according to the

major components in their oils. The first group comprises three terpenes which are terpene-phenol carvacrol, followed by p-cymene and linalool. They are the main constituents in the oils of 65 % of the species studied. The second group consists of components that are only found in a few species but when they are found are present in relatively high concentration. The compounds myrcene, g-terpinene, terpinen-4-ol, borneol, linalyl acetate, cis-sabinene hydrate, b-caryophyllene, germacrene D and bicyclogermacrene belong to this group. A typical feature of the species of this group is that all of them are oil-poor. The third group comprises compounds that are typical minor components in *Origanum* essential oils of Turkish origin such as (E)-b-ocimene, (Z)-b-ocimene, trans-sabinene hydrate, 1,8-cineole, terpinen-4-ol, a-terpineol, camphene, geranyl acetate, carvacrol methylether, spathulenol, b-bisabolene, a-cadinol, b-bourbonene, b-terpinene[11].

Origanum husnucan-baserii is included in the second group which is oil-poor. Indeed, the essential oil yield of this species was 0.13 %.

Among the *Origanum* species of Turkey studied so far, this is the only oil which contains borneol (20.3 %) as major constituent. Other major components include a-terpineol (11.46 %), trans-sabinene hydrate (10.97 %), terpinen-4-ol (8.36 %), b-caryophyllene (6.06 %) and caryophyllene oxide (5.76 %). The list of major compounds identified in the oil of *O. husnucan-baserii* is given in Table 2 [12].

This species was named after one of us, Prof. Dr. K. Hüsnü Can Baser, a pharmacognosist and essential oil scientist[1].

Sideritis gulendamii

This beautiful looking species was collected from two localities. The material collected from the type location in Eskisehir: Sivrihisar consisted of above ground parts, while the material collected from Eskisehir: Mihaliççik comprised only the flowering spikes. Therefore, different oil yields and compositions, were obtained not surprisingly, as 0.07 % and 0.14 %, respectively. Better oil yield was obtained from the inflorescence.

In the oil of herbal parts, 91 compounds were characterized representing 79.8 % of the oil with hexadecanoic acid (9.5 %), b-pinene (8.8 %), hexahydrofarnesyl acetone (6.8 %), a-pinene (4.1 %) as major constituents. The oil of the inflorescence, 67 compounds were identified representing 84.5 % of the oil with b-pinene (34.3 %), a-pinene (13.2 %), trans-pinocarveol (4.8 %), limonene (4.4%) and myrtenol (4.2 %) as main constituents.

The genus *Sideritis* has an exceptional status among the other Lamiaceae genera in Turkey. The ratio of endemism in *Sideritis* is quite high (78 %) and almost all *Sideritis* species are used in Turkey as herbal tea.

Our field studies in recent years have resulted in the description of four new species for science, one new species for Turkey. There are, at present, 45 species and 52 taxa of *Sideritis* recorded in the flora of Turkey.

All the *Sideritis* species of Turkey have been collected by our group and their oils have been analysed. They can be classified into three groups, namely "monoterpene hydrocarbon-rich", "oxygenated monoterpene-rich" and "sesquiterpene hydrocarbon-rich". 58 % of the *Sideritis* species existing in Turkey belong to the "monoterpene hydrocarbon-rich" group. *S. gulendamii* is also included in this group.

This plant was named after Assoc. Prof. Dr. Gülendam Tümen, an essential oil scientist in Balikesir University, Balikesir , Turkey [1].

Salvia aytachii

Salvia aytachii has recently been described from Turkey. It has been named after Dr. Zeki Aytaç, a taxonomist in Gazi University, Ankara [2]. This new *Salvia* species yielded an oil containing camphor (30.78 %) and 1,8-cineole (27.28 %) as major constituents[13]. Turkish *Salvia* species containing camphor and 1,8-cineole as major constituents have previously been reported, such as *S. cryptantha* [14], *S. recognita*, *S. fruticosa* (syn. *S. triloba*), *S. aucheri*, *S. blepharochlaena* [15].

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Table 1. Chemical composition of the oil of *Origanum husnucan-baserii* *(Only the major components >1% are listed here)*

Compound	%	Compound	%
γ-terpinene	1.0	α-terpineol	11.5
p-cymene	1.3	borneol	20.2
1-octen-3-ol	1.3	germacrene D	2.7
trans-sabinene hydrate	11.0	bicyclogermacrene	1.7
β-bourbonene	2.4	caryophyllene oxide	5.8
linalool	3.0	spathulenol	2.3
cis-sabinene hydrate	1.9	thymol	3.1
terpinen-4-ol	8.4	carvacrol	1.0
β-caryophyllene	6.1	a caryophylladienol*	1.2

** Correct isomer not characterized*

Table 2. Chemical composition of the oil of *Sideritis gulendamii* (*Only the major components >1% are indicated in this list*)

Compound	I (%)	H (%)	Compound	I (%)	H (%)
α-pinene	13.2	4.7	cryptone	1.3	-
β-pinene	34.3	8.8	germacrene D	-	1.8
sabinene	1.7	t	(E,Z)-2,4-decadienal*	-	1.6
limonene	4.4	2.2	myrtenol	4.2	t
p-cymene	1.7	t	(E)-geranyl acetone	-	1.8
nonanal	t	2.2	hexahydrofarnesylacetone	t	6.8
α-copaene	1.8	-	carvacrol	-	5.6
decanal	-	1.0	α-cadinol	1.0	-
pinocarvone	1.9	t	14-hydroxy-α-muurolene	t	1.3
myrtenal	3.3	t	farnesylacetone	-	1.1
trans-pinocarveol	4.8	t	tetradecanoic acid	t	5.6
(E)-β-farnesene	t	1.2	hexadecanoic acid	-	9.5

***I:** Inflorescence, **H:** Herbal part * mixed with δ-cadinene*
***t**: Compositional values >1% are denoted as traces*

Table 3. Chemical composition of the oil of *Salvia aytachii* (*Only the major components >1% are indicated in the list*)

Compound	%	Compound	%
α-pinene	4.3	camphor	30.8
camphene	6.9	bornyl acetate	1.5
β-pinene	2.5	borneol	4.8
myrcene	3.9	valeranone	4.0
limonene	2.3	β-eudesmol	1.5
1,8-cineole	27.3		

CHEMICAL ANALYSIS OF ESSENTIAL OIL AND SOLVENTS EXTRACTS OF SAGE (*SALVIA OFFICINALIS* L.) PLANT FROM POLAND

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ABSTRACT

In dry leaves of sage (*Salvia officinalis* L.) essential oil content, flavonoids and tannins were identified. Chemical compounds extraction from sage leaves was carried out, using 4 methods. By a gas chromatography was also analysed chemical composition of volatile compounds in essential oil and 70% C_2H_5OH, CH_3OH and CCl_3F extracts. There is relatively similar composition of main chemical compounds in essential oil and in extracts. Main chemical compounds are α- and β-thujone, camphor and 1,8-cineole.

KEY WORD INDEX

Salvia officinalis L (*Labiatae* Linn.), essential oil composition, solvent extracts composition, 1,8-cineole, α-, β-thujone, camphor.

INTRODUCTION

Sage (*Salvia officinalis* L.) is a very important medicinal plant, containing mainly essential oil (3, 4), flavonoids and tannins. Raw material is dried leaves (*Folium Salviae*) and herb (*Herba Salviae*). The aim of this work was to compare four methods of sage leaves extraction:

- steam distillation
- 70% C_2H_5OH extraction
- CH_3OH extraction
- CCl_3F extraction

Isolats were examined on the content and composition of volatile compounds, flavonoids and tannins, extraction reminders on the content and composition of flavonoids and tannins.

EXPERIMENTAL - MATERIALS AND METHODS

The research materials were dry leaves of sage collected in second year of plants vegetation. Plants were cultivated in collection of Department of Medicinal Plants of Warsaw Agricultural University (Poland).

- Essential oil was separated by steam distillation.
- Extraction was performed with 70% C_2H_5OH, CH_3OH, and CCl_3F at the boiling temperature of solvents during 8 hours in Soxhlet's.

  Extraction efficiency was calculated by weight method, conversed into 100 g of dry leaves (g of extract/100 g of dry leaves).

- Volatile compounds were determined by gas chromatography:
  gas chromatograph Analtical Instruments Limited model 93, (Anglia Instruments Ltd.)
  capillary column - Carbowax 20M
  detector temperature 250°C
  injector temperature 200°C
  carrier gas - helium

Column temperature was programmed as follows: 60°C (2 min.), temperature increment 4°C/min. and finally 220° (5 min).
Compounds were determined on the ground of standards retention times.

- Flavonoids were determined according to Christ-Müller (1)
- Polyphenols and polyphenols not binding hide powder (CRS), according to Deutsches Arzneibuch (2)
- Dry mass of leaves according to Pharmacopoea Polonica (6)

RESULTS AND DISCUSSION

Content of essential oil in dry leaves of sage was 2%.

Extraction efficiency conversed into 100 g of dry mass leaves was:

- 70% C_2H_5OH - 19%
- CH_3OH - 17,5%
- CCl_3F - 15,5%

Chemical compounds identified in essential oil and examined extracts are presented in Table 1.

Main chemical compounds in essential oil and extracts were α - and β-thujone, camphor and 1 8-cineole.

There is relatively similar composition of volatile compounds in essential oil obtained by steam distillation and in 70% C_2H_5OH, CH_3OH and CCl_3F extracts.

Similar dependence was observed by Langer et al. (5), when analysing by gas chromatography essential oil from sage leaves obtained by steam distillation and dichloromethane extracts.

Flavonoids and tannins content in examined samples are presented in Figure 1 and Figure 2.

Above mentioned solvents turned to be bad agents for flavonoids and tannins extraction.

Flavonoids and tannins isolation from sage leaves using these solvents is incomplete, because significant number of them is still found in extraction reminders.

Steam distillation reminders and organic solvents extraction reminders are rich source of flavonoids and tannins.

Table 1:
The compounds identified in sage (*Salvia officinalis L.*) essential oil and extracts (total amount of all components = 100%).

COMPONENT (%)	ESSENTIAL OIL	EXTRACT		
		70% C_2H_5OH	CH_3OH	CCl_3F
α-Pincne	0.4	0.1	0.1	0.3
Camphene	4.5	1.4	1.4	5.1
β-Pinene	4.2	1.2	1.2	3.9
Myrcene	0.1	0.1	0.1	0.1
α-Terpinene	0.1	-	-	-
Limonene	1.6	0.6	0.6	1.4
1,8-Cineole	12.3	7.4	7.2	9.9
p-Cymene	1.6	0.6	0.6	1.3
α-Thujone	19.0	17.9	17.4	17.3
β-Thujone	14.3	12.8	12.5	11.8
Camphor	21.9	20.7	20.1	21.0
Linalool	0.2	0.1	0.1	0.1
β-Caryophyllene	0.7	0.6	0.6	0.7
α–Humulene	6.6	5.2	5.1	5.6
Isolated chemical compounds number	57	60	55	60
PERCENTAGE SHARE OF IDENTIFIED CHEMICAL COMPOUNDS	87.5	68.8	66.9	78.5

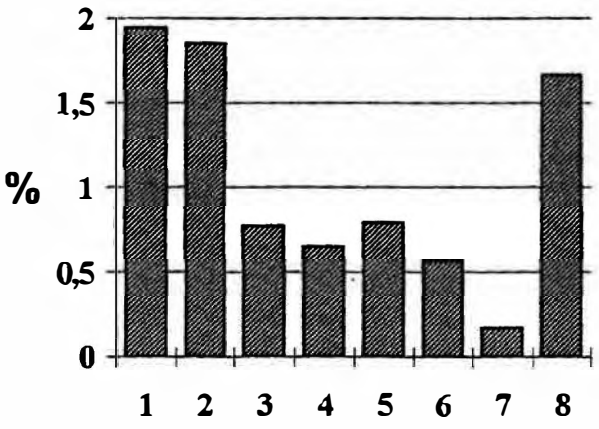

Figure 1. Flavonoids content in examined samples conversed into quercetin.

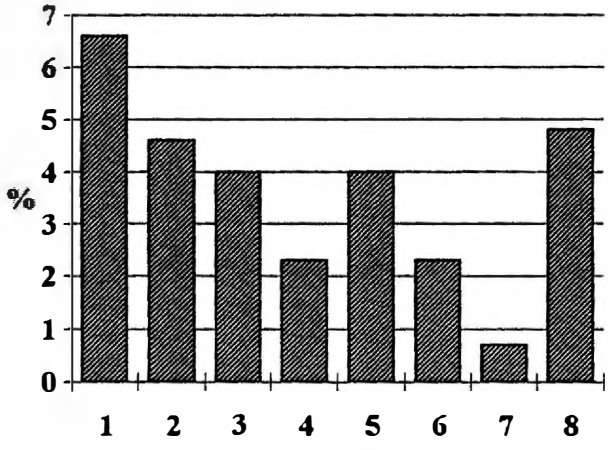

Figure 2. Tannins content in examined samples conversed into pyrogallol.

Legend:
1 - air dry sage leves
2 - after distillation reminders
3 - CH_3OH extract
4 - after CH_3OH extraction reminders
5 - 70% C_2H_5OH extract
6 - after 70% C_2H_5OH extraction reminders
7 - CCl_3F extract
8 - after CCl_3F extraction reminders.

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ESSENTIAL OIL CONTENT AND COMPONENTS OF *SALVIA OFFICINALIS* L. FROM BULGARIA

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INTRODUCTION

Garden sage (*Salvia officinalis* L.) has been known ever since the 14th century (1), being used as a medicine and spice. It is grown for its leaves which have wide application as disinfectant and anti-inflammatory means, against laryngitis, pharyngitis, pulmonic catarrh, etc. (2). Plants contain different biologically active and aromatic substances, but the essential oil is the main active ingredient (3, 4, 5). Its content, according to different authors, is highest in the leaves, being in the range of 0.5 - 2.5% (1, 3, 5), depending on the origin and time of herbage harvesting (4). Main oil components are α- and β-thujene, camphor, pinene, cineole (6, 7) and others. Most of the literature sources point to a higher α-thujene content (8, 9), which varies considerably (2.8% - 40.0%), depending on the herbage origin (10). Some authors report higher amounts of β-thujene (11), camphor (9) or borneol (1) in *Salvia officinalis* oil. Surely, the essential oil content depends on the stage of herbage harvesting. In literature, there are contradictory data about it. According to some authors, maximum yield is obtained at the stage of full bloom (2, 12), to others - at the post-flowering stage (7) or at the stage of seed ripening (5).

The objective of our study is to characterize plants from a vast garden sage population introduced from Poland, by paying a special attention to the phenological development, the habits of plants, the number of flower stalks, the flower coloration, the essential oil content and the variation in the components' values within the population.

EXPERIMENTAL

The investigation was carried out in the experimental field of the Research Institute for Roses, Aromatic and Medicinal Plants, Kazanlik, during the period 1995-1996. A cultivated garden sage population (three- and four-year-old plants), in good quality and health state was used for the experiment. Ten plants with similar external characters and the same time of flowering (early and late flowering) were selected from each chemotype. We determined the initiation time of the main phenological stages, the productive characters - the average fresh and dry herbage (leaves, stems, flowers) per tuft, the tuft height and diameter, the number of flower stalks and the number of leaves per plant. The essential oil content was determined at the time of full bloom in fresh samples of 200 g herbage in Clevenger type apparatus. The oil components were identified by a PYE UNICAM gas chromatograph, series 204, equipped with a flame-ionising detector, capillary column CARBOWAX 20M (25 m x 0.2 mm, Film thickness: 2 μm), hydrogen as a carrier gas at a flow rate of 0.8 ml/min. The GC apparatus was equipped with integrator Waters 745 B Data Module and programmed as follows: initial temperature 40^0 C for 5 min, then increasing by 12^0 per min to 150^0 where kept for 10 min. Visually, a great diversity of forms existed in the population, i.e.: different tuft habits, a wide range of flower colours - white, light and dark violet, light- and dark pink, different thickness of flower stalks, coloration and thickness of leaves - from light green to dark green and grey. Here are reported the generalised results only for seven chemotypes, which are typical representatives of the form variation and could be found in the rich population exits in the Research Institute for Roses, Aromatic and Medicinal Plants, Kazanlik.

RESULTS AND DISCUSSION

The chemotypes tested have different tuft habits (Figure 1). The greatest height (82 cm) and diameter (110 cm) are typical for the late flowering purple forms. These values vary within the population, the difference between the lowest and the highest tufts being 28 cm, and between the most and the least branched ones - 50 cm. Generally, the width of tufts is greater than their height, except the

white-flower forms. The number of flower stalks per tuft varies in the range of 32 for the white-flower forms to 152 for the pink-flower ones. The late-flowering forms have a higher number of flower stalks than the early-flowering ones, this tendency being not established only in the white-flower forms. The number of leaves per tuft varies in the range of 1256 - 1520, the lowest values being typical for the white-flower forms, and the highest - for the forms with dark violet and pink flowers from the group of the late-flowering forms. The morphological analysis on leaf blades did not establish any significant differences among the forms. In the early purple-flower forms, oblong-lanceolate outgrowths were developed at the base of leaves. The length of leaves varied from 3 to 9 cm, their width - from 2 to 3 cm. The leaf colour in the late pink-flower forms was light green, and that in the other forms varied from dark green to whitish grey.

The usable part of plants involved leaves, inflorescence and part of the flower stalks. It is seen from Figure 2 that the most-foliated chemotypes produce more fresh herbage. The late flowering forms (white-flower forms excepted) proved to be more productive, the fresh herbage amount obtained per tuft being in the range of 600-720 g, i.e. almost two times higher than the yields obtained from some of the early-flowering forms. The fresh/dry herbage ratio for most of chemotypes is in the range of 25 - 30%, with exception of white-flower forms having value of 20%. The essential oil content of the fresh herbage varies in the range of 0.15 to 0.3 %. The lowest oil content within the group of early blooming was measured in dark violet flower plants, and within the later blooming forms was found in white flowering plants.

The amount of the main component α-thujene in the oil of the different forms varies considerably (Table 1).

Table 1. Main constituents in the oils from different chemotypes (%)

Chemotypes	α-pinene	camphene	β-pinene	α-thujene	β-thujene	camphor	β-caryophylle
early blooming forms							
Dark violet flowers	0.2	1.83	0.98	19.7	1.7	0.2	7.55
Violet flowers	2.5	0.7	17.8	10.0	4.0	0.4	7.91
Pink flowers	4.2	4.4	13.8	13.9	9.4	2.3	7.41
later blooming forms							
Dark violet flowers	0.1	3.8	3.9	24.7	0.7	11.2	11.69
Violet flowers	2.5	1.0	16.7	33.3	2.5	0.3	9.78
Pink flowers	2.9	1.9	17.6	14.2	2.7	1.7	5.15
White flowers	2.5	0.52	17.9	17.4	1.8	2.4	17.10
	P=4.1	P=3.8	P=17.0	P=23.3	P=8.6	P=11.0	P=11.95

P= extent of the row

When comparing the dark violet, violet and pink flower forms from the early and late flowering groups, a tendency to increase in this component is observed. A considerable variation in the main component (α-thujene) content in the leaves of the different forms is seen, (10.02% - 33.3% (with P=23.3%) on the average for the two experimental years), being by 16 % higher than results reported by Balinova et al (6). By comparing the forms with the same flower coloration from the two groups, a tendency to increase in the α-thujene and decrease in the β-thujene amounts is established in the late-flowering forms. The highest α-thujene content is found in the oil of the late-flowering form with purple flowers, and the lowest - in the early-flowering form with the same flower coloration. The highest β-thujene content is established in the early-flowering form with pink flowers. The β-caryophyllene content in the oils of the early-flowering forms is almost the same, while in the oils of the late-flowering forms it varies from 5.15% (in the pink-flower form) to 17.1% (in the white-flower form). The lowest β-pinene content is established in the oils of the dark purple forms from

both groups, while in the oils of all other forms it is almost the same - ca. 16-17%. A similar tendency is observed on the α-pinene content. The camphene content varies within the population between 0.52% and 4.35%, and that of camphor - between 0.21% and 11.19%. Results show significant variation of investigated compounds in the population - Table 2. The highest variations were obtained for α-thujene, then β-pinene, β-thujene, β-caryophyllene and camphor, the least changeable being α-pinene and camphene.

Table 2. Comparative estimation of the essential oil constituents from all chemotypes i.e. the whole population

Constituents	Mean	S^2	S	S%
α-pinene	2.1	1.9	2.0	64.2
Camphene	2.0	2.0	1.4	69.7
β-pinene	12.6	44.4	6.7	52.8
α-thujene	19.0	53.0	7.3	38.3
β-thujene	3.2	12.4	3.5	109.3
Camphor	2.6	13.0	3.6	136.0
β-caryophyllene	9.5	13.2	3.6	38.0

S^2 = variance; S = standard variation;
S % = coefficient of variation; P = extent of the row

CONCLUSIONS

The forms (chemotypes) of *Salvia officinalis* population differ both in their appearance - size of tufts, colour of flowers and leaves, thickness of flower stalks, and their productive characters - herbage yield, essential oil content and quality. The late-flowering forms (except the white-flower ones) are most productive. The highest essential oil content is established in the chemotypes with violet and pink flowers. The lowest values of all productive characters are registered in the white-flower forms.

The highest content of the main component α-thujene is found in the late violet flower forms. The early pink-flower forms surpassed the rest in contents of α-pinene (4.23%), camphene (4.35%) and ß-thujene (9.35%), and the white flower ones - by the contents of β-caryophyllene and β-pinene.

The ultimate values, established in our study, should not be considered as threshold ones, but most representative for *Salvia officinalis* population in Bulgaria, as far as the number of possible combinations is unlimited. The richness of the garden sage population in different forms with valuable economic characters is a prerequisite for their use in the process of breeding research.

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Figure 1. Biometric measurements within different chemotypes, (mean for 20 plants)

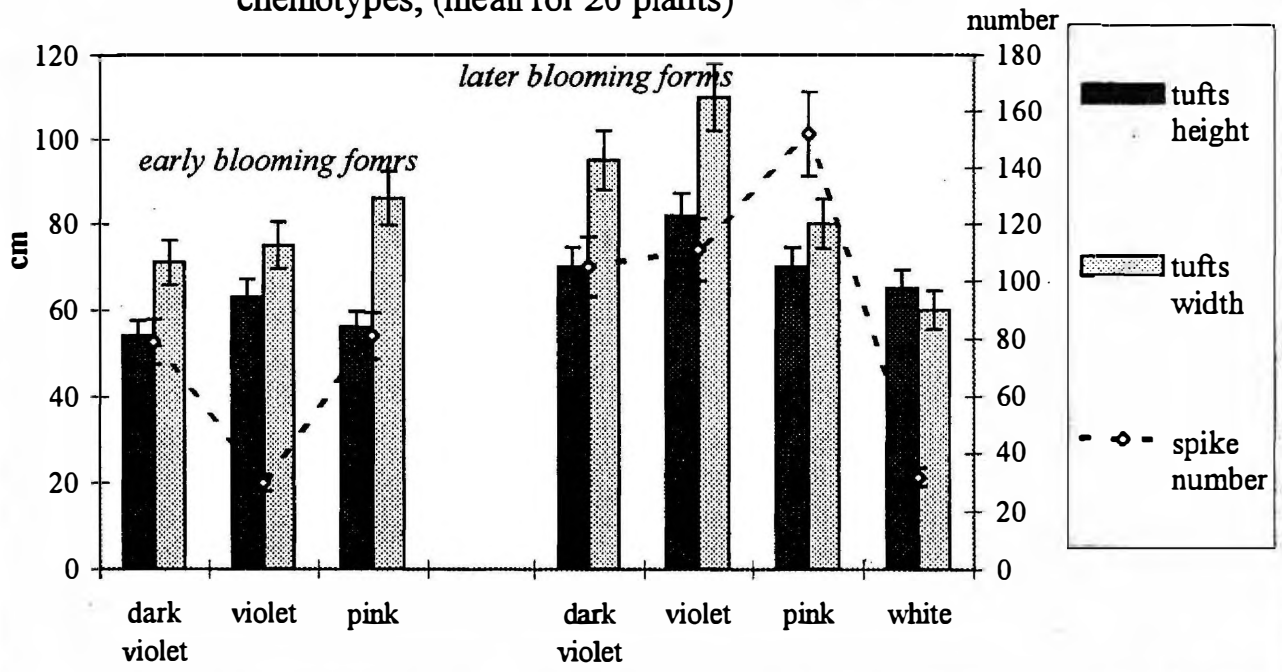
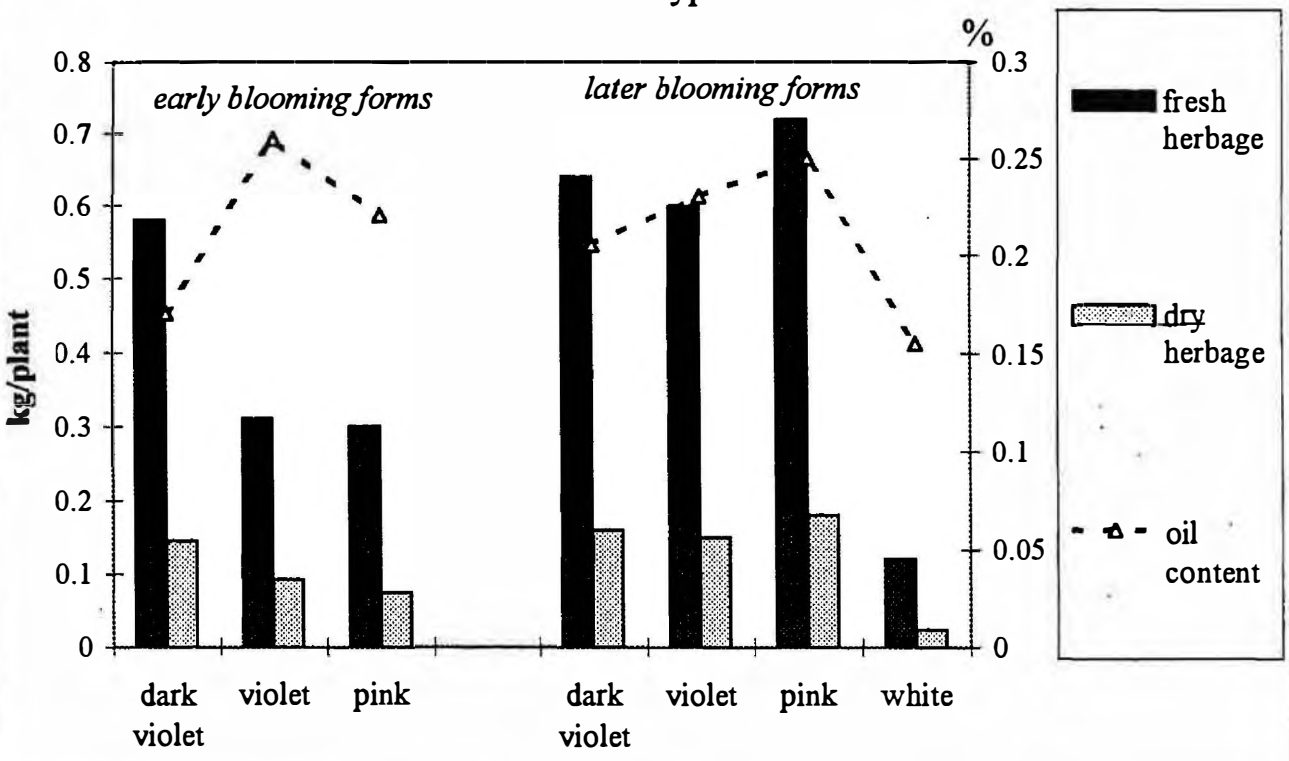


Figure 2. Yield of fresh and dry herbage and oil content in fresh herbage from different chemotypes



COMPARATIVE ANALYSIS OF *SALVIA OFFICINALIS* AND *SALVIA TOMENTOSA* ESSENTIAL OILS

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INTRODUCTION

Salvia officinalis L. (*Labiatae*) is a widely used medicinal plant, originally native to the Mediterranean Basin and produced on a large scale along the Adriatic coast and in Albania. The leaves of this species are applied as an anti-inflammatory herbal remedy, and its oil is used in perfumery, and as a raw material in the production of insecticides.

Salvia tomentosa Mill. (syn. *S. grandiflora*) is also an element of the European native flora, with limited occurrence in South-west Asia, the Crimea and the Balkan peninsula [1]. It is mostly valued as an aromatic and decorative plant.

Both species belong in the section *Salvia* (*Eusphace*, Bentham) of the genus *Salvia* and are closely related; moreover, they are quite frequently confused for each other [2, 3]. Besides the morphological differences, it is important to characterize the volatile essentials produced by the two species, which permit an unambiguous identification and provide information on the quality of the drug too. Many data have been published on the chemical composition of *S. officinalis* essential oil in recent decades [4], but there are very few records on the volatile essentials of wild-growing *S. tomentosa* [5, 6]. In our experimental field (at Vácrátót, Hungary), we have been cultivating about 60 sage species representing a range of different sections of the genus. Essential oil samples were prepared from *S. officinalis* (I) and *S. tomentosa* (II) populations and compared with *S. tomentosa* oil (III) of Bulgarian origin.

Our experiments on the characterization of the three oils were designed to yield information on the chemical differences in the compositions of these botanically close sage species. We set our to determine the chemical similarities and alterations to be found in the volatile compositions of *S. tomentosa* populations grown under different climatic conditions. Additionally, we wished to compare the volatile production of *S. tomentosa* oil samples (II and III) of cultivated populations with that of wild-growing ones reported in the literature.

MATERIALS AND METHODS

All three species were collected in June at the flowering stage, but from different places. The leaves of III were harvested from cultivated plants grown in the experimental field of the Institute of Botany of the Bulgarian Academy of Sciences near Sofia, while those of I and II were collected at the Institute of Ecology and Botany of the Hungarian Academy of Sciences in Vácrátót.

The air-dried leaves of Bulgarian *S. tomentosa* were subjected to steam-distillation for 2 h in a Clevenger apparatus, which provided volatile oils in a yield of 2.5 % (v/w). The samples grown in Hungary were steam-distilled according to the instructions of the VIIth Hungarian Pharmacopoeia.

The oils were analysed by GC and GC/MS techniques. Chromatographic analysis were carried out on a HP 5890 Series II gas chromatograph (FID) using a 30 m, 0.35 μm, 0.25 μm HP-5 fused silica capillary column which was programmed from 60 °C to 210 °C at 3 °C/min and from 210 °C to 250 °C (2-min hold) at 5 °C/min. The detector and injector temperature was 250 °C, while N_2 was used as a carrier gas at a flow rate 27 ml/min with split (60:1) introduction.

GC/MS analysis were performed on a FINNIGAN GCQ ion trap bench-top mass spectrometer. All conditions were as above except the carrier gas was He at a flow rate 31.9 ml/min and a 30 m, 0.25 μm, 0.25 μm DB-5 column was used. A positive ion electron ionization mode was used with a mass range of 40-400 amu.

RESULTS AND DISCUSSION

The oil compositions for each of the samples studied are presented in Tables 1 and 2. A total of 37 (I), 42 (II) and 44 (III) components accounting for 99.1%, 94.3% and 98.9% of the oils were identified by means of GC and GC/MS analysis. The constituents were identified by comparing their Rts and Kovats indices with those of authentic references, and also by comparison with published MS data [7] and computer library search.

Table 1. Monoterpenoid percentage composition of *S. officinalis* and *S. tomentosa* essential oils

KI	Monoterpenoids Compounds	I S. off H	II S. tom H	III S. tom B
931	α-thujene	0.2	0.2	0.3
939	α-pinene	1.1	1.5	13.6
953	camphene	2.8	2.0	6.0
976	sabinene	0.3	0.2	0.2
980	β-pinene	2.3	6.7	14.8
991	myrcene	0.9	1.4	0.6
1005	α-phellandrene	1.0		
1018	α-terpinene	0.2	0.2	0.1
1026	p-cymene	0.2	0.2	0.2
1031	limonene	1.3	1.9	1.5
1033	1,8-cineole	3.4	10.9	1.9
1040	*cis*-ocimene	0.5		1.2
1050	*trans*-ocimene	0.1		0.3
1062	γ-terpinene	0.5	0.6	4.0
1068	cis-sabinene hydrate			0.3
1088	terpinolene	0.4	0.4	0.3
1098	linalool		0.3	1.7
1102	α-thujone	35.9	7.2	tr
1114	β-thujone	9.7	3.2	tr
1140	trans-sabinol	0.3	0.2	5.0
1143	camphor	9.7	9.7	7.8
1165	borneol	8.7	8.9	9.7
1177	terpin-4-ol	0.5	0.6	0.4
1189	α-terpineol	0.1	0.4	0.4
1194	myrtenol			
1240	neral		0.1	
1255	geraniol		0.4	
1257	linalyl acetate			0.9
1270	geranial		0.1	
1285	bornyl acetate	5.0	3.2	2.5
1301	thujyl acetate	0.3		

The monoterpene and sesquiterpene hydrocarbon contents were the highest (43.1% and 22%) in the *S. tomentosa* (III) oil, whereas the corresponding values in the *S. tomentosa* (II) oil were 15.3% and 14.4%. The *S. officinalis* (I) oil has a typically high content of oxygenated compounds (81.9%). On the other hand, the contents of mono- and sesquiterpene hydrocarbons were only 11.8% and 5.4%, respectively. These results agree with data to be found in the literature [5, 6].

Table2. Sesquiterpenoid percentage composition of *S. officinalis* and *S. tomentosa* essential oils

KI	Sesquiterpenoids Compounds	I S. off H	II S. tom H	III S. tom B
1351	α-cubebene		0.1	0.8
1372	α-ylangene		tr	0.5
1376	α-copaene	tr	0.2	1.0
1384	β-bourbonene	tr	tr	0.4
1390	β-cubebene		0.1	
1394	jasmone	1.0		
1418	β-caryophyllene	0.7	6.0	5.6
1432	β-gurjunene		0.1	0.9
1439	aromadendrene			0.5
1454	α-humulene	4.3	6.4	2.9
1461	allo-aromadendrene		0.2	
1477	γ-muurolene	0.1	0.3	2.7
1480	germacrene D	0.1	0.2	
1491	valencene	0.1	0.3	1.1
1499	α-muurolene			0.6
1513	γ-cadinene			1.3
1524	δ-cadinene	0.1	0.5	3.4
1538	α-cadinene			0.3
1565	ledol			
1576	spathulenol		0.1	0.6
1581	caryophyllene oxide	0.1	0.8	0.8
1594	viridiflorol	4.6	17.1	0.7
1606	humulene 1,2-epoxide	0.7	0.5	
1659	pathohoulol			0.3
1967	sclarene	1.9	0.9	0.8

The *S. tomentosa* oil of Hungarian origin (II) contained β-pinene (6.7%), 1,8-cineole (10.9%), α-thujone (7.2%), camphor (9.7%), borneol (8.9%), and viridiflorol (17.1%) as the major constituents, while in the *S. tomentosa* oil of Bulgarian origin (III) α-pinene (13.6%), β-pinene (14.8%), camphene (6%), camphor (7.8%), and borneol (9.7%) were the main components. The main component of II was viridiflorol which was totally absent from III, and on which no data have been published before as concerns these species.

Significant differences were found in the monoterpenoid ratio of II and III: contents of 1.5% and 13.6% α-pinene, 2% and 6% camphene and 10.9% and 1.9% 1,8-cineole were recorded, respectively. *Cis*-ocimene, *trans*-ocimene, *cis*-sabinene hydrate and linalyl acetate were detected only in III, while α- and β-thujone, neral, geraniol and geranial were found in II. Our findings confirm the great variety reported earlier the α- and β-thujone contents of *S. tomentosa* oils of different origins [5, 6].

Among the monoterpenoids, α-terpinene, *cis*-sabinene hydrate, *trans*-sabinene, neral and geranial were identified for the first time in *S. tomentosa* oil. The presence of α-cubebene, α-ylangene, β-bourbonene, β-cubebene, aromadendrene, allo-aromadendrene, germacrene D,

valencene, α–muurolene, α–cadinene, caryophyllene oxide, viridiflorol, humulene 1,2-epoxide, patchoulol and sclarene in the sesquiterpene fraction had not been reported earlier, either. However, myrtenol, ledol and α–phellandrene, previously reported to be present in Bulgarian *S. tomentosa* oil, were not detected by us.

The amounts of limonene, γ-terpinene, terpinolene, camphor, borneol and bornyl acetate were very similar in I, II and III. Of the 24 monoterpene components of I, 20 and 22 were also found in II and III, respectively, while of the 13 sesquiterpene constituents of I, 12 could be detected in II and/or III too. Our findings confirm a close relationship between the two species on the basis of the chemical similarities of their oils. However, the typically high α– and β–thujone contents clearly permit the identification of *S. officinalis* oil. The significant variations in II and III can be explained by the different climatic and growing conditions.

Figure 1. Total ion chromatogram of oil (I)

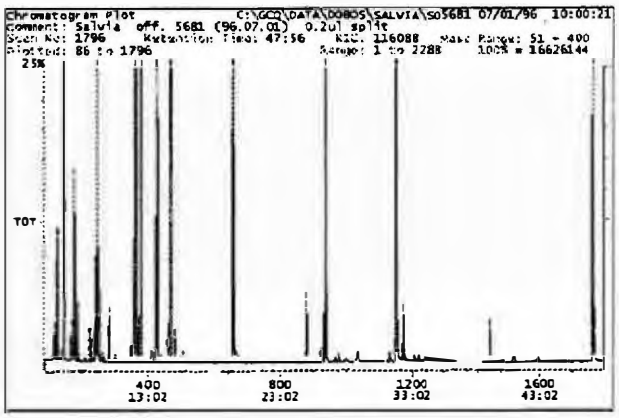


Figure 2. Total ion chromatogram of oil (II)

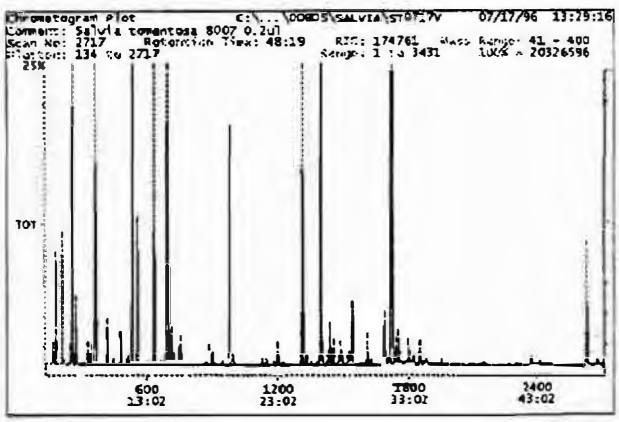
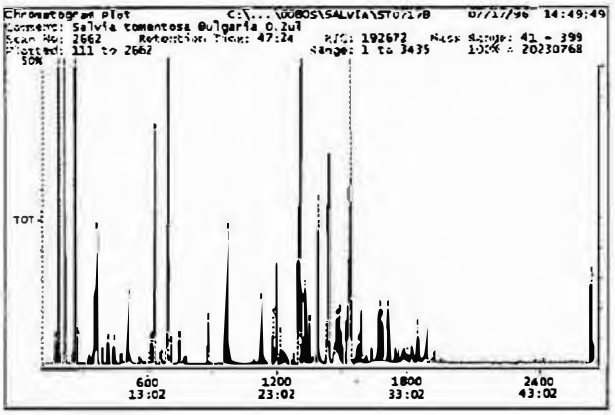


Figure 3. Total ion chromatogram of oil (III)



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COMPARATIVE STUDIES OF THE ESSENTIAL OILS OF SOME SPECIES OF *SECT. SALVIA*

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INTRODUCTION

Salvia, the largest genus of the *Family Lamiacea* belongs to the *Subfamily Nepetoideae* according to the latest classifications of the family (2, 3). Some of the most characteristic components of this subfamily, including *Salvia* species, are as follows: a./ the presence of high essential oil producing species (2, 13), b./ the occurrence of rosmarinic, caffeic acids ((2, 6, 12, 13, 19) and c./ the presence of the triterpene ursolic/oleanolic acids (6, 13). In this genus, *Section Salvia* contains those *Salvia* species richest in essential oils (13, 15). Their European representatives occur in the Mediterranean region. These species are *S. officinalis L (S. off.), S. lavandulifolia Vahl (S. lav.), S. tomentosa Mill. (S. tom.), S. fruticosa Mill.(S. frut.), S. candelabrum Boiss.(S. cand.), S. ringens Sibth. et Sm. (S. ring.)* (5). Some of them, like *S. officinalis, S. fruticosa* are of commercial importance. This fact enhances their more detailed morphological and chemical comparative studies (8, 9, 11). It also should be emphasised that the ranking, the classification of taxa (species) belonging to the Section can not be regarded definitely solved. That is also why the comparative study of their chemistry and morphology is going on (1, 4, 8, 9, 10, 13, 16, 17).

Significant questions about the stability of the chemistry of these species remained to be answered. Only sporadic information is available on the origin and time-dependent variation of the essential oil content and composition and also the non-volatile rosmarinic, caffeic, ursolic/oleanolic acid content (4, 8, 11, 13, 14, etc.). With other words, the question is how the essential oil production and composition of these plants growing under environmental conditions strange for them can change. As the species of *Sect. Salvia* are Mediterranean ones, studying their behaviour, possible cultivation under Hungarian conditions, mainly in temperate climatic belt, out of their native growing areas justifies this work.

MATERIAL AND METHODS

The plants investigated were grown in the Experimental field of the Research Institute of Ecology and Botany of the Hungarian Academy of Sciences, Vácrátót (30 km North of Budapest, Hungary). The seeds of the species were obtained via the regular seed exchange activities of botanical gardens. From among the collection of *Salvia* species growing under the same environmental conditions the leaves of the members of *Sect. Salvia* were harvested in September (and in August) for comparative essential oil studies and each month in 1995 for studying the variation of the non-volatile rosmarinic, caffeic, ursolic/oleanolic acids.

The fresh and dried weights were measured. Water steam distillation provided the oils from fresh samples which were analysed by GC (HP), GC-MS apparatus (Finnigan GC-MS). Details are given in this volume elsewhere (4).

The non-volatile components were measured by using densitometric methods. Rosmarinic, caffeic, ursolic/oleanolic acids were gained from the methanol extract of the dried (40 °C) leaves, collected monthly from the same plants. The extracts were obtained by ultrasonic extraction with diluted methanol.

After TLC separation the quantity of the compounds mentioned above was measured by densitometry (Shimadzu CS-9301 PC) in different ways. Because of the difficult separation of the closely related triterpene acids (ursolic and oleanolic acids) they were measured together. The details of the methods are published elsewhere (7).

RESULTS AND DISCUSSION

The six European *Salvia* species of the *Sect. Salvia* (5) growing in experimental field are rather similar in morphology to each others (5,9). Beside the differences in origin: *S. officinalis* occurs in Spain, South France, West Balkan; *S. lavandulifolia* in Spain, South France; *S. fruticosa* (synonym name is *S. triloba*) in South Balkan, Romania, Serbia, Bulgaria; *S. tomentosa* (synonym name is *S. grandiflora*) in Central and East Mediterranean, Sicily, Greece; *S. candelabrum* in South Spain; *S. ringens* in Balkan. It appears that, beside the differences in origin, height of the plants (maxima are 30-120 cm), form and size of the leaves together with hairiness, data on flowers etc. show only slight differences, sometimes overlapping figures. (5). The morphological differences may even be less obvious, if these species are cultivated out of their native growing areas, like in our case.

The essential oil content (quantity) only of five species out of the six could be found in measurable amount. The oil content of *S. ringens* was rather low so that it should be measured in heptane solution. For the leaves *S. officinalis* 2,5 ml/kg, *S. tomentosa* 3,9 ml/kg, *S. candelabrum* 2,5 ml/kg, *S. lavandulifolia* 8,9 ml/kg *S. fruticosa* 4,6 ml/kg essential oil could be calculated on the basis of the steam distilled samples gathered in July, 1996. These figures tend to be similar or somewhat to be lower than those in literature available on *S. officinalis, S. lavandulifolia* and *S. fruticosa* (6, 15, 16). The compositions of oils are listed in Table 1.

As far as the monoterpenoids concerned the same compounds were identified in almost all oils (Table 1.). In the ratio of the components sometimes big differences could be observed. E.g. α-thujone content was 36% in *S. officinalis*, 21.4% in *S. fruticosa*, it was below 10% in *S. ringens, S. tomentosa, S. candelabrum*. No thujone could be measured in *S. lavandulifolia*. *S. lavandulifolia* has, however, high (47.0%) 1,8-cineol content, similarly to *S. candelabrum* (50.5%). This compound was much less in the oils of the other species. Much less, but similar type of differences were found, when the other oil components were regarded. It should be emphasised that the oil content and composition, too, vary to a large extent due to the sort (e.g. origin) and time of harvest of the plants as it could be proved in the case of *S. officinalis* (13, 14, 16, 17).

Table 1. Composition (in %) of Essential Oils of some Species of *Sect. Salvia* of *Family Lamiaceae*

Terpenoids	*S. tom.*	*S. off.*	*S. frut.*	*S. lav.*	*S. can.*	*S.ring.*
Monoterpenoids:						
α-thujene	0.2	0.2	0.2	0.4	0.2	
α-pinene	1.6	1.1	1.2	2.7	6.5	2.5
β-pinene	7.2	2.3	1.7	6.8	7.4	2.9
myrcene	1.4	0.9	4.3	5.8	1.4	2.2
α-terpinene	0.2	0.2	0.1	0.4		
p-cymene	0.2	0.2	0.2	0.4	1.1	0.4
limonene	1.9	1.3	0.2			1.7
1,8-cineol	11.6	3.5	16.9	47.0	50.5	16.6
γ-terpinene	0.6	0.5	0.5	1.1		0.5
terpinolene	0.4	0.4	0.6	0.5		0.5
α-thujon	7.4	36.0	21.4		1.6	9.7
β-thujone	3.3	9.7	3.7		0.5	1.8
camphor	10.1	9.6	26.0	12.6	9.0	16.0
borneol	9.3	8.4	1.0	2.2	4.4	2.0
terpinen-4-ol	0.6	0.5	0.4	0.7	1.1	
α-terpineol	0.4	0.1	0.6	0.3	1.9	0.5
bornyl acetate	3.3	4.9	1.0	0.6	0.9	1.4
Sesquiterpenoids:						
α-gurjunene					0.1	
β-caryophyllene	5.9	0.7	1.4	4.9	0.2	2.3
α-humulene	6.2	4.2	2.8	1.0	1.1	4.8
γ-muurolene	0.3					0.3
δ-cadinene	0.5					0.7
selin (3,7)11-diene	0.6		0.1		0.2	
caryophyllene oxide	0.8	0.2	0.1	0.3		
viridiflorol	17.1	4.6	5.6	4.5	0.8	13.5
humulene 1-2 epoxid	0.5	0.8	0.2		0.4	
sclarene	0.9	1.9	0.8			2.6

More differences could be registered, if the sesquiterpenes were regarded. *S. tomentosa* was the riches in compounds and produced the highest amount of a particular compound (viridiflorol). Viridiflorol could be

detected together with β-caryophyllene and α-humulene in all the six species.

The monthly variation of the non-volatile phenolic compounds; rosmarinic, caffeic and the triterpne ursolic/oleanolic acids was investigated during the vegetation period of 1995. It has become obvious that both the ursolic/oleanolic acid and rosmarinic acid fractions vary, unlikely to the caffeic acid, significantly during the growing season. *S. lavandulifolia, S. officinalis* and *S. fruticosa* proved to be the best sources of these compounds (Table 2.). Maxima of the various compounds of particular species occurred in different months.

Table 2. Monthly Variation of some Non-volatile Components (dry wt. %) in *Salvia* Species of *Sect. Salvia*

	Apr.	May	June	July	Aug.	Sep.
Rosmarinic acid						
S. off.	0.52	0.16	0.15	0.20	0.04	0.01
S.cand		0.43		0.17		0.01
S. tom.	0.02	0.26	0.25	0.17	0.23	0.0
S. lav.	0.06	0.40	0.75	0.13		0.16
S. frut.	0.27	0.31	0.06	0.27		0.02
Caffeic acid						
S. off.	0.10	0.06	0.02	0.01	0.01	0.01
S.cand		0.08		0.02		0.01
S. tom.	0.03	0.02	0.02	0.01	0.01	0.01
S. lav.	0.05	0.01	0.03	0.02		0.02
S. frut.	0.07	0.08	0.02	0.02		0.01
Ursolic/Oleanolic acid						
S. off.	0.61	0.42	0.32	0.86	0.72	0.51
S.cand		0.24		0.77		0.08
S. tom.	0.12	0.22	0.34	0.82	0.43	0.68
S. lav.	0.51	0.38	0.40	0.65	0.24	0.17
S. frut.	0.22	0.22	0.67	0.39	0.10	0.89

Sporadic qualitative (1, 4, 12, 13, 17) and quantitative (4, 10, 13) data are available about the rosmarinic, caffeic, ursolic/oleanolic acid content and their variation in these species. Seemingly our figures on rosmarinic acid on *S. officinalis* are lower than e.g. those of Lamaison et al (8). He studied plants from the USA and obtained 3.2, 3.0% rosmarinic acid. The figures on ursolic/oleanolic acids were also higher in tendency in the literature (4). These findings suggest that the production of these compounds out of the area border should be lower than within it. Further studies should, however, be done to clarify the background of the variation of these ingredients due, among others, to climatic conditions.

CONCLUSIONS

On the basis of the many-sided chemical investigations of the *Salvia* genus, the species of *Sect. Salvia* are rather similar to each other both the essential oil content, composition, and the non-volatile triterpene and the phenolic acid (rosmarinic and caffeic acids) contents were regarded. Significant differences were, however, observed in the quantity of oils and in them the proportion of thujone and 1,8-cineol and other compounds too. The time of harvest, the strange non-Mediterranean, Hungarian climate (out of the area borders) where the plants were grown may also influence the oil content and its composition as in the case of *S. officinalis* could be experienced (13, 14, 16, 17, 18). In order to answer such questions as, how well the species (beside the well- growing *S. officinalis)* can be cultivated, how well they can be applied as oil sources under temperate climatic conditions and what kind of factors influence the chemical constituents of the species, needs further studies.

ACKNOWLEDGEMENT

The authors express their thanks for the financial support of this work to the 'OTKA' Hungarian National Grant.

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ANALYSIS OF THE ESSENTIAL OIL OF *SALVIA CARDIOPHYLLA*

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ABSTRACT: The composition of the essential oil of the leaves of *Salvia cardiophylla* (Lamiaceae), an endemic species from Paraguay, was investigated by GC/FID and GC/MS. The major constituents were found to be the sesquiterpenes ß-caryophyllene (23.1%), germacrene-D (14.3%), and ß-caryophyllene oxide (10.4%).

KEY WORD INDEX: *Salvia cardiophylla*, Lamiaceae, essential oil composition, ß-caryophyllene, germacrene-D, ß-caryophyllene oxide .

PLANT NAME: *Salvia cardiophylla* Benth., Lamiaceae.

SOURCE: *S. cardiophylla* was collected at the flowering stage in San Lorenzo (Departamento Central, Paraguay), in April 1995. A voucher specimen was included in the Herbarium of the Faculty of Chemical Sciences of the Universidad Nacional de Asunción (Paraguay), with the number Ferro 001.

PLANT PART: Air-dried leaves, subjected to hydrodistillation in a Clevenger-type apparatus according to the European Pharmacopoeia method (1), gave a low essential oil yield of 0.03% (vol/wt).

PREVIOUS WORK: To our knowledge, no reports on the composition of the volatile oil of this species have previously been reported in the literature.

PRESENT WORK: The identification of the constituents was made by means of their retention indices in the two columns, determined in relation to a homologous series of fatty acids methyl esters, and their mass spectra, which were compared with those given in the literature (2,3) and those obtained from authentic samples. GC analysis were carried out using a Hewlett-Packard gas chromatograph model 5890 A equipped with a flame ionization detector and fused silica capillary columns of two different stationary phases: Carbowax 20M (CW-20M) and methyl silicone (SE-30), (25 m x 0.2 mm, 0.25 µm film). Analytical conditions were as follows: injector temperature 250°C, detector temperature 270°C, split ratio 1:60. Oven temperature was programmed from 80 °C to 250 °C (4 °C/min), using helium as carrier gas at a working flow rate of 1 mL/min. Quantitative data were obtained from FID area values on the two columns.

GC/MS analysis were performed with a HP 5890 gas chromatograph coupled to a HP 5971 A mass selective spectrometer. Operating conditions were: columns, Supelcowax^{TM} 10 (30 m x 0.25 mm, 0.25 µm film) and methyl silicone SE-30 (25 m x 0.2 mm, 0.25 µm film); oven temperature programmed from 80 °C to 220 °C at a rate of 6 °C/min; carrier gas helium, flow rate 1 mL/min. Mass spectra were taken every 5 s over m/z 35-400, using an ionizing voltage of 70 eV.

The results of the analysis are shown in Table I. Twenty-seven different constituents, meaning a percentage of the total oil of 87.8%, were identified. It was mainly constituted by sesquiterpenes, either hydrocarbons (56%) or oxygenated (21.3%), while monoterpene rates were quite lower (1.8%, in total). The major constituents were found to be: ß-caryophyllene (23.1%), germacrene-D (14.3%), ß-caryophyllene oxide (10.4%), byciclogermacrene (6.6%), and spathulenol (7.1%).

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Table I. Composition of the essential oil the lleaves of *Salvia cardiophylla*.

Components	%	Identification Methods
Monoterpene hydrocarbons	**1.0**	
α-Thuyene	0.3	GC-MS, RI_1
Myrcene	0.1	GC-MS, RI_2
β-Phellandrene	0.3	GC-MS, RI_1, RI_2
p-Cymene	0.3	GC-MS, RI_1
Oxygenated monoterpenes	**0.8**	
Linalool	0.8	GC-MS, RI_1, RI_2
Sesquiterpene hydrocarbons	**56.0**	
γ-Elemene	0.6	GC-MS, RI_1
α-Copaene	2.0	GC-MS, RI_1, RI_2
β-Bourbonene	0.9	GC-MS, RI_1, RI_2
Longifolene	0.3	GC-MS, RI_1, RI_2
β-Elemene	2.8	GC-MS, RI_1, RI_2
β-Caryophyllene	23.1	GC-MS, RI_1, RI_2
α-Humulene	2.1	GC-MS, RI_1, RI_2
α-Guaiene	1.0	GC-MS, RI_2
D-Germacrene	14.3	GC-MS, RI_1, RI_2
Bicyclogermacrene	6.6	GC-MS, RI_1, RI_2
δ-Cadinene	2.0	GC-MS, RI_1, RI_2
γ-Gurjunene	0.3	GC-MS, RI_2
Oxygenated sesquiterpenes	**21.3**	
Isocaryophyllene oxyde	0.9	GC-MS, RI_2
Caryophyllene oxyde	10.4	GC-MS, RI_1, RI_2
Spathulenol	7.1	GC-MS, RI_1, RI_2
T-Cadinol	0.9	GC-MS, RI_1, RI_2
α-Cadinol	2.0	GC-MS, RI_1, RI_2
Others	**8.7**	
cis-2-Hexanal	0.6	GC-MS, RI_1
1-Octen-3-one	0.2	GC-MS, RI_1, RI_2
1-Octen-3-ol	0.2	GC-MS, RI_1, RI_2
β-Ionone	1.1	GC-MS, RI_1, RI_2
Hexadecanoic acid	6.6	GC-MS, RI_2

RI_1: Retention Index in Supelcowax 10.
RI_2: Retention Index in SE-30.

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THE ESSENTIAL OILS OF *SATUREJA* L. OCCURRING IN TURKEY

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Turkey has a rich aromatic flora of the *Labiatae* family. The family is represented in Turkey by 45 genera, 556 species and a total of 741 taxa (1). The genus *Satureja* is one of the some important genera of the Turkish *Labiatae*. *Satureja* species are widely used as condiment (in fresh and dried from) in folk medicine and as a source of essential oil. Some (*e.g. S. hortensis*) are cultivated but most are collected from the wild (2). *S. montana* (the species is native for Europe) has been introduced to Turkey for cultivation.

Ancestors of many species in the tribe *Saturejae* (Subfamily *Nepetoideae*) spread in the pliocene to Turkey and adjacent areas from the Irano-Turanian region. Partially through understood (and commonly accepted for all other groups as well) and partly through unknown mechanisms many new species originated (3). Most species have been for sometime in balance with all climatological and ecological factors. So, Turkey is regarded as an important gene-center for the *Labiatae* family and also for the *Satureja* species. According to some botanists, the genus *Satureja is* quite complex and several species, are considered as different genera by the other botanists (4).

No taxonomic study on *Satureja* species growing in Turkey has been performed and no chemical data on endemic species has been found in the literature. Since 1989, we have been interested in the taxonomy of the genus *Satureja* and the analysis of the essential oils from *Satureja* species growing in Turkey (5 - 9).

During our field studies, we have recently come across with two species (*S. icarica*, *S. pilosa*) which have proved to be new for Turkey (We wish to express our thanks to Prof. Dr. Snogerup for his kind advice in identifying these species). Our studies on three *Satureja* species, which may prove to be new for science, are ongoing.

This study concerns the essential oil composition of 61 samples from eleven natural and one cultivated *Satureja* species out of 15 species known to exist in Turkey (Table 1).

Table 1- *Satureja* species which grow in Turkey

Species	West - Turkey	Central Turkey	East - Turkey	South - Turkey	North - Turkey
S. hortensis	+	+	+	+	+
S. cuneifolia	+	+		+	
S. cilicica				+	
S. amani				+	
S. icarica	+				
S. wiedemanniana	+	+			
S. parnassica ssp. sipylea	+				
S. spinosa	+				
S. coerulea	+				
S. spicigera					+
S. boissieri			+	+	
S. macrantha			+		
S. aintabensis				+	
S. thymbra	+			+	
S. pilosa	+				
S. montana (cultivated)					+

EXPERIMENTAL

Plant material - All *Satureja* samples were collected from different localities during flowering. Voucher specimens are kept at the Herbarium of the Faculty of Pharmacy (ESSE) Anadolu University, Eskisehir, Turkey.

Distillation - Plant materials were hydrodistilled for 3 h using a Clevenger-type apparatus.

Analysis - The Essential Oils were analysed by GC/MS using a Hewlett-Packard GCD system. Innowax FSC column (60m x 0. 25 mm) was used with Helium as carrier gas. GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, and then kept constant at 220 °C for 10 min. Split flow was adjusted at 50 mL/min. The injector temperatures

was at 250 °C. MS were taken at 70 eV. Mass range was from m/z 10 to 425. Library search was carried out using Wiley GC/MS Library and TBAM Library of Essential Oil Constituents.

RESULTS AND DISCUSSION

We have been extensively studying the essential oils of the plants belonging to the *Labiatae* family in Turkey. Some of the most studied genera include *Satureja*, *Thymus*, *Origanum*, *Thymbra*, *Corydothymus*.

A common feature of all these genera is that the main components in the oils are either carvacrol or thymol or both (6). Meanwhile, the essential oils of some species are devoid of phenols.

Here, we report on the results of 61 analyses carried out with eleven species collected from wild sources in different localities and one cultivated species.

There are numerous studies on the essential oil of *Satureja* species growing in the various parts of the world (10 - 53). These studies have shown that the genus *Satureja* may or may not contain phenols. Phenol containing species are divided into "carvacrol type" and "thymol type", species, phenols-free species are divided into "Monoterpenic-alcohol type", "Monoterpenic-ketone type", "Monoterpenic-hydrocarbone type" and also "Sesquiterpenic hydrocarbone-type".

In Turkey, nine species are found to belong to the group of "carvacrol-type" species (Table 2). In this group the major component carvacrol is followed by p-cymene and α-terpinene. Carvacrol was found to be the main constituent in essential oils of 75 % of the species studies studied. In all cases, the phenolic compounds carvacrol and thymol are accompanied by significant amounts of p-cymene and α-terpinene. These latter compounds are considered as biogenetic precursors of the two isomeric phenols (8).

Satureja cuneifolia, which is included in the "Carvacrol -type" widely grows in west, south and inner west Anatolia. It is used to obtain "Kekik" oil by the local people in the growing regions. In Turkey, "Kekik" is a collective term for the plants containing carvacrol and/or thymol, since they smell like thyme such as *Origanum*, *Thymus*, *Satureja*, *Tymbra* and *Coridothymus*.

Table 2. „Carvacrol type" *Satureja* species in Turkey

Species	Oil Content (%)	Main components (%)	Number of Studied samples
S. cuneifolia	0.6-3.6	Carvacrol -72 p-cymene 7-21	11
S. hortensis	1.3-4.8	Carvacrol -64 γ-terpinene -42	13
S. thymbra	1-4.3	Carvacrol 47-49 γ-terpinene 18-27	6
S. spicigera	0.5	Carvacrol 26 γ-terpinene 19	1
S. cilicica	0.8	Carvacrol 38 p-cymene 14	2
S. parnassica ssp. sipylea	1.5	Carvacrol 43 p-cymene 20	1
S. icarica	2.2	Carvacrol 42 p-cymene 19	1
S. pilosa	2.7	Carvacrol 38-53 γ-terpinene 4-14	1
S. montana (cultivated)	0.03 - 2.8	Carvacrol 50-63	3

Satureja hortensis (Summer Savory) shows a very interesting situation in Turkey that *S. hortensis* of European origin is cultivated in west Anatolia, and is used as spice in both fresh and dried form. However, it grows wild in north, east and south-east Anatolia is harvested especially from the south-eastern region in tons and exported under the name "Kekik". Our results have indicated that the oils from cultivated forms of *S. hortensis* are rich in carvacrol while those from wild collections contain thymol as major constituent. It has also been observed that the plants growing in eastern Turkey were rich in thymol while those growing in the western part contained carvacrol as the main component in their oils.

S. icarica and *S. pilosa* also belong to the "Carvacrol-type" group and their essential oil yields and carvacrol contents are quite high. Both species are used as herbal tea in the regions where they grow.

S. montana of European origin is cultivated in Adana province in the Eastern Mediterranean region of Turkey. The carvacrol content in the oils of this species reaches 63 %.

S. parnassica ssp. sipylea, an endemic species of Turkey, is found only in west Anatolia. It is quite rare in the areas where it grows. It is used as herbal tea by the local population.

S. cilicia is an endemic species in South Anatolia. It is used as tea only by the local people. Although *S. thymbra* has a wide distribution stretching west to east in Southern Anatolia and contains high amounts of oil (up to 4.3 %) and carvacrol (up to 49 %), according to the literature there is no trade of this species in Turkey and the plant is hardly used by the local people because its oil is generally found not appealing. So far, Turkey seems to be poor in "thymol type" *Satureja* species. In the oils of *S. spicigera*, *S. cuneifolia* and *S. hortensis* chemotypes, the thymol content reaches 58 % (Table 3).

Table 3- "Thymol type" *Satureja* species in Turkey

Species	Oil Content (%)	Main components (%)	Number of Samples
S. spicigera	0.23-1.3	thymol 20 - 32 p-cymene 8	3
S. cuneifolia	0.6-2.8	thymol 22 - 58 p-cymene 7 - 13	8
S.hortensis	1.3-4.8	thymol 17 - 43 γ-terpinene 25-41	7

The same situation exists in the species of "Monoterpene hydrocarbon-type" group. The chemotypes of *S. spicigera* and *S. hortensis* is found in this group. The main compounds in this group are p-cymene and α-terpinene (Table 4).

Table 4. "Monoterpenic hydrocarbone type" *Satureja* species in Turkey

Species	Oil Content (%)	Main components (%)	Number of Samples
S. spicigera	1.2	p-cymene 34 γ-terpinene 9 thymol 25	1
S. hortensis	0.9-1.3	γ-terpinene 42 carvacrol 16 thymol 17	5

The oils of *S. coerulea* and *S. wiedemanniana* fall into the "sesquiterpene hydrocarbon-type" group (Table 5).

S. coerulea is listed among the endangered species in Red Data Book for Turkey (54). It is known to grow only in Bulgaria and Turkey. We have collected it from a new locality. This plant is used for the treatment of cold in the regions where it grows.

Table 5. „Sesquiterpenes hydrocarbon type" *Satureja* species in Turkey

Species	Oil Content (%)	Main components (%)	Number of Samples
S. coerulea	0.0.9	germacrene D 21 β-elemene 4.3 α-muurolene+ β-bisabolene 4	1
S. wiedemanniana	0.09	Spathulenol 13 Borneol 11 Bicyclogermacrene 6	1

S. wiedemanniana, an endemic species of Turkey, which is found in the same group, is used as spice in the regions where it grows. Also, so far only one species, *S. spinosa* is included in "monoterpenic-alcohol type" group. It contains less amount of oil (0.47 %) and high amounts of linalool (62 %). It was found to grow only in one locality in south-west Anatolia (Table 6).

Table 6. "Monoterpenic alcohol type" *Satureja* species in Turkey

Species	Oil Content (%)	Main components (%)	Number of Samples
S. spinosa	0.47	Linalool 62 bicyclogermacrene 8	1

On the other hand, none of the *Satureja* species growing in Turkey contain ketone as the main constituent. Therefore, there is no representative of the "Monoterpenic ketone-type" in Turkey. Finally, two oil- and carvacrol-rich species such as *S. hortensis* *S. cuneifolia* comprise part of the "Kekik" exported from Turkey. Our research into essential oils of *Satureja* species of Turkey has not only resulted in a better understanding of the uses and chemical compositions of wild growing *Satureja* species of Turkey, but also contributed to the discovery of species new for Turkey and possibly some species new for science.

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COMPOSITION OF THE ESSENTIAL OIL OF *TEUCRIUM HAENSELERI* BOISS. FROM PORTUGAL

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INTRODUCTION

Fifteen *Teucrium* species are recorded in the Portuguese flora, four of which are endemic (1 - 3). *T. haenseleri* Boiss. (*T. luisieri* Samp.) is endemic to the Iberian peninsula. This species is a shrub, with red or green, puberulent - glandulous stems, that normally grows in limy-calcareous soils (1, 2).

Recently, we have studied an acetone extract of the aerial parts of *T. haenseleri* Boiss. and found the previously known neo-clerodanes 19-acetyl-gnaphalin, eriocephalin, isoeriocephalin and 20-deacetyl-eriocephalin (4). In this paper we report on the composition of the essential oil of this *Teucrium* species isolated from the flowering and vegetative plant material.

EXPERIMENTAL

Plant Material

Aerial parts of *Teucrium haenseleri* Boiss. were collected from plants growing in the Serra da Arrábida (Lat. 38° 29′ N; Long. 8° 58′ W), Portugal. A voucher specimen was deposited in the Herbarium of the Instituto Botânico da Faculdade de Ciências de Lisboa (LISU: 164064).

Three samples of plant material were used: flowers (F) and leaves (LF) collected during full flowering (June 1995) and leaves (LV) collected during the vegetative phase (November 1995).

Isolation Procedure

The oils were isolated from fresh plant material by distillation-extraction, for 3 hours, using a Likens-Nickerson-type apparatus with *n*-pentane as organic solvent, and by hydrodistillation, for 3 hours, using a Clevenger-type apparatus.

The oil samples isolated by hydrodistillation were used to estimate the oil yields and those isolated by distillation-extraction to determine the percentage composition of the oils.

Gas Chromatograhy

GC analyses were performed using a Perkin Elmer 8700 gas chromatograph equipped with a FID, a data handling system and a DB-1 fused-silica column (30m x 0.25 mm i.d., film thickness 0.25μm). Oven temperature was programmed, 45-175°C, at 3°C/min, subsequently at 15°C/min up to 280°C, and then held isothermal for 10 min; injector and detector temperatures, 220°C and 240°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using the split sampling technique, ratio 1:50. The percentage composition of the oils was computed from the GC peak areas without using correction factors.

Gas Chromatography - Mass Spectrometry

The GC-MS unit consisted of a Carlo Erba 6000 Vega gas - chromatograph, equipped with a DB-1 used-silica column (30m x 0.25 mm, film thickness 0.25 μm), and interfaced with a Finnigan MAT 800 Ion Trap Detector (ITD; software version 4.1). Oven temperature was programmed as above; transfer line temperature 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; splitting ratio, 1:40; ionization energy, 70 eV; ionization current, 60μA; scan range, 40-300 u; scan time, 1s.

The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{17} n-alkanes, and mass spectra with corresponding data of components of reference oils.

Table 1. Percentage composition of the essential oils of *Teucrium haenseleri* Boiss. isolated from the flowers (F), and from the leaves collected during the flowering phase (LF) and the vegetative period (LV) of the plant

Component	Retention Index[a]	F	LF	LV
α-Thujene	924	t	t	
α-Pinene	**930**	**20.0**	**17.5**	**22.7**
Camphene	938	0.3	t	0.4
Sabinene	958	2.4	2.5	3.0
β-Pinene	**963**	**30.3**	**24.0**	**30.9**
β-Myrcene	975	3.0	2.2	3.1
α-Terpinene	1002	t	t	t
β-Cymene	1003	0.5	1.0	0.6
β-Phellandrene	1005	t	t	
Limonene	1009	3.3	2.9	3.3
γ-Terpinene	1035	t	t	t
(Z)-Linalol oxide	1045	0.5	1.1	0.6
(E)-Linalol oxide	1059	0.6	1.0	0.6
Terpinolene	1064	0.6	0.9	t
n-Nonanal	1073	t	t	
Linalol	1074	0.6	1.5	1.1
α-Campholenal	1088	3.1	4.7	0.5
(E)-Pinocarveol	1106	3.8	5.4	3.4
(Z)-Verbenol	1110	0.9	1.4	0.8
(E)-Verbenol	1114	2.3	3.4	2.1
Pinocarvone	1121	2.1	1.8	1.5
Terpinen-4-ol	1148	0.5	0.7	0.9
Myrtenal	1153	2.9	3.2	1.6
α-Terpineol	1159	0.7	0.4	
Verbenone	1164	0.9	2.4	0.5
Myrtenol	1168	1.8	2.4	2.7
E-Carveol	1189	0.6	0.6	0.5
Cumin aldehyde	1200	t	t	
Carvone	1206	0.2	t	t
Citronellol	1208	t	t	
Cumin alcohol	1260	t	t	
Bornyl acetate	1265	0.2	t	0.2
Eugenol	1327			t
α-Terpinyl acetate	1334	1.4	1.3	1.1
α-Cubebene	1345	t	t	t
β-Cubebene	1385	t	t	
α-Copaene	1375	t		t
β-Caryophyllene	1414	0.8	1.5	1.2
α-Humulene	1447	t	t	0.7
γ-Muurolene	1469	0.7	0.6	0.6
α-Muurolene	1494	0.2	t	
δ-Cadinene	1505	3.8	4.7	3.0
Bicycloelemene	1544			0.4
Spathulenol	1549	0.2	t	
Caryophyllene epoxide	1561	t	t	0.3
epi-Cubenol	1600	1.3	2.5	1.7
E-Muurolol	1616	0.4	1.3	
δ-Cadinol	1618	0.4	t	
Identified components		91.3	92.9	90.0
Grouped components				
Monoterpene hydrocarbons		60.4	51.0	64.0
Oxygen-containing monoterpenes		23.1	31.3	18.1
Sesquiterpene hydrocarbons		5.5	6.8	5.9
Oxygen-containing sesquiterpenes		2.3	3.8	2.0
Others		t	t	t

[a] Relative to C_9-C_{17} *n*-alkanes on the DB-1 column. t = trace (<0.05%).

RESULTS AND DISCUSSION

The essential oil samples showed an orange-yellowish colour and possessed a strong odour. The oil isolated from the leaves collected during the vegetative phase was obtained in a higher yield (0.8%) than those isolated from the flowers (0.5%) and from the leaves (0.3%) collected during the flowering period.

Forty-six components were identified in the oil isolated from the flowers, representing 91% of the total oil. In the oils isolated from the leaves collected during the flowering period and vegetative phase of the plant, 45 and 36 components were identified, amounting to 92% and 90% of the total oil, respectively.

The identified components and their percentages are given in Table 1, where the components are listed in order of their elution on the DB-1 column.

All the oils isolated from *T. haenseleri* consisted mainly of monoterpenes (82-84%), where α-pinene (18-23%) and β-pinene (24-31%) were the major constituents. The sesquiterpene fraction of the oils was not higher than 11%, and was dominated by δ-cadinene (3-5%).

The essential oils of *T. haenseleri* and those from *T. marum*, *T. subspinosum* and *T. flavum*, [5,6] are dominated by the monoterpene fraction, in contrast with other *Teucrium* species, which are dominated by the sesquiterpene fraction (6 - 13).

Acknowledgements

This work was partially supported by Centro de Biotecnologia Vegetal do Instituto de Biotecnologia e Química Fina (IBQF). And one of us (H.G.) thanks Junta Nacional de Investigação Científica e Tecnológica (JNICT) for a grant (PRAXIS XXI/BD/5249/95).

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QUANTITY AND QUALITY OF ESSENTIAL OIL OF *THYMUS SIBTHORPII* BENTH. CV. "KRESNA"

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INTRODUCTION

Thyme is one of the most common medicinal plants also used as spice, and source of essential oil. The polytypic nature of the genus *Thymus* provides opportunity for obtaining essential oils having various composition and aroma types. The essential oil of thyme has been reported for its expectorant, antimicrobial, anti-inflammatory, sedative, revulsive and antioxidant effect which accounts for its wide application in pharmaceutical industry, food processing and perfumery. Nowadays thyme is included in more than 12 National Pharmacopoeias as Herba Thymi vulgaris and Oleum Thymi vulgaris. Their pharmacological effect is due to the phenolic compounds - thymol (20-60%) and its isomer, carvacrol, which are strong antiseptics.

Thymus vulgaris L. does not occur in Bulgaria. That is why in our country species belonging to the polymorphous group *Thymus serpillum* L. are used as drug. The quality of Oleum Serpilli varies significantly depending on the taxonomic affiliation and environmental conditions. Its main components are carvacrol (up to 30%), p-cymol and thymol (1, 2, 3).

From 1977 to 1985 at the Institute of Botany (Bulgarian Academy of Sciences) 3 new cultivars with a stable quality of essential oil were selected: cv. "Pagane" rich in geraniol (4), cv. "Slava" rich in citronellol (5), and cv. "Kresna" rich in carvacrol. Specific quality of essential oil and aroma of these cultivars in perspective would allow to use them as additives and *corrigentia* of taste and odour of medicines, perfumes, toiletries, and food.

The subject of the current study is cv. "Kresna". Wild population of *Thymus sibthorpii* Benth. originating from the floristic region of the Strouma River Valley was used as initial material.

Cv. "Kresna" forms well-developed tufts 12-15 cm high about 55 cm in diameter. The stems are pseudocreeping (without vegetative ones). Flowering stems are 8-15 cm high, erect to ascending, slender, well-branched (about 7 shoots per a node). The leaves are up to 12 mm long and up to 2 mm wide, lanceolate to lanceolate-eliptic, leathery, glabrous. The inflorescence is 2 cm long, spicate. The beginning of the flowering stage varies from 12 till 27 June depending on weather, which ensures two harvests a year. The yield of the above-ground mass is 417 kg/dca (fresh) and 243 kg/dca (air-dried), whereas the inflorescences account for 48% of the yield. The content of essential oil is 1.68% (air-dried material) and its yield is 4.08 kg/dca. The essential oil has a strong, pure, carvacrol-type aroma (carvacrol reaches 62.36%; the most important accompanying constituents are *p*-cymene, γ-terpinene, limonene, and myrcene). Except as a substitute to Herba Thymi vulgaris in pharmaceutics, cv. "Kresna" may be used as a culinary herb, while the essential oil is especially recommended for use in food industry as a flavour and preservative.

Preliminary studies on the essential oil from cv. "Kresna" were conducted only at full flowering stage. In the process of cultivation we have observed a certain disadvantage of cv. "Kresna" - the stage of full flowering does not commence simultaneously in all plants, so on the one hand, plants at different stages of flowering contribute to the total yield, and on the other, inflorescences amount to 48% of the yield. That is why the aim of the present study is to trace the changes in the quality and yield of essential oil at different stages of flowering (budding, initial flowering, full flowering, end of flowering) and to set the most appropriate time for harvesting.

MATERIAL AND METHODS

Plant material: Cultivar "Kresna" was bred at the Experimental station of the Institute of Botany under the climatic conditions of the Sofia Plane - temperate zone, altitude 547 m a.s.l., mean temperature 9.6°C, air humidity 68.2%, precipitations 336.1 mm/sq.m, alluvial - meadow soil with the average content of N, P, K and pH = 6.1. Watering was reduced to ensure conditions close to the natural ones. The above - ground mass from plants at different sub-stages of flowering (budding, initial, full, and end of flowering) was harvested and air dried.

Quantity of essential oil:

The essential oil was extracted by hydrodistilation for 1 h using a Clevenger-type apparatus (6).

Quality of essential oil:

The composition of essential oil was determined by GC at InPaCo, Plovdiv. Carlo Erba Vega 6000 apparatus was used (OV 1-30 m; temperature programme: 80°C - 1', 80-130°C - 4°/1', 130-200°C - 10°/1', 200°C - 7'; 0.2 μl; AT=8; PW=3). The essential oil compounds were identified using their authentic samples.

RESULTS AND CONCLUSIONS

The values of essential oil content from cv. "Kresna" are presented in Table 1.

Table 1. Content of essential oil from air dried herba of cv. "Kresna" (in %)

Phenological stage	1993	1994	Average
Budding	1.67	1.67	1.67
Initial flowering	2.00	2.00	2.00
Full flowering	2.17	2.00	2.08
End of flowering	1.77	2.00	1.88
Average	1.90	1.91	1.90

In 1993 during the flowering period the essential oil content varied from 1.67 to 2.17%, in 1994, from 1.67 to 2.00%. This is an evidence of the constant intensity of essential oil synthesis. The highest content (average for the survey period) was registered at the full flowering stage (2.08%), while the lowest, at the budding stage (1.67%). Variations in the essential oil content established at different sub-stages of flowering are insignificant.

Seventy-one compounds were detected, 15 of them were identified (Table 2). The main component, carvacrol, increases during the flowering period from 63.87% (budding) to 74.81% (end of flowering). The other phenol, thymol decreases from 1.482% (budding) to 0.229% (end of flowering). The total content of phenols increases - 65.348% (budding), 65.766% (initial), 71.562% (full), 75.040% (end of flowering). The content of other components with significantly high percentage values varies as follows: p-cymene shows the slightest variations - 9.750-9.493%; γ-terpinene demonstrates the maximum values at the stage of budding (4.051%) and sharply decreases at the end of flowering (0.579%), as well as geranyl acetate (from 2.575 to 0.315%); the content of limonene is the highest at the stage of budding (1.430%) and the lowest at the stage of mass flowering (0.768%).

The most appropriate time for harvesting is when the plants at full flowering stage account for over 50% and those at the budding stage, for less than 15%. At this time the yield of the above-ground mass is the highest which ensures higher yield of essential oil; the content of phenols within this period is the highest, too.

Acknowledgements

The author thanks Mrs. M. Markova for the identification of plant material and NFSI for the financial support.

Table 2. Composition of the essential oil from *Thymus sibthorpii* Benth. cv. "Kresna"

Component	Budding	Init. flow.	Full flow.	End flow.
Hydrocarbons				
1. α-Thujene	0.98	1.01	0.83	0.61
2. α-Pinene	0.47	0.50	0.40	0.35
3. Camphene	0.20	0.25	0.25	0.23
4. β-Pinene	0.32	0.17	0.13	0.12
5. Myrcene	1.17	1.18	1.01	0.50
6. p-Cymene	9.75	9.70	9.49	9.52
7. Limonene	1.43	1.58	0.77	1.00
8. γ-Terpinene	4.05	4.05	3.55	0.58
Alcohols				
9. Linalool	0.75	0.54	0.48	0.49
10. Borneol	1.28	1.26	1.31	1.69
11. α-Terpineol	1.09	1.11	1.14	1.37
Phenols				
12. Thymol	1.48	0.48	0.27	0.23
13. Carvacrol	63.87	65.28	71.30	74.81
Acetates				
14. Geranyl acetate	2.58	1.10	0.42	0.32
Sesquiterpenes				
15. Caryophyllene	1.60	1.62	1.56	0.37

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INDOLE DERIVATIVES IN BARK ESSENTIAL OIL OF ANONIDIUM MANNII (OLIV.) ENGL. AND DIELS ANNONACEAE FROM GABON

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INTRODUCTION

The genus *Anonidium* of the Annonaceae family includes 42 species. Our study concerns the chemical composition of the essential oil from barks of *A. mannii* (Oliv.) Engl. and Diels that occurs regularly in Central Tropical Africa region where they are traditionally used for the healing of wounds.

EXPERIMENTAL

Plant material.

The barks of *Anonidium mannii* were gathered on several trees in two different areas in Gabon : near Libreville and Bitam. Voucher specimens were deposited in the National Herbarium of Libreville.

Analysis

Stem barks were submitted to hydrodistillation for 10 hours in a Clevenger type apparatus and gave essential oil yields of 0.13% and 0.10% (v/w) respectively, on the basis of air dry material.

GC analyses were performed on two fused-silica columns (25 m x 0.25 mm) coated with either OV101 or Carbowax 20M; the oven temperature was programmed from 50°C to 200°C at 5°C/min; injector and detector temperatures were 210°C and 250°C respectively; the carrier gas was nitrogen at a flow rate of 0.9 mL/min.

GC/MS investigations were carried out on a Hewlett Packard capillary GC-quadrupole MS System (Model 5970) fitted with a 25 m x 0.23 mm fused-silica column coated with DB-1; the oven temperature was programmed from 50°C to 250°C at 4°C/min; injector temperature was 220°C; the carrier gas was helium at a flow rate of 1 mL/min.

Isolation

7-isopentenylindole (1) and 3-isopentenylindole (2) were isolated by liquid/solid chromatography of *A. mannii* essential oils on silica gel 60 (Merck; 70-230 mesh ASTM) eluted with a pentane-Et_2O gradient.

7-isopentenylindole (1) MS m/z (rel. intensity) : 170(100), 185(98)$[M]^+$, 130(88), 117(42) 155 (40), 154(38).

^1H NMR (200MHz, $CDCl_3$) : δ 1.78(3H, *s*, CH_3), 1.81(3H, *s*, CH_3), 3.54(2H, *d*, J=8Hz, H1'), 5.40 (1H, *t*, J=8Hz, H2'), 6.55(1H, *d*, J=3Hz, H3), 7.00(1H, *d*, J=6Hz, H6), 7.05(1H, *t*, J=6Hz, H5), 7.14(1H, *d*, J=3Hz, H2), 7.50(1H, *d*, J=6Hz, H4), 8.10(1H, *s*, NH).

^{13}C NMR ($CDCl_3$) : δ 135.53(C_{7a}), 133.79(C3'), 128.25(C_{3a}), 124.40(C7), 124.30(C2), 122.69(C2') 121.96(C6), 120.45(C5), 119.19(C4), 103.37(C3), 31.26(C1'), 26.21(C4'), 18.44(C5').

3-isopentenylindole (2) MS m/z (rel. intensity) : 170(100), 185(93)$[M]^+$, 117(50), 130(47), 77(26) 155(24), 154(23).

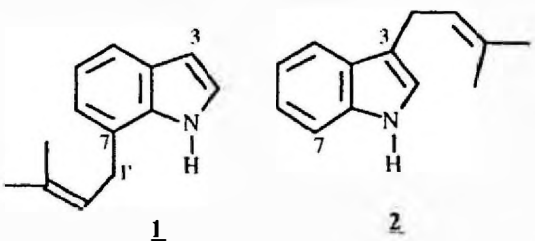
^1H NMR (200MHz, $CDCl_3$) : δ 1.68(3H, *s*, CH_3), 1.70(3H, *s*, CH_3), 3.38(2H, *d*, J=7Hz, H1'), 5.38 (1H, *t*, J=7Hz, H2'), 6.85(1H, *s*, H2), 7.03(1H, *t*, J=6Hz, H6), 7.12(1H, *t*, J=6Hz, H5), 7.25(1H, *d*, J=6Hz, H7), 7.51(1H, *d*, J=6Hz, H4).

^{13}C NMR ($CDCl_3$) : δ 136.01(C_{7a}), 131.41(C3'), 126.98(C_{3a}), 122.59(C2'), 121.44(C2), 120.66 (C5), 118.65(C4), 118.54(C6), 115.70(C3), 110.55 (C7), 25.24(C4'), 23.61(C1'), 17.31(C5').

RESULTS AND DISCUSSION

The GC and GC/MS analyses of these essential oils allowed the identification of 25 components (Table 1). Both essential oils contain unusual components having molecular masses M=185.

These products were isolated by liquid chromatography on silica gel and analyzed by ^1H and ^{13}C NMR. They have been identified as the two following indolic derivatives which are positional isomers:



1 2

The isomer 1 has been isolated and identified by Achenbach (1) in alkaloidic extracts from barks of *A. mannii*. The isomer 2 has not been identified by these authors; but it was known as a constituent of ethyl acetate extracts of barks of *Monodora tenuifolia* (2)

Both samples differ by the quantitative distribution of their constituents. The essential oil obtained from the plant material collected in the area of Bitam contains a majority of 7-isopentenylindole, accompanied by significant amounts of b-caryophyllene and its oxide; the other sample was found to be rich in sesquiterpenes (51.5%) among which germa-crene D predominates (21,4%); on the other hand, isopentenylindoles are less abundant and in inverse ratio, the 3-isomer predominating.

Chemical compositions of the bark essential oils of *Anonidium mannii* (Oliv.) Engl. and Diels from two different regions of Gabon

Components	Percentage	
	Lbv	Bm
heptan-2-ol	1.0	-
α-pinene	0.4	0.8
limonene	tr	0.5
hexa-2,4-dienyl acetate	4.8	3.0
trans-β-ocimene	1.5	0.3
2-octyl acetate	2.1	-
α-cubebene	0.7	tr
α-copaene	3.7	2.5
β-elemene	3.4	1.5
β-caryophyllene	3.7	8.0
trans-α-bergamotene	0.8	tr
α-humulene	1.1	1.9
germacrene D	21.4	0.5
γ-muurolene	1.3	-
bicyclogermacrene	2.0	-
MW 204[1]	-	1.2
γ-cadinene	1.5	-
cis-calamenene	-	0.6
δ-cadinene	1.3	tr
spathulenol	1.5	0.5
germacrene D-4-ol	1.3	-
caryophyllene oxide	6.4	14.5
humulene oxide	1.4	3.6
MW 220[2]	1.1	1.4
MW 202[3]	1.0	1.1
MW 222[4]	2.1	1.2
7-isopentenylindole	**3.1**	**50.7**
3-isopentenylindole	**28.8**	**3.0**
isopentenylindole*	0.8	1.1
MW=183[5]	0.6	1.2

* Correct isomer not characterized
tr= traces
Lbv=Libreville
Bm=Bitam

1-MS m/z : 161(100), 91(56), 105(55), 41(55), 79(25), 119(24), 55(20).
2-MS m/z : 41(100), 91(90), 123(83), 67(71), 105(69), 82(69), 120(50), 177(45).
3-MS m/z : 41(100), 91(98), 159(80), 79(69), 77(62), 117(58), 105(57), 175(55), 131(52), 67(52), 55(48), 189(23), 202(23).
4-MS m/z : 41(100), 43(73), 105(67), 91(65), 55(65), 81(63), 123(44), 193(42), 161(36).
5-MS m/z : 168(100), 167(74), 183(38)|M|+, 169(22), 141(20), 115(20).

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P-5-32

MONODORA MYRISTICA (GAERTN.) DUN. SEEDS - ANALYTICAL AND SENSORY INVESTIGATION ON CALABASH NUTMEG ESSENTIAL OILS OF DIFFERENT ORIGINS

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Introduction

Calabash nutmegs are the seeds of the tree *Monodora myristica* (Gaertn.) Dun. of the *Annonaceae* family, originating from and cultivated in West-Africa. The bean-like seeds, 10 - 30 mm long, 8 - 15 mm wide, 6 - 12 mm thick, have a glossy, smooth to slightly wrinkled, light to strongly brown surface.

The round seeds are used as savory food additive, but also for the preparation of stimulants, drugs (e.g. as a stomatic or to relieve constipation) and disinfectants. Further, rosaries and necklaces are made from the seeds.

Smell and taste of the seeds and the essential oil are stated differently. They are described as pleasant, savory, slightly citric or thymol-like, on the one hand, or as nutmeg-like, on the other hand, which explains the terms "African nutmeg", "Calabash nutmeg" and "False nutmeg".

In literature, the content of essential oil is given within the range from 3 % to 6 %. The content of fatty oils amounts to approx. 25 %. The essential oil consists of more than 75 % monoterpene hydrocarbons. The major constituents are stated differently which led Onyenekwe et al. [1] to suppose not only habitat-related differences, but also the existence of more than one variety of the plant, in Nigeria. Ekundayo *et al.* [2] have described the components p-cymene and linalool as major constituents, while Onyenekwe has analyzed the monoterpene hydrocarbons α-phellandrene (50 %), p-cymene (8.5 %) and α-pinene (5 %) as major constituents. Lamaty *et al.* [3] report a content of 48 % of α-phellandrene. Also a few sesquiterpene hydrocarbons and oxygenated compounds, such as germacrene-D-4-ol and cadinenes, are further constituents.

Experimental

The essential oils of the seeds of *Monodora myristica* seeds of four different habitats were analyzed.

Habitat of the seeds: **I** - West Nigeria
II - unknown
III - Ibandan, South-West Nigeria
IV - Zaria, East Nigeria.

The essential oils of the seeds were obtained by steam distillation, at a distillation time of 4 h. For distillation, the seeds were ground in an analytical mill with cooling.

The composition of the oil was analyzed by gas chromatography/ mass spectrometry.
The following analytical instruments were used:

Device:	GC HP 5890 II MSD HP 5971 A
Method parameters:	
Column:	DB Wax 60m, 0.25mm i.d., 0.25µm film thickness
Temperature programme:	1 min. 60°C isothermal 4 °C/min. 60-220°C 10 min. 220°C isothermal
Carrier gas:	helium 0.9 ml/min. at 110°C
Split mode:	1:60
Injection volume:	0.1µl

Method of calculation of relativ percentage

Results

The following yields of essential oils were obtained:

Habitat I	3.8 %
Habitat II	3.4 %
Habitat III	4.3 %
Habitat IV	4.8 %

Table 1 reports the composition, as relative percentage of the areas of the peaks, of the single components identified.

All the samples analyzed were composed of > 70 % monoterpene hydrocarbons, the major components being α-pinene (6 - 8 %), limonene (3 - 5 %), myrcene (2 - 3 %) and p-cymene (19 - 55 %). There are large differences in the α-phellandrene content of the samples. Samples I and II had a content of 11 % and 18 %, respectively, and sample IV had a content of 43 %, while only 0.7 % was determined in sample III.

Carvacrol was also determined in all the samples (1 - 4 %). The possible antimicrobial effect of this phenolic derivative may be an explanation of the use of the essential oil in medicine and for the preparation of disinfectants. In the essential oils of samples II and IV, the sesquiterpene germacrene-D-4-ol was analyzed as well as γ- and δ-cadinene known from other medicinal and aromatic plants.

Sabinene and β-pinene, the major constituents of the essential oil of nutmeg *(Myristica fragrans)*, are not contained in the essential oil of calabash nutmeg, nor safrole and eugenol. The hallucinogen myristicin, too, is detected only in *Myristica fragrans*.

The sensory evaluation of the essential oils and ground seeds of the four samples did not reveal any significant differences. All the samples were characterized by a strong terpenic flavour. The conception that by application of *Monodora myristica* seeds the typical nutmeg flavour will be created, is not supported.

Reference:

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Acknowledgements:

Thanks are extended to Dr. Seidemann for having made available samples of different origins.

Table 1 - Constituents of the essential oils of *Monodora myristica* (Gaertn.) Dun. seeds

Ret Time	Component	Area %			
		I	II	III	IV
6.65	α-Pinene	6.88	7.56	8.21	5.87
6.69	α-Thujene	1.96	2.81	2.97	2.39
8.00	n-Hexanal	0.30	0.05	0.03	0.06
8.69	β-Pinene	0.28	0.24	0.37	0.20
9.30	2-Carene	0.82	0.97	0.85	0.78
10.17	Myrcene	2.45	3.00	1.66	3.35
10.39	α-Phellandrene	10.76	18.18	0.65	43.42
11.35	Limonene	3.71	3.98	4.52	3.12
11.64	β-Phellandrene	0.36	0.51	0.45	0.52
12.33	cis-β-Ocimene	0.08	0.15	n.d.	0.27
12.77	γ-Terpinene	0.04	0.12	0.20	0.12
12.87	trans-β-Ocimene	0.01	0.07	n.d.	0.12
13.70	p-Cymene	43.22	41.28	54.68	18.89
19.55	δ-Elemene	0.53	n.d.	0.21	n.d.
20.27	α-Copaene	0.02	0.24	tr.	0.07
21.68	Linalool	1.90	1.67	2.00	1.21
22.34	trans-p-Menth-2-en-1-ol	0.25	0.17	0.15	0.18
22.44	α-Santalene	n.d.	n.d.	n.d.	0.22
23.39	4-Terpineol	0.07	0.07	0.38	0.02
24.05	cis-p-Menth-2-en-1-ol	0.18	0.09	0.12	0.07
25.54	γ-Muurolene	0.24	0.14	0.05	0.08
25.80	α-Terpineol	0.72	0.48	0.75	0.39
25.92	unknown (m/z: 177, 220)	0.02	1.84	0.04	0.94
26.16	Germacrene D	0.28	n.d.	n.d.	0.05
26.42	α-Muurolene	tr.	0.31	tr.	0.31
27.03	unknown (m/z: 128, 154)	n.d.	0.68	4.62	0.46
27.23	δ-Cadinene	0.14	2.16	0.06	2.31
27.35	γ-Cadinene	0.04	0.96	0.03	0.47
28.03	Cuminaldehyde	0.09	0.10	0.33	0.04
28.58	α-Phellandrene epoxide (cis or trans)	6.38	4.31	4.90	1.92
29.16	n-Hexanoic acid	0.50	tr.	tr.	n.d.
29.38	Geraniol + Thymolacetate	0.15+0.05	0.05+0.05	0.20+0.21	0.03+0.02
30.73	α-Phellandrene epoxide (cis or trans)	0.66	0.37	0.52	0.15
33.40	$C_{15}H_{24}O$ (MW 220, m/z: 119, 220)	8.54	n.d.	1.70	0.13
33.86	n-Octanoic acid	0.95	0.26	0.13	0.10
33.97	Germacrene D-4-ol	n.d.	0.58	n.d.	3.06
35.55	Spathulenol	n.d.	0.32	n.d.	0.11
36.03	n-Nonanoic acid	0.82	0.33	0.22	0.21
36.41	tau-Cadinol + Thymol	0.03+0.05	0.78+0.22	0.02+0.45	0.74+0.09
36.76	tau-Muurolol + a $C_{10}H_{14}O$ (MW 150)	0.01+0.21	0.77+0.02	0.01+0.10	0.92+0.02
37.01	Carvacrol	3.19	3.14	4.31	1.30
37.70	α-Cadinol	0.32	1.23	0.18	2.52
38.16	$C_{15}H_{24}O$ (MW 220, m/z: 119, 220)	3.27	n.d.	1.05	0.09
40.58	$C_{15}H_{24}O$ (MW 220, m/z: 159, 220)	0.63	n.d.	0.25	n.d.

tr. traces (<0,01%)
n.d. no detected

VARIABILITY OF THE ESSENTIAL OILS OF *LIPPIA GRAVEOLENS* HBK FROM GUATEMALA

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Introduction

Numerous plants in various plant families are known colloquially as oregano, origanum or similar[1]. The most important ones are: "Greek Oregano" [*Origanum vulgare* ssp. hirtum (Labiatae)], "Turkish Oregano" [*Origanum onites* (Labiatae)], "Spanish Oregano" [*Thymus capitatus* (Labiatae)] and "Mexican Oregano" [*Lippia graveolens* (Verbenaceae)]. The two main origins for oregano in commerce today are the Mediterranean and Mexico. A stronger flavour distinguishes the Mexican from the Greek, Turkish and also Spanish oregano. Strong variations have been observed in the concentration and composition of the essential oil not only between, but also within the different species dependent on their origin and genotype. Main components are, however, thymol and carvacrol.

Until now there only exist some studies on the content and composition of the essential oil of *Lippia graveolens* HBK (Verbenaceae) collected in Mexico or of unknown origin[1,2,3,4,5]. This species is also distributed in Guatemala (Fig. 1). The dried leaves are sold to a large extent on the national market as a seasoning and for medicinal purposes including stomach diseases, colics, fever, headache, diarrhea, heartburn and nausea. The raw material derives from wild collection only. Due to this fact, and the simultaneously increasing destruction of nature, mostly due to fire burning, the species might become endangered. Therefore, a domestication project was started in Guatemala in 1993. One of the first objectives was the investigation of the regional distribution and the intraspecific variability of *L. graveolens*.

Material and Methods

Plant Material: Five different wild populations of *L. graveolens* were detected in Guatemala. The natural conditions are a subtropical arid climate and stony soil.

Figure 1. Origins of the wild populations in Guatemala

All populations were found in hilly regions. A map of Guatemala showing the different areas is shown in Figure 1. Herbarium samples were collected and identified at the Universidad del Valle (Guatemala). Almost homogeneous cuttings from the geographically distinct populations were collected in 1994. To keep the age almost similar, the cuttings were taken from the apical part of the shoots. They were rooted in multi-plot boxes in a greenhouse of ICTA (Instituto de Ciencia y Tecnología Agrícola) at Chimaltenango. In April 1995, the rooted cuttings were transplanted to the field at the ICTA experimental station Cuyuta at a distance of 75 x 40 cm. Harvest was performed in July 1995 at the beginning of the flowering stage.

Analysis of the volatile oils: Mixed samples of the dried leaves of each population were steam distilled using a modified Neo-Clevenger-hydrodistillation apparatus with the following distillation conditions: 3g

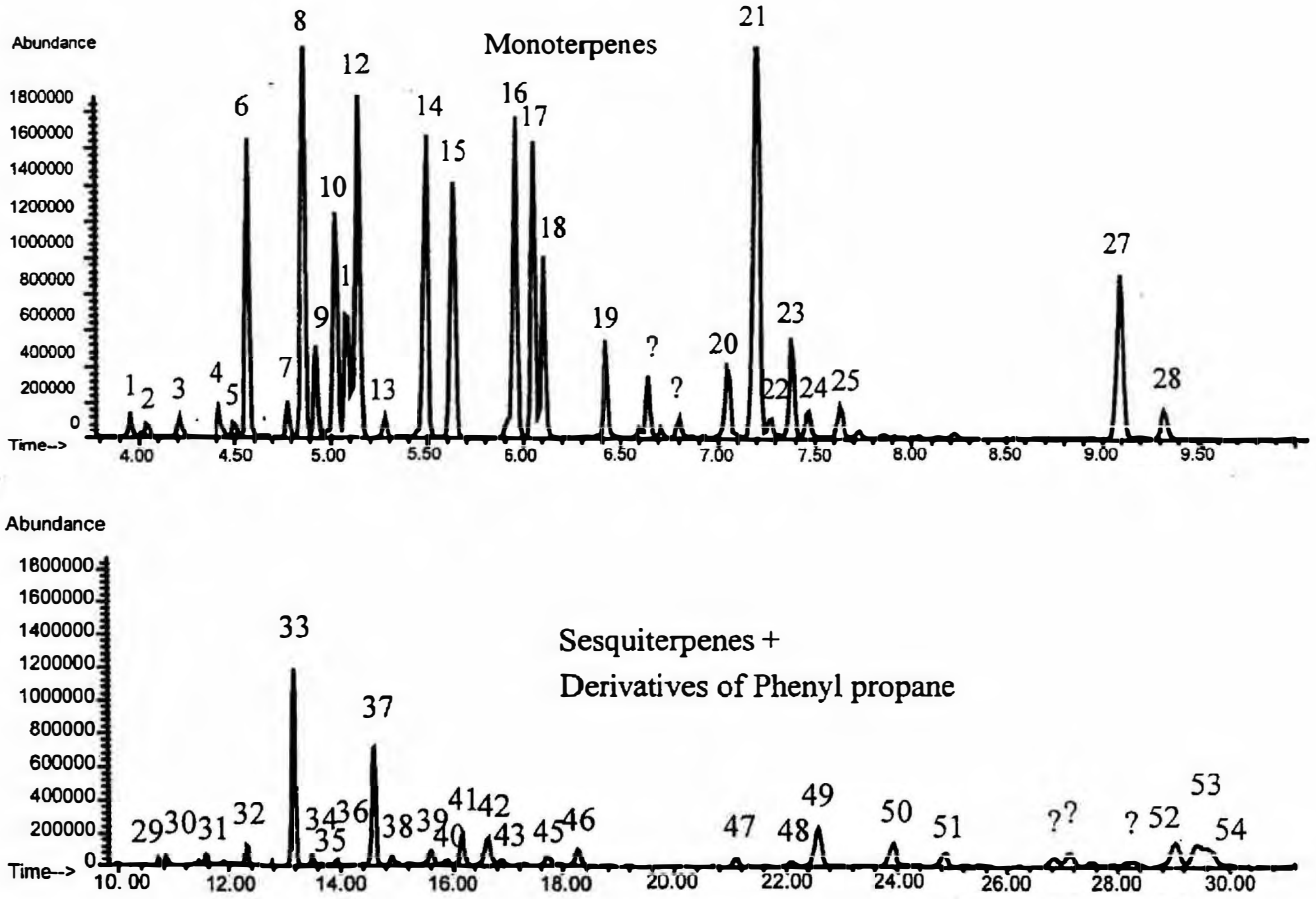

Figure 2. Gas chromatograms of the essential oil of *L. graveolens* growing in Subinal (Guatemala)

crude drug in 250 ml H_2O for 1.5 h distillation time. The vaporized oils were collected in pentane. The obtained oil-pentane mixtures were dried over anhydrous Na_2SO_4 and filtered. After removal of pentane, the yield was determined gravimetrically. The essential oils were stored in a freezer until GC/MS investigation. The volatile oils were analyzed by computerized gas chromatograph/mass spectrometry, employing a HP6890 gas-chromatograph coupled with HP5972 MSD; column: HP-5MS 30m x 0,25 mm (0,25µm film thickness); carrier gas: He (0,8 ml/min); injector T°: 250°C; transfer line T°: 280°C; ion source T°: 180°C; ionization energy: 70 eV. Temperature program: 60°C (1), gradient 1: 18°C/min 60-110°C (2), gradient 2: 28°C/min 110-130°C (27). Injection: 1µl with a split ratio of 1:50. The constituents of each distillate were identified by direct comparison with authentic samples substances as far as available, by visual or computerized matching with published mass spectra and by Kovats indices.

Results and Discussion

Concerning the oil yield, significant differences could be observed. The populations Oreganal and Agua Caliente (district Zacapa) showed with 3.24% and 3.56% (w/w) a considerably higher amount of essential oil than the other origins (1,24-2,15%). Even a survey on the investigations of *L. graveolens* from Mexico did not show much higher essential oil contents but 0.29% to 2.54%[3,5].

Also the composition of the essential oils differed remarkably between the different populations (tab. 1). 3 different oil types could be identified: a thymol, a carvacrol and a mixed type. A total of 54 different constituents could be identified. Figure 2 shows the chromatograms of a sample of *L. graveolens* found in the small village Subinal. This population differed considerably from the others. It did not contain a main component, no substance reached more than 10%. And the oil showed a higher number of substances whose proportion of sesquiterpenes was considerably high. Comparing the other four populations a similarity according to the distribution of the substances was remarkable. Though, they showed a big variation referring to their content of thymol and carvacrol. Three populations were rich in thymol, but differed significantly in overall composition. The population collected in the region Agua Caliente showed an extremely high thymol content of 80.6%, which is unique for *L. graveolens*, considering the other investigations made until now. Even other species known in having a high thymol content, rarely show such a high amount of this substance[7]. The samples of the species which

originated from San Jacinto exhibited as the main component carvacrol with an proportion of 45.2%. This is also rarely observed from this species found in Mexico.

With some exceptions *L. graveolens* from Mexico normally was shown to be a thymol type with a content of about 30-60% thymol[1,2,3,4]. Only one study found p-cymene as main compound[5] and few results showed carvacrol as the leading component in the oil[2,3]. Based on the different investigations regarding species known as oregano, it is proved that phenols are necessary components for a plant described as oregano. However, the "real" oregano being the main source of the condiment is seen to be rich in carvacrol while species types with a main content of thymol are classified as thyme types[8].

According to this classification the different origins of *L. graveolens* from Guatemala can be divided into a thyme type with significantly different thymol levels from less than 60-80%, a carvacrol type, and an undefined mixed type without any dominant substance.

Acknowledgements

This study was gratefully granted by the Austrian Academy of Sciences. Special thanks to Ing. Centeno (Universidad del Valle, Guateamala), Dr. MacVean (Universidad del Valle, Guateamala) and Lic. MacVean (Universidad del Valle, Guatemala) for the great support in Guatemala.

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Table 1. Chemical composition of essential oils of *Lippia graveolens* of 5 different wild origin in Guatemala

Peak	Constituent	Percentage of total					Identification
	Population	*Subinal*	*Oreganal*	*Agua Caliente*	*Buena Vista*	*San Jacinto*	
	Yield (%)	**1,24**	**3,35**	**3,56**	**2,06**	**2,15**	
1	α-thujene	0,3	0,4	0,2	0,2	0,3	MS, CO, KI
2	α-pinene	0,1	0,2	t	0,1	t	MS, CO, KI
3	camphene	0,3	0,1	—	0,1	t	MS, CO, KI
4	octen-3-ol + sabinene	0,3	0,3	0,2	0,3	0,2	MS, KI
5	β-pinene	0,2	0,2	t	0,2	t	MS, CO, KI
6	myrcene	2,7	2,4	1,3	1,9	1,9	MS, CO, KI
7	α-phellandrene	0,2	t	—	0,2	0,2	MS, KI,I
8	car-3-ene	3,6	0,1	t	0,3	0,1	MS, KI
9	α-terpinene	1,0	1,1	0,5	0,9	1,3	MS, CO, KI
10	p-cymene	2,8	4,9	2,7	4,2	6,9	MS, CO, KI
11	limonene	1,5	0,3	0,2	0,3	0,3	MS, CO, KI
12	1,8-cineol	5,0	2,7	0,1	5,2	0,6	MS, CO, KI
13	ocimene	0,2	0,1	t	0,1	0,1	MS, KI
14	γ-terpinene	4,1	4,1	1,7	4,0	7,3	MS, CO, KI
15	cis-p-menth-2-en-1-ol	4,6	0,8	0,6	1,1	0,6	MS, KI
16	terpinolene	3,4	0,3	0,3	0,5	0,2	MS, CO, KI
17	linalool + cis-sabinene hydrate	3,8	1,5	0,7	2,7	1,4	MS, CO, KI
18	trans-p-menth-2-en-1-ol	2,9	0,7	0,5	0,8	0,4	MS, KI
19	trans-sabinene hydrate	1,2	0,1	—	0,2	t	MS, KI
20	borneol	1,2	0,4	—	0,6	0,2	MS, CO, KI
21	4-terpineol	7,3	2,2	1,8	2,3	1,5	MS, KI
22	p-cymen-8-ol	0,3	0,2	0,1	0,2	t	MS, KI
23	α-terpineol	1,6	0,5	—	1,0	t	MS, CO, KI
24	cis-piperitol	0,4	—	—	—	—	MS, KI
25	trans-piperitol	0,5	—	—	—	—	MS, KI
26	thymol methyl ether	—	0,6	0,4	0,7	0,4	MS, KI
27	thymol	6,8	66,5	80,6	56,6	19,9	MS, CO, KI
28	carvacrol	1,1	1,1	1,3	4,4	45,2	MS, CO, KI
29	α-cubebene	0,2	—	—	—	—	MS, KI
30	eugenol	0,2	t	t	t	t	MS, CO, KI
31	α-copaene	0,3	—	—	—	—	MS, KI
32	methyl eugenol	0,5	—	—	—	—	MS, KI
33	caryophyllene	8,7	3,4	2,8	4,6	3,5	MS, CO, KI
34	β-gurjunene	0,4	—	-	-	-	MS, KI
35	z-α-trans-bergamotene	0,2	0,3	0,5	0,5	0,6	MS, KI
36	aromadendrene	t	—	—	—	—	MS, KI
37	α-humulene	5,7	2,2	1,9	2,9	2,3	MS, CO, KI
38	allo-aromadendrene	0,5	—	-	-	-	MS, KI
39	γ-muurolene	0,8	—	-	t	-	MS, KI
40	germacrene D	0,2	0,1	0,1	t	1,4	MS, KI
41	β-selinene	1,7	—	—	—	—	MS, KI
42	α-selinene	1,9	—	—	t	—	MS, KI
43	α-muurolene	t	—	—	—	—	MS, KI
44	β-bisabolene	—	0,1	0,3	t	t	MS, KI
45	γ-cadinene	0,6	—	—	—	—	MS, KI
46	δ-cadinene	0,9	—	—	0,1	—	MS, KI
47	trans-nerolidol	0,9	—	—	—	—	MS, CO, KI
48	spathulenol	t	—	—	—	—	MS, KI
49	caryophyllene oxide	3,3	0,7	0,3	0,9	0,8	MS, CO, KI
50	guaiol	2,2	—	—	0,2	—	MS, KI
51	tau-muurolol	0,2	—	—	—	—	MS, KI
52	β-eudesmol	3,0	t	—	0,5	—	MS, KI
53	α-eudesmol	4,0	t	—	0,3	—	MS, KI
54	α-cadinol	0,3	—	—	—	—	MS, KI

MS = mass identification from MS library; CO = identity confirmed by comparison with authentic compound;
KI = Kovats index; t = trace amount (<0,1%)

CONSTITUENTS OF THE HAITIAN VETIVER OIL[1]

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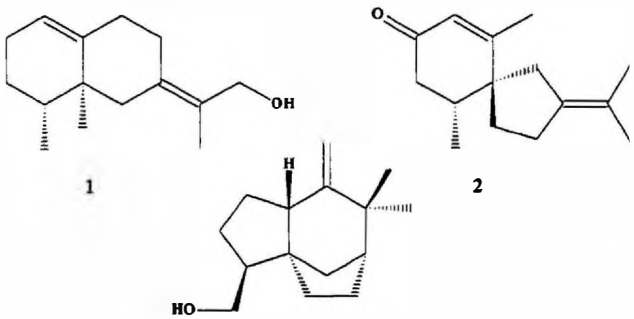
INTRODUCTION

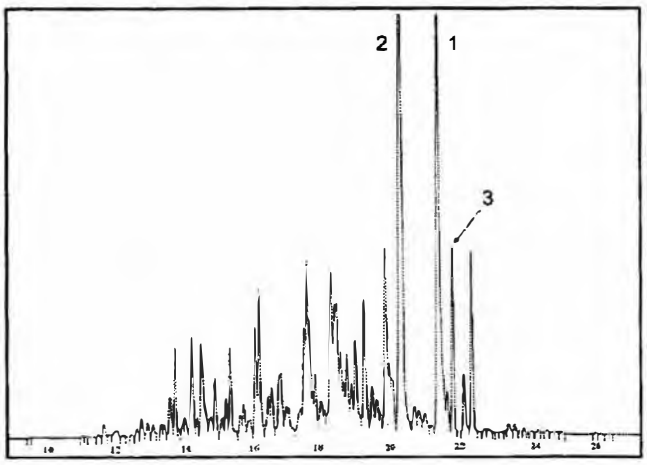
The chemical composition of Vetiver oil, obtained by steam distillation of the dried roots from *Vetiveria zizanioides*, of various geographic origins has been studied since 1901[2].

The analysis of a Haitian Vetiver oil afforded isovalencenol (**1**), β-vetivone (**2**), and khusimol (**3**) as main constituents.



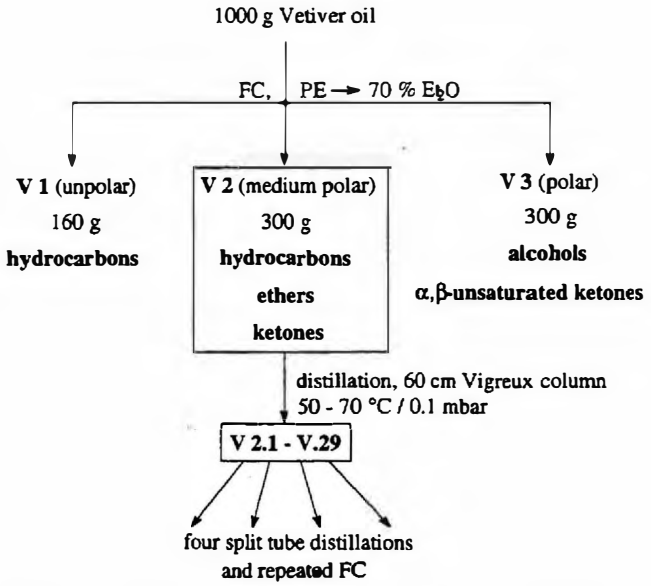
1 2 3

The pattern of the GC implies the complexity for the separation, structure elucidation, and olfactory evaluation of its constituents (see Scheme 1).



Scheme 1: GC of the Vetiver oil, 25 m CP Sil 5 CB-column, 60° C, 5° C/min to 240° C, retention time (min)

Our procedure for the separation is outlined in Scheme 2: flashchromatography (FC) of 1 kg of Vetiver oil led to the two olfactorily important fractions V2 and V3. The combination of distillation and repeated FC yielded several new compounds.



Scheme 2: Vetiver oil isolation procedure for constituents

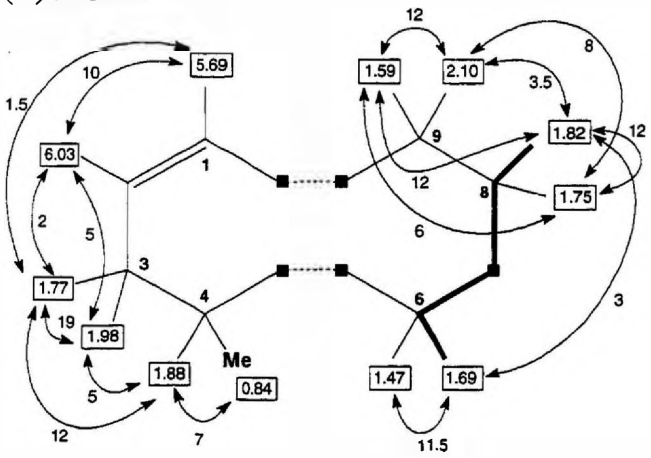
STRUCTURE ELUCIDATION OF A NEW SESQUITERPENE ETHER 4[3]

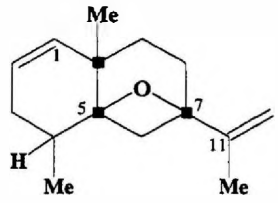
From the medium-polar fraction we isolated 30 mg of a compound with 91% purity (GC). HR-MS afforded the exact mass of 218.1671 which is consistent with the molecular formula $C_{15}H_{22}O$.

Table 1: Significant ^1H- and ^{13}C-NMR-data ($CDCl_3$)

^{13}C	^1H	*J* [Hz]	functionality
134.5 s	6.03 ddd	10, 5, 2	*cis* double bond
126.2 d	5.69 ddd	10, 2, 1.5	
109.1 t	4.94 qd	1.5, 1.5	*exo*-methylene
	4.78 qd	1.5, 1.5	group
146.4 s			
	1. 79dd	1.5, 1	allylic methyl group
	1.47 d	11.5	isolated AB-system
	1.69 dd	11.5, 3	
	0.84 d	7	secondary methyl group
	0.86 s		angular methyl group
88.5 s			ether bridge
85.6 s			(diol excluded)

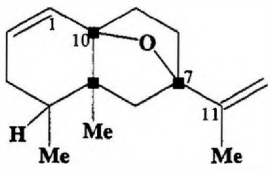
The combination of the significant ^{1}H- and ^{13}C-NMR-data (see Table 1) and the results of $^{1}H,^{13}C$-correlation and complete ^{1}H-spin-decoupling (fragments **A** and **B**, see Scheme 3) afforded two possible ethers with eudesmane (**C**) or eremophilane (**D**) skeleton.





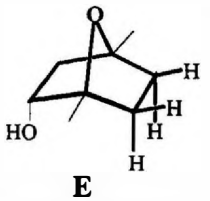
C

5,7-Epoxyeudesma-1,11-diene



D

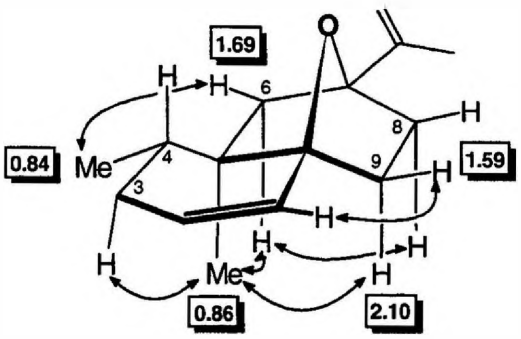
7,10-Epoxyeremophila-1,11-diene

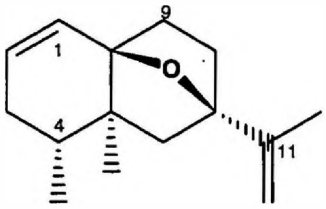


E

Scheme 3: Fragments **A** und **B**, possible structures **C** and **D**, and the reference ether **E**

The differentiation between **C** and **D** could be made by comparison of the coupling constants of the oxabicyclooctane **E**, prepared in our group. Only the eremophilane skeleton with the 5-membered ether bridge exhibits the typical pattern with the identical constants for the geminal, vicinal *cis*, and vicinal *trans* couplings. The relative configuration of the epoxyeremophiladiene **4** was confirmed by the indicated strong NOEs (see Scheme 4).

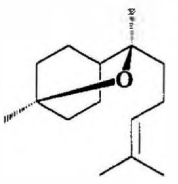




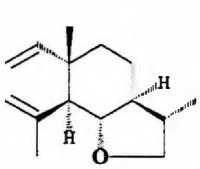
4

Scheme 4: NOEs ($CDCl_3$) of ether **4**

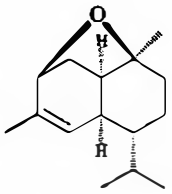
OVERVIEW ON ISOLATED ETHERS WITH MANIFOLD SKELETONS



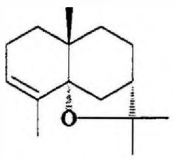
Sesquicineole



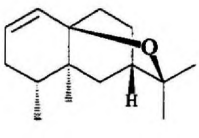
6,12-Epoxyelema-1,3-diene[5]



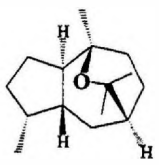
Lentideus Ether[4]



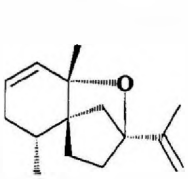
α-Agarofuran



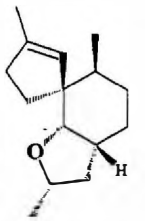
10,11-Epoxyeremophil-1-ene[3]



Kessane[6]



4,7-Epoxyspirovetiva-1,11(12)-diene[3]

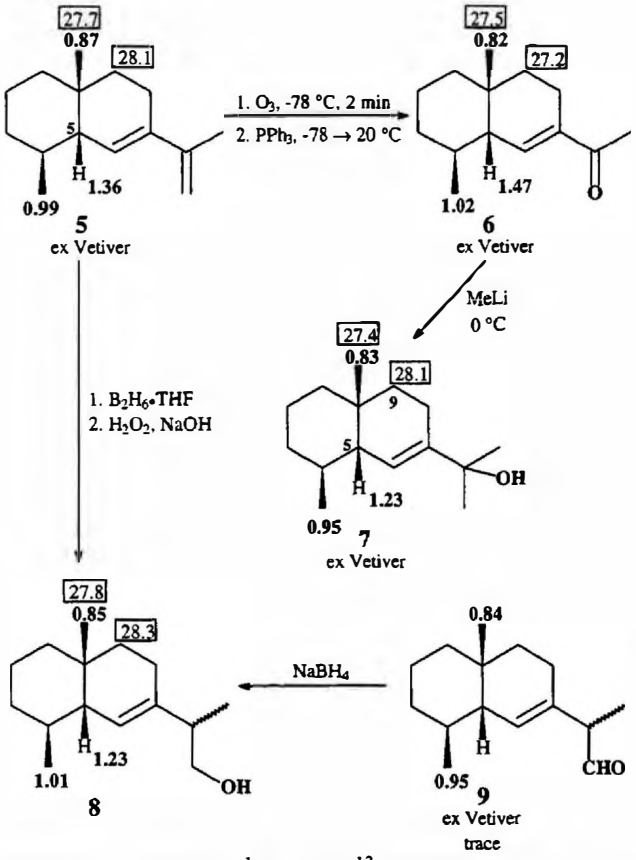


6,12-Epoxyspiroax-4-ene[3]

NEW *CIS*-EUDESM-6-ENES

To our surprise, we found a compound with a *cis*-eudesmane-skeleton **5** as one of the main sesquiterpene hydrocarbons, never isolated and analyzed before not even in the Vetiver oil. This *cis*-eudesmadiene **5** as well as three other isolated and fully characterized derivatives **6**, **7**, and **9** (see Scheme 5) exhibited the following typical signals in the ^{1}H- and ^{13}C-NMR spectra:

In the ^{1}H-NMR spectrum the angular methyl group is located at approximately δ_H 0.85 and the methyl doublet at about δ_H 1.0. Surprisingly the allylic proton at C-5 is strongly highfield-shifted to δ_H 1.3. In the ^{13}C-NMR spectrum the signal for the angular methyl group is downfield-shifted (δ_C 27) and for the methylene group at position 9 highfield-shifted (δ_C 28).

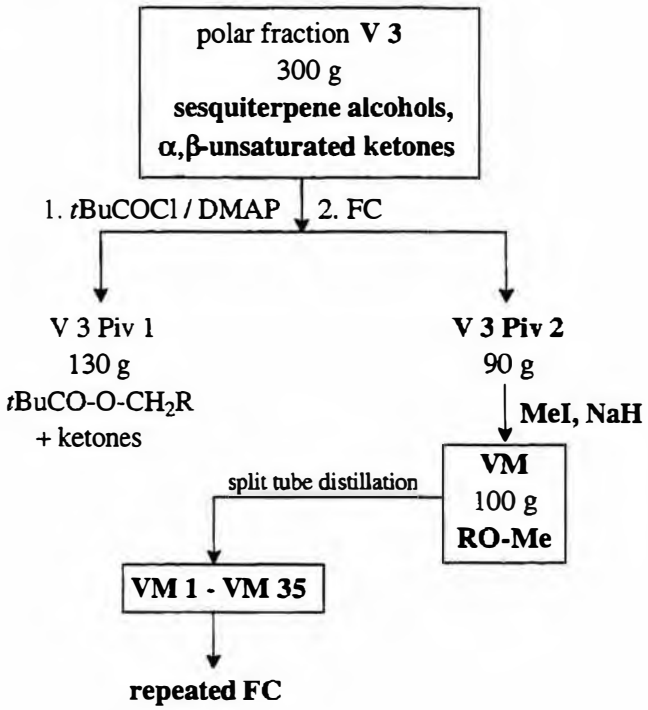


Scheme 5: Typical ^{1}H- and ^{13}C-NMR data of *cis*-eudesmanes **5-9**

The *relative* configurations and the structural similarities were confirmed by several chemical transformations, e.g. the ozonolysis of **5** to the ketone **6**, and reaction of **6** with MeLi to the alcohol **7**, or the hydroboration of **5** to the alcohol **8**, which is identical with the product obtained by the reduction of the aldehyde **9**. The high chemoselectivity at the methylene group could be explained by the sterical hindrance of the less reactive *endo* double bond.

ANALYSIS OF THE POLAR FRACTION V3 AND OLFACTORY EVALUATIONS

The polar fraction V3 was separated by the following procedure (see Scheme 6): the high amount of the two main primary alcohols isovalencenol (**1**) and khusimol (**3**) was diminished by esterification with pivaloyl chloride. Separation by FC yielded the unpolar pivaloate fraction V 3 Piv 1. The polarity of the more polar fraction V 3 Piv 2 (contains mainly secondary alcohols) was decreased by the transformation to methyl ethers with NaH / MeI. The successful split tube distillation of the methyl ether fraction VM afforded 30 new sesquiterpene methyl ethers after repeated FC.



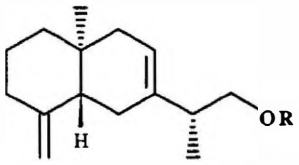
Scheme 6: Isolation procedure for the fraction V3

The transformation to methyl ethers has several advantages:

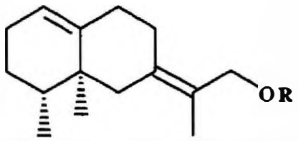
- high stability (against oxidation, water elimination)
- easier separation (boiling point - polarity)
- similar spectroscopic data (MS, ^{1}H- and ^{13}C-NMR)
- often similar odor of methyl ethers in comparison to the alcohols

The fourth point - olfactory similarities - is obvious in the evaluations of the polar fraction V3 and methyl ether fraction VM: the former is described as sour, *woody*, *grapefruit*-, sandalwood-like, sweet and the latter as *woody*, greasy, warm, *grapefruit*-, vetiver-like[7].

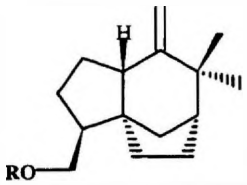
At least the comparison of the odor of several methyl ethers and their corresponding alcohols confirm this tendency.



R: -H strong, *warm*, *woody*, amber (75 % GC)
R: -Me *warm*, *woody*, khusimone-, vetiver-like (92 % GC)



1 R: -H *pleasant*, sweet, warm, *woody* (84 % GC)
R: -Me *pleasant*, *woody*, earthy, vetiver-, patchouli-like (99 % GC)



3 R: -H woody, grapefruit-, vetiver-like
R: -Me greasy, amber-like (89 % GC)

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VOLATILE COMPONENTS OF CHINESE CRUDE DRUGS, *DIOSCOREA JAPONICA*

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INTRODUCTION

Batatatis rhizoma ('Sanyaku' in Japanese), the rootstock of *Dioscorea japonica* (Dioscoreaceae), has been traditionally used in medicine in Japan for the treatment of diarrhea, asthma, polyuria, and diabetes. In previous papers, several phenanthrenes, dihydro - phenanthrenes, and bibenzyls have been isolated from *Dioscorea species*, and some of these compounds from *D. batatas* have the potency of antifungal activity [1,2]. Hikino et al. reported on the isolation of hypoglyce-mic active compounds (dioscorans A, B, C, D, E, and F) from *D. japonica* [3]. In our previous report, we found (+)-b-eudesmol and paeonol as antimutagenic compounds from *D. japonica* [4]. These components are well known as the volatile constituents [5-8], there-fore the volatile components of *D. japonica* had been interested. The volatile components of several chine-se crude drugs have been investigated in our research on the flavor compounds or flavor ingredients [9-19]. However, there is no report of the components of the volatile oils, and odor has yet been reported for this Chinese crude drug. In this report, the composition of the volatile oil from the rhizome of *D. japonica* was investigated.

RESULTS AND DISCUSSION

A gas chromatogram of the volatile oil of *D. japonica* is presented in Figure 1 in which 74 components were separated. As shown in Table 1, 68 of these were identified by direct comparison with authentic samples (on the basis of retention time and GC-MS) and confirmed by GC analyses of authentic samples from our previous work. The major constituents were fatty acids, which were palmitic acid (No. 67, 41.00 %), linoleic acid (No. 73, 28.76 %), and 9-hexadecenoic acid (No. 68, 4.47 %); the total content of fatty acids in the volatile oil was 88.45 %. The odor of these fatty acids was pungent and diffusive sour. The characteristic constituents in the volatiles from *D. japonica* were aldehydes, which were identified upon direct comparison with authentic samples, hexanal (No. 1, 0.09 %), heptanal (No. 2, 0.04 %), nonanal (No. 6, 0.18 %), furfural (No. 9, 0.05 %), decanal (No. 12, 0.09 %), benzaldehyde (No. 14, 0.04%), *trans*-2-nonenal (No. 15, 0.07 %), hyacinthin (No. 20, 0.12 %), *trans*,*trans*-2,4-nona-dienal (No. 24, 0.03 %), *trans*,*trans*-2,4-decadienal (No. 28, 0.06%), *trans*,*trans*-9,12-octadecadienal (No. 48, 0.11 %), and 2-naphthalenecarboxaldehyde (No. 52, 0.04 %). No. 6 was floral-waxy odor, while No. 20 was pungent-green, No. 12 was citrus-peel-like, and No. 15 was orris-like. Other potent odor in the oil were farnesol (No. 45, 0.16 %, sweet-oily odor), paeonol (No. 47, 0.09 %, warm aromatic), menthol (No. 19, 0.07 %, diffusive odor with sweet pungency), eugenol (No. 41, 0.06 %, deep-floral). A mixture of fatty acids (carbone no. 16-18), 8.8 %; aliphatic aldehydes (carbon no. 5-10), 0.1 % with traces of farnesol, paeonol, menthol, elemol, eugenol, and b-eudesmol in ethanol showed a characteristic odor note (slightly sweet, warm, aromatic, diffusive, and pungent) similar to that of Sanyaku.

EXPERIMENTAL

Material

Commercially available air-dried rootstock (Batatatis rhizoma) of *Dioscorea japonica* was purchased from Takasago Yakugiyo Co. (Osaka, Japan).

Isolation of the Volatile Oil

Two hundred grams of dry, coarsely powdered plant material were hydrodistilled with a Likens Nickerson-type apparatus using diethyl ether to

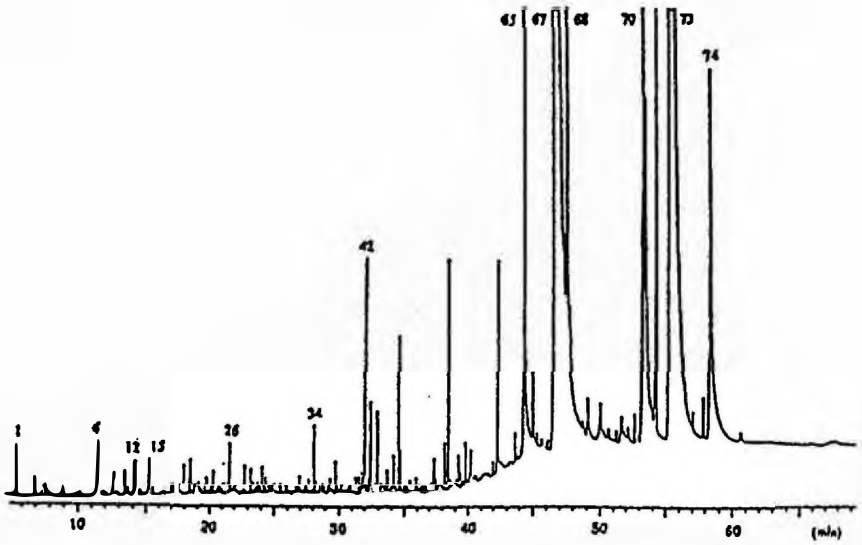

Fig. 1. Gas Chromatogram of the volatile oil from *Dioscorea japonica*. Column, TC-WAX flexible fused-silica column(60m x 0.25mm I.d.); column temperature, 60-240 °C (4°C/min); carrie gas, He at 1.0mL/min; FID

Table 1. Composition of the volatile oil from *D. japonica*.

No.[a]	Compound [b]	(%)	Identification	No.	Compound	(%)	Identification
1	Hexanal	0.09	GLC, MS	38	Methyl pentadecanoate	0.09	GLC, MS
2	Heptanal	0.04	GLC, MS	39	Nonanoic acid	0.04	GLC, MS
3	Amylfuran	0.02	GLC, MS	40	γ-Eudesmol	0.03	GLC, MS
4	Pentanol	tr.	GLC, MS	41	Eugenol	0.06	GLC, MS
5	Tetradecane	tr.	GLC, MS	42	Hinesol	0.49	GLC, MS
6	Nonanal	0.18	GLC, MS	43	Methyl palmitate	0.19	GLC, MS
7	*trans*-2-Octenal	tr.	GLC, MS	44	α-Eudesmol	tr.	GLC, MS
8	1-Octen-3-ol	0.04	GLC, MS	45	β-Eudesmol	0.16	GLC, MS
9	Furfural	0.05	GLC, MS	46	Decanoic acid	0.08	GLC, MS
10	2-Ethyl-1-hexanol	tr.	GLC, MS	47	Paeonol	0.09	GLC, MS
11	Pentadecane	tr.	GLC, MS	48	9,12-Octadecadienal	0.11	GLC, MS
12	Decanal	0.09	GLC, MS	49	Farnesol	0.22	GLC, MS
13	*l*-Camphor	tr.	GLC, MS	50	Farnesyl acetone	0.03	GLC, MS
14	Benzaldehyde	0.04	GLC, MS	51	1-Naphthalenecarboxaldehyde	tr.	GLC, MS
15	*trans*-2-Nonenal	0.07	GLC, MS	52	2-Naphthalenecarboxaldehyde	0.04	GLC, MS
16	5-Methylfurfural	tr.	GLC, MS	53	*trans*-Methyl-9-octadecenoate	0.07	GLC, MS
17	2-Undecanone	tr.	GLC, MS	54	*cis*-Methyl-9-octadecenoate	0.03	GLC, MS
18	Terpinene-4-ol	tr.	GLC, MS	55	Dodecanoic acid	0.11	GLC, MS
19	Menthol	0.07	GLC, MS	56	Methyl linoleate	0.46	GLC, MS
20	Hyacinthin	0.12	GLC, MS	57	Ethyl linoleate	0.06	GLC, MS
21	Isothiocyanatocyclohexane	0.02	GLC, MS	58	Unknown	0.06	GLC, MS
22	*endo*-Borneol	0.03	GLC, MS	59	Methyl linolenate	0.09	GLC, MS
23	*trans, trans*-2,4-Nonadienal	0.03	GLC, MS	60	Unknown	0.09	GLC, MS
24	β-Bisabolene	0.05	GLC, MS	61	Unknown	0.04	GLC, MS
25	δ-Cadinene	0.03	GLC, MS	62	Tetradecanioc acid	0.48	GLC, MS
26	(-)-ar-Curcumene	0.08	GLC, MS	63	Unknown	0.46	GLC, MS
27	*trans,trans*-2,4-Decadienal	0.06	GLC, MS	64	Phenanthrene	0.07	GLC, MS
28	*trans*-Anethole	0.05	GLC, MS	65	Pentadecanoic acid	4.90	GLC, MS
29	*trans*-Geraniol	0.02	GLC, MS	66	Unknown	0.05	GLC, MS
30	Geranylacetone	tr.	GLC, MS	67	Palmitic acid	41.00	GLC, MS
31	Guaiacol	0.06	GLC, MS	68	9-Hexadecenoic acid	4.47	GLC, MS
32	Benzylalcohol	0.03	GLC, MS	69	Unknown	0.95	GLC, MS
33	Phenol	0.03	GLC, MS	70	Elaidic acid	3.03	GLC, MS
34	Hexadecanol	0.17	GLC, MS	71	Oleic acid	2.56	GLC, MS
35	4-Methoxybenzaldehyde	tr.	GLC, MS	72	Unknown	2.43	GLC, MS
36	Cinnamaldehyde	tr.	GLC, MS	73	Linoleic acid	28.76	GLC, MS
37	Elemol	0.03	GLC, MS	74	Linolenic acid	3.02	GLC, MS

[a] Peak number refer to Figure 1.
[b] All components were identified by comparing retention time of GLC and MS with authentic samples.
tr = <0.01.

yield 0.05% of colorless oil, which was dried over anhydrous sodium sulphate.

Gas-Liquid Chromatography (GC)

The GC was carried out with a Hewlet Packard 5972A instrument fitted with a flame-ionization detector and a TC-WAX flexible fused-silica column (60m x 0.25 mm i.d.) was employed. Operating conditions were as follows; initial oven temperature, 80 °C for 5min, then 240°C at 4 °C/min and held for 30 min; injector and detector temperature, 240 °C; carrier gas He, 1.0 mL/min. Relative percentages were calculated by a HP 3396B integrator.

Gas-Liquid Chromatography-Mass Spectrometry (GC-MS)

A Hewlett-Packard 5972A GC-MS system equipped with a Wiley Library Software was used. Capillary GC conditions as above were employed. Significant MS operating parameters: ionization voltage, 70 eV; ion source temperature, 200 °C; scan mass range, 40-350u. Constituents were identified by peak matching with authentic samples, mass spectra with those recorded in the computer library and verified by comparison of their mass spectra and literature data, and confirmed by GC analyses of authentic samples from previous work.

Acknowledgment

We are grateful to the Environmental Science Institute of Kinki University for financial support. This study was also supported by a Grant-in-Aid for Science Research from Japan Private School Promotion Foundation.

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DIVERSITY OF PLANTS PRODUCING ESSENTIAL OILS IN THAILAND

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INTRODUCTION

Many plant species producing essential oils, both native and introduced ones have long been used in Thai culture. Before modern perfume, cosmetics, medicine, flavour and chemical pesticides were known, Thai people wisely used their rich plant resources to preserve and improve the flavour and fragrances of their food, as many people outside Thai culture have now realized. Many aromatic plant parts have been used to preserve and perfume clothes. Some were used traditionally as insect repellent. Most of them are still not being explored as potential use in industry and many of them are now hard to find as home gardens are forced to be of much smaller sizes or none at all due to increasing land price. The first attempt to study diversity of aromatic plants in Thailand was that of Wongkaew and Thaniswanyangur (1994) who reported botanical and ecological descriptions,of 95 aromatic species, emphasizing their cultural values, while Jirayupin (1996) emphasized fragrant flowers and Prataungsri (1994) included some essential oils bearing plants for industrial interest in her report.

Ethnobotany of 20 aromatic ornamental plants were described (Wongkaew, 1996). This report presents further investigations on aromatic plants. Due to limitation of space, only scientific names, families and growth habits of aromatic plants which are more obvious and common are reported. Many large genera consist of many aromatic members but only representatives are included. Some appeared with spp. to show many species bearing essential oil have been left out.

MATERIALS AND METHODS

The study was conducted by herbarium, literature and field investigation. The herbarium specimens of the Royal Forestry Department was used. Field investigation was designed to obtain current information. The scientific names and taxonomic classification followed those reported by Smithinan (1970). Commercial significance was assessed by export import statistics reported by Custom Department (1991 - 1995) as well as personal interview. Literature study here could be found in Jirayupin (1994), Rajnee et al. (1992)

RESULTS AND DISCUSSION

Table 1 lists the aromatic plants under investigation with families and scientific names while growth habits were abbreviated by letters in parentheses behind the respective scientific names. Table 2 gives a full survey of the abbreviated growth habits in Table 1. The species with Ex preceding the growth habits are not native.

The enriched diversity of plant bearing essential oils in Thailand could be implicated by Table 1 which showed about 279 species distributed in 177 genera and 79 families. Among the 279 species, 187 are native. Many orchid hybrids and lesser known indigenous species have to be excluded due to space limitation.A new orchid hybrid has such a high essential oil content and nice floral note that a nice perfume Udorn Sunshine is made from it. With climates favoring growing conditions, further research to explore underutilized aromatic plants for potential industrial development should be a rewarding attempt both scientifically and commercially. Essential oils of many of these species have not been studied at all.

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Table 1. List of essential oil bearing plants which are commonly grown in Thailand.
(Letters in parentheses designate growth habits of the preceding species)

FAMILY	SCIENTIFIC NAMES
AGAVACEAE	*Dracaena fragrans* Ker-Gawl. (ExS/ST), *Polianthes tuberosa* L (ExH),
ALLIACEAE	*Allium ascalonicum* L. (ExH), *A. cepa* L. (ExH), *A. sativum* L. (ExH)
ALANGIACEAE	*Alangium salviifolium* Wang. *subsp. hexapetalum* Wang. (S/ST)
ALTINGIACEAE	*Altingia excelsa* Noronha (T), *A. siamensis* Craib (T)
AMARYLLIDACEAE	*Crinum amabile* Donn (ExH), *Crinum asiaticum* L. (H)
ANACARDIACEAE	*Anacardium occidentale* Linn. (ExST), *Mangifera foetida* Lour. (T), *M. indica* L. (T), *M. spp.* (T)
ANNONACEAE	*Anaxagorea javanica* Bl. (S/ST), *Artabotrys siamensis* Miq. (C), *A. uncinatus* Merr. (ExC), *A. spp. Cananga odorata* Hook.f.&Th (ExT) *Cananga odorata* Hook. f. & Th. *var. fruticosa Corner* (S/ST), *C. latifolia* Finet & Gagnep. (T), *Desmos chinensis* Lour. (C), *Friesodielsia desmoides* Steenis (CrS), *Goniothalamus tapis* Miq. (S/ST), *Melodorum fruticosum* Lour. (S), *Rauwenhoffia siamensis* Scheff. (C), etc.
APOCYNACEAE	*Allemanda violacea* Gard. & Field (ExC), *Alstonia scholaris* R.Br. (T), *Alyxia reinwardtii Bl. var. lucida* Markgr. (C), *Beaumontia grandiflora* Wall. (ExC), *Carissa carandas* L. (ExS), *Cerbera odollum* Gaertn. (T), *C. manghas* Linn. (ST), *Ervatamia coronaria* Stapf (ExS), *Holarrhena densiflora* Ridl.(S), *Nerium indicum* Mill.(ExS), *Parameria barbata* Schum (C) *Plumeria acutifolia* Poir. (ExST), *P. obtusa* L. (ExST), *P. rubra* L. (ExST), *Strophanthus gratus* Franch. (ExC), *Vallaris glabra* Ktze. (C), *Wrightia religiosa* Benth. (S)
ARACEAE	*Alocasia odorata* C.Koch (H), *Colocasia esculenta* Schott (H)
ASCLEPIADACEAE	*Hoya ovalifolia* Wight &Arn. (C), *Stephanotis floribunda* Brongn.(C), *Telosma minor* Craib (C)
BARRINGTONIACEAE	*Barringtonia acutangula* Gaertn. (ST)
BETULACEAE	*Betula alnoides* Buch.-Ham. (T)
BIGNONIACEAE	*Dolichandrone spathacea* Schum.(T), *Millingtonia hortensis* L.f. (T)
BOMBACACEAE	*Durio zibethinus* L. (ExT)
BUDDLEJACEAE	*Buddleja davidii* Franch. (ExS), *Buddleja paniculata* Wall. (ExS)
CAESALPINIACEAE	*Bauhinia acuminata* L. (ExS), *B. purpurea* L. (ExST), *B. variegata* L. (ST), *Caesalpinia coriaria* Willd. (ExST), *C. coriaria* Willd. (ExST), *C. garrettiana* Craib (T), *Cassia javanica* Linn. (T), *Peltophorum pterocarpum* Back. ex Heyne (T), *P. dasyrachis* Kurz (T), *Saraca indica* L. (T), S. *thaipingensis* Cantley ex Prain (T)
CAPRIFOLIACEAE	*Lonicera japonica* Thunb. (ExC), *Lonicera hildebranditiana*(ExC), *Viburnum inopinatum* Craib (S)
CARICACEAE	*Carica papaya* Linn. (ExST)
COCHLOSPERMACEAE	*Cochlospermum religiosum* Alston. (ExST)
COMBRETACEAE	*Combretum punctatum* Bl. (C), *Quisqualis indica* L. (C)
COMPOSITAE	*Chrysanthemum coronarium* L. (ExH), *C. indicum* Linn. (ExUS), *Tagetes erecta* L. (ExH)
CONVOLVULACEAE	*Porana volubilis* Burm. (C)
COSTACEAE	*Costus speciosus* Smith. (H)
DILLENIACEAE	*Tetracera indica* Merr. (C), *T. loureiri* Pierre (C)
DIPTEROCARPACEAE	*Hopea odorata* Roxb. (T), *Shorea siamensis* Miq. (T), S. *roxburghii* G. Don (T), *Vatica diospyroides* Syming (T)
EBENACEAE	*Diospyros decandra* Lour. (T), *D. kaki* L. (ExST)

(Table 1. Contnd.)

FAMILY	SCIENTIFIC NAMES
EHRETIACEAE	*Ehretia laevis* Roxb. (ST)
EUPHORBIACEAE	*Hura crepitans* Linn. (ExT)
FLACOURTIACEAE	*Homalium tomentosum* Benth. (T), *Hydnocarpus anthelminthicus* Pierre (T), *Oncoba spinosa* (S/ST)
GERANIACEAE	*Pelargonium radula* L. Her (ExUS)
GESNERIACEAE	*Episcia cupreata E. fulgens* Hook. (ExH)
GRAMINEAE	*Cymbopogon citratus* Stapf (ExG), *C. nardus* Rendle (ExG), *Oryza sativa* L. cv. *Kao Mali* (G), *Vetiveria zizanoides* Nash(G)
GUTTIFERAE	*Calophyllum inophyllum* L. (T), *Cratoxylum cochinchinense* Bl.(T), *C. formosum* Dyer (T), *Garcinia* cowa Roxb. (ST), Mesua *ferrea* L. (T) *Mammea siamensis* Kosterm. *or Ochrocarpus siamensis* (T)
ILLICIACEAE	*Illicium anisatum* Lour. (ExT), *I. verum* Hook. (ExT)
LABIATAE	*Mentha arvensis* L. (ExH), *M. cordifolia* Opiz. (ExH) *M.piperata* L. (ExH), *M. spicata* L. (ExH), *Ocimum basilicum* L. (ExUS), *O. sanctum* L. (US), *O. canum* Sims. (ExH), *O. gratissimum* L. (ExS), *Pogostemon spp.*
LAURACEAE	*Cinnamomum camphora* Th. Fries (ExT), *C. iners* Bl. (T), *C. porrectum Kosterm* (T), *C. zeylanicum* Linn. (ExT), *C. spp. Litsea cubeba* Pers. (ST), *L. petiolata* Hook. f. (T), *L. spp.*
LECYTHIDACEAE	*Couroupita guianensis* Aubl. (ExT), *Gustavia gracillima* Miers (ExT)
LILIACEAE	*Asparagus racemosus* Willd.(C), *Dianella ensifolia* Red. (H)
LYTHRACEAE	*Lawsonia inermis* L. (ExS)
MAGNOLIACEAE	*Magnolia coco* DC. (ExS), *Manglietia garrettii* Craib (T), *Michelia alba* DC.(T) *Michelia champaca* L. (ST) *Michelia figo* Spreng. (ExS) *Paramichelia baillonii* Hu. (T), *Talauma candollei* Bl. *or Talauma mutabilis* Bl. (S)
MALPIGHIACEAE	*Hiptage bengalensis* Kurz. (C), *Tristellateria australasiae* A.Rich (C)
MALVACEAE	*Abelmoschus moschatus* Medic. (H)
MELIACEAE	*Aglaia odorata* Lour. (S/ST), *Azadirachta indica* Juss (ExT), *Melia azedarach* Linn. (T)
MENISPERMACEAE	*Tiliacora triandra* Diels (C)
MIMOSACEAE	*Acacia auriculaeformis* Cunn. (ExT), *A. Catechu* Willd. (T), *A. farnesiana* Willd. (ExS/ST), *A. leucophloea Willd.* (T), *A. podalyriaefolia* Cunn. (ExS), *A. tomentosa Willd.* (T), *Albizia lebbeck* Benth. (T), *A. myriophylla* Benth. (C), *A. odoratissima* Benth. (T), *Parkia speciosa* Hassk. (T), *Samanea saman* Merr. (T), *Xylia xylocarpa* Taub. (T)
MYRTACEAE	*Eucalyptus citriodora* Hook. (ExT), *E.globulus* Labill. (ExT), *Eugenia caryophyllus* Bullock & Harrison (ExST), *E. jambos* L. (S/ST), *E. macrocarpa* Roxb.(ST) *Maleleuca cajuputi* Powell.(T)
MYRISTICACEAE	*Horsfieldia irya* Warb. (T), *Myristica cinnamomea* King (T), *M. fragrans* L. (ExS)
NELUMBONACEAE	*Nelumbo nucifera* Gaertn. (AqH)
NYCTAGINACEAE	*Mirabilis jalapa* Linn. (ExH), *Pisonia alba gradis* R.Br.(ExST)
NYMPHAEACEAE	*Nymphaea nouchali* Burm. (AqH), *N. stellata* Willd. (AqH)
OLEACEAE	*Jasminum adenophyllum* Wall. (C), *J. auriculatum* Vahl (ExC), *J. grandiflorum* L. (ExC), *J. magniferum or J. nitidum* (C), *J. multiflorum* Burman f. Andrews (C), *J. nervosum* Lour. (C), *J. pubescens* Willd.(C) *J.* rex Craib.(C), *J. sambac* Ait. (ExC), *J. undulatum* Ker-Gawl.(C), *Osmanthus fragrans* Lour (ExS),

(Table 1. Contnd)

FAMILY	SCIENTIFIC NAMES
ORCHIDACEAE	*Aerides crassifolia* Par. & Reichb. f. (EO), *A. odorata* Lour. (EO), *A. spp., Cattleya Queen Sirikhit, Dendrobium cariniferum* Reichb. f. (EO), *D. lindleyi* Steud. *longicornu* Lindl. (EO), *D. ochreatum* Lindl. (EO) *D. scabrilingue* Lindl. (EO), *D. spp., Vanda denisoniana var. hebraica* Reichb. f. (EO), *Eria ornata* Lindl. (EO), *Vanilla fragrans or V. planifolia* Andr. (ExO)
PALMAE	*Areca catechu* L. (P), *A. triandra* Roxb.(P), *Borassus flabellifer* L.(ExP), *Eleiodoxa conferta* Bur. (P)
PANDANACEAE	*Pandanus amaryllifolius* Roxb. (ExS), *P. odoratissimus* L. f. (ST), *P. tectorius* Bl. (S/ST)
PAPILIONACEAE	*Dalbergia cochinchinensis* Pierre (T), *Parkinsonia aculeata* Linn.(ExS), *Pterocarpus indicus* Willd. (T), *P. macrocarpus* Kurz. (T), *Sesbania grandiflora* Desv. (ExST)
PASSIFLORACEAE	*Passiflora edulis* Sims. (ExC), *P. laurifolia* Linn. (ExC), *P. x. alatocaerulea* Lindleyg (ExC)
PEDALIACEAE	*Sesamum indicum* L. (ExH)
PERIPLOCACEAE	*Myriopteron extensum* Schum. (C)
PINACEAE	*Pinus kesiya* Royle ex Gordon (T), *P. merkusii* Jungh. & de Vriese (T)
PIPERACEAE	*Piper betel* L. (C), *P. chaba* Hunt. (C) *P. nigrum* L. (ExC), *P. sarmentosum* Roxb. (CrH)
POTALIACEAE	*Fagraea acuminatissima* Merr. (E), *F. fragrans* Roxb. (T)
RANUNCULACEAE	*Clematis paniculata* Thunb. (ExC), *C. smilacifolia* Wall. (C)
ROSACEAE	*Rosa chinensis Jacq. var* minima Voss (ExS), *R. chinensis Jacq. var. semperflorens* Koehne (ExS), *R. damascena* Mill. (ExS), *R. odorata Sweet var. gigantea* Rehd. & Wils. (C)
RUBIACEAE	*Coffea arabica* L. (ExS/ST), *Gardenia coronaria* Ham. (S/ST), *G. collinsae* Craib (ST), *G. hygrophila* Kurz (S), *G. jasminoides* Ellis. (ExS), *Hymenodictyon excelsum* Wall. (T), *Ixora finlaysoniana* Wall. (ST), *Mitragyna brunonis* Craib (T), *Randia siamensis* Craib (C), *Tarenna fragrans* K & V. (T), *Tarmarix stellulata* Ridl. (S), *Uncaria roxburghiana* Korth. (C)
RUTACEAE	*Aegle marmelos Corr.* (T), *Citrus aurantifolia* Swing. (ExST), *C. aurantium* L. (ExST), *C. hystrix* DC. (ST), *C. maxima* Merr. (ExST), *C. medica* Linn. (ExS/ST), *C. microcarpa* Bunge (ExS), *C. limon* Burm. f. (ExST), *C. reticulata* Blanco. (ExST), *C. sinensis* Osb. (ExST), *Euodia roxburghiana* Benth. (ST), *Hesperethusa crenulata* Roem. (ST), *Merope angulata* Swingle (ScanS), *Micromelum falcatum* Tanaka (S/ST), *Murraya paniculata* Jack. (S/ST), etc.
SAPINDACEAE	*Lepisanthes rubiginosa* Leenh (ST)
SAPOTACEAE	*Manilkara hexandra* Dubard. (T), *Mimusops elengi* L. (T)
SCROPHULARIACEAE	*Angelonia salicariifolia* Humb. & Bonpl. (ExH), *Digitalis salicariifolia* (ExH)
SOLANACEAE	*Brunfelsia americana* L. (ExS), *B. hopeana* Benth. (ExS), *Capsicum amuum* L. (ExUS), *C. frutescens* L. (ExUS), *Cestrum aurantiacum* Lindl. (ExS), *C. nocturnum* L. (ExS), *Nicotiana tabacum* Linn. (ExH), *Petunia hybrida* Hort.(ExH)
STERCULIACEAE	*Mansonia gagei* Drumm. (T)
STYRACACEAE	*Styrax benzoin* Dry. (T)
SYMPLOCACEAE	*Symplocos racemosa* Roxb. (ST)
THUNBERGIACEAE	*Thunbergia fragrans* Roxb. var. vestita Nees (C)
THEACEAE	*Anneslea fragrans* Wall. (ST)

FAMILY	SCIENTIFIC NAMES
TILIACEAE	*Schoutenia glomerata* King.*subsp. peregrina* Roekm. (T)
VERBENACEAE	*Citharexylum arbor-tristis* Linn. (ExS/ST), *C. spinosum* Linn.(ExS)
VIOLACEAE	*Viola odorata* L. (ExH)
ZINGIBERACEAE	*Alpinia conchigera* Griff. (H), *A. galanga* Sw. (H), *A. mutica* Roxb. (H), *A. oxymitra* Schum. (H), *Amomum krervanh* Pierre (H), *A. xanthioides* Wall. (H), *A. uliginosum* Koen. (H), *Boesenbergia pandurata* Holtt. (H), *Curcuma longa* L. (H), *Elettaria cardamomum* Maton (ExH), *Hedychium longicornutum* Bak. (EH), *Kaemferia galanga* L. (H), *Zingiber cassumunar* Roxb. (H), *Z. officinale* Roscoe (H), *Z. zerumbet* Smith (H)

Table 2. Diversity of growth habits of plants bearing essential oils shown in Table 1

Abbreviation	Type of Growth Habits	Number of Species	Abbreviation	Type of Growth Habits	Number of Species	Abbreviation	Type of Growth Habits	Number of Species
AqH	Aquatic Herb	3	ExG	Exotic Grass	2	G	Grass	2
C	Climber	34	ExH	Exotic Herb	21	H	Herb	19
Cr	Creeper		ExO	Exotic Orchid	1	P	Palm	3
CrH	Creeping Herb	1	ExP	Exotic Palm	1	S	Shrub	8
CrS	Creeping Shrub	1	ExS	Exotic Shrub	25	S/ST	Shrub/ Shrubby Tree	13
E	Epiphyte	1	ExS/ST	Exotic Shrub/ Shrubby Tree	5	ST	Shrubby Tree	17
EH	Epiphyte Herb	1	ExST	Exotic Shrubby Tree	19	T	Tree	62
EO	Epiphytic Orchid	8	ExT	Exotic Tree	13	US	Under - Shrub	1
ExC	Exotic Climber	14	ExUS	Exotic Under Shrub	5			

Production and Processing of Essential Oils

PRODUCTION OF ESSENTIAL OILS

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INTRODUCTION

Essential oils are mixtures of volatile compounds isolated from plant- and animal materials.
It as been published that about 350,000 different plant species should exist (1), and from these plant species approximately 60,000 should be medicinal plants and about 17,500 (5%) should be aromatic plants (2). The practical use of medicinal plants is estimated at a number of 10,000.

About 300 different plant species are used for the production of essential oils for the food, flavour and fragrance industry. The annual world production of volatile oils is estimated at around 50,000 tons (3). This production, however, could be up to 100,000 tons with a value of about 1 bilion $, based on the figures of the world consumption (2, 3).

More than 50% of the quantity of all the essential oils are citrus and mint oils. Especially sweet orange oil is produced in thousands of tons. Apart from the production of volatile natural oils 250,000 - 300,000 tons of turpentine is produced, from which about 100,000 tons are used for the productions of terpenoids for the flavour and fragrance industry.

Excellent reviews about the production of essential oils have been written by Meyer-Warnod, (4), Arnaudo (5) and Lawrence (6). Flores and Segredo (7) published on the citrus oil recovery during juice extraction. Boucard and Serth (10) wrote about a continuous steam stripping process for the distillation of essential oils (Figure 4). Boelens et al. (11) published about ten years of hydrodiffusion of oils. Moyler (14) reviewed thoroughly ten years of carbondioxide extracted oils.

With respect to the production of essential oils the following subjects will be treated: the preparation of the plant material, the isolation methods, yield and economics, and quality control.

PREPARATION OF THE PLANT MATERIAL

General

Various pre-preparations of the plant materials are often necessary before the essential oils can be isolated. Sometimes the desired volatile products are even not present as such in the fresh material, therefore hydrolysis (mosses) or fermentation (orris root) is necessary.

Harvesting

All plant material must be harvested before the volatiles can be isolated. The harvesting may simple concern picking of fruits or flowers, or cutting branches of the trees. In practice, however, modern apparatus have been developed, as for example for the cutting of the flowertops of lavandula species.

Threshing

It will be clear that seeds and fruits must be threshed before the oil can be isolated.
Special apparatus have been developed for the threshing of for instance umbelliferous fruits, such as anisseed (see grinding).

Drying

Often the plant material is dried before the volatiles are produced. Some herbs, as for example mint plants, are only partly dried before production of the oil. Others, as for instances spices, are more thoroughly dried as a type of conservation before the oils are isolated.

Grinding

Some fruits and seeds are sometimes ground before isolation of the volatiles as for instance with some umbelliferous fruits. Often the yields of the oil increase after grinding of the fruits, especially with wet grinding directly into the apparatus.

Chipping

Woody plant material, such as cedarwood, sandalwood etc., are chipped before steam-distillation. The chipping of cedarwood has been described in detail by Boucard and Serth (10) (Figure 4).

Hydrolysis

In some plant material the volatiles are not present as such but are formed after hydrolysis of less - or non-volatile compounds. An example of these materials are oak - and treemoss, which contain non-volatile depsides. These depsides are polyfunctional dimeric benzene-derivatives, which hydrolyse into monomers after treatment with hot water or steam, or in an enzymatic process.

Fermentation

Some volatiles, as for instance the irones from orris root, are only obtained after fermentation of the dried roots. During this fermentation process, hydrolysis and oxidation may occur.

ISOLATION METHODS

General

The methods for the production of essential oils have been modernized during the last 15 years. Most modern continuous distillations and extractions have been introduced during the last decenium. The following isolation methods will be treated in more detail: expression of citrus fruits, steam-distillation of labiate oils, hydrodistillation of flower oils, hydrodiffusion of leaf oils, solvent extraction of mosses, and supercitrical carbondioxide extraction of flower concretes.

Expression

For the expression of citrus fruits there are mainly four methods in use, the Italian pellatrice and sfumatrice method, the American Brown oil extraction and the FMC Corporation process. The methods will be discussed more in detail. One should notice that expressed oil always contain non-volatile residu, which can vary in concentration in the oil from 2 to 7%.

PELLATRICE METHOD

The pellatrice expression of citrus fruits concerns the abrasion of the surface of entire fruits.
The fruits are rotating against an abrasive surface of a moving Archimedes' screw, covered with abrasive parts. During this movement the oilcells burst and the oil is released with a water spray. An oil-water emulsion is obtained and the oil is isolated by centrifugal separators. The advantages of the method are: good yield and quality with more oxygen compounds. Some drawbacks may be a darker oil and slightly more residu.

SFUMATRICE METHOD

Before treatment the peel and pulp are separated from the fruits, and the peel hardened in a lime bath. With the sfumatrice method the oil is isolated from the peel by a ribbed roller pressing and a water spray (8). The oil-water emulsion is centrifuged. Advantages of the method are: the separated pulp, lighter product and less residu. Drawbacks are the peeling and lime treatment, and no optimal yield.

BROWN OIL EXTRACTOR

In the Brown method the whole fruit is used. The fruits move on a bed of rolls covered with needles, and a water spray removes the oil-water emulsion. There are drying rolls and a solid eliminator for the solid materials. The oil is centrifuged. Advantages are the low solid content and water recycle, a drawback is the waste of juice (8).

FMC CORPORATION METHOD

The most ingenious apparatus for the expression of citrus fruits is the FMC apparatus (see: Figure 1). More than 50% of the quantities of citrus oils are isolated by this method. The method is based on the whole fruit extraction principle. The recovery of the oil occurs during juice extraction (7). During the extraction cycle the components of the apparatus interact to separate the various parts of the fruit instantaneously. The citrus oil glands burst and release their oil when the peel is deflected by the pressure created between the cup fingers during the extraction cycle. Recycle water is introduced during extraction, through a special ring located at the upper cup, to capture the oil. The oils is finally isolated by centrifuging. Advantages of the method are: fully automatic, minimum labour costs, juice and oil production. Minor drawbacks are: grading of the fruits, high capital costs (leasing is possible), yields up to 85% of the oil content.

Distillation

Another important method for the isolation is the distillation of essential oils. One can distinguish batch or continuous steam-distillation (Figure 2), hydro-distillation and hydrodiffusion.
A modern approach to essential oils distillation from the herb has been described by Denny (9). The different distillation methods will be discussed below.

STEAM-DISTILLATION

Steam-distillation is featured by the fact that the plant material is extracted by direct steam (produced in the still) or by indirect steam. The still often has a grill at the bottom and the plant material sometimes is in a perforated basket. Steam-distillation is used for the production of labiate leaf and flower oils, laurel leaf oil, eucalyptus leaf, bitter orange leaf oil and umbelliferous fruit oils etc. For yields of the steam-distillation of umbelliferous fruit oils see Table 3. The bulk of the essential oils is, apart from the expression of citrus oils, still manufactured by steam-distillation.

HYDRODISTILLATION

Hydrodistillation are mostly carried out with flowers, e.g. bitter orange flower, rose or jasmine. The flowers are in a perforated basket and they are heated in 2 - 3 times their weight of water with indirect steam (from outside the still). A volume of water equal to the weight of the flowers is distilled. Yield of the separated oils is in general below 0.1% and the distillate water is saturated with more soluble oxygen-derivatives (see Analysis).

HYDRODIFFUSION

Hydrodiffusion is carried out with low pressure steam (< 0.1 bar) replacing the volatiles from the intact (uncomminuted) plant material by osmotic action. In the hydrodiffusor the low-pressure steam flow goes, according to the law of gravity, from the top through the vegetable load down to the condenser at the bottom. The isolation sequence of the volatile components is determined to a great extent by their water solubilities. As a consequence, the condensate water is more or less saturated with the polar constituents of the oil (11). Test results of a hydrodiffusor are shown in Table 1.

Extraction

A third method for the isolation of essential oils is the extraction of plant material, which for instance can be solvent extraction, subcritical liquid carbopndioxide and supercritical fluid carbon dioxide extraction. One has to keep in mind that with every type of extraction a certain amount of non-volatile compounds will be extracted.

SOLVENT EXTRACTION

Solvent extraction can be carried out in two types, namely by percolation and by immersion. By percolation runs the solvent through the raw material, and by immersion covers the solvent completely the plant material and moves the solvent from the bottom to the top. A wide range of solvents are in use, such as alkanes,

Table 1. Test results of Schmid Hydrodiffusor LS 500 compared with hydrodistillation
(according to Schmid Hydrodiffusion SA, Switzerland, May 1981)

Product (Origin)	Hydrodiffusion		Hydrodistillation	
	Time (hr)	Yield (%)	Time (hr)	Yield (%)
Cistus leaves (France)	8	0.13	16	0.04
Cistus leaves (Spain)	8	0.15	16	0.05
Lavender (France)	0.5	0.73	1	0.75
Lavandin (France)	0.5	1.7	1	1.4
Cumin fruits (Poland)	4	5.0	12	3.7
Caraway fruits (Poland)	4	3.6	10	4.5

haloalkanes, benzenoids, ethers, ketones etc. The most usual solvent is hexane.

Yields of the extraction of oakmoss lichen with various solvents and transesterification of the depsides is shown in Table 2.

Table 2. Yields of extraction and transesterification of oakmoss lichen

Solvent	Method	Yield (%)
hexane	extraction	2
dichloromethane	extraction	5
benzene	hydrolysis/extraction	7
acetone	hydrolysis/extraction	10
methanol	alcoholysis/extraction	15
benzene/methanol	transesterification	10

SUBCRITICAL LIQUID CARBONDIOXIDE EXTRACTION

The subcritical liquid carbondioxide extraction is carried out at 50 to 80 bar and a temperature of 0 to 10° C. Moyler (14) has published in detail about this extraction.
The most practical extraction of this type is with hop cones (fruit cones of *Humulus lupulus* L.). The yield of a steamdistilled oil of hop cones is about 0.5%, whereas the yield of the carbondioxide extraction is ca 12%, due to the fact that nonvolatile polyfunctional diterpenes (humulones) are soluble in carbondioxide.
About fifty essential oils obtained by liquid carbondioxide extraction are commercially available.

SUPERCRITICAL FLUID CARBONDIOXIDE EXTRACTION

The supercritical fluid carbondioxide extraction is carried out at pressures over 80 bar and in general with temperatures above roomtemperature (14). The supercritical fluid extraction (SFE) comes more and more into practice during the last five years. One can extract rather fresh plant material (labiates) with this method. One may also make first a solvent extraction (e.g. hexane) to prepare a concrete, and subsequently cary out a SFE-extraction, as for instance with flower concretes. Some analyses of examples of bitter orange flower concrete and rose concrete are shown in Table 4. and 5.

YIELDS AND ECONOMICS

General

Yields and economics are imported for succesful production of an essential oil.

Yields

One can sometimes find quite a range of yields for one and the same essential oil. This range may be due to several explainable reasons, as there are climate, soil, isolation method etc. More often, however, published yields are too optimistic and not reproducible.

Table 3. demonstrates the variation in the yields of umbelliferous fruit oils as collected by Moyler (14). In general the yield of carbondioxide and of ethanol extractions are higher than those of steam-distillation, these differences are mainly due to the fact that the extracts contain non-volatile residu.

Table 3. Yields (%) of umbelliferous fruit oils

	steam-distillation	liquid CO_2 -extraction	fluid	ethanol extract
Angelica	0.3-0.8	3	-	-
Anis	2.1-2.8	-	7	15
Caraway	3 - 6	3.7	-	20
Carrot	0.2-0.5	1.8	3.3	3.3?
Celery	2.5-3.0	3	-	13
Coriander	0.5-1.0	1.5	-	-
Cumin	2.3-3.6	4.5	-	12
Fennel	2.5-3.5	5.8	-	15
Parsley	2.0-3.5	3.6	-	20

Economics

The production costs of an essential oil may concern: the raw material costs, capital costs, labour costs, and energy costs. The raw material costs comprise the plant material, solvents etc. The capital costs are the investments (leasing), depreciation and interest. Labour costs concern working, maintenance and quality controll costs.

Because many aromatic plants grow in developing countries and they are used as raw materials in industrialized countries there is often a controverse between financial (capital/profit) economy and social (work) economy (6).

Raw material costs may be relatively high with flower oils (also working hours). Certain apparatus (CO_2-extraction, FMC-apparatus) have high capital costs. Steam - and hydro-distillation have increase energy costs. CO_2-extraction and hydrodiffusion need more labour costs.

QUALITY CONTROL

General

The quality control of essential oils may concern the physicochemical standards, the chromatographic and spectroscopic analysis, the sensory analysis, and the quality assessment in essential oil studies.

Physicochemical Standards

The physicochemical standards can comprise acid -, alcohol -, carbonyl -, and ester number; specific gravity, optical rotation, refractive index, freezing/concealing and flash point, moisture content and the evaporation residu. A lot is known about the physicochemical

properties of essential oils. One can find physicochemical standards in publications of the International Organization for Standardization (ISO), Essential Oil Association of the United States (EOA), in the Food Chemicals Codex (1996-IV), Monographs of the Research Institute Fragrance Materials (RIFM), the Pharmacopoeias (EP, BP, USP, DAB, etc.) and in the published country standards (AFNOR, DIN etc.).

Analysis

Analyses of essential oils are carried out using the most modern gaschromatographic and spectroscopic techniques. For gas-chromatographic analyses high resolution, high precision fused silica capillary columns are in use. Some examples are given in Table 4, 5 and 6. with the headspace and oil analyses of rose flowers and of bitter orange flowers.

Table 4. Headspace analysis of Rosa damascena variation in composition (%) after picking

Compounds	Living	Picked
Monoterpene hydrocarbons	28	57
2-Phenylethanol	40	2
Citronellol	8	2
Geraniol	2.5	+
Nerol	2.5	+
Phenylethyl acetate	3	1
Eugenol	+	2
Methyleugenol	0.5	2
cis-Rose oxide	+	1
trans-Rose oxide	+	0.5

These analyses were carried out in the rose fields near Isparta in central Southern Turkey by Robin Clery, Quest International, Ashford, Kent, UK.

Table 5. Chemical composition (%) of rose oils

Compounds	hydrodistilled	carbondioxide extract
Citronellol	30	8
Geraniol	18	4
Nerol	9	2
2-Phenylethanol	2	67
Rose oxides	0.5	0.15
Methyl eugenol	2	0.7
beta-Damascenone	0.015	<0.005

From these analyses it is clear that a significant variation exist in the concentrations of the main constituents or rose headspaces and oils. Especially the concentration of 2-phenylethanol can vary from 2 - 67 %.

Quality assessment in essential oil studies

The quality in essential oil studies has been discussed (16). It was stated that the identification of a component of an essential oil

Table 6. Chemical composition (%) of bitter orange flower oils

Compounds	hydrodistilled	carbondioxide extract
Monoterpene hydrocarbons	38	28
Linalyl acetate	4	24
Linalool	38	35
Nitrogen compounds	0.5	2
Sesquiterpene alcohols	4	2

with a GC-MS instrument with an automatic library was not a rigorous scientific exercise. And that compounds identified with a mass spectrum of that of a closest match with a library should be indicated as a tentative identification. Moreover GC-MS identifications should be supported by retention indices on two columns, and that the accuracy of quantitative analyses of essential oils is only reproducible to one place of decimals.

There are several reasons for deviations in essential oil composition, such as decomposition during isolation (terpenes from linalyl acetate), oxidation during aging (formation of linalool oxides and caryophyllene oxide), adulteration with synthetics (d/l-linalool, dihydrolinalool, dehydrolinalool, plinols), and mis-interpretation of analytical data (isoborneol in stead of borneol).

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ACKNOWLEGDEMENTS

The author is greatly indebted to Arantxa Bordas of Destilaciones Bordas Chinchurreta, Jean Francois Arnaudo of Biolandes Technologies, Robin Clery of Quest International, Jose Flores of FMC Corporation, Piet Traas and Pieter de Valois of Quest International for their valuable information, photographs, slides, sensory and GC-MS analyses.

Figure 1. Citrus Oil Recovery During Juice Extraction *(Ref. 7)*

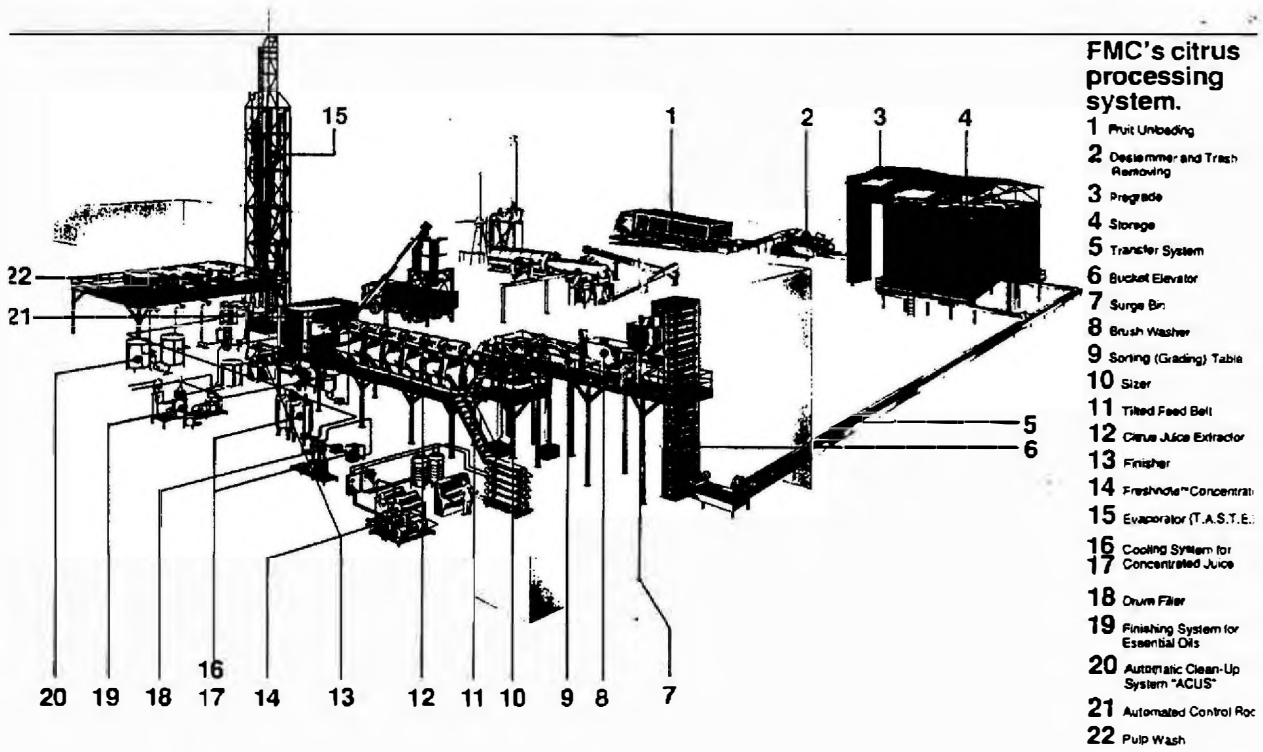


Figure 2. Scheme of Continuous Distillation *(Ref. 5)*

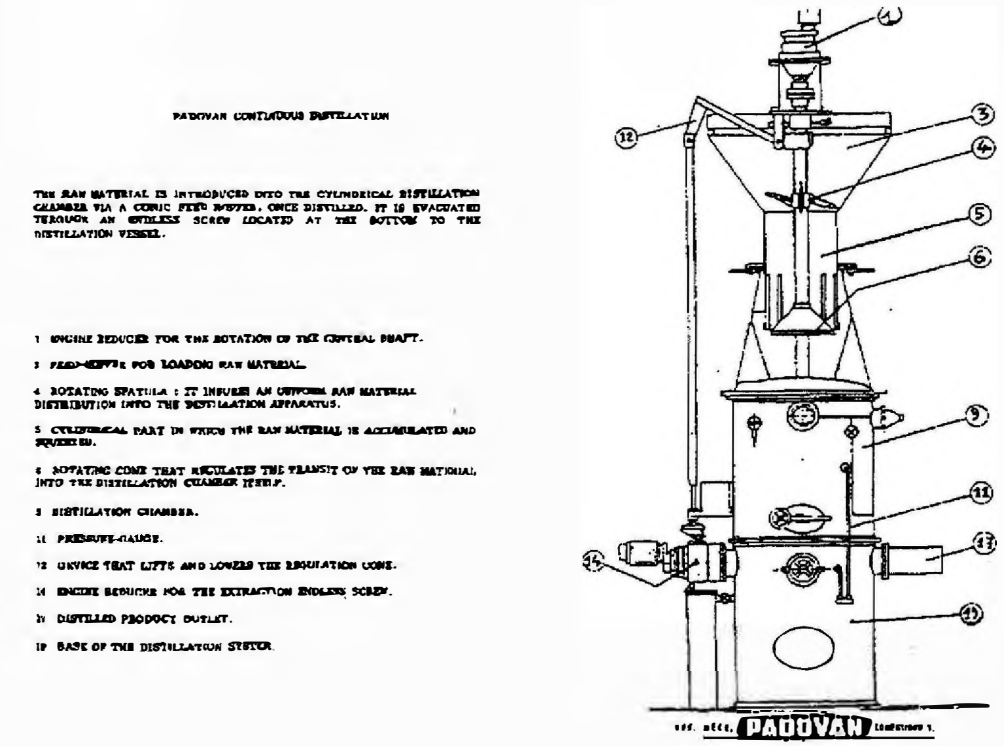


Figure 3. Scheme of a Modernized Rose Oil Production Equipment *(Ref. 5)*

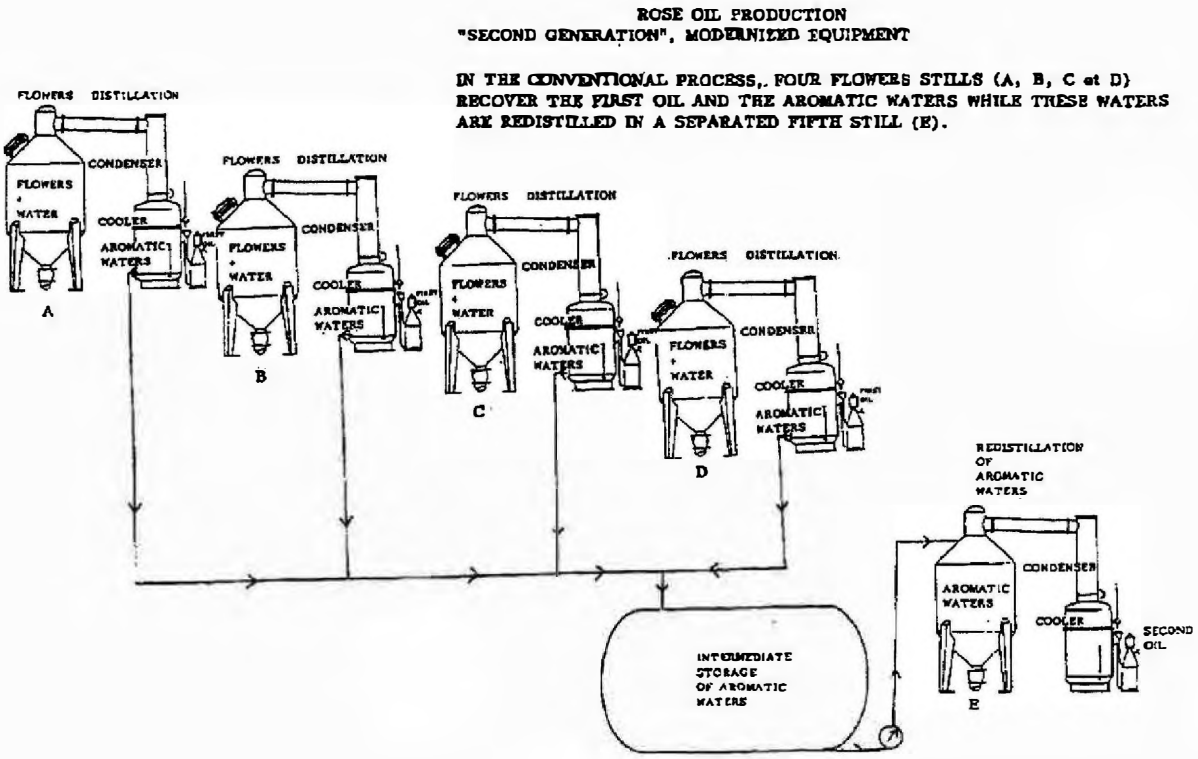


Figure 4. A Continuous Steam Stripping Process for the Distillation of Essential Oil *(Ref. 10)*

TEXAPOME'S
CONTINUOUS CEDARWOOD OIL DISTILLATION PLANT

THE PRIMARY INNOVATION IS THAT,

FROM THE HAMMERMILL (2) TILL THE BOILER FURNACE (15),

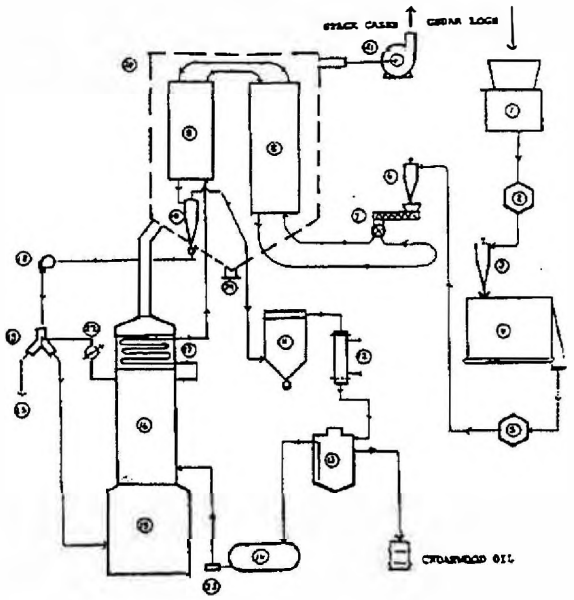
AND THROUGH THE PULVERIZER (5) THE GAS-SOLID CONTACTORS (8), (9),

THE RAW MATERIAL (CEDARWOOD) IS PNEUMATICALLY CONVEYED.

THIS PNEUMATIC CONVEYING RESULTS INTO AN INTIMATE CONTACT BETWEEN THE GAS (SUPERHEATED STEAM) AND THE SOLIDS (CEDARWOOD) IN THE DISTILLATION CHAMBER (20) WHERE ARE LOCATED THE GAS-SOLID CONTACTORS (8) AND (9).

THE OTHER INNOVATION IS THE USE OF THE SPENT CEDARWOOD RESIDUES AS FUEL IN A SUSPENSION-TYPE FURNACE (16), (18), (17).

1.- Wood chipper
2.- Second stage hammermill
3.- Air cyclone
4.- Storage bin
5.- Third stage pulverizer
6.- Feeder cyclone
7.- Plug feeder
8.- First stage counter-current contactor
9.- Second stage counter-current contactor
10.- Discharge cyclone
11.- Steam/oil filter
12.- shell and tube condenser
13.- Oil decanter
14.- Boiler feedwater tank
15.- Boiler furnace
16.- Firetube boiler
17.- Steam superheater
18.- Fuel blower
19.- Automatic stream divider
20.- Insulated plenum
21.- Boiler induced draft fan
22.- Pressure regulated fuel controler
23.- Boiler feedwater pump
24.- Ash discharge
25.- Incinerator (Not shown)



DESIGN AND CONSTRUCTION OF FIELD DISTILLATION EQUIPMENT IN EL SALVADOR

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CONTEXT

For many developing countries, regional and international trade in non-traditional, but high value added agricultural products, like essential oils, are an important source of foreign exchange.

For local consumption in the Primary Health Care System essential oil plants are normally powdered and sold in tea bags, tablets or capsules. The hygroscopic powder attracts the humidity to form patches of mould and fungi in the respective dosage form with adverse health effects.

However, the lack of technical know-how often means to many small and medium - sized enterprises that controlled wild collection and cultivation of herbal raw material cannot be followed by the subsequent on-site processing of essential oils to achieve the desired value added in the country of origin itself.

Resulting from a different level of productivity development the controlled transfer of distillation technology has put the local production of technology at a disadvantage. It has consolidated the traditional pattern of specialisation, value addition and trade.

The newly emerging local and regional markets for genuine and authentic essential oils in regional cosmetics, pharmaceuticals and aromatherapy are providing incentives to local NGOs and entrepreneurs.

THE GTZ-PROJECT

Since 1994 the GTZ-project of "Promotion of Small- and Medium Scale Industries" implements activities of trade and trade fair promotion for the Natural Products Industry within the " Integration- and Employment Programme". The objectives include technical assistance to the existing private sector as well as the creation of rural processing units for high value added natural products within the national program of integration and employment. The technical assistance aims at an improved level of information and technology for production, processing and marketing available to the productive sector in El Salvador. At present the project is organizing the essential oils producers within the Association of non-traditional Natural Products Industries.

THE NATURAL PRODUCTS INDUSTRIES IN EL SALVADOR

The following 3 examples illustrate facets of availability of information and technology and the consequences to export oriented Natural Products Industries in this small country of Central America:

Nobs Hydrodiffusion, a private sector company in San Salvador, has a well established production of Vetiver oil employing the system of Hydrodiffusion. The units had been constructed in Switzerland, Brasil and El Salvador. Experiments had been carried out on trial cultivation and processing for more than 15 local and exotic plant species during the years of the civil war. Today Nobs Hydrodiffusion has more than 200 ha of Vetiver under cultivation and is building its sales activities in USA and Europe. The company is providing advisory service to other small scale distillers as local consultants.

Traditional and herbal cosmetics are produced by Shuchil using standardized essential oils from abroad. Shuchils own distillation units had been destroyed during the civil war. Shuchil new regional and international markets are rewarding genuine products from El Salvador. Joint projects with groups of indigenous indians and with ex-guerillas allow Shuchil direct access to ingredients and their history of use. Explaining the context of tradtional knowledge and guerilla experience in product formulation and application provides the present transparency of Shuchil products as marketing arguments.

Little attention has been paid to the proper processing of Peru Balsam resulting in quality and price decline and finally in claims of allergic properties of the product. Local products from

genuine and authentic sources of Peru Balsam are not known for their allergic effects.

LOCAL NEEDS OF THE POST CIVIL WAR PERIOD

During the past 10 years APROCSAL has dedicated its services in the Primary Health Care to communities in conflictive zones. The use of local medicinal and aromatic plants provides inputs for the local systems of traditional medicine. The rural extension programme includes applied research, training in production and processing of medicinal and aromatic plants, decentralized small scale production of simple dosage forms and their use in the rural health posts. APROCSAL is interested in the income generating effects and the medical use of essential oils in the rural areas. Cultivating and processing a wide range of essential oil plants in different parts of El Salvador requires adapted technology for decentralized distillation units. The sales of essential oils and the hydrolates in the urban areas provide additional sources of income and recognition.

The FUSAL Foundation in collaboration with a cooperative in the Island El Jobal is utilizing the abandoned citrus plantations for the future distillation of neroli oils and cold pressed citrus oil. FUSAL is utilizing the employment and the income generating effects for its Children Day Care Centre in El Jobal. The essential oils shall be utilized in the FUSAL rural Primary Health Care Service in other parts of the country.

Lippia graveolens, the oregano of the eroded north-eastern highlands, is a potential high value added product for rural communities in that part of El Salvador. The Non-Government Organization (NGO) FE Y TRABAJO is assisting farmers in Corinto/Perquin to organize the controlled wild collection and processing. Several NGOs are assisting to market oregano as a spice and essential oil.

THE CONSTRUCTION OF THE DISTILLATION EQUIPMENT

Earlier publications on distillation technology from the Government of Québec (1977) and Denny (1987) had not included any measured designs. Munoz (1987) presented in his book "Plantas Medicinales y Aromaticas" for the first time designs with measurements for selected equipment.

The construction of distillation equipment in El Salvador uses an open access to technology options and information. The Protrade manufacturing and plant construction handbook "The Distillation of Essential Oils" (1993) has been well received by the productive sector in El Salvador.

Local workshops had been capable to construct distillation units according to the specific needs of the different groups as portable units in mild steel or permanent units in stainless steel.

All 7 locally constructed distillation units had been financed without financial engagement of the project, which provided the technical advisory for construction and start up. International distillation experts join local advisors to adapt the units to the user and plant species characteristics.

The national counterpart personnel of the project keeps permanent contact with the production units to attend to emerging problems in an early stage.

During the last 3 years the project has created the markets and transferred the technology and know-how for the production of essential oils as high value added natural products in small- and medium scale rural industries.

Local mechanical workshops had been strengthened and suppliers for lab-equipment and glassware identified.

For the local market the application of essential oils in aromatherapy is promoted in rural and urban areas. At present regional and international markets are interested in the oregano and especially in the forthcoming production of citrus oils.

THE RESULTS AND CONSEQUENCES

The production of distillation technology had been -with few exceptions- the monopoly of developed and industrialized nations.

At the First World Congress in Medicinal and Aromatic Plants for Human Welfare - WOCMAP in Maastricht, Netherlands, in 1992, the workshop on "Industrial Aspects, Economics and Marketing" recommended that "since medicinal and aromatic plants frequently increase in value with processing, special attention should be paid to such opportunities...".

One of the final recommendations of WOCMAP reads "More financing and research should be directed towards enabling producer countries to locally process medicinal and aromatic plant material".

Within some Country Projects of Integrated Industrial Advisory Service IBD, implemented by GTZ and DEG, as part of the German Development Program the Protrade Designs have been welcomed especially because of the set of drawings accompanied by exact and praxis-oriented measurements.

Other countries in Latin America, like Ecuador, have also shown interest to control the transfer of technology and create own capacity of design and construction of distillation technology.

For the industrial development of Non-Wood Forest Products in Malaysia forestry projects utilize the Protrade designs to establish forest based industries to serve the needs of local, regional and international markets.

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SORPTION PROCESSES AT THE EXTRACTION OF LAVANDULA VERA D. C.

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It was proved that the extraction of lavender is accompanied by a sorption of aromatic substances from miscella to the stems of the raw material. It is due to the uneven distribution of the extractable substances in the different parts of the lavender racemes.

The extraction of lavender carried out by a closed periodical process should be repeated 2 or 3 times beginning with a short first extraction to cause the process to run at high rate and to reduce the influence of the sorption phenomena.

INTRODUCTION

Lavender is one of the main essential oil plants cultivated in Bulgaria. Because of that the investigation of its technological processes is of considerable importance.

The examination of the extraction dynamics of the lavender racemes established that the main quantity of the extracted substances is taken out from the plants during the first extractions. When they are of short time duration the process proceeds at higher rates and diffusion coefficients (1). Even after 24 hours the equilibrium state of the process was not reached (2). These data and the uneven distribution of the extracted substances in the different parts of the lavender (3) gave us the base to suppose that a sorption of aromatic substances proceeds simultaneously with the extraction. The presence of a sorption of aromatic substances at the extraction of rose flowers was written by Tourisheva and Shljapnikov. Tourisheva reported that the sorption leads to a substantially reduced yield of concrete (4). The sorption phenomena prevail at the combined extraction of the petals and calyces according to Shljapnikov (5).

The duration of the extraction is one of the main factors for obtaining the maximum yield of concrete. Some authors recommend a long-time duration extractions (6,7) and others — short-time duration to obtain a clear scent (4,5,8).

The present study aims to establish the availability or the absence of a sorption phenomena when lavender is extracted and a proper duration and numbers of the extractions to receive a higher concrete yield and a good quality parameters.

EXPERIMENTAL

An investigation was carried out with fresh lavender racemes of Lavandula vera — cv "Karlovo" at 70% degree of blossom and 65% moisture. The petroleum ether — a traditional raw material is used as a solvent (3,6,7). The scheme of the experiments is shown in the corresponding tables.

To determine the quality indices of the received extracts they are analysed by traditional methods.

The direct chromatography of the calyces and stems is done by a supplementary chamber (9), Fractovap gas chromatograph model C, with FID. The analysis are made by a program for lavender oil — initial evaporator temperature is 240°C, than the temperature increases with 23.5°/min to 150°C, goes on the isotherm up to the terpinen — 4-ol, than temperature is changed again with 23.5°/min up to 170°C. The column length is of 2 m, internal diameter 2 mm, fillness BDS over chromosorp W. The gascarrier rate nitrogen is 12 ml/min.

RESULTS AND DISCUSSIONS

For the production of a lavender concrete the whole blooming racemes are used. The majority of the essential oil glands are concentrated over the calyces of the blossoms. They are nearly absent on the stems.

It is established a difference in the composition of the aromatic substances in the different parts of the racemes by a direct gas chromatography. The chromatogram of the calyces is very similar to that of the lavender oil — linalyl acetate (28.7%) and linalool (26.4%) are the main components in it. These characteristic components are not found out in the chromatogram of the stems (Fig. 1).

Products from calyces and stems are characterised with different odour — the obtained from calyces reminds lavender and this from the stems is non-typical with wax and green note.

The quantity of the extractable substances in the

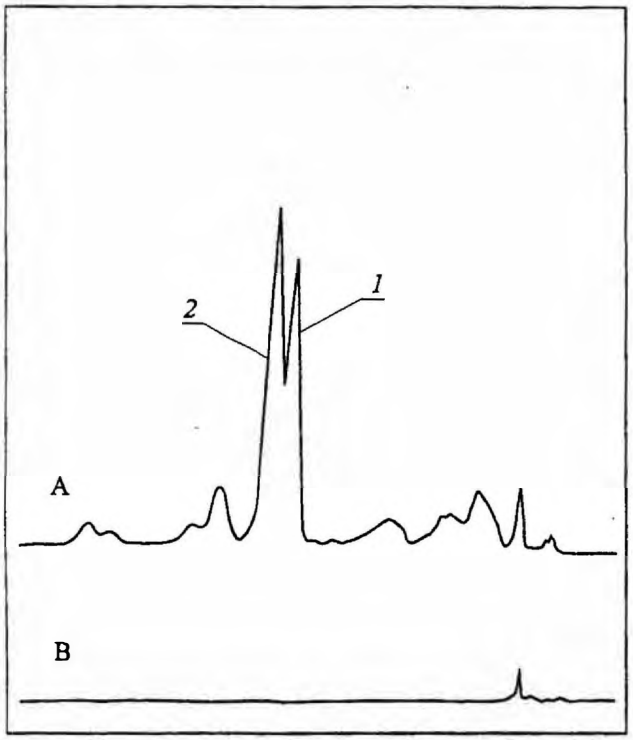

Fig 1. Chromatograms of a calyx (A) and a stem (B)
1 — linalool, *2* — linalyl acetate

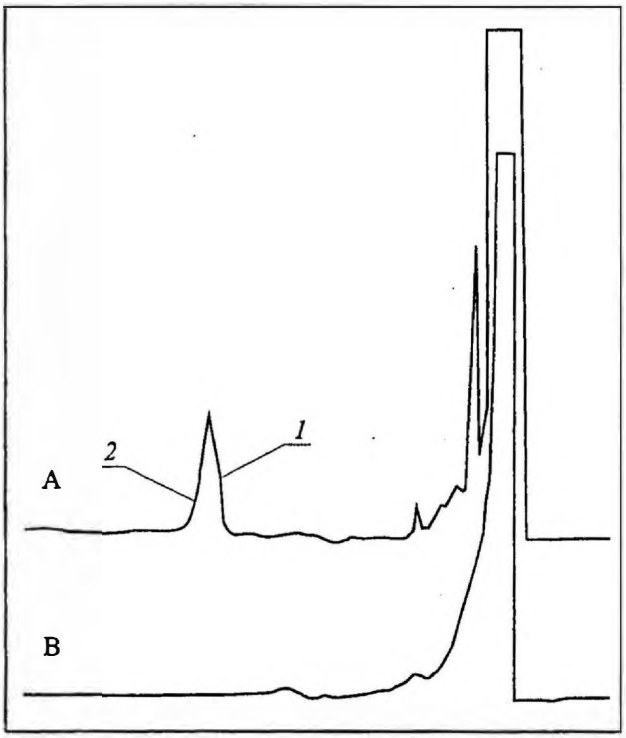

Fig 2. Chromatograms of stems extracted alone (A) and with racemes (B); *1* — linalool, *2* — linalyl acetate

parts of the racemes when they are extracted alone is 0.92% for the calyces and 0.56% for the stems (Table 1). The calculated yield of concrete on the base of these results and the correlation between calyces and stems shown in Table 1, is 0.81%. The real yield of concrete when the whole racemes are extracted is 0.59% or with 26% lower than the calculated yield.

Table 1. Yield of concrete from different parts of Lavender racemes

Part of racemes	Correlation between parts, %	Yield of concrete, %
Calyxes	69	0.92 ± 0.02
Stems	31	0.56 ± 0.07
Racemes	100	0.59 ± 0.01
Racemes (calculated)		0.81

To establish the reason for this difference between the yields of concrete stems are extracted alone and together with the racemes. The obtained results show that after 60 min contact between stems and petroleum ether, no changes appear in the chromatogram. A different chromatogram is obtained at the extraction of the stems together with the racemes for the same period. It is found out an availability of the both typical for the lavender oil components in it — linalool and linalyl acetate (Fig. 2). These results show that at once after the extraction of the aromatic substances from the calyces they are sorpt by the poor to these components stems.

Basing on these results we decided to define the duration and the number of the extractions of the lavender racemes to obtain a maximum yield of concrete by decreasing the influence of the sorption. The experiment scheme is shown in Table 2. The total duration for the variants is 110 min. The durations of the first extractions are 5, 15, 30 and 60 min. It is established that 0.17% extract is obtained when first extraction lasts 5 min. The yields of the aromatic substances for the first extractions of the other variants are 0.23% for the second, 0.26% for the third and 0.31% for the forth.

It is proved that as shorter is the first extraction as higher is the product yield of the second one at the same duration for the three variants. The total quantities of the extract for the first and second extractions for the forth variants are nearly the same, never mind the various continuance — from 65 min to 95 min. The third extractions are with continuance of 15 min to 45 min. The content of the obtained aromatic substances increases proportionally to the duration of the contact between the racemes and the solvent — from 0.05% to 0.10%. Because of the low quantity of the extract it concerns not much the total yields.

The rates of the extraction differs substantially. It is the highest for the shortest first extractions —

Table 2. The effects of the duration of the first extraction of Lavandula vera D. C. on the yield and quality indices of the concrete

Variant	Duration min	Yield of concrete, % For the plant	Yield of concrete, % To the total yield	Rate of the extraction g/gmin.10^{-5}	Quality indices Melting point	Acid number	Ester number	Linalyl acetate
	5	0.17 ± 0.040	34.7	91.9	46.5	10.4	91.4	32.0
I	60	0.22 ± 0.015	44.9	9.9	38.2	16.3	169.9	59.5
	45	0.10 ± 0.017	20.4	6.0	44.0	15.1	163.2	57.1
	110	0.49 ± 0.029	100.0		41.2	14.0	141.3	49.4
	15	0.23 ± 0.006	45.1	41.4	49.0	10.5	131.3	45.9
II	60	0.19 ± 0.020	37.3	8.5	51.0	18.0	172.2	60.3
	35	0.09 ± 0.045	17.6	6.9	48.0	18.0	152.0	53.2
	110	0.51 ± 0.031	100.0		48.6	14.1	150.2	52.6
	30	0.26 ± 0.002	52.0	23.4	46.1	12.2	140.2	49.1
III	60	0.17 ± 0.008	34.0	7.6	46.0	15.1	146.4	51.2
	20	0.07 ± 0.007	14.0	9.4	51.0	17.4	159.9	55.9
	110	0.50 ± 0.012	100.0		46.1	13.8	145.1	50.7
	60	0.31 ± 0.007	64.6	14.0	46.0	13.4	153.7	53.8
IV	35	0.12 ± 0.006	25.0	9.3	48.3	13.4	155.4	54.4
	15	0.05 ± 0.006	10.4	9.0	47.0	14.5	118.9	41.6
	110	0.48 ± 0.019	100.0		46.7	13.4	150.5	52.6

91.9×10^{-5} g/gmin for 5 min and 41.4×10^{-5} g/gmin for 15 min. The extraction proceeds at 6.5 times lower rate — 14.0×10^{-5} g/gmin — during the 60 min first extraction for the forth variant. The process rate of the second extractions is several times lower than this of the first one. The highest difference is for the first variant. The high process rate and the decreased influence of the sorption at the short time first extraction may be form the approximately equal total yield for the first and second extractions nevertheless the difference in the duration.

The quality indices of the obtained extracts show that with prolongation of the duration of the first extraction increases the quantity of the linalyl acetate as well as the acid number and the ester number. The extracts obtained within 5 to 30 min duration of the first extractions — are with the most typical lavender odour. The acid and ester number and the quantity of the linalyl acetate at the second extraction are higher than the same indices for the first extraction. There isn't clearly expressed differences in the physicochemical indices of the total yields of concretes between the four variants.

Having in mind the obtained results for the yield and quality indices, we accept that the most proper duration for the first extraction of lavender racemes is 15 min.

In order to establish the duration of the II-nd extraction an experiment was held out with its variation within 30 min to 90 min (Table 3). The concrete yield in this case increases from 0.12% for 30 min to 0.24% for 90 min. It was proved the statistical difference between 30–60 min and 30–90 min; as it could not be defined the one between 60–90 min. The received results could be concluded that at the duration of the first extraction 15 min, the second one must be 60 min.

Table 3. Influence the second extraction time over the yield of concrete of Lavandula vera D. C.

Variant	Duration min	Yield of concrete, % For the plant	Yield of concrete, % To total
	15	0.29 ± 0.010	55.8
I	30	0.12 ±0.009	23.1
	15	0.11 ± 0.016	21.1
		0.52 ± 0.043	100.0
	15	0.29 ± 0.010	48.3
II	60	0.21 ± 0.012	35.0
	15	0.10 ± 0.010	16.7
		0.60 ± 0.015	100.0
	15	0.29 ± 0.010	46.8
III	90	0.24 ± 0.010	38.7
	15	0.09 ± 0.017	14.5
		0.62 ± 0.018	100.0

During the experiments for the establishment the continuance of the III-rd extraction it was found out that with its time variation from 15 to 60 min a non-substantial increase of the extract quantity is achieved — from 0.10 to 0.14%. This difference

presents around 6.2% in respect to the total yield of the variants and could not be proved statistically. That gives us a base to recommend a short time duration of III-rd extraction.

The results of this research work show that the extraction of the lavender racemes with petroleum ether at a closed periodical process must be repeated two or three times beginning with a short-time duration of the first one to cause the process to run at high rate and to reduce the influence of the sorption phenomena.

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ON THE EXTRACTION OF *ROSA DAMASCENA* MILLER

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INTRODUCTION

Bulgaria is famous as a country of roses and rose oil all over the world. Process of rose flowers take a principle place in the essential oil industry of our country. The produced products essential oil, concrete and rose water are used to provide the requirements of home market and for export as well.

The subject of the present work is examination of the equilibrium state of the extraction of rose flowers (*Rosa damascena* Miller) and the influences of some technological factors on the yield of rose concrete.

The extraction in a closed periodical process is carried out using miscella with decreased concentration that means that fresh roses are extracted with the most concentrated miscella. A deflection of the real curves of equilibrium from the ideal ones was established when flowers of Krimska red rose were extracted. It was caused by sorption of essential oil from miscella to the calyces of the flowers.

The duration of the extraction of aromatic plants is very important for the yield and quality of concrete. It is specific for every one plant. Some authors recommended a prolonged extraction (2, 3). Others were for a shorter one (1, 4, 5, 6). The structure of the plants, the way of deposition and the composition of the extractable substances are very important for the extraction process. Rose flowers are characterized by an uneven distribution of the rose oil in their different parts (petals and calyces). The main quality of the essential oil is localized mainly in petals in the cells of their epidermis and parenchyma, while calyces contain the biggest amount of alcohol soluble substances (1, 7).

EXPERIMENTAL

The research was carried out with fresh flowers of Rosa damascena Miller. Hexane was used as a solvent.

The equilibrium state of the extraction of flowers was studied by single extractions with durations from 5 min to 24 hours. In order to determine the total amount of the extractable substances of the rose flowers (Mo) they were extracted (after the experiment) till full exhausting of this substances. The experiments were carried out three times at ambient temperature.

The parameters of the experiments for the influence of the duration and number of extractions are shown in the corresponding tables.

Influence of the use of miscella on the yield of concrete was studied by threefold extraction with duration 5–15–5 min and miscella with concentration 0.53 g/l and 0.90 g/l. Plant solvent ratio was 1:6 for the first and 1:4 for the second and third extractions.

RESULTS AND DISCUSSION

The results for the equilibrium state of the extraction of rose flowers show that the main quantity of the concrete is obtained for the first 5 min — 0.45% which is 51.7% of the yield for 24 hours and 37.5% of the total amount of extractable substances in the plant (Table 1). The yield of subsequent extract for the extractions with longer duration gradually increases but differences between them are small. For example the quantity of concrete obtained after 10 min extraction is only 0.06% higher than that obtained by 5 min extraction. The same tendency of increasing retains for all the variants independently of the greater difference in the process duration after 30 min. The extract obtained after 24 hours (0.87%) is 72.5% of the total amount of the extractable substances (Mo) of the rose flowers i.e. about a 1/4 part of them remains in the plant material without reaching the equilibrium state of the system.

The results show that the extraction of rose flowers runs at decreasing speed. It is highest for the 5 min extraction 36.0×10^{-5} g/gmin and decreases about two time (20.4×10^{-5} g/gmin) for 10 min when extraction is only 5 min longer. The speed decreasing goes on for every subsequent variant.

In order to define the duration of the first extraction basing on the obtained results we examined three variants with duration of the first extraction from 5–25 min and the same total — 45 min for all variants (Table 2). Hexane and miscella were used as solvents.

Table 1. Influence of the duration of single extraction of ROSA DAMASCENA Miller on the yield of concrete

Duration	Yield of concrete, %				Speed of process
min	To plant	Difference	To yield for 24 h	To Mo	g/gmin.10^{-5}
5	0.45 ± 0.009		51.7	37.5	36.0
10	0.51 ± 0.013	0.06	58.6	42.5	20.4
15	0.56 ± 0.037	0.05	64.4	46.7	14.9
20	0.57 ± 0.020	0.01	65.5	47.5	11.4
30	0.61 ± 0.021	0.04	70.1	50.8	8.1
60	0.66 ± 0.013	0.05	75.9	55.0	4.4
120	0.70 ± 0.038	0.04	80.4	58.3	2.3
8 h	0.82 ± 0.038	0.12	94.3	68.3	0.7
24 h	0.87 ± 0.043	0.05	100.0	72.5	0.02
Mo	1.20 ± 0.029	0.33		100.0	

Table 2. The effect of the duration of the first extraction of *Rosa damascena* Miller

			Solvent					
			Hexane		Miscella			
			The yield of concrete, %		The yield of concrete, %			
Variant	Extraction No	Duration min	To plant	To the total yield	Total	Amount of the introduced substances	Real	To the total variant
I	1	5	0.45	60.0	1.22	0.90	0.32	43.8
	2	25	0.17	22.4	0.27	0.00	0.27	37.0
	3	15	0.13	17.3	0.14	0.00	0.14	19.2
			0.75	100.0	1.63	0.90	0.73	100.0
II	1	15	0.61	67.8	1.20	0.90	0.30	44.3
	2	25	0.16	17.8	0.27	0.00	0.27	38.6
	3	5	0.13	14.4	0.12	0.00	0.12	17.1
			0.90	100.0	1.59	0.90	0.69	100.0
III	1	25	0.63	67.7	1.25	0.90	0.35	48.6
	2	15	0.16	17.2	0.25	0.00	0.25	34.7
	3	5	0.14	15.1	0.12	0.00	0.12	16.7
			0.93	100.0	1.62	0.90	0.72	100.0

The yields of concrete when hexane is used correspond to that when equilibrium state was studied — the yield gradually increases as the main quantity (0.45%) is obtained for the first 5 min extraction. The quantity of the extract for the second and the third extractions are approximately equal for all the variants — correspondingly 0.16–0.17% and 0.13–0.14% independently of the differences between the duration.

The yield for the first variant after 45 min extraction is 0.75% which is about 82% to that obtained for second and third variant which are equally the same.

Results are quite different when miscella is used for first extraction and extractable substances are introduced with solvent by this way. The yield for the first extraction are the same for the three variants— 1.20–1.25%. The real yields independently of the difference in the duration are nearly the same too, 0.31–0.35%. In comparison with the previous experiment the quantity of concrete for first variant is about 30% and for second and third variants about 50% less than when hexane is used as a solvent.

The quantities of the extract obtained for the second extraction with miscella is 0.25–0.27% for the three variants. It is higher than that for the previous experiment with more than 50%. By this way some of the loses for the first extractions are recovered. There are not considerable differences between yields for the third extractions for all the variants. They are nearly the same as for the previous experiment.

The results when miscella is used as a solvent as compared to the obtained by hexane show that the yield is nearly the same in the both cases when the duration of the first extraction is 5 min. The yields for the other two variants are about 22% lower.

Table 3. Influence of the second extraction time over the yield of concrete of *Rosa damascena* Miller

Variant	Duration	Yield of concrete, %	
	min	To plant	To total yield for variant
	5	0.69	59.0
I	15	0.34	29.0
	5	0.14	12.0
		1.17	100.0
	5	0.69	57.5
II	25	0.35	29.2
	5	0.16	13.3
		1.20	100.0
	5	0.71	60.2
III	35	0.33	28.0
	5	0.14	11.8
		1.18	100.0

Table 4. Influence of the concentration of MISCELLA on the yield of concrete

Variant	Extraction	Concentration of miscella	Amount of introduced	Yield of concrete		
				To plant	Real yield	To control
	No	g/l	concrete, g	g	g	%
	1	0.00	0.00	0.78	0.78	100.0
I	2	0.00	0.00	0.32	0.32	
	3	0.00	0.00	0.23	0.23	
			0.00	1.33	1.33	100.0
	1	0.53	0.80	1.36	0.56	71.8
II	2	0.00	0.00	0.37	0.37	
	3	0.00	0.00	0.25	0.25	
			0.80	1.98	1.18	88.7
	1	0.53	0.80	1.35	0.55	70.5
III	2	0.90	0.90	0.80	0.10	
	3	0.00	0.00	0.20	0.20	
			1.70	2.35	0.65	48.9
	1	0.90	1.35	1.52	0.17	21.8
IV	2	0.53	0.53	0.66	0.13	
	3	0.00	0.00	0.25	0.25	
			1.88	2.43	0.55	41.3

On the basis of this we conclude that the first extraction of rose flowers by close periodical process must be with minimal duration to decrease the negative effect of miscella on the yield of concrete.

In order to establish the time duration of the second extraction we carried out experiments where the first extraction lasts 5 min and the second one 15 min to 35 min (Table 3). The third extraction is 5 min for all the variants. The results confirm that the difference of 20 min do not affect the increase of the extractable substances — it is 0.33–0.35% for all the variants.

We examined the effect of the concentration of miscella on the yield of concrete by threefold extractions with duration 5–15–5 min and concentration of miscella 0.53 g/l and 0.90 g/l. The scheme of the experiment is shown in Table 4.

When miscella with concentration 0.53 g/l is used for first extraction than the real yield for the variant decreases with about 28% to the yield for the control (Variant I). If second and third extractions are done with hexane than higher yields are received for them. By this way the real yield for the second variant is only 12% lower than that for the first variant.

If second extraction is carried out also with miscella (concentration 0.9% for the experiment) real yield is not received at all (Variant 3). Some of the extractable substances introduced with the miscella are even kept by the plant material e.g. the quantity of the inserting with miscella substances for the second extraction of this variant are 0.90 g and the real yield is 0.80 g.

It is not possible to restore the losses of the first and second extractions using pure solvent for the third one. That's why the total real yield for the third variant is 48.9% to that for control.

When the concentration of miscella for the first extraction is higher (0.90 g/l) and the quantity of the inserting substances with miscella are 1.35 g than the real yield is smaller — 0.17 g. This is 21.8% to that obtained for the first variant and 30.9% to the obtained for the third variant. The inserting of the extractable substances with miscella for the second extraction irrespective of its lower concentration effect negatively on the extraction of concrete. The real yield for it is only 0.13 g. The yield for the third extraction with hexane is higher than that obtained for the first two extractions 0.25%. The total yield is 0.55 g which is 41.3% to that obtained for the first variant when pure solvent is used for the threefold extraction.

The results of this experiment show that the use of miscella for the extraction of rose flowers effect considerable on the extent of the derivation of the extractable substances from the plant. This is especially clear expressed when miscella with higher concentration for the two extractions is used.

The decrease of the yield of concrete in this case is due to the lowering of the concentration gradient as well as to the sorption of the extractable substances from miscella to the calyces of the flowers probably. This is caused by the uneven contribution of the extractable substances in the different parts of the rose flower.

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PIPER BETLE LEAF - OIL : FACTORS AFFECTING PRODUCTION AND COMPOSITION

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INTRODUCTION

The wonder trailing and climbing herb Betel, presents itself as an important cash crop. Extensive use in day today life is for chewing its leaves with some ingredients. The use of Betel leaves is a part of convention, hospitality, a habit and an innocent after-meal breath-sweetening practice; involving over one-fourth of human race of Asia, Africa and Gulf countries. Betel leaf is rich in eugenol (a high-valued perfumery and medicinal aroma), methyl chavicol etc. and also in protein and mineral matters. It is rich in vitamins and aminoacids.

The value of Betel leaf and its oil in traditional medicines has long been recognised. It is releiver of thirst, cerebral congestion and a stimulant. Besides Betel - leaf extracts can be used in treatment of respiratory catarrh and as an antiseptic. The oil has marked activity against Gram-positive and Gramnegative bacteria.

The chemical composition of essential oils from Betel leaves have been reported by Guenther (]952), Nigam *et al.* (1962) and Sharma *et al.* (1982). Chatterjee *et al.* (unpublished) made detailed studies on formation of the oil in the plants and concluded that its optimum content is dependent on position of leaf on the plant, its maturity and on different agrotechnological practices. There exists a marked difference in the content of oil amongst different varieties but common characteristic of most Betel leaf oils is the presence of compounds like chavicol, chavi-betel, chavibetol, cadinene and allyl pyrocatechol. High eugenol content in some of the varieties of Betel, particularly growing in Western India offer chemotaxonomically interesting areas of studies.

EXPERIMENTAL STUDIES & DISCUSSION

I. Agronomical factors for improved cultivation of Betel vine

Although the crop favours tropical humid conditions, it can also be grown in other places where sufficient irrigation facilities are available and a cool, humid, steady atmosphere can be artificially created.

Temperature

Betel-vine is very sensitive to temperature; below 10 °C, it causes winter-injury and above 40 °C, it causes heat injury. Favourable range is 22 °C - 30 °C.

Light and shade

Being a shade-loving plant, the betelvine requires diffused sunlight; 30-40 % incident tropical light being ideal. The leaves at positions 6 - 8 from the apex of vines recently matured, register higher photosynthetic rate than tender and over matured leaves occurring at higher positions.

Humidity

Normally 60-80 % . The vines grow faster under high humidity and continual flushing of leaves are produced during July-October. In winter months, growth practically stops.

Soil

Exceptionally well-drained, fertile soil rich in humus is the best for betelvine. Ph 7-7.5. Silt to clay loamy soil with good drainage and slopy terrain favours optimum growth of vine.

Betel-vine is usually grown in conservatory or Baroj, as it is locally called. It is made with bamboo supports and covered with coconut fronds and the like.

A. Some salient points on yield factor of betelvine

i) Optimum growth period is July to October and plucking every 15 days interval; 2-3 leaves every vine.

ii) Yield becomes maximum during 2nd year; on average, 75-90 quintals of leaves per hectare; yield declines after 2nd year but remains economic upto 4th year; leaf yield also depends on method of cultivation: open /baroj; variety, location and age of vines; spacing and training; cultural practices.

B. Some salient points on vine cultivation in Baroj

i) Baroj means 2M high mounds with slopes; 4 sides are made with efficient drainage; planting is done in rows 60-75 cm apart; 8-10 M long; 1100-1150 rows/hectare; 8-10 cm deep furrows; single node cuttings 30-35 cm apart; support by jute-stick or live Erythrina/Sesbania.

ii) Training of vine is made by support 3-3.5 M high; live Sesbenia/Erythrina grown upto 3.5 M are also used. Vines attain a height of 3-3.5 M and when 8-10 months old, needs rejuvination.iii) Rejuvination (stage I) is done by harvesting existing leaves and vines coiled carefully into rings 15-20 cm diameter, leaving 0.5 to 0.75 M top-shoot uncoiled.

iv) Rejuvination (stage II) follows when coils are placed on ridges; partially covered with soil; sprouting takes place within 10-15 days and as many primary vines are produced.

v) Lowering is generally done in monsoon months and repeated 2-3 times.

II. Requirement of Nutrients

Out of the three elements viz. nitrogen, phosphorus and potassium that are vital for yield, nitrogen proves to be being the most important one. Nitrogen produces striking responses in growth and yield of leaves. Potassium - requirement by Betel-vine is also important because potassium has been specifically found to increase the vigour and keeping quality of leaves (Table 1).

Table 1. Leaf nutrient as index to productivity of Betel-vine

Leaf Nutrient	% Content	Productivity analysis
N	2.6-3.2	High productivity vine
	2.3 & Less	Low productivity vine
K	1.5-1.9	High productivity vine
	1.4 & Less	Low productivity vine
P	0.21-0.24	High productivity vine
	Less	Low productivity vine

It has also been found that high nitrogen makes the vine prone to diseases whereas increasing potassium makes the vine more disease resistant (Table 2).

Table 2. Foliar analysis as index to disease incidence in Betelvine

Leaf nutrient	% Content	Observation
N	> 2.80	Prone to diseases
	< 2.60	Resistant to disease
K	< 2.00	Prone to disease
	> 2.90	Resistant to disease

III. Chemistry of the Betel leaf oil

Chemical constituent of Betel oil is of interest because of the medicinal uses of the latter and in relationship to its taste. Of obvious importance are the compounds that contribute to the pungency, flavour and stimulating properties of the leaf. Fresh Betel leaves generally have a moisture content ranging from 85 to 87 percent (Table 3)

Table 3. Main chemical constituents of Betel leaf growing in West Bengal

Constituents	% Contents	Constituents	% Contents
Moisture	85.86	Reducing Sugar	1.4 - 3.2
Protein	3.0 - 3.3	Non-Reducing Sugar	0.6 - 1.9
Fat	0.7 - 0.8	Total Sugar	2.2 - 5.2
Carbohydrate	6.1 - 6.6	Starch	1.0 - 1.2
Fibre	2.2 - 2.3	Tannin	1.0 - 1.3
Mineral matters	2.0 - 2.5	Essential Oil	0.25 - 0.90

IV. Varietal difference in chemical constituents of Betel leaf

The nomenclature of Betel vine cultivars is most confusing. The general craze with growers is to name the varieties after their villages or towns. It can readily be imagined how such names multiply, resulting in a plethora of synonyms and adding to the confusion already existing regarding true identity of cultivars (Balasmbramannyam et al, 1994). Thus more than one hundred fifty types/varieties are grown and recognised. Three major cultivars viz. Bangla, Mitha and Sanchi are commonly grown in West Bengal (India) (Table 4).

Table 4. Varietal difference in leaf characters of Betel

Leaf	Bangla	Sanchi	Mitha
Shape	Cordate to roundish	Narrow ovate	Cordate to broadly ovate; base assymetric
Margin	Smooth, entire	Rough entire	Slightly undulated
Venation	Multicostate; 9 prominent veins	Multicostate; 6 - 7 porminent veins	Multicostate; 5 - 6 prominent veins
Taste	Highly pungent High eugenol (50 - 70 %)	Pungent Eugenol (13 - 14 %), Phenolic ethers	Sweetish with fennel aroma, Anethole (19 - 20 %) aroma

These three varieties differ in essential oil composition of leaves (Table 5).

Table 5. Effect of age and maturity of leaves and of seasons on essential oil content in Betel leaves (Varietal difference)

Parameters		Essential Oil Content (% FW)		
		Sanchi	Bangla	Mitha
Seasons	Winter	0.4	0.4	0.9
	Rainy	0.20	0.16	0.65
	Summer	0.30	0.20	0.70
Leaves	2-leaf	0.25	0.20	0.40
	8-leaf	0.50	0.45	1.00
	14-leaf	0.48	0.45	0.90

Physicochemical characteristic of essential oil also generally differ, variety wise (Tables 6 and 7)

Table 6. Physicochemical characteristic of essential oil of Betel vine cultivars

	Varieties		
Parameters	Sanchi	Bangla	Mitha
Refractive index	1.529	1.504	1.521
Specific gravity	1.001	1.034	0.999
Specific rotation in alcohol	-15.41	+11.70	-1.401
Sap value	-	7.99	-
Phenol content %	45.00	82.50	21.00
Solubility in 90 % ethanol	1 : 1	1 : 1	1 : 1

Table 7. Varietal difference in hydrocarbons and oxygenated compounds of essential oil content in Betel leaves

Cultivars	Mono terpene hydrocarbons	Sesquiterpene hydrocarbons	Oxygenated compounds (%)
Bangla	Nil	Nil	91.00 - 92.00
Mitha	1.80 - 2.00	31.00 - 33.50	48.00 - 50.00
Sanchi	11.00 - 13.00	20.50 - 22.00	42.00 - 45.00

V. Effective distillation of essential oil of Betel leaf

The essential oil of Betel leaf, being a highly prized item and rich in flavour should not be distilled in ordinary distillation units since without incorporation of the :

i) False bottom in the body of , the plant material gets charred during distillation and gives a burnt note in the oil.

ii) Feed-back mechanism, the slow and steady fall in ratio of water with respect to the plant material in the still, leads to over cooking and spoils the final note of the oil.

Optimum period of distillation is 3-4 hrs and optimum ratio of still capacity, quantity of leaves and volume of water is 10 : 5 : 2.

CONCLUSION

Much progress has been made towards identification of components of Betel-leaf essential oil. Out of more than 50 compounds identified so far, eugenol, anethole, terpenyl acetate, iso-eugenol are found to be of importance to flavour industry. Its medicinal value is re-inforced due to the presence of chavicol, chavibetol, allylpyrocatechol and others. Betel - leaf is rich in minerals, proteins, vitamins and amino - acids; imparting its stimulant property.

An effective and economic method of distillation for maximum recovery of oil will go a long way in exploiting betel leaves, otherwise going waste for remunerative returns. Steps to promote the use of oil in flavour industry will also ensure better economic returns to the traditional cultivators who are sticking to the profession of betelvine cultivation inspite of heavy odds.

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CUTTING TIME, YIELD AND ESSENTIAL OIL COMPOSITION IN THREE CULTIVARS OF PARSLEY.

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INTRODUCTION

Parsley [*Petroselinum crispum* (Miller) Nyman ex A.W.Hill] biennial plant native of Mediterranean areas, was already known as medicinal plant by Romans who diffused it throughout Europe (Moschini, 1960, Quagliotti *et al.*, 1990). Nowadays it is cultivated in Europe and North America for its aromatic and attractive leaves used, fresh or dried, as a condiment, garnish and flavoring ingredient.

In 1995 the parsley was grown in Italy on 1.300 ha and the yield was about 230.000 t (ISTAT, 1995). Csizinszky (1993) and Simon (1993) reported that in USA the consumption and the production of parsley were increasing.

The essential oil is mainly used as an aromatic agent in food products (salads, fresh cheese, curds, canned fish, dry soups) or fragrance in perfumes, soaps and creams (Simon and Quinn, 1988; Dachler and Pelzmann, 1989; Thomann *et al.*, 1993;). Antimicrobial effects of the oil on damaging bacteria (Kivanc and Akgül, 1986; Thomann *et al., l.c.*), and activity of anethole (Kubo, 1993) and thymol (Brunelli *et al.*, 1996) against fungi, are reported.

These effects could be interesting in the future for the preparation of antioxidizers, antimicrobial agents, repellents in ecologically compatible products (Thomann *et al., l.c.*; Simon, *l.c.*).

The essential oil is contained in all parts of the plant; the oil obtained from the leaves and the flowering tops is of the finest quality, but it is not generally extracted and used because the yield is too low, about 0.06 %; usually commercial essential oil is derived from mature seeds, yield up to 7 % (Pruthi, 1976). The characterization of the essential oil of the parsley leaves has been previously reported by Kasting *et al.*, 1972; Freemann *et al.*, 1975; Vernon and Richard, 1983; MacLeod *et al.*, 1985; Simon and Quinn (*l.c.*); Nitz, 1989; Porter, 1989; Lamarti. *et al.*, 1991.

Although the yield and the oil composition are influenced by genotype, environmental conditions and cultural systems, few information are available on parsley. The aim of this study was to evaluate the influence of cutting time on yield and oil composition in different cultivars.

MATERIALS AND METHODS

The trial was carried out in Bari (41°North lat.), in southern Italy. Three cultivars, different for size and form of leaves: common, cv. Plain, Asgrow (CL); giant, cv. Prezzemolo Gigante d'Italia, Asgrow (GL) and curled, cv. Robust, Clause (CCL) were directly seeded in the field on 12 September 1992 in single rows 20 cm apart using 1g of seed m^{-2}.

Plots of 6 m long and 2 m large, each containing 10 rows, with 4 replications in randomized block design were arranged.

The soil was fertilized with 120 and 100 kg ha^{-1} respectively of P_2O_5 and K_2O spread before seeding, while 200 kg ha^{-1} of N were applied three times during the growing season. Sprinkler irrigation, weed control with a blend of cycluron and chlorbufam (15.8 and 10.7%) were used.

The cuts were made on the center rows 135, 215 and 260 days after sowing; the plants were excised 1 cm above the soil, yellow and crushed leaves were discarded.

Total yield, height, number, weight, dry matter and area of leaves were collected at each cut. Data were processed by analysis of variance.

The essential oil was obtained by steam distillation for 60 minutes of 1 kg of fresh leaves and petioles and oil content determined on a volume to fresh weight basis. The values of essential oils content of two replications were averaged, and the samples stored in silica vials at 4°C in absence of light, before analyzing.

The oil composition was determined by GC/FID using Carlo Erba Mega 5360 GC unit; GC/MS analyses were carried out on Hewlett Packard 5968 A GC/MS system provided with Hewlett Packard 5890 GC unit.

The GC/FID conditions were: single injection/dual column/dual detection/ personal computer system. Carrier gas: hydrogen, flow rate 1.5 ml/min. Injection: split; split ratio 1:20; temperature 250 °C. Detectors: FID, temperature 260 °C. Column 1: 25m HTS-FS OV-1 column; i.d.: 0.25 mm; df: 0.3 µm. Column 2: 25 m HTS-FS Carbowax 20m column; i.d.: 0.25 mm; df: 0.3 µm. Temperature program: from 50 °C (1 min) to 200 °C (20 min) at 3 °C/min.

GC/MS analyses were carried out with the same columns and under analogous conditions to those reported in the previous paragraph. Carrier gas: helium, flow rate 1.5 ml/min.

RESULTS AND DISCUSSION

Total green yield was of 40, 32 and 22 t ha^{-1} respectively in the cultivars GL, CL and CCL, (Table 1); the green yield progressively decreased from the 1st to the 3rd cut: the 1st harvest gave about 50 % of total yield in all cultivars, while the 3rd cut only 20 %.

Total dry yield was of 4.8, 3.2 and 2.3 t ha^{-1} in the cultivars GL, CL and CCL; in the 1st cut the yield of the cultivar GL and CL was on average, 1.7 t ha^{-1}, while in the cv. CCL was only 0.9 t ha^{-1}.

Essential oil content increased from 0.06 % of fresh weight in 1st and 2nd cuts to 0.1% in the last cut in all cultivars. The highest oil production, about 27 l ha^{-1}, was obtained by cultivar GL; the lower, 15 l ha^{-1}, in CCL (Table 1). These results are in agreement with previous studies reporting essential oil content between 0.04 to 0.15 % of fresh weight (Vernon and Richard, (*l.c.*), Simon and Quinn (*l.c.*), Lamarti. *et al.* (*l.c.*), Thomann *et al.* (*l.c.*).

The cultivar GL produced 20 and 50% of green weight and oil more respectively of the cultivar CL and CCL. Dry matter in the 1st cut was, on average, 9.3% and reached 14.8% in the 3rd cut; the highest content of dry matter was found in the cv. GL, from 10.6 to 15.9%, while in the others it changed, on average, from 8.7 to 14.2%. Height and leaf area of the plants progressively decreased in all cultivars from the 1st to the last cut. Taller plants, 25 cm, with higher leaf area, 88 cm^2, were observed in the cv. GL; lower values resulted in the cv. CCL.

The major components of the essential oil of the evaluated parsley cultivars were: *p*-1,3,8-menthatriene, myristicin, apiol, β-phellandrene, myrcene, and 1-methyl-4-isopropenylbenzene (Figure 1), in agreement with the findings of Kasting *et al.* (*l.c.*), Freemann *et al.* (*l.c.*), Vernon and Richard (*l.c.*), MacLeod *et al.* (*l.c.*), Thomann *et al.* (*l.c.*). In addition, a small amount of thymol and anethole were found in all cultivars.

Thymol was detected in parsley leaf oil for the first time by Simon and Quinn (*l.c.*); the same Authors reported that the presence of thymol was unexpected, but not surprising (less then 10 % of total accessions screened) because it was found in many essential oil bearing plant species.

With regard to the important characteristic aroma components, Kasting *et al.* (*l.c.*), reported that *p*-1,3,8-menthatriene, β-phellandrene, and a mixture of terpinolene and 1-methyl-4-isopropenylbenzene gave a parsley like aroma. Freemann *et al.* (*l.c.*), separated the latter two constituents and suggested 1-methyl-4-isopropenylbenzene as being responsible for leaf aroma. In contrast, Vernon and Richard (*l.c.*), affirmed that 1,3,8-menthatriene, β-phellandrene and myristicin were not characteristic of parsley flavour. MacLeod *et al.* (*l.c.*), claimed that the only constituent with desiderable parsley aroma was apiol. In addition, Simon and Quinn (*l.c.*), found a significant variability of aromatic components in a germoplasm collection of parsley.

These results were confirmed by our research that showed the different content of myristicin and apiol in relation to the cultivar and the cutting time: in the 3rd cut, myristicin content of the cv. CCL, and apiol amount of the cv. GL and CL, was respectively 6 and 10 times higher than the former two cuts (Figure 2); the cultivar CCL was richer in myristicin, while CL and GL in apiol.

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Table 1. Yield and morphological characteristics.

	Yield											
	Green weight *(t ha^{-1})*				Dry weight *(t ha^{-1})*				Oil *(l ha^{-1})*			
	Cuts				*Cuts*				*Cuts*			
	1st	2nd	3rd	Total	1st	2nd	3rd	Total	1st	2nd	3rd	Total
Cultivar												
CL	18.1 a	8.5 b	5.5 b	32.1 b	1.5 a	0.9 b	0.8 b	3.2 b	10.9 a	5.1 b	5.5 b	21.5 b
GL	17.8 a	13.6 a	8.4 a	39.8 a	1.9 a	1.6 a	1.3 a	4.8 a	10.7 a	8.2 a	8.4 a	27.3 a
CCL	9.6 b	8.1 b	4.1 b	21.8 c	0.9 b	0.8 b	0.6 b	2.3 c	5.8 b	4.9 b	4.1 b	14.8 c

	Plant								
	Dry matter *(%)*			Height *(cm)*			Leaf area *(cm^2)*		
	Cuts			*Cuts*			*Cuts*		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Cultivar									
CL	8.5 a	10.1 b	14.1 b	21.7 b	20.1 b	15.6 a	76.1 b	67.2 b	45.6 b
GL	10.6 b	11.5 a	15.9 a	25.1 a	23.3 a	17.1 a	88.0 a	83.5 a	58.9 a
CCL	8.9 a	9.7 b	14.3 a	10.8 c	9.6 c	8.8 b	56.6 c	50.1 c	38.7 c

Mean separation within the columns using Student-Neuman-Keuls multiple range test at P=0.05

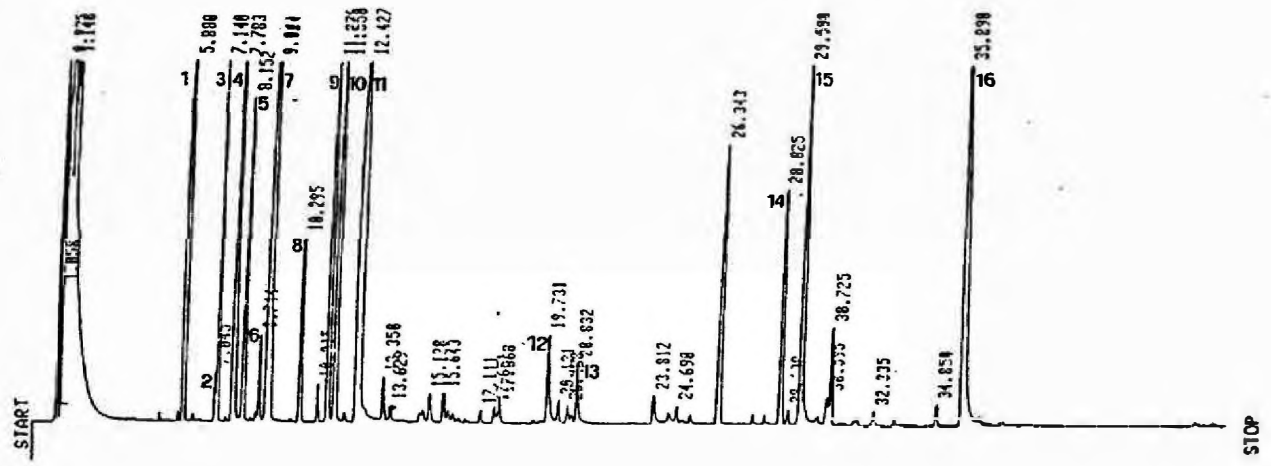

Figure 1. Gas chromatogram of volatile components of parsley leaves and petioles oil

Identification of peaks: 1 = α-pinene; 2 = sabinene; 3 = β-pinene; 4 = myrcene; 5 = α-phellandrene; 6 = cymene; 7 = β-phellandrene; 8 = terpinene; 9 = 1-methyl-4-isopropenylbenzene; 10 = terpinolene; 11 = p-1,3,8-menthatriene; 12 = anethole; 13 = thymol; 14 = germanecred; 15 = myristicin; 16 = apiol.

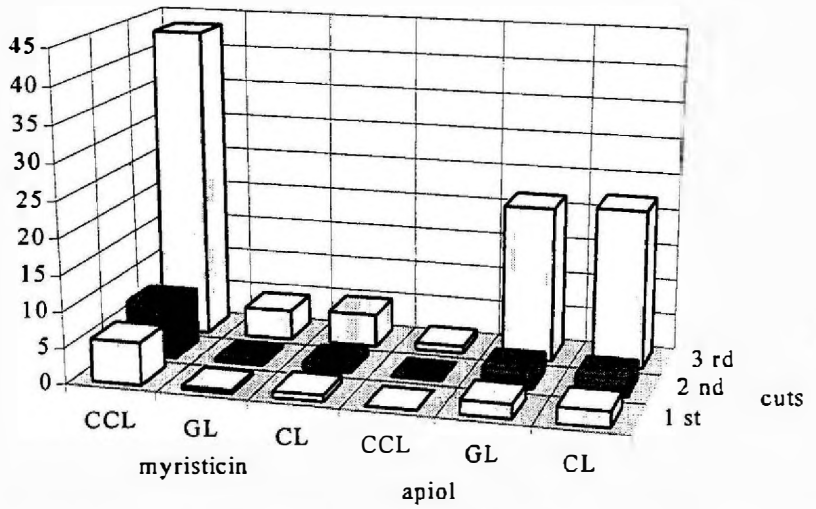

Figure 2. Percentage of myristicin and apiol in different cultivars and cuts.

STORAGE STABILITY AND RELATED ASPECTS OF *JUNIPERUS COMMUNIS* L.

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INTRODUCTION

In general, uncertain storage stability of herbal drugs containing essential oil presents a well - known problem.

However, only few systematic investigations concerning this subject have been performed (1, 2). Therefore, many Pharmacopoeias are failing in exact specifications of durability or the declarations are very general (e.g. DAB 10, 1996).

The aim of our studies was to establish declarations of stability for some of the most common drugs containing essential oil, dependent on the variables cutting size, storing conditions (T, % rel. hum.) and packing material. The choice of parameters was performed according to the conditions of storage and delivery of crude drugs in public pharmacies.

As an example, the qualitative and quantitative changings in content and composition of essential oil of *Juniperus fructus* are presented.

In Juniper berries, the essential oil is mainly stored in excretion reservoirs with a length up to 2000 µm (3). Already the slightly grinded berries loose their oil quickly.

EXPERIMENTAL PART

The samples were stored at 26 °C / 30 % rel. hum. in tin boxes as usual in public pharmacies.

Determination of essential oil yield was carried out according to the prescriptions in the DAB 10 1996 (4).

The obtained oil / Xylol - mixtures were examined without further dilution by GC and GC / MS.

Some authors reported serious changes in the composition of the essential oil during distillation (5, 6). Thereby, the two main components a-Pinene and Sabinene are of importance for *Juniperi fructus* above all.

"Stress - conditions" like extreme pH or temperature can result in the following compounds (5, 7):

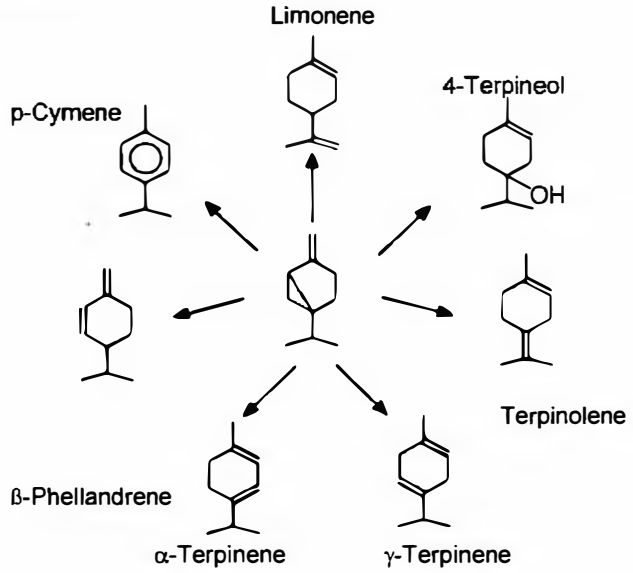

Figure 1. Possible compounds of decomposition of Sabinene.

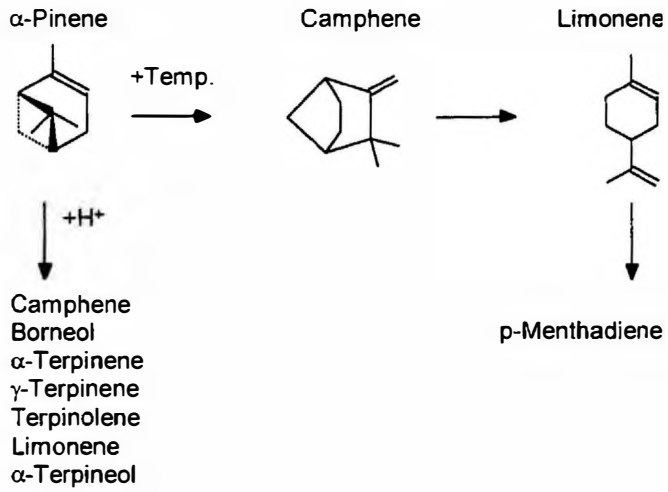

Figure 2. Some of the possible compounds of decomposition of α-Pinene.

After 1 hour boiling a-Pinene in water of pH =1 (HCl), the following compounds were identified by GC:

Camphene	ß-Phellandrene	4-Terpineol
α-Terpinene	Limonene	α-Terpineol
γ-Terpinene	p-Cymene	(Unknown)
Myrcene	Terpinolene	

The influence of temperature alone seems to increase especially the formation of 4-Terpineol (*Juniperus fructus* conc. / 50 °C).

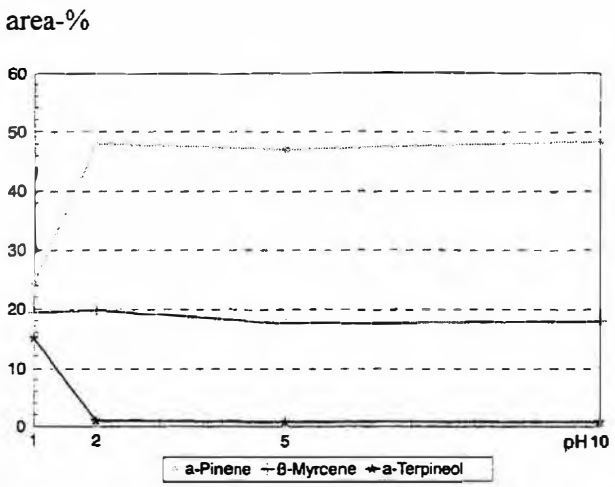

Figure 3. Dependent on the pH of the distillation water, these alternations in the composition of the major compnents [area %] were observed.

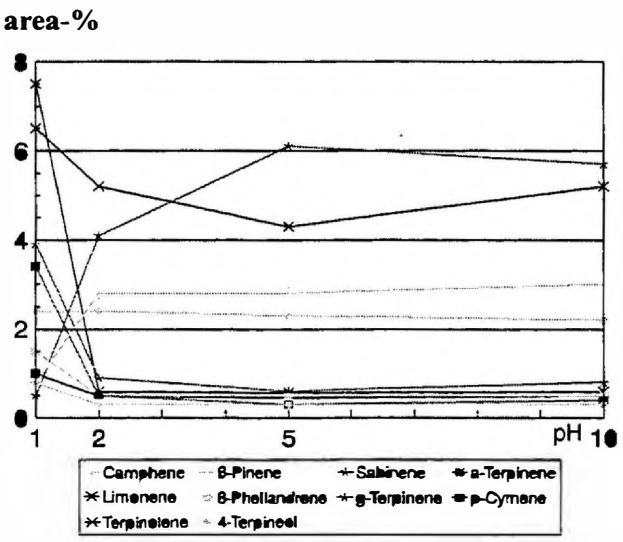

Figure 4. Alternations of the minor components dependent on the pH.

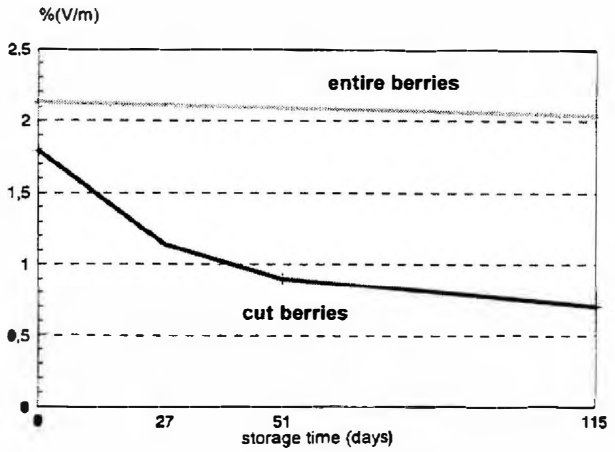

Figure 5. Stability of the essential oil content - entire and cut berries of *Juniperus fructus* (Fa. Martin Bauer, Ch.B.:3201780) during 4 months of storage.

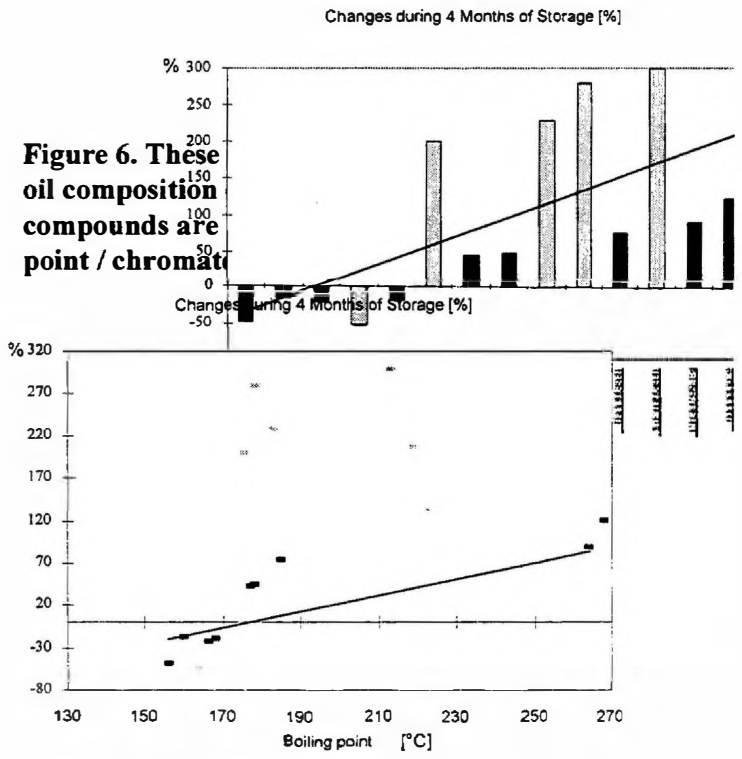

Figure 6. These oil composition compounds are point / chromat

Figure 7. Changes in relation to the boiling points. In addition to volatility, reactions of decomposition play an important role for some components (grey coloured).

CONCLUSIONS

1) Whereas the content of the entire berries is nearly constant during the storage of 4 months, that of the cut berries strongly decreases.
2) There is obviously a distinct correlation between boiling point of an essential oil component and its quantitative alternations during storage.

3) The composition of the oil is changing to a higher portion of less volatile compounds during storage.
4) Dependent on the lability of the main components α-Pinene and Sabinene of *Juniperus fructus*, alternations due to decom-position have to be considered.

5) The composition of the distillate of *Juniperus fructus* strongly depends on postharvest treatment of the berries, the conditions of distillation respectively.

According to Schilcher et al. (8), the quality of Juniperus communis oils increases with decreasing quotient of monoterpenehydro-carbons to 4-Terpineol (kidney-irritation-factor). This quotient can be positively influenced by modifying the above mentioned points.

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Acknowledgement
These investigations were supported by a grant from PhytoLab GmbH & Co KG, Vestenbergsgreuth

ESSENTIAL OILS AS PHYTOGENIC FEED ADDITIVES (PFA)

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INTRODUCTION

In the plant kingdom 24 Families are reported to contain more than 1, and further 40 Families only 1 essential oil producing genera (Protzen, 1993).

The biological activity of essential oil-plants has been known and utilized since ancient times (e.g. in food seasoning, ethnomedicine, etc.), nonetheless, a search in the veterinary medicinal records of the databases BEAST and AGRICOLA has revealed a very limited scientific approach to the application of essential oils, or in general, essential-oil plants, in animal feeding.

This observation is underlined by the fact that even a recent comprehensive compilation on the non-conventional agricultural uses of essential oils by Palevits (1994) completely ignores this topic (Table 1), despite the great importance of animal feeding for agriculture.

Table 1. Non-Conventional Uses of Volatile Oils in Agriculture

1. Botanical pesticides
2. Botanical Insecticides
   2.1. Stored Products
3. Fungicidal effects
   3.1. Stored products
   3.2. Post-harvest treatments
   3.3. Field fungi
4. Herbicidal effects
5. Nematocididal effects
6. Honeybee pathogens
7. Potato sprouting
8. Bovine aromatherapy

Source: Palevits, D. (1994): Proceedings of 4eme Rencontres Internationales - Nyons

Essential oils are, however, important commercial items, especially with a main area of utilization in the food industry (55 %). Their market share, as so called *Biological Products* (including their possible feed-applications) is rather meager and amounts to only 5 % (Protzen, 1993).

The ongoing changes in the nutritional habits of the human population, the increased concern for the environment have brought about an upsurge of interest towards the consumption and production of natural foods. To achieve this goal, similarly to organic agriculture, the food producing '*animal industry*' will also have to reduce the application of synthetic chemicals and turn towards the more healthy natural ways and means of production for which Phytogenic Feed Additives (PFA) can offer a solution.

FEED ADDITIVES VS. PHYTOGENIC FEED ADDITIVES (PFA)

A feed additive - as defined by the European Feed Additive Directive 70/524 - is *any substance, or preparation containing any substance which when incorporated into a feeding stuff, is likely to affect its characteristics or livestock production*". Phytogenic Feed Additives are of plant origin.

In the practice, various supplements are used to improve the nutritive balance or performance of the total feed. The role of such feed additives (e.g.: protein supplements, vitamins, etc.) is vital from the viewpoint of feed utilization efficiency.

To improve efficiency is, however, only one aspect of up-to-date animal production. The matters and the methods applied should contribute not only to the production of superior meat quality but they should be also conform with the increasingly severe food safety regulations. From this point of view, feed ingredients of natural origin, especially medicinal and aromatic plants containing biologically active substances seem to open up a favorable prospective. Owing to the manifold and mostly safe[1] effect of their active principles and aromatic components, their

[1] *GRAS = Generally Recognized as Safe*

use in the form of feed additives (*phytogenic feed additives)* seems to be continuously increasing. Trade trends in the feed additive market as summarized in Table 2. are also promising.

Table 2. EC 12 Feed Additive Market in 1988 and Forecast to 1994

Group of Feed Additives	1988		1994	
	$ Million	%	$ Million	%
Nutritional additives	936	74	1035	77
Auxiliary substances	69	5	85	6
Performance additives	198	16	150	11
Disease preventing agents	65	5	75	6
Total feed additives:	1268	100	1345	100

(Source: Frost & Sullivan, Inc. 1990): The European Market for Animal Feed Additives within the EEC, Fall 1990.

BIOLOGICALLY ACTIVE PRINCIPLES IN MAPs

Basically, feed additives can be ranked into the following four categories: nutritional additives *(a)*, auxiliary substances *(b)*, performance additives *(c)* and disease preventing agents *(d)*. According to the biological activity of substances contained by them, this classification can, however, be rendered even more elaborate (Figure 3)

Table 3. Main Groups of Feed Additives

1. Antibiotics
2. Antioxidants
3. Aromatic and appetizing substances
4. Coccidiostats and other medicinal substances
5. Emulsifiers, stabilizers, thickening and gelling agents
6. Colorings and pigments
7. Preservatives
8. Vitamins and pro-vitamins, etc.
9. Trace elements
10. Growth promoters
11. Non-protein nitrogen substances
12. Binders, anti-caking agents and coagulants

Regarding their biologically active properties, essential oil plants, as feed additives, are most versatile and as such they can offer an alternative for most feed additive categories. It should be mentioned that some of the species (e.g. garlic) seem to be extremely potent from this point of view (Table 4).

Table 4. Some important flavoring spice spices as sources of biologically active compounds

Name of the spice	Number of identified biologically active compounds				
	Anti-oxidant	*Sedative*	*Anti-epressant*	*Anti-viral*	*Bactericide*
Bay	3	5	-	5	0
Black Mustard	4	-	-	4	6
Black Pepper	4	7	-		14
Caper	3	-	-	3	2
Cassia	3	-	-	3	3
Cayenne	9	7	7	6	8
Clove	3	-	3		
Coriander	7	8	-	12	20
Cumin	5	6	-	7	11
Garlic	9	5	5	5	13
Ginger	6	11	5	6	17
Licorice	10	6	-	8	20
Oregano	14	-	-	11	19
Poppyseed	3	-	5		
Rosemary	12	6	-	10	19
Saffron	2	-	-		
Sage	7	-	-		6
Sesame	7	-	7		5
Thyme	4	-	3	3	5
Turmeric	3	-	-	3	8
Vanilla	7	-	-	3	7

After: Beckstrom-Sternberg, St.M. and Duke, J. (1994)

Antioxidants

All five groups i.e. antioxidants, anti-depressants, antivirals, bactericides, sedativa have obvious relevance for the animal husbandry. From the nutrition physiological point of view, however, the antioxidants merit special attention, since undesirable oxidation can produce uninviting changes in color, flavor, aroma and other quality factors of meat, as well as food. In the case of fats and oils a rancid taste and odor can develop, which not only impairs the nutritional value of the

product but might also form the grounds of toxic effects (Kanner, 1994).

Natural antioxidants, as compared to synthetic products have the advantage that they are readily accepted by the consumers (they are considered to be safe and not a 'chemical'), consequently no safety test is required by legislation (Hyland and Poulev, 1995).

Essential oil crops have a huge potential as antioxidants, offering an unexploited great choice of species and essential oil components. Some of the characteristic essential oil compontents with identified potential antioxidant properties are summarized in Table 5. Especially species of the Families *Apiaceae* and *Lamiaceae* have been identified to possess significant antioxidative properties (Deans and Waterman, 1993).

Antifungal Activity

Essential oils have been shown to exhibit antifungal activity, even at very low concentrations in the growth medium. As an example, Deans and Svoboda (1990) established that 1 - 10 μl^{-1} marjoram oil in the culture broth reduced the growth of the filamentous fungus species *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *A. parasiticus* and *Trichoderma viride* by up to 89 % . In optimal cases essential oils interfere already with spore germination, whereby the inhibitory effect of essential oil components has been demonstrated to vary substantially (Table 6.).

Table 5. Essential Oil Components with Potential Antioxidant Properties

Oil Component	*Monarda citriodora*	*Myristica fragrans*	*Origanum vulgare*	*Pelargonium sp.*	*Thymus vulgaris*
α-pinene	√	√			√
β-pinene	√	√			√
α-terpineol	√	√			
α-phellandrene				√	
α-terpinene	√				√
γ-terpinene	√		√		√
β-caryophyllene	√		√		√
p-cymene	√		√	√	√
1,8-cineole	√				√
terpinene-4-ol	√	√			√
isoeugenol		√			
isomenthone				√	
methyl eugenol				√	
geranyl acetate		√			
geranyl formate				√	
citronellic acid				√	
borneol					√
camphene					√
carvacrol	√		√		√
citronellol				√	
elemicin		√			
eugenol		√			
geraniol				√	
limonene	√	√			
linalool		√	√	√	√
myrcene	√				√
neral				√	
sabinene		√	√		
safrole		√			
thymol	√		√		√

(Source: Damien Dorman, H. J. et al. (1995): J. Essent. Oil Res., 7, pp. 645 - 651

Table 6. Effect of essential oil components on spore germination, Minimum Inhibitory Concentration (MIC) (ppm)

	A	B	C	D	E	F	G
carvacrol	250	125	62	250	250	250	125
***p*-anis-aldehyde**	1000	1000	250	500	250	250	1000
***(-)*-carvone**	-	1000	250	-	-	-	-
***(E)*-anethole**	-	-	1000	-	-	-	-

A= *B. cinerea,* B= *Monilia laxa,* C=*Mucor piriformis,* D= *Penicillium digitatum,* E= *P. expansum,* F= *P. italicum,* G= *Rhizopus stolonifer check after 24 h at 20 ^{0}C, data are the average of five replications*

Sedative and Antidepressant Activity

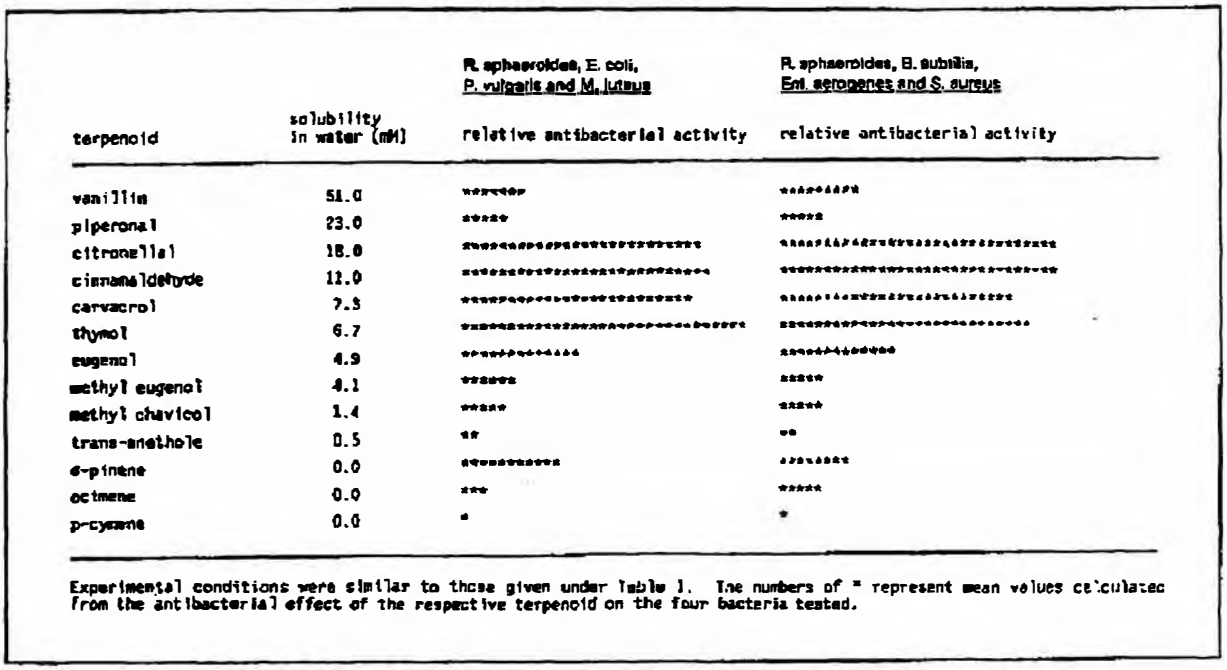
The beneficial value of some essential oil plants (e.g. *Valeriana officinalis, Melissa officinalis, Lavandula angustifolia, etc.)* in the treatment of nervous instabilities, sleep disorders (Weiss and Fintelmann, 1997) has been utilized by phytotherapy for a long time. Although, obviously, PFAs and among them essential oil plants are expected to have analogous effects on the animal organism, too, the ways and means of application need to be cleared in scientific experiments.

Antibacterial and Antiviral Activity

Antibacterial activity seems to be equally determined by both the concentration and the composition of essential oils. Remarkably, according to some authors (Knobloch *et al.*, 1989) the aseptic physiological potency (capacity) of terpenoid compounds is positively related to their water solubility (Figure 1).

Although, there is less scientific evidence, certain species e.g. garlic are generally recognized to possess antibiotic (antiviral) properties. In a recent feeding experiment with poultry, *Achillea millefolium*, *Hypericum peforatum* and *Levisticum officinale* were efficiently used as a substitute for antibiotics and it was also established that the diet favorably affected the sensory characteristics of meat (Fritz *et al.*, 1993).

Figure 1. Comparison of the water solubility of terpenoids to their antibacterial activities in plate tests



Knobloch et al. (1989)

It should be noted, however, that feedstuffs applied with the aim to deal with a certain pathological or metabolic condition fall into the category of 'dietetic feedstuffs' (European Community Council Directive, No. 8130/93 (8. Sept, 1993.) This term stands for the feedstuff category between standard (normal diets) and medicinal feeds (Wolter, 199...) and implies the objectives outlined in Table 7..

Table 7. Animal Husbandry Objectives for Dietetic Feedstuffs

Disorder	Occurrence
Hypocalcaemia	Diary cows
Ketosis	Dairy cows and sheep
Hypomagnesemia	Ruminants
Acidosis	Ruminants
Urinary caculi	Ruminants
Water and electrolyte balance	Calves, piglets, lambs, young goats, foals
Stress response	Pigs
Digestive disorders	Piglets and pigs
Constipation	Sows
Hepatic steatosis	Laying hens
Malabsorption syndrome	Poultry

(Source: Wolter, R. (1995)

PHYSIOLOGICAL EFFECTS IN ANIMALS

Nutritional products used as additives/supplements to bulk feedstuffs (e.g. grains, oilseeds, forage, etc.) are meant to improve performance, or in certain cases to cure nutritional deficiency and/or metabolic disorders. To date, mainly synthetics have been used to this end. It has, however, been established that phytogenic feed additives can be used with similar efficacy, since they are capable of influencing important physiological processes in the animal organism (Table 8).

Table 8. Physiological Effect of Essential Oils

1. ***Intensification of the impulses*** sent by the taste- and smelling-nerves in the nasal cavity area towards the central nervous system
2. ***Increasing the secretion of digestive juices***, e.g. saliva, gastric juice, gall, pancrease and intestinal-secretion
3. Intensification of the ***activity of digestive enzymes*** in the gastro-intestinal area
4. Increasing ***nutrient absorbtion*** by activating the transport mechanisms
5. ***Inhibition of oxidation-processes*** of intermediary metabolism, e.g. amino-acids
6. ***Inhibiting the growth of bacteria and fungi*** within the alimentary-tract and stabilization of the microbial flora
7. ***Inhibition of mould growth*** on feed-stuffs (fungicide effect)

Source: Günther, K.D. (1990): Gewürzstoffe können die Leistung erhöhen. Kraftfutter, 73, pp. 469 - 474.

PFAs IN THE ANIMAL PRODUCTION TECHNOLOGIES

Some companies (e.g. 'DELACON', 'INDIAN HERBS', *etc.*) make already use of MAPs in their everyday production technologies and there is accumulating scientific evidence demonstrating the benefits of PFAs for animal production.

As a good example, Adiarto (1993) in Germany has established a remarkable decrease in milk urea content, in a feeding experiment with a product 'Crinarom 898' containing medicinal and aromatic plants (Table 9).

Table 9. Average Urea content of milk, in a feeding experiment with 'Crinarom 898' (*CRINA, Switzerland)*

Trait	Experiment - Stage	Control - Stage
Experimental Period I.		
Milk urea content (*mg/1000 ml*)	227 ± 31.6	274 ± 15.2
Experimental Period II.		
Milk urea content (*mg/1000 ml*)	214 ± 15.6	254 ± 24.4

Another product, the 'De-Odorase' has proved to decrease the ammonia levels in large pig units, thus reducing ist negative consequences both on animal performance and what is equally important, on the environment (Table 10)

Table 10. Environmental ammonia levels and pig performance with and without dietary 'De-Odorase', on a large pig unit (Cole and Tuck, 1991)

	Control	'De-Odorase'
NH_3, at start (*ppm*)	31.2	30.1
NH_3, at finish (*ppm*)	30.0	19.6
Average NH_3, in first 4 weeks (*ppm*)	30.5	32.1
Average NH_3, in 2nd 3 weeks (*ppm*)	30.7	19.9
Daily liveweight gain (*g/day*)	817.3	847.3

Based on the feed back from the production practice, the general main advantages of PFA-application are summarized in Table 11.

Table 11. Some Advantages of Phytogenic Feed Additives for Animal Production

1. **Increased feed uptake,**
2. **Improved weight increase and feed-utilization,**
3. **Increase in egg-laying-capacity,**
4. Substantial **reduction of diarrhoea-risk,** especially of young animals,
5. **Reduction of losses** (drop-out),
6. **Improving the acceptance of feed** with unpleasant taste (e.g. rape-cake, protein-concentrates, mineral mixtures, etc.,
7. **Reduction** in the application **of chemotherapeuticals,**
8. **Quality improvement** of the product (better texture, taste and color),
9. **Higher vitality and better health-potential,**
10. **Better barn-climate** incl. the reduction of unpleasant odor,
11. In most cases, application is unlimited and does not involve any problems,
12. **The meat does not contain any harmful residues,** etc.
13. **Anti-stress activity** during transport.

Source: DELACON GmbH, Austria

There have also been numerous attempts to patent the application of PFAs in animal husbandry. As an example, a European Patent Application (No. 94401672.4/1994) describes a method for improving meat and fat obtainable form livestock and poultry, in which the animal feed contains at least one spice from 14 plant families, among them important essential oil containing families (e.g. *Cruciferae, Compositae, Umbelliferae, Solanaceae, Labiatae, etc.).* Although the fate of patents is sometimes difficult to assess, the values and possibilities offered by PFAs are relevant and have been recognized by many. Furthermore, it is also to be expected that the scientific knowledge accumulating on the safe and efficient use of medicinal and aromatic plants in the form of phytopharmaceuticals will also substantially contribute to the more effective and secure application of feed additives.

SUMMARY

To improve the efficiency and profitability of animal production, especially in view of meat quality, by means of animal feeding (nutrition), remains to be an economic priority. To achieve these goals, by virtue of their biologically active principles, several essential oil plants can offer a natural and healthy alternative.

These plants offer not simply an alternative for the presently still mostly synthetically produced feed additives, but owing to their not yet discovered synergistic effect of chemical

components, they can envision an even perhaps more environment friendly, positive prospective in improving production efficiency, meat quality, including longer shelf-life.

One of the main advantages of MAPs to be applied in this area is that they fall within the scope of the GRAS-List, i.e. their use as food/feed additives is generally recognized as safe. Solely the fact that the list maintained by the FDA contains some 250 herbs (Tyler, 1994), implies nearly inexhaustible resources.

It is however to be emphasized that the exploitation of these vast resources should not be, anymore, based on mere empiricism but on accurate scientific experience.

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FEEDING HERBAL FEED INGREDIENTS PRODUCES A PERFORMANCE ENHANCEMENT IN FATTENING SWINE

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TRIAL 1.

MATERIALS AND METHODS

A commercial UK farm producing 2000 fat pigs a month was selected to test the effect of herbal feed ingredients on pig production.

The unit is a breeder/fattener but the fattening enterprise is geographically separate from the breeding site. Pigs enter the fattening unit at approximately 37 kg live weight, where they are fed twice daily a liquid diet via a pipeline system. Pigs leave the unit to be slaughtered at 92 kg live weight.

The study period was 14 months. During this time a herbal feed ingredient, **LIVOL**, a product of Indian Herbs International, was included in the ration for two separate periods, once in January and February 1995, and again for four months October-January 1996. **LIVOL** was included in the diet at the rate of 2 kg per tonne of dry matter equivalent. For the other 8 months a ration without **LIVOL** was fed, whilst all other factors were as near as possible identical. **LIVOL** was included in the ration at the times of the year when productivity usually falls because of the climatic conditions of Winter.

LIVOL is a polyherbal preparation of 16 ingredients that are recognised in the ancient Indian system of herbal medicine called Ayurveda for their beneficial effects on the liver and digestive system in general. More recent investigations into the product have demonstrated its usefulness in all species of animal, including human.

The following parameters were noted:

- Number of pigs entering the fattening unit,
- Number of pigs sold fat,
- Feed usage,
- Mortality,
- Feed Conversion Ration,
- Daily Liveweight Gain.

RESULTS

The daily liveweight gain (DLWG) on a monthly basis, varied widely with an average value of 712 g/pig/day. The months in which **LIVOL** was included in the ration showed a DLWG higher than 712g in every case with an average of 825 g/pig/day. The months in which **LIVOL** was not included in the ration showed without exception a DLWG lower than 712 g with an average of 627 g/pig/day. The total number of pigs sold fat in the 14 months of the trial were 26274.

The mortality of pigs in the fattening period was on average for the 14 months 3.12 %. In general the mortality for the months when **LIVOL** was fed was lower (2.82 % for 6 months) than when **LIVOL** was not fed (3.35 % for 8 months). It must be remembered however that generally mortality is lower in the Summer months, when in this trial **LIVOL** was not fed, than in the Winter months, when in this trial **LIVOL** was fed and that this fact may have reduced the difference between the two treatments significantly. The difference in mortality figures between the two treatments would represent a reduction in mortality of 10 pigs per month in favour of the **LIVOL** treated group. Over the 14 months of the trial the average Feed Conversion Ratio (FCR) was 2.45 kg feed/kg liveweight gain. For the period when **LIVOL** was fed the FCR was 2.49 as compared with 2.53 for the 8 months when **LIVOL** was not fed. The same winter/summer argument applied to the FCR figures as to the figures for mortality, and the advantage shown for the period when **LIVOL** was fed is indeed surprising.

DISCUSSION

The inclusion of **LIVOL** in the ration fed to fattening swine produces a beneficial response despite the seasonal pressures to produce a negative effect. During the months when **LIVOL** was fed there was a 24 % increase in

daily liveweight gain a 5% reduction in FCR and a 19 % reduction in mortality.

The position of the UK pig-meat market during the period of this trial puts a conservative value on the effect of the herbal feed supplement of over £7000 per month. This shows a cost effectiveness ratio in favour of the use of **LIVOL** as a feed supplement of greater than 8 : 1 at UK prices.

Table 1. - Ingredients of LIVOL

	Ingredients	Classification	Pharmacological Action
1.	*Andrographis paniculata*	Acanthanceae	Bitter, Tonic, useful in debility
2.	*Boerhaavia diffusa*	Nyctaginaceae	Stomachic, useful in jaundice
3.	*Terminalia arjuna*	Combretaceae	Tonic, useful in bilious affections
4.	*Citrullus colocynthis*	Cucurbitaceae	Bitter, Cholagogue
5.	*Eclipta alba*	Compositae	Tonic, Deobstruent
6.	*Terminalia chebula*	Combreataceae	Tonic, alterative
7.	*Aphanamixis rohituka*	Meliaceae	Useful in spleen liver diseases
8.	*Ichnocarpus frutescens*	Apocynaceae	Diuretic, Tonic
9.	*Phyllanthus niruri*	Euphorbiaceae	Useful in jaundice, Stomachic
10.	*Fumaria parviflora*	Fumariaceae	Alterative, Tonic, Diuretic
11.	*Achyranthes aspera*	Amaranthaceae	Diuretic, useful in colic and dropsy
12.	*Azadirachta indica*	Meliaceae	Antiseptic, Bitter Blood purifier
13.	*Sida Cordifolia*	Malvaceae	Tonic digestive system
14.	*Swertia chirata*	Gentianaceae	Bitter, Tonic, stomachic
15.	*Tephrosia purpurea*	Papilionaceae	Tonic, cholagogue, diuretic
16.	*Canscora decussata*	Gentianaceae	Nervine tonic and alterative

Table 2 - Other published trial data on feeding with LIVOL

FISH Carp LIVOL increased growth (weight gain) by **26%**

GOATS LIVOL increased growth (DLWG) by **33%**

CHICKEN

		DLWG	FCR
LIVOL (1)	UP 5%	DOWN 4%	
produce (2)	UP 5%	DOWN 3%	
the following	(3) UP 7%	DOWN 6%	
in	(4) UP 23%	DOWN 12%	
10 separate	(5) UP 9%	DOWN 6%	
trials	(6) UP 12%	DOWN 9%	
	(7) UP 24%	DOWN 8%	
	(8) UP 30%	DOWN 14%	
	(9) UP 7%	DOWN 5%	
	(10) UP 12%	DOWN 11%	

CALVES LIVOL increased DLWG by 17-25%

TRIAL 2. - PERFORMED AT AN INDEPENDENT RESEARCH ESTABLISHMENT IN THE UNITED KINGDOM

The object was to investigate the possibility of producing synchronous ovulatory oestrus in a group of cows.

Animals were treated with Sychro strength Prajana a Herbal Feed Supplement mixed with a little feed for 3 days on the 22nd, 23rd and 24 July 1995. There were three groups of animals comprised as follows:

- 9 cows 3 heifers
- 2 heifers
- 3 cows

Group 1 were given the full supplement for 3 days.

Group 2 were given 50% of the supplement for 3 days,

Group 3 were given unsupplemented feed.

Animals in group 1 were all served by AI between 25th and 30th of July 1995.

One animal in Group 2 was served by AI on 1st August 1995 and the other on 10th August 1995.

The animals in Group 3 did not display oestrus and were turned out with the bull, they were served late August by natural service.

2 cows from Group 1 repeated 21 days later and were served by natural service.

All animals were confirmed pregnant by rectal palpation in November.

The results of this Trial indicate that Prajana sychro strength can indeed be used as an aid to controlled breeding programmes where synchronous oestrus is desirable. The oestrus that occurs is ovulatory and that following insemination all but 2 animals became pregnant (86%). The other 2 animals became pregnant following natural service 21 days later.

The product is composed of 100 % herbal material and the answer to its' mode of action lies in the material extracted by organic solvents (lipid). It is thought at present that the active ingredients are some special tri-enoic fatty acids that act at the hypothalamic level, possibly by acting as precursors for prostaglandia biosythesis. (Goshal Personal communication).

The product has been used for many years and has been shown in numerous studies to be a fertility aid in cattle and pig (see references).

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ANTIMICROBIAL AND ANTIOXIDANT PROPERTIES OF PLANT ESSENTIAL OILS

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INTRODUCTION

Aromatic plants, used since antiquity in food, cosmetics and pharmaceuticals, make important contributions to odour and flavour due to the presence of essential oils and other secondary metabolites. Secondary metabolites from aromatic plants have also exhibited a range of biological activities including antimicrobial and antioxidant properties. The preservative nature of plants has been realized for centuries, as herbs and spices have been used to extend the shelf-life of foods besides enhancing their flavour.

The antimicrobial activity of essential oils, complex mixtures of organic volatile compounds, has been known for more than 50 years. Only in recent time has the biological activity associated with plant natural products been subject to close scrutiny as a result of advances in analytical techniques that now permit their chemical characterization and biological evaluation of their activities either *in vitro* or *in vivo* (1–3).

While many studies on the effect of essential oils against the growth of various organisms have been published, these often disagree about the effects of the same essence. Such variability is due to the low solubility and high volatility of the oils in addition to their chemical composition which is determined by several factors, namely geographic origin, agronomic conditions, seasonal and climatic changes, harvest period, plant tissues and extraction technique.

Recently in developing countries attention has been paid to exploiting natural substances as substitutes for synthetic compounds.

We investigated the properties of 20 essential oils, some from aromatic plants typical of the Italian and Mediterranean flora and some from species originating in tropical countries. The study evaluated the minimal inhibitory concentrations (MICs) against 53 microbial strains as well as the antioxidant properties.

EXPERIMENTAL

Plant oils

Twenty essential oils obtained from the Departments of Agronomy, Bologna University, and Biorganic Chemistry, Pisa University, were used in this work (Table 1). Of the plants chosen, some are common culinary herbs and spices (sage, thyme, rosemary, clove), other are more useful in the manufacture of perfumes and cosmetics (cinnamon, lavender, peppermint) and some have medicinal applications (geranium, boldo, clove, fennel). Some are short season annuals (basil), others perennial shrubs (juniper) or evergreen trees (cypress). The oils were extracted from leaves (sage, peppermint), flowers (lavender), buds (clove), fruits (fennel, juniper), bark (cinnamon) or other specialized structures, depending on plant species. The oils were obtained by steam distillation of plant material in a Clevenger-type apparatus.

Test organisms and growth conditions

The antimicrobial properties of essential oils were studied against 45 bacteria including both Gram-positive and Gram-negative and both aerobic and anaerobic strains. In addition a group of 8 species of yeasts was tested. The bacterial strains were taken from the culture collection of the Institutes of Agricultural Microbiology and Plant Pathology of Bologna University . The yeasts were supplied by the Industrial Yeast Collection (Perugia University).

Test bacteria were chosen for their diversity and represented a range of different categories which have an important role in agriculture, food and pharmaceutical industries.

All the bacteria were cultured in liquid specific broth. TPY medium (4) was used for the strains of the genera *Clostridium*, *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Lactococcus* and *Enterococcus*. Meat extract (0.3%) and peptone (0.5%) was used as medium for the strains of the genera *Bacillus*, *Xanthomonas*, *Pseudomonas*, *Agrobacterium* and *Erwinia*. The yeasts were cultured in a medium with the following composition: 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose. Appropriate growth conditions were adopted for the different groups of microorganisms.

Assessment of antimicrobial activity

MICs were determined by broth dilution method. Oils were prepared as 10% (v/v) solutions in ethanol, due to the insolubility of oils, and stored at 4°C until analysis.

Antimicrobial properties were tested in the range 200–2000 ppm with intervals of 200 ppm. Test ethanolic solutions were added to tubes with appropriate broth for each test microbial group making a final volume of 10 ml. Final solutions were mixed on a Vortex mixer, poured into petri dishes and distributed in Corning Cell Wells 96-well plates with a round bottom using a "Multipette Eppendorf" dispenser. This method proved to be especially suitable for this study which involved a wide assortment of plant essential oils and microorganisms. Growth controls without the ethanolic solution and with the upper limit of ethanol added in the test media, were included in each Corning plate.

Plates with wells containing 100 µl/l of solution were inoculated with 10µl of 1 or 2-day-old culture of the test microorganisms by "StepperTM Repetitive Pipette". Inoculated plates were incubated for 48 h, in different conditions of temperature and aeration, to suit the test microbial groups. The lowest concentration in which no growth occurred was taken as the minimum inhibitory concentration of the test solution. The growth was evaluated by the amount of cells on the bottom of the microwell; the reduction of pH and the colour variation of bromocresol indicator were considered for fermentative organisms.

Assessment of antioxidant activity

Agar plates with 1.2% agar were prepared. Two millilitres of linoleic acid in ethanol (2 mg/l) and 10 ml ß-carotene in acetone (2 mg/) were dispensed in 100 ml of molten agar and then this mixture was poured into four petri dishes and allowed to solidify. Five 6-mm diameter Antibiotica-Testblattchen filters were put on the agar plate's surface and soaked with a drop of test oil (18 µl). Plates were incubated at 45°C for about 4 h - the requisite time required to fade the background colour of ß-carotene. The zone of colour retention around each filter was the region of antioxidant activity and was measured with a vernier calipers.

RESULTS AND DISCUSSION

The antimicrobial and antioxidant activities of the 20 essential oils tested are reported in Table 1.

Oils from cinnamon, clove, oregano and Spanish oregano, winter savory, thyme and geranium were the most inhibitory to the growth of all bacteria. Basil, French tarragon, peppermint, sage, juniper, boldo and rosemary oils possess antibacterial properties with different degrees of effectiveness: oils from cypress, bitter and sweet fennel were not inhibitory to all the tested bacteria. All the oils were inhibitory to at least one yeast. Cinnamon, Spanish oregano, juniper, winter savory, thyme and geranium oils were the most active while sweet fennel was the least effective. Spanish oregano oil has the greatest antioxidant activity. Oils from clove, thyme and oregano were also very active. On the other hand basil, rosemary, French tarragon, sweet and bitter fennel, cypress, lavender "Abrialis" and "Grosso" did not have antioxidant activity. This research revealed that numerous aromatic plants and their secondary metabolites could be of biological interest as sources of flavouring and drugs, insecticides, antimicrobials and antioxidants besides cosmetic and medicinal compounds. The prospect of an increased adoption of natural plant products in food, perfumery or pharmaceutical industries makes their exploitation fundamental for developing countries. Plant biotechnology to control composition and yield of volatile oils may

improve the application of new antimicrobial agents that are safe for man and his environment and come from available and renewable sources as plant secondary metabolites..

Acknowledgements

The excellent technical assistance of M. Albertoni is gratefully acknowledged. This research, supported by a grant of the National Research Council, is part of a project on "Biological activity of vegetable substances".

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Table I. Antimicrobial[a] and antioxidant[b] activities of plant essential oils

Microbial species	Essential oil[c]									
	A	B	C	D	E	F	G	H	I	J
Bacillus thuringiensis	>2000	>2000	400	>2000	>2000	>2000	>2000	800	1400	>2000
B. brevis	600	>2000	200	>2000	400	>2000	>2000	200	400	>2000
B. laterosporus	800	>2000	200	>2000	200	>2000	>2000	NT	200	>2000
B. subtilis	1200	>2000	200	>2000	1400	>2000	>2000	800	1000	>2000
B. polymyxa	800	>2000	400	>2000	1400	>2000	>2000	800	800	>2000
B. licheniformis	1200	>2000	400	>2000	>2000	>2000	>2000	800	800	>2000
B. cereus	1200	>2000	400	>2000	>2000	>2000	>2000	800	1000	>2000
B. amylolyticus	800	>2000	200	1600	600	>2000	>2000	400	600	>2000
Lactococcus lactis subsp.lactis	400	>2000	NT	>2000	600	>2000	>2000	800	600	>2000
L.lactis subsp. cremoris	1000	1200	400	>2000	1600	>2000	>2000	800	800	1800
Enterococtus faecium	>2000	>2000	800	>2000	>2000	>2000	>2000	1400	1400	>2000
E. faecalis	>2000	>2000	1200	>2000	>2000	>2000	>2000	1400	1400	>2000
Streptococcus salivarius	600	NT	NT	NT	1000	NT	NT	600	400	NT
S. salivarius subsp. thermophilus	800	>2000	600	>2000	1400	>2000	>2000	600	600	>2000
Lactobacillus. helveticus	1000	>2000	200	>2000	>2000	>2000	>2000	800	600	>2000
L. acidophilus	1000	>2000	600	>2000	>2000	>2000	>2000	800	800	>2000
L. brevis	1000	>2000	600	>2000	>2000	>2000	>2000	1000	1000	>2000
L. casei	>2000	>2000	600	>2000	>2000	>2000	>2000	1200	1400	>2000
L. reuteri	NT	>2000	600	>2000	NT	>2000	>2000	1600	1400	>2000
L. delbrueckii subsp. bulgaricus	1000	>2000	NT	>2000	1400	>2000	>2000	800	800	>2000
L. delbrueckii subsp. delbruecki	>2000	>2000	200	>2000	>2000	>2000	>2000	1400	1600	>2000
Clostridium. sporogenes	400	>2000	200	>2000	1000	>2000	>2000	200	400	>2000
C. beijerinckii	200	>2000	200	>2000	400	>2000	>2000	400	400	>2000
C. butyricum	800	>2000	200	>2000	>2000	>2000	>2000	600	600	>2000
C. acetobutylicum	200	>2000	200	>2000	600	>2000	>2000	200	400	>2000
C. bifermentans	>2000	>2000	200	>2000	>2000	>2000	>2000	1000	800	>2000
C. pasteurianum	600	>2000	200	>2000	1400	>2000	>2000	600	200	>2000
C. tyrobutyricum	600	>2000	200	>2000	>2000	>2000	>2000	800	400	>2000
C. putrefaciens	1000	>2000	200	>2000	NT	>2000	>2000	800	600	>2000
Bifidobacterium. adolescentis	>2000	>2000	200	>2000	>2000	>2000	>2000	1600	NT	>2000
B. bifidum	2000	>2000	NT	>2000	>2000	>2000	>2000	1000	NT	>2000
B. breve	>2000	>2000	200	>2000	2000	>2000	>2000	1000	800	>2000
B. infantis	>2000	>2000	200	>2000	>2000	>2000	>2000	1000	1000	>2000
B. longum	>2000	>2000	200	>2000	>2000	>2000	>2000	1200	1000	>2000
B. catenulatum	>2000	>2000	200	>2000	>2000	>2000	>2000	1000	1000	>2000
B. pseudocatenulatum	>2000	>2000	200	>2000	>2000	>2000	>2000	1200	1200	>2000
Erwinia carotovora subsp.carotovora	1400	>2000	400	>2000	>2000	>2000	>2000	800	1400	>2000
Xanthomonas pruni	1200	>2000	200	>2000	>2000	>2000	>2000	400	600	>2000

Microbial species	Essential oil[c]									
	A	B	C	D	E	F	G	H	I	J
Pseudomonas viridiflava	>2000	>2000	800	>2000	>2000	>2000	>2000	1800	>2000	>2000
P. syringae pv. *tomato*	>2000	>2000	600	>2000	>2000	>2000	>2000	1200	>2000	>2000
P. syringae pv. *syringae*	1400	>2000	400	>2000	NT	>2000	>2000	800	>2000	>2000
P. syringae pv. *atrofaciens*	>2000	>2000	600	>2000	>2000	NT	NT	NT	NT	>2000
P. fluorescens	>2000	>2000	1000	>2000	>2000	>2000	>2000	1000	>2000	>2000
P. corrugata	>2000	>2000	600	>2000	>2000	>2000	>2000	800	>2000	>2000
Agrobacterium vitis	NT	NT	NT	NT	>2000	>2000	>2000	800	>2000	NT
Candida sake	1800	1600	200	>2000	1000	1400	>2000	600	800	400
Kluyveromices marxianus	>2000	1800	200	1200	>2000	1600	>2000	800	1000	200
Pichia membranaefaci	1600	600	200	1000	1200	800	>2000	400	400	200
Zygosaccharomyces bailii	1000	200	200	400	800	1000	1400	400	400	200
Torulaspora delbrueckii	1600	800	200	600	1200	1400	>2000	400	600	200
Schizosaccharomyces japonicus	1600	1200	200	800	800	1800	>2000	500	600	200
S. pombe	1000	1000	200	600	800	800	>2000	400	600	200
Saccharomyces cerevisiae	1400	600	200	600	800	1600	>2000	400	600	200
Antioxidant activity	**6**	**12**	**20**	**6**	**6**	**6**	**6**	**24**	**13**	**17**

Microbial species	Essential oil[c]									
	K	L	M	N	O	P	Q	R	S	T
Bacillus thuringiensis	>2000	>2000	>2000	1200	600	600	>2000	1200	400	400
B. brevis	800	600	800	400	400	200	600	600	200	400
B. laterosporus	800	600	1200	800	400	200	NT	600	400	NT
B. subtilis	>2000	>2000	1800	1400	400	400	>2000	800	400	400
B. polymyxa	1000	1000	600	1800	400	400	>2000	1000	200	400
B. licheniformis	>2000	>2000	>2000	1800	400	400	>2000	1000	400	400
B. cereus	>2000	>2000	>2000	1200	600	600	>2000	1000	400	400
B. amylolyticus	1400	1000	1000	400	400	400	800	600	200	400
Lactococcus lactis subsp.lactis	>2000	>2000	>2000	400	400	NT	>2000	600	400	600
L.lactis subsp. cremoris	>2000	>2000	>2000	200	200	200	>2000	1200	200	600
Enterococtus faecium	>2000	>2000	>2000	>2000	600	800	>2000	>2000	800	1000
E. faecalis	>2000	>2000	>2000	>2000	1000	600	>2000	>2000	800	1000
Streptococcus salivarius	>2000	>2000	>2000	400	400	NT	>2000	1200	200	400
S. salivarius subsp. thermophilus	1000	1200	1400	400	400	400	1200	1200	400	600
Lactobacillus. helveticus	>2000	>2000	>2000	1200	400	400	>2000	>2000	400	600
L. acidophilus	>2000	>2000	>2000	1200	600	400	>2000	>2000	600	600
L. brevis	>2000	>2000	>2000	1200	600	600	>2000	>2000	600	800
L. casei	>2000	>2000	>2000	>2000	800	600	>2000	>2000	800	1000
L. reuteri	>2000	>2000	>2000	>2000	1000	600	>2000	NT	1400	1200

Microbial species	Essential oil[c]									
	K	L	M	N	O	P	Q	R	S	T
L. delbrueckii subsp. bulgaricus	>2000	>2000	>2000	800	600	NT	>2000	1400	400	400
L. delbrueckii subsp. delbruecki	>2000	>2000	>2000	1200	600	NT	>2000	>2000	600	600
Clostridium. sporogenes	800	200	600	400	200	400	>2000	1600	200	400
C. beijerinckii	1000	200	800	600	400	200	>2000	1400	400	200
C. butyricum	>2000	>2000	>2000	600	400	400	>2000	1600	400	600
C. acetobutylicum	800	800	200	400	400	200	>2000	1200	200	400
C. bifermentans	>2000	>2000	>2000	>2000	800	800	>2000	>2000	800	1600
C. pasteurianum	>2000	>2000	NT	600	600	400	>2000	1200	200	400
C. tyrobutyricum	>2000	>2000	>2000	400	600	400	>2000	1000	400	600
C. putrefaciens	>2000	>2000	>2000	NT	400	400	NT	NT	400	NT
Bifidobacterium. adolescentis	NT	NT	NT	1200	NT	1000	>2000	>2000	600	NT
B. bifidum	>2000	>2000	>2000	1400	600	NT	>2000	>2000	600	600
B. breve	>2000	>2000	>2000	1400	600	400	>2000	>2000	600	400
B. infantis	>2000	>2000	1200	1400	600	600	>2000	>2000	400	800
B. longum	>2000	>2000	>2000	1800	800	600	>2000	>2000	600	1200
B. catenulatum	>2000	>2000	>2000	>2000	800	600	>2000	>2000	600	1200
B. pseudocatenulatum	>2000	>2000	>2000	>2000	1200	1000	>2000	>2000	1000	>2000
Erwinia carotovora subsp.carotovora	>2000	>2000	>2000	>2000	400	600	>2000	1200	600	400
Xanthomonas pruni	>2000	>2000	>2000	>2000	400	400	>2000	1000	400	400
Pseudomonas viridiflava	>2000	>2000	>2000	>2000	>2000	1000	>2000	>2000	>2000	>2000
P. syringae pv. *tomato*	>2000	>2000	>2000	>2000	2000	600	>2000	>2000	>2000	>2000
P. syringae pv. *syringae*	>2000	NT	>2000	>2000	400	400	>2000	1200	400	400
P. syringae pv. *atrofaciens*	>2000	>2000	>2000	NT	NT	1000	NT	NT	1600	NT
P. fluorescens	>2000	>2000	>2000	>2000	>2000	1600	>2000	>2000	>2000	1000
P. corrugata	>2000	>2000	>2000	>2000	1000	1000	>2000	>2000	1400	1200
Agrobacterium vitis	NT	>2000	NT	>2000	800	NT	>2000	1400	NT	800
Candida sake	>2000	>2000	1600	400	200	200	>2000	1000	400	400
Kluyveromices marxianus	>2000	>2000	1800	1000	400	200	1600	2000	400	400
Pichia membranaefaci	800	1200	1400	400	200	200	1400	1200	200	200
Zygosaccharomyces bailii	600	1200	1200	400	200	200	800	1000	200	200
Torulaspora delbrueckii	600	1400	1400	400	200	200	1400	1000	200	200
Schizosaccharomyces japonicus	>2000	>2000	1400	800	200	200	1000	1000	200	200
S. pombe	600	1200	1400	600	200	200	1200	1000	200	200
Saccharomyces cerevisiae	800	1600	1200	400	200	200	800	600	200	200
Antioxidant activity	**7**	**6**	**18**	**14**	**22**	**26**	**7**	**20**	**18**	**22**

[a] MICs range 200-2000 ppm. [b] Diameter of colour retention (filter size 6mm). [c] Key to essential oils: A basil, B boldo, C cinnamon, D cypress, E french tarragon, F bitter fennel, G sweet fennel, H clove, I geranium, J juniper, K lavender "abrialis", L lavender "grosso", M lavender "supera", N peppermint, O oregano, P spanish oregano, Q rosemary, R sage, S winter savory, T thyme.

Technical Communications

ESSENTIAL OIL YIELD FROM DIFFERENT PLANT ORGANS AND VARIOUS CORIANDER - ACCESSIONS

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INTRODUCTION

Essential oil from *Coriandrum sativum* L. seeds, commonly known as coriander seed oil, is one of the most widely used essential oils in the fragrance and flavour industry. The composition of coriander seed oil has been the subject of numerous studies (1, 2, 3, 4).

Recently, however, the essential oil obtained from the whole plant (commercially known as cilantro oil) has appeared on the market. Analytical data on this oil seem to be meagre. In one of the relevant publications Lawrence (4) reviewed the work by Potter and Fagerson on the content of volatile extracts from freshly harvested plants which was difficult to compare with FID or TCD. Lawrence (5) found differences in the composition of essential oil obtained from coriander fruits and the vegetative parts of the plant. To our knowledge, there is virtually no data on the yield of different plant organs incl. the aerial plant parts.

This fact has directed our attention to the necessity to examine the essential oil yield of different plant organs In order to filter out the influence of possible within species variations of *Coriandrum sativum* different accessions will be considered for evaluation.

EXPERIMENTAL

Different accessions of *Coriandrum sativum* L. collected from various regions of Ethiopia were cultivated at Wondo Genet Experimental Station. The soil is sandy loam, slightly acidic in nature, characterized by good fertility rate. 4 x 8 m plots were designed, with 8 rows (o.5 m between rows) and seeds were drilled in the row and latter thinned to approximately 15 - 20 cm between plants. The planting was carried out during the rainy season to make sure adequate moisture during the growth period. No fertilizers, pesticides or herbicides were used. Two manual weedings were carried out.

One part of the plants was harvested in full flowering. Biomass yield of the individual plant organs, i.e. stem, leaves, flower and arial parts was determined. Subsequently, the plant parts were distilled by using a Clevenger-apparatus to quantify the essential oil content. The second half of the plants remaining in the plots were allowed to ripen seeds. The dried seeds were crushed and distilled to determine their essential oil content.

GC-MS Analysis

GC-MS analysis was carried out using a Fison Model 8000 series chromatograph coupled to MD 800 mass detector (70 ev) with Helium as carrier gas. DB-17 fused silica capillary column (30 m x 0.25 mm I.D.) was used, program: 50 - 210 °C at a rate of 4 °C/min with a final hold of 4 min. Identification of the components was based on the result of a library search in the NIST and WILEY MS database.

For analytical purposes the accession 208966 was used due to its higher yield.

RESULTS AND DISCUSSION

Essential oil production

The maximum of oil yield was produced by the aerial parts of plants, in all of the seven accessions investigated. Its amount varied between 15.73 - 34.57 kg/ha (Table 1). This was followed by the oil- - yield of leaves (6.61 - 21.16 kg/ha) and seeds ((2.25 - 9.13 kg/ha). The rate of essential oil production has proved to be mainly a function of the plant dry-mass, which was highest in the case of aerial parts (78.65 - 85.9 dt/ha), followed by the leaves (44.00 - 60.00 dt/ha).

In all accessions, the relative essential oil content was highest in the flowers (0.4 - 0.7 %) followed by the seeds (0.2 - 0.54 %) (Table 2). Lowest essential oil amounts were contained by the stems 0.08 - 0.30 %).

Table 1. Total essential oil yield (*(kg/ha)* of coriander accessions according to plant organs

Number of	Essential Oil Yield				
Accession	Leaves	Stems	Flowers	Arial Parts	Seeds
223114	6.61	1.76	1.32	34.57	5.57
211503	8.01	2.85	1.75	17.93	2.25
223289	16.41	2.20	0.99	15.73	3.68
208966	21.16	7.50	1.61	27.27	9.13
219806	7.80	2.30	1.16	25.77	4.24
223066	11.60	2.60	1.60	17.60	6.80
223068	8.80	4.90	1.44	16.40	5.70

Table 2. Relative essential oil content (%)of coriander accessions according to plant organs

Number of	Relative Essential Oil Content				
Accession	Leaf	Stem	Flower	Aerial Parts	Seed
223114	0.12	0.08	0.4	0.43	0.35
211503	0.15	0.1	0.5	0.21	0.25
223289	0.3	0.1	0.51	0.2	0.2
208966	0.4	0.3	0.7	0.34	0.54
219806	0.13	0.1	0.4	0.3	0.35
223066	0.2	0.1	0.4	0.2	0.5
223068	0.2	0.14	0.48	0.2	0.3

Although it was established that the relative essential oil content of accession 208966 was superior in terms of all plant organs, still owing to the higher flower and aerial plant part production of accessions 211503 and 223114 its total essential oil yield has not been ultimately the highest.

Essential oil composition

The essential oil components as identified by GC-MS are listed in Table 3. (Only leaf, stem and dried seed oils were analysed.)

Among the 34 components detected, only p-cymene and linalool were found in all oil samples. The major components of leaf-oils were trans-2-decenol (49.2 %), decanal (27.3 %), trans-2-dodecenal (5.8 %) and decyl alcohol (5.1 %). In the stem trans-2- decenol (50.8 %), decyl alcohol (16.6 %), trans-2-dodecenal (8.8 %), trans-2-undecenal (6.8 %) have been identified as main components.

Remarkably, the components identified in the seed oils were quite different. In these oils linalood was the major components (85.7 %).

Camphor and geranyl acetate were present in slightly lower quantities, 6.2 % and 3.2 %, respectively. The linalool content of this accession is higher than mentioned in the special literature (4).

In terms of other components, we have established that the aliphatic aldehyde decanal was one of the major components of leaf-oils (27. 3 %). This compound is used in low concentrations in blossom fragrance and also in the production of artificial citrus oils.

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Table 3. Coriander essential oil composnents identified according to plant organs

RT	Compounds	Peak area percent		
		Leaves	Stems	Dried seeds
5.3	n-Nonae	0.1	-	-
7.7	α-Pinene	-	-	0.9
8.6	Camphene	-	-	0.2
9.7	ß-Pinene	-	-	0.1
10.1	ß-Myrcene	-	-	0.4
11.6	D-Limonene	-	-	1.1
12.0	n-Caprylaldehyde	0.6	0.3	-
12.3	ß-Phellandrene	-	-	0.1
12.7	p-Cymene	0.3	0.2	0.3
13.3	r-Terpinene	-	-	1.0
14.1	Unknown	0.2	0.3	-
14.5	Linalool oxide	0.6	0.3	-
14.6	α-Terpinene	-	-	0.4
15.5	Linalool	0.6	4.0	85.7
15.6	Nonaldehyde	0.4	0.3	-
18.0	Unknown	-	-	0.1
18.3	Cis-2-Nonenal	0.1	-	-
19.3	n-Decanal	27.3	-	-
19.5	Camphor	-	-	6.2
19.8	Linalool-Z-pyranic oxide	0.4	-	-
21.6	Decyl alcohol	5.1	16.6	-
22.1	trans-2-Decenol	49.2	50.8	-
22.3	trans-Geraniol	-	-	t
22.9	n-Undecanal	1.5	3.5	-
23.6	Neral	-	-	0.1
25.2	Sabinyl acetate	-	-	0.1
25.3	trans-2-Undecenal	3.3	6.8	-
26.2	Lauraldehyde	1.2	1.4	-
26.8	Geranyl acetate	-	0.2	3.2
28.1	Unknown	0.2	-	-
28.6	trans-2-Dodecenal	5.8	8.8	-
31.7	Unknown	0.3	0.4	-
32.5	Unknown	-	0.2	-
34.7	trans-2-Tridecenal	2.5	5.3	-

QUALITATIVE - QUANTITATIVE CHARACTERISTICS OF AUTOCHTONOUS CHAMOMILE POPULATIONS ON THE EAST - SLOVAKIAN LOWLANDS

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INTRODUCTION

Chamomile, *Chamomilla recutita* (L.) Rauschert belongs to the most popular medicinal plants. Composition of the essential oil (0.2 - 1.0 % in dry matter, polyploid cultivated varieties as far as 1.5 %) is the most important for medicinal use.

Our research was aimed at the etheric oil content and variability in the composition of naturally growing population on the East-Slovakian lowlands.

MATERIAL AND METHODS

Plant material (chamomile anthodia) was collected from natural sites on the East-Slovakian lowland. The list of sites is included in the Table 1.

The flower drug was dried after harvest under room-temperature conditions.

Chamomile essential oil was isolated by hydrodistillation. Distillation lasted for 2 hours, sample weight was 2 g of ground dry anthodium-matter. The modified 'Coocking and Middlelon' distillation apparatus was used.

Composition of the oils was determined by capillary GC analysis: HEWLET-PACKARD 5890 Series II with the flame ionization detector and Split-splitless system for injection. The column used was fused silica HP-5 (50 m x 0.20 mm).

The following temperature program was used: 90 °C (0 min.), then 10 °C/min. to 150 °C (5 min.), then 5 °C/min. to 180 °C (3 min.), then 7 °C/min. to finally isothermal 250 °C for 25 min.. Injection port was at 150 °C, detector 280 °C, nitrogen was used as carrier gas.

Peak areas and retention times were measured by electronic integration with HP 3396 Series II integrator. The main components of the etheric oil were determined by using a standard pure compound on the basis of the technical literature.

RESULTS AND DISCUSSION

Percentage of essential oil content in the dry chamomile matter collected from the certain natural sites of the East-Slovakian lowland are presented in Table 2. The table also presents the qualitative-quantitative characteristics of the oil (main components) as determined by gas chromatography.

Chamomile, *Chamomilla recutita* (L.) Rauschert can be found in the secondary plant communities on the East-Slovakian lowland, such as trodden societies of dry and moist soils, weed societies and dump societies.

The large scale cultivation of chamomile is based on bred diploid and tetraploid cultivars, in Slovakia. The essential oil content of these varies between 0.99 % to 1.35 %. The crude drug collected from natural sites has a lower percentage of the essential oil, in average.

There are various provenances and different dry matter quality chamomiles on the world market. Until the 1970ies, the etheric oil quantity and its chamazulene content were important for quality evaluation. The present improvement of analytical methods indicate the importance of the other components of chamomile etheric oil, such as - I - Alpha - bisabolol. Comparison of etheric oil quality of chamomile from natural habitats with large-scale cultivated chamomile shows that cultivated chamomile has a better quality. Diploid variety „BONA" has from 40.00 to 44.74 % of highly phytotherapeutically effective compound -I- Alpha-bisabolol, tetraploid variety „GORAL" about 30 %. Chamomile collected from natural habitats has-I-Alpha-bisabolol content from 3.37 to 9.62% (Table 2). Bred varieties have a high content of chamazulene, about 7 % in average. A high content of less important compounds, such as-I-Alpha-bisabololoxides A and B is typical for chamomile populations on the East-Slovakian lowland (Table 2). It was also ascertained that the bisabololoxide chemotype of chamomile is present on the East - Slovakian lowlands.

Table 1. List of natural sites of chamomile collection

1.	Vybuchanec- ESL - altitude 123 m, field road beside wheat stand, 1st June 1995
2.	Nizný Hrabovec- ESL - hilly region, alt. 131 m, near a road, a border of a wheat stand
3.	Vranov n.Toplou-ESL - hilly region, alt. 132 m, near a viaduct directed to Sacurov, 1st June 1995
4.	Moravany - ESL - lowland region, alt. 110 m, near a wheat stand 1st June 1995
5.	Michalovce - ELS - lowland region, alt. 140 m, around a city border, 8th June 1995
6.	Bajany No. 1 ESL - lowland area, alt. 107 m, Lucerne stand, near the road Vysoka to Bajany, 1st June 1995
7.	Bajany no. 2 ESL - lowland area, alt. 107 m, a boarder of a wheat stand close to the road to Velké Kapusany, 8th June 1995
8.	Vysoká nad Uhom - experimental research base of the Research Inst., lowland area, alt. 109 m, the bank of river Uh, 1st June 1995
9.	Vojany - ELS lowland region, alt. 109 m, the road to Královský Chlmec, 1st June 1995
10.	Sírnik - ELS -lowland region, alt. 125 m, near the bridge of river Ondava, 1st June 1995

Table data are presented as follows: site number, name of locality, altitude, site characteristic, and date of collection

486

Table 2. Essential oil content and qualitative - quantitative composition (in % of the oil) of chamomile plants collected from natural sites of the East - Slovakian Lowlands

No.	Location	Ess. oil % (dry flower)	Farnesene	Bisabolol oxide β	α - Bisabolol	Chamazulene	Bisabolol oxide - A	En-yn-dicycloethers trans-	cis-
1.	VYBUCHANEC	0.97	6.03-6.06	17.00-17.09	6.60-6.64	5.45-5.51	43.09-43.65	5.39-5.44	4.19-4.23
2.	N. HRABOVEC	0.61	3.03-3.86	22.70-23.42	4.82-4.96	10.17-10.80	34.10-34.60	09.26-10.31	1.03-1.07
3.	VRANOV n/T	0.82	4.06-4.14	10.30-10.35	5.23-5.23	7.60-7.61	48.20-48.32	11.16-11.52	1.41-1.43
4.	MORAVANY	0.93	4.68-4.71	15.27-15.47	9.36-9.62	9.87-9.93	37.96-38.07	11.14-11.21	-
5.	MICHALOVCE	0.91	1.20-1.21	21.37-21.48	5.14-5.16	7.55-7.58	40.44-40.45	7.50-7.58	6.52-6.61
6.	BAJANY*1	0.74	1.49-1.50	0.38-0.39	5.10-5.11	7.42-7.43	52.83-52.90	10.45-10.51	5.26-5.32
7.	BAJANY*2	0.67	11.47-11.44	0.70-0.70	4.93-4.95	4.51-4.52	43.32-43.85	9.48-9.70	2.17-2.23
8.	VYSOKÁ n/U	0.96	7.91-8.15	0.60-0.62	5.13-5.22	10.09-10.19	48.92-48.94	8.34-8.79	0.33-0.40
9.	VOJANY	0.60	10.51-10.57	0.57-0.58	3.37-3.38	4.99-5.03	48.35-48.41	13.04-13.05	1.02-1.02
10.	SÍRNIK	0.86	4.05-4.08	0.41-0.41	6.20-6.26	10.60-10.80	43.10-44.07	7.70-7.98	-

ESSENTIAL OILS AT DIFFERENT DEVELOPMENT STAGES OF ETHIOPIAN *TAGETES MINUTA* L.

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INTRODUCTION

Tagetes minuta L. is a common (noxious) weed that grows in tropical and subtropical countries. Originating from South America it grows widely spread in different parts of Ethiopia, causing serious damage in cultivated areas. The essential oil from *T. minuta* is an established perfumery raw material. The introduction of this plant species to produce essential oil aside from bringing addition income, will also lessen the problem of weed from cultivated crops. The plant thrives well without any particular attention. It has been reported that the chemical composition of the oils vary according to the seed origin and local conditions (1). Thappa *et al.* (2) reported that different oil composition can be obtained by harvesting the crop at different growth stages. Thus a desired composition of essential oils can be obtained.

In this paper we examined the total oil yield obtainable and the composition of the oil at different stages of development.

EXPERIMENTAL

The experiment was conducted at Wondo Genet 7°3' N latitude, 38 °39' E longitude and 1850 m altitude, with 12 h photoperiod.

Seeds were drilled 50 cm spacing. At about 15 cm plant height they were thinned to 15 - 20 cm in the row. Since it was in the dry period, plants were irrigated according to necessity.

Yield: Plants were harvested at different stages of maturity before flower budding, at flower budding, at full flowering and at immature seed development. Total yield and leaf yield and the oil content per plant has been determined using Clevenger-type apparatus.

Analysis: The GC-MS analysis was carried out using Fison GV Model 8000 series chromatograph coupled to MD 800 mass detector (70 eV) with Helium as carrier gas. The column used was DB-17 fused silica capillary column (30 m x 0.25 mm I.D.) programmed at 50 - 210 °C at a rate of 4 °C/min with a final hold time of 4 min. Identification of the components was based on a library search of NIST and WILEY MS database.

RESULTS AND DISCUSSION

Preliminary studies showed none or trace amounts of oils in stems, therefore leaf and flower parts were considered for the evaluation. As it was indicated in Table 1. the total leaf and flower yield was in the range of 84.7 and 90.5 qt/ha. However, after full flowering the total quantity of leaves and flowers decreased drastically due to leaf shedding and also water loss from the whole plant.

Table 1: *Tagetes minuta* L. herbage yield (excluding stem) at different stages of maturity

Plant organs	Before flower budding	At flowering	Full flowering	Immature seeds
		(*qt/ha*)		
Leaf	90.5	51.5	43.5	9.5
Flower		29.9	41.2	36.4
Total	90.5	81.4	84.7	45.9

Before flower budding, the oil yield was 0.45 % (Table 2), it was at its minimum when flower buds appear (0.3 %). Essential oil yield culminated at the immature seed stage (1.34 %).

This result slightly differs from data by Thappa *et al.* (2),who studied the essential oil yield at the phenophases flower buds through seed shedding.

Table 2: Oil yield of *Tagetes minuta* at different stages of maturity

	Oil content (%)	Oil yield (*kg/ha*)
Before flower budding	0.5	40.7
Flower budding	0.3	24.4
Full flowering	0.5	42.3
Immature seed	1.34	61.5

They have indicated a four-fold oil yield increase according to the developmental stages of the plant. Minimum was recorded at the flower bud stage (0.33 %) and maximum at seed shedding stage (1.25 %).

Variations in the essential oil yield and composition of *T. minuta* has been documented also by other studies (3, 4, 5).

Table 3 shows the major components found in the four *T. minuta* oils analyzed in the present study. The percentage of cis-Ocimene has shown an increase from 7.2 % to 37.5 % as the plant matures which is in agreement with the study of Lawrence (1). In our analysis only traces of dihydro-Tagenone was found which does not agree with the results of Chalchat *et al.* (5). We have found low content of cis- and trans-Tagetone in general. However, cis- and trans-Ocimenone were found in larger concentration than that given in the special literature (1, 5). The concentration of cis-Ocimenone has shown a continuous decrease from 37.1 % to 13.1 % as the plant matures which was confirmed by other authors (1). In general, the chemical composition of the *Tagetes*-essential oil investigated is in agreement with the previous reports (1, 2, 6).

Based on these results, it seems to be possible to get a desired essential oil composition and yield altering the harvesting time.

ACKNOWLEDGEMENTS

The financial assistance provided by Swedish International Development Cooperation Agency (SIDA) to run the essential oil project is greatly acknowledged. Authors express their special thanks to Dr. Ermias Dagne for his GC-MS analyses.

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Table 3: Components identified from four oils obtained at different stages of development of *Tagetes minuta* L.

RT	Compounds	Peak area (%)			
		Before flower budding	**At flower budding**	**Full flower**	**Immature seed**
14.0	Limonene	2.2	3.8	1.6	t
14.9	cis Ocimene	7.2	10.4	26.0	37.5
15.3	Eucalyptol	t	t	2.5	t
15.9	Dihydro-Tagetone	t	t	0.6	t
18.2	Linalyl-acetate	t	t	1.1	t
21.0	cis-Tagetone	5.1	3.1	4.7	2.4
21.8	trans-Tagetone	2.7	3.2	4.7	3.4
22.6	Camphor	-	-	1.0	-
25.8	cis-Ocimenone	37.1	31.2	14.4	13.1
26.5	trans-Ocimenone	42.4	46.7	35.0	41.3
33.0	Methyl-Cinnamate	-	-	1.4	-

t = in traces

Index-1.doc 97-12-15 18:05
p. 1

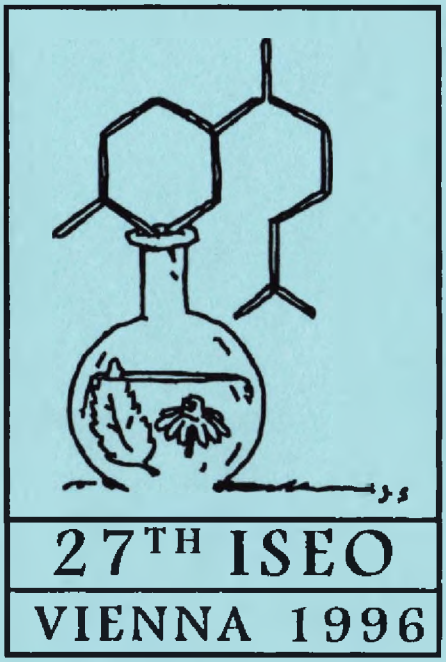
Index

27th INTERNATIONAL SYMPOSIUM ON ESSENTIAL OILS

(27. Internationales Symposium über Ätherische Öle)

September 8-11, 1996 - Wien/Vienna, Austria

Final Program



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Scientific Program

1. Biological activity of essential oil(s) (compounds)
2. Genetic aspects of essential oil formation
3. Physiological aspects of essential oil production
4. Analytics - technical aspects
5. Analytics - chemical aspects
6. Production of essential oils
7. Essential oils as phytogenic feed additives

Sunday, 08th September

18:00 Get together

Monday, 09th September

09:00 Opening

09:15 Chairperson: G. Franz

W. Kubelka, *Vienna, Austria*
Essential Oils - some remarks on problems and achievements

10:00 Coffee Break

Chairpersons: H. P. Muenzing, J. J. C. Scheffer

10:30 Plenary Lecture

1 - 1 **S. v. Toller**, *Warwick, United Kingdom*
A psychologist examines the use of essential oils in aromatherapy techniques

11:00 Short Communications

1 - 2 **J. Ilmberger**, *Munich, Germany*
Essential oils and human vigilance

1 - 3 **A. Lenhardt**, *Vienna, Austria*
Analysis of the chiral fragrance compounds (+)/(-)-carvone in biological fluids and tissues

1 - 4 **M. Lis-Balchin**, *London, United Kingdom*
Correlation of the chemical profiles of essential oil mixes with their relaxant or stimulant properties in man and smooth muscle preparations in vitro

12:00 Lunch

Monday, 09th September

Chairpersons: Y. Asakawa, R. Hiltunen

14:00 Plenary Lecture

2 - 1 **R. Croteau**, *Pullman, USA*
Biochemical and molecular genetic aspects of monoterpene formation

14:30 Short Communications

2 - 2 **A. Yuba**, *Kyoto, Japan*
Limonene synthase from *Perilla frutescens*

2 - 3 **J. W. de Kraker**, *Wageningen, The Netherlands*
Germacrene biosynthesis in caraway (*Carum carvi*) and chicory (*Cichorium intybus* L.)

2 - 4 **Y. Holm**, *Helsinki, Finland*
Variation and inheritance of monoterpenes in *Larix* species

15:30 Coffee Break

Chairpersons: G. Lamaty, I. Máthé

16:00 Short Communications

2 - 5 **C. Bischof**, *Hamburg, Germany*
Multivariate statistical analysis as a tool for the definition of chemotypes in essential oil plants - potentials and limitations

2 - 6 **A. A. Tawfik**, *Assiut, Egypt*
Selection for seed yield and earliness in fennel (*Foeniculum vulgare* Mill.) and correlated response in seed-oil yield

1 - 5 **K. H. C. Baser**, *Eskisehir, Turkey*
The chemistry and pharmacology of *Origanum* (Kekik) Water

1 - 6 **K. G. Tkachenko**, *St. Petersburg, Russia*
Antivirus activity of the essential oils of some *Heracleum* L. species

4 - 2 **K. H. Kubeczka**, *Hamburg, Germany*
New approaches in essential oil analysis using polymer-coated silica fibers

evening free disposal

Tuesday, 10th September

Chairpersons: A. Baerheim-Svendsen, K. H. C. Baser

9:00 Plenary Lecture

3 - 1 **A. C. Figueiredo**, *Lisbon, Portugal*
Physiological aspects of essential oil production

9:30 Short Communications

3 - 2 **J. Bernáth**, *Budapest, Hungary*
Variability of essential oil accumulation in fennel affected by ecological conditions and development

3 - 3 **M. R. Kolalite**, *St. Petersburg, Russia*
Essential oil storage and secretion in glands of some *Lamiaceae* (an ultrastructural study)

3 - 4 **I. Hook**, *Dublin, Ireland*
Inulin as carbon source in the culture of edelweiss hairy roots

10:30 Coffee Break

Chairpersons: A. Mosandl, R. Tabacchi

11:00 Plenary Lecture

4 - 1 **A. Nikiforov**; *Vienna, Austria*
Fragrance analysis using semiautomatic spectra (MS, ~~NMR~~ IR) interpretation and olfactoric data

11:30 Short Communications

4 - 3 **C. Bicchi**, *Torino, Italy*
Supercritical fluid extraction (SFE) as a fractionation technique for vegetable matrices

4 - 4 **J. Casanova**, *Ajaccio, France*
Carbon-13 NMR as a tool for enantiomeric differentiation of terpenes in essential oils

12:15 Lunch

Tuesday, 10th September

Chairperson: D. Joulain

14:00 Plenary Lecture

5 - 1 **C. Menut**, *Montpellier, France*
Chemical exploration of Brazilian aromatic species belonging to the *Myrtaceae* family

14:30 Poster Session

Introduction: J. Jurenitsch, H. Greger

Chairpersons: C. Bicchi, R. Naef

16:15 Short Communications

5 - 2 **Y. Asakawa**, *Tokushima, Japan*
Volatile components of selected liverworts

5 - 3 **W. A. König**, *Hamburg, Germany*
Identification of new sesquiterpenes in liverworts

5 - 4 **D. Wolf**, *Berlin, Germany*
Constituents of the Haitian Vetiver oil

5 - 5 **G. Tümen**, *Balikesir, Turkey*
The essential oils of *Satureja* occurring in Turkey

4 - 5 **H. Schilcher**, *Berlin, Germany*
New method for the determination of organochlorine pesticides in essential oils and pesticide residues data from 110 essential oil samples

20:00 Symposium Dinner

"Restaurant zur Schönen Aussicht", Pfarrplatz, Heiligenstadt

Wednesday, 11th September

Chairpersons: J. Iglesias, E. Stahl-Biskup

9:00 Plenary Lecture

7 - 1 **A. Mathé**, *Budapest, Hungary*
Essential Oils as phytogenic feed additives

9:30 Short Communications

7 - 2 **G. Wheeler**, *Priddy Wells, UK*
Feeding herbal ingredients produces a performance enhancement in fattening swine

6 - 2 **K. P. Svoboda**, *Ayr, Scotland*
Influence of storage on quantity and quality of essential oil yield from twenty herb species

10:15 Coffee Break

Chairpersons: G. Schmaus, M. A. Foglio
Short Communications

10:45 6 - 3 **T. De Silva**, *Vienna, Austria*
Development of essential oil industries in developing countries: Prospects and Constraints

6 - 4 **S. K. Chatterjee**, *Calcutta, India*
Piper betle leaf oil: Factors affecting production and composition

6 - 5 **K. Dürbeck**, *Raubling, Germany*
Design and construction of field destillation equipment in El Salvador

11:30 Plenary Lecture

6 - 1 **M. H. Boelens**, *Huizen, The Netherlands*
Production of essential oils

12:00 Closing Session

B. M. Lawrence, *Winston-Salem, USA*
A critical summary

Final Discussion
Closing Remarks

Wednesday, 11th September

14:00	Professional tour to AKRAS Essences, Heiligenkreuz Abbey, Mayerling Castle, Southern Wienerwald Botanical Excursion and "Heuriger" in Gumpoldskirchen
	For ICMAP Members only:
14:30	ICMAP Board Meeting at the Symposiums Venue

Accompanying Persons Program: Please contact the Registration Desk !

- On the trail of the Habsburgs
- Art Nouveau in Vienna
- Historical Vienna

Social Program

- Get together
- Symposium Dinner (offered by a sponsor)

The Symposium's Organizers are not responsible for any accommodation problems and not liable for losses, thefts or damages during the Conference.

Schedule

MONDAY	TUESDAY	WEDNESDAY
09:00 - 10:00	*09:00 - 10:30*	*09:00 - 09:50*
Opening, Plenary	3. Physiological aspects of essential oil production	7. Essential oils as phytogenic feed additives
Break	Break	
10:30 - 12:00	*11:00 - 12:15*	*09:50 - 10:10*
1. Biological activity of essential oil(s) (compounds)	4. Analytics - technical aspects	6. Production of essential oils
Lunch	Lunch	Break
14:00 - 15:30	*14:00 - 14:30*	*10:30 - 12:00*
2. Genetic aspects of essential oil formation	5. Analytics - chemical aspects	6. Production of essential oils
Break		
16:00 - 16:40	*14:30 - 16:00*	*12:00 - 12:30*
2. Genetic aspects of essential oil formation	Poster Session	Summary
16:40 - 17:30	*16:15 - 17:35*	Lunch
1. Biological activity of essential oil(s) (compounds)	5. Analytics - chemical aspects	
17:30 - 18:00	*17:35 - 18:05*	*14:00 - 21:00*
4. Analytics - technical aspects	4. Analytics - technical aspects	Excursion
20:00	*20:00*	*14:30*
FFJ Sci. Board Meeting	Symposium Dinner	ICMAP Board

ABOCA - SANSEPOLCRO (AR)

MED'AROM WIEN

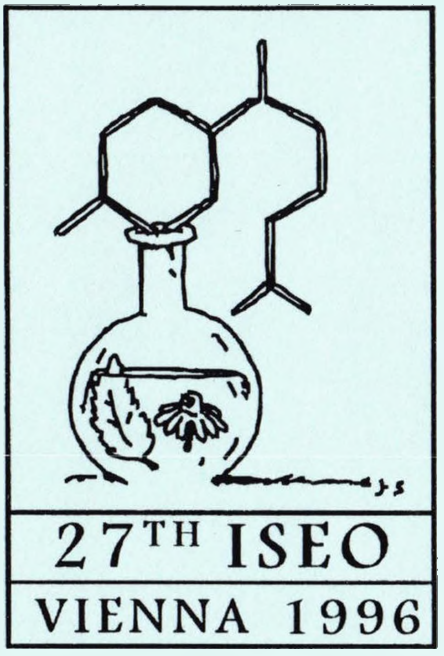
27th INTERNATIONAL SYMPOSIUM ON ESSENTIAL OILS

(27. Internationales Symposium über Ätherische Öle)

September 8-11, 1996 - Wien/Vienna, Austria

Final Circular

Provisional Program



Symposium Secretariat and Mailing Address:

Prof. Dr. Ch. Franz Mrs. E. Michel

Institut für Botanik und Lebensmittelkunde
der Veterinärmedizinischen Universität Wien
Josef-Baumann-Gasse 1
A-1210 Wien

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during the Symposium only	0664 1608363
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R. Eberhardt
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H. Greger
J. Jurenitsch

Organizing Commitee:

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T. Langer
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J. Novak
M. Hinterreiter
U. Koller
P. Lebada
A. Sunara

the staffs of the Institute for Botany and Food Science, VMU and the Institute of Pharmaceutical Chemistry, Vienna

Venue: Pharmaziezentrum (UZA II), University of Vienna, Althanstraße 14, A-1090 Wien

Access: Underground U4 or U6 (station "Spittelau")
Tram D or Bus 35A (station "Liechtenwerder Platz")

Registration

The registration desk is located in the Entrance Hall of the Pharmaziezentrum and will be opened: Sunday from 5 to 8 p.m., Monday and Tuesday from 8 a.m. to 6 p.m.

Sponsors

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Bionorica
Dragoco
Krems Chemie
Merck
Spectronex
Waldland

Preliminary Program

Sunday, 08th September

18:00 Get together

Monday, 09th September

09:00 Opening

09:15 **W. Kubelka**, *Vienna, Austria*
Essential Oils - some remarks on problems and achievements

10:00 Coffee Break

Plenary Lecture

10:30 1 - 1 **S. v. Toller**, *Warwick, United Kingdom*
Biological activity of essential oil(s)

Short Communications

11:00 1 - 2 **J. Ilmberger**, *Munich, Germany*
Essential oils and human vigilance

1 - 3 **A. Lenhardt**, *Vienna, Austria*
Analysis of the chiral fragrance compounds (+)/(-)-carvone in biological fluids and tissues

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Herbal feed ingredients as an aid to livestock production in the United Kingdom

Plenary Lecture

11:30 6 - 4 **M. H. Boelens**, *Huizen, The Netherlands*
Production of essential oils

12:00 Closing Session
Final Discussion
Announcement of the Poster Price Winners

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General Information

Vienna is located at the northeastern end of the Alps at the river Danube. In September warm summer temperatures around 20-25°C can be expected, but also a cloudy and rainy weather is possible.

Participation fee (ATS)

until 15th July 1996	after	
1.300,--	1.500,--	full participants
600,--	700,--	accompanying persons
500,--	600,--	students (with legitimation)

The participation fee includes

- Get together on Sunday evening
- Access to all lectures and presentations
- Abstract booklet and Proceedings (full participants)
- Refreshments during breaks
- 1 (one) sightseeing tour Vienna (accomp. persons)

Payment

Symposium fee must be paid in advance by cheque or remittance in ATS made payable to:

Institut für Botanik u. Lebensmittelkunde, 27th ISEO

	bank code	account
Creditanstalt Bankverein	11000	0864-60011/00
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Cancellation

If cancellation notice is received before 15th August 70% of the fee will be refunded. Afterwards no refund will be possible.

Accompanying Persons Program

- On the trail of the Habsburgs
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AUSTRIAN AIRLINES

OFFICIAL CARRIER

27th INTERNATIONAL SYMPOSIUM ON ESSENTIAL OILS

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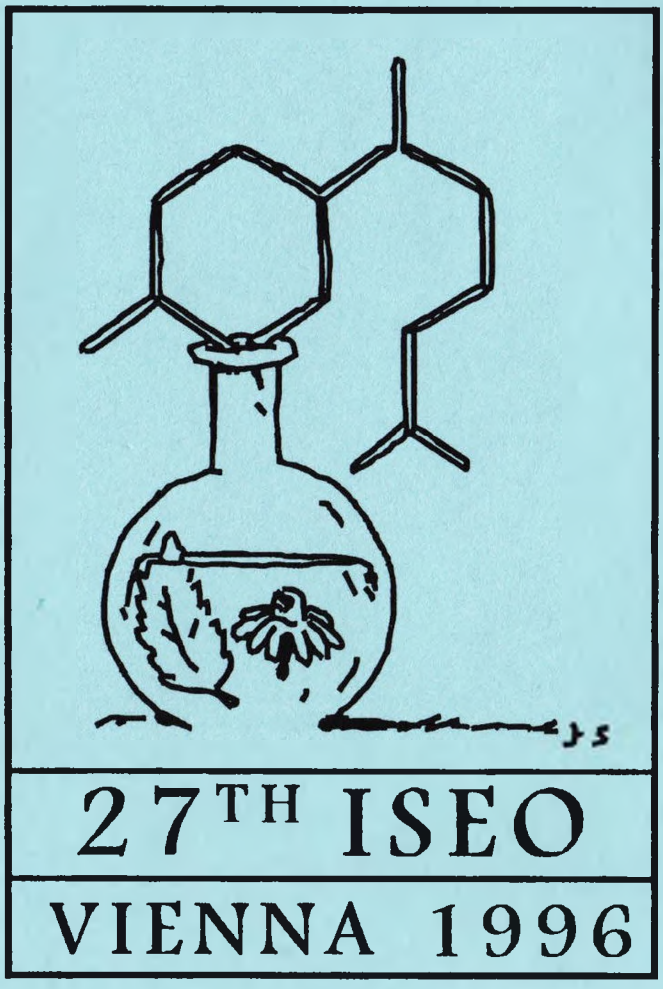
Poster Titles

List of Participants



POSTER

BIOLOGICAL ACTIVITY OF ESSENTIAL OIL(S) (COMPOUNDS)

P1-01	A. Pauli K.H. Kubeczka	Evaluation of inhibitory data of essential oil constituents obtained with different microbiological testing methods
P1-02	G. Brandi B. Biavati M. Pesenti P. Matarelli	Essential oils and antibacterial activity against Helicobacter pylori
P1-03	N.V. Kazarinova L.M. Muzychenko K.G. Tkachenko A.M. Snurgaya O.V. Pavlova N.G. Kolosov V.P. JijinO.D. Bondarenko	Essential oils as effective sanators of hospital infections
P1-03	F. Crociani B. Biavati A. Alessandrini G. Zani	Growth inhibition activity of essential oils and other antimicrobial agents towards Bifidobacteria from dental caries
P1-05	D. Kalemba D. Kusewicz K. Swader J. Góra	Antimicrobial properties of the essential oil of Artemisia molinieri Quzél
P1-06	I. Chinou V. Roussis D. Perdetzoglou A. Loukis	Chemical and biological studies on the essential oils of four Helichrysum species growing in Greece
P1-07	R. Piccaglia M. Marotti M. Pesenti P. Mattarelli B. Biavati	Chemical composition and antibacterial activity of Tagetes erecta and Tagetes patula essential oils
P1-08	O. Tzakou E. Verykokidou V. Roussis I. Chinou	The chemical composition and antibacterial properties of Thymus longicaulis subsp. Chaubardii oils: three chemotypes in the same population
P1-09	M. M. Miyazawa H. Shimamura S. Nakamura H. Kameoka	Antimutagenic activity of (+)-ß-eudesmol and paeonol from Dioscorea japonica
P1-10	A.A. Abena J.M. Ouamba A. Keita	Anti-inflammatory, analgesic and antipyretic activities of essential oil of Ageratum conyzoides

P1-11	M. Lis-Balchin	The effect of essential oils on the uterus compared to that on other muscles
P1-12	G. Buchbauer P. Weiß-Greiler P. Wolschann	3-D Studies on Odour Molecules
P1-13	A. Sunara G. Buchbauer	Structure-odour relationships of sandalwood odorants: synthesis of doublebond-modified sanaloles
P1-14	R. Obara C. Wawrzenczyk S. Lochynski R. Gancarz J. Góra	Studies on the odor structure relationship of some terpene substituted dioxanes and dioxolanes
P1-15	I. Valterová B. Kalinová K. Sjödin	Monoterpenes from Cuban pines and their possible role in the host-plant recognition by Dioryctria horneana

GENETIC ASPECTS OF ESSENTIAL OIL FORMATION

P2-01	H.J. Bouwmeester M.C.J.M. Konings	Regulation of essential oil formation in Caraway and possibilities for modification
P2-02	K. Umemoto	Stereochemistry of four 1,2-epoxymenthyl acetates isolated from S1 oils of Mentha rotundifolia
P2-03	A. Bélanger S. Khanizadeh	Classification of 92 strawberry genotypes based on their leaf essential oil composition
P2-04	A. Woloszyn J. Góra T. Majda	Comparison of the content and chemical composition of essential oils of two dill cultivars from Poland
P2-05	T. Worku Ch. Franz	Essential oil yield from different plant organ and different accessions of coriander
P2-06	N. Calabrese G. Circella	Cutting time, yield and essential oil composition in three cultivar of parsley
P2-07	K. Veres E. Varga Á. Dobos Zs. Hajdú I. Máthé	Investigation on the composition of essential oils obtained from several populations of Hyssopus officinalis L.
P2-08	S. Stanev V. Zheljazkov	Study on the content of some terpene compounds in the essential oil of some Bulgarian varieties populations and perspective clones from Peppermint (Mentha piperita Huds.)
P2-09	C. Dascalova	Seed production in male sterile plants in Salvia sclarea L. (Lamiaceae)

PHYSIOLOGICAL ASPECTS OF ESSENTIAL OIL PRODUCTION

P3-01	L. Bini Maleci B. Tirillini L. Gentili A. Pinetti G. Tani O. Servettaz	Observations on glandular trichomes and essential oils in Rosmarinus officinalis L.
P3-02	I. Fortunato Morone G. De Mastro	Histological approach for the assessment of essential oils' potential yield on Origanum vulgare ssp. Hirtum
P3-03	B. Bradu	Evaluation of lignin synthesis in relation to essential oil and eugenol contents in clocimum-2 (Ocimum gratissimum L.)
P3-04	M. Ram R. Singh R.P. Bansal A.A. Naqvi S. Kumar	Effect of salicylic acid on the yield and quality of essential oil in aromatic crops
P3-05	T. Worku M. Bertoldi	Development stages of Ethiopian Tagetes minuta L.
P3-06	V. Zheljazkov N. Kovatcheva S. Stanev	Effect of heavy metal polluted soils on some qualitative and quantitative characters of mint and cornmint

ANALYTICS - TECHNICAL ASPECTS

P4-01	D. Juchelka A. Mosandl	Advances in the authenticity assessment of Citrus oils
P4-02	D. Bartschat B. Maas S. Smietana A. Mosandl	3-Buthylphthalide: Chirospecific analysis, structure and properties of the enantiomeres
P4-03	M. Schwarz D.H. Paper G. Franz	GC-Analysis of essential oils on chiral columns - relevant for the pharmacopoeia
P4-04	S.G. Claude R. Tabacchi A. Saxer	Comparison between heptakis (6-O-thexyldimethylsilyl)-2,3-di-O-acetyl-ß-Cyclodextrin and heptakis (6-O-tert.-butyildimethylsilyl)-2,3-di-O-acetyl-ß-cyclodextrin as Chrial Startionnary Phase in Gas Chromatography
P4-05	C. Blum K.H. Kubeczka K. Becker	SFC/MS Investigation of Spice Extracts
P4-06	B. Ronyai B. Simándi M. Then S. Perneczky P. Csantó K. Szentmihály	Supercritical fluid extraction of clary sage and study of sclareol and elements content in parts of plant
P4-07	R. Ochocka H. Lamparczyk	Evaluation of essential oils on the basis of chromatographic data using principal component analysis
P4-08	K.H. Kubeczka J.A. Protzen	Investigations of tea infusions and distillation waters using solid phase microextraction (SPME) technique
P4-09	H. Krüger B. Zeiger	Evaluation of a Fennel collection by classical extraction and SPME-headspace-analysis
P4-10	B. Oberhofer A. Nikiforov	Analysis of essential oil compounds used in fragrance lamps
P4-11	K.P. Svoboda R. Caddy J. Hampson J. Parker F. Davidson S. Price	Analysis of essential oil mixes used in aromatherapy

ANALYTICS - CHEMICAL ASPECTS

P5-01	V. Roussis M. Couladis O. Tzakou P. Petrakis A. Loukis N. Dukic	The essential oil composition of three Abies species growing in South Balkans
P5-02	J. Karlsen G. Fladseth J. Remme A. Baerheim-Svendsen Y. Holm R. Hiltunen	The enantiomeric composition of the monoterpene hydrocarbons occuring in the needles of Juniperus communis L. var. saxatilis Pall. (Norwegian mountain juniper)
P5-03	J.R. Ochocka M. Asztemborska D.R. Zook D. Sybilska	Enantiomeric composition of monoterpenic hydrocarbon in various Juniperus communis specimens
P5-04	D. Kustrak A. Baerheim-Svendsen G. Fladseth J. Karlsen	Composition of the essential oil of the berries of Juniperus communis L. of Croatian origin
P5-05	M. Stoyanova	Comparative study on the needle essential oils of Juniperus communis L. and J. communis var. nana Wild in Bulgaria
P5-06	C. Cavaleiro L. Salgueiro A.C. Figueiredo J.G. Barroso O. Roque A. Proenca da Cunha	Composition of the essential oil of Juniperus phoenicea L.
P5-07	F. Bucar U. Schweiger	The essential oil of Aegopodium podagraria L.
P5-08	Y. Holm P. Vuorela R. Hiltunen	Monoterpene enantiomers of Angelica root and seed oil
P5-09	L.G. Pedro A.C. Figueiredo J.G. Barroso S.G. Deans J.J.C. Scheffer	Composition of the essential oil of Chaerophyllum azoricum Trel., an endemic species of the Azores
P5-10	S. Kusmenoglu	Analysis of essential oil of Achillea setacea Waldst. et Kit.
P5-11	M. A. Foglio A.J. Masaioli V. Ferracini	A study of the essential oil of Artemisia annua L. adapted to Brazilian climate

P5-12	M. Usai C.D. Maoddi V. Picci M. Satta C. Tuberoso	Preliminary analysis of essential oil from Sardinian Helichrysum italicum G. Don subspecies Microphyllum
P5-13	N. Bülow A. König	Germacrene D-A source of rare sesquiterpene hydrocarbones
P5-14	K. Thefeld P. Weyerstahl H. Marschall A. Rustaiyan	The essential oil of Tanacetum fruticulosum LEDEB.
P5-15	M. Usai A. Atzei V. Picci A. Furesi	Essential oil from Sardinian Melissa officinalis L. and Melissa romana Mill.
P5-16	G. Kökdil S. Kurucu G. Topcu	Composition of the essential oils of some Turkish Nepeta species
P5-17	K.H.C. Baser B. Demircakmak	The essential oil composition of some Nepeta species of Turkey
P5-18	K.H.C. Baser M. Kürkcüoglu	The essential oil of three new Labiatae Taxa from Turkey: Origanum husnuncan-baserii, Sideritis gülendamii and Salvia aytachii
P5-19	I. Olszewska-Kaczynska K. Milkowska	Chemical analysis of Salvia officinalis L. plant and extracts
P5-20	N. Kovatcheva V. Zheljazkov	Study on the essential oil content and components of Salvia officinalis L.
P5-21	Á. Dobos G. Nagy E.M. Genova V.V. Miklóssy I. Máthé	Comparative analysis of Salvia off. and Salvia tomentosa essential oils
P5-22	G. Nagy Á. Dobos I. Máthé V.V. Miklóssy G. Janicsák	Comparative studies of the essential oils of some species of sect. Salvia
P5-23	R. Vila M. Clos E. Ferro J. Iglesias S. Canigueral	Analysis of the essential oil of Salvia cardiophylla

P5-24	H. Gaspar F.M.S. Brito Palma M.C. De La Torre B. Rodriguez A.C.S. Figueiredo J.M.G. Barroso	Composition of the essential oils from leaves and flowers of Teucrium haenseleri Boiss.
P5-25	T. Stoeva	Quantity and quality of essential oil from Thymus sibthorpii CV. Kresna
P5-26	L.M.R.P. Salqueriro R. Vila X. Tomàs S. Canigueral A. Proenca da Cunha T. Adzet	Composition and infraspecific variability of the essential oil of Thymus villosus subsp. lusitanicus
P5-27	N. Asfaw A. Baerheim-Svendsen G. Fladseth J. Karlsen	The essential oil composition of Thymus species from Ethiopia
P5-28	U. Fischer Ch. Franz	Variability of the essential oils of Lippia graveolens HBK from Guatemala
P5-29	A.P. Martins L. Salgueiro R. Vila F. Tomi S. Canigueral J. Casanova A. Proenca da Cunha T. Adzet	Composition of the essential oils of some Piper used in traditional medicine in S. Tomé and Principe
P5-30	M. Mundina R. Vila J. Cicció C. Ibánez T. Adzet S Canigueral	The essential oil composition of leaves and fruits of Hedyosmum mexicanum
P5-31	C.E. Mvé-Mba Ch. Menut G. Lamaty J.M. Bessière	Indole derivatives in bark essential oil of Anonidium manni (Oliv.) Engl. and Diels (Annonaceae) from Gabon
P5-32	U. Bauermann J. Seidemann	Monodora myristica seeds - analytical and sensory investigation of essential oil of calabash nutmeg of different origin

P5-33	G. Buchbauer L. Jirovetz A. Nikiforov M.P. Shafi T.K. Bindu	Volatiles of the essential oils of the leaves and root bark of Uvaria narum Wall. (Annonaceae)
P5-34	M. Miyazawa H. Shimamura H. Kameoka A. Takahata	Volatile components of Chinese crude drugs, Dioscorea japonica
P5-35	M. Miyazawa A. Takahata T. Urano	Flavour components of Japanese traditional food, newly treated curl "Nori" (Porhyla Yezoensis F. Narawansis).
P5-36	L.H. Hu Z.L. Chen	The chemical constituents of Chinese Tradition medicine Chrysanthemum morifolium Ramat

PRODUCTION OF ESSENTIAL OILS

P6-01	R.S. Rohella T. Rath S.C. Paul B.C. Mishra J.R. Sahu J.S. Murty	Distillation of essential oils - a kinetic study
P6-02	A. Balinova-Tzvetkova E. Georgiev	Sorption processes at the extraction of Lavandula vera
P6-03	A. Balinova-Tzvetkova	On the extraction of Rosa damascena Miller
P6-04	Y. Noma Y. Asakawa	Biotransformation of terpenoids and related compoundes by microorganisms - production of biologically active substances
P6-05	M. Miyazawa H. Nankai H. Kameoka	Enantioselective cyclization of (+)lavanduol to (-)-(2S,4S)-1,5-epoxy-5-methyl-2-(1-methylethenyl)-4-hexanol by Glomerella cingulata
P6-06	B.E. Kiefl S. Alban G. Franz	Storage stability of herbal drugs containing essential oils
P6-07	I. Salamon J. Danielovic	Qualitative-quantitative characteristics of the autochtonous populations of chamomile on the East-Slovakian lowland

ESSENTIAL OILS AS PHYTOGENIC FEED ADDITIVES

P7-01	B. Biavati S. Franzoni H. Ghazvinizadeh R. Piccaglia	Antimicrobial and antioxidant properties of plant essential oils
P7-02	C.C. Chyau S.S. Liu J.D. Su	Volatile components and antioxidant properties of Myristica fragrans H.
P7-03	E. Simpson M. Lis-Balchin S.G. Deans	The impact of Levisticum officinalis L. (lovage) volatile oil on mammalian lipid metabolism

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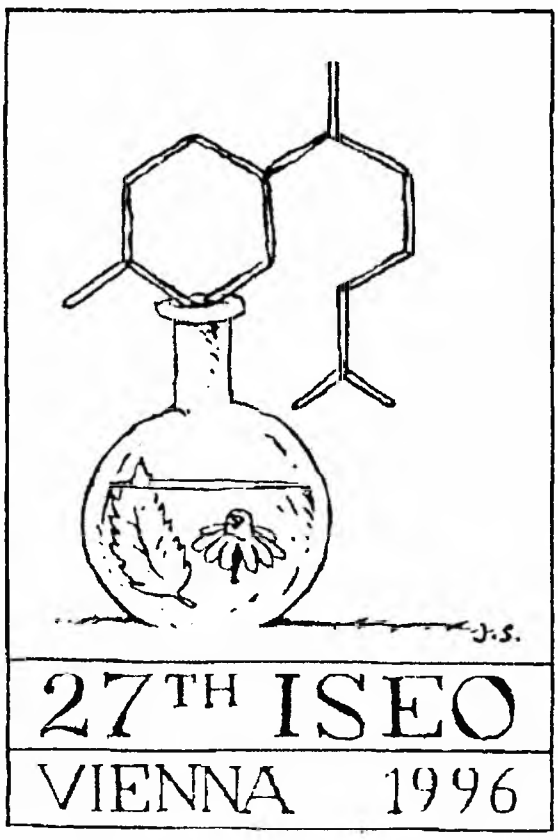

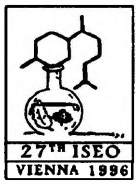

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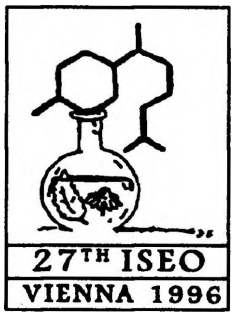
Basic and Applied Research



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Proceedings of the 27th International Symposium on Essential Oils

Ch. Franz, Á. Máthé, G. Buchbauer (editors)

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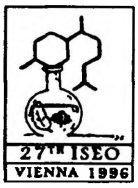

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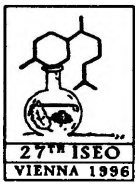

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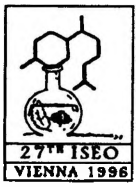

Essential Oils:
Basic and Applied Research

ANALYSIS OF THE ESSENTIAL OIL OF *SALVIA CARDIOPHYLLA*

Roser Vila [1], Montserrat Clos [1], Esteban Ferro [2], José Iglesias [1] and Salvador Cañigueral [1].

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SUMMARY

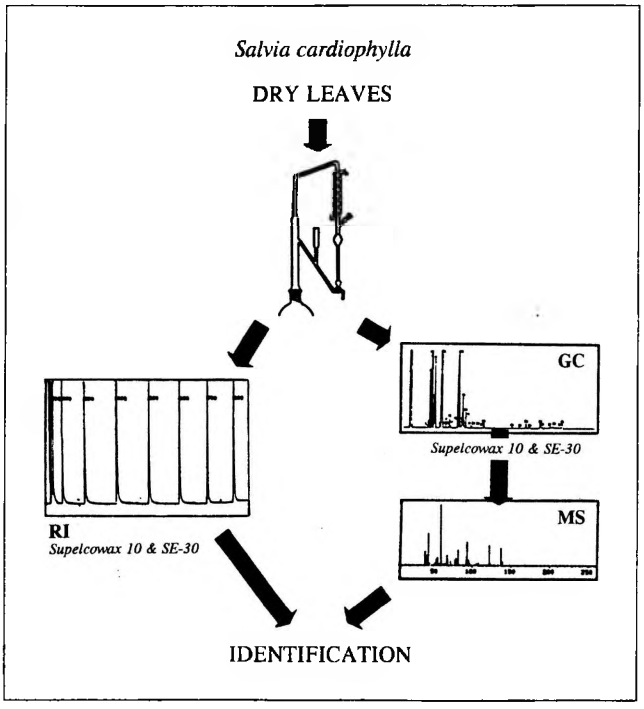
The composition of the essential oil of the leaves of *Salvia cardiophylla* (Lamiaceae), an endemic species from Paraguay, was investigated by GC-FID and GC-MS using two capillary columns (Supelcowax 10™ and methylsilicone SE-30). The identification of the constituents was made by means of their retention indices in the two columns, and their mass spectra. The quantification of each component was done in the basis of its GC-FID peak areas.

EXPERIMENTAL

Plant material

Leaves of *Salvia cardiophylla* Benth. were collected at the flowering stage, in April 1995, in San Lorenzo (Departamento Central, Paraguay). A voucher specimen was included in the Herbarium of the Faculty of Chemical Sciences of the Universidad Nacional de Asunción (Paraguay), with the number Ferro 001.

Analysis



DISCUSSION

The air-dried leaves of *Salvia cardiophylla* gave, by hydrodistillation, a low essential oil yield of 0.03%. No previous reports on the composition of the volatile oil of this species were found in the literature.

The analysis of this oil allowed the identification of 27 different constituents, meaning a percentage of the total oil of 87.8%. It was mainly constituted by sesquiterpenes, either hydrocarbons (56%) or oxygenated (21.3%), while monoterpene rates were quite lower (1.8%, in total). The major constituents were found to be: ß-caryophyllene (23.1%), germacrene-D (14.3%), ß-caryophyllene oxide (10.4%), bicyclogermacrene (6.6%), and spathulenol (7.1%).

RESULTS

COMPONENTS	%	IDENTIFICATION METHODS
Monoterpene hydrocarbons	**1.0**	
α-Thuyene	0.3	GC-MS, RI_1
Myrcene	0.1	GC-MS, RI_2
ß-Phellandrene	0.3	GC-MS, RI_1, RI_2
p-Cymene	0.3	GC-MS, RI_1
Oxygenated monoterpenes	**0.8**	
Linalool	0.8	GC-MS, RI_1, RI_2
Sesquiterpene hydrocarbons	**56.0**	
τ-Elemene	0.6	GC-MS, RI_1
α-Copaene	2.0	GC-MS, RI_1, RI_2
ß-Bourbonene	0.9	GC-MS, RI_1, RI_2
Longifolene	0.3	GC-MS, RI_1, RI_2
ß-Elemene	2.8	GC-MS, RI_1, RI_2
ß-Caryophyllene	23.1	GC-MS, RI_1, RI_2
α-Humulene	2.1	GC-MS, RI_1, RI_2
α-Guaiene	1.0	GC-MS, RI_2
D-Germacrene	14.3	GC-MS, RI_1, RI_2
Bicyclogermacrene	6.6	GC-MS, RI_1, RI_2
δ-Cadinene	2.0	GC-MS, RI_1, RI_2
τ-Gurjunene	0.3	GC-MS, RI_2
Oxygenated sesquiterpenes	**21.3**	
Isocaryophyllene oxide	0.9	GC-MS, RI_2
Caryophyllene oxyde	10.4	GC-MS, RI_1, RI_2
Spathulenol	7.1	GC-MS, RI_1, RI_2
T-Cadinol	0.9	GC-MS, RI_1, RI_2
α-Cadinol	2.0	GC-MS, RI_1, RI_2
Others	**8.7**	
cis-2-Hexanal	0.6	GC-MS, RI_1
1-Octen-3-one	0.2	GC-MS, RI_1, RI_2
1-Octen-3-ol	0.2	GC-MS, RI_1, RI_2
ß-Ionone	1.1	GC-MS, RI_1, RI_2
Hexadecanoic acid	6.6	GC-MS, RI_2

RI_1: Retention Index in Supelcowax 10.
RI_2: Retention Index in SE-30.

UNIVERSITAT DE BARCELONA

27th INTERNATIANOL SYMPOSIUM ON ESSENTIAL OILS; September 8-11, 1996, Wien; Austris

SFC/MS INVESTIGATION OF SPICE EXTRACTS

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Introduction

Essential oils and spice extracts are used as an alternative to spices and mixtures of spices in food industry. The main advantage is the microbiological stability of these products. Common extraction methods are solvent extraction, supercritical fluid extraction (SFE) and the recently developed accelerated solvent extraction (ASE) by means of ASE 200™ (Dionex).
Spice extracts are composed of volatile and less or non volatile compounds. The investigation of spice extracts by GC is only confined to volatile compounds.
A new method to investigate the total extracts is the capillary supercritical fluid chromatography (SFC). This method is usable for volatile and non volatile compounds.
The SFE extracts and ASE extracts and in addition the respective essential oils of black pepper and other spices were investigated by GC and SFC/MS (Lit. 1; 2). Capillary supercritical fluid chromatography - mass spectrometry has been used successfully to identify components of commercial supercritical fluid extracts (Fa. Raps) and ASE extracts of black pepper.

Experimental

Freshly milled black pepper was filled in 33 mL extraction cells and extracted by the ASE 200™ system (Dionex).

solvent	ethanol
temperature	100°C
pressure	140 bar
heating up time	5 min
static extraction time	5 min
static cycles	1
wash out (ethanol)	60%
wash out (nitrogene)	200 s

table 1: ASE conditions

The SFC/MS analyses were performed on a Dionex, Series 600, supercritical fluid chromatograph equipped with a Rheodyne 7256 pneumatic controlled loop injector with 0,2 µl loop size, a SFC column (Dionex SB-Biphenyl-30 10 m × 50 µm ID, 0.25 µm film), a Mplus SFC-MS interface (70°C; restrictor 250°C) and a Finnigan 4500 mass spectrometer. The applied pressure programm at 70°C oven temperature was:

- 100 bar, 1 min isobar
- 10 bar/min to 200 bar; 2 min isobar
- 20 bar/min to 450 bar; 5 min isobar

Results and Discussion

The extraction of black pepper with SFE (CO_2) produced comparable results to the extraction with ASE (ethanol). The extracts were composed of about 10% volatile components (mono- and sesquiterpenes) and about 40% low, respectively non-volatile hot tasted components (piperidides, pyrrolidides and isobutyl amides). In each case piperine was the main component.
Figure 1 shows the SFC/MS RIC of the ASE extract. The terpenes were well separated of the piperidides.The hot tasted components are separated at low temperature (70°C), so that column bleeding remains negligible.
The SFC/MS EI-mass spectra of the volatile components (e.g. figure 2: α-humulene) were comparable to GC/MS EI-mass spectra. However, the SFC/MS EI-mass spectra of the hot tasted components (e.g. figure 3 - 5) were of higher quality.

Conclusion

SFE and ASE are well-suited extraction methods for black pepper. The applied SFC/MS method proved to be useful especially for investigation of non-volatile constituents of spice extracts

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SFC/MS RIC

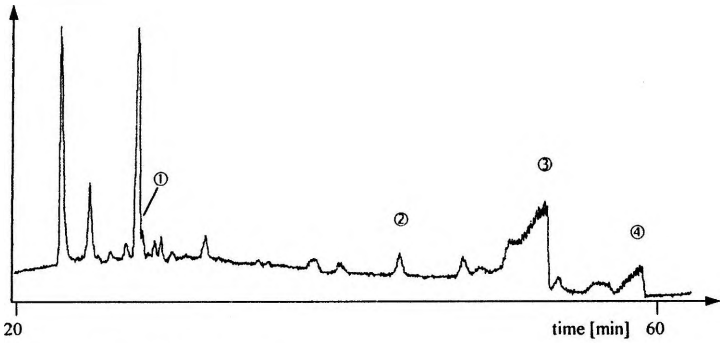

figure 1: ASE extract of black pepper

SFC/MS EI-mass spectra

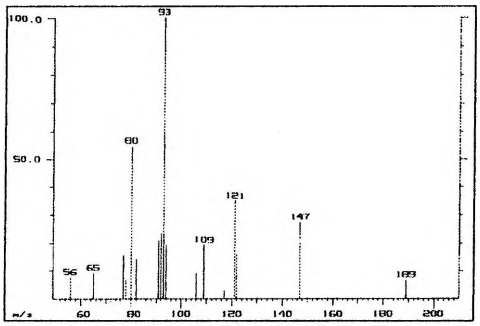

figure 2: α-humulene ①

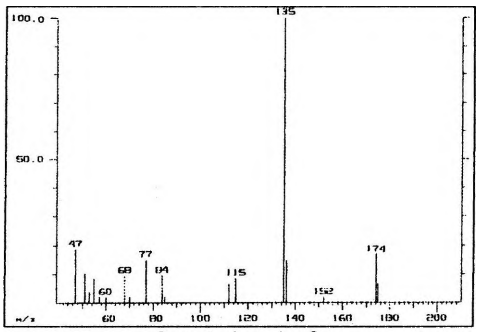

figure 3: piperanine ②

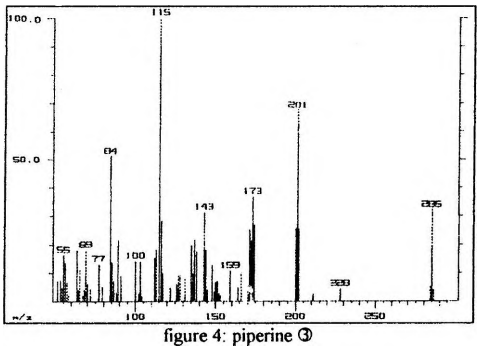

figure 4: piperine ③

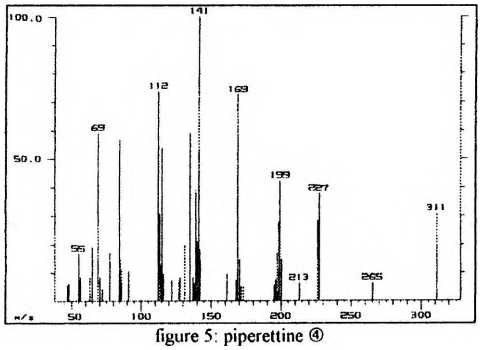

figure 5: piperettine ④