

28th ISEO

28th INTERNATIONAL SYMPOSIUM ON ESSENTIAL OILS

**1-3 September 1997
Eskişehir-TURKEY**

ABSTRACTS



**Anadolu University
Medicinal and Aromatic Plant and Drug Research Centre
(TBAM)**

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Medicinal and Aromatic Plant and Drug Research Centre
(TBAM)

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PLENARY LECTURES

SCENTS FROM RAIN FORESTS

Roman Kaiser

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Since the dawn of time, the secretive and fragile kingdom of the rain forest, which covers 11-13% of all emerged land and shelters eight to nine tenths of all forms of known and unknown life, harbors extraordinary biological treasures worth to be treated with utmost care. According to a further estimation, 70-80 % of these species, meaning around 60% of the entire biodiversity, is found for ecological reasons in the canopy region of the rain forests.

Having been convinced to find this richness also reflected on the olfactory level we searched during the past years in the understory as well as in the canopy of rain forests for new attractive scents. This communication opens the discussion of chemical, biological and olfactory aspects of such scents.

THE ESSENTIAL OIL COMPOSITION OF *PIMPINELLA* SPECIES

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The genus *Pimpinella*, belonging to the Apiaceae (Umbelliferae) subfamily Apioideae comprises about 150 species, which occur largely in Europe and Asia extending to China. It is a slightly uniform genus among the species-rich genera of umbellifers and has no generic morphological character, which can be found in all of its species. Due to the different features which have been employed, the limitation of the genus *Pimpinella*, as well as its infra-generic division differ considerably. Considering those difficulties, it seemed promising to investigate the essential oils of different *Pimpinella* species and to compare the obtained results with each other and with analytical results gained from other species from the Apiaceae. Considering the large size of the family, we decided to restrict our investigations to the subfamily Apioideae neglecting the other subfamilies. Within the Apioideae, we concentrated our attention on *Pimpinella* and related genera.

All the essential oils which have been investigated contain considerable amounts of phenylpropane and sesquiterpene derivatives, whereas monoterpenes occur only in small amounts. Most of the phenylpropanoids found exhibit the unusual substitution pattern 2,5 of the aromatic ring, which has not been found elsewhere up till now. In addition to these phenylpropanoids, all plants of the genus *Pimpinella* contain trinorsesquiterpenoids. In view of the extremely restricted occurrence of these compounds in the plant kingdom, the possible taxonomic value of these substances with regard of the delimitation of the genus *Pimpinella* will be discussed.

**AROMATHERAPY - A COMPILATION OF SCIENTIFIC
FACTS IN FAVOUR OF ITS CORRECT USE**

G. Buchbauer

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of Pharmacy, Althanstrasse 14, A-1090 Vienna, Austria

In this review a survey of scientific methods in aromatherapy research is given. Facts obtained by serious scientific investigations are presented in order to stress the fact that aromatherapy has nothing to do with holistics, or esoterics, or magics. Data obtained by animal experiments as well as by human studies clearly show that fragrance compounds behave like pharmaca despite their psychic properties. Therefore the apt description of fragrance compounds and essential oils (as their mixtures) when used in a therapeutic manner should be **AROMATHERAPEUTICS**. The influence of chirality of fragrance compounds on certain pharmacokinetic parameters is discussed likewise.

PL4

RECENT DEVELOPMENTS IN PHYTOCHEMISTRY OF BRYOPHYTES

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A number of bryophytes have been used as medicinal plants in North America, China and Europe to treat burns, bruises, external wounds, *etc.* Some liverworts contain diuretic, antimicrobial, antifungal, and antitumor active substances. Some bryophytes show characteristically fragrant odors and intensely hot and bitter or saccharine-like taste. Generally, bryophytes are not damaged by insects, snails, slugs and other small animals. Furthermore, some liverworts cause intense allergenic contact dermatitis and allelopathy. We have been interested in these Bioactive substances found in bryophytes and have studied about 800 species of Hepatica with respect to their chemistry, pharmacology, and application as sources of cosmetics, and medicinal or agricultural drugs. It has been demonstrated that most of the Hepaticae contain mainly sesqui- and diterpenoids and lipophilic aromatic compounds which constitute the oil bodies. The biological activities of liverworts are due to these substances. At present more than 300 new compounds have been isolated and their structures elucidated. The present lecture shows the structures of novel terpenoids and aromatic compounds and their biological activity (fragrant odor, pungency, antimicrobial, antifungal, antifeedant, antitumor, anti-HIV-1, piscicidal, molluscicidal, cytotoxic, neuritic sprouting, muscle relaxing and 5-lipoxygenase, calmodulin, DNA polymelase, cathepsins B and L inhibitory activities).

PL5

USE OF ESSENTIAL OILS IN THE FORMULATION OF NATURAL FLAVOURS

D. Joulain

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Outline:

Natural mixtures derived from plants: definition of an essential oil. Regulatory aspects: the FEMA/GRAS list in the USA; the case of Europe. Processing of essential oils: concentration and fractionation; natural isolates from essential oils. Purity assessment of essential oils in relation with safety regulations. Authenticity and natural character assessment: some analytical considerations. Application of essential oils in sweet flavours. The interest of using essential oils as such for implementing isolate-based formulations. The potential of essential oils in savory and fatty flavour formulations.

PL6

SYNESTHESIA

A. Baydar ^{1*} and T. Mc Gee ²

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Although fragrance is something invisible it is strongly felt, and has always excited interest and has certain magical power assigned to it. In addition to their aromachological aspects, the psychological effect of a perfume can help in differentiation of a product from its competitors and strengthen its position. A lot of work has been done in trying to understand the influence of fragrance on behavior and perception. However, the understanding of these effects is still in its infancy. At Givaudan-Roure, our research program covers synesthesia, odor imagery, language, odor classification and sensory mapping. It is directed to providing insight for perfumers to help them create fragrances that complement or reinforce the concept of the product, especially their sensory psychological benefits.

Synesthesia is the interrelationship of the senses. Certain people can describe music and numbers in terms of color. It is a condition in which a person experiences a sensation in one sensory modality and it triggers an involuntary and automatic sensation in a different modality. Synesthesia was investigated using the Rader and Tellengen test, which classify it into two components : experiential and translation. This methodology has enabled us to understand the cross modal correspondence between vision and olfaction. Data bases of odor-color and odor-mood associations have been developed for a large number of aroma chemicals. The basic protocol involves subject smelling an odor or fragrance and distributing five point across eleven mood words and eleven color words, the tests being carried out in custom designed sensory psychology booths.

**IDENTIFICATION OF RARE SESQUITERPENE
STRUCTURES IN ESSENTIAL OILS**

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Sesquiterpene derivatives substantiate a major portion and may often be responsible for a specific sensoric impression of essential oils. In addition, an extraordinary structural variability, caused by numerous skeletal rearrangements during biosynthesis, is observed for sesquiterpenes. The elucidation of the structure and stereochemical properties of unknown sesquiterpenes is therefore a great challenge. The detection of a series of structurally related sesquiterpenes may also serve to recognize biogenetic correlations. The isolation of some novel sesquiterpene constituents from essential oils with the rarely found africanane, brasilane, nardosinane and asteriscane skeletons by preparative chromatographic methods and their identification with modern NMR techniques, mass spectrometry, enantioselective gas chromatography, and by investigating thermally and acid catalyzed rearrangements and microchemical reactions will be demonstrated.

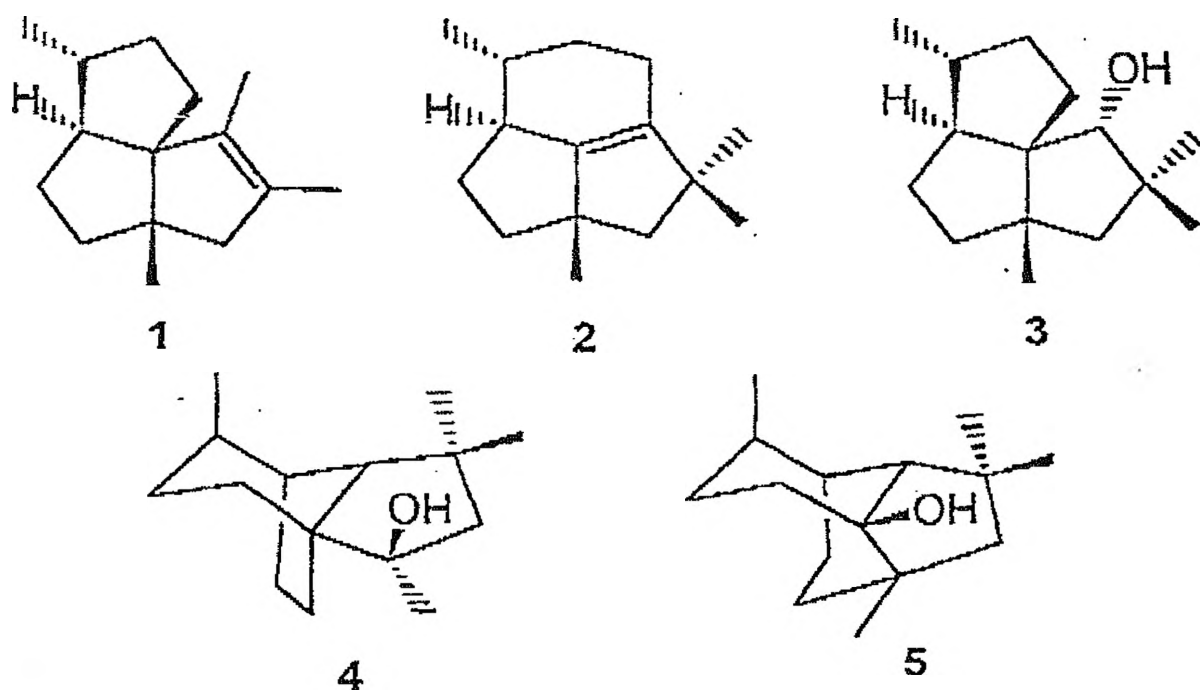
SOME NOVEL TRICYCLIC SESQUITERPENE SKELETONS FROM THE ESSENTIAL OIL OF *ECHINOPS GIGANTEUS*

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The essential oil from the roots of *Echinops giganteus* var. *lelyi* from Cameroon contains almost exclusively tricyclic sesquiterpenes (77%) with presilphiperfolane, silphiperfolane, isocomane and modhephane skeletons^[1]. The main constituents are silphiperfol-6-ene (1, 27%) and presilphiperfol-7-ene (2, 9%). The structures of seven new sesquiterpene alcohols were elucidated by detailed NMR spectroscopy. Three alcohols with new skeletons named cameroonan-7-ol (3), nopsan-4-ol (4) and echinan-8-ol (5) were isolated. Two of them (3 and 5) contribute decisively to the strong woody, patchouli- and amber-like odor of the oil.



^[1] C. Menut, G. Lamaty, P. Weyerstahl, H. Marschall, I. Seelmann, P. H. Amvam Zollo, *Flavour Fragr. J.*, 12, in press (1997).

ORAL PRESENTATION

ANTIFUNGAL PROPERTIES OF ESSENTIAL OIL COMPONENTS

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Although considerable information on the antifungal activity of spices and essential oils is available, data about the antimicrobial properties of their constituents, especially the terpenes, is very limited.

Previously we described the bioconversion of some terpenes by fungi. Nerol, geraniol and citral were completely converted by sporulated surface cultures of *P. digitatum* to methylheptenone, and by *A. niger* to linalool. Linalool on the other hand was transformed to linalool oxides by submerged liquid cultures of *A. niger*. The biotransformation of citronellol to isopulegol and α -terpineol, was carried out by sporulated surface cultures of *A. niger* and *P. digitatum*, but didn't take place when the organisms were grown as submerged liquid cultures. The bioconversion of isoeugenol to vanillin by *A. niger* was also investigated.

In this study, the inhibitory effects of the phenolic compounds ferulic acid, eugenol and isoeugenol, the terpenes geraniol, nerol, citral, citronellol and linalool and the biotransformation products vanillin, 6-methyl-5-hepten-2-one and furanoid linalool oxides towards the fungi *Aspergillus niger* and *Penicillium digitatum* were compared. It was shown that the terpene alcohol citronellol and the phenolic compounds eugenol and isoeugenol were the most inhibitory substrates, whereas the products formed through biotransformation by the fungi were the least antifungal: high concentrations of methylheptenone and linalool oxide could be tolerated by the fungi, without loss of germination and sporulation capacity. From these results, it was postulated that biotransformation of terpenes might be a detoxification mechanism of the fungi.

The Effect of the Essential Oil of *Melaleuca alternifolia* on *Escherichia coli*.

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The essential oil of *Melaleuca alternifolia* (tea tree) exhibits broad-spectrum antimicrobial activity. We have further investigated this activity by examining the effect of tea tree oil on the survival, growth rate and physiology of *Escherichia coli* strain AG100. Here we report that tea tree oil-induced cell death is accompanied by an inhibition of respiration and the formation of extracellular blebs on the cell surface observable in electron micrographs.

Kill rate data indicated that 0.125 % (v/v) tea tree oil inhibited glucose-dependent respiration and was lethal to cells in the assay conditions used. Below 0.125 %, no inhibition of either respiration or the growth rate of cells was detected.

Our results also show that stationary phase cells are more tolerant to tea tree oil than cells harvested in logarithmic phase. At 0.25 %, tea tree oil completely reduced cell numbers in suspensions from a 5 hour culture within 20 minutes, whereas, cells grown for 48 hours died more slowly, with complete die-off taking 60 minutes. Similarly, tea tree oil-induced inhibition of respiration was less extensive for stationary phase cells. These findings indicate that longer exposure times were required to cause a lethal degree of physiological damage to *E.coli* AG100 cells in stationary phase.

Overall, the inhibitory effects of tea tree oil on respiration and on cell structure, as observed by electron microscopy, in *E.coli* are consistent with a disruption of membrane structure and function related to the accumulation of tea tree oil monoterpenes. The dose-dependence observed for these effects is most likely a product of the rate and extent of partitioning of monoterpenes between the aqueous phase of cell suspensions and the hydrophobic regions of the cells.

BIOSYNTHESIS AND HYDROLYSIS OF MONOTERPENE GLYCOSIDES

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The objective of this work was to study the biosynthesis of ^{14}C -monoterpenol glycosides (geranyl glucoside) in the leaves and the berries of Muscat of Alexandria. This was accomplished by (a) reaction between the enzyme MUGT (isolated from the Muscat leaves) and natural geraniol in the presence of UDPG- ^{14}C and (b) by reaction of glucosyl transferase and ^{14}C -labeled geraniol which was prepared by incubating the leaves of Geranium plant in ^{14}C - CO_2 containing atmosphere. The other objective was to increase the yield of β -glycosidase (which cleaves the β -glycosidic bond of the monoterpenol glycosides of wine and increase significantly the aroma (free terpenols) of wine. This was accomplished by introducing the inducer IPTGlc (Isopropylthio- β -glucoside) into the strain of *Aspergillus niger* B-1, grown on bran as carbon source. The enzyme (β -glycosidase) was also capable of hydrolyzing cyanogenic glycosides and thus has the potential of detoxification of foods, such as cassava.



**THE BIOSYNTHESIS OF ESSENTIAL OIL COMPONENTS
IN AROMATIC PLANTS.**

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The unique fragrant properties of essential oils are determined by the chemical structure and relative abundance of their volatile components. These volatile compounds belong to several chemical groups, such as mono- and sesquiterpenes, phenylpropanoids and others. Plants produce and store essential oils in specialized anatomical structures, such as oil cells, glands and ducts, and possess dedicated enzymatic pathways that biosynthesize the essential oil components from metabolic precursors. The presence or absence of particular biosynthetic enzymes in a given plant tissue, ultimately determines the composition of the essential oils produced and stored. A few examples demonstrating the several biosynthetic pathways to mono- and sesquiterpenes, phenolic compounds and other important essential oil components will be explained, and some of their postulated ecological roles will be discussed.

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Biological roles: protection / Recognition / Unknown
 herbivores Symb
 diseases herbivory
 Competi pollination
 Harmful Radiation Seed dispersal

when wounded the plant reacts ^{only} locally increasing enzymatic activity

SUPERCRITICAL FLUID EXTRACTION OF TERPENE HYDROCARBONS FROM CITRUS OILS

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The separation of terpene hydrocarbons from citrus oils has been studied using supercritical CO₂ as solvent. While conventional separation processes show disadvantages regarding organic solvent residues or high temperatures, supercritical fluid extraction (SFE) allows fractionation at moderate temperatures and solvent free products. Publications in the field of deterpenation by SFE are mainly about vapor-liquid phase equilibrium (VLE) data of pure components in supercritical CO₂ or about separations of model mixtures.

To study the behavior of real multicomponent mixtures orange peel oil was used for VLE measurements and multistage countercurrent extraction. This method gives the best product quality in a continuous mode depending on operating conditions and the height of the column. Experiments have been carried out in a pressure range from 8 to 13 MPa to observe the two-phase region at 323, 333, and 343 K. VLE experiments gave loadings of the CO₂ phase from 10 to 100 g extract/kg CO₂. To investigate the composition dependency of the phase behavior fractionated products from countercurrent experiments were also used for VLE measurements. Samples from both phases were analysed by GC. To evaluate the separation of (mono-)terpenes from oxygenated and other low volatile components peaks were assembled to obtain two groups, "terpene" and "aroma" group. The distribution factors of linalool and decanal were also used as separation indicators.

The best separation was observed at low temperatures and pressures far from the one-phase region of the system. Unfortunately the high operating costs at those low loading conditions make the process unfavorable from an economical point of view because of very high solvent-to-feed ratios. Therefore, higher temperatures and pressures resulting in higher loadings allow much lower operating costs. The optimal extraction temperature will be limited by the thermal stability of the feed material. Regardless of economic evaluations deterpenation by means of SFE will give high quality products of the desired composition.

"The Evolution of a new Generation of Fine Natural Plant Oils"

Peter F Wilde

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ABSTRACT

Over the past ten years, our successful harnessing of the remarkable properties of the solvent 1,1,1,2 - tetrafluoroethane and the perfection of economical, industrial scale equipment to exploit them, has allowed us to develop over 300 new fine plant extracts.

Many of these extracts are proving to be of major interest in the preparation of functional skin care products, fragrances, flavours and phyto-pharmaceuticals.

We initially became well known for our preparation of the first English Rose Oil for 250 years (prepared from our own roses grown in North Yorkshire, England), but now hundreds of freshly harvested herbs and flowers of all kinds have yielded up their precious, fragile natural components without loss of "volatiles", without thermal damage or mutilation with customary high vacuum stripping, without the use of hexane or alcohol and without damage to the environment.

This extraction technique may still be un-familiar to some and a demonstration of both the process (using hand-held equipment) and of some of the exquisite oils prepared by it, will be given.

This technology was recently praised as being "...the most significant advance in this field for 1000 years....".

AUSTRALIAN TEA TREE OIL: EFFICACY, TOXICITY, IRRITANCY AND STABILITY

David N. Leach

**Australian Tea Tree Oil Research Institute, Southern
Cross University, Lismore, NSW, Australia 2480**

The Australian Tea Tree Oil Research Institute (ATTORI) was created in 1996 to provide a focal point for coordinated research on all aspects of this commercially important oil. An overview of current research into plant genetics, product formulation, efficacy *in vitro* and *in vivo* and clinical trials will be discussed. The use of tea tree oil for topical applications is supported by cell-line cytotoxicity studies. Of the five cell-lines tested with tea tree oil and various pure components, epithelial cells were most robust, whilst the liver cells were the most susceptible. Recent results from a skin irritancy and sensitivity trial conducted on 151 panelists revealed an average daily score of 0.1922, thereby confirming that tea tree oil is an extremely low irritant when applied to the skin, even in neat (100%) concentrations. In various formulation bases at concentrations up to 25% the oil showed virtually zero irritancy (ranging from 0.0000 0.0060). Tea tree oil must be considered a mild allergen as 2% of the panelists showed an allergic reaction. Issues surrounding production, formulation and stability of low irritancy oil will be addressed.

CHROMATOGRAPHIC STUDY OF ESSENTIAL OILS FROM *ARTEMISIA* SPECIES

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Essential oils of the plants which contain a great range of terpene compounds constitute an important group of medicines having spasmolytic, sedative, wound-healing, anti-inflammatory activities used in medical practice for the treatment of different diseases.

Asteraceae family plants are the richest ether-bearing species, especially *Artemisia* genus. Though a great number of papers regarding chemical study of *Artemisia* have been published, still, their chemical composition has not been studied properly.

This paper lists chemical studies of *Artemisia* essential oils for complex usage of medicinal material and development of wasteless technology for their processing and also in order to study the chemical composition of *Artemisia* species which are endemic in Central Kazakhstan. Four *Artemisia* species of Absinthium section were the subjects of this study: *Artemisia glabella*, *A.radicans*, *A.filatovii* and *A.austriaca*. All species were harvested in Karaganda region of Kazakhstan in 1995-96.

Composition and quantitative determination of the oils of four *Artemisia* species were studied by GC.

PACKED COLUMN SFC IN THE ANALYSIS OF TERPENOIDS

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Packed column supercritical fluid chromatography (PC-SFC) has recently stimulated interest as an alternative to HPLC. This has mainly been due to the introduction of variable and programmable restrictors, on-line flow rate controllers and high pressure cells for UV detectors, which have afforded reliable quali-quantitative results. PC-SFC has now sample capacity similar to HPLC, operates with the same columns, and in general requires shorter analysis times because of the higher theoretical plate number per unit time.

A project aiming to evaluate PC-SFC/UV in the analysis of biologically active components in plant extract has been begun. This communication aims to focus on the advantages and disadvantages of PC-SFC, and the strategy adopted in the authors' laboratory to develop new SFC methods are illustrated through some examples involving terpenoids related to aromatic plants (monoterpeneoids, sesquiterpene lactones, diterpenoids, triterpenoids, carotenoids and related glucosides).

CONTROL OF STORAGE DISEASES OF POTATO AND SPROUT INHIBITION BY PLANT VOLATILES.

Ulla Bång

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Experiments to control storage rots and skin blemishing diseases on tubers of potato (*Solanum tuberosum* L.) by using plant volatiles have been carried out during 4 storage seasons. Naturally or artificially infected tubers have been kept at +10°C in an aromatic atmosphere obtained from homogenised fresh plant material or from essential oils (EO), produced by steam distillation. The treatment period has mostly been 2-3 weeks in order to imitate the warmer wound-healing period performed after harvest in practise. The application dose of volatiles and the start of treatments relative to wounding of tubers and the time of the year have been varied. The effects on diseases as well as on sprouting ability have been recorded. In addition, *in vitro*-experiments on agar plates have been carried out to study the fungistatic/fungicidal nature of the vapours. Treatment doses were then similar to the ones of the *in vivo*-experiments.

Significant reductions of one or more of the following diseases: storage rots, caused by *Phoma foveata* Foister and *Fusarium solani* var. *coeruleum* (Sacc.) Booth, silver scurf (*Helminthosporium solani* Dur. et Mont), skin spot *Polyscytalum pustulans* (Owen & Wakef.) Ellis, and black scurf *Rhizoctonia solani* Kühn have been achieved after treatments of contaminated tubers with volatiles from plants of various families such as *Liliaceae*: garlic (*Allium sativum*) and onion (*Allium cepa*); *Lamiaceae*: sage (*Salvia officinalis*), basil (*Ocimum basilicum*) and spearmint (*Mentha spicata*); *Apiaceae*: caraway (*Carum carvi*). The far best, and consistent, control of most of the mentioned fungi, has been obtained by the vapours of garlic. A treatment period of 3 weeks has often been sufficient. Better control of silver scurf was obtained when volatiles were added immediately after harvest, compared with 2 weeks afterwards. Autumn treatment of tubers have not had any dramatic effect on the sprouting ability in spring, whereas the same aromatic compounds, applied in January and onwards, have almost completely inhibited sprouting.

BIOCONVERSION OF ALKENE PHENOLS FROM AROMATIC PLANTS TO NATURAL AROMA COMPOUNDS

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Natural flavor and fragrance compounds are of great value to the food, cosmetics, and medical industries. The price for these natural chemicals can be extremely high due to their low concentration in the plant material. Many herbs and spices contain relatively high concentrations of essential oils that resemble desirable flavor and aroma compounds. By using a single or a few enzymatic steps, it is possible to convert these essential oils components into natural flavor and aroma chemicals. In this work, we describe the microbial conversion of alkene phenols from aromatic plants to natural aroma compounds.

Using enrichment culture technique, microorganisms were isolated based on their ability to degrade specific alkene phenols. These microbial strains were capable of growing on *t*-anethole, estragole, isosafrole or isoeugenol as the sole carbon source. The degradation pathway of *t*-anethole (the major component of anise seed, star anise, and sweet fennel oils) was studied by isolation of blocked mutants, identification of metabolic intermediates, and detection of specific enzymatic activities. Aroma compounds such as anisic acid, anisyl alcohol, 4-methoxybenzyl-methyl-ketone, and anisaldehyde are likely to be metabolic intermediates in the degradation pathway of *t*-anethole. Some of the blocked mutants accumulated intermediates in yields that are close to the maximal theoretical yields. To the best of our knowledge, the conversion efficiencies obtained have never been reported for these essential oil components. The results of this research may support further development of economical bioconversion processes for the production of natural aroma compounds from alkene phenols.

METHYLTRANSFERASES INVOLVED IN THE BIOSYNTHESIS OF METHYL-CHAVICOL AND METHYL-EUGENOL IN SWEET BASIL.

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Sweet basil, *Ocimum basilicum* L., Lamiaceae, is an economically important crop. The major components of basil essential oils are linalol and methyl-chavicol (estragole). The biosynthetic pathway to methyl-chavicol in basil is poorly understood. Enzymatic assays using radioactively labeled SAM (*S*- of methyltransferase activity in young leaves (up to 15 mm length). Chavicol methyltransferase activity in inflorescences was only ~20% of that present in young leaves, while mature leaves, stalks and flowers had much lower levels (5 to 1%). No methyltransferase activity was detected in roots. These results indicate that young leaves are the primary site of methyl-chavicol formation in sweet basil.

Different basil varieties differ in their essential oil composition. Varieties that their oil contains high levels of methyl-chavicol, also display high *in vitro* methyltransferase activity towards chavicol but very little activity towards eugenol. Varieties containing a high methyl-eugenol content display high methyltransferase activity towards eugenol but are also able to act upon chavicol. One variety that contains mostly eugenol in its oil, lacks methyltransferase activity, towards eugenol or chavicol. Our results indicate the presence of different substrate-specific methyltransferases in basil varieties, that might partially explain the differences in their essential oil composition.

POSTER PRESENTATION

COMPOSITION OF THE ESSENTIAL OILS OF *ACINOS* MILLER SPECIES IN TURKEY

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The genus *Acinos* Miller (Lamiaceae) is represented by 5 species in the Flora of Turkey. Two subspecies of *A. troodi* being endemic, as follows:

1. *Acinos troodi* (Post) Leblebici subsp. *vardaranus* Leblebici
2. *Acinos troodi* (Post) Leblebici subsp. *grandiflorus* Hartvig & Strid
3. *Acinos alpinus* (L.) Moench
4. *Acinos suaveolens* (Sm.) G. Don fil
5. *Acinos arvensis* (Lam.) Dandy
6. *Acinos rotundifolius* Pers.

Essential oils from aerial parts of *Acinos* species were obtained by hydrodistillation. The essential oils were analysed by a GC/MS system. Main components were determined as follows:

Germacrene D and hexadecanoic acid for *A. troodi*, *A. alpinus*, *A. arvensis* and *A. rotundifolius*; pulegone and isomenthone for *A. suaveolens*.

The results have shown that *A. suaveolens* is a distinct species among the others in that it has the richest oil content and the oil contains considerable amounts of pulegone and isomenthone. The compositions of the essential oils of *A. alpinus* and *A. rotundifolius* are of a complex nature.

THE ESSENTIAL OILS OF *LEPECHINIA BULLATA* (BRIQ.) EPL. (LAMIACEAE) FROM VENEZUELA

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Lepechinia bullata (Briq.) Epl. (Lamiaceae) is a shrub or even a small tree with a foliage similar to *Salvia*. The species is endemic in the Andean cordillera of Venezuela and Columbia and inhabits the relatively moist upper Andean forest and subparamo from 1700 to 3900 m (1). The common plant name „*Salvia negra*“ indicates its use similar to sage in traditional medicine (2).

40 samples were collected from different sites in Mérida (Edo.) (Venezuela) during the flowering. The essential oil was obtained by hydrodistillation of the air-dried leaves with a yield of approximately 1 %.

GC and GC-MS proved the oils to consist of monoterpene hydrocarbons (0.3 - 50.6%; mainly α,β -pinene, camphene, limonene, β -phellandrene), oxygenated monoterpenes (0.1 - 10.1%; borneol, bornyl acetate), sesquiterpene hydrocarbons (23.2 - 84.1%; β -caryophyllene, α -farnesenes, eremophilene) and oxygenated sesquiterpenes (2.4 - 40.1%; palustrol, ledol, viridiflorol, spathulenol, T-cadinol). Two groups of plants can be distinguished. One group with a high level of monoterpene hydrocarbons and another group with a very low level. In addition the variability of the sesquiterpene alcohols indicates a considerably high chemical polymorphism of *Lepechinia bullata*.

- (1) J. A. Hart: Peripheral isolation and the origin of diversity in *Lepechinia* sect. *Parviflorae*, Syst. Bot. 10, 134-146 (1985)
- (2) H. G. Barriga: Flora Medicinal de Colombia, Tomo III, pp 17, Tercer Mundo, Bogotá (1992)

CHEMICAL COMPOSITION OF THE ESSENTIAL OILS OF SOME *MENTHA* SPP., FROM IRAN

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The volatile constituents of *Mentha aquatica* (L.), *Mentha pulegium* (L.) and *Mentha X piperata* were isolated by steam distillation using a KAISER & W. LANG apparatus and analyzed by GC and GC MS. GC analyses were carried out using a Shimadzu GC-9A chromatograph provided with flame ionization detector (FID), and chromatopac C-R3A as data processor. separation was carried out on two different polarity columns (DB-1 & DB-wax, each one 60m x 0.25 mm, film thick. 0.25 micron). Composition calculated by area normalization method neglecting response factors. GC MS analyses were carried out on a Saturn II model Varian with 70 ev ionization energy on column DB-1 temp. program mode the same as GC analyses. The major constituent was menthofuran (52.6% in *M. aquatica* and 44% in *M. X piperata*) and for *M. pulegium* was pulegone (with 66.5%). Chromatograms and tables of compositions (including name of compound, its Kovats retention indices and percentage) will be presented.

***EFFECT OF DIFFERENT PLANTING TIMES ON
ESSENTIAL OIL COMPONENTS OF DIFFERENT
MINT (Mentha spp.) VARIETIES.***

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This study was conducted to determine the effect of different planting times on essential oil components of different mint varieties (*Mentha arvensis* var. *piperascens*, *M. piperita* Mitcham, *M. piperita* Eskişehir and *M. piperita* Prilubskaja). The field trials have been carried out at GAP Agricultural Research Station of Çukurova University Agriculture Faculty in Harran Plain condition in years of 1993 and 1994. α -Pinen, β -Pinen, 1,8-Cineol, Menthon, Menthofuran, Menthol, Pulegon, Menthylacetat and β -Caryophyllen were determined in essential oil of mint. Essential oil components were effected by planting times and mint varieties. Autumn transplants showed higher menthol's ratio than plants transplanted in spring and was appeared on *M.arvensis* var. *piperascens* mint variety.

Evaluation of single plant descendants of *Origanum vulgare* ssp.

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Plants descending from open pollinated single plants of 5 different populations of *Origanum vulgare* ssp., planted in 1995 in Großenzersdorf, Austria, have been analysed in 1996.

Besides a huge variability in morphological traits like e.g. plant height (from 20 to 87cm) and the colours of different flower parts, also a huge variability in oil content and composition could be observed not only between but also within the offsprings.

The average content of essential oils was 1,24% with one offspring only containing 0,64%. Regarding the oil components 33 components could be identified allowing a classification in 6 different chemotypes:

chemotype	no. of plants per population				
	pop 1	pop 2	pop 3	pop 4	pop 5
carvacrol	-	5	8	1	5
thymol	2	13	10	2	-
γ -terpinen	10	1	5	-	-
linalool	-	1	3	1	4
cis-sabinene hydrate	-	2	1	3	-
mainly sesquiterpenes	10	-	3	3	-

Furthermore, 8,5% of the plants were male sterile and several partial male sterile confirming once more the frequent occurrence of male sterility in Lamiaceae.

THE ESSENTIAL OILS OF *THYMUS* SPECIES GROWING THRACE

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Thrace is the name given to the European part of Turkey. Although the area covered by Thrace is only three percent of Turkey, it has a very rich flora. Eight out of sixty seven taxa of the genus *Thymus* recorded in Turkey grow in Thrace.

Essential oils from aerial parts of the following *Thymus* taxa have been analyzed by GC/MS.

T. atticus Celak.

T. aznavourii Velen.

T. comptus Friv.

T. longicaulis C. Presl ssp. *chaubardii* (Boiss. et Heldr. ex Reichb. fil.) Jalas var. *chaubardii*,

T. longicaulis C. Presl ssp. *longicaulis* var. *subisophyllus* (Borbas) Jalas

T. sibthorpii Bentham,

T. striatus Vahl var. *interruptus* Jalas

T. zygioides Griseb. var. *zygioides*

T. zygioides Griseb. var. *lycaonicus* (Celak.) Ronniger

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1. Davis, P.H., Flora of Turkey and the East Aegean Islands, Vol. 7, pp 349-382, Edinburg University Press, Edinburg (1982).

EFFECTS OF SOME FACTORS ON QUANTITY AND QUALITY OF THYME ESSENTIAL OIL

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The environmental factors have significant effect on the quantity and quality of plants containing essential oil. In this investigation, we have studied the effect of N-fertilizer and harvesting time on the essential oil and thymol content of *Thymus vulgaris* L. cultivated in Tehran.

Optimized conditions were:

1- 100 kg nitrogen per hectare

2- Harvesting at the fruit formation stage in July

The detail of the study will be mentioned in the paper.

MULTIVARIATE STATISTICAL ANALYSIS OF ESSENTIAL OIL DATA OF *THYMUS PRAECOX* SSP. *ARCTICUS* AND *THYMUS PULEGIOIDES* FROM THE SOUTH OF ENGLAND.

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Continuing our research in the field of chemical polymorphism in the genus *Thymus*, studies in the South of England were undertaken. There two species occur, namely *Thymus praecox* (OPIZ) ssp. *arcticus* (E. DURAND) JALAS (syn. *Thymus drucei* RONN.) and *Thymus pulegioides* L.. 388 individual plants were collected at 45 different sampling sites. 85 of these samples belong to *Thymus pulegioides* and 303 to *Thymus praecox* ssp. *arcticus*. The essential oils were obtained from air-dried material of individual plants by hydrodistillation. The composition of the oils was determined on the basis of GC results. The essential oil data of *Thymus praecox* ssp. *arcticus* was statistically analysed using factor analysis, cluster analysis, and discriminant analysis *Thymus pulegioides* couldn't be examined by multivariate statistical analysis because there were too few cases in comparison to the number of variables. Though 5 chemotypes could be separated with the main components linalool, thymol, thymol/linalool, carvacrol resp. geraniol/geranial. These results were similar to those obtained in other European countries. In contrast *Thymus praecox* ssp. *arcticus* proved to be strongly chemically polymorph. On a high level 4 clusters could be found, but depending on the method used 11 to 20 clusters were visible. The cluster characterising components were: γ -terpinene, trans-sabinene hydrate, linalool, linalyl acetate, α -terpinyl acetate, α -terpineol, geranyl acetate, geraniol, geranial, trans-nerolidol, germacrene-1(10),4-dien-6-ol, hedycaryol, τ -cadinol, thymol, α -cadinol, germacrene-1(10),5-dien-4-ol, trans,trans-farnesol, one unknown component and combinations of these components. The differences between the various North European populations will be discussed.

COMPOSITION OF THE ESSENTIAL OIL OF *ZATARIA MULTIFLORA* BOISS.

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GC and GC/MS analysis were carried out on the commercial oil of *Zataria multiflora* Boiss. (Lamiaceae) Iranian origin.

30 components were identified representing 97.71% of the total oil. Thymol (37.57%), carvacrol (21.19%), p-cymene (8.96%), γ -terpinene (7.52%), α -pinene (4.79%) were found as the major components.

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RESEARCHES ON YIELD, ESSENTIAL OIL AND ESSENTIAL OIL COMPOSITION OF SAGE (*SALVIA OFFICINALIS* L.) LINES

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Four cultivated sage lines selected according to flower color and leaf shape were used in this study. PercentAge of essential oils and their compositions were determined as follows:

Oil yields: Fresh herb (0.50-0.55%), Dried herb (1.30-1.60%)

Main components: α -thujone, β -thujone, 1,8-cineole, borneol and α -humulene

**THE ESSENTIAL OILS OF TWO ENDEMIC SALVIA
SPECIES : *S. PISIDICA* AND *S. POTENTILLIFOLIA***

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The genus *Salvia* is represented in Turkey by more than 80 species of which 50 % are endemic.

Water distilled essential oils from aerial parts of two endemic *Salvia* species, *S. potentillifolia* Boiss. et Heldr. ex Benth and *S. pisidica* Boiss. et Heldr. ex Benth have been analyzed by GC/MS.

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THE ESSENTIAL OIL COMPOSITION OF *SALVIA* SPECIES

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The composition of the essential oil from the aerial parts of six *Salvia* species growing wild in Iran were investigated by GC and GC/MS.

The main components of these volatile oils were as follows:

<i>Salvia verticillata</i>	β -caryophyllene	(24.7%)
	γ -muurolene	(22.8%)
	limonene	(8.9%)
<i>Salvia santolinifolia</i>	α -pinene	(70.7%)
	β -pinene	(14.8%)
	limonene	(4.5%)
<i>Salvia officinalis</i>	β -pinene	(16%)
	borneol	(9.4%)
	globulol	(9.3%)
<i>Salvia nemorosa</i>	β -caryophyllene	(44%)
	germacrene B	(26.4%)
	caryophyllene oxide	(6.6%)
<i>Salvia hypoleuca</i>	β -caryophyllene	(45%)
	β -pinene	(11%)
	germacrene D	(10%)
<i>Salvia reuterana</i>	ocimene	(39.6)
	gurjunene	(12.5%)
	germacrene D	(10.7%)

COMPOSITION OF ESSENTIAL OILS FROM THREE NEW *SIDERITIS* SPECIES

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The genus *Sideritis* (Lamiaceae) is represented by 40 species in the Flora of Turkey (1, 2). During recent field studies, five *Sideritis* species have been characterized and recorded as new species, increasing the number to 45. Four of them are new for science (3, 4).

We report on the essential oil compositions of these species: *Sideritis vuralii*, *S. caesarea* and *S. akmanii*. The water distilled oils were analyzed by GC/MS. Main compounds characterized in each oil were as follow:

Sideritis vuralii Duman et Başer: β -pinene (35%), β -phellandrene (14%)

S. caesarea Duman, Aytaç et Başer: β -caryophyllene (8%), caryophyllene oxide (7%)

S. akmanii Z.Aytaç, M.Ekici et A.Dönmez: *ar*-curcumene (17%), spathulenol (15%)

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ESSENTIAL OILS OF ANNUAL *SIDERITIS* SPECIES GROWING IN TURKEY

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Annual *Sideritis* species are represented in Anatolia by four species and altogether five taxa. These are *Sideritis lanata*, *S. romana* subsp. *romana*, *S. curvidens*, *S. montana* subsp. *montana* and *S. montana* subsp. *remota*. Essential oils from aerial parts of these species obtained by hydrodistillation were analysed by GC/MS. Main compounds of these essential oils were as follow:

Sideritis lanata L. : hexadecanoic acid (10%), spathulenol (9%)

S. romana L. subsp. *romana* : thymol (24%), 1-octen-3-ol (12%)

S. curvidens Stapf : bicyclogermacrene (20%), spathulenol (12%)

S. montana L. subsp. *montana* : germacrene D (25%), bicyclogermacrene (11%)

S. montana L. subsp. *remota* (d' Urv.)P.W. Ball ex Heywood: bicylogermacrene (13%), germacrene D (10%).

References:

1. P.H.Davis, Flora of Turkey and the East Aegean Islands. Vol 7, p 178- 199, University Press, Edinburgh (1972).

**ESSENTIAL OILS OF FOUR LABIATAE HERBS
GROWING WILD IN TURKEY**

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The essential oils obtained by hydro distillation of four Labiatae herbs, namely *Salvia cryptantha* Montbr. et Auch., *Satureja cuneifolia* Ten., *Thymbra spicata* L. and *Thymus cilicicus* Boiss. et Bal. growing wild in Central Anatolian provinces of Turkey, were analysed by GC and GC/MS. Fifty-six, thirty-five, thirty-three and thirty-eight compounds were identified of which major ones were camphor (18.05%), 1,8-cineole (17.79%) and bornyl acetate (11.35%); thymol (65.54%), p-cymene (9.84%) and carvacrol (7.23%); p-cymene (22.98%), γ -terpinene (21.37%), carvacrol (21.14%) and thymol (11.77%); α -terpineol (16.42%), camphor (9.66%), sesquiterpene alcohol (8.79%) and 1,8-cineole (7.78%), respectively.

**Morphological and phytochemical characterization
of *Teucrium lucidum* L. and *T. chamaedrys* L.**

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Teucrium lucidum L., an endemit from Maritimes Alps, belongs to the Sect. Chamaedrys, being rather similar to *T. chamaedrys* L. itself. However, owing possibly to the very small area of distribution, the former taxon had been scarcely studied. In this work we have compared both micromorphological and phytochemical (volatile compounds) characters of *T. lucidum* and *T. chamaedrys* growing in the same site. In both species glandular (peltate and capitate types) and non glandular trichomes are present, but in *T. lucidum* non glandular trichomes are scarce and short. In *T. chamaedrys* non glandular trichomes are numerous and long so that the whole plant appears nearly hairy. Therefore the two taxa are well distinguishable relying on the macro- and micromorphological characters.

The composition of essential oils, already known for *T. chamaedrys* because of its use in etnomedicine, do not differ notably. Particularly interesting is, in both taxa, the presence of two cyclooptenones, unusual compounds in essential oils.

CHEMICAL ANALYSES OF IRANIAN AND FRENCH ESSENTIAL OILS OF *LAVANDULA* BY CAPILLARY GAS CHROMATOGRAPHY METHOD

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The essential oils were prepared by steam distillation. A Shimadzu GC-9A with chromatopac C-R3A as data processor and FID as detector were used.

Relative percentAges were calculated by area normalization method neglecting response factors. Identification was carried out by coinjection of standards, using their kovats retention indices and also BY comparison with known chromatograms.

Two columns with different polarities (DB-WAX and DB-1; each one 60 m. length, 0.25 mm. ID, 0.25 micron film thick.) were used for confirmation of the results.

Linear temperature programming modes were used.

NINETEEN compounds were identified in each essential oil among them linalool was the major one (39.5% in French and 38.9% in Iranian oils, respectively).

Four chromatograms (two for each essential oil) and two tables of compositions (including name of compound, its kovats indices and percentage) will be presented.

SECOND COLUMN CONFIRMATION AS A POWERFUL METHOD FOR QUALITATIVE/QUANTITATIVE ANALYSES OF ESSENTIAL OILS

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Essential oils are constituted of various natural organic compounds with different molecular structures and many functional groups (such as hydrocarbons, aldehydes, ketones, esters, oxides, phenolic ethers) some of them are polar and others are nonpolar compounds.

In general, mass spectra (MS) and Kovats retention indices (RI) are used for identification of compounds which are separated by capillary gas chromatography. References show identity of Kovats retention indices for some pairs of compounds on nonpolar columns and identity of some other pairs of compounds on polar columns.

In this method we used two columns of different polarities (DB-1, Dimethylsiloxane 100% methyl and DB-WAX, polyethyleneglycol each one 60 mx0.25 mm, 0.25 micron film thick.).

Kovats retention indices calculated using basic Kovats equation and also from a third order equation as the following:

$$I=A+Bt(r)+Ct(r)^2+Dt(r)^3 (*)$$

where, I= Kovats index t(r)= retention time

A,B,C,D= constants

Softwares written separately for each column for calculating Kovats from basic equation and determining constants in eq. (*).

Results showing good resolution of some compounds (for example trans-ocimene and γ -terpinene) on DB-1 and good resolution of some other compounds (for example, limonene and 1,8-cineole) on DB-WAX.

So in essential oil analyses it will be necessary using two columns of different polarities simultaneously to obtain desired resolution.

RELATION BETWEEN LINALOL CONTENT AND FRAGRANCE OF DIFFERENT ESSENTIAL OILS

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The high or substantial concentration of linalol of essential oils contributes to the antiseptic, antiviral properties, as well as uplifting effect, however their fragrances are differring, as it will be presented by special fragrance-samples. The correlation between linalol concentration and retention time, as well as relative retention time differences has been studied by GC analysis.

The GC retention times of linalol of linalol standards, levant wormseed (*Artemisia maritima*) and lemon-scented mint (*Mentha citrata*) oils (linalol content: 90-95%) have shifted, depending on the linalol quantity. This shifting range of retention times - 0,17 minute - has been reduced in case of determination of relative retention times onto 0,02 minute (internal standard was artemisia ketone as the main component of tansy oil (*Tanacetum vulgare* in 85%). The retention time of linalol constituent of other essential oils such as coriander (*Coriandrum sativum* - 75%), basil (*Ocimum basilicum* - 65%), bergamot (*Citrus bergamia* - 20-27 %), lavender (*Lavandula officinalis* - 34-41%), petitgrain (*Citrus aurantium* var. *amara* - 16-24%) and clary sage (*Salvia sclarea* - 30-55%) have shown much less shifting range in retention times since the less amount of linalol.

It might be concluded, that the difference in retention time of linalol or any other component of essential oils could be a result of much or less quantity. In support of retention time shifting relative retention times should be determined before it would be supposed to discover another chemical structure, eventually an optical isomer, than it was expected.

ESSENTIAL OIL COMPOSITION AT DIFFERENT BASIL (*OCIMUM BASILICUM* L.) ORIGINS FROM MEDITERRANEAN REGION

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Ocimum basilicum L. has a pleasant aromatic odour and many subspecies, varieties and chemotypes. The chemical composition of its essential oil changes widely depending on the origin of the plant material. This study was conducted to determine the effect of different harvesting times on essential oil yields and components of basil from different localities of the Mediterranean Region. Plant materials were collected from K.Maraş, Adana and Hatay. During the experimental year, three harvests (June, August, September respectively) were done at full flowering period. The essential oil components were analysed by GC and GC/MS. Ratio of essential oil and components showed wide differences depending on origins and harvesting times. The highest essential oil ratio 37% and yields (3.09 l/da) in fresh flowers were obtained from Hatay basil and from the second harvest. Increase of temperature in the region enhanced the essential oil yield of basil. 1,8-cineole, linalool, methyl eugenol and methyl cinnamate were determined as the main components in essential oils. Although Adana, K.Maraş and Hatay basil had linalool + methyl cinnamate as the main components in all harvest, two oil samples from Hatay basil contained high amounts of 1,8-cineole (30-38%) in the first harvest.

COMPOSITION OF THE ESSENTIAL OIL OF THREE *OCIMUM* SPECIES FROM S. TOMÉ AND PRÍNCIPE

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The aim of the present paper is to present some of the results already achieved in the study of the essential oil of three species of *Ocimum* from the medicinal aromatic flora of S. Tomé and Príncipe. This genus is widely used for the preparation of remedies in African traditional medicine. In S. Tomé the most representative species are *Ocimum gratissimum* L., *Ocimum camum* L. and *Ocimum minimum* L. All of them are used as febrifuge, for the treatment of respiratory diseases (colds and sinusitis), as well as food flavours.

The essential oils of these plants were obtained by hydrodistillation of the aerial parts. They were analysed by GC and GC-MS, using two fused silica capillary columns of different stationary phases (carbowax and methylsilicone). Identification of the compounds with a percentage higher than 1% was also performed by ¹³C-NMR. The major compounds in the volatile oil of *Ocimum gratissimum* were thymol (48.1%) and p-cymene (11.9%). *Ocimum camum* essential oil was characterised by its high content of methyl cinnamate (80.6%), whereas the most important constituent of *Ocimum minimum* essential oil was linalool (52.9%).

COMPOSITION OF THE ESSENTIAL OIL FROM TWIGS OF *JUNIPERUS NAVICULARIS* GROWN IN PORTUGAL

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Continuing our investigations on the genus *Juniperus* from Portugal we now report on the composition of the twig essential oils from three populations of *Juniperus navicularis* Gand (*Juniperus oxycedrus* subsp. *transtagana* Franco).

The essential oils were isolated by hidrodistillation (yield 0,37-0,66%) and submitted to qualitative and quantitative analysis by GC on two columns of different polarities and by GC-MS. The identity of the oil components was assigned by comparison of their R.I., relative to a homologous series of fat acid methyl esters, and MS with those of authentic samples.

More than 40 components were identified amounting to 83.8-97.3% of the total oils. Monoterpene hydrocarbons were the main components in the oil of all populations (70.3-90.2%), limonene (17.9-33.2%), α -phellandrene (12.3-15.2%) and α -pinene (10.2-25.4%) being the major constituents.

**COMPOSITION OF THE ESSENTIAL OIL OF THE FRUIT
OF *AFRAMOMUM DANIELLI*, A PLANT USED USED IN
TRADITIONAL MEDICINE IN S.TOMÉ AND PRÍNCIPE**

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Continuing our study on the essential oils of the medicinal aromatic plants from S.Tomé and Príncipe we now present the chemical composition of the essential oil of the fruit of *Aframomum danielli* L. It is used by the native population for its medicinal properties (for fevers and placenta expulsion) as well as for its edible spicy flavour. To our knowledge, this is the first time that the composition of the essential oil of this taxon is studied.

The essential oil of this plant was obtained by hydrodistillation of the air-dried fruit. It was analysed by GC and GC-MS using two fused silica capillary columns of different stationary phases (carbowax and methylsilicone). Identification of the compounds with a percentage higher than 1% was also performed by ¹³C-NMR.

The volatile oil was characterised by its high content of oxygenated monoterpenes and monoterpene hydrocarbons, being 1,8-cineol (37%), α -terpineol (11.9%) and β -pinene (13.9%) the most important ones.

***IN VITRO* SELECTION FOR SALT TOLERANCE OF
ROSEMARY (*Rosmarinus officinalis* L.)**

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Leaf segments of rosemary plants were cultured on callus induction medium (CIM) supplemented with 0, 1000, 3000, 5000, 7000 ppm of salt in a form of NaCl and CaCl₂ at ratio of 1:1 (w/w). The induced calli were cultured on regeneration medium (RM) containing the same levels of salt. Salt levels significantly affected the callus induction and its texture. Shoot number decreased by increasing the salt levels. Shoots formed roots in a medium supplemented with indole-3-butyric acid (IBA). Similarly in both the regenerated plants under salinity stress and mother plants, ten monoterpenes were identified in the essential oil extracts.

Influence of Phytohormones on Essential Oil composition of regenerated plants of *Melissa officinalis*

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From callus cultures regenerated plants of lemon balm (*Melissa officinalis*, L., Lamiaceae) have been cultivated under in-vitro-conditions (1). Thereby the growth of genetically uniform plant material was made secure under controlled conditions.

The effect of different phytohormones in various concentrations and combinations was studied on essential oil accumulation. For example some cytokinins cause a significant change of the composition of the essential leave oil (2). Alloaromadendren, a sesquiterpenhydrocarbon and trace component in regenerated control plants, accumulates up to 10% of the total amount of the essential oil. Important changes in monoterpene constitution can be established, too. So with proper phytohormones a new and unusual type of essential oil can be induced (alloaromadendren-type).

Some extremely volatile components have been detected in regenerated plants of *Melissa officinalis* for the first time, by the application of headspace-extraction with "tenax-trapping" instead of water steam distillation.

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Phytohormones and processes of the second metabolism of
peppermint and moldavian dragonhead

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The actions of exogenous plant growth regulators (mixture of gibberellins, chlorocholine chloride, 2-chloroethylphosphonic acid) on the balance of endogenous plant hormones (abscisic acid, 3-indolylacetic acid, zeatin, zeatinriboside and gibberellins), contents and composition of essential oils in five varieties of peppermint (*Menta piperita* L.) and moldavian dragonhead (*Dracocephalum moldavica* L.) were investigated.

The treatment of aromatic plants as well the mixture of gibberellins as retardants blocked up biosynthesis of endogenous gibberellins, increased yield of essential oils, stimulated menthol formation (mint) and (or) increased the level of abscisic acid (mint, dragonhead).

Type and direction of biosynthesis of plant hormones and essential oil compounds are determined by the ability of genotype reaction, variety and species of aromatic plants.

The hormonal regulation of the productivity of sklerocies spurred in
biothechnological system fungus-plant.

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The influence of endogenous gibberellines and gametocydes on IV-V stage of organogenesis fall rye have led to increasing of crop capacity of sklerocies spurred and rising of contents ergoalkaloids (five times as much) in them. The gametocydes promoted the activisation of sfaciales stades fungus development of konidiogenesis. Gibberellins ingibit konidiogenesis and promote the acceleration of the approach of sklerociesal stage. This stage is a responsible for accumulation of ergoalkaloids in spurred. Exogenous gibberellins are induced the increasing of the levels of endogenous cytokinins and IAA in rye spikes and sklerocies spurred developmenting on them.

COMPOSITION OF THE ESSENTIAL OIL OF THREE AROMATIC SPECIES FROM IRAN

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The essential oils of *Heracleum persicum* Desf. (dried leaf), *Bunium persicum* Boiss (seed) and *Stachys lavandulifolia* Vahl. (flower) have been analyzed by GC and GC/MS.

The oils were dominated respectively by trans-anethol (61%), β -pinene (7%) and trans-ocimene (4.7%) for *Heracleum persicum*. The components in the essential oil of *Bunium persicum* were γ -terpinene (40.2%), cuminyl aldehyde (13.8%) and terpinen-7-al (10.5%). Myrcene (20%) α -pinene (18%) and γ -muurolene (13%) were main constituents in the essential oil of *Stachys lavandulifolia*.

SOME ESSENTIAL OIL PLANTS IN TURKEY

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In the present study we determined the essential oil content and composition in selected plants growing in Turkey.

Coriandrum sativum L. - annual herb. The seeds contain 2.5% essential oil.

Pimpinella anisum - annual herb. The seeds contain 4 to 5% essential oil.

Foeniculum vulgare Mill. - annual and perennial herb. The fruits contain 6% essential oil, 20% fixed oil and 22% proteins.

Mentha piperita L. - perennial herb. The leaves contain essential oil from 2 to 5.5%.

Melissa officinalis L. - perennial herb. The leaves and flowering stems contain essential oil.

Salvia officinalis L. - perennial herb. The leaves contain 2.5% (per dry weight) essential oil.

HAIRY ROOT CULTURES OF *ANETHUM GRAVEOLENS* L.: ESTABLISHMENT AND COMPOSITION OF THE ESSENTIAL OIL

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Transformed root cultures of dill were induced by inoculation of 3-4 weeks-old aseptic plantlets with *Agrobacterium rhizogenes* LBA 9402 carrying plasmid pRi 1855. The plantlets were carefully wounded with the tip of a sterile hypodermic needle and injected with one drop of the bacterial suspension grown for 48h. The plantlets were transferred to an MS/2 medium [= half strength MS medium with 30g/l of sucrose] and co-cultivated with the bacteria for 48h, in a 16h light/8h dark photoperiod, at 24°C. After this period they were transferred to fresh MS/2 medium supplemented with cefotaxime and carbenicillin, 250µg/ml each. Roots developed were transferred to liquid MS/2 medium without antibiotics, maintained in darkness at 24°C on orbital shakers (80 r.p.m.), and subcultured every month.

The essential oil from the hairy roots maintained in liquid MS/2 medium, and the oils from the fruits, and from roots and aerial parts of the parent plant in the vegetative phase were analysed by GC and GC-MS and their compositions compared. In the oils from the parent plant the monoterpene fraction was always dominant, although in different amounts, whereas phenylpropanoids were the major components of the oil isolated from the hairy roots. These compounds were also present in the parent plant, except in its fruits. The main component of the oils from the fruits and from the roots of the parent plant was carvone, whereas α -phellandrene was that from the aerial parts and apiole that from the hairy roots.

Acknowledgement - We are grateful to JNICT for scholarships to P.M.L. Lourenço and P.A.G. Santos. This study was partially funded by JNICT under research contract PBIC/C/BIO/1989/95.

THE PRODUCTION OF CUMIN SEED OIL

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Steam distillation of cumin seed oil was studied to show the effects of particle size, steam rate and batch size on oil recovery. Fractionation of the oil produced was also performed to obtain cumin seed oil high in cumin aldehyde composition of the products were analysed by GC/GC-MS. Experiments were carried out at bench and pilot plant scale.

THE GERMINATION BIOLOGY OF INDIGENOUS POPULATIONS OF *FOENICULUM VULGARE* L. var.

VULGARE IN ISRAEL- PRELIMINARY STUDIES

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Foeniculum vulgare var. *vulgare* (bitter fennel), is an aromatic hemicryptophyte. In Israel, plants occur in extremely diversified habitats: in the Central Negev Desert under 100mm of rainfall and up to the Northern regions with 1200mm. Variability of seed weight and germination biology of mericarps (so called 'seeds'), of some populations, have been studied along a transect from the Mediterranean coast at Tel Aviv, to inland areas of the Judean Mountains, ca. 60 Km from the coastal area. Data indicated that seed weight of the Nachshon population (ca 40 km from the coast), was 3.5mg/seed and significantly heavier compared to all other populations (an average increase of 15%); When planted in soil, seeds of this population exhibited also highest emergence of $73 \pm 9.3\%$. However, the time needed to attain 50% germination was significantly longer, as for all mountainous populations ($G_{50} = 11.0-13.0$ days), compared to 8.5 d of the coastal population of Tel Aviv.

To enhance germination of the least germinating population (15.3% during 6.0d) of Kissalon (ca 50Km from the coast), seeds were immersed in KNO_3 solutions (100-1000mM) during 24h, prior setting to germinate. Data indicated that pre-immersion in 500mM KNO_3 had elevated final germination rate by almost 3 times ($G_t = 43.8\%$). This approach could be perhaps implied, when new chemotypes with a low germination rate are introduced into agriculture practice. At present, essential oils, amount, and composition in various populations, are determined.

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ANALYSIS OF THE ESSENTIAL OIL FROM *FERULA ASSA-FOETIDA* L.

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Ferula assa-foetida is one of the most important plant in Iran due to its several attributes is exported to many countries of the world.

The essential oil composition from the gum of *Ferula assa-foetida* growing in two regions of Iran (Kerman and Yazd) were identified by GC and GC/MS. The yield of oil were 16-17 %. The main constituents of both oils were as follows:

Kerman:

- 1- trans propenyl S-butyl disulfide
- 2- cis-ocimene
- 3- trans-ocimene

Yazd:

- 1- methyl 2-methyl-1,3-oxothiolan-2-yl ketone
- 2- trans-propenyls-butyl S-butyl disulfide
- 3- cis-propenyl S-butyl disulfide

GALBANUM

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Botanical source	<i>Ferula galbaniflua</i> Boiss & Buhse and other Ferula species
Botanical family	Umbelliferae
Foreign names	Galbanum (Fr.), Galbanum (Ger.), Galbano (Sp.), Galbano (It.)

Description: Herbaceous, tall plant that grows in northern Persia (*F.galbaniflua*) or in southern Persia (*F.rubricaulia* and *F.ceratophylla*). *F.galbaniflua* is widespread also in Turkey and Lebanon. The plant yields a resinous exudate distinguished commercially in two types: Levant galbanum (soft) and Persian galbanum (hard). The resin has a characteristic aromatic odor and bitter, warm, acrid taste. It contains approximately 16-26% essential oil.

Physical-chemical characteristics of Essential oil: The essential oil is obtained by steam distillation of the dried resinous gum. It is a pale-yellow to yellow liquid with balsamic, somewhat spicy, characteristic odor. Its main constituents include myrcene, cadinene, α -pinene, β -pinene and sesquiterpene alcohols.

Specific gravity at 20°C	:	0.867-0.916
Refractive index at 20°C	:	1.4780-1.4870
Optical rotation at 20°C	:	+3 to +20
Acid value	:	2 max
Solubility	:	1:6 in 90% ethanol

Determination of Essential Oil Content and of Main Oil Components in Umbelliferae Collections by NIRS

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For the breeding of grain-producing plants it is of special importance to investigate intact seed samples. In this context near infrared spectroscopy (NIRS) is well suitable in principle. Because collections of gene banks show a large variability in regard to concentrations of individual plant compounds they build up a suitable basis for the calibration and validation of this analytical method.

Therefore the aim of this study was to investigate whether the determination of essential oils and their main constituents can be performed by NIRS.

The calibration was set up upon solvent extracts received from the fruits of 77 coriander, 63 dill, 42 fennel and 20 caraway cultivars. The reference oil values were calculated by addition of the principal oil substances determined by GC analysis. Oil contents varied for coriander in the range of 0.1 to 1.0 %, for dill in the range of 3.0 to 9.1 %, for fennel in the range of 1.5 to 9.4 % and for caraway in the range of 2.4 to 8.1 %. Furthermore samples of running breeding projects were investigated. A partial least squares (PLS) algorithm was applied to all calibrations and the individual error between predicted and reference values was described by the standard error of cross validation (SECV). Additionally, the coefficient of determination (r^2) was calculated to describe the relationship between reference method and NIR analysis.

In contrast to the usually applied gas chromatography measurement, the described NIRS method allows to predict the individual essential oil content as well as main oil constituents already after an analysis time of approx. 3 - 5 minutes.

THE ESSENTIAL OIL COMPOSITION OF *ACHILLEA MILLEFOLIUM* L.

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Achillea millefolium (Asteraceae) has been used in traditional Turkish medicine as diuretic, appetizer, emmanagogue, carminative, diaphoretic. The essential oil Composition of *A. millefolium* growing in Turkey has not been reported. The essential oil was obtained by hydrodistillation from aerial parts and analyzed by using GC and GC/MS.

99 components were identified representing 80.84% of the oil. Artemisia ketone (10.35%), piperitone (7.47%), chrysanthenone (7.1%), 1,8-cineole (6.61%) were found as major components.

THE ESSENTIAL OIL COMPOSITION OF *ACHILLEA COARCTATA* POIR.

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The genus *Achillea* represented 40 species in Turkey is used in folk medicine as diuretic, appetizer, emmenagogue, carminative, diaphoretic. The essential oil has been obtained by hydrodistillation of *Achillea coarctata* and analyzed by using GC and GC/MS.

Two sample were collected from different localities in Ankara and Eskişehir in Turkey. 1,8-cineole and camphor were found as main components in both samples.

ANTIMICROBIAL PROPERTIES OF THE ESSENTIAL OIL OF *ARTEMISIA ASIATICA* NAKAI

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Artemisia asiatica Nakai cultivated in the Medical Plants Garden, Institute of Biology and Botany, Medical Academy in Wrocław, Poland, yields 1.0 % of the green essential oil. Its composition differs from those observed for the oil obtained from *A. asiatica* from natural origin.

The main constituents are 1,8-cineole (40%) and selin-11-en-4 α -ol (12%). The essential oil was separated by vacuum distillation into the following fractions:

1. 67 % 1,8-cineole
2. 30 % terpinen-4-ol, 21 % borneol and 11 % α -terpineol
3. residue, 75 % selin-11-en-4 α -ol

From the 1st fraction 1,8-cineole and from the 3rd fraction selin-11-en-4 α -ol were isolated by flash chromatography in pure forms (>98% purity).

The antimicrobial properties of the total oil, second fraction consisting of oxygenated monoterpene as well as 1,8-cineole and selin-11-en-4 α -ol were studied against following fungi: *Candida albicans*, *Rhodotorula rubra* and *Aspergillus fumigatus*, by using well test.

C. albicans was more resistant than other fungi.

There occur significant differences in activity of the oil and its components. Selin-11-en-4 α -ol showed the highest activity and 1,8-cineole the lowest one.

This study was supported by State Committee for Scientific Research No. 5 PO6B 103 08.

Constituents of The Essential Oil of *Artemisia absinthium* Grown in Egypt

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Artemisia absinthium L. (Asteraceae) has been used as an anthelmintic to destroy intestinal worms, hence the name "wormwood". Its leaves and flowering tops are used as an aromatic bitter or tonic, a diaphoretic and as a flavoring agent. It is employed in small amounts to flavor some of the aromatic alcoholic beverage and to impart a fragrance to certain liniments (1).

The hydrodistilled essential oil from fresh aerial parts of *Artemisia absinthium* L. (0.78 yield) cultivated in the Medicinal and Aromatic plants station of the Faculty of Pharmacy, Cairo University, was analysed by CGC-MS. Identification of the components of the oil was established by their retention times and mass spectra which were compared with the respective ones reported in literature. The oil was found to contain 72.3% mono- and sesquiterpene hydrocarbons and 27.7% oxygenated compounds. Monoterpene hydrocarbons (61.24%) comprised α -phellandrene (50.52%), γ -terpinene (2.99%) α -pinene (1.92%), tricyclene (1.82%), α -terpinene (1.57%) and limonene (1.12%) as the major compounds. Sesquiterpene hydrocarbons amounted to 11.05% of the oil, caryophyllene (4.12%) and *trans*-isoelemicine (3.56%) being the major compounds. Oxygenated compounds were mainly alcohols; terpinene-4-ol (11.98%) and linalool (10.48%) being the major ones. Chamazulenes and thujones known to be present in oils obtained from *A. absinthium* grown abroad, were not detected in the oil obtained from the plant grown in Egypt. Several chemotypes were recognized for the plant grown in Italy, France, Siberia and Romania (2). *A. absinthium* L. under investigation represents another chemotype.

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**Composition of The Essential Oil of *Artemisia vulgaris* L.
Grown in Egypt**

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Several species of the genus *Artemisia* (Asteraceae) have been the subject of numerous chemical studies (1). Analysis of the essential oil from *Artemisia vulgaris* L. was reported (2,3). In this study, we report on the CGC-MS analysis of the hydrodistilled essential oil from fresh *A.vulgaris* (0.65% yield) cultivated in the Medicinal and Aromatic Plants Station of the Faculty of Pharmacy, Cairo Univ. The analysis was carried out on a BA-70 OV31-OH capillary column. Compounds were identified by their retention times and mass spectra which were compared with the respective ones reported in literature. Hydrocarbons constituted 66.12% of the oil comprising monoterpene hydrocarbons (38.42%) and sesquiterpene hydrocarbons (27.70%). The main monoterpene hydrocarbons are α -phellandrene (17.30%), Δ^3 carene (4.27%) and camphene (4.19%). The main sesquiterpene hydrocarbons are: *trans*-isoelemicin (15.14%) and γ -elemene (8.82%). Oxygenated compounds amounted to 31.46% of the oil, lylatol (15.14%) being the major compound. Several compounds were found in traces and constituted together 2.56% of the oil.

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QUALITATIVE - QUANTITATIVE COMPOSITION OF CHAMOMILE OIL IN EGYPT

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Cooperation between the Egyptian herb specialists and researchers from Slovakia was started in 1993. Our mutual study of Chamomile, *Chamomilla recutita* (L.)Rauschert, chemotypes belongs to a very important field of a research. The purpose of this contribution is presented the qualitative - quantitative characteristics of Chamomile, which is cultivated in Egypt.

The results are presented in table:

production area	the qualitative - quantitative composition (in%) of Chamomile essential oil						
	Fa	BoB	BnA	Bo	Ch	BoA	Dc-s
El-Faiyum	18.2	4.9	9.8	5.5	2.0	40.1	9.7
El-Kanather Shalaka	5.1	2.7	8.7	2.4	1.7	50.7	8.7
El-Tahrir Sahra	3.5	2.4	7.3	11.2	2.0	51.1	5.9
Giza Cairo	2.4	1.6	2.7	3.6	2.6	68.2	7.6

Fa: Farnesene, BoB: γ -Bisabolol B, BnA: γ -Bisabolol A, Bo: γ -Bisabolol, Ch: Chamazulene, BoA: γ -Bisabolol A, Dc-s: En-in-dicycloethers

In regard to these GC-results the Chamomile, which is cultivated in Egypt, belongs to the bisabololoxide chemotype. The % composition of γ -Bisabolol and Chamazulene in essential oil is from 4.1 to 14.2.

THE COMPOSITION OF ESSENTIAL OILS FROM SOME *TANACETUM* SPECIES OF TURKEY

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Water distilled essential oils from some *Tanacetum* (Compositae) species of Turkey were analyzed by GC/MS. A summary of the results are as follows:

Species	Province	Plant part	Oil yield (%)	Main components (%)
<i>T.argyrophyllum</i> var. <i>argyrophyllum</i>	Gümüşhane	leaf	1.03	α -thujone (51.8)
<i>T.argyrophyllum</i> var. <i>argyrophyllum</i>	Gümüşhane	flower	0.96	α -thujone (62.8)
<i>T.armenum</i>	Ankara	leaf	0.62	1,8-cineole (31.3), camphor (8.6)
<i>T.armenum</i>	Eskişehir	herb	0.67	camphor (26.7), 1,8-cineole (11.3), borneol (10.6)
<i>T.balsamita</i>	Culture	herb	0.38	carvone (52.4, α -thujone (11.7)
<i>T.haradjani</i>	Adana	Leaf	0.55	camphor (15.9), 1,8-cineole (10.0)
<i>T.praeteritum</i> subsp. <i>praeteritum</i>	Muğla	herb	1.09	borneol (28.1), 1,8-cineole (12.3), bornyl acetate (10.0%)
<i>T.praeteritum</i> subsp. <i>massicyticum</i>	Antalya	herb	0.92	α -thujone (51.1), β -thujone (10.0)

Composition of the Essential Oil of *Cotinus coggygia* Scop. From Turkey

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Water distilled essential oil (0.87%) from the leaves of *Cotinus coggygia* Scop. (Anacardiaceae) was analysed by GC/MS. Forty two components were characterized representing 99.6 % of the total components. The major constituents were identified as limonene (48.5%), (Z)- β -ocimene (27.9%) and (E)- β -ocimene (9.7%).

CHEMICAL STUDIES ON *BOSWELLIA* AND *COMMIPHORA* SPECIES OF AFRICA

E.Dagne

Department of Chemistry, Addis Ababa University, P.O.Box 30270, Addis Ababa, Ethiopia

Boswellia and *Commiphora* species (family Burseraceae) are mainly found in Africa although a few species are known to occur in Arabia and India. These plants are sources of the culturally and economically important resins olibanum (frankincense) and myrrh respectively. The resins are used as incense, for fragrance and flavour purposes not only in the countries of origin but in many parts of the world. Somalia and Ethiopia are by far the biggest producers of these resins.

The chemistry of frankincense and myrrh has attracted the attention of several workers for many years. Limitation in the taxonomic and botanical knowledge of the genus has hindered chemical work on resins from properly authenticated species. Earlier reports that were based on these resins obtained from commerce lacked consistency and could not be reproduced. Thus it is clear that there is need to determine the composition of these resins from botanically defined species. In our laboratory chemical investigation is in progress on resins obtained from botanically authenticated *Boswellia* and *Commiphora* species.

The pressure of increasing population and accelerated urbanisation is leading to increased destruction of forests and people tend to cut trees for fuel and charcoal-making rather than tapping the resins and benefiting from these resources. The resins in the producing countries are very cheap because they are exported unprocessed to far away lands such as Far East and Europe where value adding takes place. It is therefore important to take measures to conserve these resources and at the same time increase ways and means of adding value to the resins in the countries of origin so that the producing communities derive more benefits from these species and become aware of their sustainable utilisation.

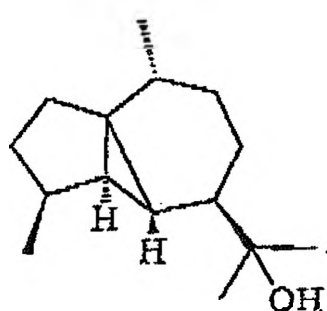
Results of our investigations on the above resins as well as on several other essential oil bearing plants of Ethiopia will be presented.

SOME NEW SESQUITERPENES FROM LABDANUM OIL

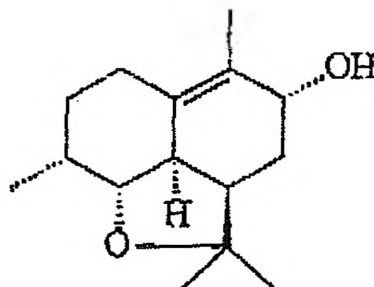
K. Thefeld*, P. Weyerstahl, H. Marschall and M. Weirauch

Technische Universität Berlin, D-10623 Berlin, Germany

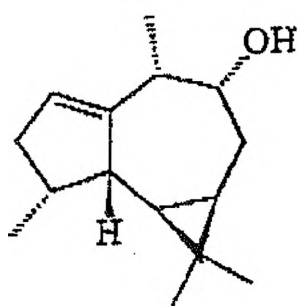
The commercial oil was separated by distillation and repeated flash chromatography. 180 components representing about 95% of the oil could be identified. The main constituents are ledene (9%), viridiflorol (4%), copaborneol (2%), ledol (1%) and the new cubeban-11-ol (1, 4%). Typical for this oil are oxidized cadinane (2) and aromadendrane (3-5) derivatives. The structures of the new compounds 1-5 were elucidated by GC-MS, ^1H - and ^{13}C -NMR spectra.



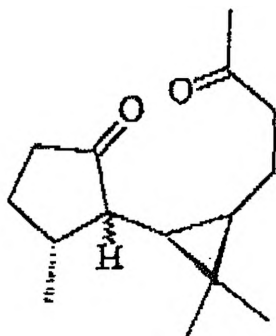
1



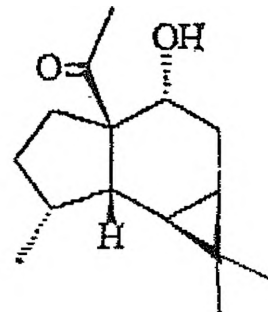
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3



4



5

SESQUITERPENOIC CONSTITUENTS OF THE ESSENTIAL OIL OF SOME *CYPERUS* SPECIES

M. Mekem Sonwa, Y. Saristas and W. A. König,

Institut für Organische Chemie, Universität Hamburg, Hamburg / Germany

Previous studies of the essential oil of the *Cyperaceae* revealed that the major sesquiterpenoic representents in this family are those of the classes of eudesmane and patchoulane^{1,2}. The first part of our work³ was in agreement with these results. The present contribution also confirms this tendency, with the isolation and identification of new eudesmane and rotundane type sesquiterpenes. The compounds were isolated from the hydrodistillated extract of the rhizomes of the plants by preparative gas chromatography. The structure elucidation was done by spectroscopic methods and chemical transformations.

1) Satinder K. Uppal, B. R. Chhabra and P.S. Kalsi *Phytochemistry* (1984), **23**, 2367.

2) B. Nyasse, T. Ghogomu Thi, B. L. Sodengam, M.T. Martin and B. Bodo *Phytochemistry* (1988), **27**, 179.

3) M. Mekem Sonwa, W. A. König, K. H. Kubeczka and O. Moll *Phytochemistry* (1997), **45**, 1435.

COMPOSITION OF ESSENTIAL OILS OBTAINED BY
WATER-DISTILLATION AND HEAD-SPACE OF
PELARGONIUM ENDLICHERIANUM FENZL.

B.Bozan, T.Özek, M.Kürkçüoğlu, N.Kırimer and K.H.C.Başer

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26470 Eskişehir, TURKEY

Pelargonium endlicherianum (Geraniaceae) is an endemic plant of Turkey. Water-distilled essential oil and head-space volatiles from fresh flowers of *Pelargonium endlicherianum* were subjected to GC and GC/MS analysis.

More than 100 components were identified, representing over 75% of the water-distilled oil. The main constituents of the oil were germacrene-D (15.8%), phenylethyl-2-methyl butyrate (12.5%), tricosane (5.5) and T-cadinol (4.7%), while germacrene-D (27.1%), β -caryophyllene (11.3%), α -phellandrene (9.8%), β -pinene (7.3%) and α -pinene (4.9%) were of head-space volatiles.

**HISTOCHEMICAL LOCALIZATION OF CITRAL
ACCUMULATION IN LEMONGRASS LEAVES
(*CYMBOPOGON CITRATUS* (DC.) STAPF., POACEAE).**

E. Lewinsohn*¹, N. Dudai¹, Y. Tadmor², U. Ravid¹, E. Putievsky¹
and D. M. Joel³

¹Aromatic, Medicinal and Spice Crops Unit, ²Corn Breeding Unit,
and ³Department of Weed Research, Newe Yaar Research Center,
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30095 ISRAEL.

Lemongrasses (*Cymbopogon* spp., Poaceae) are a group of commercially important C₄ tropical grasses. Their leaves contain up to 1.5% (dry weight) essential oils with a typical lemon-like aroma, consisting mainly of citral: a mixture of the isomeric acyclic monoterpene aldehydes geranial and neral. In order to specifically locate the sites of citral accumulation in lemongrass we have employed the Schiff's reagent, that stains aldehydes. Using this technique, single oil-accumulating cells were detected in the abaxial side of leaf mesophyll, commonly adjacent to the non-photosynthetic tissue, and between vascular bundles. The cell walls of these oil cells are lignified.

Water Stress effects on aromatic plants of commercial importance growing in India.

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Research Laboratory, WBPPDCL, 1 & 3 MBT Sarani, Illaco House,
Calcutta-700 001, India

One of the recent trends in plant-soil-water management research is the possibility of commercial cultivation of crops under water-stress conditions. In the present study the formation of essential oil in relation to developmental physiology of three important spices of Cymbopogon viz. C. winterianus (Citronella), C. flexuosus (Lemon grass) and C. martini (Palmarosa) under water-stressed condition and its alleviation by a bio-regulator of growth, n-Triacontanol have been investigated.

Water stress condition in all the three species lowers per hectre yield of essential oil; disturbing the proper correlation between different growth entities. Stress inhibiting effects on essential oil yield are manifested through shortening the duration of particularly reproductive and post reproductive phases. n-Tria application releives the water-stress inhibiting effects on oil yield by strengthening the specific correlation between different functional entities as well as of different developmental phases.

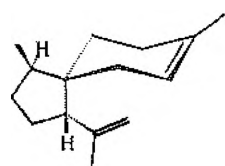
The study claims that inhibition of essential oil synthesis by water stress is monitored through modification of durations of different developmental phases and revival of inhibition by n-Tria is manifested through suitable adjustments of different developmental phases towards normalcy. The functional parameters like linear and leaf growth, flower formation, contents of nitrogenous and CHO fractions are disturbed in a specific way by water stress condition which can be modulated by n-Tria application to the advantage of the species.

Acorane-Type Sesquiterpenoids from the Liverwort *Jungermannia lanciolata*

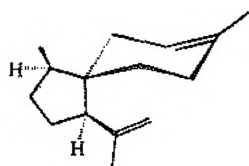
Yücel Saritas, Mesmin Mekem Sonwa and Wilfried A. König

Institut für Organische Chemie, Universität Hamburg, Martin-Luther-King-Platz 6, D-20146 Hamburg, Germany

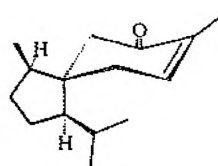
The bryophyte plant group produces a remarkable variety of rare and unknown natural compounds. In particular, liverworts (*Hepaticae*) contain a large number of structurally diverse sesquiterpenes, which are the major constituents of their essential oils¹⁾. Recently we studied the chemical constituent of the liverwort *Jungermannia lanciolata* and isolated compounds **1-4**. The structure of the isolated compounds was determined by GC-MS, 1D- and 2D-NMR techniques²⁾. The stereochemical aspects of these compounds were investigated by two-dimensional enantioselective gas chromatography and by conversion into known compounds.



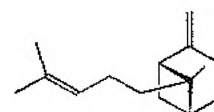
(+)-β-Acoradiene
1



(-)-α-Neocallitropsene
2



(+)-Acorenone B
3



(-)-trans-β-Bergamotene
4

1) Asakawa, Y. (1995) in *Progress in the Chemistry of Organic Natural Products* (Herz, W., Moore, R. E., Steglich, W., Tamm, Ch. and Kirby, G.W. eds.) Vol. 65, p. 1, Springer, Wien

2) We thank Prof. K. H. Kubeczka and Dr. D. Joulain for helpful discussions.

The Composition of the Essential Oils of Some *Hypericum* Species Growing in Turkey

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Guttiferae - St. John's Wort family is represented throughout the world by 40 genera and 1300 species. 400 of them are *Hypericum* species and distributed mainly in temperate regions, tropical mountains.

The genus *Hypericum* is represented in Turkey by 77 species including 30 endemics. *Hypericum* species have a wide range of use as dye, flavouring, as medicine in wound healing, astringent, for anti-viral, anti-depressive and anti-hepatotoxic properties in Turkish folk medicine as well in the world. Anti-AIDS properties of *Hypericum* extracts are being studied in USA.

In this initial study three different species from different localities: *H. perforatum* L. (Bozdağ-Eskişehir), *H. calycinum* L. (İnegöl-Bursa), *H. cardiophyllum* Boiss. (Kilis-Diyarbakır) were investigated. Air dried flowers and leaves were water distilled to obtain essential oils which were then analysed by GC/MS. The main components were identified and reported in detail for the first time for Turkish *Hypericum* species.

***EUCALYPTUS* SPECIES CULTIVATED AT UNIVERSITY COLLEGE DUBLIN: CUT FOLIAGE PRODUCTION AND CHARACTERISTICS OF VOLATILE OILS.**

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Native to Australia, the genus *Eucalyptus* (Myrtaceae) comprises some 500 species, varying in stature from low shrubs to large trees. *Eucalyptus* is grown for timber, biomass, essential oil and cut foliage. There are many chemotypes of *Eucalyptus*, the most common being 1,8-cineole, citronellal, α - and β -pinene, limonene, eudesmol and trans-methyl cinnamate. It is a low yielding species, with oil content between 0.1-1.2%, rarely with 1.2-2.5%. The plants in this experiment were cultivated in grey-podzolic soil of pH 6.0-6.9, in temperate climate as a coppiced plantation. Marketable size of foliage is achieved after 2 years. Adult and juvenile foliage was harvested, distilled and analysed by GC and GCMS. *E. globulus*, *E. rubida* and *E. viminalis* belong to the same chemotaxonomical groups, producing alpha-pinene (12-46%) and 1,8-cineole (18-68%), with significant differences between juvenile and mature leaves. *E. gunii* contained *p*-cymene (15%), bicyclogermacrene 1 and 2 (8% and 14%), ent-spathulenol (27%) and viridiflorol (7%). These results compare favourably with production of oil scented species in tropical climate. The foliage is becoming more popular and strong scent of leaves is an important characteristic. *E. gunii* proved to be a promising species for cut foliage, with yield of 5.4kg/plant and 7.5 kg/plant in 2nd and 3rd year respectively. *E. globulus* has too large leaves for cut foliage, *E. viminalis* with narrow leaves and broad side shoots does not appeal to florists and *E. rubida*, although very attractive, has a low yield (2.1 kg/plant).

**ESSENTIAL OILS OF LEAVES AND FRUITS OF
JUNIPERUS FOETIDISSIMA Willd.**

Z. Tunalier, N. Kırimer, K.H.C. Başer

Anadolu University Medicinal and Aromatic Plant and Drug Research Center 26470
Eskişehir, Turkey

Juniperus foetidissima Willd. (Cupressaceae) is known in Turkey as "Kokar Ardıç". Two samples were collected from different localities in Eskişehir-Turkey. Leaves, fruits, seedless fruits, seeds and branches of the two collections were subjected to hydrodistillation for 3 h using a Clevenger-type apparatus to produce oils in 0.32-1.37% yields. The essential oils were analyzed by GC and GC/MS techniques. The main components of these parts were as follow:

Leaves	:	β -thujone (21-22 %), sabinene (11-21 %)
Fruits	:	sabinene (31-35 %), α -pinene (23-39 %)
Seedless fruits	:	sabinene (13-17 %), α -pinene (10-24 %), terpinen-4-ol (5-13 %)
Seeds	:	sabinene (10-20 %), α -pinene (14-17 %)
Branches	:	α -pinene (56-71 %)

THE CHEMICAL COMPOSITION OF THE VOLATILE OILS OF *PINUS BRUTIA*, *PINUS NIGRA*, *PINUS SYLVESTRIS* GROWING IN TURKEY

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² Anadolu University, Medicinal and Aromatic Plant and Drug Research Centre (TBAM), 26470 Eskişehir, Turkey

The genus *Pinus* is represented by five species in Turkey. Among these species only *P.brutia*, *P.nigra* and *P.sylvestris* are used to produce timber by the Forestry Department. The needles of these three species were collected periodically from the Antalya, Bursa, Denizli, İçel, Kastamonu, Kütahya, Muğla, Samsun and Sinop provinces.

The composition of volatile oils were analysed by means of GC and GC/MS.

Forty seven constituents were identified in the essential oil of *P.brutia*. The constituents identified in high amounts and their percentages are as follows: α -pinene (10.98-33.45%), β -pinene (8.60-51.97%), Δ^3 -carene (0.05-11.45%), β -caryophyllene (2.95-10.87%), germacrene-D (2.26-17.63%).

Forty two constituents were identified in the essential oil of *P.nigra*. The constituents identified in high amounts and their percentages are as follows: α -pinene (4.51-49.53%), β -pinene (1.15-43.10%), β -caryophyllene (5.26-21.48%), germacrene-D (1.81-21.38%).

Forty three constituents were identified in the essential oils of *P.sylvestris*. The constituents identified in high amounts and their percentages are as follows: α -pinene (19.44-56.88%), β -pinene (2.87-17.09%), camphene (0.44-16.84%).

This is the first detailed report on the volatile constituents of the needles of *P.brutia*, *P.nigra* and *P.sylvestris* growing in Turkey

**PRODUCTION OF ROSE ABSOLUTE FROM ROSE
CONCRETE AND DETERMINATION OF IT'S PROPERTIES**

M. Kürkçüoğlu¹, K.H.C. Başer¹, M. Tutaş², M. Aydınli²

¹ Medicinal and Aromatic Plant and Drug Research Centre (TBAM), Anadolu University, 26470, Eskişehir, Turkey

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Turkey is one of the most important rose oil and rose concrete producer in the world. Especially in the region of Isparta and Burdur, rose-cultivation is densely done and the grown oil-rose flowers (*Rosa damascena* M.) are processed with two different methods (steam distillation and volatile solvent extraction) by domestic and foreign firms.

The products obtained by steam distillation and solvent extraction are named as rose oil and rose concrete, respectively. Neither of the products are consumed domestically, but are exported.

In this study the conditions of absolute production and the yields of rose absolute from rose concretes of four different firms were examined and the physicochemical properties and chemical composition of the product, rose absolute, has been determined by GC and GC/MS.

CALIFORNIA ROSE CONCRETE

Karl Lee

Spagyrica, Partington, Big Sur

California 93920, USA

California, a state rich in agricultural diversity, a world-class producer of fine fruits, flowers and produce with a growing international market and appreciation, lacks an agricultural aroma industry of the same world-class quality.

Thirty years ago, California had no varietal wine or grapes available. Innovative thinkers imported and planted the truly great varietals of today's California wine industry. My work suggests an aroma industry is possible in California that is equal in quality to Grasse.

The environment for my study is the Central Coast of California above the fog, lined with well-drained hills of sandy-clay soil high in limestone. The exposure is severe with east, south and west sun--a natural site for a rose study.

Three rose varietals of importance were selected following a twenty-year acclimation evaluation. It is not surprising that after twenty years of neglect and harsh environment, the survivors are: *Rosa Damascena Trigintipetala*, the standard of Rose Otto; *Rosa Gallica Officinalis*, the rose of medicine; and, a moss rose so oily it coats one's fingers with sticky resin.

Evaluation of these rosa chemotypes will hopefully stimulate a more extensive study of these roses with detailed component analysis and comparison with the Bulgarian, French and Moroccan concretes.

Renewable botanical resources are the wave of the future.

THE ESSENTIAL OILS OF *DICTAMNUS ALBUS* L. FROM THREE DIFFERENT REGIONS OF TURKEY

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A single species of the genus *Dictamnus* (Rutaceae) is recorded in the flora of Turkey. *D. albus* L. grows in the north and west Anatolia (1). This species is locally known as "akgiritotu" and "gazelotu" and is used in folk medicine as antipyretic, nervetonic and digestive.

Aerial parts of *D. albus* collected from Samsun, Bursa and Safranbolu provinces of Turkey were subjected to hydrodistillation for 3h using a Clevenger-type apparatus. The essential oils so obtained were analysed by GC and GC/MS techniques.

Oil from the Samsun material contained dictagymnine, feniculine, methyl chavicol and anethole as major constituents (2), while β -pinene, limonene, cis- β -ocimene, γ -terpinene were main compounds in the Bursa and Safranbolu materials.

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INVESTIGATIONS ON THE EXTRACTION OF ESSENTIAL OIL FROM DIFFERENT HERBAL DRUGS DURING AQUEOUS EXTRACTION

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Reliable indications and recommendations for an appropriate dosage should be the basis of a rational phytotherapy. For that reason especially traditional formulations of crude drugs, like aqueous extracts, have to be examined critically. According to a market research for the year 1994, concerning the western part of the Federal Republic of Germany, about one 1.2 billion DM have been turned over for tea and related products in retail trade (1). About 300 million DM of this amount were spent for „non black tea“.

After extensive investigations about storage stability of herbal drugs, it was very interesting for us to know, if the crude drugs' content of essential oil is important for the quality of the aqueous extract prepared out of it. Only little is known about the extraction yield of the lipophilic essential oil components into aqueous extracts.

Different methods in view of a quantitative isolation of the essential oil compounds of aqueous extractions were tested for an easy and rapid GLC analysis. A new microdistillation technique (Capillary Distiller 5325, Fa. Eppendorf) was shown to produce good results.

Sage leaves (*Salviae folium*, DAB 1996) and Peppermint leaves (*Menthae pip. folium*, DAB 1996) are commonly used for the preparation of aqueous extracts for oral application. The German Pharmacopoeia explicitly demands a quality of sage, which is rich in thujon. As a consequence of its well known toxicity, the grade of aqueous extraction for this substance is of considerable importance.

Depending on the mode of extraction, the particle size and the quality of the herbal drug, ranges of extraction from 15 % to nearly 50 % of the essential oil can be found in the aqueous extracts. Remarkable differences between the total essential oil and the lipophilic components of the aqueous extract were found (see also 2,3). In contrast to other components of the essential oil of *Salviae folium*, thujon can be found in higher proportions.

Literature:

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² F. Duband, A.P. Carnat; Ann pharmac. franc., 50 (3), 146-155, 1992

³ H. Miething, W. Holz; PZ, 133 (1), 16-17, 1988

We are grateful for financial support by PhytoLab GmbH & Co.KG, Vestenbergsreuth and BfArM, Berlin

GC-ANALYSIS OF NEROLI OIL ON CHIRAL COLUMNS

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In recent years a large number of racemic adulterations of different essential oils has been detected by GC-analysis on cyclodextrin phases (Ref. 1/2). If this method is to be used in the European Pharmacopoeia, general applicability is necessary. A two-step temperature gradient must enable the separation of all important components by a single one-dimensional GC-run. It must be possible to separate these components on commercially available columns and to check them by easily accessible substances as standards.

The development of an appropriate a chiral GC-method is difficult, due to the overlapping peaks of different ingredients in these oils. The elution sequence of the single components with cyclodextrin phases shows a strong variability depending on temperature (Ref. 3). A number of cyclodextrin phases have been examined, and the results with Neroli oil are reported. Different amounts of the enantiomeres of the main component linalool are detected in Neroli oils of different commercial origin. Similar results are found in so-called "artificial" Neroli oils. Three different adulterations must be taken into account:

- addition of racemic linalool,
- addition of (-)-linalool from sources rich in (-)-linalool (e.g. the essential oil from the wood of *Bursera delpechiana*),
- the artificial "composition" of Neroli oil.

A chiral GC-analysis represents a convenient method for a Neroli oil monograph.

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2. P. Werkhoff, S. Brennecke, W. Bretschneider, R. Hopp, H. Surburg; Z Lebensm Unters Forsch 196, 307-328 (1993)
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Acknowledgement: This investigation was supported by a grant from the Bundesinstitut für Arzneimittel und Medizinprodukte, Berlin.

ESSENTIAL OIL ANALYSIS OF PLANTS FROM CAMEROUN BY GC/FID AND GC/MS

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Ngaoundere, Cameroun

The essential Oils of various plants from Cameroun were analyzed by GC/FID and GC/MS to identify the volatiles responsible for applications in traditional folk medicine of Cameroun as well as their interesting odour impressions for perfumery.

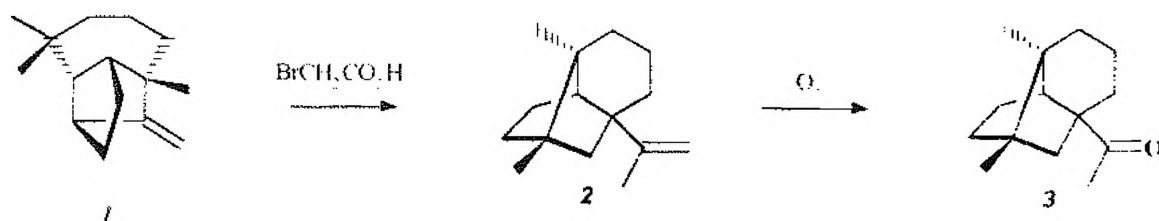
Using different GC/MS systems (GC-QP5000, GC-MSD, GCQ) the essential oils of *Callistemon rigidus* (Myrtaceae, leaves and seeds), *Dorstenia psilurus* (Moraceae, roots), *Hyptis suaveolens* (Lamiaceae, leaves and flowers), *Irvingia gabonensis* (Irvingiaceae, fruits) and *Lagdera pterodonta* (Asteraceae, leaves) were investigated. Especially mono- and sesquiterpenes are dominating among the nearly 200 different constituents of these oils.

After olfactoric evaluation (by professional perfumers) and quantification (by GC/FID measurements) each compound was correlated to a possible odour impression as well as to a reported medical application of the corresponding oil. Also the composition of the investigated essential oils is presented and discussed for a possible use of them in fine perfumery applications.

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Using a longifolene-rich fraction isolated by distillation for the residue of Polish turpentine oil, the three-step synthesis of ketone **3** from longifolene **1** via acid-catalysed isomerisation, followed by ozonolysis of alloisolongifolene **2** is presented.



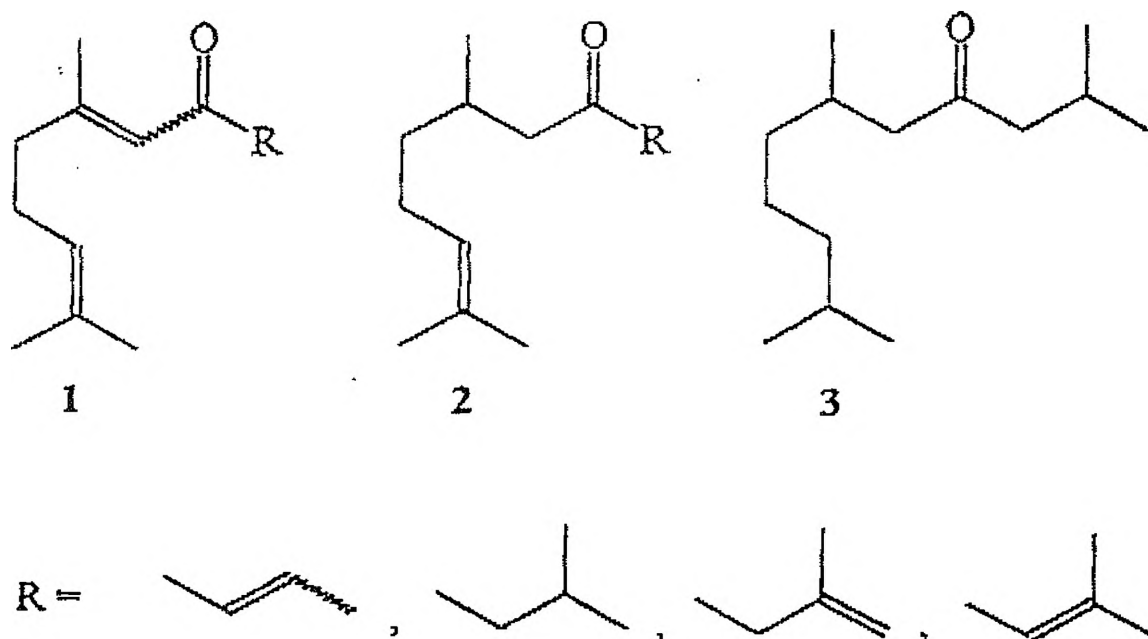
The title ketone **3** possess very interesting olfactory activity by showing an amber-like odor, which belongs to the most appreciated scents in perfumery. Synthesis, structure and spectral details are presented.

SYNTHESIS AND ODOR-STRUCTURE-RELATIONSHIP OF SEVERAL SATURATED AND UNSATURATED KETONES

A. Köhne* and P. Weyerstahl

Technische Universität Berlin, D-10623 Berlin, Germany

The mixture of sesquiterpene ketones identified from solitary bees^[1] was found to be pleasantly smelling. For the investigation of odor-structure-relationship these compounds of type 1-3 were synthesised and olfactorily evaluated.



^[1] W. Francke, *Pure & Appl. Chem.*, 61, 539 (1989)

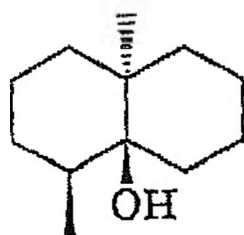
SYNTHESES AND OLFACTORY PROPERTIES OF ALCOHOLS AND KETONES WITH DECALIN SKELETON

B. Volkert* and P. Weyerstahl

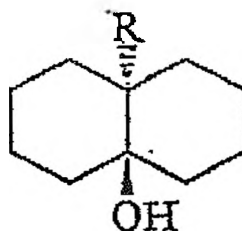
Technische Universität Berlin, D-10623 Berlin, Germany

Geosmin (1), a compound with earthy odor, is one of the strongest fragrance chemicals which is known today (threshold 0.1 ppb). It is formed by various soil microorganisms of the *Streptomyces* family and *Myxobacteria*.

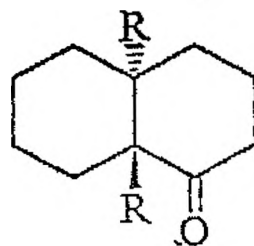
For better knowledge of the relationship of structure and earthy odor, the syntheses and olfactory properties of the two *nor*-geosmins 2 and 3 and several derivatives with decalin skeleton and different functional groups such as the saturated 4 and 5 and the α,β -unsaturated ketones 6-9 are presented.



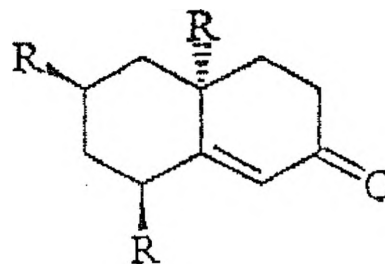
1



2, 3



4, 5



6-9

R = H or Me

**ANTIHEPATOTOXIC EFFECT OF LIPOSOMIZED PREPARATION CONTAINING
VOLATILE OIL OF *CURCUMA XANTHORRHIZA* AND CURCUMINOIDS OF
CURCUMA DOMESTICA ON LIVER DAMAGE INDUCED BY CCL₄**

Sidik

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Bandung, Indonesia

Curcuma xanthorrhiza Roxb and *Curcuma domestica* Val. have been used long in traditional medicine. The rhizomes are used traditionally for tonic, stomache, rheumatism, and liver disorder. The main components are volatile oil and curcuminoids.

The effect of antihepatotoxic effect of Curcuma Complex and Curcuma Complex plus on mice liver damage induced by CCl₄ has been carried out. Curcuma Complex (CC) consisted of Volatile oil (isolated from *Curcuma xanthorrhiza*) and curcuminoids (isolated from *Curcuma domestica*). Curcuma Complex Plus (CCP) is liposomized preparation of CC. Antihepatotoxic effect were examined histopathologically

Ninety mice were randomly divided into 6 groups. Group I was natural control group; group II received CCl₄ only; group III received CCl₄ and CC of dosis 3; Group IV received CCl₄ and CCP of doses 1 and group V received CCl₄ and CCP of dosis 2 and group VI received CCl₄ and CCP of dosis 3.

The results showed that CCP of doses 2 (liposomized CC preparation of 0,14 mg volatile oil/kg b.w and 0,572 mg curcuminoid/kg b.w) had the best antihepatotoxic effect compared with CC (0,28 mg volatile oil/kg b.w and Curcuminoid 1,144 mg/kg b.w) and CCP of dosis 1 (0,07 mg vol.oil/kg b.w and Curcuminoid 0,286 mg/kg b.w) and CCP of dosis 3 (0,28 mg vol.oil/kg b.w and Curcuminoid 1,144 mg/kg b.w).

However the antihepatotoxic effect of CCP can not stop the process of liver fibrosis caused by CCl₄ toxicity twice a week for three weeks.

GLOBAL VISION LEADERSHIP TRAINING

Kathryn Degraff-Keady

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California 95006, USA**

This paper is a survey of the efforts and progress made in the USA since 1988 to develop university level training in the growing and processing of aromatic and medicinal plants for use in fragrance, flavor and pharmaceutical applications, and how TBAM might be instrumental in helping the Americas obtain the kind of education in aromatic extraction technology and basic training in perfumery that is needed everywhere on this continent.

Both Aromatherapy and Perfumery are a combination of Art and Science. Therefore the Aroma Trades need staff with broad cultural background and inter-disciplinary perspective. In France today there are two schools of perfumery, Roure-Givaudan in Grasse, and at Versailles, a government and fragrance industry sponsored school. Both accept French speaking students with broad liberal arts backgrounds from all over the world. However, despite great need, there is no university associated training in perfumery and aromatics available to the general public in the USA.

Following World War II, the global fragrance industry became overloaded with chemists and technicians, trained in scientific discipline, but lacking in history, philosophy and the arts. By the mid sixties the industry realized that chemistry alone was not adequate foundation for perfumery. Therefore, Stephan Arctander, a visionary leader, began recruiting aspiring perfumers with liberal arts backgrounds for the two year course on perfumery & essential oils he taught at Rutgers University, College of Pharmacy in Newark, New Jersey, USA.

The course was taught until 1969. No high level training in perfumery has been available in the USA since then. Consequently, there is now a backlog of pent-up demand for training in perfumery in North America, as well as in all the countries of the Middle East, Asia, Africa, S.E. Asia and the Far East where English is a *lingua franca*. As France is the only country where there is a school of perfumery open to the public, they are inundated with requests for some kind of instruction in the English language. It is time the English speaking world took action to meet this demand.

ESSENTIAL OIL INDUSTRY IN IRAN

S.F. Mirbaha

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Karaj/Iran

The world production of the essential oils is estimated to be 45000 tons a year. The developing countries have an active role in this field. We are going to discuss crucial problems in Iran and suggest suitable methods in this regard. The main point are as follows.

1- Information

In the Essence industry the dispersion of information seems more evident. Each plant needs an information pyramid containing different species, their geographical condition, manner of cultivation, processing and harvesting and also their application, suitable technology and their quantity and effective substances and so on...

1- Industrial Structure

The main points to be covered thoroughly are as follows:

- a) The existence of internal and foreign markets.
- b) The rationalization of production lines and quality control.
- c) Requirements for the acquisition of tools and equipments and final products in rural production units and cultivation, industrial complexes.

3- Training

The manner of promoting the culture of application of technology and information through an efficient system. Special organizations are engaged in this section. Some general methods are suggested to be applicable from rural up to the university level.

THE USES OF ESSENTIAL OILS IN FOOD INDUSTRY

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Ege University, Engineering Faculty, Food Engineering Department, Bornova, İzmir

Essential oils are the volatile oils obtained from plants or from parts thereof by (1) water distillation (2) steam distillation (3) enzymatic action followed by steam distillation, or (4) water and steam distillation.

In Food Industry, essential oils are widely used as a herb and spice extract instead of direct usage of plants. The main purpose of this is to render food products more attractive to human taste. Essential oils are widely used in food production due to their antimicrobial, antioxidative, coloring and flavoring properties.

Some examples of spices that contain important essential oils used in Food Industry are cloves, turmeric, cumin, caraway, cardamom, coriander, cinnamon, fennel, fenugreek, nutmeg and mace.

ESSENTIAL OILS AND PLANT SUBSTANCES - AN ALTERNATIVE OF SYNTHETIC GROWTH ENHANCERS IN ANIMAL FEEDING

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The administration of synthetic growth enhancers came under criticism because of the suspected development of vancomycin-resistant enterococci. In some EU countries, the administration was prohibited (Avoparcin Germany, 1997). As with growth enhancers the daily growth rate of the animals can be increased by 5-9% the farmers demand secure and economic alternatives. Traditional compounds of active substances (Bio-tonic, Livol) as well as new preparations with essential oils (CRINA, Micro-plus) are commercially available. Tests in Brandenburg have shown that it is possible to store freshly harvested grain (18% moisture) with an addition of 0.2% *Origanum*, *Caraway* and *Clove* oil, resp. In fodder acceptance tests grain which was stabilized by essential oils was added. Forty days pig fattening tests (38 kg-72 kg) have demonstrated the following fodder utilization.

	Control	<i>Origanum</i> oil 0.1%	<i>Cinnam.</i> oil 0.1%
Daily weight increase, g	882	882	892
Feed per animal and day, kg	2.50	2.36	2.42
Feed per kg weight increase, kg	2.83	2.69	2.71

Alternative preparations of high efficiency and acceptance will be developed by the combined use of essential oils and prebiotic inulin.

IGV is interested in integrating the work into European research projects and is searching for partners within the 4th framework programme of EU.

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 Wyllie, S.G., O2
 Ziv-Raz,I., O12

IFEAT

The International Federation of Essential Oils and Aroma Trades is a leading sponsor of the 28th International Symposium on Essential Oils, 1-3 September, Eskişehir, Turkey.

Founded in 1977 to counteract unfavourable trading practices experienced by the industry at the time, IFEAT has grown in membership numbers, breadth of member interest and activities over the past 20 years. The federation now has a truly worldwide membership of companies involved in all aspects of essential oil production, extraction, distribution, brokerage, information supply and the use of essential oils, and aroma chemicals.

IFEAT is committed to improving the worldwide climate for trade issues in essential oils and has organised over 10 successful conferences and meeting in the past 14 years providing an unequalled forum for the discussion of current issues affecting the industry.

IFEAT is an organisation committed to training and education and runs an international distance learning course, the IFEAT Diploma in Perfumery. IFEAT is an active supporter of Plymouth University, England, the leading centre for perfumery education in Europe.

The principle objectives of IFEAT are:

- * To advance and promote the commercial viewpoint of the essential oils and aroma chemicals industry worldwide,
- * To encourage the production of essential oils and to co-operate with international associations representing the compounders of essential oils and aromatics,
- * To organise international meetings and to develop a constructive dialogue among producers, dealers and users of fragrances and flavour raw materials.

For further information on the IFEAT Conference (19-23 October 1997, Seville, Spain), to receive a membership application form or an Annual Report, please contact:

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ANADOLU ÜNİVERSİTESİ
BASİMEVİ – ESKİŞEHİR

28th ISEO

28th INTERNATIONAL SYMPOSIUM ON ESSENTIAL OILS

1-3 September 1997
Eskişehir-TURKEY

PROGRAMME



Anadolu University
Medicinal and Aromatic Plant and Drug Research Centre
(TBAM)

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Dr. Daniel Joulain, Grasse, France

Venue

Anadolu University, Atatürk Culture and Art Centre

1 SEPTEMBER 1997

- 08.30-10.00 **Registration**
Atatürk Kültür ve Sanat Merkezi
(Atatürk Culture and Art Centre)
- 10.00-10.15 **Opening Ceremony**
- K.H.C. Başer, President, Organizing Committee
- A. Öcal, Rector, Anadolu University, Eskişehir, Turkey
- Chamber Music Concert
- 10.15-10.45 **Tea Break**
Chairpersons K.H.C. Başer - E. Stahl-Biskup
- 10.45-12.00 **Opening Lecture**
Scents from Rain Forests, R. Kaiser
- 12.00-12.45 **Plenary Lecture**
The Essential Oil Composition of Pimpinella Species,
K.H. Kubeczka
- 12.45-14.30 **Lunch, Yemekhane (Cafeteria)**
Chairpersons K.H. Kubeczka - R. Ikan
- 14.30-15.15 **Plenary Lecture**
Aromatherapy- A Compilation of Scientific Facts in Favour of its Correct Use, G. Buchbauer
- Oral Presentations**
- 15.15-15.35 *Antifungal Properties of Essential Oil Components*,
J.C.R. Demyttenaere
- 15.35-15.55 *The Effect of the Essential Oil of Melaleuca alternifolia on Escherichia coli*, J. L. Markham
- 15.55-16.30 **Tea Break**
- 16.30-18.30 **Poster Session** - Kütüphane "Sergi Salonu"
(Library- Exhibition Hall)
- 19.00-21.00 **Welcome Reception**- Yemekhane (Cafeteria)

2 SEPTEMBER 1997

Chairpersons D.Joulain - W A König

09.00-09.45

Plenary Lecture

Recent Developments in Phytochemistry of Bryophytes,
Y. Asakawa

Oral Presentations

09.45-10.05

Biosynthesis and Hydrolysis of Monoterpene Glycosides,
R. Ikan

10.05-10.25

The Biosynthesis of Essential Oil Components in Aromatic Plants, E. Lewinsohn

10.25-11.00

Tea Break

Chairpersons P. Weverstahl - C. Bicchi

11.00-11.45

Plenary Lecture

The Use of Essential Oils in the Formulation of Natural Flavours, D. Joulain

Oral Presentations

11.45-12.05

Supercritical Fluid Extraction of Terpene Hydrocarbons from Citrus Oils, M. Budich

12.05-12.25

The Evolution of a New Generation of Fine Natural Plant Oils, P.F. Wilde

12.30-14.00

Lunch, Yemekhane (Cafeteria)

Chairpersons G. Buchbauer - E. Sezik

14.00-14.45

Plenary Lecture

Synesthesia, A. Baydar

Oral Presentations

14.45-15.05

Australian Tea Tree Oil: Efficacy, Toxicity, Irritancy and Stability, D.N. Leach

15.05-15.25

Chromatographic Study of Essential Oils from Artemisia Species, S.M. Adekenov

15.25-16.00	Tea Break
16.00-18.30	Poster Session - Kütüphane "Sergi Salonu" (Library- Exhibition Hall)
20.00 -	Symposium Dinner- Turkuaz Restaurant

3 SEPTEMBER 1997

Chairpersons Y. Asakawa - E. Levinsohn

09.00-09.45 **Plenary Lecture**

Identification of Rare Sesquiterpene Structures in Essential Oils. W.A. König

Oral Presentations

09.45-10.05 *Packed Column SFC in the Analysis of Terpenoids.* C. Bicchi

10.05-10.25 *Control of Storage Diseases of Potato and Sprout Inhibition by Plant Volatiles.* U. Bang

10.25-11.00 **Tea Break**

Chairpersons Ch. Franz - A. Baydar

11.00-11.45 **Plenary Lecture**

Some Novel Tricyclic Sesquiterpene Skeletons from the Essential Oil of Echinops giganteus. P. Weverstahl

Oral Presentations

11.45-12.05 *Bioconversion of Alkene Phenols from Aromatic Plants to Natural Aroma Compounds.* E. Shimon

12.05-12.25 *Methyltransferases Involved in the Biosynthesis of Methylchavicol and Methyl-eugenol in Sweet Basil.* I. Ziv-Raz

12.25-13.00 **Closing Session**

13.00-14.00 **Lunch,** Yemekhane (Cafeteria)

14.00-18.00 **Botanical Excursion** (Optional)

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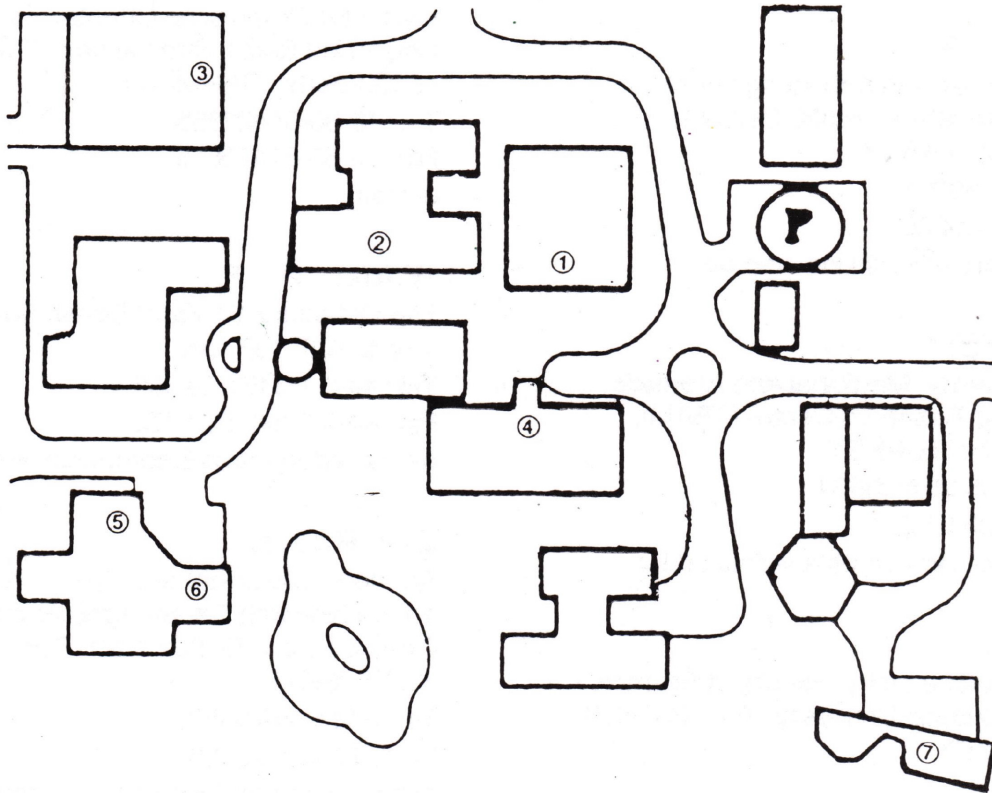
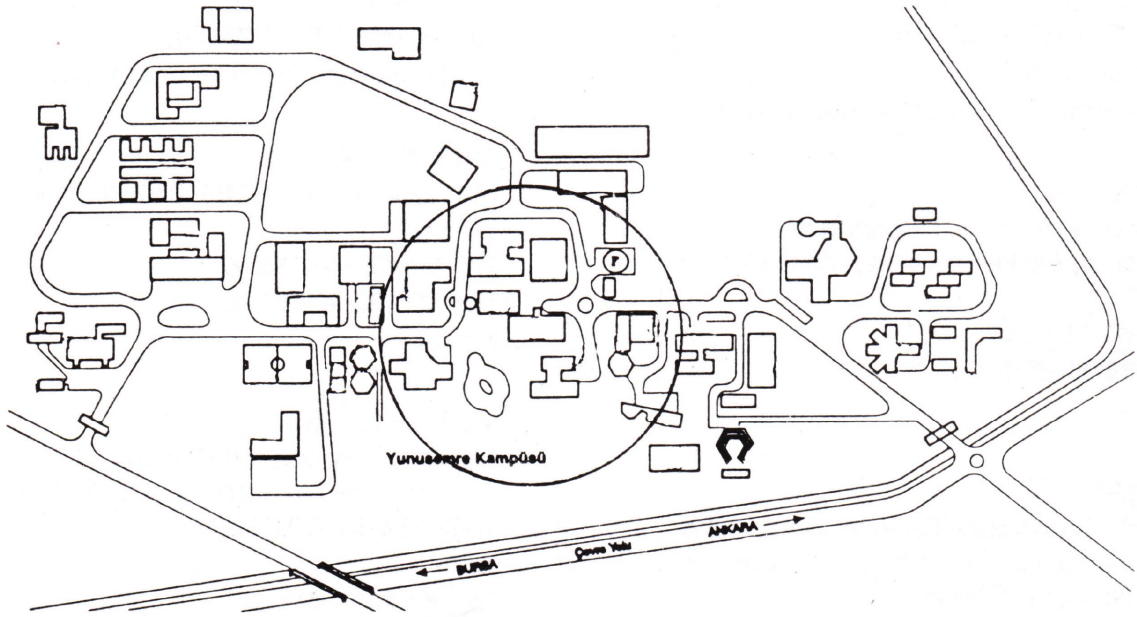
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ESSENTIAL OIL COMPOSITION AND BIOLOGICAL ACTIVITY STUDIES OF *LAVANDULA STOECHAS* SSP. *STOECHAS*

G. Topçu¹, M.N. Ayrar^{1,2}, and A. Aydın¹

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Hydrodistilled oil of *Lavandula stoechas* ssp. *stoechas* was analysed by GC-MS and camphor and fenchone were found as main components besides bornyl acetate, α -thujene, α -pinene, camphene, limonene, eucalyptol, fenchone, terpinen-4-ol, fenchyl acetate, α -terpineol, 2-norpinen-2-carboxyaldehyde, aromadendrene, cadinene, palustrol, ledol, globulol, γ -cadinol and levomenol.

GC-MS analysis of the essential oil obtained by supercritical fluid extraction of the same plant, led to the identification of eucalyptol, fenchone, camphor, bornyl acetate, aromadendrene, γ -muurolen, calamene, ledol and globulol.

The essential oils obtained by hydrodistillation and supercritical fluid extraction were evaluated for their antibacterial and cytotoxic activities and found to be moderately active against *Staphylococcus aureus* and *Proteus mirabilis*, and weakly active against COL 2 (9.8 μ g/ml) and LNCaP (17.6 μ g/ml) cancer cell lines.

THE ESSENTIAL OIL COMPOSITION OF *EUCALYPTUS CAMALDULENSIS* FROM MOZAMBIQUE

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The composition of essential oil, obtained by hydrodistillation of the leaves of *Eucalyptus camaldulensis*, using a Clevenger apparatus, have been investigated.

It was observed that the yield oil obtained from the older growth leaves of 4 and 11 years old trees yield of varied in the range of 1.3-1.6%.

GC-MS analysis of the oil revealed, as major components: α -pinene (4.63-0.78%), β -pinene (9.20-0.05%), limonene (2.88-1.05%), γ -terpinene (5.27-10.39%), p-cymene (4.68-11.60%), alloaromadendrene (1.56-0.92%), terpinen-4-ol (12.79-5.45%), α -terpinol (5.45-2.15%), epiglobulol (1.09-2.15%), globulol (5.39-9.61%) and viridiflorol (1.05-1.75%). It was also found that the 4 and 11 years old samples contain 1,8-cineole (39.9-37.09%) respectively, as the major constituent.

Keywords: 1,8-cineole, *Eucalyptus camaldulensis*, Clevenger, GC-MS

THE EFFECT OF DIFFERENT SOWING TIME ON ESSENTIAL OIL OF *CORIANDER*

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Coriander (*Coriandrum sativum* L.) is naturally grown in the Southeastern Anatolia Project (GAP) area, which covers Adıyaman, Diyarbakır, Gaziantep, Mardin, Siirt, Şanlıurfa, Batman and Şırnak provinces. The area is very rich in terms of biological diversity.

Coriander is traditionally used as a spice and medicinal. Its essential oil has analgesic, sedative, tonic, aperitif, anthelmintic, carminative, aphrodisiac and diuretic effect.

This study was conducted to find out the most suitable sowing time for essential oil production in GAP region in 1996 and 1997. Field trials were done in Diyarbakır province, representing the GAP region; 5 different sowing times (November 15, December 15, January 15, February 15, March 15) were investigated; as a result, the highest essential oil content was found as 0.36 %.

Essential oil content, no significant difference observed from sowing times in the first year (0.29-0.34%) In the second year yes (0.06-0.36%)
trial

THE LIPID ACTIVE EFFECTS OF ESSENTIAL OIL EXTRACTED FROM OREGANO (*ORIGANUM VULGARE* SSP. *HIRTUM*) IN ADIPOSE, HEART AND KIDNEY TISSUES FROM A PREGNANT RAT EXPERIMENTAL MODEL

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Essential oil extracted from oregano (*Origanum vulgare* ssp. *hirtum*) was administered orally at three concentration levels: 165 mg/kg (a), 334 mg/kg (b) and 835 mg/kg (c), to pregnant rats to investigate its capacity to change the fatty acid (FA) % composition in the four lipid fractions in a number of organs: cholesteryl (CE), triacylglyceride (TG), free fatty acid (FFA) and phospholipid (PL) fractions.

Two fractions were analysed from adipose tissue. C16:0, 31.04 ± 2.15 n=4 to $23.81 \pm 0.41^*$ n=4 (b) and $24.74 \pm 0.22^*$ n=3 (c); C22:5(N-3), 0.63 ± 0.02 n=2 to $0.48 \pm 0.02^{**}$ n=4 (b) and C22:6(N-3), 0.63 ± 0.02 n=2 to $1.21 \pm 0.04^{**}$ n=2 (b) all differed statistically from the control animal samples taken from the TG fraction. In the PL fraction, C14:0, C18:0, C18:1(N-9), C18:1(N-7), C20:1(N-9), C20:4(N-6) and C22:6(N-3) FAs differed from the control FAs significantly over the treatment levels.

In the heart, C16:0, C18:0, C18:2(N-6), C20:4 (N-6) and C22:6(N-3) in the CE fraction all differed significantly, relative to the control at one or more concentration levels.

In the kidney CE fraction, C18:1(N-9), 14.79 ± 0.71 n=2 to $19.49 \pm 0.57^{**}$ n=3; C18:2(N-6), 14.57 ± 0.17 n=2 to $25.37 \pm 1.33^{**}$ n=3 and C20:4(N-6), 28.08 ± 0.87 n=2 to $10.87 \pm 1.23^{***}$ n=4, changed at the highest treatment level. In the TG fraction, C14:0, 0.93 ± 0.08 n=4 to $1.31 \pm 0.04^{**}$ n=3 (a), $1.28 \pm 0.08^*$ n=4 (b) and $1.29 \pm 0.06^*$ n=2 (c); C14:1(N-5), 0.09 ± 0.01 n=2 to $0.02 \pm 0.00^{***}$ n=4 (b); C18:1(N-7), 2.56 ± 0.11 n=4 to $0.08 \pm 0.01^{***}$ n=3 (a) and $0.08 \pm 0.00^{***}$ n=3 (b). Other FAs which demonstrated significant changes included C18:3(N-6), C20:1(N-9) and C22:6(N-3). Only three FAs in the FFA fraction showed significant changes: C17:0, 0.33 ± 0.01 n=4 to $0.25 \pm 0.00^{**}$ n=2 (a); C18:1(N-9), 11.31 ± 0.27 n=4 to $12.91 \pm 0.34^{**}$ n=3 (b) and C18:2 (N-6), 14.91 ± 0.27 n=4 to $16.03 \pm 0.29^*$ n=4 (c). In the PL fraction C16:0, 28.86 ± 0.33 n=3 to $24.08 \pm 0.83^{**}$ n=2 (a), $24.69 \pm 1.15^*$ n=4 (b) and $22.20 \pm 0.59^{***}$ n=2 (c); C17:1(N-7) 1.03 ± 0.08 n=2 to $0.24 \pm 0.02^{**}$ n=2 (c); C20:5(N-3), 0.69 ± 0.01 n=2 to $0.90 \pm 0.01^{**}$ n=2 (a), $0.87 \pm 0.04^*$ n=2 (c) and C22:5(N-3), 4.04 ± 0.14 n=4 to $0.52 \pm 0.24^{***}$ n=2 (a), $1.11 \pm 0.40^{***}$ n=2 (b) and $1.19 \pm 0.06^{***}$ n=2 (c) all showed significant changes.

These experimental data would suggest that the essential oil extracted from oregano may cause significant changes in the FA % composition profile in different lipid fractions of adipose, heart and kidney organs under these experimental conditions and concentration levels.

EFFECT OF THYME OIL AND THYMOL ON PHOSPHOLIPID C20:4(N-6) AND C22:6(N-3) LEVELS IN AGEING RATS TISSUES.

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Ageing can be defined as a progressive accumulation of changes with time, associated with or responsible for the ever increasing likelihood of disease and death that accompanies advancing age. Free radicals can account for a number of the ageing phenomena, but it is often the direct role that free radicals play on our cell membranes particularly the polyunsaturated fatty acids (PUFA) that contribute to most of the changes occurring. This study shows that supplementing the diets of rats during their lifespan, with thyme oil (*Thymus vulgaris* L.) and thymol, has a beneficial impact on phospholipid PUFA in the rat liver and brain.

Results showed no significant change with age in the mean liver C20:4 concentrations from 7-28 months of age. Likewise, individual comparisons between the aged (28 months) control, thyme and thymol values (20.03 ± 0.38 , 21.59 ± 0.22 , 21.76 ± 0.49 ; weight percentage of total fatty acids present), with the young (7 months) control value (22.24 ± 0.96), were also not significant. However, both thyme and thymol at 28 months were significantly different from the aged matched control ($P < 0.05$). For C22:6 levels, there was a significant increase with age ($P < 0.001$). There were also significant changes between the aged control (9.22 ± 0.40), thyme (10.27 ± 0.20) and thymol (10.25 ± 0.25) with the young control value (7.43 ± 0.48) at $P < 0.05$, $P < 0.01$, $P < 0.01$ respectively. No level of significance was reported between any treatments at 28 months. In the brain, like that of the liver there was no significant change with age in the C20:4 concentrations. Also, no significant differences was found between the aged control, thyme and thymol values (9.58 ± 0.28 , 10.52 ± 0.25 , 10.58 ± 0.25) and that of the young control (10.13 ± 0.47). However, significant differences were found between the aged thyme and thymol values and that of the aged control value ($P < 0.05$). Finally the brain C22:6 levels showed significant changes with age ($P < 0.01$). The aged control (10.95 ± 0.32) decreased significantly from the young control (13.23 ± 0.31) ~ $P < 0.01$, where as the aged thyme and thymol concentrations (13.40 ± 0.29 & 12.75 ± 0.36) showed no significant changes. However, comparisons between the aged concentrations found both thyme and thymol to differ significantly from the control at $P < 0.001$ and $P < 0.01$ respectively. In conclusion, as the rats age, there appears to be significant changes with age in both liver and brain phospholipid C22:6 concentrations, but not C20:4 concentrations. Both dietary supplements have significant effects on C20:4 and C22:6 levels in the liver and brain, the most significant being in the brain C22:6 levels.

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*Editors***

**Proceedings of
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on Essential Oils
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PROGRESS IN ESSENTIAL OIL RESEARCH

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N. Kırimer
*Editors***

**Proceedings of
The 28th International Symposium
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1 - 3 September 1997
Eskişehir, Turkey**

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Proceedings of the 28th International Symposium on Essential Oils, 1-3 September 1997, Eskişehir, Turkey.

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III. E.a. IV. Dizi: Anadolu Üniversitesi. Tıbbi ve Aromatik Bitki ve
İlaç Araştırma Merkezi yayınları no. 1
TP958 .I59 1998

ISBN 975-492-794-4

98-019

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Printed in Anadolu University Press, Eskişehir, Turkey

PREFACE

28th International Symposium on Essential Oils (28th ISEO) was held between 1-3 September 1997 in Eskişehir, Turkey.

The Symposium took place at the modern campus of Anadolu University and was attended by 153 participants from 27 countries. The symposium programme consisted of 8 plenary lectures, 12 oral and 57 poster presentations.

This book of proceedings include full texts of seven plenary and twelve oral papers presented at the symposium. Abstract of one plenary lecture has also been included.

We would like to extend our gratitude to the staff of the Medicinal and Aromatic Plant and Drug Research Centre (TBAM) for their help and assistance in the organization of the symposium, to the Office of the Rector of Anadolu University for the provision of conference facilities and to the members of the Organizing and Scientific Committees through their care and efforts the 28th ISEO has become a successful symposium.

Our special thanks go to Dr.Temel Özek, Mr.Ayhan Altıntaş, Mr.Fatih Demirci and Ms.Handan Kahraman for their help during formatting the book and to Anadolu University Press for printing it in a short time.

August 1998

Prof. Dr. K. Hüsnü Can BAŞER
and
Prof. Dr. Neşe KIRIMER

Editors

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PLENARY LECTURES

SCENTS FROM RAIN FORESTS

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ABSTRACT

Since the dawn of time, the secretive and fragile kingdom of the Rain Forest, which covers 11-13% of all emerged land and shelters eight to nine tenths of all forms of known and unknown life, harbors extraordinary biological treasures worth to be treated with utmost care. According to a further estimation, 70-80% of these species, meaning around 60% of the entire biodiversity, is found for ecological reasons in the canopy region of the Rain Forests.

Having been convinced to find this richness also reflected on the olfactory level we searched during the past years in the understory as well as in the canopy of Rain Forests for new attractive scents. This communication opens the discussion of chemical, biological and olfactory aspects of such scents.

INTRODUCTION

There are no other places with so much light, warmth and humidity as in West Africa, South East Asia, on the Western Pacific Islands and in South America, from South Mexico across the Amazonian Basin down to South Brazil. Based on these conditions, at least 2'000 mm of precipitations homogeneously spread over the total year, an average humidity between 75-80% and an annual average temperature between 26-27°C, the most diverse vegetation has developed in these regions forming as a whole the Rain Forest. In total it covers today around 11-13% of the emerged land and hosts, according to a rough estimation, 80-90% of all species of plants, mush-

rooms and animals (Figure 1). In Amazonia, for example, over 100 big tree species may be encountered per hectare which give home to an enormous number of other plant species and animals. The majority of all these species, according to another estimation 70-80% (corresponds to at least 60% of the entire biodiversity), are living in the canopy region of the Rain Forest, until recently nearly inaccessible and still belonging to the least explored biospheres. All these made it most likely that the canopy would also be an olfactory treasury.

During the past 10 years, modern technical systems have been developed to get direct access to the canopy, the fulfilment of many botanists and biologists dreams. As I will illustrate during the second part of my lecture, one of the most efficient systems was available to us to search in the canopy of Lower Amazonia for new scents.

From various scent-oriented expeditions to tropical regions - we call them "ScentTreksTM" - I would like to discuss in the following some parts of our studies in the Atlantic Rain Forest in the region of Morato, near Guaraqueçaba, in the Brazilian State of Paraná, and in the Rain Forest of Lower Amazonia in French Guiana.

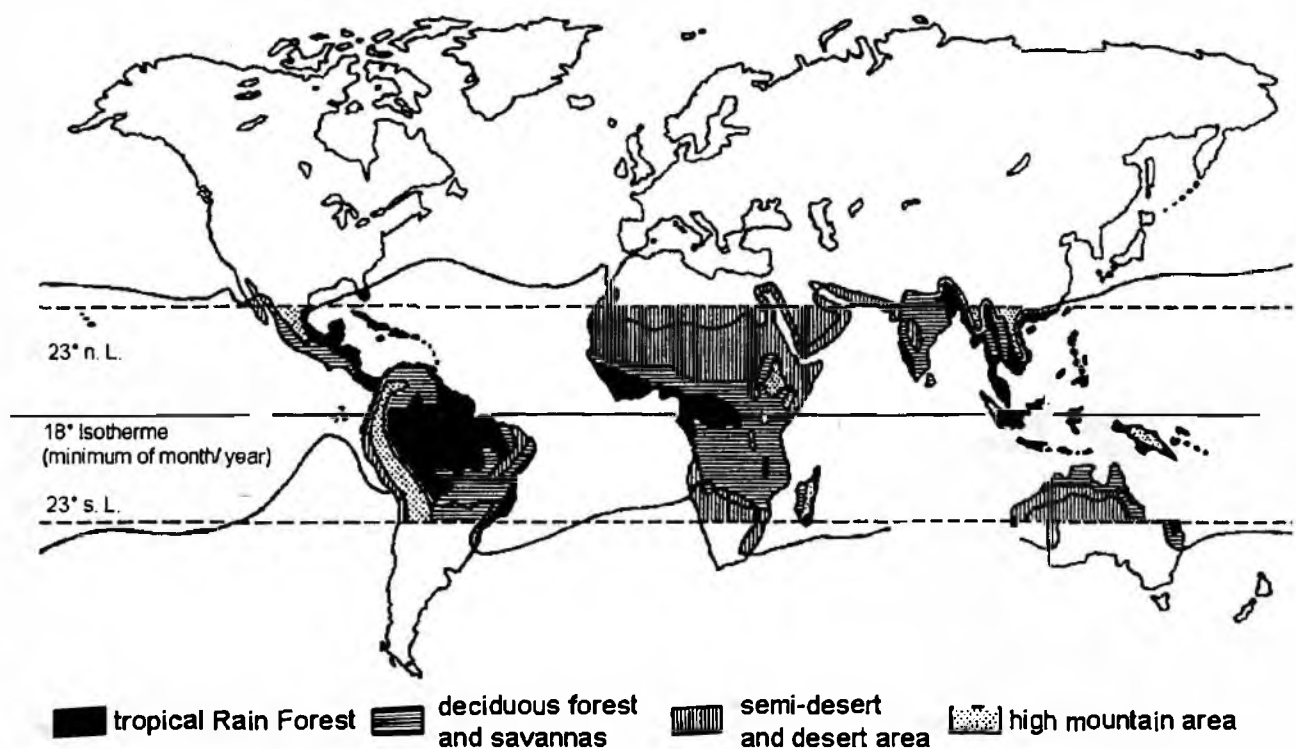


Figure 1 Climatic Zones of the Tropics

SCENTS FROM THE ATLANTIC RAIN FOREST

At the time Brazil was discovered, the Atlantic Rain Forest stretched along the entire eastern coast, from the states of Rio Grande do Norte to Rio Grande do Sul, corresponding to 12 % of the Brazilian territory. Today, it is one of the world's most threatened ecosystems representing only 4% of the original forest cover (Figure 2). Fortunately, the 1988 Brazilian Constitution declared it to be a part of the National Heritage and the Foundation of the Atlantic Rain Forest Consortium has found in the meantime also vital support from Brazilian private companies.

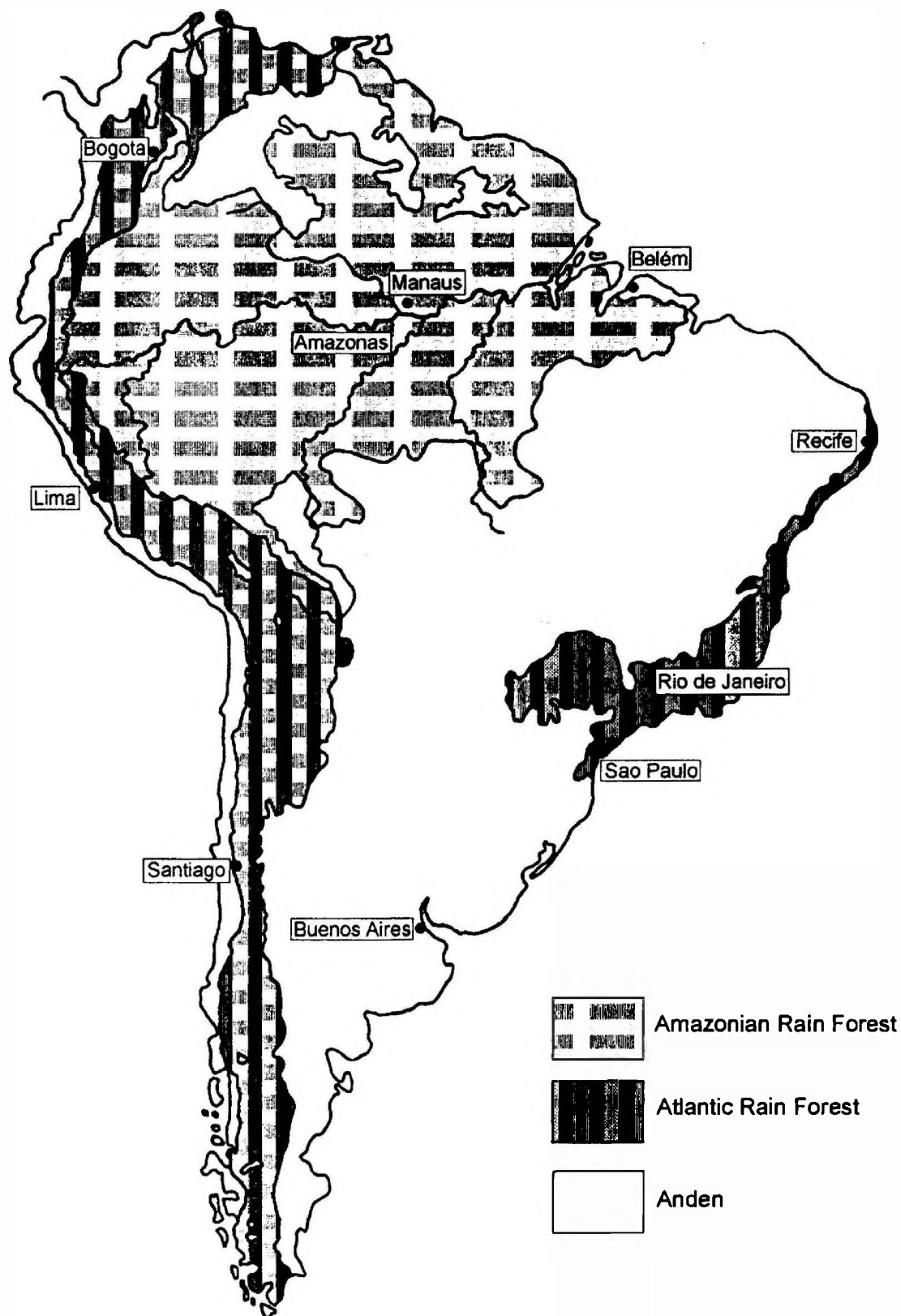
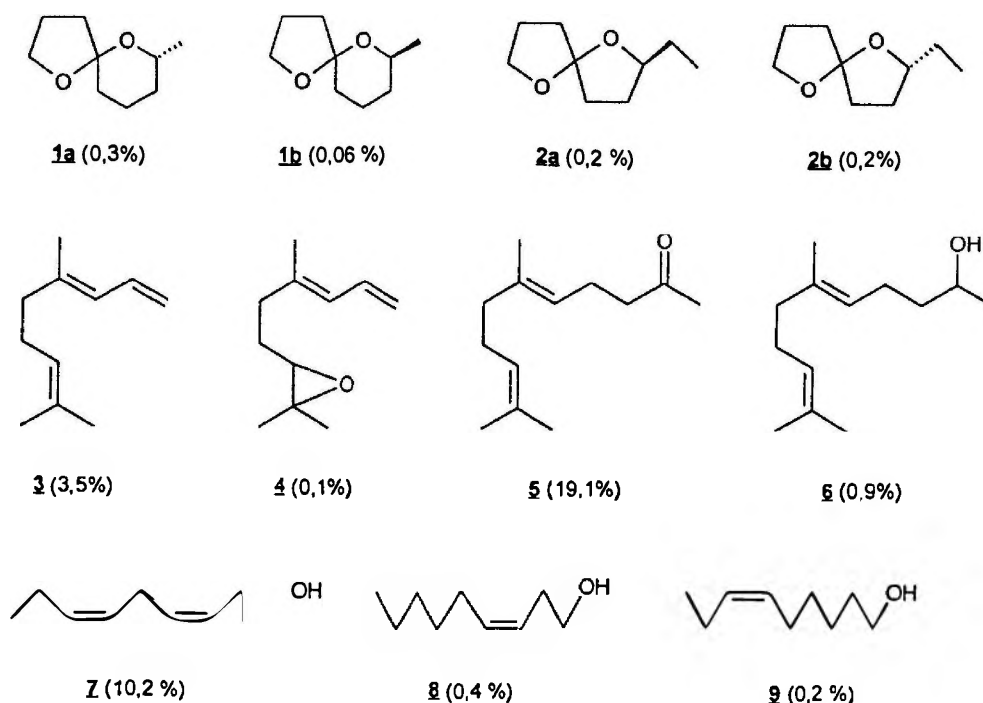


Figure 2 Rain Forest of South America

The visited region of Morato, near Guaraqueçaba, is characterized by a rather low average annual temperature of 22°C, but on the other side by a very high average relative humidity of over 80 %. These conditions favor the growth of an enormous amount of epiphytes. Especially the trees at the border to open land are often so heavily covered with species of Gesneriaceae, Bromeliaceae, Araceae, Orchidaceae and even Cactaceae, especially *Rhipsalis* and *Epiphyllum* species, that branches occasionally break. It is not only very humid, everywhere is water, as creeks flowing down the slopes and entering soon the mangrove belt, or as impressive falls as at Salto Morato. The ground vegetation is tighter here than in Amazonia, consisting e.g. of large stand of *Heliconias* and various understory palms. At transitions to open land or wherever light comes to the ground various Solanaceae can be found. During my visit in early spring, this means second part of September, *Solanum variabile* Mart., the so-called Jurubeba, was the most abundant species in bloom. Their white flowers look similar to those of *Solanum tuberosum* (potato) and they emit a very characteristic melony, watery scent, accompanied by rosy and aromatic-floral notes.

Responsible for these attributes are especially the (Z,Z)-3,6-Nonadienol (**7**), accompanied by its dihydro derivatives **8** and **9**, and (E)-Geranylacetone (**5**) together with the corresponding alcohol **6** (Figure 3). Remarkable is also the occurrence of the epoxide **4**, which is derived from the C₁₁ homoterpene **3** the latter occurring in an extremely high num-



Main Components: Limonene 12,8%, β -Phellandrene 22,2%, Linalool 1%, β -Bisabolene 9,1%, 3,5-Dimethoxy toluene 0,4%, Indole 0,2 %

^{a)} trapped on Porapak, 10.45-17.00, Sept. 9, 1996, Salto Morato

Figure 3 Floral Scent of *Solanum variabile* (Solanacea)

ber of natural scents and being in certain species involved in highly fascinating biological systems of communication [1]. Also the spiroketals **1a/b** and **2a/b** are known to be of semiochemical importance. The mixture of the latter two isomers is known under the name chalcogran as a part of the aggregation pheromone of the bark beetle [2] while **1a/b** have been found by Franke a co-author [3] in female workers of *Paravespula vulgaris*. These compounds have been identified by us as minor constituents already in various flower scents [4] but it is quite remarkable to note that the (E)- and (Z)-7-methyl -1,6-dioxaspiro[4.5]decanes (**1a/b**) could now be even

found as dominating constituents. Thus, they represent around 10 % (E:Z - 9:1) of the floral scent of a *Dorstenia* species (Moraceae) occurring in the Morato region. In the Amazonian *Dorstenia turnerifolia* Fisch. & Mey. **1a/b** occur to even around 30 % (Figure 4). Olfactorily, this scent is strongly dominated by geosmin (**10**), which is accompanied by a trace of dehydrogeosmin (**11**). Among a large number of plant species analytically investigated in our laboratory as well as in others [5] geosmin (**10**) and dehydrogeosmin (**11**) were only identified in a series of Cactaceae species and, as an interesting exception, in *Dorstenia turnerifolia* [6] (Figure 5). Geosmin creates together with the esters **12** - **15** an extremely unusual musty-fruity scent.

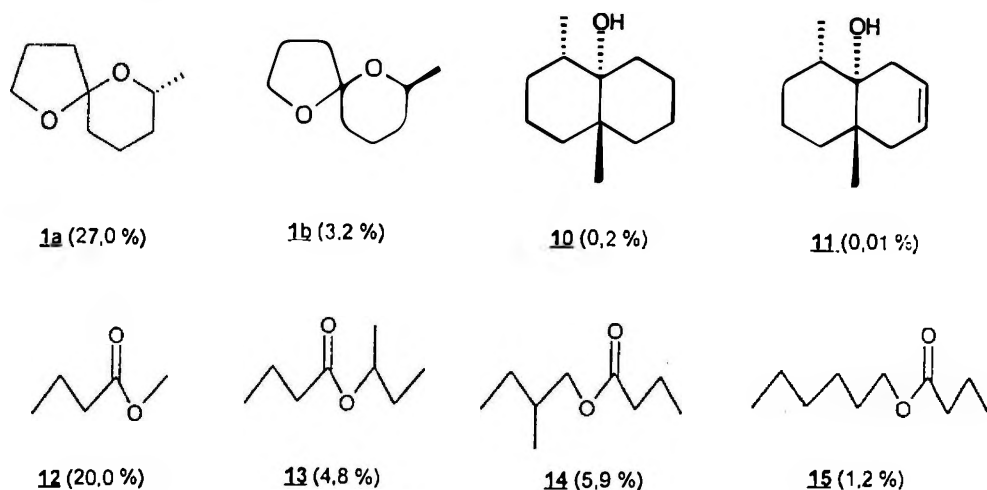
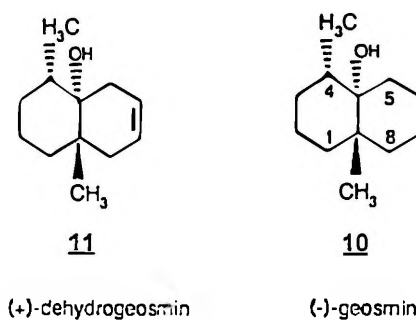


Figure 4 Floral Scent of *Dorstenia turnerifolia* (Moraceae)

Acnistus arborescens (L.) Schlecht. the so-called "Maria Mole" - is another Solanaceae typical to the Southern Atlantic Rain Forest which is in bloom during early spring. This species occurs often together with *Solanum variabile*, is



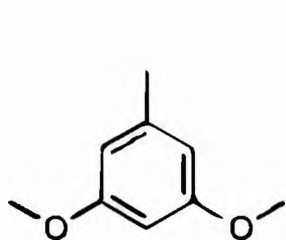
		%	%
<i>Rebutia marsoneri</i>	Werd.	33.0	0.01
<i>Rebutia narvaecense</i>	(Card.) Donald	26.5	
<i>Echinopsis coronata</i>	Card.	15.0	
<i>Sulcorebutia kruegeri</i>	(Card.) Ritt.	7.6	
<i>Echinopsis mamillosa</i>	Gurke. var. <i>tamboensis</i>	6.0	
<i>Dolichothele longimanma</i>	Br. et R.	5.5	
<i>Pereskia aculeata</i>	Mill.	0.05	1.3
<i>Dorstenia turneraefolia</i>	Fisch. et Mey.	0.01	0.2

Figure 5 Occurrence of Dehydrogeosmin and Geosmin

of shrub-like to tree-like growth habit and develops along twigs and branches a great number of white flowers which are reminiscent of a *Cestrum* species. They emit a characteristic sweet aromatic-floral scent, which is based on an accord generated by 3,5-dimethoxy toluene (**16**), jasmone, anis aldehyde and methyl anthranilate (Figure 6)

The most characteristic early spring scent in the Guaraqueçaba area has its origin, however, in blooming trees of *Inga marginata* Will. (Leguminosae) The bottle-brush-like white inflorescences covering these trees emit an extremely diffusive fruity-floral scent which is characterized by a strong black-currant note the latter showing a tendency to

Main Components	Area % ^{*)}
Limonene	2,9
β -Pihllandrene	4,6
(Z)-Ocimene	1,2
(E)-Ocimene	46,0
Linalool	1,0
Methyl benzoate	1,6
3,5-Dimethoxy toluene	32,0
Jasmone	1,1
Anis aldehyde	0,2
Methyl anthranilate	0,2



16 (32,0%)



17 (0,03%)



18 (0,03%)

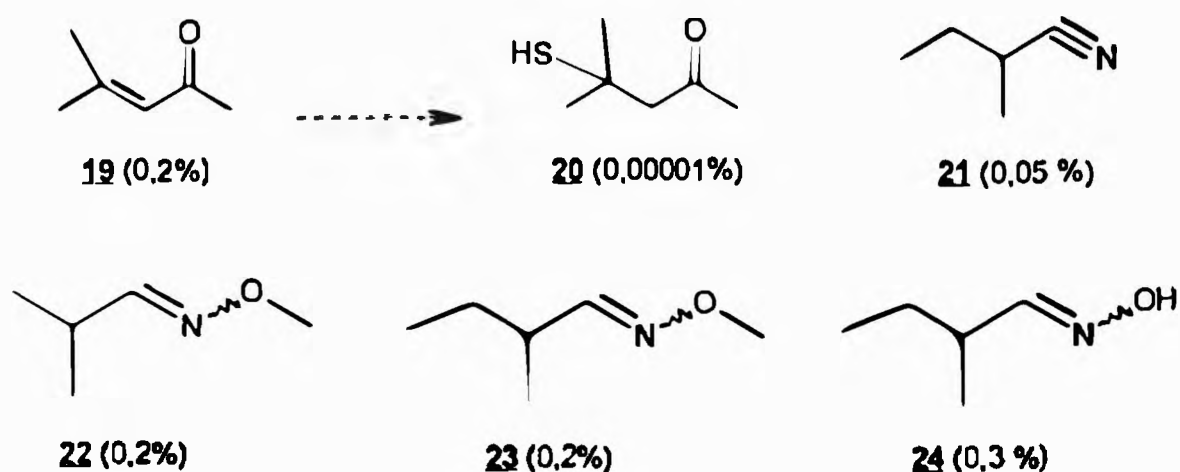
^{*)} trapped on Porapak, 09.50-14.00, Sept. 23, 1996, Salto Morato

Figure 6 Floral Scent of *Acnistus arborescens*
the so-called "Maria Mole"

Main Components

Area %^{*)}

(E)-Ocimene	17,0
trans-Linalool oxide (furanoid)	5,8
2,6,6-Trimethyl-2-vinyl-THP-5-one	2,6
cis-Linalool oxide (furanoid)	2,9
Linalool	6,9
Caryophyllene	47,2
Humulene	3,0
Linalool oxides (pyranoid)	7,0
2,6-Dimethyl-3,7-octadiene-2,6-diol	0,9
Caryophyllene epoxide	0,3



^{*)} trapped on Porapak, 15.00-19.00, Sept. 25, 1996, Salto Morato

Figure 7: Floral Scent of *Inga marginata*
the so-called "Inga Feijão"

cat urine if the nose is to close. This note could be localized by sniffing at the exit of the GC and, after pre-concentration of the corresponding region, a mass spectrum could be recorded which was identical with that of 4-mercapto-4-methylpentan-2-one (**20**), the adduct of hydrogen sulfide to mesityl oxide (**19**) (Figure 7). This mercapto-ketone shows an extremely low perception threshold of around 0,05 ng/l air and has been recently described as an aroma impact product of sauvignon wines [7]. Furthermore, **20** has been identified as one of the many compounds formed during Maillard reactions involving cysteine, ribose and phospholipid [8]. In the scent of *Inga marginata* it is accompanied by the structurally and olfactorily interesting isobutyraldoxime-O-methyl ether (**22**) and 2-methyl-butyraldoxime-O-methyl ether (**23**) (E:Z for **22** and **23** ~ 15:1).

Over the last 15 years the oximes of isobutyraldehyde, 2-methylbutyraldehyde (**24**), and isovaleraldehyde (**26**) have been identified in a great variety of natural scents [5]. In contrast, the oxime ethers are less common as natural products. Thus, the methyl ether **22** was so far only detected in the headspace above some microorganisms [9] and the two methyl ethers **23** and **25** have been recently described as occurring in the headspace volatiles of spider mite-infested cucumber leaves [10]. Undamaged leaves do not produce these oxime ethers.

As illustrated by Figure 8 we could identify these very

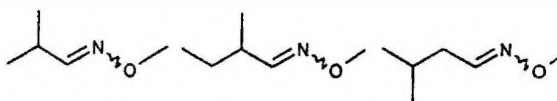
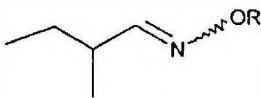
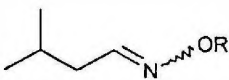
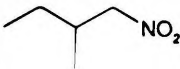
				
		<u>22</u>	<u>23</u>	<u>25</u>
		%	%	%
<i>Carissa macrocarpa</i>	Apocynaceae		0,6	
<i>Cerbera odollam</i>	Apocynaceae		0,2	
<i>Coffea arabica</i>	Rubiaceae		1,5	
<i>Curcumis melo</i>	Curcubitaceae		0,2	0,05
<i>Dioclea reflexa</i>	Papilionaceae		1,1	
<i>Inga marginata</i>	Leguminosae	0,2	0,2	
<i>Hedychium gardnerianum</i>	Zingiberaceae			0,07
<i>Lonicera caprifolium</i>	Caprifoliaceae			0,02
<i>Nicotiana alata</i>	Solanaceae		0,03	0,4
<i>Nicotiana suaveolens</i>	Solanaceae			0,2
<i>Posoqueria latifolia</i>	Rubiaceae		0,1	0,1
<i>Salix caprea</i>	Salicaceae		0,6	
<i>Tabernaemontia ventricosa</i>	Apocynaceae		0,08	

Figure 8 Occurrence of the Oxime-O-methyl ethers 22, 23 and 25 in Natural Scents

volatile components in a whole series of floral scents [11]. They occur, for example, together with high amounts of the corresponding oximes 24 and 26 in the white-floral scent of *Posoqueria latifolia*, a Rubiaceae occurring in Central America as well as in the southern part of Brazil (Figure 9). This small tree growing not higher than 7 m has its habitat preferentially along river banks in low lands. The inflores-

cences terminate the branches and consist of a cluster of white flowers with extremely long corolla tubes, well adapted to pollination by hawk moths. In fact the flowers begin to open an hour before dusk, and shortly thereafter they fill the night with a very enjoyable intense spicy white-floral scent.

Main Components	Area %
(E)-Ocimene	2,2
1-Nitro-2-methylbutane (27)	0,8
2-Methylbutyraldoxime (24)	9,0
Isovaleraldoxime (26)	10,1
Methyl benzoate	6,7
Germacrene D	34,1
Benzyl acetate	1,5
Methyl salicylate	1,0
Benzyl alcohol	0,6
Benzyl tiglate	0,9
Eugenol	1,2
Benzyl salicylate	2,5

		
23 (R = CH ₃ , 0,1%)	25 (R = CH ₃ , 0,1%)	27 (0,8 %)
24 (R = H, 9,0%)	26 (R = H, 10,1%)	

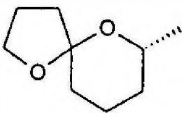
	
1a (0,1%)	2a/b (0,2 %, 1:1)

Figure 9 Floral Scent of *Posoqueria latifolia*

SCENTS FROM THE RAIN FOREST OF LOWER AMAZONIA

In a unique project we had the opportunity to search in the canopy as well as in the understory of the Amazonian Rain Forest of French Guiana for new natural scents. The project was divided into two missions: the first mission took place from September 30 to October 16, 1996 in the region of Camp Parakou, 30 km west of Kourou; the second mission was from November 29 to December 11, 1996 in the region of Camp Voltaire, 80 km south of Saint Laurent du Maroni.

The technical infrastructure to get access to the canopy consisted of a dirigible of 50 m x 20 m holding 8'500 m³ air and additionally equipped with a diesel engine for the fine approach, which is designed to carry either

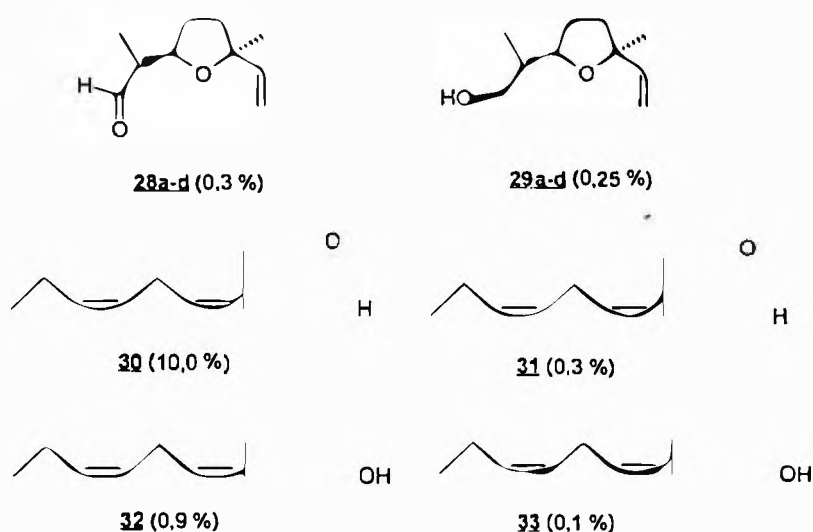
a raft (the so-called radeau des cimes) of hexagon shape measuring 600 m² to be placed on the canopy for stationary studies over a few days
or

a so-called skimmer or sledge (triangle with sides of 5,5 m) with up to three researchers on it being directly brought to the trees of interest.

This highly efficient system was developed and is operated under the guidance of Francis Hallé, professor for tropical botany at the University of Montpellier, Dany Cleyet-Marrel, designer and pilot of the dirigible, and Gilles Ebersolt, inventor and designer of the raft.

One of our first discoveries in the canopy of Parakou

Main Components	Area % ^{*)}
Sabinene	4,3
1,8-Cineole	8,2
trans-Linalool oxide (furanoid)	21,1
cis-Linalool oxide (furanoid)	1,4
α -Cubebene	2,4
Linalool	31,1
Germacrene D	6,4
trans-Linalool oxide (pyranoid)	7,2
Benzyl alcohol	2,9



^{*)} trapped on Porapak, 10.00-14.00, Oct. 10, 1996, Camp Parakou

Figure 10 Floral Scent of *Macoubea guianensis*

was *Macoubea guianensis* Aubl., a rather frequent Apocynaceae in Lower Amazonia, which blooms in September and October with clusters of white star-like flowers similar to those of *Tabernaemontana* species. In the late evening up to early morning flowering trees are perceivable from far by their very ex-

traordinary scent of yoghurt and milk, which turned bad, calamus, algae and "white flowers". Linalool and the linalool oxides, accompanied by the structurally related isomers of lilac alcohol (29a-d) and lilac aldehyde (28a-d), represent the main components of this scent (Figure 10). Much more relevant to the olfactory characteristics are, however, the uncommon C₁₀-lipid metabolites **30** - **33**. The dienal **30** and the corresponding alcohol **32** we recently also found in the floral scent of *Brassavola flagillaris* [11], an orchid native to Minas Gerais in Brazil, where they represent not only regarding their olfactory contribution but also regarding their quantities dominating constituents (Figure 11).

C₁₀-Lipid Metabolites in *Brassavola flagillaris* (Orchidacea)

(Z,Z)-4,7-Decadienal (30)	2,6 %
(Z)-4-Decenol	0,2 %
(Z)-7-Decenol	0,3 %
(E,Z,Z)-2,4,7-Decatrienal (31)	0,05 %
(Z,Z)-4,7-Decadienol (32)	18,4 %
Decan-4-olide	0,1 %
(Z)-7-Decen-4-olide	0,2 %

C₁₀-Lipid Metabolites in *Rodriguezia refracta* (Orchidacea)

(E,Z)-2,4-Decadienal (34)	85,0 %
(E,E)-2,4-Decadienal	3,7 %
(E,Z,Z)-2,4,7-Decatrienal (31)	2,4 %
(E,Z)-2,4-Decadienol	2,5 %
(E,E)-2,4-Decadienol	0,1 %
(E,Z,Z)-2,4,7-Decatrienol (33)	0,03 %

Figure 11 C₁₀-Lipid Metabolites in *Brassavola flagillaris* and *Rodriguezia refracta*

The (Z,Z)-4,7-decadienal (**30**) has been described as a minor compound with character impact in the oil of *Acorus calamus* [12] and the (E,Z,Z)-2,4,7-decatrinal as an off-flavor of strongly autoxidized oils [13] and other food as well as a lipoxygenase cleavage product in freshwater diatoms (*Melosira varians*) [14].

On the other side we found in the most unusual scent of the Venezuelan orchid *Rodriguezia refracta* Rchb. f. [4b] around 85 % of (E,Z)-2,4-decadienal (**34**), accompanied by 2,4 % of its precursor (E,Z,Z)-2,4,7-decatrinal (**31**) contributing a typical codliver aspect and the corresponding alcohol **33**, which is also present in *Macoubea guianensis* (Figure 10). It may be assumed that the C₁₀-lipid metabolites of all these floral scents are formed from linolenic acid via the common intermediate (E,Z,Z)-2,4,7-decatrinal (**31**) (Figure 12).

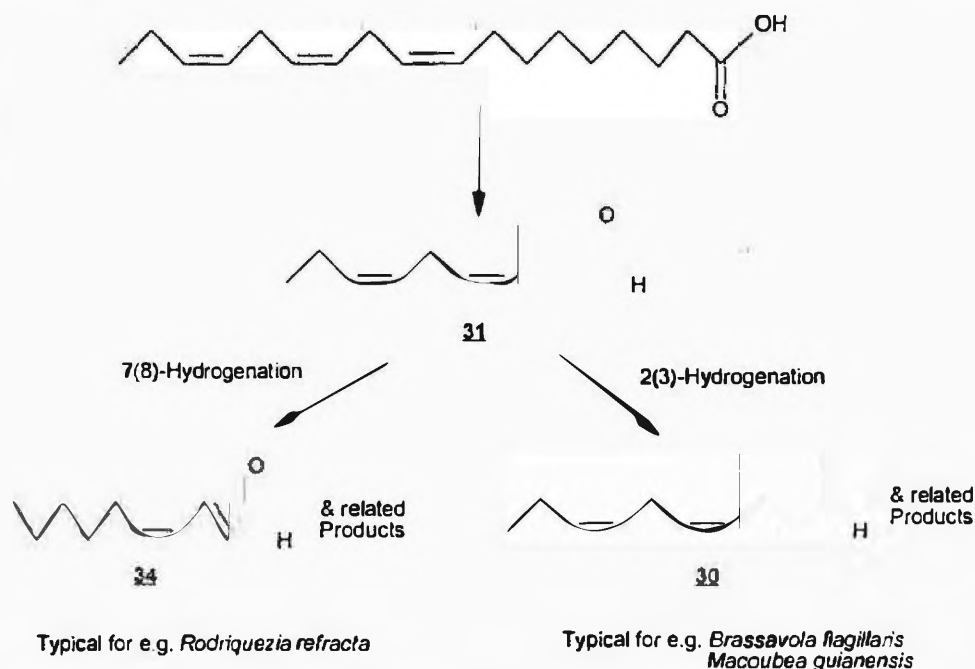


Figure 12 (E,Z,Z)-2,4,7-Decatrinal, the Intermediate of many C₁₀-Lipid Metabolites

Preferred reduction of the C7(8)-double bond leads to the typical constituents of *Rodriguezia refracta*, that of the C2(3)-double bond to those of *Brassavola flagillaris* and *Macoubea guianensis*, respectively. The structural prove for **31** was given by synthesis following in principle the procedure described by Meizboom and Stroink [13] by coupling 1-bromo-2-

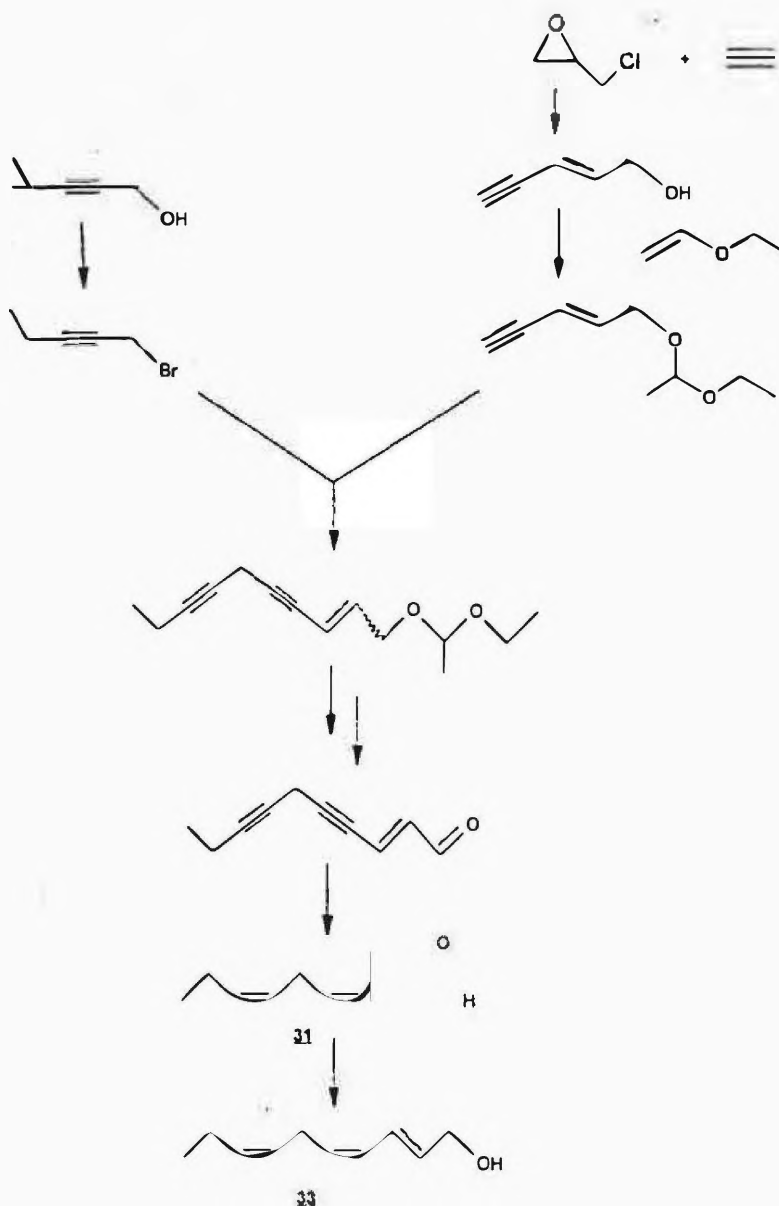
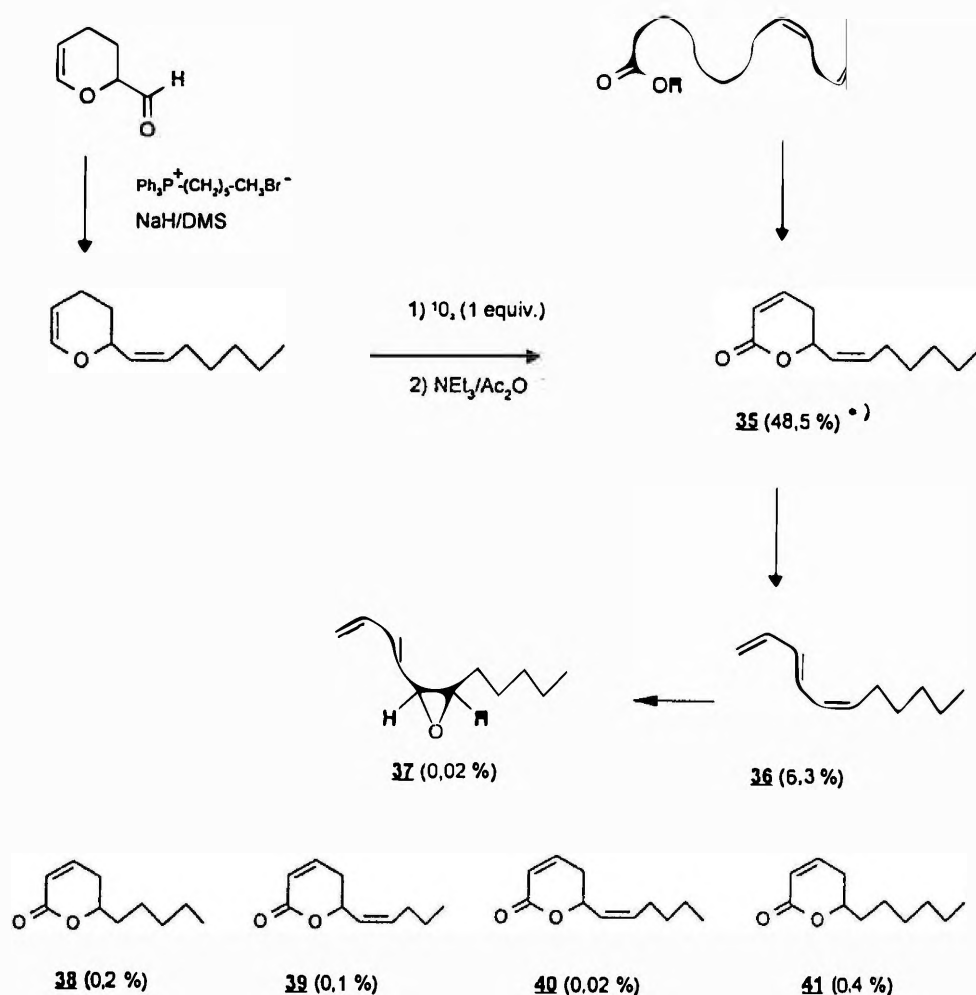


Figure 13 Synthesis of (E,2,2)-2,4-7-Decatrienal (**31**)

pentynol with (E)-2-penten-4-ynol and subsequent selective oxidation and hydrogenation using Lindlar's catalyst (Figure 13). Reduction of **31** with lithium aluminium hydride finally gave the trienol **33**, which was not yet known as natural product.

Some other highly interesting lipid metabolites we could identify in the scent of *Coryanthes elegantium* Linden Rchb. f., a rare epiphyte native to the hot and humid Rain Forests of Western Ecuador and Western Colombia. Besides the olfactorily dominating (E,Z)-1,3,5-undecatriene (**36**) the scent trapping consisted to around 50 % of an oxygen-containing compound of molecular weight 194 showing a much longer retention time (Figure 14). Based on its mass fragmentation, a 4- or 5-dodecadienolide could be assumed. Fortunately, the absolute amount of the trapping allowed to isolate 20 µg by preparative capillary gas chromatography and to elucidate its structure by NMR to be the (Z)-2,6-dodecadien-5-olide (**35**). Having a look at Figure 14 it becomes clear that **35** has to be the biological precursor of the (E,Z)-1,3,5-undecatriene (**36**) which is present in high isomeric purity. This is in contrast to galbanum oil which contains the (E,Z)- and (E,E)-isomere in a ratio of approximately 3:1 [15]. A literature search revealed that **35** was described already in 1977 by Priestap and co-authors [16] as a constituent of the rhizomes of *Aristolochia argentina* and that it was synthesized 4 years later by Fehr and co-authors [17] together with a series of other 6-substituted 5,6-dihydro-2(2H)-pyranones using the approach



*) Percentage refer to the trapped scent *Coryanthes elegantium*

Figure 14 (E,Z)-1,3,5-Undecatriene (**36**) and its Precursor (**35**) in *Coryanthes* Species

outlined in Figure 14. This possible metabolite of linolic acid is accompanied in the scent of *Coryanthes elegantium* on the level of minor constituents by the (Z)-5(6)-epoxy-(E)-1,3-undecadiene (**37**), massoia lactone (**38**), (Z)-2,6-decadien-5-olide (**39**), (Z)-2,6-undecadien-5-olide (**40**) and the 2-dodecen-5-olide (**41**). The dienolides **39** and **40**, which are not yet known as occurring in nature, have been synthesized by ap-

plying the same method as for **35**. The epoxide **37**, which is also a new natural product, was obtained by epoxidation of **36**. We found **35** and **36** in a whole series of *Coryanthes* species all belonging to the section *Coryanthes*. According to Dr. D. Joulain [18] (Z)-2,6-dodecadien-5-olide and the corresponding (E)-isomer are present in minor amounts also in massoia bark oil, where they are accompanied by similar amounts of (E,Z)- and (E,E)-1,3,5-undecatriene in a ration of ca. 1:1. He considers the later two isomers as being formed in massoia bark oil by thermolytic decarboxylation of **35** and the corresponding (E)-isomer.

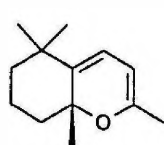
Main Components	Area % ^{*)}
(E)-Ocimene	12,0
Linalool	1,0
Neral	1,7
Geranial	1,6
Neryl acetate	13,8
Geranyl acetate	2,6
Nerol	31,8
Geraniol	2,5
Phenylethyl alcohol	2,5
Eugenol	7,5
(E)-Isoeugenol	3,5
(E,E)-Farnesol	2,5

^{*)} trapped on Porapak, 10.00 - 14.00, Oct. 11, 1996, Camp Parakou

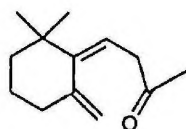
Figure 15 Floral Scent of *Couroupita guianensis* (Lecythidaceae), the so-called Cannon Ball Tree

Let us come back to the Amazonian Rain Forest of French Guiana, in which the family of Lecythidaceae (Brazil nut family) is often among the most abundant and specious tree families. Correspondingly, we trapped a respectable series of fragrant representatives at Camp Parokou and Camp Voltaire. The majority of species turned out to be dominated by a single floral scent component (e.g. 1,8-cineol, ocimene, linalool, α -farnesene, methyl salicylate, indole; compare [19]), a few, however, give off more equilibrated fragrances as, for example, the most impressive flowers of the famous Cannon Ball Tree (*Couroupita guianensis*), with their unmistakable rosy-floral and spicy scent. As summarized in Figure 15 it is based on nerol and neral and related products including derivatives and eugenol and isoeugenol (compare also [19]). Also the yellow flowering *Lecythis* sp. FG 44 (voucher FH 4397), which we trapped also at Camp Parakou, shows a more complex scent composition worth to be briefly discussed. Olfactorily, this scent is dominated by ionones, especially ionone (**44**), which is accompanied by comparatively high amounts of its photo cyclization product **42** and its intermediate, the (Z)-retro- γ -ionone (**43**) (Figure 16).

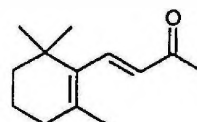
Main Components	Area % ^{*)}
(E)-Ocimene	31,1
Caryophyllene	49,0
Methyl benzoate	1,6
Cyclic- β -ionone (42)	1,3
Humulene	0,7
Methyl salicylate	3,8
Dihydro- β -ionone	1,8
β -Ionone (44)	1,0
Caryophyllene epoxide	1,1



42 (1,3 %)



43 (0,05 %)



44 (1,0 %)

^{*)} trapped on Porapak, 10.00-15.00, Oct. 15, 1996, Camp Parakou

Figure 16 Floral Scent Composition of *Lecythis* sp. FG 44

Correspondingly, caryophyllene (**46**), the main component of *Lecythis* sp. FG 44, seems to undergo partial photo-isomerization to isocaryophyllene (**45**) (Figure 17), a component all too often described as naturally occurring. Among 900 plant species investigated so far in our laboratory, caryophyllene (**46**) was found in 300, isocaryophyllene (**45**) only in 6 representatives. Important to the woody aspect of *Lecythis* sp. FG 44 are the caryophyllene epoxides **48** and **49** including

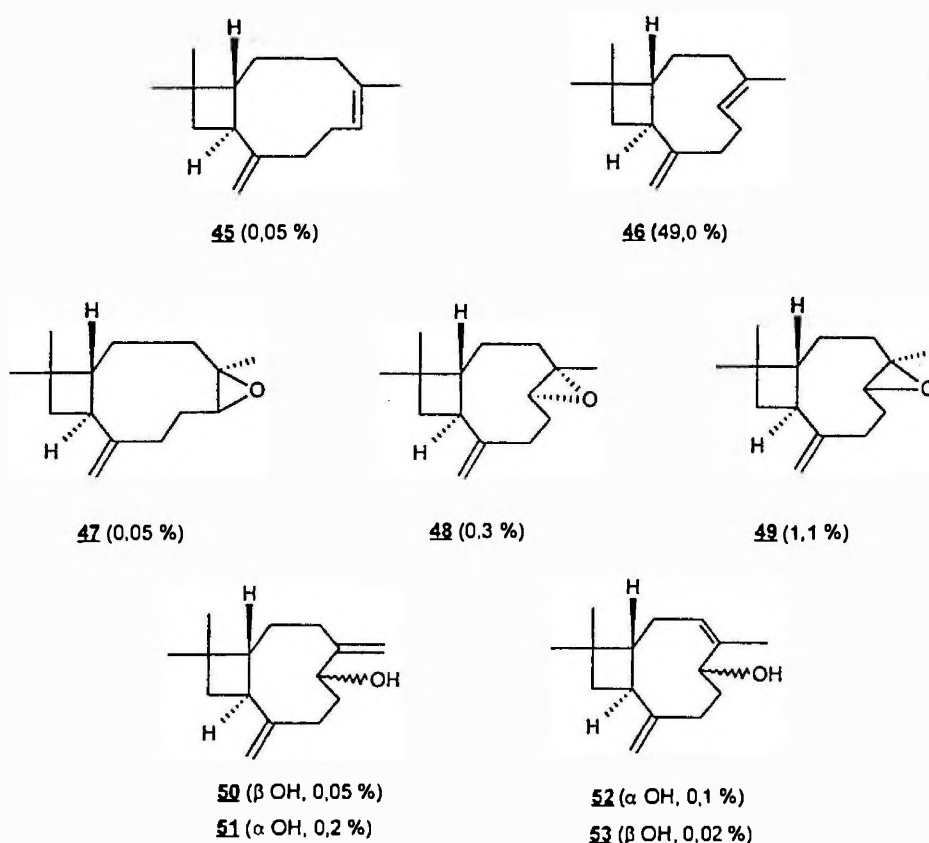


Figure 17 Caryophyllene Derivatives in *Lecythis* sp. FG 44/1

the isocaryophyllene epoxide **47** as well as the caryophylladienols **50** - **53**.

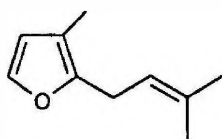
Ocimene and caryophyllene, accompanied by an astonishing high amount of indole, are also the main constituents of the magical aquamarine blue *Lecythis persistens* subsp. *persistens* which we discovered during our mission at Camp Voltaire.

In this scent, the main component caryophyllene is accompanied by its epoxide and the caryophyll-5-en-2- α -ol (**55**) (Figure 18).

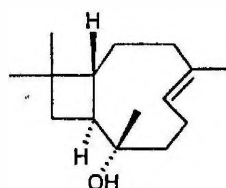
Main Components	Area % ^{*)}
Myrcene	8,1
(Z)-Ocimene	1,7
(E)-Ocimene	25,0
(E)-3(4)-Epoxy-3,7-dimethyl-1,6-octadiene	0,8
Linalool	19,0
Germacrene D	7,0
Phenylacetonitrile	1,4
Caryophyllene epoxide	0,2
1-Nitro-2-phenylethane	0,2
Indole	21,1



17 (0,8 %)



54 (0,1 %)



55 (0,1 %)

^{*)} trapped on Porapak, 10.00-12.30, Dec.9, 1996, Camp Voltaire

Figure 18 Floral Scent of *Lecythis persistens* subsp. *persistens*

Up to now we found **55** only in the floral scent of the Colombian orchid *Bollea coelestis* [4c], but in this case as main constituent totally characterizing its distinct woody fragrance. The tertiary alcohol **35** is formed to about 0.5 %, together with other side products, by the oxymercuration / cyclization / reduction of caryophyllene (**46**) to the caryophyllan-2,6- α -oxide (**56**). Selectively, it is accessible from

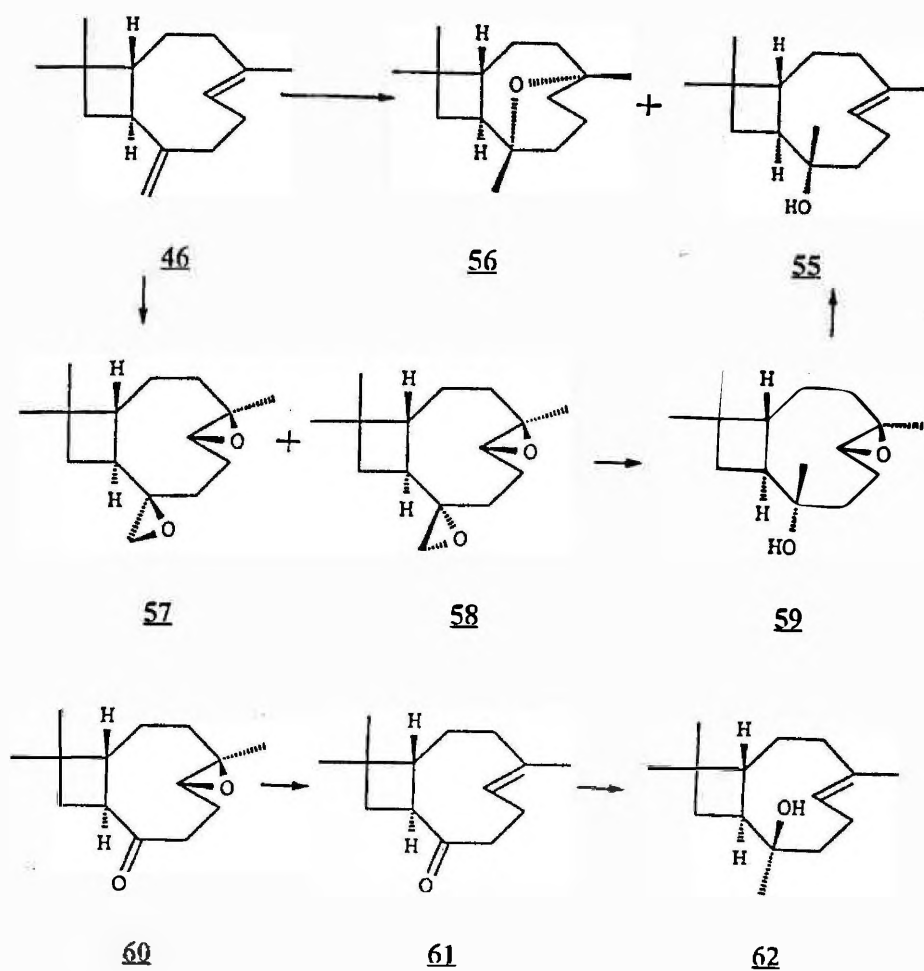


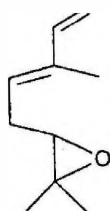
Figure 19 Caryophyll-5-en-2- α -ol, a Minor Scent Constituent of *Le-cythis persistens* subsp. *persistens*

the described diepoxide **58** [21] by reductive cleavage of the 2(12)-epoxy group with lithium aluminium hydride to the epoxy alcohol **59** followed by desepoxidation with Zn / Cu couple (Figure 19).

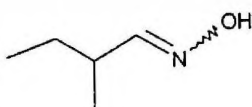
In 30 minutes hiking distance westward Camp Voltaire is, surrounded by the intact Rain Forest, a small savanna-like biotope of about 300 x 200 m with an extreme biodiversity. It was generated by a landslide which blocked a creek and, as a consequence, inundates now during the rainy season the entire place. The trees already partially lost their leaves and are

now heavily covered by epiphytes, parasitic plants, vines and lianas, which were blooming here even near the ground. In this biotope we had the chance to find many spectacular tropical plants in bloom, such as *Philodendron nobile*, a *Clitorea* species with a very delicate creamy vanilla-like scent, *Passiflora maliformis*, emitting an enjoyable aromatic-floral scent elegantly rounded off by a note of tropical

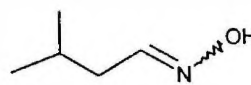
Main Components	Area % ^{*)}
(E)-Ocimene	8,5
(E)-Ocimene epoxide	10,2
2-Methylbutyraldoxime	8,2
Isovaleraldoxime	0,6
Linalool	53,0
Methyl benzoate	1,8
Germacrene D	2,4
(E,E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	6,9



18 (10,2 %)



24 (8,2 %)



26 (0,6 %)

^{*)} trapped on Porapak, 20.00-22.00, Dec. 10, 1996, Camp Voltaire

Figure 20 Floral Scent of *Epidendrum ciliare* (Orchidaceae)

fruits, or *Catasetum barbatum*, one of the amazing euglossine orchids, attracting with a balsamic aromatic-floral, indolic fragrance, the respective euglossine bees or *Epidendrum cili-* are growing as big clusters on the trunks of palm trees. This commonly occurring species has spread from Central America down to Amazonia and has, in contrast with many other orchids, adapted itself to widely differing living conditions. As a typical moth orchid it emits its appealing refreshing white-floral scent, which is based on high amounts of linalool, (E)-ocimene epoxide (**18**) and 2-methylbutyraldioxide (**24**) (Figure 20), exclusively during night. As illustrated by Figure 21 the absolute amount of scent given off from one flower per hour dramatically increases after sunset, reaching a peak at between 10 pm and 11 pm, and gradually subsiding

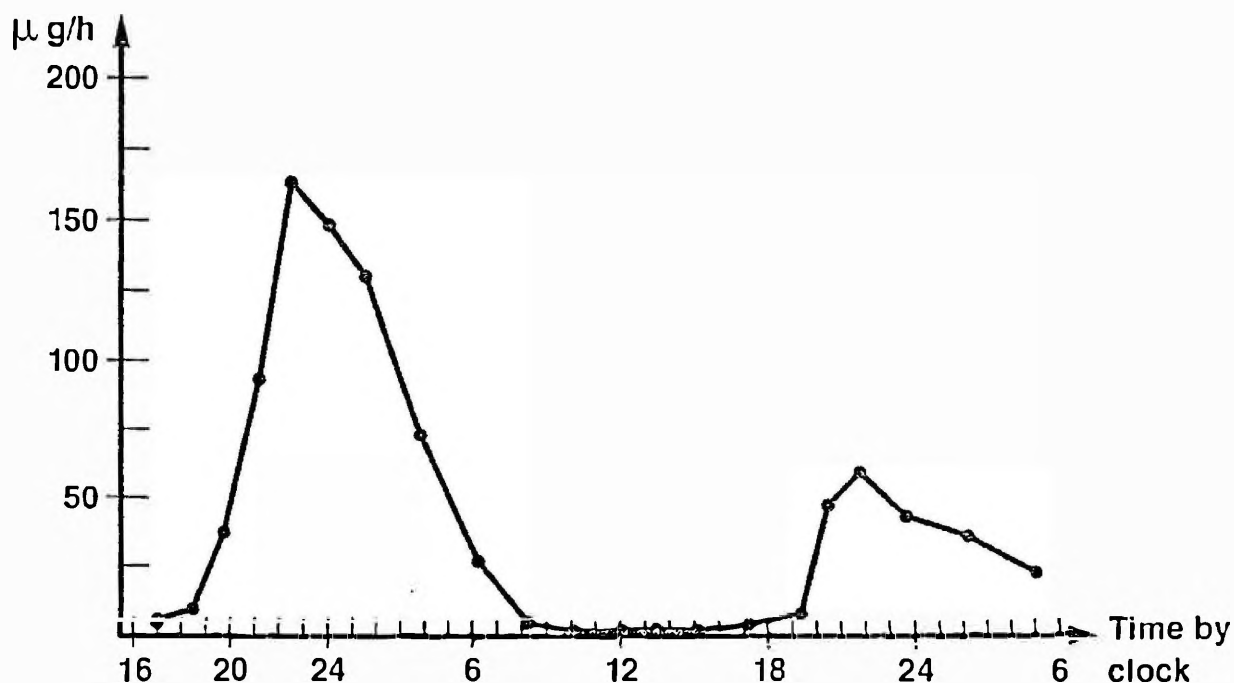


Figure 21 *Epidendrum ciliare*: Emanation of Scent in the Course of two Subsequent Nights

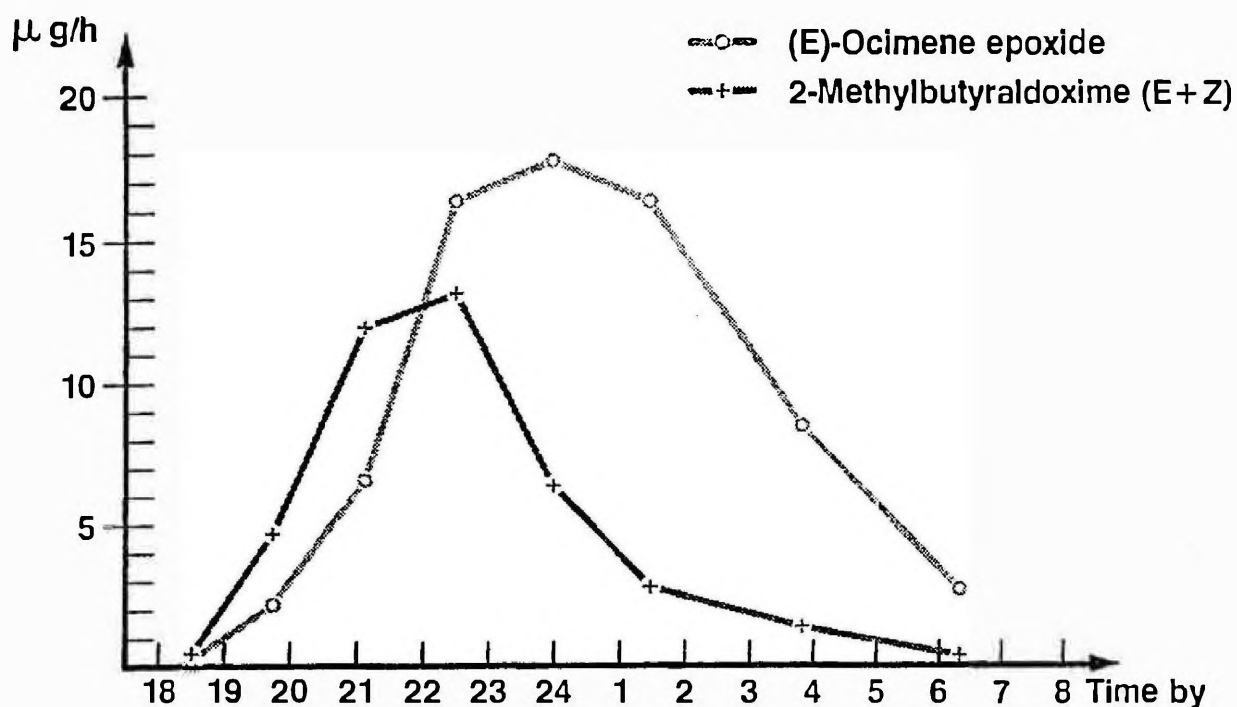
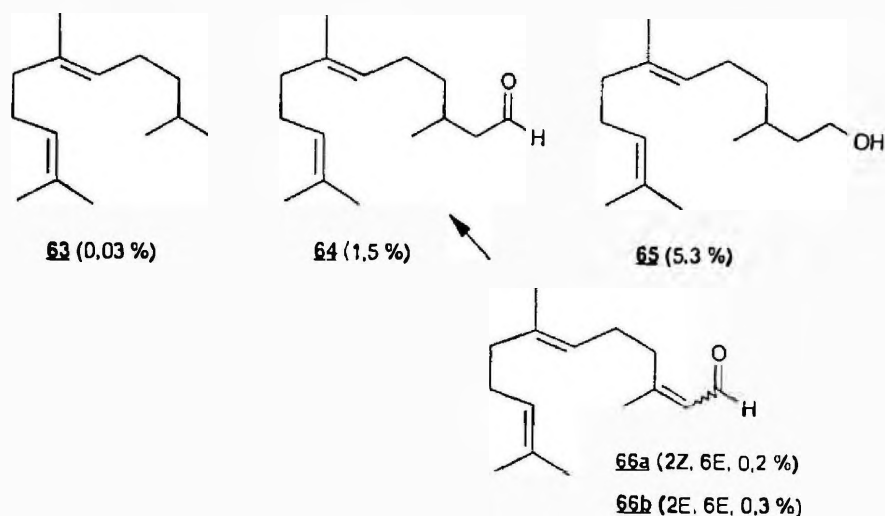


Figure 22 *Epidendrum ciliare*: Changes in the Amounts of two Scent Constituents during one Night

during the early morning hours. If the analogous graphs are plotted for the individual scent constituents as illustrated by Figure 22, it becomes clear that the maxima do not have to coincide. This explains the well-known fact that not only the quantity but also the quality of the scent given off by a flower or plant may be time-dependent, an aspect very important to us in connection with the reconstitution of such scents.

With another highly scented epiphyte typical for such biotopes, with *Cochleostema odoratissima*, I would like to conclude my discussion of some selected scents encountered in neotropical Rain Forests. This Commelinaceae emits a very distinct fragrance, which is composed of rosy-floral, ionone-floral and refreshing hesperidic notes and a high portion of

Main Components	Area % ^{*)}
(E)-Ocimene	5,0
6-Methyl-5-hepten-2-one	2,8
Linalool	5,0
Neral	1,0
Geranial	2,0
Citronellol	3,0
Nerol	39,5
Dihydro- β -ionone	2,0
(E)-Geranylacetone	6,8
(E)-6,10-Dimethyl-5,9-undecadien-2-ol	0,5
(E)-2(3)-Dihydrofarnesal (64)	1,5
(E)-2(3)-Dihydrofarnesol (65)	5,3
Indol	11,5



^{*)} trapped on Porapak, 11.00-15.00, Dec. 11, 1976, Camp Voltaire

Figure 23 Floral Scent of *Cochleostema odoratissima* (Commelinaceae)

indole. Especially worth mentioning is the occurrence of the (E)-2(3)-dihydrofarnesal (**64**) (Figure 23), which adds an attractive fresh aldehydic "lily of the valley"-type note to this rich floral scent, and that of the corresponding alcohol **65** rounding it off by a tender note of cyclamen. As we re-

cently described **64** plays also an important role in the floral scent of *Citrus limon* [21]. This product has found in the meantime its way to the perfumer's shelf and helps to give modern fragrances a unique natural floral freshness. With a last glance back to this biotope full of death and thriving life I would like to end my presentation and to thank you for your attention.

ACKNOWLEDGEMENTS

I am grateful to Dr. M. Whitten, Florida Museum of Natural History, Gainesville, FL. for samples of the trapped scent of *Brassavola flagillaris*; Dr. CH. Weymuth, University of Zürich, for samples of the trapped scent of *Coryanthes elegantium*; to Mr. Miguel Krigsner for his hospitality during my studies in the Morato region; to Prof. Francis Hallé for his help in identifying the plant species encountered in Amazonia; to the entire team of "Canopy 1996" to make the unique missions in French Guiana possible; to Mr. K. Lombard, Applied Research Fragrances of Givaudan Roure, for his help during the mission at Camp Parakou; to Mr. J. Guichard, Director of Fine Fragrances of Givaudan Roure, for his help during the mission at Camp Voltaire; to my colleagues at Corporate Research at Givaudan Roure for discussions and assistance, in particular to Dr. J. Schmid for many discussions on the mass spectra of new components, to Mr. M. Rothaupt and

Mr. E. Senn for the isolation of new compounds in the μg range by preparative capillary GC; to Mr. J. Märki for many indepth NMR-studies, to Dr. D. Helmlinger for the synthesis of the (E,Z,Z)-2,4,7-decatrienal (**31**), to Dr. Katja Schultz for discussions and synthetic help and last but not least to Mr. V. Clerc and Mr. M. Jenzer for their skillful assistance throughout the investigations summarized in this paper.

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THE ESSENTIAL OIL COMPOSITION OF *PIMPINELLA* SPECIES

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Introduction

The genus *Pimpinella* belongs to the plant family Apiaceae (Umbelliferae), which was one of the first families of flowering plants recognized as such. The dominant features of the family are the umbellate inflorescence, the reduction in flower size and the specialized fruit consisting of two one-seeded mericarps suspended from a common stalk, the carpophore. Due to the uniformity of characters within the umbellifers the family can be easily differentiated from other families. On the other hand, this has the effect of obscuring relationships within the family and of making the subdivision into tribes, subtribes and genera difficult.

The genus *Pimpinella* comprises about 150 species (1), which largely occur in Europe, extending to Russia, Asia and China. The highest density of 23 species is found in Turkey, whereas in the middle of Europe only 3 species are found. *Pimpinella* is a slightly uniform genus among the species-rich genera of umbellifers and has no generic character, which can be found in all of its species. Only the occurrence of more than one vitta between each pair of vascular bundles in the pericarp of the fruit seems to be characteristic for nearly all species. Due to the different features, which have been employed, the limitation of the genus *Pimpinella* differs considerably. The same can be said of the infra-generic division. The preferred and most often accepted division of the genus is that proposed by Wolff (2), who classifies 3 sections. The section *Reutera* comprises species with yellow petals, whereas the petals of the species belonging to the sections *Tragium* and *Tragoselinum* are white, rarely pink or purplish. The latter 2 sections are characterized by hairy fruits (*Tragium*) and by the absence of hairs on the fruit surface in the section *Tragoselinum*.

As the generic limitations and the infra-generic separation of the species-rich genus *Pimpinella* are still subject to speculation and not clearly defined by conventional morphological characters, it seemed promising to investigate the chemical constituents of *Pimpinella* species, especially the volatiles, which have proven useful as taxonomic characters.

Results

Pimpinella anisum

Our first investigations on essential oils of *Pimpinella* species date back more than 20 years (3). At that time we looked for the composition of the essential fruit oil from *Pimpinella anisum*, which is mainly used as a spice, in flavours, alcoholic beverages and in pharmaceutical preparations. The commercial available so called "Anise Seed Oil" (4) contains about 80-90% trans-anethole and minor amounts of estragole, anise aldehyde, cis-anethole and some sesquiterpene hydrocarbons, above all γ -himachalene. A more detailed gc analysis of the hydrocarbon fraction is given in **Figure 1**, showing numerous sesquiterpene hydrocarbons besides some monoterpenes **1-8**. Dominating compounds are the mentioned sesquiterpene γ -himachalene (**20**) together with the respective α - and β -isomers (**19**, **21**), α -zingiberene (**22**), β -bisabolene (**23**), δ -elemene (**12**) and β -elemene (**17**) as well as the thermally instable trinorsesquiterpene pregeijerene (**15**), which undergoes during the distillation process and the gc-separation a Cope-rearrangement forming geijerene (**10**) and isogeijerene. These C_{12} -compounds have been found in nearly all *Pimpinella* oils as will be seen later on and seem to be a useful taxonomic character for the delimitation of the genus *Pimpinella*. However, the percentage of these compounds (**Table 1**) is rather low (ca. 2 %) in the hydrocarbon-fraction of the fruit oil.

Figure 1. GC of *Pimpinella anisum* fruit oil

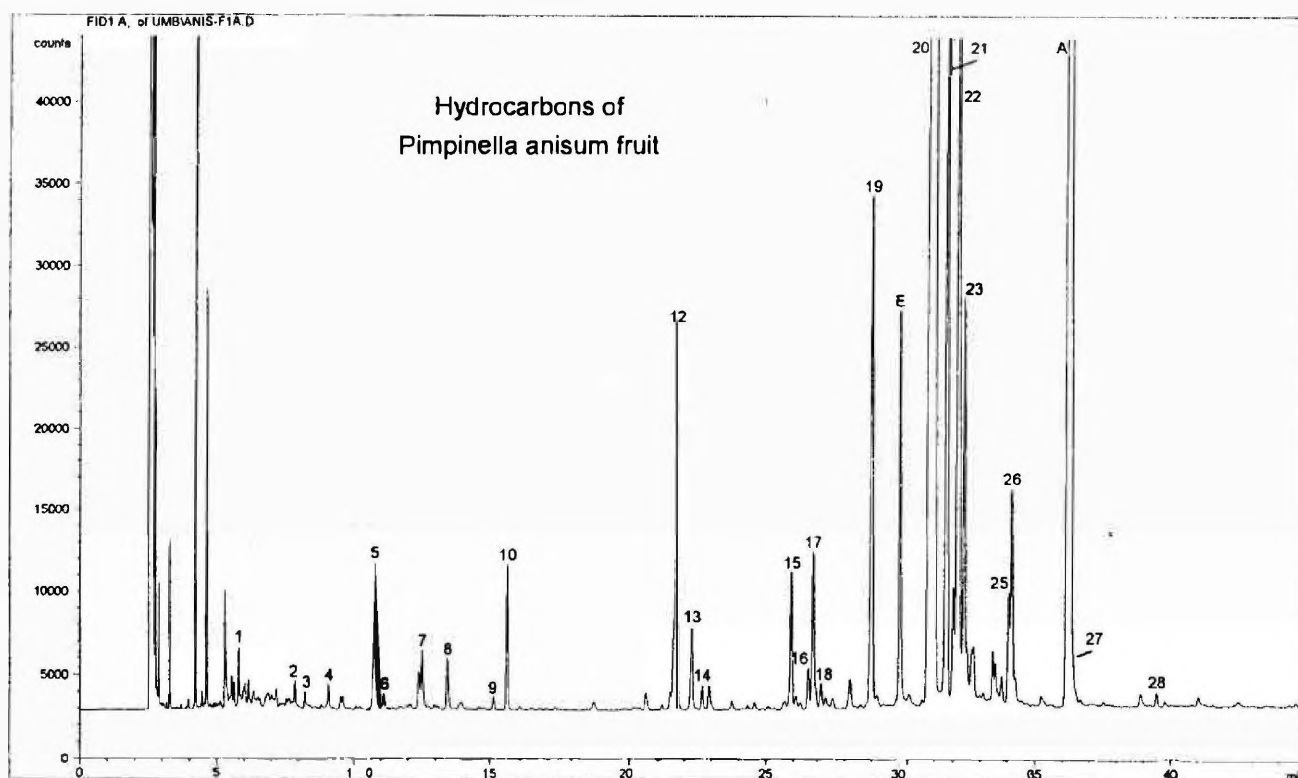


Table 1. Hydrocarbons of *Pimpinella anisum* fruit oil

No.	Compound	%
1	α -Pinene	0.24
2	β -Pinene	0.12
3	Sabinene	0.06
4	3-Carene	0.14
5	Limonene	1.11
6	β -Phellandrene	0.11
7	γ -Terpinene	0.46
8	p-Cymene	0.42
9	Isogeijerene	0.09
10	Geijerene	0.99
11	α -Longipinene	0.65
12	δ -Elemene	2.96
13	α -Ylangene	0.78
14	α -Copaene	0.20
15	Pregeijerene	1.14
16	<i>trans</i> - α -Bergamotene	0.36
17	β -Elemene	1.43
18	β -Caryophyllene	0.24
19	α -Himachalene	4.79
20	γ -Himachalene	50.47
21	β -Himachalene	8.05
22	α -Zingiberene	10.21
23	β -Bisabolene	3.83
24	δ -Cadinene	0.42
25	β -Sesquiphellandrene	0.90
26	α -Curcumene	1.85
27	Germacrene B	0.37
28	α -Calacorene	0.12

Besides these components we have isolated at that time an unknown compound, which was present up to 5 % in the fruit oil of *Pimpinella anisum* (5). By MS-, IR- and especially by NMR spectroscopy the structure of the unknown has been established as 2-methyl-butyric acid ester of 2-hydroxy-5-methoxy-*trans*-propenyl-benzene and many years later we discovered in addition the respective epoxide (6). This type of unusual 2,5-substituted phenylpropane derivatives, which have been found in all of the investigated *Pimpinella* species, can be considered as a second important marker for the delimitation of the genus *Pimpinella*.

Recent studies concerning the biosynthesis of these unusual phenylpropanoids by Reichling and co-workers (7), who have named that type of skeleton "Pseudoisoeugenol" (8), have proved the steps indicated in **Figure 2** by using labelled compounds. Starting with L-phenylalanine, *p*-coumaric alcohol is obtained by stepwise reduction via *trans*-cinnamic and *p*-coumaric acid. Prior to reduction of the alcoholic function to a methyl group, the *p*-hydroxy-group was methylated yielding *trans*-anethole an important intermediate. The next step, the introduction of the phenolic hydroxy group caused the side chain to shift to the adjacent ring position, a metabolic process known as NIH-shift (9). The postulated mechanism of this key steps in the formation of 2,5-substituted phenylpropanoids can be taken from **Figure 2**, exhibiting the possible intermediates in the formation of pseudoisoeugenol derivatives.

Figure 2a. Biosynthesis of pseudoisoeugenol derivatives

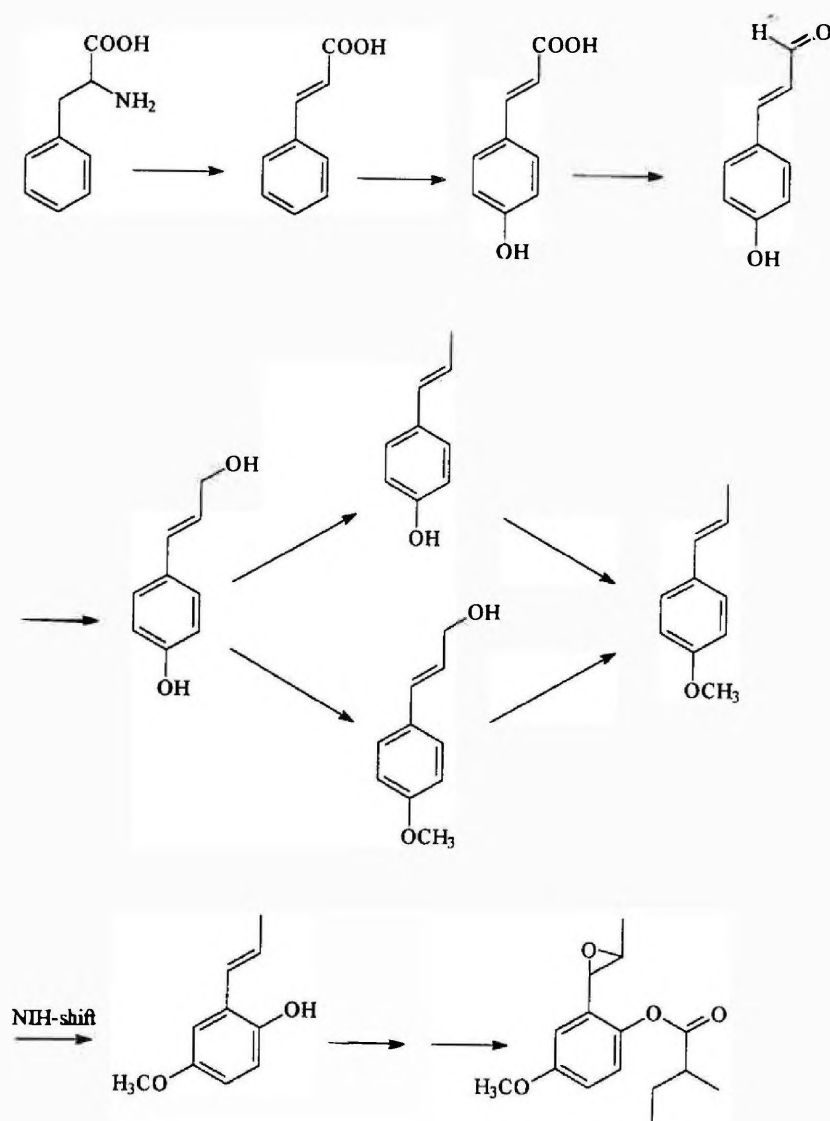
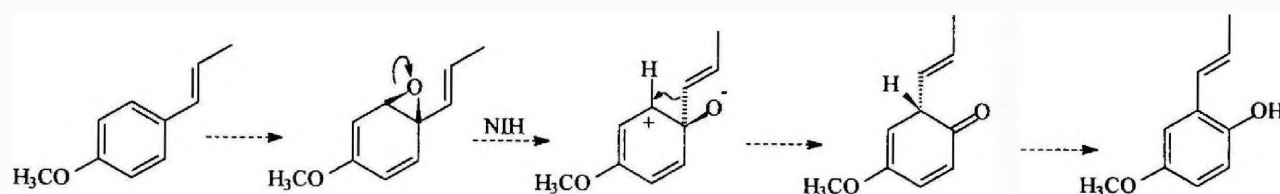


Figure 2b. Postulated mechanisms of NIH-shift



Some years ago, we continued to examine *Pimpinella* species and in course of these analyses, the essential oils from different parts of *Pimpinella anisum* have been investigated. In contrast to the fruit oil, the essential oil of the herb contained considerably higher amounts of sesquiterpene hydrocarbons (Table 2). Among these, germacrene D (**21**) is dominating besides minor amounts of γ -himachalene (**20**), α -zingiberene (**23**) and β -bisabolene (**24**). The content of *trans*-anethole has decreased to about 55 %, whereas the pseudoisoeugenyl 2-methylbutanoate and its epoxide have increased. The latter compound is the main constituent of root oil, which shows the lowest concentration of *trans*-anethole. In addition to β -bisabolene (**24**), the C₁₂-hydrocarbons pregeijerene (**15**) and geijerene (**8**) as well as trinoranastreptene (**9**) shall be mentioned. The percentual compositions of the fruit, herb and root oil given in **Table 2**, show the decreasing proportions of *trans*-anethole from the fruit, the herb to the root. In contrast, the percentages of the epoxy-*trans*-pseudoisoeugenyl 2-methylbutanoate are increasing from 0.11 to 75 %.

Table 2. Composition of the essential oils from different parts of *Pimpinella anisum* L.

No.	Compound	fruit	herb	root
1	α -Pinene	0.01	-	trace
2	n-Heptanal	-	0.10	0.18
3	Limonene	0.01	trace	trace
4	γ -Terpinene	0.01	trace	trace
5	p-Cymene	0.01	-	trace
6	n-Octanal	-	0.03	0.18
7	Isogeijerene	trace	trace	0.20
8	Geijerene	0.02	0.28	2.14
9	Trinoranastreptene	-	-	0.53
10	α -Longipinene (?)	0.01	-	0.14

11	δ -Elemene	0.06	0.12	trace
12	α -Ylangene	0.01	0.05	-
13	α -Copaene	trace	0.05	trace
14	Linalool	0.09	0.07	trace
15	Pregeijeren	0.02	0.82	3.75
16	β -Elemene	0.02	0.06	trace
17	β -Caryophyllene	trace	0.13	0.05
18	α -Himachalene	0.11	0.32	trace
19	Estragole	1.79	2.21	0.05
20	γ -Himachalene	0.76	3.07	0.10
21	Germacrene D	-	11.57	trace
22	β -Himachalene	0.11	0.18	0.04
23	α -Zingiberene	0.12	2.28	0.32
24	β -Bisabolene	0.04	2.99	8.10
25	Bicyclogermacrene	-	0.69	-
26	<i>cis</i> -Anethole	0.18	0.28	trace
27	β -Sesquiphellandrene	trace	0.12	0.50
28	α -Curcumene	trace	0.21	0.62
29	<i>trans</i> -Anethole	95.32	54.00	1.16
30	Germacrene B	trace	0.14	-
31	Anisaldehyde	0.22	trace	-
32	<i>trans</i> -Pseudoisoeugenyl-2-methylbutanoate	0.66	8.76	3.51
33	Epoxy- <i>cis</i> -pseudoisoeugenyl-2-methylbutanoate	-	0.37	0.22
34	Epoxy- <i>trans</i> -pseudoisoeugenyl-2-methylbutanoate	0.11	5.31	75.21

Sesquiterpene hydrocarbons have shown their highest concentration in the herb oil, as can be taken more easily from Table 3, showing the percentages of the most important groups of components in the essential oils from the different parts of *Pimpinella anisum*. The phenylpropanoids have been splitted into 2 groups, the 4-substituted and the "unusual" 2,5-substituted aromatic components which are the dominating compounds in the root oil.

Table 3. Groups of components in different parts of *Pimpinella anisum* L.

Group	fruit	herb	root
Tri-nor-sesquiterpenes	0.04	1.10	6.62
Sesquiterpenes	1.16	21.98	9.87
4-substituted phenylpropanoids	97.29	56.49	1.21
2,5-substituted phenylpropanoids	0.77	14.44	78.94

These observed significant differences in the oil composition of the individual parts of one plant induced us to reinvestigate the previously analyzed *Pimpinella* species and to study in addition to the root oils the essential oils of the herbs and fruits.

Pimpinella peregrina

The next species from the genus *Pimpinella* which has been examined was *Pimpinella peregrina*. This biennial plant, native to the south of Europe extending to Asia Minor and Egypt is cultivated in the south of Germany erroneously as a medicinal plant to yield the drug "Pimpinella Radix" whereas the true drug should be obtained either from *Pimpinella major* or from *Pimpinella saxifraga*. Although the discrimination of the living plant and its hairy fruits is no problem, the dried roots can hardly be distinguished from the true crude drugs. Therefore, the root oil composition could be useful to distinguish the individual drugs. The constituents of the essential root oil of *Pimpinella peregrina* (Table 4) belong mainly to 2,5-substituted phenylpropane derivatives, representing more than 70 % of the total volatiles.

Table 4. Composition of the root oil from *Pimpinella peregrina* L.

No.	Compound	%
1-11	Monoterpenes	0.92
12	Isogeijerene	0.05
13	Geijerene	0.53
14	Pregeijerene	1.00
15	Methylthymol	0.11
16	<i>trans</i> - α -Bergamotene	2.28
17	Methylcarvacrol	0.16
18	<i>trans</i> - β -Farnesene	1.03
19	Germacrene D	0.06
20	Zingiberene	0.18
21	β -Bisabolene	1.22
22	Bicyclogermacrene	0.24
23	β -Sesquiphellandrene	10.66
24	<i>trans</i> -Anethole	0.08
25	Spathulenol	0.13
26	mw 178	0.35
27	<i>trans</i> -Anyl 2-methylbutanoate	0.38
28	<i>trans</i> -Pseudoisoeugenyl 2-methylpropanoate	0.74
29	Epoxy- <i>trans</i> -anil 2-methyl butanoate	0.96
30	Epoxy- <i>cis</i> -pseudoisoeugenyl 2-methylpropanoate	0.13
31	<i>trans</i> -Pseudoisoeugenyl 2-methylbutanoate	1.81

32	Epoxy- <i>trans</i> -pseudoisoeugenyl 2-methylpropanoate	30.65
33	Epoxy- <i>cis</i> -pseudoisoeugenyl 2-methylbutanoate	0.23
34	Epoxy- <i>trans</i> -pseudoisoeugenyl 2-methylbutanoate	41.79

Main constituents are two epoxy-pseudoisoeugenol esters, the 2-methylbutanoate (**34**) and the 2-methylpropanoate (**32**), and the respective non-epoxidized products (**31,28**). In the group of sesquiterpene hydrocarbons the most abundant constituent is β -sesquiphellandrene (**23**). Additionally, the presence of small amounts of two anol derivatives can be observed: the anyl 2-methylbutanoate (**27**) and the respective epoxide (**29**).

Although all these phenylpropanoids can be found in the herb oil from *Pimpinella peregrina*, too, the dominating constituents of that part of the plant are sesquiterpene hydrocarbons (Table 5), above all *trans*- α -bergamotene (**18**) with ca. 70 %. The herb oil and more pronounced the fruit oil with more than 80 % of this sesquiterpene hydrocarbon are - to our knowledge - by far the richest natural source of *trans*- α -bergamotene.

Table 5. Composition of *Pimpinella peregrina* herb and fruit oil

No.	Compound	herb	fruit
1-8	Monoterpenes	1.78	0.02
10	Geijerene	0.06	-
11	(<i>Z</i>)-3-Hexenol	0.18	trace
12	(<i>Z</i>)-3-Hexenyl 2-methylpropanoate	0.11	-
13	1-Octen-3-ol	0.05	-
14	α -Copaene	0.08	-
15	β -Bourbonene	0.10	-
16	1-Nonen-3-ol	0.12	trace
17	Pregeijerene	0.08	-
18	<i>trans</i> - α -Bergamotene	69.12	82.09
20	<i>trans</i> - β -Farnesene	0.82	0.15
21	γ -Muurolene	0.15	0.17
22	Germacrene D	4.43	0.02
23	β -Bisabolene	0.95	0.96
24	(<i>E,E</i>)- α -Farnesene	1.96	-
25	β -Sesquiphellandrene	0.24	0.27
27	mw 154 (68,43,82,95,57)	0.76	-
28	2-Methyl-5-methoxybenzofuran	0.15	0.39
29	mw 174	0.16	0.32
30	<i>trans</i> -Anyl 2-methylbutanoate	0.05	0.04
31	<i>trans</i> -Pseudoisoeugenyl 2-methylpropanoate	0.50	0.02
32	Epoxy- <i>trans</i> -anyl 2 methylbutanoate	0.32	0.33

33	Epoxy- <i>cis</i> -pseudoisoeugenyl 2-methylpropanoate	0.10	0.04
34	<i>trans</i> -Pseudoisoeugenyl 2-methylbutanoate	0.11	-
35	Epoxy- <i>trans</i> -pseudoisoeugenyl 2-methylpropanoate	11.57	9.42
36	Epoxy- <i>cis</i> -pseudoisoeugenyl 2-methylbutanoate	0.03	trace
37	(<i>E</i>)-Phytol	1.49	-
38	Epoxy- <i>trans</i> -pseudoisoeugenyl 2-methylbutanoate	1.03	0.11

Pimpinella saxifraga

The third *Pimpinella* species, which shall be discussed is *Pimpinella saxifraga sensu stricto*. This perennial plant, growing throughout most of Europe extending to Asia, is very variable in morphological characters, especially in the leaf dissection. The same is true for the phytochemical features. Recent investigations have shown, that the essential root oil composition differs considerably from plants with simple segmented and dissected leaves, respectively (10). The results of our reinvestigation account for plants with dissected leaves.

The essential root oil from plants harvested in spring is characterized by several sesquiterpene hydrocarbons, C₁₂-hydrocarbons such as geijerene (9), trinoranastreptene (11), and pregeijerene (16), aliphatic aldehydes and pseudoisoeugenol derivatives. The detailed results are given in Table 6 from which the percentages of the identified components can be taken.

Table 6. *Pimpinella saxifraga* L. (root)

No.	Compound	%
1	α -Pinene	0.06
2	n-Undecane	0.33
3	Myrcene	0.37
4	n-Heptanal	0.22
5	γ -Terpinene	0.68
6	p-Cymene	0.11
7	n-Octanal	2.99
8	Isogeijerene	0.53
9	Geijerene	5.58
10	n-Nonanal	2.09
11	Trinoranastreptene	3.38
13	δ -Elemene	7.64
14	Longicyclene	0.20
15	<i>trans</i> -2-Nonenal	0.40
16	Pregeijeren	8.43

17	Methylthymol	0.44
20	Methylcarvacrol	1.10
21	β -Elemene	0.50
22	β -Caryophyllene	0.49
24	unknown	1.86
25	γ -Elemene	0.28
26	<i>trans</i> -2-Decenal	3.60
28	<i>trans</i> - β -Farnesene	0.35
29	α -Humulene	0.30
30	Germacrene D	1.04
32	β -Bisabolene	0.71
34	β -Sesquiphellandrene	2.37
36	3,10-Dihydro-1,4-dimethylazulene	3.94
37	Germacrene B	4.47
38	2,5-Dimethoxy-p-cymene (?)	0.18
39	C ₁₅ H ₂₄ O	0.68
40	mw 178	0.41
41	<i>trans</i> -Anyl 2-methylbutanoate	0.94
42	C ₁₅ H ₂₄ O	1.66
43	C ₁₅ H ₂₄ O	0.97
44	C ₁₅ H ₂₄ O	0.30
46	<i>trans</i> -Pseudoisoeugenyl 2-methylbutanoate	5.04
50	Epoxy- <i>trans</i> -pseudoisoeugenyl 2-methylpropanoate	12.12
52	Falcarinol	1.51

The most abundant constituents are geijerene and pregeijerene, δ -elemene, β -sesquiphellandrene, 3,10-dihydro-1,4-dimethylazulene, germacrene B and the phenylpropane derivatives pseudoisoeugenyl 2-methylbutanoate as well as its epoxide. In course of plant development oxidation processes take place resulting in an increase of higher oxidized products at the expense of hydrocarbons and lower oxidized products. Especially the occurrence of a high amount of germacrone and the epoxidized pseudoisoeugenol ester shall be mentioned (10).

The essential oil of the herb from *Pimpinella saxifraga* (Table 7) exhibits a quite different composition: phenylpropanoids are found only in small quantities.

Table 7. Composition of *Pimpinella saxifraga* L. herb and fruit oil

No.	Compound	herb	fruit
1	α -Pinene	1.91	0.36
2	Hexanal	trace	trace
3	n-Undecane	0.99	0.23
4	β -Pinene	2.40	0.77

5	Sabinene	0.38	0.11
6	Myrcene	48.94	10.78
7	Limonene	0.90	0.21
8	β -Phellandrene	0.66	0.14
9	<i>cis</i> - β -Ocimene	0.43	trace
10	γ -Terpinene	5.39	1.26
11	<i>trans</i> - β -Ocimene	8.08	0.78
12	p-Cymene	0.14	0.15
13	(<i>Z</i>)-3-Hexenyl acetate	0.94	-
14	(<i>Z</i>)-3-Hexenol	0.24	-
15	β -Ylangene	0.19	-
16	β -Caryophyllene	1.17	0.46
17	(<i>E</i>)- β -Farnesene	0.27	28.81
18	α -Humulene	0.16	-
19	Germacrene D	12.35	0.97
20	(<i>Z,E</i>)- α -Farnesene	3.39	2.47
	β -Bisabolene	-	5.37
21	Bicyclogermacrene	2.52	0.28
22	(<i>E,E</i>)- α -Farnesene	0.25	0.36
23	δ -Cadinene	0.15	-
24	n-Dodecanol	0.55	-
28	<i>trans</i> -Anyl 2-methylbutanoate	0.39	5.53
	<i>trans</i> -Anyl 3-methylbutanoate	-	0.61
29	<i>trans</i> -Pseudoisoeugenyl 2-methylbutyrate	trace	0.20
32	(<i>E</i>)-Phytol	2.86	-
33	Epoxy- <i>trans</i> -pseudoisoeugenyl 2-methylbut.	0.80	7.89

The dominating constituents in that oil are mono- and sesquiterpene hydrocarbons, above all myrcene, γ -terpinene and (*Z,E*)- α -farnesene together with β -bisabolene and bicyclogermacrene. The observed (*E*)-phytol with nearly 3 % might be regarded as a result of hydrolysis of chlorophyll in the leaf during the hydrodistillation process. It is completely absent in the other parts of the plant such as the roots and the ripe fruits, which do not contain any chlorophyll. Interesting to notice is the considerably different composition of the fruit oil in which germacrene D, one of the main constituents of the leaves is lacking. The most abundant components of the fruit oil are the farnesenes (*Z,E*)- α -farnesene and (*E*)- β -farnesene, β -bisabolene, epoxy-pseudoisoeugenyl 2-methylbutanoate and the 4-substituted phenylpropanoids *trans*-anil-2- and 3-methylbutanoate as well as their epoxides. These results are in agreement with the basal observation mentioned before, that the fruit oil of a *Pimpinella* species contains

higher amounts of 4-substituted phenylpropanoids than the other parts as has been demonstrated impressively at *Pimpinella anisum*.

Pimpinella nigra

Pimpinella nigra Mill., which is considered by several authors to be a subspecies of *Pimpinella saxifraga*, yields an essential root oil which differs considerably with respect to the qualitative composition from the before mentioned oil. This plant can be determined easily by cutting the fresh root from which a blue oil emerges, indicating the formation of an azulene derivative. Main constituents of the essential oil obtained from roots are several C₁₂-hydrocarbons which represent more than 70 % of the oil (Table 8). These components, which have been isolated and identified previously (10) may be considered to be intermediates, arising by stepwise dehydrogenation and cyclization during the formation of 1,4-dimethylazulene, the constituent responsible for the blue colour of the root oil.

In contrast to several other blue coloured essential oils it is remarkable, that this azulene derivative is not a distillation artifact as e.g. in the chamomile oil. This is worth to mention, because in the volatile oils from *Thapsia garganica* and *Ruta graveolens* 1,4-dimethylazulene is a decomposition product formed during hydrodistillation.

Phenylpropane derivatives are found only in relatively low quantities, only the epoxy-pseudoisoeugenyl 2-methylbutanoate has been identified in a higher proportion. In contrary, the non-coloured oil from *Pimpinella nigra* fruits consists of approximately 50 % phenylpropanoids. The dominating constituents of this class are 4-substituted epoxy-anol esters of 2- and 3-methylbutanoic acid. In addition, small amounts of the respective non-epoxidized products have been identified, accompanied by the 2'-oxopropyl derivatives, probably formed during hydrodistillation by opening of the oxirane ring in the acidic medium.

In addition to these mentioned constituents, the essential fruit oil of *Pimpinella nigra* exhibits relatively high amounts of sesquiterpene hydrocarbons, above all β -bisabolene, (Z,E)- α -farnesene, germacrene D and two up till now unknown sesquiterpenes.

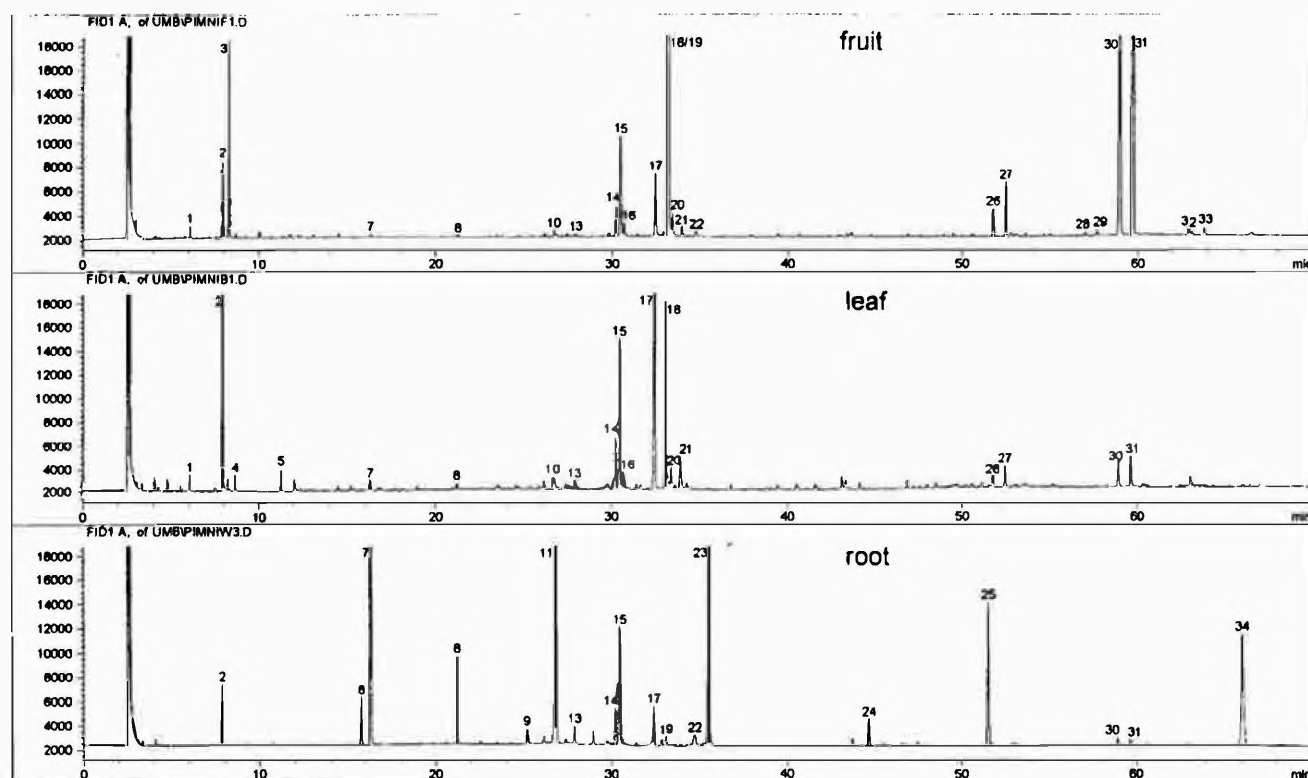
Table 8. Composition of the essential oils from different parts of *Pimpinella saxifraga* ssp. *nigra* Mill.

No.	Compound	fruit	herb	root
1	α -Pinene	0.24	0.58	-
2	n-Undecane	1.89	15.42	1.50
3	β -Pinene	4.76	0.60	-
4	Sabinene	0.10	0.73	-
5	Limonene	-	1.16	-
6	Isogeijerene	-	-	1.69
7	Geijerene	0.09	0.63	17.44
8	Trinoranastreptene	0.06	0.37	3.18
9	mw 162	-	-	0.57
10	<i>cis</i> - α -Bergamotene	0.24	0.74	0.60
11	Pregeijerene	-	-	19.58
12	<i>trans</i> - α -Bergamotene	-	0.32	0.21
13	β -Caryophyllene	0.09	0.67	0.71
14	C ₁₅ H ₂₄	1.10	3.51	1.45
15	C ₁₅ H ₂₄	3.61	10.21	4.56
16	(<i>E</i>)- β -Farnesene	0.46	1.16	trace
17	Germacrene D	2.66	23.86	1.66
18	(<i>Z,E</i>)- α -Farnesene	6.99	14.69	-
19	β -Bisabolene	21.90	-	0.45
20	Bicyclogermacrene	0.98	1.74	-
21	(<i>E,E</i>)- α -Farnesene	0.47	2.78	-
22	β -Sesquiphellandrene	0.28	-	0.88
23	3,10-Dihydro-1,4-dimethylazulene	-	0.64	23.90
24	mw 178	-	-	1.13
25	1,4-Dimethylazulene	-	-	6.61
26	<i>trans</i> -Anyl 2-methylbutanoate	1.02	0.84	-
27	<i>trans</i> -Anyl 3-methylbutanoate	2.02	1.50	-
28	Epoxy- <i>cis</i> -anil 2-methylbutanoate	0.14	-	-
29	Epoxy- <i>cis</i> -anil 3-methylbutanoate	0.19	-	-
30	Epoxy- <i>trans</i> -anil 2-methylbutanoate	20.57	2.13	0.33
31	Epoxy- <i>trans</i> -anil 3-methylbutanoate	25.75	2.49	0.26
32	4-(2'-oxopropyl)phenyl 2-methylbutanoate	0.33	-	-
33	4-(2'-oxopropyl)phenyl 3-methylbutanoate	0.40	-	-
34	Epoxy- <i>trans</i> -pseudoisoeugenol 2-methylbut.	-	-	8.61

Although these two sesquiterpene hydrocarbons were found in significant higher proportion in the herb oil, accompanied by other constituents of this class of substances, especially by germacrene D and (*Z,E*)- α -farnesene, the isolation failed due to the very low yield of essential oil from the leaves. **Figure 3**, in which the gas chromatogram of the fruit, the leaf and the root

oil from *Pimpinella nigra* are confronted, shows impressively the variation in the essential oil composition obtained from the different parts of a plant.

Figure 3. GC of essential oils from different Parts of *Pimpinella saxifraga* ssp. *nigra* Mill.

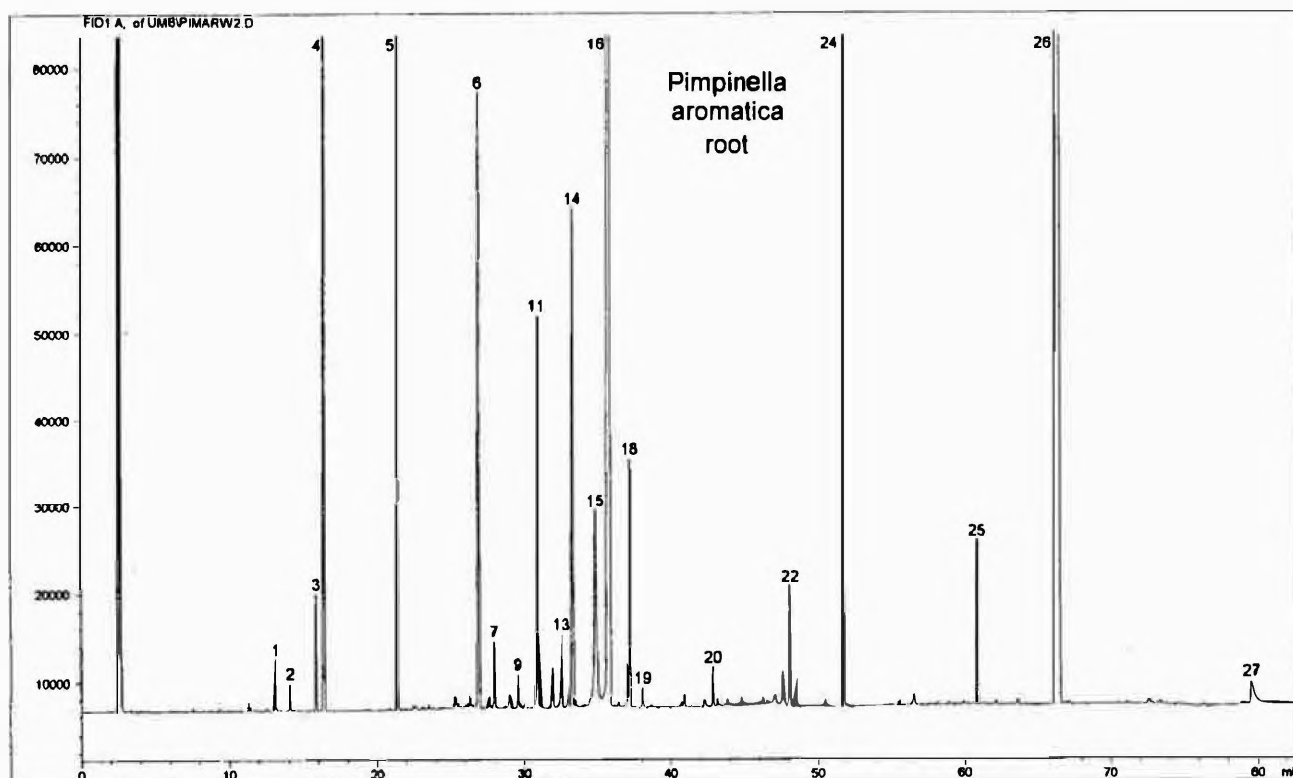


Pimpinella aromatica

A closely related oil composition with respect to the qualitative make up of the *Pimpinella nigra* root oil has been observed in course of the investigation of the root oil from *Pimpinella aromatica*, a biennial plant endemic to Easternmost Anatolia and the Caucasus. A sample of the dark blue oil, kindly provided by Prof. Baser, was analyzed and yielded the gas chromatogram given in **Figure 4**. By GC/MS and comparison of the obtained mass spectra with those of our own respective data-base including retention data, the following main constituents could be identified: the C_{12} -hydrocarbons geijerene (4), isogeijerene, pregeijerene (6), trinoranastreptene (5), two dihydro-1,4-dimethylazulenes (15,16) and 1,4-dimethylazulene (24), which is responsible for the dark blue colour of the oil. In addition four constituents with the molecular weight 178 (19-23), which seem to be oxygenated derivatives of C_{12} -constituents with the empirical formula $C_{12}H_{18}O$ and which have also been found in some other *Pimpinella* oils, remain up till now unknown. The group of phenylpropane derivatives is represented by

pseudoisoeugenyl 2-methylbutanoate (**25**) and a high amount of its epoxide (**26**), as well as by small amounts of *trans*-anethole (**17**) and significant higher proportions of estragole (**11**).

Figure 4. GC of *Pimpinella aromatica* root oil



The latter compound was found by Baser and his co-workers (11) to be the main constituent with 92 % of the aerial parts from *Pimpinella aromatica* collected in Turkey at the same place where the investigated roots came from.

The percentages of the individual components from our investigation of *Pimpinella aromatica* root oil are given in **Table 9**. As can be taken from this table sesquiterpene hydrocarbons were detected besides the before mentioned constituents only in minor concentrations, above all germacrene B, its cope-rearrangement product γ -elemene and germacrene D.

Table 9. Composition of *Pimpinella aromatica* root oil

No.	Compound	%
1	γ -Terpinene	0.27
2	p-Cymene	0.14
3	Isogeijerene	0.71
4	Geijerene	7.07

5	Trinoranastreptene	5.13
6	Pregeijerene	4.84
7	β -Caryophyllene	0.46
8	mw 160	0.12
9	γ -Elemene	0.23
10	unknown	0.25
11	Estragole	2.49
12	mw 204 (93,105,91,133,41)	0.32
13	Germacrene D	0.53
14	mw 204 (41,69,93,67,79)	4.29
15	Dihydro-1,4-dimethylazulene	2.93
16	3,10-Dihydro-1,4-dimethylazulne	29.89
17	<i>trans</i> -Anethole	0.26
18	Germacrene B	1.78
19	mw 178	0.13
20	mw 178	0.27
21	mw 178	0.18
22	mw 178	0.78
23	mw 178	0.23
24	1,4-Dimethylazulene	7.62
25	<i>trans</i> -Pseudoisoeugenyl 2-methylbutanoate	1.27
26	Epoxy- <i>trans</i> -pseudoisoeugenyl 2-methylbutanoate	26.83
27	Hexadecanoic acid	0.48

Pimpinella isaurica

The oil of a further *Pimpinella* species, which was also kindly provided by Prof. Baser was obtained from *Pimpinella isaurica*, a bushy perennial plant which is a Turkish endemism and which has been allied to *P. tragium*. The gc of the essential oil obtained from the respective herb exhibited numerous peaks, most of which were identified by GC/MS (Table 10). Beside the commonly found monoterpene hydrocarbons (1-7), the sesquiterpene hydrocarbons β -caryophyllene (12), germacrene D (26), α -zingiberene (17), α -curcumene (23) and germacrene B (25) could be identified. The main constituent in the higher boiling range of the gc displayed a mass spectrum similar to that of anil angelate, which was also present in this oil (39), with the same molecular peak at 216 and the same two strong signals with the masses 55 and 83. This suggested the unknown to be an isomer of anil angelate. In order to proof this suggestion, a ^{13}C NMR investigation of the total oil was performed, in which the unknown was represented with 75 %. From the obtained spectrum the unknown constituent could be easily identified to be the

angelic acid ester of chavicol, a compound which has not been found up till now in any pimpinella oil.

The percentages of the main constituents found in the herb oil of *Pimpinella isaurica*, which are summarized in **Table 10**, exhibit, that a second derivative of chavicol, the 2-methylbutanoate and non-esterified chavicol have also been identified, however, in relatively small concentrations. Comparing the herb oil of *P. isaurica* with the herb oil of the before mentioned *Pimpinella aromatica*, which contains estragole as the main constituent, a similar biosynthetic pathway can be postulated which includes isomerization of the 1-propenylic side chain to form allylic substituted phenylpropanoids.

Albovia tripartita

A third species endemic to Asia Minor and the Caucasus which we have investigated is *Albovia tripartita* Schischk., a perennial plant which is treated by taxonomists in different ways and included by some of them into the genus *Pimpinella* as *Pimpinella rotundifolia* M.B.. It was therefore interesting to see, whether this species contains any trinosesquiterpenoids and pseudoisoeugenol derivatives which have been found up to till now only in the species of the genus *Pimpinella* and none of the other species of the umbellifers (12).

The gc of the essential root oil from *Albovia tripartita* exhibits a number of peaks most of which could be identified by GC/MS (**Table 11**). The main constituent with approximately 50 % is the epoxy-pseudoisoeugenyl angelate. In addition, the respective tiglate (**25**) and 2-methylbutanoate (**23**) as well as the non-epoxidized angelate could be identified. In addition, some common monoterpene hydrocarbons, the geijerenes, the methylethers of thymol and carvacrol and some up till now unknown compounds with the molecular weight 178 and 180 were detected. The latter constituents **18**, **20** and **21** seem to be oxygene containing C₁₂-derivatives with the emperical formula C₁₂H₁₈O and C₁₂H₂₀O, which we are going to isolate for structural elucidation.

The percentages of the individual root oil constituents given in **Table 11** are confronted to the very preliminary results obtained from the fruit oil, which consists mainly of pregeijerene and geijerene whereas sesquiterpene hydrocarbons and phenylpropanoids are found only as minor constituents. Although the given analytical data must be considered as preliminary results, they point out unambiguously, that this species belongs to the genus *Pimpinella* because

of the extremely restricted occurrence of geijerenes and pseudoisoeugenol derivatives in umbellifers, which have only been found up till now in *Pimpinella* species.

Table 11. Composition of the essential oils from *Albovia tripartita* roots and fruits

No.	Compound	root	fruit
1	α -Pinene	0.09	-
2	β -Pinene	0.05	-
3	Sabinene	0.08	-
4	Limonene	0.39	-
5	β -Phellandrene	0.03	-
6	γ -Terpinene	0.15	-
7	p-Cymene	1.39	-
8	Isogeijerene	1.11	3.02
9	Geijerene	13.17	36.61
10	mw 162 (147,91,162,43,105)	0.66	0.70
11	Pregeijerene	9.98	51.35
12	Methylthymol	1.23	-
13	Methylcarvacrol	1.58	-
14	β -Caryophyllene	0.82	0.47
15	mw 204 (204,133,105,93,91)	0.52	-
15a	Germacrene D	-	0.14
16	Isohumulene	1.20	-
16a	β -Bisabolene	-	2.48
17	<i>trans</i> -Anethole	0.17	0.39
18	mw 178 (105,43,79,91,92)	0.63	0.06
19	2-Methyl-5-methoxybenzofuran	0.32	trace
20	mw 180 (43,95,162,93,122)	0.87	0.51
21	mw 178 (43,91,105,79,120)	6.81	0.27
22	<i>trans</i> -Pseudoisoeugenyl angelate	2.02	0.02
23	Epoxy- <i>trans</i> -pseudoisoeugenyl 2-methylbutanoate	1.28	0.03
24	Epoxy- <i>trans</i> -pseudoisoeugenyl angelate	48.29	0.61
25	Epoxy- <i>trans</i> -pseudoisoeugenyl tiglate	1.15	1.58

Pimpinella villosa

The last investigations to be discussed refer to *Pimpinella villosa*, a perennial umbellifer endemic to the Iberian peninsula, which has been collected in Spain Western to Salamanca. The main constituent of this oil with more than 50 % (**Table 12**) has been identified to be the epoxide of *trans*-pseudoisoeugenyl angelate (**30**). This compound is accompanied by the respective tiglate (**31**) and the non-epoxidized angelate (**28**). In addition to the homologous n-aldehydes from C₆ to C₉ several sesquiterpene hydrocarbons were found. The main constituent

of this group δ -elemene (8) points to the occurrence of germacrene C, which undergoes thermal rearrangement during gas chromatographic separation which can be recognized by the raising base line. In addition, the occurrence of the seldomly found "kessane" (21), a bicyclic sesquiterpene oxide is worth to mention. The same is true for the 2-methyl-5-methoxybenzofuran (25), probably a decomposition product formed from pseudoisoeugenol. The more detailed results are shown in Table 12 in which the percentages of the individual components are given.

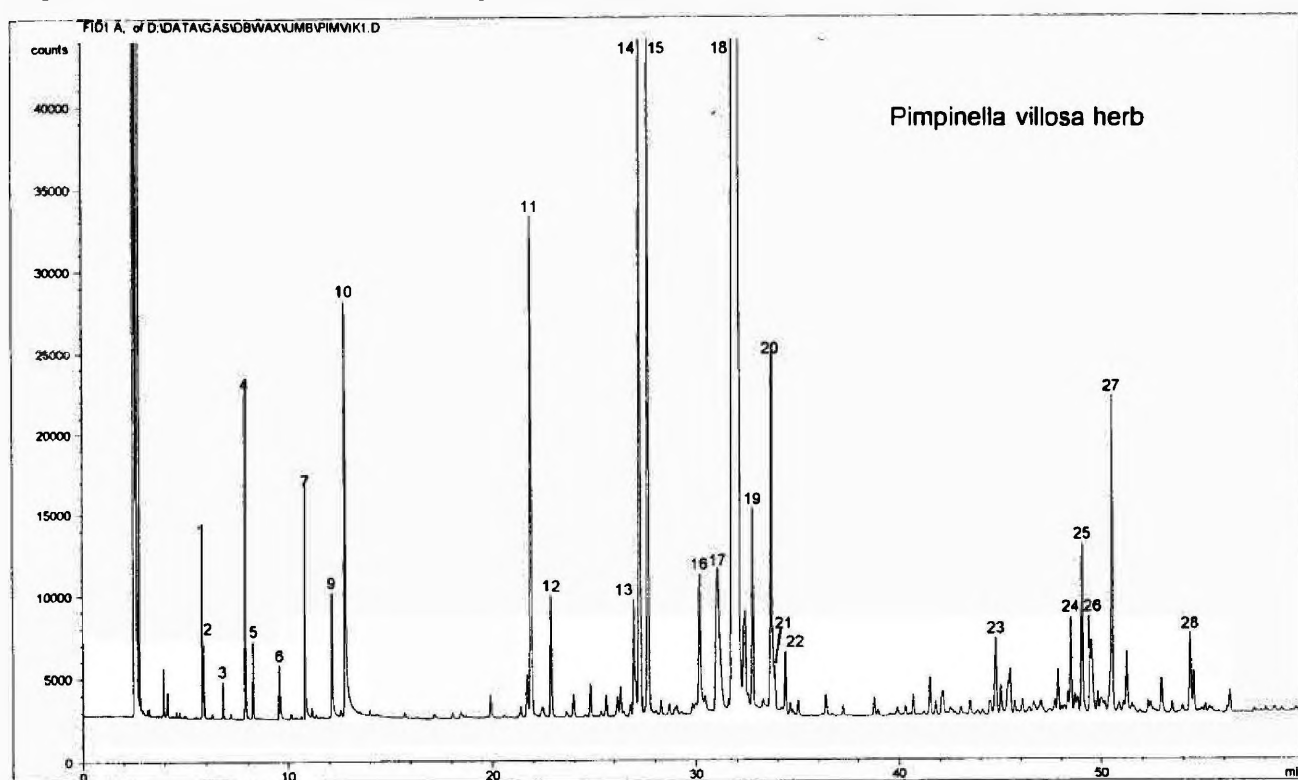
Table 12. Composition of the root oil from *Pimpinella villosa*

No.	Compound	%
1	Hexanal	0.07
2	n-Heptanal	1.04
3	n-Octanal	2.51
4	Geijerene	0.22
5	Nonanal	0.03
6	Trinoranastreptene	0.48
7	δ -Elemene epimer	0.53
8	δ -Elemene	11.86
9	<i>trans</i> -2-Nonanal	0.36
10	Pregeijerene	0.13
11	Cascarilladiene	0.18
12	β -Elemene	0.47
13	6,9-Guaiadiene	0.52
14	<i>trans</i> -2-Decenal	1.12
15	C ₁₅ H ₂₄	0.65
16	α -Humulene	0.27
17	γ -Muurolene	0.12
18	C ₁₅ H ₂₆ O	0.48
19	Germacrene D	3.21
20	δ -Cadinene	0.18
21	Kessane	3.09
22	Angelica acid	1.64
23	Germacrene B	0.33
24	unknown	0.80
25	2-Methyl-5-methoxy benzofuran	1.83
26	C ₁₅ H ₂₄ O	3.12
27	Anyl angelate	1.08
28	<i>trans</i> -Pseudoisoeugenyl angelate	1.87
29	Epoxy- <i>cis</i> -pseudoisoeugenyl angelate	0.67
30	Epoxy- <i>trans</i> -pseudoisoeugenyl angelate	52.42
31	Epoxy- <i>trans</i> -pseudoisoeugenyl tiglate	0.79

Due to the high proportion of the two main constituents, the percentages of most of the remaining components are close to, or below 1 %.

The gc of the essential oil from the herb of *Pimpinella villosa* (Figure 5) shows three separated groups of constituents: at the beginning several monoterpene hydrocarbons, in the middle diverse sesquiterpene hydrocarbons and at higher retention times several oxygenated sesquiterpenes. Main constituent is by far germacrene D (18) with nearly 60% followed by two peaks with significant lower concentrations, the first of which was identified as β -caryophyllene (14). The second (15) displayed a mass spectrum of an uncommon sesquiterpene, which was not found in our ms-database and other commercially available libraries.

Figure 5. GC of herb oil from *Pimpinella villosa*



Therefore we decided to isolate this constituent by preparative gc to perform structural elucidation. From the ^1H - and ^{13}C NMR-spectra a bicyclic compound with 4 methyl groups and 2 conjugated double bonds in one of the 2 rings could be deduced, which led us finally to suggest the unknown to be a acora-6,8-diene. In order to proof this suggestion, we isolated from two *Chaerophyllum hirsutum* chemotypes species, which we have investigated before (13), the two epimeric spiro-ketones acorenone and acorenone-B. The acora-6,8-diene obtained from

acorenone after reduction of the oxo-group with lithium-aluminium-hydride forming two acorenols and successive dehydratization finally proved the obtained product to be identical with the natural component. The detailed percentual proportions of the individual constituent found in herb and fruit oil from *Pimpinella villosa* are given in Table 13.

Table 13. Composition of the herb and fruit oil from *Pimpinella villosa*

No.	Compound	herb	fruit
1	α -Pinene	0.49	trace
2	α -Thujene	0.21	-
3	Camphene	0.11	-
4	β -Pinene	1.12	trace
5	Sabinene	0.26	trace
6	Myrcene	0.23	trace
7	Limonene	0.97	21.06
8	β -Phellandrene	0.04	-
9	<i>cis</i> -Ocimene	0.63	-
10	<i>trans</i> -Ocimene	2.50	-
11	δ -Elemene	0.06	4.05
12	α -Copaene	0.67	0.66
13	β -Elemene	0.83	2.05
14	β -Caryophyllene	4.40	2.73
15	Acora-6,8-diene	4.38	3.83
16	α -Humulene	1.04	0.81
17	γ -Muurolene	2.20	2.29
18	Germacrene D	58.81	42.25
19	Bicyclogermacrene	1.17	1.06
20	δ -Cadinene	2.29	2.08
21	γ -Cadinene	0.23	0.45
22	Cadina-1,4-diene	0.35	0.22
23	Cubebol	0.11	0.10
24	T-Cadinol	0.56	0.49
25	T-Muurolol	1.00	0.65
26	δ -Cadinol	0.30	-
26a	α -Bisabolol	0.16	2.25
27	α -Cadinol	2.05	1.35
27a	C ₁₅ H ₂₄ O	0.37	6.67
27b	C ₁₅ H ₂₄ O	0.06	1.34
28	(2 <i>E</i> ,6 <i>E</i>)-Farnesol	0.56	-
29	C ₁₅ H ₂₄ O	0.24	2.10

In the fruit oil, which exhibited a composition very similar to that of the herb oil, the acoradiene was also present in nearly the same concentration. However, a remarkable difference of both oils is the high proportion of limonene and δ -elemene in the herb oil.

Finally it is worth to mention that both oils of the above ground parts of *Pimpinella villosa* do not contain geijerene and phenylpropane derivatives in remarkable quantities. Therefore, in general only the root oils from *Pimpinella* species seem to be suitable for the delimitation of the genus *Pimpinella* using these types of components.

Acknowledgements

The author is indebted to Prof. K.H.C. Baser, Eskisehir/Turkey for providing oil samples from *Pimpinella aromatica* and *P. isaurica*, to Prof. M. Grande, Salamanca/Spain for plant material of *P. villosa*, to Dr. W. Schultze, Hamburg/Germany for performing gc/ms measurements, and the DAAD for travel grants (314-Al-e-dr) to Spain for the collection of plant material.

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METHODS IN AROMATHERAPY RESEARCH

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ABSTRACT

In this review a survey of scientific methods in aromatherapy research is given. They are designed to enhance the knowledge about effects of fragrance compounds and essential oils and the reaction of the human body upon treatment with these aroma chemicals. New results in brain research as to the effects of fragrance compounds are described and discussed as well as some exemplary and interesting animal experiments. The correct definition of the term aromatherapy is stressed again.

INTRODUCTION

At the beginning of this short review on methods in aromatherapy research some comments on the history of the term aromatherapy will be given as well as the new, modern, up-to-date and – from the standpoint of recent research results – correct definition of this word.

The use of essential oils and also of single fragrance compounds for healing purposes is known in folk medicine since ancient times and is discussed nowadays because of the trend back to natural drugs and therapies in medicine¹. The term *aromatherapy* has been coined in the late twenties of our century by the French cosmetic chemist *R.M. Gattefossé* who noticed the excellent antiseptic properties and skin permeability of essential oils and defined it as any therapy with essential oils². Using the trends of the eighties, "New Age" and esoterics *R. Tisserand* revived this term by including it into a general natural healing method with a lot of wholistic, cosmic, anthroposophic and other phenomena³. Nowadays many of the so-called aromatherapists devoid of any serious scientific training use this definition for their own purposes combining it with mystic elements⁴ and uttering such nonsenses, like "essential oils are the soul, the spirit, the personality of the plant"⁵. All this leads to a confusion or even misinterpretation of the word aromatherapy which is a combination of *aroma*, which means "fragrance, sweet smell, a subtle quality"⁷ and *therapy*, which implies a handling only by doctors or persons trained in medical sciences. Many aromatherapists also deny the close correlation of aroma to volatiles, although volatility is the main quality of fragrance compounds and essential oils!

Therefore a universal definition of the term aromatherapy on a scientific basis has been proposed by the author, as follows:

Therapeutic uses of fragrances or at least mere volatiles to cure and to mitigate or to prevent diseases, infections and indispositions only by means of inhalation^{8,9}

Following this definition serious scientific work on the biological effects of volatiles, especially essential oils, was compiled with the aim to put a clear line of demarcation between a scientifically based aromatherapy and such an aromatherapy as widely practiced today. The methods which have been applied in these studies to establish clear scientific facts of the efficiency of inhaled fragrance compounds are summarized and discussed in this paper.

DISCUSSION

The methods to establish proofs of the physiological, not psychological (!), and pharmacological activities of fragrance compounds can be divided in two groups:

- animal experiments and
- experiments with human volunteers.

Animal Experiments

Tests with various animals by administering fragrance compounds either perorally or parenterally to check their toxicity and their pharmacological behaviour are as common as skin trials to assay their allergenic potency or healing properties. But such experiments are not the topic of this review, because the main point in aromatherapy is the inhalative uptake of the fragrance molecules, their resorption by the nasal and/or pulmonal mucosa.

In this respect only two groups of scientists are known in literature up to now, who studied the behaviour of test animals under the influence of fragrance compounds. The method of choice is to examine the motor activity of test animals, preferably mice, in a cage with a light barrier. The first were *Kövar* and *Ammon et al.* in Tübingen, Germany, who showed interest in establishing an unambiguous proof of the efficiency of the essential oil of rosemary¹⁰, used in phytotherapy as an activating, refreshing remedy against exhaustion. It could be shown that by means of the main constituent of this essential oil, namely the bicyclic ether 1,8-cineole, this phytotherapeutical treatment is justified. The locomotor activity of the test animals increased significantly by inhalation of this ether or this oil. And above all these scientists succeeded in showing a clear pharmacokinetic dependance of this activating efficiency of 1,8-cineole: The higher the concentration of this fragrance in the blood of the mice is, the higher is also the rate of interruptions of the light barrier indicating an increased motor activity of the test animals. *Ammon* finally showed by similar studies that the essential oils of balm leaves and valerian roots act

calming and relaxing, whereas the essential oils of dwarf pine needles and juniper berries acted in the same way as the oil of rosemary¹¹.

Buchbauer's team is the second one known in literature, who studied the effects of single fragrance compounds and essential oils upon inhalation by test animals (mice). In accordance with the relevant literature^{12,13} fragrances and essential oils were chosen and tested in an animal model under standardized conditions^{10,14,15}. In total 44 fragrances and essential oils with ascribed sedative properties on humans have been investigated up to now by inhalation experiments on untreated and pretreated (over-agitation by intraperitoneal dosage of caffeine) mice. As a result of these studies it was found that lavender oil, its main constituents linalol and linalyl acetate as well as neroli oil, citronellal, 2-phenylethyl acetate, α -terpineol, benzaldehyde and East Indian Sandalwood oil decreased the motility of mice to an extent of 40–78% compared with 0% of the control. Tests with pretreated mice also showed a significant decrease of activity in the "caffeine over-agitated" animals in the case of lavender oil, linalol, isoeugenol, maltol, methyl salicylate, carvone and linalyl acetate (a decrease of 46–91%). In additional investigations, blood samples obtained from mice having inhaled such fragrance compounds were analysed by means of GC-spectroscopic systems to detect and identify possible active compounds responsible for the motility decrease (see also ref.¹⁰). In some cases volatiles could be identified in the blood in the range of 10.7 ppb to 0,12 ppb (benzaldehyde after inhalation) (see also ref.¹).

Finally it should be pointed out that fragrance compounds really are resorbed and are able to cross the blood brain barrier. After a 15-minute inhalation of a certain fragrance compound it could be detected in the blood and in the cortex of the test animals, but not in the medulla¹⁶.

Experiments with Human Volunteers

In order to set a clear line of demarcation between aromatherapy – where physiological and pharmacological activities of fragrances are dealt with – and aromachology – where the interrelationships between psychology and odors are discussed¹⁷ – only those methods which offer a better insight into the complex reaction of the human body upon inhaling fragrance compounds are reviewed in this paper, according to the modern definition of aromatherapy (as already defined in the introduction). Naturally it is very difficult to abstract the reflectoric answer of the human body upon a fragrance stimulus from the biological event in the body when fragrance molecules are resorbed. But nevertheless it is very important to do this, because there is a great difference in the way of getting a body-response: Either effects which are achieved through stimulation of the olfactory pathways in the brain (the field of aromachology¹⁷), or reactions which are obtained by introduction of the fragrance molecule into the blood stream by nasal and/or pulmonal resorption, – the field of aromatherapy.

Regarding the methods which have been applied to put more light on the problem of the body response upon an inhalative uptake of fragrance molecules, it is interesting to notice that especially Japanese scientists are at the front of aromatherapy research. An excellent review on the effects of odors, already published in 1990 by *Kawasaki*¹⁸, makes it easier for the author to compile the research work done up to now on aromatherapy.

Torii investigated the effects of odors upon skin potential levels (SPL)¹⁹. SPL are related to mental perspiration and correspond well with the arousal level of the subject: -40mV upon awakening, -60mV at times of excitement and almost 0mV during sleep. Upon *Torii's* findings the psychophysiological effects induced by the scents of chamomile oil and jasmin are sedative in the first case and stimulating with the latter oil. Measurements of the SPL via the CNV (=contingent negative variation) clearly showed the variation of the SPL in parallel with the level of activity of the sympathetic nervous system. Such tests indicated - according to *Niimi*²⁰ - that pleasant as well as unpleasant odors raise arousal levels and that especially the latter cause relatively large potential fluctuations.

The above mentioned contingent negative variation (CNV) is a slow upward shift in brain waves recorded by the electroencephalogramm (EEG), occurring in situations where the subject is in attention, expecting that something will happen. The first to use this method - already known in brain research since *Walter et al.*²¹ - was *Torii* and his team, who studied the CNV and the physiological effects of odors in humans²². The results obtained so far showed that jasmine oil brought about an effect similar to the administration of caffeine, namely an increase in CNV after inhalation, whereas lavender oil caused CNV to decrease, similar to the administration of tranquilizers, thus leading to a sedation. Other essential oils were also studied and their effects upon inhalation documented.

Recently also *Kubota et al.*²³ reported on similar investigations in this respect using essential oils too, not only several fractions of them obtained by vacuum distillation, but even single fragrance compounds. In some cases he was also able to show distinct differences between chiral fragrance molecules as to their ability to influence the CNV, f.e. l-carvon elicited a sedative activity, whereas its caraway-like-smelling antipode d-carvon acted as a stimulant.

Measurements of fragrance effects by plethysmography have been performed by *Konishi et al.*²⁴. This is a method which allows to observe the changes in peripheral blood stream caused by the sympathetic part of the autonomic nervous system. Stress situations cause a constriction of the peripheral vasculature, hence increasing the peripheral blood pressure. Under the influence of fragrance compounds - peppermint and jasmine oil - the obtained results demonstrate that these odors possess neurophysiological activities which lead to a loosening of an induced stress situation and consecutively to a relaxation of the maximum constriction of the peripheral vasculature. Exposure to the odor of peppermint

diminished the peripheral blood pressure to a somewhat lesser extent than it was noticed with the jasmine fragrance.

Another physiological event can be used to observe the effects of odors: The heart rate. *Oguri et al.*²⁵ showed that pleasant odors activating the CNS increase the heart rate variations, whereas the sedative odors decrease them. Also *Kikuchi et al.*²⁶ carried out such experiments and found that rose odor suppressed the deceleration of the heart rate, whereas lemon odor enhanced it, indicating activation. Thus the pattern of changes in the heart rate appears to show differences between stimulant odors and sedative ones.

Two other methods to examine the effects of odors on humans have been applied by the German fragrance chemist *W. Steiner*. Measurements of the changes of the steady current between two points at the skin surface by electrodermal activity furnished differences between 12 test odors enabling the scientist to rank them either at the sedative side or at the activating one²⁷. Another method applied by him measures the dilatation or constriction of the pupils²⁸: The obtained results showed that all odors under investigation caused a pupil enlargement indicating arousal. *Miyazaki et al.*²⁹ used *Steiner's* procedure in a cognitive task and found that orange oil increased the parasympathetic nervous system activity (indicated by a distinct acceleration of the constriction rate of the pupil), whereas the sympathetic nervous system activity was found to be decreased.

Coming back to the phenomenon of measurements of the electrical brain activity the CNV-method and its usefulness in aromatherapy-research has already been discussed earlier. Other methods to investigate the influence of fragrance compounds on brain waves still remain to be mentioned. Especially the teams of *Lorig* at Washington, *van Toller* at Warwick (UK) and *Kobal* at Erlangen (Germany) are famous for their pioneer work carried out in order to study the effects of odors on mood or feelings³⁰⁻³⁵. But these investigations cover more the field of aromachology than aromatherapy, especially when hedonic qualities exert an influence on the outcome of the results.

Nevertheless Japanese scientists again showed that evoked potentials and brain wave studies can be used also in aromatherapy-research. Steady-state waves can be distinguished by their frequency: alpha-waves are characterized by a frequency of 8-13 Hz, while those with larger frequencies are called beta -waves and those with lower frequencies theta- and delta-waves. Beta-waves are dominant when the subjects brain is engaged in concentrated thought or in a highly emotional mental state. An alpha-wave dominancy is characteristic for a mentally relaxed state or in meditation. The fragrance of lavender now is able to increase the alpha-wave activity in the EEG as well as sandalwood oil, whereas an increase of the beta-waves activity has been noticed by *Sugano* upon presentation of jasmine odor^{36,37}. Finally, *Lorig* and *Schwartz* reported that spiced apple aroma relieves stress and promotes a sense of relaxation, as could be demonstrated by a

reduction of the higher frequency-EEG activity and an increase of the slow wave EEG one³⁸.

Sugano also reported on a totally new method quasi to look into the brain^{37,39}. Using the fragrances of lavender, jasmine, sandalwood and α -pinene as olfactory stimuli he used Xe-positron-emission tomography to measure the local cerebral blood flow in order to observe the events in the brain upon inhalation of these fragrances. He compared his findings with the results he obtained by means of measurements of the alpha-brain waves activity as well as with those ones obtained by CNV-experiments and found a great similarity among all these data achieved by different methods: α -pinene, sandalwood and lavender elicited a distinct sedation, whereas jasmin exerted a stimulating effect^{37,39}.

Finally also two new methods in aromatherapy-research introduced by the author's team should be discussed. As has been mentioned earlier, *Ammon* stated that 1,8-cineole must affect the motor centres in the brain¹¹. *Ch.* and *B. Nasef* succeeded in proving this results performing Xe-computer-tomographic studies on healthy volunteers with the view to observe the cerebral blood flow upon inhalation of the fragrant 1,8-cineole⁴⁰. While *Sugano* used the positron generating radioactive ^{133}Xe in his PET-method³⁷, the Viennese group used stable, gaseous Xenon (not more than 30% in the normal breathing air) which serves as a contrast medium for computed-tomography-measurements during the examination procedure. This is a three-dimensional, high resolution method to study the regional cerebral blood flow (rCBF) for clinical routine, which allows direct analysis of the events in the blood supply within the investigated brain structures⁴¹ and which seems to become ever more irreplaceable in neurology. Eight neurologically healthy volunteers aged between 20 and 30 years and in addition one clinically tested anosmic person thus were examined before and after inhaling 1,8-cineole. In all cases the CBF of these subjects was increased upon inhaling this fragrance compound, showing an enhanced cortical activity. The anosmic woman reacted exactly in the same way. If, therefore, even an anosmic, a person who really is unable to create a pleasant feeling upon sniffing a fragrance, shows an increased CBF, it cannot be the result of a reflectoric effect. In addition the concentration of this fragrant in the blood was determined. Resorption is very fast as could be shown by an almost linear increase of the 1,8-cineole concentration from 4 to 20 minutes up to a maximum value of about 275 ng/ml serum⁴². When the inhalation was stopped, also the concentration of the fragrance in venous blood dropped immediately.

With transcranial *Doppler* (TCD) a sonographical, noninvasive imaging of vessel flow velocity is possible. TCD is an ultra sound method and is based on the *Doppler* shift principle which means a frequency shift of a sound signal when either the source or the receiver is moving. A movement towards each other is defined as a positive, a movement away from each other as a negative shift. In such TCD measurements "speed" is expressed by flow velocity. Practically an ultra sound signal is sent into the tissue and is reflected by the moving blood particles, mainly the erythrocytes⁴³. This new method is the second

innovation introduced by the *Nasel's* of *Buchbauer's* team into aromatherapy-research. The results obtained upon examination of some healthy volunteers and the anosmic subject again while inhaling 1,8-cineole are nearly the same as with Xe-CT⁴⁴.

Coming to a conclusion a lot of methods have been applied in aromatherapy research in order to verify the so-called healing effects of fragrance compounds and essential oils. Even if it is very difficult to separate the psychological influence furnishing the desired healing effect via a reflectoric action from a pharmacological, direct interaction of the fragrance molecules with appropriate receptor systems, many of these afore mentioned methods are used in aromachology research as well as in aromatherapy research. But it was due to the author that he proved for the first time that fragrance compounds – aside from creating a mostly happy feeling – do behave like pharmacologically active substances by applying new investigation methods on an anosmic person. For the first time it was possible to show that fragrance molecules really get resorbed, are transported in the blood stream indeed and by this way exert distinct effects in human subjects.

ACKNOWLEDGEMENTS

The author is indebted to many colleagues of his team for their precious cooperation, especially to: *Mag.pharm. Dr. Walter Jäger, Mag.pharm. Dr. Leopold Jirovetz, Mag.pharm. Barbara Nasel, Dr. med. Christian Nasel and Dr. med.vet. Hermann Dietrich*. He also acknowledges the support by the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (projects Nr. P7587 CHE and P8299 CHE) and the support by the Bürgermeister-fund of the City of Vienna. Finally the generosity and the interest of *Dragoco Comp.*, Vienna, should be gratefully mentioned.

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RECENT DEVELOPMENTS IN PHYTOCHEMISTRY OF BRYOPHYTES

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ABSTRACT

A number of new terpenoids and aromatic compounds have been isolated from bryophytes, particularly from liverworts. Some biological activity of sesqui- and diterpenoids and phenolic compounds isolated from bryophytes has been summarized.

INTRODUCTION

The bryophytes are placed taxonomically between algae and pteridophytes; there are about 24000 species in the world. They are further divided into three classes, Musci (mosses), Hepaticae (liverworts) and Anthocerotae (hornworts). The Hepaticae contain oil bodies which are easily extracted with organic solvents whilst the other two classes do not contain oil bodies. A number of bryophytes have been used as medicinal plants in North America, China and Europe to treat burns, bruises, external wounds, *etc.* The following mosses and liverworts are medicinal plants and are said to possess certain biological activity and effect. [**Musci**] *Bryum argenteum* (antidotal, antipyretic, antirhinitic activity; for bacteriosis), *Cratoneuron filicinum* (for malum cordis), *Ditrichum pallidum* (for convulsions, particularly in infants), *Fissidens japonicum* (diuretic activity, for growth of hair, burns and choloplasia), *Funaria hygrometrica* (for hemostasis, pulmonary tuberculosis, hematemesia, bruises, athlete's foot dermatophytosis), *Haplocladium catillatum* (antidotal, antipyretic activity; for adenopharyngitis, uropathy, mastitis, erysipelas, pneumonia, urocystitis and tympanitis), *Leptodictyum riparium* (antipyretic; for choloplasia and uropathy), *Mnium cuspidatum* (for hematostasis and nose bleed), *Oreas martiana* (for anodyne, hemostasis, external wounds, epilepsy, menorrhagia and

neurasthenia), *Philonotis fontana* (antipyretic, antidotal activity; for adenopharyngitis), *Plagiopus oederi* (as a sedative, and for epilepsy, apoplexy and cardiopathy), *Polytrichum* species (diuretic activity; for growth of hair), *Polytrichum commune* (antipyretic, antidotal; for hemostasis, cuts, bleeding from gingivae, hematemesis and pulmonary tuberculosis), *Rhodobryum giganteum* (antipyretic, diuretic, antihypertensive; for sedation, neurasthenia, psychosis, cuts, cardiopathy and expansion of hear blood vessels), *Rhodobryum roseum* (as a sedative and for neurasthenia and cardiopathy), *Taxiphyllum taxirameum* (antiphlogistic; for hemostasis and external wounds) and *Weissia viridula* (antipyretic, antidotal; for rhinitis). [Hepaticae] *Conocephalum conicum* (antimicrobial, antifungal, antipyretic, antidotal activity; used to cure cuts, burns, scalds, fractures, swollen tissue, poisonous snake bites and gallstones), *Frullania tamarisci* (antiseptic activity), *Marchantia polymorpha* (antipyretic, antihepatic, antidotal, diuretic activity; used to cure cuts, fractures, poisonous snake bites, burns, scalds and open wounds), *Reboulia hemisphaerica* (for blotches, hemostasis, external wounds and bruises).

Some bryophytes show characteristically fragrant odors and intensely hot and bitter or saccharine-like taste. Generally, bryophytes are not damaged by insects, snails, slugs and other small animals. Furthermore, some liverworts cause intense allergenic contact dermatitis and allelopathy. We have been interested in these bioactive substances found in bryophytes and have studied about 800 species of Hepaticae collected in North and South America, North and South Africa, India, Pakistan, Nepal, Taiwan, Australia, New Zealand, Europe and Japan with respect to their chemistry, pharmacology, and application as sources of cosmetics, and medicinal or agricultural drugs. It has been demonstrated that most of the Hepaticae contain mainly sesqui- and diterpenoids and lipophilic aromatic compounds (bibenzyls, bis-bibenzyls, benzoates, cinnamates, long chain alkyl phenols, naphthalenes,

phthalides, isocoumarins, benzyl and β -phenethyl acrylates and prenyl indoles) which constitute the oil bodies. The biological activities of liverworts are due to these substances⁵⁾ At present more than 300 new compounds have been isolated and their structures elucidated.^{1,5)}

The chemical structures of new terpenoids and aromatic compounds found in several liverworts and their biological activity will be summarized in this paper.

1) Typical terpenoids and aromatic compounds found in liverworts

Liverworts produce a number of different terpenoids, acetogenins and aromatic compounds as shown in Charts 1-29. They are homomonoterpenoids, tris-norsesquiterpenoids and sesquiterpenoids [africane, norafricane and secoafricanes (1-12), 2,3-secoaromadendranes (13-38), azulenoids (39-52), barbatanes (=gymnomitranes) (53-65), bazzananes (66-71), bicyclogermacrane, isobicyclogermacrane (72-82), brasilane (83a), lepidozane (84), chiloscyphanes, oppositanes (85-87), herbertanes (=isocuparanes) (89-99), monocyclofarnesanes (103-108), myltaylanes, cyclomyltaylanes (111-116), pacifigorgiane (117), pinguisanes (118-153), norpinguisanes (154-157), vitranes, omphalane (158), riccardiphenols (159, 160), serpentiphenol (161), olivacane (163), trifaranes (164-166), chenopodane (169) and zieranes, diterpenoids [cembranes, clerodanes (170-181), dolabellanes (182-191), fusicoccanes (192-203), *ent*-kauranes (204-209), labdanes (210-222), chettaphanins, phytanes, sacculatanes (223-236), sphenolobanes (237-242), tracchylobanes, verrucosanes (243-253) and verticillanes (254a-259)] and aromatic compounds [bibenzyls (260-326), bis-bibenzyls (327-383), long chain alkylphenols (384-395), naphthalenes (396, 397), isocoumarin (398), prenyl ethers (399, 400), sulfur- (401, 402) and nitrogen-containing compounds (403, 404),

neolignane (**405**), phenanthrenes (**406-411**) and phthalides (**412-416**)]^{1,5)}

On the other hand, mosses biosynthesize a number of highly unsaturated fatty acids (**417-427**) and a few diterpenoids. Neither sesqui- nor monoterpenoids have been isolated from the Musci. The Anthocerotae (hornworts) produce typical cinnamic acid derivatives, megacerotonic acid (**428**), anthocerotonic acid (**429**) and rosmarinic acid (**430**). No terpenoids have been isolated from the Anthocerotae. An important endogenous character of the Hepaticae is that most of the sesquiterpenoids isolated from liverworts are enantiomeric to those found in higher plants. It is also interesting that some different *Frullania* species produce both enantiomeric sesquiterpenoids. Table 1 shows several biological activity (including characteristic odor and tasting) of the isolated compounds from bryophytes. Some biologically active compounds will be surveyed.

Table 1. Biological activities of terpenoids and aromatic compounds isolated from liverworts

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1. Characteristic odor
 2. Hot(pungent), bitter and sweet tasting
 3. Allergenic contact dermatitis
 4. Cytotoxic activity
 5. Antimicrobial and antifungal activity
 6. Insect antifeedant and molluscicidal activity
 7. Piscicidal activity
 8. Plant growth regulatory activity
 9. Superoxide anion radical release inhibitory activity
 10. 5-Lipoxygenase, calmodulin, hyaluronidase and cyclooxygenase inhibitory activity
 11. Vasopressin antagonist and cardiotonic activity
 12. Neurite sprouting activity
 13. Muscle relaxing activity
 14. Antitumor promoting activity

- 15 DNA polymerase B inhibitory activity
 - 16. Cathepsins B and L inhibitory activity
 - 17. Anti-HIV-I activity
-

2) Biological activity

2.1. Characteristic scents

The bryophytes, particularly liverworts, emit intense sweet-woody, intense turpentine, sweet-mossy, fungal-like, carrot-like, mushroomy or seaweed-like scents when upon being crushed. Among these are *Asterella odora* (resembling the higher plant, *Houttuynia cordata*), *Chiloscyphus pallidus* (intense smell reminiscent of stink bug), *Conocephalum conicum* (*Houttuynia cordata*-like, strong mushroomy), *Conocephalum japonicum* (also *Houttuynia cordata*-like), *Frullania* species (turpentine-like), *Funaria hygrometrica* (sea-star like), *Geocalyx graveolens*, *Jungermannia* and *Lejeunea* species (turpentine-like), *Leptolejeunea elliptica* (very intense ! mixed odor of naphthalene and dried bonito), *Lophocolea heterophylla* (strongly and distinctly mossy), *L. minor* (strongly mossy), *Lophozia vicrenata* (pleasant resembling cedar oil), *Moerkia* species (intensely unpleasant), *Pellia endiviifolia* (dried seaweed-like), *Plagiochila sciophila* (= *P. acanthophylla* subsp. *japonica*) (sweet mossy and woody), *Plagiochila ovalifolia* etc. (turpentine-like), *Porella cordaeana* (dried seaweed-mossy like), *Porella vemica* complex (pungent and turpentine-like), *Riella* species (anise-like), *Solenostoma obovata* (carrot-like), *Takakia lepidozoides* (mixed odor of cinnamon and burnt wheat powder), *Trichocolea tomentella* (sulfur-like) and *Wiesnerella denudata* (strong sweet mushroomy).

Almost all liverworts which smell of mushrooms contain 1-octen-3-ol and its acetate but the strong sweet-mushroomy scent of the ether extract of *Wiesnerella denudata* is due to (+)-bornyl acetate and mixtures of the monoterpene hydrocarbons, α -terpinene, β -phellandrene, terpinolene, α -pinene, β -pinene and camphene. The odor of the steam distillate of *W. denudata* is weaker than that of its ether extract. The steam distillate contains nerol (14%), neryl acetate (27%) and γ -terpinene (31%), but the content of 1-octen-3-ol (7%) and its acetate (2%) are very low. A small thalloid liverwort, *Asterella* species grown in Pulau Dayang Bunting island, Malaysia emit very intense unpleasant odor which is due to skatole.⁶⁾ The stink bug smell of *C. pallidus* is attributable to (*E*)-dec-2-enal, (*Z*)-dec-2-enal, (*E*)- and (*Z*)-pent-2-enals.⁷⁾ The characteristic fragrance of *Leptolejeunea elliptica* is due to *p*-ethylanisol (431), *p*-ethylphenol (432) and *p*-ethyl phenylacetate (433).⁸⁾ Bicyclohumulenone (83b) isolated from *Plagiochila sciophila* (= *P. acanthophylla* subsp. *japonica*) possesses an aroma reminiscent of a variety of scents based on a strong woody note, resembling the odor of patchouli, vetiver, cedar wood, orris, moss and carnations. Tamariscol (117) from *Frullania tamarisci* subsp. *tamarisci*, *F. tamarisci* subsp. *obscura*, *F. nepalensis* and *F. asagrayana* similarly possesses a remarkable aroma reminiscent of the woody and powdery green notes of mosses, foin, fluvia, costus, violets leaf and seaweeds. Both compounds are highly esteemed in commerce. They are used as perfumes as such or perfume components of the powdery floral-, oriental bouquet-, fantastic chypre-, fancy violet- and white rose-types in various cosmetics. After it had been shown that both the tertiary alcohol and the 2-methyl-1-propenyl group attached to the cyclohexane ring of tamariscol were necessary for the characteristic scent of (117), thirteen mini-tamariscols

were synthesized by Grignard reactions of 2,7-dimethylcyclohexanone, 2-methylcyclohexanone, 4-methylcyclohexanone, cyclohexanone and cyclopentanone with vinylmagnesium bromide, 2-methyl-1-propenylmagnesium bromide and 2-methyl-2-propenylmagnesium bromide, respectively. 1-Hydroxy-1-(2-methyl-1-propenyl)-cyclohexane (**434a**) had a similar sweet mossy aroma to that of tamariscol itself. A thalloid liverwort *Mannia fragrans* emits a pleasant and intense scent which can be detected at a distance of many meters. This specific fragrance is caused by grimaldone (**305**).⁹⁾ The strong milk-like fragrance of *Cheilojejeunea imbricata* is due to a mixture of (R)-dodec-2-en-1,5-olide (**434a**) and (R)-tetradec-2-en-1,5-olide (**434b**).⁸⁾ (-)-Ambrox (**443**) has been prepared by chemical transformation of diterpenediol (**438**) isolated from the liverwort *Porella perrottetiana* as shown in Chart 32 (Asakawa *et al.* unpublished results).

2.2. Pungency and bitterness

Some genera of the Hepaticae produce intensely pungent and bitter substances which exhibit interesting biological activities described in subsequent sections. *Porella vernicosa* complex contained very pungent substances and that *Jamesoniella autumnalis* contained an intense bitter principle whose taste resembles that of the leaf of lilac and *Swertia japonica* or the root of *Gentiana scabra* var. *orientalis*. The strongly hot taste of *Porella vernicosa* complex is due to polygodial (**100a**).

We reported that sesquiterpene lactones, secoaromadendrane-type sesquiterpene hemiacetals and sacculatane-type diterpene dialdehyde possessing an intensely pungent taste had been isolated from some *Chiloscyphus* and *Wiesnerella*, *Plagiochila*, and *Pellia* and *Trichocoleopsis* species, respectively.¹⁾ The hot taste of *Pallavicinia levieri* is due to sacculatal (**223**). Polygodial and sacculatal have been obtained from cell suspension

cultures from each liverwort.^{10,11)} Plagiochiline A (**13**) from *Plagiochila* species showed a strong pungent taste. Enzymatic treatment of (**13**) with amylase in phosphate buffer or with human saliva produced two 2,3-secoaromadendrane-type aldehydes, plagiochilal B (**24**) and furanoplagiochilal (**34**). Dialdehyde possesses a strong pungent taste which implies that the intense pungency is not due to plagiochiline A (**13**) itself, but to plagiochilal B (**24**) whose partial structure is similar to that of the drimane-type very pungent sesquiterpene dialdehyde, polygodial (**100a**).

Gymnocolea inflata is amazingly persistent bitter and induces vomiting when one chews a few leaves for several seconds. The earlier review already mentioned that this is due to gymnocolin A (**171**).¹⁾ *Jungemannia infusca* has an exceedingly intense bitter taste. This is due to the presence of the infuscasides A-E (**204-208**).

2.3. Allergenic contact dermatitis

Frullania species (Hepaticae) are notable as liverworts that cause allergenic contact dermatitis. Allergy-inducing substances are sesquiterpene lactones, (+)-(**101a**) and (-)-frullanolides (**101b**) and their related α -methylene- γ -butyrolactones which have been isolated from *Frullania dilatata* and *F. tamarisci*, respectively. *F. asagrayana*, *F. bolanderi*, *F. eboracensis*, *F. franciscana*, *F. inflata*, *F. kunzei*, *F. nisquallensis*, *F. riparia* and the other *Frullania* species which contain sesquiterpene with an α -methylene- γ -butyrolactones cause strong allergenic contact dermatitis. *Schistochila appendiculata* causes allergenic contact dermatitis. The allergens are alkylphenols (C11, C13, C15 and C17) (**384**, **387**, **390**, **394**), long chain alkyl salicylic acids (C11, C13, C15) (**385**, **388**, **391**) and their potassium salts (**386**, **389**, **392**) as well as a long chain catechol (**393**).

Such allergenic contact dermatitis is very similar to that caused by the long chain alkylphenols of the fruit of *Ginkgo biloba* and Anacardiaceae. The following liverworts and mosses also cause strong allergenic contact dermatitis: *Marchantia polymorpha*, *Metzgeria furcata* (liverworts); *Eurhynchium oreganum*, *Isohecia stramineum*, *Leucolepis menziesii*, *Mnium cuspidatum*, *Polytrichum juniperium*, *Rhytidadelphus loreus* and *Sphagnum palustre*. Their allergens have not been isolated.

2.4. Antitumor Activity

As mentioned in the earlier review¹⁾, a few eudesmanolides and a germacranolide possessing inhibitory activity against KB cells have been isolated from some liverworts. *Conocephalum conicum* and *Wiesnerella denudata* contain some guaianolides which exhibited antitumor activity against P-388 lymphocytic leukemia. The crude ether extract (20µg/ml) of the following liverworts showed cytotoxicity against P-388 *in vitro*. They are *Bazzania pompeana*, *Kurzia makinoana*, *Lophocolea heterophylla*, *Makinoa crispata*, *Marsupella emarginata*, *Plagiochila fruticosa*, *P. ovalifolia*, *Porella caespitans*, *P. japonica*, *P. perrottetiana*, *P. verrucosa* and *Radula perrottetii*. *Frullania diversitexta*, *F. ericoides*, *F. muscicola*, *F. tamarisci* subsp. *obscura*, *Lepidozia vitrea*, *Pallavisinia subciliata*, *Plagiochila sciophila*, *Supraceanthus semirepandus* and *Trocholejeunea sandvicensis* were not active against P-388. We tried to isolate the active compounds from *P. ovalifolia* and *P. fruticosa* and *Marsupella emarginata*. 2,3-Secoaromadendrane-type sesquiterpenoids (**35**, **36**) isolated from *P. ovalifolia* showed antitumor activity (ID₅₀ 0.05µg/ml) against P-388. Marsupellone (**109**) and acetoxymarsupellone (**110**) from *M.*

emarginata showed antitumor activity (ID_{50} 1 μ g/ml) against P388. Plagiochiline A (**13**) from *P. fruticosa* and *P. ovalifolia* showed as antitumor activity (ID_{50} 6 μ g/ml) against P388 and its T/C(100%) value against P388 was estimated as 115 Riccardins A (**327**) and B (**329**) from *Riccardia multifida* inhibited KB cells at a concentration of 10 and 12 μ g/ml, respectively. The following compounds isolated from liverworts also showed cytotoxic activity against KB cells: values in the parentheses show the concentration in μ g/ml: Plagiochiline A (**13**) (0.28) from several *Plagiochila* species and perrottetin E (**366**) (12.5) from *Radula perrottetii* and a few species of Marchantiales. 3-Methoxy-4'-hydroxybibenzyl (**267**) from *Plagiochila* species showed cytotoxicity against monkey kidney cells (BSC) at 60 μ g/well.¹²⁾

The thalloid liverwort, *Marchantia polymorpha* can cause allergenic contact dermatitis, shows inhibitory activity against Gram-positive bacteria, and has diuretic activity. The methanol extract (100 g) of Japanese *M. polymorpha* was chromatographed on silica gel and Sephadex LH-20 to give cyclic bis-bibenzyls, marchantin A (MA) (**335**, 30 g) and its analogues (MB-G). Yield of MA (**335**) is depend upon *Marchantia* species. 100 to 120 g of pure MA has been isolated from 2kg of dried *M. paleacea* var. *diptera*. MA (**335**) showed cardiotonic [increase coronary blood flow (2.5ml/min. at 0.1mg], antimicrobial (see later) and cytotoxicity (ED_{50} 8.29 μ g/ml for KB cells), and its T/C value against P388 was estimated as 117. *Blasla pusilla* produces pusilatin A-D (**380-383**) which showed cytotoxicity against KB cell (ED_{50} 13.1-20 μ g/ml), and DNA polymerase β (IC_{50} 5.16-38.5 μ g and weak HIV-RT inhibitory activity.¹³⁾

2.5. Antimicrobial and antifungal activity

Several liverworts (*Bazzania* species, *Conocephalum conicum*, *Dumortiera hirsuta*, *Marchantia polymorpha*, *Metzgeria furcata*, *Pellia endiviifolia*, *Plagiochila* species, *Porella vernicosa* complex, *P. platyphylla*, *Radula* species etc.) and mosses (*Atrichum antustatum*, *A. undulatum*, *Dicranum scoparium*, *Isothecium stoloniferum*, *Mnium punctatum*, *Orthotrichum rupestre*, *Polytrichum commune*, *P. juniperinum*, *Thuidium recognitum* var. *delicatulum*, *Tortula muralis*, *Sphagnum fimbriatum*, *S. palustre* and *S. strictum*.) show antimicrobial activity. Several liverworts, such as *Bazzania* species, *Conocephalum conicum*, *Diplophyllum albicans*, *Lunularia cruciata*, *Marchantia polymorpha*, *Plagiochila* species, *Porella vernicosa* complex, *Radula* species and mosses, *Atrichum undulatum*, *Bryum pallens*, *Dicranella heteromalla*, *D. scoparium*, *Microbryum delicatulum*, *Mnium homum*, *Oligotrichum hercynicum*, *Plagiothecium denticulatum*, *Pogonatum aloides*, *P. urnigerum*, *Polytrichum commune*, *Pseudoscleropodium purum*, *Sphagnum nemoreum*, *S. portoricense*, *S. strictum* and *S. subsecundum* display antifungal activity.

Marchantin A (335) from many *Marchantia* species, *Marchantia chenopoda*, *M. polymorpha*, *M. paleacea* var. *diptera*, *M. plicata* and *M. tosa*, shows antibacterial activity against *Acinetobacter calcoaceticus* (MIC 6.25 µg/ml), *Alcaligenes faecalis* (100), *Bacillus cereus* (12.5), *B. megaterium* (25), *B. subtilis* (25), *Cryptococcus neoformans* (12.5), *Enterobacter cloacae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* (100) and *Staphylococcus aureus* (3.13-25) and

antifungal activity against *Alternaria kikuchiana*, *Aspergillus fumigatus* (MIC 100 µg/ml), *A. niger* (25-100), *Candida albicans*, *Microsporium gypseum*, *Penicillium chrysogenum* (100), *Piricularia oryzae* (12.5), *Rhizoctonia solani* (50), *Saccharomyces cerevisiae*, *Sporothrix schenckii* (100), *Trichophyton mentagrophytes* (3.13) and *T. rubrum* (100).

2.6. Insect Antifeedant

As mentioned earlier plagiochiline A (**13**) found in several *Plagiochila* species, is a very strong antifeedant against the African army worm (*Spodoptera exempta*).¹⁾ The pungent sacculatal (**223**) from *Pellia endiviifolia* and *Trichocoleopsis sacculata*, eudesmanolides from *Chiloscyphus polyanthos* and gymnocolin (**171**) from *Gymnocolea inflata* also have antifeedant activity against larvae of Japanese *Pieris* species. A series of natural drimanes and related synthetic compounds was tested for antifeedant activity against aphids. Polygodial (**100a**) from the *Porella vernicosa* complex and a higher plant, *Polygonum hydropiper* and warburganal (**435**) from the African tree *Warburgia ugandensis* were the most active substances. Natural (-)-polygodial (**100a**) and the synthetic (+)-enantiomer (**436**) showed similar levels of activity as aphid antifeedants.

2.7. Superoxide Release Inhibitory Activity

Excess superoxide anion radical (O_2^-) in organisms causes various angiopathies, such as cardiac infarction, arterial sclerosis *etc.* Infuscaic acid (=clerod-3,13(16)-14-trien-17-oic acid) (**170**) from *Jungermannia infusca* inhibited the release of superoxide from rabbit PMN at IC_{50} 15 µg/ml and from guinea pig peritoneal macrophage induced by formyl

methionyl leucyl phenylalanine (FMLP) at IC_{50} 2 μ g /ml. Norpinguisone methyl ether (**155**) from *Porella elegantula* exhibited 50% inhibition at 35 μ g of the release of superoxide from the guinea pig peritoneal macrophage. The same activity (IC_{50} 7.5 μ g/ml) has been found in cyclomylytaylyl-3-caffeate (**115**) from *Bazzania japonica*. Other sesquiterpenoids, plagiocbilide (**19**), bicyclogermacrenal (**73**), herbertenediol (**93**), isocuparene-3,4-diol (**95**) and norpinguisone (**154**), and the diterpenoids, infuscaside A (**204**), infuscaside B (**205**) and perrottetianal A (**232**) from liverworts also inhibited superoxide release from guinea pig peritoneal macrophage (IC_{50} 12.5-50 μ g/ml). Radulanin K (**325**) from *Radula javanica* inhibited the release of superoxide anion radical from guinea pig macrophage (IC_{50} 6 μ g/ml).

2.8. Lipoxygenase, calmodulin, hyaluronidase and cyclooxygenase inhibitory activity
 Marchantin A (**335**) from several *Marchantia* species showed 5-lipoxygenase inhibitory activity [(89% at 10^{-5} mol, 94% at 10^{-6} mol, 45% at 10^{-7} mol, 16% at 10^{-8} mol) against LTB₃ (=5*S*,12*R*-dihydroxy-6,8,10,14-eicosatetraenoic acid)], (99% at 10^{-5} mol, 97% at 10^{-6} mol, 70% at 10^{-7} mol, 40% at 10^{-8} mol) against 5-HETE (=5-hydroxy-6,8,11,14-eicosatetraenoic acid)] and calmodulin inhibitory activity at ID_{50} 1.85 μ g/ml. Perrottetins A (**311**) and D (**308**) from *Radula perrottetii* and prenyl bibenzyls (**280**, **292**, **317**, **320**), also from *Radula* species, riccardin A (**327**) from *Riccardia multifida* and marchantins D (**342**) and E (**343**) from *Marchantia* species had calmodulin inhibitory activity (ID_{50} 2.0-95.0 μ g/ml). The simple bibenzyls (**263**, **268**, **272**, **276**) from *Radula* and *Frullania*

species also showed weak calmodulin inhibitory activity (ID_{50} 100 $\mu\text{g/ml}$) as did the labdane-type diterpene diol (**438**) (ID_{50} 82 $\mu\text{g/ml}$). Perrottetin A (**311**), prenylbibenzyls (**283**, **272**, **309**, **319**), marchantins D (**342**) and E (**343**) and riccardin A (**327**), also inhibited 5-lipoxygenase (76-4% at 10^{-6} mol).

Lunularic acid (**260**) which is found in almost all liverworts as a very minor amount has anti-hyaluronidase activity (IC_{50} 0.13nM). This activity is stronger than that of tranilast (N-3',4'-dimethoxy-cinnamoylanthranilic acid) which is anti-allergenic agent developed in Japan for oral administration. Marchantin A (**335**) and its analogues showed cyclooxygenase¹⁴⁾, DNA polymerase and HIV inhibitory activity (Asakawa et al. unpublished results). and its analogues showed cyclooxygenase inhibitory activity. MA also showed DNA polymerase inhibitory and anti-HIV activity (Asakawa et al. unpublished results).

2.9. Piscicidal activity

The strongest piscicides are the pungent (-)-polygodial (**100a**) from *Porella vernicosa* complex and sacculatal (**223**) from *Pellia endiviifolia*, *Pallavicinia levieri*, *Riccardia robata* var. *yakushimensis* and *Trichocoleopsis sacculata*¹⁾ Killie-fish (= *Oryzia latipes*) are killed within 2 hr by 0.4 ppm solution of (**100a**) and (**223**), and within 20 min at 7 ppm. Killie-fish are also killed within 2 hr by a 0.4 ppm solution of synthetic very pungent (+)-polygodial (**436**). Hence piscicidal activity is not affected by the chirality of polygodial. Polygodial is also very toxic to fresh water bitterlings which are killed within 3 min by a 0.4 ppm solution. On the other hand, isopolygodial (**100b**) from cultured cells of *Porella vernicosa* and the higher plant, *Polygonum hydropiper* and isosacculatal (**224**)

from *Pellia*, *Riccardia* and *Trichocoleopsis* species showed neither piscicidal nor molluscicidal activity even at 10000 ppm.

2.10. Neuritic sprouting activity

Mastigophorenes A (96), B (97) and D (99) from *Mastigophora diclados* exhibited neurotrophic properties at 10^{-5} - 10^{-7} M, greatly accelerating neuritic sprouting and network formation in the primary neuritic cell culture derived from the fetal rat hemisphere. Plagiochilide (19) and plagiochilal B (24) from *Plagiochila fruticosa* showed not only acceleration of neurite sprouting but also enhancement of choline acetyl transferase activity in a neuronal cell culture of the fetal rat cerebral hemisphere at 10^{-5} M. Plagiochin A (358) also showed the same activity at 10^{-6} M.

2.11. Muscle relaxation activity

Marchantin A (335) and the related cyclic bis-bibenzyis are structurally similar to bis-bibenzyl-isoquinoline alkaloids such as tubocurarine (437) which are pharmacologically important muscle relaxing active drugs. Surprisingly, marchantin A (335) and its trimethyl ether (336) also showed muscle relaxing activity.¹⁵⁾ Nicotine in frog Ringer solution effects maximum contraction of rectus abdominis in frogs (RAF) at a concentration of 10^{-6} M. After preincubation of marchantin A trimethyl ether (336) (at a concentration of 2×10^{-7} ~ 2×10^{-4} M) in Ringer solution, nicotine (10^{-8} ~ 10^{-4} M) was added. At a concentration of 10^{-6} M, the contraction of RAF decreased by about 30%. *d*-Tubocurarine (437) exhibits similar effects as does (336) using acetyl choline and the same results as described above obtained. Although the mechanism of action of marchantin A (335) and its methyl ether (336) in effecting muscle relaxation is still unknown, it is interesting that these cyclic bis-bibenzyis from liverworts possessing no nitrogen atoms cause concentration dependent decrease of

contraction of RAF. Marchantin A and its trimethyl ether also had muscle relaxing activity *in vivo* in mice. MM2 calculations indicate that the conformation of marchantin A and its trimethyl ether and the presence of an *ortho* hydroxyl group in (335) and an *ortho* methoxyl group in (336) contribute to the muscle relaxation activity.

2.12. Cathepsin L and cathepsin B inhibitory activity

Cathepsin L is correlated with osteoporosis¹⁶⁾ and allergy.¹⁷⁾ We are currently searching for this enzyme inhibitors from natural products to develop chemopreventive drugs for these diseases. Marchantin series showed both cathepsins L and B inhibitory activity. Isomarchantin C (354) was the strongest inhibitor against both enzymes (95% for cathepsin L and 93% for cathepsin B at 10^{-5} M). Infuscaic acid (170) exhibited the same activity as mentioned above (63% and 32% at 10^{-5} M).

At present only 5% of total bryophytes have been studied chemically. Although bryophytes are small plant groups, there are a number of new terpenoids and phenolic compounds several of which show interesting biological activity.

ACKNOWLEDGMENTS

The author thanks Professor N. Katsunuma and Miss Y. Tada (TBU), Profs. J. M. Pezzuto, T. Pengsuparp and G. A. Cordell (The University of Illinois at Chicago), Otsuka Pharmaceutical Co. Ltd. and Kirin Brewer Co. Ltd. for bioassay of the crude extracts and isolated compounds from liverworts. A part of this work was supported by a Grant-in-Aid of Cancer Research from the Ministry of Health and Welfare, Japan.

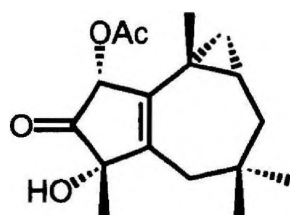
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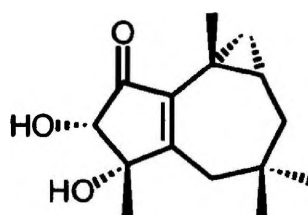
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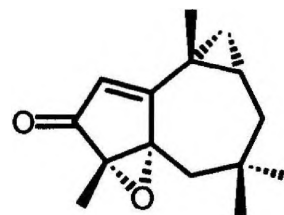
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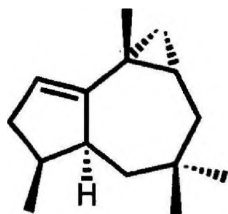
(1) 3 α ,4 α -Dihydroxyafrican-2(6)-en-5-one



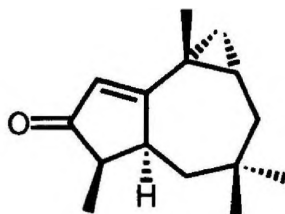
(2) 3 α -Hydroxy-5 α -acetoxy-african-2(6)-en-4-one



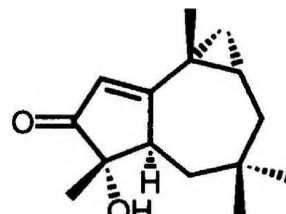
(3) Caespitenone



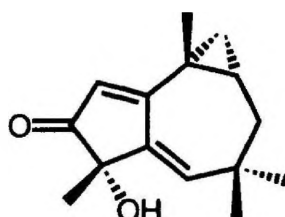
(4) Swartzianin A



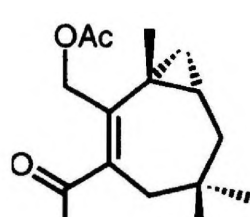
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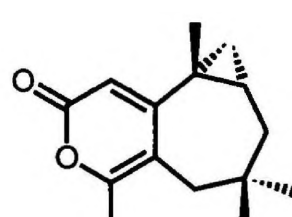
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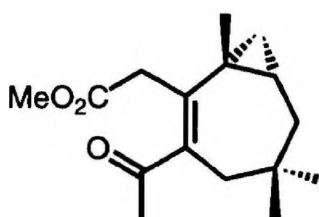
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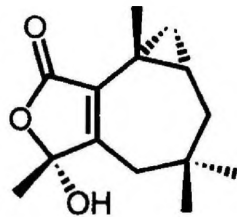
(8) Norafricanone



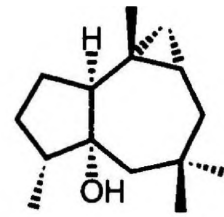
(9) Secoswartzianin A



(10) Secoswartzianin B

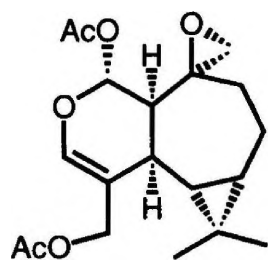


(11) Norswartzianin

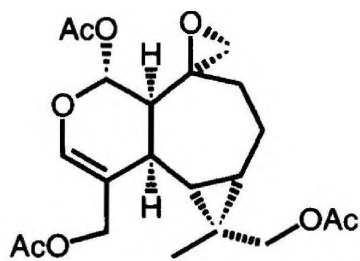


(12) Isoafricanol

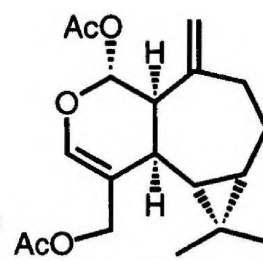
Chart 1. Africanes, norafricanes and secoafricanes found in the Hepaticae



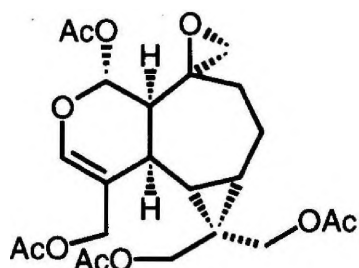
(13) Plagiochiline A



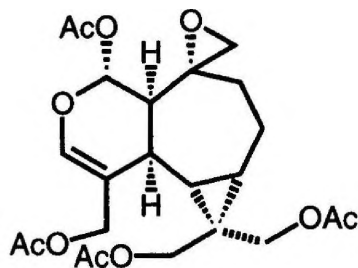
(14) Plagiochiline B



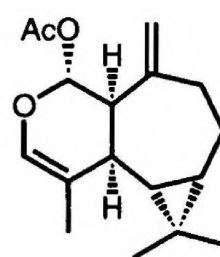
(15) Plagiochiline C



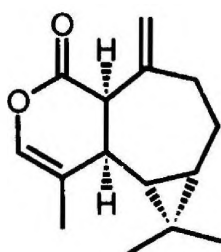
(16) Plagiochiline D



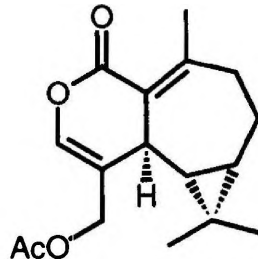
(17) Plagiochiline E



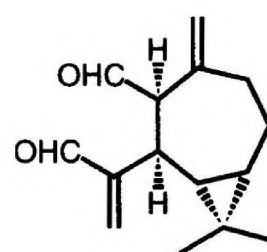
(18) Plagiochiline H



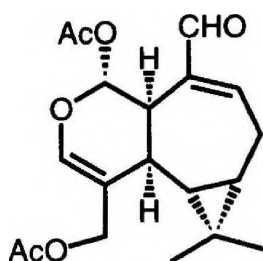
(19) Plagiochilide



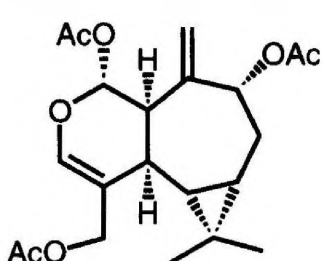
(20)
Acetoxyisoplagiochilide



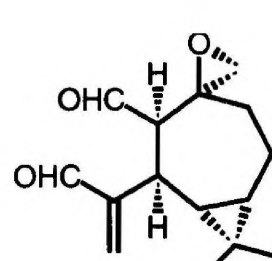
(21) Hanegokedial
(=Plagiochilal A)



(22) Ovalifolienal



(23) 9α-Acetoxyovalifoliene



(24) Plagiochilal B

Chart 2. Secoaromadendranes found in the Hepaticae

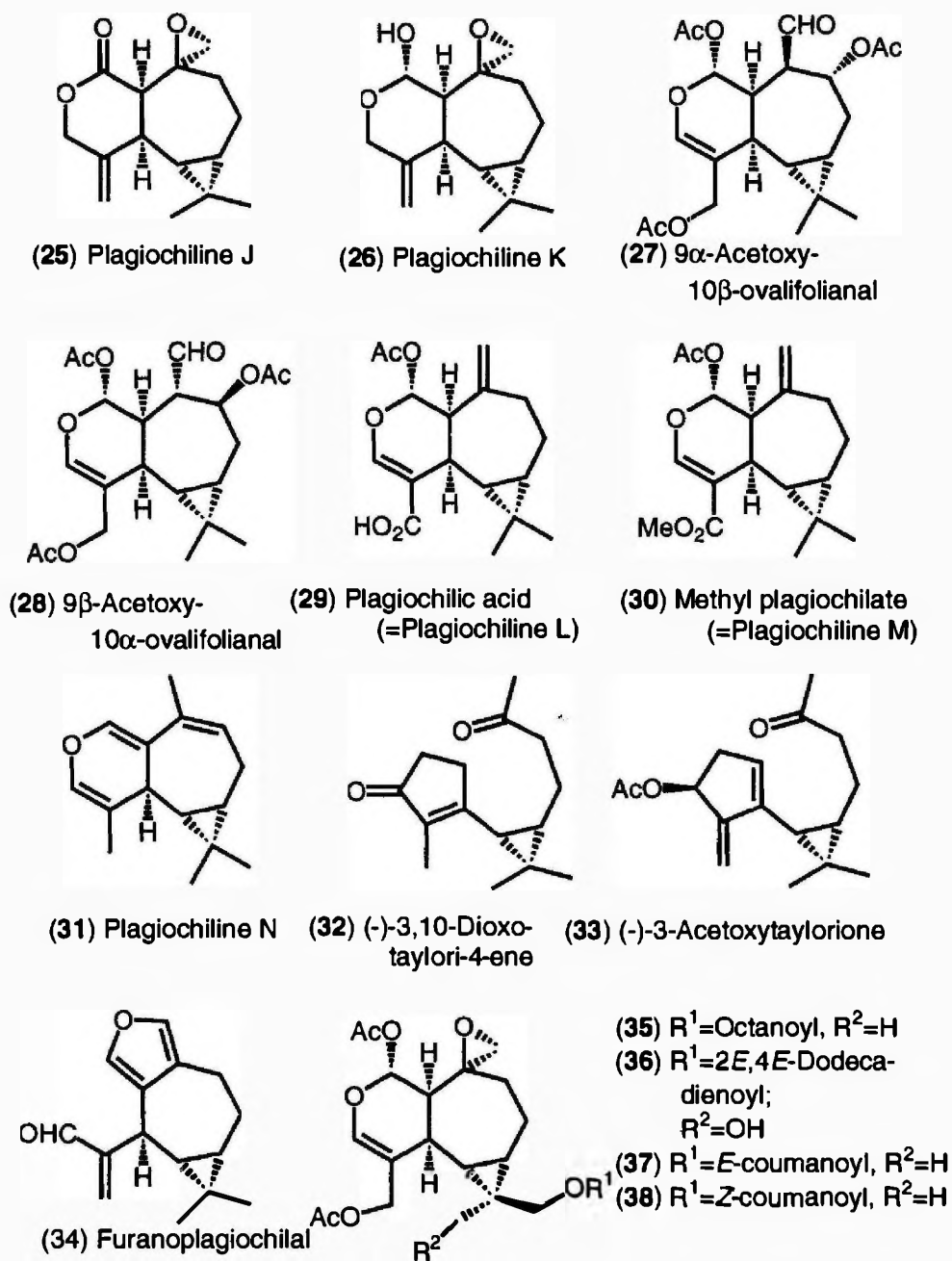
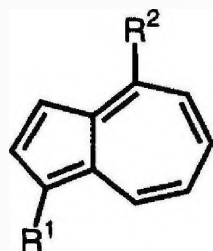
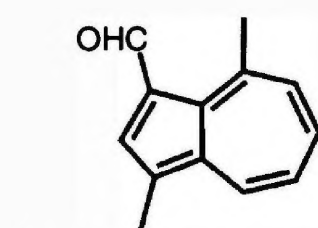


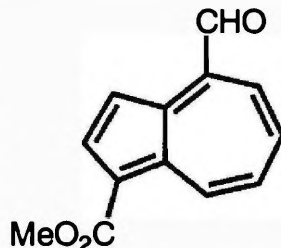
Chart 3. Secoaromadendranes and norsecoaromadendranes found in the Hepaticae



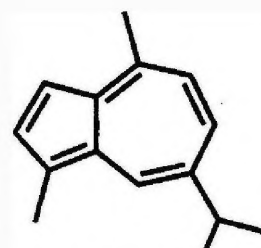
- (39) 1,4-Dimethylazulene ; $R^1=R^2=Me$
 (40) 4-Methyl-1-methoxycarbonylazulene ;
 $R^1=CO_2Me, R^2=Me$
 (41) 4-Methylazulene-1-carbaldehyde
 (=4-Methyl-1-formylazulene) ;
 $R^1=CHO, R^2=Me$
 (42) 4-Methyl-1-carboxyazulene ;
 $R^1=CO_2H, R^2=Me$
 (43) 4-Methoxymethyl-1-carboxyazulene;
 $R^1=CO_2H, R^2=CH_2OMe$
 (44) 1,4-Dicarboxyazulene ; $R^1=R^2=CO_2H$
 (45) 4-Hydroxymethyl-1-methoxycarbonyl-
 azulene; $R^1=CO_2Me, R^2=CH_2OH$
 (46) 4-Carboxy-1-methoxycarbonylazulene ;
 $R^1=CO_2Me, R^2=CO_2H$



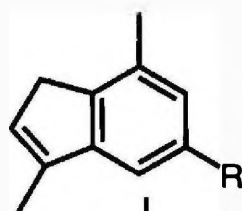
(47) 1,4-Dimethyl-3-formylazulene



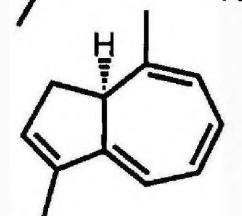
(48) 4-Formyl-1-methoxycarbonylazulene



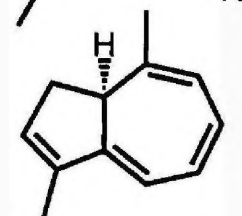
(49) Guaiazulene



(50) 3,7-Dimethyl-5-methoxy-carbonylindene ;
 $R=CO_2Me$



(51) 3,7-Dimethylindene-5-carbaldehyde ; $R=CHO$



(52) (+)-1,8a-Dihydro-3,8-dimethylazulene
 (=3,10-Dihydro-1,4-dimethylazulene)

Chart 4. Azulenes and indenenes found in the Hepaticae

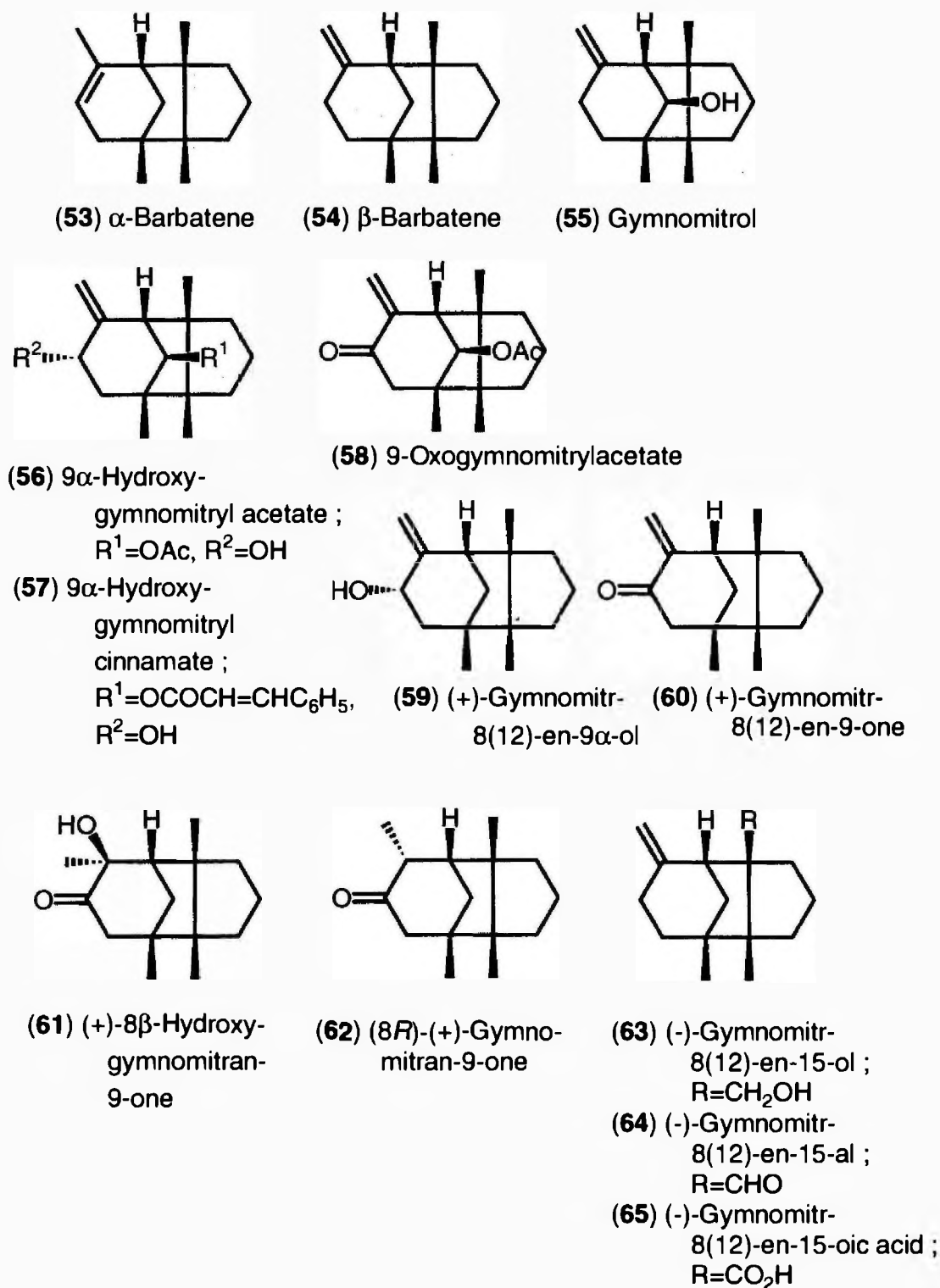


Chart 5. Barbatanes (=gymnomitranes) found in the Hepaticae

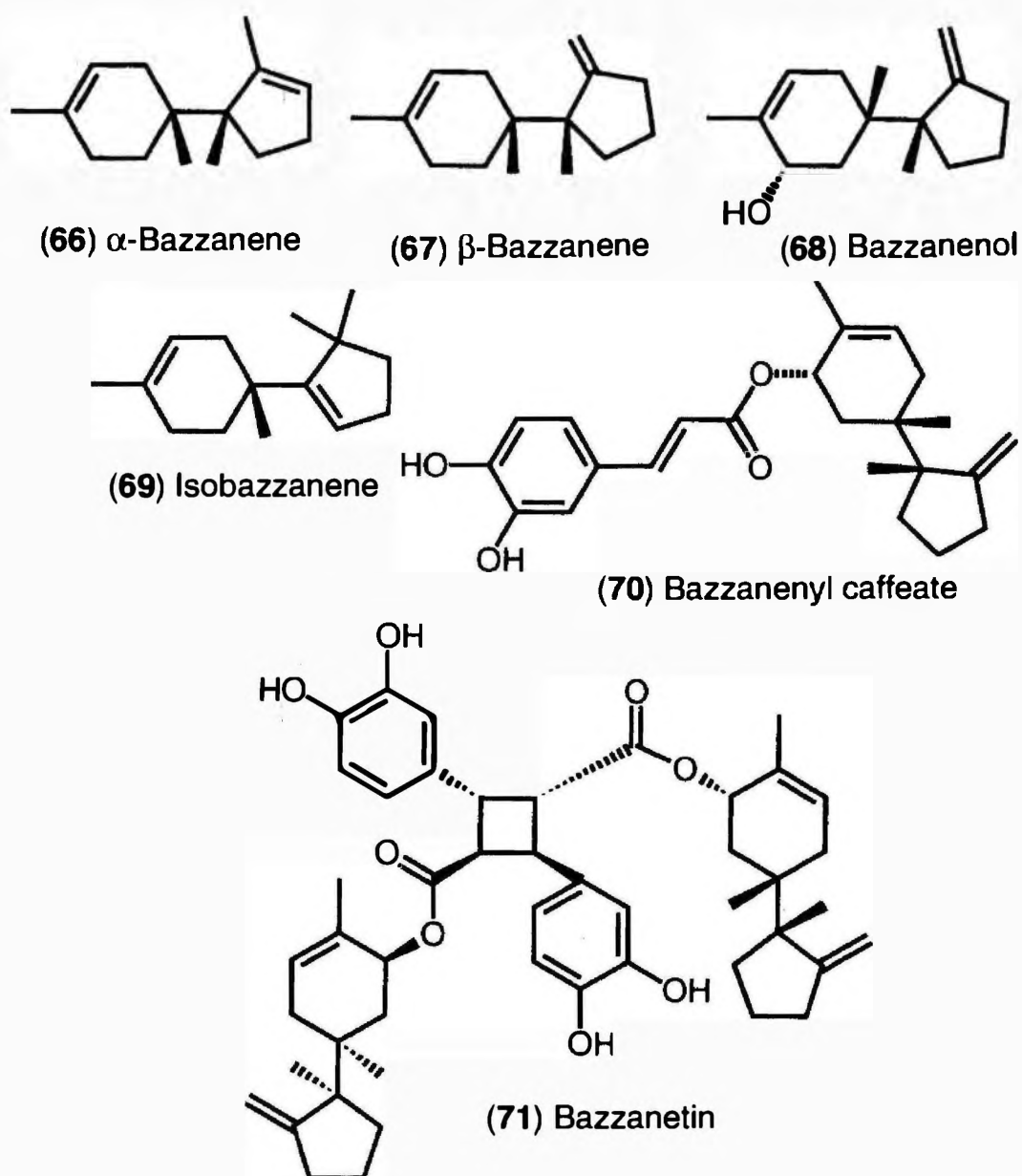


Chart 6. Bazzananes found in the Hepaticae

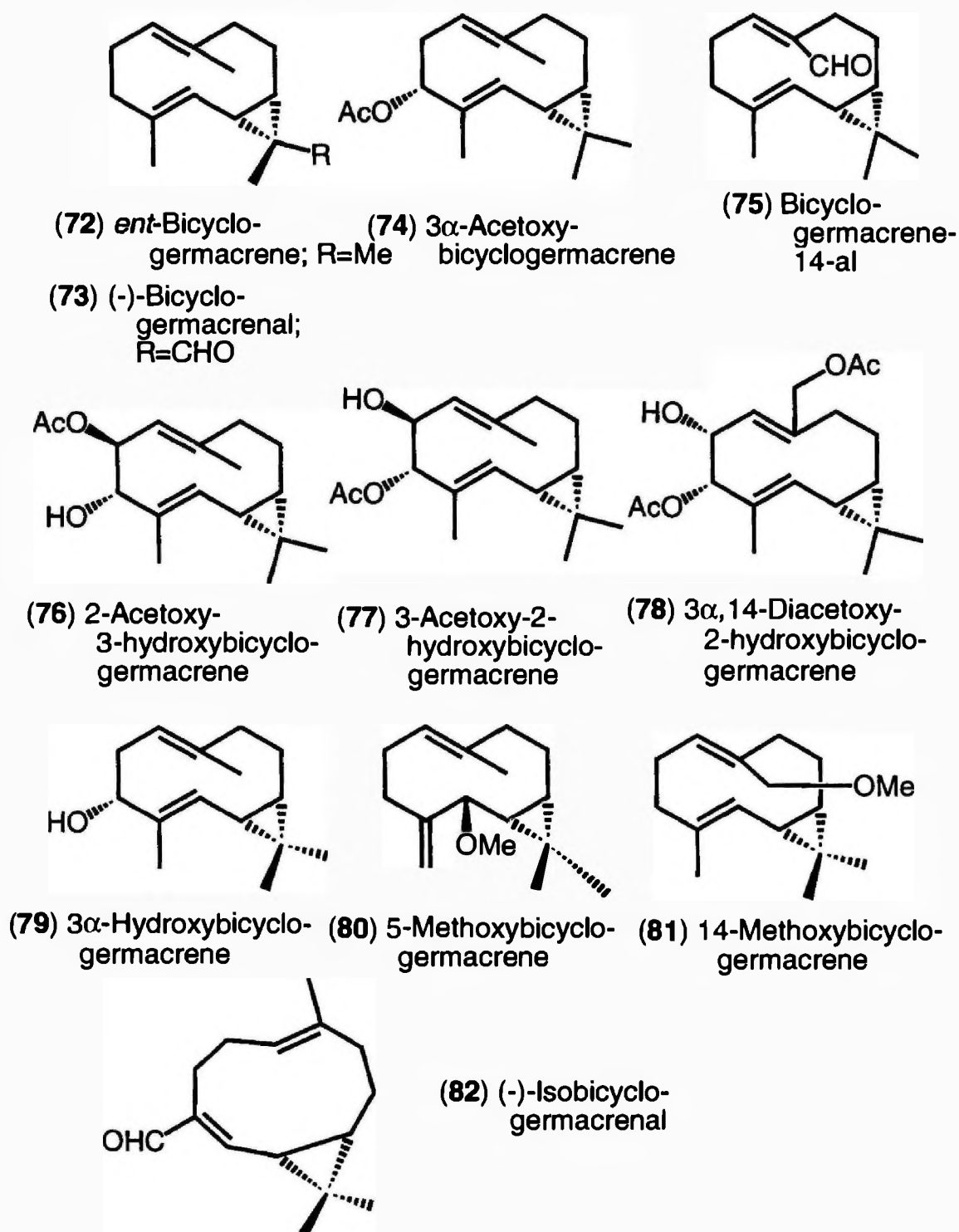
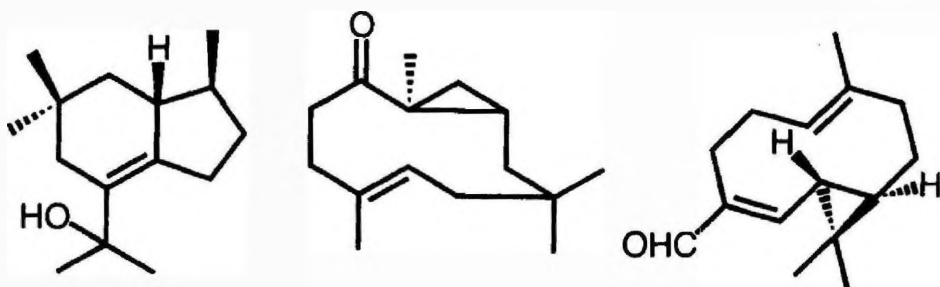
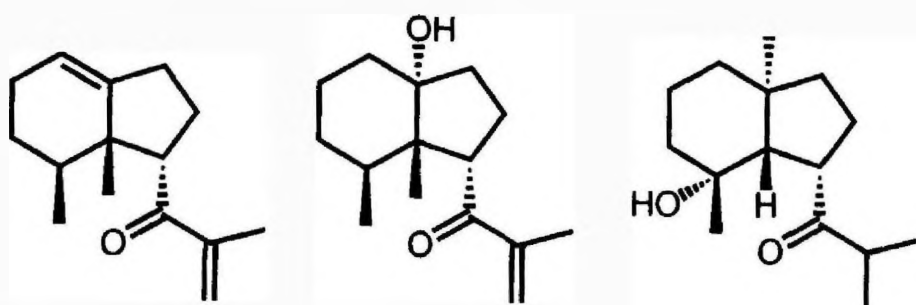


Chart 7. Bicyclogermacranes and isobicyclogermacranes found in the Hepaticae



(83a) Conocephalenol (83b) Bicyclohumulenone (84) Lepidozenal



(85) Chiloscypnone (86) Chiloscypnone (870) *ent*-(5*R*,6*S*,9*R*)-4α-Hydroxyoppositan-10-one

Chart 8. Brasilane, humulane, lepidozane, chiloscypnanes, oppositane found in the Hepaticae

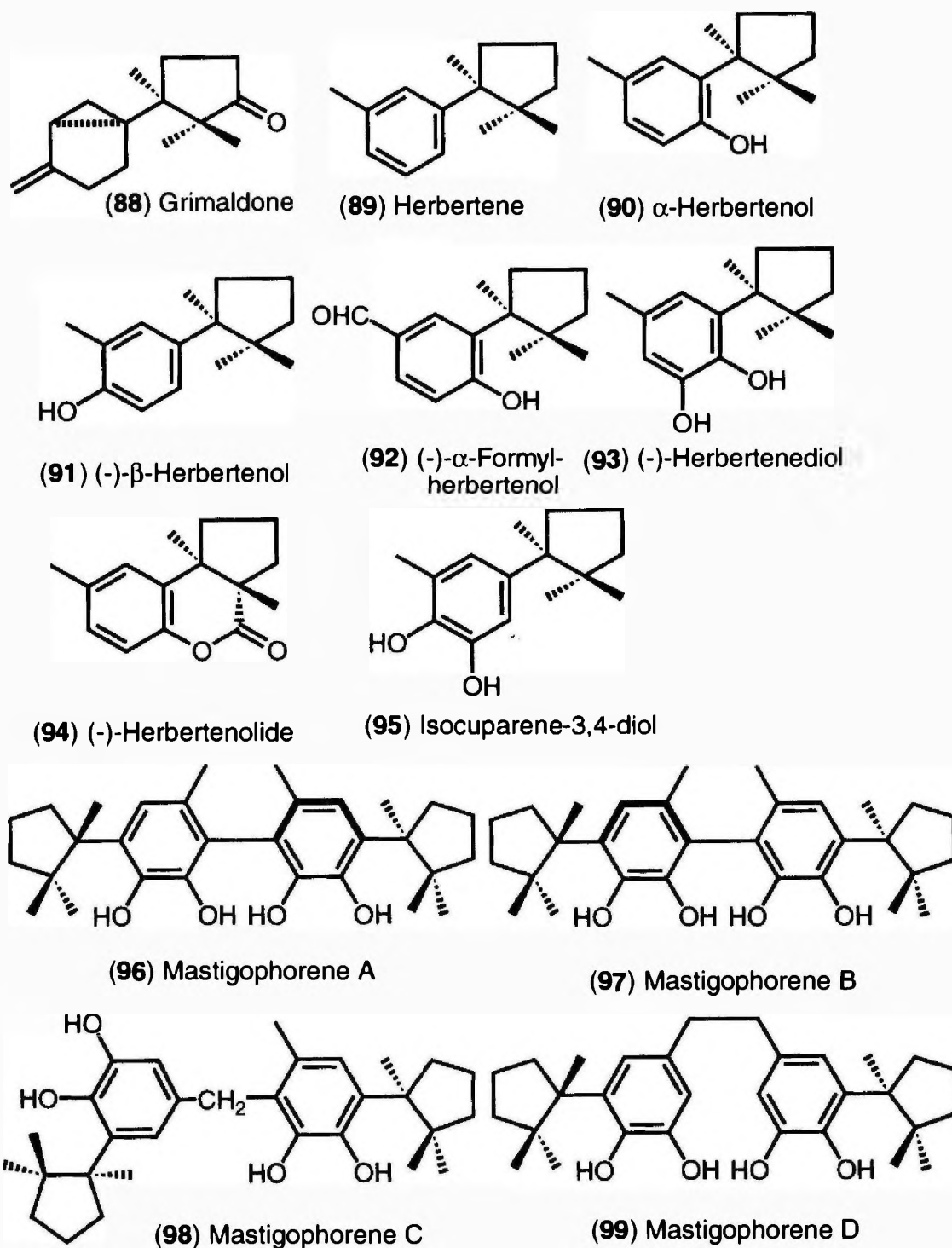


Chart 9. Cuparane and herbertanes (isocuparanes) found in the Hepaticae

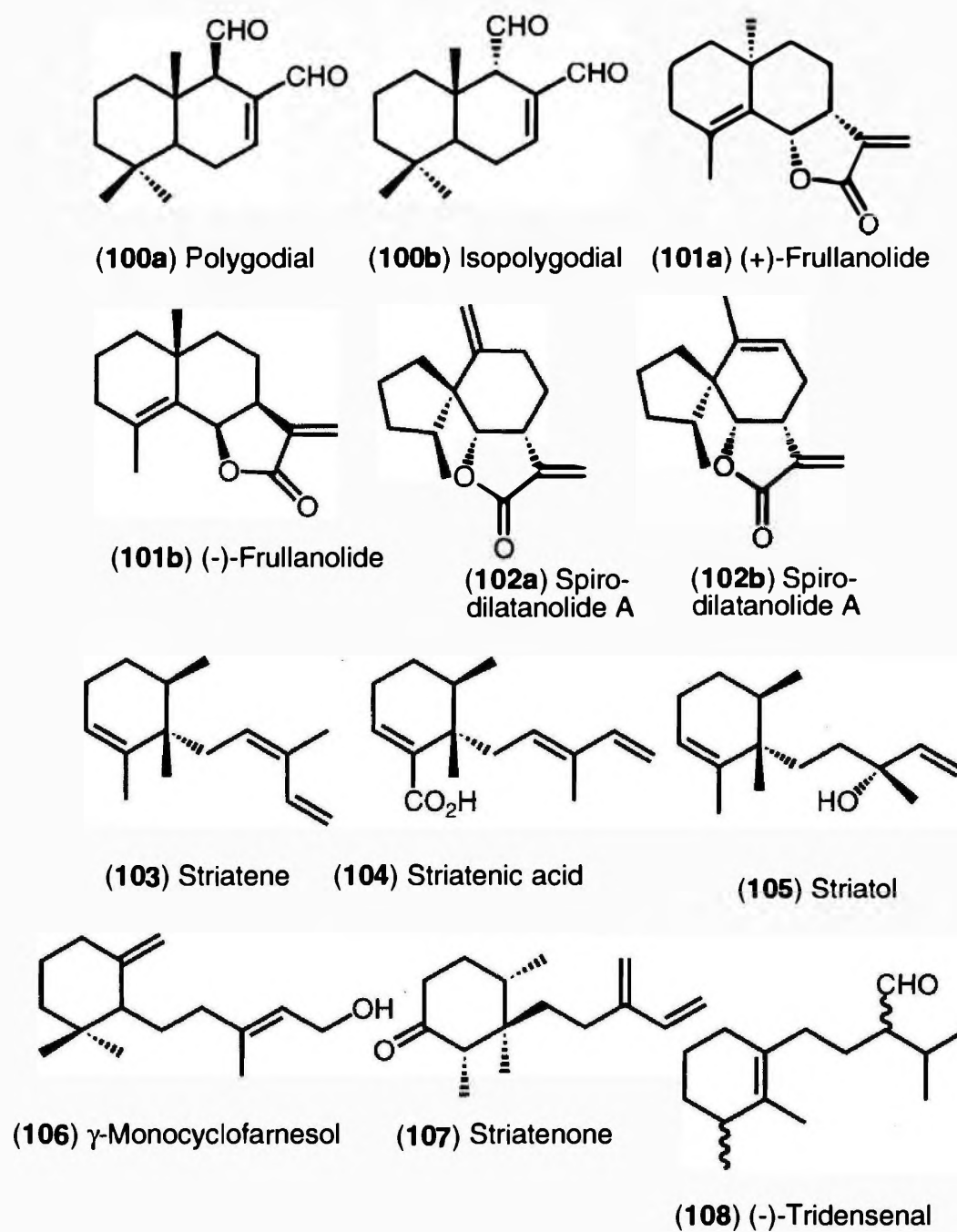
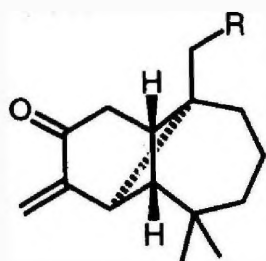
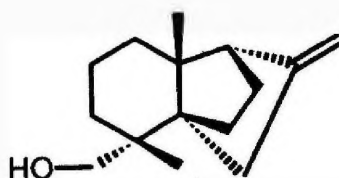


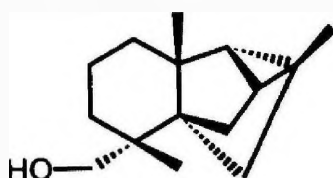
Chart 10. Drimanes, eudesmanes and monocyclofarnesanes found in the Hepaticae



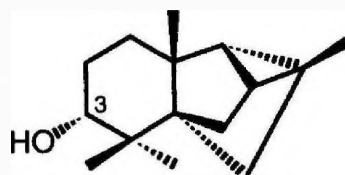
(109) Marsupellone; R=H
(110) Acetoxymarsupellone; R=OAc



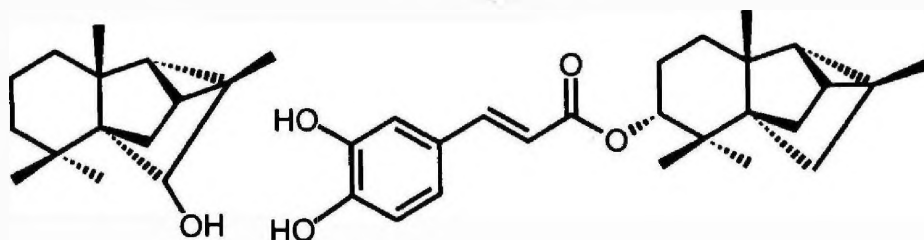
(111) Myltaylenol
(=Myltayl-4(12)-en-15-ol)



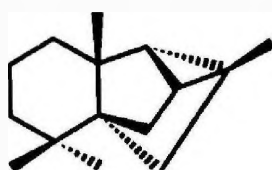
(112) Cyclomyltaylenol



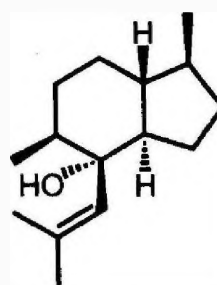
(113) Cyclomyltaylane-3-ol



(114) Cyclomyltaylane-5-ol (115) Cyclomyltayl-3-caffeate

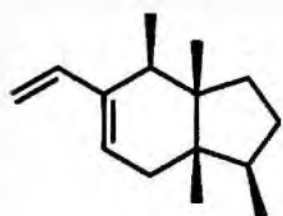


(116) Cyclomyltaylane
(=Tridensene)

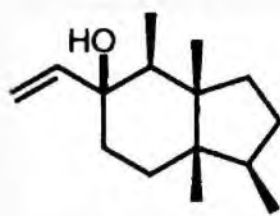


(117) Tamariscol

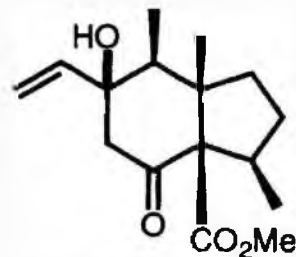
Chart 11. Longipinanes, myltaylanes, cyclomyltaylanes and pacifigorgiane found in the Hepaticae



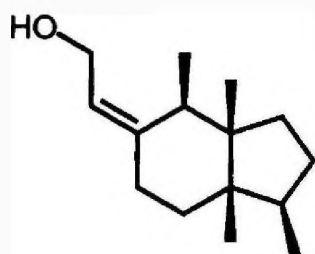
(118) α-Pinguisene



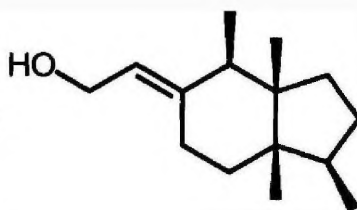
(119) Pinguisenol



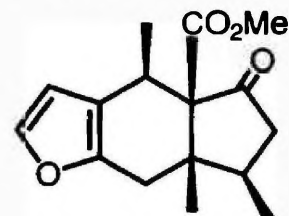
(120) 7-Keto-8-carbomethoxy-pinguisenol



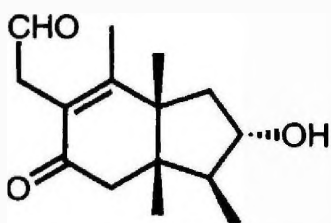
(121) Naviculol



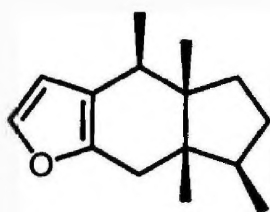
(122) Isonaviculol



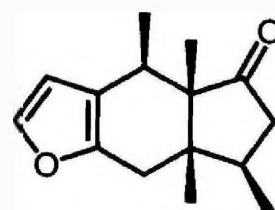
(123) Pinguisone methyl ester



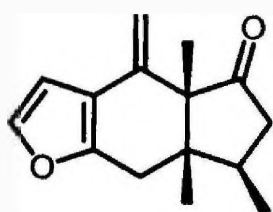
(124) Porella-pinguisenone



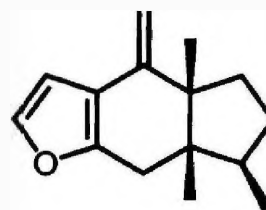
(125) Deoxopinguisone



(126) Pinguisone

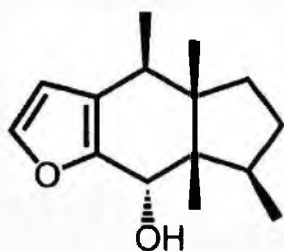


(127) Dehydropinguisone

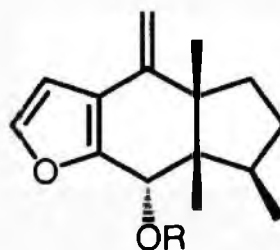


(128) Dehydrodeoxopinguisone
(=Pinguisenene)

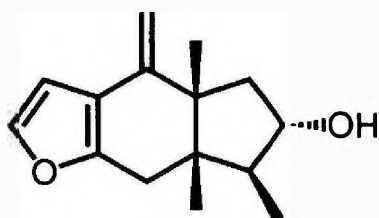
Chart 12a. Pinguisanes found in the Hepaticae



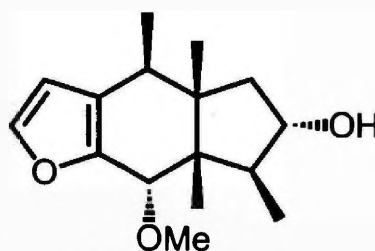
(129) Furanopinguisanol



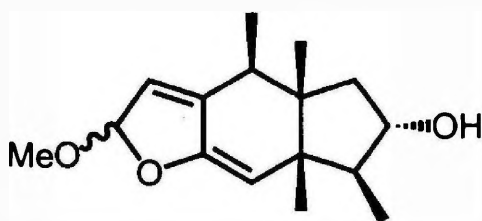
(130) Dehydropinguisenol ; R=H
(131) Dehydropinguisenol methyl ether;
R=Me



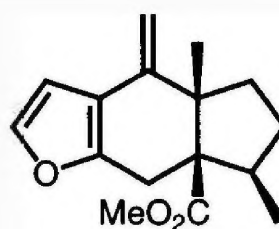
(132) 2-Hydroxypinguisenene



(133) 2-Hydroxy-7-methoxy-
deoxopinguisone

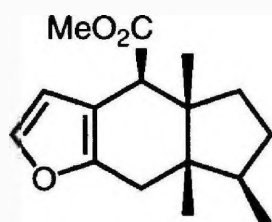


(134) 2-Hydroxy-11 ζ -methoxy-
pinguis-5(10),6-diene

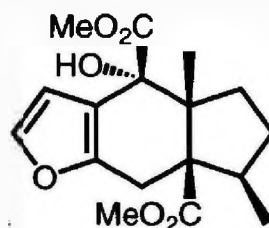


(135) Pinguisenene
methyl ester
(=Bryopterin A)

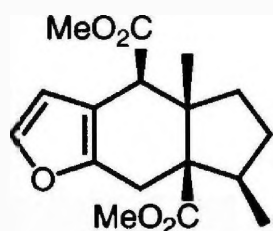
Chart 12b. Pinguisanes found in the Hepaticae



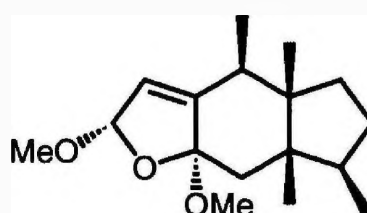
(136) Deoxopinguisone methyl ester



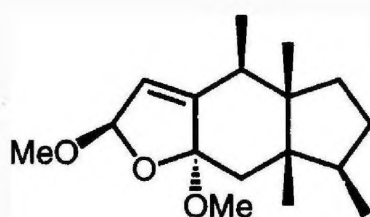
(137) 4-Hydroxydeoxopinguisone-12,15-dimethyl ester
(=Bryopterin C)



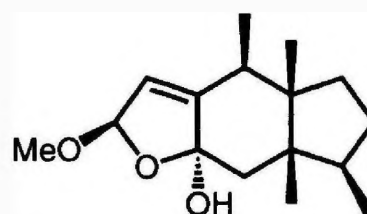
(138) Deoxopinguisone-12,15-dimethyl ester
(=Bryopterin B)



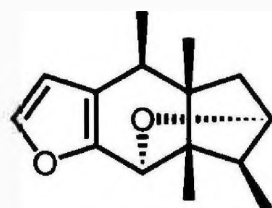
(139) 6 α ,11 α -Dimethoxy-pinguis-5(10)-ene



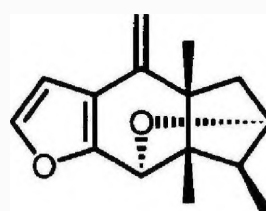
(140) 6 α ,11 β -Dimethoxy-pinguis-5(10)-ene



(141) Ptychanolactone

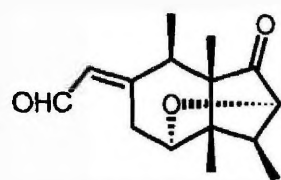


(142) Pinguisanine

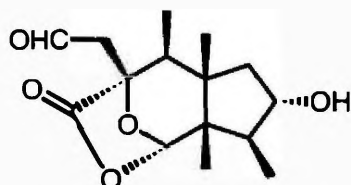


(143) Dehydropinguisanine

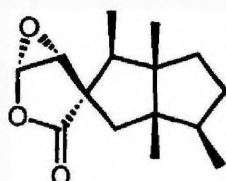
Chart 12c. Pinguisanes found in the Hepaticae



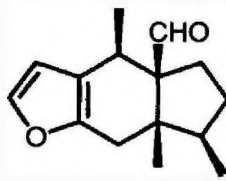
(144) Pinguisenal



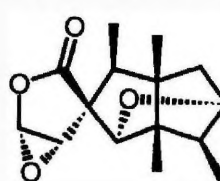
(145) Porellapinguisanolide



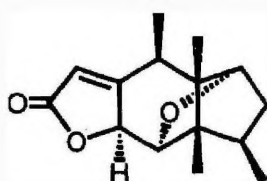
(146) Ptychanolide



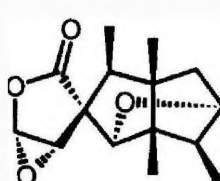
(147)



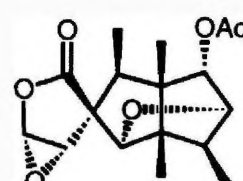
(148) Pinguisanolide



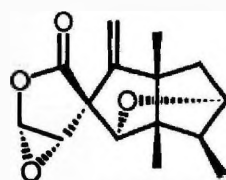
(149)



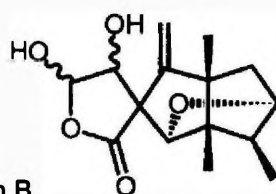
(150) Isopinguisanolide



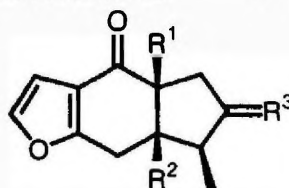
(151) Spirodensifolin A



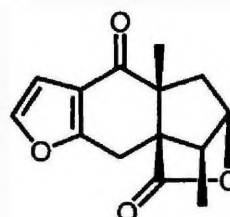
(152) Spirodensifolin B



(153) Spiro-pinguisanin



(154) Norpinguisone ; $R^1=R^2=Me$, $R^3=H_2$

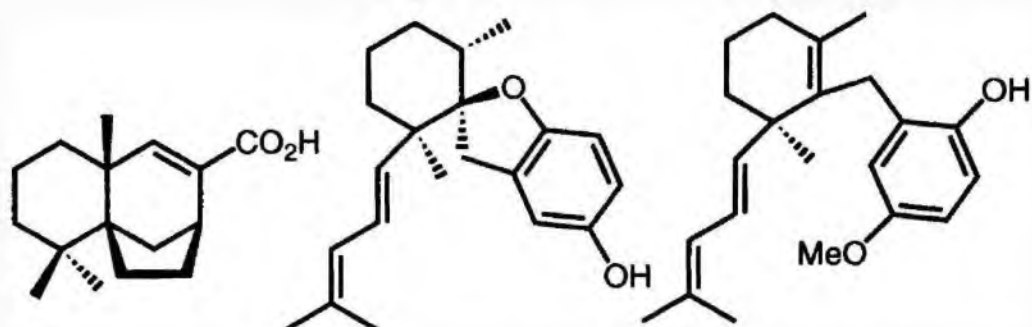


(155) Norpinguisone methyl ester ;

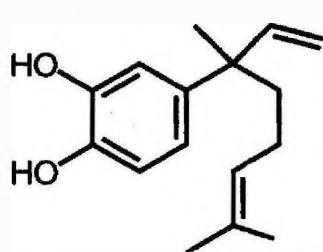
$R^1=Me$, $R^2=CO_2Me$, $R^3=H_2$

(156) Bryopterin D ; $R^1=Me$, $R^2=CO_2Me$, $R^3=O$

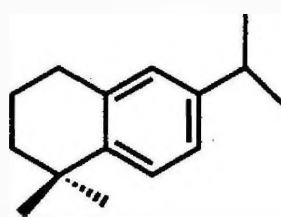
Chart 12d. Pinguisanes and norpinguisanes
found in the Hepaticae



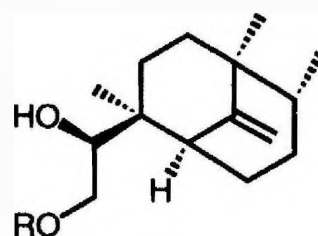
(158) Omphalic acid (159) Riccardiphenol A (160) Riccardiphenol B



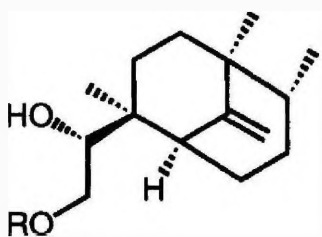
(161) Serpentiphenol



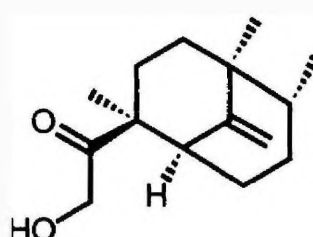
(162) Olivacene



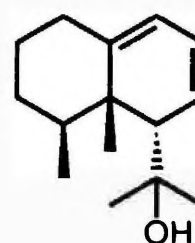
(163) Trifarienol A; R=H
(165) Trifarienol C; R=Ac



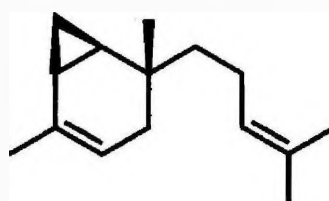
(164) Trifarienol B; R=H
(166) Trifarienol D; R=Ac



(167) Trifarienol E



(168) Rebouliadienol



(169) Chenopodene

Chart 13. Miscellaneous sesquiterpenoids found in the Hepaticae

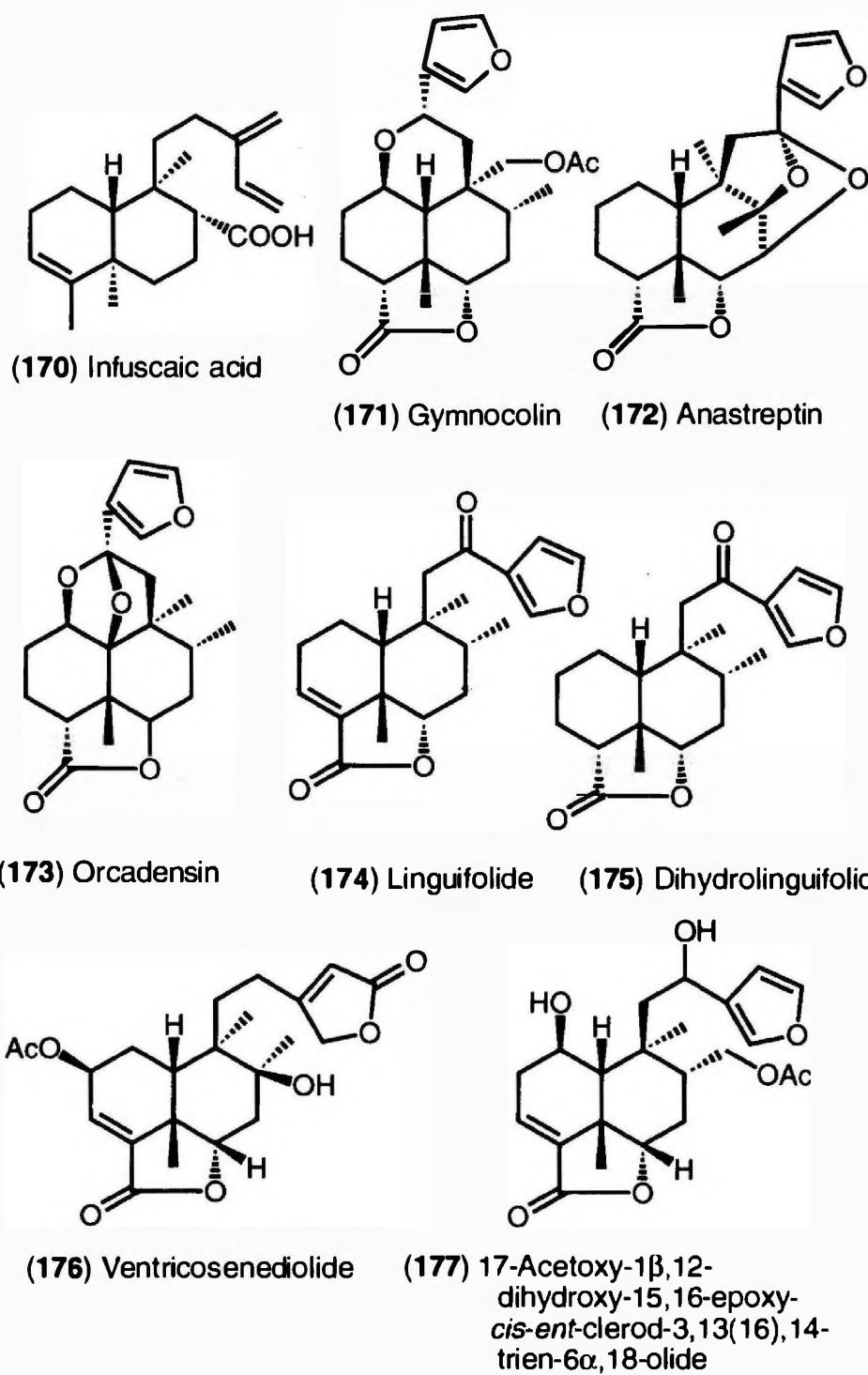
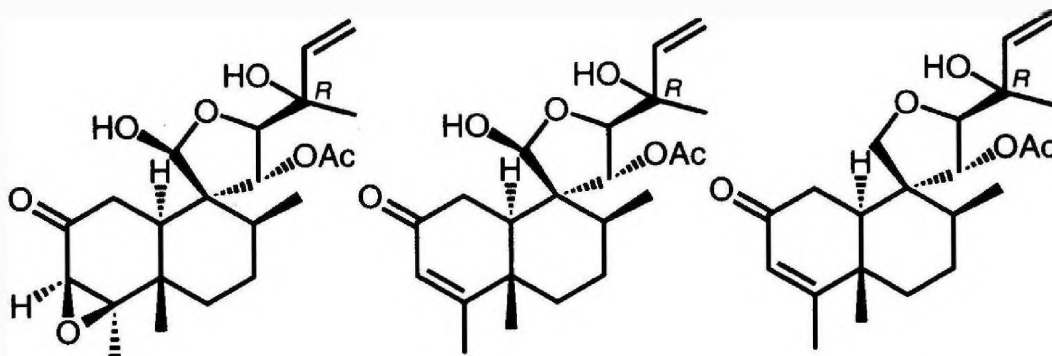
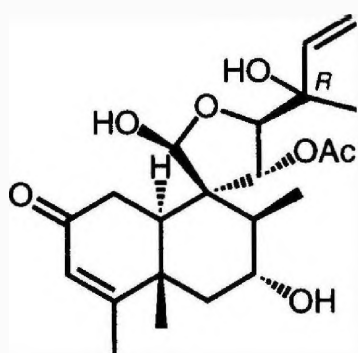


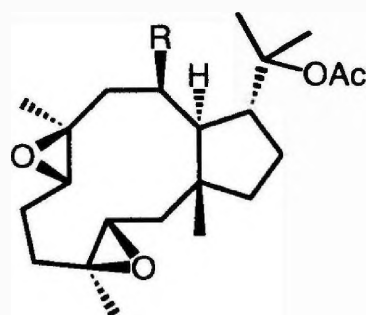
Chart 14. Clerodanes found in the Hepaticae



(178) Heteroscyphone A (179) Heteroscyphone B (180) Heteroscyphone C

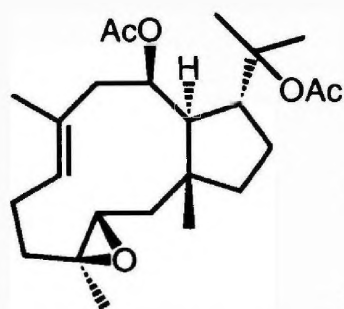


(181) Heteroscyphone D

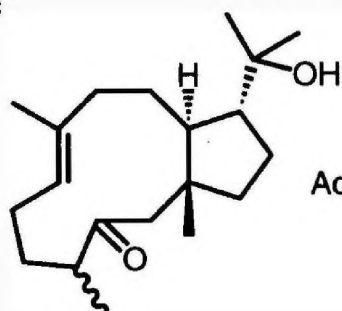


(182) Barbilycopodin (= 10*R*,18-Diacetoxy-3*S*,4*S*,7*S*,8*S*-diepoxydolabellane); R=OAc

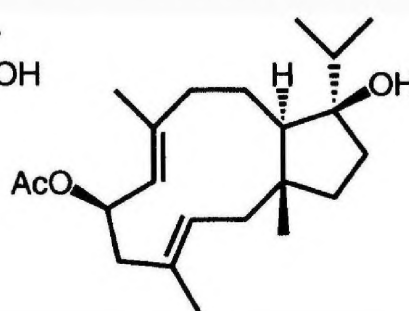
(183) 10-Deacetoxybarbilycopodin (= 18-Acetoxy-3*S*,4*S*,7*S*,8*S*-diepoxydolabellane); R=H



(184) 10*R*,18-Diacetoxy-3*S*,4*S*-epoxydolabell-7*E*-ene

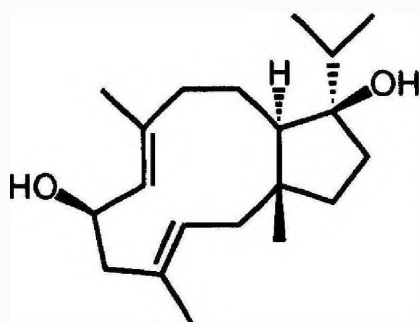


(185) 18-Hydroxydolabell-7*E*-en-3-one

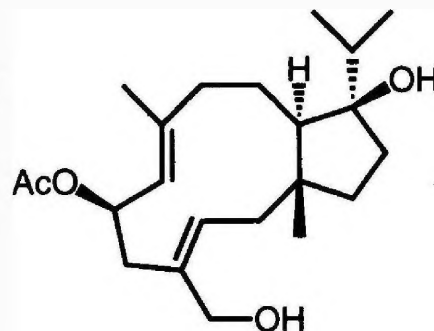


(186) Acetoxydontoschismenol

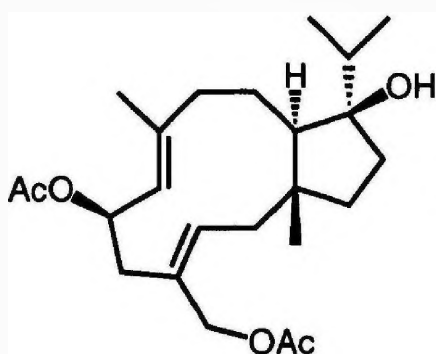
Chart 15. Spiroclerodanes and dolabellanes found in the Hepaticae



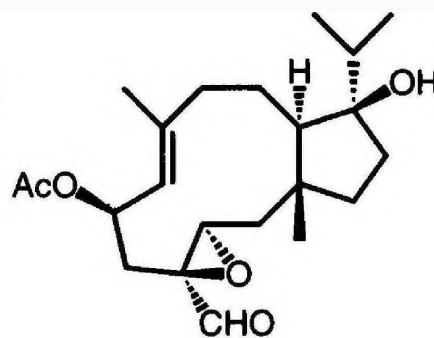
(187) 6,12-Dihydroxydolabella-3*E*,7*E*-diene



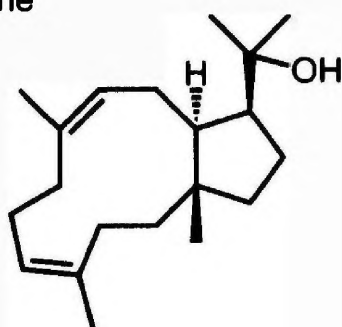
(188) 6-Acetoxy-12,16-dihydroxydolabella-3*E*,7*E*-diene



(189) 6,16-Diacetoxy-12-hydroxydolabella-3*E*,7*E*-diene

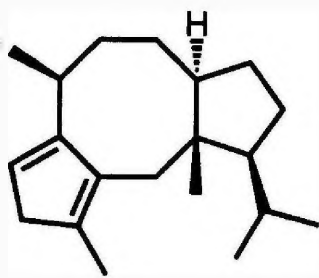


(190) 6-Acetoxy-3,4-epoxy-12-hydroxydolabell-7*E*-en-16-al

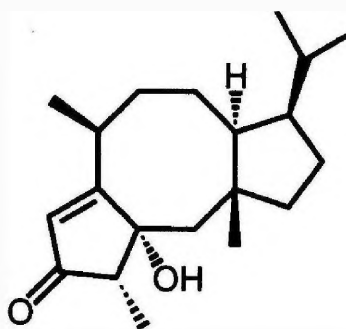


(191) 18-Hydroxy-4,8-dolabelladiene

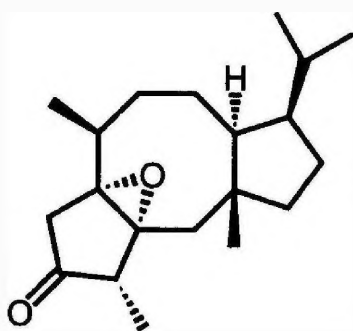
Chart 16. Dolabellanes found in the Hepaticae



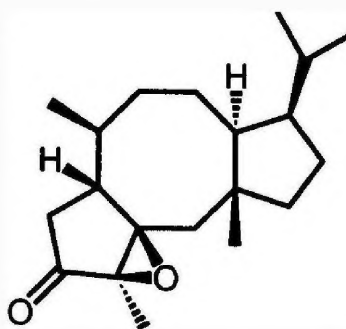
(192) Fusicoccadiene



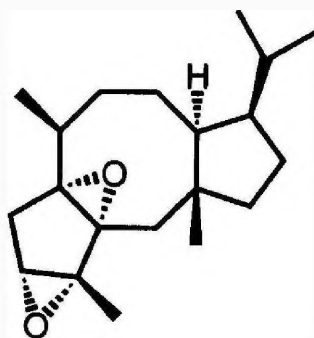
(193) Anadensin



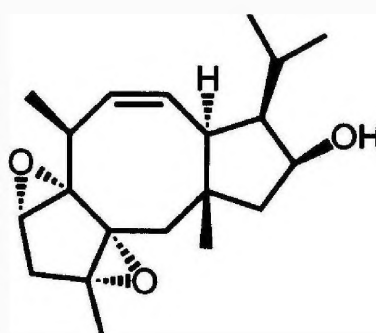
(194) Fusicogigantone A



(195) Fusicogigantone B

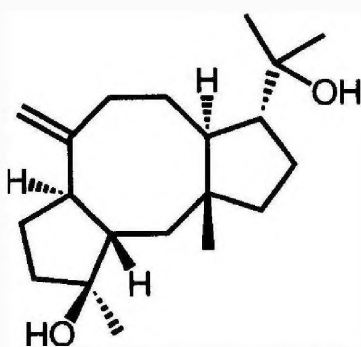


(196) Fusicogigantepoxide

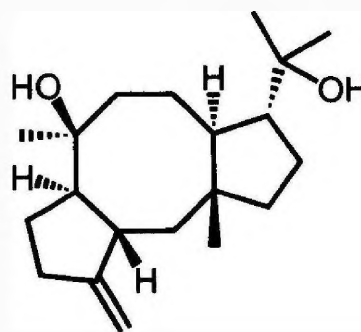


(197) Fusicorrugatol

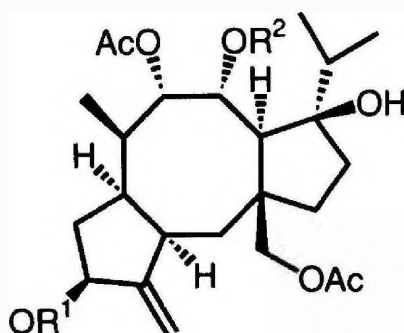
Chart 17a. Fusicoccanes found in the Hepaticae



(198) Barbifusicoccine A

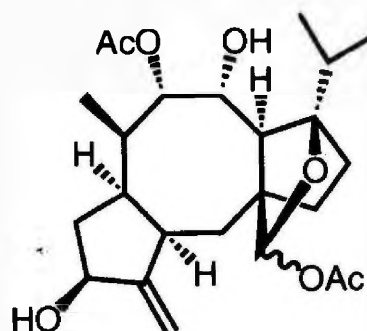


(199) Barbifusicoccine B

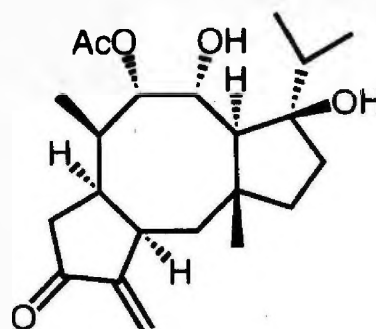


(200) Fusicoplagin A;
 $R^1=R^2=H$

(201) Fusicoplagin B;
 $R^1=H, R^2=Ac$

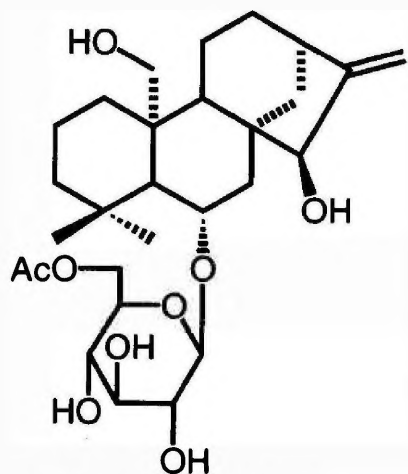


(202) Fusicoplagin C

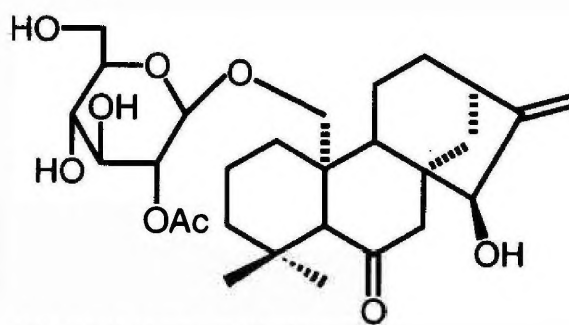


(203) Fusicoplagin D

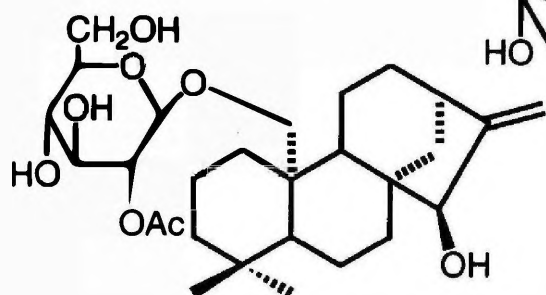
Chart 17b. Fusicoccanes found in the Hepaticae



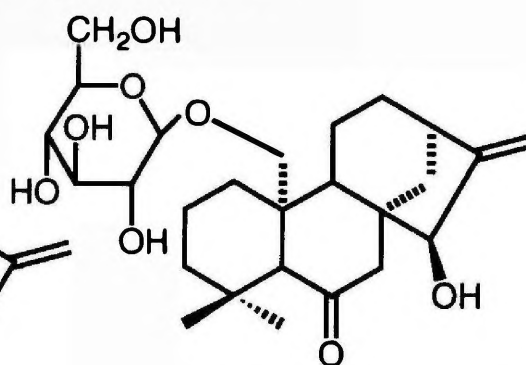
(204) Infuscaside A



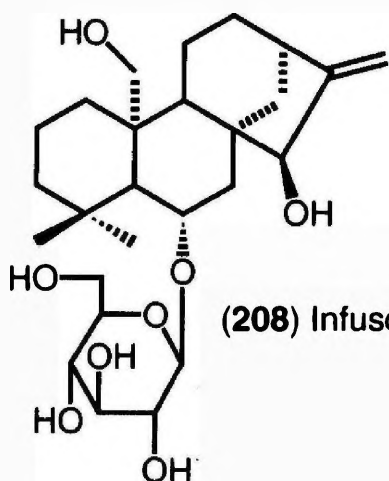
(205) Infuscaside B



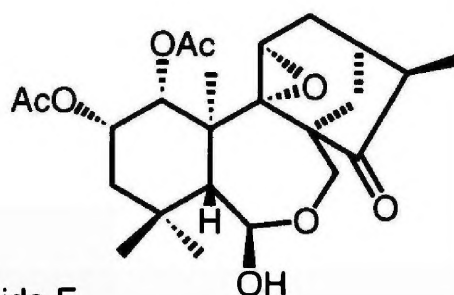
(206) Infuscaside C



(207) Infuscaside D

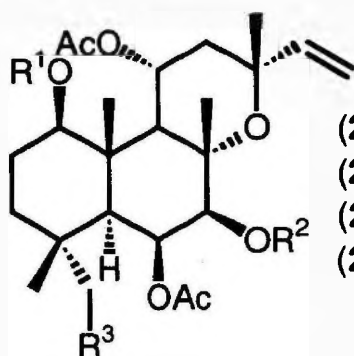


(208) Infuscaside E



(209) Secoexsertifolin A

Chart 18. Kauranes found in the Hepaticae

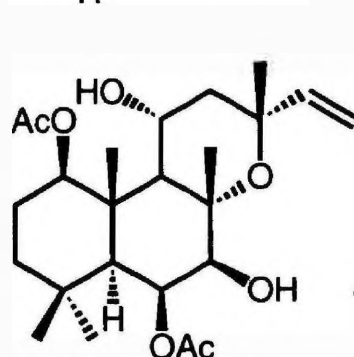


(210) Ptychantin A ; $R^1=R^2=Ac$, $R^3=H$

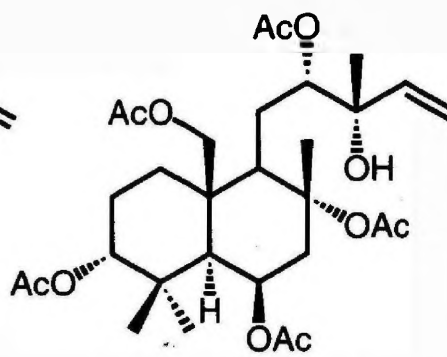
(211) Ptychantin B ; $R^1=Ac$, $R^2=R^3=H$

(212) Ptychantin C ; $R^1=R^2=R^3=H$

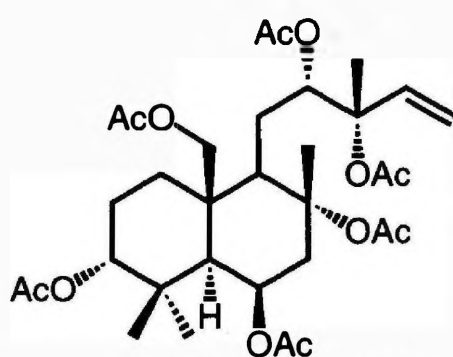
(213) Ptychantin D ; $R^1=Ac$, $R^2=H$, $R^3=OH$



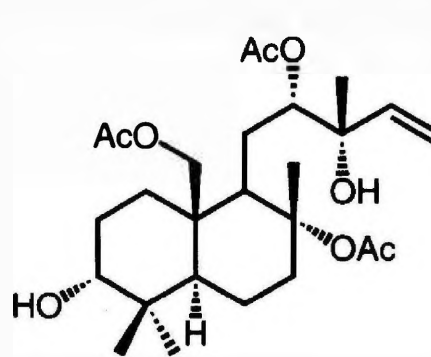
(214) Ptychantin E



(215) Ptychantin F

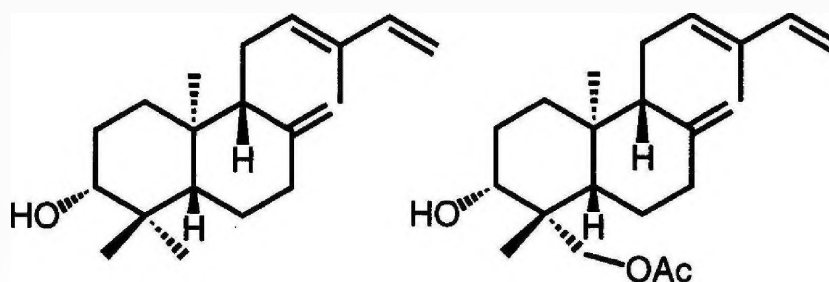


(216) Ptychantin G



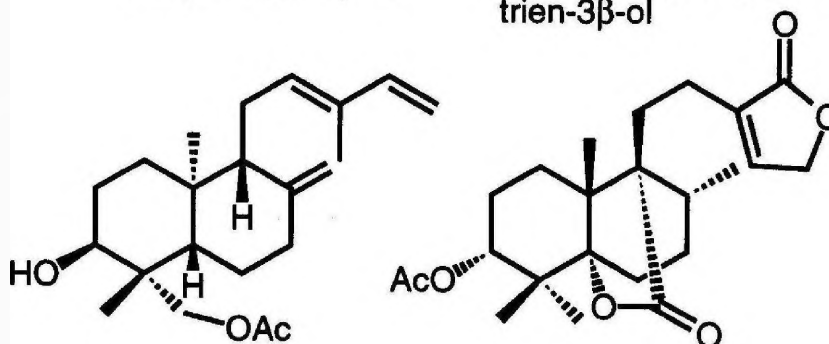
(217) Ptychantin H

Chart 19a. Labdanes found in the Hepaticae



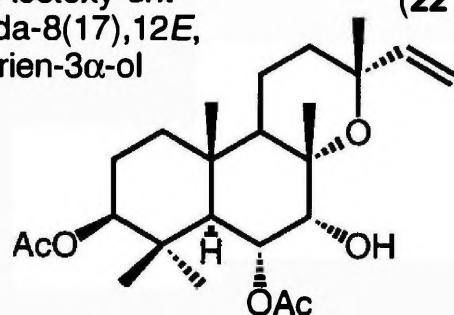
(218) *ent*-Labda-8(17), 12*E*,14-trien-3 β -ol

(219) 19 α -Acetoxy-*ent*-labda-8(17),12*E*,14-trien-3 β -ol



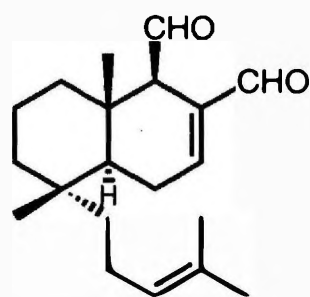
(220) 19 α -Acetoxy-*ent*-labda-8(17),12*E*,14-trien-3 α -ol

(221)

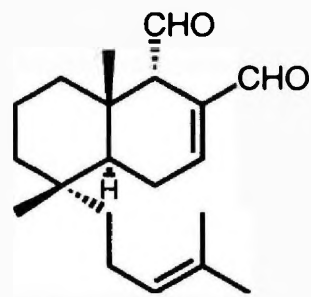


(222) Hamatilobene A
(= (3*S*,6*R*)-Diacetoxy-(7*R*)hydroxy-manoyloxide)

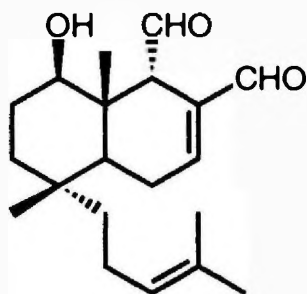
Chart 19b. Labdanes and halimane (221) found in the Hepaticae



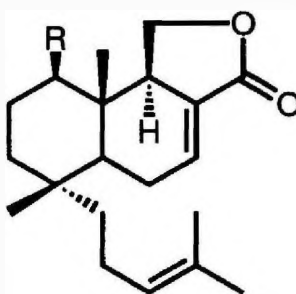
(223) Sacculatal



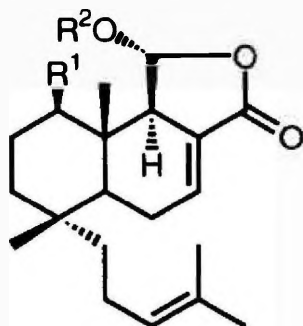
(224) Isosacculatal



(225) 1β-Hydroxyisosacculatal

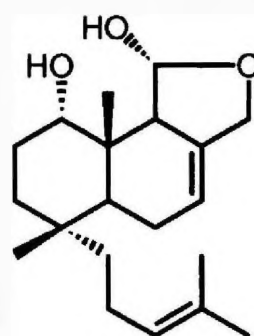


(226) Sacculatanolide ;
R=H



(227) 1β-Hydroxy-
sacculatanolide ;
R=OH

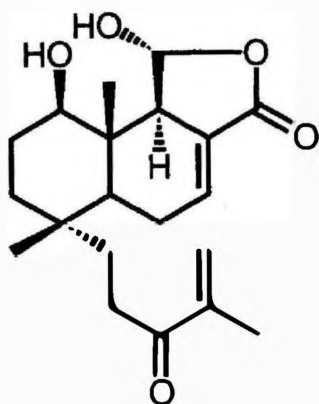
(228) 11α-Hydroxy-
sacculatanolide ;
R¹=R²=H



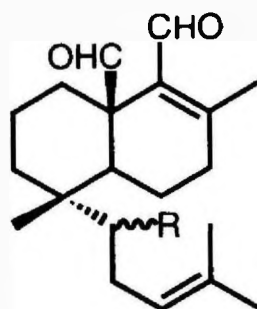
(229) 1β,11α-Dihydroxy-
sacculatanolide ;
R¹=OH, R²=H

(230) 12-Deoxy-1β,
11α-dihydroxy-
sacculatanolide

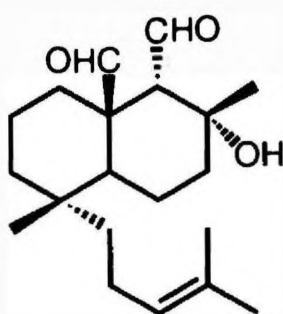
Chart 20a. Sacculatanes found in the Hepaticae



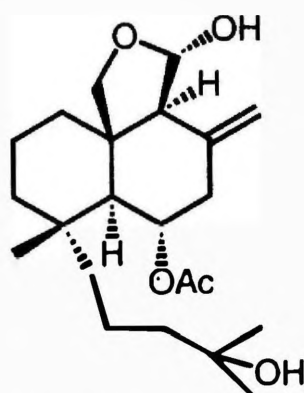
(231) 1 β ,11 α -Dihydroxy-sacculatenolide ;



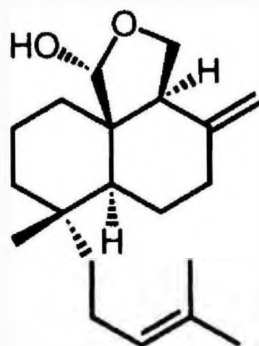
(232) Perrottetianal A ; R=H
(233) Perrottetianal B ; R=OH



(234) 8-Hydroxy-9-hydro-perrottetianal

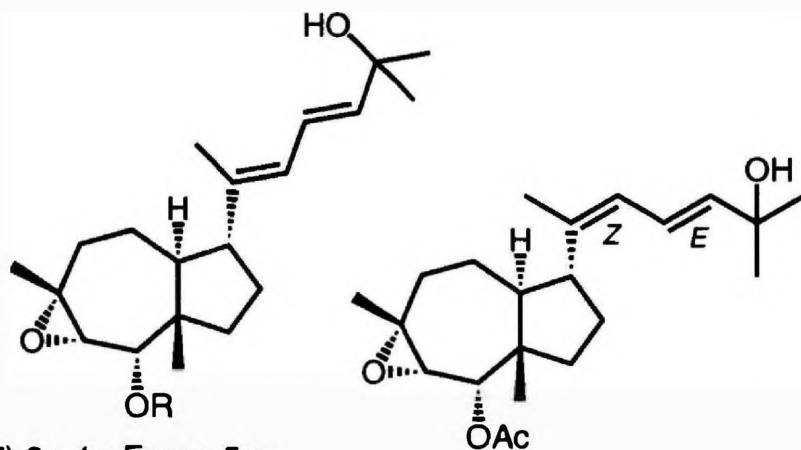


(235) Sacculaplagin



(236) Sacculaporellin

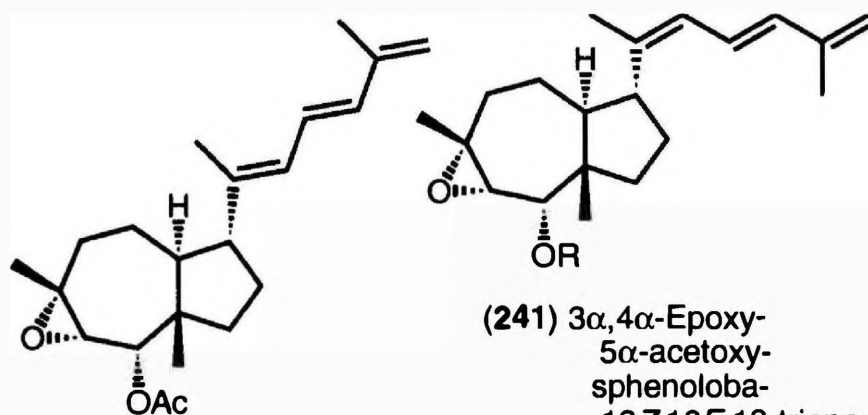
Chart 20b. Sacculatanes found in the Hepaticae



(237) 3 α ,4 α -Epoxy-5 α -
acetoxy-
18-hydroxy-
sphenoloba-
13 E ,16 E -diene ;
R=Ac

(239) 3 α ,4 α -Epoxy-
5 α -acetoxy-
18-hydroxy-
sphenoloba-
13 Z ,16 E -diene

(238) 3 α ,4 α -Epoxy-5 α ,18-
dihydroxysphenoloba-
13 E ,16 E -diene ; R=H



(240) 3 α ,4 α -Epoxy-
5 α -acetoxy-
sphenoloba-
13 E ,16 E ,18-triene

(241) 3 α ,4 α -Epoxy-
5 α -acetoxy-
sphenoloba-
13 Z ,16 E ,18-triene ;
R=Ac

(242) 3 α ,4 α -Epoxy
5 α -hydroxy-
sphenoloba-
13 Z ,16 E ,18-triene ;
R=H

Chart 21. Sphenolobanes found in the Hepaticae

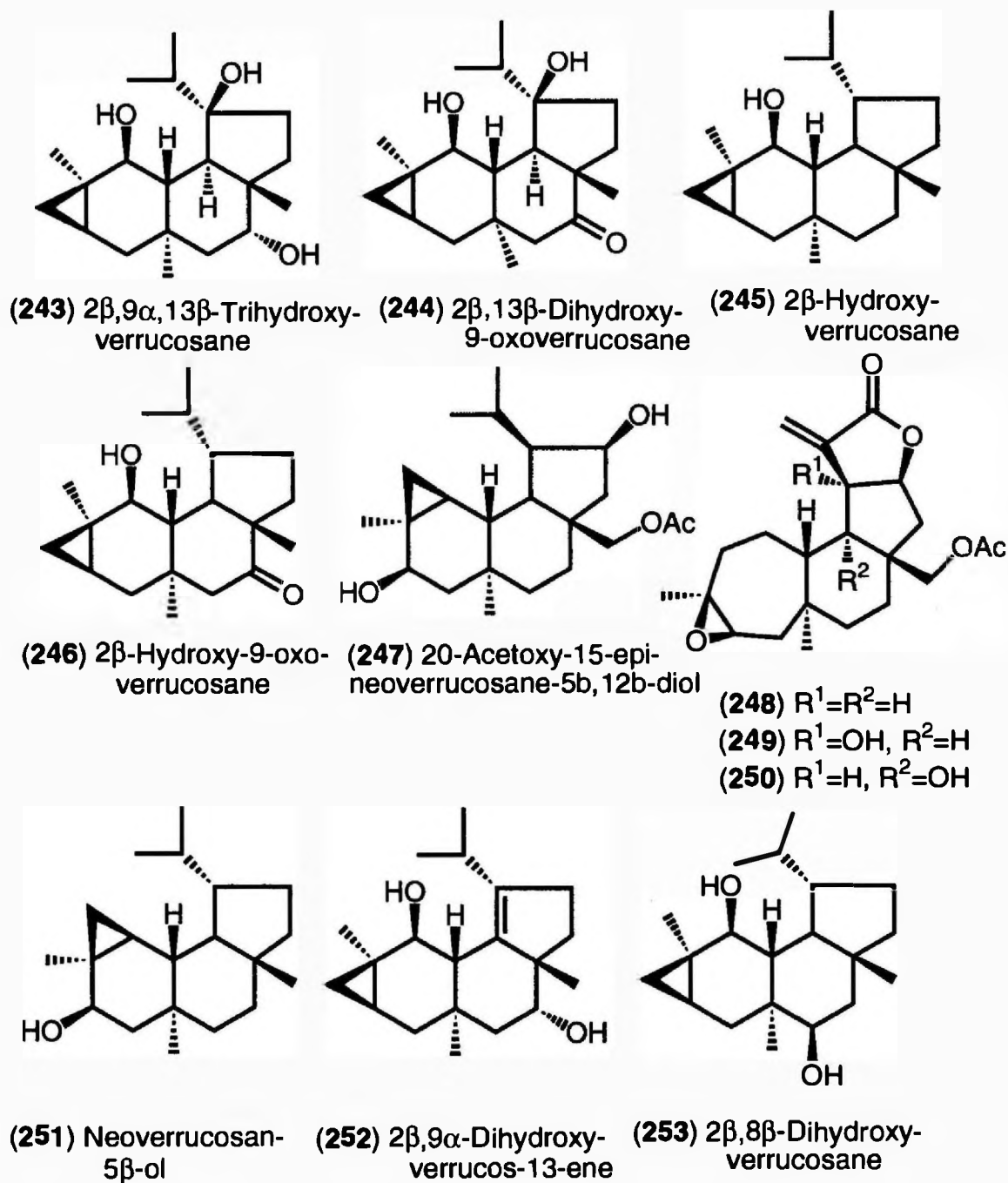
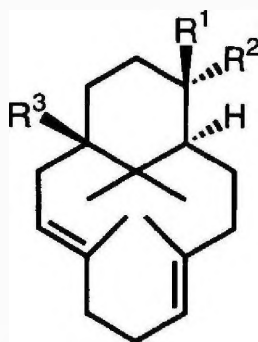


Chart 22. Verrucosanes found in the Hepaticae

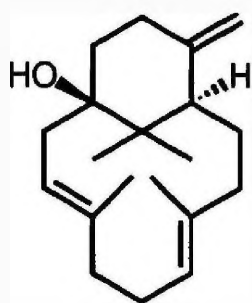


(254a) *ent*-Verticillol ; $R^1=\text{Me}$, $R^2=\text{OH}$, $R^3=\text{H}$

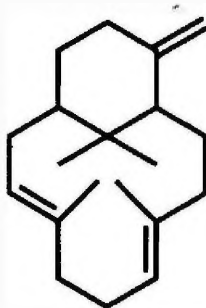
(254b) *ent*-13-*epi*-Verticillol ; $R^1=\text{OH}$, $R^2=\text{Me}$, $R^3=\text{H}$

(255) *ent*-Verticillanediol ; $R^1=\text{Me}$, $R^2=R^3=\text{OH}$

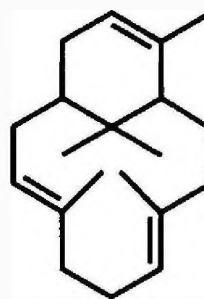
(256) *ent*-13-*epi*-Verticillanediol ; $R^1=R^3=\text{OH}$, $R^2=\text{Me}$



(257) *ent*-Iso-verticillenol



(258) Verticillene



(259) Isoverticillene

Chart 23. Verticillanes found in the Hepaticae

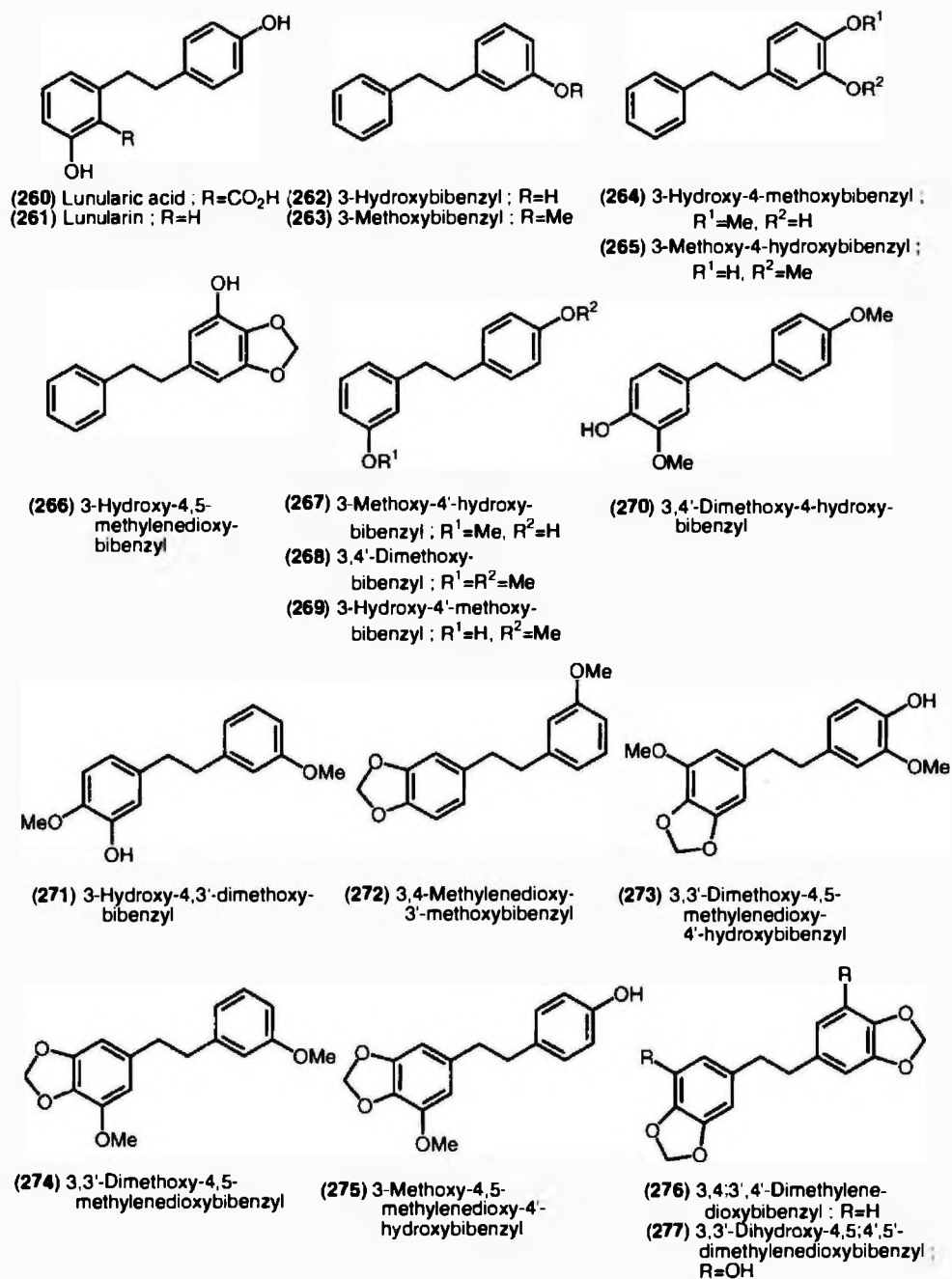
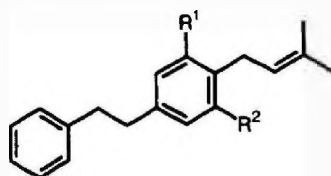
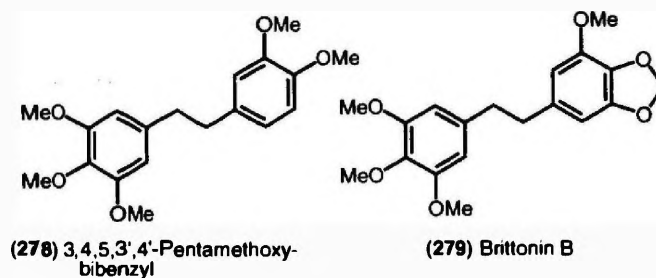
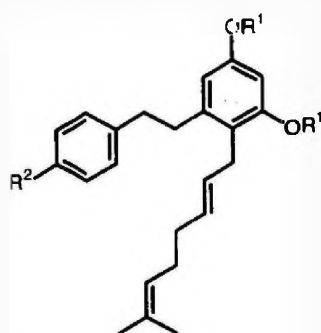


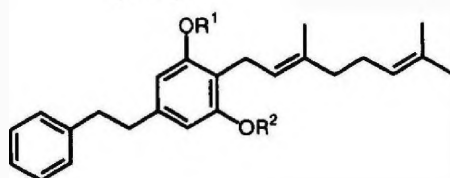
Chart 24a. Bibenzyls found in the Hepaticae



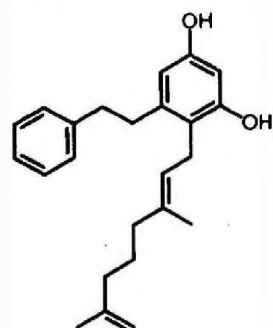
- (280) 3-Hydroxy-5-methoxy-4-(3-methyl-2-butenyl)biphenyl ; $R^1=OH$, $R^2=OMe$
 (281) 3,5-Dihydroxy-4-(3-methyl-2-butenyl)biphenyl ; $R^1=R^2=OH$
 (282) 3,5-Dimethoxy-4-(3-methyl-2-butenyl)biphenyl ; $R^1=R^2=OMe$



- (283) 2-Geranyl-3,5-dihydroxybiphenyl ; $R^1=R^2=H$
 (284) 2-Geranyl-3,5-dimethoxybiphenyl ; $R^1=Me$, $R^2=H$
 (285) 2-Geranyl-3,5,4'-trihydroxybiphenyl ; $R^1=H$, $R^2=OH$

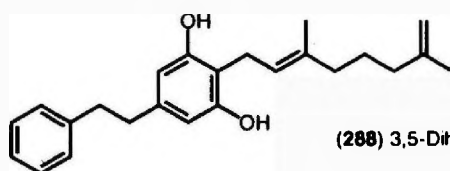


- (286) 3,5-Dihydroxy-4-geranylbiphenyl (=3,5-Dihydroxy-4-(3,7-dimethyl-2,6-octadienyl) biphenyl) ; $R^1=R^2=H$

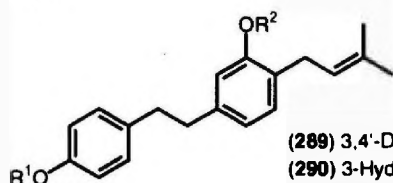


- (287) 2-(3,7-Dimethyl-2,7-octadienyl)-3,5-dihydroxybiphenyl

Chart 24b. Bibenzyls found in the Hepaticae



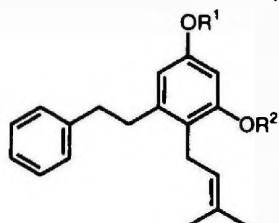
(288) 3,5-Dihydroxy-4-(3,7-dimethyl-2,7-octadienyl) bibenzyl



(289) 3,4'-Dihydroxy-4-(3-methyl-2-butenyl)biphenyl ; $R^1=R^2=H$

(290) 3-Hydroxy-4-(3-methyl-2-butenyl)-4'-methoxybiphenyl ; $R^1=Me$, $R^2=H$

(291) 3-Methoxy-4-(3-methyl-2-butenyl)-4'-hydroxybiphenyl ; $R^1=H$, $R^2=Me$

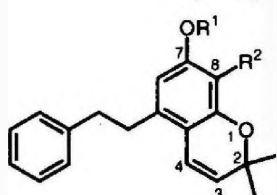


(292) 3, 5-Dihydroxy-2-(3-methyl-2-butenyl) biphenyl ; $R^1=R^2=H$

(293) 3-Methoxy-5-hydroxy-2-(3-methyl-2-butenyl) biphenyl ; $R^1=H$, $R^2=Me$

(294) 3-Hydroxy-5-methoxy-2-(3-methyl-2-butenyl)biphenyl ; $R^1=Me$, $R^2=H$

(295) 3, 5-Dimethoxy-2-(3-methyl-2-butenyl) biphenyl ; $R^1=R^2=Me$



(296) 2,2-Dimethyl-7-hydroxy-5-(2-phenylethyl)chromene ;

$R^1=R^2=H$

(297) 2,2-Dimethyl-7-methoxy-5-(2-phenylethyl)chromene ;

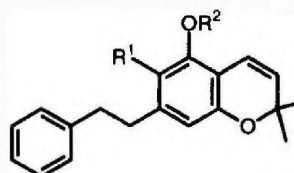
$R^1=Me$, $R^2=H$

(298) 2,2-Dimethyl-7,8-dihydroxy-5-(2-phenylethyl)chromene ;

$R^1=H$, $R^2=OH$

(299) 2,2-Dimethyl-7,8-dimethoxy-5-(2-phenylethyl)chromene ;

$R^1=Me$, $R^2=OMe$



(300) 2,2-Dimethyl-5-hydroxy-7-(2-phenylethyl)chromene ;

$R^1=R^2=H$

(301) 2,2-Dimethyl-5-methoxy-7-(2-phenylethyl)chromene ;

$R^1=H$, $R^2=Me$

(302) 2,2-Dimethyl-5-hydroxy-6-carboxy-7-(2-phenylethyl)-

chromene ; $R^1=CO_2H$, $R^2=H$

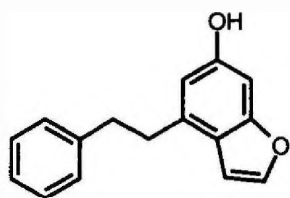


(303) 2(S)-2-Methyl-2-(4-methyl-3-pentenyl)-7-hydroxy-5-(2-phenylethyl)chromene ; $R^1=R^2=H$

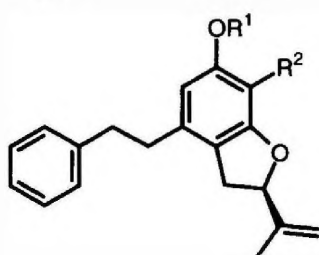
(304) 2(S)-2-Methyl-2-(4-methyl-3-pentenyl)-7-methoxy-5-(2-phenylethyl)chromene ; $R^1=H$, $R^2=Me$

(305) 2(S)-2-Methyl-2-(4-methyl-3-pentenyl)-6-carboxy-7-hydroxy-5-(2-phenylethyl)chromene ; $R^1=CO_2H$, $R^2=H$

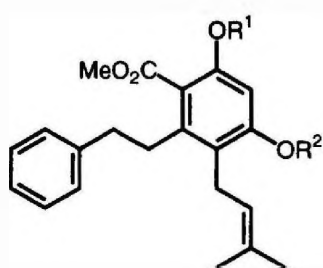
Chart 24c. Bibenzyls found in the Hepaticae



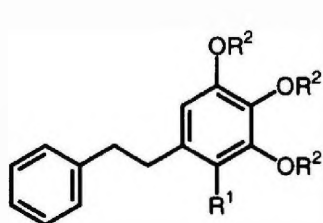
(306) 6-Hydroxy-4-(2-phenylethyl)benzofuran



(307) 2-(*R*)-Isopropenyl-6-hydroxy-4-(2-phenylethyl)dihydrobenzofuran ; $R^1=R^2=H$
(308) Perrottetin D ; $R^1=H$, $R^2=OH$



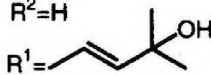
(309) 3, 5-Dihydroxy-6-carbomethoxy-2-(3-methyl-2-butenyl)biphenyl ; $R^1=R^2=H$
(310) 3, 5-Dimethoxy-6-carbomethoxy-2-(3-methyl-2-butenyl)biphenyl ; $R^1=R^2=Me$



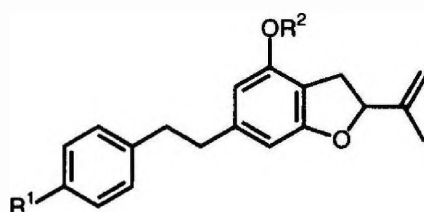
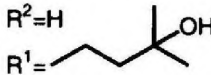
(311) Perrottetin A ;



(312) Perrottetin B ;

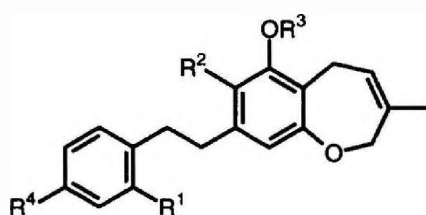


(313) Perrottetin C ;



(314) Tylimanthin A ; $R^1=H$, $R^2=Me$
(315) Tylimanthin B ; $R^1=R^2=H$
(316) Tylimanthin C ; $R^1=OMe$, $R^2=Me$

Chart 24d. Bibenzyls found in the Hepaticae



(317) Radulanin A ; $R^1=R^2=R^3=R^4=H$

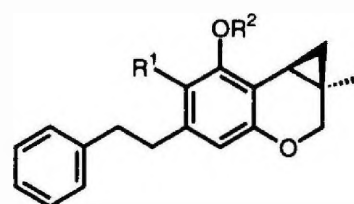
(318) Radulanin B ; $R^1=R^2=R^4=H$, $R^3=Me$

(319) Radulanin C ; $R^1=R^2=R^3=H$, $R^4=OH$

(320) Radulanin H ; $R^1=R^3=R^4=H$, $R^2=CO_2H$

(321) 4'-Hydroxyradulanin H ; $R^1=R^3=H$,
 $R^2=CO_2H$, $R^4=OH$

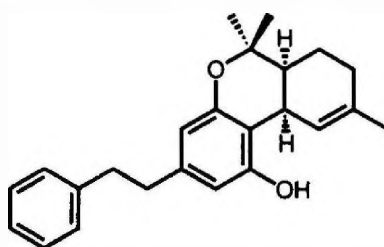
(322) Radulanin L ; $R^1=OH$, $R^2=R^3=R^4=H$



(323) Radulanin I (=3-Methyl-6-hydroxy-8-(2-phenylethyl)-3, 5-cyclopropanochroman) ;
 $R^1=R^2=H$

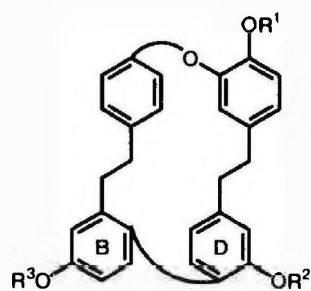
(324) Radulanin J (=3-Methyl-6-methoxy-8-(2-phenylethyl)-3, 5-cyclopropanochroman) ;
 $R^1=H$, $R^2=Me$

(325) Radulanin K (=3-Methyl-6-hydroxy-7-carboxy-8-(2-phenylethyl)-3, 5-cyclopropanochroman) ; $R^1=CO_2H$, $R^2=H$

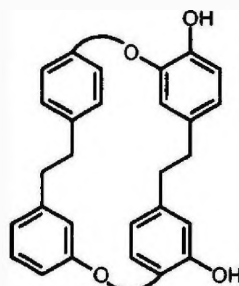


(326) Perrottetinene

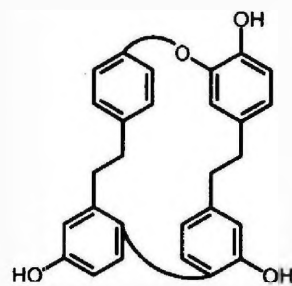
Chart 24e. Bibenzyls found in the Hepaticae



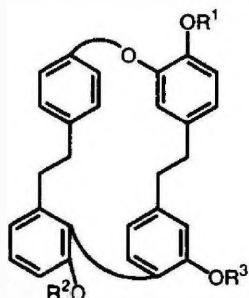
(327) Riccardin A ;
 $R^1=R^2=H, R^3=Me$
 (328) Riccardin F ;
 $R^1=Me, R^2=R^3=H$



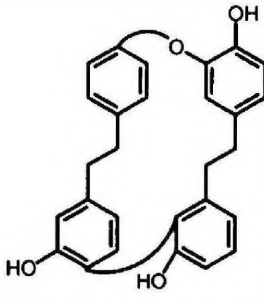
(329) Riccardin B ; $R=H$



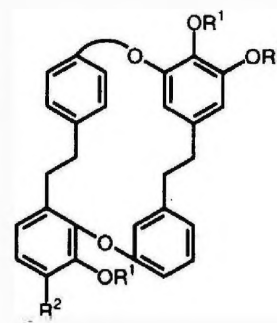
(330) Riccardin C



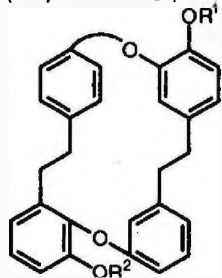
(331) Riccardin D ; $R^1=R^2=R^3=H$
 (332) Riccardin E ; $R^1=R^3=H, R^2=Me$
 (333) Riccardin G ; $R^1=Me, R^2=R^3=H$



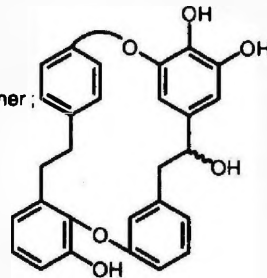
(334) Isoriccardin C ;



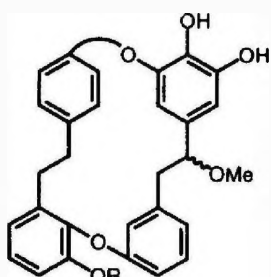
(335) Marchantin A ; $R^1=R^2=H$
 (336) $R^1=Me, R^2=H$
 (337) Marchantin B ; $R^1=, R^2=OH$



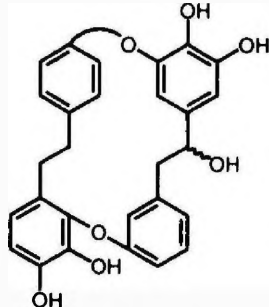
(338) Marchantin C ; $R^1=R^2=H$
 (339) Marchantin C dimethyl ether ;
 $R^1=R^2=Me$
 (340) Marchantin C monomethyl ether
 (= Marchantin O) ;
 $R^1=H, R^2=Me$
 (341) Marchantin P ;
 $R^1=Me, R^2=H$



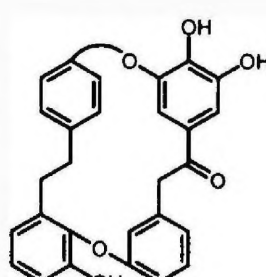
(342) Marchantin D



(343) Marchantin E



(344) Marchantin F



(345) Marchantin G

Chart 25a. Bis-benzyls found in the Hepaticae

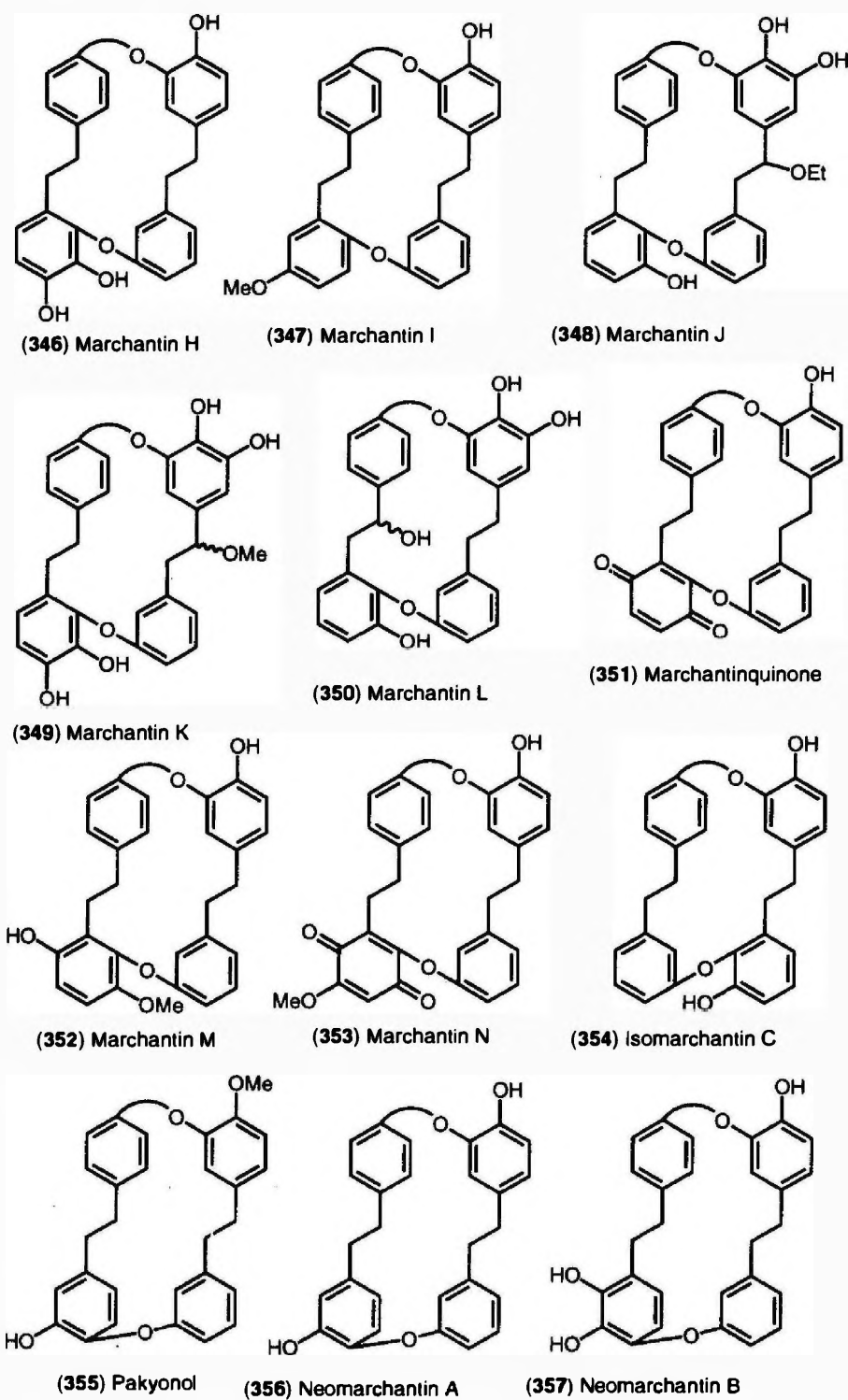


Chart 25b. Bis-benzyls found in the Hepaticae

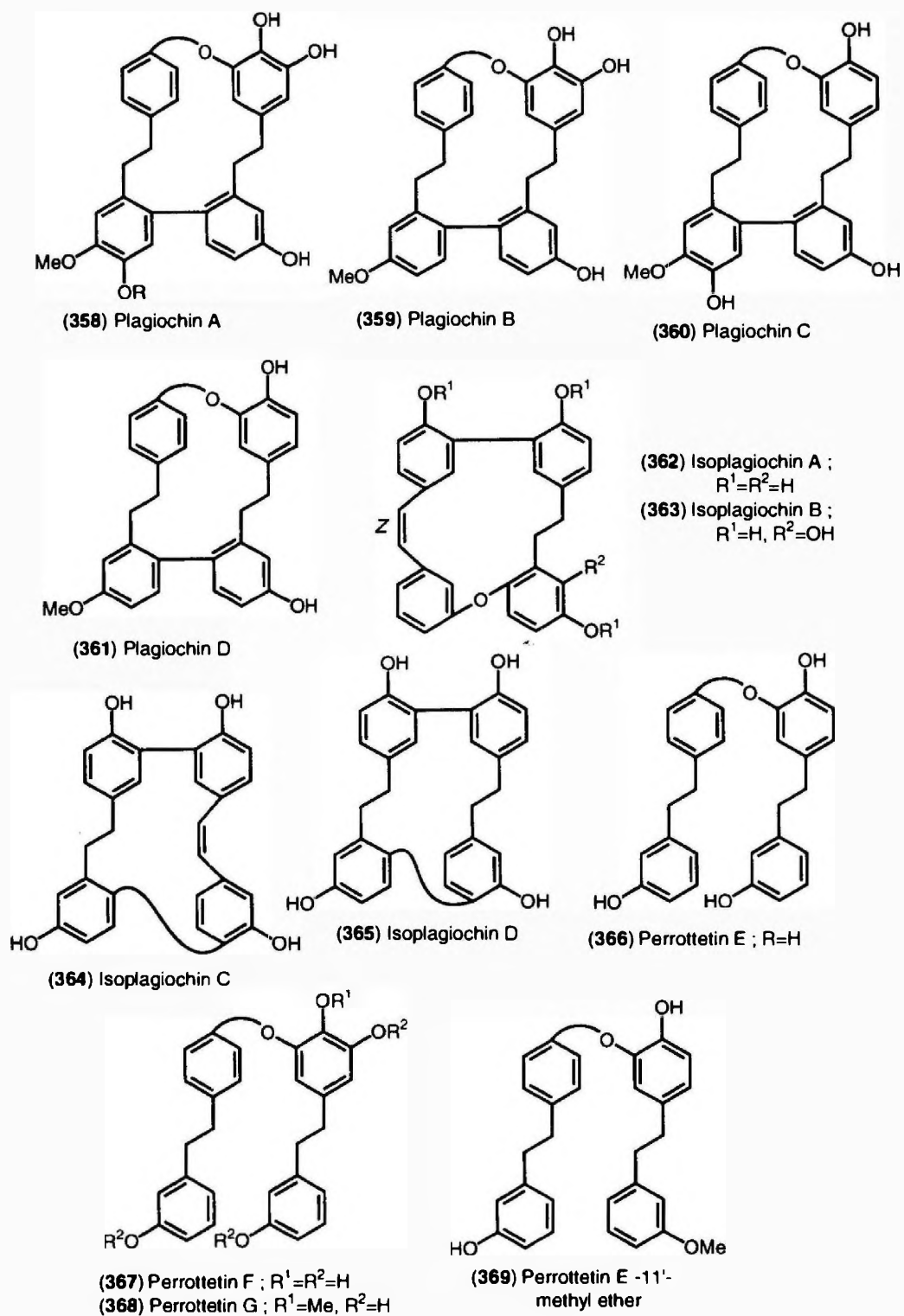


Chart 25c. Bis-benzyls found in the Hepaticae

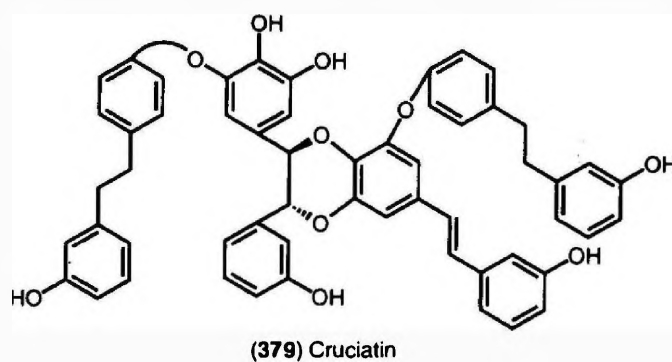
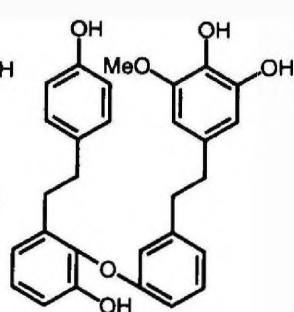
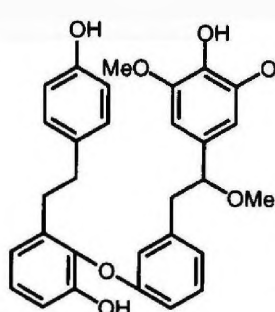
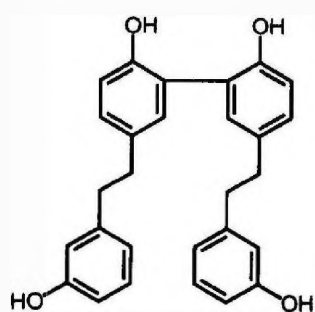
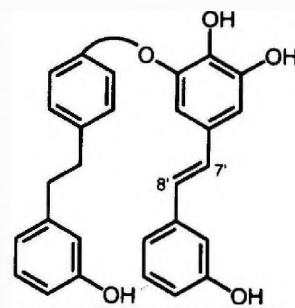
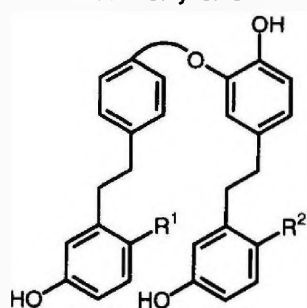
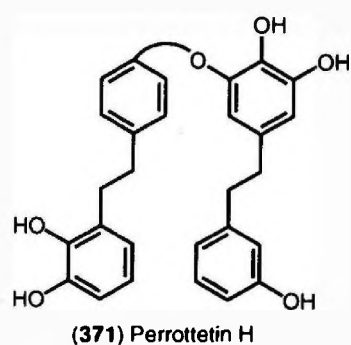


Chart 25d. Bis-bibenzyls found in the Hepaticae

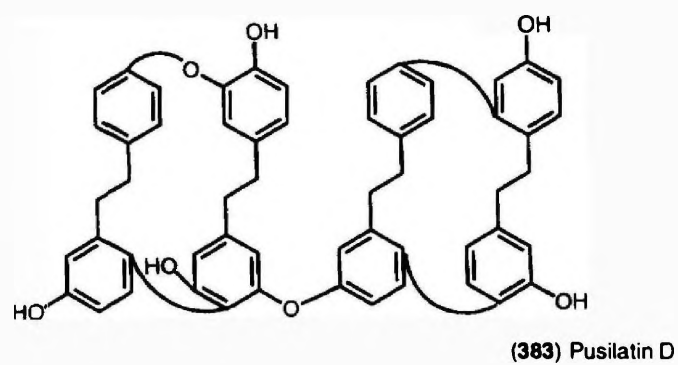
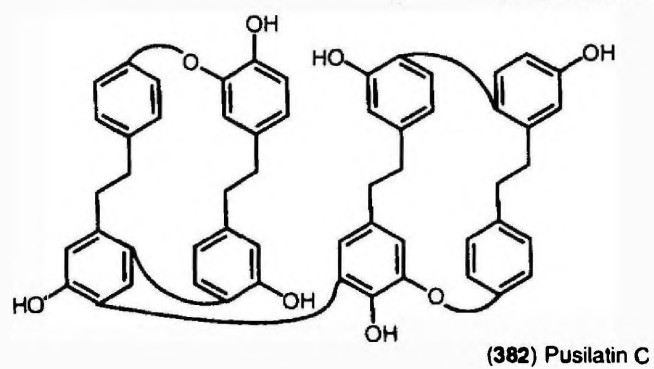
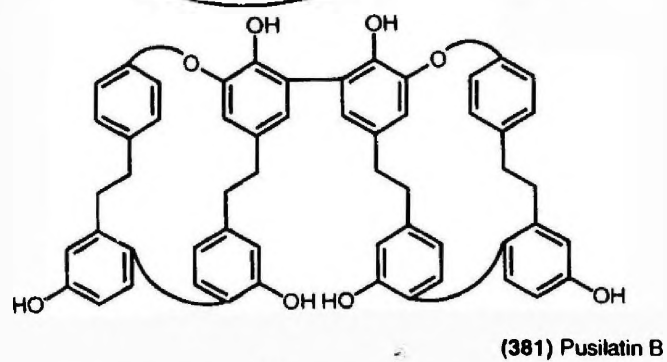
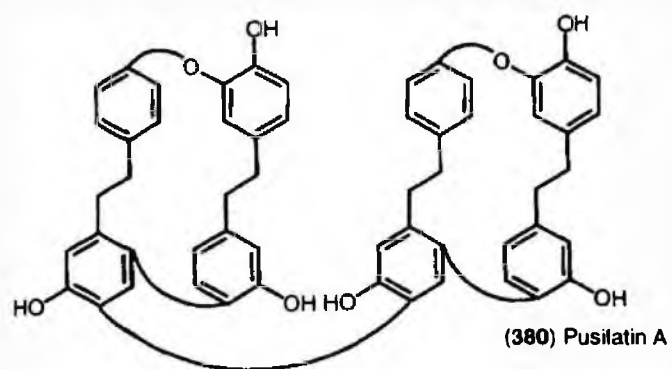
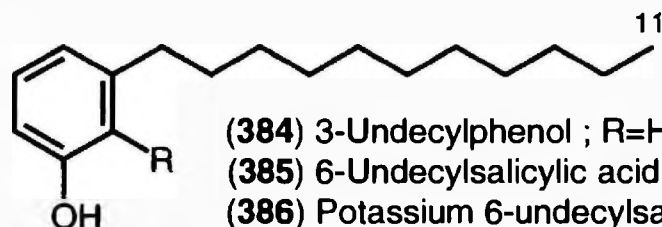


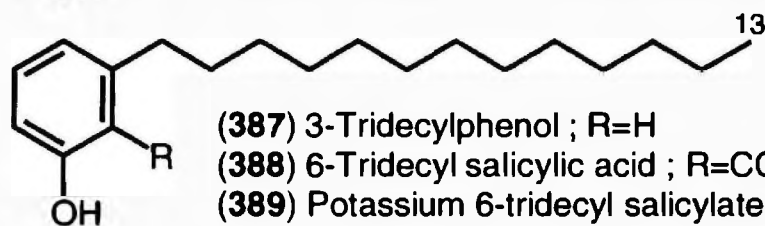
Chart 25e. Bis-bibenzyls found in the Hepaticae



(384) 3-Undecylphenol ; R=H

(385) 6-Undecylsalicylic acid ; R=CO₂H

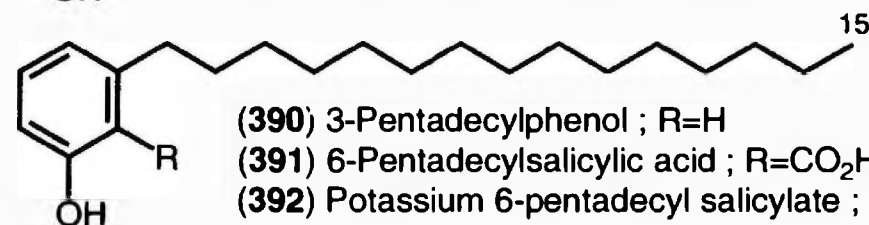
(386) Potassium 6-undecylsalicylate ; R=CO₂K



(387) 3-Tridecylphenol ; R=H

(388) 6-Tridecyl salicylic acid ; R=CO₂H

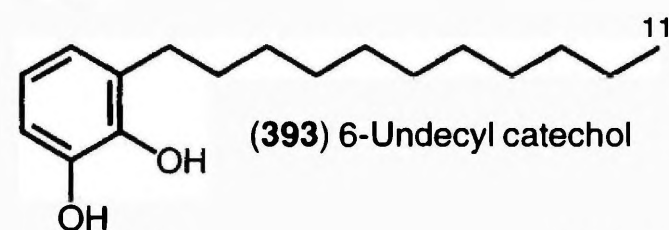
(389) Potassium 6-tridecyl salicylate ; R=CO₂K



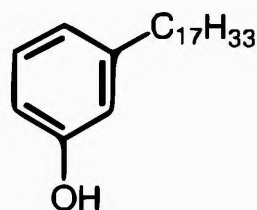
(390) 3-Pentadecylphenol ; R=H

(391) 6-Pentadecylsalicylic acid ; R=CO₂H

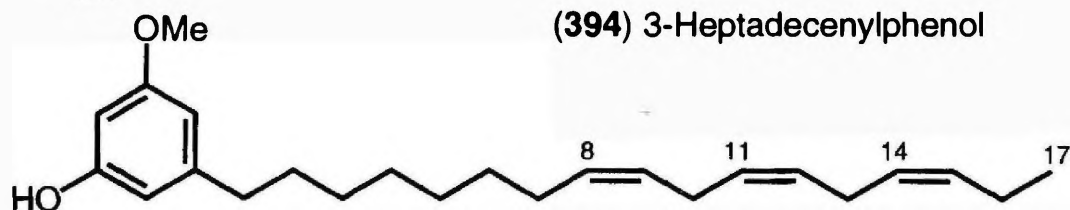
(392) Potassium 6-pentadecyl salicylate ; R=CO₂K



(393) 6-Undecyl catechol

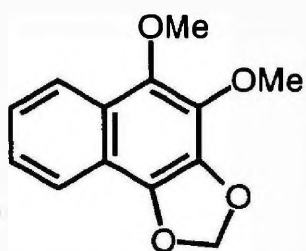


(394) 3-Heptadecenylphenol

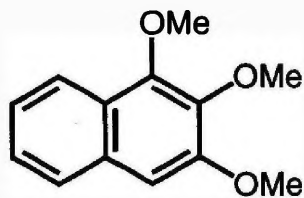


(395) 5-Heptadeca-8(Z),11(Z),14(Z)-trienylresorcinol monomethyl ether

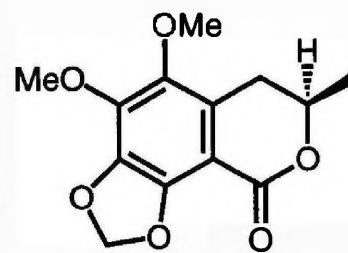
Chart 26. Long chain alkyl phenols found in the Hepaticae



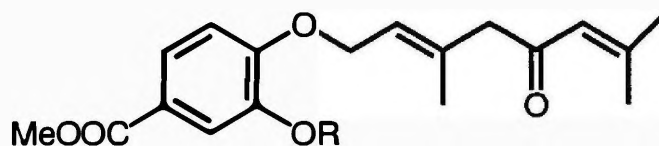
(396) Wettstein A



(397) Wettstein C

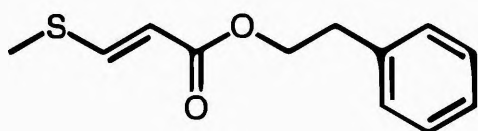


(398) Wettsteinolide

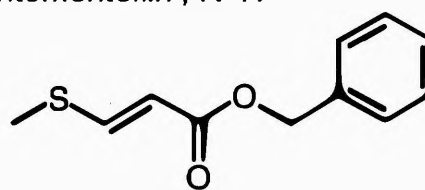


(399) Tomentellin ; R=Me

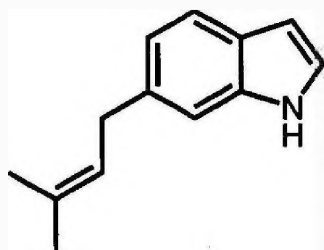
(400) Demethyltomentellin ; R=H



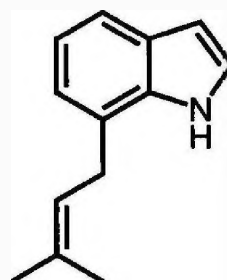
(401) Isotachin A



(402) Isotachin B

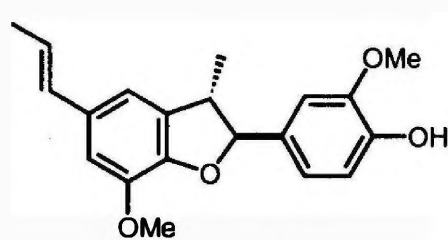


(403) 6-(3-Methyl-2-butenyl)-indole

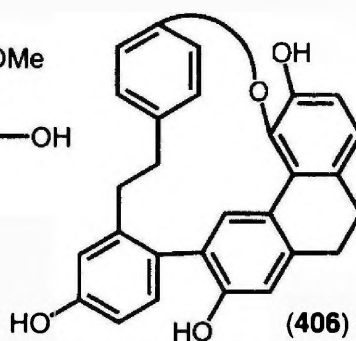


(404) 7-(3-Methyl-2-butenyl)-indole

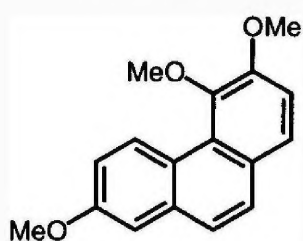
Chart 27. Naphthalenes, isocoumarin, prenyl ethers, sulfur- and nitrogen-containing compounds found in the Hepaticae



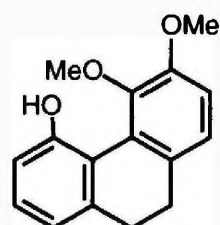
(405) (-)-Licarin A



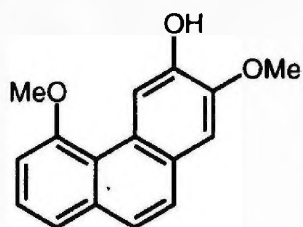
(406) Cavicularin



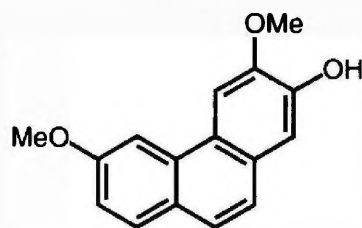
(407) 3,4,7-Trimethoxy-9,10-dihydrophenanthrene



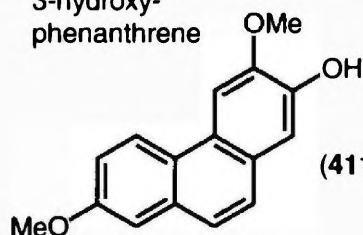
(408) 3,4-Dimethoxy-5-hydroxy-9,10-dihydrophenanthrene



(409) 2,5-Dimethoxy-3-hydroxyphenanthrene

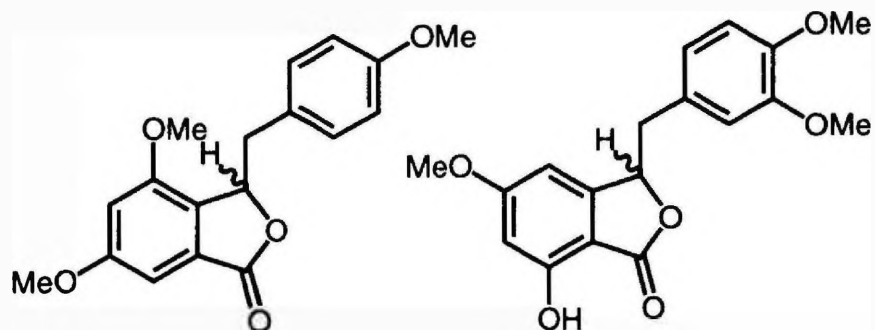


(410) 2-Hydroxy-3,6-dimethoxyphenanthrene



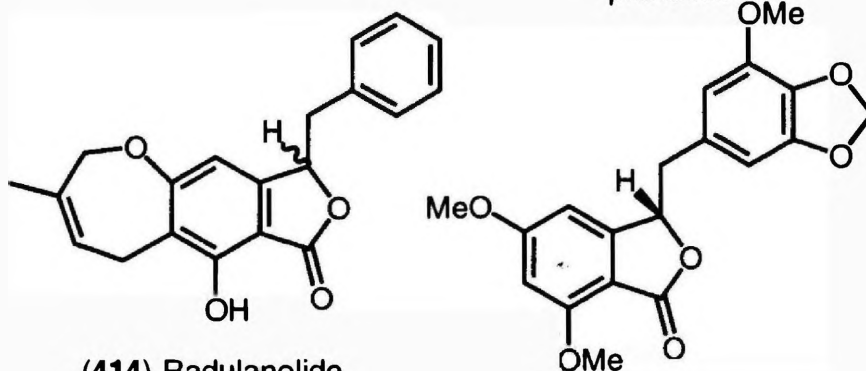
(411) 2-Hydroxy-3,7-dimethoxyphenanthrene

Chart 28. Neolignan and phenanthrenes found in the Hepaticae



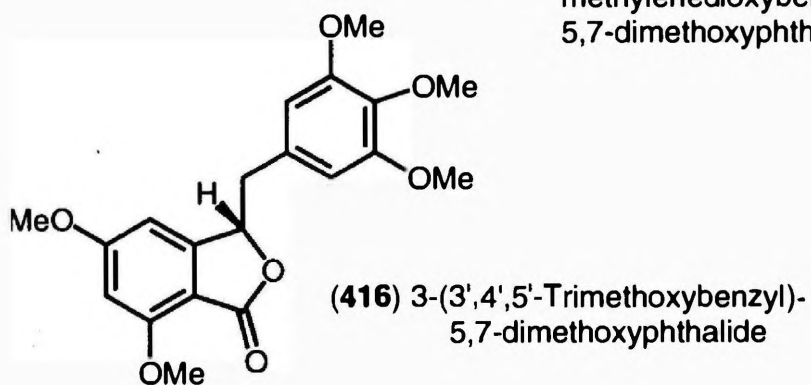
(412) 3-(4'-Methoxybenzyl)-
4,6-dimethoxyphthalide

(413) 3-(3',4'-Dimethoxybenzyl)-
7-hydroxy-5-methoxy-
phthalide



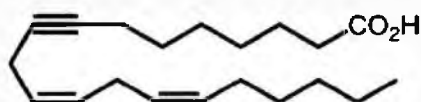
(414) Radulanolide

(415) 3-(3'-Methoxy-4',5'-
methylenedioxybenzyl)-
5,7-dimethoxyphthalide



(416) 3-(3',4',5'-Trimethoxybenzyl)-
5,7-dimethoxyphthalide

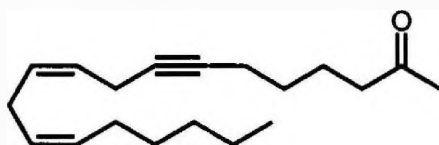
Chart 29. Phthalides found in the Hepaticae



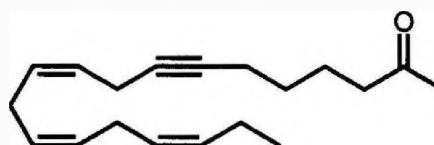
(417) 11,14-Eicosadien-8-ynoic acid



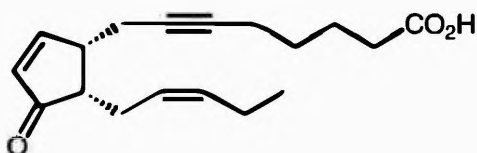
(418) 7,10,13,16,19-Docosapentaenoic acid



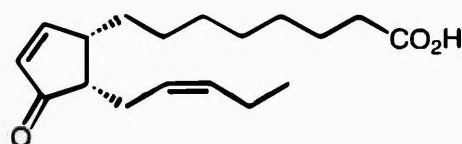
(419) 10,13-Nonadecadien-7-yn-2-one



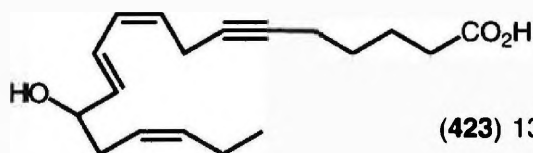
(420) 10,13,16-Nonadecatrien-7-yn-2-one



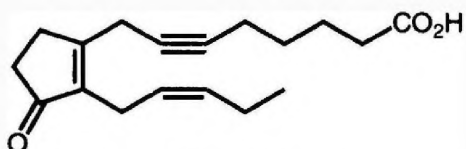
(421) Dicranenone A



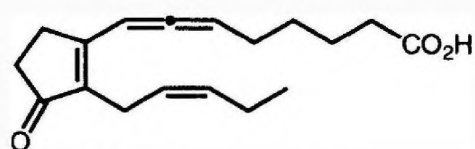
(422) 12-Oxo-PDA 1



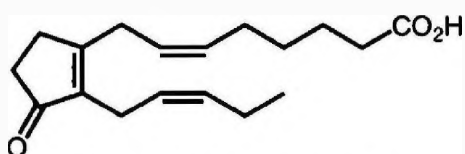
(423) 13-Hydroxy-9,11,15-Octadecatrien-6-ynoic acid



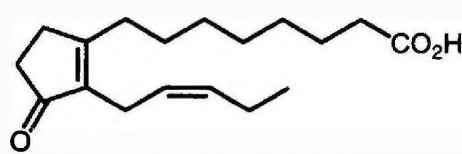
(424) Dicranenone B



(425) Dicranenone B1

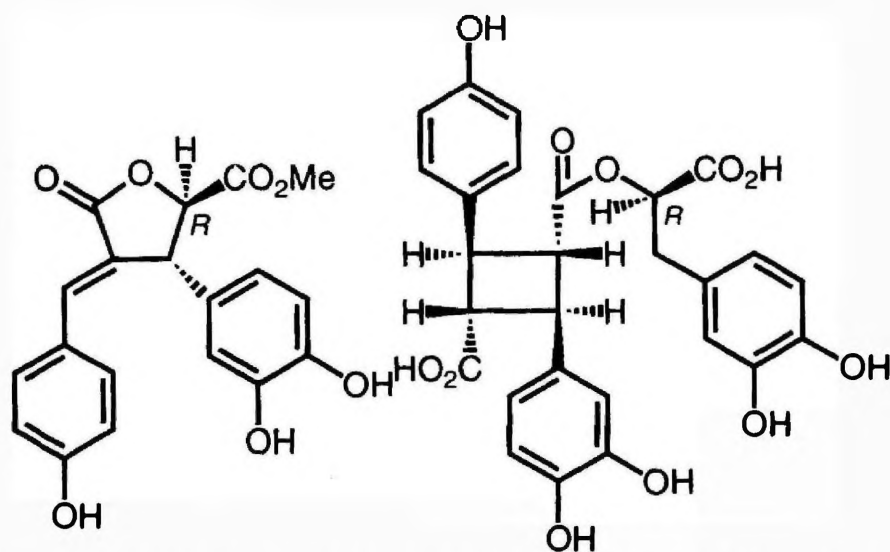


(426) Dihydrodicranenone B



(427) 12-Oxo-PDA 3

Chart 30. Fatty acids found in the Musci

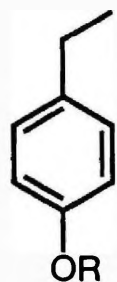


(428) Megacerotonic acid (429) Anthocerotonic acid ; R=H

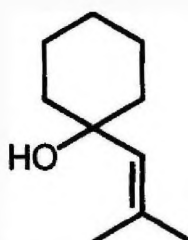


(430) (*R*)-Rosmarinic acid

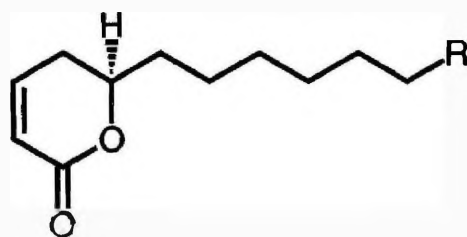
Chart 31. Lignans found in the Anthocerotae



(431) *p*-Ethylanisol;
R=Me



(434a)

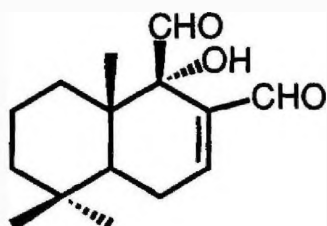


(434b) R=Me

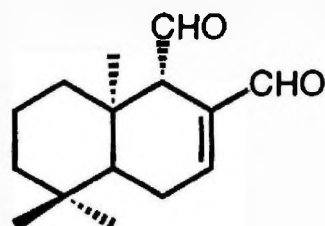
(434c) R=(CH₂)₂CH₃

(432) *p*-Ethylphenol;
R=H

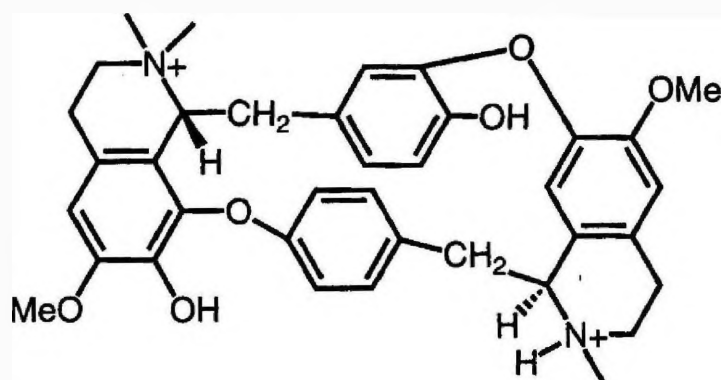
(433) *p*-Ethylphenyl
acetate; R=Ac



(435) Warburganal



(436) (+)-Polygodial



(437) α -Tubocurarine

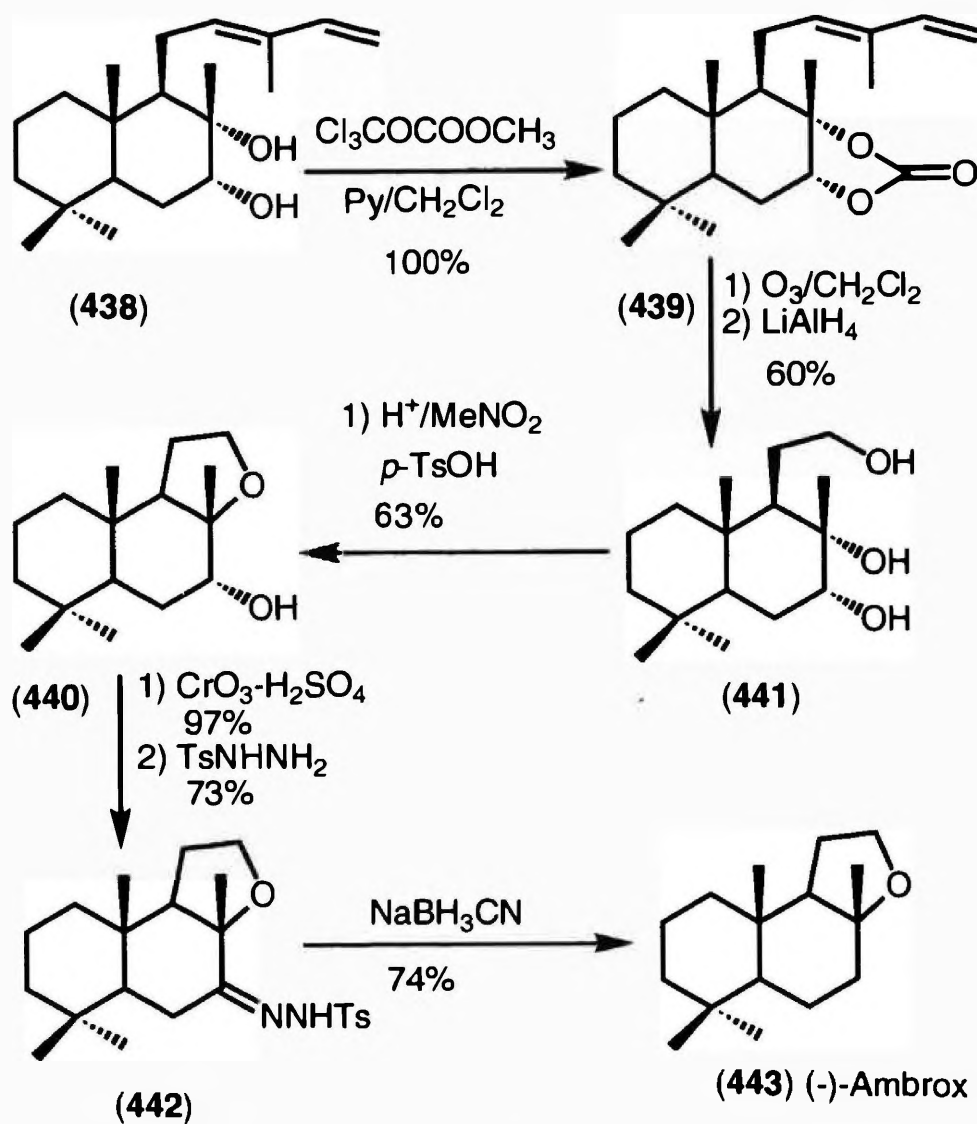


Chart 32. Chemical conversion of labdane diterpenediol (438) obtained from the liverwort *Porella perrottetiana* into (-)-Ambrox

THE USE OF ESSENTIAL OILS IN THE FORMULATION OF NATURAL FLAVOURS

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SOME BACKGROUND INFORMATION AND DEFINITIONS

Essential oils are obtained:

- 1) by distillation of a plant material, either in the presence of water (water distillation), or using steam; or
- 2) by dry distillation; or
- 3) by cold pressing;
- 4) by concentration of fruit juices.

This corresponds to the strict definition of essential oils according to ISO. Flavouring products obtained by other means are not essential oils. If the material is distilled in the presence of water, it can be stirred or not. If it is crushed and stirred inside the boiler, the process is called turbodistillation. When steam enters from the top of the vessel and the oil and water mixture is condensed from the bottom, the process is called hydrodiffusion. One can use

steam under pressure, or not; today, steam distillation under reduced pressure is very seldom used. Processes can be continuous or not. Some technologies that are used for fruit juice concentration can thus yield essential oils. Dry distillation, sometimes called destructive distillation as in the case of cade oil (*Juniperus oxycedrus*), involves the direct distillation of the natural material. The cold pressing process mainly involves the mechanical treatment of the epicarp of citrus fruits. It is thus limited to citrus fruits.

If a raw material is extracted with a solvent (e.g., hexane, acetone, ethyl acetate, dichloromethane, fluorocarbons, ethanol...), and the solvent is subsequently eliminated by concentration, the product obtained is called an oleoresin, a resinoid or a concrete, depending on the type of raw material. The latter product may be further processed into an absolute. In the case where a food grade solvent (such as ethanol) has been used as the extracting solvent and the extract solution is more or less concentrated, an infusion or a tincture are produced, depending on whether heat has been used or not. When a plant material is distilled with aqueous ethanol, various alcoholic distillates (so-

called alcoholates) are produced, depending on the water and alcohol proportions.

Therefore, it is clear that none of these products can be called essential oils. The same is true for extracts obtained by extracting plant matter with supercritical fluids, or by microwave radiation in the presence of inert solvents. The recently developed vacuum microwave hydrodistillation (VMHD) deserves special attention, but this is out of the realm of this presentation.

It is important to make these definitions clear early in this presentation since often, even in recent textbooks, review articles and research reports, extracts are confused with essential oils. I am not making this distinction because essential oils need to be defended against possible competition from products obtained by other technologies, albeit the latter sometimes display superior performance in some flavour formulations, but because essential oils are the only natural products of this type which are described using internationally accepted norms. They are manufactured from defined botanicals according to a standard procedure, whereas extracts may be obtained through a variety of processes which would make standardisation extremely difficult. In what follows, I will speak about essential oils only.

Raw materials than can be processed into essential oils are diverse: fresh or dry aerial parts of plants, rhizome, root, fruit (not seed!), almond, exudate, balsam, leaf, bark, heartwood, flower and even flower bud. Yields can be as low as 0.1% in the case of lemon balm oil (*Melissa officinalis*), or as high as 18% in the case of clove oil (*Syzygium aromaticum*). Most frequent yields are in the 0.4-1% range, as in the typical cases of peppermint and spearmint. It is sometimes preferable to refer to the yield per hectare, for example 30 Kg in the case of basil (estragole type, *Ocimum basilicum*). Flowers are generally not processed into essential oils but rather solvent extracted. The only exceptions concern: rose, ylang and orange blossom.

SOME REGULATORY ASPECTS

In the US, the FDA defines natural flavour as: « ... the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate, or any product of roasting, heating, or enzymolysis, which contains the flavouring constituents derived from a spice, fruit or fruit juice, edible yeast, hulls, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation

products thereof, whose significant function in food is imparting flavouring rather than nutrition. » US Code of Federal Regulations, Title 21, Section 101-22 a 3, Federal Register Vol. 38, No 231, 33284-33290 (December 3, 1973).

To formulate a natural flavour in the US, it is possible to use essential oils provided they are registered on the FEMA/GRAS list, which, therefore, is a positive list. By strict definition of essential oils, most of them can be considered Kosher. As of today, out of more than 1800 substances registered on this list, 120 are essential oils. It is interesting to note that since the release of the 13th FEMA/GRAS list (published in 1984), only one essential oil has been approved. This is massoya which appeared on the 1985 14th FEMA/GRAS list. During the same period, one essential oil has been withdrawn: calamus. More significantly, out of the 146 new items which have been added since 1979 on the FEMA/GRAS list, only three are natural complex mixtures: massoya essential oil, osmanthus absolute and jambu oleoresin. The other products are either nature-identical and artificial chemicals, or a very small number of defined natural products such as thaumatin, an extremely powerful sweetening agent. Today, it is very difficult to get a new natural product (such as an essential oil) approved. The main reason

is the necessity, totally controlled by the FEMA Expert Panel, to develop methods and define proper criteria for reviewing these products. Such procedures are still under study.

Concerning essential oils, the FEMA/GRAS list is not free of oddities and errors. For example, the essential oil of *Anthemis nobilis* is referenced twice. Once it is called «English camomile» (2272) and later «Roman camomile» (FEMA 2275). It should not be confused with either «German » or «Hungarian camomile», *Matricaria camomilla* (FEMA 2273) or «Moroccan camomile», *Ormenis multicaulis*, the latter not being listed. The essential oil obtained by steam distilling orris rhizomes is improperly called a «concrete liquid oil» (FEMA 2829), which should not be confused with an extract from rhizomes (FEMA 2830), wrongly designated as roots. Conversely, the purified irone fraction from the iris essential oil, improperly called «absolute iris», is currently not FEMA/GRAS listed. Essential oils of peppermint, *Mentha piperita*, spearmint, *M. spicata* and pennyroyal *M. pulegium* are registered (FEMA 2848, 3032 and 2839, respectively). Registration of cornmint, *M. arvensis*, is not yet

officially confirmed, whereas it represents one of the most important tonnage in the world !

Some essential oils which are approved for use may nevertheless be subject to restrictions because they contain a component which is controlled by a particular regulation. Examples in Europe include essential oils which contain substances such as: β -asarone, coumarin, pulegone, safrole, the thujones, and hydrogen cyanide. The International Organisation of the Flavour Industry (IOFI) currently works to define analytical protocols to determine the concentrations of these substances in the finished products which reach consumers. These methods are taken into account by the Council of Europe Committee of Experts on Flavouring Substances. Each defined substance which has just been cited is a problem for the flavour chemist operating in Europe or elsewhere and who intends to market his flavours in the US. The converse also applies as well to the American flavour chemist marketing his/her products in other countries. This problem can be illustrated by the following examples. Since the utilisation of calamus oil is forbidden in the US, any trace of β -asarone, wherever it comes from, must be excluded in the finished US product. Conversely, the essential oils of

sage (*Salvia officinalis*), Greek sage (*S. triloba*), Spanish sage (*S. lavandulaefolia*), wormwood (*Artemisia absinthium*) and cedar leaf (*Thuja occidentalis*), are all authorised in the US provided the finished product is "thujone-free". Interestingly, wormwood oil may be specifically cleared "thujone free" when tested by a method sensitive to at least 10 ppm in the finished food. The regulation in Europe imposes a more precise limit on the use of these essential oils in flavour applications, as the thujone content in a finished product cannot exceed 0.5 ppm in food products and non-alcoholic beverages, with higher levels permitted in alcoholic beverages. Curiously, up to 25 mg/Kg are allowed in « foodstuffs containing preparations based on sage ».

Essential oils can be highly complex mixtures as in the case of Cascarilla *Croton eluteria*, or very simple, as in the case of Bois de rose, *Aniba rosaeodora*, that virtually contains linalol only. Whatever their complexity, various criteria are linked to essential oils and their uses including purity, authenticity, alteration and sensory properties influenced by ageing.

Purity

Purity criteria deal with the presence of foreign substances which may have entered the product during the industrial processing of the oil. This may be fortuitous contamination, for example:

safrole may be present in massoya essential oil because the Papuans in Irian Jaya unfortunately may mix the bark of certain *Cinnamomum* species with *Cryptocarya masseyi* bark.

- Residual solvents may contaminate an essential oil when they are added to facilitate the separation (decantation) of an essential oil from the distillate. Solvent addition may be necessary if some essential oil constituents are soluble in hot water, if the oil has a density close to that of water, or if the product gives a low yield of essential oils. Strictly speaking, however, the product cannot be called any longer an essential oil if a solvent has been used, but rather an "absolute from the distillation water".

In each of these cases, an efficient investigation of the different processing steps including raw material supply and distillation, appreciably limits these risks.

Authenticity

Authenticity criteria are important when essential oils or isolates are used in natural flavour formulations. The cases of benzaldehyde and methyl salicylate, abusively named bitter almond oil and wintergreen oil respectively, would deserve comment if I had more time. The various analytical techniques for determining the genuineness of such natural products are certainly familiar to many of you. In my opinion, chirospecific gas chromatography coupled to carbon or nitrogen isotope mass spectrometry, is today one of the most powerful tools (when it applies) in this domain. It also offers very good efficiency / cost ratio. Let us note, however, that the availability of sophisticated techniques often leads to the neglect of other more modest techniques. For example, gas chromatography allows one to determine that a so-called Spanish red thyme essential oil is in fact made of synthetic thymol simply by observing the presence of di-isopropyl-metacresol ! This product is a side product in the Friedel-Crafts reaction of meta-cresol with propylene, that is very difficult to remove totally when purifying thymol.

In some applications, an isolate or essential oil fraction is preferred to the total oil. Terpeneless oils (for example bay oil) and folded citrus oils are well known examples of fractionated essential oils for an increased performance. Sometimes, fractionation also allows the reduction of undesirable notes. This is the case of anethole-containing essential oils such as anise (*Pimpinella anisum* L.), star anise (*Illicium verum* Hooker) and fennel (*Foeniculum vulgare* Miller) which are used for the flavouring of certain *apéritifs* in Europe. Other processing steps may be applied to reduce the instability of certain essential oils. This is the case of lemon oil, for example, which is known to be relatively unstable in soft drinks. Robertet has developed a proprietary process which allows the production of lemon oil containing less than 0.5% citral while maintaining remarkable organoleptic qualities and a noticeably improved shelf life in beverage applications. Contrary to what was formerly thought, citral is not essential to lemon flavour. However, it is well known to be the main culprit, although not alone, contributing to the off-flavour development during the ageing of lemon flavoured beverages.

HOW ARE ESSENTIAL OILS USED IN FLAVOUR FORMULATIONS TODAY?

In 1950, a collaborator of the French company Chiris, at that time a world leader, recommended the use of orris oil in a raspberry flavouring, sweet orange oil and neroli in a strawberry formulation and Bourbon clove bud oil, Ceylon cinnamon and lie-de-vin oils in a cherry flavour. Indeed, these natural elements were at that time only destined to provide original nuances to artificial flavour formulations largely built with synthetic products.

Today, natural flavours for the European and North American markets involve formulations with natural raw materials: essential oils, isolates and various extracts which are blended with a matrix coming from the generic natural product. WONF compound regulations in effect in the USA require that the generic natural product is unique, and that the added natural aroma compounds do not exceed 50%. (WONF stands for "With Other Natural Flavor").

Fruit flavours are widely used today in soft drinks, milk products and confectioneries. For marketing reasons related to consumer preference, soft drink flavours are practically all natural. This is, therefore, a very high tonnage activity. « Red berries »: strawberry and raspberry are the most popular, together with blackberry, blueberry, cranberry

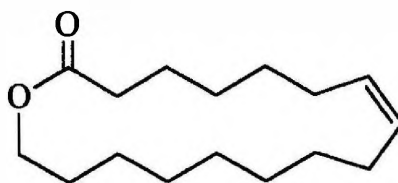
etc. Outside this category, apple, peach and apricot are among the best sellers. One should also note certain tropical fruits such as passionfruit, guava and papaya that are increasingly accepted by the consumers. Sophisticated combinations of these flavours with melon-like or even cucumber notes are appearing now on the market.

To a large extent, red berry formulations use defined natural substances, either isolated from essential oils and natural extracts, or obtained through biotechnological processes. For a strawberry flavour, it is easy to mention methyl cinnamate and *cis*-3-hexenol, isolated from certain basil and mint essential oils, respectively. The (*S*)-2-methylbutyric acid that is prepared by bio-oxidation of natural 2-methylbutanol is a key element for creating a cranberry flavour. The δ -deltadecalactone, which can be obtained by baker's yeast bio-reduction of massoialactone, is a character impact element in white peach flavour. Isolates are, therefore, particularly convenient building blocks because they provide at will and in a neat manner a characteristic effect such as green, fatty lactonic, flowery etc. which strongly intensifies the natural character provided by the juice or the pulp of the fruit serving as the aromatic

matrix. However, as necessary as they may be, they cannot suffice to create all the flavours of interest for at least two reasons :

Not all constituents of a flavour formulation are always available as isolates.

Blackberry flavour is a good example since blackberry requires a musky note of the macrocyclic type (in fact, macrocyclic lactones with a musk character have been effectively identified among the blackberry fruit volatiles). The only way to give such an effect in a formulation is to introduce ambrette seed essential oil, or, better, a fraction of this oil containing higher amounts of ambrettolide, an unsaturated macrocyclic lactone with a delicate and pronounced musk odour.



AMBRETTOLIDE

Ionones are indispensable constituents in every raspberry flavour. Unfortunately, no essential oil has been found

(yet) with a significant amount of α - and/or β -ionone, although some derivatives of this type exist in certain amounts in the commercial extracts of *Osmanthus fragrans* and *Boronia megastigma* flowers. The (quite expensive) absolute of the latter contains 5% β -ionone at best. The essential oil from henna flowers (*Lawsonia inermis*) does contain high levels of β -ionone, but it has no commercial importance. The only possibility available to the flavour chemist for bringing a characteristic violet note to the flavour is to resort to orris essential oil as such. Orris essential oil contains 5 to 10% irones (structurally and organoleptically related to the ionones) and when the myristic acid is removed it will contain 65 to 75% irones.

In a tropical fruit flavour such as passionfruit, the characteristic sulphury note is only provided by natural raw materials that already contain sulphur derivatives. In this respect, the usefulness of buchu essential oil (*Barosma betulina*) is evident. However, there are few choices available to the creative flavour chemist today for imparting natural sulphury notes.

Formulations solely based on defined substances are rarely balanced.

Essential oils as well as other natural complex mixtures such as absolutes, infusions etc., provide rich nuances which modify or complete, sometimes through contrast, the « rough » aromatic character which has been made with natural isolates. Generally used in low dosages, the essential oils contribute to the body, the roundness and the fullness of a flavour formulation. Noteworthy, this effect seems to be even more pronounced when the chemical composition of the oil is more complex. Thus, davana essential oil (*Artemisia pallens*), which is particularly rich in oxygenated sesquiterpenoids, enhances dried fruit flavours, e.g. fig and raisin. Roman camomile oil, rich in variety and unique aliphatic esters, enriches numerous and various formulations, such as those of « yellow » fruits (peach, apricot), red berries, honey, tea etc.

In the area of savoury flavours, essential oils are unavoidable elements for seasonings, including sauces and condiments for cooked meats (grilled, roasted, broiled...) or delicatessen items. Essential oils of parsley, celery, thyme, rosemary, bay leaf, sariette, laurel, mustard, nutmeg etc...

are clearly useful for those who know a bit of cooking! Conversely, they are of no practical interest in the formulation of actual meat or seafood flavours which require very specific substances. These foods generally depend upon heterocyclic molecules for their sensory properties which cannot exist in essential oils for biogenetic reasons. The creation of natural roasted meat flavours (chicken, beef, mutton, pork...) with a quality comparable to that of « sweet flavours », still remains a challenge today, and amply justifies research that is conducted on process flavors (sometimes called « reaction flavours »).

Compounding natural vegetable flavours is, in most cases, an easy task thanks to essential oils. Vegetable flavours of the *Allium* species (onion, garlic, leek, chive, shallot, and scallion) depend heavily upon onion and related essential oils which are used in seasoning flavours. Additionally, one can not omit lovage and carrot oils or *Asa foetida* contributions in this flavour area. Flavours of cruciferous and in particular *Brassica* vegetables, e.g., various cabbages, including broccoli and cauliflower, can possibly involve the same oils, including, of course mustard oil (*Raphanus sativus*). As a reminder, the particular aroma

of these vegetables is to a large extent the result of glucosinolate autolysis during blanching. Particular mention should be made for certain vegetable flavours that require a green note, such as green pea, spinach or cucumber. In these cases galbanum essential oil (*Ferula galbaniflora*) can sometimes find an application.

date, there are practically no essential oils which can be used for compounding flavours of milk-derived products, including butter, yoghurt and cheeses, or fatty materials such as oils and margarines. To a certain extent, massoya essential oil is an exception. However, it is seldom used as such because it contains some minor constituents, such as benzyl benzoate, which are responsible for off-flavours. For this reason, one prefers to use the massoyalactone (C10) after isolation and proper purification. We now know that the homologous massoyalactone C12 possesses very interesting organoleptic properties for flavour applications of the hot milk type. For several years Robertet has been conducting a research program directed at acquiring an in-depth knowledge of massoya essential oils and securing the supply of this natural raw material. As a result of these efforts, we recently found some essential oils that contain a high proportion of the massoyalactone C12 and no benzyl benzoate, and

consequently are directly suitable for use in flavourings for milk products and margarines.

Based on this presentation, I believe it is clear that essential oils are very precious elements for the formulation of natural flavours. This is why I regret the difficulty to get a new essential oil registered as FEMA/GRAS. This task is so formidable that it may discourage further investigations to find new raw materials of this type for direct application in food systems. Today, the main goal of such investigations is to find new sources of natural isolates. The following requirements apply to these isolates:

- 1) They must have a very strong and characteristic organoleptic impact with a high added value. Damascenone and 1-octen-3-ol are typical examples. One must be aware, however, that these substances may result from biogenetic pathways that may not readily occur in essential oil-bearing plants;

- 2) When it is the case, they must have a high enantiomeric purity to ensure the highest organoleptic performance;

- 3) The oil must have a favourable chemical composition for an easier separation and purification of the product of interest; and

4) The oil must be free of minor compounds which may be undesirable from a toxicological as well as an organoleptic (off-flavour) standpoint.

CONCLUSION

In summary, it is possible to distinguish three groups of essential oils:

1) The citrus orange, lemon, tangerine, lime, mandarin, grapefruit. This group has found, by far, the largest number of important applications, in tonnage as well as variety of flavours. In this group, we can also include the mint oils, although they are sometimes destined for more specific applications, such as tobacco, chewing gum and toothpaste flavouring.

2) The seasoning oils: clove bud, celery seed, cinnamon, garlic, nutmeg, onion etc..This group is involved in more selected applications and in lesser quantities.

3) The «exotic» oils, which are used in even lower quantities, but in a more targeted manner, either because of their very high impact or their specificity (costus, buchu, rose, ambrette seed...), or for other, more *subjective* reasons (neroli, roman camomile, davana...). *Subjectivity* is

the sometimes very narrow path which allows the creative flavourist to give his/her personal signature to the flavour, between the constraints of formulation and those of cost.

SYNESTHESIA

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ABSTRACT

Fragrance has always excited interest and has had certain magical powers assigned to it. It is an invisible communicator. In addition to aromachological effects the psychological aspects of a perfume help it to differentiate a product from its competitors. This property is enhanced when the fragrance is coherent with the sensory characteristics of the product. To gain a better understanding of the sensory psychology of fragrance, Givaudan Roure is researching synesthesia, odor imagery, language, odor classification and sensory mapping techniques. This paper discusses our work on synesthesia, which is the interrelationship of the senses. We use a modified Rader and Tellegen methodology, which was developed to research the cross modal correspondence of mood, color and line elements. Specifically, this paper covers our work on odor-color and odor-mood associations.

INTRODUCTION

Smell is our oldest sense. It connects through the olfactory bulb directly to the part of the brain involved in emotional experiences, that is, the limbic system. Although the sense of smell has been superseded in the higher cognitive processes by vision and hearing, it is still an important contributor, both consciously and subconsciously, to our perception and feelings.

Fragrance is something invisible yet strongly felt. It has always excited interest and has had a certain magical power assigned to it. Aromatic substances are found in many ancient pharmacopoeia. Many cultures claim they are calmatives, stimulants and aphrodisiacs. These medicinal properties have given rise to the development of aromatherapy. Aromatherapy in the classic meaning is the treatment of a disease or illness by essential oils viz. aroma chemicals. Two schools of treatment exist. The French School treats

health problems by ingestion of the relevant essential oil, whilst the English School treatment is based on massage with the essential oil. Both methods of treatment cause relatively large amounts of the aroma chemical to enter the bloodstream and could act pharmacologically.

More recently the term aromatherapy has also been used to describe the effects of fragrance inhaled from products. However, these usually refer to the way fragrance influence mood rather than to its aromatherapeutic effects and is based primarily on an individual's emotional and psychological association with the specific odor. This effect can be likened to that of music. For example, listening to calming music can calm the listener.

"Music has charms to soothe a savage breast"

William Congreve 1697

To avoid confusion with classic aromatherapy, the Fragrance Foundation introduced the term aromachology. It specifically refers to the associative effect of inhaled fragrance and does not imply any therapeutic effects.

In addition to the aromachological effects, fragrance also has a sensory psychological role in a product and is a powerful communicator. These aspects of a fragrance have been used successfully by Marketers to differentiate a product from its competitors. The effect of fragrance on product acceptance is especially strong if the fragrance is coherent with the other sensory properties, such as color. However, despite the long history of a fragrance's effect on influencing perception and behavior, our understanding of these effects is still at its infancy.

Thus our research program seeks to gain a better understanding of the sensory psychology of fragrance. Research has been carried out on synesthesia, odor imagery,

language, odor classification and sensory mapping techniques. This paper focuses on synesthesia, which in its broad meaning is the translation of attributes of sensation from one sensory domain to another. .. This phenomenon is important in designing fragrances. Our work, therefore, examines the synesthetic interrelationship between the sense of smell and our other. An extensive amount of research has been done into the relationship between audition and vision (1), and some on the cross modal association between colors and emotions (2). However, the cross modal relationship of olfaction, the other senses and emotions has not been systematically studied.

At the beginning of our program we examined the available techniques with potential to study synesthesia. We were specifically interested in non-verbal techniques. Several instrumental techniques have been put forward e.g., skin conductivity, contingent negative variation, functional magnetic resonance imaging and the like. We concluded that while they are capable of showing a response to aroma, the interpretation of the observations, especially from mixtures of aroma chemicals is currently not possible. We, therefore, decided to base our studies on psychological testing techniques. We selected method developed by Rader and Tellegen (3), to study the synesthetic relationship between mood, color and line elements. We then adapted and validated the technique to study cross sensory relationships such as odor-color (4) and odor-mood.

This paper reviews our work to date. It will briefly describe our methodology and illustrate with some examples, the cross modal correspondence between odors and color and odors and mood. This data is used by our perfumers to design and optimize fragrances that complement or reinforce the concept of a product, especially in its sensory psychological benefits.

METHODOLOGY

The Rader and Tellegen method is a questionnaire based protocol that uses the assignment of 5 points to one or more of 11 color to describe a stimulus. They found that this approach reflects individual differences more sensitively than asking a person to select just one color. We first replicated the Rader and Tellegen study and found the method to be reliable. We then adapted and validated the technique to use with odors and subsequently with complex fragrances.

In our initial studies with raw materials, we first matched the odor intensities using the method of magnitude estimation. Analysis of the results were carried out using the Friedman two way analysis of variance by ranks. The basic protocol involved the subject smelling an odor and distributing 5 points across 11 color words (white, pink, yellow, red, orange, green, blue, purple, brown, gray and black) and 11 mood words (angry, tired, proud, peaceful, surprised, sad excited, tender, ashamed, joyful, an scared). The colors were identified only by name to avoid problems of hue and intensity. The modified Rader and Tellegen technique was shown to be robust and produce replicable results.

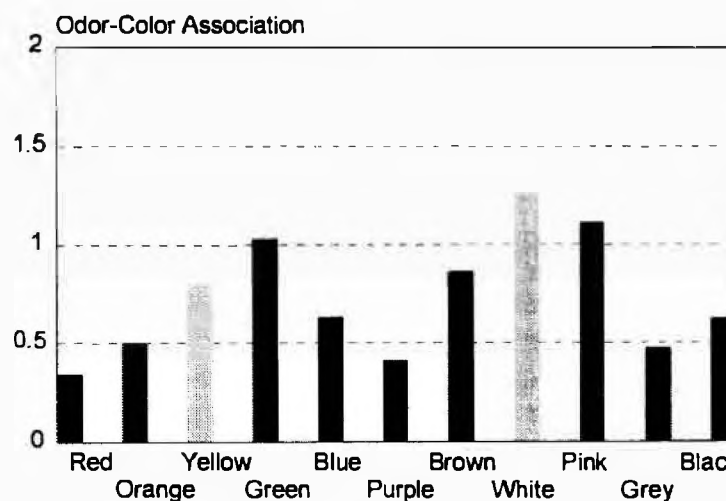
Our studies are carried out in custom designed sensory psychology facilities. All panelists are screened for their olfactive acuity, ability to use scales and color blindness to ensure that they are able to perform the experimental task.

RESULTS AND DISCUSSION

Unless otherwise stated, the results are from the work carried out in the custom designed sensory psychology facilities at Givaudan Roure, USA.

Odor-Color Association

Allyl Cyclohexyl Propionate



Star Anise Oil

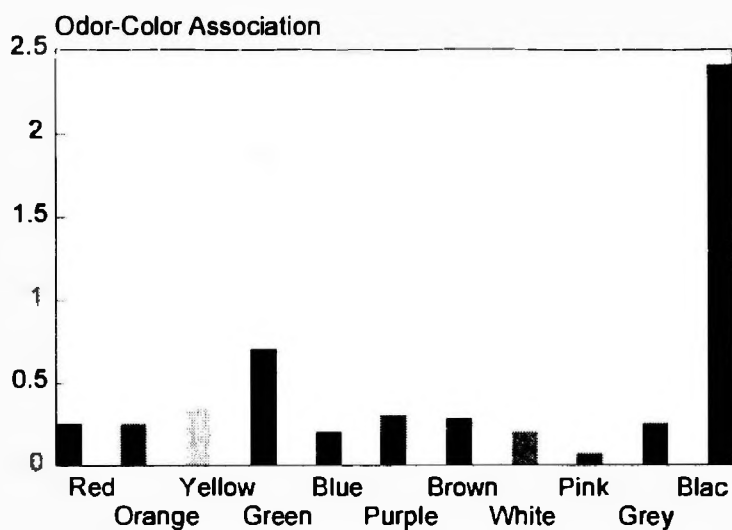


Figure 1 Odor-Color Association of two Aroma Chemicals

In the first series of experiments we examined odor-color associations for raw materials to provide a data base for perfumers. We showed that that associations are. Figure 1 shows examples of two color profiles, Allyl Cyclohexyl Propionate and Star Anise Oil.

The Allyl Cyclohexyl Propionate, a fruity, predominantly pineapple odor, is strongly associated with white, pink and green and moderately associated with brown, yellow and black. The Star Anise Oil, which is a licorice, aniseed, fresh, fruity odor, is strongly associated with black and moderately with green colors.

In the second series of experiments we examined odor-color associations of naturals, which are mixtures of aroma chemicals. Validation studies showed that this methodology is Laos appropriate for mixtures. Figure 2 shows an example of Juniper Berry oil. which is a fresh-piney, gin-like odor with woody resinous undertones. The color profiles indicates a strong associated with orange and green.

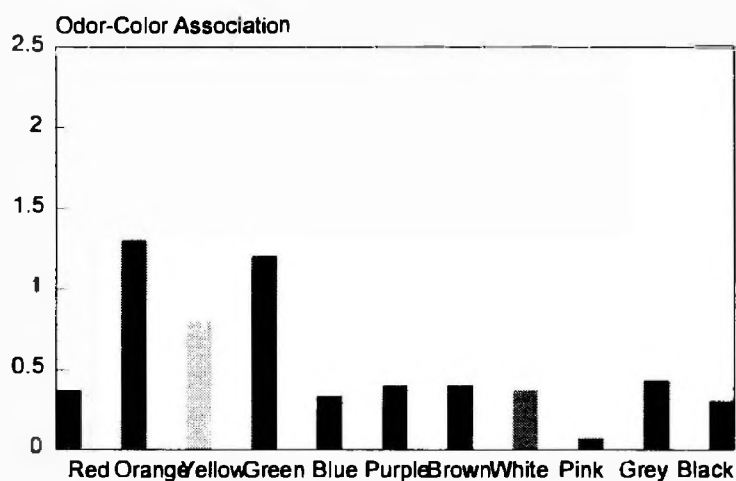


Figure 2. Odor-Color Profile of Juniper Berry Oil

The Rader and Tellegen methodology has enabled us to developed a large database of odor-color correspondences for aroma chemicals and naturals. These are used by our perfumers to create fragrances that have strong odor-color congruence with the product mix.

Additionally to studying the synesthetic relationship between odors and colors we have further examined whether perceptual dimensions of odor vary systematically with those of vision. Subjects matched Munsell color chips, which have color in a three dimensional space, hue, chroma and value, to odors presented at different concentrations. The results show that stronger odors were associated with darker colors thus suggesting cross-modal relationship between vision and olfaction is dimensional (3).

Increasingly we developed cross regional fragrances. To establish the influence of race and culture on synesthetic correspondences we carried out studies on Caucasian, Afro-, Japanese- Americans and Japanese born and raised in Japan (4). We found that cultural experiential learning determines color congruence with odor. For example, all Americans associate cinnamic aldehyde with red regardless of their ethnic background. However, the Japanese raised in Japan do not have this association.

An example of how cultural experiential learning affects odor-color association is shown by the USA and French color profiles of Star Anise Oil. This is shown in Figure 4.

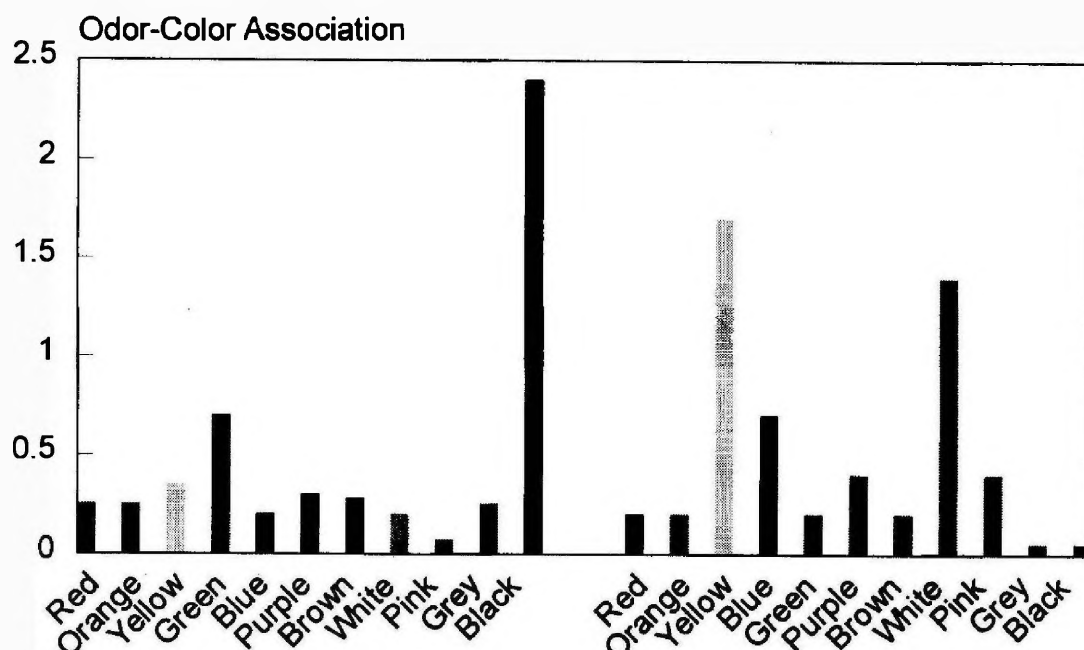


Figure 4. Odor-Color Association of Star Anise Oil in USA and France.

The odor-color profiles of Star Anise Oil in the USA and France are markedly different. The dominant color association in the USA is black. This may be due to the association with the color of children's licorice candies, which is black. In France, however, this smell is associated with white and yellow colors, probably as the French encounter this smell predominantly via Pastis drinks, which are mainly yellow and whitish in color. We are building up our knowledge base on synesthetic differences between different countries for use in perfume creation.

ODOR-MOOD ASSOCIATIONS

Using the modified Rader Tellegen method we have also determined the mood correspondence of key aroma chemicals and mixtures. An example of two mood profiles are shown below. The aroma chemical Lilial™, which is a floral, primarily muget,

are shown below. The aroma chemical Lilial™, which is a floral, primarily muget, aldehydic odor, profiles as tender and peaceful. The profile of a mixture, exemplified by Bergamot Oil, a fresh, citrus, fruity, floral note, profiles as joyful and excited, , is shown in Figure 6

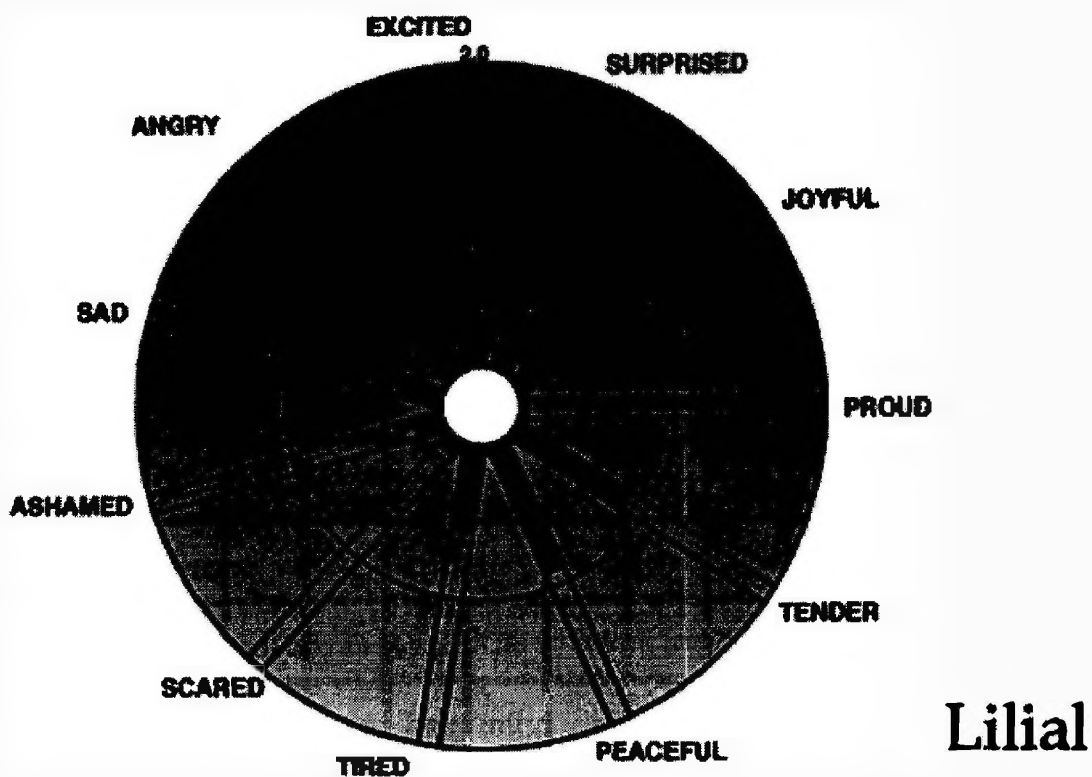


Figure 5. The mood profile of Lilial™.

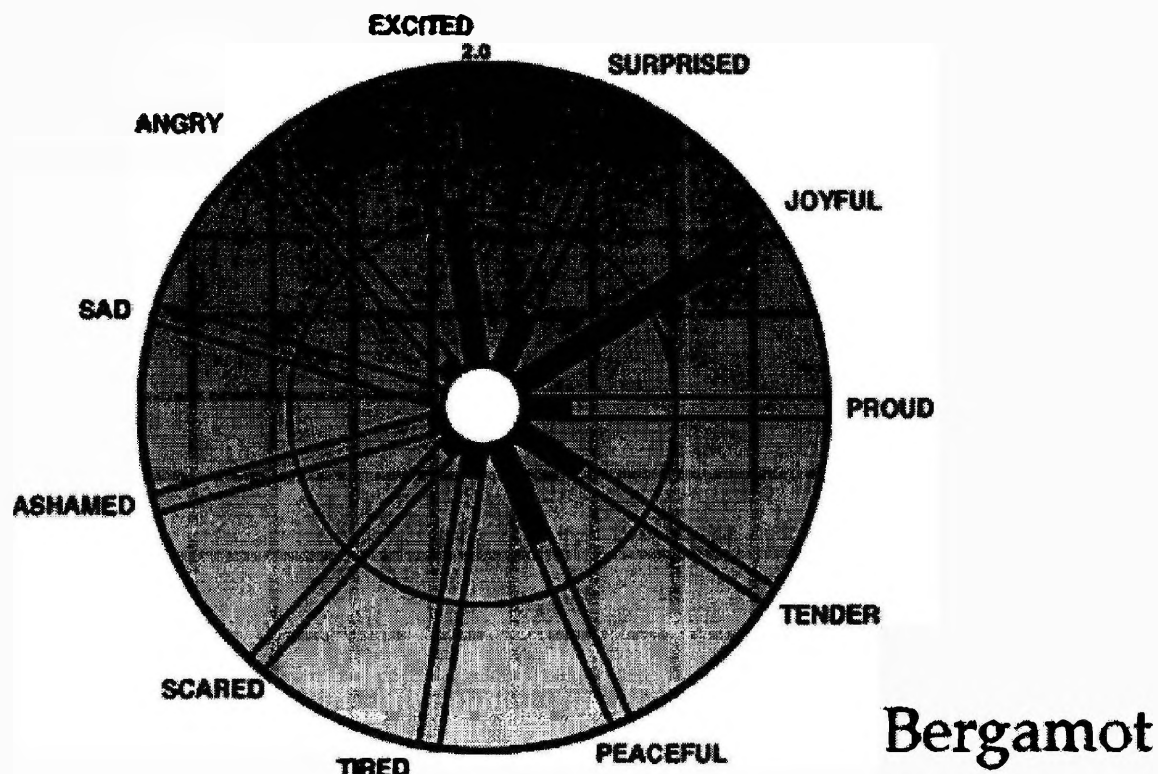


Figure 6. The Odor-Mood profile of Bergamot Oil

We have extended the Rader and Tellegen mood list into product specific moods. This enables us to develop fragrances highly associated with specific moods that are relevant to the product category. The data base on odor-mood association helps perfumers design and select fragrances that are either congruent with the mood of the product's concept or develop a fragrance with an associative aromachology effect.

Similarly to color, mood associations result from cultural experiential learning and thus also require understanding of the culture. The strong influence odor has on our senses and moods is reflected in the following;

"In the fifth month

When orange trees

Fill all the world with scent

I think of the sleeve

Of a girl who loved me."

Love Songs of Asia

translated by Powys Mathers

GIVAUDAN ROURE recognizes the importance of understanding how odors affect our feelings and moods. It is committed to a research program in Sensory Psychology that continuously refines and develops new techniques to study the psychology of fragrance.

ACKNOWLEDGMENT

The authors would like to thank Dr. K. Rankin for help in preparing this paper.

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Proceedings of the 13th International Congress of Flavor, Fragrances and Essential Oils,

SOME NOVEL TRICYCLIC SESQUITERPENE SKELETONS FROM THE ESSENTIAL OIL OF *ECHINOPS GIGANTEUS*

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ABSTRACT

The essential oil from the roots of *Echinops giganteus* var. *lelyi* from Cameroon contains almost exclusively tricyclic sesquiterpenes (77%) with presilphiperfolane, silphiperfolane, isocomane and modhephane skeletons. The main constituents are silphiperfol-6-ene (27%) and presilphiperfol-7-ene (9%). The structures of seven new sesquiterpene alcohols were elucidated by detailed NMR spectroscopy. Three alcohols with new skeletons named cameroonan-7-ol, nopsan-4-ol and echinan-8-ol were isolated.

Cameroonan-7-ol and echinan-8-ol contribute decisively to the strong woody, patchouli- and amber-like odor of the oil.

INTRODUCTION

Echinops giganteus var. *lelyi* C. D. Adams (Compositae) is an endemic species of Cameroon and Nigeria that grows in the high savannahs. The plant is a perennial herb, erect branched, 60-150 cm high, with bluish-white flowers. In the western province of Cameroon its rhizomes are used as an ingredient in different culinary preparations such as 'yellow sauce' which are consumed with maize and taro meals. The plant is also used in folk medicine as a remedy against numerous diseases. The fresh rhizomes of *Echinops giganteus* were bought on the market of Bafoussam (Western Cameroon) and

identified at the National Herbarium of Yaoundé where voucher specimens are deposited. 12 hours of steam distillation yielded 1.7% of a colorless oil, $[\alpha]_D -42^\circ$ (neat). The oil smells strong woody, patchouli-like, heavy-floral with undertones of bacon and lemon peel. A first analysis of this oil was reported by Lamaty et al. and our group.¹

RESULTS

The GC¹ and GC-MS shows that the *Echinops* oil contains only sesquiterpenes, hydrocarbons (CP Sil 5 CB column: RRI 1330-1450, obviously ball-shaped or tricyclic) as well as oxygenated derivatives (RRI 1500-1650). Flash chromatography (FC on silica gel) of 8.0 g of the oil gave four main fractions: 4.1 g unpolar (hydrocarbons), 1.6 g medium polar (less polar alcohols), 1.8 g polar (more polar alcohols), 0.4 g very polar (diols, not investigated). The three main fractions were further separated by repeated FC, FC on AgNO₃ impregnated silica gel and PGC on Sil 5 and Wax columns. Four trivial constituents (together 12.5%) could be identified in the oil (Figure 1). The main part is composed of six rare hydrocarbons with silphiperfolane, modhephane and isocomane skeletons (Figure 2). Silphiperfol-6-ene is the main constituent (27%). All these compounds were described by previous authors² and their structures could be completely confirmed. A new hydrocarbon, silphiperfola-5,7-diene, was isolated as a minor compound and its structure elucidated by the NMR spectra and comparison of these data with methyl cantabradienoate (Figure 3).³ Presilphiperfol-7-ene was found as a naturally occurring compound for the first time (Figure 4). On the other hand, dehydration of presilphiperfolan-8 α -ol yielded a mixture of this hydrocarbon with the rearrangement product silphiperfol-6-ene, as observed by Bohlmann⁴ (Figure 5).

From the polar fraction of the oil some new alcohols were isolated. One of these, modheph-2-en-8-ol shows typical NMR data (Figure 6). Furthermore, four alcohols possessing almost identical MS and similar ^1H - and ^{13}C -NMR data were identified. One of these compounds had data identical with those of a silphiperfolan-6-ol described by Coll (Figure 7).⁵ Detailed investigation of the stereochemistry including NOED spectra in pyridine- D_5 evidenced that the sterical assignment in the literature⁵ was wrong, and that the diastereomers **A-D** should possess the stereochemistry as shown in Figure 7 and 8.

The secondary alcohol cameroonan-7-ol (6%), a major constituent, is the first naturally occurring representative with this skeleton, called 'cameroonane' by us (Figure 9), although the corresponding cation was discussed as biogenetic intermediate in the literature^{2a}, and the 7-trifluoro acetate was isolated previously as a rearrangement product of presilphiperfolan-8 α -ol (Figure 10).⁶

Cameroonan-7-ol was oxidized to the cameroonan-7-one smelling woody, warm, khusimone-like. LiAlH_4 reduction leads to both the epimeric alcohols (Figure 11).

A minor compound $\text{C}_{15}\text{H}_{26}\text{O}$ was isolated from a medium polar fraction by PGC. The NMR data revealed a tricyclic saturated tertiary alcohol, named nopsan-4-ol, containing the two sequences shown in Figure 12. These sequences are typical for a presilphiperfolane as well as for the biogenetically related skeletons **A** and **B** in Figure 13. Structure **B** was confirmed by a COLOC spectrum. But also a representative with skeleton **A** was found, this additional tricyclic saturated tertiary alcohol was called echinan-8-ol by us (Figure 14).

Biogenetic consideration should be compared with these findings. Already Bohlmann many years ago postulated a biogenetic pathway starting with farnesyl pyrophosphate

providing the presilhiperfolanyl cation (Figure 15 and 16). Further rearrangements lead either to the silphinanyl or to the cameroonanyl cation (Figure 17). Subsequent reactions of the silphinanyl cation give the isocomanyl and modhephanyl cation (Figure 18) and the corresponding hydrocarbons (Figure 19). Analogous rearrangements of the cameroonanyl cation result in the silhiperfolanyl and echinanyl cation (Figure 20). Silhiperfola-5,7(14)-diene, the silhiperfolenes, the silhiperfolanols and silhiperfol-6-en-5-one are derivatives of the silhiperfolanyl cation (Figure 21). Finally, a 1,2-shift of the echinanyl cation gives the nopsanyl cation. These cations are precursors of echinan-8-ol and nopsan-4-ol (Figure 22).

All three new sesquiterpene skeletons - cameroonane, echinane, nopsane - (for naming we used the country where the plant grows and we splitted the name *Echi-nops*) are biogenetically related as shown by three *abeo*-steps starting from presilhiperfolane (Figure 23).

The fragrance of some alcohols, particularly of cameroonan-7-ol and echinan-8-ol contributes decisively to the strong, woody, patchouli-like and floral odor complex of the *Echinops* oil (Figure 24).

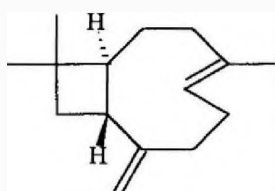
ACKNOWLEDGEMENT

The results were obtained by a fruitful cooperation of the group in Montpellier (G. Lamaty, C. Menut) and the group in Berlin (H. Marschall, P. Weyerstahl). I. Seelmann, who performed his Ph. D. thesis at the Technical University Berlin had the opportunity to work at Montpellier for three months (DAAD grant). - We are grateful to Dr. H. Surburg, Haarmann & Reimer GmbH, D-37601 Holzminden, for the olfactory evaluations.

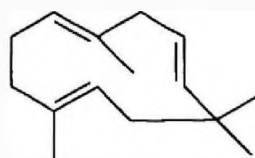
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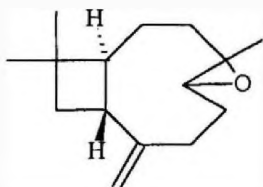
TRIVIAL CONSTITUENTS OF THE *ECHINOPS* - OIL



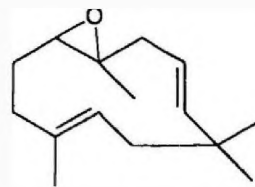
β -Caryophyllene 8.3 %



α -Humulene 2.0 %



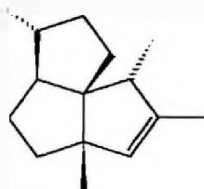
Caryophyllene epoxide 1.8 %



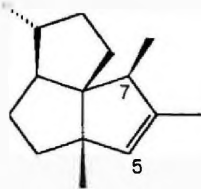
Humulene epoxide II 0.4 %

All the other constituents are tricyclic compounds.

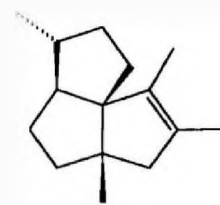
IDENTIFICATION OF SOME RARE HYDROCARBONS



Silphiperfol-5-ene 2.4 %



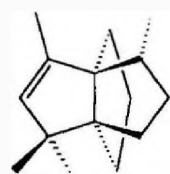
7-*epi*-Silphiperfol-5-ene 6.5 %



Silphiperfol-6-ene 27 %

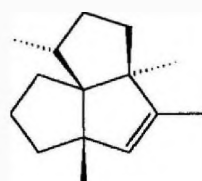
F. Bohlmann 1980; P. A. Wender 1985; P. Weyerstahl 1991

F. Bohlmann 1980



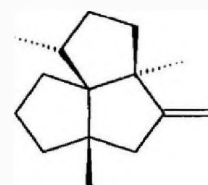
Modheph-2-ene 4.9 %

L. H. Zalkow 1978



α -Isocomene 3.1 %

F. Bohlmann 1977
L. H. Zalkow 1977

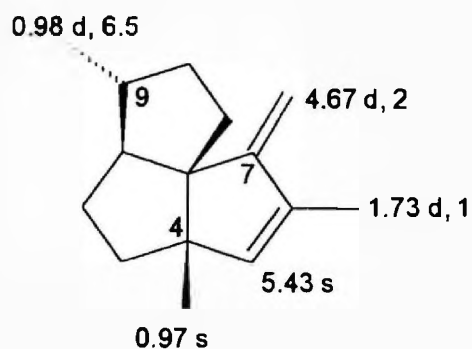


β -Isocomene 2.5 %

F. Bohlmann 1979

Identification: Comparison of MS, $^1\text{H-NMR}$, $^{13}\text{C-NMR}$

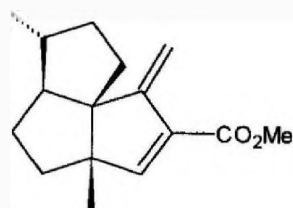
A NEW DIENE : SILHIPERFOLADIENE



Silhiperfoladiene-5,7(14)-diene
or Cantabra-5,7(14)-diene

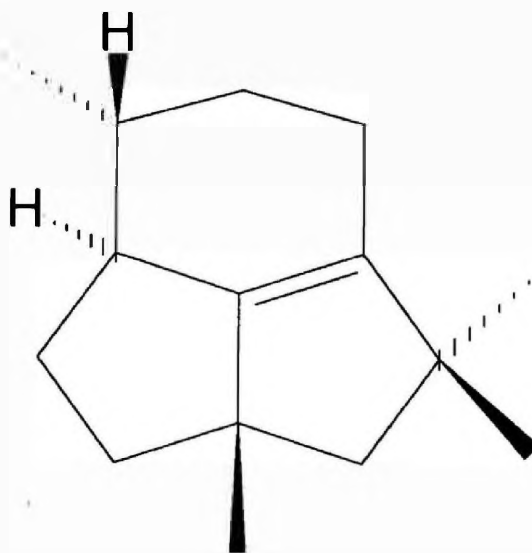
0.4 %

Comparison of the ^{13}C -NMR data
with those of methyl cantabradienoate



isolated from *Artemisia cantabrica*
by A. San Feliciano 1986

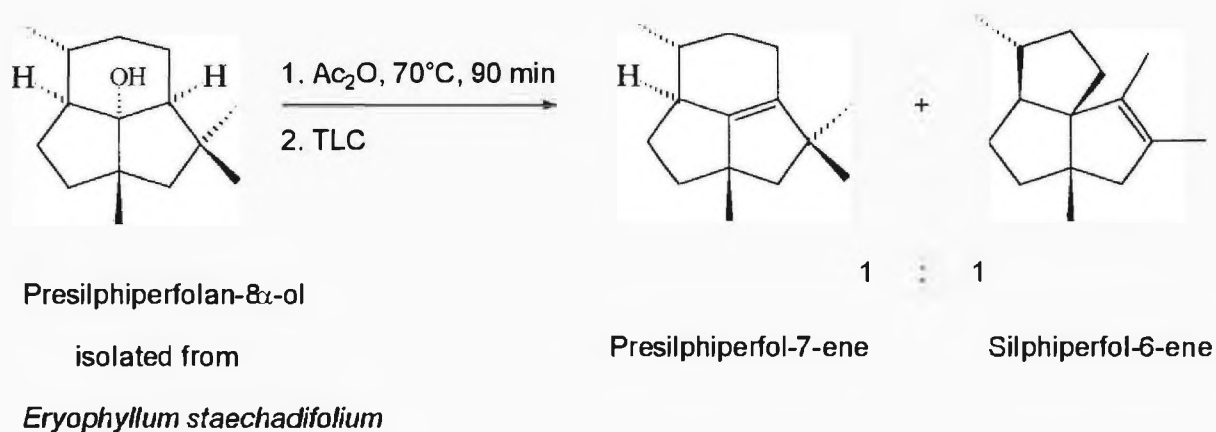
PRESILHIPERFOL-7-ENE, A NEW NATURAL COMPOUND



9.4 %

$[\alpha]_{\text{D}}^{20} -85.2^\circ$ (C_6D_6 , GC: 91 %)

BOHLMANN'S RESULTS ON DEHYDRATION OF PRESILPHIPERFOLAN-8 α -OL



F. Bohlmann 1981

MODHEPH-2-EN-8 β -OL

C₁₅H₂₄O HR-MS

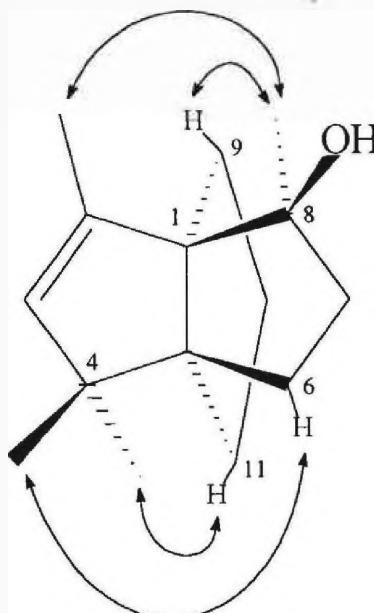
¹H-NMR

5.22 q, 1.5
1.66 d, 1.5
1.31 s
1.05 s
1.01 s

¹³C-NMR

142.8 d
134.2 s
79.7 s
no further doublets

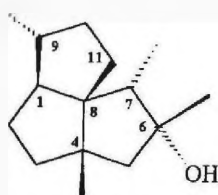
¹H, ¹³C-COSY COLOC



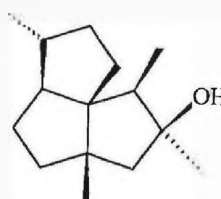
important NOEs

Comparison of
the NMR data
with those of
modheph-2-ene

SILHIPERFOLAN-6-OLS I



A, 1.0 %

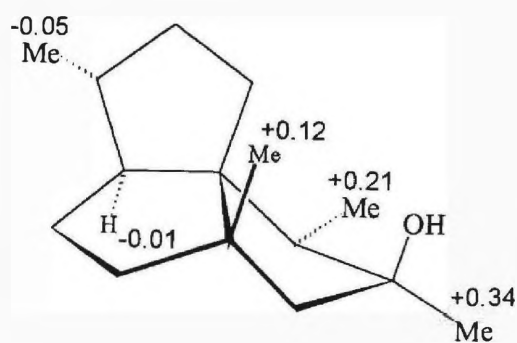
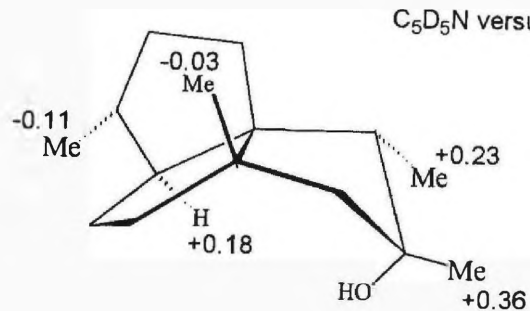


B, 1.0 %

J. C. Coll 1990
¹H- and ¹³C-NMR

¹H-NMR shifts in

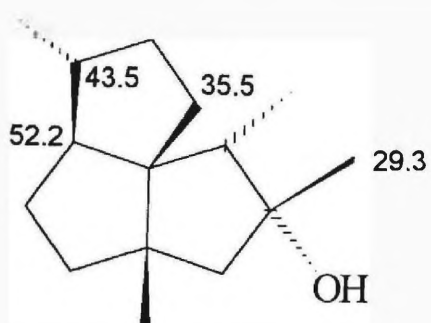
C₅D₅N versus C₆D₆



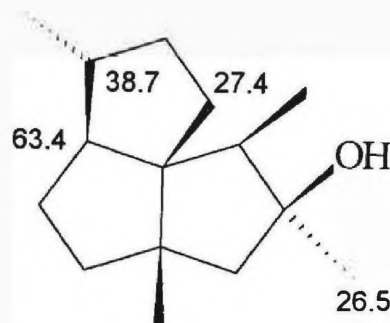
Confirmation by NOED

SILHIPERFOLAN-6-OLS II

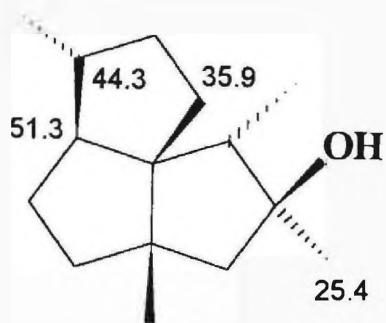
most
 unpolar



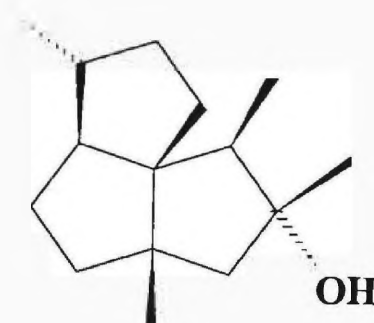
A



B



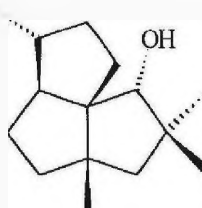
C, 1.4 %



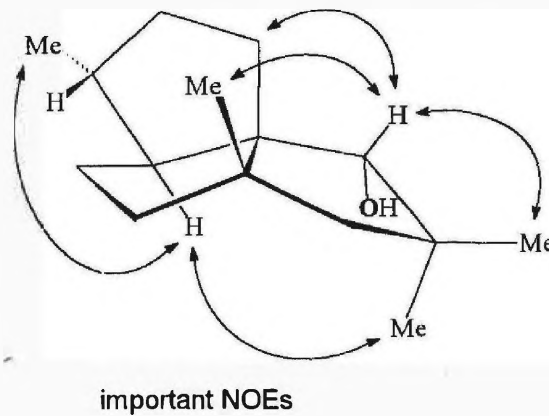
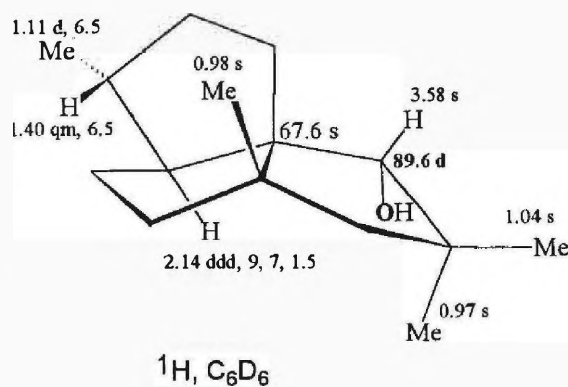
D

most
 polar

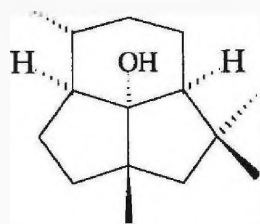
CAMEROONAN-7 α -OL



6 %



FORMATION OF A CAMEROONANE TRIFLUOROACETATE



Presilphiperfolan-8 α -ol

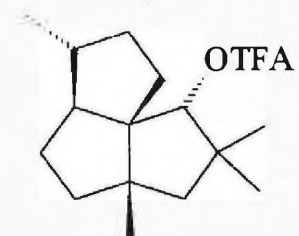


Silphiperfol-6-ene (40 %)

+

Presilphiperfol-7-ene (20 %)

+

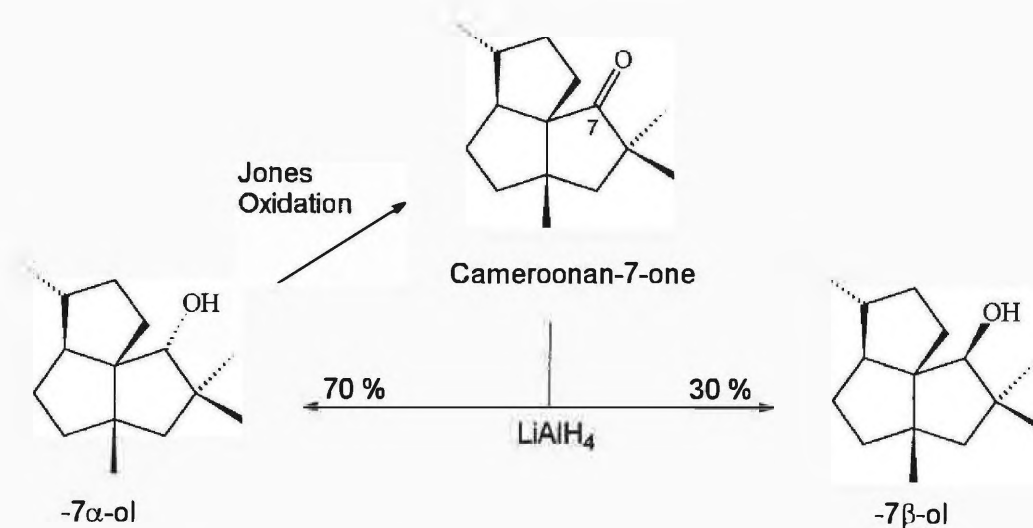


(11 %)

Cameroonane-7 α -yl
trifluoroacetate

R. M. Coates 1996

CAMEROONAN-7-ONE



odor: woody, warm, khusimone

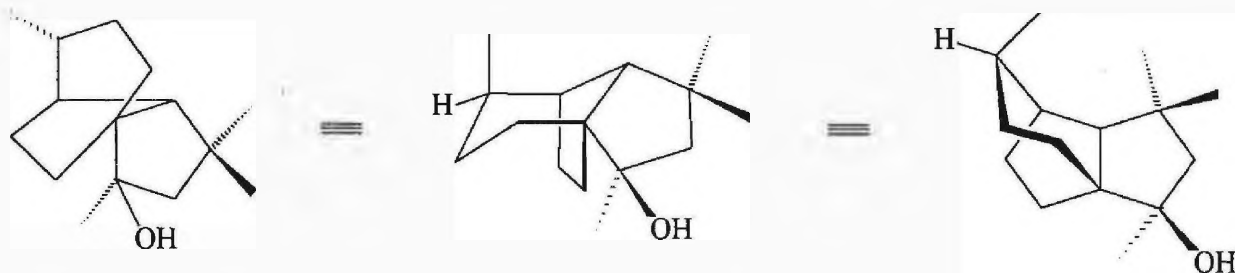
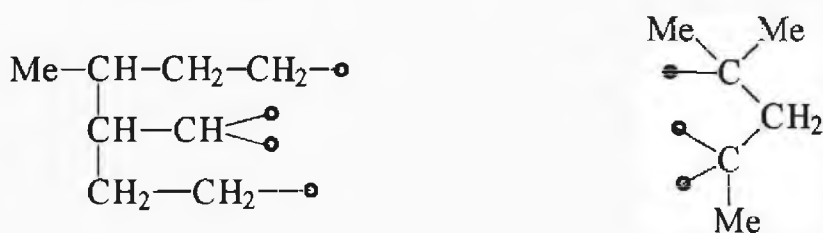
NOPSAN-4-OL

$C_{15}H_{26}O$ HRMS, 0.9 %

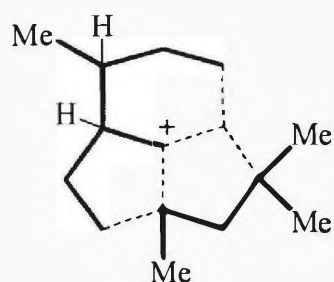
1H : 4 Me δ 0.94, 1.12, 1.15 (3 s), 0.95 (d)

^{13}C : 81.5 (s), 60.3 (s), 37.1 (s), no double bond

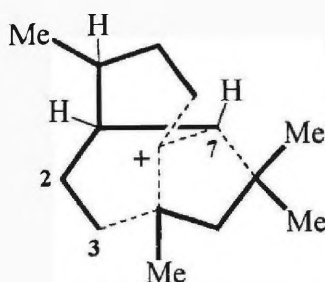
Selective decoupling and COLOC furnished two sequences



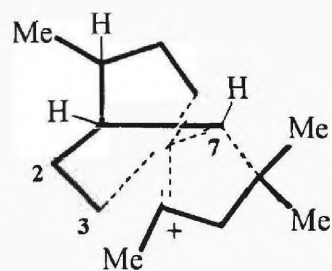
SKELETAL CONSIDERATIONS



Presilphiperfolane



A



B

Decision for skeleton **B** by COLOC:

3J correlation of 7-H to C-2 and C-3

ECHINAN-8-OL

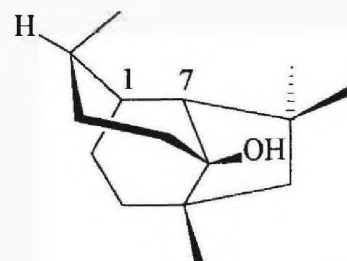
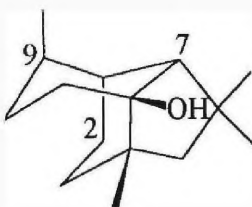
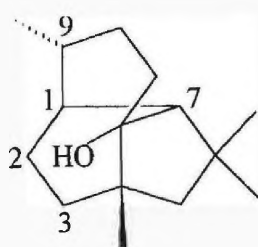
$C_{15}H_{26}O$ HRMS, 2.2 %

1H : 4 Me δ 0.92, 1.12, 1.32 (3 s), 1.00 (d)

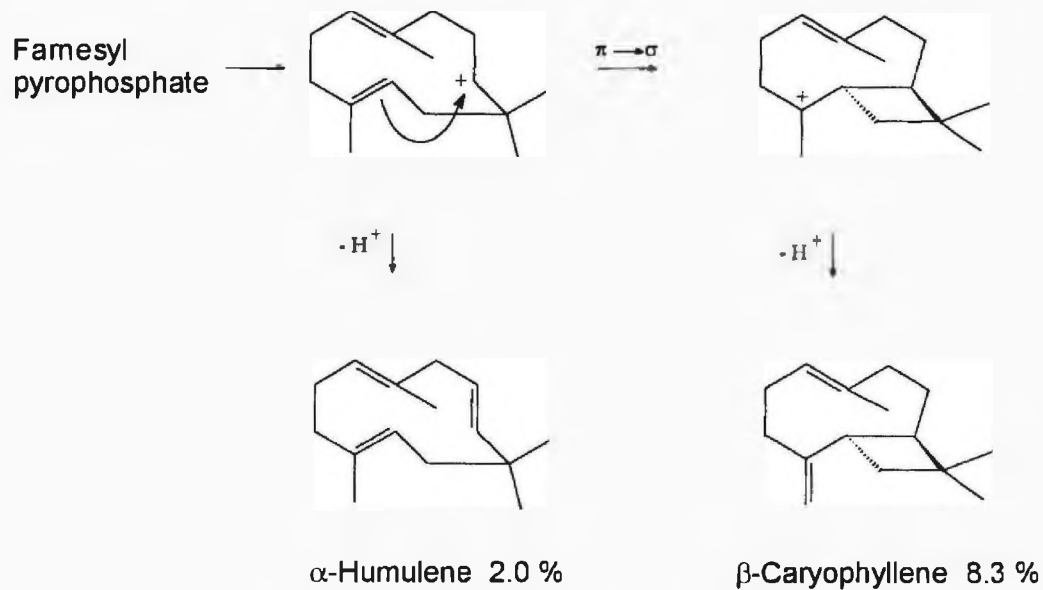
^{13}C : 82.0 (s), 48.3 (s), 36.8 (s), no double bond

COLOC of 7-H with C-2, but not with C-3

Same sequences as found for Nopsan-4-ol

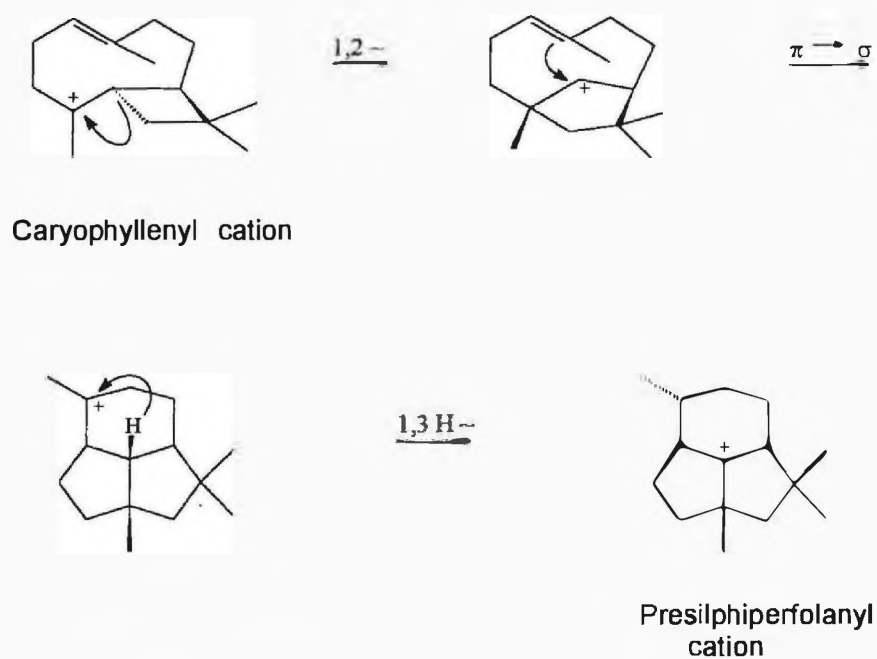


HUMULENYL AND CARYOPHYLLENYL CATIONS



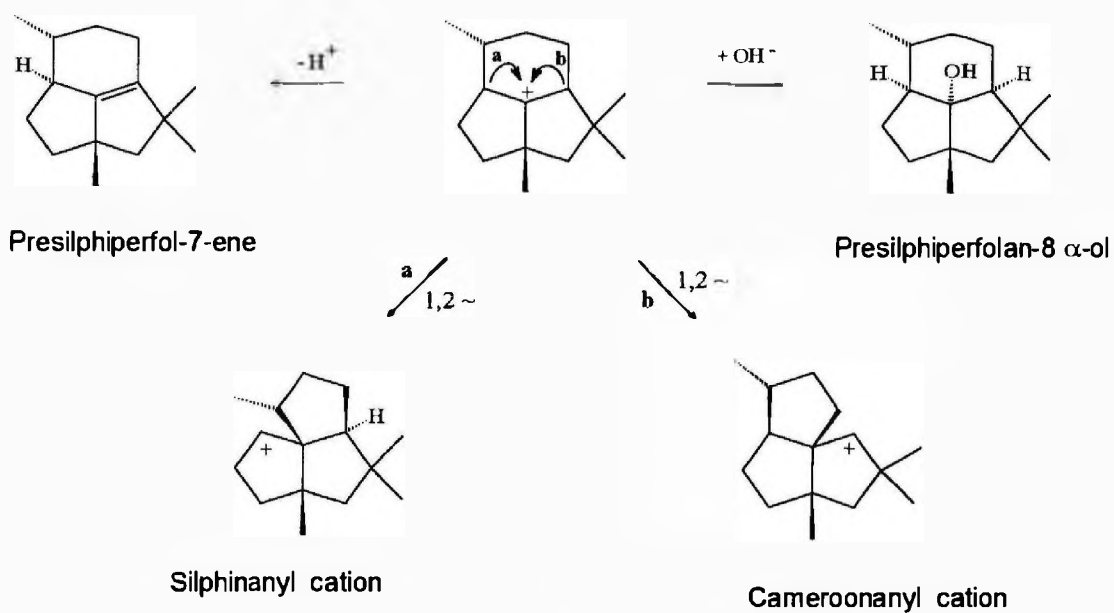
F. Bohlmann 1980

FORMATION OF THE 8-PRESILPHIPERFOLANYL CATION



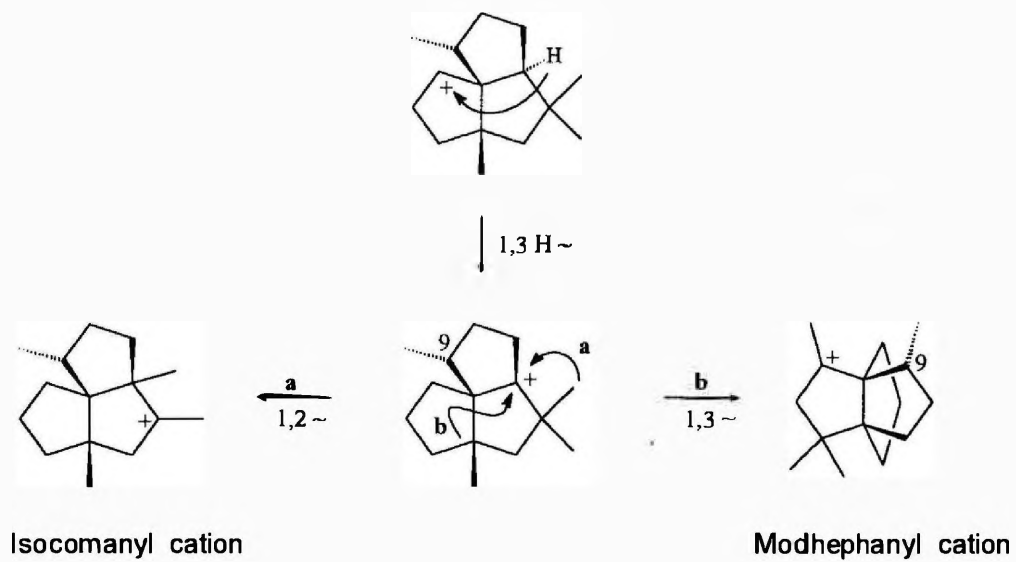
F. Bohlmann 1980

SUBSEQUENT REACTIONS OF THE 8-PRESILPHIPERFOLANYL CATION



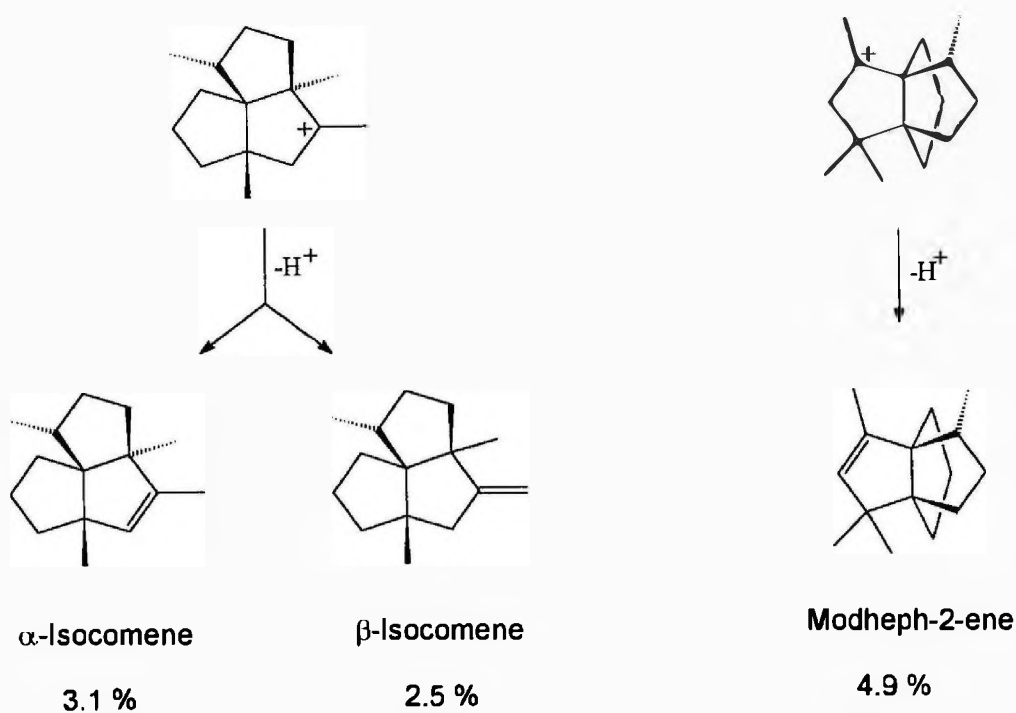
F. Bohlmann 1980

REARRANGEMENTS OF THE SILPHINANYL CATION

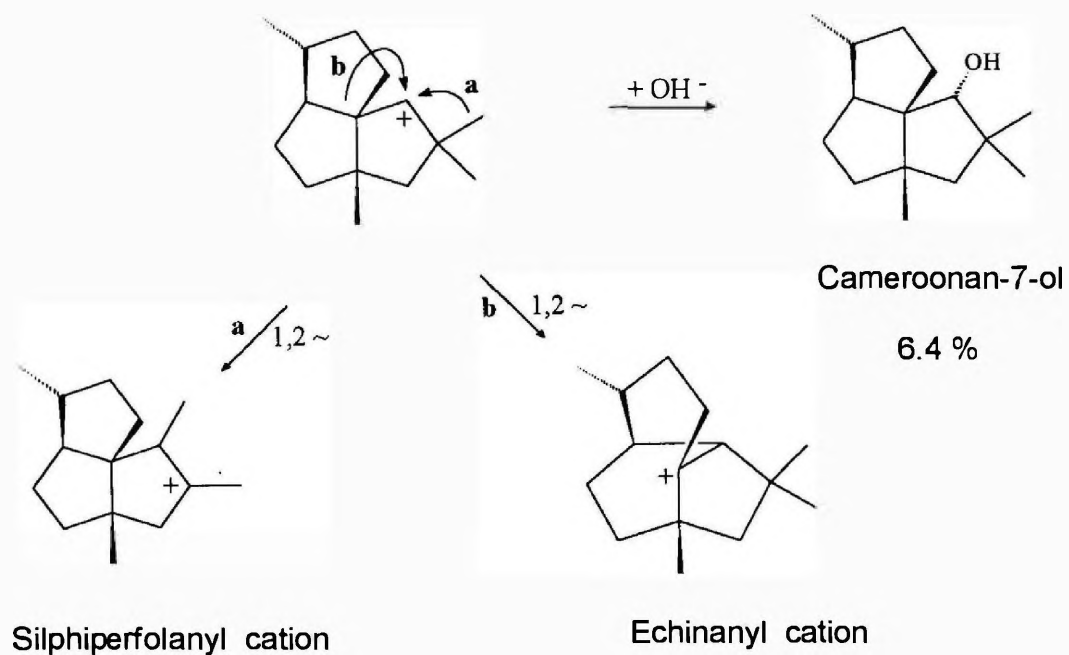


L. H. Zalkow 1978

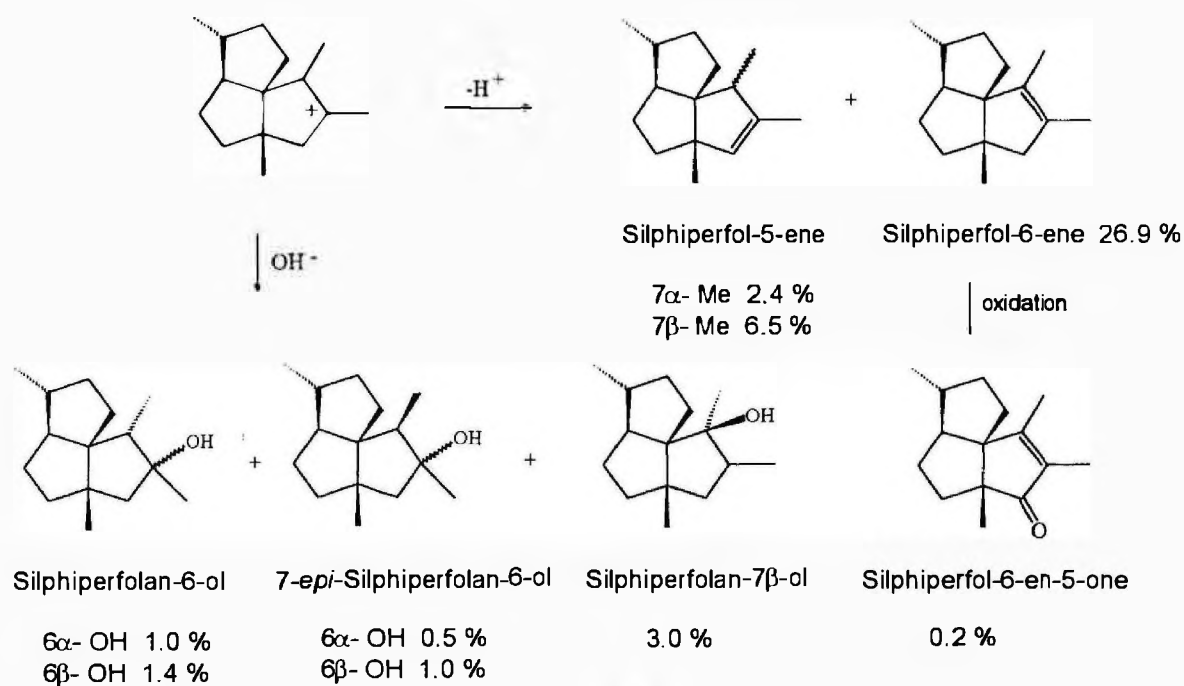
REACTIONS OF THE ISOCOMANYL AND MODHEPHANYL CATION



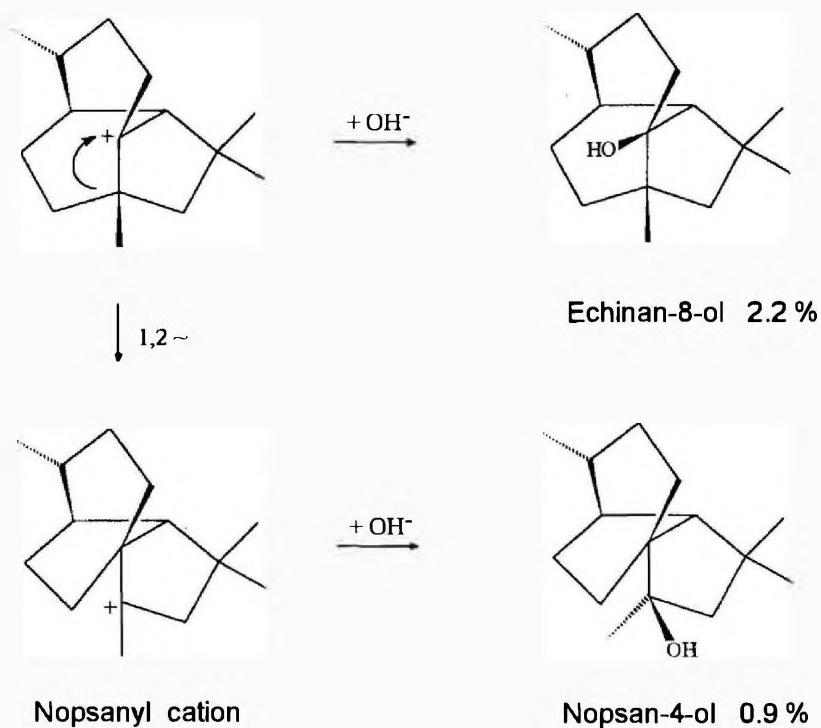
REACTIONS OF THE CAMEROONANYL CATION



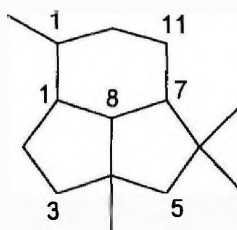
REACTIONS OF THE SILHIPERFOLANYL CATION



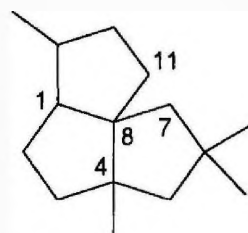
REACTIONS OF THE ECHINANYL CATION



THREE NEW SESQUITERPENE SKELETONS

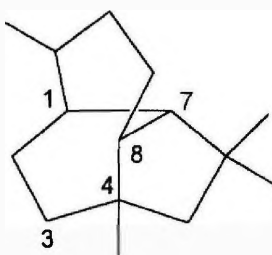


Presilphiperfolane

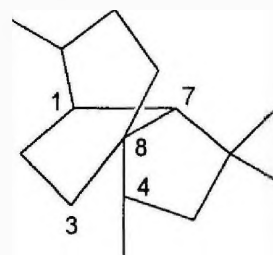


Cameroonane

11(7→8)-*abeo*-Presilphiperfolane



Echinane

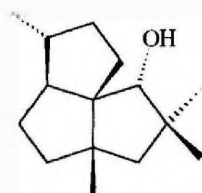


Nopsane

1(8→7)-*abeo*-Cameroonane

3(4→8)-*abeo*-Echinane

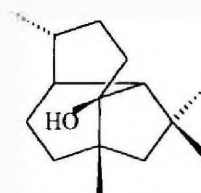
OLFACTORY EVALUATIONS



Cameroon-7-ol (GC: 95 %)

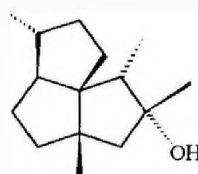
strong woody,
ambergris, patchouli,
dry dusty,
heavy floral

most important



Echin-8-ol (GC: 88 %)

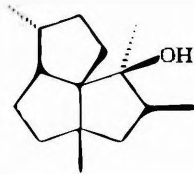
very strong woody,
slightly musty wet



Silphiperfolan-6α-ol (GC: 84 %)

woody, camphoraceous

less important



Silphiperfolan-7β-ol (GC: 92 %)

strong fresh, camphoraceous, woody

total oil

strong woody,
patchouli,
slightly greasy,
heavy floral,
citrus peel

IDENTIFICATION OF RARE SESQUITERPENE STRUCTURES IN ESSENTIAL OILS

W. A. König, C. Fricke, S. Melching, K. Wihstutz
Institut für Organische Chemie, Universität Hamburg, Deutschland.

Sesquiterpene derivatives substantiate a major portion and may often be responsible for a specific sensoric impression of essential oils. In addition, an extraordinary structural variability, caused by numerous skeletal rearrangements during biosynthesis, is observed for sesquiterpenes. The elucidation of the structure and stereochemical properties of unknown sesquiterpenes is therefore a great challenge. The detection of a series of structurally related sesquiterpenes may also serve to recognize biogenetic correlations. The isolation of some novel sesquiterpene constituents from essential oils with the rarely found africanane, brasilane, nardosinane and asteriscane skeletons by preparative chromatographic methods and their identification with modern NMR techniques, mass spectrometry, enantioselective gas chromatography, and by investigating thermally and acid catalyzed rearrangements and micro-chemical reactions will be demonstrated.

ORAL PRESENTATIONS

ANTIFUNGAL PROPERTIES OF ESSENTIAL OIL COMPONENTS

J.C.R. Demyttenaere*, H.M. Willemen, M. del C. Herrera and R. Verhé
Gent University, Faculty of Agricultural and Applied Biological Sciences, Department of
Organic Chemistry, Coupure Links 653, B-9000 Gent, Belgium

ABSTRACT

The antifungal properties of some essential oil components were tested. The inhibitory effect of the phenolic compounds ferulic acid, vanillin, eugenol and isoeugenol, the terpenes geraniol, nerol, citral, citronellol and linalool and the biotransformation products 6-methyl-5-hepten-2-one and furanoid linalool oxides towards the fungi *Aspergillus niger*, *Penicillium digitatum* and *Penicillium* sp. was compared. It was shown that the terpene alcohol citronellol and the phenolic compounds eugenol and isoeugenol were the most inhibitory substrates for all fungal cultures tested, whilst the products formed through biotransformation by the fungi are the least antifungal: high concentrations of methylheptenone and linalool oxide can be tolerated by the fungi, without loss of germination and sporulation capacity. It was postulated that biotransformation of terpenes might be a detoxification mechanism of the fungi.

INTRODUCTION

Although considerable information on the antifungal activity of spices and essential oils is available [1-5], data about the antimicrobial properties of their constituents, especially the terpenes, is very limited [6-9]. In some cases, the composition of the essential oil was studied [9-13], but only few authors also investigated the inhibitory effects of the components of these oils [9-10].

A well studied group of compounds is that of the phenolic derivatives eugenol, thymol, carvacrol, *trans*-anethol (see Fig. 1). Curtis *et al.* (1996) [14] compared the inhibitory and lethal effects of synthetic versions of compounds found in common herbs and spices on a food spoilage yeast *Debaromyces hansenii*. Maritini *et al.* (1996) [9] determined the concentration of eugenol and carvacrol in ethanolic extracts of cloves and savory resp. by

GC. In their study, they compared the antifungal activities of the GC-analysed ethanolic extracts of both spices with those of eugenol and carvacrol alone in similar concentrations. It was found that carvacrol in savory and eugenol in clove were exclusively responsible for the antifungal activity of these spices against *Cladosporium herbarum* and *Penicillium glabrum*.

Carvacrol was also found in the essential oil of *Thymus broussonettii* (53.3%) together with *p*-cymene (13.5%) and α -pinene (8.6%) [12]. Examination of the antimicrobial activity of this oil and two others (*T. zygis* containing 50.6% *p*-cymene and *T. satureioides* containing 31.2% borneol as main constituent) against three bacteria, two yeasts and two molds, revealed that the oil of *T. broussonettii* was most efficient in both killing the microorganisms and inhibiting their growth. To growth inhibition, the molds were more sensitive than the yeasts and the bacteria, but *A. niger* was shown to be more difficult to destroy than the most resistant bacterium, *E. coli*. The inhibitory effect of carvacrol alone was not tested by these authors.

Morozumi *et al.* (1988) [10] investigated the inhibitory effect of 60 commercial foods and spices on the growth and toxin production of common toxigenic fungi such as *Aspergillus flavus*, *A. ochraceus*, *A. versicolor* and *Fusarium sporotrichioides*. Of these, 8 foods showed potent antifungal activity, and 9 antifungal substances were isolated and identified. Apart from the minimal inhibitory concentration (MIC, expressed in $\mu\text{g/ml}$) of foods on growth and mycotoxin production of toxigenic fungi, also the inhibitory effect of some of the main compounds (*o*-methoxycinnamaldehyde, cinnamaldehyde, eugenol,

thymol, anethol, geraniol, citronellol, etc.) was tested. *o*-Methoxycinnamaldehyde was most inhibitory, whilst geraniol and anethol showed the lowest activity.

Another essential oil rich in phenolic compounds is the one of *Origanum syriacum* L. It exists in two phenolic chemotypes, one containing thymol as the major constituent, while the other is rich in its stereoisomer, carvacrol. The percentage of carvacrol in the essential oil increased to 62% after flowering and maturation, while the concentration of thymol decreased to 14% [13]. *Origanum* oil extracted from plants collected during midseason was evaluated for its antifungal activity against *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium* species. The oil exhibited strong inhibitory action against the three fungi tested. The antifungal properties of the pure compounds thymol and carvacrol was not tested.

The inhibitory effect of phenolic compounds as eugenol, thymol and cumin aldehyde compared to that of the terpenes carvone, borneol and thujone was studied by Farag *et al.* (1989) [11]. They investigated the influence of spice essential oils such as thyme, cumin, clove, caraway, rosemary and sage on *Aspergillus parasiticus* growth and aflatoxin production. The essential oils caused complete inhibition of both mycelial growth and aflatoxin production. Thyme, cumin and clove essential oil containing thymol, cumin aldehyde and eugenol resp. were more effective than caraway, rosemary and sage, containing carvone, borneol and thujone resp. It was concluded that there was a relationship between the chemical structures of the most abundant compounds in essential oils and the antifungal effects. The inhibition of the oils was attributed to the presence of an aromatic nucleus containing a polar functional group. This explained why borneol and

thujone had little inhibitory effect compared to thymol which has an aromatic nucleus. The higher inhibitory action of thymol could also be due to the presence of a phenolic hydroxyl group. It is well known that the HO-group is much more reactive and can easily form hydrogen bonds with active sites of enzymes.

The structure-activity relationship was also studied by Knobloch *et al.* (1988) [6]. A relation between the water solubility of terpenes (mM) and antibacterial properties was described. The antimicrobial activity of essential oils was found to be due to their solubility in the phospholipid bilayer of cell membranes [7].

The higher antifungal activity of phenolic compounds compared to that of terpenes was also shown in a study of the antimicrobial activity of essential oils from Sicilian aromatic plants [15]. The highest and broadest activity, both antibacterial and antifungal, was shown by the oils of *Origanum onites* and *Thymus capitatus* both having a high carvacrol content (62 resp. 86%) and commercial oregano (contg. 16% γ -terpinene, 25% terpinen-4-ol, 12% thymol), while oils from commercial sage (contg. 56% 1,8-cineole, 20% limonene) and *Laurus nobilis* (contg. 35% 1,8-cineole, 27% linalool) were almost inactive.

Only few examples are known where terpenes rather than phenolic compounds are responsible for the antimicrobial properties of essential oils and related natural products. Some of the flavour components responsible for the antimicrobial activity of green tea are terpenes [16]. Most of the tea volatiles tested inhibited the growth of one of the most important cariogenic bacteria, *Streptococcus mutans*. Among them, nerolidol was the most potent, linalool was the least effective.

The antimicrobial properties of the leaf essential oil of *Clausena anisata* were also related to its high terpene composition: 33% sabinene and 17% germacrene-D [17]. The antifungal activity of essential oils from aromatic plants of Argentina against the phytopathogenic fungi *Alternaria solani*, *Sclerotium cepivorum* and *Colletotrichum coccodes* could again be related to the high mono- and sesquiterpene content of the essential oils [18]. It was found that the antifungal activity of oils containing more terpene ketones and alcohols or oxides was higher than that of the hydrocarbon rich essential oils, which shows that oxygen contg. compounds are more fungitoxic.

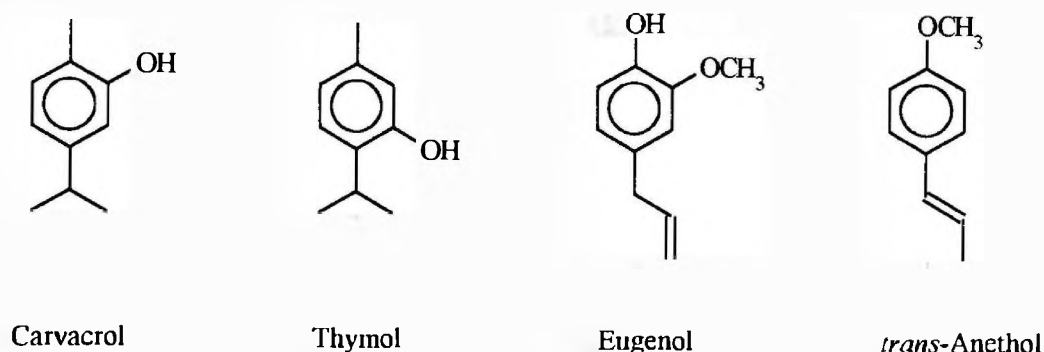


Fig. 1: Phenolic compounds with antifungal properties

It was shown that fungi as *Aspergillus niger* and *Penicillium* sp. are able to biotransform terpenoids [19-20]. In this paper the antifungal properties of the substrates (terpenes) are compared to the inhibitory effects of the biotransformation products formed, because it is believed that biotransformation of terpenes by fungi acts as a detoxification

mechanism. In this study, the test substrates are dissolved in the agar-medium, using ethanol as co-solvent prior to solidification and inoculation of the medium [2]. This method is based on the diffusion of the test substrates in agar and yields MIC (minimum inhibitory concentration) values, whereas the agar overlay technique, using filter paper discs, is also based on the diffusion of the products in agar, but gives inhibition zone diameters [1]. Although the agar overlay technique is the most often employed one for screening of antimicrobially active oils or constituents [21] and is the easiest to use, we believe that the method used in this study mimics best the practical conditions to carry out biotransformation experiments, and hence has the strongest predictive value. However, since bioconversion experiments should always be carried out at sub-inhibitory concentration of the substrate, in the next inhibition tests, the maximum allowed concentration rather than the MIC will be searched for.

RESULTS AND DISCUSSION

The cultures were inoculated on Petri dishes filled with medium to which an ethanolic terpene solution was added before solidification took place. The concentration of ethanol was always 1%, the first concentration of the test substrate was 0.05% (v/v). Dependent on the growth of the cultures the second concentration was higher (0.1%) or lower (0.025%). An overview of all tested substrates is shown in Fig. 2.

The inhibition of 1% (v/v) ethanol was also checked. All strains grown in the presence of 1% ethanol showed germination after 1 day and normal growth. After 3 to 4 days, complete sporulation had occurred for all cultures on the entire surface of the media.

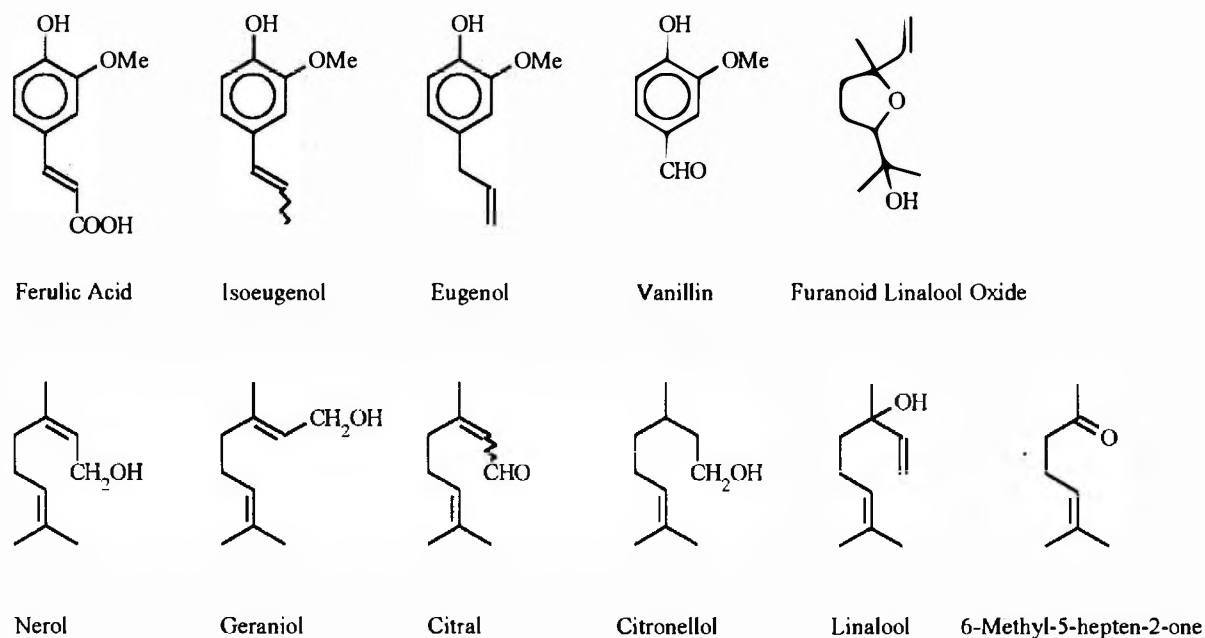


Fig. 2: Structures of the used compounds

The general results for all test substrates at different concentrations applied are displayed below:

- ferulic acid
 - * 0.1 %: The *A. niger* strains germinated after 1 day and had completely sporulated after 4 days. The *Penicillium* strains germinated only on a small part of the surface. Once germination had occurred, the growth and sporulation was completed after 4-6 days.
 - * 0.2 %: The *A. niger* strains germinated after 1 day and had completely sporulated after 4-5 days. The *Penicillium* strains hardly germinated and only after 3-4 days. Growth and sporulation was normal but only on a very small part of the surface.

- vanillin
 - * 0.05 %: For all fungal strains, germination started after 2 days and not on the entire surface. Only after a week almost the complete surface of the cultures had sporulated.
 - * 0.1 %: For most strains, germination was inhibited; during the first week the germination was only noticed on a small part of the surface with ANG and WEL. These local parts had sporulated after a week. The other fungi only showed germination after a week and not on the entire surface.

- isoeugenol
 - * 0.025 %: The first week there was no germination at all. After 8 days the only strain that showed some germination was CLE. It is possible that by then the concentration of substrate had decreased because of evaporation.

- eugenol
 - * 0.05 %: There was no germination shown at all for the first 11 days, meaning complete inhibition.
 - * 0.025 %: For the *A. niger* strains, germination started on a few spots after 5 days and then growth was very slow. CLE started germinating after 4 days and had fully grown and sporulated after a week.

- citral
 - * 0.05 %: Most strains showed no germination at all. Only CLE started after 5 days and had grown and sporulated after 8 days, however not on the entire surface.
 - * 0.025 %: CLE showed normal growth. ANG, COR and ROQ showed germination after 6 days and the other strains only after 12 days. Once germinated the growth was normal.

- geraniol
 - * 0.05 %: Most strains showed no germination at all. Only CLE started after 2 days, however not on the entire surface and had grown and sporulated after a week.
 - * 0.025 %: Of all *A. niger* strains only ANG germinated after 6 days and on a small part of the surface. CLE showed normal growth. The other *Penicillium* strains started germinating after 4-5 days and had sporulated after 9 days, except for CCH. It only started after 10 days.

- nerol
 - * 0.05 %: Most strains showed no germination at all. Only CLE started after 3 days and had grown and sporulated after 7 days.
 - * 0.025 %: The *A. niger* strains started germination after 3 days and had grown and sporulated after 8 days, only ANT not on the entire surface. The *Penicillium* strains started germinating after 2 days and had sporulated after 6 days.
- 6-methyl-5-hepten-2-one
 - * 0.05 %: Germination started after 1-2 days for all strains. They had completely grown and sporulated after 5 days.
 - * 0.1 %: All strains started germination after 1-2 days, however not immediately on the entire surface. But in the next days this improved and after 5-7 days all cultures showed growth and sporulation on almost the complete surface.
- linalool
 - * 0.05 %: Germination started for all fungi after 2 days. The growth and sporulation was normal and was completed after 6-7 days. The sporulation of ANA on the other hand took a few days longer.
 - * 0.1 %: The *A. niger* strains started germination only after 7-8 days and had grown and sporulated after 11 days. The germination of the *Penicillium* strains started after 4 days.

CLE had sporulated after 7 days, the others after 10 days. So the growth was slower than normal.

- citronellol

- * 0.05 %: The first week there was no germination of most of the cultures. Only CLE showed a weak germination after two days, and had sporulated after one week, but only on parts of the medium.
- * 0.025 %: After one week a weaker germination than normal could be observed in most of the cultures, followed by further sporulation. CLE showed normal germination, growth and sporulation.
- * 0.0125 %: The strains of *A. niger* and also CLE and PIN were germinated after one day and had completely sporulated after 4-5 days. The other strains germinated the first day but not on the whole surface. After one week all cultures had completely sporulated.

- linalool oxide

- * 0.05 %: Germination started for all fungi after one day. The growth was normal and sporulation took place after one week.
- * 0.1 %: The *A. niger* strains showed a normal germination after one day, followed by sporulation. The other strains showed germination after 2-3 days. ORA had a slower germination during the first days, but all cultures had completely sporulated after 4-5 days.

* 0.2 %: The germination of most of the strains took place after 2-3 days, being faster with the *A. niger* strains. After one week all strains had completely sporulated.

The results show that inhibition of the fungi is mostly inhibition of germination. Once germinated, growth and sporulation are quiet normal. Table 1 enlists the maximum concentrations per compound per fungal strain at which germination occurs within a week.

Table 1: Maximum tolerated conc (%) for several compounds on 5 *A. niger* and 5 *Penicillium* strains

strain	ferulic acid	vanillin	(iso-) eugenol	citral	geraniol & nerol	methyl- heptenone	linalool	citronellol	linalool oxide
ANG	> 0.2	0.1	< 0.025	0.025	0.025	> 0.1	0.05	0.0125	> 0.2
ANA	> 0.2	0.05	< 0.025	< 0.025	< 0.025	> 0.1	0.05	0.0125	> 0.2
WEL	> 0.2	0.1	< 0.025	< 0.025	< 0.025	> 0.1	0.05	0.0125	> 0.2
COR	> 0.2	0.05	< 0.025	0.025	< 0.025	> 0.1	0.05	0.0125	> 0.2
ANT	0.2	0.05	< 0.025	< 0.025	< 0.025	> 0.1	0.05	0.0125	> 0.2
CLE	0.1	0.05	0.025	0.05	0.05	> 0.1	0.1	0.050	> 0.2
PIN	0.1	0.05	< 0.025	< 0.025	0.025	> 0.1	0.1	>0.025	> 0.2
CCH	0.1	0.05	< 0.025	< 0.025	< 0.025	> 0.1	0.1	0.0125	> 0.2
ROQ	0.1	0.05	< 0.025	0.025	0.025	> 0.1	0.1	0.0125	> 0.2
ROK	0.1	0.05	< 0.025	< 0.025	0.025	> 0.1	0.1	0.0125	> 0.2

For abbreviations of the fungal cultures tested: see experimental section

It can be concluded that the terpene alcohol citronellol and the phenolic compounds eugenol and isoeugenol are the most inhibitory substrates for all fungal cultures tested, whilst the products formed through biotransformation by the fungi are the least antifungal: high concentrations of methylheptenone and linalool oxide can be tolerated by the fungi, without loss of germination and sporulation capacity. From these data, it can be postulated that biotransformation of terpenes might be a detoxification mechanism of the fungi. It is also noteworthy that the more fungitoxic substrates, geraniol, nerol and citral are better transformed by spores than by submerged cells, since spores are more resistant, whilst the less antifungal substrate, such as linalool can readily be converted by submerge liquid cultures. These data give also important information to carry out biotransformation experiments: very inhibitory compounds, such as isoeugenol, eugenol and citronellol should not be added to the growth medium at the time of inoculation. The applied concentration should be lower than the inhibition concentration, or the substrate should be added after growth has taken place, mostly after 24-36 hr. The biotransformation with sporulated surface cultures on the other hand can take place with higher substrate concentrations.

EXPERIMENTAL

Microorganisms. One strain of *Penicillium digitatum* was used, one *Penicillium lividum*, one *P. chrysogenum*, 2 *Penicillium roqueforti* and 5 strains of *Aspergillus niger*.

The *P. digitatum* strain was identified by the MUCL (Mycothèque de l'Université Catholique de Louvain, Laboratoire de Mycologie Systématique et Appliquée, Belgium) as *P. digitatum* (Persoon: Fries) Saccardo. It was assigned as *P. digitatum* ATCC 201167. Earlier research showed that this strain was able to transform geraniol, nerol and citral to 6-methyl-5-hepten-2-one [19-20]. During the experiments, this strain was marked as CLE. The *A. niger* strains were marked as follows: ANG (*A. niger* isolated from a Cypress branch), ANA (*A. niger* DSM 821, obtained from Deutsche Sammlung von Mikroorganismen), WEL (isolated from an African *Welwitschia* plant), COR (isolated from a coriander branch), ANT (*A. niger* ATCC 9142 obtained from the American Type Culture Collection). Two *Penicillium* strains were isolated in the lab: PIN from a Pinus branch and CCH from a contaminated agar plate. One *P. roqueforti* ROQ was isolated from a piece of Roquefort cheese, another *P. roqueforti* ROK from a commercial blue cheese. The cultures were conserved by periodic replications (every 2 weeks) on malt extract agar (MEA).

Growth medium. For the isolation, growth and conservation of the fungi as surface cultures, the medium used was: malt extract agar (MEA) (OXOID®) (malt extract 2%, bacteriological peptone 0.1%, glucose 2% and agar 2%). Fungi were stored in Petri dishes, filled with 20 ml sterile medium.

Inhibition experiments. For the experiment a solution of test substrate in ethanol at 2.5%, 5% or 10% (v/v) was prepared. Every day 20 Petri dishes were filled with 20 ml medium (MEA) to which the ethanolic solution of the test substrate was added prior to solidification of the medium. The concentration of ethanol in the medium was always 1%

(v/v). For each substrate two concentrations were tested. The first concentration of substrate in the medium was usually 0.05% (v/v). The test plates were then inoculated with the fungal strains, using an inoculation needle. The growth was then followed for a week. The second substrate concentration was dependent on those results. When germination and growth of the cultures were normal, the concentration was increased to 0.1% (v/v). When no growth took place or germination was very weak, the second substrate concentration was 0.025% (v/v). All experiments were carried out in duplicate. As a control experiment, medium with only 1 % ethanol was also inoculated with all test organisms: this was done to test the inhibition of ethanol alone.

Test substrates. The following compounds were used as test substrates: ferulic acid (99%, Acros Chimica), isoeugenol (mixt. Z (5%)/E (95%): Janssen Chimica), eugenol (> 99%, Fluka), vanillin (99%, Acros Chimica), geraniol (> 99.5%, Fluka), nerol (90-95%, Fluka), citral (99%, Janssen Chimica), 6-methyl-5-hepten-2-one (99%, Janssen Chimica), (±)-linalool (97%, Aldrich), (±)-citronellol (97%, Aldrich), furanoid linalool oxide (mixt. *cis/trans*, > 97%, Fluka).

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THE EFFECT OF THE ESSENTIAL OIL OF *MELALEUCA ALTERNIFOLIA* ON *ESCHERICHIA COLI*

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ABSTRACT

The essential oil of *Melaleuca alternifolia* (tea tree) exhibits broad spectrum antimicrobial activity. We have further investigated this activity by examining the effect of tea tree oil on the survival, growth rate and physiology of *Escherichia coli* strain AG100. Here we report that tea tree oil-induced cell death is accompanied by an inhibition of respiration and the formation of extracellular blebs on the cell surface observable in electron micrographs.

Kill rate data indicated that 0.125% (v/v) tea tree oil inhibited glucose-dependent respiration and was lethal to cells in the assay conditions used. Below 0.125%, no inhibition of either respiration or the growth rate of cells was detected.

Our results also show that stationary phase cells are more tolerant to tea tree oil than cells harvested in logarithmic phase. At 0.25%, tea tree oil completely reduced cell numbers in suspensions from a 5 hour culture within 20 minutes, whereas, cells grown for 48 hours died more slowly, with complete die-off taking 60 minutes. Similarly, tea tree oil-induced inhibition of respiration was less extensive for stationary phase cells. These findings indicate that longer exposure times were required to cause a lethal degree of physiological damage to *E. coli* AG100 cells in stationary phase.

Overall, the inhibitory effects of tea tree oil on respiration and on cell structure, as observed by electron microscopy, in *E. coli* are consistent with a disruption of membrane structure and function related to the accumulation of tea tree oil monoterpenes. The dose-dependence observed for these effects is most likely a product of the rate and extent of partitioning of monoterpenes between the aqueous phase of cell suspensions and the hydrophobic regions of the cells.

INTRODUCTION

The steam distilled oil of *Melaleuca alternifolia* is commonly known as tea tree oil. Its antimicrobial properties were first described in the literature by Penfold and Grant (1925) and during the following decade claims were made for its success in the treatment of many conditions including fungal infections of the skin and nails, vaginal infections, tonsillitis, catarrh, mouth ulcers and dirty wounds (Humphrey, 1930; Lassak and McCarthy, 1983). However, because of inconsistent quality and quantity of oil production, the demand for the oil declined for several decades.

Tea tree oil has regained popularity over the past twenty years because of its reputation as a safe, natural and effective antiseptic and changes in production methods have lead to a more reliable supply. It is now incorporated as the principal antimicrobial or as a natural preservative in many pharmaceutical, cosmetic or 'cosmoceutic' products for external use, including face washes, pimple gels, vaginal creams, veterinary skin care products, foot powders, shampoos and conditioners.

The chemical composition of tea tree oil has been well defined and consists largely of monoterpenes (Brophy *et al.* 1989). There is a large natural variation in oil composition with the concentrations of two significant components, terpinen-4-ol and 1,8-cineole being inversely proportional to each other (Southwell *et al.* 1996). The draft standard ISO/DIS 4730 Oil of *Melaleuca* - Terpinen-4-ol type (1994) requires a minimum concentration of 30%

terpinen-4-ol and a maximum of 15% 1,8-cineole. These parameters were selected to exclude the higher cineole chemical varieties of *M. alternifolia*, as such oils are less potent antimicrobials (Southwell *et al.* 1993).

The broad antimicrobial activity of tea tree oil has been reported in a number of studies (reviewed by Markham, 1997), and this activity attributed principally to terpinen-4-ol (Southwell *et al.* 1993; Carson and Riley, 1995). Although the oil is well accepted in the marketplace for its antimicrobial properties little is known about its effect on the physiology of microorganisms. An understanding of the mode of action will elucidate the interaction between the various components and is a necessary foundation for the determination of the optimal composition of the oil. In addition, it will provide insights for formulators into the effects of combining oil with other ingredients in cosmetic and pharmaceutical products.

This paper reports the preliminary results of a study into the effect of tea tree oil on the physiology of the Gram-negative bacterium, *Escherichia coli* strain AG100. The results demonstrate changes to membrane function and to cell ultrastructure, with differences in tolerance to tea tree oil of logarithmic and stationary phase cells.

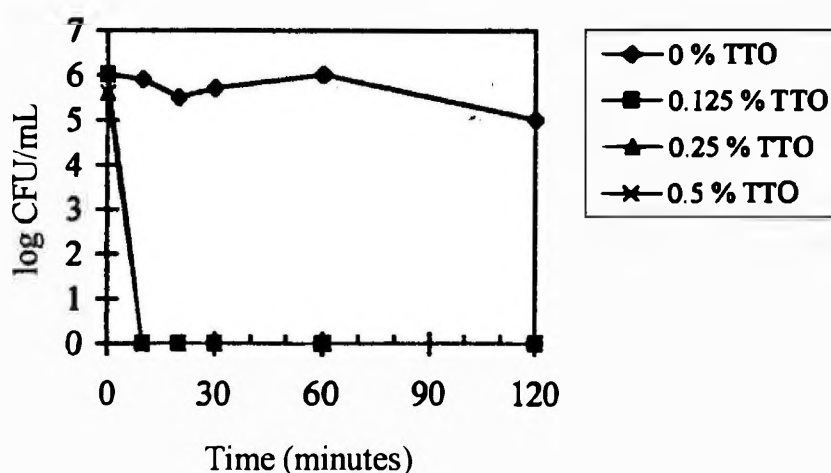
RESULTS AND DISCUSSION

Minimum inhibitory concentration (MIC)

In order to establish an appropriate range of concentrations for subsequent experiments, the Minimum Inhibitory Concentration (MIC) of the oil against *E. coli* AG100 was determined. After 24 h incubation, the MIC was determined to be 0.25% (v/v).

Effects of tea tree oil on cell viability

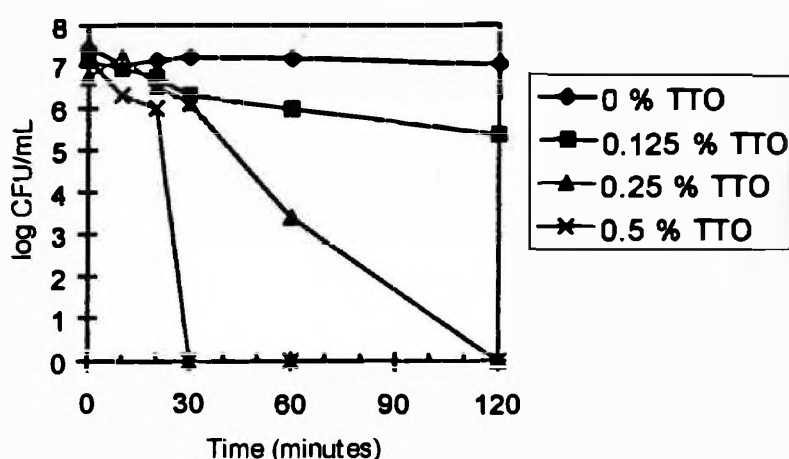
Fig. 1 Viability of logarithmic phase cells of *Escherichia coli* AG100 after exposure to tea tree oil



Following exposure to tea tree oil, cells of *E. coli* AG100 which had been harvested in early logarithmic phase, showed a rapid decline in viability, expressed as the number of colony-forming units per mL (cfu/mL) (Figure 1). No cells were culturable after 10 m exposure to concentrations of oil ranging from 0.125-0.5%. Cells harvested in stationary phase were less

susceptible to tea tree oil (Figure 2). In the presence of 0.25% (v/v) tea tree oil (the MIC) no viable cells were detected after 2h exposure. The kill rate increased at higher oil concentrations with loss of viability after 30 minutes exposure to 0.5% oil, but was markedly reduced at oil concentrations below the MIC. Exposure to an oil concentration of 0.125% (v/v) for 2h resulted in a reduction of cell viability of approximately one-and-a-half orders of magnitude.

Fig. 2 Viability of stationary phase cells of *Escherichia coli* AG100 after exposure to tea tree oil



The determination of MIC, MBC and kill rates are important because they enable the correlation of biochemical and physiological effects with inhibitory and lethal concentrations of the antimicrobial. However, it is more difficult to pinpoint the time of death of the cells,

as what is measured in these experiments is, in fact, the time required for the irreversible uptake of a lethal dose of the oil.

Effects of tea tree oil on respiration

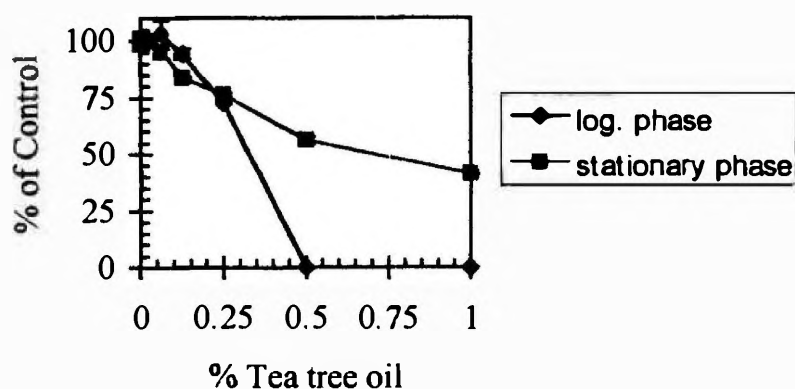
The results in Figure 3 show that at the MIC there is a 25% inhibition of respiration in both logarithmic and stationary phase cells. With cells harvested at logarithmic phase, increasing the concentration of tea tree oil to 0.5% (v/v) resulted in total inhibition of respiration. Stationary phase cells were more tolerant, although inhibition of respiration was dose dependent. At an oil concentration of 1% (v/v) the rate of oxygen uptake was approximately 45% that of the control. At concentrations of oil below the MIC the effects on respiration were slight. In the presence of tea tree oil, the time taken for a maximal rate of oxygen consumption to be reached upon addition of glucose showed an obvious delay when compared to assays that contained no oil (data not shown). This effect was apparent even in the presence of oil levels that did not reduce the maximum rate of oxygen uptake. These observations suggest that the effects of tea tree oil on respiration are not confined to an inhibition of the electron transport processes which lead to oxygen consumption.

Concentrations of tea tree oil which cause rapid loss of viability (Figures 1 and 2) correlate with the concentrations of oil which inhibit oxygen uptake (Figure 3). The fact that inhibition of respiration was not observed below an oil concentration of 0.125% (v/v), the minimum concentration causing a loss of viability of the more susceptible logarithmic phase

cells, suggests that the plasma membrane, the site of the electron transport complex in bacteria, may

be a primary site of action of tea tree oil in the cell. The reported experiments do not distinguish which component(s) of the respiratory chain are affected, but the lack of significant inhibition below oil concentrations determined in other assays to be inhibitory, suggest an effect on cellular processes related to a physical disruption of membrane structure.

Fig. 3 Effect of tea tree oil on respiration in suspensions of *Escherichia coli* AG100



Electron microscopy

Consistent with the above hypothesis, preliminary results of transmission electron microscope studies have demonstrated a disruption to cell ultrastructure following treatment with tea tree oil. Two major changes were observed in cells cultured for one hour in the

presence of tea tree oil at concentrations of both 0.125% and 0.25% (v/v). Coagulation of intracellular material was observable in the majority of treated cells and extracellular blebs were present on some cells. Such blebs may result from leakage of intracellular material due to disruption of the cell membrane and / or wall, or may be due to interference with other physiological processes in the cell, such as cell division. As in the other assays reported, the effects were more marked with logarithmic phase cells than with stationary phase cells. The technique used did not, however, demonstrate significant disruption to the membrane structure.

Conclusions

This study provides some preliminary evidence that the antimicrobial activity of tea tree oil is due to a disruption of membrane structure and function. The hydrophobicity of the major active ingredients of tea tree oil suggest that accumulation is likely to occur in the hydrophobic regions of the cell such as in the membrane bilayer. In a study of the uptake of radiolabelled cyclic hydrocarbons, including toluene, carvone and tetralin into liposomes, Sikkema *et al.* (1994) have reported the partitioning of such compounds into the lipophilic membrane. These authors also report that the accumulation of cyclic hydrocarbons in the membranes is proportional to the concentration of the compound in the aqueous phase. This observation could explain the greater activity of the oxygenated monoterpenes, such as terpinen-4-ol, which have limited water solubility, in comparison to the hydrocarbon

monoterpenes, which are considered insoluble in water. Additionally, the increased rate at which cells lose viability as oil concentration increases, is probably a result of an increase in both the rate and extent of monoterpene accumulation in the membrane. This bioavailability of the active ingredients in tea tree oil is important to consider during the development of formulations containing the oil.

The experimental evidence reported in this paper all demonstrates that stationary phase cells are more tolerant to tea tree oil than cells harvested in the logarithmic phase. In the natural environment, bacteria are seldom in conditions which enable exponential growth: cell structure and metabolism will more frequently resemble that of laboratory-cultured stationary phase cells. The greater resistance of such cells to a variety of environmental stresses has frequently been reported in the literature (for example, Kolter *et al.* 1993). A number of genes which are induced under conditions of stress, such as nutrient deprivation, have been identified in *E. coli*, but the mechanisms of increased tolerance are not well understood. It is possible that changes in the surface structures of stationary phase cells of *E. coli* limit the uptake of the monoterpenes in tea tree oil, or that there are variations in the lipids of the membrane which render them more tolerant to the deleterious effects of tea tree oil.

EXPERIMENTAL

Tea tree oil

Tea tree oil used in all experiments was from a single batch of Main Camp pharmaceutical grade oil, containing 39.8% terpinen-4-ol and 4.5% 1,8-cineole.

Growth of bacteria

Inocula for all experiments were twice passaged in Isosensitest broth. A 1% inoculum was transferred to 500mL of Isosensitest broth, incubated at 37°C with shaking (200rpm) and harvested at either 5 hours (logarithmic phase) or at 48 hours (stationary phase) by centrifugation for 10 minutes at 5000 rpm at 13°C. Cell pellets were washed in sterile distilled water and resuspended in 100mM sodium phosphate buffer, pH7, containing 0.5% Tween 20. Cell density was adjusted to approximately 10^6 cells per mL for kill rate studies and 10^{10} cells per mL for determination of respiration rate. Cell densities were confirmed by the plate count method.

MIC determination

MIC values were determined in triplicate by an agar dilution method (Mann and Markham, 1997).

Kill rate studies

Tea tree oil-induced reduction in cell viability was determined in continuously stirred suspensions containing a range of tea tree oil concentrations in 100mM sodium phosphate

buffer, pH 7, containing 0.5% Tween 20 at 22°C. Suspensions were sampled at 10m, 20m, 30m, 60m and 120m and transferred to an inactivation broth containing 0.1% peptone, 5% lecithin and 5% yeast extract. After 10m dilutions were prepared in 0.1% peptone and viable counts were performed on Trypticase Soy Agar to encourage resuscitation of sub-lethally impaired organisms. All experiments were repeated on three separate occasions.

Oxygen consumption

Oxygen consumption was measured using a Clark-type oxygen electrode and oxygen meter (Microelectrodes, Inc.) connected to a chart recorder. Cell suspensions (2mL final volume in 100mM sodium phosphate buffer, pH 7, containing 0.5% Tween 20) were treated with a range of tea tree oil concentrations and pre-incubated with stirring (5 minutes, 25°C). The change in oxygen concentration was measured after the addition of 25 μ moles of D-glucose. Oxygen concentration was calculated as nmoles O₂ consumed per 2mL suspension and respiration rates are expressed as a percentage of the control activity which contained no tea tree oil. Measurements were carried out in duplicate and the experiment repeated on three separate occasions.

Electron microscopy

Logarithmic phase cells in Luria broth (Gibco BRL) were treated with 0.125% or 0.25% (v/v) tea tree oil for one hour, and untreated cells were included as controls. Cells were harvested by centrifugation and resuspended in 2.5% glutaraldehyde in 0.05M cacodylate buffer, pH

7.2, and fixed at room temperature for two hours. The cells were then washed in two changes of cacodylate buffer and stained in tannic acid solution overnight. Stained cells were embedded in 1.25% (w/v) agar followed by dehydration and embedding in increasing concentrations of alcohol, followed by epoxy propane and Epon 812. Polymerisation of the resin was carried out overnight at 70°C. Sections were stained with uranyl acetate followed by lead citrate.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support of the Australian Tea Tree Oil Research Institute (ATTORI) and thank Main Camp Pty Ltd for supplying samples of tea tree oil.

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Biosynthesis and Hydrolysis of Monoterpene Glycosides

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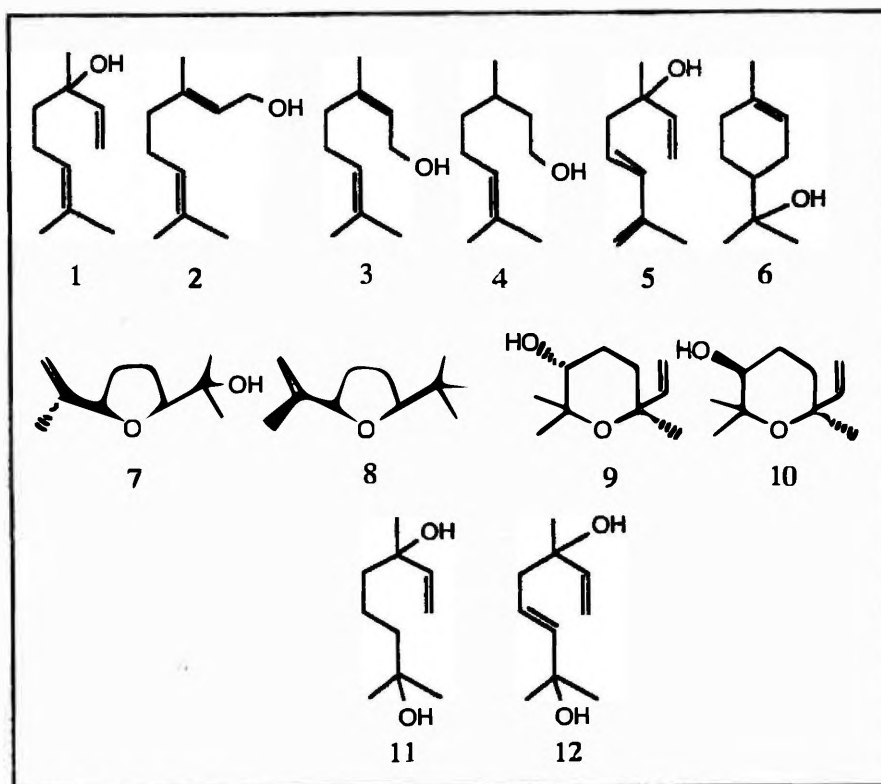
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Introduction

Monoterpenes play an important role in grape and wine flavor (1,2). Cordonnier and Bayonove (3) were the first to suggest that grapes of *Vitis vinifera* cv Muscat of Alexandria contain non-volatile forms of monoterpene glycosides. In grapes and wines of that variety, as well as of variety Rhine Riesling, Williams et al. (4) identified a series of glycosides of monoterpene alcohols.

The glycosides are not hydrolyzed during the fermentation process and remain as a non-utilized potential source of aroma (5).

The ratio between the potential (bound) and the free monoterpenes varies between 5:1 in Muscat of Alexandria and 1:1 in some non-muscat varieties (6).



1 - Linalool; 2 - Geraniol; 3 - Nerol; 4 - Citronellol; 5 - Hortienol; 6 - p-Menth-1-ene-9-ol; 7 - cis-Furan linalool oxide; 8 - trans-Furan linalool; 9 - cis-Pyran linalool oxide; 10 - trans-Pyran linalool oxide; 11 - 3,7-Dimethylocta-1-ene-3,7-diol; 12 - 3,7-Dimethylocta-1,5-diene-3,7-diol (and also Benzyl- and 2-phenylethyl-alcohols).

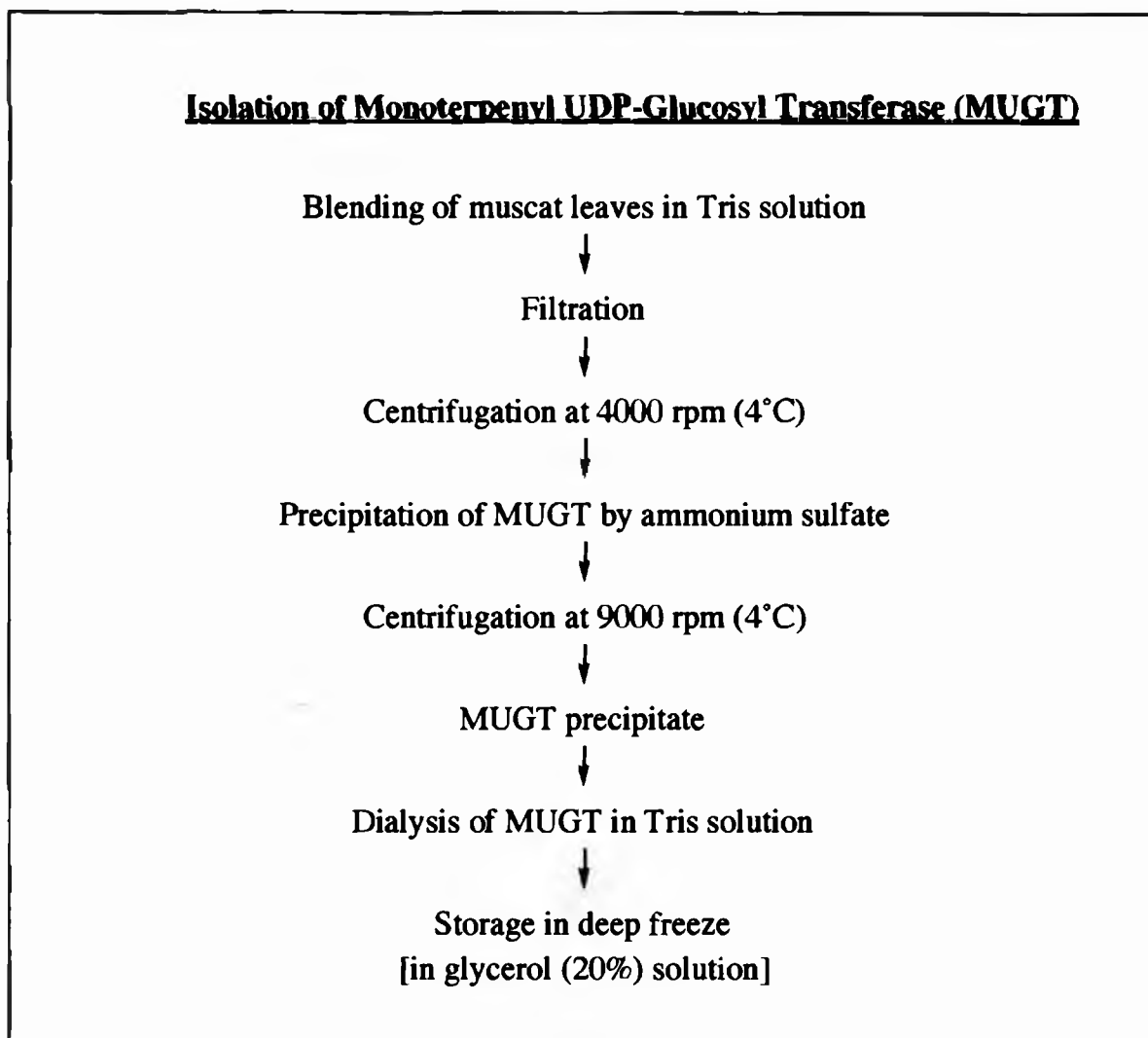
The free monoterpenes are present in different quantities in different grape varieties: in muscat in particular, they form the major part of the aroma and consist essentially of terpenols in various states of oxidation and of terpenoid polyols (7).

The above figure shows the main aroma compounds in wine.

The non-volatile glycosidically bound terpenols in wines have been isolated and characterized by several investigators (1,5). The distribution of monoterpene glycosides in various plants has been summarized in a review by Stahl-Biskup (8).

Biosynthesis

In order to clarify the mechanism of formation and transportation of glycosidically bound monoterpenes in the leaves and the berries of grapes, we have accomplished a biosynthetic study of the formation of ^{14}C -monoterpenyl glucoside (geranyl glucoside) in the leaves of Muscat of Alexandria. This was accomplished by two pathways (9):

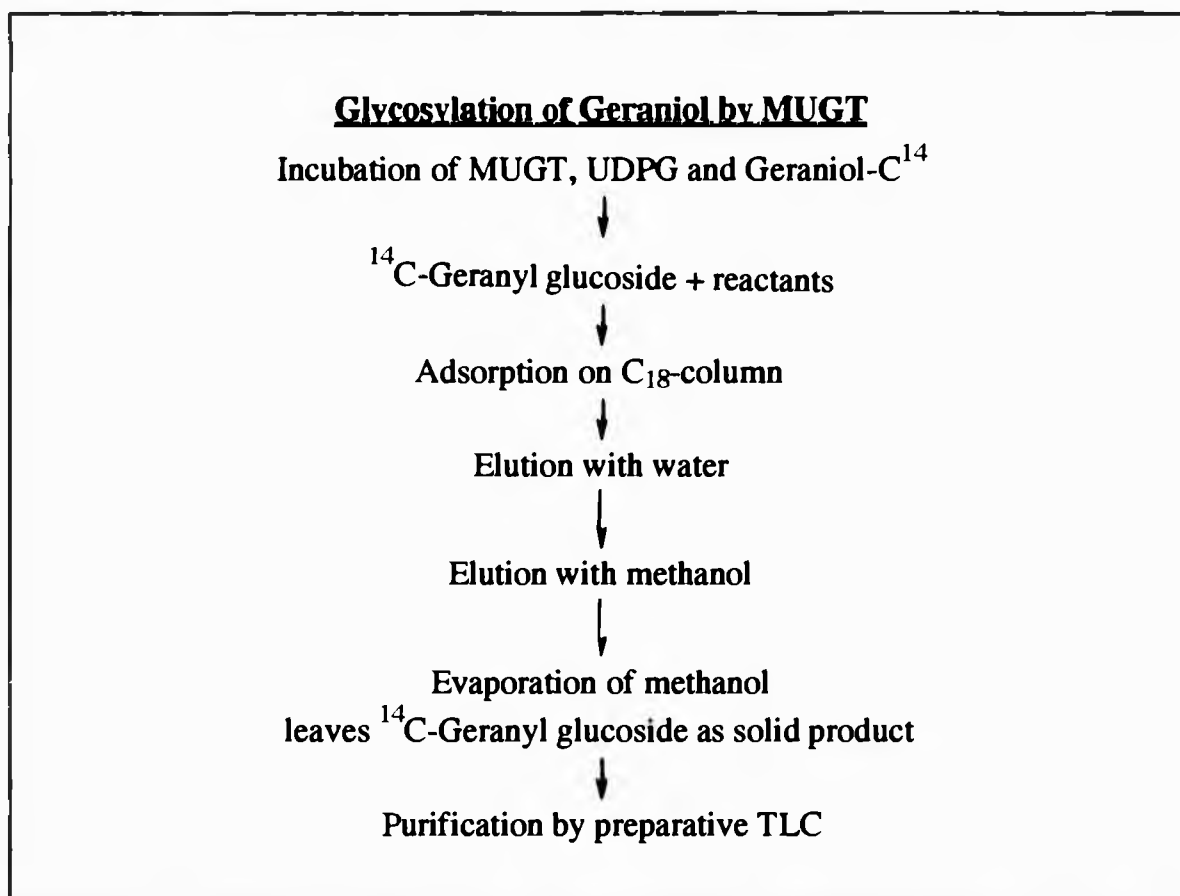


(a) By the reaction of the enzyme MUGT (Monoterpenyl Uridine

Diphosphate Glucosyl Transferase), which was isolated from the leaves of Muscat of Alexandria (see the above flowsheet), and natural geraniol in the presence of ^{14}C -UDPG (Uridine Diphosphate Glucose). The biosynthesis was carried out in a solution of Bis-tris propane (pH 6.9) containing magnesium chloride and DTT, at 32°C , for periods of 1 and 24 hours. Upon termination of the incubation ^{14}C -geranyl glucoside and the remaining reactants were adsorbed on a C_{18} -column. After elution with distilled water (which eluted the unreacted reactants), the column was eluted with methanol which eluted the glycosidic fraction. Removal of methanol yielded ^{14}C -geranyl glucoside as a solid product.

It was analyzed by thin-layer chromatography (TLC). The reference compound (geranyl glucose) was synthesized by treating glucose pentaacetate and geraniol, followed by a mild alkaline hydrolysis of geranyl glucoside pentaacetate with sodium methoxide.

The elution solvent for TLC was *n*-propanol-ethyl acetate 9:3). The spots on the chromatogram were detected by spraying with a solution of diphenylamine (2% in acetone) - 5 parts, aniline (2% in acetone) - 5 parts and phosphoric acid (85%) - 2 parts followed by heating the plate at 120°C . R_f of geranyl glucoside was 0.7. The identity of ^{14}C -labeled geranyl glucoside was also detected by means of autoradiogram.



(b) The alternative biosynthetic approach for the formation of geranyl glucoside was by the reaction of glucosyl transferase (MUGT) with ^{14}C -labeled

geraniol in the presence of UDPG. (See the above flowsheet).

The yield of ^{14}C -labeled geranyl glucoside was lower by this pathway, probably due to the low specific activity of ^{14}C -geraniol which was formed by incubating leaves of Geranium plant in ^{14}C - CO_2 containing atmosphere for 3 days, followed by steam distillation and extraction with a mixture of freon 11 and dichloromethane. Its specific activity and identity were measure by scintillation counter and verified by gas chromatography.

Enzymatic hydrolysis of monoterpenyl glycosides

It has been reported (6) that terpene flavorants of grapes are derived from a pool of non-volatile monoterpene glycosides which are regarded as "hidden" aromatic potentials. The glycosidic part was found to be mostly diglycosidic, whereas the aglycone moiety was terpenolic such as linalool, α -terpineol, citronellol, geraniol, hotrienol, linalool pyranic oxides and aromatic alcohols such as benzyl and 2-phenylethyl.

In order to determine the bound fraction of the aroma of glycosidic hydrolysis, two methods were attempted. Acid hydrolysis was discarded because it induces considerable structural modifications of the free terpenols (3, 10). Enzyme treatment of grape juices and wines to increase the concentrations of volatile monoterpene flavorants could provide considerable benefit to wine making. Commercial enzymes such as rohapect C and other available pectinases were inactive for cleaving the glycosidic bonds at vinification temperature and pH levels, as well as in the presence of glucose and ethanol (2).

Recently we have isolated an endo-b-glycosidase from a unique strain of *Aspergillus niger*. Its optimum pH was 3.4 and its optimum temperature was 65°C (11). The enzyme's activity was not inhibited by fructose, sucrose, sulfur dioxide and was enhanced by ethanol. This enzyme was utilized during juice fermentation and also in wines for the enrichment of aroma by the hydrolysis of monoterpene glycosides.

Banthorpe et al (11) and Skopp and Horster (12) have attempted to clarify the possible involvement of monoterpene glycosides as the transport form of the free monoterpenes.

Feeding experiments with Muscat of Alexandria with ^{14}C -labeled mevalonic acid indicated that monoterpene glycosides were transformed from the leaves to the berries (13).

The present study is in agreement with that proposal.

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THE BIOSYNTHESIS OF ESSENTIAL OIL COMPONENTS IN AROMATIC PLANTS

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ABSTRACT

The unique fragrant properties of essential oils are determined by the chemical structure and relative abundance of their volatile components. These volatile compounds belong to several chemical groups, such as mono- and sesquiterpenes, phenylpropanoids and others. Plants produce and store essential oils in specialized anatomical structures, such as oil cells, cavities, glands and ducts, and possess dedicated enzymatic pathways that biosynthesize the essential oil components from metabolic precursors. The presence or absence of particular biosynthetic enzymes in a given plant tissue, ultimately determines the composition of the essential oils produced and stored. This short review presentation focuses on a few examples demonstrating the several biosynthetic pathways to mono- and sesquiterpenes, phenolic compounds and other essential oil components. Some of the postulated ecological roles of essential oils are also discussed.

INTRODUCTION

The ecological roles of essential oils

Essential oils are volatile secondary metabolites largely responsible for the odors of aromatic plants. Most of them have biological roles that are still unknown, but in a few cases, their ecological roles, such as defense and biological recognition, is well established. For example, many plant pests are repelled by plant volatiles, while sometimes pest

predators are attracted by the same volatiles (Takabayashi *et al.*, 1994). Essential oils inhibit stored-product insect development, and studies are currently underway to test if they could replace chemical fumigants to preserve grain during storage (Shaaya *et al.*, 1991). Essential oils also have allelopathic properties and inhibit plant germination (Dudai *et al.*, 1994). It is also well known that plant volatiles attract pollinators and entomophages (Dobson 1993, Langenheim 1994), and serve as precursors for insect pheromones (Gijzen *et al.*, 1993).

Anatomical Specialization for Essential Oil Accumulation

Since essential oils are often water-insoluble products and toxic to the plants that produce them, they are usually secreted into and stored in special metabolic compartments. Anatomical specialization to form unique secretory structures such as oil cells, oil glands, oil cavities and oil ducts are often encountered in aromatic plants. For example, in lemongrass (*Cymbopogon citratus*) the essential oil is stored in individual cells, scattered in the mesophyll tissues of leaves (Lewinsohn *et al.*, *In Press*). Other aromatic plants, such as those belonging to the mint family (Lamiaceae) store and produce their essential oils in multicellular secretory glands (also called glandular trichomes), that are normally located on the surfaces of both sides of the leaves (Werker 1993). Other plants, such as citrus and patchouli store their essential oils in internal multicellular cavities (Fahn 1979). Many conifers store their resin in interconnected systems of 'resin ducts' by which the resin can be transported from one site of the plant to another (Fahn 1979). All these specialized tissues

not only store the essential oils, but in most cases investigated, these tissues are also found to be the sites of their biosynthesis, and contain the enzymes involved in the special biosynthetic pathways to the different essential oil components (Croteau 1986, Gershenzon 1994).

The biosynthetic pathways to essential oil components

The components of the essential oils are formed by discrete biochemical pathways, and therefore, they can be classified according to their biochemical origin. Highly refined biosynthetic pathways that consist of arrays of specific enzymes that concertedly divert the metabolic flow through well defined biosynthetic pathways. The biochemical and molecular regulation of these enzymes often follows well defined and discrete patterns. For example, the production of monoterpenes in grand fir (*Abies grandis*, Pinaceae) is regulated by wounding (Lewinsohn *et al.*, 1992). In wounded grand fir stem tissues, the levels of several of the biosynthetic enzymes to mono- and diterpenes are expressed in very high levels, corresponding to between 10 and 100 fold from the levels normally found in non-wounded tissues (Funk *et al.*, 1994).

Many of the enzymes involved in menthol biosynthesis in peppermint, are active only during discrete stages during leaf development (Gershenzon 1994), and linalool emission from *Clarkia* flowers seems regulated by an increase of linalool biosynthetic activity and

linalool-synthase gene expression both in discrete flower tissues and during flower development and senescence (Pichersky *et al.*, 1994, Dudareva *et al.*, 1996).

Volatile metabolites are formed by distinct biochemical pathways and they are grouped according to their biochemical origin:

The terpenes comprise the largest biochemical group of natural products known. More than 22,000 different terpenic chemical structures are known, and they share a common biosynthetic origin (McGarvey and Croteau 1995). Terpenes are derived from the enzymatic fusion of five-carbon isopentenyl pyrophosphate units (Croteau and Karp 1991), that are normally go to processes of cyclization and further modification to give rise to the vast majority of terpenes known. Many of the enzymes involved in these reactions have been studied and characterized (Croteau 1986, Gershenzon and Croteau 1993, McGarvey and Croteau 1995).

Other essential oil components, such as the short chain alcohols and aldehydes (1-octen-3-ol, hexanal) are formed by metabolic conversion or degradation of phospholipids and fatty acids (Croteau and Karp 1991).

The phenylpropanoids are important components of essential oils. This group includes fragrant compounds such as estragole, (found in tarragon and basil); eugenol (clove), myristicin (nutmeg), cinnamaldehyde (cinnamon), and *l*-anethole (anise). Phenylpropanoids are all derived from the amino-acid phenylalanine by the action of the key enzyme phenylalanine ammonia lyase (PAL) that removes the amino group of phenylalanine to

generate the key metabolic precursor *trans*-cinnamic acid, which is also a precursor of lignin, and the anthocyanin pigments in flowers and fruits. *t*-Cinnamic acid is modified by series of enzymes, including hydroxylases, methyltransferases, oxidoreductases and ligases that concertedly catalyze the reactions needed for the biosynthesis of the specific compounds (Croteau and Karp 1991). Methyltransferase enzymes that catalyze the specific methylation of the *p*-hydroxyl groups of eugenol and chavicol generating methyl eugenol and methyl chavicol respectively, have been described in this symposium (Ziv-Raz et al., 1997)

Conclusion

Essential oils are a unique class of secondary metabolites. They are responsible for the aromas and flavors of many plants and serve as chemical defenses against pathogens and pests. They are stored and biosynthesized in special anatomical organs, such as oil cells, oil cavities, ducts and glands. Arrays of enzymes normally present in these anatomical structures act upon their corresponding precursors in a well regulated manner to form the final products stored in such cavities or cells. Small chemical modifications, such as the degree or position of oxidation, often have profound effects on the aroma characteristics of the essential oil components. Thus, evolutionary related enzymes catalyzing analogous reactions can give rise to products with diverse chemical and biological characteristics. This is reflected in the large diversity of the essential oils and their components found in nature.

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SUPERCRITICAL FLUID EXTRACTION OF TERPENE HYDROCARBONS FROM CITRUS OILS

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ABSTRACT

The separation of terpene hydrocarbons from oxygenated aroma components was investigated using supercritical carbon dioxide for the fractionation of orange peel oil. To understand the process behavior of this multicomponent system, phase equilibrium measurements and countercurrent column experiments were carried out. Extraction conditions were set within the temperature range from 323 to 343 K and pressures from 8 MPa up to 13 MPa yielding loadings from 10 to 120 g extract/kg CO₂. All samples were analyzed by gas chromatography. Comparing conditions at low and high loadings, a decrease in selectivity is observed with increasing loading at a constant temperature. At constant solvent-to-feed ratios, the separation is improved at high loading conditions using external reflux. Short-cut stage calculations with the method of Jänecke enabled the estimation of process conditions depending on the desired product quality.

INTRODUCTION

Citrus oils, obtained by cold-pressing of citrus peels during juice processing, are usually fractionated into terpene hydrocarbons and oxygenated aroma components to meet special demands for use in food, detergent, or other chemical industry. Since monoterpenes tend to decompose at high temperatures, separation processes at moderate temperatures such as vacuum distillation, solvent extraction, and adsorption are used commercially. All of them show disadvantages like toxic solvent residues or high process costs. One of the most promising alternatives is gas extraction, also called supercritical fluid extraction (SFE). Fractionation may be performed at moderate temperatures and without any remaining organic solvent in the products. Carbon dioxide is preferred as a supercritical solvent because it is nontoxic, nonflammable, inexpensive, and chemically stable [1].

Up until the present, many researchers have studied the separation behavior of different aroma components of citrus oils in supercritical CO₂. Most of the papers report binary data for systems containing CO₂ and a pure component with the objective to find suitable correlation methods based on equations of state. Studying the separation of real or model mixtures, it was found that supercritical CO₂ is suitable to selectively extract the more volatile and non-polar monoterpene fraction [10].

There are only few studies on real multicomponent mixtures. Some were carried out at low loading conditions [6] which are unfavorable from an economical point of view. Most of the results found in literature are qualified for estimations but not sufficient to elaborate a large scale process. Therefore, to get reliable data for citrus oil deterpenation by means of supercritical gases, the system CO₂+orange peel oil was studied with the objective to enable scale-up calculations.

MATERIALS AND ANALYTICAL METHODS

Valencia orange peel oil from Brazil was used in this study. Carbon dioxide was used with a purity higher than 99.95 wt.% (KWD, Germany). Ethanol p.a. (Merck, Germany) was utilized for sample preparation.

Gas chromatographic analysis was established using a Perkin Elmer (Germany) glass capillary GC type 8320 with FID detection. A non-polar capillary column of type XTI-5 (30 m, 0.25 mm ID, 0.25 µm df, by Restek, Germany) was used to enable the elution of high-boiling components like flavonoids. Furthermore, retention behavior of this type of column is similar to the fractionation behavior of CO₂, and peaks within a certain retention

time range can be lumped to pseudocomponents [2]. Helium (Messer Griesheim, Germany) served as carrier gas at an inlet pressure of 145 kPa. 2 μ l of diluted sample (10 mg sample in 1 ml ethanol) were injected using a split ratio of 1:100. The following temperature program was established: 313 K, 5 min constant, 3 K/min to 423 K, 5 K/min to 593 K, 15 min constant; injector: 593 K, detector: 623 K. Samples with a high concentration of oxygenated aroma components were diluted at 2 mg per ml ethanol to enable a good peak resolution. Due to the variety of components, all response factors were set equal to one. The feed material contained around 95 wt.% d-limonene, 3.25 wt.% of components with a lower boiling point than d-limonene, and 1.75 wt.% of low volatile components (oxygenated aroma components, sesquiterpenes, flavonoids). To simplify the study, two pseudocomponents were defined. D-limonene together with the more volatile components, mostly other monoterpenes, are referred to as a “terpene” group. All other components with a higher retention time than d-limonene are called “aroma fraction” and include oxygenated components as well as sesquiterpenes, waxes, and flavonoides.

PHASE EQUILIBRIA

The temperature and pressure range for supercritical deterpenation was defined with respect to the following considerations. Terpene solubilities below 1 wt.% in supercritical CO₂ at extraction conditions should be avoided in order to allow economic processing. To enable loadings above 1 wt.%, temperatures of 323 K and up are required due to the shape of the phase diagram. Concerning product stability, a temperature limit may be around 343 K [10], but it was never investigated under inert CO₂ atmosphere. The pressure range

was set starting from 8 MPa up to the one-phase region of the quasi-binary system CO₂+orange peel oil. Since the critical pressure for a mixture changes with temperature, the pressure limit for VLE measurements for the system CO₂+orange peel oil was around 9.8 MPa at 323 K, 11.5 MPa at 333 K, and 13.2 MPa at 343 K. Deterpenation using supercritical CO₂ has to be carried out below these critical pressures.

Two different apparatus were used in the study of vapor liquid equilibrium (VLE) data and are described elsewhere [9, 5]. Both are of the static-analytical type, and sampling of the two phases is realized by means of capillaries, using cold traps to collect all condensable material, and a burette system to measure the amount of CO₂. Additionally, one apparatus was designed to sample a large amount of the vapor phase using a second autoclave in series with the equilibrium cell. After circulating the vapor phase, this sampling autoclave contained 300 ml of the vapor phase in equilibrium with the liquid phase. The autoclave was separated from the apparatus by means of valves, and the entire content was withdrawn yielding larger amounts of extract compared to the cold-trap method. However, results from both sampling procedures are in good agreement.

The mutual solubility of the quasi-binary system can be illustrated in a P-x diagram with reference to the CO₂ content of both phases in equilibrium. Figure 1 shows VLE data measured at 323, 333, and 343 K. The measured vapor phase composition is in clear contrast to previously published data for the system CO₂+orange peel oil [10]. The latter study reported no significant change in vapor phase composition for 323, 333, and 343 K. The reason for the discrepancy is assumed to be the unsuitable experimental method, using a dynamic CO₂ flow through just 10 ml of feed.

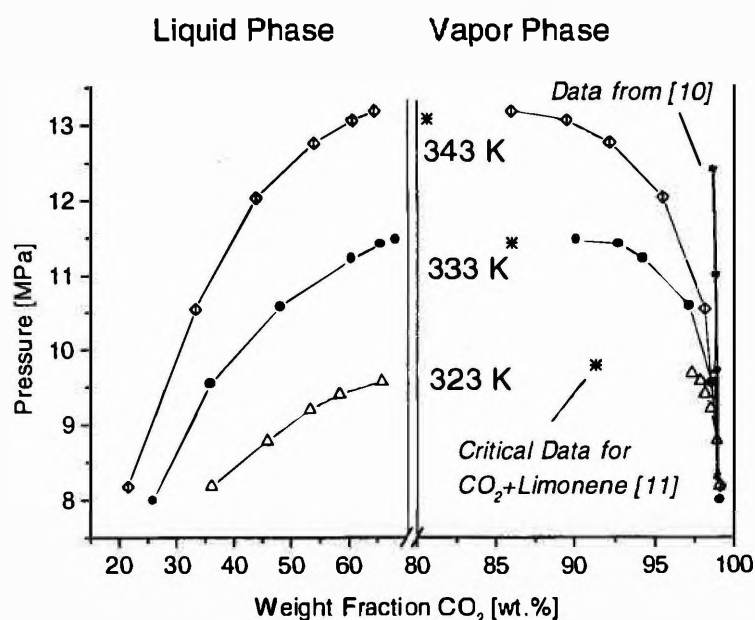


Figure 1: VLE data for the system CO₂+orange peel oil

A more detailed comparison with literature data for the system CO₂+limonene is given elsewhere [3], and it confirmed the accuracy of the data measured in this study. The phase behavior of the system CO₂+limonene is very similar to that of CO₂+orange peel oil due to its high d-limonene content. This is shown here by the good agreement of the critical points for the binary system CO₂+limonene [11] with the phase boundary line for the real system data. However, the binary system's critical points are located at lower pressures than those for any pseudoternary system CO₂+terpenes+aroma. Critical points will shift to higher pressures with an increasing amount of aroma in the mixture [3].

It can be seen from Figure 1 that extract solubilities in CO₂ at 323 K are rather low. Therefore, high solvent-to-feed ratios are required to produce highly concentrated aroma fractions at 323 K. Even if loadings around 25 g extract/kg CO₂ can be realized at 323 K

and 9.6 MPa, this pressure is too close to the critical point of the mixture. Besides the similarity in density of both phases, what is unfavorable in countercurrent processing because of flooding, the major disadvantage to work close to a mixture's critical point are the huge changes of mutual solubilities with very small changes in pressure.

To evaluate the selectivity of supercritical CO₂, all samples were analyzed by GC and treated like pseudobinary mixtures of “terpenes” (T) and “aroma” (A) as explained above.

The separation of both groups is expressed by the separation factor $\alpha_{T/A}$ in a solvent-free representation:

$$\alpha_{T/A} = \frac{y_T/x_T}{y_A/x_A} \quad \text{with} \quad \begin{array}{l} y_i = \text{concentration of } i \text{ in liquid dissolved in the vapor phase} \\ x_i = \text{concentration of } i \text{ in the CO}_2\text{-free liquid phase} \end{array}$$

Figure 2 illustrates the dependency of the separation factor on loading at the three different temperatures for the system CO₂+orange peel oil. While pressure is only an operation variable, the influence of the loading on selectivity was chosen due to its economical significance. Additional data from previous measurements are included in this figure [3].

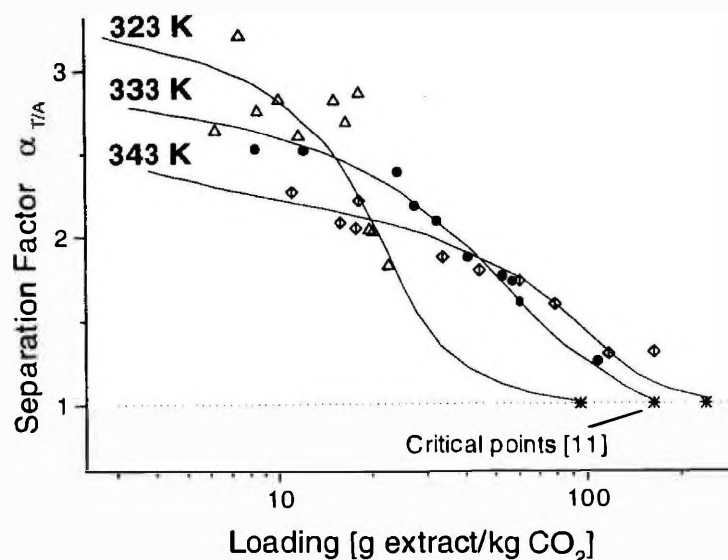


Figure 2: Selectivity of supercritical CO₂ on the deterpenation of orange peel oil

Algebraic relationships were selected to describe the dependency of both separation factor and loading on pressure for each temperature. The lines in Figure 2 are calculated from these pressure depending functions. Since supercritical CO₂ shows a higher affinity to terpenes than to aroma components, separation factors $\alpha_{T/A}$ exceed the value of 1. It is clear that a high separation factor represents a good selectivity and is favored in multistage processing. On the other hand, high loading conditions are requested to minimize the solvent-to-feed ratio. Unfortunately, selectivity decreases with increased loading due to the fact that very close to a mixture's critical point, both phases become very similar resulting in separation factors close to 1. This was observed for all temperatures.

At low loading conditions, the selectivity is best for low temperatures. This can be explained by the different increase in vapor pressures of the mixture's components with increasing temperature. The relative change of vapor pressures is more distinct for high-

boiling oxygenated aroma components than for monoterpenes. With an increasing amount of aroma components in the vapor phase, the separation factor decreases. The vapor pressure ratio of d-limonene and linalool, decanal, and geranial is shown in Figure 3.

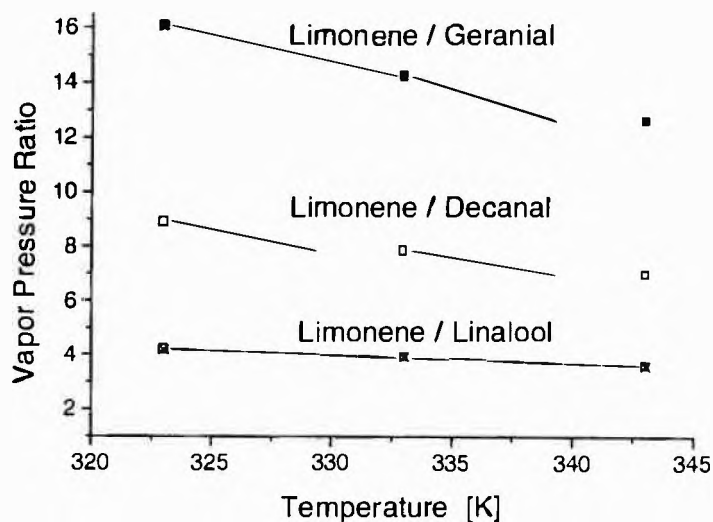


Figure 3: Vapor pressure ratio for orange oil components (data from [8])

Another effect predominates at higher loadings. Since the critical loading increases with temperature (see Figure 1), there is still a reasonable selectivity at a loading around 100 g/kg at 343 K while the system's critical point is around 100 g/kg for 323 K. Therefore, the selectivity is best for high temperatures at high loading conditions, and the curves of Figure 2 have to intersect. The scattering in data at 323 K can be explained by the difficulty in sampling due to the low loading and distinctive sensitivity of solubility on pressure.

No significant change in liquid phase composition was observed for all measurements using a large amount of liquid feed (200 ml) and a solvent-to-feed mass ratio around 1:1.

However, there is a change in phase behavior with changing composition of the liquid phase. To enable stage calculations for a countercurrent process, both the dependency of the selectivity on composition and the change in mutual solubility of solvent and liquid has to be known. Assuming a constant separation factor will give no reliable scale-up calculation.

Since the McCabe-Thiele method does not account for the solvent, the stage calculation method of Jänecke was applied in this study. Both short-cut methods are described in literature [1]. Figure 4 gives an example for the change of separation factor with liquid phase composition at 333 K and 10 MPa for the quasi-ternary system CO₂+terpenes+aroma. Measurements were carried out using both terpene fractions and concentrated aroma fractions obtained from countercurrent column experiments.

The decrease in selectivity with increasing terpene content is due to the different contribution of the variety of aroma components to the separation factor. Since high molecular weight components like flavonoids, sesquiterpenes, and its derivatives are hardly dissolved in the vapor phase, they contribute to a relatively high selectivity of CO₂ for monoterpenes when using the feed material or aroma fractions. Fractions rich in terpenes only contain some volatile oxygenated aroma components like linalool and decanal, and these are more difficult to separate from monoterpenes. Consequently, the separation factor becomes smaller resulting in many theoretical stages needed to produce a terpene fraction of high purity.

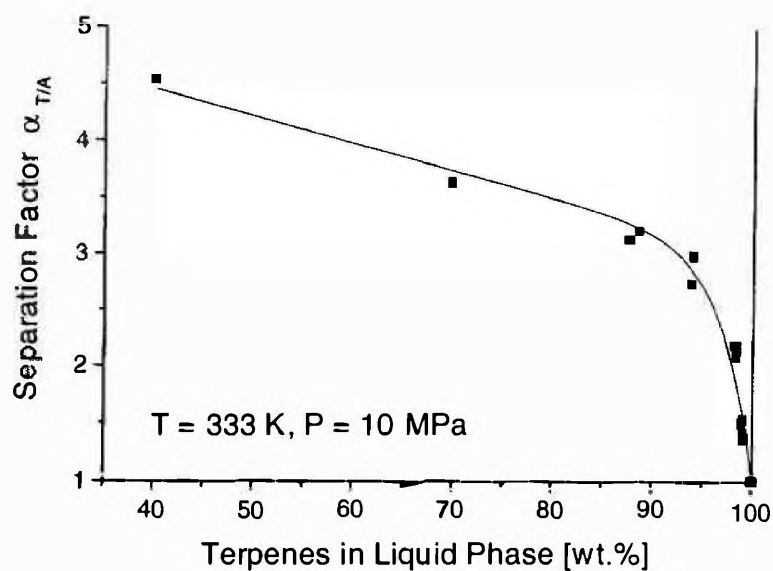


Figure 4: Selectivity of CO₂ on the separation of terpenes and aroma components

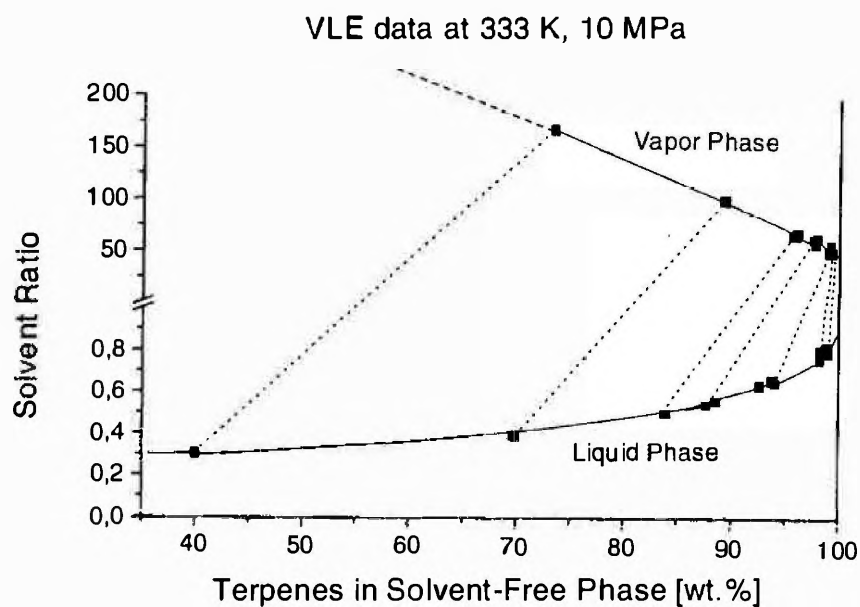


Figure 5: Jänecke diagram for the system CO₂+terpenes+aroma at 333 K and 10 MPa

The corresponding change in mutual solubility is illustrated in Figure 5 using Jänecke coordinates. The composition of both phases is expressed by the ratio of solvent to

condensable liquid, which is either extract or raffinate. Due to the low solubility of extract in the solvent, the solvent ratio for the vapor phase is high and changes significantly with composition. The tie lines connect points in equilibrium. Using analytical expressions for the three curves shown in both figures, equilibrium phase composition can be calculated easily. This enables the estimation of stages needed for a given separation task.

MULTISTAGE COUNTERCURRENT EXTRACTION

A typical example for a deterpenation process is the production of a 5-, 10-, or 25-fold aroma concentrate from orange peel oil. The concentration factor is defined as the mass flow ratio of feed and raffinate. Processing 100 kg orange oil and withdrawing 90 kg terpenes with the solvent will give 10 kg of a 10-fold aroma concentrate. Due to the loss of small amounts of oxygenated aroma components with the terpene fraction, the real aroma concentration factor is less than 10 for a 10-fold product. Aroma loss can be greatly reduced by establishing extract reflux at the top of the column. For example, a reflux ratio of 5 will result in a reflux flow of 450 kg/h, and 540 kg of terpenes have to be dissolved in the vapor phase. At low loading conditions of 10 g/kg, the solvent flow needed to dissolve this amount of terpenes is easily estimated to be around 54000 kg/h. With increasing solubility, the solvent-to-feed ratio decreases and is around 100 for loading conditions of 50 g/kg. This explains the need of high solubilities in order to reduce process costs.

The data shown in Figure 6 are calculated with the short-cut method of Jänecke based on VLE data measured in this study. Additionally, data for the binary system CO₂+limonene were taken from literature [7]. Some other assumptions have been made to enable the

calculation. A linear decrease of the separation factor from $x_T=98.25$ wt.% to $x_T=100$ wt.% was assumed using a minimum separation factor of 1.1, while the larger value for $x_T=98.25$ wt.% was calculated in analogy to that shown in Figure 2. The separation factor was set constant to this value at lower terpene contents. This is believed not to affect the calculation procedure since a high number of stages is only calculated in the enriching section at very low separation factors. At a constant temperature only pressure was used as a variable, and the solvent-to-feed ratio was calculated for the following examples.

Example	A 10-fold concentrate	B 10-fold concentrate	C 25-fold concentrate
Feed flow, terpene content	100 kg/h, 98.25 wt. %	100 kg/h, 98.25 wt. %	100 kg/h, 98.25 wt. %
Raffinate flow, terpene content	10 kg/h, 84.3 wt. %	10 kg/h, 83.4 wt. %	4 kg/h, 61.05 wt. %
Extract flow, terpene content	90 kg/h, 99.8 wt. %	90 kg/h, 99.9 wt. %	96 kg/h, 99.8 wt. %
Number of stages in enriching section	10	20	9 or 8
Number of stages in stripping section	5	5	6 or 7
Total number of stages	15	25	15

Table 1: Parameters for stage calculations

When the phase behavior is known in the temperature and pressure range of interest, the parameters influencing stage calculations are the compositions of the products and the solvent-to-feed ratio. Product composition was set constant here, and therefore pressure is the key variable that influences the phase behavior. With the number of theoretical stages chosen to be constant, the solvent-to-feed ratio was determined.

A number of 15 equilibrium stages was set to be suitable for both examples limited to an aroma loss of 0.2 wt.% with the terpene fraction. Reducing the amount of oxygenated aroma components in the extract to 0.1 wt.% required a much larger amount of equilibrium stages in the enriching section of the column due to small separation factors at high terpene concentrations (Figure 4).

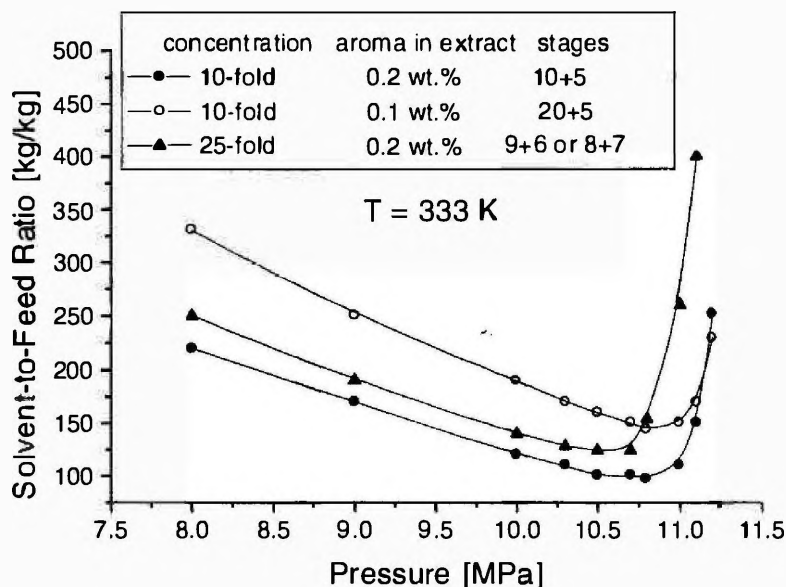


Figure 6: Short-cut calculation of the countercurrent deterpenation process at 333 K

An increase in pressure enables a higher solubility combined with a decrease in selectivity. As long as the increase in solubility predominates, the process becomes more economic with increasing pressure. This is observed for all examples calculated here. The minimum solvent-to-feed ratio at 333 K is around 10.8 MPa. For 25-fold concentrates just one or two more stages are needed in the stripping section. Reducing the enriching section in order to work with a total number of 15 stages leads to a slightly lower minimum and a steeper increase at 11 MPa compared to the examples for 10-fold concentrates. At 11 MPa the

loading is 40 g/kg and the separation factor still around 1.9 for a liquid of 98.25 wt.% terpenes (Figure 2).

The countercurrent column used in this study is 25 mm in inner diameter, 4 m high, and equipped with a structured packing of type EX (Sulzer, Switzerland). Detailed information can be found elsewhere [4]. Figure 7 shows experimental results from countercurrent column experiments at 333 K for two different solvent-to-feed ratios. All experiments were carried out to obtain a 5-fold concentrate. With a constant feed composition, the terpene content of the bottom product can be calculated by a mass balance depending on the composition of the extract. While at 50 kg CO₂/kg feed the terpene content of the extract was limited to 99.55 wt.% (0.45 wt.% aroma loss), the aroma loss was reduced to 0.3 wt.% using 100 kg CO₂/kg feed.

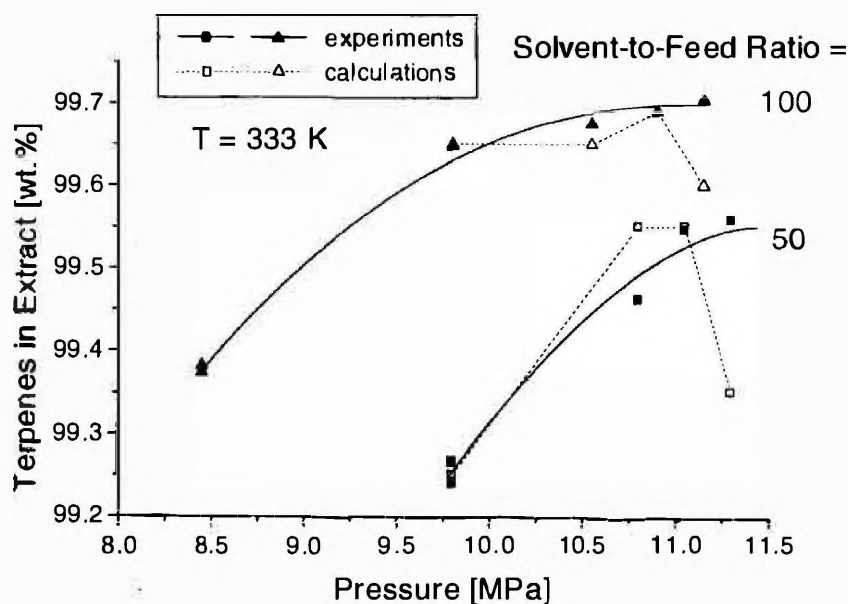


Figure 7: Comparison of countercurrent column experiments with stage calculations

Those are the results obtained in a 4 m column, and therefore, the number of stages has been limited. From a comparison of experiments and stage calculations the height of one theoretical stage can be estimated, and it was found to be in the range of 0.5 m. The hollow symbols in Figure 7 represent the calculated extract compositions assuming 4 stages in the stripping section and 4 stages in the enriching section. In analogy to Figure 6, a change in separation efficiency is predicted at 10.8 MPa, but it was not confirmed by the experiments. This may be due to some inaccuracies of the simplified model. On the other hand, it is well known from literature that the height of a theoretical stage changes with hydrodynamic conditions [2]. Explaining the difference in calculation and measurement in Figure 7, the height of a theoretical stage decreases with increasing pressure, since reflux ratio is increased simultaneously with pressure at constant solvent-to-feed ratios and enables a better mixing of the phases.

SUMMARY AND OUTLOOK

To study the deterpenation of orange peel oil VLE measurements and countercurrent column experiments were carried out in a wide range of temperature and pressure in order to work at high loading conditions. Stage calculations with the simple short-cut method of Jänecke allow a first estimation of optimum process conditions. Since the height of a theoretical stage depends on hydrodynamics, pilot plant experiments are needed to enable a reliable estimation of the design of an industrial scale process. However, the data presented in this study are the basis for scale-up calculations, and additional evaluation of column experiments at 323 and 343 K will complete the project's objectives.

The temperature stability of citrus oil in dense CO₂ is still not known. In order to find the most suitable operation temperature, stability analysis in dependence of residence time of the product should be studied. It will also be a future objective to investigate other citrus oils like lemon oil and bergamot oil to study differences and similarities in phase behavior. With an increased knowledge of the influence of specific groups of aroma components the prediction of phase behavior might become more reliable.

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The HFC 134a Extraction of Fine Natural Products from Plants. A review of recent world-wide progress.

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ABSTRACT

Since I discovered the remarkable solvent properties of HFC 134a to extract fine natural oils from flowers, in my garage in 1990, the use of this new technology has been expanding around the world. Hundreds of new oils, pharmaceuticals and industrial products are now being extracted with this solvent, in purpose built plant and equipment, in several countries. The European Parliament has now added HFC 134a to the "Solvents Directive" list and approved its use for the extraction of natural oils for food flavouring.

INTRODUCTION

The British have a traditional and legendary difficulty with their "inventors".

Typically there are two choices open to British innovators: a) emigrate (usually to the USA or Japan) or b). starve.

As for me, I don't yet know which it will be. Meantime, life could not be described as dull.

In the late 1980s I simply wanted to prepare the world's best quality English Rose oil. Most people are now familiar with this story ^(1 and 2) so I won't go into that now. However, I did find that it was necessary to "invent" new technology for its extraction.

At that time, there was no existing technique which would provide me with oils which were:

- natural and **not** "cooked" (as are those obtained with steam distillation).
- without risk of personal incineration and
- with low solvent residues (which ruled out hexane/gasoline extraction),
- without the necessity to "vacuum strip" (and risk losing "volatiles")
- and then refine with alcohol (which ruled out hexane and DCM extraction)
- non-damaging to the environment (which ruled out all the above methods).
- produced at neutral pH, not pH 2 (which ruled out SCF extraction with CO₂).
- made in equipment that I could afford (which again ruled out SCF Extraction)

Since describing this new HFC 134a extraction technology ^(3, 4 and 5) and spending several years developing equipment necessary to handle it, rose oil has become only one of the

hundreds of plant (and some animal) materials successfully extracted, which are now in commercial scale production in several countries.

Some examples of successful products produced in this way are reviewed below.

RESULTS AND DISCUSSION

SIGNIFICANT PROPERTIES OF HFC 134a

HFC 134a is a highly selective solvent that does not remove waxes, triglycerides or high molecular weight fatty acids from plant bio-mass. It is not flammable nor toxic. It has a boiling point of - 26 °C at atmospheric pressure. It has a vapour pressure of only 6 BarG at 25°C. All this makes it a simple solvent to handle, in extremely inexpensive equipment.

These properties also make it ideal for the extraction of flavour oils from fresh herbs and spices, fragrant oils from fresh flowers and pharmaceutical extracts of high purity and potency.

In addition, industrially significant products such as insecticides and plasticisers can be extracted. Drugs of abuse can be assayed in body fluids and environmentally harmful chemicals such as carcinogenic poly-cyclic hydrocarbons and poly-chlorinated biphenyl's can also be removed from contaminated soils with this process.

EXAMPLES

Some of the plant bio-mass from which excellent oils have been experimentally or are now routinely extracted using HFC 134a as a solvent include:

AGAR WOOD - "Oud" (<i>Aquillaria agallocha</i>)	AJOWAIN SEED (<i>Tachyspermum copticum</i>)
AMBRETTE SEED (<i>Abelmoschus moschatus</i>)	ANISE SEED (<i>Illicium verum</i>)
ASAFOETIDA (<i>Ferula asa-foetida</i>)	BASIL (<i>Ocimum basilicum</i>)
BASIL (SACRED) "Tulsi" (<i>O. sanctum</i>)	BAY LEAF (<i>Laurus nobilis</i>)
BLACK PEPPER (<i>Piper nigrum</i>)	BLACK TEA
CARDAMOM SEED (<i>Elettaria cardomomum</i>)	CARAWAY SEED (<i>Carum carvi</i>)
CASSIA BARK (<i>Cinnamomum cassia</i>)	CHILLI (RED)
CHILLI (GREEN)	CHOCOLATE (both Milk and Dark)
CINNAMON BARK (<i>Cinnamomum zeylanicum</i>)	CLOVE BUD (<i>Syzygium aromaticum</i>)
COFFEE (both Roasted and Green)	CORIANDER SEED (<i>Coriandrum sativum</i>)
CUMIN SEED (<i>Cuminum cyminum</i>)	DILL SEED (<i>Anethum graveolens</i>)
ENGLISH ROSE FLOWERS	FENNEL SEED (<i>Foeniculum vulgare</i>)
FENUGREEK (<i>Trigonella foenum-graecum</i>)	FRANKINCENSE (<i>Boswellia carteri and freena</i>)
GINGER ROOT (<i>Zingiber Sp.</i>)	HOP FLOWERS (<i>Humulus lupulus</i>)
HYACINTH FLOWERS (<i>Hycinthus orientalis</i>)	JASMINE (Flowers and Concrete) (<i>Jasminum off.</i>)
JUNIPER BERRIES (<i>Juniperus communis</i>)	KALONJI SEED (<i>Nigella sativa</i>)
<i>Lipia citriadora</i>	LOVAGE SEED (<i>Levisticum officinale</i>)
MARIGOLDS (<i>Calendula off.</i>)	MACE
MEADOW-SWEET (<i>Filipendula ulmaria</i>)	MELISSA (<i>Melissa officinalis</i>)

MUGUET (<i>Convallaria majalis</i>)	MYRRH (<i>Commiphora molmol</i>)
NARCISSUS (<i>Narcissus poeticus</i>)	NEEM SEEDS (<i>azadirachta indica</i>)
NUTMEG (<i>Myristica fragrans</i>)	ORANGE (Peel and Flowers) (<i>Citrus aurantium</i>)
ORRIS - ROOT (<i>Iris pallida</i>)	PAPRIKA FRUIT
<i>Pimpinlla anisum</i>	SAW PALMETTO (<i>Serenoa repens</i>)
SALVIA (<i>Salvia sclarea and officinalis</i>)	TOBACCO LEAF (<i>Nicotiana tobaccum</i>)
TUBEROSE (<i>Polianthes tuberosa</i>)	THYME LEAF (<i>Thymus vulgaris</i>)
TURMERIC ROOT ("Fingers" and "Potatoes" - Cooked and Raw) (<i>Curcuma longa</i>)	WHITE PEPPER
VANILLA POD (<i>Vanilla planifolia</i>)	

Apart from all the above (fairly obvious) materials, interesting and unexpected products have been obtained using this technology to extract other bio-masses. For example:

1. *Artemissia anua and A. absinthum* (WORMWOOD)

This herb has been known for many years to be beneficial in the treatment of malaria. In clinical trials, some examples of complete cures of (normally terminal) cerebral malaria in children have been obtained using refined extracts of this herb. Two of the active principles are "Artemisinin" and "Artemisinic acid". Extraction of the carefully dried herb with HFC 134a has yielded extracts with between 35% and in some cases as high as 70% content of these active ingredients. These extracts were very viscous, sticky, glutinous masses, probably because most of the light essential oils had been driven off by the drying process.

However, using freshly harvested *Artemissia absinthum* (a close relative - better known as "Wormwood" and employed as a flavouring for "Vermouth") a light mobile clear oil was obtained (see sample).

2. *Dypteryx odorata* (TONKA BEANS)

Tonka Beans (*Dypteryx odorata*) are well known to contain copious quantities of Coumarin, an ingredient used in most perfumes. Extraction of Tonka Beans with HFC 134a yields a pure white crystalline solid comprising 93% Coumarin. Curiously enough, no oil phase was obtained and the crystals were easily crushed into a powder (see sample). This is curious, because "super-critical carbon dioxide" extraction of Tonka beans has been reported as yielding a TWO PHASE mixture of crystals suspended in an OIL phase ⁽⁶⁾. (see samples).

3. *Azadirachta indica* (NEEM)

Insecticides from the seeds, leaves and bark of the Neem Tree (*Azadirachta indica*) have been known and respected in India for thousands of years. Several of the active principles: Azadirachtin, Meliantriol, Salanin, Nimbin and Nimbidin have been observed in HFC 134a extracts "in clean and highly potent form" from these seeds.

4. *Caleolaria andina*

Perhaps a new generation of novel insecticides will emerge from experimental work now being undertaken. Active insecticidal components are now being extracted from the Chilean wild plant *Calceolaria andina*. The purest and highest titre of potency has been observed in extracts made using HFC 134a.

5. *Chrysanthemum coccineum* (PYRETHRUM)

The oldest natural insecticide in commercial use at present is "Pyrethrin" from the flowers of Kenyan or Tasmanian grown *Chrysanthemum coccineum*. These flowers have hitherto been dried and pelletised prior to extraction with hexane. The "oleo-resin" which results from this treatment requires a great deal of refinement before the extract is of any value in a domestic retail insecticide, as waxes, resins and triglycerides block the nozzles of domestic aerosol sprays.

Extraction of pelletised flowers (and also of the oleo-resin) with HFC 134a resulted in the production of a pale green mobile clear oil with a high titre of "Pyrethrin" and a low wax content (see sample).

6. *Serenoa repens* (SAW PALMETTO)

The "Saw Palmeto" is a valuable source of progestagens which are used in the treatment of prostatic carcinoma. HFC 134a has produced excellent extracts from dried Saw Palmetto

7. *Trigonella foenum-graecum* (FENUGREEK)

The eating of copious amounts of fenugreek has been widely reported to have cholesterolaemic activity. Unfortunately, the penetrating aroma of fenugreek imparts a strong personal body odour to the eater. This has delayed the exploitation of its therapeutic advantages. HFC 134a has been shown to be able to remove this aroma and provide a "de-odourised" fenugreek product which still retains its full compliment of cholesterolaemic activity.

8. Organic Lanolin

In Britain, it is the law that all sheep are dipped in organo-phosphorous insecticides once a year. The residues of these chemicals are found in the lanolin traditionally "scoured" from the raw fleece (sheeps' wool). This has ruined the lanolin industry in the UK. In South Africa, sheep are not treated in this way and "organic sheep" still yield good quality lanolin.

Extraction of raw South African "organic fleece" with HFC 134a yielded a light, mobile, clear fragrant oil with many of the characteristic advantages of traditional lanolin, but

without many of its unpleasant characteristics (stickiness, high viscosity, opacity). A market for this novel product has yet to be found.

EXPERIMENTAL

The essential equipment used for all these extractions conforms to simple principles. Extractor(s), capable of withstanding 6 BarG at room temperature, are provided with a reservoir of liquid HFC 134a. Bio-mass is introduced into an extractor and this is then sealed. Solvent is allowed to percolate (upwards or downwards) through the bio-mass contained in the extractor. The solution of extract so formed is metered into an evaporator, where it is encouraged to boil (at no more than ambient temperature - of course). The resulting solvent gas is condensed and returned to the reservoir. This process is continued until no more active ingredient is washed from the bio-mass or until commercial considerations determine the extraction.

Finally, all the solvent is recovered from both extract and bio-mass and returned to the reservoir, where it is available for future extractions.

SUMMARY

Techniques for the extraction of valuable products with HFC 134a, from an apparently limitless range of bio-masses, are no longer a laboratory curiosity, they are commercial fact.

Two world class chemical companies are now seriously beginning to view this new market for their HFC 134a.

Seven or eight commercial or semi-commercial plants are now operating in five countries around the world. Several more are at advanced stages of design.

Firms of expert technologists and engineers have established facilities which offer to design, specify, fabricate, procure, erect, build, commission and maintain either turn-key skid mounted plant packages or larger scale bespoke equipment produced to the customers own requirements. They also carry out laboratory scale testing and produce samples for market testing.

The products of this process are now being traded.

A range of fine oils of exquisite quality is gaining an increasing share of the lucrative aromatherapy market, where a growing confidence in these excellent products is apparent in USA, Japan and Europe.

The process is now

competitively extracting exquisite flavours from fresh herbs
extracting fine fragrance oils from fresh flowers
producing excellent natural aromatic flavour oils from culinary spices
refining "concretes", natural resins and "oleo-resins" to yield high value oils
extracting many industrially significant products
producing a wide range of pharmaceutically important extracts
with residue levels routinely 1/1000 of those normally experienced with
hexane extracts.

The equipment operating now

is economical to purchase (costing less than 1/50th of that of a similar sized SCF
Extraction plant),
uses practically no energy (a solar powered plant is at the concept stage)
can be portable and taken to the crop
can be automatic or manual
is without damage to the environment or hazard to the operating personnel.

One day all oils will be prepared this way.

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AUSTRALIAN TEA TREE OIL: EFFICACY, TOXICITY, IRRITANCY AND STABILITY

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ABSTRACT

Australian tea tree oil quality is defined by the composition of 14 terpenes as set out in the ISO 4730 standard. Whilst much is known about the chemistry, *in vitro* efficacy and toxicity of this oil, additional data is still required if the oil is to become a recognised and creditable therapeutic agent. To address some of these key issues the Australian Tea Tree Oil Research Institute (ATTORI) was created in 1996 to provide a focal point for co-ordinated research on all aspects of this commercially important oil. ATTORI has established collaborative and contractual research with various institutions to further research into plant genetics, product formulation, efficacy *in vitro* and *in vivo* and clinical trials for topical applications. The use of tea tree oil for topical applications is supported by cell-line cytotoxicity studies. Of the five cell-lines tested with tea tree oil and various pure components, epithelial cells were most robust, whilst liver cells were the most susceptible. Recent results from a skin irritancy and sensitivity trial conducted on 151 panellists revealed that tea tree oil is an extremely low irritant when applied to the skin, even in neat (100%) concentrations. Tea tree oil added to various formulation bases, at concentrations up to 25%, was not significantly different in irritancy from the placebo.

However, tea tree oil must be considered a mild allergen as 2% of the panellists tested to date showed an allergic reaction. Issues surrounding production, formulation and stability of low irritancy oil are also addressed.

BACKGROUND

The essential oil obtained from *Melaleuca alternifolia* is now clearly defined by ISO 4730 "Oil of Melaleuca - Terpinen-4-ol Type". This standard identifies a set of chemical parameters (Table 1) that stipulate the range of fourteen key terpenes identified in the definitive study of the terpinen-4-ol type tea tree oil (Brophy *et al.*, 1989). It should be stressed that this standard is primarily a quality assurance tool for trade in pure tea tree

oil and does not provide a definitive composition for optimum concentrations of principal bioactive compounds. The issue of optimal bioactivity of tea tree oil has been addressed (Southwell, *et al.*, 1993, Carson and Riley, 1995) and must be linked ultimately with quality assurance.

TABLE 1

Normative Values of Tea Tree Oil Constituents

Constituent	% Maximum	% Minimum
1,8-cineole	15	-
α -terpinene	13	5
γ -terpinene	28	10
p-cymene	12	0.5
terpinen-4-ol	-	30
α -terpineol	8	1.5
α -pinene	6	1
terpinolene	5	1.5
limonene	4	0.5
sabinene	3.5	trace
aromadendrene	7	trace
δ -cadinene	8	trace
globulol	3	trace
viridiflorol	1.5	trace

Sabinene hydrate occurs in high levels in the flush growth leaves of *M. alternifolia* (Southwell and Stiff, 1989) which rapidly converts to terpinen-4-ol during leaf ageing or hydrodistillation. Our research (Cornwell *et al.*, 1995) on this transformation indicates there is no biochemical control during the ageing process as the same chiral ratio of terpinen-4-ol (Leach *et al.*, 1993) and the profile of related p-menthane products can be generated from sabinene hydrate by simple acid-catalysed hydrolysis. This pathway is not

unique to *M. alternifolia* (Cornwell *et al.*, 1997) and parallels the chemistry of marjoram as elucidated by Fischer *et al.*, (1987, 1988).

To develop a greater understanding of the many facets of tea tree oil, the Australian Tea Tree Oil Research Institute (ATTORI), a privately funded research centre, was set up in 1996 on the campus of Southern Cross University (SCU), Lismore with initial funding of AUS\$60,000,000. The primary aim of the Institute is to conduct fundamental research into tea tree oil and to develop value-added products based on a better understanding of its properties and mode of action. ATTORI has established excellent research facilities in chemistry with an array of GCs, GCMS, GCAED, SFEGC, HSGC, and HPLC equipment; formulation with pilot batch manufacturing facilities up to 100kg capacity and collaborative interactions with microbiology and pharmacology staff. Over 300 tea tree oil based products are currently under development for end-use in the areas of acne, hospital and household antiseptics and disinfectants, oral hygiene, tinea, candida infections, dandruff control, hair care and personal care products. To achieve this goal ATTORI currently out-sources a significant proportion of the research work. This will change in 1998 when the Institute will have over twenty scientists accommodated in the new ATTORI building due to be completed in late 1997.

Through the Institute's close association with SCU and working in collaboration with the university academic staff, ATTORI intends to grant postgraduate scholarships as well as postdoctoral and visiting fellowships in the areas of natural product chemistry,

plant genetics, microbiology and pharmacology. The Institute's longer term aims are to investigate the pharmacological properties of other Australian native plants and to develop sustainable commercial production. This has already been extended to research into other natural substances and to the development of new and better applications for them.

EFFICACY

Today's consumers are seeking their health care needs more and more from natural sources. The expectations of fewer side effects and dissatisfaction with conventional medicine has led to an increasing demand for plant based health care products.

Unfortunately, very few properly controlled efficacy studies have been conducted on natural products and without these studies, the benefits of such preparations have remained anecdotal at best. This was certainly true for tea tree oil which has over the past decade received considerable attention culminating in some excellent *in vitro* data (Carson *et al.*, 1993, 1995, 1996, Southwell *et al.*, 1993). To advance the credibility of tea tree oil and its acceptance as a recognised therapeutic agent, major research into products that satisfy the demands of full clinical trials are required. ATTORI's funding was raised to address this financial hurdle and the Institute now has external research projects being conducted at Curtin University, Royal Perth Hospital, Royal North Shore Hospital and Monash Medical Centre. These centres are currently involved in multi-centred clinical trials on human volunteers on acne, tinea and dandruff with combined patient numbers of over 500. These trials should all be completed by early 1998 and will provide statistically significant data

to clarify the hitherto anecdotal claims for tea tree oil. A joint industry/government funded clinical trial on infection control is being conducted for MRSA (Methacillin Resistant *Staphylococcus aureus*). Pilot studies are also underway on oral and vaginal *Candida* infections and Vancomycin Resistant *Enterococci* (VRE).

To optimise the efficacy of formulations it is necessary to determine the mode of action of tea tree oil against micro-organisms. Work is being performed at two centres using sensitive probes to determine both physical and biochemical effects of tea tree oil on a range of micro-organisms. Linked to this study is a detailed fractionation of tea tree oil and investigation of its components. The aim is to identify the potency of each component against bacteria and to determine if there is synergy within the mixture. There is also anecdotal evidence that tea tree oil has anti-inflammatory, skin penetration and insecticidal properties. Such claims are under investigation by ATTORI and contracts are being negotiated and in some cases have been exchanged to commence work in these areas.

As the use of tea tree oil as a therapeutic agent increases there is an increasing need for validated scientific data that addresses important issues surrounding toxicity and skin irritancy and sensitivity.

TOXICITY

Tea tree oil has an acute oral toxicity in rats of 1.9-2.6 mL/kg body weight and an acute dermal toxicity in rabbits of >2g/kg body weight (Altman, 1991). These findings place tea tree oil in a moderate toxicity category and are in line with known toxicity levels

for pure terpenic compounds, in the order of 2-5g/kg body weight. Following on from preliminary cytotoxicity data reported by Sonderberg *et al.* (1996) we have recently completed a more detailed study of the cytotoxicity of tea tree oil and some of its major components using a range of human cell lines (Hayes *et al.*, 1997). The assay employed MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) in a colourimetric method to measure cell proliferation based on mitochondrial dehydrogenase activity. Two exposure times, 4 and 24 hours, are used for the assay against five different human cell lines. Results of the 4 hour MTS assay indicate the acute effect of the test compounds on the cellular activity of the cell lines tested, while MTS with 24 hour exposure showed further cumulative toxicity on the cells.

Results of the cytotoxicity study for IC₅₀ values for only the liver (HepG2) and epithelial (HeLa) cell lines at 4 and 24 hour incubation periods for 100% tea tree oil and its major oxygenated monoterpenes terpinen-4-ol, α -terpineol and 1,8-cineole are presented in Table 2 and Table 3 respectively. Aspirin and mercuric chloride were chosen as the controls. Aspirin is available as an OTC drug and is regarded as a moderately safe compound with extensive toxicity data available on its usage and a LD₅₀ value of approximately 1.3 g/kg. Mercuric chloride is described as a 'violent poison' and 'highly toxic' with 1-2 g often resulting in fatalities.

TABLE 2

IC₅₀ Values (g/L) of Tea Tree Oil Components and Standards of Aspirin and Mercuric Chloride at an Incubation Period of 4 Hours Against Two Cell Lines

CELLS	HeLa	Hep G2
Tea Tree Oil	2.80±0.90	2.80±0.30
1,8-Cineole	3.80±0.40	3.00±0.65
Terpinen-4-ol	2.70±0.30	0.50±0.09
α-Terpineol	1.10±0.20	0.80±0.22
Mercuric Chloride	0.05±0.01	0.05±0.01
Aspirin	2.40±0.42	2.10±0.40

IC₅₀ values expressed as Mean ±SD, where a minimum of two repeats with six measurements per dilution were performed on each test compound.

TABLE 3

IC₅₀ Values (g/L) of Tea Tree Oil Components and Standards of Aspirin and Mercuric Chloride at an Incubation Period of 24 Hours Against Two Cell Lines

CELLS	HeLa	Hep G2
Tea Tree Oil	2.70±0.07	0.02±0.006
1,8-Cineole	2.5±0.50	0.14±0.06
Terpinen-4-ol	0.14±0.012	0.06±0.01
α-Terpineol	0.15±0.01	0.02±0.008
Mercuric Chloride	0.05±0.01	0.01±0.005
Aspirin	1.40±0.27	0.25±0.03

IC₅₀ values expressed as Mean ±SD, where a minimum of two repeats with six measurements per dilution were performed on each test compound.

The results indicate that α -terpineol is the most cytotoxic of the tea tree oil components tested. Tea tree oil and terpinen-4-ol have similar toxicity with the exception of their affect on Hep G2 cells at 4 hours, but at 24 hours the IC₅₀ values are again similar. 1,8-Cineole is the least toxic in most cell lines tested and is comparable to that of aspirin in its toxic rating. All test compounds other than 1,8-cineole at 4 hours showed an increase in cell death when compared to that of the standard control aspirin, while mercuric chloride at both 4 and 24 hour time periods with respect to all components tested had the most cytotoxic effect on all cell lines. The overall rating for toxicity of tea tree oil and its components was α -terpineol>tea tree oil>terpinen-4-ol>1,8-cineole and with reference to tea tree oil and the controls used the toxicity rating was mercuric chloride>tea tree oil>aspirin.

The IC₅₀ range of tea tree oil in the human cell lines tested was 0.02-2.8g/L and compares favourably with the LD₅₀ *in vivo* of rats of 1.9-2.6 mL/kg (Altman, 1991). The IC₅₀ range of 1,8-cineole in the cell lines tested was 0.14-4.20g/L which is also comparable to the published acute toxicity (rabbit dermal and rat oral) value of 2-5g/kg body weight (Villar *et al.*, 1994). These results indicate that human cells show a greater sensitivity to tea tree oil than rats, suggesting a greater susceptibility of cells in a closed experimental culture system than similar cells involved with the metabolic dynamics of an experimental animal.

Of the five cell lines tested, epithelial like cells (HeLa) at a time period of 4 hours were the most robust cells with respect to the monoterpene alcohol based tea tree oil components while the liver derived cells (Hep G2) at 24 hours were the most susceptible. HeLa cells have been used extensively as a good model for toxicity studies (Ekwall, 1983; Ekwall *et al.*, 1990) and are phenotyped similar to human skin fibroblasts. Our

cytotoxicity results support the use of tea tree oil in topical applications but not for ingestion purposes. These differences in cytotoxicity of cell lines could indicate a metabolic difference between the HeLa and the Hep G2 cell lines. As the liver is the major organ for metabolism these observations for increased cytotoxicity of the oxygenated monoterpenes terpinen-4-ol, 1,8-cineole and α -terpineol could be a result of direct inhibition of liver cells by these compounds or by their resulting metabolites.

The results of this study indicate the usefulness of the *in vitro* cytotoxicity assay MTS as an excellent, rapid technique for elucidating the toxic properties of essential oils such as tea tree oil and its components without the ethical problem of using experimental animals

IRRITANCY

Tea tree oil is used as a topical disinfectant and as its use increases there has been growing concern about skin irritancy and sensitivity (Southwell *et al.*, 1997). To address this issue ATTORI has contracted the Skin and Cancer Foundation, Australia, to conduct irritancy and sensitisation studies on 300 volunteers. On the 150 volunteers tested to date, irritation scores were not significantly different from the placebo for formulations using a variety of bases including gels, creams and lotions, containing up to 25% Main Camp Pharmaceutical Grade tea tree oil. For neat (100%) oil an average daily score of 0.1922 was observed (based on a scale of 0 = no reaction, 1 = erythema, 2 = erythema & odema, 3 = vesiculation and 4 = bulla formation), thereby confirming that tea tree oil is an extremely low irritant when applied to the skin. However, tea tree oil must be considered

a mild allergen as 2% of the panellists showed an allergic reaction. Possible reasons for the adverse reactions to tea tree oil could include:-

- 1) The inherent irritancy of one or more of the 100+ components of tea tree oil.
- 2) Irritation from degradation products formed during the manufacturing process (e.g. uncontrolled distillation and poor storage conditions).
- 3) Allergens and irritants introduced from weeds harvested with the tea tree biomass.
- 4) Adulteration of tea tree oil with cheaper oils.

ATTORI is following up this irritancy/sensitivity study with additional trials on panellists that displayed sensitisation. The oil used in the preliminary study has been fractionated and the fractions subjected to detailed chemical analysis and resubmitted to the Skin and Cancer Foundation for further assessment. It is anticipated that the results will elucidate the compound(s) that cause the sensitisation and that such compound(s) can then be minimised or eliminated from the oil.

STABILITY

Tea tree oil is susceptible to oxidation and this is highlighted by the analytical data (Table 4) presented for two oils stored at room temperature in the dark in glass bottles. Several important trends are apparent. Firstly terpinen-4-ol concentration can increase significantly over time whilst the 1,8-cineole level remains constant. Secondly, the monoterpene hydrocarbons α -terpinene, γ -terpinene and α -terpinolene decrease, in some cases markedly, over time.

TABLE 4**Stability Data for Tea Tree Oil Components Over Two Years**

Batch No	#1			#2		
Time (years)	0	1	2	0	1	2
COMPONENT						
terpinen-4-ol	40.6	45.9	45.1	40.3	41.4	40.8
1,8-cineole	2.1	2.0	1.9	2.7	2.8	2.6
α -terpinene	8.2	7.0	5.8	8.5	9.1	2.9
γ -terpinene	17.0	15.1	12.8	18.5	21.1	10.6
α -terpinolene	3.3	2.7	2.5	3.3	3.4	2.1
p-cymene	5.7	5.2	4.7	2.5	2.9	10.1

Finally, p-cymene concentration can increase to levels approaching its upper limit of 12% in the ISO 4730 standard. Two pathways are operating here (Figure 1), one involving hydrolysis of the pi bond at C-4 to produce terpinen-4-ol, the other involving oxidation of the p-menthane skeleton to its benzene analogue, p-cymene. The first pathway must involve water present in the oil through the steam distillation extraction process and presumably trace volatile organic acids that may catalyse the reaction. Ageing processes that enhance oil quality must be thoroughly understood through the monitoring of simple parameters such as moisture and pH. The second pathway is well known in

terpene chemistry however the various oxidising agents and catalysts that are involved in tea tree oil degradation require further investigation.

The key chemical indicator of oil treatment during processing and storage is p-cymene and elevated levels are usually an indication of poor storage conditions, old oil or harsh treatment during extraction. An upper level of 12% in the draft standard may need closer scrutiny and could be revised downwards to provide greater quality assurance.

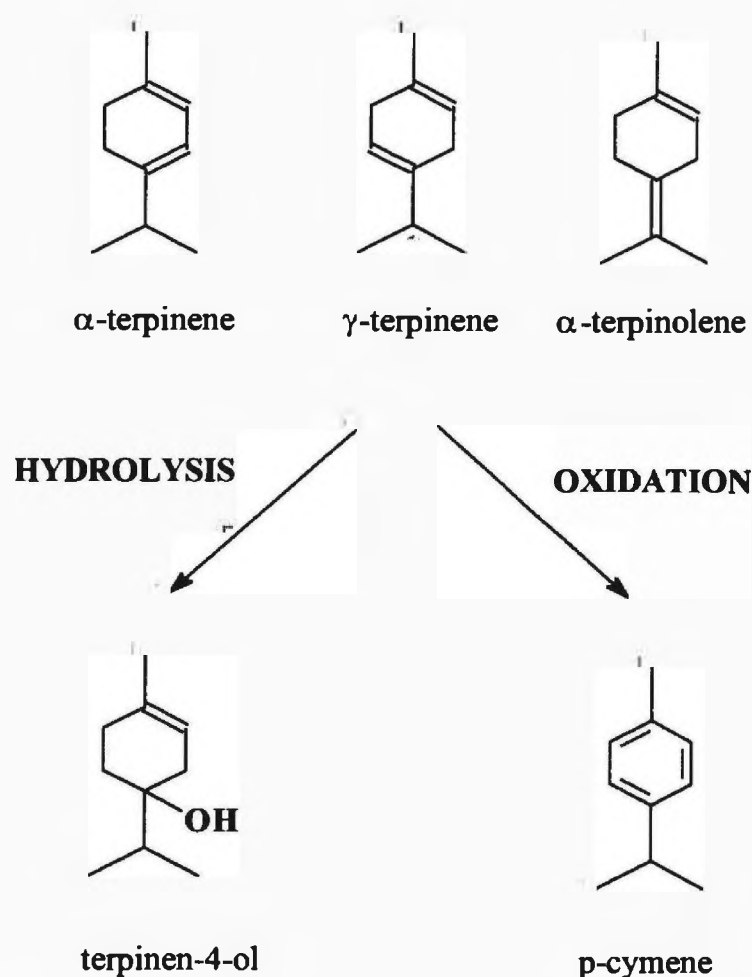


FIGURE 1. Degradation Pathways of Some Monoterpene Hydrocarbons

Tea tree oil has excellent penetration properties, a plus for its therapeutic activity but a problem for storage and packaging. The oil's affinity for simple olefinic plastics renders these containers unsuitable. Stainless steel is far superior to galvanised iron and epoxy lined steel containers and is the container of choice for medium to long term storage (up to two years) and shipping of bulk quantities. Fluorinated HDPE and coloured glass bottles (which must incorporate an aluminium lined plastic cap) are satisfactory for short to medium term storage for smaller quantities of oil. These simple packaging guidelines apply equally well to formulated products, although the lower levels of tea tree oil and the formulation matrix of some products may permit adequate stability in common plastics such as HDPE and polypropylene. This is not always the case as the data in Table 5 reveals. Using glass as the reference storage container losses over 50% can be observed at temperatures of 30°C over three months.

TABLE 5

Stability of Terpinen-4-ol and 1,8-Cineole at 30°C in Different Containers.

Container Type	Product Batch No	Terpinen-4-ol (mg/100g)	1,8-Cineole (mg/100g)
Glass	16	569	62.5
HDPE	16	417	40.6
Polypropylene	16	375	26.6

OIL PRODUCTION

As part of the process of improving tea tree oil, ATTORI, in conjunction with Southern Cross University, has collected leaf and seed samples from 600 *M. alternifolia* trees across its natural distribution. This material will be profiled using microsatellite DNA markers that will be correlated with oil yield and oil quality parameters. Such data will be used to identify trees for breeding programs that will be directed towards specific oil quality and yield characteristics. This research is linked to another investigation into the biosynthetic pathway of tea tree oil formation. As part of its ISO 9000 oil production process Main Camp plantation has a patented post-distillation process that improves water separation, oil clarity and odour. This process may be a significant contributor to the production of low irritancy oil whilst ensuring uniformity of product from batch to batch and season to season.

ACKNOWLEDGMENTS

I would like to acknowledge the significant contributions of my colleagues at ATTORI; Craig Bell, Bill Eickhoff, Kellie Shepherd, Ross Macdougald and at the University of Western Sydney, Hawkesbury; Shane Griffin, Julie Markham, Grant Wyllie, Charles Cornwell, and Amanda Hayes, in the Centre for Biostructural & Biomolecular Research, without whose input this work would not have come to fruition. I would also like to acknowledge the support of Don Priest, Main Camp Tea Tree Oil Group, Ballina, NSW; Geoff Holmes and Susan Freeman, Skin & Cancer Foundation, Sydney, NSW; Bob

Dean, Blackmores Ltd, Sydney, NSW and Boban Markovic, University of NSW for their input and expert advice that has been used in this work.

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PACKED COLUMN SFC IN THE ANALYSIS OF TERPENOIDS

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SUMMARY

Packed column supercritical fluid chromatography (*p*-SFC) has recently stimulated interest as an alternative to HPLC. This has mainly been due to the introduction of variable and programmable restrictors, on-line flow rate controllers and high pressure cells for UV detectors, which have afforded reliable quali-quantitative results. *p*-SFC has now sample capacity similar to HPLC, operates with the same columns, and in general requires shorter analysis times because of the higher theoretical plate number per unit time.

This communication aims to focus on the advantages and disadvantages of *p*-SFC, and the strategy adopted in the authors' laboratory to develop new *p*-SFC methods are illustrated through some examples involving terpenoids related to aromatic plants (in particular sesquiterpene lactones and triterpenoids).

INTRODUCTION.

Since Klesper introduced it in 1962, supercritical fluid chromatography (SFC) has continued to experience some difficulties in becoming really popular. This may be because SFC lies between gas- and liquid-chromatography, and most people probably assume that these two alone already cover all fields of applications. This brought to underestimate the possibilities of SFC very seriously.

A fluid is in a supercritical state when it is above its critical pressure and temperature. The characteristics of a supercritical fluid are in between those of a liquid and those of a gas, since they have the density and the solvating power of a liquid, the viscosity of a gas, while their diffusivity is intermediate between liquids and gases.

The most popular supercritical fluid is CO₂, because it is non-toxic and environmentally safe, and it can be brought to a supercritical state under relatively mild conditions (31°C and 73 bar). Supercritical CO₂ polarity is medium-to-low, and this limits its use in the analysis of highly polar compounds. On the other hand, at present, no polar supercritical fluid has been found with

characteristics comparable to that of supercritical CO₂. But, the polarity of a supercritical fluid can be varied not only chemically, as is usual for a liquid, through a miscible soluble polar chemical modifier such as methanol, ethanol, or acetonitrile, but also physically, through its density, that is pressure and temperature.

Since the beginning, SFC has continued to evolve along two general lines: capillary columns and packed columns. Recently there has been a resurgence of interest in the use of packed columns in SFC, thanks to the introduction of variable and programmable restrictors, on-line flow rate controllers and high pressure cells for UV detectors. As a consequence, packed column SFC (*p*-SFC) has again gained interest as an alternative to HPLC, since a) it has similar sample capacity; b) it uses the same columns as in HPLC; c) it is easier than is HPLC to couple with mass spectrometry, or with general detectors, such as FID, or evaporative light scattering detector; and d) it has a higher theoretical plate number per unit time, which means shorter analysis times and reliable qualitative results. This last is the best known characteristic of SFC, and in general most people look for quick analysis results, without considering several others important properties of SFC, such as selectivity and efficiency.

EXAMPLES OF *p*-SFC APPLICATIONS TO ANALYSIS OF TERPENOIDS EXTRACTS

The possibilities of *p*-SFC/UV, mainly in comparison with HPLC will be illustrated with the following examples.

The first example aims to illustrate the best known property of SFC/UV, that is high analysis speed, together with another important point for routine analysis, that is column conditions stability over a series of analyses. It concerns the analysis of iridals in *Iris pallida* rhizome extracts [1].

Iridals are a class of triterpenoids characteristic of the *Iris* genera which have been demonstrated to be the precursors of irones. Irones are a group of compounds that are prized in the perfume industry because of their attractive violet smell (Fig. 1). Iridals are generally analysed by HPLC with a C18 column and methanol-water as mobile phase and the method is well established. But, when a high number of injections per day are required, a one hour analysis is too long. Moreover, in routine HPLC analysis of iridals, after a number of injections (10 to 15), column

pressure often abnormally increases probably because of the coextracted fatty acids. As a consequence, column must very often be reconditioned.

SFC/UV analysis with a cyano column and supercritical CO₂ modified with methanol as mobile phase halved the analysis time, but, even more important, column conditions during repeated analyses were constant, thus allowing us to run 80 to 100 injections before column reconditioning became necessary (Fig.2)

The next application aims to stress efficiency, selectivity and flexibility of *p*-SFC/UV [2]. Sesquiterpene lactones are a group of about 500 compounds which are present in several plant families and which show interesting biological or therapeutic activity. These compounds, whose molecular mass generally ranges from 230 to 500, are close in polarity, thermolabile, and their volatility is low, therefore up to now, they have generally been analysed by reverse phase HPLC.

This example aims to emphasize the role of pressure in SFC selectivity. Eighteen sesquiterpene lactones with different carbon skeletons belonging to the groups of the germacranolides, guaianolides, eudesmanolides, *seco*-eudesmanolides, and helenanolides were analysed. Fig. 3 reports the structures of the lactones investigated. The most interesting results were obtained with a cyano column and supercritical CO₂ modified with MeOH/H₂O 95/5 as mobile phase. Under these conditions, the eighteen lactones were well separated, by analysing them in two sub-groups according to vegetable origin, skeleton and polarity. The two sub-groups were differentiated by varying the CO₂ elution strength which was varied only by pressure: the first one which contains the less polar lactones was analysed at 14 MPa, while the second one, containing the more polar ones, was analysed at 20 Mpa (Fig. 4 and 5).

Several lactonic extracts from many plants were then analysed.

The analysis of SL extracts from *Artemisia umbelliformis* Lam. is here reported. *A. umbelliformis* is one of the main ingredients for typical alpine liqueurs, because of its aromatic and sesquiterpene lactones-related bitter properties, and it contains two hydroperoxytelekines, AU1 and AU2, and umbellifolide, among others. Fig. 6 reports the SFC/UV pattern of the *Artemisia umbelliformis* extract carried out at 20MPa. It is again interesting to remark the role that pressure plays on the selectivity of the chromatographic system: umbellifolide and AU2 reverse their elution

order when analysed at different pressures, without varying the other analysis parameters: at 20MPa AU2 elutes before umbellifolide, while at 30MPa the contrary occurs.

CONCLUSIONS:

In conclusion, *p*-SFC is a technique that is very useful in the field of natural extracts, and that is alternative and/or complementary to HPLC. In addition to its quickness, efficiency and selectivity, one of the main characteristics of *p*-SFC is that, because of the low polarity of supercritical CO₂, it mainly works by normal phase, while most HPLC applications (i.e. over 90%) are made in reverse phase mode. SFC is therefore particularly useful for the analysis of slightly or moderately polar compounds, as is the case of several classes of terpenoids related to our field.

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IRIDALS AND IRONES

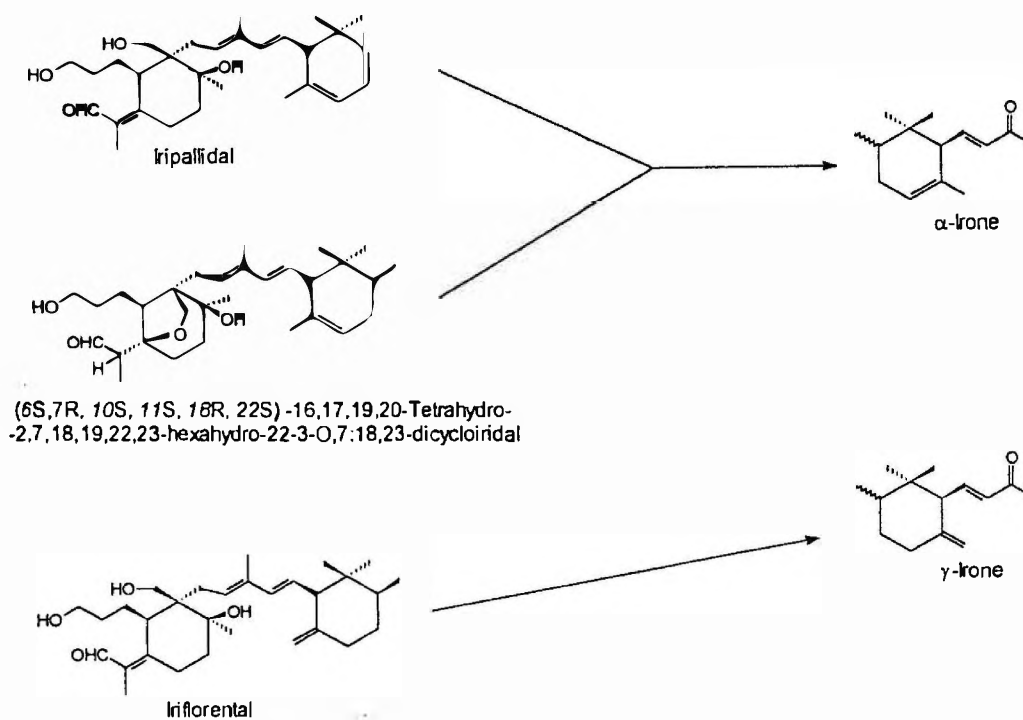


Fig. 1: Iridals and irones

IRIDAL FRACTION FROM *IRIS PALLIDA* L.

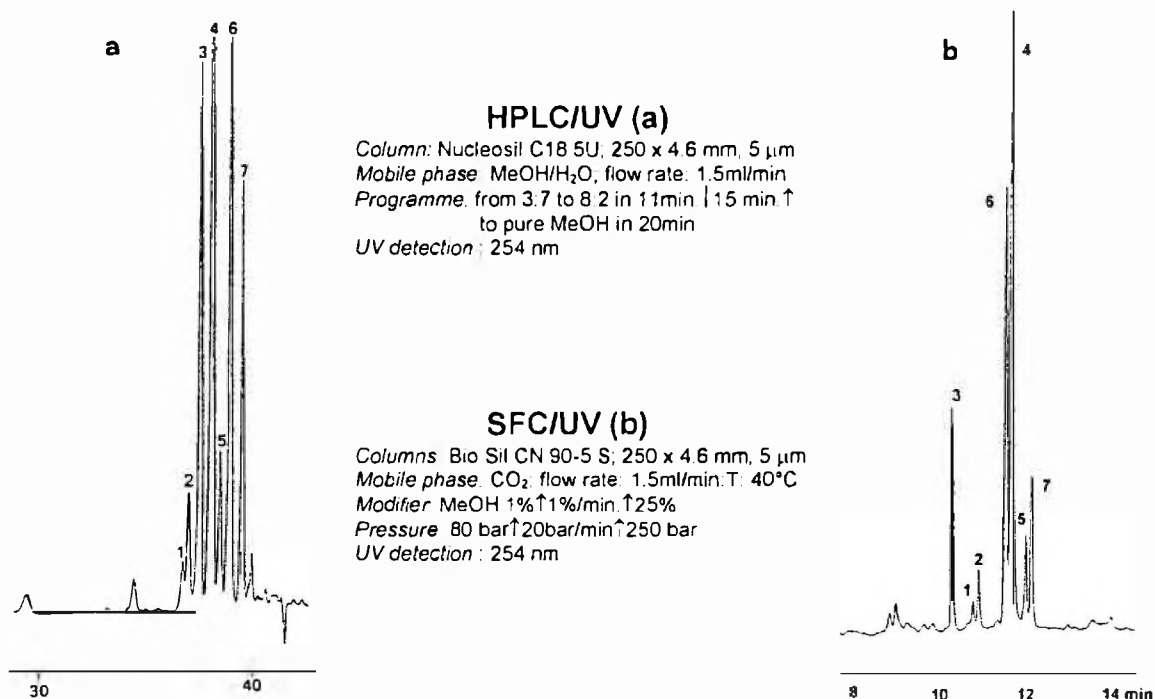


Fig.2 HPLC and *p*-SFC analysis of iridals

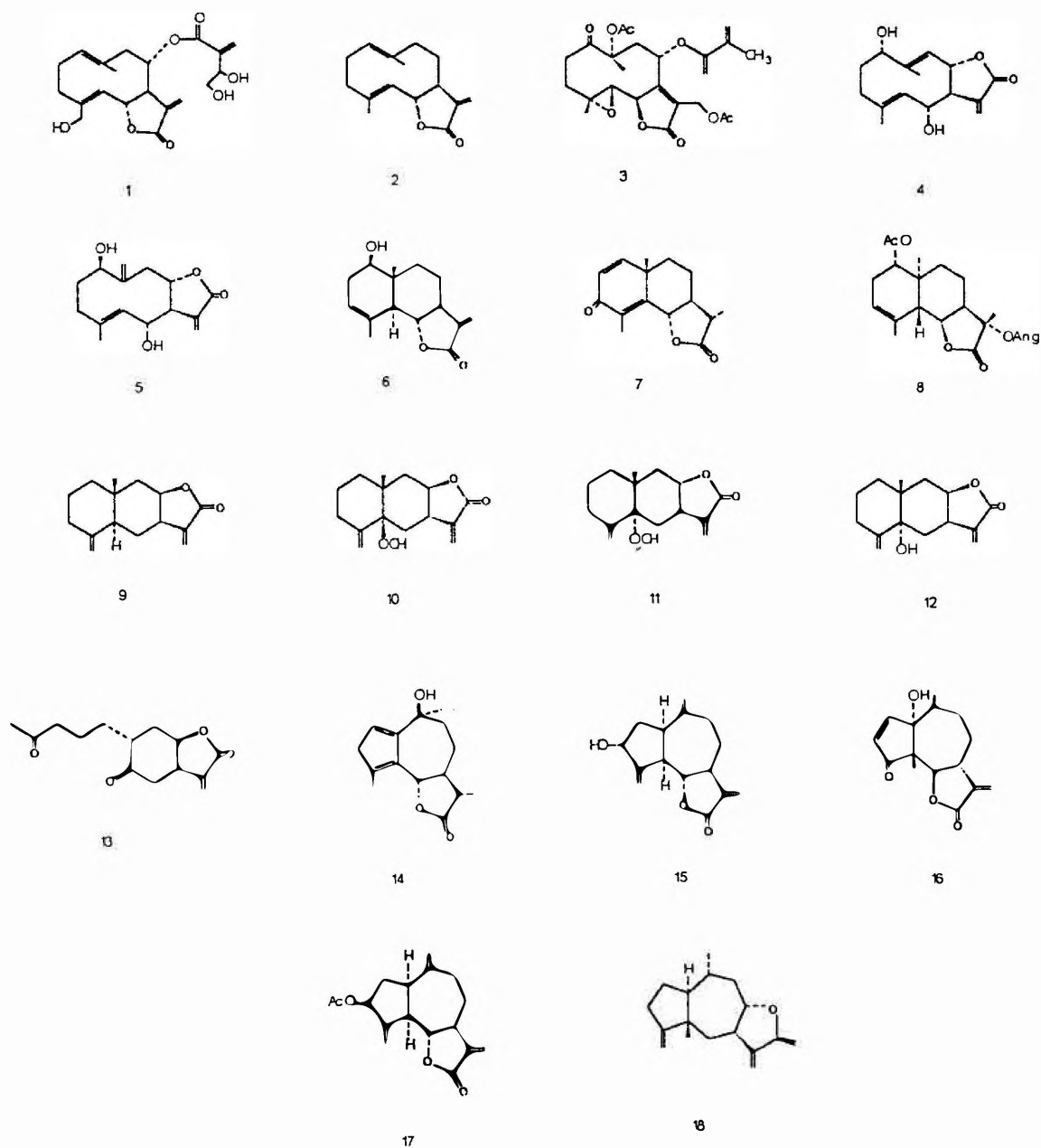


Fig. 3 Structures and names of the lactones investigated

(1) cnicin, (2) costunolide, (3) glaucolide A, (4) tatridine A, (5) tatridine B, (6) santamarine, (7) α -santonine, (8) isosilerolide, (9) isoallantolactone, (10) 5-deoxy-5-hydroperoxy-5-epitelekinine (AU1), (11) 5-deoxy-5-hydroperoxy-5-telekinine (AU2), (12) telekinine, (13) umbellifolide, (14) artabsine, (15) epizaluzanine C, (16) parthenine, (17) zaluzanine D, (18) graveolide

SESQUITERPENE LACTONES STANDARD MIXTURE 1

Costunolide (3), isosilerolide (10), zaluzanine D (21), graveolide (22), artabsine (18), santamarine (8) and glaucolide (4)

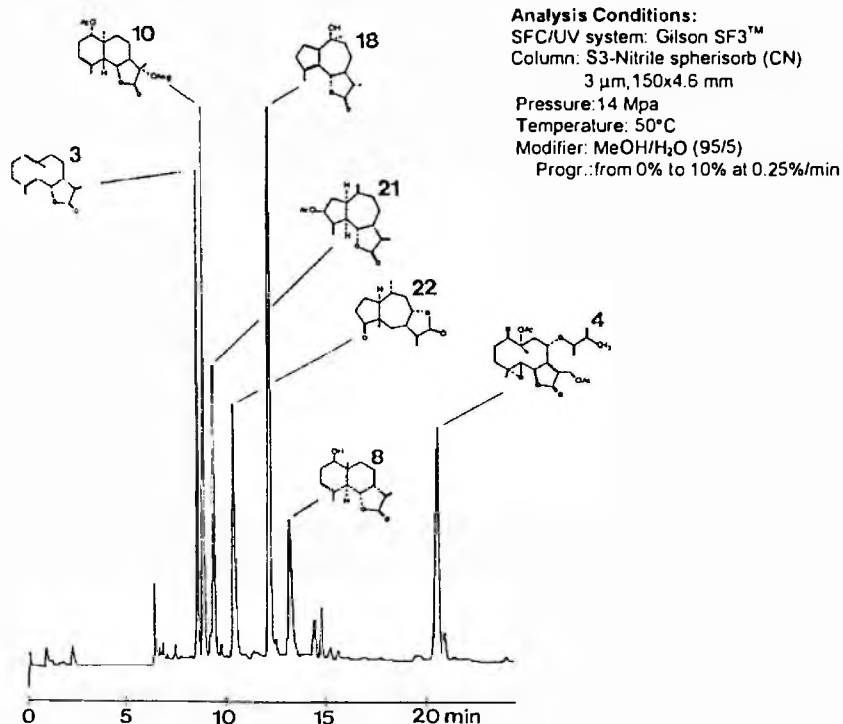


Fig. 4 Analysis of sesquiterpene lactones eluting at 14 Mpa

SESQUITERPENE LACTONES STANDARD MIXTURE 2

Isoallantolactone (12), telekine (15), umbellifolide (16), α -santonine (9), epizaluzanine C (19), latridine A (6), latridine B (7), parthenine (20) and cnicin (2).

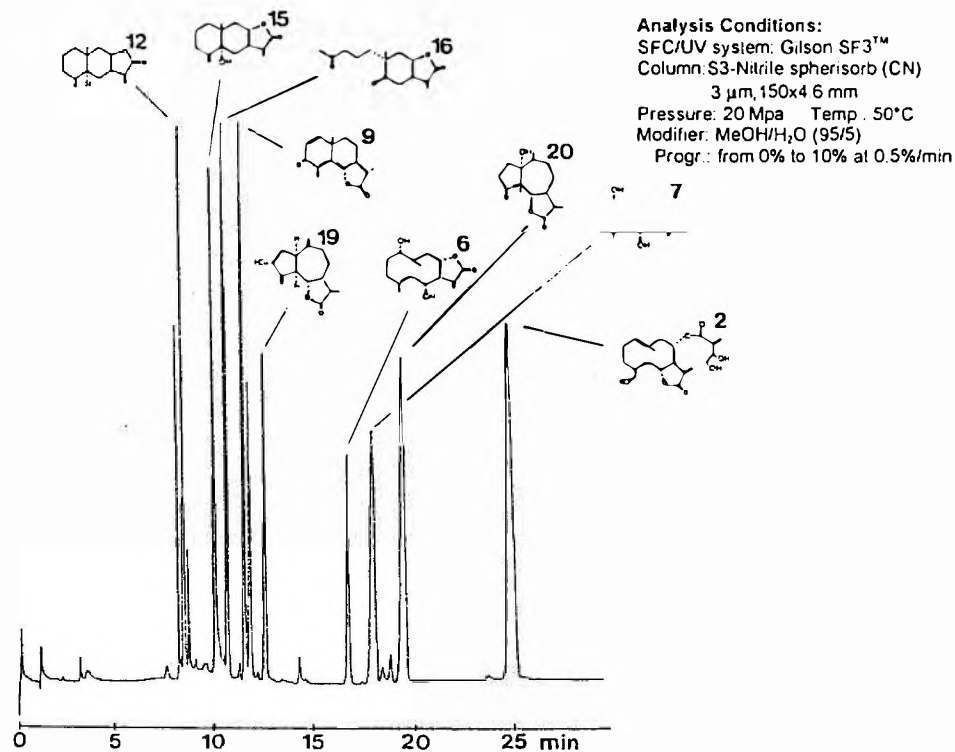


Fig. 5 Analysis of sesquiterpene lactones eluting at 20 Mpa

SESQUITERPENE LACTONE EXTRACT FROM *ARTEMISIA UMBELLIFORMIS*

AU1(13), AU2 (14) and umbellifolide (16).

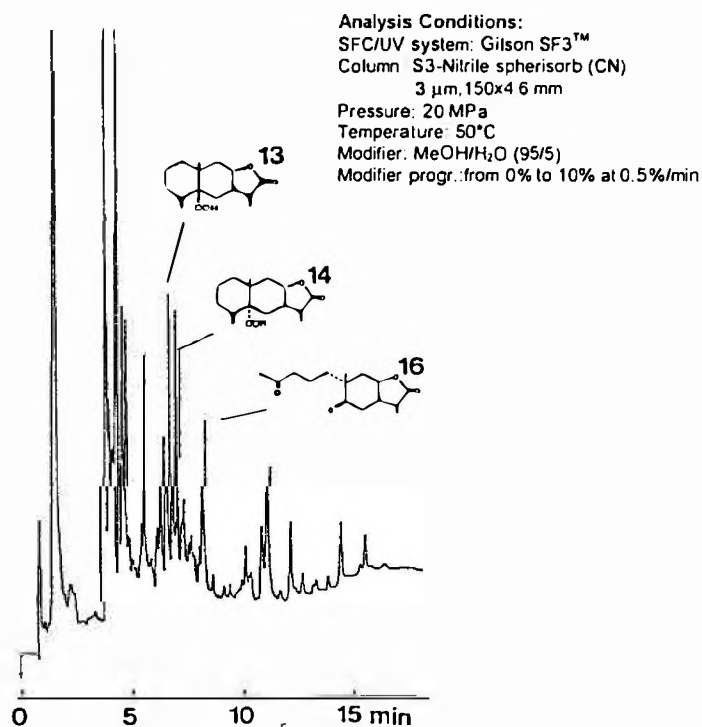


Fig. 6 SFC/UV pattern of the *Artemisia umbelliformis* extract

SESQUITERPENE LACTONES STANDARD MIXTURE 3

AU1(13), AU2 (14) and umbellifolide (16)

Analysis Conditions: SFC/UV system: Gilson SF3™; Column: S3-Nitrile spherisorb (CN) 3 µm, 150x4.6 mm;
 Temperature: 50°C; Modifier: MeOH/H₂O (95/5); Modifier progr.: from 0% to 10% at 0.5%/min

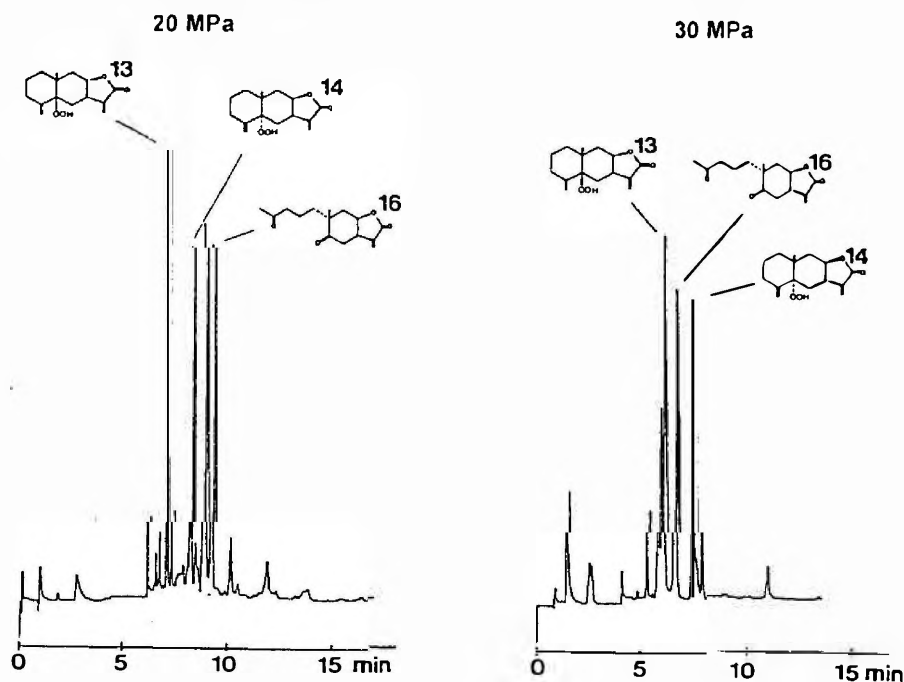


Fig. 7 SFC/UV patterns of the *Artemisia umbelliformis* extract carried out at 20mpa and 30MPa.

CONTROL OF STORAGE DISEASES OF POTATO AND SPROUT INHIBITION BY PLANT VOLATILES.

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ABSTRACT

Experiments to control storage rots caused by *Fusarium solani* var. *coeruleum* (Sacc.) Booth and the silver scurf organism, *Helminthosporium solani* Dur. et Mont, on artificially inoculated tubers of potato (*Solanum tuberosum* L.) by using plant volatiles are described. Tubers were kept at +10°C during 2-3 weeks in an aromatic atmosphere obtained from essential oils (EO) or from homogenised plant material. In addition, *in vitro*-experiments on agar plates were carried out to study the fungistatic/fungicidal nature of the vapours and to compare the results from the two experimental systems. Also, the effect of some volatiles on sprouting of tubers is reported.

Significant reductions of both pathogens were achieved *in vitro* and *in vivo* with vapours of garlic EO (*Allium sativum*). Groundcloves of garlic and onions (*Allium cepa*) were tested against *F. solani* *in vivo* and found effective. At the doses used, caraway EO (*Carum carvi*) was effective against *H. solani*, only. Essential oil of sage (*Salvia officinalis*) significantly controlled the attack of both fungi on tubers, although its fungitoxic effect in *in vitro* experiments were lacking or very weak. Basil EO (*Ocimum basilicum*) and balsam poplar EO (*Populus balsamifera*) increased the incidence of *F. solani* and *H. solani*, respectively. The *in vitro* experiments revealed no or only fungistatic effects of these oils.

Sprouting of tubers, cv Bintje, was influenced by many plant extracts already after 3 wks vapour treatment in spring. An almost complete sprout suppression was achieved in cv Hertha by many volatiles after a continuous treatment during the summer months in 1996.

INTRODUCTION

Both synthetic fungicides and as well as sprout-inhibitors may be found as residues in treated potato tubers (*Solanum tuberosum* L.) and products thereof. Also, various fungal potato pathogens have developed resistance against the former. These drawbacks and the scrutiny by consumers and food safety regulators, has increased the interest to develop alternative control methods based on environmentally sound, natural substances. This is not a new idea. In the country of origin of the potato, the Inkas put leaves of Muña plants (*Minthostachys* spp.) in the potato stores to prevent sprouting. This method is still practised in the Andes and its inhibiting activities on sprouting as well as on rotting has recently been confirmed (1). In experiments in Greece, the vapours from several other

aromatic herbs, growing wild in the Mediterranean district, and their essential oils were shown to possess sprout-inhibiting properties of potatoes (8). Various single volatile plant constituents have been demonstrated to suppress sprouting (3, 6, and references therein). In the Netherlands, special interest has been directed towards the monoterpene carvone, produced from caraway seeds. Its sprout-suppressing properties as well as its activity on wound-healing processes of potatoes has been thoroughly investigated (4, 5)

The antimicrobial effects of essential oils, or their constituents, on plant pathogens have been quite extensively studied on artificial media in *in vitro*-trials. There are, however, not many reports from *in vivo*-experiments comprising a complete host-pathogen-system. Considering potato storage diseases, some control is reported by Aliaga & Feldheim (1) but the causing organisms are not specified. In one series of experiments, Hartmans et al. (4) showed that carvone controlled rotting caused by *Fusarium sulphureum* and *Phoma foveata*, but not that caused by *F. solani* var *coeruleum* in artificially inoculated tubers. Carvone treatment also reduced the attack of silver scurf in naturally infected ones. Menthol somewhat reduced the incidence of rotting in tubers, artificially inoculated with *F. sulphureum* (7).

The experiments, presented here, report the effects of vapours of essential oils or ground plant parts, on the development of two fungal storage pathogens: *Fusarium solani* var. *coeruleum* (Sacc.) and *Helminthosporium solani* Dur. et Mont in artificially inoculated potato tubers and also their fungitoxic actions in *in vitro* trials. They are parts of a greater project, including studies of other artificially inoculated pathogens as well as vapour treatments of naturally contaminated, newly harvested tubers. The influence on tuber sprouting has also been studied. The screening of plant extracts has been biased, as the plants should preferably be possible to grow in a temperate climate. If found suitable as producers of natural fungicides, they would offer an alternative use of agriculture land for non-food production. This project has been carried out during 4 storage seasons, starting in the autumn of 1993. Some of the results from the first year of experiments have been reported in English (2).

RESULTS AND DISCUSSION

Results from experiments to control *F. solani* are presented in Table 1. The effects of garlic treatments both in agar and tuber tests were comparable and showed a strongly fungicidal action. The vapour treatments with ground cloves of garlic or onion also significantly reduced disease development. Interestingly, the sage treatment, which did not prevent the spore-germination and subsequent mycelial growth in agar tests, decreased the rotting in the tuber experiment at the lowest application used. The basil treatment, on the other hand, which seemed to have some fungistatic action at the higher doses *in vitro*, actually significantly increased the rotting of tubers at all doses. Obviously, it is not always possible to foresee the practical action as a fungicide of a substance, by making *in vitro* trials only. Many volatiles probably affect the host as well as the pathogen.

Table 1. No. of colonies of *Fusarium solani* on agar plates and rot-index (1-15) in artificially inoculated potato tubers, cv. Escort, in parallel *in vitro* and *in vivo* experiments after exposure to vapours of various aromatic plant extracts during 3 wks, applied in various initial amounts. The number of colonies on agar plates were counted at two occasions: a) at removal of plant extracts, and b) 5 wks thereafter. The *F. solani* rot-index was registered 7 wks after inoculation. Index values, significantly different from control values at $P \leq 0.05$ are in bold figures. Means of 5 (*in vitro*) and 3 (*in vivo*) replicates.

Essential oils	40 ppm			80 ppm			120 ppm			160 ppm			9.1 g/l	
	<i>in vitro</i>		<i>in vivo</i>	<i>in vitro</i>		<i>in vivo</i>	<i>in vitro</i>		<i>in vivo</i>	<i>in vitro</i>		<i>in vivo</i>	<i>in vivo</i>	
	no. colonies	Rot-index	Rot-index	no. colonies	Rot-index	Rot-index	no. colonies	Rot-index	Rot-index	no. colonies	Rot-index	Rot-index	Rot-index	
Control ¹⁾	a	b		a	b		a	b		a	b			
	200	200	5.19	200	200	5.19	200	200	5.19	200	200	5.19		
<i>Allium sativum</i>	0	0	1.88	0	0	2.00	0	0	2.13	0	0	2.46		
<i>Carum carvi</i>	200	200	4.73	165.8	200	4.94	0	200	6.46	0	200	5.96		
<i>Carum carvi</i> ²⁾			5.38			4.06			5.42			5.08		
<i>Salvia officinalis</i>	200	200	3.46	200	200	4.65	200	200	4.00	200	200	4.60		
<i>Mentha piperita</i>	200	200	4.90	200	200	5.67	200	200	5.08	141.	200	6.5		
										2				
<i>Ocimum basilicum</i>	200	200	7.77	182.0	200	8.46	95.8	200	9.27	12.6	200	7.90		
ground plant material														
<i>Allium sativum</i>													1.42	
<i>Allium cepa</i>													3.10	

1) The same controls were used for comparisons at all concentrations

2) cold-pressed oil

Table 2. No. of colonies of *Helminthosporium solani* on agar plates, Exp 1, and *H. solani* - index (0-20) in artificially inoculated potato tubers, cv. Bintje, Exp 2, in parallel experiments in spring -96 after exposure to vapours of various essential oils during 3 wks. The number of colonies on agar plates were counted at two occasions: a) at removal of plant extracts, and b) 5 wks thereafter. The *H. solani*-index was registered at two occasions: c) 2 wks after removal of EO:s and d) 3 wks thereafter = date b. After an additional storage period of 3 months in the dark at +10°C the no.of sprouts and their weight was registered. In Exp 3 the sprout weight of tubers, cv Hertha, was registered after continous vapour treatment from June 05 until August 21 1996. Mean values, significantly different from control values at $P \leq 0.05$ are in bold figures. Means of 2 (Exp 1), 4 (Exp 2), and 3 (Exp 3) replicates.

Essential oils	Exp 1, <i>in vitro</i> 163 ppm		Exp 2, <i>in vivo</i> 77 ppm				Exp 3 77 ppm
	No. colonies		<i>H. solani</i> index		No. sprouts	Spr. w. g	Spr. w. g
	date a	date b	date c	date d			
Control ¹⁾	100.0	112.0	3.88	4.81	24.25	25.69	28.44
<i>Allium sativum</i>	0	0	0.15	0.68	36.0	21.48	2.77
<i>Carum carvi</i>	0	136.0	0.37	1.47	30.25	17.87	0.04
<i>Salvia officinalis</i>	31.5	80.5	0.94	2.10	41.0	6.74	8.14
<i>Salvia lavandulifolia</i>							3.24
<i>Lavandula angustifolia</i>	0	68.5	0.97	2.65	26.75	23.62	0.29
<i>Minthostachys verticillata</i> , Muña	0	91.0	0.36	2.82	34.25	19.32	0.15
<i>Mentha piperita</i>	0	107.5	0.59	4.22	29.25	10.08	0.01
<i>Mentha arvensis</i>	0	98.0	0.98	5.98	32.0	11.40	0.02
<i>Mentha spicata</i>	0	92.5	1.07	3.92	30.0	19.07	0.13
<i>Thymus vulgaris</i> (thymol-type)	0	0	1.44	4.12	25.75	24.33	27.5
<i>Rosmarinus officinalis</i>	83.0	107.0	1.46	3.97	41.5	13.21	
<i>Populus balsamifera</i>	116.0	122.0	2.69	9.67	23.75	25.19	23.7
<i>Anethum vulgaris</i>							0.11
<i>Ocimum basilicum</i>							0.21

In the *in vivo* experiment, all vapours, except balsam poplar, reduced the incidence of *H. solani*, at the registration two weeks after the removal of essential oils (date c), indicating a potential as controlling agents, Table 2. However, three weeks thereafter (date d) only the garlic, caraway and sage treatments still reduced the disease significantly. At that date,

there was an index twice as high as the control value after the balsam poplar treatment. No fungistatic/fungicidal action by this oil was detected in the agar experiment. The result seems similar to that of basil presented in Table 1.

Again, the sage oil did not perform very promisingly in the agar tests, where it did not completely prevent spore germination during the treatment period and the final reduction of colonies was not very great. Yet, this oil was one of the best in controlling disease in the *in vivo* experiment, where less oil was applied. Also, the caraway treatment performed better in the *in vivo* experiment than could be expected from the agar tests.

On agar, the garlic and thyme oils were fungicidal and completely inhibited the germination of fungal spores. On tubers however, the thyme treatment was not as effective as the garlic one. Maybe, the applied amount of thyme oil was not sufficient in the tuber experiment. The mints, the *Minthostachys*, and lavender oils were more or less fungistatic both in agar and tuber tests under the given circumstances. Had the treatment period been longer or continuous and/or the applied amounts of oils higher, the effects would probably have been stronger. The agar tests were performed with twice the initial amount of oil per volume treatment chamber, compared with the tuber trial, and comparisons of results should therefore be made with this in mind. At half the dose one would expect the *in vitro* effects to be less fungistatic/fungicidal.

All oils except lavender, thyme, and balsam poplar influenced the sprouting ability to some extent in Exp 2, with a treatment period of 3 wks, mostly by reducing the total weight and increasing the number of sprouts. This indicates that the apical sprout was damaged during the treatment, leading to delayed development of the secondary buds. In Exp 3, with continuous vapour treatment, all essential oils, except those of thyme and balsam poplar, suppressed the sprouting significantly. Garlic and the two salvia oils were not among the most effective in this respect.

In the above presented experiments, the vapours of some essential oils increased the attack of the pathogens in *in vivo* experiments. They had no detected antimycotic or stimulating actions in the agar experiments at the doses used. The reason for increased disease can not be explained by the available data. The fungi might have been stressed in a way that they became more aggressive or the defence mechanisms of the host might have been impaired. In two different experiments, treatment with vapours of sage essential oil reduced both diseases although the antimycotic effect, when tested on agar, was weak or lacking. The results indicate that sage might in some way initiate the tuber defence systems and possibly induce resistance. Obviously, the actions of these substances must be carefully studied in the biological system where they are supposed to be used. *In vitro* studies only are not sufficient. In the case of induced resistance, one could risk to reject an active substance as it might not seem promising on artificial media. More detailed studies are needed to clarify these complicated events.

EXPERIMENTAL

Artificial inoculations. Surface sterilised tubers were artificially inoculated, *F. solani* in standard wounds, two on each tuber, and *H. solani* on the tuber skin without wounding in three circular spots per tuber, using newly prepared spore suspensions. These were prepared from cultures, obtained from naturally infected tubers. The inoculated tubers were put into double-bottomed treatment chambers of 3.25l. There were 5 - 8 tubers in each replicate and they rested on a perforated disc above the aromatic plant material. The application doses are given as volume essential oil, or g ground material, added per volume empty container. Controls were stored likewise, but without volatiles. The boxes were closed with a lid. During the treatment period of 3 wks, the boxes were ventilated twice a week during 2 min. to keep the oxygen and carbon-dioxide concentrations at normal levels. All *in vivo* experiments were carried out at +10°C in the dark. After treatment, tubers were put into new boxes without lids and stored long enough for the controls to develop disease symptoms. The incidence of attack was then registered on each tuber and mean indexes of the samples calculated. The *F. solani* index is in a scale from 1 - 15, based on the extent of rotting, where a rot-index of 1 means a healthy tuber and 2 means very small, arrested rots, whereas indexes of 3 and more are fully developed rots of increasing sizes, horizontally and vertically. The *H. solani* index is based on the number of conidophores, times 0.1, within the three rings per tuber, where inoculation took place. The registrations are performed under magnification. Maximum index value is 20, as more than 200 is very difficult to distinguish.

Agar experiments. The same spore-suspensions as in the *in vivo* experiments, although diluted, were used for platings in 9 cm Petri-dishes on agar adapted to fit the two pathogens. After absorption of the suspension into the agar during 60 min. the dishes were turned upside down and the appropriate amount of essential oil was added to a small filter paper disc in the middle of the lid, now bottom. The dishes were sealed with parafilm and stored at room temperature. Replicates of the same treatment were put into a plastic bag which was sealed.

Sprout suppression. The reported experiment, aimed at studying the influence on sprouting ability, was handled as the *in vivo* experiments described above, but without inoculating the tubers. Ventilation was only made once a week and the plant extracts were not removed until the final registration when sprouts were counted and weighed.

Aromatic plant material. Most of the essential oils were bought from CreArom, Gammelstad, Sweden. Some were kindly provided from other laboratories, see below. The garlic and onions used as ground materials were bought from "ecological" Swedish farmers.

Statistics. Statistical calculations were performed using GLM-ANOVA of NCSS, copyright Dr. Jerry L. Hintze, Kaysville, Utah 84037.

ACKNOWLEDGEMENTS

These investigations were supported by the Swedish Farmers Foundation for Agricultural Research, SLF. The essential oil of *Minthostachys verticillata* was kindly provided from Dr Vincenzo DeFeo, University of Salerno, those of *Populus balsamifera*, *Tagetes mexicana*, and *T. Rosola* from Dr Lars Björk, Swedish University of Agricultural Sciences, Balsgård, and that of *Mentha sachalinensis* from Dr T. H. Iversen, University of Trondheim, Norway.

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BIOCONVERSION OF ALKENE PHENOLS FROM AROMATIC PLANTS TO NATURAL AROMA COMPOUNDS

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ABSTRACT

Natural flavor and fragrance compounds are of great value to the food, cosmetics, and medical industries. The price for these natural chemicals can be extremely high due to their low concentration in the plant material. Many herbs and spices contain relatively high concentrations of essential oils that resemble desirable flavor and aroma compounds. By using a single or a few enzymatic steps, it is possible to convert these essential oils components into natural flavor and fragrance chemicals. In this work, we describe the isolation of microbial strains capable of converting alkene phenols from aromatic plants.

Using enrichment culture technique, transforming microorganisms were isolated based on their ability to either survive toxic concentrations of alkene phenols or use them as carbon source. Isolated strains were capable of converting isoeugenol to vanillin, and *t*-anethole or estragole to anisaldehyde. Some of these strains were also capable of growing on *t*-anethole, estragole, isosafrole or isoeugenol as the sole carbon source. Using mutagenesis and penicillin enrichment, blocked mutants, not capable of utilizing *t*-anethole were isolated. These mutants accumulate intermediates in yields that are close to the maximal molar yields. The results of this research may support further development of economical bioconversion processes for the production of natural aroma compounds from alkene phenols.

INTRODUCTION

Taste and aroma chemicals are of great importance for the food, cosmetic and pharmaceutical industries. Natural flavors and fragrances are usually extracted from plants that are expensive to cultivate, contain only low concentrations of the active compounds, and are available only for a short season during the year. Because of their limited availability, the price of some natural flavor and fragrance compounds can be over a 100-fold higher than that of the synthetic chemicals. As a result, most flavor and fragrance chemicals are chemically synthesized. To date, flavor and fragrance industries are actively seeking new flavors and novel technologies for the production of new and natural compounds. One of the emerging fields for the production of flavor and aroma compounds is biotechnology, and in particular the use of enzymes or microorganisms for biocatalysis (Berger, 1997; Berger, 1995; Gabelman, 1994).

Why bioconversion? Microbial and enzymatic conversions possess several intrinsic properties that can be, in some cases, advantageous over traditional chemical synthesis:

(i) Biocatalysis is highly regio- and stereo- selective. In many cases, flavor and fragrance perception is based on the precise stereochemical properties of the molecule, thus, stereochemical reactions are of great advantage. (ii) Biocatalysis can be carried out under mild conditions, thus, reducing undesired byproducts, energy and safety costs. (iii) Enzymes can be produced at low cost, less than \$10 per kg of catalyst. (iv) With the emerging technology of protein engineering, enzymes can be modified via rational amino acid replacements that result in improved and novel properties. (v) In many cases, enzymes can act on similar substrates providing a broad range of products. (vi) If the starting substrate for the enzymatic conversion is natural, the resulting product is also considered natural (Reference 13: Code of Federal Regulations, 1993). Thus, enzymatic conversion can be used for the production of natural products. Although these natural compounds will not resemble in all cases the properties of natural extracts, they are valuable for natural high-volume food manufactures.

Bioconversion is a procedure in which an enzyme or a microorganism catalyzes a specific chemical modification in a selected compound. There are currently 50 to 100 flavor materials and chemicals that are produced by fermentation (Cheetham, 1997). Bioconversions are highly selective for functional groups (chemoselectivity), reaction sites (regioselectivity), and specific stereo isomers or enantiomers (stereo selectivity). As a result, desired molecular forms can be produced with the required taste and odor. Many essential oils contain relatively high amounts of alkene phenols. Alkene phenols that possess a chemical structure resembling that of flavor and fragrance compounds may serve as an excellent starting material for the

bioproduction of aroma chemicals. A number of aromatic aldehydes and acids are important components of natural flavors and fragrances, including vanillin, vanillic acid, anisaldehyde, piperonal, and benzaldehyde.

Suitable essential oils for the production of alkene phenols. Essential oils are obtained from plant materials by distillation with water or steam. They consist of volatile, lipophilic substances that are mainly oxygenated compounds derived from the metabolism of mono- and sesquiterpenes, alkene phenols, amino acids, and fatty acids (Bauer et al., 1990). Many alkene phenols that are present in high concentrations in essential oils, have similar structures to commercial flavor and aroma compounds.

Based on their structure (substitutions in position 3 and 4 on the benzene ring) and availability, several essential oil components are excellent potential starting materials for the production of natural aromatic aldehydes via biotransformation. Potential biotransformations include: eugenol or isoeugenol to vanillin, *t*-anethole or estragole to anisaldehyde, and safrole or isosafrole to piperonal. The starting compounds are present in many essential oils, for example: clove oil (*Syzigium aromaticum*) contains eugenol as the major component, and minor amounts of isoeugenol. Eugenol is also a major component of bay oil (*Pimenta racemosa*), cinnamon leaf oil (*Cinnamomum zeylanicum*), and pimento berry oil (*Pimenta dioica*) (Bauer et al., 1990). *Trans*-anethole is the major component of star anise (*Illicium verum*) (80-90%), anise seed oil (*Pimpinella anisum*) (>95%), and the essential oil of sweet fennel (*Foeniculum vulgare* Mill. var. dulce) (63-83%) (Ravid et al., 1983). Estragole is the major component of Reunion basil (*Ocimum basilicum*) oil (>80%) (Conan, 1977), the essential oil of sweet basil contains estragole and linalol as major components (and eugenol as a minor one) (Werker et al., 1993), French tarragon (*Artemisia dracuncululus*) contains 60-80% estragole (Gramshaw et al., 1988; Werker et al., 1993), and sweet fennel (*Foeniculum vulgare* Mill. var. dulce) contains 3% estragole (Ravid et al., 1983). Safrole and isosafrole are the major components (>90%) of Brazilian Sassafras oil (*Ocotea pretiosa*). Safrole is also the main component (>80%) of brown camphor oil (Bauer et al., 1990).

Microbial routes for the degradation of alkene phenols. Some of the metabolic intermediates in the degradation pathways of alkene phenols may have economical importance. Several aromatic aldehydes and acids are intermediates in the degradation pathways of alkene phenols by microorganisms. Due to its importance, the production of vanillin in the course of phenylpropanoid degradation has been extensively investigated. Vanillin appears as an intermediate in the microbial degradation of several substrates, including: ferulic acid, phenolic stilbenes, lignin, eugenol and isoeugenol.

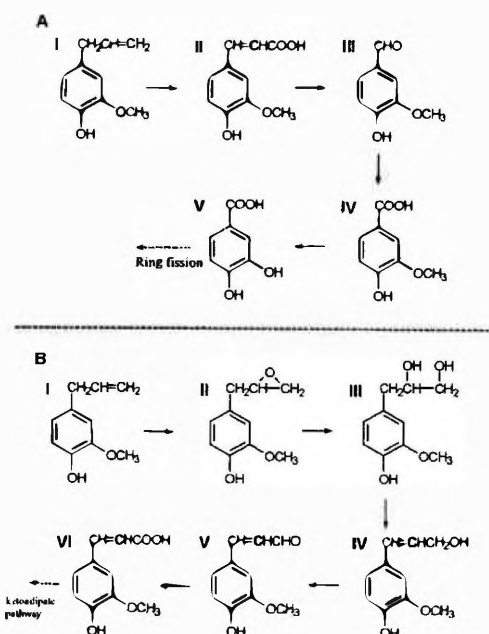
Eugenol and isoeugenol are alkene phenols, derived from essential oils, that can be used as substrates for the production of vanillin by biotransformation. In one of the earliest works on eugenol biotransformation to vanillin, Tadasa isolated a strain of *Corrynebacterium* that grew on eugenol and accumulated low levels of ferulic acid and vanillin (Tadasa, 1977). Later, Tadasa and Kayahara (1983) identified vanillin, vanillic acid, ferulic acid, coniferyl alcohol, and eugenol epoxide, as intermediates in eugenol degradation by a strain of *Pseudomonas* (Figure 1). The transformation of eugenol to coniferylaldehyde by a mutant of *Arthrobacter globiformis* DSM397 indicated that coniferylaldehyde is a true intermediate in eugenol metabolism (Cooper, 1987). Recently, Rabenhorst (1996) reported the isolation of a *Pseudomonas* sp. capable of growing on eugenol as the sole carbon source. Vanillic acid, ferulic acid, and coniferyl alcohol were detected as metabolic intermediates in eugenol degradation by this organism. However, the enzymes involved in eugenol degradation were not characterized or purified. Ferulic acid, a lignin monomer, is metabolized via vanillin by many microorganisms (Henderson and Farr, 1955; Cartwright and Buswell, 1967; Turner and rice, 1975; Rosazza, 1995), and some strains also produce vanillin acid as an intermediate in ferulic acid metabolism (Cartwright and Buswell, 1967; Toms and Wood, 1970; Sutherland et al., 1983; Otuk, 1985; Rahouti et al., 1989; Andreoni et al., 1995). A strain of *Bacillus subtilis*, isolated by Gurujeyalakshmi and Mahadevan (1987), utilized ferulic acid and its intermediates as a sole carbon source. The enzymes of the ferulic acid degradative pathway (deacetylase, vanillin oxidase, vanillate-*O*-demethylase, and protocatechuate 3,4-dioxygenase) are induced by these intermediates,

Figure 1: Proposed early steps of degradation pathways of eugenol.

A. *Corrynebacterium* sp. (Tadasa, 1977):

Eugenol (I) is metabolised via ferulic acid (II), vanillin (III), vanillic (IV) and protocatechuic (V) acids;

B. *Pseudomonas* sp. (Tadasa and Kayahara, 1983): Eugenol (I) is metabolised via eugenol-epoxide (II), eugenol-diol (III), coniferyl alcohol (IV), coniferyl aldehyde (V), and ferulic acid (VI).



suggesting that ferulic acid is catabolized by this strain via vanillic acid, vanillin, and protocatechuic acid.

In many cases aromatic aldehydes and alcohols are further degraded to their acid form. Vanillin is oxidized to vanillic acid by *Serratia marcescens* (Perestelo et al., 1989), *Pseudomonas fluorescens* (Bare et al., 1992), and *Streptomyces viridosporus* (Pometto and Crawford, 1983). The degradation of vanillic acid involves a monooxygenase that demethylates aromatic methyl ethers (Dagley, 1978). The demethylation occurs via hydroxylation of the methyl ether, and its decomposition to formaldehyde and protocatechuic acid (Cartwright and Smith, 1967; Cartwright and Buswell, 1967; Ribbons, 1970). Two of the genes (*vanA* and *vanB*) involved in the demethylation of vanillic acid by *Pseudomonas* sp. strain ATCC 19151 were cloned and sequenced, but the enzymes were not purified (Brunell and Davison, 1988). Another aromatic aldehyde, anisaldehyde, is an intermediate in the degradation pathway of anisyl alcohol by *Penicillium simplicissimum* (Fraaje et al., 1997). However, it is further degraded to *p*-methoxybenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid.

Bioprocesses for the production of aroma chemicals from alkene phenols.

So far, reported biotransformation processes for the production of vanillin from eugenol and isoeugenol gave poor yields with very low productivities. *Aspergillus niger* ATCC 9142 is capable of transforming isoeugenol to vanillin in only 10% efficiency, since vanillin is further degraded to vanillyl alcohol and vanillic acid (Abraham et al., 1988). Scientists from Haarman & Reimer (Rabenhorst and Hopp, 1991; Hopp, 1993) developed a process for the production of vanillin by biotransformation of isoeugenol and eugenol, using *Serratia marcescens* DSM 30126. With isoeugenol as a substrate, the initial yield was 5%, and following optimization, the yield increased to 20% (3.8gr/l). However, the productivity of the process was low (0.018 gr/l per hr). Eugenol can also be transformed by this microorganism but the final yield of vanillin was much lower (0.018 gr./l). Washisu et al. (1993) obtained a patent for producing vanillin from eugenol using a strain of *Pseudomonas* sp. TK2102 (FERM P-12689). This strain can utilize eugenol as a sole carbon source, but not vanillin, vanillic acid, or vanillyl alcohol. In this process, the final concentration of vanillin was 280 mg/l. Labuda et al. (1992, 1993) reported the transformation of eugenol to vanillin by strains of *Aspergillus niger*, *Pseudomonas putida*, and *Corynebacterium glutamicum*. The authors used thiol reagents, such as dithiothreitol, to increase the level of vanillin (18.5 mg/l). Markus et al. (1992), developed a process for production of vanillin using the enzyme lipooxygenase (Sigma L8383). The yield of isoeugenol conversion was 10-15%, and of eugenol 0.3-0.5%.

Recently, Lesage-Meessen et al. (1995, 1996), reported a two step bioconversion process for the production of vanillin from ferulic acid. In the first step, *Aspergillus niger* converted ferulic acid to vanillic acid, and in the second step vanillic acid was reduced to vanillin by *Pycnopus cinnabarinus*. Vanillic acid production by *A. niger* from ferulic acid reached 920 mg/l with a molar yield of 88%, and vanillin production by *P. cinnabarinus* from vanillic acid attained 237 mg/l with a molar yield of 22%. Thus, the overall yield of the process was about 16% conversion in 14 days. Audras and More used a *Streptomyces* strain that accumulated vanillin from benzene precursors, especially ferulic acid (Audras and More, 1995). In a batch process (90 hours), vanillin was accumulated up to a concentration of 1 gr/l (molar yield 66%) when ferulic acid was added in a concentration of 2.2 gr/l.

Objectives of the present research. The overall long term objective of this research is to develop bioconversion processes of alkene phenols to natural aroma compounds. Several potential transformations are illustrated in figure 2. In the present communication, we describe the isolation of bacterial strains capable of degrading alkene phenols, and the construction of blocked mutants capable of accumulating valuable metabolic intermediates.

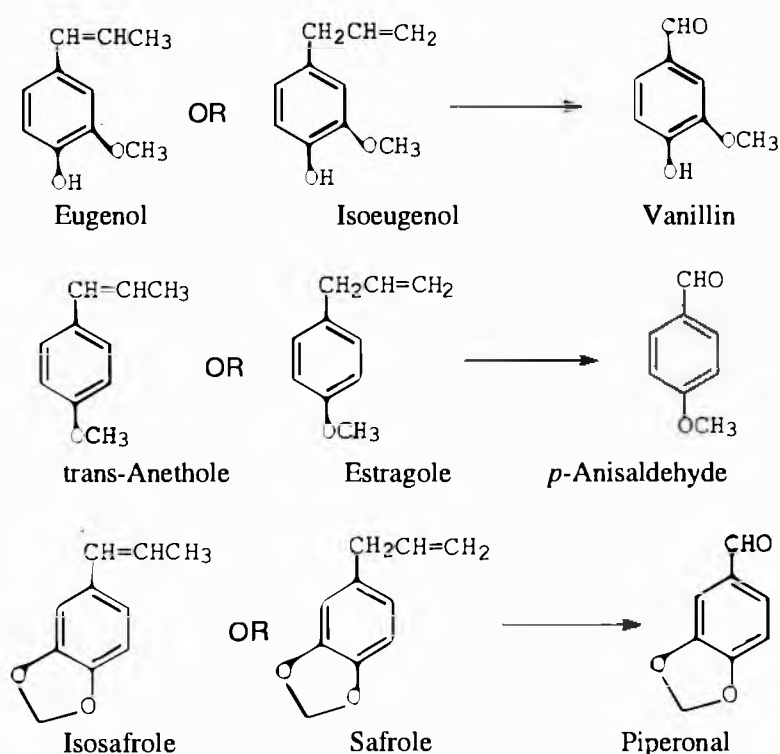


Figure 2: Potential biotransformations of alkene phenols to natural aroma compounds.

RESULTS AND DISCUSSION

Isolation of transforming strains

Our logical path for obtaining transforming strains was to isolate bacterial strains based on their ability either to degrade or utilize alkene phenols. We reasoned that strains that survive the toxic concentrations of the substrates or capable of degrading them may be able to execute transformations of alkene phenols.

Bacterial strains that are tolerant for high concentrations of alkene phenols were isolated from samples of soils collected from the aromatic plant greenhouses at Newe Ya'ar Research Center (Agricultural Research Organization, P.O.Box 1021, Ramat Yishay, 30095 Israel), and from the ecological garden of the Technion (Technion City, Haifa 32000, Israel). The soil samples were used as inoculum for the enrichment cultures in MMYG medium containing up to 1% of a specific alkene phenol. Using this method, 15 different strains were isolated (Table 1).

Table 1: strains isolated based on their ability to survive toxic concentrations of alkene phenols.^a

Strain	Origin of Soil	Tolerant for the alkene phenol [%]
IE1, IE2	Basil greenhouse (Newe Ya'ar)	Isoeugenol
E1, E2,	Basil greenhouse (Newe Ya'ar)	Eugenol
E70, E71	Ecological garden (Technion)	Eugenol
IS1, IS2	Basil greenhouse (Newe Ya'ar)	Isosafrole
ES,	Tarragon greenhouse (Newe Ya'ar)	Estragole
ES71, ES701, ES702	Ecological garden (Technion)	Estragole
tA,	Tarragon greenhouse (Newe Ya'ar)	<i>t</i> -Anethole
tA71, tA72	Ecological garden (Technion)	<i>t</i> -Anethole

^a Strains were isolated from soil samples using rich MMYG enrichment medium containing 1% alkene phenol. Cultures were incubated at 30°C and shaken in 150 rpm. Following several transfers, cultures were plated on LB agar plates, and single strains were isolated based on their morphology.

Alkene phenols degrading bacteria were isolated from soil samples using a modified enrichment culture procedure. Since many alkene phenols are highly toxic to microorganisms, they were added to the medium adsorbed on hydrophobic resin (amberlite XAD-2) (10%w/w). This way, only sublethal concentrations of the compound were released to the medium, allowing microbial growth. Following the enrichment culture, different bacterial strains capable of using alkene phenols as the sole carbon source were isolated. A typical growth curve on estragole of one of these strains is presented in figure 3. Strain LS13 exhibited a logarithmic growth curve (doubling time=5.9 hours), and reached a turbidity of 1.7

OD. The concentration of estragole in the medium decreased rapidly during the first 9 hours of growth, and continued to decrease moderately.

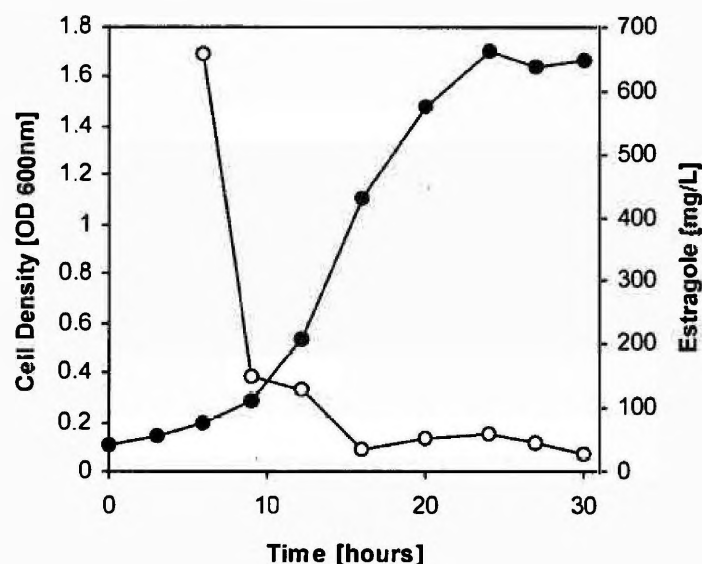


Figure 3: Estragole degradation by isolated strain LS13. Growth (●) and estragole (○) degradation in a fermentation system. Culture was grown in 20 ml modified M9 medium + 0.1% estragole adsorbed on XAD-2, at 30°C, shaken in 100 ml flasks at 200 rpm.

The ability of the isolated strains to produce aromatic aldehydes was examined by growing the cells in rich medium that contained alkene phenols. Following fermentation, the transformation products were extracted and analyzed by TLC. The aldehydes were specifically detected by color reaction with 2,4-dinitrophenylhydrazine. Using this screening procedure we detected 6 strains (IE1, IE2, tA, tA71, tA72, ES) that produce aldehydes, and quantitative assessment of the products with GC revealed detectable amounts of aromatic aldehydes (Table 2). Vanillin and anisaldehyde were detected in the presence of either isoeugenol, *t*-anethole or estragole. However, the bioconversion efficiencies obtained under these fermentation conditions were rather low (1-6%, based on molar yields).

One possible reason for the low yields, is that the aromatic aldehydes are further degraded and utilized by the strains. To test this possibility, we examined whether these strains can grow on alkene phenols. The growth of strains IE1 and IE2 on isoeugenol is shown in figure 4. Both strains were capable of utilizing isoeugenol as the sole carbon source, however, the growth was non logarithmic, very slow and the yield was poor. Thus the aldehydes can probably be degraded, and therefor, only small amount of aldehydes was detected.

Table 2: Biotransformation of essential oil components. ^a

Strain	Substrate ^b	Product ^c	Product Final Concentration ^d [mg/l]	Theoretical Biotransformation Efficiency ^e [%]	Productivity ^f [mg/l/day]
IE1	isoeugenol	vanillin	570	6.15	190
IE2	isoeugenol	vanillin	493	5.32	164
tA	<i>t</i> -anethole	anisaldehyde	58	0.63	19
tA71	<i>t</i> -anethole	anisaldehyde	98	1.06	33
tA72	<i>t</i> -anethole	anisaldehyde	44	0.47	15
ES	estragole	anisaldehyde	30	0.32	10

^a Cultures were grown in 20 ml (100 ml flasks) containing MMYG medium 30°C and 150 rpm. Substrate was added to the cultures following 48 hours of fermentation.

^b Substrates were added up to a concentration of 1% to a stationary phase culture.

^c Products were extracted with ethyl acetate 3 days after the addition of the substrates.

^d Final concentration of the products was determined by GC using SPB-1 column (Supelco, 30m, 0.25mm, 0.25µm).

^e Theoretical biotransformation efficiency = $100 \times \text{mole product} / \text{mole substrate}$.

^f Productivity = mg product / liter / day.

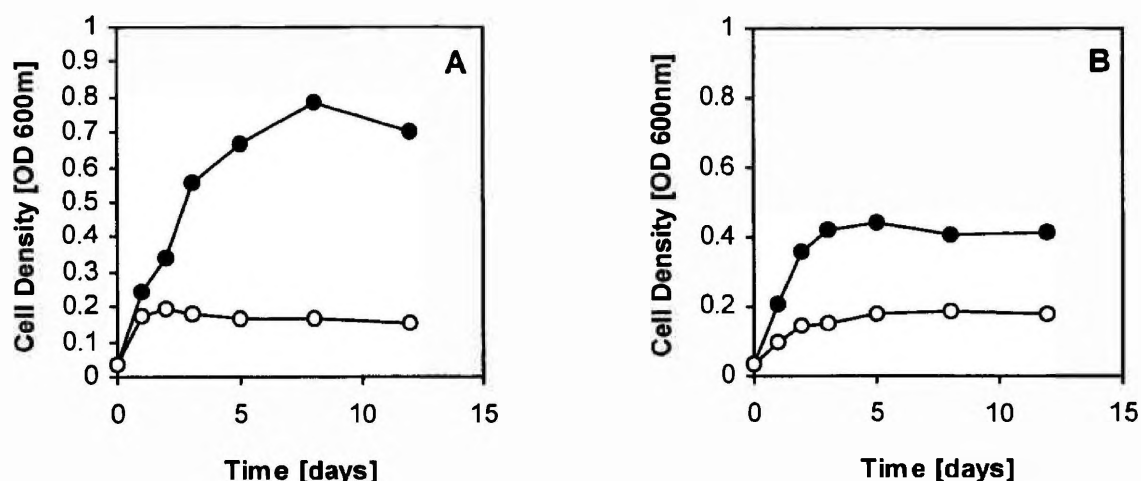


Figure 4: Growth of strains IE1 (A) and IE2 (B) on isoeugenol as the sole carbon source (●), the control represents growth without isoeugenol (○). Cultures were grown in 20 ml of MMY medium + 0.1% estragole adsorbed on XAD-2, at 30°C, shaken in 100 ml flasks at 200 rpm (MMY medium = MMYG minus the glycerol).

Obtaining blocked mutants, not capable of degrading alkene phenols. Some of the isolated strains grow on *t*-anethole as the sole carbon source. We induced mutagenesis, and isolated blocked mutants that accumulate metabolic intermediates. *t*-Anethole blocked mutants were obtained following *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MeNNG) mutagenesis (Che et al., 1996) and penicillin enrichment (Queener and Lively, 1986). Several

of these mutants accumulated, in the presence of *t*-anethole, metabolic intermediates, such as anisic acid in high yields (Figure 5). In a 24 hours fermentation, most of the *t*-anethole was degraded, and anisic acid accumulated up to a concentration of 255 mg/L. The theoretical molar efficiency of *t*-anethole conversion to anisic acid was 49% (anisic acid produced / *t*-anethole degraded). Thus, it is evident that blocked mutants obtained from the isolated strains may prove useful in developing a bioconversion processes for the production of aroma chemicals.

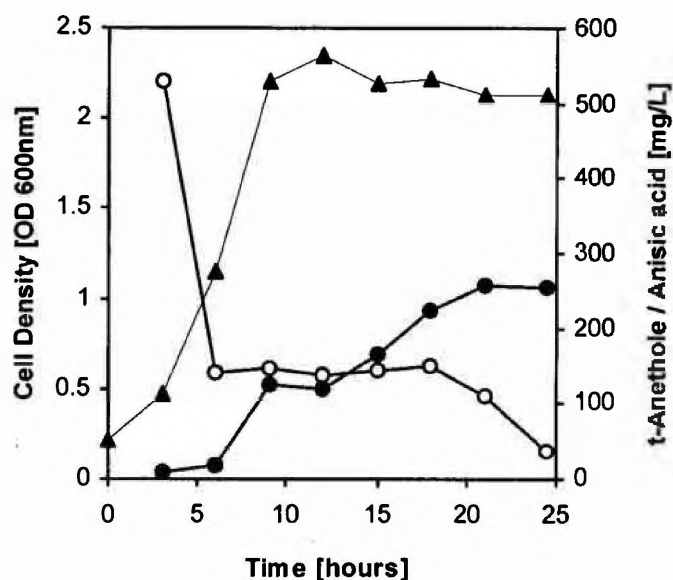


Figure 5: Bioconversion of *t*-anethole to anisic acid by blocked mutant A. Growth (▲) and anisic acid (●) formation from *t*-anethole (○) in a fermentation system. Strain A was grown in modified M9 medium containing 0.1% glucose and 0.1% *t*-anethole adsorbed on XAD-2. Culture were grown in 20 ml medium (100 ml flasks) shaken at 200 rpm in 30°C.

EXPERIMENTAL

Isolation of bacterial strains. Strains were isolated using enrichment culture procedure (Queener and Lively, 1986). Soil samples were taken from a greenhouse of spices and herbs at ARO Newe Ya'ar, and the Technion's ecological garden. Ten (10) g of soil were dispersed in 100 ml saline, and 2ml of the dispersion were used as inoculum to 20 ml (in 100 ml flasks) enrichment medium. Two procedures were used: (i) Enrichment medium contained MMYG medium + casamino acids 0.3%, and 0.1% alkene phenol (*MMYG medium*: 0.5% yeast extract + 1% glycerol + 0.14% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.2 g/l K_2HPO_4 , 1.0 g/l $(\text{NH}_4)_2\text{SO}_4$, 0.02 g/l $\text{Ca}(\text{NO}_3)_2$, 0.368 mg/l CaCl_2 , 0.624 mg/l $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 0.604 mg/l $\text{FeSO}_4 \times 7\text{H}_2\text{O}$, 0.594 mg/l $\text{MnSO}_4 \times 4\text{H}_2\text{O}$, 0.422 mg/l $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 0.788 mg/l $\text{CoCl}_2 \times 6\text{H}_2\text{O}$, 0.696 mg/l Na_2MoO_4). Cultures were incubated at 30°C and shaken in 150 rpm. (ii) The medium contained minimal Inorganic Basal Media (IBM= Solution A: $(\text{NH}_4)_2\text{SO}_4$ 1.2 g, $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ 0.1 g, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 0.1 g, $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ 0.01 g, distilled water 1000 ml; Solution B: K_2HPO_4 0.2 g, KH_2PO_4 0.1 g, distilled water 200 ml; Solutions A and B were sterilized separately and mixed when cold). Alkene phenols were added to the culture adsorbed on amberlite XAD-2 (1:10 w/w) up to a concentration of 0.1%, and the culture was incubated at 30°C (200 rpm). When turbidity was detected, 50 μl of the cultures were transferred to 20 ml fresh medium and the purity of the culture was tested by streaking samples on LB and IBM agar plates that contained 0.1% glucose as the carbon source.

Following several transfers, samples from the culture were diluted and plated on LB agar plates. Bacterial strains were isolated based on their morphology on agar plates.

Isolation of blocked mutants. Blocked mutants were obtained by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MeNNG) followed by penicillin enrichment (Queener and Lively, 1986) for mutants incapable of using *t*-anethole as a carbon source. More specifically, dilutions of culture in LB broth were plated on LB agar plates, and MeNNG-crystals were placed on the agar. Following incubation (30°C, 24 hours), colonies from the boundaries of the clear halo around the MeNNG crystal were collected, suspended in 10 ml modified M9 medium, and washed twice with the same medium (Modified M9= 1000 ml Solution A: Na_2HPO_4 6.8 g/l, KH_2PO_4 3.0 g/l, NaCl 0.5 g/l, NH_4Cl 1.0 g/l; 5 ml Solution B: FeSO_4 2.0 g/l, CaCl_2 4.0 g/l; 5 ml Solution C: $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 200 g/l (58)). The treated cells were diluted in 25 ml M9 medium + 0.2% glucose and incubated for 24 hours (30°C, 200 rpm). This overnight culture was the inoculum of a fresh culture, containing 10 ml M9 medium + 0.1% *t*-anethole in 100 ml flasks, shaken in 200 rpm at 30°C. At turbidity of 0.15

O.D. 600nm, penicillin was added up to a concentration of 10000 U/ml. Following a sharp absorbance decrease, culture was diluted and plated on LB agar plates (30°C). Blocked mutants were isolated by transfer of single colonies to triplicate modified M9 agar plates, that contained: (i) no carbon source, and (ii) glucose or (iii) *t*-anethole as the sole carbon source. Mutants were isolated from colonies that did not grow on *t*-anethole.

Analytical procedures. Thin layer chromatography (TLC) was performed using silica gel 60 F254, 0.25 mm, plates (Merck). Plates were developed by chloroform (85): ethyl acetate (15) : formic acid (1). Following development, the products were detected by UV 254 nm, sulfuric acid (50% in methanol), aromatic aldehydes were specifically stained by 2,4-dinitrophenylhydrazine (0.4% in 2N HCl), and phenolic compounds were stained by solution of 0.5% K₃Fe(CN)₆ + 0.5% FeCl₂×6H₂O (Krebs et al., 1969).

Gas chromatography analysis was performed on a Hewlett-Packard 5890 gas chromatograph, equipped with SPB1 capillary column (30m, 0.25mm id, 0.25µm (Supelco Inc., Bellefonte, PA, USA)), carrier gas N₂ (1.7 ml min⁻¹), split ratio 35, injection temp. 250°C, detection temp. 300°C, linked to a computerized integrator (PC Integration pack, Kontron instruments, Milano, Italy). The initial temperature was 100°C (2 min.), and then programmed in rate of 5°C/min. to 160°C.

ACKNOWLEDGMENTS

This research was supported by grants from the Israeli Ministry of Agriculture, and the Technion V. P. R. Fund - Elite Food Engineering Fund. Technical support was provided by the Technion Otto Meyerhof Biotechnology Laboratories established by the Minerva Foundation, Federal Republic of Germany.

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METHYLTRANSFERASES INVOLVED IN THE BIOSYNTHESIS OF METHYL EUGENOL AND METHYL CHAVICOL IN SWEET BASIL

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ABSTRACT

Sweet basil, *Ocimum basilicum* (Lamiaceae), is an economically important essential oil crop. The major components of sweet basil essential oils are linalol and methyl chavicol (estragole). Some varieties also contain eugenol and methyl eugenol. The biosynthetic pathway to methyl chavicol, eugenol and methyl eugenol is poorly understood. We describe here that basil cell free extracts, incubated with radioactively labeled *S*-adenosyl-L-methionine (SAM) and an allyl-phenolic derivative substrate as a methyl acceptor, convert chavicol and eugenol to radiolabeled methyl chavicol and methyl eugenol respectively. The activity was absent after boiling the cell-free extracts. The above findings indicate the presence of allyl-phenol methyltransferase activities in sweet basil leaves.

INTRODUCTION

The genus *Ocimum* includes about a dozen of species and subspecies native to the tropical and sub-tropical regions of the world. Sweet basil (*Ocimum basilicum* L.) is an important medicinal plant and culinary herb which is also used for essential oil production (Bremness 1994). Despite the importance of its essential oil, little is known about the biochemistry of the formation of its components. Basil chemotypes with different essential oil profiles are known (Grayer *et al.*, 1996). The major components include linalol, an acyclic monoterpene alcohol, and the allyl-phenol derivatives eugenol, methyl eugenol and methyl chavicol (estragole) (Grayer *et al.*, 1996, Werker *et al.*, 1993). Eugenol, also a major component of clove oil, is used in the perfumery industry, and as an analgesic in dentistry and veterinary medicine. The methylation of the *p*-hydroxyl

group in eugenol dramatically changes the smell of the molecule: eugenol is pungent, while methyl eugenol has a pleasant smell, resembling fresh cut green grass. Methyl chavicol is also used in the perfumery industry and has a smell resembling that of fennel and anise. Its smell is not very different from that of chavicol.

Methyltransferases are common enzymes found in several plants and tissues (Poulton, 1981). They catalyze the transfer of a methyl group from a donor substrate such as *S*-adenosyl-L-methionine (SAM), to various acceptor substrates, to form N-, O-, C-, and S-methyl linkages (Luckner 1990). Methyltransferases acting upon DNA (cytosine) (Kutueva *et al.*, 1996), or caffeic and 5-hydroxyferulic acids (Dumas *et al.*, 1988, De Carolis and Ibrahim 1989) are well known. An *O*-methyltransferase that converts *t*-anol (1-hydroxy-4-(1-propenyl) benzene) to *t*-anethole (1-methoxy-4-(1-propenyl) benzene) in *Pimpinella anisum* has also been described recently (Kemmerer and Reichling 1996). In this report we present preliminary evidence that methyltransferase activities are involved in the biosynthesis of methyl chavicol and methyl eugenol in basil leaves (Fig 1).

RESULTS AND DISCUSSION

Incubation of cell-free extracts with radioactively labeled *S*-adenosyl-L-methionine (SAM) and chavicol, resulted in the formation of hexane-soluble radioactive products (Fig 2A). Control experiments, in which boiled cell-free extracts were used as an enzyme source were devoid of hexane-soluble radioactive products. This indicates that a methyltransferase activity might be present in basil cell-free extracts. The omission of chavicol resulted in a lower but still significant rate of formation of the radioactive products (Fig 2A). In order to ascertain whether the activity observed in the absence of

exogenous chavicol was due to methylation of endogenous substrate, the cell-free extracts were partially purified by Sephadex G-25 chromatography, prior to the assays. Complete assays, containing SAM and chavicol utilizing Sephadex G-25 partially purified enzyme displayed a higher enzymatic activity level as compared to crude extracts, both per fresh weight (2B), and on a protein basis (not shown). Additionally, the omission of chavicol resulted in virtually no formation of hexane-soluble radioactive products during the enzymatic assays (Fig 2B). To confirm the identity of the radioactive compounds formed, the hexane extracts were evaporated and analyzed by TLC-autoradiography. Only one radioactive substance was formed during the enzymatic assays and its R_f co-incided with that of methyl chavicol. These results indicates that a methyltransferase activity is able to convert chavicol to methyl chavicol in cell-free extracts. Comparable results were obtained when eugenol (instead of chavicol) was used as a substrate, but in this case, methyl eugenol was apparently the product formed as determined by TLC-autoradiography. At present, it is unknown whether only one enzymatic activity with a broad substrate specificity is able to transfer methyl groups to either eugenol or chavicol, or whether two or more separable enzymatic activities are involved in these biochemical conversions. The purification and biochemical characterization of the enzymatic activities are currently under investigation.

EXPERIMENTAL

Plant material

O. basilicum L. plants were raised from seed and grown in pots for periods of 3.5 to 15 weeks in a greenhouse at the Newe Ya'ar Research Center, Israel.

Enzyme extraction

Cell-free extracts were prepared as follows: Fresh plant leaves (1-2 gr.) were weighed, frozen in liquid nitrogen, and placed in a cold mortar. The leaves were then ground with a pestle in the presence of liquid nitrogen, ≈ 0.6 gr. sand and ≈ 0.1 gr. polyvinyl-polypyrrolidone (PVPP) to absorb phenolic materials. Ice cold extraction buffer (100 mM MOPS [pH 6.5], 10% glycerol [v/v], 6.6 mM dithiothreitol, 1% polyvinylpyrrolidone [w/v], 5 mM $\text{Na}_2\text{S}_2\text{O}_5$) were added (5 volumes to fresh weight tissue), and the powder was further extracted for ≈ 30 sec. The slurry was centrifuged twice at 20,000 g, for 10 min at 4°C, and the supernatant (crude extract) was used in the enzymatic assays. One ml of the crude extracts were further desalted using Sephadex G-25 columns (1×3 cm) and eluted with 1.2 ml of running buffer (50 mM HEPES [pH 7.5], 5% [v/v] glycerol, 3.3 mM dithiothreitol).

Enzyme Assay

Assays were prepared by mixing: 40 μL of the crude extract (about 20 μg protein), 40 μL running buffer, 5 μM allyl-phenol substrate (either eugenol or chavicol from 0.1 mM solution in 0.5% ethanol), 25 μM *S*-[methyl- ^3H]-adenosyl-L-methionine (6 Ci/mol) (in a total volume of 100 μL). The assays were incubated at 21°C for 1.5 h, and then 1 ml

hexane was added to each tube, vigorously vortexed and spun for 13 sec. at 20,000 g to separate the phases. The hexane layers containing the radioactive labeled enzyme products, were transferred to scintillation tubes containing 3 ml scintillation fluid [4 gr./L 2,5-diphenyloxazol (PPO) and 0.05 gr./L 2,2'-*p*-Phenylen-*bis*(5-phenyloxazol) (POPOP) in toluene]. The radioactivity was quantified using a Packard Tri-carb liquid scintillation counter. To confirm the identity of the biosynthetic products, similar incubations were performed, except that ^{14}C -labeled SAM was used, and the hexane layer was evaporated to a volume of 20 μL using a gentle stream of N_2 , and analyzed by TLC-autoradiography using Silica gel 60 F₂₅₄ plates developed with chloroform. Spots were visualized by U.V. light and radioactive spots detected by autoradiography on Kodak X-OMAT paper.

ACKNOWLEDGMENTS

Technical support was provided by the Technion Otto Meyerhof Biotechnology Laboratories established by the Minerva Foundation, Federal Republic of Germany. This work was supported by a grant from the Chief Scientist of the Ministry of Agriculture of the State of Israel.

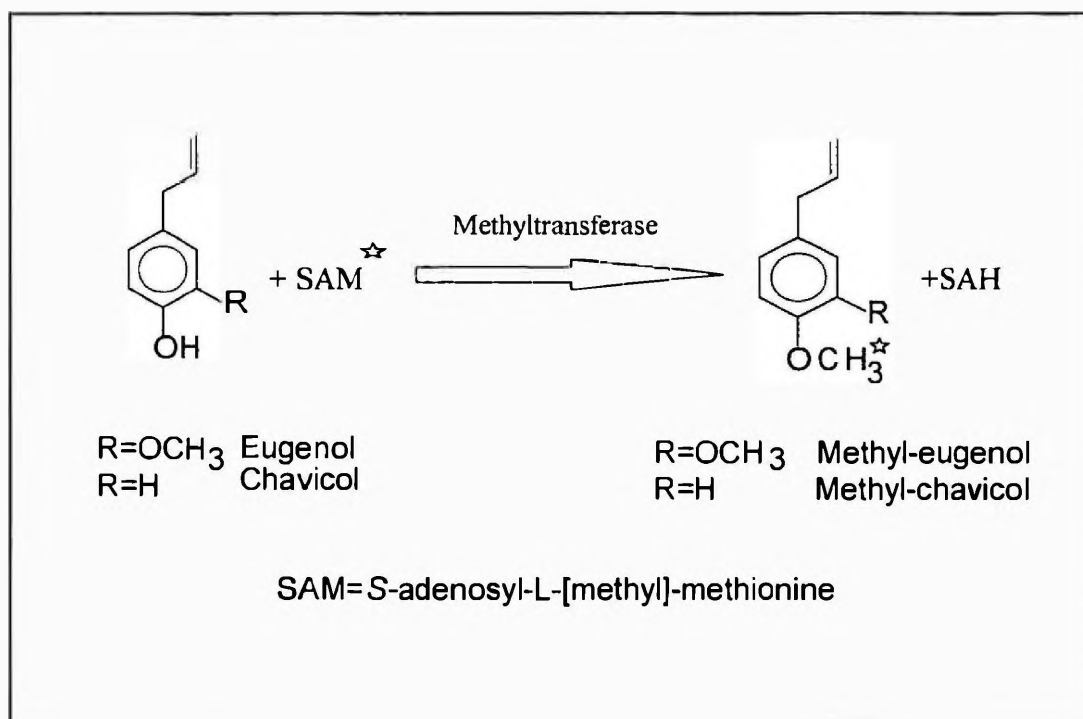


Fig 1: Methyltransferases from *O. basilicum* catalyze the *p*-methylation of the allylphenol derivatives chavicol and eugenol to methyl chavicol and methyl eugenol, respectively.

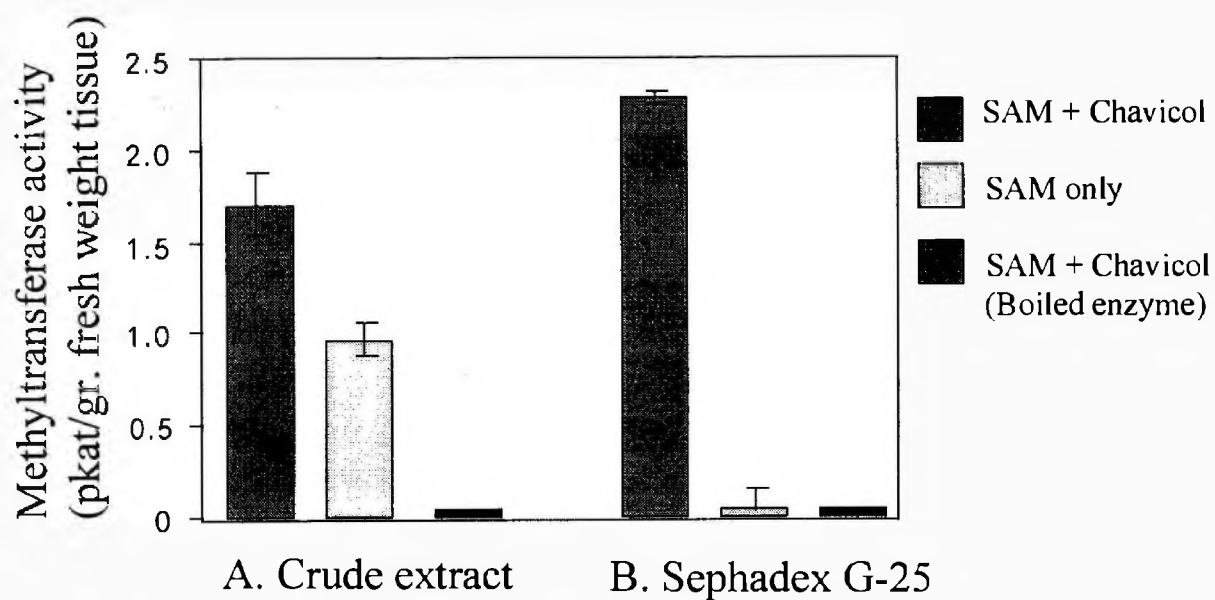


Fig 2: Methyltransferase activity in cell-free extracts of *O. basilicum* leaves.

A. Crude cell-free extracts were prepared from fresh *O. basilicum* leaves, and incubated with 5 μ M chavicol and 25 μ M SAM as indicated. B. The cell-free extracts were partially purified by Sephadex G-25 chromatography before the assays, and incubated as above.

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