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SEMIPREPARATIVE SEPARATION OF TERPENOIDS FROM ESSENTIAL OILS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THEIR SUBSEQUENT IDENTIFICATION BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

After mobile phase optimization (composition and elution profile) on an analytical scale with a standard mixture of mono- and sesquiterpenoids, the prefractionation of an essential oil has been carried out by semipreparative liquid chromatography. Reversed-phase chromatography on an octadecyl-bonded silica was used and the separation of the essential oil into several fractions was achieved within 20 min in a single experiment. The fractions were oxygenated monoterpenes, monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated sesquiterpenoids. Thus, liquid chromatographic separations of essential oils can be achieved, at room temperature, avoiding the risk of thermal rearrangement and decomposition which can occur in gas chromatographic separations. The fractions obtained were easily analysed by gas chromatography–mass spectrometry and several minor components, in particular sesquiterpenoids, were identified.

INTRODUCTION

Essential oils are complex mixtures of terpene compounds containing around 100 components. Whereas gas chromatography–mass spectrometry (GC–MS) is the method of choice for analysing the volatile components of these essential oils, the presence of so many components makes GC analysis in a single experiment very

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difficult, due to the overlap of some peaks and the loss of resolution particularly with minor compounds.

Traditional methods for prefractionation of terpene mixtures, *e.g.*, vacuum distillation and preparative GC cannot be achieved without thermal degradation[1,2], and column liquid chromatography is time consuming and has a low resolution[3].

The high-performance liquid chromatography (HPLC) technique is well adapted to thermosensitive compounds like terpenoids; experiments are done at room temperature directly on plant extracts, without derivatization procedures. Moreover, the molecular interactions are increased at room temperature and the selectivity of the separation is improved. The lack of an universal and sensitive detector is however a serious limitation in the HPLC separation of these compounds which, unfortunately, have no good chromophoric groups.

For instance, O'Connors and Goldblatt[4] studied the UV absorption spectra of several monoterpene hydrocarbons. The spectra of the saturated compounds (*cis*- and *trans-p*-menthane, pinane and tricyclene) exhibit no characteristic absorption above 200 nm. The spectra of unsaturated compounds (α- and β-pinenes, camphene, Δ^3-carene, limonene, terpinolene and γ-terpinene) exhibit some absorption only at 220 nm with no characteristic bands; their absorptivity is low at this wavelength ($\varepsilon \approx 100$–200 l mol^{-1} cm^{-1} for monounsaturated and $\varepsilon \approx 250$–400 l mol^{-1} cm^{-1} for polyunsaturated compounds).

Many papers have been published on the analytical separation of terpenoids by HPLC (Table I), but most concern separations of synthetic mixtures. In a general paper on the separation of terpenoids by HPLC[5], the main chromatographic methods (adsorption, reversed-phase, exclusion, ligand exchange) used for separation of terpene compounds are discussed and detection systems (UV–visible absorption, Fourier transform IR, fluorimetry and electrochemical detection) are compared in respect of their selectivity and sensitivity.

Otherwise, few fundamental works have been published on the semipreparative

TABLE I

ANALYTICAL SEPARATIONS OF TERPENOIDS BY HPLC

Terpenoids	*UV detection wavelength (nm)*	*Chromatographic method*	*Ref.*
Limonene, menthone, citral	215	Reversed phase	6
Nerol, geraniol, linalol, citral, farnesol	254	Reversed phase	7
Sesquiterpene hydrocarbons	220	Reversed phase	8
Sesquiterpene lactones	210	Reversed phase	9
	215	Reversed phase	10
Diterpenes	230	Reversed phase	11
	220	Reversed phase	12
Diterpene acids	220	Reversed phase	13
Triterpenols	215	Reversed phase	14
Isoprenoid benzoates and naphthoates	214	Reversed phase	15
Monoacid sesquiterpenes	220	Adsorption	16
Eugenol, cinnamaldehyde	260	Adsorption	17
Triterpenes	210	Adsorption	18

(or preparative) liquid chromatography of terpenoids[22–24,28]; some of them are described in Table II.

The present study describes the usefulness of semipreparative liquid chromatography for the prefractionation of complex mixtures of components with a large range of polarity. In this way, minor components are more easily identified by GC–MS.

EXPERIMENTAL

Liquid chromatography

Analytical separations were performed on a 250 mm × 4.6 mm I.D. columns packed with octadecyl-bonded silica Nucleosil C_{18} (7 μm), LiChrosorb RP-18 (10 μm) or Zorbax C_{18} (7–8 μm); semipreparative separations were performed on a 250 mm × 9.2 mm I.D. column packed with 7–8 μm octadecyl-bonded silica (Zorbax C_{18}), using a Model 5060 pump (Varian, Palo Alto, CA, U.S.A.). Injections were made through a sample loop (volume: 10 μl in analytical and 420 μl in semipreparative work) with a conventional Model 7010 injection valve (Rheodyne, Berkeley, CA, U.S.A.). Detection was performed with a UV spectrophotometer (Pye Unicam, Cambridge, U.K.) at 220 nm.

Gas chromatography

The HPLC fractions were extracted with pentane–diethyl ether (1:1), dried over sodium sulphate and the organic extract was evaporated with a rotating bath. The extracts were directly analysed with a Carlo Erba chromatograph, Fracto Vap Series 4160, equipped with a split injector (1:100), a flame ionization detector and a 25 m × 0.32 mm I.D. fused-silica DB 5 (J & W) column. The oven temperature was programmed from 60 to 220°C at 4°C/min and held for 10 min at 220°C.

Gas chromatography–mass spectrometry

The different fractions of essential oils were analysed using an Hewlett-Packard capillary GC–quadrupole MS system (Model 5995 B) fitted with a 25 m × 0.32 mm I.D. fused-silica DB 5 (J & W) column coupled to the source. The GC oven was programmed from 60 to 220°C at 4°C/min with helium as carrier gas. Typical MS operating conditions were used and mass spectra were obtained by scanning from 40 to 400 a.m.u. with a selected ion monitoring (SIM) technique on the fragment at *m/e* 43. The mass spectrometer was operated at 70 eV. Peak assignments were made by comparing spectra of unknown terpenoids with those present in our mass spectra library.

Solvents and reagents

Mobile phases were prepared by mixing water and acetonitrile. The water used was specially twice distilled and the solvent was obtained from SDS (Peypin, France). The capacity factors, k', were calculated with a column dead volume, t_0, measured by injection of nitrate.

Solutes

The different samples of terpenoids (Table III) were purchased from several

TABLE II

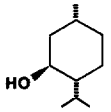
SEMIPREPARATIVE OR PREPARATIVE SEPARATIONS OF TERPENOIDS BY LC

Terpenoids	*Chromatographic method*	*Stationary phase*	*Column dimensions*	*Flow-rate (ml/min)*	*Sample size*	*Ref.*
Linalyl acetate purification	Adsorption	Spherosil XOA-400	50 cm × 23 mm I.D.	23	500 mg	19
Farnesol isomers	Adsorption	Silica gel	30 cm × 5 cm I.D.	250	3 g	20
Sesquiterpene lactones	Reversed phase	Ultrasphere ODS with an Altex precolumn	25 cm × 10 mm I.D.	4	–	21
Mono- and sesquiterpenoids	Reversed phase	LiChroprep RP-18 (40 μm)	24 cm × 10 mm I.D.	8	0.5 ml	16
Farnesol isomers	Exclusion	Styrene–divinylbenzene copolymer gel	60 cm × 21 mm I.D.	6	200 mg	25
	Adsorption	Prep-Pak 500 silica cartridge	–	350	2.4 g	26
(−) Menthone, (+)isomenthone	Exclusion	Partisil 10 (Whatman)	30 cm × 7.7 mm I.D.	–	50 mg	27

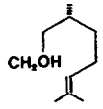
TABLE III

FORMULAE OF THE MOLECULES SEPARATED BY HPLC OR GC

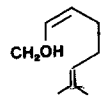
Monoterpene alcohols



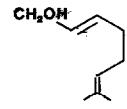
isomenthol



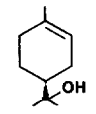
(+)citronellol



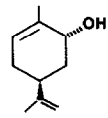
nerol



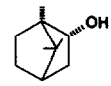
geraniol



α-terpineol



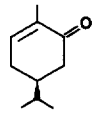
cis-carveol



borneol

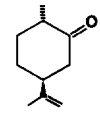
Monoterpene ketones

(−)piperitone

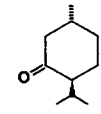


(+) carvone

fenchone

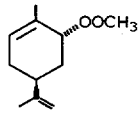


(−)dihydrocarvone

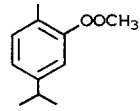


(−) menthone

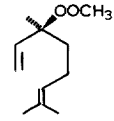
Monoterpene esters



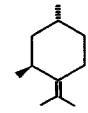
carvyl acetate



carvacryl acetate

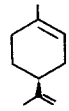


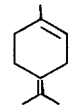
linalyl acetate

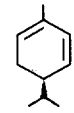

isopulegyl acetate

Monoterpene hydrocarbons

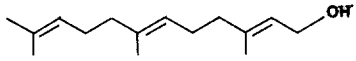

α-pinene


limonene

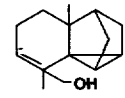

terpinolene


α-phellandrene

Sesquiterpene alcohols

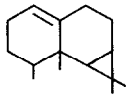


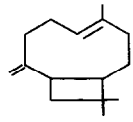
farnesol

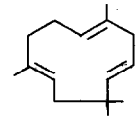


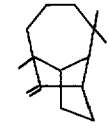
santalol

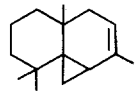
Sesquiterpene hydrocarbons

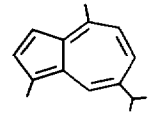

calarene


caryophyllene


humulene


longifolene


thujopsene


guiazulene


longipinene

companies; isomenthol, citronellol, carveol, fenchone, dihydrocarvone, menthone, carvyl acetate, isopulegyl acetate, guiazulene, humulene from EGA-Chimie France (Aldrich, Strasbourg, France); nerol, borneol, geraniol, terpineol, carvone, linalyl acetate, calarene, α-pinene, limonene, α-phellandrene, farnesol, caryophyllene, longifolene, thujopsene, longipinene from Fluka (Buchs, Switzerland).

The Vervain essential oil (29) was a generous gift from Dr. Garnero (Ets Robertet, Grasse, France) who has identified by classical methods (column LC, GS–MS) the main components of this essential oil[30].

RESULTS AND DISCUSSION

Two chromatographic techniques can be used: adsorption or reversed-phase chromatography. HPLC on silica columns is well known to be a convenient method for resolving compounds with different functional groups[31], while HPLC on octadecyl-bonded silica is well adapted for resolving compounds according to their hydrophobic properties.

An essential oil contains merely monoterpenoids (C_{10} chemical compounds) and sesquiterpenoids C_{15}) which differ in their hydrophobicities. So, reversed-phase chromatography has been chosen.

Analytical separation

A preliminary study with synthetic mixtures containing mono- and sesquiterpenoids was carried out and each group was resolved in order to determine the optimum mobile phase composition for semipreparative separation.

The variation of the capacity factors of several terpenoids, factors on octadecyl-bonded silica *vs.* the binary solvent (acetonitrile–water) composition is shown in Fig. 1. In reversed-phase liquid chromatography, the elution order is generally related to the solute hydrophobicity. Unfortunately, it is difficult to link the retention of terpenoids, to their hydrophobic characteristics, as calculated by Rekker's hydrophobic fragmental constants[32]; terpenoids have generally complex skeletons. However, the retention behaviour follows the classical rule of reversed-phase chromatography and the low selectivity exhibited by monoterpene ester–sesquiterpene alcohol groups was due to the small difference in polarity between these compounds.

Isocratic elution of terpenoids with the same functionality. The analytical separation of a standard mixture of sesquiterpene hydrocarbons, different from those in Fig. 1, containing guiazulene, humulene, caryophyllene, thujopsene, longifolene, calarene and longipinene was achieved on a C_{18} silica phase under isocratic conditions in about 40 min with a fair resolution (Fig. 2).

Gradient elution of terpenoids with various functionalities. The polarity range of terpenoids practically precludes the analysis of an essential oil by HPLC in a single experiment, except with a very long microbore column, packed with small diameter particles, which yields several hundred thousand theoretical plates[33]. Scott and Kucera[34] used this method for the analysis of an essential oil at the expense of long analysis time (32 h).

The retention behaviour of synthetic mixtures of terpenoids was investigated in the binary solvent system (acetonitrile–water) on Zorbax C_{18} (7 μm) silica. Acetonitrile–water (70:30) gives a satisfactory resolution of all the components, but

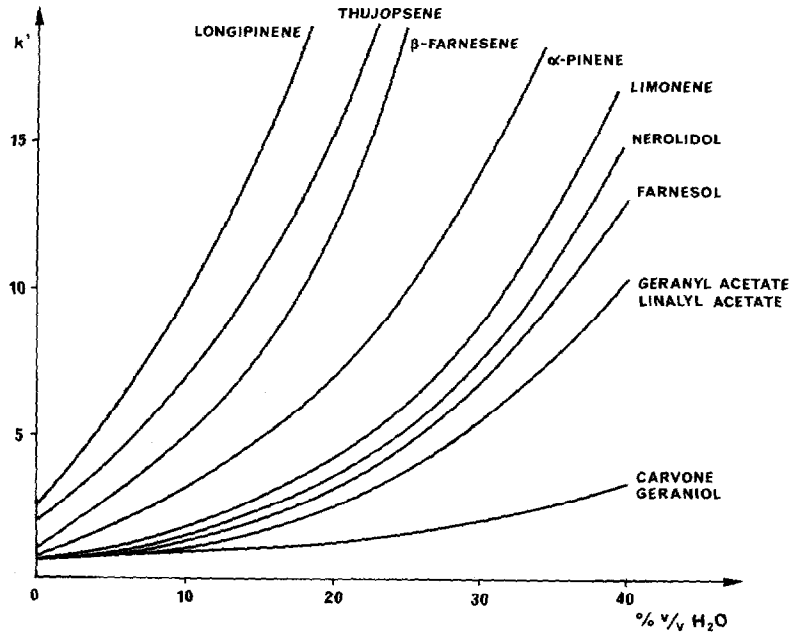


Fig. 1. Variations of the capacity factor, k', for some terpenoids *versus* the mobile phase composition. Column: 250 mm × 4.6 mm I.D. Stationary phase: LiChrosorb RP-18 (10 μm). Mobile phase: acetonitrile–water. Flow-rate: 2 ml min^{-1}. UV detection: 220 nm.

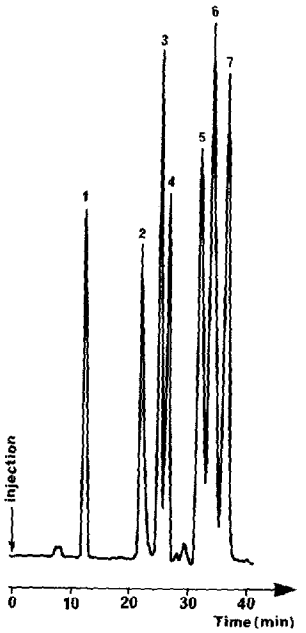


Fig. 2. HPLC resolution of a standard mixture of sesquiterpene hydrocarbons. Column: 250 mm × 4.6 mm I.D. Stationary phase: Nucleosil C_{18} (7 μm). Mobile phase: acetonitrile–water (77.5:22.5). Flow-rate: 2 ml min^{-1}. UV detection: 220 nm. Solutes: 1 = guiazulene; 2 = humulene; 3 = caryophyllene; 4 = thujopsene; 5 = longifolene; 6 = calarene; 7 = longipinene.

TABLE IV

CAPACITY FACTORS OF TERPENOIDS IN THE BINARY SYSTEM ACETONITRILE–WATER ON ZORBAX C_{18} SILICA

Solutes	*Acetonitrile–water*		
	77.5:22.5	*70:30*	*Elution gradient**
Monoterpene alcohols	0.4	0.9	0.9
ketones	0.5–0.7	1.0–1.2	1.0–1.2
esters	0.9–1.2	2.0–2.4	1.9–2.2
Sesquiterpene hydrocarbons	2.6	4.6	4.6
Monoterpene hydrocarbons	3.5–6.3	7.0–12.0	6.5–8.9
Sesquiterpene hydrocarbons	13–17	22–30	11.6–13.8

* For elution gradient profile, see Fig. 3.

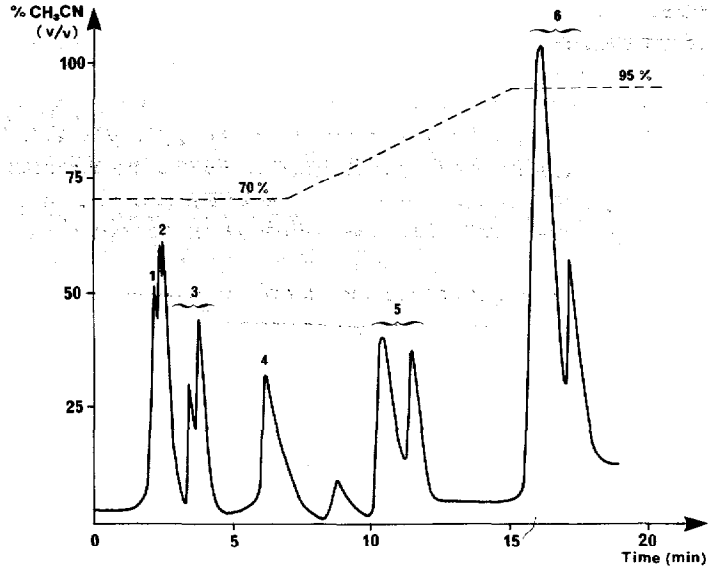


Fig. 3. Analytical HPLC separation of a standard mixture of terpenoids. Column: 250 mm × 9.2 mm I.D. Stationary phase: Zorbax C_{18} (7 μm). Mobile phase: acetonitrile–water; the gradient profile is shown as a dashed line. Flow-rate: 6 ml min^{-1}. Injection volume: 50 μl. UV detection: 220 nm. Solutes: 1 = monoterpene alcohols (isomenthol, citronellol, nerol, borneol, α-terpineol, carveol); 2 = monoterpene ketones (piperitone, carvone, fenchone, dihydrocarvone, menthone); 3 = monoterpene esters (carvyl acetate, carvacryl acetate, linalyl acetate, cinnamyl isobutyrate, isopulegyl acetate); 4 = sesquiterpene alcohols (nerolidol, santalol, patchoulol, farnesol); 5 = sesquiterpene hydrocarbons (caryophyllene, longifolene, bisabolene, β-patchoulene, guiazulene).

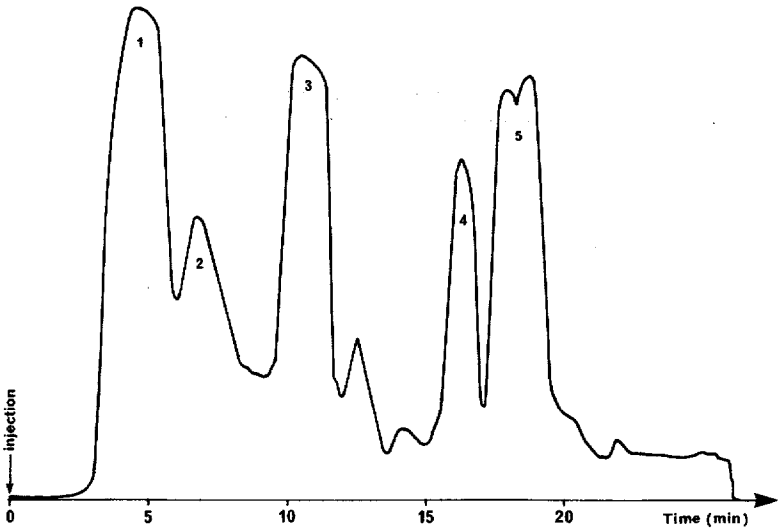


Fig. 4. Semipreparative HPLC separation of a Vervain essential oil. For experimental conditions, see Fig. 3, except: quantity injected, 0.5 ml, and concentration, 50 mg/100 ml.

the peaks eluted last (sesquiterpene and monoterpene hydrocarbons) are too broad and the analysis time too long (Table IV). So, this complex separation requires the use of gradient elution in order to keep the capacity factors in the optimum range (Fig. 3). The peaks are generally broad, but we must bear in mind that several compounds are present within each peak.

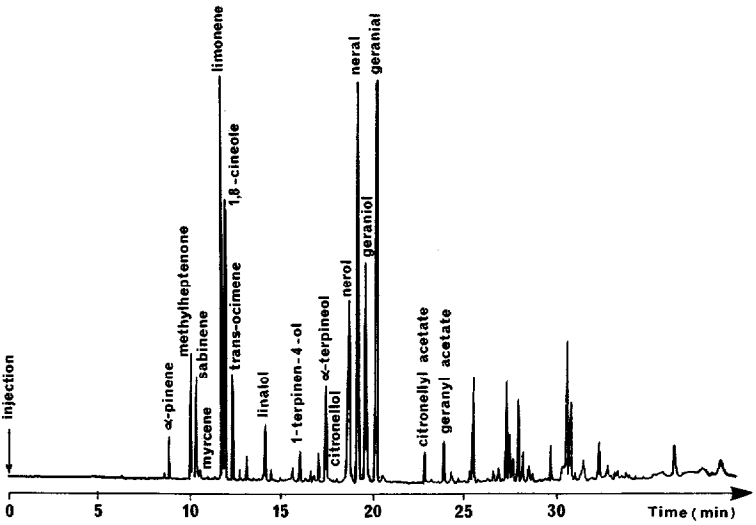


Fig. 5. GC resolution of a Vervain essential oil. Fused-silica capillary column: 25 m × 0.32 mm I.D. Stationary phase: DB 5 (J & W). The oven temperature was increased from 60 to 220°C at 4°C/min and held for 10 min at the upper limit. Detection: flame ionization.

Semipreparative separation

We selected a semipreparative column packed with the same packing material (Zorbax C_{18}, 7 μm). Each sample of essential oil was dissolved in a mixture of the eluent and tetrahydrofuran (THF) (50:50) at a concentration of 50 mg/100 ml. The mobile phase composition (acetonitrile–water) varies from 70:30 to 95:5 as shown in Fig. 3. This separation, into the main classes of terpenoids, is accomplished in about 20 min (Fig. 4) and allows five main fractions to be collected.

If monoterpene components of an essential oil can be analysed by GC–MS in

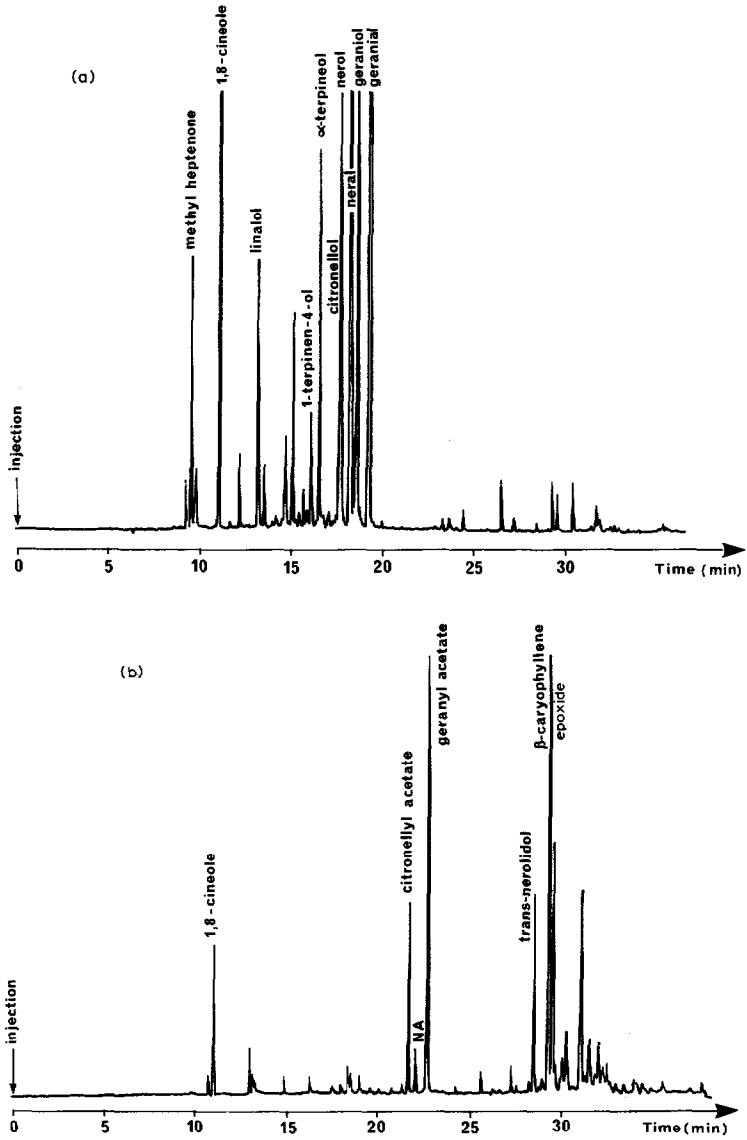


Fig. 6.

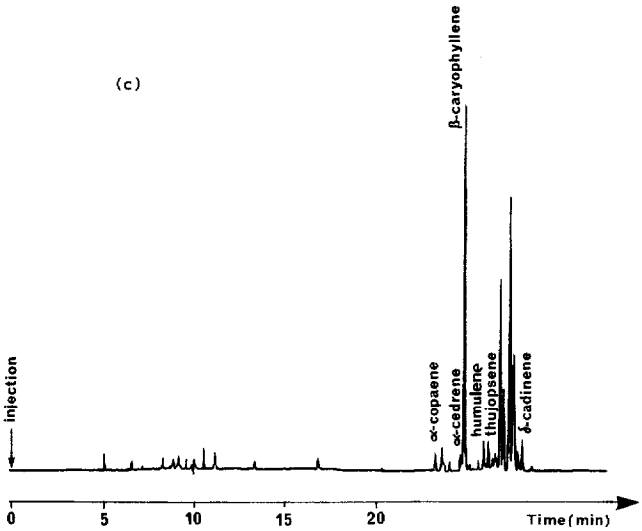


Fig. 6. GC resolution of HPLC fractions from the Vervain essential oil. For experimental conditions, see Fig. 5. Solutes: (a) fraction 1; (b) fraction 2; (c) fraction 5. NA = neryl acetate.

a single experiment, it is more convenient to perform an HPLC prefractionation for minor and thermosensitive components such as sesquiterpenoids.

The different HPLC fractions were collected and the terpenoids recovered by liquid–liquid extraction with diethyl ether–pentane (50:50). The organic layer was dried over anhydrous sodium sulphate and the solvent was removed at 30°C under vacuum with a rotary evaporator, to yield an oil which was analysed by fused-silica (DB 5) capillary GC. The characterization of the products was carried out by comparison of their mass spectra and retention data with those of authentic samples or with those present in our mass spectra library.

TABLE V

GC COMPOSITION OF VERVAIN ESSENTIAL OIL

Compound	*Content (%)*	*Compound*	*Content (%)*
α-Pinene	0.6	Geraniol	6.6
Methylheptenone	2.3	Geranial	19.9
Sabinene	1.8	Citronellyl acetate	0.6
Myrcene	0.4	Geranyl acetate	1.0
Limonene	13.5	α-Cedrene	0.2
1,8-Cineole	4.9	β-Caryophyllene	2.0
trans-Ocimene	1.7	Humulene	0.20
Linalol	1.2	Thujopsene	0.30
1-Terpinen-4-ol	0.6	Aromatic curcumene	2.4
α-Terpineol	2.1		
Citronellol and nerol	5.9	Nerolidol	0.7
Neral	14.4	β-Epoxycaryophyllene	2.1

TABLE VI

GC COMPOSITION OF HPLC FRACTIONS

Fraction no.	*Terpenoids*	*Content (%)*	*Fraction no.*	*Terpenoids*	*Content (%)*
1	1-Octen-3-ol	0.33	2	Neryl acetate	1.10
	Methylheptenone	1.48		Geranyl acetate	11.30
	3-Octanol	0.33		Citronellyl acetate	4.40
	1,8-Cineole	5.83		Nerolidol	4.50
	Linalol	1.56		β-Epoxycaryophyllene	7.50
	1-Terpinen-4-ol	1.13		1,8-Cineole	2.70
	α-Terpineol	2.47	4	Aromatic curcumene	74.0
	Citronellol	1.25	5	α-Cubebene	traces
	Nerol	3.72		α-Copaene	0.92
	Neral	28.22		α-Cedrene	0.95
	Geraniol	4.63		β-Caryophyllene	22.7
	Geranial	40.46		Humulene	2.15
				Thujopsene	2.65
				δ-Cadinene	2.4

GS–MS analysis of HPLC fractions

The chromatograms of a Vervain essential oil and HPLC fractions are shown in Figs. 5 and 6. The compounds identified in these mixtures are summarized in Tables V and VI. Fraction 1 comprises the most polar compounds, *i.e.*, monoterpene alcohols (3-octanol, 1-octen-3-ol), monoterpene oxide (1,8-cineole), monoterpene aldehydes (neral, geranial, citronellal) and aliphatic ketone (methylheptenone). In fraction 2 are present the medium polarity solutes such as monoterpene alcohol esters (neryl acetate, geranyl acetate and citronellyl acetate) and oxygenated sesquiterpenoids (nerolidol, β-epoxycaryophyllene). The apolar fractions 3, 4 and 5 contain respectively monoterpene hydrocarbons (α-pinene, sabinene, limonene, myrcene, α-terpinene, *trans*-ocimene), a sesquiterpene hydrocarbon of intermediate polarity, aromatic curcumene, and sesquiterpene hydrocarbons (α-copaene, α-cedrene, β-caryophyllene, humulene, thujopsene, δ-cadinene).

The comparison of the data in Tables V and VI demonstrates the advantages of the LC–GC–MS technique over direct GC–MS. The LC prefractionation step allows the concentration of minor compounds like sesquiterpene hydrocarbons. For instance, the humulene content in Vervain essential oil is about 0.2%, whereas its concentration is ten times greater in fraction 5. This enrichment gives a better precision and accuracy for GC and GC–MS experiments and allows identification of a greater number of terpenoids. In addition, LC prefractionation of essential oil, at room temperature, avoids the thermal degradations of sesquiterpenoids which occur in the traditional method, where monoterpene hydrocarbons and sesquiterpene hydrocarbons are separated by vacuum distillation of the *n*-hexane fraction.

CONCLUSION

Essential oil analysis by HPLC in a single experiment is difficult due to the complexity of the mixture (great number of compounds and large polarity range)

and the insufficient resolution for minor compounds. Semipreparative liquid chromatography and fraction analysis by GC–MS solves this problem. First, the essential oil is separated into several main fractions by semipreparative reversed-phase chromatography; these are subsequently analysed by GC–MS. In this way, a greater number of terpenoids can be resolved and identified.

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