On-line HPLC–HRGC–MS for the Analysis of Natural Complex Mixtures

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Abstract

A fully automated high-performance liquid chromatographic–high-resolution gas chromatographic–mass spectrometric system (ion-trap detection) is used for the analysis of hydrocarbon and oxygenated fractions of bitter orange, sweet orange, lemon, and mandarin leaf oils. The fractions are isolated by liquid chromatography and separated by gas chromatography. Component identification is by mass spectrometry (ITD). The qualitative and quantitative composition of the oils is discussed. Addition of or contamination by sweet orange, lemon, and mandarin leaf oils to bitter orange oil is detectable because of qualitative and quantitative differences.

Introduction

The analysis of complex matrices is often laborious because more than one chromatographic step is required. The best approach is to fractionate the sample before gas chromatographic analysis. The simpler mixtures thus obtained, which may be homogeneous, are easier to resolve.

Off-line methods, such as vacuum distillation, preparative gas chromatography, solvent extraction, and classical column liquid chromatography, are disadvantageous because they are time consuming and liable to sample contamination and/or loss at the fraction collection stage (1–3). In comparison with off-line methods, on-line liquid chromatography (LC) coupled with gas chromatography (GC) overcomes some of the drawbacks of off-line preseparation. In on-line high-performance LC–high-resolution GC (HPLC–HRGC), the sample is first separated by HPLC using a single column or a combination of columns to isolate the components of interest and then to directly transfer them to a capillary column where a further separation is carried out using the high efficiency and sensitivity of HRGC.

The two principal techniques of eluent evaporation that allow transfer of large LC fractions into GC are concurrent eluent evaporation (4) and the retention gap (5). Concurrent eluent evaporation means complete evaporation of the eluent during its introduction into GC. It allows the analysis of solutes with intermediate to high elution temperatures, depending on the volatility of the eluent and the volume of the LC fraction transferred. In this case, the temperature difference between transfer and the elution of the first sharp peaks is 60–100°C. In spite of the restrictions concerning elution temperatures, concurrent eluent evaporation is applied for most samples. This technique is preferred to the retention gap techniques due to its simplicity and the possibility of transferring very large LC fractions (1).

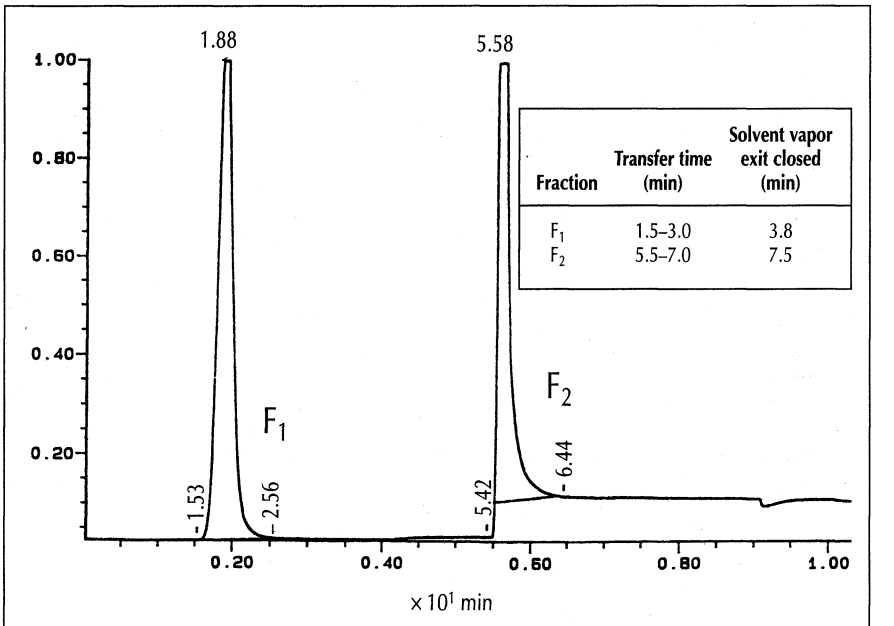

Figure 1. LC chromatogram of bitter orange petitgrain oil.

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The retention gap method represents the best approach in the case of qualitative and quantitative analysis of samples containing highly volatile compounds. In fact, the retention gap technique allows analysis of substances eluting immediately after the solvent peak due to reconcentration of those components by the so-called solvent effects (primarily solvent trapping) (6). On the other hand, this method is restricted to fractions of only modest volumes and the use of long uncoated precolumns. Working under conditions that still produce a zone flooded by the eluent (providing solvent trapping) but that cause a large amount of eluent to evaporate during its introduction allows us to work with a shorter uncoated precolumn or to transfer larger fraction volumes. This method is called partially concurrent evaporation. In fact, part of the eluent is evaporated concurrently, that is, during its introduction into the GC. The introduction of an early vapor exit greatly improves partially concurrent evaporation and protects the GC detector.

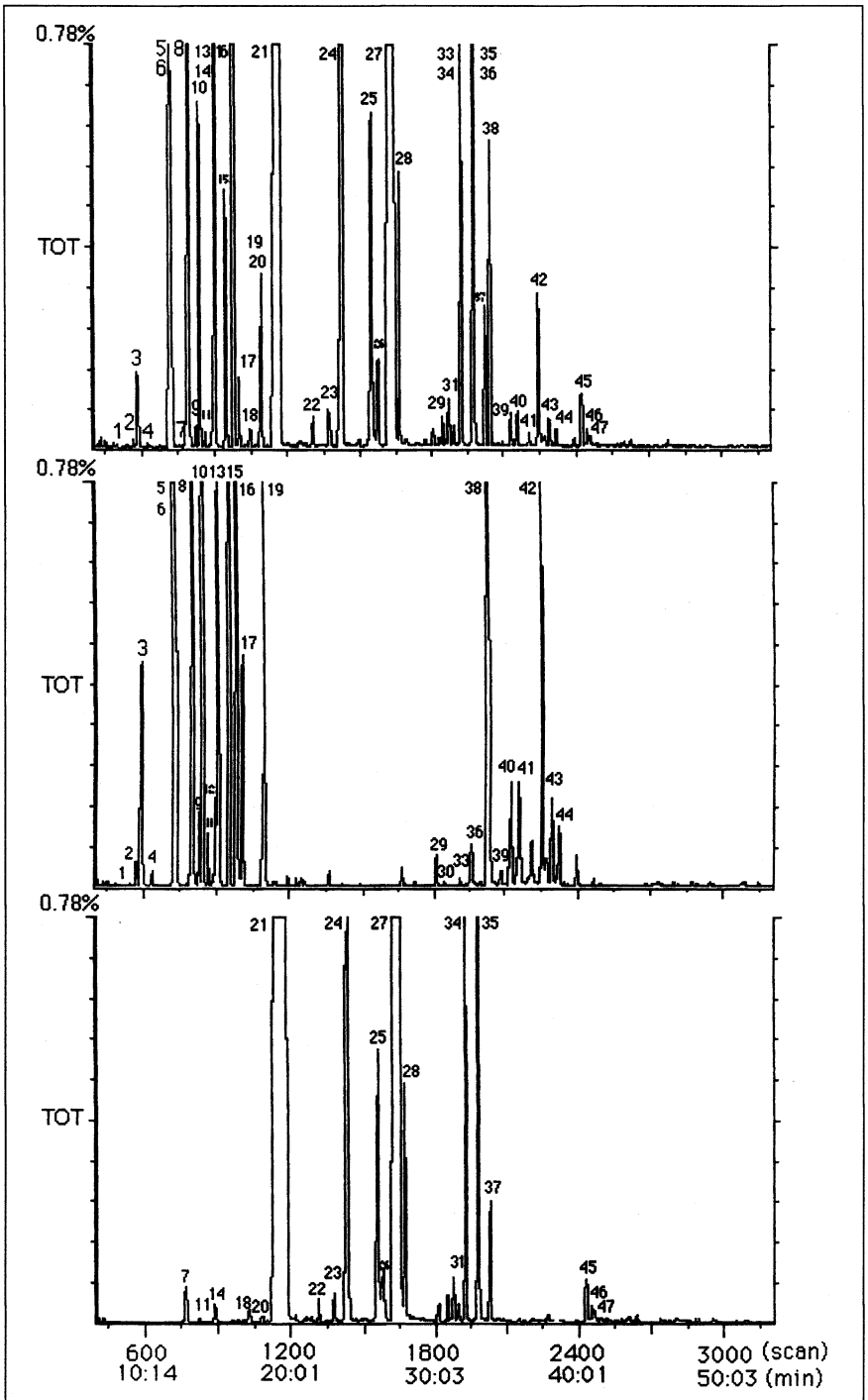

Figure 2. Total ion chromatogram of a bitter orange petitgrain oil (A) and of the F1 (hydrocarbons) (B) and F2 (oxygenated compounds) (C) fractions from its LC separation. Peak identification appears in Table I.

Essential oils consist of mixtures of monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives. The analysis of these oils often presupposes fractionation of the samples prior to GC analysis (7) due to substantial overlap between peaks. Moreover mass spectra of the components of the same class (monoterpenes or sesquiterpenes) are often similar, and it is necessary to have the spectrum of an extremely pure compound to obtain an unambiguous identification using library matching. The further combination of the HPLC–HRGC system with a mass spectrometer allows components to be reliably identified (8–10).

Petitgrain oils are obtained by steam distillation of leaves, twigs, and little unripe fruits of some citrus species. The bitter orange (bigarade) petitgrain oil shows organoleptic characteristics better than other petitgrain oils obtained from other citrus trees (lemon, mandarin, sweet orange) whose production is limited and sometime used to adulterate petitgrain bigarade. The chemical composition of petitgrain oils is not as well established as that of the oils obtained from the peel of citrus fruits. The available data are often relative to commercial and unstandardized samples, due to the different geographical origin, the age of the leaves used, their freshness, the time of the harvest, etc. Moreover, sometimes petitgrain oils are obtained in the laboratory by solvent extraction of the leaves (11,12).

The composition of the leaf oils has been proposed for chemotaxonomy (11,13). The analysis of citrus leaf oils seems to be more useful than that of citrus peel oils because leaf samples of new hybrids can be obtained much earlier than fruits (11). Leaf oil analysis has been also carried out with the aim of studying the origin of the volatile constituents in citrus trees (14,15).

A thorough qualitative and quantitative knowledge of these oils can be useful to define their composition and to identify possible adulterations; such studies are essential for increased use of these products.

The qualitative and quantitative differences, which are sometimes remarkable

among the results reported in the literature regarding leaf oils, are probably due to the different materials or extraction techniques used. The results reported in the literature for bitter orange leaf oil, relative to the content of the main components, are similar to each other, with the exception of those results obtained by Lin and co-workers (16), who found a very high amount of myrcene, and those obtained by Ortiz and co-workers (13), who found a very small amount of linalyl acetate for some varieties of bitter orange.

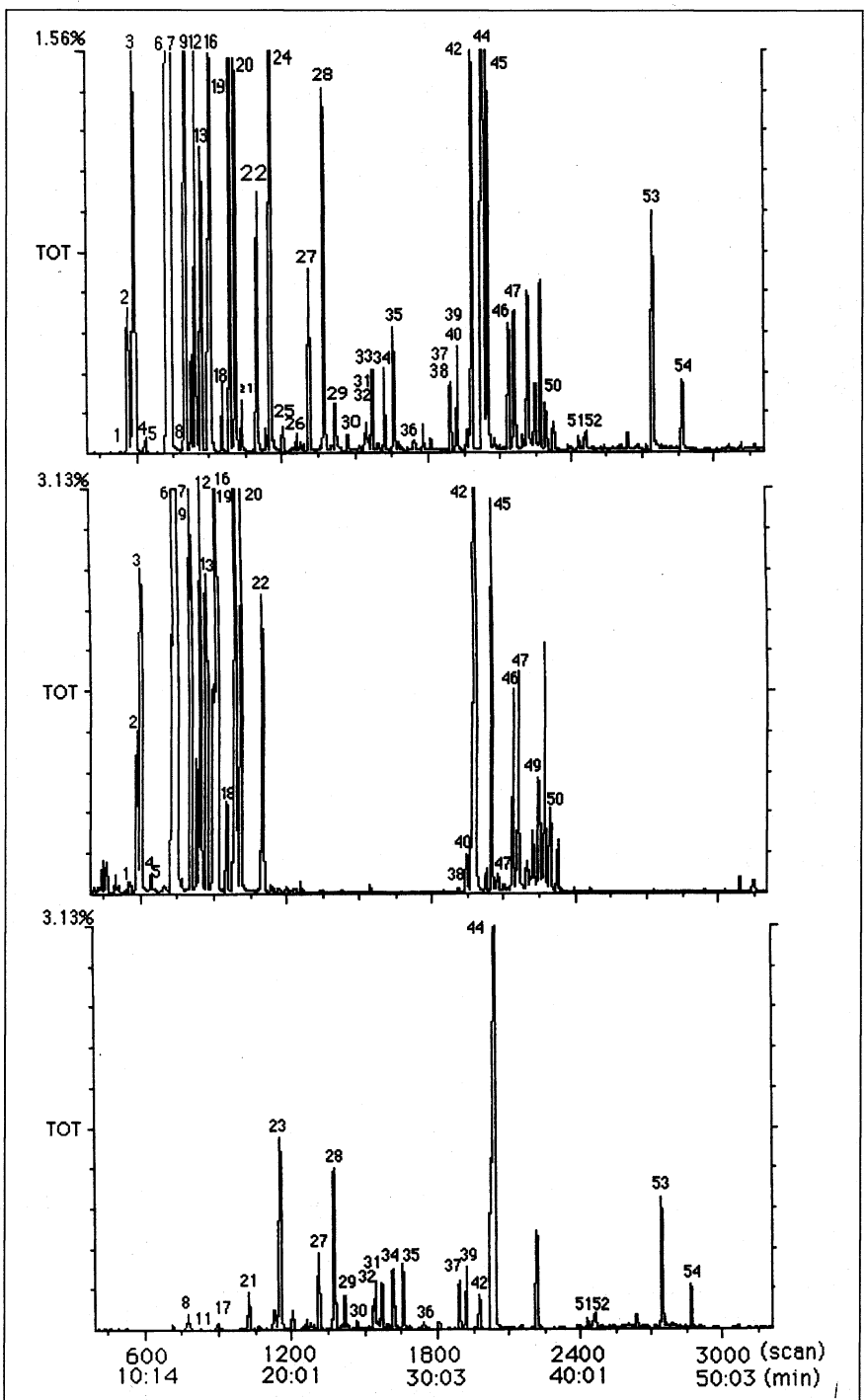

Figure 3. Total ion chromatogram of a sweet orange petitgrain oil and the F1 and F2 fractions from its LC separation. Peak identification appears in Table I.

Experimental

Analyses were carried out on Sicilian bitter orange, sweet orange, lemon, and mandarin distilled leaf oils.

A fully automated LC–GC instrument (Dualchrom 3000 Series, Fisons) was used for on-line preseparation by HPLC and further separation by capillary GC. The instrument was set up to use an on-column type interface that permitted partially concurrent solvent evaporation. The system was equipped with an early solvent vapor exit system for the reduction of the mobile phase evaporation time. Analysis was carried out under computer control throughout, with step gradient elution to separate and transfer the fractions to GC under the following HPLC conditions. Twenty microliters of solution (0.1 % v/v essential oil/pentane) was injected into a 10-cm × 2-mm i.d. column packed with Spherisorb 5-µm silica (Stagroma; Tübingen, Germany). The mobile phase consisted of eluent A (pentane; Carlo Erba; Milan, Italy) and eluent B (diethyl ether, Carlo Erba). The HPLC analyses were performed using eluent A for 5 min for hydrocarbon elution; the column was then backflushed with 1 mL diethyl ether for elution of oxygenated compounds. The flow rate was 180 µL/min, and detection was by Micro UVIS at 220 nm × 0.50 AUFS. The transfer time was 1.5–3.0 min for hydrocarbons and 5.5–7.0 min for oxygenated compounds. The GC system was equipped with a 10-m × 0.53-mm i.d. fused-silica uncoated precolumn. It was deactivated by phenyldimethyl silylation (retention gap). The retaining precolumn consisted of a 4-m × 0.32-mm i.d. SE-52 column with a 0.40–0.45-µm film thickness (MEGA; Legnano, Italy) connected to the retention gap by a press fit connection (MEGA). A capillary fused-silica SE-52 column (30 m × 0.32-mm i.d.; 0.40–0.45-µm film thickness) (MEGA) was used for separation. A butt connector attached to the purge line and fitted with a flow control valve automatically switched from high purging flow (26 mL/min) to low purging flow (0.2 mL/min) during analysis. The temperature during transfer of LC fractions was kept at 45°C for 6 min and then increased to 240°C at a rate of 3°C/min. The carrier gas (He) was delivered at a constant pressure of 120 kPa (linear velocity 36 cm/s). The eluent evaporation rate (140 µL/min) was determined as described elsewhere (17,18). A flame was held close to the solvent vapor exit. The time from ignition of the gas emerging from the vapor exit to extinction gave an exact measurement of the solvent evaporation time. The solvent vapor exit was switched to low flow shortly after the end of GC transfer time.

All components were identified using the retention time of standards and by means of coupled LC–GC–MS. Mass spectra were obtained on a Finnigan ion-trap mass spectrometer (Model 800) directly coupled to the LC–GC system. The tuning values for the ITD were 100, 100, 100, 100 using FC43 as a tuning standard; The tune sensitivity was 9000. The acquisition parameters were as follows: full scan; scan range, 41–300 amu; scan time, 1.0 s; threshold, 1 count. AGC mode was as follows: on; micro scans, 5; filament delay, 240 s. The multiplier was set at 2200 V depending on multiplier condition. The temperatures of the transfer line, exit nozzle, and manifold were all 250°C.

Quantitative results were also obtained with the same system by using a flame-ionization detector and by measuring the peak area (relative percentage).

Results and Discussion

Figure 1 shows the LC chromatogram of the bitter orange petitgrain oil. The transferred fractions are marked F1 and F2, and the transfer times of each fraction are listed with the time of the vapor exit closure. Figures 2–5 show the total ion chromatograms (TIC) of the LC fractions for each oil. The on-column GC chromatograms obtained with the same column system are shown above the chromatograms of the transferred LC fractions. Compound identification is reported in Table I. The on-column chromatograms of the whole essential oil samples show some overlap between peaks of monoterpenes and oxygenated compounds, but HPLC preseparation allowed the separation of all detectable components present in the oils.

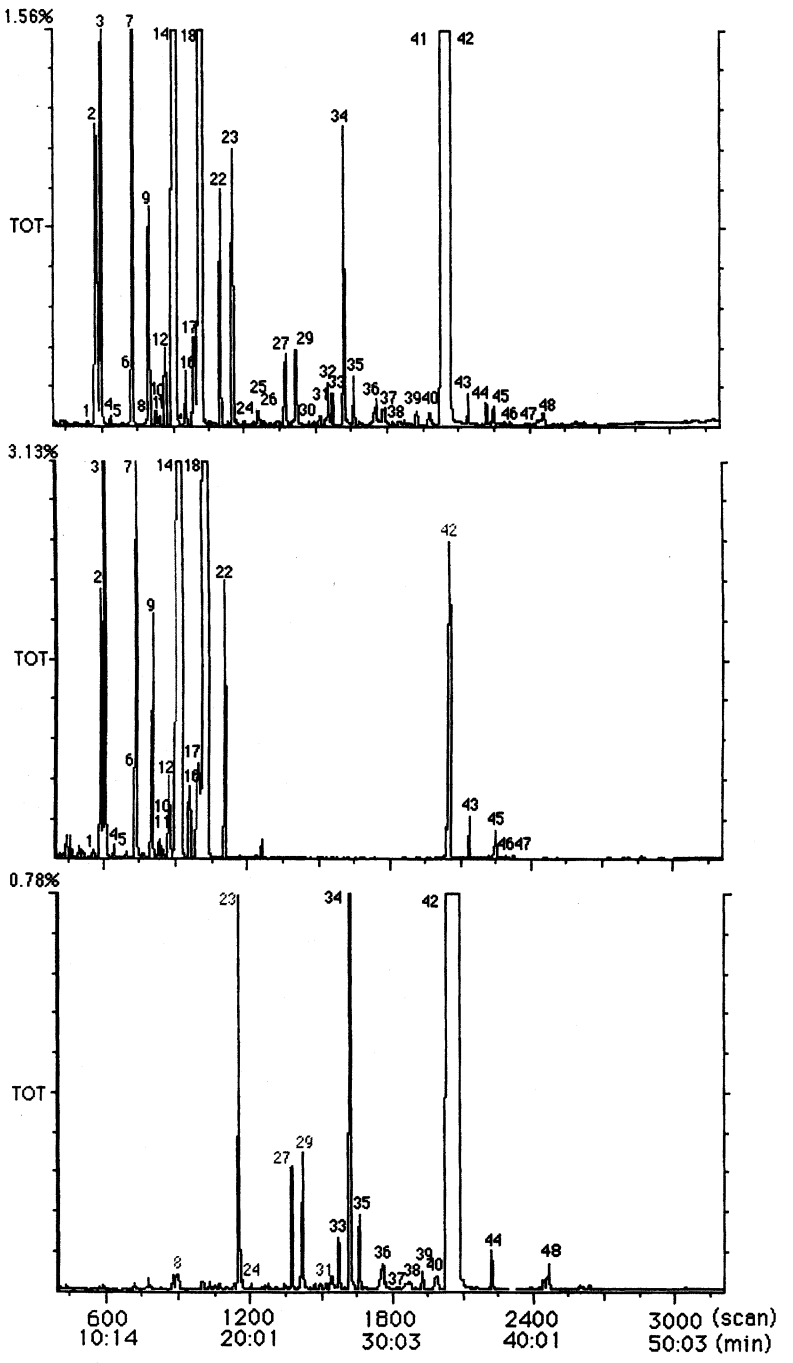

Figure 4. Total ion chromatogram of a mandarin petitgrain oil and its F1 and F2 fractions from its LC separation. Peak identification appears in Table I.

Bitter orange petitgrain oils

Bitter orange petitgrain oil is characterized by its high content of oxygenated compounds. Among the most abundant compounds present in bitter orange petitgrain oil are esters (46.4% of the oil), alcohols (36.8%), aldehydes (1.1 %), and monoterpenes (13.8%). (*E*)-Caryophyllene is the principal component (0.7%) of the sesquiterpene fraction, which represents little more than 1% of the oil.

The chromatogram of the whole oil sample shows overlap between the following pairs of peaks: 6-methyl-5-hepten-2-one and myrcene; 1,8-cineole and limonene; *trans*-linalool oxide and terpinolene; δ-elemene and an unknown oxygenate; α-copaene and citronellyl acetate; β-elemene and geranyl acetate. The amount of 6-methyl-5-hepten-2-one, and of all the oxides in general, is an important parameter in the evaluation of the freshness of the oil and the more or less drastic conditions under which distillation was carried out.

Sweet orange petitgrain oils

Sweet orange petitgrain oil consists of 75.1% terpene hydrocarbons (67.1% monoterpenes and 8.0% sesquiterpenes) and 22.3% oxygenated compounds. In detail, this fraction contains 11.7% esters, 7.7% alcohols, 2.9% aldehydes, and very small amounts of oxides (0.1%). The chromatogram of the whole sample shows that the following components of different classes co-eluted: 6-methyl-5-hepten-2-one and myrcene; octanal

and α-phellandrene; 1,8-cineole and limonene; α-copaene and citronellyl acetate; β-cubebene and neryl acetate; *cis*-α-bergamotene and *N*-methyl methylanthranilate.

Mandarin petitgrain oils

The monoterpene fraction of mandarin petitgrain oil represents 54.0% of the oil; the major component is γ-terpinene (27.6%). The sesquiterpene fraction constitutes 1.0% of the oil. Among the oxygenated compounds, esters are the best represented class at 42.7%; *N*-methyl methylanthranilate is the main component of this fraction (41.6%). Alcohols represent 0.8% of the oil, and aldehydes and oxides represent only 0.2% and 0.1%, respectively. The chromatogram of the whole oil shows that 6-methyl-5-hepten-2-one coelutes with myrcene, and the peaks for (*E*)-caryophyllene and *N*-methyl methylanthranilate overlap.

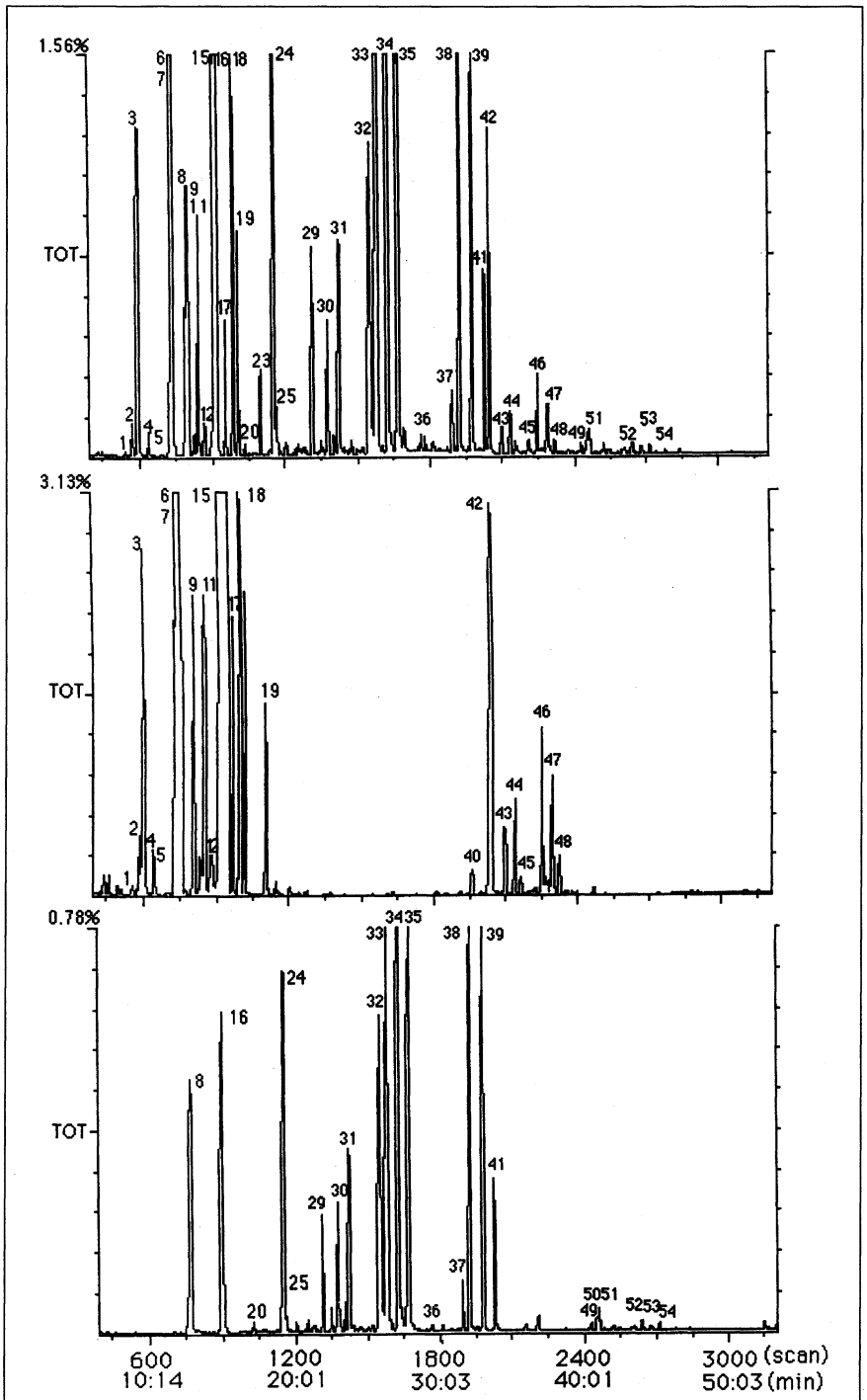

Figure 5. Total ion chromatogram of a lemon petitgrain oil and its F1 and F2 fractions from its LC separation. Peak identification appears in Table I.

Lemon petitgrain oils

Lemon petitgrain oil is characterized by its high content of monoterpene hydrocarbons (51.8%). Sesquiterpenes constitute 1.5%. Aldehydes are most prominent in the oxygenated fraction (20.7%). Esters and alcohols represent 14.7% and 8.7% of the oil, respectively.

The chromatogram of the oil show some co-elutions: 6-methyl-5-hepten-2-one and myrcene; and β-elemene and geranyl acetate.

Contamination and adulteration of bitter orange petitgrain oil by sweet orange petitgrain oil

These two oils show some significant qualitative and quantitative differences. The presence of α-fenchene, *o*-cymene, *cis*- and *trans*-*p*-menth-2-en-1-ol, thymol, β-cubebene, α-selinene, valencene, β-sinensal, and α-sinensal, which are not present in bitter orange petitgrain, allow the detection of sweet orange petitgrain.

The presence of sweet orange petitgrain oil may be due to deliberate addition, because it is less valuable than bitter orange petitgrain, or may be due to contamination of the raw material to be distilled. Moreover, the presence of sweet orange petitgrain oil may be detected by the remarkable quantitative differences of the following components in the two oils : α-thujene, α-pinene, sabinene, α-phellandrene, α-terpinene, γ-terpinene, citronellal, terpinen-4-ol, and β-elemene.

Contamination and adulteration of bitter orange petitgrain oil by mandarin petitgrain oil

Quantitative differences between the two oils are observed for the following components: α-thujene, α-pinene, α-terpinene, limonene, γ-terpinene, and *N*-methyl methylanthranilate. These compounds are present in larger amounts in mandarin petitgrain oil than in bitter orange petitgrain oil. α-Fenchene, octanol, nonanal, *trans*-*p*-menth-2-en-1-ol, thymol, and α-selinene were only found in mandarin petitgrain oil. Moreover methyl anthranilate and *N*-dimethyl methyl anthranilate were found only in mandarin petitgrain oil among all the petitgrain oils analyzed.

Table I. Components Identified by HPLC–HRGC–MS and Quantitative Composition for the Four Oils Analyzed ***(Continued on page 180)***

Compound	Bitter orange (g/100 g)	Peak in Figure 2	Sweet orange (g/100 g)	Peak in Figure 3	Mandarin orange (g/100 g)	Peak in Figure 4	Lemon (g/100 g)	Peak in Figure 5
Tricyclene	tr*	1	tr	1	0.01	1	tr	1
α-Thujene	0.02	2	0.39	2	0.98	2	0.07	2
α-Pinene	0.17	3	1.51	3	2.16	3	0.87	3
α-Fenchene	–	–	0.01	4	0.01	4	0.01	4
Camphene	tr	4	0.04	5	0.02	5	0.05	5
Sabinene	0.45	5	38.26	6	0.30	6	3.01	6
β-Pinene	2.20	6	2.62	7	2.31	7	12.57	7
6-Methyl-5-hepten-2-one	0.08	7	0.05	8	0.01	8	1.06	8
Myrcene	2.31	8	2.88	9	0.79	9	0.83	9
Octanal	–	–	0.01	10	–	–	–	–
α-Phellandrene	0.05	9	0.23	11	0.04	10	0.03	10
δ-3-Carene	1.15	10	3.46	12	0.02	11	0.75	11
α-Terpinene	0.03	11	0.65	13	0.24	12	0.04	12
o-Cymene	–	–	0.05	14	–	–	0.01	13
p-Cymene	0.12	12	2.89	15	5.19	13	0.51	14
Limonene	2.02	13	5.85	16	12.59	14	30.66	15
1,8-Cineole	0.06	14	0.03	17	0.02	15	1.34	16
(*Z*)-β-Ocimene	0.89	15	0.21	18	0.18	16	0.30	17
(*E*)-β-Ocimene	3.64	16	4.49	19	0.59	17	1.50	18
γ-Terpinene	0.18	17	2.62	20	27.64	18	0.42	19
cis-Sabinene hydrate	–	–	0.23	21	0.01	19	0.03	20
cis-Linalool oxide	0.05	18	–	–	tr	20	0.01	21
Octanol	–	–	–	–	tr	21	0.01	22
Terpinolene	0.59	19	0.98	22	0.97	22	0.19	23
trans-Linalool oxide	0.03	20	–	–	–	–	–	–
Linalool	29.80	21	4.34	23	0.93	23	3.09	24
Nonanal	–	–	0.04	24	0.01	24	0.08	25
cis-Limonene oxide	–	–	–	–	tr	25	tr	26
cis-*p*-Menth-2-en-1-ol	–	–	0.09	25	–	–	0.02	27
trans-*p*-Menth-2-en-1-ol	–	–	0.07	26	0.02	26	–	–
Isopulegol	–	–	–	–	–	–	tr	28
Citronellal	0.05	22	0.43	27	–	–	0.78	29
Terpinen-4-ol	0.12	23	2.36	28	0.24	27	0.51	30
p-Cymen-8-ol	–	–	–	–	0.02	28	–	–
α-Terpineol	5.39	24	0.21	29	0.26	29	0.96	31
Decanal	–	–	0.03	30	0.01	30	–	–
Citronellol	–	–	0.03	31	0.01	31	–	–
Nerol	1.28	25	0.26	32	0.10	32	2.66	32
Neral	0.40	26	0.28	33	0.06	33	8.13	33
Linalyl acetate	39.75	27	0.40	34	0.96	34	5.44	34
Geranial	0.67	28	0.59	35	0.10	35	11.67	35
Undecanal	–	–	–	–	–	–	0.04	36
Thymol	–	–	0.05	36	0.11	36	–	–
δ-Elemene	0.02	29	–	–	–	–	–	–
α-Cubebene	0.02	30	–	–	–	–	–	–
Methyl anthranilate	–	–	–	–	0.01	37	–	–
α-Terpinyl acetate	0.06	31	–	–	0.01	38	–	–
Citronellyl acetate	0.11	32	0.25	37	–	–	0.21	37
α-Copaene	0.01	33	0.01	38	–	–	–	–
Neryl acetate	2.27	34	0.38	39	0.03	39	5.89	38
β-Cubebene	–	–	0.10	40	–		–	–
Geranyl acetate	4.22	35	0.28	41	0.05	40	2.92	39
β-Elemene	0.04	36	3.80	42	–	–	0.03	40
cis-α-Bergamotene	–		tr	43	–	–	–	–
N-Methyl methyl anthranilate	0.17	37	10.29	44	41.61	41	0.24	41
(*E*)-Caryophyllene	0.71	38	2.47	45	0.92	42	0.96	42

Table I. Components Identified by HPLC–HRGC–MS and Quantitative Composition for the Four Oils Analyzed *(Continued from page 179)*

Compound	Bitter orange (g/100 g)	Peak in Figure 2	Sweet orange (g/100 g)	Peak in Figure 3	Mandarin orange (g/100 g)	Peak in Figure 4	Lemon (g/100 g)	Peak in Figure 5
trans-α-Bergamotene	0.01	39	–		–		0.06	43
α-Humulene	0.07	40	0.60	46	0.07	43	0.09	44
(*Z*)-β-Farnesene	0.07	41	0.58	47	–	–	0.02	45
N-Dimethyl methyl anthranilate	–	–	–	–	0.03	44	–	–
Bicyclogermacrene	0.28	42	-	–	-	–	0.17	46
α-Selinene	–	–	0.04	48	0.02	45	–	–
Valencene	–	–	0.02	49	–	–	–	–
α-Farnesene	0.05	43	0.12	50	tr	46	0.14	47
δ-Cadinene	0.04	44	–	–	tr	47	0.02	48
(*E*)-Nerolidol	0.06	45	0.05	51	–	–	0.01	49
Sphatulenol	0.03	46	–	–	–	–	0.04	50
Caryophyllene oxide	0.02	47	0.06	52	0.02	48	0.05	51
2,3-Dimethyl-3-(4-methyl-3-pentenyl-bis)-2-norbornanol	–	–	–	–	–	–	0.02	52
Campherenol	–	–	–	–	–	–	0.01	53
α-Bisabolol	–	–	–	–	–	–	0.01	54
β-Sinensal	–	–	1.25	53	–	–	–	–
α-Sinensal	–	–	0.24	54	–	–	–	–

*tr = transferred.

Table II. Components Not Found in Bitter Orange Petitgrain Oil

Sweet orange petitgrain	Mandarin petitgrain	Lemon petitgrain
α-Fenchene	α-Fenchene	α-Fenchene
o-Cymene	Octanol	o-Cymene
cis-*p*-menth-2-en-1-ol	Nonanal	*cis*-Sabinene hydrate
trans-*p*-menth-2-en-1-ol	*trans*-*p*-Menth-2-en-1-ol	Nonanal
Thymol	Thymol	*cis*-Limonene oxide
β-Cubebene	α-Selinene	Isopulegol
α-Selinene	Methyl anthranilate	Undecanal
Valencene	*N*-Dimethyl methyl anthranilate	2,3-Dimethyl-3-(4-methyl-3-pentenyl)-2-norbornanol
β-Sinensal		Campherenol
α-Sinensal		α-Bisabolol

Contamination and adulteration of bitter orange petitgrain oil by lemon petitgrain oil

Lemon petitgrain oil differs greatly from bitter orange petitgrain oil in qualitative and quantitative composition. Characteristic components of lemon petitgrain, which are completely absent in bitter orange petitgrain, are α-fenchene, *o*-cymene, *cis*-sabinene hydrate, nonanal, and *cis*-limonene oxide. Moreover, among the four oils under investigation, the following compounds were only found in lemon petitgrain: isopulegol, undecanal, 2,3-dimethyl-3-(4-methyl-3-pentenyl-2-norbornanol), campherenol, and α-bisabolol. Quantitative differences are observed for the following components: α-pinene, sabinene, β-pinene, limonene, 1,8-cineole, citronellal, neral, and geranial.

Conclusion

With this method, it was possible to identify and quantitate most of the compounds present in the four oils (see Table I). Moreover, it was possible to determine the identity and amount of compounds whose presence is characteristic for each oil (see Tables II and III). The preseparation of hydrocarbons and oxygenated compounds allowed us to obtain interpretable mass spectra more easily. These mass spectra permit the positive identification of compounds that belong to the same class, for example, monoterpene hydrocarbons (acyclic, mono-, bi- or tricyclic) which have the same molecular formula ($C_{10}H_{16}$) and the same molecular weight (MW=136). Figure 6 shows how it is possible to identify even two monoterpene hydrocarbons of the same type. Mass spectra of two monocyclic monoterpene hydrocarbons, α-phellandrene and γ-terpinene, were compared with the library mass spectra. By comparison of the library algorithms for purity, fit and reverse fit, it was determined that a reliable identification was obtained.

Table III. Components Present in Larger Amounts in Sweet Orange, Mandarin, and Lemon Petitgrain Oils Than in Bitter Orange Petitgrain Oil (g/100 g oil)

	Bitter orange petitgrain	Sweet orange petitgrain	Mandarin petitgrain	Lemon petitgrain
α-Thujene	0.02	0.39	0.98	–
α-Pinene	0.17	1.51	2.16	0.87
Sabinene	0.45	38.26	–	3.01
β-Pinene	2.20	–	–	12.52
α-Phellandrene	0.05	0.23	–	–
α-Terpinene	0.03	0.65	0.24	–
Limonene	2.02	–	12.59	30.66
1,8-Cineole	0.06	–	–	1.34
γ-Terpinene	0.18	2.62	27.64	–
Citronellal	0.05	0.43	–	0.78
Terpinen-4-ol	0.12	2.36	–	–
Neral	0.40	–	–	8.13
Geranial	0.67	–	–	11.67
β-Elemene	0.04	3.80	–	–
N-Methyl methyl anthranilate	0.17	–	41.61	–

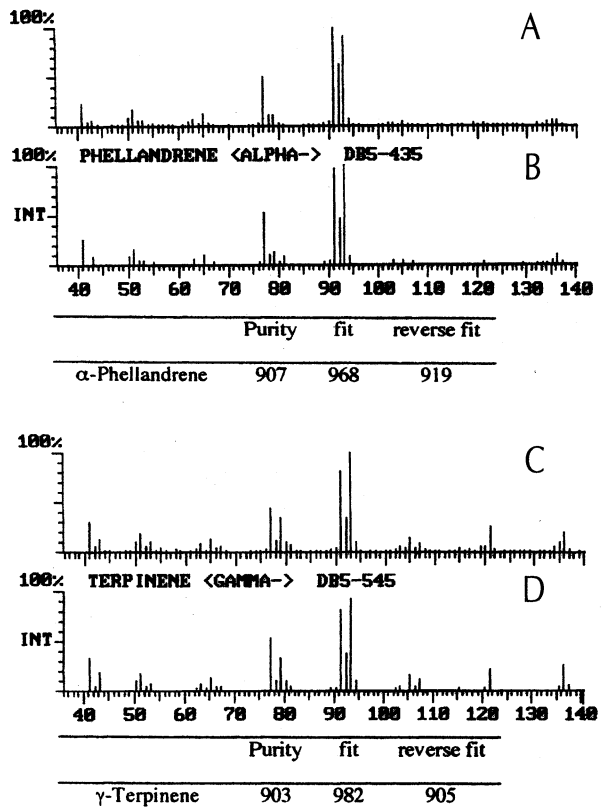

Figure 6. The comparison of a-phellandrene mass spectrum obtained by HPLC–HRGC-MS (A) with the library spectrum (B) and comparison of γ-terpinene mass spectrum obtained by HPLC–HRGC-MS (C) with the library spectrum (D). The results obtained from the library search for each spectrum are reported. DB5-435 and DB5-535 are the retention times (in seconds) carried out on a DB5 column, as reported by the mass spectral library.

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