By Luigi Mondello, Giovanni Dugo, and Keith D. Bartle

Coupled HPLC-HRGC-MS: A new method for the on-line analysis of real samples

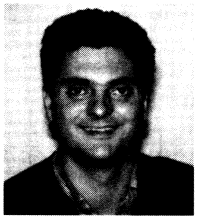
THE COMPOSITION of the minor components that are present in real, complex samples provides highly specific information. In general, the analysis of minor components often presupposes the fractionation of the sample prior to GC analysis. The simpler mixtures thus obtained are easier to resolve without problems of peak overlap. Because of the ease of collecting and handling liquids, classical LC followed by injection into a GC system is often used for the analysis of trace components in environmental and food samples. LC allows preseparation and concentration of the components of interest, and high-resolution gas chromatography (HRGC) is used to analyze the fractions. Other off-line methods (e.g., vacuum distillation, preparative GC, and solvent extraction) are laborious, very slow, and prone to sample contamination and/or sample loss at the fraction collection stage.

Food samples, like almost all real samples, are very complex mixtures, with components belonging to different classes and showing very different distribution. Additional difficulties arise when the component of interest is present only at trace levels. On-line coupling permits the separation and identification of compounds of the same polarity in mixtures of compounds of different polarity even when the concentration of the various classes of compounds is considerably different.

The two principal techniques of eluant evaporation that allow the transfer of large LC fractions into GC are concurrent eluant evaporation[1] and the retention gap method.[2]

Concurrent eluant evaporation (conventional and with cosolvent trapping)

The total eluant evaporation during GC transfer of the eluant[3] is more satisfactory if carrier gas is used to drive eluant into the GC. To prevent the liquid from passing the inlet of the GC column, the oven temperature should be slightly above the boiling point of the solvent, corrected for the inlet pressure. In this way, the liquid can be transferred at a speed corresponding to the evaporation speed. The loop interface,[4] shown schematically in *Figure 1*, allows the concurrent elu-



Dr. Mondello *(top) is an Assistant Professor in the Dipartimento Farmaco-Chimico of the University of Messina (Italy). He received a degree in chemistry from the University of Messina in 1991. He worked for one year postdoctorally at the University of Leeds, under the supervision of Prof. K.D. Bartle, with whom he still collaborates. His research interests include the development of coupled techniques such as LC-GC-MS and GC-GC and their applications in the study of essential oils and other natural complex matrices. He is the author of approximately 45 scientific papers, and he has been chairman and invited lecturer in national and international congresses and symposiums.*

Prof. Dugo *(middle) is Full Professor of Food Chemistry of the University of Messina (Italy) and Vice-Rector of the same university. He received his degree in chemistry from the University of Messina in 1964. He was chief of the group of Food Chemistry of the Italian Chemical Society for many years. He is a member of the working group of the Italian and of the European Pharmacopoeia and of the committee of the Italian Society of Food Science. Since 1964, he has been involved in chromatographic techniques, directing his attention to different problems related to the chemistry of natural products. Lately, his interest has been directed toward all the aspects of citrus chemistry to resolve analytical and technological problems. He coordinated many research projects within his country in the food chemistry field; now, he is the coordinator for a research project between Italy and Uruguay, leading to the improvement of the value of Uruguayan agricultural products and essential oils. He organized the first and the second Italian Food Chemistry Congresses. He coordinates a large group that carries out research on the analysis of essential oils with different analytical separation techniques. He is the author of approximately 150 scientific papers.*

Dr. Bartle *(bottom) is a Professor in the Leeds University School of Chemistry and a Technical Director of Express, a company specializing in applications of supercritical fluid technology and separation. He has been involved in chromatographic and related techniques since working as a technician at the Coal Tar Research Association while studying part-time for a degree at the Bradford Technical College. After his Ph.D. from Leeds University, he had a research fellowship in Stockholm before joining the Leeds staff and has since held visiting faculty positions at Indiana and Brigham Young Universities. Dr. Bartle was awarded a Jubilee Medal by the Chromatographic Society in 1991 and received the Royal Society of Chemistry Award for Analytical Separations in 1993, both for developing supercritical analysis techniques. Current research interests are still centered on the mainly analytical uses of supercritical analysis fluids and also on the development and applications of microchromatography with gas, supercritical fluid, and liquid mobile phases.*

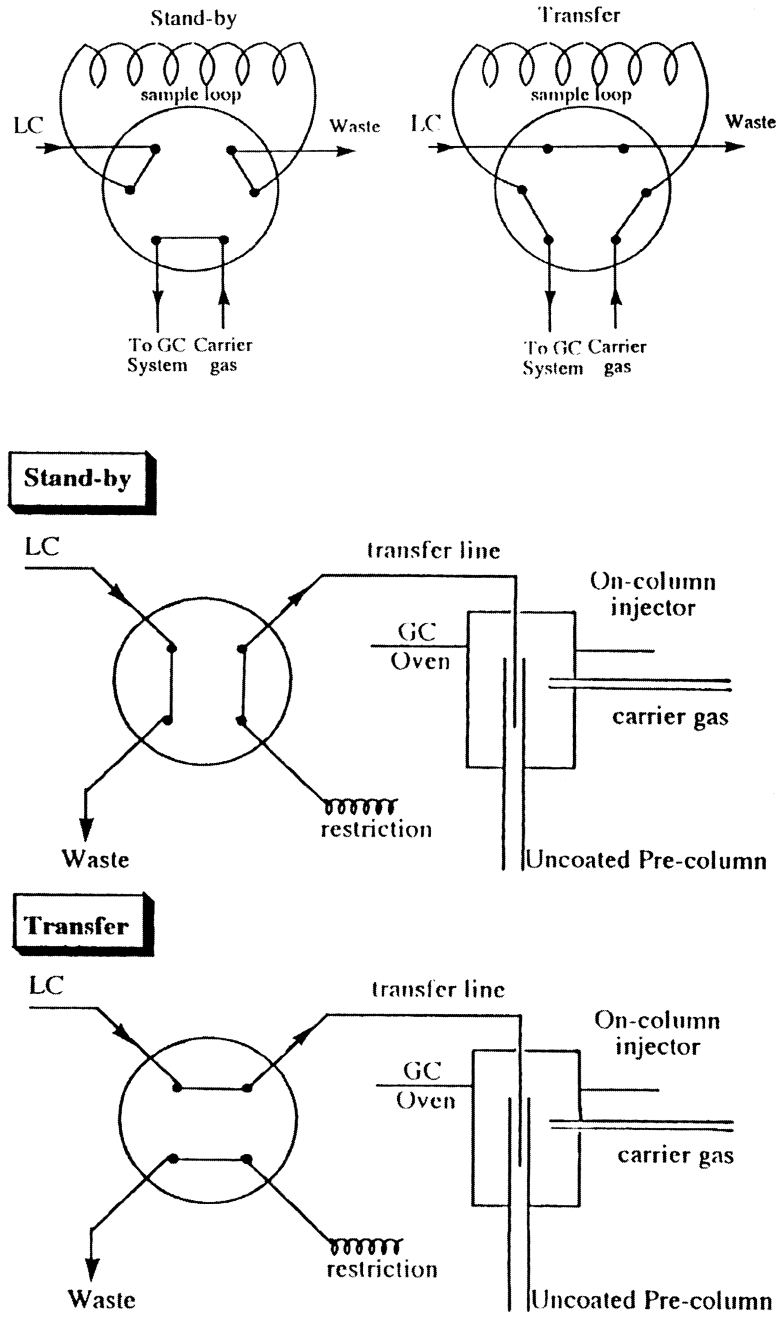


Figure 2 *On-column interface.*

ant evaporation described above, and the liquid is driven by the carrier gas instead of the LC pump.

The fraction to be analyzed is collected in a loop placed in a switching valve; the opening of the valve allows the sample contained in the loop to be driven by the carrier gas into the GC. Usually, an early vapor exit, located after a few meters of deactivated pre-column and a 3–4 m piece of retaining pre-column[5] (cut from the separation column), is opened during solvent evaporation to reduce the amount of solvent vapor that goes through the detector and at the same time increases the solvent evaporation rate.[6] The drawback of the concurrent eluant evaporation method is the band broadening at the beginning of the chromatogram. In fact, as the sample solvent evaporates at the front end of the liquid, no condensed phase remains ahead of the evaporation site. Solutes liberated from the retaining solvent envelope immediately start moving into the separation column. The consequence of solute coevaporation with the solvent is the loss of volatile solute material resulting either from coelution with solvent or from venting through the early vapor exit. In practice, the first perfect peaks are eluted about 69–80 °C above the transfer temperature. This means that concurrent eluant evaporation is only applicable if the elution temperatures of the compounds of interest are above 120–150 °C if the eluant is pentane or diethyl ether.

The partial solution to the problem of producing sharp peaks at low elution temperatures is to use the auxiliary technique of concurrent eluant evaporation with cosolvent trapping. In fact, conventional concurrent eluant evaporation suffers from the fact that there is no barrier to prevent volatile components from coevaporating with the eluant. To overcome this drawback, a small amount of a higher boiling cosolvent is added to the main solvent to build up a layer of condensed liquid ahead of the main evaporation site, retaining volatile solutes by solvent trapping.[7,8]

Retention gap (conventional and partially concurrent evaporation)

The retention gap method represents the best approach in the case of qualitative and quantitative analysis of sample containing highly volatile compounds. The retention

gap technique (*Figure 2*) allows the analysis of substances eluting immediately after the solvent peak due to the reconcentration of those components by the so-called solvent effects (primarily solvent trapping).[9] A layer of condensed eluant is built up ahead of the evaporation site to act as a thick layer of retaining stationary phase, blocking the further movement of all but the most volatile compounds into the column. Solvent evaporation, therefore, proceeds from the rear toward the front of the sample layer. However, working with the conventional retention gap technique, due to the limited capacity of uncoated pre-columns to retain liquid, the technique is only suited to the transfer of small fractions and involves the use of long, uncoated pre-columns.

Partially concurrent solvent evaporation allows the transfer of larger fractions working under conditions that still produce a zone flooded by eluant (providing solvent trapping).[10] This is a typical retention gap technique, but part of the solvent is evaporated concurrently during its introduction into the GC. The introduction of an early solvent vapor exit[6] improves partially concurrent solvent evaporation. In theory, the solvent vapor exit could be placed between the uncoated pre-column and the analytical column, but this makes the closure critical for partial losses of peaks eluted very early. Additional retention power after the solvent evaporation is necessary. A section of the analytical column, a "retaining pre-column,"[11] is installed after the uncoated pre-column but before the solvent vapor exit.

Composition of the volatile fraction of citrus essential oils

The volatile fraction of citrus essential oils consists of mixtures of monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives. The analysis of these oils often presupposes fractionation of the sample prior to GC analysis[12] due to the substantial overlap be-

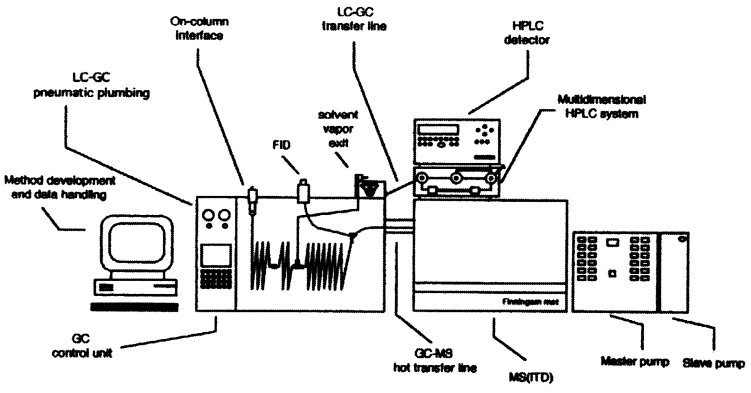


Figure 3 *Schematic of the HPLC-HRGC-MS instrument.*

tween 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.[13–15]

Experimental

The research was carried out on genuine petitgrain and neroli essential oils obtained from F.lli Di Bartolo Derivati Agrumi (Calatabiano, Catania, Italy). A fully automated instrument (Dualchrom 3000 series, Fisons, Rodano, Milan, Italy) was used for on-line preseparation by LC and further separation by capillary GC. The instrument was configured to use an on-column interface, permitting partially concurrent solvent evaporation with an early solvent vapor exit system for the reduction of the mobile-phase evaporation time. A schematic diagram of the HPLC-HRGC-MS interface is shown in *Figure 3*. A 10-port valve permits cleaning of the LC column by backflushing from time to time. Analyses were carried out under computer control throughout, with step gradient elution to separate and transfer the fractions to GC with the following HPLC conditions: 20 µL of solution (0.2% vol/vol essential oil/pentane) was injected into a 10 cm × 2 mm i.d. column packed with Spherisorb 5 µm silica (Stagroma, Tubingen, Germany). The HPLC analyses were performed using eluant pentane (Carlo Erba, Milan, Italy) for 5 min for hydrocarbon elution; the column was then backflushed with 1 mL diethyl ether (Carlo Erba) for elution of oxygenated compound; the flow rate was 180 mL/min; and detection was at 220 nm × 0.50 absorbance units full scale (aufs) using a Micro UVis (Fisons, Rodano, Milan, Italy).

The GC system was composed of the following (*Figure 4*): 10 m × 0.53 mm i.d. fused-silica uncoated pre-column, deactivated by phenyldimethyl silylation (retention gap); retaining pre-column consisting of 4 m of the separation column, connected to the retention gap by a press fit connection (MEGA, Legnano, Italy); separation column, capillary fused-silica coated with SE 52, 0.4–0.45 µm film thickness, 0.32 mm i.d., 25 m length (MEGA); and butt connector with purge line

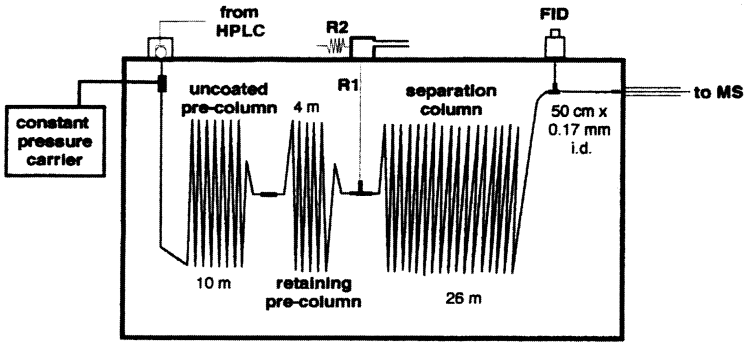


Figure 4 *Column scheme of the HRGC. R2 = 46 cm silica tubing, 0.05 mm i.d. R1 = 35 cm of fused-silica tubing, 0.32 mm i.d.*

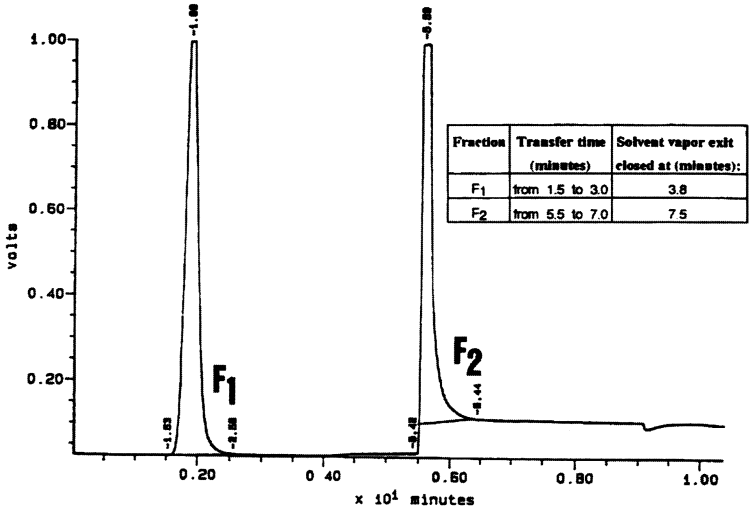


Figure 5 *HPLC chromatogram of bitter orange petitgrain oil.*

fitted with flow-control valve, which automatically switched from high purging flow (23 mL/min) to low purging flow (0.2 mL/min) during analysis. The temperature during transfer of the LC fraction was kept at 45 °C for 6 min and then increased to 220 °C at a rate of 3 °C/min; detection was by flame ionization detection (FID). The carrier gas (helium) was delivered at a constant pressure of 120 KPa. The eluant evaporation rate was 151 µL/min. A flame was held close to the solvent vapor exit. The time from ignition of the gas emerging from the vapor exit to extinction gives an exact measurement of the solvent evaporation time. The vapor exit was switched to low flow shortly after the end of GC transfer time.

All components were identified by retention time of standards and also by means of coupled LC-GC-MS. Mass spectra were obtained on a model 800 ion trap detector (ITD) mass spectrometer (Finnigan MAT, San Jose, CA) directly coupled to the LC-GC system under the following conditions: tuning values for the ITD were 100, 100, 100, 100 using FC_{43} as a tuning standard. Tune sensitivity was 9000. Acquisition parameters were as follows—full scan, scan range: 41–3000 amu; scan time: 1.0 sec; threshold: one count; automatic gain control (AGC) mode: on; micro scans: five; filament delay: 240 sec; multiplier: 1500–2300 V, depending on multiplier condition temperatures; transfer line: 250 °C; exit nozzle: 250 °C; and manifold: 250 °C.

Results and discussion

Figure 5 shows the HPLC 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. *Figure 6* shows the total ion chromatogram (TIC) of the LC fractions. The on-column GC chromatogram obtained with the same column system is shown above the chromatograms of the transferred LC fractions. Compound identification is reported in Table 1.

Bitter orange petitgrain oil is characterized by its high content of oxygenated compounds. The most abundant compounds present in bitter orange oils are esters (46.4% of the oil) and alcohols (36.8%). Monoterpenes (13.8%) are followed by aldehydes (1.1%), and the sesquiterpene fraction 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 oxygenated compound; α-copaene and citronellyl acetate; and δ-elemene and geranyl acetate. HPLC preseparation allowed the separation of all detectable components present in the oil.

Figure 7a shows the on-column GC-FID chromatogram of the neroli essential oil obtained with the same system as that used in the on-line HPLC-HRGC-MS. Figures 7*b* and *c* show the GC-FID chromatograms of the HPLC transferred fractions of hydrocarbons and oxygenated compounds, respectively. Compound identifications are reported in Table 2. The most abundant compounds present in neroli oil are monoterpene

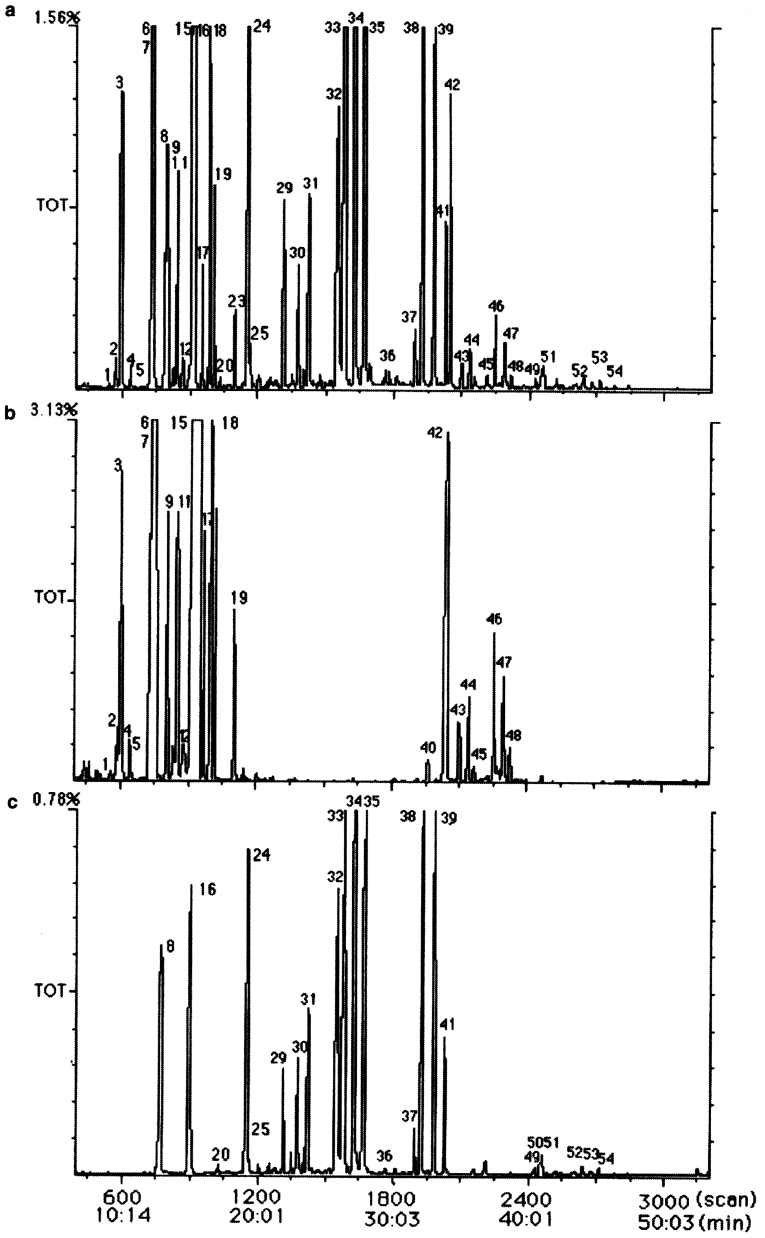


Figure 6 *TIC of a) bitter orange petitgrain oil, b) hydrocarbons, and c) oxygenated compounds from the HPLC separation. Peak identification appears in* Table 1 (TOT = total).

hydrocarbons (59.1% of the oil), followed by monoterpene alcohols (19.7%), esters (14.1%), sesquiterpene hydrocarbons (3.1%), and sesquiterpene alcohols (2.8%).

The on-column chromatogram of the whole essential oil also shows substantial overlap between peaks of monoterpenes and sesquiterpenes and some oxygenated compounds. For example, there is complete overlap between limonene and 1,8-cineole, *trans*-linalool oxide and terpinolene, and δ-elemene and methyl anthranilate, and partial overlap between geranyl acetate and β-elemene. In addition, the HPLC preseparation permits clearer chromatograms to be obtained where all the peaks are well resolved.

For extremely complex matrices, such as neroli and petitgrain essential oils, which contain different classes of components, GC-MS does not provide such clear results in the identification and quantitation of all of the compounds in the mixture; it is not possible to separate all of these compounds with only one analysis no matter which GC column is used; the mass spectra of the components of the same class are very similar, and it is necessary to have an extremely pure spectrum to obtain an unambiguous identification from the library matching. A certain identification may only be obtained using the mass spectrum together with the retention time obtained from the injection of pure standards. This is not easy for the compounds considered here, because only a few of them are available commercially.

To demonstrate the power of the HPLC-HRGC-MS system, the authors selected two representative components of the hydrocarbon fraction (camphene and α-phellandrene) of the neroli essential oil marked 4 and 10 in *Figure 8*, an enlargement of the chromatogram obtained by HPLC-HRGC-MS of the first part of the hydrocarbon fraction. *Figures 9* and *10* show the comparison of the mass spectra of these compounds (camphene and α-phellandrene) by HPLC-HRGC-MS (Figures 9*a* and 10*a*) with a library spectrum (Figures 9*b* and 10*b*) and the mass spectra for the same peaks obtained by GC-MS of the whole oil (Figures 9*c* and 10*c*). The figures show that the spectra obtained after LC preseparation have a much greater purity than those obtained for the same compound by GC-MS of the whole oil; comparison with the library spectrum is certainly more reliable. The program allows a

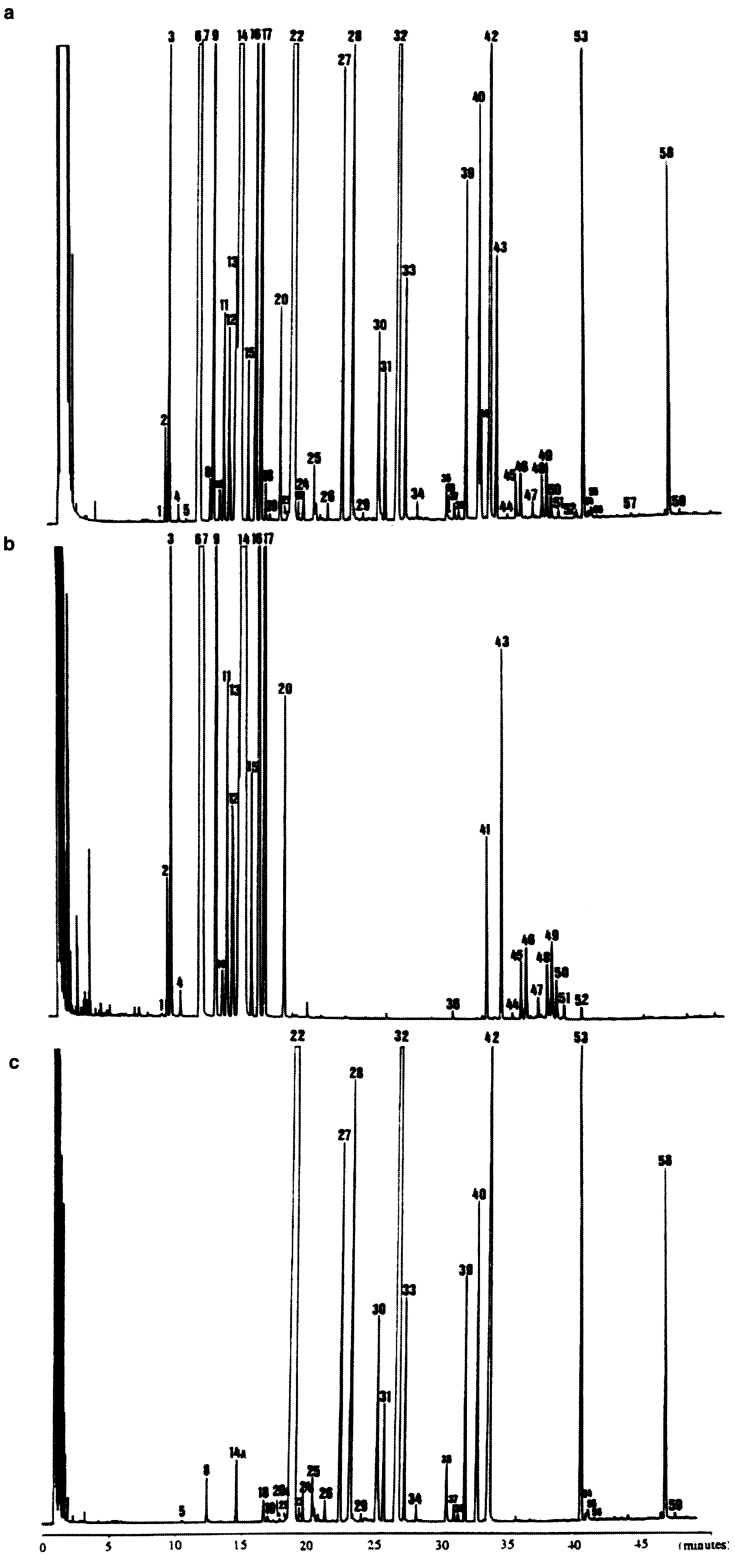


Figure 7 *TIC of a) neroli oil, b) hydrocarbons, and c) oxygenated compounds from the HPLC separation. Peak identification appears in* Table 2.

Figure 8 *Enlargement of part of the chromatogram of the hydrocarbon fraction obtained by HPLC-HRGC-MS.*

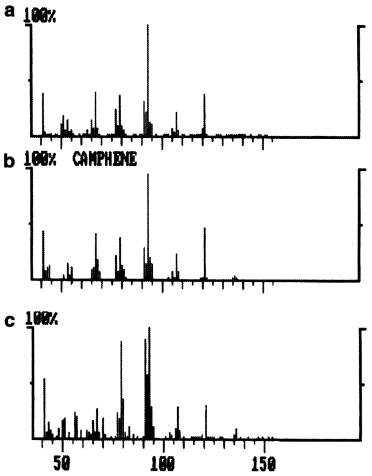


Figure 9 *Comparison of the mass spectra of peak 4 (camphene) a) by HPLC-HRGC-MS, b) with a library spectrum, and c) by GC-MS.*

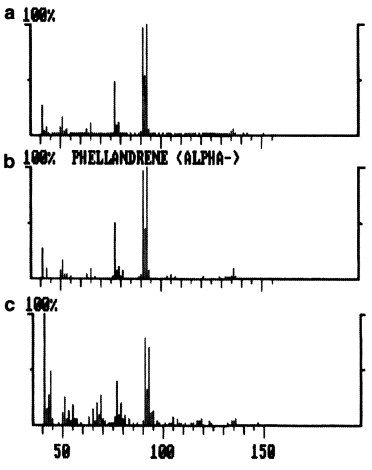


Figure 10 *Comparison of the mass spectra of peak 10 (α-phellandrene) a) by HPLC-HRGC-MS, b) with a library spectrum, and c) by GC-MS.*

search of a library of mass spectra for compounds that resemble the current data spectrum. For any unknown spectrum and a library spec-

Table 1

Components identified by HPLC-HRGC-MS for bitter orange petitgrain oil

No.	Compound	g/100 g oil	No.	Compound	g/100 g oil
1	Tricyclene	tr	25	Nerol	1.28
2	α-Thujene	0.02	26	Neral	0.40
3	α-Pinene	0.17	27	Linalyl acetate	39.75
4	Camphene	tr	28	Geranial	0.67
5	Sabinene	0.45	29	δ-Elemene	0.02
6	β-Pinene	2.20	30	α-Cubebene	0.02
7	6-Methyl-5-hepten-2-one	0.08	31	α-Terpinyl acetate	0.06
8	Myrcene	2.31	32	Citronellyl acetate	0.11
9	α-Phellandrene	0.05	33	α-Copaene	0.01
10	δ-3-Carene	1.15	34	Neryl acetate	2.27
11	α-Terpinene	0.03	35	Geranyl acetate	4.22
12	*p*-Cymene	0.12	36	β-Elemene	0.04
13	Limonene	2.02	37	N-Methyl methyl anthranilate	0.17
14	1,8-Cineole	0.06	38	(E)-Caryophyllene	0.71
15	(Z)-β-Ocimene	0.89	39	*trans*-α-Bergamotene	0.01
16	(E)-β-Ocimene	3.64	40	α-Humulene	0.07
17	γ-Terpinene	0.18	41	(Z)-β-Farnesene	0.07
18	*cis*-Linalool oxide	0.05	42	Bicyclogermacrene	0.28
19	Terpinolene	0.59	43	α-Farnesene	0.05
20	*trans*-Linalool oxide	0.03	44	δ-Cadinene	0.04
21	Linalool	29.80	45	(E)-Nerolidol	0.06
22	Citronellal	0.05	46	Spathulenol	0.03
23	Terpinen-4-ol	0.12	47	Caryophyllene oxide	0.02
24	α-Terpineol	5.39			

Table 2

Components identified by HPLC-HRGC-MS for neroli oil

No.	Compound	g/100 g oil	No.	Compound	g/100 g oil
1	Tricyclene	0.01	31	Neral	0.41
2	α-Thujene	0.25	32	Linalyl acetate	9.76
3	α-Pinene	1.31	33	Geranial	0.65
4	Camphene	0.04	34	Indole	0.06
5	Benzaldehyde	0.01	35	Methyl anthranilate	0.11
6,7	Sabinene+β-Pinene	20.22	36	δ-Elemene	–
8	6-Methyl-5-hepten-2-one	0.11	37	α-Terpenyl acetate	0.05
9	Myrcene	2.33	38	Citronellyl acetate	0.03
10	α-Phellandrene	0.09	39	Neryl acetate	0.92
11	δ-3-Carene	0.52	40	Geranyl acetate	–
12	α-Terpinene	0.51	41	β-Elemene	1.63
13	*p*-Cymene	1.04	42	N-Methyl methyl anthranilate	3.18
14	Limonene	24.57	43	(E)-Caryophyllene	0.72
14A	1,8-Cineole		44	*trans*-α-Bergamotene	0.02
15	(Z)-β-Ocimene	0.34	45	α-Humulene	0.10
16	(E)-β-Ocimene	3.60	46	(Z)-β-Farnesene	0.14
17	γ-Terpinene	3.71	47	Germacrene D	0.05
18	*trans*-Sabinene hydrate	0.09	48	Germacrene B	0.13
19	*cis*-Linalool oxide	0.02	49	Unknown sesquiterpene	0.16
20	Terpinolene	0.53	50	α-Farnesene	0.07
20A	*trans*-Linalool oxide	–	51	δ-Cadinene	0.03
21	*cis*-Sabinene hydrate	0.03	52	*cis*-Nerolidol	tr
22	Linalool	15.59	53	*trans*-Nerolidol	1.76
23	Phenyl ethyl alcohol	0.01	54	Spathulenol	0.02
24	*cis-p*-Menth-2-en-1-ol	0.09	55	Caryophyllene oxide	0.04
25	*trans-p*-Menth-2-en-1-ol	0.19	56	Globulol	0.01
26	Citronellal	0.06	57	α-Cadinol	0.02
27	Terpinen-4-ol	1.20	58	*cis,trans*-Farnesol	0.98
28	α-Terpineol	1.79	59	*trans,trans*-Farnesol	0.01
29	*trans*-Piperitol	0.03			
30	Nerol	0.69			

Table 3

Results obtained from the library search for peak 4 (camphene)

GC-MS	Purity	Fit	Rfit
1 δ-3-Carene	643	878	700
2 α-Fenchene	634	873	699
3 Camphene	629	842	681
4 2-Carene	629	837	710
5 α-Pinene	626	825	684
6 β-Phellandrene	622	850	661
7 (E)-β-Ocimene	622	869	683
8 Sabinene	617	855	652
9 Santolina triene	617	853	686
10 β-Pinene	616	806	699
HPLC-HRGC-MS			
	Purity	**Fit**	**Rfit**
1 Camphene	849	931	876
2 Santolina triene	780	870	808

Table 4

Results obtained from the library search for peak 10 (α-Phellandrene)

GC-MS	Purity	Fit	Rfit
1 β–Phellandrene	836	944	858
2 Sabinene	803	939	824
3 α-Pinene	799	899	830
4 (Z)-β-Ocimene	774	923	803
5 Limonene	771	884	779
6 (E)-β-Ocimene	761	908	792
7 δ-3-Carene	758	908	792
8 β-Pinene	753	856	790
9 Tricyclene	752	935	784
10 Myrcene	752	864	758
HPLC-HRGC-MS			
	Purity	**Fit**	**Rfit**
1 α-Phellandrene	883	977	892
2 α-Thujene	761	853	770

trum, the algorithm provides purity, fit, and reverse fit, each with a value of between 0 and 1000. A purity search measures the resemblance of the currently selected data to the specified library entry. A fit search measures the degree to which the library spectrum is included in the unknown spectrum. The reverse fit (Rfit) search measures the degree to which the unknown spectrum is included in the library spectrum. A high fit with a lower purity suggests that the unknown spectrum is included in the library spectrum. A high fit with a lower purity suggests that the unknown spectrum is a mixture that includes the compounds selected from the library or that the two compounds have some major substructure in common. *Tables 3* and *4* list the first 10 compounds in the library that best compare with the

two peaks in the GC-MS chromatogram of the whole oil (see Figures 8 and 9). For camphene, the library shows that the 10 most similar compounds are all monoterpenes and gives camphene only as the third choice. Moreover, all 10 compounds have a high fit with lower degree purity. The library does not find α-phellandrene in the first 10 most similar spectra, but only an isomer. HPLC-HRGC-MS with LC prefractionation of the oil allows the library to find both the components as first choices, with a much higher degree of purity (camphene—purity: 849, fit: 931, Rfit: 876; α-phellandrene—purity: 883, fit: 977, Rfit: 892). Additionally, this library reports the retention time of compounds on a DB-5 column. Since the column used in this work is equivalent to a DB-5 column, it follows that, by using data obtained from the mass spectra and the retention time, a more certain identification of the components of the oil was achieved.

Conclusion

The preseparation of hydrocarbons and oxygenated compounds for these two oils allowed interpretable mass spectra to be obtained 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 ($CH_{10}H_{16}$) and the same molecular weight (MW = 136).

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