# On-Line HPLC-HRGC in the Analytical Chemistry of Citrus Essential Oils

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The analysis of very complex matrices can often prove to be laborious, since more than one chromatographic step is required due to the complexity of the sample. The best approach is to fractionate the sample prior to gas chromatographic analysis. The simpler mixtures thus obtained, which may be homogeneous, are easier to resolve without problems of peak overlap.

Off-line methods (such as vacuum distillation, preparative gas chromatography, solvent extraction and classical column liquid chromatography) are laborious, very slow 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-gas chromatography (LC-GC) offers some advantages: the amount of sample required is less, no sample work-up is needed, and very complex sample pre-treatment is possible in a fully automated way. In on-line HPLC-HRGC, the sample is first separated by HPLC (high performance liquid chromatography) 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 (high resolution gas chromatography).

The two principal techniques of eluent evaporation which 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 some 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 technique due to its simplicity, and the possibility of transferring very large LC fractions.[1]

The *retention gap* method represents the best approach in the case of qualitative and quantitative analysis of samples containing highly volatile compounds. In fact, retention gap allows 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*).[6] On the other hand, this method is restricted to fractions of only modest volumes, and the use of a long uncoated precolumn.

Working under conditions which still produce a zone flooded by the eluent (providing solvent trapping), but which cause a large amount of eluent to evaporate during its introduction, we are able to work with a shorter uncoated precolumn or to transfer larger fraction volumes. This method is the so-called *partially concurrent evaporation*: part of the eluent is evaporated concurrently, that is, during its introduction into GC. The introduction of an early-vapor exit greatly improves partially concurrent evaporation and protects the GC detector.

The volatile fraction of citrus essential oils is a mixture of monoterpene and sesquiterpene hydrocarbons and the oils' oxygenated derivatives. The analysis of these oils often

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presupposes fractionation of the samples prior to GC analysis[7] due to substantial overlap between peaks. Mass spectra of the components of the same class (monoterpenes or sesquiterpenes) are often very similar, and it is necessary to have a spectrum of an extremely pure compound to obtain an unambiguous identification from library matching. The combination of the HPLC-HRGC system with mass spectrometry (MS) allows components to be reliably identified.[8-10]

In this paper we describe some applications of on-line HPLC-HRGC and HPLC-HRGC-MS in the analysis of citrus essential oils. In particular, we used HPLC-HRGC to determine the enantiomeric ratio of monoterpene alcohols in lemon, mandarin, bitter orange and sweet orange. We used HPLC-HRGC-MS to determine the following: the aldehydes of sweet orange oil; the composition of the mono- and sesquiterpene hydrocarbon fraction of bergamot, lemon, mandarin, sweet orange, bitter orange, clementine, grapefruit and lime essential oils; and the composition of the oils of bergamot, neroli and bitter orange, sweet orange, mandarin and lemon petitgrain oils.

## General Experimental Conditions

A fully automated HPLC-HRGC instrument (Dualchrom 3000 Series, Fisons) was used for on-line pre-separation by HPLC and further separation by capillary GC. A solution of essential oil in pentane (0.2-1% v/v, 20 µL) was injected into a 10 cm or 25 cm x 2 mm i.d. LC column packed with 5 µm Spherisorb (Stagroma). Detection was by micro UVIS (spectrometric ultraviolet visible detector with a micro cell) at 220 nm. The retention window was determined using standard substances. A schematic diagram of the LC-GC-MS instrument is shown in Figure 1. The instrument was configured to use an on-column interface, permitting partially concurrent solvent evaporation, with an early solvent vapor exit system. Analyses were carried out under computer control. The GC system is shown in Figure 2.

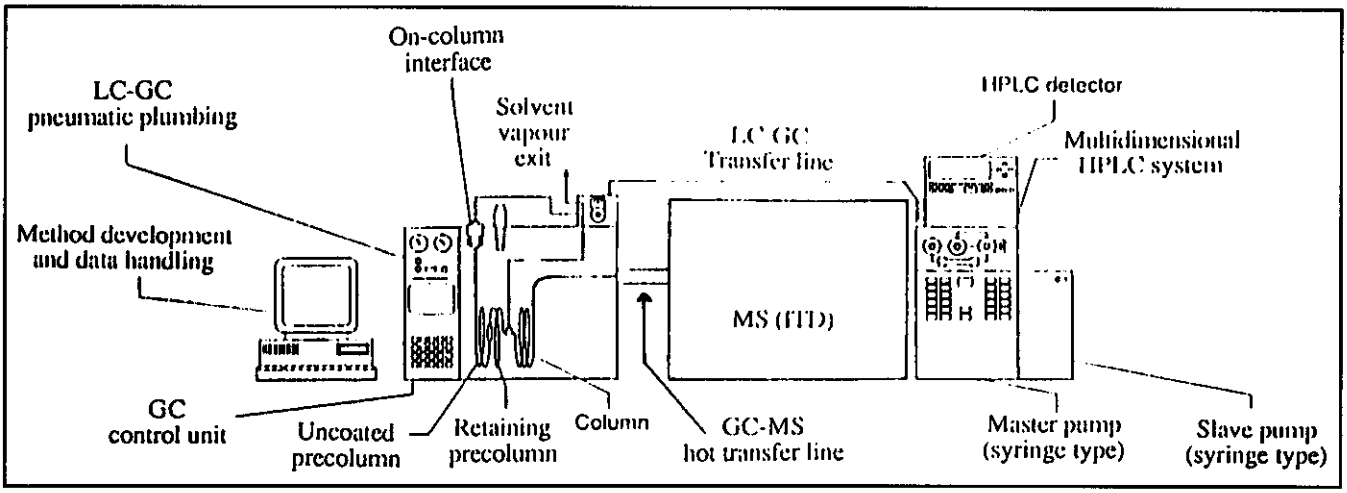


**Figure 1. Schematic of the HPLC-HRGC-MS Instrument**

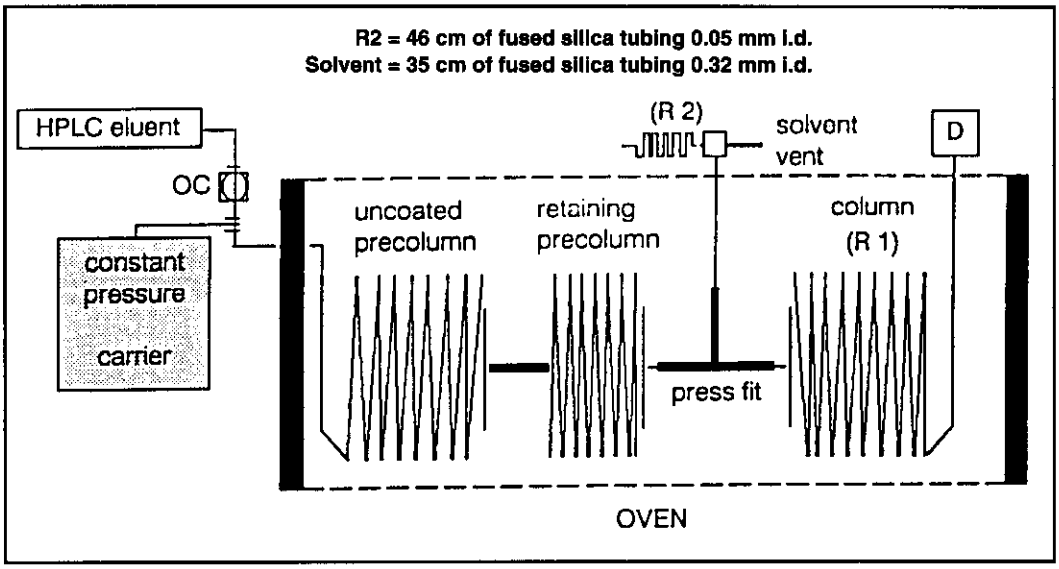


**Figure 2. Schematic of the HPLC-HRGC-MS Interface**

The column inlet was connected, by means of a butt connector with purge line, to a retaining precolumn[11] (4 m of the separation column); the precolumn was connected, by means of a press-fit connector (MEGA, Legnano, Italy), to a 10 m x 0.53 mm i.d. uncoated fused silica precolumn (retention gap). The purge line of the butt connector, between the separation column and the retaining precolumn, was fitted with a flow-control valve that automatically switched from high purging flow to low purging flow during analysis (Figure 1). The GC column oven tempera-

**Table I. HPLC conditions**

| | Time interval (min) | Eluents | Flow (µL/min) | Fraction | Transfer time (min) | Solvent vapor exit closed at (min) |
|---|---|---|---|---|---|---|
| aldehydes of sweet orange oil | 0-2 | pentane | 180 | | | |
| | 3-12 | pentane:diethyl ether, 97:3 | | $F_1$ | 9.5-10.5 | 10.9 |
| | 13-17 | pentane:diethyl ether, 90:10 | | $F_2$ | 13.0-14.3 | 15.0 |
| | 18-23 | pentane:diethyl ether, 85:15 | | $F_3$ | 16.5-17.8 | 18.5 |
| | back-flush with diethyl ether (1 ml) | | | | | |
| monterpene and sesquiterpene hydrocarbons of citrus oil | 0-5 | pentane | 180 | | 1.6-3.6 | |
| | back-flush with diethyl ether (1 ml) | | | | | |
| bergamot oil | 0-3 | penane | 180 | $F_1$ | 1.0-3.0 | 3.8 |
| | 4-13 | pentane:diethyl ether, 97:3 | | $F_2$ | 12.0-15.0 | 16.5 |
| | 14-19 | pentane:diethyl ether, 90:10 | | $F_3$ | 17.0-19.0 | 19.9 |
| | 20-30 | pentane:diethyl ether, 80:20 | | $F_4$ | 21.0-23.5 | 24.9 |
| | back-flush with diethyl ether (1 ml) | | | | | |
| neroli oil | 0-5 | pentane | 180 | $F_1$ | 1.5-3.5 | 4.0 |
| | back-flush with diethyl ether (1 ml) | | | $F_2$ | 7.5-9.5 | 10.1 |
| enantiomeric distribution of linalool and terpinen-4-ol* | 0-3 | pentane | 220 | $F_1$ | 17.0-19.0 | |
| | 4-15 | pentane:tert-butylmethyl ether, 95:5 | | | | |
| | 16-30 | pentane:tert-butylmethyl ether, 90:10 | | | | |
| | 31-50 | pentane:tert-butylmethyl ether, 70:30 | | | | |
| petitgrain oils | 0-5 | pentane | 180 | $F_1$ | 1.5-3.0 | |
| | back-flush with diethyl ether (1 ml) | | | $F_2$ | 5.5-7.0 | |

* LC column 25 cm x 2 mm i.d., Spherisorb, 5 !mm

**Table II. GC conditions**

| | Separation column | Carrier | GC | Eluent evaporation rate (µL/min) |
|---|---|---|---|---|
| aldehydes of sweet orange oil | 25 m x 0.32 mm i.d. fused silica SE-52, 0.4-0.45 µm | He, 120 kPa | 45°C (6 min) to 220°C at 3°C/min | 151 |
| monoterpene and sesquiterpene hydrocarbons of citrus oil | 21 m x 0.25 mm i.d. fused silica DB-5, 0.25 µm | He, 140 kPa 32 cm/s | 45°C (6 min) to 240°C at 3°C/min | 138 |
| bergamot oil | 21 m x 0.32 mm i.d. fused silica SE-52, 0.4-0.45 µm | He, 120 kPa | 45°C (6 min) to 220°C at 3°C/min | 145 |
| neroli oil | 26 m x 0.32 mm i.d. fused silica SE-52, 0.4-0.45 µm | He, 120 kPa 33 cm/s | 45°C (6 min) to 250°C at 3°C/min | 150 (pentane) 139 (diethyl ether) |
| enantiomeric distribution of linalool and terpinen-4-ol | 25 m x 0.32 mm i.d. fused silica 2,3-dimethyl-6-pentyl-β-cyclodextrin 30% and OV-1701 70%, 0.25 µm | He, 120 kPa | 45°C (3 min) to 150°C at 1°C/min | 155 |

ture was maintained at 45°C for 6 minutes during transfer of the LC fraction, and then increased. The rate of evaporation of the eluent was determined as described elsewhere.[12,13] The solvent evaporation time was determined by igniting the gas as soon as it began to emerge from the solvent vapor exit and then measuring elapsed time from ignition until the flame went out. The vapor exit was switched to low flow shortly after the end of GC transfer time.

All components were identified by comparing GC retention times with those of standards, and also by means of coupled LC-GC-MS. Mass spectra were obtained with a Finnigan Ion Trap (ITD) mass spectrometer, model 800, directly coupled to the LC-GC system described above. Tuning values for the ITD were 100, 100, 100, 100 using $FC_{43}$ as a tuning standard; tune sensitivity was 9,000. Full scans were acquired between 41 and 3,000 amu; the scan time was 1.0 second and the threshold was 1 count. AGC mode was on; micro scans, 5; and filament delay, 240 seconds. The multiplier voltage was from 1,500 to 2,300 V, depending on multiplier condition. Transfer line, exit nozzle and manifold temperatures were 250°C.

For each determination, the particular HPLC and GC experimental conditions used are shown in Tables I and II, respectively.

## Aldehydes of Sweet Orange Oil[14]

In sweet orange oil the aliphatic aldehyde content may furnish useful information concerning the origin of the oil and the ripeness of the fruit at harvest time.[15-23] Identification and quantitative calculations of aldehydes in sweet orange oils by gas chromatographic analysis of the whole oil is very difficult because of the possible overlapping of peaks. For example, overlaps of some aldehydes with some esters may occur using the most common stationary phases, such as SE-52, SE-54, DB-5. These interferences may be eliminated if the mixture is subjected to a preliminary

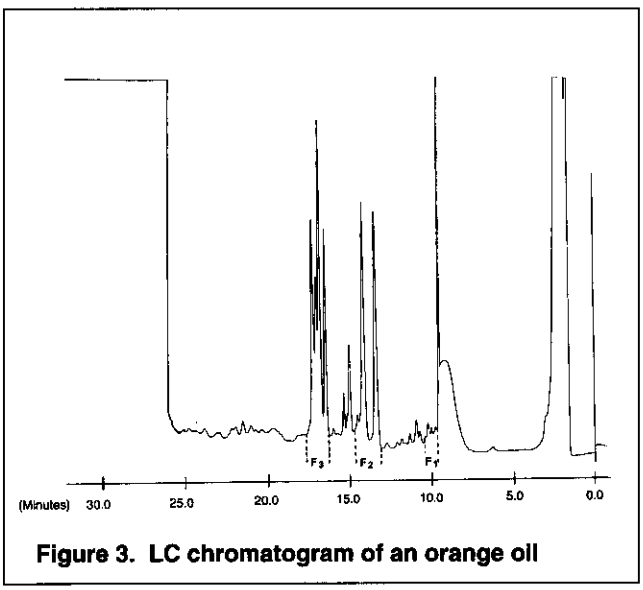


**Figure 3. LC chromatogram of an orange oil**

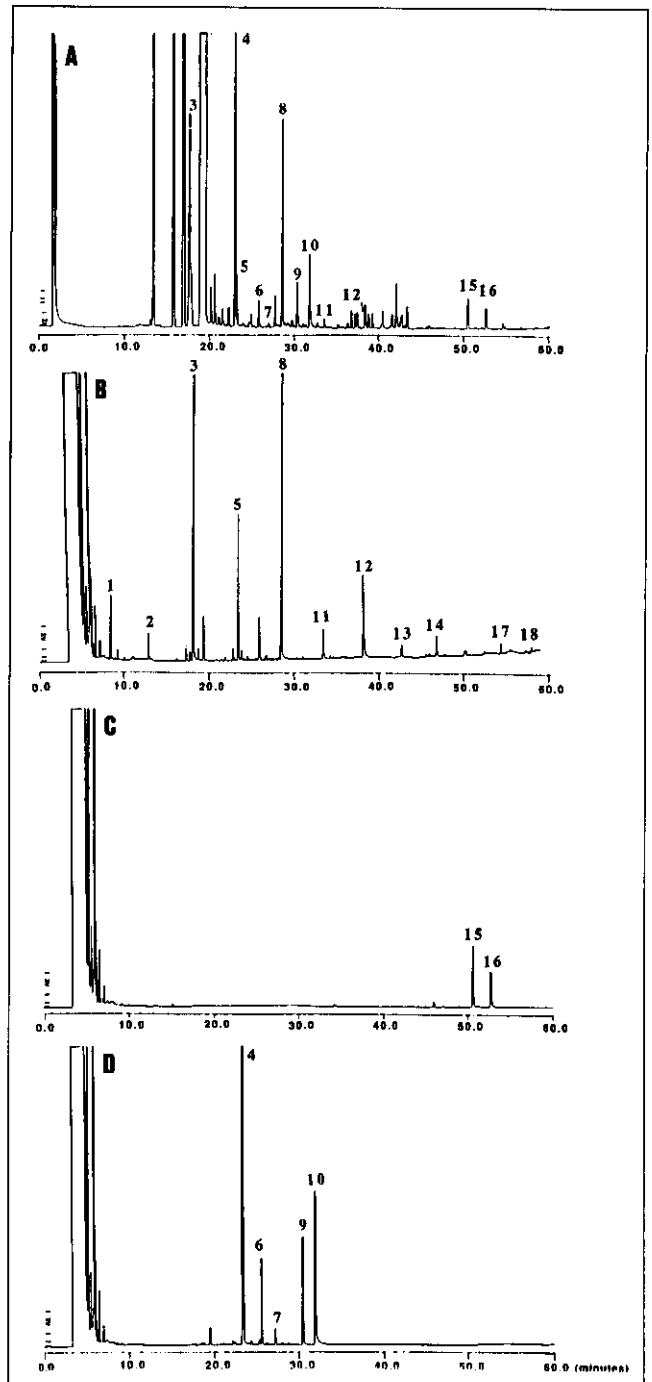


**Figure 4. Chromatogram of an orange essential oil and of the fractions from its LC separation**

A) whole essential oil (on-column injection)
B) aliphatic aldehydes (fraction $F_1$)
C) sesquiterpene aldehydes (fraction $F_2$)
D) monoterpene aldehydes (fraction $F_3$)

| | | | |
|---|---|---|---|
| 1 | hexanal | 10 | geranial |
| 2 | heptanal | 11 | undecanal |
| 3 | octanal | 12 | dodecanal |
| 4 | linalool | 13 | tridecanal |
| 5 | nonanal | 14 | tetradecanal |
| 6 | citronellal | 15 | β-sinensal |
| 7 | terpinen-4-ol | 16 | α-sinensal |
| 8 | decanal | 17 | hexadecanal |
| 9 | neral | 18 | heptadecanal |

separation before the gas chromatographic analysis.

The HPLC pre-separation of sweet orange oil under the experimental conditions given above produces a chromatogram in which the aliphatic, monoterpene and sesquiterpene aldehydes are separated into three fractions, marked $F_1$, $F_2$ and $F_3$ in Figure 3. The GC chromatograms of these three fractions are shown in Figures 4B, 4C and 4D; these chromatograms are much simpler than that of the unfractionated oil (Figure 4A). Fractions $F_1$ and $F_2$ contain only aliphatic and sesquiterpene aldehydes, respectively. Fraction $F_3$, however, contains principally the monoterpene aldehydes, but also the alcohols linalool and terpinen-4-ol. This is because in HPLC these two alcohols are eluted after neral and geranial but before citronellal, so that the fraction passed from HPLC to GC must contain these alcohols unless the cut is made earlier and the citronellal is lost. It is apparent from Figures 4B-4D that the aldehydes are all clearly identifiable. This presents the possibility of developing a method for quantitative analysis free from interferences, even for components present in trace amounts, such as hexanal, heptanal, hexadecanal and heptadecanal.

## Terpene Hydrocarbons[24]

The hydrocarbon fraction does not have a fundamental role in determining the olfactory character of the essential oils, but it has an important characterizing function, and some of the components of this fraction can provide useful information about the genuineness and quality of the oils.

For example, δ-3-carene is a characteristic monoterpene of sweet orange essential oil; its presence in lemon, mandarin and bitter orange oils, in amounts above trace level, is a sign of adulteration;[25-28] the qualitative and quantitative composition of the sesquiterpene fraction is very different for various oils[26,29-31] and can be used to differentiate between them and to detect addition or contamination of different oils.

While the composition of the monoterpene fraction is well known, the composition of the sesquiterpene fraction is subject to many contradictions.[24]

The complexity of the mixtures and the resemblance of many of the sesquiterpene structures make it very difficult to identify the components of these mixtures, even with GC-MS.

The mono- and sesquiterpene fraction of cold-pressed bergamot, lemon, sweet orange, bitter orange, mandarin, clementine, grapefruit and Mexican lime have been analyzed using HPLC-HRGC-MS. All components were identified by retention times of standards, when available, and also by means of their MS spectra.

***Monoterpene hydrocarbons:*** Table III reports the relative percentage composition of the monoterpene fraction for each essential oil.

The following monoterpene hydrocarbons were found in all the oils analyzed: α-thujene, α-pinene, camphene, sabinene, β-pinene, myrcene, α-phellandrene, δ-3-carene (except for grapefruit oil), α-terpinene, p-cymene, limonene, (Z)-β-ocimene, (E)-β-ocimene, γ-terpinene and terpinolene. Moreover, tricyclene was found in the bergamot, lemon and Mexican lime oils. Allo-ocimene, previously reported in bergamot oil,[32-33] was not found in any of the oils examined. Limonene is the most abundant component of this fraction for all the oils examined; its content varies

**Table III. Percentage composition of monoterpene hydrocarbon fractions of the citrus oils analyzed**

| | | Bergamot | Lemon | Mandarin | Sweet orange | Bitter orange | Grapefruit | Clementine | Mexican lime |
|---|---|---|---|---|---|---|---|---|---|
| 1 | tricyclene | 0.01 | 0.01 | - | - | - | - | - | 0.01 |
| 2 | α-thujene | 0.56 | 0.43 | 0.68 | 0.01 | 0.01 | 0.01 | 0.03 | 0.41 |
| 3 | α-pinene | 2.24 | 1.90 | 1.93 | 0.50 | 0.51 | 0.48 | 0.53 | 2.57 |
| 4 | camphene | 0.10 | 0.06 | 0.02 | t | 0.01 | t | t | 0.12 |
| 5 | sabinene | 1.95 | 1.99 | 0.23 | 0.67 | 0.23 | 0.27 | 0.50 | 3.43 |
| 6 | β-pinene | 11.93 | 12.40 | 1.40 | 0.04 | 0.75 | 0.02 | 0.08 | 22.85 |
| 7 | myrcene | 1.64 | 1.51 | 1.77 | 1.93 | 1.81 | 1.87 | 1.90 | 1.47 |
| 8 | α-phellandrene | 0.05 | 0.04 | 0.07 | 0.04 | 0.02 | 0.02 | 0.05 | 0.06 |
| 9 | δ-3-carene | t | t | t | 0.10 | t | - | 0.04 | 0.01 |
| 10 | α-terpinene | 0.12 | 0.21 | 0.38 | t | t | 0.01 | 0.03 | 0.22 |
| 11 | p-cymene | 0.68 | 0.27 | 0.35 | - | t | t | t | 0.21 |
| 12 | limonene | 65.71 | 69.93 | 74.53 | 96.63 | 96.31 | 97.21 | 96.03 | 57.80 |
| 13 | (Z)-β-ocimene | 0.03 | 0.10 | 0.01 | 0.01 | 0.02 | 0.01 | 0.01 | 0.17 |
| 14 | (E)-β-ocimene | 0.42 | 0.16 | 0.02 | 0.03 | 0.28 | 0.08 | 0.05 | 0.46 |
| 15 | γ-terpinene | 14.13 | 10.56 | 17.80 | 0.02 | 0.04 | 0.01 | 0.67 | 9.61 |
| 16 | terpinolene | 0.43 | 0.43 | 0.81 | 0.02 | 0.01 | 0.01 | 0.08 | 0.50 |

t = trace (<0.01%)

**Table IV. Percentage composition of the sequiterpene hydrocarbon fractions of the citrus oils analyzed**

| | Compounds | Bergamot | Lemon | Mandarin | Sweet orange | Bitter orange | Grapefruit | Clementine | Mexican lime |
|---|---|---|---|---|---|---|---|---|---|
| 1 | δ-elemene | - | - | - | - | - | - | - | 4.4 |
| 2 | α-cubebene | - | - | - | - | - | - | 0.3 | - |
| 3 | unknown sesquiterpene | - | - | - | - | - | - | 0.5 | - |
| 4 | α-copaene | - | - | 3.7 | 8.5 | - | 13.4 | 12.9 | - |
| 5 | β-cubebene | - | - | 2.5* | 7.0 | - | 13.3 | 12.6 | - |
| 6 | β-elemene | - | - | - | 0.7 | 1.0 | - | - | 2.3 |
| 7 | unknown sesquiterpene | - | - | - | - | - | - | 0.6 | - |
| 8 | unknown sesquiterpene | - | - | - | - | - | - | 0.7 | - |
| 9 | (Z)-cis-α-bergamotene | 2.5 | 2.7 | - | - | - | - | - | 3.0 |
| 10 | β-caryophyllene | 24.0 | 13.8 | 32.2 | 6.8 | 27.8 | 38.2 | 4.0 | 11.8 |
| 11 | unknown sesquiterpene | - | - | - | 7.5 | - | - | 7.4 | t |
| 12 | (Z)-trans-α-bergamotene | 21.9 | 24.5 | 0.3* | - | - | - | - | 16.5 |
| 13 | unknown sesquiterpene | - | - | - | - | - | - | - | 0.2[c] |
| 14 | (E)-epi-β-santalene | - | - | - | - | - | - | - | 0.1* |
| 15 | unknown sesquiterpene | - | - | - | 0.5 | - | - | - | - |
| 16 | α-humulene | 2.0 | 1.2 | 3.1 | 1.5 | 2.7 | 5.2 | 2.2 | 1.6 |
| 17 | cis-β-farnesene | 4.1* | 1.6 | - | 9.0 | 3.7 | 1.4 | 9.5* | 1.7 |
| 18 | (Z)-β-santalene | 1.2 | 1.3 | - | - | - | - | - | 0.9 |
| 19 | γ-muurolene | - | - | - | 0.8 | - | - | 1.1[a] | - |
| 20 | γ-curcumene | - | 0.7* | - | - | - | - | - | - |
| 21 | germacrene D | 4.9 | - | 2.8 | 6.3 | 51.8 | 9.8* | 9.3 | 4.0* |
| 22 | ar-curcumene | - | - | - | 0.5[a] | - | - | - | - |
| 23 | unknown sesquiterpene | 1.5 | 2.1 | - | - | - | - | - | 1.1 |
| 24 | unknown sesquiterpene | - | - | - | - | - | - | - | t |
| 25 | valencene | - | 1.7[b] | - | 26.5 | - | - | 1.0 | - |
| 26 | α-selinene | - | - | 10.6 | - | - | - | - | 1.0* |
| 27 | unknown sesquiterpene | 0.7 | 8.6 | - | 1.5 | 3.2 | 3.2 | 3.4 | - |
| 28 | α-muurolene | - | - | - | 1.0 | 0.3[a] | 1.2[a] | 2.1 | - |
| 29 | unknown sesquiterpene | - | - | - | - | - | - | - | 1.7 |
| 30 | unknown sesquiterpene | 2.8 | 2.7 | - | 4.3 | 1.8 | - | 1.9 | 2.1 |
| 31 | α-farnesene | - | - | 40.9 | 5.4 | 0.9* | 1.3[a] | 14.6 | - |
| 32 | β-bisabolene | 32.7 | 37.1 | - | - | - | - | - | 38.5 |
| 33 | γ-cadinene | - | - | - | 1.3 | - | - | - | - |
| 34 | cis-γ-bisabolene | 0.1[a]* | 0.5[a]* | - | - | - | - | - | 0.3* |
| 35 | unknown sesquiterpene | 0.2 | - | - | - | 1.8 | - | - | 0.6 |
| 36 | δ-cadinene | - | - | 3.9 | 10.9 | 0.9[a] | 13.0 | 15.9 | - |
| 37 | trans-β-farnesene | 0.1[a] | 0.4[a] | - | - | - | - | - | - |
| 38 | β-sesquiphellandrene | 0.1[a] | - | - | - | - | - | - | - |
| 39 | trans-γ-bisabolene | - | 0.4[a] | - | - | - | - | - | 0.2 |
| 40 | unknown sesquiterpene | 0.7 | 0.7 | - | - | - | - | - | 0.6 |
| 41 | unknown sesquiterpene | 0.5* | - | - | - | 4.1 | - | - | 7.4 |

[a] tentative
[b] tentative, probably eluted with another unknown sesquiterpene
[c] a farnesene
t = trace (<0.1%)
* identified for the first time

from 58% in the lime oil to about 97% in the grapefruit oil. δ-3-Carene is absent from the grapefruit oil, and is present in greater than trace amounts only in the lime, clementine and sweet orange oils. Myrcene is present in almost the same percentage in all the oils, and is the only monoterpene, apart from limonene, present at percentages greater than 1% in the sweet orange, bitter orange, grapefruit and clementine oils. In the other oils, γ-terpinene, and α- and β-pinene are present in considerable amounts. Sweet orange oil is the only one of the oils considered that does not contain p-cymene. The compositions of the monoterpene fraction of lemon and bergamot oils are very similar. Lime oil differs from these two oils especially in the ratio of β-pinene to γ-terpinene. In these last three oils the ratio of the two isomers of ocimene is different. The compositions of the monoterpene fraction of sweet orange, bitter orange, grapefruit and clementine are very similar; the sweet orange and clementine oils, however, are characterized by the presence of δ-3-carene, which is absent or only present as a trace component in the other two oils. Bitter orange oil differs from the other three oils in that it contains a higher percentage of β-pinene. The monoterpene fraction of mandarin oil has some resemblance to that of lemon and bergamot oils, for example in the limonene and γ-terpinene contents, but differs in that the β-pinene content is ten times less. The monoterpene fraction of the mandarin oil contained the highest relative percentage of terpinolene among all the oils considered.

In summary, it is possible to conclude that, even if the qualitative composition of the monoterpene fraction of the citrus essential oils does not show many differences, the quantitative composition allows a clear differentiation of the oils.

***Sesquiterpene hydrocarbons:*** Table IV reports the relative percentage composition of the sesquiterpene fractions for each essential oil. Figure 5 shows the sesquiterpene region of the GC chromatogram, obtained from the LC transfer of the corresponding fraction of the different essential oils. In Table IV, the sesquiterpenes identified for the first time in the various oils are indicated with an asterisk. Table V reports, for each oil, sesquiterpenes previously reported as present in the oil, but not found in this study.

β-Caryophyllene and α-humulene are present in all the oils analyzed. Some sesquiterpenes were found in only one essential oil (Table VI). cis-β-Farnesene is absent from only mandarin oil, and germacrene D from only lemon oil. α- and β-Ylangene were found in none of the oils. It is possible that their reported presence in some citrus oils, as noted in the literature, was based on the erroneous identifications,[34-36] later corrected,[37] made by Hunter and Brogden. Bergamot and lemon oils are both characterized by large amounts of β-bisabolene, trans-α-bergamotene and β-caryophyllene; mandarin oil by a high concentration of α-farnesene and α-selinene; sweet orange oil by a large percentage of valencene; and bitter orange oil by germacrene D, which represents more than 50% of the whole sesquiterpene fraction. The sesquiterpene fraction of grapefruit oil contains the highest relative percentage of α-humulene, and is similar to sweet orange and clementine oils in its α-copaene, β-cubebene, germacrene D and δ-cadinene contents; however, it differs from these two oils in the lower content of cis-β-farnesene, and especially in the absence of valencene. Mexican lime is the only oil that shows the simultaneous presence of β- and δ-elemene. In total, in the eight essential oils studied, 28 sesquiterpenes were identified, and the presence of another 13 unknown sesquiterepnes was confirmed. Sometimes the composition of the

**Table V. Sesquiterpenes previously reported in citrus oils but not found in this study**

| Bergamot | Mandarin | Sweet orange | Bitter orange |
|---|---|---|---|
| acoradiene | β-bisabolene | allo-aromadendrene | trans-α-bergamotene |
| α-bisabolene | γ-cadinene | cis-α-bergamotene | α-bisabolene |
| β-bourbonene | β-copaene | α-cadinene | β-bisabolene |
| δ-cadinene | β-elemene | β-copaene | α-copaene |
| ar-curcumene | γ-elemene | α-cubebene | β-copaene |
| δ-elemene | δ-elemene | β-humulene | δ-elemene |
| (E,E)-α-farnesene | β-humulene | longifolene | (E)-β-farnesene |
| β-humulene | longifolene | nootkatene | β-humulene |
| α-muurolene | β-selinene | γ-selinene | γ-muurolene |
| α-santalene | γ-selinene | α-ylangene | β-santalene |
| (E)-epi-β-santalene | β-sesquiphellandrene | β-ylangene | selinene |
| α-selinene | α-ylangene | | α-ylangene |
| β-selinene | β-ylangene | | β-ylangene |
| | valencene | | valencene |

| Lemon | Grapefruit | Clementine | Mexican lime |
|---|---|---|---|
| acoradiene | trans-α-bergamotene | δ-cadinene | α-bisabolene |
| α-copaene | β-bisabolene | β-elemene | β-copaene |
| α-cubebene | γ-cadinene | β-gurjunene | α-elemene |
| β-elemene | β-copaene | | α-farnesene |
| β-humulene | α-cubebene | | β-humulene |
| α-selinene | β-elemene | | β-sesquiphellandrene |
| | β-humulene | | |
| | α-ylangene | | |
| | valencene | | |

**Table VI. Sesquiterpenes found in only one essential oil**

| | |
|---|---|
| β-sesquiphellandrene* | bergamot oil |
| γ-curcumene | lemon oil |
| ar-curcumene,* γ-cadinene | sweet orange oil |
| α-cubebene | clementine oil |
| δ-elemene, (E)-epi-β-santalene | lime oil |

* tentative identification

sesquiterpene fraction of these oils (Table IV) does not agree with literature data, but the methods used here are considered effective and reliable.

Our procedure has the advantage of affording mass spectra of the components of a fraction containing no oxygenated compounds, eliminating many interferences that can occur when the whole essential oil is analyzed. The method operates, apart from the initial dilution of the sample, without any sample treatment which could allow losses, contamination or formation of artifacts.

The application of the same method to all the essential

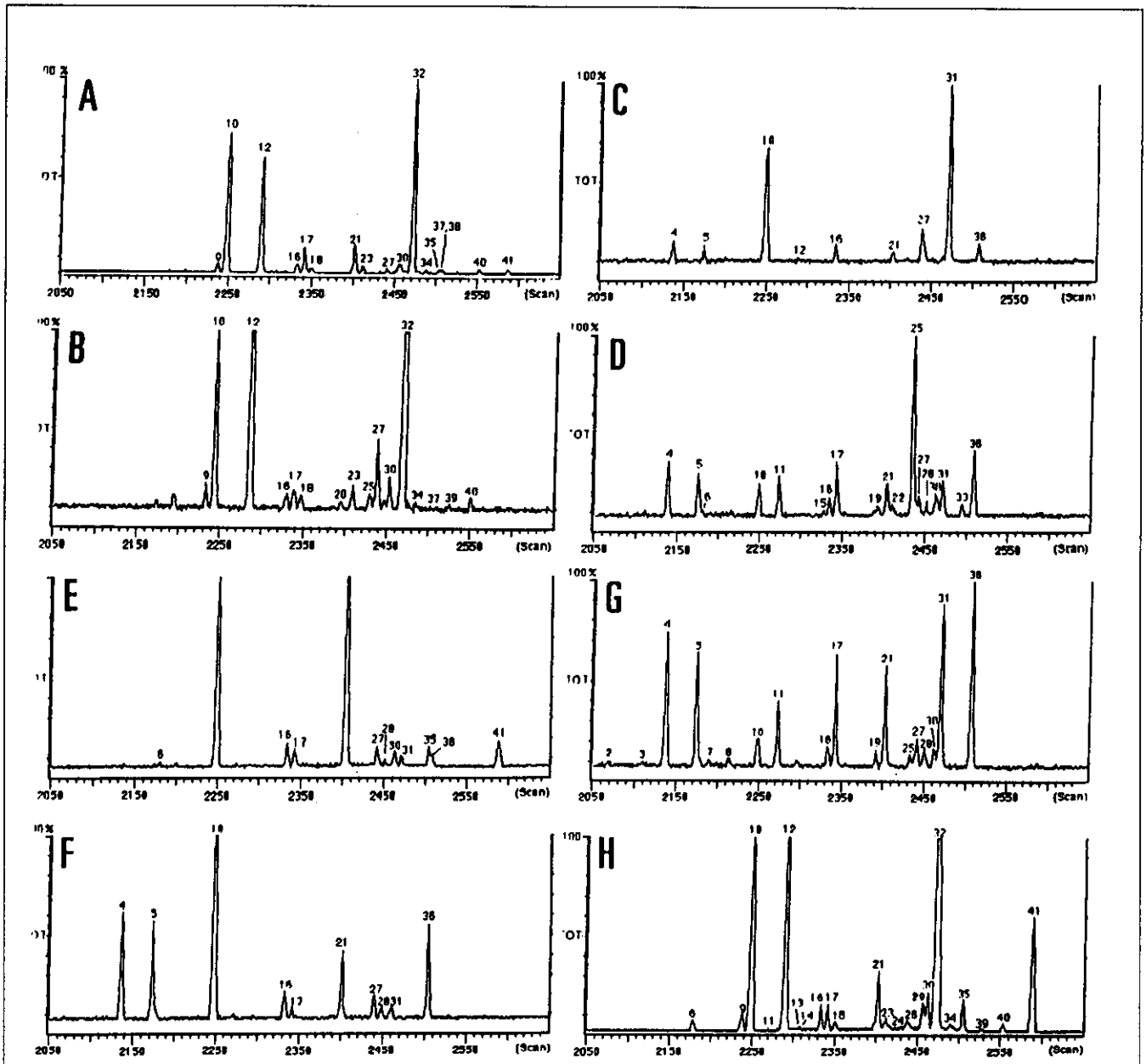


**Figure 5. Sesquiterpene region of the GC chromatogram obtained from LC transfer of the corresponding fraction for**

**A bergamot** **C mandarin** **E bitter orange** **G clementine**
**B lemon** **D sweet orange** **F grapefruit** **H lime**

oils analyzed has allowed an easier and more certain interpretation of the data, in comparison with the results obtained for each oil individually. The results reported should constitute a useful reference point for the characterization of the citrus essential oils.

## Bergamot, Neroli and Petitgrain Essential Oils

The study of the composition of an essential oil by LC-GC-MS may be carried out by LC pre-separating the oil into two fractions: the first containing the terpene hydrocarbons and the second the oxygenated compounds, to be analyzed by GC-MS. Otherwise the oil may be separated, according to its composition, into more than two fractions: the first containing the terpene hydrocarbons, and the following fractions containing the oxygenated compounds separated in accordance with the different polarity of compounds which belong to different classes.

The first of these two methods has been applied to the study of the volatile fraction of a neroli and petitgrain oil, and the second to the study of a bergamot essential oil.

***Bergamot oil:***[38] Figure 6 shows the LC chromatogram of the bergamot essential oil. The transferred fractions are marked with $F_1$, $F_2$, $F_3$ and $F_4$, and the transfer times for each fraction are listed in Table I with the time of the vapor exit closure.

Figures 7 and 8 show the GC chromatograms of the LC fractions. Above each chromatogram of the transferred fraction is shown the on-column GC chromatogram of the whole oil with the same column systems. Compound identification is reported in Table VII.

The on-column chromatogram of the whole essential oil shows some overlap between peaks of monoterpenes and the more volatile oxygenated compounds. Such interferences between peaks of compounds in mixtures as complex as essential oils are inevitable, and occur regardless of the stationary phase.

Figures 7 and 8 show that the LC pre-separation of the

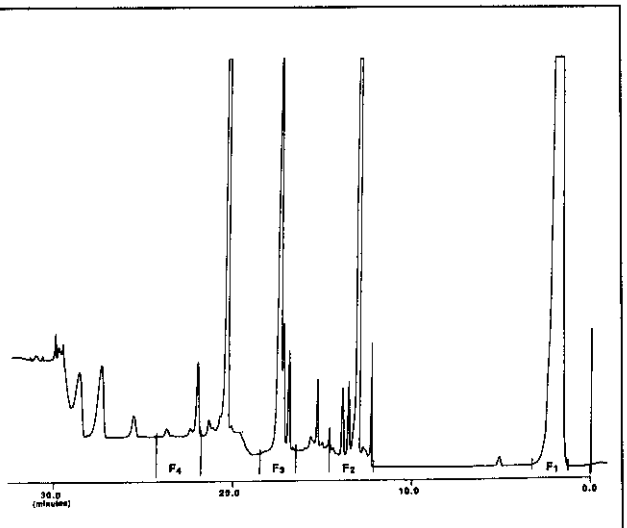


**Figure 6. LC chromatogram of a bergamot oil**

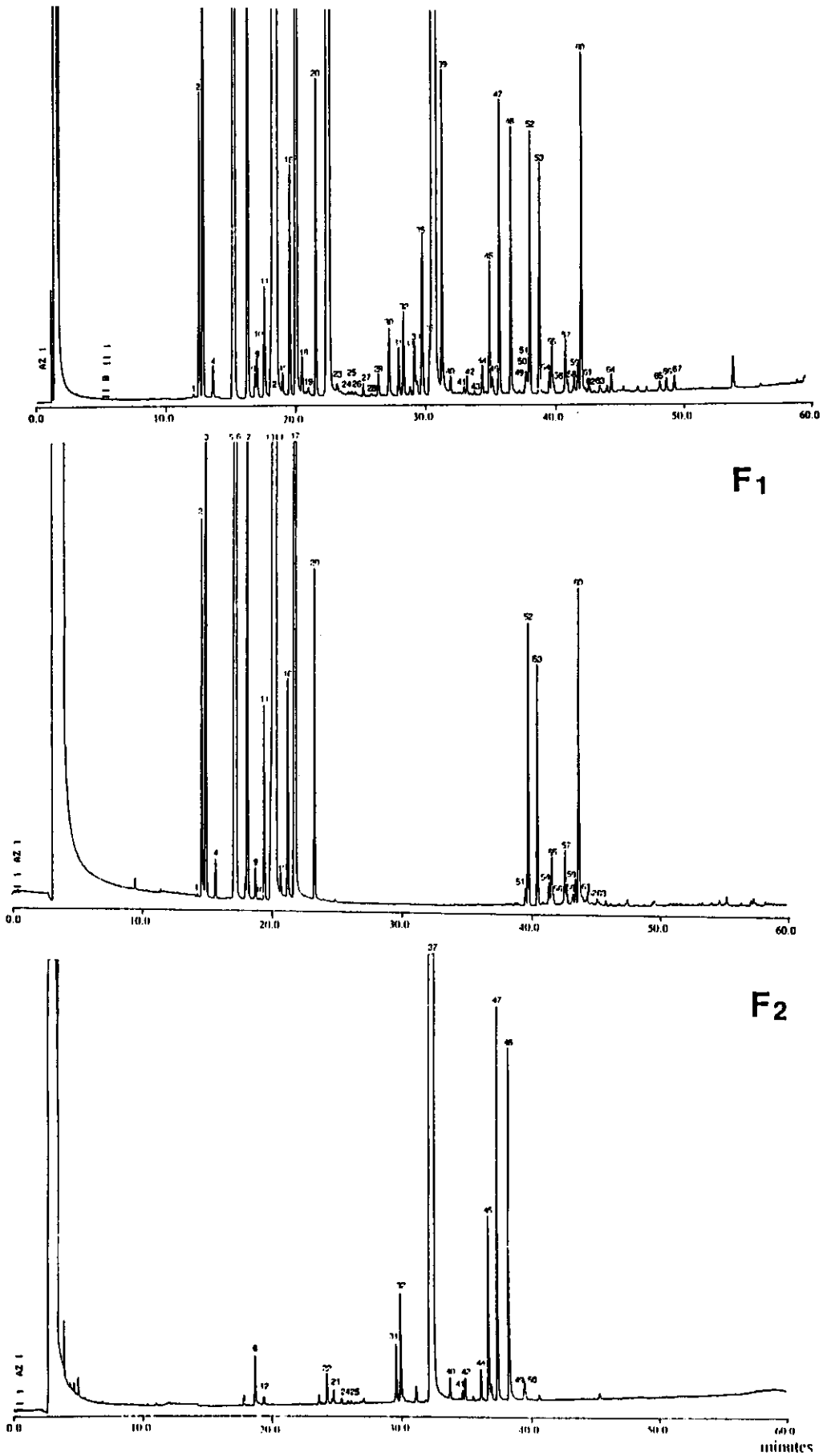


**Figure 7. GC chromatogram of a bergamot essential oil and of the $F_1$ and $F_2$ fractions from its LC separation**

**Table VII. Components identified in the essential oil of bergamot**

| | |
|---|---|
| 1 | tricyclene |
| 2 | α-thujene |
| 3 | α-pinene |
| 4 | camphene |
| 5 | sabinene |
| 6 | β-pinene |
| 7 | myrcene |
| 8 | octanal |
| 9 | α-phellandrene |
| 10 | δ-3-carene |
| 11 | α-terpinene |
| 12 | hexyl acetate |
| 13 | p-cymene |
| 14 | limonene |
| 15 | (Z)-β-ocimene |
| 16 | (E)-β-ocimene |
| 17 | γ-terpinene |
| 18 | cis-sabinene hydrate |
| 19 | octanol |
| 20 | terpinolene |
| 21 | linalool |
| 22 | nonanal |
| 23 | heptyl acetate |
| 24 | cis-limonene oxide |
| 25 | trans-limonene oxide |
| 26 | isopulegol |
| 27 | camphor |
| 28 | citronellal |
| 29 | terpinen-4-ol |
| 30 | α-terpineol |
| 31 | decanal |
| 32 | octyl acetate |
| 33 | nerol |
| 34 | citronellol |
| 35 | neral |
| 36 | cis-sabinene hydrate acetate |
| 37 | linalyl acetate |
| 38 | geraniol |
| 39 | geranial |
| 40 | bornyl acetate |
| 41 | undecanal |
| 42 | nonyl acetate |
| 43 | methyl geranoate |
| 44 | linalyl propionate |
| 45 | α-terpenyl acetate |
| 46 | citronellyl acetate |
| 47 | neryl acetate |
| 48 | geranyl acetate |
| 49 | dodecanal |
| 50 | decyl acetate |
| 51 | cis-α-bergamotene |
| 52 | β-caryophyllene |
| 53 | trans-α-bergamotene |
| 54 | α-humulene |
| 55 | cis-β-farnesene |
| 56 | β-santalene |
| 57 | germacrene D |
| 58 | a germacrene or an elemene |
| 59 | unknown sesquiterpene |
| 60 | β-bisabolene |
| 61 | cis-γ-bisabolene |
| 62 | unknown sesquiterpene |
| 63 | germacrene B |
| 64 | trans-nerolidol |
| 65 | 2,3-dimethyl-3-(4-methyl-3-pentenyl)-2-norbornanol |
| 66 | campherenol |
| 67 | α-bisabolol |

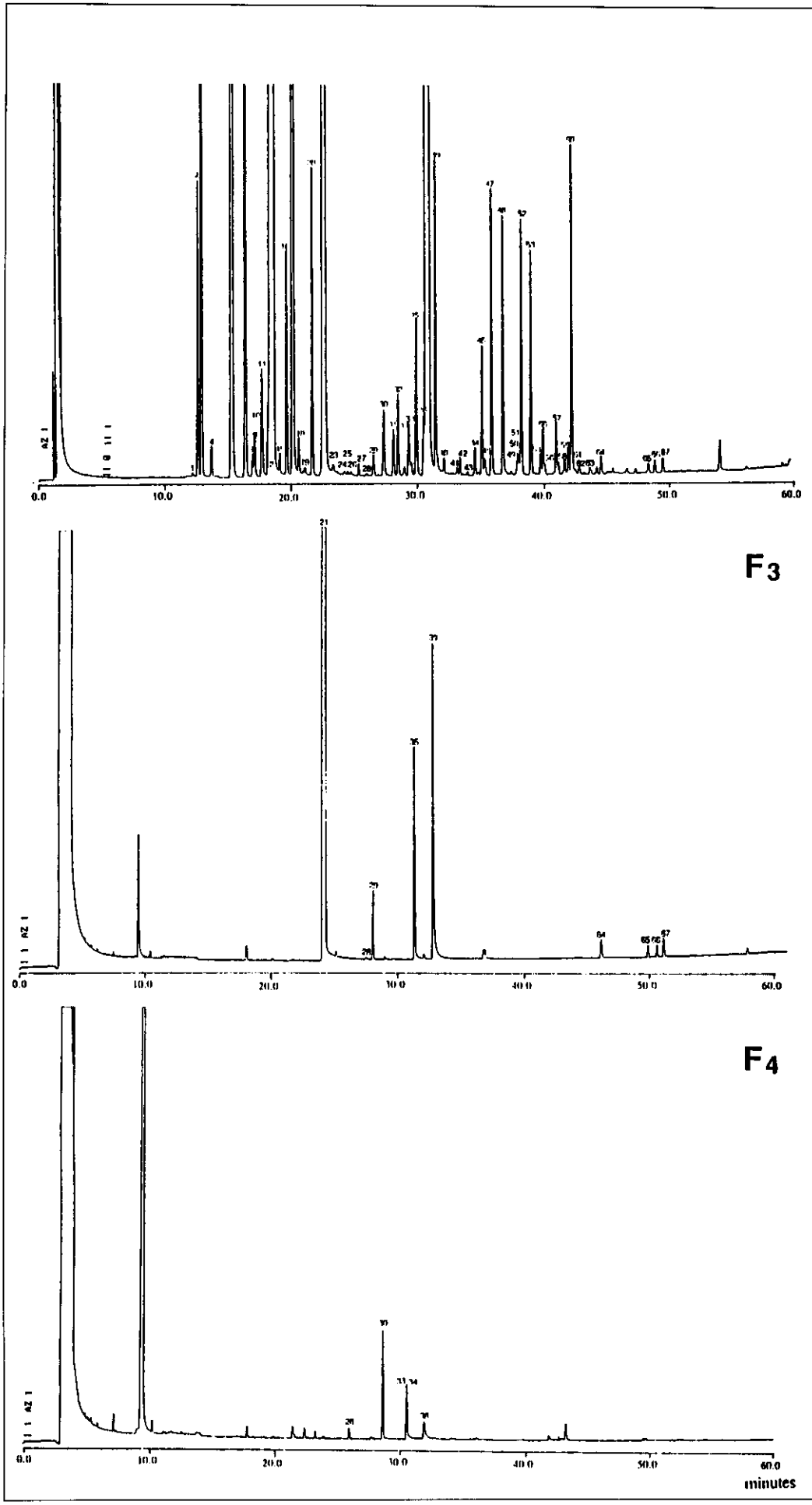


**Figure 8. GC chromatogram of a bergamot essential oil and of the $F_3$ and $F_4$ fractions from its LC separation**

oil gives fractions that are much simpler than the whole oil, and also chemically more homogeneous. These fractions give chromatograms in which all the peaks are better separated and thus easier to identify by retention times or MS. In the on-column chromatogram of the whole oil there is overlap between some monoterpenes and hexyl acetate and octanal, and between linalool and nonanal. Using the LC pre-separation, mono- and sesquiterpenes are eluted in fraction $F_1$, aliphatic aldehydes and esters are grouped

**Table VIII. Components identified in the essential oil of neroli**

| | | | |
|---|---|---|---|
| 1 | tricyclene | 30 | nerol |
| 2 | α-thujene | 31 | neral |
| 3 | α-pinene | 32 | linalyl acetate |
| 4 | camphene | 33 | geranial |
| 5 | benzaldehyde | 34 | indole |
| 6 | sabinene | 35 | methyl anthranilate |
| 7 | β-pinene | 36 | δ-elemene |
| 8 | 6-methyl-5-hepten-2-one | 37 | α-terpenyl acetate |
| 9 | myrcene | 38 | citronellyl acetate |
| 10 | α-phellandrene | 39 | neryl acetate |
| 11 | δ-3-carene | 40 | geranyl acetate |
| 12 | α-terpinene | 41 | β-elemene |
| 13 | p-cymene | 42 | methyl N-methyl anthranilate |
| 14 | limonene | | |
| 14A | 1,8-cineole | 43 | β-caryophyllene |
| 15 | (Z)-β-ocimene | 44 | trans-α-bergamotene |
| 16 | (E)-β-ocimene | 45 | α-humulene |
| 17 | γ-terpinene | 46 | cis-β-farnesene |
| 18 | trans-sabinene hydrate | 47 | germacrene D |
| 19 | cis-linalool oxide | 48 | unknown sesquiterpene |
| 20 | terpinolene | 49 | unknown sesquiterpene |
| 20A | trans-linalool oxide | 50 | (E,E)-α-farnesene |
| 21 | cis-sabinene hydrate | 51 | δ-cadinene |
| 22 | linalool | 52 | (Z)-nerolidol |
| 23 | phenyl ethyl alcohol | 53 | (E)-nerolidol |
| 24 | cis-p-menth-2-en-1-ol | 54 | spathulenol |
| 25 | trans-p-menth-2-en-1-ol | 55 | caryophyllene oxide |
| 26 | citronellal | 56 | globulol |
| 27 | terpinen-4-ol | 57 | α-cadinol |
| 28 | α-terpineol | 58 | cis,trans-farnesol |
| 29 | trans-piperitol | 59 | trans,trans-farnesol |

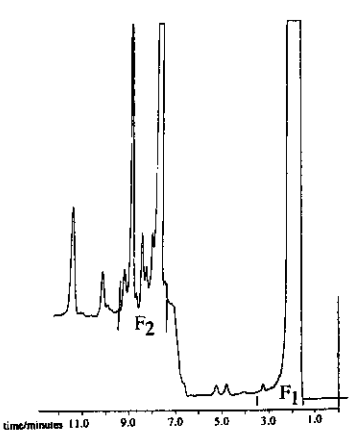


**Figure 9. HPLC chromatogram of the neroli essential oil**

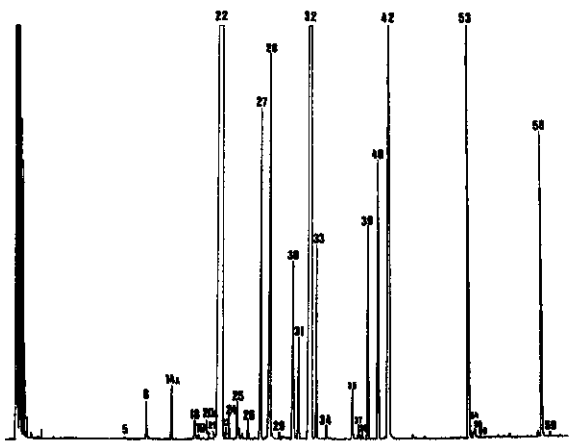

**Figure 10. On-column GC-FID chromatogram of the whole neroli essential oil (for peak identification see Table VIII)**

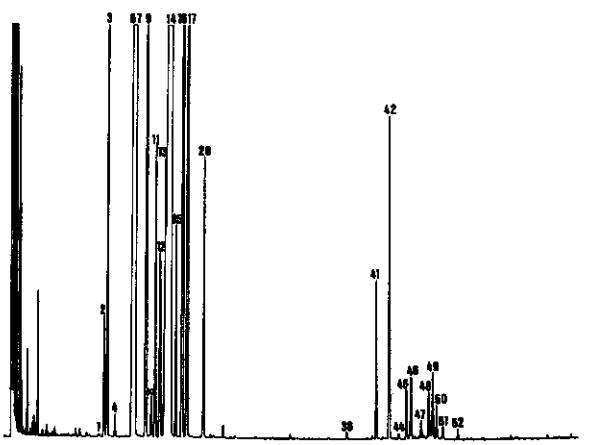

**Figure 11. Chromatogram of the hydrocarbon fraction ($F_1$) of neroli oil obtained by HPLC-HRGC-FID (for peak identification see Table VIII)**

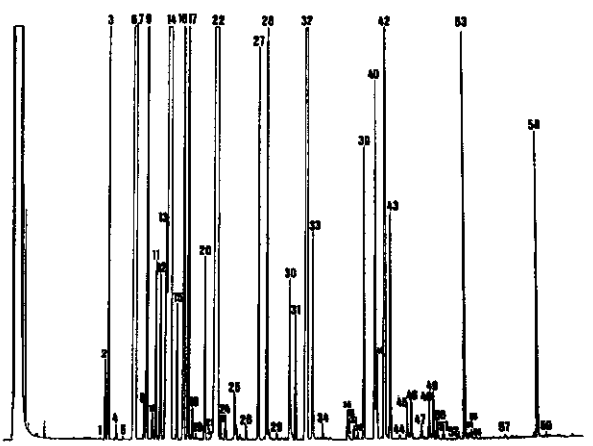

**Figure 12. Chromatogram of the oxygenated fraction ($F_2$) of neroli oil obtained by HPLC-HRGC-FID (for peak identification see Table VIII)**

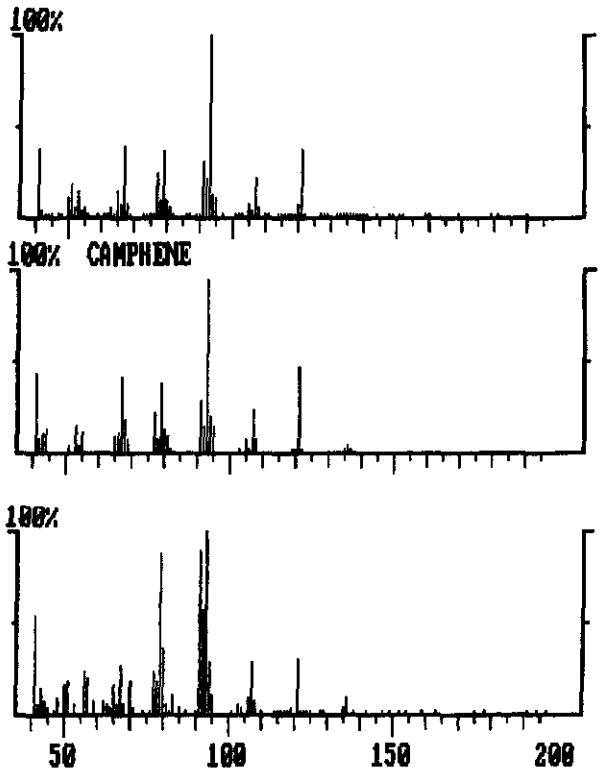


**Figure 13. Comparison of the mass spectra of the neroli oil peak 4 (camphene) obtained by HPLC-HRGC-MS (upper) and by GC-MS (lower) with a library spectrum (middle)**

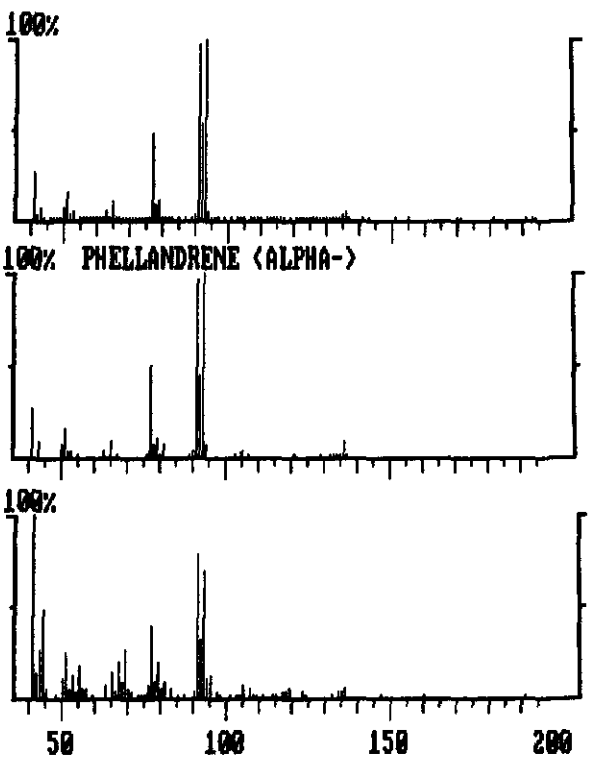


***Figure 14. Comparison of the mass spectra of the* neroli oil peak 4 (α-phellandrene) obtained by HPLC-HRGC-MS (upper) and by GC-MS (lower) *with a library spectrum (middle)***

**Table IX. Comparison between data obtained from the whole neroli oil and data obtained from the hydrocarbon fraction**

| | % Components in the whole oil (on-column injection) | % Components in the hydrocarbon fraction | % Modified from the analysis of hydrocarbon fraction* |
|---|---|---|---|
| tricyclene | 0.01 | 0.01 | 0.01 |
| α-thujene | 0.25 | 0.42 | 0.25 |
| α-pinene | 1.31 | 2.22 | 1.34 |
| camphene | 0.04 | 0.07 | 0.04 |
| benzaldehyde | 0.01 | - | - |
| sabinene + β-pinene | 20.22 | 33.31 | 20.15 |
| 6-methyl-5-hepten-2-one | 0.11 | - | - |
| myrcene | 2.33 | 4.02 | 2.43 |
| α-phellandrene | 0.09 | 0.15 | 0.09 |
| δ-3-carene | 0.52 | 0.89 | 0.54 |
| α-terpinene | 0.51 | 0.87 | 0.52 |
| p-cymene | 1.04 | 0.18 | 0.71 |
| limonene | 24.57 | 41.13 | 24.27 |
| 1,8-cineole | - | - | - |
| (Z)-β-ocimene | 0.34 | 0.58 | 0.35 |
| (E)-β-ocimene | 3.60 | 6.18 | 3.74 |
| γ-terpinene | 3.71 | 6.20 | 3.75 |
| trans-sabinene hydrate | 0.09 | - | - |
| cis-linalool oxide | 0.02 | - | - |
| terpinolene | 0.53 | 0.91 | 0.55 |
| trans-linalool oxide | - | - | - |
| cis-sabinene hydrate | 0.03 | - | - |
| linalool | 15.59 | - | - |
| phenyl ethyl alcohol | 0.01 | - | - |
| cis-p-menth-2-en-1-ol | 0.09 | - | - |
| trans-p-menth-2-en-1-ol | 0.19 | - | - |
| citronellal | 0.06 | - | - |
| terpinen-4-ol | 1.20 | - | - |
| α-terpineol | 1.79 | - | - |
| trans-piperitol | 0.03 | - | - |
| nerol | 0.69 | - | - |
| neral | 0.41 | - | - |
| linalyl acetate | 9.76 | - | - |
| geranial | 0.65 | - | - |
| indole | 0.06 | - | - |
| δ-elemene | - | 0.02 | 0.02 |
| methyl anthranilate | 0.11 | - | - |
| α-terpenyl acetate | 0.05 | - | - |
| citronellyl acetate | 0.03 | - | - |
| neryl acetate | 0.92 | - | - |
| geranyl acetate | - | - | - |
| β-elemene | 1.63 | 0.52 | 0.31 |
| methyl N-methyl anthranilate | 3.18 | - | - |
| β-caryophyllene | 0.72 | 1.19 | 0.72 |
| trans-α-bergamotene | 0.02 | 0.02 | 0.01 |
| α-humulene | 0.10 | 0.16 | 0.10 |
| cis-β-farnesene | 0.14 | 0.22 | 0.13 |
| germacrene D | 0.05 | 0.08 | 0.05 |
| unknown sesquiterpene | 0.13 | 0.22 | 0.13 |
| unknown sesquiterpene | 0.16 | 0.27 | 0.17 |
| α-farnesene | 0.07 | 0.13 | 0.08 |
| δ-cadinene | 0.03 | 0.05 | 0.03 |
| (Z)-nerolidol | t | - | - |
| (E)-nerolidol | 1.76 | - | - |
| spathulenol | 0.02 | - | - |
| caryophyllene oxide | 0.04 | - | - |
| globulol | 0.01 | - | - |
| α-cadinol | 0.02 | - | - |
| cis,trans-farnesol | 0.98 | - | - |
| trans,trans-farnesol | 0.01 | - | - |
| hydrocarbons | 60.48 | | |
| oxygenated | 39.52 | | |

* data % obtained from: % fraction x 60.48/100
t = trace <0.01%

in fraction $F_2$, while alcohols are eluted in fractions $F_3$ and $F_4$. The chromatograms of fractions $F_1$ and $F_2$ show that there is no overlap between component peaks which could compromise the identification. The only exception is the incomplete separation between dodecanal and decyl acetate.

As can be seen from Table VII, this method has allowed unambiguous identification of 65 components in the bergamot oil. The method is general for complex mixtures such as essential oils. The reduced complexity of the chromatograms of the GC fractions allows easier identification of the different components and, of course, their quantitation. This is practically impossible during a direct GC analysis for some esters and aldehydes which may be very important for the olfactory characteristics of the essential oils and whose content represents a fundamental quality parameter.

***Neroli oil:***[39] Neroli oil was separated into two fractions, marked $F_1$ (terpene hydrocarbons) and $F_2$ (oxygenated compounds) in Figure 9, which shows the HPLC chromatogram of the neroli essential oil. Figure 10 shows the on-column GC-FID chromatogram with the same column system as was used in the on-line HPLC-HRGC-MS. The compound identifications are reported in Table VIII. The on-column chromatogram of the whole essential oil shows substantial overlap between the peaks of monoterpenes and sesquiterpenes and some oxygenated compounds. For example, there is a 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.

Figures 11 and 12 show the GC-FID chromatograms of the HPLC transferred fractions $F_1$ (hydrocarbons) and $F_2$ (oxygenated compounds), respectively. These figures show that HPLC pre-separation of the oil results in mixtures that are

much simpler than the original oil and chemically more homogeneous. Moreover, this transfer technique provides efficient solvent effects, and the solute peaks in GC immediately after the solvent peak are well shaped and of the correct intensity. The first column of Table IX contains the percentage composition of the oil, the second column the percentage composition of the hydrocarbon fraction and the third column the percentage composition considering the percentage of hydrocarbons in the whole oil (60.48%). In Table X the same calculations for the oxygenated compounds are reported. These two tables show that the percentage composition for the fractions is very similar to the percentage composition for the whole oil. These data prove this method is very effective and that there is a quantitative transfer of the compounds of interest.

To demonstrate the power of the HPLC-HRGC-MS system, we have chosen two representative components of the hydrocarbon fraction (camphene and α-phellandrene), marked 4 and 10, respectively, in Figure 11. Figures 13 and 14 compare the mass spectra of these peaks (camphene and α-phellandrene) obtained by HPLC-HRGC-MS (upper), with a library spectrum (middle) and the mass spectra for the same peaks obtained by GC-MS of the whole oil (lower). The figures show that the spectra obtained after LC pre-fractionation (upper) have a much greater purity than those obtained for the same compounds by GC-MS of the whole oil (lower); the comparison with the library (middle) is certainly more reliable. Tables XI and XII list the first ten compounds in the library which compare best with the two peaks in the GC-MS chromatogram of the whole oil (Figures 13 and 14). For camphene, the library shows that the ten most similar compounds are all monoterpenes, and gives camphene only as third choice. Moreover, all ten compounds have a high fit with lower degree of pu-

**Table X. Comparison between data obtained from the whole neroli oil and data obtained from the fraction for oxygenated compounds**

| | % Components in the whole oil (on-column injection) | % Components in the oxygenated fraction | % Modified from the analysis of oxygenated fraction* |
|---|---|---|---|
| tricyclene | 0.01 | - | - |
| α-thujene | 0.25 | - | - |
| α-pinene | 1.31 | - | - |
| camphene | 0.04 | - | - |
| benzaldehyde | 0.01 | 0.02 | 0.01 |
| sabinene + β-pinene | 20.22 | - | - |
| 6-methyl-5-hepten-2-one | 0.11 | 0.28 | 0.11 |
| myrcene | 2.33 | - | - |
| α-phellandrene | 0.09 | - | - |
| δ-3-carene | 0.52 | - | - |
| α-terpinene | 0.51 | - | - |
| p-cymene | 1.04 | - | - |
| limonene | 24.57 | - | - |
| 1,8-cineole | ** | 0.44 | 0.18 |
| (Z)-β-ocimene | 0.34 | - | - |
| (E)-β-ocimene | 3.60 | - | - |
| γ-terpinene | 3.71 | - | - |
| trans-sabinene hydrate | 0.09 | 0.25 | 0.10 |
| cis-linalool oxide | 0.02 | 0.06 | 0.02 |
| terpinolene | 0.53 | - | - |
| trans-linalool oxide | *** | 0.03 | 0.01 |
| cis-sabinene hydrate | 0.03 | 0.08 | 0.03 |
| linalool | 15.59 | 39.16 | 15.48 |
| phenyl ethyl alcohol | 0.01 | 0.02 | 0.01 |
| cis-p-menth-2-en-1-ol | 0.09 | 0.19 | 0.08 |
| trans-p-menth-2-en-1-ol | 0.19 | 0.47 | 0.19 |
| citronellal | 0.06 | 0.17 | 0.07 |
| terpinen-4-ol | 1.20 | 3.11 | 1.23 |
| α-terpineol | 1.79 | 4.63 | 1.83 |
| trans-piperitol | 0.03 | 0.09 | 0.03 |
| nerol | 0.69 | 1.76 | 0.70 |
| neral | 0.41 | 1.17 | 0.46 |
| linalyl acetate | 9.76 | 25.03 | 9.89 |
| geranial | 0.65 | 1.64 | 0.65 |
| indole | 0.06 | 0.16 | 0.06 |
| δ-elemene | **** | - | - |
| methyl anthranilate | 0.11 | 0.26 | 0.10 |
| α-terpenyl acetate | 0.05 | 0.13 | 0.05 |
| citronellyl acetate | 0.03 | 0.07 | 0.03 |
| neryl acetate | 0.92 | 2.09 | 0.83 |
| geranyl acetate | 1.63 | 3.49 | 1.38 |
| β-elemene | - | - | - |
| methyl N-methyl anthranilate | 3.18 | 8.06 | 3.19 |
| β-caryophyllene | 0.72 | - | - |
| trans-α-bergamotene | 0.02 | - | - |
| α-humulene | 0.10 | - | - |
| cis-β-farnesene | 0.14 | - | - |
| germacrene D | 0.05 | - | - |
| unknown sesquiterpene | 0.13 | - | - |
| unknown sesquiterpene | 0.16 | - | - |
| (E, E)–α-farnesene | 0.07 | - | - |
| δ-cadinene | 0.03 | - | - |
| (Z)-nerolidol | t | 0.01 | t |
| (E)-nerolidol | 1.76 | 4.46 | 1.76 |
| spathulenol | 0.02 | 0.09 | 0.04 |
| caryophyllene oxide | 0.04 | - | - |
| globulol | 0.01 | 0.02 | 0.01 |
| α-cadinol | 0.02 | 0.05 | 0.02 |
| cis,trans-farnesol | 0.98 | 2.49 | 0.99 |
| trans,trans-farnesol | 0.01 | 0.04 | 0.02 |
| hydrocarbons | 60.48 | | |
| oxygenated | 39.52 | | |

* data % obtained from: % fraction x 39.52/100
** coeluted with limonene in the whole sample
*** coeluted with terpinolene in the whole sample
**** coeluted with methyl anthranilate in the whole sample
t = trace <0.01%

**Table XI. Results obtained from the library search for peak 4 (camphene) of neroli oil**

| | 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 | trans-ocimene | 622 | 869 | 683 |
| 8 | sabinene | 617 | 855 | 652 |
| 9 | santolina triene | 617 | 853 | 686 |
| 10 | β-pinene | 616 | 806 | 699 |
| | **HPLC-HRGC-MS** | | | |
| 1 | camphene | 849 | 931 | 876 |
| 2 | santolina triene | 780 | 870 | 808 |

**Table XII. Results obtained from the library search for peak 10 (α-phellandrene) of neroli oil**

| | GC/MS | Purity | Fit | Rfit |
|---|---|---|---|---|
| 1 | β-phellandrene | 836 | 944 | 858 |
| 2 | sabinene | 803 | 939 | 824 |
| 3 | α-pinene | 799 | 899 | 830 |
| 4 | cis-ocimene | 774 | 923 | 803 |
| 5 | limonene | 771 | 884 | 779 |
| 6 | trans-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** | | | |
| 1 | α-phellandrene | 883 | 977 | 892 |
| 2 | α-thujene | 761 | 853 | 770 |

rity. The library does not find α-phellandrene in the first ten most similar spectra, only an isomer. HPLC-HRGC-MS with LC pre-fractionation 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). Moreover, this library reports the retention time of compounds on a DB-5 column. Since the column used in our 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.

***Petitgrain oils:***[40] Bitter orange, sweet orange, mandarin and lemon were analyzed by HPLC-HRGC-MS. Each oil was separated into two fractions; one containing terpene hydrocarbons and the other containing oxygenated compounds. Figures 15-18 show the total ion chromato-

**Table XIII. Components identified by HPLC-HRGC-MS in the petitgrain oils. The number indicates peak assignment in the chromatogram.**

| | Bitter orange | Sweet orange | Mandarin | Lemon |
|---|---|---|---|---|
| tricyclene | 1 | 1 | 1 | 1 |
| α-thujene | 2 | 2 | 2 | 2 |
| α-pinene | 3 | 3 | 3 | 3 |
| α-fenchene | - | 4 | 4 | 4 |
| camphene | 4 | 5 | 5 | 5 |
| sabinene | 5 | 6 | 6 | 6 |
| β-pinene | 6 | 7 | 7 | 7 |
| 6-methyl-5-hepten-2-one | 7 | 8 | 8 | 8 |
| myrcene | 8 | 9 | 9 | 9 |
| octanal | - | 10 | - | - |
| α-phellandrene | 9 | 11 | 10 | 10 |
| δ-3-carene | 10 | 12 | 11 | 11 |
| α-terpinene | 11 | 13 | 12 | 12 |
| o-cymene | - | 14 | - | 13 |
| p-cymene | 12 | 15 | 13 | 14 |
| limonene | 13 | 16 | 14 | 15 |
| 1,8-cineole | 14 | 17 | 15 | 16 |
| (Z)-β-ocimene | 15 | 18 | 16 | 17 |
| (E)-β-ocimene | 16 | 19 | 17 | 18 |
| γ-terpinene | 17 | 20 | 18 | 19 |
| cis-sabinene hydrate | - | 21 | 19 | 20 |
| cis-linalool oxide | 18 | - | 20 | 21 |
| octanol | - | - | 21 | 22 |
| terpinolene | 19 | 22 | 22 | 23 |
| trans-linalool oxide | 20 | - | - | - |
| linalool | 21 | 23 | 23 | 24 |
| nonanal | - | 24 | 24 | 25 |
| cis-limonene oxide | - | - | 25 | 26 |
| cis-p-menth-2-en-1-ol | - | 25 | - | 27 |
| trans-p-menth-2-en-1-ol | - | 26 | 26 | - |
| isopulegol | - | - | - | 28 |
| citronellal | 22 | 27 | - | 29 |
| terpinen-4-ol | 23 | 28 | 27 | 30 |
| p-cymen-4-ol | - | - | 28 | - |
| α-terpineol | 24 | 29 | 29 | 31 |
| decanal | - | 30 | 30 | - |
| citronellol | - | 31 | 31 | - |
| nerol | 25 | 32 | 32 | 32 |
| neral | 26 | 33 | 33 | 33 |
| linalyl acetate | 27 | 34 | 34 | 34 |
| geranial | 28 | 35 | 35 | 35 |
| undecanal | - | - | - | 36 |
| thymol | - | 36 | 36 | - |
| δ-elemene | 29 | - | - | - |
| α-cubebene | 30 | - | - | - |
| methyl anthranilate | - | - | 37 | - |
| α-terpinyl acetate | 31 | - | 38 | - |
| citronellyl acetate | 32 | 37 | - | 37 |
| α-copaene | 33 | 38 | - | - |
| neryl acetate | 34 | 39 | 39 | 38 |
| β-cubebene | - | 40 | - | - |
| geranyl acetate | 35 | 41 | 40 | 39 |
| β-elemene | 36 | 42 | - | 40 |
| cis-α-bergamotene | - | 43 | - | - |
| methyl N-methyl anthranilate | 37 | 44 | 41 | 41 |
| (E)-caryophyllene | 38 | 45 | 42 | 42 |
| trans-α-bergamotene | 39 | - | - | 43 |
| α-humulene | 40 | 46 | 43 | 44 |
| (Z)-β-farnesene | 41 | 47 | - | 45 |
| methyl N-dimethyl anthranilate | - | - | 44 | - |
| bicyclogermacrene | 42 | - | - | 46 |
| α-selinene | - | 48 | 45 | - |
| valencene | - | 49 | - | - |
| α-farnesene | 43 | 50 | 46 | 47 |
| δ-cadinene | 44 | - | 47 | 48 |
| (E)-nerolidol | 45 | 51 | - | 49 |
| spathulenol | 46 | - | - | 50 |
| caryophyllene oxide | 47 | 52 | 48 | 51 |
| 2,3-dimethyl-3-(4-methyl-3-pentenyl)-2-norbornanol | - | - | - | 52 |
| campherenol | - | - | - | 53 |
| α-bisabolol | - | - | - | 54 |
| β-sinensal | - | 53 | - | - |
| α-sinensal | - | 54 | - | - |

grams (TIC) of the LC fractions transferred for each oil. Above the chromatograms of the two fractions, the on-column GC chromatogram with the same column system is reported. Table XIII identifies the compounds in the four oils analyzed.

As can be seen from Figures 15-18, the chromatograms of the whole oils showed many overlaps between pairs of peaks, but the HPLC pre-separation overcomes these disadvantages.

Bitter orange petitgrain oil is characterized by a high content of oxygenated compounds: 54–67% esters, 27–40% alcohols and 0.6–1.2% aldehydes. Monoterpene hydrocarbons represent 3.3-8.2% of the oil, while sesquiterpenes are about 1%.

Sweet orange petitgrain oil contains 69–79% terpene hydrocarbons and 19–29% oxygenated compounds.

Mandarin petitgrain oil contains 47–55% terpene hydrocarbons. Among the oxygenated compounds, esters are the best represented, with a percentage of 43–51%.

Lemon petitgrain oil is characterized by a high content

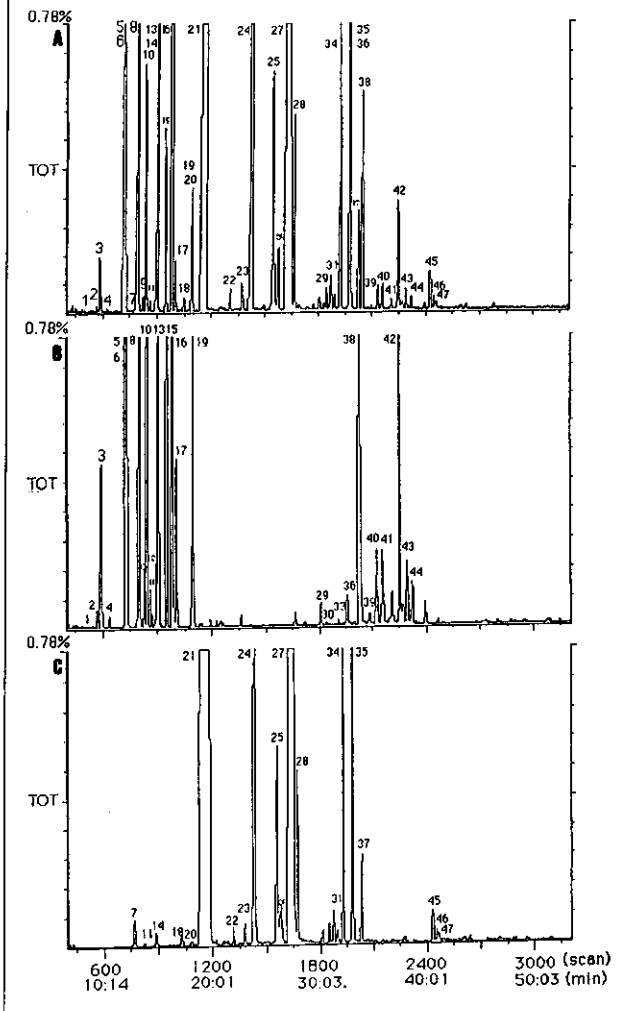


**Figure 15. Total ion current chromatogram of a bitter orange petitgrain oil (A) and of the fractions of its LC separation: hydrocarbons (B) and oxygenated compounds (C)**

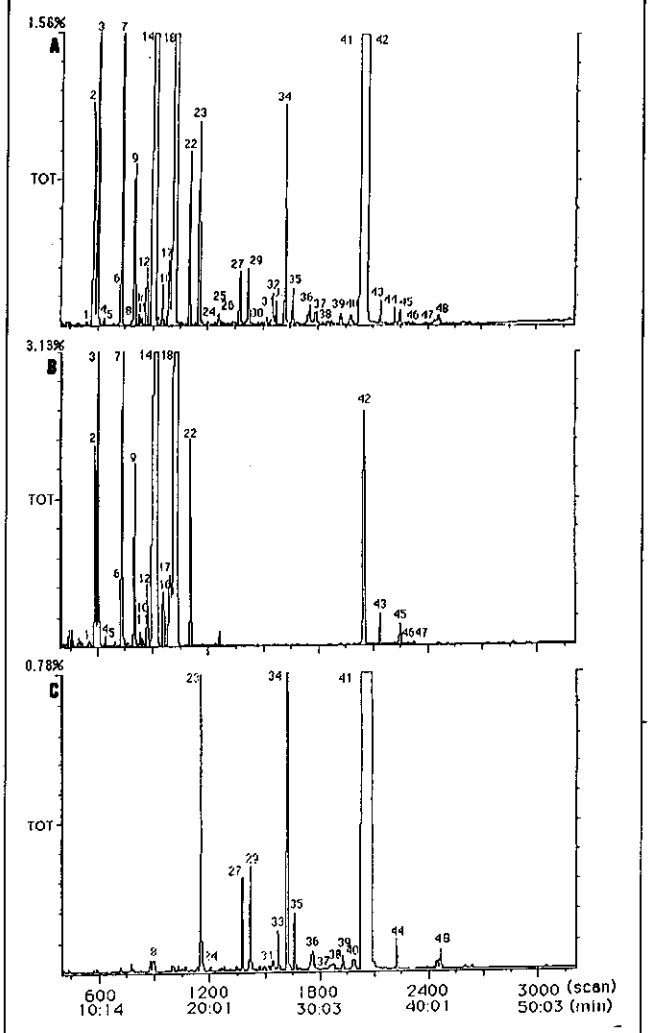


**Figure 16. Total ion current chromatogram of a sweet orange petitgrain oil (A) and of the fractions of its LC separation: hydrocarbons (B) and oxygenated compounds (C)**

**Table XIV. Relative percentages of compounds responsible for the main qualitative differences among the petitgrain oils**

| | Bitter orange (%) | Sweet orange (lab. distld) (%) | Mandarin (%) | Lemon (%) |
|---|---|---|---|---|
| linalyl n-propanoate | 0.02-0.04 | - | - | - |
| δ-elemene | 0.01 | - | - | - |
| thymol methyl ether | - | - | 0.10-0.16 | - |
| carvacrol | - | - | 0.01 | - |
| methyl anthranilate | - | - | t-0.03 | - |
| methyl N-methyl anthranilate | - | - | t-0.04 | - |
| isopulegol | - | - | - | t-0.03 |
| undecanal | - | - | - | 0.02-0.08 |
| geranyl propanoate | - | - | - | 0.03-0.04 |
| 2,3-dimethyl-3-(4-methyl-3-pentenyl)-norbornanol | - | - | - | 0.02-0.06 |
| campherenol | - | - | - | 0.01-0.02 |
| α-bisabolol | - | - | - | 0.01-0.03 |
| cis-piperitol | - | 0.05-0.06 | - | - |
| trans-piperitol | - | 0.07-0.16 | - | - |
| β-sinensal | - | 1.27-1.44 | - | - |
| δ-sinensal | - | 0.08-0.46 | - | - |

t = trace

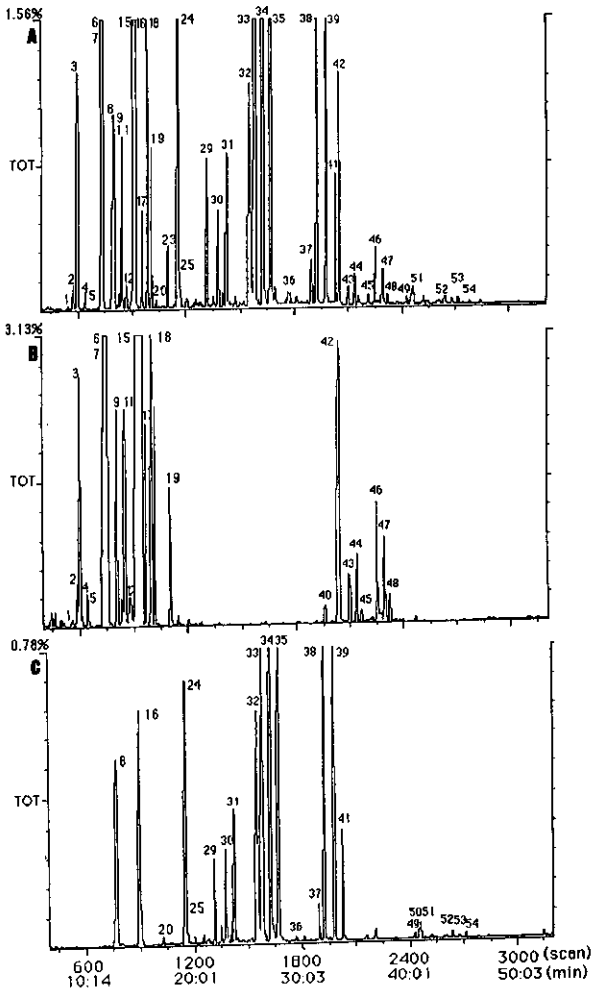


**Figure 17. Total ion current chromatogram of a mandarin petitgrain oil (A) and of the fractions of its LC separation: hydrocarbons (B) and oxygenated compounds (C)**

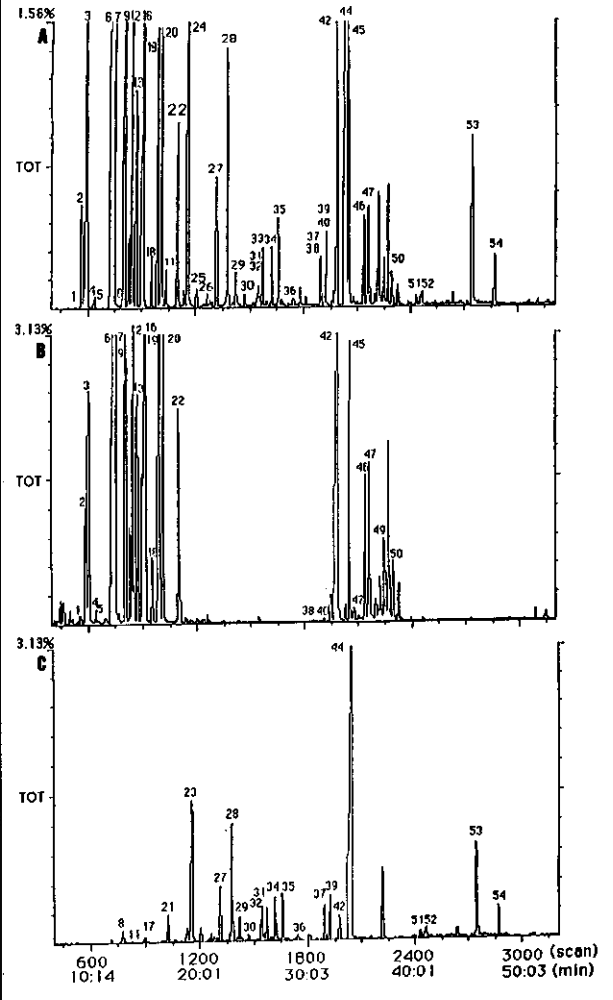


**Figure 18. Total ion current chromatogram of a lemon petitgrain oil (A) and of the fractions of its LC separation: hydrocarbons (B) and oxygenated compounds (C)**

**Table XV. Relative percentages of compounds responsible for the main quantitative differences among the petitgrain oils**

| | Bitter orange (%) | Sweet orange (%) | Mandarin (%) | Lemon (%) |
|---|---|---|---|---|
| sabinene | 0.13-0.23 | 38.46-48.52 | 0.22-0.90 | 2.99-3.81 |
| β-pinene | 0.65-1.15 | 1.87-2.33 | 1.90-2.45 | 11.96-16.03 |
| δ-3-carene | 0.21-0.67 | 4.45-10.28 | 0.01-0.10 | 0.63-1.08 |
| p-cymene | 0.03-0.08 | 0.59-1.68 | 2.96-4.84 | 0.04-0.51 |
| limonene | 0.44-2.17 | 2.90-4.04 | 7.18-11.65 | 28.41-34.82 |
| β-phellandrene | 0.03-0.04 | 0.65-0.74 | 0.03-0.05 | 2.22-2.60 |
| (E)-β-ocimene | 0.57-1.76 | 4.99-9.73 | 0.42-0.72 | 1.50-2.43 |
| γ-terpinene | 0.01-0.09 | 1.41-2.43 | 23.94-28.48 | 0.34-0.70 |
| 1,8-cineole | 0.02-0.05 | - | 0.01-0.02 | 1.12-2.13 |
| neral | 0.21-0.43 | 1.04-1.79 | t-0.03 | 6.64-10.78 |
| geranial | 0.38-0.64 | 1.37-2.17 | t-0.03 | 9.87-14.07 |
| linalool | 21.70-32.75 | 6.95-15.12 | 0.27-0.64 | 0.88-3.87 |
| terpinen-4-ol | 0.05-0.08 | 3.75-7.33 | 0.20-0.26 | 0.25-0.59 |
| α-terpineol | 3.09-5.63 | 0.36-0.93 | 0.16-0.21 | 0.53-1.00 |
| linalyl acetate | 50.68-62.57 | 0.07-0.10 | 0.04-0.10 | 0.31-0.42 |
| neryl acetate | 1.04-1.73 | 0.04-0.15 | t-0.04 | 3.75-6.74 |
| geranyl acetate | 1.90-3.16 | 0.04-0.16 | t-0.02 | 2.17-2.92 |
| methyl N-methyl anthranilate | t-0.14 | t | 43.19-51.93 | t-0.39 |

t = trace

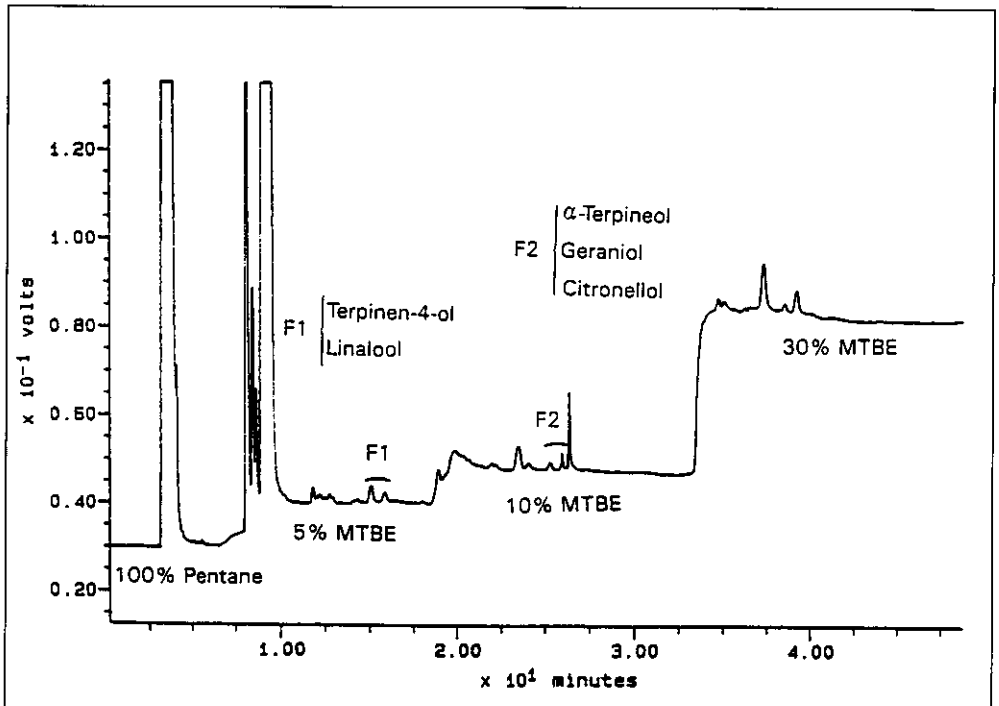


**Figure 19. HPLC chromatogram of lemon oil**

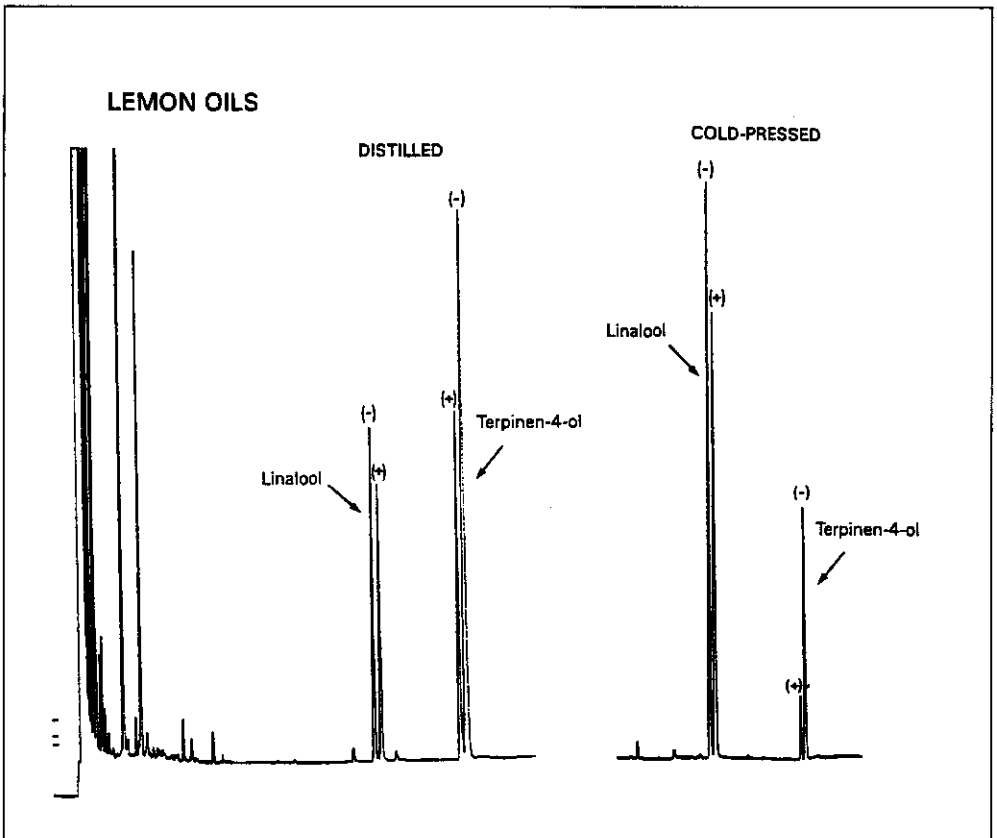


**Figure 20. Enantiomeric distribution of linalool and terpinen-4-ol for lemon oils obtained by HPLC-HRGC**

of monoterpene hydrocarbons (52–68%). Among the oxygenated compounds, aldehydes show the highest percentage (18–27%), followed by alcohols and esters.

Many qualitative and quantitative differences are observed among the four petitgrain oils. These differences allow us to distinguish the various oils and to determine their mixtures or reciprocal contaminations. Tables XIV and XV report the compounds responsible for the main qualitative and quantitative differences among the four oils. [*Editor's note: The complete composition for each of these oils will be reported in four future articles in* ***The Journal of Essential Oil Research***.]

## Enantiomeric Distribution of Monoterpene Alcohols in Citrus Oils[41,42]

The direct gas chromatographic determination of enantiomers of complex mixtures, such as essential oils, is often difficult, especially for compounds present in small quantities, because of the possible overlapping of peaks. It has therefore, proved useful to resort to a multidimensional system.[43-46] Altogether, monoterpene alcohols represent about 0.5% of the volatile fraction of lemon, mandarin, sweet and bitter orange oils. The determination of their enantiomeric distribution can give useful information about the genuineness, quality and origin of the essential oils.

Figure 19 shows the HPLC chromatogram of a lemon oil where the peaks relative to the monoterpene

alcohols are identified.

Figures 20 and 21 show the HRGC chromatograms of the HPLC-transferred fraction containing linalool and terpinen-4-ol of cold-pressed and distilled lemon and mandarin oils, respectively.

As can be seen from Figures 20 and 21, the analyzed HPLC fraction contains only linalool and terpinen-4-ol, making it possible to evaluate exactly the enantiomeric ratios of these alcohols without any interferences.

Table XVI reports the enantiomeric distribution of linalool and terpinen-4-ol for the same lemon and mandarin oils.

Linalool content does not show significant differences in cold-pressed and distilled oils, while terpinen-4-ol content is higher in distilled than in cold-pressed oils (Table XVII). These differences are due to processing technology. Distilled oils are obtained from the acid-aqueous residues of the cold extraction, with the temperature and pH causing the hydration of monoterpenes and, consequently, the increase of alcohols, especially terpinen-4-ol and α-terpineol. Linalool content is not influenced to a great extent.[47,48]

Because the monoterpene hydration is not a stereospecific reaction, it modifies the enantiomeric ratio of the alcohols, leading this ratio toward the racemate.

In accord with previous statements and as can be seen from Figures 20 and 21 and Table XVI, the enantiomeric distribution of linalool is approximately the same in cold-pressed and distilled oils. In contrast, the

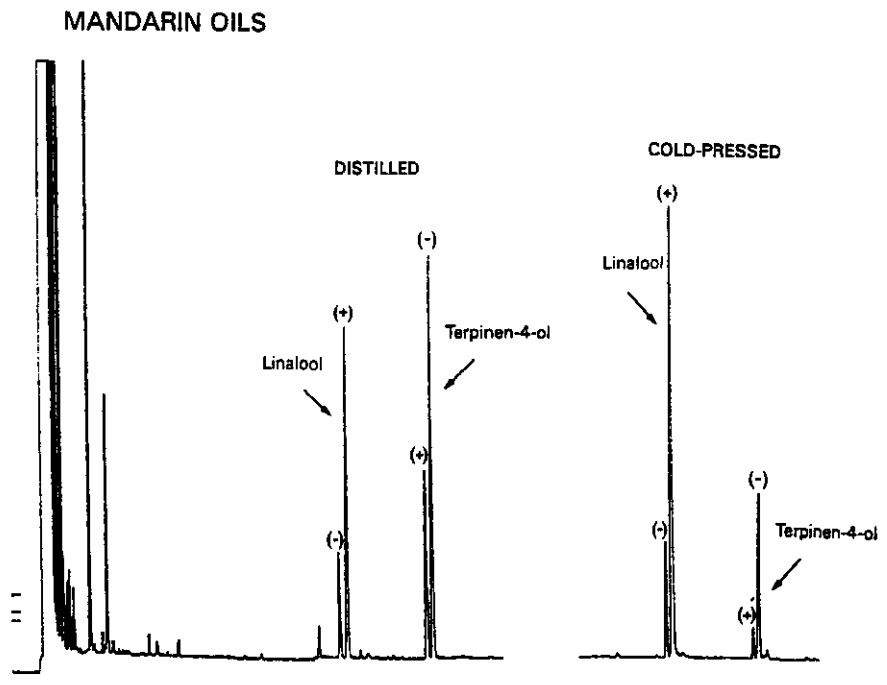


**Figure 21. Enantiomeric distribution of linalool and terpinen-4-ol for mandarin oils obtained by HPLC-HRGC**

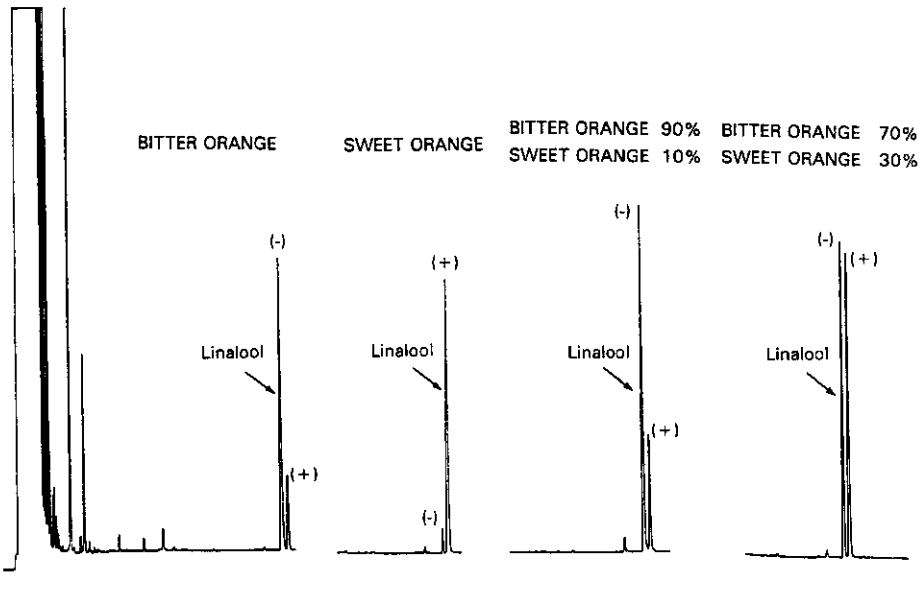


**Figure 22. Enantiomeric distribution of linalool for Italian bitter and sweet orange oil and for samples of bitter orange oil with sweet orange oil added**

**Table XVI. Enantiomeric ratios of linalool and terpinen-4-ol for lemon and mandarin oils**

| | Lemon oils | | Mandarin oils | |
|---|---|---|---|---|
| | Cold-pressed | Distilled | Cold-pressed | Distilled |
| (–)/(+)-linalool | 54/46 | 53/47 | 17/83 | 17/83 |
| (+)/(–)-terpinen-4-ol | 20/80 | 28/12 | 13/87 | 27/73 |

**Table XVII. Percentage content of linalool and terpinen-4-ol for lemon and mandarin oils**

| | Lemon oils | | Mandarin oils | |
|---|---|---|---|---|
| | Cold-pressed | Distilled | Cold-pressed | Distilled |
| linalool | 0.10 | 0.19 | 0.13 | 0.21 |
| terpinen-4-ol | 0.02 | 0.35 | 0.04 | 0.35 |

**Table XVIII. Enantiomeric ratios of linalool for bitter orange oils, sweet orange oils, mandarin oils and mixtures of bitter and sweet orange oils**

| | (–) | (+) |
|---|---|---|
| **Bitter and sweet orange oils and mixtures** | | |
| bitter orange oils (Italy) | 83 | 17 |
| sweet orange oils (Italy) | 7 | 93 |
| bitter orange oils 95% sweet orange oils 5% | 75 | 25 |
| bitter orange oils 90% sweet orange oils 10% | 68 | 32 |
| bitter orange oils 80% sweet orange oils 20% | 58 | 42 |
| bitter orange oils 70% sweet orange oils 30% | 48 | 52 |
| bitter orange oils (Spain) | 82 | 18 |
| bitter orange oils (Brazil) | 68 | 32 |
| bitter orange oils (Ivory Coast) | 67 | 33 |
| **Italian sweet orange oils** | | |
| *Blond oranges* | | |
| Biondo comune | 8 | 92 |
| Navelina | 9 | 91 |
| Washington navel | 8 | 92 |
| Valencia late | 9 | 91 |
| Ovale | 5 | 95 |
| *Red oranges* | | |
| Moro | 6 | 94 |
| Tarocco | 5 | 95 |
| Sanguinello | 5 | 95 |
| **Mandarin oils** | | |
| Mandarin oils (Italy) | 16 | 84 |
| Mandarin oils (Uruguay) | | |
| Malvasio | 12 | 88 |
| Ellendale | 14 | 86 |
| Ortanique | 16 | 84 |
| Comun | 21 | 79 |
| Malaquina | 30 | 70 |

enantiomeric distribution of terpinen-4-ol is different in cold-pressed and distilled mandarin and lemon oils. These differences, therefore, distinguish the more valuable cold-pressed oils from the distilled versions.

Figure 22 shows the HRGC chromatogram of the linalool-containing HPLC-transferred fraction for a bitter orange oil, a sweet orange oil and for two mixtures obtained by adding sweet orange oil to bitter orange oil.

Table XVIII reports the enantiomeric distributions of linalool for bitter orange oils, sweet orange oils, mandarin oils and mixtures of bitter and sweet orange oils.

Bitter orange oil is more valuable than sweet orange oil. It happens sometimes that bitter orange oil contains a small amount of sweet orange oil, either deliberately added or due to the involuntary presence of sweet orange fruits among bitter oranges processed industrially.

The enantiomeric ratio between (–)-linalool and (+)-linalool in sweet orange oil is about 7:93, while that of bitter orange oil is about 83:17. These very different ratios allow the detection of contamination by as little as 5% of sweet orange oils in the more valuable bitter orange oils.

The linalool enantiomeric ratio is similar for Italian and Spanish bitter orange oils, and for Brazilian and Ivory Coast bitter orange oils, but considerable differences are evident between the two groups of oils.

The linalool enantiomeric distribution for Italian sweet orange oils and Uruguayan mandarin oils depends on the cultivar of the fruits. In Italian oils, the (–)-linalool/(+)-linalool ratio is generally higher in blond oils, except for the blond oil from cv. Ovale, which shows values similar to the red oils. The (–)-linalool/(+)-linalool ratio for Uruguayan mandarin oils ranges from 12:88 (cv. Malvasio) to 30:70 (cv. Malaquina). The oils obtained from cv. Ortanique show a ratio similar to that of Italian mandarin oils (16:84).

## Conclusion

Coupled LC-GC has been demonstrated to be an excellent method for the analysis of complex mixtures, such as essential oils. The further coupling of the LC-GC system with a mass spectrometer enhances the power of the system, and permits a more reliable identification of components than a GC/MS system.

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