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MULTIDIMENSIONAL ADVANCED TECHNIQUES FOR THE ANALYSIS OF BERGAMOT OIL

RIASSUNTO - *Vengono riportati i risultati relativi allo studio della composizione dell'olio essenziale di bergamotto ottenuti con i seguenti metodi di analisi:*
- *un sistema HPLC-HRGC/MS (Cromatografia Liquida ad Alte Prestazioni-Cromatografia Gassosa ad alta Risoluzione/Spettrometria di Massa) che ha permesso la pre-separazione HPLC dell'olio in classi di sostanze seguita dal trasferimento di queste frazioni in gascromatografia e rivelazione FID e MS.*
- *un sistema HRGC/MS (Cromatografia Gassosa ad alta Risoluzione/Spettrometria di Massa) munito di una libreria costruita in laboratorio che contiene sia spettri di massa che gli indici di ritenzione lineare di circa 200 composti presenti negli olii essenziali. Gli indici di ritenzione lineare sono stati calcolati sia su una colonna polare che su una apolare e possono essere usati interattivamente con la ricerca degli spettri di massa.*
- *un sistema accoppiato HRGC-HRGC (Cromatografia Gassosa ad Alta Risoluzione-Cromatografia Gassosa ad Alta Risoluzione) per trasferimenti multipli.*

Parole chiave: *Bergamotto, HPLC-HRGC/MS, HRGC-HRGC*

RÉSUMÉ - *On reporte ici les résultats de l'étude sur la composition de l'huile essentielle de bergamote. Ils ont été obtenus avec les méthodes qui suivent:*
- *on a utilisé un système "on-line" de CLHP-CGHR/SM (Chromatographie Liquide Haute Performance - Cromatographie Gazeuse Haute Resolution/Spectrométrie de Masse) qui permet la pré-séparation de l'huile en classes de substance par gradient d'élution, suivie du transfert de cette fraction dans une colonne capillaire et de la détermination par spectrométrie de masse.*
- *on a aussi utilisé un système CGHR/SM fourni d'une collection de spectres de 200 composés différents, présents dans les huiles essentielles, et des valeurs des Indices de Rétention Linéaires calculés soit pour colonnes polaires que apolaires, qui peuvent être utilisés interactivement avec le données de la spectrométrie de masse.*
- *enfin, on a utilisé un système completement automatisé de CGHR-CGHR.*

Mots clés: *Bergamote, HPLC-HRGC/MS, HRGC-HRGC.*

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SUMMARY - *Are reported the results of the study of the composition of bergamot essential oil obtained with the following methods of analysis:*
- *an on-line HPLC-HRGC/MS (High performance Liquid Chromatography-High Resolution Gas Chromatography/Mass Spectrometry) system that allows the pre-separation by HPLC of the oil into classes of compound by gradient elution followed by the transfer of these fraction into a capillary GC column and detection by MS and FID.*
- *a HRGC/MS (High Resolution Gas Chromatography/Mass Spectrometry) system equipped with an home-made library which contains the spectra of about 200 compounds present in essential oils and the values of Linear Retention Indices calculated both on a polar and on an apolar column, to be used interactively with mass data.*
- *a fully automated HRGC-HRGC (High Resolution Gas Chromatography-High Resolution Gas Chromatography) system for multitransfer operations.*
Key words: *Bergamot, HPLC-HRGC/MS, HRGC-HRGC.*

INTRODUCTION

Citrus essential oils obtained from the peel of fruit are used in the food and perfume industries. They are mixtures of more 200 components (1), that can be grouped essentially into two fractions:

- a volatile fraction, that constitutes 93-96% of the whole oil, and contains the monoterpenes and sesquiterpenes hydrocarbons and their oxygenated derivatives along with aliphatic aldehydes, alcohols and esters;
- a non volatile residue, that ranges from 4% to 7% of the whole oil, and contains hydrocarbons, fatty acids, sterols, carotenoids, waxes, coumarins, psoralens and flavanoids.

The analysis of the components of the volatile fraction of an essential oil is characterised by the complexity of the separation of the components which belong to different classes of compounds and are present in a wide range of concentrations. Many components cannot be resolved in a single GC analysis and the best approach is to fractionate the essential oil before the gas chromatographic analysis. The simpler, more homogeneous mixtures so obtained are easier to resolve without problems of co-elution. Prefractionating methods, such as open column liquid chromatography on silica gel or alumina, HPLC, vacuum distillation or preparative gas chromatography, have been described. All these methods have the drawbacks of an off-line pre-separation: they entail numerous steps which introduce the danger of contamination or loss of sample. Whatever off-line method is employed for solvent evaporation before the injection of the sample into GC, loss of even high boiling analytes is likely.

This paper reports the use of different multidimensional techniques that are HPLC-HRGC/MS, HRGC/MS with Linear Retention Indices, HRGC-HRGC. These techniques were successfully applied to the analysis of bergamot essential oil.

HPLC-HRGC/MS

High Performance Liquid Chromatography (HPLC) coupled to High Resolution Gas Chromatography (HRGC) is one of the most current powerful analytical techniques because of its selectivity and sensitivity in the analysis of complex mixtures. On-line coupling (2-8) permits the separation and identification of compounds of the same polarity in mixtures of compounds of different polarity even when the concentrations of the various classes of compounds are considerably different. Moreover, there is no sample pre- or post-treatment as the separation analysis is fully automated. In comparison with off-line methods, on-line high performance liquid chromatography-high resolution gas chromatography (HPLC-HRGC) 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 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 an automated HPLC-HRGC system the analysis with the so-called retention gap transfer technique is possible. This technique is based on the mechanical stabili-

sation of the liquid using an uncoated pre-column at the entrance of the GC system until all solvent is evaporated. The retention gap method (9) 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*) (10). 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 short uncoated pre-column or to transfer larger fraction volumes. This method is the so-called *partially concurrent evaporation* (11): part of the eluent is evaporated concurrently, that is, during its introduction into GC. The introduction of an early-vapour exit greatly improves partially concurrent evaporation and protects the GC detector.

The coupling of a mass spectrometer to an HPLC-HRGC instrument greatly enhances the detection capabilities of the system and allows components to be reliably identified. The HPLC preseparation into classes of compounds eliminates interferences from co-eluted peaks and produces better resolved gas chromatograms. The mass spectra obtained with this system can be more easily interpreted than those obtained with a simple HRGC/MS system.

HRGC/MS with Linear Retention Index

The main approaches to the identification of an "unknown" from its mass spectrum consist in searching in a library of reference mass spectra for the list of compounds whose mass spectrum is similar or very close to that of the "unknown" spectrum. Commercially available libraries may not be the most reliable for several reasons, such as (i) inaccurate or non-existence of experimental conditions. This is particularly the case with certain data banks whose spectra come from the literature or from different instruments. According to whether a quadrupole, a magnetic, or an ion trap apparatus is used, significant differences can be observed; or (ii) very different spectra for the same product. Significant differences between various spectra of the same product may not be ascribed to experimental procedures, but to erroneous interpretation and sampling; or (iii) display of several spectra for the same product reported more than one time with different names (systematic and/or common) or CAS number. Difficulties increase when a complex mixture is analysed because of the presence of components with similar structures corresponding to very similar spectra. Hence, identification by MS should always be accompanied by information on retention time that may support the MS result.

The retention time of a solute varies with temperature and with flow rate, and it is almost impossible to reproduce the retention time for a solute.

The measurement of relative retention time may be done using a reference solute.

The most commonly used homologous series consist of n-paraffin hydrocarbons as first applied by Kovats (12) for isothermal analysis. Because in isothermal analysis the logarithm of the adjusted retention times is proportional to the carbon number of homologues, while the relationship is linear in temperature programming, Van Den Dool and Kratz (13) applied a linear equation for temperature programming GC analyses.

Our approach (14) has been to build a MS library using about 200 pure standards found on the market or separated in the laboratory. This data bank has been augmented with Linear Retention Indices calculated on two GC columns (SE-52 and Carbowax 20M). The calculated Linear Retention Indices were used interactively as a filter in conjunction with the spectra library.

HRGC-HRGC and HRGC-HRGC-MS

Multi-Dimensional Gas Chromatography (MDGC) has been used for many years in many different ways. According to Bertsch (15), the techniques which combine two separately controlled gas chromatographic separation systems can be defined as "two dimensional gas chromatographic techniques". The transfer of one or more selected groups of compounds eluted from a gas chromatographic column onto a second column is usually referred to as "heart-cutting". The main application of heart-cutting is the optimisation of chromatographic resolution, where non-resolved sample components eluted from the first column are selectively diverted to a second column of higher efficiency or better selectivity. At the same time, it is possible to collect information on the retention time in two different stationary phases, which facilitates the identification of the compounds. We have built a multidimensional GC system (16-18) for multitransfer purposes, based on a high temperature valve to heart-cut fractions from the first capillary column to a second capillary column with a hot transfer line and a system to maintain a constant flow during the transfer.

EXPERIMENTAL

The research was carried out on a genuine cold-pressed bergamot essential oil obtained from " Consorzio del Bergamotto", Reggio Calabria, Italy.

The oil has been analysed by HPLC-HRGC/MS and by HRGC/MS with Linear Retention Indices to determine its qualitative composition.

Moreover, the oil has been analysed by HRGC-HRGC using a chiral column in the second GC oven, to determine the enantiomeric distribution of some monoterpene hydrocarbons and monoterpene alcohols.

HPLC-HRGC/MS

A fully automated instrument (Dualchrom 3000 Series, CE Instruments, Rodano, Milan, Italy) was used for on-line pre-separation by HPLC and further separation by capillary HRGC. The instrument was configured to use an "on-column" interface, permitting "partially concurrent solvent evaporation", with an early solvent vapour exit system for the reduction of the mobile phase evaporation time.

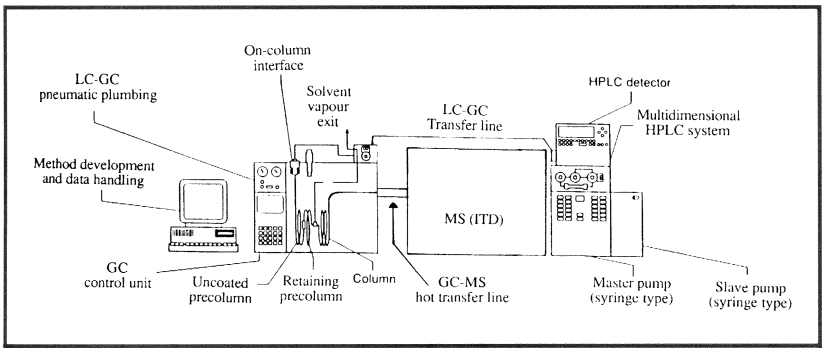
A schematic diagram of the HPLC-HRGC/MS interface is shown in Scheme 1. A ten-port valve allows cleaning of the HPLC column by backflushing from time to time. Analysis was carried out under computer control throughout, with step gradient elution to separate and transfer the fractions to HRGC. 20 µl of solution (0.2% v/v essential oil/pentane) were injected into a 10 cm x 2 mm i.d. column packed with Spherisorb 5 µm silica (Stagroma, Tubingen, FRG).

Chromatography was started with pentane as mobile phase for 3 min; the composition of the mobile phase was then changed to pentane-ether, 97:3, for 9 min, then 90:10 for 5 min, then 85:20 for 10 min; the column was then back-flushed with diethyl ether (1 ml). The mobile phase flow rate was 180 µl/min. Detection was by Micro UVis at 220 nm x 0.5 AUFS. The retention window was determined using standard substances.

Gas chromatography was performed on a 21 m x 0.32 mm i.d. fused silica capillary column coated with a 0.40-0.45 µm film of SE-52 (MEGA, Legnano, Italy). The column inlet was connected, by means of a butt connector with purge line, to a 'retaining precolumn' comprising 4 m of the separation column; the pre-column was connected, by means of a press-fit connector (MEGA) to a 10 m x 0,53 mm i.d. uncoated fused silica pre-column (retention gap), deactivated by phenyldimethyl silylation.

The column oven temperature was maintained at 45°C for 6 min during transfer of the LC fraction, and then increased to 220°C at 3°C/min; detection was by flame ionisation and mass spectrometry. The carrier gas (He) was delivered at a constant pressure of 120 kPa.

Mass spectra were obtained on a Finnigan ITD mass spectrometer,



Scheme 1 - Schematic of the HPLC-HRLC/MS instrument.

Model 800, directly coupled to the LC-GC system described above 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 full scan, 41-300 amu scan range, 1.0 s scan time, 1 count threshold, AGC mode "on" 5 micro scans, 240 s filament delay, 2200 V multiplier depending on multiplier condition, 250°C transfer line, 250°C exit nozzle, and 250°C manifold.

HRGC/MS with Linear Retention Indices

The analyses were carried out by HRGC/MS (EI) on a CE Instruments MD 800 (Rodano, Milan, Italy) system coupled with commercially library and a home-made library (FFC). HRGC conditions for the apolar column were: HRGC column SE-52, 60 m x 0.32 mm i.d. 0.40-0.45 µm film thickness (MEGA, Legnano, Italy); the temperature was kept at 45°C for 6 min, then increased to 111°C at a rate of 3°C/min, then to 160 °C at 2°C/min and at 3°C/min to 300°C and held for 15 min. Carrier gas (He) was delivered at constant pressure of 70 kPa (40.5 cm/s). HRGC conditions for the polar column were: HRGC column Carbowax 20 M (Megawax), 60 m x 0.32 mm i.d. 0.40-0.45 µm film thickness (MEGA, Legnano, Italy); the temperature was kept at 45°C for 3 min, then increased to 300°C at a rate of 3°C/min and held for 20 min. Carrier gas (He) was delivered at constant pressure of 70 kPa (36.3 cm/s). For both columns 1 µl of solution (0.02% v/v standard/pentane) and 1 µl of solution (0.33% v/v essential oil/pentane) were injected on a cold on-column system fitted with an automated actuator. The MS scan conditions were: source temperature: 200°C; interface temperature: 260°C; E energy: 70 eV; mass scan: 39.00-350.00 amu.

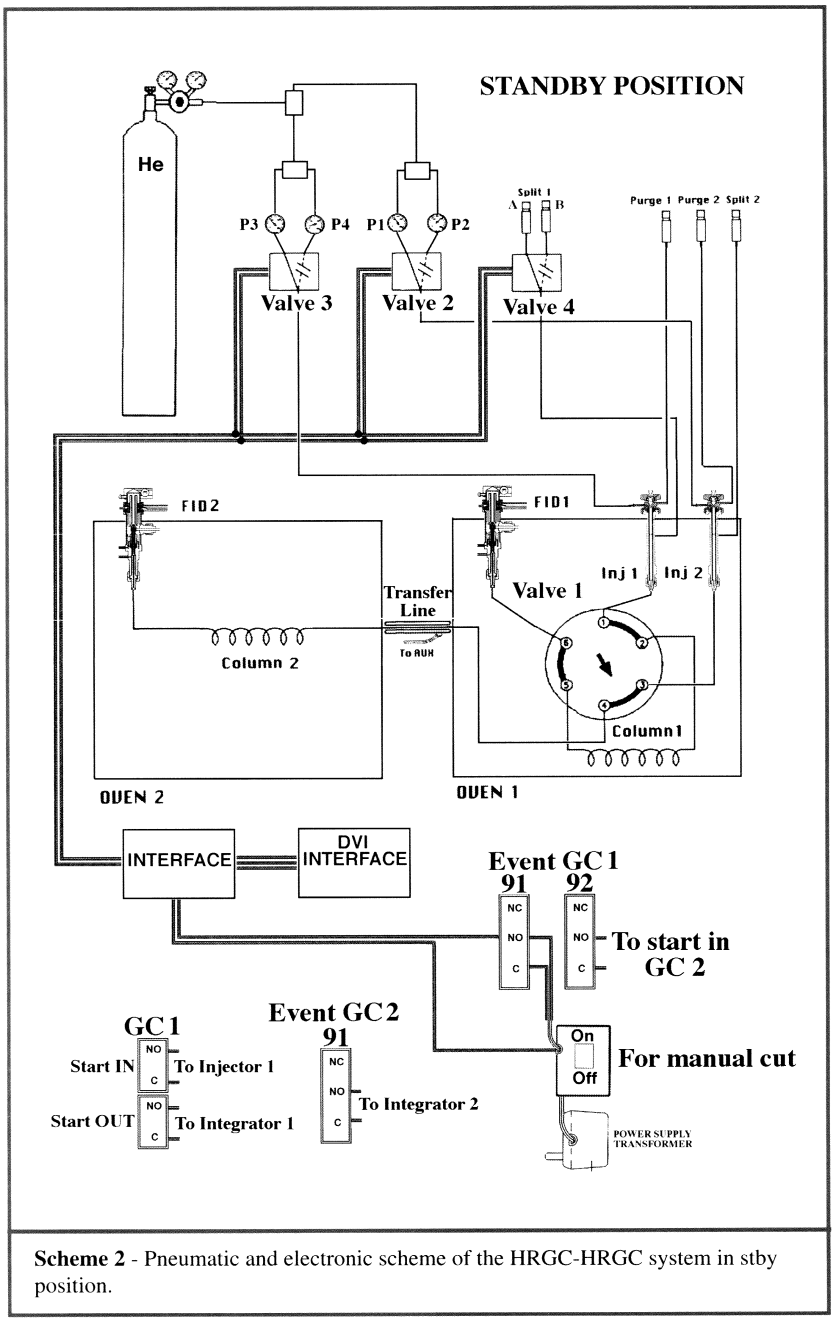
HRGC-HRGC

The sample of bergamot oil was analysed by multidimensional GC injecting 1 µl of a 10% (v/v) solution of essential oil in pentane with a split ratio of 1:10.

The multidimensional system used in this study was a developmental model, formerly described in detail (16-17), which consisted of two gas chromatographs Shimadzu 17A, a transfer line and two integrators Shimadzu C-R3A. The instrumental set-up and the experimental conditions used were as follows (see Schemes 2 and 3):

Gas chromatograph 1

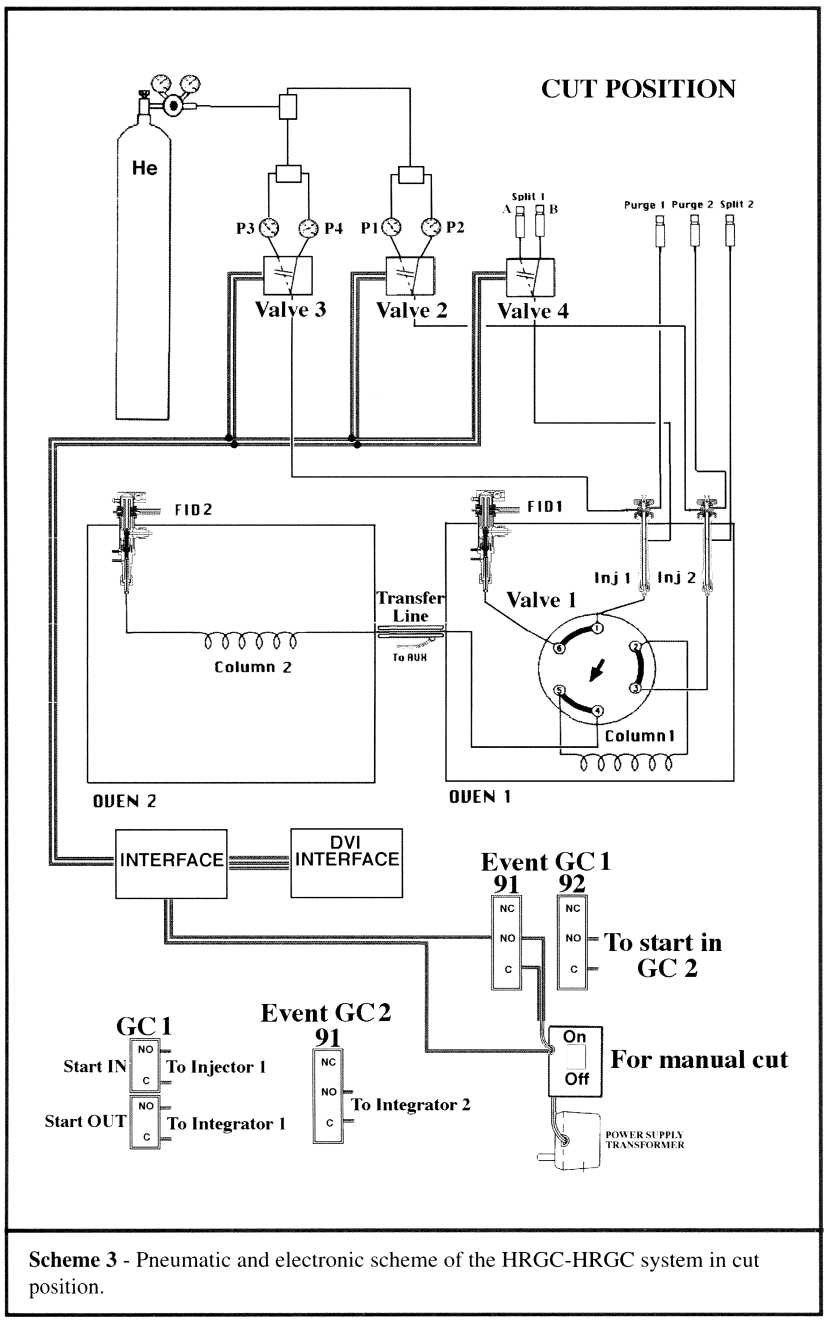
- Two split/splitless injectors at 250°C with two manual flow controllers (injectors 1, 2) and a FID at 250°C (FID 1);
- a SE-52 capillary column 30 m x 0.32 mm i.d. 0.40-0.45 µm film thickness (Mega, Legnano, Italy). Temperature program.: 45°C for 6 min then to 240°C at 2.0°C/min; carrier He 90 KPa (2.7 ml/min);
- a Valco 6-ports (1/16") two-position UW type valve (Valve 1) with a right angle drive (A3RADN6WT) (Valco Europe); the valve has a rotor made of fluorocarbon-filled crosslinked polyimide, a port diameter of 0.40 mm and can be operated up to



Scheme 2 - Pneumatic and electronic scheme of the HRGC-HRGC system in stby position.

350°C. Moreover, this valve is designed for use with fused silica columns using a special adapter (Valco FSR.5-5) consisting of a liner which slides over the fused silica tubing and a ferrule which makes up on the liner;

- a digital valve interface (DVI-220) (Valco Europe) connected to EVENT 91 on gas chromatograph 1;
- 1/16" removable fused silica adapters (FSR.5-5 and FSR.4-5) (Valco Europe) to connect the valve and the fused silica tubing;
- a solenoid valve (Valve 2) to change the carrier pressure (P1, 110 KPa) (stby position, column 2) to higher pressure (P2, 195 KPa) (cut position, column 1 and 2) connected to EVENT 92 on gas chromatograph 1. This ensures that the right retention time, are obtained on column 1, even for those components eluted after more than on transfer;
- a solenoid valve (Valve 3) to change the carrier pressure (P3, 90 KPa) (stby position, column 1) to lower pressure (P4, 5 KPa) (cut position, injector 1 and FID 1) connected to EVENT 91 on the gas chromatograph 1. This allows to maintain a constant flow in detector FID 1 and protecting it from flow surges due to absence of column 1 in the flow path when the system is in the cut position;
- a solenoid valve (valve 4) which allows the use of two splitter valves (A and B) with different ratios in the



Scheme 3 - Pneumatic and electronic scheme of the HRGC-HRGC system in cut position.

injector 1. This valve allows splitter 1A to be used in the stby position (split ratio for the sample introduction) and splitter 1B in the cut position (high split ratio to rapidly establish pressure P4);
- an integrator Shimadzu C-R3A connected to start and out signals on gas chromatograph 1.

TRANSFER LINE

An aluminium thermoregulated block equipped with a heater assay and a thermocouple assy connected to the AUX2 exit on gas chromatograph 1.

Gas chromatograph 2

- a MEGADEXDETTBSß (Diethyl-tert-butylsilyl-ß-cyclodextrin) 25 m x 0.25 mm i.d. 0.25 µm film thickness (Mega, Legnano, Italy). Temperature program.: 45°C x 6 min then to 180°C at 2.0°C/min; carrier He 110 KPa (1.9 ml/min); the GC program started with the first cut.
- a FID at 250°C (FID 2);
- an integrator Shimadzu C-R3A connected to start and out signals on gas chromatograph 2.

As shown in Schemes 2 and 3, the system is completely automated by the use of the external events of the gas chromatograph. The time at which the valve should be switched to begin the cuts can be determined from a preliminary analysis. After this a fully-automated analysis is possible by programming the valve events.

HRGC-HRGC/MS

The HRGC-HRGC/MS analyses were carried out using the same system described above. In this case the second column was a Carbowax 20 M 25 m x 0.25 mm with a 0.25 µm film thickness. The temperature program for this column was 45°C for 6 min then increased to 180°C for 10 min. Moreover, the acquisition was by MS using a Shimadzu QP 5000 apparatus with the following experimental conditions: interface temperature, 250°C; mass range, 41.00-300.00; scan interval, 1.00 s; threshold, 500; detector gain, 1.5 kV.

RESULTS AND DISCUSSION

HPLC-HRGC and HPLC HRGC/MS analysis

Figure 1 shows the LC chromatogram of the bergamot essential oil. The transferred fractions are marked with F1, F2, F3, and F4, and the transfer times for each fraction are listed in Table 1 with the time of the vapour exit closure. Figures 2 and 3 show the HRGC chromatograms of the HPLC fractions. Above each chromatogram of the transferred fraction is shown the on-column HRGC chromatogram of the whole oil obtained with the same column systems. The compounds identified are reported in Table 2. As results, the HPLC pre-separation of the oil gives mixtures much simpler

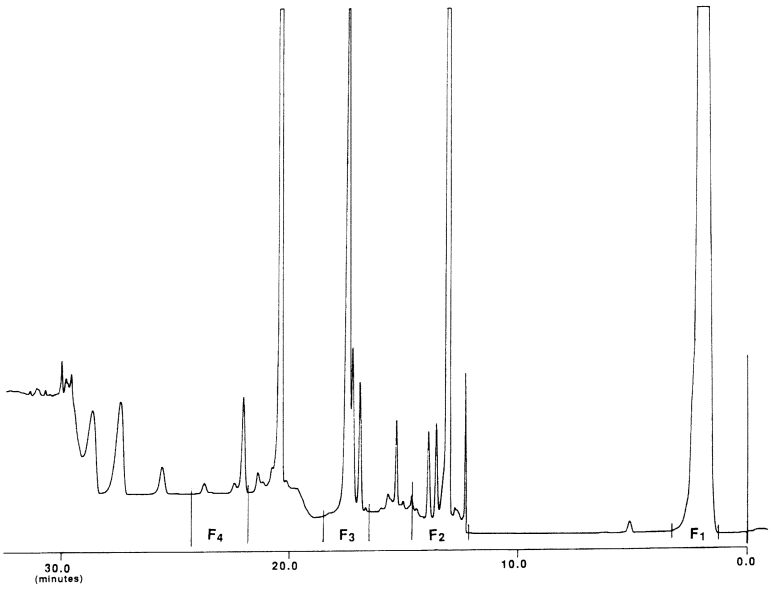


Figure 1 - HPLC chromatogram of a bergamot oil.

Fraction	Transfer time (min)	Time at which solvent vapor exit was closed (min)
F1 (hydrocarbons)	from 1.0 to 3.0	3.8
F2 (aliphatic aldehydes and esters)	from 12.0 to 15.0	16.5
F3 (monoterpene aldehydes and alcohols)	from 17.0 to 19.0	19.9
F4 (alcohols)	from 21.0 to 23.5	24.9

Table 1 - HPLC retention windows of the fractions transferred to the HRGC with the time of the vapor exit closure for each fraction.

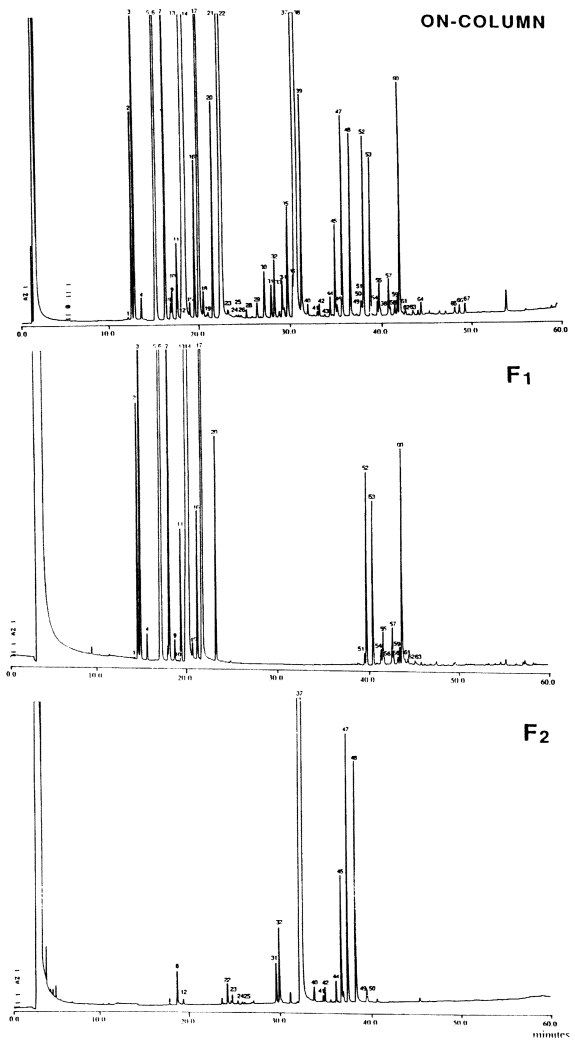


Figure 2 - HRGC chromatogram of a bergamot oil and of the F_1 and F_2 fractions from its HPLC separation.

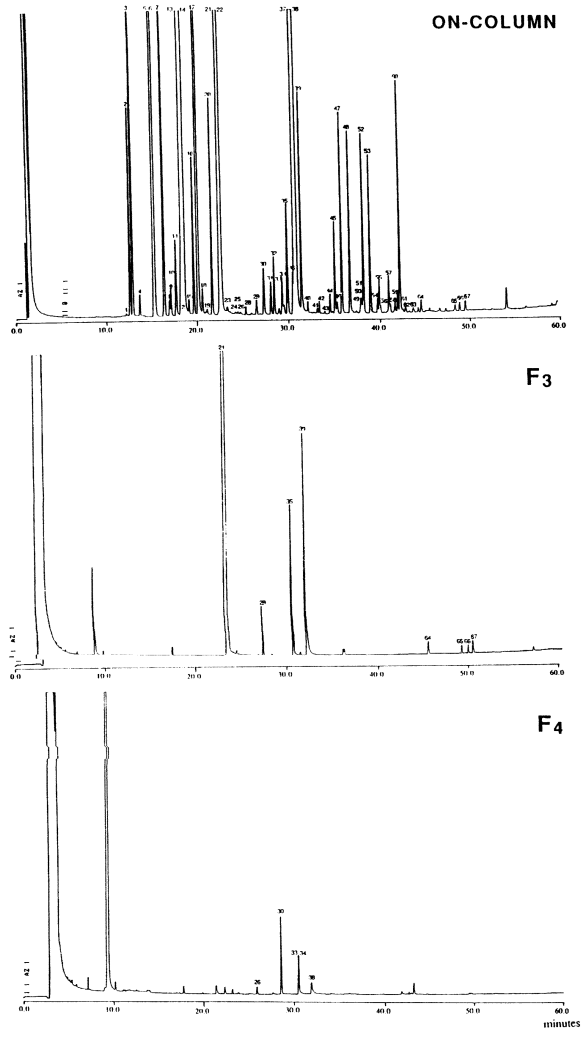


Figure 3 - HRGC chromatogram of a bergamot oil and of the F_3 and F_4 fractions from its HPLC separation.

Peak N°	Compound	Peak N°	Compound
1	Tricyclene	35	Neral
2	a-Thujene	36	*cis*-Sabinene hydrate acetate
3	a-Pinene	37	Linalyl acetate
4	Camphene	38	Geraniol
5	Sabinene	39	Geranial
6	ß-Pinene	40	Bornyl acetate
7	Myrcene	41	Undecanal
8	Octanal	42	Nonyl acetate
9	a-Phellandrene	43	Methyl geranoate
10	d-3-Carene	44	Linalyl propionate
11	a-Terpinene	45	Terpinyl acetate
12	Hexyl acetate	46	Citronellyl acetate
13	*p*-Cymene	47	Neryl acetate
14	Limonene	48	Geranyl acetate
15	(Z)-ß-Ocymene	49	Dodecanal
16	(E)-ß-Ocymene	50	Decyl acetate
17	g-Terpinene	51	*cis*-a-Bergamotene
18	*trans*-Sabinene hydrate	52	ß-Caryophyllene
19	Octanol	53	*trans*-a-Bergamotene
20	Terpinolene	54	a-Humulene
21	Linalol	55	*cis*-ß-Farnesene
22	Nonanal	56	ß-Santalene
23	Heptyl acetate	57	Germacrene D
24	*cis*-Limonene oxide	58	Germacrene B
25	*trans*-Limonene oxide	59	Unknown sesquiterpene
26	Isopulegol	60	ß-Bisabolene
27	Camphor	61	*cis*-g-Bisabolene
28	Citronellal	62	Unknown sesquiterpene
29	Terpinen-4-ol	63	g-Elemene
30	a-Terpineol	64	*trans*-Nerolidol
31	Decanal	65	2,3-Dimethyl-3-(4-methyl-3-pentenyl)-2-norbornanol
32	Octyl acetate	66	Campherenol
33	Nerol	67	a-Bisabolol
34	Citronellol		

Table 2 - List of the compounds identified in bergamot oil by HPLC-HRGC/MS.

than the oil, which are also chemically more homogeneous. These fractions give chromatograms in which all of the peaks are better separated and more easily identified by retention times or MS. Figure 4 presents an enlargement of the first part of the total ion current chromatogram of fraction F1, showing the separation of the monoterpenes with the HPLC-HRGC/MS system. Figure 5 shows the comparison of the mass spectrum of peak 9, which is a-phellandrene, obtained by HPLC-HRGC/MS with the library spectrum; it can be seen that the two spectra are very similar once the interfering peak (octanal) is removed by the pre-separation by HPLC. As can be seen in Table 2, with the HPLC-HRGC/MS analysis, 67 components have been identified in the bergamot essential oil by comparison of the mass spectra with those of commercial libraries.

HRGC/MS with Linear Retention Indices analysis

Figure 6 shows the total ion current chromatogram of the bergamot essential oil obtained with the HRGC/MS system equipped with a SE-52 column. The identified compounds are reported in Table 3. In Figure 7 the total ion current chromatogram of the bergamot essential oil obtained with the same system but on a Carbowax 20 M column is shown, with peak identification in Table 4. As can be

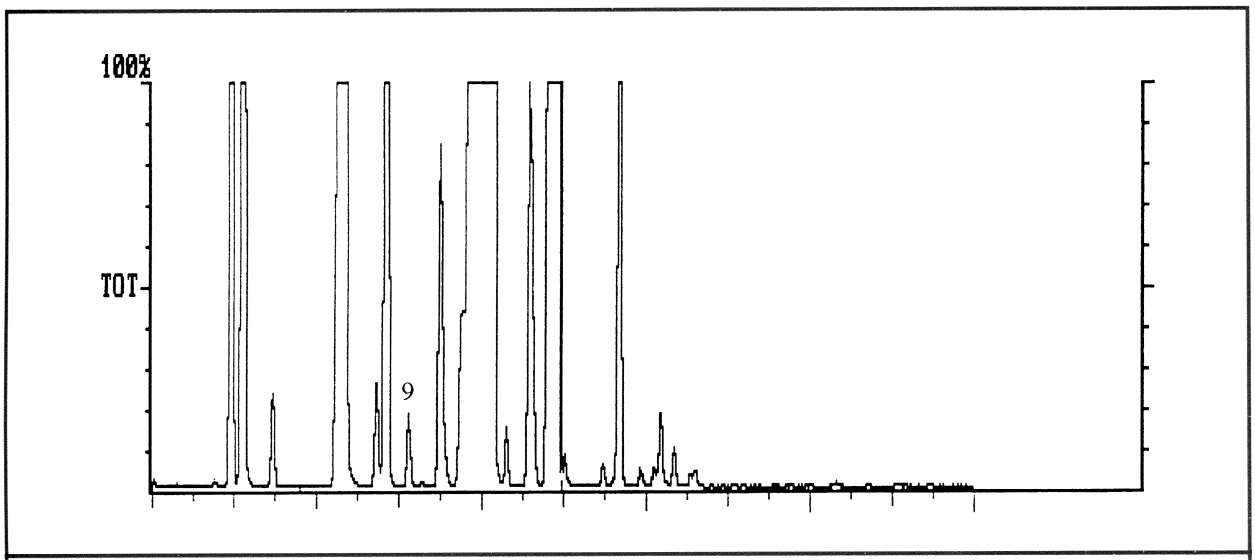


Figure 4 - Total ion current chromatogram obtained by HPLC-HRGC/MS of the first part of the F1 fraction.

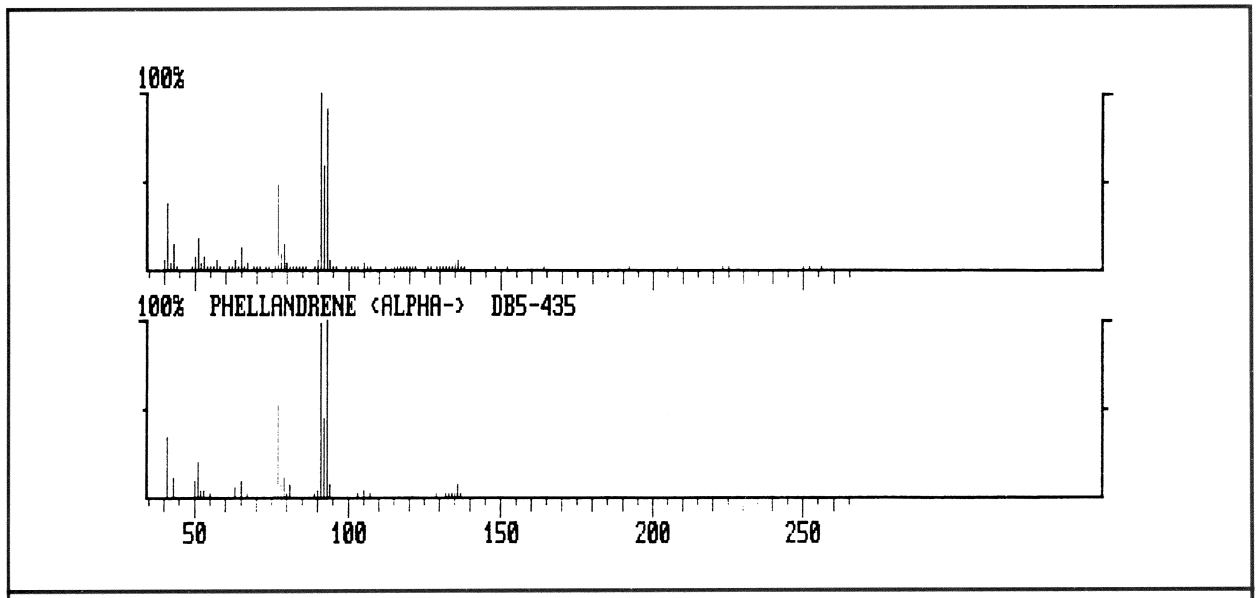


Figure 5 - MS (ITD) spectra of a-phellandrene [9]. (A) Observed and (B) library spectrum.

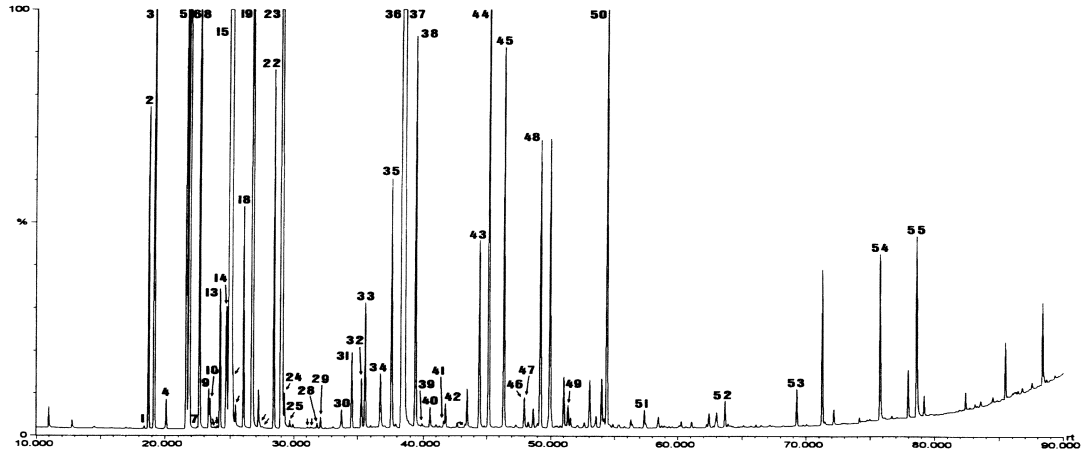


Figure 6 - Total ion current chromatogram of a bergamot oil on SE-52 column.

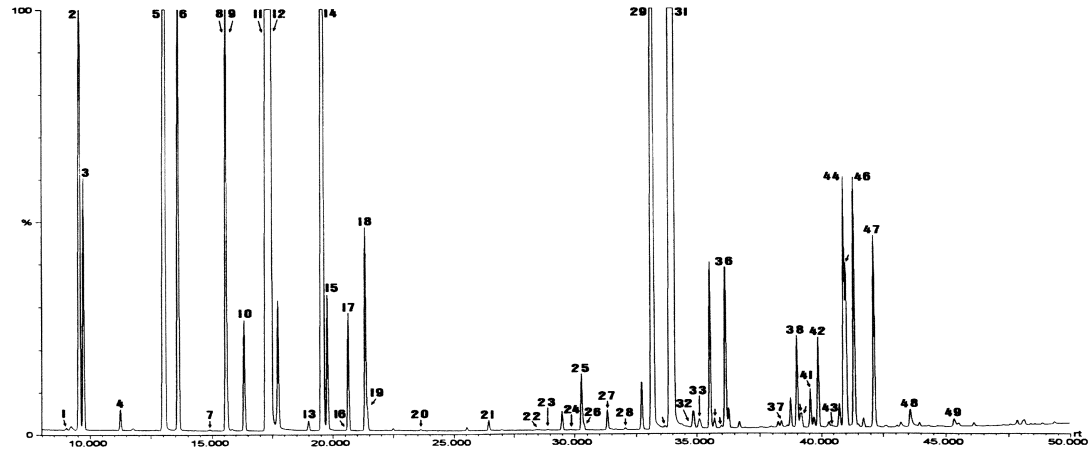


Figure 7 - Total ion current chromatogram of a bergamot oil on Carbowax 20 M column.

Peak N°	Compound	Peak N°	Compound
1	Tricyclene	29	Citronellal
2	a-Thujene	30	Terpinen-4-ol
3	a-Pinene	31	a-Terpineol
4	Camphene	32	Decanal
5	Sabinene	33	Octyl acetate
6	ß-Pinene	34	Nerol
7	6-Methyl-5-hepten-2-one	35	Neral
8	Myrcene	36	Geraniol
9	Octanal	37	Linalyl acetate
10	a-Phellandrene	38	Geranial
11	d-3-Carene	39	Perilla aldehyde
12	Hexyl acetate	40	Bornyl acetate
13	a-Terpinene	41	Undecanal
14	*p*-Cymene	42	Nonyl acetate
15	Limonene	43	Terpinyl acetate
16	1,8-Cineole	44	Neryl acetate
17	(Z)-ß-Ocymene	45	Geranyl acetate
18	(E)-ß-Ocymene	46	Dodecanal
19	g-Terpinene	47	Decyl acetate
20	Octanol	48	(E)-Caryophyllene
21	(Z)-Linalool oxide (furanoid form)	49	a-Humulene
22	Terpinolene	50	ß-Bisabolene
23	Linalol	51	*trans*-Nerolidol
24	Nonanal	52	a-Bisabolol
25	Heptyl acetate	53	Nootkatone
26	*cis*-Limonene oxide	54	Citropten
27	*trans*-Limonene oxide	55	Bergapten
28	Camphor		

Table 3 - Compound identifications on the SE-52 column by HRGC/MS analysis for bergamot oil.

seen by comparing Figures 6 and 7, and Tables 3 and 4 the apolar column offers a better resolution than the polar column. In Figure 6 it is possible to distinguish four different zones: the first (from about 18-29 min) represents the monoterpene hydrocarbons; the second (from about 29-49 min) the oxygenated monoterpenes and the aliphatic oxygenated compounds; the third (from about 49-60 min) the sesquiterpene hydrocarbons; and the fourth (from about 60-90 min) the sesquiterpene alcohols and other some high-boiling oxygenated compounds. The chromatogram obtained with the Carbowax 20 M column (Figure 7) shows only two zones: the first (from about 6-23 min), where the monoterpenes hydrocarbons are eluted, and the second (from about 23-50 min) corresponding to co-eluted sesquiterpene hydrocarbons and oxygenated compounds. This is probably because sesquiterpene hydrocarbons have about the same retention times on both the columns while the oxygenated compounds are eluted later in the polar column. For these reasons we suggest the use of the apolar column, using the polar column to check peaks that are overlapped on the apolar column. We have equipped the HRGC/MS system with an home-made library, called FFC ("Flavour and Fragrance Compounds") by injecting on the two different columns (SE-52 and Carbowax 20 M) about 200

Peak N°	Compound	Peak N°	Compound
1	Tricyclene	26	Citronellal
2	a-Pinene	27	Decanal
3	a-Thujene	28	Camphor
4	Camphene	29	Linalol
5	ß-Pinene	30	Octanol
6	Sabinene	31	Linalyl acetate
7	d-3-Carene	32	Nonyl acetate
8	Myrcene	33	Bornyl acetate
9	a-Phellandrene	34	Terpinen-4-ol
10	a-Terpinene	35	Undecanal
11	Limonene	36	ß-Caryophyllene
12	1,8-Cineole	37	Citronellyl acetate
13	(Z)-b-Ocymene	38	Neral
14	g-Terpinene	39	a-Humulene
15	(E)-ß-Ocymene	40	Decyl acetate
16	Hexyl acetate	41	a-Terpineol
17	*p*-Cymene	42	Terpinenyl acetate
18	Terpinolene	43	Dodecanol
19	Octanal	44	Neryl acetate
20	6-Methyl-5-hepten-2-one	45	Geranial
21	Nonanal	46	ß-Bisabolene
22	*cis*-Linalool oxide (furanoid form)	47	Geranyl acetate
23	*cis*-Limonene oxide	48	Nerol
24	*trans*-Linalool oxide (furanoid form)	49	Geraniol
25	Octyl acetate		

Table 4 - Compound identifications on the Carbowax 20 M column by HRGC/MS analysis for bergamot oil

standard compounds usually present in essential oils. Figure 8 reports an entry example of the FFC library. For each standard, the mass spectrum, the synonym, the systematic name used by Chemical Abstract, the Chemical Abstract Service Number (CAS), the molecular weight, the molecular formula, the structure and Linear Retention Indices on apolar column (value 1) and on polar column (value 2) have been appended. Commercial compounds and those separated in the laboratory have been checked by FT-IR and ^{1}H-HMR to determine the correct isomer before they were included in the FFC library. Table 5 reports the list of all the standard compounds injected, with the values of Linear Retention Indices on the two columns. Table 6 is an example of how the commercial libraries sometimes cannot furnish reliable results. It contains the list obtained by comparing the spectral data of compound 44 (see Figures 6 and 7) of bergamot essential oil with the commercial library. As can be seen from the upper part of this table (A), the compounds with a higher degree of purity are: (E)-3,7-dimethyl-2,6-octadien-1-ol acetate (geranyl acetate) with a forward fit of 924 and a reverse fit of 935; (Z)-3,7-dimethyl-2,6-octadien-1-ol acetate (neryl acetate) with a forward fit of 875 and a reverse fit of 889; followed by a monoterpene ester with a forward fit of 892 and a reverse fit of 838.

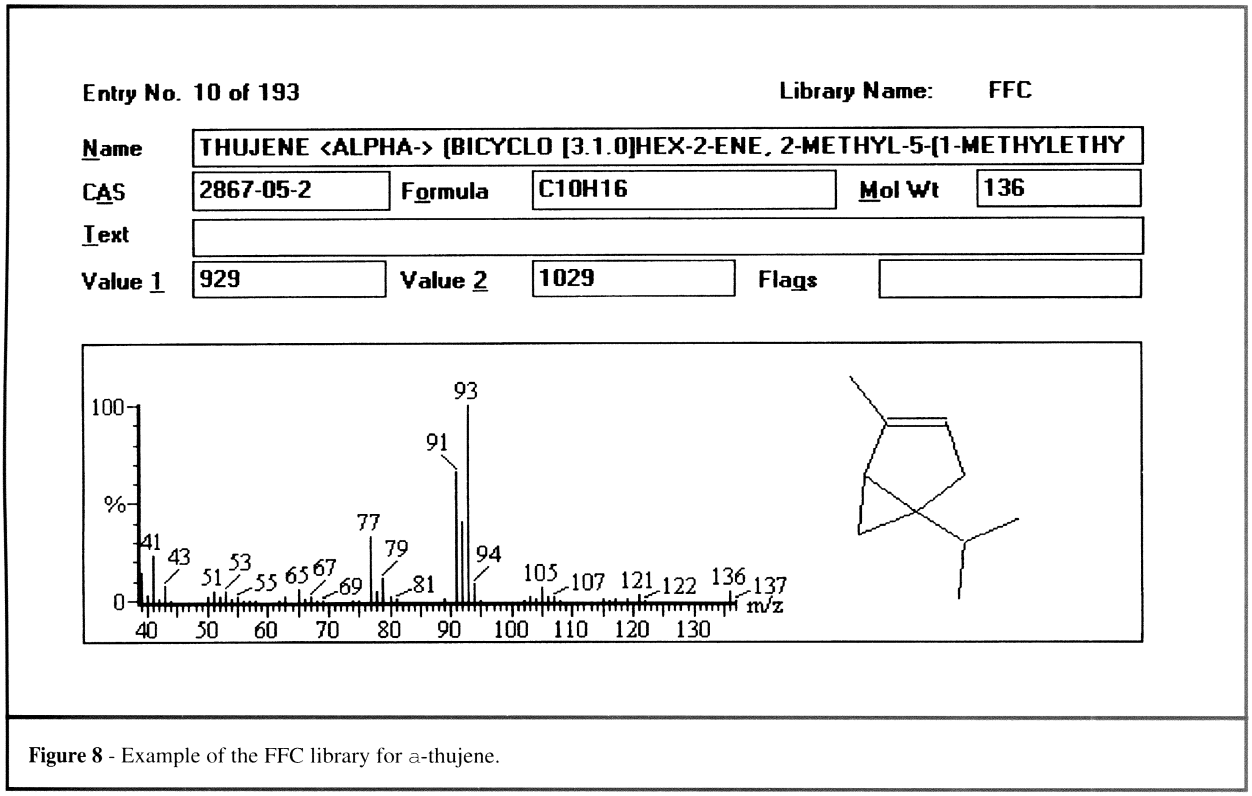


Figure 8 - Example of the FFC library for a-thujene.

Without any other information it is practically impossible to be sure which compound corresponds to the unknown compound, mostly for the first two choices. The same result is obtained using our library (Table 6, middle part (B)). The first two choices are still the same.

The lower part (C) of Table 6 shows the list obtained for the library search (for peak 44) using simultaneously the Linear Retention Index (on SE-52) and the mass spectra using a window of ±3 Linear Retention units for the match. The choice is reduced to only one compound (neryl acetate) quite different from the commercial library or the FFC library without using Linear Retention Indices, which both gave geranyl acetate as first choice. We have decided to use a window of ±3 Linear Retention units for the interactive search after a preliminary test with a selected component that was a-thujene. In fact, five subsequent injections of a-thujene gave a Linear Retention Index of 931.

Using an automatic system to open the port of the injector and to start the acquisition, a good repeatability is obtained.

Table 7 reports Linear Retention Indices for a-thujene in 8 different essential oils. Each essential oil contains a different amount of a-thujene, so the peak maximum changes a little from one chromatogram to another. The difference is very small (1 Linear Retention Unit).

Compound	$_{I}$T a	$_{I}$T b	Compound	$_{I}$T a	$_{I}$T b
2-Methyl-propanol	*	1075	*cis*-Linalol oxide (furanoid form)	1073	1430
2-Butanol	*	1009	Neroli aldehyde	1074	1365
3-Methyl-butanal	*	909	*trans*-Linalol oxide (furanoid form)	1088	1460
Butanol	*	1126	Terpinolene	1088	1280
Pentanal	*	968	Fenchone	1090	1391
2-Methyl-butanol	734	1193	Methyl benzoate	1095	1607
Pentanol	762	1235	Tetrahydro linalool	1097	1420
Hexanal	798	1075	Linalol	1098	1531
Ethyl lactate	813	1325	Nonanal	1102	1386
(E)-3-Hexen-1-ol	850	1349	a-Thujone	1108	1416
(Z)-3-Hexen-1-ol	853	1370	2,5-Dimethyl-2-octen-6-one	1110	1381
(E)-2-Hexen-1-ol	865	1388	2-Phenyl ethanol	1117	1886
(Z)-2-Hexen-1-ol	868	1399	Fenchyl alcohol	1117	1571
Hexanol	868	1338	(Z)-Limonene oxide	1133	1439
Isoamyl acetate	876	1119	(E)-Limonene oxide	1138	1451
Heptanal	899	1176	Isopulegol	1147	1555
Pentyl acetate	913	1167	Camphor	1148	1508
Tricyclene	922	1007	Citronellal	1151	1472
a-Thujene	925	1024	Nerol oxide	1155	1463
a-Pinene	933	1020	Menthone	1156	1458
Ethyl-3-hydroxybutyrate	933	1499	Pulegol	1158	1561
Camphene	948	1063	Isoborneol	1160	1659
a-Fenchene	949	1054	3-Phenyl-propionaldehyde	1162	1761
Benzaldehyde	960	1508	Menthofuran	1165	1478
Heptanol	969	1440	Lavandulol	1167	1663
Sabinene	972	1118	Isomenthone	1168	1484
ß-Pinene	976	1105	Borneol	1168	1507
6-Methyl-5-hepten-2-one	985	1327	Nonanol	1170	1644
Myrcene	989	1160	Menthol	1175	1627
Ethyl caproate	998	1230	Isopinocampheol	1180	1703
2-Carene	1000	1130	Terpinen-4-ol	1180	1592
Octanal	1001	1281	Diethylsuccinate	1181	1661
a-Phellandrene	1003	1161	Myrtanal	1188	1543
d-3-Carene	1009	1146	a-Terpineol	1192	1680
Hexyl acetate	1012	1267	1-Terpineol	1192	1683
1,4-Cineole	1014	1175	Isomenthol	1192	1655
a-Terpinene	1017	1176	Ethyl caprylate	1196	1431
p-Cymene	1024	1266	(Z)-Dihydro carvone	1200	1600
Limonene	1029	1197	Myrtenal	1204	1621
1,8-Cineole	1033	1206	Decanal	1204	1490
Benzyl alcohol	1037	1849	(E)-Dihydro carvone	1207	1618
Phenylacetaldehyde	1044	1628	Octyl acetate	1209	1468
g-Terpinene	1059	1242	(E)-Carveol	1220	1815
Octanol	1070	1541	Fenchyl acetate	1221	1466
Dihydro myrcenol	1072	1455	Citronellol	1224	1747

Compound	$_{I}$T a	$_{I}$T b	Compound	$_{I}$T a	$_{I}$T b
Nerol	1228	1782	Longifolene	1414	1580
(Z)-Carveol	1232	1845	N-Methyl methyl anthranilate	1415	2055
Neral	1241	1666	a-Gurjunene	1418	1540
Cumin aldehyde	1244	1771	a-Cedrene	1421	1576
Carvone	1246	1721	ß-Caryophyllene	1427	1603
Geraniol	1251	1825	ß-Cedrene	1429	1606
(Z)-Anethol	1254	1746	Thujopsene	1441	1628
(E)-Myrtanol	1256	1847	Aromadendrene	1447	1613
2-Phenyl-ethylacetate	1258	1800	Isoeugenol	1452	2322
Piperitone	1260	1717	a-Humulene	1462	1673
Myrcenyl acetate	1261	1571	2-Dodecenal	1464	1849
Linalyl acetate	1264	1548	Dodecanol	1471	1948
Decanol	1269	1745	Valencene	1499	1721
Geranial	1270	1716	Tridecanal	1510	1811
Cinnamaldehyde	1274	2019	Piperonyl acetate	1514	2344
Perylla aldehyde	1279	1776	ß-Bisabolene	1515	1726
(E)-Anethol	1286	1814	Lilial	1533	2034
Lavandulyl acetate	1289	1597	*cis*-Nerolidol	1535	1984
Isobornyl acetate	1290	1580	*trans*-Nerolidol	1566	2025
Hydroxycitronellal	1290	1915	Tetradecanal	1611	1920
Thymol	1290	2156	Cedrol	1614	2112
Bornyl acetate	1290	1580	Cedrenol	1615	2115
Safrole	1293	1862	Isoeugenyl acetate	1620	2395
Menthyl acetate	1294	1561	Tetradecanol	1675	2155
Cumin alcohol	1294	2078	a-Bisabolol	1689	2202
Carvacrol	1302	2186	(Z)-Farnesol	1698	**
Undecanal	1306	1598	(E)-Farnesol	1723	**
Nonyl acetate	1309	1574	(Z)-Farnesyl acetate	1817	2214
Linalyl propanoate	1338	1604	Nootkatone	1822	2506
Citronellyl acetate	1349	1651	(E)-Farnesyl acetate	1842	2251
Terpinyl acetate	1353	1692	Hexadecanol	1877	2361
Neryl acetate	1362	1713	Citropten	1984	**
Undecanol	1371	1847	Bergapten	2075	**
Geranyl acetate	1382	1747	Octadecanol	2080	2569
Ethyl Caprate	1390	1637	(Z)-Phytol	2086	2555
Vanillin	1403	2578	(E)-Phytol	2110	2599
Dodecanal	1408	1704	Heicosanol	2280	**
Decyl acetate	1409	1676			

a Linear Retention Indices on Se-52
b Linear Retention Indices on Carbowax
* Coeluted with the solvent
** Not eluted

Table 5 - List of the standards analysed and included in the FFC bank, with linear retention indices on both columns.

Compound name	FOR	rev	CAS	M.W.
2,6-OCTADIEN-1-OL, 3,7-DIMETHYL-, ACETATE, (E)-	924	935	105-87-3	196
2,6-OCTADIEN-1-OL, 3,7-DIMETHYL-, ACETATE, (Z)-	875	889	141-12-8	196
PROPANOIC ACID, 2-METHYL-, 3,7-DIMETHYL-2,6-OCTADIENYL ESTER, (E)-	892	838	2345-26-8	224
4-HEXEN-1-OL, 2-ETHENYL-2,5-DIMETHYL-	810	828	50598-21-5	154
LINALYL 3-METHYLBUTANOATE	801	837	0-00-0	238
LINALYL 2-METHYLPROPANOATE	794	845	0-00-0	224
2,6-OCTADIEN-1-OL, 2,7-DIMETHYL	781	806	22410-74-8	154
2,6,10-DODECATRIEN-1-OL, 3,7,11-TRIMETHYL-, ACETATE, (E,E)-	776	814	4128-17-0	264
NERYL PHENYLACETATE	745	762	0-00-0	272
GERANYL ACETATE (2,6-OCTADIEN-1-OL, 3,7-DIMETHYL-, ACETATE, (E)-)	955	957	105-87-3	196
NERYL ACETATE (2,6-OCTADIEN-1-OL, 3,7-DIMETHYL-, ACETATE, (Z)-)	940	944	141-12-8	196
LAVANDULYL ACETATE	868	878	0-00-0	196
NEROL (2,6-OCTADIEN-1-OL, 3,7-DIMETHYL-, (Z)-)	816	829	106-25-2	154
GERANIOL (2,6-OCTADIEN-1-OL, 3,7-DIMETHYL-, (E)-)	799	817	106-24-1	154
LINALYL ACETATE (1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-, ACETATE)	730	757	115-95-7	196
LINALOOL (1,6-OCTADIEN-3-OL, 3,7-DIMETHYL-)	725	744	78-70-6	154
LAVANDULOL (4-HEXEN-1-OL, 5-METHYL-2-(1-METHYLETHENYL)-, (R)-)	718	731	498-16-8	154
TRANS, TRANS-FARNESYL ACETATE	705	813	4128-17-0	264
MYRCENYL ACETATE	693	711	0-00-0	0
CIS, TRANS FARNESYL ACETATE	663	705	29548-30-9	264
NERYL ACETATE (2,6-OCTADIEN-1-OL, 3,7-DIMETHYL-, ACETATE, (Z)-)	940	944	141-12-8	196

FOR Forward Fit
rev Reverse Fit
FFC Homemade "Flavor and Fragrance Components" library

Table 6 - List obtained for peak 44 matching spectral data with a commercial library (A); with the FFC library (B); and with the FFC library using linear retention indices on SE-52 with a filter of ±3 linear retention units (C).

a-Thujene	Linear Retention Indices
Bergamot oil	932
Lemon oil	931
Mandarin oil	932
Sweet orange oil	932
Bitter orange oil	932
Grapefruit oil	932
Clementine oil	931
Lime oil	932

Table 7 - Linear retention indices for a-thujene in eight different essential oils.

Table 8 (upper part (A)) reports another example obtained using the commercial library to identify peak 52 (see Figure 6). The list reports in the first and the third positions a compound with the same common name, molecular formula, and molecular weight, but with a different CAS number and structure. The second choice has the same structure of the third, but a different systematic name and MS fragmentations (see Figure 9).

Compound name	FOR	rev	CAS	M.W.
ALPHA-BISABOLOL	865	901	72691-24-8	222
3-CYCLOHEXENE-1-METHANOL.,.ALPHA.,4-DIMETHYL-, ALPHA.-(4-METHYL-	819	916	23178-88-3	222
ALPHA-BISABOLOL	810	899	515-69-5	222
LEVOMENOL	781	834	23089-26-1	222
1,6,10-DODECATRIEN-3-OL, 3,7,11-TRIMETHYL-, (E)-	723	796	40716-66-3	222
CYCLOHEXENE, 1-METHYL-4-(5-METHYL-1-METHYLENE-4-HEXENYL)-, (S)-	684	786	495-61-4	204
2,6,10-DODECATRIEN-1-OL, 3,7,11-TRIMETHYL-	645	720	4602-84-0	222
3,6-OCTADIEN-1-OL, 3,7-DIMETHYL-, (Z)-	629	725	5944-20-7	154
BICYCLO 3.1.0 HEXAN-3-OL, 4-METHYL-1-(METHYLETHYL)-	605	695	513-23-5	154
ALPHA-BISABOLOL	906	927	515-69-5	222

FOR Forward F
rev Reverse Fit
FFC Homemade "Flavor and Fragrance Components" library

Table 8 - List obtained for peak 52 matching spectral data with a commercial library (A) and with the FFC library using linear retention

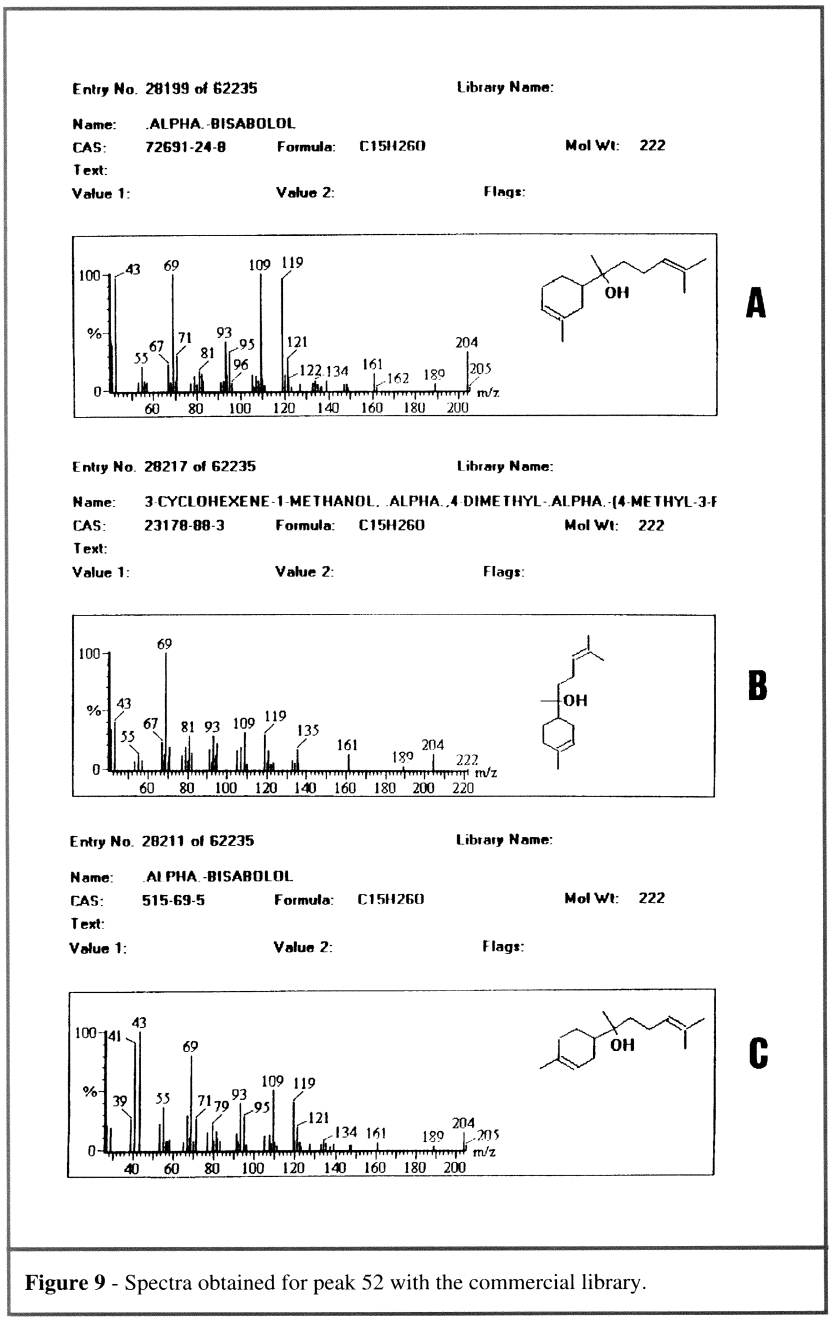


Figure 9 - Spectra obtained for peak 52 with the commercial library.

In the lower part of Table 8 is reported the only appropriate standard compound, a-bisabolol (obtained from Extrasynthese, Genay, France), using the FFC library and the Linear Retention filter (±3 Linear Retention units).

In conclusion, when a complex mixture containing components that belong to the same class is analysed, the mass spectroscopic data are insufficient. For a reliable identification, in this case, the best method is to build a library with standards previously checked by other spectroscopic techniques (e.g., FT-IR and ^{1}H-NMR) and to calculate Linear Retention Indices on two columns of different polarity. The interactive use of Linear Retention Indices and spectroscopic data has allowed the identification of 55 components in of bergamot oil, even when the molecular formula and the molecular weight are identical. For example, 16 monoterpenes with the same molecular formula ($C_{10}H_{16}$) and molecular weight (136) have been identified. As can be seen by comparing the results obtained with the HPLC-HRGC/MS method and with the HRGC/MS with Linear Retention Indices, there are some differences in the components identified in bergamot essential oils. This is due to the different power of the two techniques. HPLC-HRGC/MS permits to have a more pure MS spectrum, because of the LC prefractionation. In our study,

with the HPLC-HRGC/MS system the spectra obtained were compared with those of commercial libraries, such as NIST or Adam's.
The MS spectra obtained with the HRGC/MS system, without the LC prefractionation, can be less pure because of possible coelution or because the baseline is more disturbed. The use of Linear Retention Indices on two columns interactively with the MS search can improve the results obtained, because two other data are introduced in the matching of the unknown spectrum. One limitation of this system is that many standard compounds are not commercially available, so the number of compound identified is restricted to those of which the MS spectrum has been included in the library.

HRGC-HRGC analysis of bergamot essential oil for the determination of the enantiomeric distribution of ß-pinene, sabinene, limonene, linalol, terpinen-4-ol and α-terpinene

A bergamot essential oil was first analysed with the SE-52 pre-column to determine the concentrations of the components of interest and their retention times, maintaining the multidimensional system in the stby position. Depending on the retention times and the concentration of each single component, different transfer windows were then chosen and automatically programmed so that well-resolved peaks would be obtained in the chiral column either for components present in the oils at high concentrations and for those present at very low concentrations.
Figure 10A reports the chromatogram of a cold-pressed bergamot oil obtained with the SE-52 column and the system in the stby position; the chromatogram of the same oil obtained with the SE-52 column and the system in the cut position (on this chromatogram the cuts are shown) is reported in Figure 10B; while in Figure 10C the chromatogram obtained with the chiral column for the fractions transferred from the SE-52 pre-column is reported. Table 9 reports the enantiomeric distribution of ß-pinene, sabinene, limonene, linalol, linalyl acetate, terpinen-4-ol and a-terpineol for the 52 samples of bergamot oil analysed.

		Bergamot* (52 samples)
ß-pinene	(+)	6.8-8.9
	(-)	93.2-91.1
sabinene	(+)	14.1-16.0
	(-)	85.9-84.0
limonene	(-)	2.0-2.7
	(+)	98.0-97.3
linalol	(-)	99.5-99.7
	(+)	0.5-0.3
terpinen-4-ol	(+)	13.4-25.4
	(-)	86.6-74.6
a-terpineol	(+)	49.3-68.1
	(-)	50.7-31.9
linalyl acetate	(-)	99.7-99.8
	(+)	0.3-0.2
* preliminary results		

Table 9 - Enantiomeric ratios for some compounds of bergamot oil.

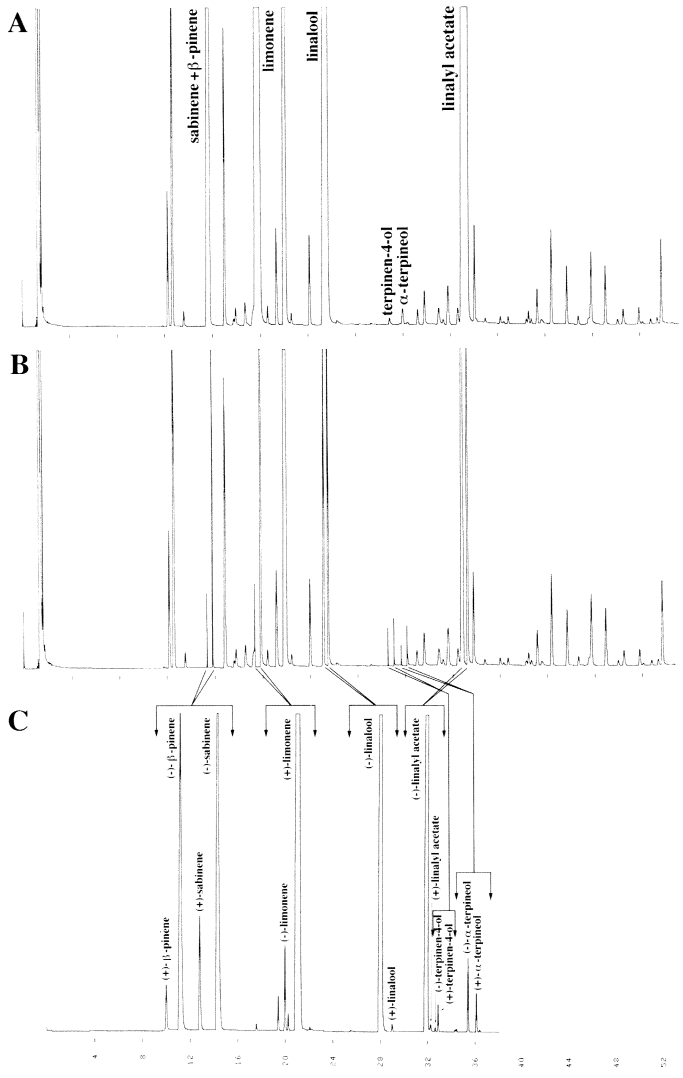


Figure 10
A) HRGC chromatogram of a bergamot oil obtained with the SE-52 column;
B) HRGC chromatogram of a bergamot oil obtained with the SE-52 column with the heart-cuts;
C) HRGC-HRGC chiral chromatogram of the transferred components.

As can be seen from the table, the enantiomeric distribution of ß-pinene, sabinene, limonene and mainly linalol and linalyl acetate vary into very narrow ranges, while terpinen-4-ol and a-terpineol present pretty wide ranges. The values obtained for the enantiomeric distribution of linalol and linalyl acetate confirm the presence of very small amounts of the (+)- isomers in the genuine bergamot oils. These ratios can represent a reference for the authenticity of bergamot oils. The enantiomeric ratios of sabinene, terpinen-4-ol and a-terpineol were not previously reported in bergamot essential oils.

The correct use of this system permits the automatic transfer even of those components that elute on the SE-52 column in a critical zone of the chromatogram. For example, linalol elutes from the SE-52 column between trans-sabinene hydrate and nonanal (Figure 10A).

It is clear that in such a case the choice of transfer window is critical as imperfect reproducibility of the retention time could cause a partial loss of linalol or the presence of trans-sabinene hydrate and/or of nonanal in the transferred fraction.

When the system is not used as a multidimensional GC, the simultaneous and independent use of the two gas chromatographs is possible without any change to the hardware configuration.

HRGC-HRGC/MS analysis of bergamot essential oil on SE-52 pre-column and Carbowax 20M analytical column.

The lower part of Figure 11 reports the chromatogram of a bergamot oil on SE-52 column. On this figure, the zone of the chromatogram that has been transferred on to the Carbowax 20M column is clearly shown; the upper part of Figure 11 shows the chromatogram of the fraction analysed on the Carbowax 20M column and acquired with a MS detector. As can be seen, the fraction transferred showed on the SE-52 column two peaks, identified as cis-a-bergamotene and (E)-caryophyllene; after the transfer and the analysis on the Carbowax 20M column, the same fraction shows seven peaks, four of which have been identified on the basis of the MS data.

CONCLUSION

The examples shown in this paper easily point out that the use of coupled analytical techniques and the chromatographic multidimensional techniques constitute a fundamental reference to obtain thorough information on the composition of complex mixtures, such as essential oils.

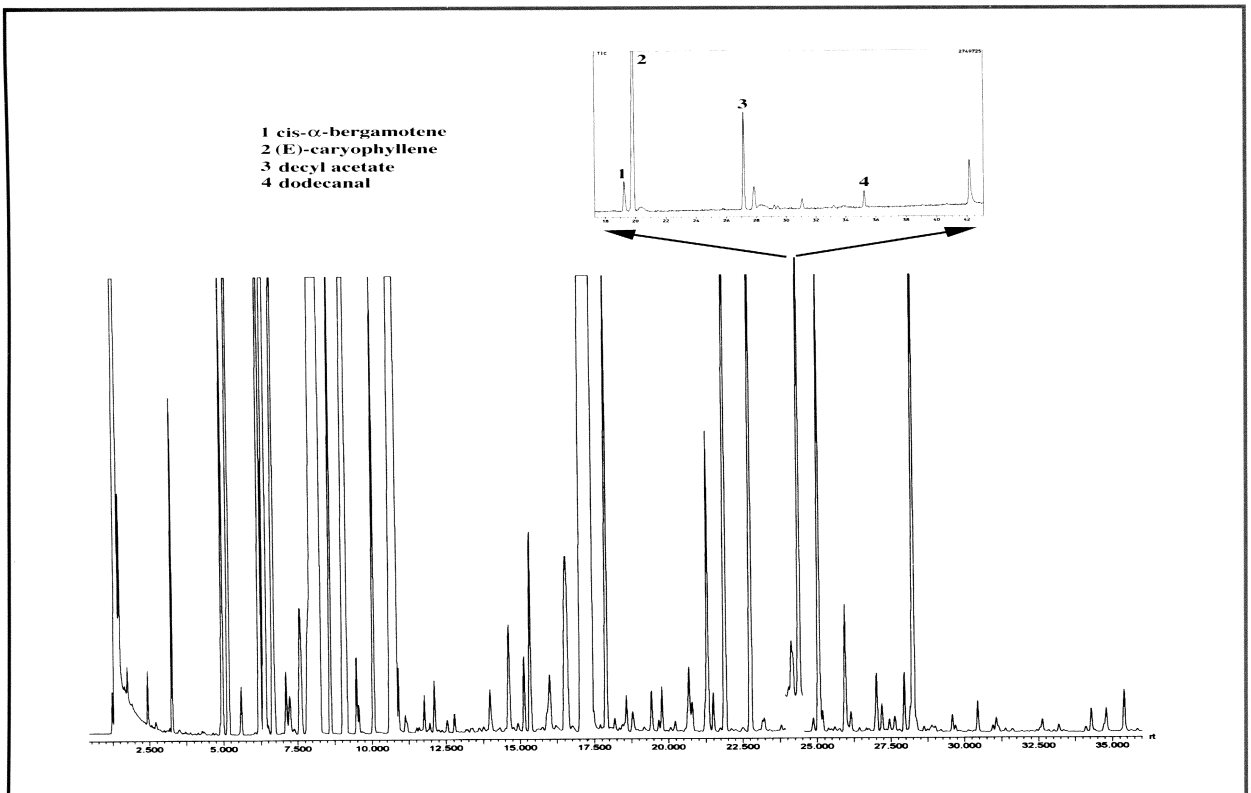


Figure 11 - Lower: HRGC-HRGC-FID chromatogram on a SE-52 column of a bergamot essential oil with the transfer heart-cut. Upper: HRGC-HRGC/MS chromatogram on a Carbowax 20 M column of the heart-cut.

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