

On the Genuineness of Citrus Essential Oils. Part II.* Chemical Characterization of the Essential Oil of New Hybrids of Lemon Obtained in Sicily

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The composition of laboratory extracted lemon oil from three new lemon hybrids is reported. The volatile fraction was analysed by HRGC–FID; the enantiomeric distribution of β -pinene, sabinene, limonene, linalol, terpinen-4-ol and α -terpineol was studied by multidimensional HRGC–HRGC; coumarins and psoralens present in the non-volatile residue were analysed by normal phase HPLC. The results were compared with those obtained for Italian industrial lemon oils. The three oils differ from each other in the qualitative and quantitative composition, and also show very significant differences compared to industrial lemon oil. © 1997 by John Wiley & Sons, Ltd.

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INTRODUCTION

In Italy, lemon-growing in almost all the traditional cultivation areas is conditioned by the devastating effect of the fungus which causes 'mal secco' (*Phoma tracheiphila* (Petri) Kanc. and Ghik). For this reason the area under cultivation is constantly decreasing. The most common cultivars are a very large and heterogeneous population of the group 'Femminello', which are extremely productive but very vulnerable to 'mal secco'. In contrast, the cultivars 'Interdonato' and 'Monachello' which are able to withstand this plant pathogen show low productivity and low fruit quality.²

The cultivar 'Femminello zagara bianca' offers a better combination of good tolerance to 'mal secco', good productive characteristics and good fruit quality. The absence of the typical violet pigmentation of the flowers and buds distinguishes this cultivar from other lemon cultivars. It is now

commonly used both for regrafting and for new plants. Since 1946, the programmes of genetic improvement of the lemon in Italy have been directed towards the achievement of cultivars that can stand up to 'mal secco', because the most productive and best quality cultivars are susceptible to this plant pathogen.

Over the last 50 years, clones resistant in different degrees to 'mal secco' have been favoured, but they have shown unsatisfactory production and qualitative characteristics.³

Today the market requires early lemon fruits. The cultivar 'Femminello Siracusano' best fulfils the requirements of productivity, fruit quality and early ripening. This cultivar was selected in the district Carrozieri near Siracusa in 1958. Its main undesirable characteristics are sensitivity to 'mal secco' and the presence of seeds.⁴

Although the programs of genetic improvement of lemon in Sicily are mainly aimed at the fresh fruit market, the quality of products such as juice and essential oil, which can be obtained from industrial processing of these new hybrids, should not be disregarded. In this paper, the characteristics of the essential oil obtained from three hybrids are reported.

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These hybrids came from a cross between the cultivar 'Femminello Siracusano' and the hybrid 51-33A-224. This last hybrid derives from a cross carried out in 1951 between the lemon cultivar 'Femminello comune' and the so-called 'Pera del commendatore', which was originally considered to be a natural hybrid of pummelo (*C. grandis* L. Osbeck), but which analysis by molecular marker RADP (Random Amplified Polymorphic DNA) has now shown to be an hybrid between pummelo, lemon and citron (*C. medica* L.).⁵

The hybrid '51-33A-224' produces large fruits, between lemon and 'Pera del commendatore' in size, and shows qualitative characteristics very similar to those of lemon. The tree is robust, productive and resistant to 'mal secco'. For these reasons it has been used as a parent plant in crosses with lemon.

EXPERIMENTAL

In 1979, from a controlled cross between the lemon cultivar 'Femminello siracusano', used as female parent, and the hybrid '51-33A-224', 340 hybrid plants were obtained. They were grafted on young and healthy rootstocks, and in 1982 were placed in the field 'Fonti Ciane' (Siracusa) of the 'Istituto Sperimentale per l'Agrumicoltura' of Acireale (Catania) to monitor their bio-agronomic behaviour.

In the 14 years since they were planted, almost all the plants have turned out to be sensitive to 'mal secco', and have died. Only six have survived. The fruits of these plants show characteristics similar but not identical to lemon, including different flavour, shape and taste.

The hybrids considered in this paper are: (1) 79-LFS-224-4; (2) 79-LFS-224-11; and (3) 79-LFS-224-17. Fruits were harvested during December 1995. The essential oil was extracted in the laboratory, by putting enough pressure on the peel of the fruits to break the oil glands and allow the essential oil to escape (the oil was collected in a watch-glass, transferred to a test-tube and centrifuged). All samples were analysed by GC-FID and GC-MS for the quantitative and qualitative analysis of the volatile fraction; by multidimensional GC for the determination of the enantiomeric distribution of some monoterpene hydrocarbons (β -pinene, sabinene and limonene) and some monoterpene alcohols (linalol, α -terpineol and terpinen-4-ol); by normal phase

HPLC for the analysis of coumarins and psoralens; and by UV spectrometry for determination of CD values.

GC Analysis

The volatile fraction was analysed by HRGC-FID as described: gas chromatograph Fisons Mega Series 5160 equipped with a Shimadzu data processor C-R3A; SE-52 fused silica column, 30 m \times 0.32 mm i.d., film thickness, 0.40–0.45 μ m; column temperature, 45°C (6 min) to 180°C at 3°C/min; injector and detector temperatures, 280°C; carrier gas He 95 kPa; injection mode, split; injected volume, 1 μ l of neat oil.

GC-MS Analysis

Some samples were analysed by GC-MS(EI) on a Fisons MD 800 (Milan, Italy) system coupled with Adams' library⁶ and FFC banks;⁷ GC conditions were: SE-52 fused silica column, 60 m \times 0.32 mm i.d.; film thickness, 0.40–0.45 μ m (Mega, Legnano (MI) Italy); column temperature 45°C (6 min) to 111°C at 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/sec). 1 μ l 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 range, 39.00–350.00 amu.

Chiral Analysis

Enantiomeric ratios of some monoterpene hydrocarbons (β -pinene, sabinene, limonene) and of some monoterpene alcohols (linalol, terpinen-4-ol, α -terpineol) were obtained by multidimensional gas chromatography, using a developmental model GC-GC⁸ set up with: two GC ovens, the first one equipped with a column coated with SE-52 and the second one with a chiral column coated with derivatized β -cyclodextrin; a hot interface; a rotary switching valve and a system to maintain a constant flow during the transfer. With this system a heart-cut of the relevant fractions can be made and these fractions transferred from the non-chiral column to the chiral one under the following experimental conditions: precolumn, SE-52 fused silica column, 30 m \times 0.32 mm i.d.,

Table 1. Composition of the volatile fraction of the oil from three lemon hybrids

Peak no.	Compound	1. 79-LFS-224-4	2. 79-LFS-224-11	3. 79-LFS-224-17	Lemon oils, 1991/92
1	Pentyl acetate	tr	tr	-	-
2	Tricyclene	-	-	tr	0.01
3	α -Thujene	0.80	0.01	0.22	0.44
4	α -Pinene	2.09	0.58	1.02	1.95
5	Camphene	0.02	0.02	0.02	0.06
6	Sabinene + β -Pinene	1.83	4.15	4.65	14.31
7	Myrcene	1.71	1.75	1.70	1.48
8	Octanal	0.03	0.03	0.04	0.05
9	α -Phellandrene	0.03	0.02	0.03	0.07
10	δ -3-Carene	tr	tr	0.01	0.01
11	α -Terpinene	0.41	-	0.12	0.18
12	<i>p</i> -Cymene	-	-	0.03	0.15
13	Limonene	68.34	90.05	81.09	65.87
14	(<i>Z</i>)- β -Ocimene	0.02	0.01	0.01	0.07
15	(<i>E</i>)- β -Ocimene	0.20	0.04	0.01	0.12
16	γ -Terpinene	21.00	0.01	5.92	9.39
17	<i>cis</i> -Sabinene hydrate	0.07	0.02	0.04	0.04
18	Terpinolene	0.88	0.01	0.26	0.38
19	<i>trans</i> -Sabinene hydrate	0.12	0.01	0.05	0.03
20	Linalol	0.14	0.14	0.15	0.11
21	Nonanal	-	0.01	0.04	0.11
22	<i>cis</i> -Limonene oxide	-	-	0.01	0.01
23	<i>trans</i> -Limonene oxide	0.01	-	0.01	0.01
24	Camphor	-	-	tr	0.01
25	Citronellal	-	-	0.08	0.10
26	Borneol	tr	-	0.01	0.01
27	Terpinen-4-ol	0.07	-	0.02	0.04
28	α -Terpineol	0.27	0.09	0.19	0.17
29	Decanal	-	0.02	0.03	0.04
30	Octyl acetate	-	-	tr	tr
31	Nerol + Citronellol	-	-	0.03	0.04
32	Neral	0.03	0.04	0.71	0.87
33	Linalyl acetate	tr	tr	-	-
34	Geraniol	-	-	0.02	0.02
35	Geranial + Perilla aldehyde	0.05	0.06	1.12	1.49
36	Bornyl acetate	-	-	tr	tr
37	Undecanal	-	0.01	0.01	0.02
38	Nonyl acetate	-	-	tr	0.01
39	Methyl geranoate	-	-	tr	tr
40	Citronellyl acetate	0.01	0.01	0.03	0.03
41	Neryl acetate	0.24	0.03	0.46	0.43
42	Geranyl acetate	0.02	0.11	0.31	0.40
43	Dodecanal	-	0.01	tr	tr
44	Decyl acetate	-	-	tr	tr
45	α - <i>cis</i> -Bergamotene	0.02	0.04	0.03	-
46	(<i>E</i>)-Caryophyllene	0.46	0.39	0.20	0.23
47	α - <i>trans</i> -Bergamotene	0.32	0.65	0.40	0.34
48	α -Humulene	0.03	0.03	0.02	0.02
49	(<i>E</i>)- β -Farnesene	0.03	0.06	0.03	0.04
50	β -Santalene	0.01	0.03	0.02	-
51	Germacrene-D	tr	0.04	-	0.01
52	Bicyclogermacrene	0.05	0.04	0.06	0.06
53	(<i>Z</i>)- α -Bisabolene	0.04	0.06	0.05	0.04

Table continues on next page

Table 1. Continued

Peak no.	Compound	1. 79-LFS-224-4	2. 79-LFS-224-11	3. 79-LFS-224-17	Lemon oils, 1991/92
54	β -Bisabolene	0.45	0.99	0.59	0.50
55	γ -Elemene	0.01	0.03	0.02	0.01
56	Tetradecanal	0.01	0.02	0.01	0.01
57	2,3-Dimethyl-3-(4-methyl-3-pentenyl)-2-norbornanol	0.02	0.04	0.02	0.02
58	Campherenol	0.02	0.04	0.02	0.02
59	α -Bisabolol	0.02	0.04	0.03	0.02
60	7-Methoxy-2H-1-benzopyran-2-one (herniarin)	–	0.18	–	–
61	Nootkatone	–	–	0.02	tr
	Hydrocarbons	99.75	99.01	96.51	95.59
	Monoterpenes	97.33	96.65	95.09	94.30
	Sesquiterpenes	1.42	2.36	1.42	1.29
	Oxygenated compounds	1.13	0.91	3.46	4.11
	Aldehydes	0.12	0.20	2.04	2.72
	Alcohols	0.73	0.38	0.58	0.51
	Esters	0.27	0.15	0.80	0.87

film thickness, 0.40–0.45 μm (Mega, Legnano (MI) Italy); column temperature 45° (6 min) to 220°C, at 2°C/min. Analytical column, fused silica column, 25 m \times 0.25 mm i.d.; coated with Diethyl-*tert*-butylsilyl- β -cyclodextrin (Mega, Legnano (MI) Italy); column temperature, 40° to 180°C, at 2°C/min; interface temperature, 200°C; detector FID, 250°C (for both chromatographs).

HPLC Analysis

Coumarins and psoralens were analysed by normal phase HPLC, using a Waters Associates instrument and a photo diode array detector (PDA) model 996 under the same experimental conditions as previously reported⁹ using a μ -Porasil column (15 cm \times 3.9 mm i.d., particle size 10 μm). Mobile phase, eluent A: hexane:ethyl acetate, 92:8; eluent B: hexane:ethyl alcohol, 9:1. Eluent program: 0–15 min, A 100%; 15–20 min, from A 100% to B 100%, with a linear gradient; 20–30 min, B 100%; flow rate: 1.25 ml/min. Peak integration and quantitative calculations were performed with the Millennium 2010 system (Waters Associates). The injection volume was 20 μl of a solution obtained by diluting about 90 mg of lemon oil and 0.1 ml of a coumarin solution of known concentration to 1 ml of hexane:ethyl acetate (75:25). Detection was by UV absorbance at 315 nm. The UV spectra

of eluting peaks were monitored in the region 200–400 nm.

CD Determination

The CD was calculated according to Sale¹⁰ from the UV spectrum between 270 and 370 nm. This value is the difference in absorbance at the maximum and the point at which a vertical line from the maximum crosses a line tangent to the two minima for solution of 250 mg of oil diluted in 100 ml of ethyl alcohol.

RESULTS AND DISCUSSION

Volatile Fraction

Table 1 reports the composition of single components and classes of components of the volatile fraction of the three oils. In the same table the average composition of industrial Sicilian lemon oil produced during the season 1991/92 is also reported.¹¹

In Figure 1 the chromatogram of the volatile fraction of the three oils is reported. As can be seen from Table 1 and Figure 1, 64 components, which represent about 99.9% of the whole volatile fraction, have been identified in the three oils.

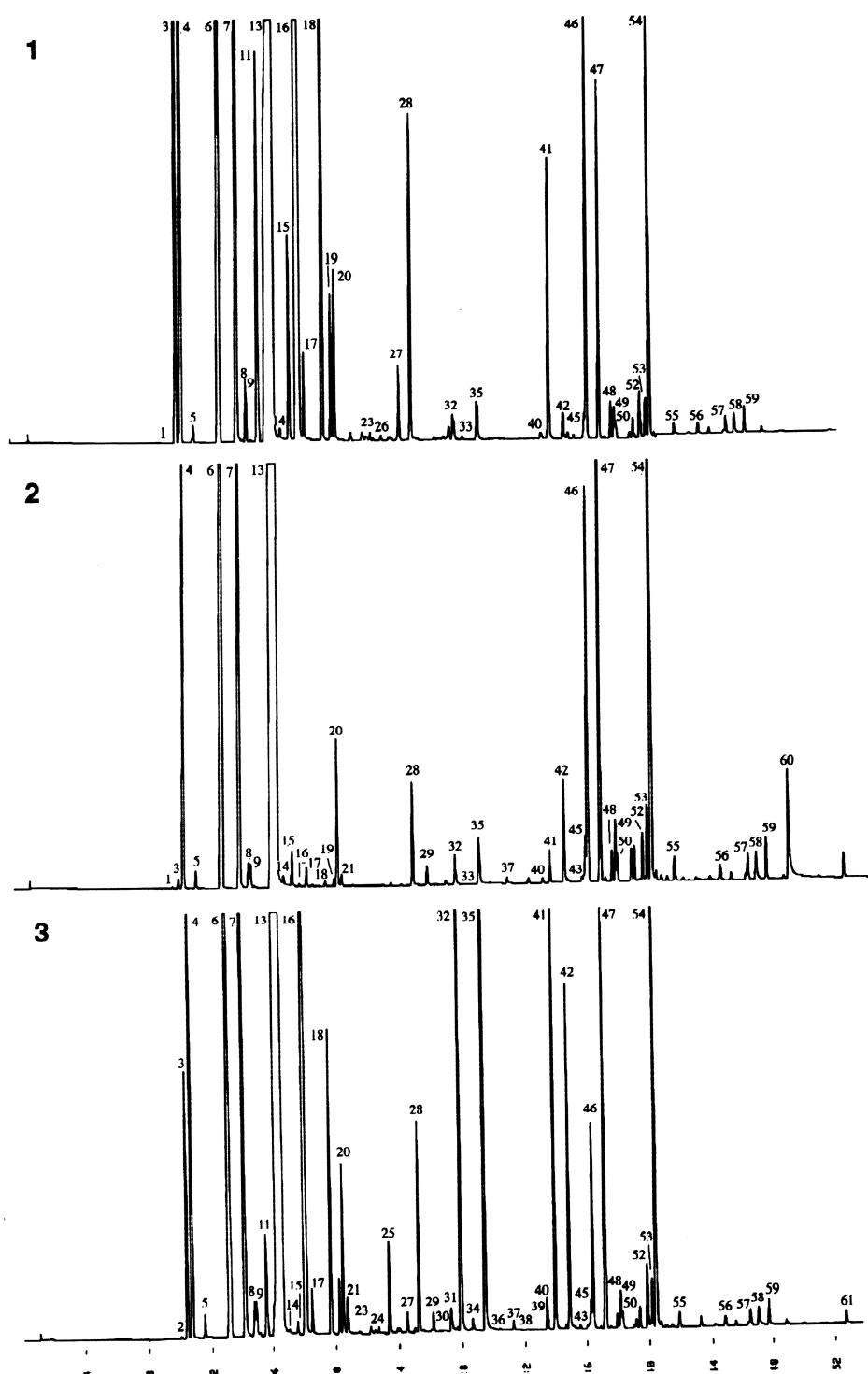


Fig. 1. Gas chromatogram of the volatile fraction of the oils of the three lemon hybrids analysed. For peak identification see Table 1.

The three oils show sometimes considerable qualitative and quantitative differences and their composition is significantly different from that of Sicilian industrial oils. The total amount of monoterpene hydrocarbons, which ranges from 95.1% (hybrid 3) to 97.3% (hybrid 1), is very similar for the three oils, and is higher than that of industrial lemon oils. The composition of this fraction shows considerable differences between the three oils. For example, hybrid 1 shows a content of limonene of little more than 68% and of γ -terpinene of 21%; hybrid 2 contains about 90% of limonene and only traces of γ -terpinene; hybrid 3 contains about 81% of limonene and about 6% of γ -terpinene. Moreover, none of these three hybrids shows a quantitative composition of the monoterpene hydrocarbon fraction similar to that of the industrial oils.

The sesquiterpene hydrocarbon fraction represents about 1.4% of the oil of hybrids 1 and 3, while it is higher in the oil of hybrid 2 (about 2.4%). The total content of sesquiterpenes and the qualitative and quantitative composition of this fraction in hybrids 1 and 3 is similar to industrial oils. The quantitative differences which occur in hybrid 2 are mainly due to the content of β -bisabolene and α -trans-bergamotene.

Oxygenated compounds represent 3.5% of hybrid 3; this value approaches that of industrial lemon oils (4.1%). In the oils of hybrids 1 and 2 the content of oxygenated compounds is lower than hybrid 3. The differences in the content of oxygenated compounds are due to the different amounts of aldehydes (mainly neral and geranial) and esters (mainly neryl acetate and geranyl acetate). Moreover, hybrids 1 and 2 do not contain citronellal and nootkatone. Hybrid 2 contains herniarin.

Enantiomeric Ratios

Enantiomeric ratios of six components were detected by four subsequent transfers (sabinene + β -pinene; limonene; linalol; terpinen-4-ol + α -terpineol) during the same analysis. Figure 2 shows the chiral chromatograms of the six components for the three oils. Table 2 shows the enantiomeric ratios of the components analysed. In the same Table values obtained for industrial lemon oils are reported.¹²

The enantiomeric ratios of the six components analysed show values which are almost always very different for the oils of the three hybrids and

different again from those of industrial lemon oils. For example, the ratio between (+) and (–) β -pinene ranges from 0.7:99.3 for hybrid 2 to 95.1:4.9 for hybrid 1; the ratio between (+) and (–) terpinen-4-ol goes from 11.2:88.8 for hybrid 1 to 70.5:29.5 for hybrid 2. The ratios between the two enantiomers of limonene are the only ones which are quite similar for the oils of the three hybrids and the industrial lemon oil. On the other hand, the enantiomeric ratios between (–) and (+)-limonene for all the citrus peel oils analysed until now^{12–15} fall within a limited range that goes from 0.5:99.5 for sweet orange oil to 2.2:97.8 for mandarin oil. The ratio value for (–) and (+)-limonene of 14:86 reported by Mosandl *et al.*¹⁵ for bergamot oil is probably referred to a commercial sample that was not genuine.

Coumarins and Psoralens

Figure 3 shows the HPLC chromatograms of the three oils, and Table 3 reports their composition in coumarins and psoralens and the CD values. The same Table gives the average composition in coumarins and psoralens of industrial lemon oil.⁹ In this Table, quantitative results are reported only for those components for which calibration curves could be constructed using the pure compounds previously isolated from lemon and lime oils. As can be seen from CD values and from the quantitative data of Table 3, the total amount of coumarins and psoralens present in the oils of the three hybrids is higher than that of industrial

Table 2. Enantiomeric distribution of some volatile components in the hybrid lemon oils

Compound	1. 79-LFS-224-4	2. 79-LFS-224-11	3. 79-LFS-224-17	Lemon oils
(+)- β -Pinene	95.1	0.7	11.2	6.3
(–)- β -Pinene	4.9	99.3	88.8	93.7
(+)-Sabinene	73.2	21.2	23.4	14.9
(–)-Sabinene	26.8	78.8	76.6	85.1
(–)-Limonene	1.5	0.7	1.1	1.6
(+)-Limonene	98.5	99.3	98.9	98.4
(–)-Linalol	65.7	50.4	52.4	52.8
(+)-Linalol	34.3	49.6	47.6	47.3
(+)-Terpinen-4-ol	11.2	70.5	30.1	27.4
(–)-Terpinen-4-ol	88.8	29.5	69.9	72.6
(–)- α -Terpineol	75.0	44.7	60.3	75.2
(+)- α -Terpineol	25.0	55.3	39.7	24.8

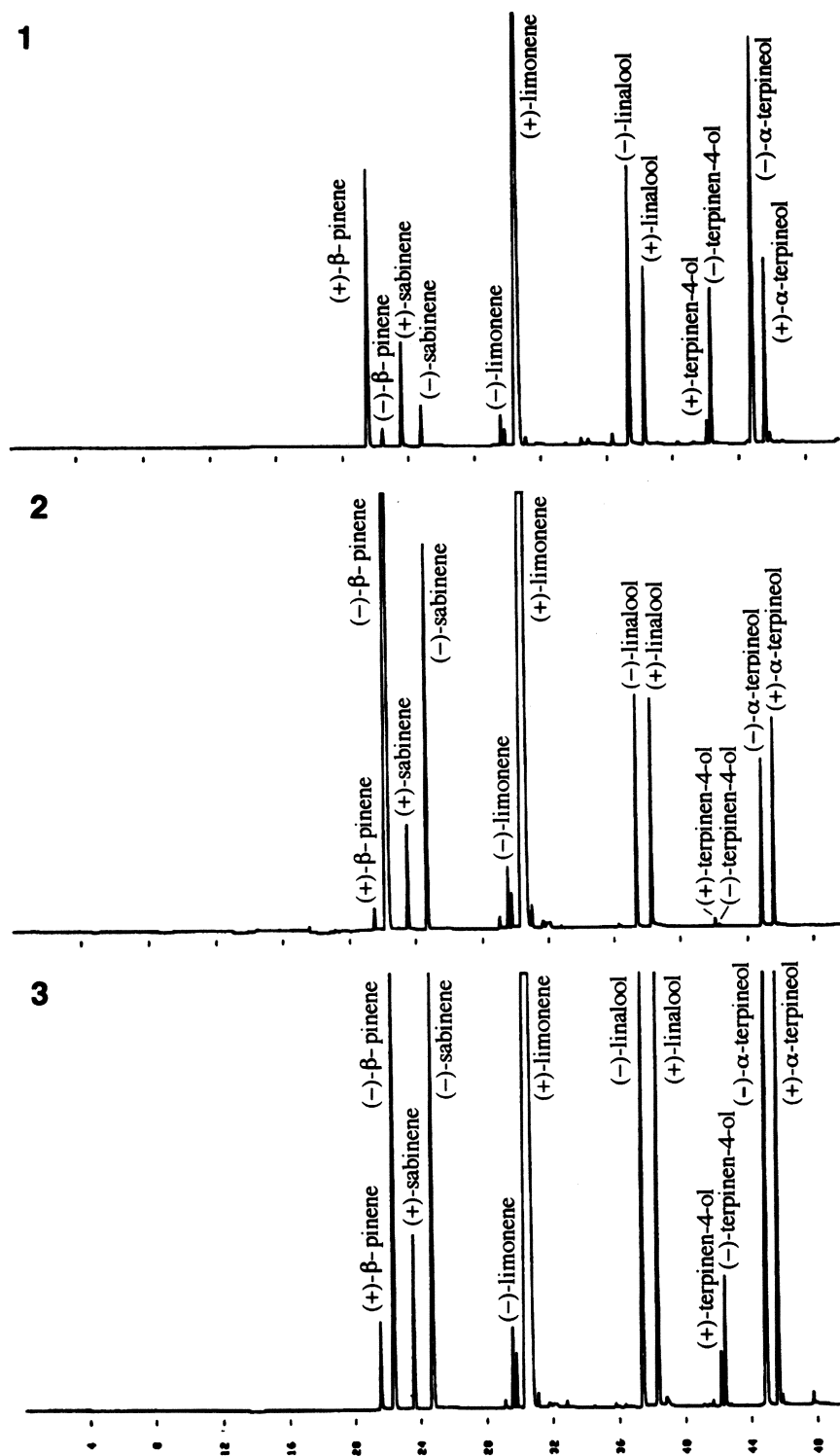


Fig. 2. Chiral gas chromatogram of the oils of the three lemon hybrids analysed.

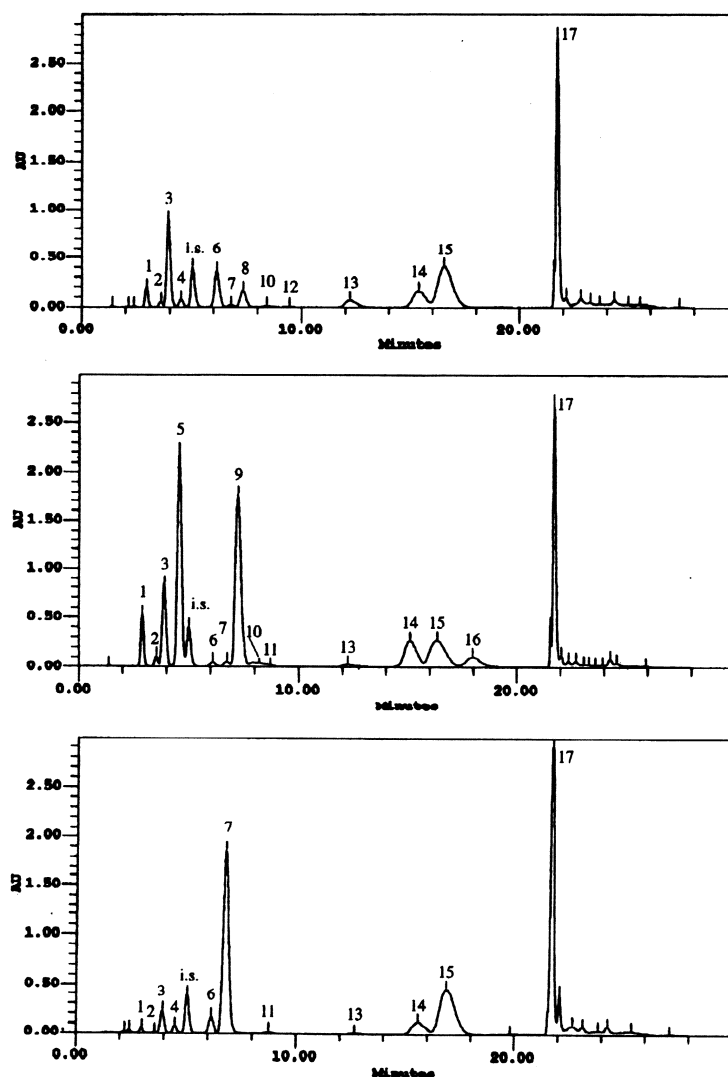


Fig. 3. HPLC chromatogram of the oils of the three lemon hybrids analysed. For peak identification see Table 3.

lemon oils. As can be seen from Figure 3 and Table 3, the three oils show significant qualitative and quantitative differences, both amongst themselves and compared with industrial lemon oils, confirming what has been previously observed for the volatile fraction and for the enantiomeric distribution of some components. Auraptin is the main component of hybrid 2 but it is absent in the other two oils; in the same oil, herniarin and an other unknown coumarin are present, but absent in the other two oils. Phellopterin is present in the oil of hybrid 1, but is absent in the other two oils. Oil

of hybrid 3 contains a high amount of citropten (493 mg/100 g of oil), which is much lower in the other two oils (6 and 14 mg/100 g of oil respectively). In comparison with industrial lemon oils, these three oils differ not only for the higher total content of the measured components, but also for presence of osthol, and some of them for the presence of herniarin absent in the industrial lemon oils. On the other hand, industrial oils contain 8-geranyloxypsoralen, oxypeucedanin hydrate and biakangelicin, that were not found in the oils of the three hybrids.

Table 3. Composition of coumarin and psoralen fraction (mg/100 g of oil) and CD values of the hybrid lemon oils

Peak no.	Compound	1. 79-LFS-224-4	2. 79-LFS-224-11	3. 79-LFS-224-17	Lemon oils, 1991/92
1	Bergamottin	54	132	15	229
2	Isoimperatorin	+	+	+	+
3	5-Geranyloxy-7-methoxycoumarin	228	204	59	213
4	5-Isopentenyl-7-methoxycoumarin	+	—	+	+
5	Aurapten	—	741	—	—
6	Osthol	281	17	54	—
7	Citropten	6	14	493	91
	8-Geranyloxy-psoralen	—	—	—	28
8	a Coumarin	+	—	—	—
9	Herniarin	—	+	—	—
10	Imperatorin*	+	+	—	+
11	a Psoralen	—	—	+	—
12	Phellopterin**	+	—	—	+
13	5-Isopent-2'-enyloxy-8-(2',3'-epoxyisopentenyl)psoralen	16	4	12	26
14	Oxypeucedanin	187	299	155	129
15	a Psoralen+a Coumarin	+	+	+	—
16	a Coumarin	—	+	—	—
17	Byacangelicol	777	663	1060	95
	Oxypeucedanin hydrate	—	—	—	+
	Byakangelicin	—	—	—	+
	CD	3.75	4.97	3.10	0.45–0.95 ¹⁶

* Tentative.

** Tentative, identified according to McHale and Sheridan.¹⁷

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