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Journal of Chromatography A, 730 (1996) 9–16

JOURNAL OF CHROMATOGRAPHY A

Analysis of bitter essential oils from orange and grapefruit by high-performance liquid chromatography with microbore columns

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Abstract

The analysis of bitter orange and grapefruit essential oils (non-volatile fraction) was carried out by HPLC in normal- and reversed-phase mode with UV detection. These oils were compared with the sweet orange and mandarin essential oils, analyzed previously. For the identification of chromatographic peaks, fractionation by RP-HPLC was carried out. The purified fractions were analyzed by GC–MS and LC–MS. Some new compounds were found, together with many others already identified in different citrus essential oils.

Keywords: Fruits; Essential oils

1. Introduction

In a previous paper the non-volatile fraction of lemon and bergamot oils [1] and orange and mandarin essential oils [2] was analyzed by HPLC and LC–MS and GC–MS. The non-volatile fraction of lemon is similar to that of bergamot, and sweet orange is similar to mandarin oil.

Most of the components of the non-volatile fractions are coumarins, flavones and flavanones. These compounds are also present in other natural products and HPLC has been used to separate them [3–10].

In the present paper bitter orange and grapefruit oils of the non-volatile fraction were examined and compared with those of sweet orange.

Analyses were carried out in normal- and reversed-phase mode with a microbore HPLC column and UV detection.

For the identification of some of the chromatographic peaks, fractionation by RP-HPLC was carried out. The collected fractions were analyzed by capillary GC–MS and, when possible, by LC–MS.

2. Experimental

2.1. Apparatus

Two Phoenix 20 (Fison) syringe pumps (master and slave) interfaced to an external computer (IBM) for remote control operations, and a rapid scanning UV–Vis detector (micro UV–Vis 20) were used. The injection valve was a Rheodyne 7520 with 1.0-μl sample loop.

The columns, 500×1.1 mm I.D. and 165×4.6 mm I.D., were slurry packed in our laboratory with Spherisorb ODS2 and NH_2 5 μm obtained from Phase Separation (Norwalk, CT, USA) [11].

For GC–MS a Hewlett-Packard 5970 HSD was

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SSDI 0021-9673(95)01135-8

used with SE-54 capillary column (25 m×0.15 mm I.D., film thickness 0.12 μm. Initial temperature of 150°C for 10 min, subsequently programmed to 280°C in 36 min and held at 280°C for 60 min. The carrier gas was helium and split–splitless injection was employed (closed for 10 min then opened at a split ratio of 10:1). LC–MS was carried out with an ODS2 column on a TS 250 (VG) mass spectrometer with plasma spray interface.

The HP 5921A atomic emission detector was used as an element specific detector of the gas chromatograph to obtain the C:H:O ratio of some unknown components.

2.2. Materials

Distilled water, stored in glass, was filtered and passed through a Millipore Norganic cartridge to eliminate organic substances.

The solvents *n*-hexane, isopropanol, acetonitrile (HPLC grade) were from C. Erba (Italy). Samples of sweet orange essential oil were kindly supplied by the Stazione Sperimentale Olii Essenziali di Reggio Calabria (Italy). The bitter orange and grapefruit oils, bergapten (5-methoxypsoralen), bergamottin (5-geranyloxypsoralen), tangeritin and aurapten (7-geranyloxycoumarin) were from Roth (Germany).

The samples of essential oils were evaporated under nitrogen at room temperature and the residue dissolved in *n*-hexane–isopropanol (60:40), up to the original volume.

2.3. Fractionation by HPLC

Column, 165×4.6 mm I.D., 5 μm ODS2; flow-rate, 1 ml/min; mobile phase: A=acetonitrile, B=distilled water with the following gradient: 0–20 min, 40% A; subsequently from 40% to 100% A in 30 min.

A 1-ml sample of grapefruit essential oil, after evaporation under nitrogen, was dissolved in 100 μl isopropanol–hexane (40:60) and aliquots of 10 μl were injected onto the liquid chromatographic system. Each collected fraction of the peaks no. 8, 9 and 10 (50–70 ml of acetonitrile–water mixture) after evaporation of acetonitrile under vacuum (below 30°C in a rotavapor), was extracted with 2×3 ml CH_2Cl_2. The collected organic layers were dried over sodium sulphate and concentrated under nitrogen to the original volume of 1 ml; approximately 1 mg was obtained for each peak.

3. Results and discussion

3.1. HPLC

Fig. 1, Fig. 2 and Fig. 3 show the chromatograms of bitter orange, sweet orange and grapefruit essential oils analyzed by HPLC with a microbore column of 500×1.1 mm I.D.

The numbered peaks in Fig. 2, previously identified [2], are compared with the retention times and the UV spectra of the peaks of the chromatograms in Fig. 1 and Fig. 3, confirming the presence of these compounds in all the analyzed essential oils except for peak 6 (tetra-O-methylscutellarein) which is not present in grapefruit essential oil.

The three essential oils were also analyzed in normal-phase HPLC (column, 500×1.1 mm I.D. of NH_2, 5 μm). By running the same essential oil in both the columns with different polarity and by comparing the ultraviolet spectra of the chromatographic peaks with the reference compounds, the identification of the peak is confirmed. Fig. 4 shows a normal-phase chromatogram of the bitter orange essential oil. As shown from the chromatogram there are large differences in the retention times for the two columns.

3.2. GC–MS and LC–MS analysis

The essential oils, and the compounds purified by HPLC (Fig. 5) were analyzed by GC–MS (Fig. 6) and LC–MS:

Peak no. 1. The CI mass spectrum obtained by LC–MS gives the base peak at m/z 261 [M+H] and peaks at m/z 243 [M+H−H_2O] and 283 [M+Na]. In NP- and RP-HPLC, this compound has the same retention time and the same UV spectrum as citropten but a greater molecular mass (citropten, M_r 206). Citropten was not found in bitter orange and grapefruit oils.

Peak no. 2. By comparison of UV, LC–MS and

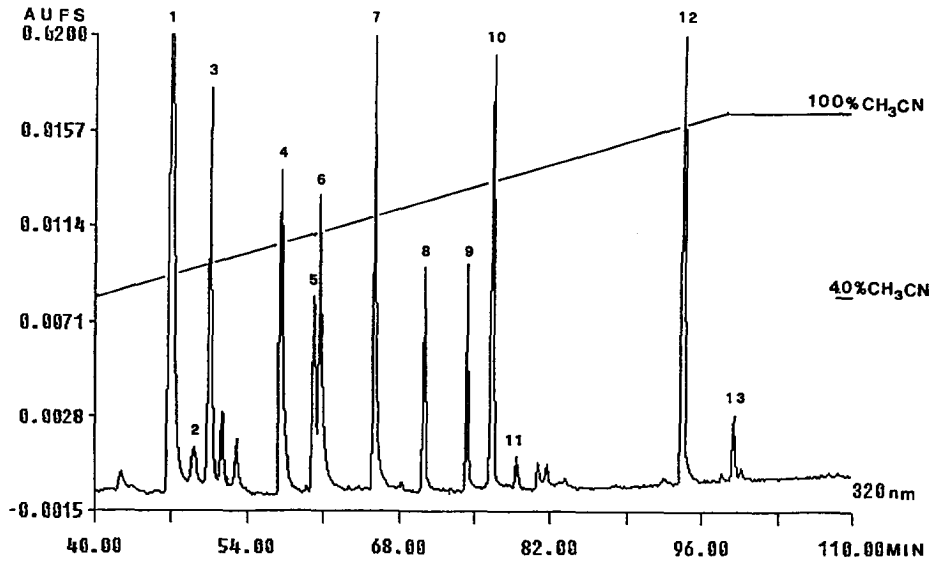

Fig. 1. RP-HPLC chromatogram of bitter orange essential oils. Column, 500×1.1 mm I.D.), 5 μm ODS2; flow-rate, 40 μl/min; mobile phase: A=acetonitrile, B=distilled water; gradient, from 40% A to 100% A in 100 min. Peaks as in Table 1.

GC–MS spectra with the standard, this peak was identified as bergapten.

Peak no. 3. The UV spectrum is similar to that of peak no. 1, and the peaks *m/z* 243 [M−H−H_2O], 261 [M+H] and 283 [M+Na] are present in the chemical ionization (CI) LC–MS mass spectrum. In the normal-phase mode peaks 1 and 3 are not well resolved.

Peak no. 4. The CI mass spectrum gives the base peak at *m/z* 403 [M+H]. The peaks *m/z* 388 [M+H−CH_3] and *m/z* 373 [M+H−2CH_3] are also present. Peak no. 4 is the same peak already iden-

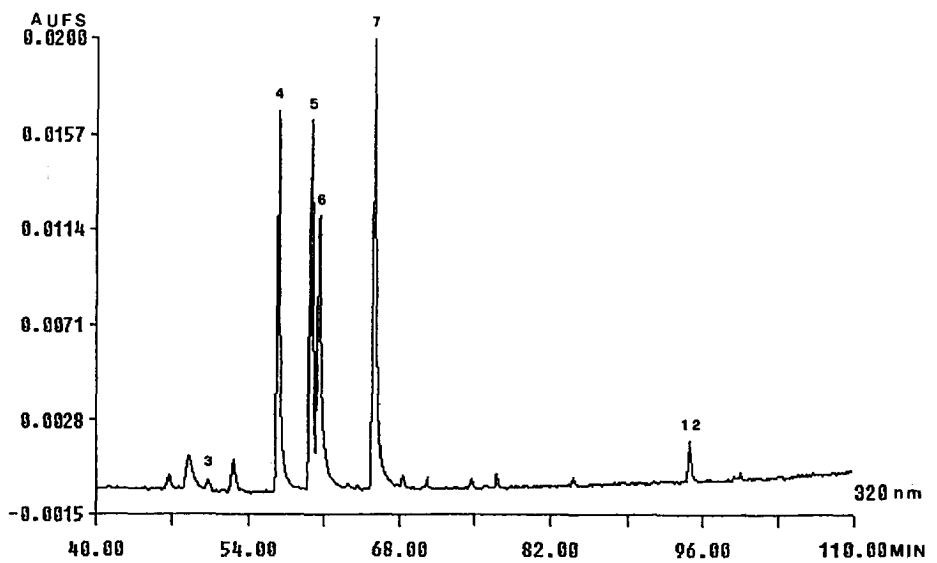

Fig. 2. RP-HPLC chromatogram of sweet orange essential oil. Conditions as in Fig. 1.

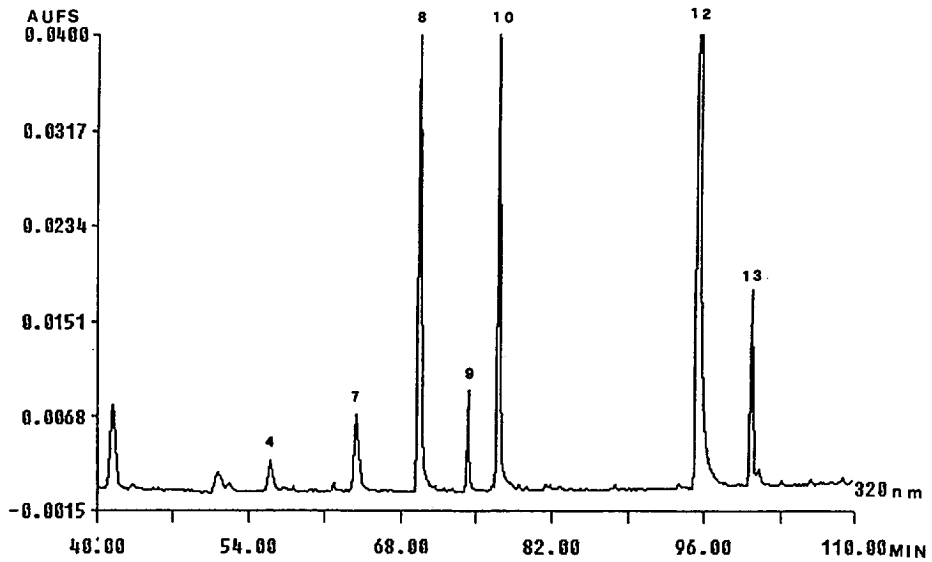

Fig. 3. RP-HPLC chromatogram of grapefruit essential oil. Conditions as in Fig. 1.

tified in sweet orange [2]. It is 3′,4′,5,6,7,8-hexamethoxyflavone or nobiletin ($C_{21}H_{22}O_8$) and, as reported in the literature [8,9,13,14], it is found in some citrus oils; however, its mass spectrum is unknown.

Peak no. 5. The CI mass spectrum gives the base peak at *m/z* 433 [M+H]. The peaks *m/z* 418 [M+H−CH_3] and *m/z* 403 [M+H−2CH_3] are also present. The electron ionization (EI)-MS spectrum is similar to that of 3,3′,4′,5,5′,6,7-heptametoxyflavone with M_r 432 and formula $C_{22}H_{24}O_9$ obtained from GC–AED. The NMR spectrum of the peak, purified by HPLC, confirmed that this compound is an heptametoxyflavone [2].

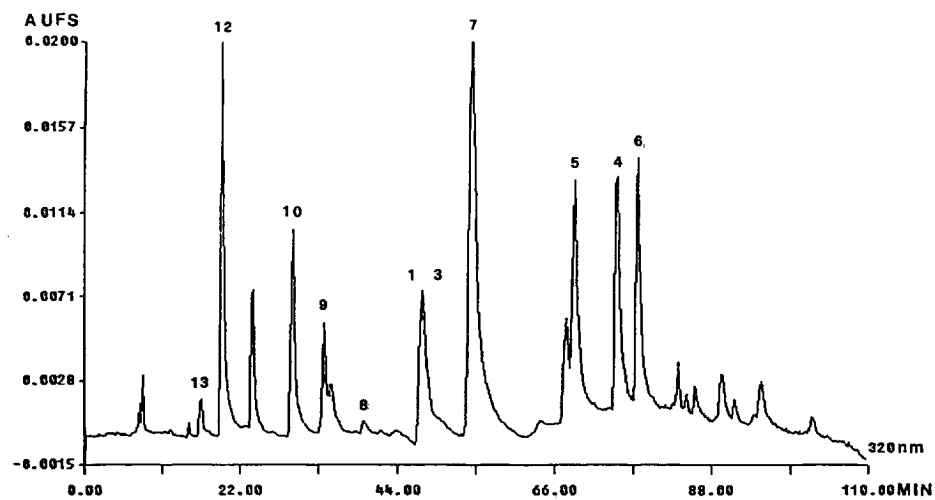

Fig. 4. NP HPLC chromatogram of bitter orange essential oil. Column (500 mm×1.1 mm I.D.), 5 μm NH_2, flow-rate 50 μl/min; mobile phase: A=isopropanol B=*n*-hexane with the following gradient: 0 min, A=5%; 50 min, A=5%; 80 min, A=30%; 120 min, A=45%. Peaks as in Table 1.

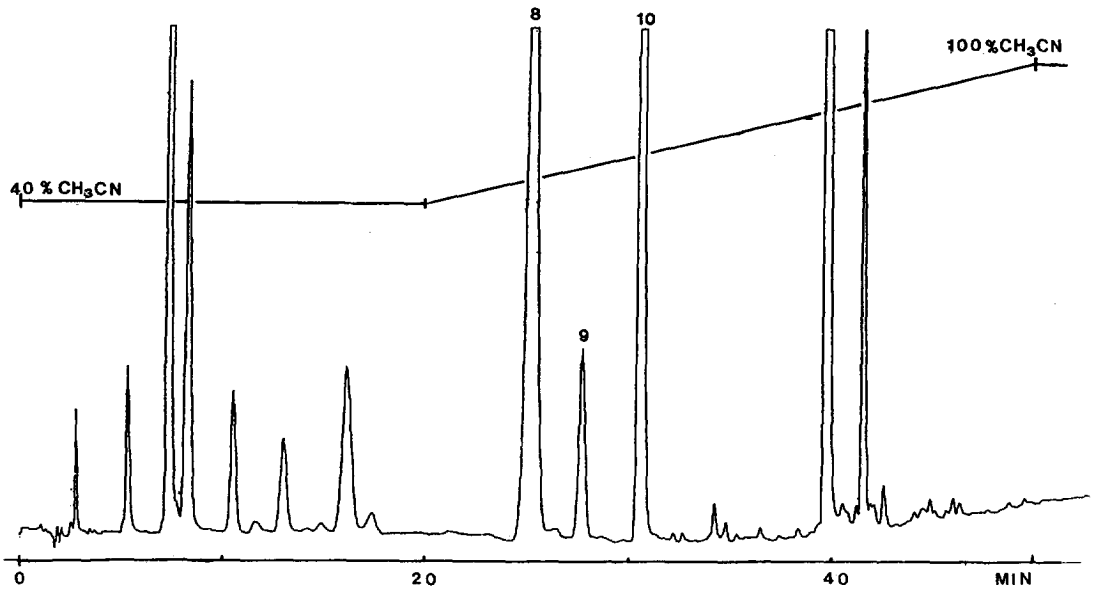

Fig. 5. Fractionation RP-HPLC chromatogram of grapefruit essential oil. Peaks 8, 9 and 10 were collected. Column, 165×4.6 mm I.D., 5 μm ODS2; flow-rate, 1 ml/min; mobile phase: A=acetonitrile, B=distilled water; gradient: 0–20 min, 40% A; subsequently from 40% A to 100% A in 30 min; UV detection, 320 nm.

Peak no. 6. This is the same peak already identified in sweet orange. It is tetra-O-methylscutellarein [2].

Peak no. 7. The UV, LC–MS and GC–MS spectra are the same as those of the tangeritin standard.

Peak no. 8. The M_r 332 was obtained by LC–MS. Fig. 7 shows the GC–EI-MS spectrum. The peak *m/z* 162 is evident in this spectrum. Loss of the allylic side chain with an hydrogen transfer, probably from allylic $C_{(3')}$-methyl group via a six-membered ring transition-state, results in the 7-hydroxycoumarin radical ion (*m/z* 162), and then expulsion of CO gives the benzofuran (*m/z* 134). Cleavage of the allylically activated $C_{(1')}$–O ether bond, with charge retention on the side chain, gives the allylic carbonium ion which further fragments in a variety of ways, among the more important of which are the loss of water from the $C_{(6')}$–$C_{(7')}$ diol system to give

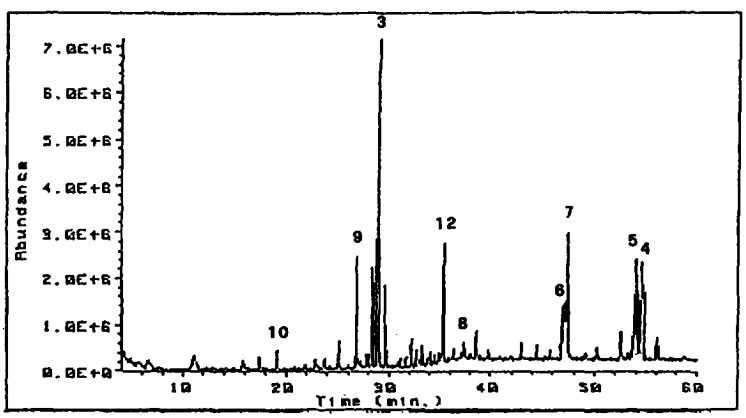

Fig. 6. GC–MS total-ion chromatogram of bitter orange essential oil. Column, SE-54, 25 m×0.15 mm, film thickness 0.12 μm); carrier gas, helium; temperature program: 150°C for 10 min, from 150° to 280°C in 26 min and 34 min at 280°C. Peaks as in Table 1.

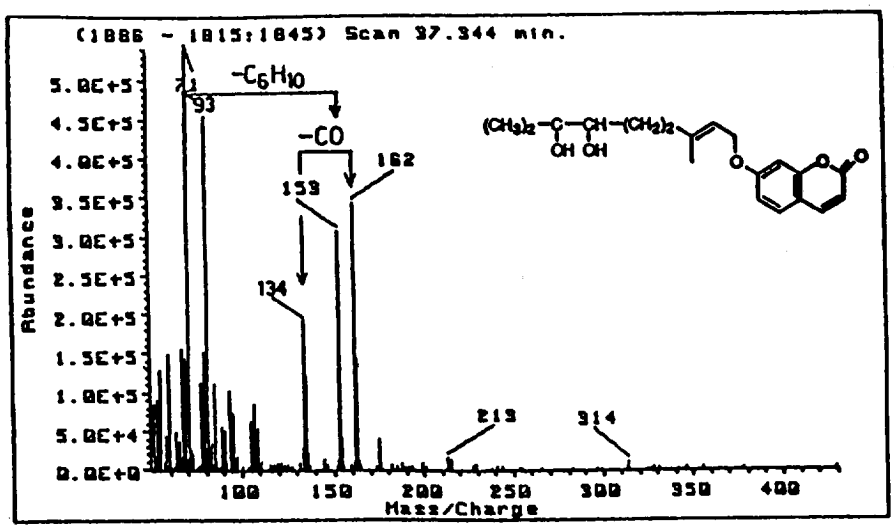

Fig. 7. GC–EI-MS mass spectrum of peak no. 8.

the ion m/z 153 and the cleavage of the $C_{(5')}$–$C_{(6')}$ bond and formation of the ion m/z 71 [14]. From this mass spectrum, this compound can be 7-(6′,7′-dihydroxy-3′,7′-dimethyl-2′-octenyloxy) coumarin.

Peak no. 9. The LC–MS gives M_r 244. In the GC–EI-MS spectrum (Fig. 8) the parent ion is the base peak. Loss of the methyl group probably occurs from the side chain and gives the peak m/z 229, then the loss of CO gives the peak m/z 201. The peak m/z 213 is due to loss of the methoxy group from the molecular peak. Fission of the β-chain to the ring to remove a C_4H_7 radical gives the tropylium-type ion m/z 189 [M−55], which subsequently loses a methoxy group to give the m/z 159, and the expulsion of CO from the pyran ring gives the ion m/z 131. From this mass spectrum and instrument library

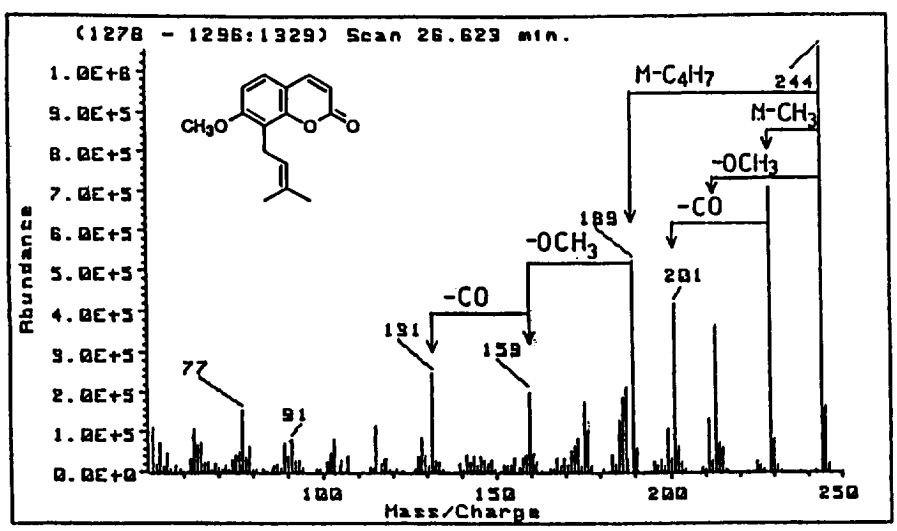

Fig. 8. GC–EI-MS mass spectrum of peak no. 9.

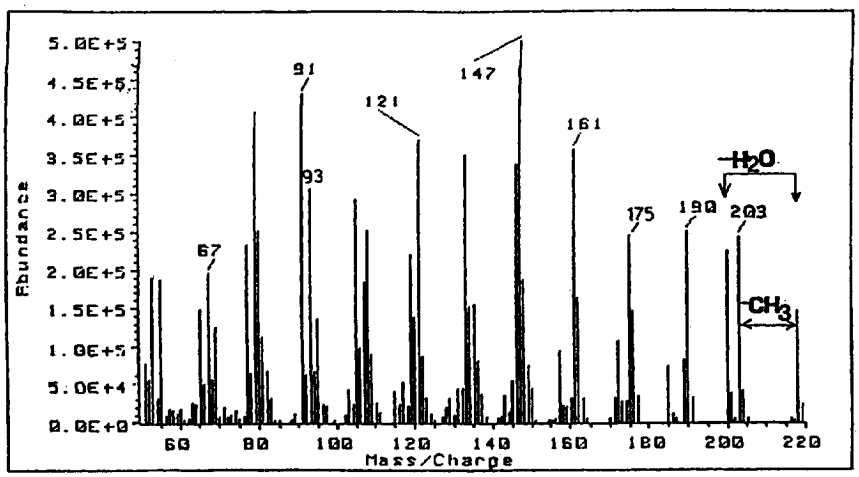

Fig. 9. GC–EI-MS mass spectrum of peak no. 10.

comparison, this peak can be osthol (7-methoxy-8-isoprenylcoumarin $C_{15}H_{16}O_3$)· The literature [7] reports that osthol is present in bitter orange essential oil.

Peak no. 10. The M_r 218 was obtained by LC–MS. In the GC–MS spectrum (Fig. 9) the base peak is *m/z* 147. The peak *m/z* 203 [M−CH_3], and the peak *m/z* 200 may be due to loss of water, and the peak *m/z* 190 to loss of CO from the molecular ion. By retro Diels–Alder reaction with hydrogen transfer, the ion *m/z* 190 gives *m/z* 121, and *m/z* 93 by loss of CO. The peak *m/z* 91 is the tropylium ion. No similar spectra were found in the instrument library or in the mass spectra atlas [12], so this compound cannot be identified.

Peaks no. 12 and 13. By comparison of the retention time, UV and MS spectra of the standard compounds these compounds were found to correspond to aurapten (7-geranyloxycoumarin) and bergamottin (5-geranyloxypsoralen), respectively. Peaks nos. 2, 7, 12, 13 were identified by LC–MS and GC–MS and confirmed by comparing with pure

Table 1
Detected compounds in the non-volatile fraction of the essential oils

Bitter orange	Sweet orange	Grapefruit	Peak no.	Identification
+		+	1	Unknown
+			2	Bergapten
+		+	3	Unknown
+	+	+	4	3′,4′,5,6,7,8-Hexamethoxyflavone (nobiletin)
+	+	+	5	Heptamethoxyflavone
+	+		6	5,6,7,4′-Tetramethoxyflavone (tetra-O-methylscutellarein)
+	+	+	7	5,6,7,8,4′-Pentamethoxyflavone (tangeritin)
+		+	8	7-(6′-7′-Dihydroxy-3′-7′-dimethyl-2′-octenyloxy) coumarin
+		+	9	Osthol
+		+	10	Unknown
+		+	11	Unknown
+	+	+	12	Aurapten
+	+	+	13	Bergamottin

standards. The other peaks were identified by LC–MS and GC–MS and with aid of literature data of the most probable compounds, since we did not have the pure standards. As shown in Table 1, many of these compounds are found in both of these oils, whilst the sweet and bitter orange oils show large differences. This fact can be very useful to detect the addition of sweet orange essential oil to the bitter orange oil, the latter being of more commercial value.

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