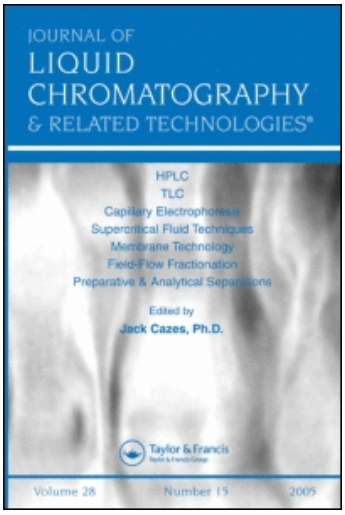

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IDENTIFICATION OF MINOR OXYGEN HETEROCYCLIC COMPOUNDS OF CITRUS ESSENTIAL OILS BY LIQUID CHROMATOGRAPHY-ATMOSPHERIC PRESSURE CHEMICAL IONISATION MASS SPECTROMETRY

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ABSTRACT

The oxygen heterocyclic compounds (coumarins, psoralens, and polymethoxylated flavones (PMFs)) present in citrus essential oils (lemon, mandarin, sweet orange, bitter orange, bergamot, grapefruit, and lime) were analysed by an HPLC-MS system equipped with an APcI source. The mass spectra obtained at different voltages of the "sample cone" have been used to build a library.

Citrus essential oils have been analysed with this system, using an optimised normal phase HPLC method, and the mass spectra were compared with those of the home made library. This method allowed the rapid identification and the characterisation of the oxygen heterocyclic compounds of citrus oils, the detection of some minor components for the first time in some oils, and the detection of authenticity and possible adulteration of the oils.

INTRODUCTION

The non volatile residue of citrus essential oils greatly influences the olfactory properties of these oils. The residue ranges from approximately 1-15% in the different oils and consists, in large part, of many oxygen heterocyclic compounds, particularly coumarins, psoralens, and polymethoxylated flavones.[1]

The qualitative and quantitative composition of this residue characterises the different citrus oils, and plays an important role in identifying the various oils, and controlling their quality and authenticity. Moreover, the oxygen heterocyclic compounds posses numerous pharmacological activities. Figure 1 shows the basic structures of the oxygen heterocyclic compounds present in citrus oils.

Data of the literature on oxygen heterocyclic compounds, often refers only to the main components of this fraction, and report some contradictions.[2-6] Literature reports many examples of isolation of oxygen heterocyclic compounds from citrus essential oils by classical chromatographic techniques (TLC, column chromatography) followed by identification by comparison of some parameters, such as R_f values, melting points, spectroscopic data, with those of reference compounds, where available.[6-8]

Although some GC method have been developed for the qualitative and quantitative analysis of this fraction,[9-10] HPLC is the most used technique, both in reserved[4,11-12] or in normal phase mode.[5,13,14]

LC detectors, such as UV or fluorescence, have been widely used. These detectors can give limited information about the identity of the analysed compounds compared to a MS detector. In fact, interfacing HPLC and MS represents one of the most powerful analytical techniques of recent times.[15-18]

The HPLC-MS analysis can be very useful for the characterisation of oxygen heterocyclic compounds, either to confirm the identity of the peaks already known, or to identify new components which are often present as traces, and are useful in the characterisation of citrus oils and for the possible pharmacological action they can have.

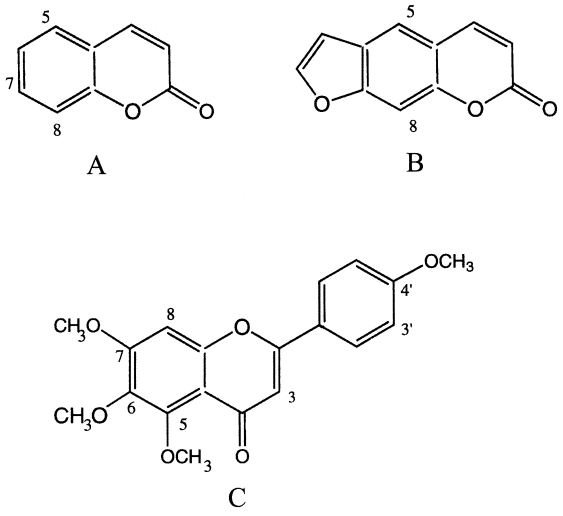


Figure 1. Chemical structure of coumarins (A), psoralens (B), and polymethoxylated flavones (C).

Some commercially available LC-MS interfaces, such as thermospray or particle-beam, presented many problems. They were critical to optimise, less sensitive then GC-MS systems, and their use was limited to a restricted number of samples.[16-18] With the introduction of atmospheric pressure ionisation (API) techniques as a means for mass spectrometric sample introduction, LC-MS has increased in popularity. Coupling HPLC-MS with an API (Atmospheric Pressure Ionisation) interface permits one to obtain a high selective and sensitive detection method, and to use the HPLC-MS technique in the routine analysis.

The analysis of the oxygen heterocyclic fraction of citrus oils by an HPLC system equipped with atmospheric pressure ionisation (API) using atmospheric pressure chemical ionisation interface in the positive mode ($APcI^+$) is reported. The compounds have been identified by comparison of the MS spectra at different cone voltages with those of a home made library. The detection and identification of some components previously not identified in some citrus oils is also reported.

EXPERIMENTAL

This research was carried out on samples of genuine cold-pressed citrus oils (mandarin, sweet orange, bitter orange, grapefruit, bergamot, lemon, and lime).

Table 1

Oxygen Heterocyclic Compounds Identified in Citrus Essential Oils

		MW	B.O.	S.O.	Lem	Lim	B	M	G
1	**Aurapten** (7-geranyloxycoumarin)	298							X
2	**Herniarin** (7-methoxycoumarin)	176			X	X			
3	**Epoxyaurapten** (7-(6',7'-epoxygeranyloxy)coumarin	314							X
4	**Meranzin** (7-methoxy-8(2',3'-epoxy)isopentenylcoumarin)	260	X						X
5	**Osthol** (7-methoxy-8-isopentenylcoumarin)	244	X						X
6	**Meranzin hydrate**(7-methoxy-8(2',3'-dihydroxy)isopentyloxycoumarin)	278	X						X
7	**Isomeranzin** (7-methoxy-8-(2'-one isopentyl)coumarin)	260	X						X
8	**Citropten** (5,7-dimethoxycoumarin)	206			X	X	X		X
9	5-Isopentenyloxy-7-methoxycoumarin	260			X	X			
10	5-Geranyloxy-7-methoxycoumarin	328			X	X	X		
11	**Bergapten** (5-methoxypsoralen)	216	X			X	X		X
12	**Epoxybergamottin** (5-(6',7'-epoxy-geranyloxypsoralen)	354	X						X
13	**Epoxybergamottin hydrate** (5-(6',7'-dihydroxy-geranyloxypsoralen)	372	X						X
14	**Bergamottin** (5-geranyloxypsoralen)	338			X	X	X		X
15	**Oxypeucedanin** (5-(2',3'-epoxy-isopentyloxy)psoralen)	286			X	X			
16	**Oxypeucedanin hydrate** (5-(2',3'-dihydroxy-isopentyloxy)psoralen)	304			X	X			
17	**Isoimperatorin** (5-isopentenyl-oxypsoralen)	270			X	X			
18	8-Geranyloxypsoralen	338			X	X			
19	**Imperatorin** (8-isopentenyloxy-psoralen	270			X	X			
20	5-Geranyloxy-8-methoxypsoralen	368			X	X			
21	**Byakangelicin** (8-(2',3'-dihydroxy-isopentyloxy)-5-methoxypsoralen)	334			X				
22	5-Isopent-2'-enyloxy-8-(2',3'-epoxyisopentyloxy)psoralen	370			X				
23	**Phellopterin** (5-methoxy-8-isopentenyloxypsoralen)	300			X				
24	**Byakangelicol** (5-methoxy-8-(2',3'-epoxy-isopentyloxypsoralen)	316			X	X			
25	**Isopimpinellin** (5,8-dimethoxy psoralen)	246				X			
26	**Cnidilin** (5-Isopentenyloxy-8-methoxypsoralen)	300				X			
27	**Tangeretin** (4',5,6,7,8-pentamethoxyflavone)	372	X	X				X	X

Table 1 (continued)

		MW	B.O.	S.O.	Lem	Lim	B	M	G
28	**Nobiletin** (3',4',5,6,7,8-hexamethoxyflavone)	402	X	X				X	X
29	3,3',4',5,6,7,8-Heptamethoxyflavone	432	X	X				X	X
30	**Sinensetin** (3',4',5,6,7-pentamethoxyflavone	372		X			X	X	
31	3,3',4',5,6,7-Hexamethoxyflavone	402		X					
32	**Tetra-O-methylscutellarein** (4',5,6,7 tetramethoxyflavone)	342	X	X			X	X	

MW = Molecular weight; B.O. = Bitter Orange; S.O. = Sweet Orange; M = Mandarin; Lem = Lemon; Lim = Lime; G = Grapefruit; B = Bergamot Isopentenyloxy = 3'-methylbut-2'-enyloxy; Geranyloxy 3',7'-dimethyloct-2',6'-enyloxy.

The oxygen heterocyclic compounds of these citrus oils were previously isolated and characterised in our laboratory,[7,14,19-20] and have been used as standard compounds to prepare a library of mass spectra. Oxygen heterocyclic compounds commercially available have been purchased from Extrasynthese (Genay, France). All the standard compounds and the citrus oils have been analyzed by normal-phase HPLC (Thermo Separation Products equipment composed of: HPLC pump model P2000, gradient controller, Rheodyne injector, spectrophotometric detector model UV 2000).

Method A

The column was a 15 cm x 3.9 mm i.d. μ-Porasil, with a particle size of 10 μm (Waters Associates). Two mobile phases were used: eluent A (hexane:ethyl acetate, 9:1) and eluent B (hexane:ethyl alcohol, 9:1). The HPLC analyses were carried out according to the following program: 0-7 min, 98% A + 2% B; 7-20 min, from 98% A + 2% B to 5% A + 95% B. The flow rate was 1.0 mL/min; pressure, 34 atm. The injection volume was 20 μL of a 5% solution of oil in hexane:ethyl acetate, 75:25. The detection was by UV absorbance at 315 nm.

Method B

Bitter orange oil, which shows a quite complex composition of the oxygen heterocyclic fraction, has been analysed also under the following conditions to separate all the components of interest: μ-Porasil column 30 cm × 3.9 i.d. (10 μm) for the first 12 min, then the flow was switched to a second column, Zorbax silica 25 cm × 4.6 mm i.d. (7 μm). Eluent A, hexane:ethyl acetate, 9:1, eluent B, hexane:ethyl alcohol, 9:1. 95% B over 23 min (2-25 min) with a concave gradient, then 20 min isocratic 95% B; flow rate 1.6 mL/min.

The HPLC was coupled to a Navigator LC-MS detector with an Atmospheric Pressure chemical Ionisation (APcI) probe. The flow at the exit of the HPLC column was split with a zero dead volume T-piece between the APcI source and the UV detector in the ratio 1:4. The MS acquisition was performed under the following conditions: Full scan (90-450m/z), $APcI^+$. Cone voltage Fragmentation: 20, 40, and 60 V. Source heater, 120°C; APcI heater, 500°C; APcI pin, 3,88 kV. Drying gas, N_2, 270 L/hr.

UV and MS data were acquired and processed using Masslab software for Windows NT™, equipped with a home made library of mass spectra of oxygen heterocyclic compounds previously isolated from citrus oils or found on the market.

RESULTS

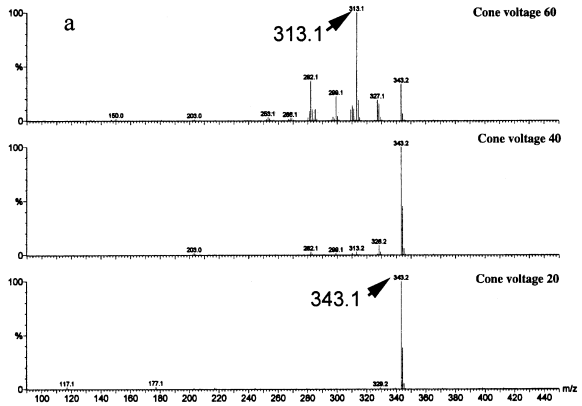
All the standard oxygen heterocyclic compounds have been injected in flow–injection (FIA), in $APcI^+$ mode, and a library has been built with MS spectra obtained at different voltages of the sample cone. The simultaneous acquisition at different values of cone voltage can give structural information in addition to the molecular weight information, because the degree of fragmentation is dependent on the magnitude of the cone voltage. The technique is called "cone voltage fragmentation". At low cone voltage (20 V, in our case), the oxygen heterocyclic compounds show the $(M+H)^+$ ion, while at higher cone voltage (40 and 60 V) there are additional fragments.

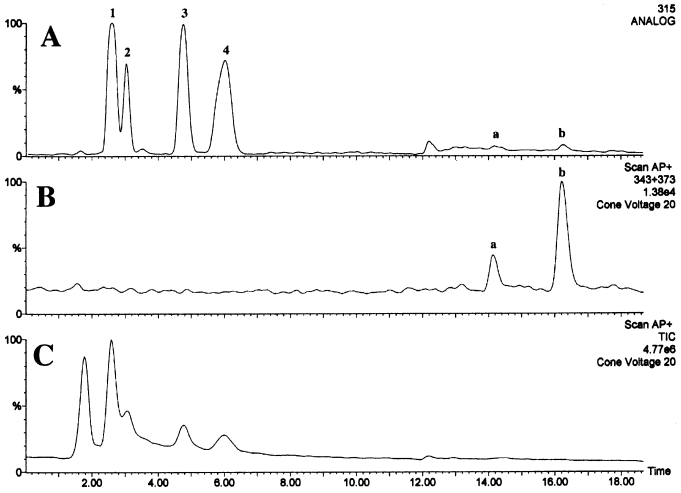
The compounds injected are reported in Table 1. They are 10 coumarins, 16 psoralens, and 6 polymethoxylated flavones. They were isolated from the various citrus oils in different stages by column chromatography, preparative thin layer chromatography, or semiprepeparative HPLC.[7,14,19-20]

The concentration of these components in the different oils may vary, so a component present in large amount in an oil may be a minor component in an other oil. The LC-MS analysis can help in the identification of some minor components, very difficult to isolate and to identify by off-line methods.

All citrus essential oils have been analysed by HPLC-MS in $APcI^+$ mode, under the same HPLC and MS conditions. The MS conditions were identical to those used to acquire MS spectra for the library.

Figure 2 (right). HPLC-UV chromatogram (A), HPLC-TIC chromatogram (C) and extracted chromatogram at m/z = 343 + 373 (B) of the coumarin fraction of bergamot oil, with cone voltage fragmentation of peak a and b, identified as tetra-O-methylscutellarein and sinensetin, respectively.





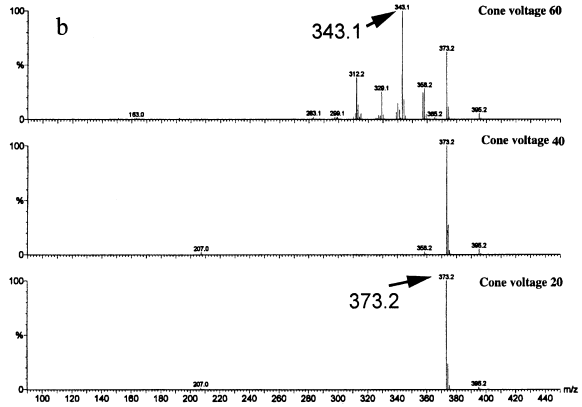


Table 1 also reports the molecular weight of the components, and their distribution in the various oils, as obtained by comparison of the single MS spectra to data of the home-made library, and retention time of the same component in the different oils. In this way, the confirmation of the components previously identified and dosed in the citrus oils was possible. Other components have been identified for the first time in citrus oil; in particular, two polymethoxylated flavones in bergamot oil, a coumarin in lemon oil, and an other coumarin in bitter orange oil.

Bergamot Oil

With the HPLC-MS coupling it was possible to confirm the presence of tetra-O-methylscutellarein and sinensetin in bergamot oil. These two PMFs were not previously reported in bergamot oil. Figure 2 shows the HPLC-UV chromatogram of a genuine bergamot oil (A), together with the HPLC TIC chromatogram acquired at cone voltage 20 V (C), and the extracted chromatogram at m/z = 343 + 373 (B). The figure also shows the cone voltage fragmentation of peaks a and b.

These mass spectra were compared to those of the library, and there was good agreement with the corresponding spectra of standard tetra-O-methylscutellarein and sinensetin. To confirm the identification, Figure 3 shows the HPLC chromatogram acquired at cone voltage 20 V of a genuine cold-pressed sweet orange oil, that certainly contains these two PMFs, compared to the HPLC-MS chromatogram of a genuine cold pressed bergamot oil, together with the extracted chromatograms at m/z = 343 and m/z = 373, corresponding to the $(M+H)^+$ ions of tetra-O-methylscutellarein and sinensetin, respectively.

Bitter Orange Oil

Figure 4 shows the HPLC-UV chromatogram of a bitter orange oil, together with the LC-MS chromatogram acquired at low cone voltage, and the extracted chromatogram at m/z=261.

Bitter orange oil contains numerous components that show the m/z = 261 ion. Meranzin ($(M+H)^+$ ion = 261), isomeranzin ($(M+H)^+$ ion = 261) meranzin hydrate, ($(M+H)^+$ -18 = 261) have been previously identified in the oil. The peak eluted before meranzin and isomeranzin has never before been identified in bitter orange oil. Figure 5 shows the cone voltage fragmentation of this peak, that was identified as citrusal. To confirm this identification, the ^{1}H-NMR spectrum of a fraction containing this compounds in mixture with meranzin and isomeranzin has been acquired. The signal (1H, s) of an aldehydic proton at 9.6 ppm was detected.

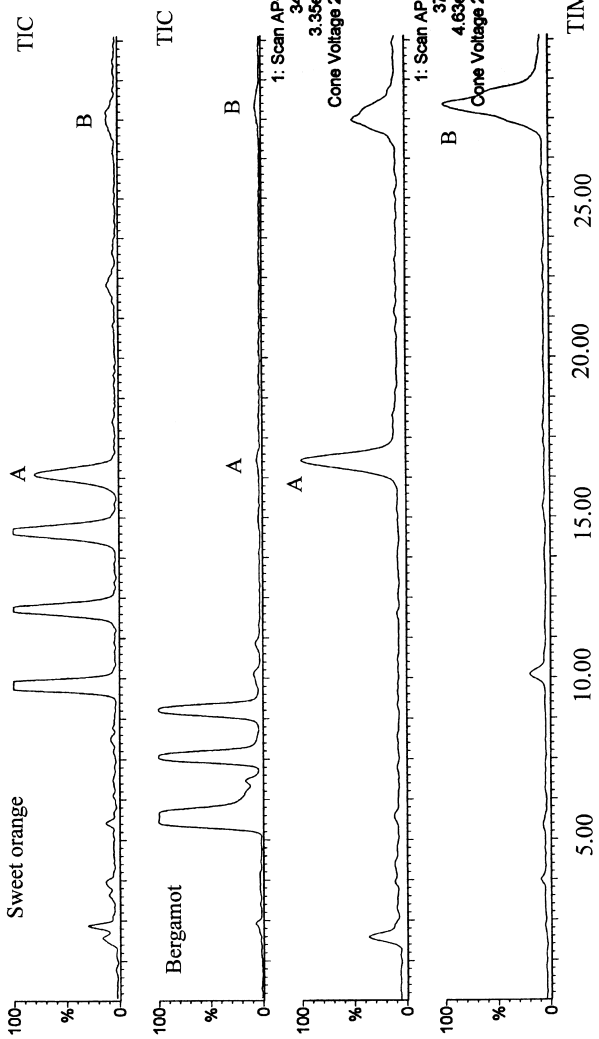


Figure 3. HPLC-TIC chromatograms of sweet orange and bergamot oils, and extracted chromatograms at m/z = 343 and 373 of the coumarin fraction of bergamot oil.

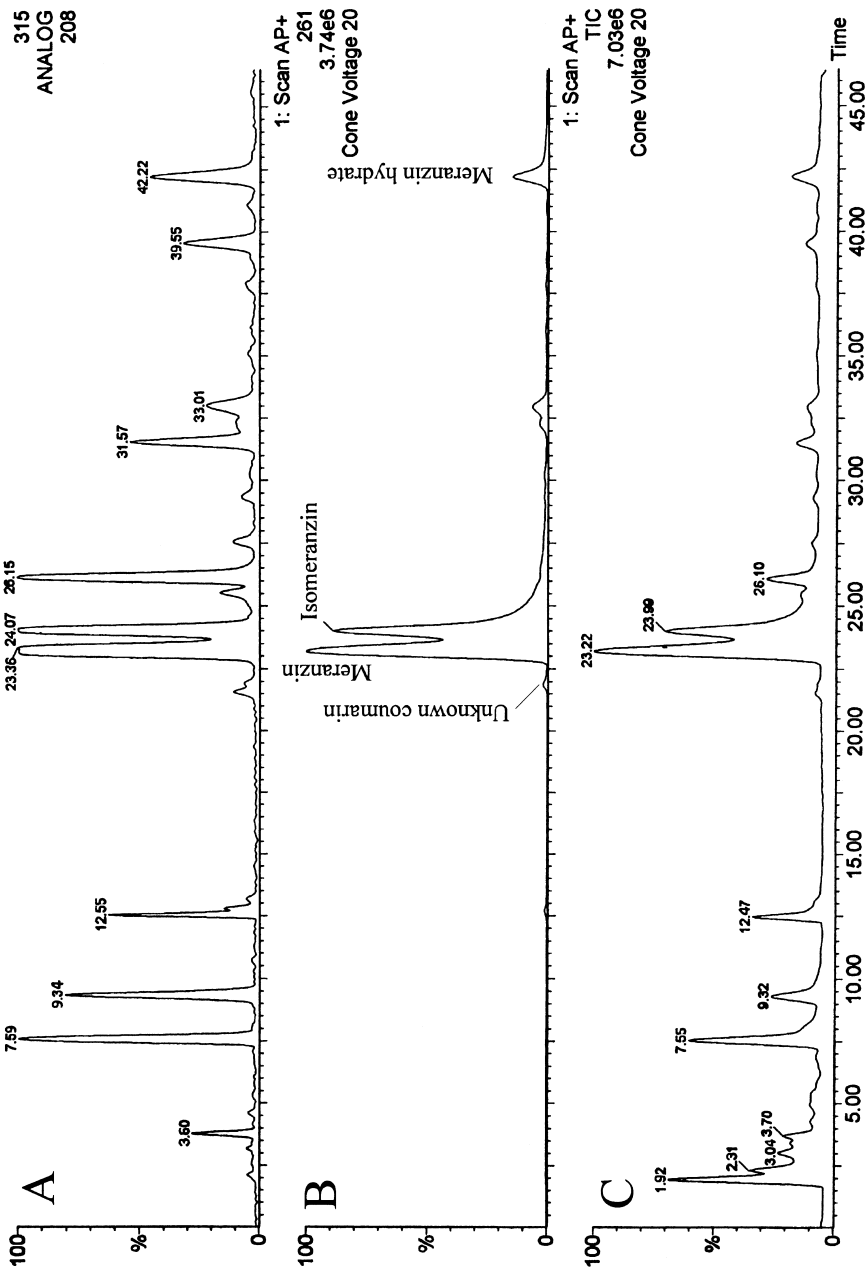


Figure 4. HPLC-UV chromatogram (A), HPLC-TIC chromatogram (C) and HPLC-TIC chromatogram extracted at m/z = 261 (B) of the coumarin fraction of bitter orange oil.

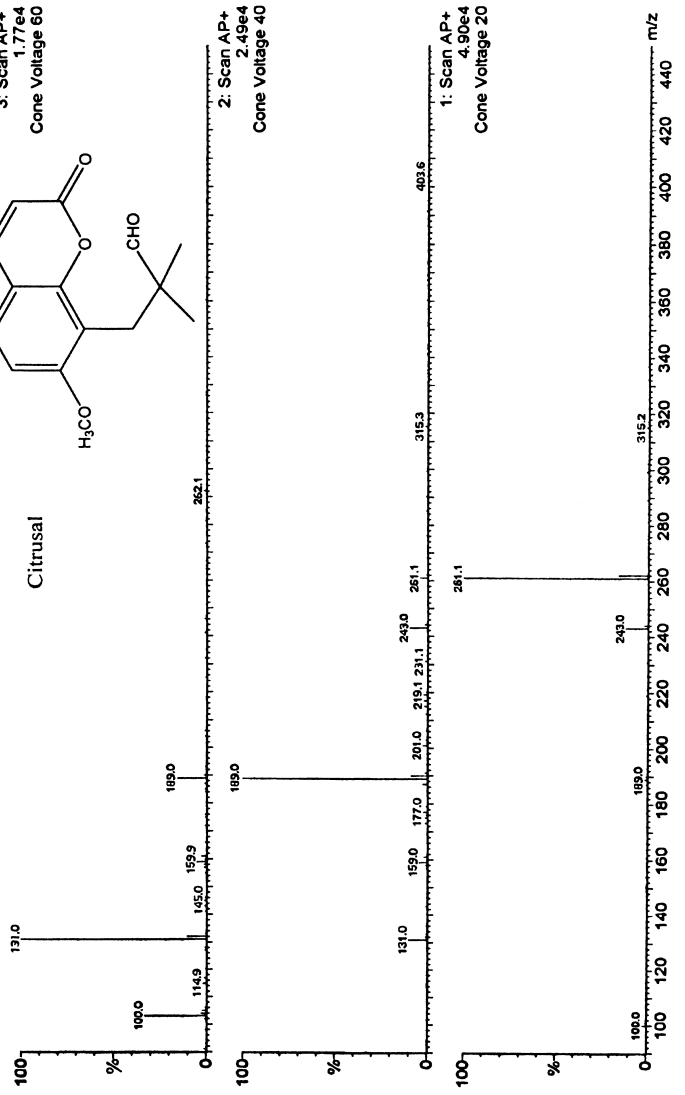


Figure 5. Cone voltage fragmentation of citrusal using APcI$^+$ ionization.

Lemon Oil

Herniarin (7-methoxycoumarin) has been reported in literature as a compound characteristic only of lime oil. Under the HPLC conditions used in this study (method A), herniarin co-elutes with 8-geranyloxypsoralen, a compound present both in lime and in lemon oil.

By HPLC-MS at different cone voltage, it was possible to detect herniarin also in lemon oil. Figure 6 shows the cone voltage fragmentation of the peak of lemon oil containing 8-geranyloxypsoralen + herniarin, compared to the MS spectra of the two standard compounds. The presence of peak at m/z = 177 in the MS spectrum of figure 6B confirms the presence of herniarin in lemon oil.

Because the addition of lime oil to lemon oil is one of the most common adulteration of lemon oil, the detection of small amounts of herniarin in the oxygen heterocyclic fraction of lemon oil does not necessarily mean that lemon oil has been added of lime oil. The composition of the volatile fraction and of the oxygen heterocyclic fraction of these two oils is quite similar, so it is difficult to detect adulteration of lemon with lime oil either by GC analysis or by HPLC-UV analysis.

HPLC-MS analysis can be very useful to identify the presence of lime in lemon oil, for example by detection of isopimpinellin (MW=246), a compound identified only in lime oil.

DISCUSSION

HPLC-MS technique with API interface, using the "cone voltage fragmentation" has been particularly useful for the study of the oxygen heterocyclic fraction of citrus essential oils. The above described method allowed the confirmation of the identification of the main components of the fraction, previously reported for the different oils, and allowed the identification for the first time of some minor components in bergamot, bitter orange, and lemon oils.

The identification of such components by off-line methods would need the isolation and purification by long and laborious procedures, followed by the spectroscopic characterization.

The use of hyphenated techniques, such as LC-MS provides great information about the content and nature of constituents of natural complex matrices, such as essential oils. The isolation of the components can be done only if further structure elucidation is needed, or for biological or pharmacological study. In this way, the unnecessary isolation of common compounds of minor interest is avoided.

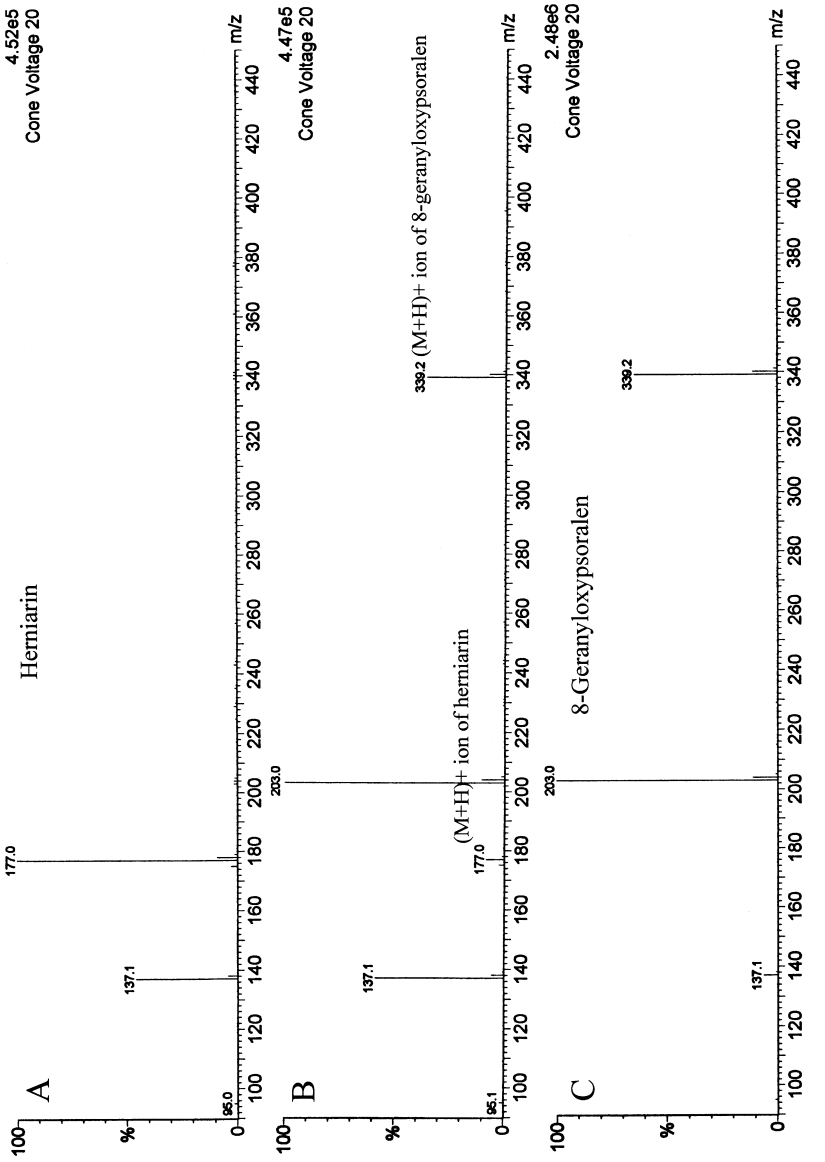


Figure 6. Cone voltage fragmentation of standard herniarin (A), standard 8-geranyloxypsoralen (C) and of the peak of lemon oil containing herniarin + 8-geranyloxypsoralen (B).

As recently stated by other authors,[21] the information generated with the use of hyphenated techniques such as HPLC-UV or HPLC-MS can be more efficiently used if all the data will be centralized for rapid pattern recognition by reference to standard components.

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