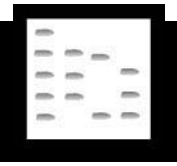
CITRUS OILS: LIQUID CHROMATOGRAPHY



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Citrus essential oils are very complex matrices which contain numerous compounds of different chemical classes. These compounds are generally divided into two fractions: the volatile fraction, which is the most representative, and ranges between 85 and 99% in the different cold-pressed citrus oils, and the non-volatile residue, which ranges between 1 and 15%.

The development of new instrumental analytical techniques, mainly chromatographic, has allowed the characterization of citrus essential oils to become more precise.

Gas chromatography is an essential tool for the study of the volatile fraction, while liquid chromatography (thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) combined with spectral absorption and fluorescence measurements) is widely used for the study of the composition of the non-volatile residue. This fraction consists largely of oxygen heterocyclic compounds (coumarins, psoralens (furanocoumarins) and polymethoxylated flavones) that exhibit strong absorption in the ultraviolet region (λ_{max} about 315 nm).

The presence of coumarin compounds is widespread in plants of the *Rutaceae* family to which the citrus species belong. Their presence is qualitatively and quantitatively different in the different citrus oils, so knowledge of the oxygen heterocyclic fraction may be useful to assess authenticity, the geographical origin and the possible adulteration of the oils.

Figure 1 shows the basic structures of the oxygen heterocyclic compounds present in citrus oils. In the numbered position, coumarins and psoralens may contain hydroxyl, methoxyl, isopentenyl, isopentenyloxy, geranyloxy groups; polymethoxylated flavones contain methoxyl groups. **Table 1** lists the oxygen heterocyclic compounds identified in citrus oils.

TLC Separations

The literature from the 1930s to the end of 1970s reports numerous TLC separations of non-volatile residue of citrus oils with the aim of isolating the components. Often these components were not identified before and were responsible for ultraviolet (UV) absorption. Knowledge of the chromatographic characteristics and the chemical structures of these compounds was considered very important in order to determine the authenticity of the oils. In fact, valuable cold-pressed oils may be adulterated with less valuable cold-pressed or distilled oils. In these cases the presence and/or the content of some oxygen heterocyclic compounds may be useful to determine the kind and also the degree of adulteration.

Many methods developed for the TLC analysis of oxygen heterocyclic compounds of citrus oils used silica gel as a stationary phase, and mixtures of hexane or cyclohexane with variable amounts of ethyl acetate as mobile phases. For example, a paper of 1965 reports the separation of coumarins of many citrus oils by TLC, obtained on silica gel plates, using mixtures of hexane with variable amounts (25–70%) of ethyl acetate, according to the different polarity of the components. The spots were detected by UV absorbance at 254 and 366 nm. The components were also isolated from the plates, extracted from silica gel and analysed by UV spectroscopy. **Table 2** provides chromatographic and spectroscopic data for the oxygen heterocyclic compounds of lemon, bergamot, mandarin, sweet orange and bitter orange. Identification was carried out by comparison of R_f values and spectroscopic data with those of standard compounds.

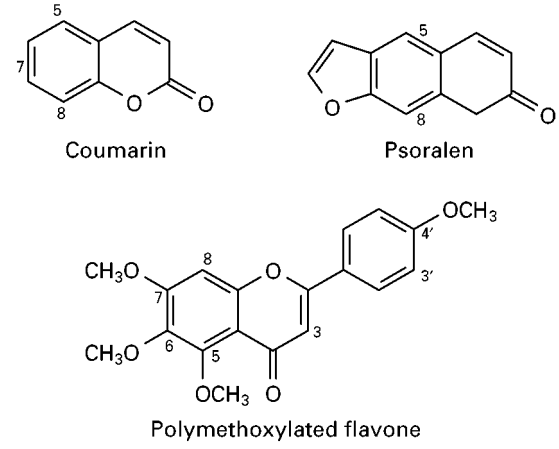

Figure 1 Structures of oxygen heterocyclic compounds present in citrus oil.

Table 1 Oxygen heterocyclic compounds identified in citrus essential oils

	Bitter orange	*Sweet orange*	*Lemon*	*Lime*	*Bergamot*	*Mandarin*	*Grape fruit*
Aurapten (7-geranyloxycoumarin)	X		X				X
Auraptenol (7-(2′-hydroxy-isopent-3′-enyloxy)coumarin	X						
Marmin (7-(6′,7′-dihydroxygeranyloxy)coumarin)							X
Umbelliferone (7-hydroxycoumarin)	X						
Herniarin (7-methoxycoumarin)			X	X			
Epoxyaurapten (7-(6′,7′-epoxygeranyloxy)coumarin)							X
7-Isopentenyloxycoumarin			X				
Meranzin (7-methoxy-8(2′,3′-epoxy)isopentenylcoumarin)	X						X
Osthol (7-methoxy-8-isopentenylcoumarin)	X						X
Meranzin hydrate (7-methoxy-8(2′,3′-dihydroxy)isopentyloxy-coumarin)	X						X
Isomeranzin (7-methoxy-8-(2′-one-isopentyl)coumarin)	X						X
7-(Methoxy-8(2′-formyl-2′-methylpropyl)coumarin)							X
Citropten (5,7-dimethoxycoumarin)	X	X	X	X	X		X
5-Isopentenyloxy-7-methoxycoumarin			X	X	X		
5-(2′,3′-Epoxy-isopentyloxy)-7-methoxycoumarin			X				
5-Geranyloxy-7-methoxycoumarin			X	X	X		
5-(2′,3′-Dihydroxy-isopentyloxy)-7-methoxycoumarin)			X				
Bergapten (5-methoxypsoralen)	X	X		X	X		X
Epoxybergamottin (5-(6′,7′-epoxy-geranyloxypsoralen)	X						X
Epoxybergamottin hydrate (5-(6′,7′-dihydroxy-geranyloxypsoralen)	X						X
Bergamottin (5-geranyloxypsoralen)			X	X	X		X
Bergaptol (5-hydroxypsoralen)	X	X	X		X		X
Oxypeucedanin (5-(2′,3′-epoxy-isopentyloxy)psoralen)			X	X	X		
Oxypeucedanin hydrate (5-(2′,3′-dihydroxy-isopentyloxy)psoralen)			X	X	X		
Pabulenol/Gosferol (5-(2′-hydroxy-3′-methylbut-3′-enyloxy)psoralen)			X				
Isoimperatorin (5-isopentenyloxypsorelen)			X	X			
8-Geranyloxypsoralen			X	X			
Heraclenin (8-(2′,3′-epoxy-isopentyloxy)psoralen)			X	X			
Heraclenol (8-(2′,3′-dihydroxyisopentyloxy)psoralen)			X				
Imperatorin (8-isopentenyloxypsoralen)			X	X			
8-(6′,7′-Epoxygeranyloxy)psoralen			X				
5-Methoxy-8(2′,3′-epoxy-isopentyloxy)psoralen			X				
5-Geranyloxy-8-methoxypsoralen			X	X			
Byakangelicin (8-(2′,3′-dihydroxy-isopentyloxy)-5-methoxypsoralen)			X		X		
Isobyakangelicol (5-methoxy-8-(2′-one-isopentyl)psoralen)				X			
5-Isopent-2′-enyloxy-8-(2′,3′-epoxy-isopentyloxypsoralen)			X				
Phellopterin (5-methoxy-8-isopentenyloxypsoralen)			X				
Byakangelicol (5-methoxy-8-(2′,3′-epoxy-isopentyloxypsoralen)			X	X	X		
Isopimpinellin (5,8-dimethoxypsoralen)				X			
Cnidilin (5-Isopentenyloxy-8-methoxypsoralen)				X			
Neobyakangelicol (5-methoxy-8-(2′-hydroxy-3′-methylbut-3′-enyloxy)psoralen			X				
5-Isopentenyloxy-8-(2′,3′-dihydroxy-isopentyloxy)psoralen			X				
Cnidicin (5,8-diisopentenyloxy)psoralen			X				
5-Methoxy-8-geranyloxypsoralen			X				
3,6,7,8,4′-pentamethoxyflavone	X						
Tangeretin (4′,5,6,7,8-pentamethoxyflavone)	X	X				X	X
Nobiletin (3′,4′,5,6,7,8-hexamethoxyflavone)	X	X				X	X
3,3′,4′,5,6,7,8-Heptamethoxyflavone	X	X				X	X
Sinensetin (3′,4′,5,6,7-pentamethoxyflavone)		X			X	X	
3,3′,4′,5,6,7-Hexamethoxyflavone		X					
Tetra-O-methylscutellarein (4′,5,6,7-tetramethoxyflavone)	X	X			X	X	

Isopentenyloxy = 3′-methylbut-2′-enyloxy; Geranyloxy = 3′7′-dimethyloct-2′,6′-enyloxy.

Figure 2 shows another example of a TLC separation of coumarins of a cold-pressed lemon oil, obtained using butyl acetate instead of ethyl acetate, in particular, hexane–butyl acetate 65 : 35 (A) and 75 : 25 (B). Detection was by UV absorbance at 254 nm. As can be seen, 21 components have been separated, but only the main components were identified: (1) bergamottin; (3) 5-geranyloxy-7-methoxy-

Table 2 Chromatographic and spectroscopic data of the oxygen heterocyclic compounds of lemon, bergamot, mandarin, sweet orange and bitter orange oils

Fluorescence	Rf_{25}*	Rf_{30}*	Rf_{40}*	Rf_{50}*	Rf_{60}*	Rf_{70}*	*λ max*	*λ min*	*λ shoulder*
Lemon oil									
1. yellow	0	0	0				315	280	–
2. yellow (bergaptol)	0.02	0.04	0.05				265, 310	260, 280	250
3. violet	–	0.08	0.15				315	280	230, 245, 255
4. red (byakangelicin)	0.14	0.16	0.20				240, 265, 310	235, 255, 285	250
5. yellow (5, 8 -... psoralen)	0.20	0.26	0.27				250, 307	235, 275	260
6. blue (citropten)	0.33	0.36	0.38				245, 325	240, 265	255
7. yellow (8-geranyloxypsoralen)									
8. blue	0.39	0.41	0.49				315	280	245, 270
9. blue (5-geranyloxy-7-methoxycoumarin)	0.44	0.46	0.55				323	277	245, 270
10. yellow (bergamottin)	0.50	0.52	0.59				250, 310	240, 278	270
Bergamot oil									
1. Yellow	0	0	0	0	0	0	–	–	–
2. Red	0	0	0	0	0	0.02	–	–	–
3. Blue	0	0	0	0.03	0.03	0.04	–	–	–
4. Blue	0	0	0	0.03	0.06	0.12	–	–	–
5. Violet	0.01	0.02	0.04	0.06	0.12	0.17	325	285	270
6. Yellow/red	–	0.03	0.09	0.14	0.20	0.26	270, 320	285	270
7. Blue	–	0.08	0.16	0.25	0.29	0.32	–	–	265
8. Green	–	0.08	0.22	0.29	0.40	0.44	270, 325	260, 305	–
9. Blue	0.14	0.17	0.28	0.32	–	–	324	275	270
10. Yellow (bergapten)	0.20	0.22	0.29	0.37	0.50	0.55	250, 260, 310	235, 255, 280	–
11. Blue (citropten)	0.33	0.36	0.38	0.42	0.53	0.59	245, 255, 270, 325	240, 250, 265, 275	–
12. Blue (5-geranyloxy-7-methoxycoumarin)	0.44	0.46	0.52	0.56	0.67	0.68	323	278	250, 270
13. Yellow (bergamottin)	0.50	0.52	0.59	0.60	0.67	0.68	240, 310	235, 280	270
14. Red	–	–	0.62	0.64	0.72	0.73	–	–	–
Mandarin oil									
1. Yellow			0	0	0	0	325	–	–
2. Blue			0	0	0.05	0.07	–	–	–
3. Blue			0.03	0.06	0.12	0.16	325	285	270
4. Yellow/green (nobiletin)			0.06	0.10	0.19	0.22	270, 333	260, 290	245
5. Yellow (tangeretin)			0.09	0.15	0.27	0.32	270, 325	245, 290	–
6. Red (a polymethoxyflavone)			0.13	0.18	0.32	0.34	270, 325	245, 290	–
7. Yellow/green (a flavanone)			0.18	0.26	0.35	0.38	275, 322	260, 300	–
8. Brown (a flavanone)			0.23	0.29	0.38	0.47	275, 332	265, 310	–
9. Red (a flavanone)			0.30	0.31	0.46	0.51	265, 275	260, 270	–
10. Blue (citropten)			0.37	0.41	0.54	0.56	–	–	–
11. Pale blue			0.53	–	–	–	–	–	–
12. Blue (methyl anthranilate)			0.56	0.61	0.63	0.66	–	–	–
13. Blue (N-methyl methylanthranilate)			0.64	0.65	0.70	0.70	253, 355	240, 290	–
Sweet orange oil									
1. Yellow			0	0	0	0	–	–	–
2. Blue			0	0.03	0.05	0.07	–	–	–
3. Blue			0.03	0.06	0.12	0.16	265, 325	260, 295	–
4. Yellow/green (nobiletin)			0.05	0.10	0.19	0.22	250, 270, 333	240, 260, 290	–
5. Yellow/red (tangeretin)			0.10	0.17	0.26	0.33	270, 324	250, 285	–
6. Green (5,8-dihydroxy-3,7,3′,4′-tetramethoxyflavone)			0.18	0.27	0.37	0.48	255, 270, 330	245, 260, 290	–
7. Blue (citropten)			0.38	0.44	0.53	0.59	–	–	–
8. Blue (methyl anthranylate)			0.56	0.61	0.63	0.66	–	–	–
9. Yellow			0.67	0.70	0.74	0.87	–	–	–

Table 2 *continued*

Fluorescence	Rf_{25}*	Rf_{30}*	Rf_{40}*	Rf_{50}*	Rf_{60}*	Rf_{70}*	*λ max*	*λ min*	*λ shoulder*
Bitter Orange oil									
1. Yellow		0	0	0	0	0	–	–	–
2. Blue		0	0.02	0.03	0.05	0.07	320	268	260
3. Blue/yellow		0	0.04	0.06	0.13	0.18	313	280	270
4. Yellow/green (nobiletin)		0.02	0.05	0.11	0.18	0.22	270, 329	265, 285	250
5. Yellow/red (tangeretin)		0.03	0.10	0.17	0.26	0.33	270, 324	250, 290	–
6. Blue (aurapten)		0.10	0.18	0.25	0.34	0.44	255, 322	250, 265	–
7. Violet (umbelliferone)		0.14	0.20	0.29	0.38	0.49	245, 255,325	240, 250, 265	320
8. Blue		0.16	0.24	0.33	0.43	0.50	320	265	255
9. Yellow (bergapten)		0.23	0.29	0.38	0.48	0.55	250, 260, 310	245, 255, 280	235, 250
10. Blue (citropten)		0.23	0.35	0.48	0.51	0.59	–	–	–
11. Yellow (isoimperatorin)		0.29	0.39	0.47	–	–	250, 310	245, 280	240
12. Blue		0.29	0.39	0.47	–	–	260, 270, 320	245, 265, 275	–
13. Violet		0.32	0.39	0.47	–	–	258, 320	240, 270	–
14. Blue (methyl anthranilate)		0.42	0.48	–	–	–	–	–	–
15. Blue		0.49	0.48	–	–	–	235	–	–
16. Yellow		0.54	0.59	0.62	0.70	0.73	–	–	230, 310
17. Blue		0.66	–	–	–	–	270	265	–

*The subscript number represents the % amount of ethyl acetate in the eluent mixture. (Reproduced with permission from D'Amore G and Calapaj R (1965) *Rassegna Chimica* 6: 264–269.)

psoralen; (7) 8-geranyloxypsoralen; (9) citropten; (12) oxypeucedanin; (14) byakangelicol. These main components were isolated by preparative column chromatography, crystallized and analysed by spectroscopic methods [infrared (IR), UV, nuclear magnetic resonance (NMR)].

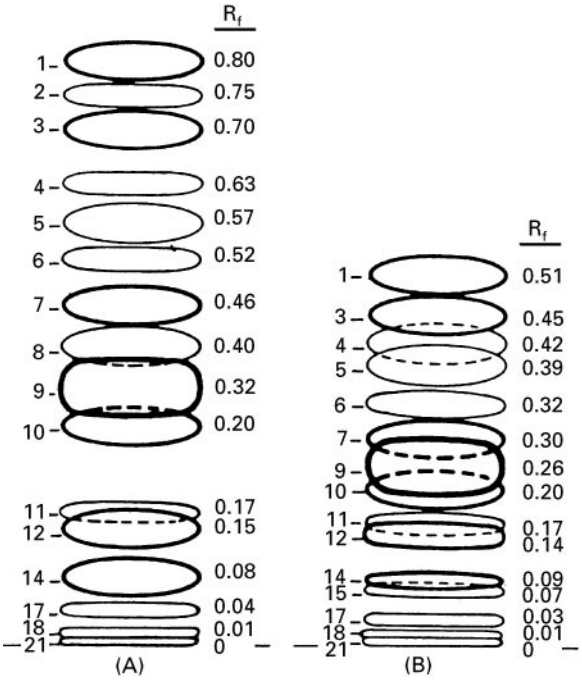

Figure 2 TLC separation of coumarins and psoralens of lemon oil. Eluent: hexane–butyl acetate, 65 : 35 (A) and 75 : 25 (B). (Reproduced with permission from Glandian R, Corneteau H, Drouet S and Rouzet M (1978) *Plantes, Medicinales et Phytoterapie*, 12 (2), 112–122.)

TLC separation of citrus oils has also been coupled with detection by *in situ* fluorimetry. This method shows the advantages of both the techniques, and a good selectivity and sensibility. As an example, the quantitative determination of 5-geranyloxy-7-methoxycoumarin and 5,7-dimethoxycoumarin (citropten) present in bergamot, lime and lemon oils, and the qualitative profile of citrus oils have been obtained by measuring the fluorescence and the fluorescence quenching profiles directly on the TLC plates. Emission monochromator wavelength settings of 403, 440 and 490 nm were used to obtain the various fluorescence emission profiles. An excitation wavelength of 272 nm and an emission wavelength of 520 nm were used to obtain the fluorescence quenching profile. These values correspond to the maximum of excitation and emission of the fluorescent indicator in the adsorbent layer.

Analytical and preparative TLC of coumarins and psoralens have been widely used. Some of the advantages of this technique are its simplicity and low cost. Disadvantages may be the difficulty of controlling the flow rate, the low resolution, the low reproducibility and the long time required for development. In recent years various modern planar chromatographic methods have been reported for the analysis of coumarins. Some of these methods use a new forced-flow TLC technique, developed by Tyihák and coworkers between 1979 and 1981, called OPLC (overpressured layer chromatography). This new planar technique combines the advantages of classical TLC and HPLC: shorter analysis times, lower solvent consumption, simultaneous analysis of a large number of

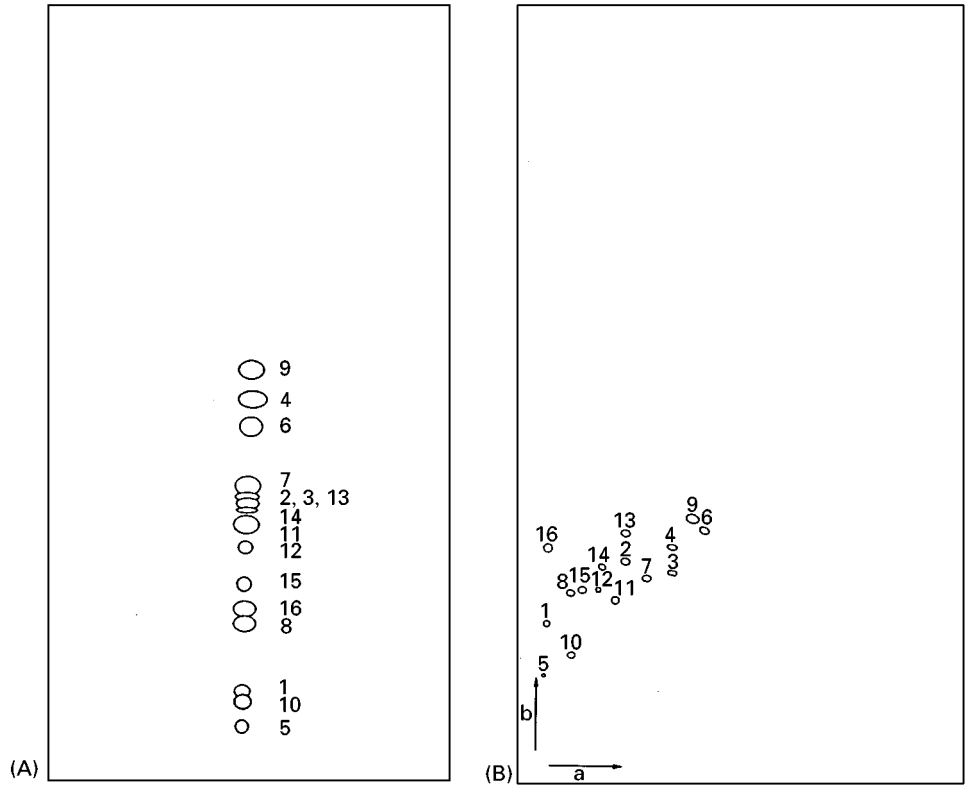

Figure 3 (A) One-dimensional OPLC development and (B) two-dimensional OPLC development of 16 closely related coumarins. (1) Umbelliferone: (2) herniarin; (3) psoralen; (4) osthol; (5) apterin; (6) angelicin; (7) bergapten; (8) oxypeucedanin; (9) isobergapten; (10) scopoletin; (11) sphondin; (12) xanthotoxin; (13) imperatorin; (14) pimpinellin; (15) isopimpinellin; (16) new archangelicin derivate. (Reproduced with permission from Harmala P, Botz L, Sticher O and Hiltunen R (1990) *Journal of Planar Chromatography* 3: 515–520.)

samples, the possibility of performing isocratic or gradient elution, control of the flow rate and higher efficiency. The literature reports some examples of the applications of OPLC technique to the analysis of coumarins and psoralens.

As shown by Härmälä *et al*. in 1990, TLC separation methods can be improved by use of two-dimensional high performance TLC (2D TLC). **Figure 3** shows the one-dimensional (A) and the two-dimensional (B) OPLC development of 16 coumarins.

Another way to increase the efficiency of TLC separation is by using the stepwise gradient technique. By increasing the strength of the mobile phase, the separation of compounds with similar R_f values is

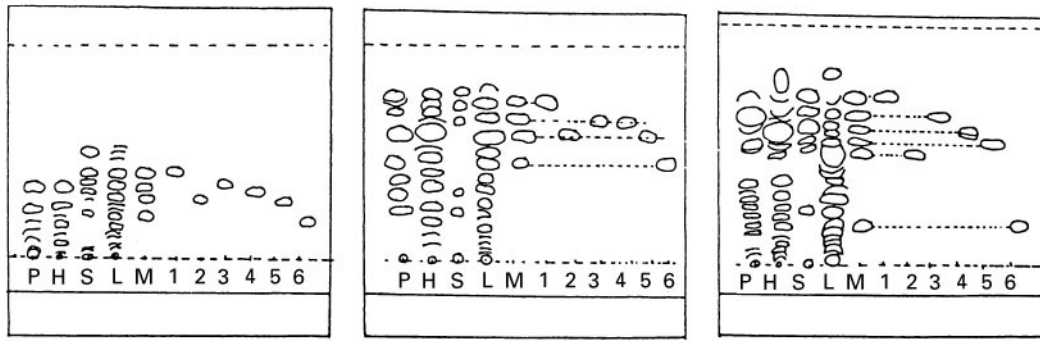

Figure 4 Chromatograms of plant extracts and references coumarins corresponding to the following stepwise gradient programs: (A) 10–50% methyl ethyl ketone in n-heptane; (B) 30–70% methyl ethyl ketone in n-heptane; (C) 2.5–15% ethyl acetate in chloroform. P = *Pastinaca sativa*; H = *Heracleum sphondylium*; S = *Sium sisarum*; L = *Libanotis intermedia*. (1) Osthol; (2) isopimpinellin; (3) imperatorin; (4) bergapten; (5) xanthotoxin; (6) umbelliferone. (Reproduced with permission from Glowniak K, Matysik G, Bieganowska M and Soczewinski E (1986) *Chromatographia* 22: 307–310.)

Table 3 R_f values obtained using chloroform–*n*-butyl acetate–hexane, 9 : 1 : 15 as eluent

Compound	*Fluorescence at 366 nm*	*Sweet orange*	*Bitter orange*	*Mandarin*	*Grape-fruit*	*Lemon*	*Bergamot*	*Mexican lime*
Bergamottin	Y				0.52	0.52	0.52	0.52
Aurapten	V				0.41			
5-Geranyloxy-7-methoxycoumarin	B					0.39	0.38	0.38
8-Geranyloxypsoralen	Y					0.34		
Osthol	V		0.38		0.38			
Bergapten + Epoxybergamottin	Y		0.30		0.30			
Bergapten	Y						0.30	0.30
Citropten	B					0.26	0.25	0.25
Meranzin + Isomeranzin	V		0.25		0.25			
Herniarin	B							0.23
Epoxyaurapten	V				0.18			
?	B					0.18		
?	P							0.17
Oxypeucedanin	Y					0.10		0.11
Epoxybergamottin hydrate	Y		0.10		0.10			
Meranzin hydrate	V		0.08		0.08			
Byakangelicol	Y					0.04		0.04
Polymethoxylated flavones	Y	0.04	0.04	0.04	0.04			
Polymethoxylated flavones	B	0.03	0.03	0.03	0.03			

B: Blue; Y: Yellow; V: Violet; P: Pink. (Reproduced with permission from Dugo P, Mondello L, Lamonica G and Dugo G (1996) *Journal of Planar Chromatography* 9: 120–125.)

Table 4 R_f values obtained using *n*-butyl acetate–hexane, 80 : 20 as eluent

Compound	*Fluorescence at 366 nm*	*Sweet orange*	*Bitter orange*	*Mandarin*	*Grape-fruit*	*Lemon*	*Bergamot*	*Mexican lime*
Bergamottin	Y				0.96			
Bergamottin + 5-Geranyloxy-7-methoxycoumarin	Y + B					0.97	0.97	0.97
8-Geranyloxypsoralen	Y					0.92		0.92
Aurapten	V				0.90			
?	B				0.82			
?	B							0.82
Osthol	V		0.74		0.74			
Citropten	B					0.71	0.71	0.71
?	B				0.71			
Epoxybergamottin	Y		0.70		0.68			
Bergapten	Y		0.65				0.65	0.66
Bergapten + epoxyayrapten	Y + V				0.65			
Herniarin	V							0.62
Oxypeucedanin	Y					0.61		0.60
?	Y				0.60			
?	B				0.56			
Byakangelicol	Y					0.49		0.49
Isomeranzin	V		0.49		0.49			
Meranzin	V		0.45		0.45			
?	B		0.41		0.40			
Tangeretin	P	0.25	0.25	0.25	0.25			
Heptamethoxyflavone	Y	0.21	0.21	0.21	0.21			
Tetra-O-methylscutellarein	P	0.20		0.20				
Hexamethoxyflavone	SB	0.16						
Nobiletin	Y	0.14	0.14	0.14	0.14			
Sinensetin	SB	0.10		0.10				
Epoxybergamottin hydrate	Y		0.10		0.10			
Meranzin hydrate	V		0.04		0.04			

B: Blue; SB: Sky-blue; Y: Yellow; V: Violet; P: Pink. (Reproduced with permission from Dugo P, Mondello L, Lamonica G and Dugo G (1996) *Journal of Planar Chromatography* 9: 120–125.)

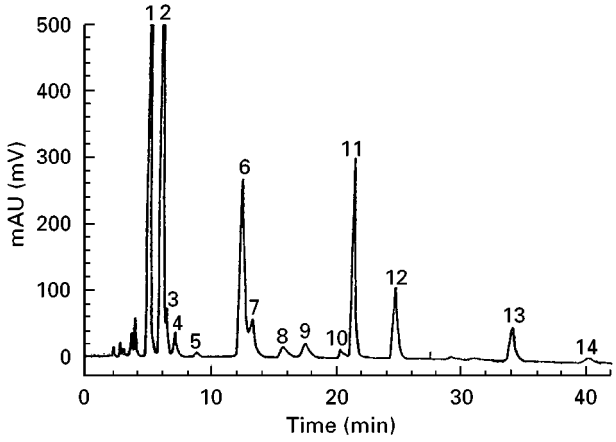

Figure 5 HPLC chromatogram of oxygen heterocyclic components of genuine cold-pressed lemon oil. Column 25 cm × 4.6 mm internal diameter Zorbax 6 μm spherical silica; gradient elution, solvent A hexane–ethyl acetate (9 : 1), solvent B hexane–ethyl alcohol (9 : 1), 2–95% B over 25 min; flow rate, 1.5 mL min^{-1}; sample volume 20 μL (20% solution of lemon oil in dichloromethane); detection UV absorbance at 315 nm. For peak assignment, see Table 5. (Reproduced with permission from McHale D and Sheridan JB (1988) *Flavour Fragrance Journal* 3: 127–133.)

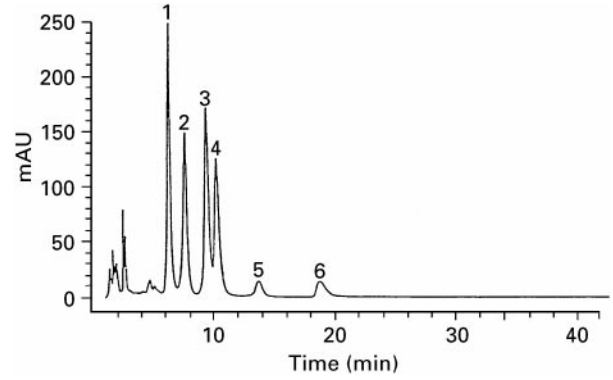

Figure 6 HPLC chromatogram of oxygen heterocyclic components of cold-pressed sweet orange oil. (1) Tangeretin; (2) heptamethoxyflavone; (3) nobiletin; (4) tetra-*O*-methylscutellarein; (5) 3,3′,4′,5,6,7-hexamethoxyflavone; (6) sinensetin. (Reproduced with permission from McHale D and Sheridan JB (1989) *Journal of Essential Oil Research* 1: 139–149.)

improved. **Figure 4** shows an example of gradient TLC of coumarins and psoralens of some plant extracts.

Tables 3 and **4** provide results obtained for the OPLC analysis applied to seven citrus oils, using silica gel 60 F254 HPTLC plates with impregnated edges (flow rate: 0.7 mL min^{-1}). Detection was by UV at 366 nm; time of analysis: 10 min. Because of its advantages, this method has been proposed as a rapid, preliminary check to evaluate the authenticity of citrus oils.

HPLC Separations

As well as TLC methods the literature reports numerous qualitative and quantitative methods for the analysis of coumarins and related compounds by both normal- and reversed-phase HPLC. Detection is commonly performed by UV absorbance, but methods which use the highly selective and sensible fluorescence detector are also reported.

Two important papers are those of McHale and Sheridan of 1988 and 1989 in which the authors developed normal-phase HPLC methods for the analysis of the most common citrus oils, making huge progress in the identification and in the quantitative determination of oxygen heterocyclic compounds. The first paper refers on the composition of

Table 5 Composition of oxygen heterocyclic fraction of cold-pressed Sicilian lemon oil reported by McHale and Sheridan (1988)

Peak no.	*Component*	*Concentration mg L^{-1} (ppm)*
1	Bergamottin	2200
2	5-Geranyloxy-7-methoxycoumarin	1600
3	Isoimperatorin	180
4	5-Isopentenyloxy-7-methoxycoumarin	80
5	Unidentified (UV 7-substituted coumarin)	10
6	Citropten	650
7	8-Geranyloxypsoralen	750
8	Phellopterin + Imperatorin	90 + 60
9	5-Isopentenyloxy-8-epoxyisopentyloxypsoralen	220
10	Unidentified (UV ill-defined)	–
11	Oxypeucedanin	1100
12	Byakangelicol	450
13	Oxypeucedanin hydrate	260
14	Byakangelicin	70

(Reproduced with permission from McHale D and Sheridan JB (1988) *Flavour Fragance Journal* 3: 127–133.)

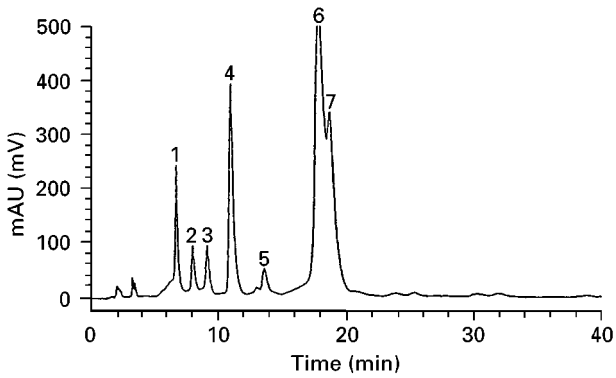

Figure 7 HPLC chromatogram of oxygen heterocyclic components of cold-pressed bitter orange oil. (1) Osthol; (2) epoxybergamottin; (3) bergapten; (4) tangeretin; (5) heptamethoxyflavone; (6) meranzin; (7) isomeranzin + nobiletin. (Reproduced with permission from McHale D and Sheridan JB (1989) *Journal of Essential Oil Research* 1: 139–149.)

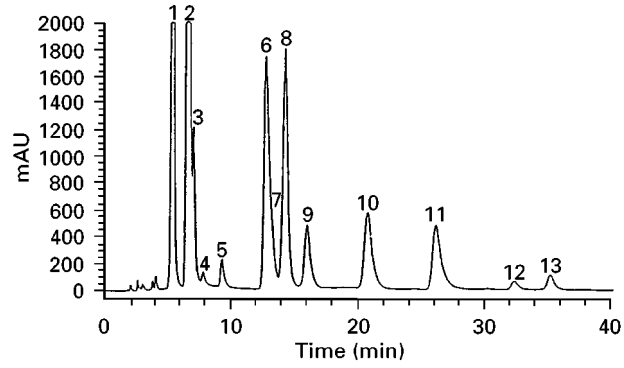

Figure 9 HPLC chromatogram of oxygen heterocyclic components of cold-pressed lime oil. (1) Bergamottin; (2) 5-geranyloxy-7-methoxycoumarin; (3) 5-geranyloxy-8-methoxypsoralen; (4) 5-isopentenyloxy-7-methoxycoumarin; (5) 5-isopentenyloxy-8-methoxypsoralen; (6) citropten; (7) 8-geranyloxypsoralen; (8) herniarin; (9) bergapten; (10) isopimpinellin; (11) oxypeucedanin; (12) isobyakangelicol; (13) byakangelicol + heraclenin. (Reproduced with permission from McHale D and Sheridan JB (1989) *Journal of Essential Oil Research* 1: 139–149.)

cold-pressed lemon oil. **Figure 5** shows the HPLC chromatogram in which 14 components were detected and quantitatively determined. The experimental conditions are shown in the figure legend. **Table 5** reports the quantitative results.

These workers identified and quantified not only the main components, but also those present in lower amounts. Moreover, they analysed commercial samples of lemon oils, and demonstrated the validity of the method to detect some adulterations practised to increase the UV absorbance of oils previously diluted with distilled ones. The method allows for the detection of *p*-dimethylaminobenzoate, grapefruit oil and/or lime oil, that are the most common substances used to increase UV absorbance. The paper of 1989 shows normal-phase HPLC chromatograms of bergamot, sweet orange, bitter orange, grapefruit and lime oils, as illustrated in **Figures 6–9**.

Table 6 reports the experimental conditions used for these analyses. The paper reported quantitative data for all the oils analysed, according to their geographical origin.

Figure 10 shows a reversed-phase HPLC chromatogram obtained in 1992 by Ziegler and Spiteller for a lemon oil under the following experimental conditions: column, Spherisorb ODS2 (C_{18} – particle size 5 μm), 250 × 4.6 mm internal diameter; flow, 1 mL min^{-1}; solvents: A, methanol–water–acetonitrile (1 : 1.35 : 0.5) B, acetonitrile. Program: 10% B to

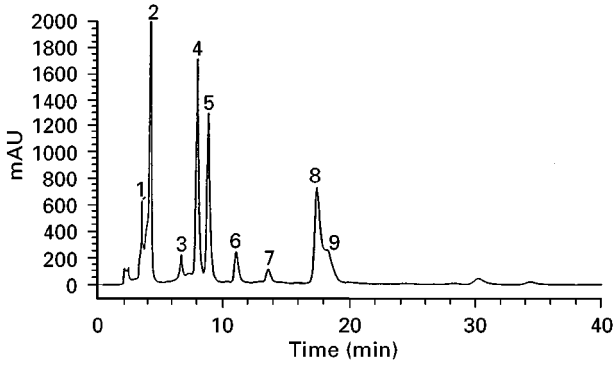

Figure 8 HPLC chromatogram of oxygen heterocyclic components of cold-pressed grapefruit oil. (1) Bergamottin; (2) aurapten; (3) osthol; (4) epoxybergamottin; (5) epoxyaurapten; (6) tangeretin; (7) heptamethoxyflavone; (8) meranzin; (9) isomeranzin + nobiletin. (Reproduced with permission from McHale D and Sheridan JB (1989) *Journal of Essential Oil Research* 1: 139–149.)

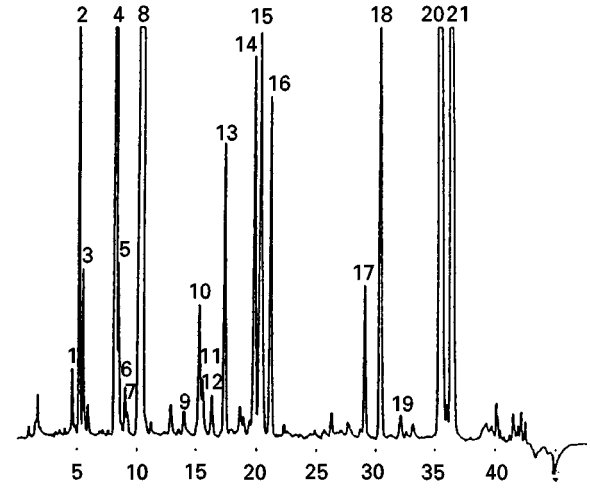

Figure 10 Reversed phase HPLC chromatogram of oxygen heterocyclic components of cold-pressed lemon oil. For peak assignment, see Table 7. (Reproduced with permission from Ziegler H and Spiteller G (1992) *Flavour Fragrance Journal* 7: 129–139.)

Table 6 HPLC conditions reported by McHale and Sheridan (1989) for the analysis of oxygen heterocyclic compounds of citrus oils

	Lemon	*Lime*	*Mandarin*	*Grape fruit*	*Bitter orange*	*Sweet orange*
Eluent	(A) hexane–ethyl acetate, 9 : 1 (B) hexane–ethyl alcohol, 9 : 1			Hexane–ethyl alcohol, 19 : 1		Hexane–ethyl alcohol, 9 : 1
Programme	From 98 A : 2 B to 5 A : 95 B over 25 min			Isocratic		Isocratic
Column	6 μm Zorbax SIL spherical, 25 cm × 4.6 mm internal diameter					
Injection volume	20 μL of a 20% solution of oil in dichloromethane					
Detection	UV absorbance at 315 nm					
Flow rate	1.5 mL min^{-1}					

40% B in 20 min; 40% B to 70% B in 15 min; 70% B to 90% B in 2 min. Injection: 100 μL of a 0.2% solution of original lemon oil in 10% B and 90% A. Detection: UV at 220 and 310 nm.

Table 7 lists the 25 components identified by HPLC, MS, GC–MS and NMR, 11 of which identified for the first time in a cold-pressed lemon oil as trace constituents. It is noteworthy that on the basis of the spectroscopic data obtained, bergapten does not result to be present in genuine lemon oil, in contrast with data previously reported in literature.

Table 7 Components identified in the oxygen heterocyclic fraction of cold-pressed Sicilian lemon oil by Ziegler and Spiteller

Peak no. in HPLC run	*Components*
1	5-(2′,3′-Dihydroxyisopentyloxy)-7-methoxycoumarin*
	Heraclenol*
2	Oxypeucedanin hydrate
3	Byakangelicin
4	Citropten
5	Heraclenin
6	Pabulenol/Gosferenol*
7	Neobyakangelicol*
8	Oxypeucedanin
	Byakangelicol
	5-(2′,3′-Epoxyisopentyloxy)-7-methoxycoumarin*
9	5-Isopentenyloxy-8-(2′,3′dihydroxyisopentyloxy)psoralen*
10	Imperatorin
11	7-Isopentenyloxycoumarin*
12	8-(6′,7′-Epoxygeranyloxy)psoralen*
13	Phellopterin
14	Isoimperatorin
15	5-Isopentenyloxy-7-methoxycoumarin
16	5-Isopentenyloxy-8-(2′,3′-epoxyisopentyloxy)psoralen
17	Cnidicin*
18	8-Geranyloxypsoralen
19	Aurapten*
	5-Methoxy-8-geranyloxypsoralen*
20	Bergamottin
21	5-Geranyloxy-7-methoxycoumarin

*Compounds previously unknown in cold-pressed lemon oil.

Citrus oils that show a quite complex composition of the oxygen heterocyclic fraction are difficult to analyse either by normal- or reversed-phase HPLC with a single column. In these cases the 'column switching' technique can be useful to improve the separation of those critical peaks. This technique has been applied successfully to the analysis of bitter orange and grapefruit oils, which show a very similar composition, to separate 17 components and, in particular, to the couple meranzin–isomeranzin. **Figures 11** and **12** show the results obtained, together with the experimental conditions and peak identification.

Most of the data found in the literature refer to the characterization of bergamot oil, and in particular to the determination of 5-methoxypsoralen (bergapten), which is known to have a higher phototoxic action than other psoralens found in citrus oils. Bergamot oil is widely used in the cosmetic and pharmaceutical

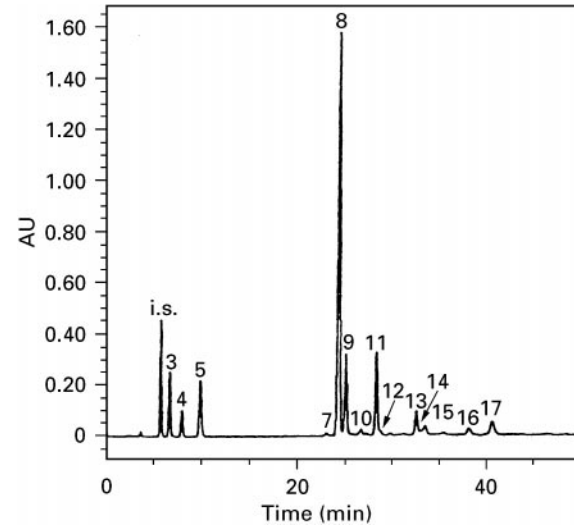

Figure 11 HPLC chromatogram of oxygen heterocyclic components of cold-pressed bitter orange oil. For peak assignment and experimental conditions see Figure 12. (Reproduced with permission from Dugo P, Mondello L, Stagno d'Alcontres I, Cavazza A and Dugo G (1997) *Perfumer and Flavorist* 22: 25–30.)

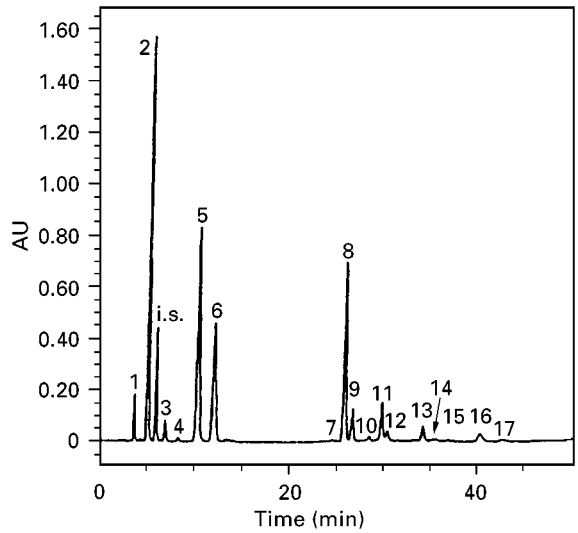

Figure 12 HPLC chromatogram of oxygen heterocyclic components of cold-pressed grapefruit oil obtained in the following conditions: μ-porasil column 30 cm × 3.9 mm internal diameter (10 μm) for the first 12 min, then the flow was switched to a second column, Zorbax silica 25 cm × 4.6 mm internal diameter (7 μm). Eluent A, hexane–ethyl acetate, 9 : 1; eluent B, hexane–ethyl alcohol, 9 : 1. 2–95% B over 23 min (2–25 min) with a concave gradient, then 20 min isocratic 95% B, flow rate 1.6 mL min^{-1}; sample volume 20 μL (5% solution of oil in hexane–ethyl acetate, 75 : 25); detection by UV absorbance at 315 nm. Peak assignment: (1) bergamottin; (2) aurapten; (3) osthol; (4) bergapten; (5) epoxybergamottin; (6) epoxyaurapten; (7) unknown coumarin; (8) meranzin; (9) isomeranzin; (10) unknown coumarin 2; (11) tangeretin; (12) 3,3′,4′,5,6,7,8-heptamethoxyflavone; (13) nobiletin; (14) tetra-*O*-methylscutellarein; (15) unknown coumarin; (16) epoxybergamottin hydrate; (17) meranzin hydrate; (i.s.) internal standard, coumarin. (Reproduced with permission from Dugo P, Mondello L, Stagno d'Alcontres I, Cavazza A and Dugo G (1997) *Perfumer and Flavorist* 22: 25–30.)

industries. Usually, genuine cold-pressed bergamot oil contains about 2000–3000 ppm of bergapten, but many industrial processes have been developed with the aim to reduce its concentration to values of only a few parts per million. The oils so obtained are known as 'bergapten-free' oils. **Table 8** summarizes some of the TLC and HPLC methods proposed for the quantitative determination of bergapten in bergamot oil.

HPLC–MS

To obtain more information on the nature and the structure of the components analysed by HPLC, an MS detector can be coupled on-line to the HPLC system. An innovative interface for the HPLC–MS coupling is the API (atmospheric pressure ionization), that differs from the traditional interfaces because the ionization takes place at atmospheric pressure.

The API technique can use two different interfaces, the electrospray (ES) or the atmospheric pressure chemical ionization (APCI), and can give different information than those obtained with conventional LC–MS interfaces. Both the techniques are classified as 'soft' ionization methods. By varying the voltage of the sample cone it is possible to obtain different degrees of fragmentation.

The HPLC–MS technique with the APCI interface has been applied to the analysis of coumarins of citrus oils to confirm the identification of some components or to obtain more information for those not identified yet. As an example, **Figure 13** shows the HPLC–UV chromatogram of a cold-pressed bergamot oil, compared to the full scan HPLC–MS chromatogram acquired at different cone voltage values.

Figure 14 shows the MS spectra obtained at different cone voltage values for one of the components of bergamot oil (bergamottin). As can be seen, at the lower cone voltage value the $(M + H)^+$ ion is visible, while at higher values additional fragmentation occurs.

The HPLC–MS technique allowed the confirmation of the presence of oxygen heterocyclic compounds previously not identified in bergamot oil, such as tetra-*O*-methylscutellarein and sinensetin. **Figure 15** shows the HPLC–UV chromatogram at cone voltage value of 20 V, and the extracted chromatogram at m/z 243 and 273, corresponding to the $(M + H)^+$ ions of tetra-*O*-methylscutellarein and sinensetin.

Supercritical Fluid Chromatography (SFC)

A few applications of supercritical fluid chromatography (SFC) to the analysis of citrus coumarins have been reported. Even though this technique is less popular in the analysis of citrus coumarins, it is interesting to compare the results obtained by SFC to those obtained by HPLC. **Figure 16** shows a normal phase HPLC chromatogram of the polymethoxylated flavones of sweet orange oil (A), compared to the packed SFC chromatogram obtained for the same oil (B).

Table 9 provides the chromatographic conditions under which the two analyses have been carried out. Both the analyses allowed a complete separation of the six polymethoxylated flavones (PMFs) known to be present in sweet orange oil. The SFC analysis was completed in less than 6 min, while the HPLC analysis took more than 25 min. This represents a reduction in the analysis time by a factor of four. Quantitative results obtained with the SFC method compared well with those obtained with the HPLC method.

Table 8 TLC and HPLC methods for the analysis of bergapten in bergamot oil.

Technique	*Stationary phase*	*Mobile phase*	*Detection method*	*Flow rate*	*Sample*
HPLC	2 cm × 2.3 mm internal diameter stainless steel tubing packed with 37-50 Corasil II	Hexane–chloroform, 75 : 25	UV abs. at 254 nm	0.25 mL min^{-1} P = 100 psi	10 μL of a solution of 0.05 g of oil in 10 mL of $CHCl_3$
TLC	Silica gel plates F-254	Isoctane–ethyl acetate, 83 : 17	UV abs. at 254 nm		Oil diluted 1 : 10 in $CHCl_3$
TLC	Silica gel plates F-254 (20 μm)	Cyclohexane–ethyl acetate–acetic acid, 80 : 20 : 2	UV abs. at 254 and 366 nm		
TLC	10 × 10 cm Silica gel HPTLC plates 60 F-254 (5 μm)	Petroleum ether or benzene followed by cyclohexane–ethyl acetate,75 : 25	Densitometer at 254 or 308 nm or Fluorescence (λ_{exc} 330 nm, λ_{em} 450 nm)		
TLC	10 × 10 cm RP 18 TLC plates (7 μm)	Methanol–water, 80 : 20	Densitometer at 254 or 308 nm		Oil diluted in methanol
HPLC	Lichrosorb Si 60 (5 μm) 23 cm × 4.35 mm internal diameter	Heptane–isopropanol, 93 : 7	UV abs at 254 nm	0.85 mL min^{-1}, P = 23 bar	
HPLC	Lichrosorb RP 18 (5 μm) 17 cm × 4.35 mm internal diameter	Methanol–water, 9 : 1	UV abs at 254 nm	1.1 mL min^{-1}, P = 24 bar	
HPLC	Lichrosorb Si 60 (5 μm) 25 cm × 4.6 mm internal diameter	Hexane–ethyl acetate–propan-2-ol, 88 : 10 : 2 (isocratic)	UV absorbance at 305 nm	1 mL min^{-1}	Undiluted for oil with less than 40 mg L^{-1} of bergapten, or diluted in the range 20–40 mg L^{-1} with $CHCl_3$ · 20 μL inj

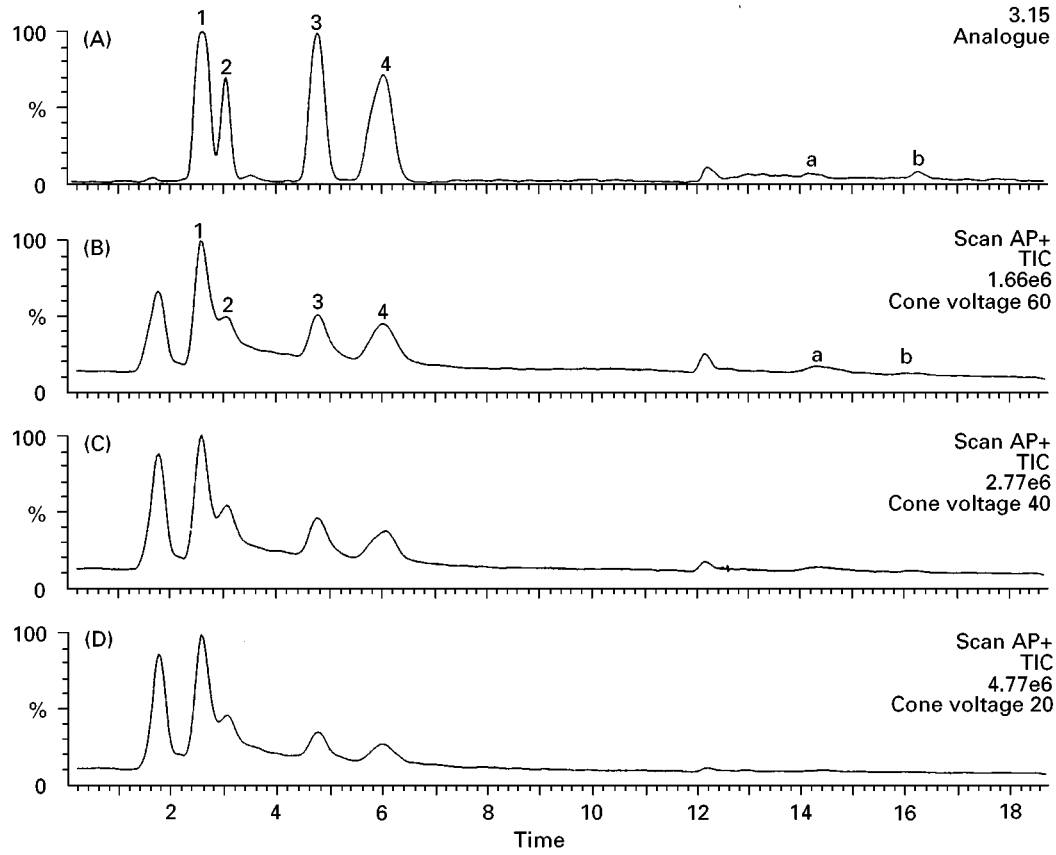

Figure 13 (A) HPLC–UV chromatogram and (B), (C) and (D) total ion current (TIC) HPLC chromatograms acquired at cone voltage values of 60, 40 and 20 V, respectively, of coumarin fraction of a genuine bergamot essential oil. (1) Bergamottin; (2) 5-geranyloxy-7-methoxycoumarin; (3) citropten; (4) bergapten; (a) tetra-*O*-methylscutellarein; (b) sinensetin; (i.s.) internal standard.

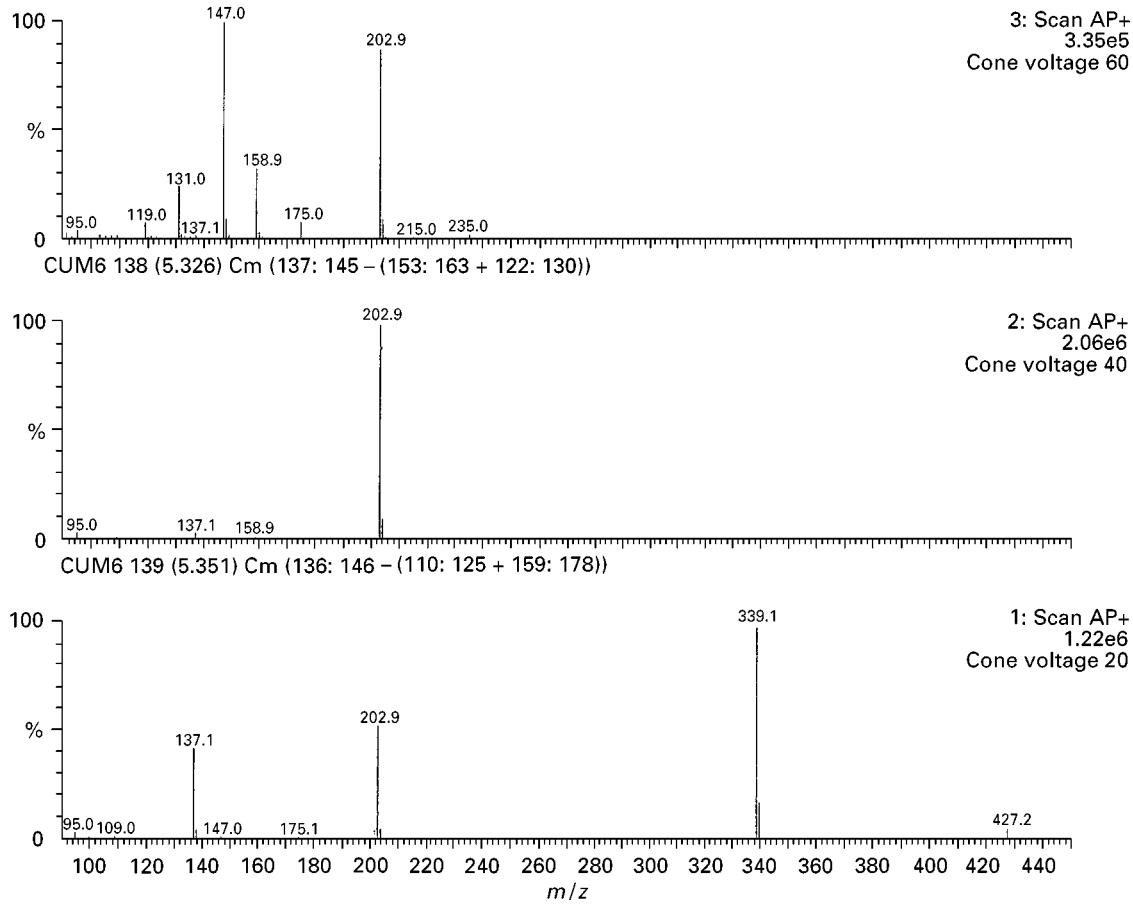

Figure 14 Cone voltage fragmentation of bergamottin using APCI ionization.

Conclusion

Citrus essential oils show a characteristic composition of their oxygen heterocyclic fraction. These components make it possible to differentiate the individual oils and to detect mixtures or mutual contamination. Quantitative analysis is often necessary to assess authenticity or geographical origin of an oil. Sometimes, quantitative analysis has to be preceded by isolation of single components for structure elucidation and also to obtain pure standard compounds to be used for preparation of standard solutions, since they are not always commercially available. Moreover, these components possess numerous pharmacological activities, so isolation may be necessary for testing specific biological activities.

Both analytical and preparative analyses can be carried out with planar and column liquid chromatographic methods (TLC, OPLC, HPLC). Usually, the preparative separations, that may be long and laborious, are followed by further purification steps and by spectroscopic analyses for identification.

Working with natural products, efficient detection and rapid characterization are often essential. Furthermore, the achievement of structural information on unknown constituents of a complex mixture is a strategic element for guiding an efficient and selective isolation procedure.

A big advantage can be achieved by using hyphenated techniques. In the last few years, LC-MS is becoming more and more popular, because if the introduction of API (atmospheric pressure ionization) techniques as a means for mass spectrometric sample introduction. This interface permits a highly selective and sensitive detection method, to be obtained and use of HPLC-MS in routine analysis. Recently, LC-NMR has been introduced as another powerful complementary technique for on-line structural identification, even though it is much less sensitive than LC-MS. Application of planar chromatography coupled with mass spectrometric (MS) or Fourier transformed infrared (FT-IR) have also been developed.

Looking to the future, it is reasonable to expect a much wider use of hyphenated techniques that will allow the rapid structural determination of constituents of complex matrices requiring only a small amount of samples, and the use of shorter HPLC columns packed with smaller particles that will

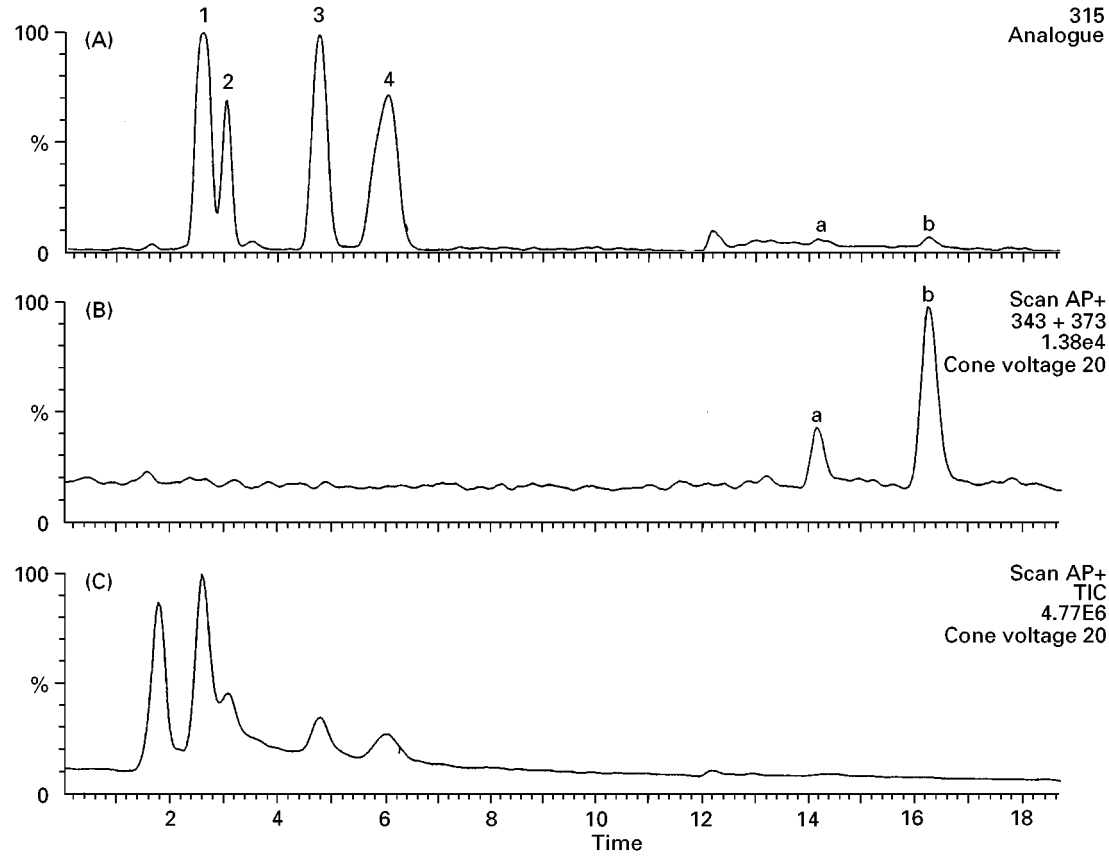

Figure 15 (A) HPLC–UV chromatogram, (C) TIC HPLC chromatogram acquired at cone voltage of 20 V and (B) TIC HPLC chromatogram extracted at m/z 343 + 373, of coumarin fraction of a genuine bergamot oil.

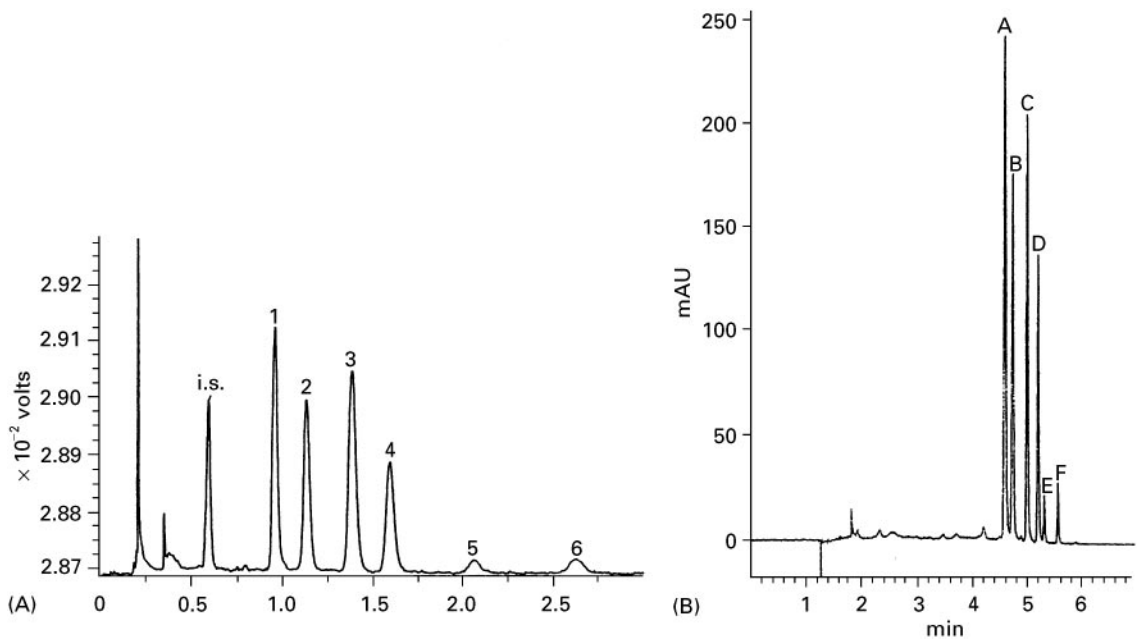

Figure 16 (A) HPLC chromatogram and (B) packed SFC chromatogram of polymethoxylated flavones of sweet orange oil. 1 = (A) tangeretin; 2 = (B) heptamethoxyflavone; 3 = (C) nobiletin; 4 = (D) tetra-*O*-methylscutellarein; 5 = (E) 3,3′,4′,5,6,7-hexamethoxyflavone; 6 = (F) sinensetin. ((A) Reproduced with permission from Dugo P, Mondello L, Cogliandro E, Stagno d'Alcontres I and Controneo A (1994) *Flavour and Fragrance Journal* 9: 105–111 and (B) reproduced with permission from Dugo P, Mondello L, Dugo G, *et al.* (1996) *Journal of Agricultural and Food Chemistry* 44: 3900–3905.)

Table 9 Experimental conditions of the HPLC and of the SFC analyses of polymethoxylated flavones of sweet orange oil

	HPLC	*SFC*
Column	Zorbax silica, 25 cm × 4.6 mm internal diameter (7 μm)	S5W uncoated silica, 25 cm × 4.6 mm internal diameter (5 μm)
Eluent	Hexane–ethyl acetate, 95 : 5	CO_2 modified with small amounts of methanol
Flow rate	1.6 mL min^{-1}	2 mL min^{-1} for 4 min, then gradient of 2 up to 5 mL min^{-1} thereafter held constant
Programme	Isocratic	$P = 100$ atm, $T = 40°C$; modifier, 1.5% min^{-1} from 10% to 30% thereafter held constant
Injected amount	20 μL of a 5% solution of oil in ethyl acetate	100 μL of a solution obtained by diluting 0.71 g of oil to 20 mL of ethyl acetate
Detection	UV absorbance at 315 nm	UV absorbance at 315 nm

provide faster separations with the same resolution as that observed in longer columns packed with particles of larger diameter.

See also: **II/Chromatography: Liquid:** Detectors: Mass Spectrometry. **Chromatography: Thin-Layer (Planar):** Densitometry and Image Analysis; Modes of Development: Forced Flow, Over Pressured Layer Chromatography and Centrifugal; Preparative Thin-Layer (Planar) Chromatography. **III/Essential Oils:** Gas Chromatography; Thin-Layer (Planar) Chromatography; Distillation.

Further Reading

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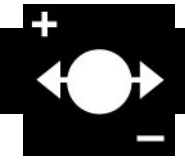
CLINICAL APPLICATIONS



Capillary Electrophoresis

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Introduction

The area of clinical applications of capillary electrophoresis (CZE) is such a rapidly growing field that it would be impossible here to cover it in detail. We thus offer a list of major reviews to which the reader is referred for a more comprehensive coverage of the literature. Such reviews can be divided into:

1. Broad-coverage reviews, such as those of Lehmann *et al.* (1997), Perrett (1999; CZE in clinical chemistry) and Guzman *et al.* (1997; dedicated also to on-line analyte concentration and microreaction). Also of interest are special issues of the *Journal of Chromatography B* dedicated to CZE in the life sciences (Krstulovic 1997) and of *Electrophoresis* devoted to CZE in the clinical sciences (Landers 1997) and in forensic science (McCord, 1998).
2. Specialized reviews, such as those of Thormann *et al.* (1996, 1997; drug analysis in body fluids); Lurie (1996; analysis of seized drugs), Hong and Baldwin (1997; metabolite profiling in human urine), Righetti and Gelfi (1997a,b, 1998; CZE of