Oxygen Heterocyclic Compounds of Citrus Essential Oils

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The nonvolatile residue of citrus essential oils greatly influences the olfactory properties of these oils. The residue ranges from approximately 1-10% in the different oils and consists, in large part, of many oxygen heterocyclic compounds, particularly the coumarins, psoralens and polymethoxylated flavones.

The qualitative and quantitative compositions of this residue characterize the different citrus oils, and play an important role in identifying the various oils and controlling their quality and genuineness.

Sometimes it is also possible to find a correlation between the quantitative variations and parameters such as the production period, the kind of fruit or the extraction technology.[1-10] But data in the literature is very limited; usually the quantitative data is poor and often refers only to a limited number of samples.

In this paper we report the results on the isolation, identification and quantitative analysis of oxygen heterocyclic compounds of bergamot, sweet orange, mandarin, bitter orange, grapefruit and lemon oils.

Experiment Procedures

This research was carried out on samples of genuine industrial cold-pressed Italian bergamot, sweet orange, mandarin, bitter orange, grapefruit and lemon oils.

All samples were analyzed by normal phase HPLC, using Waters Associates equipment and photodiode array detector model 996. Peak integration and quantitative calculations were performed with the Millenium 2010 system (Waters Associates) and a calibration curve that was obtained for each previously isolated standard component against a coumarin standard. The injection volume was 20 µl of a solution obtained by diluting about 90 mg of lemon oil or 50 mg of the other oils and 0.1 ml of a coumarin solution of known concentration with 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.

Bergamot oils were analyzed using a 30 cm x 3.9 mm i.d. µ-Porasil[a] column with particle size of 10 µm. Mobile phase: eluent A—hexane:ethyl acetate, 9:1; eluent B—hexane:ethyl alcohol, 9:1. Eluent program: 0-7 min, A 100%; 7-20 min, from A 100% to A 5% + B 95% with a linear gradient; 20-25 min, A 5% + B 95%; flow rate 1 ml/min.

Sweet orange and mandarin oils were analyzed using a Zorbax[b] silica column 25 cm x 4.6 mm i.d., particle size 7 µm. Mobile phase, hexane:ethyl alcohol, 95:5; flow rate 1.6 ml/min.

Bitter orange and grapefruit oils were analyzed using a µ-Porasil column 30 cm x 3.9 mm i.d., particle size 10 µm for the first 12 minutes of the analysis, then the flow was switched to a second column, 25 cm x 4.6 mm i.d., Zorbax silica. Mobile phase: eluent A—hexane:ethyl acetate, 9:1; eluent B—hexane:ethyl alcohol, 9:1. Eluent program: 0-2 min, A 98% + B 2%; 2-25 min, from A 98% + B 2% to A 5% + B 95% with a concave gradient; 25-45 min, A 5% + B 95%; flow rate 1.6 ml/min.

The analyses of lemon oil samples were carried out using a µ-Porasil column 15 cm x 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.

Oxygen heterocyclic compounds were isolated from the various oils by column chromatography, TLC and semipreparative HPLC in recycle mode.[4,11,12] Purity was monitored by HPLC, under the same experimental conditions mentioned for the analysis of the corresponding oils, using the spectral contrast technique of the photodiode array detector.[13] The identity of the compounds isolated was confirmed by ^{1}H-NMR and mass spectrometry.

Bergamot Oil

Figure 1 shows the HPLC chromatogram of the coumarin fraction of a genuine bergamot oil. Table I reports

[a] µ-Porasil is a trade name of Waters Associates, Milford, MA
[b] Zorbax is a trade name of Phenomenex, Torrance, CA

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Table I. Average content of coumarins in 128 bergamot essential oils

Peak (Figure 1)	Coumarin	Average content (mg/100 g of oil)
1	bergamottin	1,870
2	5-geranyloxy-7-methoxycoumarin	130
3	citropten	220
4	bergapten	210

the average content (X) of the four components identified relative to all the samples of bergamot oil analyzed. The four components are bergamottin, 5-geranyloxy-7-methoxycoumarin, citropten and bergapten.

These data refer to 128 genuine Calabrian bergamot essential oils produced during the 1991-1992 season. All the samples were from the Fantastico cultivar, except for a few samples from the Castagnaro cultivar. We compared our results with previously published results relative to at least ten positively genuine oils.[14-17] The values we obtained for bergamottin, 5-geranyloxy-7-methoxycoumarin and citropten agree with those of the literature. Our results on bergapten agree with those of Calabrò et al.[15] obtained by TLC, but we show an average content lower than those reported in other papers[16,17] and obtained by HPLC. The changes in bergamot cultivation in recent years probably brought about this lowering of the bergapten content in Calabrian bergamot oils.

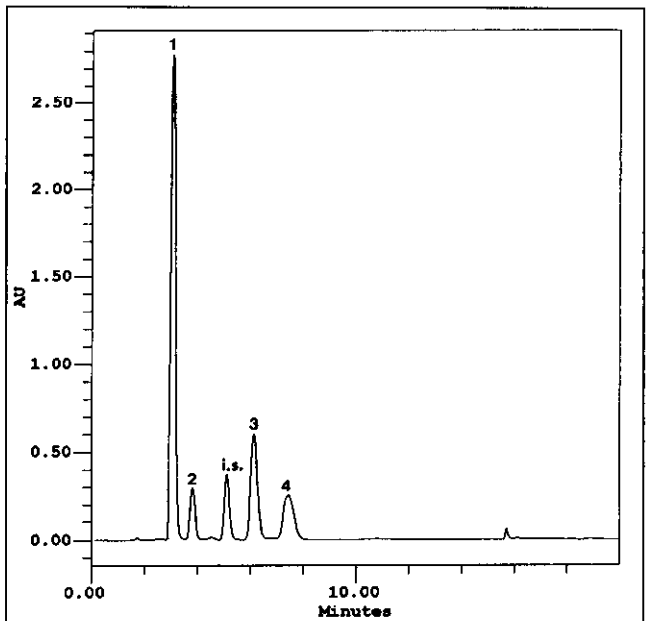

Figure 1. HPLC chromatogram of coumarins of bergamot oil (coumarin internal standard). For identification of components, see Table I.

We also tried to correlate the coumarin content with the production period. The results showed that 5-geranyloxy-7-methoxycoumarin remained constant for the whole period considered, bergamottin increased slightly, while citropten and bergapten showed a significant decrease (to about two-thirds of the original content) from January to March.

Altitude or latitude of the fruit production areas seems not to influence the coumarin content of the bergamot essential oils.

Sweet Orange and Mandarin Oils

Figure 2 shows the HPLC chromatograms of the polymethoxylated flavones in a sweet orange and a mandarin oil. Table II reports the average content (X) from the quantitative data relative to the samples of sweet orange and mandarin. Both oils contain five polymethoxylated flavones: tangeretin, 3,3',4',5,6,7,8-heptamethoxyflavone, nobiletin, tetra-O-methylscutellarein and sinensetin. Sweet orange oil also contains 3,3',4',5,6,7-hexamethoxyflavone.

The presence of the hexamethoxyflavone and the value of the ratio tangeretin/heptamethoxyflavone may be used to detect possible additions of the less expensive sweet orange oil to the more expensive mandarin oil.

This research was carried out on 190 genuine sweet orange essential oils and 66 mandarin essential oils produced during the 1991-1992 season.

The quantitative data relative to the 190 sweet orange oil samples were grouped according to the oil extraction technology. The two technologies are "Pelatrice," which is preferred for processing blond fruits, and "FMC," the

Table II. Average content of polymethoxylated flavones in 190 sweet orange and 66 mandarin essential oils

Peak (Figure 2)	Polymethoxylated flavones	Average content (mg/100 g of oil) Sweet orange	Mandarin
1	tangeretin	48	214
2	3,3',4',5,6,7,8-heptamethoxy-flavone	84	37
3	nobiletin	52	74
4	tetra-O-methylscutellarein	31	5
5	3,3',4',5,6,7-hexamethoxy-flavone	13	-
6	sinensetin	9	2

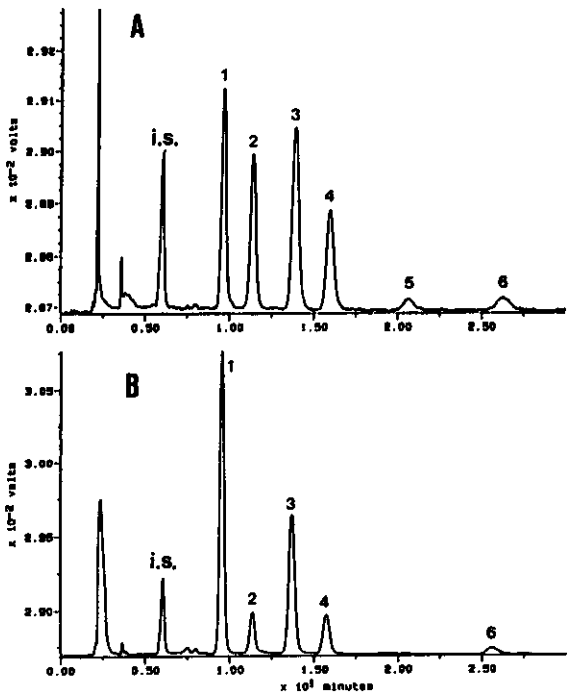

Figure 2. HPLC chromatogram of polymethoxylated flavones of sweet orange (A) and mandarin (B) oils (coumarin internal standard). For identification of components, see Table II.

preferred technology for processing blood fruits. Levels of 3,3',4',5,6,7,8-heptamethoxyflavone, nobiletin, 3,3',4',5,6,7-hexamethoxyflavone and sinensetin increased during the season. Tangeretin content decreased in the oils obtained via Pelatrice. Tetra-O-methylscutellarein content increased as the season progressed for sweet orange oils obtained via FMC processing, but it showed higher amounts at the beginning and at the end of the season than at mid-season for sweet orange oils produced via the Pelatrice technique. Tangeretin, nobiletin and tetra-O-methylscutellarein showed average values higher in the Pelatrice oils than the FMC oils of the same production period; 3,3',4',5,6,7,8-heptamethoxyflavone and 3,3',4',5,6,7-hexamethoxyflavone showed an opposite behavior. For sinensetin content, there were no differences between Pelatrice and FMC oils.

Since FMC extractors are used especially to work blood fruits, and Pelatrice for blond fruits, all the same samples were grouped according to production month and kind of fruit (blond or blood), rather than extraction technology. The aim of this grouping was to see if the differences observed above were due to extraction technique or to the kind of fruit. The data obtained demonstrate that oils obtained from blond fruits and from blood fruits show behavior that correlates with Pelatrice oils and FMC oils, respectively.

It would appear that the sweet orange essential oil's polymethoxyflavone fraction is influenced most by the time of harvest and kind of fruit (blond or blood).

The quantitative data regarding the 66 samples of mandarin essential oil were divided according to extraction technology and production month from October to February. Three extraction technologies were used: Torchi, Pelatrice and FMC. For any given technology, there were no relevant quantitative differences among oils produced in the different periods of the season. Quantitative differences were observed only in oils extracted with Torchi or Pelatrice in the first part of the season; Pelatrice oils showed a higher content of each polymethoxyflavone.

Bitter Orange and Grapefruit Oils

Figure 3 shows the HPLC chromatograms of a bitter orange and a grapefruit oil. Table III reports the average content of each oxygen heterocyclic compound detected in the two oils.

The research was carried out on six genuine bitter orange essential oils produced in the years from 1993 to 1995, and on two genuine grapefruit essential oils produced in 1993 by a Sicilian industry.

Table III. Average content of oxygen heterocyclic compounds in six genuine Italian bitter orange and two grapefruit essential oils

Peak (Figure 3)	Oxygen heterocyclic compounds	Average content (mg/100 g of oil)	
		Bitter orange	Grapefruit
1	bergamottin	-	97
2	aurapten	-	1,124
3	osthol	171	58
4	bergapten	63	11
5	epoxybergamottin	275	1,126
6	epoxyaurapten	-	930
7	unknown coumarin 1	9	6
8	meranzin	926	510
9	isomeranzin	188	81
10	unknown coumarin 2	26	21
11	tangeretin	110	68
12	3,3',4',5,6,7,8-heptamethoxy-flavone	10	37
13	nobiletin	64	46
14	tetra-O-methylscutellarein	14	2
15	unknown coumarin 3	45	4
16	epoxybergamottin hydrate	25	70
17	meranzin hydrate	34	12

The bitter orange oils contained four known coumarins (osthol, meranzin, isomeranzin, meranzin hydrate), three unknown coumarins, three psoralens (bergapten, epoxybergamottin, epoxybergamottin hydrate) and four polymethoxylated flavones (tangeretin, heptamethoxyflavone, nobiletin, tetra-O-methylscutellarein). Meranzin is the main component, with an average value of 0.9 g/100 g of oil. Coumarins are the principal compounds, followed by psoralens and polymethoxylated flavones.

The grapefruit oil contained all the compounds identified in bitter orange oil, plus two other coumarins (aurapten, epoxyaurapten) and a psoralen (bergamottin). Aurapten and epoxybergamottin are the main components. Coumarins are the main compounds of grapefruit oil, as they were for bitter orange oil, followed by psoralens and polymethoxylated flavones.

The main differences in the composition of the oxygen heterocyclic compounds of bitter orange and grapefruit oils may be observed in the first part of the chromatograms. Presence of grapefruit oil in bitter orange oil is easily detectable, due to the presence of peaks of aurapten and epoxyaurapten.

Some samples of bitter orange oil, the results of which were not used to calculate the average values reported in Table III, did not contain meranzin, and contained epoxybergamottin in only a very small amount. These anomalies of the composition are probably due to the technology used for the extraction of the oil, which sometimes allows for a more prolonged contact of the oil with an

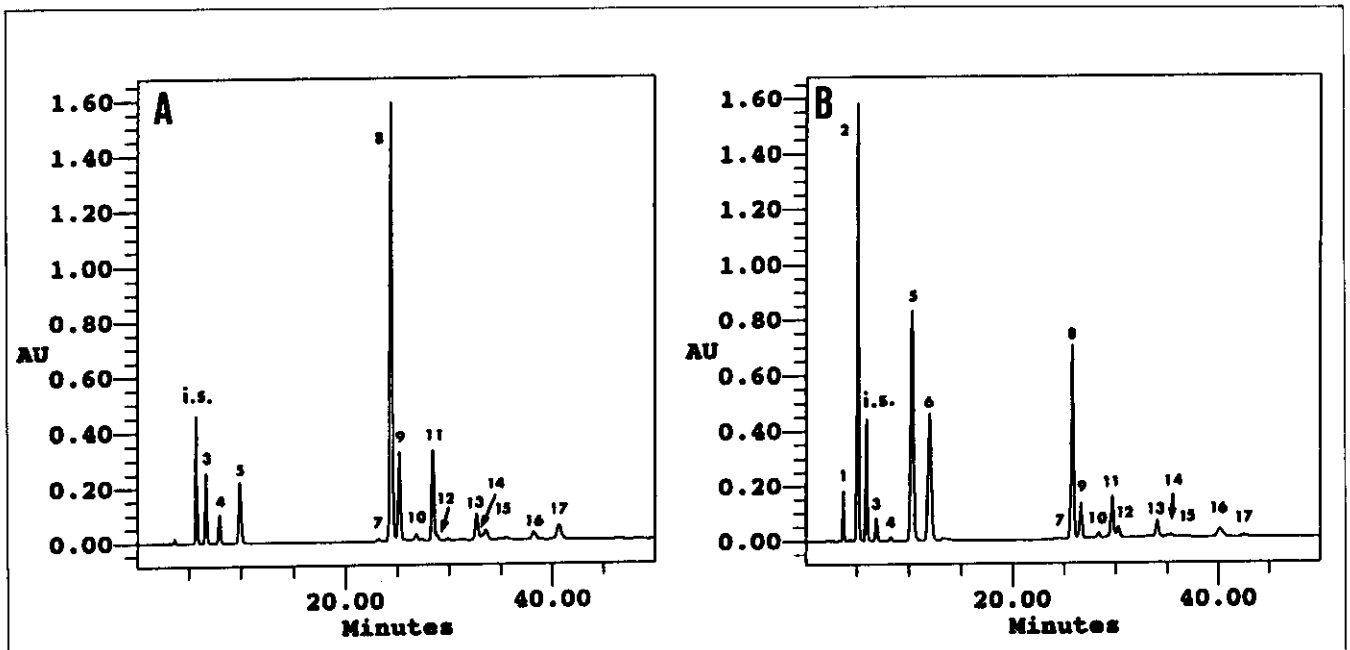

Figure 3. HPLC chromatogram of oxygen heterocyclic compounds of bitter orange (A) and grapefruit (B) oils (coumarin internal standard). For identification of components, see Table III.

acid aqueous medium. Under these conditions, the epoxy rings of meranzin and epoxybergamottin may be opened to form meranzin hydrate and epoxybergamottin hydrate, which are water soluble and are lost. To confirm this thesis, a genuine sample of bitter orange oil was mixed with orange juice or with a citric acid aqueous solution; meranzin practicially disappeared. Moreover, a mixture of meranzin hydrate and isomeranzin in diethyl ether, after treatment with a citric acid aqueous solution, showed a clearly lower amount of meranzin hydrate, while isomeranzin did not show any decrease.

Lemon Oil

Figure 4 shows the HPLC chromatogram of a lemon oil. The oil contained three coumarins (citropten, 5-geranyloxy-7-methoxycoumarin, 5-isopentenyloxy-7-methoxycoumarin), ten known psoralens (bergamottin, 8-geranyloxypsoralen, byakangelicol, oxypeucedanin, isoimperatorin, imperatorin, phellopterin, 5-isopent-2'-enyloxy-8-(2',3'epoxyisopentyloxy)psoralen, oxypeucedanin hydrate, byakangelicin) and two unknown psoralens.

The research was carried out on 37 genuine lemon oil samples produced during the 1994-1995 season. Quantitative results (Table IV) were obtained for bergamottin, 5-geranyloxy-7-methoxycoumarin, citropten, 8-geranyloxypsoralen, oxypeucedanin, byakangelicol and 5-isopent-2'-enyloxy-8-(2',3'-epoxyisopentyloxy)psoralen. For the other components, there was not a sufficient amount isolated at the required degree of purity to build a calibration curve and calculate the correction factor, so the quantitative data is not reported.

The quantitative data relative to all the samples analyzed were grouped according to production month. All six dosed compounds showed the same behavior: a slight—but not meaningful—decrease during the production season.

Our qualitative results agree well with those of McHale and Sheridan.[1] Ziegler and Spiteller[3] detected 29 compounds in the coumarin fraction of a cold-pressed Sicilian lemon oil. Many of them were trace constituents. For example, they found aurapten, a characteristic compound of grapefruit oil, present as a trace constituent of lemon oil. Under our conditions, aurapten shows the same retention time as 5-isopentenyloxy-7-methoxycoumarin, but even after pre-fractionating lemon oil on a silica gel column, aurapten was not detected. This result is in agreement with McHale and Sheridan,[1] who found aurapten only in commercial lemon oils, where grapefruit oil was probably added to enhance the ultraviolet absorbance of lemon oil. They did not find aurapten in genuine lemon oils.

Under our HPLC conditions, herniarin (7-methoxycoumarin), a characteristic compound of lime oil, co-eluted with 8-geranyloxypsoralen, which elutes immediately after citropten. Herniarin was never before detected in genuine cold-pressed lemon oils. During pre-fractionation of cold-pressed lemon oil by silica gel column chromatography, using a mixture of petroleum ether:ethyl acetate as eluent, we observed that 8-geranyloxypsoralen eluted earlier than citropten, while herniarin eluted after citropten.

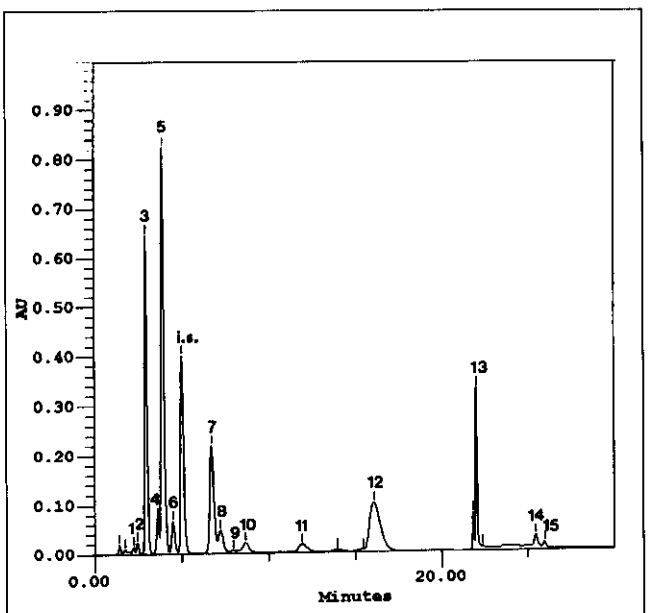

Figure 4. HPLC chromatogram of coumarins of lemon oil (coumarin internal standard). For identification of components, see Table IV.

Table IV. Average content of coumarins in 37 lemon essential oils

Peak (Figure 4)	Coumarins	Average content (mg/100 g of oil)
1	unknown psoralen	+
2	unknown psoralen	+
3	bergamottin	229
4	isoimperatorin	+
5	5-geranyloxy-7-methoxycoumarin	213
6	5-isopenteniloxy-7-methoxycoumarin	+
7	citropten	91
8	8-geranyloxypsoralen	28
9	imperatorin*	+
10	phellopterin**	+
11	5-isopent-2'-enyloxy-8-(2',3'-epoxy-isopentyloxy)psoralen**	26
12	oxypeucedanin	129
13	byakangelicol	95
14	oxypeucedanin hydrate	+
15	byakangelicin	+

* tentative
** tentative, identified according to McHale and Sheridan[1]
+ = quantitative data not reported

In one of the fractions so obtained it was possible to detect herniarin.

The analysis of some samples of lemon oil produced two or three years ago showed a total absence or very low content of oxypeucedanin and byakangelicol. As is well stated in many papers,[1,12,18] epoxypsoralens can undergo hydrolysis to the corresponding diols. The relatively low concentrations of these diols (oxypeucedanin hydrate and byakangelicin) found in some old lemon oils are probably due to their poor solubility in the oils.

Summary

We examined the composition of the oxygen heterocyclic fraction of bergamot, sweet orange, mandarin, bitter orange, grapefruit and lemon essential oils obtained by normal phase HPLC. We performed quantitative calculations using calibration curves obtained for each pure component previously isolated from the essential oils by chromatography on silica gel columns, thin layer chromatography and semipreparative HPLC with recycling.

The citrus essential oils analyzed show a characteristic composition of the oxygen heterocyclic fraction. This composition makes it possible to differentiate the individual oils and to detect mixtures or mutual contamination.

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