On the Genuineness of Citrus Essential Oils. 51. Oxygen Heterocyclic Compounds of Bitter Orange Oil (*Citrus aurantium* L.)

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The composition of the oxygen heterocyclic fraction of bitter orange essential oil, obtained by normal-phase HPLC with two on-line coupled columns (μ-Porasil and Zorbax), is reported. Genuine, industrial, cold-pressed Italian and Spanish essential oils, commercial oils, laboratory hand-extracted oils, and mixtures of bitter orange oil with sweet orange, lemon, lime, and grapefruit oils were analyzed. Four coumarins (osthol, meranzin, isomeranzin, and meranzin hydrate), three psoralens (bergapten, epoxybergamottin, and epoxybergamottin hydrate), and four polymethoxyflavones (tangeretin, 3,3′,4′,5,6,7,8-heptamethoxyflavone, nobiletin, and tetra-*O*-methylscutellarein) were identified. In addition, three unknown coumarins were found. Meranzin was the main component isolated. Meranzin hydrate, the formation of which was probably due to the hydration of meranzin during the industrial extraction, was not found in the laboratory hand-extracted samples. Meranzin was not present in some industrial samples, probably because of a prolonged and unusual contact of the essential oil with an acid aqueous medium during the process of extraction. Italian essential oils usually exhibited a higher content of oxygen heterocyclic compounds than the Spanish oils. The bitter orange oils adulterated by sweet orange oil were characterized by a lower content of almost all the aforementioned components; the adulteration of bitter orange oils with lemon, lime, or grapefruit oils was detected by the presence of components peculiar to added oils.

Keywords: *Citrus aurantium L.; bitter orange essential oil; coumarins; psoralens; polymethoxylated flavones; HPLC*

INTRODUCTION

Coumarin and psolaren compounds are common to plants of the Rutaceae family (Gray and Waterman, 1978; Stanley and Jurd, 1971), to which citrus species belong. Together with polymethoxylated flavones, coumarin and psolaren compounds represent a large part of the nonvolatile residue of citrus oils, which ranges from 1% in sweet orange oil to 10−15% in lime oil.

The occurrence of oxygen heterocyclic compounds in citrus species has also been widely studied (McHale and Sheridan, 1988, 1989; Ziegler and Spiteller, 1992; P. Dugo et al., 1994; Tatum and Berry, 1979; Mondello et al., 1993). Because of their structural diversity and diverse occurrence in different citrus peel oils, oxygen heterocyclic compounds may have an important role in the identification of the various oils and also in their quality and genuineness control (McHale and Sheridan, 1988; P. Dugo et al., 1994; Mondello et al., 1993; Di Giacomo, 1990). Oxygen heterocyclic compounds exhibit strong absorption in the ultraviolet (UV) region and cold-pressed citrus oils show characteristic absorption curves and characteristic CD values, as measured by the method of Sale (1953) in the region from 260 to 375 nm. Distilled oils (peratoner), obtained from residues of cold extraction, and terpenes, obtained by distillation of the whole oils, do not contain the components of the nonvolatile residue and are transparent in the UV region (Calvarano, 1966). CD values lower than the minimum found for genuine oils can indicate that less valuable products were added to cold-pressed oils. Extraneous substances or other oils are sometimes added to adulterated lemon or bitter orange oils with low CD values for the purpose of raising this value. The correct CD value does not ensure that the oil is genuine; chromatographic analyses of the volatile and nonvolatile fractions of the oil are needed to verify genuineness.

Bitter orange oil is widely used in perfumery and for flavoring candies, soft drinks, and baked goods (Calvarano, 1966). Its production is limited, however, because of the inconstancy of demand and the limited acreage of the plantations. The fruits used for the extraction of bitter orange oil are sometimes mixed with a small number of sweet orange fruits, and sometimes the industries use the same production line previously used for extraction of lemon oil (G. Dugo et al., 1993). Bitter orange oil, therefore, may be contaminated by sweet orange or lemon oils. Moreover, sweet orange oil or terpenes are sometimes added deliberately.

Many studies have been conducted on the volatile fraction of bitter orange oil, see reviews by Lawrence (1982) and Boelens (1991). Little information is available on the qualitative and quantitative composition of the heterocyclic oxygen compounds of the nonvolatile residue of bitter orange oil, however, and the data often refer to a limited number of samples (Stanley and Jurd, 1971; McHale and Sheridan, 1989; Fisher and Trama, 1979; D'Amore and Calapaj, 1965). In this paper we report the isolation, identification, and quantitation of oxygen heterocyclic compounds of bitter orange oil.

MATERIALS AND METHODS

Materials. This research was carried out on samples of genuine, industrial, cold-pressed Italian bitter orange oils; commercial bitter orange oils; laboratory extracted bitter orange oils, obtained by putting enough pressure on the peel

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of the fruits to break the oil glands and to allow the essential oil to get out (the oil is collected into a watch glass, transferred into a test-tube, and centrifuged); mixtures of genuine bitter orange oils with lemon, lime, grapefruit, and sweet orange oils; and spanish bitter orange oils.

Methods. All samples were analyzed by normal-phase HPLC [model 519 pump; 600 E gradient controller; Rheodyne 9125 injector; photodiode array detector (PDA), model 996; Waters Associates]. 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 column used for the first 12 min of analysis was a 30 cm × 3.9 mm i.d. μ-Porasil with a particle size of 10 μm (Waters Associates). Then, the flow was switched onto a second column (25 cm × 4.6 mm i.d., Zorbax silica; Phenomenex) with a particle size of 7 μm. Two mobile phases were used: eluent A (hexane:ethyl acetate, 9:1) and eluent B (hexane:ethyl alcohol, 9:1). The HPLC analyses of bitter orange oil samples were performed according to the following program: 0−2 min, 98% A + 2% B; 2−25 min, from 98% A + 2% B to 5% A + 95% B, with a concave gradient; 25−45 min, 5% A + 95% B; 45−50 min, from 5% A + 95% B to 98% A + 2% B. The flow rate was 1.6 mL/min; the pressure from 0 to 12 min on the μ-Porasil column was 444 psi, and from 12 to 50 min on the μ-Porasil + Zorbax columns was 780 psi; and the column temperature was 30 °C. The injection volume was 20 μL of a solution obtained by diluting ∼50 mg of the essential oil and 0.1 mL of a coumarin solution of known concentration to 1 mL of hexane:ethyl acetate (75:25). Detection was by UV absorbance at 315 nm. The UV spectra of eluting peaks were monitored with the PDA detector in the region 200−400 nm.

The values of some parameters significant for the genuineness of the bitter orange oil were also determined: the content of δ-3-carene and α-terpinene by HRGC (G. Dugo et al., 1993), the enantiomeric distribution of linalool by HPLC-HRGC (G. Dugo et al., 1994), and the CD value by the method of Sale (1953).

Osthol (7-methoxy-8-isopentylcoumarin), bergapten (5-methoxypsoralen), epoxybergamottin [5-[(3,7-dimethyl-6-epoxy-2-octenyl)oxy]psoralen], meranzin [7-methoxy-8-(2,3-epoxyisopentyl)coumarin], isomeranzin [7-methoxy-8-(2-one-isopentyl)coumarin], tangeretin (4′,5,6,7,8-pentamethoxyflavone), 3,3′,4′,5,6,7,8-heptamethoxyflavone, nobiletin (3′,4′,5,6,7,8-hexametoxyflavone), meranzin hydrate [7-methoxy-8-(2,3-dihydroxyisopentyl)coumarin], epoxybergamottin hydrate [5-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen], and three other unknown coumarins were isolated from a sample of bitter orange oil by column chromatography, TLC, and semipreparative HPLC in recycle mode. The method for the isolation was as follows: 300 mL of genuine bitter orange oil were treated in a rotatory evaporator (5 Torr, 25 °C) to remove most of the volatile fraction; and the residue was fractionated on a glass column (30 × 6 cm i.d.) filled with 400 g of silica gel (0.063−0.200 mm, Baker Analyzed) with a gradient of a mixture of hexane and ethyl acetate [from hexane:ethyl acetate (9:1) to 100% ethyl acetate] as eluent.

The fractions were monitored by TLC [5 × 10 cm plates coated with 0.25 mm SIL-254 UV 254; (Aldrich); eluent, hexane:ethyl acetate (70:30)], and HPLC under the conditions already mentioned. The fractions were gathered, according to their composition, into eight groups that contained the following compounds: fraction 1, osthol (95%); fraction 2, bergapten (40%), epoxybergamottin (60%); fraction 3, unknown coumarin 1 (8%), meranzin (46%), isomeranzin (44%); fraction 4, unknown coumarin 2 (60%), tangeretin (15%); fraction 5, tangeretin (40%), heptamethoxyflavone (4%), meranzin hydrate (33%); fraction 6, meranzin hydrate (72%); fraction 7, nobiletin (30%), epoxybergamottin hydrate (40%); and fraction 8, nobiletin (10%), tetra-*O*-methylscutellarein + unknown coumarin 3(7%), epoxybergamottin hydrate (20%). Osthol, unknown coumarin 2, and meranzin hydrate were isolated directly by crystallization from the corresponding fractions. Fractions 2, 3, and 7 needed further chromatographic separation. Tetra-*O*-methylscutellarein, tangeretin, and heptamethoxyflavone were previously isolated from sweet orange essential oil (P. Dugo et al., 1994). Unknown coumarin 3 was not obtained at the required degree of purity, so quantitative results were obtained using the correction factor of meranzin.

Separation of Bergapten and Epoxybergamottin. Compounds of fraction 2 were separated by semipreparative HPLC, in the recycle mode, with a Waters Associates setup composed of a 519 pump with 225 μL heads, a gradient controller (600E), a manual injector (U6K), a spectrophotometric detector (model 484), two 25 × 100 mm PrepPak cartridges (Porasil; 15−20 μm, 125 Å) inserted in a Waters RCM 25 × 200 module, compression solvent (isopropyl alcohol at 1500 psi), and a three-port recycle valve (Valco). The mobile phase for bergapten was hexane:ethyl acetate (95:5), and the injection volume was 2 mL of an ethyl acetate solution containing approx 40 mg of fraction 2. Epoxybergamottin was also obtained by column chromatography on silica gel of grapefruit oil, with a mobile phase of petroleum ether:ethyl acetate (80:20).

Separation of Meranzin, Isomeranzin, and Unknown Coumarin 1. Compounds of fraction 3 were separated by preparative TLC in four runs hexane:acetone (83:17) was the eluent for the first three runs, and methylene chloride:ethyl ether (94:6) was the eluent for the last run. Moreover, we obtained isomeranzin by column chromatography on silica gel (mobile phase, petroleum ether:ethyl acetate, 70:30) from a sample of bitter orange oil that did not contain meranzin.

Separation of Nobiletin and Epoxybergamottin Hydrate. Compounds of fraction 6 were separated by column chromatography on silica gel with an eluent of a mixture of methylene chloride and ethyl acetate (80:20).

Coumarins were crystallized by cooling a hexane solution, psoralens were crystallized by addition of hexane to a solution of ethyl acetate, and polymethoxylated flavones were crystallized by addition of hexane to a solution of methylene chloride. Purity was monitored by HPLC, with the same experimental conditions mentioned for the analysis of bitter orange oils. The spectral contrast technique of the photodiode detector, which makes it possible to detect co-elution by matching all spectra within a peak (Millenium 2010, 1993), was used. All the compounds isolated were spectrally pure. The identity of each compound isolated was confirmed by ^{1}H NMR (300 MHz; Varian) and electron-impact (EI) mass spectrometry (70 eV; Finnigan, Mass 90). Structural assignment was obtained by comparison with data of authentic samples or with literature data.

RESULTS AND DISCUSSION

δ-3-Carene and α-terpinene content in the volatile fraction, the enantiomeric distribution of linalool, and the CD value for all the oils analyzed are presented in Table 1. Samples 1−6 show values, for the measured parameters, in agreement with genuine bitter orange oils. A δ-3-carene value less than 0.03% excludes the possibility of contamination or addition of oils or terpenes of sweet orange (G. Dugo et al., 1993); an α-terpinene value less than 0.003% excludes contamination or addition of lemon or lime oils or terpenes (G. Dugo et al., 1993); the ratio between (−)- and (+)-linalool greater than 80/20 excludes the presence of sweet orange oil or, at least, of synthetic linalool (G. Dugo et al., 1994); and CD values are always high enough to exclude dilutions of the oils by terpenes or distilled oils.

The laboratory extracted oils (samples 7−12) show values in accord with those of genuine industrial oils. Samples 13−17 show high values of δ-3-carene, indicating the presence of sweet orange oils or terpenes; the (−)/(+)-linalool ratio shows the presence of sweet orange oils, which have a ratio of ∼7/93, or of synthetic linalool. The low value of CD means dilutions with products with a low content of nonvolatile residue. Sample 18, shows a high percentage of α-terpinene together with a high content of δ-3-carene; its CD value is low, and the

Table 1. Values of Some Genuineness Parameters for Bitter Orange Oils

	Italian genuine oils						Italian laboratory extracted oils					
parameter	1	2	3	4	5	6	7	8	9	10	11	12
CD[a]	1.60	2.10	1.90	1.89	1.81	1.70	1.93	1.60	1.90	1.43	1.56	1.71
(−)/(+)-linalool	80/20	87/13	87/13	86/14	83/17	81/19	80/20	88/12	79/21	80/20	87/13	83/17
δ-3-carene[b]	tr	0.003	0.002	0.002	tr	0.004	tr	tr	tr	tr	tr	tr
α-terpinene[b]	0.003	0.002	0.002	0.002	0.005	tr	tr	tr	0.002	0.002	tr	tr

	adulterated oils							mixtures			
	13	14	15	16	17	18	19	20[c]	21[d]	22[e]	23[f]
CD[a]	0.98	0.65	0.69	0.59	0.54	0.68	0.54	1.40	1.44	1.83	1.79
(−)/(+)-linalool	59/41	64/36	58/42	57/43	58/42	84/16	65/35	60/40	66/34	79/21	79/21
δ-3-carene[b]	0.027	0.035	0.032	0.033	0.030	0.028	0.003	0.012	tr	tr	tr
α-terpinene[b]	tr	tr	0.005	0.005	0.005	0.010	0.020	tr	0.045	0.019	0.004

	Spanish oil			Italian anomalous oils			Spanish anomalous oils		
	24	25	26	27	28	29	30	31	32
CD[a]	1.44	1.42	1.37	0.67	0.60	0.68	0.79	0.71	0.76
(−)/(+)-linalool	76/24	76/24	76/24	80/20	86/14	79/21	81/19	83/17	83/17
δ-3-carene[b]	tr	tr	tr	0.003	0.003	0.003	tr	tr	tr
α-terpinene[b]	0.005	0.004	0.002	0.002	0.004	0.003	0.005	0.003	0.003

[a] 250 mg of oil/100 mL of ethanol. [b] Relative percentage in volatile fraction. [c] 20% sweet orange oil. [d] 20% lemon oil. [e] 10% lime oil. [f] 10% grapefruit oil.

enantiomeric distribution of linalool is in agreement with the values shown by genuine bitter orange oils. Sweet orange, lemon, or lime terpenes must have been added to the sample and have determined variations in the values of carene, α-terpinene, and CD, but the sample doesn't show the presence of extraneous linalool. In fact, citrus terpenes do not contain linalool at all or in very small amounts. Sample 19 has probably been adulterated by lemon or lime terpenes, because of the values of CD and α-terpinene. Enantiomeric distribution of linalool also indicates the presence of extraneous linalool.

The mixture that contains 20% sweet orange oil (sample 20) presents, as expected, a high value of δ-3-carene and a low (−)/(+)-linalool ratio; the mixture containing 20% lemon oil (sample 21) presents a high value of α-terpinene and a low (−)/(+)-linalool ratio; and the mixture containing 10% lime oil (sample 22) shows a high value of α-terpinene. The presence of grapefruit oil (sample 23) does not affect any of the parameters we considered. Spanish bitter orange oils (samples 24−26), compared with the genuine Italian oils, show lower values of CD and slightly lower (−)/(+)-linalool ratios. The HPLC chromatogram of the oxygen heterocyclic fraction of a genuine bitter orange oil is shown in Figure 1. Four coumarins (osthol, meranzin, isomeranzin, and meranzin hydrate), three furocoumarins (bergapten, epoxybergamottin, and epoxybergamottin hydrate), four polymethoxylated flavones (tangeretin, heptamethoxyflavone, nobiletin, and tetra-*O*-methylscutellarein) were identified. In addition, three unknown coumarins, two of which (unknown coumarins 1 and 2) have UV spectra very similar to those of meranzin and meranzin hydrate, were found.

The composition of the fraction containing oxygen heterocyclic compounds for the oils to be considered genuine, according to their origin and data reported in Table 1, is shown in Table 2. Meranzin is the main component, and its content, in 100 g of oil, ranges from 0.8 to 1.2 g. Significant amounts of epoxybergamottin, isomeranzin, and osthol are also present. The total content of the oxygen heterocyclic fraction ranges from 1.6 to 2.3 g in 100 g of oil. Coumarins are the principal compounds, followed by psoralens and polymethoxylated flavones.

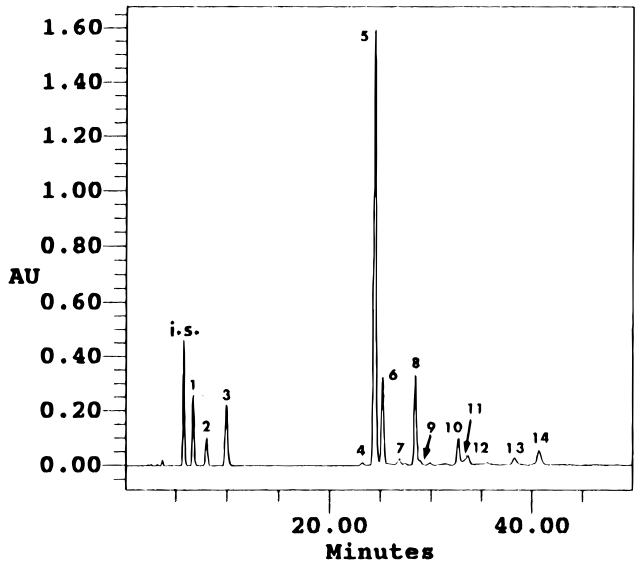

Figure 1. HPLC chromatogram of an Italian genuine bitter orange oil [sample 2; internal standard (i.s.)]. For identification of components, see Table 2.

The contents of oxygen heterocyclic compounds of the laboratory hand-extracted oils are shown in Table 3. The results are similar to those obtained for the genuine commercial oils (see Table 2). It is noteworthy that none of these samples contain meranzin hydrate, which may be formed during the industrial process of extraction. Values obtained from commercial bitter orange oils are shown in Table 4. These commercial oils may be considered adulterated according to the data reported in Table 1. Almost all the components of these oils are lower than those of genuine oils. Heptamethoxyflavone is the exception because all the samples from 13 to 17, which are clearly adulterated with sweet orange oil or related products, show a higher content than the genuine oils. Samples 13−17 show only quantitative differences from the genuine oils. Thus, the adulteration was carried out with products not containing an oxygen heterocyclic fraction at all or in very small quantities and which do not contain compounds absent in the bitter orange oils. The adulterants may have been sweet orange terpenes or oils.

Table 2. Oxygen Heterocyclic Compounds in Genuine Italian Bitter Orange Oils (mg/100 g of Oil)

component	samples 1	2	3	4	5	6
osthol	159	184	181	174	154	172
bergapten	54	70	73	65	52	66
epoxybergamottin	246	307	319	322	188	271
unknown coumarin 1	12	2	9	8	13	12
meranzin	788	1172	1005	941	823	827
isomeranzin	175	211	191	154	204	193
unknown coumarin 2	23	29	35	24	24	20
tangeretin	59	156	122	146	85	93
heptamethoxyflavone	5	13	8	14	11	12
nobiletin	34	87	87	78	47	51
tetra-*O*-methyl-scutellarein	10	12	18	16	12	15
unknown coumarin 3	45	40	48	39	46	50
epoxybergamottin hydrate	15	35	18	22	13	45
meranzin hydrate	12	70	10	31	21	62
coumarins	1214	1708	1479	1371	1285	1336
psoralens	315	412	410	409	253	382
polymethoxylated flavones	108	268	235	254	155	171
total	1637	2388	2124	2304	1693	1889

Table 3. Oxygen Heterocyclic Compounds in Laboratory Extracted Italian Bitter Orange Oils (mg/100 g of Oil)

component	samples 7	8	9	10	11	12
osthol	135	192	115	99	122	142
bergapten	55	92	46	36	70	65
epoxybergamottin	173	186	141	119	149	182
unknown coumarin 1	14	16	15	10	15	13
meranzin	1403	836	1345	635	825	883
isomeranzin	296	215	299	172	254	293
unknown coumarin 2	20	21	41	26	21	53
tangeretin	122	86	82	66	83	114
heptamethoxyflavone	3	21	4	12	4	21
nobiletin	70	89	61	54	64	151
tetra-*O*-methyl-scutellarein	6	9	9	6	5	10
unknown coumarin 3	30	28	30	24	31	36
epoxybergamottin hydrate	15	16	12	9	10	26
meranzin hydrate	–	–	–	–	–	–
coumarins	1898	1308	1845	966	1268	1420
psoralens	243	306	199	164	229	273
polymethoxylated flavones	201	205	156	138	156	296
total	2342	1807	2200	1268	1653	1989

Samples 18 and 19 contain high amounts of bergamottin and 5-(geranyloxy)-7-methoxycoumarin. This result may indicate the presence of lemon or lime oil, both of which contain a high amount of these compounds (McHale and Sheridan, 1988, 1989).

The HPLC chromatogram of mixtures obtained in the laboratory of genuine bitter orange oil and lime, lemon, grapefruit, and sweet orange oils are shown in Figure 2. These chromatograms, and their comparison with the chromatogram of a genuine bitter orange oil, shown in Figure 1, easily point out that the presence of sweet orange oil makes only quantitative differences, because of the composition of the two oils. The presence of lemon or lime oil is easily detectable, due to the presence of bergamottin and 5-(geranyloxy)-7-methoxycoumarin, and the presence of grapefruit oil is indicated by aurapten and epoxyaurapten.

5-(Geranyloxy)-7-methoxycoumarin, aurapten, and epoxyaurapten were identified according to McHale and Sheridan (McHale and Sheridan, 1989). The Spanish oils (Table 5) show a qualitative composition very

Table 4. Oxygen Heterocyclic Compounds in Adulterated Bitter Orange Oils (mg/100 g of Oil)

components	samples 13	14	15	16	17	18	19
bergamottin	–	–	–	–	–	+	+
5-(geranyloxy)-7-methoxy-coumarin	–	–	–	–	–	+	+
osthol	114	105	109	117	113	140	197
bergapten	37	39	31	34	31	27	45
epoxybergamottin	127	100	72	70	84	60	-
unknown coumarin 1	8	6	7	4	7	4	11
meranzin	401	314	122	135	133	205	-
isomeranzin	110	116	77	90	69	126	108
unknown coumarin 2	9	8	–	–	–	–	3
tangeretin	78	70	72	53	62	53	34
heptamethoxyflavone	25	32	24	30	27	5	4
nobiletin	46	45	34	40	33	18	16
tetra-*O*-methylscutellarein	24	31	18	22	16	7	–
unknown coumarin 3	–	–	4	–	–	4	–
epoxybergamottin hydrate	26	34	38	32	43	24	24
meranzin hydrate	53	73	55	32	96	31	98
coumarins	695	622	374	378	418	510	417
psoralens	190	173	141	136	158	111	69
polymethoxylated flavones	173	178	148	145	138	83	54
total	1058	973	663	659	714	704	540

Table 5. Oxygen Heterocyclic Compounds in Spanish Bitter Orange Oils (mg/100 g of Oil)

component	samples 24	25	26
osthol	369	366	371
bergapten	71	71	71
epoxybergamottin	328	318	304
unknown coumarin 1	13	13	12
meranzin	332	313	307
isomeranzin	209	208	213
unknown coumarin 2	24	23	16
tangeretin	95	95	99
heptamethoxyflavone	24	23	25
nobiletin	84	85	76
tetra-*O*-methylscutellarein	8	5	8
unknown coumarin 3	57	48	55
epoxybergamottin hydrate	24	33	42
meranzin hydrate	18	39	38
coumarins	1022	1010	1012
psoralens	423	422	417
polymethoxylated flavones	211	208	208
total	1656	1640	1637

similar to that of Italian oils (Table 2), but Spanish oils show a higher content of osthol and a lower content of meranzin than the Italian oils. The entire heterocyclic fraction is quantitatively less represented, and this finding agrees with the lower CD value compared with the Italian oils.

Results for samples of Italian and Spanish commercial oils that we named “anomalous” are shown in Table 6. They did not seem contaminated or adulterated on the basis of values reported in Table 1, except for the lower value of CD. The chromatogram of one of these samples is shown in Figure 3. The results in Table 6 and Figure 3 indicate that meranzin, usually the main component of the oxygen heterocyclic fraction of the bitter orange oil, is absent and epoxybergamottin is present in a very small amount; the other components are present in smaller amounts than those of the oils reported in Table 2.

These anomalies of the composition are probably due to the technology used for the extraction of the oil. Sometimes bitter orange oil is extracted by “Torchi”, directly from the whole fruit. This technique allows for a more or less prolonged contact of the oil with the juice,

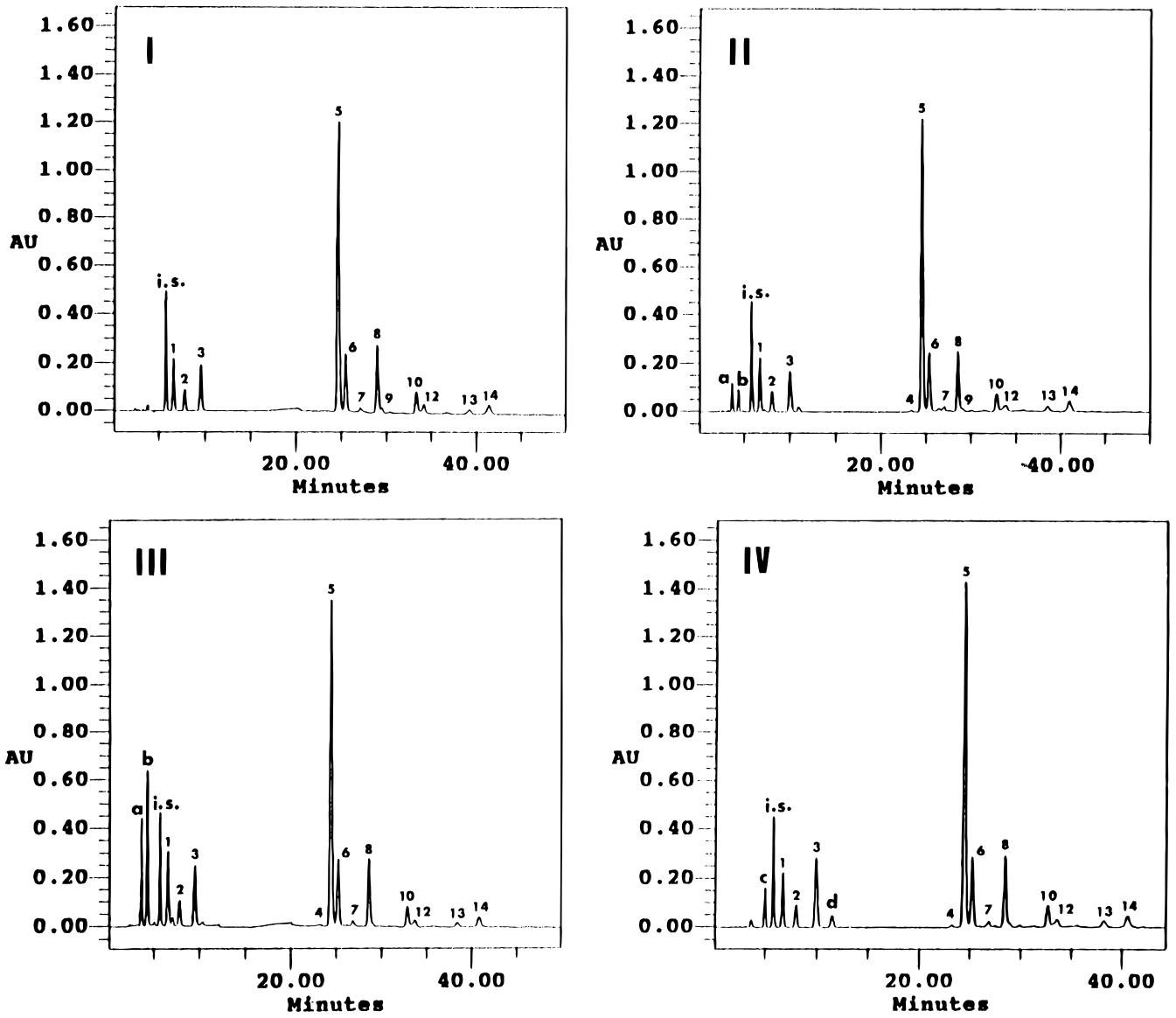

Figure 2. HPLC chromatograms of mixtures of bitter orange oil with (I) sweet orange, (II) lemon, (III) lime, and (IV) grapefruit oils [(i.s.) internal standard]. Key: (a) bergamottin; (b) 5-(geranyloxy)-7-methoxycoumarin; (c) aurapten; (d) epoxyaurapten. For identification of other components, see Table 2.

that has an acid pH. Moreover, regardless of the technology used, some juice or a citric acid aqueous solution is sometimes added to break the emulsion between the oil and the water. Under these conditions, meranzin and epoxybergamottin may undergo some transformations that cause their drastic reduction or their complete disappearance; for example, the epoxy ring is opened by hydrolysis to form meranzin hydrate and epoxybergamottin hydrate, which are water soluble (McHale and Sheridan, 1988; Radford and Olansky, 1994). Two tests was done to verify this hypothesis. First, a sample of bitter orange oil was mixed with bitter orange juice and stirred for 4 h. After this period, HPLC analysis of the oil showed the meranzin content decreased from 749 to 12 mg/100 g of oil, meranzin hydrate increased from 0.9 to 5 mg, and epoxybergamottin decreased from 215 to 160 mg. Treatment of the oil with an aqueous acid solution gave the same results. In the second test, 40 mg of a mixture containing 88% meranzin and 12% isomeranzin dissolved into diethyl ether was treated with a citric acid aqueous solution overnight. HPLC analysis of the organic phase showed that

Table 6. Oxygen Heterocyclic Compounds in "Anomalous" Italian and Spanish Bitter Orange Oils (mg/100 g of Oil)

	Italian oils			Spanish oils		
component	27	28	29	30	31	32
osthol	141	156	178	236	232	239
bergapten	5	40	6	77	75	76
epoxybergamottin	18	3	–	8	7	7
unknown coumarin 1	9	11	5	4	2	2
meranzin	–	–	–	–	–	–
isomeranzin	172	172	201	229	214	218
unknown coumarin 2	4	–	10	6	7	6
tangeretin	56	58	94	73	70	72
heptamethoxyflavone	7	6	–	19	15	12
nobiletin	25	17	65	63	75	69
tetra-*O*-methylscutellarein	8	5	6	10	tr	16
unknown coumarin 3	–	–	–	–	tr	–
epoxybergamottin hydrate	41	23	31	61	49	50
meranzin hydrate	132	70	108	7	6	6
coumarins	458	409	502	482	461	471
psoralens	64	66	37	146	131	133
polymethoxylated flavones	96	86	165	165	160	169
total	618	561	704	793	752	773

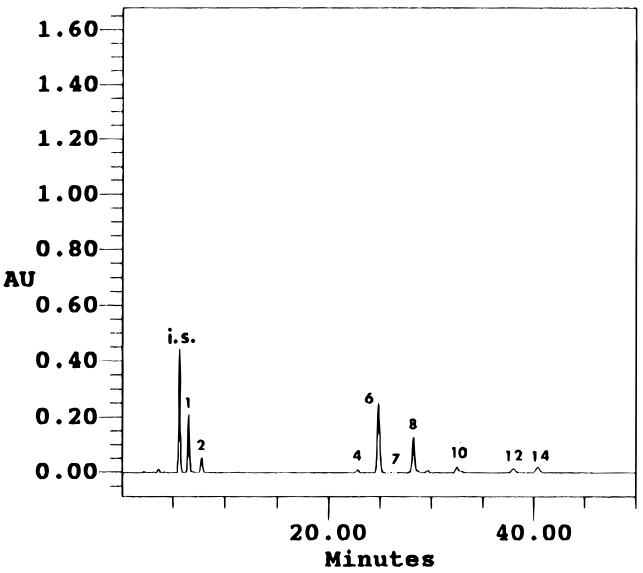

Figure 3. HPLC chromatogram of an "anomalous" bitter orange oil [sample 28; internal standard (i.s.)]. For identification of components, see Table 6.

isomeranzin did not undergo any transformation andmeranzin was present only in trace amount. However, 6.6 mg of meranzin hydrate was found. The total amount of meranzin hydrate is clearly lower than the amount of meranzin that disappeared because most of the formed hydrate is lost in the aqueous phase.

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