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On the genuineness of citrus essential oils. Part LIII.† Determination of the composition of the oxygen heterocyclic fraction of lemon essential oils (*Citrus limon* (L.) Burm. f.) by normal-phase high performance liquid chromatography

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ABSTRACT: The oxygen heterocyclic fraction of cold-pressed lemon essential oils has been studied by normal-phase HPLC. The components of the fraction have been isolated by column chromatography, TLC and semi-preparative HPLC with recycle. The identification of the isolated components has been carried out by ^{1}H-NMR and mass spectrometry. Three coumarins (5-geranyloxy-7-methoxycoumarin, citropten, 5-isopentenyloxy-7-methoxycoumarin) and ten psoralens (bergamottin, 8-geranyloxypsoralen, oxypeucedanin, byakangelicol, oxypeucedanin hydrate, byakangelicin, imperatorin, phellopterin, isoimperatorin, 5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen) have been isolated and identified. The main components were bergamottin (160–291 mg/100 g of oil) and 5-geranyloxy-7-methoxycoumarin (180–250 mg/100 g of oil). Moreover, herniarin, a coumarin characteristic of lime essential oil, has been detected. Herniarin had been reported previously in essential oils obtained from *Citrus limon* L. var. Eureka. © 1998 John Wiley & Sons, Ltd.

KEY WORDS: *Citrus limon* (L.) Burm. f.; lemon oil; coumarins; psoralens; HPLC

Introduction

Coumarins and psoralens are present in the non-volatile residue of lemon essential oil, which represents 1.5–4% of the oil. The residue also contains carotenoids, fatty acids and sterols.[2] Coumarins and psoralens, because of their differentiated occurrence in citrus peel oil, may have an important role to play in the identification of the various oils, and in the quality control of genuine oils.[3] Moreover, isolation of these compounds, some of which are not commercially available, paves the way for further study of their potential as pharmacological agents.[4] Numerous studies on the qualitative composition of the oxygen heterocyclic fraction of lemon oil can be found in the literature, but there is little agreement from one to another. Many authors[3,5,6] have found coumarins which were not confirmed in other studies.[7] Quantitative data are poor and often related only to the main components and to a limited number of samples.[4,5,7–9]

In this paper we report results on the isolation, identification and quantitative determination of oxygen heterocyclic compounds of lemon oils.

Experimental

This research was carried out on 37 samples of genuine industrial cold-pressed Italian lemon oils produced during the season 1994–95. All samples were analysed by normal-phase HPLC, using Waters Associates (Milan, Italy) equipment consisting of a model 519 pump, a model 600 E gradient controller, a Rheodyne model 9125 injector and a photodiode array (PDA) detector model 996. Peak integration and quantitative calculations were performed by a Waters Millennium 2010 system, using a calibration curve obtained for each previously isolated standard component against a coumarin standard. The column was a 15 cm × 3.9 mm i.d. μ-porasil (Waters Associates) with a particle size of 10 μm. Mobile phases were: eluent A, hexane : ethyl acetate 92:8; eluent B, hexane : ethyl alcohol, 90 : 10. Eluent A was pumped for 15 min, then a linear gradient

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to eluent B in 5 min, with a final hold for 10 min. The flow rate was 1.25 ml/min; column pressure was 204 psi; column temperature was 30°C, and the injection volume was 20 µl of a solution obtained by diluting 90 mg, accurately weighed, of essential oil with 0.80 ml of hexane:ethyl acetate, 75:25, and adding 0.1 ml of coumarin solution (0.99 mg/ml) as internal standard. Detection was by UV absorbance at 315 nm. The UV spectra of eluting peaks were monitored with the PDA detector in the region 220–400 nm. For detection of herniarin, the samples of lemon oil were analysed by normal-phase HPLC, with the same equipment as described above, under the following conditions: two 15 cm × 3.9 mm i.d. NovaPak silica columns with a particle size of 4 µm (Waters Associates, Milan, Italy). Two mobile phases were used: eluent A (hexane:ethyl acetate, 93:7) and eluent B (hexane:ethyl alcohol, 90:10); eluent A for 15 min, then under a linear gradient to 95% eluent B in 15 min, with a subsequent hold for 5 min. Flow rate, 1 ml/min; column pressure, 600 psi; column temperature, 30°C.

5-Geranyloxy-7-methoxycoumarin, bergamottin (5-geranyloxypsoralen), citropten (5,7-dimethoxycoumarin), 8-geranyloxypsoralen, oxypeucedanin [5-(2′,3′-epoxy-3′-methylbutyloxy)-psoralen], byakangelicol [5-methoxy-8-(2′,3′-epoxy-3′-methylbutyloxy)-psoralen], oxypeucedanin hydrate [5-(2′,3′-dihydroxy-3′-methylbutyloxy)-psoralen], byakangelicin [5-methoxy-8-(2′,3′-dihydroxy-3′-methylbutyloxy)-psoralen], imperatorin [8-(3′-methylbut-2′-enyloxy)-psoralen], phellopterin [5-methoxy-8-(3′-methylbut-2′-enyloxy)-psoralen], isoimperatorin [5-(3′-methylbut-2′-enyloxy)-psoralen], 5-isopentenyloxy-7-methoxycoumarin and 5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen were isolated from a sample of lemon oil by column chromatography, TLC and semi-preparative HPLC in a recycle mode.

Isolation was carried out as follows: 200 ml of lemon oil were concentrated in a rotary evaporator (5 Torr, 25°C) removing most of the volatile fraction. The residue was fractionated on a glass column (30 × 6 cm i.d.) filled with silica gel (0.063–0.200 mm, Baker Analysed (Milan, Italy), using as eluent light petroleum:ethyl acetate, 80:20; flow rate, 1 ml/min. Light petroleum refers to the fraction b.p. 30–50°C. The fractions were monitored by TLC (plates 5 × 10 cm, coated with SIL-254 UV 254, 0.25 mm (Aldrich, Milan, Italy); eluent light petroleum:ethyl acetate, 80:20), and normal-phase HPLC under the conditions mentioned above. The fractions were gathered, according to their composition, into seven groups, which contained the compounds in the following relative percentages:

Fraction 1: bergamottin, 41%; 5-geranyloxy-7-methoxycoumarin, 41%.

Fraction 2: bergamottin, 27%; 5-geranyloxy-7-methoxycoumarin, 32%; isoimperatorin, 5%; 5-isopentenyloxy-7-methoxycoumarin, 5%.

Fraction 3: citropten, 50%; 8-geranyloxypsoralen, 6%; imperatorin, 1%; phellopterin, 1%; 5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen, 4%.

Fraction 4: citropten, 94%.

Fraction 5: oxypeucedanin, 99%.

Fraction 6: byakangelicol, 63%.

Fraction 7: byakangelicol, 35%, oxypeucedanin hydrate, 38%, byakangelicin, 13%.

Citropten, oxypeucedanin and byakangelicol were isolated by crystallization from the correspondent fractions 4, 5 and 6. The other fractions needed further chromatographic separation.

Separation of Components of Fraction 2

Fraction 2 was fractionated by column chromatography on silica gel using hexane:ethyl acetate, 98:2 as eluent. Two fractions were obtained, which contained the compounds in the following relative percentages:

Fraction 2A: bergamottin, 89%; isoimperatorin, 4%.

Fraction 2B: 5-geranyloxy-7-methoxycoumarin, 74%; 5-isopentenyloxy-7-methoxycoumarin, 6%.

Compounds of fraction 2A were separated by semi-preparative HPLC using a Waters Associates set-up composed of: model 519 pump with 225 µl heads; gradient controller 600 E; manual injection U6K; spectrophotometric detector model 484; PrepPak cartridge Porasil (15–20 µm, 125 Å) 25 × 100 mm, inserted into a Waters RCM 25 × 100 mm module; compression solvent, isopropyl alcohol at 1600 psi. The mobile phase was hexane:ethyl acetate, 96:4; the injection volume was 2 ml of a hexane solution containing about 30 mg of fraction 2A.

Compounds of fraction 2B were separated by semi-preparative HPLC in recycle mode, using the equipment as previously described equipped with a three-port recycle valve (Valco Europe, Schenkon, Switzerland). The mobile phase was hexane:ethyl acetate, 9:1.

Separation of Components of Fraction 3

Fraction 3 was separated by column chromatography on silica gel using light petroleum:ethyl acetate, 9:1, as eluent. Two fractions were obtained, which contained the compounds in the following relative percentages:

Fraction 3A: citropten, 78%; 8-geranyloxypsoralen, 15%.

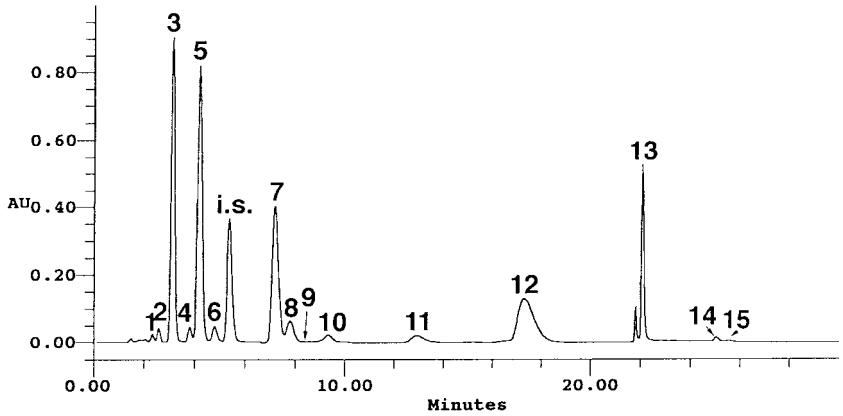

Figure 1. HPLC chromatogram of an Italian genuine lemon oil. i.s., internal standard. For identification of components, see Table 1

Fraction 3B: citropten, 5%; phellopterin, 3%; 5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen, 88%.

Compounds of fraction 3A were separated by preparative thin layer chromatography (plates 20 × 20 cm, coated with silica gel F254, 2 mm thickness; Aldrich, Milan, Italy). Mobile phase: light petroleum:ethyl acetate, 70:30. Alternatively, citropten and 8-geranyloxypsoralen may be separated by semi-preparative HPLC, in the recycle mode, using the same equipment as previously described. The mobile phase was hexane : ethyl acetate, 9:1. The flow rate was 20 ml/min.

5-Isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen was crystallized from fraction 3B.

Separation of Oxypeucedanin Hydrate and Byakangelicin

Compounds of fraction 7 were separated by semi-preparative HPLC using hexane:ethyl acetate, 55:45. A fraction containing oxypeucedanin hydrate 74% and byakangelicin 26% was obtained.

Bergamottin and oxypeucedanin were crystallized by cooling a hexane solution; 5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen by adding light petroleum to a solution of diethyl ether; the other compounds were crystallized by addition of hexane to a solution of ethyl acetate.

Purity was monitored by HPLC, under the same experimental conditions mentioned for the analysis of the oils, using the spectral contrast technique of the photodiode array detector, which makes it possible to detect co-elution by matching all spectra within one peak.[10]

The identity of each compound isolated was confirmed by ^{1}H-NMR (300 MHz, Varian (Milan, Italy)) and mass spectrometry (EI, 70 eV, Finningan (Milan, Italy), Mass 90).

Results

Figure 1 shows the HPLC chromatogram of a lemon oil. Three coumarins — citropten, 5-geranyloxy-7-methoxycoumarin and 5-isopentenyloxy-7-methoxycoumarin — and ten psoralens — bergamottin, 8-geranyloxypsoralen, byakangelicol, oxypeucedanin, isoimperatorin, imperatorin, phellopterin, 5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen, oxypeucedanin hydrate and byakangelicin — were identified in lemon oil. In addition, two unknown psoralens were detected.

Quantitative results (Table 1) were obtained for bergamottin, 5-geranyloxy-7-methoxycoumarin, citropten, 8-geranyloxypsoralen, 5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen, oxypeucedanin and byakangelicol. Quantitative data are not reported for the minor components, because a sufficient amount at the required degree of purity could not be isolated to prepare a calibration curve and calculate the correction factor. Bergamottin (160–291 mg/100 g of oil) and

Table 1. Content (mg/100 g of oil) of coumarins in lemon essential oils

1	Unknown psoralen	+
2	Unknown psoralen	+
3	Bergamottin	160–291
4	Isoimperatorin	+
5	5-Geranyloxy-7-methoxycoumarin	180–250
6	5-Isopentenyloxy-7-methoxycoumarin	+
7	Citropten	52–142
8	8-Geranyloxypsoralen	19–36
9	Imperatorin*	+
10	Phellopterin**	+
11	5-Isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen	19–37
12	Oxypeucedanin	89–157
13	Byakangelicol	66–123
14	Oxypeucedanin hydrate	+
15	Byakangelicin	+

* Tentative. ** Tentative, identified according to McHale and Sheridan.[7]
+ = Present.

5-geranyloxy-7-methoxycoumarin (180–250 mg/100 g of oil) were the principal components of the fraction, followed by oxypeucedanin (89–157 mg/100 g of oil), byakangelicol (66–123 mg/100 g of oil) and citropten (52–142 mg/100 g of oil).

The quantitative data relative to all the samples analysed were grouped according to the month of production. All the dosed compounds showed the same behaviour: a slight but not meaningful decrease during the productive season.

Our qualitative results are in agreement with those of McHale and Sheridan.[7] Ziegler and Spiteller[3] detected 29 compounds in the coumarin fraction of a cold-pressed Sicilian lemon oil. Many of them were trace constituents. Eleven of these compounds were previously unknown as constituents of lemon oil, and their presence has not so far been confirmed by other authors. The authors did not find either bergapten (5-methoxypsoralen) or herniarin (7-methoxycoumarin) in the Sicilian lemon oil. They found aurapten (7-geranyloxycoumarin), a characteristic compound of grapefruit oil, as a trace constituent of lemon oil. Under our conditions, aurapten shows the same retention time as 5-isopentenyloxy-7-methoxycoumarin, but even pre-fractionating lemon oil on a silica gel column, aurapten was not detected. This result is in agreement with McHale and Sheridan,[7] who found aurapten in commercial lemon oils, where grapefruit oil was probably added to enhance the ultraviolet absorbance of the lemon oil, but not in genuine lemon oils. Glandian *et al.*[11] identified bergapten in a sample of cold-pressed lemon essential oil from the Ivory Coast (*Citrus limon* L. var. Eureka). Chouchi and Barth[12] identified, in addition to citropten, phellopterin and oxypeucedanin, bergapten, scoparone (6,7-dimethoxycoumarin) and herniarin in a sample of lemon essential oil from the Ivory Coast (var. Eureka). No-one else has reported the presence of herniarin in lemon oil.

Under our HPLC conditions, herniarin, a characteristic compound of lime oil, co-eluted with 8-geranyloxypsoralen, which eluted immediately after citropten. During pre-fractionation of cold-pressed lemon oil by silica gel column chromatography, using a mixture of light petroleum : ethyl acetate as eluent, 8-geranyloxypsoralen elutes earlier than citropten, while herniarin elutes after citropten. In one of the fractions so obtained it was possible to detect herniarin.

By using the 8-geranyloxypsoralen/herniarin separation procedure described above, we were able to confirm the presence of small amounts of herniarin in all the samples analysed. Figure 2 shows the HPLC chromatogram obtained under these conditions. Further confirmation of the presence of herniarin in lemon oil was obtained by analysing two samples of lemon oil extracted in the laboratory; one sample was extracted from lemons 'verdelli' and the other from winter lemons. The fruits were selected from batches destined for industrial processing. Figures 3 and 4 show the chromatograms of the two laboratory extracted oils. As can be seen from these figures, herniarin is clearly present in both oils, and the oil extracted from 'verdelli' shows a higher concentration of herniarin than the oil extracted from winter lemons. The presence of herniarin has also been confirmed by gas chromatography–mass spectrometry.

Table 2 reports the comparison of our quantitative results with those of the literature. There is good overall agreement, except that we found a smaller amount of 8-geranyloxypsoralen and a higher amount of byakangelicol than McHale and Sheridan[7] and Glandian.[11]

The analysis of some samples of lemon oil produced two or three years ago, showed the total absence or a very low content of oxypeucedanin and byakangelicol. As many authors have pointed out,[7,13,14] epoxy-

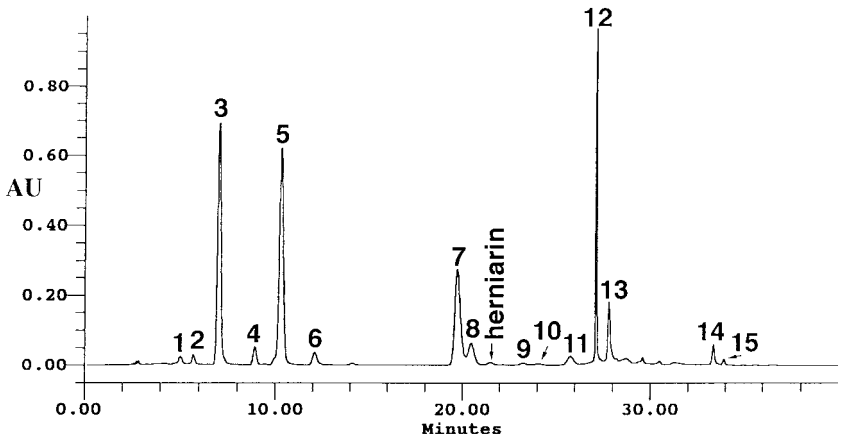

Figure 2. HPLC chromatogram of an Italian genuine lemon oil obtained under conditions that allow separation of 8-geranyloxypsoralen and herniarin. For identification of other components, see Table 1

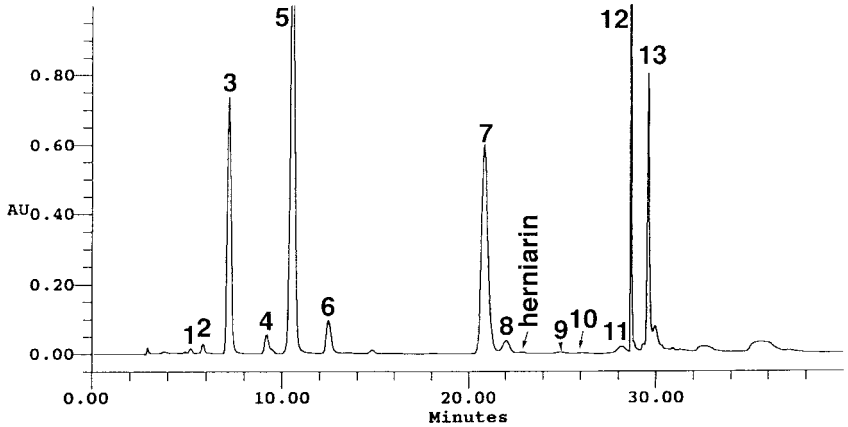

Figure 3. HPLC chromatogram of a laboratory extracted lemon oil from winter lemons obtained under the same conditions used for Figure 2. For identification of other components, see Table 1

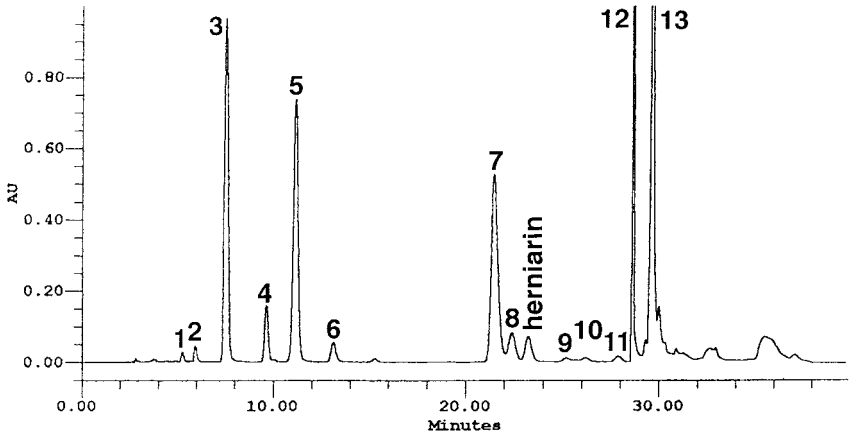

Figure 4. HPLC chromatogram of a laboratory extracted lemon oil from 'verdelli' lemons obtained under the same conditions used for Figure 2. For identification of other components, see Table 1

Table 2. Comparison of our quantitative results with those of the literature

	Our results (mg/100 g)	McHale and Sheridan[7] (mg/100 ml)	Glandian *et al.*[11] (mg/100 g)	Calabrò and Currò[5] (mg/100 g)
Bergamottin	160–291	220	200	270
5-Geranyloxy-7-methoxycoumarin	180–250	160	170	200
Isoimperatorin	+	18	–	–
5-Isopentenyloxy-7-methoxycoumarin	+	8	6	–
Phellopterin	+	9	–	–
Imperatorin	+	6	–	–
5-Isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy)-psoralen	19–37	22	–	–
Citropten	52–142	65	180	120
8-Geranyloxypsoralen	19–36	75	100	–
Bergapten	–	–	10	–
Oxypeucedanin	89–157	110	100	–
Byakangelicol	66–123	45	50	–
Oxypeucedanin hydrate	+	26	30	–
Byakangelicin	+	7	10	–

+ = present.

psoralens can hydrolyse to their corresponding diols. The relatively low concentrations of these diols (oxypeucedanin hydrate and byakangelicin) found in some old lemon oils were probably due to their poor solubility in the oils.

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