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Determination of Oxygen Heterocyclic Components in Citrus Products by HPLC with UV Detection

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The study of oxygen heterocyclic compounds (coumarins, psoralens, polymethoxylated flavones) in natural matrices such as citrus oils is not easy due to the difficulty of obtaining standards at the required level of purity and the diversity of structures present in each kind of sample. In this work, standards were either isolated by preparative LC methods from citrus oils or synthesized, then characterized by their physicochemical parameters and spectroscopic techniques, and further used for qualitative and quantitative calculations in citrus essential oils and products made with them (Earl Grey tea, liquors, juices). An HPLC method using an innovative partially porous particle HPLC column enabled baseline separation of all analytes. The method developed was validated in terms of detection limit, quantitation limit, linearity, and precision as repeatability and intralaboratory reproducibility.

KEYWORDS: HPLC-DAD; furocoumarins; coumarins; polymethoxylated flavones; citrus products

INTRODUCTION

Coumarins form a vast class of natural products widely occurring as secondary plant metabolites (*1*). More than 1800 different natural coumarins have been discovered and described to date, many of them exhibiting high levels of biological activity (*2*−*6*). Coumarins are also used as food additives, in cosmetics (*7*), as optical brightening agents (*8*), and as dispersed fluorescent and laser dyes (*9*). In addition, some coumarins are of concern because of their toxicity (*10*), anticarcinogenicity (*11*), and photodynamic effects (*12*).

Coumarins also act as intermediates for the synthesis of their furan derivatives (*13*). Furocoumarins are toxic secondary metabolites that occur in various plant species (*14*). At present, a high number of furocoumarins (*14*) are known and, considering their chemical structure, two main subgroups can be recognized: the first involves linear furocoumarins (e.g., psoralen, bergapten, bergamottin), whereas the second is represented by angular furocoumarins (e.g., angelicin, pimpinellin, isobergapten). Furocoumarins are contained in the nonvolatile fraction of cold-pressed *Citrus* oils together with other oxygen heterocyclic components (coumarins and polymethoxylated flavones) (*15*); the residue also contains carotenoids, waxes, flavonoids, fatty acids, and sterols. Citrus oils represent the biggest potential contribution to furocoumarin content in fragrance products (*16*).

Currently available data indicate that naturally occurring furocoumarins possess diverse activities in terms of inducing various xenobiotic metabolizing enzymes based on their chemical structure (*17*, *18*). Although all of these coumarins, furocoumarins, and polymethoxylated flavones occur in plants, many are not available in sufficient quantity and purity for confident biological investigations. Moreover, the lack of some oxygen heterocyclic standards limits the correct estimation of benefit/risk factor in relation to the exposure of these compounds in foodstuffs and beverages.

Because of these considerations, the development of a versatile, simple, rapid, and economic method to isolate, identify, and quantify oxygen heterocyclic compounds in foodstuffs and beverages is urgently required. A fast and efficient RP-HPLC method for the analysis of oxygen heterocyclic components in citrus essential oils (lemon, bergamot, lime, bitter orange, grapefruit, mandarin) and products made with them (juices, teas, and liquors) is here illustrated. The use of a C18 HPLC column with partially porous particles of 2.7 μm and of a ternary mobile phase containing tetrahydrofuran (THF) allowed the development of a method that permits the separation of all of the sample components, preventing coelution phenomena.

With the purpose of creating an efficient library of coumarin and furocoumarin standards, useful for peak identification and quantification, a method based on the medium-pressure liquid chromatography (MPLC)/RP-HPLC separation of oxygen heterocyclic components from citrus oil residue has been developed. Crude lemon essential oil residue has been selected as a source of coumarins and furocoumarins not available in the laboratory. All of the isolated compounds have been identified by NMR spectroscopy, and the most relevant data are reported under Materials and Methods.

MATERIALS AND METHODS

Samples. This research was carried out on six genuine cold-pressed essential oils (lemon, lime, bergamot, grapefruit, mandarin, and bitter orange), two laboratory-made juices (lemon and bergamot), a homemade

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limoncello, two commercial "bergamino" liquors, and a commercial Earl Grey tea. The label of the Earl Grey tea declared it a fine black tea scented with a citrus bergamot flavor (4.3%). For its analysis, one filter bag were weighed and steeped in 100 mL of hot water and then left to brew for 5 min.

Citrus essential oils were analyzed without any pretreatment: ca. 20 mg of oil was accurately weighed and diluted in 1 mL of ethanol. Before HPLC analysis, 50 μL of internal standard (I.S.) coumarin (56.8 mg in 50 mL of ethanol) was added.

Juice, liquor, and tea samples have been subjected to solvent extraction prior to HPLC analysis. The extraction procedure was carried out on 10 mL of each sample, with 50 μL of psoralen (10.1 mg in 10 mL of ethanol) added as internal standard, and extracted with three aliquots of 10 mL each of ethyl acetate. The extracts were gathered, dried with anhydrous sodium sulfate, filtered on filter paper, and brought to dryness in a rotary vacuum evaporator; the extract thus obtained was dissolved in 1 mL of ethanol, and 50 μL of coumarin (56.8 mg in 50 mL of ethanol) was added prior to HPLC analysis.

Reagents and Materials. THF was purchased from Carlo Erba Reagenti (Milan, Italy), and water, acetonitrile, methanol, *n*-hexane, ethyl acetate, ethanol, dichloromethane, cyclohexane, and 3-cloroperbenzoic acid were purchased from Sigma-Aldrich (Milan, Italy). All solvents were of HPLC grade. Anydrous sodium sulfate and sodium carbonate were purchased from Fluka (Milan, Italy). Psoralens and coumarins (citropten, byakangelicol, oxypeucedanin, bergamottin, cnidicin, 8-geranyloxypsoralen, 5-geranyloxy-7-methoxycoumarin, isoimperatorin, 5-isopentenyloxy-7-methoxycoumarin, 5-(isopent-2′-enyloxy)-8-(2′,3′-epoxy)isopentenyloxypsoralen) were obtained by MPLC and preparative HPLC separation from lemon oil residue; epoxybergamottin was synthesized starting from bergamottin; meranzin, isomeranzin, isopimpinellin, tangeretin, nobiletin, heptamethoxyflavone, imperatorin, phellopterin, epoxyaurapten, osthol, aurapten, and 5-geranyloxy-8-methoxypsoralen were previously isolated in our laboratory (*19*−*21*). The other components were purchased as standards from Fluka (Milan, Italy).

MPLC/Preparative RP-HPLC Separation. Three grams of nonvolatile residue obtained by evaporating lemon essential oil on a water bath for 6 h have been subjected to MPLC on a silica gel column (5 g) using, as eluent, a mixture of cyclohexane/ethyl acetate (from 9:1 to 5:5), and the eluted fractions have been detected by their UV absorbance at 254 nm. Flow rate was 12 mL/min; the fractions were monitored by thin layer chromatography (TLC), (hexane/ethyl acetate 5:5) and then collected into seven main groups (**A**−**G**), which were identified by ^{1}H NMR spectroscopy. Each fraction was separated by preparative RP-HPLC using an XTerra MS C_{18} column (5 μm, 150 × 19 mm i.d.) (Waters SpA, Vimodrone, Milan, Italy) with a linear gradient of water/acetonitrile as mobile phase. The fractions collected in correspondence with each peak were evaporated on an Genevac EZ-2 evaporator to obtain compounds citropten (50 mg), byakangelicol (30 mg), oxypeucedanin (70 mg), bergamottin (70 mg), cnidicin (4 mg), 8-geranyloxypsoralen (24 mg), 5-geranyloxy-7-methoxycoumarin (61 mg), isoimperatorin, 5-isopentenyloxy-7-methoxycoumarin (8 mg), and 5-(isopent-2′-enyloxy)-8-(2′,3′-epoxy)isopentenyloxypsoralen (40 mg), the structures of which were confirmed by ^{1}H and ^{13}C NMR data.

Synthesis of Epoxybergamottin (19). 3-Chloroperbenzoic acid (360 mg, 2 mmol) was added to a solution of bergamottin (**26**) (500 mg, 1.5 mmol) in dichloromethane (10 mL) at −10 °C in an ice−methanol bath, and the solution was stirred for 3 h. The solution was washed with aqueous sodium carbonate, and the organic layer was dried on sodium sulfate and evaporated under reduced pressure. The residue was subjected to column chromatography using ethyl acetate/cyclohexane 4:1 as eluent. The removal of solvent under reduced pressure furnished a translucent oil, which was recrystallized from *n*-hexane to afford colorless crystals in 81% yield.

4-[(*E*)-3,7-Dimethylocta-2,6-dienyloxy]-7*H*-furo[3,2-*g*]chromen-7-one (Bergamottin, 26): white solid from hexane; mp 55−56 °C (lit. mp 55−56 °C (*22*, *23*)); ^{1}H NMR ($CDCl_3$) δ 1.58 (s, 3H), 1.66 (s, 3H), 1.67 (s, 3H), 2.08 (m, 4H), 4.93 (d, 2H, J = 6.8 Hz), 5.10 (m, 1H), 5.54 (t, 1H, J = 6.8 Hz), 6.25 (d, 1H, J = 9.8 Hz), 6.94 (d, 1H, J = 2.5 Hz), 7.13 (s, 1H), 7.57 (d, 1H, J = 2.5 Hz), 8.14 (d, 1H, J = 9.8 Hz); ^{13}C NMR ($CDCl_3$) δ 17.7, 18.7, 26.7, 27.3, 40.6, 70.8, 95.3, 106.8, 108.6, 113.6, 115.2, 119.9, 124.5, 133.0, 140.7, 144.1, 145.9, 150.0, 153.7, 159.2, 162.3.

5-[(*E*)-3,7-Dimethylocta-2,6-dienyloxy)]-7-methoxy-2*H*-chromen-2-one (5-geranyloxy-7-OMe-coumarin, 27): white solid from hexane; mp 86−87 °C (lit. mp 86−87 °C (*24*)); ^{1}H NMR ($CDCl_3$) δ 161 (s, 3H), 1.70 (s, 3H), 1.75 (s, 3H), 2.15 (m, 4H), 3.90 (s, 3H), 4.62 (d, 2H, J = 6.9 Hz), 5.10 (m, 1H), 5.55 (t, 1H, J = 6.9 Hz), 6.18 (d, 1H, J = 9.8 Hz), 6.29 (d, 1H, J = 0.8), 6.42 (d, 1H, J = 0.8), 8.05 (d, 1H, J = 9.8); ^{13}C NMR ($CDCl_3$) δ 16.7, 17.7, 25.6, 26.2, 39.5, 55.7, 65.7, 92.4, 95.7, 104.2, 110.7, 118.5, 123.5, 131.9, 139.0, 142.05, 156.2, 156.3, 161.6, 163.5.

4,9-Bis(3-methylbut-2-enyloxy)-7*H*-furo[3,2-*g*]chromen-7-one (cnidicin, 22): light yellow solid from hexane; mp 53−57 °C (lit. mp 76 °C (*25*)); ^{1}H NMR ($CDCl_3$) δ 1.65 (s, 3H), 1.70 (s, 3H), 1.81 (s, 3H), 1.85 (s, 3H), 4.85 (d, 2H, J = 6.8 Hz), 5.92 (d, 2H, J = 6.8 Hz), 5.55 (t, 1H, J = 6.8 Hz), 5.65 (t, 1H, J = 6.8 Hz), 6.32 (d, 1H, J = 9.8 Hz), 6.99 (d, 1H, J = 2.9 Hz), 7.65 (d, 1H, J = 2.9), 8.18 (d, 1H, J = 9.8 Hz); ^{13}C NMR ($CDCl_3$) δ 18.1, 25.8, 70.3, 70.4, 105.1, 111.1, 111.4, 112.8, 119.3, 119.5, 139.0, 139.7, 140.5, 141.3, 145.2, 166.5.

5-(3-Methylbut-2-enyloxy)-7-methoxy-2*H*-chromen-2-one (5-isopentenyloxy-7-methoxycoumarin, 20): colorless needles from hexane; mp 88−91 °C (lit. mp 88−91 °C (*26*)); ^{1}H NMR ($CDCl_3$) δ 1.76 (s, 3H), 1.82 (s, 3H), 3.95 (s, 3H), 4.60 (d, 1H, J = 6.7 Hz), 5.50 (t, 1H, J = 6.7 Hz), 6.14 (d, 1H, J = 9.8 Hz), 6.29 (d, 1H, J = 2.0 Hz), 6.40 (d, 1H, J = 2.0 Hz), 8.0 (d, 1H, J = 9.8 Hz); ^{13}C NMR ($CDCl_3$) δ 25.7, 29.7, 55.8, 69.8, 97.1, 103.8, 110.7, 111.7, 113.5, 119.1, 139.6, 144.9, 156.6, 156.8, 161.4

9-[(*E*)-3,7-Dimethylocta-2,6-dienyloxy]-7*H*-furo[3,2-*g*]chromen-7-one (8-geranyloxy-psoralen, 23): colorless crystals from hexane; mp 53−54 °C (lit. mp 53−54 °C (*18*)); ^{1}H NMR ($CDCl_3$) δ 1.57 (s, 3H), 1.64 (s, 3H), 1.70 (s, 3H), 2.01 (m, 4H), 5.0 (t, 1H, J = 3.0 Hz), 5.03 (d, 2H, J = 7.2 Hz), 5.60 (t, 1H, J = 7.2 Hz), 6.38 (d, 1H, J = 9.6 Hz), 6.82 (d, 1H, J = 2.2 Hz), 7.37 (s, 1H), 7.69 (d, 1H, J = 2.2 Hz), 7.68 (d, 1H, J = 9.6 Hz); ^{13}C NMR ($CDCl_3$) δ 14.9, 16.0, 24.0, 24.7, 37.9, 68.4, 105.1, 111.6, 113.0, 114.8, 117.8, 122.1, 124.2, 129.9, 130.1, 141.5, 142.3, 142.7, 145.0, 147.1, 148.9.

4-(3-Methylbut-2-enyloxy)-7*H*-furo[3,2-*g*]chromen-7-one (isoimperatorin, 13): light yellow solid from hexane; mp 108−109 °C (lit. mp 108−109 °C (*23*)); ^{1}H NMR ($CDCl_3$) δ 173 (s, 3H), 1.81 (s, 3H), 4.92 (d, 2H, J = 6.5 Hz), 5.54 (t, 1H, J = 6.5 Hz), 6.30 (d, 1H, J = 9.8 Hz), 6.97 (d, 1H, J = 2.4 Hz), 7.19 (s, 1H), 7.60 (d, 1H, J = 2.4 Hz), 8.20 (d, 1H, J = 9.9 Hz); ^{13}C NMR ($CDCl_3$) δ 25.7, 29.7, 69.8, 94.2, 105.0, 108.2, 112.5, 114.2, 119.1, 139.6, 139.8, 144.9, 152.2, 152.7, 158.1, 161.3.

5,7-Dimethoxy-2*H*-chromen-2-one (citropten, 4): white crystals from *n*-hexane; mp 143−144 °C; for ^{1}H NMR and ^{13}C NMR data, see ref *27*.

9-[(3,3-Dimethyloxiran-2-yl)methoxy]-4-(3-methylbut-2-enyloxy)-7*H*-furo[3,2-*g*]chromen-7-one (5-(isopent-2′-enyloxy)-8-(2′,3′-epoxy) isopentenyloxy-psoralen, 21): lit. (*28*); ^{1}H NMR ($CDCl_3$) δ 1.26 (s, 3H), 1.34 (s, 3H), 1.62 (s, 3H), 1.80 (s, 3H), 3.38 (t, 1H, J = 5.5 Hz), 4.46 (d, 2H, J = 5.5 Hz), 4.82 (d, 2H, J = 6.5 Hz), 5.52 (t, 1H, J = 6.5 Hz), 6.30 (d, 1H, J = 9.8 Hz), 6.95 (d, 1H, J = 2.5 Hz), 7.65 (d, 1H, J = 2.5 Hz), 8.14 (d, 1H, J = 9.8 Hz); ^{13}C NMR ($CDCl_3$) δ 18.8, 18.2, 24.6, 25.8, 61.3, 70.4, 72.7, 105.2, 108.6, 112.9, 116.2, 119.2, 139.7, 144.0, 145.2, 145.3, 150.2, 161.0.

4-[(3,3-Dimethyloxiran-2-yl)methoxy]-7*H*-furo[3,2-*g*]chromen-7-one (oxypeucedanin, 11): pale yellow crystal from *n*-hexane; mp 141−142 °C (lit. mp 141−142 °C (*23*)); ^{1}H NMR ($CDCl_3$) δ 1.35 (s, 3H), 1.43 (s, 3H), 3.26 (dd, 1H, J = 4.4 and 6.5 Hz), 4.46 (dd, 1H, J = 6.5 and 10.8 Hz), 4.65 (dd, 1H, J = 4.4 and 10.8 Hz), 6.36 (d, 1H, J = 9.8 Hz), 6.97 (d, 1H, J = 2.3 Hz), 7.23 (s, 1H), 7.64 (d, 1H, J = 2.3 Hz), 8.23 (d, 1H, J = 9.8 Hz); ^{13}C NMR ($CDCl_3$) δ 19.0, 24.5, 58.3, 72.3, 94.9, 104.5, 107.5, 113.15, 113.4, 139.0, 145.3, 152.0, 158.1.

9-[(3,3-Dimethyloxiran-2-yl)methoxy]-4-methoxy-7*H*-furo[3,2-*g*]-chromen-7-one (byakangelicol, 9): light yellow crystals from *n*-hexane; mp 53−60° (lit. mp 105 °C (*25*)); ^{1}H NMR ($CDCl_3$) δ 1.30 (s, 3H), 1.40 (s, 3H), 3.35 (t, 1H, J = 6.8 Hz), 4.21 (s, 3H), 4.51 (d, 2H, J = 6.8 Hz), 6.35 (d, 1H, J = 9.8 Hz), 7.05 (d, 1H, J = 2.2 Hz), 7.65 (d, 1H, J = 2.2 Hz), 8.20 (d, 1H, J = 9.8 Hz); ^{13}C NMR ($CDCl_3$) δ 18.7, 24.5, 55.8, 60.7, 61.3, 72.7, 105.2, 110.9, 111.1, 112.9, 127.3, 131.4, 139.4, 142.8, 143.6, 143.8, 145.2, 166.5.

4-[(*E*)-3-Methyl-5-(3,3-dimethyloxiran-2-yl)pent-2-enyloxy]-7*H*-furo-[3,2-*g*]chromen-7-one (epoxybergamottin, 19): colorless crystals from

n-hexane; mp 69−70 °C (lit. mp 69−70 °C (*23*)); ^{1}H NMR ($CDCl_3$) δ 128 (s, 3H), 1.70 (m, 2H), 1.72 (m, 2H), 2.70 (dd, 1H, J = 5.5 and 5.6 Hz), 4.95 (d, 2H, J = 6.7 Hz), 5.61 (t, 1H, J = 6.7 Hz), 6.28 (d, 1H, J = 9.8 Hz), 6.96 (d, 1H, J = 2.3 Hz), 7.17 (s, 1H), 7.61 (d, 1H, J = 2.3 Hz), 8.17 (d, 1H, J = 9.8 Hz); ^{13}C NMR ($CDCl_3$) δ 16.9, 19.0, 25.0, 27.4, 36.6, 59.5, 64.0, 69.9, 94.6, 105.3, 111.5, 111.6, 113.0, 119.8, 139.7, 142.4, 145.2, 151.2, 152.5, 156.9, 159.5.

HPLC Analyses. HPLC separation was carried out on a Shimadzu system equipped with two LC 10 AD *Vp* pumps, an SPD-M10 A*vp* UV detector, an SCL-10-A*vp* controller, a CTO-20AC column oven thermostated at 30 °C, and a DGU-14A degasser. The column used was an Ascentis Express C18, 150 × 4.6 mm i.d., with particle size of 2.7 μm (Supelco, Bellefonte, PA). The injection volume was 2 μL; mobile phase consisted of water/acetonitrile/THF (85:10:5) (solvent A) and acetonitrile/methanol/THF (65:30:5) (solvent B). The linear gradient profile was as follows: 0−5 min, 0% B; 5−25 min, 0−40% B; 25−45 min, 40−90% B; 45−55 min, 90% B; 55−60 min, 0% B. Flow rate was 1.0 mL/min. Data were acquired using a photodiode array detector in the range pf 190−370 nm, and the chromatograms were extracted at 315 nm. Time constant was 0.64 s and sample frequency, 1.5625 Hz. Data acquisition was performed by Shimadzu LCsolution software ver 3.3.

Method Validation. The validation process of the RP-HPLC method was carried out following EURACHEM guidelines (*29*).

The detection limit (LOD, y_D) and the quantitation limit (LOQ, y_Q) were expressed for each analyte as signals based on the mean value (y_b) and the standard deviation (s_b) of the blank signal as

$$y_D = \bar{y}_b + 2ts_b \quad y_Q = \bar{y}_b + 10s_b$$

where t is a constant of the t-Student distribution (one-tailed) dependent on the confidence level and degrees of freedom (df). A 95% confidence level was chosen. For y_b and s_b determination, nine blank measurements were performed by injection of 2 μL of sweet orange essential oil (20 mg roughly of oil was accurately weighed and diluted in 1 mL of ethanol).

The concentration values of the detection limit (LOD) and quantitation limit (LOQ) were obtained by projection of the corresponding signals y_D and y_Q through the calibration plot $y = f(x)$ onto the concentration axis.

The instrumental intraday repeatability and the recovery were calculated on six replicated injections at one concentration level for four analytes (citropten, bergapten, bergamottin, and 5-geranyloxy-7-methoxycoumarin: 1 mg/L). The intralaboratory reproducibility was calculated by performing six replicated injections of the same solution used for intraday repeatability into two different HPLC instruments run under identical experimental conditions. The interday repeatability was calculated as repeatability limit (r) on two replicates (x_1 and x_2) of a sample analogous to that used for intraday precision. Interday repeatability was then calculated using the formula

$$|X_1 - X_2| \le r \quad r = \sqrt{2} \times t \times s_r$$

where s_r is the standard deviation measured under repeatability conditions.

Linearity of the method was established over 0.1−100 mg/L by performing three HPLC replicate runs for each concentration level (five equispaced concentration levels).

Quantitative Calculation. To quantify the content of oxygen heterocyclic fraction present in citrus oils and other products, calibration curves were constructed by using each single standard previously isolated or synthesized, if not commercially available. Five different concentrations of each component, in the 100−0.1 mg/L concentration range, were prepared by diluting a stock solution of about 1000 mg/L, using ethanol as a solvent, and analyzed in triplicate by HPLC under the same chromatographic conditions optimized for the oil samples. Before injection, 50 μL of internal standard (I.S.) coumarin (56.8 mg/ 50 mL) was added to 1 mL of each standard solution. Correction factors were then calculated using the following formula:

$$CF = \frac{[\text{analyte}] \times \text{I.S.} \times \text{area}}{[\text{I.S.}] \times \text{analytearea}}$$

At the same time, from HPLC analysis of each component, UV spectra of each standard were extracted and included in a homemade UV spectra library useful to identify oxygen heterocyclic components present in real samples.

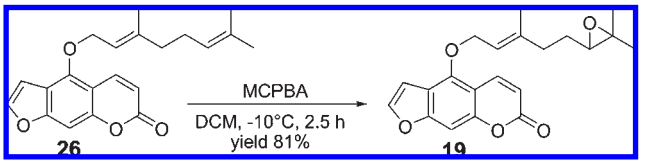

Figure 1. Scheme of the synthesis of epoxybergamottin.

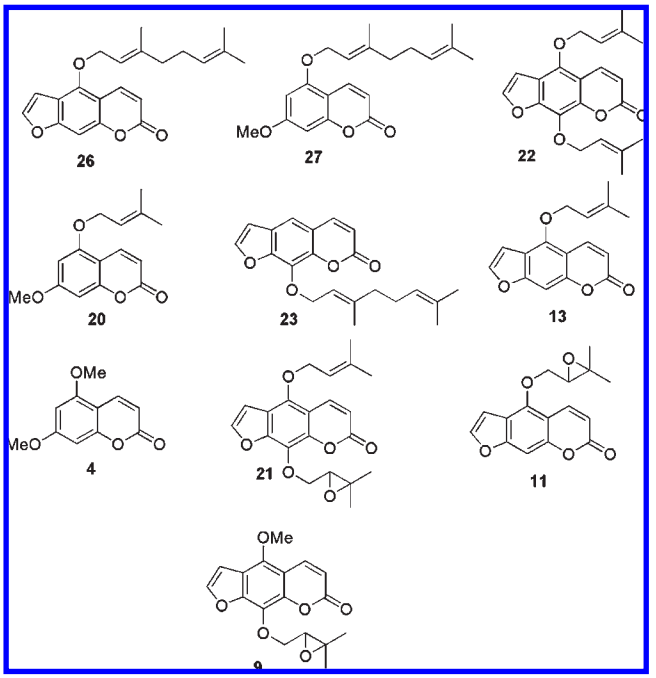

Figure 2. Structures of components isolated from the residue of lemon oil by MPLC.

RESULTS AND DISCUSSION

Preparative Separation. The residue of cold-pressed lemon essential oil was chosen as the source of standards because it contains in satisfactory amounts a complete series of coumarins and furocoumarins of our interest, not commercially available. Seven main fractions (**A**−**G**) were collected and identified by ^{1}H NMR spectroscopy.

The ^{1}H NMR spectrum of the first eluted fraction, **A**, shows the presence of the furocoumarin bergamottin as the main compound and 5-geranyloxy-7-methoxycoumarin as a minor product. As described under Materials and Methods, preparative HPLC were used to obtain pure bergamottin (**26**) and 5-geranyloxy-7-methoxycoumarin (**27**), the structures of which were confirmed by ^{1}H and ^{13}C NMR and UV data. All other fractions **B**−**G** were separated and identified following the above-described procedure. The content of each fraction and the relative amounts are reported under Materials and Methods (MPLC/preparative RP-HPLC separation).

Epoxybergamottin (**19**), not present in the lemon residue oil, was synthesized starting from bergamottin by reaction with *m*-chloroperbenzoic acid in dichloromethane at −10 °C. Under these conditions, reaction occurs with a high level of chemoselectivity, because epoxydation takes place only at the terminal alkene (**Figure 1**). All of the standards obtained from the lemon oil residue are reported in **Figure 2**.

Other oxygen heterocyclic components not present in lemon oil residue and not commercially available were isolated from

Table 1. Correction Factors and Limits of Detection (LOD) and Quantification (LOQ) of Oxygen Heterocyclic Compounds

trivial name	systematic name	CF	LOD (mg/L)	LOQ (mg/L)	source
coumarins					
herniarin (**1**)	7-methoxy-2*H*-chromen-2-one	0.48	0.014	0.019	Sigma-Aldrich
citropten (**4**)	5,7-dimethoxy-2*H*-chromen-2-one	0.68	0.021	0.029	lemon oil residue
5-geranyloxy-7-methoxycoumarin (**27**)	5-[(*E*)-3,7-dimethylocta-2,6-dienyloxy)]-7-methoxy-2*H*-chromen-2-one	1.15	0.036	0.050	lemon oil residue
5-isopentenyloxy-7-methoxycoumarin (**20**)	5-(3-methylbut-2-enyloxy)-7-methoxy-2*H*-chromen-2-one				lemon oil residue
aurapten (**24**)	7-((*E*)-3,7-dimethylocta-2,6-dienyloxy)-2*H*-chromen-2-one	0.82	0.025	0.034	grape oil
epoxyaurapten (**16**)	7-((*E*)-3-methyl-5-(3,3-dimethyloxiran-2-yl)pent-2-enyloxy)-2*H*-chromen-2-one				grape oil
osthol (**18**)	7-methoxy-8-(3-methylbut-2-enyl)-2*H*-chromen-2-one				bitter orange oil
meranzin (**5**)	7-methoxy-8-((3,3-dimethyloxiran-2-yl)methyl)-2*H*-chromen-2-one				bitter orange oil
isomeranzin (**8**)	7-methoxy-8-(3-methyl-2-oxobutyl)-2*H*-chromen-2-one				bitter orange oil
psoralens					
oxypeucedanin (**11**)	4-[(3,3-dimethyloxiran-2-yl)methoxy]-7*H*-furo[3,2-*g*]chromen-7-one	1.51	0.040	0.056	lemon oil residue
oxypeucedanin hydrate (**2**)	4- (2,3-dihydroxy-3-methylbutoxy)-7*H*-furo[3,2-*g*]- chromen-7-one	1.51[a]	0.040[a]	0.056[a]	lemon oil residue
byakangelicol (**9**)	9-[(3,3-dimethyloxiran-2-yl)methoxy]-4-methoxy-7*H*-furo[3,2-*g*] chromen-7-one	1.51	0.045	0.062	lemon oil residue
byakangelicin (**3**)	9-(2,3-dihydroxy-3-methylbutoxy)-4-methoxy-7*H*-furo[3,2-*g*]-chromen-7-one	1.51[a]	0.045[a]	0.062[a]	lemon oil residue
bergamottin (**26**)	4-[(*E*)-3,7-dimethylocta-2,6-dienyloxy]-7*H*-furo[3,2-*g*]chromen-7-one	1.39	0.039	0.054	lemon oil residue
epoxybergamottin (**19**)	4-[(*E*)-3-methyl-5-(3,3-dimethyloxiran-2-yl)pent-2-enyloxy]-7*H*-furo[3,2-*g*]chromen-7-one	1.03	0.032	0.044	synthesis
xanthotoxin	9-methoxy-7*H*-furo[3,2-*g*]chromen-7-one	0.88	0.028	0.039	Sigma-Aldrich
psoralen	7*H*-furo[3,2-*g*]chromen-7-one	0.96	0.028	0.038	Sigma-Aldrich
bergapten (**7**)	4-methoxy-7*H*-furo[3,2-*g*]chromen-7-one	0.65	0.018	0.025	Sigma-Aldrich
isopimpinellin (**6**)	4,9-dimethoxy-7*H*-furo[3,2-*g*]chromen-7-one	0.85	0.026	0.037	lime oil
8-geranyloxypsoralen (**23**)	9-[(*E*)-3,7-dimethylocta-2,6-dienyloxy]-7*H*-furo[3,2-*g*]chromen-7-one	1.99	0.056	0.078	lemon oil residue
imperatorin (**15**)	9-(3-methylbut-2-enyloxy)- 7*H*-furo[3,2-*g*]chromen-7-one	1.27	0.039	0.053	Extrasynthese
isoimperatorin (**13**)	4-(3-methylbut-2-enyloxy)-7*H*-furo[3,2-*g*]chromen-7-one	0.94	0.029	0.040	lemon oil residue
cnidicin (**22**)	4,9-bis(3-methylbut-2-enyloxy)-7*H*-furo[3,2-*g*]chromen-7-one	1.23	0.040	0.055	lemon oil residue
5-(isopent-2′-enyloxy)-8-(2′,3′-epoxy) isopentenyloxypsoralen (**21**)	9-[(3,3-dimethyloxiran-2-yl)methoxy]-4-(3-methylbut-2-enyloxy)-7*H*-furo[3,2-*g*]chromen-7-one				lemon oil residue
polymethoxyflavones					
nobiletin (**10**)	5,6,7,8-tetramethoxy-2-(3,4-dimethoxyphenyl)-4*H*-chromen-4-one				mandarin oil
tangeretin (**14**)	5,6,7,8-tetramethoxy-2-(4-methoxyphenyl)-4*H*-chromen-4-one				sweet orange oil
heptamethoxyflavone (**12**)	3,5,6,7,8-pentamethoxy-2-(3,4-dimethoxyphenyl)-4*H*-chromen-4-one				sweet orange oil

[a]CF, LOD, and LOQ of oxypeucedanin and byakangelicol.

different citrus oils in previous research (*19*−*21*). **Table 1** summarizes the oxygen heterocyclic components used in this work. For those available in sufficient amount and purity for quantitative calculation, values of correction factor (CF), limit of detection (LOD), and limit of quantification (LOQ) are also reported in this table (structures of compounds **1**, **5**−**8**, **10**, **12**, **14**, **16**, **18**, and **24** can be found in the Supporting Information).

HPLC Method Optimization. Due to their nonvolatile nature, oxygen heterocyclic component in citrus oils and in other natural matrices (*15*, *16*, *19*−*21*) have been traditionally analyzed by means of liquid chromatography. Both normal phase and reversed phase HPLC modes have been used, in combination with UV, fluorescence, and MS detection.

Because of the structural diversity of oxygen heterocyclic components, HPLC analysis is not easy, due to the frequent coelutions between sample components. Moreover, each natural matrix shows a characteristic qualitative and quantitative composition in oxygen heterocyclic components, and a general HPLC method is therefore difficult to optimize, although many different stationary phases with specific selectivity are today available for HPLC analysis.

Recently, it has been demonstrated that the use of a ternary mixture of water, THF, and methanol under gradient conditions permits one to obtain good resolution of coumarins and furocoumarins (*16*, *30*) on C18 columns. On the basis of the method optimization carried out by Frérot et al. (*16*), we used the same mobile phase mixtures to optimize a gradient program able to provide baseline resolution of critical pairs of psoralens, such as bergapten−isopimpinellin (both present in lime oil) or oxypeucedanin−byakangelicol (both present in lemon oil), and also coumarins from psoralens.

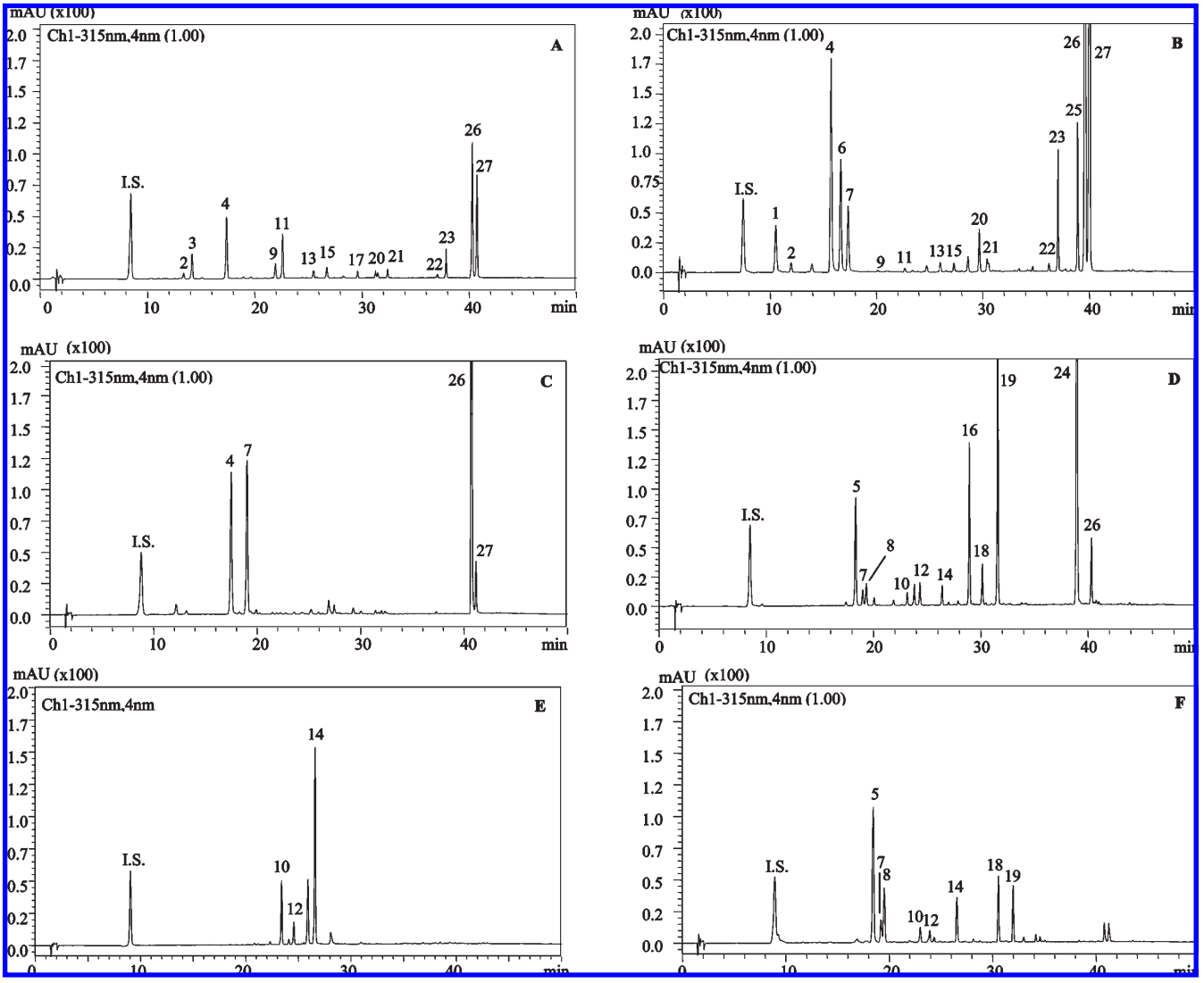

Figure 3. RP-HPLC chromatograms of citrus essential oils: (**A**) lemon oil; (**B**) lime oil; (**C**) bergamot oil; (**D**) grapefruit oil; (**E**) mandarin oil; (**F**) bitter orange oil. For peak identification, see **Table 2**.

The method was optimized so that all of the citrus oils were analyzed under the same experimental conditions. Separation was achived on a Ascentis Express column packed with partially porous particles of 2.7 μm based on Fused-Core technology. A major benefit of these particles, consisting of 1.7 μm solid core and a 0.5 μm porous shell, is the small diffusion path, compared to that of a totally porous particle, which reduces axial dispersion of solutes and minimizes peak broadening. In this way, the mass transfer component of the van Deemter curve becomes small, allowing for higher resolving power to be obtained (*31*−*33*).

The validation process provided the results shown in **Table 1** for LOD and LOQ. LOD and LOQ values in the low milligrams per liter range attested to the applicability of the method to the analysis of oxygen heterocyclic components in trace level in foodstuffs.

Excellent linearity was obtained for all of the analytes, as confirmed by the correlation coefficient r^2, ranging from 0.997 to 1.000.

Concerning the intraday repeatability and intralaboratory reproducibility, coefficient of variation (CV) values of $<8\%$ demonstrated good precision at the concentration level tested. Concerning recovery, values ranging from 80 to 97% were obtained for the four analytes at the concentration level tested, thus demonstrating good accuracy (*34*).

HPLC Analysis of Citrus Oils. Figure 3 shows the chromatograms obtained for the different citrus oils analyzed: lemon oil (**A**), lime oil (**B**), bergamot oil (**C**), grapefruit oil (**D**), mandarin oil (**E**), and bitter orange oil (**F**). The components were identified on the basis of their retention times, compared with those of pure standards and previously isolated components, and with the aid of spectroscopic data. In fact, UV spectra are characteristic for each class of components and further reflect their substitution pattern (see **Figures 4** and **5** for coumarins and polymethoxylated flavones spectra; see Frérot et al. (*16*) for furocoumarins spectra). Peak identification is reported in **Table 2**.

As can be seen from the chromatograms, all of the identified peaks were well resolved, allowing an easy identification and quantification of each component. The qualitative and quantitative data obtained for the six analyzed oils were compared with those reported in the literature (*15*, *35*−*37*). Qualitative results were in good agreement with those previous data, whereas the quantitative values for some components were out of the ranges reported in the literature. However, only one sample of each oil was analyzed, because the aim of this work was the optimization of a single HPLC method useful for the separation of all the analytes in real samples. It is well-known that the amount of oxygen heterocyclic components in citrus oils may vary in relation to geographical origin of the oil, period, and technology of extraction.

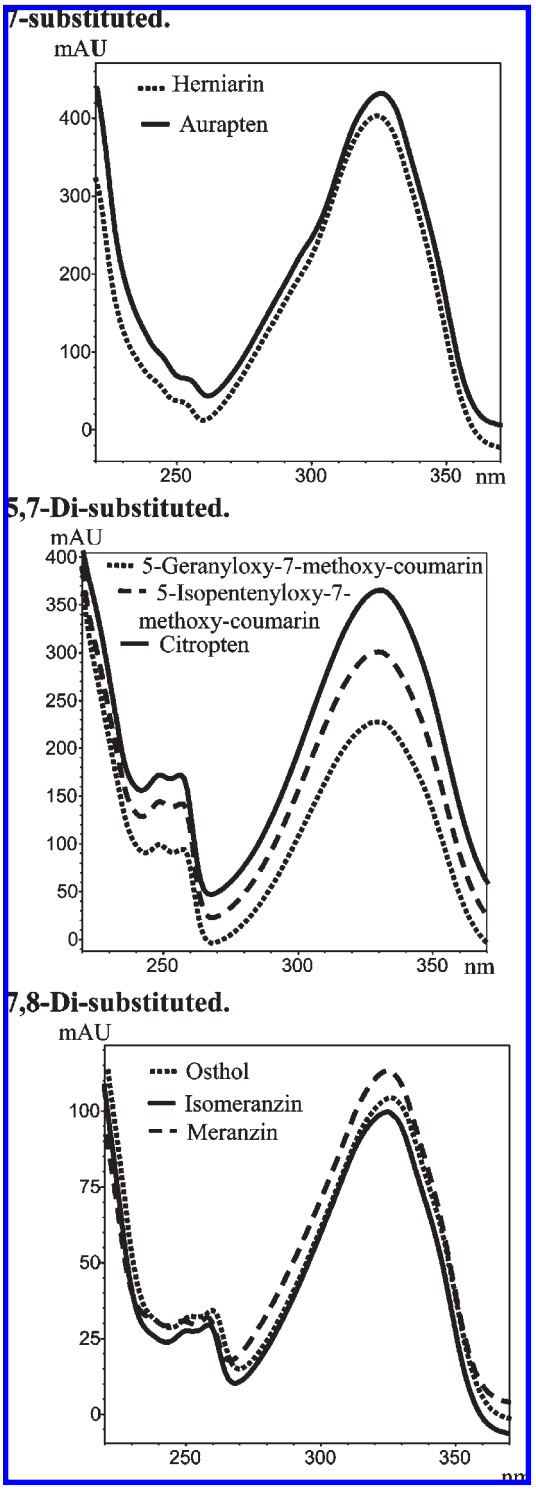

Figure 4. UV spectra of coumarins detected in citrus essential oils.

HPLC Analysis of Teas, Juices, and Liquor Samples. Oxygen heterocyclic components present in samples of Earl Grey tea, lemon and bergamot juices, "limoncello" (lemon liquor), and "bergamino" (bergamot liquor) were studied, and the results are reported in **Table 3**. Samples were extracted with ethyl acetate as described under Materials and Methods. For tea and juice samples, recovery of the oxygen heterocyclic components was determined by carrying out the extraction procedure on a sample of orange juice fortified of known amounts of citropten, bergapten, bergamottin, and 5-geranyloxy-7-methoxycoumarin. Orange juice normally does not contain coumarins and psoralens.

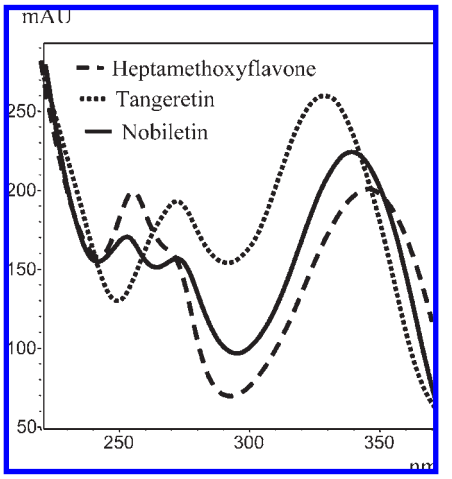

Figure 5. UV spectra of polymethoxylated flavones detected in citrus essential oils.

Moreover, a sample of bergamot liquor fortified with known amounts of citropten, bergapten, bergamottin, and 5-geranyloxy-7-methoxycoumarin was extracted to determine recovery for the liquor samples. In both cases, fortification was performed on five samples, and each extract was analyzed in duplicate.

Recovery was calculated using the formula

$$\text{recovery } \% = [(\text{conc sample fortified} - \text{conc sample unfortified})/\text{fortification}] \times 100$$

and the results are reported in **Table 3**.

The content of oxygen heterocyclic components in lemon juice and Earl Grey tea was below the limit of detection, whereas the other samples showed the presence of these components.

Data found in the literature report a case of intoxication with Earl Grey tea consumed daily in very high quantities (*38*). Health problems due to the high consumption of Earl Grey tea have been related to the presence of bergamot oil, which contains bergamottin, bergapten, and citropten. In this work, the analysis of an extract obtained from a cup of Earl Grey tea prepared following the traditional recipe did not show the presence of coumarins and furocoumarins. The same results were obtained from the analysis of a more concentrated tea prepared using two tea bags instead of one bag.

According to these findings, lemon juice hand-squeezed in the laboratory contained only trace amounts of bergamottin and 5-geranyloxy-7-methoxycoumarin, the main components of the oxygen heterocyclic fraction of lemon essential oil.

Figure 6 shows the HPLC chromatograms obtained for the homemade lemon juice (**A**), the bergamot juice (**B**), the limoncello (**C**), and the bergamino (**D**).

Bergamot juice hand-squeezed in the laboratory was found to contain all of the oxygen heterocyclic components detected in bergamot essential oil. Bergamottin was the main component (with about 61 ppm), followed by bergapten (about 30 mg/L). In the juice, the ratio between these two furocoumarins was about 2:1. In bergamot oil this ratio is about 10:1. This ratio is very different, probably due to the lower solubility of bergamottin in the aqueous juice. Coumarins, citopten, and 5-geranyloxy-7-methoxycoumarin were all present in comparable, small amounts (about 1 mg/L). Gardana et al. (*39*) reported the presence of bergamottin and bergapten in the same ratio of 2:1 in bergamot juice analyzed after dilution and filtration. Gattuso et al. (*40*) reported values in the ranges of 6.4−11.8 mg/L for bergapten and 22.5−43.7 mg/L for bergamottin in bergamot juices. Coumarins have not been reported. In this case, the ratio

Table 2. Retention Times (Rt) and Concentrations (Milligrams per Liter ± CV %) of Oxygen Heterocyclic Compounds in Essential Oil

compound	Rt (min)	lemon	lime	bergamot	grapefruit	mandarin	bitter orange
herniarin (**1**)	12.27		1258 ± 2.3				
oxypeucedanin hydrate (**2**)	13.39	249 ± 2.9	784 ± 3.2				
byakangelicin (**3**)	14.16	706 ± 0.9					
citropten (**4**)	17.38	950 ± 0.8	5725 ± 6.1	2582 ± 0.8			
meranzin (**5**)	18.45				1971 ± 5.9		1923 ± 1.8
isopimpinellin (**6**)	18.53		7422 ± 6.6				
bergapten (**7**)	19.03		2402 ± 2.3	2374 ± 3.7	263 ± 3.4		388 ± 3.9
isomeranzin (**8**)	19.52				412 ± 8.0		1041 ± 1.1
byakangelicol (**9**)	21.95	755 ± 0.8	83 ± 2.7				
nobiletin (**10**)	22.83				na[b]	na	na
oxypeucedanin (**11**)	22.89	1900 ± 0.8	243 ± 5.7				
heptamethoxyflavone (**12**)	24.17				na	na	na
isoimperatorin (**13**)	25.49	173 ± 8.2	384 ± 5.4				
tangeretin (**14**)	26.54				na	na	na
imperatorin (**15**)	26.73	326 ± 2.3	805 ± 5.0				
epoxyaurapten (**16**)	28.94				na		
phellopterin[a] (**17**)	29.60	na					
osthol (**18**)	30.14				na		na
epoxybergamottin (**19**)	31.58				5317 ± 5.5		592 ± 4.9
5-isopentenyloxy-7-methoxycoumarin (**20**)	31.81	na	na				
5-(isopent-2′-enyloxy)-8-(2′,3′-epoxy)isopentenyloxypsoralen (**21**)	32.41	na	na				
cnidicin (**22**)	37.05	82 ± 1.7	252 ± 6.9				
8-geranyloxypsoralen (**23**)	37.87	916 ± 0.1	6722 ± 8.7				
aurapten (**24**)	38.97				9271 ± 5.8		
5-geranyloxy-8-methoxypsoralen[a](**25**)	40.15		na				
bergamottin (**26**)	40.64	4056 ± 0.5	45699 ± 8.1	21419 ± 2.9	1791 ± 5.2		
5-geranyloxy-7-methoxycoumarin (**27**)	41.07	2319 ± 0.5	42000 ± 9.6	1120 ± 3.8			

[a] Tentative identification. [b] na, standard was not available in sufficient amount for quantitative calculation.

Table 3. Concentrations (Milligrams per Liter ± CV %) and Values of Recovery of Oxygen Heterocyclic Compounds in Foods

				commercial bergamino				recovery (%)	
no.	compound	lemon juice	bergamot juice	I	II	limoncello	Earl Grey tea	juice	liquor
2	oxypeucedanin hydrate					3.9 ± 15.31			
3	byakangelicin					3.3 ± 9.45			
4	citropten		0.8 ± 10.55	2.6 ± 6.96	0.9 ± 8.45	3.6 ± 1.18		51 ± 8.8	80 ± 7.0
7	bergapten		30.4 ± 3.42	12.9 ± 5.65	1.9 ± 8.61			51 ± 4.9	68 ± 5.6
9	byakangelicol					0.5 ± 8.44			
11	oxypeucedanin					0.5 ± 6.52			
23	8-geranyloxypsoralen					0.6 ± 0.55			
26	bergamottin	<LOQ	61.0 ± 7.23	12.8 ± 8.47	1.5 ± 4.45	1.9 ± 10.46		51 ± 10.1	74 ± 8.5
27	5-geranyloxy-7-methoxycoumarin	<LOQ	1.1 ± 5.47	0.9 ± 7.49	<LOQ	1.2 ± 4.66		49 ± 2.9	91 ± 7.5

between bergamottin and bergapten was about 4:1. These different findings may be due to the different preparation and extraction procedures of the juice prior to HPLC analysis. It is relevant to note that we found the extraction step strictly necessary to detect and quantify oxygen heterocyclic components in aqueous samples such as teas or juices. In fact, a juice sample filtered only through a 0.45 μm nylon filter was analyzed, and coumarins and furocoumarins were not detected. This is possibly due to the fact that these components are poorly soluble in the aqueous juice and are therefore lost in the filtration process.

With regard to the analysis of citrus liquors, the literature reports some data on the presence of oxygen heterocyclic components in limoncello samples (*41*, *42*), whereas no data can be found regarding bergamino.

From the data reported in the literature (*42*) for commercial lemon liquors, it can be noted that the amounts of coumarins and psoralens can be very different on the basis of the recipe used to prepare the liquor. In this work, only one sample of homemade liquor was analyzed, prepared following the classical recipe based on the alcoholic maceration of the external part (flavedo) of lemon peels followed by a mix of the filtered infusion with a syrup made with water and sugar (1:1, v/w). The total amount of coumarins and psoralens detected in this sample was of 17.44 ppm. This value is in good agreement with that previously reported for a homemade limoncello (*42*).

From the data reported in **Table 3**, it is possible to note the presence of diols (oxypeucedanin hydrate, byakangelicin), formed from the corresponding epoxides (oxypeucedanin and byakangelicol), for the presence of water and an acid pH (*43*). Citropten was the main component, followed by bergamottin and 5-geranyloxy-7-methoxycoumarin, these two being the most representative components in lemon oil.

Two commercial bergamino liquors were also analyzed, and very different values were found between them. In both cases, the four components detected in bergamot oil were found in the liquors. One of them (bergamino I) presented comparable values of bergapten and bergamottin, but lower amounts of citropten and 5-geranyloxy-7-methoxycoumarin. The other bergamino sample (bergamino II) presented comparable values for bergapten, citropten, and bergamottin, but always lower than those of sample I.

In conclusion, the reversed phase HPLC method that uses innovative partially porous particles HPLC columns permitted us

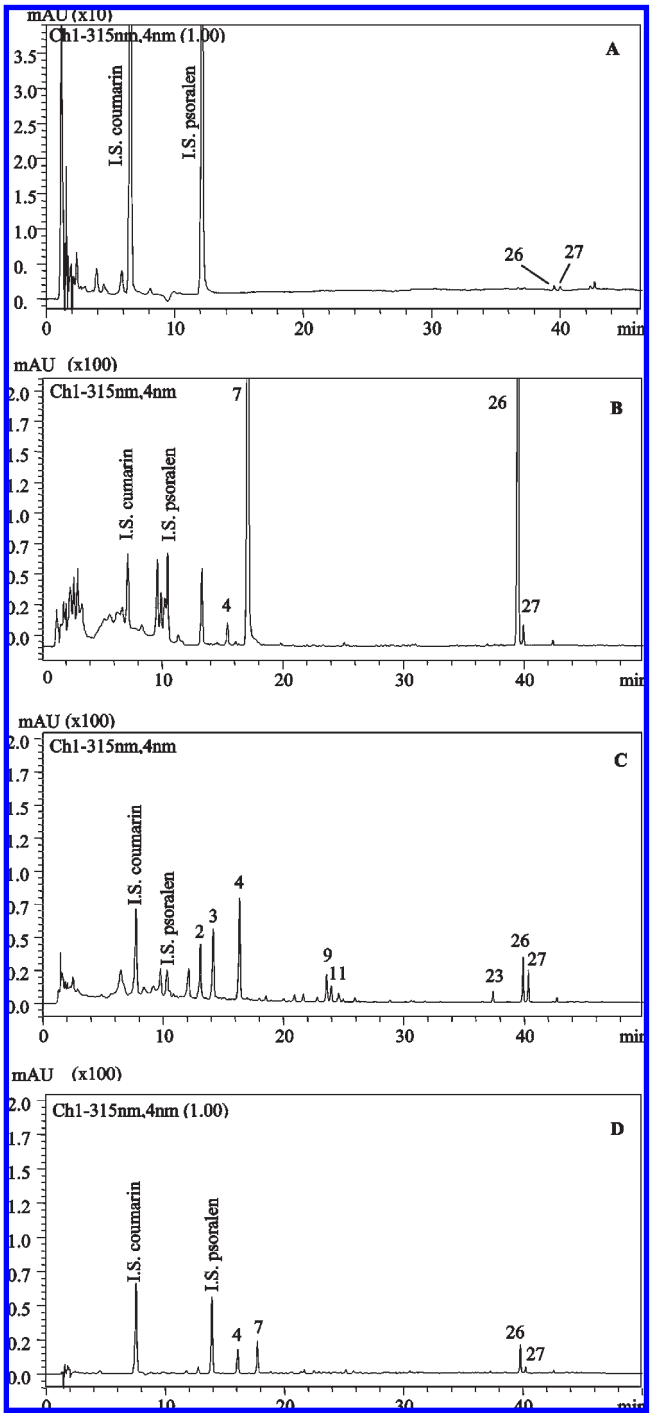

Figure 6. RP-HPLC chromatograms of (**A**) lemon juice, (**B**) bergamot juice, (**C**) limoncello, and (**D**) bergamino. For identification of the components, see **Table 3**.

to obtain baseline separation of all the identified components in different citrus oils. A photodiode array detector was successfully used for the qualitative and quantitative analysis. As stated already (*44*), this detector could be less informative and versatile than the MS detector, but presents the advantages of lower cost and wide diffusion in analytical laboratories.

Among the food samples containing citrus oils analyzed in this work, Earl Grey tea and lemon juice did not present oxygen heterocyclic components. Bergamot juice was found to be the richest in coumarins and psoralens, with a total amount of about 90 mg/L. Bergamot juice is today considered a byproduct of the bergamot essential oil industry. However, it is known to contain a high amount of flavonoids that can exert beneficial effects on human health due to their antioxidant properties. It has been proposed as a fortifier of fruit juices or as a possible adulterant of other citrus juices, mainly lemon.

For this reason, knowledge of the oxygen heterocyclic components present in this juice is important, taking into consideration the restrictions in the presence of these components in other products due to their possible toxic effects.

Liquors made both from lemon (limoncello) and bergamot (bergamino) were found to contain coumarins and psoralens. Due to the limited number of samples analyzed, no conclusions can be drawn. However, for the same reasons described for bergamot juice, a deeper knowledge of the commercial products that may contain coumarins and psoralens is advisible, both to determine the authenticity and quality of the liquor and to correlate its intake to beneficial or negative effects on human health.

The HPLC-DAD method developed in this work allowed for the first time the baseline separation of all oxygen heterocyclic components present in different oils, under the same chromatographic conditions.

The validated method allowed the quantification of components in different samples such as oils, juices, teas, and liquors down to values of <1 mg/L. Its applicability could be easily extended to the analysis of other matrices containing oxygen heterocyclic components.

ACKNOWLEDGMENT

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Supporting Information Available: Molecular structures of components (**1**, **5**−**8**, **10**, **12**, **14**, **16**, **18**, **24**) and their characterization by ^{1}H NMR and ^{13}C NMR. This material is available free of charge via the Internet at http://pubs.acs.org.

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