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Quantification of Total Furocoumarins in Citrus Oils by HPLC Coupled with UV, Fluorescence, and Mass Detection

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Furocoumarins or psoralens represent a class of photosensitizers whose use level is likely to be restricted to 1 ppm in cosmetic products by the EU. A reversed-phase HPLC method was developed to separate the 15 main furocoumarins present in citrus oils. Quantification by UV, fluorescence, or mass detectors was compared in terms of linearity and limit of detection. Cold-pressed oils of different citrus species were analyzed using this method. This method could be implemented in quality control laboratories equipped with an HPLC system and a UV diode array detector. Because of possible coelutions, the UV-spectral data should be carefully examined to avoid misleading interpretations of peaks.

KEYWORDS: Furocoumarins; psoralens; UV−DAD; HPLC; LC−MS; quantification citrus oil

INTRODUCTION

The European Cosmetics Directive 76/768/EEC was recently modified, introducing for the first time a limit on the presence and use of the photosensitizing furocoumarins in cosmetics (*1*). Furocoumarins constitute a highly diverse family of natural chemicals in different plant extracts used as ingredients in fragrances. So far, only the alkoxyfurocoumarins having alkyl groups of 11 carbons or less have been identified, but it is completely plausible that other furocoumarins exist in natural essences, which have so far escaped detection. Even for those that we know about, it would not be practical to have to quantify them in all cosmetic products. In cases such as this (e.g., polyaromatic hydrocarbons), it is normal practice to identify one or maybe two individual members that can be used as markers to indicate the level of the whole family (in this case, the total furocoumarin content).

The citrus oils present the biggest potential contribution to furocoumarin content in fragranced products (*2*, *3*). 5-Methoxypsoralen (bergapten) is the major furocoumarin in some of these but not all. Furthermore, the ratio of bergapten to what seems to be "total furocoumarins" varies from one species to another and probably is not even constant for different preparations made from the same species. As an example of this, the ratio of bergapten to the total of 10 furocoumarins measured in cold-pressed lime oils vary considerably. These are for key lime oils from Dominica, 2000/31 800 ppm (6.29%); Mexico, 2400/32 840 ppm (7.3%); Peru, 1200/32 330 ppm (3.71%); Haiti, 1800/29 540 ppm (6.09%) and for Persian lime oils from Mexico, 1500/18 850 ppm (7.96%); Brazil, 1400/203 390 ppm (6.87%); and Florida, 2200/21 980 ppm (10.01%) (*4*). It should also be pointed out that some citrus oils (particularly cold-pressed lemon oil), oxygenated coumarins, and furocoumarins are added to dissimulate adulteration with cheaper steam-stripped oils (*5*). These authors maintain that bergapten is not one of the 9 furocoumarins found in cold-pressed Sicilian lemon oil (5160 ppm in all).

Bergapten is not always detected in the furocoumarin fraction of some oils. When it is, its relative amount varies according to the type and origin and is probably seasonal. Relative proportions of different furocoumarins might also vary because of adulteration. When those facts are taken into account, it was necessary to develop a method for the quantification of as many furocoumarin derivatives as possible. In this paper, we describe the separation and quantification of 15 furocoumarins previously isolated and identified in various citrus oils (*3*, *6*−*9*). Relationships between individual furocoumarin contents and the total furocoumarins were evaluated.

Although it is possible to analyze furocoumarins by GC−MS (*10*), some products need to be derivatized to enhance their volatility. This explains why HPLC has always been the technique of choice for the analysis of furocoumarins and coumarins in citrus oils (*2*). Normal-phase HPLC was first used to characterize oils from different species (*4*) and to detect adulterations (*5*). Different gradient programs with hexane, ethyl acetate, and ethanol were necessary to achieve acceptable separations for each type of oil. UV detection at 315 nm was used for quantification of furocoumarins and also coumarins in the parts per million range. Normal phase is still used, e.g., for the authentication of lemon oils from different geographical origins (*11*−*13*). Reversed-phase HPLC was also used for the separation of closely related furocoumarins (*14*). Ziegler and Spiteller (*10*) used different techniques, including reversed-phase HPLC to characterize Sicilian lemon oil and provided many useful spectroscopic data for psoralens. Brown and Thompson (*15*) compared normal and reversed phases for the separation

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of 67 coumarins including 19 furocoumarins. Their conclusion was that the two techniques were complementary. Recently, LC−MS with electrospray ionization or atmospheric pressure chemical ionization (APCI) was used to detect and quantify the major coumarins, psoralens, and polymethoxyflavones in various citrus oils (*16*). Finally, LC−NMR has been applied for the identification of these products in lemon peel oil (*17*). There are only a few descriptions of quantification parameters (linearity, range of calibration, and limit of detection) in the literature. Recently, Kaminski et al. (*18*) described in detail the quantification of selected coumarins in plant extracts. They used reversed-phase HPLC to obtain a good resolution with a ternary gradient of water, THF, and methanol. They also used time-programmed wavelength with a DAD detector at 340, 325, and 300 nm. Linearity was good from 0.2 to 10 ppm with a detection limit of 0.1 ppm. Unfortunately, only three furocoumarins: psoralen, bergapten, and xanthotoxin were studied.

In the present paper, we describe the preparation of a high-purity calibration standard containing the 15 major furocoumarins present in citrus oils. We describe the separation of these furocoumarins by reversed-phase HPLC. We also compare UV absorbance, fluorescence, and mass spectrometry for the detection, identification, and quantification of these compounds in various citrus oils.

MATERIALS AND METHODS

HPLC Analyses. HPLC-grade THF was purchased from SDS (Peypin, France), freshly filtered over basic alumina, and distilled. Methanol and acetonitrile, both HPLC grade were purchased from Carlo Erba (Rodano, Italy). Water was freshly produced by a Millipore Synergy 185 system (Molsheim, France). HPLC separations were carried out with two Agilent 1100 Series systems (Meyrin, Switzerland). System 1 consisted of a G1312A binary pump, G1329A autosampler, G1316A thermostated column compartment, and G1315B diode array detector. LC−MS experiments were performed on a Finnigan LCQ Classic ion-trap mass spectrometer (Spectronex, Basel, Switzerland). HPLC system 2 was the same as system 1 except for a G1365B multiwavelength UV detector, G1321A fluorescence detector, and G1364A automated fraction collector.

Analytical HPLC Method 1. The gradient separations were carried out using an Interchim MS Uptisphere 3 ODB (3 μm, 120A) cartridge column (150 × 2.1 mm inside diameter) purchased from Laubscher Labs (Miecourt, Switzerland). Solvent A was water−acetonitrile−THF (85:10:5), and solvent B was acetonitrile−methanol−THF (65:30:5). The gradient profile was as follows: 0−5 min, 0% of B (isocratic); 5−20 min, 0−32% of B (linear); 20−24 min, 32% of B (isocratic); 24−38 min, 32−55% (linear); 38−40 min, 55−90% (linear); rinse at 90% of B for 10 min; and then equilibrate at 0% of B for 10 min. Flow rate was 0.3 mL/min. Total analysis time was 60 min. Wavelength was set at 310 nm (bandwidth = 4 nm, and slit = 4 nm) for UV detection, and excitation/emission wavelengths were 310 and 490 nm, respectively, for fluorescence detection. LC−MS were recorded on the LCQ using the APCI source (vaporizer at 450 °C, heated capillary at 165 °C, pin voltage at 5 kV, sheath gas N_2 at 45 arbitrary units, and aux. gas at 5 arbitrary units).

Analytical HPLC Method 2. In this method, solvent A was water and solvent B was acetonitrile. The gradient profile was as follows: 0−5 min, 10% of B (isocratic); 5−35 min, 10−90% of B (linear); rinse at 90% of B for 10 min; and then equilibrate at 0% of B for 10 min. Flow rate was 0.3 mL/min. Total analysis time was 55 min. Other parameters were the same as in method 1.

Semipreparative HPLC Purifications. Semipreparative HPLC purifications were performed with a Microsorb C18 Prep, 8 μm, 60A, 250 × 10 mm inside diameter, Varian (Zug, Switzerland) using water and acetonitrile at 4.5 mL/min (isocratic). Collections were triggered by the UV signal using the automated fraction collector.

Chemicals. Psoralen, bergapten, and xanthotoxin were purchased from Fluka (Buchs, Switzerland). Byakangelicin, oxypeucedanin, imperatorin, and isoimperatorin were obtained from Apin (Abingdon, U.K.). Bergamottin and isopimpinellin were obtained from Carl Roth (Karlruhe, Germany). 8-Geranyloxypsoralen was obtained from Indofine (Hillsborough, NJ) under the name xanthotoxol geranyl ether. It is important to check for the purity of these products before preparing the calibration solutions. In one batch of imperatorin (Apin 05127i control *N*° 22510), we found about 25% of a later eluting product, which was purified by semipreparative HPLC (water/acetonitrile, 65:35, isocratic) and identified as phellopterin by HPLC−MS and ^{1}H (*17*) and ^{13}C NMR (*18*). A NOESYPopt experiment showed an Overhauser effect between the OCH_3 and the furan H−C(6) protons, which ascertained the structure. Opportunely, we used this purified phellopterin in our study. Otherwise, this compound is difficult to obtain. We obtained oxypeucedanin hydrate from a citrus byproduct (Sunkist, Van Nuys, CA) containing byakangelicin as a main component. This powder was washed with pentane and ethyl acetate. Chloroform washings of the insoluble material afforded almost pure byakangelicin, whereas the soluble material was concentrated and crystallized several times with ethyl acetate to afford pure oxypeucedanin hydrate. However, this compound could be obtained in a much simpler manner by hydration of commercial oxypeucedanin (*20*).

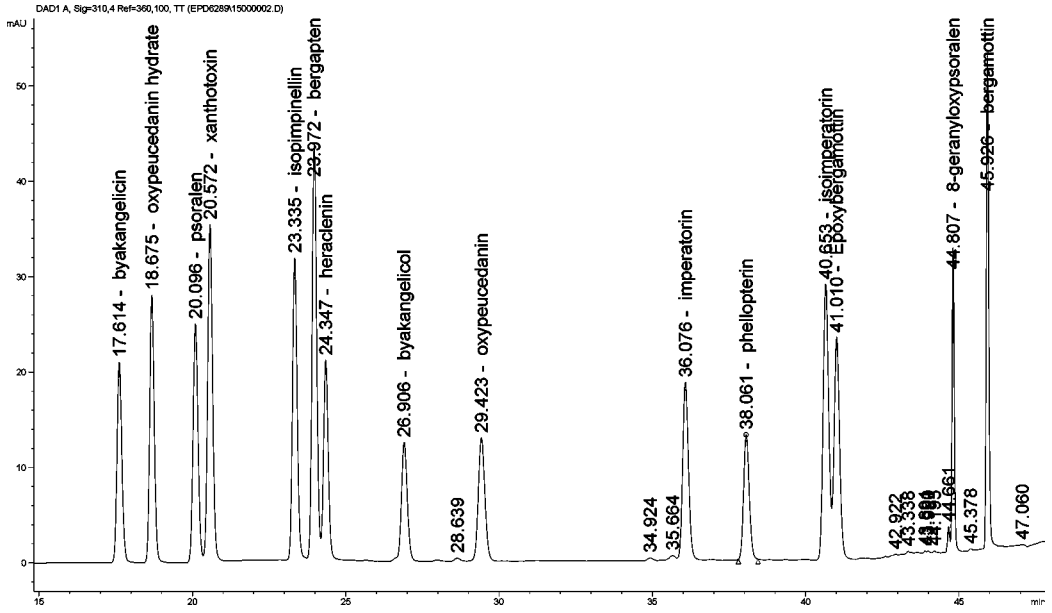
The remaining furocoumarins were synthesized. Heraclenin and epoxybergamottin were synthesized from commercial imperatorin and bergamottin, respectively, by oxidation with *m*-chloroperbenzoic acid (*21*). Heraclenin was purified by semipreparative HPLC (water/acetonitrile, 60:40, isocratic). Epoxybergamottin was purified by flash chromatography over silica gel (cyclohexane/ethyl acetate, 60:40) and recrystallized from cyclohexane−ethyl acetate.

Byakangelicol was synthesized in two steps from byakangelicin (Sunkist, see above). Toluene-4-sulfonyl chloride (6.00 g) was added portion-wise to a solution of byakangelicin (1.80 g) in 100 mL of pyridine at 0 °C. The mixture was heated to 40 °C during 28 h. The mixture was poured onto ice and then extracted with CH_2Cl_2. The organic layer was separated and concentrated to dryness. The product was dissolved in 400 mL of CH_2Cl_2, washed twice with water, dried over Na_2SO_4, and concentrated to yield 3.72 g (96%) as a yellowish solid. A total of 100 mg of the byakangelicin tosylate was stirred in triethylamine (5 mL) during 24 h at 40 °C. The reaction mixture was concentrated to dryness and purified first by flash chromatography (cyclohexane/ethyl acetate, 70:30) and then by semipreparative HPLC (water/acetonitrile, 60:40). After freeze-drying and recrystallization from dichloromethane/pentane, 40 mg of fine yellow needles were obtained. ^{1}H and ^{13}C NMR spectra (*19*) and APCI−MS spectrum (*17*) were consistent with the expected structure.

Purities of compounds **1**−**15** were checked by TLC using three different eluent systems: (I) dichloromethane; (II) toluene/THF, 60:40; and (III) cyclohexane/ethyle acetate, 70:30, on kieselgel 60 F_{254} HPTLC plates (Merck). All products showed only one spot when revealed with UV (254 nm) and with phosphomolybdic acid. Melting points were measured using a B-540 unit (Büchi, Switzerland). Purities of all products were measured by HPLC−UV at 310 nm from 100 ppm solutions using the analytical HPLC method 2.

Psoralen (**1**): R_f (I, II, and III), 0.50, 0.80, and 0.41; mp 163−164 °C [lit. (*22*) 165−166 °C]; HPLC−UV (310 nm), 100%. Bergapten (**2**): R_f, 0.49, 0.79, and 0.41; mp 191−193 °C [lit. (*22*) 188−190°C]; HPLC−UV (310 nm), 100%. Xanthotoxin (**3**): R_f, 0.39, 0.79, and 0.37; mp 148−149°C [lit. (*22*) 146−148 °C]; HPLC−UV (310 nm), 99.3%. Isopimpinellin (**4**): R_f,0.34, 0.79, and 0.37; mp 149−150 °C [lit. (*7*) 150−151 °C]; HPLC−UV (310 nm), 99.2%. Oxypeucedanin (**5**): R_f, 030, 0.80, and 0.37; mp 140−141 °C [lit. (*20*) 142−143 °C]; HPLC−UV (310 nm), 95.7%. Oxypeucedanin hydrate (**6**): R_f, 0.03, 0.42, and 0.04; mp 124−126 °C [lit. (*7*) 129−132 °C]; HPLC−UV (310 nm), 99.1%. Byakangelicol (**7**): R_f, 0.16, 0.75, and 0.28; mp 84−85 °C [lit. (*23*) mp 87−89 °C]; HPLC−UV (310 nm), 99.4%. Byakangelicin (**8**): R_f, 0.02, 0.44, and 0.04; mp 112−115 °C [lit. (*24*) 115−116 °C]; HPLC−UV (310 nm), 100%. Heraclenin (**9**): R_f, 0.20, 0.76, and 0.35; mp 101−103 °C [lit. (*21*) 113−115 °C]; HPLC−UV (310 nm), 93.9%. 8-Geranyloxypsoralen (**10**): R_f, 0.59, 0.87, and 0.51; mp 52−54 °C [lit. (*20*) 53°C]; HPLC−UV (310 nm), 96.2%. Bergamottin (**11**): R_f, 0.64, 0.96, and 0.74; mp 62−63°C [lit. (*7*) 59−60 °C]; HPLC−UV (310 nm), 100%. Imperatorin (**12**): R_f, 0.53, 0.83, and 0.46; mp 97−

Chart 1. All 15 Furocoumarins at 310 nm, with 5 μL of a 10 ppm Solution Injected HPLC Conditions (Analytical Method 1, see the Materials and Methods).



98 °C [lit. (*22*) 104 °C]; HPLC−UV (310 nm), 100%. Isoimperatorin (**13**): R_f, 0.60, 0.90, and 0.63; mp 109−110 °C [lit. (*7*) 110−111 °C]; HPLC−UV (310 nm), 100%. Phellopterin (**14**): R_f, 0.46, 0.85, and 0.44; mp 102 °C [lit. (*7*) 104 °C]; HPLC−UV (310 nm), 95.7%. Epoxybergamottin (**15**): R_f, 0.19, 0.86, and 0.41; mp 76−77 °C [lit. (*8*) 67−70 °C, lit. (*9*) 83−84 °C]; HPLC−UV (310 nm), 97.0%. For this compound, the ^{1}H NMR spectrum did not show any impurity signals that exceeded the height of the ^{13}C satellite signals of the product.

Peak Identification and Quantification. Retention times were obtained by injection of the reference material. UV and MS spectra obtained from the diode-array detector and the mass spectrometer were used to identify peaks, when necessary.

Each compound was diluted to 1 mg/mL in acetonitrile. The mother solution (50 ppm) was prepared by mixing 1 mL of each solution and completing to 20 mL with water/acetonitrile (1:1). Then, the mother solution was diluted in water/acetonitrile (1:1) to 25, 10, 5, 1, 0.5, 0.2, 0.1, 0.05, and 0.01 ppm calibration solutions.

Calibration curves were obtained by the analytical methods 1 and 2 by injecting 5 μL of calibration solutions. External calibration was performed using the Agilent HPChemstation (UV, fluorimetry) and the Finnigan Xcalibur (module LCQuan 2.0) softwares.

Citrus Oils. All citrus oils were purchased from various companies. Bergamot oil was specified as the product of the extraction of the fruits of *Citrus aurantium* L. subsp. *bergamia*, followed by alkaline treatment to remove bergapten. Lemon oil was obtained by the cold expression of the fresh peel of the ripe fruit of Californian lemon [*Citrus limonum* (L.) (N. L. Burman)]. Grapefruit oil was obtained by the expression of the fresh peel of the Israeli grapefruit (*Citrus paradisis macfayden* or *Citrus decumana* L.). Mandarin oil was obtained by pression ("sfumatrice" method) of the peel of the mandarin fruit (C*itrus reticulata blanco*). Tangerine oil was obtained by the cold expression of the peel of tangerine (*Citrus reticulata blanco* var. *tangerine*). Bitter orange oil was obtained by the cold expression of the peel of bitter orange (*Citrus aurantium* L. subsp. *amara*). For the HPLC analyses, these oils were diluted in ethanol to 5 mg/mL (bergamot), 10 mg/mL (lemon and grapefruit), 20 mg/mL (bitter orange), or 100 mg/mL (tangerine and mandarin) before injection.

RESULTS AND DISCUSSION

Standard Solution. A total of 10 of the 15 furocoumarins used in this study were commercially available. However, some of them needed to be purified before their use as a calibration reference. As described in the Materials and Methods, a compound was obtained from a batch of commercial **12** (Apin Chemicals) and identified as **14** on the basis of ^{1}H and ^{13}C spectroscopy (*19*). However, the ^{13}C NMR spectrum of cnidilin (8-isoprenyloxy-5-methoxypsoralen) as described by Schmidt (*25*) is very close to that of phellopterin described by Sterner (*19*). A NOESY experiment showed an Overhauser effect between the methoxy protons and the H−C(6) of the furan ring of our product. Furthermore, the epoxidation of the same compound furnished **7**. This proved the occurrence of **14**. Cnidilin would not have given the same epoxide.

Some compounds had to be prepared synthetically. Fortunately, the syntheses (see the Materials and Methods) were generally effected in only one step from commercial products. The preparation of **7** from **8** required two chemical steps. This represents a drawback for the implementation of such quantification in routine analytical laboratories. The purity of each compound was checked by TLC using three different solvent systems and by measuring the melting points. HPLC−UV purity of the 15 compounds was also measured at 310 nm from 100 ppm solutions. All purities were higher than 99% except for **5**, **10**, **9**, **14**, and **15**, in which the purities ranged from 93.9 to 97%.

HPLC Separation. Using only water and acetonitrile as the solvents (see the analytical method 2), a good separation was obtained for only 12 of the 15 products. In particular, isopimpinellin and bergapten could not be separated. As described earlier (*18*), the presence of THF was necessary to separate those two compounds. We noticed that the presence of acetonitrile in the beginning of the separation was important for the separation of bergapten and heraclenin. Methanol helped in the separation of psoralen and xanthotoxin. **Chart 1** shows the separation of the 15 furocoumarins (10 ppm each) obtained with the analytical method 1 (see the Materials and Methods). A few artifact peaks, e.g., at 35.6 min, were also present in blank samples, as well as the baseline drift after 42 min. These artifacts were not present when only water and acetonitrile were used.

While the column that we employed gave excellent results, in particular regarding the peak shape, other C_{18} columns could also be employed. Sometime after about 100 citrus sample

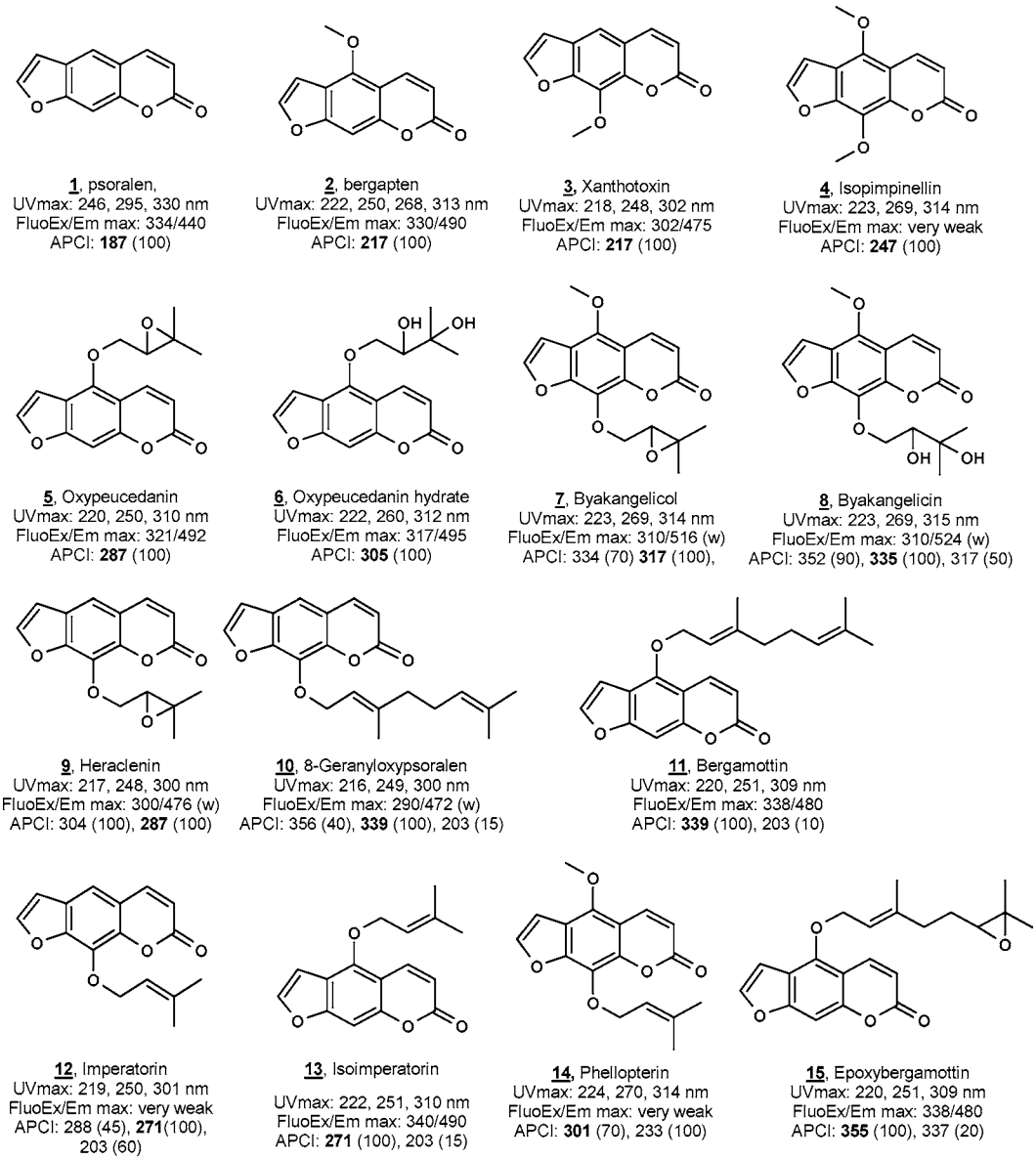


Figure 1. Structure and spectral data of the 15 furocoumarins.

analyses, the column degraded, giving peak tailing or splitting. However, it was fully regenerated by sequential back flushing with methanol, dichloromethane, and again methanol.

Product Identification. Besides furocoumarins, citrus oils or other plant extracts contain other products including coumarins or flavonoids, which could coelute with the components of our study. Therefore, we evaluated the possibility of using UV, fluorescence, and mass detectors. This would minimize a possible misinterpretation of the results. UV spectra of a few furocoumarins have already been described (*3*, *18*). **Figures 1**−**5** show that UV spectra recorded with the UV diode array detector could be clustered in four families of furocoumarins according to their substitution pattern (*3*). **Figure 2** represents the UV spectrum of psoralen, the unsubstituted furocoumarin. Parts a and b of **Figure 3** show superimposed and normalized UV spectra of furocoumarins monosubstituted in position 5 (bergamottin, bergapten, epoxybergamottin, isoimperatorin, oxypeucedanin, and oxypeucedanin hydrate). **Figure 4** shows UV spectra of furocoumarins monosubstituted in position 8 (8-geranyloxypsoralen, heraclenin, imperatorin, and xanthotoxin). **Figure 5** shows UV spectra of furocoumarins disubstituted in position 5 and 8 (byakangelicin, byakangelicol, isopimpinellin,

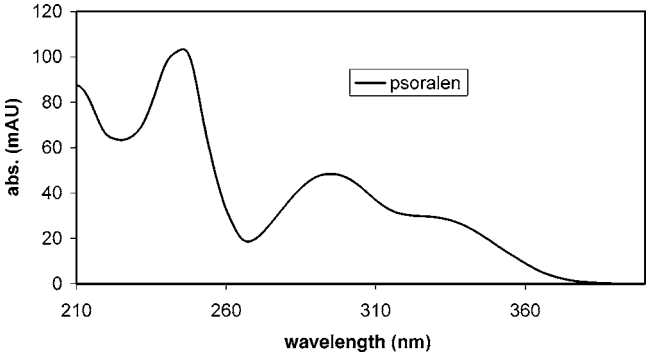


Figure 2. UV spectrum of psoralen (unsubstituted furocoumarin).

and phellopterin). Using these data, it should be easy to identify an unknown furocoumarin. It is obvious that UV−DAD is a necessary detector for the analysis of complex citrus oils. As a good compromise between sensitivity and selectivity, 310 nm was the most suitable wavelength.

Figure 1 gives the best excitation/emission wavelength for the 15 compounds obtained with the fast-scanning fluorimeter in two runs (one for excitation spectra and one for emission

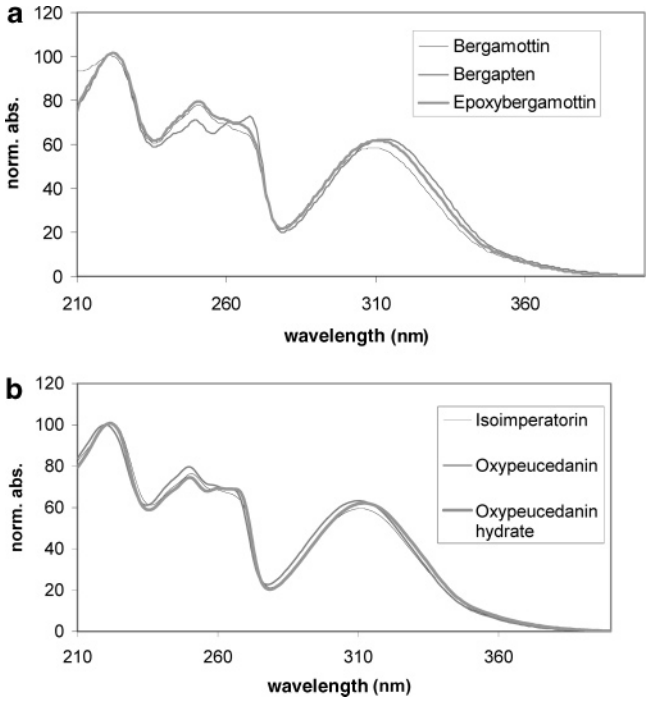


Figure 3. (a) UV spectra of 5-substituted furocoumarins. (b) UV spectra of 5-substituted furocoumarins.

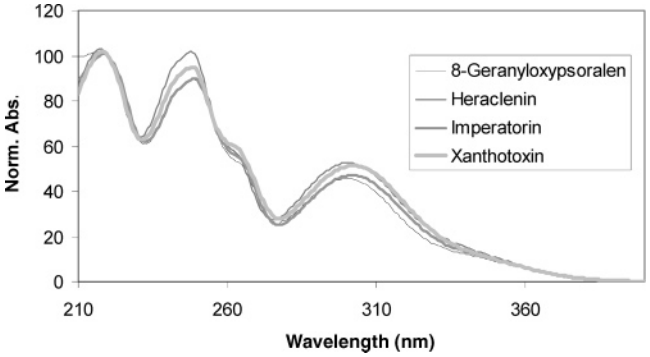


Figure 4. UV spectra of 8-substituted furocoumarins.

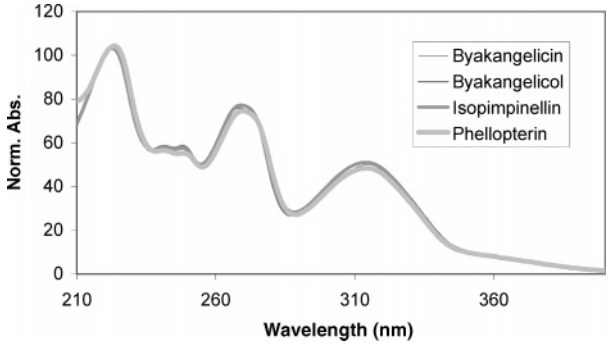


Figure 5. UV spectra of 5,8-disubstituted furocoumarins.

spectra). As described earlier (*26*), the presence of an alkoxy group in position 5 or 8 influences both the absorbance and fluorescence of the furocoumarins. In particular, we noticed a much lower fluorescence in 8-substituted furocoumarins as opposed to those bearing a hydrogen atom in this position (**1**, **2**, **3**, **5**, **6**, **11**, **13**, and **15**). The use of 310 nm in excitation and 490 nm in emission was a good compromise. It allowed the quantification of 8 furocoumarins with the same sensitivity as UV detection. Fluorescence detection seems to have no advantage over UV detection. It is only slightly more sensitive for certain furocoumarins, whereas the others could not be quantified in the low parts per million range. However, a dual detection could be useful in case of coelution problems happening during the analysis of citrus oils.

We also recorded the APCI−MS spectra, in which the protonated molecular ion was the main peak. All spectral data for the 15 furocoumarins are summarized in **Figure 1**.

Calibration. UV at 310 nm was the best detection method for the quantification in terms of convenience, sensitivity, linearity, and reproducibility. UV response factors defined as the average ratio between the injected concentration (ppm) and the UV peak area were in the same range for all of the compounds. This was not the case for the other detectors. Table 1 summarizes the results obtained in the calibration of the 15 compounds by UV and fluorescence detection. Because the calibration range included 4 orders of magnitude, we used a log−log regression. In the **Table 1**, we present the average relative deviation between calculated and specified amounts for each compound. In the given usable range, the relative deviation was less than 5%. We also give the average response factor for each of the 15 studied furocoumarins. Thus, another laboratory equipped with its own HPLC system could obtain the response factors for the commercial reference compounds and then estimate those of missing compounds using our data. The data presented in **Table 1** are related to standard solutions and not to real citrus oil solutions.

For those laboratories equipped with LC−MS, quantification is also possible in the low parts per million range. We found it more convenient to use quadratic log−log curves, which better fit the lower levels of calibration in the range of 0.1−50 ppm. Thus, using this type of equation, Table 2 shows that the difference between calculated and actual amounts was below 5% for most compounds and within the usable range. It should be noted that our mass spectrometer was an ion trap. Other mass spectrometers, in particular, those equipped with a quadrupole, would give different results regarding sensitivity and linearity.

Quantification in Citrus Oils. The main difficulty of the analysis of furocoumarins in citrus oils is due to the presence of numerous products including coumarins and flavonoids. Even the best HPLC method could not always avoid coelution problems. We analyzed six oils from different citrus species: bergamot, lemon, bitter orange, grapefruit, mandarin, and tangerine. The results obtained with the different types of detectors are summarized in **Tables 3**−**6**.

Mandarin and tangerine oils did not contain any of the 15 referenced furocoumarins, as seen by LC−MS and by examining UV−DAD spectra. However, UV and fluorimetry traces displayed peaks at the expected retention times of some furocoumarins.

In bergamot oil, bergamottin accounted for more than 99% of the total furocoumarin (**Table 3**). The alkaline treatment of this particular oil explains the far lower than expected level of bergapten. McHale and Sheridan (*4*) found 1830 mg/100 g of bergamottin and 270 mg/100 g of bergapten in bergamot oil. Although it could not be detected by LC−MS, oxypeucedanin gave an UV peak corresponding to 53 ppm. The LC−MS trace showed a compound having a molecular weight of 342 at the expected retention time. Similarly, coelution with a compound of molecular weight 274 resulted in an overestimation of epoxybergamottin by UV−DAD. Fluorimetry gave consistent results for bergapten, epoxybergamottin, and bergamottin. In this case, fluorescence detection was useful to show the absence of oxypeucedanin. We see that the total furocoumarin content

Table 1. Statistical Data for UV and Fluorescence Calibration Curves

product *N*° (R.T. in min)	UV–DAD 310 nm (ppm) usable range (% deviation) [log(amount) versus log(area)]	UV–DAD 310 nm (ppm) limit of detection ($S/N = 8$) average response factor (=amount/area)	fluo 310/490 nm (ppm) usable range (% deviation)	fluo 310/490 nm (ppm) limit of detection ($S/N = 8$) average response factor (=amount/area)
byakangelicin (**8**) (17.6 min)	0.05–50 (2.6%)	0.05 (0.043 59)		1
oxypeucedanin hydrate (**6**) (18.7 min)	0.1–50 (0.8%)	0.05 (0.035 22)	0.05–50 (1.0%)	<0.01 (0.049 93)
psoralen (**1**) (20.1 min)	0.1–50 (1.1%)	0.05 (0.037 13)	0.05–50 (1.5%)	<0.01 (0.054 79)
xanthotoxin (**3**) (20.6 min)	0.1–50 (1.2%)	0.05 (0.026 49)	0.1–50 (1.5%)	<0.01 (0.110 72)
isopimpinellin (**4**) (23.4 min)	0.1–50 (3.6%)	0.05 (0.031 77)		5
bergapten (**2**) (24.0 min)	0.1–50 (0.9%)	0.01 (0.023 23)	0.1–50 (1.8%)	<0.01 (0.030 18)
heraclenin (**9**) (24.3 min)	0.1–50 (0.9%)	0.05 (0.044 77)		0.05
byakangelicol (**7**) (26.9 min)	0.1–50 (2.9%)	0.05 (0.040 24)		0.5
oxypeucedanin (**5**) (29.4 min)	0.05–50 (1.9%)	0.05 (0.052 93)	0.1–50 (0.7%)	<0.01 (0.039 91)
imperatorin (**12**) (36.1 min)	0.1–50 (1.6%)	0.01 (0.044 87)	0.2–50 (2.9%)	0.1
phellopterin (**14**) (38.1 min)	0.1–50 (1.1%)	0.05 (0.060 96)		0.5
isoimperatorin (**13**) (40.7 min)	0.05–50 (2.3%)	0.01 (0.029 22)	0.1–50 (1.0%)	<0.01 (0.017 92)
epoxybergamottin (**15**) (41.0 min)	0.1–50 (2.2%)	0.05 (0.034 06)	0.1–50 (2.7%)	<0.01 (0.024 06)
8-geranyloxypsoralen (**10**) (44.8 min)	0.2–50 (2.2%)	0.05 (0.062 40)		1
bergamottin (**11**) (45.9 min)	0.05–50 (1.0%)	0.05 (0.035 95)	0.1–50 (1.1%)	<0.01 (0.017 37)

Table 2. Statistical Data for APCI–MS Calibration Curves

	extracted ion	APCI–MS (r^2) for equation $\log y = m(\log x)^2 + n(\log x) + b$; variations between specified/calculated amounts [usable range ppm]	APCI–MS LOD (ppm) ($S/N = 8$)
byakangelicin (**8**)	335	(0.9995) from –5 to +9% [0.1–50 ppm]	0.1
oxypeucedanin hydrate (**6**)	305	(0.9990) from –5 to +13% [0.05–50 ppm]	0.05
psoralen (**1**)	187	(0.9997) from –3 to +7% [0.1–50 ppm]	0.1
xanthotoxin (**3**)	217	(0.9998) from –3 to +6% [0.05–50 ppm]	0.01
isopimpinellin (**4**)	247	(0.9999) from –3 to +5% [0.05–50 ppm]	0.01
bergapten (**2**)	217	(0.9998) from –4 to +6% [0.05–50 ppm]	0.01
heraclenin (**9**)	287	(0.9997) from –5 to +6% [0.05–50 ppm]	0.05
byakangelicol (**7**)	317	(0.9998) from –4 to +5% [0.05–50 ppm]	0.05
oxypeucedanin (**5**)	287	(1.0000) from –1 to +2% [0.05–50 ppm]	0.05
imperatorin (**12**)	271	(0.9999) from –3 to +1% [0.5–50 ppm]	0.1
phellopterin (**14**)	301	(0.9997) from –3 to +7% [0.2–50 ppm]	0.1
isoimperatorin (**13**)	271	(0.9999) from –1 to +3% [0.05–50 ppm]	0.01
epoxybergamottin (**15**)	355	(0.9996) from –7 to +4% [0.05–50 ppm]	0.05
8-geranyloxypsoralen (**10**)	339	(0.9992) from –6 to +10% [0.1–50 ppm]	0.1
bergamottin (**11**)	339	(0.9998) from –4 to +5% [0.05–50 ppm]	0.05

calculated from the UV−DAD is very close to that given by the LC−MS (difference of 3.3%).

In lemon oil, none of the furocoumarins accounted for more than 35% of the total. No or little coelution occurred, and results obtained from UV−DAD and MS detectors were in accordance. A fluorescence detector gave inconsistent results probably because of traces of highly fluorescent coumarin derivatives. McHale and Sheridan (*4*) mentioned the presence of cnidilin (5-isoprenyloxy-8-methoxypsoralen) in key lime and Persian lime oils. On the other hand, Sommer (*17*) recently identified

Table 3. Furocoumarins in Bergamot Oil (in ppm)

N°	product	UV–DAD	MS	fluo
2	bergapten	8.0	7.7	8.1
6	oxypeucedanin	53.5	nd[a]	nd
15	epoxybergamottin	70.3	17.8	11.9
11	bergamottin	16 312.0	15 810.0	16 487.5
	total furocoumarins	16 443.8 (16 373.5)[b]	15 835.5	16 507.5

[a] nd = not detected. [b] In brackets, total furocoumarins excluding the false oxypeucedanin peak.

Table 4. Furocoumarins in Lemon Oil (in ppm)

N°	products	UV–DAD	MS	fluo
8	byakangelicin	97.89	72.20	
5	oxypeucedanin hydrate	131.91	124.20	92.40
2	bergapten	15.69	18.50	35.10
9	heraclenin	27.46	29.60	130.60
7	byakangelicol	197.36	193.20	
6	oxypeucedanin	18.86	5.20	18.30
12	imperatorin	16.24	17.00	78.20
14	phellopterin	155.95	153.00	
13	isoimperatorin	42.46	36.70	
10	8-geranyloxypsoralen	807.15	883.40	1567.80
11	bergamottin	1253.37	1386.60	503.80
	total furocoumarins	2764.3	2919.6	2426.2

Table 5. Furocoumarins in Grapefruit Oil (in ppm)

N°	products	UV–DAD	MS	fluo
2	bergapten	513.2 (75.0)[a]	69.2	254.4
6	oxypeucedanin	700.2	nd[b]	99.6
15	epoxybergamottin	1732.1	1757.2	1722.2
11	bergamottin	428.6	410.3	365.7
	total furocoumarins	3374.1 (2235.7)[c]	2236.7	2441.9

[a] Bergapten quantified using analytical method 2 (H_2O/ACN). [b] nd = not detected. [c] In brackets, result excluding the false oxypeucedanin and including the right bergapten.

Table 6. Furocoumarins in Bitter Orange Oil (in ppm)

N°	products	UV–DAD	MS	fluo
2	bergapten	1671.1 (315.1)[a]	294.8	797.2
6	oxypeucedanin	457.8	nd[b]	1115.4
14	phellopterin	46.3	nd	
15	epoxybergamottin	814.2	814.0	784.5
10	8-geranyloxypsoralen	119.0	nd	
11	bergamottin	111.6	64.6	128.6
	total furocoumarins	1548.9 (1240.9)[c]	1173.4	2825.7

[a] Bergapten quantified using analytical method 2 (H_2O/ACN). [b] nd = not detected. [c] In brackets, results excluding false oxypeucedanin and phellopterin and including right bergapten.

14 and not cnidilin in lemon oil with LC−NMR and NOE experiments. In this work, lemon oil displayed a peak having the same retention time, the same mass spectrum, and the same UV spectrum as our reference **14**. Therefore, we confirmed the work of Sommer concerning the presence of phellopterin in lemon oil. This is logical because bykangelicol present in lemon oil is an epoxide corresponding to phellopterin.

In grapefruit oil, bergamottin and epoxybergamottin accounted for 97% of the total. Bergapten was almost negligible. However, coelution with a compound of molecular weight 260, a coumarin, probably meranzin or isomeranzin (*4*), made it impossible to quantify with UV or fluorimetry. Bergapten could be quantified using only water and acetonitrile as eluents (analytical method 2, see the Materials and Methods). With this method, bergapten eluted just between meranzin and isomeranzin. Oxypeucedanin was not present in our grapefruit oil, as shown by the LC−MS. A coelution occurred probably with a pentamethoxyflavone ($M + H^+ = 373$). These coelutions were obvious when examining UV−DAD spectra. When the right values are taken into account, the total furocoumarin contents in grapefruit oil calculated from the UV−DAD and the MS detector were the same.

In bitter orange oil, bergapten and epoxybergamottin accounted for 26 and 68% of the total furocoumarins, respectively. As in the case of grapefruit oil, bergapten coeluted with a coumarin, meranzin, or isomeranzin and quantification was impossible with UV or fluorescence detection using the analytical method 1. As for grapefruit oil, method 2 (H_2O/ACN) allowed the quantification of bergapten. Oxypeucedanin was not present in LC−MS. The peak at the expected retention time was a pentamethoxyflavone ($M + H^+ = 373$). Again, the coelutions were easily detected by examination of UV−DAD spectra. If we exclude the false results, the total furocoumarin contents obtained from the UV−DAD and from the MS detector were very close.

Because of its selectivity, LC−MS was clearly the best technique for the quantification of furocoumarins in citrus oils. However, the total furocoumarin content could be determined using an UV−DAD detection. A careful examination of UV spectra would be necessary in all cases. Although we did not analyze different oils of the same species, some guidelines could be drawn taking literature data into account (*3*−*13*, *27*). For bergamot oils, the quantification of bergamottin and bergapten would be sufficient to obtain the total furocoumarin content. For grapefruit oils (*9*), epoxybergamottin and bergamottin represented 97% of the total content. Lemon oils would require a whole range of different furocoumarins to be determined. Fortunately, no major coelution problems occurred during this analysis; therefore, the UV−DAD gave the total furocoumarin content with a good precision. In bitter orange oils, bergapten would have to be determined by using an alternative elution program or by LC−MS. In general, fluorimetry gave inconsistent results and would not be very useful except for avoiding misleading peak identifications. For those analyses where a legal problem might occur, LC−MS would be the most reliable technique.

We have developed a method for the calibration and quantification of 15 different furocoumarins by reversed-phase HPLC coupled with UV, fluorescence, and mass spectrometric detectors. This method would theoretically allow for the quantification of these products at very low levels (0.1 ppm in the injected solution) with an excellent precision using UV, fluorescence, and mass spectrometric detectors. However, when we applied our method to real cold-pressed oils, some coelution problems occurred. The UV diode array detector easily detected such coelutions. Provided that the analyst would carefully examine the UV−DAD spectra of each peak, the result obtained would be accurate enough to obtain the total furocoumarin content. A fluorescence detector was not very useful, and ambiguities could only be resolved using a mass spectrometric detector. It should be noted that cold-pressed oils generally contain a high amount of total furocoumarins and would have to be distilled prior to their use in cosmetic products to fall below the permitted level of 1 ppm.

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