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Quantitative Evaluation of Auraptene and Umbelliferone, Chemopreventive Coumarins in *Citrus* Fruits, by HPLC-UV-FL-MS

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Supporting Information

ABSTRACT: An analytical strategy, based on the development of two HPLC methods with spectrophotometric (UV), spectrofluorometric (FL), and mass spectrometric (MS) detection, has been developed to investigate the presence of and to quantitate two important chemopreventive coumarins, auraptene and umbelliferone, in foodstuffs. The analytes were determined in fruits, and fruit parts, of plants belonging to the *Citrus*, *Poncirus*, and *Fortunella* genera, to test their nutraceutical potential. The method validation has been carried out according to international guidelines, with good results in terms of precision (RSD < 6.9%) and extraction yields (>91%). Application to the quantitative analysis of auraptene and umbelliferone in several kinds of citrus fruits was successful, providing reliable and consistent data. Exploiting three different kinds of detection, the analytical methodology proposed herein has been demonstrated to be sound but versatile, as well as reliable. Performances and results were compared and always found in good agreement among themselves. Thus, this approach is suitable for the identification and simultaneous quantitation of auraptene and umbelliferone in citrus fruits, with the aim of evaluating their nutraceutical potential.

KEYWORDS: *auraptene, umbelliferone, nutraceutical, quali-quantitative analysis, citrus fruits, HPLC-UV-FL, HPLC-MS*

INTRODUCTION

The fruits of many plants belonging to the *Citrus* genus, and related genera such as *Poncirus* and *Fortunella*, are rich in health-promoting substances, such as vitamins, folates, and fibers. However, other compounds found at lower (and even trace) levels can significantly contribute to the nutraceutical potential of citrus fruits. Among these compounds, coumarins represent an important class of active substances possessing a range of different biological properties, including anticancer, antioxidant, anti-inflammatory, anticoagulant, antibacterial, and analgesic effects.[1] In recent years, some of these coumarins have been investigated as possible therapeutic or nutraceutical compounds.

Auraptene (7-((*E*)-3,7-dimethylocta-2,6-dienyloxy)-2*H*-chromen-2-one, **1**, Figure 1) is the main coumarin, which has been demonstrated to act as an anti-inflammatory, antibacterial, and immunomodulatory agent[2] and to have positive effects on cholesterol metabolism.[3] It has been shown to protect rodents against chemically induced carcinogenesis[4,5] and to possess antiproliferative and proapoptotic activities in vitro on cancer cell lines from human hepatocellular carcinoma, colorectal adenocarcinoma, and breast adenocarcinoma.[4−7] However, the first trials to test auraptene chemopreventive effects, with substance administration to animals and even human beings, are just starting to appear in the scientific literature.[8]

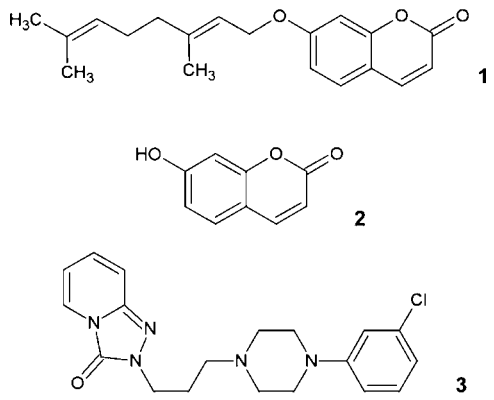

Figure 1. Chemical structures of auraptene (**1**), umbelliferone (**2**), and trazodone (**3**).

Umbelliferone (7-hydroxychromen-2-one, **2**, Figure 1) is another interesting coumarin, chemically related to auraptene,

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well-known for its strong UV absorbance, and also seems to possess some potential chemopreventive activity as well, for instance, against hepatocarcinoma in rats.[9] Moreover, both auraptene and umbelliferone have shown interesting antioxidant properties. For example, auraptene suppresses superoxide generation in leukocytes,[10] blocks the activation of the NADPH oxidase system,[11] and inhibits reactive oxygen species (ROS) formation;[12] umbelliferone decreases lipid peroxidation markers, increases endogenous antioxidant concentrations in rats,[13] and inhibits ROS generation caused by γ radiation exposure.[14]

To correctly evaluate the real nutraceutical potential of auraptene and umbelliferone, an analytical approach is necessary, to reliably identify and quantitate both compounds in foodstuffs, such as fruits belonging to different species (or hybrids) of the *Citrus*, *Poncirus*, and *Fortunella* genera, and also in their different parts (exocarp or flavedo, mesocarp or albedo, endocarp, seeds). This information could drive agronomic research toward the most promising species and cultivars to be hybridized, in the hope of obtaining fruits with pleasant taste and smell and also containing optimal levels of both coumarins.

In the past few years, much attention has been directed to the analysis of citrus and in particular of coumarins (not including auraptene or umbelliferone) in citrus fruits, with different purposes, for example, for the enantioselective analysis of chiral coumarins and psoralenes;[15] for the control of coumarin absence in bergamot essential oil;[16] for the determination of furocoumarins in citrus products;[17] and for the investigation of supercritical fluid extraction as an alternative to solvent extraction.[18]

Several analytical methods based on HPLC can be found in the literature for the analysis of auraptene[19−21] or, alternatively, umbelliferone[22,23] in a variety of citrus plants.

These methods are based on HPLC with diode array detection (HPLC-DAD),[19,21−23] except one that exploits an HPLC-MS technique.[20] Other papers describe the simultaneous determination of both analytes.[24−26] However, of the latter group, two methods analyzed auraptene and umbelliferone in animal matrices, that is, rat organs[24] and colorectal cell lines,[25] and just one method,[26] based on GC-MS, was applied to the original plant matrices, but in particular to a very limited selection of fruits (Egyptian cultivars of red grapefruit and sweet orange).

The aim of this study was the development and comparison of analytical methods, based on HPLC-UV-fluorescence (FL) and HPLC-MS, for the identification and simultaneous determination of auraptene and umbelliferone in vegetal matrices such as citrus fruits and their parts, to test their nutraceutical potential. Because the analytes possess native fluorescence, the use of spectrofluorometric detection could give many advantages: it is as feasible and almost as inexpensive as HPLC-UV or HPLC-DAD but grants much higher sensitivity and selectivity; however, none of the available methods is based on this technique.

This is the first methodology that exploits and compares three detection means for auraptene and umbelliferone analysis with nutraceutical purposes and, in particular, the only one that features HPLC with spectrofluorometric detection. The availability of different analytical methods allows the choice of the one best suiting specific research needs.

MATERIALS AND METHODS

Chemicals and Solutions. Auraptene was prepared from umbelliferone and geranyl bromide in the presence of K_2CO_3 as the base in acetone at 80 °C for 1 h following the reported methodology.[27,28] The compound was obtained in 95% yield and a purity >98%. Umbelliferone powder (≥98%) was purchased from Fluka (Milan, Italy). Trazodone (≥99.5% purity, 2-{3-[4-(3-chlorophenyl)piperazin-1-yl]propyl}[1,2,4]triazolo[4,3-*a*]pyridin-3(2*H*)-one, **3**, Figure 1), used as the internal standard (IS), HPLC grade methanol and acetonitrile, phosphoric acid (85−87%, w/w), monobasic potassium phosphate (≥98%), formic acid (98%, w/w), and 0.1 M sodium hydroxide were manufactured by Sigma-Aldrich (Milan, Italy). Ultrapure water (18.2 MΩ cm) was obtained by means of a Milli-Q apparatus from Millipore (Milford, MA, USA). Stock solutions (1 mg/mL) of the analytes and the IS were prepared by dissolving 5 mg of each pure substance in 5 mL of methanol. Standard solutions were obtained by diluting stock solutions with the mobile phase (for HPLC-UV-FL) or with methanol (for HPLC-MS). When stored at −20 °C in the dark, stock solutions were stable for at least 1 month (as assessed by HPLC-UV); standard solutions were prepared fresh every day.

Apparatus and Chromatographic Conditions. *HPLC-UV-FL.* The chromatographic system was composed of a Jasco (Tokyo, Japan) PU-980 isocratic pump, equipped with a Jasco UV-975 spectrophotometric detector and a serially connected Varian (Walnut Creek, CA, USA) 9075 fluorescence detector.

The stationary phase was a 100 mm × 3.0 mm i.d., 3 μm, Pack Pro C18 column with a 4 mm × 3 mm i.d. guard column of the same material (YMC, Kyoto, Japan). The mobile phase was composed of a mixture of acetonitrile (60%, v/v) and a pH 2.7, 50 mM, aqueous phosphate buffer (40%, v/v). The flow rate was 0.5 mL/min, and the injections were carried out through a 20 μL loop. UV absorbance was monitored at 330 nm, and fluorescence intensity was monitored at 390 nm, with excitation at 330 nm.

HPLC-MS. The chromatographic apparatus was a Waters (Milford, MA, USA) Alliance e2695 system coupled to a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer. Separations were obtained on a 50 mm × 2.1 mm i.d., 1.8 μm, Zorbax Rapid Resolution SB-C18 column with a 4 mm × 2.1 mm i.d. guard column of the same material (Agilent, Waldbronn, Germany), using a mobile phase composed of 0.1% (v/v) formic acid in acetonitrile (A) and 0.1% (v/v) formic acid in water (B), flowing at 0.3 mL/min. The gradient program of the mobile phase composition started with a 5:95 (v/v) A/B ratio and then ramped up linearly to 95% (v/v) of A over 4 min; this ratio was maintained for 2 min and then ramped down linearly to 5% (v/v) of A over 1 min. The injection volume was 5 μL, and injections were carried out through the autosampler integrated into the Alliance system.

Tandem mass spectrometry acquisition was carried out in multiple reaction monitoring (MRM) scan mode, using an electrospray ionization source operating in positive mode (ESI+). The working conditions were as follows: ion source voltage, 3.4 kV; ion source temperature, 120 °C; desolvation temperature, 150 °C; desolvation gas flow, 150 L/h; cone gas flow, 50 L/h; extractor potential, 9 V; RF lens voltage, 0.7 V; collision entrance potential, 1 V; collision exit potential, 2 V; gas cell Pirani pressure, 4.4×10^{-3} mbar. Cone voltage was 24 V for auraptene and 56 V for umbelliferone. Collision energy was 12 V for auraptene and 54 V for umbelliferone. Nitrogen was used as the desolvation gas and was generated from pressurized air by an N_2 LC-MS (Claind, Lenno, Italy) nitrogen generator; collision gas was 99.995% argon (SIAD, Bergamo, Italy). The chosen analyte and IS transitions were m/z 299.4 → 163.1 for auraptene, m/z 163.1 → 77.3 for umbelliferone, and m/z 372.9 → 176.1 for the IS. The dwell times were set at 300 ms for each transition.

Citrus Fruit Pretreatment. Samples of fruits belonging to the following species and hybrids were analyzed: *Citrus* × *aurantium* L. (bitter orange), white and pink *C.* × *paradisi* Macfad. (grapefruit), *C.* × *aurantifolia* (Christm.) Swingle (lime), *C.* × *bergamia* Risso (bergamot), *C.* × *sinensis* Osbeck (sweet orange), *C. reticulata* Blanco

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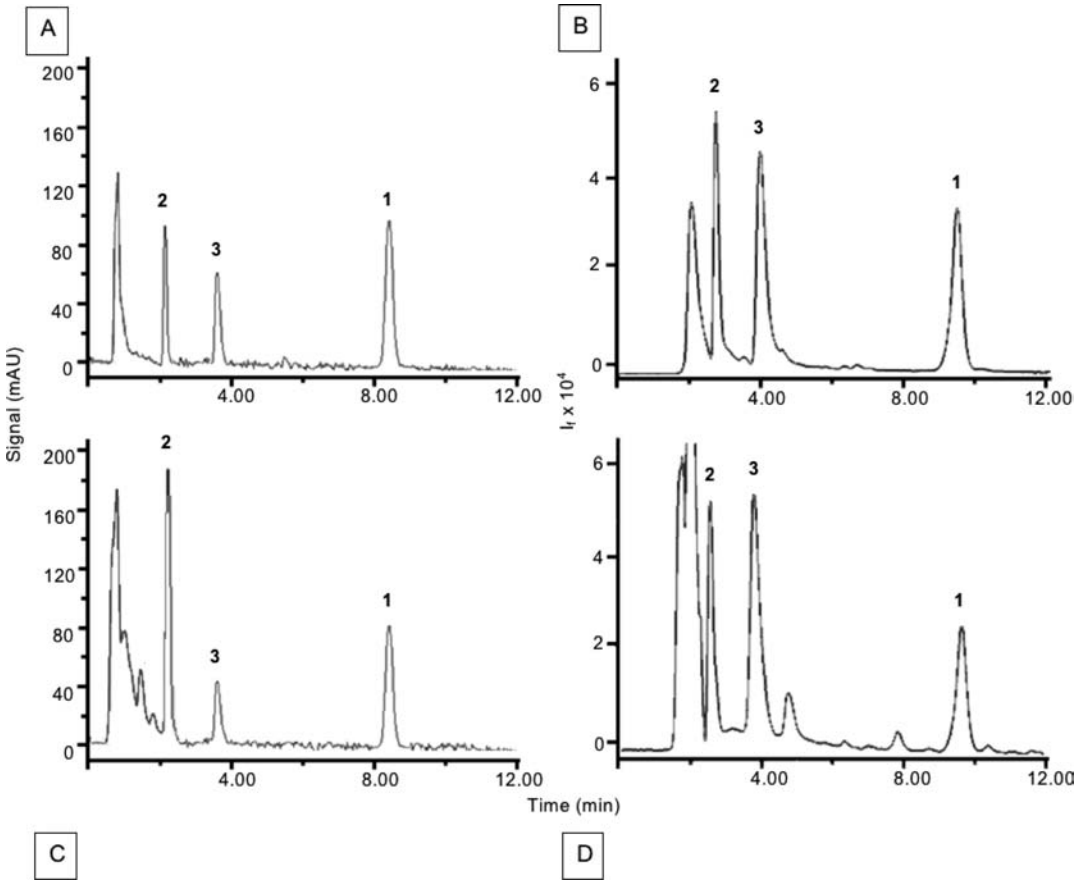

Figure 2. Chromatograms obtained from (A) the HPLC-UV analysis of a standard solution (50 ng/mL of each analyte), (B) the HPLC-FL analysis of a standard solution (50 ng/mL of each analyte), (C) the HPLC-UV analysis of a *P. trifoliata* exocarp sample, and (D) the HPLC-FL analysis of a white *C.* × *paradisi* endocarp sample. Peak identification: 1, auraptene; 2, umbelliferone; 3, internal standard.

(tangerine), *C.* × *limon* (L.) Osbeck (lemon), *Poncirus trifoliata* (L.) Raf. (trifoliate orange), and *Fortunella japonica* Swingle (kumquat). Some of these fruits had different geographic origins: *C.* × *aurantium* fruits were collected in Bologna, Bari, and Cosenza (Italy); *C.* × *paradisi* fruits in Catania (Italy); *C.* × *aurantifolia* fruits in Bari, Cosenza, and Palermo (Italy); *C.* × *bergamia* fruits in Reggio Calabria (Italy); *C.* × *sinensis*, *C. reticulata*, and *C.* × *limon* fruits in Bari and Bologna (Italy); *P. trifoliata* fruits in Bologna, Ferrara, and Catania (Italy); and *F. japonica* fruits from China.

At least three fruits of each kind were analyzed, from the same source and at the same apparent ripeness stage, to evaluate also the natural variability of analyte content. All procedures were carried out using light-absorbing (high-actinic) containers and away from direct light sources. The fruits were individually weighed, then accurately peeled, removing (if possible) only the external, colored exocarp (flavedo); then, the white mesocarp (albedo) was removed, leaving the endocarp (i.e., the "flesh" part, including the juice sacs and segment membrane) with the seeds. The endocarp was cut in small pieces (collecting the juice and the pieces in the same glass bowl), and the seeds (if present) were removed and stored separately. However, it was not possible to separate all fruits in these different parts. The sample pretreatment was the same for all fruit parts: they were weighed, dried to constant weight in a ventilated oven, in the dark at 40 °C, and finely ground to a powder. An amount of 100 mg of powder was extracted with 2 mL of methanol, vortexed for 10 min, and centrifuged at 1400*g* for 3 min; the supernatant was separated. The extraction was repeated with the same volume of solvent, and the supernatants were combined, dried (rotary evaporator), redissolved in 100 μL of mobile phase, suitably diluted with the mobile phase, and injected into the chosen HPLC system. The two coumarin concentrations were obtained by interpolation on the respective calibration curves.

Method Validation. *Calibration Curves.* Analyte standard solutions at seven different concentrations, containing the IS at a constant concentration, were injected into the HPLC system. The procedure was carried out in triplicate for each concentration. The analyte concentrations were as follows: HPLC-UV, 10, 25, 50, 75, 100, 250, and 500 ng/mL for auraptene and 15, 30, 50, 75, 100, 250, and 500 ng/mL for umbelliferone; HPLC-FL, 1, 5, 10, 50, 100, 250, and 500 ng/mL for auraptene and 2, 10, 20, 50, 100, 250, and 500 ng/mL for umbelliferone; HPLC-MS, 0.5, 2, 5, 10, 50, 100, and 250 ng/mL for both auraptene and umbelliferone. The analyte/IS peak area ratios (pure numbers) obtained were plotted against the corresponding concentrations of the analyte (expressed as ng/mL) and the calibration curves constructed by means of the least-squares method.

The values of the limit of quantitation (LOQ) and the limit of detection (LOD) (see the Supporting Information) were calculated according to 3rd AAPS/FDA Bioanalytical Workshop[29] guidelines, as the analyte concentrations that give rise to peaks of heights of 10 and 3 times the baseline noise, respectively.

Extraction Yield (Absolute Recovery). Representative fruit part samples (*C.* × *aurantium* mesocarp, *C.* × *paradisi* endocarp, and *P. trifoliata* exocarp) were subjected to the extraction procedure and analyzed. Then, the same materials were subjected to the extraction and analysis procedure again. The results (analyte/IS peak area ratios) of the first extraction and analysis were compared with the corresponding results obtained from the sum of all extractions. The extraction yield was considered to be complete when a further extraction, upon injection, produced analyte amounts lower than the LOD.

Precision. A fruit sample was analyzed six times within the same day to obtain repeatability (intraday precision) and six times over different days to obtain intermediate precision (interday precision), both expressed as percentage relative standard deviation (RSD%) values.

Accuracy. Method accuracy was tested by means of percentage recovery assays: known amounts of standard solutions of the analytes and the IS were added to real fruit part samples, which had been already analyzed. The added concentrations corresponded to the lower limit, an intermediate value, and a high value of the respective calibration curves. The percentage recovery was obtained by comparing the added analyte concentration to the difference between the total concentration obtained from the analysis and the original analyte concentration.

Theoretical log*P* values of the analytes were calculated using Advanced Chemistry Development's (Toronto, ON, Canada) ACD/LogP v.11.0 software (copyright 1994−2011 ACD/Laboratories).

RESULTS AND DISCUSSION

HPLC-UV-FL Method Development. The two analytes possess widely different lipophilicity characteristics, with auraptene (log*P* = 5.2) being much more lipophilic than umbelliferone (log*P* = 1.6) due to its geranyl side chain. For this reason, it was decided to use reversed-phase HPLC with a C18 column to simultaneously analyze both compounds within acceptable run times. Using this kind of sorbent and a mobile phase relatively rich in organic modifier (acetonitrile), both analytes and the IS (trazodone) are eluted within 9 min. The corresponding HPLC-UV chromatogram of a standard solution (containing 50 ng/mL of each analyte and 100 ng/mL of the IS) is shown in Figure 2A. As can be seen, the analytes and the IS are baseline resolved, and symmetrical peaks were obtained. The use of a column with small diameter (3.0 mm) and particle size (3 μm) grants good column efficiency and relatively short run times while also requiring reduced volumes (0.5 mL/min) of mobile phase and, thus, of organic solvents.

Preliminary spectrofluorometric assays ascertained that both analytes are natively fluorescent under the experimental conditions, and this characteristic was exploited to obtain a HPLC method with tandem UV-FL detection. The spectrofluorometric detection grants higher sensitivity and selectivity than UV while avoiding the need for complicated derivatization procedures. The chromatogram of a standard solution (containing 50 ng/mL of each analyte and 100 ng/mL of the IS) obtained by HPLC-FL analysis is reported in Figure 2B: the increase in sensitivity is apparent from the higher signal-to-noise ratio, and the other chromatographic performance parameters remain substantially satisfactory, although significant dead volume, with some efficiency loss, was introduced into the system when the two detectors were connected in series.

HPLC-MS Method Development. With regard to the HPLC-MS method, due to the extreme selectivity of the technique, a fast, linear composition gradient was set up to further shorten run times, which were thus reduced to 6 min. The chromatogram of a standard solution containing 10 ng/mL of each analyte and 20 ng/mL of the IS is reported in Figure 3A; again, peaks are symmetrical and complete resolution is achieved; although not strictly necessary in HPLC-MS, analyte peak resolution is still desirable.

Sample Pretreatment Procedure Development. Sample pretreatment was carried out on the dried matrix to reduce the water sample content, which is a source of variability; using the dried sample also greatly simplifies the application of water-miscible solvents. It was verified that all specimens were dry enough to be ground after being treated in a ventilated oven at 40 °C. Higher temperatures were tried to reduce the drying time, but significant analyte losses were observed. Light was also taken into account, because umbelliferone and other

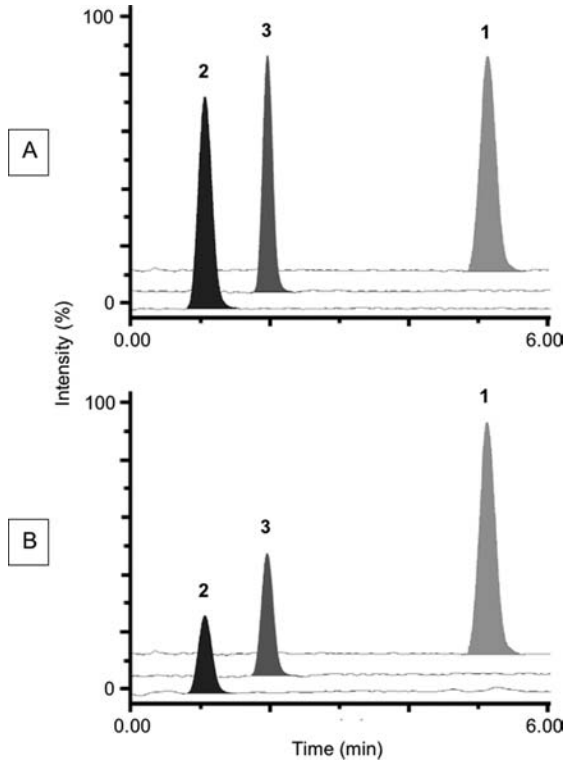

Figure 3. Chromatograms obtained from (A) the HPLC-MS analysis of a standard solution (10 ng/mL of each analyte) and (B) the HPLC-MS analysis of a whole *F. japonica* fruit. Peak identification: 1, auraptene; 2, umbelliferone; 3, internal standard.

coumarins are known to be photolabile;[30] it was found that typical exposure of the analytes to normal laboratory light during sample handling causes a significant loss of umbelliferone. For this reason, all procedures were carried out away from direct light sources and using light-absorbing (high-actinic) containers. A solvent extraction was first tried as the sample pretreatment step, using different solvents and solvent mixtures, such as diethyl ether, ethyl acetate, methanol/ethyl acetate, methanol, methanol/water, and water. No pH adjustment was tried, because the analytes do not possess easily ionizable functions. Most solvents proved to be unsatisfactory, due to either unequal extraction of the two analytes or insufficient purification of the extract (detected as interference in the HPLC-UV-FL method and as ionic suppression in the HPLC-MS method). However, methanol gave the most promising results: its relatively high hydrophilicity granted similar extraction yields for both analytes. Extraction assays proved that two steps (with 2 mL of methanol per 100 mg of dried matter) were sufficient to completely solubilize both auraptene and umbelliferone: in fact, the third extraction step with methanol contained negligible analyte concentrations.

Some fruit parts contained very high concentrations of the analytes, which would fall outside the calibration ranges if the corresponding extracts were injected as such; for this reason, the most concentrated samples were suitably diluted before injection.

Method Validation. The methods were separately validated according to international regulatory guidelines (e.g., those of the U.S. Food and Drug Administration,[31] as refined by the third AAPS/FDA Bioanalytical Workshop).[29,32] Satisfactory linearity values (r^2 > 0.9990) were obtained on standard solutions for all analytes. Extraction yield (absolute

Table 1. Extraction Yield and Precision Data for Auraptene and Umbelliferone in Citrus Fruit Parts

compound	method	fruit/part	mean extraction yield,[a] %	repeatability,[a] RSD%	intermediate precision,[a] RSD%
auraptene	HPLC-UV	*C.* × *aurantium*/mesocarp	93.4	2.0	2.8
		C. × *paradisi*/endocarp	92.7	3.2	4.1
		P. trifoliata/exocarp	91.1	5.5	6.5
	HPLC-FL	*C.* × *aurantium*/mesocarp	100.8	3.2	3.5
		C. × *paradisi*/endocarp	99.3	4.8	5.0
		P. trifoliata/exocarp	97.7	5.5	6.8
	HPLC-MS	*C.* × *aurantium*/mesocarp	100.5	2.4	3.5
		C. × *paradisi*/endocarp	100.5	0.4	2.8
		P. trifoliata/exocarp	96.5	4.9	4.7
umbelliferone	HPLC-UV	*C.* × *aurantium*/mesocarp	98.0	1.8	2.3
		C. × *paradisi*/endocarp	98.2	4.0	4.6
		P. trifoliata/exocarp	99.4	4.2	5.5
	HPLC-FL	*C.* × *aurantium*/mesocarp	92.8	5.2	6.0
		C. × *paradisi*/endocarp	93.6	4.3	4.7
		P. trifoliata/exocarp	92.2	5.2	6.7
	HPLC-MS	*C.* × *aurantium*/mesocarp	99.0	0.9	3.8
		C. × *paradisi*/endocarp	98.8	0.5	1.5
		P. trifoliata/exocarp	100.2	2.2	4.4
IS	HPLC-UV	100 ng/mL[b]	99.1	1.8	2.0
	HPLC-FL	100 ng/mL[b]	99.3	1.3	3.9
	HPLC-MS	20 ng/mL[b]	97.2	2.4	4.4

[a] $n = 6$. [b] IS concentration added to the fruit part sample.

recovery) and precision assays were carried out on fruit samples, and the complete results for the two methods are reported in Table 1. As one can see, mean extraction yield values were good, always >91% for all analytes (>97% for the IS). Precision results, expressed as RSD, were always <5.6% (2.5% for the IS) for repeatability and <6.9% (4.5% for the IS) for intermediate precision.

Analysis of Real Samples. After development and validation, the method was applied to the analysis of the parts of the following *Citrus*, *Fortunella*, and *Poncirus* fruits: bitter orange, grapefruit, lime, bergamot, sweet orange, tangerine, lemon, trifoliate orange, and kumquat.

The results were very satisfactory: the two coumarins were identified and quantitated in all of them. As representative examples, the chromatograms obtained from the analysis of a *P. trifoliata* exocarp (by HPLC-UV), of a white *C.* × *paradisi* endocarp (by HPLC-FL), and of a whole *F. japonica* fruit (HPLC-MS) are shown in panels C and D of Figure 2 and panel B of Figure 3, respectively. As one can see, the matrices are remarkably clean, and no evident interference is present.

The results obtained with the different proposed methods were always in good agreement: the differences in the concentration estimates were always <7%. The complete data are reported in Table 2.

Although the data set was very limited, some simple statistical comparisons (independent two-sample Student t tests with $p < 0.01$) were made, and the following results were obtained: (1) the exocarp, mesocarp, and endocarp of *P. trifoliata* grown in warm climates contain auraptene levels that are significantly higher than those of all other studied fruit parts; (2) the differences in *P. trifoliata* auraptene content between warm and cold climates are significant; (3) *P. trifoliata* seeds contain significantly lower auraptene levels than the other fruit parts in all cases, without significant differences among climates; (4) *C.* × *aurantium* endocarp contains auraptene levels significantly higher than those of other fruits, excluding *P. trifoliata* (the auraptene content of other parts is not significantly different from that of other fruits); (5) the umbelliferone content of *C.* × *aurantium* exocarp is significantly higher than those of all other fruits and parts (including other *C.* × *aurantium* parts), but no significant difference exists between climates.

In absolute terms, the highest concentration of auraptene (>5 mg/g) has been found in the trifoliate orange mesocarp and endocarp (i.e., the "flesh" part). On the other hand, the fruit part that contains the highest levels of umbelliferone (>1 mg/g) is bitter orange endocarp. These concentrations correspond to >10 mg of auraptene in a whole trifoliate orange fruit (mean weight = 30 g) and >4 mg of umbelliferone in a whole bitter orange fruit (mean weight = 80 g). It seems that warmer climates (Catania vs Bologna, Ferrara) contribute to the high levels of auraptene in trifoliate orange. Interestingly, the analyzed white grapefruits seem to contain much higher coumarin levels than pink grapefruits (about 10 times, $p < 0.001$), although the highest auraptene levels are still much lower than those found in *P. trifoliata* fruits.

Of course, these are just preliminary results, and it is possible that the analyte concentrations will show different behaviors when a larger variety of samples and/or other kinds of citrus fruits are analyzed.

Accuracy. Method accuracy was evaluated by means of recovery assays, by adding three different concentrations of each analyte to already analyzed samples and calculating analyte recovery values. Mean recovery values were always between 95 and 105% for all matrices. Thus, method accuracy is satisfactory.

Method Comparison. The comparison of performances and results obtained with the proposed methodology gives interesting insight into the options available for the analysis of auraptene and umbelliferone in citrus fruits. In particular, UV

Table 2. Analysis of Different Citrus Fruits and Parts for Auraptene and Umbelliferone

fruit	fruit part	analyte concentration[a] (μg/g) ± SD	
		auraptene	umbelliferone
trifoliate orange (*P. trifoliata*) from Bologna	exocarp	1211 ± 20	113 ± 4
	mesocarp + endocarp	1072 ± 21	19 ± 0.8
	seeds	663 ± 19	12 ± 0.5
trifoliate orange (*P. trifoliata*) from Ferrara	exocarp	1247 ± 27	103 ± 6
	mesocarp + endocarp	1807 ± 33	31 ± 0.1
	seeds	978 ± 22	14 ± 0.2
trifoliate orange (*P. trifoliata*) from Catania	exocarp	3432 ± 59	44 ± 2
	mesocarp + endocarp	5786 ± 102	29 ± 0.8
	seeds	980 ± 15	15 ± 0.8
grapefruit (*C.* × *paradisi*), white, from Catania	exocarp	18 ± 1	11 ± 0.6
	mesocarp	262 ± 11	122 ± 8
	endocarp	715 ± 12	222 ± 12
grapefruit (*C.* × *paradisi*), pink, from Catania	exocarp	1 ± 0.05	0.8 ± 0.05
	mesocarp	25 ± 1	17 ± 1
	endocarp	83 ± 4	190 ± 12
kumquat (*F. margarita*), from China	whole fruit	317 ± 24	151 ± 10
lime (*C.* × *aurantifolia*) from Bari	exocarp	243 ± 10	35 ± 1
	mesocarp + endocarp	6 ± 0.2	3 ± 0.1
lime (*C.* × *aurantifolia*) from Cosenza	exocarp	331 ± 29	44 ± 0.8
	mesocarp + endocarp	3 ± 0.2	0.7 ± 0.03
lime (*C.* × *aurantifolia*) from Palermo	exocarp	395 ± 33	59 ± 0.9
	mesocarp + endocarp	8 ± 0.4	5 ± 0.1
bergamot (*C.* × *bergamia*) from Reggio Calabria	exocarp	9 ± 0.5	0.9 ± 0.5
	mesocarp + endocarp	0.4 ± 0.01	0.2 ± 0.01
bitter orange (*C.* × *aurantium*) from Bologna	exocarp	338 ± 18	31 ± 2
	mesocarp	244 ± 10	72 ± 3
	endocarp	1205 ± 66	1038 ± 54
bitter orange (*C.* × *aurantium*) from Bari	exocarp	267 ± 9	14 ± 0.7
	mesocarp	183 ± 11	89 ± 5
	endocarp	1184 ± 41	972 ± 40
bitter orange (*C.* × *aurantium*) from Cosenza	exocarp	441 ± 31	50 ± 2
	mesocarp	363 ± 14	99 ± 5
	endocarp	1201 ± 41	991 ± 44
sweet orange (*C.* × *sinensis*) from Bari	exocarp	0.3 ± 0.01	0.1 ± 0.01
	mesocarp	0.03 ± 0.01	0.1 ± 0.01
	endocarp	0.4 ± 0.01	0.1 ± 0.01
sweet orange (*C.* × *sinensis*) from Bologna	exocarp	3.3 ± 0.1	1.0 ± 0.04
	mesocarp	0.2 ± 0.01	0.3 ± 0.01
	endocarp	0.6 ± 0.02	0.2 ± 0.01
tangerine (*C. reticulata*) from Bari	exocarp + mesocarp	0.1 ± 0.01	0.1 ± 0.02
	endocarp	0.2 ± 0.04	0.1 ± 0.04
tangerine (*C. reticulata*) from Bologna	exocarp + mesocarp	0.2 ± 0.07	0.2 ± 0.04
	endocarp	0.2 ± 0.07	0.1 ± 0.03

Table 2. continued

fruit	fruit part	analyte concentration[a] (μg/g) ± SD	
		auraptene	umbelliferone
lemon (*C.* × *limon*) from Bari	exocarp	0.3 ± 0.08	0.1 ± 0.04
	mesocarp	1 ± 0.02	0.1 ± 0.03
	endocarp	1 ± 0.02	0.5 ± 0.01
lemon (*C.* × *limon*) from Bologna	exocarp	0.2 ± 0.02	0.2 ± 0.03
	mesocarp	0.8 ± 0.01	0.4 ± 0.01
	endocarp	0.5 ± 0.04	0.1 ± 0.01

[a]Each value is the mean of the results obtained with the three detection means: μg of analyte per g of fruit part.

detection is feasible and inexpensive, but suitable only when coumarins are present at middle−high concentrations, being limited with regard to sensitivity and intrinsic selectivity. Thus, it could be best used for preliminary assays or when analyte levels are expected (or already known) to be relatively high.

Coupling the HPLC method to spectrofluorometric detection surely grants much better sensitivity and selectivity thanks to the native fluorescence of the analytes. Moreover, the HPLC-FL method retains satisfactory speed because there is no need for complicated and time-consuming derivatization procedures: thus, it could be a good choice for routine analysis and for basic research in this field. It should be noted that this is the first and only methodology that features HPLC-FL for simultaneous auraptene and umbelliferone quantitation with nutraceutical purposes. Finally, the HPLC-MS method granted the best results for most validation parameters, and its high sensitivity and selectivity are great advantages in several situations. The fast chromatographic system combined with a fully automated setup achieves higher throughput and outstanding performance, at the cost of higher acquisition and maintenance expenses. The method could be used for advanced research purposes, granting the best results in many respects. Exploiting three different detection means, the developed methodology covers a wide range of needs and economic and scientific possibilities.

The two methods, based on HPLC-UV-FL and HPLC-MS, have been developed and validated for the identification and quantitation of auraptene and umbelliferone in several fruits (and parts) of the *Citrus*, *Fortunella*, and *Poncirus* genera having different origins. The sample pretreatment procedure, based on a fast and feasible solvent extraction, grants very good extraction yields (>91%) and matrix purification; validation assays also provided satisfactory results in terms of linearity and precision (RSD < 6.9%). It should be noted that, to the best of our knowledge, just one GC-MS method[26] is available for the simultaneous analysis of both auraptene and umbelliferone in fruits of two *Citrus* species (not in *Poncirus* or *Fortunella* fruits). With respect to this method, those proposed herein have been applied to many more kinds of fruits and also to their different parts; moreover, being based on three different detection principles, they can provide a wider choice to scientists and analysts. All other published methods either considered just one of the two analytes[19−23] or were not applied to plant materials.[24,25] Application of the proposed methods to real fruits gave interesting results: *P. trifoliata* fruits grown in warm climates are one of the best sources of auraptene, and the highest umbelliferone concentrations are found in *C.* × *aurantium*. Because these fruits are seldom eaten as such, different approaches should be explored (e.g., preparation of processed foods and beverages, such as jams, candies, and juices) to take full advantage of their nutraceutical potential. The study of new hybrids with *Citrus* species having more pleasant taste and relatively high coumarins content could also be a viable strategy.

The proposed analytical methodology is a significant improvement and useful tool for further research on the nutraceutical properties of citrus fruits and foods prepared from them. The study of possible synergies between the biological activities of the two coumarins when present in the same foodstuff is one example of this kind of research; another example could be the evaluation of the long-term health effects of diets including fruits having a known content of auraptene and/or umbelliferone or the effectiveness of food supplements as sources of these compounds.

A study is currently in progress for the evaluation of the changes in auraptene and umbelliferone levels during fruit ripening and in perspective for the application to other potentially nutraceutical coumarins and to other kinds of citrus fruits.

■ ASSOCIATED CONTENT

Supporting Information

Table 1s, Calibration Data and LOQ and LOD Values on Standard Solutions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ REFERENCES

(1) Wu, L.; Wang, X.; Xu, W.; Farzaneh, F.; Xu, R. The structure and pharmacological functions of coumarins and their derivatives. *Curr. Med. Chem.* **2009**, *16*, 4236−4260.

(2) Epifano, F.; Genovese, S.; Curini, M. Auraptene: phytochemical and pharmacological properties. In *Phytochemistry Research Progress*; Matsumoto, T., Ed.; Nova Science Publishers: New York, 2008; pp 145−162.
(3) De Medina, P.; Genovese, S.; Paillasse, M.; Caze-Subra, S.; Bystricky, K.; Curini, M.; Silvente-Poirot, S.; Epifano, F.; Poirot, M. Auraptene is an inhibitor of cholesterol esterification and a modulator of estrogen receptor. *Mol. Pharmacol.* **2010**, *78*, 827−836.
(4) Tanaka, T.; de Azevedo, M. B.; Durán, N.; Alderete, J. B.; Epifano, F.; Genovese, S.; Tanaka, M.; Tanaka, T.; Curini, M. Colorectal cancer chemoprevention by 2β-cyclodextrin inclusion compounds of auraptene and 4-geranyloxyferulic acid. *Int. J. Cancer* **2010**, *126*, 830−840.
(5) Krishnan, P.; Yan, K. J.; Windler, D.; Tubbs, J.; Grand, R.; Li, B. D.; Aldaz, C. M.; McLarty, J.; Kleiner-Hancock, H. E. Citrus auraptene suppresses cyclin D1 and significantly delays *N*-methyl nitrosourea induced mammary carcinogenesis in female Sprague-Dawley rats. *BMC Cancer* **2009**, *9*, 259; URL http://www.biomedcentral.com/1471-2407/9/259 (July 12, 2012).
(6) Zheng, Q.; Hirose, Y.; Yoshimi, N.; Murakami, A.; Koshimizu, K.; Ohigashi, H.; Sakata, K.; Matsumoto, Y.; Sayama, Y.; Mori, H. Further investigation of the modifying effect of various chemopreventive agents on apoptosis and cell proliferation in human colon cancer cells. *J. Cancer Res. Clin. Oncol.* **2002**, *128*, 539−546.
(7) Ohnishi, H.; Asamoto, M.; Tujimura, K.; Hokaiwado, N.; Takahashi, S.; Ogawa, K.; Kuribayashi, M.; Ogiso, T.; Okuyama, H.; Shirai, T. Inhibition of cell proliferation by nobiletin, a dietary phytochemical, associated with apoptosis and characteristic gene expression, but lack of effect on early rat hepatocarcinogenesis in vivo. *Cancer Sci.* **2004**, *95*, 936−942.
(8) ARKRAY, Inc. The anti-metabolic syndrome and cholesterol improving effects from *Citrus hassaku* oil. 66th Annual Meeting of the Japanese Society of Nutrition and Food Science, Sendai, May 18−20, 2012; URL http://www.arkray.co.jp/english/ex/release24.html (July 12, 2012).
(9) Senthilkumar, G. P.; Ashokkumar, B.; Suresh, R.; Mazumder, U. K.; Gupta, M. Anticarcinogenic potential and biomarker enzymes level of umbelliferone against diethylnitrosoamine induced hepatocarcinoma in rats. EASL 2009, the 44th Annual Meeting of the European Association for the Study of the Liver, Copenhagen, April 22−26, 2009; URL http://www.kenes.com/easl2009/posters/Abstract419.htm (July 12, 2012).
(10) Murakami, A.; Kuki, W.; Takahashi, Y.; Yonei, H.; Nakamura, Y.; Ohto, Y.; Ohigashi, H.; Koshimizu, K. Auraptene, a *Citrus* coumarin, inhibits 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion in ICR mouse skin, possibly through suppression of superoxide generation in leukocytes. *Jpn. J. Cancer Res.* **1997**, *88*, 443−452.
(11) Tanaka, T. Chemoprevention of oral carcinogenesis. *Oral Oncol. Eur. J. Cancer* **1995**, *31B*, 3−15.
(12) Sciullo, E. M.; Vogel, C. F.; Wu, D.; Murakami, A.; Ohigashi, H.; Matsumura, F. Effects of selected food phytochemical in reducing the toxic actions of TCDD and *p,p′*-DDT in U937 macrophages. *Arch. Toxicol.* **2010**, *84*, 957−966.
(13) Ramesh, B.; Pugalendi, K. V. Antioxidant role of umbelliferone in STZ-diabetic rats. *Life Sci.* **2006**, *79*, 306−310.
(14) Kanimozhi, G.; Prasad, N. R.; Ramachandran, S.; Pugalendi, K. V. Umbelliferone modulates γ-radiation induced reactive oxygen species generation and subsequent oxidative damage in human blood lymphocytes. *Eur. J. Pharmacol.* **2011**, *672*, 20−29.
(15) Dugo, P.; Russo, M.; Sarò, M.; Carnovale, C.; Bonaccorsi, I.; Mondello, L. Multidimensional liquid chromatography for the determination of chiral coumarins and furocoumarins in *Citrus* essential oils. *J. Sep. Sci.* **2012**, *35*, 1828−1836.
(16) Belsito, E. L.; Carbone, C.; Di Gioia, M. L.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C.; Viscomi, M. C. Comparison of the volatile constituents in cold-pressed bergamot oil and a volatile oil isolated by vacuum distillation. *J. Agric. Food Chem.* **2007**, *55*, 7847−7851.
(17) Gorgus, E.; Lohr, C.; Raquet, N.; Guth, S.; Schrenk, D. Limettin and furocoumarins in beverages containing citrus juices or extracts. *Food Chem. Toxicol.* **2010**, *48*, 93−98.
(18) Teng, W.-Y.; Chen, C.-C.; Chung, R.-S. HPLC comparison of supercritical fluid extraction and solvent extraction of coumarins from the peel of *Citrus maxima* fruit. *Phytochem. Anal.* **2005**, *16*, 459−462.
(19) Ogawa, K.; Kawasaki, A.; Yoshida, T.; Nesumi, H.; Nakano, M.; Ikoma, Y.; Yano, M. Evaluation of auraptene content in citrus fruits and their products. *J. Agric. Food Chem.* **2000**, *48*, 1763−1769.
(20) Chen, H.-F.; Zhang, W.-G.; Yuan, J.-B.; Li, Y.-G.; Yang, S.-L.; Yang, W.-L. Simultaneous quantification of polymethoxylated flavones and coumarins in *Fructus aurantii* and *Fructus aurantii immaturus* using HPLC-ESI-MS/MS. *J. Pharm. Biomed.* **2012**, *59*, 90−95.
(21) Mondello, L.; Herrero, M.; Kumm, T.; Dugo, P.; Cortes, H.; Dugo, G. Quantification in comprehensive two-dimensional liquid chromatography. *Anal. Chem.* **2008**, *80*, 5418−5424.
(22) Chu, J.; Li, S.-L.; Yin, Z.-Q.; Ye, W.-C.; Zhang, Q.-W. Simultaneous quantification of coumarins, flavonoids and limonoids in *Fructus Citri Sarcodactylis* by high performance liquid chromatography coupled with diode array detector. *J. Pharm. Biomed.* **2012**, *66*, 170−175.
(23) Chuang, C.-C.; Wen, W.-C.; Sheu, S.-J. Origin identification on the commercial samples of *Aurantii Fructus*. *J. Sep. Sci.* **2007**, *30*, 1235−1241.
(24) Kuki, W.; Hosotani, K.; Ohigashi, H.; Murakami, A. Metabolism and absorption of auraptene (7-geranyloxylcoumarin) in male SD rats: comparison with 7-ethoxycoumarin. *Nutr. Cancer* **2008**, *60*, 368−372.
(25) Murakami, A.; Wada, K.; Ueda, N.; Sasaki, K.; Haga, M.; Kuki, W.; Takahashi, Y.; Yonei, H.; Koshimizu, K.; Ohigashi, H. In vitro absorption and metabolism of a citrus chemopreventive agent, auraptene, and its modifying effects on xenobiotic enzyme activities in mouse livers. *Nutr. Cancer* **2000**, *36*, 191−199.
(26) Mohamed, T. K. Chemical constituents and antioxidant activity of *Citrus paradisi* (star-ruby red grapefruit) and *Citrus sinensis* (blood sweet orange) Egyptian cultivars. *Asian J. Chem.* **2004**, *16*, 1753−1764.
(27) Curini, M.; Epifano, F.; Maltese, F.; Marcotullio, M. C.; Prieto Gonzales, S.; Rodriguez, J. C. Synthesis of collinin, an antiviral coumarin. *Aust. J. Chem.* **2003**, *56*, 59−60.
(28) Curini, M.; Epifano, F.; Maltese, F.; Marcotullio, M. C.; Tubaro, A.; Altinier, G.; Gonzales, S. P.; Rodriguez, J. C. Synthesis and anti-inflammatory activity of natural and semisynthetic geranyloxycoumarins. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2241−2243.
(29) Viswanathan, C. T.; Bansal, S.; Booth, B.; DeStefano, A. J.; Rose, M. J.; Sailstad, J.; Shah, V. P.; Skelly, J. P.; Swann, P. G.; Weiner, R. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *Pharm. Res.* **2007**, *24*, 1962−1973.
(30) Fink, D. W.; Koehler, W. R. pH effects on fluorescence of umbelliferone. *Anal. Chem.* **1970**, *42*, 990−993.
(31) U.S. Food and Drug Administration, Center for Drug Evaluation and Research (CDER). Guidance for industry: bioanalytical method validation, May 2001; URL http://www.fda.gov/CDER/GUIDANCE/4252fnl.pdf (July 12, 2012).
(32) Bansal, S.; DeStefano, A. Key elements of bioanalytical method validation for small molecules. *AAPS J.* **2007**, *9*, E109−E114.