JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Coumarin and Furanocoumarin Quantitation in Citrus Peel via Ultraperformance Liquid Chromatography Coupled with Mass Spectrometry (UPLC-MS)

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Supporting Information

ABSTRACT: Coumarins and furanocoumarins are secondary metabolites commonly found in citrus plants. These molecules are allelochemical compounds in plants that have controversial effects on humans, such as phototoxicity and the commonly described interactions with drugs, referred to as the "grapefruit juice effect". Thus, it is important to develop a reliable method to identify and quantitate the coumarins and furanocoumarins in citrus extracts. For this purpose, we herein describe an ultraperformance liquid chromatography coupled with mass spectrometry (UPLC-MS)-based method. We first developed a rapid UPLC method (20 min) to separate the isomers of each furanocoumarin. A subsequent single ion monitoring MS detection method was performed to distinguish between the molecules, which were possibly coeluting but had different molecular weights. The method was successfully used to separate and quantitate 6 coumarins and 21 furanocoumarins in variable amounts within peel extracts (flavedo and albedo) of 6 varieties of *Citrus* (sweet orange, lemon, grapefruit, bergamot, pummelo, and clementine). This method combines high selectivity and sensitivity in a rapid analysis and is useful for fingerprinting *Citrus* species via their coumarin and furanocoumarin contents.

KEYWORDS: *coumarin, furanocoumarin, quantitation, citrus, UPLC-MS, Rutaceae*

INTRODUCTION

Citrus is one of the most important fruit crops grown in the world, with 129 million tons in 2011.[1] Citrus production can be divided into four primary groups: sweet oranges, mandarins (including clementine and tangerine), grapefruit (including pummelo), and lemons/limes. The consumption of citrus fruits has many beneficial effects on human health such as improvements in lipid profile and inflammation markers in patients suffering from metabolic syndrome,[2] the reduction of cardiovascular risk,[3,4] possible neuroprotective effects,[5] and, more generally, strong antioxidant effects.[6] These beneficial effects are supported by different classes of compounds found in citrus fruits that include microconstituants such as vitamin C or secondary metabolites such as carotenoids and polyphenolic constituents.

Among the polyphenolic compounds in citrus, coumarins have attracted attention. In plants, coumarins display important allelochemical functions such as defense against pathogens.[7] Many studies have also focused on their beneficial effect on human health. For example, antitumor activities have been reported for prenylated coumarins such as auraptene (7-geranyloxycoumarin) or 5-geranyloxy-7-methoxycoumarin.[8,9] Furanocoumarins constitute a subclass of coumarins that are characterized by the presence of an additional furan ring linked at the C6/C7 or C7/C8 position of the coumarin core molecule.[10]

However, furanocoumarins are known to exhibit toxic effects. For example, molecules such as psoralen, bergapten, and xanthotoxin can lead to dermatitis, blisters, and hyperpigmentation reactions upon contact with citrus plants followed by photoactivation of the molecules subsequent to sun exposure.[11,12] Several studies have focused on the threshold dose of furanocoumarins that leads to phototoxic effects. Brickl and colleagues determined that the lowest dose of xanthotoxin combined with UVA that led to detectable phototoxic effects in human adults was 14 mg (corresponding to approximately 0.23 mg/kg bw for a 60 kg adult).[13] Schlatter et al. later established that a dose combining 10 mg of xanthotoxine and 10 mg of bergapten (0.25 mg/kg bw for a 60 kg adult) was equivalent to a 15 mg xanthotoxin dose in regard to phototoxic effects.[14] Only a few studies have addressed the phototoxic effects due to the consumption of beverages or food containing furanocoumarins. In 2010, Gorgus and collaborators concluded that in a Western diet, exposure to furanocoumarins primarily came from

Received: June 24, 2013
Revised: October 11, 2013
Accepted: October 12, 2013
Published: October 12, 2013

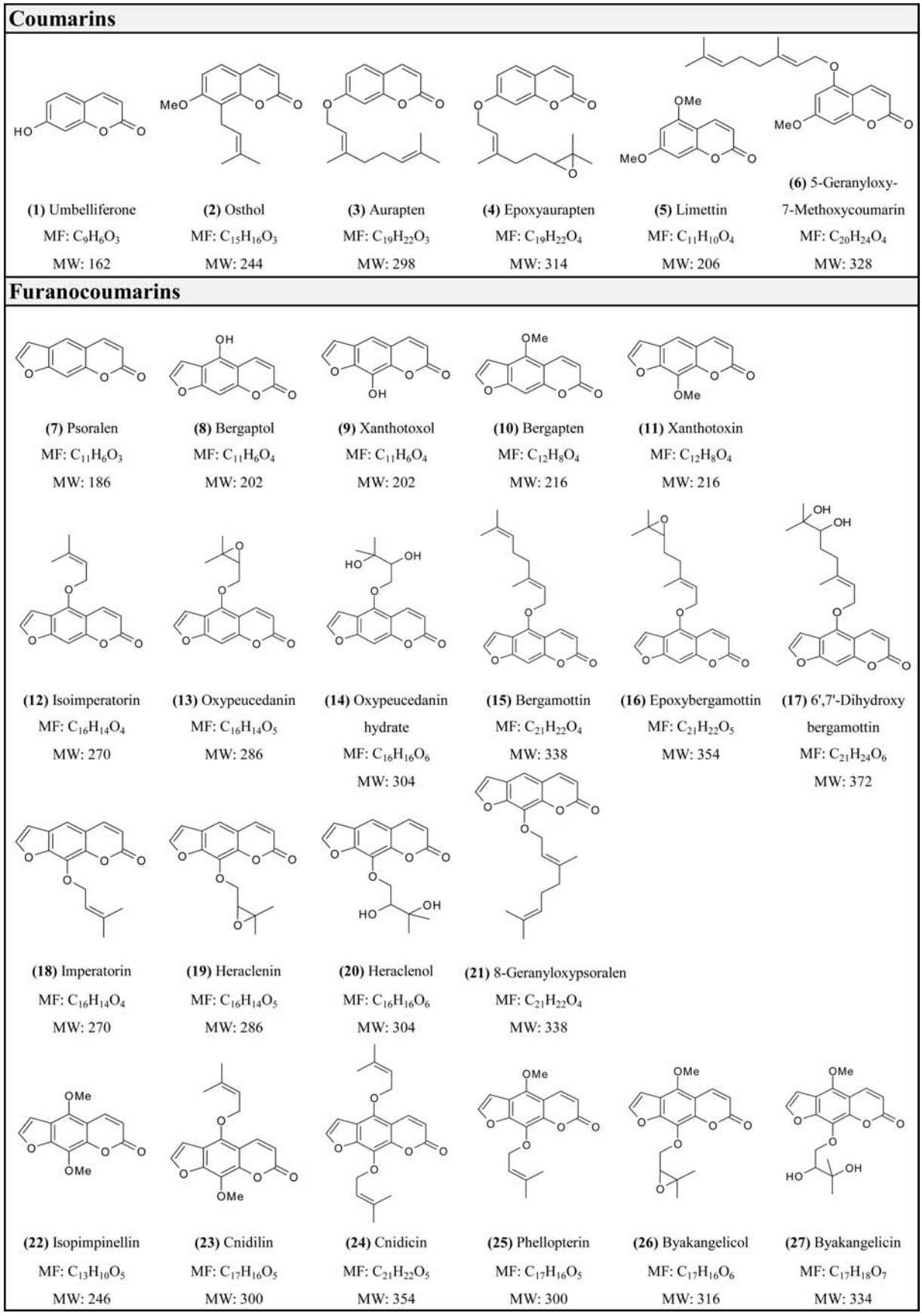

Figure 1. From the study, 6 coumarins and 21 furanocoumarins and their characteristics: molecular formula (MF) and molecular weight (MW).

grapefruit juice. They determined that the amount of grapefruit juice containing 20 mg of bergamottin was 1.2 L.[15] The daily consumption of such an amount of grapefruit juice is not realistic, and the phototoxic effects are unlikely.

Furanocoumarins can also inhibit cytochrome P450s of the 3A4 family through mechanism-based inactivation[16,17] or the CYP73A family.[18] Moreover, for patients submitted to medication, this inhibition of cytochrome P450s by furanocoumarins can lead to an increased drug concentration in the blood,[19] which can cause deleterious side effects.[20] This phenomenon was first demonstrated with grapefruit juice[19] and is often referred to as the "grapefruit juice effect" by physicians. This CYP3A4 inhibition process is primarily attributed to bergaptol and its derivatives (bergapten, isoimperatorin, bergamottin, 6′,7′-dihydroxybergamottin, and paradisins A, B, and C).[21,22] However, these compounds do not have the same inhibitory potential, and furanocoumarin dimers (spiroesters) such as paradisins are considered stronger CYP3A4 inhibitors than the monomers.[21,23]

There have been several published methods for the identification and/or quantitation of coumarins and furanocoumarins in plants. For example, to identify 21 oxygen heterocyclic compounds of *Citrus* essential oils, Bonaccorsi and McNail created a rapid (8 min) HPLC method.[24] Dugo and colleagues established a 60 min HPLC-DAD method to identify and quantitate 27 oxygen heterocyclic compounds including coumarins and furanocoumarins in *Citrus* products.[25] One of the most comprehensive studies on furanocoumarin quantitation was published by Frérot and Decorzant, who developed a 60 min method using HPLC coupled with DAD, fluorescence, and MS detection to quantitate 15 linear furanocoumarins in *Citrus* essential oils.[26]

None of these methods allowed for the identification and quantitation of a complete set of coumarins and furanocoumarins by combining the following parameters: high selectivity (no coelution problems), high sensitivity (well-resolved signal), and a short time of analysis. The coelution of coumarins and furanocoumarins in liquid chromatography, especially isomers, is a major problem[26] due to their similar physicochemical properties. This coelution frequently leads to a lack of selectivity when using UV absorption-based detection. Finally, the length of an analysis is a limiting step in performing a large set of analyses in a short period of time.

In this paper, we describe a rapid UPLC-based separation method followed by single ion monitoring MS detection that enables an unambiguous determination and quantitation with high selectivity and sensitivity of 27 coumarins and linear furanocoumarins (see Figure 1 for structures) found in *Citrus* plants in a single 20 min run. The method was developed for six different *Citrus* fruits that were chosen for their variable richness in coumarins and furanocoumarins: sweet orange (var. Washington Navel, *Citrus sinensis*), lemon (var. Eureka, *Citrus limon*), grapefruit (var. Duncan, *Citrus paradisi*), bergamot (var. Castagnaro, *Citrus bergamia*), pummelo (var. Chandler, *Citrus maxima*), and the clementine (var. Commune, *Citrus clementina*). The method was developed using the citrus peel, which is the part of the fruit that has the greatest diversity and concentration of coumarins and furanocoumarins.[27] Our objective was to rapidly discriminate between *Citrus* species for their abilities to synthesize the selected compounds. This new method was validated using the following parameters: linearity, limits of detection and quantitation, specificity, precision (including repeatability and intermediate precision), accuracy, and robustness. This method can therefore provide information on the species exhibiting potential phototoxicity or causing the grapefruit juice effect. Finally, this method also constitutes a valuable tool for chemotyping various *Citrus* species and may help the scientific community fingerprint these plants.

MATERIALS AND METHODS

Chemicals. Umbelliferone, psoralen, xanthotoxin, and bergapten (purity ≥ 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xanthotoxol (purity ≥ 90%), bergaptol, osthol, isopimpinellin, and 5-geranyloxy-7-methoxycoumarin (purity ≥ 99%) were bought from Extrasynthese (Genay, France). Angelicin, limettin, aurapten, epoxyaurapten, isoimperatorin, oxypeucedanin, oxypeucedanin hydrate, bergamottin, epoxybergamottin, 6′,7′-dihydroxybergamottin, imperatorin, heraclenin, heraclenol, 8-geranyloxypsoralen, cnidilin, cnidicin, phellopterin, byakangelicol, and byakangelicin (purity = 99%) were obtained from Herboreal Ltd. (Edinburgh, Scotland). Stock solutions of each standard at a concentration of 10 mmol/L were prepared by diluting the powder in dimethyl sulfoxide (DMSO).

HPLC-grade methanol and formic acid were purchased from Carlo Erba Reagents (Milan, Italy). Ultrapure water was freshly produced in the laboratory using a PURELAB Ultra system (Veolia Water S.T.I., Antony, France).

Plant Accession. This study was performed using citrus extracts taken from samples of the peel of six varieties including 'Washington Navel' sweet orange SRA 203, 'Eureka lemon' SRA 2, 'Duncan' grapefruit SRA 470, 'Castagnaro' bergamot SRA 612, 'Chandler' pummelo SRA 608, and 'Commune' clementine SRA 92. The fruits were collected from the Agronomic Research Station INRA/CIRAD of San Giuliano in Corsica (France).

Sampling. To limit variations due to uncontrolled environmental conditions, the fruits were harvested on the south side of the trees (exposed to the sun) and on the extremities of branches between 9:00 and 10:00 a.m. The samples were collected when the fruits were at commercial maturity. The harvest dates for clementine, sweet orange, grapefruit, bergamot, and pummelo were, respectively, as follows: December 19, 2011; February 17, 2012; February 20, 2012; February 24, 2012; and February 27, 2012. The lemons were harvested at the same date as the sweet oranges. For each variety, five fruits were picked from the tree.

The samples consisted of citrus peel (albedo and flavedo) collected from the equatorial region of the fruit. The thickness of the albedo varied from a few millimeters (mandarin) to several centimeters (pummelo). We decided not to exceed a thickness of 4 mm for the albedo fraction, as we assumed that this part of the peel contained fewer coumarins and furanocoumarins. Each fresh sample was weighed before being frozen in liquid nitrogen and stored at −80 °C until extraction.

Extraction of Compounds. The extraction process was adapted from that of Royer et al.[28] Fresh citrus peel samples were lyophilized in a Christ Beta 1-8 LD apparatus (Christ, Osterode am Harz, Germany) under dark conditions over a period of 5 days. The dried samples were weighed and ground in a ball mill (Retsch, Haan, Germany) at a frequency of 30 s^{-1} for 4 min. The resulting powder was stored at −80 °C.

To extract both coumarins and furanocoumarins, 20 mg of dried powder was mixed with 850 μL of an 80:20 methanol HPLC-grade/water solution. Incubation at room temperature was performed for 1 h in a Reax 2 overhead shaker at 30 rpm (Heidolph, Schwabach, Germany). After 10 min of centrifugation at 4550*g*, the supernatant was transferred to a new microtube, and the pellet was resuspended in 800 μL of 80:20 methanol HPLC-grade/water. A second extraction was performed for 2 min in an Eppendorf Thermomixer compact (Eppendorf, Hamburg, Germany) at room temperature and at 1400 rpm before being centrifuged for 10 min at 4550*g*. The two supernatants were mixed and dried overnight in an evaporator centrifuge (Jouan, Nantes, France). The pellet was resuspended in 75:25 methanol HPLC-grade/ultrapure water, mixed for another 2 min

Table 1. Linearity of the UPLC-MS Method (Equation and Coefficient of Determination, r^2), Limits of Detection (LOD) and Quantitation (LOQ) of the Coumarins and Furanocoumarins, and Accuracy of the UPLC-MS Method (Mean and Standard Deviation (SD))

	linearity		LOD and LOQ (mg/kg fresh weight)		accuracy (μmol/L)	
compound	equation	r^2	LOD	LOQ	mean	SD
coumarins						
umbelliferone (**1**)	$y = 0.0418x + 0$	0.9928	0.16	0.55	7.10	0.32
limettin (**5**)	$y = 0.3264x + 0$	0.9961	0.04	0.12	6.62	0.81
epoxyaurapten (**4**)	$y = 0.0800x + 0$	0.9900	0.37	1.22	8.61	1.22
osthol (**2**)	$y = 0.6263x + 0$	0.9906	0.03	0.11	7.66	0.74
aurapten (**3**)	$y = 0.2882x + 0$	0.9942	0.03	0.10	5.28	0.43
5-geranyloxy-7-methoxycoumarin (**6**)	$y = 0.8044x + 0$	0.9903	0.02	0.06	4.32	0.36
linear furanocoumarins						
xanthotoxol (**9**)	$y = 0.1792x + 0$	0.9922	0.11	0.38	7.60	0.94
heraclenol (**20**)	$y = 0.0201x + 0$	0.9927	0.16	0.55	5.28	0.72
psoralen (**7**)	$y = 0.1986\ x + 0$	0.9933	0.06	0.18	6.16	0.70
bergaptol (**8**)	$y = 0.0899x + 0$	0.9922	0.26	0.88	6.63	0.64
xanthotoxin (**11**)	$y = 0.2542x + 0$	0.9901	0.05	0.17	5.82	0.61
oxypeucedanin hydrate (**14**)	$y = 0.1387x + 0$	0.9908	0.07	0.23	8.36	0.99
byakangelicin (**27**)	$y = 0.0004x + 0$	0.9927	1.96	6.54	7.72	1.02
isopimpinellin (**22**)	$y = 0.3175x + 0$	0.9916	0.03	0.11	7.46	0.90
heraclenin (**19**)	$y = 0.0313x + 0$	0.9904	0.08	0.26	7.56	0.91
bergapten (**10**)	$y = 0.3322x + 0$	0.9925	0.07	0.23	7.60	0.81
byakangelicol (**26**)	$y = 0.1318x + 0$	0.9998	0.07	0.23	8.13	0.88
oxypeucedanin (**13**)	$y = 0.1564x + 0$	0.9968	0.05	0.16	7.61	0.83
6′.7′-dihydroxybergamottin (**17**)	$y = 0.0060x + 0$	0.9951	0.45	1.51	7.65	0.98
imperatorin (**18**)	$y = 0.0029x + 0$	0.9986	0.40	1.33	7.64	1.54
phellopterin (**25**)	$y = 0.0050\ x + 0$	0.9905	0.18	0.59	8.77	1.12
cnidilin (**23**)	$y = 0.0333x + 0$	0.9923	0.06	0.19	7.10	0.75
epoxybergamottin (**16**)	$y = 0.0043x + 0$	0.9991	0.71	2.38	7.09	1.42
isoimperatorin (**12**)	$y = 0.0117x + 0$	0.9991	0.19	0.63	7.72	0.86
cnidicin (**24**)	$y = 0.0144x + 0$	0.9901	0.17	0.55	9.57	0.94
8-geranyloxypsoralen (**21**)	$y = 0.0483x + 0$	0.9901	0.23	0.78	8.19	0.64
bergamottin (**15**)	$y = 0.2939x + 0$	0.9910	0.03	0.11	3.80	0.26

in the Thermomixer at room temperature and at 1400 rpm, and centrifuged for 10 min at 13360*g* to remove any remaining debris.

UPLC-MS Analyses. *Equipment.* The coumarin and furanocoumarin analyses were performed using a NEXERA UHPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a photodiode array (PDA) detector (SPDM20A, Shimadzu) combined with a mass spectrometer (single quadrupole, LCMS 2020, Shimadzu).

UPLC Separation. The separation was achieved on a C18 reversed-phase column (ZORBAX Eclipse Plus), 150 × 2.10 mm, particle size = 1.8 μm (Agilent Technologies, Santa Clara, CA, USA) protected with an Agilent Technologies 1290 infinity filter and thermostated at 40 °C. The solvents consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The compounds were eluted using the following mobile phase composition gradient (A:B; v/v): 90:10 at 0 min, 80:20 at 0.74 min, 40:60 at 5.88 min, 10:90 at 10 min, 0:100 between 12 and 16 min, and 90:10 from 16.01 to 20 min. The analyses were performed at a flow rate of 0.2 mL/min, and the injection volume was 3 μL. The total analysis duration was 20 min.

MS Detection. The UPLC system was connected to the MS by a dual ion source (DUIS), a mix between electrospray ionization (ESI) (here operating in positive mode (ESI+)), and atmospheric pressure chemical ionization (APCI). The inlet, desolvation line, and heating block temperatures were set at 350, 250, and 400 °C, respectively. The capillary voltage was set at 4.5 kV. For each compound, the voltages of the desolvation line (DL) and Qarray were optimized to increase the detection sensitivity. For this, each standard was directly introduced in the MS at the concentration of 1 mmol/L. The detection was performed in single ion monitoring mode (SIM) to increase selectivity.

Data Acquisition. The data were acquired and processed on LabSolution software version 5.52 sp2 (Shimadzu).

Peak Identification and Quantitation. Three nanomoles of each standard molecule was individually injected in the UPLC-MS. These preliminary analyses allowed for the completion of the software database (retention time and m/z ratio), which was further used to perform semiautomatic research for the identification of each molecule in any citrus extract.

The quantitation of each molecule was based on the signal obtained from the MS detection. However, the signal intensity in a mass spectrometer is not repeatable, as the ion source is prone to clogging. Additionally, the volume injected in the apparatus can change somewhat between two samples. To address these problems, the quantitation was performed using angelicin, an angular furanocoumarin missing from *Citrus* plants, as an analytical internal standard. Angelicin was added at the same concentration (5 μmol/L) in the samples as well as in seven calibration solutions. The calibration solutions contained all of the standard molecules at the same concentrations ranging from 1 to 30 μmol/L (1, 3, 6, 12, 18, 24, and 30 μmol/L). Calibration curves were drawn for each compound by linking its relative peak area (compound area divided by the angelicin area) and its concentration. Each curve fit type was linear and forced to pass through 0.

RESULTS AND DISCUSSION

Method Validation. *Linearity.* Calibration curves were constructed using seven calibration solutions ranging from 1 to 30 μM. As described in Table 1, the curve fits were linear

Table 2. Specificity (Equation and Coefficient of Determination, r^2), Precision (Repeatability and Intermediate Precision Expressed in Relative Standard Deviation (RSD%)), and Robustness (Expressed in RSD% and Based on Oven Temperature and Flow Rate Changes) of the UPLC-MS Method

	specificity		precision (RSD%)		robustness (RSD%)	
compound	equation	r^2	repeatability	intermediate precision	oven temperature	flow rate
coumarins						
limettin (**5**)	$y = 54402x$	0.995	8.89	9.59	0.52	8.79
5-geranyloxy-7-methoxycoumarin (**6**)	$y = 49722x$	0.995	10.51	33.75	3.77	10.59
linear furanocoumarins						
oxypeucedanin hydrate (**14**)	$y = 2507x$	0.995	9.56	14.13	1.83	10.26
byakangelicol (**26**)	$y = 16605x$	0.994	8.29	18.99	1.50	6.51
oxypeucedanin (**13**)	$y = 37500x$	0.992	8.17	13.50	0.36	7.83
8-geranyloxypsoralen (**21**)	$y = 2434x$	0.995	5.47	18.44	2.06	5.42
bergamottin (**15**)	$y = 24006x$	0.993	11.87	32.85	10.97	13.88

(passing through 0) with optimal linear correlation coefficients in all cases ($r^2 > 0.99$).

Limits of Detection and Quantitation. The limits of detection (LOD) and quantitation (LOQ) were determined by calculating the concentration and the signal/noise (S/N) ratio of each compound in a lemon (var. Eureka, *C. limon*) peel extract enriched in coumarins and furanocoumarins, as lemon does not synthesize them all. The limits of detection and quantitation correspond to S/N ratios of 3 and 10, respectively. In most cases, these limits are extremely low (Table 1). Only epoxyaurapten, bergaptol, byakangelicin, 6′,7′-dihydroxybergamottin, imperatorin, epoxybergamottin, and 8-geranyloxypsoralen have higher limits of detection and quantitation.

Specificity. To assess a possible matrix effect, a lemon peel extract was prepared and injected in the UPLC-MS system to determine its content in coumarins and furanocoumarins. In the extract, only seven molecules could be quantitated: limettin, 5-geranyloxy-7-methoxycoumarin, oxypeucedanin hydrate, byakangelicol, oxypeucedanin, 8-geranyloxypsoralen, and bergamottin. Specificity was assessed by preparing four more samples (from the same lemon peel) spiked with these seven compounds at 25, 50, 75, and 100% of the initial concentration, respectively. The equations linking the compound areas and the percentages of the initial concentration and their linear correlation coefficients ($r^2 > 0.99$) (Table 2) show that no matrix effect could be observed.

Precision. The repeatability and intermediate precision were calculated by injecting six lemon peel extracts from the same fruit six times on six different days. For each extract, the areas of the seven compounds quantifiable in lemon were divided by the area of the analytical internal standard (angelicin). The relative standard deviations (RSD) of the ratios were close to 10% (Table 2), which allows us to conclude that the method is repeatable. Concerning the intermediate precision, the RSDs are generally below 20%, except for 5-geranyloxy-7-methoxycoumarin and bergamottin, for which the RSDs are approximately 30%. Thus, the intermediate precision of this method is generally good but less for the two compounds previously cited.

Accuracy. In this study, six tomato leaf extracts (var. Micro-Tom, *Solanum lycopersicum*) were prepared according to the extraction protocol described before. These extracts represent complex matrices without coumarins or furanocoumarins. They were spiked with the 27 compounds at a concentration of 7.5 μmol/L and with angelicin at a concentration of 5 μmol/L. The UPLC-MS quantitation results showed that the concentration values are generally close to 7.5 μmol/L (Table 1), which demonstrates the accuracy of our method.

Robustness. The robustness of the method was tested by changing two parameters: the column oven temperature and the flow rate. Three temperatures (35, 40, and 45 °C) and three flow rates (0.15, 0.20, and 0.25 mL/min) were tested. The peaks of the seven compounds quantifiable in lemon were integrated in all cases, and the RSDs were calculated. The RSD values were similar to those of the repeatability section (around 10%, as seen in Table 2), providing evidence that the method is robust.

Optimization of the UPLC Separation Conditions. The best separation method was obtained with an elution gradient based on five different steps of water/methanol ratios. The complete elution was performed in 16 min and, to avoid any contamination between samples, the column was washed and equilibrated with the starting solvent (90:10) for an additional 4 min between each analysis. Because we decided to proceed to the detection and quantitation with MS, the objective of the separation method was not necessarily to obtain a full resolution of all of the peaks but rather to simply separate between two isomers, thus enabling further unambiguous MS processing. With this method, the 27 coumarins and furanocoumarins could be efficiently separated for subsequent MS detection (Table 3 and Supporting Information Figure S1). In comparison, Frérot and Decorzant developed two complementary HPLC methods based on analyses that were 3-fold longer in duration to separate 15 furanocoumarins.[26]

Optimization of the MS Detection. Coumarins and furanocoumarins are prone to coelute in HPLC/UPLC systems, which leads to a lack of selectivity but also to quantitation mistakes with a PDA detection system due to overlapping UV spectra between structurally related molecules.[26] Mass spectrometry, which has recently become increasingly accessible at the bench scale, is a powerful tool to distinguish between unsubstituted coumarins/furanocoumarins and closely matching derivatives such as hydroxylated/methoxylated compounds. Hence, MS was chosen as the detection method in our work.

MS detection was optimized by increasing the selectivity and the sensitivity of the apparatus. The ions were detected in SIM mode, which increases the selectivity of the MS detection. This mode is convenient for data processing because each mass signal can be individually identified and integrated. The sensitivity of the MS could be increased by modifying several parameters. First, the chosen interface between the UPLC and MS systems was DUIS, which was used in positive mode. The compounds were ionized softly at atmospheric pressure, and the

Table 3. Analyzed Molecules and Their Characteristics: Ion Species, Mass/Charge Ratio (m/z), Retention Time (t_R), Desolvation Line Voltage (DL), and Qarray Voltage

compound	ion species	m/z	t_R (min)	DL (V)	Qarray (V)
coumarins					
umbelliferone (**1**)	$[M + H]^+$	163	7.01	80	0
limettin (**5**)	$[M + H]^+$	207	9.51	0	0
epoxyaurapten (**4**)	$[M + H]^+$	315	11.32	0	0
osthol (**2**)	$[M + H]^+$	245	11.66	0	0
aurapten (**3**)	$[M + H]^+$	299	13.46	80	0
5-geranyloxy-7-methoxycoumarin (**6**)	$[M + H]^+$	329	14.00	0	90
linear furanocoumarins					
xanthotoxol (**9**)	$[M + H]^+$	203	7.92	80	0
heraclenol (**20**)	$[M + H]^+$	305	8.45	0	0
psoralen (**7**)	$[M + H]^+$	187	8.85	0	0
bergaptol (**8**)	$[M + H]^+$	203	8.85	80	0
xanthotoxin (**11**)	$[M + H]^+$	217	8.90	0	0
oxypeucedanin hydrate (**14**)	$[M + H]^+$	305	8.99	0	0
byakangelicin (**27**)	$[M + H]^+$	335	9.04	80	30
isopimpinellin (**22**)	$[M+H]^+$	247	9.49	80	0
heraclenin (**19**)	$[M + H]^+$	287	9.60	0	30
bergapten (**10**)	$[M + H]^+$	217	9.62	0	0
byakangelicol (**26**)	$[M + H]^+$	317	10.10	80	0
oxypeucedanin (**13**)	$[M + H]^+$	287	10.22	0	30
6′,7′-dihydroxybergamottin (**17**)	$[M + H]^+$	373	10.77	0	0
imperatorin (**18**)	$[M + H]^+$	271	11.06	80	30
phellopterin (**25**)	$[M + H]^+$	301	11.38	80	30
cnidilin (**23**)	$[M + H]^+$	301	11.69	80	30
epoxybergamottin (**16**)	$[M + H]^+$	355	11.88	0	30
isoimperatorin (**12**)	$[M + H]^+$	271	11.90	80	30
cnidicin (**24**)	$[M + H]^+$	355	12.88	0	30
8-geranyloxypsoralen (**21**)	$[M + H]^+$	339	13.01	80	0
bergamottin (**15**)	$[M + H]^+$	339	13.88	80	0
standard					
angelicin	$[M + H]^+$	187	9.12	0	0

generated signals were more intense than when using ESI or APCI ion sources alone. Finally, for each mass, the optimum voltages at the entry of the MS were determined (Table 3) to increase the sensitivity of the detection and the resolution of the peaks. More precisely, these voltages were applied in the desolvation line and in the Qarray parts of the MS.

Identification and Quantitation of the Coumarins and the Furanocoumarins in Citrus Extracts. To validate the method, citrus peel extracts from six different varieties were prepared and analyzed: 'Washington Navel' sweet orange, 'Eureka' lemon, 'Duncan' grapefruit, 'Castagnaro' bergamot, 'Chandler' pummelo, and 'Commune' clementine. Preliminary analyses were performed on 27 standard molecules, each characterized by their respective m/z ratio and associated retention time. Thanks to the highly selective MS detection performed in SIM mode, each peak could be reliably and individually identified. These analyses highlighted that a limited number of the 27 investigated molecules were present in each sample (Figure S2 in the Supporting Information). With compounds found at the level of traces taken into account, only 4 molecules could be detected in orange, 6 in clementine, 12 in bergamot and pummelo, 13 in grapefruit, and finally 15 in lemon (Table 4).

These analyses highlighted two main groups of samples. The first group, comprising clementine and orange, is characterized by low amounts of coumarins and furanocoumarins, whereas the second group composed of the other varieties synthesizes a broader diversity of these molecules; some of these molecules are significantly more concentrated than in clementines and oranges. Among the 27 coumarins and furanocoumarins investigated, seven could not be detected in any of the six *Citrus* varieties: xanthotoxol, xanthotoxin, heraclenol, byakangelicin, imperatorin, cnidilin, and cnidicin. Xanthotoxol and xanthotoxin correspond, respectively, to 8-hydroxy and 8-methoxy derivatives of the parent psoralen compound. This specialized subgroup of molecules is less frequently found in *Citrus* fruits than the corresponding 5-hydroxy/methoxy/geranyloxy derivatives (bergaptol, bergapten, and bergamottin).[26,29] Heraclenol and cnidilin constitute butoxy and methylbutenyloxy furanocoumarin end-products, respectively, which have been specifically reported in Apiaceae plants.[30,31] Of the seven molecules undetected in our citrus samples, only byakangelicin, imperatorin, and cnidicin, which are methylbutoxy and butenyloxy derivatives, have already been reported in *Citrus* oils.[25,26] This nondetection of byakangelicin, imperatorin, and cnidicin could be explained by higher detection limits but also by low amounts or the absence of these molecules in the citrus extracts.

In sweet orange peel, only limettin could be quantitated, although traces of osthol, bergapten, and oxypeucedanin were highlighted. Bonaccorsi and McNair could detect these four compounds in *Citrus* essential oils using an HPLC-DAD method.[24] Nonetheless, they could not detect these compounds in sweet orange oil. The confirmed occurrence of these four compounds in our sweet orange sample is in agreement with the higher sensitivity of the MS detection method. However, this occurrence may be due to the peculiar set of plant tissues analyzed, as our samples include the flavedo (and subsequently the essential oil cavities) but also a part of the albedo.

In lemon peel, limettin, 5-geranyloxy-7-methoxycoumarin, oxypeucedanin hydrate, byakangelicol, oxypeucedanin, 8-geranyloxypsoralen, and bergamottin could be quantitated, as already achieved by Dugo and colleagues.[25] In addition, umbelliferone, heraclenin, phellopterin, osthol, aurapten, isopimpinellin, and bergapten could also be detected but not quantitated. Among these, the last four were not identified by Dugo et al.,[25] possibly due to a higher sensitivity in our MS detection method. Byakangelicin, imperatorin, and cnidicin could not be detected in the lemon samples or in any other variety, although they have already been reported in lemon essential oils.[25] This may be related to their high detection limits but also to their poor content in this variety.

In grapefruit peel, limettin, epoxyaurapten, osthol, aurapten, bergapten, 6′,7′-dihydroxybergamottin, epoxybergamottin, and bergamottin were quantitated, as already described by Dugo and collaborators in grapefruit essential oils[25] (except for limettin, which could be identified but not quantitated). Compounds detected at the trace level were umbelliferone, bergaptol, isopimpinellin, oxypeucedanin, and 5-geranyloxy-7-methoxycoumarin.

In bergamot peel, limettin, 5-geranyloxy-7-methoxycoumarin, bergapten, bergamottin, and psoralen were quantitated, which is in agreement with other results from bergamot essential oils[25,26,29] (except for psoralen, which was not identified or even investigated). Epoxybergamottin could not be quantitated, as in the study by Frérot and Decorzant,[26] which is likely related

Table 4. Concentration (in Milligrams per Kilogram Fresh Weight ± Standard Deviation) of the Coumarins and the Furanocoumarins in the Citrus Peel Extracts[a]

compound	varieties					
	orange	clementine	lemon	grapefruit	bergamot	pummelo
coumarins						
umbelliferone (**1**)			traces	traces	traces	
limettin (**5**)	0.77 ± 0.20	2.15 ± 0.37	24.58 ± 12.57	0.87 ± 0.09	102.43 ± 14.85	1.24 ± 0.15
epoxyaurapten (**4**)				64.00 ± 21.93	traces	50.16 ± 8.88
osthol (**2**)	traces		traces	3.18 ± 0.25	traces	1.12 ± 0.30
aurapten (**3**)		traces	traces	69.14 ± 12.89	traces	30.76 ± 6.37
5-geranyloxy-7-methoxycoumarin (**6**)			9.67 ± 4.41	traces	28.63 ± 11.26	
linear furanocoumarins						
xanthotoxol (**9**)						
heraclenol (**20**)						
psoralen (**7**)					1.95 ± 0.98	
bergaptol (**8**)				traces	traces	traces
xanthotoxin (**11**)						
oxypeucedanin hydrate (**14**)			8.58 ± 5.49			
byakangelicin (**27**)						
isopimpinellin (**22**)		1.40 ± 0.27	traces	traces	traces	traces
heraclenin (**19**)			traces			
bergapten (**10**)	traces	0.96 ± 0.16	traces	2.01 ± 0.60	500.51 ± 177.60	38.21 ± 14.05
byakangelicol (**26**)			21.50 ± 5.16			
oxypeucedanin (**13**)	traces	1.30 ± 0.22	22.30 ± 8.94	traces	traces	traces
6′,7′-dihydroxybergamottin (**17**)				157.52 ± 58.02		106.83 ± 14.70
imperatorin (**18**)						
phellopterin (**25**)			traces			
cnidilin (**23**)						
epoxybergamottin (**16**)				96.16 ± 41.40		69.01 ± 20.42
isoimperatorin (**12**)			traces			
cnidicin (**24**)						
8-geranyloxypsoralen (**21**)			15.48 ± 9.29			traces
bergamottin (**15**)		traces	17.57 ± 13.30	11.79 ± 1.67	146.18 ± 60.68	2.00 ± 0.38

[a]"Traces" means that the compound could be detected but not quantitated (3 < S/N < 10).

to variations in plant material between the two studies. The umbelliferone, epoxyaurapten, osthol, aurapten, bergaptol isopimpinellin, and oxypeucedanin contents were above the limits of detection, whereas they were not mentioned in the other studies.

Pummelo peel allowed the quantitation of limettin, epoxyaurapten, osthol, aurapten, bergapten, 6′,7′-dihydroxybergamottin, epoxybergamottin, and bergamottin. Bergaptol, isopimpinellin, oxypeucedanin, and 8-geranyloxypsoralen were detected only in the pummelo samples.

As for 'Commune' clementine peel, limettin, bergapten, isopimpinellin, and oxypeucedanin could be quantitated, whereas aurapten and bergamottin were only detectable within the samples.

For these two last varieties, our data were not compared with those of other studies, as we did not find any study quantitating coumarins or furanocoumarins in pummelo and clementine.

In conclusion, this study presents a new method to identify and quantitate 27 coumarins and furanocoumarins in citrus peel extracts. This method has the advantage of combining selectivity, sensitivity, and rapidity, allowing efficient and large-scale analyses. Taking advantage of the MS detection method (SIM), the UPLC separation method is based on a solvent gradient specifically designed only to separate compounds with the same m/z. Our method also takes less time than quantitation methods using UV absorption, for which a longer gradient is required for adequate molecule separation. The sensitivity of our method makes it possible to identify compounds at very low concentrations, and its selectivity enables the accurate identification of compounds in *Citrus* species containing a high diversity of coumarins and furanocoumarins. This method was developed using citrus peel extracts, which contain the highest concentrations and the greatest diversity of coumarins and furanocoumarins, making the peel the best tissue to optimize this method. This new method can be used to deepen our knowledge of citrus phototoxicity and of the grapefruit juice effect related to furanocoumarin monomers. Its application to many citrus varieties will allow assessments of their possible toxicity on the basis of their quantitative results. As an example, the present study has focused on six *Citrus* varieties for which bergamot peel appeared the richest in total furanocoumarin content (sum of means = 648.64 mg/kg). In this case, to reach the 20 mg threshold dose of furanocoumarins that leads to phototoxic effects, a daily consumption of approximately 31 g of bergamot peel is necessary (roughly equivalent to three to four fruits peel). This consumption level seems unrealistic because bergamot peel is scarcely used as a food ingredient. Another use for this method is the fingerprinting of *Citrus* species that produce coumarins and furanocoumarins, as the rapidity of this method allows for the analyses of many varieties.

■ ASSOCIATED CONTENT

Supporting Information

Mass chromatogram of a standard mixture (Figure S1) and of citrus peel extracts (Figure S2) after UPLC separation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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