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A Fast Method Coupling Ultrahigh Performance Liquid Chromatography with Diode Array Detection for Flavonoid Quantification in *Citrus* Fruit Extracts

Alexander Medina-Remón,[†,‡] Sara Tulipani,[†,§] Maria Rotchés-Ribalta,[†,§] Maria De Lourdes Mata-Bilbao,[†] Cristina Andres-Lacueva,[†,§] and Rosa M. Lamuela-Raventos[*,†,‡]

[†]Nutrition and Food Science Department, XaRTA, INSA, Pharmacy School, University of Barcelona, Barcelona, Spain

[‡]CIBER 06/003 Physiopathology of Obesity and Nutrition, (CIBEROBN), and RETICS RD06/0045/0003, Instituto de Salud Carlos III, Spain

[§]Ingenio-CONSOLIDER program, FUN-C-FOOD, CSD2007-063, Barcelona, Spain

ABSTRACT: Flavonoids are a widely distributed group of polyphenolic compounds present in an extensive range of edible plants, notably *Citrus* species. This article reports a rapid, optimized, and validated method for the separation and quantification of flavonoids in three *Citrus* fruit extracts by ultrahigh performance liquid chromatography (UHPLC) using a photodiode array detector. This new procedure allowed the simultaneous separation and quantification of 11 selected flavonoids in 5.5 min, 8.2 times faster than that by HPLC analysis. The solvent consumption for each individual analysis was also reduced almost 6.2-fold. The most abundant component in the analyzed samples was naringin (299.06−544.36 mg 100 g^{-1}), followed by rutin (116.60−256.33 mg 100 g^{-1}) and quercetin (7.78−251.49 mg 100 g^{-1}). Isoquercitrin was found in a lower proportion (60.05−81.88 mg 100 g^{-1}). The method was completely validated, providing a sensitive analysis for flavonoid detection and showing satisfactory data for all the parameters tested. This methodology is cheaper, more environmentally friendly, and easier to perform than others previously described.

KEYWORDS: *Citrus*, flavonoids, *Citrus* fruit extracts, UHPLC, LC/MS/MS

INTRODUCTION

Flavonoids are a widely distributed group of polyphenolic compounds that have been reported to act as antioxidants in various biological systems.[1] They are present in a wide range of edible plants, fruits, vegetables, teas, wines, and fruit juices, especially those from *Citrus* species.[2,3] Flavonoids in plants usually occur in a glycosylated form, mainly with glucose or rhamnose, but they can also be linked with galactose, arabinose, xylose, glucuronic acid, or other sugars.[1,4] Because the solid parts of *Citrus* fruit have a very high flavanone content, the whole fruit may contain up to 5 times as much as a glass of orange juice.[5]

The amount of flavonoids present in *Citrus* fruit extracts varies according to the genetic background, the processing methods and maturity since immature fruits have higher amounts of polyphenols than mature fruits.[6] The concentrations also depend on the age of the plant, as the highest levels are detected in tissues showing pronounced cell divisions.[2,3,7] Because of the beneficial health effects of flavonoids present in fruits and vegetables,[8] their simultaneous identification and quantification is very important for many areas of science.

Liquid chromatographic (LC) methodologies represent, to date, the most widely used approach to phenolic analysis.[9] High performance liquid chromatography (HPLC) of *Citrus* fruit juices shows peaks for flavanone glycosides that vary from one fruit to another. Numerous methods for the detection and quantification of flavonoid compounds in *Citrus* fruit have already been developed.[10−13] According to Molnár-Perl et al.[14] and Kocevar et al.,[15] the number of flavonoids detected simultaneously by most HPLC methods are separated in 45 or 50 min.

However, Chen et al.[16] have developed a rapid ultrahigh performance liquid chromatography (UHPLC) method for the simultaneous determination of flavonoids in different species of *Epimedium* using a 12 min gradient elution. Spácil et al.[17] compared a conventional HPLC system and a UHPLC system, equipped with columns containing similar stationary phases, in the analysis of wine and tea samples. While both analytical methods gave good results, the UHPLC system appeared to be superior. UHPLC methods not only are faster, more sensitive, and more efficient but also are more reliable and ecological.[17]

This article reports a rapid, optimized, and validated method for the separation and quantification of flavonoids in *Citrus* fruit extracts by UHPLC using a photodiode array (PDA) detector with a very rapid sample preparation in order to decrease the time and cost of sample analysis; its many advantages include high resolution, speed, an exceptionally small sample volume, and short running time for the separation.[18] This method was applied in three *Citrus* fruits extracts to characterize the flavonoids with an efficient separation.

MATERIALS AND METHODS

Standards and Reagents. All samples and standards were handled with no exposure to light. Naringin (naringenin-7-*O*-rhamnoglucoside),

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quercetin, hesperidin (hesperetin-7-*O*-rhamnoglucoside), hesperetin, and rutin (quercetin 3-*O*-rutinoside) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); narirutin (naringenin-7-*O*-rutinoside), prunin (naringenin-7-*O*-glucoside), naringenin, tangeretin, sinensetin, neohesperidin (hesperetin -7-*O*-neohesperidoside), poncirin (isosakuranetin-7-*O*-neohesperidoside), didymin (isosakuranetin-7-*O*-rutinoside), neoeriocitrin (eriodictyol-7-*O*-neohesperidoside), and eriocitrin (eriodictyol-7-*O*-rutinoside) were purchased from Extrasynthèse (Genay, France). Isoquercitrin (quercetin 3-D-glucoside) was obtained from ChromaDex (LGC Promochem) (Barcelona, Spain). Methanol, acetonitrile, and formic acid were obtained from Scharlau Chemie, S.A. (Barcelona, Spain), and ultrapure water (Milli-Q) was obtained from Millipore System (Bedford, MA, USA). Naringin and quercetin standard solutions were prepared with acetonitrile at 0.1% of formic acid: water at 0.05% formic acid (30:70) with 30% of methanol; rutin and isoquercitrin were prepared with 100% methanol; and the rest of standards were prepared with water at 0.05% formic acid.

***Citrus* Fruit Extract Samples, Preparation, and Quantification.** The three *Citrus* fruit extracts Leben FC, Biocitro, and Trinken were manufactured by Quinabra (Química Natural Brasileira Ltd.a., Sao José dos Campos-SP-Brazil). The products are made from natural ingredients and do not contain genetically modified organisms, being composed of a mixture of four *Citrus* species: grapefruit (*C. paradisi*), sweet orange (*C. sinensis*), bergamot (*C. aurantium L.* subspecies *bergamia*), and mandarin (*C. white reticulate*). The fruits were crushed, fermented, and ultracentrifuged to remove the solid phase and keep the liquid phase, which was concentrated by evaporation with heat at 80 °C to obtain the final syrup. After these processes, vegetable glycerin (at 50%) and organic acids (citric and lactic acids, both at 1%) were added. Each extract contained the four *Citrus* species.

The samples were coded, packed, and preserved at 4 °C until analysis. They were transported from Brazil in a polystyrene box with dry ice to maintain their temperature. For flavonoid quantification, the different dilutions were prepared with Milli-Q water (4 g L^{-1} for Biocitro and 2 g L^{-1} for Leben FC and Trinken). Prior to UHPLC injection, they were filtered with 13 mm polytetrafluoroethylene (PTFE) and 0.22 μm filters from Waters (Milfords, MA, USA).

Instrumentation and Chromatography Conditions. Flavonoid quantification was accomplished with a Waters Ultra Performance Liquid Chromatographic Acquity system (UPLC) (Milford, MA, USA) equipped with a binary solvent delivery module, an autosampler cooler, a column heater, and a 2996 PDA detector, version 4.1 firmware a degassing system, and driven by Waters Empower software from Waters Corporation. Optimum separation was achieved with a binary mobile phase which consisted of (A) water at 0.05% of formic acid and (B) acetonitrile at 0.1% of formic acid, with a flow rate of 0.6 mL min^{-1}. The 5.5 min gradient was as follows: (t (min), % A), (0, 90), (0.1, 80), (1.7, 80), (1.8, 70), (2.1, 70), (2.3, 60), (3.8, 60), (3.9, 50), (4.5, 50), (4.6, 0), (5.4, 0), and (5.5, 90). The equilibration time for bringing the column to the initial conditions after gradient analysis was 1 min. All solvents were passed through a 0.22 μm pore diameter filter before use.

The autosampler was cooled to 15 °C, and 6 μL of samples were injected into the Waters Acquity UPLC system, combined with an Acquity UPLC BEH C_{18} column (1.7 μm, 2.1 × 100 mm) and protected with an Acquity UPLC BEH C_{18}Van Guard Precolumn (1.7 μm, 2.1 × 5 mm, 3/pk) (Waters, Milford, MA, USA); column temperature was thermostatted at 40 °C.

Detection was at 280 nm for narirutin, naringin, prunin, hesperidin, naringenin, hesperetin, sinensetin, and tangeretin, at 365 nm for rutin, isoquercitrin, and quercetin. They were identified by comparing the retention time and spectral characteristics of their peaks with those of standards, and they were quantified using the standards of each one.

Validation of the UHPLC Method. The analytical method was validated with naringin, quercetin, hesperidin, hesperetin, rutin, narirutin, prunin, naringenin, tangeretin, sinensetin, and isoquercitrin standards according to the recommendations of AOAC International,[19] and the following criteria were determined: linearity, precision, accuracy, and sensibility. To evaluate the linearity of the assay, a series of calibrators at different concentrations were prepared in Milli-Q water. The areas obtained were plotted against the corresponding standard concentrations, prepared in duplicate, in the range of 1−100 μg/mL for rutin, naringin, hesperidin, and hesperetin, 1−90 μg/mL for isoquercitrin, 1−80 μg/mL for narirutin, quercetin, and naringenin, 1−60 μg/mL for sinensetin, and 1−50 μg/mL for tangeretin, and 1−30 μg/mL for prunin.

Accuracy and precision were evaluated by repeatedly spiking the matrix of the *Citrus* fruit extracts, Trinken and Leben, with known levels of 11 standards at 3 different concentrations (80%, 100%, and 120% of expected value) of each flavonoid. The replicates were injected into the UHPLC system. The percentage of added analyte recovered from a *Citrus* fruit extract matrix was used as the index of accuracy. Precision is a function of concentration, and it was calculated by dividing the standard deviation (SD) by the means of concentration to obtain the coefficient of variation (CV), which when expressed on a percentage basis gives the relative standard deviation (RSD). The use of the RSD values facilitates comparisons of variabilities at different concentrations.

The sensibility of the method evaluated determining the limits of detection (LOD) and limits of quantification (LOQ). The LOD is the smallest quantity of analyte that can be shown to be significantly greater than the measurement (random) error of the blank at the prescribed level of confidence (usually 95%). It is the lowest concentration of analyte in a sample that can be detected but not necessarily quantified, and it was calculated as 3 times the SD of the background noise. The LOQ is the smallest amount of analyte in a test sample that can be quantitatively determined with suitable precision and accuracy under previously established methods and conditions. It was taken as 10 times the SD of the background noise.[19]

UPLC-MS/MS Confirmation Analysis. The phenolic compounds present in the *Citrus* extracts were first identified by UPLC-MS/MS analysis. Online coupled to the UPLC system, an API 3000 triple quadrupole mass spectrometer (ABSciex, Concord, Ontario, Canada) equipped with a Turbo Ionspray source was used both in negative and positive ion modes to obtain mass spectrometry (MS) and MS/MS information. Turbo Ionspray source settings were as follows: capillary voltage, −3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); and drying gas (N_2), heated to 400 °C and introduced at a flow rate of 8000 cm^3/min. The system was controlled by software Analyst, version 1.4.2, supplied by Applied Biosystems (Foster City, CA, USA).

Prior to the UPLC-MS/MS analysis of the samples, direct infusion experiments were carried out with each standard compound commercially available. Briefly, 50:50 (v/v) [A, B] individual standard solutions (10 μg/mL) were infused into the mass spectrometer using a model syringe pump (Harvard Apparatus, Holliston, MA, USA), at a constant flow rate of 5 μL/min, and manual tuning was performed in positive and negative modes. For each standard compound, the molecular ion (MI) ($[M - H]^-$ and/or $[M + H]^+$) and the most abundant fragment ions (m/z) were easily detected, and the optimal focusing potential (FP), declustering potential (DP), and collision energy (CE) for the MI fragmentation were recorded.

Citrus phenolic compounds were identified on the basis of comparison of retention times (Rt), full scan (FS) data, and neutral loss (NL) and product ion scan (PIS) MS/MS spectra, with the commercial standards. Full data acquisition was performed scanning from 100 to 800 u in profile mode and using a cycle time of 2 s, with a step size of 0.1 u and a 2 ms pause between each scan. NL scans of 162 u (for the detection of glucosides) and 308 u (for the detection of rutinosides/rhamnosides) were carried out by scanning within the m/z range from 200 to 800 u.

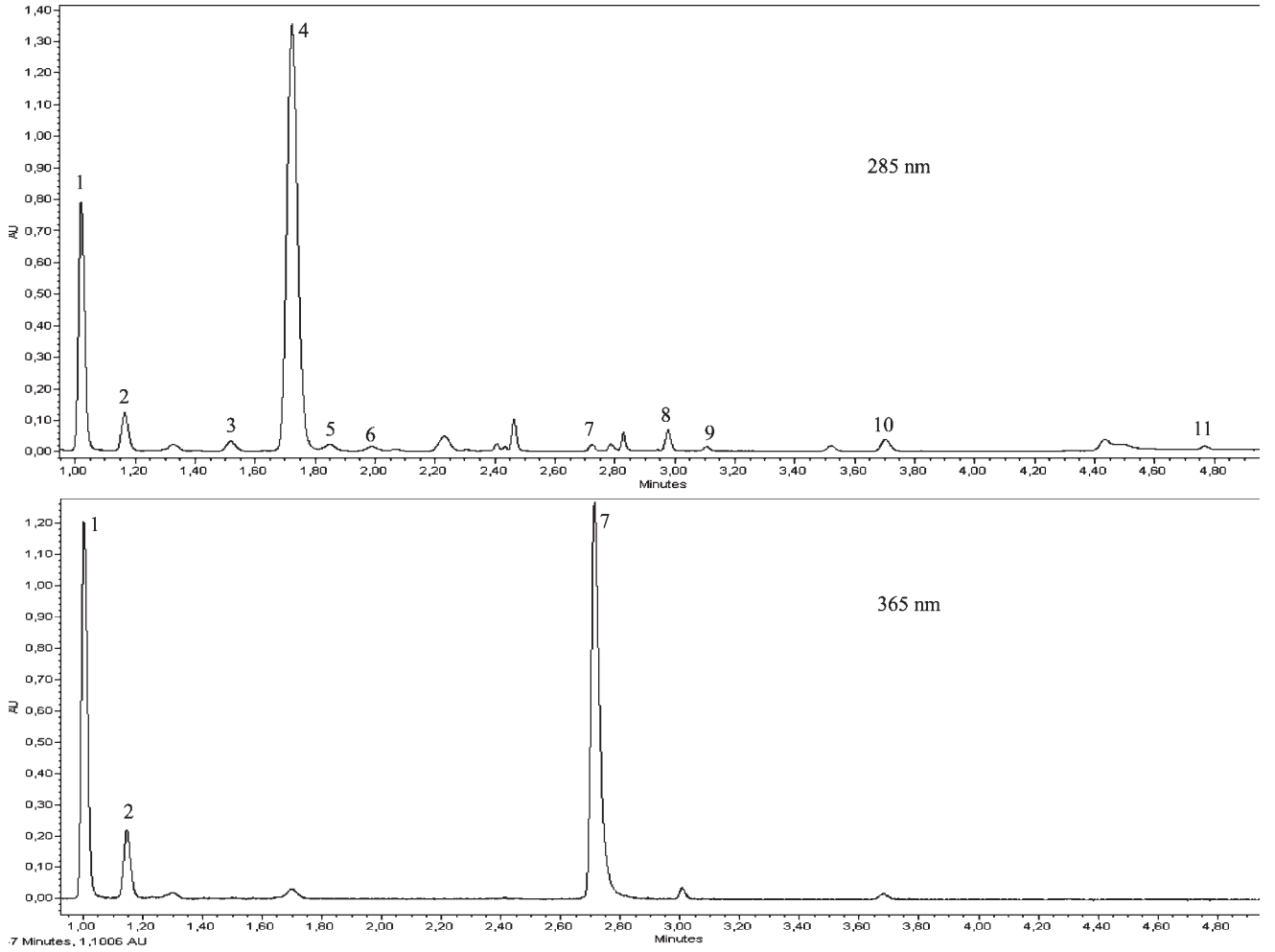

Figure 1. Chromatogram corresponding to Leben and Trinken extracts at 280 and 365 nm, respectively. Rutin (1), isoquercitrin (2), narirutin (3), naringin (4), prunin (5), hesperidin (6), quercetin (7), naringenin (8), hesperetin (9), sinensetin (10), and tangeretin (11).

PIS experiments were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass analyzed using the instrument's second analyzer. In all the experiments, both quadrupoles (Q1 and Q3) operated at unit resolution.

RESULTS AND DISCUSSION

In this work, the UHPLC method developed for the quantification of flavonoids in *Citrus* fruit extracts was found to be capable of giving a faster analysis with good resolution than that achieved with conventional HPLC. We developed a very rapid method especially adapted to analyze large batches of samples, with the potential for proportionally greater sensibility and with a methodology that is much friendlier to the environment due to its low consumption of organic solvents compared to that of other analytical techniques.

Flavonoid Samples. We separated the 11 selected flavonoids in only 5.5 min. The chromatograms for Leben and Trinken samples at 280 and 365 nm, respectively, are shown in Figure 1. The peaks separated for rutin, isoquercitrin, and quercetin reached their maximum at 365 nm, while narirutin, naringin, prunin, hesperidin, naringenin, hesperetin, sinensetin, and tangeretin reached their maximum at 280 nm. The classes of flavonoids that characterize *Citrus* species have their maximum absorption at specific wavelength ranges: flavanones (280−290 nm), flavones (304−350 nm), and flavonols (352−385 nm).[9]

Fujita et al.[6] measured the flavonoids of 12 immature *Citrus* fruit extracts using HPLC. In this assay, the samples were refluxed 3 times during 30 min at 80 °C, concentrated under vacuum, and dissolved in methanol. This laborious method required 45 min to obtain the chromatogram. Pupin et al.[20] used HPLC to analyze flavones in Brazilian orange juice. This technique is useful to distinguish orange juice (single strength or concentrated) from a second-pressure extract and can identify mixtures and determine the authenticity of pigmented orange juice.

Mata-Bilbao et al.[13] quantified flavonoids in a similar *Citrus* fruit extract composed of four species using HPLC with a photo diode array detector. This method required 45 min for the gradient elution and 15 min to equilibrate the HPLC column. Our UPLC analysis was faster in two steps: the run time was reduced, and the equilibration time for bringing the column to the initial conditions after gradient analysis was much shorter, allowing the separation of 11 selected flavonoids in only 5.5 min after the injection of less volume (6 μL).

In some European countries, there is a legal barrier against marketing some *Citrus* juice mixtures under the label orange juice, which the FDA conditionally allows for pasteurized and

Table 1. Linear Regression Data, LOD, and LOQ of the Investigated Compounds[a]

	parameter					
	linearity range (μg mL^{-1})	slope (b) ± SD	y-intercept (a) ± SD	correlation coefficient (R^2)	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
rutin	1−100	12378.50 ± 587.61	4237.65 ± 94.40	0.9996	0.02	0.08
isoquercitrin	1−90	14378.00 ± 33.94	10651.05 ± 1101.60	0.9994	0.23	0.77
narirutin	1−80	19511.00 ± 599.63	1146.00 ± 981.46	0.9995	0.15	0.50
naringin	1−100	16139.00 ± 257.39	11527.50 ± 1117.94	0.9996	0.21	0.69
hesperidin	1−100	15024.00 ± 728.32	18208.50 ± 440.53	0.9988	0.09	0.29
prunin	1−30	23451.00 ± 1112.98	9952.15 ± 394.35	0.9994	0.05	0.17
quercetin	1−80	35516.00 ± 1364.72	11574.50 ± 843.58	0.9992	0.07	0.24
naringenin	1−80	28722.50 ± 663.97	10378.05 ± 1098.77	0.9998	0.11	0.38
hesperetin	1−100	28298.50 ± 1802.42	10511.00 ± 493.56	0.9985	0.05	0.17
sinensetin	1−60	13743.00 ± 468.10	6797.70 ± 182.01	0.9992	0.04	0.13
tangeretin	1−50	22994.50 ± 409.41	35479.00 ± 1144.10	0.9969	0.15	0.50

[a] SD, standard deviation; LOD, limit of detection; LOQ, limit of quantification.

canned orange juice and frozen concentrated orange juice. A simultaneous LC method for the separation of flavonoid compounds allows *Citrus* juice to be characterized by its phenolic profile.[21] HPLC technology allows adulterated *Citrus* juices to be identified.[22,23] Ooghe et al.[24] established a procedure using HPLC equipped with a PDA detector to determine the juice addition to *C. sinensis* of up to 10% *C. reticulata* and hybrids thereof and up to 5% *C. aurantium*.

Spácil et al.[17] compared a conventional HPLC system and a UHPLC system and found that for flavonoid compounds (rutin, naringin, quercitrin, daidzein, quercetin, hesperetin, kaempferol, rhamnetin, and galangin), analysis duration was 2.5 times shorter with UHPLC, and solvent consumption was decreased by 5.5 times. The UHPLC system also showed very good sensitivity (1.7 times higher), allowing the injection of only 1.5 μL volume for reliable analysis results. Its peak capacity number was also significantly higher than that in the HPLC system. Cooper et al.[25] described a UHPLC method that can separate and quantify six of the major polyphenols in a wide range of chocolates from different countries in 3 min. UHPLC makes it possible to perform very high-resolution separations in short periods of time with little solvent consumption. Because of the hardware adjustments, it also allows work at extreme pressures of up to 100 MPa.[17,26,27] The run-time of analysis and the equilibration time for bringing the column to the initial conditions after gradient analysis are much shorter.[17] This new UHPLC analysis was performed 8.2 times faster than those by HPLC,[13] and solvent consumption for each individual analysis was reduced by almost 6.2 times. The pressure reached was 665 bar at 90% of mobile phase A and 332 bar at 100% of mobile phase B; hence, the pressures in the complete process were between these values at the flow rate of 0.6 mL min^{-1}.

Method Validation. For the validation of the method under the optimized conditions, the 7-point calibrator concentration showed a linear and reproducible curve for all standards, with good correlation coefficients (Table 1) indicated by $R^2 > 0.99$ values. LOD and LOQ were very low, less than 0.23 and 0.77 μg mL^{-1}, respectively, which confirmed the high sensitivity of the proposed method.

Precision was evaluated at three different concentration levels (Table 2) for all the standards found in the samples. Repeated aliquots were prepared for each concentration level. The spiked *Citrus* fruit extracts were prepared according to the general procedure and analyzed by the proposed method. Three recovery values were less than 80% but higher than 66%, while the others were between 80.11% and 107.00%; the AOAC International specifies that recovery should be within the range of 80−110%. The values obtained for precision RSD (%) were lower than those established by the AOAC International,[19] which would be 7.3% at this concentration. Obviously, the low RSD confirmed the high precision of the proposed method.

Similarly, levels of accuracy were determined by recovery studies of flavonoids from Trinken and Leben matrices spiked with different concentrations of flavonoids (80%, 100% and 120% of the expected value) covering the linear range of the method. Each flavonoid peak area versus the concentration of each flavonoid was then interpolated from the linear regression equation. The recovery values (presented as percentages) were calculated using the formula: calculated experimental concentration/nominal concentration × 100. The mean recovery value was found to be 94.99 ± 6.03%, indicating the accuracy of the proposed method (Table 2).

In our method, the speed of the analysis was improved in two steps: the run-time was reduced by 5.5 min, and the equilibration time for bringing the column to the initial conditions after gradient analysis was significantly shortened by 1 min. Solvent consumption was decreased by a factor of 8 in comparison with that in HPLC methods.[13] The UHPLC system also showed very good sensibility, allowing the injection of only 6 μL volume for reliable analysis results.

Confirmation Analysis. Ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-QqQ-MS/MS) was applied to confirm the results obtained by UPLC/PDA analysis (Table 3). As with UPLC-PDA, 11 *Citrus* flavonoids were identified in the extracts: quercetin (m/z^- 301), isoquercetrin (m/z^- 463), rutin (m/z^- 609), naringenin (m/z^- 271), narirutin (m/z^- 579), naringin (m/z^- 579), hesperitin (m/z^- 301), hesperidin (m/z^- 609), tangeretin (m/z^+ 373), sinensetin (m/z^- 373), and prunin (m/z^- 433), with this last one only found in Biocitro and Leben *Citrus* extracts, showing an intense molecular ion $[M - H]^-$ at m/z 433 and the corresponding aglycone molecular ion at m/z^- 271 $[433 - 162]^-$. However, no neohesperidin (hesperitin-7-*O*-neohesperidoside) was observed in the extracts. Table 4 specifies the MRM transition and collision energy (CE) for each target analyte. Additional important MS parameters, namely, DP, FP, EP, and collision cell exit potential (CXP) optimized for each compound are also included in this table.

Table 2. Evaluation of Precision and Recovery Obtained from UHPLC for Different Compounds in Two *Citrus* Fruit Extracts[a]

	Trinken				Leben			
standard	added conc. mg g^{-1}	measured conc. (n = 3) mg g^{-1}	recovery (error) (%)	precision (RSD) (%)	added conc. mg g^{-1}	measured conc. (n = 3) mg g^{-1}	recovery (error) (%)	precision (RSD) (%)
rutin	3.39	3.07	90.55	5.69	3.54	3.79	107.00	5.66
	4.42	4.50	101.59	3.57	4.45	4.35	97.83	1.37
	5.77	6.00	103.99	1.45	5.09	4.78	93.83	1.72
isoquercitrin	0.69	0.56	80.66	2.37	0.73	0.72	99.75	3.66
	0.92	0.81	87.43	4.62	0.89	0.87	98.11	1.15
	1.15	1.19	102.75	2.91	1.02	0.97	94.92	2.44
narirutin	5.75	5.64	98.09	1.55	9.15	8.90	97.23	2.19
	7.19	7.05	98.09	0.86	11.44	11.27	98.51	3.59
	8.62	8.37	97.01	1.83	13.73	13.30	96.90	1.50
naringin	3.73	2.47	66.26	2.48	4.06	3.19	78.72	5.80
	5.05	4.03	79.74	2.49	4.90	4.03	82.14	2.24
	6.35	5.26	82.86	2.47	5.60	4.48	80.11	4.38
hesperidin	1.16	1.05	91.19	6.24	1.13	1.12	99.17	2.87
	6.24	1.37	94.92	6.59	1.41	1.16	82.61	2.72
	1.73	1.57	90.40	2.90	1.70	1.40	82.37	1.9
prunin	0.90	0.87	96.53	5.24	1.07	1.00	93.60	3.04
	1.13	1.07	94.99	4.94	1.34	1.17	87.37	3.63
	1.35	1.22	90.60	5.26	1.60	1.43	89.20	6.53
quercetin	4.51	4.27	94.45	6.06	4.72	4.77	101.0	3.72
	5.97	5.93	99.52	5.66	5.79	5.79	98.96	2.40
	7.50	7.58	100.96	2.92	6.61	6.66	100.59	1.69
naringenin	0.93	0.98	105.07	2.48	0.64	0.75	102.23	3.89
	1.16	1.16	100.22	3.23	1.00	0.98	97.92	2.41
	1.40	1.27	90.51	3.98	1.36	1.61	94.75	1.16
hesperetin	1.02	1.07	104.61	4.80	0.75	0.73	97.81	3.61
	1.27	1.13	89.36	2.63	0.93	0.82	88.39	5.42
	1.53	1.28	83.61	6.02	1.12	1.00	89.74	2.55
sinensetin	4.88	4.54	93.05	1.80	4.51	4.54	100.70	4.32
	6.10	5.62	92.21	3.22	5.63	5.72	101.53	1.99
	7.32	6.55	89.49	3.27	6.76	6.23	92.22	1.95
tangeretin	0.45	0.45	100.87	5.53	0.49	0.47	95.95	5.87
	0.57	0.56	98.96	6.96	0.62	0.62	99.79	6.66
	0.68	0.68	99.29	4.95	1.00	0.79	102.62	5.64

[a] SD, relative standard deviation.

The samples analyzed were composed of four *Citrus* species: *C. paradisi*, *C. sinensis*, *C. aurantium L.* subspecies *bergamia*, and *C. white reticulata*; each one is characterized by particular flavanone glycosides.[9] The presence of a relatively large number of flavonoids in *Citrus* juices is a result of the many different possible combinations between polyhydroxylated aglycones and a limited number of mono- and disaccharides.

Each species of *Citrus* is characterized by a particular flavanone glycoside pattern that can be separated by HPLC. The most abundant component in sweet orange juices (*C. sinensis*), regardless of variety, is by far hesperidin (286 mg L^{-1}), followed by narirutin (52 mg L^{-1}) and didymin (18.9 mg L^{-1}). Mandarin juice (*C. reticulata*) is quite similar to sweet orange juice, hesperidin (243 mg L^{-1}) again being the main component, followed by narirutin (39.2 mg L^{-1}) and didymin (14.4 mg L^{-1}).[9,28] Polymethoxyflavones have been reported over the years as minor components of orange juice, including sinensetin (3.7 mg L^{-1}), nobiletin (3.3 mg L^{-1}), tangeretin (0.4 mg L^{-1}), and 3,3′,4′,5,6,7,8-heptamethoxyflavone (0.8 mg L^{-1}).

Contrary to sweet orange, mandarin, and their closely related species, *C. aurantium* (Bergamot) is not generally suitable for direct consumption. Its juice composition is very different from that of sweet orange but quite similar to grapefruit, being rich in naringin (19.6 mg L^{-1}), neohesperidin (8.7 mg L^{-1}), and neoeriocitrin (7.7 mg L^{-1}).[9] Grapefruit (*C. paradisi*) juice can generally be found in three color varieties: red, pink and white, depending on the presence or absence of lycopene.[29] The main components of grapefruit juice are naringin (230 mg L^{-1}) and naringenin (27.0 mg L^{-1}), with narirutin also present in good amounts (76.0 mg L^{-1}).[9] Citrus extracts such as *C. paradisi* contain naringin and furanocoumarins in abundance.[6] *Citrus* flavonoid composition appears to vary greatly depending on genetic origin, the time of fruit collection, and the different parts of the fruit used (peel and edible parts).[30]

Table 3. List of the Flavonoid Compounds Identified in the *Citrus* Extracts[a]

			ion full scan MS		MS/MS data		comparison	Biocitro	Leben	Trinken
compound	*Rt* (min)	ion mode	MI	fragment ions	PIS	NL	with standard	extract	extract	extract
rutin	0.99	−	609.3	300.9	609	308	yes	yes	yes	yes
isoquercitrin	1.13	−	463.3	300.9, 179.1	463	162	yes	yes	yes	yes
narirutin	1.48	−	579.5	271.2, 151.1	579	308	yes	yes	yes	yes
naringin	1.68	−	579.5	459.6, 372.8, 271.3, 150.8	579	308	yes	yes	yes	yes
prunin	1.81	−	433.6	271.1	433	162	no	yes	yes	no
hesperidin	1.95	−	609	301.1	609	308	yes	yes	yes	yes
quercetin	2.70	−	301.3	178.9, 151.1, 121.3, 107.1	301	−	yes	yes	yes	yes
naringenin	2.96	−	271.1	150.9, 118,9, 107.1, 93.1	271	162	yes	yes	yes	yes
hesperetin	3.10	−	300.9	285.9, 242.3, 164.0, 150.9	301	−	yes	yes	yes	yes
sinensetin	3.70	+	373.1	358.1, 343.1, 329.1, 312.3	373	−	yes	yes	yes	yes
tangeretin	4.73	+	373	358.2, 342.9, 325.0, 297.3	373	−	yes	yes	yes	yes

[a] Rt: retention time; NL, neutral loss; PIS, precursor ion scan.

Table 4. MRM Transition, Declustering Potential (DP), Focusing Potential (FP), Entrance Potential (EP), Collision Energy (CE), and Collision Cell Exit Potential (CXP) Optimized for Each Analyte Searched

compound	ion mode	MRM (Q1→Q3)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
rutin	−	609→301	−60	−210	−10	−30	−15
isoquercetrin	−	463→301	−70	−230	−10	−30	−15
narirutin	−	579→271	−120	−200	−10	−40	−15
naringin	−	579→459	−130	−200	−10	−35	−15
prunin	−	433→271	−70	−200	−10	−30	−15
hesperidin	−	609→301	−70	−250	−10	−35	−15
quercetin	−	301→151	−60	−200	−10	−30	−15
naringenin	−	271→151	−80	−200	−10	−25	−15
hesperetin	−	301→286	−60	−220	−10	−35	−15
sinensetin	+	373→312	−50	−200	−10	−30	−15
tangeretin	+	373→343	−50	−190	−10	−30	−15

Table 5. Quantity of Each Flavonoid Identified in Different Samples[a]

flavonoids	Biocitro	Leben	Trinken
	Mean (mg 100 g^{-1} of sample) ± SD		
rutin	116.60 (5.75)	256.33 (26.79)	248.24 (9.64)
isoquercitrin	60.05 (0.75)	81.88 (7.92)	80.85 (2.32)
narirutin	5.54 (0.46)	14.17 (1.12)	11.51 (2.00)
naringin	299.06 (5.12)	544.36 (48.43)	515.78 (3.55)
hesperidin	1.27 (0.21)	2.20 (0.38)	2.31 (0.13)
prunin	1.89 (0.37)	1.85 (0.25)	nd
quercetin	251.49 (2.02)	7.78 (0.63)	179.88 (25.07)
naringenin	2.80 (0.55)	2.82 (0.32)	4.64 (0.56)
hesperetin	3.30 (0.28)	1.44 (0.14)	2.55 (0.17)
sinensetin	168.76 (10.06)	7.73 (1.58)	9.77 (1.18)
tangeretin	0.17 (0.01)	0.42 (0.07)	0.41 (0.24)

[a] SD, standard deviation; RSD, relative standard deviation; nd, not detected.

Table 5 shows the quantity of each flavonoid identified in three different samples (mg 100 g^{-1} of sample); the means ± SD represent the average of six replicated measures for each sample. The most abundant component in the analyzed samples was naringin (between 299.06 and 544.36 mg 100 g^{-1}) followed by rutin (between 116.60 and 256.33 mg 100 g^{-1}) and quercetin (between 7.78 and 251.49 mg 100 g^{-1}). Isoquercitrin was found in a lower proportion, between 60.05 and 81.88 mg 100 g^{-1}, similar to a previous study.[13] This is the second study to report the presence of isoquercitrin in samples of grapefruit (*C. paradisi*), sweet orange (*C. sinensis*), bergamot (*C. aurantium L. bergamia*), and mandarin (*C. white reticulate*).

In summary, we have developed a fast method combining UHPLC with a diode array detector, which was successfully applied in *Citrus* fruit extracts for the simultaneous quantification of flavonoids in only 5.5 min. This method required a rapid gradient that provides the flavonoid separation from the extracts. It is cheaper, more environmentally friendly, and simpler than other previously described methodologies. The excellent retention time and peak area repeatability of the UHPLC method are of particular importance for use in routine food analyses. The method was completely validated, providing a sensitive analysis for flavonoid detection and showing satisfactory data for all the parameters tested. Good results were obtained with respect to linearity and recovery as well as an excellent level of precision. UHPLC methods can also be rated as green methods.

AUTHOR INFORMATION

Corresponding Author

*Tel: +34-934034843. Fax: +34-934035931. E-mail: lamuela@ub.edu.

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■ ABBREVIATIONS USED

CV, coefficient of variation; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; LOD, limits of detection; LOQ, limits of quantification; PDA, photodiode array; RSD, relative standard deviation; SD, standard deviation; UHPLC, ultrahigh performance liquid chromatography; UPLC, Waters Acquity ultraperformance liquid chromatography; UV, ultraviolet.

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