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Rapid Determination of Phenolic Compounds and Alkaloids of Carob Flour by Improved Liquid Chromatography Tandem Mass Spectrometry

Nàdia Ortega,[†,‡] Alba Macià,[†] Maria-Paz Romero,[†] Esther Trullols,[‡] Jose-Ramón Morello,[‡] Neus Anglès,[‡] and Maria-Jose Motilva*[,†]

[†]Food Technology Department, XaRTA-UTPV, Escuela Técnica Superior de Ingeniería Agraria Universidad de Lleida, Av/Alcalde Rovira Roure 191, 25198 Lleida, Spain, and [‡]R+D+i Department, La Morella Nuts, S.A., Camí Ample s/n, 43392 Castellvell del Camp, Spain

An improved chromatographic method was developed using ultra-performance liquid chromatography-tandem mass spectrometry to identify and quantify phenolic compounds and alkaloids, theobromine and caffeine, in carob flour samples. The developed method has been validated in terms of speed, sensitivity, selectivity, peak efficiency, linearity, reproducibility, limits of detection, and limits of quantification. The chromatographic method allows the identification and quantification of 20 phenolic compounds, that is, phenolic acids, flavonoids, and their aglycone and glucoside forms, together with the determination of the alkaloids, caffeine and theobromine, at low concentration levels all in a short analysis time of less than 20 min.

KEYWORDS: Phenols; alkaloids; carob flour; ultra performance liquid chromatography; tandem MS

INTRODUCTION

Carob (*Ceratonia siliqua* L.) is a typical tree in the Mediterranean area, whose pods and seeds are harvested mainly for use as ingredients in the food industry. The commercial value of the carob has been mainly attributed to the seeds, which have several applications in the food and cosmetic industries. As a byproduct, the pods have usually been used for animal feed, given their high content of carbohydrates and dietary fiber and their low fat content (*1*, *2*). Furthermore, recent studies have attributed an antioxidant capacity to the carob pod due to the presence of polyphenols, principally condensed tannins (*3*, *4*).

The process of producing carob flour starts by deseeding carob pods to yield the kibbles. These kibbles are first ground to different sizes and then roasted. Finally, the samples are milled into a fine powder called flour. The time and temperature conditions during the roasting process could affect the stability of the polyphenols by reducing the hydrolyzable tannins into an increased content of simple phenolic compounds. However, there is still little information about the characterization of the phenol compounds, such as flavonoids and phenolic acids, after processing the carob pods.

The possibility of obtaining carob flour by roasting and milling the pods has increased its relevance as a food ingredient. For example, several applications have been assigned to carob flour, that is, as in the preparation of cocoa substitute drinks and as a sweetener for children (*2*, *5*, *6*). Moreover, carob flour has also attracted interest due to its high fiber content (*7*). Because of the increasing demand for healthy food products, recent studies have also used carob flour to enrich functional products, such as biscuits (*8*, *9*).

*To whom correspondence should be addressed. Tel: +34 973 702817. Fax: +34 973 702596. E-mail: motilva@tecal.udl.cat.

With regard to the alkaloids, caffeine and theobromine, they have been found to be absent from the carob pod and derivatives (*10*), which is important for its use as a cocoa substitute in the formulation of infant foods. Nevertheless, as carob flour could be proposed as a cocoa substitute, it should guarantee the absence or a minor content of caffeine and theobromine. In this sense, the use of improved chromatographic techniques and sensitive detector systems is necessary to detect and confirm the absence or presence of these alkaloids in trace levels in the carob flour samples.

There is very limited data about the chromatography methods performed by high-performance liquid chromatography (HPLC) for determining polyphenols in carob derivatives, such as carob flour, and these have been found to require a long analysis time, ranging from 40 to 102 min (*7*, *11*, *12*). Because of the complexity of the carob matrix and the need to determine a wide range of phenols and alkaloids in the carob samples at low concentration levels, the development of an efficient, fast, and reliable analytical method is quite a challenge. As efficiency and speed of analysis have become of great importance in many applications of liquid chromatography, especially in the area of functional food formulation, it is important to increase throughputs and reduce analysis costs. In these sense, ultra-performance liquid chromatography (UPLC) could play a significant role. UPLC uses analytical columns packed with 1.7 μm particles, which offers the advantages of increasing speed and improving sensitivity, selectivity, and specificity as compared to conventional HPLC. The higher efficiency of small particles enables shorter columns to be used, reducing analysis time and solvent consumption.

Recent studies have used UPLC to improve the phenol analysis in beverages, with the detection of 17 phenolic compounds in 9.5 min (*13*). Moreover, coupling UPLC to tandem mass spectrometry (MS/MS) allows a better identification and confirmation of

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some polyphenol compounds, as compared to HPLC, for example, the determination of procyanidins up to nonamers in different cocoa sources in less than 12.5 min (*14*). Furthermore, this technique has also been used to analyze marine toxins (*15*) and pesticides residues (*16*, *17*) in foods in response to the demand to monitor a wide range of compounds in a single analysis, to determine analytes at lower concentration levels in a shorter analysis time, and to avoid the use of environmentally hazardous solvents.

Taking into account that carob flour could be used as a natural ingredient in the formulation of new functional foods, based on its high content in fiber and low levels of fat, as well as the low content of alkaloids (caffeine and theobromine) (*10*), the aim of this study was focused on developing a rapid and sensitive methodology based on UPLC-MS/MS to determine the different classes of phenolic compounds, such as phenolic acids, flavonoids, and alkaloids (caffeine and theobromine). The quality parameters of the developed method [linearity, calibration curve, reproducibility, detection limits (LODs) and quantification limits (LOQs)] were studied. Then, the method was applied to determine polyphenols in carob flour samples.

MATERIALS AND METHODS

Chemicals and Reagents. Caffeine, theobromine, syringic acid, naringenin, and myricetin were obtained from Sigma Aldrich (St. Louis, MO). Kaempferol came from Fluka Co. (Buchs, Switzerland). Ferulic acid, *o*-coumaric acid, *p*-coumaric acid, cinamic acid, quercetin, apigenin, genistein, and luteolin were purchased from Extrasynthese (Genay, France). Gallic acid was obtained from Panreac (Barcelona, Spain).

For each phenol, a stock standard solution of 1 mg/mL was prepared in methanol. Then, standard working solutions, containing a mixture of all standard phenols at increasing concentrations, were prepared weekly by diluting the stock standard solution in methanol. These solutions were stored in dark-glass flasks at 4 °C.

Chromatographic Analysis. *UPLC*. The chromatographic analyses were performed using a Waters Acquity Ultra-Performance liquid chromatography system (Waters, Milford, MA), equipped with a binary pump system (Waters). The column used was a 100 mm × 2.1 mm i.d., 1.8 μm, Acquity High Strength Silica (HSS) T3 from Waters. The mobile phase was Milli-Q water/acetic acid (0.2%), as solvent A, and acetonitrile, as solvent B. The gradient elution was as follows: 0−2 min, 5% B isocratic; 2−6 min, 5−18% B; 6−7 min, 18−21% B; 7−10 min, 21% B isocratic; 10−12 min, 21−21% B; 12−16 min, 21−40% B; 16−16.1 min, 40−100% B; 16.1−18 min, 100% B isocratic; 18−18.1 min, 100−5% B; 18.1−20 min, 5% B isocratic. The flow rate was 0.4 mL/min, and the temperature was set at 30 °C.

The UPLC was coupled to an Acquity UPLC PDA detector and a TQD mass spectrometer (Waters). The wavelengths in the PDA detector were set at 278 and 339 nm.

MS/MS. The tandem MS analyses were carried out on a TQD mass spectrometer (Waters) equipped with a Z-spray electrospray (ESI) interface. The analyses were done in a single run, in negative mode for all of the phenolic compounds and in positive mode for the alkaloids, and the data were acquired by selected reaction monitoring (SRM). The ionization source working conditions were as follows: capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow rate, 80 L/h; desolvation gas flow rate, 800 L/h; and desolvation temperature, 400 °C. Nitrogen (>99% purity) and argon (99% purity) were used as nebulizing and collision gases, respectively. Cone voltages and collision energies were optimized by infusion of a standard solution of 10 mg/L of each standard in a mixture of acetonitrile:water (50:50, v/v) at a flow rate of 10 μL/min. First, full-scan mode was acquired to select the most abundant *m*/*z* value, and the cone voltage was optimized. In all cases, $[M-H]^-$ or $[M-H]^+$ ions were found to be the most abundant. These ions were selected as the precursor ions, and afterward, the collision energies were studied to find the most abundant product ions. Therefore, the most sensitive transition for each phenol was selected for quantification purposes and the second one for confirmation (Supporting Information). For cinamic acid, no second transition SRM was available for its confirmation, and to consider only one ion is a problem for its correct identification according to EU criteria. In this work, one transition and LC retention time were the criteria applied for the purposes of identifying and quantifying this phenolic acid. The dwell time established for each transition was 30 ms. Data acquisition was carried out with the MassLynx v 4.1 software.

Quality Parameters. The quality parameters of the developed method were studied by analyzing a serial dilution of the standard solutions, which contained the phenolic compounds and the two alkaloids, by UPLC-MS/MS. The parameters considered were linearity, calibration curve, reproducibility, LOD, and LOQ.

The calibration curves (based on the integrated peak area) were calculated using five points at different concentrations (from 0.0005 to 62.5 μg/mL), and each standard solution was injected three times. The reproducibility, expressed by relative standard deviations (RSDs) of the concentration, was studied at two concentration levels, 1 and 10 μg/mL, on three different days and with one injection per day (interday). The LODs and LOQs were experimentally calculated using signal-to-noise criteria of 3 and 10, respectively.

Carob Flour Phenol Extraction. Commercial carob flour sample was kindly provided by La Morella Nuts, S. A. (Reus, Catalonia, Spain). To obtain the flour, carob pods were crushed mechanically using a kibbler, which provided two important products: the carob kernels (10% of the pod's weight) and the kibbles (the remaining pulp, 90% of the pod's weight). The kibbles were roasted (between 150 and 250 °C) and milled to obtain a fine powder, the carob powder, or carob flour.

The extraction of phenolic compounds and alkaloids, caffeine and theobromine, from the carob flour was similar to the method reported in our previous work for cocoa sources (*14*). Briefly, the sample was defatted once for 20 min at a ratio of 1:5 with hexane in an orbital shaker (Selecta, Barcelona, Spain) at 200 rpm and subsequently centrifuged for 15 min at 15000*g*. The obtained residue (defatted carob flour) was extracted four times with a 70% acetone−aqueous solvent mixture at a ratio of 1:5. After the addition of the extraction solvent mixture, the sample was extracted by Polytron (Branson, Danbury, CT) for 5 min and then centrifuged for 8 min at 12500*g* at 4 °C. The supernatants (four) were combined, and the organic solvent (acetone) was removed by rotary evaporation (Büchi, Labortechnick AG, Switzerland) under a partial vacuum at 30 °C. Finally, the phenolic extract was freeze-dried (BMG Labtech, Offenburg, Germany) and stored at −80 °C in an N_2 atmosphere.

Identification and Quantification of Carob Flour Phenolic Fraction. The lyophilized phenolic extract was dissolved in 70% acetone aqueous at a concentration of 10 mg/mL and filtered through 0.22 μm nylon filters before the chromatographic analysis by UPLC, as described before. The sample volume injected was 2.5 μL.

Apart from the phenolic compounds included in the validation method (flavonoids, phenolic acids, and alkaloids), the application of the method to analyze the carob flour phenolic extract revealed the presence of methyl gallate and flavonoids in their glucoside form. To identify and quantify them, analyses in MS (full-scan mode) and MS/MS (based on product ion scan mode) from carob flour phenolic extract were performed. Thus, two transitions were obtained.

The quantification of the different phenols was performed by using external calibration. The results of the quantification of the phenolic acids, the aglycone flavonoids, and the alkaloids (caffeine and theobromine) were expressed as μg standard per g of carob flour. Methyl gallate was quantified as gallic acid. All of the flavonoid glucosides were quantified as μg of the corresponding aglycone. The results of the quantification were expressed as μg per g of carob flour. All data were expressed as means ± standard deviations.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Method. Several studies have been focused on the characterization of carob derivatives, like pods, kibbles, and fiber fraction (*7*, *11*, *12*), but there are a few reports based on the identification and quantification of phenol compounds in carob flour. The quantification of phenols in carob products has shown that gallic acid is the main phenolic acid (*11*), while myricetin, quercetin, and kaempferol and their glucoside forms were the major compounds quantified from the flavonoids group (*11*). Thus, two different phenolic groups (phenolic acids and flavonoids) were selected in the present study to develop and validate the chromatographic method. Because of the interest in

Table 1. Retention Time (RT), Linearity, Calibration Curve, RSD (%) in Concentration, LOD, and LOQ for the Analysis of the Studied Compounds by UPLC-MS/MS in Standard Solutions

				% RSD (n = 3)			
compound	RT[a] (min)	linearity (μg/mL)	calibration curve[b]	10 μg/mL	1 μg/mL	LOD (μg/mL)	LOQ (μg/mL)
theobromine	1.78	0.05−20	$y = 6592.5x + 756.0$	0.9	1.9[c]	0.03	0.09
caffeine	2.69	0.002−20	$y = 39062x + 6351.7$	1.9	2.9	0.001	0.003
gallic acid	1.47	0.25−12.5	$y = 2157.8x - 616.9$	1.0	1.8	0.1	0.7
syringic acid	3.14	0.05−10	$y = 1255.5x + 105.2$	2.1	1.4	0.03	0.09
ferulic acid	4.84	0.04−10	$y = 4437.8x + 153.4$	1.0	0.6	0.02	0.05
o-coumaric acid	7.16	0.04−50	$y = 6424.1x + 1964.1$	3.0	2.4	0.02	0.06
p-coumaric acid	4.14	0.04−10	$y = 7522.1x + 1045.2$	1.1	1.6[c]	0.02	0.06
myricetin	8.57	0.6−12.5	$y = 448.1x - 179.4$	2.9	5.3	0.3	1.0
cinamic acid	12.35	0.025−62.5	$y = 2848.7x + 2124.4$	1.2	2.5	0.015	0.05
quercetin	13.51	0.1−10	$y = 3141.7x - 463.3$	2.9	3.6[c]	0.04	0.15
luteolin	13.69	0.004−50	$y = 4409.1x + 2851.1$	2.7	2.5	0.002	0.007
naringenin	15.24	0.0005−50	$y = 7947.9x + 3861.8$	2.0	1.0	0.0003	0.0010
genistein	15.41	0.005−62.5	$y = 1143.9x + 1318.5$	2.5	3.9	0.003	0.010
apigenin	15.52	0.003−5	$y = 14368x + 1005.1$	1.8	0.3[d]	0.001	0.005
kaempferol	15.78	0.2−10	$y = 476.8x - 69.3$	1.0	1.5[c]	0.1	0.4

[a] RT, retention time. [b] Calibration curve: $y = mx + b$, where y is the integrated peak area and x is the concentration in μg/mL. [c] A 2 μg/mL amount was used to calculate the % RSD. [d] A 0.5 μg/mL amount was used to calculate the % RSD.

carob flours for use as a cocoa substitute, caffeine and theobromine, two common alkaloids in cocoa derivatives, were also included.

The initial experiments were based on the chromatographic conditions described by Owen et al. (*7*). First, the HPLC elution gradient reported by these authors was converted to UPLC elution gradient by using the Acquity UPLC Columns calculator (Waters). Two UPLC columns were tested for the stationary phase, the conventional C_{18} and the HSS T3. The flow rate was changed from 1 to 0.4 mL/min to be the optimum in these columns, whose particle size is 1.8 μm. In our study, the solvent B methanol was replaced with acetonitrile because the use of methanol led to pressures higher than the maximum permitted in the equipment (15000 psi). By using the conventional C_{18} column, some analytes coeluted at the end of the LC gradient, such as the flavonoids. In contrast, the resolution of these phenolic compounds was improved when the HSS T3 column was used. This column has the advantage that it retains the polar compounds better than the conventional C_{18}, thus improving the resolution of the peaks. Afterward, the elution gradient, obtained in the Acquity UPLC Columns calculator, was slightly modified and optimized to improve the resolution of some peaks of flavonoids using the HSS T3 column. This methodology allows the studied compounds to be analyzed within 20 min.

Quality Parameters. Once the UPLC-MS/MS method was developed, its quality parameters, including linearity, reproducibility, LODs, and LOQs, were studied. The results of these quality parameters for the analysis of the studied compounds in standard solutions are shown in **Table 1**.

For each compound, the linearity range was determined by analyzing different dilution solutions. The calibration curves were obtained based on the integrated peak area, and these were linear over the range of study. The determination coefficients (R^2) of these calibration curves were higher than 0.995 for all of the compounds.

The reproducibility, expressed by % RSDs of the concentration, was studied at two concentration levels, namely, 10 and 1 μg/mL, except for theobromine, *p*-coumaric acid, quercetin, and kaempferol, which were studied at 2 μg/mL, and for apigenin at 0.5 μg/mL. As **Table 1** shows, good RSDs values were achieved in all of the compounds studied, ranging from 0.9 to 3 for 10 μg/mL and from 0.6 to 5.3 for 1 μg/mL.

The LODs ranged from 0.0003 to 0.3 μg/mL, and the LOQs ranged from 0.001 to 0.10 μg/mL, depending on the compound. The obtained results (LODs and LOQs) were lower than those reported in the literature for the analysis of these compounds by HPLC-PDA (*12*, *18*, *19*). For example, it should be noted that the LODs and LOQs obtained in our study for phenolic acids and flavonoids were lower than those reported by Muñoz et al. (*18*) and Kumar et al. (*19*), respectively, while for theobromine and caffeine, the values obtained in our study were between 25 and 200 times lower than those described by Peng et al. (*20*).

One significant drawback of ESI mass spectrometer is that the ionization source is highly susceptible to matrix signal suppression effects, in which the coextracted matrix components can alter the signal response, causing either suppression or enhancement, resulting in poor analytical accuracy. Therefore, the detector response obtained from standard solutions and food matrix samples may differ significantly. To evaluate this matrix effect, the detector responses of the phenolic compounds spiked in elution solvent (70% acetone aqueous) were compared to those spiked in phenolic extract of carob flour at different concentrations. Either a positive or a negative effect of less than 20% was observed (data not shown), which meant an increase or decrease in the detector response. To minimize this effect, the extract was diluted 1:2 to prevent it, but this dilution produced a decrease of sensitivity. Then, no dilution of the sample was done because this effect (20%) could be considered small in the complex matrix of carob flour.

Application of the Developed Method to the Analysis of Carob Flour Phenols. The UPLC-MS/MS developed method was applied to identify and quantify the phenolic fraction of carob flour. **Figure 1** shows the extracted ion chromatograms of the phenolic compounds studied and the two alkaloids, caffeine and theobromine, from the phenolic carob flour extract. Apart from the phenols included in the validation of the chromatographic method, flavonoid glucosides and a gallic acid derivative, such as methyl gallate, were also identified in carob samples, similarly to that previously reported by other authors (*7*, *11*). Some of the flavonoid glucosides are not available as commercial standards. To identify and quantify these in phenolic extracts, full-scan mode and product ion scan experiments were performed. In these experiments, first the precursor ion and the cone voltage were selected, and in the second experiment, the most sensitive product ions and the collision energy were obtained. Thus, two transitions were obtained, and then, these phenolic compounds were analyzed by SRM at the highest selectivity and sensitivity mode in LC-MS/MS. Seven flavonoid glucosides were identified, these

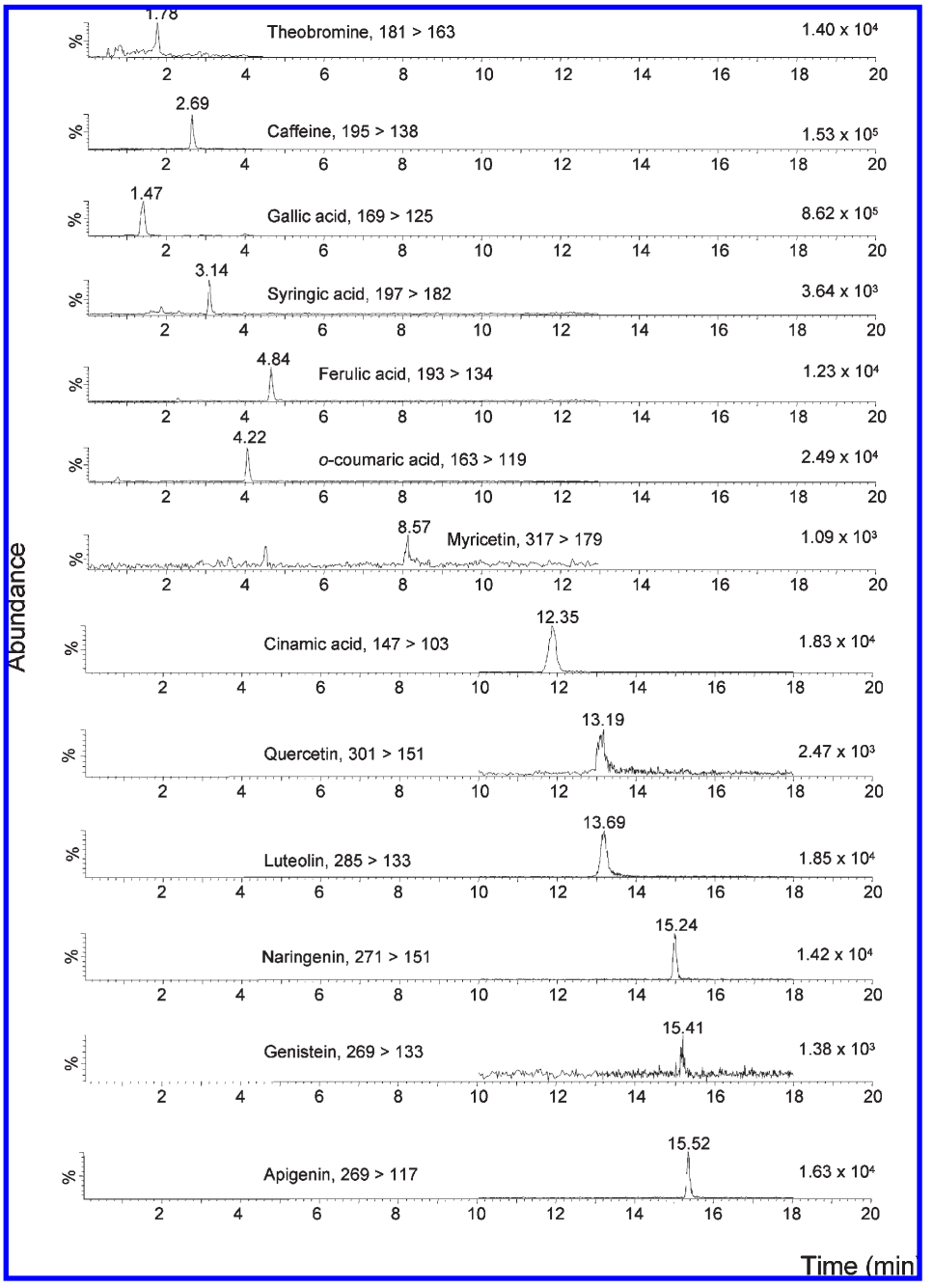

Figure 1. Extracted ion chromatograms of the studied phenolic compounds in carob flour sample. The concentration of the sample was 10 mg/mL.

being apigenin glucoside, kaempferol rhamnoside, quercetin arabinoside, quercetin rhamnoside, quercetin glucoside, myricetin rhamnoside, and myricetin glucoside and methyl gallate as a gallic acid derivative. **Figure 2** shows the extracted ion chromatograms of the flavonoid glucosides and the gallic acid derivative (methyl gallate) identified and their MS spectra.

The spectra generated by ESI-MS in negative acquisition mode for the compound eluting at 4.66 and 4.89, 5.53, 5.92, and 6.47, and 7.00 min showed an intense ion at *m*/*z* 463, 433, and 447, respectively, and a product ion at *m*/*z* 301 and 151, which demonstrated the presence of the quercetin molecule. Therefore, these ions could be explained by the loss of the glucose molecule (162 units), the arabinose molecule (132 units), and the rhamnoside molecule (146 units), respectively.

Two ions with precursor ions of *m*/*z* 479 and 463, which eluted at 3.56 and 4.50 min and whose product ions were *m*/*z* 317 and *m*/*z* 179, were identified as myricetin glucose and myricetin rhamnoside, respectively. These could be explained by the loss of 162 (*m*/*z* 317) and 146 units (*m*/*z* 317) that could correspond to the glucose and rhamnoside molecules, respectively.

The two ions with a precursor ion of *m*/*z* 431 that eluted at 5.29 and 7.39 min were identified as apigenin glucose, due to the loss of 162 units (*m*/*z* 269) that corresponded to the glucose molecule. On the other hand, the precursor ion of *m*/*z* 431 that eluted at 9.25 min was identified as kaempferol rhamnoside due to the loss of 146 units (*m*/*z* 269) that corresponded to the rhamnoside molecule. Finally, methyl gallate was identified to give a precursor ion of *m*/*z* 184 and a product ion of *m*/*z* 124. This product ion is explained by the loss of the methyl group (14 units).

Besides the sensitivity, the UPLC-MS/MS developed method allows the phenolic compounds and the alkaloids, caffeine and theobromine, in carob flour samples to be determined in 20 min. This analysis time is between two (*7*, *12*) and five times (*11*) shorter than the other methods reported in the literature for the analysis

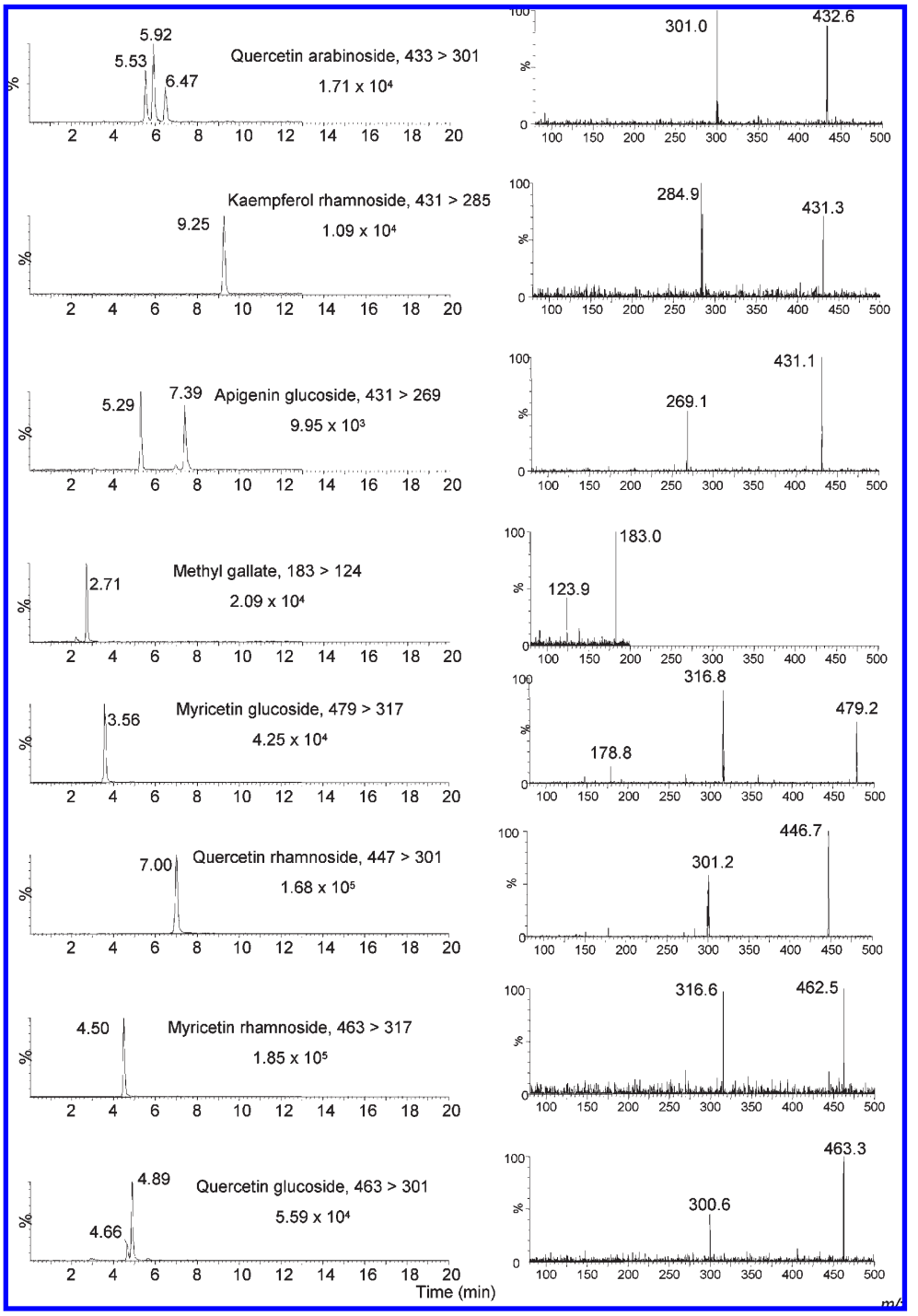

Figure 2. Extracted ion chromatograms and mass spectra of the flavonoids glucosides and methyl gallate identified in the carob flour sample.

of similar samples. This significant reduction in the analysis time was due to the improvements in the LC technique, which uses shorter columns that are packed with smaller size particles (1.8 μm) than the conventional HPLC technique (5 μm).

Table 2 shows the composition and quantification of the phenols in the carob flour. During the characterization, three different phenolic groups were distinguished, phenolic acids, flavonoid aglycones, and glucosides. Among the phenolic acids, gallic acid was the predominant compound, and its concentration was 2675 μg/g of carob flour. Owen et al. (*7*) have described 1647 μg of gallic acid/g of carob fiber, which supposes 50% of the carob flour composition. On the other hand, the values obtained in our study are four times higher than those reported by Papagiannopoulos et al. (*11*) in different roasted carob flours, which ranged from 264.1 to 685.6 μg/g of flour. These authors attributed the differences in the gallic acid content between samples to the roasting process, mainly to the air and temperature treatment, used to obtain the carob flour (*11*). Among all of the flavonoid aglycones, myricetin and luteolin were the compounds with the highest concentration, at around 108 and 41 μg/g of flour, respectively (**Table 2**). With regard to the flavonoid glucosides, attention should be paid to the high concentration of myricetin rhamnoside, at 1665 μg/g of flour.

The results shown for the quantification of polyphenols in carob flour in our work are partially in agreement with those reported by Papagiannopoulos et al. (*11*). The differences mainly

Table 2. Quantification by UPLC-MS/MS of Phenolic Compounds (Phenolic Acids, Flavonoid Aglycones, and Glucosides) and Alkaloids (Caffeine and Theobromine) in Carob Flour[a]

compounds	μg/g carob flour
cinamic acid	44.0 ± 14.9
p-coumaric acid	9.9 ± 2.7
gallic acid	2675.6 ± 143.3
ferulic acid	38.0 ± 1.1
syringic acid	11.0 ± 3.3
total phenolic acids	2778 ± 145
genistein	3.8 ± 2.2
apigenin	1.6 ± 0.6
naringenin	9.3 ± 2.2
luteolin	41.3 ± 9.3
kaempferol	0.0 ± 0.0
quercetin	17.6 ± 1.65
myricetin	108.3 ± 11.6
total flavonoid aglycones	182.6 ± 11.8
methyl gallate	54.5 ± 8.2
apigenin glucose	0.5 ± 0.0
kaempferol rhamnoside	174.4 ± 45.7
quercetin arabinoside	88.0 ± 13.8
quercetin rhamnoside	470.3 ± 120.4
quercetin glucose	140.7 ± 28.1
myricetin rhamnoside	1665.4 ± 252.3
myricetin glucose	530.3 ± 121.6
total flavonoid glucosides	3123.7 ± 254.4
total polyphenols	6081.9 ± 238.3
caffeine	38.5 ± 4.8
theobromine	24.8 ± 3.3
total alkaloids	63.3 ± 4.9

[a] Values are expressed as means ± standard deviations (n = 3).

concern the high concentration of free phenolic acids, such as gallic acid. Curiously, in our work, no condensed tannins were found in the carob flours, although these have been described in other carob fruit and pods (*2*−*4*, *12*). This could be due to the technological process for obtaining the carob flour. For instance, the roasting temperature could hydrolyze these types of polyphenols, resulting in an amount of simple phenolic acids. This could explain the higher concentration of the simple phenolic acids observed in our study in comparison with other carob byproducts reported in the literature (*11*).

Finally, the two alkaloids, caffeine and theobromine, were also identified and quantified. Their concentrations were 38.5 and 24.8 μg/g of carob flour, respectively, and these values were significantly lower (around 50 times) than the amount found in cocoa derivates (*14*). The high sensitivity of the developed chromatographic methods allowed the quantification of these alkaloids by the first time in carob flour samples. This low content of alkaloids, especially theobromine, could reinforce interest in carob flour as a substitute for cocoa ingredients in the food formulation.

Supporting Information Available: Table of SRM conditions for the analysis of the phenolic compounds and the alkaloids by UPLC-MS/MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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