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Identification and Quantification of Soluble Free, Soluble Conjugated, and Insoluble Bound Phenolic Acids in Durum Wheat (*Triticum turgidum* L. var. *durum*) and Derived Products by RP-HPLC on a Semimicro Separation Scale

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ABSTRACT: A straightforward semimicro separation scale RP-HPLC method was developed for the identification and quantification of phenolic acids (PAs) occurring as soluble free, soluble conjugated, and insoluble bound compounds, which were independently extracted from wholemeal of durum wheat and from its derived products coarse bran, semolina, and dried pasta. A narrow bore column and a semimicro photodiode array detector (PDA) cell, in conjunction with a single quadrupole mass spectrometer, equipped with an electrospray ionization source (ESI-MS), were employed. The method was validated in terms of linearity of calibration graphs, limits of detection, limits of quantification, repeatability, and accuracy, which was evaluated by a recovery study. In each sample (wholemeal, coarse bran, semolina, and dried pasta), the total amounts of the three different forms of PAs were in the order bound > conjugated > free, with bound PAs accounting for 61.0−83.6% of the total PAs. Ferulic acid was the most abundant PA in both soluble free and insoluble bound forms, whereas sinapic acid predominated in the conjugated ones. The highest PA content, calculated as the sum of total PAs quantified in the three forms, was found in coarse bran, followed by wholemeal, semolina, and dried pasta.

KEYWORDS: *phenolic acids, durum wheat, Triticum turgidum L. var. durum, wholemeal, coarse bran, semolina, dried pasta, HPLC, semimicro analytical scale, PDA detection, ESI-MS detection*

INTRODUCTION

Durum wheat (*Triticum turgidum* L. var. *durum*) is one of the most important cereal crops used in the Mediterranean-type temperate zones for the production of staple food, such as pasta, couscous, bulgur, and various types of bread. In Italy, durum wheat is one of the most cropped species, with a plant area of about 1.30 million hectares in 2012,[1] mainly used for production of pasta for which Italy is the world leader.

Increasing evidence from clinical and epidemiological studies suggests that the regular consumption of wheat as whole grain and whole-grain products might reduce the risk of developing chronic illnesses such as cardiovascular diseases, type-2 diabetes, and certain types of cancer.[2−4] These health benefits have been partly attributed to the occurrence in wheat, as well as in other cereals, of phytochemicals with antioxidant activity, which include phenolic acids (PAs) belonging to hydroxycinnamic acids and hydroxybenzoic acid derivatives, such as ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanillic, and syringic acids. These PAs are mainly concentrated in the outermost layers of the wheat grains, such as aleurone, pericarp, and embryo cell walls.[5] They occur as soluble free acids, as soluble conjugate PAs that are esterified to sugars and other low molecular mass compounds, and as insoluble bound PAs that are mostly ester-linked to cell wall polymers such as polysaccharides and lignin. Accordingly, the soluble forms are those extracted either as free or as esterified or glycosylated PAs, whereas the insoluble forms refer to bound PAs that are solubilized after alkaline hydrolysis.[6]

Due to the fact that PAs occur in the outer parts of the grain, they may be lost during the milling processes, which results in reduced nutrients and phytochemical content in refined grain products.[7,8] On the other hand, PAs as well as dietary fiber, vitamins, minerals, and other antioxidants appear to be preserved in wholemeal products, in which all components of the kernel are present as in the intact grain.[9]

The literature is not univocal on the content of PAs found in durum wheat. Differences in the content of PAs reported in the literature might be due to the differentiations in the cultivar and/or environmental conditions.[6,10−12] Dissimilarities in the reported data can also be attributed to the different analytical methods employed for the investigations and to the fact that some studies discuss data related to each insoluble bound acids

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or soluble free forms or total PAs, whereas other investigations considered only few PAs (mainly ferulic acid).[13]

The assessment of the total amount of PAs in wheat is currently performed by spectrophotometric methods based on the Folin−Ciocalteau reaction using either gallic[14] or ferulic acid[9] as the standard, whereas reversed-phase high-performance liquid chromatography (RP-HPLC), using analytical size columns (4.0−4.6 mm i.d.) and photodiode array detection, has been widely reported for the identification and quantification of individual PAs.[15−17] The qualitative and quantitative analyses mentioned above are performed after the extraction of PAs, in either free or bound forms, carried out by a variety of techniques,[18] including pressurized solvent extraction,[19] microwave-assisted extraction,[20] ultrasound-assisted extraction,[21] and solid-phase extraction using different sorbents and liquid mixtures at various pH values as the eluent.[22]

This paper reports the results of a study carried out to develop a straightforward HPLC method for the simultaneous identification and quantification of PAs occurring as soluble free, soluble conjugated, and insoluble bound PAs in durum wheat wholemeal and in its main products (coarse bran, semolina, and dried pasta), using a narrow-bore reversed-phase column (2.0 mm i.d.) and detection by in sequence UV photodiode array spectrophotometry, by means of a semimicro detection cell, and electrospray ionization mass spectrometry (ESI-MS). The selection of a narrow-bore column, in combination with a microvolume (2.5 μL) detection cell, was motivated by the positive impact on the environment and analysis costs, due to the reduced consumption of organic solvents, flow rate compatibility with ESI-MS detection, and the expected higher sensitivity of PDA detection, owing to the minor dilution of samples during separation, in comparison to using a conventional analytical size column.

Understanding the distribution of phenolic compounds in different milling fractions might have important implications in ensuring their health benefits to consider grains as a good source of health beneficial phytochemicals and functional foods. Therefore, the developed method has been applied to investigate the occurrence of soluble free, soluble conjugated, and insoluble bound forms of PAs in durum wheat wholemeal, in the main milling fractions (coarse bran and semolina), and in pasta made from semolina.

■ MATERIALS AND METHODS

Chemicals and Standards. Authentic standards of the PAs *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, sinapic acid, and ferulic acid, as well as 3,5-dichloro-4-hydroxybenzoic acid (DHB), which was employed as an internal standard (IS), were obtained from Sigma-Aldrich (Milan, Italy). Analytical-reagent grade formic acid, ethanol, hexane, hydrochloric acid, acetic acid, natrium hydroxide, ethyl acetate, and HPLC grade acetonitrile and methanol were purchased from Carlo Erba Reagents (Milan, Italy). Deionized water was produced by a Milli-Q unit (Millipore, Bedford, MA, USA).

Samples. A widespread Italian durum wheat cultivar (Duilio) was grown in the 2010−2011 cropping season at CRA experimental field of Montelibretti (Rome). After harvesting, the collected grain (7 kg) was stored at 4 °C before milling processes. An aliquot (1 kg) of the harvested grain sample was ground in a laboratory mill (Cyclotec, VWR International PBI, Milan, Italy), using a sieving of 1 mm. To study the influence of technological processes, another aliquot of the grain sample (5 kg) was ground using a milling pilot plant (Buhler MLU 202, Uzwill, Switzerland) obtaining semolina and coarse bran; this last fraction was milled again on the Cyclotec laboratory mill to obtain smaller particles with a maximum particle size of 1 mm. Wholemeal and milling fractions (coarse bran and semolina) were stored before the analyses at −80 °C to avoid degradation of bioactive compounds. Pasta was produced by mixing 3.5 kg of semolina with tap water to obtain a total dough water content of 32−33%, which was processed into traditional spaghetti by an experimental press (NAMAD, Rome, Italy) and an experimental dryer (AFREM, Lyon, France) applying a low-temperature drying cycle (50 °C). For all samples the moisture was measured every time just before the beginning of the analysis on 3 g of milled sample by a MA35 Sartorius (Göttingen, Germany) moisture analyzer.

Extraction of Phenolic Acids. The extraction of PAs in three separated forms (soluble free, soluble conjugated, and insoluble bound PAs) was performed in triplicate, according to the procedure proposed by Li et al.[6] with some modifications. A common treatment was performed to extract free, conjugated, and bound PAs (step 1). A 250 mg portion of either wholemeal or milling fractions or finely ground spaghetti was weighted and mixed with 1 mL of 80:20 (v/v) ethanol−water solution, containing DHB as an internal standard at a concentration of either 37.5 or 150 μg/mL for the extraction of free and conjugated PAs, respectively, whereas no IS was added at this stage for the extraction of the insoluble bound PAs. The resulting mixtures were sonicated for 10 min, maintaining the temperature at 4 °C to avoid starch gelatinization, and then centrifuged for 10 min at 10 000 rpm at 4 °C. The supernatant containing the solubilized free and conjugated PAs was transferred in a new vial. The extraction was repeated twice with the 80:20 (v/v) ethanol−water solution, without the incorporation of the IS, and the supernatants were combined, evaporated to dryness with gaseous nitrogen, and then lyophilized to avoid the oxidation of the extracted compounds. For the extraction of insoluble bound PAs, the supernatants containing the solubilized free and conjugated PAs were discharged, whereas the pellet containing the insoluble bound PAs was collected.

Extraction of Soluble Free Phenolic Acids. A 500 μL volume of 2% (v/v) aqueous acetic acid solution was added to the lyophilized sample produced by step 1, which was subsequently acidified to pH 2.0 with 12 N HCl (2.5 μL) to enable extraction into organic solvent. After mixing, 500 μL of ethyl acetate was added and mixed at room temperature. The resulting mixture was centrifuged at 10 000 rpm for 2 min, and the upper organic layer was collected in a clean vial. The extraction with ethyl acetate was repeated twice, and the combined supernatants were evaporated to dryness with gaseous nitrogen, stored at −20 °C, and then reconstituted in 100 μL of 80:20 (v/v) methanol−water solution, containing 2% (v/v) formic acid, just before HPLC analysis.

Extraction of Soluble Conjugated Phenolic Acids. The lyophilized sample produced by step 1 was hydrolyzed with 2 M sodium hydroxide (400 μL) for 4 h under continuous agitation at 4 °C. After acidification to pH 2 with 12 N HCl (80 μL), conjugated PAs were extracted with 500 μL of ethyl acetate, and after centrifugation at 10 000 rpm for 2 min the upper organic layer was collected in a clean vial. The extraction with ethyl acetate was repeated twice, and the combined supernatants were evaporated to dryness with gaseous nitrogen, stored at −20 °C, and then reconstituted in 100 μL of 80:20 (v/v) methanol−water solution, containing 2% (v/v) formic acid, just before HPLC analysis.

Extraction of Insoluble Bound Phenolic Acids. The pellet remaining after the extraction with ethanol−water solution lacking the IS (see step 1) was mixed with 20 μL of 7.5 mg/mL of the IS solution (in 80:20 (v/v) ethanol−water) and then hydrolyzed for 4 h under continuous agitation at 4 °C by adding 400 μL of 2 M sodium hydroxide aqueous solution, with the exception of the coarse bran sample that required the addition of 800 μL of 2 M sodium hydroxide, because this matrix tended to absorb more water. After acidification to pH 2 with 12 N HCl (120 μL for all samples and 240 μL for coarse bran sample), bound PAs were extracted in duplicate with 800 μL of ethyl acetate (1600 μL for coarse bran) and then centrifuged at 10 000 rpm for 2 min. The combined supernatants were evaporated to dryness with gaseous nitrogen, stored at −20 °C, and then reconstituted in 100 μL of 80:20 (v/v) methanol−water solution, containing 2% (v/v) formic acid, just before HPLC analysis.

Equipment for HPLC Analysis. Qualitative and quantitative analyses of PAs were carried out by RP-HPLC using a Shimadzu (Milan, Italy) LC-10A$_{VP}$ system consisting of an SCL-10A$_{VP}$ system controller, two LC-10AD$_{VP}$ solvent delivery units, a SPD-M10A spectrophotometric diode array (PDA) detector, a CTO-10AS$_{VP}$ column oven, a DGU-14A online vacuum membrane degasser, and a Rheodyne (Cotati, CA, USA) model 8125 semimicro injection valve with a 5 μL sample loop. Instrument control and data acquisition and processing were performed by either the Shimadzu Class VP 5.6 HPLC data system or the Shimadzu LCMS Solution software, both running on a Pentium IV personal computer (Gigabyte, Milan, Italy). A Polaris C18-A column (150 × 2.0 mm i.d., 5 μm; Varian Inc. Lake Forest, CA, USA) with a C18 (30 × 2 mm i.d., 5 μm) guard cartridge column was employed at controlled temperature of 30° ± 1 °C. Soluble free PAs were separated by a multisegment gradient of increasing concentration of acetonitrile in water acidified with 2.0% (v/v) formic acid, at a flow rate of 0.2 mL/min, according to the following program: 15 min linear gradient from 5 to 7% (v/v) acetonitrile, followed by 5 min isocratic elution with 7% (v/v) acetonitrile, 10 min linear gradient from 7 to 20% (v/v) acetonitrile, 5 min linear gradient from 20 to 25% (v/v) acetonitrile, 5 min isocratic elution with 25% (v/v) acetonitrile, and 1 min steep gradient from 25 to 90% (v/v) acetonitrile and subsequent 5 min isocratic elution with 90% (v/v) acetonitrile to ensure complete elution of any strongly retained components of the extracted samples. The gradient used for the separation of conjugated and bound PAs differed from that described above in the steepness of the last gradient segment, which had a duration of 5 min instead of 1 min, and by the introduction of a further segment gradient from 90 to 95% (v/v) in 1 min, followed by 15 min isocratic elution with 95% (v/v) acetonitrile. At the end of both gradient elution programs described above, the composition of the mobile phase was brought to the initial condition in 1 min, and the column equilibrates for 20 min (first gradient profile) and 23 min (second gradient profile), respectively, before the next injection. UV spectra were recorded in the 210−400 nm range, and the chromatograms were acquired at 254, 280, and 320 nm.

To confirm peak identification by ESI-MS in the single ion monitoring (SIM) detection mode, the above HPLC instrument was hyphenated with a Shimadzu single quadrupole model LCMS-2010 mass spectrometer equipped with an ESI interface, which was operated in the negative ionization mode at the following conditions: nebulizing gas nitrogen at a flow rate of 4.5 L/min; temperature of block heater, 200 °C; temperature of the curved desolvation line (CDL), 225 °C; probe voltage, −3.5 V; CDL voltage, 25 V; Q-array voltages, 0, −15, −60 V; Q-array RF, 150. The column effluent was first passed through the PDA detector before being introduced into the ESI interface without flow rate splitting.

Method Development and Validation. Soluble free, soluble conjugated, and insoluble bound PAs were separated by RP-HPLC using the multisegment elution gradient program described above, which was optimized as described in the Results and Discussion section. The individual PAs liberated from the three different forms occurring in wholemeal, coarse bran, semolina, and dried pasta were identified by ESI-MS in the negative single ion monitoring mode and by comparison of retention times and UV spectra with those of authentic standards. To this end, stock solutions of authentic standards of the major PAs expected to occur in durum wheat were prepared by dissolving weighted amounts of each standard in 80% (v/v) methanol−water solution and subjected to RP-HPLC by the optimized method to make libraries comprising retention times, UV spectra, and MS spectra in the negative ion mode. The recorded UV spectra were used by the Shimadzu Class VP software to calculate a Similarity Index (SI) employed to evaluate how closely spectra of standard and corresponding PAs separated in wheat extracts resemble each other. Negative ion mass spectra were recorded to select the target ions for the detection of PAs by ESI-MS in negative SIM mode.

The quantitative analysis of the identified PAs was based on calibration graphs obtained with solutions of authentic standards at five different concentration levels within the range of concentration over which the response of the UV detector was linear (see Table 4). Calibration graphs of each PA were constructed by plotting the ratio of the peak area of the authentic standard to that of the IS as a function of the concentration of the standard solutions that, before being injected onto the HPLC column, had undergone the same extraction procedure employed for the real samples to ensure that losses due to the extraction method were accounted for. The peak areas of PAs (both standards and analytes) and of IS were collected at the wavelength of maximum absorbance of each identified PA, which were determined by the PDA spectra acquired in the wavelength range 210−400 nm. All standard solutions and samples were analyzed in triplicate, and the concentrations of individual PAs in real samples were expressed in milligrams per kilogram (mg/kg) of dry matter (dm).

The RP-HPLC method was validated by assessing precision, linearity, limit of detection (LOD), limit of quantification (LOQ), and accuracy. Precision was evaluated by intraday and interday repeatability, as relative standard deviation (RSD) of both retention time and ratio of the analyte response to that of the IS for all PAs considered in the study, which were analyzed by the proposed method in triplicate during the same day and over three consecutive days.

Linearity was evaluated by analyzing mixtures of PA standard solutions at five equally spaced concentrations within appropriate ranges, employing linear least-squares regression analysis to calculate slope, intercept, and correlation coefficient of the calibration graphs constructed as reported above. The limit of detection (LOD) and the limit of quantification (LOQ) were estimated as the concentrations of PAs producing chromatographic peaks with a height at least three times and ten times as high as the baseline, respectively. The accuracy of the method was evaluated by recovery studies performed by adding known amounts of the examined PA to the sample, which was subjected to the extraction process and RP-HPLC analysis described above in parallel to a nonspiked sample of the same source. Each sample was spiked at three different concentration levels corresponding to 80%, 100%, or 120% of the concentration determined in the nonspiked sample. All samples were analyzed in triplicate, and the average concentrations were determined by the quantitative method described above.

RESULTS AND DISCUSSION

Optimization of the HPLC Method. Initial experiments were devoted to optimize the HPLC method employed for the identification and quantification of PAs extracted from wholemeal, milling fractions (coarse bran and semolina), and dried pasta. PAs were extracted in the three different forms (soluble free, soluble conjugated, and insoluble bound) by the method reported by Li et al.[6] with the minor modifications described in the Materials and Methods and subsequently analyzed by RP-HPLC, using a narrow-bore C-18 column (2.0 mm i.d.). The selection of a narrow-bore column, in combination with a microvolume (2.5 μL) detection cell, was motivated by the expected higher sensitivity of PDA detection, due to the minor dilution of samples during separation in comparison to using a conventional analytical size column (4.0 or 4.6 mm i.d.). Additional advantages of narrow-bore columns, compared with conventional analytical size ones, include sharper peaks, due to minor radial dispersion, minor impact of heat effects on separation performance as a consequence of better heat dissipation,[23] and flow rate compatibility with mass spectrometry detection.[24] In addition, the use of a narrow-bore column implies reduced consumption of toxic and expensive organic solvents and of the other reagents employed for elution, with a positive impact on the environment and on the cost of the analysis.[25] Formic acid was selected as the additive of the aqueous component of the mobile phase to suppress the ionization of PAs and to avoid peak broadening caused by the

simultaneous presence of protonated and ionized forms of these analytes.

A study was carried out with the purpose of investigating the influence of the concentration of formic acid in the mobile phase on the separation performance of PAs by RP-HPLC. Increasing the concentration of formic acid within the range 0.5−10% (v/v) decreased the retention times of almost all analytes, as a consequence of the expected interactions between protonated PAs and formic acid via hydrogen-bonding formation,[26] which are believed to increase the polarity of the analytes that, therefore, are expected to establish weaker interactions with the hydrophobic stationary phase. On the other hand, increasing the concentration of formic acid had the effect of raising its efficacy in controlling the protonic equilibrium with consequent decreasing of band broadening and peak tailing. A good compromise between these two opposite effects was obtained incorporating formic acid in the starting eluent at the concentration of 2% (v/v), even though better sensitivity in ESI-MS was obtained with mobile phases containing a lower concentration of formic acid, in accordance with the observation that the negative-ion ESI response is expected to decrease with increasing concentration of electrolytes in the mobile phase.[27]

Acetonitrile was selected as the organic modifier because its results were superior to methanol in separating the PAs potentially present in wheat and, due to the lower viscosity, it is more suitable than methanol to be used with a narrow-bore HPLC column and ESI interface, both sources of high back pressure associated with their low permeability.

Validation of the HPLC Method. The optimized RP-HPLC method allowed the concomitant resolution of all PAs potentially present in our samples in less than 40 min, as depicted by the chromatograms displayed in Figures 1−3, showing the separations of soluble free, soluble conjugated, and insoluble bound PAs extracted from durum wheat coarse bran. The significant differences in both peak size and values of absorbance units at full scale displayed by these chromatograms reflect the diverse content of PAs in the three forms occurring in durum wheat coarse bran. Analogous separations were obtained for the samples extracted from wholemeal flour, semolina, and dried pasta made from semolina, all produced with the durum wheat cultivar Duilio.

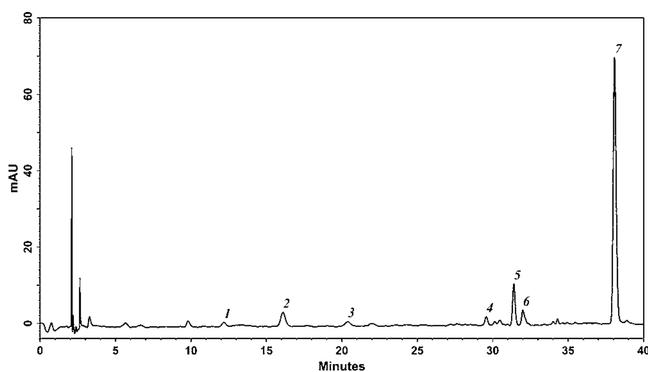

Figure 1. Separation of soluble free PAs extracted from coarse bran. Experimental conditions as reported in the text. Sample detection at 280 nm. Peak identity: (1) *p*-hydroxybenzoic acid; (2) vanillic acid; (3) syringic acid; (4) *p*-coumaric acid; (5) ferulic acid; (6) sinapic acid; (7) 3,5-dichloro-4-hydroxybenzoic acid (IS).

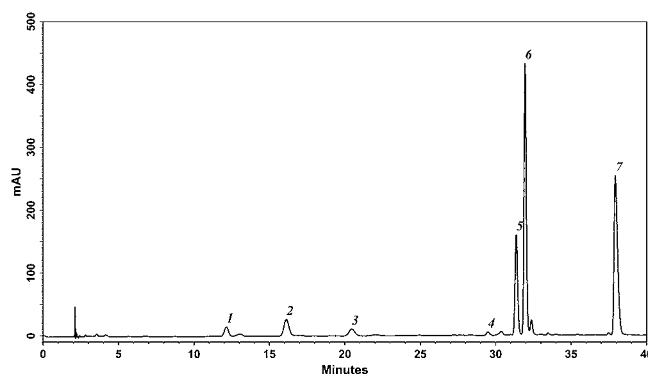

Figure 2. Separation of soluble conjugated PAs extracted from coarse bran. Experimental conditions, detection wavelength, and peak identity as in Figure 1.

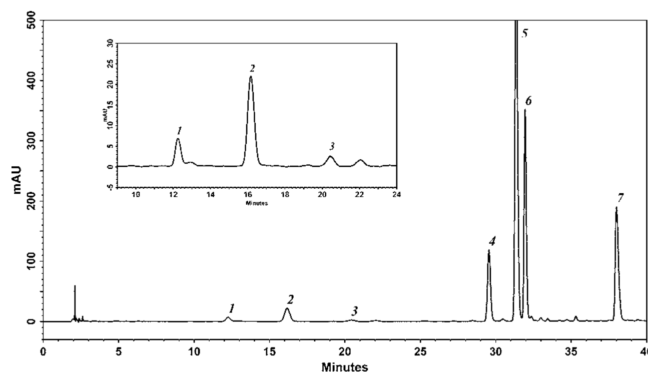

Figure 3. Separation of insoluble bound PAs extracted from coarse bran. All conditions and peak identity as in Figure 1, except detection at 254 nm for the three peaks displayed in the inset.

The intraday and interday repeatability of peak area ratio (analyte/IS) resulted to be better than 8.4% and 9.9%, respectively, whereas that of retention times was better than 0.9% and 1.5%, respectively (see Tables 1−3). Linear calibration graphs with correlation coefficients better than 0.990 were obtained for all PA standards, which were subjected to the same extraction procedure employed to liberate these compounds from the three forms occurring in real samples. The calibration equations and correlation coefficients calculated by linear least-squares regression analysis are reported in Table 4, which also displays LOD, LOQ, and detection wavelength for each analyte.

The accuracy of the method was evaluated by a recovery study, which was carried out by spiking the samples with known amounts of ferulic acid and comparing the resulting amount with that determined in the nonspiked sample, analyzed in parallel. All samples were analyzed in triplicate. Average recoveries ranging from 90% to 112% (RSD < 5.4%) were determined for ferulic acid in the soluble free and conjugated forms, whereas the recovery was much lower for the determination of insoluble bound ferulic acid, whose average value was 73.3% (RSD 6.1%). These values compare positively with the recent results of Brandolini et al.,[28] presenting recoveries of 88.5 ± 0.7% and 80.9 ± 4.7% for insoluble bound ferulic and *p*-coumaric acids, respectively, and of Stracke et al.,[29] reporting recoveries for free, conjugated, and bound PAs ranging from 87 to 72 ± 10%. Recoveries for insoluble bound ferulic acid as low as 63% have also been reported and related to the partial degradation of PAs during alkaline hydrolysis.[30]

Table 1. Intraday and Interday Repeatability of Retention Time and Peak Area Analyte/Peak Area IS for PAs Occurring in Soluble Free form

	repeatability retention time (min)					
	intraday (n = 3)			interday (n = 3 replicates over 3 days)		
analyte	average	SD	RSD (%)	average	SD	RSD (%)
1. p-hydroxybenzoic acid	12.263	0.112	0.9	12.277	0.181	1.5
2. vanillic acid	16.142	0.125	0.8	16.136	0.164	1.0
3. syringic acid	20.366	0.151	0.7	20.311	0.169	0.8
4. p-coumaric acid	29.540	0.089	0.3	29.520	0.123	0.4
5. ferulic acid	31.342	0.059	0.2	31.320	0.070	0.2
6. sinapic acid	31.904	0.037	0.1	31.879	0.043	0.1
	repeatability peak area[a]/IS area					
	intraday (n = 3)			interday (n = 3 replicates over 3 days)		
analyte	average	SD	RSD (%)	average	SD	RSD (%)
1. p-hydroxybenzoic acid	0.021	0.001	6.9	0.021	0.001	4.9
2. vanillic acid	0.033	0.001	2.6	0.031	0.002	4.9
3. syringic acid	0.019	0.000	0.2	0.019	0.000	2.3
4. p-coumaric acid	0.029	0.002	7.9	0.027	0.002	8.4
5. ferulic acid	0.315	0.010	3.0	0.031	0.008	2.5
6. sinapic acid	0.007	0.001	8.4	0.007	0.001	9.6

[a]Arbitrary units.

Table 2. Intraday and Interday Repeatability of Retention Time and Peak Area Analyte/Peak Area IS for PAs Occurring in Soluble Conjugated Form

	repeatability retention time (min)					
	intraday (n = 3)			interday (n = 3 replicates over 3 days)		
analyte	average	SD	RSD (%)	average	SD	RSD (%)
1. p-hydroxybenzoic acid	12.018	0.033	0.3	12.153	0.155	1.3
2. vanillic acid	15.883	0.038	0.2	15.997	0.134	0.8
3. syringic acid	20.039	0.050	0.3	20.153	0.140	0.7
4. p-coumaric acid	29.280	0.028	0.1	29.400	0.134	0.5
5. ferulic acid	31.154	0.022	0.1	31.220	0.078	0.3
6. sinapic acid	31.755	0.011	0.0	31.791	0.049	0.2
	repeatability peak area[a]/IS area					
	intraday (n = 3)			interday (n = 3 replicates over 3 days)		
analyte	average	SD	RSD (%)	average	SD	RSD (%)
1. p-hydroxybenzoic acid	0.019	0.000	0.5	0.021	0.002	9.9
2. vanillic acid	0.017	0.000	0.6	0.019	0.002	8.4
3. syringic acid	0.008	0.000	0.4	0.009	0.001	9.6
4. p-coumaric acid	0.024	0.000	0.6	0.025	0.002	8.5
5. ferulic acid	0.169	0.001	0.5	0.181	0.013	7.3
6. sinapic acid	0.215	0.001	0.5	0.229	0.017	7.2

[a]Arbitrary units.

Application. The identification of PAs liberated from the three different forms occurring in wholemeal, coarse bran, semolina, and dried pasta was performed by the developed RP-HPLC method on the basis of their retention times, UV spectra, and ESI-MS detection in single ion monitoring mode. Authentic standards of p-hydroxybenzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, and sinapic acid, detected by ESI-MS in negative ionization mode, produced $[M - H]^-$ ions of m/z values 136.0, 167.0, 197.0, 162.9, 192.9, and 223.0, respectively, which, when extracted during RP-HPLC analysis of real samples, displayed SIM chromatograms with peaks in correspondence to those identified as the above PAs on the basis of their retention times and UV spectra (see Materials and Methods).

The occurrence of soluble free, soluble conjugated, and insoluble bound PAs in wholemeal, coarse bran, semolina, and dried pasta is reported in Table 5, which also displays, for each of the 12 analyzed samples, the total concentration of PAs (total PAs) calculated by the addition of the content of the individual PAs determined in the corresponding fraction. The full amounts of PAs occurring in wholemeal, coarse bran, semolina, and dried pasta were calculated by the addition of the total PAs, determined for each of them in the three separated forms (soluble free, soluble conjugated, and insoluble bound). The highest full amount of PAs was found in coarse bran (2993.0 mg/kg dm), followed by wholemeal, semolina, and dried pasta (969.9, 154.7, and 105.1 mg/kg dm, respectively), in accordance with the expectation that PAs are mostly concentrated in the outermost layers of wheat grains. It is worth

Table 3. Intraday and Interday Repeatability of Retention Time and Peak Area Analyte/Peak Area IS for PAs Occurring in Insoluble Bound Form

	repeatability retention time (min)					
	intraday (n = 3)			interday (n = 3 replicates over 3 days)		
analyte	average	SD	RSD (%)	average	SD	RSD (%)
1. *p*-hydroxybenzoic acid	12.107	0.060	0.5	12.002	0.113	0.9
2. vanillic acid	15.982	0.093	0.6	15.848	0.137	0.9
3. syringic acid	20.141	0.087	0.4	19.982	0.160	0.8
4. *p*-coumaric acid	29.365	0.059	0.2	29.264	0.102	0.3
5. ferulic acid	31.214	0.048	0.2	31.143	0.069	0.2
6. sinapic acid	31.852	0.097	0.3	31.774	0.079	0.2
	repeatability peak area[a]/IS area					
	intraday (n = 3)			interday (n = 3 replicates over 3 days)		
analyte	average	SD	RSD (%)	average	SD	RSD (%)
1. *p*-hydroxybenzoic acid	0.005	0.000	2.9	0.005	0.000	3.7
2. vanillic acid	0.004	0.000	6.7	0.004	0.000	6.2
3. syringic acid	0.222	0.000	1.5	0.002	0.000	5.0
4. *p*-coumaric acid	0.005	0.000	2.0	0.005	0.000	2.9
5. ferulic acid	0.210	0.003	1.4	0.207	0.004	2.0
6. sinapic acid	0.037	0.002	4.3	0.035	0.002	6.0

[a]Arbitrary units.

Table 4. Linear Regression Analysis of Calibration Graphs Based on UV Absorbance at the Reported Wavelength (λ)

	analyte	λ (nm)	linear range (mg/L)	equation[a] free form	correlation coefficient	LOD (mg/L)	LOQ (mg/L)
soluble free PAs	1. *p*-hydroxybenzoic acid	254	0.2−4.0	$y = 0.0046x + 0.0014$	0.9959	0.1	0.3
	2. vanillic acid	254	0.6−12.0	$y = 0.0026x + 0.00007$	0.9983	0.2	0.7
	3. syringic acid	280	2.0−8.0	$y = 0.0022x + 0.0011$	0.9996	0.7	2
	4. *p*-coumaric acid	320	0.4−8.4	$y = 0.0049x + 0.0007$	0.9984	0.07	0.2
	5. ferulic acid	320	4.0−80.0	$y = 0.0046x + 0.0051$	0.9989	0.06	0.2
	6. sinapic acid	320	0.5−10.8	$y = 0.0030x + 0.0008$	0.9984	0.2	0.55
	analyte	λ (nm)	linear range (mg/L)	equation[a] conjugated form	correlation coefficient	LOD (mg/L)	LOQ (mg/L)
soluble conjugated PAs	1. *p*-hydroxybenzoic acid	254	1.2−60	$y = 0.0016x + 0.0038$	0.9972	0.06	0.21
	2. vanillic acid	254	2.0−100	$y = 0.0009x - 0.0012$	0.9955	0.18	0.60
	3. syringic acid	280	1.0−50	$y = 0.0009x - 0.0003$	0.9961	0.35	1.01
	4. *p*-coumaric acid	320	1.2−60	$y = 0.0021x - 0.0013$	0.9956	0.06	0.20
	5. ferulic acid	320	10.5−525	$y = 0.0016x + 0.0015$	0.9989	0.10	0.35
	6. sinapic acid	320	20.0−1000	$y = 0.0014x - 0.0315$	0.9944	0.16	0.48
	analyte	λ (nm)	linear range (mg/L)	equation[a] bound form	correlation coefficient	LOD (mg/L)	LOQ (mg/L)
insoluble bound PAs	1. *p*-hydroxybenzoic acid	254	2.4−24	$y = 0.0014x + 0.005$	0.9900	0.20	0.50
	2. vanillic acid	254	2.0−40	$y = 0.0009x + 0.00007$	0.9927	0.41	1.25
	3. syringic acid	280	1.0−20	$y = 0.0008x + 0.0002$	0.9932	0.20	0.71
	4. *p*-coumaric acid	320	2.4−220	$y = 0.0009x + 0.0003$	0.9991	0.31	0.60
	5. ferulic acid	320	63−2400	$y = 0.0010x + 0.0936$	0.9910	0.08	0.25
	6. sinapic acid	320	12.5−480	$y = 0.0110x - 0.0163$	0.9934	0.06	0.18

[a]y expresses the detection response in arbitrary units (peak area analyte/peak area IS) and x the concentration of the standard PAs in mg/L.

noting that the full amount of PAs determined in wholemeal (969.9 mg/kg dm) is comprised within the concentration range of 536−1086 mg/kg dm reported by Li et al.[6] for wholemeal samples of ten different varieties of durum wheat, determined by an approach similar to that reported in this paper, i.e., the addition of the concentrations of PAs separately quantified by HPLC in the soluble free, soluble conjugated, and insoluble bound fractions.

Free PAs. The most abundant PA in the soluble free fraction of the investigated samples was ferulic acid, which varied from 11.76 mg/kg dm in dried pasta to 2.38 mg/kg dm in semolina, followed by sinapic acid (ranging from 3.23 mg/kg dm in coarse bran to 0.42 mg/kg dm in semolina) and by *p*-coumaric acid (varying from 1.51 mg/kg dm in coarse bran to 0.24 mg/kg dm in semolina). Soluble free syringic acid (1.44 mg/kg dm) and *p*-hydroxybenzoic acid (0.92 mg/kg dm) were only quantified in the coarse bran sample, whereas vanillic acid was determined in coarse bran (4.80 mg/kg dm) and wholemeal (1.56 mg/kg dm). The total concentrations of soluble free PAs in wholemeal, coarse bran, semolina, and dried pasta were 11.20, 19.92, 3.04, and 13.15 mg/kg dm, respectively, representing the smallest contribution to the full amounts of PAs occurring in all samples, as it was previously found in wholemeal of other varieties of wheat.[15,16]

Conjugated PAs. For conjugated forms, the most abundant PA was sinapic acid, whose concentration was 198.86, 90.99,

Table 5. Content of Free, Conjugated, and Bound PAs in Wholemeal, Milling Fractions, and Dried Pasta (Mean ± SD)

		p-hydroxybenzoic acid	vanillic acid	syringic acid	p-coumaric acid	ferulic acid	sinapic acid	total PAs[a]
		mg/kg dm	mg/kg dm	mg/kg dm	mg/kg dm	mg/kg dm	mg/kg dm	mg/kg dm
soluble free PAs	wholemeal	nq[b]	1.56 ± 0.07	nd[c]	0.69 ± 0.04	7.77 ± 0.38	1.18 ± 0.04	11.20
	coarse bran	0.92 ± 0.07	4.80 ± 0.07	1.44 ± 0.06	1.51 ± 0.02	8.06 ± 0.12	3.23 ± 0.11	19.92
	semolina	nq	nq	nd	0.24 ± 0.01	2.38 ± 0.03	0.42 ± 0.04	3.04
	dried pasta	nq	nq	nd	0.69 ± 0.03	11.76 ± 1.03	0.70 ± 0.04	13.15
soluble conjugated PAs	wholemeal	5.00 ± 0.35	12.44 ± 0.44	1.72 ± 0.13	2.22 ± 0.05	35.42 ± 1.29	90.99 ± 0.41	147.79
	coarse bran	9.46 ± 0.05	32.56 ± 0.37	10.15 ± 0.12	4.93 ± 0.08	64.65 ± 0.73	198.86 ± 3.20	320.61
	semolina	nq	3.23 ± 0.09	nq	0.81 ± 0.06	14.64 ± 0.47	26.10 ± 0.48	44.78
	dried pasta	1.20 ± 0.15	3.18 ± 0.09	nq	0.80 ± 0.04	4.49 ± 0.10	17.77 ± 0.34	27.44
insoluble bound PAs	wholemeal	nq	4.01 ± 0.06	1.83 ± 0.07	22.42 ± 0.20	752.56 ± 5.14	30.08 ± 0.26	810.90
	coarse bran	2.90 ± 0.13	15.02 ± 0.59	0.60 ± 0.02	73.27 ± 3.24	2447.49 ± 24.73	113.16 ± 5.49	2652.44
	semolina	nd	nd	nd	4.20 ± 0.23	91.88 ± 3.08	10.82 ± 0.19	106.90
	dried pasta	nd	nd	nd	3.73 ± 0.11	47.55 ± 1.06	13.20 ± 0.23	64.48

[a]Calculated by the addition of the concentrations of PAs determined in each sample. [b]Amount lower than LOQ. [c]Amount lower than LOD.

26.10, and 17.77 mg/kg dm in coarse bran, wholemeal, semolina, and dried pasta, respectively. The same order of occurrence was also determined for ferulic acid, which varied from 64.65 mg/kg dm in coarse bran to 4.49 mg/kg dm in dried pasta. Also quantified in all samples were vanillic and p-coumaric acids, whose highest concentrations were found in coarse bran (32.56 and 4.93 mg/kg dm, respectively) and in wholemeal (12.44 and 2.22 mg/kg dm, respectively), whereas almost coincident contents of vanillic acid (3.23 and 3.18 mg/kg dm) and p-coumaric acid (0.81 and 0.80 mg/kg dm) were determined in semolina and dried pasta, respectively. Syringic acid was determined only in wholemeal (1.72 mg/kg dm) and coarse bran (10.15 mg/kg dm); p-hydroxybenzoic acid was quantified in wholemeal, coarse bran, and dried pasta (5.00, 9.46, and 1.20 mg/kg dm, respectively); whereas in semolina occurred at a concentration lower than LOQ. The total concentrations of soluble conjugated PAs in wholemeal, coarse bran, semolina, and dried pasta were 147.79, 320.61, 44.78, and 27.44 mg/kg dm, respectively, and, therefore, resulted to be a significant portion of the full amounts of PAs, occurring as soluble free, soluble conjugated, and insoluble bound compounds, in agreement with previous findings.[6,16]

Bound PAs. The bound form gave the highest contribution to the full amount of PAs quantified in all investigated samples, with a determined occurrence of 810.90, 2652.44, 106.90, and 64.48 mg/kg dm in wholemeal, coarse bran, semolina, and dried pasta, respectively, and contributed for 61.0−83.6% of entireties PAs across the investigated samples. Ferulic acid was the predominant bound PA in all tested samples and accounted for about 73.8−92.3% of the total identified bound PAs on a per weight basis. Its content varied from 2447.49 mg/kg dm in coarse bran to 47.55 mg/kg dm in dried pasta.

The second most abundant bound PA was sinapic acid, whose content was 30.08, 113.16, 10.82, and 13.20 mg/kg dm in wholemeal, coarse bran, semolina, and dried pasta, respectively. Also determined in all samples was the bound form of p-coumaric acid, whose content varied from 73.27 mg/kg dm in coarse bran to 3.73 mg/kg dm in dried pasta. Vanillic acid and syringic acid were quantified only in wholemeal (4.01 and 1.83 mg/kg dm, respectively) and coarse bran (15.02 and 0.60 mg/kg dm, respectively); however, p-hydroxybenzoic acid was determined only in coarse bran, and its concentration was 2.90 mg/kg dm, corresponding to 0.1% of total bound PAs in this milling fraction.

Our study has evidenced that RP-HPLC on a semimicro separation scale offers both economical and environmental benefits, while maintaining separation performance and reliability of traditional HPLC methods, which use conventional analytical size columns (4.0−4.6 mm i.d.), generally eluted at flow rate of 1.0 mL/min and, therefore, requiring larger amounts of expensive organic solvents and generating greater volumes of hazardous waste to be disposed of. In addition to 5-fold savings in mobile phase waste generation, the developed HPLC method allows the accurate estimation of the individual PAs occurring as soluble free, soluble conjugated, and insoluble bound compounds in wholemeal of durum wheat and in its products coarse bran, semolina, and dried pasta. In each of these samples, the total content of the three different forms of PAs is in the order bound > conjugated > free. Also common among samples is the relative abundance of the individual PAs, with ferulic acid prevailing in the soluble free and insoluble bound forms, whereas sinapic acid is the predominant component of the soluble conjugated one.

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Notes

The authors declare no competing financial interest.

ABBREVIATION USED

PAs, phenolic acids; RP-HPLC, reversed-phase high-performance liquid chromatography; PDA, photodiode array; ESI, electrospray ionization; MS, mass spectrometry; DHB, 3,5-dichloro-4-hydroxybenzoic acid; SD, standard deviation; RSD, relative standard deviation; IS, internal standard

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