



A HPLC-UV Method for the Quantification of Phenolic Acids in Cereals

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Abstract

Cereals are a good source of phenolic acids, most of which are present in bound form. The aim of this study was to develop a method for quantifying total phenolic acids in cereals that includes a robust step for hydrolysis of bound forms. Different hydrolysis procedures were evaluated. Acid hydrolysis, even with subsequent use of enzymes, proved unsuitable for releasing bound phenolic acids from the cereal matrix. Base hydrolysis (3 M, 90 min) resulted in the highest extractability, with average recoveries of 88–108% for cereal phenolic acids. The phenolic acid content in cereals (two cultivars each of rye, barley, and oats, and eight cultivars of wheat) varied up to 2-fold between cereal genotypes and 1.5-fold within genotypes. The highest content was found in rye, followed by wheat, barley, and oats. Ferulic acid dominated in all cereals, amounting to 48–72% of total phenolic acid content.

Keywords Phenolic acids · Extraction · Cereals · HPLC-UV

Introduction

A recent meta-analysis provided evidence that whole grain intake is associated with a reduced risk of cardiovascular disease and cancer (Aune et al. 2016). This has been attributed partly to the high content of phenolic compounds present in whole grain cereals (Dykes and Rooney 2007), which function as antioxidants (Slavin et al. 1997; Thompson 1994). The term phenolic acids refers to a large number of naturally occurring compounds in plant-derived foods which, based on C1–C6 and C3–C6 backbone, are divided into two main groups, benzoic acid and cinnamic acid derivatives. Several compounds are reported in each of these two groups. The structural dissimilarities derive from the number and position of the hydroxyl and methoxy groups on the aromatic ring.

A wide range of phenolic acid concentrations (197–3907 µg/g) in cereals has been reported, depending on genotype,

cultivar, growing conditions, and cereal fraction (Dykes and Rooney 2007). The majority (up to 90%) of the phenolic acids in cereals are found in bound form (Acosta-Estrada et al. 2014; Robbins 2003; Shahidi and Yeo 2016). They are mainly ester-linked to cell wall components (Acosta-Estrada et al. 2014; Robbins 2003; Shahidi and Yeo 2016), which makes extraction difficult. Therefore, hydrolysis of the esters to carboxylic acid is considered an important prerequisite in any analytical method for determining bound phenolic acid content in cereal grains. There are two main procedures to cleave the ester bond for release of the phenolic acids from the food matrix, acid hydrolysis and base hydrolysis. A third, less prevalent, technique is cleavage using enzymes (esterases) (Acosta-Estrada et al. 2014; Robbins 2003; Shahidi and Yeo 2016). To the best of our knowledge, no previous study has systematically compared these different hydrolysis methods. Moreover, no definitive method for hydrolysis of bound phenolic acids in foods has been established to date and thus, there is a need to develop a simple method that quantitatively releases the phenolic acids from food matrices.

A number of methods have been developed for quantification of phenolic acids in foods, including the Folin-Ciocalteu assay and chromatographic approaches (HPLC-UV, GC-MS, LC-MS) (Acosta-Estrada et al. 2014; Robbins 2003; Shahidi and Yeo 2016). The Folin-Ciocalteu assay, which provides an estimate of total phenolic content by quantifying the antioxidant capacity, is not specific to phenolic groups and suffers from interference by ascorbic acid and reducing sugars (Stalikas 2007). Chromatographic methods have been widely

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used to separate and characterize individual phenolic acids (Stalikas 2007). However, the relative profile of phenolic acids differs depending on the food type (Manach et al. 2004). Therefore, there is a need for development of a chromatographic (HPLC) method that can separate and measure simultaneously the prominent phenolic acids in various foods in a single run. Accurate quantification of phenolic acid content in foods would enable selection of foods with a high content of these health-promoting compounds. The aims of this study were thus (1) to develop a simple and robust hydrolysis method for releasing the bound phenolics from cereals and (2) to use the method to quantify the content of phenolic acids in different cereal genotypes.

Materials and Methods

Chemicals, Enzymes, and Standards

All chemicals and phenolic acid standards (18 phenolic acids, as listed in Table 1) were purchased from Sigma-Aldrich (St. Louis, USA) except for acetonitrile, methanol, and sodium hydroxide, which were purchased from VWR International (Stockholm, Sweden). All reagents were of p.a. grade except acetonitrile and methanol, which were of HPLC grade. Water was purified using a Milli-Q water purification system (Merck, Darmstadt, Germany).

Standard stock solutions of phenolic acids (100 µg/mL for ellagic acid, 1000 µg/mL for all other phenolic acids) were prepared in methanol under subdued light and stored under nitrogen atmosphere at – 80 °C for 4 months. A standard mixture of the 18 phenolic acids (6 µg/mL of ellagic acid, 20 µg/mL of all other phenolic acids) was prepared in 40% aqueous methanol under subdued light and stored under nitrogen atmosphere at – 80 °C for 1 month. This standard mixture was used for optimization of the chromatographic conditions.

Thermostable α -amylase (from *Bacillus amyloliquefaciens*, ≥ 250 units/g) and cellulase (from *Aspergillus niger*, 1.44 units/mg) were obtained from Sigma-Aldrich (St. Louis, USA). Protease (from *Bacillus licheniformis*, 25 mg/mL) was purchased from Megazyme (Bray, Ireland). Suspensions of α -amylase and protease were used without any additional preparation. Before extraction, a cellulase solution (20 µg/mL) was prepared by dissolving cellulase powder in 0.1 M aqueous sodium acetate.

Cereal Samples

A total of 14 cereal grain samples from different genera and cultivars were tested. Grain from two cultivars each of rye (genus *Secale*), barley (genus *Hordeum*), and oats (genus *Avena*) and eight cultivars of wheat (genus *Triticum*), harvested during 2015 and 2016, was obtained from different mills and producers. Grain samples (250–500 g) were vacuum-

Table 1 Calibration parameters, limits of detection and quantification, detection wavelengths, and molecular masses of phenolic acid standards used in the current study

Phenolic acid	Slope	Intercept	R^2	LOD (µg/mL)	LOQ (µg/mL)	λ (nm)	Mass
Galic acid	59.4	– 31.9	0.9996	0.01	0.03	270	170.12
2,3,4-Trihydroxybenzoic acid	82.6	– 59.1	0.9992	0.02	0.06	270	170.12
3,4-Dihydroxybenzaldehyde	86.0	– 45.7	0.9996	0.01	0.03	275	138.12
<i>p</i> -Hydroxybenzoic acid	114.8	– 54.7	0.9997	0.01	0.04	260	138.12
Gentisic acid	37.3	– 21.6	0.9997	0.04	0.13	325	154.12
Vanillic acid	104.9	– 54.8	0.9996	0.02	0.08	270	168.15
Caffeic acid	131.4	– 70.8	0.9996	0.02	0.06	325	180.16
Vanillin	78.1	– 44.5	0.9997	0.03	0.10	278	152.15
Syringic acid	55.4	– 36.8	0.9995	0.04	0.13	278	198.17
Chlorogenic acid	78.4	– 54.5	0.9995	0.05	0.16	325	354.31
Benzoic acid	7.0	– 6.4	0.9993	0.26	0.86	275	122.12
<i>p</i> -Coumaric acid + syringaldehyde	234.8	– 94.6	0.9998	0.02	0.08	310	164.15 182.17
Ferulic acid	125.1	– 71.8	0.9995	0.01	0.03	325	194.18
Sinapic acid	119.6	– 68.3	0.9996	0.01	0.04	325	224.21
<i>o</i> -Coumaric acid	168.5	– 87.8	0.9996	0.01	0.03	275	164.16
Cinnamic acid	172.7	– 31.9	0.9999	0.01	0.03	275	148.16

R^2 correlation coefficient, *LOD* limit of detection, *LOQ* limit of quantification, established by signal-to-noise (1:10) approach. It was difficult to generate an accurate calibration curve for ellagic acid in the standard mixture because of the poor stability

packed in polyethylene bags and stored at $-20\text{ }^{\circ}\text{C}$ until analysis. Before analysis, the grain was milled using an ultracentrifuge mill, with a 0.5-mm ring sieve (ZM 200, Retsch, Hannover, Germany).

Optimization of the Hydrolysis Procedure

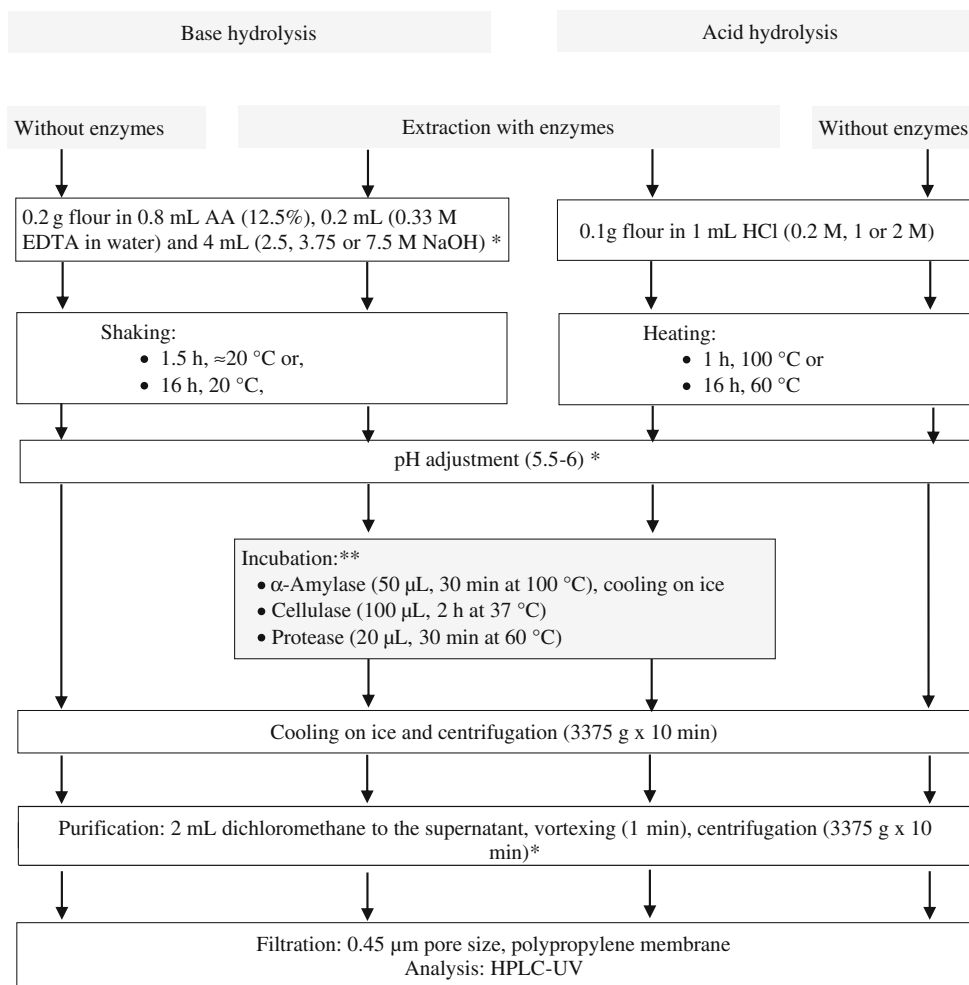
Milled Emmer wheat (*Triticum dicoccum*, cultivar (cv.) Västgötarna) was used for optimization of the hydrolysis procedure. Two main approaches were tested for hydrolysis of total phenolic acids (acid hydrolysis and base hydrolysis, with/without thermostable α -amylase, cellulase, and protease), based on methods described by others (Fuentelba et al. 2017; Ross et al. 2009; Yu et al. 2001). Different conditions with respect to hydrochloric acid (HCl) and sodium hydroxide (NaOH) concentrations, incubation time, and use of enzymes during hydrolysis were tested, as outlined in Fig. 1. The need for antioxidants (EDTA and ascorbic acid) during hydrolysis, acidification after base hydrolysis (different hydrogen ion concentrations at pH 2, 4, and 6), and purification with dichloromethane was also examined (Fig. 1). After

hydrolysis, sample extracts were filtered through a syringe filter (0.45 μm pore size, polypropylene membrane, Agilent Technologies, Santa Clara, USA). All hydrolysis procedures were performed in duplicate under nitrogen atmosphere and subdued light. The phenolic acid content of the extracts was measured under optimized chromatographic conditions using HPLC-UV. Phenolic acid content was quantified as the total of free, conjugated, and matrix-bound forms of the individual compound.

Application of Optimized Procedure to Cereal Cultivars

The optimized hydrolysis method for phenolic acid extraction from cereal samples was as follows: milled cereal grains (0.2 g) were homogenized ($n = 3$) in 0.8 mL aqueous ascorbic acid solution (12.5%), 0.2 mL aqueous EDTA solution (0.33 M), and 4 mL 3.75 M NaOH (to give a final concentration of 3 M). The mixture was shaken for 90 min at ambient temperature under nitrogen atmosphere protected from light. After base hydrolysis, the pH of the extract was adjusted to ≈ 6 using

Fig. 1 Flow chart of different extraction procedures. Single asterisks mean extraction without enzyme; addition of ascorbic acid (AA) and EDTA, pH adjustment, and purification were also tested. Double asterisks mean incubation with individual enzymes was also tested



HCl (2 M). The mixture was vortexed for 1 min under nitrogen atmosphere and subdued light. After centrifugation (10 min, 3375g), an aliquot of the supernatant was filtered through a syringe filter (0.45 µm pore size) and stored under nitrogen atmosphere at –20 °C until analysis within 1 week by HPLC-UV using the optimized chromatographic conditions.

Chromatographic Conditions

Phenolic acids were quantified by HPLC with UV detection (Agilent 1260, Agilent Technologies, Santa Clara, USA). Phenolic acid compounds in standards and samples were separated on a reverse-phase C18 column (Luna, 250 × 4.6 mm, 3 µm, 100 Å; Phenomenex, Torrance, USA) with a guard column (3.0 × 4.0 mm; Phenomenex, Torrance, USA) containing the same packing material. The column was thermostatically controlled at 25 °C. The flow rate was set at 1.0 mL/min, the injection volume was 20 µL, and the total run time was 60 min. On testing different gradient conditions and formic acid concentrations (0.1%, 0.5%, and 1%), it was found that nearly complete separation was achieved using the following mobile phase composition: solvent A (1% aqueous formic acid) and solvent B acetonitrile/methanol/water (8:1:1, v/v/v). The gradient started at 6% solvent B and was maintained for 34 min, after which solvent B was increased to 23% within 1 min. After 10 min, solvent B was further increased to 50% within 1 min and held at 50% for 4 min. Solvent B was then decreased to 6% over 1 min and maintained there for a further 9 min to recondition the column before the next injection.

Identification and Quantification of Phenolic Acids

The detection wavelengths were set at 260 (*p*-hydroxybenzoic acid, vanillic acid, ellagic acid), 270 (gallic acid, 2,3,4-trihydroxybenzoic acid), 275 (3,4-dihydroxybenzaldehyde, vanillin, syringic acid, benzoic acid, *o*-coumaric acid, cinnamic acid), 310 (*p*-coumaric acid, syringaldehyde), and 325 nm (gentisic acid, caffeic acid, chlorogenic acid, ferulic acid, sinapic acid) (Table 1). Aqueous calibration standard solutions were prepared by serial dilution to contain 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, 5, and 10 µg/mL ellagic acid and 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 50, and 100 µg/mL of all other phenolic acids to determine the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were estimated by the signal-to-noise (*S/N*) ratio of 3 for LOD and 10 for LOQ for each compound.

Quantification was based on multilevel external calibration curves with a linear range over 0.03–100 µg/mL for gallic acid and 3,4-dihydroxybenzaldehyde; 0.04–100 µg/mL for *p*-hydroxybenzoic acid and sinapic acid; 0.06–100 µg/mL for

caffeic acid and 2,3,4-trihydroxybenzoic acid; 0.08–100 µg/mL for vanillic acid and *p*-coumaric acid + syringaldehyde; 0.1–100 µg/mL for vanillin; 0.13–100 µg/mL for caffeic acid, gentisic acid, and syringic acid; 0.16–100 µg/mL for chlorogenic acid; and 0.86–100 µg/mL for benzoic acid.

Quality Control of Analytical Methods

To control for within- and between-day variation, an in-house control sample (Emmer, *T. dicoccum*, cv. Västgötarna), milled and stored in subsamples at –20 °C under nitrogen atmosphere, was included in triplicate in each extraction batch (*n* = 4). The coefficient of variation (CV) for within- and between-day variation was calculated from the replicates.

Recovery tests were performed by standard addition of vanillic acid, caffeic acid, vanillin, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid to the in-house control sample (Emmer, *T. dicoccum*, cv. Västgötarna (in triplicate)). Standard was added aiming at two concentrations of each phenolic acid: for ferulic acid, 300 µg/g dry matter (DM) and 600 µg/g DM; for sinapic acid, 100 µg/g DM and 200 µg/g DM; for all other compounds, 50 µg/g of DM and 100 µg/g DM. The recovery (*R*) was calculated as:

$$R (\%) = 100 \times (C_{\text{found}} - C_{\text{sample}}) / C_{\text{added}}$$

where C_{found} is the measured content in the spiked sample, C_{sample} is the measured content in the sample before spiking, and C_{added} is the added concentration.

In order to check the stability of chlorogenic acid during base hydrolysis, 50 µL of chlorogenic acid standard (1000 µg/mL in methanol) was treated (*n* = 2) as a sample according to the optimized procedure to cereal cultivars.

Calculations and Statistics

The values presented are mean (µg/g dry matter (DM)) ± standard deviation (SD) of duplicate samples during method development, or of triplicate samples during recovery and analysis of cereal samples except for the in-house control sample Emmer (Västgötarna) which was included into every batch during analysis (*n* = 12). The sum of individual phenolic acids was expressed in µg ferulic acid/g DM, after conversion using the molecular mass values listed in Table 1. *p*-Coumaric acid and syringaldehyde coeluted and were expressed as *p*-coumaric acid, as only traces of syringaldehyde have been reported in cereals (Li et al. 2008). One-way ANOVA and Tukey's pair-wise comparison, with the level of significance set at *P* < 0.05, were used to analyze differences between hydrolysis methods, and between and within different cereal

species. All statistical analyses were carried out using Minitab (Minitab Ltd, Coventry, UK).

Results

Optimization of Chromatographic and Extraction Conditions

Separation of phenolic acids is mainly performed on a C18 column (see review by Robbins (2003)). A standard C18 column with a 3 μm particle size was chosen in the present work, to be consistent with the aim of developing a robust method. With the exception of *p*-coumaric acid, which coeluted with syringaldehyde acid, 16 phenolic acids were successfully separated (Fig. 2). Addition of formic acid to the mobile phase was required for both separation and peak shape, which improved with increasing formic acid concentration from 0.1 to 1% (Fig. 2).

Up to 5-fold higher extractability was observed after base hydrolysis compared with acid hydrolysis (Fig. 3), depending on the hydrolysis conditions. This indicates that base hydrolysis was more effective in releasing the esterified and conjugated phenolic acids. Incubation with enzymes, i.e., α -amylase, protease, and cellulase, after acid hydrolysis improved the extractability by 4-fold, but was half as efficient as base hydrolysis (Fig. 3 (II and III)). On increasing the molarity of HCl from 0.2 to 1 or 2 M, the stability of compounds was negatively affected and extraction resulted in 48% and 72% lower phenolic acid content, respectively (Fig. 3 (III)).

The highest extractability of phenolic acids was achieved after base hydrolysis (3 M NaOH, 90 min). Higher (6 M) or lower (2 M) base concentration did not affect the extractability (Fig. 3 (III)). Moreover, increasing the incubation time from 1.5 to 16 h and addition of hydrolyzing enzymes (α -amylase, protease, and cellulase) did not result in any significant increase in phenolic acids (Fig. 3 (I and II)). Enzymatic treatment was therefore

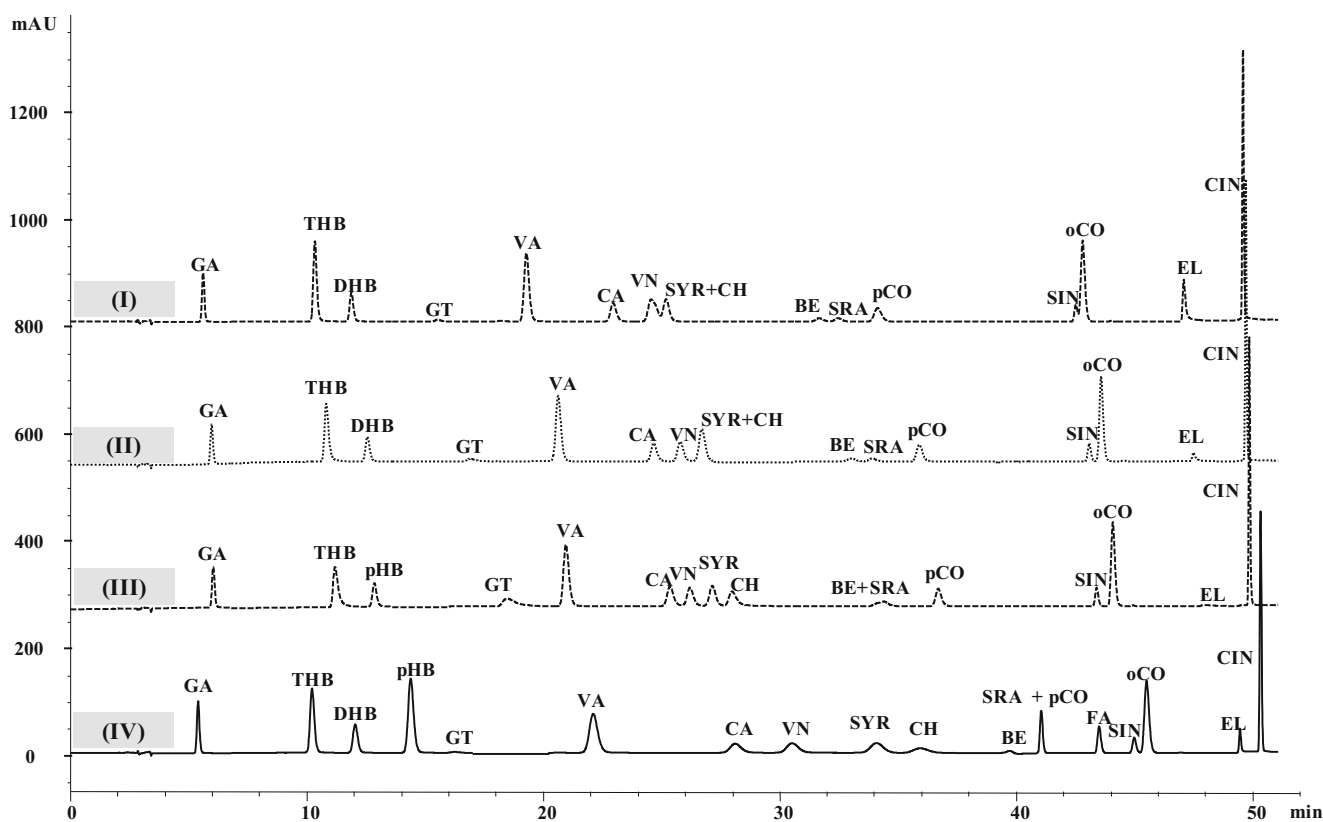


Fig. 2 Chromatograms of a standard mixture of 18 phenolic acids at 260 nm using different concentrations of formic acid in the mobile phase: (I) 0.1% formic acid, (II) 0.5% formic acid, (III, IV) 1% formic acid. Solvent A consists of different formic acid concentrations in water and solvent B of acetonitrile/methanol/water (8:1:1, v/v/v). In I, II, and III, the gradient for solvent B started at 6% and was then increased to 12% over 30 min, to 30% over 15 min, and to 50% over 1 min. It was held at 50% for 2 min, and then lowered to 6% over 1 min and kept there for 6 min to recondition the column before the next injection. Total run time

was 55 min. In panel IV, the optimized gradient conditions were used. GA gallic acid, THB 2,3,4-trihydroxybenzoic acid, DHB 3,4-dihydroxybenzaldehyde, *p*HB *p*-hydroxybenzoic acid, GT gentisic acid, VA vanillic acid, CA caffeic acid, VN vanillin, SYR syringic acid, CH chlorogenic acid, BE benzoic acid, SRA syringaldehyde, *p*CO *p*-coumaric acid, FA ferulic acid, SIN sinapic acid, *o*CO *o*-coumaric acid, EL ellagic acid, CIN cinnamic acid. The concentration used was 6 $\mu\text{g}/\text{mL}$ for EL and 20 $\mu\text{g}/\text{mL}$ for all other phenolic acids

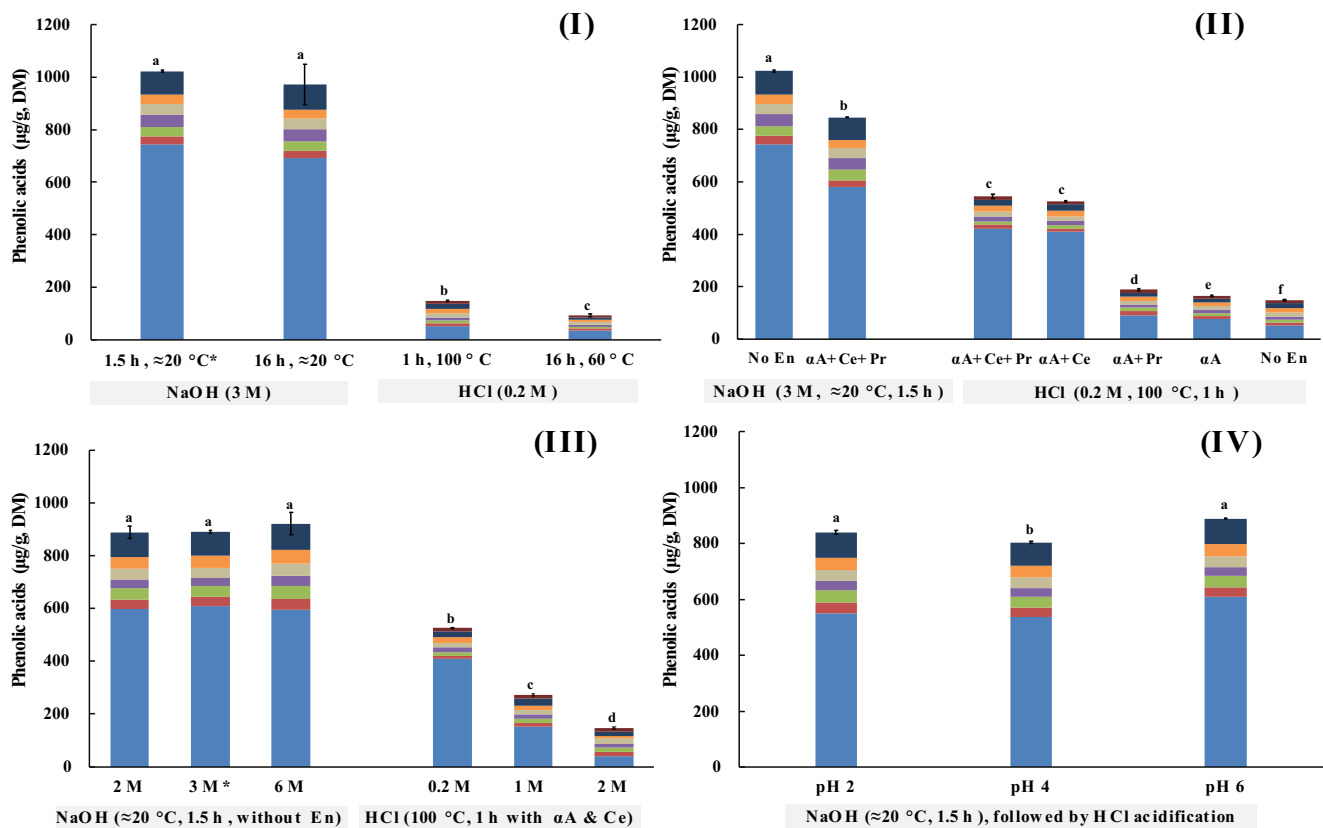


Fig. 3 Effect of different extraction procedures in terms of (I) extraction time, (II) use of enzymes, (III) acid or base concentration during hydrolysis, and (IV) acidification after base hydrolysis on the extractability of phenolic acids ($\mu\text{g/g}$, DM) in whole grain wheat flour (Emmer, cv. Västgötarna). Columns represent means of duplicate samples ($n = 2$). Error bars were calculated for the sum of phenolic acids. Different superscripts represent significant differences ($P < 0.05$). Single asterisks mean

experiments summarized in each panel were partly carried out at different occasions; data are therefore underlying the day-to-day variation of $\leq 10\%$. ■ Ferulic acid, ■ *p*-hydroxybenzoic acid, ■ vanillic acid, ■ caffeic acid, ■ syringic acid, ■ *p*-coumaric acid, ■ sinapic acid, ■ 3,4-dihydroxybenzaldehyde. No En no enzymes, αA α -amylase, Ce cellulase, Pr protease

omitted during hydrolysis, to simplify the extraction procedure and reduce costs. However, acidification to pH 5.5–6 after base hydrolysis was shown to be important, especially when quantifying syringic acid, as $\approx 15\%$ of the content was lost if the pH was not adjusted. Decreasing the pH further to 4 or 2 after base hydrolysis did not significantly alter the measurable content (Fig. 3 (IV)), indicating that bound phenolic acids were fully hydrolyzed during base hydrolysis.

Caffeic acid was only determined in cereal samples after addition of EDTA and ascorbic acid to improve the stability during extraction. However, the sum of the individual phenolics expressed as ferulic acid was not significantly affected ($P = 0.13$) due to the low content of caffeic acid in the samples. Furthermore, purification of hydrolysates with dichloromethane or ethyl acetate (to eliminate impurities) did not affect the results (data not shown).

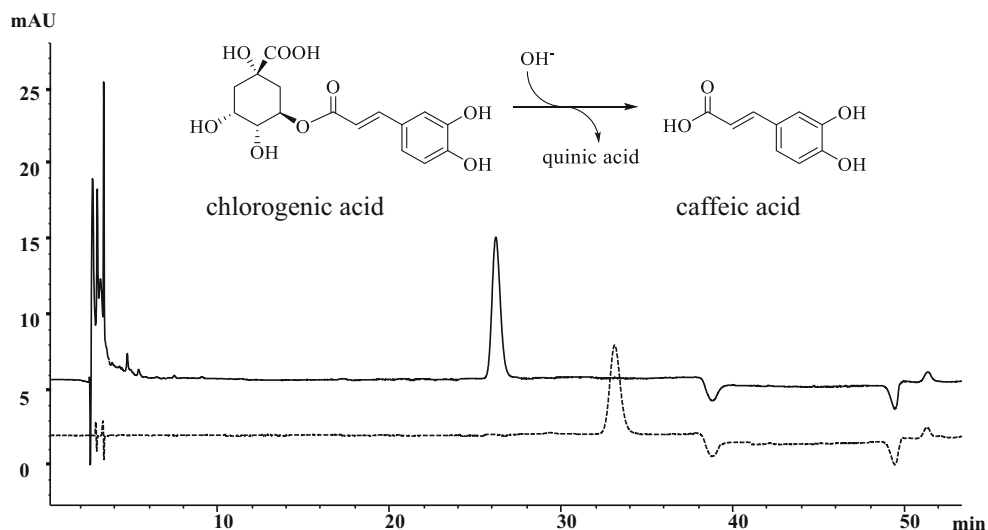
The stability of phenolic acids during base hydrolysis was checked by treating a standard mix of all phenolic acids ($n = 18$) as a sample. All individual phenolic acids

showed stability above 90% (data not shown) with the exception of ellagic acid and chlorogenic acid (which is completely converted to caffeic acid at alkaline pH) (Fig. 4).

Quality Control of the Analytical Method

The calibration curves of all phenolic acids except ellagic acid were linear up to $100 \mu\text{g/mL}$ ($R^2 \geq 0.9992$) (Table 1). It was impossible to generate an accurate calibration curve for ellagic acid in a standard mixture including the other 17 phenolic acid compounds, due to the low stability of ellagic acid. The within-day ($n = 3$) and between-day ($n = 12$) CV for the control sample (Emmer, *T. dicoccum*, cv. Västgötarna) was $\leq 10\%$ in both cases for each individual phenolic acid (Table 2). The relative recovery of individual phenolic acids after standard addition to the control sample at two concentrations ($n = 3$, triplicate samples at two addition levels) ranged from 88% (caffeic acid) to 108% (sinapic acid) (Table 3).

Fig. 4 Conversion of chlorogenic acid (dotted line chromatogram) into caffeic acid (solid line chromatogram) during the base hydrolysis



Content of Phenolic Acids in Cereal Samples

The phenolic acids *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, vanillin, syringic acid, ferulic acid, sinapic acid, and *p*-coumaric acid/syringaldehyde were found in all cereals tested (Table 4). In addition to these, both rye cultivars contained 3,4-dihydroxybenzaldehyde. The sum of these compounds (Table 4) was expressed as ferulic acid ($\mu\text{g/g}$, DM) due to differing molecular masses, as shown in Table 1. A typical chromatogram of a cereal sample (Emmer, *T. dicoccum*, cv. Västgötarna) after extraction using the optimized method is shown in Fig. 5. The average content of phenolic acids in the cereal samples varied significantly depending on cereal genus and species (Table 4). Rye contained the highest amounts, followed by wheat, barley, and oats, with concentrations ranging from $\approx 600 \mu\text{g/g}$ (barley) to $1150 \mu\text{g/g}$ (rye). The distribution of individual

phenolic acids was similar in the different cereal samples (Table 4), with ferulic acid dominating (48–72% of total phenolic acid content (Table 4)). Sinapic acid was the second most dominant form, representing up to 11% of total phenolic acid content (Table 4).

Discussion

Our aim was to develop a simple method for analysis of total phenolic acids in cereals, including free, bound, and conjugated forms, since the free forms only comprise 1–4% of the total content (Andersson et al. 2008; Li et al. 2008; Nyström et al. 2008; Shewry et al. 2008). Our results showed that base hydrolysis is more effective for extraction of phenolic acids from the cereal matrix than acid hydrolysis, with or without use of sequential enzyme hydrolysis. Base and acid hydrolysis have

Table 2 Precision of the quantification method for phenolic acids in the wheat in-house control sample (Emmer, *T. dicoccum*, cv. Västgötarna)

Phenolic acid	Mean content ($\mu\text{g/g}$, DM)	RSD (%), $n = 3$, intra-assay	RSD (%), $n = 12$, inter-assay
<i>p</i> -Hydroxybenzoic acid	27 ± 0.3	0.27	1.1
Vanillic acid	33 ± 0.7	0.11	2.1
Caffeic acid	41 ± 1.5	1.59	3.6
Vanillin	32 ± 0.8	0.02	2.5
Syringic acid	37 ± 0.4	0.40	1.1
<i>p</i> -Coumaric acid + syringaldehyde	37 ± 2.0	2.75	5.5
Ferulic acid	591 ± 59.8	5.00	10.1
Sinapic acid	82 ± 6.6	2.94	8.1
Total, expressed in ferulic acid*	901 ± 68.8	3.74	7.6

The following phenolic acids were not detected: gallic acid, 2,3,4-trihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, gentisic acid, chlorogenic acid, benzoic acid, *o*-coumaric acid, ellagic acid, and cinnamic acid

*The sum of individual phenolic acids expressed in ferulic acid using the molecular masses listed in Table 1
 n refers to sample replicates, for intra-assay as triplicate on the same day, for inter-assay on 12 independent days

Table 3 Recovery of phenolic acids during quantification in Emmer (*T. dicoccum*, cv. Västgötarna)

Phenolic acid	C_{sample} ($\mu\text{g/g DM}$)	C_{added} ($\mu\text{g/g DM}$)	C_{found} ($\mu\text{g/g DM}$)	Recovery (%)	Mean recovery (%)
Vanillic acid	32 ± 0.1	50	78 ± 0.8	91	93
		100	126 ± 0.8	94	
Caffeic acid	39 ± 0.6	50	83 ± 1.6	88	88
		100	127 ± 1.7	88	
Vanillin	32 ± 0.1	50	80 ± 0.3	96	98
		100	132 ± 1.6	100	
Syringic acid	37 ± 0.1	50	88 ± 0.8	102	104
		100	142 ± 1.1	105	
<i>p</i> -Coumaric acid	36 ± 1.0	50	81 ± 1.6	90	90 ^a
		100	126 ± 2.5	90	
Ferulic acid	518 ± 25.9	300	802 ± 14.3	94	92
		600	1053 ± 15.8	89	
Sinapic acid	76 ± 2.2	100	185 ± 3.9	109	108
		200	292 ± 5.1	108	

C_{found} mean content in the spiked sample, C_{sample} mean content in the sample without spiking, C_{added} content added to the sample

^a Recovery was calculated only for *p*-coumaric acid (not for syringaldehyde). Concentrations are means of triplicate samples (± standard deviation)

different effects on plant cell wall compounds (Acosta-Estrada et al. 2014). Base hydrolysis is reported primarily to break the ester bonds, while acid hydrolysis is reported to break glycosidic bonds, solubilizing sugars without affecting the ester

bonds (Acosta-Estrada et al. 2014). The bound phenolic acids in cereal grain are mainly ester-linked to cell wall components such as hemicellulose (e.g., arabinoxylans), cellulose, lignin, and pectin (Acosta-Estrada et al. 2014; Rhodes et al. 2002).

Table 4 Phenolic acid content ($\mu\text{g/g DM}$) in cultivars of rye (*Secale*), wheat (*Triticum*), barley (*Hordeum*), and oats (*Avena*)

Cereal	Cultivar (origin)	Phenolic acid content ($\mu\text{g/g DM}$)								
		<i>p</i> HB	VA	CA	VN	SYR	<i>p</i> CO	FA	SIN	**Sum as ferulic acid
Rye*	Schmidt rye (Västgötarna)	26 ± 0.1	28 ± 0.4	49 ± 0.5	35 ± 1.0	33 ± 0.6	48 ± 1.2	762 ± 34.0	104 ± 2.0	1148 ± 41.6 ^A
	Rye (Saltå mill)	26 ± 0.4	27 ± 0.2	45 ± 1.2	37 ± 1.5	33 ± 0.4	52 ± 1.2	696 ± 30.3	120 ± 4.6	1096 ± 47.8 ^{AB}
Wheat	Spelt (Warbro mill)	27 ± 0.2	31 ± 0.4	38 ± 0.6	30 ± 0.6	37 ± 0.1	30 ± 0.3	592 ± 17.7	61 ± 1.2	869 ± 23.6 ^F
	Spelt (Saltå mill)	26 ± 0.2	31 ± 0.3	41 ± 1.1	30 ± 0.1	38 ± 0.4	31 ± 0.6	703 ± 43.2	69 ± 2.6	991 ± 58.0 ^{BCD}
	Öland spring wheat (Isgårde farm)	27 ± 0.1	31 ± 0.1	38 ± 0.2	31 ± 0.1	36 ± 0.1	41 ± 0.3	590 ± 9.0	72 ± 1.3	891 ± 12.8 ^{EF}
	Wheat (Saltå mill)	26 ± 0.1	29 ± 0.4	36 ± 0.4	29 ± 0.2	36 ± 0.2	35 ± 0.5	639 ± 19.8	69 ± 1.5	923 ± 26.0 ^{DEF}
	Jacoby Borst lantvete (Nibble farm)	29 ± 0.1	36 ± 0.1	40 ± 0.8	31 ± 0.1	41 ± 0.4	47 ± 1.9	695 ± 53.2	80 ± 3.0	1025 ± 72.7 ^{BCD}
	Wheat Dala lantvete (Västgötarna)	27 ± 0.1	32 ± 0.1	38 ± 0.1	32 ± 0.6	38 ± 0.2	33 ± 0.1	770 ± 17.3	72 ± 0.5	1065 ± 19.7 ^{ABC}
	Emmer (Västgötarna)	27 ± 0.3	33 ± 0.7	41 ± 1.5	32 ± 0.8	37 ± 0.4	37 ± 2.0	591 ± 59.8	82 ± 6.6	901 ± 68.8 ^{EF}
Emmer (Warbro mill)	27 ± 0.1	33 ± 0.2	40 ± 0.1	30 ± 0.1	37 ± 0.4	39 ± 0.6	596 ± 6.4	90 ± 0.6	915 ± 8.3 ^{DEF}	
Barley	Naked barley (Warbro mill)	25 ± 0.1	34 ± 0.1	64 ± 0.5	31 ± 0.3	35 ± 0.3	29 ± 0.1	682 ± 8.1	42 ± 0.4	971 ± 9.6 ^{CDEF}
	Barley (Saltå mill)	24 ± 0.1	30 ± 0.1	41 ± 0.5	29 ± 0.2	33 ± 0.2	45 ± 1.0	340 ± 9.4	36 ± 0.2	607 ± 12.8 ^G
Oat	Naked oat (Warbro mill)	29 ± 0.5	32 ± 0.7	57 ± 3.9	30 ± 0.3	49 ± 2.4	30 ± 0.6	283 ± 30.5	61 ± 4.6	597 ± 53.4 ^G
	Oat (Saltå mill)	30 ± 0.1	32 ± 0.3	54 ± 0.8	32 ± 0.6	49 ± 0.4	27 ± 0.2	293 ± 9.6	73 ± 0.8	614 ± 13.4 ^G

*3,4-Dihydroxybenzaldehyde (DHB) only detected in rye samples: 29 ± 0.4 $\mu\text{g/g}$ in Västgötarna and 28 ± 0.3 $\mu\text{g/g}$ in Saltå mill

**The sum of individual phenolic acids, expressed as ferulic acid, using the molecular masses listed in Table 1. *p*HB *p*-hydroxybenzoic acid, VA vanillic acid, CA caffeic acid, VN vanillin, SYR syringic acid, *p*CO^a *p*-coumaric acid (may also contain syringaldehyde), FA ferulic acid, SIN sinapic acid

Individual phenolic acids are expressed as mean ± standard deviation ($n = 3$ for all cereal samples except for the in-house control sample Emmer (Västgötarna) with $n = 12$ which was included into every batch during analysis)

Means with different letters are significantly different ($P < 0.05$)

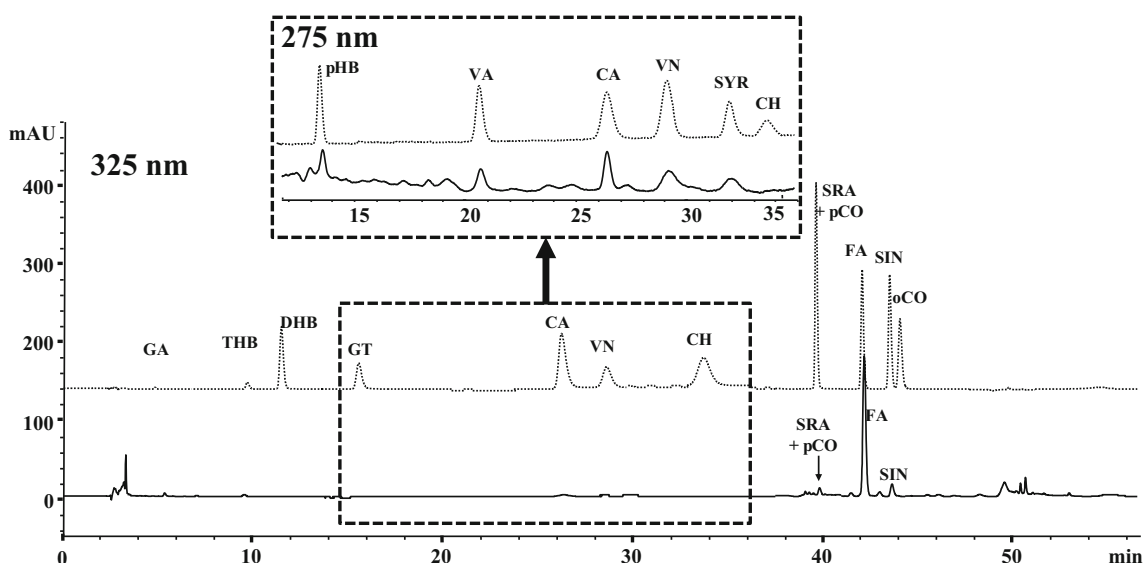


Fig. 5 Chromatograms of the in-house control sample Emmer (*T. dicoccum*, cv. Västgötarna) (solid line) after extraction using the optimized procedure and of the standard mixture of 18 phenolic acids (dotted line, 20 $\mu\text{g}/\text{mL}$ except ellagic acid with 6 $\mu\text{g}/\text{mL}$). Different wavelengths used in identification are listed in Table 1. GA gallic acid, THB 2,3,4-

trihydroxybenzoic acid, DHB 3,4-dihydroxybenzaldehyde, *p*HB *p*-hydroxybenzoic acid, GT gentisic acid, VA vanillic acid, CA caffeic acid, VN vanillin, SYR syringic acid, CH chlorogenic acid, SRA syringaldehyde, *p*CO *p*-coumaric acid, FA ferulic acid, SIN sinapic acid, *o*CO *o*-coumaric acid

For example, the hydroxycinnamic acids (e.g., ferulic acid, the major phenolic acid in cereals) are largely ester-linked to arabinose molecules in the arabinoxylan chain in cereal cell walls (Rhodes et al. 2002). This explains the ineffectiveness of acid hydrolysis for release of bound phenolic acids, as it leaves ester bonds intact (Fazary and Ju 2007). Increasing the acid concentration from 0.2 to 1 or 2 M was found to negatively affect the stability of the phenolic acids and therefore decreased the measurable content, with losses being higher after treatment with 2 M acid compared with 1 M. This confirmed the lower stability of phenolic acids under strong acidic conditions reported by others (Krygier et al. 1982; Verma et al. 2009). Combined acid and enzymatic hydrolysis with amylase, protease, and cellulase increased the extractability of phenolic acids to some extent, confirming findings by others (Yu et al. 2001). Nonetheless, the yield was still up to 50% lower than after base hydrolysis. To effectively release bound phenolic acids, specific esterases have to be used (Fazary and Ju 2007; Shahidi and Yeo 2016). This might explain the lower extractability observed in this study and others on using amylase, protease, and cellulase.

The highest extractability of phenolic acids was achieved after base hydrolysis (3 M, 90 min). An increase in the base concentration or in the incubation time did not result in higher extractability. Ross et al. (2009) reported higher extractability after extraction using 10 N NaOH compared with mild base hydrolysis using 2 N NaOH. In their study, the 10 N NaOH (5 mL) was diluted with 10 mL water before extraction and therefore, the final concentration was 3.3 N NaOH, which is similar to that used in the present study. No significant changes in the content of phenolic acids were detected on using

different acidification levels (pH 2, 4, and 6) after base hydrolysis (see Fig. 3 (IV)). This may confirm that the hydrolysis was caused by the alkali and not the acid.

Addition of ascorbic acid and EDTA to prevent losses of phenolic acids during base hydrolysis resulted only in improved stability of caffeic acid and did not significantly ($P = 0.13$) affect the sum of phenolic acids expressed as ferulic acid. Other studies have reported high stability of phenolic acids on using ascorbic acid and EDTA during extraction (Nardini et al. 2002; Ross et al. 2009). Under alkaline conditions, complete hydrolysis of chlorogenic acid to caffeic acid was observed (Fig. 5), by breaking the ester bond between caffeic and quinic acid, confirming findings by others (Nardini et al. 2002).

A limitation of this study was the co-elution of *p*-coumaric acid and syringaldehyde and the poor calibration curve for ellagic acid. However, these phenolic acids have not previously been detected in cereals, so this limitation is not expected to affect the measured phenolic acid content in cereals.

The high content of phenolic acids in cereal foods, in addition to their high consumption rate, makes them a significant source of phenolic acids in the European diet. Hence, information regarding the effects of genotype and species on the content of phenolic acids can be helpful in efforts to improve dietary intake. The results in this study confirmed that cereals are a rich source of phenolic acids (concentration range 600 to > 1000 $\mu\text{g}/\text{g}$), although the concentration of phenolic acids quantified varied significantly between cereal genotypes (up to 2-fold variation) and within the same genotype (1.5-fold variation). However, these data should be regarded as indicative rather than definitive, since only a few cereals were analyzed. Variation due to genotype and growing conditions in

the concentration of phenolic acid in wheat (422–1257 µg/g, DM), rye (1093–1357 µg/g, DM), barley (969–1132 µg/g, DM), and oats (351–874 µg/g, DM) has been reported previously (Andersson et al. 2008, 2000; Fernandez-Orozco et al. 2010; Gawlik-Dziki et al. 2012; Li et al. 2008; Ndolo and Beta 2014; Nyström et al. 2008; Shewry et al. 2008).

The distribution of individual phenolic acids was similar in the different cereal samples tested (Table 4), with ferulic acid being the dominant form, followed by sinapic acid. The other phenolic acids (*p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, vanillin, and syringic acid) were only found in minor amounts. Similar distributions have been reported by others (Andersson et al. 2008, 2000; Fernandez-Orozco et al. 2010; Gawlik-Dziki et al. 2012; Li et al. 2008; Ndolo and Beta 2014; Nyström et al. 2008; Shewry et al. 2008). The compound 3,4-dihydroxybenzaldehyde was only detected in the two rye cultivars.

To summarize, a robust HPLC method was developed for simultaneous quantification of 16 individual phenolic acids deriving from free, conjugated, and bound forms in cereals. The method involves a short, cost-efficient hydrolysis step (no enzymes required). Acid hydrolysis, even followed by enzyme treatment, proved ineffective. The method was successfully applied in quantifying phenolic acids in different cereal genotypes and species. The results confirm that cereals are a good source of phenolic acids, but that the content is highly variable depending on cereal genotype and species.

Conclusions

A HPLC-UV method suitable for quantifying and identifying simultaneously 16 phenolic acids was developed. Up to nine phenolic acids were quantified in the tested cereal matrices at reduced cost and sample preparation time. Among the cereals, rye was the best source of phenolic acids. However, since only a few cultivars were analyzed, the findings should be regarded as indicative rather than definitive.

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Compliance with Ethical Standards

Conflict of Interest Mohammed E. Hefni declares that he has no conflict of interest. Laura S. Amann declares that she has no conflict of interest. Cornelia M. Witthöft declares that she has no conflict of interest.

Ethical Approval This study does not contain any studies with human or animal subjects.

Informed Consent Not applicable

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