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Phenolic Acids from Wheat Show Different Absorption Profiles in Plasma: A Model Experiment with Catheterized Pigs

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ABSTRACT: The concentration and absorption of the nine phenolic acids of wheat were measured in a model experiment with catheterized pigs fed whole grain wheat and wheat aleurone diets. Six pigs in a repeated crossover design were fitted with catheters in the portal vein and mesenteric artery to study the absorption of phenolic acids. The difference between the artery and the vein for all phenolic acids was small, indicating that the release of phenolic acids in the large intestine was not sufficient to create a porto-arterial concentration difference. Although, the porto-arterial difference was small, their concentrations in the plasma and the absorption profiles differed between cinnamic and benzoic acid derivatives. Cinnamic acids derivatives such as ferulic acid and caffeic acid had maximum plasma concentration of 82 ± 20 and 200 ± 7 nM, respectively, and their absorption profiles differed depending on the diet consumed. Benzoic acid derivatives showed low concentration in the plasma (<30 nM) and in the diets. The exception was *p*-hydroxybenzoic acid, with a plasma concentration (4 ± 0.4 μM), much higher than the other plant phenolic acids, likely because it is an intermediate in the phenolic acid metabolism. It was concluded that plant phenolic acids undergo extensive interconversion in the colon and that their absorption profiles reflected their low bioavailability in the plant matrix.

KEYWORDS: *phenolic acids, dietary fiber, plasma, pigs, wheat*

INTRODUCTION

Phenolic acids are considered ubiquitous among vascular plants, where they occur in most tissues. In cereals, phenolic acids are present in all cell tissues but with much higher concentrations in the aleurone and pericarp/testa layers compared to the endosperm.[1] Epidemiological studies have linked whole-grain cereal consumption with a reduced risk of developing colonic and breast cancer, arteriosclerosis, and type 2 diabetes.[1−3] The underlying physiological mechanism behind the protective effects of whole-grain, however, are unclear but is most likely assigned to a concerted action of a wide variety of bioactive compounds, many of which are associated with the dietary fiber (DF) matrix.[1] Among the possible mechanisms, the antioxidative capacity of phenolic acids most likely play a role even though the concentration level induced in vitro and ex vivo to obtain an effect appears much higher than detected in vivo in both animals and humans.[4−7]

Phenolic acids are hydroxylated derivatives of cinnamic and benzoic acids, Figure 1. Cinnamic acid derivatives found in wheat are ferulic acid, caffeic acid, *p*-coumaric acid, and sinapic acid, whereas protocatechuic acid, *p*-hydroxybenzoic acid, salicylic acid, vanillic acid, and syringic acid are derivatives of benzoic acid. Ferulic acid is the predominant phenolic acid in wheat, accounting for 70−90% of total phenolic acid content.[8] A small proportion of free phenolic acid is located in the outer layer of the pericarp. Free phenolic acids are absorbed in the small intestine and conjugated in the intestinal epithelium or in the liver.[9] Most phenolic acids in cereals, however, occur bound to plant cell walls, which consist of cellulose, arabinoxylan, and β-glucan. Ferulic acid, for instance, is linked to the O-5 position of the arabinofuranose substitutes in the arabinoxylan but may also be linked by ester and ether bonds to lignin.[10] In contrast to the free phenolic acids, bound phenolic acids have to be released from the plant matrix by intestinal esterases in the mucosa or, the main part, by bacterial esterases in the colon.[11] The bioavailability of phenolic acids depends largely on their bioaccessibily in the plant matrix.[8,12] In the process of microbial fermentation, however, plant phenolic acids are further metabolized to, i.e., derivatives of phenylpropionic, phenylacetic, hippuric, and benzoic acids with different hydroxylation patterns. For instance, ferulic and caffeic acids can be de-esterified to 3-(3-hydroxyphenyl)-propionic acid and subsequently β-oxidized to benzoic acid. The efficiency of release by intestinal and bacterial esterases and further metabolism of phenolic acids affect their absorption pattern and their route of excretion, i.e., through feces or urine.[11,9] More knowledge concerning absorption and bioavailability of phenolic acids in plasma is therefore required to fully understand their physiological properties and potential health effects in vivo.

To our knowledge, previous studies on bioavailability and absorption of phenolic acids from wheat have primarily been performed with rats.[13,4] However, because the digestive physiology of pigs is more similar to humans than it is the case for rats, we used porto-arterial catheterized pigs to measure the net absorption of the nine phenolic acids present in wheat.

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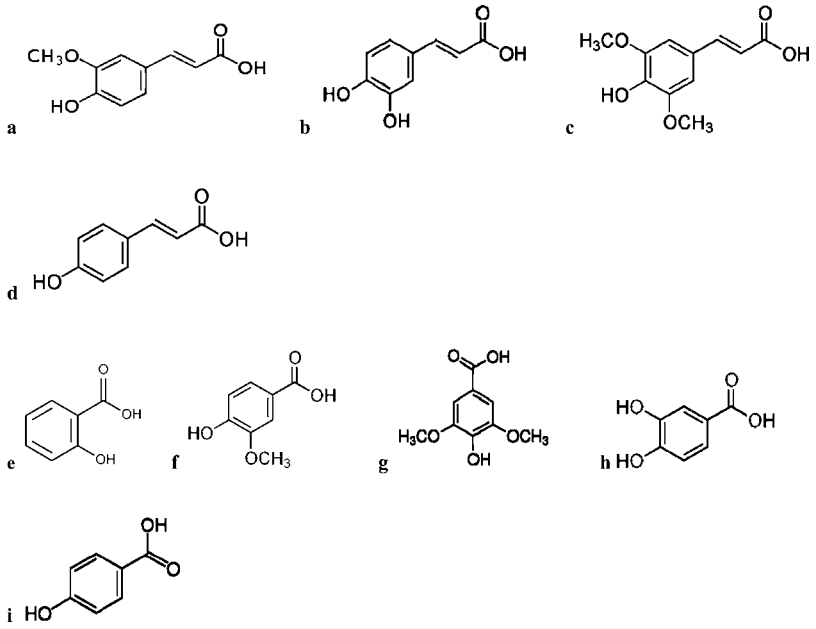

Figure 1. Chemical structures of cinnamic acid derivatives: a, ferulic acid; b, caffeic acid; c, sinapic acid; d, *p*-coumaric acid and benzoic acid derivatives; e, salicylic acid; f, vanillic acid; g, syringic acid; h, protocatechuic acid; i, *p*-hyroxybenzoic acid.

We hypothesized that bound phenolic acids were released during active fermentation in the large intestine and that the absorption profiles may differ between the phenolic acids after consumption of whole grain wheat and wheat aleurone.

MATERIALS AND METHODS

Chemicals. Acetonitrile and methanol of HPLC grade were from Rathburn (Mikrolab Aarhus A/S, Denmark). Acetic acid was from J. T. Baker (Deventer, Holland), and formic acid was from Merck (Darmstadt, Germany). Sulfatase (EC 3.1.6.1) type H-1 from *Helix pomatia* was purchased from Sigma-Aldrich (S. Louis, MO, USA) and *β*-glucuronidase (EC 3.2.1.31) from *Escherichia coli* K12 was purchased from Roche (Mannheim, Germany). Sodium acetate was obtained from Prolabo (Leuven, Belgium). The following phenolic acid standards were used: syringic acid, salicylic acid, and caffeic acid (Sigma-Aldrich, S. Louis, MO, USA), vanillic acid, sinapic acid, protocatechuic acid, *p*-coumaric acid, ferulic acid, and *p*-hydroxybenzoic acid (Fluka, Sigma-Aldrich, S. Louis, MO, USA). Water was purified with an A10 system from Millipore (Merck Millipore, MA, USA).

Diets. Breads were made of standard refined wheat flour (RWF), whole grain wheat (WGW), and wheat aleurone flour (WAF). The RWF and WGW breads were produced at Holstebro Technical College (Holstebro, Denmark), and the WAF breads were baked in a local bakery (Konditor-Bager Ørum, Denmark). The ingredients for the RWF breads were refined wheat flour (*Triticum aestivum* L. Cv. Tiger), purified wheat fiber (Vitacel R200; J. Rettenmaier and Söhne GmbH, Rosenberg, Germany), rapeseed oil, sugar, salt, wheat gluten (LCH A/S, Frederiksberg, Denmark), and yeast, while in WGW and WAF breads, whole wheat grain (BFEL Karlsruhe, Germany) and wheat aleurone flour (Bühler AG, Uzwil, Switzerland) replaced the refined wheat flour and purified wheat fiber on an iso-DF basis. After production, the breads were cut into pieces. After weighing, meal portions were frozen at −20 °C and thawed immediately before consumption. Information on the ingredients content of the breads can be found in Table 1. The breads were balanced with regard to starch, proteins, fat, and DF, but they varied in DF characteristics and the ratio between DF and phenolic acids as shown in Table 2.

Study Design and Animals. Experiments were in compliance with the guidelines of the Danish Ministry of Justice and regulations for humane care and use of animals in research (The Danish Ministry of Justice, Animal Testing ACT (Consolidation Act no. 726 of 9 September 1993 as amended by Act no. 1081 of 20 December 1995)). The pigs were fed three times daily at 09.00 (breakfast), 14.00 (lunch), and 19.00 h (dinner) with an amount of 40, 40, and 20% of the daily supply, thereby mimicking the diurnal variation in cereal intake experienced by humans. Daily feed allowance supplied 193-211 g DF/d and 76−779 mg/d of phenolic acids, Table 3. The pigs used in the study were from the swineherd at Aarhus University, Department of Animal Science. Six female pigs (Landrace x Yorkshire) with a bodyweight of 56.5 kg (±1.8 kg) were included in the experiment. The pigs were adapted to the pen for 5 days, and then the animals were surgically fitted with a flow probe (Transonic, 20A probe, 20 mm; Transonic System, Inc., Ithaca, NY, USA) around the portal vein, a catheter in the portal vein, and a catheter in the MA. After the surgery, the animals were allowed a 5−7 days recovery period before entering the experimental crossover design. In each experimental week, the pigs were fed the wash-out diet, RWF on day 1−3 (Friday-Sunday), and then the pigs were fed either WGW or WAF on days 4−7 (Monday−Thursday). The pigs had access to water ad libitum, whereas no straw

Table 1. Ingredients List of Experimental Diets

ingredients (g/kg, as-fed basis)	RWF	WGW	WAF
wheat whole grain		813	
standard wheat flour	711		
wheat aleurone 2[a]			214
wheat starch[b]			516
wheat gluten[b]	59	36	116
Vitacel R200 (99.5% cellulose)[c]	69		
rapeseed oil	86	79	79
sugar	15	15	15
baker's yeast	15	15	15
vitamin-mineral mixture[d]	4	4	4

[a]ASP02, Bühler AG, Uzweil, Switzerland. [b]LCH A/S, Peter Bangs Vej 33, DK-2000 Frederiksberg. [c]J. Rettenmaier and Söhne GmbH, Rosenberg, Germany. [d]Supplying per kg diet: retinol 660 μg, cholecalciferol 12.5 μg, α-tocopherol 30 mg, menadione 11 mg, thiamin 1 mg, riboflavin 2 mg, D-pantothenicacid 5.5 mg, niacin 11 mg (available), biotin 27.5 μg, cyanocobalamin 11 μg, pyridoxine 1.65 mg, Fe 25 mg, Cu 10 mg, Zn 40 mg, Mn 13.9 mg, Co 0.15 mg, I 0.01 mg, Se 0.15 mg and maize $Ca_2(PO_4)_3$, K_2PO_3, NaCl, $CaCO_3$ as a carrier (SolivitMikro 106, Løvens Kemiske Fabrik, Vejen, Denmark).

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Table 2. Chemical Composition and Phenolic Acid Content of the Experimental Diets

	RWF	WGW	WAF
Chemical Composition (g/kg DM)			
DM (g/kg as is)	639	654	704
ash	37	43	41
protein (Nx6.25)	168	173	177
fat	37	43	58
carbohydrates	641	630	589
sugars	11	19	14
starch	513	506	470
total NSP[a]	117	105	105
cellulose	66	17	14
arabinoxylan	26	61	62
β-glucan	1	4	8
Klason lignin	10	22	23
total dietary fiber	125	124	125
Phenolic Acid (mg/kg DM)			
total phenolic acids	57 (11)[b]	327 (21)	550 (33)
ferulic acid	28 (2)	204 (8)	353 (20)
sinapic acid	21 (1)	97 (1)	164 (3)
p-coumaric acid	1 (1)	6 (b.d)	12 (1)
caffeic acid	bd[c]	bd	bd
p-hydroxybenzoic acid	4 (4)	5 (5)	5 (2)
protocatechuic acid	1 (1)	1 (1)	1 (1)
vanillic acid	2 (2)	11 (5)	11 (4)
syringic acid	bd	2 (1)	3 (2)
salicylic acid	bd	1 (bd)	1 (bd)

[a]Non-starch polysaccharides (NSP). [b]Values in brackets are free phenolic acids. [c]Below detection limit (bd).

Table 3. Intake of Feed and Phenolic Acids When Fed the Refined Wheat Flour (RWF), Whole Grain Wheat (WGW), or Wheat Aleurone (WAF) Diets

	RWF	WGW	WAF
Intake of Feed (g/d)			
feed	2333	2550	2185
DM	1543	1669	1539
dietary fiber	193	207	211
Intake of Phenolic Acids (mg/d)			
total phenolic acids	76	501	779
ferulic acid	37	312	500
sinapic acid	28	149	233
p-coumaric acid	1	10	17
caffeic acid	0	0	0
p-hydroxybenzoic acid	6	8	6
protocatechuic acid	1	2	2
vanillic acid	0	16	16
syringic acid	0	2	5
salicylic acid	1	1	1

was supplied. Fasting blood samples (30 min before the first daily meal) were collected from the portal vein and the mesenteric artery on days 4−7 (Monday−Thursday). Blood samples were collected in 9 mL Na-heparinized vacutainers, centrifuged, and plasma harvested and stored at −20 °C.

Proximate Analyses of the Breads. The dry matter content was measured by drying to a constant weight for approximately 20 h at 103 °C, and ash was analyzed by AOAC method.[14] Nitrogen was measured according to DUMAS,[15] and calculation of protein was performed using $N \times 6.25$. Fat was extracted by Bligh & Dyer[16] after hydrochloric acid hydrolysis. Sugars such as glucose, fructose, sucrose, and fructans were analyzed as described by Larsson & Bengtsson,[17] and starch and NSP were measured as described by Bach Knudsen.[18] Klason lignin was analyzed as the sulphuric acid-insoluble residue according to Theander and Åman.[19]

Extraction of Bound Phenolic Acids in Breads. To 0.1 g of finely milled bread was added 5 mL of 0.08 M Na phosphate buffer pH 6 and shaken for 30 s. Afterward, 5 mL of 4 M NaOH was added and the solution was shaken again for 30 s and placed in the tailored mixer for approximately 20 h at room temperature. After incubation, the solution was adjusted to pH 2 with HCl and placed in the freezer overnight and freeze-dried afterward. The freeze-dried samples were extracted using accelerated solvent extraction.

Accelerated Solvent Extraction (ASE). Free and bound phenolic acids were extracted using ASE. The samples were extracted on an ASE 350 system from Thermo Scientific Dionex (Hvidovre, Denmark) as described previously by Petersen et al.[20] Briefly, after inserting a cellulose filter, 5 g of glowed chemically inert Ottawa sand (particle size 20−30 mesh, Fisher Chemicals) was added to the 33 mL extraction cells. Approximately 0.1 g of pulverized flour or fine milled bread were weighed, sample was transferred to the extraction cell followed by 5 g of glowed chemically inert Ottawa sand. A filter was placed on top of the sample, and the extraction cell was filled with glowed glass balls. The cycles for the ASE extraction was as following: temperature 80 °C, preheat for 5 min, heat for 5 min, static extraction for 3 min, flush volume 80% of cell volume, purge with N_2 for 60 s, pressure 1500 psi, totally four cycles. Phenolic acids were eluted with 80% methanol in water containing 1% acetic acid (v/v). The extracts were collected in vials and stored at −20 °C until analysis. All extractions were performed in duplicate. The extracted samples were diluted 1:1 with water and subjected to LC-MS/MS analyses.

Recovery of Phenolic Acids after ASE. The recovery of nine phenolic acids was measured in the breads spiked with standard mix with the concentration of 2 μg/g. The recovery experiments were repeated six times and gave mean values for syringic acid 67.7 ± 0.49%, salicylic acid 109.67 ± 0.13%, vanillic acid 94.5 ± 0.30%, sinapic acid 58.9 ± 0.31%, protocatechuic acid 54.3 ± 0.43%, *p*-coumaric acid 76.9 ± 0.31%, ferulic acid 61.8 ± 0.59%, caffeic acid 72.8 ± 0.10%, and *p*-hydroxybenzoic acid 103.9 ± 0.28%.

Deconjugation of Phenolic Acid in Plasma. Hydrolysis of plasma samples were performed according to Penalvo et al.,[21] with minor modifications. The method is briefly as follows. An enzyme mix containing β-glucuronidase with β-glucuronidase activity of ≥140 U/mg and sulfatase type H-1 with sulfatase activity of ≥10 U/mg was used. Enzymes were dissolved in 0.1 M NaOAc buffer with pH 5. Plasma (0.5 mL) was incubated at 37 °C for 2 h with 2 mL of enzyme mix containing 1.3 U/mL sulfatase and 0.13 U/mL β-glucuronidase.

Solid Phase Extraction (SPE). SPE of phenolic acids was performed on OASIS HBL column from Waters (Milford, MA, USA) using an extraction chamber equipped with a pump. Before the extraction of phenolic acids, 0.5 mL of plasma was added 4 mL of 0.2% of formic acids in water. Hydrolyzed plasma samples were centrifuged before the extraction on OASIS HBL columns. The OASIS HBL columns were equilibrated with 2 mL of methanol and 2 mL of water. After addition of the sample, the column was washed with 5% methanol and then sucked to dryness. The elution of phenolic acids was performed twice with 1 mL of 80% methanol containing 1% acetic acid. The elution solution was added to the column, and the valve was closed. After 2.5 min, the valve was opened and the eluent was collected. The procedure was repeated one more time. The extracted samples were diluted 1:1 with water and subjected to LC-MS/MS analyses.

Recovery of Phenolic Acids in Plasma. Recovery of phenolic acids were determined by spiking 0.5 mL of blank plasma (plasma of pig after consumption of refined wheat) with 400 ng/mL standard stock solution to obtain the concentration of 20 ng/mL and then subjecting the samples to the described SPE procedure. The recovery experiments were repeated six times and gave mean values for syringic acid 69.9 ± 6.2%, salicylic acid 82.5 ± 8.1%, vanillic acid 91.7 ± 5.1%, sinapic acid 58.9 ± 12.6%, protocatechuic acid 56.4 ± 2.3%, *p*-coumaric acid 94.4 ± 10.6%, ferulic acid 80.5 ± 2.0%, caffeic acid 81.2 ± 10.5%, and *p*-hydroxybenzoic acid 84.9 ± 13.1%. The influence of

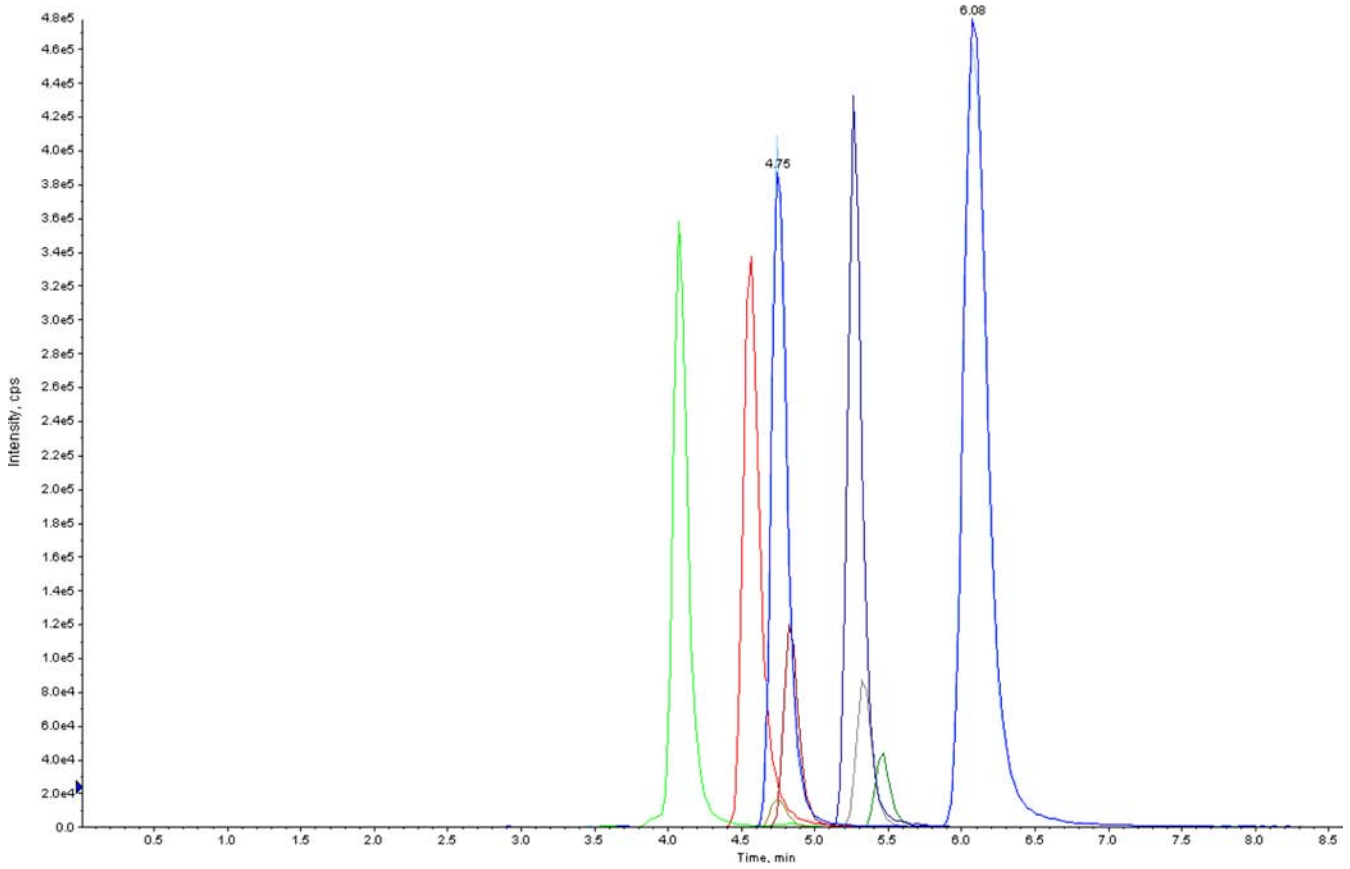

Figure 2. Representative chromatogram of phenolic acids standards in the concentration 100 ng/mL. The retention time of salicylic acid and *p*-hydroxybenzoic acid was 6.08 and 4.75 min, respectively.

Table 4. Compound-Dependent LC MS/MS Parameters, Declustering Potential (DP), Entrance Potential (EP), Cell Entrance Potential (CEP), Collision Energy (CE), and Cell Exit Potential (CEP)

	Q1 mass	Q3 mass	DP (V)	EP (V)	CEP (V)	CE (V)	CEP (V)
ferulic acid	193.0	149.0	−22	−2	−18.80	−16	−1.0
sinapic acid	223.1	164.0	−24	−3	−19.94	−22	−1.0
p-coumaric acid	163.2	119.2	−22	−5	−17.69	−20	−2.0
caffeic acid	179.0	135.0	−22	−4	−18.28	−22	−3.0
p-hydroxybenzoic acid	137.2	92.8	−24	−4	−16.73	−23	−1.5
protocatechuic acid	153.1	109.0	−28	−10	−17.32	−22	−2.0
vanillic acid	167.2	152.0	−24	−2	−17.84	−20	−3.0
syringic acid	197.2	121.2	−22	−2	−18.95	−22	−1.0
salicylic acid	137.2	92.8	−24	−4	−16.73	−23	−1.5

hydrolyses on the recovery of phenolic acids was also investigated by spiking the blank plasma samples prior to enzymatic hydrolysis and subjecting the samples to SPE. The values for recovery of phenolic acids after the hydrolysis were similar to the values without the enzymatic hydrolysis.

Calibration Curves. Calibration curves were prepared from standards with concentration ranging from 0.195 to 100 ng/mL. The calibration curves were linear with the mean r^2 of 0.998 for nine phenolic acid standards Figure 1.

LC MS/MS Analysis. Measurements were performed on LC-MS system from Agilent Technologies (UK, Workinham). Chromatographic separation was performed on HPLC Agilent 1200 equipped with Polar-RP column (Polar-RP 80 Å 250 mm × 2.0 mm) from Phenomenex (Torrance, CA, USA) and with column oven set to 30 °C. The flow of the system was 0.200 μL/min. Solvent A consisted of 7% acetonitrile containing 20 mM acetic acid and solvent B consisted of 78% acetonitrile containing 20 mM acetic acid. The gradient started at 16% of solvent B and continued to 30% of solvent B during 17 min, and then it continued from 30% to 50% of solvent B during 8 min and from 50% to 70% of solvent B during 2 min. It was kept isocratically for 3 min at 70% of solvent B. The column was equilibrated for 5 min in the beginning of each injection, and 20 μL of sample was injected each time. The chromatographic separation of phenolic acids is shown in Figure 2. Because salicylic acid and *p*-hydroxybenzoic acid have similar mass, they had different retention during chromatographic separation, salicylic acid (6.08 min), and *p*-hydroxybenzoic acid (4.75 min). HPLC system was connected to a model 3200 linear ion trap quadrupole mass spectrometer (Agilent Technologies, UK, Workinham). The eluent was introduced into the mass spectrometer by a TurboIonSpray probe operating at 475 °C, with ion spray voltage set in negative mode to −4500 V. Both the nebulizer gas pressure (GS1) and turbo heater gas (GS2) were set to 60 psi. The curtain gas flow was set to 12 L/min. The MS data were collected in MRM scan mode with compound-dependent parameters listed in Table 4.

Calculations and Statistics. The net absorption of phenolic acids into the portal vein was calculated from the porto-arterial (AV)

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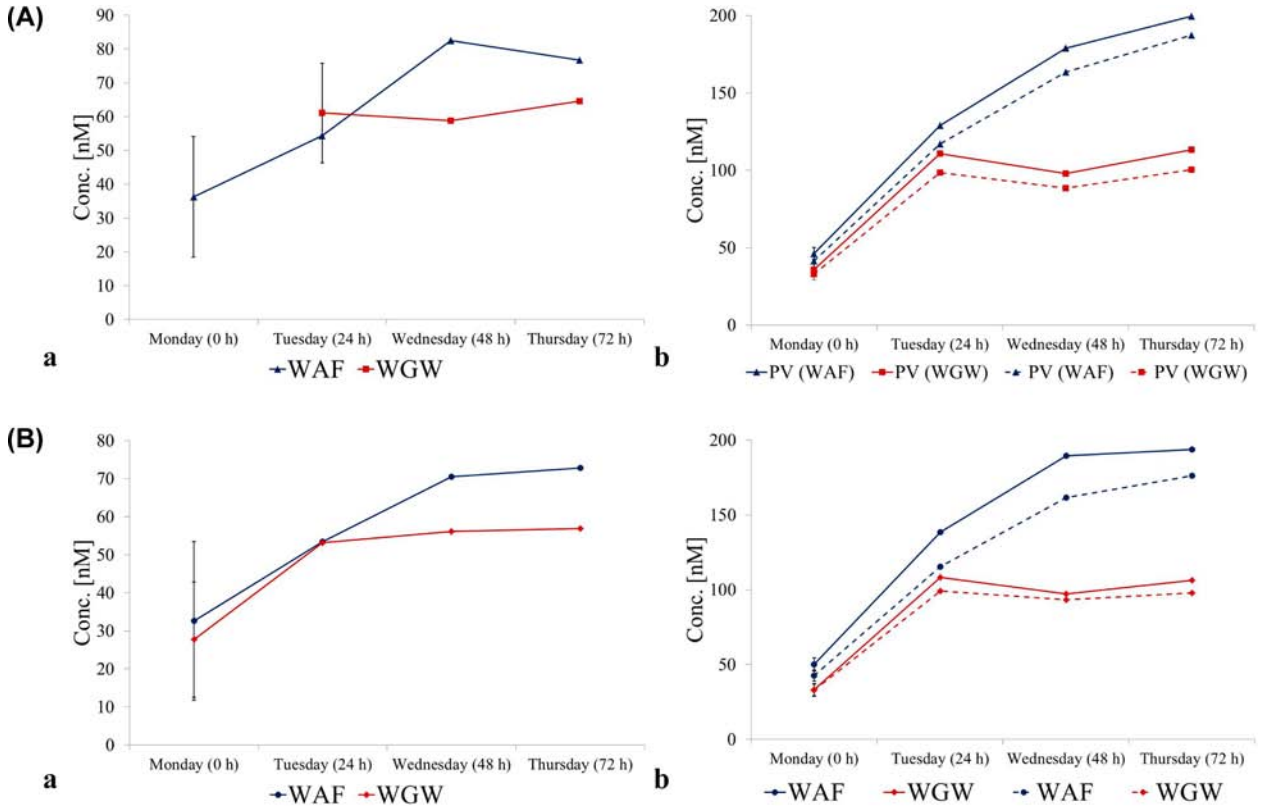

Figure 3. (A) Concentration of cinnamic acid derivatives in the portal vein, ferulic acid (a), caffeic acid (b) in the plasma of pigs after overnight fermentation, taken as a fasting value Monday (0 h), Tuesday (24 h), Wednesday (48 h), and Thursday (72 h). Monday is represented by the wash-out diet (RWF), the total acid concentration (solid line), and the free acid concentration (dash line). Wheat aleurone-rich flour (WAF) diet is represented with (blue) and whole-wheat grain (WGW) diet is represented with (red). Values are least-squares means with standard error (SEM), n = 6. Standard error is illustrated Monday using error bars. In the case of ferulic acid, a standard error for portal vein (WGW) is given on Tuesday due to missing values. (B) Concentration of cinnamic acid derivatives in the mesenteric artery, ferulic acid (a), caffeic acid (b) in the plasma of pigs after overnight fermentation, taken as a fasting value Monday (0 h), Tuesday (24 h), Wednesday (48 h), and Thursday (72 h). Monday is represented by the wash-out diet (RWF), the total acid concentration (solid line), and the free acid concentration (dash line). Wheat aleurone-rich flour (WAF) diet is represented with (blue), and whole-wheat grain (WGW) diet is represented with (red). Values are least-squares means with standard error (SEM), n = 6. Standard error is illustrated Monday using error bars.

differences and the portal flow measurements according to Rérat et al.[22] using the following equation:

$$q = (C_p - C_a)\cdot F(dt)$$

$$Q = \sum_{t0}^{tn} q$$

where q is the amount of phenolic acids absorbed within the time period dt, C_p is the concentration (nM) of phenolic acids in the portal vein, C_a is the concentration (nM) of phenolic acids in the mesenteric artery, F is blood flow (L/min) in the portal vein, and Q is the amount absorbed (nmol/h) from $t0$ to tn.

Statistical analyses were performed in SAS (version 9.2; SAS Institute Inc., Cary, NC, USA). The effects of diet and time on the concentration of phenolic acids in plasma were examined by PROC MIXED repeated measurements.[23]

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (U)_{ikl} + \varepsilon_{ijkl}$$

Where Y_{ijkl} is the dependent variable, α_i denotes the effect of diet (i = WGW, WAF), β_j is the effect of time (j = Mon, Tue, Wed, Thu), γ_k is the effect of week (k = 1, 2), $(\alpha\beta)_{ij}$ is the interaction between diet and time, $(U)_{ikl}$ is the term accounting for repeated measurements being performed on the same pig within week, whereas ε_{ijkl} describes the random error. The level of significance was set at $P < 0.05$. The concentration results were reported as the least-squares means (LSMeans) with standard errors of the means (SEM).

RESULTS AND DISCUSSION

The concentration of total phenolic acids varied substantially between the three diets, from 57 mg/kg DM in RWF bread to 550 mg/kg DM in the WAF bread and the WGW bread being in between with 327 mg/kg DM, Table 2. It was expected that WGW and WAF breads would have a higher content of phenolic acids than RWF bread because the bran and aleurone layer account for the highest concentration of phenolic acids in the grain.[1] The higher concentration of phenolic acids in WAF than WGW is a reflection of a higher concentration of phenolic acids as a proportion of the DF in the aleurone cells relative to the whole grain. It was also clear that the main phenolic acids detected in the breads were ferulic acid and sinapic acid and that these phenolics were mainly present as bound phenolic acids. Similar results were achieved in other studies with wheat, where bound ferulic acid was the main phenolic acid found in wheat bran and bread enriched with aleurone.[24,8]

Absorption. The bioavailability of phenolic acid in the body depends to a large extent on the bioaccessibility of these compounds in the food matrix with free phenolic acids more easily available for absorption than bound phenolic acids.[10] In the current study, the proportion of free phenolic acids account

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for only approximately 6% in the WAF and WGW diets, whereas it represented 19% of total phenolics in the RWF diet, Table 2. The majority of phenolic acids, therefore, have to be released by microbial esterases in the cecum and colon along with the fermentation of carbohydrates. Theil et al.[25] reported that the net absorption of short-chain fatty acids, the products deriving from the anaerobic fermentation of carbohydrates in the large intestine, was 33 and 39 mmol/h for WGW and WAF diets, respectively. For comparison, the absorption of ferulic acid varied from 240 nmol/h on Monday after consumption of the RWF diet to 297 and 458 nmol/h after consumption of WGW and WAF diets, respectively. These data correspond to a bioavailability of only 0.4−2.9% of ingested phenolic acids. Similar low bioavailability have been reported in studies with humans and rats.[5,26,27]

In spite of an active fermentation in the large intestine at the time of sampling, we were not able to identify a significant AV difference with any of the diets ($P > 0.05$), most likely because the load of phenolic acids was not sufficient. For instance, the daily supply of DF was approximately 200 g/d, whereas phenolic acids were in the range of 37−500 mg/d, Table 3, which may not be sufficient to create a significant concentration difference between the artery and the vein.

Cinnamic Acid Derivatives. The absorption profiles of the cinnamic acid derivatives, ferulic acid and caffeic acid, differed depending on the diet consumed in both portal vein and mesenteric artery, Figure 3A,B. When consuming the WAF diet, the absorption profile over time reflected the continuous increase until the concentration became nearly constant after 72 h (Thursday), whereas the concentration was already constant after 24 h (Tuesday) when consuming the WGW diet. This difference in the shape of the curves can be explained by the higher digestibility of the DF in WAF compared to WGW diet, which makes phenolic acids more accessible.[28,29] However, neither a time nor a diet effect was identified to be significant for ferulic acid, Table 5. Opposite to ferulic acid, diet and diet × time interaction affected plasma concentration of caffeic acid in both mesenteric artery and portal vein, Table 5. Moreover, in contrast to ferulic acid, the concentration of caffeic acid was negligible in the diet, Table 2, but in plasma the concentration of caffeic acid reached a maximum of 200 nM, more than the double of ferulic acid of 82 nM. Increase in the concentration from Monday to Thursday was observed for both caffeic acid and ferulic acid, but because no caffeic acid was detected in the breads, we speculated a metabolic link between these two compounds. Moreover, if these phenolic acids are metabolically related, the higher amount of ferulic acid in the WAF bread than WGW bread can explain the dietary concentration difference of caffeic acid found in plasma. It is known that the colon is the main site for the metabolic conversion of phenolic acids, e.g., ferulic acid can undergo reduction, demethylation, and dehydroxylation to form a majority of related phenolic acid metabolites.[30,11] Therefore, a proportion of ferulic acid may be metabolized to caffeic acid.

Table 5. *P* Values of Diet, Time, and Diet and Time Interactions for Cinnamic Acids Derivatives for Portal Vein (PV) and Mesenteric Artery (MA)[a]

		ferulic acid			caffeic acid		
		P diet	*P* time	*P* diet × time	*P* diet	*P* time	*P* diet × time
PV	total	0.35	0.069	0.32	<0.001	<0.001	0.002
	free				<0.001	<0.001	0.001
MA	total	0.38	0.086	0.81	<0.001	<0.001	0.008
	free				<0.001	<0.001	0.004

[a]*P* values were calculated using PROC MIXED repeated measurements.

Ferulic acid was only detected in the plasma as conjugates, whereas the majority of caffeic acid was present as non-conjugated. That is in agreement with in vivo and in situ studies, which reported that the most abundant form of ferulic acid in plasma and bile is glucurono- and sulfoconjugated.[26,5,13] It has also been reported that conjugation of phenolic compounds can occur in both enterocytes and hepatocytes.[9,31] Conjugation increases the hydrophilicity of these compounds, which can facilitate their biliary and urinary excretion, a feature of metabolic detoxification known for many phenolic compounds.[9] The low concentration of conjugated ferulic acid (27−82 nM) indicates that the enterohepatic ciculation did not allow the plasma concentration of this compound to remain high. It has also been reported that the half-life of caffeic acid in the plasma is longer than that of ferulic acid,[32] which is in agreement with our result indicating that free caffeic acid is more stable than free ferulic acid in the plasma of pigs.

Another cinnamic acid derivative present at high level in the diets was sinapic acid, Table 2. However, this phenolic acid was not detected in the plasma. This may be due to different factors such as its poor bioaccessability or lack of stability in plasma but also total metabolic conversion to other phenolic acids and/or rapid elimination to the urine. We cannot completely exclude that this compound could be present in the plasma at concentrations below the detection limit of our method (0.195 ng/mL). The concentration of *p*-coumaric acid in the diets was low compared to ferulic acid and sinapic acid, but the concentration in plasma of this metabolite varied largely among the pigs, from 0 nM to approximately 200 nM. The recovery of *p*-coumaric acid in the extraction procedure was high, 94.4%, indicating that the main reason for large variation in the concentration of this phenolic acid is more likely related to the diversity of the micro flora and/or hepatic conversion rather than the analytical procedure.

Benzoic Acid Derivatives. The concentration of salicylic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, and protocatechuic acid in the WGW, WAF, and RWF breads was all low or negligible compared to ferulic acid and sinapic acid, Table 2. In the plasma, the concentration of salicylic acid, vanillic acid, and protocatechuic acid after consumption of WAF and WGW diet was similarly low, ranging between 6 and 28 nM, Figure 4a,c,d. However, time and diet affected plasma concentration of salicylic acid, Table 6. In general, vanillic acid and protocatechuic acid were not affected either by diet or time. The low concentration of the free and conjugated salicylic acid, vanillic acid, and protocatechuic acid and the absence of syringic acid in the plasma can be related to their low abundance in the diets and/or to the rapid metabolism of these phenolics to the other phenolic acids and elimination to the bile and urine. The capacity to catalyze demethylation and dehydroxylation reactions of phenolic acid such as vanillic acid and protocatechuic acid is restricted to the colonic microbiota.[11]

In contrast to the other benzoic acid derivatives, the plasma concentration of *p*-hydroxybenzoic acid was high, and much higher than the concentration of any other phenolic acid

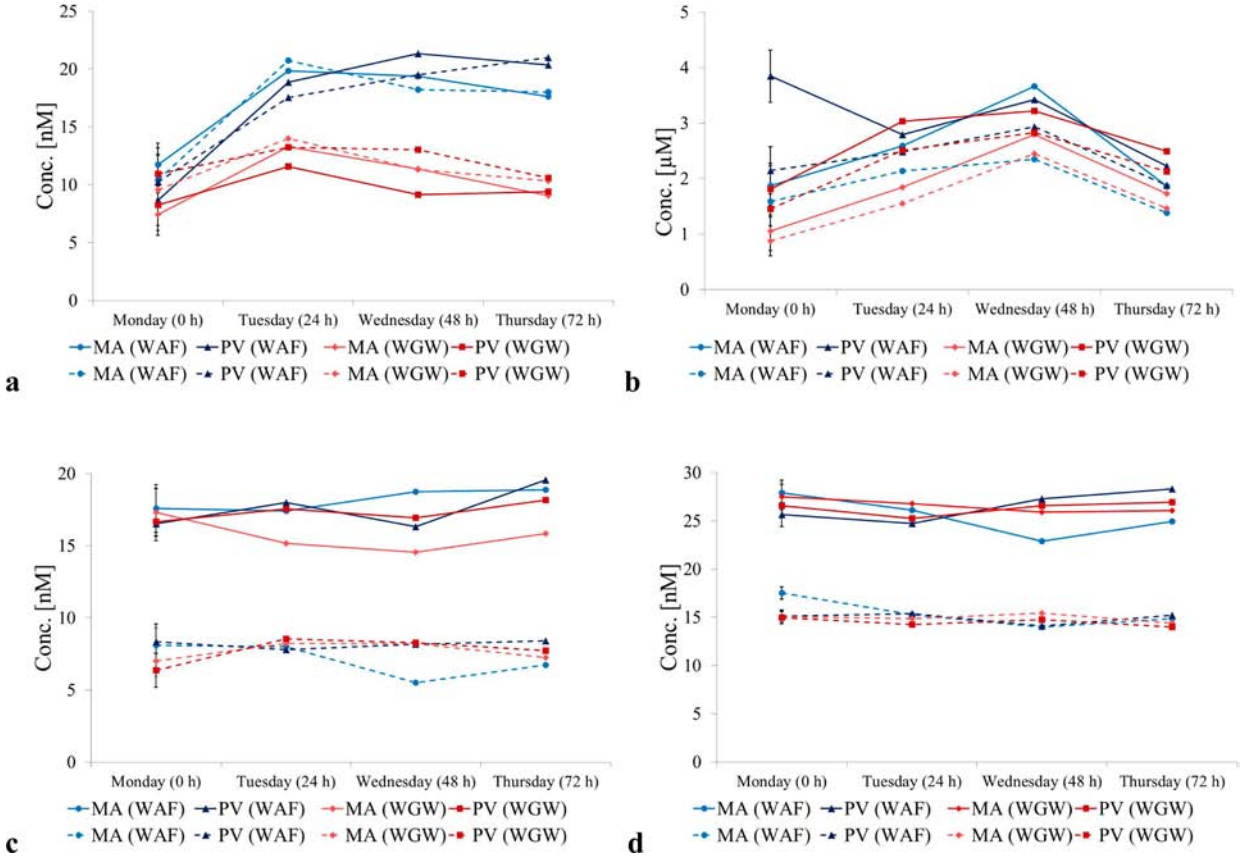

Figure 4. Concentration of benzoic acid derivatives, salicylic acid (a), *p*-hydroxybenzoic acid (b), vanillic acid (c), and protocatechuic acid (d). in the plasma of pigs after overnight fermentation, taken as a fasting value Monday, Tuesday, Wednesday, and Thursday. Monday is represented by the wash-out diet (RWF), the total acid concentration (solid line), and the free acid concentration (dash line). Wheat aleurone-rich flour (WAF) diet is represented with (blue), and whole-wheat grain (WGW) diet is represented with (red). Mesenteric artery (MA) is represented with light-red and portal vein (PV) with dark-blue. Values are least-squares means with standard error (SEM), $n = 6$. Standard error is illustrated Monday using error bars.

Table 6. *P* Values of Diet, Time, and Diet and Time Interactions for Benzoic Acids Derivatives for Portal Vein (PV) and Mesenteric Artery (MA)[a]

		salicylic acid			*p*-hydroxybenzoic acid			vanillic acid			protocatechuic acid		
		P diet	*P* time	*P* diet × time	*P* diet	*P* time	*P* diet × time	*P* diet	*P* time	*P* diet × time	*P* diet	*P* time	*P* diet × time
PV	total	0.001	0.011	0.076	0.44	0.47	0.20	0.73	0.05	0.71	0.86	0.09	0.68
	free	0.021	0.017	0.15	0.80	0.20	0.80	0.64	0.84	0.62	0.41	0.78	0.39
MA	total	0.0001	0.001	0.47	0.15	0.009	0.86	0.07	0.82	0.58	0.29	0.03	0.46
	free	0.010	0.017	0.39	0.38	0.013	0.54	0.45	0.42	0.18	0.36	0.015	0.053

[a] *P* values were calculated using PROC MIXED repeated measurements.

measured in this study, 10-fold greater than the concentration of caffeic acid and ferulic acid and 100-fold greater than the concentration of salicylic acid, vanillic acid, and protocatechuic acid. Because the diets were not the source of *p*-hydroxybenzoic acid, Table 2, the presence of this compound in plasma may be only due to the metabolism of other phenolic acids. For instance, hydroxycinnamic acids derivatives such as ferulic acid, *p*-coumaric acid, and caffeic acid can undergo β-oxidation in the colon or in the liver and be converted to hydroxybenzoic acid derivatives.[9] Although β-oxidation may take place in the colon, the main site for benzoic acid, 3-hydroxybenzoic acid and *p*-hydroxybenzoic acid formation, is the liver.[33] Thus, *p*-hydroxybenzoic acid is a phenolic acid intermediate formed from other phenolic acid and with no specific link to the experimental diets, Table 6.

Health Effects. The metabolic fate of plant phenolic acids in the body is crucial for understanding their biological function in vivo such as antioxidative activity against LDL oxidation. Their bioactivity depends on their bioavailability, and it is therefore also important to consider the concentration of the phenolic acids in the plasma. It has been shown in vitro that hydroxycinnamic acids, such as ferulic acid and caffeic acid, possess antioxidative activity against LDL oxidation at micromolar concentration (1−100 μM).[5] The antioxidative activity decreased in the order: caffeic acid > sinapic acid > ferulic acid > *p*-coumaric acid.[7] In the present study, however, the maximum concentrations of caffeic acid and ferulic acid

reached 200 and 87 nM, respectively, and no sinapic acid was detected. Thus, the concentration of caffeic acid and ferulic acid was considerably lower than the concentration required to induce the response in vitro. Too low concentration of phenolic acids in plasma was also one of the explanations for the lack of effect of rye bran on LDL oxidation, in the study of Harder et al.[6] Conjugation of phenolic acids is another factor to consider. Ohta et al.[34] reported that conjugated ferulic acid possessed higher antioxidative activity against LDL oxidation than nonconjugated ferulic acid in vitro. Of the measured phenolic acids, *p*-hydroxybenzoic acid was the only phenolic acid with plasma concentration high enough to potentially affect the LDL oxidation. However, it was reported that hydroxybenzoic acid derivatives, such as *p*-hydroxybenzoic acid, have lower antioxidative activity than hydroxycinnamic acids.[10] Thus, the levels of plant phenolic acids in plasma of pigs measured in this study was much lower than the concentration needed to induce the inhibition when measured in vitro. One may therefore suggest that the protective effect of whole grain cereals against cardiovascular diseases may not only come from the action of plant phenolic acids but as a result of a synergetic action of different phytochemicals.

In summary, the concentration of the nine phenolic acids of wheat in the plasma of fasted pigs after overnight fermentation was measured to be low. In spite of an active fermentation in the large intestine at the time of sampling, we were not able to identify any significant AV difference with any of the diets, probably because the load of phenolic acids was not sufficient to create a concentration difference between the artery and the vein. The plasma concentration of phenolic acids therefore represented the net balance between their transformation in the colon and elimination to the urine and feces. However, the concentration of ferulic acid in the plasma increased when feeding the diets with higher content of ferulic acid. More interestingly the concentration of caffeic acid was higher than that of ferulic acid even though the concentration of caffeic acid in the diets was negligible. That indicated that caffeic acid was metabolized endogenously and that the precursor to caffeic acid might have been ferulic acid. It was also clear from the results that free caffeic acid was more stable than free ferulic acid because only conjugated ferulic acid was detected in the plasma. The concentration of salicylic acid, vanillic acid, and protocatechuic acid in the plasma was low with limited time and diet effects, the exception being *p*-hydroxybenzoic acid, the only phenolic acid with plasma concentration high enough to potentially affect LDL oxidation. In conclusion, we found big differences in the plasma concentrations and the absorption profiles between the cinnamic and benzoic acid derivatives, which most likely were related to extensive interconversion in the colon.

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Notes

The authors declare no competing financial interest.

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