JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Absorption of Hydroxycinnamates in Humans after High-Bran Cereal Consumption

SANDRA M. KERN,[†,§] RICHARD N. BENNETT,[†] FRED A. MELLON,[†] PAUL ANTHONY KROON,[†] AND MARIA-TERESA GARCIA-CONESA*,[†]

Institute of Food Research, Nutrition Division, Norwich Research Park, Colney, Norwich NR4 7UA, United Kingdom, and Institute of Phytopathology, Department of Applied Biochemistry, Technical University of Munich, 85350 Freising-Weihenstephan, Germany

Hydroxycinnamic acids are a group of phenolic compounds that exhibit a wide range of in vitro chemoprotective and antioxidant properties. Cereals containing a high proportion of the bran layers are rich in ester-linked hydroxycinnamic acids, such as ferulic and diferulic acids. The present work investigated the absorption in humans of hydroxycinnamic acids from high-bran breakfast cereal (wheat). Plasma and urine samples from six volunteers were collected before and after cereal consumption and analyzed for total hydroxycinnamic acids content after β-glucuronidase/sulfatase treatment both by HPLC-DAD and by LC-MS (SIM monitoring). High-bran cereal administration resulted in increased plasma ferulic and sinapic acid concentrations (maximum levels detected of ~200 and ~40 nM, respectively) with absorption peaks between 1 and 3 h. Increases of ~4-fold in ferulic acid and ~5-fold in feruloylglycine were detected in 24-h urine after consumption of the cereal. Most of the ferulic acid detected in urine and plasma was present as conjugates (feruloylglycine and/or glucuronides). Diferulic acids were undetectable. The data show that ferulic and sinapic acids are taken up in humans from dietary high bran wheat but that absorption is limited and may originate only from the free and soluble portions present in the cereal.

KEYWORDS: Hydroxycinnamic acids; diferulic acids; bran; cereals; absorption; humans

INTRODUCTION

Epidemiological studies suggest a link between the consumption of whole grain products and the prevention of chronic diseases such as coronary heart disease (*1*) and certain forms of cancer (*2*). The exact mechanisms linking whole grains to disease prevention are not known but may include gastrointestinal effects and antioxidant protection, which can be attributed to various nutrients of the whole grain cereals (bran plus germ) such as fiber, vitamins, minerals, or phenolic compounds (*3*). Cereal bran contains significant quantities of phenolic compounds, mainly hydroxycinnamic acids (**Figure 1**), that are present mainly ester-linked to polymers in the plant cell wall (*4*, *5*). Thus, regular consumption of cereal bran and bran-enriched products would result in the ingestion of significant amounts of these plant components, in particular, ester-linked ferulic and diferulic acids (**Figure 2**). For example, an intake of 20 g of wheat bran would provide approximately 100 mg of total ferulate, 2 mg of sinapate, 3 mg of *p*-coumarate, and 20 mg of diferulates (*4*).

Hydroxycinnamic acids and diferulic acids possess significant antioxidant properties and chemoprotective effects as shown by multiple in vitro assays and animal studies (*6*−*11*), and it is suspected that they may contribute to some of the beneficial effects derived from the consumption of cereal bran. However,

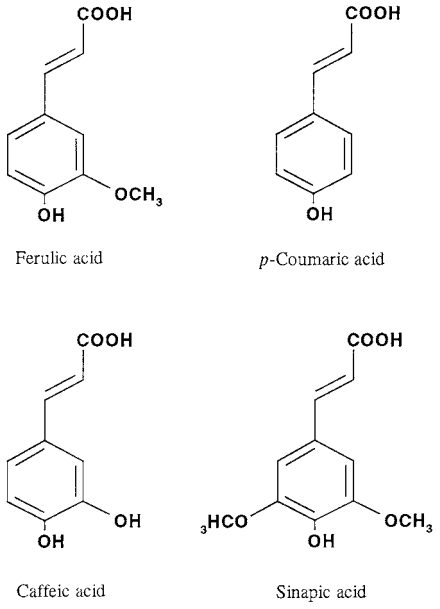

Figure 1. Chemical structures of some dietary hydroxycinnamates.

* Corresponding author [telephone +44 (0)1603 255051; fax +44 (0)-1603 255038; e-mail maria.conesa@bbsrc.ac.uk].
† Institute of Food Research.
§ Technical University of Munich.

10.1021/jf0302299 CCC: $25.00
Published on Web 08/20/2003

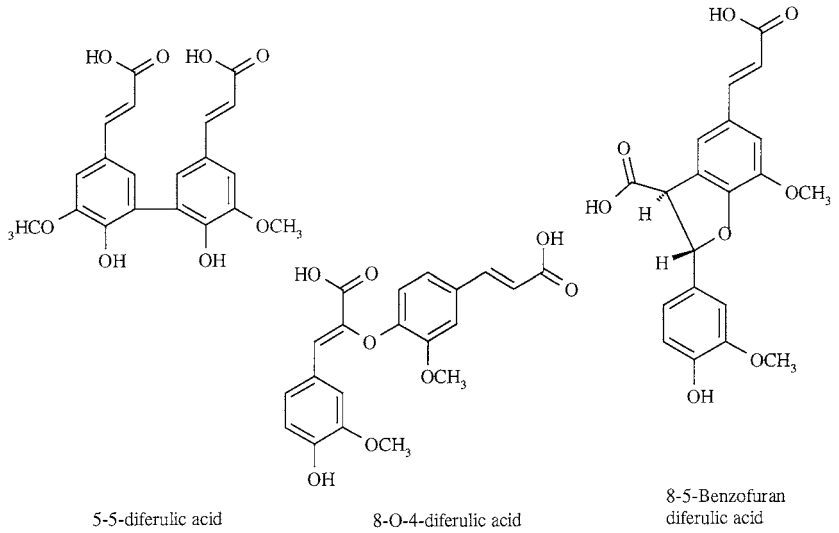

Figure 2. Structures of the main diferulates present in cereal bran.

to establish their possible in vivo effects, it is essential to determine whether these compounds are absorbed from the diet and which forms are found circulating in plasma and may reach specific tissues. Esterified hydroxycinnamates and diferulates cannot be absorbed as part of complex molecules, but esterases present in the small intestine (*12*, *13*) and in the colon of humans (*14*, *15*) are able to cleave the ester bonds and may release a proportion of the hydroxycinnamic acids and diferulic acids free into the lumen, which can then be absorbed. It has been shown in humans that some hydroxycinnamic acids (mostly ferulic acid and caffeic acid) can be absorbed from various sources such as tomatoes (*16*), beer (*17*), bark extract (*18*), herbal extract (*19*), and prunes (*20*) and that the main circulating metabolites were glucuronide and sulfate conjugates (*17*−*19*, *22*).

Our knowledge about hydroxycinnamate absorption from the diet and their metabolism in humans is incomplete, one major unresolved point consisting of the absorption of the bound compounds (ester-linked to complex polymers of the plant cell wall). The aim of this study was to investigate the bioavailability of hydroxycinnamates and diferulates from high-bran wheat (rich in the ester-linked forms of these compounds) and to determine if some of the covalently bound forms of these phenolics are absorbed.

MATERIALS AND METHODS

Chemicals. *trans*-Ferulic acid (FA), *trans*-*p*-coumaric acid (*p*-CA), *trans*-sinapic acid (SA), vanillin, ascorbic acid, pure β-glucuronidase from *Escherichia coli* (EC 3.2.1.31, type IX-A), and sulfatase from *Helix pomatia* (EC 3.1.6.1, type H-1) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). *o*-Coumaric acid was obtained from Avocado Research Chemicals Ltd. (Heysham, Lancashire, U.K.). The free diferulic acids (diFA) 8-5-diFA (open ring), 5-5-diFA, 8-*O*-4-diFA, and 8-5-benzofuran-diFA were prepared as described elsewhere (*12*). All other chemicals were of analytical or HPLC grade purity. Ultrapure water (18 Ω) was used throughout the study.

Synthesis of Feruloylglycine. The glycine conjugate of ferulic acid was prepared using a method to synthesize glycine-conjugated bile acids (*21*) as follows: 0.25 mmol of ferulic acid was dissolved in 1.0 mL of dry dimethylformamide and 0.5 mmol of glycine methyl ester hydrochloride, 0.3 mmol of diethylcyanophosphonate and 0.4 mL of triethylamine were added successively, and the mixture was incubated for 20 h at room temperature. The product of the reaction was extracted with ethyl acetate (3× vol), and the dried residue was refluxed in 5 mL of 5% methanolic KOH (1 M) for 30 min at 100 °C. The methanolic solution was then applied to a preconditioned C-18 cartridge (Strata X, Phenomenex), and the unbound fraction was collected, acidified to pH 4.0 with HCl, and extracted with ethyl acetate (3× vol). The pooled organic phases were dried by rotary evaporation to obtain the solid glycine conjugate as free acid. The final product was analyzed by HPLC-DAD, and the percentage of unreacted ferulic acid was <5%. The molecular mass of the conjugate was confirmed by LC-MS using ESI+ mode ($[M + H]^+ = 252$).

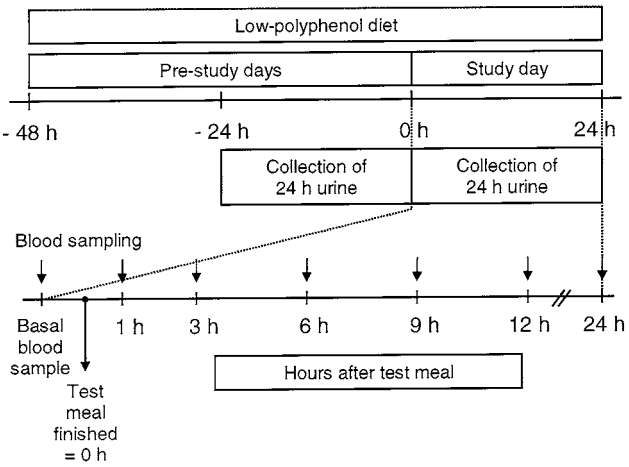

Figure 3. Scheme showing the study design.

Study Design. This study was approved by the Norwich District Research Ethics Committee and was performed under their guidelines. Six healthy volunteers, three males and three females (mean age = 32, range = 26−39; mean body mass index, 25.7 kg/m^2, range = 21.7−30.3), were recruited from the Norwich Research Park by advertisement. Volunteers were excluded from the study if they were suffering from diagnosed acute or chronic illness or taking medication, giving blood as a donor within a month (before or after) the study, outside the reference range of blood parameters, known to have a history of gastrointestinal disease, exhibiting altered gut behavior (constipation or diarrhea), pregnant, vegetarian, vegan, or known to have a specific food allergy to all bran or cereal bran. The six volunteers underwent a 2 day low polyphenol diet prior to the study day. The diet specifically avoided bran cereals, whole grain products, seeds and nuts, all fruits and vegetables, herbs and spices, and other products such as ready meals as well as specific beverages (black, green, or herbal tea; coffee; fruit juice; red wine; cider; or beer). Volunteers were allowed to eat peeled potatoes, white bread, white pasta, white rice, meat, fish, eggs, cheese, unflavored yogurt, salt, and pepper. Water, semiskimmed, or skimmed milk and white wine were the only beverages allowed. The study protocol and the collection of samples are depicted in **Figure 3**. On the study day and immediately before eating the test meal a cannula was inserted into an appropriate vein and a sample of blood (30 mL)

was taken as a baseline control. Volunteers were then asked to consume 100 g of a commercial breakfast cereal (85% wheat bran) with skimmed milk. The time the volunteer finished his/her meal was recorded as $t = 0$ (maximum = 20 min). Blood samples (30 mL each) were taken by cannulation at 1, 3, 6, 9, and 12 h after the test meal. Low-polyphenol meals and water were served during the study day. Volunteers were asked to collect urine for 24 h on the day prior to the study day and throughout the study day. The following morning the volunteers returned for giving a 24-h blood sample. The venous blood samples were immediately centrifuged at 1500*g* for 10 min in 10 mL lithium−heparin tubes (Sarstedt Ltd., Leicester, U.K.); plasma was collected, placed on dry ice, and stored at −20 °C until analysis. The 24-h urine samples were collected in plastic bottles containing 2 g of ascorbic acid and were also stored at −20 °C.

Water Extractable Hydroxycinnamates in Cereals. A water soluble fraction containing free hydroxycinnamic acids and low molecular weight feruloylated material was prepared by extracting the finely ground high-bran cereal (5.0 g) with 20 mL of water for 30 min at 50 °C with constant agitation (200 rpm) under nitrogen in the dark. The solubilized fraction was centrifuged at 13845*g* for 10 min. The remaining pellet was re-extracted once more, and the supernatants were combined (final volume = 33 mL). A 10 mL aliquot of the resulting supernatant was spiked with 4 μg of *o*-coumaric acid as internal standard, acidified to pH <2.0 with HCl, and extracted with ethyl acetate (3× vol). The combined organic phases were dried by rotary evaporation at 40 °C and dissolved in 800 μL of 50% methanol, followed by centrifugation at 15000*g* for 5 min and filtering (0.2 μm) prior to analysis of free hydroxycinnamic acids by HPLC-DAD.

Total Esterified Hydroxycinnamates in Cereals. Total esterified hydroxycinnamates in the cereal and in the water-soluble fraction were determined after alkaline hydrolysis following a protocol described previously (*12*). Samples (200 mg of ground cereal or 10 mL of the water-soluble fraction) were incubated with NaOH (1 M final concentration) for 18 h at room temperature under N_2 and in the absence of light. *o*-Coumaric acid (4 μg) was added as internal standard, and the mixture was acidified to pH <2.0 with HCl and extracted with ethyl acetate (3× vol). The combined organic phases were dried by rotary evaporation at 40 °C and dissolved in 800 μL of 50% methanol, followed by centrifugation at 15000*g* for 5 min and filtering (0.2 μm) prior to analysis of total hydroxycinnamic acids and diferulic acids by HPLC-DAD.

Plasma Sample Preparation. Plasma samples (7 mL) were spiked with 0.5 μg of *o*-coumaric acid as internal standard and processed using a slightly modified version of a method described previously (*12*). Methanol (20 mL) was added to the plasma sample, and the mixture was vortexed for 30 s and centrifuged at 1000*g* for 10 min at 4 °C. The pellet was washed twice with 20 mL of methanol and centrifuged for 10 min at 4 °C at 2000*g* and 2500*g*, respectively. The pooled methanol phases were dried by rotary evaporation at 40 °C and dissolved in 10 mL of PBS buffer. After acidification (pH 6.5, 0.5 M HCl), samples were incubated with β-glucuronidase (7.5×10^4 units/L) and sulfatase (1.5×10^4 units/L) for 1 h at 37 °C. This mixture was further acidified with HCl to pH <2 and then extracted with ethyl acetate (3× vol), and the organic phase was rotary evaporated to dryness. The residue was taken up in 600 μL of 50% methanol, centrifuged for 5 min at 15000*g*, and filtered through a 0.2 μm syringe filter prior to analysis by LC-MS and HPLC-DAD.

Urine Sample Preparation. From the 24-h urine samples an aliquot of 40 mL was mixed with 4 mL of PBS buffer (×10) and the pH was adjusted to 6.5 with 1 M NaOH. The sample solution was incubated with β-glucuronidase (7.5×10^4 units/L) and sulfatase (1.5×10^4 units/L) for 1 h at 37 °C. After the addition of *o*-coumaric acid (13 μg) as internal standard, the pH was adjusted to 2.0 with HCl, and the sample mixture was applied onto a preconditioned C-18 SPE column (TechElut, 1000 mg/6 mL) with a flow rate of 1 mL/min (*19*). The SPE cartridges were equilibrated with 10 mL of methanol followed by 10 mL of 5 mM HCl. The samples were eluted with 4 mL of methanol. An aliquot of this methanolic fraction was diluted with water (1:1), centrifuged for 5 min at 15000*g*, and filtered through a 0.2 μm syringe filter prior to analysis by LC-MS.

HPLC Analysis. Hydroxycinnamic acids and diferulic acids were analyzed by reverse-phase HPLC-DAD with a constant flow rate of 1 mL/min. The chromatographic conditions have been described elsewhere (*12*). Eluent was monitored at 325 and 280 nm, and sample identification was established by comparing retention time and absorption spectra to reference standards. Hydroxycinnamic acids and diFAs present in the cereal and feruloylglycine in the urine samples were quantitated using the response factor determined for authentic synthetic compounds.

Liquid Chromatography—Mass Spectrometry (LC-MS). Positive ion electrospray LC-MS measurements were obtained using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) coupled to a Jasco PU-1585 triple-pump HPLC with an AS-1559 cooled autoinjector, a CO-1560 column oven, and a UV detector [Jasco (U.K.) Ltd., Great Dunmow, U.K.]. The HPLC column temperature was maintained at 25 °C and the autoinjector at 4 °C. Eluent flow (1 mL/min) was split between the mass spectrometer and the UV detector in a ratio of 1:5 using an ASI 600 fixed ratio splitter valve (Presearch, Hitchin, U.K.). Approximately 800 μL of the flow split, monitored using a Humonics Optiflow 1000 flow meter (Sigma-Aldrich, Dorset, U.K.), was diverted to the UV detector to carry out detection at 325 nm. HPLC conditions were described above. All mass spectra were performed using a Micromass Z-spray ion source with the following tuning parameters: the electrospray capillary voltage was set to 3.5 kV and the cone voltage to 22 V; the source block temperature was 120 °C and the desolvation temperature 350 °C; nitrogen was used as the drying and nebulizing gas at flows of 500 and 15 L/h, respectively; full-scan spectra were obtained in positive ion mode between *m/z* 50 and 1000 with a scan rate of 1.5 s. Identification and quantitation of hydroxycinnamic acids in plasma and urine samples was based on retention time and SIM monitoring observing ions at *m/z* 195 $[M + H]^+$ and 177 $[M - H_2O + H]^+$ for ferulic acid, *m/z* 225 $[M + H]^+$ and 207 $[M - H_2O + H]^+$ for sinapic acid, *m/z* 165 $[M + H]^+$ and 147 $[M - H_2O + H]^+$ for *o*-coumaric acid, and *m/z* 387 $[M + H]^+$, 369 $[M - H_2O + H]^+$, 389 $[M + 2H + H]^+$, 371 $[M - H_2O + 2H + H]^+$, and 409 $[M + Na]^+$ for diferulic acids. Instrument control and data acquisition and processing were performed using Micromass MassLynx version 3.4 data system and software.

Calibration curves were prepared by injecting samples of standard solutions in 50% methanol for the concentration ranges of 10 nM−5 μM for ferulic and sinapic acid and 500 nM−25 μM for *o*-coumaric acid and then plotting the peak areas of selected ions monitored against concentration. The limit of detection for ferulic acid and sinapic acid was 2.5 pmol as injected (which corresponds to ∼4.5 nM concentration in plasma and ∼10 nM in urine). For the diferulic acids the limit of detection as injected was 5 pmol (approximate plasma concentration of 10 nM and urine concentration of 25 nM). A peak was identified as positive by retention time and area increase of at least two monitored ions above a signal-to-noise level of 3.

RESULTS

Composition of the Cereal. In this study high-bran breakfast cereal (wheat) was used as a dietary source of hydroxycinnamic acids because of its high content of these phenolic compounds (∼325 mg/100 g of fresh weight), particularly ferulic acid and diferulic acids. **Table 1** shows the content (milligrams per 100 g of fresh weight) of free and total (free plus ester-linked) water soluble hydroxycinnamic acids as well as total hydroxycinnamic acids (soluble plus insoluble) of a commercially available high-bran breakfast cereal. The most abundant hydroxycinnamic acid detected was *trans*-ferulic acid followed by sinapic acid (about 80% and 6%, respectively, of the total quantified phenolics). Only a small proportion of *trans*-ferulic acid was solubilized in the water extract (∼7% of the total compound), whereas just over half of the total sinapic acid was found in the water soluble fraction. Small amounts of the free soluble acids were also detected. *cis*-Ferulic acid, *p*-coumaric acid, and vanillin were present only in low quantities. Approximately 9% of the

Table 1. Hydroxycinnamate and Diferulate Composition of the High-Bran Cereal As Consumed (Milligrams per 100 g of Fresh Weight)[a]

	water soluble		total (soluble and insoluble)
	free	free and ester-linked	free and ester-linked
vanillin	1.35 ± 0.12	1.39 ± 0.02	2.72 ± 0.40
trans-*p*-coumaric acid	0.14 ± 0.06	0.47 ± 0.11	5.44 ± 0.45
trans-sinapic acid	0.19 ± 0.04	9.00 ± 1.41	19.60 ± 1.73
trans-ferulic acid	2.45 ± 0.64	18.54 ± 4.40	259.10 ± 15.63
cis-ferulic acid	0.12 ± 0.16	0.84 ± 0.71	9.22 ± 4.96
8-5-diFA (open form)			4.94 ± 0.18
5-5-diFA			6.43 ± 0.24
8-*O*-4-diFA			14.46 ± 1.92
8-5-benzofuran-diFA			2.85 ± 0.75

[a] Values are means ± SD of two independent analyses ($n = 2$).

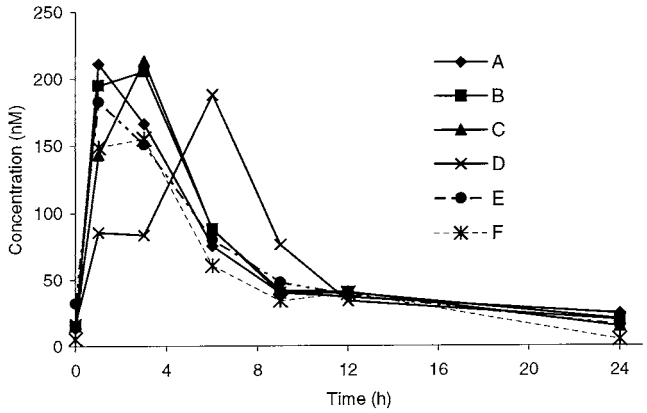

Figure 4. Concentration of free ferulic acid in plasma immediately prior to and for 24 h following consumption of 100 g of high-bran cereal for six volunteers (plasma samples treated with β-glucuronidase plus sulfatase): volunteers A (◆), B (■), C (▲), D (×), E (●), and F (∗). Time zero was taken as the point at which consumption of the dose cereal was completed.

quantified total phenolics comprise diferulic acids. 8-*O*-4-diFA was the most abundant diferulate (50% of total diferulates) followed by the 8-5-diFA (open form plus benzofuran, 27%), whereas the 5-5-dimer accounted for 22% of the diferulates. No diferulic acids were detected in the water extract obtained from the cereal, indicating that these dimeric phenolics were mainly present as ester-linked compounds in the insoluble fraction of the cereal.

Plasma Levels of Hydroxycinnamic Acids after Cereal Consumption. The major hydroxycinnamic acid detected in human plasma after the consumption of the high-bran cereal was ferulic acid. **Figure 4** shows the plasma levels of total free ferulic acid (after glucuronidase plus sulfatase treatment) measured before and after consumption of 100 g of a high-bran breakfast cereal in six volunteers. For most volunteers the plasma concentration of ferulic acid reached a maximum (150−210 nM) between 1 and 3 h after ingestion of the cereal and decreased rapidly between 3 and 6 h and then more slowly until 24 h. For one subject (volunteer D) the maximum peak of absorption was, however, detected at 6 h after the consumption of the cereal. At times 0 and 24 h, plasma concentrations between 5 and 30 nM were measured. The presence in plasma of ferulic acid before the test meal may originate from trace quantities of this compound present in the diet of the volunteers. We cannot discard, however, the presence in human plasma of minor compounds with the same retention time and spectral properties similar to those of ferulic acid.

To determine the type of ferulic conjugates in human plasma, plasma samples from two volunteers (at time points 1 and 3 h) were treated according to the following procedures: (i) no enzyme treatment (to detect free ferulic acid); (ii) β-glucuronidase treatment (the β-glucuronidase enzyme used in this study is pure (*22*); and (iii) β-glucuronidase plus sulfatase treatment. One hour after consumption of the cereal, the concentration of free ferulic acid detected in untreated plasma was 56 nM (mean value of two volunteers). After treatment with β-glucuronidase, the levels of free ferulic acid rose to 190 nM. Treatment with a combination of the two enzymes showed no substantial increase in ferulic acid (199 nM). Similar results were observed 3 h after consumption of the cereal (31 nM of ferulic acid in untreated plasma, 172 nM after treatment with β-glucuronidase, and 177 nM after treatment with the mixture of enzymes). These data indicate that ferulic acid detected in human plasma after the consumption of high-bran cereal was present mostly as a glucuronide conjugate.

Trace levels of sinapic acid were also detected and identified (retention time, LC-MS, SIM mode) in the plasma of four volunteers with the highest levels of total free compound 1 h after consumption of the cereal. Although quantitation of sinapic acid was problematic due to the presence of minor interfering peaks, we were able to estimate maximum plasma values of ∼10−40 nM. Sinapic acid was not detected in any plasma samples taken at 3 h. We were not able to detect any of the selected ions for the diferulic acids in the plasma samples after consumption of the high-bran cereal at any of the time points examined. We cannot completely exclude that traces of these compounds could be present in the samples at concentrations below the detection limit of our method (plasma concentration of ∼10 nM) or that different metabolites with molecular masses different from those selected by us were present in the plasma.

Urinary Excretion of Hydroxycinnamic Acids. Free ferulic acid, sinapic acid, and a glycine conjugate of ferulic acid were detected after enzyme treatment, in the 24-h urine collected from all subjects, both before and after consumption of high-bran cereal (as confirmed by LC-MS). Total excreted amounts in 24 h (after β-glucuronidase plus sulfatase treatment) are shown in **Table 2**. On average, the amounts of ferulic acid (3.17 ± 0.67 μmol) and feruloylglycine (5.33 ± 2.94 μmol) excreted in the 24-h urine collected after a 2 day low-polyphenol diet increased ∼4-fold (12.26 ± 3.22 μmol) and ∼5-fold (28.99 ± 15.92 μmol), respectively, after the cereal consumption. Two of the volunteers showed increases of feruloylglycine of up to ∼10-fold. Low but measurable quantities of total sinapic acid were detected in 24-h urine after the low-polyphenol diet, and only a small increase was observed for most volunteers after the consumption of the cereal. For subject D, sinapic acid decreased slightly on the study day.

Urine samples from two volunteers were treated with β-glucuronidase or β-glucuronidase plus sulfatase according to the procedures described above. Treatment with pure β-glucuronidase raised the levels of free ferulic acid in urine from 2.2 to 9.7 μmol/24-h urine. Additional treatment with sulfatase yielded a slight (but not significant) increase of free ferulic acid up to 14.4 μmol/24-h urine. Our results indicate that the major ferulic conjugates detected in the urine were feruloyl glycine (∼70% of the total ferulic acid excreted) followed by glucuronide derivatives. A small proportion of sulfate derivatives may also be present.

Table 2. Total Amounts of Ferulic Acid, Feruloylglycine, and Sinapic Acid Excreted in Urine before and after Consumption of High-Bran Cereal (Micromoles per 24 h)

	ferulic acid				sinapic acid	
	free + glucuronides + sulfates		feruloylglycine		free + glucuronides + sulfates	
volunteer	prestudy day	study day	prestudy day	study day	prestudy day	study day
A	3.19	10.45	8.94	26.81	1.24	1.88
B	4.40	15.09	3.20	11.42	1.20	2.74
C	2.60	16.07	3.78	22.61	0.54	2.78
D	2.80	7.77	2.03	24.68	2.62	1.48
E	2.69	13.79	5.19	58.89	0.78	2.94
F	3.32	10.39	8.83	29.55	1.96	2.63
mean ± SD	3.17 ± 0.67	12.26 ± 3.22	5.33 ± 2.94	28.99 ± 15.92	1.39 ± 0.77	2.41 ± 0.59

Table 3. Intake, Subsequent Excretion, and Recovery of Ferulic Acid and Sinapic Acid in Urine of All Six Volunteers after Consumption of High-Bran Cereal

	intake (mg)			total urine excretion[b]	urine excretion[a] (% intake)	
acid	total	water soluble	free	(mg/24 h)	total	water soluble
ferulic	259.10	18.54	2.45	8.10 ± 3.34 [5.15–14.63][c]	3.13 ± 1.29 [1.99–5.65]	43.68 ± 18.02 [27.77–78.92]
sinapic	19.60	9.00	0.19	0.54 ± 0.13 [0.33–0.66]	2.76 ± 0.67 [1.69–3.36]	6.00 ± 1.46 [3.69–7.32]

[a] Total excreted (mg) × 100/intake (mg). Values are means ± SD ($n = 6$). [b] Values are means ± SD ($n = 6$). [c] Range of data for all six volunteers.

DISCUSSION

Cereal bran and bran-enriched products are the most important dietary sources of cell wall bound hydroxycinnamates and diferulates (*4*, *5*). Although there are considerable data concerning the composition of plants, nothing is known of the phenolic composition of cereal-derived food products (e.g., breakfast cereals, baked products) that are consumed by humans. In this paper we report for the first time the composition in hydroxycinnamates and diferulates of a commercial high-bran breakfast cereal and show that the consumption of a 40 g portion of this product provides ~130 mg of these phenolic antioxidants. We have also shown that only a small proportion of these phenolics, specifically ferulic acid and sinapic acid, are detected in the plasma and urine of volunteers after consumption of the cereal. As shown in **Table 3**, the percentage of each of these two phenolics recovered in the urine in relation to the total amount ingested is ~3%. The amounts of ferulic and sinapic acid excreted in urine are greater than the amount of total free acids present in the test meal (approximately 2.5 and 0.2 mg, respectively; **Table 1**), indicating that some de-esterification must have taken place. It has been reported that esterases with the ability to cleave ferulate from a model substrate are present in cell free extracts from duodenum, jejunum, and ileum human mucosa (*13*). In the present study, maximum absorption of ferulic acid takes place between 1 and 3 h after the test meal, suggesting that absorption of this compound occurs primarily in the small intestine (*23*). Thus, it is likely that cleavage, release, and absorption of ferulic and sinapic acids from the high-bran breakfast cereal take place mainly in the small intestine and from the soluble compounds present in the test meal (the urinary total ferulic and sinapic acids account for approximately 44 and 6% of the respective total water soluble compounds in the cereal; **Table 3**). Further release of hydroxycinnamic acids from both the soluble and insoluble portions of the high-bran cereal is possible in the large intestine due to the action of bacterial enzymes (*12*−*15*). However, it has been shown that bioavailability of ferulic acid from a complex cereal matrix in rats is severely reduced probably due to limited access of the digestive enzymes to the phenolic substrate (*24*). In this study, the low levels of hydroxycinnamic acids found in plasma 6 h after consumption of the cereal are indicative of little or no absorption from the large intestine. If hydroxycinnamates from cereals are released in the colon, they are more likely to be further metabolized by the microflora (*25*, *26*) or excreted via feces (*14*).

Free diferulic acids can be absorbed from the gut in rats (*12*), but there are no reports on the absorption of these compounds in humans from a dietary source. In vitro assays have shown that human colonic esterases (mainly luminal and probably derived from the microflora) are capable of releasing diferulic acids from cereal brans (*12*) and, thus, it is possible that a small proportion of these cereal components may have been released into the lumen. In this study, none of the diferulic acids were detected in the plasma or urine samples from any volunteer, indicating that absorption of the ester-linked dimers, if any, is very restricted (plasma and urine levels were below the detection limits, ~10 and ~25 nM, respectively). Instead, released dimers may be further metabolized by colonic bacteria as it has been shown that diferulic acids can undergo hydrogenation reactions and opening of the hydrofuran rings by human fecal microorganisms (*27*). Among the selected ions for the detection of the diferulic compounds we included the mass corresponding to a monohydrogenated form of the dimer (389 $[M + 2H + H]^+$, 371 $[M - H_2O + 2H + H]^+$, but we were not able to detect it either in plasma or in urine. Overall, it is likely that the bulk of the diferulic acids remains covalently bound and is excreted in the feces.

It is important to consider the circulating levels of hydroxycinnamics and conjugates after the cereal intake in relation to their putative in vivo protective effects against cardiovascular diseases or cancer. For example, oxidation of lipids or low-density lipoproteins (LDL) is important in the pathogenesis of atherosclerosis, and recent in vitro studies show that hydroxycinnamic and diferulic acids can partially inhibit the oxidation process at micromolar concentrations (~1−100 μM) (*7*, *28*). Also, the anticarcinogenic properties of ferulic acid have been reported in rats using milligram doses of the free compound (*10*, *11*). In this study the maximum levels of hydroxycinnamic

acids found in plasma after the consumption of the cereal at any time point were ~200 nM for ferulic acid, ~40 nM for sinapic acid, and <10 nM for diferulic acids, considerably lower than the concentrations required to induce responses in vitro. Other studies looking at absorption in humans of dietary hydroxycinnamic acids have also reported plasma concentrations in the nanomolar range (*20*, *26*). Thus, in vitro and animal studies looking at chemoprotective and antioxidant effects of dietary hydroxycinnamic acids need to be carried out using more physiologically relevant concentrations of these compounds and their metabolic conjugates. Additionally, and given the likely presence of a proportion of free hydroxycinnamic acids in the lumen, studies looking at the biological effects of these compounds on intestinal epithelia cells are worth considering.

In conclusion, we have shown that (1) ferulic acid and sinapic acid are the major hydroxycinnamic acids taken up in humans after the consumption of a high-bran cereal (maximum levels reached in plasma in the nanomolar range), with absorption occurring mostly from the small intestine, and (2) covalently bound diferulic acids either are not absorbed or are absorbed only in very small amounts (<10 nM in plasma), indicating that the bulk of ester-linked dimeric compounds are excreted in feces or further metabolized by colonic microflora.

ACKNOWLEDGMENT

We thank all of the volunteers who took part in this study and Aliceon Blair, Lesley Maloney, and Yvonne Clements in the Human Nutrition Unit at the Institute of Food Research. We also thank John Eagles for mass spectrometry analysis.

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Received for review March 28, 2003. Revised manuscript received July 10, 2003. Accepted July 11, 2003. The work was funded by the European Union (QLK5-1999-50510) to S.K. and the Biotechnology and Biological Sciences Research Council, U.K.

JF0302299