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The Effect of In Vitro Digestion on Steryl Ferulates from Rice (*Oryza sativa* L.) and Other Grains

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ABSTRACT: Polished and cargo rice, wild rice, rice bran, corn bran, and wheat bran were subjected to a static in vitro digestion model, to monitor changes in their steryl ferulate content and composition. Free sterols, possible hydrolysis products of steryl ferulates, were also measured. Additionally, steryl ferulate bioaccessibility was calculated as the percentage of steryl ferulates liberated from the grain matrix into the digestive juice. Steryl ferulate content ranged between 6.1 and 3900 μg/g and decreased by 1−63% due to digestion. A parallel increase in free sterols of more than 70% was observed in all samples. Additionally, bioaccessibility of steryl ferulates was found to be almost negligible. These findings suggest that intestinal enzymes immediately hydrolyze steryl ferulates, which are liberated from the grain matrix, and thus they are practically unavailable for absorption in the small intestine. This further indicates that the hydrolysis products of steryl ferulates could be bioactive in the gut.

KEYWORDS: *steryl ferulate, bioaccessibility, in vitro digestion, Oryza sativa, Zizania palustris, Zea mays, Triticum aestivum*

INTRODUCTION

Grains are the most common staple foods consumed worldwide. Among them, rice feeds over half of the world's population. The outer part of most grain kernels is rich in bioactives; therefore unpolished or whole grains have numerous beneficial health effects. During rice processing, first the outermost layer (the hull or husk) is removed, providing unpolished or cargo rice. Cargo rice can either be brown, red, or black. Complete milling and polishing transforms cargo rice into polished or white rice.

Steryl ferulates are various plant sterols esterified to ferulic acid, and they can be mainly found in the outer part of the grain kernel. They belong to the bioactive compounds contributing to the health promoting effects of whole grains. Steryl ferulates have already been identified in wheat, corn, rye, triticale, Job's tears, barley, wild rice, and rice[1−3] with varying sterol composition in different grains. They were first extracted from rice bran oil in the 1950s,[4] and the mixture of rice steryl ferulates was named γ-oryzanol (Figure 1). Steryl ferulates have been shown to possess antioxidant activity and plasma cholesterol lowering effect in animals as well as in humans. These health promoting properties have recently been reviewed by Ghatak and Panchal.[5]

The antioxidant activity of steryl ferulates is due to the hydrogen donating capability of their ferulic acid constituent. As for the cholesterol lowering activity, steryl ferulates are thought to be cleaved by intestinal enzymes during digestion, most probably by cholesterol esterase. Hydrolysis liberates free sterols, which are then the bioactive compounds acting as cholesterol lowering agents.[6] The extent of hydrolysis of steryl ferulates is not yet clear.[7,8] In vitro studies comparing the affinity of different intestinal enzymes (pancreatic preparations and cholesterol esterase from various sources) to different steryl ferulate substrates demonstrated that 4-desmethyl sterols (lacking methyl groups at the C-4 position) were preferentially hydrolyzed over 4,4′-dimethyl sterols (containing two methyl groups at the C-4 position).[9−11] This suggests that 4-desmethyl sterols could be more effective in cholesterol lowering than 4,4′-dimethyl sterols.

It is generally assumed that free sterols lower plasma cholesterol by inhibiting its absorption into the systemic circulation.[12] Several hypotheses have been proposed about the inhibition mechanism. First, free sterols might block cholesterol ester hydrolysis. Second, they might coprecipitate with cholesterol and form nonabsorbable mixed crystals. Third, they may also compete with cholesterol in the incorporation into dietary mixed micelles.[12] The results of Ikeda and co-workers[13] support the theory of competition for micelles. They found that free sitosterol was more potent in cholesterol lowering than free cycloartenol in rats. Sitosterol is a 4-desmethyl sterol and shows more structural similarity with cholesterol than cycloartenol, a 4,4′-dimethyl sterol. Further, a difference between sterols and stanols (saturated counterparts of sterols) has also been observed. A mixture of nonesterified stanols from corn oil showed significantly higher cholesterol lowering ability than a mixture of nonesterified sterols.[14] The same tendency was noted when comparing pure β-sitostanol to β-sitosterol.[15,16] In addition, the cholesterol lowering potential seems to be in an inverse relationship with the absorbability of the sterol/stanol.[16,17]

To our knowledge, only a few studies have been conducted about the pharmacokinetic properties of steryl ferulates. Huang[18] investigated the digestibility of γ-oryzanol using in vitro cell culture models, while Fujiwara and co-workers[7,8] studied absorption and metabolism of γ-oryzanol in vivo, in rabbits and rats. However, none of these studies provide clear information about the exact mechanism of action of steryl ferulates, nor do they indicate where steryl ferulates are bioactive in the body.

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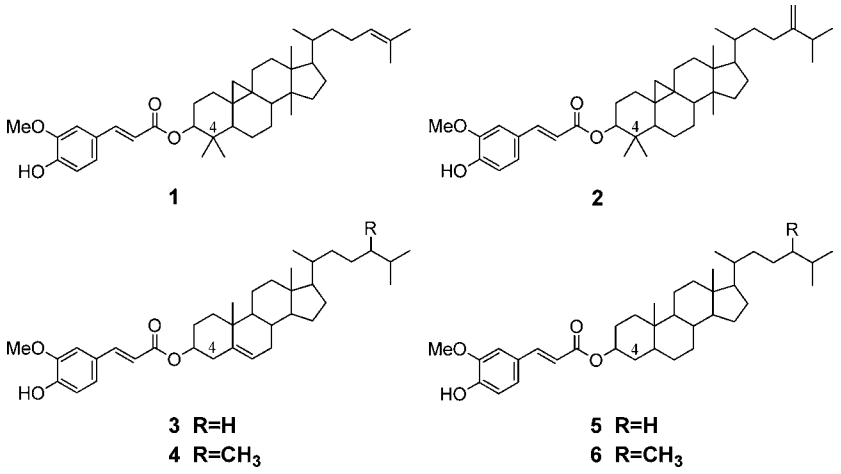

Figure 1. Chemical structures of the main constituents of γ-oryzanol: cycloartenyl ferulate (**1**), 24-methylenecycloartanyl ferulate (**2**), campesteryl ferulate (**3**), sitosteryl ferulate (**4**), campestanyl ferulate (**5**), and sitostanyl ferulate (**6**).

Determination of bioaccessibility is a good preliminary trial for absorption and metabolism studies. By definition the bioaccessible portion of a compound is the fraction that is liberated from the matrix into the digestive juice and which therefore becomes available for absorption.[19,20] The bioaccessible portion can be transported across the intestinal epithelium, it can further be metabolized in the liver, and it can also reach tissues and organs. On the other hand, the non-bioaccessible portion is not available for absorption into the systemic circulation, and hence it remains in the gastrointestinal tract. To measure bioaccessibility, samples were subjected to a static in vitro digestion model, developed by Oomen and co-workers.[21] This model has been designed to measure bioaccessibility of hydrophobic compounds, providing all the necessary conditions for the formation of mixed micelles.

The aim of our study was to investigate the effect of in vitro digestion on steryl ferulates from different cereal grains in order to gain insight into the pharmacokinetic properties of these bioactive compounds. Differences between grain sources were monitored.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin was purchased from VWR, Lutterworth, U.K., acetic acid, β-sitosterol (purity ≥95%), lipase, pancreatin, pepsin, and sodium hydroxide from Merck, Darmstadt, Germany, and cycloartenyl ferulate (purity >99%) and γ-oryzanol (purity >98%) from Wako, Osaka, Japan. Calcium chloride dihydrate, glucose, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, and uric acid were purchased from Fluka, Buchs, Switzerland, while acetone, acetonitrile, α-amylase, ammonium acetate, ammonium chloride, bile, butanol, disodium hydrogen phosphate, glucosamine hydrochloride, glucuronic acid, magnesium chloride, heptane, hydrochloric acid, isopropanol, mucin, potassium hydroxide, and urea were obtained from Sigma-Aldrich, St. Louis, MO, USA. All solvents were of HPLC grade.

Samples and Sample Preparation. Polished rice (*Oryza sativa* L.), cargo rice, wild rice (*Zizania palustris* L.), and rice bran samples were a kind gift from Riseria Taverne SA, Switzerland. Polished and cargo rice were of European and Asian origin, wild rice was from Canada, and rice bran consisted of a mixture of Italian rice cultivars. Polished rice cultivars were Carolina, S. Andrea, Carnaroli, Originario, and Baldo from Italy, Loto from Switzerland, Perfume from Thailand, Jasmine from both Thailand and Cambodia, and Basmati from both India and Pakistan. Cargo rice cultivars were S. Andrea and Baldo from Italy, Perfume from Thailand, Basmati from India, and red cargo rice from the Camargue region of France. All rice samples were grown in the year 2010. Polished, cargo, and wild rice were first premilled (particle size 1−2 mm) with a toothed disk mill (Condux LV 15M, Germany) and then milled (Retsch ZM 200, Germany, pore size of sieve: 0.5 mm, 10 000 rpm). Rice bran was provided as a milled fraction. Corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.) were grown in Switzerland in the year 2010, and the bran was provided by Swissmill, Switzerland, as milled fraction. The particle size of all milled samples was below 0.5 mm. All samples were stored at −20 °C and were analyzed within 6 months.

Moisture and Ash Content Determination. The moisture and ash content of the samples were both determined with gravimetric analysis. For moisture content determination, 2 g samples were dried overnight in preweighed dishes at 103 °C. They were weighed after cooling down for 1 h in a desiccator.[22] To determine the ash content, 2 g samples were weighed into preweighed dishes and 2 mL of ethanol was added to each of them. Ethanol was well mixed with the sample to ensure complete removal of all organic material. Samples were first incinerated in the muffle furnace and then totally reduced to ashes at 600 °C during 12 h. The residues were weighed after cooling.[23]

In Vitro Digestion. The in vitro digestion method of Oomen and co-workers[21] was implemented, with minor modifications. Digestive juices were prepared adding inorganic and organic solutions together, adding enzymes to the solutions, and then adjusting the pH. KSCN, a toxic compound and complexing agent, was left out from our experiment. Saliva was prepared from 1 mL of KCl (89.6 g/L), 1 mL of NaH_2PO_4 (88.8 g/L), 1 mL of Na_2HPO_4 (57 g/L), 0.17 mL of NaCl (175.3 g/L), 0.18 mL of NaOH (40 g/L) (inorganic) and 0.8 mL of urea (25 g/L) (organic). Gastric juice was prepared from 1.57 mL of NaCl (175.3 g/L), 0.3 mL of NaH_2PO_4 (88.8 g/L), 0.92 mL of KCl (89.6 g/L), 1.8 mL of $CaCl_2{\cdot}2H_2O$ (22.2 g/L), 1 mL of NH_4Cl (30.6 g/L), 0.83 mL of HCl (37% g/g) (inorganic) and 1 mL of glucose (65 g/L), 1 mL of glucuronic acid (2 g/L), 0.34 mL of urea (25 g/L) as well as 1 mL of glucosamine hydrochloride (33 g/L) (organic). Duodenal juice was prepared from 8 mL of NaCl (175.3 g/L), 8 mL of $NaHCO_3$ (84.7 g/L), 2 mL of KH_2PO_4 (8 g/L), 1.26 mL of KCl (89.6 g/L), 2 mL of $MgCl_2$ (5 g/L), 36 μL of HCl (37% g/g) (inorganic) and 0.8 mL of urea (25 g/L) (organic). Bile was prepared from 3 mL of NaCl (175.3 g/L), 6.83 mL of $NaHCO_3$ (84.7 g/L), 0.42 mL of KCl (89.6 g/L), 20 μL of HCl (37% g/g) (inorganic) and 1 mL of urea (25 g/L) (organic). Inorganic and organic solutions of saliva, gastric juice, and bile were augmented to 50 mL with distilled water, whereas inorganic and organic solutions of duodenal juice were augmented to 100 mL. Then, 14.5 mg of α-amylase, 1.5 mg of uric acid, and 5 mg of mucin were added to saliva. 0.1 g of BSA, 0.1 g of pepsin, and 0.3 g of mucin were added to gastric juice. 1.8 mL of $CaCl_2{\cdot}2H_2O$ (22.2 g/L), 0.2 g of BSA, 0.6 g of pancreatin, and 0.1 g of lipase were added to duodenal juice. 1 mL of $CaCl_2{\cdot}2H_2O$ (22.2 g/L),

Table 1. Moisture, Ash Content and Digestion Induced Changes in the Steryl Ferulate Content of Polished, Cargo, and Wild Rice as Well as Three Types of Bran (μg/g dry weight, n = 3)[a]

	origin	moisture content [%]	ash content [g/100 g]	content of steryl ferulates [μg/g] in rice and bran	in supernatant	in precipitate	decrease in steryl ferulates [%]	bioaccessibility[b] of steryl ferulates [%]
polished rice								
Carolina[c]	Italy	12.2 ± 0.0	0.47 ± 0.0	34.9 ± 1.8	0.01 ± 0.00	27.8 ± 1.4	20 ± 1	nd
S. Andrea[d]	Italy	12.2 ± 0.1	0.48 ± 0.0	39.6 ± 1.3	0.01 ± 0.00	23.5 ± 1.2	41 ± 2	nd
Carnaroli[d]	Italy	11.9 ± 0.0	0.42 ± 0.0	26.5 ± 1.1	0.01 ± 0.00	19.8 ± 1.1	25 ± 1	nd
Originario[d]	Italy	11.7 ± 0.0	0.53 ± 0.0	47.6 ± 0.2	0.02 ± 0.00	38.1 ± 1.3	20 ± 0	nd
Baldo[d]	Italy	12.5 ± 0.1	0.40 ± 0.0	26.7 ± 0.2	0.01 ± 0.00	19.6 ± 0.2	27 ± 0	nd
Loto[d]	Switzerland	11.8 ± 0.0	0.49 ± 0.0	27.2 ± 0.9	0.01 ± 0.00	20.7 ± 0.9	24 ± 1	nd
Perfume[c]	Thailand	11.7 ± 0.0	0.48 ± 0.0	22.1 ± 0.2	0.02 ± 0.00	14.2 ± 0.8	36 ± 0	0.1 ± 0.0
Jasmine[c]	Thailand	12.1 ± 0.1	0.33 ± 0.0	6.7 ± 0.2	0.01 ± 0.00	4.3 ± 0.2	36 ± 1	0.2 ± 0.0
Jasmine[c]	Cambodia	12.2 ± 0.1	0.33 ± 0.0	6.1 ± 0.3	0.01 ± 0.00	3.8 ± 0.1	38 ± 2	0.2 ± 0.0
Basmati[c]	India	11.3 ± 0.1	0.45 ± 0.0	22.5 ± 0.4	0.01 ± 0.00	17.7 ± 0.3	21 ± 1	nd
Basmati[c]	Pakistan	11.1 ± 0.1	0.32 ± 0.0	7.4 ± 0.4	0.01 ± 0.00	5.7 ± 0.1	23 ± 2	0.1 ± 0.0
cargo rice								
S. Andrea[d]	Italy	11.7 ± 0.1	1.31 ± 0.0	401 ± 3	1.1 ± 0.1	382 ± 4	4 ± 1	0.3 ± 0.0
Baldo[d]	Italy	11.8 ± 0.1	1.49 ± 0.1	381 ± 4	1.9 ± 0.1	368 ± 2	3 ± 0	0.5 ± 0.0
Perfume[c]	Thailand	11.2 ± 0.1	1.32 ± 0.0	216 ± 2	0.5 ± 0.0	208 ± 3	4 ± 1	0.2 ± 0.0
Basmati[c]	India	10.6 ± 0.1	1.44 ± 0.1	263 ± 4	0.9 ± 0.1	259 ± 3	1 ± 3	0.3 ± 0.0
Camargue[c]	France	12.5 ± 0.0	1.53 ± 0.0	393 ± 2	0.6 ± 0.0	391 ± 2	1 ± 3	0.2 ± 0.0
wild	Canada	9.8 ± 0.0	1.43 ± 0.0	94 ± 2	0.1 ± 0.0	90 ± 3	4 ± 2	0.1 ± 0.0
rice bran	Italy	11.4 ± 0.2	10.9 ± 0.3	3900 ± 40	59 ± 1	3710 ± 50	5 ± 0	1.5 ± 0.0
corn bran	Switzerland	12.4 ± 0.1	4.33 ± 0.1	331 ± 5	1.2 ± 0.1	156 ± 8	53 ± 1	0.4 ± 0.0
wheat bran[e]	Switzerland	7.8 ± 0.1	7.15 ± 0.1	452 ± 6	0.3 ± 0.0	165 ± 5	63 ± 1	0.1 ± 0.0

[a]Recovery rate of 97% is considered. [b]Bioaccessibility = % of steryl ferulates in the original sample liberated to the supernatant during digestion; nd = not detected. [c]Long grain cultivars. [d]Short or medium grain cultivars. [e]In-house reference sample, n = 10.

0.18 g of BSA, and 0.6 g of bile were added to bile. The pH of saliva, gastric juice, duodenal juice, and bile were adjusted respectively to 6.5, 1.07, 7.8, and 8.0. For pH adjustment, 6 M HCl and 6 M NaOH were used. All digestive juices were freshly prepared prior to the experiment and were consecutively added. A 5 g sample was first incubated for 10 min with 9 mL of saliva, then for 2 h with an additional 13.5 mL of gastric juice, and for another 2 h with the addition of 27 mL of duodenal juice and 9 mL of bile. Incubation was performed in a water bath with orbital shaking, at 37 °C. Digestion was terminated by adding 0.2 mL of 6 M HCl to the sample (pH = 3). Afterward, chyme was centrifuged for 5 min at 3000 rpm, to enable complete separation of the supernatant and the precipitate phase. Further, a recovery test was conducted to ensure that the analytes were efficiently extracted, and that they were not hydrolyzed nonenzymatically due to the chemical reaction conditions. To achieve this, γ-oryzanol was subjected to the in vitro digestion model, without the addition of enzymes. The recovery rate was found to be 97%.

Extraction and Quantitation of Total Steryl Ferulates and Free Sterols. For the extraction of steryl ferulates and free sterols, the supernatant (approximately 60 mL) was incubated at ambient temperature in a shaking water bath with 3 times 20 mL of heptane, with an incubation time of 20 min each. From the solid samples (precipitate (approximately 5 g) and nondigested samples (5 g)), steryl ferulates and free sterols were extracted at 50 °C in a shaking water bath, with 3 times 20 mL of acetone, with an incubation time of 20 min each. In the case of corn bran, acetone was replaced with heptane, according to the report of Seitz.[1] The extracts were collected in round-bottom flasks. Solvent from the extracts was evaporated using a rotary evaporator (Büchi R-114, Switzerland) at 50 °C. After this precipitates and nondigested samples were redissolved in 5 mL of heptane–isopropanol (99:1, v/v), whereas supernatants were redissolved in 0.5 mL of heptane–isopropanol (99:1, v/v). All samples were analyzed by normal phase HPLC (Agilent 1200, Switzerland), using a diode-array detector (DAD) at 315 nm for steryl ferulate analysis and a refractive index detector (RID) for free sterol determination. Separation was achieved using a diol silica column (LiChrosorb, Diol phase, 5 μm, 3.0 × 150 mm, Phenomenex, USA), at 35 °C, at a flow rate of 0.5 mL/min with isocratic elution. As eluent, heptane–isopropanol (99:1) was used (modified from Nyström and co-workers[24]). For quantitation, γ-oryzanol and β-sitosterol were used as external standards. For the γ-oryzanol analysis, the limit of detection (defined as 3 times the detector signal noise) and the limit of quantitation (3 times the limit of detection) were 0.5 ng/injection and 1.5 ng/injection, respectively. The linear range was between 0.025 and 25.1 μg/injection. In the case of free sterol analysis, the limit of detection was 0.2 μg/injection and the limit of quantitation was 0.6 μg/injection. The linear range was between 1 and 75.6 μg/injection. Wheat bran (an in-house reference sample) was analyzed within each series of measurements as a control. The steryl ferulate and free sterol contents of the control sample were 452 ± 6 μg/g and 667 ± 11 μg/g (n=10), respectively.

Purification, Separation, and Identification of Individual Steryl Ferulates. For the analysis of the sterol composition of steryl ferulates from the extracts, an aliquot sample (2.5 mL) in heptane–isopropanol (99:1, v/v) was transferred into a vial. Solvent was evaporated under nitrogen stream at 50 °C, and the samples were redissolved in 3 mL of methanol. After this, samples were subjected to base–acid cleanup according to the method of Seitz[1] in order to eliminate neutral lipids. The sterol composition of steryl ferulates was analyzed by reversed phase HPLC (Agilent 1200, Switzerland), using a DAD detector at 325 nm. Separation was achieved with an XBridge Shield C18 column (Waters), using acetonitrile–water–butanol–acetic acid (88:6:4:2, v/v/v/v) as eluent (modified from Norton[25]) and 1 mL/min flow rate at 25 °C. The identity of steryl ferulate species was confirmed based on their characteristic elution order from the literature,[26] as well as on mass spectrometric measurements. Samples were collected from the HPLC, and the solvent was changed to 10 mM ammonium acetate in methanol. After this, samples were injected into an ion trap mass spectrometer (LTQ Velos, Thermo Scientific, USA), with electrospray ionization, used in the negative ion mode. For instrument control, data acquisition, and processing, the Xcalibur software (version 2.1.0) was applied. For semiquantitative

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Table 2. Digestion Induced Changes in the Free Sterol Content of Polished, Cargo, and Wild Rice as Well as Three Types of Bran (μg/g dry weight, n = 3)[a]

	origin	free sterol content [μg/g] in rice and bran	in supernatant	in precipitate	increase in free sterols [%]
polished rice					
Carolina[b]	Italy	47.3 ± 0.9	0.6 ± 0.0	147 ± 7	213 ± 4
S. Andrea[c]	Italy	48.9 ± 1.5	0.8 ± 0.0	121 ± 1	150 ± 5
Carnaroli[c]	Italy	57.4 ± 1.9	0.7 ± 0.0	149 ± 8	160 ± 5
Originario[c]	Italy	74.7 ± 3.5	0.8 ± 0.0	174 ± 6	135 ± 6
Baldo[c]	Italy	48.2 ± 1.3	0.7 ± 0.1	167 ± 2	248 ± 7
Loto[c]	Switzerland	34.8 ± 1.9	0.8 ± 0.0	174 ± 6	404 ± 40
Perfume[b]	Thailand	79.2 ± 5.6	0.7 ± 0.0	192 ± 5	143 ± 10
Jasmine[b]	Thailand	49.5 ± 2.7	0.9 ± 0.0	174 ± 9	254 ± 16
Jasmine[b]	Cambodia	40.7 ± 2.2	0.9 ± 0.0	167 ± 2	312 ± 28
Basmati[b]	India	75.9 ± 3.6	1.4 ± 0.0	281 ± 73	272 ± 13
Basmati[b]	Pakistan	36.5 ± 0.8	1.2 ± 0.0	189 ± 3	422 ± 9
cargo rice					
S. Andrea[c]	Italy	197 ± 7	1.4 ± 0.1	370 ± 5	88 ± 3
Baldo[c]	Italy	238 ± 6	2.3 ± 0.1	402 ± 7	70 ± 2
Perfume[b]	Thailand	211 ± 10	1.1 ± 0.0	391 ± 1	86 ± 4
Basmati[b]	India	215 ± 9	3.5 ± 0.2	377 ± 11	77 ± 3
Camargue[b]	France	217 ± 8	1.5 ± 0.1	375 ± 18	73 ± 3
wild	Canada	117 ± 4	1.0 ± 0.0	286 ± 7	145 ± 5
rice bran	Italy	1110 ± 40	68.2 ± 0.2	2410 ± 20	119 ± 5
corn bran	Switzerland	2020 ± 30	44.4 ± 0.1	3420 ± 140	70 ± 0
wheat bran[d]	Switzerland	667 ± 11	21.5 ± 0.1	1138 ± 19	74 ± 1

[a]Recovery rate of 97% is considered. [b]Long grain cultivars. [c]Short or medium grain cultivars. [d]In-house reference sample, n = 10.

determination, cycloartenyl ferulate was used as an external standard. The limit of detection for cycloartenyl ferulate was 1 ng/injection, whereas the limit of quantitation was 3 ng/injection. The linear range was between 3 ng/injection and 10 μg/injection. As the UV absorbance of steryl ferulates originates from ferulic acid, the UV response of the individual molecular species is almost identical. Therefore, concentrations or mass ratios of steryl ferulate molecules can be approximated by the ratios of the HPLC peak integrals. Conclusively, relative proportions instead of exact masses of the individual steryl ferulate species are provided (Table 4).

Statistical Analysis. All analyses were performed in triplicate. All results are expressed on a dry weight basis unless otherwise stated. Presented data are means ± standard deviation (error probability 5%). ANOVA, together with Tukey's test, was performed in order to determine significant differences between mean values, using SPSS 17.0 for Windows software. The test was performed with a confidence interval of 95%.

RESULTS AND DISCUSSION

Steryl Ferulate Content before and after Digestion. Steryl ferulate content was monitored in polished rice, cargo rice, wild rice, rice bran, corn bran, and wheat bran (Table 1). The γ-oryzanol (mixture of steryl ferulates in rice) content of nondigested polished rice varied between 6.1 μg/g and 47.6 μg/g. Until now, studies have only included 1−3 varieties,[27,28] revealing a 70−120 μg/g γ-oryzanol content. Our study, however, provides a more comprehensive comparison of steryl ferulates in polished rice. No difference could be seen between short or medium and long grain cultivars, which is in accordance with the observations of Miller and Engel,[3] who measured the content of 30 different European brown rice varieties. Differences due to various growing locations could be observed in the case of Basmati rice. The γ-oryzanol content of the sample grown in India was three times higher than the γ-oryzanol content of the sample grown in Pakistan. In the case of Jasmine rice, however, the different growing locations did not induce significant changes in the γ-oryzanol content. Additionally, the γ-oryzanol content of the two Jasmine rice cultivars and the Basmati rice grown in Pakistan (6.1−7.4 μg/g) was very low compared to the rest of polished rice analyzed (22.1−47.6 μg/g). The γ-oryzanol content of the polished and the cargo (brown) form of four different cultivars was also compared. The cargo form was found to exhibit a 12-fold higher γ-oryzanol content than the corresponding polished form (27.7 μg/g compared to 315 μg/g). The γ-oryzanol content of wild rice (94 μg/g) was found to be between that of polished and cargo rice. As expected, rice bran from which γ-oryzanol is extracted at an industrial scale exhibited a 12-fold higher γ-oryzanol content than cargo rice, providing the highest content (3900 μg/g) of all analyzed samples. The steryl ferulate content of corn bran (331 μg/g) was in the same range as cargo rice (216−401 μg/g), whereas wheat bran exhibited a somewhat higher steryl ferulate content (452 μg/g). Our findings are in good agreement with the literature for the γ-oryzanol content of wild rice (91 μg/g), cargo rice (201−388 μg/g), and rice bran (2510−6860 μg/g)[2,3,29] as well as for the total steryl ferulate content of corn bran (200−250 μg/g) and wheat bran (300 μg/g).[22,30]

In all samples, a 1−63% decrease in the steryl ferulate content due to digestion could be observed (Table 1). The rate of decrease varied among different grains, which likely results from the differences in the sterol profiles between the different grains (see discussion below). Steryl ferulates in corn bran and wheat bran decreased by 53% and 63%, respectively, compared to a 1−41% decrease in different milling fractions of rice. The decrease rate was lower for cargo rice (1−4%), wild rice (4%), and rice bran (5%) and higher for polished rice (20−41%). A likely explanation for the decrease in the steryl ferulate content is the hydrolysis by cholesterol esterase, found in pancreatin, a

Table 3. Electrospray Ionization Mass Spectra of Steryl Ferulates: Cycloartenyl Ferulate (1), 24-Methylenecycloartanyl Ferulate (2), Campesteryl Ferulate (3), Sitosteryl Ferulate (4), Campestanyl Ferulate (5), and Sitostanyl Ferulate (6)

		negative CID spectra m/z (rel int, %)						
steryl ferulate	retention time [min]	precursor[a] $[M-H]^-$	$[M-H-Me]^-$	$[M-H]^-$	$[M-H-2Me]^-$	$[feruloyl]^-$	other ions from feruloyl part	ref substance used for identification
1	7.4	601	586 (100)	601 (1.9)			175 (0.3)	*b*
2	8.2	615	600 (100)	601 (1.8)			175 (0.2)	*c*
3	8.9	575	560 (100)	575 (0.9)	545 (0.4)	193 (1.5)		*c*
4	10.2	589	574 (100)	589 (1.2)	559 (0.2)	193 (1.3)		*c*
5	10.8	577	562 (100)	577 (2.1)		193 (0.3)		*c*
6	12.2	591	576 (100)	591 (2.9)		193 (1.1)		*c*

[a]Deprotonated molecular ion. [b]Cycloartenyl ferulate from Wako (Japan). [c]Substance from commercial source is not available, therefore compound is only tentatively identified.

component of duodenal juice. The γ-oryzanol content of cargo rice, wild rice, and rice bran seems to be less accessible to cholesterol esterase than the γ-oryzanol content of polished rice. Cholesterol esterase has already been demonstrated to hydrolyze γ-oryzanol in previous studies.[9−11] However reports on the γ-oryzanol hydrolyzing capacity of pancreatin (a mixture of pancreatic enzymes, containing lipase, amylase, protease, cholesterol esterase, and other enzymes) are contradictory. Miller and co-workers[9] found pancreatin to hydrolyze γ-oryzanol, while Moreau and Hicks[10] observed no hydrolysis when incubating γ-oryzanol with pancreatin.

Ours was the first study of any kind to measure bioaccessibility of steryl ferulates. In order to quantitate bioaccessibility, the steryl ferulate content of the supernatant was compared to the total extractable steryl ferulate content of the sample and it was found to be between 0.0% and 0.2% for polished rice, between 0.2% and 0.5% for cargo rice, 0.1% for wild rice and wheat bran, 0.4% for corn bran, and 1.5% for rice bran. These findings are in accordance with the literature available on the bioaccessibility of the two moieties of steryl ferulates, namely, ferulic acid and the sterol, both showing low bioaccessibility. Ferulic acid bioaccessibility from grain samples ranged from 0.4% to 5% in animal and human studies.[31−33] Sterol bioaccessibility has not been investigated from grains; data is only available for pure sterols. For these compounds absorbability ranged from 0% to 12%, depending on the side chains of the sterol.[17] In addition, Heinemann and co-workers[17] concluded that an inverse relationship exists between the absorbability and the cholesterol lowering efficiency of the sterol. Therefore, the very low bioaccessibility from grain samples suggests that steryl ferulates are practically not absorbed from the upper intestinal tract. This indicates that steryl ferulates do not necessarily need to be absorbed in order to be bioactive, hence they must exert their bioactivity in the lumen of the gut. However their exact mode of action remains unclear.

Free Sterol Content before and after Digestion. In parallel with the steryl ferulate content, the free sterol content before and after digestion was also monitored in polished rice, cargo rice, wild rice, rice bran, corn bran, and wheat bran (Table 2). Free sterol content of nondigested polished rice ranged from 34.8 μg/g to 79.2 μg/g. Free sterol content of nondigested cargo rice was found to be 4-fold higher; it ranged between 197 μg/g and 238 μg/g. Free sterol content of nondigested wild rice (117 μg/g) was between that of polished and cargo rice, similarly as in the case of γ-oryzanol. As for the differences within the same rice grown at different locations, a similar pattern could be observed as in the case of steryl ferulates. The free sterol contents of the two Jasmine rice cultivars were closer to each other than the free sterol contents of the two Basmati rice cultivars. Free sterol content of Jasmine rice from Thailand and Cambodia was 49.5 μg/g and 40.7 μg/g, compared to 75.9 μg/g and 36.5 μg/g for Basmati rice from India and Pakistan, respectively. Similarly to steryl ferulates, no difference could be seen between short or medium and long grain cultivars. Free sterol content of rice bran was found to be 1110 μg/g, whereas corn bran exhibited the highest free sterol content, namely, 2020 μg/g. The free sterol content of wheat bran was 667 μg/g. Earlier reports on these grains take into account the various steryl esters, in addition to free sterols.[34] Therefore our data are not directly comparable with previous findings.

Due to digestion, free sterol content increased by at least 70% in all samples. In corn bran and wheat bran, the increase was 70% and 74%, respectively. Polished rice exhibited an increase of 247% on average, cargo rice increased by 79%, rice bran by 119%, and wild rice by 145%. The increase in the free sterol content could be explained by the observed decrease in the steryl ferulate content, knowing that free sterols are hydrolysis products of steryl ferulates. Accordingly, a higher hydrolysis rate of γ-oryzanol in polished rice (20−41%) induced a higher increase in free sterols (135−404%), whereas a lower hydrolysis rate of γ-oryzanol in cargo rice (1−4%) induced a lower increase in free sterols (70−86%). Nevertheless free sterols are also the hydrolysis products of other steryl conjugates present in grains, such as steryl glycosides, acylated steryl glycosides, and steryl fatty acid esters, which might also have hydrolyzed during the in vitro digestion. However, steryl glycosides were recently shown not to be cleaved into their glucose and free sterol moieties during digestion in mice.[35] This suggests that the increase in free sterols, which does not result from the hydrolysis of steryl ferulates, would originate from the hydrolysis of steryl fatty acid esters. Steryl fatty acid esters are also known to be good substrates for cholesterol esterase.[36] Additionally, digestion induced increase in the free sterol content might also occur due to an increased extractability of free sterols. To our knowledge, digestion of steryl ferulates has only been investigated by Huang,[18] who observed a decrease in the steryl ferulate content but no increase in free sterols, concluding that free sterols were further degraded during digestion. In our study, however, it seems more probable that steryl ferulates were hydrolyzed to free sterols, which were not (or at least not fully) degraded in further processes.

Identification of Steryl Ferulate Species. The identity of cycloartenyl ferulate was confirmed by mass spectrometric

Table 4. Digestion Induced Changes in the Sterol Composition of Steryl Ferulates in Polished, Cargo and Wild Rice as Well as in Three Types of Bran: Cycloartenyl Ferulate (1), 24-Methylenecycloartanyl Ferulate (2), Campesteryl Ferulate (3), Sitosteryl Ferulate (4), Campestanyl Ferulate (5), and Sitostanyl Ferulate (6)[a]

	before digestion						after digestion					
	1	2	3	4	5	6	1	2	3	4	5	6
polished rice												
Carolina[b]	38.3	29.2	9.5	5.5	12.3	5.2	41.9	28.3	7.8	4.6	12.5	4.9
S. Andrea[b]	30.4	33.1	15.0	5.2	12.2	4.1	36.1	33.6	10.3	4.2	11.3	4.5
Carnaroli	34.0	32.8	11.5	2.8	12.6	6.3	33.6	32.0	11.8	3.5	12.3	6.8
Originario	26.4	34.3	17.8	5.8	10.7	5.0	29.6	33.5	17.8	4.8	10.0	4.3
Baldo	37.8	33.6	11.6	3.2	10.1	3.7	39.7	33.4	11.0	3.4	8.8	3.7
Loto	34.1	28.7	13.2	5.9	13.2	4.9	36.4	27.4	12.9	5.2	13.0	5.1
Perfume[b]	16.5	54.0	14.5	9.9	3.1	2.0	17.2	56.4	12.1	9.1	2.7	2.5
Jasmine (Thai)[b]	21.0	48.0	16.3	9.1	3.4	2.2	21.7	50.2	12.2	9.8	3.4	2.7
Jasmine (Cam)[b]	17.9	48.4	18.1	9.9	3.2	2.5	19.7	49.9	13.3	10.2	3.8	3.1
Basmati (Ind)[b]	30.9	28.5	24.6	3.1	10.2	2.7	36.2	26.3	21.9	11.8	2.5	1.3
Basmati (Pak)	28.7	40.7	18.0	2.7	8.9	1.0	29.1	38.6	17.6	9.4	3.1	2.2
cargo rice												
S. Andrea	32.2	32.0	14.4	4.1	12.1	5.2	29.1	32.7	10.7	3.8	15.9	7.8
Baldo	37.1	32.9	11.8	4.6	9.7	3.9	37.1	32.3	11.5	3.4	11.2	4.5
Perfume	16.2	54.4	13.8	10.1	3.0	2.5	15.1	53.2	12.7	11.3	3.2	4.5
Basmati	32.2	32.4	21.9	3.1	9.3	1.1	33.5	31.9	20.3	2.7	10.1	1.5
Camargue	39.3	25.1	12.2	4.6	13.5	5.3	36.9	25.8	11.0	4.9	15.0	6.4
wild	19.6	24.2	24.4	5.9	14.4	11.5	18.7	23.7	23.7	6.1	15.8	12.0
rice bran	27.9	35.1	10.3	6.9	11.8	8.0	27.5	36.1	8.6	6.8	12.1	8.9
corn bran			8.8	4.4	28.2	58.7			14.8	5.4	21.9	58.0
wheat bran[c]			20.7	3.9	45.6	29.8			20.1	5.3	44.7	29.9

[a]Values were calculated as the ratios of peak integrals [%]. For all values SD $\leq \pm 1.0$. [b]Change in the ratio of 4,4′-dimethyl and 4-desmethyl steryl ferulates due to digestion is statistically significant. [c]In-house reference sample, $n = 10$.

determination, based on a reference substrate. Due to lack of reference material available, other steryl ferulate species were only tentatively identified (Table 3). We found the same order of the six steryl ferulate species in question as Hakala and co-workers,[26] who used similar HPLC conditions to ours. As for the mass spectrometric measurement, the deprotonated molecular ions of all individual steryl ferulates could be detected in the negative ion mode. Additionally, collision-induced decomposition (CID) of deprotonated molecular ions yielded characteristic fragment ions, such as $[M - H - Me]^-$, $[M - H - 2Me]^-$, deprotonated ferulic acid at m/z 193, and a fragment derived from deprotonated ferulic acid at m/z 175. These findings were well in accordance with the observations of Fang and co-workers.[37]

Sterol Composition of Steryl Ferulates before and after Digestion. The sterol composition of steryl ferulates was determined in polished rice, cargo rice, wild rice, rice bran, corn bran, and wheat bran before and after digestion (Table 4). Our study was the first to investigate the sterol composition of steryl ferulates in wild rice, a species not closely related, however belonging to the same family as rice, the family of Poaceae. To our knowledge, this study was also the first to investigate the steryl ferulate composition of both the polished and the brown form of a rice cultivar. The most remarkable difference within the various grains was that corn bran and wheat bran contained only 4-desmethyl sterols, while rice milling fractions and wild rice contained both 4,4′-dimethyl and 4-desmethyl sterols. This could eventually explain the differences in the digestion induced changes in their steryl ferulate contents, namely, that steryl ferulate content of corn bran and wheat bran decreased by 53% and 63% respectively, whereas γ-oryzanol content of rice bran decreased only by 5%.

In corn, sitostanyl ferulate was most abundant (approximately 60%), whereas in wheat campestanyl ferulate accounted for almost 50% of all ferulated sterols. These results were in good accordance with earlier findings.[1] Polished rice, cargo rice (including brown and red rice), and rice bran all showed similar results for the 4,4′-dimethyl/4-desmethyl sterol ratio, namely, 65:35, on average, which was also in good agreement with earlier reports.[3,38] In wild rice, the 4,4′-dimethyl/4-desmethyl sterol ratio was found to be 44:56. Sterol composition of steryl ferulates did not significantly vary within the polished and the brown form of a cultivar (S. Andrea, Baldo, Perfume, and Basmati). Although the ratio of 4,4′-dimethyl and 4-desmethyl sterols was found to be similar for polished rice, cargo rice, and rice bran, differences in the ratio of the two 4,4′-dimethyl steryl ferulates did occur. In almost all samples, cycloartenyl ferulate ranged from 26.4% to 39.3% and 24-methylenecycloartanyl ferulate accounted for 25.1−40.7% of steryl ferulates. Two varieties, however, namely, Perfume (from Thailand) and Jasmine (from both India and Pakistan), showed a different sterol composition. In these, cycloartenyl ferulate ranged from 16.5% to 21.0% and 24-methylenecycloartanyl ferulate ranged between 48.0% and 54.0%. Interestingly, in addition to the difference in the sterol composition, Jasmine rice was already found to be exceptional due to its very low γ-oryzanol content.

During digestion, the sterol composition of steryl ferulates in wheat bran remained unchanged (Table 4). In corn bran, the proportion of campesteryl ferulate significantly increased (by 5.9%), while the proportion of its saturated counterpart, campestanyl ferulate, significantly decreased (by 6.2%). This is in compliance with the findings that nonesterified stanols from corn oil have a higher ability to significantly lower cholesterol than nonesterified sterols.[14] Thus, our results suggest that

hydrolysis of steryl ferulates is needed for their cholesterol lowering effect to take place. In rice samples and wild rice, where both 4,4′-dimethyl and 4-desmethyl sterols are present, we expected a decrease in the proportion of 4-desmethyl sterols and an increase in the proportion of 4,4′-dimethyl sterols. In 6 samples out of 18, the change in the ratio was significant (Table 4). In all other samples, the change in the ratio was not significant. It only altered by 1−2% in both directions, which could also result from the analytical method used and not necessarily from the difference in hydrolysis of different molecular species. These findings, together with the changes in the overall steryl ferulate and free sterol content, seem to indicate that hydrolysis of steryl ferulates did occur due to digestion and that in most samples 4-desmethyl sterols were hydrolyzed to a higher extent than 4,4′-dimethyl sterols. Taking into account that hydrolysis might be a key step in the plasma cholesterol lowering mechanism of steryl ferulates, this compositional difference could be of great importance. It would mean that although the steryl ferulate content of wheat and corn is lower, they could be more efficient in lowering cholesterol than steryl ferulates from rice, which contains a high proportion of 4,4′-dimethyl sterols.

A digestion induced steryl ferulate decrease as well as free sterol increase could be seen in all examined samples. This likely originated from the hydrolysis of steryl ferulates. The sterol composition of steryl ferulates was also measured, and in addition to the differences among various grains, a difference in the hydrolysis rate of 4,4′-dimethyl and 4-desmethyl sterols was found. In the majority of samples, 4-desmethyl sterols showed a higher hydrolysis rate than 4,4′-dimethyl sterols, suggesting that 4-desmethyl steryl ferulates could play a more important role in cholesterol lowering than 4,4′-dimethyl steryl ferulates. In addition, bioaccessibility of steryl ferulates from different grain samples was found to be between 0.0 and 1.5%. This result indicates that steryl ferulates are virtually not available for absorption in the upper gastrointestinal tract. Instead, they hydrolyze into free sterol and ferulic acid, which exert cholesterol lowering and antioxidative activities. Based on our findings, further studies could be conducted with more elaborate dynamic in vitro digestion models, or even with animal or human models. These approaches might provide improved understanding of the exact mechanism of action of steryl ferulates.

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Notes

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