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Steryl Phenolic Acid Esters in Cereals and Their Milling Fractions

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The steryl ferulate contents of rye and wheat grains and their milling fractions were analyzed using a reversed-phase high-performance liquid chromatographic (HPLC) method. HPLC−mass spectrometry was used for identification. In addition, steryl ferulates of some selected milling byproducts were determined. The total steryl ferulate contents of rye and wheat grains were 6.0 and 6.3 mg/100 g, respectively. Uneven distribution of steryl ferulates in the grains led to considerable differences in the milling products; their steryl ferulate contents ranged from trace amounts in flours with low ash content to 20 and 34 mg/100 g in rye and wheat brans, respectively. Campestanyl ferulate and sitostanyl ferulate were the main components, followed by campesteryl ferulate and sitosteryl ferulate, whereas sitosterol was the main component in total sterols. Among the other samples, a byproduct of rice milling (pearling dust) was the best source of steryl ferulates, its total steryl ferulate content being 119 mg/100 g, whereas no measurable amounts of steryl ferulates were measured in oat bran or pearling dust of barley. The results indicated that rye and wheat and especially their bran fractions are comparable to corn as steryl ferulate sources.

KEYWORDS: Plant sterols; phytosterols; steryl phenolic acid ester; steryl ferulate; wheat; rye; milling fraction; byproducts

INTRODUCTION

Plant sterols occur in plants as free sterols and conjugated forms, that is, steryl esters of fatty or phenolic acids, steryl glycosides, and acylated steryl glycosides (*1*). These lipids have received increasing attention in recent years because of their positive effects on health. They have been shown to reduce serum total cholesterol in several studies (e.g., refs *2*−*5*). They may also inhibit development of certain types of cancer (*6*). The focus has mainly been on steryl fatty acid esters and free sterols. However, the significance of steryl phenolic acid esters has also aroused research interest.

Research into steryl phenolic acid esters has mainly focused on rice, which has been shown to contain considerable amounts of steryl ferulates (*7*−*9*). This interest has led to the development of a commercial product named γ-oryzanol, which is a mixture of steryl ferulates from rice bran oil. A number of components have been identified in γ-oryzanol: cycloartenyl, 24-methylenecycloartanyl, campesteryl, Δ^7-campestenyl, sitosteryl, Δ^7-sitostenyl, stigmasteryl, Δ^7-stigmasteryl, and sitostanyl and campestanyl ferulates (*7*, *8*, *10*−*12*). The chemical structures of the main steryl ferulates are shown in **Figure 1**.

Earlier results on health benefits associated with γ-oryzanol include decreasing plasma cholesterol (*13*, *14*), platelet aggregation (*15*), cholesterol absorption from high-cholesterol diets (*16*, *17*), and aortic fatty streaks (*17*). In these studies both human and animal tests have been used. A mild but clear hypocholesterolemic effect was observed when 300 mg of γ-oryzanol was administered daily to 67 subjects for 3 months (*14*). The significance of γ-oryzanol in decreasing cholesterol absorption and aortic fatty streaks was investigated in hamsters by feeding chow-based diets with 0.5−1.0% of γ-oryzanol for 7−10 weeks (*17*). This study showed that γ-oryzanol induced lower cholesterol absorption without changing de novo cholesterol synthesis in the intestinal mucosa. In a recent human study, in which the subjects received 30 g/day of margarine enriched with various plant sterols for 3.5 weeks, it was found that γ-oryzanol was less effective than the fatty acid esters of the common 4-desmethyl sterols and stanols (i.e., sitosterol, campesterol, and the respective stanols) in lowering total and low-density lipoprotein (LDL) cholesterol (*18*). The authors suggested that the difference may be partly due to the lower intake

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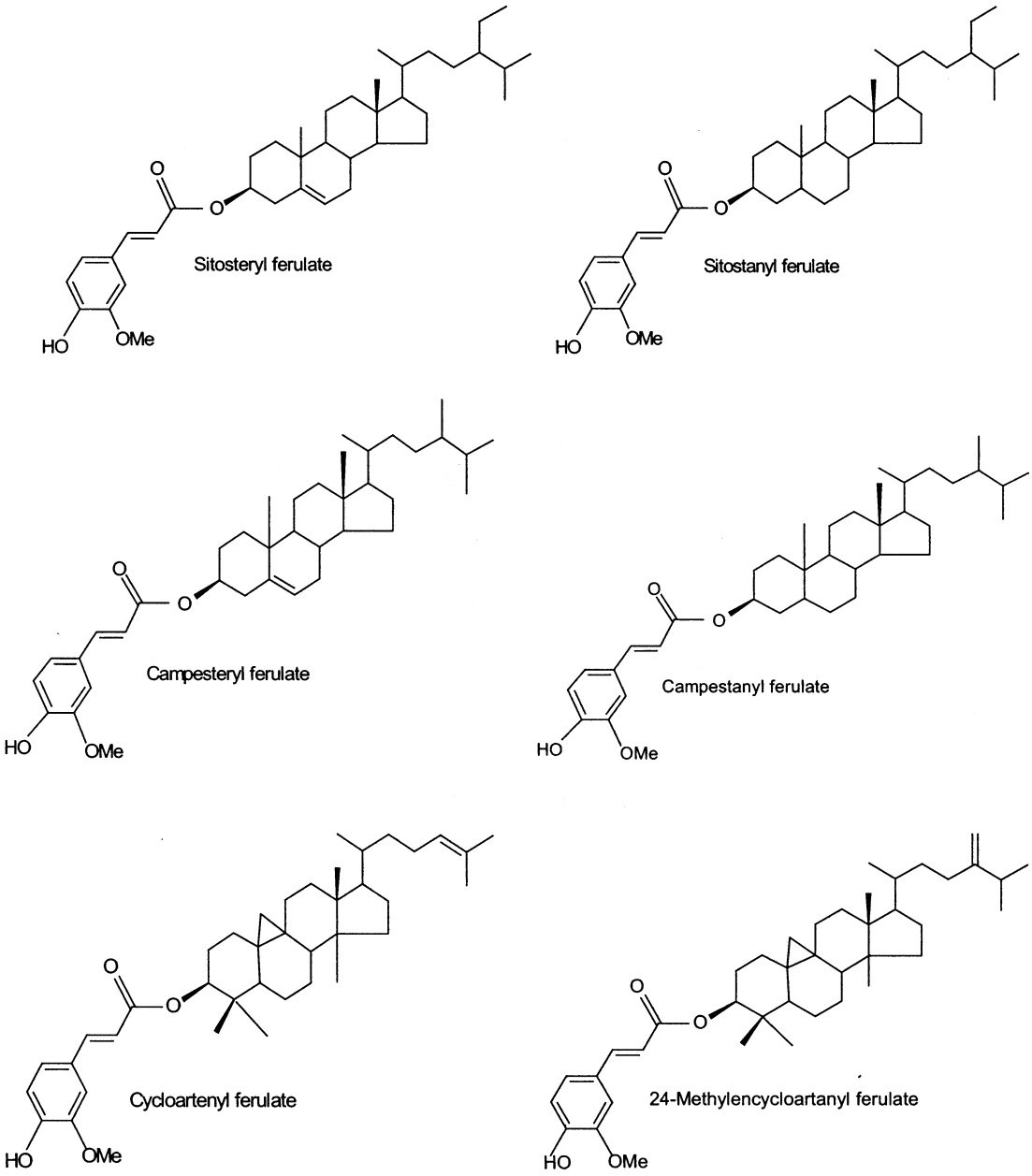

Figure 1. Molecular structures of sitosteryl, sitostanyl, campesteryl, campestanyl, cycloartenyl, and 24-methylenecycloartanyl ferulates.

of sterols from the γ-oryzanol-enriched margarine (1.5 g/day) than from the margarines enriched with other plant sterols (3.3 g/day) and partly due to the structure of the sterols in γ-oryzanol that may be less effective in lowering cholesterol absorption. Furthermore, the cycloartenyl ferulate component of γ-oryzanol has been shown to inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in two-stage carcinogenesis in mouse skin (*19*).

Moreover, γ-oryzanol is a potential antioxidant due to its phenolic moiety, which is able to donate a hydrogen atom and form a peroxyl radical in a similar way to other phenolic antioxidants (*20*). It has been shown to retard oxidation in various lipid systems as a purified preparation (e.g., refs *21* and *22*) and as a constituent of the rice bran unsaponifiable fraction (*23*). Moreover, campesteryl ferulate has also shown to possess antioxidant activity (*24*). The large molecular size and highly nonpolar structure of steryl ferulates may affect their partitioning in matrices and thus their accessibility and potential as antioxidants.

Other studies on steryl phenolic acid esters in cereals have mainly dealt with utilization of corn (*8*, *25*−*29*). Studies on other cereals are rare and mainly focused on identification of steryl ferulate structures (*25*). In corn bran, sitosteryl, sitostanyl, stigmasteryl, campesteryl, campestanyl, Δ^7-sitosteryl, and Δ^7-campesteryl ferulates were identified (*8*, *27*). Furthermore, Seitz (*25*) found minor amounts of sitostanyl and campestanyl *p*-coumarates from corn. In wheat, rye, and triticale grains, he identified sitostanyl, campestanyl, sitosteryl, and campesteryl ferulates. In addition, Moreau et al. (*28*) found minor amounts of steryl ferulates in barley grains but none in oats, flax, and millet grains.

The concentrations of steryl phenolic acid esters in corn grain and in some grain fractions have been determined (*8*, *9*, *25*), but quantitative studies on cereals other than corn and rice are rare. For wheat, barley, oats, and rye only whole grains have been studied (*9*, *25*). Steryl phenolic acid esters are known to be highly localized within corn kernels (*8*, *25*). Therefore, data on the quantitative distribution of steryl ferulates in these other

Table 1. Steryl Ferulate and Total Sterol Contents in Milling Fractions of Cereals (Milligrams per 100 g Wet Basis)[a]

sample[b]	campesteryl ferulate	sitosteryl + campestanyl ferulate	sitostanyl ferulate	total steryl ferulates	total sterols
wheat grain					
A	1.2 ± 0.1	3.3 ± 0.1	1.8 ± 0.1	6.3 ± 0.3	63.8 ± 0.4
B	1.1 ± 0.1	3.2 ± 0.2	1.9 ± 0.1	6.2 ± 0.4	59.2 ± 0.4
wheat bran					
A	6.4 ± 0.5	15.6 ± 1.2	9.1 ± 0.7	31.1 ± 2.4	159.6 ± 2.4
B	5.7 ± 0.4	14.8 ± 3.6	9.2 ± 1.2	29.7 ± 0.4	150.3 ± 1.8
C	6.8 ± 0.3	18.3 ± 0.8	11.4 ± 0.5	36.5 ± 1.6	147.9 ± 1.9
C	7.9 ± 0.4	20.0 ± 0.8	11.2 ± 0.6	39.0 ± 1.8	166.8 ± 4.9
wheat enriched flour (ash content = 4.5%)					
A	4.4 ± 0.2	11.0 ± 0.7	6.2 ± 0.4	21.6 ± 1.3	182.3 ± 5.3
B	3.8 ± 0.1	9.8 ± 0.1	5.8 ± 0.1	19.4 ± 0.2	167.9 ± 2.1
wheat flour (ash content = 0.6%)					
A	Tr[c]	Tr	Tr	Tr	34.9 ± 0.4
B	Tr	Tr	Tr	Tr	33.0 ± 0.7
rye grain					
A	0.8 ± 0.1	2.9 ± 0.1	1.8 ± 0.1	5.5 ± 0.3	84.5 ± 1.2
B	1.1 ± 0.1	3.3 ± 0.2	2.1 ± 0.2	6.4 ± 0.4	82.3 ± 0.4
rye bran					
A (ash content = 4.5%)	2.5 ± 0.4	7.6 ± 1.0	4.9 ± 0.7	15.0 ± 2.1	147.8 ± 1.0
B (ash content = 4.5%)	3.7 ± 0.1	10.6 ± 0.3	6.8 ± 0.2	21.0 ± 0.6	151.8 ± 0.9
C	5.4 ± 0.2	12.7 ± 0.4	7.0 ± 0.3	25.1 ± 0.9	147.8 ± 1.0
rye flour (ash content = 0.7%)					
A	Tr	Tr	Tr	Tr	42.5 ± 0.5
B	Tr	Tr	Tr	Tr	40.9 ± 0.1
oat bran					
C	–[d]	–	–	–	56.1 ± 1.3
C (with β-glucan)	–	–	–	–	57.3 ± 0.5
C	–	–	–	–	55.7 ± 1.1
C	–	–	–	–	62.0 ± 0.6
pearling dust of barley					
C	–	–	–	–	150.8 ± 2.0

[a] Mean ± SD (n = 3). [b] A and B represent two sets of milling fractions. C represents individual samples (see Materials and Methods). [c] Tr, steryl ferulate content < 0.5 mg/100 g wb. [d] –, not detected.

cereals are needed. Furthermore, even the results for corn are inconsistent. This may be due to differences in the samples and the analytical methods used. In previous studies, the validation of the method, for example, the recovery of steryl ferulates after the extraction or cleanup procedures, has not been documented.

We have been interested in cereals as natural sources of sterols and their conjugates (*30*). In Finland cereal products were shown to be the main natural dietary plant sterol sources (*31*). The aim of this study was to investigate the significance of cereals and their milling fractions as steryl ferulate sources. The focus was on rye and wheat and their milling process. In addition, steryl ferulates in some milling byproducts known to contain considerable amounts of total plant sterols were investigated. Steryl ferulates were determined by a validated reversed-phase high-performance liquid chromatographic method (HPLC). Their identification was confirmed using an HPLC−mass spectrometric (HPLC-MS) method with atmospheric pressure chemical ionization (APCI).

MATERIALS AND METHODS

Samples. Cereal samples were obtained from three food companies (Oy Karl Fazer Ab, Oululainen Mill, Helsinki's Mill, and Melia Co., all located in Finland). Grains and corresponding milling fractions of rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) were purchased on two occasions from one milling company (**Table 1**, samples A and B). The milling fractions included brans and flours, and furthermore wheat flours were enriched with the aleurone layer. Sampling was supplemented by purchasing a collection of wheat, rye, and oat (*Avena sativa* L.) bran samples, and, in addition, two milling byproducts, pearling dust of rice and barley (*Hordeum vulgare* L.), were included (**Table 1**, samples C). Samples of 500 g−2 kg were delivered to the laboratory. Grains of rye and wheat were milled to pass a 0.8 mm sieve (pin-mill KT-30, Koneteollisuus Oy). Cereal samples were stored at − 20 °C before sterol analysis.

Chemicals and Materials. All organic solvents were of HPLC grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Potassium hydroxide (Eka Nobel Ab, Surte, Sweden), hydrochloric acid (36−38%, J. T. Baker), acetic acid (100%, J. T. Baker), ethanol (from Primalco Oy), and water (purified by Milli-Q Plus, Molsheim, France) were used. Cycloartenyl ferulate was used as an external standard in quantitative steryl ferulate analyses. It was kindly provided by Dr. P. Kochhar (Good-Fry International n.v., Rotterdam, The Netherlands). Cholesteryl ferulate and sitosteryl ferulate were synthesized by Dr. K. Wähälä (Department of Chemistry, University of Helsinki, Finland) and used to optimize the instrumental parameters of the HPLC-MS. Sterol composition of cholesteryl and sitosteryl ferulates was confirmed by a gas chromatographic (GC) method, which has been described earlier (*30*, *32*). For total sterol analysis by GC dihydrocholesterol (3β-hydroxy-5α-cholestane, Sigma Chemical Co., St. Louis, MO) was used as an internal standard.

Extraction and Base—Acid Cleanup of Steryl Ferulates. Steryl ferulates were extracted from cereal samples according to the method of Seitz (*25*) with some modifications (**Figure 2**). Cereal samples (about 2 g) were weighed into screw-capped 100 mL extraction bottles and extracted with 20 mL of acetone by shaking with a wrist shaker at ambient temperature for 60 min. After extraction, the samples were filtered through a GFA filter paper into 250 mL round-bottom flasks. The cake was rinsed with acetone and transferred back to the extraction bottle. The extraction was repeated as described above, and the acetone extracts were collected into the same round-bottom flask. After the solvent was completely evaporated in a rotavapor at 50 °C, the residue was dissolved in 6 mL of methanol and was made basic (pH ∼10) by adding 1 mL of 0.6% KOH. Under these conditions, the steryl ferulates were anionic and remained in the alkaline aqueous phase, whereas the neutral lipids, which were present in huge excess, were partitioned into hexane (*10*). The washing of the neutral lipids was repeated.

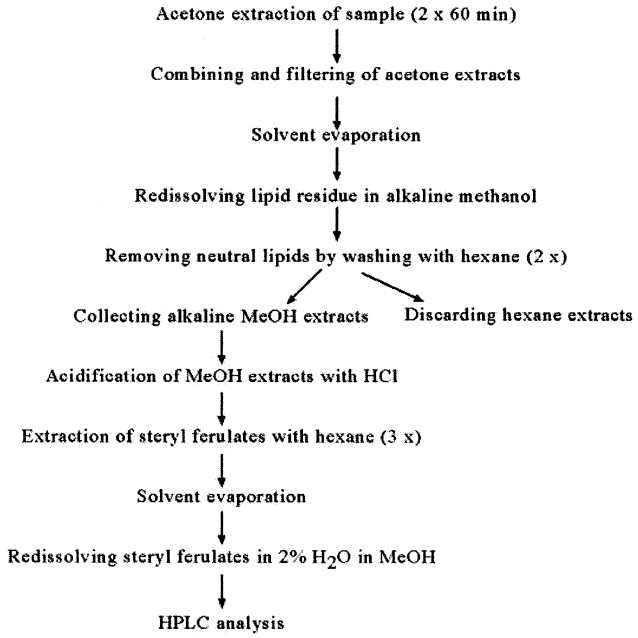

Figure 2. Outline of procedure for extracting steryl ferulates from cereal samples.

During method development, it was confirmed that hexane washing of the alkaline methanolic extract did not remove steryl ferulates. The hexane extracts were extracted twice with 6 mL of methanol and 1 mL of 0.6% KOH, and the methanolic extracts were analyzed by HPLC. Because no steryl ferulates could be extracted from the hexane layers, they could be discarded without any loss of steryl ferulates.

The combined alkaline aqueous phases were acidified (pH ~1) with 0.6 mL of 6 M HCl and partitioned three times with hexane (total of 5 mL) to transfer the steryl ferulates into hexane. The hexane extracts were combined and evaporated under nitrogen flow at 50 °C. Finally, the residue was redissolved in 5 mL of methanol/H_2O (98:2, v/v) and filtered, using a 0.45 μm, Gelman GHP Acrodisc syringe filter, before analysis by HPLC. Each sample was extracted in triplicate, and each extract was analyzed by HPLC using duplicate injections.

HPLC Analysis of Steryl Ferulates. Steryl ferulates were analyzed by a high-performance liquid chromatograph (model 1090, Hewlett-Packard, Böblingen, Germany) using a C18 reversed-phase column (5 μm, 4.6 × 250 mm, ODS-2, Waters Spherisorb, Wexford, Ireland) and methanol/water/acetic acid (97:2:1) as the mobile phase. The flow rate was 1.5 mL/min, and the column temperature was 50 °C. The effluent was monitored using a diode array detector set at 325 nm, and the spectrum was measured between 220 and 400 nm with a step wavelength of 2 nm.

The steryl ferulate contents of the samples were quantified by an external standard method using cycloartenyl ferulate as the standard. To study the linearity of the HPLC response, duplicate injections of cycloartenyl ferulate at six concentration levels between 2 and 100 μg/mL were determined. The correlation coefficient of the calibration curve was 0.99965. In addition, a standard curve at three concentration levels was determined daily. The concentration of the cycloartenyl ferulate stock solution was confirmed daily using a spectrophotometrical method (ferulic acid $\epsilon = 19500$ L mol^{-1} cm^{-1}, $\lambda = 328$ nm; *10*).

Validation of the HPLC Method. The accuracy of the method was monitored by determining the recoveries of cycloartenyl ferulate and cholesteryl ferulate from a rye bran sample. The ferulates were added to rye bran samples (2 g) at two levels (0.2 and 0.5 mg) before extraction. After the extraction and cleanup procedures, the recoveries of added cycloartenyl and cholesteryl ferulates were very similar, 83.5 ± 4.1% ($n = 6$) and 83.8 ± 3.3% ($n = 5$), respectively, which indicates that differences in the steryl ferulate structure have no effect on the recoveries. Thus, the method is capable of analyzing various steryl ferulates in the same way.

The analytical level of the method was confirmed by determining steryl ferulates in the same rye bran sample on each day of analysis. The day-to-day variation of steryl ferulates in this rye bran sample was low, the total content being 35.5 ± 2.0 mg/100 g ($n = 16$); thus, the level of analysis was stable. In addition, an in-house reference extract of rye bran, which was stored at −20 °C, was monitored daily for variation in the detector response and retention times of the HPLC system. The steryl ferulate contents of the in-house reference extract were stable, 35.9 ± 1.4 mg/100 g ($n = 16$); the variation was similar to the day-to-day variation of the rye bran sample. The limit of detection (3 times the height of the noise level) and quantification (twice the level of the detection limit) determined with cycloartenyl ferulate were 0.01 and 0.02 μg/injection, respectively.

Identification of Steryl Ferulates by HPLC-MS. The identification of steryl ferulates was confirmed by an HPLC-MS analysis, in which the separation was based on the HPLC procedure indicated above. The HPLC conditions were similar except that the mobile phase was methanol/water (98:2). The mass spectrometric detection was carried out with an ion-trap mass spectrometer (Esquire-LC, Bruker Daltonik, Bremen, Germany) using APCI in positive ion (PI) and negative ion (NI) mode. The eluent flow of liquid chromatography was split (1:10). The daily variation in the APCI procedure was monitored by measuring the 609.7 *m/z* value of protonated reserpine ($[M + H]^+$, an 82 μM solution of reserpine dissolved in 2-propanol). The reserpine solution was applied to the ion source using a direct flow injection technique, the flow rate being 200 μL/min. Collision-induced dissociation (CID) was produced with helium (99.96%, AGA) at a pressure of ~10^{-6} mTorr (130 nPa) in the ion trap. Furthermore, the applicability of the method was tested by cholesteryl and sitosteryl ferulates.

In the NI mode, the temperature and flow rate of the dry gas (nitrogen) were set at 350 °C and 7 L/min, respectively, and the APCI interface temperature was 400 °C. The pressure of the nebulizer gas (nitrogen) was 50 psi (340 kPa), and the voltage of the corona discharge needle was −1.5 kV. Skimmer 1, the trap drive, and the capillary exit offset were set at −55.0, 50.0, and −55.0 V, respectively. In the PI, the temperature and flow rate of the dry gas were set at 350 °C and 5 L/min, respectively. The APCI interface temperature was 350 °C, and the pressure of the nebulizer gas was again 50 psi. The voltages of the corona discharge needle, skimmer 1, trap drive, and capillary exit offset were 2.3 kV and 30.0, 45.0, and 60.0 V, respectively.

Analysis of Total Plant Sterols by GC. To relate the amounts of steryl ferulates to those of total plant sterols, we determined plant sterol contents of all samples by GC. The GC method used was described earlier (*30*). Briefly, the cereal sample was acid hydrolyzed with 6 M HCl and saponified with KOH, and the nonsaponifiables were extracted with cyclohexane. The sterol extract was further purified by SiOH solid-phase extraction. Finally, free sterols were derivatized to their trimethylsilyl ethers (TMS) and analyzed by GC. The GC conditions and parameters were similar to those described by Piironen et al. (*30*) except that the carrier gas velocity was 1.4 mL/min.

RESULTS AND DISCUSSION

In this study, the total steryl ferulate contents of rye and wheat and their milling fractions and those of some milling byproducts of other cereals were analyzed using a validated HPLC method for their quantification and HPLC-MS for identification. In addition, the total plant sterol contents were determined by GC in order to be able to relate the steryl ferulate contents to those of the total sterols.

Identification of Steryl Ferulates. As illustrated by typical reversed-phase HPLC chromatograms of rye bran, wheat bran, and pearling dust of rice (**Figure 3**), the steryl ferulates of the rye and wheat milling fractions were separated to three peaks and those of pearling dust of rice to three major and some minor peaks by the chromatographic method used. The steryl ferulate profile in the rye and wheat bran samples resembled those obtained previously for whole grain rye and wheat using a reversed-phase HPLC system (*25*). Moreover, the ultraviolet spectra were similar (data not shown). On the basis of GC-MS analyses, Seitz (*25*) proposed that the three major steryl ferulates

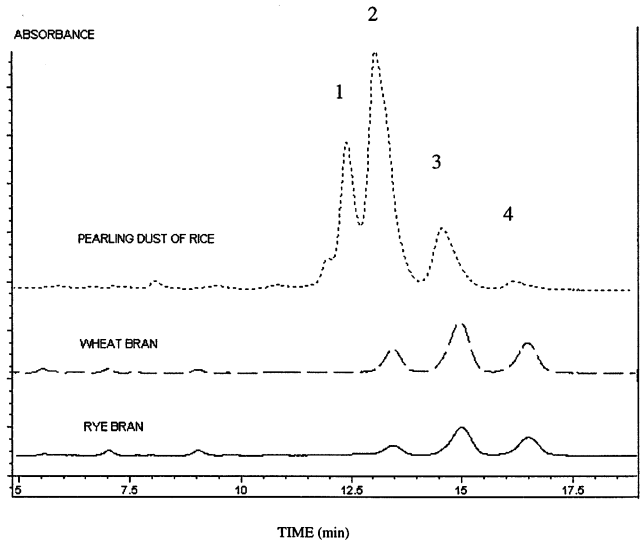

Figure 3. HPLC chromatograms of rye bran, wheat bran, and pearling dust of rice (column, reversed-phase C18; mobile phase, methanol/water/acetic acid (97:2:1); flow rate, 1.5 mL/min; diode array detector, $\lambda = 325$ nm; for details see Materials and Methods): 1, cycloartenyl ferulate; 2, 24-methylenecycloartanyl ferulate and campesteryl ferulate; 3, campestanyl ferulate and sitosteryl ferulate; 4, sitostanyl ferulate.

in rye were campestanyl (54%), sitostanyl (31%), and campesteryl (15%) ferulates. In this study, the second peak of the three major ones in the rye and wheat bran samples was tentatively identified to contain sitosteryl ferulate by comparing its retention time to that of the synthesized compound.

Pearling dust of rice was analyzed to assist in identifying steryl ferulates of cereal samples by comparing the chromatograms to those previously obtained from rice bran or rice bran oil. Up to 10 compounds have been identified in rice bran oil (*7*, *8*, *22*). In our study, the two major compounds, that is, cycloartenyl and 24-methylenecycloartanyl ferulates, were eluted first with retention times of 12.4 and 13.0 min, respectively, followed by a third ferulate with a retention time of 14.6 min (**Figure 3**). A clearly smaller peak eluted with a retention time of 16.2 min. The similar retention behavior of the cycloartenyl ferulate standard compound as compared to the first peak of the pearling dust of rice extract supported the identification of this compound.

Because coelution of some steryl ferulates present in the samples might occur, positive identification of the steryl ferulates with only HPLC-DAD could not be done. Therefore, the identities of the steryl ferulates were confirmed by HPLC-MS using both NI and PI APCI. By using the NI mode, specific detection of each steryl ferulate was possible because a characteristic deprotonated anion $[M - H]^-$ was formed from each compound (**Figure 4a**; **Table 2**). Thus, the molecular mass of the sterol conjugate could be resolved. The PI mode was further used to confirm the identification. It allowed resolution of the sterol moiety of the steryl ferulates, because during ionization ferulic acid was cleaved from the ester yielding a cation $[M + H -$ ferulic acid$]^+$ (**Figure 4b**; **Table 2**). Moreover, a typical ion for ferulates (*m*/*z* 177) was present in all of the spectra studied. It corresponds to a positive ion of protonated ferulic acid minus water as a neutral fragment (*7*).

In earlier studies, identification of steryl phenolic acid esters in rice bran oil and cereal samples had been done by GC-MS. Generally, when using GC-MS the method includes alkaline saponification of the sample when the sterol and the phenolic acid moieties are separately analyzed (e.g., refs *12*, *25*, and *27*) or derivatization of the intact molecules to trimethylsilyl (TMS) ethers (*7*, *10*). The benefit of HPLC-MS used in this study was that it allowed us to confirm the identification of native steryl ferulates under conditions similar to those under which the compounds were analyzed using the quantitative HPLC-DAD method.

On the basis of the HPLC-MS analyses in NI and PI mode APCI, the first eluting compound of the milling fractions of rye and wheat was campesteryl ferulate, after which sitosteryl ferulate and campestanyl ferulate coeluted, followed by sitostanyl ferulate (**Figure 5**). Campestanyl ferulate was the main compound in the second peak contributing to ~85% of its ions. The PI mode APCI data confirmed a similar ratio of the two coeluting compounds, campestanyl and sitosteryl ferulates. Consequently, campestanyl ferulate was the major compound in the entire steryl ferulate fraction, and the proportion of sitostanyl ferulate was the second highest. The use of HPLC-MS made it possible to conclude that in the pearling dust of rice 24-methylenecycloartanyl and campestanyl ferulates coeluted. The other compounds were identified as cycloartenyl ferulate, sitostanyl ferulate, and sitosteryl ferulate (**Figure 5**).

Milling Process of Rye and Wheat. The total steryl ferulate contents of rye and wheat grains were of the same level, 5.5−6.4 and 6.2−6.3 mg/100 g, respectively (**Table 1**). Our result for wheat is in line with previous results; the result of Seitz (*25*) was 6−12 mg/100 g (depending on the variety) and that of Moreau et al. (*9*), 5.3 mg/100 g. On the other hand, only 3 mg/100 g of steryl ferulates was measured in rye grain (*25*). This study indicates that rye and wheat are comparable to corn as steryl ferulates sources; corn was shown to contain 3.1−7.0 mg/100 g (*25*) and 9.8−11.3 mg/100 g (*9*) of steryl ferulates.

Previously, Seitz (*25*, *26*) showed that steryl ferulates of corn grains were highly localized within the inner pericarp layer. Therefore, steryl phenolic acid esters were regarded as candidates for kernel pathogen resistance compounds for both fungi and insects (*27*). Recent studies of Moreau et al. (*33*) indicate that all steryl ferulates in corn are present in the single-cell aleurone layer. Our results also clearly showed the localization within rye and wheat grains. The range in the contents was considerable; the total steryl ferulate contents of rye and wheat milling products ranged from trace amounts (flours with low ash content) to average contents of 22 and 34 mg/100 g (bran), respectively (**Table 1**). The results showed that the higher the ash content of the sample, the higher was also the steryl ferulate content. For both the wheat and rye milling fractions the bran was thus shown to be the best source of steryl ferulates. Wheat bran contained about a quintuple level of steryl ferulates compared to the whole grain. The content of rye bran was a bit lower, although the levels of rye and wheat grains were similar. On the other hand, the distributions of the different steryl ferulates were similar in the different fractions and in the whole grains. To our knowledge, no previous data are available for the rye or wheat milling processes.

It was interesting to note that the total steryl ferulate contents of the bran samples varied considerably when the sampling was repeated; this variation was more pronounced when the samples were obtained from different companies. In the rye bran samples the total steryl ferulate content varied between 15 and 25 mg/100 g, and those of wheat bran varied between 31 and 39 mg/100 g. This indicated that in addition to differences in the grain raw material, also the way the milling process is carried out may affect the contents. If steryl ferulates are highly localized within the grain, even slight differences in separation of the fractions may have significant effects.

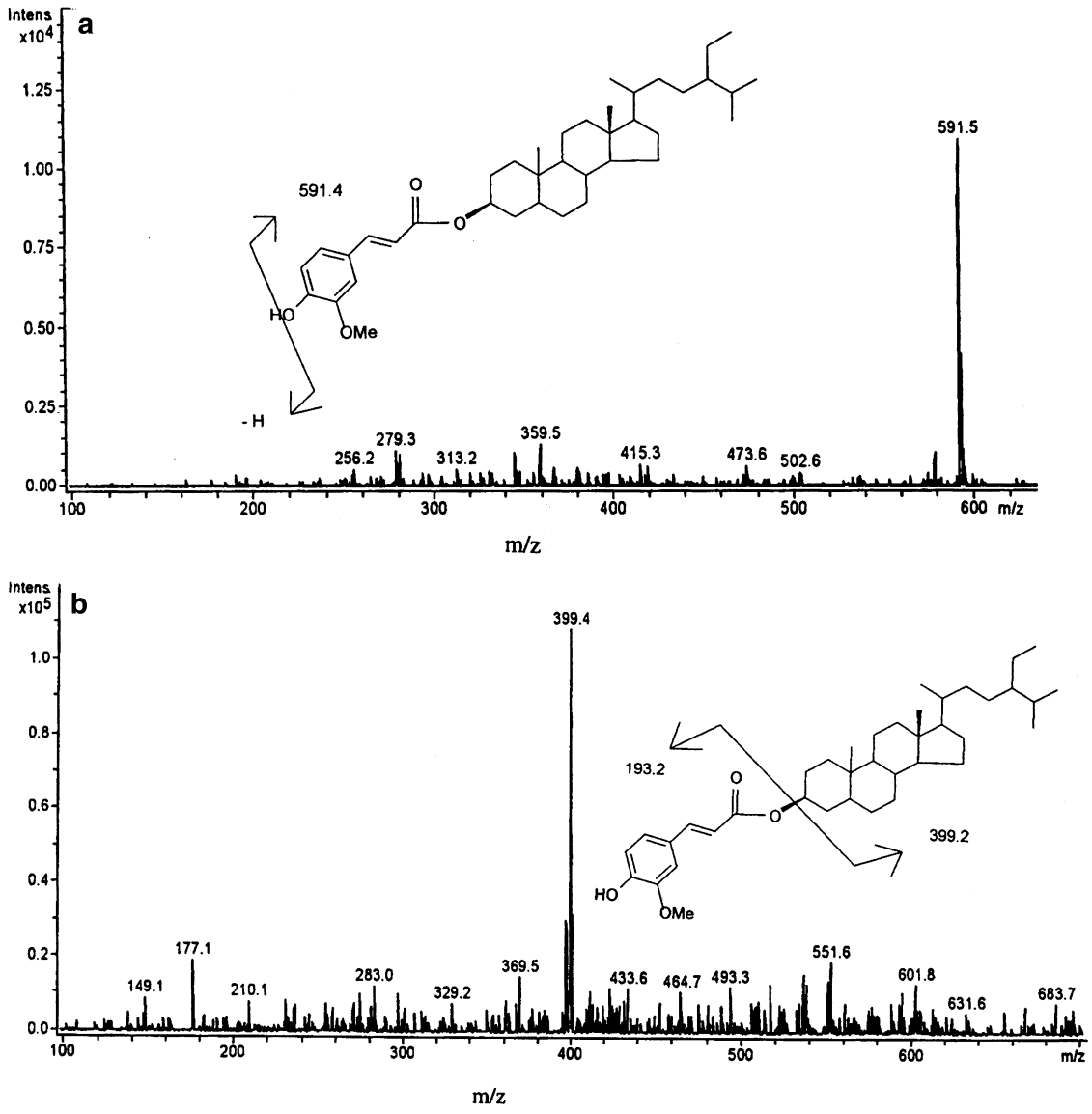

Figure 4. APCI mass spectra for sitostanyl ferulate using (a) negative ion and (b) positive ion mode after reversed-phase HPLC separation.

Table 2. Negative Ion (NI) and Positive Ion (PI) Chemical Ionization Mass Spectra of Steryl Ferulates

	characteristic *m/z* values	
steryl ferulate	NI $[M-H]^-$	PI $[M+H-\text{ferulic acid}]^+$
campesteryl ferulates	575.4	383.2
sitosteryl ferulates	589.4	397.2
campestanyl ferulate	577.4	385.2
sitostanyl ferulate	591.4	399.2
cycloartenyl ferulate	601.4	409.2
24-methylenecycloartanyl ferulate	615.4	423.2

The results in **Table 1** also show that the proportion of steryl ferulates from the total sterols differs in different parts of the kernel. The total sterol contents in the milling fractions of rye and wheat varied from 42 to 147 mg/100 g and from 35 to 182 mg/100 g, respectively, depending on the milling fractions. This is in agreement with the findings of Piironen et al. (*30*). In this study, 4−17% of the total plant sterol content was shown to consist of steryl ferulates. In wheat grain the proportion of steryl ferulates was 6−7%, whereas it was considerably higher, 13−17%, in wheat bran. For rye, the difference was smaller and more variable in repeated samplings; the corresponding figures were 4−5 and 6−11%, respectively.

Furthermore, the results (**Table 1**) showed that the plant sterol composition of the steryl ferulate fraction differs markedly from the total sterol composition of the corresponding milling fraction. Stanols predominated in the steryl ferulates (**Figure 4**), whereas their proportion of the total sterols was only 14−30% (data not shown). Campestanol was the main sterol in the steryl ferulates, whereas its proportion in total sterols was lower than that of sitostanol. In the total sterols, sitosterol was the main component. Previously, Norton (*8*) reported that stanols predominate in corn ferulates. Accordingly, the sterol composition of the ferulate fraction of rice bran differs considerably from the total sterol composition; the major sterols are 4,4-dimethylsterols cycloartenol and 24-methylenecycloartanol instead of sitosterol and campesterol, which dominate in total sterols (*8*, *12*).

Milling Byproducts of Other Cereals. Oat bran and byproducts of rice and barley milling (pearling dust) were also analyzed in this study. Neither oat bran nor the pearling dust of barley contained measurable amounts of the steryl ferulates, although the total sterol content of the pearling dust of barley (150

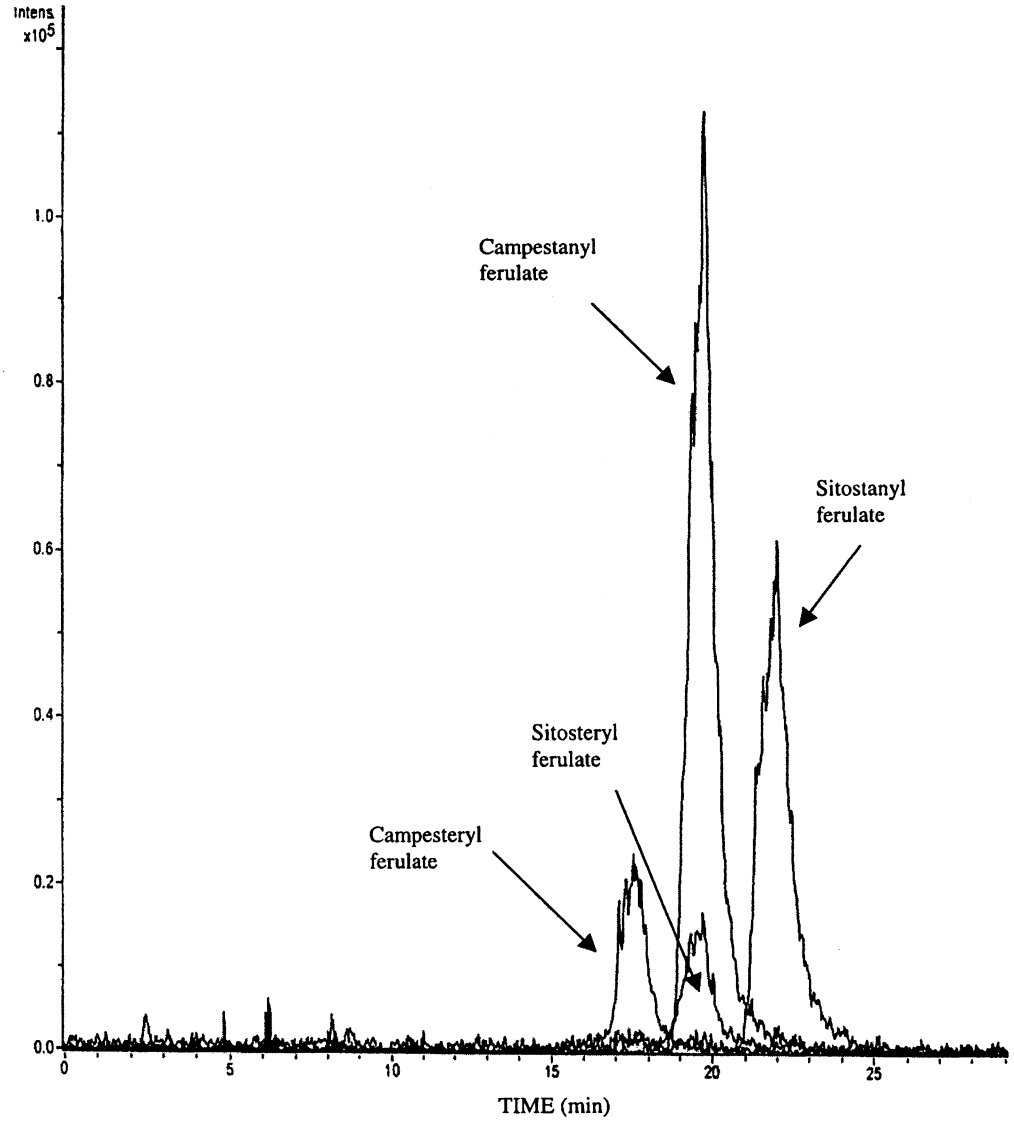

Figure 5. HPLC chromatogram of rye bran extract with selective ion monitoring (SIM) mass spectroscopy using APCI in negative ion mode (column, reversed-phase C18; mobile phase, methanol/water (98:2); flow rate, 1.5 mL/min; for detector parameters see Materials and Methods). Characteristic ions (*m/z*) used for SIM: 575.4, campesteryl ferulate; 589.4, sitosteryl ferulate; 577.4, campestanyl ferulate; 591.4, sitostanyl ferulate.

mg/100 g) was of the same level as those of rye and wheat bran. The total sterol content of oat bran was lower, 56.1−62.0 mg/100 g. The sampling of oat bran was repeated four times, and the samples were obtained from three different companies to confirm this finding. Previously, Moreau et al. (*9*) reported that barley grains (without hull) contained small amounts (0.39 mg/100 g) of steryl ferulates.

On the other hand, the byproduct of rice processing, pearling dust of rice, was the richest source of steryl ferulates of all the analyzed samples. Its total steryl ferulate content was 119 mg/100 g, and those of the individual components were as follows: cycloartenyl ferulate, 33.5 ± 1.4 mg/100 g; 24-methylenecycloartanyl and campesteryl ferulate, together 67.1 ± 3.1 mg/100 g; sitosteryl ferulate, 18.5 ± 0.9 mg/100 g; and sitostanyl ferulate, 3.2 ± 0.1 mg/100 g. 24-Methylenecycloartanyl ferulate was the main component. Previously, the reported total steryl ferulate contents for rice bran was 340 mg/100 g (*8*).

On the basis of the results, rye and wheat and especially their bran fractions are interesting steryl phenolic acid sources. The levels we determined were lower than found in rice but of the same level as determined earlier for corn bran, which has been actively studied in countries where corn is widely cultivated. Furthermore, their sterol composition in the steryl ferulate fraction may be more beneficial in regard to some in vivo effects, such as cholesterol absorption, than that of rice (*18*). On the other hand, in regard to in vitro effects, such as antioxidativity, the phenolic acid part of the molecule may be regarded as the active part of the molecule (*20*). Therefore, rice, corn, rye, and wheat steryl ferulates can be assumed to be comparable antioxidants. Our future studies will focus on investigating the properties of the steryl ferulates of wheat and rye in more detail.

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