50, 99-86-5; 51, 99-87-6; 52, 555-10-2; 53, 138-86-3; 54, 99-85-4; 55, 586-62-9; 56, 109-66-0; 57, 110-54-3; 58, 142-82-5; 59, 111-65-9; 60, 111-84-2; 61, 124-18-5; 62, 1120-21-4; 63, 112-40-3; 64, 629-50-5; 65, 629-59-4; 66, 629-62-9; 67, 544-76-3; 68, 591-76-4; 69, 592-27-8; 70, 3221-61-2; 71, 871-83-0; 72, 6975-98-0; 73, 7045-71-8; 74, 1560-97-0; 75, 589-34-4; 76, 589-81-1; 77, 2216-33-3; 78, 5911-04-6; 79, 13151-34-3; 80, 1002-43-3; 81, 17312-57-1; 82, 589-53-7; 83, 2216-34-4; 84, 17301-94-9; 85, 2847-72-5; 86, 2980-69-0; 87, 6117-97-1; 88, 15869-85-9; 89, 13151-35-4; 90, 1632-70-8; 91, 17453-93-9; 92, 17302-33-9; 93, 6044-71-9; 94, 540-84-1; 95, 584-94-1; 96, 589-43-5; 97, 592-13-2; 98, 2213-23-2; 99, 4032-94-4; 100, 2801-84-5; 102, 17312-80-0; 107, 1640-89-7; 108, 108-87-2; 109, 1678-91-7; 110, 1678-92-8; 111, 1678-93-9; 112, 4292-92-6; 113, 4292-75-5; 114, 91-17-8; 115, 2958-76-1; 116, 592-41-6; 117, 25264-93-1; 119, 111-66-0; 120, 111-67-1; 121, 592-99-4; 122, 63597-41-1; 123, 71-43-2; 124, 108-88-3; 125, 100-41-4; 126, 106-42-3; 127, 108-38-3; 128, 100-42-5; 129, 95-47-6; 130, 98-82-8; 131, 103-65-1; 132, 620-14-4; 133, 622-96-8; 134, 611-14-3; 135, 108-67-8; 136, 95-63-6; 137, 526-73-8; 138, 108-68-9; 139, 100-52-7; 140, 100-47-0; 141, 140-67-0; 142, 104-46-1; 143, 534-22-5; 144, 3208-16-0; 145, 4229-91-8; 146, 4466-24-4; 147, 3777-69-3; 148, 3777-70-6; 149, 624-92-0; 150, 109-99-9; 151, 75-09-2; 152, 67-66-3; 153, 107-06-2; 154, 79-01-6.

LITERATURE CITED

Kovats, E. Gas-chromatographische Charakterisierung organischer Verbindungen. Teil 1: Retentionsindices aliphatischer Halogenide, Alkohole, Aldehyde und Ketone. *Helv. Chim. Acta* **1958**, *41*, 1915–1932.

Manjunath, T. M.; Bhatnagar, V. S.; Pawar, C. S.; Sitanantham, S. Economic importance of Heliothis spp. in India and an assessment of their natural enemies and host plants. Proceedings of a Workshop on Biological Control of *Heliothis*, ICRISAT, Patancheru, India, 1985.

Nitz, S.; Drawert, F.; Jülich, E. Trace analysis by intermediate trapping with a modified programmable temperature vaporizer. *Chromatographia* **1984**, *18*, 313–317.

Rembold, H.; Wallner, P.; Singh, A. K. Attractiveness of volatile chickpea (*Cicer arietinum* L.) seed components to *Heliothis armigera* larvae. *Z. Angew. Entomol.* **1989**, *107*, 65–70.

Saxena, K. N.; Rembold, H. Attraction of *Heliothis armigera* (Hübner) larvae by chickpea seed powder constituents. *Z. Angew. Entomol.* **1984**, *97*, 145–153.

van den Dool, H.; Kratz, P. D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatog.* **1963**, *11*, 463–471.

van Straten, S.; Maarse, H. Volatile compounds in food. Qualitative data. *Div. Nutr. Food Res. TNO, Rep.* **1983**.

Wächter, H.; Drawert, F.; Nitz, S. Erprobung von Verfahren zur qualitativen und quantitativen Erfassung von Geruchsstoffen. *VDI Ber.* **1986**, *No. 561*, 469–486.

Received for review June 20, 1988. Accepted November 1, 1988.

Stanol and Sterol Esters of Ferulic and *p*-Coumaric Acids in Wheat, Corn, Rye, and Triticale

Larry M. Seitz

Sitostanyl and campestanyl ferulates, and lesser amounts of sitosteryl and campesteryl ferulates, were found in corn, wheat, rye, and triticale grains. Corn also contained minor amounts of sitostanyl and campestanyl *p*-coumarates. Identification of individual esters isolated by thin-layer and high-performance liquid chromatography (HPLC) was confirmed mainly by ultraviolet and ^1H nuclear magnetic resonance spectra of the esters and by gas chromatography–mass spectroscopy of products from transesterification using potassium carbonate in methanol. A reversed-phase (C_{18}, methanol–water) HPLC system equipped with a photodiode array detector was used to determine the esters in extracts cleaned by a base–acid procedure. Analyses of dissected tissues from corn and wheat indicated that the esters were associated mostly with inner pericarp. The ferulates alone did not stimulate *Aspergillus amstelodami* spore germination and in the presence of nutrient did not inhibit its spore germination or mycelial growth.

The stanols (saturated sterols) corresponding to cholesterol, campesterol, and sitosterol are found only rarely in tracheophytes (Nes, 1977). Among the cereal grains, stanols have been reported only in corn (Knights, 1967; Kemp and Mercer, 1968), wheat (Knights, 1967), rye (Knights, 1967), triticale (Dominguez et al., 1972), and oats (Knights and Laurie, 1967). Campestanol and sitostanol were found in nonsaponifiable extracts of the whole grains and wheat flour (MacMurray and Morrison, 1970). Concerning dissected grain fractions, relatively little information is available on sterol content and essentially none on stanol content (Barnes, 1983).

The presence of ferulic (4-hydroxy-3-methoxycinnamic), *p*-coumaric (4-hydroxycinnamic), and other hydroxycinnamic acids (mainly in the trans form) in cereal grains is well documented (Collins, 1986; Sosulski et al., 1982). Ferulic acid, the most abundant, is associated with autofluorescence of aleurone cell walls (Fulcher, 1982) and is an indicator of nonendosperm tissues in wheat milling fractions (Pussayanawin et al., 1988). Ferulic and *p*-coumaric acids are known to be esterified to cell wall polysaccharides (Hartley and Jones, 1977). Ferulic acid bound to carbohydrate in wheat bran cell walls is released with a cellulase (Smith and Hartley, 1983). Rice bran oil contains the ferulates cycloartenyl (Ohta and Shimizu, 1957), 24-methylenecycloartenyl (Ohta, 1960), an unidentified C_{28} steryl (Kato, 1961), sitosteryl (Tanaka et al., 1964) and methyl (Tanaka et al., 1971). By using a normal-phase HPLC system, Tanaka et al. (1977) found that total ferulate content in 13 rice bran oils ranged from 1.47 to 1.97%. Dihydro-β-sitosteryl ferulate was found in corn oil (Tamura et al., 1958; Nilsson et al., 1968), and dihydro-γ-sitosteryl ferulate was found in wheat oil (Tamura

U.S. Grain Marketing Research Laboratory, U.S. Department of Agriculture—Agricultural Research Service, Manhattan, Kansas 66502.

et al., 1959). No references were found concerning p-coumaric acids esterified to stanols or sterols. Most methods for determining ferulic or other hydroxycinnamic acids in grain contain steps that either preclude extraction of the stanol or sterol esters from the grain or cause the esters to be decomposed or discarded during the analysis.

This work originated from a broad project dealing with various factors that might affect susceptibility of corn and wheat to invasion by fungi during storage. Phenolic acids and related components are known to exhibit antimicrobial activity in grains and other plant systems (Baranowski and Nagel, 1982; Hahn et al., 1983; Friend, 1977). For this study, it was considered desirable to use an extraction and cleanup procedure to obtain a fraction containing acidic phenolic components not strongly bound to cell walls and other macromolecular components in the grain. From analyses of such fractions by thin-layer (TLC) and high-performance liquid chromatography (HPLC, equipped with a photodiode array detector for rapid acquisition of ultraviolet spectra), and from literature searches, it became apparent that the fractions contained major phenolic components that were not well characterized or previously reported. Tissues from kernel dissections were analyzed to determine the location of the components within the kernel. Bioassays were conducted to assess their effects on fungal spore germination and mycelial growth of *Aspergillus amstelodami*, a common grain storage fungus.

MATERIALS AND METHODS

Extraction and Base–Acid Cleanup Procedures. Ten-gram samples of ground whole kernels were extracted with 50 mL of acetone by shaking the mixture for 60 min with a wrist shaker. After particulate material was allowed to settle, a 30-mL aliquot of supernatant was transferred with a syringe to a 25 × 150 mm screw-cap (Teflon lined) culture tube. The solvent was evaporated under nitrogen and the residue dissolved in 30 mL of methanol. The methanol solution was made basic (pH ~10) by adding 10 mL of 0.6% KOH and partitioned with hexane (2 × 10 mL), and the hexane was discarded. The aqueous portion was then acidified (pH ~2) with 1 mL of 6 N HCl and partitioned again with hexane (3 × 5 mL). The final hexane extract was evaporated under nitrogen and the residue redissolved in methanol for analysis by HPLC or in chloroform for application to TLC plates.

Other solvents were tried for the initial extraction. For these tests the procedure was as described above except that chloroform, methanol, or hexane was used in place of acetone.

Because dissected fractions (described below) were small, usually only about 1 g or less, they were extracted with a few milliliters of chloroform in a small glass test tube. The mixture was stirred with a glass rod for about 1 h and then filtered. The filtrate was evaporated under nitrogen and the residue redissolved in 0.5 mL of methanol for analysis by HPLC.

Chromatographic and Spectroscopic Analyses. The high-performance liquid chromatograph (HPLC) and the photodiode array detector used were obtained from the Hewlett-Packard Co. (Avondale, PA). The chromatograph was Model 1084B equipped with an autosampler, autoinjector, integrator, column oven, and fraction collector. The detector, Model 1040A, records ultraviolet–visible (UV–vis) spectra (190–600 nm) without having to stop the flow of the mobile phase. Chromatograms are formed by monitoring the difference between absorbances of sample and reference wavelengths. For all analyses the reference wavelength was centered at 550 nm with a 100-nm bandwidth. Sample wavelength and bandwidths varied as indicated below.

The HPLC column (6.2 × 80 mm) contained C_{18} packing of 3-μm particle size (Golden Series Zorbax; Du Pont, Wilmington, DE). An in-line filter was positioned between the column and the injector. Column temperature was 50 °C. The mobile phase was methanol or methanol–water mixtures as indicated below. The flow rate was 1.2 mL/min.

Gas chromatographic–mass spectrometric (GC–MS) analyses were performed on a Hewlett-Packard system consisting of a Model 5790A GC containing a 30 m × 0.2 mm bonded-phase capillary column (DB-5, Supelco) connected to a Model 5970 mass-selective detector and Model 9816 data system. Carrier gas was helium at about 1 mL/min. Ion source voltage was 70 eV, and temperature was 250 °C.

Thin-layer chromatography (TLC) was performed by using glass-backed plates (20 × 20 cm, SIL-G coating; Brinkman Instruments, Waterbury, NY). Coating thickness was 0.25–2.0 mm, depending on the amount of material to be applied or the resolution required. Plates were usually developed in benzene–ethyl acetate (90/10, v/v). Ferulate bands were identified by blue fluorescence under long-wave UV (~366 nm). To locate sterols and stanols, plates were sprayed with 50% sulfuric acid and heated for a few minutes at 135 °C. Ferulate bands were scraped off, the silica gel was extracted with methanol, and the extract was evaporated on a steam bath with a gentle stream of nitrogen directed into the vial. The residue was dissolved in methanol and analyzed by HPLC.

In addition to spectra recorded with the HPLC photodiode array detector, a Cary Model 118 spectrophotometer was used to obtain spectra of methanolic solutions of ferulic acid, the ferulates, and the potassium salts of the ferulates.

Proton spectra of samples dissolved in chloroform-*d* (100 atom % d, Gold Label; Aldrich Chemical Co., Milwaukee, WI) were obtained with a Bruker 400-MHz nuclear magnetic resonance spectrometer.

Transesterification Procedure. The stanol and sterol esters of ferulic and p-coumaric acids were transesterified by using a saturated solution of potassium carbonate in anhydrous methanol. The solution was prepared by mixing 0.3 g of potassium carbonate (dried under vacuum) with 15 mL of anhydrous methanol. Nitrogen was bubbled through the mixture for a few minutes to remove oxygen. The reagent produced the methyl esters of the acids and was mild enough to minimize further decomposition of the methylated acids.

Typically, the transesterification reaction was carried out as follows: Sample solution (usually containing 2–5 mg of sample) was placed in a small vial, evaporated to dryness under nitrogen, and finally dried under vacuum. Of the methanolic potassium carbonate 2 mL was added and the mixture heated at 65–70 °C for about 15 h. Water (2 mL) was added and the mixture partitioned with diethyl ether (2 mL the first time and then twice more with 1 mL each). After layers separated, the ether was removed with a Pasteur pipet, making sure not to remove any of the lower aqueous layer. These ether extracts, which contained the sterols and stanols, were combined and saved. The aqueous layer was acidified (pH ~2) with a few drops of 6 N HCl and then partitioned again with ether (3 × 1 mL). These ether extracts, which contained the methyl esters of the phenolic acids, were also combined and saved. Both ether extracts were evaporated to dryness under nitrogen without added heat. The residue was redissolved in a solvent appropriate for subsequent analyses.

The ferulate components from all of the grains were resistant to acid because hydrolysis did not occur with 2 N HCl for 15 h at 85 °C. The components were hydrolyzed by KOH saponification, but that caused extensive decomposition of the released phenolic acid.

Reference Standards. Stigmastanol (sitostanol) and ferulic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Campesterol was obtained from Applied Science Laboratories (State College, PA), and sitosterol (N.F. grade) was obtained from Aldrich. GC–MS analyses indicated that the sitosterol standard was about 60% sitosterol and 40% campesterol and that the stigmastanol standard was about 60% sitostanol and 40% campestanol.

Dissection of Wheat and Corn Kernels. Previously described procedures (Carratu et al., 1985; Chrispeels and Varner, 1967) for separating aleurone layers (with attached coats) from starchy endosperm of wheat were used with slight modification.

Wheat kernels were degermed by picking out the germ with sharp-pointed, dissecting tweezers. To prevent fungal growth, the degermed kernels were surface sterilized by soaking them in 2% sodium hypochlorite for 20 min. The seeds were rinsed three times with sterile water and placed between wet paper towels in Petri dishes. The samples were incubated at 30 °C. After 6 days, seeds were removed and dissected into outer pericarp, inner pericarp, and endosperm. By working under a magnifying glass

and using tweezers, the outer pericarp was easily peeled off. The remainder of the kernel was squeezed to separate inner pericarp from endosperm. The incubation period softened the starchy endosperm enough that it could be fairly easily squeezed away from the inner pericarp. The separation between outer and inner pericarp apparently occurred at the thin-walled cells, such that the outer pericarp consisted of mainly epidermal and hypodermal cells (Bradbury et al., 1956).

The same procedure was used to dissect yellow, dent corn. However, fractions were not separated as easily and cleanly as with wheat. The outer pericarp could be removed fairly easily, but the endosperm did not soften enough to allow it to be squeezed away from the inner pericarp. Instead, the kernel (minus outer pericarp) was split open and the endosperm scraped away from the inner pericarp by using a scalpel.

Bioassays. The major steryl and stanyl ferulates from wheat (isolated by TLC as described above) were tested for effect on spore germination and fungal mycelial growth. The tests involved incubating *A. amstelodami* spores in a humid environment in the presence of the ferulates alone and in the presence of ferulates plus known nutrient.

Three glass microscope slides were prepared in duplicate as follows. Slide 1 was spotted with 2, 5, and 10 μL of the ferulates in chloroform (approximately 1 μg/μL) to give three spots ranging from about 0.5- to 1.0-cm diameter. Slide 2 was prepared from spotting with sample as for slide 1, then applying 10% malt extract (Difco Laboratories, Detroit, MI) evenly over the entire slide, and finally drying at 50 °C. Slide 3 was prepared like slide 2, except that applications of malt extract and sample were reversed. Each slide was sprayed with a suspension of *A. amstelodami* spores in water and then incubated at 83% RH and 25 °C in a walk-in chamber. After 2, 3, and 6 days, the slides were examined inside the chamber by using a light microscope (~100×).

In another test, the same ferulate sample was mixed in wells of a warm ceramic spot plate with warm Czapek-Dox agar (Difco) containing 20% sucrose. Four wells containing agar were treated with 5, 15, 20, and 60 μL of sample, and two additional wells were treated with 20 and 60 μL of chloroform for controls. The plate was cooled, sprayed with *A. amstelodami* spore suspension, and incubated as described above. After 2 and 3 days, the plates were examined with the aid of the light microscope.

RESULTS AND DISCUSSION

Acetone extracts of corn, wheat, rye, and triticale grains after cleanup by the base–acid procedure gave the typical HPLC chromatograms shown in Figure 1. Ultraviolet (UV) spectra recorded at times marked by arrows in Figure 1 were identical and had the form shown in Figure 2 for the 24.8-min peak. These spectra, together with the fact that a mobile phase with a high methanol/water ratio was required to elute the components from the C_{18} column, indicated that the materials eluted were relatively nonpolar esters of *trans*-ferulic acid. Free *trans*-ferulic acid was eluted with 25% methanol and had a UV spectrum very similar to the spectra for the components in the grain extracts. Geissman and Harborne (1955) reported that in neutral solutions the spectra of phenolic acids are almost identical with those of their esters. As discussed below, further UV results with neutral and basic solutions of the isolated component representing the 24.8-min peak (Figure 1) are consistent with a component esterified to the carboxylic acid group of *trans*-ferulic acid (hereafter simply called ferulic acid).

Spectra recorded at the times marked by the asterisk in Figure 1 were identical to each other. The spectrum for the peak at 23.0 min is shown in Figure 2. Peaks associated with these spectra were found only in corn and are consistent with spectra expected for *trans*-*p*-coumarates (Jurd, 1957). Further evidence for the presence of *trans*-*p*-coumarates (hereafter simply called *p*-coumarates) in corn is given below.

Thin-layer chromatography (TLC) of the final extracts revealed a major blue fluorescent (under long-wave UV)

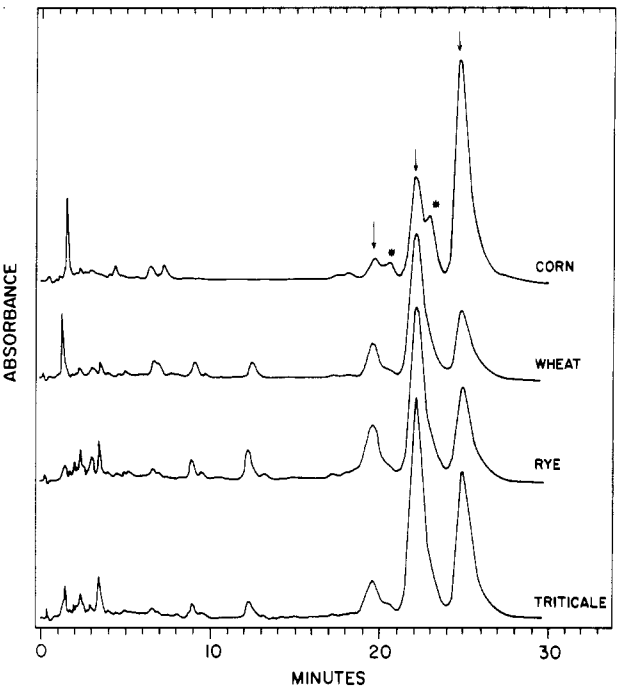

Figure 1. HPLC chromatograms of extracts (cleaned up by base–acid treatment) of ground whole kernels of corn, wheat, rye, and triticale. Arrows and asterisks indicate ferulates and *p*-coumarates, respectively, having typical ultraviolet spectra shown in Figure 2. The chromatograms were obtained by monitoring a 30-nm bandwidth centered at 325 nm. Milliabsorbance units full scale were 150 for each chromatogram. The mobile phase was 97% methanol; other conditions are given in Materials and Methods.

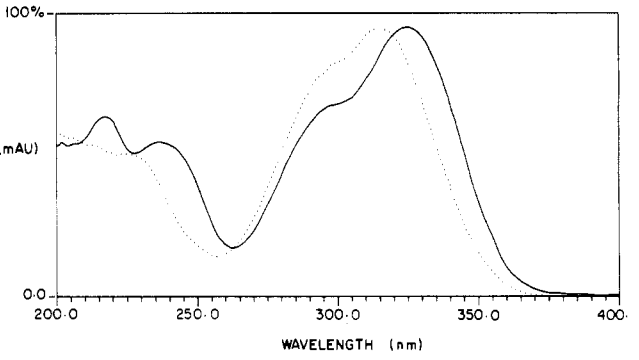

Figure 2. Ultraviolet spectra of the ferulate eluting at 24.8 min (- - -) and the *p*-coumarate eluting at 23.0 min (···) in the chromatogram for corn shown in Figure 1. Milliabsorbance units full scale were 66.8 and 20.8 for the ferulate and the *p*-coumarate, respectively.

spot at about R_f 0.65. Extracts of all the grains showed the same major spot. Removal of the spot from the plate and subsequent HPLC analysis of the extract from the removed silica showed that the major TLC spot was associated with ferulate components between 14 and 27 min (Figure 1). With corn, a nonfluorescent zone (identified by a dark zone under short-wave UV light when plates with a UV indicator were used) at about R_f 0.55 was associated with the *p*-coumarate components marked with an asterisk in Figure 1.

Ferulate components corresponding to peaks at 19.6, 22.0, and 24.8 min in HPLC chromatograms for corn and wheat (Figure 1) were collected from repeated HPLC separations of ferulates prepurified by TLC. Analyses of products from transesterification of each collected fraction showed that (1) the 24.8-min peak contained exclusively sitostanyl ferulate, (2) the 22.0-min peak consisted of

Table I. Some Diagnostic Ion Masses (m/z) and Relative Intensities for Stanols and Sterols Released by Tranesterification of Ferulates and p-Coumarates from Corn and Wheat

mass (m/z)	relative intensity, %			
	campesterol	campestanol	sitosterol	sitostanol
416				31
414			25	
402		26		
401				19
400	30			
399			14	
396			17	
387		17		
385	14			
383				7
382	20			
381			15	
369		7		
367	14			
329			19	
315	21			
303			24	
289	23			
281	14		7	
273	11		17	
255	14		12	
234		25		30
233		38		44
231	9		12	
217		17		19
216		25		27
215		51		62
213	23		17	
199	9		7	
185	7		10	
173	12		13	
166		11		11
165		32		33
163	19		16	
160	14		16	
159	20		22	
145	26		27	
143	8		14	
122		17		15
120	16		17	

mainly campestanyl ferulate (83% with corn, 77% with wheat) with some sitosteryl ferulate (17% with corn, 23% with wheat), and (3) the 19.6-min peak consisted of campesteryl ferulate (about 85% with corn and wheat) and minor amounts of other unknown ferulates (15%). Each fraction yielded methyl ferulate as verified by HPLC (eluted with 55% methanol and gave a UV spectrum like that for ferulate shown in Figure 2) and GC–MS analyses. Characteristic ion masses (m/z) observed for methyl ferulate were 208 (M^+, 100%), 177 (72%), 145 (44%), 133 (17%), 117 (18%), 105 (10%), 89 (18%), 78 (12%), 63 (8%), 51 (18%), and 39 (10%). The stanols and sterols released by transesterification were identified primarily by GC–MS, with GC retention times and MS data matching those of the reference standards. Characteristic ion masses observed for the stanols and sterols are shown in Table I. Ions with m/z 233, 234, 215, 216, 217, 166, and 165 were particularly diagnostic for stanols. The presence of stanols and sterols was further verified by TLC, with the former giving a light brown color and the latter a rose-red color after spraying the plate with sulfuric acid and heating. Only the sterols could be observed by HPLC using 100% methanol mobile phase and a sample wavelength of 202 nm (4-nm bandwidth). As expected from absence of unsaturation, the sample containing sitostanol released by transesterification of the 24.8-min peak gave no HPLC response.

Results from ^1H NMR analyses of collected HPLC fractions representing wheat components eluting at 22.0 and 24.8 min (Figure 1) provided additional evidence for stanyl ferulates. Expected ferulate resonances were essentially the same for both components: δ 7.01 (d, 1 H, C2, J = 1.9 Hz), 6.89 (d, 1 H, C5, J = 8.1 Hz), 7.04 (dd, 1 H, C6, $J_{5,6}$ = 8.1 Hz and $J_{2,6}$ = 1.9 Hz), 3.90 (s, 3 H, CH_3O–), 5.81 (s, 1 H, HO–), 7.57 (d, 1 H, Cβ, J = 16 Hz), 6.25 (d, 1 H, Cα, J = 16 Hz). Expected resonances associated with stanyl groups ranged from δ 4.8 (m, 1 H, C3) to δ 0.63 (s, 3 H, C18). Spectra from the 22.0- and 24.8-min fractions were similar (especially considering signals from side chains) to spectra from campestanol and sitostanol, respectively. With both collected fractions, the singlet from the C19 methyl group was at δ 0.82, which is consistent with the absence of a Δ^5 bond (Nes, 1977). By comparison, the C19 methyl in campesterol was observed at δ 0.99. The definite absence of a signal at about δ 5.3 (from C6 on a Δ^5 bond) also showed that the 24.8-min fraction contained no steryl ferulate; a slight signal from the 22.0-min fraction was consistent with the presence of campesteryl ferulate as indicated by the GC–MS results.

To confirm the presence of p-coumarates in corn as indicated by HPLC (Figures 1 and 2), the base–acid cleaned extract was first subjected to preparative TLC for isolation of the p-coumarate band at R_f 0.5, just below the ferulate band (see TLC discussion above). This TLC fraction was then injected into the HPLC system for collection of components corresponding to peaks at 20.6 and 23.0 min (marked by asterisks in Figure 1). To achieve better separation of p-coumarates and ferulates than shown in Figure 1, the methanol composition of the mobile phase was lowered to about 91%. The two fractions were transesterified, and the products were analyzed by HPLC and GC–MS. The 20.6- and 23.0-min peaks corresponded to p-coumaric acid esterified to campestanol and sitostanol, respectively. Ion masses of 178 (M^+, 66%), 147 (100%), 119 (32%), 91 (31%), 65 (23%), and 39 (17%) were observed as expected for methyl p-coumarate.

Because the HPLC peak at 24.8 min in Figure 1 represented a single component, stitostanyl ferulate, 2.14 mg of that component was collected for preparation of an analytical standard and for further study by UV spectroscopy. Neutral methanolic solutions of this component and ferulic acid gave UV maxima at 325 nm (ϵ 17 100) and 321 nm (ϵ 17 000), respectively, and the spectra had very similar shapes, which is consistent with previous reports that spectra of phenolic acids are almost identical with those of their esters (Geissman and Harborne, 1955). Addition of KOH to the sitostanyl ferulate solution caused a bathochromic shift of the absorption maxima to 377 nm, as expected for ionization of the phenolic hydroxyl group (Geissman and Harborne, 1955; Jurd, 1957).

HPLC analyses of fractions from the dissected wheat kernels showed that the stanyl and steryl ferulates were associated solely with inner pericarp (Figure 3). The separation between outer and inner pericarp apparently occurred at the thin-walled cells, such that the outer pericarp consisted of mainly epidermal and hypodermal cells (Bradbury et al., 1956). The aleurone was in the inner pericarp fraction because the soaking procedure was designed to separate aleurone (with attached coats) from starchy endosperm (Carratu et al., 1985; Chrispeels and Varner, 1967). Studies of wheat by fluorescence microscopy have indicated that ferulic acid or ferulates occur in aleurone cell walls and seed coat tissues, but not in significant quantities in the starchy endosperm (Fulcher, 1982). It is not known what specific tissues within the

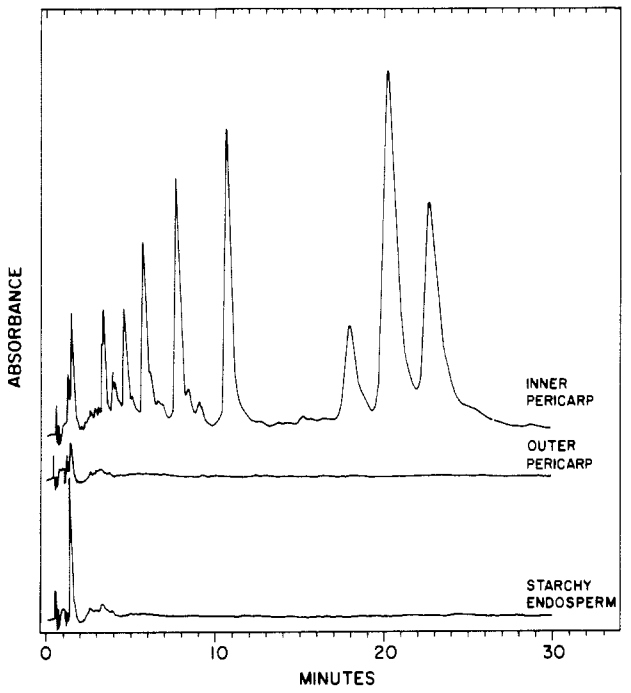

Figure 3. HPLC chromatograms of extracts of fractions from dissected wheat kernels. The chromatograms were obtained by monitoring a 4-nm bandwidth centered at 325 nm. Milliabsorbance units full scale were 45 for each chromatogram. The mobile phase was 98% methanol; other conditions are given in Materials and Methods.

Table II. Effects of Initial Extraction Solvent on Measured Total Concentrations of Stanyl and Steryl Ferulates in Wheat and Corn

solvent	measured concn,[a] μg/g	
	wheat	corn
acetone	73	32
methanol	7.3	11
hexanes	13	88
chloroform	38	28

[a] Each value is a mean of duplicate assays. Coefficient of variation of duplicates averaged 9.3%.

inner pericarp were associated with the stanol and sterol ferulates. However, the fact that ferulates were not found in the outer pericarp and endosperm fractions (Figure 3) suggests that the ferulates were located in the interior portion of the inner pericarp.

The results for dissected fractions from corn were like those for wheat except the outer pericarp and endosperm fractions contained traces of ferulates due to the difficulty in getting clean tissue separations. HPLC and UV analyses of extracts of whole corn kernels (details to be reported elsewhere) showed that the ferulates were extractable from kernel surfaces only when the pericarp was broken, which was consistent with absence of the ferulates in the outer pericarp and their presence in the inner pericarp.

The solvent used for the initial extraction significantly affected the measured total concentrations of stanyl and steryl ferulates (Table II), but not their distribution. The solvents resulting in the highest values were acetone for wheat and hexanes for corn. It is not known why hexane was so much more effective with corn than with wheat. Methanol was the least effective solvent for extracting the ferulates from wheat and corn.

The solvent used for initial extraction also affected the extraction of some unknown ferulates from wheat, particularly those associated with the five peaks between 3 and 11 min in the HPLC chromatogram of wheat inner

Table III. Concentrations and Relative Distribution of Stanyl and Steryl Ferulates in Corn, Wheat, Rye, and Triticale

grain type	class	variety or hybrid	total concn,[a] μg/g	distrib, % campesteryl	distrib, % campestanyl	distrib, % sitostanyl
wheat	SWW[b]	Newgaines	64	13	54	33
wheat	HWW	Cymmitt	62	10	60	30
wheat	HWW	KS-84AW196	79	11	55	34
wheat	HRW	Newton	73	13	55	32
wheat	HRW	Vona	94	10	56	34
wheat	SRW	Caldwell	72	13	55	32
wheat	HRS	Oslo	123	10	54	36
wheat	club	Crew	82	10	59	31
wheat	durum	Aloura	68	7	64	29
rye		Rymin	29	15	54	31
triticale		unknown	52	7	54	39
corn[c]	yellow	BoJac X603	35	6	26	68
corn	yellow	Stauffer 144+	42	5	26	69
corn	yellow	Dekalb XL395	32	3	29	68
corn	yellow	Bulk-Mix	31	3	29	68
corn	yellow	Unknown	70	5	27	68
corn	yellow	Unknown	50	4	27	69
corn	white	OPW[d]	42	3	26	71

[a] Each value is a mean of duplicate assays. Coefficient of variation of duplicates averaged 4.9%. [b] Key: SWW = soft white winter; HWW = hard white winter; HRW = hard red winter; SRW = soft red winter; HRS = hard red spring. [c] All corn samples were dent corn. [d] OPW = open-pollinated white breeding line.

pericarp (Figure 3). Ultraviolet spectra of these five peaks, which were also observed in extracts of ground whole wheat (Figure 1), indicate ferulates. These ferulates were extracted by acetone, methanol, and especially chloroform, but hexanes did not extract them at all. The relatively large size of those five peaks in Figure 3 was due to chloroform being used to extract the inner pericarp tissue and, possibly, to the compounds being more extractable from isolated tissues than from ground whole grain.

Ultraviolet spectra of other minor peaks in the chromatograms in Figure 1 suggest the presence of still more unidentified ferulates. With corn, the two small peaks between 6 and 8 min, and at least two more between 16 and 19 min, appear to be ferulates. Some wheat samples also showed minor peaks between 16 and 19 min. Preliminary HPLC studies of acetone extracts (obtained as described herein) from rough and brown rice suggest that cycloartenyl and 24-methylcycloartenyl ferulates were eluted in 18–19.5 min. Cycloartenyl (Ohta and Shimizu, 1957), 24-methylenecycloartenyl (Ohta, 1960), an unidentified C_{28} steryl (Kato, 1961), and sitosteryl (Tanaka et al., 1964) ferulates were reported as components of rice bran oil. The chromatogram for rice showed that sitostanyl ferulate was a minor component, if present at all.

Concentrations of stanyl and steryl ferulates in ground whole grain samples of corn, wheat, rye, and triticale are listed in Table III. Acetone was the initial extraction solvent with these grains. The total ferulate concentrations were generally higher in wheat than in corn, rye, and triticale. However, this difference could be due to a solvent effect (Table II), and the actual ferulate concentrations in corn and wheat could be nearly the same. Neither class nor variety/hybrid had a strong influence on total concentration or distribution of ferulates within grain type (corn or wheat). The hard red spring wheat variety Oslo had the highest total concentration, but its ferulate distribution was similar to that of the other wheats. Distribution varied considerably among grain types. In corn, sitostanyl ferulate was the predominant component followed by campestanyl ferulate, whereas the reverse was true with wheat, rye, and triticale. Within grain type, ferulate distribution varied relatively little. The durum wheat sample showed the greatest deviation from the rest

of the wheats in distribution of ferulates.

The p-coumarates were minor components in corn with estimated total concentrations (sitostanyl plus campestanyl) ranging from 6 μg/g in BoJac X603 and Stauffer 144+ to 1.5 μg/g in most of the rest of the samples. The chromatogram shown in Figure 1 represents a sample with relatively high p-coumarate concentration. With the p-coumarates, like the ferulates, the sitostanyl component was predominant. The sitostanyl/campestanyl ratios were 4 and 2.5 for p-coumarates and ferulates, respectively.

The bioassays indicated that stanyl and steryl ferulates from wheat did not stimulate fungal (*A. amstelodami*) spore germination. Spores on plates spotted with ferulates alone did not germinate on the sample spots nor on any of the plate surface away from the sample. When nutrient (malt extract or Czapek-Dox agar with 20% sucrose) was present, spore germination and mycelial growth were profuse and not inhibited by the presence of the ferulates. On each slide coated with malt extract, areas treated with ferulates had fungal growth equivalent to that of the nontreated areas. Also, with the spot-plate test using Czapek-Dox agar plus 20% sucrose as nutrient, there was essentially no difference in spore germination and mycelial growth among treated and control wells. Results from these tests cast doubt on the possibility that the stanyl and steryl ferulates regulate fungal activity in the grain. Whether these ferulates have any other function(s) in the grain is still unknown.

This work confirmed previous reports of sitostanyl ferulates in corn (Tamura et al., 1958; Nilsson et al., 1968) and wheat (Tamura et al., 1959). The presence of campesteryl, campestanyl, and sitosteryl ferulates in corn, wheat, rye, and triticale, as well as campestanyl and sitostanyl p-coumarates in corn, had not been reported previously. Because occurrence of stanols in cereal grains is low compared to that of sterols, the preferential occurrence of stanyl ferulates and p-coumarates was somewhat unexpected. The transesterification method using potassium carbonate in methanol was especially easy and effective for splitting the esters into their component parts without decomposing the phenolic acid. The extraction and HPLC methodology reported here for determining the ferulates was relatively simple, quick, and potentially applicable to other grains, oilseeds, or other plant materials. The fact that the stanyl and steryl ferulates are associated mostly with inner pericarp suggests that an assay for them could be used to measure (1) bran contamination in flour and (2) degree of damage to pericarp on whole kernels. Preliminary work on the latter application with corn has produced encouraging results.

LITERATURE CITED

Baranowski, J. D.; Nagel, C. W. Inhibition of *Pseudomonas fluorescens* by Hydroxycinnamic Acids and Their Alkyl Esters. *J. Food Sci.* **1982**, *47*, 1587–1589.

Barnes, P. J. Non-Saponifiable Lipids in Cereals. In *Lipids in Cereal Technology*; Barnes, P. J., Ed.; Academic Press: New York, 1983.

Bradbury, D.; MacMasters, M. M.; Cull, I. M. Structure of the Mature Wheat Kernel. II. Microscopic Structure of Pericarp, Seed Coat, and Other Coverings of the Endosperm and Germ of Hard Red Winter Wheat. *Cereal Chem.* **1956**, *33*, 342–360.

Carratu, G.; Colacino, C.; Giannattasio, M. Binding of Hydrolases from Wheat Aleurone to Concanavalin A- and Wheat-Germ Agglutinin-Sepharose. *Phytochemistry* **1985**, *24*, 683–688.

Chrispeels, M. J.; Varner, J. E. Gibberellic Acid-Enhanced Synthesis and Release of α-Amylase and Ribonuclease by Isolated Barley Aleurone Layers. *Plant Physiol.* **1967**, *42*, 398–406.

Collins, F. W. Oat Phenolics: Structure, Occurrence, and Function. In *Oats: Chemistry and Technology*; Weber, F. H., Ed.; American Association of Cereal Chemists: St. Paul, MN, 1986.

Dominguez, X. A.; Rodriguez-Bores, F.; Jauregui, F. Sitosterol and β-Sitostanol from Triticale. *Phytochemistry* **1972**, *11*, 2656.

Friend, J. Phenolic Substances and Plant Diseases. *Recent Adv. Phytochem.* **1977**, *12*, 557–588.

Fulcher, R. G. Fluorescence Microscopy of Cereals. *Food Microstruct.* **1982**, *1*, 167–173.

Geissman, T. A.; Harborne, J. B. The Chemistry of Flower Pigmentation in *Antirrhinum majus*. IV. The Albino (-mm-nn) Form. *Arch. Biochem. Biophys.* **1955**, *55*, 447–454.

Hahn, D. H.; Faubion, J. M.; Rooney, L. W. Sorghum Phenolic Acids, Their High Performance Liquid Chromatography Separation and Their Relation to Fungal Resistance. *Cereal Chem.* **1983**, *60*, 255–259.

Hartley, R. D.; Jones, E. C. Phenolic Components and Degradability of Cell Walls of Grass and Legume Species. *Phytochemistry* **1977**, *16*, 1531–1534.

Jurd, L. The Detection of Aromatic Acids in Plant Extracts by the Ultraviolet Absorption Spectra of Their Ions. *Arch. Biochem. Biophys.* **1957**, *66*, 284–288.

Kato, A. Isolation of Ferulates from Soapstock of Rice Bran Oil. *Yukagaku* **1961**, *10*, 679.

Kemp, R. J.; Mercer, E. I. The Sterol Esters of Maize Seedlings. *Biochem. J.* **1968**, *110*, 111–118.

Knights, B. A. In *The Gas Liquid Chromatography of Steroids*; Grant, J. K., Ed.; Cambridge University Press: Cambridge, MA, 1967; pp 211–221.

Knights, B. A.; Laurie, W. Application of Combined Gas-Liquid Chromatography-Mass Spectrometry to the Identification of Sterols in Oat Seed. *Phytochemistry* **1967**, *6*, 407–416.

MacMurray, T. A.; Morrison, W. R. Composition of Wheat-Flour Lipids. *J. Sci. Food Agric.* **1970**, *21*, 520–528.

Nes, W. R. The Biochemistry of Plant Sterols. In *Advances in Lipid Research*; Paoletti, R., Kritchevsky, D., Eds.; Academic Press: New York, 1977; Vol. 15.

Nilsson, J. L. G.; Redalieu, E.; Nilsson, I. M.; Folkers, K. On The Protection Against Infarction by Corn Oil. *Acta Chem. Scand.* **1968**, *22*, 97–105.

Ohta, G. Constituents of Rice Bran Oil. III. Structure of Oryzanol-C. *Chem. Pharm. Bull.* **1960**, *8*, 5–9; *Chem. Abstr.* **1961**, *55*, 5570.

Ohta, G.; Shimizu, M. Constituents of Rice Bran Oil. II. Structure of Oryzanol-A. *Chem. Pharm. Bull.* **1957**, *5*, 40–44; *Chem. Abstr.* **1958**, *52*, 1190.

Pussayanawin, V.; Wetzel, D. L.; Fulcher, R. G. Fluorescence Detection and Measurement of Ferulic Acid in Wheat Milling Fractions by Microscopy and HPLC. *J. Agric. Food Chem.* **1988**, *36*, 515–520.

Smith, M. M.; Hartley, R. D. Occurrence and Nature of Ferulic Acid Substitution of Cell-Wall Polysaccharides in Graminaceous Plants. *Carbohydr. Res.* **1983**, *118*, 65–80.

Sosulski, F.; Krygier, K.; Hogge, L. Free, Esterified, and Insoluble-Bound Phenolic Acids. 3. Composition of Phenolic Acids in Cereal and Potato Flours. *J. Agric. Food Chem.* **1982**, *30*, 337–340.

Tamura, T.; Sakaedmani, N.; Matsumoto, T. Isolation of dihydro-β-sitosteryl Ferulate from Corngerm Oil. *Nippon Kagaku Zasshi* **1958**, *79*, 1011–1014; *Chem. Abstr.* **1960**, *54*, 24857i.

Tamura, T.; Hibino, K.; Yokoyama, K.; Matsumoto, T. On the Occurrence of Dihydro-γ-Sitosterylferulate in Wheat Oil. *Nippon Kagaku Zasshi* **1959**, *80*, 215–217; *Chem. Abstr.* **1961**, *55*, 4889.

Tanaka, A.; Kato, A.; Tsuchiya, T. Isolation of β-Sitosteryl Ferulate from Rice Bran Oil. *Yukagaku* **1964**, *13*, 260–263.

Tanaka, A.; Kato, A.; Tsuchiya, T. Isolation of Methyl Ferulate from Rice Bran Oil. *J. Am. Oil Chem. Soc.* **1971**, *48*, 95–97.

Tanaka, A.; Tanabe, K.; Kato, A.; Muramatsu, J. Quantitative Analysis of Ferulates in Rice Bran Oil by High Performance Liquid Chromatography. *Yukagaku* **1977**, *26*, 119–122.

Received for review July 5, 1988. Accepted December 5, 1988. Mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others not mentioned.