Isolation and Identification of Steryl Cinnamic Acid Derivatives from Corn Bran

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ABSTRACT

Esters of sterols and cinnamic acid derivatives (CAD) comprise a group of compounds found in seeds of, among others, corn, wheat, rye, triticale, and rice. They have been implicated in lowering blood lipid levels. Related compounds are antioxidants and others inhibit fungal spore germination. To obtain pure compounds for bioactivity studies with corn insects and kernel-rotting fungi, an improved method of separating these compounds was developed. A high-performance liquid chromatography method, using a mixture of acetonitrile, *n*-butanol, acetic acid, and water (94:3:2:1, v/v), improved the resolution of steryl esters of CAD isolated from corn bran, which shows that this group of compounds is at least twice as complex as was previously reported. A simple method for hydrolysis and joint determination of the CAD and sterol portions of the ester is described. A total of 16 compounds that appear to be steryl CAD are reported along with relative retention times and composition percentages. Sitosteryl, sitostanyl, stigmasteryl, campesteryl, campestanyl and Δ^7-sitosteryl, and Δ^7-campesteryl ferulates were identified in corn bran. Mass spectrometry data for the trimethylsilyl derivatives of the sterols esterified to ferulic and *p*-coumaric acids are given. In addition to known *p*-coumarates, campesterol and Δ^7-campestenol forms were also found.

An ongoing topic in corn pathogen research is the effect of chemical factors on the resistance of the corn plant and maturing ears to fungal and insect pathogens. Because the pericarp is the first line of defense against kernel pathogens, the secondary metabolites in this part of the seed should be among those most likely to have a defensive function. Steryl esters of cinnamic acid derivatives (CAD) appear to be localized in the inner pericarp of corn (Seitz 1989). For this and other reasons discussed later, these compounds could be involved in pathogen resistance. As a part of a project to identify the chemical factors that might affect susceptibility of maturing corn kernels to invasion by *Aspergillus flavus* or the amount of the aflatoxin produced in infected kernels, research was initiated on steryl CAD, a group of compounds that have not been extensively examined in corn.

The steryl CAD in rice bran oil have been investigated for a number of years. The ferulates of seven sterols have been identified: cycloartenol, 24-methylene cycloartanol, β-sitosterol, stigmasterol, campesterol, cycloartanol, and an unidentified sterol later designated as cyclobranol (Endo et al 1968). The major sterol components of ferulate esters in γ-oryzanol, a commercial preparation of these esters extracted from rice bran, are campesterol, cycloartenol, and 24-methylene cycloartanol.

Sitostanyl ferulate was identified in corn in 1958 (Tamura et al 1958) and in wheat the following year (Tamura et al 1959). Evershed et al (1988) found campestanyl ferulate in whole corn seeds, and Seitz (1989) identified four compounds common to corn, rye, wheat, and triticale, including sitostanyl, campestanyl (the principal forms), sitosteryl, and campesteryl ferulate. Corn also contained small amounts of sitostanyl and campestanyl *p*-coumarate. In addition, Seitz (1990) showed that these compounds are highly localized within cereal seeds. In corn, they are associated mostly with the inner pericarp region, consisting of ". . . some tube cells, the seed coat and, probably, most of the aleurone layer" (Seitz 1990). A nonuniform distribution was suggested by Seitz's (1990) observation that when kernels are uniformly punctured on either the germinal or abgerminal sides and then surface-extracted, more sitostanyl ferulate is released from the abgerminal surface.

It seemed plausible, therefore, that steryl CAD could be candidates for kernel pathogen resistance compounds for both fungi and insects. Reasons for this include: 1) the localization of the compounds within the pericarp; 2) the specialized character of the sterol portion of the ester in corn and related cereals (they are primarily saturated forms of normal membrane sterols); and 3) preliminary data revealed that there were perhaps a dozen or more CAD esters of sterols, most at low levels, present in corn bran. This suggests the possibility of natural selection for forms with a range of biological activity. The second reason is underscored in rice, where the principal sterols in the esters are equally atypical. Preliminary work also suggested that rice and corn produced a complementary set of esters. Therefore, purification of the esters from both grains was initiated with the objective of obtaining enough of the main compounds to determine activity against corn insects and kernel-rotting fungi and enough of the minor ones for identification. The initial objectives were: to improve high-performance liquid chromatography (HPLC) separation of the steryl CAD esters of corn bran, to develop an efficient method for determining the components of the esters, to isolate and identify as many compounds as feasible, and to assess the possible importance of photoisomerization on analysis.

MATERIALS AND METHODS

Extraction and Fractionation

Corn bran flour (yellow dent, dry milled, ConAgra Grain Processing, Omaha, NE) was stored at −20°C until use. Bran was extracted according to the method of Seitz (1989). Hexane

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(1 L) was added to 200 g of bran, which was shaken at low speed on a rotary shaker for 40 min, removed, and allowed to set overnight. The extract was filtered, and the filter cake was rinsed twice with 500 ml of fresh hexane. The filtrate was evaporated to an oil under reduced vacuum at 45°C. The residue was dissolved in 200 ml of warm methanol, 200 ml of 0.6% aqueous KOH was added, and the residue was extracted twice with 200 ml of hexane in a separatory funnel. The aqueous phase was acidified with 20 ml of 6*N* HCl and partitioned three times into 100 ml of hexane. The acid extracts were combined and evaporated as before. Except as noted, the acid extract was used for all further work. Extracts were dissolved in $CHCl_3$ and stored in brown glass vials at −20°C. Rice bran oil was used without dilution. All operations were performed without artificial light when enough ambient light was available to see clearly.

Rice bran oil (BioSan, New Dimensions, Fountain Hills, AZ) was fractionated on a 4.25-cm × 50-cm silica gel column (320 g, 63–200 μm) (QC345, Alltech, Deerfield, IL) by dissolving 110 g of oil in 100 ml of hexane, applying this solution to the column, and eluting with successive 1 L aliquots of 100% hexane and hexane and ethyl ether at 9:1, 7:3, and 1:1 (v/v). Steryl CAD eluted in the 1:1 fraction. γ-Oryzanol was purchased from CTC Organics, Atlanta, GA.

Corn bran acid hexane extract was fractionated in a similar manner, except that 25 g of the oil was applied to the column in hexane and eluted successively with 250 ml each of: 100% hexane; hexane and ethyl ether at 95:5, 90:10, 80:20, 70:30, and 50:50 (v/v); 100% ether; and 100% methanol. Steryl CAD eluted in the 100% ether fraction.

The steryl ferulate fraction (which fluoresces blue on the column under long-wave UV, 365 nm) from both corn bran and rice bran oil was separated from contaminating oils, free sterols, and fatty sterol esters by use of rotation planar chromatography (Chromatotron, Harrison Research, Palo Alto, CA), which is essentially a substitute for preparative thin-layer chromatography (TLC). The ester fraction (0.6–0.85 g) was dissolved in an equal amount of hexane and applied to a 2-mm Chromatotron silica gel GF rotor (Analtech, Newark, DE), equilibrated with hexane, and eluted with hexane and benzene at ratios of 7:3 (150 ml), 5:5 (250 ml), and 2.5:7.5 (200 ml), and with benzene (150 ml) and ethyl ether (125 ml) at a flow rate of 5 ml/min. The instrument chamber was purged with N_2 before development and continually flushed with N_2 (~15 ml/min) during development. Two blue fluorescing bands (ferulates) eluted closely together, followed by a very dark purple band (*p*-coumarates). The second (lower) fluorescent blue band was considerably more intense than the first. The progress of the elution was followed by long-wave UV (365 nm), and appropriate fractions were collected. Fractions were monitored by TLC ($CHCl_3$ and acetone, 98:2), and similar fractions were combined. Cholesterol (cho) and lanosterol (lan) standards were spotted on each TLC plate; R_f cho 0.32, lan 0.44, lower ester band 0.52, upper ester band 0.56, *p*-coumarate band 0.48. Cholesterol cinnamate has an R_f of 0.88 and ergosterol has an R_f of 0.32. TLC plates were 0.25-mm silica gel 60 F_{254} (Merck). Compounds were visualized by UV or by spraying with concentrated sulfuric acid in methanol (1:1, v/v) and heating at 100°C for ~5 min. Steryl ferulate esters fluoresce bright greenish yellow after charring, and *p*-coumarate esters fluoresce orange (similar to sterol standards); the intensity increases with time.

High-Performance Liquid Chromatography

The purified steryl CAD mixtures obtained from Chromatotron fractionation were separated by semipreparative HPLC using an Alltech (Deerfield, IL) Econosil C_{18}, 250-mm × 10-mm, 10-μm, column at a flow rate of 5 ml/min and ambient temperature. A Beckman HPLC system with System Gold software was used, which included a 507 autosampler, a 168 diode array detector, a 406 analog interface module, and 110B pumps. The detector was set to monitor at 325 nm (λ_{max} for ferulate) with a 15-nm bandwidth, at 280 nm (λ_{max} for internal standards) with a 10-nm bandwidth, and at 550 nm (reference) with a 50-nm bandwidth. Repetitive runs were made, and peaks and shoulders were collected and dried with a stream of N_2. The solvent system used was a mixture of acetonitrile, *n*-butanol, acetic acid, and water at 93:4:2:1 (v/v), isocratic. Internal standards of ergosterol (Sigma) and cholesteryl cinnamate (5-cholesten-3β-ol 3-cinnamate, Research Plus, Bayonne, NJ) were added, as necessary, to samples before injection. Amounts of 5 μg each were sufficient for detection. Retention times relative to ergosterol (α_{erg}) and cholesteryl cinnamate (α_{cc}) were calculated as described by Nes (1985).

Examination of some of the peaks from the semipreparative column on the analytical column showed additional resolution. So each of the complex peaks were, in turn, analyzed and collected using a Supelco (Bellefonte, PA) Supelcosil LC_{18} 4.6-mm × 25-cm, 5-μm analytical column with a guard column of the same packing, and a flow rate of 1.2 ml/min. Other parameters were the same as those for the semipreparative column. HPLC purified peaks gave a single peak on HPLC, a single spot on TLC, and a single peak (except for stanols which gave a split peak) as the intact ester with gas chromatography-mass spectrometry (GC-MS), and two peaks after hydrolysis and silylation.

Quantitation

Quadruplicate HPLC injections of purified sitostanyl ferulate at levels of 0.01, 0.1, 1.0, and 5.0 μg/ml (0.4, 4.0, 40.0, and 200 μg total amount) were made, and a standard curve was derived by linear regression. This standard curve was used for quantitation of all steryl ferulates. Steryl *p*-coumarates were estimated by using the same standard curve and a correction factor of 1.06× for the *p*-coumarate area. The factor was derived from the ratio of areas for triplicate 2-μg injections of *p*-coumaric acid and ferulic acid monitored at 325 nm. Quadruplicate extractions of 50-g samples of bran were made, aliquots were separated by TLC, the steryl ferulates and *p*-coumarate from the corresponding bands were eluted, and composition percentages and amounts of compounds were calculated from the average for single injections of each extract.

Hydrolysis and Derivatization

Hydrolysis was adapted from the method of Warnaar (1976). Aliquots of the collected HPLC peaks containing 10–20 μg of compound were dried with a stream of N_2 and hydrolyzed with 0.1 ml of benzene and 0.2 ml of 0.5*N* NaOH in methanol by heating at 100°C for 45 min. Concurrently with the addition of base, N_2 was bubbled for 30 sec through the solution, which was then tightly capped in tubes as quickly as possible. Immediately after opening the tubes when the solution had cooled, three drops of 6*N* HCl and 0.6 ml of water were added to the solution, which was extracted twice with 1.0 ml of ethyl acetate. The combined extracts were dried with Na_2SO_4 and evaporated with N_2. The trimethylsilyl (TMS) ether derivatives were prepared by heating at 55°C for 10 min with 0.1 mL bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (1:1, v/v). The BSTFA (Sigma) contained 1% trimethylchlorosilane. After it cooled, more pyridine was added to give a final concentration of ~50 μg/ml. A mixture of *cis*- and *trans*-CAD standards was prepared by irradiating a mixture of all five CAD-TMS derivatives in a mixture of pyridine and BSTFA (4:1, v/v) using a Rayonet photochemical reactor (RMR-500, Southern New England Ultraviolet Co., Hamden, CT) with RPR-2537 Å short-wave UV lamps (253.7 nm). An equilibrium mixture was obtained in ~5 hr. To determine the effect of irradiation on underivatized sitostanyl ferulate, HPLC-purified compound was irradiated in ethanol for 5 hr and analyzed by HPLC, TLC, and GC-MS. Acetylation was performed with a pyridine and acetic anhydride mixture (1:1, v/v) at 55°C for 2 hr.

Gas Chromatography-Mass Spectroscopy

Derivatized samples were analyzed by GC-MS analysis with a Hewlett-Packard 5890 series II GC and 5970 MSD (70 eV) using a DB-5 0.25-mm × 15-m capillary column under splitless conditions with a 2-mm i.d. gooseneck inlet sleeve (Restek Corp., Bellefonte, PA). The temperature program was: 140°C isothermal for 3 min, ramping to 270°C at 20°C/min, isothermal 3 min

and ramping to 280°C at 2°C/min. The carrier gas was He at a flow rate of 1.2 ml/min. For CAD and sterol derivatives, the injection port was 250°C and the transfer line was 280°C. Injections of 1 μl were made with an HP7673 autoinjector. For analysis of intact esters, inlet and transfer line temperatures were both 300°C using a temperature program of: 170°C isothermal 1 min, ramping to 300°C at 2°C/min, and isothermal for 19 min. Standards of sterols and CAD included (in order of elution): cinnamic (Aldrich), *p*-coumaric (Fluka), ferulic (Sigma), caffeic (Sigma) and sinapic (Fluka) acids, campesterol, campestanol, stigmasterol, Δ^7-campesterol, sitosterol, stigmastanol, and cycloartenol. They were derivatized as above and run as required. Sterol standards were obtained from Sigma, except for campestanol and Δ^7-campesterol (Research Plus) and cycloartenol (a gift from W. David Nes, USDA-ARS, Athens, GA).

RESULTS

Extraction and Workup

Corn bran flour was chosen as a source of sterol esters because Seitz (1989) showed that almost all of the CAD esters are found in the pericarp and aleurone layer. As bran accounts for only about 5% of the total kernel, bran should contain a higher percentage of esters and a lower level of triglycerides, which would simplify purification.

Free caffeic acid has been found in corn flour (Sosulski et al 1982) and two caffeoyl compounds accumulate in corn leaves in response to infection by certain pathogenic fungi (Lyons et al 1990). To minimize the possibility of oxidation and destruction of compounds such as caffeic acid, a hydrolysis procedure was routinely used that would at least minimize the loss of caffeic acid. The inclusion of a 30-sec N_2 sparge of the hydrolysis mixture immediately after addition of the base is critical when recovering caffeic acid using this method. If the N_2 sparge is omitted, less than 5% of the acid is recovered for derivatization, as shown in Figure 1A and B, where a mixture of five free CAD acids were processed through the hydrolysis step with and without the N_2 sparge and then derivatized.

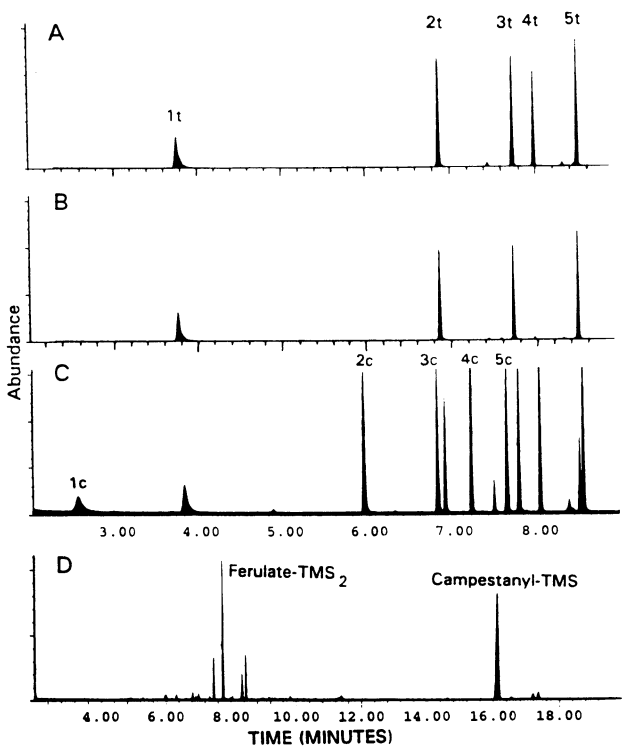

Fig. 1. Gas chromatography-mass spectrometry total ion chromatograms of cinnamic acid derivatives (CAD) after derivatization to trimethylsilyl (TMS) esters and ethers and separation on a 12-m DB-5 capillary column. Equal-weight mixture of five free CAD acids (all *trans*) derivatized after saponification with the N_2 sparge (**A**), and with no N_2 sparge (**B**). **C**, Same as **A**, except compounds are derivatized without saponification after 5-hr irradiation. **D**, Total ion chromatogram of saponified and derivatized campestanyl ferulate. Acids: 1 = cinnamic, 2 = *p*-coumaric, 3 = ferulic, 4 = caffeic, 5 = sinapic. c = *cis*. t = *trans*.

A potential problem with previous extraction schemes for this group of compounds was that they included addition of base to the hexane extract, which would destroy any caffeate esters present. The procedure used for the bulk of the work reported here employed a basification step. To determine whether esters of caffeic acid might be present, the crude hexane extract was fractionated directly on TLC plates without a partition step, and the appropriate bands were scraped, eluted, and analyzed. HPLC analysis of all the areas on the plates, except the main oil band, showed no evidence of appreciable amounts of sinapate, cinnamate, or caffeate steryl esters. This included material that migrates above the di- and triglyceride fractions on TLC—similar to cholesteryl cinnamate—which was saponified and analyzed for cinnamate esters.

Thin-Layer Chromatography

The purified steryl ferulate esters separate on TLC and the Chromatotron as two bands; the lower (later) band normally fluoresces much more intensely. To determine whether these were *cis* or *trans* isomers, a solution of sitostanyl ferulate was irradiated and separated on TLC versus a dark control. Portions of each were also analyzed by GC-MS before and after irradiation. The TLC bands for the irradiated sample were nearly equal in density after charring, but the dark control showed much more material in the lower band. GC-MS analysis confirmed that the ferulate in the upper band was identical to the material formed by irradiation of the free acids (Fig. 1C). We found 14.5% of *cis* isomer in the dark control and 40.1% in irradiated material. The faint upper band therefore is identified as the *cis* form of the esters.

The Chromatotron was useful in isolating a steryl CAD fraction free of most residual oils and sterols from the column chromatography fractions. It replaced the tedious process of streaking, scraping, and eluting bands from preparative TLC plates. One disadvantage, particularly for these types of compounds, is that, in the absence of a UV detector for the eluate, the progress of the separations must be followed by frequent use of a UV lamp, and this will cause some isomerization.

HPLC Analysis

Previous work on separating mixtures of steryl CAD esters used one- or two-part mobile phase mixtures of methanol, methanol and water, or methanol and acetonitrile with reverse-phase columns (Evershed et al 1988, Seitz 1989). In addition, a normal-phase HPLC method has been published (Tanaka et al 1977). Collins (1992) reported using ion-exchange, Sephadex LH-20, Octyl-Sepharose, and Nylon 6.6 column chromatography and two-dimensional TLC for separation.

Preliminary separations using the reverse-phase systems seemed to leave some compounds unresolved, and a better system was sought. Murphy and Stutte (1978) reported excellent resolution of free cinnamic acids with a system using a mixture of *n*-butanol, methanol, acetic acid, and water at 2.5:12.5:2:83 (v/v) and 0.018*M* ammonium acetate on a reverse-phase C_{18} column. This system was adapted for use with steryl CAD esters. Ammonium acetate was unnecessary; it was thought to prevent interaction between carboxy and ortho-hydroxyl groups (Murphy and Stutte 1978), which are not present in the ester. After we tried a number of different ratios for methanol, *n*-butanol, and water, and after we compared methanol to acetonitrile, we used a final mobile-phase composition of acetonitrile, *n*-butanol, acetic acid, and water (AnBAW) 94:3:2:1. A chromatogram of the steryl ferulate fractions (containing both *cis* and *trans* isomers) from corn using 97% acetonitrile is shown in Figure 2A along with the same material using AnBAW (Fig. 2B). Improvement is evident in the baseline separation of peaks and resolution of additional peaks. Figure 2C shows the ferulate fraction from rice bran oil under the same HPLC conditions as those of corn in Figure 2B. All of the peaks in these chromatograms have UV spectra typical

of ferulic acid. *p*-Coumarate esters were analyzed as a separate fraction that had some peaks from the ferulate bands, additional peaks corresponding to the *p*-coumarates, and unidentified compounds. The *p*-coumarate HPLC chromatogram is shown in Figure 3A superimposed on an expanded chromatogram of the ferulates to make comparison of the minor peaks easier. Typical UV spectra of each group are shown in Figure 4a and b, along with the spectra for the two internal standards used (Fig. 4d and e), and a spectrum representative of two compounds not identified (Fig. 4c). From the similarity in UV spectra of the different peaks resolved, there appeared to be at least 16 or more different compounds present in both rice and corn—if all the peaks represented steryl CAD esters. Based on the HPLC retention times and UV spectra, some of the minor compounds present in corn appeared to be major compounds in rice and vice versa.

Evershed et al (1988) reported improved separation when the esters were acetylated. Accordingly, a test mixture of ferulate esters was acetylated and run under standard conditions using AnBAW. No overall improvement in separation was seen and the acetates were not used further.

Although the *cis* and *trans* isomers separate distinctly on TLC (a normal-phase system), this is not so for the reverse-phase HPLC method employed here. To show this, the sample of UV-irradiated sitostanyl ferulate described above was analyzed by HPLC under standard conditions with internal standards. For both the dark control and the irradiated material, the peak shapes were similar and symmetric, with no suggestion of shoulders. The α_{erg} values were 2.84 for the dark control and 2.87 for the irradiated material; α_{cc} was 0.95(*trans*) and 0.96(*cis*), respectively. However, the UV spectrum taken over the peaks shows differences indicative of the two isomers present. Figure 5c shows the spectrum for the top of the peak of the control and spectra from a point about one-third up from the baseline (the estimated peak maximum for the *cis* isomer). The increase in *cis* isomer is evident in the flatter shape of the spectrum for the irradiated sample (Fig. 4a) and the intermediate shape for the control (Fig. 5b), which contains about 14% *cis* isomer (see above). Spectra from peak fronts and tops were the same for both preparations. Whether the *cis* form elutes somewhat later than the *trans* form for each of the different compounds was not determined. An unidentified compound eluting at about 7 min (Fig. 3A) with the same UV spectrum of the other ferulate compounds showed a similar pattern, except that a tailing shoulder was clearly present. Although not quantitative, this shift in the UV spectrum gives an indication of the level of *cis* isomer present.

Because the UV spectra were identical for species of ferulate esters with differing sterol moieties (no conjugated diene or higher systems were found) and compounds eluted closely together on HPLC, it was essential to have an internal standard to accurately collect and combine fractions from different runs. Cholesteryl

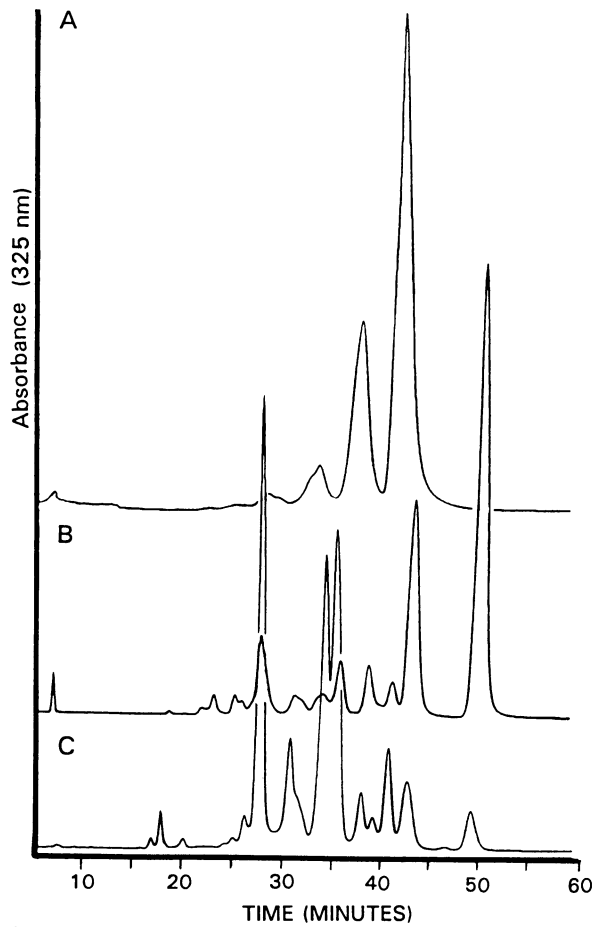

Fig. 2. Comparison of high-performance liquid chromatography solvent systems for separation of the steryl ferulate fractions for corn bran and rice bran oil. Mobile phase: acetonitrile and water (97:3, v/v) for corn bran (**A**). Mobile phase: acetonitrile, *n*-butanol, acetic acid, and water (93:4:2:1, v/v) for corn bran (**B**) and rice bran oil (**C**).

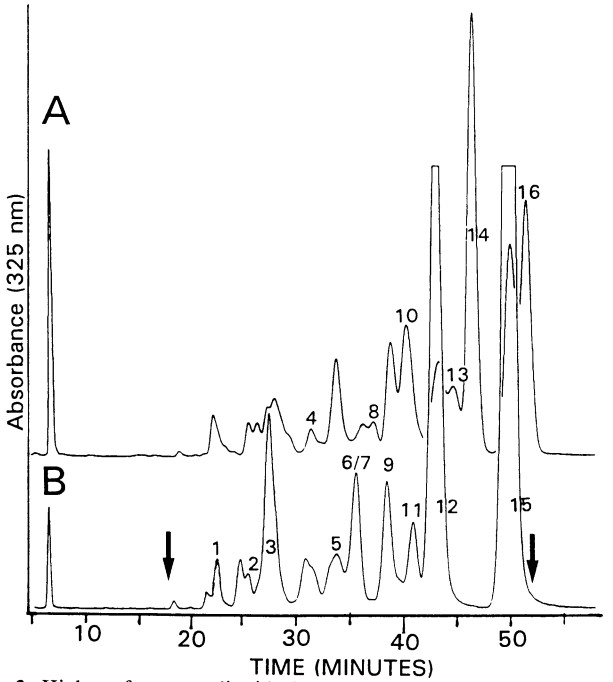

Fig. 3. High-performance liquid chromatogram of corn bran steryl *p*-coumarate (**A**) and steryl ferulate (**B**) fractions. Vertical scale expanded 2.5×. Arrows are the elution points for ergosterol (left) and cholesteryl cinnamate (right). Peaks are identified by number in Table II.

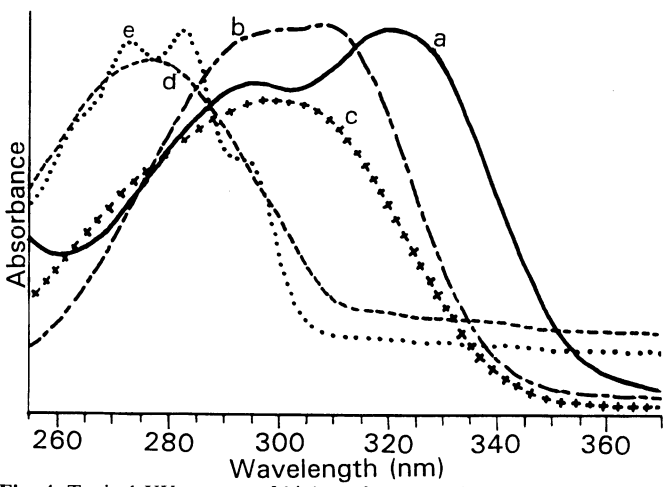

Fig. 4. Typical UV spectra of high-performance liquid chromatography peaks: a, ferulate; b, *p*-coumarate; c, unknowns; d, cholesteryl cinnamate internal standard; e, ergosterol internal standard. Spectra taken by diode array detector during elution with acetonitrile, *n*-butanol, acetic acid, and water (93:4:2:1, v/v).

cinnamate (5-cholesten-3B-ol 3-cinnamate) and ergosterol have several advantages as reference compounds: they are readily available, inexpensive, have retention times bracketing the range for CAD esters, and have UV spectra distinct from the hydroxylated CADs—having virtually no absorption at the λ_{max} for CAD (315–325 nm) but a similar λ_{max} at 280 nm (Fig. 3d and e). This allows them to be easily distinguished from other CAD esters (except cinnamates) that might be present. Comparing the chromatograms for the two wavelengths shows two extra peaks for the 280-nm detector that correspond to the two standards. The UV cutoff point for the AnBAW solvent system is ~240 nm, so most free sterols, if present, are not detected.

Gas Chromatography-Mass Spectroscopy

Because CAD are known to isomerize in light, especially light containing appreciable amounts of UV (Kahnt 1967), *cis* forms of pure *trans* standards were prepared. GC-MS analysis of the mixture showed that baseline separation was obtained for all of the isomers (Fig. 1C). This made it straightforward to monitor the ratio of *cis* to *trans* isomers in purified compounds, which helped to determine whether a shoulder or closely eluting peak was a separate compound or an isomer of the parent peak. The occurrence of *cis* CAD in *Hoya australis* was found to be an artifact produced by photoconversion (Baas and Niemann 1979). Similarly, no *cis*-ferulic acid was found in saponified cell-wall preparations of dark-grown seedlings of barley (Yamamoto and Towers 1985). However, because it is helpful to use UV to monitor different phases of the preparation of the esters (CC, chromatotron, TLC), appreciable quantities of the photoisomers could occur as artifacts. In addition, it is possible that at least some photoisomerization occurs naturally due to sunlight under normal growing and harvesting conditions. It is not clear whether the husks and outer pericarp filter out enough of the UV to entirely avoid isomerization.

To simplify and speed up the analysis of the HPLC fractions, the saponified esters were extracted only after acidification, and then twice with ethyl acetate. The extract, therefore, contained both the sterol and the phenolic portions of the ester, which were then derivatized in one step, and both portions were determined in one GC-MS run. The derivatives separated into two groups of peaks on the GC column, as shown in Figure 1A–C. Figure 1D shows a total ion chromatogram for the derivatized saponification products of campestanyl ferulate, which is representative of the results obtained from using this method.

In addition to GC-MS analysis of each of the saponified and derivatized HPLC peaks, an aliquot of each unsaponified peak was also chromatographed to ensure that no free sterol contaminant accounted for the sterol found in the saponified material. Free sterols had a molecular ion for the intact alcohol, whereas the ester yielded a mass spectrum for the Δ^2 compound remaining after thermal-1,-2 elimination of the substituent at the three position (*anhydro* form). This is known for the acetates of Δ^5-sterols (Budzikiewicz et al 1964) and has been previously noted for steryl ferulate esters by Tanaka and Kato (1975), who observed molecular ions for the TMS derivatives that were very small. Evershed et al (1988) were able to detect the molecular ions of four esters, including campestanyl and stigmastanyl ferulate, by using GC-MS at 20 eV. The TMS derivatives under the same conditions gave molecular ions as base peaks for these two esters. Molecular ions for the free alcohols or the TMS derivatives were not observed in the present work performed at 70 eV. The first appreciable ion was observed at $M^+ - (194+90)$ for ferulates; i.e., $M^+ -$(4-TMS-ferulic acid) and analogously for the *p*-coumarates .

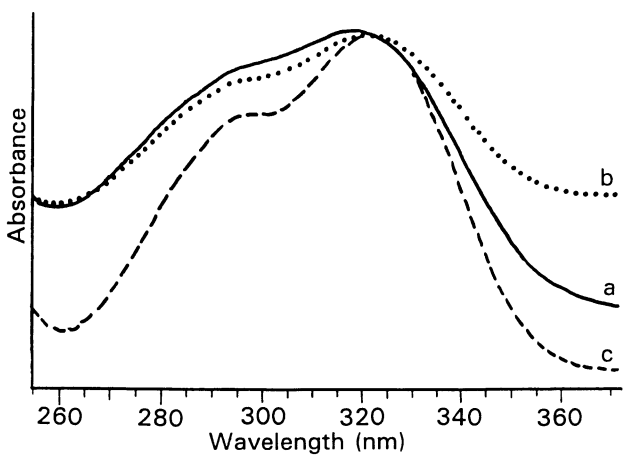

Fig. 5. UV spectra of sitostanyl ferulate before and after irradiation with short-wave UV for 5 hr and analyzed by high-performance liquid chromatography: from tail of irradiated sample (a), from tail of dark control (b), and from peak of dark control (c). Spectra taken by diode array detector during elution with acetonitrile, *n*-butanol, acetic acid, and water (93:4:2:1, v/v).

Identification and Quantitation of Esters

Identification of the compounds from the purified and saponified HPLC peaks was based on: 1) the retention time, mass spectra, and UV spectra of the CAD derivatives agreeing with standards; 2) the retention time and mass spectra of sterol derivatives agreeing with standards; and 3) observing the appropriate anhydro M^+ ions for the intact ester. Campesteryl and stigmasteryl ferulates eluted so close together on HPLC that one fraction contained campesteryl alone and the next fraction contained both. Hence, the saponified second fraction contained both compounds. It was assumed that the ferulate was esterified to both sterols. This was confirmed by finding anhydro M^+ ions for both compounds and by identifying campesteryl ferulate in the preparation in which it was the only component.

Table I lists the molecular ion and characteristic fragments for the sterol portion of the esters identified. Table II lists the

TABLE I
Electron Ionization Mass Spectra of Steryl TMS-Ether Derivatives of Saponified Steryl Ferulates and *p*-Coumarates

	Characteristic Ions: *m/z* (relative abundance)	
Sterol	**Molecular Ion**	**Fragment Ions**
Sitostanol-TMS	488 (56)	473 (63), 431 (23), 398 (29), 383 (48), 306 (24), 305 (24), 290 (13), 257 (4), 255 (4), 230 (11), 215 (79), 147 (19), 107 (29), 95 (31), 75 (100), 73 (37)
Campestanol-TMS	474 (53)	459 (69), 417 (22), 384 (31), 369 (49), 358 (5), 306 (30), 305 (26), 276 (13), 230 (14), 215 (90), 147 (21), 107 (32), 95 (27), 91 (16), 75 (100), 73 (35)
Sitosterol-TMS	486 (46)	471 (12), 396 (100), 381 (41), 357 (84), 356 (28), 329 (6), 303 (5), 275 (11), 261 (4), 255 (16), 213 (11), 145 (20), 129 (56), 105 (17), 95 (28) 73 (38)
Campesterol-TMS	472 (51)	457 (17), 382 (100), 368 (15), 367 (43), 344 (23), 343 (89), 342 (27), 315 (3), 289 (5), 261 (12), 255 (14), 213 (9), 173 (9), 161 (11), 160 (12), 159 (15), 145 (17), 129 (57), 95 (27)
Stigmasterol-TMS	484 (63)	469 (10), 394 (79), 379 (27), 372 (10), 355 (27), 354 (25), 352 (17), 351 (30), 281 (15), 255 (57), 253 (19), 213 (16), 159 (21), 147 (29), 145 (32), 129 (74), 83 (100)
Δ^7-Stigmastenol-TMS[a]	486 (100)	471 (24), 429 (3), 396 (9), 381 (19), 345 (10), 303 (8), 255 (43), 229 (19), 213 (21), 199 (9), 173 (6), 161 (10), 159 (13), 147 (14), 107 (18), 75 (21)
Δ^7-Campestenol	472 (100)	457 (24), 415 (3), 382 (6), 367 (17), 345 (7), 303 (3), 255 (33), 229 (17), 213 (21), 173 (5), 161 (6), 159 (8), 147 (10), 133 (9), 107 (11), 75 (23)

[a]Tentative identification, see text.

identified and putative esters and the relative retention values, average retention times, observed anhydro molecular ion, and percent composition by weight. Two peaks (10 and 14) were not integrated because the phenolic portion of the ester was not identified. The total amount of ester (excluding the unidentified peaks) was calculated as 93.3 μg per gram of fresh weight of bran (CV = 4.8). This is about 2.1 times as much as the average for seven lines that Seitz (1989) found for whole kernels, and it is lower by at least a factor of 5 than that expected for bran. The distribution of sterols was similar to that reported by Seitz (1989), within the range of compounds identified in that study. It is not clear why the level of esters was not higher, given that the fraction of the kernel reported to contain the bulk of the esters was used. According to the manufacturer, no heat was used in processing the bran. Possibly, milling and exposure of the finely ground bran to air or storage resulted in degradation of compounds. To check one possibility, the sterol fraction of the extract was analyzed by GC-MS. There were small amounts of the stanyls in this fraction but not the levels that would be expected if degradation released the free sterol. Perhaps the lines of corn that went into the bran had lower levels of esters than those of the lines Seitz evaluated.

The composition of the esters of corn bran, as shown in Table II, is largely (80.8%) composed of the saturated forms of sitosterol and campesterol, confirming previous studies. Only 10.7% is made up of the three sterols that are dominant in most corn tissues: sitosterol, campesterol, and stigmasterol. Also, a small percentage (2.3%) of the Δ^7-steryls was present, as well as more than 4.3% of unknowns. At least two additional *p*-coumarates were present at low levels: Δ^7-campestenyl and campesteryl *p*-coumarate. It was expected that some of the dominant sterols in rice bran oil esters would be found in corn, but campesterol was the only one that could be identified (at <5% in corn). Detectable amounts of cycloartenol or 24-methylene cycloartanol were not found. An uncharacterized compound at α_{erg} 1.56 (Fig. 3B, peak 3, MW 472) was not the latter compound, because 512 is the molecular weight for the TMS derivative of 24-methylene cycloartanol. Although there are patents referring to γ-oryzanol in corn (references in Seetharamaiah and Prabhakar 1986), it is clear that the composition of the γ-oryzanol fraction in corn is significantly different from that in rice.

A tentative assignment of Δ^7-stigmastenol was given to the sterol portion of the peak eluting at α_{erg} 2.20 (Table II) because: 1) the spectrum of Δ^7-campestenol (Table I) was very similar to that of the unknown, except for an m/z 14 difference in fragments arising from loss of the sidechain; 2) the m/z 129 peak that is characteristic of Δ^5-sterol TMS ethers was not prominent; 3) a moderately intense m/z 229 ion that is characteristic of Δ^7 compounds (Gustafsson et al 1966) was present. Dividing the retention time (R_t) of a Δ^7-campesteryl-TMS standard (16.91 min) by the R_t of campesteryl-TMS (Δ^5, 16.11 min) results in a factor of 1.05, which represents the change in R_t caused by the bond position. Multiplying this factor times the R_t of sitosteryl-TMS gives a predicted R_t of 18.17 min for Δ^7-stigmastenyl-TMS. The R_t of the unknown is 18.15 min.

The esters eluted at α_{erg} 2.26 and 2.61 (Table II) had an identical UV spectra at a λ_{max} of 299 nm (Fig. 4c); had a TLC R_f of 0.52; fluoresced dark purple with long-wave UV; and turned orange after spraying and charring. The saponification products are the sterol and *trans-p*-coumarate. The anhydro GC R_t and mass spectra were identical to those of the other *p*-coumarates. It is possible that the two compounds were epimers at about the sterol-3 position, which might account for the different chromatographic properties. However, it is not clear how that could account for the UV spectrum.

Stanyl esters gave two closely spaced GC peaks with mass spectra differing primarily in the intensity of an $M^+ - 54$ fragment of the anhydro sterol and the corresponding m/z 55 fragment. Figure 6A shows a GC-MS total ion chromatogram (TIC) for campestanyl ferulate and the corresponding mass spectra (Fig. 6B and C). The TIC for sitostanyl ferulate was similar and the spectra showed analogous differences. In this study, Δ^5-steryl ferulate or *p*-coumarate esters (cholesterol, sitosterol, stigmasterol, campesterol) produced only a single GC peak. This is in agreement with Budzikiewicz et al (1964), who showed that Δ^2-desmethyl sterols, but not Δ^5-sterols, eliminated butadiene (m/z 54) under electron impact, yielding an $M^+ - 54$ fragment of high intensity caused by a retro-Diels-Alder reaction. Thus, dehydration in the inlet resulted in the Δ^2 compound that, in stanyls, apparently underwent partial isomerization to an isomer that was much less likely to undergo the retro-Diels-Alder reaction. This is shown by the much reduced ion at $M^+ - 54$ for peak 2 (Fig. 6A and C).

TABLE II
Relative High-Performance Liquid-Chromatography Retention Values of Steryl Ferulates and *p*-Coumarates Isolated from Corn Bran with Respect to Two Internal Standards[a]

Peak	Sterol	MW[b]	CAD[c]	R_t[d]	α_{erg}[e]	α_{cc}[f]	AMW[g]	Wt. %[h]
1	U[i]	470	Fer[j]	22.57	1.27	0.436	380	0.4 (18.8)
2	U	498	Fer	26.92	1.52	0.520	408	Trace
3	U	472	Fer	27.74	1.56	0.536	382	3.9 (9.8)
4	Δ^7-Campestenol	472	*p*-Cou[k]	32.00	1.80	0.618	382	1.7 (10.3)
5	Δ^7-Campestenol	472	Fer	33.70	1.90	0.651	382	0.8 (11.0)
6	Campesterol	472	Fer	35.93	2.03	0.694	382	...[l]
7	Stigmasterol	484	Fer	36.19	2.04	0.699	394	5.1 (2.4)
8	Campesterol	472	*p*-Cou	36.97	2.09	0.714	382	Trace
9	Δ^7-Stigmastenol[m]	486	Fer	38.98	2.20	0.753	396	0.8 (11.4)
10	Campestanol	474	U[n]	40.02	2.26	0.773	384	...[o]
11	Sitosterol	486	Fer	41.68	2.35	0.805	396	5.6 (0.8)
12	Campestanol	474	Fer	43.07	2.43	0.832	384	19.3 (2.2)
13	Campestanol	474	*p*-Cou	44.47	2.51	0.859	384	0.9 (9.5)
14	Sitostanol	488	U	46.28	2.61	0.894	398	...
15	Sitostanol	488	Fer	49.91	2.82	0.964	398	60.2 (1.9)
16	Sitostanol	488	*p*-Cou	51.46	2.90	0.994	398	1.3 (13.1)

[a] Mobile phase composition of acetonitrile, *n*-butanol, acetic acid, and water (94:3:2:1).
[b] Molecular weight of ion of trimethylsilyl derivative.
[c] Cinnamic acid derivative.
[d] Average retention times.
[e] Retention values relative to ergosterol.
[f] Retention values relative to cholesteryl cinnamate.
[g] Anhydrate molecular weight of ion.
[h] Composition (%) by weight. CV in parenthesis.
[i] Identity uncertain.
[j] Ferulate.
[k] *p*-Coumarate.
[l] Peaks 6 and 7 combined.
[m] Tentative identification.
[n] UV γ_{max} 299 nm.
[o] Peaks not quantitated.

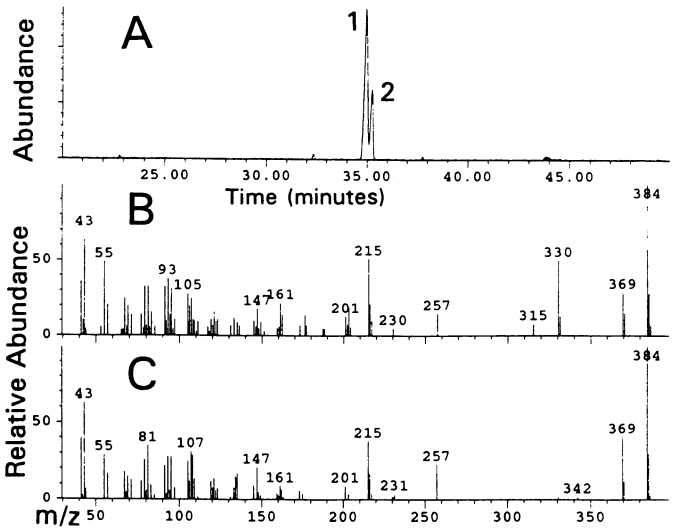

Fig. 6. Gas chromatography-mass spectrometry total ion chromatogram (**A**) of campestanyl ferulate and spectra showing its Δ^2 dehydration product isomers 1 (**B**) and 2 (**C**).

DISCUSSION

The function of steryl esters of ferulic and *p*-coumaric acid compounds in the metabolism of the plants and seeds in which they occur is unclear. There is evidence that the esters of rice bran oil have antioxidant activity against superoxide, which plays an important role in lipid peroxidation (Tajima et al 1983). Arguably, the related esters in corn have similar activity. Ferulic acid at a level of 200 ppm was reported to inhibit aflatoxin production in *A. parasiticus* NRRL-3240 by 90% (Sinha and Singh 1981). However, other investigators using the same fungal strain found that 100 and 200 ppm of ferulic acid showed only a moderate inhibition of growth (17 and 30%, respectively); it showed ~43% reduction in the aflatoxin level relative to that of the control (Chipley and Uraih 1980). Even at the lower level, this is a significant effect on the amount of aflatoxin. If a fungal, or kernel, esterase were present during infection or wounding, and it released free ferulic or *p*-coumaric acids from the esters, then it is possible that the outer region of the seed containing the esters could have an effect on the course of infection or the amount of aflatoxin produced. However, Seitz (1989) found no inhibition of spore germination or mycelium growth of *A. amstelodami* when incubated with a nutrient medium containing purified steryl CAD. *A. amstelodami* causes deterioration of grain in storage but is not known to rot kernels before harvest. Work in progress in our lab is studying the effect of pure esters and mixtures from corn on insect and fungal pathogens.

There are also effects on mammals of the related steryl CAD esters from rice. γ-Oryzanol is a commercial product that has been the subject of research and patents for a wide range of uses and activities for nearly 30 years (references in Seetharamaiah and Prabhakar 1986). A body of research shows beneficial effects of γ-oryzanol on hyperlipidemia in a variety of subjects, including humans (Yoshino et al 1989, Kahlon et al 1991). To date, there are no comparable studies on corn γ-oryzanol. There is clearly a need to determine whether these compounds offer health benefits that are comparable to those attributed to γ-oryzanol from rice. If these compounds can act as antioxidants, or if there are health benefits associated with their use, then their addition to some food products might be desirable. The results reported here will allow greater accuracy in determining the composition of this group of compounds and in isolating individual compounds for testing.

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