Antioxidant Activities of Major Components of γ-Oryzanol from Rice Bran Using a Linoleic Acid Model

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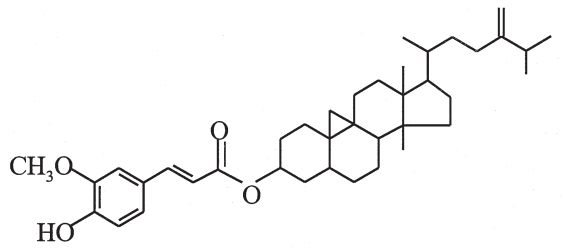
ABSTRACT: The change in hydroperoxides of linoleic acid incubated with constant micro air flow at 37°C was used to evaluate the antioxidant activities of three major components of γ-oryzanol from rice bran (cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate) compared with α-tocopherol and ferulic acid. The four hydroperoxide isomers of linoleic acid, 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid [9HPODE(*t,c*)], 9-hydroperoxy-10-*trans*,12-*trans*-octadecadienoic acid, 13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid [13HPODE(*c,t*)], and 13-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic acid, were measured using normal-phase high-performance liquid chromatography with an ultraviolet detector. The three components of γ-oryzanol evidenced significant antioxidant activity when they were mixed with linoleic acid in a molar ratio of 1:100 and 1:250 but not in a molar ratio of 1:500 ($P < 0.05$). α-Tocopherol and ferulic acid also demonstrated significant antioxidant activity at all three molar ratios ($P < 0.05$). The highest molar ratio (1:100) of α-tocopherol, however, caused greater levels of 9HPODE(*t,c*) and 13HPODE(*c,t*) than the other two less concentrated treatments.

Paper no. J9721 in *JAOCS 78,* 645–649 (June 2001).

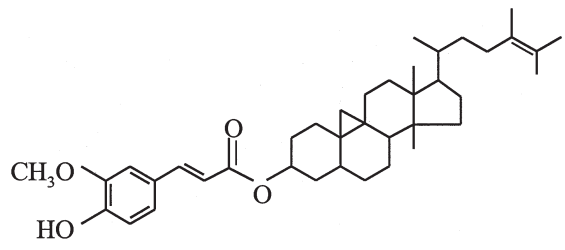
KEY WORDS: Antioxidant, hydroperoxide, linoleic acid, γ-oryzanol.

γ-Oryzanol in rice bran has been reported to lower serum cholesterol (1–3). Ten components of γ-oryzanol identified in rice bran consist of ferulic acid and triterpene derived compounds, which are combined by an ester bond (4). Cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate (Scheme 1) are the three major components and account for *ca.* 80% of γ-oryzanol in rice bran. The nutritional function of γ-oryzanol components may be related to their antioxidant property because of the ferulic acid structure. Ferulic acid is a phenolic acid antioxidant (5–8), and Ohta *et al.* (9) identified ferulic acid sugar esters as active components in corn bran hemicellulose fragments. However, the antioxidant capacities of γ-oryzanol components are not known.

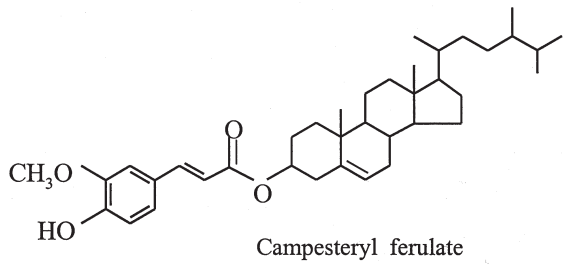
Accelerated stability methods are used extensively in evaluating the antioxidant activity of a compound. Usually, higher temperatures and oxygen concentrations are used to accelerate the oxidation in a lipid system. Frankel (10) reported the limitations of these tests and indicated that the results were unreliable when the test was done at some extreme conditions. Also, these severe models may not be appropriate to predict antioxidant activities of nutritional components in the human body. Under more gentle conditions, however, disadvantages include lower sensitivity in monitoring oxidative products and more time-consuming procedures. In this study, a mildly accelerated oxidation model was developed in which linoleic acid was incubated with micro air flow at 37°C, with continuous hydroperoxides analysis by a sensitive high-performance liquid chromatography (HPLC) method. The antioxidant activity of the three major components of γ-oryzanol was investigated and compared with that of α-tocopherol and ferulic acid under identical experimental conditions.



24-Methylene cycloartanyl ferulate



Cycloartenyl ferulate



Campesteryl ferulate

SCHEME 1

EXPERIMENTAL PROCEDURES

Chemical and materials. All solvents were HPLC grade. Hexane was obtained from Curtin Matheson Scientific Inc.

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(Houston, TX). Isopropanol was from Mallinckrodt Baker Inc. (Paris, KY), and ethyl ether and acetic acid were from Fisher Scientific Inc. (Fair Lawn, NJ). α-Tocopherol, ferulic acid, and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Hydroperoxides and hydroxides of linoleic acid were from ICN Pharmaceuticals, Inc. (Costa Mesa, CA) and were stored at −80°C before use. Rice bran was supplied from Riviana Foods, Inc. (Abbeville, LA).

Accelerated oxidation of linoleic acid. The three high-purity components of γ-oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate, were purified from rice bran using a preparative scale reversed-phase HPLC procedure (4). α-Tocopherol, ferulic acid, and the three high-purity γ-oryzanol components were prepared at concentrations of 0.08, 0.16, and 0.40 mM in hexane and isopropanol (9:1, vol/vol) as treatments. The control was hexane and isopropanol (9:1, vol/vol) without the addition of any components. Linoleic acid solution (40 mM) was prepared by dissolving 1.1202 g of linoleic acid in 100 mL of hexane. The linoleic acid solution (5 mL) and 5 mL of each treatment or control solution were mixed in a 25-mL test tube. The molar ratios of treatment to linoleic acid were 1:100 (0.40 mM treatment solution), 1:250 (0.16 mM treatment solution), and 1:500 (0.08 mM treatment solution). An aliquot of 500 μL of the solution was transferred to an HPLC vial to determine initial concentrations of hydroperoxides. Then, the test tube was incubated in a 37°C water bath. Tubing connected to compressed air (BOC Gases, Port Allen, LA) was passed though the tube cap and into the test tube with the tip of the tubing touching the bottom of the test tube. The flow rate of air was controlled at 200 μL/min. Air flow in the reaction solution produced a tiny air bubble about every 10 s. Time of sampling was scheduled at 40, 80, 120, 160, and 200 min from the beginning of incubation. The volume of reaction solution remained constant between each interval of sampling. In the sampling step, 500 μL was taken from the reaction solution and added to an HPLC vial after vortexing for 30 s.

Analysis of hydroperoxides of linoleic acid. The hydroperoxides of linoleic acid were determined using a normal-phase HPLC method. The HPLC system consisted of a Waters (Milford, MA) 510 pump, a 680 automated gradient controller, a 715 ultra WISP sample processor, a Hewlett-Packard (San Fernando, CA) diode-array detector, and a Baseline 810 chromatography workstation (Waters, Milford, MA). A Zorbax SIL (DuPont Co., Wilmington, DE) column was used with mobile phase that consisted of hexane/ethyl ether/isopropanol/acetic acid (100:15:0.1:0.1, by vol), and the flow rate (min, mL/min) was 0–18, 1.8; 17–18, 1.8–2.0; 19–39, 2.0; 39–40, 2.0–1.8. Total run time was 40 min. Absorbance at 234 nm was monitored with the detector. The concentration change of each hydroperoxide of linoleic acid at every sampling time was obtained by deducting the initial concentration from measured concentration. The change of concentration indicated the peroxide production of linoleic acid during oxidation.

Total production of hydroperoxides was obtained by summing all hydroperoxides. The rate (slope) of total production of hydroperoxides over time was used to evaluate antioxidant activity for each tested compound.

Statistical analysis. Each treatment and control were evaluated three times in the linoleic acid model. The production of each hydroperoxide of linoleic acid was analyzed using the General Linear Model procedure of the Statistical Analysis System (SAS Institute, Cary, NC). The rates of total production of hydroperoxides were analyzed using the regression model of the Statistical Analysis System. Significant difference between means was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Hydroperoxides of linoleic acid. Four hydroperoxides (HPODE) and hydroxides (HODE) of linoleic acid were separated using normal-phase HPLC (Fig. 1). Linoleic acid is susceptible to oxidation because its structure contains 1,4-pentadiene that is highly vulnerable to free radical attack (11). The hydrogen atom abstraction from the 1,4-pentadiene of linoleic acid leads to a pentadienyl radical. The pentadienyl radical generates 9-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid [9HPODE(*t,c*)] and 13-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid [13HPODE(*c,t*)] after addition of oxygen. Also, the pentadienyl radical produces a new *trans* pentadienyl radical during β-fragmentation. The *trans* pentadienyl radical produces 9-hydroperoxy-10-*trans*,12-*trans*-octadecadienoic acid [9HPODE(*t,t*)] and 13-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic acid [13HPODE(*t,t*)]. Generally, the *trans* structure has lower energy than the *cis* structure and dominates the hydroperoxides mixture (11).

Adequate resolution of each hydroperoxide was obtained after HPLC conditions were optimized. Since the structure and molecular weight of linoleic acid hydroperoxides and hy-

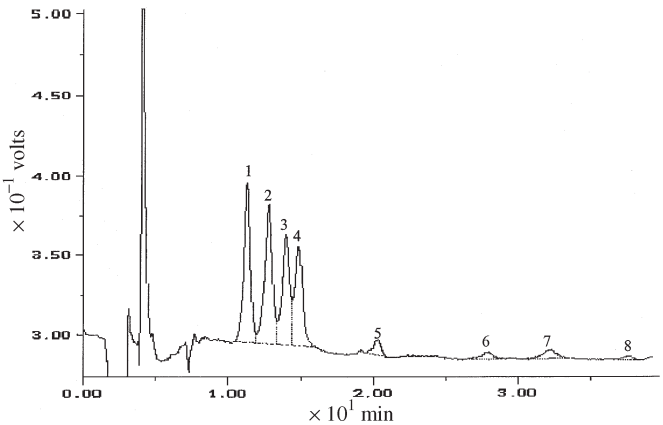

FIG. 1. Chromatogram of hydroperoxides and hydroxides of linoleic acid at 200 min of oxidation time. **1**: 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid [13HPODE(*c,t*)]; **2**: 13-hydroperoxy-9-*trans*,11-*trans*-octadecadienoic acid [13HPODE(*t,t*)]; **3**: 9-hydroperoxy-10-*trans*-12-*cis*-octadecadienoic acid [9HPODE(*t,c*)]; **4**: 9-hydroperoxy-10-*trans*,12-*trans*-octadecadienoic acid [9HPODE(*t,t*)]; **5**: 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid [13HODE(*c,t*)]; **6**: 13-hydroxy-9-*trans*,11-*trans*-octadecadienoic acid [13HODE(*t,t*)]; **7**: 9-hydroxy-10-*trans*,12-*cis*-octadecadienoic acid [9HODE(*t,c*)]; **8**: 9-hydroxy-10-*trans*,12-*trans* octadecadienoic acid [9HODE(*t,t*)].

droxides are similar, it is difficult to separate them individually within a short retention time. The 40-min run time with gradient flow rate of the mobile phase was needed to obtain quantitative resolution. In addition, the short wavelength (234 nm) used in the ultraviolet (UV) detector was very close to the UV cutoff of ethyl ether, isopropanol, and acetic acid, thus the baselines of chromatograms were slightly noisy. The order of elution of the hydroperoxides and hydroxides of linoleic acid was 13HPODE(*c,t*), 13HPODE(*t,t*), 9HPODE(*t,c*), 9HPODE(*t,t*), 13HODE(*c,t*), 13HODE(*t,t*), 9HODE(*t,c*), and 9HODE(*t,t*). The chromatogram of hydroperoxides of linoleic acid is similar to that of hydroperoxides of methyl linoleate (12). Hydroxides of linoleic acid were not as abundant as that of hydroperoxides and did not change significantly during oxidation; thus hydroxides were not sensitive indicators of the degree of lipid oxidation in this linoleic acid model system within 200 min of oxidation. Hydroperoxides of linoleic acid were used to evaluate oxidation status of linoleic acid in this system. Makinen *et al*. (12) also reported that the concentration of hydroperoxides is greater than that of hydroxides in the methyl linoleate model.

Linoleic acid model. In Table 1, the productions of hydroperoxides of the control after 120 and 200 min of oxidation are depicted. The four hydroperoxides increased significantly with increasing oxidation time. The amounts of 13HPODE(*t,t*) and 9HPODE(*t,t*) were significantly greater than that of 13HPODE(*c,t*) and 9HPODE(*t,c*) during oxidation with air. This greater production of *trans,trans* hydroperoxide isomers supported the idea that the hydroperoxides containing a *trans,trans* structure have lower energy and are more thermodynamically favorable for formation during free radical attack than those having *trans,cis* or *cis,trans* (11). Discriminate changes in concentration for each hydroperoxide in the linoleic acid model as measured by HPLC demonstrated that this system could be used in quantitative determination of the status of linoleic acid oxidation. The mild conditions of oxidation in this model, i.e., 37°C and micro air flow, overcome the limitations of accelerated stability models that use more severe or complex oxidative conditions as discussed by Frankel (10). The antioxidant activity of a component determined in this model may more closely reflect its real situation in a biological system. A similar analytical method and model were performed by Makinen *et al*. (12) after methyl linoleate was oxidized at 40°C for 24 and 48 h with or without antioxidants. The advantage of methyl linoleate model is that the hydroperoxides in methyl linoleate model are not as readily decomposed as in linoleic acid model in longer oxidation time (13). As hydroperoxides with linoleic acid are not highly stable, the linoleic acid model may only be suitable to indicate oxidation status in the early stage of oxidation.

Antioxidant activity of α-tocopherol and ferulic acid at different ratios to linoleic acid. The rates of hydroperoxide production in linoleic acid with α-tocopherol (α-tocopherol/linoleic acid molar ratio = 1:100, 1:250, and 1:500) are shown in Figures 2A, 2B, and 2C, respectively. Antioxidant activity of α-tocopherol was indicated by significantly lower rates of hydroperoxide production than control. An interesting phenomenon was noted in that there was a higher rate of formation of hydroperoxides containing *cis,trans* or *trans,cis* structure at the 1:100 ratio of α-tocopherol than at 1:250 or 1:500 (Fig. 3), even though hydroperoxides containing *trans,trans* were extensively inhibited by α-tocopherol. This agrees with Makinen *et al*. (12), who state that α-tocopherol increases the proportion of the *cis,trans* hydroperoxides while the formation of *trans,trans* hydroperoxides is inhibited. Jung and Min (14) reported that tocopherols had significant prooxidant effects at higher concentration. In this study, high concentrations of α-tocopherol inhibited the production of *trans,trans* hydroperoxides but appeared to elicit prooxidant activity by accelerating free radical reactions that produced *cis,trans* and *trans,cis* hydroperoxides. The total production of hydroperoxides was higher in 1:100 ratio of α-tocopherol than either 1:250 or 1:500 ratio. Marinova and Yanishlieva (6) also found that the antioxidant activity of α-tocopherol is altered at different temperatures. α-Tocopherol is more effective at extending the induction period and reducing the rate of autoxidation as the temperature is increased (6).

Ferulic acid exhibited antioxidant activity at all three molar ratios (ferulic acid/linoleic acid = 1:100, 1:250, and 1:500) (Figs. 2A, 2B, 2C). The production of hydroperoxides with ferulic acid at a ratio of 1:100 was significantly lower than that with the ratios of 1:250 or 1:500. Table 1 shows the

TABLE 1
Production of Hydroperoxides of Control, α-Tocopherol, Ferulic Acid, and the Three Major γ-Oryzanol Components Treatments After 120 and 200 min of Oxidation (treatment component/linoleic acid molar ratio = 1:250)[a]

Products	13HPODE(*c,t*)		13HPODE(*t,t*)		9HPODE(*t,c*)		9HPODE(*t,t*)	
Time (min)	120	200	120	200	120	200	120	200
Control	225.7 ± 36.1	316.2 ± 56.9	517.9 ± 77.7	950.4 ± 76.0	71.8 ± 8.6	134.1 ± 6.7	185.5 ± 31.5	370.8 ± 14.8
α-Tocopherol	78.7 ± 7.9	119.7 ± 9.6	41.0 ± 3.3	54.2 ± 3.8	50.7 ± 6.1	78.7 ± 6.3	26.8 ± 2.1	34.6 ± 2.8
Ferulic acid	48.9 ± 3.9	70.5 ± 7.8	151.0 ± 22.6	244.8 ± 39.2	24.7 ± 1.5	61.0 ± 7.3	60.8 ± 4.9	100.2 ± 15.0
COMP 1	182.1 ± 25.6	252.0 ± 44.1	346.8 ± 28.8	473.9 ± 53.4	86.0 ± 8.8	126.4 ± 15.2	106.0 ± 14.8	157.4 ± 26.8
COMP 2	169.2 ± 26.2	292.4 ± 37.7	292.3 ± 31.4	417.6 ± 31.4	75.8 ± 9.4	112.8 ± 10.7	84.5 ± 13.6	136.0 ± 19.1
COMP 3	163.4 ± 29.7	250.4 ± 42.8	261.9 ± 28.3	362.9 ± 41.6	89.6 ± 9.2	113.4 ± 13.6	73.8 ± 14.6	106.7 ± 19.9

[a]COMP 1, cycloartenyl ferulate; COMP 2, 24-methylene cycloartanyl ferulate; COMP 3, campesteryl ferulate; 13HPODE(*c,t*), 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid; 13HPODE(*t,t*), 13-hydroperoxy-9-*trans*-11-*trans*-octadecadienoic acid; 9HPODE(*t,c*), 9-hydroperoxy-10-*trans*-12-*cis*-octadecadienoic acid; 9HPODE(*t,t*), 9-hydroperoxy-10-*trans*-12-*trans*-octadecadienoic acid.

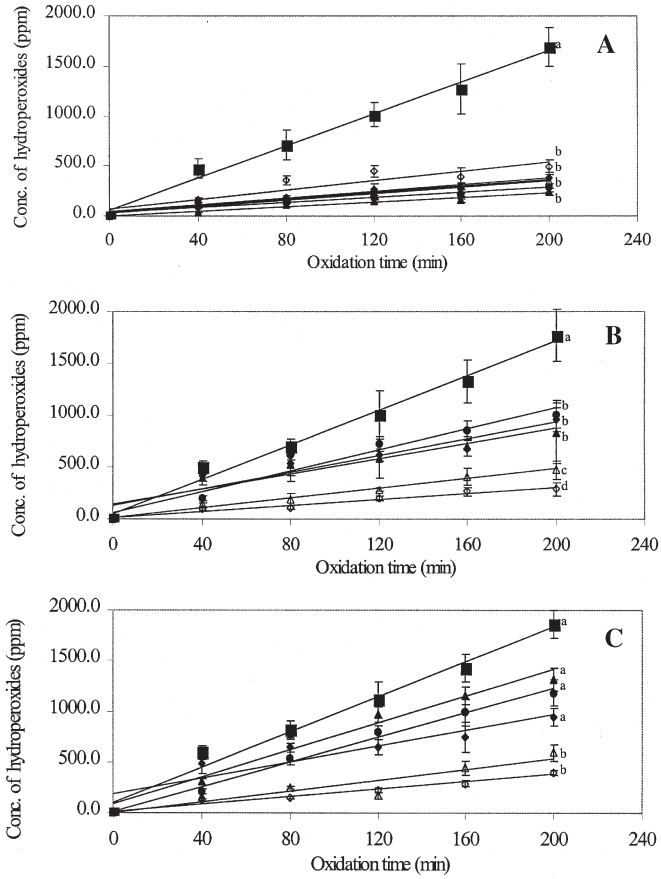

FIG. 2. Rates of production of total hydroperoxides of linoleic acid with different ratios of component to linoleic acid and control. Significant differences in rate ($P < 0.05$) are expressed by different letters in each ratio. (A) 1:100 in mole; (B) 1:250 in mole; (C) 1:500 in mole. ■ Control; ● cycloartenyl ferulate; ◆ 24-methylene cycloartanyl ferulate; ▲ campesteryl ferulate; ◇ α-tocopherol; △ ferulic acid.

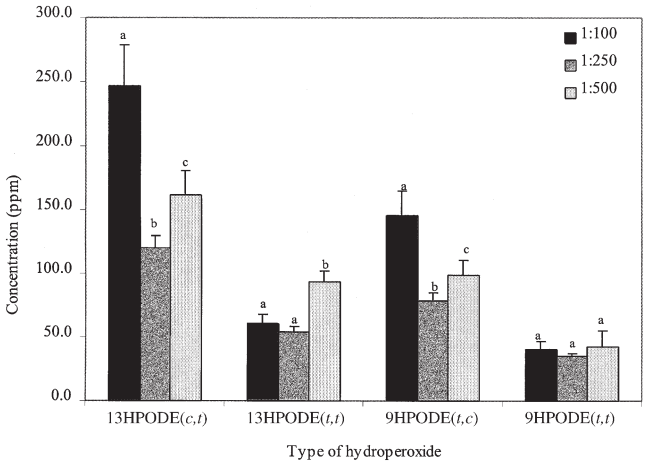

FIG. 3. Production of hydroperoxides of linoleic acid with different molar ratios of α-tocopherol to linoleic acid after 200 min oxidation. Significant differences ($P < 0.05$) are expressed by different letters on the bars in a cluster. For abbreviations see Figure 1.

production of each hydroperoxide of linoleic acid in ferulic acid treatment after 120 and 200 min of oxidation. Unlike α-tocopherol, in which *cis,trans* hydroperoxide increased to a greater extent than *trans,trans* isomers, all of the hydroperoxides with ferulic acid increased over time. The concentration of *trans,trans* hydroperoxides was higher than that of *cis,trans* or *trans,cis* hydroperoxides. It has been reported that the antioxidant activity of ferulic acid depends on the hydroxylation (–HO) in the phenolic ring (5,8). Marinova and Yanishlieva (6) investigated the antioxidant activity of ferulic acid using purified lard triacylglycerol at 25, 50, 75, and 100°C and found that antioxidant activity of ferulic acid remained constant with increasing temperature.

Antioxidant activities of the three major components of γ-oryzanol at different ratios to linoleic acid. Three major components of γ-oryzanol, cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, and campesteryl ferulate, significantly reduced the rate of hydroperoxide production at the ratios of 1:100 and 1:250 (Figs. 2A,2B) but not at 1:500 (Figs. 2C). Antioxidant activities of these components decreased significantly with decreasing concentration in the linoleic acid model. The production of each hydroperoxide in the three major oryzanol components treatments after 120 and 200 min of oxidation is listed in Table 1. The concentrations of *trans,trans* hydroperoxides were much higher than that of *trans,cis* and *cis,trans* hydroperoxides. The changes in concentration of individual hydroperoxides in linoleic acid models with each of the three components were similar to those with ferulic acid. This suggests that the mechanism of antioxidation of components of γ-oryzanol is the same as that of ferulic acid. The antioxidant activity of these ferulic components arises from the phenolic hydroxyl group in the ferulate portion of their overall structure. In this study, free ferulic acid had greater activity than did the three ferulic acid esters at the three different ratios, especially at a lower molar ratio (1:500). The triterpene portion of γ-oryzanol may affect its antioxidant activity by lowering the mobility in the system due to its relatively larger molecular structure than free ferulic acid.

Although the antioxidation activities of γ-oryzanol components were lower than that of α-tocopherol in protecting against linoleic acid oxidation, the quantity of these components is 10 times greater than α-tocopherol in rice bran (15). Because the structure of γ-oryzanol components is similar to that of cholesterol, an important component in cell membranes, they may have greater availability and accessibility in cell membranes than α-tocopherol in reducing oxidation stress and maintaining functionality of cells. We are currently evaluating this possibility in model systems. It is apparent from this study that the antioxidant function of γ-oryzanol components supports the potential nutritional value of rice bran.

ACKNOWLEDGMENT

Approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript number 99-21-0562.

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[Received August 2, 2000; accepted February 22, 2001]