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Antioxidant Activity of Steryl Ferulate Extracts from Rye and Wheat Bran

LAURA NYSTRÖM,* MARJUKKA MÄKINEN, ANNA-MAIJA LAMPI, AND VIENO PIIRONEN

Department of Applied Chemistry and Microbiology, University of Helsinki, Post Office Box 27, FIN-00014 Helsinki, Finland

Antioxidant activity of steryl ferulates from other sources than rice have not yet been studied much, despite the fact that rice steryl ferulates (γ-oryzanol) have been shown to possess good antioxidant activity. In this study, steryl ferulate extracts from wheat or rye bran were studied for their capability to inhibit hydroperoxide formation in bulk methyl linoleate and methyl linoleate emulsion. Further, their activity to scavenge DPPH radicals was analyzed. The activities were compared to synthetic steryl ferulates, rice steryl ferulates, ferulic acid, and α-tocopherol. Nonrice cereal extracts of steryl ferulates exhibited good antioxidant activity, especially in the bulk lipid system. The radical scavenging activity was similar to that of nonesterified ferulic acid, indicating that the ferulic acid moiety is responsible for the antioxidant properties. This study illustrates a new aspect to the health-promoting properties of rye and wheat.

KEYWORDS: Steryl ferulates; rye; wheat; *Secale cereale* L.; *Triticum aestivum* L.; cereal antioxidants

INTRODUCTION

Cereal antioxidants have gained substantial interest in the past years. Increasing consumption of cereals also increases the intake of components that have beneficial health effects. Further, there is a demand to find effective natural antioxidants to replace synthetic ones, which has also added to the amount of research done on cereal antioxidants such as tocopherols, tocotrienols, and phenolic acids (*1*). For example, Andreasen et al. (*2*) have demonstrated a significant antioxidant activity of ferulic acid from rye in inhibiting LDL oxidation. The content of ferulic acid in that study was 3 times higher in rye bran than in whole grain rye. Similarly, tocopherols as well as other bioactive components (phytosterols, folates, lignans, and alk(en)ylresorcinols) were shown to be localized on the outer parts of the cereal kernel, especially the bran (*3*).

Steryl ferulates (ferulic acid esters of plant sterols and their saturated form stanols) make up a significant part of sterols in cereal bran layers. The steryl ferulate molecule is a combination of a cholesterol-lowering plant sterol and an antioxidative phenolic acid (**Figure 1**). Thus far, most research has been done with γ-oryzanol that is a mixture of steryl ferulates from rice. In sources such as wheat bran, the content of steryl ferulates varies in the range of 30−39 mg/100 g, contributing 20−25% of all plant sterols (*4*). In addition to the well-documented total and LDL-cholesterol-lowering properties of plant sterols (*5*), steryl ferulates are demonstrated to have antioxidant activity (*6*, *7*) and anti-inflammatory properties (*8*) and to inhibit tumor formation (*9*).

Metabolism of steryl ferulates in humans is still quite poorly known. In rabbits, however, the level of ferulic acid in the plasma was shown to be dose-dependent to the administration of γ-oryzanol. Only very small amounts of intact γ-oryzanol were found in the plasma of the rabbits and none in the urine (*10*). It has also been shown that, to reduce cholesterol absorption, sterols must be hydrolyzed from their conjugates. Two recent studies have shown that steryl ferulates are hydrolyzed by mammalian digestive enzymes. Moreau and Hicks (*11*) demonstrated that synthetic sitostanyl ferulate is hydrolyzed by cholesteryl esterase and pancreatin, whereas steryl ferulates of γ-oryzanol were only hydrolyzed by cholesteryl esterase. Miller et al. (*12*) demonstrated that lipase preparations from different sources (animal, plant, bacteria, and fungi) cannot hydrolyze steryl ferulates of γ-oryzanol. Further, they also showed that cholesteryl esterase was only capable of hydrolyzing sitosteryl ferulate and campesteryl ferulate, which are present only in small amounts in rice γ-oryzanol but are the major steryl ferulates in other cereals such as wheat and rye. Cycloartenyl ferulate and 24-methylenecycloartanyl ferulate, which are major steryl ferulates in rice bran, were not hydrolyzed with any of the cholesteryl esterases in the study. The key difference in the steryl ferulates from rice to those from wheat or rye is the sterol structure: in rice, the sterols are 4,4-dimethylsterols with two methyl groups in carbon C4, whereas the sterols in other cereals are principally desmethylsterols that have no methyl groups in the C4 position. The study suggests that wheat and rye steryl ferulates may have an advantage in having a better suited ring structure for human metabolism, which enables the activity to lower cholesterol absorption. Vissers et al. (*13*) suggested that

* To whom correspondence should be addressed. Telephone: +358 9 19158282. Fax: +358 9 19158475. E-mail: laura.nystrom@helsinki.fi.

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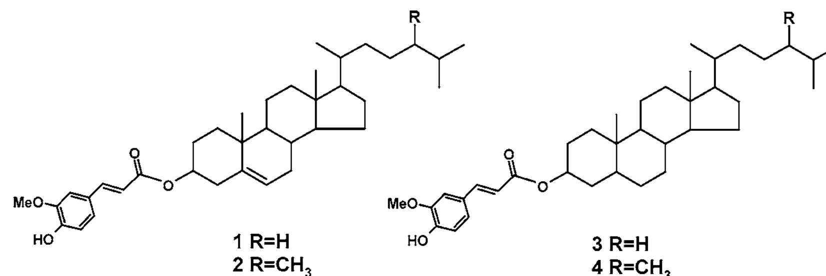

Figure 1. Chemical structures of campesteryl ferulate (**1**), sitosteryl ferulate (**2**), campestanyl ferulate (**3**), and sitostanyl ferulate (**4**).

the beneficial effects of rice bran oil on serum cholesterol levels were due to desmethylsterols and not 4,4-dimethylsterols. This is further supported by the study of Trautwein et al. (*14*) who demonstrated that 4,4-dimethylsterols had a weaker cholesterol-lowering effect than desmethylsterols. Thus, it may be prudent to refocus the research of steryl ferulates to the more active steryl ferulates with a desmethylsterol moiety.

Steryl ferulates are also considered to be potential antioxidants because of the hydrogen-donating ability of the phenolic group of ferulic acid. Campesteryl ferulate was shown to inhibit linoleic acid oxidation caused by UV irradiation (*6*). Xu and co-workers studied the antioxidative properties of rice bran γ-oryzanol components and showed that they inhibited induced-cholesterol oxidation even more than vitamin E components that are well-known for their antioxidant properties (*15*). In another study of the same authors, γ-oryzanol was shown to inhibit also linoleic acid oxidation (*16*). Further, γ-oryzanol components, namely, cycloartenyl ferulate and 24-methylenecycloartanyl ferulate, were shown to act as antioxidants in methyl linoleate bulk and multiphase lipid systems and as radical scavengers (*7*). Because most studies of the antioxidant activity of steryl ferulates so far are done with γ-oryzanol and its single constituents, there is not much information available on the antioxidant properties of steryl ferulates with a desmethylsterol structure, which are on the other hand the major steryl ferulates in sources other than rice.

Antioxidant activity of different compounds in complex food systems may vary greatly depending on the contents of water and lipid in the system. Discussions of antioxidant activity often apply to the concept of "polar paradox", which principally suggests that polar, hydrophilic antioxidants have better activities in nonpolar matrixes and vice versa. The hydrophilic nature of hydroxycinnamic acids, such as ferulic acid, inevitably changes as the acid is esterified to a nonpolar alkyl group like a fatty acid or sterol. Kikuzaki et al. (*7*) studied the partition of ferulic acid and its sterol esters between *n*-octanol and phosphate-buffered saline and concluded that esterification with cycloartenol or 24-methylenecycloartanol changed the partition coefficient from 0.13 to 0.99, indicating a better affinity of steryl ferulic acid esters to the hydrophobic phase. Therefore, it is necessary to study antioxidant capability of a compound in different surrounding environments. On the other hand, polar compounds such as ferulic acid have a limited solubility in very nonpolar lipid matrixes. Thus, steryl ferulates, owing to hydrophobicity and therefore better solubility, may be added in higher quantities, which may compensate for a possible lesser activity.

The aim of this study was to analyze the antioxidant activity of synthetic steryl ferulates and steryl ferulates extracted from rye and wheat bran and to further compare the possible differences between compounds with a different steryl moiety or a different origin of extraction. Antioxidant activity was evaluated by analyzing the ability of steryl ferulates to inhibit methyl linoleate oxidation by monitoring hydroperoxide formation in a bulk and emulsified lipid system. Further, the possible mechanism of antioxidant activity was assessed by determining the radical scavenging activity of synthetic and extracted steryl ferulates.

MATERIALS AND METHODS

Chemicals and Materials. Rye bran (*Secale cereale* L.) and wheat bran (*Triticum aestivum* L.) were obtained from two food companies (Oy Karl Fazer Ab, Oulainen Mill, Finland, and Melia Company, Finland). Sitosteryl ferulate and cholesteryl ferulate were synthesized by Dr K. Wähälä (Department of Chemistry, University of Helsinki, Finland). Cycloartenyl ferulate and γ-oryzanol were kindly provided by Dr P. Kochhar (Good-Fry International, n.v., Rotterdam, The Netherlands). Ferulic acid, pyrogallol, and diphenylpicrylhydrazyl (DPPH) were purchased from Sigma Chemicals (St. Louis, MO). α-Tocopherol (purity 98.8%) was purchased from Merck (Darmstadt, Germany). Methyl linoleate (purity > 99%) was obtained from Nu Chek Prep, Inc. (Elysian, MI). Each lot of methyl linoleate was checked not to contain tocopherols at a level that would lead to a tocopherol content greater than 1 μg/g in the final sample. The method for tocopherol analysis is described below. Methyl linoleate lots with any tocopherol residues were purified using a method previously described by Lampi et al. with minor changes (*17*). A glass column (20 × 2.0 cm inside diameter) was packed with 50 g of aluminum oxide, which had been activated (100 °C for 8 h and 200 °C for 12 h) and suspended in 85 mL of heptane. After the addition of the lipid to be purified, the column was washed with 75 mL of heptane. Solvents of HPLC grade were supplied by Rathburn Chemicals (Walkerburn, U.K.). Diethyl ether was purified prior to use by silica solid-phase extraction cartridge to remove peroxides.

Production of the Steryl Ferulate Extracts from Rye and Wheat Bran. Steryl ferulates were extracted from cereal bran based on the method described by Seitz (*18*) and further developed by Hakala et al. (*4*). Samples of cereal bran (10 g) were extracted with 100 mL of acetone under shaking for 1 h and filtered through a Whatman GF/A filter paper, after which the procedure was repeated with a fresh portion of acetone. The two extracts were combined, evaporated to dryness in a rotavapor, and dissolved to methanol (15 mL). The solution was made alkaline by adding 5 mL of 0.6% potassium hydroxide solution and washed twice with hexane (10 mL). After this, 1 mL of 6 M hydrochloric acid was added and steryl ferulates were extracted 3 times with hexane (5 mL). The combined extracts were evaporated and dissolved to 5 mL of 96% methanol. These crude extracts from a number of extractions were pooled and filtered before purification.

The crude extract was purified with solid-phase extraction columns (Supelco LC18, 20 mL, 5 g). After conditioning with 96% methanol, about 15 mL of the crude extract was filtered on the column (Gelman GHP-Acrodisc syringe filter, 0.45 μm) and the column was washed with 96% methanol. The first 100 mL was drawn to waste, after which five fractions (10 mL each) were eluted with 100% methanol. The fractions were monitored with a high-performance liquid chromatograph (Model 1090, Hewlett−Packard, Böblingen, Germany) using a C18 reverse-phase column (5.0 μm, 4.6 × 250 mm, ODS-2, Waters Spherisorb, Wexford, Ireland). The mobile phase methanol/water (98:2) was used at a flow rate of 2.0 mL/min, with column heating at 50 °C. The effluent was monitored using a diode-array detector at three

wavelengths (325, 292, and 280 nm). Quantification of steryl ferulates was done at 325 nm, purification of the extract was monitored at 280 nm to detect substances with an aromatic ring and 292 nm to detect tocopherols in particular. The fractions containing steryl ferulates were combined and concentrated. The content of steryl ferulates in the final extract was determined using the same RP-HPLC method and cycloartenyl ferulate as an external standard.

Characterization of the Steryl Ferulate Extracts. The sterol profiles of the steryl ferulates in cereal extracts were relatively similar for both extracts: rye bran extract consisted of 17% campesteryl ferulate, 53% campestanyl and sitosteryl ferulates (coeluting in the chromatogram), and 30% sitostanyl ferulate. The composition of wheat bran extract steryl ferulates was 20, 56, and 24%, respectively. On the basis of the LC−MS analysis of rye and wheat steryl ferulates by Hakala et al. (*4*), it is known that the majority of sterol moieties are the saturated stanols. Thus, the coeluting peak in the chromatogram principally consists of campestanyl ferulate.

Steryl ferulate extracts as well as γ-oryzanol and cycloartenyl ferulate were analyzed to be free of tocopherols (final concentration in an oxidation sample of less than 0.1 μg/g). For this analysis, a method reported by Ryynänen et al. (*19*) using a normal phase Si column was used (5 μm, 250 × 4.6 mm, Inertsil 5 Si, Varian Chromapack, Middelburg, The Netherlands). Mobile phase was 3% 1,4-dioxane in *n*-hexane at a flow rate of 2 mL/min. Tocopherols were detected with a fluorescence detector with excitation at 292 nm and emission at 325 nm. The HPLC apparatus was composed of an autosampler (Waters 700 Satellite WISP, Millipore Corporation, Milford, MA), pump (Waters 510), UV−vis-diode array detector (Waters 996), fluorescence detector (HP1046A, Hewlett−Packard), and a computer workstation. Data handling was done using Millennium 2010 software (Waters).

The final steryl ferulate extracts from rye and wheat were obtained from solid-phase extraction elution stream, and only fractions that contained steryl ferulates were chosen, as described above. The final extract was analyzed in three different chromatographic systems. First was a RP-HPLC analysis with a diode array detector that was used to quantify steryl ferulates. The second system was a normal phase HPLC (NP-HPLC) system with fluorescence detection, which was used for tocopherol analysis. The third chromatographic system was also a NP-HPLC system that was used for the analysis of methyl linoleate hydroperoxides (described in detail below). None of these three chromatographic systems with different detectors revealed the presence of compounds other than steryl ferulates in the extracts.

Antioxidant Activity Assays. Antioxidant activity of synthetic steryl ferulates, rye and wheat extracts, rice steryl ferulates, and ferulic acid was studied by monitoring hydroperoxide formation in bulk methyl linoleate. Because of the large number of compounds under study, samples in the methyl linoleate bulk system were divided into two analytical series. Each series included ferulic acid and α-tocopherol as reference compounds for comparison. Further, each series contained one synthetic steryl ferulate, one sample of extracted steryl ferulates from cereals, and one sample of characteristic rice steryl ferulates. Furthermore, as the emphasis of this study was on nonrice steryl ferulates, sitosteryl ferulate, wheat extract, and rye extract were chosen for methyl linoleate emulsion studies and the DPPH test. The DPPH test was also done on cholesteryl ferulate.

In the studies of methyl linoleate oxidation (both bulk and emulsion systems), the formation of hydroperoxides was determined at two levels of concentration 0.52 and 2.57 mM, corresponding to 100 and 500 μg/g of ferulic acid, respectively. These concentration levels are in the following tables and figures denoted as 1 and 2, respectively. The molecular weight of campesteryl ferulate (576.9 g/mol) was applied for the calculations of rye and wheat extracts and γ-oryzanol. α-Tocopherol was analyzed only at the lower concentration. Radical scavenging activity was determined at two final concentrations of 1.67 and 16.67 μM. All analyses were performed in triplicate.

Radical Scavenging Activity. Radical scavenging activity was measured by the method of Malterud et al. (*20*). Antioxidant solutions were prepared at two concentrations, 0.1 and 1.0 mM, in methanol, thus leading to final concentrations of 1.67 and 16.67 μM in the sample. The DPPH solution (0.45 mg/L in methanol) was pipetted into a 1-cm cuvette, and the absorbance was recorded at 517 nm (Perkin−Elmer Lambda 11/Bio UV−vis spectrometer, Überlingen, Germany). Sample solution was added (50 μL), and the absorbance was recorded immediately after addition (A_0) and further at 15-s intervals up to 5 min. The solution was stirred after the first measurement. Pyrogallol (4.0 mM) was used as a positive control to determine 100% scavenging activity. Each analysis was performed in triplicate. The percentage for radical scavenging activity (RSA %) was calculated with the following formula: RSA % $= (A_0 - A_t)/(A_0 - A_p)$ x 100, where A_0 = absorbance at the beginning of the measurement at time zero, A_t = absorbance of the sample after 5 min, and A_p = absorbance of the pyrogallol sample after 5 min.

Antioxidant Activity in Bulk Methyl Linoleate. A sample (0.5 g) of methyl linoleate was weighed in 4-mL glass vials (Ø = 12 mm), and the antioxidant was added in solutions of methanol, except α-tocopherol in ethanol. Control samples were treated similarly with pure methanol without the antioxidant. The solvent was evaporated under nitrogen stream. Samples in open vials were oxidized at 40 °C in a dark oven, and the rate of oxidation was monitored by determining the increase in the amount of hydroperoxides. Samples for HPLC analysis were taken before oxidation and later at suitable intervals by diluting a 50-mg sample with heptane in a 5-mL volumetric flask. Formation of methyl linoleate hydroperoxides was determined by following the increase in peak area (*A*) of hydroperoxides in the HPLC chromatogram. As the rate of oxidation varied between the parallel experiments, results of the different series were not combined but were analyzed separately. Results reported in this paper are means of triplicate analyses. Uninhibited oxidation served as a control in calculations of the percentage of inhibition, which was calculated using the following formula: % inhibition $= (A_{control} - A_{sample})/A_{control} \times 100$.

Antioxidant Activity in Emulsified Methyl Linoleate. Similar antioxidant studies were also performed in methyl linoleate emulsions. Methyl linoleate (0.5 g) was weighed in 20-mL vials (Ø = 25 mm), and antioxidants were added similarly as in the bulk methyl linoleate samples. After the solvent of antioxidant solution was evaporated, 50 mg of emulgator Tween 20 was added in 4.45 mL of water. Emulsion was formed by sonicating (U50 Control Ultrasonic Processor, IKA Labortechnik, Germany) the sample for 3 × 45 s in an ice bath. The particle size of the emulsion was 1−1.5 μm, determined by microscopic analysis (Zeiss Axioskop 2 plus, Carl Zeiss Microscopy, Germany). Vials were sealed with screw caps to avoid water evaporation during oxidation. Samples were oxidized in a dark oven at 40 °C under moderate magnetic stirring. Aliquot samples were taken before and during oxidation to monitor the formation of hydroperoxides. Each sample (500 mg) was weighed in a test tube, followed by 2 mL of methanol and a few drops of saturated sodium chloride solution. Methyl linoleate hydroperoxides were extracted with 2 mL of heptane. Extraction was repeated 2 times, and all three extracts were combined and diluted to final volume of 10 mL. Sample was dried with anhydrous sodium sulfate and filtrated with a GHP-syringe filter prior to HPLC analysis. Again oxidation was monitored by measuring the increase in hydroperoxide peak area.

HPLC Determination of Hydroperoxides. Formation of the four methyl linoleate hydroperoxides (methyl-13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoate, methyl-13-hydroperoxy-*trans*-9-*trans*-11-octadecadienoate, methyl-9-hydroperoxy-*cis*-10-*trans*-12-octadecadienoate, and methyl-9-hydroperoxy-*trans*-10-*trans*-12-octadecadienoate that may be abbreviated to 13-*cis*,*trans*, 13-*trans*,*trans,* 9-*cis*,*trans*, and 9-*trans*,*trans*, respectively) was monitored as previously reported by Mäkinen et al. (*21*). The HPLC apparatus was similar to the one used in tocopherol analyses described above, excluding the fluorescence detector. The mobile phase was 12% diethyl ether in heptane with a flow rate of 0.4 mL/min. A Supelcosil column LC-SI 57930 (250 × 2.1 mm, 5 μm particle size, Supelco, Bellefonte, PA), preceded by a Supelcosil precolumn (20 × 2.1 mm) was used for chromatographic separations. Hydroperoxides were detected at a wavelength of 234 nm. Functioning of the chromatographic system (retention times and detector response) was controlled with daily analysis of a purified sample of methyl linoleate hydroperoxides.

Data Analysis. Statistical differences between three different samples (hydroperoxide peak areas or inhibition percentages) or three DPPH activity measurements were tested using Kruskal−Wallis test or

Table 1. DPPH-Radical Scavenging Activities (RSA) of Antioxidants at Two Concentration Levels Using Pyrogallol (4.0 mM) as a Reference for 100% Activity

	RSA (%)[a]	
antioxidant	final concentration of 1.67 μM	final concentration of 16.67 μM
sitosteryl ferulate	3.5 ± 0.15 a	13.5 ± 0.64 a
cholesteryl ferulate	3.7 ± 0.22 a	14.2 ± 0.56 a
rye extract	4.7 ± 1.02 a	17.5 ± 0.67 b
wheat extract	5.0 ± 3.33 a	16.4 ± 0.28 c
ferulic acid	5.1 ± 1.90 a	19.8 ± 0.40 d
α-tocopherol	5.2 ± 0.15 a	34.2 ± 0.43 e

[a] RSA (%) = mean ± SD. Each value is a mean of triplicate analysis. Values with the same letter within a column are not statistically different ($p < 0.05$).

ANOVA with Statgraphics Plus 3.0 software (Manugistics, Inc., Rockville, MD) at a 95% confidence level.

RESULTS AND DISCUSSION

Radical Scavenging Activity. Radical scavenging activity was measured at two final concentrations of 1.67 and 16.67 μM. Results are represented in **Table 1**. At the lower concentration, there were no significant differences in radical scavenging activities between steryl ferulates, ferulic acid, and α-tocopherol. At the higher concentration, α-tocopherol had the highest radical scavenging activity of all studied compounds. The activity decreased in the order α-tocopherol > ferulic acid > rye extract > wheat extract > cholesteryl ferulate = sitosteryl ferulate. Ferulic acid was a slightly more active radical scavenger than its sterol esters, which is in agreement with previously published results (*7*). In other studies, radical scavenging activity of ferulic acid was determined to be 15.7 and 27.3% with antioxidant concentrations of 13.3 and 20 μM, respectively (*7*, *22*), which is in good agreement with our result for the radical scavenging activity of ferulic acid at 19.8% with a concentration of 16.67 μM. The sterol structure did not have an effect on the activity of synthetic sitosteryl ferulate or cholesteryl ferulate, which both exhibited equal radical scavenging activity. On the other hand, there was a significant difference in the activity of synthetic steryl ferulates and steryl ferulate bran extracts, with the latter being more effective. Furthermore, rye bran extract had a better radical scavenging activity than wheat bran extract. The key difference that could be thought to make a difference in the antioxidant activity between synthetic steryl ferulates and steryl ferulate extracts is the sterol composition: extracted steryl ferulates are comprised of numerous sterols (in this case four major sterols), and synthetic steryl ferulates are comprised of only one. As previously mentioned, the steryl ferulate extracts did not to our knowledge contain any other compounds than steryl ferulates that could explain the better radical scavenging activity.

Antioxidant Activity in Bulk Methyl Linoleate. All components tested in this study showed significant antioxidant activity in the bulk methyl linoleate system when studied by analyzing the capability to inhibit hydroperoxide formation (**Figure 2**) and further by calculating the inhibition percentages (**Table 2**). In moderate temperatures of autoxidation, the length of the initiation period may vary considerably between different experiments and thus the results from the two experimental series were not combined for calculations. To be able to compare the results of the different series, α-tocopherol and ferulic acid were analyzed in both experimental series along with one synthetic steryl ferulate, one cereal extract, and one steryl ferulate sample with rice origin.

The inhibition percentages for different antioxidants were calculated after 5 days at a point of oxidation where the formation of hydroperoxides in the control sample was still dominating their degradation (**Table 2**). For 5 days of oxidation, all added compounds could inhibit hydroperoxide formation substantially with inhibition percentages between 75 and 99.9% (**Table 2**). The results show that in series 1 oxidation had not proceeded as far in 5 days as in series 2, which is indicated by the inhibition percentages of the compounds analyzed in both series. The difference was especially clear in ferulic acid 1 (0.52 mM), which has an inhibition percent of 93.1% in the first series and 75.4% in the second series. Similarly, the inhibition percentages of ferulic acid 2 (2.58 mM) and α-tocopherol were significantly lower in the second experimental series, indicating that the oxidation of methyl linoleate had proceeded faster than in the first series. Nonetheless, these two antioxidants had similar behavior in both experimental series, which indicates that they may be used to standardize the results of the two series. The lower added level (0.52 mM) of free ferulic acid, synthetic sitosteryl, or cholesteryl ferulate and steryl ferulates from rice (cycloartenyl ferulate and oryzanol) had the poorest capability to retard the formation of hydroperoxides, while the lower level of extracted steryl ferulates from wheat bran or rye bran had an activity comparable to α-tocopherol. The inhibition percentages of sitosteryl ferulate, cholesteryl ferulate, cycloartenyl ferulate, and γ-oryzanol even at the higher concentration (2.58 mM) were significantly lower than that of α-tocopherol (0.52 mM), whereas extracted steryl ferulates from rye and wheat had a significantly higher inhibition percentage and ferulic acid had an equal inhibition percentage with α-tocopherol. When considering the inhibition percentages of steryl ferulates, it may be stated that natural mixtures of steryl ferulates from nonrice sources are more active antioxidants than synthetic or rice-originated steryl ferulates.

Steryl ferulates and ferulic acid acted as antioxidants at both concentration levels, and the activity increased with increasing concentration at the range used in this study (**Figure 2**). Response to increased concentration might not be linear for all antioxidants. For example, α-tocopherol was shown to be pro-oxidative in the bulk methyl linoleate system at levels above 250 μg/g (*23*). In this study, α-tocopherol was analyzed only at the lower concentration of 0.52 mM that is 222 μg/g, and therefore, as expected, the activity of α-tocopherol was anti-oxidative rather than pro-oxidative. In the course of nearly 3 weeks of oxidation, the higher concentration (2.57 mM) of cereal steryl ferulate extract was the most effective antioxidant in both series, followed by α-tocopherol (0.52 mM) and the higher concentration of ferulic acid, which were equally active. This indicates that steryl ferulates and ferulic acid are poorer antioxidants than α-tocopherol in bulk methyl linoleate, because a 5-fold concentration is needed to accomplish equal activity. Rye and wheat extracts were the most active antioxidants of steryl ferulates at the lower concentration as well. The fact that the antioxidant activity of steryl ferulates from cereals was less than that of α-tocopherol does not however reduce their importance as natural antioxidants and health-promoting compounds because the content of steryl ferulates in, e.g., wheat bran (30−39 mg/100 g) is over 10-fold the content of tocopherols (2.2 mg/100 g) (*24*).

The steryl ferulates from rice, oryzanol and cycloartenyl ferulate, did not inhibit the formation of methyl linoleate hydroperoxides quite as effectively as other steryl ferulates. In both series, oryzanol and cycloartenyl ferulate were among the least active antioxidants tested in this study. For example, the

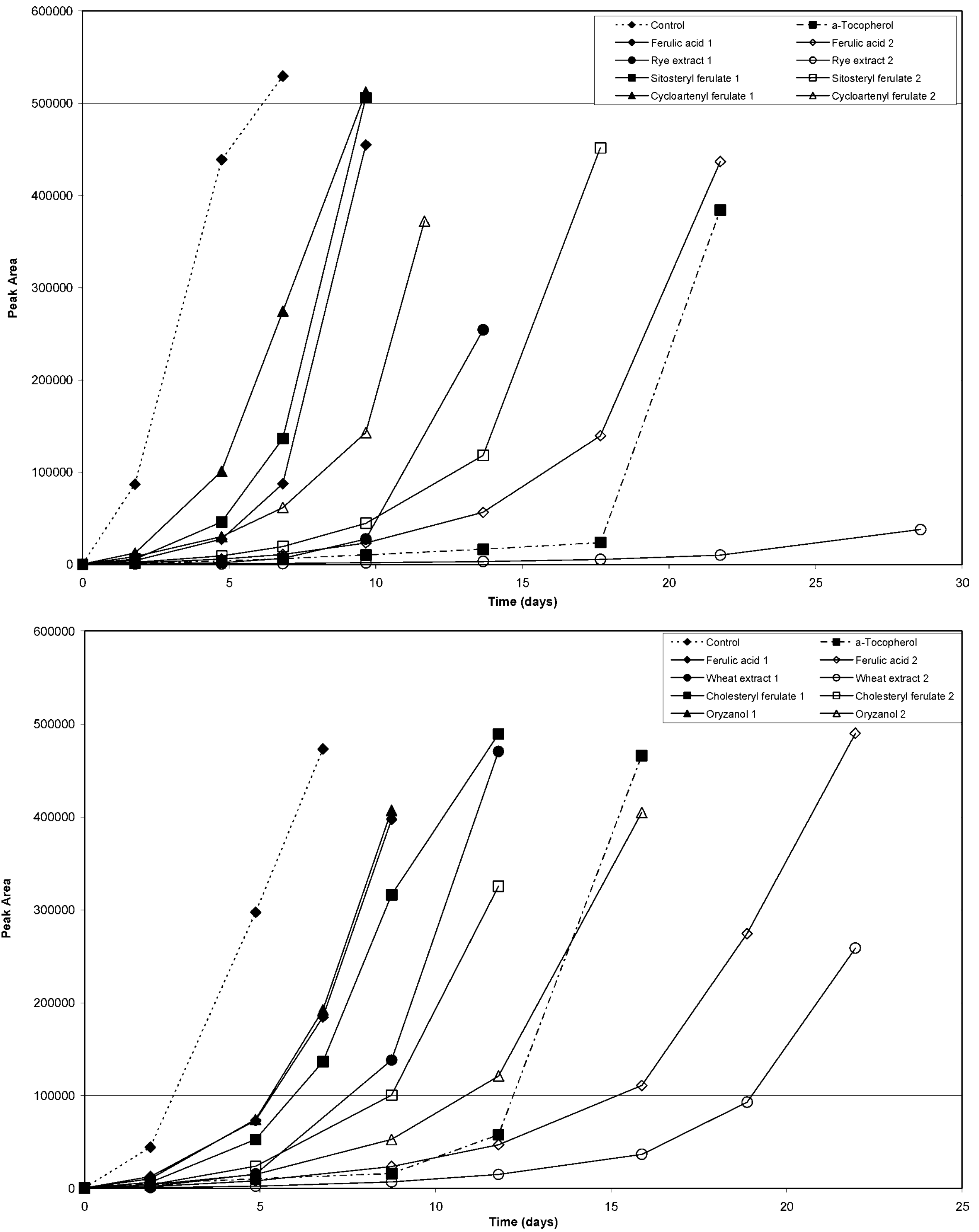

Figure 2. Antioxidant activity assays in bulk methyl linoleate at +40 °C. Sample 1 refers to the lower concentration (0.52 mM), and sample 2 refers to the higher concentration (2.58 mM). Upper graph is series 1, and lower graph is series 2.

inhibition percentages of cycloartenyl ferulate 1 and oryzanol 1 after 5 days were 76.1 and 75.0%, whereas those of sitosteryl ferulate 1 and cholesteryl ferulate 1 were 89.6 and 82.3% respectively. Similar result was seen in the study by Wang et al. (*25*), in which sitostanyl ferulate was a more effective antioxidant than oryzanol. However, this is in contrast with the study of Xu et al. (*15*) who demonstrated that in an accelerated diphase system 24-methylenecycloartenyl ferulate (dimethylsterol) was a better antioxidant than campesteryl ferulate (desmethylsterol). It may be possible that the ring structure of

Table 2. Inhibition Percentages Determined in the Bulk Methyl Linoleate System at +40 °C[a]

sample	inhibition percentage (5 days) series 1	inhibition percentage (5 days) series 2
ferulic acid 1	93.1 ± 1.1 a	75.4 ± 3.8 a,d
ferulic acid 2	98.7 ± 0.3 b	97.3 ± 0.7 b
α-tocopherol	99.1 ± 0 b	96.6 ± 0 b
rye extract 1	99.5 ± 0.2 b,c	
rye extract 2	99.9 ± 0 c	
sitosteryl ferulate 1	89.6 ± 0.6 d	
sitosteryl ferulate 2	97.8 ± 0.1 e	
cycloartenyl ferulate 1	76.1 ± 0.3 f	
cycloartenyl ferulate 2	92.6 ± 0.2 a	
wheat extract 1		94.8 ± 0.4 c
wheat extract 2		99.2 ±0.1 e
cholesteryl ferulate 1		82.3 ± 0.4 d
cholesteryl ferulate 2		92.0 ± 1.7 c
oryzanol 1		75.0 ± 1.3 a
oryzanol 2		95.0 ± 0.4 c

[a] Sample 1 refers to the lower concentration (0.52 mM), and sample 2 refers to higher concentration (2.58 mM). There is no statistically significant difference between the samples with the same letter within each column ($p < 0.05$). Each result is a mean of three independent analyses.

4,4-dimethylsterols and 4-desmethylsterols affects, for example, the solubility of the compound in a way that the desmethylsteryl ferulates are better antioxidants in nonpolar environments, whereas dimethylsterols are more active in diphasic systems.

Effects of Antioxidants on the Isomeric Distribution of Methyl Linoleate Hydroperoxides in the Bulk Lipid System. Along with the ability to retard total hydroperoxide formation, the effects of antioxidants on the ratio of different hydroperoxide isomers were studied. In methyl linoleate autoxidation, antioxidants do not only inhibit the formation of hydroperoxides but also affect the relative amounts of different hydroperoxide isomers in the system. The cis,trans isomers are the principal compounds that are first formed and later converted to trans,trans isomers. This isomerization is inhibited by effective hydrogen-donating antioxidants, thus increasing the relative abundance of cis,trans isomers (*21*). In the present bulk methyl linoleate oxidation studies in **Figure 2**, the relative amounts of different isomers were calculated for the samples with the higher antioxidant addition at a point when the oxidation had proceeded to a level similar to the control sample after 2 days of oxidation. The percentage of cis,trans isomers in the control sample was 45% and for ferulic acid and steryl ferulates, all at 43−46%. On the other hand for α-tocopherol, the percentage of cis,trans isomers was 62%, which is significantly higher than that for ferulic acid and its steryl esters. This phenomenon is supported by the radical scavenging activities of these antioxidants described above that α-tocopherol is a better radical scavenger and thus also a better hydrogen donor compared to ferulic acid and steryl ferulates, and therefore, it scavenges the hydroperoxy radical also faster principally in the cis,trans form and before it is isomerized to trans,trans hydroperoxide. Similar observations were reported by Xu and Godber (*15*), who analyzed the antioxidant activity of α-tocopherol, ferulic acid, and γ-oryzanol compounds.

Antioxidant Activity in Emulsified Methyl Linoleate. Measurements of antioxidant activity in emulsions are generally more complex than those in bulk lipid systems, and thus, two identical experimental emulsion series were carried out. Results for one of the series are shown in **Figure 3**, and the results for the other identical series were similar. All compounds in the test showed antioxidant activity in methyl linoleate emulsions. Ferulic acid, sitosteryl ferulate, and steryl ferulate extracts from rye and wheat all had equal antioxidant activity at the lower concentration (0.52 mM). This did not show the polar paradox that polar ferulic acid would have a lower activity in the polar environment compared to its nonpolar counterparts. Similar to the bulk methyl linoleate system, a higher concentration of steryl ferulates inhibited hydroperoxide formation more than the lower concentration. A major difference between the activities of these

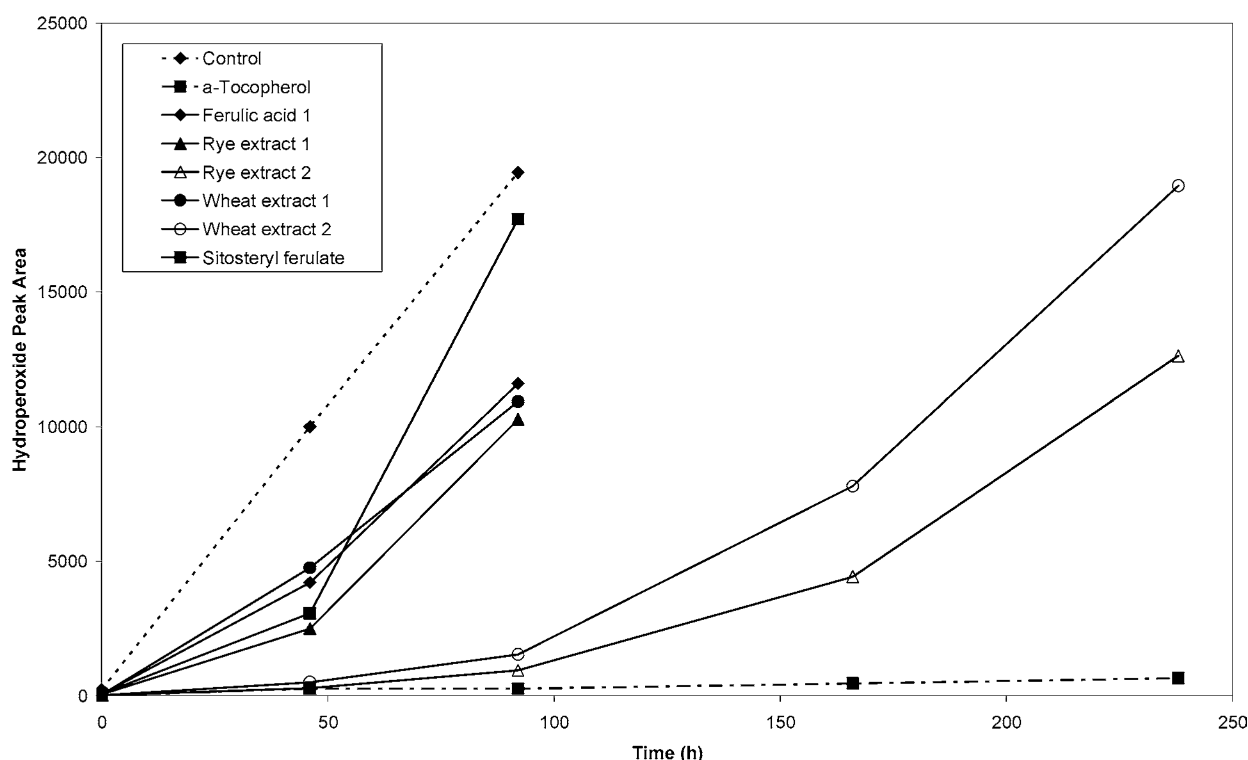

Figure 3. Antioxidant activity assays in methyl linoleate emulsions with Tween 20 emulgator at +40 °C. Sample 1 refers to the lower concentration (0.52 mM), and sample 2 refers to the higher concentration (2.58 mM).

compounds in the bulk or emulsified methyl linoleate systems was that in emulsion α-tocopherol showed the greatest antioxidant activity even if it was again added at the lower concentration. In bulk lipid systems, the cereal steryl ferulate extracts at the higher concentration exhibited better activity than α-tocopherol. In the emulsion system, the extracted steryl ferulates from rye or wheat bran did not possess better antioxidant capacity than the synthetic sitosteryl ferulate or free ferulic acid. Because the formation of hydroperoxides went over the induction period only with the ferulic acid and steryl ferulate samples, no comparison to the isomeric distribution of the hydroperoxides with the α-tocopherol samples can be made. However, it is obvious that, if the α-tocopherol sample had been monitored longer, cis,trans isomers would be expected to dominate.

In this study, the total oxidation time for emulsions was about one-third of that used in bulk lipid oxidation. Hydroperoxide analysis was done only for the time that the sample structure was uniform. After the formation of hydroperoxides had began to accelerate, the emulsion structure also started to slowly break down and thus lipid oxidation could not be expected to further proceed evenly in the sample. Uniformity and stability of the emulsion are essential for a successful antioxidant analysis but are both fairly difficult to accomplish. Choice of an emulsifying agent greatly affects the activity of antioxidants because partitioning of an antioxidant between water and lipid phases and their interface is influenced by the emulsifying agent (*26*). Studies in our laboratory have shown that with phenolic antioxidants Tween 20 emulsions tend to break up faster than lysolecithin emulsions, resulting in an uneven size and distribution of oil particles. However, lysolecithin, which is a common alternative emulsifier to Tween, is of plant origin and contains significant amount of tocopherols that retard oxidation. Thus, lysolecithin cannot be used in emulsion systems that analyze antioxidant activity because the tocopherols of the emulsifier also contribute to the oxidation system. It is possible that, as oxidation proceeded generally so fast in the emulsion, no difference between the activities of different antioxidants could be seen. Perhaps with another emulsifying agent the emulsion structure would not break as fast, and it would be possible to differentiate between the activities of ferulic acid and synthetic or extracted steryl ferulates.

Huang et al. (*27*) suggested that in emulsion systems hydrophobic antioxidants would locate in the lipid phase and the lipid−water interphase where they may inhibit lipid oxidation, whereas the more hydrophilic antioxidants would be located in the water phase and thus be less effective in the inhibition of lipid oxidation. This was not clearly the case in our study, because no difference in the antioxidant activity of free ferulic acid and its more hydrophobic steryl esters could be seen. On the other hand, the hydrophobic α-tocopherol was very active in the inhibition of lipid oxidation in the emulsion system and, in comparison with the activity of hydrophilic free ferulic acid, the theory for the activity of nonpolar antioxidants in polar systems can be seen. A difference in the antioxidant activity of ferulic acid and steryl ferulates could not be seen in the diphase system in the study of Kikuzaki et al. (*7*), where no difference was seen when compared to α-tocopherol as well. This is unlike our study in which α-tocopherol was much more effective in the inhibition of lipid oxidation than ferulic acid and its steryl esters. In that study, however, no emulsifying agent was used in the mixture of linoleic acid, ethanol, and buffer, and thus, it is hard to predict and compare the partitioning of antioxidants in the sample and further their antioxidant activity in the system.

Steryl ferulates show good antioxidant activities in both bulk and emulsion lipid systems, principally owing to the radical scavenging activity of the ferulic acid moiety of the molecule. A steryl ferulate mixture extracted from cereal bran may exert even more activity than a similar synthetic component of the mixture. Further, this study also demonstrates the better activity of steryl ferulates from rye and wheat when compared to steryl ferulates from rice. In light of their high content in cereal bran, steryl ferulates from rye and wheat are important bioactive compounds adding to the health benefits of the fiber-rich bran in the diet. Further, if metabolized more effectively than their counterparts from rice, steryl ferulates of wheat and rye may be even more beneficial to health than the already well-defined γ-oryzanol.

ABBREVIATIONS USED

DPPH, 2,2-diphenyl-1-picrylhydrazyl; concentration of antioxidant sample 1, 0.52 mM; concentration of antioxidant sample 2, 2.58 mM.

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