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Antioxidant Potential of Hydroxycinnamic Acid Glycoside Esters

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Hydroxycinnamic acids are natural antioxidants found in fruits, vegetables, and cereals. In this study, the antioxidant activity of various types of hydroxycinnamoyl glycoside esters that mimic the structure of polymeric carbohydrates was studied in different model systems prone to oxidation, namely, liposomes and emulsions. In addition, radical scavenging activity against the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was tested. It was found that the esterification in the primary hydroxyl group of the glycoside resulted in the improved radical scavenging activity of both sinapoyl and feruloyl glycosides compared to conjugation to the secondary hydroxyl group. Increased activity was also observed, particularly in the case of feruloyl glucosides in inhibiting the oxidation of liposomes emulsions. The results showed that sinapic and ferulic acid glycoside esters were as effective or more efficient antioxidants than their free forms. In conclusion, the strength of their antioxidant effect depends on the nature of conjugation.

KEYWORDS: Hydroxycinnamic acid; ferulic acid; sinapic acid; glycoside ester; conjugation; radical scavenging; antioxidant activity

INTRODUCTION

Hydroxycinnamic acid derivatives have been found in several fruits, vegetables, and cereals, in which they usually appear as the esters of quinic acid, sterol, glucose, or plant cell wall polysaccharides (*1–4*). Raspberry, coffee, and crispbread are among the best dietary sources of phenolic acids (*5*), with large amounts of phenolic acids also present in rye (*6–8*). In various fruits and vegetables the main phenolic acid component is chlorogenic acids such as 3-caffeoylquinic acid, 4-caffeoylquinic acid, or 5-caffeoylquinic acid, but other phenolic acids are also found. *p*-Coumaric acid, ferulic acid, sinapic acid, 5-hydroxyferulic acid, and dehydrodimers of ferulic and sinapic acids have been isolated from the hydrolysates of plant cell walls (*3, 4, 9*). Winter and Herrmann (*1*) identified *p*-coumaroyl-, caffeoyl-, and feruloyl-glucosides esterified to the C-1 hydroxyl group from tomatoes, bell peppers, and eggplants. In corn and barley, cell walls have been found to contain esterified *p*-coumaric and ferulic acid and also some 5-hydroxyferulic acid and sinapic acid (*10*). In cereals, the main phenolic acids are ferulic acid and *p*-coumaric acid, which are found in the different parts of grains such as wheat bran, maize bran, and rice endosperm (*12*).

Among the different hydroxycinnamic acids, ferulic acid has special importance. Ferulic acid is esterified to various cell wall polysaccharides. It is linked at position C-5 to α-L-arabinofuranosyl side groups in arabinoxylans, at position C-2 to α-L-arabinofuranosyl residues in arabinans, at position C-6 to β-D-galactopyranosyl residues in galactans (*11*), and at position C-4 to α-D-xylopyranosyl side groups in xyloglucans (*9*). Ferulic acid has a significant role in cross-linking cell wall polysaccharides (*13*), and cereals contain moderate levels of ferulic acid dehydrodimers, which are formed by oxidative coupling reactions (*4, 8*).

Phenolic acids are considered to be anti-inflammatory, anticarcinogenic, and antimicrobial agents as well as antioxidants (*14–16*). Benefits provided by phenolic acids are assumed to be due to their antioxidant activity: metal ion chelating, radical scavenging, and inhibition of prooxidant enzymes (*17*). The antiradical activity of polyphenols is ascribed to the hydroxyl groups and the availability of phenolic hydrogen for donation (*2, 15*). Hydroxycinnamic acids have been shown to exhibit singlet oxygen quenching. The electron-donating ability of the substituents and the polarity of the solvent influence the quenching rate. Charge transfer is thereby attended to singlet oxygen quenching (*18*). Phenolic compounds having one hydroxyl group on their aromatic ring are less effective antioxidants than phenolics with the second hydroxyl in the ortho or para position.

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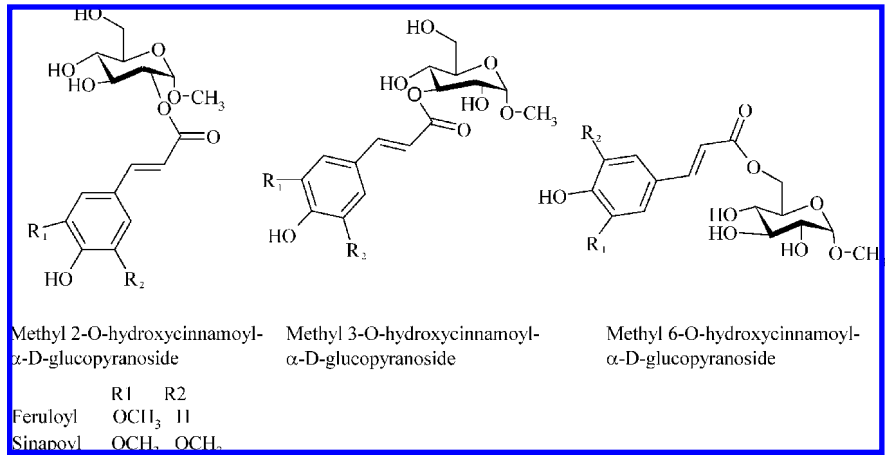
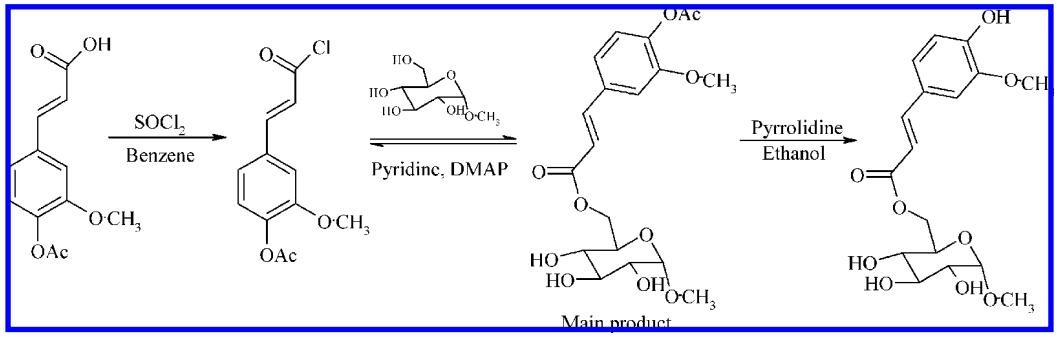


Figure 1. Hydroxycinnamic acid ester conjugates of methyl glucose.

Scheme 1. Synthesis of Methyl 6-*O*-Feruloyl-glucopyranoside Ester (*26*)



However, the presence of more than three hydroxyl groups on an aromatic ring does not improve the efficacy. Kikuzaki et al. (*19*) showed that alkyl esters of ferulic acid and gallic acid have a lower antioxidant efficacy than free acid in bulk methyl linoleate at 40 °C. Alkyl esters of ferulic acid were, however, more active than ferulic acid in ethanolic buffer. Ferulic acid dehydrodimers have been shown in vitro to be more effective antioxidants than monomeric ferulic acid in both the aqueous and lipid phases (*20, 21*).

Free hydroxycinnamic acids are widely examined, and they are known to have antioxidant potential, but the antioxidant activity of conjugated hydroxycinnamates is less investigated. The aim of this paper was to evaluate the antioxidant activity of various glycoside esters and to assess whether the nature of ester conjugation has an effect on the antioxidant activity. It was hypothesized that phenolic acids esterified to the primary hydroxyl group of the glycoside could be more effective antioxidants compared to other types of conjugates. To mimic the polymeric structure of carbohydrates, phenolic acids were esterified to primary hydroxyl groups (C-5 or C-6) and to secondary hydroxyl groups (C-2, C-3, and C-4), whereas the naturally favored position C-1 of monosaccharides was blocked by methyl or 4-nitrophenyl groups.

MATERIALS AND METHODS

Materials. Ferulic acid, pyrogallol, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) reagent were from Extrasynthèse (Genay, France). All solvents were of HPLC grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). A Milli-Q water purification system was used (Millipore, Bedford, MA). Ammonium dihydrogen phosphate, sodium dihydrogen phosphate, disodium hydrogen phosphate cupric acetate, copper sulfate, sodium chloride, and α-tocopherol were from Merck (Darmstadt, Germany). Lecithin from soybean (containing 40% phosphatidylcholine) and sinapic acid were from Sigma Chemical Co. (St. Louis, MO). Ultraflo L was provided by Novozymes (Bagsvaerd, Denmark). Emultop emulgator was from Degussa Texturants Systems Deutschland GmbH & Co. KG (Hamburg, Germany). Tocopherols were removed from rapeseed oil (Kultasula, Mildola, Finland) prior to use as described by Lampi et al. (*22*). 4-Nitrophenyl ferulate was a gift from Dr. M. Mastihubová (Institute of Chemistry, Slovak Aacdemy of Sciences, Bratislava, Slovakia).

Synthesis of Hydroxycinnamic Acid Glucoside Ester Conjugates. Ferulic acid and sinapic acid ester conjugates of methyl glucoside (**Figure 1**) were synthesized from methyl α-D-glucopyranose and *trans*-hydroxycinnamic acids as presented in **Scheme 1**. Position C1 in glycosides was blocked by methylation to prevent the esterification to that position to mimic the natural conditions in polymeric carbohydrates where the position C1 is not available. Ester conjugates were prepared in two fashions: using "batch" method to obtain isomeric monoester conjugate mixtures and, on the other hand, by synthesizing specific regioisomers of particular ester conjugates of interest (*24–27*).

Synthesis of Ferulic Acid Derivatives. Dehydrodimer of ferulic acid (**Figure 2**) was synthesized according to the method by Ralph et al. (*28*), and for the synthesis of 5-hydroxyferulic acid the methods outlined by Ellis and Lenger (*29*) and Lam et al. (*30*) were followed.

Synthesis of 4-Nitrophenyl Feruloyl Arabinofuranoside and Xylopyranosides. 4-Nitrophenyl glycoside conjugates contained another type of sugar moiety. They were used for the evaluation of the effect of addition of sugar to the hydroxycinnamic acid. 4-Nitrophenyl 2-*O*-

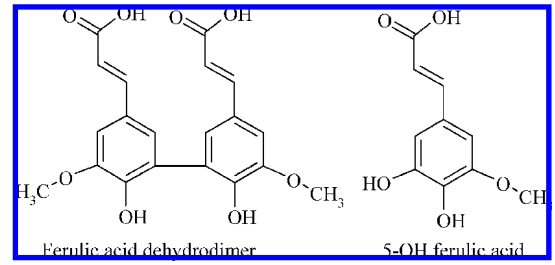


Figure 2. Structures of 5-hydroxyferulic acid and ferulic acid dehydrodimer.

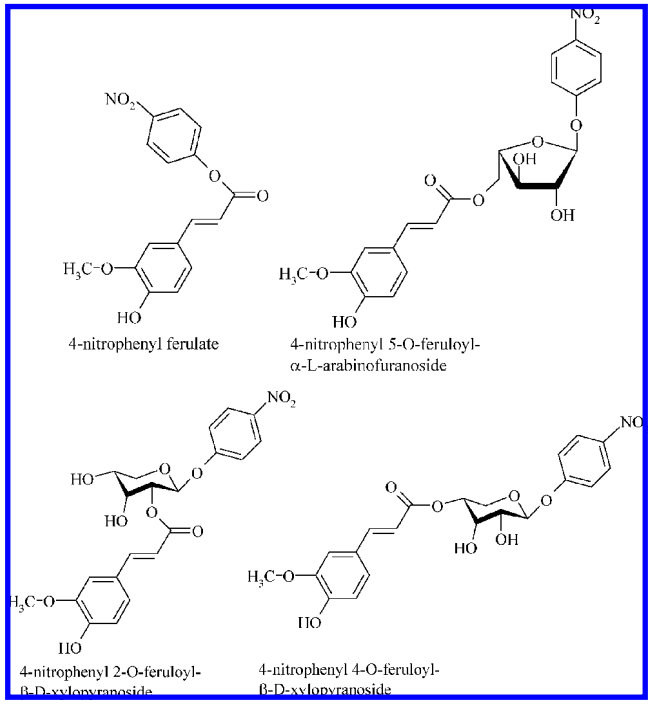


Figure 3. Structures of 4-nitrophenyl 2-*O*-feruloyl- and 4-*O*-feruloyl-β-D-xylopyranosides, 5-*O*-feruloyl-α-L-arabinofuranoside, and 4-nitrophenyl ferulate.

feruloyl-β-D-xylopyranoside, 4-nitrophenyl 4-*O*-feruloyl-β-D-xylopyranoside, and 4-nitrophenyl 5-*O*-feruloyl-α-L-arabinofuranoside (**Figure 3**) were previously prepared according to the method of Mastihubová et al. (*23*) to be used for specific substrates for feruloyl esterases and thus contained an additional 4-nitrophenyl moiety.

Analysis of Mixtures of Hydroxycinnamoyl Glucosides. For identification, NMR spectra were recorded with Varian Inova 500 and Varian Mercury Plus 300 spectrometers (^{1}H, 500 and 300 MHz; ^{13}C, 125 and 75 MHz, respectively) in acetone-d_6. The 2D NMR techniques (HSQC, HSQC-TOCSY, and HMBC) were used for the identification of products. The isomeric composition of the mixtures of acid glucosides after the synthesis was determined with HPLC. The HPLC analysis was performed using a Waters 717 plus autosampler, a Waters 600 pump, a Waters Symmetry C18, 5 μm, 4.6 × 150 mm column, and a Waters 996UV spectrophotometric detector with detection at 280 nm. Isocratic acetonitrile/water (18:82) was used as an eluent with flow rate of 0.8 mL/min. MS and HRMS were detected with Bruker microTOF using ESI.

To verify that the feruloyl and sinapoyl glucosides contain only one esterified hydroxycinnamoyl group per molecule, the glucoside ester mixtures were hydrolyzed with Ultraflo L. The hydrolysis was performed according to method by Vuorela et al. (*31*). The phenolic sample (2 mL in methanol) was evaporated to dryness, and the enzyme concentration was 0.1% (v/v) of the phenolic concentration in the final volume (1100 μL in 0.02 M ammonium dihydrogen phosphate buffer, pH 5.5). The hydrolyses were performed under nitrogen in a shaking water bath at 37 °C. The HPLC analyses before and after the enzymatic hydrolysis of hydroxycinnamic acid glucosides were performed according to the method outlined by Koski et al. (*32*). The HPLC system consisted of a 2690 separations module, a PDA 996 diode array detector, and a Millennium^{32} software data module. The column was a Nova-Pak C18 (150 × 3.9 mm, 4 μm, Waters, Millipore, Bedford, MA) equipped with a C18 guard column. For detection, 320 nm (for hydroxycinnamic acids) and 280 nm (generally for phenolics) were the wavelengths recorded.

Total Phenolic Content. Samples (0.2 mL in methanol) were evaporated to dryness. After that, 0.2 mL of deionized water, 1 mL of Folin−Ciocalteu reagent (1:10), and 0.8 mL of disodium carbonate solution (7.5%) were added. After 30 min, the total phenolic content was measured at 765 nm by a Perkin-Elmer λ 25 UV−vis spectrophotometer (Norwalk, CT) (*33*). Ferulic acid and sinapic acid were used as standard compounds.

Antioxidant Activity Testing. Antioxidant activities were tested by using three different models. The DPPH radical scavenging test was used to evaluate the theoretical antioxidant potential of hydroxycinnamoyl glycoside esters. Liposome and emulsion lipid oxidation models represent two different structures naturally occurring in foods. Tested compounds solubilized in methanol were dosed on the basis of the total phenolic content.

DPPH Radical Scavenging Test. The test was performed according to the method of Kähkönen et al. (*34*). Methanolic 0.1 mM DPPH$^•$ radical solution (2.950 mL) was mixed with 0.050 mL of the tested compound. The concentration of the sample was 1 mg/mL based on the content of total phenolics. All samples were dissolved in methanol. Molar concentrations of different hydroxycinnamic glycosides varied from 4.5 to 12.4 mM, but there was no significant difference in the molar concentration between similar types of hydroxycinnamates. The absorption was measured at 517 nm every 30 s for 4 min. The results were expressed as the percentage of radicals scavenged in 4 min of reaction time. The percentage of radical scavenging activity was calculated as

$$\text{radical scavenging activity } (\%) = 100 \times (A_1 - A_2)/A_1$$

where A_1 = the initial absorbance at beginning of the reaction ($t = 0$) and A_2 = the absorbance after 4 min of reaction time. DPPH$^•$ radical scavenging activity test was performed in triplicate measurements using pyrogallol (0.5 mg/mL) as a control sample.

Emulsion Model System. The emulsion (10% o/w) oxidation model system (*34*) was prepared from oil, water, and Emultop partially hydrolyzed soybean lecithin emulgator (2% w/w). α-Tocopherol (25 μM) was used as a reference antioxidant. The sample not containing any antioxidant was used as a control. Concentrations of the samples were 50 and 100 μg/g of oil based on the content of total phenolics. Molar concentrations for 50 μg/g were 212−727 μM and for 100 μg/g, 424−1454 μM. Samples were incubated at 37 °C for 6 days. The inhibition against emulsion oxidation was calculated at day 6 by measuring the formation of conjugated hydroperoxide dienes and hexanal. The absorbance was measured at 234 nm by a Perkin-Elmer λ 25 UV−vis spectrophotometer. For analysis of conjugated hydroperoxide dienes a sample of emulsion (25 μL) or liposome (100 μL) was dissolved in 5 mL of methanol. Measurements were done in triplicates, and the inhibition of oxidation was expressed as percentage (*35, 36*). For hexanal analysis a sample of emulsion (0.25 mL) or liposome (0.5 mL) was transferred into a headspace vial and the formation of hexanal was measured according to the method by Frankel et al. (*37*). The inhibition against formation of conjugated hydroperoxide dienes and hexanal was calculated as

$$\text{inhibition } (\%) = (A_0 - A_1)/A_0 \times 100$$

where A_0 = the absorbance for conjugated dienes and area for hexanal of a control sample and A_1 = the absorbance for conjugated dienes or area for hexanal of a tested sample.

Liposome Model System. The liposomes were prepared from soybean lecithin (containing 40% phoshatidylcholine), and the concentration of phosphatidylcholine in samples was 0.8 mg/mL. The inhibition against liposome oxidation was calculated at day 3 by measuring the formation of conjugated dienes and hexanal as described earlier. α-Tocopherol (25 μM) was used as a reference antioxidant and a sample without any antioxidant as a control. Concentrations of the samples were 4.2 and 8.4 μg/mL based on the content of total phenolics. Molar concentrations for 4.2 μg/mL were 19−61 μM and for 8.4 μg/mL, 38−122 μM. Oxidation was started by adding 3 μM cupric acetate. Samples were stored in a shaking water bath (100 rpm, 37 °C) for 4 days. The inhibition against oxidation was calculated at day 4 by measuring the formation of conjugated hydroperoxide dienes and hexanal.

Statistical Analysis. Statistical differences among antioxidant activity were tested by multivariance analysis using Statgraphics Plus 3.0 (STCC Inc., Rockville, MD). The significance level was $p < 0.05$. For the statistical analysis samples were divided into groups and variances were determined among the group. Sinapic acid and its glucoside esters

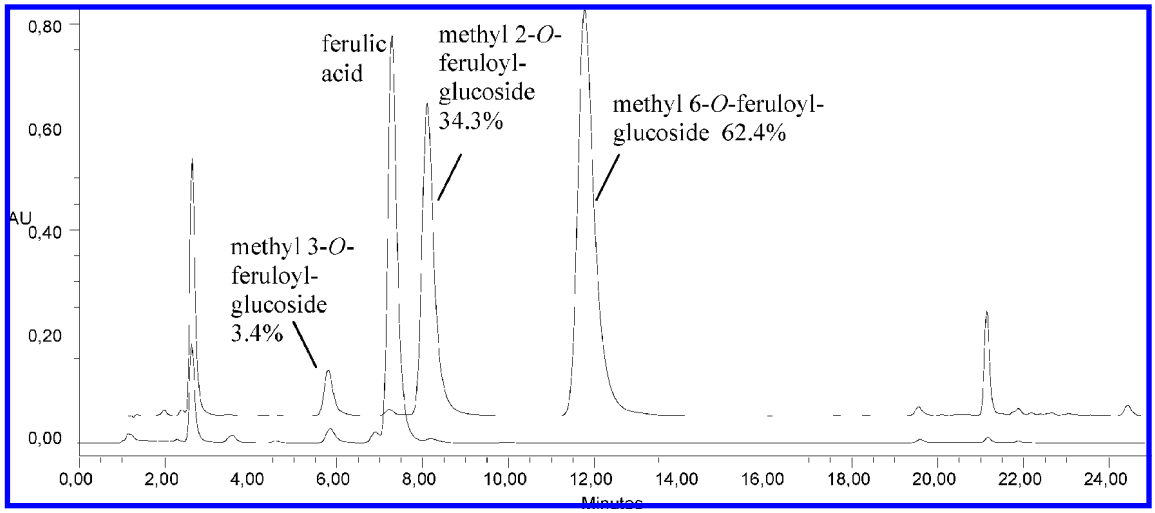


Figure 4. HPLC analysis of the mixture of methyl feruloyl-D-glucopyranosides. The upper chromatogram is obtained before the enzymatic hydrolysis and the lower one after the hydrolysis. Detection was at 320 nm. The main component in the mixture was methyl 6-*O*-feruloyl glucoside (62.4%).

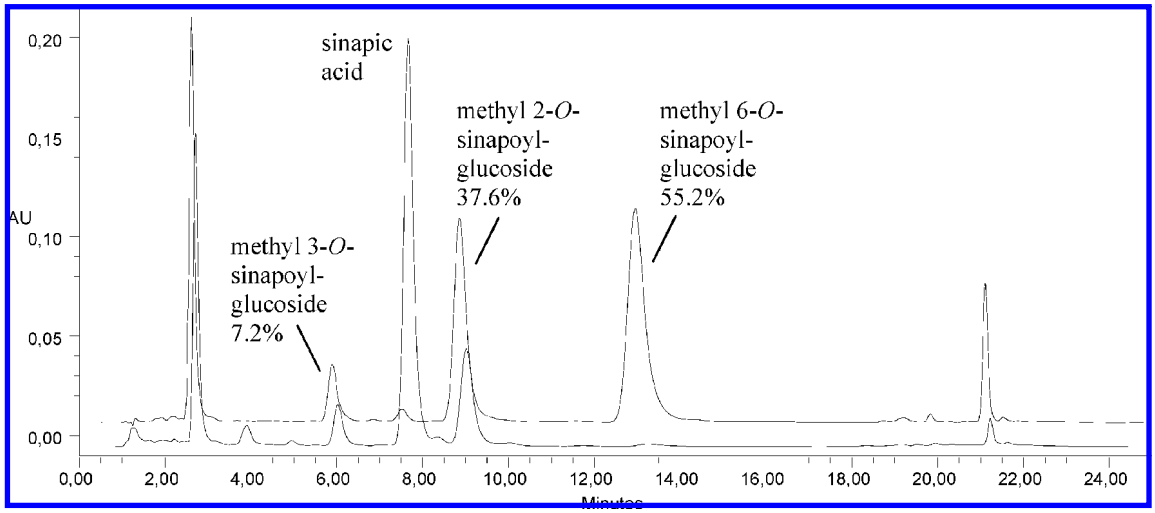


Figure 5. HPLC analysis of the mixture of methyl sinapoyl-D-glucopyranosides. Methyl 3-*O*-sinapoyl-glucosides were tentatively identified due to lack of pure isomer for peak identification. The upper chromatogram is obtained before enzymatic hydrolysis and the lower one after the hydrolysis. Detection was at 320 nm. The main component in the mixture was methyl 6-*O*-sinapoyl glucoside (55.2%).

constituted one group. The mixture of feruloyl glucosides, 2-*O*-feruloyl-glucoside, 3-*O*-feruloyl-glucoside, and 6-*O*-feruloyl-glucoside, constituted another group. A third group was formed from 5-hydroxyferulic acid, ferulic acid dehydrodimer, ferulic acid, and the mixture of feruloyl glucosides. 4-Nitrophenyl feruloyl glycosides were compared with each other and with free ferulic acid.

RESULTS

Characterization of Hydroxycinnamic Acid Glycoside Esters. The HPLC analysis of mixtures of feruloyl- and sinapoyl-glucoside esters revealed that they contained two main and two minor compounds (**Figure 4**). The percentage composition of the mixtures is shown in **Figures 4** and **5**. The mixtures were hydrolyzed with Ultraflo L, which is a multicomponent cellulolytic and xylanolytic enzyme preparation, and the hydrolysates were analyzed by HPLC. The mixtures contained no free phenolic acids before the hydrolysis. The corresponding hydroxycinnamic acids were released during the hydrolysis, and only minor amounts of feruloyl-glucoside esters remained, whereas sinapoyl-glucoside esters were not hydrolyzed as effectively. Quantification (data not shown) of the peak areas from the chromatograms as ferulic acid or sinapic acid equivalents revealed that the molar ratios of the acids before and after the hydrolysis are equal, indicating that all glucoside esters in the mixtures contained one hydroxycinnamoyl moiety per molecule. The last peak in both chromatograms is tentatively identified as 4-*O*-feruloyl- and 4-*O*-sinapoyl-6-acetyl glucoside.

In LC-MS, the observed *m*/*z* values measured with HRMS (ESI/TOF) of Na^+ adducts of all pure isomers of ester conjugates between hydroxycinnamic acids and methyl glucopyranoside were identical: 393 and 423 for feruloyl and sinapoyl conjugates, respectively. When referenced to pure regioisomers synthesized, the main esterified positions in the glucoside were 6-O and 2-O (**Figures 4** and **5**). The HRMS and NMR results will be published and discussed elsewhere.

The total phenolic content of the tested compounds was determined by the Folin−Ciocalteu method (**Table 1**). Within the group of similar structures, the total phenolic contents were the same as calculated on the molecular weight basis. The amount added of each compound to different antioxidant tests was based on the measured total phenolic group content.

Radical Scavenging Activity. The radical scavenging activity of the hydroxycinnamic acid glycoside esters was determined at the concentration of 1 mg/mL (based on the total phenolic content) (**Figure 6**). 6-*O*-Sinapoyl-glucoside had the best radical

Table 1. Total Phenolics of Hydroxycinnamic Acids and Their Derivatives[a]

compound	total phenolics (mg/g ± SD)
sinapic acid	1043 ± 14
methyl sinapoyl glucoside mixture	347 ± 58
methyl 2-*O*-sinapoyl-α-D-glucoside	407 ± 5
methyl 6-*O*-sinapoyl-α-D-glucoside	356 ± 13
ferulic acid	988 ± 6
methyl feruloyl glucoside mixture	394 ± 2
methyl 2-*O*-feruloyl-α-D-glucoside	407 ± 5
methyl 3-*O*-feruloyl-α-D-glucoside	356 ± 13
methyl 6-*O*-feruloyl-α-D-glucoside	367 ± 1
4-nitrophenyl 2-*O*-feruloyl-β-D-xyloside	232 ± 8
4-nitrophenyl 4-*O*-feruloyl-β-D-xyloside	225 ± 12
4-nitrophenyl 5-*O*-feruloyl-α-L-arabinofuranoside	194 ± 6
4-nitrophenyl ferulate	241 ± 3
5-hydroxyferulic acid	459 ± 2
ferulic acid dehydrodimer	308 ± 8

[a] Ferulic acid and sinapic acid were used as standards for respective derivatives.

scavenging activity compared with other tested compounds. It was statistically more active (96%) compared to free sinapic acid (91%), the mixture of sinapoyl glucosides (91%), and 2-*O*-sinapoyl-glucoside (91%).

The mixture of different feruloyl glucosides was more effective in the radical scavenging test compared to the free ferulic acid as well as pure isomers of glucoside esters (**Figure 6**). The mixture, which mainly contained 6-*O*- and 2-*O*-feruloyl-glucosides, had the scavenging activity of 95%. Comparison of the different pure isomers of feruloyl glucosides revealed that the esterification to the primary C-6 hydroxyl group resulted in significantly ($p < 0.05$) more effective radical scavenging (86%) than esterification to the secondary C-2 positions (74%). On the other hand, esterification to another secondary C-3 hydroxyl group resulted in much lower radical scavenging activity (48%) than conjugation to C-2. The activity of free ferulic acid was lower and of the same order of magnitude as *O*-3-feruloyl-glucoside. The effect of conjugation on the radical scavenging activity was also evaluated with 4-nitrophenyl 5-*O*-feruloyl-α-L-arabinofuranoside, 4-nitrophenyl 4-*O*-feruloyl-β-D-xylopyranoside, 4-nitrophenyl 2-*O*-feruloyl-β-D-xylopyranoside, and 4-nitrophenyl ferulate. The ferulic acid esterified to the arabinofuranosyl moiety was the most effective, scavenging activity being 61%. There was a slight difference compared to feruloyl group linked to position C-4 (52%) and C-2 (48%) of xylopyranoside or directly to 4-nitrophenol (49%). Control experiments showed that radical scavenging activities of 1-methyl glucoside and 4-nitrophenyl glucoside were negligible (2%). Overall, sinapic acid or ferulic acid esterified to the primary hydroxyl of glycoside was a better radical scavenger than esters to the secondary hydroxyl groups.

Of the ferulic acid derivatives, 5-hydroxyferulic acid scavenged the radicals most effectively, about 95%, thus showing as a good scavenging activity as the mixture of feruloyl glucosides.

Antioxidant Activity in Liposome. The antioxidant activity was measured in the liposome model system at 4.2 and 8.4 μg/mL concentrations (based on the total phenolic content). The inhibition of hexanal formation was excellent (>90%) for all tested compounds except 3-*O*-glucoside, 5-hydroxyferulic acid, and ferulic acid dehydrodimer at 8.4 μg/mL concentration (**Table 2**). The inhibition of conjugated diene formation was generally lower than the inhibition of hexanal formation. There were no statistical differences between the mixture of sinapoyl glucosides, 2-*O*-sinapoyl-glucoside, 6-*O*-sinapoyl-glucoside, and sinapic acid, except for the inhibition of conjugate diene formation with the mixture of sinapoyl glucosides.

Ferulic acid and 2-*O*- and 6-*O*-feruloyl-glucosides were significantly ($p < 0.05$) more effective antioxidants than 3-*O*-feruloyl-glucoside. 2-*O*- and 6-*O*-feruloyl-glucosides inhibited the conjugated hydroperoxide formation by over 85% and the formation of hexanal by over 90%, whereas the antioxidant effect of 3-*O*-feruloyl-glucoside was lower by 10−15%. Thus, the same trend in antioxidant activity between different feruloyl glucoside isomers, that is, 3-*O*-feruloyl-glucoside possessing lower activity than the other two isomers, was detected both in the radical scavenging test and in the liposome model system. However, the antioxidative effect of feruloyl glucoside esters in the liposome model system was not better than that of free ferulic acid.

In the liposome model system, free ferulic acid was a more potent antioxidant than 5-hydroxyferulic acid, the dehydrodimer of ferulic acid, and the mixture of feruloyl glycosides. At the concentration of 4.2 μg/mL, ferulic acid inhibited conjugate diene formation by 87% and hexanal formation by 95%. The dehydrodimer of ferulic acid and the mixture of feruloyl glucosides had approximately the same antioxidant activities; inhibition of the formation of conjugated dienes was 67−75% and of hexanal was 84%. 5-Hydroxyferulic acid had the lowest antioxidant activity toward conjugated diene (60%) and hexanal formation (70%). At higher concentration the trend was the same. The dehydrodimer of ferulic acid and the mixture of feruloyl-glucosides were more active against lipid oxidation than 5-hydroxyferulic acid for both conjugated diene and hexanal formation.

4-Nitrophenyl 5-*O*-feruloyl-arabinoside was the most powerful antioxidant among 4-nitrophenyl feruloyl glycosides at lower concentration against both hexanal and conjugated diene formation. There was no significant difference between 4-nitrophenyl glycosides of 2-*O*-feruloyl-xyloside, 4-*O*-feruloyl-xyloside, and 5-*O*-feruloyl-arabinoside at 8.4 μg/mL concentration.

Antioxidant Activity in Emulsion. The antioxidant activity of hydroxycinnamoyl glycoside esters was examined in the emulsion lipid oxidation model at 50 and 100 μg/g concentrations (based on the total phenolic content) (**Table 3**). Generally, the inhibition of hexanal formation was more efficient than the inhibition of conjugated diene formation in the emulsion model, similarly as in the liposome model. Comparison of the inhibition of hexanal formation of sinapic acid and its glucoside esters revealed that there are only minor differences between them. The inhibition was over 85% for both tested concentrations. Comparison of the inhibition of conjugate diene formation showed the difference to be greater. The mixtures of sinapoyl glucosides were the most effective against conjugated diene formation. At both tested concentrations 6-*O*-sinapoyl-glucoside was a more potent antioxidant than 2-*O*-sinapoyl-glucoside. Thus, 2-*O* and 6-*O*-sinapoyl-glucosides behave differently in the inhibition of formation of conjugated dienes in the liposome model and in the emulsion model as no difference was observed between these isomers in the liposome model. The increase of concentration had a positive effect on the conjugate diene formation, whereas the inhibition of hexanal formation was already efficient at 50 $\mu g/g_{oil}$ concentration.

At the concentration of 50 μg/g, 6-*O*-feruloyl-glucoside was the most powerful antioxidant for both hexanal and conjugated diene formation compared with 2-*O*- and 3-*O*-feruloyl-glucosides. Also at a concentration of 100 μg/g 6-*O*-feruloyl-glucoside as well as free ferulic acid was more effective than the other two glucoside isomers for hexanal formation, but not for conjugated diene formation. The mixtures of feruloyl glucosides

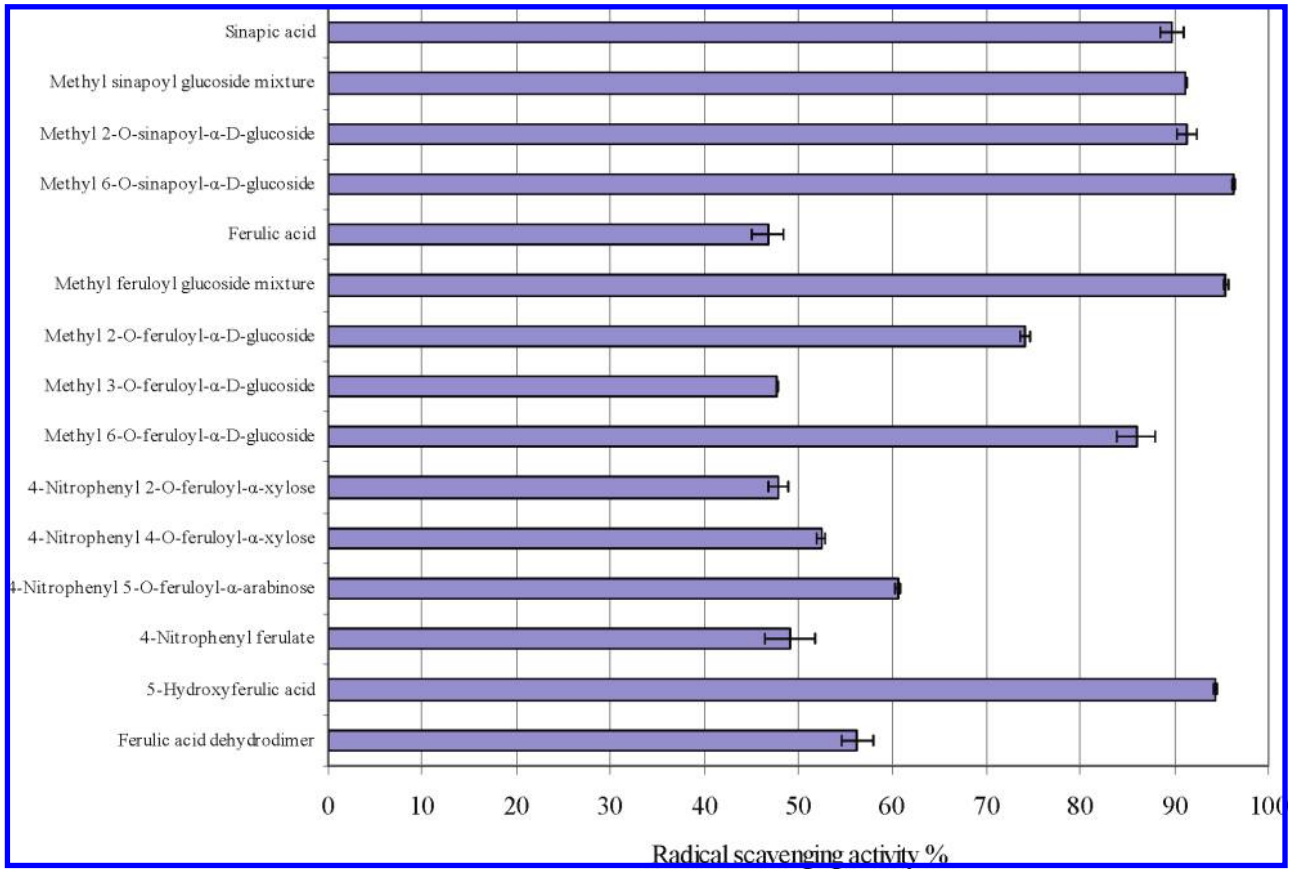


Figure 6. Radical scavenging activity (DPPH test) of hydroxycinnamic acids, their glycoside esters, and other derivatives (1 mg/mL).

Table 2. Inhibition of Lipid Oxidation As Measured by Formation of Conjugated Dienes and Hexanal in Lecithin Liposome Oxidation Model System with 4.2 and 8.4 μg/mL Hydroxycinnamic Acids and Their Derivatives[a]

	conjugated dienes (%)		hexanal (%)	
compound	4.2 μg/mL	8.4 μg/mL	4.2 μg/mL	8.4 μg/mL
sinapic acid	89.2 ± 2.1	nd	96.4 ± 0.3	nd
methyl sinapoyl glucoside mixture	84.0 ± 2.9	87.1 ± 2.1	93.4 ± 1.7	95.9 ± 0.4
methyl 2-*O*-sinapoyl-α-D-glucoside	90.8 ± 0.9	90.5 ± 1.8	93.8 ± 4.4	92.8 ± 3.1
methyl 6-*O*-sinapoyl-α-D-glucoside	91.3 ± 0.5	91.2 ± 1.8	94.0 ± 2.7	93.0 ± 2.4
ferulic acid	87.0 ± 2.7	nd	95.4 ± 1.2	nd
methyl feruloyl glucoside mixture	67.6 ± 4.9	79.9 ± 3.1	84.3 ± 3.0	92.2 ± 1.9
methyl 2-*O*-feruloyl-α-D-glucoside	84.1 ± 0.8	86.5 ± 2.2	80.6 ± 14.3	90.8 ± 4.6
methyl 3-*O*-feruloyl-α-D-glucoside	67.7 ± 7.3	73.7 ± 6.0	78.4 ± 4.1	83.8 ± 1.7
methyl 6-*O*-feruloyl-α-D-glucoside	87.0 ± 0.8	88.5 ± 2.2	89.5 ± 6.8	93.1 ± 2.4
4-nitrophenyl 2-*O*-feruloyl-β-D-xyloside	81.3 ± 4.3	89.0 ± 3.0	90.5 ± 2.4	94.3 ± 0.9
4-nitrophenyl 4-*O*-feruloyl-β-D-xyloside	85.7 ± 5.9	89.7 ± 3.0	91.0 ± 4.3	93.7 ± 1.1
4-nitrophenyl 5-*O*-feruloyl-α-L-arabinoside	88.4 ± 4.7	91.1 ± 3.5	92.5 ± 0.7	95.3 ± 1.2
4-nitrophenyl ferulate	76.3 ± 5.4	80.1 ± 4.0	87.6 ± 3.8	90.7 ± 2.1
5-hydroxyferulic acid	60.1 ± 5.1	69.6 ± 6.3	70.1 ± 3.4	76.9 ± 3.8
ferulic acid dehydrodimer	75.2 ± 5.1	82.4 ± 3.0	83.1 ± 5.0	88.6 ± 3.3

[a] Concentrations based on the total phenolic content (percent inhibition, mean ± SD).

were also effective antioxidants for both hexanal (>90%) and conjugated diene (>78%) formation.

There was no statistical difference between ferulic acid, ferulic acid dehydrodimer, and 5-hydroxyferulic acid in the inhibition of lipid oxidation. Their inhibitions were approximately 60%. 4-Nitrophenyl ferulate, 4-nitrophenyl 2-*O*-feruloyl-xyloside, and 4-nitrophenyl 5-*O*-feruloyl-arabinoside had no significant difference at 50 μg/g concentration. Their inhibition against hexanal formation was 85−93%. At higher concentration 4-nitrophenyl ferulate, 4-nitrophenyl 4-*O*-feruloyl-xyloside, and 4-nitrophenyl 5-*O*-feruloyl-arabinoside inhibited the hexanal formation at over 90%. 4-Nitrophenyl 2-*O*-feruloyl-xyloside had the lowest antioxidant activity (79%). Against conjugated diene formation, the most effective antioxidant was 4-nitrophenyl ferulate at 50 and 100 μg/g concentrations (79 and 87%).

DISCUSSION

Radical Scavenging and Antioxidant Activity of Feruloyl and Sinapoyl Glucosides. Glucoside esters were very potent radical scavengers (DPPH test), with the inhibition over 85% except for 2-*O*-feruloyl-glucoside and 3-*O*-feruloyl-glucoside. In the DPPH test only the amount of hydroxyl groups is significant in assessing radical scavenging activity. Therefore, no difference between the feruloyl glucosides was anticipated, contrary to the findings. A possible explanation for the lower

Table 3. Inhibition of Lipid Oxidation As Measured by Formation of Conjugated Dienes and Hexanal in Emulsion Oxidation Model System with 50 and 100 μg/g Hydroxycinnamic Acids and Their Derivatives[a]

compound	conjugated dienes (%)		hexanal (%)	
	50 μg/g	100 μg/g	50 μg/g	100 μg/g
sinapic acid	66.3 ± 4.2	nd	83.5 ± 6.9	nd
methyl sinapoyl glucoside mixture	90.3 ± 2.8	90.1 ± 3.8	94.6 ± 3.0	99.8 ± 0.4
methyl 2-*O*-sinapoyl-α-D-glucoside	57.6 ± 8.1	54.7 ± 9.0	96.8 ± 1.4	95.1 ± 5.2
methyl 6-*O*-sinapoyl-α-D-glucoside	83.4 ± 8.5	76.0 ± 15.0	92.0 ± 5.5	94.4 ± 7.3
ferulic acid	59.5 ± 4.2	nd	87.0 ± 7.1	nd
methyl feruloyl glucoside mixture	78.3 ± 6.5	79.0 ± 10.0	91.7 ± 7.6	95.4 ± 7.1
methyl 2-*O*-feruloyl-α-D-glucoside	50.8 ± 6.7	64.6 ± 10.1	70.4 ± 5.4	95.2 ± 6.2
methyl 3-*O*-feruloyl-α-D-glucoside	72.6 ± 5.2	78.0 ± 11.1	68.4 ± 5.2	81.5 ± 16.0
methyl 6-*O*-feruloyl-α-D-glucoside	73.4 ± 11.9	59.1 ± 7.7	89.5 ± 8.0	92.4 ± 10.9
4-nitrophenyl 2-*O*-feruloyl-β-D-xyloside	55.3 ± 7.1	76.4 ± 6.5	85.9 ± 3.7	79.0 ± 10.4
4-nitrophenyl 4-*O*-feruloyl-β-D-xyloside	49.7 ± 8.1	76.9 ± 10.9	37.2 ± 8.0	91.5 ± 2.6
4-nitrophenyl 5-*O*-feruloyl-α-L-arabinoside	64.4 ± 4.3	71.9 ± 5.4	93.2 ± 7.9	95.2 ± 5.0
4-nitrophenyl ferulate	79.2 ± 5.3	86.9 ± 4.2	88.9 ± 7.8	95.8 ± 1.7
5-hydroxyferulic acid	71.6 ± 10.5	67.7 ± 13.2	93.1 ± 0.7	94.7 ± 4.8
ferulic acid dehydrodimer	53.8 ± 19.6	66.2 ± 13.5	85.8 ± 4.5	92.7 ± 3.4

[a] Dosages based on the total phenolic content (percent inhibition, mean ± SD).

radical scavenging activity toward DPPH radical when using 2-*O*- and 3-*O*-feruloyl glucosides might be a slight difference in the solubility in the test solvent, methanol. The presence of the −CH═CH−COOH chain in hydroxycinnamic acids ensures hydrogen-donating ability and subsequent radical stabilization (*38*). Ortho substitution with electron-donating alkyl or methoxy groups increases the stability of the radical formed from the phenolic compound and its antioxidant potential (*39*). There was no difference between sinapic acid and sinapic acid conjugates. In the emulsion and liposome oxidation, ferulic acid and its derivatives had similar antioxidant activities, indicating that the introduction of a sugar moiety to the hydroxycinnamic acid does not reduce the capability for the antiradical potential. Moreover, the present results as well as our previous studies on hydroxycinnamic acids (*40*) suggest that the naturally existing glycoside esters are at least as potent as the free phenolic acids. More hydrophilic glucoside esters of hydroxycinnamic acids are more active radical scavengers compared with free forms of acids. These findings contradict earlier reports. Tominaga et al. (*41*) found that the introduction of a β-glucose moiety to caffeic and ferulic acids decreased their radical scavenging activities due to increased hydrophilicity and permeability in aqueous solutions in the DPPH test. According to Cos et al. (*42*) caffeic acid had the highest scavenging activity followed by ferulic and sinapic acids, whereas the esterification of caffeic acid with quinic acid decreased the activity.

Feruloyl-β-D-glucopyranosides, sinapoyl-β-D-glucopyranosides, and caffeoyl-β-D-glucopyranosides were reported to be weaker antioxidants than free corresponding phenolic acids toward the inhibition of oxidation of β-carotene−linoleic acid emulsions (*41, 43*). In our studies, the hydroxycinnamic acid esters were as potent antioxidants as the free acids also toward the oxidation of liposomes and emulsions. In general, 6-*O*-feruloyl-glucoside exhibited the highest antioxidant activity followed by ferulic acid, 2-*O*-feruloyl-glucoside, and 3-*O*-feruloyl-glucoside, thus complying with the original hypothesis. With sinapic acid conjugates no significant difference was seen. This may be explained by the significant antioxidant potency of sinapic acid and its derivatives compared to other hydroxycinnamates. Sinapic acid and its derivatives behave similarly in liposomes and emulsions. The mixture of sinapoyl glucosides was the most efficient at emulsions, whereas in liposomes they were less potent. Sinapic acid has been also earlier shown to be a more effective antioxidant than ferulic acid, which in turn is more effective than *p*-coumaric acid (*42*). Nyström et al. (*3*) have demonstrated that in bulk and emulsified methyl linoleate steryl ferulates had a similar activity against lipid oxidation as ferulic acid. Feruloylated arabinoxylotrisaccharides from wheat enhanced the activity toward LDL oxidation, whereas the activity was reduced compared to ferulic acid in the DPPH test (*44, 45*). Antioxidants trapped near the surface of the liposomal membrane are better antioxidants as shown with hexyl and octyl ferulates being more effective than shorter alkyl esters (*41, 43*). In emulsions the lipophilic antioxidants of low hydrophilic−lipophilic balance are favored (*37*). The lipophilic antioxidants are adequately surface active to be located at the oil−water interface, where they are able to inhibit lipid oxidation, whereas hydrophilic antioxidants are located in the aqueous phase and are consequently less effective against lipid oxidation (*46*). The oil−water interface appears to be significant for antioxidants to function as chain-breakers. Emulsifiers, which are amphiphilic, are located at the oil−water interfaces in emulsions and can solubilize antioxidants, thus enabling polar antioxidants to get in contact with lipids. Stöckmann et al. (*47*) showed that emulsions containing partially hydrolyzed soybean lecithin as emulsifier solubilized gallic acid more efficiently than its more hydrophobic derivative ethyl gallate. Feruloyl and sinapoyl glucosides are more hydrophilic than their free forms. Due to the ability of soybean lecithin to solubilize hydrophilic compounds, tested glucosides were effective antioxidants. Altering the pH changes the solubility of hydroxycinnamic acids to the aqueous phase and lowering the pH might be related to the dissociation of the functional group. Schwarz et al. (*48*) observed that the concentrations of ferulic acid and caffeic acid decreased in the aqueous phase of water/oil systems when the pH was lowered from 7.0 to 3.0. Introduction of sugar to the phenolic compounds would make them more hydrophilic and inhibit them from reaching the oil−water interface (*49*). These findings were confirmed in the present study, where the tested hydrophilic antioxidants were not as effective in emulsions as in liposomes. The pH in the present study was 5.0 in the liposomes and neutral in the emulsion.

Antioxidant Activity of 4-Nitrophenyl Feruloyl-Glycosides. To further test the hypothesis of conjugation of glycosides to hydroxycinnamic acids resulting in similar antioxidant activity as free acids, different 4-nitrophenyl feruloyl-glycosides were investigated. 4-Nitrophenyl 5-*O*-feruloyl-arabinoside was the most powerful antioxidants among the 4-nitrophenyl glycosides. 4-Nitrophenyl 4-*O*-feruloyl-xyloside was the second most effective, whereas 4-nitrophenyl 2-*O*-feruloyl-xyloside was the

weakest antioxidant in general. 4-Nitrophenyl ferulate had an activity similar to that of free ferulic acid. The 4-nitrophenyl moiety did not change the antioxidant activity of ferulic acid, although it changes the hydrophilicity. In liposomes at a concentration of 100 μg/g all 4-nitrophenyl derivatives had equal antioxidant activity. At lower concentration, the order of the activity was 4-nitrophenyl 5-*O*-feruloyl-arabinoside > 4-nitrophenyl 4-*O*-feruloyl-xyloside > 4-nitrophenyl 2-*O*-feruloyl-xyloside > 4-nitrophenyl ferulate. In emulsions 4-nitrophenyl 5-*O*-feruloyl-arabinoside and 4-nitrophenyl ferulate inhibited the most hexanal and conjugated diene formation. In all tests, there was no difference between different 4-nitrophenyl derivatives.

Antioxidant Activity of Other Ferulic Acid Derivatives. 5-Hydroxyferulic acid and ferulic acid dehydrodimer have different solubilities in the lipid phase and thus exhibit different orientations in liposome and emulsions. Paiva-Martins et al. (*50*) discovered that the oxidative attack seems to occur from the aqueous phase initiating lipid peroxidation. The antioxidant activity of phenolic compounds in liposomes depends both on the location and on the orientation of the antioxidant in the system. Lipophilic antioxidants can act both by scavenging of aqueous peroxyl radicals and by scavenging of lipid peroxyl radicals within the liposomal membrane. The more hydrophilic 5-hydroxyferulic acid has a lower hydrophilic−lipophilic balance, and therefore it was a more powerful antioxidant compared with ferulic acid and ferulic acid dehydrodimer. In liposomes, the order was reversed, with ferulic acid dehydrodimer being the most effective. In acidic conditions, phenolic acids are weak antioxidants, whereas with increasing pH their antioxidant activity increases (*17*). The explanation for the better antioxidant activity in basic conditions is due to a rapid electron transfer from anionic phenolic acids to peroxyl radicals. Hydrogen abstraction and electron transfer in the antioxidant reactions depend on the conditions, such as pH value and the stability of the intermediate radicals (*49*).

In conclusion, sinapic acid and its derivatives were the most effective antioxidants. The esterification to glycosides did not decrease the antioxidant activity of ferulic and sinapic acids. In most cases, the mixture of ferulic acid glucosides or ferulic acid esterified to the position C-6 of methyl glucopyranoside was more potent lipid oxidation inhibitor compared with esters on positions C-2 and C-3. 4-Nitrophenyl 5-*O*-feruloyl-arabinofuranoside was also the most effective among the 4-nitrophenyl glycosides. The most likely explanation is that hydroxycinnamic acids esterified to the primary hydroxyls in glucopyranoside and arabinofuranoside are able to move more freely than in other isomers, thus enabling them to function as antioxidants more efficiently. Our studies indicate that naturally existing or synthetic glycoside esters of phenolic acids are potent antioxidants and that the antioxidant activity also depends on the conjugation.

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