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Synthesis of steryl ferulates with various sterol structures and comparison of their antioxidant activity

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ABSTRACT

Steryl ferulates synthesised from commercial sterols as well as commercial oryzanol were used to better understand how structural features affect antioxidant activity *in vitro* by the $ABTS^{\cdot+}$ radical decolorization assay, by oxidative stability index (OSI) of soybean oil, and by analysis of antioxidant activity during frying. Steryl ferulates inhibited the $ABTS^{\cdot+}$ radical by 6.5–56.6%, depending on their concentration, but were less effective, especially at lower concentrations, than ferulic acid. Ferulic acid and steryl ferulates had either no effect, or lowered the OSI of soybean oil by up to 25%, depending on the concentration. In their evaluation as frying oil antioxidants, steryl ferulates with a saturated sterol group had the best antioxidant activity, followed by sterols with one double bond in the C5 position. The results indicate that a dimethyl group at C4 as well as a C9,C19 cyclopropane group, as found in oryzanol, negatively affects antioxidant activity in frying oils.

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1. Introduction

Steryl ferulates are composed of ferulic acid esterified to plant sterols and found in the inner pericarp and aleurone layer of cereals grains such as corn, wheat, rice, rye, and triticale (Moreau, Singh, Nuñez, & Hicks, 2000; Norton, 1995; Seitz, 1989). Steryl ferulates from rice bran oil, which are called γ-oryzanol (oryzanol for short), are mainly composed of two 4,4′-dimethylsterols, cycloartenol and 24-methylene-cycloartanol, as well as the 4-desmethylsterol, campesterol, and low amounts of other 4-desmethyl sterols (Xu & Godber, 1999). Corn steryl ferulates consist mainly of two saturated 4-desmethylsterols, sitostanol, and campestanol, with lower amounts of other 4-desmethylsterols such as sitosterol and campesterol (Seitz, 1989).

Ferulic acid is a well-known phenolic antioxidant, thus steryl ferulates have antioxidant activity due to the radical scavenging activity of the phenolic component (Juliano, Cossu, Alamanni, & Piu, 2005; Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002; Nyström, Achrenius, Lampi, Moreau, & Piironen, 2007; Nyström, Mäkinen, Lampi, & Piironen, 2005; Suh, Yoo, & Lee, 2007; Xu & Godber, 2001). The hydrophobic sterol group allows steryl ferulates to be soluble in oils, making them excellent candidates for antioxidants in oil-rich foods. In addition, because of the increased molecular weight of steryl ferulates compared to ferulic acid, steryl ferulates are less likely to evaporate at high temperatures such as in frying oils and in other high temperature food processes. Steryl ferulates are also less susceptible to thermal decomposition as compared to non-esterified ferulic acid (Shopova & Milkova, 2000). Nyström et al. (2007) demonstrated that 0.5% and 1.0% sitostanyl ferulate inhibited polymer formation in stripped high oleic sunflower oil (HOSUN) heated to 180 °C by 27% and 43%, respectively. In previous frying studies, we found that corn steryl ferulates inhibited polymerisation of soybean oil by up to 70%, whereas oryzanol was much less effective (Winkler-Moser, Rennick, Palmquist, Berhow, & Vaughn, 2012). Corn steryl ferulates also protected the endogenous tocopherols in soybean oil during frying, whereas oryzanol did not. It was also noted that oryzanol degraded at a faster rate during frying compared to corn steryl ferulates. It was hypothesised that higher stability was the reason for the better anti-polymerisation activity; however, in a heating study (180 °C, in soybean oil), corn steryl ferulates actually degraded at a faster rate compared to oryzanol, but still had better antipolymerisation activity (Winkler-Moser, Rennick, Hwang, Berhow, & Vaughn, 2013), indicating that the differences in activity may not be completely attributed to their stability. In fact, Nyström et al. (2005) observed stronger antioxidant activity for wheat and

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rye steryl ferulates compared to oryzanol and cycloartenyl ferulate in methyl linoleate oxidised at 40 °C, which is not likely to be related to thermal stability.

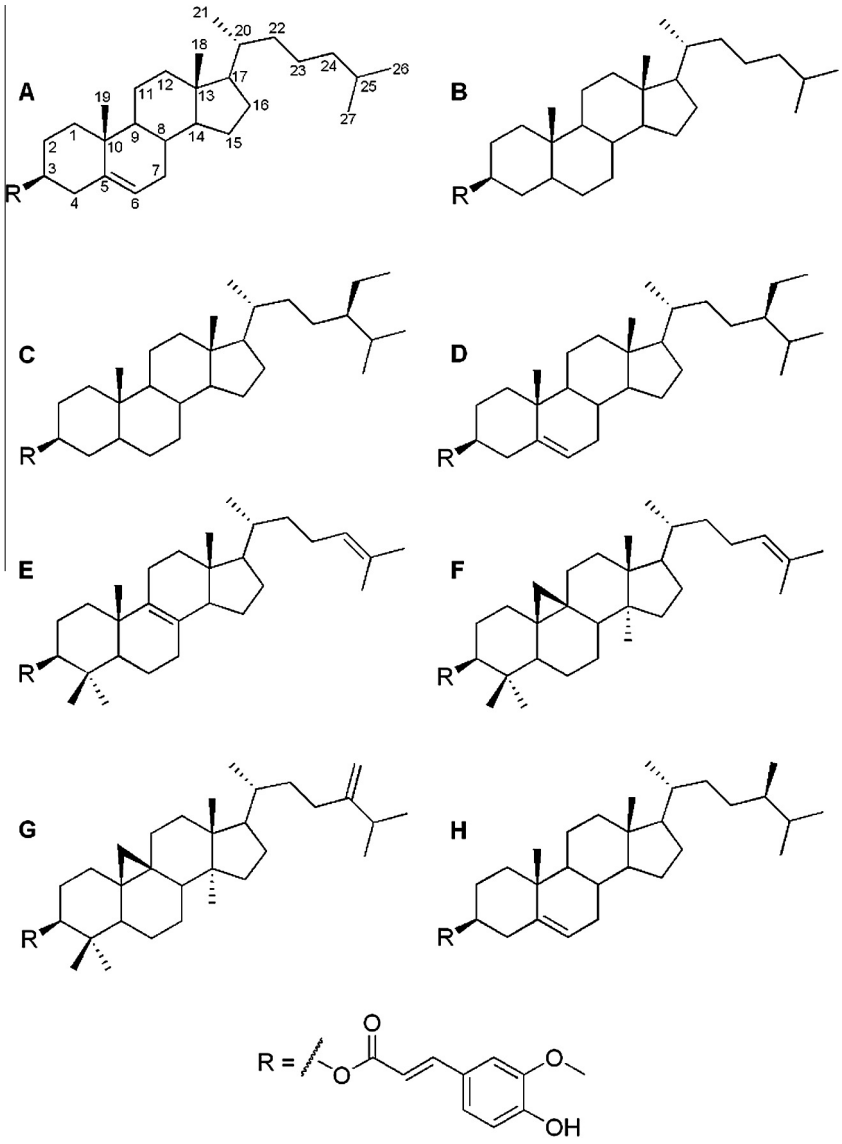
Since the only difference between oryzanol and corn steryl ferulates are in the structures of the phytosterol moieties, the objective of this research was to conduct studies to further understand the effect of structural features of the phytosterol head group on antioxidant activity. Since isolation and purification of the quantities needed to carry out these analyses would require large amounts of starting material, as well as solvents, we chose to synthesise steryl ferulates using pure phytosterols. Previously published synthesis techniques (Condo, Baker, Moreau, & Hicks, 2001; Ebenezer, 1991) were improved for time, solvent consumption, and yield, and the antioxidant activity of synthetic steryl ferulates as well as oryzanol and ferulic acid were evaluated *in vitro* and by analysis of oxidative stability index in soybean oil, as well as in frying studies in soybean oil.

The structures of the steryl ferulates that were synthesised are shown in Scheme 1. Cholesterol (A) and sitosterol (D) were chosen to represent sterols with a double bond at the C5-position, which should provide information about the effect of this double bond on steryl ferulate activity. Sitosterol, stigmasterol, campesterol, and brassicasterol are the four most common plant sterols, and they all have a C5 double bond. Since the majority of corn steryl ferulates have saturated (no double bonds) phytosterols, β-cholestanol (B) and sitostanol (C, also called stigmastanol) were chosen as model compounds for this group. Finally, lanosterol (E) was chosen to study the effect of the dimethyl group at C4, which is a structural feature that is also found in the two major steryl ferulates found in oryzanol, cycloartenol (F) and 24-methylene cycloartanol (G). These two sterols were not commercially available in the quantities needed for this study, so commercially available oryzanol was studied instead. Also shown in Scheme 1 is campesterol (H), which together with sitosterol makes up a low percentage of corn steryl ferulates (~14%), but makes up about 31% of oryzanol.

2. Materials and methods

2.1. Materials

8,24,(5-α)-Cholestadien-4,4,14-α-trimethyl-3-β-ol (lanosterol, ~65%) was purchased from Steraloids Inc. (Newport, RI). ABTS



Scheme 1. Structures of synthetic steryl ferulates: (A) cholesterol, (B) cholestanol, (C) sitostanol, (D) sitosterol, (E) lanosterol, and structures of steryl ferulates in oryzanol: (F) cycloartenol, (G) 24-methylene cycloartanol, (H) campesterol. R = *trans*-ferulic acid.

(2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)), trolox® (6-hydroxy-2,5,7,8-tetramethlchroman-2-carboxylic acid), *trans*-ferulic acid, pyridine, cholesterol (94%), cholestanol (⩾95%), β-sitosterol (from soybean, >70%), stigmastanol (>95%), 4-(dimethylamino)pyridine (DMAP), acetic anhydride, dicyclohexylcarbodiimide (DCC), and potassium carbonate were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Methanol, ethyl acetate, dichloromethane, chloroform, hexanes, and hydrochloric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Oryzanol (98%) was from Spectrum (Gardena, CA, USA). Silica gel 60 (70–230 mesh) used for column chromatography was purchased from EMD Chemical Inc. (Darmstadt, Germany). Thin layer chromatography (TLC) plates (MK6F silica gel 60 Å) were purchased from Whatman (Clifton, NJ, USA). Refined, bleached, deodorized soybean oil was kindly provided by Archer Daniels Midland (Decatur, IL, USA).

2.2. Methods

2.2.1. Synthesis of acetylferulic sterols

Acetic anhydride (18.7 mL, 0.20 mol) was added dropwise over 10 min to the stirred solution of *trans*-ferulic acid (35.0 g, 0.18 mol) and DMAP (0.14 g, 0.0011 mol) in dry pyridine (109 mL) at 0 °C under nitrogen. The mixture was stirred at 0 °C for 10 min, warmed to room temperature slowly and stirred for 4 h at room temperature. A 1:1 mixture of ice and water (100 g) was added and the mixture was stirred for 10 min maintaining the temperature at 0 °C and neutralized by slowly adding 2 N HCl. The precipitate was filtered and washed with de-ionised water. Methanol (210 mL) was added to the resulting white solid and the mixture was stirred at 55–60 °C for 30 min and then at room temperature for 1 h. The solid was filtered and dried to give the product as white solid (39.0 g, 91.6%). ^{1}H and ^{13}C NMR data were consistent with published literature values (Ebenezer, 1991).

For synthesis of 3-O-(trans-4-O-acetylferuloyl)-β-cholestanol, to a mixture of β-cholestanol (5.0 g, 12.9 mmol) and *trans*-4-*O*-acetylferulic acid (4.6 g, 19.5 mmol) in dry dichloromethane (100 mL), DMAP (0.5 g, 4.1 mmol) and DCC (4.1 g, 19.9 mmol) were subsequently added at 0 °C under nitrogen. The mixture was stirred at 0 °C for 30 min and then at room temperature for 1 h. Hexanes (300 mL) was added and the insoluble solid (1,3-dicyclohexylurea (DCU)) was removed by filtration. Solvent was evaporated and the residue was subjected to column chromatography on silica gel with dichloromethane/hexanes/ethyl acetate (4:16:1) to give the product (7.1 g, 91%). Further purification by recrystallization from methanol/dichloromethane provided 6.6 g (84%) of the product as a white solid. mp 147–149 °C; IR (cm^{-1}) 2931, 2849, 1758, 1698, 1261, 1178, 1155, 1125, 1031, 1013; ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3H), 0.86 (s, 3H), 0.88 (d, 3H, J = 2.2 Hz), 0.89 (d, 3H, J = 2.2 Hz), 0.92 (d, 3H, J = 6.7 Hz), 0.96–2.04 (m, 31H), 2.32 (s, 3H), 3.86 (s, 3H), 4.85 (m, 1H), 6.37 (d, J = 16.0 Hz, 1H), 7.05 (d, J = 8.7 Hz, 1H), 7.12 (m, 2H), 7.63 (d, J = 16.0 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 12.1, 12.3, 18.7, 20.6, 21.2, 22.6, 22.8, 23.9, 24.2, 27.6, 28.0, 28.2, 28.6, 32.0, 34.1, 35.5, 35.8, 36.2, 36.8, 39.5, 40.0, 42.6, 44.7, 53.4, 54.3, 55.9, 56.3, 56.4, 74.0, 111.2, 119.1, 121.2, 123.2, 133.5, 141.3, 143.5, 151.4, 166.3, 168.7.

For synthesis of 3-O-(trans-4-O-acetylferuloyl)-cholesterol, the same procedure used for 3-*O*-(*trans*-4-*O*-acetylferuloyl)-β-cholestanol provided 7.1 g (91%) of the product after column chromatography and 6.5 g (83%) after recrystallization from methanol/dichloromethane as a white solid. mp 167–168 °C; IR (cm^{-1}) 2934, 2867, 1759, 1701, 1635, 1262, 1175, 1155, 1124, 1031, 1006; ^{1}H NMR ($CDCl_3$) δ 0.70 (s, 3H), 0.88 (d, J = 2.2 Hz, 3H), 0.89 (d, J = 2.2 Hz, 3H), 0.94 (d, J = 6.6 Hz, 3H), 0.95–1.06 (m, 3H), 1.07(s, 3H), 1.08–2.09 (m, 24H), 2.33 (s, 3H), 2.42 (m, 1H), 3.87 (s, 3H), 4.76 (m, 1H), 5.42 (br m, 1H), 6.38 (d, J = 15.9 Hz, 1H), 7.06 (d, J = 8.6 Hz, 1H), 7.12 (m, 2H), 7.63 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) 11.9, 18.7, 19.3, 20.6, 21.1, 22.6, 22.8, 23.8, 24.3, 27.9, 28.0, 28.2, 31.9, 31.9, 35.8, 36.2, 36.6, 37.0, 38.2, 39.5, 39.8, 42.3, 50.1, 52.9, 56.2, 56.7, 74.2, 111.2, 118.9, 121.2, 122.8, 123.2, 133.5, 139.6, 141.4, 143.7, 151.4, 166.2, 168.7.

For synthesis of 3-O-(trans-4-O-acetylferuloyl)-β-sitosterol, the same procedure used for 3-O-(trans-4-O-acetylferuloyl)-β-cholestanol provided 7.3 g (96%) of the product after column chromatography and 6.9 g (90%) after recrystallization from methanol/dichloromethane as a white solid. ^{1}H and ^{13}C NMR data were consistent with published literature values (Condo et al., 2001): mp 171–172 °C [lit. 162 °C (Condo et al., 2001)]; IR (cm^{-1}) 2935, 2867, 1760, 1710, 1637, 1259, 1172, 1154, 1031, 1009; ^{1}H NMR ($CDCl_3$) δ 0.70 (s, 3H), 0.83 (d, 3H, J = 6.9), 0.85–0.89 (m, 6H), 0.95 (d, 3H, J = 6.6), 0.97–1.05 (m, 3H), 1.09–2.09 (m, 28H), 2.33 (s, 3H), 2.42 (m, 1H), 3.87 (s, 3H), 4.76 (m, 1H), 5.42 (br d, 1H), 6.38 (d, J = 16.0 Hz, 1H), 7.05 (d, J = 8.6 Hz, 1H), 7.12 (m, 2H), 7.64 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.9, 12.0, 18.8, 19.1, 19.3, 19.8, 20.6, 21.1, 23.1, 24.3, 26.1, 27.9, 28.3, 29.2, 31.9, 31.9, 34.0, 36.2, 36.6, 37.0, 38.2, 39.7, 42.3, 45.9, 50.1, 55.9, 56.1, 56.7, 74.2, 111.2, 118.9, 121.2, 122.8, 123.2, 133.5, 139.6, 141.4, 143.7, 151.4, 166.2, 168.7.

For synthesis of 3-O-(trans-4-O-acetylferuloyl)-8,24,(5-α)-cholestandien-4,4,14-α-trimethyl-3-β-ol, the same procedure used for 3-O-(trans-4-O-acetylferuloyl)-β-cholestanol provided 6.4 g (85%) of the product after column chromatography and 5.8 g (77%) after recrystallization from methanol/dichloromethane as a white solid. mp 154–165 °C; IR (cm^{-1}) 2939, 2866, 11758, 1699, 1634, 1286, 1154, 1123, 1031; ^{1}H NMR ($CDCl_3$) δ 0.72 (s, 3H), 0.85–0.89 (m, 3H), 0.90 (s, 3H), 0.91–0.94 (m, 3H), 0.95 (s, 3H), 0.99 (s, 3H), 1.05 (s, 3H), (m, 12H), 1.62 (s, 3H), 1.70 (s, 3H), 1.72–2.09 (m, 11H), 2.33 (s, 3H), 2.42 (m, 1H), 3.88 (s, 3H), 4.67 (m, 1H), 5.12 (m, 1H), 6.40 (d, J = 16.0 Hz, 1H), 7.06 (d, J = 8.0 Hz, 1H), 7.12 (m, 2H), 7.63 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 15.8, 16.7, 17.6, 18.2, 18.6, 19.2, 20.6, 21.0, 22.6, 22.8, 24.1, 24.3, 24.3, 24.9, 25.7, 26.4, 28.0, 28.2, 30.8, 31.0, 35.3, 36.3, 36.4, 36.9, 38.1, 44.5, 81.1, 111.2, 119.1, 121.2, 123.2, 125.3, 133.5, 134.3, 141.3, 143.5, 151.4, 166.6, 168.7.

For synthesis of 3-O-(trans-4-O-acetylferuloyl)-stigmastanol, the same procedure used for 3-O-(trans-4-O-acetylferuloyl)-β-cholestanol provided 6.6 g (86%) of the product after column chromatography and 6.4 g (84%) after recrystallization from methanol/dichloromethane as a white solid. mp 163–164 °C; IR (cm^{-1}) 2940, 2866, 1754, 1710, 1638, 1263, 1224, 1171, 1154, 1033, 1010; ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3H), 0.74–2.06 (m, 47H), 2.33 (s, 3H), 3.87 (s, 3H), 4.84 (m, 1H), 6.37 (d, J = 16.0 Hz, 1H), 7.05 (d, J = 8.7 Hz, 1H), 7.11 (m, 2H), 7.63 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 12.0, 12.1, 12.3, 18.7, 19.0, 19.8, 20.6, 21.2, 23.1, 24.2, 26.1, 27.6, 28.3, 28.6, 29.2, 32.0, 33.9, 34.1, 35.5, 36.2, 36.8, 40.0, 42.6, 44.7, 45.9, 54.3, 55.9, 56.2, 56.4, 74.0, 111.2, 119.1, 121.2, 123.2, 133.5, 143.5, 151.4, 166.3, 168.7.

2.2.2. Synthesis of steryl ferulates

For synthesis of 3-O-(trans-4-feruloyl)-β-cholestanol (cholestanyl ferulate), K_2CO_3 (0.24 g, 1.74 mmol) was added to the solution of 3-*O*-(*trans*-4-*O*-acetylferuloyl)-β-cholestanol (5.45 g, 9.0 mmol) in $CHCl_3$-MeOH (2:1, 150 mL) and the mixture was refluxed for 6 h. The reaction mixture was cooled down to room temperature and saturated aqueous NH_4Cl (15 mL) was added. Dichloromethane (200 mL) and water (200 mL) were added and the organic layer was separated, washed with water (200 mL) and then with brine (100 mL), dried over $MgSO_4$, and filtered. Short column chromatography (50 g silica gel) with 1:3:1 of dichloromethane/hexanes/ethyl acetate followed by recrystallization from dichloromethane-methanol gave a white solid (4.91 g, 97%). mp 132–133 °C; IR (cm^{-1}) 3402, 2931, 2866, 2849, 1706, 1636, 1263, 1172, 1154,

1032; ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3H), 0.86 (s, 3H), (0.89 (d, 3H, J = 2.2 Hz), 0.89 (d, 3H, J = 2.2 Hz), 0.92 (d, 3H, J = 6.7 Hz), 0.95–2.04 (m, 38H), 2.32 (s, 3H), 3.92 (s, 3H), 4.83 (m, 1H), 5.95 (s, 1H), 6.28 (d, J = 15.9 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 7.4 (d, J = 1.7 Hz, 1H), 7.07 (m, 1H), 7.60 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 12.1, 12.3, 18.7, 21.2, 22.6, 22.8, 23.9, 24.2, 27.6, 28.0, 28.3, 28.7, 32.0, 34.2, 35.5, 35.8, 36.2, 36.8, 39.5, 40.0, 42.6, 44.7, 54.3, 55.9, 56.3, 56.4, 73.7, 109.3, 114.7, 116.2, 123.0, 127.1, 144.4, 146.8, 147.9, 166.8. Analysis by TLC showed one spot.

For synthesis of 3-O-(trans-4-feruloyl)-cholesterol (cholesteryl ferulate), the same procedure used for cholestanyl ferulate provided 4.8 g (89%) white solid. mp 161–162 °C; IR (cm^{-1}) 3436, 2934, 2867, 2849, 1708, 1636, 1263, 1170, 1156, 1030; ^{1}H NMR ($CDCl_3$) δ 0.70 (s, 3H), 0.88 (d, J = 2.2 Hz, 3H), 0.89 (d, J = 2.2 Hz, 3H), 0.94 (d, J = 6.6 Hz, 3H), 0.96–1.05 (m, 3H), 1.07 (s, 3H), 1.08–2.08 (m, 23H), 2.42 (m, 2H), 3.93 (s, 3H), 4.76 (m, 1H), 5.41 (br m, 1H), 5.93 (s, 1H), 6.29 (d, J = 15.9 Hz, 1H), 6.93 (d, J = 8.2 Hz, 1H), 7.04 (d, J = 1.6 Hz 1H), 7.08 (m, 1H), 7.61 (d, J = 15.8 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.9, 18.7, 19.4, 21.1, 22.6, 22.8, 23.9, 24.3, 27.9, 28.0, 28.2, 31.9, 31.9, 35.8, 36.2, 36.6, 37.1, 38.3, 39.5, 39.8, 42.3, 50.0, 55.9, 56.2, 56.7, 73.9, 109.3, 114.7, 116.1, 122.7, 123.0, 127.1, 139.7, 144.5, 146.8, 147.9, 166.7. Analysis by TLC showed one spot.

For synthesis of 3-O-(trans-4-feruloyl)-β-sitosterol (sitosteryl ferulate), the same procedure used for cholestanyl ferulate provided 4.8 g (90%) white solid. ^{1}H and ^{13}C NMR data were consistent with published literature values (Condo et al., 2001): mp 131–132 °C lit. 156–157 °C (Condo et al., 2001); IR (cm^{-1}) 3377, 2935, 2867, 1701, 1633, 1593, 1267, 1157, 1030; ^{1}H NMR ($CDCl_3$) δ 0.72 (s, 3H), 0.84 (d, 3H, J = 6.9 Hz), 0.85–0.89 (m, 6H), 0.95 (d, 3H, J = 6.6 Hz), 0.96–1.05 (m, 3H), 1.06 (s, 3H), 1.07–2.08 (m, 24H), 2.42 (m, 2H), 3.93 (s, 3H), 4.76 (m, 1H), 5.41 (m, 1H), 5.92 (s, 1H), 6.29 (d, J = 15.9 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 7.04 (d, J = 1.8 Hz, 1H), 7.06 (d, J = 8.0 Hz, 1H), 7.63 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 11.9, 12.0, 18.8, 19.1, 19.4, 19.8, 21.1, 23.1, 24.3, 26.1, 27.9, 28.3, 29.2, 31.9, 31.9, 34.0, 36.2, 36.6, 37.1, 38.3, 39.8, 42.3, 45.9, 50.1, 55.9, 56.1, 56.7, 73.9, 109.3, 114.7, 116.1, 122.7, 123.0, 127.1, 139.7, 144.5, 146.8, 147.9, 166.7. Analysis by TLC showed one spot.

For synthesis of 3-O-(trans-4-feruloyl)-8,24,(5-α)-cholestadien-4,4,14-α-trimethyl-3-β-ol (lanosteryl ferulate), the same procedure used for cholestanyl ferulate provided 4.3 g (81%) white solid. mp 167–171 °C; IR (cm^{-1}) 3350, 2948, 2872, 1676, 1635, 1592, 1254, 1182, 1159, 1010; ^{1}H NMR ($CDCl_3$) δ 0.72 (s, 3H), 0.86–0.89 (m, 3H), 0.90 (s, 3H), 0.91–0.94 (m, 3H), 0.95 (s, 3H), 0.99 (s, 3H), 1.05 (s, 3H), (m, 12H), 1.62 (s, 3H), 1.70 (s, 3H), 1.71–2.15 (m, 11H), 3.95 (s, 3H), 4.67 (m, 1H), 5.12 (m, 1H), 5.90 (s, 1H), 6.31 (d, J = 15.9 Hz, 1H), 6.93 (d, J = 8.2 Hz, 1H), 7.05 (d, J = 1.7 Hz, 1H), 7.09 (m, 1H), 7.61 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 15.8, 16.7, 17.6, 18.2, 18.6, 18.7, 19.2, 21.0, 22.6, 22.8, 24.3, 24.3, 24.3, 24.9, 25.7, 26.4, 28.0, 28.0, 28.2, 28.2, 30.8, 31.0, 35.3, 36.3, 36.4, 36.5, 37.0, 38.1, 39.5, 44.5, 44.5, 49.8, 50.4, 50.6, 56.0, 80.8, 109.3, 114.7, 116.3, 123.0, 125.3, 127.1, 130.9, 134.3, 134.5, 144.3, 146.8, 147.9, 167.1. Analysis by TLC showed one spot.

For synthesis of 3-O-(trans-4-feruloyl)-stigmastanol (stigmastanyl ferulate), the same procedure used for cholestanyl ferulate provided 4.6 g (86%) white solid. mp 156–157 °C; IR (cm^{-1}) 3350, 2931, 2866, 1701, 1676, 1633, 1592, 1257, 1158, 1010; ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3H), 0.74–2.06 (m, 47H), 3.93 (s, 3H), 4.84 (m, 1H), 5.91 (s, 1H), 6.28 (d, J = 15.9 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 7.04 (d, J = 1.8 Hz, 1H), 7.07 (m, 1H), 7.60 (d, J = 15.9 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 12.0, 12.1, 12.3, 18.7, 19.1, 19.8, 21.2, 23.1, 24.2, 26.1, 27.6, 28.3, 28.7, 29.2, 32.0, 33.9, 34.2, 35.5, 36.2, 36.8, 40.0, 42.6, 44.7, 45.9, 54.3, 55.9, 56.2, 56.4, 73.7, 109.3, 114.7, 116.2, 123.0, 127.1, 144.4, 146.8, 147.8, 166.8. Analysis by TLC showed one spot.

2.2.3. Analysis of synthesised products

Melting point was measured with OptoMelt Automated melting point system (Stanford Research systems, Sunnyvale, CA, USA). IR spectrum was obtained with Nexus 470 FT-IR equipped with Smart Orbit (Thermo Electron Corporation, Madison, WI, USA). FT-IR data were analysed with Monic 8.2 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). ^{1}H NMR spectra were acquired on a Bruker (Billerica, MA, USA) Avance 500 spectrometer operating at 500 MHz. ^{13}C NMR experiment was also done at 125 MHz. SpinWorks 3.1.7 (Marat, 2011) software was used for analysis of NMR signals. Purity was determined by TLC (silica gel, dichloromethane/hexanes/ethyl acetate 4:16:1 v/v/v). Since commercial phytosterol standards often contain impurities, and because the commercial sitosterol and lanosterol that were used had manufacturer declared purities of only >70% and 65%, respectively, the content and composition of sterols in the synthetic steryl ferulates were checked by GC after saponification followed by derivatization to the trimethylsilyl ether, as described by Winkler-Moser et al. (2013).

2.2.4. Antioxidant activity assay

The antioxidant activity was determined using the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) decolorization assay as described by Re et al. (1999). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the antioxidant standard and was tested in triplicate at 2 μM, 5 μM, 10 μM, and 15 μM (final assay concentration). Steryl ferulates and ferulic acid were also tested in triplicate at 2 μM, 5 μM, 10 μM, and 15 μM. At these concentrations, the steryl ferulates were soluble in ethanol. We compared results for several steryl ferulates by first dissolving them in either dichloromethane or ethanol, but the results were the same using either solvent. In brief, 10 μl of test compound or standard, dissolved in ethanol, was pipetted into three cuvettes, while 10 μl of ethanol was added to another cuvette to serve as the control. One mL of the $ABTS^{\cdot+}$ solution, prepared as described by Re et al. (1999), was added and the cuvettes were placed into a Perkin Elmer Lambda 35 UV–vis spectrophotometer set to take automatic readings at 734 nm. Readings were started exactly 30 s after the addition of the $ABTS^{\cdot+}$ solution, and were taken every 30 s for 5 min. The percentage inhibition of absorption of each compound at 1 min and 5 min was calculated, and the percentage inhibition at both time points was plotted as a function of the concentration. The trolox equivalent antioxidant capacity (TEAC) was calculated by dividing the slope of the least-squares best-fitting line for the sample by the slope of the trolox line.

2.2.5. Oxidative stability index

Soybean oils were prepared with steryl ferulate concentrations ranging from 0.4413 to 8.85 μmol/g (~250 μg/g to ~5000 μg/g) using steryl ferulate stock solutions dissolved in chloroform. Oils prepared with ferulic acid (0.441, 0.885, and 4.41 μmol/g) were also prepared using stock solutions dissolved in ethanol. An appropriate volume of stock solution was added to amber vials and evaporated under a gentle stream of nitrogen. Oil was weighed into the vials, and the vials were sonicated for 15 min, at room temperature, to dissolve the steryl ferulates, followed by vortexing for 10 s. The control soybean oil samples were sonicated and vortexed in the same manner as the treatment oils. The OSI at 110 °C was determined in duplicate following the AOCS Official Method Cd 12b-92 (1998). A Metrohm (Herisau, Switzerland) 743 Rancimat with software control automatically controlled air flow and temperature and calculated the OSI values based on induction time. Steryl ferulates and ferulic acid were analysed at each concentration level together in duplicate, and a control soybean oil was included with each set.

2.2.6. Frying studies

In previous frying and heating studies, steryl ferulates had strong anti-polymerisation activity at a concentration of 0.5% (w/w). Using the molecular weight of cholestanyl ferulate as the arbitrarily chosen standard, 0.5% is equal to 8.85 μmol/g oil. The mass concentration of each steryl ferulate was adjusted so that each was studied at the same molar concentration, thus the weight concentration for the seven steryl ferulates ranged from 4.98 to 5.40 mg/mL. Steryl ferulates were weighed into 10 mL volumetric flasks and brought to volume with chloroform for a concentration of 20.0 mg/mL. The appropriate volume of each test compound solution was pipetted into 10 mL Pierce Reacti-therm glass vials and the solvent was evaporated under a gentle stream of nitrogen. Soybean oil (6.5 g) was added. After filling the headspace with argon, the vials were capped and stored at −20 °C for one to 5 days prior to the frying studies.

The frying studies were conducted using a miniature frying protocol that was described in detail previously (Winkler-Moser et al., 2012). The frying apparatus limited the amount of treatments that could be compared at one time, so a partially balanced, randomized incomplete block design was used where all seven treatments were studied in triplicate frying studies completed over a series of four frying experiments. After vials of oil were thawed, they were placed in a Reacti-Vap aluminum block and heated to 180 °C using a hot plate connected to a temperature controller (J-KEM Scientific, St. Louis, MO). When the oil reached 70 °C, glass pipettes were used to mix the oil to make sure that the steryl ferulates dissolved completely. Once the oil reached 180 °C, a zero-time sample was taken (500 mg), followed by the first fry cycle, which consisted of frying one 0.6 cm potato cube per vial for 2 min. Fry cycles were repeated every hour for the next seven hours, and additional oil samples (500 mg) were taken after 2, 4, 6, and 8 h. The frying experiments ended after a total of 8 h at 180 °C. Oil samples were kept in 2 mL capped vials, covered with argon, and frozen (−80 °C) until analysis could be conducted.

2.2.7. Analysis of frying oils

Polymerised triacylglycerols (PTAG) in the oil samples were determined using the Association of Official Analytical Chemists (AOAC) Official Method 993.25 (2002) with the exceptions that sample concentration was 5 mg/mL, methylene chloride was used as solvent instead of tetrahydrofuran, and that an evaporative light scattering detector (Shimadzu ELSD-LT II) was used rather than a refractive index detector. Samples were injected into a Shimadzu HPLC (model LC20AT) equipped with an autosampler, membrane degasser, column heater (30 °C) and a size exclusion column (PLGel 5 μm, 100 Å pore size, 300 × 7.5 mm Agilent Technologies, Santa Clara, CA). The ELSD was operated at a temperature of 40 °C with the nebulizer gas (ultra-pure N_2) pressure set to 3.5 Bar, and the Gain set to 1. HPLC control, data collection and analysis were performed by Shimadzu EZStart Chromatography Software Version 7.3. All samples were analysed in duplicate. Data were reported as peak area %, which was the sum of dimer, trimer, and polymer peaks divided by the total peak area including triacylglycerol monomers.

Reactions at reactive sites of SBO triacylglycerols were monitored during heating as previously described (Hwang, Winkler-Moser, & Liu, 2012). In brief, ^{1}H NMR spectra were acquired after diluting 60 mg oil sample with $CDCl_3$ as solvent using the NMR equipment and software previously described. No measurable hydrolysis affecting the peak intensity of the triacylglycerol backbone protons was observed up to 8 h of heating in a quantitative experiment in which an exact amount (60 mg) of sample dissolved in 0.5 mL solvent was used. Therefore, integration of peak area was conducted with the triacylglycerol backbone protons as the standard peak (two double doublets, 4H, 4.06–4.36 ppm). The changes in intensity of the peaks are reported as percentage of the original amount of the olefinic protons (—CH=CH—, multiplet, 5.30–5.44 ppm), bisallyllic protons (=CH—CH_2—CH=, multiplet, 2.74–2.85 ppm) and allylic protons (=CH—CH_2—CH_2—, multiplet, 1.98–2.13 ppm).

Total polar compounds were determined according to a micro method developed by Marquez-Ruiz, Jorge, Martin-Polvillo, and Dobarganes (1996) and modified in our laboratory. This method is a modification of the American Oil Chemists' Society (AOCS) official method Cd 20-91 (97) (1998). Micro methods utilising SepPaks have been shown to give similar results to the standard methods. Oils were separated into nonpolar and polar fractions on a1g silica SepPak (Waters Corporation). The column was conditioned with 10 mL of a 90:10 (v/v) petroleum ether to diethyl ether mixture, and then 1 mL of a 25 mg/mL oil solution in $CHCl_3$ was loaded onto the SepPak. The nonpolar fraction was eluted with an additional 10 mL of the 90:10 (v/v) petroleum ether to diethyl ether mixture, followed by 10 mL of a 50:50 (v/v) chloroform to methanol mixture to elute the polar fraction. During method development, separations were monitored by TLC to verify the separation of fractions. Fractions were dried under nitrogen and gentle heat for several hours and weighed upon dryness. Experiments were conducted in triplicate, and each sample was analysed in duplicate.

Steryl ferulates in soybean oil at time 0 and during the frying study were measured by HPLC as described by Winkler-Moser et al. (2013).

2.3. Statistical analysis

For statistical analysis of the treatments on antioxidant activity, total polar compounds, and NMR analysis of double bonds, data were imported to JMP (Statistical Analytical Systems, Cary, NC) for analysis of variance (ANOVA) using a level of significance of $P < 0.05$. Statistical differences between treatment means were evaluated either by Fisher's LSD or by the Tukey–Kramer HSD test using a level of significance of $P < 0.05$. For analysis of statistical significance of the formation of polymerised triacylglycerols during frying, the mean percentage PTAGs were weighted by 1/variance, and the best fitting regression curves were determined using TableCurve 2D version 5.01 (Systat Software, San Jose, CA, USA). Upper and lower 95% confidence intervals were determined at each time point using the predicted PTAGs values for each treatment. Treatment differences were considered significant ($P < 0.05$) when there was no overlap of the confidence intervals. Microsoft Excel 2007 (Redmond, WA, USA) was used to calculate means and standard deviations and to construct best-fitting lines for antioxidant assay data.

3. Results and discussion

3.1. Synthesis of steryl ferulates

The previously reported method (Ebenezer, 1991) for the synthesis of *trans*-4-*O*-acetylferulic acid requires solvent extraction using a large amount of solvent (3 × 20 mL dichloromethane for 0.66 g product) because the product has low solubility in most organic solvents. The solvent extraction process was also followed by successive washing processes with 2 N HCl, water and brine, which resulted in use of a large volume of the aqueous solution. This procedure is cumbersome for a large-scale synthesis. A larger scale synthesis (25 g) was reported (Condo et al., 2001), however, a long reaction time (overnight) and four-times repeated addition of solvent and evaporation (toluene, 4 × 50 mL) were involved. This method does not have a purification step and, therefore, the purity

was not confirmed. Low purity of *trans*-4-*O*-acetylferulic acid may result in a low yield for the next step in the synthesis. Therefore, we developed a new synthetic procedure for *trans*-4-*O*-acetylferulic acid that provides a high yield and high purity and that requires a shorter time for better suitability for a large scale preparation. In this new procedure, the reaction time is about 4 h, the excessive acetic anhydride is hydrolysed with water, and pyridine is washed with water after neutralization. For purification, impurities were dissolved in methanol and removed by filtration.

The synthesis of *trans*-ferulolyl-β-sitostanol using the coupling reaction of *trans*-4-*O*-acetylferulic acid and β-sitostanol catalyzed by DCC was previously reported (Condo et al., 2001). This method provided somewhat low yield (39–44%) for the coupling reaction and starting materials, *trans*-4-*O*-acetylferulic acid and sterols were not completely reacted. Furthermore, reaction time was relatively long (18 h) and complete removal of the byproduct, 1,3-dicyclohexylurea (DCU), took a long time (overnight) to be precipitated out from THF. A chemoenzymatic synthesis of phytosteryl ferulates was also recently reported by Tan and Shahidi (2011) where vinyl ferulate was synthesised via a vinyl interchange reaction of vinyl acetate with ferulic acid, followed by lipase catalyzed synthesis of phytosteryl ferulates using a 1:2 mol ratio of vinyl ferulate to phytosterols. Synthesis of the vinyl ferulate intermediate took 12hrand resulted in a yield of 46%. Ten lipase sources were investigated and *Candida rugosa* lipase was the only enzyme which catalyzed the esterification of phytosteryl ferulates. A yield of 90% was reported, but the reaction took 10 d. Thus, for this study a chemical synthesis route was chosen in order to obtain gram scale quantities of pure phytosteryl ferulates. In order to increase the yield and purity, some of the existing procedures (Condo et al., 2001; Ebenezer, 1991) were improved. In this new procedure, the reaction time was reduced to 1.5 h, DCU was precipitated instantly by adding hexanes, column chromatography was used for purification instead of the preparative liquid chromatography (PLC), and the final yield was improved to 77–90%. The reported procedure was followed for removal of the acetate protecting group (Condo et al., 2001).

Since commercial phytosterol standards often contain impurities, and because the commercial sitosterol and lanosterol that were used had manufacturer declared purities of >70% and 65%, respectively, the content and composition of sterols in the synthetic steryl ferulates were checked by GC. Sitosteryl ferulate was composed of 80% (peak area percentage) sitosterol, while several other sterol peaks were identified, including campesterol (8.7%), campestanol (2.9%), stigmasterol (7.2%), and sitostanol (0.8%). These contaminants were likely co-extracted from soybean oil from which the sitosterol was extracted. Campesterol and stigmasterol also have a delta-5 double bond, but stigmasterol has an additional double bond at C24 in the side chain. Lanosteryl ferulate had two sterol peaks; lanosterol made up 63.2% of the peak area, while the other peak did not elute at the retention time of any known standards, so the sample (as the trimethylsilylether) was injected on a GC–MS for identification. The minor peak (36.8%) corresponded to 24-dihydrolanosterol, which does not have a double bond at C24, but still has the main structural feature of interest, the 4,4′-dimethyl group. Sitostanyl ferulate was 97% sitostanol, cholesteryl ferulate had 95% cholesterol, and cholestanyl ferulate had 98% cholestanol.

3.2. Antioxidant activity of steryl ferulates

The antioxidant activity of the synthesised steryl ferulates, oryzanol, and ferulic acid were evaluated by the $ABTS^{\cdot+}$ radical cation decolorization assay and in soybean oil by measuring the oxidative stability index (OSI). In the $ABTS^{\cdot+}$ radical cation decolorization assay, ABTS is reacted with potassium persulfate to generate a stable $ABTS^{\cdot+}$ radical which is bright green–blue in colour and has an absorption maximum of 734 nm. The absorbance of the $ABTS^{\cdot+}$ solution decreases when an electron is donated by an antioxidant to quench the free radical; the % inhibition is proportional to the concentration of the antioxidant. When the % inhibition is plotted as a function of the concentration and the slope of the linear regression line is divided by the slope of the trolox linear regression line, the trolox equivalent antioxidant capacity (TEAC), or the μM concentration of sample that has the same activity as 1 μM trolox, can be calculated. As an example, a TEAC of 0.5 means the compound has half of the radical scavenging capacity of trolox. Table 1 shows the % inhibition after 5 min reaction for each compound at each concentration, as well as the TEAC calculated at 5 min. Ferulic acid had the highest radical scavenging activity, with a TEAC of 1.155. For ferulic acid and the steryl ferulates, the radical scavenging activity increased between 1 and 5 min, indicating that they react more slowly with the $ABTS^{\cdot+}$ radical compared to trolox, which reacts much more quickly and does not increase in antioxidant activity much between 1 and 5 min. The % inhibition by ferulic acid leveled off between 10 μM and 15 μM, so only the first three concentrations were used to determine the slope and to calculate the TEAC for ferulic acid. Others reported a slightly higher TEAC for ferulic acid, from 1.69 to 1.90 (Re et al., 1999). The fact that ferulic acid had much higher activity than the steryl ferulates indicates that either solubility or steric hindrance prevented the steryl ferulates from reacting as efficiently with the radical. This assay has been used for both hydrophilic and lipophilic antioxidants such as tocopherols and carotenoids (Re et al., 1999). Furthermore, we tested oryzanol using both methanol and methylene chloride as a solvent, and found that there was no difference in activity. Also, since we found that activity of steryl ferulates

Table 1
% Inhibition of $ABTS^{\cdot+}$ radical, and trolox equivalent antioxidant capacity (TEAC), for synthetic steryl ferulates and ferulic acid ($n = 3$).

	% Inhibition at 5 min (Ave. ± SD)					TEAC
	2 μM	5 μM	10 μM	15 μM	Overall[1]	(5 min)
Cholestanyl ferulate	8.91 ± 0.81^{b}	18.54 ± 1.74^{b}	41.47 ± 0.98^{b}	48.49 ± 2.03^{c}	29.08^{b}	0.887
Cholesteryl ferulate	7.75 ± 0.52^{bc}	17.67 ± 0.35^{b}	37.17 ± 1.55^{cd}	45.41 ± 1.69^{d}	$26.75^{b,c}$	0.829
Sitostanyl ferulate	8.03 ± 1.97^{bc}	19.51 ± 1.86^{b}	35.01 ± 3.13^{d}	43.70 ± 1.33^{d}	$26.33^{b,c}$	0.762
Sitosteryl ferulate	6.59 ± 0.41^{c}	17.33 ± 2.23^{b}	35.15 ± 0.60^{cd}	43.13 ± 1.13^{d}	25.31^{c}	0.795
Lanosteryl ferulate	6.88 ± 2.04^{bc}	14.71 ± 1.20^{c}	37.98 ± 2.11^{c}	56.56 ± 1.75^{b}	28.70^{b}	1.094
Oryzanol	7.77 ± 0.38^{bc}	18.67 ± 0.65^{b}	31.67 ± 0.52^{e}	44.52 ± 0.43^{d}	25.49^{c}	0.867
Ferulic acid	22.41 ± 1.37^{a}	43.79 ± 0.98^{a}	57.08 ± 0.65^{a}	61.02 ± 0.19^{a}	45.84^{a}	1.155

[a,b,c,d,e] Mean values within the same column with different superscript letters are significantly different at $P < 0.05$ by Fisher's LSD test.
See Section 2.2.4. to see how TEAC is calculated.
[1] Standard error of the means was 1.05.

Table 2
OSI (hr) at 110 °C, and OSI percentage of control soybean oil (in parentheses), for synthetic steryl ferulates, oryzanol, and ferulic acid added to soybean oil ($n = 2$).

	OSI ± SD (% SBO)			
Treatment	0.44 μmol/g	0.88 μmol/g	4.43 μmol/g	8.85 μmol/g
Soybean oil (control)[1]	7.89 ± 0.13	7.68 ± 0.13^{ab}	8.61 ± 0.12^{a}	8.60 ± 0.02^{a}
Cholestanyl ferulate	7.86 ± 0.01 (99.6)	7.95 ± 0.03 (104)^{a}	7.81 ± 0.11 (90.7)^{cd}	7.35 ± 0.13 (85.5)^{bc}
Cholesteryl ferulate	7.75 ± 0.13 (98.2)	7.23 ± 0.03 (94.2)^{b}	7.44 ± 0.11 (86.5)^{de}	6.44 ± 0.05 (74.9)^{d}
Sitostanyl ferulate	7.65 ± 0.01 (97.0)	7.71 ± 0.23 (100)^{ab}	8.26 ± 0.01 (96.0)^{ab}	7.32 ± 0.13 (85.2)^{bc}
Sitosteryl ferulate	7.83 ± 0.11 (99.2)	7.27 ± 0.09 (94.7)^{b}	7.38 ± 0.15 (85.7)^{e}	7.50 ± 0.06 (87.2)^{b}
Lanosteryl ferulate	7.86 ± 0.01 (99.6)	6.46 ± 0.17 (84.2)^{c}	8.13 ± 0.04 (94.5)^{bc}	5.62[2] (65.4)^{e}
Oryzanol	7.92 ± 0.06 (100)	7.61 ± 0.08 (99.2)^{ab}	8.39 ± 0.01 (97.5)^{ab}	7.15 ± 0.01 (83.2)^{c}
Ferulic acid	7.56 ± 0.01 (96.1)	6.63 ± 0.08 (86.3)^{c}	7.08 ± 2.06 (82.2)^{bcde}	ND[3]

^{a,b,c,d,e} Mean values within the same column with different superscript letters are significantly different at $P < 0.05$ by Tukey's HSD test. In columns with no superscripts, ANOVA showed no significant effect of treatment on OSI.

[1] Two different batches of soybean oil were used for the analysis at 0.44 and 0.88 μmol/g vs 4.43 and 8.85 μmol/g, explaining the difference in SBO OSI between the two sets of data.

[2] Due to machine error, only one sample was analysed.

[3] Not determined, ferulic acid was not soluble at this concentration.

increased with concentration, which would not be expected if they were insoluble at these concentrations, we concluded that the lower activity is likely due to steric hindrance by the sterol moiety interfering with the ability of the radical and the phenolic group to react. However, as concentration increased, the % inhibition by steryl ferulates increased linearly and approached the activity of ferulic acid. Although at each concentration, there were some significant differences in % inhibition between the steryl ferulates, there was no clear trend for the radical scavenging activity based on the sterol structural features. TEAC values indicated that lanosteryl ferulate had the highest antioxidant activity, but this value appears to have been highly influenced by the % inhibition at 15 μM, whereas it had lower % inhibition at all of the other concentrations. Two-factor analysis of variance indicated that there was a significant effect of sterol head group, but there was also a significant interaction between the concentration and the sterol head group. Comparison of the mean % inhibition over all concentrations indicated that cholestanyl ferulate had the best activity, but was not significantly higher than lanosteryl ferulate, cholesteryl ferulate or sitostanyl ferulate. However, cholestanyl ferulate was significantly higher in overall antioxidant activity compared to both oryzanol and sitosteryl ferulate. We have previously found (unpublished results), that corn steryl ferulates and oryzanol had similar antioxidant activities by the ABTS assay when evaluated at a concentration range from 10 to 50 μM. The TEAC measured for both was 0.600, which is lower than the current results because the activity levels off above 15 μM. Our results are somewhat in agreement with Nyström et al. (2005), who reported that ferulic acid had significantly higher radical scavenging activity, using the diphenylpicrylhydrazyl (DPPH) radical, compared to sitosteryl ferulate, cholesteryl ferulate, and steryl ferulate mixtures extracted from wheat and rye. However, they also reported that these extracts, which mostly contain sitostanyl- and campestanyl-ferulates, had better radical scavenging activity compared to pure sitosteryl and cholesteryl ferulate. In another study, Islam et al. (2009) reported similar DPPH scavenging activities for 1–60 μM cycloartenyl ferulate, 24-methylenecycloartenyl ferulate, sitosteryl ferulate, and ferulic acid. Tan and Shahidi (2011) reported improved antioxidant activity for chemoenzymatically synthesised phytosteryl ferulates compared to ferulic acid (~16 μmol trolox equivalents/μmol phytosteryl ferulates vs ~8.00 μmol troloxequivalents/μmol ferulic acid) using a lipophilic oxygen radical absorbance capacity (ORAC) assay. In this assay, lipophilic compounds are solubilised using randomly methylated β-cyclodextrin (RMCD), which allows for measurement of the antioxidant activity of lipophilic substances in the otherwise aqueous environment. However, in our experience (unpublished data), the RMCD interferes with the antioxidant activity of water soluble phenolic compounds such as trolox and ferulic acid giving them effectively lower antioxidant activity. This makes it difficult to make direct comparisons between the lipophilic ORAC assay and other antioxidant assays using trolox as a standard. Tan and Shahidi (2011) also demonstrated improved antioxidant activity of the phytosteryl ferulates compared to ferulic acid in cooked ground pork stored at 4 °C, indicating that the increased hydrophobicity of the phytosteryl ferulates increased their activity in a high-fat food system.

Previous studies had shown antioxidant activity during frying for steryl ferulates at an addition level of 5.0 mg/g in soybean oil (Winkler-Moser et al., 2012), which is approximately 8.85 μmol/g oil, so the antioxidant activity of phytosteryl ferulates was assessed by the effect on the oxidative stability index of soybean oil at two high level concentrations, 4.43 μmol/g and 8.85 μmol/g oil, which for steryl ferulates corresponded to 2.5–2.7 mg/g and 4.9–5.3 mg/g, as well as two lower concentrations levels, 0.44 and 0.89 μmol/g (~0.25 and 0.5 mg/g, respectively), which corresponds to a typical concentration range for added antioxidants. Ferulic acid was only assessed at 0.44 μmol/g, 0.89 μmol/g, and 4.3 μmol/g (0.86 mg/g) because it was not soluble in the oil at higher concentrations.

At the lowest concentration, ferulic acid and the steryl ferulates had very little impact on the OSI of soybean oil (Table 2), and in fact there was no significant treatment effect on the OSI. At 0.88 μmol/g, both ferulic acid and lanosteryl ferulate had a significant prooxidant effect on soybean oil OSI, while the other steryl ferulate treatments had no significant effect on soybean oil OSI. At a concentration of 4.43 μmol/g, it was difficult to completely dissolve the ferulic acid, and the duplicate OSI values were 8.54 h and 5.62 h, indicating an uneven distribution of the ferulic acid in the oil. At this concentration, the steryl ferulates, except for sitostanyl ferulate and oryzanol, also had a significant prooxidant effect on soybean oil, and at the highest concentration, all of the steryl ferulates had a significant prooxidant effect. Lanosteryl ferulate and cholesteryl ferulate had the greatest prooxidant effect at 8.85 μmol/g, while cholesteryl and sitosteryl ferulate were the most prooxidant at 4.4 μmol/g. Since the steryl ferulates had very little antioxidant effect in the concentration range that we tested, no conclusions could be drawn about the effect of structure on antioxidant activity in this system. The fact that steryl ferulates with one or more sterol double bonds (oryzanol, cholesteryl-, sitosteryl-, and lanosteryl-ferulate) had the most prooxidant activity at higher concentrations indicates that oxidation of the ring double bond may accentuate the prooxidant activity due to oxidation of the double bonds and interaction of resulting radicals with surrounding triacylglycerols (Winkler & Warner, 2008). However,

the prooxidant activity of ferulic acid itself indicates that the main contributor is the ferulic acid moiety. When the concentration of antioxidants that all have the same antioxidant function (i.e. chain breaking phenolic antioxidants), are increased, the effect does not always increase and in some cases, they can have a prooxidant effect. This is because when concentrations exceed their optimal levels, the phenolics and their corresponding phenolic radicals can participate in side reactions which can actually promote oxidation (Frankel, 2005, chap. 9; Yanishlieva, Kamal-Eldin, Marinova, & Toneva, 2002).

Our results, which demonstrated little to no effect of steryl ferulates on soybean oil OSI at lower concentrations, and loss of efficacy/prooxidant activity at higher concentrations, are supported by previous research (Wang, Hicks, & Moreau, 2002), where at 1.2 μmol/g oryzanol, ferulic acid, and sitostanyl-ferulate increased the oxidative stability of soybean oil at 90 °C by 7.5%, 10.8%, and 0%, respectively, while at a concentration of 2.4 μmol/g, the soybean oil oxidative stability was increased by 1.7%, 4.8%, and 3.2% for oryzanol, ferulic acid, and sitostanyl ferulate, respectively. In another study, oryzanol, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and ferulic acid (all at 0.4 μmol/g) increased the OSI of 10% methyl linoleate in mineral oil 2.25–2.98-fold (Kikuzaki et al., 2002). The difference in results might be explained by the highly oxidizable nature of the substrate, methyl linoleate, compared to soybean oil, which has some protection from its endogenous tocopherols. As an example, we previously demonstrated that the addition of 0.5% and 1% of an antioxidant extract from distillers dried grains containing steryl ferulates, tocopherols, and tocotrienols had no effect on the OSI of soybean, sunflower, and high-oleic sunflower oils, but increased the OSI of the same oils in a dose dependent manner when the oils were stripped of their native tocopherols (Winkler-Moser & Vaughn, 2009).

3.3. Effect of steryl ferulates in soybean oil during frying

One of the major products of oil oxidation and degradation during frying is the formation of triacylglycerol polymers (PTAGs), as well as a mixture of polar oxidised triacylglycerols, monoacylglycerols and diacylglycerols, which are generally referred to as total polar compounds (TPC). Measurement of these two products are used worldwide as an indicator of oil quality during frying (Gertz, 2000). Formation of PTAGs in the control soybean oil, as well as in soybean oil with added steryl ferulates, are shown in Fig. 1. Weighted regression equations (Table 3) were optimised for each treatment and they all had high correlation coefficients ($R^2 \geqslant 0.922$). For all treatments, PTAGs increased slowly between 0 h and 2 h, but after 4 h treatment differences in the rate of increase in PTAGs were apparent. Treatments were compared by non-overlap of 95% confidence intervals for the predicted % PTAGs from the equations. The synthetic steryl ferulates lowered PTAGs

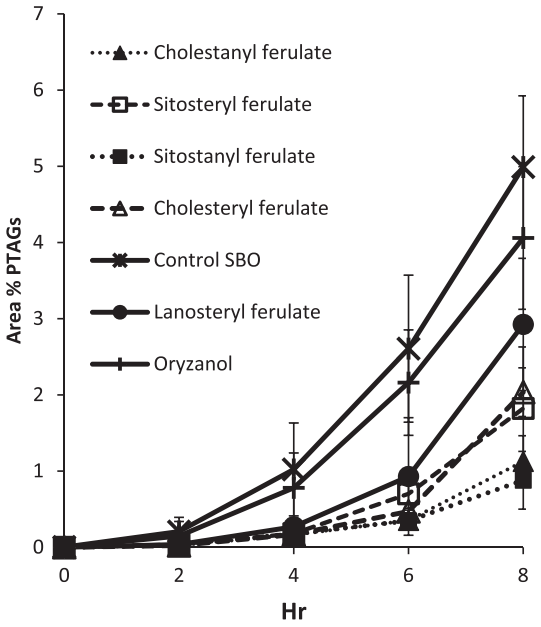


Fig. 1. Formation of polymerised triacylglycerols (PTAGs) during frying. See Table 3 for statistical analysis of PTAGs at each time point.

significantly after 6 and 8 h of frying, whereas oryzanol was only significantly lower from the control at 8 h. The most remarkable difference noted was between oryzanol and the synthetic steryl ferulates. Lanosteryl ferulate prevented polymerisation better than oryzanol, but it was significantly worse than the other steryl ferulates. Cholestanyl ferulate and sitostanyl ferulate prevented polymerisation in a similar fashion, and PTAGs were consistently lower with these two treatments compared to their C5 unsaturated counterparts, sitosteryl and cholesteryl ferulate. However, the differences between the C5 saturated vs their unsaturated counterparts were not statistically significant except that at 8 h sitosteryl ferulate was significantly higher in PTAGS compared to sitostanyl- and cholestanyl-ferulate. These results indicate that saturated phytosterols make slightly better antipolymerisation agents compared to sterols with a double bond at C5. Lanosterol, which has both the dimethyl group at C4, as well as double bonds at C8 and C24, had activity in between the other sterols with zero or one double bonds, and oryzanol. Since oryzanol is composed of a mixture of sterols with a C5 double bond, sterols with a C4 dimethyl group, a C9,C19- cyclopropane group, and a C24 double bond, these data indicate that the anti-polymerisation activity is greatly impacted by the C9,C19 cyclopropane group, since this is the major structural difference between oryzanol and lanosteryl ferulate a the other synthetic steryl ferulates.

Table 3
Weighted regression equations for mean % PTAGs (*Y*-variable) as a function of time in hours (*X*-variable), adjusted R^2, treatment differences in predicted % PTAGs at each time point, and change in % total polar compounds from 0 h to 8 h during frying ($n = 3$).

Treatment	Weighted regression equations[A]	Adj. R^2	Time					
			0	2	4	6	8	ΔTPC (%)
Control	$\ln(Y) = -4.867 + 2.309 * \sqrt{X}$	0.922	0.0077^{a}	0.2016^{a}	0.7798^{ab}	2.2020^{a}	5.2820^{a}	11.06 ± 2.66^{a}
Cholestanyl	$\ln(Y) = -6.156 + 2.127 * \sqrt{X}$	0.929	0.0021^{a}	0.0430^{a}	0.1494^{ab}	0.3886^{c}	0.8702^{e}	5.72 ± 0.55^{cd}
Cholesteryl	$\ln(Y) = -8.356 + 3.112 * \sqrt{X}$	0.964	0.0002^{a}	0.0192^{a}	0.1187^{ab}	0.4807^{c}	1.5630^{de}	7.31 ± 1.43^{bc}
Sitostanyl	$\ln(Y) = -6.326 + 2.174 * \sqrt{X}$	0.962	0.0018^{a}	0.0387^{a}	0.1383^{ab}	0.3673^{c}	0.8371^{e}	4.30 ± 1.31^{d}
Sitosteryl	$\ln(Y) = -7.392 + 2.850 * \sqrt{X}$	0.992	0.0006^{a}	0.0347^{a}	0.1842^{b}	0.6630^{c}	1.9520^{d}	5.77 ± 1.48^{cd}
Lanosterol	$\ln(Y) = -7.343 + 2.982 * \sqrt{X}$	0.987	0.0006^{a}	0.0439^{a}	0.2517^{ab}	0.9616^{b}	2.9770^{c}	10.15 ± 0.89^{ab}
Oryzanol	$\ln(Y) = -4.335 + 2.042 * \sqrt{X}$	0.972	0.0131^{a}	0.2352^{a}	0.7779^{a}	1.9480^{a}	4.2230^{b}	10.48 ± 2.30^{a}

^{a,b,c,d,e} Values within a column followed by the same letter are not significantly different based on overlap of 95% confidence intervals from weighted regression equations for predicted mean pTAGs, and based on Fisher's LSD test for ΔTPC.
^{A} Slope and intercept are significantly different from 0 at $P \leqslant 0.01$ for all treatment equations.

The antipolymerisation activity of the synthetic steryl ferulates compared to oryzanol in this study corresponded to previous results showing higher antipolymerisation activity for corn steryl ferulates, which are composed mainly of saturated sterols, compared to oryzanol (Winkler-Moser et al., 2012; Winkler-Moser et al., 2013), and also confirmed antipolymerisation activity of synthetic sitostanyl ferulate in heated high-oleic sunflower oil (Nyström et al., 2007). We also demonstrated in a previous study (Winkler-Moser et al., 2012), that ferulic acid used at the same molar concentration (8.85 μmol/g) had a prooxidant effect on soybean oil polymerisation during frying, which is confirmed by the OSI results (Table 2).

TPC were determined gravimetrically at the 0 h and 8 h time points to compare to and verify the results from PTAG analysis. It is important to note that for PTAG analysis, the area % reported is not the same as weight percentage that is reported for TPC, due to differences in the sensitivity of the evaporative light scattering detector for monomeric vs polymeric triacylglycerol peaks. The overall change in TPC from 0 to 8 h corresponded well with the PTAG analysis (Table 3). Cholestanyl-, sitostanyl-, cholesteryl-, and sitosteryl ferulate all prevented the formation of total polar compounds after 8 h compared to the control SBO. However, oryzanol and lanosteryl ferulate did not significantly prevent formation of total polar compounds, although lanosteryl ferulate showed a similar trend to the PTAG formation in that it prevented TPC formation slightly better than oryzanol.

During frying, the fatty acids in oils, especially polyunsaturated fatty acids, undergo both oxidative and thermal reactions which produce numerous compounds besides the polymerised triacylglycerols measured previously, including aldehydes, ketones, and short chain volatile compounds. These reactions result in a loss of double bonds, which can be monitored by ^{1}H NMR measurement of the peak intensities of olefinic protons (—C**H**=C**H**—, multiplet, 5.39–5.45 ppm), bisallylic protons (=CH—C**H**$_2$—CH=, multiplet, 2.71–2.85 ppm), and allylic protons (=CH—CH$_2$—CH$_2$—, multiplet, 1.97–2.13 ppm) (Hwang et al., 2012; Wanasundara, Shahidi, & Jablonski, 1995). Compared to analysis of fatty acid composition, this method is a more sensitive measure of double bond loss, but it is less specific in that it does not specify which fatty acids are affected by the loss of double bonds. As would be expected, the bis-allylic protons were lost the fastest, followed by the olefinic and allylic protons (Table 4). After 4 h, the control SBO lost an average of 2.57% of the olefinic, 3.93% of bis-allylic, and 1.94% of the allylic double bond H protons. Oils with added cholestanyl, cholesteryl, sitostanyl, and sitosteryl ferulate lost significantly fewer allylic H protons, while only cholestanyl, cholesteryl, and sitosteryl lost significantly fewer olefinic protons, and only cholestanyl lost significantly fewer bis-allylic protons. After 8 h frying, neither lanosteryl ferulate nor oryzanol significantly prevented the loss of olefinic, bis-allylic, or allylic double bonds. However, cholestanyl, cholesteryl, sitostanyl, and sitosteryl significantly prevented the loss of olefinic and bis-allylic double bond protons. For preventing loss of allylic double bond protons after 8 h, the saturated steryl ferulates cholestanyl- and sitostanyl ferulate were significantly better than their C5 unsaturated counterparts.

Table 4
NMR analysis showing the mean percentage, compared to the 0 h time point in each frying study, of olefinic, bis-allylic, and allylic double bond H protons remaining in SBO during frying (n = 3).

Treatment	% Olefinic	% Bis-allylic	% Allylic
		4 h	
Control-SBO	97.43^{cd}	96.07^{bc}	98.06^{cd}
Cholestanyl ferulate	99.08^{ab}	98.55^{a}	99.37^{ab}
Cholesteryl ferulate	99.49^{a}	98.29^{ab}	99.47^{a}
Sitostanyl ferulate	99.04^{abc}	98.15^{ab}	99.32^{ab}
Sitosteryl ferulate	99.27^{ab}	98.30^{ab}	99.37^{ab}
Lanosteryl ferulate	98.80^{abc}	97.84^{ab}	99.07^{abc}
Oryzanol	97.66^{bcd}	96.32^{abc}	98.24^{bcd}
		8 h	
Control-SBO	93.52^{cd}	90.33^{cd}	95.11^{cd}
Cholestanyl ferulate	97.03^{a}	95.89^{a}	98.70^{a}
Cholesteryl ferulate	96.39^{ab}	94.56^{ab}	97.44^{abc}
Sitostanyl ferulate	97.37^{a}	96.01^{a}	98.11^{ab}
Sitosteryl ferulate	95.97^{ab}	94.46^{ab}	97.13^{abc}
Lanosteryl ferulate	95.18^{abc}	92.76^{abc}	96.45^{abcd}
Oryzanol	94.26^{bcd}	91.45^{bcd}	95.75^{bcd}

^{a,b,c,d}Mean values within the same column with different superscript letters are significantly different at $P < 0.05$ by Tukey's HSD test.

Thus, three indicators for frying oil degradation all showed superior antioxidant activity during frying for phytosteryl ferulates composed of C4-desmethylsterols compared to C4-dimethylsterols. All three measurements indicated a trend showing that saturated C4-desmethylsterols are slightly better antioxidants during frying compared to those with a C5 double bond, however, the differences were not great enough to be statistically significant. During frying, lanosteryl ferulate was significantly less effective than the C4-desmethylsteryl ferulates, indicating that either the C4-dimethyl group, or the presence of two double bonds in the structure of 65% of its components, affected its antioxidant activity. Lanosteryl ferulate also showed a trend of better antioxidant activity compared to oryzanol, although this was only significant for PTAG formation. Lanosteryl ferulate is similar in structure to the major components in oryzanol, cycloartenyl ferulate and 24-methylene cycloartanyl ferulate, in that it has a 4,4-dimethyl group as well as a double bond at C24. However, it also has an extra ring double bond at C8. Since the minor components of oryzanol (36% by weight) are made up of desmethylsterols similar in structure to the sitosteryl and cholesteryl ferulate, it might be expected to have antioxidant activity in between that of lanosteryl ferulate and sitosteryl and cholesteryl ferulates, if the C9,C19 cyclopropane group did not impact antioxidant activity. However, its consistently lower antioxidant activity indicates that the C4-dimethyl group and the C9,C19-cyclopropane group both have a significant impact on its antioxidant activity during frying.

At this time, we cannot offer a complete explanation for why structural differences in the sterol head group lend to varying antioxidant activity during frying. The structural differences that we examined in this study, including double bonds, dimethyl substitution, and the presence of a cyclopropane group, could affect the stability of the phytosterol moiety to oxidation. In this study, the average percentages of intact steryl ferulates remaining after 8 h of frying were 74.6%, 78.5%, 78.7%, 88.0%, 88.2%, and 91.6% for oryzanol, lanosteryl-, sitosteryl-, cholesteryl-, sitostanyl-, and cholestanyl ferulate, respectively. The percentages of steryl ferulates remaining at 2, 4, 6, and 8 h were negatively correlated with PTAGs (r = −.8159) and looking at 8 h alone, were negatively correlated with PTAGs (r = −0.7805) and TPC (r = −0.6695). However, since there was on average 75% of the oryzanol remaining and 78.7% of sitosteryl ferulate remaining after 8 h, stability alone is unlikely to explain the effectiveness of sitosteryl ferulate compared to oryzanol. A previous study showed that corn steryl ferulates were more effective antioxidants compared to oryzanol and were also more stable during frying in soybean oil (Winkler-Moser et al., 2012). In a heating study at 180 °C, under conditions where soybean oil oxidation progressed more rapidly, oryzanol was slightly more stable than corn steryl ferulates, but was still less effective of an antioxidant (Winkler-Moser et al., 2013). In this study, the phytosterol moieties were measured, and it was found that 50–75% of the phytosterols remained intact, even though only 10–20% of the steryl ferulates remained intact by the end of the study. The phytosterols from oryzanol degraded to a greater extent than phytosterols from

corn steryl ferulates, so it is possible that oxidation products from the oxidation of oryzanol and lanosteryl phytosterols interacted with soybean triacylglycerols to have a prooxidant effect that counteracted, or interfered, with their antioxidant effect.

Another possibility is that ferulic acid or oxidised ferulic acid released from steryl ferulates by hydrolysis may be acting as pro-oxidants as well. Ferulic acid acted as a prooxidant in the present study by OSI analysis, and in a previous frying study (Winkler-Moser et al., 2012), showed prooxidant activity and accelerated the degradation of tocopherols. Unfortunately, our methods for steryl ferulate analysis only indicate the levels of intact steryl ferulates remaining, but do not explain if the loss is due to hydrolysis or to oxidation or a combination of both. Thus, we need to explore these and other possible explanations in order to understand the differences observed in antioxidant activity during frying.

Overall, the results from this study indicate that despite having similar antioxidant activities when compared using an *in vitro* antioxidant activity assay, as well as by analysis of the effects on OSI in soybean oil at 110 °C, steryl ferulates are dramatically affected by the structure of the sterol group when comparing their antioxidant activity in frying oils. These results indicate that saturated sterols are slightly better, within the confines of our model frying experiment, compared to more typical sterols which have a double bond at C5. In addition, both types (saturated and C5-unsaturated sterols) are superior to sterols such as those in oryzanol that have a C4 dimethyl group, and a C9,C19 cyclopropane group. At least within the parameters of this study, the 9,19 cyclopropane group appeared to have a major impact on steryl ferulate antioxidant activity during frying. Thermal and oxidative stability of the phytosterol group may play a role on the antioxidant activity of the steryl ferulates, but it may not have been the major factor involved (Winkler-Moser et al., 2012; Winkler-Moser et al., 2013). Future studies will focus on determining the reason for differences in antioxidant activity during frying, as well as to understand why steryl ferulates are not especially strong antioxidants *in vitro*, nor as assessed by OSI at 110 °C, but are very effective during frying.

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References

American Oil Chemists' Society (1998). *Official methods and recommended practices of the American Oil Chemists' Society* (5th ed.). Urbana, IL: AOCS Press.

Association of Official Analytical Chemists (2002). *Official Methods of Analysis of AOAC International* (17th ed.). AOAC International: Gaithersburg, MD.

Condo, A. M., Jr., Baker, D. C., Moreau, R. A., & Hicks, K. B. (2001). Improved method for the synthesis of trans-feruloyl-β-sitostanol. *Journal of Agricultural and Food Chemistry, 49*, 4961–4964.

Ebenezer, W. J. (1991). Colabomycin co-metabolites. Synthesis of 2880-II, a metabolite related to ferulic acid. *Synthetic Communications, 21*, 351–358.

Frankel, E. N. (2005). *Lipid oxidation* (2nd ed.). Bridgwater, England: The Oil Press P.J. Barnes & Associates.

Gertz, C. (2000). Chemical and physical parameters as quality indicators of used frying fats. *European Journal of Lipid Science & Technology, 102*, 566–572.

Hwang, H.-S., Winkler-Moser, J. K., & Liu, S. X. (2012). Structural effects of lignans and sesamol on polymerization of soybean oil at frying temperature. *Journal of the American Oil Chemists' Society, 89*, 1067–1076.

Islam, M. S., Yoshida, H., Matsuki, N., Ono, K., Nagasaka, R., Ushio, H., et al. (2009). Antioxidant, free radical-scavenging, and NF-κB-inhibitory activities of phytosteryl ferulates: Structure–activity studies. *Journal of Pharmacological Sciences, 111*, 328–337.

Juliano, C., Cossu, M., Alamanni, M. C., & Piu, L. (2005). Antioxidant activity of gamma-oryzanol: Mechanism of action and its effect on oxidative stability of pharmaceutical oils. *International Journal of Pharmaceuticals, 299*, 146–154.

Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K., & Taniguchi, H. (2002). Antioxidant properties of ferulic acid and its related compounds. *Journal of Agricultural and Food Chemistry, 50*, 2161–2168.

Marquez-Ruiz, G., Jorge, N., Martin-Polvillo, M., & Dobarganes, M. C. (1996). Rapid quantitative determination of polar compounds in fats and oils by solid-phase extraction and size-exclusion chromatography using monostearin as internal standard. *Journal of Chromatography A, 749*, 55–60.

Marat, K. (2011). *SpinWorks, version 3.1.8 beta 4*. Manitoba, Canada: University of Manitoba.

Moreau, R. A., Singh, V., Nuñez, A., & Hicks, K. B. (2000). Phytosterols in the aleurone layer of corn kernels. *Biochemistry Society Transactions, 28*, 803–806.

Norton, R. A. (1995). Quantitation of steryl ferulate and p-coumarate esters from corn and rice. *Lipids, 30*, 269–274.

Nyström, L., Mäkinen, M., Lampi, A.-M., & Piironen, V. (2005). Antioxidant activity of steryl ferulate extracts from rye and wheat bran. *Journal of Agricultural and Food Chemistry, 53*, 2503–2510.

Nyström, L., Achrenius, T., Lampi, A.-M., Moreau, R. A., & Piironen, V. (2007). A comparison of the antioxidant properties of steryl ferulates with tocopherol at high temperatures. *Food Chemistry, 101*, 947–954.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology Medicine, 26*, 1231–1237.

Seitz, L. M. (1989). Stanol and sterol esters of ferulic and p-coumaric acids in wheat, corn, rye, and triticale. *Journal of Agricultural and Food Chemistry, 37*, 662–667.

Tan, Z., & Shahidi, F. (2011). Chemoenzymatic synthesis of phytosteryl ferulates and evaluation of their antioxidant activity. *Journal of Agricultural and Food Chemistry, 59*, 12375–12383.

Shopova, N., & Milkova, T. (2000). Thermal decomposition of cholesteryl esters of cynnamic acid derivatives and their effect on the α-tetralylhydroperoxyde free-radical-induced decomposition. *Thermochimica Acta, 356*, 101–107.

Suh, M.-H., Yoo, S.-H., & Lee, H. G. (2007). Antioxidative activity and structural stability of microencapsulated γ-oryzanol in heat-treated lards. *Food Chemistry, 100*, 1065–1070.

Wanasundara, U. N., Shahidi, F., & Jablonski, C. R. (1995). Comparison of standard and NMR methodologies for assessment of oxidative stability of canola and soybean oils. *Food Chemistry, 52*, 249–253.

Wang, T., Hicks, K. B., & Moreau, R. (2002). Antioxidant activity of phytosterols, oryzanol, and other phytosterol conjugates. *Journal of the American Oil Chemists' Society, 79*, 1201–1206.

Winkler, J. K., & Warner, K. (2008). The effect of phytosterol concentration on oxidative stability and thermal polymerization of heated oils. *European Journal of Lipid Science & Technology, 110*, 455–464.

Winkler-Moser, J. K., & Vaughn, S. F. (2009). Antioxidant activity of phytochemicals from distillers dried grain oil. *Journal of the American Oil Chemists' Society, 86*, 1073–1082.

Winkler-Moser, J. K., Rennick, K. A., Palmquist, D. A., Berhow, M. A., & Vaughn, S. F. (2012). Comparison of the impact of γ-oryzanol and corn steryl ferulates on the polymerization of soybean oil during frying. *Journal of the American Oil Chemists' Society, 89*, 243–252.

Winkler-Moser, J. K., Rennick, K. A., Hwang, H.-S., Berhow, M. A., & Vaughn, S. F. (2013). Effect of tocopherols on the anti-polymerization activity of oryzanol and corn steryl ferulates in soybean oil. *Journal of the American Oil Chemists' Society, 90*, 1351–1358.

Xu, Z., & Godber, J. S. (1999). Purification and identification of components of γ-oryzanol in rice bran oil. *Journal of Agricultural and Food Chemistry, 47*, 2724–2728.

Xu, Z., & Godber, J. S. (2001). Antioxidant activities of major components of γ-oryzanol from rice bran using a linoleic acid model. *Journal of the American Oil Chemists' Society, 78*, 645–649.

Yanishlieva, N. V., Kamal-Eldin, A., Marinova, E. M., & Toneva, A. G. (2002). Kinetic study of the antioxidant activity of α- and γ-TOHs in sunflower and soybean oil triacylglycerols. *European Journal of Lipid Science and Technology, 104*, 262–270.