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ORIGINAL PAPER

Antioxidant Activity of Phytochemicals from Distillers Dried Grain Oil

Jill Kristine Winkler-Moser · Steven Francis Vaughn

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Abstract A distillate was obtained by molecular distillation of oil extracted from distillers dried grains (DDG). The distillers dried grain oil distillate (DDGD) contained phytosterols, steryl ferulates, tocopherols, tocotrienols, and carotenoids. DDGD was tested for its impact on the oxidative stability index (OSI) at 110 °C of soybean, sunflower, and high-oleic sunflower oils, as well as the same oils that were stripped of their natural tocopherols and phytosterols. In addition, the impact of added DDGD on the stability of stripped sunflower oil during an accelerated storage study conducted at 60 °C was also determined. DDGD (0.5–1% w/w) had little impact on the OSI of soybean, sunflower, and high-oleic sunflower oil, but at levels of 0.1–1% it significantly increased the OSI for stripped soybean, sunflower, and high-oleic sunflower oil in a dose-dependent manner. DDGD also delayed peroxide value, conjugated diene, and hexanal formation during accelerated storage of stripped sunflower oil. The antioxidant activity is probably due to the combination of tocopherols, tocotrienols, and steryl ferulates.

Keywords Steryl ferulates · Ferulate phytosterol esters · Oryzanol · Tocopherols · Tocotrienols · Distillers dried grain · Oxidative stability · Antioxidant · Oil stability

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

J. K. Winkler-Moser (✉) · S. F. Vaughn
National Center for Agricultural Utilization Research, Agricultural Research Service, USDA, 1815 N. University St., Peoria, IL 61604, USA
e-mail: Jill.Moser@ars.usda.gov

Abbreviations

DDG	Distillers dried grains
DDGD	Distillers dried grain oil distillate
α-T	α-Tocopherol
α-T3	α-Tocotrienol
γ-T	γ-Tocopherol
γ-T3	γ-Tocotrienol
δ-T	δ-Tocopherol
δ-T3	δ-Tocotrienol
SF	Steryl ferulates

Introduction

Distillers dried grains (DDG) are a major co-product of the dry-grinding process for ethanol fermentation from corn and other grains [1]. The principal market for this co-product at the moment is for animal feed because it is a valuable energy and protein source. We previously found that hexane, ethanol, and supercritical carbon dioxide (CO_2) extracts from corn DDG yielded oils with high levels of phytosterols, steryl ferulates, tocopherols and tocotrienols [2]. High contents of tocopherols, phytosterols, and policosanols were also found in hexane and supercritical CO_2 extracts from sorghum distillers grains [3]. Aside from these two studies, most of the available literature on DDG focuses on the impact of feed levels on animal nutrition. However, the phytochemicals in DDG oil are valued for their antioxidant activity and for various other health-promoting or disease-preventing actions. The high content of phytosterols is significant because consumption of phytosterols may lower blood cholesterol by blocking readsorption of cholesterol from the gut [4]. Numerous food products and supplements incorporating phytosterols have

recently developed to take advantage of this benefit. Tocopherols and tocotrienols each have four forms (α-, β-, γ-, δ-), which altogether comprise the family of compounds responsible for natural Vitamin E activity. Tocopherols are common in vegetable oils and are the primary antioxidants protecting most oils [5]. Tocotrienols are common in rice bran oil and palm oil, but are not abundant in most commercial vegetable oils. Tocotrienols have an antioxidant activity similar to tocopherols in bulk oil systems [6]. However, they are also gaining attention due to an increasing number of studies investigating the role that they may have in preventing cardiovascular heart disease and cancer, and in providing neuroprotection [7]. Steryl ferulates (ferulate phytosterol esters) are another group of compounds of particular interest in corn DDG oil. These compounds are composed of a mixture of phytosterols, mainly sitostanol and campestanol, each esterified to a ferulic acid moiety [8]. Steryl ferulates are not found in most vegetable oils except for rice bran oil, which contains a mixture of 24-methylene cycloartanyl-, cycloartenyl-, and campesteryl-ferulate, along with some other minor steryl ferulate components, altogether known as γ-oryzanol (or simply oryzanol) [8, 9]. Oryzanol and individual steryl ferulates have been shown to have antioxidant activity due to the ferulic acid group [10, 11], as well as cholesterol lowering properties due to the phytosterol moiety [12, 13]. Carotenoid analysis was not included in the first two studies of phytochemicals in DDG oil [2, 3], but Moreau et al. [14] showed that levels of β-carotene, β-cryptoxanthin, lutein, and zeaxanthin are higher in oil extracted from ground whole corn than from oil extracted from corn germ alone, so we expect that these carotenoids are also present in DDG oil. Beta-carotene and β-cryptoxanthin are precursors to Vitamin A [15]. Carotenoids have also been shown to have a number of beneficial physiological actions other than Vitamin A activity, including antioxidant activity, enhanced immune response, and chemoprotective activity against several types of cancer [15]. Lutein and zeaxanthin are both associated with reduced risk of cataracts and macular degeneration [16]. Beta-carotene and carotenoids have both antioxidant and prooxidant activity in vitro [17], and have also been shown to synergistically enhance the antioxidant activity of tocopherols and tocotrienols [6] in bulk oils and liposomes.

The value of ethanol co-products might be increased if the oil is extracted from DDG and the phytochemicals were concentrated or purified, especially since some of the components, i.e. the tocotrienols and the steryl ferulates, are not commonly found in most commercial vegetable oils. The remaining oil after removal of phytochemicals could be used for biodiesel production to produce two biofuels from one feedstock. In this study, phytochemicals from hexane extracted DDG oil were removed from the oil by molecular distillation. The distillate, which had concentrated phytosterols, steryl ferulates, as well as tocopherols, tocotrienols, and carotenoids, was added to vegetable oils to determine the impact of these components on oil oxidative stability.

Materials and Methods

Materials

Corn DDG were provided by Big River Resources (Burlington, IA, USA). The grains contained 10% moisture and 11% oil. Hexane extracted, alkali refined, bleached and deodorized soybean oil (SBO) and sunflower oil (SUN), with no added antioxidants were obtained from a commercial processor. The high-oleic sunflower oil (HOSUN) was mechanically expeller-pressed from Spectrum Organic Products, Inc. (Petaluma, CA, USA), and was purchased at a local supermarket. Tocopherol standards (≥95% purity) and stigmasterol were purchased from Matreya, Inc. (Pleasant Gap, PA, USA). Tocotrienol standards (≥95% purity) were purchased from EM Science (Gibbstown, NJ, USA). Gamma-oryzanol (≥98% purity) was purchased from CTC Organics (Atlanta, GA, USA). Campesterol was purchased from Steraloids (Newport, RI, USA). Sitosterol and sitostanol were from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Each phytosterol standard was ≥97% purity. *N*,*O*-Bis(trimethylsilyl)fluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) was purchased from Regis (Morton Grove, IL, USA). Beta-carotene and *tert*-butylhydroquinone (TBHQ) was obtained from Sigma. Lutein (xanthophyll), zeaxanthin, and β-cryptoxanthin were purchased from the Indofine Chemical Co. (Hillsborough, NJ, USA). All solvents used for extractions and for high performance liquid chromatography (HPLC) were HPLC-grade.

Methods

DDG Oil Extraction and Molecular Distillation

Oil was extracted from DDG using hexane in a Soxhlet extractor as previously described [2]. The DDG oil was subjected to molecular distillation using a short-path thin film evaporator unit (Incon Processing, Batavia, IL, USA) equipped with a high-vacuum pump and a diffusion pump, a Neslab (Thermo Scientific, Newington, NH, USA) model RTE-140 re-circulating chiller filled with ethylene glycol and set to 30 °C, Julabo (Vista, CA, USA) model SE-6 re-circulating heated bath filled with Thermal H (Julabo) bath fluid, and an Ika Works, Inc. (Wilmington, NC, USA) model RW20 digital mechanical overheard stirrer set to 350 rpm. In the first few passes, the objective was to distill

free fatty acids but to minimize the loss of tocopherols, tocotrienols, and phytosterols. Using only the high-vacuum pump, vacuum pressures of 50–100 mTorr were achieved. The temperature of the first pass was 85 °C, the second pass was conducted at 100 °C followed by two passes at 120 °C which appeared to remove most free fatty acids. The final pass was conducted with additional vacuum pressure achieved with the diffusion pump <1 mTorr, and at a temperature of 250 °C, which was known from previous studies [18] to remove 85–97% of the tocopherols and phytosterols. The distillate from the final pass was designated as DDGD, for "distillers dried grain oil distillate". the DDGD was transferred to a vial with argon in the headspace and was kept frozen at −20 °C.

The commercial SBO, SUN, and HOSUN were also subjected to molecular distillation to remove the tocopherols and phytosterols. These oils were subjected to one pass at 250 °C at 100 mTorr to de-gas the oils, followed by a second pass at 250 °C and vacuum <1 mTorr. This procedure removed 80% of the phytosterols and 99% of the tocopherols [18]. These oils, which are referred to as "stripped", were kept in brown screw-cap bottles, the headspace was covered with argon and they were stored at −80 °C. Prior to OSI and storage studies, the peroxide value of each oil was determined to assure that the oils did not have any prior oxidation.

Analysis of Oils and DDGD

Acid Value and Fatty Acid Composition

The acid values of the DDG oil and residues following molecular distillation were determined in duplicate by AOCS method Cd 3d-63 [19]. Fatty acid methyl esters (FAME) were prepared using the method of Ichihara et al. [20]. FAME were analyzed in triplicate by GC as previously described [18].

Tocopherol and Phytosterol Content

Tocopherols, tocotrienols, and steryl ferulates were analyzed in triplicate by HPLC with a combination of UV and fluorescence detection as previously described [2]. Phytosterols were analyzed in triplicate after saponification, extraction, and derivatization as previously described [2]. Phytosterols were manually injected onto a Varian 3400 GC equipped with an FID, and a Supelco (Bellefonte, PA, USA) SPB™-1701 (30 m × 0.25 mm × 0.25 μm) capillary column. Helium was used as a carrier gas, with a 1:50 injector split. The injector temperature was 270 °C, and detector temperature was 290 °C. The column oven initial temperature was 250 °C for 0.5 min, increased at 10 °C/min to 270 °C and held for 27 min, then increased at 10 °C/min to 280 °C and held for 3.5 min. Data collection and integration were performed using Varian Star Chromatography Software Ver. 5.3. Phytosterols were identified by comparison of their retention times (relative to the internal standard, 5α-cholestane) with those of commercially available standards. Quantitation was carried out by the internal standard method developed with available standards. For phytosterols with no available commercial standard (avenasterol, cycloartenol, 24-methylene-cycloartanol, and citrostadienol) the response factor for β-sitosterol was used for quantitation. The identity of phytosterol peaks was confirmed by GC–MS analysis performed on an Agilent (Santa Clara, CA, USA) 6890 GC–MS equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm), a 5973 mass selective detector, and an 7683 autosampler. The transfer line from GC to the MSD was set to 280 °C. The injector and oven temperature programs were the same as described above for the GC–FID instrument. MSD parameters were as follows: scan mode, 50–600 amu, ionizing voltage, 70 eV, and EM voltage, 1,823 V. Mass spectral identification was performed using the Wiley MS database combined with comparison to literature values for relative RT (compared to β-sitosterol) and mass spectra [21].

Carotenoid Content

For determination of carotenoid content, DDG oil and DDGD were diluted to 50 mg/ml in 50:50 (v/v) methanol:methyl *tert*-butyl ether (MTBE), and then injected onto a Shimadzu (Columbia, MD, USA) HPLC system with an LC20AT HPLC pump, DGU-20A membrane degasser, SIL-10AF autosampler and SPD-M20A diode array detector. Separation and analysis of β-carotene was performed as described by Sass-Kiss et al. [22] on a YMC (Kyoto, Japan) C30 column (3 μm, 250 × 4.6 mm i.d.). The same column, but a different gradient system was used for separation and quantitation of the other carotenoids. This gradient consisted of 100% Solvent A (81:15:4 methanol:MTBE:H_2O) to 100% Solvent B (8.5:87.5:4 MeOH:MTBE:H_2O) in 45 min at 0.75 mL/min. Peaks were scanned from 200 to 550 nm, the detection wavelength was 450 nm. Carotenoids in the samples were identified by retention time compared to known standards as well as by spectral characteristics; quantitation was carried out by the external standard method. Carotenoid standards were all ≥95% pure, verified by HPLC. Stock solutions of carotenoids were prepared and the content was determined spectrophotometrically using published extinction coefficients [23]. Calibration curves were made using levels of 13.3 ng/ml to 1.3 μg/ml for β-carotene, and 62.5 ng/ml to 3 μg/ml for lutein, zeaxanthin, and β-cryptoxanthin.

Determination of Oxidative Stability Index

Oil samples were prepared on the day of analysis. DDGD was added to SBO, SUN, and HOSUN at levels of 0.5 and 1.0% by weight. DDGD was weighed into clean foil-covered vials and the appropriate weight of oil was added, then the mixtures were stirred at room temperature with a Teflon-coated stir bar on a magnetic stir plate at 950 rpm for 5 min. DDGD was added to SSOY, SSUN, and SHOSUN at levels ranging from 0.1 to 1% as described above. For samples with added α-tocopherol (α-T), the standard, which was a 50 mg/ml solution in hexane, was added to vials and the hexane evaporated under a stream of argon. The appropriate amount of oil was then added, and the samples were stirred at room temperature with a Teflon-coated stir bar at 950 rpm for 5 min. The OSI at 110 °C was determined in duplicate following the AOCS Official Method Cd 12b-92 [19]. A Metrohm (Herisau, Switzerland) 743 Rancimat with software control automatically controlled air flow and temperature and calculated the OSI values based on induction time.

Accelerated Storage Study

An accelerated storage study was conducted to see if DDGD improved the stability of SSUN, since this oil had the lowest oxidative stability according to its OSI. The treatments included the control, SSUN; DDGD added to the SSUN at either 0.1, 0.5, or 1% as described above; and a positive control, SSUN with 100 ppm of TBHQ. The storage study protocol followed the AOCS Recommended Practice Cg 5–97 [19]. Oil samples (2 g) were aliquoted into 10-ml glass scintillation vials which were loosely capped. For each treatment and day, three vials were prepared. Vials were stored in completely randomized order in a dark oven held at 60 ± 1 °C. Vials were removed after 1, 2, 4, 6, and 8 days of storage. Upon removal, vial contents were immediately topped with argon, tightly capped, covered with foil and frozen (−20 °C) until analysis. Analyses were conducted either on the same day or within 1 day of removal from the oven. Peroxide values of the oils were determined in duplicate using the ferric thiocyanate method as previously described [18]. Conjugated dienes were determined in duplicate on 0, 4, and 8 days as described by AOCS Official Method Ti 1a-64 [19]. Hexanal in the headspace was analyzed in duplicate by solid-phase microextraction (SPME) and GC analysis. Oil samples (100 ± 1 mg) were weighed into 2-ml amber snap-cap headspace vials. The following procedure for preheating, extraction, and injection was automated using a Varian Combi-Pal autosampler equipped with a preheating unit. Samples were preheated to 45 °C for 5 min while rotating at 250 rpm to equilibrate, then rotation was stopped and the septum was pierced with a SPME needle with a retractable 50/30 μm divinylbenzene/Carboxen™ on polydimethylsiloxane coated fiber (Supelco, Bellefonte, PA, USA). The fiber was exposed to the headspace for 15 min, then retracted and immediately injected and desorbed at 260 °C for 7 min in a Varian 3800 GC with helium as the carrier gas (1 ml/min), and an FID detector (280 °C). Volatiles were separated on a DB-5 (30 m × 0.25 mm i.d. 0.25 μm) column (Agilent, Santa Clara, CA, USA) using a temperature program of 60 °C for 1 min, 2 °C/min to 76 °C, then 20 °C/min to 240 °C and hold for 2 min. Injection was splitless until complete desorption (7 min) followed by split (1:50). Hexanal was identified by retention time. Peaks were integrated and analyzed using Varian Galaxy Chromatography Software. Standard curves using the same extraction and injection protocol were generated by dissolving known quantities of hexanal in fresh oil at levels from 0.1 to 100 μg/g. The standard curve had a correlation coefficient of 0.999 in this range. A standard containing 10 μg/g hexanal was injected after each batch of samples to verify the procedure and to ensure that the SPME fiber was maintaining the response factor.

Statistical Analysis

All data were imported into SAS for Windows version 9.1 (Statistical Analysis Systems Inc., Cary, NC, USA) for statistical analysis. The general linear models procedure (GLM) was used to conduct analysis of variance for the effect of treatment (DDGD or other added antioxidants) on OSI, PV, CD, and hexanal. Mean values for those variables were compared using Tukey's studentized range test. Treatment effects were considered significant when the p-value was <0.05.

Results

Isolation and Composition of DDGD

Molecular distillation has been used in our laboratory to remove native phytosterols and tocopherols from vegetable oils, leaving a residue of stripped vegetable oil triacylglycerols that are useful for studying the antioxidant efficacy of various compounds [18]. This technique has also been used to recover phytosterols and tocopherols from deodorizer distillate [24]. Corn DDG oil has high concentrations of phytosterols, steryl ferulates, tocopherols, and tocotrienols, compared to most commercial vegetable oils [2]. Therefore, it seemed that molecular distillation might also be useful as a solvent-free method for concentrating these valuable phytochemicals. The crude DDG oil initially had a high content of free fatty acids (FFA) (6.8%, data not

shown), which because of their lower molecular weight, would be co-distilled with other phytochemicals at the high temperature and vacuum conditions that are used for their removal. Therefore, the first three passes were performed to remove free fatty acids. At a vacuum pressure of 100 mTorr, temperatures ranging from 85 to 100 °C were not high enough to remove all of the free fatty acids from the DDG oil (data not shown). However, at a temperature of 120 °C, the content of free fatty acids in the residue was reduced to 0.7%. A second pass at 120 °C did not further reduce the remaining free fatty acids. Once the free fatty acids were removed, the temperature was increased to 250 °C and the diffusion pump was utilized to reduce the vacuum to ≤1 mTorr. The resulting distillate is referred to as DDGD, for distillers dried grain oil distillate. The starting oil weight for the final distillation described above was 24.4 g, and the DDGD yield was 7.96 g, or 36.6% of the starting weight.

DDGD is higher in phytosterols and steryl ferulates than DDG oil, while the tocopherol and tocotrienols content is similar to DDG oil, and the concentration of carotenoids in DDGD was lower than in the DDG oil (Table 1). The phytosterol content of DDGD, which includes the steryl ferulates, is 10–40 times higher than found in typical vegetable oils, and is 5.75-fold higher than in the DDG oil. The composition of phytosterols was also very similar to the DDG oil (2), thus was composed mainly of sitosterol (46%), sitostanol (18.4%), campesterol (12.4%), campestanol (7.4%), as well as smaller quantities of stigmasterol, avenasterol, cycloartenol, 24-methylene-cycloartanol, and citrostadienol. The phytosterol composition of corn DDG oil and DDGD is unique in that it is relatively high in the saturated sterols (phytostanols) sitostanol and campestanol, which are minor components in most other vegetable oils. The high content of sitostanol and campestanol are contributed mainly by the steryl ferulates, where these phytostanols seem to be preferentially bound in corn [25]. The steryl ferulate content increased from 3.8 mg/g in the DDG oil to 10.5 mg/g in DDGD (Table 1), however, the residue still contained some steryl ferulates that were not completely removed by distillation. In the HPLC method for determining steryl ferulate content, they elute as a single peak and are not separated by the sterol constituents, therefore we could not ascertain whether the composition of the steryl ferulates in DDGD were the same as in the DDG oil. However, considering that the total phytosterol composition was similar in both DDG oil and DDGD, we assumed that the steryl ferulate composition is likely also very similar, and that there was no preferential distillation based on the sterol constituents. The tocopherol and tocotrienol content was similar to the starting DDG oil (Table 1), and the relative composition of these components was also similar to the DDG oil, being mainly composed of α-T, α-tocotrienol (α-T3), as well as γ-tocopherol (γ-T) and γ-tocotrienol (γ-T3). Analysis of the distillates from the lower temperatures (85–120 °C) revealed that some of the tocopherols and tocotrienols were distilled off at these lower temperatures. However, after the distillation at 250 °C , no detectable tocopherols or tocotrienols remained in the residue.

Table 1 Phytochemicals in corn DDG oil and DDGD

Component	Amount	
	DDG Oil	DDGD
Phytosterols (mg/g)[a]	16.2 (0.7)	93.2 (3.2)
Steryl ferulates (mg/g)[b]	4.0 (0.0)	10.4 (0.3)
α-Tocopherol (μg/g)	194 (5)	273 (7)
α-Tocotrienol (μg/g)	155 (3)	241 (8)
γ-Tocopherol (μg/g)	948 (17)	910 (23)
γ-Tocotrienol (μg/g)	453 (3)	429 (12)
δ-Tocopherol (μg/g)	51 (2)	41 (2)
β-Carotene(μg/g)	4.5 (0.1)	0.97 (0.0)
Lutein (μg/g)	34.6 (1.8)	11.9 (0.9)
Zeaxanthin (μg/g)	23.3 (0.0)	14.2 (1.3)
β-Cryptoxanthin (μg/g)	3.9 (0.1)	4.0 (0.1)

Results shown are the average of triplicate measurements with the standard deviation in parentheses*DDG* dried distillers grains; *DDGD* dried distillers grain oil distillate

[a] Total phytosterols including free sterols, steryl-fatty acid esters and steryl ferulate esters as determined by saponification and GC analysis

[b] Steryl ferulates as determined by HPLC analysis, which contribute to the total phytosterol content shown in the row above

The carotenoid content of DDG oil was 66 μg/g oil (Table 1), and was mainly composed of β-carotene, lutein, zeaxanthin, and β-cryptoxanthin. The carotenoid content in DDG oil is 33 times higher than reported in oil extracted from corn germ using hexane, 5.5-fold higher than in hexane extracted corn fiber oil, and 2.5-fold higher than found in hexane extracted ground corn [14]. Content and composition of carotenoids in corn kernels vary significantly depending on the corn variety [26]. Thus, DDG oil is a good source for carotenoids, especially of lutein and zeaxanthin. Though all of the carotenoids did not distill over into the DDGD, probably because of their high molecular weights, its content of 31 μg/g is still higher than in most commercial oils.

These data indicate that molecular distillation could be used at a lower temperature to sequentially remove FFA from DDG oil, followed by higher temperatures for removal of desirable phytochemical components. Further work needs to be done so that this method can be optimized to a two-stage operation, and to improve the recovery of tocopherols, steryl ferulates, and carotenoids into the second distillate.

Effect of DDGD on Oil Oxidative Stability Index

DDGD was tested for its ability to increase the OSI of vegetable oils at 110 °C. The OSI is an accelerated method used to measure of the inherent resistance of an oil or fat to oxidation due to its degree of unsaturation, presence of natural or added antioxidants, or prior level of oxidation. We studied the effect of adding DDGD on the OSI of regular soybean (SBO), sunflower (SUN), and high-oleic sunflower oil (HOSUN), which vary in their content and composition of polyunsaturated fatty acids (Table 2) as well as tocopherols (data not shown). We also studied the effects of adding DDGD on the OSI of the same oils that had been subjected to molecular distillation to remove the native tocopherols and phytosterols: stripped soybean oil (SSBO), stripped sunflower oil (SSUN), and stripped high-oleic sunflower oil (SHOSUN). This is because these phytochemical components, mainly the tocopherols, have antioxidant activity, which could interfere with, or obscure, any activity of DDGD.

The initial peroxide value (in mequiv./kg oil) for the SBO was 0.1, SSBO was 0.3, SUN was 0.3, SSUN was 0.4, HOSUN was 4.0 and SHOSUN was 0.3. The high initial PV for the HOSUN indicates that this oil, which was minimally refined and purchased at a local grocery store, was slightly oxidized. However, the distillation treatment to strip the oil appears to have removed most of the peroxides. The addition of DDGD did not significantly affect the PV of the three oils (data not shown). When added to the SBO, SUN, and HOSUN, DDGD at levels of 0.5–1% had very little impact on the OSI (Table 3). The OSI of SBO improved from 8.48 to 8.76 h with 1% addition, which was statistically significant ($p < 0.05$) but is not practically significant, since it only represents about a 3% increase. However, DDGD increased the OSI of the SSBO, SSUN, and SHOSUN in a linear, dose-dependent fashion when added at 0.1–1% levels (Fig. 1). DDGD had greatest impact in SHOSUN, which was the most stable of the stripped oils due to its lower content of PUFA, while it had the least pronounced impact, though still significant, in SSUN. In practical terms, 1% DDGD essentially doubled the OSI of the stripped oils, so the impact is both practically and statistically significant. Stripping the oils of their native tocopherols reduced the OSI by 75–84%, thus, the addition of DDGD did not improve the OSI of the oils to the level of the native oils. Extrapolation of the dose–response curves in Fig. 1 indicate that 2.4% DDGD would improve the OSI of SHOSUN to the level of HOSUN; while 5.7 and 5.2% DDGD would be required to return the OSI of SSBO and SSUN, respectively, to the level of the unstripped oil. However, this assumes that the dose–response curve is still linear at higher concentrations.

Since DDGD improved the OSI of the stripped oils, it was expected that it would also improve the OSI of the unstripped oils, unless the components in DDGD act antagonistically with the native tocopherols or some other components in the oil. However, DDGD had little impact on the OSI of the unstripped oils, as seen in Table 3. Therefore, in order to better determine the interaction between DDGD and tocopherols, an experiment was designed whereby the OSI of SHOSUN was determined either alone, with α-T (the major tocopherol component in HOSUN), with DDGD, and with combined α-T and DDGD (Fig. 2). Two different levels of α-T were used: 950 μg/g was chosen because this is approximately the original content of total tocopherols in the HOSUN [18], of which about 90% were α-T; 500 μg/g was chosen as a slightly lower level to see if there was any influence of concentration on the interaction. The OSI of SHOSUN was 3.0 h, but was increased to 6.4 h by adding 0.5% DDGD, to 13.5 h by adding 500 μg/g α-T, and to 14.4 h by 950 μg/g α-T (Fig. 2). The OSI of SHOSUN with the addition of 950 μg/g α-T is similar to the OSI of HOSUN (Table 3). The OSI of the SHOSUN with the added combination of DDGD and α-T was about 97% of the expected OSI based on the fraction of improvement that each individual component contributed (see "expected" values in Fig. 2).

Table 2 Fatty acid composition (%) of oils

	SBO	SSBO	SUN	SSUN	HOSUN	SHOSUN
16:0	11.6	11.3	6.2	6.1	3.8	4.0
18:0	4.4	4.4	4.4	4.5	3.3	3.3
18:1	25.1	25.3	25.8	25.8	80.9	80.4
18:2	52.2	52.3	63.4	63.4	11.6	12.3
18:3	6.7	6.7	0.2	0.2	0.4	0.0

SBO soybean oil; *SSBO* stripped soybean oil; *SUN* sunflower oil; *SSUN* stripped sunflower oil; *HOSUN* high-oleic sunflower oil; *SHOSUN* stripped high-oleic sunflower oil. Results are the average of triplicate measurements for each oil. Coefficient of variation was <5% for all values

Table 3 Oxidative stability index (OSI) of soybean, sunflower, and high-oleic sunflower oils blended with DDGD

	OSI (h) for:		
	SBO	SUN	HOSUN
Control (no additive)	8.5 (0.1)[b]	5.5 (0.01)[a]	14.7 (0.04)[a]
0.5% DDGD	8.8 (0.15)[a]	5.7 (0.26)[a]	14.4 (0.04)[a]
1% DDGD	8.8 (0.02)[a]	5.7 (0.06)[a]	14.8 (0.26)[a]

Results are the average of two measurements for each treatment, standard deviations are shown in parentheses

SBO soybean oil; *SUN* sunflower oil; *HOSUN* high-oleic sunflower oil; *DDGD* distillers dried grain oil distillate

Within each column, mean OSI values with the same superscript letter were not significantly different from each other

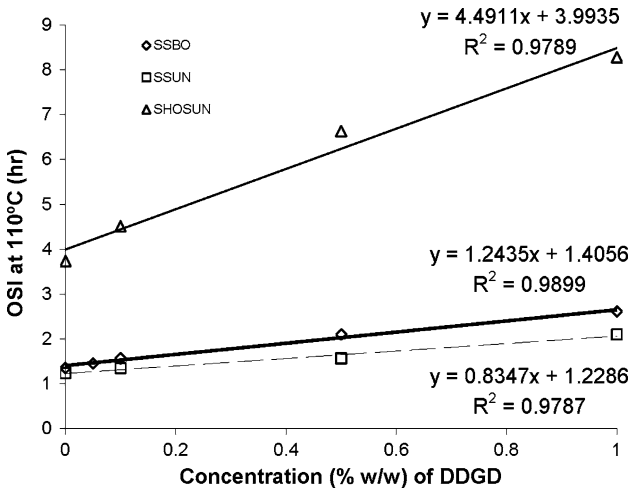

Fig. 1 Impact of added DDGD on the oxidative stability index (OSI) of stripped soybean (SSBO), stripped sunflower (SSUN), and stripped high-oleic sunflower (SHOSUN) oils. Each data point shows the average of two OSI measurements for each treatment

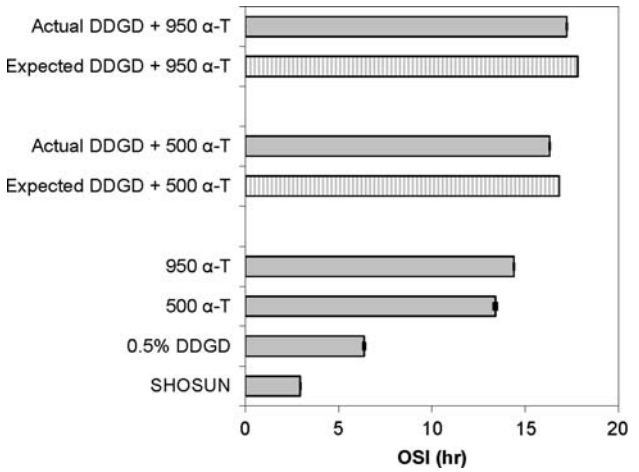

Fig. 2 Interaction between DDGD and α-tocopherol in the improvement of the oxidative stability index (OSI) of stripped high-oleic sunflower oil (SHOSUN). From the *bottom* of the graph, the first set of *bars* shows the OSI for SHOSUN alone, SHOSUN with added 0.5% DDGD, SHOSUN + 500 μg/g α-T, and SHOSUN + 950 μg/g α-T. In the second (*middle*) set of *bars*, the expected OSI for SHOSUN + 0.5% DDGD and 500 μg/g α-T, based on the partial increase in OSI contributed by each component alone, is compared with the measured OSI. In the final (*top*) set of *bars*, the expected OSI for SHOSUN + 0.5% DDGD and 950 μg/g α-T is compared with the measured OSI. *Bars* show the average (±SD) of two measurements for each treatment. Error calculations were not made for theoretical data

There was no difference in this percentage based on the two different tocopherol concentrations. Unlike the addition of DDGD to the unstripped oils, the combination of DDGD and α-T in SHOSUN improved the OSI significantly compared to either additive alone. Thus, an antagonistic interaction between DDGD and tocopherols does not seem to explain the reason for the reduced activity of DDGD in the unstripped oils.

Storage Study

The impact of DDGD on oxidation of SSUN during an accelerated storage study at 60 °C was also determined. SSUN was chosen since its OSI was least affected by DDGD concentration, as determined by the slope of OSI increase over DDGD concentration in Fig. 2. During storage, lipid hydroperoxides and conjugated diene hydroperoxides, which are produced during the primary phase of lipid oxidation [17], were analyzed (Figs. 3, 4). Lipid peroxides decompose to form short chain fatty acids, aldehydes, and ketones, which are considered secondary oxidation products [17]. Hexanal, which is primarily produced from the decomposition of linoleic acid hydroperoxides, was analyzed as an indicator of secondary products of lipid oxidation in stripped sunflower oil (Fig. 5).

The initial peroxide value of the SSUN and SSUN with added DDGD or TBHQ ranged between 0.4 and 0.5. The PV of SSUN increased after 1 day storage with no apparent lag period, then the PV started to decline after 4 days storage, indicating that the rate of peroxide decomposition was beginning to exceed the rate of peroxide formation. SSUN with added 0.1% DDGD had a significantly lower PV than SSUN after 1 day storage, but it also had no lag period, and in subsequent days, followed the same pattern for peroxide formation as SSUN. In SSUN with 0.5% DDGD, peroxide formation showed a lag period of 1 day,

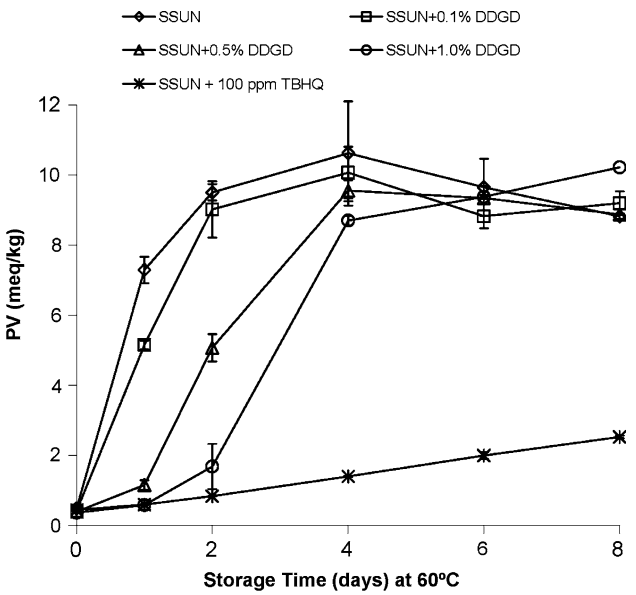

Fig. 3 Peroxide value (PV) of stripped sunflower oil with added DDGD or TBHQ during storage at 60 °C. Each data point represents the average (±SD) PV for the three replicate samples ($n = 3$), each replicate was measured for PV in duplicate

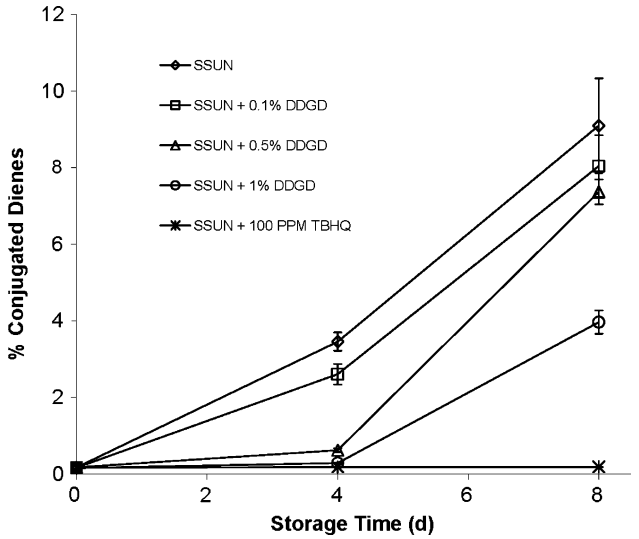

Fig. 4 Conjugated diene (CD) analysis of stripped sunflower oil with added DDGD or TBHQ during storage at 60 °C. Each data point represents the average (±SD) CD for the three replicate samples ($n = 3$), each replicate was measured for CD in duplicate

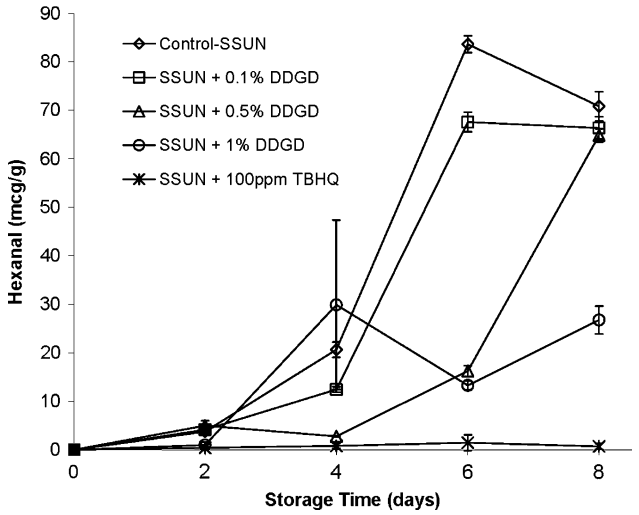

Fig. 5 Hexanal formation in oils with added DDGD or TBHQ during storage at 60 °C. Each data point represents the average (±SD) hexanal content for the three replicate samples ($n = 3$), each replicate was measured for hexanal in duplicate

then increased rapidly to maximum PV after 4 days storage, after which the PV declined. In SSUN with 1% DDGD, the lag period was increased to around 2 days, then rapidly increased between 2 and 4 days. However, peroxide formation continued to slowly increase in SSUN with 1% DDGD from 4 to 8 days of storage, indicating that at this level, DDGD may have not only slowed peroxide formation, but also slowed the rate of peroxide decomposition. CD increased in all samples between 0 and 8 days storage, but samples with DDGD (at all levels) had significantly lower CD than in SSUN alone at 4 days. After 8 days, only SSUN with 1% DDGD was significantly lower than SSUN. In SSUN with added TBHQ, the rates of PV increase were much lower than in all other samples, and CD were seen to increase only slightly after 8 days storage.

There was no detectable hexanal in any of the samples on day 0. Hexanal content increased most in the control SSUN and the SSUN + 0.1% DDGD between 2- and 6-day storage, after which hexanal content either leveled off or decreased slightly. This trend seems to correlate with the drop in PV accumulation after 4-day storage in these samples. The hexanal content of SSUN + 0.1% DDGD was significantly lower than in SSUN except for 0 and 2-day. The lag period for hexanal production in SSUN with 0.5% DDGD was extended until 4 days storage, and hexanal was significantly lower than both SSUN and SSUN + 0.1% DDGD on days 4 through 8. In SSUN with 1% DDGD, the average hexanal content at 4 days storage was higher than in all other samples due to high content measured in two out of the three replicate samples, which also accounts for the high standard deviation. Repeated measurement confirmed that the values were indeed, correct. However, SSUN + 1% DDGD was significantly lower in hexanal content on days 2, 6, and 8 compared to SSUN and SSUN with 0.1 or 0.5% DDGD, indicating that 1% DDGD seemed to be the most effective concentration for slowing hexanal formation. The reason for the spike in hexanal content in 4-day samples is unknown, but may be due to contamination of the sample vials or perhaps due to a hot spot in the oven. However, the variation in these samples was not detected by either the PV or CD analysis conducted within the same time frame.

Possible Components Contributing to DDGD Antioxidant Activity

DDGD has several components that may be contributing to the antioxidant activity seen in the OSI tests and storage study, including tocopherols, tocotrienols, carotenoids, and steryl ferulates. Tocopherols are common in most vegetable oils and are well-known for their antioxidant activity [27]. In bulk oil systems, γ-T and δ-T are usually found to be better antioxidants than α-T [27], but these observations appear to be dependent on the concentration of each individual component. DDGD has 1.2 μg/mg total tocopherols, of which about 75% are composed of γ-T, 22.5 are α-T, and 2.5% are δ-T. At the levels of DDGD addition that were used in this study, the resulting tocopherol content in the oils ranged from 1.2 to 12 μg/g. This is lower than the effective concentrations usually observed and tested for tocopherols (50–1,000 μg/g), however, levels as low as 5 μg/g have been observed to have antioxidant activity in stripped rapeseed oil triacylglycerols [28].

Tocotrienols are common in rice bran oil and palm oil, but are not abundant in most other vegetable oils. Total tocotrienol content of DDGD is 0.67 μg/mg, of which 64% is γ-T3 and 36% is α-T3. Tocotrienol concentrations in the oils with added DDGD ranged from 3 to 6 μg/g, which again, is 10–100-fold lower than effective concentrations used in most studies of tocotrienol antioxidant activity in bulk lipids or emulsions [6, 29]. However, several studies have shown that tocopherols and their respective tocotrienols have similar antioxidant activity [6, 29], therefore, tocotrienols may also have antioxidant activity at 5–10 μg/g.

There was a total of 66 μg/g carotenoids in the DDG oil, but most of these did not carry over into DDGD during distillation, resulting in less than half the concentration (31 μg/g) in DDGD. Carotenoids have been shown to have both antioxidant and prooxidant activity, and have also been shown at concentrations of about 100 μg/g to act synergistically with tocopherols and tocotrienols to extend the induction period for conjugated diene formation in phospholipid liposomes [6]. In this study, dilution of DDGD in the oils resulted in carotenoid content lower than 0.3 μg/g, which may have been too low to contribute much towards the antioxidant activity.

Steryl ferulates were present in DDGD at 10.2 μg/mg, which is about 2.5-fold higher than in the original DDG oil, and 5.5-fold higher than the tocopherol and tocotrienol content. Steryl ferulates are not typically found in most vegetable oils, but a mixture known as γ-oryzanol is present in rice bran oil (commercially refined) at concentrations ranging from 3 to 8 μg/mg [9]. Oryzanol and individual steryl ferulates have been shown to have antioxidant activity by a variety of tests [10, 11]. In a study similar to the present storage study, oryzanol, sitosteryl ferulate, and cycloartenyl ferulate at concentrations ranging between 0.3 and 1.5 μg/mg inhibited peroxide formation in bulk methyl linoleate stored at 40 °C [10]. In the present study, dilution of DDGD in SSUN resulted in steryl ferulate concentrations ranging between 0.01 and 0.1 μg/mg.

The depletion of tocopherols, tocotrienols, and steryl ferulates in the storage study samples was followed (Fig. 6) in order to investigate their possible contribution to the antioxidant activity of DDGD. Carotenoid levels were too low, even at the 1% addition level to follow their depletion. At the addition level of 0.1% DDGD, only γ-T and the steryl ferulates were in a detectable range; α-T and α-T3 as well as γ-T3 were below levels of detection by our HPLC system. At all three DDGD concentration levels, the tocopherols and tocotrienols were completely depleted before the steryl ferulates. Rather than gradually depleting over time, in most cases the tocopherols, tocotrienols and steryl ferulates remained within 90–100% of their original level

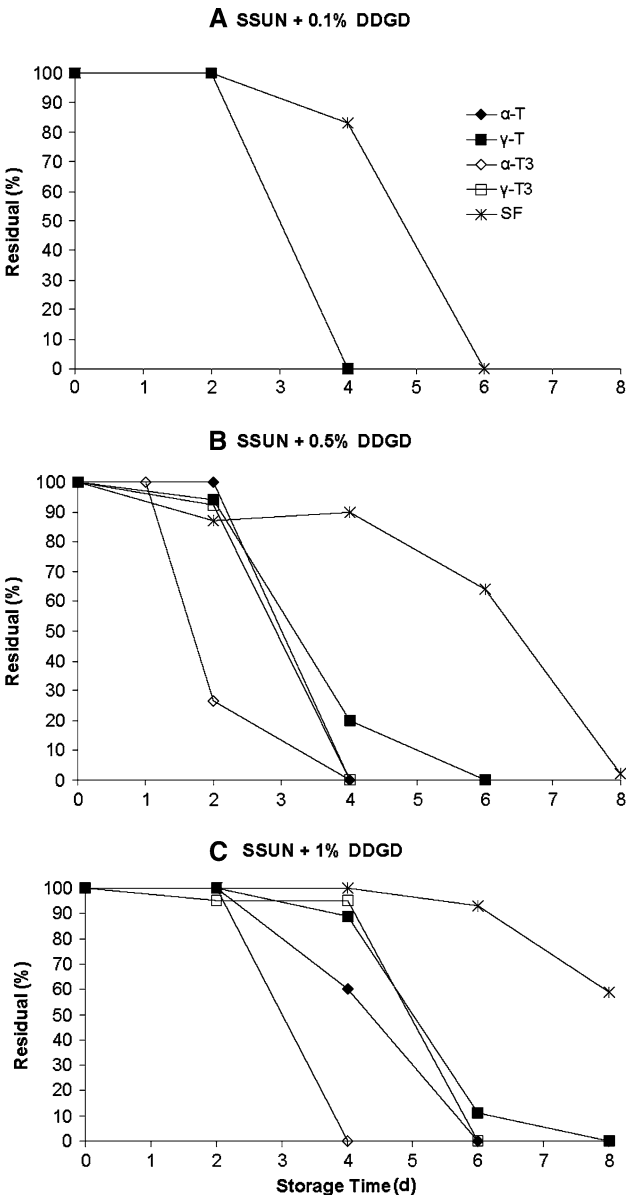

Fig. 6 Depletion of antioxidant components in SSUN with added DDGD (**a** 0.1%, **b** 0.5%, and **c** 1%) during storage at 60 °C. *SF* steryl ferulates, see text for other abbreviations. Each point shows the average retention (% of original content) for the three replicate samples

for a lag period followed by rapid decomposition. At the 0.5% level of DDGD addition, this lag period was 1 day for α-T3, 2 days for α-T, γ-T, and γ-T3, and 4 days for SF. At the 1% level, the lag period was 2 days for α-T and α-T3, 4 days for γ-T, and γ-T3, and 6 days for SF. Comparing α-T and α-T3, which were present at similar concentrations, it appears that α-T was slightly more stable than α-T3. Gamma-tocopherol lasted longer than the other tocopherols and tocotrienols, but this may partially be due to the fact that it was present at higher concentrations. The fact that with increasing DDGD concentration there was an

increase in the lag period for tocopherol, tocotrienol, and steryl ferulate depletion concomitant with an increased lag period for PV, CD, and hexanal formation, indicates that despite their relatively low concentrations, all three components contributed to the antioxidant activity of DDGD. The exact contribution of each component and possible synergisms would be impossible to elucidate under these conditions. However, the fact that the tocopherols and tocotrienols were degraded much faster than the steryl ferulates indicates that the tocopherols may have protected the steryl ferulates from degradation. This would be consistent with the study by Nyström et al. [30], who found that α-T degraded more rapidly in heated (100 and 180 °C) purified (tocopherols removed) high-oleic sunflower oil when it was combined with sitosteryl ferulate, compared to the degradation rate of α-T when tested alone. Sitosteryl ferulate, on the other hand, degraded more slowly in combination with α-T than when tested alone. The results of this study indicate that components extracted from DDG oil have antioxidant activity when added to stripped vegetable oils, even when added at very low concentrations. While the depletion of tocopherols, tocotrienols, and steryl ferulates indicates that they participated in the antioxidant action of DDGD, there may be some other unidentified components present in DDGD that contribute to its antioxidant activity.

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