Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

A Simple Two-Step Method for Simultaneous Isolation of Tocopherols and Free Phytosterols from Soybean Oil Deodorizer Distillate with High Purity and Recovery

Novy S. Kasim[a]; Setiyo Gunawan[ab]; Maria Yuliana[a]; Yi-Hsu Ju[a]
[a] Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan [b] Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Kampus ITS Keputih Sukolilo, Surabaya, Indonesia

Online publication date: 29 November 2010

To cite this Article Kasim, Novy S. , Gunawan, Setiyo , Yuliana, Maria and Ju, Yi-Hsu(2010) 'A Simple Two-Step Method for Simultaneous Isolation of Tocopherols and Free Phytosterols from Soybean Oil Deodorizer Distillate with High Purity and Recovery', Separation Science and Technology, 45: 16, 2437 — 2446
To link to this Article: DOI: 10.1080/01496391003789171
URL: http://dx.doi.org/10.1080/01496391003789171

Separation Science and Technology, 45: 2437–2446, 2010

ISSN: 0149-6395 print/1520-5754 online
DOI: 10.1080/01496391003789171

Taylor & Francis
Taylor & Francis Group

A Simple Two-Step Method for Simultaneous Isolation of Tocopherols and Free Phytosterols from Soybean Oil Deodorizer Distillate with High Purity and Recovery

Novy S. Kasim,[1] Setiyo Gunawan,[1,2] Maria Yuliana,[1] and Yi-Hsu Ju[1]
[1]*Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan*
[2]*Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Kampus ITS Keputih Sukolilo, Surabaya, Indonesia*

Most of the methods reported for isolating phytosterols and tocopherols from soybean oil deodorization distillate (SODD) involved complicated steps or failed to obtain high content and recovery. In this study, we succeeded in isolating α-, δ-, γ-tocopherols, and free phytosterols (campesterol, stigmasterol, and β-sitosterol) from SODD with high content and recovery. Our protocol involved a simple two-step method. SODD was separated into non-polar and polar fraction by modified soxhlet extraction. Cold saponification was chosen instead of conventional saponification which is carried out at a temperature no lower than 60°C. This study of cold saponification was applied to the polar fraction to remove free fatty acids and acylglycerols. SODD contains 9.13 ± 0.28% tocopherols and 9.75 ± 0.12% free phytosterols, was converted to a final product which contains 38.08 ± 0.36% tocopherols and 55.51 ± 0.56% free phytosterols. The total recovery of tocopherols and free phytosterols is 94 ± 0.19%.

Keywords cold saponification; modified soxhlet extraction; phytosterol; soybean oil deodorizer distillate; tocopherol

INTRODUCTION

Phytosterols and tocopherols are valuable bioactive compounds. Phytosterols have many applications in the food industry and in medicine because of their serum cholesterol level-lowering effect (1,2). They are found in cosmetics as an alternative way for the phytosterols intake into the human body (3). Tocopherols have vitamin E activity and are natural antioxidants and have been used in supplements as nutraceuticals or functional foods. Recently, a modified form of tocopherols, e.g., d-α -tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS) has been used as a novel emulsifier as well as a novel material when blended with nanoparticles of biodegradable polymers for the formulation of anticancer drugs as well as protein drugs (4,5). Soybean oil deodorizer distillate (SODD) is a side product from refined soybean oil with a total tocopherols and free phytosterols content of *ca.* 20%. Three kinds of free phytosterols were found in SODD: campesterol, stigmasterol, and β-sitosterol (6). Four kinds of tocopherol compounds were also found in SODD: δ-tocopherol, γ-tocopherol, α -tocopherol, and a trace amount of β-tocopherol (6).

SODD has a high content of bioactive compounds (18 ∼ 32 wt%). It is a promising starting material for isolating squalene, fatty acid steryl esters (FASEs), tocopherols, and free phytosterols. Past studies have reported several methods for isolating either tocopherols or free phytosterols from SODD. Martins et al. (7) succeeded in removing 96.16% free fatty acids (FFAs) and isolating tocopherols. They reported a 81.23% recovery by molecular distillation at 160°C and a feed flow rate of 10.4 g min^{-1}. This flow rate is too low for commercial production and scale-up is still the main obstacle for its commercial application. Fizet (8) esterified phytosterols with FFAs into FASEs, and distilled the reaction product to obtain residue containing tocopherols and FASEs. The residue was distilled again to obtain tocopherols in the distillate and FASEs in the residue. FASEs were transformed back into free phytosterols by an acid-catalyzed transesterification. Watanabe at al (9). applied short-path distillation to concentrate tocopherols and free phytosterols in SODD. Free phytosterols and acylglycerols were transformed into FASEs and fatty acid methyl esters, respectively. The reaction mixture was distilled to obtain a product that contains 76.4% tocopherols (89.6% recovery) and 97.2% FASEs (86.3% recovery). Torres et al. (10) used a two-step enzymatic process to obtain sterol esters, tocopherols, and fatty acid ethyl esters (FAEEs) from SODD. Free phytosterols were esterified to become FASEs (90% conversion) in 5 h; FFAs were

Received 23 November 2009; accepted 18 March 2010.
Address correspondence to Yi-Hsu Ju, Department of Chemical Engineering, National Taiwan University of Science and Technology. 43, Sec. 4, Keelung Road, Taipei 106-07, Taiwan. Tel.: +886 2 27376612; Fax: +886 2 27376644. E-mail: yhju@mail.ntust.edu.tw

esterified to become FAEEs (>95% conversion). The final products were used as starting materials for the purification of FASEs, tocopherols, and FAEEs via supercritical fluid extraction.

We reported (11,12) isolating squalene and FASEs from SODD, both in high content and recovery. One key principle to separate bioactive compounds is by exploiting the difference in their polarities. This is best done by modified soxhlet extraction (MSE). The main advantage of the MSE method is that by coating SODD onto silica gel, higher selectivity can be achieved than that of liquid-liquid extraction. MSE is a simple and promising method for the isolation of nonpolar lipid fraction (NPLF) and polar lipid fraction (PLF) from SODD. Under optimized MSE operational conditions, we have successfully concentrated squalene and FASEs in NPLF while FFAs, acylglycerols, tocopherols, and free phytosterols were concentrated in PLF. MSE is an efficient isolation technique. It only requires a small amount of solvent and is performed at atmospheric pressure. In this study, we report a simple method for obtaining tocopherols and free phytosterols from SODD with high content and recovery by combining MSE and cold saponification techniques. An important objective of this study is to reduce FFAs content in the final product to less than 0.05%. The identification of compounds other than tocopherols and free phytosterols in the final product was also investigated.

EXPERIMENTAL SECTION

Materials and Reagents

SODD was donated by TTET Union Corporation (Tainan, Taiwan). 1-Oleoyl-rac-glycerol, cholesteryl stearate, δ-tocopherol, and decanal standards were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Dioleylglycerol, linoleic acid, and stigmasterol standards were supplied by Acros Organics (New Jersey, USA). Tripalmitin standard was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Potassium hydroxide (KOH) solution 1 N in ethanol was obtained from Fluka Analytical (Steinheim, Germany). Reagents used in the isolation of free phytosterols and tocopherols were of industrial grade and those used in the analyses (GC and TLC) were of either HPLC grade or analytical grade. Silica gel (pore size: 60 Å, 70–230 mesh) was purchased from Silicycle (Quebec, Canada) while analytical TLC silica gel plates (0.25 mm thickness, pore size: 60 Å, specific surface: 480–540 m^2/g) were obtained from Merck KGaA.

Isolation of PLF From SODD

Figure 1 shows the flow chart for the isolation of tocopherols and free phytosterols from SODD. Foreign particulates were removed from SODD at 50°C by using a 7 μm Advantec filter paper (Toyo Roshi Kaisha Ltd., Tokyo, Japan). Silica gel was activated by heating in an oven at 150°C for 1–2 h prior to use. A soxhlet extractor, modified by constructing an additional jacket outside the extraction chamber and is connected to a condenser system, was set up as described by Gunawan et al. (11). This design allowed the temperature inside the extraction chamber to be controlled easily. SODD (20 g) was dissolved in hexane (150 mL). Activated silica gel (60 g) was added to this solution. The mixture was magnetically stirred at 300 rpm, 25°C for one hour. SODD was coated onto the silica gel by removing the hexane at 60°C and 160 mmHg. The SODD-loaded silica gel was then packed into an extraction thimble. The top surface of the thimble was covered with cotton to prevent spillage. A thermocouple was inserted into the thimble and the thimble was placed inside the soxhlet extractor. Less-polar lipids were extracted with hexane (350 mL) for approximately 12 h. The temperature inside the extraction chamber was set at −6°C. Ethyl alcohol flowing through the jacket was the refrigerant in the refrigerated circulating bath. The hexane extract was referred to as the NPLF. More polar lipids, still adsorbed on the silica gel, were then extracted with ethyl acetate for 3 h at 71 ± 1°C. This extract was designated as the PLF.

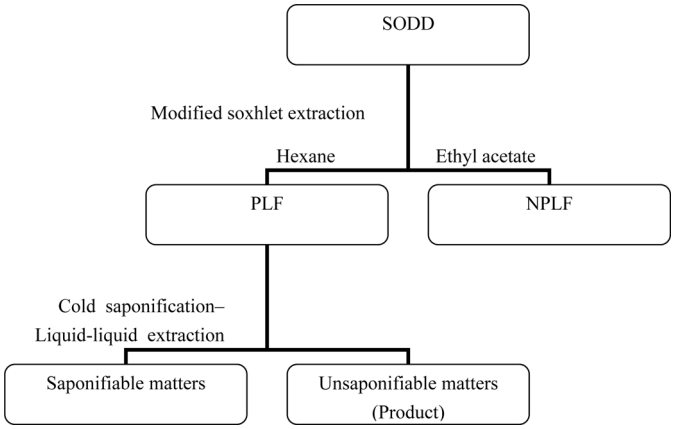

FIG. 1. Flow diagram of tocopherols and free phytosterols isolation.

Isolation of Tocopherols and Free Phytosterols from PLF

The PLF was subjected to cold saponification to remove FFAs and acylglycerols. The method used is based on the American Society for Testing and Materials (ASTM) D 5553 test method (13) with some modifications as this standard method describes a conventional saponification carried out at a temperature no lower than 60°C. The reactions (cold saponification) were carried out in a closed system under nitrogen atmosphere with a magnetic stirrer (400 rpm) at various temperatures (25, 40, and 50°C). Ascorbic acid was tested for its effectiveness in preventing the decomposition of tocopherols in cold saponification.

The ratios of PLF-to-KOH solution (1 N) investigated in this study were 1:5, 1:12.5, and 1:25 (g/mL).

GC Analysis

A Shimadzu GC-2010 equipped with a split/splitless injector and a FID was used. The fused silica column used was a DB-5HT (15 m × 0.32 mm i.d., 0.1 μm film thickness, Agilent Tech. Palo Alto, CA). The initial column temperature was 80°C and then was raised to 180°C at 15°C/min, to 185°C at 5°C/min, to 215°C at 15°C/min, to 250°C at 2°C/min, to 325°C at 15°C/min, to 365°C at 5°C/min, and held at 365°C for 6.83 min. The total analysis time is 51 min. The temperature of the injector and detector was 370°C. The linear velocity of N_2 at 80°C was 30.0 cm/sec.

External standard calibration curves were obtained by using 0.2–20 mg pure standards. Regression analysis of every calibration curve was further carried out to obtain a correlation coefficient between peak area and concentration of standard. This correlation coefficient was then termed as the calibration factor. Cholesteryl stearate was selected for the determination of the FASEs' calibration factor and was used for all FASEs. The calibration factor of squalene, stigmasterol, γ-tocopherol, monooleylglycerol, diolein, and tripalmitin was used to quantify squalene, free phytosterols, tocopherols, monoacylglycerols (MAGs), diacylglycerols (DAGs), and triacylglycerols (TAGs), respectively.

TLC Analysis

For TLC analysis, a specific reagent used to detect free phytosterol spot, was prepared by dissolving 50 mg $FeCl_3 \cdot 6H_2O$ in 90 mL H_2O, along with 5 mL acetic acid and 5 mL sulfuric acid. The desired spot will yield a red-violet color. The mobile phase used in the TLC analyses was HPLC grade *n*-hexane/ethyl acetate/acetic acid (90/10/1 v/v/v). The reagent was sprayed onto the TLC plate, and then the plate was heated at approximately 100°C until the desired spot appeared. The spot was also observed by ultraviolet light at 366 nm as confirmation.

Statistical Analysis

The reliability of the results was checked by statistical analyses. Standard deviation of the measures (SD) was calculated from the difference between the value of the individual experiment and the mean value of the three independent experiments.

The differences in mean values were evaluated by analysis of variance (ANOVA) followed by Fisher's LSD (Least Significant Difference) method and cross-checked by using Tukey's method. Differences associated with $p < 0.05$, based on a two-tailed test, were considered significant. ANOVA test is carried out by assuming that the errors have normal distribution i.e., $N(0, \sigma^2)$. Normality assumption was checked using a normal probability plot of the studentized residuals (14) and showed that the errors distribution are approximately normal (data not shown), which means the assumption is not violated.

RESULTS AND DISCUSSION

Isolation of PLF From SODD

The SODD used in this study was a brownish semi-solid at room temperature. The objective of MSE was to separate polar components in SODD from non-polar ones by adjusting the mass ratio of silica gel-to-SODD and the extraction temperature.

The effects of silica gel-to-SODD mass ratio and extraction temperature on separation were reported by Gunawan et al. (11). The mass ratio of silica gel-to-SODD used in the study was 3, 4, 5, or 6; the extraction temperature used in the isolation of NPLF was 65°C, 55°C, 7°C, 3°C or −6°C. The extraction temperature applied in isolating PLF was 60–70°C. Higher silica gel-to-SODD mass ratio resulted in lesser amount of NPLF extracted, higher number of extraction cycles and longer extraction time to recover 100% squalene and >95% FASEs in NPLF. At higher silica gel-to-SODD mass ratio the larger adsorption area of silica gel resulted in higher adsorption capacity of more compounds onto silica gel, and thus more numbers of cycles and longer times are needed to extract all squalene and HCs, and the most FASE from the silica gel.

Lower silica gel-to-SODD mass ratio (less than 3) did not result in higher amounts of NPLF extraction due to insufficient adsorption area and less complete binding between compounds and silica gel. A successful separation between NPLF and PLF compounds could not be achieved. Gunawan (15) reported that because PLF adsorption onto silica gel is an exothermic process, an increase in extraction temperature resulted in the increase of the desorption rate of compounds in PLF; hence in poor separation of NPLF and PLF.

It was found that at a silica gel-to-SODD mass ratio of 3 and an extraction temperature of −6°C, a NPLF which recovered 100% squalene and 89.40% FASEs and a PLF which recovered 92.26% tocopherols and 99.54% free phytosterols can be obtained. Most FFAs (~80% recovery) and acylglycerols (~99% recovery) were concentrated in the PLF. The compositions of SODD, NPLF and PLF are shown in Table 1.

The MSE method is comparable to supercritical fluid extraction in terms of efficiency in isolating bioactive components from vegetable oil deodorization distillate. Vázquez et al. (16) modified sunflower oil deodorizer distillate (SuODD) into ethylated SuODD (60–70 wt% FAMEs and FAEEs) and then pre-concentrated tocopherols, free phytosterols, and FASEs by removing FAMEs and FAEEs via supercritical CO_2. They succeeded in recovering 98% tocopherols, 98% free phytosterols, and 97% FASEs in

TABLE 1
Compositions of SODD, NPLF, and PLF

Compounds	SODD (%)	NPLF (%)	PLF (%)
FFAs	41.15 ± 0.39	25.29 ± 0.30 (18.81 ± 0.49)[a]	48.14 ± 0.30 (81.19 ± 0.52)
Squalene	1.17 ± 0.13	3.84 ± 0.77 (100.00 ± 0.04)	ND[b]
MAGs	0.15 ± 0.01	ND	0.22 ± 0.01 (99.98 ± 0.01)
Total tocopherols	9.13 ± 0.28	2.31 ± 0.02 (7.74 ± 0.38)	12.14 ± 0.28 (92.26 ± 0.44)
δ-Tocopherol	2.41 ± 0.17	0.85 ± 0.23	3.10 ± 0.28
γ-Tocopherol	5.81 ± 0.01	1.03 ± 0.20	7.92 ± 0.13
α-Tocopherol	0.90 ± 0.10	0.41 ± 0.01	1.11 ± 0.13
Total free phytosterols	9.75 ± 0.12	0.15 ± 0.21 (0.48 ± 0.43)	13.98 ± 0.03 (99.54 ± 0.35)
Campesterol	2.57 ± 0.04	ND	3.70 ± 0.01
Stigmasterol	2.19 ± 0.02	ND	3.16 ± 0.03
β-Sitosterol	4.99 ± 0.06	0.16 ± 0.01	7.12 ± 0.01
DAGs	2.44 ± 0.03	0.30 ± 0.04 (3.80 ± 0.23)	3.39 ± 0.01 (96.20 ± 0.23)
FASEs	6.76 ± 0.08	19.75 ± 0.10 (89.40 ± 0.02)	1.06 ± 0.19 (10.90 ± 3.78)
TAGs	5.90 ± 0.01	ND	8.50 ± 0.41 (100.00 ± 0.02)
Others[c]	23.54 ± 0.84	48.35 ± 2.16 (62.86 ± 1.77)	12.56 ± 0.32 (37.04 ± 0.81)
Amount (g)	21.64 ± 0.01	6.62 ± 0.14	15.01 ± 0.13

[a]Recovery.
[b]Not detected.
[c]Hydrocarbons, aldehydes, ketones, pesticides, herbicides, and the degradation products of tocopherols and free phytosterols.

raffinate product. However, if using SODD as starting material, which contains more bioactive compounds such as squalene, supercritical CO_2 extraction is more difficult to employ (17,18). The MSE method has some drawbacks; a relatively more prolonged extraction period compared to supercritical CO_2 method; and it may be difficult to upscale for industrial application due to problems of uneven silica gel packing and reusability. To overcome this shortcoming, Fabian et al. (19) constructed a stirred batch-wise silica gel adsorption-desorption vessel and overcame the problem of uneven silica gel packing and separating SODD into NPLF and PLF in less time. The performance of their batch vessel is comparable to that of a modified soxhlet extractor. The large amount of silica gel used in separation of SODD increases the cost and may hinder its commercial application. Cheng et al. (20) showed that silica gel can be regenerated, reused, and still achieved the same degree of separation between NPLF and PLF in MSE.

Isolation of Tocopherols and Free Phytosterols from PLF

The GC chromatograms, Fig. 2, show that after MSE, most FFAs along with tocopherols, and free phytosterols were concentrated in the PLF. Table 1; the main impurity in the PLF is FFAs (48.14%). One of the quality criteria of refined soybean oil is its FFAs contents must not exceed 0.05 wt% (21). Purification methods such as molecular distillation and supercritical CO_2 normally require multiple steps in order to meet this stringent requirement on low FFAs contents. Martins et al. (22) used a five-stage molecular distillation in order to reduce FFAs content from 57.80% in SODD to 1% in the distillate.

Methods such as solvent extraction, low temperature crystallization, enzymatic esterification to transform FFAs into fatty acid methyl esters (FAMEs), and cold saponification were tested for their effectiveness in removing FFAs. Except for cold saponification, all methods tested failed to

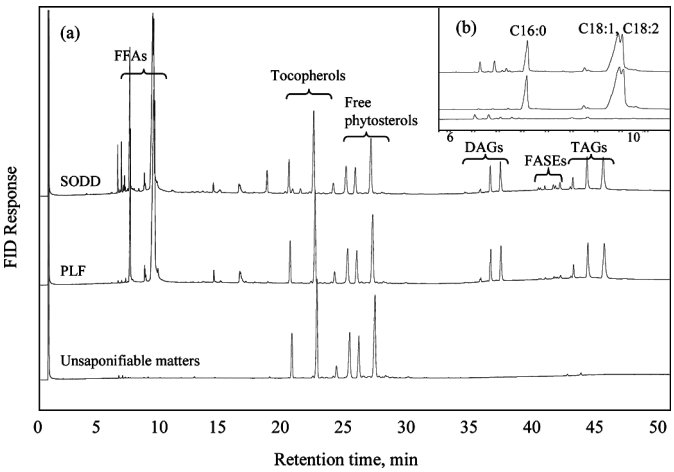

FIG. 2. Typical results of the HT-GC analysis. (a) The GC chromatogram of SODD, PLF, and unsaponifiable matters resulted from cold saponification at 40°C, 2 h, 10 mL KOH, 0.1 g ascorbic acid. (b) Enlargement of FFA region in (a).

reduce FFAs contents to less than 2%. After cold saponification, tocopherols and free phytosterols can be recovered in the unsaponifiable matters via liquid-liquid extraction using water-diethyl ether as the solvent. Diethyl ether is the best solvent as it dissolves organic compounds with a wide range of polarity. Water, a highly polar solvent, ensures that complete separation of saponifiable matter from unsaponifiable matter takes place. Some small amount of neutral lipids, such as FFA, DAG, TAG, and FASE, still remained in the unsaponifiable matter which was possibly caused by incomplete cold saponification reaction.

The identification of FFA peaks in GC chromatogram is important for ensuring the complete removal of FFAs. Three FFA peaks appeared in GC chromatogram (see Fig. 2b), which were identified as palmitic, oleic, and linoleic acids. All three appear at retention time of less than 11 min. An increase in the recovery of free phytosterol (to higher than 100%) after being subjected to cold saponification was observed and detected in the unsaponifiable matters. This result agrees with the previous observation that FASEs can be hydrolyzed into FFAs and free phytosterols under alkaline condition (23).

Effects of Temperature

The effects of saponification temperature on the contents and recoveries of FFAs, acylglycerols, tocopherols, and free phytosterols were investigated and the results are shown in Table 2. It can be seen from Table 2 that the recoveries of FFAs, acylglycerols, tocopherols, FASEs, and free phytosterols all decrease with increasing temperature. The ANOVA and Fisher's LSD methods were applied to determine the significance of differences in recovery. Change in temperature resulted in significant difference ($p < 0.05$) in contents and recoveries of FFAs, tocopherols, and free phytosterols, but did not give significant difference ($p > 0.05$) in contents and recoveries of acylglycerols and FASEs. FFAs can be removed in 2 h by saponification at 40°C or 50°C. At 25°C, the reaction product still contains 0.18% FFAs at a reaction time of 2 h. However, by extending the reaction time to 4 h, FFAs can be removed completely.

TABLE 2
Effects of saponification temperature on the composition of unsaponifiable matters in the polar fraction of SODD[a]

Compounds	PLF (%)	Unsaponifiable matters (%) 25°C	40°C	50°C
FFAs	48.14	0.18 ± 0.21 (0.11 ± 0.12)[b]	ND[c]	ND
MAGs	0.22	ND	ND	ND
Total tocopherols	12.14	39.05 ± 0.97^{e} (92.25 ± 3.06)^{e}	38.08 ± 0.36^{e} (89.79 ± 1.29)^{f}	36.43 ± 1.26^{e} (84.86 ± 2.18)^{g}
δ-Tocopherol	3.10	10.36 ± 0.32	10.07 ± 0.18	9.79 ± 0.26
γ-Tocopherol	7.92	25.23 ± 0.56	24.65 ± 0.28	23.40 ± 0.93
α-Tocopherol	1.11	3.46 ± 0.12	2.98 ± 0.65	3.25 ± 0.09
Total free phytosterols	13.98	56.80 ± 0.68^{e} (116.50 ± 1.23)^{e}	55.51 ± 0.56^{e} (113.67 ± 0.96)^{e}	54.20 ± 0.76^{e} (109.66 ± 1.11)^{e}
Campesterol	3.70	15.47 ± 0.19	15.16 ± 0.11	14.79 ± 0.21
Stigmasterol	3.16	12.67 ± 0.18	12.38 ± 0.08	12.07 ± 0.18
β-Sitosterol	7.12	28.66 ± 0.35	27.97 ± 0.38	27.34 ± 0.38
DAGs	3.39	ND	ND	ND
FASEs	1.06	0.64 ± 0.10^{e} (17.35 ± 2.44)^{e}	0.66 ± 0.07^{e} (17.91 ± 1.83)^{e}	0.81 ± 0.13^{e} (21.77 ± 3.70)^{e}
TAGs	8.50	0.21 ± 0.01 (0.71 ± 0.01)	0.22 ± 0.01 (0.74 ± 0.03)	0.22 ± 0.02 (0.72 ± 0.08)
Others[d]	12.56	3.11 ± 1.30 (7.10 ± 2.96)	5.53 ± 0.80 (12.61 ± 1.83)	8.08 ± 1.90 (18.21 ± 4.37)
Amount (g)	2.02	0.58 ± 0.00	0.56 ± 0.02	0.57 ± 0.01

[a]Reaction conditions: 2 g PLF, 25 mL KOH, 0.1 g ascorbic acid, reaction time 2 h.
[b]Recovery.
[c]Not detected.
[d]FAEEs, ketones, degradation products of tocopherols and free phytosterols.
[e,f,g]Data with the same letter indicate no statistical difference ($p > 0.05$).

By comparing the effect of every temperature level (25, 40, and 50°C) using Fisher's LSD method, it was found that contents and recoveries of free phytosterols, and content of tocopherols were not significantly different between various temperature levels. Meanwhile at 50°C, tocopherols recoveries were significantly lower ($p < 0.05$) than those obtained at 25°C and 40°C. If it is desirable to concentrate tocopherols and free phytosterols in the final product, a lower temperature is preferred because it minimizes the degradation of bioactive compounds in the PLF. Therefore, 40°C was chosen as the saponification temperature in this study because at this temperature FFAs contents were not detected after 2 h and tocopherols recoveries in the final product obtained was not significantly different ($p > 0.05$) from those at 25°C.

Effects of Ascorbic Acid

Citric acid and phosphoric acid are used as antioxidants because of their metal ions-binding or chelating property. Butylated hydroxyanisole, butylated hydroxytoluene, and propyl gallate possess antioxidant property due to their radical scavenger ability. Ascorbic acid is a preferred antioxidant; it is an effective, cheap, and one of the best oxygen scavengers. The mechanism of ascorbic acid as oxygen scavenger was proposed by Cort (24), as shown in Fig. 3. Cort (24) reported that in the presence of O_2, H_2O, or both, ascorbic acid will react to become dehydroascorbic acid. Its solubility in KOH solution is higher than tocopherols, so, ascorbic acid will be degraded before tocopherols during saponification. The addition of ascorbic acid is expected to prevent the loss of tocopherols during cold saponification. Krukovsky (25) showed that the addition of ascorbic acid significantly increased the recovery of tocopherols in milk fat.

The effects of adding ascorbic acid on the recoveries of tocopherols and free phytosterols in cold saponification were investigated in this study. Variable ascorbic acid gives significant effect on the contents and recovery of tocopherols but not on the contents and recoveries of free phytosterols, FFAs, acylglycerols, and FASEs. No addition of ascorbic acid gives significantly lower recoveries of tocopherols compared to those of ascorbic acid. As the amount of ascorbic acid added increases from 0.05 g (2.5 wt%) to 0.1 g (5 wt%); the recovery of tocopherols increases significantly from 81.25% to 89.22%. Greater amounts of ascorbic acid (0.3 g or 15 wt%) did not result in increasing the recovery of tocopherols. It is possible that ascorbic acid competes with FFAs in reacting with sodium hydroxide resulting in some unreacted FFAs being left in the unsaponifiable matter, as shown in Table 3. The oxidized form of ascorbic acid, e.g., dehydroascorbic acid, in high concentration may also generate an oxidative potential. In this study, 5 wt% ascorbic acid was added in cold saponification and resulted in the increase of the recovery of tocopherols and free phytosterols of about 15% and 7% respectively, as shown in Table 3. Tukey's procedure, that analyzed the effect of ascorbic acid amount, showed that the difference between the means of the largest and the smallest samples is significantly different. This indicates that ascorbic acid amounts significantly affected the content and recovery of tocopherols.

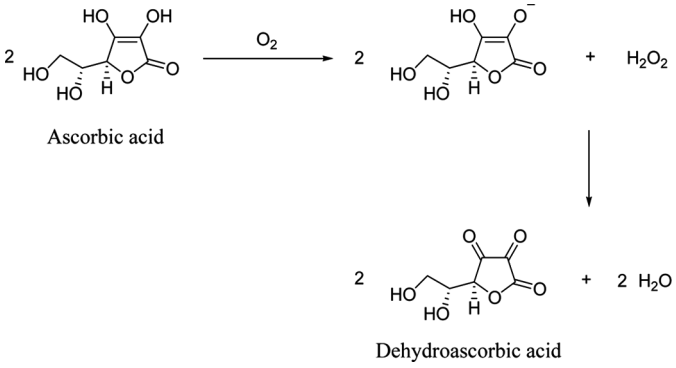

FIG. 3. Proposed mechanism of ascorbic acid as oxygen scavenger.

Effects of Alkali Amount

The role of tocopherols as an antioxidant is due to its free radical scavenger property. Free radical reaction is often initiated by oxygen, light/UV, acid, or base catalyst. For this reason, it is important to investigate the effects of alkali amount on the removal of FFAs and on the recovery of tocopherols. Table 4 shows the effects of the amount of KOH (mL KOH per 2 g PLF) used in cold saponification on the removal of FFAs and the recoveries of tocopherols and free phytosterols. The theoretical amount of KOH needed for the complete conversion of all the fatty acids (including FFAs and those in the forms of DAGs and TAGs, and FASEs) contained in the PLF into fatty acid potassium salts is calculated to be 8.94 mL, based on an average fatty acid molecular weight of 278.43.

The ASTM method (13) recommends using 25 mL KOH for 2 g PLF. Table 4, shows even the minimum amount of KOH used (10 mL) is more than enough to remove all FFAs. Tocopherols, free phytosterols, FFAs, FASEs, and acylglycerols contents and recoveries were not significantly different ($p > 0.05$) at 10 mL KOH from that obtained using 25 mL KOH. These may be the results of a short reaction time (2 h) and the addition of ascorbic acid.

As the KOH amount increases from 10 mL to 50 mL or 25 mL to 50 mL, both contents and recoveries of tocopherols and free phytosterols decrease significantly ($p < 0.05$). The KOH amount needed for complete saponification of 2 g PLF is about 10 mL. The addition of 50 mL KOH, five times the theoretical amount needed, resulted in the decrease of recovery of tocopherols as well as that of free

TABLE 3
Effects of ascorbic acid on the composition of unsaponifiable matters in the polar fraction of SODD[a]

		Unsaponifiable matters (%)			
			Ascorbic acid (wt%)		
Compounds	PLF (%)	No ascorbic acid	2.5	5	15
FFAs	48.14	ND[b]	ND	ND	4.36 ± 2.70 (2.71 ± 1.69)[c]
MAGs	0.22	ND	ND	ND	ND
Total tocopherols	12.14	31.67 ± 1.65^{e} (73.76 ± 3.19)[e]	34.96 ± 1.18^{e} (81.91 ± 0.67)[e]	37.66 ± 0.51^{f} (89.22 ± 0.08)[g]	34.49 ± 3.06^{e} (84.61 ± 6.75)[h]
δ-Tocophe rol	3.10	9.29 ± 0.38	10.31 ± 1.19	9.85 ± 0.18	10.52 ± 0.67
γ-Tocopherol	7.92	19.69 ± 1.88	22.01 ± 0.60	24.51 ± 0.29	21.38 ± 2.99
α-Tocopherol	1.11	2.69 ± 0.15	2.64 ± 0.27	3.31 ± 0.04	2.58 ± 0.66
Total free phytosterols	13.98	51.74 ± 2.20^{e} (104.69 ± 5.38)[e]	52.43 ± 2.45^{e} (106.65 ± 2.27)[e]	54.25 ± 0.22^{e} (111.63 ± 1.87)[e]	53.25 ± 1.17^{e} (113.49 ± 2.36)[e]
Campesterol	3.70	14.15 ± 0.59	14.55 ± 0.83	15.80 ± 0.05	14.82 ± 0.37
Stigmasterol	3.16	11.55 ± 0.54	11.40 ± 0.27	12.04 ± 0.04	11.54 ± 0.48
β-Sitosterol	7.12	26.05 ± 1.07	26.48 ± 1.35	27.42 ± 0.13	26.90 ± 0.50
DAGs	3.39	ND	ND	ND	ND
FASEs	1.06	0.79 ± 0.22^{e} (20.98 ± 5.95)[e]	0.79 ± 0.52^{e} (21.16 ± 13.60)[e]	0.56 ± 0.16^{e} (15.26 ± 4.22)[e]	0.71 ± 0.15^{e} (19.89 ± 4.45)[e]
TAGs	8.50	0.26 ± 0.04 (0.86 ± 0.11)	0.49 ± 0.34 (1.64 ± 1.10)	0.23 ± 0.02 (0.78 ± 0.06)	0.37 ± 0.15 (1.31 ± 0.54)
Others[d]	12.56	15.54 ± 0.73 (34.98 ± 1.33)	11.32 ± 4.09 (25.81 ± 10.07)	7.30 ± 0.47 (16.72 ± 1.29)	10.56 ± 3.00 (25.09 ± 7.35)
Amount (g)	2.02	0.57 ± 0.01	0.58 ± 0.02	0.58 ± 0.01	0.60 ± 0.01

[a]Reaction conditions: 2 g PLF, 10 mL KOH, reaction time 2 h, reaction temperature 40°C.
[b]Not detected.
[c]Recovery.
[d]FAEEs, ketones, degradation products of tocopherols and phytosterols.
[e,f,g,h]Data with the same letter indicate no statistical difference ($p > 0.05$).

phytosterols. Overall, Tukey's method shows that the difference between means of the largest and the smallest samples is significantly different. This implies that the alkali amount significantly affected the contents and recoveries of tocopherols and free phytosterols.

Time Courses

The effect of saponification time on the removal of FFAs was investigated in order to determine the shortest time required to remove FFA completely. Saponifications at 40 and 50°C can effectively remove all FFAs within 2 h while it requires 4 h to remove all FFAs at 25°C. The times required in this study are much shorter than the $12 \sim 48$ h reported in the literature (26–28) for cold saponification carried out at room temperature. This may happen because most of neutral lipid contained in PLF is FFAs. Reaction rates of KOH with FFAs are faster than that with TAGs. TAGs have to be hydrolyzed to become FFAs and then react with KOH. This is supported by the results of soybean oil saponification at 40°C for 2 h with the addition of 25 mL KOH in ethanol and 0.1 g ascorbic acid. TAGs and DAGs were removed completely, while some FFAs were detected in the unsaponifiable matters (data not shown).

In this study, the reaction vessel was immersed in a thermal bath at constant temperature. Heat released by the exothermic saponification reaction only induced an increase of 1–3°C inside the vessel as measured by a thermocouple immersed in the reaction mixture. Insignificant thermal degradation of tocopherols was observed as evidenced by the high recovery obtained at 25 and 40°C as shown in Table 2. Resistance of tocopherols to oxidation is due to the –OH group position in tertiary carbon, which has no hydrogen atoms on the carbinol carbon atom. Oxidation occurs if the carbon–carbon bonds in the tocopherol ring structure are broken (29). Tocopherols will

TABLE 4
Effects of KOH amount on FFA and bioactive compounds[a]

Compounds	PLF (%)	Unsaponifiable matters (%)		
		10 mL KOH	25 mL KOH	50 mL KOH
FFAs	48.14	ND[b]	ND	ND
MAGs	0.22	ND	ND	ND
Total tocopherols	12.14	37.68 ± 0.36^{e}	38.08 ± 0.36^{f}	27.51 ± 3.00^{g}
		$(89.24 \pm 0.06)^{c,\ e}$	$(89.79 \pm 1.29)^{f}$	$(63.75 \pm 7.48)^{g}$
δ-Tocopherol	3.10	9.83 ± 0.13	10.07 ± 0.18	8.34 ± 0.23
γ-Tocopherol	7.92	24.54 ± 0.21	24.65 ± 0.28	16.51 ± 3.07
α -Tocopherol	1.11	3.32 ± 0.04	2.98 ± 0.65	2.66 ± 0.14
Total free phytosterols	13.98	54.63 ± 0.68^{e}	55.51 ± 0.56^{e}	47.71 ± 2.85^{e}
		$(112.35 \pm 1.82)^{e}$	$(113.67 \pm 0.96)^{f}$	$(95.92 \pm 5.12)^{g}$
Campesterol	3.70	14.89 ± 0.16	15.16 ± 0.11	12.99 ± 0.77
Stigmasterol	3.16	12.12 ± 0.16	12.38 ± 0.08	10.67 ± 0.73
β-Sitosterol	7.12	27.62 ± 0.36	27.97 ± 0.38	24.05 ± 1.35
DAGs	3.39	ND	ND	ND
FASEs	1.06	0.60 ± 0.13^{e}	0.66 ± 0.07^{e}	1.04 ± 0.36^{e}
		$(16.25 \pm 3.44)^{e}$	$(17.91 \pm 1.83)^{e}$	$(27.46 \pm 9.40)^{e}$
TAGs	8.50	0.23 ± 0.02	0.22 ± 0.01	1.44 ± 0.54
		(0.78 ± 0.05)	(0.74 ± 0.03)	(1.44 ± 0.54)
Others[d]	12.56	7.30 ± 0.47	5.53 ± 0.80	23.30 ± 1.18
		(15.71 ± 1.98)	(12.61 ± 1.83)	(52.16 ± 2.59)
Amount (g)	2.01	0.58 ± 0.01	0.56 ± 0.02	0.56 ± 0.00

[a]Reaction conditions: 2 g PLF, reaction time 2 h, reaction temperature = 40°C, 0.1 g ascorbic acid.
[b]Not detected.
[c]Recovery.
[d]FAEEs, ketones, degradation products of tocopherols and phytosterols.
[e,f,g]Data with the same letter indicate no statistical difference ($p > 0.05$).

be degraded if there are free radicals available in the system because it will react with free radical and form tocopheryl radical compounds. These will react with another free radical to form tocopheryl quinine (30). In this study, the presence of peroxyl and hydroperoxyl radicals, which are formed in the presence of oxygen, UV radiation, or both, was avoided by sealing the reaction vessel and carrying out the reaction in a short time; reducing the loss of tocopherols. With an extended reaction time of 12 h at 40°C, no significant changes in the content and recovery of tocopherols, free phytosterols, and other compounds in the unsaponifiable matters were observed.

Identification of Other Compounds in Unsaponifiable Matters

The total content of tocopherols and free phytosterols in the final product (unsaponifiable matters) is about 95% under optimum conditions (2 h, 10 mL KOH, 40°C, and 0.1 g ascorbic acid). This value drops to 75–80% if harsher conditions (e.g., 2 h, at 40°C, 50 mL KOH, and without ascorbic acid) were used (data not shown). The identification of other compounds in the final product is important for its possible commercial application. Before subjection to cold saponification, the other compounds that may exist in PLF are ketones, aldehydes, oxidation products of free phytosterols, pesticides, and herbicides (31). After saponification, a small amount of FAEEs was detected in the GC chromatogram (data not shown). Due to the presence of ethanol during saponification, some FFAs and acylglycerols reacted with ethanol in base condition to produce FAEEs as evidenced by TLC analysis (Fig. 4a) and gas chromatography. In Fig. 4a, PLF fraction (lane I) shows no spot at position correspond to FAEE standard (ethyl stearate) spot (lane II), while spot correspond to the unsaponifiable matters (lane III) does appear.

Aldehydes were also found in saponifiable matters after acidification. Aldehydes might be reacted or oxidized in alkaline conditions and the oxidation products of aldehyde were removed along with the saponifiable matters. After acidification using HCl, FFAs, and aldehydes were discovered in the saponifiable matters (see Fig. 4b). By comparing both the unsaponifiable matters (lane I) and

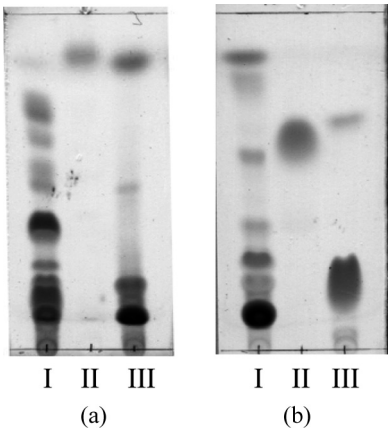

FIG. 4. TLC analysis of the other compounds developed by using *n*-hexane/ethyl acetate/acetic acid (90/10/1) as mobile phase: (a) Lane I: PLF, lane II: FAEE standard (ethyl stearate), and lane III: unsaponifiable matters; (b) Lane I: unsaponifiable matters, lane II: aldehyde standard (decanal), and lane III: saponifiable matters.

the saponifiable matters (lane III) with aldehyde standard (decanal) at lane II, it can be seen that the spot at lane III appears at the same position as that of the aldehyde standard. Both spots give the same color and absorbance after being sprayed with a specific reagent, whereas no aldehyde spot appears in the unsaponifiable matters.

The final contents of tocopherols and free phytosterols in the unsaponifiable matters are $38.08 \pm 0.36\%$ and $55.51 \pm 0.56\%$, respectively, while the total recoveries of tocopherols and free phytosterols are $83 \pm 0.57\%$ and $113 \pm 0.34\%$, respectively. The recovery of this two-step procedure is comparable to the result of Fizet (8), in which high yields of tocopherols (88%) and free phytosterols (90%) were obtained. The time required to complete the process in both studies are about the same (16–17 h). However, at least 4 steps were required in the work of Fizet (8). Only two steps were needed in this work. Only moderate operational temperatures were needed in this work which avoid damaging the heat labile biocompounds, especially tocopherols.

To fulfill the demands of commercial industry, it is important to know the prospect of scale-up of the two-step procedure developed in this work. The modified soxhlet extractor is somewhat difficult to scale-up. However, it was demonstrated that the function of a modified soxhlet extractor can be accomplished by using a stirred batch-wise silica gel adsorption-desorption vessel which can overcome the drawback of uneven silica gel packing and long extraction time associated with modified soxhlet extraction, yet still yield comparable separation efficiency (19). Furthermore, silica gel can be regenerated and reused up to three times without significant loss of its performance in adsorption and desorption (20). It is straightforward to up scale cold saponification.

CONCLUSIONS

We demonstrated in this study that a lipid mixture with a total tocopherols and free phytosterols content of 95% can be obtained by applying MSE to SODD to obtain the PLF, and then removing more than 99.9% of FFAs contents from PLF by cold saponification. The total recoveries for tocopherols and phytosterols are 83% and 113%, respectively. Impurities that come with the lipid product are FAEEs, ketones, and the degradation products of tocopherols and free phytosterols. Similar to methods such as molecular distillation, most bioactive compounds are not affected by this cold saponification. However, the process developed in this study is simpler because it involved only two relatively simple steps as compared to the other method which typically involves multiple molecular distillation and other reactions.

ACKNOWLEDGEMENTS

This work was supported by the National Science Council of Taiwan through the project NSC94-2214-E011-004. The authors thank Dr. Fred Quarnstrom for his assistance during the preparation of this manuscript.

LIST OF NONSTANDARD ABBREVIATIONS

ASTM::	American Society for Testing and Materials
DAGs:	Diacylglycerols
FAEEs:	Fatty Acid Ethyl Esters
FASEs:	Fatty Acid Steryl Esters
FFAs:	Free Fatty acids
FID:	Flame-Ionization Detector
HT-GC:	High Temperature Gas Chromatograph
MAGs:	Monoacylglycerols
MSE:	Modified Soxhlet Extraction
NPLF:	Non Polar Lipid Fraction
PLF:	Polar Lipid Fraction
SODD:	Soybean Oil Deodorizer Distillate
SuODD:	Sunflower Oil Deodorizer Distillate
TAGs:	Triacylglycerols

REFERENCES

1. Rozner, S.; Garti, N. (2006) The activity and absorption relationship of cholesterol and phytosterols. *Colloids Surf. A*, 282–283: 435–456.
2. Weber, N.; Weitkamp, P.; Mukherjee, K.D. (2002) Cholesterol-lowering food additives: Lipase-catalyzed preparation of phytosterol and phytostanol esters. *Food Res. Int.*, 35: 177–181.
3. Tonello, A.; Poli, G. (2006) Serum phytosterols not only from dietary intake. *Br. J. Nutr.*, 96: 791–792.
4. Mu, L.; Feng, S.S. (2003) A novel controlled release formulation for the anticancer drug Paclitaxel (Taxol®): PLGA nanoparticles containing vitamin E TPGS. *J. Controlled Release*, 86: 33–48.
5. Lee, S.H.; Zhang, Z.; Feng, S.-S. (2007) Nanoparticles of poly(Lactide)-tocopheryl polyethylene glycol succinate (PLA-TPGS) copolymers for protein drug delivery. *Biomaterials*, 28: 2041–2050.
6. Verleyen, T.; Verhe, R.; Garcia, L.; Dewettinck, K.; Huyghebaert, A.; De Greyt, W. (2001) Gas chromatographic characterization of vegetable oil deodorization distillate. *J. Chromatogr. A*, 921: 277–285.

7. Martins, P.F.; Ito, V.M.; Batistella, C.B.; Maciel, M.R.W. (2006) Free fatty acid separation from vegetable oil deodorizer distillate using molecular distillation process. *Sep. Purif. Technol.*, 48: 78–84.
8. Fizet, C. Process for Tocopherols and Sterols from Natural Sources. U.S. Patent 5,487,817, January 30, 1996.
9. Watanabe, Y.; Nagao, T.; Hirota, Y.; Kitano, M.; Shimada, Y. (2004) Purification of tocopherols and phytosterols by a two-step in situ enzymatic reaction. *J. Am. Oil Chem. Soc.*, 81: 339–345.
10. Torres, C.F.; Torrelo, G.; Senõrans, F.J.; Reglero, G. (2007) A two steps enzymatic procedure to obtain sterol esters, tocopherols and fatty acid ethyl esters from soybean oil deodorizer distillate. *Process Biochem.*, 42: 1335–1341.
11. Gunawan, S.; Kasim, N.S.; Ju, Y.-H. (2008) Separation and purification of squalene from soybean oil deodorizer distillate. *Sep. Purif. Technol.*, 60: 128–135.
12. Gunawan, S.; Fabian, C.; Ju, Y.-H. (2008) Isolation and purification of fatty acid steryl esters from soybean oil deodorizer distillate. *Ind. Eng. Chem. Res.*, 47: 7013–7018.
13. D 5553 Test method for determination of the unsaponifiable nonvolatile matter in sulfated oils (2001) In: *Annual Book of Astm Standards*, ASTM International: Pennsylvania.
14. Montgomery, D.C. (2005) *Design and Analysis of Experiments*, 6th Ed.; John Wiley and Sons, Inc.: New York.
15. Gunawan, S. (2008) Isolation and purification of squalene and fatty acid steryl esters from soybean oil deodorizer distillate, Chemical Engineering Department, National Taiwan University of Science and Technology, Taipei.
16. Vázquez, L.; Torres, C.F.; Fornari, T.; Grigelmo, N.; Señoráns, F.J.; Reglero, G. (2006) Supercritical fluid extraction of minor lipids from pretreated sunflower oil deodorizer distilates. *Eur. J. Lipid Sci. Technol.*, 108: 659–665.
17. Gast, K.; Jungfer, M.; Saure, C.; Brunner, G. (2005) Purification of tocochromanols from edible oils. *J. Supercrit. Fluids*, 34: 17–25.
18. Mendes, M.F.; Pessoa, F.L.P.; Coelho, G.V.; Uller, A.M.C. (2005) Recovery of the high aggregated compounds present in the deodorizer distillate of the vegetable oils using supercritical fluids. *J. Supercrit. Fluids*, 34: 157–162.
19. Fabian, C.; Gunawan, S.; Kasim, N.S.; Chiang, C.-L.; Ju, Y.-H. (2009) Separation of nonpolar lipid from soybean oil deodorizer distillate by stirred batch-wise silica gel adsorption-desorption. *Sep. Sci. Technol.*, 44: 1621–1637.
20. Cheng, B.-R.; Gunawan, S.; Ju, Y.-H. (2009) Silica gel regeneration for separation of soybean oil deodorizer distillate. *Adsorption*: Submitted.
21. Debruyne, I. (2004) Soybean oil processing; Quality criteria and flavor reversion, *IUPAC-AOCS Workshop on Fats, Oils, and Oilseeds Analysis and Production*: Tunis, Tunisia.
22. Martins, P.F.; Batistella, C.B.; Maciel-Filho, R.; Wolf-Maciel, M.R. (2006) Comparison of two different strategies for tocopherols enrichment using a molecular distillation process. *Ind. Eng. Chem. Res.*, 45: 753–758.
23. Fernandes, P.; Cabral, J.M.S. (2007) Phytosterols: Applications and recovery methods. *Bioresour. Technol.*, 98: 2335–2350.
24. Cort, W.M. (1974) Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid and their mode of action. *J. Am. Oil Chem. Soc.*, 51: 321–325.
25. Krukovsky, V.N. (1964) Milk fat analysis, protection of milk fat tocopherols during saponification with ascorbic acid. *J. Agric. Food Chem.*, 12: 289–292.
26. Williams, L.D.; Pearson, A.M.; Dugan, L.R. (1964) A comparison of two saponification methods. *J. Am. Oil Chem. Soc.*, 41: 336.
27. Lampi, A.-M.; Juntunen, L.; Toivo, J.; Piironen, V. (2002) Determination of thermo-oxidation products of plant sterols. *J. Chromatogr. B*, 777: 83–92.
28. King, A.J.; Paniangvait, P.; Jones, A.D.; German, J.B. (1998) Rapid method for quantification of cholesterol in turkey meat and products. *J. Food Sci.*, 63: 382–385.
29. Wade, L.G. (2006) Reactions of Alcohols. In: *Organic Chemistry*, Pearson Prentice Hall: New York, 461–465.
30. Sies, H.; Stahl, W. (1995) Vitamins E and C, β-carotene, and other carotenoids as antioxidants. *Am. J. Clin. Nutr.*, 62: 1315S–1321S.
31. Bockisch, M. (1998) *Fats and Oils Handbook*; American Oil Chemist Society: Champaign.