Isolation and Quantitative Determination of Sterol Oxides in Plant-Based Foods: Soybean Oil and Wheat Flour

Jaffar Nourooz-Zadeh* and **Lars-Åke Appelqvist**
Department of Food Hygiene, Swedish University of Agricultural Sciences, S-750 07, Uppsala, Sweden

Soybean oil and wheat flour were analyzed for the content of sitosterol oxides. The method involved chromatography on a Lipidex-5000 column and enrichment on a disposable NH_2-column, yielding a sterol fraction and a sterol oxide fraction. Each fraction was separated as trimethylsilyl-ethers on a methyl silicone capillary column. Analysis of crude and freshly refined soybean oil showed no detectable levels of the isomeric 5,6-epoxysitosterols, the epimeric 7-hydroxysitosterols and 5,6-dihydroxysitosterol at the detection limit of 0.2 ppm. Storage of a refined soybean oil for one year at 4°C caused no significant increase in the level of free sitosterol oxides when compared to the freshly refined soybean oil. Analysis of three wheat flours (at 2, 8 and 36 months) revealed that the samples contained variable levels of 5α,6α-epoxysitosterol (5.4–55 ppm in the lipids), 5β,6β-epoxysitosterol (0.2–29 ppm), 7α-hydroxysitosterol (9.3–118 ppm) and 7β-hydroxysitosterol (9.7–126 ppm).

KEY WORDS: Sitosterol oxides, sterol oxides.

Sitosterol is susceptible to spontaneous oxidation. The reaction is preferentially initiated by allylic hydrogen abstraction at position C-7. Hydrogen abstraction can also take place at the tertiary carbons in the side chain. A number of autoxidation products have been characterized and identified (1–3). In this context, exposure of plant-based food to heat during manufacturing and/or long-term storage at ambient temperature leads to an accumulation of sitosterol oxides.

Occurrence of cholesterol oxides in food has been considered to play an important role in the etiology of atherosclerosis (4,5). However, information regarding the biological activity of sitosterol oxides is limited (6). By virtue of their structural similarity to cholesterol oxides, it may be assumed that high intake of sitosterol oxides also could be associated with the development of undesirable biological effects, such as the inhibition of cholesterol biosynthesis from other lipids, cytotoxicity, angiotoxicity, carcinogenicity and mutagenicity. The previously mentioned biological properties of the cholesterol oxides are discussed elsewhere (7–9).

Sitosterol is the major sterol in vegetable oils (10). Isolation and accurate quantitation of trace levels of sitosterol oxides from a complex matrix, containing several plant sterols, is a difficult and tedious procedure. Finocchiaro (6) analyzed commercial wheat flour samples for the content of sitosterol oxides (the isomeric 5,6-epoxysitosterols, the epimeric 7-hydroxysitosterols and the 5,6-dihydroxysitosterol). The total lipids were saponified and the unsaponifiable material was separated by preparative thin-layer chromatography. The sitosterol oxides were quantitated by comparison of the areas of thin-layer chromatography spots. French fries and potato chips were analyzed by Lee *et al.* (11). The method involved saponification, followed by $AgNO_3$ column chromatography. The sitosterol oxides (the isomeric 5,6-epoxysitosterols and the epimeric 7-hydroxysitosterols) were separated by high-performance liquid chromatography (HPLC) with refractive index detection.

The aim of the present study was to determine the levels of sitosterol oxides in soybean oil and wheat flour with simultaneous estimation of the sterol profile of such products. In order to achieve this goal, substantial modification of existing methods suitable for cholesterol oxides had to be undertaken.

MATERIALS AND METHODS

Samples. Virgin olive oil was purchased from a local supermarket. Crude and industrially refined soybean oil samples with background information were obtained from Margarinbolaget (Helsingborg, Sweden). Wheat flour samples were obtained from Nord Mills (Uppsala, Sweden) or purchased from a local supermarket. The wheat flour samples were stored in sealed paper bags at room temperature. Potato granular samples were provided by SIK, the Swedish Institute for Food Research (Göteborg, Sweden). The samples were stored in sealed plastic bags at 35°C for 13 and 36 months, respectively.

Reagents. Campesterol, sitosterol, stigmasterol, cholestane, 5α,6α-epoxycholesterol and 5,6-dihydroxycholesterol were purchased from Sigma Chemical Co. (St. Louis, MO), while epimeric 7-hydroxycholesterols were obtained from Steraloids Inc. (Wilton, NH). The commercially available sterols and sterol oxides were used without further purification. Acetic anhydride, chloroform, dichloromethane, diethyl ether, heptane, methanol, and *p*-toluene sulfonic acid were purchased from E. Merck (Darmstadt, Germany). 1,2-Dichloroethane and pyridine were obtained from Fischer Scientific (Fair Lawn, NJ). *m*-Chloroperbenzoic acid (98%) was purchased from Kodak Laboratory and Research Products (Rochester, NY). Tri-Sil was purchased from Pierce Chemicals (Rockford, IL). The Lipidex-5000 was purchased from Packard Instruments Inc. (Downers Grove, IL), and aminopropyl-cartridges (NH_2-sorbent, 100 mg) were obtained from Analytichem International (Harbor City, CA). ^{14}C-labelled sitosterol and phase-combining liquid scintillation (PCS) were purchased from Amersham Inc. (Amersham, U.K.). All chemicals and reagents were of the highest quality available commercially.

Synthesis of sitosterol oxides. 5α,6α-Epoxysitosterol was synthesized as described elsewhere (12) after a slight modification. Briefly, sitosterol (1 g) was dissolved in 30 mL dichloromethane and stirred, and then one equivalent of *m*-chloroperbenzoic acid was gradually added. After stirring for 4 hr, the solvent was removed under a stream of nitrogen and the residue was redissolved in diethyl ether. The ether solution was washed sequentially with 10% aqueous sodium bicarbonate and water, and was dried over sodium sulfate. The solvent was removed under reduced pressure, and the 5,6-epoxysitosterol was purified

*To whom correspondence should be addressed at William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London EC1 M6EQ, U.K.

by preparative thin-layer chromatography (prep-TLC) as described below. This gave a ratio of 9:1 of 5α,6α-epoxysitosterol over the 5β,6β-epoxy isomer, judged by normal-phase high-performance chromatography on a CN column or by gas chromatography (GC) as trimethylsilyl (TMS)-ether derivatives (13). The 5α,6α-epoxysitosterol was used without further purification. 5,6-Dihydroxysitosterol was prepared by treating the 5α,6α-epoxysitosterol with periodic acid (14). The product was isolated from starting material by prep-TLC and purity was checked by HPLC and GC. 5β,6β-Epoxysitosterol was synthesized according to Davis and Petrow (15) after a slight modification. Briefly, 10 mg 5,6-dihydroxysitosterol was incubated with 0.5 mL pyridine and 0.3 mL acetic anhydride overnight at room temperature. The solvent was removed under a stream of nitrogen. The residue (5,6-diacetylsitosterol) was redissolved in 5 mL acetic acid anhydride containing 30 mg *p*-toluene sulfonic acid, and the mixture was refluxed at 100°C for 30 min. The reaction was terminated by adding water (5 mL), and the triacetylsitosterol was partitioned with petroleum ether/diethyl ether (2:1, v/v). The organic layer was sequentially washed with 10% sodium bicarbonate and water, and the solvent was removed under reduced pressure. The residue was redissolved in 10 mL 0.5 M potassium hydroxide in dry methanol and the mixture was refluxed at 75°C for 1 hr. The 5β,6β-epoxysitosterol was extracted with diethyl ether, and the solvent was removed by rotary evaporation. The 5β,6β-epoxysitosterol was purified by prep-TLC and subsequently analyzed by HPLC and GC. Epimeric 7-hydroxysitosterols were prepared by autoxidation of sitosterol. A detergent was prepared by dissolving 250 mg stearic acid and 300 mg trisodium phosphate in 37% ethanol (16). Eighty milliliters of the detergent were transferred into a beaker, diluted with water to 500 mL, and the content was stirred at room temperature. Subsequently, a hot ethanol solution (10 mL) containing 1 g sitosterol was added slowly. After stirring for 48 hr, chloroform/methanol (2:1, v/v; 500 mL) was added and the reaction mixture was agitated. The organic layer was transferred into an evaporation flask and the solvent was removed. The residue was resuspended in hot ethanol and kept at 4°C to crystallize the native sitosterol. The crystals were removed by filtration and the filtrate (a sitosterol oxide-rich fraction) was dried under reduced pressure. The epimeric 7-hydroxysitosterols were purified by prep-TLC and subsequently analyzed by HPLC and GC.

Synthesis of radio-labelled sitosterol oxides. 5α,6α-Epoxysitosterol and 5,6-dihydroxysitosterol were prepared from ^{14}C-labelled sitosterol, as described above.

Lipid extraction. One hundred grams of wheat flour were transferred into an Erlenmeyer flask and suspended in 200 mL water. *Iso*-propanol (300 mL) was added and the suspension was shaken vigorously for 5 min. Heptane (300 mL) was added and the mixture was agitated for another 5 min. The mixture was centrifuged for 5 min at 3000 g, and the heptane layer was transferred into a separatory funnel. The residue (pellet and aqueous phase) was transferred into the Erlenmeyer flask and heptane (300 mL) was added. Thereafter, partitioning and centrifugation procedures were repeated as described above. The heptane layer was transferred into the separatory funnel. *Iso*-propanol (100 mL), followed by 100 mL 1.0 M aqueous sodium sulfate, was added and the suspension was shaken. The upper layer, mainly heptane, was transferred into an evaporation flask and the solvent was removed. The residue was dissolved in chloroform and transferred into a glass-stoppered test tube. The sample was centrifuged for 5 min and the supernatant was then transferred into another test tube and stored at −20°C until analysis.

Saponification. A 250-mg sample of wheat flour lipids or a 100-mg sample of soybean oil was transferred into a glass-stoppered test tube and 2 μg 7α-hydroxycholesterol was added as an internal standard. The solvent was removed under a stream of nitrogen. The lipids were dissolved in 1 M potassium hydroxide in dry methanol (20 mL) and incubated at room temperature for 18 hr. Unsaponified material was then extracted with diethyl ether as described elsewhere (17). Prior to Lipidex-5000 chromatography, the solvent was removed under a stream of nitrogen, and the sample was redissolved in 0.3 mL hexane/1,2-dichloroethane (19:1, v/v).

Column packing. Conditioning of Lipidex-5000 with heptane/1,2-dichloroethane (19:1, v/v) and the column packing procedure were the same as described earlier (18).

Thin-layer chromatography. Samples and reference mixtures were dissolved in chloroform and applied on the TLC plate (Silica 60, 20 × 20 cm, 0.5 mm thickness, E. Merck) with the automatic applicator Linomat-III (CAMAG, Muttenz, Germany). The TLC-plate was developed in diethyl ether/cyclohexane (9:1, v/v). For color development, the TLC plate was sprayed with 50% sulfuric acid and heated at 110°C for 5 min (19). In the case of preparative TLC, the reference portion was removed by cutting the plate and spraying with sulfuric acid to locate the sitosterol oxide. The corresponding zone was scraped off, the gel was washed twice with an appropriate volume of chloroform/methanol (2:1, v/v), and the solvent was removed under reduced pressure. Finally, sitosterol oxide was stored in chloroform at −70°C.

With the wheat flour samples, the sterol oxide fraction from NH_2-enrichment was dried under a stream of nitrogen. The sample was then redissolved in chloroform and applied in a 1-cm band on the TLC plate. Sitosterol oxides (5α,6α-epoxy, the epimeric 7-hydroxy and 5,6-dihydroxy) were applied on one side as references, and the plate was developed as described above. The reference part was removed by cutting the plate and spraying with sulfuric acid to localize the 5,6-dihydroxysitosterol and the 5α,6α-epoxysitosterol. The sterol oxide zone, ranging in polarity from the 5,6-epoxides to the 5,6-dihydroxysitosterol, was then scraped off rapidly and transferred to a test tube. The gel was washed with 20 mL chloroform/methanol (2:1, v/v) and the solvent was removed in a rotary evaporator. The residue was redissolved in chloroform and transferred into a glass-stoppered test tube. The sample was kept at −20°C until derivatization.

Gas chromatography and gas chromatography-mass spectrometry (GC/MS). Sterols and sterol oxides were derivatized to TMS-ethers as described earlier (18). GC analysis were carried out on a Varian 3700 gas chromatograph equipped with a falling-needle injector and a flame ionization detector (Varian Associates, Palo Alto, CA). The TMS-ether derivatives were separated on a chemically bonded methyl silicone capillary column (25 m × 0.25 mm, film thickness 0.25 μ, Quadrex Corp., New Haven, CT).

A temperature program was used for sterol analysis: Initial temperature 200°C; initial time 15 min; programming rate 15°C/min; final temperature 275°C; final time 20 min; detector temperature 310°C. Prior to GC analysis, an internal standard (cholestane) was added for quantitative determination of the individual sterols. The percentage composition was calculated after direct normalization. No response factors were determined for the different plant sterols.

Sterol oxide analysis was performed under isothermal conditions at a column temperature of 270°C. Response factors were not estimated for the different TMS-ether derivatives of the sitosterol oxides. They are assumed to be close to 1.00, based on the response factor study for some of the TMS-ether derivatives of the cholesterol oxides (20).

GC-MS analysis was performed on a Finnigan 4000 GC-MS MAT equipped with a falling-needle injector and the above-mentioned capillary column. Operation conditions: Column temperature, 260°C; interface temperature, 280°C; ion source temperature, 280°C. Spectra were obtained by electron impact ionization at 40 eV and a scan rate of 1 sec/scan.

High-performance liquid chromatography. The HPLC system consisted of a Spectra Physics (SP) model 8700 solvent delivery system, a model SP 8700 pump, a model R 401 Waters differential refractometer, and a Rheodyne injector (Spectra Physics, San Jose, CA) fitted with a 50-μL sample loop. The analysis was performed on a Lichrosorb CN column (250 × 4 mm, 5 μ, E. Merck) with hexane/*iso*-propanol (95:5, v/v) at a flow rate of 0.5 mL/min.

Liquid scintillation. The samples were transferred into 10-mL plastic vials, and the solvent was removed under a stream of nitrogen. Six milliliters PCS/xylene (2:1, v/v) were added, and radioactivity was measured with a Beckman L-230 Liquid Scintillation System (Fullerton, CA).

RESULTS

Characterization of synthetic sitosterol oxides. Sitosterol oxides, including the epimeric 7-hydroxysitosterols, the isomeric 5,6-epoxysitosterols and 6,5-dihydroxysitosterol, were prepared as described under Materials and Methods. The compounds were chromatographed as single spots on a Silica 60 TLC plate with diethyl ether/cyclohexane (9:1, v/v). The sitosterol oxides and the corresponding cholesterol oxides had similar R_f values when analyzed as single compounds or as a mixture (2,21,22). HPLC on a CN column with hexane/*iso*-propanol (95:5, v/v) also indicated that the sitosterol oxides were virtually pure. Figure 1 shows the chromatographic pattern of the synthetic sitosterol oxides as TMS-ether derivatives obtained by capillary GC.

Structure assignments of the synthetic sitosterol oxides are supported by GC-MS analysis. In the mass spectra of the isomeric 5,6-epoxysitosterols, the highest signal at *m/z* 502 assigned as the molecular ion [M^+]. The peaks at 487, 484, 412, 397 and 369 are equivalent to [M − CH_3], [M − H_2O], [M − TMS], [M − CH_3 − TMS] and [M − CH_3 − C_2H_5 − TMS]. Mass spectra of the epimeric 7-hydroxysitosterols contained only a few distinct signals. These were 574, 484, 469, 394 and 379, referring to [M^+], [M − TMS], [M − CH_3 − TMS], [M − 2TMS], and [M − CH_3 − 2TMS], respectively. In the case of 5,6-dihydroxysitosterol, no signal was observed at *m/z* 664 = [M^+]. However, the mass spectra displayed several distinct fragments at *m/z* 574, 559, 484, 469, 433 and 394, corresponding to [M − TMS], [M − CH_3 − TMS], M − 2TMS], [M −CH_3 − 2TMS], [M − C_2H_5 − TMS] and [M − 3TMS], respectively. The fragmentation patterns for the sitosterol oxides were identical to those obtained for the corresponding cholesterol derivatives; however, the signals were 28 mass units higher. Similar observations

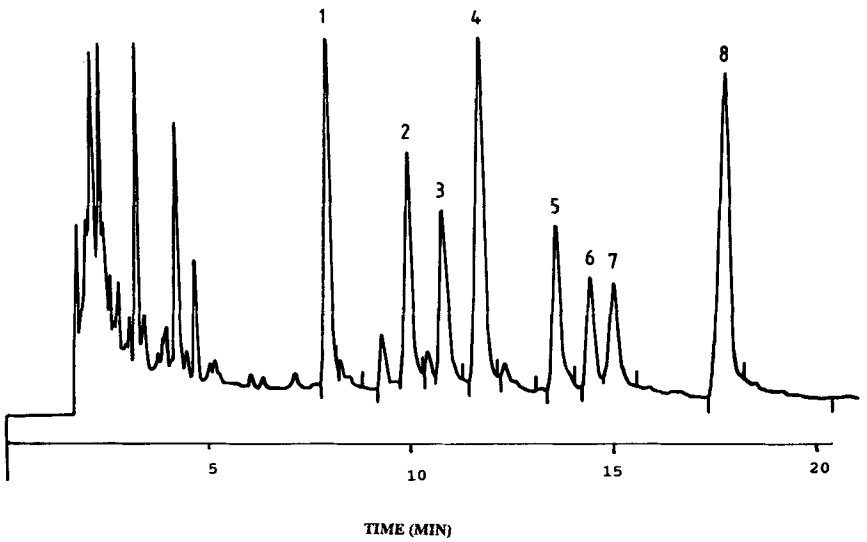

FIG. 1. Separation of sitosterol oxides as TMS-ether derivatives on a capillary methyl silicone column. 1, Cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol); 2,24α-methyl-cholest-5-ene-3β-ol (campesterol); 3,24α-ethyl-cholest-5-ene-3β,7α-diol (7α-hydroxy-β-sitosterol); 4,24α-ethyl-cholest-5-ene-3β-ol (β-sitosterol); 5,24α-ethyl-cholest-5-ene-3β,7β-diol (7β-hydroxy-β-sitosterol); 6,24α-ethyl-5,6β-epoxy-5β-cholestane-3β-ol (5β,6β-epoxy-β-sitosterol); 7,24α-ethyl-5,6α-epoxy-5α-cholestane-3β-ol (5α,6α-epoxy-β-sitosterol); and 8,24α-ethyl-5α-cholestan-3β,5,6β-triol (5,6-dihydroxy-β-sitosterol).

are reported by other investigators (21–23).

Identities of the relevant peaks in the food samples were established by comparing the relative retention time values and mass spectra (200–750 *m/z*) to those of synthetic sitosterol oxides. In the present study, mass spectra of all sitosterol oxides in food samples were apparently identical to those of the corresponding synthetic compounds. This implies that the signals recorded by the flame ionization detector predominantly (if not completely) correspond to the TMS-ether derivative of the sitosterol oxides.

Modification of a method for sterol oxide analysis. In a previous study, complete removal of the triacylglycerols and the major part of cholesterol from egg yolk lipids was attained by using a hydrophobic derivative of Sephadex, Lipidex-5000 (18). In the present study, an olive oil sample, spiked with 5α,6α-epoxysitosterol, was analyzed. Olive oil was selected as a model system for method development because sitosterol is the predominant sterol and 90% of the total sterols are in the free form (24). GC analysis of the sterol oxide fraction revealed the presence of substantial levels of the major "native" sterols (sitosterol, stigmasterol, and campesterol). Therefore, different proportions of heptane/1,2-dichloroethane were explored to obtain a sufficient separation between the native sterols and the pair of 5,6-epoxysitosterols, which is the critical part of the method. TLC analysis of the eluents of the Lipidex-5000 column indicated that heptane/1,2-dichloroethane in the proportion 19:1 (v/v) gave optimal separation of the compounds of interest. The efficiency of the chromatographic system was established with ^{14}C-sitosterol and ^{14}C-5,6-epoxysitosterol. Radioactivity measurement revealed that 95% (n = 2) of the sitosterol was recovered from the native sterol fraction. With the ^{14}C-5α,6α-epoxysitosterol, 99% (n = 2) of the labelled compound was detected in the sterol oxide fraction. TLC analysis of the sitosterol oxide fraction indicated the presence of a substantial amount of polar compounds at the origin. Hence, further purification of the sterol oxide fraction was of great importance in increasing the sensitivity during GC analysis and in prolonging the life of the capillary column.

A rapid enrichment of the sterol oxide fraction from the Lipidex-5000 chromatography was obtained with a NH_2-cartridge (13). Gravimetric analysis of the eluent showed that 70% of the load was retained on the column during this enrichment step. Efficiency of the combined Lipidex-5000 and NH_2-cartridge system was studied with ^{14}C-5α,6α-epoxysitosterol and ^{14}C-5,6-dihydroxysitosterol. Recovery of the 5α,6α-epoxysitosterol was 85% (n = 2), while that for 5,6-dihydroxysitosterol was 88% (n = 2). Thus, it appears that the use of Lipidex-5000 chromatography followed by NH_2-enrichment is an effective procedure for the quantitative determination of the composition of the free sterols and the autoxidation products from such sterols.

Sitosterol oxides in plant-based food. For analysis of the autoxidation products from free sterols, 2 μg 7α-hydroxycholesterol (internal standard) was added to 100 mg lipids. The solvent was removed under a stream of nitrogen and the residue was then redissolved in 3 mL of the actual chromatographic solvent. A scheme for the isolation of the native sterol and sterol oxide fractions is shown in Figure 2.

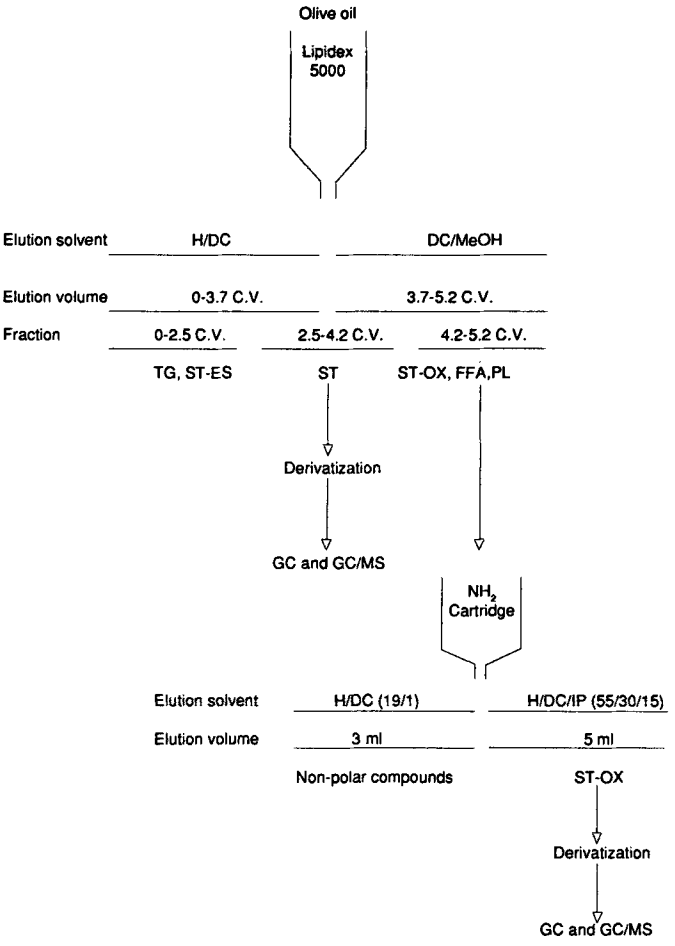

FIG. 2. Isolation and final determination of sitosterol oxides from plant-based food. H, heptane; DC, dichloroethane; MeOH, methanol; C.V., column volume; TG, triacylglycerols; ST-ES, sterol esters; ST, sterols; ST-OX, sterol oxides; FFA, free fatty acids; PL, phospholipids.

The effect of industrial processing, such as refining, deodorization and storage, on the level of sitosterol oxides was studied in different batches of soybean oil. Analysis of crude soybean oil (peroxide value = 0.4; anisidine value = 0.6) revealed no detectable levels of any of the sitosterol oxides of interest at the detection level of 0.2 ppm. The peroxide value (PV) is a measure of the primary oxidation products from fatty acids of the triacylglycerols, whereas the anisidine value (AV) is a measure of the secondary oxidation products. Freshly refined soybean oil under conditions similar to industrial processing (PV = 0.0; AV = 1.1) showed no increase in the levels of the sitosterol oxides when compared to the control. Analysis of a refined soybean oil sample (stored for one year at 4°C, PV = 21; AV = 4) revealed no quantifiable levels of any of the sitosterol oxides.

The effect of cold saponification on formation of artifacts and/or release of bound sterols was studied with freshly refined soybean oil. GC analysis of the sterol oxide fraction revealed no detectable levels of any of the sitosterol oxides of interest at the detection level of 0.2 ppm. However, an unknown peak was detected immediately after the elution region of the 5α,6α-epoxysitosterol. Mass spectra of the unknown compound indicated

the absence of peaks at *m/z* higher than 576. In addition, the mass spectrum contained several characteristic peaks for sitosterol oxides analyzed in this study. No further attempt was made to elucidate the structure of the unknown compound. Analysis of the free sterol fraction revealed that sitosterol (54%), campesterol (24%), and stigmasterol (22%) were the major components, followed by Δ5-avenasterol (1%). The ratio of esterified to free sterol was 1:2.7, as calculated from analysis of free and total sterols. The sterol pattern and the ratio of esterified to free sterols are in close agreement with an earlier report (10).

To study the effect of manufacturing and storage on the generation of the sitosterol oxides in wheat flour, three different samples, at 2, 8 and 36 months, were analyzed. The 2 and 8 months samples contained quantifiable levels of the isomeric 5,6-epoxysitosterols and epimeric 7-hydroxysitosterols (Table 1). The 36 month sample contained substantial amounts of total oxides. The 7β-hydroxysitosterol was the major product followed by the α epimer, the 5α,6α-epoxysitosterol, and the 5β,6β-epoxyisomer. The 7-keto- and 5,6-dihydroxysitosterols were not analyzed. It is worth noting that peaks that could correspond to the epimeric 7-hydroxycampesterols, based on R_t and GC-MS spectra, were observed in the chromatograms of wheat flour. However, no attempt was made to quantitate any of the campesterol oxides in the wheat flour samples.

Earlier data concerning the sterol pattern of wheat lipids are conflicting (25). In the present study, analysis of the sterol profile showed that sitosterol was the major sterol, followed by campesterol and Δ5-avenasterol, at 67%, 22% and 8%, respectively. The ratio of esterified to free sterol was 1:1 as calculated from analysis of free and total sterols. Other minor compounds were present at less than one percent. The data regarding sterol pattern and the ratio of esterified to free sterols are in line with those reported by Morrison (26).

Two olive oil samples and two potato granular samples were analyzed by TLC after chromatography on Lipidex-5000 and the NH_2-cartridge system. A freshly opened olive oil sample contained no detectable levels of any of the sitosterol oxides, whereas traces of the epimeric 7-hydroxysterols were detected in a sample stored at room temperature for approximately 30 months. Analysis of the potato granular samples, stored at 35°C for 13 and 36 months, respectively, indicated the presence of substantial amounts of the epimeric 7-hydroxysterols. The sitosterol oxides in the potato granular samples were not quantitated due to the high content of galactolipids, which were 20% of total lipids (27).

TABLE 1

Content of Sitosterol Oxides in Wheat Flour

Age (months)	Sitosterol oxides, ppm, in lipids[a,b]			
	5α,6α-epoxy	5β,6β-epoxy	7α-hydroxy	7β-hydroxy
2	7.1/6.0	3.0/3.5	11.2/12.6	13.6/13.4
8	5.6/4.8	TR[c]/TR	9.9/8.7	8.3/11.2
36	57/53	29/29	119/118	124/129

[a]Data represents two single analyses.

[b]Fat content was 1.4%.

[c]TR, trace, detection limit 0.2 ppm in lipids.

DISCUSSION

The analytical data presented herein indicate that industrial processing (*viz.*, chemical refining and deodorization) of soybean oil causes no increase in the level of the autoxidation products of the free sitosterol compared to the crude soybean oil at the detection level of 0.2 ppm. Furthermore, the present investigation suggests that free sitosterol is stable during prolonged storage of soybean oil at 4°C. However, if sitosterol undergoes autoxidation, the process is probably slow compared to that of unsaturated fatty acids.

Wheat flour contains substantial amounts of sterylglucoside and esterified sterylglucoside (25,26). These are not hydrolyzed under alkaline conditions. In order to remove the sterylglucosides and the esterified sterylglucoside derivatives, it was necessary to include preparative TLC as a final purification step before GC analysis. A 2-month-old wheat flour sample was found to contain 35 ppm of total oxide, including the isomeric 5,6-epoxysitosterol and the epimeric 7-hydroxysitosterols in the lipids (Table 1). Eight- and 36-month samples contained 24 and 328 ppm of total sitosterol oxides in the lipids, respectively. The 7-keto- and 5,6-dihydroxysitosterols were not analyzed. It should be noted that the samples were not from a planned storage experiment but that they represent different batches. Nine wheat flour samples were analyzed after hot saponification followed by preparative TLC (6). The levels of the isomeric 5,6-epoxysitosterols were 100 to 200 ppm in the lipids, and the levels of the epimeric 7-hydroxysitosterols ranged from 120 to 400 ppm. The 5,6-dihydroxysitosterol was not analyzed. Information concerning the age of samples and storage conditions was not presented (6). Comparison of the data reported in the present study to those reported in (6) should be done with great caution because of differences in the analytical procedures used. The relative prevalence of the major sitosterol oxides in wheat flour is similar to those reported for the autoxidation products from cholesterol in dehydrated egg yolk products and milk powder products, with the epimeric 7-hydroxy derivatives as dominating compounds (18,28). However, further studies should be conducted to study the effect of different varieties, manufacturing parameters and storage conditions on the levels of sitosterol oxides in dry foods of vegetable origin, such as flours and other cereal products.

Quantitation of the total sterol oxides is important in estimating the contribution from the esterified sterol oxides to the total oxide level. Nourooz-Zadeh (13) developed a mild enzymatic method for the hydrolysis of the triacylglycerols followed by enzymatic release of the esterified sterols. The method is too cumbersome and tedious to be used for routine purposes. Hot saponification causes destruction and/or artifact formation (29,30). On the other hand, Park and Addis (17) reported a complete recovery of cholesterol oxides after incubation with 1 M potassium hydroxide in dry methanol for 18 hr at room temperature. This investigation reveals that cold saponification can be suitable as a preconcentration step before column chromatography. However, the presence of an unknown compound with mass spectra indicative of a hydroxysterol derivative makes it premature to fully recognize cold saponification of esterified "plant sterols" as safe.

ACKNOWLEDGMENTS

We would like to thank the Swedish Council for Agricultural and Forestry Research for financial support; Margarinbolaget AB, Helsingborg, Sweden (Hans Gran) for providing the soybean oil samples and carrying out the peroxide value and anisidine value analyses; and Nord Mills, Uppsala, Sweden for the wheat flour samples.

REFERENCES

1. Yanishlieva-Maslarova, N., H. Schiller and A. Seher, *Fette Seifen Anstrichm. 84*:308 (1982).
2. Daly, G.C., T. Finocchiaro and T. Richardson, *J. Agric. Food Chem. 31*:46 (1983).
3. Yanishlieva, N., and H. Schiller, *J. Sci. Food Agric. 35*:219 (1983).
4. Imai, H., N.T. Werthessen, V. Subramanyan, P.W. LeQuesne, A.H. Soloway and M. Kanisawa, *Science 207*:651 (1980).
5. Peng, S.K., C.B. Taylor, E.H. Mosbach, W.Y. Huang and B. Mikkelson, *Atherosclerosis 41*:395 (1982).
6. Finocchiaro, T., Ph.D. Thesis, University of Wisconsin-Madison, 1983, pp. 109–141.
7. Smith, L.L., *Cholesterol Autoxidation*, Plenum Press, NY, 1981, pp. 369–458.
8. Peng, S.K., and C.T. Taylor, *World Rev. Nutr. Diet. 44*:117 (1984).
9. Addis, P.B., *Food Chem. Toxic. 24*:1021 (1986).
10. Kochhar, S.P., *Prog. Lipid Rev. 22*:161 (1983).
11. Lee, K., A.M. Herian and N.H. Highly, *J. Food Prot. 48*:158 (1985).
12. Fieser, L.F., and M. Fieser, *Reagents for Organic Synthesis*, Vol. 1, Wiley, NY, 1967, pp. 136–137.
13. Nourooz-Zadeh, J., *J. Agric. Food Chem. 38*:1667 (1990).
14. Fieser, L.F., and S. Rajagopalan, *J. Am. Chem. Soc. 71*:3938 (1949).
15. Davis, M., and V. Petrow, *J. Chem. Soc. London 4*:2536 (1949).
16. Chicoye, E., W.D. Powrie and O. Fennema, *Lipids 3*:335 (1968).
17. Park, S.W., and P.B. Addis, *J. Agric. Food Chem. 34*:653 (1986).
18. Nourooz-Zadeh, J., and L.A. Appelqvist, *J. Food Sci. 52*:57 (1987).
19. Smith, L.L., W.S. Mathews, J.C. Price, R.C. Bachman and S.B. Reylond, *J. Chromatography 27*:187 (1967).
20. Park, S.W., and P.B. Addis, *Anal. Biochem. 149*:275 (1985).
21. Aringer, L., and P. Eneroth, *J. Lipid Res. 14*:563 (1973).
22. Aringer, L., and P. Eneroth, *Ibid. 15*:389 (1974).
23. Aringer, L., and L. Nordstrom, *Biomed. Mass Spectrometry 8*:183 (1981).
24. Dimitrios, B., and V. Ioanna, *Lebensm. Wiss. Technol. 19*:156 (1986).
25. MacMurray, T.A., and Morrison, W.R., *J. Sci. Food Agric. 21*:520 (1970).
26. Morrison, W.R., *Cereal Chem. 55*:548 (1978).
27. Lilja, M., and H. Lingnert, *Food Chem. 31*:267 (1989).
28. Nourooz-Zadeh, J., and L.A. Appleqvist, *J. Food Sci. 53*:74 (1988).
29. Tsai, L.S., K. Ijichi, C.A. Hudson and J.J. Meehan, *Lipids 15*:124 (1980).
30. Maerker, G., and J. Unruh, *J. Am. Oil Chem. Soc. 63*:767 (1986).

[Received March 3, 1991; accepted December 19, 1991]