ANALYSIS OF STERYL GLUCOSIDES IN FOODS AND DIETARY SUPPLEMENTS BY SOLID-PHASE EXTRACTION AND GAS CHROMATOGRAPHY

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ABSTRACT

A method to quantify β-sitosterol (BSS) and β-sitosteryl glucoside (BSSG) in dietary supplements containing added BSS and BSSG was developed and also validated for the analysis of naturally occurring free and esterified sterols and steryl glucosides in foods. Steryl glucosides were extracted with hexane-diethyl ether (1 : 1, v/v) after the alkaline saponification of a powdered supplement or of total lipid extracts of whole wheat flour, granola bars, soybeans, flaxseed, dried figs, peanut butter, lyophilized mixed vegetables, almonds, pine nuts and a diet homogenate. An oil-based supplement was analyzed directly. Steryl glucosides were isolated by solid-phase extraction, derivatized and quantified as trimethylsilyl ethers using capillary gas chromatography (GC) with a 5% diphenyl–95% dimethylpolysiloxane column. Recovery studies using a commercially available mixed steryl glucoside standard and gas chromatography-mass spectrometry (GC-MS) analyses validated the method. Approximately 2–36 mg/100 g (9–37%) of total sitosterol, campesterol and stigmasterol in the foods was contributed by the glucosides.

INTRODUCTION

Phytosterols have been the subject of much interest with respect to their hypocholesterolemic effect and have also demonstrated anticarcinogenic and

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immune-modulating properties (Ling and Jones 1995; Pegel 1997; Bouic 2001; Moreau *et al.* 2002). Sterols in foods exist as free sterols, glycosides, fatty acid esters and acylated steryl glycosides (Grunwald and Huang 1989). Data from Jonker *et al.* (1985) suggest that up to 80% of total phytosterols are glycoslyated in foods. Toivo *et al.* (2001) showed that whole grains have a high proportion of steryl glycosides, for example, 25% in whole wheat flour and 55% in cornmeal. Sterol glucosides have also been added to dietary supplements designed to improve lipid metabolism and immune function (Bouic *et al.* 1999; St.-Onge and Jones 2003). However, routine analytical methods which rely on alkaline saponification followed by the measurement of free sterols fail to detect steryl glycosides, because the acetal bond is stable under those hydrolytic conditions. Resulting food composition data, therefore, have the potential to underestimate the total phytosterol content of many foods.

While acid hydrolysis will cleave the glycosidic bond and has been used by some researchers to analyze total sterols in foods (Jonker *et al.* 1985; Normen *et al.* 1999; Piironen *et al.* 2002), the liberated free sterols are not distinguished from free or esterified sterols in the subsequent analysis of total sterols. When relatively low levels of glucosides are present, their quantitation as the difference in total sterols with and without acid hydrolysis may be quite unreliable. Step-wise solid-phase extraction can be used to preseparate glycosidic, free and esterified sterols (Breinhölder *et al.* 2002), but if the glycoside fractions are subsequently subjected to acid hydrolysis before analysis as free sterols, isomerization of some sterols occurs (Kamal-Eldin *et al.* 1998). This limitation is particularly relevant for foods containing Δ^5-avenasterol – for example, wheat germ (Piironen *et al.* 2002), ginseng seed (Beveridge *et al.* 2002) and walnuts (Amaral *et al.* 2003).

In the 1970s, Elbein *et al.* reported on the direct gas chromatographic (GC) analysis of steryl glycosides using a packed column and mass spectrometry (MS) for detection (Laine and Elbein 1971; Elbein and Forsee 1975), and more recently Gutierrez and Del Rio (2001) applied GC-MS to the analysis of steryl glycosides in wood products. The direct analysis of steryl glycosides avoids acid hydrolysis and associated acid-catalyzed isomerization. Furthermore, keeping the glycosides intact permits the use of MS for the characterization of the molecular species. The goal of the present study was to develop and validate a precise and accurate method to quantify β-sitosterol (BSS) and β-sitosteryl glucoside (BSSG) in dietary supplements containing added BSS and BSSG and to extend the methodology to quantitation of naturally occurring free and esterified sterols and steryl glycosides in foods.

MATERIALS AND METHODS

Samples

Capsules of a powdered dietary supplement (Moducare®, Essential Phytosterolins Inc., Ontario, Canada) and an oil-based dietary supplement (Moducare-enriched olive oil, Essential Phytosterolins Inc., Ontario, Canada), both reported to contain a 1 : 100 ratio of BSSG : BSS, were analyzed. To ensure a representative subsampling of the oil, a stock solution was first prepared from each of the three bottles (each containing approximately 30 mL product). After vigorously shaking the bottle for ~30 s, a 5-g aliquot was transferred to a 250-mL volumetric flask, diluted to volume with 2 : 1 (v/v) chloroform-methanol and mixed thoroughly. Each stock solution (20 mg/mL) was dispensed in 2-mL aliquots among fifteen 50-mL test tubes, blanketed with nitrogen and stored up to 23 days at 0–8C before analysis. The remaining stock solution was discarded.

Food samples were prepared as described below, dispensed in 15- to 30-g aliquots among 30-mL or 60-mL glass jars with Teflon™-lined lids, capped under nitrogen and stored at −60 ± 5C. Whole wheat flour (General Mills, Minneapolis, MN) and peanut butter (Peter Pan® creamy, ConAgra Brands, Inc., Hunt-Wesson, Inc, Fullerton, CA) were purchased locally and used without further preparation. Ground flaxseed (*Linum usitatissimum*) was a composite of samples from two different brands (Bob's Red Mill, Milwaukie, OR and Hodgson Mill, Effington, IL) sampled from 5 retail outlets across the U.S. Granola bars (Nature Valley® 100% Natural Crunchy Oat and Honey, General Mills, Minneapolis, MN), dried California mission figs (*Ficus carica*) (Sunmaid®, Kingsburg, CA), pine nuts (*Pinus koraiensis*) (Nature's Finest, Rema Foods, Englewood Cliff, NJ) and others from bulk grocery purchase were composites of samples from 2 to 11 retail outlets across the U.S. and were frozen in liquid nitrogen, then homogenized using a 6-L commercial food processor (Blixer, Robot Coupe U.S.A., Jackson, MS). Raw almonds (*Prunus dulcis*) were a composite from three sources (Poindexter Nut Co., Selma, CA; Hines Nut Co., Dallas TX; and Kroger, Cincinnati, OH) and were ground with a KnifeTec 1095 sample mill (Foss Tecator AB, Hillerød, Denmark) after being frozen in liquid nitrogen. Raw soybeans (*Glycine max*) (Dahlgren and Company, Inc., Crookston, MN) were frozen in liquid nitrogen and ground with a coffee mill. A diet homogenate was prepared from a mixture of daily menus (Svetkey *et al.* 1999) using methods described elsewhere (Phillips *et al.* 2001). Canola oil (Smart Beat®, GFA brands, Inc., Cresskill, NJ) was purchased and dispensed in 5-mL aliquots into 12-mL test tubes that were blanketed with nitrogen and stored at −15 ± 5C; one aliquot was analyzed with each batch of samples as an in-house control sample for free sterols.

Reagents and Standards

Reagents and standards used for lipid extraction and sterol analysis have been described elsewhere (Phillips *et al.* 2002). A mixed steryl glucoside standard was obtained from Matreya (Pleasant Gap, PA).

Reference materials were purchased from RT Corporation in Laramie, WY and included anhydrous milkfat fortified with phytosterols (CRM 164; Pocklington *et al.* 1993) and lyophilized mixed vegetables (sweet corn, carrots and tomatoes) (CRM 485; Finglas *et al.* 1998).

Extraction and Analysis of Free Sterols and Steryl Glucosides

Solid phase extraction (SPE) was used to separate free and esterified sterols from steryl glucosides. Total lipids were extracted from food samples (0.85–6.0 g) (Phillips *et al.* 1997), and a suitable portion of the extract (10–50 mL) was dried under nitrogen at 50 ± 2C and reconstituted with 1-mL chloroform before SPE. In some cases (dried figs, peanut butter and lyophilized mixed vegetables), the lipid extract aliquot was filtered before SPE using a 0.45-µm syringe filter (Varian, Inc., Harbor City, CA). The powdered dietary supplement was directly saponified, as described below, because of fine particulates which made the recovery of the total lipid extract impractical. The oil-based dietary supplement was taken through SPE directly. A silica SPE cartridge (Varian, Inc., Harbor City, CA) was conditioned with one-column-volume chloroform, then the sample was quantitatively transferred to the cartridge using 2 × 0.5 mL chloroform rinses. An additional 1-mL chloroform was used to complete the elution of free sterols, and steryl glucosides were then eluted with 2 × 1 mL methanol.

The steryl glucoside fraction was dried under nitrogen at 50 ± 2C, derivatized with 0.500-mL 1 : 1 (v/v) pyridine/bis (trimethylsilyl) trifluoroacetamide with 1% (w/v) trimethylchlorosilane (BSTFA/TMCS) (Thompson and Merola 1993) and assayed by GC with flame ionization detection (GC-FID) for steryl glucosides under the following conditions: RTX-5 column (5% diphenyl–95% dimethylpolysiloxane; 15 m, 0.32-mm ID, 0.25-µm film; Restek Corporation, Bellefonte, PA); 1.0-µL injection volume; helium carrier gas at 0.98 mL/min (~20 cm/s linear velocity); 10 : 1 split ratio; oven temperature 290C; injector temperature 280C; detector temperature 310C. A 4-point external standard calibration curve was prepared for sitosteryl glucoside using a BSTFA/TMCS-derivatized mixed steryl glucoside standard. The quantitation of all steryl glucosides was based on the sitosteryl glucoside standard curve.

The free sterol fraction was diluted and subsampled if necessary (to place analytes within the range of the standard curve), combined with 25-µg epicholesterol (internal standard), then saponified at 85–89C for 20 min in 8.5-mL aqueous 1.3-N potassium hydroxide containing 2.8% (w/v) pyrogallol as

an antioxidant (Phillips *et al.* 1999). Unsaponifiables were extracted with 20-mL cyclohexane (12-mL water added), dried under nitrogen at 50 ± 2C, derivatized using 0.25-mL BSTFA/TMCS (described above for glucoside fraction), then assayed by GC-FID under the following conditions: RTX-5 column (Restek); 0.5-µL injection volume; helium carrier gas at 0.58 mL/min (19.7-cm/s linear velocity); 17 : 1 split ratio; oven temperature 270C; injector temperature 280C; detector temperature 300C. A 7-point sitosterol calibration curve was used for quantification, with all sterols calibrated by reference to sitosterol.

After the direct saponification of the powdered dietary supplement, the unsaponifiables were extracted twice with 20-mL cyclohexane (12-mL water added) for BSS or twice with 20-mL (1 : 1, v/v) diethyl ether/hexane (12-mL water added) for BSSG. The cyclohexane extract was diluted and subsampled if necessary, transferred to a tube with 25-µg epicholesterol (internal standard), dried under nitrogen at 50 ± 2C and then derivatized and analyzed by GC as described above for BSS. The diethyl ether/hexane extract was dried under nitrogen at 50 ± 2C and then derivatized and analyzed by GC as described above for BSSG.

Gas Chromatography-Mass Spectrometry of Steryl Glucosides

Steryl glucosides were analyzed by GC-MS using a Hewlett Packard 5890 series 2B GC and Hewlett Packard 5972 mass selective detector with electron impact ionization, with the following conditions: RTX-5MS column (5% diphenyl–95% dimethylpolysiloxane; 15-m, 0.25-mm ID, 0.1-µm film; Restek); 1.0-µL injection volume; helium carrier gas at 1.05 mL/min; split ratio 1 : 10; injector temperature: 290C; oven temperature: 275–290C at 2C/min, hold 40 min; transfer line temperature 290C, electron energy: 70 eV; scan range: 35–550 amu at 1.4 scan/s.

Determination of Sitosteryl Glucoside Content of Mixed Steryl Glucoside Standard

A mixed steryl glucoside standard from Matreya (catalog #1117, lot #21094; Matreya, Inc., Pleasant Gap, PA) was used to prepare calibration standards for analyses. This standard was reported by the manufacturer to contain 98% minimum total steryl glucosides but was not provided with an exact concentration of sitosteryl glucoside. The sitosteryl glucoside content was thus quantitatively determined as follows: The trimethylsilyl derivatization and GC analysis of the material was done as described above for 19 individual GC runs of the standard at two different concentrations. The GC-MS confirmed the identity of sitosteryl glucoside. Three peaks (sitosteryl, campesteryl and stigmasteryl glucosides) were expected on the basis of the supplier's data; however, a fourth peak was evident at the higher concentra-

tions and eluted immediately after sitosteryl glucoside and comprised 1.4% of the total peak area. The GC-MS analysis confirmed that this compound was a steryl glucoside and was presumed to be Δ^5-avenasteryl glucoside (see Results section). On the basis of the peak area of the sitosteryl glucoside as percent of total steryl glucosides, the sitosteryl glucoside was calculated to constitute 55.92% (0.2% relative standard deviation [RSD]) by weight of the mixed glucoside standard. The concentration of sitosteryl glucoside in standards prepared from the mixed steryl glucoside product was thus calculated by adjusting for 98% purity and then by multiplying by 0.5592.

Method Validation and Quality Control

Recovery studies using sitosterol standard or canola oil added to selected sample matrices and canola oil added to almonds, as well as an analysis of commercial reference materials, were performed to validate the recovery of free sterols. Given the lack of commercially available reference materials for individual steryl glucosides, the validation of the sitosteryl glucoside determination relied on recovery studies using the mixed steryl glucoside standard added to each sample matrix.

With selected exceptions, 2–7 replicates of each material were assayed. An in-house quality control material (canola oil) was included in each assay batch to monitor the run-to-run precision and accuracy of the free sterol analysis. For the glucoside assay, an aliquot of a separately prepared stock solution of the mixed steryl glucoside standard was derivatized and analyzed in each GC run as a quality control check to validate the quantitation by the external calibration curve. For the analysis of the foods, a sample of the peanut butter was included in each assay batch as a control material to monitor the run-to-run precision of the glucoside assay.

Statistical Analysis of Data

Means, standard deviations and standard errors were calculated using Quattro® Pro (version 8.8.0.393; Corel Corporation, Ottawa, ON). To evaluate whether additional sterols were recovered when steryl glucosides were included in the analysis, a paired *t*-test was performed on the data for each food, using the SAS univariate procedure (SAS System for Windows, Release 8.02 TS Level 02M0; SAS Institute, Cary, NC).

RESULTS

Method Validation and Quality Control

The sitosterol concentration assayed in the commercial reference material (anhydrous milkfat, BCR 164 [Community Bureau of Reference,

TABLE 1.
RECOVERY OF SITOSTEROL AND SITOSTERYL GLUCOSIDE FROM DIETARY SUPPLEMENTS AND SELECTED FOODS

Product	Sitosterol glucoside (% recovery) Mean	SD (*n*)	Sitosterol (% recovery) Mean	SD (*n*)
Dietary supplement, powdered	102	1.4 (4)	96	3.1 (3)
Dietary supplement, oil-based	101	7.3 (5)	99	1.1 (4)
Whole wheat flour	96	6.2 (2)	n/a	n/a
Granola bars	99	1.5 (2)	n/a	n/a
Lyophilized mixed vegetables*	98	3.0 (2)	n/a	n/a
Almonds†	n/a	n/a	100	2.8 (4)

* CRM 485, Finglas *et al.* (1998).
† Almonds were spiked with a canola oil that was used as an in-house control material for free sterol analysis.
SD, standard deviation; n/a, not available.

European Commission, Brussels, Belgium]) (mean, 53.0 mg/100 g; 1.29 mg/100 g standard deviation [SD], $n = 11$) was within the certified 95% confidence interval of 48.9–56.9 mg/100 g. The data for sitosterol in the canola oil in-house control material were consistent over the entire period of sample analysis with an RSD of 1.2% ($n = 16$). The recovery of sitosterol and sitosteryl glucoside from the dietary supplements and from the selected food matrices was >96% in all cases (Table 1). The results for the separately prepared standard stock solution of steryl glucosides (that was analyzed in each GC run) for sitosteryl glucoside ranged from 0 to 5.3% deviation from the expected concentration (mean 2.2, $n = 9$), indicating acceptable quantitation by the external calibration curve.

Sterol Composition of Samples

Representative GC-FID traces for the selected samples are shown in Fig. 1. The concentrations of BSS and BSSG measured in the powdered and oil dietary supplements and food samples are summarized in Table 2. The assayed concentrations yielded ratios of 0.0090 and 0.0094 for BSSG : BSS, confirming the expected ratio of 1 : 100 within acceptable analytical tolerance limits. The concentrations of individual phytosterols contributed by glucosides in the food products are summarized in Table 3, where the assayed values for the steryl glucosides have been converted to equivalent free sterol concentration. The nut, seed and legume samples had the highest concentration of steryl glucosides, with sitosteryl glucoside ranging from 23 to 46 mg/100 g. Whole wheat flour and granola bars also had a notable content of sitosteryl glucoside (9–10 mg/100 g) and other steryl glucosides. The proportion

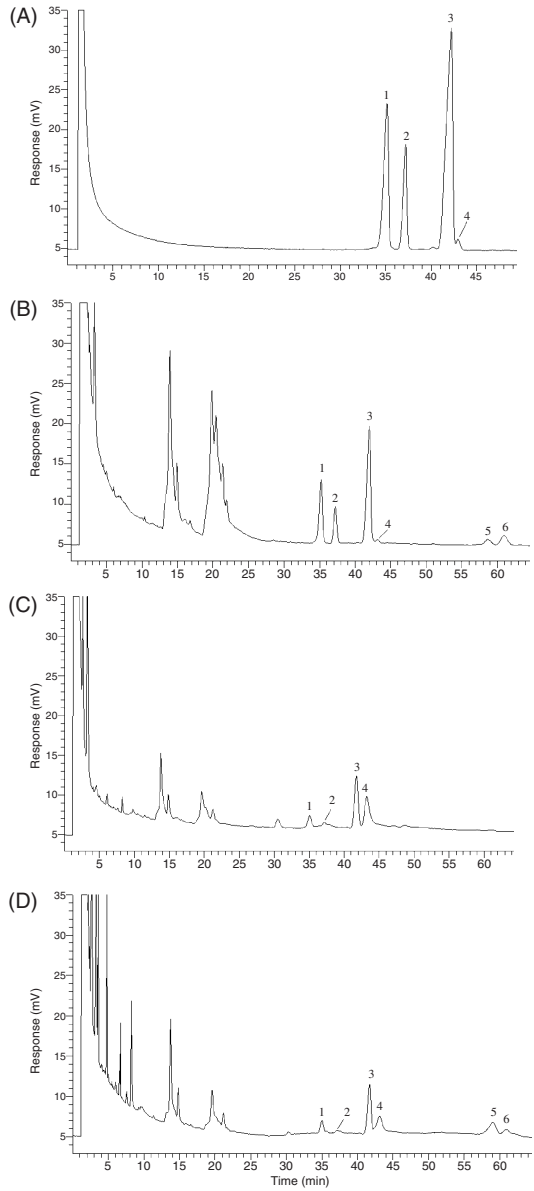

FIG. 1. REPRESENTATIVE GC-FID CHROMATOGRAMS FOR STERYL GLUCOSIDES IN (A) STANDARD MIXTURE, (B) SOYBEANS, (C) GRANOLA BAR AND (D) WHOLE WHEAT FLOUR

(1) Campesteryl glucoside, (2) stigmasteryl glucoside, (3) sitosteryl glucoside, (4) Δ^5-avenasteryl glucoside (presumptive ID), (5) and (6) unidentified glucose derivatives.

TABLE 2.
β-SITOSTEROL (BSS) AND β-SITOSTERYL GLUCOSIDE (BSSG) CONTENT OF DIETARY SUPPLEMENTS

Product	BSS (mg/100 g)		BSSG (mg/100 g)		BSSG : BSS ratio
	Mean	SD (*n*)	Mean	SD (*n*)	
Dietary supplement, powdered	3584	197 (12)	33.7	1.3 (3)	0.0094
Dietary supplement, oil-based	5902	159 (9)	52.9	1.3 (5)	0.0090

SD, standard deviation.

of glycosylated conjugates was similar for all sterols, comprising 9–37% of the total sterol content in the foods analyzed.

GC-MS Analysis of Steryl Glucosides

The primary focus of this work was the major naturally occurring food sterols, sitosterol, campesterol and stigmasterol. The GC-MS analysis of steryl glucoside fractions confirmed the identity of sitosteryl, campesteryl and stigmasteryl glucoside in all foods analyzed. Each compound was identified on the basis of mass spectrum and retention time comparison. Figures 2–4 show the mass spectra of steryl glucoside peaks in soybeans. Masses 451, 361, 305, 217, 204 and 147 were observed, all of which are characteristic tetratrimethylsilyl glucose ion fragments resulting from the cleavage of the (C—O) bond in steryl glucosides (Laine and Elbein 1971). Masses of 383, 395 and 397 are parent ions for the campesterol, stigmasterol and sitosterol moieties, respectively, after cleavage of the (C—O) bond. Ions at 255 and 129, which are characteristic fragments of the steroid moiety (Laine and Elbein 1971), were also observed in the spectra.

A fourth peak eluting immediately after sitosteryl glucoside was evident in FID chromatograms of the mixed steryl glucoside standard and all of the foods analyzed (Fig. 1) and thus, a GC-MS analysis of this component was pursued. The mass spectrum (Fig. 5) was consistent with that of a steryl glucoside as described above. Although absolute identification of the compound was not possible, it may well be Δ^5-avenasteryl glucoside based on both the presence of mass 395, which is the expected parent ion and the expected GC elution order and retention time.

FID chromatograms of the food samples displayed peaks eluting after the steryl glucosides, which were initially suspected to be steryl polyglycosides (Fig. 1, peaks 5 and 6). The retention times for these peaks were the same for all of the foods. Mass spectral analysis suggested that these compounds have a glucose (tetratrimethylsilyl) component as described above for the steryl glucosides. However, because no sterol parent ion could be

TABLE 3.
STEROL COMPOSITION OF SELECTED FOODS

Food	Sitosterol (mg/100 g)		Campesterol (mg/100 g)		Stigmasterol (mg/100 g)		Δ^5-Avenasterol mg/100 g)	
	F + E*†	G*‡	F + E*†	G*‡	F + E*†	G*‡	F + E*†	G*‡§
Whole wheat flour	32.2 (0.16)	6.64 (0.01)	9.11 (0.06)	1.38 (0.03)	1.35 (0.01)	<0.2	2.41 (0.02)	3.41 (0.12)
Granola bar	41.0 (0.76)	7.20 (0.46)	19.6 (0.42)	1.77¶	1.35 (0.04)	0.43¶	4.33 (0.08)	5.52 (0.27)
Mixed vegetables, lyophilized††	60.5 (0.62)	6.07 (0.38)	20.9 (0.08)	1.94 (0.12)	7.92 (0.03)	1.08 (0.14)	7.98 (0.04)	1.91 (0.13)
Figs, dried	14.4 (2.36)	1.85 (0.24)	1.11 (0.18)	0.11 (0.02)	1.87 (0.28)	0.18 (0.02)	12.06 (1.73)	0.53 (0.08)
Flaxseed	69.3 (1.90)	20.1 (0.58)	34.1 (0.71)	11.0 (0.11)	8.53 (0.42)	2.89 (0.08)	21.93 (0.40)	1.07¶
Soybeans	37.1 (1.04)	20.4 (0.35)	13.6 (0.01)	10.4 (0.03)	12.0 (0.12)	5.54 (0.32)	1.70 (0.18)	0.7¶
Peanut butter	72.0 (0.51)	15.1 (0.73)	14.5 (0.21)	2.43 (0.17)	8.78 (0.05)	2.10 (0.09)	14.58 (0.13)	2.42 (0.15)
Diet homogenate	7.7 (0.04)	1.72 (0.06)	2.26 (0.02)	0.26 (0.02)	1.22 (0.01)	0.23 (0.04)	0.53 (0.01)	0.54 (0.03)
Almonds	111 (0.47)	32.9 (0.37)	4.28 (0.06)	1.19 (0.11)	4.25 (0.05)	1.17 (0.01)	17.76 (0.07)	3.95 (0.06)
Pine nuts	109 (0.64)	16.9 (0.74)	18.6 (0.18)	1.51 (0.11)	<0.1	<0.2	38.23 (0.26)	2.48 (0.14)

* Mean values, with standard error in parentheses.
† mg/100 g free sterol after alkaline saponification of total lipid extract (contributed by free and esterified sterols).
‡ mg/100 g free sterol contributed by the steryl glucoside, calculated from assayed steryl glucoside concentrations using molecular weights.
§ Presumptive identification of avenasteryl glucoside based on mass spectral and GC-FID data (see text).
¶ $n = 1$.
†† Mixture of sweet corn, carrots and tomatoes (CRM 485; Finglas *et al.* 1998).

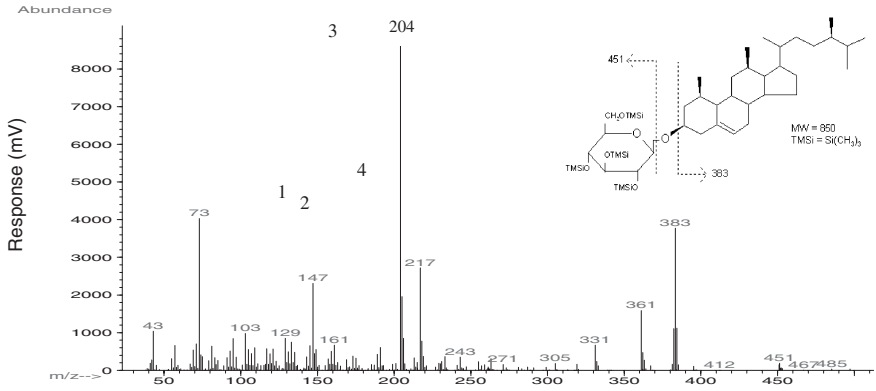

FIG. 2. MASS SPECTRUM FOR TRIMETHYLSILYL ETHER DERIVATIVE OF CAMPESTERYL GLUCOSIDE IN SOYBEANS (1, FIG. 1B)

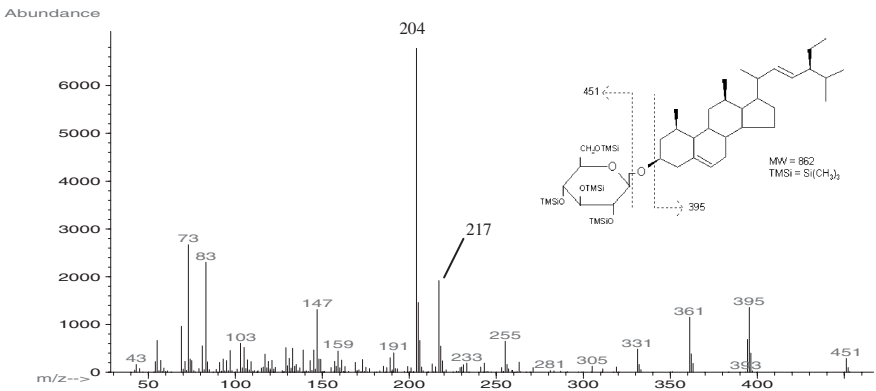

FIG. 3. MASS SPECTRUM FOR TRIMETHYLSILYL ETHER DERIVATIVE OF STIGMASTERYL GLUCOSIDE IN SOYBEANS (2, FIG. 1B)

observed, possibly because of lack of adequate sensitivity, the identification as steryl polyglucosides could not be confirmed. Figure 6 is a representative mass spectrum for the larger of the two late eluting peaks in whole wheat flour (Fig. 1D, peak 5). Masses 361, 217, 204 and 143 were present in the spectrum, while ions at 451 and 305, also expected for tetratrimethylsilyl-glucose, were absent, possibly because of low concentrations.

DISCUSSION

Direct determination of steryl glucosides was undertaken in the present study for the analysis of dietary supplements specifically formulated to con-

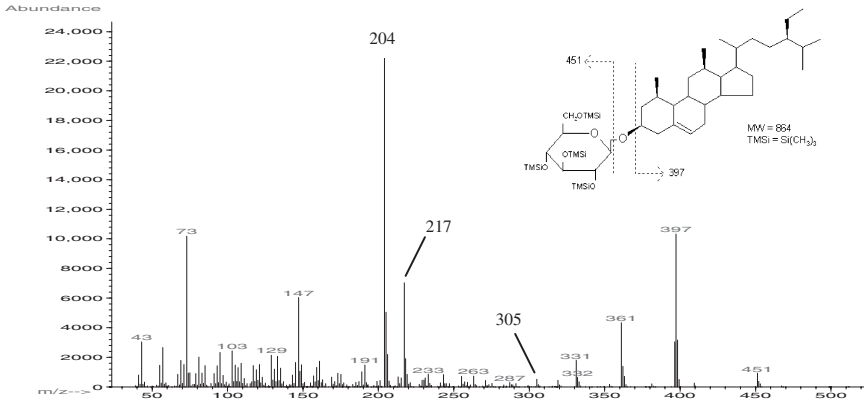

FIG. 4. MASS SPECTRUM FOR TRIMETHYLSILYL ETHER DERIVATIVE OF SITOSTERYL GLUCOSIDE IN SOYBEANS (3, FIG. 1B)

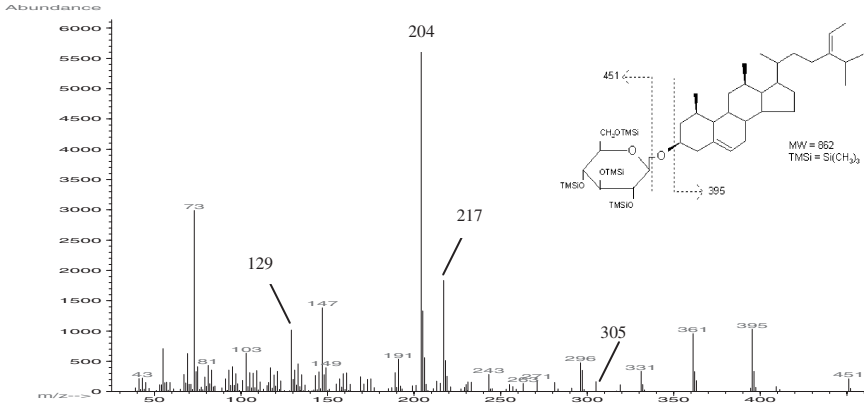

FIG. 5. MASS SPECTRUM FOR TRIMETHYLSILYL ETHER DERIVATIVE WITH PRESUMPTIVE IDENTIFICATION OF Δ^5-AVENASTERYL GLUCOSIDE IN GRANOLA BAR (4, FIG. 1C)

tain a 1 : 100 ratio of sitosteryl glucoside to sitosterol. The very low concentration of sitosteryl glucoside in these products precluded the determination of the glucoside by a difference from the analysis of the total sitosterol with and without acid hydrolysis of the material, for example, as described by Toivo *et al.* (2001). The method also yielded accurate and precise results for a wide range of food samples. Excellent precision was achieved through careful analytical technique, but the ruggedness of the method could be improved by the inclusion of an internal standard in the steryl glucoside analysis. No appropriate commercially available standards have been identified, but cho-

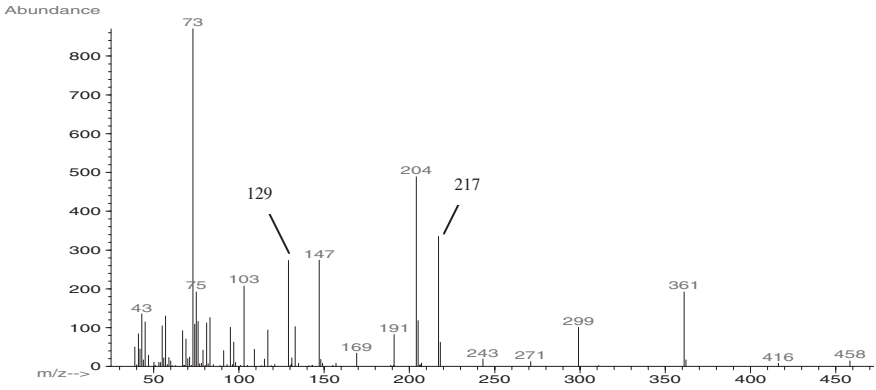

FIG. 6. MASS SPECTRUM FOR LATE ELUTING PEAK IN WHOLE WHEAT FLOUR DISPLAYING TRIMETHYLSILYL GLUCOSE DERIVATIVE (5, FIG. 1D)

lesteryl glucoside has been synthesized and used as an internal standard by others (Breinhölder *et al.* 2002), although additional cost is certainly incurred in that process. Quality control measures that were probably essential to achieving accurate and precise results in the present study included GC injector-liner replacement every 40–50 food extract injections (to minimize the buildup of nonvolatile residue, which adversely effects the steryl glucoside response), the choice of 0.5-mL minimum final derivatized sample volume to limit the effect of sample residue on the actual final volume which is critical for sample quantitation and the use of a 0.500-mL volumetric pipet for the precise measurement of this critical volume.

Although direct saponification of food samples rather than the use of a total lipid extract can be employed for sterol analysis, the latter offers some advantages. A total lipid extract allows a larger sample size, which can compensate for lesser homogeneity of some food samples and allow subsequent flexibility in optimizing the final analyte concentrations for GC. Also, separation of free and glycosidic sterols by SPE before saponification allows the glucoside fraction to be derivatized directly, thereby reducing potential incomplete recovery during postsaponification extraction. The recovery data for the dietary supplements, granola bar, lyophilized mixed vegetables and whole wheat flour (Table 1) indicate that total lipid extraction in the present study fully recovered monoglucosylsterols. Further work would be necessary to validate the recovery of polyglucosylsterols; Grunwald and Huang (1989) report that with total lipid extraction using chloroform/methanol, if the solvent to sample ratio is too high, the lower water content can cause reduced extraction efficiency for the more polar polyglucosides.

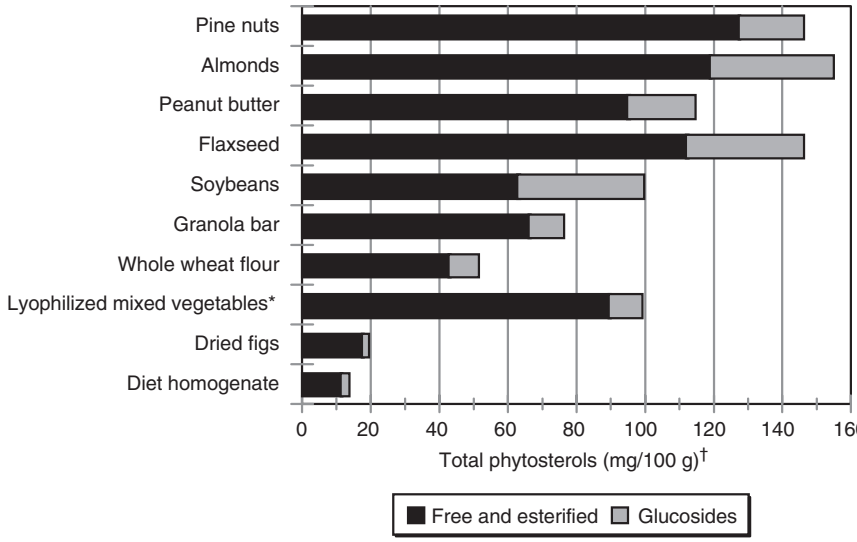

FIG. 7. PROPORTION OF TOTAL SITOSTEROL, CAMPESTEROL AND STIGMASTEROL AS GLUCOSIDES IN DIFFERENT FOODS
*CRM 485, FINGLAS *et al.* (1998); †Campesterol + stigmasterol + sitosterol.

Figure 7 illustrates the underestimation of the total phytosterol content of the foods studied that would occur if only free and esterified sterols were quantified, as in typical analytical methods using only alkaline saponification followed by the quantitation of free sterols. Sitosteryl, campesteryl and stigmasteryl glucosides comprised 9–37% (2–36 mg/100 g) of total sterols among these foods, with the highest proportion occurring in the nuts, seeds and legumes. The highest concentration of total sterols from these glucosides was in soybeans. For all foods, the additional recovery of steryl glucosides resulted in a significantly higher total sterol content (pine nuts, $P < 0.025$; almonds, $P < 0.007$; peanut butter, $P < 0.0003$; flaxseed, $P < 0.015$; soybeans, $P < 0.012$; whole wheat flour, $P < 0.002$; lyophilized mixed vegetables, $P < 0.005$; dried figs, $P < 0.085$; diet homogenate, $P < 0.002$; no test was possible for granola bar, $n = 1$). These results for a range of food products suggest that the phytosterol data generated by methods that do not include glycosides meaningfully underestimate the total sterol content, especially when applied to seeds, legumes and whole grains. Further research is underway to compare the total sterol levels determined by the direct GC analysis of the glycosides and the free plus esterified sterols versus acid hydrolysis, followed by the quantitation of total free sterols (e.g., Toivo *et al.* 2001), for the purpose of determining the total phytosterol content of foods. Additionally, while the

focus of this study was to quantify free sitosterol and sitosteryl glucoside, the SPE method could be modified to also separate free and esterified sterols if desired (e.g., Phillips *et al.* 2002) to obtain further information on individual sterol classes.

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