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Phytosterol Composition of Nuts and Seeds Commonly Consumed in the United States

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Phytosterols were quantified in nuts and seeds commonly consumed in the United States. Total lipid extracts were subjected to acid hydrolysis and then alkaline saponfication, and free sterols were analyzed as trimethylsilyl derivatives by capillary GC-FID and GC-MS. Δ^5-Avenasterol was quantified after alkaline saponification plus direct analysis of the glucoside. Sesame seed and wheat germ had the highest total phytosterol content (400−413 mg/100 g) and Brazil nuts the lowest (95 mg/100 g). Of the products typically consumed as snack foods, pistachio and sunflower kernel were richest in phytosterols (270−289 mg/100 g). β-Sitosterol, Δ^5-avenasterol, and campesterol were predominant. Campestanol ranged from 1.0 to 12.7 mg/100 g. Only 13 mg/100 g β-sitosterol was found in pumpkin seed kernel, although total sterol content was high (265 mg/100 g). Phytosterol concentrations were greater than reported in existing food composition databases, probably due to the inclusion of steryl glycosides, which represent a significant portion of total sterols in nuts and seeds.

KEYWORDS: Phytosterols; plant sterols; stanols; nuts; seeds; food composition; analysis; almonds (*Prunus dulcis*); Brazil nuts (*Bertholletia excelsa*); cashews (*Anacardium occidentale*); chocolate; flaxseed (*Linum usitatissimum)*; hazelnuts; filbert (*Corylus* spp.); macadamia nuts; peanuts (*Arachis hypogaea*); peanut butter; pecans (*Carya illinoensis)*; pine nuts (*Pinus* spp.); pinon; pinyon; pistachios (*Pistacia vera*); poppy seed (*Papaver somniferum*); pumpkin seed (*Cucurbita* spp.); sesame seed (*Sesamum indicum*); sunflower seed (*Helianthus annuus*); walnut (*Juglans regia*); black walnut (*Juglans nigra*); wheat germ

INTRODUCTION

Phytosterols (plant sterols) are similar in structure to cholesterol, having the same basic cyclopentanoperhydrophenanthrene ring structure but differing in the side chain at C24 and/or the position and configuration of unsaturated double bonds and the optical rotation at chiral carbons (*1*). Phytosterols have been classified on the basis of the number of methyl groups at the C4 position [4-desmethyl sterols ("sterols"), 4-methyl-, and 4,4-dimethylsterols] (*2*), but for food composition purposes the term "phytosterol" commonly refers to the 28- and 29-carbon 4-desmethyl sterols. β-Sitosterol, campesterol, and stigmasterol predominate in higher plants (*2*) and in typical diets (*3*). Phytosterols have been shown to reduce blood cholesterol, as well as to decrease the risk of certain types of cancer and enhance immune function (*4*−*8*). Whereas most intake studies have involved relatively high doses of phytosterols (from 2 to several grams per day) using specially formulated products (*4*), recent research by Ostlund and co-workers has demonstrated the effectiveness of much lower levels of phytosterols, such as those that occur naturally in diets rich in plant foods, in reducing cholesterol absorption (*9*, *10*). Therefore, comprehensive and accurate data on the naturally occurring phytosterol content of foods are essential to facilitate epidemiological studies, formulation of research diets, and development of dietary recommendations related to the health impact of phytosterols.

Nuts and seeds are rich sources of phytosterols. Nuts have also been consistently associated in both epidemiological studies and clinical feeding trials with reduced blood cholesterol levels and decreased incidence of cardiovascular disease (*11*−*15*). Besides having a favorable fatty acid profile, nuts contain many micronutrients and bioactive constituents ("phytochemicals") (*11*). Although data on the fatty acid and nutrient contents of commonly consumed nuts and seeds are readily available [e.g., the USDA National Nutrient Database for Standard Reference (USNDB) (*16*)], quantitative data on their phytochemical composition are limited, diffuse, or dated. A summary of the phytosterol content of several nuts and seeds was included in a 1978 review by Weihrauch and Gardner (*17*). However, this publication is now over 25 years old and also does not specify sample origin, sample preparation, or precise analytical methods for each value reported, making it uncertain how the data relate to products available in the current marketplace. Other literature reports (e.g., refs *18*−*22*) are useful, but confined to single nuts

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or a more limited range of products not necessarily representative of those in the U.S. market.

Additionally, advances in the analysis of sterols suggest that values determined using the standard method of alkaline hydrolysis underestimate the total phytosterol content of some foods by failing to recover glycosidic sterols (*23*, *24*). That is, total phytosterols exist not only as free sterols but also as fatty acyl esters, glycosides, and fatty acyl glycosides, with these substitutions occurring at C3 (*2*). The latter two classes of conjugates are not quantified by analytical methods that do not involve acid hydrolysis to cleave the acetal bond or direct analysis of the intact glycosides. Exclusion of glycosidic sterols has been shown to underestimate the total sterol content of nuts and seeds by as much as 37% (*25*). It is also common for routine nonresearch analysis of phytosterols (e.g., at commercial laboratories) to measure only β-sitosterol, campesterol, and stigmasterol. Although overall these are the major phytosterols in plants, others may be present at significant levels in particular products, including nuts and seeds.

It is also important that the samples analyzed to generate food composition data for the purpose of assessing dietary intake are representative of the products available to the consumer. The U.S. Department of Agriculture's National Food and Nutrient Analysis Program (NFNAP) is an ongoing project with the goal of updating and increasing the reliability of food composition data in the USNDB (*26*, *27*). Products are collected according to a probability-based statistical sampling plan reflecting current U.S. food consumption data, composited, and then analyzed for a broad range of nutrients. As part of the NFNAP, a number of commonly consumed tree nuts, seeds, and related products (e.g., peanuts, peanut butter) were sampled. The goal of the present work was to apply new analytical methodology that measures total free, esterified, and glycosidic sterols to determine the phytosterol content and composition of these products.

MATERIALS AND METHODS

Reagents and Standards. Hydrochloric acid (12.1 N) (certified ACS), methanol, and hexane (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). Butylated hydroxytoluene (BHT) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), and diethyl ether (anhydrous, certified ACS) was purchased from VWR International (West Chester, PA). All other reagents and standards are described in the references cited.

Samples. Products were collected according to a statistical sampling plan designed to generate nationally representative composites (*28*, *29*). Briefly, the strategy involved dividing the United States into four regions, each with roughly equal populations based on data derived from the U.S. Census Bureau 1990 census. Each region was then divided into three strata of approximately equal population. The primary sampling units in each stratum were generalized Consolidated Metropolitan Statistical Areas (gCMSA). The gCMSA were defined as standard CMSA or individual counties for areas not in a CMSA, and one gCMSA was chosen in each stratum proportional to population size. Two counties were then chosen, proportional to urbanicity, from each selected gCMSA. For gCMSA that were made up of a single county, the county was chosen twice. Selected gCMSA were also supplemented with surrounding counties when the gCMSA contained fewer than 10 grocery stores. Grocery store lists, which contained all retail store names (for stores of $2 million or more in sales per year), contact information, and value of sales data were obtained from Trade Dimensions for 12 selected counties. A sample of size one was selected proportional to value of sales from each county (two in cases when the same county was chosen twice). Alternative outlets were also selected in case the primary outlet was inaccessible or products were unavailable. Within selected stores, brand name products were chosen proportional to package size, market share, or consumption patterns. Loose nuts were sampled from retail outlet bins or as prepackaged brands. Samples for most nuts and seeds were combined by region, yielding in most cases a total of four composite samples. For selected products (including poppy seed, sesames seed, and flaxseed), all samples were combined into a single national composite. Peanuts, peanut butter, and baking chocolate were also collected for the NFNAP, and although not nuts or seeds, they were also assayed due to the similarity in matrix and the interest in the phytochemical composition of these widely consumed foods.

Exceptions to the above sampling scheme were as follows. Black walnuts and USDA commodity peanut butter were received directly from suppliers. Roasted pinon nuts were obtained from American Indian vendors on the Navajo reservation in Kayenta, AZ, Shiprock, NM, according to sampling plans developed for analysis of American Indian foods (*30*), from sites that approved the study. Sunflower seed kernels from two growing locations (North Dakota and Kansas) and high-oleic sunflower seed kernels were provided by the National Sunflower Association (Bismarck, ND), as previously described (*31*). Additionally, to supplement data for nationwide samples from the NFNAP, several composites of locally procured nuts and seeds were prepared. One to two kilograms (3−7 units of packaged product) each of oil-roasted sunflower seed kernels, dry roasted peanuts, wheat germ, raw cashews, and raw sunflower seed kernels were purchased from retail outlets in Blacksburg, VA, and dry-roasted pumpkin seed kernels were purchased from Vitacost.com (www.vitacost.com).

Table 1 describes the nuts and seeds analyzed. Only Brazil nuts and pistachios were obtained in the shell. The meat from these nuts was removed and used to prepare composites, with no special attempt to eliminate or retain the "skin".

Composite Preparation. With the exceptions noted below, equal weights (~300−500 g) of sample from each outlet in each region were combined for analysis. Each composite was frozen in liquid nitrogen and then ground using a 6 L commercial food processor (Blixer, Robot Coupe USA, Jackson, MS) except oil-roasted peanuts were frozen in liquid nitrogen and processed in an analytical sample mill (KnifeTec 1095; Foss Tecator AB, Sweden); peanut butter and pine nuts were homogenized using a Robot Coupe food processor without liquid nitrogen; sesame seeds, poppy seeds, and flaxseeds (whole and ground) were stirred thoroughly. Each composite was dispensed in 6−15 g aliquots among 30 or 60 mL glass jars with Teflon-lined lids, capped, and stored in darkness at −60 ± 5 °C until analyzed. In the cases of the sesame seed, poppy seed, and whole flaxseed, a small portion of the frozen composite (12−15 g) was ground in an electric mill (Girmi model TR30, Omegna, Italy) prior to a subsample being taken for analysis.

Extraction and Quantitation of Sterols. Total lipids were extracted from 1−2 g of each composite using a previously described method (*32*). An aliquot of the total lipid extract was analyzed for total free, esterified, and glycosidic sterols after acid hydrolysis, using hydrolysis conditions reported by Nagy and Nordby (*33*), as follows. A 10 mL portion of the total lipid extract was evaporated to dryness with nitrogen at 50 ± 2 °C, combined with 6 mL of 0.5 N methanolic HCl (with 0.1% w/v BHT added as an antioxidant) in a 50 mL Teflon screw-cap test tube with a leak-proof cap (Fisher Scientific), and then heated for 22 h at 75 ± 2 °C with constant shaking at 145 rpm. The hydrolysate was extracted twice with 20 mL of 1:1 (v/v) diethyl ether/hexane with 12 mL of water added to facilitate phase separation. The diethyl ether/hexane extracts were diluted, if necessary, to place the analytes within the working range of the gas chromatographic method (below), combined with 25 μg of epicholesterol (internal standard), and then subjected to alkaline hydrolysis, derivatized to trimethylsilyl (TMS) ethers, and assayed for free sterols as described previously (*25*).

For quantitation of Δ^5-avenasterol, a separate portion of the total lipid extract was subjected to alkaline hydrolysis and then derivatized and assayed for sterols derived from the free and esterified sterols as described above. Steryl glucosides were isolated from another aliquot of the total lipid by solid-phase extraction and assayed directly by GC, as described previously (*25*). For the steryl glucosides quantified in this manner, the molar equivalent of free sterol derived from the glucoside was added to the free sterol concentration determined after alkaline hydrolysis, to obtain total sterol concentration for that

Table 1. Description of Products Analyzed

product common name	NDB no.[a]	scientific name[b]	description of product (as purchased)[c]	no. of composites and type[d]
almond	12061	*Prunus dulcis*	raw, shelled	4, regional
Brazil nut	12078	*Bertholletia excelsa*	raw, in shell	4, regional
cashew, oil roasted	12586	*Anacardium occidentale*	oil-roasted (containing primarily peanut and/or cottonseed oils, with soybean and/or sunflower oils in some products); salted	6, regional
cashew	12087	*Anacardium occidentale*	raw, shelled	1, local
chocolate	19078		unsweetened solid baking chocolate	1, national
flaxseed, ground	12220	*Linum usitatissimum*	packaged, ground flaxseed	1, national
flaxseed, whole	12220	*Linum usitatissimum*	packaged, whole flaxseed	1, national
hazelnut (filbert)	12120	*Corylus* spp.	raw, shelled	4, regional
macadamia nut	12632	*Macadamia integrifolia M. tetraphylla*	dry-roasted, salted	4, regional
peanut, oil roasted, salted	16089	*Arachis hypogaea* spp.	oil-roasted (unspecified vegetable oils), salted ("cocktail" peanuts)	4, regional
peanut, dry-roasted	16390	*Arachis hypogaea* spp.	dry roasted, unsalted	1, local
peanut butter, retail, smooth	16098		containing partially hydrogenated vegetable oils (type not specified)	4, regional
peanut butter, USDA commodity, smooth	16167		containing partially hydrogenated vegetable oils (type not specified)	4, from supplier
peanut butter, retail, chunk style	16097		containing partially hydrogenated vegetable oils (type not specified)	4, regional
pecan	12142	*Carya illinoensis*	raw, shelled	3, regional
pine nut	12147	*Pinus* spp.	raw, shelled	4, regional
pinon[e] nut	NA[f]	*Pinus* spp.	roasted; purchased from vendors on Navajo Indian reservations in Kayenta, AZ, and Shiprock, NM	1, from supplier
pistachio	12652	*Pistacia vera*	roasted, salted, in shell, edible portion	4, regional
poppy seed	02033	*Papaver somniferum*	raw	1, national
pumpkin seed kernel	12516	*Cucurbita* spp. (Shine Skin variety)	organic pumpkin seed kernels, sea salt	1, local
sesame seed	12201	*Sesamum indicum*	raw, hulled	1, national
sunflower seed kernel	12036	*Helianthus annuus*	raw, shelled	4, from supplier and local
sunflower seed kernel, high-oleic	NA	*Helianthus annuus*	raw, shelled	1, from supplier
sunflower seed kernel, oil-roasted	12538	*Helianthus annuus*	oil-roasted (cottonseed and/or sunflower oil), salted	1, local
walnut, black	12154	*Juglans nigra*	raw, shelled	1, from supplier
walnut, English	12155	*Juglans regia*	raw, shelled	4, regional
wheat germ	NA		raw, packaged	1, local

[a] U.S. National Nutrient Database (NDB) number (*16*). [b] Scientific names given are based on those designated for the product with the NDB number indicated, not as a result of specific morphological or genetic analysis of samples, and are provided as additional descriptive information in case the common name of a product may not be universally understood. [c] Detailed information on brands, suppliers, outlets, and sampling dates was documented, but is not presented. [d] See description of sampling in text and refs *28–30*. [e] Also spelled piñon or pinyon according to USDA's GRIN taxonomy (*44*) and the Northern Nut Growers' Association (*45*). [f] Not available.

component. In these cases, acylated steryl glycosides were not quantified, but the contribution to total sterols in any given product by excluding this fraction (if present) for only Δ^5-avenasterol and one or two other sterols is expected to be <3%.

β-Sitosterol, campesterol, stigmasterol, brassicasterol, Δ^5-avenasterol, sitostanol, and campestanol were routinely analyzed by GC-FID based on retention times previously established (*25*), but the identity and purity of the peak for each of these components in one sample of each type of nut and seed were verified by gas chromatography−mass spectrometry (GC-MS) as described below. Additionally, any peak in the GC-FID chromatogram of an acid-hydrolyzed sample that had a retention time in the sterol region and represented at least ~2% of the sitosterol area was evaluated by GC-MS, as were peaks that appeared in chromatograms of sample extracts subjected to alkaline hydrolysis but not acid hydrolysis (which suggested a potentially acid-labile sterol, as is Δ^5-avenasterol). If a component was confirmed to be a sterol, its concentration was estimated from the β-sitosterol/epicholesterol standard curve. For acid-labile peaks comprising >15 mg/100 g in a given sample, the steryl glucosides chromatogram was examined by GC-MS and if the corresponding glucoside could be identified, then the concentration was estimated as described above for Δ^5-avenasterol.

GC-MS. The TMS-sterols were analyzed by GC-MS using an Agilent (Wilmington, DE) 5890 series 2B gas chromatograph and an Agilent 5972 mass selective detector using electron impact ionization, under the following conditions: RTX-5MS column (5% diphenyl−95% dimethylpolysiloxane; 30 m, 0.25 mm i.d., 0.1 μm film; Restek, Bellefonte, PA); 1.0 μL injection volume; helium carrier gas at 1.05 mL/min; split ratio, 1:10; injector temperature, 290 °C; oven temperature, 275 °C, hold for 45 min, then raised at 2 °C/min to 290 °C; transfer line temperature, 290 °C; electron energy, 70 eV; and scan range, 35−550 amu at 1.4 scan/s. A 60 m column was used in selected cases in which additional resolution was needed.

Each peak was evaluated via detection of the parent molecular ion and fragmentation pattern of the TMS derivatives. For example, the campesterol spectrum showed the molecular ion of *m*/*z* 472 and fragmentation of $M^+ - ROH$ (*m*/*z* 382), $M^+ - Me - ROH$ (*m*/*z* 367), $M^+ - 129$ (*m*/*z* 343), and $M^+ - SC - ROH$ (*m*/*z* 255), where R is $(CH_3)_3Si$ and SC refers to the side chain (*35*). In addition to the presence of specific ion fragments, the relative intensity of ion fragments was considered in the process of identification. Although in most cases the precise identity could not be determined on the basis of GC-MS alone, it was possible to characterize unknowns as sterol or non-sterol on the basis of the presence or absence of a molecular ion and fragmentation pattern characteristic of sterols, as described in the example for campesterol, above. For purposes of this study, phytosterols were considered to be the 28- and 29-carbon desmethyl sterols, and only sterols with a molecular ion of *m*/*z* 470−486 were included. Any unknowns confirmed as phytosterols are reported with GC-FID retention

Table 2. Results for Peanut Butter Control Material Assayed with Each of 20 Batches of Samples

component	mean	range	SD[a]	RSD[b] (%)
β-sitosterol	88.7	86.6–92.5	1.48	1.7
campesterol	16.3	15.6–16.7	0.31	1.9
stigmasterol	11.4	11.1–11.9	0.22	1.9
Δ^5-avenasterol	17.8	16.9–18.5	0.40	2.2
sitostanol	1.05[c]	0.98–1.20	0.06	5.9
campestanol	2.20	1.86–2.39	0.13	5.9
total phytosterols	137	134–142	2.2	1.6

[a] Standard deviation. [b] Relative standard deviation. [c] $n = 13$; does not include values less than the limit of quantitation (1 mg/100 g).

times noted. If a component was not present at a high enough concentration (~3 mg/100 g) to be evaluated by GC-MS, but had a GC-FID retention time matching a peak confirmed as a phytosterol in another sample, then it was reported as that component.

Assay Validation and Quality Control. To verify that sterols quantified after acid hydrolysis were not degraded during the acid treatment, the recovery of sterols from a well-characterized sample of canola oil added to ground almonds was evaluated. Duplicate test portions of unspiked almonds, canola oil, and almonds with a precisely measured amount of canola oil added were assayed according to the method described above. The percent recovery of the expected concentration of each of β-sitosterol, campesterol, stigmasterol, brassicasterol, and sitostanol was calculated by subtracting the expected individual endogenous sterol contribution (determined from the unspiked almonds taken through the same analysis) from the individual sterol concentrations measured in the spiked samples and then comparing these values to the expected individual sterol contribution from the canola oil spike. Expected sterol concentrations in the canola oil were calculated from the amount of oil added and the individual sterol concentrations measured after alkaline hydrolysis only, using the mean values from 16 replicate analyses in 9 assay batches over a 4 month period. Recovery of Δ^5-avenasterol and Δ^5-avenasteryl glucoside from the alkaline hydrolysis and direct assay of sterol glucosides has been previously reported (*25*).

A peanut butter control material was analyzed with each set of samples to monitor interassay precision. Additionally, at least one of the composites of each type of nut and seed was analyzed in duplicate in a separate assay batch to obtain a matrix-specific estimate of analytical variability.

Presentation of Results. Because the intent of this study was to determine phytosterol concentrations in statistically representative U.S. nationwide sampling of commonly consumed nut and seeds, the values for each product are presented as the mean of results for the four regional composites assayed. For each sample matrix, an estimated analytical standard error for each component was calculated using data from the interassay replicates, along with the range of values reported for separate composites of the same product.

RESULTS AND DISCUSSION

Assay Validation and Quality Control. The mean percent recoveries of β-sitosterol, campesterol, stigmasterol, brassicasterol, and sitostanol from the spiked almonds were 100.0, 95.0, 100.2, 96.6, and 106.8, respectively [overall mean = 99.7%; $n = 10$, standard deviation (SD) = 4.4], suggesting no significant loss of these phytosterols from this type of food matrix under the acid hydrolysis conditions used.

Results for the peanut butter control sample are summarized in **Table 2**. Excellent interassay precision was obtained for each component, with a relative standard deviation (RSD) ≤2.2% for total phytosterols and for individual sterols present at >3 mg/100 g and an RSD <6% for sitostanol and campestanol, which were present at <3 mg/100 g.

Results of interassay replicate analyses of each type of nut/seed are summarized in **Table 3**. These replicates were evaluated to check the analytical precision for each type of matrix, on the premise that extraction or homogeneity might vary depending on product characteristics that differed from the control material (peanut butter). The analytical standard error for all types of samples and all components suggests excellent precision, including reproducibility of extraction and quantitation as well as homogeneity of each type of nut/seed composite.

Table 3. Average Analytical Standard Error (SE) for Selected Sterols Assayed in All Types of Nuts and Seeds

component	av SE	range of SE	product with lowest SE	product with highest SE
β-sitosterol	1.46	0.01–4.84	pinon nut	sunflower seed kernel
campesterol	0.33	0.08–1.06	pecan	wheat germ
stigmasterol	0.15	0.03–0.26	pecan	chocolate
Δ^5-avenasterol	0.36	0.045–1.4	flaxseed, ground	cashew, oil-roasted
sitostanol	0.04	0.13–0.34	almond	pumpkin seed kernel
campestanol	0.27	0.015–1.54	sesame seed	wheat germ
sterol A[a]	0.19	0.025–0.62	macadamia nut	sesame seed
sterol B[a]	0.20	0.005–0.61	black walnut	flaxseed, ground
sterol C[a]	0.17	0.01–0.47	flaxseed, whole	pistachio
total phytosterols	1.85	0.5–8.9	flaxseed, whole	pumpkin seed kernel

[a] See **Table 5**.

Sterol Composition of Nuts and Seeds. The sterol composition of the nuts, seeds, and related products analyzed is summarized in **Table 4**. Representative chromatograms are shown in **Figure 1**. Overall, the predominant sterols were β-sitosterol, Δ^5-avenasterol, and campesterol. Δ^5-Avenasterol comprised 40 mg/100 g (17% of total sterols) in pine nut and sesame seed (10% of total sterols) and was also high (≥20 mg/100 g and >9% total sterols) in pistachio, black walnut, almond, and flaxseed. Stigmasterol was not present or <10 mg/100 g in all but sunflower kernel, peanuts and peanut butter, sesame seed, chocolate, and flaxseed and was more prominent in chocolate (39.8 mg/100 g) than in the nuts, seeds, and peanut products. Brassicasterol was monitored but found at trace level (<2 mg/100 g) or not detected in all samples (data not shown). Pumpkin seed kernel was extremely unusual, insofar as β-sitosterol was a minor constituent (4.9% of total sterols), although β-sitosterol is the predominant naturally occurring sterol in virtually all plant foods. This very low β-sitosterol content is consistent with published data for another variety of pumpkin seed kernel (*22*, *36*).

Wheat germ was richest in saturated sterols overall (19.6 mg/100 g). Campestanol was the major stanol, present in all products and ranging from 1 to 12.7 mg/100 g. Sitostanol was >2 mg/100 g only in almond, Brazil nut, hazelnut, pine nut, pumpkin seed kernel, and wheat germ. Almond, Brazil nut, hazelnut, and pine nut had similar concentrations of sitostanol and campestanol. Interestingly, pine nut contained a relatively high concentration of saturated sterols (10 mg/100 g), but a related product, pinon nut (roasted), had only 3 mg/100 g campestanol and no sitostanol.

GC-MS analysis confirmed several sterols other than those identified in **Table 4**. The high concentration of these compounds in some of the samples facilitated detection of the molecular ion and also a fragmentation pattern consistent with a sterol structure (*35*). Definitive identification by GC-MS alone was not possible due to the unavailability of reference standards and the lack of a comprehensive library of mass spectra for phytosterols. Because fragmentation is affected by the form in which the sterol exists (i.e., as the free alcohol or as the acetate or TMS derivative), it is necessary to have a reference spectrum for the same derivative to achieve an accurate comparison (*4*). Identification was further complicated by the fact that fragmen-

Table 4. Phytosterol Composition of Nuts and Seeds in Milligrams per 100 g of Product[a,b]

product[c]	β-sitosterol	campesterol	stigmasterol	Δ5-avenasterol	sitostanol	campestanol	other sterols[d]	total phytosterols[e]
almond	143.4	4.9	5.0	19.7	3.2	3.3	19.6	199 (193–208; 3.2)
Brazil nut	65.5	2.0	6.2	13.6	4.1	2.0	3.4	95 (92–101; 2.2)
cashew	112.6	8.9	<1.2	13.7	<1.2	2.0	13.3	150 (2.7)
cashew, oil-roasted	119.3	10.0	<1.7	13.6	<1.7	2.8	12.6	158 (157–165; 6.7)
chocolate	103.9	14.1	39.8	3.6	<1.7	2.7	3.0	167 (1.2)
flaxseed, ground	83.6	40.2	8.6	21.6	<1.3	2.7	26.0	183 (2.2)
flaxseed, whole	96.0	49.7	14.2	20.9	<1.3	2.9	26.5	210 (0.3)
hazelnut	102.2	6.6	<2.5	2.6	4.0	3.0	2.5	121 (117–124; 2.1)
macadamia nut	143.7	9.6	nd	13.3	nd	2.9	17.0	187 (181–198; 1.9)
peanut, oil roasted	74.9	14.2	13.0	16.7	<1.3	2.0	14.0	135 (129–146; 0.3)
peanut, dry roasted	76.8	13.2	12.1	17.8	<1.2	1.6	15.0	137 (3.7)
peanut butter, USDA commodity, smooth	85.5	16.4	12.5	15.5	<1.0	2.3	12.9	146 (129–167; 1.2)
peanut butter, retail, smooth	77.7	15.0	11.4	16.0	<1.0	2.1	13.0	135 (132–138)
peanut butter, retail, chunk-style	77.2	14.8	11.2	15.0	<1.0	2.1	12.0	132 (130–134)
pecan	116.5	5.9	2.6	14.6	<1.7	2.8	14.1	157 (154–159; 0.9)
pine nut	132.0	19.8	<1.7	40.3	5.9	3.8	34.2	236 (235–237; 1.0)
pinon nut	104.4	13.7	<1.7	13.9	<1.7	2.6	12.3	147 (0.1)
pistachio	209.8	10.1	2.3	26.2	1.2	5.0	24.6	279 (274–287; 4.2)
poppy seed	109.3	29.0	6.8	17.7	<1.3	2.6	19.6	185 (0.8)
pumpkin seed kernel	13.1	3.4	nd	3.5	3.5	0.8	241.0	265 (252–278; 13.1)
sesame seed	231.7	53.1	22.2	40.4	nd[f]	5.6	47.4	400 (0.7)
sunflower seed kernel, high-oleic	168.5	30.0	16.6	11.7	<2.5	4.6	39.5	271
sunflower seed kernel	165.1	22.8	18.0	17.0	nd	3.6	43.2	270 (265–273; 6.6)
sunflower seed kernel, oil-roasted	178.3	23.8	19.3	19.9	nd	3.6	44.0	289 (2.1)
walnut, black	114.4	4.7	<1.7	29.5	<2.5	2.6	25.8	177 (1.1)
walnut, English	88.9	4.9	nd	7.3	<1.7	2.4	9.1	113 (106–120; 3.1)
wheat germ	228.6	78.7	3.9	16.1	6.9	12.7	66.2	413 (6.5)

[a] Each value is the mean for all composites/replicates (see text); "nd" indicates less than the limit of detection (which ranged from 0.3 to 1.0 mg/100 g, depending on product, unless otherwise noted); values reported as "<" indicated cases when a peak matching the GC-FID retention time was detected, but the concentration was below the limit of quantitation indicated. [b] Brassicasterol was monitored but found at trace level (<2 mg/100 g) or not detected (<0.5 mg/100 g) in all samples. [c] All products are raw and shelled, unless otherwise noted; see **Table 1** for product descriptions. [d] See **Table 5**. [e] In parentheses: range for all composites; analytical standard error. If no range is given, only one composite was analyzed. [f] <2.4 mg/100 g.

tation ion ratios are dependent on analytical conditions, making it challenging to compare data obtained under different conditions (*35*). Differentiation between Δ^5 and Δ^7 sterols was possible when major ions matching published spectra (*35*) were evident; however, many spectra displayed characteristics of both Δ^5 and Δ^7 sterols and were difficult to classify. In many products (including both nuts and seeds), compounds with a characteristic sterol fragmentation pattern but displaying a parent ion at *m*/*z* 498 or ion fragments at *m*/*z* 422 and 408 were found and probably represent 4,4-dimethylsterols (including cycloartenol and 24-methylenecycloartanol) (*37*).

Table 5 summarizes the concentrations and GC-FID retention times of the unknown sterols; selected mass spectra are shown in **Figure 2**. The identity of some of the unknowns could be postulated (**Table 5**) by inference from literature reports. Δ^7-Avenasterol, spinasterol, poriferasta-7,25-dienol, poriferasta-7,-22,25-trienol, and Δ^7-stigmastenol have been reported in pumpkin seed oil by Wenzl et al. (*36*) and Breinholder et al. (*22*), and on the basis of the GC-MS spectra and elution order and/or relative concentrations, it seems likely that sterol C (**Figure 2a**) is poriferasta-7,25-dienol, sterol I is Δ^7-avenasterol, and sterol E (**Figure 2b**) is Δ^7-stigmastenol. Unknown sterol L (**Figure 2c**), found in pumpkin seed extracts subjected to acid hydrolysis, matched the retention time of stigmasterol but did not display a fragmentation pattern characteristic of Δ^5 sterols (*35*), most notably a prominent $M^+ - ROH$ ion (*m*/*z* 394), and was therefore determined not to be stigmasterol. This finding is consistent with the negligible level of stigmasterol found in pumpkin seed by Breinholder et al. (*22*). Although less certain due to coelution, sterol(s) JK, which had the same retention time as sitosterol (**Figure 1a**-ii), is likely a mixture of spinasterol and poriferasta-7,22,25-trienol, as reported by Strobl (*38*) and also consistent with findings of Breinholder et al. (*22*), who found these components to elute sequentially using a more polar column. Other minor phytosterols have been found at low levels in nuts and seeds in other studies, such as clerosterol in walnuts (*18*) or oils derived from them (*17*), and may correspond to some of the unknown sterols in the present study. Although further work with nuclear magnetic resonance could assist in the identification of these sterols, it was beyond the scope of the present study. Spectroscopic data for many sterols have been compiled by Goad (*1*) and Rahier and Benveniste (*35*); data for Δ^7-sterols in particular have been reported by Strobl (*38*); physical properties can be found in the review by Bergmann (*39*).

Figure 3 illustrates the relative total phytosterol content of the nuts and seeds analyzed. Sesame seed and wheat germ had the highest total sterol contents (400 and 413 mg/100 g, respectively). Black walnut had a notably higher concentration of phytosterols than English walnut (177 versus 113 mg/100 g). Of the products commonly consumed as snack foods, pistachio, sunflower seed kernel, and pumpkin seed kernel were richest in phytosterols (265−289 mg/100 g), equivalent to 74−81 mg per 1 oz (28 g) serving. Most other products contained 135−200 mg/100 g total phytosterols (38−56 mg/28 g serving), with the lowest levels in Brazil nut (95 mg/100 g) and English walnut (113 mg/100 g; 27−32 mg/28 g serving).

β-Sitosterol, campesterol, and stigmasterol on average are the predominant sterols in foods and are therefore often the only sterols routinely measured for some applications. However, **Figure 3** shows that sterols other than these comprise a significant portion of total phytosterols in most nuts and seeds

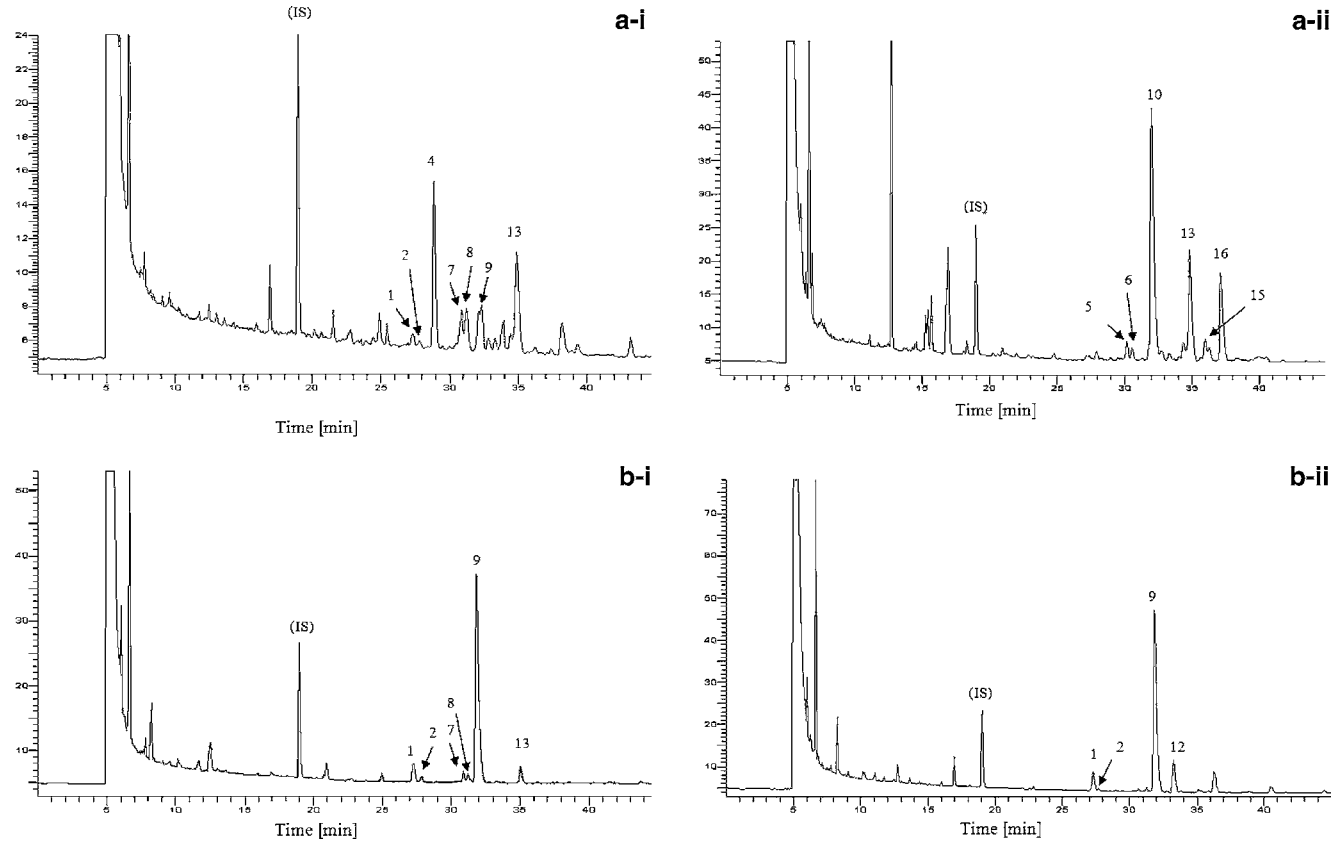

Figure 1. Representative GC-FID chromatograms for (**a**) pumpkin seed kernel, (i) with acid hydrolysis and (ii) alkaline hydrolysis only; (**b**) cashew, (i) with acid hydrolysis and (ii) alkaline hydrolysis only. Key: (IS) internal standard (epicholesterol); (1) campesterol; (2) campestanol; (3) stigmasterol; (4) sterol L; (5) sterol G; (6) sterol H; (7) sterol A; (8) sterol B; (9) β-sitosterol; (10) sterol(s) JK; (11) sitostanol; (12) Δ^5-avenasterol; (13) sterol C; (14) sterol F; (15) sterol E; (16) sterol I. Lettered sterols refer to unknowns summarized in **Table 5**, with mass spectra shown in **Figure 2**. Chromatography conditions: 5% diphenyl-95% dimethylpolysiloxane column, 60 m, 0.25 mm i.d., 0.1 μm film; 0.5 μL injection volume; helium carrier gas at 0.58 mL/min; split ratio, 1:17; injector temperature, 280 °C; detector temperature, 300 °C; oven temperature program, 270 °C, hold for 45 min, then 5 °C/min to 285 °C, hold for 12 min.

and, in fact, 93% of the sum in pumpkin seed kernel. On average, the contribution of sterols other than β-sitosterol, stigmasterol, and campesterol was 50 mg/100 g in the products tested and was generally higher in seeds. Furthermore, the proportion of these other sterols was not the same in all products. Therefore, the sum of β-sitosterol, campesterol, and stigmasterol will likely underestimate the total phytosterol content of nuts and seeds and also not provide a reliable indicator of the relative sterol content among different products. Also, it was notable that GC-MS analysis of pumpkin seed kernel extracts undergoing only the usual alkaline hydrolysis (i.e., no acid hydrolysis) contained a significant peak matching the FID retention time of β-sitosterol (**Figure 1a**-ii), corresponding to 82.8 mg/100 g, but GC-MS analysis revealed this component (**Table 5**, sterol JK) was not β-sitosterol.

For most of the products there was a very small difference ($\leq$10 mg/100 g) in total phytosterols among different samples of the same product (see last column of **Table 4**). The largest variability was in the USDA commodity peanut butter, with a range of 129−167 mg/100 g. It is possible that variation in the type of vegetable oils used in the peanut butter contributed to differences in total phytosterol content; only "vegetable oil" was specified in the ingredient listing on the product labels. Because sampling and composite preparation in most cases was based on key foods and food intake data and therefore incorporated multiple variables (e.g., brand, geographical sampling location, production lot) (*28*), it is not possible to determine the precise reason for any differences observed among individual composites. Interestingly, pine nut had a much higher phytosterol content than roasted pinon nut (236 versus 147 mg/100 g). Pine nuts in the marketplace come from a variety of sources and species (*40*), and the difference between "pinon nut" and "pine nut" is that pinon refers specifically to pine species in the southwestern United States and traditionally prepared by Navajo and Zuni Indians (*41*). The large difference in phytosterol content of pine nut and pinon nut in the present study could be due to difference in species or in processing (raw versus roasted), and further studies would be interesting.

Comparison of Results to Existing Data. The main source of food composition data in the United States is the USDA National Nutrient Database for Standard Reference (USNDB) (*16*), and data for phytosterols in nuts and seeds are limited. In fact, the present study was conducted as part of the USDA's National Food and Nutrient Analysis Program (*26*, *27*) to improve the database content for phytosterols by using newer methods as well as nationally representative samples. **Figure 4** summarizes current phytosterol data in the USNDB compared to results from the present study, as well as to values that appeared in the compilation of Weihrauch and Gardner (*17*). For all products, the value for total phytosterols was lower in the USNDB and the Weihrauch and Gardner compilation, except in the case of sunflower kernel, for which the value in the present study was markedly lower. The likely reason for the higher values in the present study is that existing values include only the major dietary phytosterols (β-sitosterol, stigmasterol, campesterol) that are typically quantified in routine analyses,

Table 5. Unknown Sterols (Milligrams per 100 g of Product)[a]

	unknown sterol									
	A	B	C	E[d]	F[d]	G[d]	H[d]	I[d]	JK[d]	L
tentative ID:			poriferasta-7,25-dienol	Δ^7-stigmastenol				Δ^7-avenasterol	spinasterol and poriferasta-7,22,25-trienol	
RRT:[b]	1.62	1.64	1.84	1.89	1.87	1.59	1.61	1.95	1.68	1.52
product[c]										
almond	5.0	4.6	10.1							
chocolate			3.0							
Brazil nut			1.4							
cashew	3.2	3.2	6.9							
cashew, oil-roasted	2.9	3.0	6.9							
flaxseed, ground	4.4	4.5	14.8		2.4					
flaxseed, whole	4.5	4.8	14.7		2.4					
hazelnut			2.5							
macadamia nut	3.4	3.5	6.9					3.3		
peanut, oil-roasted	3.4	3.4	7.3							
peanut, dry-roasted	3.7	3.4	7.9							
peanut butter, USDA commodity, smooth	3.1	3.0	6.7							
peanut butter, retail, smooth	3.1	3.2	6.7							
peanut butter, retail, chunk-style	2.9	2.8	6.3							
pecan	3.3	3.3	7.6							
pine nut	8.7	7.9	17.7							
pinon nut	2.9	2.7	6.6							
pistachio	5.8	6.2	12.6							
poppy seed	4.5	4.7	8.9		1.5					
pumpkin seed kernel	13.4	13.7	41.0	5.7		4.7	2.9	36.2	94.8	28.2
sesame seed	9.4	9.2	20.4		8.5					
sunflower seed kernel, high-oleic	3.4	3.7	9.9	22.5						
sunflower seed kernel	5.5	5.2	11.7	20.8						
sunflower seed kernel, oil-roasted	5.7	6.6	14.3	17.5						
walnut, black	6.4	5.9	13.5							
walnut, English	1.8	1.9	5.4							
wheat germ	6.0	6.2	13.6	11.6	6.0	3.8	5.7	13.3		

[a] Confirmed to be phytosterols by GC-MS, but the exact structure could not be identified; selected mass spectra are given in **Figure 3**. No entry indicates the component was not detected. [b] GC-FID relative retention time, under the analytical conditions described in the text, expressed as (retention time analyte)/(retention time IS), where IS is the epicholesterol internal standard. (Lettering sequence does not correspond to elution order.) [c] All products are raw and shelled, unless otherwise noted; see **Table 1** for product descriptions. [d] Present only in sample extracts not subjected to acid hydrolysis.

whereas nuts and seeds contain significant levels of other sterols (e.g., Δ^5-avenasterol; **Table 4**), and also that the data were obtained by methods that do not recover steryl glucosides (e.g., ref *42*). Previous work has shown that steryl glucosides comprise 13, 17, and 23% of total sterols in pine nut, peanut butter, and almond, respectively (*25*). Given the many possible mechanisms of action of phytosterols on processes that affect cholesterol metabolism, carcinogenesis, and other biological activities that are the subject of continuing research (*43*), it is important to have quantitative estimates of total phytosterol content that comprise all forms in which these compounds exist in foods. In the case of sunflower kernel, further investigation revealed that the source of the total phytosterol value in the USNDB was actually the Weihhrach and Gardner publication (*17*), which provides no information about the precise source of the value, but suggests that it may have been inferred using sterol data reported for sunflower oil, so it is not possible to determine the reason for the discrepancy. These results highlight differences that may exist between published phytosterol values and the composition of marketplace samples of apparently the same product. It is expected that data from the present study will be used to update phytosterol values in a subsequent release of the current USNDB (*16*).

The results of this study also illustrate the complexity that can be involved in evaluating food phytochemical levels. Determination of the phytosterol composition of nuts and seeds is not amenable to "production scale" analysis of predetermined components using existing standard methods. The use of carefully validated analytical methodology and GC-MS verification of analyte identities on a product-specific basis was essential to avoid errors in quantitation of phytosterols. Further method development would be desirable to simplify the procedure used to recover and quantify total free, esterified, and glycosidic sterols, especially acid labile components.

ABBREVIATIONS AND NOMENCLATURE

The following trivial names for sterols were used, with systematic nomenclature noted in parentheses: β-sitosterol (24*R*-ethylcholest-5-en-3β-ol); campesterol (24*R*-methylcholest-5-en-3β-ol); stigmasterol (22*E*-24*S*-ethylcholesta-5,22-dien-3β-ol); Δ^5-avenasterol [24*Z*-ethylidenecholesta-5,24(28)-dien-3β-ol]; sitostanol (24*R*-ethylcholestan-3β-ol); campestanol (24*R*-methylcholestan-3β-ol); brassicasterol (22*E*-24*R*-methylcholesta-5,22-dien-3β-ol); Δ^7-avenasterol [24*Z*-ethylidenecholesta-7,24(28)-dien-3β-ol]; spinasterol (22*E*-24*S*-ethylcholesta-7,22-dien-3β-ol); Δ^7-stigmastenol (24*S*-ethylcholest-7-en-3β-ol); clerosterol (24*S*-ethylcholesta-5,25-dien-3β-ol); epicholesterol (cholest-5-en-3α-ol). A general discussion of sterol nomenclature has been published by Goad (*1*).

SAFETY

Usual laboratory precautions should be taken when working with hydrochloric acid and solvents (e.g., chloroform, methanol,

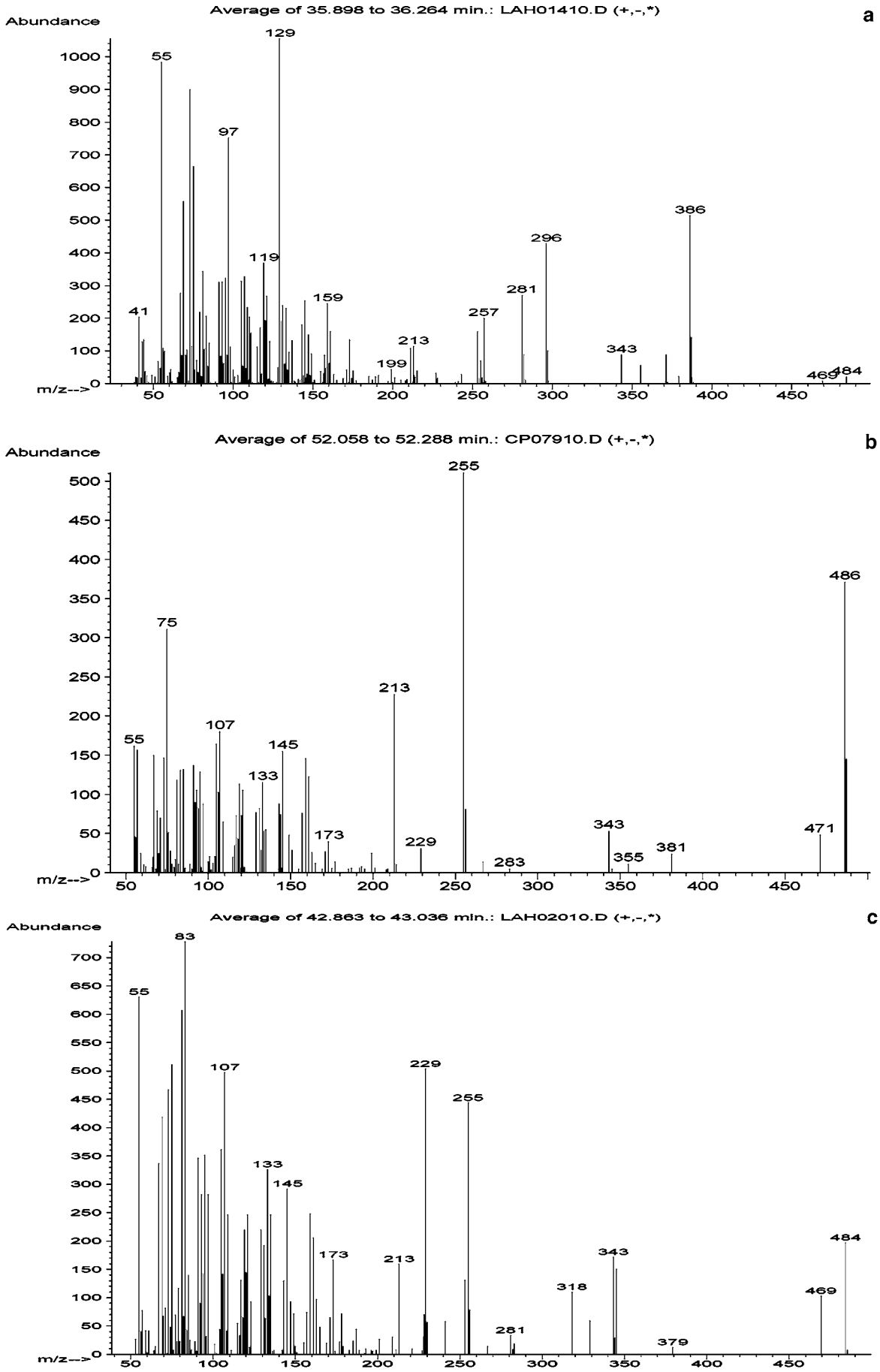

Figure 2. Mass spectra for selected unknown sterols (**Table 5** and **Figure 1**), as trimethylsilyl derivatives: (**a**) sterol C, from poppy seed; (**b**) sterol E, from pumpkin seed kernel; (**c**) sterol L, from pumpkin seed kernel. For GC-MS conditions, see GC-MS under Materials and Methods.

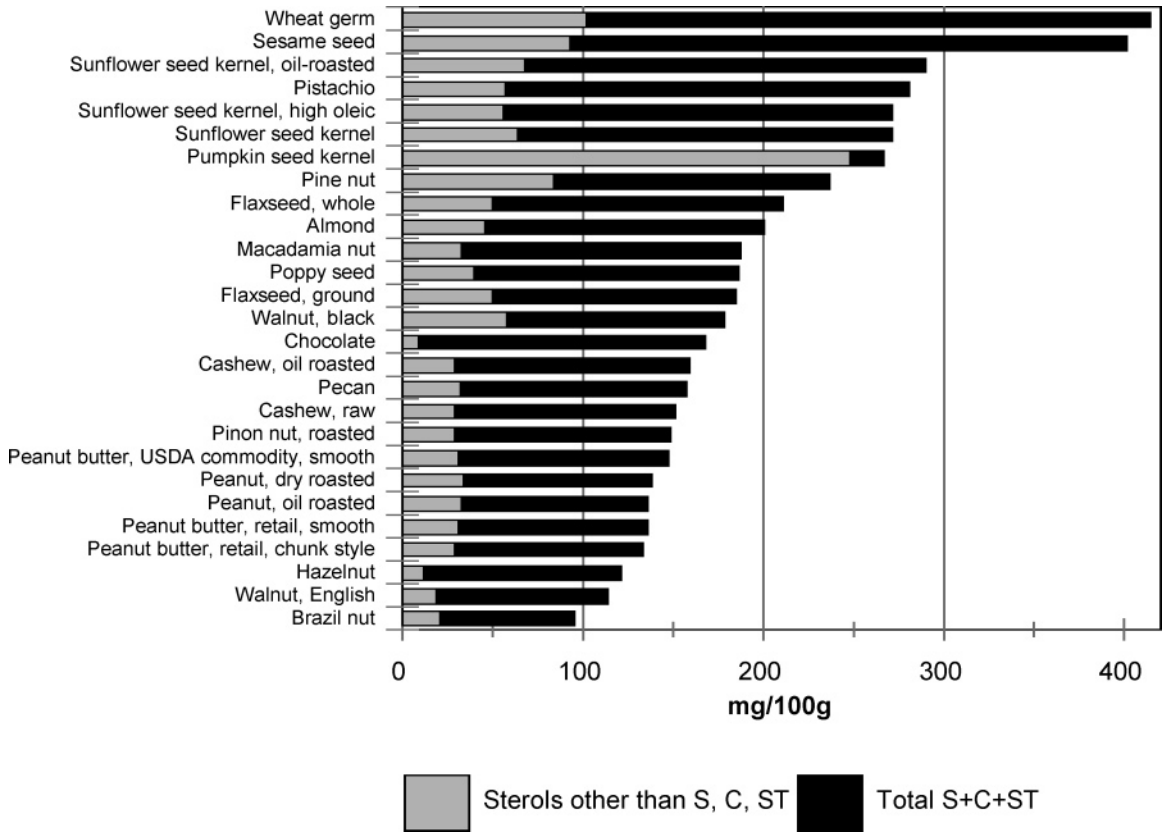

Figure 3. Relative content of phytosterols in nuts, seeds, and some related products [β-sitosterol (S), campesterol (C), and stigmasterol (ST)].

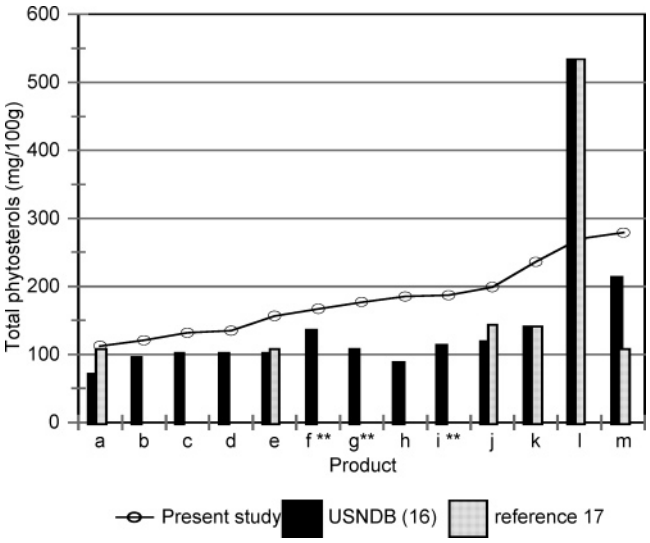

Figure 4. Comparison of total phytosterol concentrations determined in present study to previous data [USNDB, release 17 (*16*), and Weihrauch and Gardner (*17*)]: a, English walnut; b, hazelnut; c, chunk-style peanut butter; d, smooth peanut butter; e, pecan; f, chocolate; g, black walnut; h, poppy seed; i, macadamia nut; j, almond; k, pine nut; l, sunflower seed kernel; m, pistachio. Total phytosterols reported in refs *16* and *17* are either not specified or the sum of β-sitosterol, campesterol, and stigmasterol only. In the present study, total phytosterols included free, esterified, and glycosidic β-sitosterol, campesterol, stigmasterol, Δ^5-avenasterol, sitostanol, campestanol, and 10 unknown sterols (**Tables 4** and **5**). The double asterisk (**) indicates cases in which the same composites were assayed to yield the values from the USNDB (*16*) and the present study.

ether, hexane); consult manufacturer's materials safety data sheets for all chemicals for specific cautionary measures and storage information.

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Supporting Information Available: Mass spectra for additional unknown sterols (**Table 5**) are available free of charge via the Internet at http://pubs.acs.org.

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