Commercial Peanut (*Arachis hypogaea* L.) Cultivars in the United States: Phytosterol Composition

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Phytosterols in commercially grown Runner, Virginia, and Spanish peanuts (*n* = 221) from 2005 and 2006 were quantified by a combination of acid hydrolysis and alkaline saponification steps followed by gas chromatography−flame ionization detection and gas chromatography−mass spectrometry analysis of the trimethylsilyl derivatives. Δ^5-Avenasterol, which partially degrades during acid hydrolysis, was quantified after alkaline saponification plus direct analysis of the steryl glucosides isolated by solid-phase extraction. β-Sitosterol, Δ^5-avenasterol, campesterol, and stigmasterol were identified in peanut lipid extracts as the dominant sterols by retention time mapping and mass spectra with recoveries ∼99%. Clerosterol, $\Delta^{5,24(25)}$-stigmastadienol, Δ^7-sitosterol + cycloartenol, and one unidentified sterol were also present but at low levels. Free and esterified phytosterols accounted for ∼80% of the total sterols determined; the remainder was attributed to steryl glucosides. The total sterol level in Spanish market type peanuts (144.1 ± 5.3 mg/100 g) was significantly greater than both Runners (127.5 ± 6.3 mg/100 g) and Virginias (129.3 ± 6.9 mg/100 g) (*P* < 0.05). Tamspan 90 (146.9 mg/100 g) followed by OLIN (138.5 mg/100 g) showed the highest total sterol content among the cultivars examined. Cultivar effects were strongly significant (*P* < 0.001) for all phytosterols, whereas production year effects were strongly significant (*P* < 0.001) for Δ^5-avenasterol, $\Delta^{5,24(25)}$-stigmastadienol, and the combined quantities of Δ^7-sitosterol + cycloartenol, which coeluted. Cultivar × year interactions were strongly significant (*P* < 0.001) in all sterols except for Δ^7-sitosterol + cycloartenol (*P* < 0.01). Total phytosterol contents were markedly higher than those reported in the existing literature for Runner and Virginia type peanuts, partially attributed to the inclusion of steryl glucosides in the analysis.

KEYWORDS: Peanuts; phytosterols; chemical composition; phytochemicals; U.S. Department of Agriculture National Nutrient Database for Standard Reference; GC-MS; mass spectrometry; cluster analysis

INTRODUCTION

The production of Runner, Virginia, Spanish, and Valencia market type peanuts (*Arachis hypogaea* L.) in the United States comprises ∼10% of the world's supply (*1*). Utilization of peanuts by the food industry and per capita consumption in the United States are greater than those of all tree nuts combined (*2*); moreover, the peanut, which is actually a legume and not a nut, represents over 50% of all nuts consumed by adults who eat nuts as a snack (*3*). Even though peanuts and tree nuts are high in fat, their consumption is viewed as a significant contribution to a healthy diet. They contain important nutrients and bioactive constituents that can provide a wide range of health benefits; many of these have been reviewed (*3−5*). Important attributes of peanuts include protein with very high arginine content, relatively high levels of soluble and insoluble fiber, healthful fatty acids,

vitamins including high folate and vitamin E levels, phenolics, and phytosterols (*6*). In August 2002, the International Tree Nut Council Nutrition Research and Education Foundation submitted a petition to the Food and Drug Administration (FDA) requesting a health claim for nuts (both peanuts and tree nuts) and heart disease. The strength of research reports documenting the health benefits on serum lipids from nut consumption led the U.S. FDA to issue a category B qualified health claim: "Scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (*7*).

Phytosterols are one of the important bioactive constituents in peanuts. They are unsaponifiable triterpenes found in the lipid fraction, possessing a cyclopentanoperhydrophenanthrene ring structure, and are sometimes referred to as 4-desmethyl sterols. More than 250 phytosterols and related compounds have been described in the literature (*8*). They are structurally similar to cholesterol but differ in the side chain at C_{24} and/or the position

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and configuration of double bonds. In foods, phytosterols can exist in four forms: free sterols, steryl esters of fatty or phenolic acids, steryl glucosides, and acylated steryl glucosides (*9, 10*). Phytostanols are a fully saturated subgroup of phytosterols, which are normally present at trace levels in plants but are fairly abundant in cereal grains. Campestanol and β-sitostanol are also typically found in partially hydrogenated vegetable oils.

The health benefits of phytosterol/stanols consumption as natural components of a regular diet or from fortification in functional foods include anticarcinogenic and hypocholesterolemic effects (*11, 12*). Clinical studies have shown the capacity of phytosterols to inhibit HT-29 colon cancer cells, prostate cancer LNCaP cells, and MDA-MB-231 breast cancer cells (*13*). However, it is the documented research demonstrating a reduction in serum cholesterol levels, among other positive effects on blood lipid profiles, that led the FDA to authorize a health claim in 2000 on the relationship between plant sterol and stanol esters and a reduction in the risk of coronary heart disease (21 CFR § 101.83, September 8, 2000). Questions still exist as to the optimum levels and frequency of phytosterol consumption required each day to attain these benefits. Investigations by Ostlund and co-workers (*14, 15*) have demonstrated the effectiveness of low levels of phytosterols, like those occurring naturally in diets rich in plant foods, at reducing the absorption of dietary cholesterol. This bodes well for peanuts, as they are consumed as a snack throughout the day by many. Surprisingly, few studies exist on the characterization and quantification of phytosterols among present day peanut cultivars grown in the United States, and no data exist for raw peanuts in the U.S. Department of Agriculture National Nutrient Database for Standard Reference, Release 22 (*16*).

Studies within the past 5 years concerning the phytosterol content of peanuts and peanut products include those of Jonnala et al. (*17, 18*), Normén et al. (*19*), and the excellent investigations by Phillips et al. (*10, 12*). Phillips et al. (*12*) reported on the phytosterol contents of nut- and seed-containing products for composite samples obtained as part of the U.S. Department of Agriculture National Food and Nutrient Analysis Program (*20*). The total phytosterol levels were found to be 135 and 137 mg/100 g in oil- and dry-roasted peanuts, respectively, and ranged from 132 to 135 mg/100 g in retail chunky and smooth peanut butters. The researchers stressed the importance of including steryl and acetylated steryl glucoside levels in the "total sterol" determination, as exclusion of these can markedly underestimate the total sterol content of nuts and seeds. To our knowledge, the study by Phillips et al. (*12*) is the first to report the contribution of steryl and acetylated steryl glucosides to "total sterol" content in a peanut product. Recently, Lin et al. (*21*) investigated the nutritional effects of steryl glucosides in humans. In a randomized crossover study design, participants received ~300 mg of added phytosterols in the form of phytosterol glucosides or esters or a placebo. The findings indicated that phytosterol glucosides reduced cholesterol absorption by 37.6% ($P < 0.0001$) and phytosterol esters by 30.6% ($P = 0.0001$) when compared to the placebo test. This study demonstrates that steryl glucosides in plant-based foods ought to be measured and results included in nutritional databases of food phytosterol contents. Using a validated method, Normén et al. (*19*) documented the phytosterol levels of fatty foods consumed in Sweden and The Netherlands. From this study, a peanut sample (most likely roasted peanuts) was found to contain 116 mg total phytosterols/100 g sample, and two samples of peanut oil contained total phytosterol levels of 251 and 315 mg/100 g oil. Using transgenic peanut lines, Jonnala et al. (*17, 18*) reported very high, however, variable phytosterol contents for genetically modified peanuts. β-Sitosterol was found

to be the major phytosterol in all of the genetically modified peanuts and constituted over 90% of the total sterol content; this is atypical as compared to the 60−70% level determined in classically plant-bred peanut cultivars. Furthermore, β-sitosterol concentrations ranged from 190 to 649 mg/100 g oil, which is considerably higher than those reported by either Phillips et al. (*12*) or Normén et al. (*19*). Reasons for the high values given by Jonnala et al. (*17, 18*) are not clear.

The methodology chosen to quantify food sterols greatly influences analytical values. Approaches employing the direct injection of an oil or lipid extract into a gas chromatograph without first subjecting it to acid or alkaline hydrolysis can only measure the content of free sterols (*22*). Methods based on saponification (i.e., alkaline hydrolysis) determine both free and esterified sterols but not steryl glucosides, as the acetal bonds are stable under these hydrolytic conditions (*1, 13, 17, 18, 23*). Because of the fact that sterols exist in free, esterified, and glycosidic forms, the best approach seems to be a combination of acid and alkaline hydrolysis (i.e., double hydrolysis) to release and convert all forms of sterols to free sterols, which can be extracted with nonpolar extractants, such as hexanes, and then quantified. Jonker et al. (*24*) first described such an approach. More recent studies by Phillips et al. (*10, 12*) clearly demonstrated the importance of including acylated steryl and steryl glucosides in the total sterol value for foods, as methods employing only saponification underestimate the total phytosterol content. Analysis of a wide variety of food products indicated that between 9 and 37% of the total β-sitosterol, campesterol, and stigmasterol in foods was contributed by the glucosides (*10*). For instance, in peanut butter, the inclusion of steryl glucosides increased the phytosterol levels of campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol by 14, 19, 17, and 14%, respectively.

Although peanuts are recognized as an excellent source of phytosterols, the aforementioned data in the literature on the phytosterol content of raw peanuts are variable and difficult to interpret; many of these studies report phytosterol levels based on experimental growth conditions that do not reflect commercial production employing present-day agronomic practices. Moreover, data for retail products often represent peanuts from unknown cultivars and, in some studies, are based on small sample sizes. At the request of the U.S. peanut industry, a study was designed to re-examine and update compositional information, including the levels of key bioactive constituents, for major peanut cultivars in the United States. This paper, only a portion of the complete study, reports on the phytosterol composition of Runner, Virginia, and Spanish peanut types. To ensure the best possible data, an intensive sampling program was designed and implemented by the industry to provide cultivars in current production by accepted agricultural practices employed in the United States sampled over two harvest seasons. The strength of this research initiative lies in the care and detail that went into the sampling effort of peanut types and cultivars. A number of lipid constituents were analyzed, and results for fatty acids and vitamin E have been reported elsewhere (*25, 26*). Data resulting from the analyses of 221 peanut samples using validated analytical methods distinguish this work from all other peanut phytosterol investigations.

The objective of the present study was to quantify the total phytosterol contents in raw peanuts (Runner, Virginia, and Spanish market types) of present-day cultivars grown in the United States using modern agronomic practices. Acid hydrolysis, saponification, and solid-phase extraction (SPE) steps were employed to isolate the total free, esterified, and glycosidic forms of phytosterols from peanut lipid extracts, which were then chromatographed, identified, and quantified by gas chromatography−flame

ionization detection (GC-FID) and gas chromatography−mass spectrometry (GC-MS) techniques using validated methods. Findings from this study will be submitted to the U.S. Department of Agriculture for consideration of inclusion in the National Nutrient Database for Standard Reference, as no data for phytosterols in raw peanut types exist in the current database.

MATERIALS AND METHODS

Collection of Samples. Runner, Virginia, and Spanish type peanut samples (n = 221) comprising 13 normal (six Runner, six Virginia, and one Spanish), one mid oleic (Tamrun OL01, Runner), and four high oleic cultivars (three Runner and one Spanish) collected from three different regions in the country (i.e., Southeast, Southwest, and Virginia/Carolinas) for the 2005 and 2006 production years were provided by The Peanut Institute (Albany, GA), the U.S. Department of Agriculture−Agricultural Research Service National Peanut Laboratory (Dawson, GA), and personnel from the peanut industry. Although Valencia is a common peanut market type, it was not possible to obtain a sufficient number of samples; so, it was not included in this study to maintain balanced sampling among cultivars. In brief, sampling involved the development of a uniform plan that accurately defined major Runner, Virginia, and Spanish cultivars grown in the fields of U.S. peanut farmers from the three peanut-growing regions. The cultivars for study were selected based on sheller input and seed sales to reflect present-day percentages of peanuts in the U.S. market, which explained the inclusion of fewer Spanish cultivars as compared to Runner and Virginia peanut types.

Peanuts were taken from seed wagons, after initial drying to a moisture content between 8 and 10%, and an official sample was pulled and graded. The sheller obtained the back half of the official-grade sample and subdivided it down to 3 lb. This sample was then cleaned using a grade-room Farmerstock cleaner and forwarded to the U.S. Department of Agriculture−Agricultural Research Service National Peanut Research Laboratory for further processing. There, the samples were then shelled and sent to the Department of Food Science and Technology, University of Georgia (UGA), in Athens, GA. Upon arrival at UGA, the raw peanuts with skins on were packaged in labeled vacuum pouches (Prime Source, Kansas City, MO) with a vacuum system (Henkelman 600, Henkelman, 's-Hertogenbosch, The Netherlands) to prevent oxidative degradation. The vacuum-packaged peanuts were stored at −40 °C until analyzed.

Chemicals and Glassware. Hydrochloric acid (ACS-grade), potassium hydroxide (ACS-grade), pyridine, and toluene were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ). Methanol, chloroform, and hexanes were ACS-grade and purchased from Fisher Scientific Co., LLC (Suwanee, GA). Denatured ethanol (Chromasolv, for HPLC) and pyrogallol (99%, ACS-grade) were obtained from Sigma Chemical Co. (St. Louis, MO). A plant sterol mixture (purity 98%), stigmasterol (purity 95%), and 5α-cholestane [i.e., the internal standard (IS) for this work, purity > 98%] were acquired from Matreya, LLC (Pleasant Gap, PA), whereas β-sitosterol (purity > 97%) and campesterol (purity ∼98%) were purchased from Sigma Chemical Co. The silanizing reagent, 5% dimethyl-dichlorosilane (DMDCS) in toluene, was obtained from Supelco Chemical Co. (Bellefonte, PA), and *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) was acquired from Pierce Chemical Co. (Rockford, IL). Reacti-vial small reaction vials (5 mL), septa, and caps were purchased from Fisher Scientific.

Silanization of Glassware. All glassware was silanized by coating the glass surfaces with the 5% DMDCS in toluene for ∼15 s, after which the surfaces were rinsed 2× with toluene followed by 3× with anhydrous methanol. The glassware was allowed to air dry.

Extraction of Lipids from Peanuts. Raw peanuts were removed from the freezer and tempered until they reached room temperature. Twenty grams was finely ground in a commercial coffee mill (Tipo 203, Krups, New York, NY). Lipids were extracted in duplicate from 5 g of each peanut cultivar according to Shin et al. (*25*). Briefly, ground peanuts were homogenized with $CHCl_3/CH_3OH$. The $CHCl_3$ layer, containing lipids, was collected and evaporated to dryness using a nitrogen evaporator (N-EVAP 111 with an aluminum bead dry bath, Organomation Associates, Inc., Berlin, MA). The resulting lipid extract was transferred to an amber-colored vial and stored under a N_2 headspace at −80 °C until analyzed for phytosterols.

Acid Hydrolysis and Saponification. As recommended by Phillips et al. (*12*), a combination of acid hydrolysis and saponification was employed for the extraction of total phytosterols. The acid hydrolysis step was employed to liberate sterols from steryl and acylated steryl glucosides (*27*). Briefly, 1 mL of the IS solution (0.5 mg 5α-cholestane/mL in hexanes) was added to a 25 mm × 200 mm glass culture tube with a screw cap and then evaporated to dryness using the N-EVAP. A portion (∼300−500 mg) of the peanut lipid extract was accurately weighed into the culture tube via a Pasteur pipet. Absolute ethanol (1 mL) was added, and the tube was shaken manually and then sonicated for 2 min in a water bath to dissolve the oil and IS. After this, 5.0 mL of 6 M HCl was added. The sample tube was flushed with N_2 to help prevent lipid oxidation, capped tightly, and sealed with Teflon tape. The mixture was heated in a shaking water bath (BT-23, American Scientific Products LLC, Columbus, OH) at 80 °C for 1 h. After it was heated, the hydrolysis tube was cooled at room temperature for ∼15 min. More absolute ethanol (5 mL) was added, and the tube was vortexed for 30 s. To facilitate phase separation, 12 mL of deionized water was added to the tube after which the liberated sterols were extracted 3× with 7 mL portions of 1:1 (v/v) diethyl ether:hexanes. The organic solvent layers were collected, pooled, and evaporated to dryness at 40 °C using the N-EVAP.

The dried residue from acid hydrolysis was then subjected to alkaline hydrolysis according to Jekel et al. (*28*). The sample was dissolved in 8.0 mL of denatured ethanol containing 3% (w/v) pyrogallol, and then, 0.5 mL of a saturated KOH solution was added. The sample tube was flushed with N_2 and capped tightly, and the contents were saponified at 80 °C for 30 min. To facilitate phase separation, 12 mL of deionized water was added to the tubes after which the unsaponifiables were extracted 3× with 7 mL portions of hexanes. The pooled hexane fraction was evaporated to dryness at 40 °C using the N-EVAP. Peanut lipid extracts were also saponified without an acid hydrolysis step. Quantification of phytosterols recovered by saponification alone provides values only for the free and esterified sterols but not the steryl and acetylated steryl glucosides. Duplicate analyses of each lipid extract were completed.

Preparation of Trimethylsilyl (TMS)-Ether Derivatives. After hydrolysis, free sterols were assayed by GC-FID as their TMS-ether derivatives following the procedure of Toivo et al. (*27*). The entire portion of the dried residue, after the combination of acid hydrolysis and alkaline saponification, was quantitatively transferred with hexanes (and then evaporated to dryness) to a 5 mL Reacti-vial containing a Reacti-vial magnetic stirrer. For preparing TMS derivatives, 250 μL each of anhydrous pyridine and BSTFA + 1% TMCS reagent were added. The vial was capped and placed in a Reacti-Block B-1 aluminum block within a Reacti-Therm III Heating/Stirring Module (Thermo Fisher Scientific, Rockford, IL) set at 70 °C with gentle stirring for 1 h. After derivatization, samples were cooled to room temperature and evaporated to dryness with N_2 using the N-EVAP. The TMS ethers were redissolved in 1.5 mL of hexanes and transferred to 2 mL wide-opening crimp top vials (Agilent Technologies, Wilmington, DE). Vials were capped with 11 mm silver aluminum caps, clear PTFE/red rubber septa, and then crimped with an electronic crimper.

Quantificiation of Δ^5-Avenasterol. As documented by Phillips et al. (*12*), Δ^5-avenasterol is an acid-labile sterol. Steryl glucosides were isolated from the peanut lipid extract by SPE. Briefly, a Sep-Pak classic silica cartridge (500 mg, 6 cc, 80 μm particle size; Waters Corp., Milford, MA) was conditioned with 2 vol of $CHCl_3$, and then, 100 mg of the lipid extract was quantitatively transferred to the cartridge using 2× $CHCl_3$ rinses. A further 2 mL of $CHCl_3$ was employed to ensure complete elution of the free and esterified sterols; steryl glucosides were then eluted with 3× 2 mL aliquots of CH_3OH. The steryl glucoside fraction was dried with the N-EVAP at 50 °C and then derivatized using an excess (500 μL) of the BSTFA + 1% TMCS reagent, a reaction temperature of 75 °C, and a 2 h incubation. The TMS ethers were analyzed by GC-FID as described below. A separate portion of the peanut lipid extract was subjected to alkaline hydrolysis alone, derivatized, and then assayed for free and esterified sterols. The total content of Δ^5-avenasterol in the peanut lipid extract was calculated by summing the quantity of steryl glucosides determined by the SPE approach with that by saponification. As there is no commercial standard for Δ^5-avenasterol or its derivatives, Δ^5-avenasterol was quantified by constructing a calibration curve using a steryl glucoside standard mixture containing sitosteryl, campesteryl, and stigmasteryl glucosides (catalog no. 1117, purity 98%) from Matreya LLC.

Five different concentrations of the standard ranging from 0.1 to 1.0 $\mu g/1$ μL injection were derivatized as described above. On the basis of the peak area of the β-sitosteryl glucoside as a percent of the total steryl glucosides, the sitosteryl glucoside was calculated to comprise 60.12% (0.2% relative SD) by weight of the mixed glucoside standard. The concentration of β-sitosteryl glucoside in the mixed steryl glucoside standard was calculated by adjusting for 98% purity and then by multiplying by 0.6012; the quantity of Δ^5-avenasterol was calculated against the β-sitosteryl glucoside peak.

GC-FID Analysis. An Agilent Technologies 6890N Network gas chromatograph system [configuration: capillary split/splitless inlet with electronic pneumatic control (EPC) and a FID with EPC, for packed and capillary column] equipped with a 7683 autosampler tray module, 7683B autoinjector module, and GC ChemStation software (Rev. A.08.03) was employed for phytosterol analysis profiling. Operating conditions were as follows: The column was a (5% phenyl) methylpolysiloxane fused-silica HP-5 capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness; Agilent Technologies); ultrahigh purity helium was the carrier gas at a flow rate of 1.9 mL/min, and analyses were performed in constant flow mode; a split linear with glass wool was installed in the injector; the injector temperature was set at 300 °C for split injection at a split ratio of 50:1; the FID temperature was set at 320 °C; ultrahigh purity hydrogen and scientific-grade air were the fuel gases for the FID and set at 40 and 450 mL/min, respectively, with 25 mL/min of N_2 as the makeup gas; the initial oven temperature was set at 260 °C and ramping up at 3 °C/min to 300 °C; this temperature was maintained for an additional 5 min. One microliter of sample was injected per run, and analyses were performed in triplicate or quadruplicate.

GC-MS Analysis. GC-MS was employed to confirm peak identity. The TMS-sterols were analyzed by a Hewlett-Packard 5890A gas chromatograph with a 5971A mass selective detector using electron impact ionization under the following conditions: an EC-5 Alltech Econo-Cap fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Alltech Associates, Inc., Deerfield, IL); a 1.0 μL injection volume; ultrahigh purity helium as the carrier gas; a split ratio of 17:1; an injector temperature of 290 °C; a detector temperature of 290 °C; an initial oven temperature of 260 °C that was ramped at 3 °C/min to a final oven temperature of 290 °C and held for 15 min; a transfer line temperature of 290 °C; an electron ionization energy of 70 eV; a scan range and rate of 50−550 amu at 1.4 scan/s; and an inlet pressure of 12 psi.

TMS-sterol peaks were identified by comparison of retention times to those of TMS-derivatized standards of campesterol, stigmasterol, β-sitosterol, and the plant sterol mixture. The Wiley 1994 Registry of Mass Spectral Data library (6th ed.) was used to match mass spectral peaks of phytosterol standards to those found in peanut TMS-sterol samples. Comparisons of parent molecular ion (M^+) and fragmentation patterns were employed to assist with elucidation of the identities of the phytosterols. Additional sterols, for which commercial standards are not available, were tentatively identified based on comparison of relative retention times (RRTs), M^+ values, and fragmentation patterns of TMS-sterols to those reported in the literature for peanut oil and plant oils.

Quantification of Phytosterols. Quantification was carried out using 5α-cholestane as the IS. To accurately quantify the sterols in raw peanuts, relative response factors (RRFs) to 5α-cholestane were determined based on five replicate analyses (*22*). The RRFs were calculated according to the following equation:

$$\text{RRF} = \frac{\text{PA}_{\text{sterol}}}{W_{\text{sterol}}} \times \frac{W_{\text{IS}}}{\text{PA}_{\text{IS}}}$$

where, $\text{PA}_{\text{sterol}}$ is the peak area of the sterol, W_{sterol} is the mass (mg) of the sterol, PA_{IS} is the peak area of the IS, and W_{IS} is the mass (mg) of the IS.

On the basis of the RRFs, sterol contents were calculated using the following equation (*22*):

$$\text{sterols (mg/100 g lipid extract)} = \frac{\text{PA}_{\text{sterol}}}{\text{PA}_{\text{IS}}} \times \frac{1}{\text{RRF}} \times \frac{W_{\text{IS}}}{W_{\text{sample}}} \times 100$$

where $\text{PA}_{\text{sterol}}$ is the peak area of the sterol, PA_{IS} is the peak area of the IS, W_{IS} is the mass (mg) of the IS, and W_{sample} is the mass (g) of the peanut lipid extract. Because of unavailability of commercial standards, Δ^5-

avenasterol, Δ^7-avenasterol, and other minor sterol compounds were quantified using the FID response of β-sitosterol.

Method Validation. Standard curve linearity was determined using the IS, campesterol, stigmasterol, and β-sitosterol. For each compound, standard solutions were prepared at five different concentrations in the ranges of 0.2−1.0 for 5α-cholestane, 0.025−0.125 for campesterol, 0.12−0.60 for stigmasterol, and 0.2−1.0 $\mu g/\mu L$ injection for β-sitosterol. All assays were replicated three times. Peanut lipid extracts were also spiked with known concentrations of the sterols: Final spiking levels were 32.4, 9.4, and 64.6 mg/100 g lipid extract for campesterol, stigmasterol, and β-sitosterol, respectively. Recovery was calculated using the following equation:

$$R\ (\%) = \frac{C_{\text{s}} - C_{\text{p}}}{C_{\text{a}}} \times 100$$

where R (%) is the percent recovery of added standard, C_{s} is the phytosterol content in the spiked sample, C_{p} is the phytosterol concentration in the unspiked sample, and C_{a} is the amount of phytosterol standard added.

For campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol, repeatability precision (i.e., % RSD_{r}) based on 10 replicates analyses was evaluated using a Runner peanut lipid extract and calculated with the following equation:

$$\%\text{RSD}_{\text{r}} = \text{standard deviation/mean} \times 100$$

The limits of detection (LODs) and limits of quantitation (LOQs) were determined on the basis of a minimal accepted value of the signal-to-noise ratio of 3 and 10, respectively.

Statistical Analysis. Phytosterol contents of raw peanuts were reported as mg per 100 g of kernel weight [means ± standard deviation (SD)]. Differences in the means of each cultivar were determined by Tukey's multiple range test at $P < 0.05$ using the Statistical Analysis System software, Version 9.0 (SAS Institute, Cary, NC). Cultivar, production year, and cultivar × production year effects were evaluated by a two-way factorial analysis of variance. Georgia-01R, Georgia-03L, AP-3, and NC-12 cultivars were eliminated from this factorial analysis because only one production year was available. The experimental factorial design contained 14 × 2 levels for each of two variables, cultivar, and production year. Cultivar and production year interactions were determined at $P <$ 0.05, 0.01, and 0.001, respectively.

Hierarchical cluster analysis (HCA) was performed to identify relative similarity among cultivars and reported as a dendrogram. Each similarity in the dendrogram was achieved on the basis of the Euclidean distance between cultivars using Ward's algorithm as the agglomerative method (*29*). The hierarchical algorithm constructs the nested grouping of patterns and similarity levels at which groupings change. From 1.0 to 0.0 on similarity, a value for the nearest neighbor distance at which two clusters from a preceding level merged was calculated. Similarity 0.0 was determined at the last clustering in the dendrogram. HCA was completed using the XLSTAT program (Version 2009) for the Window program.

RESULTS AND DISCUSSION

Method Validation. The RRFs for the TMS ether derivatives of the campesterol, stigmasterol, and β-sitosterol standards were 1.02, 1.03, and 0.98, respectively. Linearity of the FID responses for the IS, campesterol, stigmasterol, and β-sitosterol was excellent ($r^2 > 0.999$) over the concentration ranges ($\mu g/1\ \mu L$ injection) of 0.2−1.0 (5α-cholestane), 0.025−0.125 (campesterol), 0.12−0.6 (stigmasterol), and 0.2−1.0 (β-sitosterol). The mean percent recoveries of campesterol, stigmasterol, and β-sitosterol from spiked samples were 98.4 ± 2.2, 97.5 ± 4.3, and 98.7 ± 4.7%, respectively, thereby suggesting no significant loss of these phytosterols from raw peanut samples subjected to acid hydrolysis. Repeatability precision (% RSD_{r}) from intraday analyses of the Runner peanut sample was found to be 1.8, 1.4, 1.4, and 1.5% for campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol, respectively. The LOD values were 0.19, 0.21, and 0.23 $\mu g/mL$ for

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campesterol, stigmasterol, and β-sitosterol, respectively, whereas the respective LOQ values were 0.59, 0.51, and 0.52 μg/mL.

Identification of Phytosterols by GC-FID and GC-MS. Figure 1 depicts a representative GC-FID chromatogram of the TMS-sterols isolated from raw peanuts. A total of 10 peaks representing four major sterols, three minor ones, one unidentified sterol, and two additional peaks were resolved. Peak 9 in the chromatogram was confirmed to contain two minor sterol components that could not be resolved. Phytosterols were identified by comparison of retention times to those of commercial standards, MS parent
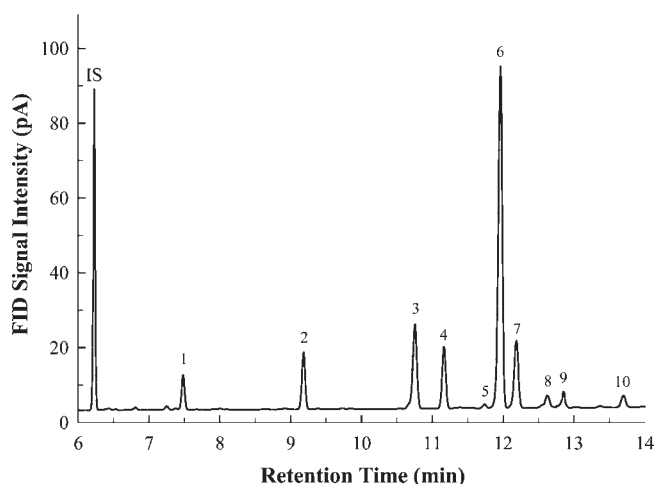
ions (M$^+$), and key fragmentation ions at m/z of [M − 15; {CH$_3$}]$^+$, [M − 90; {(CH$_3$)$_3$−Si−OH}]$^+$, [M − 105; {CH$_3$ + (CH$_3$)$_3$−Si−OH}]$^+$, and [M − 129; {(CH$_3$)$_3$−Si−O$^+$=CH−CH=CH$_2$}]$^+$ (**Table 1**). The mass spectra of TMS ethers of nonepimeric sterols are fairly unique. Peaks 1 and 2 were confirmed by the GC-MS library, based on their M$^+$ values and key fragmentation ions/patterns, to be fatty acids. The peaks came about from traces of residual fatty acids in the unsaponifiable fraction due to incomplete saponification. For the major sterols, confirmation of the identities of peak 3 (campesterol; ergost-5-en-3β-ol), peak 4 (stigmasterol; 5α-stigmasta-5,22-dien-3β-ol), and peak 6 (β-sitosterol; 5α-stigmast-5-en-3β-ol) was also achieved by comparing retention times and MS fragmentation patterns with those from the three authentic commercial standards.

Mass spectra for TMS ethers of 4-desmethyl sterols characteristically yield peaks with an m/z of [M − 129]$^+$ and 129. The peak at m/z of 129 (see chemical structure above) has been identified as the fragment originating from the breakdown of ring A along with the TMS moiety (*30*). In this work, [M − 129]$^+$ fragmentation ions were found at m/z of 343, 355, 355, 357, 355, and 355 in the mass spectra for TMS derivatives of the IS, stigmasterol, clerosterol, β-sitosterol, Δ^5-avenasterol, and $\Delta^{5,24(25)}$-stigmastadienol, respectively. Peak 7 (**Figure 1**) was identified as Δ^5-avenasterol {5α-stigmasta-5,24(28)-dien-3β-ol (24Z)}. The RRT and MS fragmentation data matched those reported by Kamal-Eldin et al. (*31*) and Beveridge et al. (*32*). Another possibility for peak 7 is fucosterol, an isomer of Δ^5-avenasterol, but these two compounds can be clearly distinguished from their mass fragmentation ions at m/z of 394 [M − (CH$_3$)$_3$−Si−OH]$^+$ and 296. Observed here and as reported by Moreau et al. (*9*), Δ^5-avenasterol gives a weaker ion signal at an m/z of 394 and a more abundant one at m/z of 296 than that of fucosterol. On the basis of these fragmentation patterns, peak 7 is assigned as Δ^5-avenasterol.

Figure 1. Representative chromatogram of TMS ether derivatives of phytosterols from raw peanuts of Georgia Green (IS, 5α-cholestane; peaks 1 and 2, fatty acids; peak 3, campesterol; peak 4, stigmasterol; peak 5, clerosterol; peak 6, β-sitosterol; peak 7, Δ^5-avenasterol; peak 8, $\Delta^{5,24(25)}$-stigmastadienol; peak 9, coelution of Δ^7-sitosterol and cycloartenol; and peak 10, unidentified sterol).

Table 1. RRTs and Fragmentation Ions of the Peanut Phytosterols as TMS Ethers

compound	RRT[a]	peak no.[b]	key fragmentation ions, m/z (RI)[c]					additional fragmentation ions
			M$^+$	[M − 15]$^+$	[M − 90]$^+$	[M − 105]$^+$	[M − 129]$^+$	
5α-cholestane (IS)	0.52							
campesterol	0.90	3	472 (46)	457 (11)	382 (71)	367 (28)	343 (66)	444 (14), 426 (1), 315 (4), 289 (5), 255 (12), 213 (8), 201 (13), 159 (16), 129,[d] 95 (34), 73 (64), 55 (40)
stigmasterol	0.93	4	484 (40)	469 (6)	394 (31)	379 (12)	355 (15)	343 (8), 309 (2), 255 (34), 228 (3) 215 (7), 161 (10), 129 (69), 107 (19), 83,[d] 55 (82)
clerosterol	0.98	5	484 (21)		394 (11)	379 (13)	355 (18)	343 (9), 331 (9), 285 (13), 281 (15), 253 (15), 207,[d] 191 (14), 135 (22), 129 (41), 73 (59), 55 (68)
β-sitosterol	1	6	486 (41)	471 (11)	396 (74)	381 (28)	357 (69)	444 (1), 386 (0.4) 343 (1), 329 (4), 303 (4), 296 (0.3), 275 (9), 255 (13), 213 (9), 173 (9), 145 (22), 129,[d] 73 (55), 57 (37)
Δ^5-avenasterol	1.02	7	484 (7)	469 (5)	394 (3)	379 (4)	355 (7)	386 (83), 371 (13), 343 (6), 296 (44), 281 (36), 257 (21), 218 (92), 189 (19), 145 (21), 129 (73), 73 (91), 55[d]
$\Delta^{5,24(25)}$-stigmastadienol	1.05	8	484 (8)				355 (14)	386 (25), 371 (11), 343 (11), 296 (22), 281 (44), 257 (8), 255 (14), 253 (14), 207 (83), 173 (13), 129 (40), 55[d]
Δ^7-sitosterol	1.08	9	486 (8)					412 (12), 386 (23), 359 (11), 296 (10), 281 (40), 255 (11), 207,[d] 193 (15), 173 (10), 147 (19), 121 (23), 97 (34), 73 (52), 55 (76)
cycloartenol	1.08	9	498 (8)	483 (7)	408 (42)	393 (36)		422 (69), 365 (24), 339 (16), 297 (6), 286 (12), 271 (11), 189 (14), 69[d]

[a] The RRT is the retention time for elution of the phytosterol derivatives relative to the β-sitosterol TMS ether, which eluted at 11.7 min. [b] Peak number assigned based on elution order by GC-FID (see **Figure 1**). [c] Relative intensity (RI) to base peak (%). [d] Base peak (relative intensity = 100%). M$^+$, molecular ion.

Table 2. Phytosterol Contents of Runner, Virginia, and Spanish Market Type Peanuts (mg per 100 g of Kernels)[a]

type	campesterol	stigmasterol	clerosterol	β-sitosterol	Δ^5-avenasterol	$\Delta^{5,24(25)}$-stigmastadienol	Δ^7-sitosterol + cycloartenol	total[b]
				Runner				
normal (n = 81)	13.6 ± 1.1 b[c]	10.0 ± 0.9 b	1.4 ± 0.4 a	72.0 ± 6.4 a	19.6 ± 2.9 a	1.4 ± 0.3 a	3.6 ± 2.0 a	127.0 ± 6.7 a
mid oleic (n = 15)	13.7 ± 0.9 b	10.3 ± 0.3 b	1.1 ± 0.1 a	75.7 ± 3.9 a	16.5 ± 1.2 b	1.1 ± 0.1 b	3.5 ± 0.7 a	124.5 ± 5.9 a
high oleic (n = 55)	14.9 ± 0.7 a	11.3 ± 0.9 a	1.2 ± 0.3 a	72.5 ± 3.9 a	18.6 ± 2.6 ab	1.2 ± 0.3 ab	3.8 ± 1.1 a	126.9 ± 5.8 a
mean ± SD	14.0 ± 1.1 B[d]	10.4 ± 1.0 A	1.3 ± 0.3 A	73.6 ± 6.4 B	19.0 ± 2.8 A	1.3 ± 0.3 A	3.6 ± 1.7 A	127.5 ± 6.3 B
% F + E[e]	84	84		77	74			80
				Virginia				
normal (n = 50)	15.1 ± 2.2 B	10.5 ± 0.8 A	1.5 ± 0.3 A	72.8 ± 5.8 B	19.8 ± 3.2 A	1.4 ± 0.4 A	3.0 ± 0.8 AB	129.3 ± 6.9 B
% F + E	82	86		75	73			79
				Spanish				
normal (n = 12)	19.8 ± 0.6 a	11.6 ± 0.4 a	1.3 ± 0.1 b	88.6 ± 1.8 a	17.3 ± 0.3 a	1.3 ± 0.2 a	3.1 ± 0.4 a	148.8 ± 3.0 a
high oleic (n = 8)	18.7 ± 0.2 b	7.9 ± 0.2 b	1.4 ± 0.1 a	84.5 ± 1.4 b	17.9 ± 2.8 a	1.3 ± 0.3 a	2.5 ± 0.7 b	140.7 ± 1.4 b
mean ± SD	19.3 ± 0.7 A	9.8 ± 2.0 B	1.4 ± 0.2 A	86.5 ± 2.7 A	17.6 ± 1.9 A	1.3 ± 0.3 A	2.8 ± 0.6 B	144.1 ± 5.3 A
% F + E	87	87		83	80			84
total (n = 221)	14.7 ± 1.9	10.4 ± 1.1	1.4 ± 0.3	73.0 ± 7.5	19.1 ± 3.0	1.4 ± 0.3	3.3 ± 1.4	128.5 ± 8.6
% F + E	84	85		83	75			82

[a] n = number of samples; data represent the mean ± SD of each sample assayed in triplicate or quadruplicate. [b] Total includes the sum of all identified sterols plus a minor unidentified one shown by GC-MS to be a sterol; the latter was quantified as β-sitosterol equivalents. [c] Means with different small case letters indicate significant differences among normal, mid, and high oleic peanut cultivars within types (Runner and Spanish) by Tukey's multiple range test ($P < 0.05$). [d] Means with different capital letters indicate significant differences among Runner, Virginia, and Spanish types by Tukey's multiple range test ($P < 0.05$). [e] Percentage of the sum of free (F) and esterified (E) sterols assayed by saponification alone relative to the total sterols by the combination of acid hydrolysis + saponification.

Furthermore, Pelillo et al. (*33*) distinguished Δ^5-avenasterol from fucosterol on the basis of RRTs: Relative to β-sitosterol, Δ^5-avenasterol exhibited a RRT of 1.03, while fucosterol showed 1.07. In this work, the RRT for peak 7 was 1.02.

On the basis of parent ions and comparisons to mass spectral data reported in the literature, peaks 5, 8, and 9 (**Figure 1**) are tentatively identified as minor sterols. No identification was made from the Wiley library. Peak 5 is most likely clerosterol (5α-stigmasta-5,25-dien-3β-ol) with a RRT of 0.96, TMS parent ion at an *m/z* of 484, and primary fragmentation ions corresponding to those reported by Beveridge et al. (*32*), Pelillo et al. (*33*), Li et al. (*34*), and Yorulmaz et al. (*35*). Moreover, Reina et al. (*36*) found clerosterol to be present in peanut oil by GC. This minor sterol has been reported in ginseng (*32*), sea buckthorn (*34*), and hazelnut (*35*) and olive (*37*) oils. Further confirmation of its presence in the peanut lipid extracts was provided when the mass spectrum of this sterol was compared against the spectra of the olive and hazelnut oils from the aforementioned works. The retention time of the peak identified as clerosterol in the present investigation corresponded to peaks identified as clerosterol in both the olive and the hazelnut oil studies, as a similar nonpolar stationary phase was employed in this research. We, therefore, feel confident that peak 5 is correctly identified as clerosterol.

On the basis of the M$^+$ at an *m/z* of 484 and a RRT similar to that reported by Li et al. (*34*) for sea buckthorn oil, Yorulmaz et al. (*35*) for hazelnut oil, and Temine et al. (*37*) for olive oil, peak 8 (**Figure 1**) was identified as $\Delta^{5,24(25)}$-stigmastadienol. Peak 9 (**Figure 1**) was assigned to be Δ^7-sitosterol (5α-stigmast-7-en-3β-ol) on the basis of matching the parent molecular ion (*m/z* of 486) and a RRT similar to that reported for ginseng oil (*32*) and sea buckthorn oil (*34*). Δ^7-Sterols characteristically show an intense fragment ion at an *m/z* of 255, as was noted for peak 9 (*38*). Additionally, both peaks 8 and 9 exhibited fragmentation ions at an *m/z* of 386 and 296. According to Beveridge et al. (*32*), these are indicative of Δ^5 and Δ^7-sterols. The RRT and mass fragmentation pattern of peak 9 were also characteristic of cycloartenol (cycloartan-3β-ol) as reported by Phillips et al. (*10*). Cycloartenol shows a TMS parent molecular ion at an

m/z of 498 and key fragmentation ions at an *m/z* of 422 and 408. Thus, peak 9 most likely contains coeluting Δ^7-sitosterol and cycloartenol. Unfortunately, attempts made to modify the GC program to resolve these two coeluting compounds were not fruitful. Mass spectral data for peak 10 were typical of a sterol based on its TMS parent molecular ion (*m/z* of 498) and RRT 1.15; however, this compound is considered as an unidentified sterol due to the lack of corroborating information for its proper elucidation.

Phytosterol Levels in Raw Peanuts. The mean phytosterol contents in Runner (*n* = 151), Virginia (*n* = 50), and Spanish (*n* = 20) market type peanuts are given in **Table 2**. As expected in all types, β-sitosterol was the dominant sterol (> 60% of the total) followed by campesterol, Δ^5-avenasterol, stigmasterol, and the minor sterols of peak 5 (clerosterol), peak 8 ($\Delta^{5,24(25)}$-stigmastadienol), and peak 9 (Δ^7-sitosterol and cycloartenol). The total phytosterol content was significantly greater in Spanish peanuts (144.1 mg/100 g) as compared to Runner (127.5 mg/100 g) and Virginia (129.3 mg/100 g) types (*P* < 0.05). The higher levels noted for Spanish types were primarily due to significantly greater levels of campesterol and β-sitosterol (*P* < 0.05). In Spanish types, normal cultivars contained significantly higher total phytosterol levels (148.8 mg/100 g) as compared to high oleic cultivars (140.7 mg/100 g) (*P* < 0.05). In Runner types, no significant (*P* > 0.05) differences were found in total phytosterol content of normal, mid, and high oleic peanuts.

The percentage of free and esterified (% F + E) sterols relative to the total phytosterol content determined in raw peanuts ranged from 79% in Runner and Virginia types to 84% in Spanish types. **Table 2** gives a breakdown of the % F + E for the four dominant phytosterols: campesterol, stigmasterol, β-sitosterol, and Δ^5-avenasterol. For each of these sterols assayed, levels in all samples were higher in the lipid extracts treated by the combination acid hydrolysis and saponification as compared to quantities found after treatment by saponification alone. Jonker et al. (*24*) observed that acid hydrolysis with saponification markedly increased measured levels of campesterol, stigmasterol, and β-sitosterol in mixed diet samples as compared to levels found only in saponified samples: that is, 13−23% for campesterol, 9−39%

Article

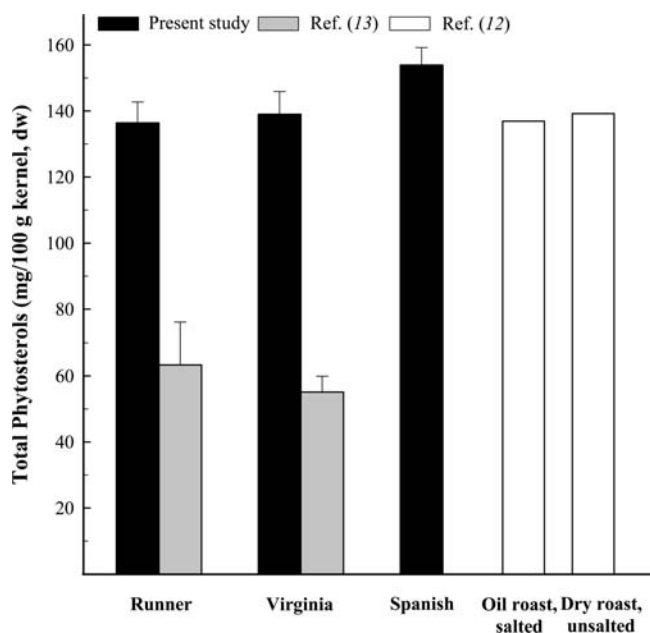*J. Agric. Food Chem.*, Vol. 58, No. 16, 2010 **9143**

Figure 2. Comparison of phytosterol levels determined in the present study with those reported in the literature. All sterol values were normalized to total mg/100 g peanut kernels, dry weight (dw) basis. Error bars represent the SD of the mean.

for stigmasterol, and 22–42% for β-sitosterol. Kesselmeier et al. (*39*) reported that acid hydrolysis decomposed labile Δ^5- and Δ^7-avenasteryl glucosides; however, we found higher levels of Δ^5-avenasterol in the peanut oil (results not shown) after treatment with the combined acid and alkaline hydrolysis procedure. Nevertheless, Phillips et al. (*12*) recommendation to use SPE to isolate and quantify Δ^5-avenasteryl glucosides was adopted in this study.

Figure 2 compares the phytosterol contents determined in this study with those reported by Awad et al. (*13*) and Phillips et al. (*12*) after normalizing all data to a dry weight (dw) basis. Other published phytosterol studies on peanuts were not appropriate for comparison, because the data are presented as a percentage of each individual phytosterol to the total quantity of phytosterols measured (i.e., mg/100 g total sterols) (*1*, *23*). Awad et al. (*13*), on the other hand, give the contents of individual phytosterols in raw Runner and Virginia market types as mg/100 g edible portion. Most interesting is that these researchers did not employ an acid hydrolysis step to recover steryl glucosides from the raw peanut samples before saponification: Total phytosterol contents were 63 and 55 mg/100 g peanut for Runner and Virginia peanuts, respectively (the Spanish market type was not studied). These levels are > 50% less than those reported in this work, thereby reaffirming the position of Phillips et al. (*12*) that steryl glucosides comprise a significant proportion of naturally occurring phytosterols in some foods and must be included in the analysis. Even though Phillips et al. (*12*) do not list details pertaining to the specific peanut types examined because retail products were studied, the data provide phytosterol levels for dry- and oil-roasted peanuts extracted by the combination of acid hydrolysis and alkaline saponification. For a realistic comparison, phytosterol values were normalized to the total mg/100 g peanut kernel (dw). Experimentally determined moisture contents by AOAC gravimetric determination (Method 925.40) for raw Runner, Virginia, and Spanish peanuts were 6.50, 6.91, and 6.39%, respectively, while those for oil-roasted and salted as well as dry-roasted and unsalted were 1.45 and 1.55%, respectively, based on values taken from the U.S. Department of Agriculture National Nutrient Database for Standard Reference (*16*). Comparing data

of the present study with that of Awad et al. (*13*), the contents of total sterols in Runner and Virginia peanuts were 115 and 152% greater. The marked differences in the values are due to the inclusion of steryl glucosides liberated by acid hydrolysis, the identification and quantification of minor sterols, and perhaps a more efficient/exhaustive extraction of phytosterols from the raw peanut matrix; Awad et al. (*13*) reported the total phytosterol content only as the sum of β-sitosterol, campesterol, and stigmasterol. Relating the data of Phillips et al. (*12*) with those reported in this study, the contents of total sterols in dry- and oil-roasted peanuts were basically the same as those in raw Runner and Virginia peanuts but lower than in raw Spanish peanuts (**Figure 2**). The only dissimilarity between the two studies is that Phillips et al. (*12*) analyzed two roasted peanut samples while the present work measured the phytosterols in raw peanuts. Two important observations are evident: First, the thermal processing operations to which the peanuts were subjected in the products analyzed by Phillips et al. (*12*) did not degrade/oxidize the phytosterols; this is not always the case for bioactive constituents. Second, the slightly higher sterol value for dry-roasted peanuts compared to that for raw Runners suggests that roasting may have released sterol compounds bound to the carbohydrate/protein/lipid constituents of the peanut matrix, which were unavailable for extraction with the lipophilic solvent system ($CHCl_3$/CH_3OH) employed for raw peanuts. There is also a possibility that phytosterols can be absorbed by peanuts during oil roasting, as peanut oil contains higher concentrations of phytosterols relative to raw peanuts, but this does not explain why the phytosterols in dry-roasted peanuts are at basically the same level as their oil-roasted counterparts.

Cultivar and production year variation are shown by the data in **Table 3**. In Runner cultivars, total phytosterol levels ranged from 113.2 mg/100 g in Georgia-03L to 135.8 mg/100 g in Tamrun 96. Significant differences between the 2005 and 2006 production years were noted for Georgia Green, Tamrun 96, OL01, Flavorunner-458, and Georgia-02C ($P < 0.05$). In Virginia cultivars, total phytosterol levels ranged from 116.6 (Perry) to 130.6 mg/100 g (Gregory). Significant year-to-year variation in total phytosterol content was noted for NC-7 and VA-98R ($P < 0.05$). For the Spanish cultivars, the normal variety, Tamspan 90, contained higher total phytosterol levels (146.9 mg/100 g) than for the high oleic cultivar, OLIN (138.5 mg/100 g) ($P < 0.05$). Again, significant differences between production years were found for Tamspan 90 ($P < 0.05$).

Factorial analysis (**Table 3**) revealed that cultivar ($n = 14$) effects were strongly significant ($P < 0.001$) for all phytosterols. Production year ($n = 2$) effects were significant ($P < 0.001$) for the major sterols but not for Δ^5-avenasterol, $\Delta^{5,24(25)}$-stigmastadienol, and the coeluted sterols in peak 9. Significant year × cultivar interaction effects were noted for all phytosterols ($P < 0.001$) except for Δ^7-sitosterol and cycloartenol coeluted in peak 9.

Cluster Analysis. Figure 3 depicts the dendrogram representing cultivar clusters grouped by similarities in phytosterol contents by Ward's algorithm. The segregation produced two primary clusters (A and B). Cluster A contained cultivars of Runner and Virginia type peanuts, while cluster B contained Spanish cultivars and three Runners Tamrun 96 (2005 and 2006), OL01 (2005), and AP-3 (2006). The mean total phytosterol content was significantly greater in cluster B relative to cluster A (139.5 vs 126.1 mg/100 g) ($P < 0.05$). Significant differences were noted for campesterol (14.6 and 17.5 mg/100 g for cluster A and B, respectively) and β-sitosterol (71.8 and 84.8 mg/100 g for cluster A and B, respectively) ($P < 0.05$). No significant ($P > 0.05$) differences were noted for other sterols presented in the cultivars in cluster A and B.

Cluster A was segregated into subclusters A1 and A2. The mean total phytosterol level in cluster A1 (127.7 mg/100 g) was

Table 3. Phytosterol Contents in Runner, Virginia, and Spanish Peanut Cultivars (mg per 100 g of Kernels)[a]

cultivar	campesterol	stigmasterol	clerosterol	β-sitosterol	Δ^5-avenasterol	$\Delta^{5,24(25)}$-stigmastadienol	Δ^7-sitosterol + cycloartenol	total[b]
				Runner				
normal								
Georgia Green								
2005 (*n* = 22)	12.8 ± 1.0 b[c]	9.8 ± 0.6 a	1.3 ± 0.2 a	70.2 ± 2.4 b	21.4 ± 0.7 b	1.4 ± 0.2 a	3.6 ± 1.3 a	122.6 ± 4.3 b
2006 (*n* = 22)	14.7 ± 0.2 a	9.3 ± 0.3 a	1.5 ± 0.4 a	75.0 ± 0.4 a	24.7 ± 0.6 a	1.5 ± 0.1 a	4.8 ± 1.5 a	128.9 ± 1.5 a
mean ± SD	13.7 ± 1.2 CD[d]	9.6 ± 0.5 C	1.4 ± 0.2 AB	72.6 ± 3.0 C	22.7 ± 1.7 A	1.4 ± 0.1 A	4.1 ± 1.5 A	128.7 ± 4.5 BC
Tamrun 96								
2005 (*n* = 5)	15.0 ± 0.2 b	10.2 ± 0.1 b	1.1 ± 0.2 a	71.4 ± 0.6 b	18.5 ± 0.1 a	1.1 ± 0.2 b	2.3 ± 0.5 b	131.4 ± 1.7 b
2006 (*n* = 3)	15.4 ± 0.3 a	11.2 ± 0.1 a	1.2 ± 0.1 a	87.4 ± 0.1 a	16.9 ± 0.1 b	1.6 ± 0.4 a	3.9 ± 0.3 a	140.5 ± 0.6 a
mean ± SD	15.2 ± 0.4 A	10.7 ± 0.5 B	1.1 ± 0.1 B C	84.3 ± 3.3 A	17.7 ± 0.9 BC	1.3 ± 0.4 AB	3.1 ± 0.9 A	135.8 ± 4.6 A
C99-R								
2005 (*n* = 9)	13.7 ± 0.2 a	10.4 ± 0.1 a	0.9 ± 0.2 b	67.8 ± 0.2 a	18.4 ± 0.1 a	0.9 ± 0.2 b	2.4 ± 0.3 a	117.5 ± 0.3 a
2006 (*n* = 15)	12.8 ±0.3 b	10.0 ± 0.1 b	1.5 ± 0.1 a	66.2 ± 0.4 b	18.4 ± 0.2 a	1.3 ± 0.2 a	3.2 ± 1.1 a	116.9 ± 1.4a
mean ± SD	13.3 ± 0.5 D	10.2 ± 0.2 BC	1.2 ± 0.3 BC	66.9 ± 0.9 D	18.4 ± 0.1 B	1.1 ± 0.3 B	2.8 ± 0.8 A	117.2 ± 1.3 D
Georgia-01R								
2005 (*n* = 1)	12.9 ± 0.3	10.0 ± 0.1	2.3 ± 0.2	73.1 ± 0.5	15.5 ± 0.1	2.0 ± 0.1	1.6 ± 0.2	120.0 ± 1.2
Georgia-03L								
2006 (*n* = 1)	12.3 ± 0.2	11.2 ± 0.3	1.3 ± 0.1	56.6 ± 0.8	17.7 ± 0.2	1.4 ± 0.1	2.4 ± 0.3	113.2 ± 1.3
AP-3								
2006 (*n* = 3)	13.6 ± 0.1	8.3 ± 0.1	1.2 ± 0.1	77.8 ± 0.5	16.7 ± 0.1	1.3 ± 0.1	4.1 ± 0.8	125.2 ± 0.5
mid oleic								
OL01								
2005 (*n* = 2)	14.7 ± 0.2 a	10.7 ± 0.1 a	1.2 ± 0.1 a	79.5 ± 0.3 a	17.6 ± 0.2 a	1.1 ± 0.1 a	2.9 ± 0.1 b	129.9 ± 0.7 a
2006 (*n* = 13)	13.1 ± 0.2 b	10.1 ± 0.2 b	1.1 ± 0.2 a	73.1 ± 0.2 b	15.3 ± 0.3 b	1.1 ± 0.3 a	4.0 ± 0.3 a	119.4 ± 0.2 b
mean ± SD	13.9 ± 0.9 B−D	10.4 ± 0.3 BC	1.1 ± 0.1 BC	74.7 ± 2.9 B	16.5 ± 1.2 C	1.1 ± 0.1 B	3.5 ± 0.7 A	124.8 ± 4.2 C
high oleic								
OL02								
2005 (*n* = 5)	14.6 ± 0.1 a	12.0 ± 0.1 a	0.8 ± 0.1 a	69.3 ± 0.3 a	16.9 ± 0.1 b	0.9 ± 0.2 b	3.4 ± 1.4 a	120.1 ± 1.5 a
2006 (*n* = 17)	14.9 ± 0.7 a	11.9 ± 0.9 a	1.0 ± 0.2 a	68.0 ± 3.6 a	17.7 ± 0.1 a	1.2 ± 0.1 a	3.7 ± 1.0 a	123.0 ± 2.7 a
mean ± SD	14.8 ± 0.6 A−C	11.9 ± 0.7 A	1.0 ± 0.2 C	68.4 ± 2.9 CD	17.4 ± 0.4 BC	1.1 ± 0.2 B	3.6 ± 1.1 A	122.1 ± 2.1 CD
Flavorunner-458								
2005 (*n* = 3)	14.8 ± 0.0 b	10.3 ± 0.1 b	1.4 ± 0.1 a	74.6 ± 0.4 b	16.5 ± 0.1 a	1.3 ± 0.1 a	3.2 ± 0.9 a	121.1 ± 1.5 b
2006 (*n* = 3)	15.9 ± 0.1 a	11.8 ± 0.0 a	1.0 ± 0.2 b	75.6 ± 0.2 a	16.4 ± 0.1 a	1.2 ± 0.1 a	4.0 ± 0.2 a	124.7 ± 0.5 a
mean ± SD	15.4 ± 0.6 A	11.1 ± 0.8 B	1.2 ± 0.3 BC	75.2 ± 0.6 B	16.4 ± 0.1 C	1.3 ± 0.1 AB	3.6 ± 0.7 A	122.9 ± 2.3 BC
Georgia-02C								
2005 (*n* = 3)	14.7 ± 0.1 b	10.2 ± 0.1 b	1.6 ± 0.1 a	75.7 ± 0.1 a	21.5 ± 0.6 b	1.4 ± 0.1 b	2.9 ± 0.2 b	131.0 ± 0.5 b
2006 (*n* = 24)	15.3 ± 0.2 a	11.0 ± 0.1 a	1.5 ± 0.2 a	74.8 ± 0.5 a	23.6 ± 0.1 a	1.6 ± 0.0 a	5.4 ± 0.4 a	132.0 ± 1.1 a
mean ± SD	15.0 ± 0.4 AB	10.6 ± 0.4 B	1.5 ± 0.1 A	75.2 ± 0.6 B	22.5 ± 1.1 A	1.5 ± 0.1 A	4.1 ± 1.4 A	131.3 ± 2.1 AB
				Virginia				
normal								
NC-V11								
2005 (*n* = 9)	13.4 ± 0.2 a	10.9 ± 0.2 a	1.3 ± 0.2 a	65.3 ± 0.6 a	21.0 ± 0.1 b	1.5 ± 0.1 a	2.8 ± 0.1 a	118.5 ± 0.4 a
2006 (*n* = 6)	13.0 ± 0.1 b	10.4 ± 0.1 b	1.5 ± 0.1 a	64.0 ± 0.4 b	23.8 ± 0.2 a	1.5 ± 0.2 a	2.9 ± 0.8 a	119.5 ± 1.5 a
mean ± SD	13.2 ± 0.3 C	10.6 ± 0.3 B	1.4 ± 0.2 B	64.6 ± 0.7 CD	22.6 ± 1.5 B	1.5 ± 0.1 B	2.9 ± 0.6 A	118.9 ± 1.2 C
NC-7								
2005 (*n* = 3)	15.1 ± 0.3 b	9.7 ± 0.1 b	1.2 ± 0.1 a	73.9 ± 0.5 b	17.6 ± 0.4 a	1.0 ± 0.2 b	1.9 ± 0.2 b	124.7 ± 1.2 b
2006 (*n* = 3)	16.0 ± 0.2 a	10.5 ± 0.4 a	1.3 ± 0.2 a	75.9 ± 0.6 a	16.3 ± 0.2 b	1.4 ± 0.1 a	3.6 ± 1.0 a	127.8 ± 0.4 a
mean ± SD	15.6 ± 0.6 A	10.0 ± 0.5 CD	1.3 ± 0.1 B	75.0 ± 1.2 A	17.0 ± 0.6 C	1.2 ± 0.2 C	2.7 ± 1.1 A	126.3 ± 2.9 B
NC-12								
2005 (*n* = 1)	15.1 ± 0.9	9.8 ± 0.1	1.6 ± 0.2	75.1 ± 0.4	15.5 ± 0.5	1.1 ± 0.2	4.8 ± 0.3	128.1 ± 0.7
Gregory								
2005 (*n* = 1)	14.3 ± 0.1 a	9.9 ± 0.1 a	2.1 ± 0.2 a	71.3 ± 0.1 a	24.6 ± 0.4 a	2.1 ± 0.1 a	2.7 ± 0.2 a	130.5 ± 0.4 a
2006 (*n* = 3)	13.9 ± 0.2 b	9.8 ± 0.3 a	2.0 ± 0.1 a	70.8 ± 0.3 b	25.1 ± 0.7 a	2.3 ± 0.2 a	3.0 ± 0.1 a	130.6 ± 0.8 a
mean ± SD	14.1 ± 0.3 B	9.9 ± 0.1 D	2.0 ± 0.2 A	71.1 ± 0.4 B	24.9 ± 0.4 A	2.2 ± 0.1 A	2.9 ± 0.2 A	130.6 ± 0.5 A
Perry								
2005 (*n* = 7)	14.9 ± 0.2 a	10.7 ± 0.2 a	1.3 ± 0.5 a	65.8 ± 0.3 a	15.3 ± 0.2 b	1.1 ± 0.3 a	3.0 ± 1.3 a	116.8 ± 1.7 a
2006 (*n* = 6)	13.7 ± 0.1 b	10.0 ± 0.1 b	1.4 ± 0.3 a	65.6 ± 0.4 a	18.1 ± 0.1 a	1.2 ± 0.2 a	2.4 ± 0.4 b	116.3 ± 0.6 a
mean ± SD	14.4 ± 0.7 B	10.4 ± 0.4 BC	1.3 ± 0.1 B	65.7 ± 0.2 C	16.7 ± 1.4 C	1.2 ± 0.2 C	2.7 ± 0.9 A	116.6 ± 1.2 D
VA-98R								
2005 (*n* = 5)	14.1 ± 0.3 a	11.6 ± 0.2 a	1.3 ± 0.6 a	65.0 ± 0.3 a	21.0 ± 0.2 b	1.6 ± 0.2 a	2.7 ± 0.5 a	120.6 ± 1.0 a
2006 (*n* = 6)	13.5 ± 0.1 b	11.1 ± 0.1 b	1.6 ± 0.1 a	62.1 ± 0.1 b	21.6 ± 0.1 a	1.7 ± 0.3 a	2.9 ± 0.1 a	119.0 ± 0.3 b
mean ± SD	13.9 ± 0.3 BC	11.4 ± 0.3 A	1.5 ± 0.1 B	63.6 ± 1.5 D	21.3 ± 0.3 B	1.6 ± 0.1 B	2.8 ± 0.3 A	119.6 ± 1.7 C
				Spanish				
normal								
Tamspan 90								
2005 (*n* = 6)	20.2 ± 0.1 a	11.9 ± 0.2 a	1.2 ± 0.1 a	90.6 ± 0.1 a	17.4 ± 0.1 a	1.1 ± 0.3 a	3.5 ± 0.2 a	148.5 ± 0.3 a
2006 (*n* = 6)	19.3 ± 0.5 b	11.3 ± 0.3 b	1.4 ± 0.2 a	88.1 ± 1.5 b	17.3 ± 0.4 a	1.4 ± 0.2 a	2.8 ± 0.3 b	146.3 ± 2.7 b
mean ± SD	19.8 ± 0.6 A	11.6 ± 0.4 A	1.3 ± 0.1 B	89.3 ± 1.7 A	19.0 ± 0.3 A	1.3 ± 0.2 A	3.1 ± 0.4 A	146.9 ± 3.0 A

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Table 3. Continued

cultivar	campesterol	stigmasterol	clerosterol	β-sitosterol	Δ^5-avenasterol	$\Delta^{5,24(25)}$-stigmastadienol	Δ^7-sitosterol + cycloartenol	total[b]
high oleic								
OLIN								
2005 ($n = 3$)	18.6 ± 0.3 a	8.1 ± 0.1 a	1.6 ± 0.1 a	86.2 ± 0.9 a	15.4 ± 0.3 b	1.5 ± 0.4 a	2.7 ± 0.6 a	137.2 ± 1.5 a
2006 ($n = 5$)	18.9 ± 0.2 a	7.7 ± 0.2 b	1.4 ± 0.1 b	84.0 ± 0.1 b	20.5 ± 0.2 a	1.1 ± 0.2 a	2.3 ± 0.7 b	139.2 ± 0.9 a
mean ± SD	**18.8 ± 0.2 B**	**7.9 ± 0.2 B**	**1.5 ± 0.1 A**	**86.2 ± 1.3 B**	**17.9 ± 2.8 A**	**1.3 ± 0.3 A**	**2.5 ± 0.7 B**	**138.5 ± 1.6 B**
factorial analysis[e]								
cultivar[f]	***	***	***	***	***	***	***	***
year[g]	NS[i]	NS	*	NS	***	***	***	**
cultivar × year[h]	***	***	***	***	***	***	**	***

[a] n = number of samples; data represent the mean ± SD of each sample assayed in triplicate or quadruplicate. Bold face indicates the mean of all samples for each cultivar for production years 2005 and 2006. [b] The total includes the sum of all identified sterols plus a minor unidentified one shown by GC-MS to be a sterol; the latter was quantified as β-sitosterol equivalents. [c] Small case letters that are different for production years within a cultivar indicate that the means are significantly different by Tukey's multiple range test ($P < 0.05$). [d] Means ($n = 14$) for production years 2005 and 2006 with different capital letters indicate significant differences of each cultivar except for Georgia-01R, Georgia-03 L, AP-3, and NC-12 ($P < 0.05$). [e] A two-way factorial analysis was used. [f] All cultivars ($n = 14$) except for Georgia-01R, Georgia-03 L, AP-3, and NC-12. [g] Production years ($n = 2$; 2005 and 2006). [h] The interaction between cultivar × production year. [i] NS, not significant; *, **, and *** correspond to significance at $P < 0.05$, 0.01, and 0.001, respectively.
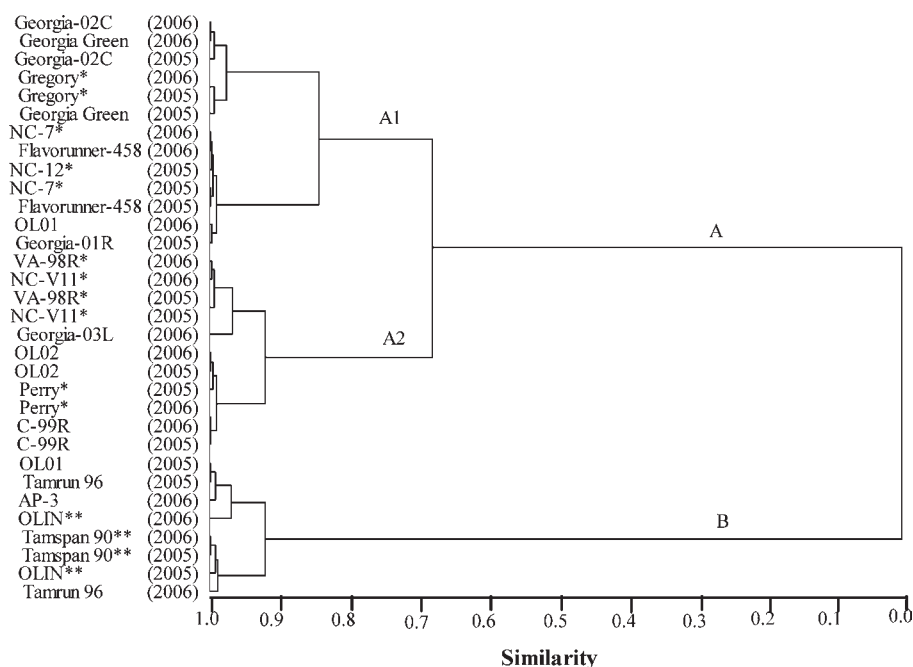

Figure 3. Dendrogram of phytosterol contents in raw peanut cultivars. * and ** represent Virginia and Spanish types, respectively.

significantly greater than the level in A2 (118.9 mg/100 g) ($P < 0.05$). As noted for the segregation of cluster A and cluster B, significant differences were primarily due to variations in levels of campesterol (15.2 vs 13.9 mg/100 g in subclusters A1 and A2, respectively) and β-sitosterol (73.6 and 66.2 mg/100 g in subcluster A1 and A2, respectively) ($P < 0.05$). As shown here, HCA is a valuable tool to differentiate cultivars of peanuts or other food commodities based on compositional properties.

To summarize, the contents of phytosterols were assessed in commercial Runner, Virginia, and Spanish market type peanuts obtained from a well-designed sampling plan that provided cultivars representative of those in current production by accepted farming practices. The cultivar richest in total phytosterols is Tamspan 90 (146.9 mg/100 g), a Spanish market type peanut. Overall, Spanish type peanuts possessed significantly higher total phytosterol contents than Runner and Virginia peanuts ($P < 0.05$). The study also shows that the combination of analytical data with chemometric analysis can be used by peanut breeders to give more information on peanut types than is possible with the experimental data alone. Cultivar effects were highly significant for the individual and total phytosterol levels ($P < 0.001$). Cluster analysis segregated Runner and Virginia market types and Spanish market types by their campesterol and β-sitosterol levels. Employing saponification alone, only ~80% of the sterols present in raw peanuts could be accounted for. An acid hydrolysis step prior to saponification (i.e., double hydrolysis) was critical to liberate steryl glucosides from the peanut matrix so that accurate phytosterol levels could be determined. The strength of the validated analytical methodology and aforementioned sampling plan makes this the first study to report phytosterol levels for raw peanuts that are worthy for inclusion in the U.S. Department of Agriculture National Nutrient Database for Standard Reference. As a snack food eaten at various times throughout the day, peanuts and peanut butter offer a valuable dietary source of phytosterols; these phytosterols can inhibit intestinal cholesterol absorption and help regulate whole body cholesterol excretion and balance.

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