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# Update on the Healthful Lipid Constituents of Commercially Important Tree Nuts

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**ABSTRACT:** Uncharacteristic of most whole foods, the major component of tree nuts is lipid; surprisingly, information on the lipid constituents in tree nuts has been sporadic and, for the most part, not well reported. Most published papers focus on only one nut type, or those that report a cultivar lack a quality control program, thus making data comparisons difficult. The present study was designed to quantify the healthful lipid constituents of 10 different types of commercially important tree nuts (i.e., almonds, black walnuts, Brazil nuts, cashews, English walnuts, hazelnuts, macadamias, pecans, pine nuts, and pistachios) according to standardized, validated methods. The total lipid content of each nut type ranged from 44.4 ± 1.9% for cashews to 77.1 ± 1.7% for macadamias. As expected, the major fatty acids present in the tree nuts were unsaturated: oleic (18:1 $\omega$9) and linoleic (18:2 $\omega$6) acids. A majority of the lipid extracts contained <10% saturated fatty acids with the exceptions of Brazil nuts (24.5%), cashews (20.9%), macadamias (17.1%), and pistachios (13.3%). The total tocopherol (T) content ranged from 1.60 ± 1.27 mg/100 g nutmeat in macadamias to 32.99 ± 0.78 in black walnuts. The predominant T isomers in the nut types were $\alpha$- and $\gamma$-T. Tocotrienols were also detected, but only in 6 of the 10 nut types (i.e., Brazil nut, cashews, English walnuts, macadamias, pine nuts, and pistachios). In most cases, total phytosterol contents were greater in the present study than reported in peer-reviewed journal papers and the USDA National Nutrient Database for Standard Reference, which is attributed to total lipid extraction and the inclusion of steryl glucosides in the analysis; the levels were highest for pistachios (301.8 ± 15.4 mg/100 g nutmeat) and pine nuts (271.7 ± 9.1 mg/100 g nutmeat). Minor sterols were also quantified and identified using GC-FID and GC-MS techniques.

**KEYWORDS:** tree nuts, fatty acids, tocopherols, vitamin E, phytosterols, chemical composition, health benefits, USDA National Nutrient Database for Standard Reference

## INTRODUCTION

Numerous health benefits attributed to the consumption of tree nuts in one's daily diet have been reported.[1−6] In 2002, tree nuts received a qualified health claim from the U.S. Food and Drug Administration, which stated "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] These benefits were again documented in the 2010 U.S. Dietary Guidelines: Moderate evidence indicates that eating peanuts and certain tree nuts (i.e., walnuts, almonds, and pistachios) reduces risk factors for cardiovascular disease when consumed as part of a diet that is nutritionally adequate and within calorie needs.[8] Claims pertaining to the health benefits afforded by tree nuts are also being published in other countries such as Canada and Spain.[2] These health claims have been partially attributed to the lipid constituents in tree nuts, including unsaturated fatty acids, the isomers of tocopherols along with tocotrienols (vitamin E), and phytosterols.[3]

Tree nuts have a high total lipid content, ranging from 45 to 75% depending on nut type,[9] and the lipid profile is composed mainly of heart-protective monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs).[4] For most nut types, oleic acid (18:1 $\omega$9) is the prominent fatty acid; moreover, $\omega$-6 (linoleic acid, 18:2 $\omega$6) and $\omega$-3 ($\alpha$-linolenic acid, 18:3 $\omega$3) fatty acids are also represented.[10] Of all tree nuts, walnuts possess the largest quantity of the $\omega$-3 fatty acid $\alpha$-linolenic acid (18:3 $\omega$3).[5] The lipid profile of nuts has been reported to contribute significant positive effects on one's lipoprotein panel.[4] It should be noted that these benefits are attributed not only to the fatty acids but also to other nutrients, such as tocopherols and tocotrienols.[6]

For most foods, as the amount of fat increases so does the bioavailability of vitamin E.[11] Because of the high percentage of lipids found in tree nuts, not only are they a good source of vitamin E, but also the nutrient is present in a more bioavailable form compared to other food matrices. Two different classes of the vitamin exist: tocopherols (T) and tocotrienols (T3). There are four isomers ($\alpha$, $\beta$, $\gamma$, and $\delta$) of each class, and the two classes differ in that the latter contains unsaturated side chains.[12] Vitamin E contents can be quite variable depending upon horticultural aspects such as cultivar type,[13] growing location/conditions,[14] and harvest year,[15] as well as the storage, processing, and assay conditions employed.[12] Vitamin E is a fat-soluble vitamin that has been noted for its strong antioxidant properties. Studies have linked vitamin E consumption with a decrease in coronary heart disease (CHD)-related deaths,[16,17] along with a reduction in the incidence of Alzheimer's disease and some cancers.[18] The most important isomer to health is $\alpha$-T: it is retained in the human body by action of the $\alpha$-T transfer protein in the liver, which preferentially incorporates this isomer into lipoproteins that are circulated in the blood and hence delivers

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α-T to various tissues/organs.[18] The function of γ-T is somewhat unclear. Levels of γ-T in the blood are ca. 10 times lower than that of α-T; nevertheless, its antioxidant properties to scavenge reactive nitrogen oxide species (RNOS), which are produced by phagocytes during inflammatory events in the body,[19−21] are believed to be important.

Phytosterols are a class of compounds comprising plant sterols and stanols; their composition in plant tissue is typically in a ratio of ∼98% sterols to ∼2% stanols.[22] Phytosterols are necessary in plants to maintain cell wall fluidity. In addition, they resemble cholesterol in structure, but possess a different side chain at $C_{24}$ and different numbers and locations of double bonds throughout the molecules. Plant stanols are chemically similar to sterols, but are fully saturated molecules. In foods, phytosterols can exist in four different forms: free sterols, steryl glucosides, acylated steryl glucosides, and esterified sterols.[23] To ensure that all forms are quantified during analysis, both an acid hydrolysis and a saponification step must be employed.[24] In the majority of phytosterol studies, the acid hydrolysis step usually is not carried out. This obligatory step cleaves the glycosidic bonds, changing the steryl glucosides and acylated steryl glucosides to free phytosterols, which can then be quantified after saponification. An alkaline saponification step is still used to separate the unsaponifiable phytosterols from the fatty acids present in the oil. Phillips et al.[22] reported that $\Delta^5$-avenasterol is an acid-labile sterol and must be quantified using a different procedure on account of its degradation during acid hydrolysis. Noteworthy is that in years prior to the research of Toivo et al.[24] and Phillips et al.,[22] most phytosterol analyses underestimated the total sterol contents of lipid extracts and unrefined oils by not including the vital acid hydrolysis step.

Inclusion of phytosterols in the diet reduces the risk of CHD. Andersson et al.[25] investigated the effect of diets rich in plant sterols on the level of cholesterol in the body. They found that phytosterol intake reduced total and low-density lipoprotein (LDL) cholesterol without interfering with cholesterol-lowering medications. It is postulated that the structural similarities of phytosterols to cholesterol allow competition for micellarization, thus reducing intestinal cholesterol absorption and the transport of cholesterol-laden chylomicrons through the lymphatic system to the bloodstream. Cholesterol absorption is much faster than absorption of phytosterols, 50 and 5−10%, respectively.[26] Ostlund et al.[27] performed a randomized, double-blind crossover clinical trial in which participants were fed diets containing corn oil with and without plant sterols added. These authors found that when 300 mg/test meal of phytosterols was included in the diet, there was a ∼28% reduction in the level of cholesterol absorption. What is more, phytosterols have also been reported to confer anti-inflammatory, antibacterial, and antitumor properties.[28] von Holtz et al.[29] pointed out that a diet fortified with phytosterols also tends to supply marked levels of other nutrients/bioactives, such as fiber and flavonoids. Possibly a favorable interaction between all of these nutrients is contributing toward the noticeable difference in incidence of cancers among various diets.

There are numerous studies and reviews in the literature emphasizing the health benefits afforded by nuts, but surprisingly, very few identify the nature and quantity of key nutrients/bioactives present in multiple types of commercially important tree nuts. Those that do exist tend to present only data found in the USDA National Nutrient Database for Standard Reference,[9] perform incomplete analyses, or lack proper quality control/method validation and statistical analysis protocols. With an increasing concern by the American public on the role of diet to one's health and wellness, it is important to ensure that accurate and reliable/validated data of selected health-beneficial constituents are readily available. This study investigates the fatty acid, tocopherol/tocotrienol isomers, and phytosterol contents of 10 different commercially important tree nut types.

## MATERIALS AND METHODS

**Chemicals and Glassware.** Hydrochloric acid (ACS grade), potassium hydroxide (ACS grade), pyridine, toluene, and anhydrous magnesium sulfate powder were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Methanol, chloroform, hexanes, carbon disulfide, anhydrous sodium sulfate, sulfuric acid, and sodium chloride (all ACS grade) were purchased from Fisher Scientific Co., LLC (Suwanee, GA). In addition, HPLC grade hexanes, ethyl acetate, and isopropanol were acquired from Fisher Scientific. Denatured ethanol (Chromasolv, for HPLC) and pyrogallol (99%, ACS grade), heptadecanoic acid (98% purity), and hydroquinone were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). A variety of lipid standards were ordered from Nu-Chek Prep (Elysian, MN). For vitamin E analyses, butylated hydroxytoluene (BHT), ethanol, and tocopherol standards (α-, β-, γ-, and δ-T) were purchased from Sigma-Aldrich. Reacti-vial small reaction vials (5 mL), septa, and caps were bought from Fisher Scientific. For the phytosterol analyses, 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-Aldrich. The silanizing reagent, 5% dimethyldichlorosilane (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).

**Collection of Samples.** Ten commercial tree nut samples (i.e., almonds, black walnuts, Brazil nuts, cashews, English walnuts, hazelnuts, macadamias, pecans, pine nuts, and pistachios) were accrued from various grocery stores in Athens, GA ($n = 3$), over an 18-month period. All nut types were prepackaged, national brand samples processed at each company's main manufacturing plant throughout the United States. The nuts were raw and unsalted, with the exception of cashews, which were oil roasted, and macadamias as well as pistachios, which were dry roasted. When collected, the tree nut samples were placed in labeled pouches (Prime Source, Kansas City, MO), vacuum packed (Henkelman 600, Henkelman BV, The Netherlands) to prevent any oxidative degradation, and stored at −40 °C until analyzed. On the day of analysis, each tree nut type was removed from the freezer, and ∼40 g was ground in a commercial coffee mill (Tipo 203, Krups, New York, NY), while still partially frozen, to a very fine powder using an intermittent pulsing technique. In this manner, oils were not expressed from the nuts during the grinding process.

**Fatty Acid Analysis.** *Lipid Extraction.* The extraction of total lipids was conducted following the classical Bligh−Dyer method[30] with slight modifications as described by Shin et al.[31] A ∼5 g portion of ground nut sample was used in each extraction. Sufficient lipids were collected for all analyses (i.e., fatty acids, tocopherols, and phytosterols), and the lipid extracts were stored in amber vials with a $N_2$ headspace at −80 °C.

*Fatty Acid Methylation.* Fatty acid methyl esters (FAMEs) were prepared from the extracted tree nut lipids according to the method of Dhanda et al.[32] with slight modifications as described by Shin et al.[31] The internal standard (IS) selected for this work was heptadecanoic acid. After methylation, the FAMEs were redissolved in 1.5 mL of $CS_2$ and transferred to 2 mL wide-opening crimp-top vials (Agilent Technologies,

Wilmington, DE). Vials were capped and crimped. Analyses of triplicate samples were performed in duplicate.

*Gravimetric Determination.* The content of total lipids in each nut type was determined by gravimetric analysis. After the total volume of the collected $CHCl_3$ extract had been recorded, 5.0 mL aliquots were dispensed, in triplicate, with a volumetric transfer pipet into preweighed aluminum pans. Under a fumehood, the pans were placed on hot plates and gently heated to drive off the $CHCl_3$. Then to remove residual traces of solvent/water, the aluminum pans were put in a mechanical convection oven (model 28, Precision Scientific Group, Chicago, IL) set at 104 °C for 30 min. The masses of the pans containing the lipids were recorded, and the percent total lipids extracted from each nut sample was calculated using the equation

$$\text{total lipid } (\%) = (W_{p+l} - W_p)/W_{nut} \times V_t/V_a \times 100$$

where $W_{p+l}$ is the mass (g) of the aluminum pan plus lipids, $W_p$ is the mass (g) of the empty aluminum pan, $V_t$ is the total volume (mL) of the $CHCl_3$ collected, $V_a$ is the volume (mL) of the $CHCl_3$ aliquot taken, and $W_{nut}$ is the mass (g) of the nut sample used for lipid extraction.

*Gas Chromatographic Analysis.* An Agilent Technologies 6890N Network GC system [configuration: capillary split/splitless inlet with electronic pneumatic control (EPC) and an FID with EPC, for packed and capillary columns] equipped with a 7683 autosampler tray module, a 7683B autoinjector module, and GC ChemStation software [Rev. A0803 (847); Agilent] was employed for fatty acid analysis. The column was a (50%-cyanopropyl)-methylpolysiloxane J&W fused silica DB-23 capillary column (60 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies); runs were made in constant flow mode; the injector temperature was set at 250 °C for split injection at a split ratio of 50:1; the FID temperature was set at 250 °C; and an initial oven temperature of 130 °C was held for 5 min before ramping up at 4 °C/min to 240 °C, and then held for an additional 15 min.

*Identification of Fatty Acids.* Individual FAMEs from tree nut samples were identified and quantified according to the method of Shin et al.[31] The Nu-Chek Prep GLC-463 FAME reference standard was used for retention time mapping and quantification of each FAME present in the tree nut samples.

**Vitamin E Analysis.** *Direct Solvent Extraction.* Vitamin E [i.e., the tocopherol (T) and tocotrienol (T3) isomers] was extracted from the tree nut samples using the direct solvent method developed by Landen and modified by Lee et al.;[33] the method is described by Shin et al.[13] The residue was redissolved in 1 mL of the mobile phase (0.85% isopropanol in hexanes) prior to injection into the HPLC. Yellow light (devoid of UV and blue light by a filter; λ range from 460 to 800 nm) was employed throughout the extraction to prevent photooxidation of the Ts and T3s. Analyses of triplicate samples were performed in duplicate.

*HPLC Quantitation.* The lipid extracts were injected into an HPLC system comprising a Shimadzu LC-6A pump equipped with an RF-10$A_{XL}$ fluorescence detector (Shimadzu Corp., Columbia, MD), a SpectraSERIES AS 100 autosampler (Thermo Separation Products, Inc., San Jose, CA), a Waters 746 Data Module integrator (Waters-Millipore Inc., Milford, MA), a normal-phase LiChrosorb Si 60 column (4 mm × 250 mm, 5 μm particle size; Hibar Fertigsäule RT, Merck, Darmstadt, Germany) connected to a LiChroCART 4-4 guard column packed with LiChrospher Si 60 (5 μm), and an isocratic mobile phase comprising 0.85% (v/v) isopropanol in hexanes at a flow rate of 1.0 mL/min. Before use, the mobile phase was vacuum filtered through a 0.45 μm nylon membrane filter (MSI, Westboro, MA) and degassed by stirring under vacuum. The excitation and emission wavelengths for fluorescent determination of the T and the T3 isomers were 290 and 330 nm, respectively. Twenty microliters of sample extract or 20 μL of tocopherol standard solution were injected per run.

*Preparation of Standards.* Tocopherol standards were prepared as described by Lee et al.[33] Extinction coefficients of the T homologues, first published by Scott[34] and presented by Eitenmiller et al.,[35] were used to establish the percent purity. After this, stock solutions of the standards were prepared at concentrations of 1.96, 1.65, 3.65, and 1.80 mg/mL for α-, β-, γ-, and δ-T, respectively.[13] As described by Chun et al.,[12] running a palm oil sample through the HPLC system was chosen as the method for determining T3 retention times instead of acquiring T3 standards due to their high cost. Palm oil contains a large amount of γ-T3 and lesser quantities of α-T3 and δ-T3; it provides excellent cross-reference for determining the retention times of endogenous T3s in the tree nut samples.

*Calculations.* Concentrations of the Ts were calculated from each peak area determined by the Waters 764 integrator on the basis of the fluorescence response of each T isomer in a 20 μL injection of the working standard stock solution.[13] Concentrations of the T3s were calculated on the basis of peak responses observed from the respective T isomer.[36]

**Phytosterol Analysis.** *Silanization of Glassware.* Glassware was silanized before use following the method employed by Shin et al.[37]

*Acid Hydrolysis and Saponification.* As recommended by Phillips et al.,[38] both acid hydrolysis and saponification steps were employed to ensure total extraction of phytosterols. Acid hydrolysis was performed on the lipid extracts of each tree nut sample according to the method of Toivo et al.[24] Alkaline hydrolysis was then followed according to the method of Jekel et al.[39]

*Preparation of Trimethylsilyl (TMS) Ether Derivatives.* After saponification, sterols were assayed as their TMS ether derivatives by GC-FID according to the method of Toivo et al.[24] Briefly, the entire portion of the dried residue resulting from saponification was redissolved in ∼1 mL of hexane, transferred to a Reacti-vial, and evaporated to dryness with an N-EVAP (Organomation Associates, Inc., Berlin, MA). Then, 250 μL each of anhydrous pyridine and BSTFA + 1% TMCS reagent were added to Reacti-vials containing the sterols and a Reacti-vial magnetic stirrer. Each 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 75 °C with gentle stirring for ∼2 h. After derivatization was complete, the samples were cooled and evaporated to dryness using the N-EVAP. The TMS ethers were redissolved in 1.5 mL of hexanes and transferred to GC vials.

*Quantification of $\Delta^5$-Avenasterol.* As expounded by Phillips et al.,[22] $\Delta^5$-avenasterol is an acid-labile sterol that degrades during acid hydrolysis and thus its steryl glucosides must be analyzed separately. Steryl glycosides were isolated from the tree nut lipid extracts using solid phase extraction following the method employed by Shin et al.[37] Once the steryl glucoside fractions were isolated, they were dried with the N-EVAP and then derivatized using 500 μL each of pyridine and the BSTFA + 1% TMCS reagent, a reaction temperature of 75 °C, and a 2 h reaction time. TMS ethers were transferred to GC vials and immediately analyzed by GC-FID. The standards and quantity of $\Delta^5$-avenasterol were determined according to the method of Shin et al.[37]

*GC-FID Analysis.* The same instrument was employed for the phytosterol analyses as for the FAMEs. 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); 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; the initial oven temperature was set at 260 °C and ramped up at 3 °C/min to 300 °C, and this temperature was maintained for an additional 5 min. One microliter of sample was injected per run. Analyses of triplicate samples were performed in duplicate.

*GC-MS Analysis.* GC-MS was employed to elucidate the TMS ether phytosterol derivatives present in each nut type. The TMS ether derivatives were analyzed by an Agilent Technologies 7890A gas chromatograph with a 5975C mass selective detector using electron impact ionization. The conditions employed were as follows: a (5% phenyl) methylpolysiloxane fused-silica HP-5 ms capillary column

Table 1. Fatty Acid Composition of 10 Different Commercially Important Tree Nuts ($n = 3$)[a]

| nut | total lipid (g/100 g) | fatty acids (wt % of total lipid) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 14:0 | 16:0 | 16:1 $\omega$7 | 18:0 | 18:1 $\omega$9 | 18:2 $\omega$6 | 18:3 $\omega$3 | 20:0 | 20:1 $\omega$9 | 20:2 $\omega$6 | 22:0 |
| almond | 50.1 ± 1.1 | nd[b] | 6.45 ± 0.26 | 0.43 ± 0.35 | 1.47 ± 0.15 | 67.62 ± 0.90 | 24.03 ± 1.28 | nd | nd | nd | nd | nd |
| black walnut | 60.0 ± 1.8 | nd | 3.42 ± 0.07 | nd | 3.06 ± 0.10 | 28.42 ± 0.59 | 61.21 ± 0.28 | 3.57 ± 0.12 | nd | nd | nd | nd |
| Brazil nut | 68.6 ± 2.8 | nd | 14.71 ± 0.79 | 0.29 ± 0.30 | 9.79 ± 1.08 | 38.36 ± 2.94 | 36.84 ± 4.35 | nd | nd | nd | nd | nd |
| cashew | 44.4 ± 1.9 | nd | 11.14 ± 0.29 | nd | 9.08 ± 0.08 | 56.87 ± 0.83 | 22.22 ± 0.61 | nd | 0.68 ± 0.01 | nd | nd | nd |
| English walnut | 67.0 ± 4.0 | nd | 7.11 ± 0.13 | nd | 2.72 ± 0.08 | 16.96 ± 0.65 | 59.78 ± 0.81 | 13.43 ± 0.16 | nd | nd | nd | nd |
| hazelnut | 61.6 ± 1.3 | nd | 5.78 ± 0.38 | 0.16 ± 0.02 | 2.36 ± 0.31 | 79.64 ± 1.94 | 11.78 ± 2.26 | nd | 0.12 ± 0.01 | 0.15 ± 0.02 | nd | nd |
| macadamia | 77.1 ± 1.7 | 0.83 ± 0.13 | 8.78 ± 0.22 | 17.95 ± 0.23 | 3.74 ± 0.54 | 60.08 ± 0.25 | 2.32 ± 0.42 | nd | 2.88 ± 0.17 | 2.53 ± 0.23 | nd | 0.87 ± 0.05 |
| pecan | 73.1 ± 2.6 | nd | 6.15 ± 0.69 | nd | 2.54 ± 0.16 | 62.36 ± 7.75 | 27.69 ± 6.92 | 1.25 ± 0.18 | nd | nd | nd | nd |
| pine nut[c] | 68.9 ± 2.4 | nd | 5.22 ± 0.06 | nd | 2.36 ± 0.04 | 27.67 ± 0.91 | 45.02 ± 0.16 | nd | 0.42 ± 0.02 | 1.38 ± 0.03 | 0.74 ± 0.04 | 1.08 ± 0.03 |
| pistachio | 49.8 ± 3.1 | nd | 11.79 ± 0.42 | 1.07 ± 0.08 | 1.50 ± 0.32 | 56.75 ± 1.70 | 28.56 ± 2.24 | 0.33 ± 0.26 | nd | nd | nd | nd |

[a] $n$ = number of independent original samples; data represent the mean ± standard deviation. [b] nd = not detected (below the limit of detection for the GC-FID system). [c] In addition to these fatty acids, pine nuts also possess two unique $\Delta^5$-olefinic fatty acids: 2.13 ± 0.05% of taxoleic acid [5(Z),9(Z)-octadecadienoic acid] and 13.99 ± 0.37% of pinolenic acid [5(Z),9(Z),12(Z)-octadecatrienoic acid].

(30 m × 0.25 mm i.d., 0.25 $\mu$m film thickness; Agilent Technologies); a 1.0 $\mu$L injection volume; ultrahigh-purity helium as the carrier gas; an inlet pressure of 162 kPa (i.e., 23.5 psi) with runs performed in constant flow mode; an injector temperature of 230 °C; a source detector temperature of 230 °C and a quadrupole temperature of 150 °C; an initial oven temperature of 230 °C, which after 1 min was ramped at 5 °C/min to a final oven temperature of 310 °C and then held for 9 min; a transfer line temperature of 280 °C; and an electron ionization energy of 70 eV.

From the GC-FID chromatograms, the TMS ether phytosterol derivative peaks were tentatively identified by comparison of retention times to those of a TMS ether derivatized standard plant sterol mixture. Confirmation of these structures was achieved by GC-MS using the National Institute of Standards and Technology (NIST; Gaithersburg, MD) 2008 library to match mass spectral peaks of phytosterol standards to those found in tree nut TMS ether samples. Comparisons of parent molecular ion ($M^+$) and fragmentation ions/patterns were employed to assist in elucidating the identities of the phytosterols. Additional sterols, for which commercial standards are not available, were tentatively identified on the basis of comparison of relative retention times (RRTs), $M^+$ values, and fragmentation patterns of TMS ether phytosterol derivatives to those reported in the literature for tree nut oils and plant oils.

*Quantification of Phytosterols.* To accurately quantify the sterols in raw tree nuts, relative response factors (RRFs) to 5$\alpha$-cholestane were determined on the basis of five replicate analyses.[40] The RRFs were calculated with the equation

$$\mathrm{RRF} = \frac{\mathrm{PA_{sterol}}}{W_{\mathrm{sterol}}} \times \frac{W_{\mathrm{IS}}}{\mathrm{PA_{IS}}}$$

where $\mathrm{PA_{sterol}}$ is the peak area of the sterol, $W_{\mathrm{sterol}}$ is the mass (mg) of the sterol, $\mathrm{PA_{IS}}$ is the peak area of the IS, and $W_{\mathrm{IS}}$ is the mass (mg) of the IS. On the basis of the RRFs, sterol contents were calculated using the equation[40]

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

where $\mathrm{PA_{sterol}}$ is the peak area of the sterol, $\mathrm{PA_{IS}}$ is the peak area of the IS, $W_{\mathrm{IS}}$ is the mass (mg) of the IS, and $W_{\mathrm{sample}}$ is the mass (g) of the tree nut lipid extract. Because of unavailability of commercial standards, $\Delta^5$-avenasterol, $\Delta^7$-avenasterol, and other minor sterol compounds were quantified using the FID response of $\beta$-sitosterol.

**Statistical Analysis.** Fatty acid contents were reported as weight percents of the total oil in each tree nut. Tocopherols and phytosterols were reported as milligrams per 100 g of nutmeat. Three samples of each nut type were assayed in duplicate for each analysis. All values were reported as the mean ± standard deviation ($n = 3$). A two-sample $t$ test was carried out when the means reported in this work were compared to those presented in the USDA National Nutrient Database for Standard Reference, release 24; the level of significance was determined at $P < 0.05$.

**Quality Control/Method Validation.** Because there is no tree nut standard reference material (SRM) available from NIST, the fatty acid methodology was validated for accuracy and interday precision using SRM 2387 peanut butter purchased from NIST. Five replications were completed for each assay, and the data were compared against the certified values. Relative standard deviation, bias, and percent accepted value were calculated as follows:

$$\%\ \mathrm{RSD_r} = \text{standard deviation/mean} \times 100$$

$$\text{bias} = \text{accepted value} - \text{analytical value}$$

$$\%\ \text{accepted value} = (\text{analytical value} \times 100)/\text{accepted value}$$

**Table 2. Tocopherol (T) and Tocotrienol (T3) Contents of 10 Different Commercially Important Tree Nuts (Milligrams per 100 g of Kernels, $n = 3$)[a]**

| nut | $\alpha$-T | $\beta$-T | $\gamma$-T | $\delta$-T | $\alpha$-T3[b] | $\gamma$-T3 | $\delta$-T3 | total |
|---|---|---|---|---|---|---|---|---|
| almond | 23.21 ± 1.11 | 0.16 ± 0.03 | 0.57 ± 0.04 | nd[c] | nd | nd | nd | 23.94 ± 1.10 |
| black walnut | 2.35 ± 0.25 | 0.01 ± 0.01 | 29.07 ± 0.54 | 1.57 ± 0.06 | nd | nd | nd | 32.99 ± 0.78 |
| Brazil nut | 5.44 ± 2.33 | 0.03 ± 0.02 | 14.63 ± 4.24 | 0.21 ± 0.10 | nd | 0.16 ± 0.11 | nd | 20.47 ± 5.73 |
| cashew | 1.48 ± 0.33 | nd | 5.56 ± 0.63 | 0.37 ± 0.03 | nd | 0.07 ± 0.01 | nd | 7.48 ± 0.73 |
| English walnut | 1.14 ± 0.26 | 0.06 ± 0.02 | 20.44 ± 1.74 | 1.70 ± 0.26 | nd | 0.06 ± 0.01 | nd | 23.40 ± 2.07 |
| hazelnut | 26.46 ± 2.49 | 0.93 ± 0.16 | 4.11 ± 0.61 | 0.20 ± 0.02 | nd | nd | nd | 31.70 ± 2.09 |
| macadamia | nd | nd | 0.01 ± 0.02 | nd | 1.57 ± 1.21 | 0.03 ± 0.04 | nd | 1.60 ± 1.27 |
| pecan | 1.21 ± 0.30 | 0.78 ± 0.29 | 21.94 ± 1.79 | 0.60 ± 0.22 | nd | nd | nd | 24.53 ± 1.48 |
| pine nut | 12.46 ± 0.87 | nd | 11.64 ± 0.26 | 0.03 ± 0.03 | nd | 0.74 ± 0.07 | nd | 24.87 ± 0.83 |
| pistachio | 1.45 ± 0.24 | 0.05 ± 0.01 | 23.01 ± 1.09 | 0.57 ± 0.06 | 0.07 ± 0.01 | 1.79 ± 0.22 | 0.12 ± 0.01 | 27.05 ± 0.73 |

[a] $n$ = number of independent original samples; data represent the mean ± standard deviation. [b] Tocotrienol (T3) retention times were determined using a palm oil reference sample and calibration was made with respect to each T isomer. All samples were devoid of $\beta$-T3, and no limits of detection (LODs) were determined for the T3s. [c] nd = not detected. The LODs were 0.15, 0.14, 0.16, and 0.11 ng/20 $\mu$L injection for $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T, respectively.

The tocopherol methodology was validated by spiking samples of the peanut butter SRM with known quantities of each T isomer. Final spiking levels employed were 21.17, 14.74, 39.43, and 19.48 $\mu$g/g sample for $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T, respectively. Recovery ($R$) was calculated from the equation

$$R\,(\%) = \frac{C_s - C_p}{C_a} \times 100$$

where $R$ (%) is the percent recovery of added standard, $C_s$ is the T content in the spiked sample, $C_p$ is the T concentration in the unspiked sample, and $C_a$ is the amount of T standard added. Bias and accuracy were evaluated by the same equations described for the fatty acids.

To validate the phytosterol methodology, 5$\alpha$-cholestane (IS), campesterol, stigmasterol, and $\beta$-sitosterol were used to determine standard curve linearity.[37] For each compound, solutions of the standards were prepared at five different concentrations in the ranges of 0.2−1.0 for 5$\alpha$-cholestane, 0.025−0.125 for campesterol, 0.12−0.60 for stigmasterol, and 0.2−1.0 $\mu$g/1 $\mu$L injection for $\beta$-sitosterol. All of the standard assays were replicated three times. Repeatability precision (% $RSD_r$) was calculated on the basis of 10 replicates employing the same equation as for tocopherol precision. The limits of detection (LODs) and limits of quantification (LOQs) for each sterol were calculated on the basis of a minimal accepted value of the signal-to-noise ratio of 3 and 10, respectively. Phytosterol recoveries were calculated in the same manner as described above for tocopherols.

## RESULTS

**Method Validation.** For fatty acids, the values for bias were quite small. The main fatty acids, that is, oleic acid and linoleic acid, had percent accepted values of 100.51 ± 0.10 and 98.52 ± 0.29, respectively. This indicates that there was close agreement between the true and analytical values for the samples. The percent recoveries for the tocopherols (T) ranged from 101.2 to 105.4 for the tree nut samples and from 97.0 to 105.8 for NIST SRM 2387. For more details concerning this SRM, the authors recommend accessing the certificate of analysis data sheet available online. The LOD values were 0.15, 0.14, 0.16, and 0.11 ng/20 $\mu$L injection for $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T, respectively, and the LOQ values were 0.28, 0.26, 0.28, and 0.13 ng/20 $\mu$L injection, respectively. The linearities of the FID responses for the IS, campesterol, stigmasterol, and $\beta$-sitosterol were outstanding ($r^2 > 0.9999$) over the measured concentration ranges. The mean percent recoveries of samples spiked with campesterol, stigmasterol, and $\beta$-sitosterol were 98.4 ± 2.2, 97.5 ± 4.3, and 98.7 ± 4.7, respectively, thereby demonstrating no significant loss of phytosterols when subjected to acid hydrolysis. Repeatability precision (% $RSD_r$) from intraday analyses of the samples was found to be between 1.4 and 1.8%. The LOD values were 0.19, 0.21, and 0.23 $\mu$g/mL for campesterol, stigmasterol, and $\beta$-sitosterol, respectively, and the LOQ values were 0.59, 0.51, and 0.52 $\mu$g/mL, respectively.

**Fatty Acid Contents.** The fatty acid profiles of the 10 commercially important tree nuts are presented in Table 1. The total lipid of each nut type ranged from 44.4 ± 1.9% for cashews to 77.1 ± 1.7% for macadamias. The majority of the other nut types had oil contents of ∼60%. Most of the tree nuts had a relatively low percentage, <10%, of saturated fatty acids (14:0, 16:0, 18:0, 20:0, and 22:0). Only Brazil nuts (24.5%), cashews (20.9%), macadamia nuts (17.1%), and pistachios (13.3%) were higher in saturated fatty acids. This is one reason Brazil nuts, cashews, and macadamia nuts were excluded from the USDA qualified health claim for tree nuts. Oleic (18:1 $\omega$9) and linoleic (18:2 $\omega$6) acids comprised the majority of the lipids present in all of the nut types with a combined weight percentage ranging from 62.4% in macadamias to ∼90% in almonds, black walnuts, hazelnuts, pecans, pine nuts, and pistachios.

Macadamia nuts were the only nut type to have a detectable amount of myristic acid (14:0), with a content of 0.83 ± 0.13%, whereas palmitic acid (16:0) was detected in all samples. The mean values for 16:0 ranged from 3.42 ± 0.07% in black walnuts to 14.7 ± 0.79% in Brazil nuts. Palmitoleic acid (16:1 $\omega$7) was present in minute, or undetectable, quantities for all nut types studied except macadamias, which contained 17.95 ± 0.23%. Stearic acid (18:0) was detected in all nuts at levels ranging from 1.47 ± 0.15% in almonds to 9.79 ± 1.08% in Brazil nuts. The only $\omega$-3 fatty acid detected in a nut sample was $\alpha$-linolenic acid (18:3 $\omega$3). It was found in black walnuts, English walnuts, pecans, and pistachios. The levels ranged from 0.33 ± 0.26% in pistachios to 13.43 ± 0.16% in English walnuts. The other fatty acids measured (20:0, 20:1 $\omega$9, 20:2 $\omega$6, 22:0) were all determined in trace amounts (<3%) for all nut types. Pine nut was the only nut type to possess $\Delta^5$-olefinic fatty acids: pinolenic and taxoleic acids were determined at 13.99 ± 0.37 and 2.13 ± 0.05%, respectively.

**Tocopherol and Tocotrienol Contents.** Table 2 gives the total T levels as well as the $\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T and $\alpha$-, $\gamma$-, and $\delta$-tocotrienol contents of the 10 commercially important tree nut

**Table 3. Phytosterol Contents of 10 Different Commercially Important Tree Nuts (Milligrams per 100 g of Kernels, $n = 3$)[a]**

| nut | campesterol | stigmasterol | clerosterol | $\beta$-sitosterol | $\Delta^5$-avenasterol + $\beta$-sitostanol | other sterols[b] | total[c] |
|---|---|---|---|---|---|---|---|
| almond | 5.3 ± 0.3 | 3.3 ± 1.1 | 4.1 ± 1.2 | 161.4 ± 2.4 | 22.0 ± 1.1 | 14.6 ± 2.3 | 210.7 ± 4.6 |
| black walnut | 4.3 ± 0.5 | 0.2 ± 0.3 | 2.4 ± 0.3 | 116.1 ± 3.5 | 26.3 ± 0.2 | 25.8 ± 2.7 | 173.8 ± 2.6 |
| Brazil nut | 1.2 ± 0.2 | 5.5 ± 0.7 | 1.0 ± 0.1 | 62.7 ± 3.2 | 22.0 ± 2.5 | 68.4 ± 13.8 | 145.6 ± 9.9 |
| cashew | 8.6 ± 0.2 | 0.7 ± 0.1 | 2.3 ± 0.5 | 111.0 ± 6.3 | 7.5 ± 0.9 | 15.1 ± 3.2 | 145.3 ± 6.2 |
| English walnut | 5.2 ± 0.8 | 0.9 ± 0.3 | 1.6 ± 0.3 | 103.1 ± 11.1 | 9.2 ± 1.1 | 44.1 ± 4.3 | 164.1 ± 15.7 |
| hazelnut | 7.0 ± 0.4 | 1.5 ± 0.2 | 2.0 ± 0.4 | 124.8 ± 3.2 | 3.8 ± 0.4 | 12.1 ± 2.4 | 147.9 ± 4.3 |
| macadamia | 9.5 ± 1.5 | 0.0 ± 0.0 | 3.0 ± 0.5 | 151.5 ± 11.0 | 16.0 ± 2.9 | 11.6 ± 0.6 | 192.7 ± 12.1 |
| pecan | 6.0 ± 0.5 | 2.4 ± 0.2 | 2.5 ± 0.4 | 130.1 ± 4.1 | 12.6 ± 1.4 | 31.4 ± 6.3 | 184.9 ± 8.6 |
| pine nut | 22.1 ± 1.1 | 0.9 ± 0.3 | 3.1 ± 0.2 | 136.3 ± 5.3 | 45.6 ± 1.3 | 63.8 ± 5.0 | 271.7 ± 9.1 |
| pistachio | 10.8 ± 0.7 | 1.9 ± 0.2 | 4.0 ± 0.2 | 219.6 ± 10.5 | 22.2 ± 3.5 | 43.3 ± 3.6 | 301.8 ± 15.4 |

[a] $n$ = number of independent original samples; data represent the mean ± standard deviation. [b] Identification of each minor sterol was confirmed by GC-MS and quantified as $\beta$-sitosterol equivalents, as no standards exist. [c] Total includes the sum of all dominant sterols plus the minor ones.

**Table 4. Minor Sterol Contents of 10 Different Commercially Important Tree Nuts (Milligrams per 100 g of Kernels, $n = 3$)[a]**

| | minor sterols[b] | | | | | |
|---|---|---|---|---|---|---|
| ID[c] | peak A $\Delta^{5,24(25)}$-stigmastadienol | peak B $\alpha$-amyrin | peak C $\Delta^7$-stigmastenol | peak D cycloartenol | peak E 24-methylenecycloartenol | peak F citrostadienol |
| RRT[d] | 1.05 | 1.06 | 1.07 | 1.08 | 1.15 | 1.20 |
| almond | 4.63 ± 0.29 | nd[e] | 4.65 ± 0.89 | nd | 2.40 ± 0.61 | 2.88 ± 0.50 |
| black walnut | 4.37 ± 0.73 | nd | nd | 14.8 ± 2.3 | 5.29 ± 0.45 | 1.28 ± 0.05 |
| Brazil nut | nd | 53.1 ± 9.7 | nd | 10.8 ± 3.2 | 2.14 ± 0.73 | 2.33 ± 0.32 |
| cashew | 2.51 ± 0.32 | 0.76 ± 0.74 | nd | 7.94 ± 2.32 | 2.59 ± 1.24 | 1.34 ± 0.57 |
| English walnut | 2.23 ± 0.22 | nd | nd | 36.8 ± 3.9 | 1.75 ± 0.15 | 3.30 ± 0.33 |
| hazelnut | 1.42 ± 0.05 | nd | 3.00 ± 1.51 | nd | 0.89 ± 0.17 | 2.92 ± 0.71 |
| macadamia | 1.96 ± 0.24 | nd | 4.27 ± 0.89 | nd | 2.77 ± 0.53 | 3.61 ± 0.19 |
| pecan | 2.68 ± 0.39 | nd | nd | 15.3 ± 4.4 | 2.07 ± 0.35 | 9.24 ± 1.28 |
| pine nut | 4.58 ± 0.59 | nd | 8.18 ± 0.76 | nd | 20.4 ± 1.3 | 19.0 ± 2.0 |
| pistachio | 3.15 ± 0.67 | nd | nd | 11.3 ± 1.1 | 17.0 ± 1.3 | 8.19 ± 1.36 |

[a] $n$ = number of independent original samples; data represent the mean ± standard deviation. [b] Identification (ID) of each minor sterol was confirmed by GC-MS and quantified as $\beta$-sitosterol equivalents, as no standards exist. [c] IDs based on elution order from the GC-FID and GC-MS systems, relative retention times (RRT), and parent as well as fragmentation ions with comparison to literature and library values. [d] RRTs are relative to the retention time of the $\beta$-sitosterol peak in the GC-FID chromatogram. [e] nd = not detected (below the limit of detection for the GC-FID system). The LOD value for $\beta$-sitosterol was 0.23 $\mu$g/mL.

lipid extracts tested. The total T content ranged from 1.60 ± 1.27 mg/100 g nutmeat in macadamias to 32.99 ± 0.78 mg/100 g nutmeat in black walnuts. Most other nut types exhibited values in the upper third of the listed range. The major T isomers present were $\alpha$- and $\gamma$-T. An interesting observation is whether $\alpha$- or $\gamma$-T predominated; this was dependent upon the nut type. Specifically, almonds and hazelnuts had dominant $\alpha$-T contents, whereas marked $\gamma$-T levels were found in black walnuts, Brazil nuts, cashews, pecans, and pistachios. Pine nut was the only tree nut that possessed a fairly equal level of both $\alpha$- and $\gamma$-T.

Tocotrienols (T3) were detected in only six of the nut types sampled (i.e., Brazil nuts, cashews, English walnuts, macadamias, pine nuts, and pistachios). $\gamma$-T3 was the chief T3 in all nuts except for macadamias, which contained a higher quantity of $\alpha$-T3. In fact, macadamias are the only nut type that contained an $\alpha$-T3 content of >1 mg/100 g nutmeat. The mean levels of $\gamma$-T3 in the nutmeats ranged from 0.03 ± 0.04 to 1.79 ± 0.22 mg/100 g nutmeat in macadamias and pistachios, respectively. Pistachios were the only nut type to contain $\delta$-T3 with a content of 0.12 ± 0.01 mg/100 g nutmeat. All nut types were devoid of $\beta$-T3.

**Identification and Quantification of Phytosterols.** Table 3 provides the phytosterol content of the 10 different commercially important tree nut lipid extracts. As expected, $\beta$-sitosterol (5$\alpha$-stigmast-5-en-3$\beta$-ol) was the primary phytosterol among the tree nut types investigated. Other 4-desmethylsterols of importance included campesterol (ergost-5-en-3$\beta$-ol), stigmasterol (5$\alpha$-stigmasta-5,22-dien-3$\beta$-ol), clerosterol (5$\alpha$-stigmasta-5,25-dien-3$\beta$-ol), and $\Delta^5$-avenasterol [5$\alpha$-stigmasta-5,24(28)-dien-3$\beta$-ol (24$Z$)], which possibly coeluted with $\beta$-sitostanol. Pistachios and pine nuts possessed the highest total phytosterol contents of 301.8 ± 15.4 and 271.7 ± 9.1 mg/100 g nutmeat, respectively. Brazil nuts, cashews, and hazelnuts exhibited the lowest total phytosterol contents of ~145 mg/100 g nutmeat. All other nut types fell within these ranges. Minor sterols, which are often excluded in reportings, were quantified using GC-FID and further identified by GC-MS. Surprisingly, these contributed in various degrees to the total phytosterol content for each tree nut. In fact, these variations facilitated the identification of the minor sterols by GC-MS. Of note, the minor sterols of Brazil nuts accounted for 47.0% of the total sterols, whereas for macadamias their contribution was only 6.0%.

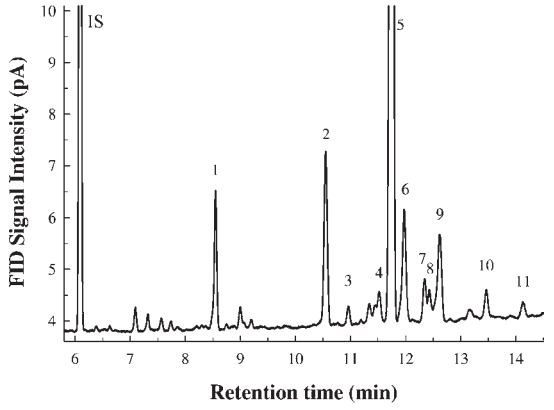


**Figure 1.** Representative chromatogram of TMS ether derivatives of phytosterols in cashews [internal standard (IS), 5$\alpha$-cholestane]. Peaks: 1, fatty acid; 2, campesterol; 3, stigmasterol; 4, clerosterol; 5, $\beta$-sitosterol; 6, $\Delta^5$-avenasterol + $\beta$-sitostanol (possible coelution); 7, $\Delta^{5,24(25)}$-stigmastadienol; 8, $\alpha$-amyrin; 9, cycloartenol; 10, 24-methylenecycloartenol; 11, citrostadienol.

Table 4 lists the six minor sterols that were identified in the tree nut lipid extracts by comparing their elution order and relative retention time (RRT) to the $\beta$-sitosterol peak, and MS fragmentation patterns to those found in the literature. Figure 1 depicts a representative GC-FID chromatogram of the TMS ether phytosterol derivatives isolated from cashews. Cashew was the only nut type possessing all of the minor sterols elucidated with the exception of $\Delta^7$-stigmastenol. An additional minor sterol was present in the pecan, pine nut, and pistachio tree nut lipid extracts, but its identity could not be confirmed using the methods described in this work and, therefore, was omitted from Table 4.

With reference to Table 4: Sterol peak A (peak 7 in Figure 1) was present in all tree nut lipid extracts except for Brazil nut and was identified as $\Delta^{5,24(25)}$-stigmastadienol due to its elution order immediately following $\Delta^5$-avenasterol + $\beta$-sitostanol, as seen in hazelnut oil[41] and olive oil.[42] The quantity of sterol A ranged from 1.42 mg/100 g nutmeat in hazelnuts to 4.63 mg/100 g nutmeat in almonds. Peak A's RRT of 1.05 was comparable to that found in peanut oils by Shin et al.[37] This tentative identification was further supported by examining the MS fragmentation patterns found in peanut oil,[37] sea buckthorn oil,[28] and ginseng seed oil.[43] The key fragmentation ions present were at $m/z$ 484 ($M^+$), 394 {i.e., M − 90; $[(CH_3)_3 - Si - OH]^+$}, 379 {i.e., M − 105; $[CH_3 + (CH_3)_3 - Si - OH]^+$}, and 355 {i.e., M − 129; $[(CH_3)_3 - Si - O^+{=}CH{-}CH{=}CH_2]^+$}. Mass spectra for TMS ethers of 4-desmethylsterols characteristically yield peaks with $m/z$ of $[M - 129]^+$ and 129. The peak at $m/z$ 129 $[(CH_3)_3 - Si - O^+{=}CH{-}CH{=}CH_2]^+$ has been identified as the fragment originating from the breakdown of ring A along with the TMS moiety.[37] In this work, $[M - 129]^+$ fragmentation ions were found at $m/z$ 343, 355, 355, 357, 355, and 355 in the mass spectra for TMS derivatives of the IS, stigmasterol, clerosterol, $\beta$-sitosterol, $\Delta^5$-avenasterol, and $\Delta^{5,24(25)}$-stigmastadienol, respectively.

Sterol peak B (peak 8 in Figure 1) was present only in Brazil nuts and cashews. The peak was identified as the 4,4′-dimethylsterol $\alpha$-amyrin (5$\alpha$-urs-12-en-3$\beta$-ol). This identification was based mainly on the MS fragment ions. As reported by Li et al.,[28] there are significant peaks at the fragmentation ions at $m/z$ 218 (base peak), 498 ($M^+$), and 393 {i.e., M − 105; $[CH_3 + (CH_3)_3 - Si - OH]^+$}. In Brazil nuts, $\alpha$-amyrin represented a large portion of the quantified minor sterols with a mean content of 53.1 mg/100 g nutmeat comprising nearly 78% of the total minor sterols present in the Brazil nut lipid extract.

Sterol peak C (not detected in cashews, so absent from Figure 1) was tentatively identified as $\Delta^7$-stigmastenol (stigmast-7-en-3$\beta$-ol). The RRT was calculated to be ∼1.07, and the compound was found in almond, hazelnut, macadamia, and pine nut lipid extracts. This identification was determined on the basis of comparison of the elution order and RRT with those reported in hazelnuts[41] and olive oils.[42] Pine nut oil contained ca. 2 times the amount of the other three tree nut oils with a quantity of 8.18 mg/100 g nutmeat.

Sterol peak D (peak 9 in Figure 1) was a prominent peak among the minor sterols and has been identified as the 4,4′-dimethylsterol cycloartenol (9$\beta$,19-cyclo-5$\alpha$-lanost-24-en-3$\beta$-ol); however, it might be coeluting with $\Delta^7$-sitosterol (5$\alpha$-stigmast-7-en-3$\beta$-ol) as reported by Shin et al.[37] Cycloartenol was detected in 6 of the 10 tree nut oils (i.e., black walnuts, Brazil nuts, cashews, English walnuts, pecans, and pistachios). Its identification was based on a similar RRT, 1.07, as noted by Shin et al.[37] The MS fragmentation ions observed were also compared to those found in the literature. Similar in nature to the fragments reported in peanut lipids[37] and sea buckthorn oil,[28] the key fragmentation ions were at $m/z$ 498 ($M^+$), 483 [i.e., M − 15; $(CH_3)^+$], 408 {i.e., M − 90; $[(CH_3)_3 - Si - OH]^+$}, and 393 {i.e., M − 105; $[CH_3 + (CH_3)_3 - Si - OH]^+$} along with a base peak at $m/z$ 69.

Similar in structure, sterol peak E (peak 10 in Figure 1) was identified to be the 4,4′-dimethylsterol 24-methylenecycloartenol (24-methylene-9$\beta$,19-cyclo-5$\alpha$-lanost-24-en-3$\beta$-ol). Peak E was found to have a RRT of ∼1.15 and was present in the chromatograms for all 10 tree nut lipid extracts. The important MS fragment ions were $m/z$ 73 (base peak), 497 {i.e., M - 15; $[CH_3]^+$}, and 379. These ions matched up with those reported in sea buckthorn oil by Li et al.[28] Sterol peak F (peak 11 in Figure 1) eluted with a RRT of 1.20 and was tentatively identified to be the 4-monomethylsterol citrostadienol. The key MS fragmentation ions were $m/z$ 498 ($M^+$), 483 [i.e., M − 15; $(CH_3)^+$], 400, and 357 (base peak). These ions matched up well with those reported by Li et al.[28] for the citrostadienol found in sea buckthorn oil.

## DISCUSSION

The total lipid contents of each nut type determined in this study were compared against those reported in the USDA National Nutrient Database for Standard Reference, release 24.[9] Even though the values presented in this work were slightly higher than those cited in the database, most of the fatty acid percentages were within 4% of the database total lipid values. Pistachios, however, are the exception, with a content within 11% of the reported value. Cashews were the only nut studied to have a value lower, ∼8%, than the database reported value. The slightly greater lipid quantities noted are attributable to extraction of the lipids with chloroform/methanol/water, which includes the presence of phospholipids in the total lipid percentage.[44] Marked differences do exist between the predominant fatty acids; that is, palmitic, oleic, and linoleic acids. The differences between the values reported in this work and those of the database were at times quite pronounced. In several instances, our values are >20% of those found in the database. A majority of

these distinctions can be attributed to variations among the selection of cultivar, growing year, and growing locations for the tree nuts in the present study.

Even though the health significance of low levels of saturated fatty acids and high levels of MUFAs/PUFAs in tree nuts are reported in guidance documents and claims, few data actually exist in the literature highlighting these beneficial attributes. Of the data that have been published, most studies fail to include method validation steps that ensure accurate reporting of data values. For instance, Miraliakbari and Shahidi[10] investigated the lipid compositions of seven different types of tree nuts (i.e., almonds, Brazil nuts, hazelnuts, pecans, pine nuts, pistachios, and walnuts) using either hexane or chloroform/methanol extractions, but comparisons could be made for only six of the seven tree nuts listed due to the suspected combination of English and black walnut types into one category. Of the six comparable nut types, three nut types fell into the listed range of their data. The values reported for almonds, pistachios, and pine nuts in the present study were slightly lower (<6%) than those given by Miraliakbari and Shahidi.[10] Among the predominant fatty acids investigated in both studies, linoleic acid (18:2 $\omega 6$) exhibited the largest variation between the two data sets. Almonds, hazelnuts, and pistachios were 5, 1, and 1.5% higher, respectively, whereas pecans and pine nuts were 3% lower in our findings. The lack of standard deviations in the tables makes a true comparison difficult. This was also the case for a study conducted by Ryan et al.,[45] in which the fatty acid, tocopherol, and phytosterol contents of Brazil nuts, pecans, pine nuts, pistachios, and cashews were investigated. Direct comparisons or the concept of natural variation were not possible due to the lack of reported variance in the collected data.

Pine nuts contain a unique class of unsaturated fatty acids, the $\Delta^5$-unsaturated polymethylene-interrupted fatty acids ($\Delta^5$-UPIFA or $\Delta^5$-olefinic acids). This special class of fatty acids can be found in all gymnosperm seed, leaf, and wood lipids.[46] It is suspected that the rare location of the double bond comes from a desaturation reaction at carbon 5 of oleic, linoleic, and linolenic fatty acids. Another possible mechanism would be a desaturation reaction of one of the elongation products of those acids.[47] The $\Delta^5$-olefinic fatty acids are not included in the USDA National Nutrient Database for Standard Reference, release 24,[9] and thus were not included in the body of Table 1 to make for easier comparisons between data. Two $\Delta^5$-olefinic acids, namely, 5($Z$),9($Z$)-octadecadienoic acid (taxoleic acid) and 5($Z$),9($Z$),12($Z$)-octadecatrienoic acid (pinolenic acid), were found in the pine nut oils. These two fatty acids comprised ~16% of the total fatty acid profile of pine nut. When our data values were compared to those of the literature, it was noted that most papers investigating the fatty acid content of multiple nut types exclude these unique acids. The fatty acid profile of pine nut determined in this work compared well to that reported by Kim and Hill, Jr.,[48] who included the $\Delta^5$-olefinic fatty acids in their analysis. They reported contents of 2.2 and 16.8% for taxoleic and pinolenic acids, respectively, compared to our values of 2.1 and 14.0%, respectively.

For vitamin E there were some significant ($P < 0.05$) differences noted between database values and those generated in this work. Apropos $\alpha$-T, five of the nut types investigated showed higher levels than those cited in the database. The differences ranged from a ~31% increase for black walnuts to ~76% greater content for hazelnuts. Interestingly, the $\alpha$-T contents from the HPLC chromatograms for almonds and pistachios were significantly ($P < 0.05$) lower by 13 and 67%, respectively, than database values. With regard to the other T isomers determined, similar results were noted. When T data reported in the literature were compared, it was a mixed bag; that is, for selected nut types we found higher levels than the database for some of the isomers, and for other nut types the opposite was true. This "lack of a difference trend" can be attributed to possible variations in horticultural characteristics of the samples such as cultivar, growing conditions, and harvest year.[13]

Kornsteiner et al.[49] examined the T content in 10 different tree nuts. Our findings are in agreement with these authors for a majority of the nut types. They reported null values for several T isomers, whereas we detected quantities for all four. It would have been helpful if the LOD in their analytical methodology had been given, because it makes it difficult to draw conclusions from the observed differences. Furthermore, variations existed between the analytical techniques: in the present study, the direct solvent extraction method using isopropanol/ethyl acetate/hexane was used, but for Kornsteiner et al.[49] only hexane was employed. This disparity in solvent system might account for the inability to detect the minute levels of some of the T isomers. Maraliakbari and Shahidi[50] determined the T content of seven different types of tree nuts. Of the six comparable types, four (i.e., almonds, hazelnuts, pine nuts, and pistachios) gave higher values than those reported in this work. The values for the T isomers of pine nuts were markedly different from each other in all Ts presented. It is suspected that this might be due to the extreme differentiation observed in pine nut cultivars, particularly when pinyon nuts are included in the sampling.[38] Other differences ranged from 10.7% in almonds to 50.4% in pecans. Variations in the horticultural and storage practices of the nuts might account for the observed differences.

No T3s are reported in the USDA National Nutrient Database for Standard Reference, release 24,[9] for any of the tree nuts investigated. Even though the T3s are present only in minute quantities in some tree species, it would be beneficial to include these values in the database. As the American public's interest in healthful lipid constituents of food increases, it is important that they be provided with the most accurate and all-encompassing data. A review of the scientific literature in preparation for this study found no investigations reporting T3 levels for the nut types examined. Possible reasons for this may be the nonavailability of T3 standards or fluorescence detection capabilities with its increased sensitivity.

Of the three lipid constituents examined, the phytosterol contents determined were the most similar to those reported in the database. This, however, is only a recent development because the database was updated from release 23 to 24 in late September 2011. A marked change in the database was the inclusion of phytosterol contents for all tree nuts. Previously, Brazil nuts, cashews, and pine nuts had no phytosterol contents listed in the database; therefore, no comparisons could have been made. The updated values now include the contribution of steryl glucosides, released via an acid hydrolysis step prior to saponification, for each nut type. As stated by Phillips et al.,[22] "Sterol glucosides can account for as much as 37% of the total sterols found in the nut." Therefore, the updated values are more representative of the total phytosterol contents in tree nuts. With regard to the predominant sterol, $\beta$-sitosterol, our values were greater than the revised levels reported in the database for all nut types except for cashews and Brazil nuts. In almonds, hazelnuts, and pecans the differences were found to be significant

($P < 0.05$). Similar observations were also noted for the other two major sterols, namely, campesterol and stigmasterol. The three major phytosterols described above are the only ones included in the body of the database, whereas the contribution of minor sterols is listed as a footnote. Even though these three sterols represent the lion's share of the total, in some cases, as can be seen in Tables 3 and 4, clerosterol, $\Delta^5$-avenasterol + $\beta$-sitostanol, and minor sterols do contribute significantly to the overall phytosterol level. In the present study, for example, minor sterols accounted for ~41 and ~63% of the total phytosterol contents of pine nuts and Brazil nuts, respectively.

On the other hand, our findings are not dissimilar when compared to other studies reported in the literature that did include an acid hydrolysis step, as well as the additional SPE assay for $\Delta^5$-avenasterol. A case in point is Phillips et al.:[38] the main differences between our findings, when direct comparisons could be made for selected tree nut species, came in the quantification of the minor sterols. This could be attributed to an increased knowledge in recent years of mass spectrum fragmentation patterns, differences in horticultural aspects of the nuts, and perhaps better LODs in the GC-FID analyses.

To summarize, the contents of lipid constituents (i.e., fatty acids, vitamin E, and phytosterols) were assessed in 10 commercially important tree nut types using validated methods and a quality control program to confirm the accuracy of replicate analyses. The results for fatty acids and phytosterols were comparable in most cases to those reported in the USDA National Nutrient Database for Standard Reference, release 24, but significant variations existed for vitamin E. Six of the nut types investigated showed higher levels of $\alpha$-T than those cited in the database, whereas two exhibited lower values. Furthermore, the present study reports the presence of T3s, albeit at levels <2 mg/100 g kernels, in 6 of the 10 nut types. Of greatest value, this study shows that when saponification alone is employed, only ~80% of the phytosterols present in most of the tree nuts can be accounted for. An acid hydrolysis step prior to saponification (i.e., double hydrolysis) is critical to liberate steryl glucosides from the tree nut matrix so that accurate phytosterol levels can be determined. The strength of the validated analytical methodology and employment of SRMs in the quality control program make the findings from this study worthy for inclusion in the USDA National Nutrient Database for Standard Reference.

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