Journal of Food Composition and Analysis 75 (2019) 61–69

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

Identification and quantification of phytosterols in black walnut kernels

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ARTICLE INFO

Keywords:
Food analysis
Food composition
Black walnut
English walnut
Juglans nigra
Phytosterols
Sitosterol
Lowering cholesterol
Health-promoting
Metabolomics

ABSTRACT

This study aimed to identify and quantify phytosterols of six black walnut (*Juglans nigra* L.) varieties, and compare the levels of these phytosterols between black walnuts and English walnut (*Juglans regia* L.). Totally, 13 phytosterols were identified in the black walnut kernels, with β-sitosterol predominating over the other sterols. The analysis also revealed the presence of 3 phytosterols, $\Delta^{5,23}$-stigmastadienol, cycloeucalenol and 28-methylobtusifoliol, which have never been reported in black walnuts. The average levels of total sterols ranged from 1236.0 mg/kg to 1542.3 mg/kg. No significant differences were noted in total sterol levels between the studied black walnut varieties and English walnut. Through untargeted metabolomics analysis, five additional glycosylates and hydroxycinnamates of phytosterols were putatively identified in the black walnuts. The findings from this research suggest black walnuts are rich in phytosterols most of which have been demonstrated to exert bioactivities. Future studies should be focused on seasonal and geographic variations in phytosterol content of black walnuts, and bioassay-guided purification to assess potential health-promoting properties of these phytosterols.

1. Introduction

Black walnut trees (*Juglans nigra* L.), belonging to the walnut family (Juglandaceae), are one of the most valuable species native to North America. The trees produce not only high-quality lumber and veneer, but also high-value nuts with unique flavor. Black walnuts are used all over the United States in ice cream, candies, baked goods, and walnut oil production. The use of cold press extraction allows for production of high-quality walnut oil (Martínez et al., 2010; Cuesta et al., 2017; Rabadán et al., 2018). Black walnuts contain a diverse mixture of nutritionally and medicinally important components (Câmara and Schlegel, 2015; Vu et al., 2018). Among these, phytosterols have attracted considerable attention for their health-promoting activities which have been reported to prevent lifestyle-related diseases such as obesity, diabetes mellitus, hyperlipidemia, hypertension (Marinangeli and Jones, 2010; Misawa et al., 2012; Mohamed, 2014). Phytosterols have been demonstrated to inhibit the absorption of dietary and endogenously generated cholesterol from intestinal cells, and thus reduce circulating levels of cholesterol (Ostlund, 2007; Jones and Abu-Mweis, 2009). Since cholesterol is one of the major risk factors for cardiovascular disease, the property of lowering cholesterol absorption potentially leads to a reduced risk of heart diseases. The efficacy of phytosterols as cholesterol-lowering agents has also been shown in the case of phytosterol-supplemented foods (Abu-Mweis et al., 2008). This has resulted in the commercial availability of many dietary supplements and food products fortified with phytosterols, which were considered as alternatives to cholesterol-lowering drugs. Phytosterols have also been proven to have many other health-promoting effects such as anti-inflammatory, antioxidant, and anticancer activities (Awad and Fink, 2000; Akihisa et al., 2000; Vivancos and Moreno, 2005). Therefore, these reviewed health-promoting effects of phytosterols make black walnuts as well as black walnut-containing products a very attractive diet option due to the nutritional and medicinal values.

Despite the growing recognition of high values of black walnuts, limited information is available about their phytosterol compositions. Prior studies on phytosterols neither compared the phytosterol profiles between black walnut cultivars, nor specified cultivars selected for their studies (Phillips et al., 2005; Robbins et al., 2011). They were often described as the generic black walnuts collected from various grocery stores. That could result in ambiguous scientific information about the phytosterol profiles and their concentrations. Advances in phytosterol analysis suggest that both acid hydrolysis and saponification steps are required to facilitate total extraction of these compounds. Otherwise, it would lead to underestimation of phytosterol levels because the

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https://doi.org/10.1016/j.jfca.2018.09.016
Received 23 April 2018; Received in revised form 18 September 2018; Accepted 21 September 2018
Available online 26 September 2018

presence of total phytosterols in plants are closely related to their free alcohol, fatty acid ester, hydroxycinnamic ester, steryl glycoside, and acetylated steryl glycoside forms (Moreau et al., 2002).

To date, there has not been any study that systematically characterizes and compares the phytosterol levels among black nut varieties. The objectives of this study were to 1) identify and characterize phytosterol composition of six black walnut varieties, 2) compare the levels of these phytosterols between black walnuts and English walnut (*Juglans regia* L.). Systematic characterization of health-promoting phytosterols will help identify new industrial applications of the black walnuts and give a better understanding of how this class of chemicals contributes to prevention and treatment of diseases.

2. Materials and methods

2.1. Sample preparation

Six black walnut varieties were selected in this study, including Emma K, Kwik Krop, Mystry, Schessler, Sparks 147, and Tomboy. The nuts for each variety were all collected from the trees planted at the University of Missouri Horticulture and Agroforestry Research Center (HARC) in New Franklin, Missouri, the United States, in September 2016. Black walnut hulls were mechanically removed following harvest. After hulling, the nuts were cured in a cool, dry, well-ventilated area out of direct sunlight for two weeks. After curing, the unshelled nuts were stored in freezer (−20 °C) until analysis. Immediately before the analysis, each nut was shelled to obtain the kernel which was afterwards ground and homogenized in a commercial coffee grinder (Black & Decker) to 20–40 mesh sizes.

The HARC, located at 39°0'55"N 92°45'5"W, is the primary research site for the Center for Agroforestry at the University of Missouri. The area is about 185 m above sea level. This 270-hectare farm sits on seven recognized soil associations. The most common soil type (rich, fertile, well-drained, windblown silt-loam known as loess) covers the bulk of the property and is approximately 1 m deep at its center. The black walnut trees are grown on 90 cm deep, well-drained soils with a nearly neutral pH. The black walnut plantation at the HARC has been established for nut production. Therefore, the land management activities include the typical practices (lime, pruning, fertilization, weed control) for black walnut production (Reid et al., 2009). No reports on insect or disease damages during the studied crop year were given. Climatic conditions at the experimental site in which the black walnuts were harvested were documented (Supplemental Table S1).

2.2. Chemicals and reagents

Hydrochloric acid, 0.5 N potassium hydroxide in ethanol, pyridine (99.8%), and HPLC-grade solvents including methanol, n-hexane, cyclohexane, diethyl ether, absolute ethanol (99.5%, ACS grade) were purchased from Fisher Scientific (Pittsburg, PA, USA). Analytical phytosterol standards (β-sitosterol, campesterol, stigmasterol, stigmastanol), α-cholestanol and the silylation reagent N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Analysis of phytosterol composition

2.3.1. Acid hydrolysis and saponification

The extraction of phytosterols was achieved by acid hydrolysis in ethanol followed by saponification. The samples underwent acid hydrolysis to break down ester and glycosidic bonds in conjugated phytosterols, releasing free phytosterols. Following the acid hydrolysis, the samples were saponified with alkali to remove the saponifiable triglycerides. Acid hydrolysis was performed on each of the ground black walnut samples according to Toivo et al. (2001) with minor modification. In brief, 2.0 g of ground black walnut were weighed into a glass culture tube, then 1 mL of absolute ethanol was added. The mixture was shaken vigorously. 50 μg of α-cholestanol (in 2.5 mL of ethanol) as an internal standard was added. After addition of 5 mL of 6 M HCl, the mixture was shaken vigorously and heated in a water bath (80 °C for 60 min.). The tube was shaken every 10 min. After acid hydrolysis, the tube was cooled with water over its bottom. 5 mL of ethanol were added and then the mixture was vortexed. 20 mL of hexane-ethyl ether (1:1) were then pipetted into the tube. The sample was tube-rocked for 10 min. before centrifuged at 1000 rpm for 10 min. The upper phase was separated using pipette, and an aliquot of 15 mL was transferred to a clean tube, then evaporated until dryness under a clean stream of N_2.

The dried residue obtained from the acid hydrolysis step was dissolved in 7 mL of 0.5 M KOH in ethanol to perform saponification. The mixture was heated in a water bath at 80 °C for 60 min. During heating, the mixture was shaken and cooled with water as described in the acid hydrolysis step. The remaining unsaponifiable phytosterols were extracted by liquid-liquid extraction using 20 mL of cyclohexane and 12 mL of deionized water. The mixture was tube-rocked for 10 min., and then centrifuged at 1000 rpm for 10 min. (Toivo et al., 2000). A 1 mL aliquot from the upper phase (i.e., cyclohexane fraction) was evaporated until dryness under a clean stream of N_2, then redissolved in 1 mL of methanol. All samples were analyzed in triplicate.

2.3.2. Solid phase extraction

The phytosterols in methanol were further extracted using solid phase extraction (SPE). A C18 SPE (Bond-Elut LRC-C18, 500 mg, Agilent, Santa Clara, CA, USA) cartridge was activated with 5 mL of methanol followed by conditioning process with 5 mL of deionized water. A Thermo Fisher PrepSep 12-port Vacuum Manifold was used for the SPE step. The phytosterols were eluted with 10 mL of 5% MeOH/chloroform at flow rates of 2 mL/min. The extract was evaporated until dryness under a clean stream of N_2.

2.3.3. Preparation of trimethylsilyl (TMS) ether derivatives

Derivatization of phytosterols was carried out to convert free phystosterols into hydrophobic derivatives which are suitable for GC analysis. 250 μL of anhydrous pyridine and BSTFA + 1% TMCS reagent (1:1, v/v) were added into a GC vial containing the dried sample after the SPE step. The mixture was heated in a water bath set at 60 °C for 1 h prior to injection into GC-MS.

2.3.4. GC–MS analysis

GC-MS was used to identify and quantify the phytosterols in the samples. The analysis of phytosterol-TMS derivatives was performed using gas chromatography coupled with mass spectrometry (GC-MS). A Varian CP-3400CX gas chromatograph (Walnut Creek, CA, USA) equipped with a 5% phenyl 95% dimethylarylene siloxane DB-5MS capillary column (30 m × 0.25 mm I.D.), is interfaced to an ion-trap mass spectrometer (Varian Saturn 2000) and an autosampler (Varian 8200). The injector temperature was held at 290 °C. The GC temperature program was initially set at 95 °C for 1 min, then was ramped at 30 °C/min to a final oven temperature of 320 °C, which was maintained for an additional 25 min. Mass spectra were obtained, with the *m/z* range from 50 to 520, using electron impact ionization (70 eV). Identification of TMS-sterol signals was performed by comparison of their retention times to those of TMS-derivatized α-cholestanol, campesterol, stigmasterol, β-sitosterol, and stigmastanol standards. The National Institute of Standards and Technology (NIST/EPA/NIH, 2009) mass spectral library was used to help confirm mass spectra of phytosterol standards to those detected in black walnut TMS ether samples. Comparison of molecular ion (M^+) and product ions was made to facilitate elucidation of the identities of phytosterols. For additional phytosterols whose standards were not commercially available, their identification was performed based on comparison of relative retention times (RRT), M^+ values, and product ions of TMS phytosterol derivatives to those reported in the literature for walnut oils and plant oils.

2.4. Untargeted metabolomics analysis of conjugated phytosterols

Each ground black walnut sample (2.5 g) was extracted with 15 mL of HPLC-grade methanol (Fisher Scientific, PA, USA) in a capped test tube placed in a cooled water bath (4 °C). The mixture was sonicated for 60 min., and then centrifuged for 10 min. at 8000 rpm. The supernatant was filtered through a 0.2 µm Whatman Anotop filter (GE Healthcare, Germany), and transferred to an LC vial prior to injection into UPLC-MS (ultra-performance liquid chromatography-mass spectrometry).

2.4.1. Analysis of UPLC-MS

The UPLC-MS analysis was performed on a Bruker maXis impact quadrupole-time-of-flight mass spectrometer (QTOF-MS) coupled with a Waters ACQUITY UPLC system. Separation was achieved on a Waters C18 column (2.1 × 100 mm, BEH C18 column with 1.7 µm particles) using a linear gradient of 95%:5% to 30%:70% eluent A:B (A: 0.1% formic acid, B: acetonitrile) over 30 min. Between 30–33 min, the linear gradient was increased from 70% to 95% B, and maintain at 95% B for 3 min. The percentage of B was maintained at 5% from 36 to 40 min. The flow rate was 0.56 mL/min and the column temperature was 60 °C. Mass spectrometry was performed in the negative (or positive) electrospray ionization mode with the nebulization gas pressure at 43.5 psi, dry gas of 12 L/min, dry temperature of 250 °C and a capillary voltage of 4000 V. Mass spectral data were collected from *m/z* 100 and 1000, and were auto-calibrated using sodium formate after data acquisition.

2.4.2. Data analysis

The UPLC-MS data were processed using XCMS Online in multi-group mode (https://xcmsonline.scripps.edu/landing_page.php?pgcontent = multiJob). The. cdf files obtained from UPLC-MS were directly loaded into XCMS Online. The data files were automatically read and converted for peak detection, peak grouping, spectra extraction, and retention time (RT) correction/alignment. The multi-group job allowed for the comparison of multiple groups of black walnut extracts. The XCMS parameters set for UPLC-QTOFMS were as follows: the centWave method for feature detection (with *m/z* tolerance = 5 ppm); the Obiwarp method for retention time correction (profStep = 0.5); and chromatograms were aligned using the following parameters: mzwid = 0.01, minfrac = 0.5, bw = 2. Metabolite features (i.e., ions with characteristic *m/z* and retention times) were listed in a feature list table containing their integrated intensities (extracted ion chromatographic peak areas). Putative identification of conjugated phytosterols as metabolites was performed by the integration of the METLIN into XCMS Online.

2.5. Statistical analysis

The chromatographic data obtained in the analysis of phytosterols were processed with Varian MS Workstation software (service pack 1, version 6.5). One-way analysis of variance (ANOVA) was performed on the quantitative data generated from the analysis in order to determine variation of the phytosterol concentrations between black walnut varieties. Comparisons of means were achieved using the XLSTAT program (XLSTAT Premium 19.5, Addinsoft, Paris, France). When significant differences were found, multiple pair-wise comparisons were determined using the Tukey's Studentized Range HSD test at a level of significance of $p \leq 0.05$.

Principal component analysis (PCA) was used to examine patterns in phytosterol data, and to underscore similarities and dissimilarities in phytosterol contents of walnut cultivars. Hierarchical cluster analysis (HCA) was carried out to determine relative similarity among walnut samples, and the results were displayed as a dendrogram. Sample similarities in the dendrogram were calculated based on the Euclidean distance between the cultivars using Ward's algorithm. The PCA and HCA were performed using the XLSTAT program.

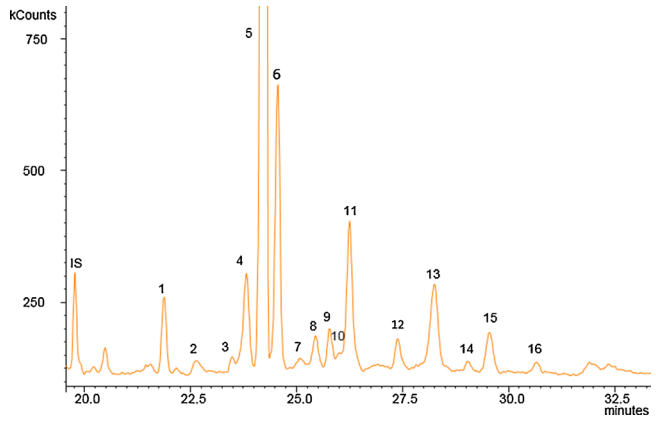

Fig. 1. Representative GC–MS chromatogram of TMS-derivatized phytosterols from a black walnut. The peak numbers in the chromatograms refer to the following components: 1 = campesterol, 2 = stigmasterol, 3 = $\Delta^{5,23}$-stigmastadienol, 4 = clerosterol, 5 = β-sitosterol, 6 = stigmastanol and Δ^{5}-avenasterol, 7 = unidentified, 8 = $\Delta^{5,24(25)}$-stigmastadienol, 9 = unidentified, 10 = cycloeucalenol, 11 = cycloartenol, 12 = 28-methylobtusifoliol; 13 = 24-methylenecycloartenol; 14 = unidentified, 15 = citrostadienol, 16 = unidentified, IS = α-cholestanol (internal standard).

3. Results and discussion

3.1. Identification of phytosterols by GC-MS

Fig. 1 demonstrated a representative GC–MS chromatogram of the TMS-ether phytosterols isolated from a black walnut kernel. The list of the identified phytosterols with their RRT and mass spectral fragmentation patterns is described in Table 1. The results indicated that the chromatographic peaks numbered from 1 to 16 consisting of major and minor sterols eluted from 21.66 min to 30.36 min. Peak 6 in the chromatogram was composed of two coeluting sterols. The results also showed that these derivatized phytosterols can be chromatographically separated using a DB-5MS capillary column (30 m length x 0.25 mm I.D. x 1 µm film thickness, Agilent Technologies, Santa Clara, CA). The data of RRT and mass spectral fragmentation patterns of phytosterols are described in Table 1. The utilization of GC–MS has resulted in excellent separation resolution and symmetric peaks with high signal-to-noise ratios. More importantly, GC–MS combined with NIST mass spectral library has facilitated identification of the additional phytosterols, of which analytical standards are not available. The GC–MS analysis has covered a much wider range of sterols. In addition, the NIST mass spectral libraries provide the fingerprint spectra needed for identification of the TMS-ether phytosterols. The approach used in this study could be used for identification and quantification of a wide range of sterols in walnuts.

As summarized in Table 1, fragmentation patterns of the TMS-derived phytosterols identified in black walnut kernels were constructed using phytosterol standards, NIST, and those previously reported in the literature. Commercial sterol standards only available for campesterol, stigmasterol, β-sitosterol and stigmastanol were employed for the identification of peaks 1, 2, 5 and 6a, respectively. Other sterol peaks (without analytical standards commercially available) were identified by comparison of RRT, molecular ion (M^+) and main 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$\}]^+$, [M – 129; $\{(CH_3)_3$–Si–O^+ = CH–CH = $CH_2\}]^+$.

Peak 3 was identified as $\Delta^{5,23}$-stigmastadienol (Fig. 1), based on RRT and mass spectral data previously reported in the literature (Benitez-Sánchez et al., 2003; Gwatidzo et al., 2014). The mass spectrum of the $\Delta^{5,23}$ sterol characteristically yields peak at *m/z* of 283 generated by allylic cleavage of the $C_{20\text{-}22}$ bond and loss of TMS-OH (Gwatidzo et al., 2014). In their study on manketti nut oil, those authors reported that this sterol exhibited an RRT of 0.95. Our result showed

Table 1
Relative retention time and fragmentation ions of the phytosterols as TMS-ethers identified in black walnuts.

Compound	RRT[a]	Peak	Main fragment ions, m/z (RI)[b]					Additional fragment ions
			M^+	M - 15	M - 90	M - 105	M - 129	
campesterol	0.90	1	472 (55)	457 (9)	382 (100)	367 (32)	343 (47)	415 (2), 315 (5), 289 (3), 261 (8), 255 (21), 207 (9), 129 (26), 73 (16), 55 (12)
stigmasterol	0.93	2	484 (80)	469 (11)	394 (100)	379 (25)	355 (17)	373 (12), 343 (11), 282 (13), 255 (29), 228 (5), 213 (12), 129 (35), 105 (17), 83 (55), 55 (37)
$\Delta^{5,23}$-stigmastadienol	0.96	3	484 (100)	469 (20)	394 (65)	379 (27)	355 (12)	386 (17), 283 (51), 253 (31), 213 (34), 208 (92), 129 (35)
clerosterol	0.97	4	484 (23)	469 (12)	394 (24)	379 (14)	355 (13)	386 (8), 373 (15), 343 (7), 296 (9), 253 (5), 207 (100), 129 (23), 55 (35)
β-sitosterol	1	5	486 (51)	471 (14)	396 (100)	381 (26)	357 (32)	329 (4), 255 (15), 213 (6), 129 (27), 55 (9)
stigmastanol	1.01	6a	488 (82)	473 (72)	398 (88)	383 (68)	359 (7)	431 (32), 373 (71), 306 (24), 215 (100), 75 (98)
Δ^5-avenasterol	1.01	6b	484 (8)	469 (8)	394 (12)	379 (4)	355 (4)	386 (100), 371 (29), 296 (66), 281 (38), 257 (9), 255 (9), 129 (20), 73 (16), 55 (25)
unidentified	1.03	7	486 (2)	–	–	–	355 (3)	441 (100), 386 (2), 207 (22), 55 (16)
$\Delta^{5,24(25)}$-stigmastadienol	1.04	8	484 (10)	469 (11)	394 (13)	379 (12)	355 (1)	386 (100), 371 (28), 343 (8), 296 (56), 281 (23), 257 (8), 255 (8), 253 (12), 213 (14), 129 (22), 73 (28), 55 (44)
unidentified	1.06	9	498 (23)	–	408 (100)	–	355 (63)	379 (2), 355 (3), 207 (3), 73 (24), 55 (15)
cycloeucalenol	1.07	10	498 (7)	483 (20)	408 (31)	393 (46)	–	365 (23), 339 (9), 325 (7), 297 (10), 286 (10), 283 (6), 269 (10), 189 (9), 55 (100)
cycloartenol	1.08	11	498 (4)	483 (19)	408 (75)	393 (61)	–	365 (67), 339 (64), 297 (15), 286 (8), 189 (16), 69 (100)
28-methylobtusifoliol	1.13	12	512 (12)	497 (41)	–	407 (64)	383 (3)	309 (15), 295 (25), 281 (58), 225 (34), 215 (9), 213 (16), 69 (48), 55 (100)
24-methylenecycloartenol	1.16	13	512 (2)	497 (7)	422 (76)	407 (94)	383 (2)	379 (55), 353 (28), 339 (5), 323 (7), 297 (16), 269 (21), 255 (16), 241(12), 201 (19), 73 (100), 69 (61)
unidentified	1.19	14	498 (3)	483 (6)	408 (5)	–	369 (7)	396 (8), 355 (29), 269 (19), 241 (8), 255 (17), 207 (100)
citrostadienol	1.21	15	498 (4)	483 (5)	408 (4)	393 (2)	–	400 (29), 385 (6), 357 (100), 310 (7), 295 (10), 267 (24), 241 (6), 227 (8)
unidentified	1.26	16	488 (7)	–	–	–	–	422 (43), 408 (37), 379 (63), 207 (100), 191 (63), 73 (10), 55 (20)

[a] Retention time relative to β-sitosterol TMS-ether, which eluted at 24.054 min, using a DB-5MS 30 m column.
[b] Intensity relative to base peak (%).

the RRT for $\Delta^{5,23}$-stigmastadienol peak was 0.96 (Table 1). Abdallah et al. (2015) found it to be present in English walnuts grown in Tunisia.

Peak 4 was identified to be clerosterol. This sterol is observed typically as a peak slightly preceding β-sitosterol (peak 5). Fig. 1 and Table 1 showed that the RRT of 0.97, TMS molecular ion at *m/z* of 484, and the primary fragmentation ions of this sterol corresponded to those reported by Shin et al. (2010), Li et al. (2007), and Beveridge et al. (2002). In a study conducted by Robbins et al. (2011), clerosterol was found to be present in all 10 commonly consumed nuts in the US, including black walnut.

The extracted ion chromatograms with *m/z* 488 and *m/z* 484 allow for separation of the coeluting stigmastanol and Δ^5-avenasterol, which are described as peak 6a (RRT = 1.01) and peak 6b (RRT = 1.01), respectively. As discussed above, the ion at *m/z* 488 (peak 6a) of the coeluting stigmastanol was confirmed by analytical standard. Peak 6b was assigned as Δ^5-avenasterol with an RRT and fragmentation data (Table 1) matching those reported by Li et al. (2007) and Pelillo et al. (2003).

While prior studies showed that it was challenging to resolve these two phytosterols with conventional approaches, such as GC flame ionization detector (FID), our approach successfully distinguished stigmastanol at low concentrations from the coleuted Δ^5-avenasterol by its unique mass to charge ratios *m/z*. Although the fucosterol, isomer of Δ^5-avenasterol, could be the possible candidate, similar to the previous findings by Shin et al. (2010) and Pelillo et al. (2003), we found Δ^5-avenasterol yielded much higher signal ions at *m/z* of 386 and 296 than those of fucosterol. Our results showed that TMS-ether avenasterol produced intense ion signals at *m/z* of 386 and 296, corroborating that the peak was Δ^5-avenasterol.

Peak 8 was assigned as $\Delta^{5,24(25)}$-stigmastadienol (Fig. 1 and Table 1) with an RRT of 1.04, the M^+ ion at *m/z* of 484, and other main fragmentation ions similar to those reported by Gwatidzo et al. (2014) in manketti nut oil, and Li et al. (2007) in sea buckthorn oil, and Beveridge et al. (2002) in ginseng seed oil. This sterol was also detected in English walnut and black walnut oils (Robbins et al., 2011).

Fig. 1 and Table 1 give information about peak 11 which was identified as cycloartenol based on an RRT and mass spectral fragmentation data resembling those reported by Robbins et al. (2011) and Yang et al. (2001). The molecular ion M^+ at *m/z* of 498, two abundant fragment ions at *m/z* of 408 and 393, and the base peak at *m/z* of 69 were found to be associated with the presence of TMS-derived cycloartenol. A minor peak on the leading edge of peak 11 provides mass spectrum data similar to those reported by Yang et al. (2001) and Li et al. (2007). The RRT and mass spectral data suggest this peak was cycloeucalenol.

Peaks 12 and 13 were identified as 28-methylobtusifoliol and 24-methylenecycloartenol (Fig. 1 and Table 1) with RRT's and fragmentation patterns corresponding to those reported in the literature (Yang et al., 2001; Gwatidzo et al., 2014). Their TMS-ether forms characteristically yield molecular ion M^+ at *m/z* 512. The latter was found to be present in black walnut oil (Robbins et al., 2011).

Peak 15 (Fig. 1) which has been detected in oils extracted from ginseng, hazelnut, olive, black walnut, English walnut and other common nuts was assigned as citrostadienol (Beveridge et al., 2002; Benitez-Sánchez et al., 2003; Robbins et al., 2011). Our results showed that the mass spectrum of peak 15 (Table 1) depicts two diagnostic fragment ions at *m/z* 357 (identified as the base peak) and 400, which are in agreement with those described by Pelillo et al. (2003).

Among the peaks demonstrated in this study, there are four peaks which were not identified. They include peak 7, 9, 14, and 16 (Fig. 1) with their fragmentation data described in Table 1, assigned as "unidentified" (Table 1).

3.2. Calibration, sensitivity, precision and recovery

In this study, we constructed calibration curves for four sterol standards (campesterol, stigmasterol, β-sitosterol and stigmastanol) as stated above. The linear relationship was obtained by fortifying 1 mL of 5 to 150 μg/mL mixed standards to achieve the concentration of 7, 14, 35, 71, 106, 142, 212 mg/kg (Supplemental Table S2). The recovery rates of all the spiked phytosterols at the levels of 71 and 142 mg/kg ground black walnut ranged between 92.5 and 103.1%. All the recovery values for the four sterol standards in this study fall within 85–110% as recommended by AOAC (Horwitz, 2002). The LOD, LOQ and precision

Table 2A
Phytosterol contents (mg/kg of kernel, n = 3)[1] in different black walnut varieties.

Black walnuts	campesterol	stigmasterol	β-sitosterol	stigmastanol	Additional sterols	Total phytosterols[2]
Emma K	43.3 ± 4.1^{bc}	< LOD*	1016.9 ± 68.5^{ac}	14.2 ± 2.7^{a}	267.0 ± 10.1	1329.0 ± 78.9^{ab}
Kwik Krop	31.9 ± 2.4^{a}	< LOD	1148.1 ± 56.9^{ac}	15.3 ± 2.6^{ab}	307.8 ± 28.9	1489.9 ± 81.1^{b}
Mystry	41.4 ± 2.8^{bc}	3.1 ± 0.1^{a}	988.3 ± 68.7^{a}	12.3 ± 2.3^{a}	203.2 ± 29.2	1236.0 ± 94.8^{a}
Schessler	47.7 ± 0.9^{c}	< LOD	1223.9 ± 84.3^{c}	20.2 ± 1.4^{bc}	270.6 ± 21.5	1542.3 ± 104.9^{b}
Sparks 147	36.4 ± 0.5^{ab}	3.7 ± 0.1^{ab}	1199.8 ± 109.1^{ac}	16.6 ± 1.0^{ac}	273.7 ± 21.7	1513.6 ± 89.8^{b}
Tomboy	46.3 ± 4.8^{c}	4.0 ± 0.2^{b}	1211.4 ± 86.5^{bc}	21.5 ± 1.2^{c}	261.2 ± 15.8	1522.9 ± 104.5^{b}
English walnut[5]	49.5 ± 3.0^{c}	5.9 ± 0.5^{c}	1007.2 ± 35.7^{ab}	13.9 ± 2.2^{a}	370.0 ± 32.1	1432.7 ± 66.8^{ab}

values for each of the four phytosterols were calculated and shown in Table S2. The LOD and LOQ values ranged from 2.0 to 3.0 and from 6.8 to 11.0 mg/kg, respectively. The repeatability precision expressed as % RSD for the four sterols ranged between 1.3 and 5.4%.

3.3. Phytosterol compositions in black walnut varieties

Thirteen sterols have been successfully identified in our study (Table 1). The mean phytosterol levels in six black walnut varieties and an English walnut (provided by Nuts.com, Inc., New Jersey, USA) are demonstrated in Tables 2A and 2B. The six black walnut varieties varied significantly on the concentrations of the phytosterols ($p < 0.05$). The concentrations of campesterol, stigmasterol, β-sitosterol and stigmastanol were determined using calibration equations developed by analytical standards. The relative concentrations of the additional 9 phytosterols of which we did not have access to their reference standards, were quantified as β-sitosterol equivalents following the similar approach described previously by Laakso (2005). Our results indicated significant variation in the concentrations of campesterol among the varieties ($p < 0.05$). Particularly, Schessler was composed of approximately 50% higher concentration of this sterol as compared to Kwik Krop (Table 2A). Campesterol is believed to inhibit the absorption of cholesterol, and thereby reduce serum cholesterol levels (Moreau et al., 2002). This common phytosterol has also been proposed to play an influential role in respect of protection against colon cancer (Awad and Fink, 2000).

As shown in Table 2A, the level of stigmasterol was found 29% higher in Tomboy than that in Mystry ($p < 0.05$). This sterol was not detected in three black walnuts (Emma K, Kwik Krop, and Schessler) in this study. Along with campesterol, stigmasterol is believed to inhibit cholesterol absorption, and reduce serum cholesterol levels (Moreau et al., 2002). Gabay et al. (2010) pointed out that the sterol produced inhibitory effect on the pro-inflammatory mediator PGE2 (prostaglandin E2) involved in osteoarthritis-induced cartilage degradation, resulting in potential anti-osteoarthritic activity.

Among the phytosterols reported, β-sitosterol with concentrations ranging from 988.3 to 1223.9 mg/kg was found to be the most abundant sterol (76.5–80.0% of the total, see Supplemental Table S3) in all the investigated walnuts. The result is consistent with prior studies showing that β-sitosterol accounted for more than 60% of total sterols in walnuts and other nuts such as almond, cashew, hazelnut, macadamia, pecan, and pistachio (Phillips et al., 2005; Robbins et al., 2011; Abdallah et al., 2015). As demonstrated in Table 2A, Schessler and Tomboy contained significantly higher amounts of β-sitosterol as compared to Mystry ($p < 0.05$). β-sitosterol has shown multiple bioactivities potentially important to human health. This sterol was previously reported to reduce oxidative stress by increasing the activity of antioxidant enzymes (Vivancos and Moreno, 2005). In addition, β-sitosterol may serve as an effective apoptosis-promoting agent, making it a good candidate for use in alleviation of certain cancers such as colon, breast and prostate cancers (Awad and Fink, 2000; Awad et al., 2007).

In addition to the sterols, a stanol, namely stigmastanol, was found in the black walnut samples. The levels of stigmastanol varied among the studied black walnut cultivars ($p < 0.05$), with Tomboy and Schessler containing 75% and 64% higher amounts of this sterol as compared to Mystry (Table 2A). Stigmastanol was proven to possess cholesterol-lowering property (Heinemann et al., 1991). Moreover, those researchers showed that this stanol produced a reduction of intestinal cholesterol absorption by approximately 85% as compared to a 50% decline in cholesterol absorption achieved by β-sitosterol. Based on the understanding of bioactivities and the concentration levels of these four phytosterols in the studied black walnuts, it is presumable that consumption of Tomboy and/or Schessler could be superior to that of Mystry in cholesterol-lowering effect.

As described in Fig. 2, the levels of the four sterols quantitatively

Table 2B
Additional phytosterol contents[3] (n = 3) in different black walnut varieties.

Black walnut	$\Delta^{5,23}$-stigmastadienol	clerosterol	Δ^{5}-avenasterol[4]	$\Delta^{5,24(25)}$-stigmastadienol	cycloeucalenol	cycloartenol	28-methylobtusifoliol	24-methylene-cycloartenol	citrostadienol
Emma K	12.3 ± 0.4^{c}	65.9 ± 2.4^{bc}	68.4 ± 5.7^{c}	30.3 ± 1.2^{d}	5.8 ± 0.6^{bc}	67.0 ± 3.8^{ab}	5.8 ± 0.2^{a}	5.9 ± 0.5^{b}	5.5 ± 0.3^{a}
Kwik Krop	10.1 ± 2.9^{bc}	88.2 ± 6.0^{d}	66.5 ± 13.1^{c}	27.6 ± 2.6^{cd}	3.7 ± 0.7^{ac}	80.7 ± 13.0^{ab}	6.5 ± 1.2^{a}	11.9 ± 1.9^{c}	12.6 ± 2.8^{c}
Mystry	6.4 ± 0.5^{ab}	59.3 ± 8.1^{b}	42.0 ± 8.6^{ab}	19.4 ± 1.3^{b}	2.1 ± 0.2^{ab}	50.9 ± 9.9^{a}	5.1 ± 0.3^{a}	6.6 ± 0.9^{b}	11.3 ± 0.7^{bc}
Schessler	5.9 ± 0.5^{a}	62.4 ± 5.0^{b}	46.1 ± 0.8^{ab}	23.1 ± 1.9^{bc}	7.6 ± 1.4^{c}	93.9 ± 7.5^{b}	13.6 ± 2.3^{b}	12.2 ± 1.7^{c}	5.8 ± 0.6^{ab}
Sparks 147	5.0 ± 0.7^{a}	81.7 ± 7.2^{cd}	60.7 ± 6.3^{bc}	21.2 ± 1.4^{b}	nd	88.7 ± 6.6^{b}	nd	6.1 ± 0.2^{b}	10.5 ± 0.5^{ac}
Tomboy	4.7 ± 2.5^{a}	77.6 ± 11.5^{bd}	68.1 ± 3.4^{c}	21.0 ± 2.0^{b}	3.9 ± 1.2^{ac}	77.0 ± 9.1^{ab}	nd	2.4 ± 0.5^{a}	6.5 ± 1.2^{ab}
English walnut[5]	nd**	6.4 ± 0.7^{a}	37.5 ± 3.8^{a}	8.6 ± 0.2^{a}	24.6 ± 3.8^{d}	273.5 ± 20.5^{c}	nd	nd	19.5 ± 4.3^{d}

Different letters for the same phytosterol indicate significant difference among the varieties ($p < 0.05$).
[1]n is the number of independent original samples; data are shown as mean ± standard deviation.
[2]Not including stigmastanol concentrations individually quantified using its analytical standard.
[3]Relative phytosterol concentrations (mg/kg of kernel) quantified as β-sitosterol equivalents.
[4]Peak area of Δ^{5}-avenasterol is based on the peak consisting of two co-eluting components (stigmastanol and Δ^{5}-avenasterol).
[5]English walnut (*Juglans regia* L.).
*LOD = 3.0 mg/kg.
**indicates no detection based on signal-to-noise ratio of the peak below 3.

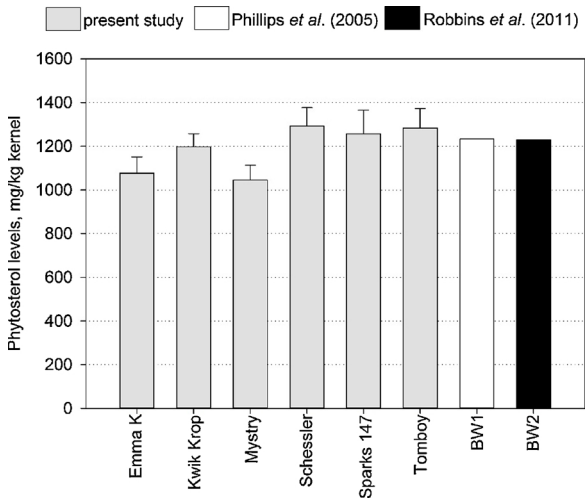

Fig. 2. Comparison of the levels of the four phytosterols analyzed in the present study with those reported in the literature. BW1 and BW2 represent black walnut samples from the prior studies conducted by Phillips et al. (2005) and Robbins et al. (2011), respectively. Phytosterol levels were normalized to mg/kg of black walnut kernels. Error bars indicate standard deviation of the mean.

identified using available analytical standards (i.e., campesterol, stigmasterol, β-sitosterol, and stigmastanol) were compared with the levels reported in the literature. The phytosterol values in the present study range from 1045.1 to 1291.9 mg/kg of kernels, which are comparatively consistent with the results reported by Robbins et al. (2011) (1228 mg/kg) and Phillips et al. (2005) (1233 mg/kg), respectively. No significant differences were noted for the phytosterol levels of Kwik Krop, Schessler, Sparks 147, and Tomboy cultivars as compared to those previously reported by Phillips et al. (2005) and Robbins et al. (2011). In contrast, those of Emma K and Mystry were found to be significantly different from the results presented by Phillips et al. (2005) and Robbins et al. (2011). Moreau et al. (2002) stated that plant sterols exist in the forms of alcohol, fatty acid ester and glycosides. The two latter forms constitute a significant portion of naturally occurring phytosterols in nuts. As suggested by Phillips et al. (2005), a combination of acid hydrolysis and saponification can insure breakdown of the two conjugated forms of phytosterols, giving rise to total extraction of phytosterols. In the present study, all the black walnut samples were subjected to both acid hydrolysis and saponification, yielding the results consistent with those reported by Robbins et al. (2011) and Phillips et al. (2005). Notably, Robbins et al. (2011) used three sterol standards (not including stigmastanol) while Phillips et al. (2005) and the present study employed four sterol standards (as listed above) for quantification of phytosterols. The results from the two latter studies showed that stigmastanol levels in black walnut kernels accounted for negligible proportions (2% and 1.3%, respectively) of the total four phytosterol levels, explaining why the results from these three studies were found to be consistent. Therefore, the comparison of the phytosterols between these studies is completely acceptable.

The results showed that other major sterols including clerosterol, Δ^5-avenasterol, $\Delta^{5,24(25)}$-stigmastadienol, and cycloartenol were found in all the studied black walnut cultivars. These sterols in black walnut kernels were also found in commonly consumed nuts by Robbins et al. (2011). The aforementioned data in the literature indicate that several cytoprotective activities of the plant extracts have been ascribed to clerosterol. For examples, clerosterol naturally occurring in a seaweed species (*Codium fragile*) has been reported to have cytotoxic effects on human melanoma cells (Kim et al., 2013). The results also yielded the evidence of several minor sterols such as $\Delta^{5,23}$-stigmastadienol, cycloeucalenol, 28-methylobtusifoliol and 24-methylenecycloartenol which were found in most of the studied black walnut cultivars. To our knowledge, this is the first time that the former three have been identified and quantified in black walnut kernels.

As demonstrated in Table 2A, the mean levels of total sterols varied between 1236.0 mg/kg (Mystry) and 1542.3 mg/kg (Schessler). The total sterol concentrations of Kwik Krop, Schessler, Sparks 147, and Tomboy were significantly higher than that of Mystry ($p < 0.05$). The significant contribution of β-sitosterol to phytosterol contents in black walnuts offers an explanation for the lowest total phytosterol level in Mystry among all the varieties. Our study also compared mean total phytosterol levels of black walnuts and their English walnut counterpart. Table 2A showed that no significant differences in total sterol contents between the black walnuts and English walnut were noted ($p > 0.05$), corroborating the result presented previously by Robbin et al. (2011). While the English walnut contained significantly higher levels of stigmasterol, cycloeucalenol, cycloartenol, and citrostadienol, its β-sitosterol was not significantly different from those of the black walnuts (except Schessler). This explains why no significant differences in total phytosterol concentrations between the two species of walnut were noted.

Altogether, these phytosterols have received considerable attention due to their potential health promoting properties (Supplemental Table S4). As natural constituents of black walnuts, this class of compounds helps underscore the nutritional and medicinal values of black walnuts and black walnut-containing products.

Phytosterols are isoprenoid-derived compounds that play important roles in plant growth and development (Benveniste, 2004). They are also important in mediating plant stress tolerance (Posé et al., 2009). Interestingly, the fact that β-sitosterol is reportedly the most abundant sterol in black walnuts and many other plants could be attributed to the plant biodefense mechanism in which plants accumulate this compound to combat abiotic stresses such as UV radiation and air pollution (Obata and Fernie, 2012; Wang et al., 2012). It has been demonstrated that conversion between phytosterols occurs under biotic stress (such as pathogens) and abiotic stress (Sun et al., 2010; Wang et al., 2012). This may contribute to explanation of variations in phytosterol compositions among black walnut varieties. Indeed, ecological factors such as water stress (Cohen et al., 1996) and high rainfall (Lynch et al., 2016) were previously identified as having impacts on English walnut and black walnut quality, respectively. Prior study showed walnuts from different countries appeared to exhibit higher variations in phytosterol composition than those with genetic differences (Martínez et al., 2010). This suggests that growing conditions and geographic locations may play more important roles than genetic traits in sterol composition in walnuts. In the present study, all the studied black walnut varieties are grown in the same location as stated above. This could explain that genetic factors might be responsible for the variation in phytosterol composition, in addition to ecological factors.

As shown in Fig. 3, principal component 1 explaining up to 56.28% of the total variance is contributed mainly by stigmasterol, $\Delta^{5,23}$-stigmastadienol, clerosterol, $\Delta^{5,24(25)}$-stigmastadienol, cycloeucalenol, cycloartenol, 24-methylenecycloartenol, and citrostadienol. As discussed earlier, the concentrations of these sterols in the English walnut were found to be significantly different from those in the black walnuts. That expounds the distant positioning of English walnut from the black walnut samples in the PCA graph. Principal component 2 explains 15.39%, and is characterized by 28-methylobtusifoliol. The PCA graph shows 71.67% of the total data variance. It also graphically summarizes the close distribution of Tomboy and Sparks 147, as well as Emma K and Kwik Krop samples, indicating similarities in their phytosterol contents. As seen in Fig. 4, the dendrogram shows four clusters of walnut samples grouped by similarities in phytosterol contents using Ward's algorithm. It is important to note that the dendrogram and the PCA plot convey similar information in different forms. Also, these mathematical tools appear to be useful for differentiation of the black walnut varieties as well as two walnut species based on their phytosterol contents.

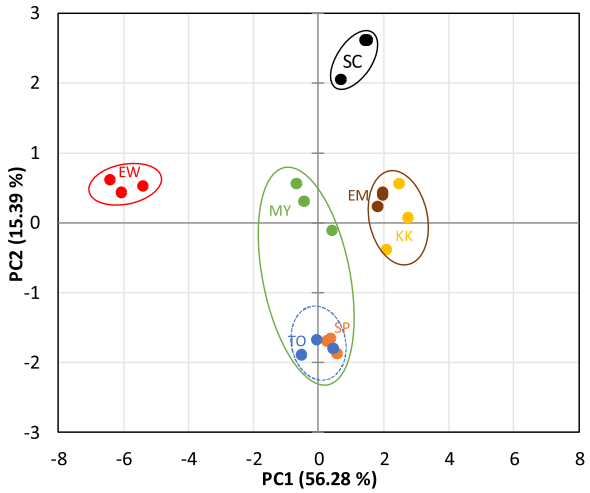

Fig. 3. Principal component analysis of phytosterol data of the studied black walnuts and English walnut. Abbreviations: EM (Emma K), EW (English walnut), KK (Kwik Krop), MY (Mystry), SC (Schessler), SP (Sparks 147), TO (Tomboy).

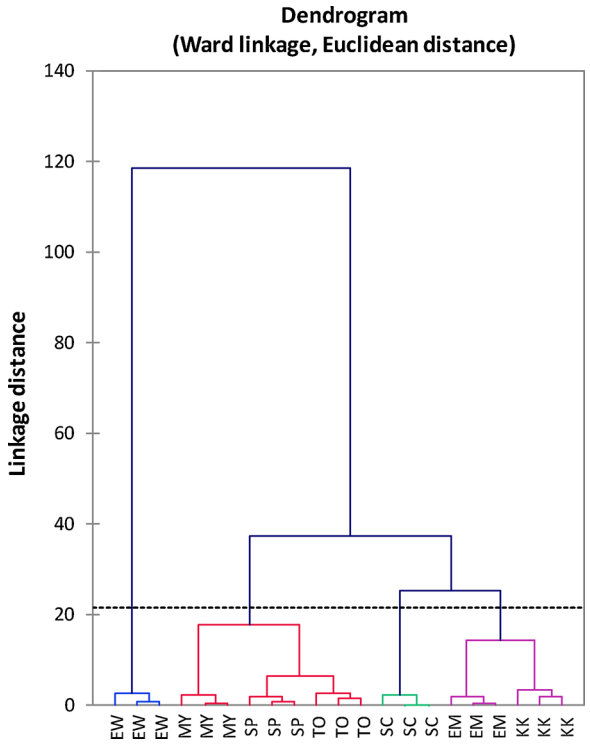

Fig. 4. Dendrogram for the hierarchical cluster analysis of the studied black walnuts and English walnut. Abbreviations: EM (Emma K), EW (English walnut), KK (Kwik Krop), MY (Mystry), SC (Schessler), SP (Sparks 147), TO (Tomboy).

3.4. Untargeted analysis of conjugated phytosterols

In the untargeted analysis, black walnut extracts were analyzed for conjugated phytosterols by using XCMS Online, a web-based application to process untargeted metabolomic data (Tautenhahn et al., 2012). A multi-group job allowed for the comparison of multiple groups of black walnut extracts. We searched for features with intensities > 10,000. Putative identification of glycosylated and esterified phytosterols was carried out by the integration of the METLIN into XCMS Online. The monoisotopic accurate mass (theoretical mass) of a metabolite was searched using the Human Metabolome Database (HMDB) and PubChem database. As summarized in Table 3, the multi-group comparison enabled the detection of five differentially expressed metabolite features (p-value < 0.05, intensity > 10,000) putatively identified as conjugated phytosterols in positive mode. Metabolites were identified on the basis of their accurate mass (mass error ≤ 5 ppm). The conjugated phytosterols were found in the forms of glycosides and hydroxycinnamates (i.e., caffeates and ferulates). Among these, three compounds are the conjugates of campesterol while the other two are cycloartenol derivatives. Significant differences were noted for the relative concentrations of all these conjugated phytosterols (Fig. S1, Supplemental data). To our knowledge, the presence of these compounds has never been reported in black walnuts. Prior studies reported the presence of these phytosterol hydroxycinnamates in rice and rice bran (Akihisa et al., 2000; Fang et al., 2003; Aladedunye et al., 2013; Kim et al., 2015). Particularly, phytosterol ferulates which naturally occur in rice and corn have received considerable attention for their health-promoting properties, such as cholesterol lowering effects, prevention of lifestyle-related diseases, anti-inflammatory and anticancer properties (Akihisa et al., 2000; Berger et al., 2005; Luo et al., 2005; Islam et al., 2009). In the aforementioned works, the abundant presence of conjugated phytosterols and small amount of free phytosterols in plant species were underscored. Identification of the conjugates has been difficult due to lack of commercially available standards. Recently, the web-based platform XCMS Online, which is equipped with an enormous database (METLIN) consisting of more than 960,000 metabolites, has been released to perform untargeted global metabolomics analysis, facilitating the tentative identification of conjugated phytosterols. As summarized by Sumner et al. (2007), there are four levels of metabolite identifications which can be found in the published metabolomics literature. In the present study, the putatively annotated compounds were reported based upon the similarities of their mass spectra with public spectral libraries (METLIN, HMDB, and PubChem) without the use of chemical reference standards, denoting that the identification level is 2.

4. Conclusions

The phytosterol compositions of black walnuts were successfully assessed using GC–MS based analytical method in our study. Totally, 13 phytosterols in six different black walnut varieties (Emma K, Kwik Krop, Mystry, Sparks 147, Schessler, Tomboy) have been identified and quantified. The six black walnut varieties and English walnut varied significantly on the concentrations of 13 phytosterols. β-sitosterol was found to be the most abundant sterol in all the investigated nuts. Several minor sterols, including $\Delta^{5,23}$-stigmastadienol, cycloeucalenol and 28-methylobtusifoliol, were identified and quantified in black walnut kernels for the first time. Total phytosterol concentrations of all the black walnuts were not significantly different from that of the English walnut used in our study. The PCA revealed more than 71% of the variance in the phytosterol data. The HCA classified the walnut samples into four groups based on phytosterol contents. The use of UPLC-MS and XCMS Online platform for untargeted global metabolomics analysis facilitated the tentative identification of glycosylates and hydroxycinnamates of phytosterols to differentiate black walnut extracts. Most of the phytosterols and their conjugates identified in the black walnuts have received considerable attention for their potential health-promoting properties. Future studies should be focused on seasonal and geographic variations in phytosterol compositions of black walnuts, and bioassay-guided purification to assess the potential health-promoting activities of these phytosterols in black walnuts.

Conflict of interest

The authors declare no conflict of interest.

Table 3
Conjugated phytosterols tentatively identified in black walnut extracts.

Putative identification	Retention time, min	Formula	Adducts	Theoretical mass	Observed mass	Δm, ppm	ID database
Campesteryl caffeate	30.92	$C_{37}H_{54}O_4$	M + H–H_2O	545.3989	545.4002	2.38	METLIN91429 HMDB36284
Caffeoylcycloartenol	30.95	$C_{39}H_{56}O_4$	M + H	589.4251	589.4263	2.04	METLIN95271 HMDB40807 PubChem102444973
Campesteryl glucoside	34.55	$C_{34}H_{58}O_6$	M + H–H_2O	545.4201	545.4191	−1.83	METLIN103408 PubChem70699334
Campesteryl ferulate	36.07	$C_{38}H_{56}O_4$	M + H–H_2O	559.4145	559.4146	−0.18	METLIN91430 HMDB36285 PubChem15056832
Cycloartanyl ferulate	36.07	$C_{40}H_{60}O_4$	M + H–H_2O	587.4459	587.4477	3.06	METLIN91440 HMDB36295 PubChem59271038

Acknowledgment

The authors would like to thank the Center for Agroforestry, University of Missouri for supporting this research.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2018.09.016.

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