

# Identification and Characterization of Phenolic Compounds in Black Walnut Kernels

Danh C. Vu,<sup>†,‡,§</sup> Phuc H. Vo,<sup>†</sup> Mark V. Coggeshall,<sup>§</sup> and Chung-Ho Lin<sup>\*,†,‡</sup>

<sup>†</sup>Center for Agroforestry, School of Natural Resources and <sup>‡</sup>Department of Forestry, School of Natural Resources, University of Missouri, Columbia, Missouri 65211, United States

<sup>§</sup>Hardwood Tree Improvement and Regeneration Center, United States Department of Agriculture, Forest Service, Northern Research Station, 715 State Street, West Lafayette, Indiana 47907, United States

## Supporting Information

**ABSTRACT:** Black walnuts (*Juglans nigra* L.) are highly valued for producing phenolic-enriched nuts. The objectives of this study were to identify and characterize the phenolic contents of 11 different black walnut cultivars and compare the levels of these phenolics between black walnuts and English walnut (*Juglans regia* L.). Totally, 16 phenolics including phenolic acids, flavonoids, and catechins were identified in the black walnut kernels, with ellagic acid predominating over the other phenolics. Significant differences were noted for the levels of quinic acid, gallic acid, 1,3,6-trigalloylglucose, catechin, and penta-*O*-galloyl- $\beta$ -D-glucose between the studied black walnuts and English walnut. Through principal component analysis, 51.54% of the variance in the phenolic data was explained. The hierarchical cluster analysis results showed three groups to which each walnut sample belongs. Most of the phenolics identified in this study have been reported to exert potential health-promoting activities. The findings of this study will provide critical information for consumers, nutritional therapy practitioners, researchers, and producers.

**KEYWORDS:** black walnut, English walnut, phenolic compounds, ellagic acid, health-promoting

## INTRODUCTION

Black walnut (*Juglans nigra* L.), belonging to the walnut family (Juglandaceae), is a hardwood tree species native to the central and eastern United States. Black walnut is valued for high-quality wood which is used throughout the world in making furniture, cabinet-work, interior woodwork, and flooring.<sup>1,2</sup> The tree also produces nuts with distinctive flavor. Black walnut is used all over the USA in ice cream, candies, and baked goods. It is believed that black walnut kernel contains a diverse phytochemical profile.<sup>3</sup> Among the phytochemicals, phenolic compounds have attracted considerable attention for their potential health-promoting activities. Phenolic compounds belong to one of the most widely occurring groups of phytochemicals. They have been categorized into different subclasses such as phenolic acids, flavonoids, stilbenes, coumarins, and tannins. Phenolic compounds are of possible importance for human health due to their antioxidant, antibacterial, antifungal, anti-inflammatory, antiaging, anticarcinogenic, and neuroprotective effects.<sup>4,5</sup> Antioxidant activity is regarded as a fundamental property important for life. Biological functions such as antimutagenicity, anticarcinogenicity, and antiaging arise from this property.<sup>6,7</sup> For example, phenolic antioxidants counter the damaging effects of reactive oxygen species (ROS) by suppressing multiple ROS-associated process steps in metabolite pathways in living cells, resulting in a reduced risk of certain cancers, such as skin and prostate cancers.<sup>8</sup> In addition, the presence of ROS initiates oxidative stress which is involved in modulating skin alteration due to aging or UV exposure. Treatment with antioxidants such as phenolic compounds helps improve endogenous oxidative stress-eliminating systems, resulting in prevention of skin aging.<sup>9</sup>

Prior research into phenolic compounds in black walnuts reported the presence of caffeoylquinic acids and a few glycosylated forms of quercetin in acetone and methanolic extracts.<sup>10</sup> The authors showed that the black walnut extracts exhibit in vitro antioxidant capacity due to these flavonol components. To our knowledge, analytical data about phenolic composition in black walnuts are very limited while those of English walnuts (*Juglans regia* L.) abound in the literature. As a result, attempts have been made to address potential health benefits associated with black walnut phytochemical content through extrapolation from those of English walnuts.<sup>3</sup>

Despite the growing recognition of nutritional and medicinal values of black walnuts, limited information has been documented about their phenolic compositions. Furthermore, researchers neither compare the phenolic profiles between the cultivars nor specify black walnut cultivars selected for their research. The studied samples were often described as the generic black walnuts collected from various grocery stores. That could result in ambiguous scientific information about the phenolic profiles and their concentrations.

To date, there has not been any study that systematically characterizes and compares the phenolic profiles among black walnut cultivars. Thus, the aim of this study was to gain further insight into the phenolic composition of black walnut kernels through identification and characterization of phenolic composition of 11 different black walnut cultivars. Systematic characterization of phenolic compounds with potential health-

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**Table 1. Retention Times, Molecular and Product Ions, Linear Correlation Coefficients, LOD, and LOQ of the Screened Phenolic Compounds**

Compound	Retention time, min	Molecular ion $[M - H]^-$ , $m/z$	Product ions $MS^2$ , $m/z$	Linear equation <sup>a</sup>	Correlation coefficient $R^2$	LOD, <sup>b</sup> $\mu\text{g/g}$	LOQ, <sup>c</sup> $\mu\text{g/g}$
Quinic acid	2.07	191	85	$y = 44008x$	0.994	0.38	1.25
Gallic acid	2.12	169	125	$y = 43081x$	0.983	0.38	1.28
Neochlorogenic acid	2.12	353	191, 179	$y = 53274x$	0.992	3.68	12.25
Procyanidin B1	5.86	577	425, 407, 289	$y = 8969x$	0.993	4.42	14.73
Chlorogenic acid	5.92	353	191	$y = 52360x$	0.999	3.68	12.25
Procyanidin B2	6.11	577	425, 289	$y = 2128x$	0.998	4.42	14.73
(+)-Catechin	6.30	289	205, 151, 109	$y = 12024x$	0.996	0.32	1.06
1,3,6-Trigalloylglucose	6.33	635	na <sup>d</sup>	$y = 2819x$	0.996	5.04	16.81
<i>p</i> -Hydroxybenzoic acid	6.34	137	93	$y = 192315x$	0.999	0.91	3.04
(-)-Epicatechin	6.37	289	205, 151, 109	$y = 21066x$	0.994	1.06	3.54
Vanillic acid	6.54	167	123	$y = 4984x$	0.990	6.38	21.28
Syringic acid	6.60	197	na	$y = 3203x$	0.977	6.19	20.64
Caffeic acid	6.65	179	135	$y = 373017x$	0.971	0.21	0.69
Rutin	6.67	609	301	$y = 2360x$	0.967	1.24	4.12
Penta- <i>O</i> -galloyl- $\beta$ -D-glucose	6.75	939	na	$y = 3291x$	0.983	6.38	21.28
Quercetin-3- $\beta$ -D-glucoside	6.88	463	300, 301	$y = 23215x$	0.977	1.06	3.54
(-)-Epicatechin gallate	6.89	441	331, 289, 271	$y = 3051x$	0.996	1.85	6.18
Ellagic acid	7.10	301	229	$y = 22166x$	0.990	0.77	2.55
Naringin	7.15	579	271	$y = 27832x$	0.991	0.21	0.69
<i>p</i> -Coumaric acid	7.24	163	119	$y = 212184x$	0.997	0.14	0.47
Ferulic acid	7.26	193	178, 134	$y = 62111x$	0.998	0.18	0.61
Resveratrol	8.00	227	143, 159, 185	$y = 27501x$	0.986	0.15	0.49
Quercetin	8.37	301	179, 151, 106	$y = 320950x$	0.980	0.26	0.85
Cinnamic acid	8.51	147	103	$y = 7178x$	0.999	2.61	8.70
Chrysin	9.80	253	209	$y = 106699x$	0.994	2.51	8.38

<sup>a</sup>Linear equations represent the relationship between phenolic peak area and concentration ( $\mu\text{g/mL}$ ) in standard solutions. <sup>b</sup>LOD: limit of detection. <sup>c</sup>LOQ: limit of quantitation. <sup>d</sup>na: compounds were analyzed in SIR (single ion recording) mode.

promoting properties will not only help identify new industrial applications of the black walnuts but also give a better understanding of how this class of chemicals contributes to prevention and treatment of diseases.

## MATERIALS AND METHODS

**Sample Preparation.** The black walnut samples used in this study include 11 cultivars (Daniel, Davidson, Emma K, Hay, Jackson, Kwik Krop, Mystry, Sparks 147, Schessler, Surprise, Tomboy). They were all collected from the trees grown at the University of Missouri Horticulture and Agroforestry Research Center in New Franklin, Missouri, the USA. 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 2 weeks. After curing, the unshelled nuts were stored in freezer ( $-20\text{ }^{\circ}\text{C}$ ) until analysis. The English walnut samples were purchased from Nuts.com, Inc. located in New Jersey, USA. On the day of analysis, each nut cultivar was shelled to obtain the kernel which was afterward ground in a commercial coffee grinder (Black & Decker) to 20–40 mesh sizes.

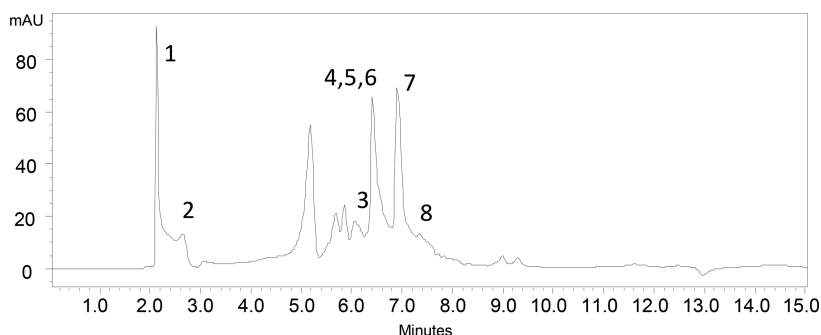
**Chemicals and Reagents.** Analytical phenolic standards (gallic acid, ellagic acid, *p*-coumaric acid, ferulic acid, chlorogenic acid, neochlorogenic acid, (+)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2, quercetin, quercetin-3-*O*-glucoside, cinnamic acid, *p*-hydroxybenzoic acid, naringin, chrysin, rutin, caffeic acid, syringic acid, 1,3,6-trigalloylglucose, penta-*O*-galloyl- $\beta$ -D-glucose hydrate, (-)-epicatechin gallate, quinic acid, vanillic acid, resveratrol) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol (HPLC-grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA).

**Analysis of Phenolic Composition. Extraction of Phenolic Compounds.** Each ground black walnut sample (2.5 g) was extracted with 15 mL of methanol in a capped test tube placed in a cooled water

bath ( $4\text{ }^{\circ}\text{C}$ ). The mixture was sonicated for 60 min and then centrifuged for 10 min at 8,000 rpm (9100g). The supernatant was filtered through a  $0.2\text{ }\mu\text{m}$  Whatman Anotop filter (GE Healthcare, Germany) and transferred to an LC vial prior to injection into HPLC-MS/MS.

**LC-MS/MS Analysis.** The concentration of each phenolic compound was determined using a Waters Alliance 2695 High Performance Liquid Chromatography system coupled with a Waters Acquity TQ triple quadrupole mass spectrometer (HPLC-MS/MS). The phenolic compounds were chromatographically separated by a Phenomenex (Torrance, CA, USA) Kinetex C18 ( $100\text{ mm} \times 4.6\text{ mm}$ ;  $2.6\text{ }\mu\text{m}$  particle size) reverse-phase column. The mobile phase consisted of 10 mM ammonium acetate and 0.1% formic acid in water (A) and 100% acetonitrile (B). The gradient conditions were 0–0.5 min, 2% B; 0.5–7 min, 2–80% B; 7.0–9.0 min, 80–98% B; 9.0–10.0 min, 2% B; 10.0–15.0 min, 2% B at a flow rate of  $0.5\text{ mL/min}$ . The MS/MS system was operated using electrospray ionization (ESI) in the negative ion mode with capillary voltage of 1.5 kV. The ionization source was programmed at  $150\text{ }^{\circ}\text{C}$ , and the desolvation temperature was programmed at  $750\text{ }^{\circ}\text{C}$ . The MS/MS system was operated in the multiple reaction monitoring (MRM) modes, and the collision energy was 30 eV. The molecular ions were screened, and the product ions used for the quantifications were determined from the spectra obtained from injecting  $30\text{ }\mu\text{L}$  of a  $10\text{ }\mu\text{g/mL}$  solution of the analytical standards.

The MS/MS system was operated in the MRM mode with the optimized collision. The ionization energy, MRM and SIR (single ion recording) transition ions (molecular and product ions), capillary and cone voltage, desolvation gas flow, and collision energy were optimized by a Waters IntelliStart optimization software package. Analytical data were processed using Waters Empower 3 software (Waters, CA, USA).



**Figure 1.** An HPLC-PDA chromatogram of black walnut (the cultivar of Mystry) extract recorded at 280 nm. The peak numbers in the chromatogram refer to the following components: 1 = quinic acid, 2 = gallic acid, 3 = 1,3,6-trigalloyl glucose, 4 = vanillic acid, 5 = syringic acid, 6 = rutin, 7 = ellagic acid, 8 = naringin.

**Calibration, Sensitivity, and Recovery.** Calibration curves were constructed using seven concentrations (0.1, 0.5, 1, 5, 10, 25, 50  $\mu\text{g/mL}$ ) of the phenolic standards. Assessment of the sensitivity of the analytical method was conducted by calculating the limit of detection (LOD) and limit of quantification (LOQ). Signal-to-noise ratios of three and ten were employed to calculate the LOD and LOQ for each phenolic compound, respectively. The extraction efficiencies of the extraction procedure were determined based on the extraction recover rates of the fortified internal standards colchicine and  $\beta$ -naphthylsulfate. For the extraction procedure, 100  $\mu\text{g}$  of each internal standard was fortified. The recovery rates were greater than 90%.

**Statistical Analysis.** The chromatographic data obtained were processed with a Waters Empower 3 (Waters, MA, USA). One-way analysis of variance (ANOVA) was performed on the quantitative data generated from the analysis in order to determine the variation of the phenolic concentrations between black walnut varieties and English walnut. Comparisons of means were drawn using the XLSTAT program (XLSTAT Premium 19.5, Addinsoft, Paris, France). Multiple comparisons were determined using the Tukey's Studentized Range HSD test at a significance level of  $p < 0.05$ .

Principal component analysis (PCA) as a statistical procedure was used to investigate patterns in phenolic data and to highlight similarities and dissimilarities in phenolic contents of walnut cultivars. Hierarchical cluster analysis (HCA) was performed to identify relative similarity among walnut samples, and the results were graphically presented as a dendrogram. Sample similarities in the dendrogram were calculated on the basis of the Euclidean distance between the cultivars using Ward's hierarchical agglomerative method. The PCA and HCA were implemented using the XLSTAT program.

## RESULTS

**Identification of Phenolic Compounds.** In total, we have screened for 25 phenolic compounds (Table 1). The results of retention times, molecular ions, characteristic fragment ions, optimized source condition, and MRM transition are summarized in Table S1 (see Supporting Information). The optimized ionization parameters for a representative phenolic compound (naringin) are shown in Figure S1 and S2 (Supporting Information). All the monitored phenolic compounds produced symmetric peaks with widths less than 0.4 min, and they were eluted within the first 10 min. The retention time of quinic acid was as early as 2.07 min while chrysin was the last compound to elute (at 9.80 min).

**Calibration and Sensitivity.** As demonstrated in Table 1, the linear relationship successfully fitted every calibration curve over the concentration range (0.1–50  $\mu\text{g/mL}$ ). In order to illustrate the results of calibration, the calibration curve for a representative phenolic compound (naringin) is shown in Figure S3 (Supporting Information). The developed calibration equation for every compound in the study had correlation

coefficients ( $R^2$ ) greater than or equal to 0.990, except for resveratrol, rutin, caffeic acid, gallic acid, syringic acid, quercetin-3- $\beta$ -D-glucoside, and penta-O-galloyl- $\beta$ -D-glucose with  $R^2$  ranging from 0.967 to 0.986 (Table 1). The LOD and LOQ values for each of the phenolic compounds were calculated and shown in Table 1. The LOD and LOQ values ranged from 0.14 to 6.38, and from 0.47 to 21.28  $\mu\text{g/g}$ , respectively.

**Phenolic Compositions in Black Walnut Cultivars.** In total, 16 phenolic compounds were successfully identified and quantified in the studied black walnut kernels in our study. The HPLC-PDA chromatograms of standards of these phenolic compounds recorded at 220, 280, and 330 nm are demonstrated in Figure S4 (Supporting Information). Quinic acid eluting at 2.07 min produced molecular ion at  $m/z$  191  $[\text{M}-\text{H}]^-$  with the predominant fragmented product ion at  $m/z$  85. Gallic acid (RT = 2.12) displayed the deprotonated molecule at  $m/z$  169 fragmenting on  $\text{MS}^2$  to yield at  $m/z$  125 due to a loss of  $\text{CO}_2$  (–44 Da). Two phenolic acids which are *p*-hydroxybenzoic acid (RT = 6.34) and vanillic acid (RT = 6.54) with the respective molecular ions at  $m/z$  137 and 167 produced  $\text{MS}^2$  fragment ions at  $m/z$  93 and 123, respectively, due to loss of  $\text{CO}_2$  (–44 Da) from their molecular ions. The catechin epimers, namely (+)-catechin and (–)-epicatechin, coeluting at 6.35 min, presented the same  $m/z$  289 for their molecular ions. The same product ions at  $m/z$  205, 151, and 109 were observed for both molecular ions. Two gallic acid esters of glucose, namely 1,3,6-trigalloylglucose (RT = 6.33) and penta-O-galloyl- $\beta$ -D-glucose (RT = 6.75), with  $[\text{M}-\text{H}]^-$  at  $m/z$  635 and 939, respectively, were analyzed in SIR mode. Similarly, syringic acid (RT = 6.60) with  $[\text{M}-\text{H}]^-$  at  $m/z$  197 was identified in SIR mode. The compound eluting at 7.11 min presented its deprotonated molecule at  $m/z$  301, yielding characteristic ions at  $m/z$  229 upon dissociation. The flavonoid glycoside (rutin) eluting at 6.67 min demonstrated molecular ion at  $m/z$  609 and product ion at  $m/z$  301. Its  $\text{MS}^2$  spectrum showed the ion at  $m/z$  301, indicating a loss of rutinoside. Another flavonoid glycoside, namely quercetin-3- $\beta$ -D-glucoside, eluting at 6.88 min, produced the deprotonated molecule at  $m/z$  463. The product ion spectrum presented the ion at  $m/z$  301, corresponding to a glucoside loss. The compound eluting at 7.10 min produced  $[\text{M}-\text{H}]^-$  at  $m/z$  579 which fragmented on  $\text{MS}^2$  to the ion at  $m/z$  271 due to a loss of rhamnoglycoside, identified as naringin (Figure S5, Supporting Information). Two coeluting hydroxycinnamic acids, namely ferulic acid (RT = 7.24) and *p*-coumaric acid (RT = 7.26), displayed the same fragmentation pattern with a  $\text{CO}_2$  loss, resulting in the



Table 2. Phenolic Contents ( $\mu\text{g/g}$  of Dry Weight Kernel,  $n = 3$ )<sup>a</sup> in Different Black Walnut Cultivars

Black walnuts	Quinic acid	Gallic acid	1,3,6-Trigalloylglucose	<i>p</i> -Hydroxy-benzoic acid	Catechin <sup>b</sup>	Vanillic acid	Syringic acid	Rutin
Daniel	4.69 ± 0.20 <sup>d</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	7.26 ± 1.34 <sup>b</sup>	<LOD <sup>a</sup>
Davidson	1.07 ± 0.08 <sup>a</sup>	0.56 ± 0.08 <sup>b</sup>	<LOD <sup>a</sup>	1.38 ± 0.10 <sup>b</sup>	<LOD <sup>a</sup>	7.20 ± 1.64 <sup>b</sup>	14.26 ± 1.37 <sup>c</sup>	<LOD <sup>a</sup>
Emma K	7.83 ± 0.42 <sup>f</sup>	0.47 ± 0.10 <sup>b</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	7.56 ± 2.60 <sup>b</sup>	1.63 ± 0.12 <sup>b</sup>
Hay	2.35 ± 0.08 <sup>b</sup>	1.37 ± 0.16 <sup>d</sup>	7.15 ± 0.52 <sup>b</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	9.92 ± 2.64 <sup>b</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>
Jackson	1.42 ± 0.21 <sup>a</sup>	0.72 ± 0.17 <sup>bc</sup>	7.71 ± 1.93 <sup>bc</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	8.66 ± 1.69 <sup>b</sup>	7.69 ± 1.35 <sup>b</sup>	<LOD <sup>a</sup>
Kwik Krop	4.21 ± 0.18 <sup>cd</sup>	0.52 ± 0.03 <sup>b</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	1.65 ± 0.62 <sup>b</sup>
Mystery	2.39 ± 0.31 <sup>b</sup>	4.29 ± 0.34 <sup>f</sup>	11.41 ± 2.17 <sup>c</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	6.90 ± 1.16 <sup>b</sup>	9.49 ± 2.14 <sup>bc</sup>	4.18 ± 1.33 <sup>c</sup>
Schessler	3.72 ± 0.10 <sup>c</sup>	0.49 ± 0.05 <sup>b</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	1.32 ± 0.56 <sup>b</sup>
Sparks 147	2.53 ± 0.41 <sup>b</sup>	3.26 ± 0.83 <sup>e</sup>	9.38 ± 2.34 <sup>bc</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	6.43 ± 1.88 <sup>b</sup>	<LOD <sup>a</sup>
Surprise	3.88 ± 0.47 <sup>c</sup>	1.01 ± 0.03 <sup>cd</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	0.59 ± 0.01 <sup>c</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>
Tomboy	2.86 ± 0.08 <sup>b</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	0.48 ± 0.01 <sup>b</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	2.40 ± 0.95 <sup>b</sup>
English walnut	6.77 ± 0.43 <sup>e</sup>	8.05 ± 0.68 <sup>g</sup>	38.20 ± 2.88 <sup>d</sup>	1.21 ± 0.50 <sup>b</sup>	47.91 ± 3.45 <sup>d</sup>	7.32 ± 1.80 <sup>b</sup>	7.25 ± 2.20 <sup>b</sup>	2.68 ± 0.27 <sup>b</sup>
Black walnuts	PGDG <sup>c</sup>	QDG <sup>d</sup>	(-)-Epicatechin gallate	Ellagic acid	Naringin	Ferulic acid	<i>p</i> -Coumaric acid	
Daniel	<LOD <sup>a</sup>	<LOD <sup>a</sup>	13.22 ± 0.48 <sup>f</sup>	30.4 ± 1.03 <sup>bc</sup>	0.47 ± 0.10 <sup>bd</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	
Davidson	<LOD <sup>a</sup>	<LOD <sup>a</sup>	4.61 ± 0.38 <sup>cd</sup>	51.62 ± 1.98 <sup>de</sup>	1.28 ± 0.32 <sup>e</sup>	<LOD <sup>a</sup>	0.26 ± 0.00 <sup>bc</sup>	
Emma K	<LOD <sup>a</sup>	1.78 ± 0.21 <sup>bc</sup>	2.26 ± 0.48 <sup>b</sup>	48.74 ± 2.91 <sup>d</sup>	0.70 ± 0.04 <sup>cd</sup>	0.89 ± 0.13 <sup>c</sup>	0.19 ± 0.03 <sup>b</sup>	
Hay	<LOD <sup>a</sup>	3.20 ± 0.13 <sup>d</sup>	7.01 ± 0.60 <sup>e</sup>	40.54 ± 5.86 <sup>c</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	0.24 ± 0.03 <sup>bc</sup>	
Jackson	<LOD <sup>a</sup>	1.55 ± 0.19 <sup>bc</sup>	3.60 ± 0.66 <sup>bc</sup>	61.07 ± 3.65 <sup>ef</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	0.22 ± 0.03 <sup>bc</sup>	
Kwik Krop	<LOD <sup>a</sup>	<LOD <sup>a</sup>	<LOD <sup>a</sup>	11.35 ± 1.96 <sup>a</sup>	0.32 ± 0.10 <sup>b</sup>	0.74 ± 0.04 <sup>bc</sup>	0.21 ± 0.05 <sup>bc</sup>	
Mystery	15.24 ± 2.52 <sup>b</sup>	2.10 ± 0.27 <sup>c</sup>	1.95 ± 0.22 <sup>b</sup>	65.07 ± 4.77 <sup>f</sup>	0.54 ± 0.21 <sup>bd</sup>	0.61 ± 0.06 <sup>b</sup>	0.29 ± 0.02 <sup>bc</sup>	
Schessler	<LOD <sup>a</sup>	1.02 ± 0.21 <sup>b</sup>	<LOD <sup>a</sup>	18.97 ± 3.23 <sup>ab</sup>	0.39 ± 0.05 <sup>bc</sup>	1.25 ± 0.11 <sup>d</sup>	0.24 ± 0.06 <sup>bc</sup>	
Sparks 147	7.88 ± 1.29 <sup>c</sup>	4.06 ± 0.93 <sup>d</sup>	3.82 ± 1.03 <sup>bc</sup>	63.89 ± 4.40 <sup>f</sup>	0.80 ± 0.08 <sup>d</sup>	1.29 ± 0.15 <sup>d</sup>	0.29 ± 0.04 <sup>bc</sup>	
Surprise	<LOD <sup>a</sup>	1.78 ± 0.29 <sup>bc</sup>	6.02 ± 0.68 <sup>de</sup>	72.05 ± 8.33 <sup>fg</sup>	0.83 ± 0.13 <sup>d</sup>	4.85 ± 0.08 <sup>e</sup>	0.31 ± 0.07 <sup>cd</sup>	
Tomboy	<LOD <sup>a</sup>	<LOD <sup>a</sup>	2.72 ± 0.22 <sup>bc</sup>	9.05 ± 1.69 <sup>a</sup>	0.52 ± 0.04 <sup>bd</sup>	<LOD <sup>a</sup>	0.20 ± 0.01 <sup>b</sup>	
English walnut	58.55 ± 7.71 <sup>d</sup>	3.67 ± 0.23 <sup>d</sup>	4.93 ± 1.50 <sup>cd</sup>	98.41 ± 20.58 <sup>g</sup>	0.30 ± 0.04 <sup>b</sup>	0.94 ± 0.14 <sup>cd</sup>	0.48 ± 0.09 <sup>d</sup>	

<sup>a</sup> $n$  is the number of independent original samples; data are shown as mean  $\pm$  standard deviation. Different letters for the same phenolic compound indicate significant difference among the cultivars ( $p < 0.05$ ). <sup>b</sup>Catechin = (+)-catechin + (-)-epicatechin. <sup>c</sup>Penta-O-galloyl- $\beta$ -D-glucose. <sup>d</sup>Quercetin-3- $\beta$ -D-glucoside.

fragment ions  $[\text{M} - \text{CH}_3 - \text{CO}_2 - \text{H}]^-$  at  $m/z$  134 and  $[\text{M} - \text{CO}_2 - \text{H}]^-$  at  $m/z$  119, respectively. An HPLC-PDA chromatogram of phenolic compounds detected in a black walnut extract is demonstrated in Figure 1.

The phenolic levels in the black walnut cultivars are demonstrated in Table 2. Quinic acid and ellagic acid were detected in all the samples. As shown in Table 2, ellagic acid with mean concentrations ranging from 9.05 to 72.05  $\mu\text{g/g}$  is the dominant phenolic compound. Surprise, Sparks 147, and Mystry contained significantly higher levels of ellagic acid as compared to the other cultivars except Jackson ( $p < 0.05$ ). The concentration of quinic acid in Emma K (7.83  $\pm$  0.42  $\mu\text{g/g}$ ) was found to be the highest among the studied cultivars. Davidson and Jackson ranked the lowest for concentrations of quinic acid. Our study indicates the presence of three hydroxybenzoic acids, *p*-hydroxybenzoic acid, vanillic acid, and syringic acid. The former was reported only in Davidson (1.38  $\pm$  0.10  $\mu\text{g/g}$ ) while both the two latter were detected in several cultivars such as Davidson, Jackson, and Mystry. As seen in Table 2, no significant differences in the concentrations of vanillic acid were found among the four cultivars containing this compound ( $p > 0.05$ ). The level of syringic acid was significantly higher in Davidson (14.26  $\pm$  1.37  $\mu\text{g/g}$ ) as compared to the other cultivars except Mystry ( $p < 0.05$ ). Two important hydroxycinnamic acids, ferulic acid and *p*-coumaric acid, were reported in our study. The concentration of ferulic acid in Surprise (4.85  $\pm$  0.08  $\mu\text{g/g}$ ) was found to be the highest among the studied cultivars ( $p < 0.05$ ). *p*-Coumaric acid was detected in all the black walnuts except Daniel. Our results demonstrated the presence of two gallic acid esters of glucose,

namely 1,3,6-trigalloylglucose and penta-O-galloyl- $\beta$ -D-glucose (PGDG). The trigallic acid ester of glucose was detected in four black walnut cultivars (i.e., Hay, Jackson, Mystry, and Sparks 147) with the mean concentration values ranging from 7.15 to 11.41  $\mu\text{g/g}$ . The pentagallic acid ester of glucose was found only in Mystry (15.24  $\pm$  2.52  $\mu\text{g/g}$ ) and Sparks 147 (7.88  $\pm$  1.29  $\mu\text{g/g}$ ). The cultivar of Mystry also contained significantly higher levels of gallic acid (4.29  $\pm$  0.34  $\mu\text{g/g}$ ) and rutin (4.18  $\pm$  1.33  $\mu\text{g/g}$ ) as compared to the other cultivars. Along with rutin, two other glycosylated phenolic compounds, namely naringin and quercetin-3- $\beta$ -D-glucoside, are reported in this study. A representative LC-MS/MS chromatogram of naringin is shown in Figure S6 (Supporting Information). Davidson was found to contain the highest level of naringin (1.28  $\pm$  0.32  $\mu\text{g/g}$ ). Hay and Sparks 147 were the two black walnuts with significantly higher levels of quercetin-3- $\beta$ -D-glucoside as compared to the other cultivars ( $p < 0.05$ ). As demonstrated in Table 2, catechin, which is representative of the two coeluting flavanols, namely (+)-catechin + (-)-epicatechin, was found only in Surprise and Tomboy with significantly different concentrations ( $p < 0.05$ ). The other flavanol, which is (-)-epicatechin gallate, was found to be significantly greater in Daniel than in the other cultivars ( $p < 0.05$ ). In general, the 11 black walnut cultivars significantly differed on the concentrations of 16 phenolic compounds ( $p < 0.05$ ). Our study also compared phenolic compositions of black walnuts and English walnut. Table 2 showed that English walnut contains significantly higher concentration levels of quinic acid, gallic acid, 1,3,6-trigalloylglucose, catechin, and PGDG as compared to the black walnut cultivars ( $p < 0.05$ ). For ellagic acid, the

Table 3. Comparison of Concentrations of Phenolic Compounds ( $\mu\text{g/g}$  Walnut Kernel)<sup>a</sup> between Different Studies

Compounds	Colaric et al. (2005)	Slatnar et al. (2015)	Figueroa et al. (2017)	The present study (2017)	
				Black walnuts	English walnut
Quinic acid	na <sup>b</sup>	na	na	1.07–4.69	6.77
Gallic acid	na	5.3–9.6	115.8–219.2	0.47–4.29	8.05
1,3,6-Trigalloyl glucose	na	na	na	7.15–11.41	38.20
<i>p</i> -Hydroxybenzoic acid	na	342.6–525.2 <sup>c</sup>	na	1.38	1.21
Catechin	na	44.2–62.8	14.8–82.0	0.48–0.59	47.91
Vanillic acid	na	na	na	6.90–9.92	7.32
Syringic acid	165.7–574.5	na	151.9–445.9	6.43–14.26	7.25
Rutin	na	na	na	1.63–4.18	2.68
Penta- <i>O</i> -galloyl- $\beta$ -D-glucose	na	na	na	15.24	58.55
Quercetin-3- $\beta$ -D-glucoside	na	4.1–8.3	na	1.02–4.06	3.67
(–)-Epicatechin gallate	na	na	0.2–0.6	2.72–13.22	4.93
Ellagic acid	33.6–97.7	31.8–51.5	217.3–704.7	11.35–72.05	98.41
Naringin	na	na	na	0.32–1.28	0.30
<i>p</i> -Coumaric acid	0.5–2.9	na	1.6–2.6	0.20–0.31	0.48
Ferulic acid	0.4–1.1	na	na	0.61–4.85	0.94

<sup>a</sup>Data expressed as mean concentration values. <sup>b</sup>na: not reported. <sup>c</sup>Total hydroxybenzoic acids.

concentration of this phenolic was found to be significantly higher in English walnut than those in the black walnuts, except Surprise ( $p < 0.05$ ). As seen in Table 2, no significant difference was noted for ellagic acid concentrations between English walnut and Surprise ( $p < 0.05$ ).

## DISCUSSION

**Identification of Phenolic Compounds.** As stated above, among the 25 screened phenolic compounds (Table 1), 16 were quantified and confirmed by comparing their retention times and MS and MS<sup>2</sup> mass spectral data corresponding to those of analytical standards. The list of phenolics includes phenolic acids, flavonoids, and catechins. To the best of our knowledge, this present work is the first study that reports the levels of a wide range of phenolic compounds in kernels of different black walnut cultivars. The developed HPLC–MS/MS method offers the sensitivity and selectivity required for rapid and accurate quantification of the phenolic compounds in organic-rich matrices and plant materials. Additionally, optimization of the SIR and MRM modes allows addressing several analytical challenges during the method development.

**Phenolic Compositions of Black Walnuts.** As demonstrated in Table 2, the 11 black walnut cultivars significantly differed on the concentrations of 16 phenolic compounds. Ellagic acid was found to be the most abundant phenolic compound in all the black walnut extracts. Our results indicated significant variation in concentrations of ellagic acid among the cultivars. Particularly, Surprise was composed of approximately eight times higher level of this compound as compared to Tomboy. Ellagic acid is biosynthesized from penta-*O*-galloyl- $\beta$ -D-glucose generated by esterification of gallic acid and glucose.<sup>11</sup> This free phenolic acid was also found in English walnuts<sup>12,13</sup> and pecans.<sup>14</sup> Slatnar et al. (2015) showed that ellagic acid levels averaged from 31.8 to 51.5  $\mu\text{g/g}$  of kernel with respect to five different English walnut cultivars,<sup>13</sup> falling within the range reported in our study (Table 3). We also performed untargeted metabolomics analysis of black walnut extracts. The methanolic black walnut extracts obtained from the same extraction protocol as described earlier were injected into UPLC–HRMS, and the chromatographic data were then processed using a multigroup job by XCMS Online platform (<https://xcmsonline.scripps.edu/>). Through the untargeted

global metabolomics analysis, Surprise and Sparks 147 were also found to contain significantly higher levels of ellagic acid as compared to the other cultivars (unpublished data).

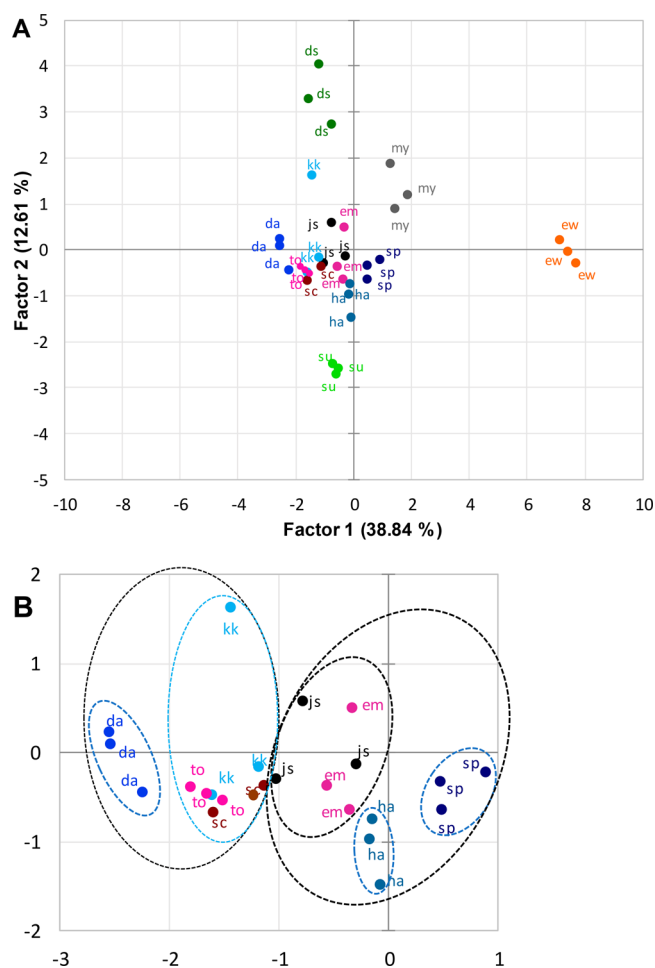
Quinic acid, which was found in all the samples along with ellagic acid, is not considered a phenolic compound due to a lack of phenolic ring in its molecule. As a secondary metabolite in the shikimate pathway, quinic acid contributes to biosynthesis of aromatic amino acids and many phenolic compounds.<sup>15</sup> Catechin such as (+)-catechin and (–)-epicatechin was commonly found in tea, Chinese medicinal plants, and oil nuts such as hazelnut and English walnut.<sup>16–18</sup> Notably, the amount of catechin in black walnuts determined in our study was significantly lower than that in English walnuts reported in prior studies<sup>13,18,19</sup> (Table 3). The analysis of an English walnut sample carried out in our study as stated earlier helped verify those findings. Our results indicated that English walnut was composed of approximately 100-fold higher level of catechin as compared to black walnuts, corroborating the results presented by Slatnar et al. (2015).<sup>13</sup> The low level of catechin detected in black walnuts could be due to catechin much more commonly existing in the forms of glycosides and gallic acid conjugates. The detection of (–)-epicatechin gallate, a gallic acid conjugate of epicatechin, in the nine black walnuts (Table 2) could further support our findings. Along with other flavones, catechin is derived from phenylalanine.

Our study reported the detection of gallic acid as free form in the black walnuts and English walnut. As seen in Table 2, English walnut contains almost twice as much amount of gallic acid as Mystry which was identified as the cultivar with the highest level of gallic acid. The concentration of gallic acid in English walnut in our study is comparatively consistent with the results reported by Slatnar et al. (2015) (Table 3). Moreover, gallic acid was found both in free acid and as part of hydrolyzable tannins in English walnut, with the latter far more abundant than the former.<sup>13</sup> On the basis of these findings, it is suggested that gallic acid could mostly exist in the form of hydrolyzable tannins in black walnuts. As reviewed earlier, gallic acid is derived from the Shikimate pathway and contributes to formation of ellagic acid. Gallic acid was also found in hazelnut<sup>20</sup> and pecan.<sup>14</sup>

The four phenolic acids (i.e., vanillic acid, syringic acid, ferulic acid, and *p*-coumaric acid) and the flavonoid glycosides

(i.e., rutin, QDG, and naringin) are reported in the studied black walnuts and English walnut. The first group of phenolic compounds not including vanillic acid was also found in different English walnut cultivars<sup>21</sup> while, to our knowledge, the second group has never been reported in both two species of walnuts. Notably, the study by Colaric et al. (2005) showed that syringic acid was the most abundant phenolic compounds in English walnut kernels with concentrations ranging from 165.7 to 574.5  $\mu\text{g/g}$ <sup>21</sup> (Table 3).

The principal component analysis (PCA) was applied to assess the data on phenolic contents in the black walnuts and English walnut determined by LC-MS/MS. As demonstrated in Figure 2A, principal component 1 (Factor 1) explains up to



**Figure 2.** Principal component analysis of phenolic data of 11 black walnuts and an English walnut. Figure 2A depicts a scatter plot (PC1 vs PC2) obtained from the phenolic data in which all the studied black walnut cultivars and English walnut were included. Abbreviations: da (Daniel), ds (Davidson), em (Emma K), ew (English walnut), ha (Hay), js (Jackson), kk (Kwik Krop), my (Mystery), sc (Schessler), sp (Sparks 147), su (Surprise), to (Tomboy). Figure 2B illustrates a zoomed-in scatterplot on the cluster containing the cultivars of da, kk, to, sc, js, em, ha, sp.

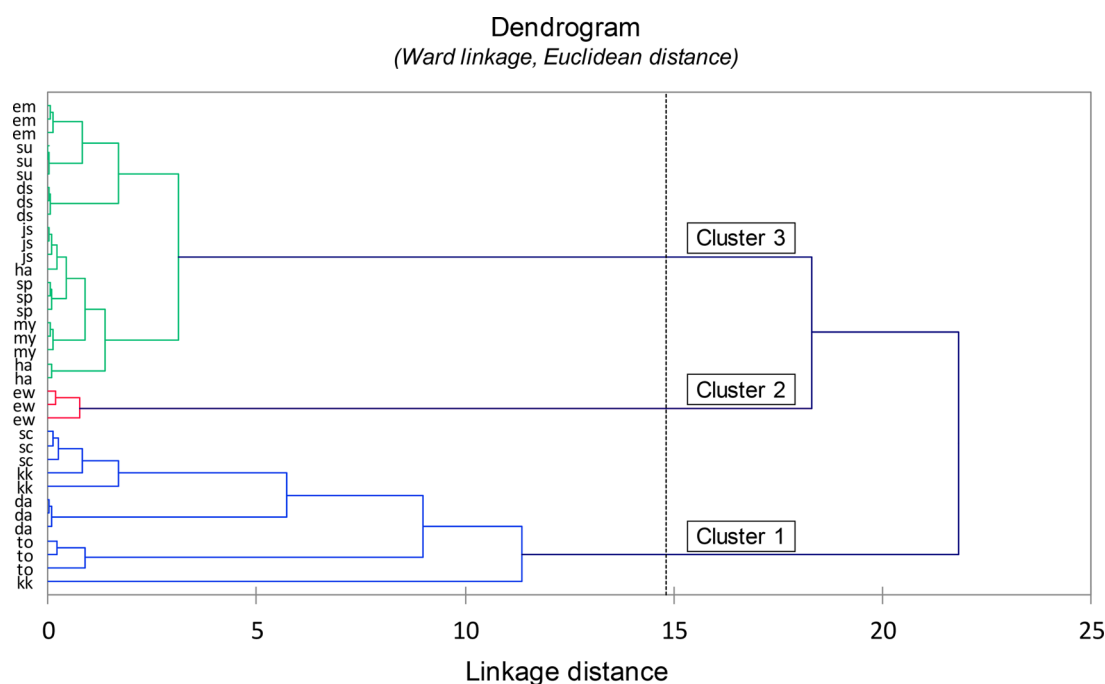
38.84% of the total variance and is characterized mainly by trigalloyl glucose, gallic acid, catechin, ellagic acid, and PGDG. Particularly, English walnut which contained significantly higher levels of these phenolic compounds clusters separately from black walnuts. Principal component 2 (Factor 2) explaining 12.61% is contributed mainly by syringic acid, *p*-hydroxyben-

zoic acid, and vanillic acid. The PCA scatterplot showed 51.54% of the total variability in the phenolic data set. Through PCA, it is important to note that the cultivars of Kwik Krop, Schessler, and Tomboy cluster together, indicating insignificant differences in their phenolic contents (Figure 2B).

Figure 3 depicts the dendrogram for three clusters of walnut samples grouped by similarities in phenolic contents using Ward's method. The arithmetic mean concentrations and standard deviations of each phenolic compound were calculated among the three clusters and are shown in Table 4. The nonparametric Kruskal–Wallis test was performed to compare the three walnut groups in order to test the null hypothesis that all the walnut groups originate from the same distributions. A significant level of  $p < 0.05$  was used to reject the null hypothesis. Multiple pairwise comparisons were determined using the Dunn test. As demonstrated in Table 4, Cluster 1 consists of the samples originating from four black walnut cultivars (i.e., Schessler, Kwik Krop, Daniel, and Tomboy) with significantly lower mean concentrations of vanillic acid, quercetin-3- $\beta$ -D-glucoside, ellagic acid, and *p*-coumaric acid. Cluster 2 comprises only English walnut samples with significantly higher mean concentrations of gallic acid and catechin as compared to the other clusters. As delineated in the HCA graph (Figure 3), Cluster 2 and Cluster 3 present similar characteristics in 73% (11/15) of the total phenolic constituents reported.

It is proposed that ecological factors could be responsible for the variation in phenolic composition among the studied black walnuts. Phenolic compounds as secondary metabolites are known to be involved in physiological processes of fruit tree growth and development. Prior research has revealed that these compounds act as defense factors against biotic and abiotic stresses,<sup>22</sup> which consequently affect phenolic contents. By contrast, in cases in which the stress intensity and/or duration is high, the rate of depletion of phenolic compounds is higher than their biosynthesis and phenolics cannot cope with stress. One study by Król et al. (2014) has showed that long-term and continuous drought stress inhibits total biosynthesis of phenolic compounds in leaves and roots.<sup>23</sup> Indeed, ecological factors such as water stress<sup>24</sup> and high rainfall<sup>25</sup> were previously identified as having impacts on English walnut and black walnut quality, respectively. This could be further supported by prior studies which pointed out the relationship between phenolic contents and walnut quality characteristics (i.e., aroma and color).<sup>26,27</sup> Black walnuts possess wide genetic variation that helps them grow and survive when environmental factors change. The variation is partially related to geographic origin.<sup>28</sup> For examples, Davidson originated from Ohio, while Kwik Krop began in Kansas, USA. This difference in geographic origin may have affected kernel quality and thereby phenolic compositions of the studied black walnuts in our study. Therefore, genetic factors might be accountable for the variation in phenolic composition, in addition to ecological factors.

**Phenolic Compounds as Potential Health-Promoting Components.** Phenolic compounds constitute one of the largest and most ubiquitous groups of phytochemicals in plants and plant-derived foods. Accordingly, a significant quantity of phenolic compounds is daily consumed in our diet. As reviewed earlier, antioxidant properties of phenolic compounds which have been recognized for decades are believed to give rise to other important bioactivities such as anticarcinogenicity and antimutagenicity. The biological functions of phenolic anti-



**Figure 3.** Dendrogram for black walnuts and English walnut obtained from the hierarchical cluster analysis.

**Table 4.** Walnut Samples Grouped by Phenolic Compounds<sup>a</sup>

Compounds	Cluster 1 (n = 12)	Cluster 2 (n = 3)	Cluster 3 (n = 21)
Quinic acid	3.87 ± 0.72 <sup>ab</sup>	6.77 ± 0.43 <sup>b</sup>	3.07 ± 2.19 <sup>a</sup>
Gallic acid	0.40 ± 0.13 <sup>a</sup>	8.05 ± 0.68 <sup>b</sup>	1.67 ± 1.45 <sup>a</sup>
1,3,6-Trigalloyl glucose	<LOD <sup>a</sup>	38.20 ± 2.88 <sup>b</sup>	5.62 ± 4.46 <sup>ab</sup>
<i>p</i> -Hydroxybenzoic acid	<LOD <sup>a</sup>	1.21 ± 0.50 <sup>a</sup>	0.46 ± 0.49 <sup>a</sup>
Catechin	0.17 ± 0.21 <sup>a</sup>	47.91 ± 3.45 <sup>b</sup>	0.13 ± 0.22 <sup>a</sup>
Vanillic acid	<LOD <sup>a</sup>	7.32 ± 1.80 <sup>b</sup>	5.60 ± 3.58 <sup>b</sup>
Syringic acid	5.37 ± 4.88 <sup>a</sup>	7.25 ± 2.20 <sup>a</sup>	7.76 ± 3.51 <sup>a</sup>
Rutin	1.31 ± 1.10 <sup>ab</sup>	2.68 ± 0.27 <sup>b</sup>	0.83 ± 1.57 <sup>a</sup>
Penta-O-galloyl- $\beta$ -D-glucose	<LOD <sup>a</sup>	58.55 ± 7.71 <sup>b</sup>	5.09 ± 5.07 <sup>ab</sup>
Quercetin-3- $\beta$ -D-glucoside	0.43 ± 0.40 <sup>a</sup>	3.67 ± 0.23 <sup>b</sup>	2.07 ± 1.27 <sup>b</sup>
(-)-Epicatechin gallate	4.86 ± 5.43 <sup>a</sup>	4.93 ± 1.50 <sup>a</sup>	4.18 ± 1.89 <sup>a</sup>
Ellagic acid	17.20 ± 9.28 <sup>a</sup>	98.41 ± 20.58 <sup>b</sup>	57.57 ± 11.20 <sup>b</sup>
Naringin	0.43 ± 0.11 <sup>a</sup>	0.30 ± 0.04 <sup>a</sup>	0.59 ± 0.46 <sup>a</sup>
<i>p</i> -Coumaric acid	0.15 ± 0.11 <sup>a</sup>	0.48 ± 0.09 <sup>b</sup>	0.27 ± 0.05 <sup>b</sup>
Ferulic acid	0.42 ± 0.51 <sup>a</sup>	0.94 ± 0.14 <sup>a</sup>	1.09 ± 1.64 <sup>a</sup>

<sup>a</sup>Data are shown as mean ± standard deviation ( $\mu\text{g/g}$  dry weight kernel). Different letters for the same phenolic compound indicate significant differences among the clusters ( $p < 0.05$ ).

oxidants have aroused much interest of researchers and the food and pharmaceutical industries.

Among the identified phenolic compounds in our study, ellagic acid has exerted multiple bioactivities potentially important to human health. For example, Papoutsis et al. (2008) reported that ellagic acid in walnut extracts exhibits osteoblastic and antiatherogenic activities, showing the beneficial effect of walnut consumption on cardioprotection and bone loss.<sup>29</sup> This phenolic compound (10  $\mu\text{mol/L}$ ) was demonstrated to inhibit vascular endothelial growth factor

(VEGF) and platelet-derived growth factor (PDGF) receptors in vitro.<sup>30</sup> The combined inhibitory effects on these two receptors may result in potential antitumor property in vivo. One recent study shows that ellagic acid at nontoxic dose levels (2.5 to 20  $\mu\text{mol/L}$ ) exhibits potent antiangiogenesis activities through specifically targeting VEGF receptor (VEGFR-2) and its signaling pathway in breast cancer.<sup>31</sup> Due to its natural inhibitory effects on VEGFR-2 and PDGFR, ellagic acid could be developed as an antiangiogenesis agent which is useful for the prevention and treatment of cancer.

Hydroxycinnamic acids such as ferulic acid and *p*-coumaric acid are among the phenolic acids detected in the studied black walnuts. Of these, *p*-coumaric (100 mg/kg body weight) shows an anti-inflammatory effect in adjuvant-induced arthritic rats by diminishing the expression of inflammation-related factor TNF- $\alpha$ .<sup>32</sup> This compound also demonstrates in vivo immunosuppressive property due to reductions in cell-mediated immune responses and macrophage phagocytic index observed in control rats treated with *p*-coumaric acid. It is proposed that the use of ferulic acid could be helpful for the treatment of several age-related diseases such as neurodegenerative disorders, cardiovascular diseases, diabetes, and cancer.<sup>33</sup> However, further research into therapeutic use of ferulic acid needs to be performed.

In addition, other phenolic acids found in the black walnuts include *p*-hydroxybenzoic acid, vanillic acid, and syringic acid. Of these, vanillic acid and syringic acid were found to inhibit the activation of cultured hepatic stellate cells, which are involved in the formation of scar tissue in response to liver damage and maintain hepatocyte viability.<sup>34</sup>

Naringin, which is a flavanone-7-O-glycoside, has been reported to exert antioxidant and antiproliferative effects.<sup>35</sup> The authors showed that induction of autophagic cell death by naringin would lead to development of anticancer agent for human gastric carcinoma.

Our study results indicate the detection of several flavanols such as catechin, epicatechin, and epicatechin gallate as



described earlier. Flavanols, a class of phenolic compounds present in certain plants, have attracted much attention for their potential anticancer activities. Notably, recent studies have revealed that combinations of this phytochemical group and other anticancer agents will provide significant enhancement in growth inhibition and apoptosis of lung, breast, prostate, and colon human cancer cells.<sup>16,36,37</sup> Interestingly, catechin and epicatechin gallate have also been reported to improve the antibacterial effect of  $\beta$ -lactam antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) in vitro and in vivo.<sup>38</sup>

Quercetin glycosides such as rutin and quercetin-3- $\beta$ -D-glucoside (also known as isoquercetin) have been shown to produce multiple health-promoting effects in vitro and/or in vivo such as antioxidant, anti-inflammatory, antidiabetic, antithrombotic, antiplatelet, anticancer, neuroprotective, and vasodilatory actions.<sup>39,40</sup>

The polyphenol named penta-*O*-galloyl- $\beta$ -D-glucose is a naturally occurring phenolic compound commonly found in numerous herbal plants such as tree peony (*Paeonia suffruticosa*) and nutgall tree (*Rhus chinensis*). One systematic review by Zhang et al. (2009) shows that this compound exerts a variety of biological activities in vitro and in vivo such as antioxidant, antimutagenic, anticancer, antidiabetic, anti-inflammatory, antiallergic, and cardiovascular protective actions.<sup>41</sup> Due to these multiple health-promoting activities, this compound could be developed as a promising and novel drug candidate for prevention and treatment of cancer, diabetes, or other diseases.

Altogether, phenolic compounds have received considerable attention due to their potential health promoting properties as summarized in Table S2 (Supporting Information). Prior studies showed that consumption of walnut and other oil nuts provided a variety of health benefits including the lowering of serum LDL and total cholesterol levels, anti-inflammation, neuroprotective effects, and reduced risk of cardiovascular diseases, diabetes, and certain cancers.<sup>42</sup> These health benefits have been ascribed to their phytochemical compositions enriched in phenolic compounds. Our study shows that phenolic compounds abound in black walnut. As natural components of black walnuts, this class of compound helps highlight the nutritionally and medicinally important values of black walnuts as well as black walnut containing products.

In conclusion, phenolic compositions were successfully assessed in black walnuts using the LC-MS/MS based analytical method. Sixteen phenolic compounds in 11 different black walnut cultivars (Daniel, Davidson, Emma K, Hay, Jackson, Kwik Krop, Mystry, Schessler, Sparks 147, Surprise, Tomboy) have been identified and quantified. The 11 black walnut cultivars significantly differed on the concentrations of 16 phenolic compounds. Ellagic acid was found to be the most abundant phenolic compound in all the investigated black walnuts. The comparison of phenolic compositions of black walnuts and English walnut was made. Significant differences were noted for the concentrations of quinic acid, gallic acid, 1,3,6-trigalloylglucose, catechin, and penta-*O*-galloyl- $\beta$ -D-glucose between the studied black walnuts and their English walnut counterpart used in our study. Through PCA, more than 50% of the variance in the phenolic data was explained, and a visible differentiation in phenolic profiles between the studied walnuts was obtained. The HCA results showed three groups to which each walnut sample belongs. The analysis also highlighted the samples from the black walnut cultivars of Kwik Krop, Daniel, Schessler, and Tomboy with lower phenolic

contents than those of other black walnuts. In contrast, the samples originating from Davidson, Emma K, Hay, Mystry, Sparks 147, and Surprise shared similar characteristics in 73% of the total phenolic compounds detected in our study. All the phenolic compounds identified in our study have attracted considerable attention due to their potential health promoting activities. Therefore, understanding of phenolic compositions and their potential health benefits is of importance for contribution to development of new strategies for prevention and treatment of diseases. It also helps identify new industrial applications of the black walnuts. Future research should be focused on bioassay-guided purification to evaluate the potential health-promoting properties of these phenolic compounds in black walnuts.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b01181.

Table S1. Optimized ionization parameters of phenolic compounds identified by LC-MS/MS. Table S2. Summary of potential health-promoting properties of several phenolics identified in black walnuts. Figure S1. Cone voltage optimization for identification of naringin. Figure S2. Collision energy optimization for identification of naringin. Figure S3. Calibration curve of naringin. Figure S4. HPLC-PDA chromatograms of phenolic standards recorded at 220, 280, and 330 nm. Figure S5. MS spectrum of naringin (A) and MS/MS spectrum of naringin (B). Figure S6. A representative LC-MS/MS chromatogram of naringin in a black walnut kernel. (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

\*Phone: 573-882-6283. Fax: 573-882-1977. E-mail: [linchu@missouri.edu](mailto:linchu@missouri.edu).

### ORCID

Danh C. Vu: 0000-0003-0854-8444

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### Notes

The authors declare no competing financial interest.

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