Journal of Agricultural Chemistry and Environment, 2021, 10, 222-256
https://www.scirp.org/journal/jacen
ISSN Online: 2325-744X
ISSN Print: 2325-7458

Estimation of Total Phenolic Compounds and Non-Targeted Volatile Metabolomics in Leaf Tissues of American Chestnut (*Castanea dentata*), Chinese Chestnut (*Castanea mollissima*) and the Backcross Breeding Generations

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How to cite this paper: She, J.Y., Mohottige, C.U.G., King, M., Jiang, Y., Mlsna, M., Clark, S., Baird, R. and Mlsna, T. (2021) Estimation of Total Phenolic Compounds and Non-Targeted Volatile Metabolomics in Leaf Tissues of American Chestnut (*Castanea dentata*), Chinese Chestnut (*Castanea mollissima*) and the Backcross Breeding Generations. *Journal of Agricultural Chemistry and Environment*, **10**, 222-256.
https://doi.org/10.4236/jacen.2021.102015

Received: March 10, 2021
Accepted: May 28, 2021
Published: May 31, 2021

Abstract

The American chestnut (*Castanea dentata*) was once a dominant tree species in the Appalachian Mountains and played a critical role in the ecological system. However, it was nearly eliminated by chestnut blight caused by the Ascomycetous fungus *Cryphonectria parasitica*. Identification of compounds specific to species and backcross hybrids may help further refine disease resistance breeding and testing. Phenolic compounds produced by plants are significant to their defense mechanisms against fungal pathogens. Therefore, an analytical platform has been developed to estimate the total phenolic content in leaf tissues of the American chestnut, Chinese chestnut (*Castanea mollissima*), and their backcross breeding generations (B_3F_2 and B_3F_3) using the Folin-Ciocalteu reagent assay with UV/Vis spectrophotometry which may be used to predict blight resistance. Adsorption (765 nm) results from leaf tissue extraction in methanol/water (95%:5% v/v) and pH 2, show that the variations among these four tree species are significant (ANOVA $p = 2.3 \times 10^{-7}$). The kinetics of phenolic compound solid-liquid extraction was elaborated using Peleg, second order, Elovich, and power law models. In addition, extensive analysis using headspace solid phase microextraction (SPME) gas

chromatography and mass spectrometry was conducted to identify volatile organic compounds (VOCs) from the leaf of American chestnut, Chinese chestnut, and their backcross hybrids B_3F_2 and B_3F_3. A total of 67 VOCs were identified among all chestnut types. Many of the metabolites associated with the Chinese chestnut have been reported to have antifungal properties, whereas the native and hybrid American chestnut metabolites have not. Most of the antifungal metabolites showed the strongest efficacy towards the Ascomycota phylum. A partial least squares discriminant analysis (PLS-DA) model ($R^2X = 0.884$, $R^2Y = 0.917$, $Q^2 = 0.584$) differentiated chestnut species and hybrids within the first five principal component (PCs).

Keywords

Total Phenolic Compounds, Plant VOCs, HS-SPME-GC/MS, Chemometric Analysis, Chestnut Tree Hybrids

1. Introduction

The American chestnut tree (*Castanea dentata*) was a dominant native species in the eastern forests of the United States. Due to its rapid growth, large size, and nut production, it was critical to the U.S forest ecosystem and economy [1]. However, this tree species was devastated by a fungal pathogen, *Cryphonectria parsitica* [2] starting in 1904 and nearly wiped out by 1945. The virulent strains of the fungus attack the tree through penetration of wounds and bark fissures, killing the bark and cambium layer, preventing the transport of water and nutrients, and reducing the tree to short-statured sprouts or causing mortality [3] [4] [5]. It is difficult to know all the consequences however the loss of these dominant tree species likely significantly changed a wide range of ecosystem services dependent on this species [6]. Because the tree was nearly wiped out before modern forest research there is only speculation on how it affected soil chemistry and food webs. However, the chestnut was likely a more stable and abundant resource for wildlife than other tree species [7]. In addition, the long-term impact of the loss of a dominant tree species could affect local climate regulation [8], regional carbon balance [9], and flood control [10], as well as reducing biodiversity [11] and threatening the sustainable ecosystem [12]. Therefore, the restoration of American chestnuts has received attention and efforts have ensued to restore the species [13].

In the late 20th century, backcross breeding programs were implemented to produce hybrids from pathogen resistant Asian chestnut species, primarily Chinese chestnut (*Castaneamollissima*) and American chestnut [14]. The hope is that the sixth generation (B_3F_3) carries American chestnut morphology characteristics [15] but also resistance to the blight from Chinese chestnut [16]. However, the selection of trees with resistance is costly, labor intensive and time-consuming, as the determination of blight resistance can take up to 15 years and may change

over time due to enhanced juvenile resistance. Traditionally, resistance tests involve pathogen inoculation in plantations, forest test plantings that receive natural blight pressure [17], or complex genomic sequencing [18]. Therefore, feasible and reliable alternative analysis methods are desired.

Natural phenolic compounds play a critical role in plant defensive systems against herbivores, pests, and pathogens [19]. Plant phenolics are secondary metabolites containing one or more aromatic rings with hydroxyl groups [20] and are toxic to numerous invaders [21]. According to Galili *et al.*, many of these metabolites are generated from the pentose phosphate, shikimate, and phenyl propanoid metabolism pathways [22]. Reported plant phenolics include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, and quinones [23] [24] [25] [26].

As indicated by Lafka *et al.* each plant has a unique composition of phenolics, therefore the development of optimal condition regarding phenolic extraction is critical [27]. To date, although plant phenolic extraction techniques such as supercritical fluid extraction (SFL) [28] and pressurized liquid extraction (PLE) [29] have been developed, the most commonly applied procedure is the organic solvent extraction. This is likely because both SFL and PLE require high pressure equipment not available in many labs. Common solvents like methanol, ethanol, ethyl acetate, and acetone have been applied to many plant materials [20]. However, the extracting solvent choice affects the recovery of phenolics. For example, methanol was shown to be more efficient in the extraction of lower molecular weight polyphenols [30], while aqueous acetone is better for higher molecular weight compounds such as flavanols [31]. Furthermore, the extracted phenolic profile could also be affected by solvent-to-solid ratio, extraction time, temperature, and extraction pH [32].

The complex, volatile organic compounds (VOCs) profiles emitted from plants have been successfully used in differentiating plant species [33] [34] [35] [36] [37]. A recent study further expanded VOCs pattern analysis to discriminate modified plant hybrids from their parents [38]. To date, many VOC sampling techniques have been developed. Extraction techniques such as steam distillation (SD), simultaneous distillation extraction (SDE), purge and trap, or dynamic headspace have been used in the research. In particular, nondestructive sampling methods such as solid-phase microextraction (SPME) have been extensively applied to sampling VOCs [39]. Furthermore, mass spectrometry based non-targeted metabolomics or metabolic profiling, which can screen biological alterations that correlate to phenotypic perturbations [40], is finding application in a wide range of research including plant biology [41]. For example, Zhao *et al.* applied metabolic profiling with gas chromatography-mass spectrometry and successfully revealed plant growth interaction between carbon and nitrogen metabolism [42].

In this study, a Folin-Ciocalteu reagent assay was employed to optimize extraction conditions such as liquid/solid ratio, time, and pH to maximize the yield of total phenolic compounds from chestnut tree leaf tissues. In addition, the to-

tal phenolic content (TPC) of species was compared. Extensive analysis using headspace SPME gas chromatography and mass spectrometry was conducted to identify VOCs from the leaf of American chestnut, Chinese chestnut, and their B_3F_2 and B_3F_3 hybrids.

2. Materials and Methods

2.1. Chemicals and Reagents

Caffeic acid (CA), analytical reagent grade methanol, ethanol, acetone, isopropanol, n-propanol, and ethyl acetate, HPLC grade hexane, and the Folin-Ciocalteau phenol reagent were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, USA).

2.2. Plant Material

American chestnut, Chinese chestnut, and their backcrossing generations B_3F_2, and B_3F_3 were obtained from The American Chestnut Foundation (TACF) via the United States Department of Agriculture Forest Service who is collaborating with TACF on chestnut reintroduction [13]. Backcross hybrid (B_3F_2 and B_3F_3) experimental material consisted of progeny from orchard trees (open-pollinated) at TACF's Meadowview Orchard [43]. The BC_3F_2 material consisted of progeny from one mother tree, and the BC_3F_3 consisted of progeny from three mother trees, which were bulked together for this experiment. Chinese chestnut experimental material was collected from one open-pollinated tree in Asheville, NC that was surrounded by other Chinse chestnut trees, and progeny were thus deemed pure Chinese chestnut (Paul Sisco, The American Chestnut Foundation, personal communication). American chestnut experimental material was collected from two open-pollinated native wild trees in Virginia that were surrounded by other native American chestnuts and were thus deemed pure American chestnut (Fred Hebard and Jeff Donahue, The American Chestnut Foundation, personal communication).

Two independent experiments were performed. In each test, 33 nuts of each chestnut family were grown in the research greenhouses of the Department of Plant Pathology, Mississippi State University, MS. The nuts were stratified at 3˚C for 60 days prior to planting. The seedlings were randomized in greenhouse placement to overcome any environmental gradients that might exist and grew in 2.83 L pots (Stuewe & Sons, Tangent, OR) with Premix, Vermiculite, Perlite (1:1:1) mixture and uniformly fertilized using a water-soluble Osmocote concentrations fertilizer for woody plants at the manufacturers recommended rate weekly (1 Tsp fertilizer per liter of water) (J. R. Peters Inc, Allentown, PA) and watered daily. The following growth conditions were used: daylight, 8 - 14 hours (winter-summer), temperature, 18˚C - 35˚C (winter-summer). Plant leaf tissue was collected as fully developed leaves (23 - 26 cm in length and 8 - 12 cm width) which usually occur at five to six months of growth. All samples from two independent tests were collected at the same time of the day (at 9-11 am) under

uniform light conditions. For each sampling date, leaves from each species were randomly selected to harvest for chemical analysis.

Analysis of volatile organic compounds from leaves, SPME and GC/MS, were made on the same date as the sampling. VOCs samples preparation procedure was based on Chang *et al.* with modifications [34]. Fresh leaves were immediately chopped into small pieces, and 5 g samples were placed in 1 L glass jars (Environmental Sampling Supply, San Leandro, CA) and sealed with aluminum foil and parafilm. Samples were heated for 30 min in a 35˚C incubator before SPME extraction. SPME was done for 2 hours at room temperature and desorbed at a GC inlet for 5 min at 300˚C. VOCs samples labelled replication 1 to replication 6 were all from the first test, and replication 7 to replication 12 were from a second independent test. For the total phenolic determination, fresh leaves were collected and immediately frozen in liquid nitrogen and stored at −80˚C until they were freeze-dried for total phenolic measurements.

2.3. Solvent Extraction

The extraction of phenolic compounds from chestnut tree leaves was based on the procedures described by Lafka *et al.* with modifications [28]. Briefly, 20 mg of ground tree leaf tissue was placed in a sample vial (Hach, Loveland, CO) with n = 6 for each type of solvent. Samples were extracted with 2 mL of methanol/water (v/v 95%/5%), ethanol, ethyl acetate, and acetone/water (v/v, 70%/30%) in a shaker (C-24 model, New Brunswick Scientific Co., INC. Edison, NJ) for different extraction times (30 min to 24 hours) at room temperature. The extract was centrifuged at 6000 G for 30 min and the supernatant was transferred and evaporated to dryness in a rotary evaporator (Organomation Associates, Inc, Berlin, MA). The solution was then dissolved in 4 mL ice-cold methanol for F-C assay analysis. For the pH study, prior to organic solvent extraction, the leaf tissue was acidified with HCl to pH range 2 to 6, and n-hexane was added 5:1 (v/w) for 20 min at room temperature. Six replicates (n = 6) of each chestnut tree family in two independent tests were made and analyzed under the same conditions.

2.4. Total Phenolic Content (TPC) Determination

The TPC procedure was based on Ainsworth *et al.* with changes [44]. In general, 10% (v/v) of F-C reagent was made with deionized water. The extracts were then mixed with 200 μL of the F-C reagent and vortexed thoroughly in a sample vial (Hach, Loveland, CO) for 1 minute. The solution was incubated for 10 min at room temperature before adding 0.8 mL of 700 mM Na_2CO_3 solution into the tube. The mixture was then kept in the dark for 2 hours. The absorbance of the solution was measured at 765 nm using a GENESYS 30 UV/Visible spectrophotometer (Thermo Scientific, Waltham, MA) against deionized water blank. The value of the TPC was determined via a calibration curve (with $R^2 = 0.99$) prepared with a series of caffeic acid standards (0, 20, 40, 60, 80, 100, and 120 mg/L). The TPC results were expressed as mg of caffeic acid equivalents per gram of fresh weight leaf tissue (mg CA/g FL).

2.5. Headspace SPME Extraction

The SPME technique is a non-destructive sampling method that can be used to collect VOCs in the headspace above samples. Commercially available SMPE fibers coated with 85 μm Carboxen-polydimethylsiloxane (CAR/PDMS) (Supelco, Sigma-Aldrich, PA, USA) were selected. All fibers were conditioned based on the supplier's recommendations at 300˚C for one h before first use. SPME extraction was performed by exposing fibers to the headspace above ground leaf samples for two hours in order to reach chemical and thermal equilibration. Six replicates (N = 6) of VOCs extraction of each chestnut tree family in two independent tests were made and analyzed under the same conditions.

2.6. GC/MS Condition

Extracted VOCs were analyzed with an Agilent gas chromatograph (Agilent Technologies 7890A) coupled with Agilent mass spectrometer (Agilent Technologies 5975C) system (GC/MS) with ChemStation software. Separations were done using a DB-1 capillary column (60 m × 320 μm × 1 μm) (Agilent J&W column, Santa Clara, CA). Samples were analyzed in splitless mode. The injector temperature was kept at 270˚C, and was equipped with a 0.7 mm ID SPME inlet liner (Supelco, Bellefonte, PA). The GC oven temperature operation conditions were applied as in previous studies [45]. Briefly, 45˚C for 9 min, 10˚C·min^{-1} to 85˚C, hold for 3 min, 3 min^{-1} to 110˚C, hold for 3 min, 3˚C·min^{-1} to 120˚C, hold for 3 min, and 10˚C·min^{-1} to 270˚C, hold for 5 min. The carrier gas supplied to the column was helium (99.9999% purity) at a constant flow rate of 2 mL/min. For the MS detection, the electron impact (EI) was set as 70 eV with the ion source temperature at 230˚C and quadrupole temperature at 150˚C, respectively. The analytes were characterized by full-scan acquisition from 35 - 350 atomic mass unit (amu). Library matching identified chromatographic peaks to the reference spectra (NISTT05a.L, Agilent Technologies, Inc.).

2.7. Data Analysis

GC-MS data files were preprocessed for noise filtering, baseline correction, and converted to CDF format with ChemStation(Agilent Technologies, Inc. Santa Clara, CA). The output files were uploaded to XCMS [46] software to process the peak detection, matching, and alignment with the default setting. The data set was then filtered by removing peaks with 75% missing values. The intensity of resultant peaks was further normalized with respect to the sum of the intensities, in which each peak intensity was divided by the sum of all peak intensities in the fraction. The final peak tables were uploaded to MetaboAnalysis [47] software for statistical analysis.

Prior to analysis, all variables were logarithm transformed and mean centered. Nonparametric univariate method, Kruskal-Wallis Test, was performed to analyze the significance (p-value < 0.05) of peaks among the samples. The false discovery rates (FDR) test with p-values less than 0.05 (pFDR < 0.05) was applied to

the results for further adjustment. Spearman correlation rank test was used to generate correlation matrices for the volatile metabolites. Differentiation of plant emissions was analyzed using principal component analysis (PCA) and partial lease squares discriminant analysis (PLS-DA). In addition, clustering techniques, such as K-means and Hierarchical cluster analysis (HCA) were applied.

3. Results

3.1. Optimization of Solvent Extraction Variables

Prior to final analysis of phenolic content from the chestnut tree leaf, the organic solvent extraction procedure was optimized using pooled samples. 20 mg of chestnut leaf tissues were extracted with methanol, ethanol, ethyl acetate, and acetone/water (v/v 7/3) with different solvent to mass ratios (20:1, 40:1, 60:1, 80:1, 100:1, 150:1 and 200:1; v:w; mL/g) at room temperature for 24 hours. Results are shown in **Figure 1**.

The amount of extracted phenolics was affected by the type of organic solvent as well as the solvent to mass ratio. In general, the concentration of extraction (mg of phenolics/g of leaf) increases as the solvent/mass ratio increases. The highest extraction for methanol occurred at 100:1 (12.2 mg/g). For ethanol, the highest was at 200:1 (15.1 mg/g). Ethyl acetate showed the greatest amount of extraction at 60:1 (14.7 mg/g). Finally, acetone/water showed its highest extraction at 150:1 (14.4 mg/g). Overall, the ethyl acetate extraction led to a maximum phenol content of 14.2 mg/g on average. However, this maximum extraction from ethyl acetate is not significantly different ($p > 0.05$) from the extractions with other types of organic solvent. Although it varied from solvent to solvent, the best extraction solvent/mass ratio was 150:1 with an average extraction of 14.2 mg/g of phenolics.

The extraction of phenolics was further studied by varying extraction pH from 2 to 6 at room temperature for 24 hours. In addition, a set of non-acidified control samples were also extracted under the same experimental condition. As shown in **Figure 2**, acidification has a significant impact on phenolic extraction. The addition of acid improved the extraction from methanol and ethanol with the highest extraction content of 14.7 mg/g and 14.3 mg/g respectively. However, acidification caused the loss of phenolic content in ethyl acetate and acetone/water solvents. Based on the high extraction yield and low sample variations, methanol was employed for the phenolic extraction of chestnut varieties.

3.2. The Extraction Kinetics

The kinetics of total phenolic extraction was analyzed to determine the extraction rate and appropriate time range. The solid/liquid extraction processes from plant materials were investigated with mathematical models [48] [49] [50]. The empirical models such as Pelog, second order, Elovich, and power law are commonly used to fit the experimental data (Section 1 Supplementary Material (SM)).

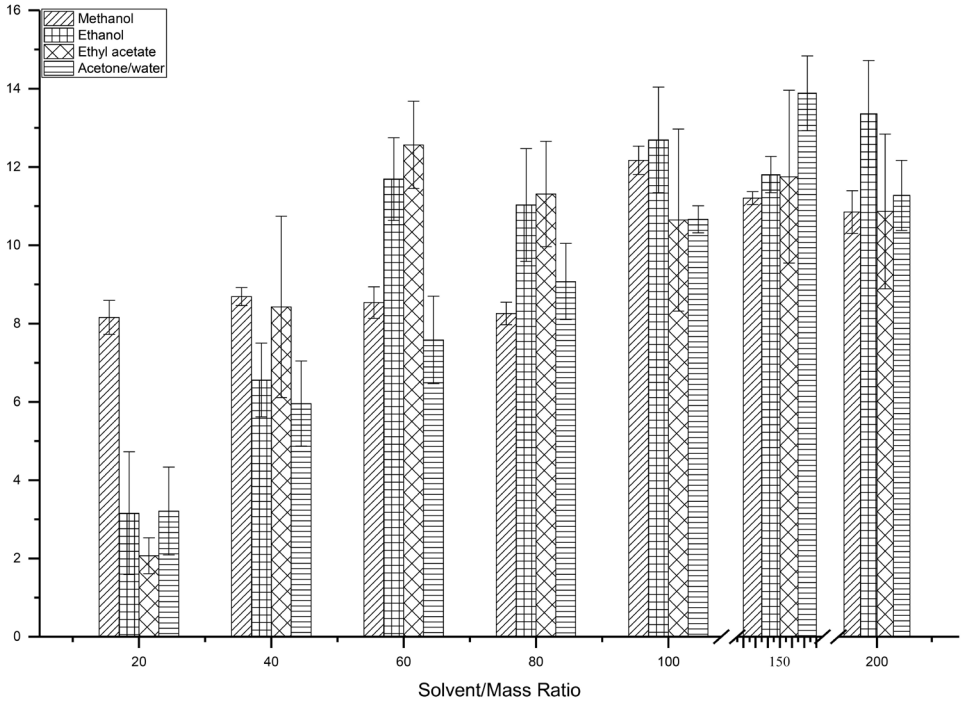

Figure 1. The total phenolic content (mg/g) extracted from pooled chestnut leaf samples using methanol, ethanol, ethyl acetate, and acetone/water at solvent to mass ratios (mL/g) ranging from 20:1 to 200:1 (n = 3). Point and error bar in the chart indicate means ± s.d.

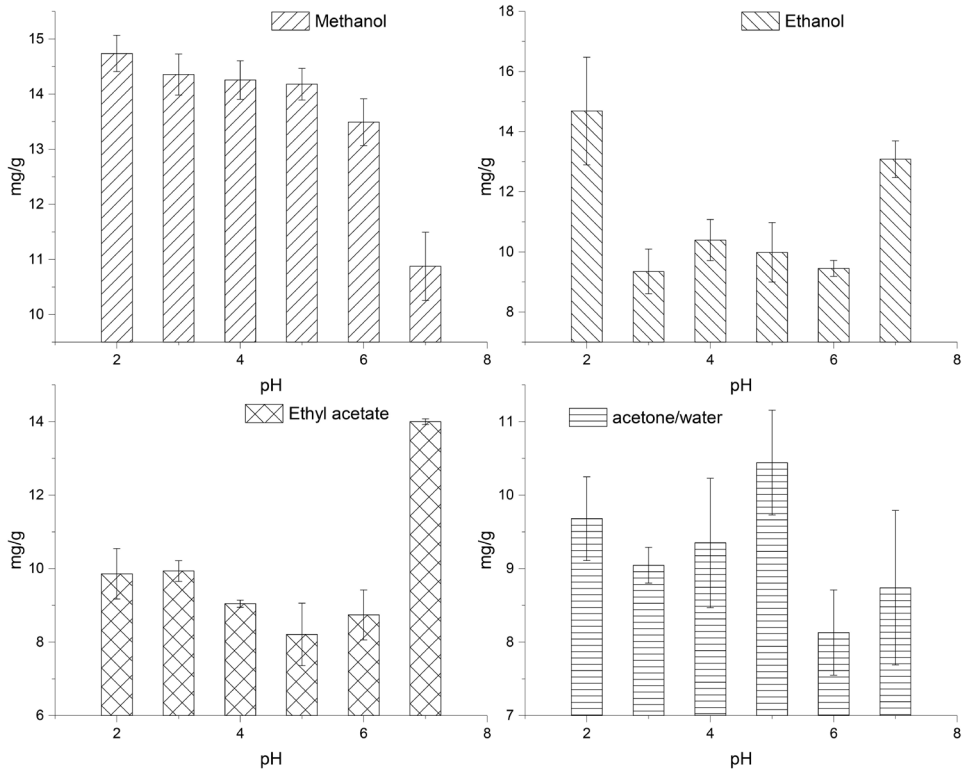

Figure 2. The total phenolic content extraction (mg/g) of pooled chestnut leaves using methanol, ethanol, ethyl acetate, and acetone/water (n = 3) with adjusted pH (range from 2 - 7) and 100:1 (liquid/solid ratio). Point and error bar in the chart indicate means ± s.d.

The extraction of total phenolics vs time is shown in Figure 3. Extraction kinetics could generally be considered to take place in two phases. A high initial rate of extraction can be observed for all solvents from 0.5 to about 3 hours followed by a slower extraction rate. It should be noted that previous studies suggest that long extraction time could cause degradation of phenolics, however, no decline was observed in extraction yield during 24 hours extraction for all solvents in this study. Several models were used to describe the experimental data with the empirical parameters shown in Table SM1. The R^2 (Table SM1) from model fitting indicates that the phenolic extraction process from chestnut tree leaf tissue is second-order for all solvents that were tested. The R^2 in second-order models for methanol, ethanol, ethyl acetate, and acetone/water were 0.990, 0.998, 0.999, and 0.996, respectively.

The total phenolic content of tree leaf tissues varies from American, Chinese, and their hybrids B_3F_2, and B_3F_3. As shown in Figure 4, the highest quantity of total phenolic compounds was found in Chinese chestnut (14.125 mg/g; n = 12), and the lowest was in American chestnut (9.774 mg/g; n = 12). The phenolics found in B_3F_2 and B_3F_3 were 9.931 mg/g and 10.884 mg/g, respectively. The ANOVA ($p = 2.3 \times 10^{-7}$) based on the overall value indicated the phenolics variation among tree species as significant. Student's t-test ($p < 0.05$) confirmed the difference between American and Chinese ($p = 1.26 \times 10^{-6}$), Chinese and B_3F_2 ($p = 1.16 \times 10^{-6}$), and Chinese and B_3F_3 ($p = 5.35 \times 10^{-6}$) as significant. However, substantial difference was not found between American chestnut and B_3F_2 ($p = 0.778$), or American and B_3F_3 ($p = 0.053$), and the hybrids B_3F_2 and B_3F_3 ($p = 0.528$).

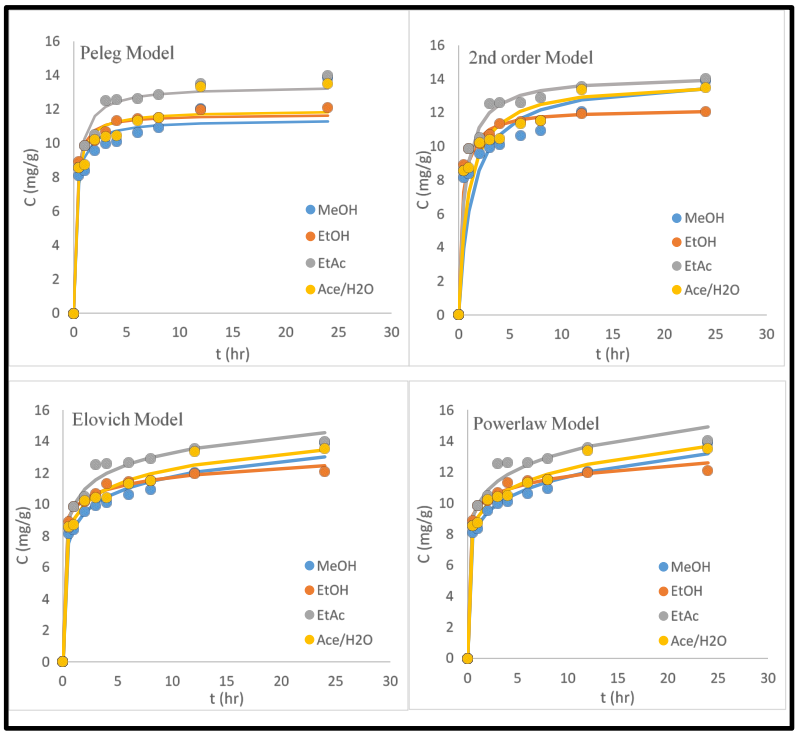

Figure 3. Four kinetic models for extraction of total phenolic compounds from chestnut leaves using a 100:1 solvent/extract ratio at pH = 2 at 25˚C.

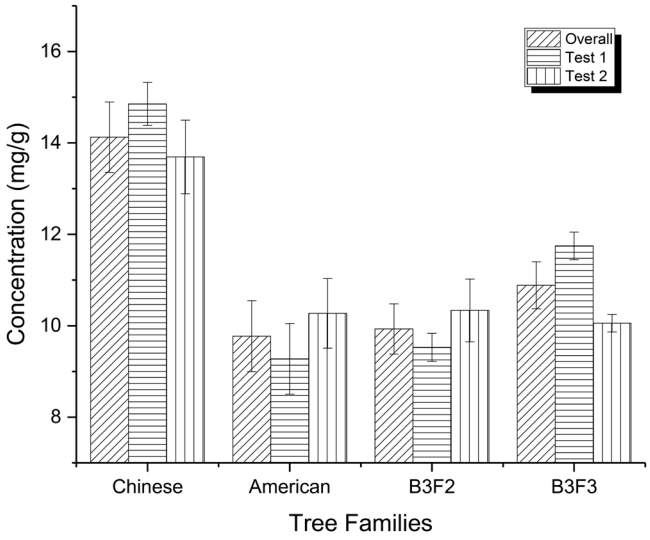

Figure 4. The total phenolic contents from two independent tests of American chestnut, (9.8 ± 0.8 mg/g, 9.3 ± 0.8 mg/g, and 10.3 ± 0.8 mg/g; overall, test 1, and test 2 respectively), Chinese chestnut (12.1 ± 0.8 mg/g, 14.9 ± 0.5 mg/g, and 13.7 ± 0.8 mg/g), B_3F_2 (9.9 ± 0.5 mg/g, 9.5 ± 0.3 mg/g, and 10.3 ± 0.7 mg/g), and B_3F_3 (10.9 ± 0.5 mg/g, 11.7 ± 0.3 mg/g, and 10.1 ± 0.2 mg/g). (test 1, n = 6; test 2, n = 6; overall n = 12). Point and error bar in the chart indicate means ± s.d.

Table 1. Differential VOCs that accountable for the discrimination among chestnut species.

No.	Chemicals[6]	KI[1]	$KI_{reference}$[2]	VIP[3]	p-value[4]	FDR[5]	Anti-Microbial	Citation#
1	(4E)-4-hexenyl acetate	1021	992	3.303	0	0	+	Mar *et al.* 2016 [51]
2	ylangene	1367	1221	2.835	0	0.00003	+[7]	Hernandez *et al.* 2014 [52]
3	caryophyllene	1426	1494	2.332	0.00094	0.00508	+	Ahamed *et al.* 2015 [53]
4	pentyl acetate	858	884	1.737	0	0	+	Ando & Kishimoto 2015 [54]
5	2-methyl-1-phenylbut-3-en-1-ol	1153	1280	1.676	0	0	-	None
6	seychellene	1277	1464	1.658	0.00589	0.0225	+	Yang *et al.*, 2013 [55]
7	(E)-2-hexen-1-ol	861	848	1.637	0.0004	0.00326	+	Nyiligira *et al.*, 2005 [56]
8	2-methyl-propanal	539	543	1.592	0.00111	0.00557	+[7]	Matebie *et al.*, 2019 [57]
9	methyl acetate	502	517	1.592	0.00227	0.0105	-	None
10	squalene	2611	2818	1.545	0.00273	0.0118	+	Wu *et al.*, 2019 [58]
11	2-nonen-1-ol	1028	1051	1.420	0.00009	0.00093	+	Dan *et al.*, 2017 [59]
12	*β*-phellandrene	1047	1030	1.357	0.00073	0.00476	+	Zhang *et al.* 2017 [60]
13	vinylfuran	701	713	1.311	0.00502	0.0204	+[7]	Balachari & O'Doherty, 2000 [61]

[1]the Kovats retention index calculated based on n-alkanes (C8-C20) on a DB-1 non-polar stationary phase. [2]KIreference is the Kovats retention index value obtained from the NIST Chemistry WebBook. [3]variable importance in projection. [4]p-value fromKruskal-Wallis Test. [5]false discovery rate. [6]Compound identified based on a comparison of RI value and mass spectra using the NIST database. [7]Component of essential oil that was tested for antimicrobial activity.

3.3. Volatile Organic Compounds Analysis

HS-SPME was employed for VOC extraction from fresh leaves followed by GC/MS analysis to investigate VOCs emitted from four chestnut species (*Casta-*

nea dentata, *Castanea mollissima*, and their hybrids B_3F_2 and B_3F_3). The identification of the VOCs in samples was based on the comparison of mass spectrum reference NIST MS library and assisted with the calculated Kovats index comparison with literature. The identified VOCs, their retention times, and the relative abundance (peak intensity) are detailed in Supplementary Information SM Table 2.

ChemStation software used on SPME-CC/MS chromatograms (Figure 5) showed 52 peaks on average were detected in American chestnut, 30, 71, 40 peaks were found in Chinese, B_3F_2 and B_3F_3 respectively. The major constituents of VOCs of chestnut in all samples were cis-3-hexenyl acetate, 3-hexen-1-ol, 2,4-hexendienal, and trans-2-hexenyl acetate. It should be noted that the strong emission of cis-3-hexenyl acetate was found in all tree species. XCMS was applied for the deconvolution, detection, and alignment of signal peaks in multiple GC/MS experiments.

In all, 67 VOCs were selected from amongst trees studies. These VOCs included 9 alcohols, 14 sesquiterpenes, 4 alkanes, 10 alkenes, 1 alkyne, 12 esters, 3 furans, 1 organic acid, and 4 ketones, and 1 monterpene (*α*-pinene). The VOCs identified from integrated GC/MS peak areas of major constituents showed that the leaf of the American chestnut contained 1.27% - 8.47% alcohols, 1.81% - 17.78% esters, and 0.31% - 2.79% sesquiterpenes. The VOCs emitted from Chinese chestnut leaves contained 3.98% - 12.11% alcohols, 0 - 11.41% aldehydes, 0.55% -3.84% alkanes, 0.029% - 3.05% alkenes, 0.55% - 22.04% of esters, and 0.22% - 5.67% sesquiterpenes. For VOCs from B_3F_2consisted of 1.89% - 7.39% alcohols, 2.18% - 8.42% aldehyde, 5.03% - 17.10% esters, 0.10% - 3.91% alkanes, 0.080% - 3.73% monterpene, and 0.29% - 8.52% sesquiterpenes. As for B_3F_3 contained 1.45% - 6.91% alcohols, 2.56% - 5.74% aldehydes, 10.85% - 25.17% esters, and 0.34% - 1.24% sesquiterpenes.

Table 2. Differential VOCs that accountable for the discrimination among Chestnut species.

	Samples in each cluster				
Cluster (1)	B3F3_REP9	B3F3_REP12	CC_REP9[4]	CC_REP11	
Cluster (2)	AC_REP5[1]	B3F2_REP1[2]	B3F2_REP2	B3F2_REP3	B3F2_REP4
	B3F2_REP5	B3F2_REP6	B3F3_REP1[3]	B3F3_REP2	B3F3_ REP3
	B3F3_REP4	B3F3_REP5	B3F3_REP6		
Cluster (3)	AC_REP1	AC_REP2	AC_REP3	AC_REP4	AC_REP5
	AC_REP6	AC_REP7	AC_REP8	AC_REP9	AC_PRP10
	AC_REP11	AC_REP12	B3F2_REP7	B3F2_REP8	B3F2_REP9
	B3F2_REP10	B3F2_REP11	B3F2_REP12	B3F3_REP7	B3F3_REP8
	B3F3_REP10	B3F3_REP11	B3F3_REP11		
Cluster (4)	CC_REP1	CC_REP2	CC_REP3	CC_REP4	CC_REP 5
	CC_REP6	CC_REP7	CC_REP8	CC_REP10	CC_REP12

[1]American chestnut (AC), replicate number (REP); [2]B_3F_2 (B3F2); [3]B_3F_3 (B3F3); [4]Chinese chestnut (CC).

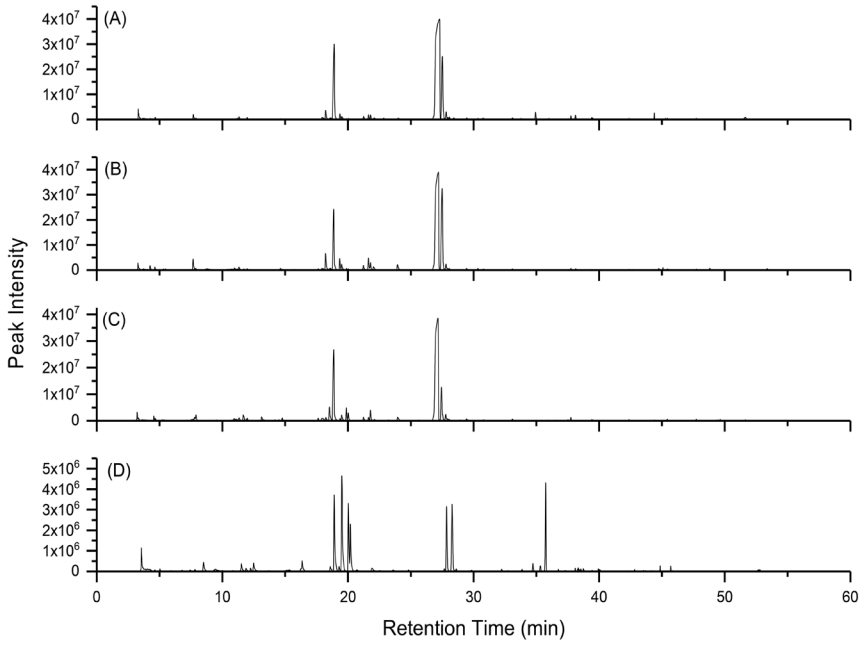

Figure 5. Representative HS-SPME-GC/MS chromatogram of VOCs of leaves from chestnut tree species: (A) American chestnut; (B) B_3F_2; (C) B_3F_3; and (D) Chinese chestnut.

As shown in **Table SM2**, a large portion of the identified VOCs were found in the four chestnut hybrids. However, chemicals such as ethyl 2-methylbutanoate, 2-nonen-1-ol, and γ-elemene were primarily detected in parental chestnuts tree leaves samples. Furthermore, compared to VOCs from parental Chinese chestnut, methyl acetate, heptanal, 4-hexen-1-ol acetate, β-phellandrene, p-xylene, caryophyllene, seychellene, and ylangene were identified mainly in American tree leaf samples. Regarding hybrids, (3E)-3,7-dimethyl-1,3,7-octatriene, γ-cadinene, (E)-2-pentene, and 2-methyl-furan was only discovered in B_3F_2. To verify whether the VOCs profiles can be used to discriminate the breeding generations and their parental species and to discover potential volatile metabolites that differ between hybrids and their parental Chestnut, chemometric analysis such as univariate (ANOVA) and multivariate analysis are required.

3.4. Chemometric Analysis of VOC Profiles from Chestnut Tree Species and Hybrids

Preliminary visual inspection of the TICs of GC/MSleaf VOCs profiles of American, Chinese, B_3F_2, and B_3F_3 revealed a high degree of similarity (**Figure 5**). Feature identification and peak alignment was obtained using XCMS to show the variation among tree species. Initially, a total of 138 features were identified amongst all samples. After the data filtering with 75% rule, 67 VOCs were selected. Then 18 chemicals were defined as statistically significant ($p < 0.05$) using nonparametric ANOVA in Univariate statistical analysis. However, ANOVA only provides a preliminary overview of possible significant features under the

experimental conditions. Therefore, multivariate analysis was employed for further interpretation of overall VOC profiles.

The PCA model derived from GC-MS spectra of all the samples was applied to the full data set, in which five principal components cumulatively account for 61.7% of the data variation (Supplementary Material SM Figure 1). To maximize the separation of chestnut tree leaf VOC profiles produced from different chestnut species and hybrids, PLS-DA was further performed. The best separation amongst four chestnut tree species was made by supervised models PLS-DA with 5 components ($R^2X = 0.884$ $R^2Y = 0.917$, $Q^2 = 0.584$) and was validated with 1000 times permutation tests (Figure 6).

In the PLS-DA model, the variable importance in projection (VIP) scores was determined to further differentiate four chestnut tree species from their VOC profiles. The VOCs that contributed to the most variance amongst four tree species with VIP > 1 from PLS-DA combined with nonparametric ANOVA ($p < 0.05$) are listed in Table 1.

The K-means algorithm divides the data into a defined number of clusters (K). In this study, four clusters were defined (K = 4) and listed in Table 2. Cluster (1) only included two samples from B_3F_3 and two Chinese chestnut samples from the second independent test. Cluster (2) only contains the mixed samples of hybrids from the first independent test. Cluster (3) included the samples of hybrids from the second test and American chestnut leaf samples. Finally, cluster (4) only contains the VOCs samples of Chinese chestnut leaves.

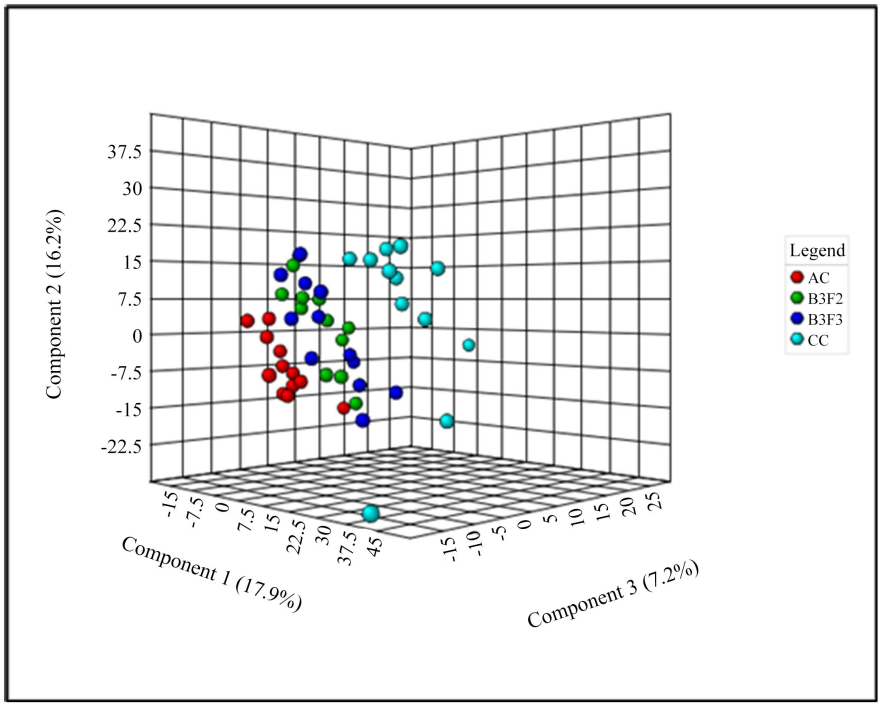

Figure 6. The PLS-DA model with the first 3 components ($R^2X = 0.884$ $R^2Y = 0.917$, Q2 = 0.584) applied to separation VOC profiles from American chestnut (Red, n = 12), B_3F_2 (Green, n = 12), B_3F_3 (Blue, n = 12), and Chinese chestnut (Light blue, n = 12).

K-means clusters help to suggest that VOCs profiles of Chinese chestnut differ from both American chestnut and hybrids. Furthermore, the similarity between hybridized generations is considerably high. However, K-means does not reveal VOCs profile differences between American chestnut and hybrids. In addition to K-means, Hierarchical Cluster Analysis (HCA), aimed to uncover latent structure in terms of a hierarchy of embedded group clusters, was further applied to the dataset [50]. The resulting dendrogram shown in **Figure 7** implies three major clusters organized by HCA, namely A, B, and C with some subclusters.

The first group (A) includes VOCs samples from hybrids, especially from the first independent test, and implied that B_3F_2 and B_3F_3 shared similar VOCs profiles. The second group (B) contains two subclusters, B_1 and B_2. B_1 was mainly composed of American chestnut leaf samples, while B_2 primarily consisted of the hybrids. Finally, cluster C included mostly samples of Chinese chestnut except for a few B_3F_2 samples from the second independent test and an American chestnut sample.

4. Discussion

Phenolic compounds are important secondary metabolites involved in plant protection against pathogens and pathogen resistance [62]. Therefore, knowing the total phenolic content of American, Chinese, B_3F_2, and B_3F_3 could aid in the restoration of American chestnut and improve understanding of the differences between parental species and hybrids bred for resistance. However, an optimized extraction method needed to be established that takes into the chestnut's phenolic structural complexity and distribution diversity.

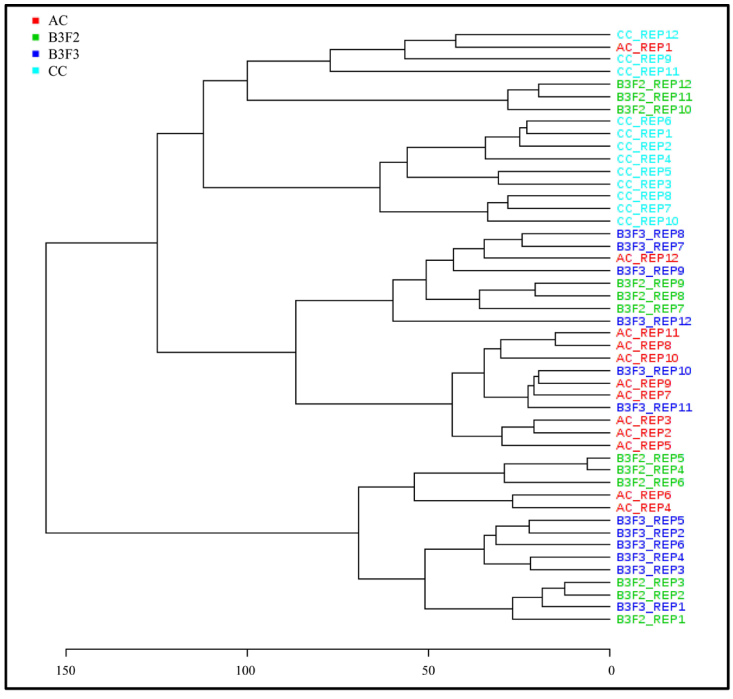

Figure 7. The dendrogram of VOCs from chestnut tree leaf tissue. VOC profiles from American chestnut (Red, n = 12), B_3F_2 (Green, n = 12), B_3F_3 (Blue, n = 12), and Chinese chestnut (Light blue, n = 12).

In this study, extraction variables including solvent type, solvent/mass ratio, pH, and extraction kinetics were examined by colorimetric reaction with the Folin-Ciocalteu reagent and UV/Vis spectrophotometric method. It should be noted that the higher temperature and longer extraction time yield greater phenolic content [48]. However, according to Dai *et al.*, phenolic compounds can be easily hydrolyzed and oxidized over time [20]. Therefore, in this study, the time of extraction was not extended beyond 24 h, and the extraction temperatures above ambient were not considered.

Conventional organic solvents such as methanol, ethanol, ethyl acetate, and acetone/water were selected using a solvent/mass ratio from 20:1 to 200:1 [44] [63] [64]. The highest phenolic extraction content was obtained using ethyl acetate. However, this extraction with ethyl acetate is not significantly different ($p > 0.05$) from the extractions with other solvents. The best extraction solvent/mass ratio was 150:1 among all the tested solvents.

Acidification enhanced plant phenolic extractions when using solvents such as methanol, ethanol, propanol, and ethyl acetate. Lafka *et al.* argued that acidification of the extraction system can increase the solubility of phenolics, increase the disintegration of plant tissue cell walls, and improve the stability of phenolics [28]. Our study indicated that acidification improved the extraction capacity of methanol, however, it negatively impacted the extraction from both ethyl acetate and the acetone/water systems.

Kinetic studies are often used to describe phenolic extraction properties. For example, Cevallos-Casals *et al.* indicated the phenolic antioxidants extracted from Andean purple corn and sweet potato follows second-order kinetic [64], Spigno *et al.* investigated extraction kinetic in their study of grape marc phenolics [65], and Bucic-Kojic *et al.* employed Peleg's kinetic in solid/liquid polyphenols extraction from grape seeds [48]. In this study, Peleg's, second-order, Elovich and power models were applied to interpret extraction kinetics with second-order kinetics providing the best fits (R^2 over 0.99) for all tested solvents.

Plant phenolic extraction that follows a second-order kinetic process takes place in two subsequent phases. The fast phase occurs early, where the majority of phenolics get extracted quickly to the washing by the solvent. This is followed by a slower phase where the extraction process becomes steady and driven by diffusion [64]. The power law model describes the diffusion process during the extraction. The diffusion exponent n calculated from experimental data was less than 0.5 ($n < 0.5$), which indicates that Fickian diffusion [66] predominates during leaf tissue extraction.

The total phenolic content from American chestnut, Chinese chestnut, and their backcrossing generations B_3F_2 and B_3F_3 has been determined. The highest phenolic content was found in Chinese chestnut trees, the lowest was in the American chestnut, and the two backcross hybrids showed intermediate levels (**Figure 4**). Student t-test suggests that the difference between Chinese chestnut, and other tree species were significant ($p < 0.05$). This suggests that phenolic

content may be used to distinguish Chestnut species and hybrids, that are differentiated by blight resistance, Steiner *et al.* and Diskin *et al.* found patterns of blight resistance rankings and morphological distinctions among species and hybrids similar to our findings of phenolic compound content, notably that the backcross hybrids had intermediate characteristics to the parental species [67] [68].

This study also extended the investigation of VOCs profiles from different tree species and hybrids by untargeted volatile metabolomics. Studies have demonstrated VOCs emitted from plants can be used for discrimination of species. For example, Rambla *et al.* showed that the volatile compounds from hybrids significantly differ from their parents [38]. In the present study, a total number of 66 volatile compounds were identified from all tree species.

It should be noted that strong emission of cis-3-hexenyl acetate, a compound that plays an important role in a plants' defense system, was found amongst all samples. Frost *et al.* showed that emission of cis-3-hexenyl acetate induces jasmonic acid synthesis and activates upstream hydroperoxidation which primes leave for rapid response to subsequent herbivory [69]. In addition, grass alcohols 3-hexen-1-ol and (E)-2-hexen-1-ol and a number of sesquiterpenes were identified in the volatile extract, including β-phellandrene, limonene, δ-elemene, α-farnesene, caryophyllene, sequalene, aristolene, seychellene, dihydromyrcene, cis-α-bisabolene, β-pinene, β-humulene, β-panasinsene, ylangene, and α-cubebene. Previous studies suggest the primary function of these varied sesquiterpenes is to deliver messages. This signaling function was found in both plant-microbe interactions [70] and in plant-plant interactions [71].

Almost all volatile metabolites associated with Chinese chestnut were reported to have antifungal or antimicrobial activity individually or constituent components of essential oil extracts (see **Table 1**). However, the 3 native chestnut metabolites did not show direct antifungal activity except for 2-nonen-1-ol, (E)-2-pentene, and y-cardinene from the hybrid chestnuts were reported to have antifungal efficacy properties.

The detection of variation in VOCs profiles from four tree species was highly dependent on robust statistical methods. The ANOVA test, which is a univariant analysis method, initially identified 18 VOCs as significantly different among tree species. Further analysis required chemometric methods, and the results from this multivariate modeling suggest that the separation of VOCs profiles can be established between American and Chinese chestnut, American chestnut and the hybrids, and Chinese chestnut and the hybrids. However, poor separations were obtained from the hybrids' (B_3F_2 and B_3F_3) VOC profiles. This supports the hypothesis that VOCs from hybrids are different from the parental species.

The differences between the parental species and the B_3F_3 generation supports previous evidence that traditional breeding has not yet reached the goal of obtaining American chestnut characteristics while maintaining blight resistance of the Chinse chestnut [43] [57]. Similar behavior in VOCs profiles involving hy-

brids, and their parental species have been observed in Citrus fruit [38] and peach tree siblings [72]. Moreover, the combination of univariate and PLS-DA approaches can be utilized when handling biological data [73] [74]. Therefore, the VIP from PLS-DA and p-values < 0.05, and the false discovery rates were calculated. As a result, 13 volatile chemicals showed significantly higher or lower levels among species.

Xuan *et al.* argued that using a set of data from complex biological samples may provide better discrimination power and more useful information [75]. Therefore, the identification of biomarkers was not considered in this study. The algorithms of cluster analysis focused on dividing data objects into groups or clusters based on shared common characteristics [76].

Two extensively applied cluster analysis methods in metabolomics, non-hierarchical K-means, and HCA were used in this study. The observation of K-means (**Table 2**) suggested the existence of a high similarity of VOCs collected from hybridized generations. In addition, compared to the other tree species, the VOCs profiles of Chinese chestnut leaves differ. However, K-means does not explain the variation between American chestnut leaves VOCs and the hybrids. This phenomenon could be explained by the genotype intimacy between American chestnut and the hybrids since both B_3F_2 and B_3F_3 carried 15/16 portion of American chestnut genes.

HCA was further employed for clustering the individual sample using Euclidian distance and Ward's linkage method. The resulting clusters were represented in a dendrogram to indicate the similarity and distance of each sample in the dataset. HCA results suggest that VOC samples collected from parental species differ from their breeding generations. Clear separation of VOCs profiles can also be made between American chestnut and Chinese chestnut. However, a high similarity of VOCs profiles was found in the two hybrids. HCA was consistent with the PLS-DA results, where clear separation can be made between parental chestnut tree species while poor separation was made between hybrids.

5. Conclusions

In the present study, we have established an extraction method to determine the total phenolic content from chestnut tree leaf samples. Extraction conditions were optimized for solvent type, solvent/mass ratio, pH and extraction time. Methanol was selected as an appropriate solvent for the extraction. The optimized solvent/mass ratio, extraction time, and pH were 100:1, 24 hours, and pH = 2, respectively. The highest conventional phenolic content was found in the Chinese chestnut, while the lowest was in the American chestnut. The simple analytical methods described here along with chemometrics have proven to be a powerful tool in chestnut hybrid discrimination.

This study also demonstrated the potential of using plant leaf tissue VOCs profiles to discriminate between American, Chinese, and their hybrids B_3F_2 and B_3F_3 via non-destructive headspace SPME sampling with untargeted volatile

metabolomics. A total of 67 VOCs was identified from all tree species. A strong emission of cis-3-hexenyl acetate was found in all tested samples. Although there were high similarities among tree species' VOCs profiles, distinctions can be approached using chemometric analysis. A PLS-DA model showed that, compared with their parents, the VOCs from hybrids plant leaf is significantly different. The variations of thirteen VOCs among tree leaf samples were considered significant. The similarities of samples were analyzed and visualized by clustering analysis such as K-means and HCA. Results from this study provide a feasible and useful method to rapidly classify four chestnut tree species using a small amount of leaves. The results from the study indicate that the advanced breeding generation (BC_3F_3) had markedly lower phenolic compounds than the Asian parent, which may be indicative of a reduced disease defense mechanism, as has been exhibited in other species. The BC_3F_3 did not exhibit VOC leaf chemistry similar to the American parent, suggesting a departure from desired traits of having similar physiology/morphology of the American chestnut in all ways except blight resistance. However, results indicated slight improvements from traditional breeding in phenolic compound content. Future research using leaf chemistry may provide a better understanding of breeding effects on American chestnut restoration.

Acknowledgements

Appreciation is extended to Highlands Biological Station for financial support as grants-in-aid for Richard Baird from 2014-2016.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplemental Material

Section 1

Extraction Kinetics

The hyperbolic model, Peleg's model is expressed as:

$$C = \frac{t}{K_1 + K_2 t} \tag{1}$$

where C is the concentration at time t, K_1 is the initial extraction rate at $t = 0$, and K_2 is the maximum extraction yield.

The second-order kinetic model can be described by Equation (2):

$$\frac{dC_t}{dt} = k\left(C_s - C_t\right)^2 \tag{2}$$

where: k is the extraction rate constant, C_s is the extraction capacity, and C_t is the concentration of phenolics in the solution at any time. Its linear form can be expressed:

$$\frac{C_t}{t} = \frac{1}{\left(\frac{1}{kC_s^2}\right) + \left(\frac{t}{C_s}\right)} \tag{3}$$

The initial extraction rate is represented as h, where

$$h = kC_s^2 \tag{4}$$

Finally, Equation (4) can be rearranged as

$$C_t = \frac{t}{\frac{1}{h} + \frac{t}{C_s}} \tag{5}$$

The Elovich model is shown in Equation (6):

$$\frac{dq}{dt} = \alpha e^{-\beta q} \tag{6}$$

where q is the amount of absorbance at time t, and α, β are constants. Its linear form can be expressed as

$$q = \frac{1}{\beta}\ln \alpha\beta + \frac{1}{\beta}\ln\left(t\right) \tag{7}$$

Under the diffusion controlled mechanism, the extracted amount can be described by the power law model:

$$C = Bt^n \tag{8}$$

where C is a dimensionless quantity, B is a constant that describes the particle-active substance system, and n is the diffusion exponent.

Table SM1. Kinetic models of phenolics extraction from chestnut tree leaves.

Model	Methanol		Ethanol		Ethyl Acetate		Acetone/water	
Peleg	Value	R^2	Value	R^2	Value	R^2	Value	R^2
K_1	0.0216	0.7174	0.0146	0.904	0.0229	0.923	0.0201	0.729
K_2	0.0877		0.0854		0.0747		0.0837	
Second-order	Value	R^2	Value	R^2	Value	R^2	Value	R^2
C_s	14.144	0.99	12.24	0.999	14.245	0.999	13.908	0.996
h	10.905		35.971		25.316		15.291	
k	0.055		0.24		0.125		0.079	
Elovich	Value	R^2	Value	R^2	Value	R^2	Value	R^2
α	5.78E+02	0.932	7.93E+04	0.949	1.45E+03	0.924	9.64E+02	0.932
β	0.706		1.173		0.693		0.722	
Power	Value	R^2	Value	R^2	Value	R^2	Value	R^2
B	8.579	0.966	9.737	0.937	9.907	0.905	9.09	0.949
n	0.135		0.081		0.129		0.128	

Table SM2. (a) VOCs profiles of chestnut genotypes; (b) VOCs profiles of chestnut genotypes for CC; (c) VOCs profiles of chestnut genotypes for B_3F_2.

(a)

rt	Chemical name	Category	B3F3_REP1	B3F3_REP2	B3F3_REP3	B3F3_REP4	B3F3_REP5	B3F3_REP6	B3F3_REP7	B3F3_REP8	B3F3_REP9	B3F3_REP10	B3F3_REP11	B3F3_REP12
			B3F3	B3F3	B3F3	B3F3	B3F3	B3F3	B3F3	B3F3	B3F3	B3F3	B3F3	B3F3
3.773	Ethanol	alcohol	0.017	0.018	0.007	0.011	0.006	0.009	0.004	0.001	0.013	0.007	0.010	0.000
4.609	Acetone	ketones	0.037	0.070	0.031	0.019	0.014	0.011	0.009	0.005	0.026	0.008	0.009	0.000
4.634	2-Butynal	aldehyde	0.294	0.349	0.550	0.965	0.261	0.314	0.030	0.027	0.024	0.034	0.030	0.000
4.888	(E)-1,3-Pentadiene	alkene	0.004	0.002	0.007	0.008	0.000	0.002	0.001	0.001	0.000	0.001	0.000	0.000
5.778	Methyl acetate	ester	0.064	0.073	0.120	0.216	0.064	0.058	0.010	0.014	0.006	0.005	0.008	0.000
5.904	(Z)-1,3-Pentadiene	alkene	0.056	0.047	0.098	0.082	0.021	0.033	0.006	0.032	0.013	0.054	0.064	0.000
6.808	2-Methylbutadiene	alkene	0.026	0.033	0.022	0.030	0.037	0.025	0.014	0.016	0.040	0.017	0.005	0.000
7.472	Isobutyraldehyde	aldehyde	0.018	0.012	0.065	0.107	0.032	0.014	0.002	0.003	0.000	0.004	0.010	0.000
7.956	Acetic acid	Organic Acid	0.158	0.111	0.384	0.722	0.110	0.263	0.058	0.060	0.032	0.126	0.193	0.000
8.311	Ethyl Acetate	ester	0.479	0.349	1.120	0.430	0.117	0.085	0.109	0.133	0.045	0.010	0.024	0.000
8.749	Furan, 2-methyl-	Furan	0.012	0.000	0.012	0.002	0.000	0.007	0.010	0.002	0.000	0.000	0.000	0.000
10.933	3-Methyl-3-buten-1-ol	alcohol	0.008	0.004	0.032	0.027	0.005	0.011	0.003	0.002	0.000	0.005	0.004	0.000
10.950	2,4-Hexadiene, Z,Z-	alkene	0.118	0.051	0.420	0.312	0.083	0.089	0.040	0.024	0.014	0.060	0.069	0.000
11.724	1-Penten-3-one	ketones	0.183	0.076	0.633	1.200	0.355	0.405	0.037	0.029	0.011	0.068	0.093	0.003
11.777	1-Penten-3-ol	alcohol	0.074	0.074	0.058	0.103	0.062	0.037	0.016	0.003	0.000	0.004	0.000	0.000
11.893	1,3-Hexadien-5-yne	alkyne	0.142	0.084	0.348	0.552	0.189	0.076	0.038	0.035	0.000	0.085	0.069	0.000
12.495	1,3,5-Hexatriene, Z-	alkene	0.174	0.127	0.333	0.354	0.129	0.077	0.180	0.140	0.070	0.350	0.401	0.007
12.909	3-Vinyl-1-cyclobutene	alkene	0.007	0.000	0.025	0.021	0.003	0.011	0.009	0.000	0.000	0.015	0.011	0.000
14.104	3-Pentanone	ketones	0.002	0.000	0.007	0.000	0.000	0.000	0.007	0.000	0.000	0.007	0.005	0.000
14.630	3-Pentanol	alcohol	0.011	0.008	0.030	0.031	0.013	0.011	0.000	0.000	0.000	0.006	0.008	0.000
14.780	Furan, 2-ethyl-	furan	0.021	0.016	0.026	0.018	0.012	0.000	0.005	0.007	0.005	0.006	0.003	0.000

Continued

15.215	Vinylfuran	Furan	0.049	0.042	0.039	0.036	0.000	0.020	0.043	0.027	0.015	0.050	0.044	0.000
15.728	1,4-Pentadiene	alkene	0.017	0.015	0.032	0.042	0.014	0.013	0.046	0.024	0.023	0.035	0.049	0.004
16.226	ethyl 2-methylbutanoate	ester	0.006	0.000	0.000	0.000	0.000	0.000	0.044	0.032	0.087	0.033	0.029	0.000
17.634	Cyclohexane	alkane	0.102	0.038	0.223	0.287	0.117	0.174	0.000	0.000	0.000	0.016	0.020	0.000
17.913	3-Hexenal	aldehyde	0.105	0.027	0.182	0.138	0.042	0.042	0.061	0.055	0.113	0.031	0.012	0.079
18.877	3-Hexen-1-ol	alcohol	5.840	6.050	6.140	6.290	5.550	5.540	4.490	3.950	3.750	4.970	5.580	1.240
18.900	(E)-2 hexenal	aldehyde	0.581	0.493	0.561	0.384	0.323	0.334	0.499	0.439	0.946	0.498	0.352	0.146
19.226	heptanal	aldehyde	0.180	0.083	0.322	1.200	0.683	0.665	0.015	0.013	0.012	0.029	0.042	0.009
19.509	Octane	alkane	0.508	0.427	0.600	0.493	0.325	0.388	0.573	0.417	0.391	0.477	0.681	0.227
20.059	alph-pinene	monterpene	0.310	0.231	0.289	0.162	0.102	0.151	0.634	0.562	1.020	0.373	0.375	0.255
21.220	4-Hexen-1-ol, acetate	ester	0.109	0.077	0.105	0.198	0.209	0.000	0.010	0.000	0.000	0.009	0.018	0.059
21.645	Pentyl acetate	ester	0.411	0.365	0.402	0.216	0.191	0.236	0.116	0.125	0.085	0.124	0.112	0.067
21.854	1-octen-3-ol	Alcohol	0.060	0.000	0.097	0.081	0.000	0.000	0.098	0.012	0.000	0.066	0.023	0.050
22.103	.beta.-Phellandrene	sesquiterpenes	0.037	0.024	0.041	0.013	0.014	0.016	0.034	0.038	0.012	0.016	0.000	0.000
22.296	1,3,7-Octatriene, 3,7-dimethyl-	alkene	0.000	0.000	0.005	0.000	0.000	0.000	0.004	0.006	0.000	0.000	0.000	0.008
22.524	ethyl hexanoate	ester	0.377	0.276	0.390	0.661	0.645	0.687	0.114	0.109	0.041	0.124	0.139	0.063
22.857	(2Z)-2-Pentenyl acetate	ester	0.027	0.007	0.049	0.010	0.000	0.002	0.000	0.000	0.000	0.124	0.000	0.036
22.904	2-Pentene, E-	alkene	0.016	0.000	0.026	0.000	0.000	0.007	0.000	0.000	0.000	0.000	0.000	0.009
23.556	p-Xylene	aromatic hydrocarbons	0.000	0.000	0.000	0.000	0.000	0.011	0.193	0.116	0.000	0.175	0.139	0.007
24.773	Styrene	aromatic hydrocarbons	0.205	0.236	0.136	0.193	0.320	0.256	0.005	0.000	0.000	0.012	0.022	0.006
25.187	3-Methyl-3-buten-1-ol, acetate	ester	0.032	0.019	0.039	0.027	0.018	0.013	0.006	0.009	0.014	0.005	0.009	0.044
27.756	2,3-pentanedione	ketones	0.052	0.067	0.091	0.242	0.049	0.054	0.027	0.019	0.059	0.030	0.026	0.139

Continued

27.797	1-Octanol	alcohol	0.312	0.413	0.334	0.333	0.373	0.379	0.171	0.151	0.083	0.211	0.206	0.149
28.026	cis-3-Hexenyl acetate	ester	9.700	11.100	8.280	8.380	11.500	10.500	9.580	9.790	21.200	13.800	14.100	16.900
28.352	2-hexen-1-ol, acetate	ester	1.750	1.750	1.290	0.698	0.963	1.100	4.980	3.830	3.690	2.960	3.510	2.740
28.418	2,4-Hexadienal, E,E-	aldehyde	2.680	2.580	1.860	1.060	1.400	1.550	3.110	5.200	2.160	3.010	2.110	3.310
28.762	ethyl octanoate	ester	0.006	0.017	0.021	0.012	0.037	0.000	0.000	0.006	0.000	0.011	0.009	0.017
29.395	2-Nonen-1-ol	alcohol	0.034	0.018	0.011	0.012	0.021	0.037	0.014	0.010	0.000	0.007	0.025	0.007
29.847	4-Methyl-1,5-Heptadiene	alkene	0.014	0.011	0.025	0.015	0.030	0.030	0.000	0.000	0.000	0.008	0.006	0.000
31.644	.gamma.-Elemene	sesquiterpenes	0.053	0.065	0.025	0.000	0.000	0.056	0.042	0.046	0.000	0.035	0.036	0.014
34.670	.alpha.-Farnesene	sesquiterpenes	0.007	0.007	0.015	0.010	0.005	0.008	0.001	0.005	0.009	0.003	0.004	0.017
34.930	Caryophyllene	sesquiterpenes	0.009	0.010	0.000	0.000	0.000	0.000	0.027	0.085	0.199	0.128	0.194	0.025
35.675	geranyl acetate	ester	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.004	0.000
36.768	(-)-Aristolene	sesquiterpenes	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.003	0.003	0.000
37.186	2-Methyl-1-phenylbut-3-en-1-ol	alcohol	0.054	0.057	0.017	0.018	0.060	0.061	0.008	0.012	0.011	0.009	0.009	0.003
37.516	Dodecane	alkane	0.042	0.076	0.072	0.134	0.055	0.049	0.052	0.073	0.056	0.255	0.215	0.231
37.804	Seychellene	sesquiterpenes	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.004	0.005	0.000
38.337	.alph.humulene	sesquiterpenes	0.319	0.309	0.104	0.094	0.279	0.237	0.207	0.247	0.735	0.154	0.175	0.127
38.733	valencene	sesquiterpenes	0.005	0.009	0.000	0.000	0.005	0.003	0.019	0.017	0.050	0.005	0.016	0.000
39.917	cis-.alpha.-Bisabolene	sesquiterpenes	0.074	0.089	0.019	0.022	0.097	0.071	0.010	0.018	0.052	0.006	0.008	0.017
42.643	.gamma.-cadinene	sesquiterpenes	0.017	0.014	0.004	0.000	0.013	0.010	0.000	0.000	0.000	0.000	0.023	0.000
42.827	.beta.-Humulene	sesquiterpenes	0.066	0.069	0.060	0.199	0.066	0.044	0.059	0.052	0.072	0.072	0.079	0.326
43.936	.beta.-Panasinsene	sesquiterpenes	0.001	0.000	0.001	0.001	0.001	0.000	0.001	0.000	0.002	0.000	0.001	0.002
45.083	Ylangene	sesquiterpenes	0.033	0.043	0.000	0.020	0.054	0.000	0.017	0.028	0.039	0.055	0.105	0.135
45.838	.alpha.-Cubebene	sesquiterpenes	0.184	0.094	0.073	0.352	0.091	0.057	0.239	0.034	0.067	0.022	0.042	0.324
47.730	Octadecane	alkane	1.390	0.094	0.073	0.390	0.097	0.055	0.143	0.000	0.000	0.000	0.000	0.208

(b)

rt	Chemical name	Category	CC_REP1	CC_REP2	CC_REP3	CC_REP4	CC_REP5	CC_REP6	CC_REP7	CC_REP8	CC_REP9	CC_REP10	CC_REP11	CC_REP12
			CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC
3.773	Ethanol	alcohol	0.081	0.016	0.009	0.028	0.014	0.083	0.003	0.018	0.010	0.050	0.086	0.028
4.609	Acetone	ketones	0.494	0.053	0.004	0.124	0.000	0.448	0.052	0.078	0.000	0.099	0.000	0.134
4.634	2-Butynal	aldehyde	0.184	0.166	0.132	0.094	0.566	0.081	0.023	0.000	0.000	0.000	0.000	0.185
4.888	(E)-1,3-Pentadiene	alkene	0.005	0.000	0.006	0.004	0.013	0.004	0.008	0.006	0.000	0.011	0.000	0.000
5.778	Methyl acetate	ester	0.023	0.015	0.025	0.014	0.000	0.032	0.000	0.000	0.000	0.000	0.000	0.000
5.904	(Z)-1,3-Pentadiene	alkene	0.053	0.050	0.140	0.048	0.153	0.034	0.017	0.022	0.000	0.063	0.000	0.033
6.808	2-Methylbutadiene	alkene	0.018	0.021	0.061	0.032	0.589	0.032	0.025	0.023	0.000	0.018	0.029	0.091
7.472	Isobutyraldehyde	aldehyde	0.006	0.001	0.001	0.012	0.150	0.005	0.000	0.000	0.000	0.000	0.000	0.122
7.956	Acetic acid	Organic Acid	0.095	0.039	0.266	0.135	2.460	0.117	0.022	0.033	0.063	0.061	0.000	0.540
8.311	Ethyl Acetate	ester	0.345	0.077	0.000	0.136	0.000	0.287	0.012	0.012	0.000	0.033	0.000	0.010
8.749	Furan, 2-methyl-	Furan	0.015	0.000	0.003	0.020	0.000	0.002	0.003	0.007	0.000	0.000	0.000	0.000
10.933	3-Methyl-3-buten-1-ol	alcohol	0.008	0.000	0.017	0.031	0.050	0.006	0.005	0.000	0.000	0.010	0.000	0.028
10.950	2,4-Hexadiene, Z,Z-	alkene	0.106	0.065	0.304	0.596	0.340	0.111	0.092	0.106	0.135	0.096	0.000	0.394
11.724	1-Penten-3-one	ketones	0.068	0.053	0.213	0.643	0.287	0.114	0.057	0.053	0.012	0.022	0.000	0.207
11.777	1-Penten-3-ol	alcohol	0.057	0.047	0.057	0.094	0.671	0.068	0.008	0.000	0.000	0.010	0.000	0.000
11.893	1,3-Hexadien-5-yne	alkyne	0.089	0.041	0.056	0.341	0.096	0.118	0.028	0.032	0.055	0.047	0.000	0.170
12.495	1,3,5-Hexatriene, Z-	alkene	0.356	0.298	1.030	0.552	1.770	0.266	0.258	0.269	0.218	0.739	0.000	0.573
12.909	3-Vinyl-1-cyclobutene	alkene	0.011	0.008	0.049	0.023	0.104	0.011	0.004	0.007	0.035	0.054	0.000	0.000
14.104	3-Pentanone	ketones	0.002	0.000	0.000	0.015	0.000	0.006	0.008	0.014	0.000	0.046	0.000	0.008
14.630	3-Pentanol	alcohol	0.011	0.012	0.034	0.011	0.028	0.011	0.003	0.002	0.000	0.007	0.000	0.000
14.780	Furan, 2-ethyl-	furan	0.028	0.015	0.027	0.015	0.045	0.017	0.003	0.003	0.047	0.008	0.000	0.056
15.215	Vinylfuran	Furan	0.021	0.019	0.000	0.069	0.000	0.017	0.010	0.020	0.000	0.057	0.000	0.055

Continued

15.728	1,4-Pentadiene	alkene	0.029	0.079	0.143	0.133	0.081	0.064	0.287	0.557	0.271	0.796	0.000	0.467
16.226	ethyl 2-methylbutanoate	ester	0.020	0.035	0.000	0.061	0.000	0.023	0.033	0.038	0.000	0.421	0.000	0.078
17.634	Cyclohexane	alkane	0.009	0.016	0.031	0.099	0.023	0.012	0.005	0.005	0.000	0.000	0.000	0.000
17.913	3-Hexenal	aldehyde	0.129	0.095	0.153	0.063	0.771	0.097	0.099	0.049	0.223	0.059	0.000	0.246
18.877	3-Hexen-1-ol	alcohol	5.980	5.680	6.890	5.380	3.180	6.030	3.950	4.180	4.150	11.500	9.930	4.330
18.900	(E)-2 hexenal	aldehyde	0.553	1.010	0.183	1.210	0.207	1.320	1.760	3.490	2.120	1.430	0.000	5.020
19.226	heptanal	aldehyde	0.005	0.020	0.029	0.206	0.317	0.014	0.000	0.000	0.000	0.000	0.000	0.000
19.509	Octane	alkane	0.823	0.681	1.130	0.921	0.170	0.813	3.440	3.810	1.020	1.620	0.553	2.450
20.059	alph-pinene	monterpene	0.235	0.223	0.081	0.850	0.051	0.586	0.925	0.782	1.260	0.725	0.000	3.690
21.220	4-Hexen-1-ol, acetate	ester	0.000	0.000	0.000	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.000	0.000
21.645	Pentyl acetate	ester	0.049	0.055	0.026	0.113	0.000	0.037	0.008	0.010	0.000	0.006	0.000	0.000
21.854	1-octen-3-ol	Alcohol	0.061	0.112	0.065	0.224	0.000	0.111	0.229	0.196	0.000	0.472	0.000	0.267
22.103	.beta.-Phellandrene	sesquiterpenes	0.003	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
22.296	1,3,7-Octatriene, 3,7-dimethyl-	alkene	0.000	0.000	0.000	0.001	0.000	0.000	0.016	0.009	0.000	0.000	0.000	0.000
22.524	ethyl hexanoate	ester	0.266	0.194	0.162	0.497	0.059	0.570	0.174	0.148	0.000	0.085	0.000	0.041
22.857	(2Z)-2-Pentenyl acetate	ester	0.000	0.051	0.022	0.065	0.000	0.044	0.090	0.000	0.000	0.337	0.000	0.071
22.904	2-Pentene, E-	alkene	0.000	0.024	0.019	0.063	0.000	0.021	0.084	0.000	0.000	0.521	0.000	0.000
23.556	p-Xylene	aromatic hydrocarbons	0.000	0.000	0.000	0.131	0.000	0.039	0.148	0.120	0.000	0.000	0.000	0.034
24.773	Styrene	aromatic hydrocarbons	0.037	0.035	0.000	0.086	0.000	0.074	0.012	0.009	0.000	0.020	0.000	0.000
25.187	3-Methyl-3-buten-1-ol, acetate	ester	0.027	0.022	0.037	0.033	0.162	0.021	0.011	0.004	0.000	0.007	0.000	0.000
27.756	2,3-pentanedione	ketones	0.050	0.043	0.070	0.045	0.180	0.000	0.034	0.029	0.183	0.050	0.000	0.146
27.797	1-Octanol	alcohol	0.415	0.575	0.472	0.165	0.030	0.281	0.070	0.057	0.000	0.049	0.000	0.000

Continued

28.026	cis-3-Hexenyl acetate	ester	8.790	10.900	9.950	8.360	0.000	9.670	11.100	11.500	2.250	14.000	12.200	3.540
28.352	2-hexen-1-ol, acetate	ester	1.980	2.300	1.850	0.000	0.290	1.490	10.600	8.100	1.260	0.628	0.000	0.000
28.418	2,4-Hexadienal, E,E-	aldehyde	2.370	2.350	0.000	0.808	0.000	2.200	6.310	7.880	1.590	0.000	0.000	1.650
28.762	ethyl octanoate	ester	0.025	0.050	0.019	0.000	0.038	0.031	0.006	0.000	0.000	0.000	0.000	0.030
29.395	2-Nonen-1-ol	alcohol	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000
29.847	4-Methyl-1,5-Heptadiene	alkene	0.032	0.038	0.017	0.066	0.003	0.030	0.004	0.000	0.055	0.000	0.000	0.059
31.644	.gamma.-Elemene	sesquiterpenes	0.082	0.060	0.037	0.030	0.000	0.062	0.010	0.000	0.000	0.000	0.000	0.000
34.670	.alpha.-Farnesene	sesquiterpenes	0.003	0.005	0.011	0.006	0.051	0.005	0.005	0.002	0.026	0.004	0.000	0.013
34.930	Caryophyllene	sesquiterpenes	0.000	0.019	0.000	0.029	0.026	0.000	0.022	0.000	2.420	0.000	0.000	4.480
35.675	geranyl acetate	ester	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.180	0.000	0.000	0.026
36.768	(-)-Aristolene	sesquiterpenes	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.202	0.000	0.000	0.124
37.186	2-Methyl-1-phenylbut -3-en-1-ol	alcohol	0.002	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
37.516	Dodecane	alkane	0.049	0.084	0.102	0.306	0.299	0.078	0.018	0.028	0.201	0.067	0.000	0.251
37.804	Seychellene	sesquiterpenes	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.244	0.000	0.000	0.110
38.337	.alph.humulene	sesquiterpenes	0.973	0.783	0.383	0.431	0.047	0.613	0.146	0.191	0.116	0.072	1.000	0.145
38.733	valencene	sesquiterpenes	0.045	0.023	0.000	0.064	0.000	0.081	0.009	0.018	0.000	0.003	0.000	0.198
39.917	cis-.alpha.-Bisabolene	sesquiterpenes	0.186	0.197	0.060	0.178	0.032	0.278	0.020	0.028	0.084	0.028	0.112	0.131
42.643	.gamma.-cadinene	sesquiterpenes	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
42.827	.beta.-Humulene	sesquiterpenes	0.043	0.026	0.102	0.407	0.306	0.113	0.053	0.063	0.231	0.036	0.000	0.104
43.936	.beta.-Panasinsene	sesquiterpenes	0.001	0.000	0.001	0.003	0.003	0.001	0.000	0.000	0.000	0.000	0.000	0.001
45.083	Ylangene	sesquiterpenes	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.270
45.838	.alpha.-Cubebene	sesquiterpenes	0.087	0.030	0.151	0.480	0.421	0.214	0.019	0.030	0.000	0.079	0.000	0.098
47.730	Octadecane	alkane	0.504	0.043	0.185	0.459	0.469	0.245	0.000	0.000	0.000	0.027	0.000	0.000

(c)

rt	Chemical name	Category	B3F2_REP1	B3F2_REP2	B3F2_REP3	B3F2_REP4	B3F2_REP5	B3F2_REP6	B3F2_REP7	B3F2_REP8	B3F2_REP9	B3F2_REP10	B3F2_REP11	B3F2_REP12
			B3F2	B3F2	B3F2	B3F2	B3F2	B3F2	B3F2	B3F2	B3F2	B3F2	B3F2	B3F2
8.311	Ethyl Acetate	ester	0.297	0.997	0.172	2.170	1.660	0.557	0.006	0.000	0.000	0.090	0.045	0.040
8.749	Furan, 2-methyl-	Furan	0.041	0.007	0.026	0.009	0.004	0.011	0.013	0.000	0.000	0.036	0.017	0.045
10.933	3-Methyl-3 -buten-1-ol	alcohol	0.014	0.010	0.013	0.085	0.104	0.049	0.002	0.000	0.000	0.018	0.017	0.013
10.950	2,4-Hexadiene, Z,Z-	alkene	0.159	0.106	0.104	0.982	0.874	0.411	0.050	0.029	0.021	0.137	0.233	0.211
11.724	1-Penten-3-one	ketones	0.097	0.077	0.050	1.370	0.776	0.384	0.026	0.019	0.055	0.199	0.200	0.159
11.777	1-Penten-3-ol	alcohol	0.042	0.040	0.037	0.174	0.047	0.085	0.000	0.000	0.001	0.039	0.000	0.000
11.893	1,3-Hexadien -5-yne	alkyne	0.244	0.223	0.171	1.240	1.150	0.751	0.047	0.044	0.056	0.145	0.226	0.175
12.909	3-Vinyl-1-cyclobutene	alkene	0.008	0.000	0.000	0.018	0.027	0.007	0.006	0.000	0.000	0.025	0.006	0.017
14.104	3-Pentanone	ketones	0.023	0.014	0.020	0.013	0.010	0.000	0.009	0.000	0.000	0.063	0.050	0.019
14.630	3-Pentanol	alcohol	0.006	0.004	0.006	0.020	0.022	0.011	0.000	0.000	0.000	0.004	0.004	0.000
14.780	Furan, 2-ethyl-	furan	0.011	0.007	0.009	0.027	0.028	0.020	0.003	0.005	0.003	0.009	0.019	0.015
15.215	Vinylfuran	Furan	0.112	0.094	0.082	0.120	0.089	0.109	0.050	0.019	0.011	0.094	0.107	0.080
15.728	1,4-Pentadiene	alkene	0.026	0.020	0.023	0.088	0.066	0.074	0.031	0.011	0.008	0.283	0.473	0.175
16.226	ethyl 2-methylbutanoate	ester	0.020	0.005	0.012	0.000	0.000	0.000	0.064	0.069	0.033	0.166	0.173	0.118
17.634	Cyclohexane	alkane	0.141	0.083	0.074	0.749	0.662	0.631	0.000	0.000	0.000	0.039	0.036	0.047
17.913	3-Hexenal	aldehyde	0.166	0.048	0.101	0.144	0.165	0.152	0.004	0.031	0.025	0.010	0.032	0.149
18.877	3-Hexen-1-ol	alcohol	4.490	3.910	3.800	3.680	3.450	3.290	2.680	2.250	1.430	6.780	5.090	4.290
18.900	(E)-2 hexenal	aldehyde	1.220	1.400	1.150	1.050	1.060	1.130	0.417	0.358	0.245	5.850	5.910	3.000
19.226	heptanal	aldehyde	0.147	0.072	0.081	1.840	2.160	1.710	0.000	0.004	0.018	0.318	0.213	0.187
19.509	Octane	alkane	0.407	0.267	0.281	0.449	0.434	0.427	0.137	0.070	0.058	3.820	3.620	1.800
20.059	alph-pinene	monterpene	1.210	0.972	0.940	0.709	0.680	0.756	0.287	0.165	0.089	2.800	3.730	3.130

Continued

21.220	4-Hexen-1-ol, acetate	ester	0.135	0.270	0.217	0.173	0.238	0.330	0.036	0.040	0.029	0.059	0.036	0.022
21.645	Pentyl acetate	ester	0.849	0.647	0.759	0.743	0.717	0.814	0.144	0.159	0.133	0.075	0.034	0.092
21.854	1-octen-3-ol	Alcohol	0.109	0.000	0.062	0.119	0.122	0.119	0.107	0.040	0.049	0.489	0.377	0.161
22.103	.beta.-Phellandrene	sesquiterpenes	0.026	0.021	0.017	0.000	0.000	0.021	0.017	0.018	0.009	0.000	0.000	0.000
22.296	1,3,7-Octatriene, 3,7-dimethyl-	alkene	0.016	0.008	0.003	0.004	0.012	0.001	0.004	0.003	0.001	0.018	0.006	0.008
22.524	ethyl hexanoate	ester	0.448	0.325	0.314	0.344	0.353	0.625	0.075	0.075	0.170	0.079	0.041	0.052
28.026	cis-3-Hexenyl acetate	ester	8.060	8.350	9.080	8.600	10.100	8.800	12.100	14.700	14.200	5.250	2.010	3.370
28.352	2-hexen-1-ol, acetate	ester	3.610	3.160	2.550	1.900	1.960	2.790	0.000	1.980	1.780	1.080	2.370	2.710
28.418	2,4-Hexadienal, E,E-	aldehyde	1.940	2.200	1.790	0.000	0.000	0.000	1.770	1.620	3.320	2.220	1.340	2.540
28.762	ethyl octanoate	ester	0.009	0.024	0.017	0.014	0.004	0.000	0.000	0.010	0.003	0.000	0.017	0.000
29.395	2-Nonen-1-ol	alcohol	0.014	0.007	0.016	0.000	0.000	0.000	0.023	0.000	0.003	0.025	0.000	0.000
31.644	.gamma.-Elemene	sesquiterpenes	0.048	0.048	0.068	0.000	0.000	0.000	0.032	0.025	0.040	0.000	0.000	0.000
34.670	.alpha.-Farnesene	sesquiterpenes	0.006	0.007	0.003	0.022	0.010	0.009	0.005	0.003	0.004	0.005	0.005	0.009
34.930	Caryophyllene	sesquiterpenes	0.019	0.026	0.019	0.041	0.018	0.093	0.043	0.211	0.102	1.250	4.430	6.880

Chemometric analysis of VOCs profiles from chestnut tree species and hybrids

The PCA model derived from GC-MS spectra of all the VOCs samples was applied to the full data set, in which five principal components cumulatively account for 61.7% of the data variation (Figure SM1)

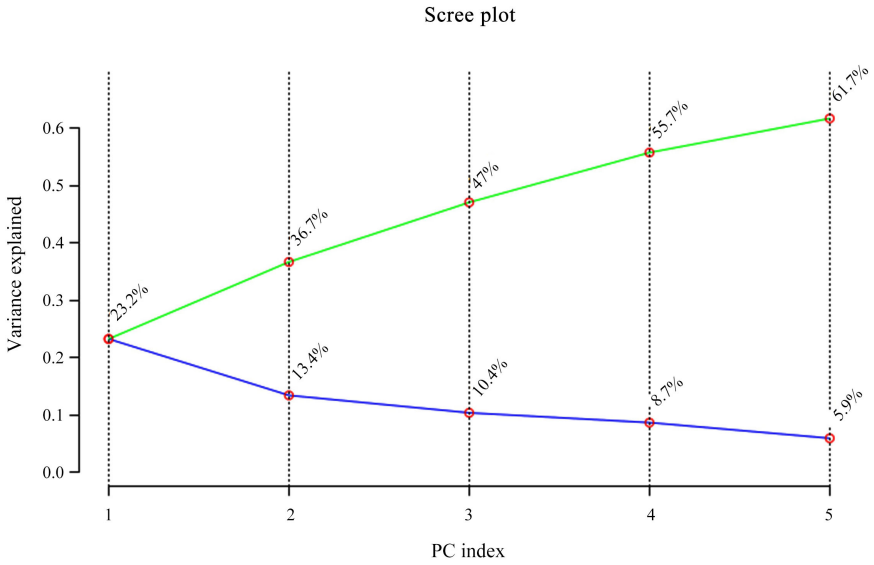

Figure SM1. The variations that explained by PCs from PCA modeling of VOC profiles from American, Chinese and two backcross hybrids generations of chestnut.