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Building a Metabolite Database of Walnuts for Authenticity and Quality Analysis

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Building a Metabolite Database of Walnuts for Authenticity and Quality Analysis

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ABSTRACT

The California walnut industry faces some challenges, including walnut quality deterioration during the supply chain, cultivar variation, origin fraud, kernel skin darkening, and safety concerns of the byproducts. Walnuts have a high lipid content and most lipids in walnuts contain polyunsaturated fatty acids that are readily oxidized. A higher degree of oxidation is commonly associated with lower quality and increased rancidity. To understand the level of quality degradation during the supply chain, some Californian walnuts sold at retail, in the United States and overseas, were collected and analyzed, and then their quality parameters were compared to those sourced directly from handlers. The data showed that many Californian walnuts sold at retail have undergone oxidation. Many showed higher concentrations of primary oxidation products, indicated by a higher K_{232} index, than those from handlers. Some also showed higher levels of secondary oxidation products, indicated by a high K_{268} index and high concentrations of some volatiles. Several samples even showed high concentrations of volatile 2-pentyl-furan, a tertiary oxidation product. Decreased antioxidant capacity is also apparent in many walnuts sold at retail. These findings suggested that a guideline for minimizing oxidation in walnuts during the supply chain was needed for quality improvement.

Because Californian walnuts comprise various cultivars, particularly Chandler, Howard, and Tulare, a thorough assessment of their quality was needed to help farmers and handlers select cultivars and to determine if the metabolites could be used for cultivar authenticity. The three cultivars were compared for their quality index, lipids, and phenol metabolites. They were similar in free fatty acidity (FFA), peroxide value (PV), oxidative stability, and the concentrations of total fat, volatiles, delta- and alpha-tocopherols, most of the phenols, most $\delta^{13}C_{\text{amino acids}}$, all $\delta^{15}N_{\text{amino acids}}$, all $\delta^{13}C_{\text{fatty acids}}$, and all $\delta^{2}H_{\text{fatty acids}}$. The data demonstrated that the overall quality of Chandler,

Howard, and Tulare was similar. They differed in the concentrations of fatty acids, triacylglycerols, gamma-tocopherol, and chlorogenic acid. Cultivar authenticity was possible when linear discrimination analysis was used on the triacylglycerol data.

Like many other agricultural products, walnuts' geographical origin can be subjected to fraud. To help detect the fraud, a database of multiple metabolites and quality indicators of walnuts from various countries was developed. Our data showed that walnuts from varying origins had different $\delta^{13}C_{\text{amino acids}}$, $\delta^{2}H_{\text{amino acids}}$, $\delta^{13}C_{\text{fatty acids}}$, and $\delta^{2}H_{\text{fatty acids}}$. They also had different fatty acid, phenol, triacylglycerol, and tocopherol profiles.

Skin darkening in walnuts is not preferred by the farmers and handlers because walnuts with dark skin receive lower scores during the grading process. Investigating if the skin darkening is associated with quality degradation is necessary. Therefore, we compared the quality index and relevant metabolites of walnuts of light and dark skin. Our observation demonstrated that walnuts with dark skin had significantly lower weight and fat content and higher oxidative stability and volatile concentrations than light walnuts. The dark kernels had larger concentrations of some phenolics like gallic acid, quercetin, and naringenin and lower concentrations of procyanidin B1 and epicatechin gallate than the light walnuts.

The walnut industry creates some byproducts, including shells and wood chips. They can be utilized in various products like furniture, fuel, cat litter, and mulch. Because juglone is a quinone in walnut plants that can exert toxicity and allelopathy at high concentrations, it is essential to know its concentration in walnut shells and wood chips. Two methods, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and liquid chromatography connected to a diode array detector (LC-DAD), were compared for their sensitivity, and the LC-DAD method was selected for sample analysis for higher sensitivity. Juglone concentrations in walnut shells and

wood chips were lower than what could exert toxicity or allelopathy effects, so they should be considered safe for value-added products.

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CHAPTER 1

INTRODUCTION

California produces approximately 99% of the United States (US) walnuts. The walnut industry in California involves more than 4800 farmers and around 100 handlers. California walnuts dominate the market in the US and many other countries(California Walnut Commission, 2024). There are some challenges that the California walnut industry faces, including minimal understanding of the quality of walnuts at retail, minimal understanding of cultivar variation effects on walnut quality and chemical characteristics, potential origin fraud in the international market that can lower its export, minimal knowledge of how pre-harvest extreme heat that causes kernel skin darkening affects quality, and safety concern over their byproducts.

California supplies most of the walnut demands in the US. California walnuts are also exported to other countries, including Canada, the United Kingdom, Japan, South Korea, Indonesia, and Australia. The lengthy supply chain process, including transportation, storage, and display, may lead to quality degradation, particularly oxidation of their lipid fractions. Lipid accounts for approximately 50% - 70% of walnut kernel mass. Lipids in walnuts are primarily triacylglycerols with a high proportion of polyunsaturated fatty acids (PUFAs) that are susceptible to oxidation(Geng et al., 2021; Kerrihard et al., 2015). Oxidation products in walnuts develop over time, and the concentration is higher when walnuts are exposed to high oxygen exposure, temperature, light, and humidity(Adkison et al., 2021). Temperature, humidity, and light exposure of walnuts vary with manufacturers and retailers. These variations may result in differences in product qualities. A survey is needed to understand the quality index ranges of

walnuts sold at retailers in the US or overseas. This information will help in developing quality standards for walnuts.

Californian walnuts are primarily of varieties Chandler, Howard, and Tulare. A comparison of them, in several previous works, is mainly done on agricultural and processing aspects, e.g., leafing date, yield, bearing habit, tree rigor, nut weight, shell hardness, and kernel weight and color(Vahdati et al., 2019). It needs to be fully understood how their quality and relevant metabolites differ. The data should inform handlers and prospective farmers in cultivar selection. In addition, the data can also be used for cultivar authenticity.

Walnuts are cultivated in various countries besides the United States (US). It is grown in China, Turkey, Chile, India, Poland, Iran, and others. China and the US are the first and second largest producers, respectively (Figure 1.1). The origin of agricultural products has long been associated with specific perspectives regarding their quality(Chousou et al., 2018; Sidali et al., 2021). The varying perspectives are commonly accompanied by different pricing that can lead to origin fraud. Origin fraud can harm the walnut industry by inflicting financial loss on honest producers. Because walnuts from various origins look alike, it is difficult to compare them visually. Therefore, analytical methods that can help origin authentication are needed.

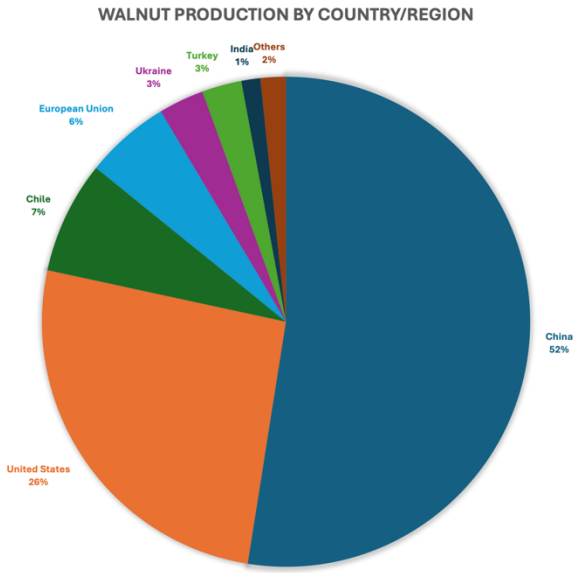

Figure 1.1. Percentage of walnut production by countries/regions that dominate the global supply, adopted from (USDA Foreign Agricultural Service, 2024)

Currently, walnuts (Figure 1.2) are graded using some parameters, i.e., cleanliness, presence of shriveling and mold, kernel size, and kernel skin/pellicle color. Kernels with light skin receive better grades, while kernels with dark skin are given lower grades or even rejected (Figure 1.3) (USDA, 2017). Despite the widespread practice, little is known about how the quality and the metabolites of the light vs. dark skin kernels differ.

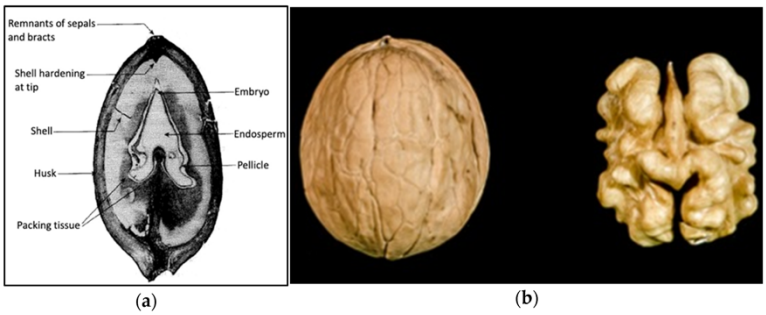

Figure 1.2. Walnut structure (a), in-shell walnut (b-left) and shelled walnut half (b-right), adopted from (Crisosto et al., 2023)

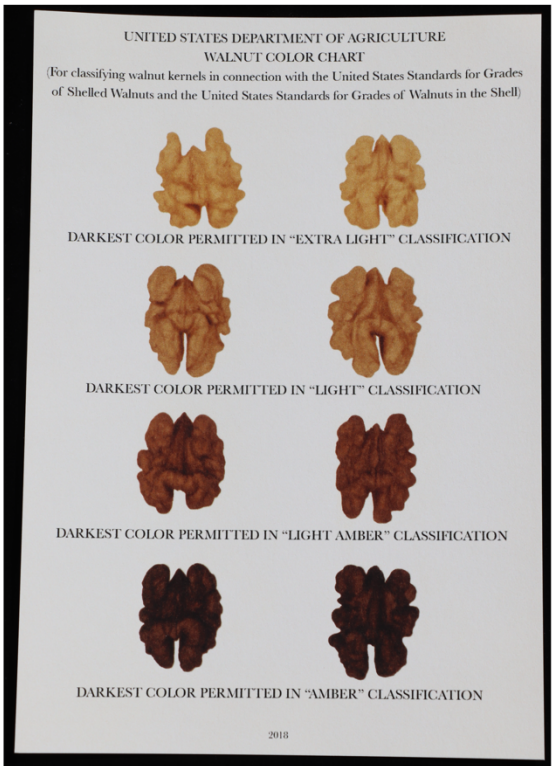

Figure 1.3. USA standards for grade of shelled walnuts, adopted from (Crisosto et al., 2023; USDA, 2017)

Walnuts contain juglone, a phenolic compound that has long been associated with toxicity, either to plants or animals, when it is present at high concentrations. However, therapeutic effects of juglone are found when they are at low concentrations (Babula et al., 2014; Chobot & Hadacek, 2009; Jin et al., 2016). Due to the importance of concentrations on its effect, it is essential to determine the concentration in walnut byproducts, i.e., shells and wood chips. This information would help increase the utilization of the products.

This dissertation aimed to investigate if variations in retailers, cultivars, geographical origins, kernel skin lightness, and plant parts are associated with changes in walnut quality and metabolite profiles. The specific objectives of this study were:

1. Understanding the quality of California walnuts sold at retail in the US and foreign countries.
2. Investigating the effects of cultivar variations on walnut quality and lipid and phenol metabolites.
3. Describing how the quality and chemical profiles of walnuts vary with origins.
4. Determining analytical methods that could differentiate walnuts from various geographical origins.
5. Comparing the quality and chemical characteristics of walnuts with light skin vs. dark skin.
6. Determining the concentrations of juglone in walnut industry byproducts (shells and wood chips).

The quality assessment conducted in this research mainly includes free fatty acidity, peroxide value, oxidative stability, volatiles, and antioxidant capacity. Free fatty acids are breakdown products of triacylglycerols. Polyunsaturated fatty acids, which comprise 70% of walnut fatty acids(Amaral et al., 2003), are prone to oxidation(Kerrihard et al., 2015). The oxidation reaction generates hydroperoxides that can break into various volatile compounds affecting the walnut sensory profile, including rancidity. Because the types of free fatty acids primarily affect oxidation susceptibility(Kerrihard et al., 2015), the assessment of fatty acid composition and proportion in the samples is required. Besides on free fatty acids, oxidation can also occur on triacylglycerols(Kato et al., 2018). Understanding triacylglycerol composition may help understand their oxidative stability because the composition and positions of fatty acids in triacylglycerols are linked to different oxidation rates(Neff et al., 1992). In addition, the

oxidative stability of high-lipid foods like walnuts can also be assessed using accelerated oxidation in a Rancimat apparatus.

Walnuts contain some antioxidants, mainly phenolic compounds(Wu et al., 2021). The concentration of phenolics in walnuts surpasses the values in many other tree nuts, such as almond, Brazil nut, chasew, hazelnut, macadamia, peanut, pine nut, and pistachio(Bolling et al., 2010). These compounds are beneficial for health improvement(Kang et al., 2016) and may also limit oxidation reactions(Labuckas et al., 2008). Concentrations of the phenolic compounds may vary with cultivars and origins because they are affected by genetics(Trandafir & Cosmulescu, 2020) and biotic and abiotic stressors(Tuladhar et al., 2021). The types and levels of biotic and abiotic stressors likely vary with growing locations.

Stable isotope ratios of walnuts from different origins are evaluated for origin classification. Stable isotope ratios of hydrogen, nitrogen, and carbon are affected by multiple factors with potential association with origins. Hydrogen stable isotope is influenced by types of water sources, the distance of growing locations to the nearest coast, altitude, and latitude(Gori et al., 2015; Meng et al., 2023). Nitrogen stable isotope ratios are affected by agricultural practices. Nitrogen stable isotope ratio is linked to the types of nitrogen sources for the plants, with animal manure leading to higher values of nitrogen isotope ratio(Paolini et al., 2015). Carbon stable isotope is affected by the type of land used for cultivation(Chung et al., 2017). Bulk stable isotope analysis (BSIA) was compared to compound-specific stable isotope analysis (CSIA) for their effectiveness in walnut origin discrimination, with CSIA predicted to result in better effectivity than BSIA because the values of different compound groups may vary and even out the bulk values, resulting in minimum variation among samples from various origins.

The study in Chapter 2 focused on the quality assessment of California walnuts sold at retailers, either in the US or in foreign countries. The hypothesis was that Californian walnuts sold in various retailers in the US and overseas would have lower quality than those sourced directly from handlers (that have not undergone lengthy transportation, storage, and display). The ones sold overseas were predicted to have lower quality than the ones sold domestically because they were transported longer distances than those sold in the US. Extended transportation and handling time likely results in more intense oxidation, yielding lower quality.

In Chapter 3, we compared Californian walnuts of Chandler, Howard, and Tulare varieties. A comprehensive comparison of their quality and metabolites has yet to be done. We hypothesized significant differences among them regarding their quality, lipids, and phenolic compounds. We also predicted that they could be differentiated using CSIA on their amino acids and fatty acids.

In Chapter Four, we investigated the effectiveness of stable isotope analysis (BSIA and CSIA) in distinguishing walnut origins. The hypothesis was that BSIA and CSIA were able to discriminate walnut samples based on their origins. The discriminating power of CSIA should be higher than that of BSIA.

In Chapter Five, we extensively compared the quality and metabolites of walnuts sourced from various origins. We hypothesized that there were differences in their quality and metabolites, and some chemical parameters could be used as markers for origin authenticity. Because CSIA was found useful for origin authentication in the study in Chapter Four, in this chapter, it was tested again in samples collected from a different year to confirm its effectiveness.

In Chapter Six, the quality and metabolite profiles of walnuts with dark vs. light kernel skin were compared. We hypothesized that dark-skinned walnuts were of lower quality and had different concentrations of various metabolites than light-skinned walnuts.

Chapter Seven is an overview of the extraction and analysis of juglone from some walnut industry byproducts. Juglone extraction procedures from walnut wood chips and shells were developed. The analysis procedures using LC-MS/MS and LC-DAD were also developed and compared.

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CHAPTER 2

QUALITY OF CALIFORNIA WALNUTS SOLD AT RETAIL IN THE UNITED STATES AND VARIOUS OTHER COUNTRIES

Abstract

Walnut is one of the five leading export commodities of California. Around two-thirds of California walnuts productions are exported worldwide. In addition, California walnuts account for around 99% of the US production. The complex and lengthy supply chains may result in the degradation of the quality of walnuts, and there has been minimal information on the quality of California walnuts sold to consumers. To assess the quality, walnuts were collected from various markets in the US and overseas and then subjected to chemical analysis related to lipid and phenol oxidation. The data were then compared with those of reference (samples sourced directly from handlers). Our data showed that the ranges of K_{232} (index for primary lipid oxidation products) for domestic and foreign-sourced California walnuts were 0.945 – 2.871 and 1.018 – 2.869, respectively. The values for K_{268}, indicative of secondary lipid oxidation products, ranged from 0.055 to 0.613 and 0.017 to 0.246 for the domestic and foreign samples, respectively. The values for %DPPH inhibition, associated with antioxidant capacity, ranged from 26.84 to 81.68 and 42.22 to 86.00 in the domestic and foreign walnuts, respectively. The reference samples' K_{232}, K_{268}, and %DPPH inhibition values were 0.910 - 1.350, 0.015 - 0.092, and 57.11 - 88.84, respectively. Significant differences were found in the K_{232}, K_{268}, and antioxidant capacity when the domestic and foreign-sourced samples were compared to a referenced handler. These data suggested that guidelines to minimize lipid and phenol oxidation during walnut distribution are necessary for improved quality.

Introduction

California walnuts dominate the market for walnuts in the United States (US) and some other countries. California produces around 99% of walnuts in the US, and around 67% of them are exported worldwide(California Walnuts, 2021). California walnuts can be found in markets in America, Asia, Australia, Europe, and Africa. The trading involves a complex supply chain from handlers in California to retailers in the US or overseas. Quality degradation of walnuts during the supply chain is expected because many nutritional components in walnuts are not stable, and the distribution involves lengthy periods and conditions that favor chemical degradation, i.e., moderate or high temperature. However, information regarding the extent of the degradation and the quality of the products sold by retailers is lacking. The information would serve as baseline knowledge that may help in developing standards for walnut quality and guidelines for quality improvement. This chapter focused on the quality of walnuts that were not roasted nor mixed with other ingredients.

The quality parameters that were evaluated were primarily associated with lipid, which is the most dominant nutrition group in walnuts. Walnuts contain 57-70 g lipid/100 g weight(Caglarirmak, 2003). The lipids predominantly comprise polyunsaturated fatty acids (77% of all fatty acids)(Nogales-Bueno et al., 2021). Polyunsaturated fatty acids are prone to oxidation because the allylic C-H bonds adjacent to methylene groups are weak and vulnerable to radical attacks followed by singlet oxygen attachment. The reaction generates hydroperoxides that can be detected using titration and spectrophotometry methods. Hydroperoxides are considered intermediate or primary products of lipid oxidation because they are generally unstable. Hydroperoxides can decompose into aldehyde esters and aldehydes (through carbon-carbon cleavage), isomerized unsaturated hydroperoxides (through carbon-oxygen cleavage), and

alkoxyl and hydroxyl radicals (through oxygen-oxygen cleavage). These compounds are known as considered secondary products of oxidation(Kontogianni & Gerothanassis, 2022). Many of the secondary oxidation products are volatiles and some of them (hexanal, 1-hexanol, 1-octen-3-ol, and 2-pentyl-furan) are considered oxidation markers in walnuts because the concentrations increase as walnuts become more oxidized(Grilo & Wang, 2021). The secondary oxidation products can be detected using multiple techniques, including spectrophotometry and gas chromatography coupled with mass spectrometry.

Besides having a high concentration of lipids, walnuts also contain a high concentration of phenolic compounds. Many of these compounds have antioxidant capacity and various health benefits(Cerdá et al., 2005; Kang et al., 2016). They may limit the lipid oxidation of walnuts, based on a study showing that walnut oils that had been added with walnut cake phenolic extracts had lower amounts of secondary lipid oxidation products(Bakkalbaşı, 2019). Generally, antioxidant capacity decreases as walnuts undergo oxidation upon storage(Hao et al., 2020).

Materials and Methods

Sampling

California walnut samples were collected from December 2023 to March 2024. They were sourced from two handlers (Table 2.1), various retailers in twelve states within the United States (Table 2.2), and eight countries outside the United States (Table 2.3). All of them were shelled, except for two samples sold in the United Kingdom (under brands Wonderland) and two samples sold in India (brands Nutraj and Yum Yum) that were sold as in-shell.

Table 2.1. Codes, sources, cultivar, and collection time of California walnut samples sourced directly from handlers

No	Sample Code	Source	Cultivar	Collection Time
1	K005717	Handler 1	na[1)]	Dec-23
2	K005695	Handler 1	na	Dec-23
3	K005713	Handler 1	na	Dec-23
4	K005765	Handler 1	na	Dec-23
5	K005735	Handler 1	na	Dec-23
6	27322	Handler 2	Ashley	Jan-24
7	7158	Handler 2	Ashley	Jan-24
8	28245	Handler 2	Chandler	Jan-24
9	28163	Handler 2	Chandler	Jan-24
10	29177	Handler 2	Chandler	Jan-24
11	27614	Handler 2	Chandler	Jan-24
12	28766	Handler 2	Chandler	Jan-24
13	29462	Handler 2	Chandler	Jan-24
14	28973	Handler 2	Chandler	Jan-24
15	28039	Handler 2	Hartley	Jan-24
16	27206	Handler 2	Hartley	Jan-24
17	27553	Handler 2	Hartley	Jan-24
18	26959	Handler 2	Hartley	Jan-24
19	27924	Handler 2	Howard	Jan-24
20	26875	Handler 2	Howard	Jan-24
21	27277	Handler 2	Howard	Jan-24
22	28854	Handler 2	Howard	Jan-24
23	26672	Handler 2	Ivanhoe	Jan-24
24	28137	Handler 2	Mixed	Jan-24
25	4091	Handler 2	Solano	Jan-24
26	26817	Handler 2	Tulare	Jan-24
27	27384	Handler 2	Vina	Jan-24
28	26769	Handler 2	Vina	Jan-24
29	27384	Handler 2	Vina	Jan-24

[1)]na: not available

Table 2.2. Sources, brand/manufacturer, collection time, and best-by-date of California walnut samples sourced from various states (within the United States)

No	Source		Brand/Manufacturer	Collection Time	Best by date
	State	Retailer			
1	NV	Raley's	DSD Merchandises	Feb or Mar -24	7/16/24
2	CA	Costco	Kirkland	Feb or Mar -24	na[1)]
3	CA	Costco	Kirkland	Feb or Mar -24	9/18/24
4	CA	Raley's	DSD Merchandises	Feb or Mar -24	na
5	CA	Raley's	Raley's	Feb or Mar -24	6/7/24
6	NV	Raley's	DSD Merchandises	Feb or Mar -24	8/26/24
7	GA	Publix	Diamond of CA	Feb or Mar -24	3/18/25
8	NV	Trader Joe's	Trader Joe's nuts	Feb or Mar -24	9/20/24
9	CA	Trader Joe's	Trader Joe's organics	Feb or Mar -24	9/27/24
10	OR	Safeway	Open Nature	Feb or Mar -24	11/1/24
11	OR	Safeway	Diamond	Feb or Mar -24	12/10/24
12	OR	Grocery Outlet	Primavera	Feb or Mar -24	9/11/24
13	OR	Safeway	Signature	Feb or Mar -24	11/4/24
14	OR	Walmart	Great Value	Feb or Mar -24	9/1/24
15	OR	Grocery Outlet	Sun Tree	Feb or Mar -24	9/14/24
16	CA	Sprouts	Sprouts	Feb or Mar -24	9/21/24
17	CA	Food for Less	DSD Merchandises	Feb or Mar -24	na
18	CA	Nugget	DSD Merchandises	Feb or Mar -24	10/2/24
19	CA	Save Mart	Mariani	Feb or Mar -24	na
20	CA	Sprouts	Sprouts	Feb or Mar -24	8/25/24
21	FL	Walmart	Fisher	Feb or Mar -24	12/4/24
22	FL	Walmart	Great Value	Feb or Mar -24	10/11/24
23	FL	Publix	Mariani	Feb or Mar -24	1/15/25
24	MI	Kroger	Simple Truth Organic	Feb or Mar -24	9/27/24
25	GA	Whole Foods	Point Dexternut	Feb or Mar -24	na
26	GA	Walmart	Great Value	Feb or Mar -24	10/21/24
27	GA	Food Lion	Food Lion	Feb or Mar -24	9/27/24
28	GA	Harveys	SE Grocers	Feb or Mar -24	8/7/24
29	MA	BigY	Diamond	Feb or Mar -24	12/24/24
30	WA	Costco	Kirkland	Feb or Mar -24	na
31	WA	IGA	Diamond	Feb or Mar -24	2/28/25
32	SC	Costco	Kirkland	Feb or Mar -24	na
33	SC	Piggly Wiggly	Diamond	Feb or Mar -24	3/16/25

34	SC	Publix	Publix	Feb or Mar -24	2/28/25
35	SC	Food Lion	Fisher	Feb or Mar -24	10/1/24
36	ME	Hannaford	Bulk Mariani	Feb or Mar -24	na
37	ME	Hannaford	Fisher	Feb or Mar -24	7/2/25
38	ME	Job Lots	Point Dexternut	Feb or Mar -24	7/12/24
39	ME	Hannaford	Mariani	Feb or Mar -24	10/2/24
40	CO	Safeway	Open Nature	Feb-24	11/9/24
41	CO	Walmart	Fisher	Feb-24	11/19/24
42	CO	Walmart	Diamond	Feb-24	11/7/24
43	CO	Amazon	I'm a Nut	Feb-24	3/22/25
44	CO	King Soopers	Fisher	Feb-24	12/7/24
45	NE	Amazon	I'm a Nut	Feb-24	3/22/25
46	NE	Walmart	Food To Live Organic	Feb-24	Sep-25
47	NE	Walmart	Fisher	Feb-24	12/4/24
48	NE	Amazon	Fisher	Feb-24	11/14/24
49	NE	Hyvee	Fisher	Feb-24	11/14/24

[1)]na: not available

Table 2.3. Sources, brand/manufacturer, collection time, and best-by-date of California walnut samples sourced from various countries outside the United States (US)

No	Country	Brand/Manufacturer	Collection Time	Best by date
1	Japan	Jona	Jan-24	Nov-25
2	Japan	Kotobuki	Jan-24	5/30/24
3	Japan	Seijoshii	Feb-24	7/16/24
4	Canada	Yupik Organic	Feb-24	3/14/25
5	Canada	Yupik	Feb-24	7/24/25
6	Canada	The Nut Shoppee	Feb-24	1/29/25
7	Canada	Clevera	Feb-24	Aug-24
8	UK	Kirkland	Jan-24	na[1)]
9	UK	Wonderland (1 kg)	Feb-24	na
10	UK	Wonderland (0.5 kg)	Feb-24	na
11	France	Earth Best	Feb-24	1/22/25
12	Hongkong	HKJEBN	Feb-24	9/6/24
13	Indonesia	Indian Grocery	Feb-24	na
14	Indonesia	Sales by Summer (SBS)	Feb-24	Jun-24
15	Indonesia	Jia Jia	Feb-24	na

16	India	Ambrosia	Mar-24	May-24
17	India	Tulsi	Mar-24	8/8/24
18	India	Nutraj (shelled)	Mar-24	8/6/24
19	India	Nutraj (in shell)	Mar-24	8/15/24
20	India	Yum Yum	Mar-24	12/8/24
21	Australia	McKenzie	Mar-24	1/3/25
22	Australia	Natural Californian	Mar-24	2/12/25
23	Australia	Fresh Harvest	Mar-24	8/25/24
24	Australia	Horn of Plenty	Mar-24	4/26/25
25	Australia	Premium Nut	Mar-24	1/13/25

[1)]na: not available

Oil extraction

Approximately 250 g of walnuts were chopped using a food processor. The chopped walnuts were later fed into an oil extraction machine (Vevor, China) and cold-pressed. The resultant slurry was centrifuged at 10,000 g for 5 min to separate oil from the solid particles. The oil was then collected and stored at -20 °C until analysis. The oil was used for free fatty acidity, peroxide value, and UV index assays.

Free fatty acidity (FFA)

The oil samples (±2.82 g) were added with 70 mL of ethanol. The solution was then titrated with 0.1 N sodium hydroxide using an autotitrator (888 Titrando, Metrohm, Florida, US). The FFA values were determined using the formula $FFA = \frac{VxcxM}{10Xm}$, with V, c, M, and m defined as the volume of titrated sodium hydroxide after blank correction (mL), the molarity of sodium hydroxide solution, molar mass per gram of oleic acid (282 g/mol), and the mass of the sample (g), respectively. The values were expressed as %oleic acid.

Peroxide value (PV)

Oil samples (±5.00 g) were added to 50 mL of isooctane: acetic acid (2:3, v/v). Saturated potassium iodide solution (0.5 mL) was added to react with hydroperoxides in the samples, and after one minute, the reaction was stopped by adding 30 mL of water. The mixture was then titrated with sodium thiosulfate (0.02 N) using an autotitrator 888 Titrando (Metrohm, Florida, US). PV was determined as $\frac{VxTx1000}{m}$, with V as the volume of sodium thiosulfate solution used for the test (with blank correction), T as the exact molarity of the sodium thiosulfate solution, and m as the weight of the oil samples. The values were expressed as meq O_2/kg.

Ultraviolet (UV) index

The UV index assay followed American Oil Chemistry Guidelines Ch 5-91(09) as described in a previous study (Grilo & Wang, 2021). Approximately 0.1 g oil was solubilized in 10 mL isooctane. The solution was then measured for its UV absorption at wavelengths 232 and 268 nm. K_{232} or K_{268} was calculated using a following equation: $K_\lambda = E_\lambda/(CxS)$, where K_λ, E_λ, C, and S were specific extinction at wavelength λ, extinction measured at wavelength λ, concentration of the solution (g/100 mL), and thickness of the cuvette (cm), respectively.

Oxidative stability

Approximately 15 g of walnuts were chopped finely (particle size of 1-2 mm). An aliquot (0.5 g) of the sample was placed in a vial and then the oxidative stability assay was done following a published method(Ampofo et al., 2022). The measurement was done using a Rancimat apparatus 617 (Metrohm AG, Herisau, Switzerland). The vials were heated at 110 °C, and the air gas flow was 20 L/h.

2,2,'-diphenyl-1-picrylhydrazyl (DPPH) radical assay

Walnut samples (±15 g) were ground using a food processor until the particles were about 1-2 mm wide. The ground samples (3 g) were defatted by mixing them with 15 mL of hexane followed by sonication for 5 min. The mixture was centrifuged (10,000 g for 5 min), and later the hexane portion containing lipids was removed. The sediment was then mixed with 10 mL acetone: water (7:3, v/v). The mixture was sonicated for 30 min, followed by centrifugation (10,000 g, 5 min). The extract was then collected and stored at -20 °C until analysis.

The DPPH assay followed a published method(Zhao et al., 2023). An aliquot of the extracts (4 µL) was added with 96 µL of acetone: water (7:3, v/v). The DPPH solution (4.7 mg/100 mL) was added, and the mixture was incubated for 30 min at dark. Later, the absorbance at 515 nm was measured. The percentage of DPPH inhibition was determined as $\frac{(A_0-A)}{A_0} x100\%$ where A_0 was the absorbance of a blank solution, and A was the absorbance of a sample extract.

Volatile analysis

Volatile analysis was done following a previous study on walnuts(Grilo & Wang, 2021). Approximately 20 g of walnuts were chopped finely using a food processor (particle size was 1-2 mm). An aliquot of the samples (3 g) was placed in a 20-mL vial. Solid-phase microextraction (SPME) of the headspace volatile was done using divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber after the samples were incubated at 45 °C for 20 min in an autosampler (Agilent PAL RSI 85). The volatiles were desorbed from the fiber in the inlet of a gas chromatography (GC, Agilent 7820A). Compound separation was achieved using a Supelcowax 10 column (30 mm x 0.25 mm x 0.25 µm, Sigma Aldrich) and helium as the carrier gas with a flow rate of 1 mL/min. The initial GC oven

temperature was 40 °C, then after 10 min it was increased at 3 °C/min to 200 °C. Outlet of the GC was connected to a mass-spectrometer (Agilent 5977B). The volatiles were identified using NIST 08 library.

Statistical analysis

The quality of walnuts from two handlers was compared using a t-test. The samples from the handler with superior quality were used as references for comparing the quality of walnuts from various sources. The presence of significant differences between means of various groups was determined using analysis of variance (ANOVA) if the requirements for normality and equal variances were met. When a significant difference was found in ANOVA, Dunnet test was performed to compare the quality of the samples from varying sources to the reference. When equal variances were not achieved, Welch test was used instead of ANOVA and Dunnet tests to compare the means of a group to that of the reference. The statistical analysis was performed on groups that had three or more replicates and conducted using R 4.3.3 and R Studio 2023.03 (Posit software, PBC, MA, US).

Results and Discussion

Comparison of the quality of California walnuts sourced from two handlers

The handler samples were sourced from two companies. There were some significant differences among them regarding peroxide value (PV) and UV index (K_{232} and K_{268}), but not in free fatty acidity (FFA), oxidative stability, and antioxidant capacity. Those from handler 1 had higher PV, K_{232}, and K_{268} than those from handler 2 (Figure 2.1a-f). PV is a common method to measure the concentration of lipid hydroperoxides (LOOHs), the primary nonradical oxidation products. UV index should support the PV and volatile data. K_{232} measures the levels of conjugated peroxides,

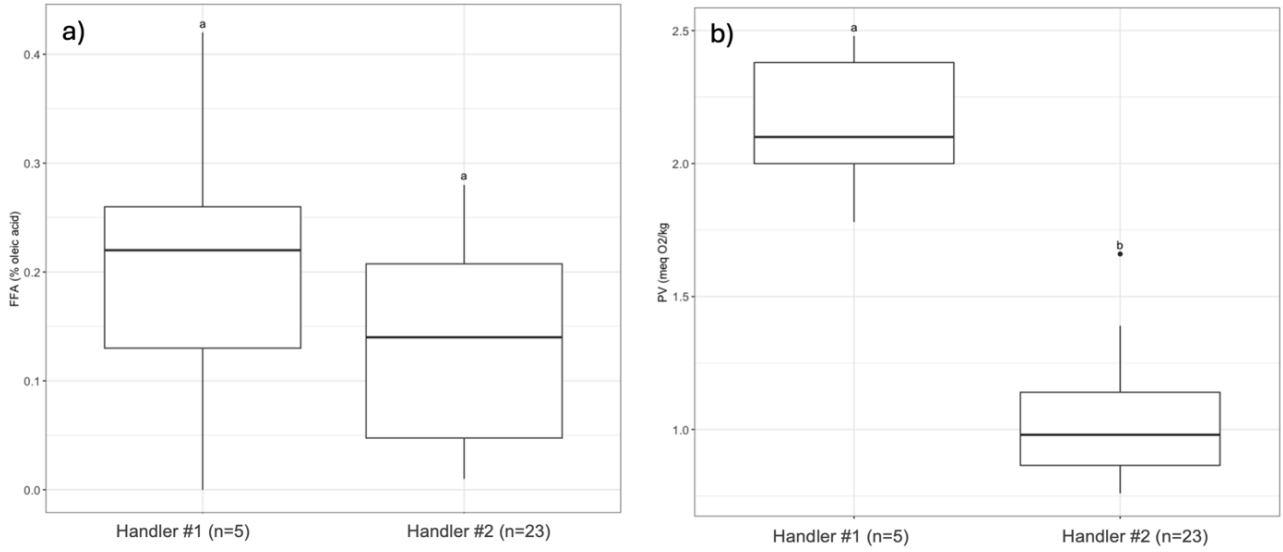
which are included as primary oxidation products of lipids. Another UV index, K_{268}, measures aldehydes, ketone dienes, and epoxides, which are secondary oxidation products of lipids(Tarapoulouzi et al., 2022). Higher K_{268} values usually indicate that the samples have reached an advanced oxidation stage. The higher PV, K_{232}, and K_{268} values of the samples from handler 1 indicate that they were more oxidized than those from handler 2.

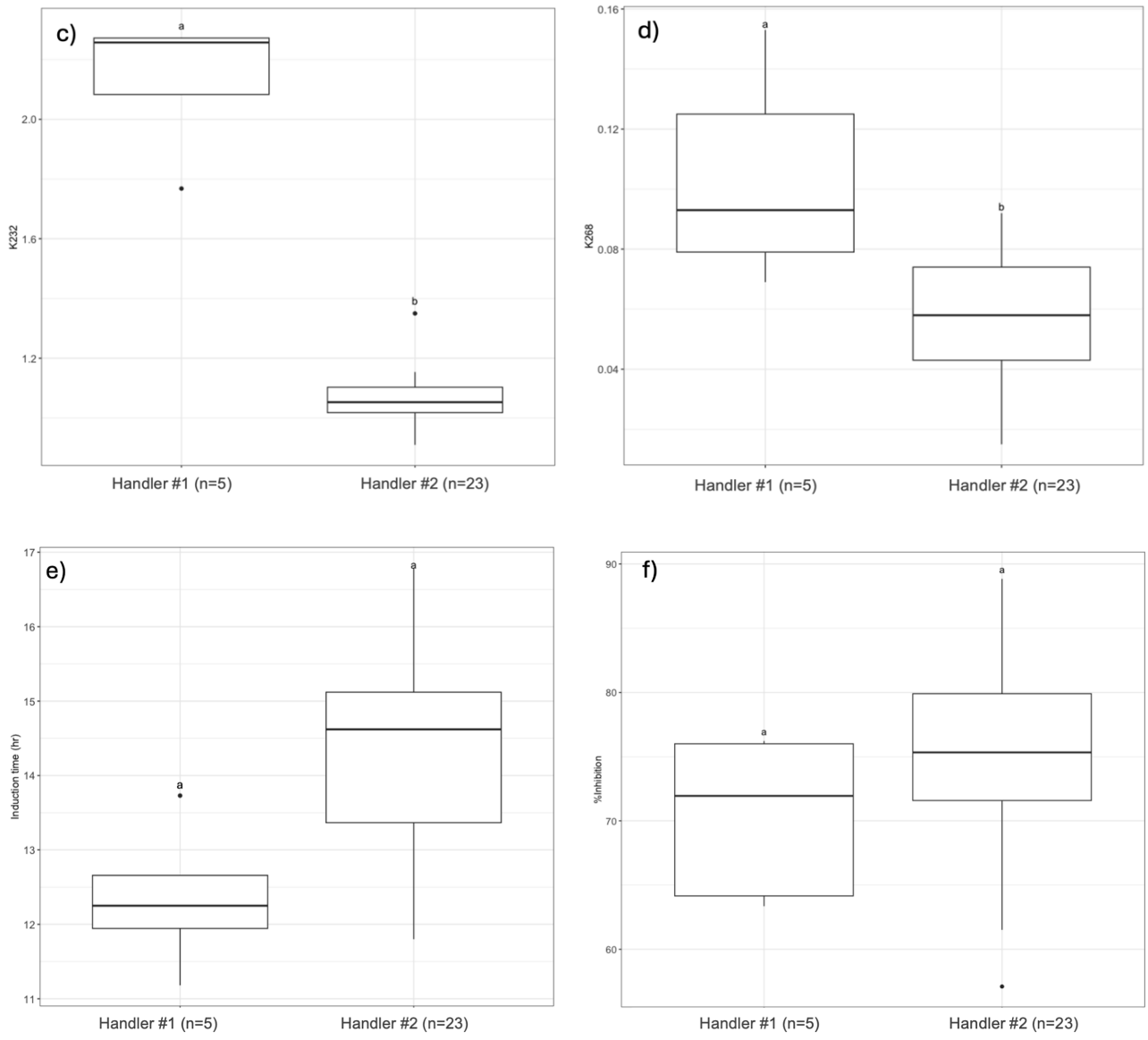
The samples from handler 1 also had higher concentrations of the four volatiles that are considered oxidation markers in walnuts: hexanal, 1-hexanol, 1-octen-3-ol, and 2-pentyl-furan(Chakraborty et al., 2023; Grilo & Wang, 2021) (Figure 2.1g-j). Hexanal is produced from linoleic acid with lipoxygenase and hydroperoxide lyase enzyme activity (Hatanaka et al., 1983). Lipoxygenase enzyme catalyzes fatty acid peroxidation, and hydroperoxide lyase cleaves the resultant hydroperoxides into smaller compounds like hexanal. Hexanal may be reduced into hexanol by alcohol dehydrogenase(Hashem et al., 2022). 1-Octen-3-ol is also synthesized from linoleic acid. First, linoleic acid is oxidized, producing 10-hydroperoxide (10-HPOD), and the compound later breaks down into 1-octen-3-ol and 10-oxodecanoic acid(Su et al., 2022). 2-Pentyl-furan likely also originates from linoleic acid. The compound can be generated from the reaction of conjugated diene radical of linoleic acid with oxygen. This reaction produces a vinyl hydroperoxide that later undergoes cyclization by loss of hydroxy radical(Frankel, 1983).

Peak areas of all volatiles found in the samples, along with the total peak areas of various volatile groups, were presented in Figure 2.2. The peak area of hexanal was the largest among the other volatiles because it is the main volatile generated from linoleic acid(Frankel, 1983), the most dominant fatty acid in walnuts(Goodarzi et al., 2023). The higher concentrations of volatiles in handler 1 than in handler 2 were not limited to the four main oxidation markers. It also occurred

on many other volatiles, including furans, ketones, carboxylic acids, and esters. In fact, the mean peak area of total volatiles in handler 1 was about four times that of handler 2.

The volatile data confirmed the PV, K_{232}, and K_{268} results - the samples from handler 1 were more oxidized. The higher level of lipid oxidation in the samples from handler 1 could be due to a combination of longer storage and darker kernel skin. Those from handler 1 were collected/harvested in 2022, and the kernel skin was darker than that of handler 2. Considering the lighter kernel skin of the samples from handler 2, they were likely harvested in 2023, at which no extreme heat occurred prior to walnut harvest time, resulting in sunburn damage in walnuts, as in the case of 2022(California Agriculture News Today, 2023). Because of the better quality of the samples from handler 2, they were selected as a reference for determining the quality of the samples from various sources.




c)
a
b
2.0
1.6
1.2
K232
Handler #1 (n=5)
Handler #2 (n=23)
d)
a
b
0.16
0.12
0.08
0.04
K268
Handler #1 (n=5)
Handler #2 (n=23)
e)
a
a
17
16
15
14
13
12
11
Induction time (hr)
Handler #1 (n=5)
Handler #2 (n=23)
f)
a
a
90
80
70
60
%Inhibition
Handler #1 (n=5)
Handler #2 (n=23)

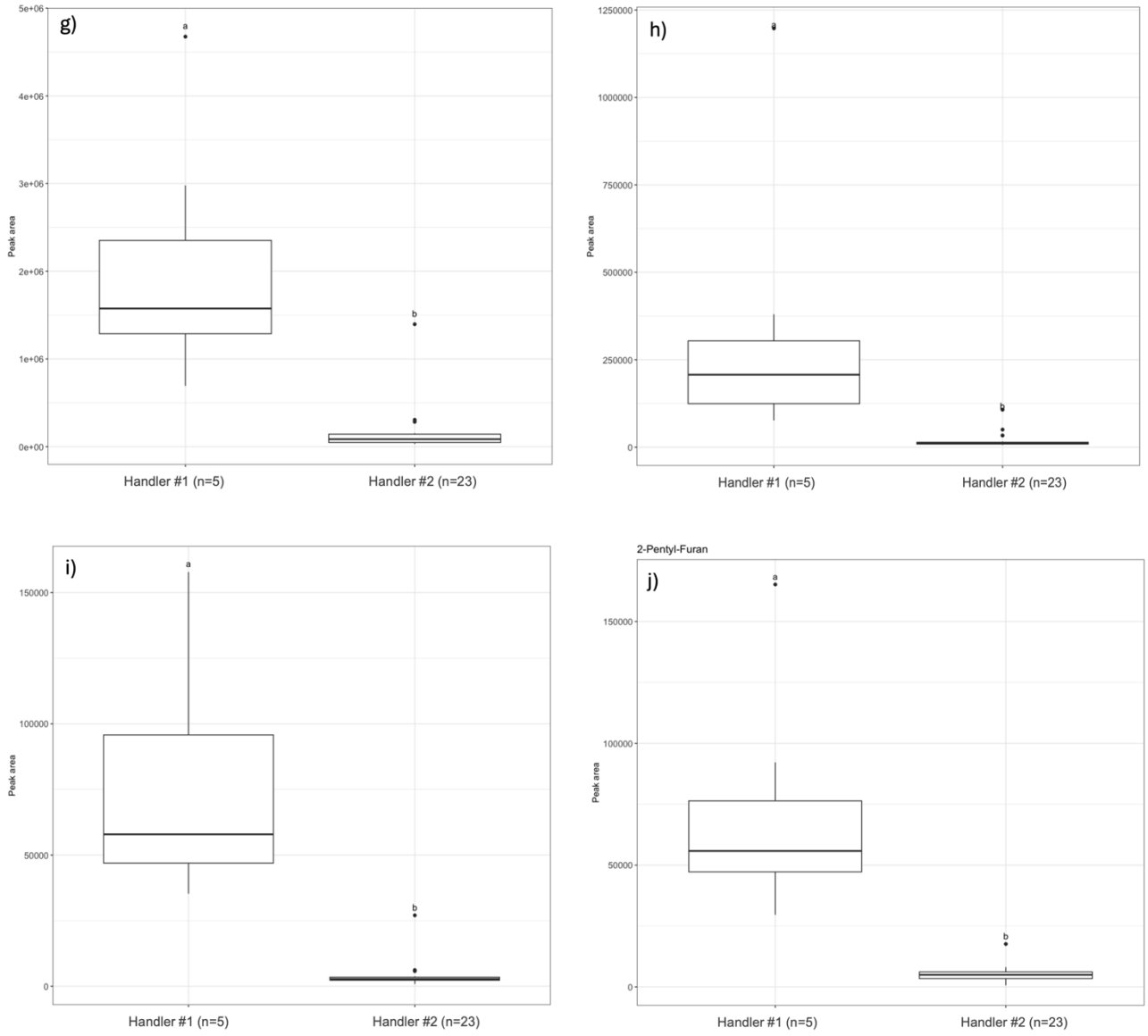


Figure 2.1. Boxplots of FFA (a), PV (b), K_{232} (c), K_{268} (d), oxidative stability (e), %DPPH inhibition (f), and peak areas of hexanal (g), 1-hexanol (h), 1-octen-3-ol (i), and 2-pentyl-furan (j) of California walnuts sourced from two handlers

[1)]Significant difference is indicated by different letters

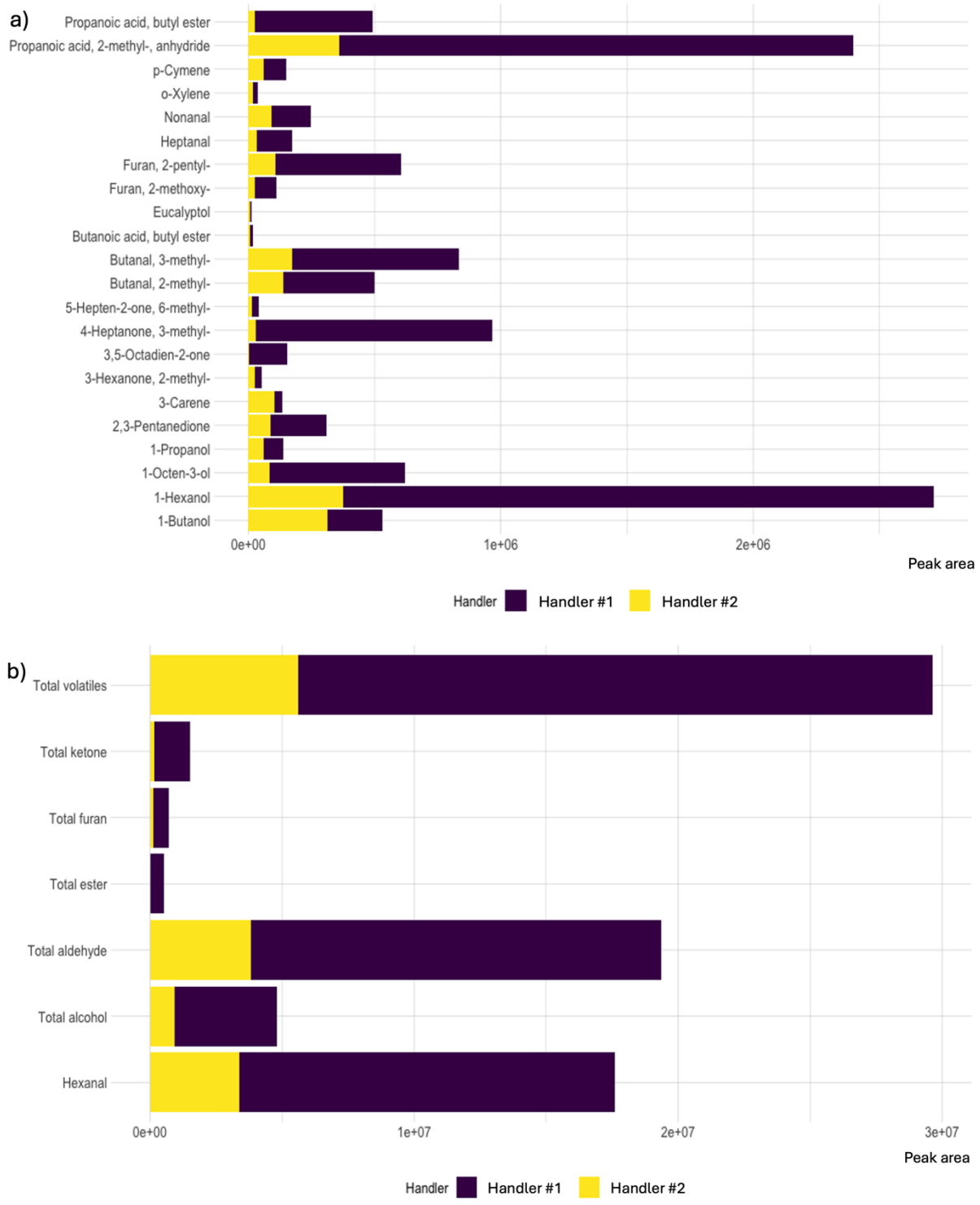


Figure 2.2. Mean peak areas of various volatiles (a) and hexanal and total volatiles (b) of California walnuts sourced from two handlers

Quality of California walnuts sourced from domestic and foreign markets

The domestic and foreign samples had significantly larger UV indexes (K_{232} and K_{268}) and lower antioxidant capacity (measured as %DPPH inhibition) than the reference. However, they were similar to the reference regarding PV, FFA, and oxidative stability (Table 2.4). The non-significant difference in PV might be because PV is less sensitive than the UV index(Shahidi et al., 2017). The non-significant difference in FFA is likely because the level is influenced by some factors that have competing effects, i.e., the activity of lipase enzymes (that produce free fatty acids from hydrolysis of triacylglycerols)(Pournik et al., 2019) and oxidation (that depletes free fatty acids). Oxidative stability does not seem very sensitive in detecting walnut oxidation, based on the non-significant difference when the other quality parameters showed otherwise.

Higher K_{232} and K_{268} values suggest elevated levels of the primary and secondary products of lipid oxidation, respectively. In the present study, the maximum K_{232} and K_{268} values of the commercial walnuts were 2.871 and 0.613, respectively. Walnuts' K_{232} value may exceed 2.871, as demonstrated in an earlier report(Grilo & Wang, 2021). The maximum K_{268} in the current study (0.613) was much larger than the previously reported value in walnuts that had been stored at 25 °C and 50% for 28 weeks(Grilo & Wang, 2021). This data suggested that many of the walnuts sold in retailers had been stored for longer than 28 weeks.

The lower antioxidant capacity suggested a reduced concentration of phenols because many phenols in walnuts demonstrate antioxidant capacity. Walnuts contain abundant phenolic compounds(Wu et al., 2021). Ellagic acid, which is present in large quantities in walnuts, can deactivate peroxyl radicals, hydroxyl radicals, nitrogen dioxide, and peroxynitrite. It also can prevent oxidation triggered by metals such as copper, iron, nickel, and cadmium by chelating them. The chelating ability should be due to its four hydroxyl groups (Sharifi-Rad et al., 2022).

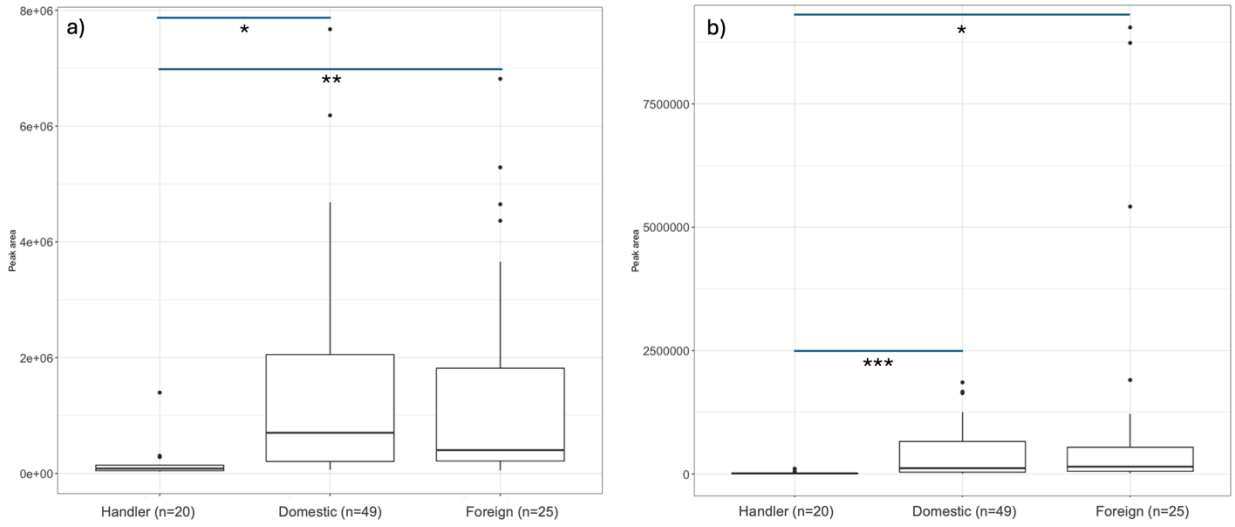
The reduction of %DPPH inhibition of walnut extracts might be linked to structural changes in the phenolics caused by oxidation during the lengthy distribution process. A decrease in total phenol and DPPH inhibition of walnuts upon storage had been reported in a previous study. The study also demonstrated that storage at refrigeration temperature and the application of synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) minimized antioxidant activity reductions in walnuts during storage(Ahad et al., 2020). It was unclear if the samples sold in the domestic and foreign markets were refrigerated during storage, but walnuts are generally displayed at ambient temperature in supermarkets. The addition of BHA and BHT in commercial walnuts has not been reported. However, adding rosemary, as a natural antioxidant, inside walnut packaging has been observed, although rarely.

In addition to having a higher UV index and lower antioxidant capacity, the domestic and foreign samples also had higher concentrations of hexanal, 1-hexanol, 1-octen-3-ol, and 2-pentyl-furan than the reference (Figure 2.3). As compared to the reference, the increased concentrations of these volatiles in the domestic and foreign markets were also apparent in all the other volatiles (Figure 2.4). The higher concentrations of volatiles supported the K_{268} data that the oxidation has advanced to produce a significant amount of secondary oxidation products. The evidence of oxidation in the samples from domestic and foreign markets is expected because they have been subjected to prolonged light and oxygen exposure that promotes oxidation.

Table 2.4. Range and mean ± standard deviations of FFA (%oleic acid), PV (meq O_2/kg), induction time (hr), K_{232}, K_{268}, and %DPPH inhibition of California walnut samples sourced from a handler, domestic markets and foreign markets

Parameter	Handler		Domestic		Foreign	
	Range	Mean ± sd	Range	Mean ± sd	Range	Mean ± sd
FFA (%oleic acid)	0.01 – 0.28	0.14 ± 0.09	0.00 – 0.86	0.21 ± 0.19	0.00 – 0.67	0.25 ± 0.18
PV (meq O_2/kg)	0.76 – 1.66	1.03 ± 0.23	0.15 – 4.62	1.29 ± 0.88	0.51 – 3.98	1.36 ± 0.86
Induction time (hr)	11.80 – 16.79	14.32 ± 1.30	5.23 – 18.11	13.66 ± 2.57	7.72 – 18.75	13.63 ± 2.51
K_{232}	0.910 – 1.350	1.063 ± 0.086	0.945 – 2.871	**1.747 ± 0.476**	1.018 – 2.869	**1.596 ± 0.472**
K_{268}	0.015 - 0.092	0.056 ± 0.019	0.055 – 0.613	**0.123 ± 0.081**	0.017 – 0.246	**0.103 ± 0.049**
%DPPH inhibition	57.11 – 88.84	74.76 ± 8.35	26.84 – 81.68	**58.22 ± 10.95**	42.22 – 86.00	**63.23 ± 8.37**

[1)]Bold values indicate significant differences from the values of the handler



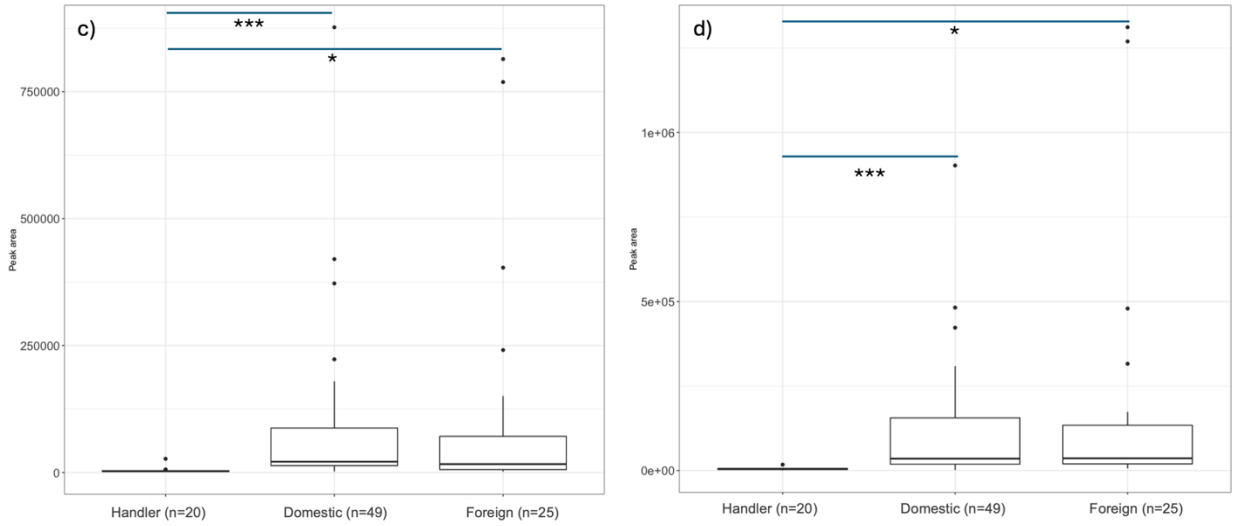
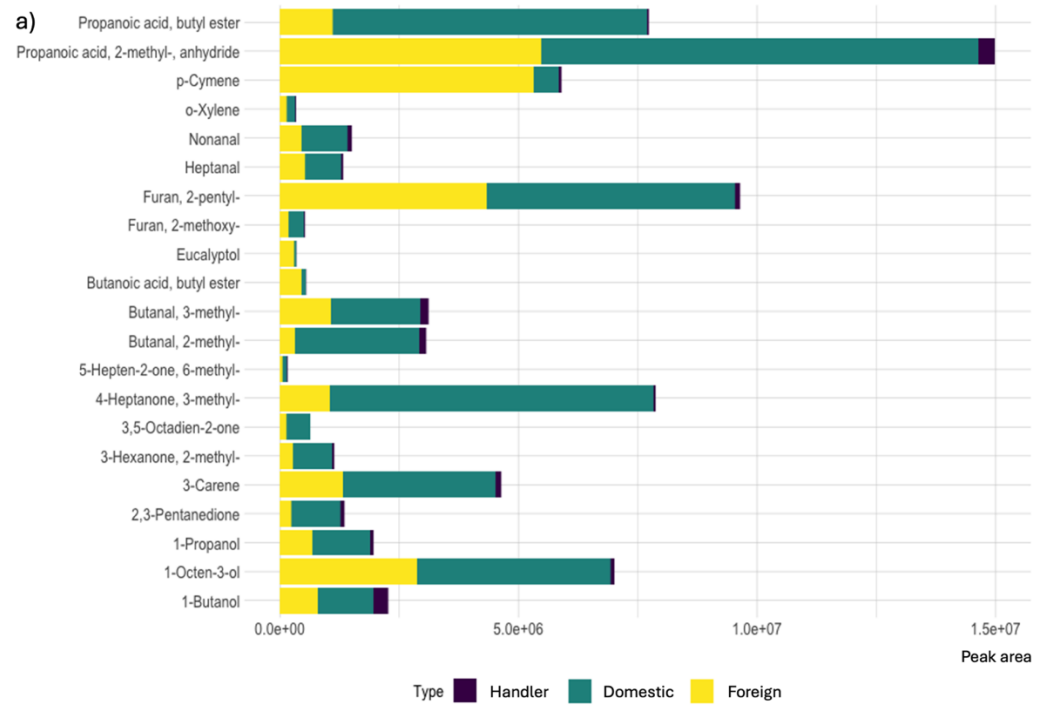


Figure 2.3. Boxplots of peak areas of hexanal (a), 1-hexanol (b), 1-octen-3-ol (c), and 2-pentyl-furan (d) of California walnuts sourced from a handler (reference), domestic markets, and foreign markets

[1)] Blue lines connecting two groups indicate significant differences (p-values < 0.05). (*) indicates that p-values were between 0.05 and 0.01. (**) indicates that p-values were between 0.01 and 0.001. (***) indicates that p-values were less than 0.001.



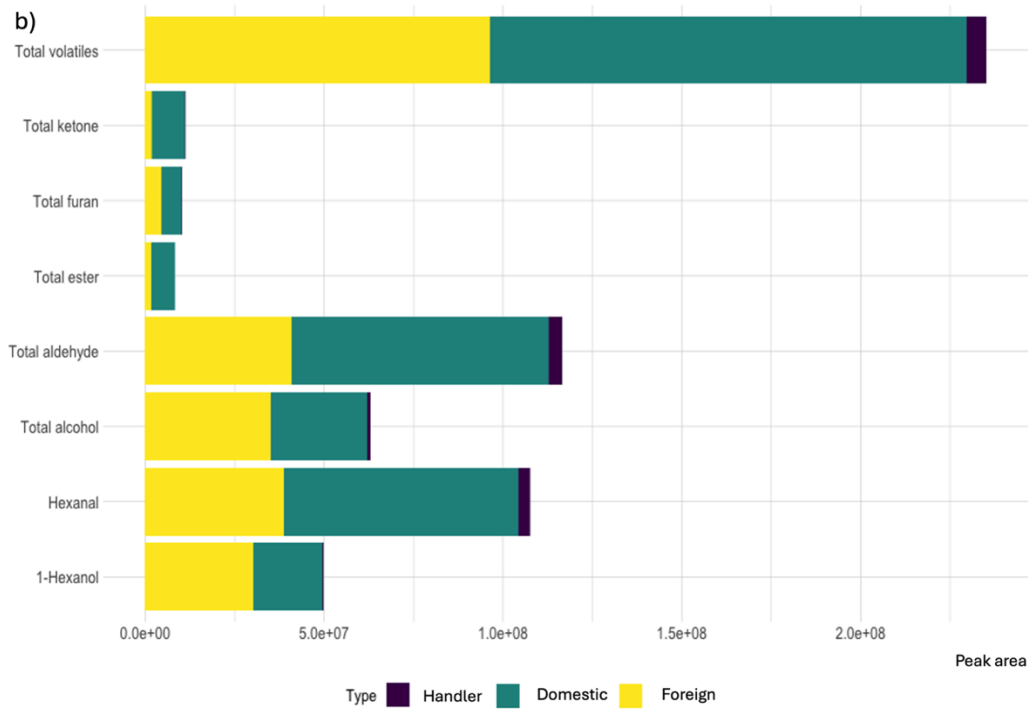


Figure 2.4. Mean peak areas of various volatiles (a) and hexanal, 1-hexanol, and total volatile groups (b) of California walnuts sourced from a handler, domestic markets, and foreign markets

Linear discriminant analysis (LDA) was conducted on all the parameters evaluated, and it almost separated the reference samples from the domestic market samples entirely. A small intersection between the handler and foreign-sold samples was noted. A large intersection was observed between the domestic and foreign markets ellipses (Figure 2.5). These results suggested that the domestic samples largely had different quality characteristics than the handler, and the foreign-sourced samples shared a few similarities with the handler. A large similarity between the quality profiles of the domestic and foreign markets was noted. Considering that the walnuts might be subjected to similar storage and retail conditions, it was expected.

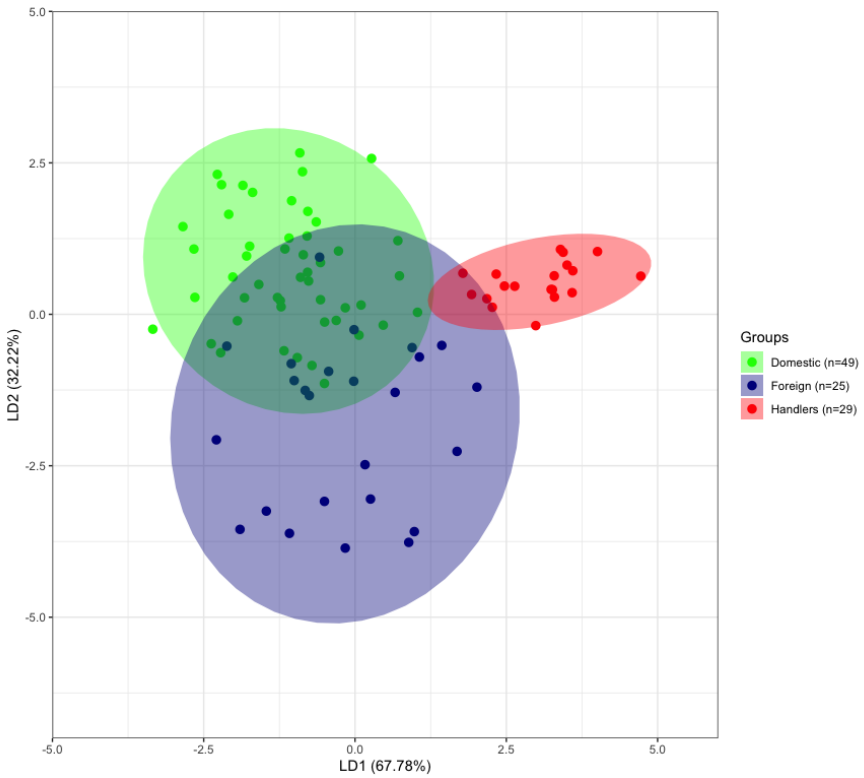


Figure 2.5. LDA plot of the quality profiles of California walnuts sourced from a handler, domestic markets, and foreign markets

Quality of California walnuts sold in multiple states in the United States

Samples collected from some stores in Colorado, Florida, and Nebraska had similar values of FFA, PV, K_{232}, K_{268}, induction time, and %DPPH inhibition with those of the handler. Those sold in California and Nevada had larger K_{232} and K_{268} and lower %DPPH inhibition than the handler. The samples sold in Maine and Oregon had larger K_{232} and lower %DPPH inhibition than the handler. The samples from South Carolina only had a lower %DPPH inhibition than the handler. The samples from Georgia showed the most differences from the handler. They had larger PV, K_{232}, and K_{268} and lower %DPPH inhibition (Table 2.5). These findings suggested that no evidence of oxidation was found for the samples sold in Colorado, Florida, and Nebraska but the samples sold in California, Georgia, Maine, Nevada, Oregon, and South Carolina showed

signs of oxidation. The samples sold in California, Georgia, and Nevada were likely more oxidized than the others based on their significantly higher K_{268} values than the reference since K_{268} values indicate secondary oxidation products. The volatile data partially supported this finding. The samples from California and Oregon had significantly larger concentrations of 1-hexanol than the handler. The samples sold in California also had more 2-pentyl-furan than the handler (Figure 2.6).

The finding suggested that the oxidation level in walnuts was not directly linked to the distance from the source (handlers in California) to the point of sale. While some samples sold in Georgia, which is very far from California, had high oxidation levels, some sold in California also showed intense oxidation. The quality of walnuts is affected by some factors like oxygen pressure/packaging types(Shojaei et al., 2023), temperature(Adkison et al., 2021; Shojaei et al., 2023), humidity(Adkison et al., 2021), and antioxidant addition(Hao et al., 2020).

Table 2.5. FFA (% oleic acid), PV (meq O_2/kg), K_{232}, K_{268}, induction time (hr), and %DPPH inhibition of California walnuts sourced from a handler and various retailers in multiple states in the United States

State	FFA (% oleic acid)	PV (meq O_2/kg)	K_{232}	K_{268}	Induction time (hr)	%DPPH Inhibition
Handler	0.14 ± 0.09	1.03 ± 0.23	1.063 ± 0.086	0.056 ± 0.019	14.32 ± 1.30	74.76 ± 8.35
CA (n=10)	0.22 ± 0.16a	1.41 ± 0.70	**1.885 ± 0.571**	**0.126 ± 0.042**	13.12 ± 2.55a	**61.61 ± 13.62**
CO (n=5)	0.24 ± 0.16a	0.67 ± 0.43	1.363 ± 0.338	0.075 ± 0.014	15.41 ± 2.63a	63.72 ± 12.51
FL (n=3)	0.14 ± 0.11a	0.90 ± 0.08	1.559 ± 0.554	0.126 ± 0.023	13.96 ± 2.28a	61.78 ± 7.09
GA (n=5)	0.13 ± 0.16a	**2.89 ± 1.60**	**2.220 ± 0.141**	**0.155 ± 0.031**	12.93 ± 2.30a	**48.74 ± 13.23**
ME (n=4)	0.22 ± 0.28a	1.20 ± 0.22	**1.813 ± 0.432**	0.110 ± 0.020	14.56 ± 2.47a	**59.02 ± 7.32**
NE (n=5)	0.07 ± 0.08a	0.73 ± 0.32	1.444 ± 0.238	0.105 ± 0.061	14.03 ± 1.88a	64.51 ± 6.33
NV (n=3)	0.31 ± 0.34a	0.96 ± 0.39	**2.039 ± 0.744**	**0.270 ± 0.298**	12.79 ± 2.97a	**52.11 ± 8.41**

OR (n=6)	0.34 ± 0.07a	1.65 ± 0.62	**1.892 ± 0.430**	0.114 ± 0.038	11.75 ± 3.59a	**53.80 ± 9.94**
SC (n=4)	0.33 ± 0.36a	0.94 ± 0.24	1.570 ± 0.336	0.082 ± 0.018	14.74 ± 1.38a	**56.12 ± 8.75**
MA (n=1)	0.25	0.82	1.762	0.106	15.86	65.47
MI (n=1)	0.00	0.86	1.429	0.133	10.49	46.34
WA (n=2)	0.12	0.92	1.381	0.112	15.90	57.48 ± 9.12

[1)]Bold values indicate significant differences from the values of the handler

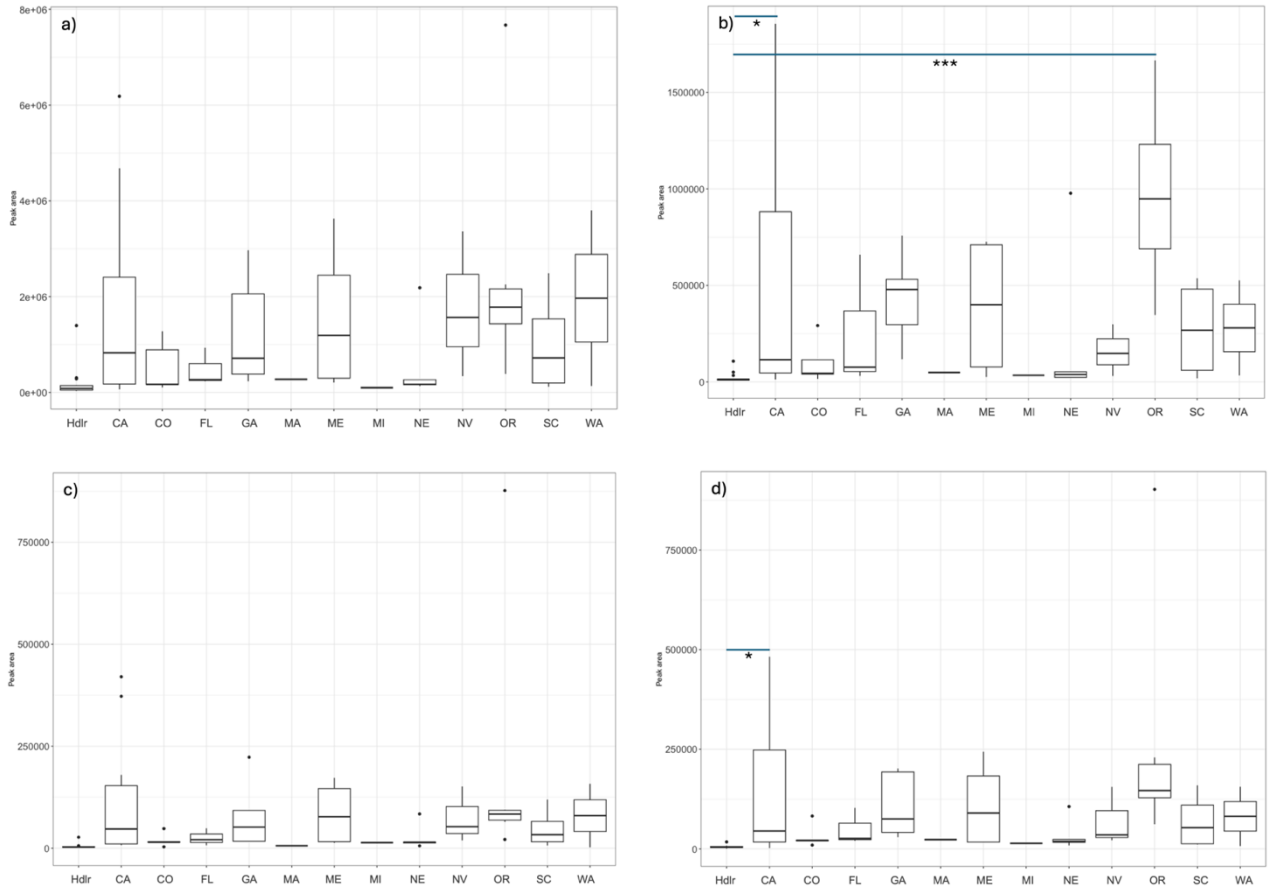


Figure 2.6. Boxplots of peak areas of hexanal (a), 1-hexanol (b), 1-octen-3-ol (c), and 2-pentyl-furan (d) of California walnuts sourced from a handler (Hdlr) and various states in the United States

[1)] Blue lines connecting two groups indicate significant differences (p-values < 0.05). (*) indicates that p-values were between 0.05 and 0.01. (**) indicates that p-values were between 0.01 and 0.001. (***) indicates that p-values were less than 0.001.

Quality of California walnuts sold in multiple retailers in the United States

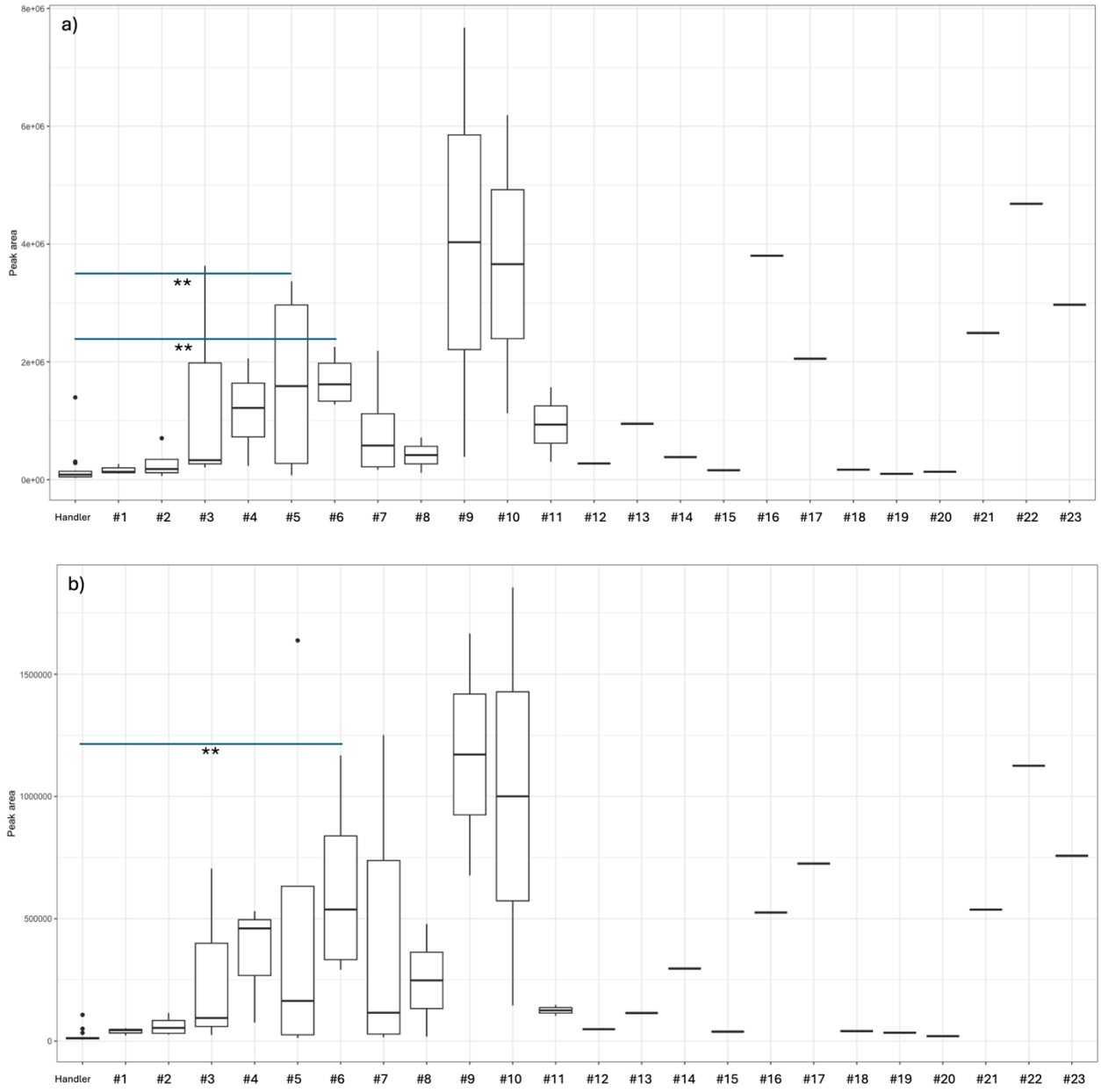
The California walnuts sold in retailers 1 and 2 did not seem to be oxidized, based on their non-significant differences to the reference in FFA, PV, K_{232}, K_{268}, induction time, %DPPH inhibition, and volatile concentrations. Signs of oxidation were found in the samples sold in retailers 3, 4, 5, 6, and 7. The samples sold in retailers 3 and 4 had higher K_{232} values than the handler. The samples sold in retailers 6 and 7 had larger K_{232} and lower %DPPH inhibition than the reference samples. The samples sold in retailer 5 had higher K_{232}, K_{268}, hexanal, and 1-octen-3-ol values and lower %DPPH inhibition than the reference samples (Table 2.6). The samples sold in retailer 6 had more hexanal, 1-octen-3-ol, and 2-pentyl-furan than the handler. The retailer 3 samples had more 1-octen-3-ol, and the retailer 7 samples had more 2-pentyl-furan than the handler (Figure 2.7). Based on the data, the quality of the samples from retailers 5 and 6 was likely among the lowest based on the more significant differences to the reference.

Table 2.6. FFA (% oleic acid), PV (meq O_2/kg), K_{232}, K_{268}, induction time (hr), and %DPPH inhibition of California walnuts sourced from a handler and multiple retailers in the United States

Source	FFA (% oleic acid)	PV (meq O_2/kg)	K_{232}	K_{268}	Induction time (hr)	%DPPH Inhibition
Handler	0.14 ± 0.09	1.03 ± 0.23	1.063 ± 0.086	0.056 ± 0.019	14.32 ± 1.30	74.76 ± 8.35
Retailer 1 (n=3)	0.13 ± 0.09	0.43 ± 0.29a	1.180 ± 0.263	0.075 ± 0.012	16.10 ± 2.85a	66.72 ± 4.77
Retailer 2 (n=4)	0.17 ± 0.12	0.81 ± 0.13a	1.217 ± 0.150	0.089 ± 0.022	15.27 ± 2.37a	63.05 ± 11.06
Retailer 3 (n=3)	0.08 ± 0.08	1.14 ± 0.22a	**1.708 ± 0.463**	0.103 ± 0.017	14.81 ± 2.96a	61.07 ± 7.42
Retailer 4 (n=3)	0.10 ± 0.12	1.19 ± 0.32a	**1.637 ± 0.600**	0.112 ± 0.018	15.20 ± 1.03a	57.87 ± 10.76
Retailer 5 (n=4)	0.24 ± 0.22	1.24 ± 0.67a	**2.054 ± 0.708**	**0.241 ± 0.253**	13.36 ± 2.51a	**55.57 ± 9.13**
Retailer 6 (n=4)	0.27 ± 0.18	1.29 ± 0.37a	**2.087 ± 0.298**	0.096 ± 0.039	13.31 ± 2.41a	**54.28 ± 10.46**
Retailer 7 (n=8)	0.18 ± 0.18	1.43 ± 1.36a	**1.673 ± 0.360**	0.112 ± 0.041	13.31 ± 2.33a	**57.96 ± 16.12**

Retailer 8 (n=2)	0.07	2.64	1.961	0.133	13.26	47.03
Retailer 9 (n=2)	0.31	1.81	1.454	0.105	9.06	59.83
Retailer 10 (n=2)	0.09	1.91	2.07	0.125	12.54	63.64
Retailer 11 (n=2)	0.46	0.89	1.577	0.108	12.18	62.37
Retailer 12 (n=1)	0.25	0.82	1.762	0.106	15.87	65.47
Retailer 13 (n=1)	0.37	2.61	2.548	0.157	9.40	34.17
Retailer 14 (n=1)	0.19	1.21	2.212	0.123	15.20	59.69
Retailer 15 (n=1)	0.17	0.86	1.699	0.214	13.49	55.83
Retailer 16 (n=1)	0.04	1.00	1.641	0.128	15.40	63.93
Retailer 17 (n=1)	0.62	1.37	2.125	0.132	13.82	52.86
Retailer 18 (n=1)	0.19	0.86	1.431	0.094	13.64	49.69
Retailer 19 (n=1)	0.00	0.86	1.429	0.133	10.49	46.34
Retailer 20 (n=1)	0.21	0.85	1.529	0.095	14.20	63.88
Retailer 21 (n=1)	0.86	1.04	1.651	0.089	16.73	64.19
Retailer 22 (n=1)	0.29	1.41	2.765	0.196	12.04	62.08
Retailer 23 (n=1)	0.39	2.66	2.306	0.163	12.34	56.58

[1)]Bold values indicate significant differences from the values of the handler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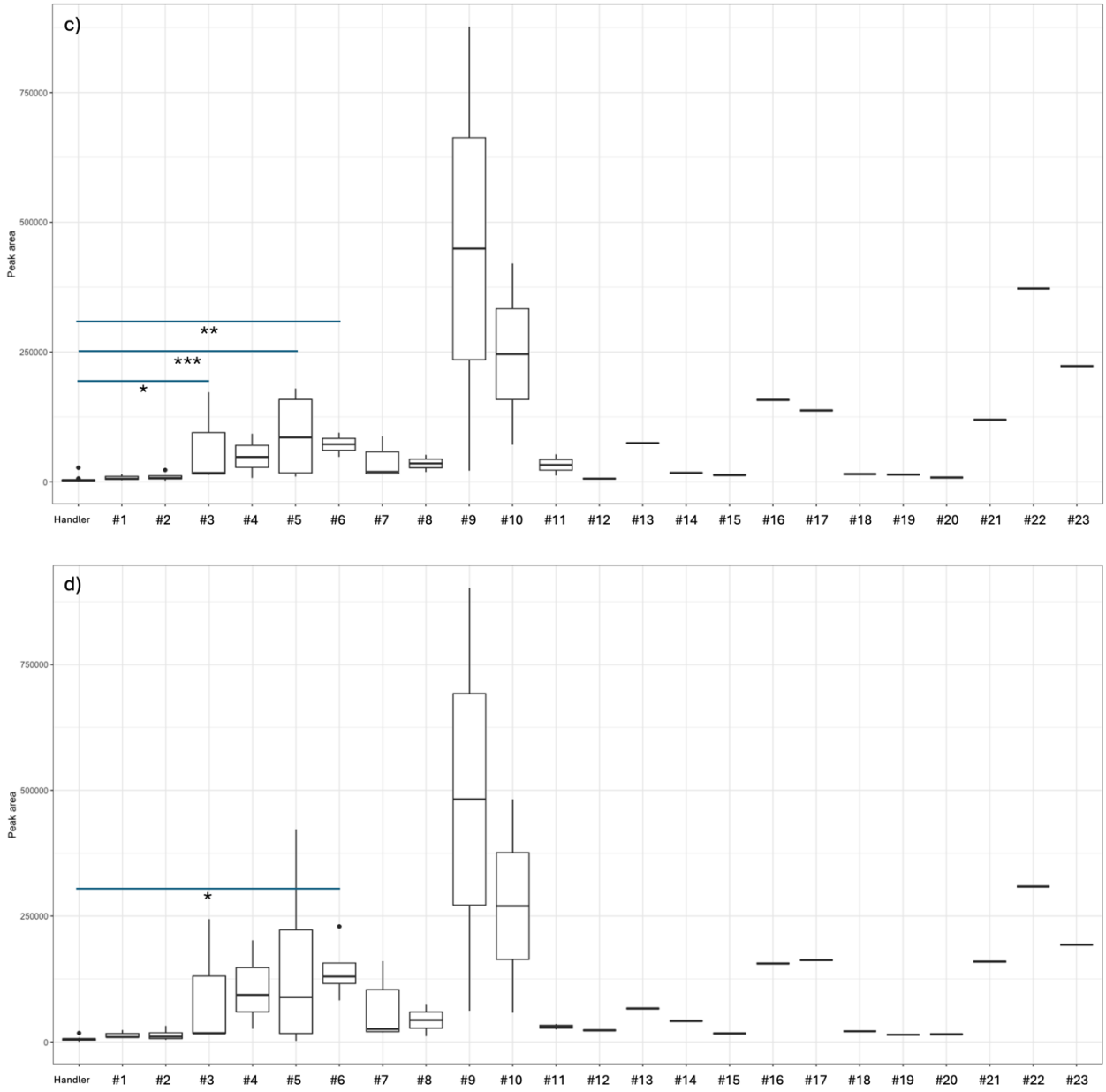

Figure 2.7. Boxplots of peak areas of hexanal (a), 1-hexanol (b), 1-octen-3-ol (c), and 2-pentyl-furan (d) of California walnuts sourced from a handler and multiple retailers in the United States

[1)] Blue lines connecting two groups indicate significant differences (p-values < 0.05). (*) indicates that p-values were between 0.05 and 0.01. (**) indicates that p-values were between 0.01 and 0.001. (***) indicates that p-values were less than 0.001.

Quality of California walnuts sold in multiple countries outside the United States

Except for the California walnuts sold in the United Kingdom, all the samples (with the number of replicates larger than two) showed signs of oxidation. The samples sold in Australia showed larger K_{232} and K_{268}, lower %DPPH inhibition, and higher concentrations of hexanal than the reference samples. The samples sold in Canada had higher FFA, K_{232}, and lower %DPPH inhibition than the reference samples. The India samples had larger K_{268} and lower %DPPH inhibition. The Indonesia samples had higher K_{232}, K_{268}, and hexanal concentrations than the reference samples. The Japan samples had higher K_{232} and lower %DPPH inhibition than the reference samples (Table 2.7 and Figure 2.8). These results suggested that, in most cases, the international distribution led to the oxidation of lipids and phenols in walnuts.

Higher K_{232} and K_{268} values were often accompanied by lower antioxidant capacity. However, in the Indonesia samples with high UV indexes, the antioxidant capacity was not significantly different from the reference (Table 2.7). The rare disagreement between the DPPH assay result and UV index may be attributable to the diversity in the phenolic compounds and their antioxidant capacity. Phenol composition is affected by a variety of factors, including genetics(Trandafir & Cosmulescu, 2020), kernel skin color(Athaillah & Wang, 2024), ripening stages(Amin et al., 2017) and storage condition and period(Ma et al., 2021). In addition, the phenolic compounds have varying antioxidant capacities(Apak et al., 2007).

Preserving walnut quality can be done by minimizing oxygen exposure and storing them at low temperatures. A study showed that vacuum packaging (at 5% vacuum level in polyethylene bags) resulted in higher antioxidant capacity of walnuts than those packaged with air when stored at 25 °C for 6 months for Stone and Paper cultivars grown in Iran. A similar vacuum treatment coupled with refrigeration (4 °C) improved antioxidant capacity in cultivar Stone but not in

cultivar Paper when compared to the respective samples packaged with air. For both cultivars, storage at 4 °C yielded significantly higher antioxidant capacity than the samples stored at 25 °C(Shojaei et al., 2023). This study demonstrated that refrigeration alone is adequate in preserving the antioxidant capacity of walnut phenolics upon 6-month storage, while the effect of vacuum treatment depends on cultivar selection and temperature(Shojaei et al., 2023). Another study showed that limiting oxygen by vacuum packaging in aluminum foil with the addition of iron deoxidizer helped preserve the quality of hot air-dried walnuts during accelerated storage at 60 °C for 30 days. The quality of walnuts from that treatment was better than those vacuum packaged and added with food preservatives tert-butylhydroquinone (TBHQ), citric acid, and sodium dihydrogen phosphate(Hao et al., 2020).

Table 2.7. FFA (% oleic acid), PV (meq O_2/kg), K_{232}, K_{268}, induction time (hr), and %DPPH inhibition of California walnuts sourced in various countries outside the United States

Country	FFA (% oleic acid)	PV (meq O_2/kg)	K_{232}	K_{268}	Induction time	%DPPH Inhibition
Handler	0.14 ± 0.09	1.03 ± 0.23	1.063 ± 0.086	0.056 ± 0.019	14.32 ± 1.30	74.76 ± 8.35
Australia (n=5)	0.24 ± 0.14	1.84 ± 1.37	**1.833 ± 0.288**	**0.126 ± 0.044**	13.44 ± 1.83	**61.32 ± 3.26**
Canada (n=4)	**0.34 ± 0.22**	0.95 ± 0.27	**1.623 ± 0.580**	0.100 ± 0.028	15.48 ± 1.83	**55.98 ± 9.34**
India (n=5)	0.25 ± 0.15	1.73 ± 0.96	1.418 ± 0.318	**0.105± 0.034**	11.73 ± 2.32	**63.86 ± 5.85**
Indonesia (n=3)	0.00 ± 0.01	1.76 ± 0.69	**2.108 ± 0.823**	**0.146 ± 0.104**	13.63 ± 3.60	72.91 ± 13.93
Japan (n=3)	0.25 ± 0.10	1.15 ± 0.19	**1.663 ± 0.106**	0.082 ± 0.006	13.14 ± 2.76	**60.53 ± 2.26**
UK (n=3)	0.27 ± 0.09	0.60 ± 0.12	1.100 ± 0.089	0.054 ± 0.034	15.20 ± 3.32	61.69 ± 7.60
France (n=1)	0.67	0.87	1.172	0.059	12.2	71.37
Hongkong (n=1)	0.14	0.86	1.370	0.127	14.76	74.16

[1)]Bold values indicate significant differences from the values of the handler

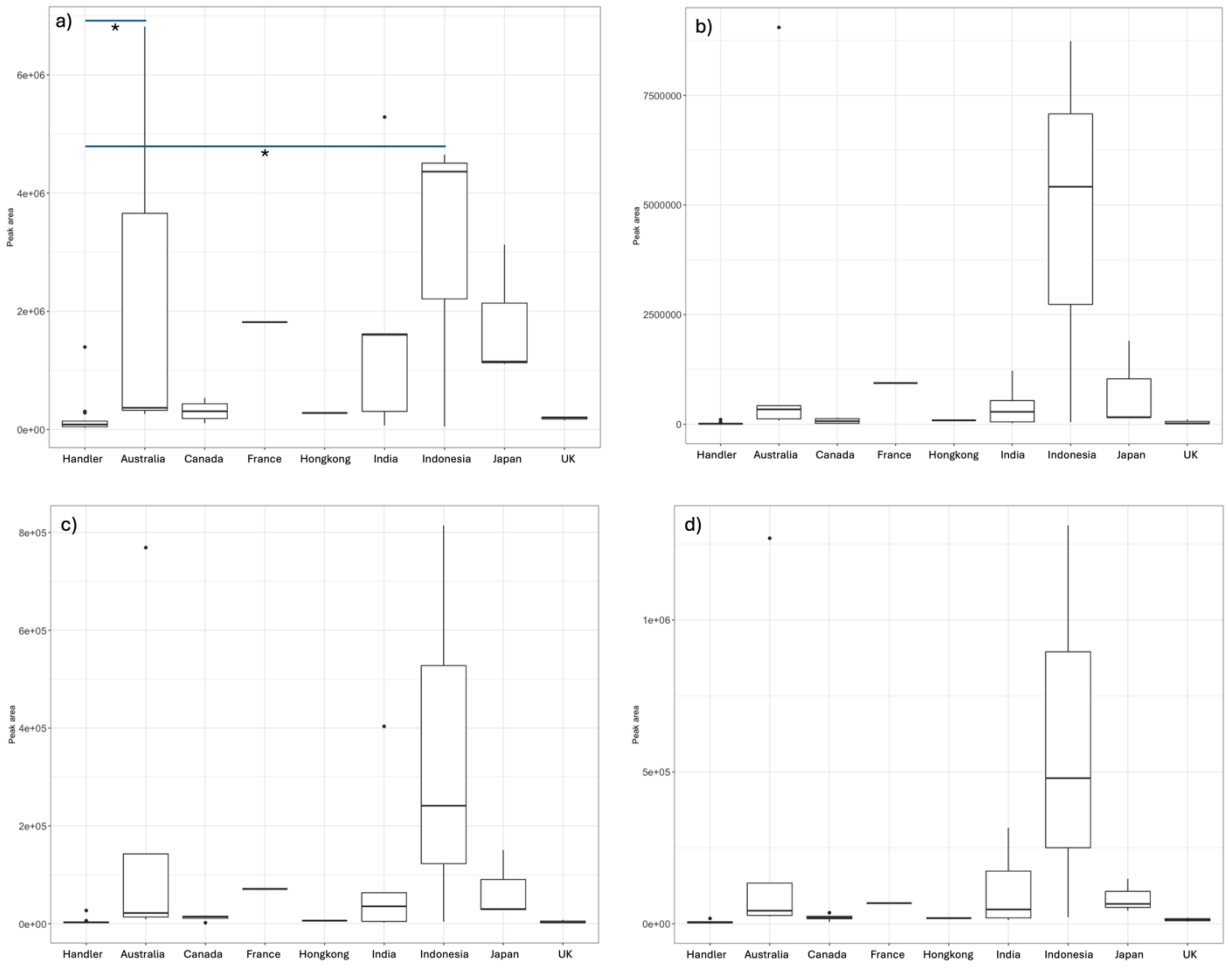

Figure 2.8. Boxplots of peak areas of hexanal (a), 1-hexanol (b), 1-octen-3-ol (c), and 2-pentyl-furan (d) of California walnuts sold in various countries outside the United States

[1)] Blue lines connecting two groups indicate significant differences (p-values < 0.05). (*) indicates that p-values were between 0.05 and 0.01. (**) indicates that p-values were between 0.01 and 0.001. (***) indicates that p-values were less than 0.001.

Comparison and correlation of walnut quality parameters

Our data demonstrated that the UV index, antioxidant capacity, and volatiles were more sensitive in detecting differences between commercial walnuts and the reference than FFA, PV, and oxidative stability (Table 2.4-7). While FFA values may increase during storage as triacylglycerols break down into diacylglycerols, monoacylglycerols, and free fatty acids, they also can deplete as the free fatty acids are oxidized. Peroxide values may not fully correspond to

oxidation level because the values increase in the initial oxidation stage and decrease as oxidation advances(Charles Bai et al., 2022; Li et al., 2014). Concentrations of conjugated dienes, however, keep increasing during the oxidation of sunflower and rapeseed oil at 40 and 60 °C for up to 60 days(Amft et al., 2023). The concentration of volatiles included as secondary oxidation products, i.e., hexanal, keeps increasing as rapeseed oil with an initial PV of 4 meq O_2/kg is heated at 160 °C for 120 min. However, hexanal concentration was steady after a maximum value was reached for rapeseed oil with an initial PV of 20 meq O_2/kg heated at the same temperature and period(Grebenteuch et al., 2021). In some cases, low concentrations of hexanal (and other aldehydes and alkenals) do not mean that the samples were less oxidized. The volatiles may form tertiary oxidation products, including alkyl furans and aldol adducts(Grebenteuch et al., 2021). Therefore, the presence of alkyl furans, i.e., 2-pentyl furan, suggests a later stage of oxidation.

Correlations between the quality parameters were evaluated. FFA did not show any significant correlations with the other parameters. PV showed moderate negative correlations to induction time and %DPPH inhibition and moderate positive correlations to K_{232}, 1-propanol, and 6-methyl-5-hepten-2-one. K_{232} highly corresponded to K_{268}, total esters, total ketones, propanoic acid butyl ester, 3-methyl-4-heptanone, 6-methyl-5-hepten-2-one, and 3,5-octadien-2-one. K_{268} also showed strong positive correlations to propanoic acid butyl ester, 3-methyl-4-heptanone, 3,5-octadien-2-one, total esters, and total ketones but at a lesser intensity than K_{232}. Oxidative stability demonstrated moderate negative correlations to heptanal, 1-octen-3-ol, 2-methoxy-furan, total ketones, total furans, and total volatiles. DPPH inhibition levels had strong negative correlations to propanoic acid butyl ester, 3-methyl-4-heptanone, total esters, and total ketones. The peak areas of many volatiles highly correspond to those of the other volatiles (Figure 2.9).

Some of the correlations mentioned above could be explained by connections between those parameters reported in previous studies. The high correlation between antioxidant capacity and concentrations of ketones and esters could be explained by the ability of some antioxidant phenolics, i.e., ferulic acid and caffeic acid, to minimize hydroperoxide production(Yanishlieva & Marinova, 2003), that are substrates for ketone (through OH^- elimination)(Xia & Budge, 2017) and ester formation(Frankel, 1980). The high correlation between the peak areas of ketones and K_{232} values confirmed that ketones are produced through the decomposition of hydroperoxides(Xia and Budge 2017). The high correlation between ketones and K_{268} indicates that the head-space volatile measurement corroborated the spectrophotometry analysis of ketones.

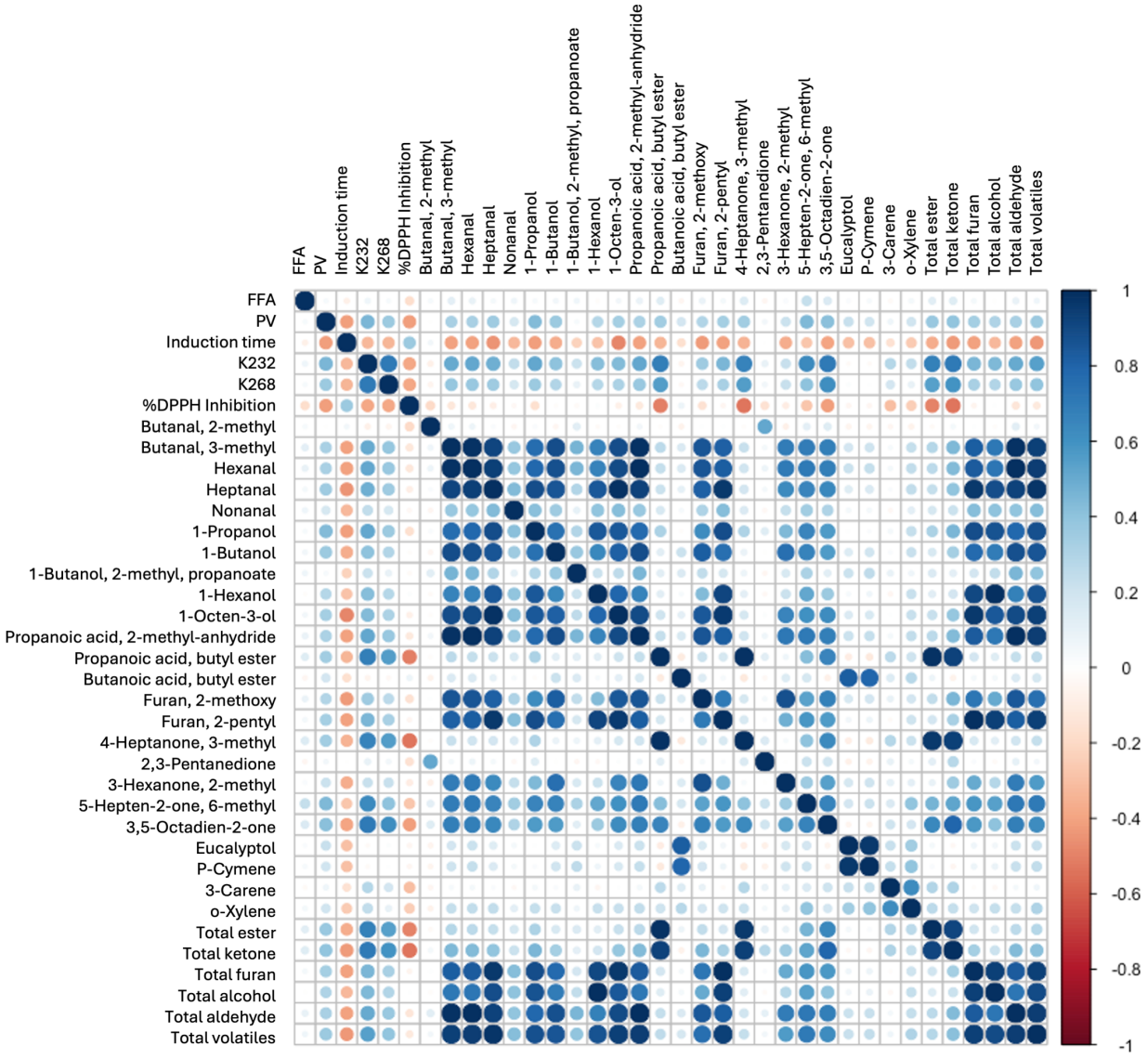

Figure 2.9. Correlation plot between all the parameters studied

Conclusions

Many California walnut samples sold at retail, either in the US or overseas, showed signs of lipid and phenol oxidation, evidenced by higher K_{232}, K_{268}, some volatiles and lower antioxidant capacity compared to the reference. The samples sourced from the domestic markets had quality profiles that were almost entirely different than the reference while those sourced from overseas shared a few similarities with the reference. The quality of the samples from several states and

retailers or those sold in the United Kingdom was comparable to that of the reference. This suggested that preserving the quality of walnuts during their supply chain is possible with the application of proper packaging, storage and protection from oxidation.

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CHAPTER 3

Lipid and Phenol Metabolites of Californian Walnuts (*Juglans regia* L.) Varieties: Chandler, Howard, and Tulare

Abstract

Californian walnuts are dominated by cultivars Chandler, Howard, and Tulare. To obtain a thorough comparison of their quality and methods to classify them, lipid and phenol metabolites were evaluated. Chandler, Howard, and Tulare were sourced from multiple growers, and for some parameters, the samples were collected from two harvest years. Our data demonstrated that Chandler, Howard, and Tulare were similar regarding the concentrations of total fat, some triacylglycerols (i.e., trilaurin, tricaprylin, tristearin, tripalmitin, trilinolein, 1,2-olein-3-stearin, and 1,2-laurin-3-miristin), several fatty acids (i.e., margaric, stearic, oleic, and linoleic acids), delta- and alpha-tocopherols, free fatty acids (FFA), peroxide values (PV), all the volatiles, and many phenolic compounds (i.e., 3,4-dihydroxybenzoic acid, catechin, procyanidin B1, caffeic acid, p-coumaric acid, vanillic acid, ferulic acid, sinapic acid, ellagic acid, quercetin, and naringenin). They also had similar values in oxidative stability and most compound-specific stable isotope of amino acids and fatty acids. However, they differed in the levels of 1-olein-2,3-linolein, triolein, 1,2-linolein-3-linolenin, 1,2-palmitin-3-linolein, 1-palmitin-2-olein-3-stearin, 1-palmitin-2-stearin-3-olein, 1,2-linolein-3-stearin, trilinolenin, palmitic acid, palmitoleic acid, linolenic acid, arachidic acid, eicosenoic acid, beta-sitosterol, gamma-tocopherol, chlorogenic acid, and $\delta^{13}C_{Ser}$. Classification of the cultivars was possible when linear discriminant analysis (LDA) was employed on the triacylglycerol (TAG) data. The connection between Chandler and

Howard seemed closer than Chandler with Tulare or Howard with Tulare, based on their TAG profile, which their phylogeny could explain.

Keywords: walnut (*Juglans regia* L.), quality, cultivar, authenticity

Introduction

In the United States (US), around 99% of walnuts are cultivated in California, particularly in Central Valley(OSU, 2024). Californian walnuts are not only essential for US consumption but also for many other countries because they are exported worldwide(Agricultural Marketing Resource Center, 2006). Chandler, Howard, and Tulare are the major varieties of walnuts planted in California(Volk & Preece, 2021). They are released from various breeding programs in the region. Chandler and Howard are derived from crossing varieties #56-224 and Pedro(#53-113). Tulare, on the other hand, was generated by crossing Tehama and Serr. Varieties #56-224, Pedro, Tehama, and Serr can be traced back to cultivar Payne(Vahdati et al., 2019).

Many studies related to the comparison of these cultivars mainly focus on the agronomical, processing, economy, or sensory aspects, e.g., leafing date, yield, bearing habit, tree rigor, nut weight, shell hardness, and kernel weight and color(Vahdati et al., 2019). However, a comprehensive comparison of their quality and chemical profiles is lacking.

Walnut quality is linked to lipid degradation and oxidation because lipids comprise 60-70% of walnut kernel mass(Amaral et al., 2003). Walnut lipids primarily consist of triacylglycerols (TAGs)(Yan et al., 2021) and they can undergo oxidation, forming triacylglycerol peroxides that can later transform into secondary oxidation products like aldehydes and carboxylates(Kato et al., 2018). TAG oxidative stability is affected by the composition and position of their constituent fatty acids(Neff et al., 1992). Variability in the fatty acid composition of different

walnut cultivars is expected because it has been observed previously(Kafkas et al., 2020). TAGs with a polyunsaturated fatty acid (PUFA) in the 1st or 3rd carbon positions have less oxidative stability than TAGs with PUFA in the 2nd position(Kato et al., 2018), probably because in the 2nd position the fatty acid is more protected from radicals. TAGs can also be hydrolyzed, and the resultant free fatty acids are oxidized into hydroperoxides. The hydroperoxides can then be cleaved, generating various volatiles that affect sensory profiles, including rancidity. The oxidation susceptibility of free fatty acids is influenced by their structure, particularly the unsaturation degree. The more double bonds there are, the higher the susceptibility to oxidation(Shahidi & Zhong, 2010). While polyunsaturated fatty acids are readily oxidized, they have various health effects, including improving antioxidant enzyme activity, reducing DNA damage, and reducing the risks of cardiovascular disease and diabetes(Marangoni et al., 2020; Pal & Ghosh, 2012).

In addition to using the concentrations of triacylglycerols, fatty acid profile, free fatty acids, hydroperoxides, and volatiles to determine walnut stability, an oxidative stability test was also conducted. This test has been widely used to predict food products' shelf life and stability, particularly oils or foods with high lipid content. In some previous works, the oxidative stability index strongly correlates with peroxide value(Gordon & Mursi, 1994) or rancidity onset determined by a sensory panel(Coppin & Pike, 2001).

Besides evaluating lipids that are substrates or products of oxidation, lipids with antioxidant capacity, i.e., sterols and tocopherols, were also measured. Beta-sitosterol, the most abundant sterol in walnuts, can reduce methyl linoleate oxidation(Yoshida & Niki, 2003). Similarly, tocopherols are also antioxidants; for instance, they reduce the rate of hydroperoxide and hexanal formation(Huang et al., 1994). Besides functioning as antioxidants for food systems, sterols and

tocopherols provide beneficial health effects. Phytosterol intake is linked to reduced intestinal cholesterol absorption, decreased onset or growth of lung, stomach, ovarian, and breast cancer, and increased activity of antioxidant enzymes(Woyengo et al., 2009). Tocopherols promote cell membrane repair, inhibit platelet aggregation, and enhance immune activity(Rizvi et al., 2014).

Besides lipids, walnut kernels also have high concentrations of phenolic compounds(Wu et al., 2021). These compounds are expected to reduce the rate of lipid oxidation because many of them have antioxidant capacity(Labuckas et al., 2008). Total phenol was measured in the current study to provide an overview of the phenolic content in the samples. To delve further, identification and quantifications of the phenolic compounds were also conducted.

The efficacy of compound-specific stable isotope analysis (CSIA) for cultivar authentication was also assessed. This method has successfully differentiated walnut origins but has yet to be tested for cultivar classification of walnuts from the same origin, so we explored its potential in this work.

All the parameters were analyzed to help us better understand the metabolites and quality profiles of Chandler, Howard, and Tulare. Variability in harvest years was included for some parameters to consider variations in agroclimatic effects and postharvest handling that may affect the cultivar comparison. The information provided from this work could be helpful for walnut growers in selecting cultivars and for handlers to consider ensuring the quality and nutrient density of the walnuts.

Materials and Methods

Sampling

Walnuts (0.5– 2 kg) were sourced from various growers or handlers in Central Valley in California, USA. The samples were collected in 2020 and 2022. The samples collected in 2020 could be from the harvest of that year or prior. The samples sourced in 2022 were harvested in September and October of that year.

Some tests were conducted on samples from the two collection years, while the other tests were only conducted on the samples sourced in 2022. In 2020, the samples comprised nine Chandler, ten Howard, and ten Tulare. In 2022, they comprised eight Chandler, three Howard, and three Tulare (Table 3.1). Samples were stored at 0-5 °C before testing or oil extraction.

Table 3.1. Collection year (CY), cultivar, and condition upon received of the walnut samples

No	CY	Cultivar	Condition
1	2020	Chandler	In-shell
2	2020	Chandler	In-shell
3	2020	Chandler	In-shell
4	2020	Chandler	In-shell
5	2020	Chandler	In-shell
6	2020	Chandler	In-shell
7	2020	Chandler	In-shell
8	2020	Chandler	In-shell
9	2020	Chandler	In-shell
10	2020	Howard	In-shell
11	2020	Howard	In-shell
12	2020	Howard	In-shell
13	2020	Howard	In-shell
14	2020	Howard	In-shell
15	2020	Howard	In-shell
16	2020	Howard	In-shell
17	2020	Howard	In-shell
18	2020	Howard	In-shell
19	2020	Howard	In-shell
20	2020	Tulare	In-shell

21	2020	Tulare	In-shell
22	2020	Tulare	In-shell
23	2020	Tulare	In-shell
24	2020	Tulare	In-shell
25	2020	Tulare	In-shell
26	2020	Tulare	In-shell
27	2020	Tulare	In-shell
28	2020	Tulare	In-shell
29	2020	Tulare	In-shell
30	2022	Chandler	Shelled
31	2022	Chandler	In-shell
32	2022	Chandler	In-hull
33	2022	Chandler	In-shell
34	2022	Chandler	In-shell
35	2022	Chandler	In-shell
36	2022	Chandler	Shelled
37	2022	Chandler	In-shell
38	2022	Howard	In-shell
39	2022	Howard	Shelled
40	2022	Howard	In-shell
41	2022	Tulare	In-shell
42	2022	Tulare	In-shell
43	2022	Tulare	Shelled

Oil extraction

Oil was extracted from the samples for analysis of triacylglycerols, fatty acid profile, sterols, tocopherols, free fatty acidity, peroxide value, and CSIA-fatty acids. Walnut kernels (±400 g) were fed into a cold-press machine (KK Oil Prince F Universal, Reut, Germany). The expeller screw was rotated at 22 rpm, and a die number 7 was used. The resultant slurry was centrifuged at 10,000 g for 15 min to separate the oil from solid particles. The oil samples were then kept at -20 °C until analysis.

Lipid analysis

Total fat

Walnut kernels (10-15 kernel halves) were ground using a food processor, and 3 ± 0.1 g of them was added into a cellulose thimble (33 × 150 mm, Whatman 603 Cellulose thimbles). The thimbles were placed in a Buchi Universal Extractor E-800 (Buchi Corporation, New Castle, United States). The fat was extracted 30 times using n-hexane. After the hexane was evaporated, the fat mass was recorded. Three replicates were analyzed for each sample.

Triacylglycerols (TAG) and beta-sitosterol

Approximately 80 mg of oil samples were mixed thoroughly with 10 mL of chloroform: methanol (1:1, v/v). One μL of the solution was then injected into a liquid chromatography (LC) system (Agilent 1260 Infinity II, Agilent Technologies Inc., California, USA) that was connected to tandem mass spectrometry (6470 triple quadrupoles, Agilent Technologies Inc., California, USA). The LC settings followed a previous study(Matsuzawa et al., 2021) with modifications in the column and detector used. In the current study, separation was done on a Thermo Scientific™ Accucore™ C18 column (100 mm x 2.1 mm, 2.6 μm). Mobile phase A was acetonitrile: water: ammonium acetate 1 M: formic acid (200:800:10:1, v/v). Mobile phase B was acetonitrile: isopropanol: ammonium acetate 1 M: formic acid (100: 900: 10:1, v/v). The flow rate was 0.20 mL/min, and the LC compartment was maintained at 55 °C. At the start, the mobile phase B was 35%, then the proportion was changed to 70% at 3 min, 85% at 7 min, and 90% at 10 min. The 90% proportion was held for 2 min, then ramped again to 97% at 12.5 min. Later, it was decreased to 35% at 18 min, and that percentage remained constant until 23 min. The mass spectrometry (MS) was operated at MS2 scan mode and positive ionization. The scan

mode searched for ions from 100 to 1600 m/z with scan time 200 ms and fragmentor energy of 200 V. The cell accelerator voltage was set at 5 V. Compound identification was done by comparing the retention time and mass/charge (m/z) to those of standards. TAGs were identified using $[M+NH_4]^+$ ions, whereas sterols were identified using $[M+1-H_2O]^+$ ions. A list of retention time and target m/z values was supplied (Supporting Information, Table S3.1). TAG quantitation was done by calculating the percentage of peak area of a TAG compound to total peak area of TAGs studied. For free sterol, the actual concentration was quantified. The analysis was conducted in triplicates.

Fatty acid profile

Walnut oil (±20 µL) was dissolved in 3 mL of hexane. Fatty acids in the samples were derivatized using methanolic potassium hydroxide (0.2 mL, 2 M) to produce fatty acid methyl esters (FAMEs). The mixture was vortexed and then left to stratify until the upper solution became clear. The upper phase was subjected to a gas chromatography-flame ionization detector, GC-FID (Agilent 7890A GC, Agilent Technologies, California, USA). The GC-FID analysis was done following this method(Green & Wang, 2020). Peak identification was performed using a 37-FAME mix, certified reference material (TraceCERT, Millipore Sigma, USA). This analysis was done in triplicates.

Tocopherols

The cold-pressed oil from each sample (0.2 g) was mixed thoroughly with 0.9 mL of isopropanol and 0.1 mL of internal standard (α-tocopherol acetate). The mixture was then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and filtered using a nylon filter (0.45 µm). The samples were then analyzed using high-performance liquid chromatography coupled to a

diode array detector (HPLC-DAD) (Agilent 1290 Infinity II LC System, Agilent Technologies, California, United States). Reverse phase LC followed a published method(Górnaś, 2015) with several modifications, particularly in the detector type and flow rate. The injection volume was 5 µL. Separation was done using a PFP column (150 mm x 4.6 mm, 3 µm) with methanol: water (93:7, v/v) as the mobile phase). The flow followed isocratic mode at 0.8 mL/min. The DAD wavelength was set at 290 nm. This analysis was done in triplicates.

Free fatty acidity (FFA)

Free fatty acid analysis was conducted on 10 g of oil samples following European Union Commission Regulation (EEC) no 2568/91. Three replicates were analyzed for each sample.

Peroxide value (PV)

The peroxide value analysis was performed as illustrated in the AOCS official method Cd 8b-90. Approximately 5 g of oil was used for the analysis, and two or three replicates were analyzed for each sample.

Oxidative stability

Oxidative stability of the kernels (0.5 g) was measured following a published study on walnut(Grilo & Wang, 2021) using a Rancimat apparatus 617 (Metrohm AG, Herisau, Switzerland). The analysis was done in triplicates.

Volatile organic compounds

Walnut kernels (10-15 kernel halves) were ground using a food processor, and 3 ± 0.1 g of them was placed in a 20-mL vial. Volatile organic compounds were analyzed using headspace-solid phase microextraction (HS-SPME) connected to a gas chromatography-mass spectrometry (GC-

MS), following a published method(Grilo & Wang, 2021). Three replicates were analyzed for each sample.

Phenol analysis

Total phenol assay

Walnut kernels (10-15 kernel halves) were ground using a food processor. Then, ±3 g of the ground kernel was placed in a centrifuge tube. The lipid portion was removed through hexane extraction. Hexane (25 mL) was added, and the mixture was vortexed for 5 s and sonicated for 5 min at room temperature. The mixture was centrifuged at 2,000 g for 5 min, and the hexane phase was removed. This lipid extraction was done one more time with 15 mL of hexane. Afterward, 5 mL of aqueous acetone (acetone: water, 7:3, v/v) was added to the defatted sample for phenol extraction. The mixture was vortexed for 2 min and sonicated for 30 min. The mixture was centrifuged at 10,000 g for 5 min. The aqueous acetone phase was transferred into a centrifuge tube. Phenol extraction was repeated, and the aqueous acetone layer was combined with that of the first extraction.

Total phenol was analyzed using the Folin Ciocalteu assay following a published method(Polari & Wang, 2020) with few modifications in the concentration and volume of the reagent. An aliquot of the extract (0.2 mL) was diluted with water to 5 mL. Folin Ciocalteu reagent (0.5 mL) was added, followed by 3 mL of 17.5% (w/v) sodium carbonate. After adding 1.5 mL of water, the mixture was mixed and then incubated for 30 min in the dark. Later, the absorbance at 725 nm was measured, and the concentration of total phenolic compounds was calculated using an external calibration curve prepared with gallic acid.

Individual free phenols

Phenol extracts were prepared the same way as those used for the total phenol assay. The extract was then filtered using a nylon filter (45 μm), and two μL of it was injected into a liquid chromatography coupled to a tandem mass spectrometry (LC-MS/MS). The LC-MS/MS method followed a previous study(Athaillah & Wang, 2024)

CSIA of amino acids and fatty acids

The sample preparation and analytical procedure were similar to those in a previous study(Athaillah et al., 2023) and followed some earlier studies(Eder, 1995; Meier-Augenstein, 2002).

Statistical Analysis

An analysis of variance (ANOVA) test was conducted to determine if the means of fat content, relative concentrations of fatty acids, concentrations of volatile organic compounds, total phenol content, and concentrations of individual free phenols differed across cultivars (Chandler, Howard, and Tulare), collection years (2020 and 2022), and the interaction between them. It was also used to determine if the means of triacylglycerol relative concentrations, beta-sitosterol concentration, tocopherol concentrations, free fatty acidity, peroxide value, oxidative stability, and CSIA of amino acids and fatty acids differed among the cultivars (without considering collection year) for the samples collected in 2022. Tukey HSD test was selected for pairwise comparison. Linear discriminant analysis (LDA) was conducted to reduce the dimensionality of triacylglycerol data. All the statistical analysis was performed using R Studio 2022.07.01 (Posit software, PBC, Boston, MA, USA) and R 4.2.2. (The R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

Lipids

Total fat

A significant difference in total fat was only observed between Tulare collected in 2022 and Howard sourced in 2020. The other combinations of cultivar and year did not result in considerable variations in the fat content values (Table 3.2). Within the same harvest year, the fat content was similar among Chandler, Howard, and Tulare. This finding agreed with an earlier report - no significant difference was noted between the total fat of Chandler, Howard, Tulare, Franquette, and Lara, either when the automated Soxhlet technique (similar to our method) or near-infrared hyperspectral imaging was used for the analysis(Nogales-Bueno et al., 2021). A significant difference was, however, observed when other cultivars, i.e., Scharch-Franquette, Hartley, Hugget, Mayette, Mollar de Nerpio, Parisienne, Payne, Pedro, and Serr, were compared(Rabadán et al., 2019).

No significant variability between the fat contents of walnuts from the two harvest years suggested that the fat content was relatively stable upon climatic changes. It agreed with another study showing that the fat content of walnuts did not vary significantly between harvest years(Rabadán et al., 2019). The non-significant difference in the fat content in the current study also indicated that the harvest time of the samples from each cultivar was similar between the harvest years because harvest time is one of the factors affecting the fat contents of walnuts(Matthäus et al., 2018).

Table 3.2. Total fat (g/100 g) of Chandler, Howard, and Tulare walnuts sourced in 2020 and 2022

CY	Cultivar	Total fat (g/100 g)
2020	Chandler	67.61 ± 0.96ab
	Howard	66.87 ± 1.79b
	Tulare	68.66 ± 1.92ab
2022	Chandler	68.31 ± 2.62ab
	Howard	70.56 ± 4.87ab
	Tulare	71.22 ± 2.15a

*CY: collection year
**Significant difference (p-value < 0.05) between combinations of the cultivars and collection years was marked as different letters.

Triacylglycerols

Chromatograms of a standard mixture and a sample were provided in Figure S3.1 of the Supporting Information. Fifteen TAG compounds were analyzed. TAGs with the highest concentrations were trilinolein (±29%), 1,2-linolein-3-linolenin (±25%); 1-olein-2,3-linolein (±15%), and 1,2-linolein-3-stearin (±13.5%) (Table 3.3). A high abundance of TAGs containing linoleic acids was expected as linoleic acid is walnuts' most dominant fatty acid. The high abundance of linoleic acid-containing TAGs agreed with the observation of Wang et al.(Wang et al., 2022) and Bouabdallah et al.(Bouabdallah et al., 2014).

Differences in the TAG profile of walnuts from Chandler, Howard, and Tulare varieties were observed. Tulare had a lower concentration of TAGs containing linolenic fatty acid (i.e., 1,2-linolein-3-linolenin and trilinolenin) than Chandler and Howard (Table 3.3). Effects of genetics on TAG composition have been noted before. Some TAGs, e.g., 1,2-linolein-3-linolenin, trilinolein, and 1-olein-2,3-linolein, varied among several cultivars of walnuts grown in an

experimental orchard in Tunisia(Bouabdallah et al., 2014) and China(Yan et al., 2021). The lower concentration of linolenic acid-containing TAGs in Tulare corresponded with the lower concentrations of linolenic acid in the cultivar, based on the fatty acid profile (Table 3.4). Similar trends of fatty acid and TAG profiles were expected because fatty acids are the building blocks of TAGs. Linolenic acid is a precursor for jasmonic acid that plays a vital role in defense response when plants are exposed to herbivores and microbial pathogens(Li et al., 2005). The decrease of linolenic acid in Tulare could manifest as a lower ability to defend against some biotic stress, such as pests and microbes. It was also possible that linolenic acid concentration in Tulare decreased because it had been converted into jasmonic acid for stress adaptation. In addition, the lower concentration of linolenic acid in Tulare could also suggest lower health benefits. Linolenic acid intake reduces total cholesterol, low-density lipoprotein (LDL) cholesterol, triacylglycerols, and blood pressure(Sala-Vila et al., 2022). On the other hand, linolenic acid, owing to its three double bonds, is highly susceptible to oxidation. Its concentration negatively correlates with oxidative stability(Mao et al., 2020).

Table 3.3. Percentage of peak areas of triacylglycerols of Chandler, Howard, and Tulare walnuts sourced in 2022

TAG	Chandler	Howard	Tulare
Trilaurin	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a
1-Olein-2,3-linolein	15.3 ± 0.38a	14.8 ± 0.33ab	14.2 ± 0.34b
Tricaprylin	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a
Triolein	8.52 ± 0.53ab	7.87 ± 0.65b	9.38 ± 0.61a
Tristearin	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a
Tripalmitin	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a
Trilinolein	29.0 ± 0.83a	29.6 ± 1.08a	29.6 ± 0.84a
1,2-Linolein-3-linolenin	25.1 ± 0.25a	25.5 ± 0.18a	24.0 ± 0.81b

1,2-Palmitin-3-linolein	0.48 ± 0.05b	0.53 ± 0.01b	0.67 ± 0.02a
1,2-Olein-3-stearin	3.07 ± 0.19a	3.30 ± 0.74a	3.66 ± 0.54a
1-Palmitin-2-olein-3-stearin	0.03 ± 0.00b	0.03 ± 0.00ab	0.04 ± 0.00a
1-Palmitin-2-stearin-3-olein	0.64 ± 0.05b	0.69 ± 0.15ab	0.87 ± 0.13a
1,2-Laurin-3-miristin	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a
1,2-Linolein-3-stearin	14.2 ± 0.50b	13.9 ± 0.22b	15.2 ± 0.36a
Trilinolenin	3.56 ± 0.30a	3.72 ± 0.25a	2.31 ± 0.13b

*Significant difference (p-value < 0.05) among Chandler, Howard, and Tulare for each parameter was marked as different letters.

Linear discriminant analysis (LDA) could discriminate the samples from Chandler, Howard, and Tulare varieties (Figure 3.1). LD 1 and LD 2 together explained 100% of the variables. The LDA plot demonstrated that Chandler was closer in its TAG profile to Howard than to Tulare. This confirmed that Chandler and Howard had similar parental varieties(Vahdati et al., 2019).

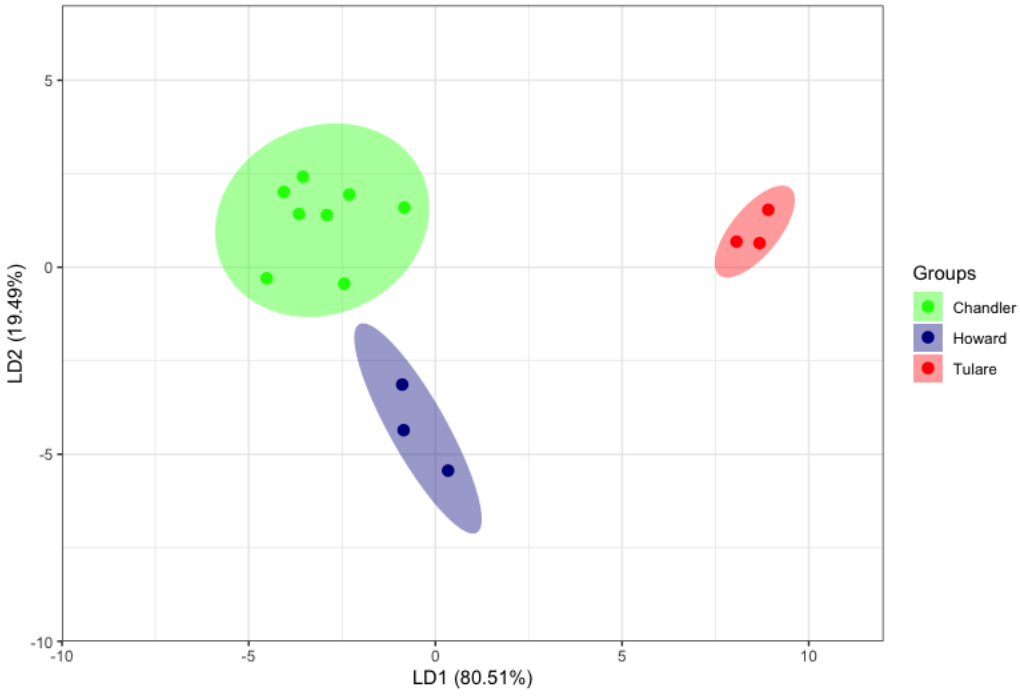

Figure 3.1. LDA plot of triacylglycerols of Chandler, Howard, and Tulare walnuts sourced in 2022

Fatty acid profile

Cultivar and collection year yielded some significant differences in the fatty acid composition. The concentration of palmitic acid was the highest in Tulare and the lowest in Chandler; the concentration of linolenic acid was the highest in Chandler and the lowest in Tulare, consistently in 2020 and 2022. Howard sourced in 2022 had a lower value for palmitoleic acid than Howard and Tulare collected in 2020 and Chandler collected in 2022. The highest concentration for stearic acid was shown in Tulare received in 2022, and the lowest in Howard collected in 2020. For arachidic acid, the highest was found in Tulare collected in 2022 and the lowest was in Chandler, Howard, and Tulare collected in 2020. For eicosenoic acid, the highest concentration was found in Chandler and Howard sourced in 2022 and the lowest was in Howard sourced in 2020. Within the same harvest year, Chandler, Howard, and Tulare had similar margaric, stearic, oleic, and linoleic acid concentrations (Table 3.4).

Considering the two collection years, the consistently significant difference between Chandler and Tulare was noted for palmitic acid and linolenic acid, whereas Howard and Tulare differed in the concentrations of palmitic acid and eicosenoic acid. The variations between Chandler and Tulare or Howard and Tulare were expected as Chandler and Howard have different parental varieties from Tulare(Vahdati et al., 2019). The same parental varieties of Chandler and Howard should explain their fatty acid profile similarities, shown as no consistent difference between the two collection years.

Table 3.4. Percentages of peak areas of fatty acids of Chandler, Howard, and Tulare walnuts sourced in 2020 and 2022

Fatty acid	CY: 2020			CY: 2022		
	Chandler	Howard	Tulare	Chandler	Howard	Tulare
C16:0	6.20 ± 0.25c	6.62 ± 0.23b	7.19 ± 0.33a	6.06 ± 0.19c	6.25 ± 0.23bc	7.41 ± 0.24a
C16:1	0.06 ± 0.01ab	0.07 ± 0.01a	0.06 ± 0.01a	0.06 ± 0.00a	0.04 ± 0.03b	0.06 ± 0.00ab
C17:0	0.04 ± 0.00a	0.04 ± 0.00a	0.04 ± 0.01a	0.03 ± 0.02a	0.02 ± 0.03a	0.04 ± 0.01a
C18:0	2.59 ± 0.15ab	2.43 ± 0.17b	2.74 ± 0.29ab	2.62 ± 0.32ab	2.60 ± 0.08ab	3.03 ± 0.05a
C18:1	13.17 ± 0.75a	13.62 ± 0.98a	13.82 ± 0.92a	14.14 ± 0.71a	13.66 ± 1.33a	13.77 ± 1.22a
C18:2	63.00 ± 0.53a	61.95 ± 1.39a	63.25 ± 1.00a	61.75 ± 0.78a	61.41 ± 2.62a	62.78 ± 1.36a
C18:3	14.81 ± 0.54ab	15.14 ± 1.92a	12.75 ± 0.87c	15.00 ± 0.58a	15.69 ± 0.70ab	12.59 ± 0.18bc
C20:0	0.05 ± 0.01c	0.05 ± 0.01c	0.05 ± 0.01c	0.09 ± 0.00b	0.08 ± 0.01b	0.11 ± 0.00a
C20:1	0.08 ± 0.01cd	0.08 ± 0.00d	0.09 ± 0.02c	0.24 ± 0.01a	0.24 ± 0.00a	0.21 ± 0.01b

*CY: Collection year

**Significant difference (p-value < 0.05) between combinations of the cultivars and collection years was marked as different letters

Beta-sitosterol

The concentrations of beta-sitosterol, the most prevalent sterol in walnuts(Crews et al., 2005), varied among Chandler, Howard, and Tulare (p-value 0.008). The concentration in Chandler was significantly lower than that of Howard (p-value 0.006) but not significantly different from that of Tulare (Figure 3.2). The variation of beta-sitosterol concentrations among cultivars was also demonstrated in an earlier study that compared the sterol composition of four introduced cultivars (Franquette, Hartley, Lauzeronne, and Parisienne) to two local varieties in Tunisia(Abdallah et al., 2015).

The relationship between sterols and the oxidative stability of lipids is complex. An experimental study shows that sterols can limit the oxidation of methyl linoleate in acetonitrile solution and beta-linoleoyl-gamma-palmitoyl phosphatidylcholine (PLPC)(Yoshida & Niki, 2003). However, an observational study comparing the oxidative stability of various olive oil cultivars does not demonstrate any correlation between the parameter and beta-sitosterol concentrations(Noorali et al., 2014). This might be linked to sterols being oxidized when the matrices are exposed to air; hence, the antioxidant capacity was likely reduced. Sterol oxidation generates various ketones, alcohols, and hydrocarbons(Raczyk et al., 2017). The development of the oxidation products increases with time and temperature(Soupas et al., 2004) and it is limited by catechin, alpha-tocopherol, and quercetin(Xu et al., 2009).

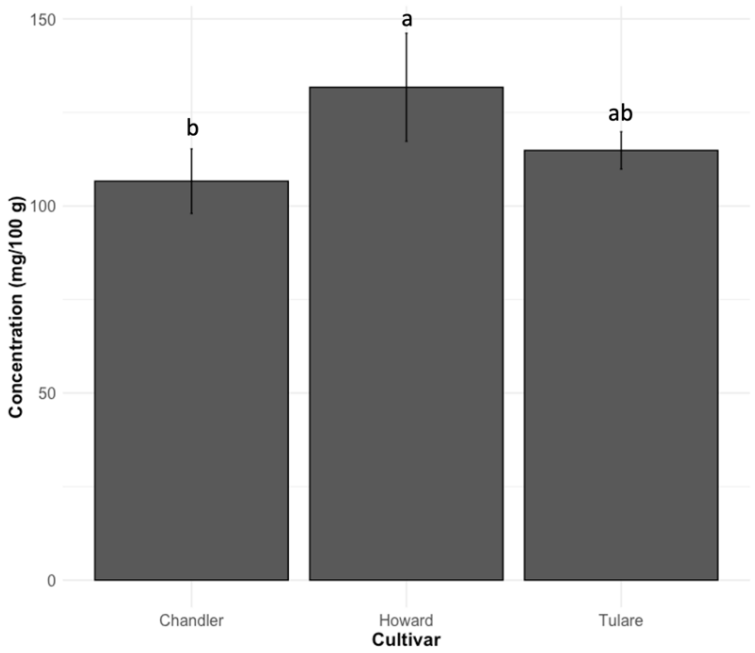

Figure 3.2. Beta-sitosterol concentrations of Chandler, Howard, and Tulare walnuts sourced in 2022*

*Significant difference (p-value < 0.05) among Chandler, Howard, and Tulare was marked as different letters.

Tocopherols

Delta-, alpha-, and gamma-tocopherols were detected, but beta-tocopherol was not found. Among Chandler, Howard, and Tulare grown in California, there was no significant difference in delta- and alpha-tocopherol concentrations, likely because the three cultivars originate from the same cultivar, Payne(Vahdati et al., 2019). Chandler's concentration of gamma-tocopherol was significantly higher than that of Tulare but similar to that of Howard. Because gamma-tocopherol dominates the total tocopherol in walnuts(Liu et al., 2023), the variations in total tocopherol among the cultivars followed that of gamma-tocopherol (Table 3.5). The higher concentration of gamma-tocopherol in Chandler collected in 2022 should counter its disadvantage of having a

higher concentration of linolenic acid (Table 3.4) that is susceptible to oxidation. Larger linoleic acid concentrations usually correspond to higher peroxide value (PV) and lower oxidative stability(Mao et al., 2020; Park & Kim, 2016). Despite having more linolenic acid, Chandler had similar PV and oxidative stability (Table 3.6) and concentrations of most of the volatiles (Table 3.7) with Howard and Tulare.

Table 3.5. Tocopherol concentrations (mg/kg) of Chandler, Howard, and Tulare walnuts sourced in 2022

Cultivar	Delta	Alpha	Gamma	Total
Chandler	19.69 ± 4.13a	32.27 ± 4.76a	434.55 ± 48.10a	486.52 ± 50.85a
Howard	18.16 ± 2.14a	33.39 ± 6.32a	391.98 ± 8.12ab	443.53 ± 11.11ab
Tulare	16.95 ± 2.03a	24.93 ± 2.65a	349.35 ± 19.01b	391.23 ± 18.54b

*Significant difference (p-value < 0.05) among Chandler, Howard, and Tulare was marked as different letters.

Free fatty acidity (FFA)

There was no difference in FFA among Chandler, Howard, and Tulare (p-value 0.656) (Table 3.6). Although Adkison et al. observe a higher FFA in Chandler than Howard and Tulare when they are stored at 60% relative humidity (RH) and 25 °C for one year, there is no significant difference among them for other combinations of RH (40, 60, and 80%) and storage temperature (5, 15, and 25 °C)(Adkison et al., 2021). Another study demonstrates that initially, the FFA values of Chandler, Hartley, Franquette, and Ioli are similar, but after 12 months of storage with air exposure at 1 or 20 °C, the FFA value of Chandler is lower than those of Hartley, Franquette, and Ioli(Christopoulos & Tsantili, 2015). Our FFA measurement is done within two months after harvest. The samples are kept at 5 °C during the storage (before oil extraction) or -20 °C (after

oil extraction), accompanied by low humidity and limited air exposure. Limited hydrolysis had likely occurred, resulting in no observable differences in FFA.

Peroxide values (PV)

Like FFA, no significant difference was found among the three main cultivars grown in California: Chandler, Howard, and Tulare (Table 3.6). The non-significant difference could be due to the short time between sample harvest and analysis. An earlier study shows that recently harvested Chandler, Howard, and Tulare walnuts have similar PV values. After 12 months of storage at 60% RH and 5 °C, Tulare has significantly more PV than Howard, but not from Chandler. The value of Tulare is similar to that of Chandler. At 80% RH and 5 °C, the PV value of Tulare is higher than those of both Howard and Chandler. Storing at 40% RH and 25 °C for 12 months leads to higher PV values of Chandler than those of Howard and Tulare. However, Tulare has more PV than Chandler for the same temperature and period but 80% RH, but the value is similar to Howard's. The report suggests that Tulare more often develops a larger concentration of peroxides under the experimental settings(Adkison et al., 2021).

Oxidative stability

Chandler, Howard, and Tulare had similar induction times (Table 3.6). Generally, samples with larger concentrations of linoleic and linolenic acids have shorter induction times, while those with a higher concentration of saturated fatty acids and oleic acid have longer induction times(Nosratpour et al., 2017; Szterk et al., 2010). The three cultivars had similar concentrations of oleic and linoleic acids. Even though the palmitic acid and linolenic acid concentrations varied between Chandler and Tulare (Table 3.4), the differences (1.3 and 2.41%, respectively) were not large enough to induce changes in oxidative stability.

Table 3.6. FFA (g/100 g), PV (meq O_2/kg), and induction time (hr) of Chandler, Howard, and Tulare walnuts sourced in 2022

Cultivar	FFA	PV	Induction time
Chandler	0.04 ± 0.01a	0.34 ± 0.16a	11.58 ± 1.02a
Howard	0.05 ± 0.01a	0.33 ± 0.09a	13.70 ± 2.41a
Tulare	0.04 ± 0.01a	0.32 ± 0.19a	13.47 ± 0.47a

*Significant difference (p-value < 0.05) between Chandler, Howard, and Tulare for each parameter was marked as different letters.

Volatiles

Volatiles in the walnut samples consisted of aldehydes, ketones, alcohols, carboxylic acid, ester, and furan. Volatiles with the largest peak areas were hexanal (in the samples collected in 2020) and acetone (in the samples sourced in 2022). The variations might be attributed to the pre-harvest heat extreme occurring before the harvest time of the 2022 samples(Athaillah & Wang, 2024). The increasing concentration of acetone and other compounds like monoterpene with higher temperatures is also noted in Norway spruce. This is likely because of the increased activity of enzymes involved in its synthesis(Filella et al., 2007).

Variation of volatile profiles among Chandler, Howard, and Tulare was minimal. There was no significant difference among the three cultivars that were consistent in the 2020 and 2022 collection years (Table 3.7). The harvest years had more influence than the cultivar, likely because of some changes in the climatic properties of the cultivation area(Athaillah & Wang, 2024). A previous study evaluating the volatiles of walnuts from Bleggiana, Blegget, and Lara varieties collected in 2017, 2018, and 2019 in Trentino, Italy(Di Pierro et al., 2022) also shows that harvest year makes a more significant impact on volatile profiles than cultivars.

The concentrations of 2-butenal, 2-butenal-3-methyl, 2-hexenal, 2-heptenal, 2-heptanone, 1-penten-3-one, 1-octen-3-ol, 2-pentanol-4-methyl, pentanoic acid, and ethyl acetate were similar among all combinations of cultivar and collection years. The similarity confirmed that Chandler, Howard, and Tulare shared genetic similarities. However, among the samples harvested in 2022, there was a significant difference in acetone concentrations among the three cultivars. Tulare had less acetone than Chandler and Howard. This should correspond to the lower concentration of linolenic acid-containing TAGs and linolenic acid in Tulare (Table 3.3 and Table 3.4) because acetone is produced from the oxidation and degradation of linolenic acid(Cao et al., 2014). The trend was not observed among the samples sourced in 2020 because likely the factors that may trigger acetone production, e.g., preharvest high temperature, did not occur.

Table 3.7. Peak areas of volatiles of Chandler, Howard, and Tulare walnuts sourced in 2020 and 2022

Cultivar	CY: 2020			CY: 2022		
	Chandler	**Howard**	**Tulare**	**Chandler**	**Howard**	**Tulare**
Pentanal	27.89 ± 22.62a	7.41 ± 9.04b	11.57 ± 10.69ab	2.43 ± 1.44b	3.43 ± 1.32ab	2.00 ± 0.66b
Hexanal	343.46 ± 288.97a	98.56 ± 138.98b	142.49 ± 155.89ab	71.28 ± 41.40b	84.18 ± 29.42ab	84.57 ± 32.36ab
Octanal	0.62 ± 0.32a	0.39 ± 0.26a	0.43 ± 0.30a	0.76 ± 1.06a	0.44 ± 0.19a	0.74 ± 0.67a
Nonanal	0.79 ± 0.42b	0.53 ± 0.21b	0.59 ± 0.35b	2.33 ± 2.31a	1.36 ± 0.30ab	2.32 ± 0.32a
2-Butenal	0.75 ± 0.58a	9.22 ± 10.05a	7.72 ± 12.73a	16.39 ± 22.44a	5.48 ± 7.13a	1.03 ± 0.45a
2-Butenal,3-methyl	0.91 ± 0.45a	1.030 ± 0.79a	0.40 ± 0.20a	1.25 ± 1.98a	1.11 ± 0.31a	0.12 ± 0.07a
2-Hexenal, (E)	1.50 ± 1.20a	1.95 ± 1.33a	0.92 ± 0.63a	2.37 ± 3.72a	1.09 ± 1.11a	0.26 ± 0.15a
2-Heptenal, (E)-	1.86 ± 1.45a	0.99 ± 0.61a	1.22 ± 0.95a	1.25 ± 0.90a	2.14 ± 0.97a	0.84 ± 0.22a
2-Pentenal, (E)-	0.74 ± 0.60a	0.31 ± 0.28a	0.36 ± 0.25a	0.39 ± 0.63a	0.19 ± 0.10a	0.06 ± 0.04a
2,4-Heptadienal, (E,E)-	0.23 ± 0.16ab	0.15 ± 0.14b	0.16 ± 0.14b	0.44 ± 0.19a	0.51 ± 0.04a	0.40 ± 0.21ab
Acetone	75.21 ± 24.07b	97.82 ± 138.15b	90.74 ± 58.55b	1842.09 ± 912.03a	1893.97 ± 1078.07a	1676.16 ± 792.08b
2-Heptanone	2.82 ± 2.08a	1.94 ± 2.19a	1.82 ± 1.34a	1.81 ± 1.44a	2.24 ± 1.16a	1.61 ± 0.48a
2-Butanone	19.05 ± 7.37bc	14.19 ± 12.33c	12.67 ± 6.12c	37.90 ± 13.05ab	66.14 ± 35.31a	51.85 ± 37.78a
1-Penten-3-one	2.72 ± 1.33a	0.94 ± 0.93a	1.39 ± 1.47a	1.82 ± 1.55a	3.56 ± 2.41a	2.02 ± 0.87a
5-Hepten-2-one, 6-methyl-	0.94 ± 0.28ab	0.56 ± 0.23b	0.61 ± 0.21b	1.39 ± 0.82a	1.23 ± 0.28ab	1.38 ± 0.21ab
3,5-Octadien-2-one	1.96 ± 0.74a	1.10 ± 1.03ab	1.23 ± 0.97ab	0.66 ± 0.37b	1.10 ± 0.28ab	0.89 ± 0.41ab
3-Buten-2-ol-2-methyl	3.15 ± 1.87bc	2.47 ± 1.33c	2.75 ± 1.47bc	18.77 ± 20.11ab	30.21 ± 30.22a	8.62 ± 4.61abc
1-Octen-3-ol	5.31 ± 4.10a	2.52 ± 2.24a	3.13 ± 3.05a	3.53 ± 2.48a	3.62 ± 1.22a	1.83 ± 0.13a
1-Penten-3-ol	54.94 ± 39.38a	13.81 ± 15.72b	23.61 ± 23.68ab	7.59 ± 3.31b	8.37 ± 1.36b	7.82 ± 2.05b

2-Pentanol-4-methyl	35.85 ± 9.14a	31.95 ± 16.18a	42.85 ± 27.59a	20.98 ± 8.73a	23.39 ± 6.79a	22.26 ± 6.44a
Pentanoic acid	12.16 ± 13.54a	4.62 ± 5.28a	12.29 ± 15.78a	1.43 ± 0.79a	0.81 ± 0.17a	1.25 ± 1.28a
Ethyl acetate	851.75 ± 886.10a	621.06 ± 690.63a	900.29 ± 899.30a	150.98 ± 221.44a	256.34 ± 145.17a	727.61 ± 1124.48a
Furan,2-ethyl	7.18 ± 5.42a	2.89 ± 2.38ab	3.93 ± 3.91b	2.12 ± 0.80b	2.45 ± 1.87ab	2.18 ± 1.35ab

*CY: Collection year

**Significant difference (p-value < 0.05) between combinations of the cultivars and collection years was marked as different letters

Phenols

Total phenol

The total phenol of Tulare collected in 2020 was significantly higher than those of Chandler and Howard sourced in 2022 and Chandler sourced in 2020 (Figure 3.3). This finding was similar to the trend of gallic acid concentrations, quantified using an LC-MS/MS, among the three cultivars (Table 3.8). Previous studies comparing total phenols among Chandler, Howard, and Tulare are lacking. There are studies comparing the total phenols of Chandler and Howard, but the results are inconclusive. Two studies show that Howard has more phenols than Chandler(Kafkas et al., 2020; Tapia et al., 2013), but another study suggests similar values(Kafkas et al., 2017). Another study indicates that Chandler walnuts that are gently shelled have less total phenol than Howard walnuts with the same treatment, but the opposite is observed for harsh shelling treatment(Ortiz et al., 2019). The variability in the comparison results may be due to some factors affecting phenol concentrations, including sample maturity(Wei et al., 2022), kernel skin lightness(Athaillah & Wang, 2024), mass ratio of skin to kernel, and post-harvest handling(Ahad et al., 2020).

Our data demonstrated that harvest year did not significantly impact total phenol, signaling that the parameter was relatively stable. However, it should be noted that the samples were all from Central Valley, CA, where the agricultural practices are likely more controlled. Therefore, it might not apply to other regions.

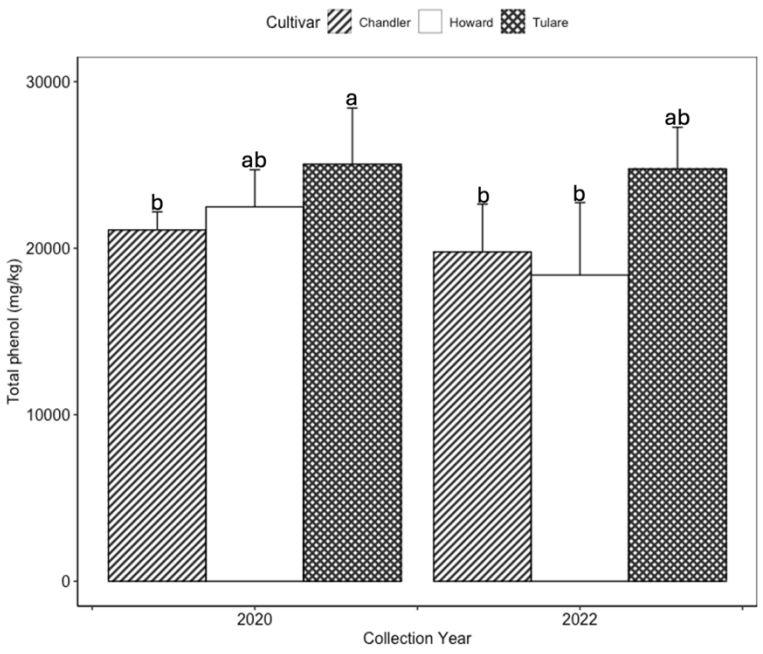

Figure 3.3. Total phenol content of Chandler, Howard, and Tulare walnuts sourced in 2020 and 2022

*Significant difference (p-value < 0.05) between combinations of the cultivars and collection years was marked as different letters.

Individual Free Phenols

Concentrations of catechin, procyanidin B1, caffeic acid, p-coumaric acid, and quercetin were similar among all combinations of cultivar and harvest years, indicating that their similarities were consistent. Within the same harvest year, concentrations of vanillic acid, ferulic acid, sinapic acid, ellagic acid, and naringenin were similar among Chandler, Howard, and Tulare. Among the samples from the same cultivar, vanillic, ferulic, sinapic, and ellagic acid concentrations differed with harvest years. The variations were expected because the factors affecting phenol metabolism, e.g., biotic and abiotic stressors, change occasionally. Consistent variation among Chandler, Howard, and Tulare was only observed in chlorogenic acid

concentration. This compound was detected in Howard but not in Chandler and Tulare (Table 3.8). Chlorogenic acid's function is protection against oxidative stress and bacterial infection(Niggeweg et al., 2004), and the increase in its concentration might reflect adaptation to the stress and cultivar sensitivity. Because only chlorogenic acid, from fourteen compounds studied, showed consistent differences among Chandler, Howard, and Tulare, its variation did not seem to impact the walnut quality much, based on the data of FFA, PV, oxidative stability (Table 3.6), and volatiles (Table 3.7).

Chandler, Howard, and Tulare were released from the UC Davis breeding program. Howard and Chandler were derived from a crossing between #56-224 and Pedro varieties, while Tulare is a cross between Tehama and Serr. Some similarities in the phenolic profiles of Chandler, Howard, and Tulare might be because they originated from the cultivar Payne(Brown et al., 2020; Vahdati et al., 2019). In addition, it could also originate from similar rootstocks. The majority of Californian walnuts use hybrid seedlings of *Juglans hindsii* x *J. regia* (Paradox), clonal selections of *J. hindsii* x *J. regia*, or a clonal selection of *J. microcarpa* x *J. regia* as rootstocks(Kluepfel et al., 2021). A study on pistachio reveals that rootstock selection affects concentrations of phenols, e.g., caffeic acid hexoside, kaempferol-O-dihexoside, and polymeric procyanidins(Noguera-Artiaga et al., 2018).

Table 3.8. Concentrations (mg/kg) of individual free phenol of Chandler, Howard, and Tulare walnuts sourced in 2020 and 2022

Cultivar	CY: 2020			CY: 2022		
	Chandler	**Howard**	**Tulare**	**Chandler**	**Howard**	**Tulare**
Gallic acid	10.71 ± 2.33b	14.28 ± 4.03ab	18.46 ± 5.88a	8.70 ± 3.20b	9.08 ± 1.39b	11.58 ± 7.81ab
3,4 Dihydroxybenzoic acid	0.10 ± 0.04a	0.06 ± 0.04a	0.06 ± 0.03a	0.09 ± 0.02a	0.09 ± 0.01a	0.03 ± 0.03a
Catechin	61.41 ± 9.68a	78.43 ± 12.53a	62.55 ± 11.86a	63.90 ± 32.84a	65.71 ± 7.53a	48.10 ± 43.83a
Chlorogenic acid	nd c	3.37 ± 1.48a	nd c	nd c	1.84 ± 0.83ab	nd bc
Procyanidin B1	27.66 ± 6.18a	31.22 ± 10.26a	22.30 ± 4.25a	30.39 ± 16.27a	25.08 ± 1.00a	20.26 ± 0.80a
Vanillic acid	0.53 ± 0.51b	0.41 ± 0.58b	0.38 ± 0.58b	1.08 ± 0.35ab	1.04 ± 0.34a	2.40 ± 1.46a
Caffeic acid	0.06 ± 0.04a	0.06 ± 0.03a	0.06 ± 0.03a	0.09 ± 0.01a	0.07 ± 0.01a	0.09 ± 0.03a
Epicatechin gallate	0.36 ± 0.08c	0.68 ± 0.23ab	0.87 ± 0.29a	0.43 ± 0.12bc	0.56 ± 0.03abc	0.44 ± 0.10abc
p-Coumaric acid	0.10 ± 0.01a	0.10 ± 0.04a	0.12 ± 0.02a	0.12 ± 0.02a	0.19 ± 0.16a	0.13 ± 0.01a
Ferulic acid	0.10 ± 0.05b	0.08 ± 0.06b	0.12 ± 0.08b	0.28 ± 0.04a	0.31 ± 0.10a	0.35 ± 0.17a
Sinapic acid	0.19 ± 0.04b	0.27 ± 0.08b	0.30 ± 0.08b	0.33 ± 0.08ab	0.53 ± 0.14a	0.37 ± 0.26ab
Ellagic acid	312.96 ± 68.33b	387.54 ± 96.37b	412.67 ± 83.92b	731.88 ± 236.48a	552.14 ± 95.01ab	819.95 ± 360.45a
Quercetin	0.48 ± 0.18a	0.41 ± 0.14a	0.50 ± 0.12a	0.37 ± 0.06a	0.38 ± 0.03a	0.35 ± 0.15a
Naringenin	0.34 ± 0.10a	0.27 ± 0.09ab	0.27 ± 0.07ab	0.22 ± 0.04ab	0.12 ± 0.03b	0.13 ± 0.04b

*CY: Collection year
**Significant difference (p-value < 0.05) between combinations of the cultivars and collection years was marked as different letters.
***nd: not detected

Compound-Specific Stable Isotope (CSIA)

CSIA of fatty acids was conducted on all samples, but two Howard and two Chandler were not analyzed for CSIA of amino acids because of insufficient quantity. Because only one replicate (one source) of Howard was used for comparing CSIA of amino acids, only Chandler and Tulare were included for statistical analysis for that parameter.

The $\delta^{15}N_{amino\ acid}$ values of our samples were close to 0, indicating minimal isotope fractionation and the use of synthetic fertilizer(van Leeuwen et al., 2014). The data was reasonable because many walnut orchards in California apply conventional farming techniques, and organic walnut is estimated to be only 2% of the total production(OPN, 2021). There were no significant variations in $\delta^{15}N_{amino\ acids}$ in Chandler and Tulare (Table 3.9), indicating that the cultivars did not affect nitrogen isotope fractionation in amino acids. A similar trend was observed for $\delta^{13}C_{amino\ acids}$, except for $\delta^{13}C_{Ser}$. The variation in $\delta^{13}C_{Ser}$ but not in other $\delta^{13}C_{amino\ acids}$ suggested that the cultivars had minimum effects on the carbon isotope fractionation of amino acids.

All $\delta^{2}H_{fatty\ acids}$ and $\delta^{13}C_{fatty\ acids}$ values were identical among Chandler, Howard, and Tulare (Table 3.9). The similarity in $\delta^{13}C_{fatty\ acids}$ among cultivars has also been observed in rape oil(Richter et al., 2010). While studies on the use of $\delta^{2}H_{fatty\ acids}$ for cultivar classification are lacking, we found a study that demonstrated an overlapping of $\delta^{2}H_{bulk}$ of Greek olive oils from three cultivars(Tarapoulouzi et al., 2021). The similar values among the cultivars in all $\delta^{2}H_{fatty\ acids}$ and $\delta^{13}C_{fatty\ acids}$ in the current study indicated uniformity in the geographical factors of their growing locations, i.e., altitude, latitude, precipitation, and irrigation, and a minimal, almost non-existent, impact of cultivars.

Table 3.9. $\delta^{15}N_{amino\ acids}$, $\delta^{13}C_{amino\ acids}$, $\delta^{2}H_{fatty\ acids}$, and $\delta^{13}C_{fatty\ acids}$ (‰) of Chandler, Howard, and Tulare walnuts sourced in 2022

Parameter	Chandler	Tulare	Howard
Amino acids			
$\delta^{15}N_{Ala}$	1.01 ± 1.21a	2.16 ± 1.21a	2.73
$\delta^{15}N_{Asx}$	2.37 ± 1.50a	3.75 ± 1.09a	4.08
$\delta^{15}N_{Glx}$	1.56 ± 1.40a	2.65 ± 0.80a	3.19
$\delta^{15}N_{Gly}$	1.25 ± 1.63a	3.37 ± 1.55a	3.33
$\delta^{15}N_{His}$	-2.00 ± 2.00a	-0.13 ± 1.34a	-0.64
$\delta^{15}N_{Ile}$	1.24 ± 1.40a	2.18 ± 1.08a	2.49
$\delta^{15}N_{Leu}$	0.15 ± 1.35a	1.02 ± 0.91a	1.46
$\delta^{15}N_{Lys}$	1.76 ± 1.87a	3.69 ± 1.74a	4.35
$\delta^{15}N_{Met}$	0.57 ± 1.50a	2.23 ± 1.32a	0.24
$\delta^{15}N_{Phe}$	-0.66 ± 1.64a	0.17 ± 1.25a	1.88
$\delta^{15}N_{Pro}$	3.95 ± 1.46a	5.23 ± 1.22a	5.40
$\delta^{15}N_{Ser}$	0.87 ± 1.91a	3.17 ± 1.50a	2.92
$\delta^{15}N_{Thr}$	0.32 ± 1.88a	0.29 ± 0.98a	1.96
$\delta^{15}N_{Tyr}$	2.24 ± 1.76a	2.69 ± 2.11a	6.02
$\delta^{15}N_{Val}$	3.26 ± 1.47a	4.62 ± 1.29a	5.06
$\delta^{13}C_{Ala}$	-19.88 ± 0.69a	-19.04 ± 0.48a	-18.5
$\delta^{13}C_{Asx}$	-20.69 ± 0.30a	-20.67 ± 1.39a	-20.09
$\delta^{13}C_{Glx}$	-22.12 ± 0.14a	-22.03 ± 0.82a	-21.59
$\delta^{13}C_{Gly}$	-20.36 ± 1.23a	-18.99 ± 1.17a	-18.14
$\delta^{13}C_{His}$	-21.98 ± 2.42a	-21.23 ± 0.99a	-20.80
$\delta^{13}C_{Ile}$	-22.60 ± 0.38a	-22.69 ± 0.74a	-21.64
$\delta^{13}C_{Leu}$	-27.44 ± 0.38a	-27.73 ± 1.05a	-26.55
$\delta^{13}C_{Lys}$	-19.64 ± 0.15a	-19.54 ± 1.23a	-19.43
$\delta^{13}C_{Met}$	-33.48 ± 1.31a	-35.10 ± 1.72a	-32.93
$\delta^{13}C_{Phe}$	-30.81 ± 0.33a	-30.64 ± 0.86a	-29.63

$\delta^{13}C_{Pro}$	-24.00 ± 0.16a	-23.97 ± 1.36a	-22.85
$\delta^{13}C_{Ser}$	-16.00 ± 0.48b	-14.95 ± 0.71a	-14.94
$\delta^{13}C_{Thr}$	-15.02 ± 0.56a	-14.68 ± 0.59a	-14.92
$\delta^{13}C_{Tyr}$	-28.88 ± 0.44a	-28.62 ± 0.07a	-27.84
$\delta^{13}C_{Val}$	-28.37 ± 0.28a	-28.00 ± 1.18a	-27.13
Fatty acids			
$\delta^{2}H_{C16:0}$	194.18 ± 6.64a	-198.90 ± 8.24a	-194.02 ± 5.54a
$\delta^{2}H_{C18:1w9c}$	-123.23 ± 7.05a	-127.39 ± 6.84a	-120.33 ± 14.84a
$\delta^{2}H_{C18:2w6}$	-209.03 ± 6.98a	-212.89 ± 7.86a	-207.66 ± 7.46a
$\delta^{2}H_{C18:3w3}$	-217.39 ± 6.75a	-218.87 ± 8.27a	-214.88 ± 7.59a
$\delta^{13}C_{C16:0}$	-30.56 ± 0.60a	-30.82 ± 0.35a	-31.00 ± 1.11a
$\delta^{13}C_{C18:1w9c}$	-28.84 ± 0.74a	-29.25 ± 0.31a	-29.40 ± 0.76a
$\delta^{13}C_{C18:2w6}$	-29.74 ± 0.50a	-30.05 ± 0.45a	-30.11 ± 1.31a
$\delta^{13}C_{C18:3w3}$	-29.85 ± 0.50a	-29.99 ± 0.48a	-30.20 ± 1.25a

*Significant difference (p-value < 0.05) among Chandler, Howard, and Tulare for each parameter was marked as different letters.

Conclusions

Chandler, Howard, and Tulare walnuts were similar in their overall quality, as determined by FFA, PV, and oxidative stability. The investigation of the metabolites suggested that they were also similar in the concentrations of some triacylglycerols (i.e., trilaurin, tricaprylin, tristearin, tripalmitin, trilinolein, 1,2-olein-3-stearin, 1,2-laurin-3-miristin), some fatty acids (i.e., margaric, stearic, oleic, and linoleic acids), most volatiles, alpha- and delta-tocopherols, and most of the phenolic compounds. They differed in the concentrations of some triacylglycerols (i.e., 1-olein-2,3-linolein, triolein, 1,2-linolein-3-linolenin, 1,2-palmitin-3-linolein, 1-palmitin-2-olein-3-stearin, 1-palmitin-2-stearin-3-olein, 1,2-linolein-3-stearin, and trilinolenin), some fatty acids (i.e., palmitic, palmitoleic, linolenic, arachidic, and eicosenoic acids), beta-sitosterol, and gamma-tocopherol. Combined with LDA, TAG data could separate Chandler, Howard, and

Tulare samples. They also could explain the closer association between Chandler and Howard, compared to Chandler or Howard with Tulare. On the other hand, $\delta^{15}N_{\text{amino acids}}$, $\delta^{13}C_{\text{amino acids}}$, $\delta^{2}H_{\text{fatty acids}}$, and $\delta^{13}C_{\text{fatty acids}}$ were not able to distinguish the cultivars. Some effects of harvest year, potentially associated with agroclimatic events, were demonstrated on some metabolites, particularly volatiles.

Nomenclature

FFA: free fatty acidity; PV: peroxide value; TAG: triacylglycerol; CSIA: compound-specific stable isotope analysis; Ala: alanine; Asx: asparagine or aspartic acid; Glx: Glutamate or glutamine; Gly: glycine; His: Histidine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Thr: Threonine; Tyr: Tyrosine; Val: Valine.

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Conflict of Interest

The authors declare no conflict of interest in this study.

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Supporting Information

Table S3.1. Retention time (min), molecular weight (M), and target mass/charge (m/z) and ionization mode for identification of triacylglycerols and sterols

Lipid species	Retention time (min)	Molecular weight (M)	Target m/z	Ionization mode
Triacylglycerols				
Trilaurin	13.9	639	657	$[M + NH_4]^+$
1-Olein-2,3-linolein	16.6	881.4	899	$[M + NH_4]^+$
Tricaprylin	10.5	470.7	489	$[M + NH_4]^+$
Triolein	17.8	885.4	903	$[M + NH_4]^+$
Tristearin	16.6	891.5	909	$[M + NH_4]^+$
Tripalmitin	17.9	807.3	825	$[M + NH_4]^+$
Trilinolein	16.1	879.4	897	$[M + NH_4]^+$
1,2-Linolein-3-linolenin	15.7	877.4	895	$[M + NH_4]^+$
1,2-Palmitic-3-linolein	17.3	831.3	849	$[M + NH_4]^+$
1,2-Olein-3-stearin	18.6	887.4	905	$[M + NH_4]^+$
1-Palmitin-2-olein-3-stearin	17.1	861.4	879	$[M + NH_4]^+$
1-Palmitin-2-stearin-3-olein	18.9	861.4	879	$[M + NH_4]^+$
1,2-Laurin-3-myristin	14.4	667.1	685	$[M + NH_4]^+$
1,2-Linolein-3-stearin	17.4	883.4	901	$[M + NH_4]^+$
Trilinolenin	14.7	873.3	891	$[M + NH_4]^+$
Sterols				
Beta sitosterol	11.9	414.7	398	$[M + 1 - H_2O]^+$

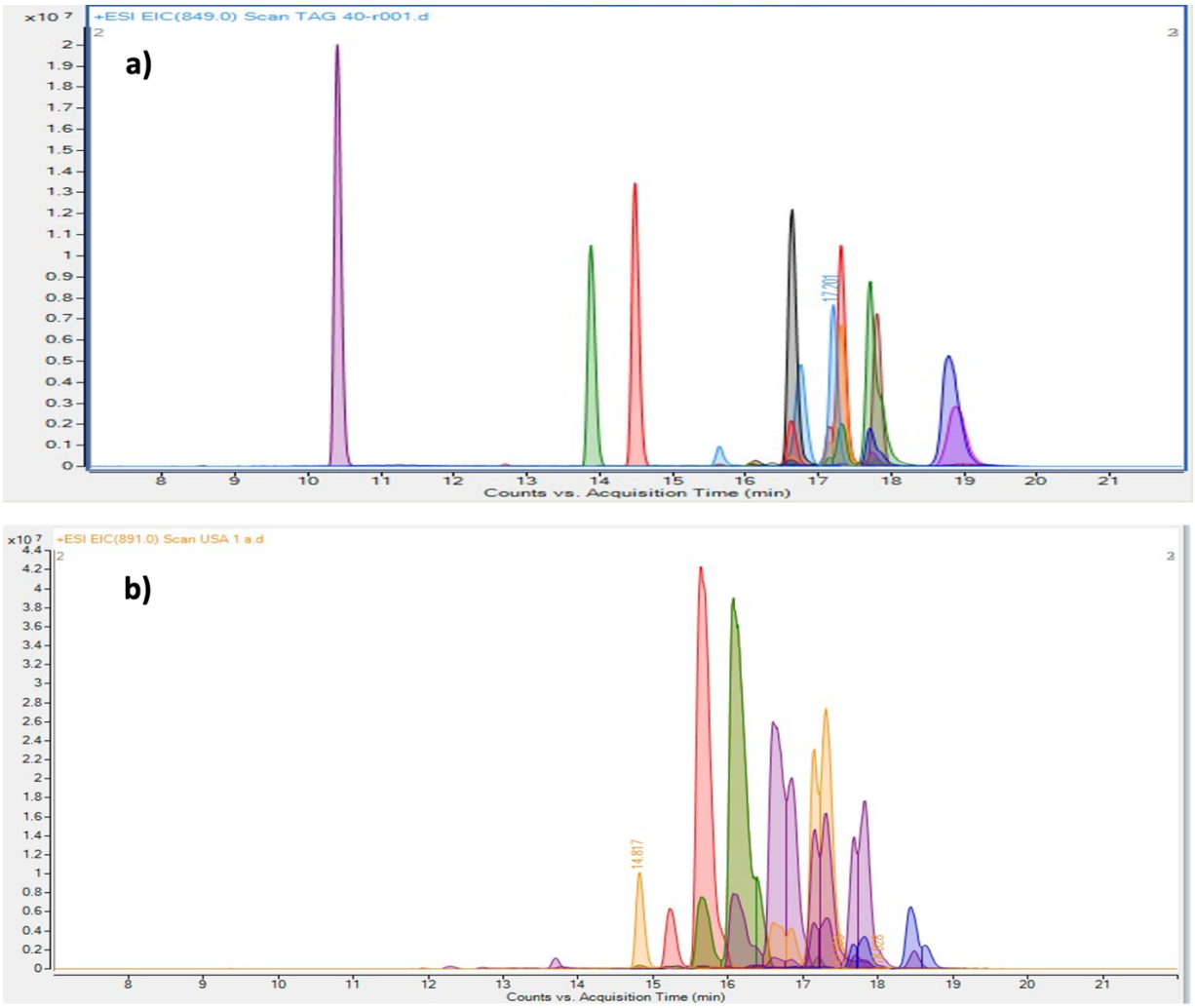

Figure S3.1. LC MS chromatograms of TAG mixture (a) or a sample (b)

CHAPTER 4

BULK AND COMPOUND-SPECIFIC STABLE ISOTOPE ANALYSIS FOR AUTHENTICATION OF WALNUTS (*JUGLANS REGIA*) ORIGINS

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Abstract

Walnuts are grown in various countries and as product origin information is becoming more important to consumers, new techniques to differentiate walnut geographical authenticity is needed. We conducted bulk stable isotope analysis (BSIA) and compound-specific stable isotope analysis (CSIA) on walnuts grown in seven countries. The BSIA consisted of $\delta^{13}C_{bulk}$, $\delta^{15}N_{bulk}$, and $\delta^{34}S_{bulk}$ and CSIA covered $\delta^{2}H_{fatty\ acid}$, $\delta^{13}C_{fatty\ acid}$, $\delta^{13}C_{amino\ acid}$, $\delta^{15}N_{amino\ acid}$, and $\delta^{2}H_{amino\ acid}$. ANOVA and linear discriminant analysis (LDA) were used for statistical analysis to compare samples from the USA and China. Parameters that yielded significant variations are: $\delta^{2}H_{C18:1n-9}$, $\delta^{13}C_{C18:2n-6}$, $\delta^{13}C_{C18:3n-3}$, $\delta^{13}C_{Gly}$, $\delta^{13}C_{Leu}$, $\delta^{13}C_{Val}$, $\delta^{2}H_{Glu}$, $\delta^{2}H_{Ile}$, $\delta^{2}H_{Leu}$, and $\delta^{2}H_{Thr}$.. Our findings suggested that CSIA of fatty acids and amino acid can be useful to differentiate geographical provenance of walnuts.

Keywords: walnut, stable isotope, bulk, compound-specific, origin authentication

Introduction

Walnuts are known to have many health-promoting properties, namely fatty acids, including high concentrations of linoleic acid; tocopherols; ellagitannins; and urolithins. Walnuts help protect against several neurological disorders(Hosseini Adarmanabadi et al., 2023). Walnut intake is also associated with reduced breast tumor incidence, multiplicity, and size(Hardman et al., 2011); improved diastolic functions and cardiovascular risk factors(Steffen et al., 2021); and its oil can lower cholesterol production, increase cholesterol efflux, and reduce total cholesterol and triacylglycerols in HepG2 cells(Gao et al., 2022). Walnut consumption has shown to increase gut microbiome diversity as well(Byerley et al., 2017). A study indicated that walnut oils may be useful as a plant-based infant formula ingredient because of the similar major fatty acids as human milk(Hussain et al., 2023).

Walnuts are grown in various countries, including China, the USA, Chile, Iran, Ukraine, Moldova, Poland, India, and Romania. Similar to many other crops and food products, such as wines and olive oils, their geographical origin is often linked with specific quality perception and consumers regard some countries produce better quality products than the others(D'Alessandro & Pecotich, 2013; Lans, 2001; Veale & Quester, 2008). This leads to economically motivated adulteration (EMA) by intentional origin mislabeling to increase profits. Currently, origin information is obtained from suppliers through simple documentation, therefore its falsification is uncomplicated.

Stable isotope ratio measurements have been used to accurately differentiate geographical origin or agricultural practices associated with food commodities(Chung et al., 2017). For example, variation in nitrogen stable isotope ratio has been associated with the types of fertilizer used (organic and synthetic). Plants cultivated using conventional methods generally have lower $\delta^{15}N$

values than their organic counterparts(Bontempo et al., 2020; Novak et al., 2019) because synthetic fertilizer used in conventional farms did not undergo isotope fractionation, relative to the reference standard which is air nitrogen(van Leeuwen et al., 2014). Among organically-grown vegetables, those received animal manure have higher $\delta^{15}N$ than those given green manure or catch crops(Novak et al., 2019) because the increased ^{15}N enrichment accompanying higher trophic level of animals, compared to plants(Schmidt et al., 2015).

Isotope fractionation of hydrogen has been linked to evaporation, condensation, and precipitation(Kelly et al., 2005) that specific to regions of cultivation. For example, $\delta^{2}H$ values exhibit correlation with distance of growing location to coast. Precipitation that occurs at an increasing distance from the coast towards the inner continent is more depleted in $\delta^{2}H$, due to isotope fractionation through Rayleigh distillation processes ("continental" effect) as water vapor is transported away from the oceans(Gori et al., 2015). In addition,$\delta^{2}H$ has been used to indicate milk that had been diluted with local water(Hamzić Gregorčič et al., 2020), as well as to distinguish the geographical provenance of sake(Kuribayashi et al., 2017) and milk(Chesson et al., 2010).

Variation in carbon isotope ratios is significantly affected by photosynthetic types (C3 or C4)(Kelly et al., 2005) through additional environmental (e.g. elevation), and physiological variables (e.g. water use efficiency). Similar to nitrogen and hydrogen stable isotope ratios, there have also been several reports linking variation in carbon isotope ratios to geographical provenance(Hrastar et al., 2009). Carbon stable isotope of fatty acids could also identify maize oil that had been adulterated with oil from some C3 plants because of the difference mechanism in CO_2 fixation(Mottram et al., 2003).

Stable isotope of sulfur can help identify pollution from mining activities(Gałuszka et al., 2020; Li et al., 2022). Plants exposed to acid mine water had depleted ^{34}S (negative values), compared to non-affected plants that had positive ^{34}S values because sulfur from the mining area had minimum isotopic fractionation(Gałuszka et al., 2020).

Stable isotope analysis can be divided into bulk stable isotope analysis (BSIA) and compound-specific stable isotope analysis (CSIA). BSIA is conducted on whole samples, meanwhile CSIA is done on a specific group of compounds extracted from the samples. BSIA is done using an elemental analyzer connected with an isotope ratio mass spectrometry (EA-IRMS). Whole tissues of samples undergo either combustion (i.e., for detection of C and N) or thermal conversion (i.e., for detection of H) to convert the small elements of interests to gases. The gases are then ionized and detected using a sector field mass spectrometer(Kaklamanos et al., 2020).

In this study, CSIA was performed on both fatty acids and amino acids. Multi-element CSIA of fatty acids and amino acids have been associated with numerous environmental factors relevant to geographical provenance. Carbon and hydrogen stable isotope of fatty acids have been known to reflect the geographical properties (e.g., elevation and precipitation, respectively), while stable isotope of carbon and nitrogen of amino acids correspond to fertilizer and pesticide use. Hydrogen stable isotope of amino acids and fatty acids have been linked with the source of water used by plants or animals.

While BSIA has been widely used for diverse purposes and requires simple sample preparation and less cost, some studies show that it may be less useful than CSIA, e.g., in differentiating organic from conventional shiitake mushrooms(Kim et al., 2022) and in tracing the geographical origin of wines(Paolini et al., 2016). It is reasonable that CSIA may provide more detailed isotopic information than BSIA, because BSIA data represent a mass balance of molecular

constituents which results in information from the isotope fractionation of the individual components to be diluted or masked by co-occurring molecules.

In this study, we applied both BSIA and CSIA to compare their effectivity in distinguishing walnut origins. BSIA was performed on three elements (carbon, sulfur, and nitrogen), while CSIA was measured on fatty acids (hydrogen and carbon) and amino acid (carbon, nitrogen, and hydrogen). This is the first comprehensive stable isotope study on walnuts, in regards of extensive parameters and sample origins. A previous study using stable isotopes $\delta^{13}C_{bulk}$, $\delta^{15}N_{bulk}$, and $\delta^{2}H_{bulk}$ were done on kernels from Germany and France(Krauß et al., 2020); and another one using $\delta^{13}C_{fatty\ acid}$ on walnut oil from China(Zhang et al., 2021).

To process our data, analysis of variance was applied to see if the parameters significantly differ across the regions while multivariate analysis was applied to reduce data dimensionality and evaluate whether the samples can be classified based on the origins and which parameter, or combination of parameters, that generates the most discrimination. An emphasis of comparing samples from China and the USA was performed because these two countries are the major walnut producers.

Materials and Methods

Sample

Walnuts (±2 kg) from seven countries: the USA (29 samples), China (5 samples), Poland (2 samples), Moldova (2 samples), India (1 sample), Romania (1 sample), and Ukraine (1 sample) were collected between July and September 2020. The USA samples were all from California and consisted of three cultivars commonly grown in the region: Chandler (9 samples), Howard (10 samples), and Tulare (10 samples). The China samples were sourced from Xinjiang (2 samples), Shaanxi (1 sample), Yunnan (1 sample), and unknown province (1 sample). We did

not have information on which province/state the remaining samples were grown. Each sample represented different suppliers or sources. For the USA, it also reflected different combinations of cultivars and growers.

For bulk and amino acid specific analysis, five kernel halves were peeled to remove the skin (pellicle) manually with a knife and then ground and later dried at 45 °C to remove water (initial moisture content was ±4%, w/w). For fatty acid analysis, oil was extracted from each sample using an expeller (KK Oil Prince F Universal, Reut, Germany). Approximately 500 g of walnut kernels were fed into the expeller. A screw was rotated at 45 rpm. Walnut slurry containing oil came out from a perforated tube because of the pressing action. Walnut cake, the byproduct, was extruded from a die #10. The slurry was centrifuged at 10,000 g for 15 min to separate walnut oil that was later used for CSIA-fatty acids.

BSIA - $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$

Samples were analyzed on an elemental analyzer-isotope ratio mass spectrometer (EA-IRMS) for $\delta^{13}C$ and $\delta^{15}N$, following a published study(Sieper et al., 2006). Samples were combusted at 950 °C in a reactor packed with chromium oxide and silvered copper oxide. Oxygen was dosed during sample introduction for complete combustion. Following complete combustion, residual oxygen and nitrogen oxides were removed by passing the combustion products over reduced copper at 650 °C. CO_2 and N_2 were separated by an adsorption trap in the Elementar EA (Elementar vario EL cube EA). After separation, an aliquot of the analyte gases was carried out to the IRMS, an Elementar VisION IRMS (Elementar Analysensysteme GmbH, Langenselbold, Germany), for measurement.

At least six laboratory reference materials were interspersed with samples and used for finalizing sample data and assessing analytical quality. All of the laboratory references had been calibrated

against international reference materials. Final delta (δ) values were expressed relative to international standards Vienna Pee Dee Belemnite (VPDB) for carbon and atmospheric nitrogen (Air) for nitrogen, respectively. Mean measurement error of replicates was ±0.14 and ±0.07 for $\delta^{13}C$ and $\delta^{15}N$, respectively, while measurement accuracy, as determined by quality control materials was within ±0.07 and ±0.04, for $\delta^{13}C$ and $\delta^{15}N$.

Samples were analyzed for $\delta^{34}S$ following an earlier study(Sieper et al., 2006) on an Elementar vario ISOTOPE cube elemental analyzer (Elementar Analysensysteme GmbH, Langenselbold, Germany) interfaced to an Elementar PrecisION isotope ratio mass spectrometer (Cheadle Hulme, Cheadle, England). Samples were combusted at 1150 °C in a reactor packed with tungsten oxide. Immediately following combustion, sample gases were reduced with an elemental copper at 880 °C and subsequently pass through a buffering reactor filled with quartz chips held at 900 °C. SO_2 and CO_2 were then separated by adsorption columns, allowing for full separation and peak focusing. Following separation, the SO_2 adsorption trap was heated, and the sample SO_2 passed directly to the IRMS for measurement.

Multiple instances of at least four laboratory reference materials were interspersed with samples and used for finalizing sample data and assessing analytical quality. Mean measurement error of $\delta^{34}S$ was ±0.21, while measurement accuracy, as determined by quality control materials was within ±0.07.

CSIA - $\delta^{2}H$ and $\delta^{13}C$ of fatty acids

Sample preparation and analytical procedure followed some studies(Eder, 1995; Meier-Augenstein, 2002). Fatty acids in walnut oil were subjected to a derivatization step to generate fatty acid methyl esters (FAMEs). The FAMEs were dissolved in heptane and injected at 290 °C (splitless, 1 min). The separation was performed on an Agilent DB-5ms Ultra Inert

column (60 m x 0.25 mm ID x 1 μm film thickness) at a constant flow rate of 1.2 ml/min. The column temperature program was 80 °C (hold 1 min); 220 °C (4 °C/min); and 290 °C (10 °C/min; hold 30 min).

Gas chromatography-combustion-pyrolysis (GC-C-P-IRMS) was performed on a Thermo Trace GC 1310 gas chromatograph connected to a Thermo Finnigan MAT 253 isotope-ratio mass spectrometer via a GC IsoLink II combustion interface. For ^{13}C analysis, individual FAMEs were converted to CO_2 within a combustion reactor composed of a NiO tube containing CuO and NiO wires maintained at 1000 °C. Water was subsequently removed using a Nafion dryer before the analyte gases were transferred to the IRMS. For ^{2}H analysis, individual FAMEs were converted to H_2 within a high-temperature thermal conversion reactor of graphitized Al_2O_3 tube maintained at 1425 °C.

One of every eight samples were analyzed in duplicate; further replicates were analyzed if initial measurements fell outside the expected measurement error. Replicates of the quality control and assurance reference materials were measured every eight samples.

Quality control and assurance mixtures were composed of pure FAMEs (and FAs) that had been calibrated separately by EA- and high temperature conversion (TC)/EA-IRMS using certified reference materials (e.g., NBS-22 and IAEA-CH-7) distributed by the United States Geological Survey (USGS), National Institute of Standards and Technology (NIST), and the International Atomic Energy Agency (IAEA), and all were directly traceable to the primary isotopic reference material for each element (i.e., VPDB for $\delta^{13}C$ and VSMOW for $\delta^{2}H$). Calibration procedures for CSIA of FAMEs were applied identically across reference and sample materials. First, the provisional isotopic value for each FAME was obtained by normalization to an isotopically calibrated internal reference compound (e.g., c13:0). Isotopic values of the individual FAMEs

were then scale-normalized to the primary reference materials using FMIX1, an external mixture composed of FAMEs with a broad range of calibrated $\delta^{13}C$ and $\delta^{2}H$ values. Final quality assessment was based on the accuracy and precision of an unbiased quality control material, a second $\delta^{13}C$- and $\delta^{2}H$-calibrated FAMEs mixture, FMIX2. Mean measurement error of sample replicates was ±0.16 and ±2.3 for $\delta^{13}C$ and $\delta^{2}H$, respectively, while measurement accuracy, as determined by quality control materials was within ±0.43 and ±3.1, for $\delta^{13}C$ and $\delta^{2}H$.

CSIA - $\delta^{13}C$ and $\delta^{15}N$ of amino acids

The experimental procedure followed several previous works(Corr et al., 2007; Docherty et al., 2001; Yarnes & Herszage, 2017). Peeled walnut kernels were hydrolyzed (6 M HCl for 70 min at 150 °C under N_2 headspace). The amino acids were later derivatized as *N*-acetyl methyl esters (NACME). NACME was analyzed via GC-combustion-IRMS (GC-C-IRMS, Thermo Trace GC 1310 gas chromatography coupled to a Thermo Scientific Delta V Advantage isotope-ratio mass spectrometer via a GC IsoLink II Combustion Interface).

Sample injection was done at 260 °C with splitless mode for 1 min. The separation was performed on an Agilent DB-35 column (60 m x 0.32 mm ID x 1.5 µm film thickness) with constant flow rate of 2 mL/min. The column temperature was set as the following: 70 °C (hold 2 min); 140 °C (15 °C/min, hold 4 min); 240 °C (12 °C/min, hold 5 min); and 255 °C (8 °C/min, hold 35 min).

The combustion reactor was a NiO tube containing CuO and NiO wires maintained at 1000 °C. Water is removed through a Nafion dryer before the analyte gases were transferred to the IRMS. During ^{15}N analysis, CO_2 was removed from the post-combustion carrier stream using a liquid nitrogen trap to prevent isobaric interferences within the ion source. For ^{2}H analysis, individual

AAs were converted to H_2 within a high-temperature thermal conversion reactor of graphitized Al_2O_3 tube maintained at 1425 °C.

All samples were analyzed in duplicate; further replicates were analyzed if initial measurements fell outside the expected measurement error. Replicates of the quality control and assurance reference materials were measured every five samples.

The pure amino acids used in quality control and assurance mixtures had been calibrated separately by EA-IRMS and were directly traceable to the primary isotopic reference material for each element (i.e., VPDB for $\delta^{13}C$, Air for $\delta^{15}N$, VSMOW for $\delta^{2}H$). EA/HTC-IRMS was performed using secondary reference materials calibrated against certified standard reference materials from USGS, NIST, and the IAEA (i.e., IAEA-600, USGS40, USGS41, USGS42, USGS43, USGS61, USGS64, and USGS65). Calibration procedures for CSIA of amino acids were applied identically across reference and sample materials. First, initial isotopic values for all amino acids were adjusted such that the known isotopic composition of an internal reference material (e.g., Nor) was obtained. Next, the isotopic values of the individual amino acids were adjusted based on the first quality assurance reference mixture, UCD AA1, such that the known isotopic composition of each amino acid within the mixture was obtained. Finally, measurements were scale-normalized to the primary reference materials for $\delta^{15}N$ (i.e., Air) using the second quality assurance reference mixture, UCD AA2; in the case of $\delta^{13}C$ analysis, calibration then proceeded by accounting for the influence of exogenous carbon and potential kinetic isotope effects following derivatization. For ^{2}H analysis, scale-normalization of individual AAs was performed using a certified *n*-alkanes supplied by Indiana University (IU A7). Final quality assessment was based on the accuracy and precision of unbiased quality control materials, which included a calibrated amino acid mixture, UCD AA3, and multiple natural materials.

Mean measurement error of sample replicates was ±0.26 and ±0.61, for $\delta^{13}C$ and $\delta^{15}N$, respectively, while measurement accuracy, as determined by quality control materials was within ±0.56 and ±0.53, for $\delta^{13}C$ and $\delta^{15}N$.

Statistical Analysis

The stable carbon isotope values were expressed in δ notation which illustrated the deviations of the isotope ratio of a sample relative to a standard, for instance:

$$\delta^{13}C = \left[\frac{(R_{sam} - R_{std})}{R_{std}}\right] x10^3$$

where R_{sam} and R_{std} were the $^{13}C/^{12}C$ of the sample and standard, respectively.

Analysis of variance (ANOVA) was used to determine if means differed significantly between the USA and China. Linear discriminant analysis (LDA) was performed for dimensionality reduction. All the analysis was performed using R Studio 2022.07.01 (Posit software, PBC, Boston, MA, USA) and R 4.2.1. (The R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

BSIA - $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$

BSIA of walnut kernels from multiple countries had ranges of -29.30 – -24.81‰ and -2.00 – 7.34‰ for $\delta^{13}C_{bulk}$ and $\delta^{15}N_{bulk}$, respectively (Table 4.1). These ranges were similar to those reported on samples from Germany and France(Krauß et al., 2020). $\delta^{34}S_{bulk}$ was -0.64 – 16.70‰ (Table 4.1). No data has been reported on $\delta^{34}S$ of walnuts in the literature.

Table 4.1. $\delta^{13}C_{bulk}$, $\delta^{15}N_{bulk}$, and $\delta^{34}S_{bulk}$ (‰) of walnut kernels from China (Ch), Moldova (Mo), Poland (Po), the USA (US), India (In), Romania (Ro), and Ukraine (Uk)

Element	$\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ (‰)						
	US	Ch	Mo	Po	In	Ro	Uk
S	9.46 ± 4.34a	5.54 ± 3.95a	5.52	5.54	3.36	3.54	5.85
C	-27.23 ± 1.00a	-26.67 ± 1.39a	-25.56	-27.35	-26.14	-27.08	-24.81
N	1.29 ± 1.59a	1.28 ± 1.50a	6.60	4.67	6.90	6.41	6.95

*Different letters for the same parameter indicating significant difference among the countries ($p \leq 0.05$).

Our data suggested that $\delta^{13}C_{bulk}$ and $\delta^{34}S_{bulk}$ were not significantly different ($p > 0.05$) between the samples from the USA and China (Table 4.1). $\delta^{13}C_{bulk}$ is mainly used to distinguish photosynthetic pathways (C_3, C_4, or CAM)(Simon Kelly et al., 2005), with C_3 plants having significantly lower values than C_4 plants. C3 plants use Calvin cycle; C4 plants employ Hatch-Slack cycle; while CAM plants can utilize both depending on the conditions(van Leeuwen et al., 2014). All of the samples in this study belong to the same species (*Juglans regia* L.) and are C_3 plants(Krauß et al., 2020), therefore the lack of intraspecific variation in $\delta^{13}C_{bulk}$ values is not surprising. While there have been studies indicating that $\delta^{13}C_{bulk}$ may respond to geographical features like altitude, climatic moisture and temperature of the growing locations in whitebark pines(Mahalovich et al., 2016) and wheat(Branch et al., 2003), it was not noticeable in this study and some others(Rodrigues et al., 2009).

While Mahalovich et al.[26] found a correlation between $\delta^{34}S_{bulk}$ values and climate (summer mean annual temperature, frost-free period, and annual precipitation)(Mahalovich et al., 2016), the $\delta^{34}S_{bulk}$ values of walnuts were not significantly different among the countries, whereas variation within countries (i.e., within China or USA,) was high. The intense within-country variation has also been observed in tomato and its paste(Bontempo et al., 2020). The sources of

$\delta^{34}S_{bulk}$ variability can stem from fertilizer types and quantity(Chung et al., 2017), with further modification via enzymatic sulfur isotope fractionation.

Like $\delta^{13}C_{bulk}$ and $\delta^{34}S_{bulk}$, $\delta^{15}N_{bulk}$ values of China and the USA samples were not significantly different ($p > 0.05$). However, they were lower than those from other countries (Table 4.1). Many previous studies have demonstrated that reduced $\delta^{15}N_{bulk}$ values are associated with inorganic fertilizers common to conventional agriculture, whereas higher $\delta^{15}N_{bulk}$ results from the use of organic sources of N, such as manures, in organic farming(Chung et al., 2017). This finding is expected, as the majority of walnuts grown in the USA are produced though conventional practice(OPN, 2021) and many walnut orchards in China also rely on synthetic fertilizer(Bai et al., 2020). The lower $\delta^{15}N_{bulk}$ values associated with synthetic fertilizer corresponded to minimum or no isotope fractionation occurring during the production of the fertilizer via the Haber-Bosch process(van Leeuwen et al., 2014).

High standard deviations within the Chinese and the USA samples (Table 4.1) indicated the variation in the level of synthetic fertilizer usage and availability of other primary nitrogen sources besides synthetic fertilizer, i.e., soil nitrogen(Werner & Schmidt, 2002). $\delta^{13}C$ and $\delta^{15}N$ could also be influenced by cadmium exposure to plants, e.g., in castor(Zhu et al., 2022) but it was not evident in our study.

These finding reflected that the BSIA data of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ were not sufficient for reliable discrimination of the walnut samples based on the geographical provenance. This could result from fluctuation of the values among different chemical groups present in the samples that eventually compensated the BSIA values. Therefore, CSIA should be employed as it has been proved to be more sensitive in responding to geographical changes. Another point to consider is analyzing BSIA of different portions of walnut kernels or walnut plants. For example, a study on

peanuts showed that utilizing defatted portions improved the classification(Wadood et al., 2022). This way, the risk of values becoming evened out, e.g., by the values of lipid compounds, is less than in the whole kernel, although still not as specific as CSIA.

CSIA - Fatty Acids

CSIA - δ^2H of fatty acids

Lipids are the dominant nutrient in walnuts, with concentrations reaching 75 g/100 g dry matter. Fatty acids are key components of glycoglycerolipids and glycerophospholipids, the major components of walnut lipids(Wang et al., 2022). Stable isotope analysis of fatty acids has been used in other food products to successfully determine geographical provenance(Richter et al., 2010) and differentiate organic from conventional produce(Chung et al., 2019). This is possible due to environmental effects on isotope fractionation during lipid metabolism.

Isotope fractionation of hydrogen in lipids reflects biochemical, physiological, and environmental influences. To the best of our knowledge, this is the first study analyzing $\delta^2H_{fatty\ acids}$ in walnuts from multiple regions. A sample chromatogram of $^2H_{fatty\ acid}$ was given in Figure S4.2a of the Supporting document. The range of $\delta^2H_{fatty\ acids}$ (-249.89 to -80.01‰) (Table 4.2) was wider than the range for δ^2H_{bulk} of walnuts grown in Germany and France (-186 to -147‰)(Krauß et al., 2020). This is unsurprising, as δ^2H_{bulk} reflects the sum of CSIA values of all compounds and the variation among them were high and can compensate the BSIA values. Wider variation in isotope values of constituent molecules compared to the bulk tissue is tied to their specific biochemistries and one of the reasons why CSIA is commonly more well-suited for discrimination purposes.

Table 4.2. δ^2H (‰) of fatty acids in walnuts sourced from seven countries: China (Ch), Moldova (Mo), Poland (Po), the USA (US), India (In), Romania (Ro), and Ukraine (Uk)

Fatty acid	δ^2H (‰) of fatty acids						
	US	Ch	Mo	Po	In	Ro	Uk
C16:0	-189.22 ± 9.59Ab	-189.87 ± 11.48Ab	-200.48	-212.09	-170.62	-199.77	-202.75
C18:1w9c	-109.08 ± 12.30Aa	-131.02 ± 16.42Ba	-143.06	-162.44	-118.68	-154.43	-173.96
C18:2w6c	-218.76 ± 9.31Ac	-221.72 ± 12.76Ac	-227.84	-242.44	-202.32	-225.15	-238.18
C18:3w3c	-228.46 ± 15.38Ad	-230.13 ± 10.31Ac	-247.59	-232.19	-218.96	-250.63	-257.98

*Different capital letters for the same parameter indicating significant difference among the countries ($p \leq 0.05$).
**Different small letters for the same country indicating significant difference among the parameters ($p \leq 0.05$).

Significant difference ($p < 0.05$) between the USA and China samples was only observed on $\delta^2H_{C18:1n-9}$. For $\delta^2H_{C16:0}$, $\delta^2H_{C18:2n-6}$, and $\delta^2H_{C18:3n-3}$, the sample from India had the highest $\delta^2H_{fatty\ acids}$ value, followed by the samples from the USA and China. Interestingly, $\delta^2H_{C18:1n-9}$ showed a distinct profile: the USA samples had the highest average value than the others, followed by the Indian and Chinese samples (Table 4.2).

δ^2H values are influenced by several geographical factors, including precipitation/rainfall level, temperature, elevation, distance from coast(Bowen, 2010), and whether surface water and/or groundwater is used in cultivation(Dawson & Pate, 1996). Using surface water (e.g., irrigation) led to increased δ^2H values whereas more negative values were associated with groundwater use(Dawson & Pate, 1996). Many walnut orchards in the USA and China are irrigated(Xue et al., 2021), therefore the higher values of δ^2H of the USA and Chinese samples, as compared to those of Moldova, Poland, Romania, and Ukraine, are logical. The average values for the USA samples were slightly larger than those of China, probably due to more intense irrigation.

For $\delta^2H_{C16:0}$, $\delta^2H_{C18:2n-6}$, and $\delta^2H_{C18:3n-3}$, the value of the India sample exceeded those of the USA. We did not have any information whether it was irrigated during its cultivation. The higher value could not be explained by the continental effect because the northwestern Himalayan

regions, particularly Jammu and Kashmir, are the main areas of walnut cultivation in India(Shah et al., 2021). These areas are at a farther distance from the nearest coast (±1200 km), compared to the Central Valley-CA, where all the USA samples originated, to the nearest coast (±140 km). Precipitation that occurs at a farther distance from a coast is generally more depleted in δ^2H than the precipitation near a coast. Altitude effects may be more reasonable in explaining this finding. A previous study reported a positive correlation between altitude and δ^2H(Kong & Pang, 2016). This theory supported our data because the approximate altitude/elevation of the Central Valley and northwestern Himalaya are 50 m and 1200-3500 m, respectively.

The fact that oleic acid showed distinct characters for country comparison, at which the USA samples were more enriched than the India sample and those of the other countries, could be related to more intense desaturation process of oleic acid to generate more polyunsaturated fatty acids. This was evidenced by the lower ratio of oleic acid (the main monounsaturated fatty acid) to polyunsaturated fatty acids of the USA samples, as compared to those of other countries (Supporting document, Figure S4.1).

Oleic acid was more enriched in δ^2H than linoleic acid and linoleic acid was more enriched than linolenic acid for most of the samples (Table 4.2). This is not unexpected, as numerous studies have found biochemical isotope fractionation results in substrates that are more enriched than their products(Schmidt et al., 2015). Oleic acid acts a substrate for linoleic acid synthesis, whereas linoleic acid is the substrate for linolenic acid. Serial isotopic depletion of oleic acid desaturation products has also been observed elsewhere(Wang et al. 2022).

CSIA - $\delta^{13}C$ of fatty acids

We analyzed $\delta^{13}C_{\text{fatty acid}}$ and compared $\delta^{13}C_{\text{fatty acid}}$ in samples sourced from multiple countries. A sample chromatogram of $^{13}C_{\text{fatty acid}}$ was provided in Figure S4.2b of the Supporting document.

The predominant fatty acids of walnuts were C16:0, C18:0, C18:1*n-9*, C18:2*n-6*, and C18:3*n-3*, and had a range of carbon stable isotope values typical of C_3 plants (Table 4.3). The values for $\delta^{13}C_{C16:0}$, $\delta^{13}C_{C18:0}$, and $\delta^{13}C_{C18:1n-9}$, were within the range of $\delta^{13}C$ previously reported on walnut oils from China. The $\delta^{13}C_{C18:2n-6}$ values were also mostly within the reported range, with a few exceptions. For $\delta^{13}C_{C18:3n-3}$, many of our data were slightly higher than those reported in that study(Zhang et al., 2021).

Table 4.3. $\delta^{13}C$ (‰) of fatty acids in walnuts sourced from seven countries: China (Ch), Moldova (Mo), Poland (Po), the USA (US), India (In), Romania (Ro), and Ukraine (Uk)

Fatty acid	$\delta^{13}C$ (‰) of fatty acids						
	US	Ch	Mo	Po	In	Ro	Uk
C16:0	-30.51 ± 0.69Ab	-30.15 ± 0.56Ab	-30.19	-30.94	-30.13	-30.21	-29.96
C18:0	-31.20 ± 0.75Ac	-30.56 ± 0.56Ab	-30.35	-30.22	-30.21	-30.44	-30.58
C18:1w9c	-29.93 ± 0.73Aa	-29.38 ± 0.78Aab	-29.07	-29.86	-29.18	-29.13	-29.38
C18:2w6c	-29.68 ± 0.70Ba	-28.42 ± 0.72Aa	a-28.67	-29.33	-28.39	-28.30	-28.18
C18:3w3c	-30.88 ± 0.79Bbc	-29.8 ± 0.85Ab	-29.89	-30.95	-30.32	-29.62	-29.72

*Different capital letters for the same parameter indicating significant difference among the countries ($p < 0.05$).
**Different small letters for the same country indicating significant difference among the parameters ($p < 0.05$).

Minor variation was observed among fatty acids from the same country of origin. For the USA samples, $\delta^{13}C_{C18:2n-6}$ had the highest level, followed by $\delta^{13}C_{C18:1n-9}$, $\delta^{13}C_{C16:0}$, $\delta^{13}C_{C18:3n-3}$, and then $\delta^{13}C_{C18:0}$. A similar pattern was noticed in Chinese samples, except that $\delta^{13}C_{C18:3n-3}$ had slightly higher value, albeit insignificant, than $\delta^{13}C_{C16:0}$ (Table 4.3). A previous study of olive oils showed that in oils from unripe olives, the carbon stable isotope ratios of oleic and linoleic acids were significantly different whereas no noticeable difference was found in oils from ripe fruits(Royer et al., 1999). We predict that in ripe walnuts, linoleic and linolenic acids had been converted into volatiles, based on a report demonstrating that advanced ripening led to a lower proportion of polyunsaturated fatty acids(Amin et al., 2017).

Due to isotope fractionation, elongation from palmitic to stearic acid yielded a slight $\delta^{13}C$ depletion of the later compound in most of the samples (Table 4.3). However, $\delta^{13}C_{\text{fatty acids}}$ of the desaturation products of stearic acid were confounded because the products were slightly more enriched ($\delta^{13}C_{C18:2n-6} > \delta^{13}C_{C18:1n-9} > \delta^{13}C_{C18:0}$). Similar trends have been observed in pumpkin oil(Spangenberg & Ogrinc, 2001) and various seed oils(S Kelly et al., 1997) because unsaturated fatty acids are prone to oxidation.

Our data showed that there was significant difference in $\delta^{13}C_{C18:2n-6}$ and $\delta^{13}C_{C18:3n-3}$ between the USA and China samples but not for the other fatty acids (Table 4.3). This confirmed earlier findings that cultivation locations had some effects on the carbon stable isotopes of individual fatty acids(Zhang et al., 2021). The earlier study on walnut oils showed significant difference in $\delta^{13}C$ values of all dominant fatty acids among samples from different regions in China. Noticeable difference in $\delta^{13}C_{C18:2n-6}$ and $\delta^{13}C_{C18:3n-3}$ values between China and the USA samples may originate from varying intensity of the fatty acid oxidation because they are both polyunsaturated fatty acids. Relatively higher values in the China samples could indicate that they were oxidized at a faster rate.

These two fatty acids were likely more sensitive to environmental factors affecting $\delta^{13}C$ values, e.g., temperature, water availability. These factors can influence stomatal aperture and CO_2 diffusion into leaves(van Leeuwen et al., 2014). The higher sensitivity of these fatty acids was reasonable, considering that their synthesis included more reactions than the others.

CSIA – Amino Acids

CSIA - $\delta^{13}C$ of amino acids

CSIA of amino acids had previously been used in other matrices to evaluate resource utilization and trophic connections among organisms in ecosystems(Takizawa et al., 2020) and authenticity

of organic produce(Bontempo et al., 2020). This is the first study reporting CSIA-amino acids in walnuts. A sample chromatogram of $^{13}C_{amino\ acid}$ was provided in Figure S4.2c of the Supporting document. Most $\delta^{13}C_{amino\ acid}$ values were higher than $\delta^{13}C_{fatty\ acids}$, agreeing with previous studies showing that $\delta^{13}C_{carbohydrate} > \delta^{13}C_{protein} > \delta^{13}C_{lignin} > \delta^{13}C_{lipid}$(van Leeuwen et al., 2014).

For walnut kernels, $\delta^{13}C_{His}$, $\delta^{13}C_{Hyp}$, and $\delta^{13}C_{Met}$ were present at concentrations lower than the limit of quantitation for all the samples and are not included here. Among all the walnut amino acids, $\delta^{13}C_{Ser}$ had the highest value. In general, $\delta^{13}C_{amino\ acid}$ values were moderately higher than $\delta^{13}C_{bulk}$, except for leucine, phenylalanine, tyrosine, and valine. $\delta^{13}C_{Phe}$ and $\delta^{13}C_{Val}$ were more depleted while $\delta^{13}C_{Leu}$ and $\delta^{13}C_{Tyr}$ were like the $\delta^{13}C_{bulk}$ (Table 4.1 and Table 4.4). The difference between $\delta^{13}C_{amino\ acid}$ and $\delta^{13}C_{bulk}$ is not surprising, as bulk were not lipid-extracted. Because there are no previous studies on walnuts on this parameter, we compare the data to those of other food products. The range of our $\delta^{13}C_{amino\ acid}$ values overlapped with that reported for rice in South Korea(Chung et al., 2019) and tomatoes in Italy(Bontempo et al., 2020). While some values were similar, the other $\delta^{13}C_{amino\ acid}$ of walnuts were higher than the values for tomatoes and rice. This was reasonable because of the genetic variation.

ANOVA results showed that no significant difference ($p > 0.05$) was observed among the means of $\delta^{13}C_{amino\ acid}$ between the USA and China samples, except for glycine, leucine, and valine (Table 4.4). Interestingly, these three compounds are grouped into the same category: aliphatic amino acid. Similar finding was observed in a shiitake authentication study. $\delta^{13}C_{leucine}$ discriminated shiitake mushrooms that were grown organically to those grown conventionally, at which the values were more negative. Meanwhile, $\delta^{13}C_{valine}$ of organic shiitake was significantly lower than the pesticide-free and conventional shiitake(Kim et al., 2022). A study on rice demonstrated no significant difference on $\delta^{13}C_{leucine}$ and $\delta^{13}C_{valine}$ among organic, pesticide-free

and conventional rice but the values for $\delta^{13}C_{isoleucine}$, $\delta^{13}C_{lysine}$, and $\delta^{13}C_{tyrosine}$ were significantly different. The organic sample had more negative values than the conventional ones, except for $\delta^{13}C_{tyrosine}$ that showed a reverse effect. A study on tomatoes found a significant difference in $\delta^{13}C_{Glx}$ when organic and conventional tomatoes were compared, but no differences in the remaining $\delta^{13}C_{amino\ acid}$.

Table 4.4. $\delta^{13}C$ (‰) of amino acids in walnuts sourced from 7 countries: China (Ch), Moldova (Mo), Poland (Po), the USA (US), India (In), Romania (Ro), and Ukraine (Uk)

Amino acid	$\delta^{13}C$ (‰) of amino acids						
	US	Ch	Mo	Po	In	Ro	Uk
Ala	-20.32 ± 1.31Ae	-19.99 ± 1.67Acd	-19.85	-20.35	-19.04	-20.65	-19.5
Asx	-18.73 ± 1.16Ad	-18.18 ± 1.04Abc	-18.18	-18.31	-18.41	-18.87	-17.4
Glx	-20.55 ± 1.02Ae	-20.64 ± 1.04Acd	-20.25	-21.41	-20.55	-21.16	-20.11
Gly	-20.74 ± 1.71Be	-19.47 ± 1.64Acd	-19.69	-21.04	-19.85	-20.10	-19.32
Ile	-22.79 ± 1.22Af	-21.75 ± 0.99Ad	-21.75	-22.37	-21.28	-22.75	-21.31
Leu	-28.10 ± 1.12Bg	-26.55 ± 1.04Ae	-26.58	-27.58	-27.76	-27.75	-25.98
Lys	-16.05 ± 2.88Ac	-17.91 ± 0.97Abc	-18.36	-18.15	-18.36	-19.1	-17.66
Phe	-31.11 ± 1.21Ah	-30.83 ± 1.52Ag	-30.45	-31.15	-29.01	-31.64	-28.64
Pro	-19.74 ± 1.20Ade	-20.03 ± 1.36Acd	-19.92	-20.66	-19.84	-20.45	-19.22
Ser	-12.50 ± 1.61Aa	-13.35 ± 1.26Aa	-12.52	-13.27	-12.56	-13.28	-11.31
Thr	-13.95 ± 1.77Ab	-15.43 ± 1.66Aab	-14.96	-16.16	-14.17	-16.25	-14.68
Tyr	-27.62 ± 1.93Ag	-27.22 ± 1.11Aef	-26.71	-27.63	-27.16	-27.93	-25.96
Val	-30.56 ± 1.25Bh	-29.45 ± 1.07Afg	-29.60	-30.23	-30.6	-30.67	-29.37

*Different capital letters for the same parameter indicating significant difference among the countries ($p \leq 0.05$).
**Different small letters for the same country indicating significant difference among the parameters ($p \leq 0.05$).

The tomato study described that samples grown in two locations had similar $\delta^{13}C_{amino\ acids}$. $\delta^{13}C_{amino\ acids}$ of tomatoes grown in different years were also not significantly different(Bontempo et al., 2020). Taken together, this may indicate that location and cultivation time (and possible climate variability associated with it) had a minimum effect on $\delta^{13}C_{amino\ acids}$. However, in our study, there was small but significant differences in the $\delta^{13}C_{amino\ acids}$ profile between samples

from China and the USA. It showed that this parameter had some utility for origin discrimination, similar to $\delta^{13}C_{fatty\ acid}$, but the reasons need to be explored more. It could be related to geographical factors affecting the stomatal aperture and subsequent photosynthesis.

CSIA - $\delta^{15}N$ of amino acids

Nitrogen stable isotope analysis of amino acids had been studied in other food matrices like rice(Chung et al., 2019) and tomatoes(Bontempo et al., 2020), mainly to distinguish organic vs conventional or pesticide-free products. A sample chromatogram of $^{15}N_{amino\ acid}$ was displayed in Figure S4.2d of the Supporting document.

Similar to the carbon amino acids, $\delta^{15}N_{His}$, $\delta^{15}N_{Hyp}$, and $\delta^{15}N_{Met}$ were present in amounts below the limit of quantification in all of the samples and are not included here. Some $\delta^{15}N_{amino\ acids}$, for instance $\delta^{15}N_{Phe}$, $\delta^{15}N_{Leu}$, were lower than $\delta^{15}N_{bulk}$ while others (e.g., $\delta^{15}N_{Asx}$, $\delta^{15}N_{Lys}$, and $\delta^{15}N_{Pro}$) were higher than $\delta^{15}N_{bulk}$ (Table 4.5), similar to a study in shiitake mushroom(Kim et al., 2022). The difference among $\delta^{15}N_{amino\ acid}$ reflected well-known differences in nitrogen isotope fractionation during nitrogen assimilation, as well as synthesis and metabolism of amino acids. Enrichment, relative to the $\delta^{15}N_{bulk}$ values, might indicate that the amino acid is further converted into other compounds, e.g., catabolism of lysine in response to biotic and abiotic stress, or increased concentration of this compound. Proline accumulation occurred when plants were exposed to stress. Depletion, relative to the bulk, might signal the lengthy process during their synthesis(Trovato et al., 2021).

Table 4.5. $\delta^{15}N$ (‰) of amino acids in walnuts sourced from seven countries: China (Ch), Moldova (Mo), Poland (Po), the USA (US), India (In), Romania (Ro), and Ukraine (Uk)

Amino acid	$\delta^{15}N$ (‰) of amino acids						
	US	Ch	Mo	Po	In	Ro	Uk
Ala	0.86 ± 1.58Acdef	1.78 ± 1.86Aa	5.85	3.72	3.96	6.15	6.08
Asx	2.2 ± 1.49Aabc	2.75 ± 2.03Aa	7.27	5.74	6.34	7.72	7.98
Glx	1.74 ± 1.48Abcde	2.06 ± 2.08Aa	6.73	5.11	6.33	7.2	7.31
Gly	1.43 ± 1.77Acde	2.53 ± 2.88Aa	7.46	3.89	5.39	7.41	8.09
Ile	0.38 ± 1.87Aef	0.41 ± 2.34Aa	5.42	3.01	3.71	5.2	5.6
Leu	-0.57 ± 1.47Afg	-0.57 ± 2.23Aa	4.21	2.05	3.19	4.35	4.32
Lys	1.96 ± 1.78Aabcd	2.93 ± 3.24Aa	7.73	6.06	8.05	8.38	7.75
Phe	-1.31 ± 1.97Ag	-0.55 ± 2.37Aa	3.51	2.05	1.41	3.68	4.89
Pro	3.41 ± 1.57Aa	3.9 ± 2.37Aa	8.65	7.30	7.45	8.6	8.78
Ser	1.38 ± 1.66Acde	1.86 ± 2.97Aa	6.21	2.97	4.15	5.77	6.15
Thr	0.56 ± 1.92Adef	1.22 ± 3.67Aa	6.02	7.06	5.03	6.64	5.52
Tyr	1.04 ± 1.85Acde	2.42 ± 2.60Aa	6.06	5.79	5.11	7	8.57
Val	2.93 ± 1.55Aab	3.32 ± 2.60Aa	8.72	6.92	8.27	8.89	9.11

*Different capital letters for the same parameter indicating significant difference among the countries ($p \leq 0.05$).
**Different small letters for the same country indicating significant difference among the parameters ($p \leq 0.05$).

In general, the USA samples had the lowest values for all of the amino acids while Moldova had the highest values. For all the amino acids investigated, Chinese samples were not significantly different from the USA samples ($p > 0.05$). This follows the $\delta^{15}N_{bulk}$ data indicating that most walnuts from these two countries were grown mainly using a similar method at which synthetic fertilizer was used. This is supported by a prior finding in tomato and shiitake where $\delta^{15}N_{amino\ acid}$ values were lower in tomatoes grown using conventional method(Kim et al., 2022). Synthetic fertilizer is commonly produced via the Haber-Bosch reaction that relies on nitrogen fixation from atmosphere; therefore, the $\delta^{15}N$ values are close to 0‰. Green manure from legumes also exhibit nitrogen values near atmophere, however animal manure and compost undergo more complex biochemical reactions resulting in higher $\delta^{15}N$(Mie et al., 2022). Based on this, we

predicted that the samples from the other countries besides China and the USA used compost or animal manure.

Across all amino acids, valine had the highest $\delta^{15}N$ values, except for proline in some cases (Table 4.5). In contrast, phenylalanine and leucine had the lowest $\delta^{15}N$ values among walnut $\delta^{15}N_{amino\ acid}$. This is similar to previous findings from tomato, komatsuna, and spinach(Bontempo et al., 2020; Yoneyama & Tanaka, 1999). This complex process involves the shikimate pathway and other amino acids(Yoo et al., 2013).

Positive values of $\Delta_{amino\ acid\ -\ Glx}$ ($\delta^{15}N_{amino\ acid}$ - $\delta^{15}N_{Glx}$) occurred for aspartate, lysine, proline, and valine (for the USA samples) and aspartate, glycine, lysine, proline, tyrosine, and valine (for the China samples). Negative fractionation occurred during the synthesis of alanine, glycine, isoleucine, leucine, phenylalanine, serine, threonine, and tyrosine (for the USA samples) and alanine, isoleucine, leucine, phenylalanine, serine, and threonine (for the China samples). This profile shared some similarity with rice(Chung et al., 2019) and shiitake(Kim et al., 2022) but some contrasts were also noticed that supported the idea that isotope fractionation of amino acids is influenced by genetics(Kim et al., 2022).

Similar to carbon of the same compound group, nitrogen stable isotope of amino acids is rarely used to distinguish geographical origins. They are mainly used to investigate agricultural practices, particularly types of fertilizer use. In this study, $\delta^{15}N_{amino\ acids}$ showed very strong correlation with $\delta^{15}N_{bulk}$, indicating that $\delta^{15}N_{bulk}$ data was sufficient if investigation of fertilizer type (i.e., N source) was the sole purpose of the study.

CSIA - δ^2H of amino acids

$\delta^2H_{amino\ acids}$ had been evaluated on the samples from China and the USA but not from those of the other countries. A sample chromatogram was given in Supporting document, Figure S4.2e.

There were four amino acids with significant difference among the USA and China samples: glutamate, isoleucine, leucine, and threonine (Table 4.6). For the first three compounds, the values in the China samples were lower but for threonine, the opposite trend was observed. Lower $\delta^2H_{amino\ acids}$ values generally corresponded to higher distance from the nearest coast (continental effect) as precipitation that occurs at farther area from the sea is more depleted in ^2H(van Leeuwen et al., 2014). Central Valley, the main walnut growing area in the USA, is around 140 km from the nearest coast whereas Yunnan, Shaanxi, and Xinjiang, the sources of our China samples, are at approximately 900, 1100, and 3000 km from the nearest coast, respectively. The reverse trend on threonine could be confounded by its conversion into other amino acids.

Table 4.6. δ^2H (‰) of amino acids in walnuts sourced from China (Ch) and the USA (US)

Amino acid	δ^2H (‰) of amino acids	
	US	Ch
Ala	-106.29 ± 4.63A	-106.89 ± 13.08A
Asx	-106.03 ± 3.87A	-109.94 ± 9.36A
Glx	-96.71 ± 4.56A	-103.89 ± 13.15B
Gly	-111.87 ± 4.06A	-116.01 ± 8.94A
Ile	-246.08 ± 7.10A	-258.50 ± 11.69B
Leu	-170.08 ± 5.57A	-179.73 ± 14.71B
Lys	-155.55 ± 9.85A	-157.80 ± 9.74A
Met	-127.18 ± 18.57A	-132.17 ± 9.98A
Phe	-93.58 ± 7.60A	-95.07 ± 11.38A
Pro	-84.86 ± 7.14A	-92.69 ± 14.27A
Ser	-78.80 ± 5.32A	-82.53 ± 10.64A
Thr	-192.09 ± 4.03B	-186.80 ± 6.97A
Tyr	-104.04 ± 5.81A	-98.65 ± 9.17A
Val	-190.69 ± 7.45A	-197.05 ± 14.38A

*Different capital letters for the same parameter indicating significant difference among the countries ($p < 0.05$).

Our data demonstrates that $\delta^2H_{amino\ acids}$ were generally more enriched than $\delta^2H_{fatty\ acids}$, agreeing with a previous study on avocado(Muñoz-Redondo et al., 2022). A plausible reason was that less isotope fractionation occurred during amino acid synthesis or more intense catabolism of the group.

Combination of multiple parameters

Our finding demonstrated that BSIA data had limited use in geographical provenance. The values of $\delta^{15}N_{bulk}$ of the USA and China samples were very different from those of other countries, consistent with modern agricultural practice, i.e., synthetic fertilizer usage. It was not useful, however, to distinguish samples from China to the USA.

CSIA, of both amino acids and fatty acids, showed promising results with significant difference between samples of the two countries for the following parameters: $\delta^2H_{C18:1n-9}$, $\delta^{13}C_{C18:2n-6}$, $\delta^{13}C_{C18:3n-3}$, $\delta^{13}C_{Gly}$, $\delta^{13}C_{Leu}$, $\delta^{13}C_{Val}$, δ^2H_{Glu}, δ^2H_{Ile}, δ^2H_{Leu}, and δ^2H_{Thr}. Similar to the $\delta^{15}N_{bulk}$, $\delta^{15}N_{amino\ acid}$ profiles of the samples from the USA and China were identical, likely due to the same fertilizer type.

A Pearson correlation test showed that strong positive correlation was observed between $\delta^{15}N_{bulk}$ and $\delta^{15}N_{amino\ acids}$; $\delta^{13}C_{bulk}$ and $\delta^{13}C_{Gly}$; $\delta^{13}C_{bulk}$ and $\delta^{13}C_{Ile}$; δ^2H_{Leu} and $\delta^2H_{C18:2n-6}$; and $^2H_{Leu}$ and $\delta^2H_{C18:3n-3}$. High positive correlation was also observed among majority, if not all, $\delta^{15}N_{amino\ acids}$ and $\delta^{13}C_{fatty\ acids}$ and some of $\delta^2H_{amino\ acid}$; $\delta^{13}C_{amino\ acid}$; and $\delta^2H_{fatty\ acid}$ values (Figure 4.1). High correlation between $\delta^{15}N_{bulk}$ and $\delta^{15}N_{amino\ acids}$ and among $\delta^{15}N_{amino\ acids}$ was expected considering that synthesis of all amino acids involves either nitrate as the substrate or other amino acid(Tcherkez, 2010). Meanwhile, the trend in $\delta^{13}C_{fatty\ acids}$ had been found in olive oil(Royer et al., 1999), indicating the relationship among them during their synthesis.

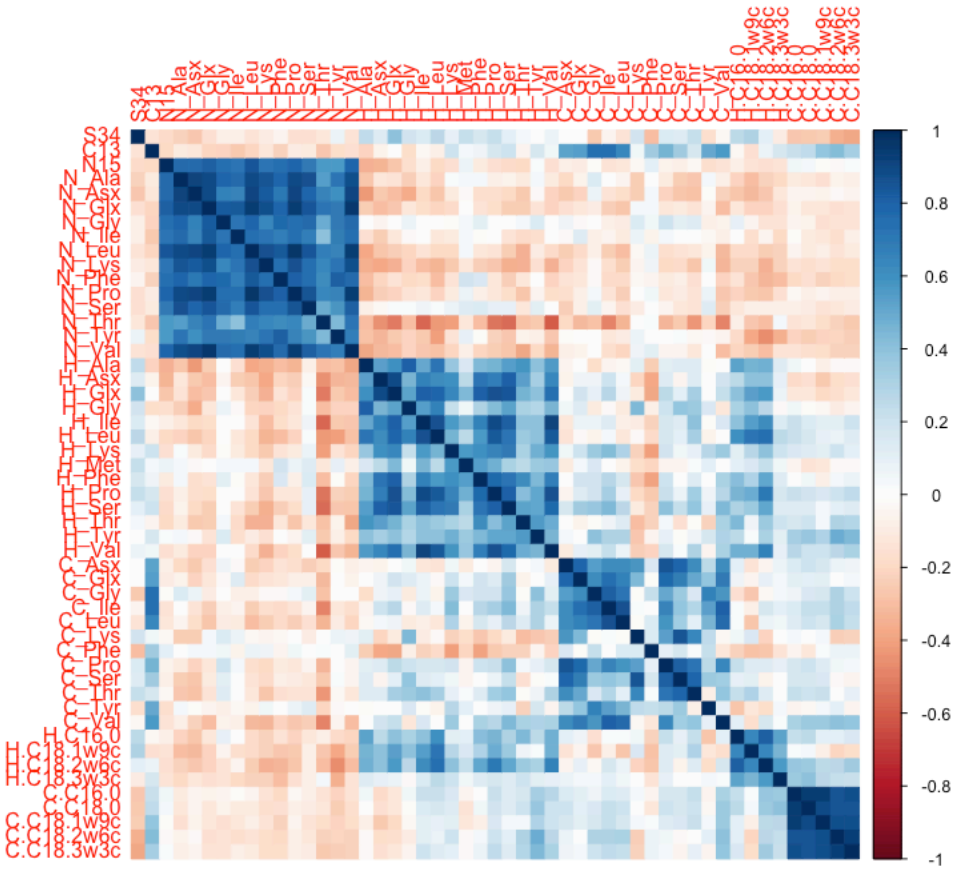

Figure 4.1. Correlation plot for all the parameters studied with a significance level of 0.05.

Linear discriminant analysis (LDA) was used to evaluate if the stable isotope profile of the USA and China samples are distinguishable. First, we conducted the test on the CSIA-fatty acid data and found that the profile of the two countries were different, indicated as separate peaks (no overlapping). There were at least two peaks representing the profile of China samples, suggesting high variability. The high variability was common for areas as diverse as China (e.g., desert areas in Xinjiang and humid regions of Yunnan). The variations, however, did not compensate the contrast to the USA samples. When LDA was conducted on either $\delta^{2}H_{\text{fatty acids}}$ or $\delta^{13}C_{\text{fatty acids}}$, overlapping was observed, indicating their insufficiency for the geographical provenance purpose when used separately.

$\delta^2H_{\text{amino acids}}$, on the other hand, was enough to separate the samples from the two countries. Identical to the LDA density plot for $\delta^2H_{\text{fatty acids}}$ combined with $\delta^{13}C_{\text{fatty acids}}$, there were at least two peaks for the China samples. When $\delta^{13}C$, $\delta^{15}N$, and $\delta^2H_{\text{amino acids}}$ were combined, the discrimination power became larger, expressed as a larger distance between the peaks. When all CSIA values were used together, the LDA density plot showed a clear distinction, but the distance of the USA and China peaks were smaller than that of combination of $\delta^{13}C_{\text{amino acids}}$, $\delta^{15}N_{\text{amino acids}}$, and $\delta^2H_{\text{amino acids}}$. In addition, we also performed LDA on parameters with significant variation only ($\delta^2H_{C18:1n-9}$, $\delta^{13}C_{C18:2n-6}$, $\delta^{13}C_{C18:3n-3}$, $\delta^{13}C_{\text{Gly}}$, $\delta^{13}C_{\text{Leu}}$, $\delta^{13}C_{\text{Val}}$, δ^2H_{Glu}, δ^2H_{Ile}, δ^2H_{Leu}, and δ^2H_{Thr}) and a striking contrast was found but at a lesser extent (shown as a smaller distance), compared to those of a combination of $\delta^{13}C_{\text{amino acids}}$, $\delta^{15}N_{\text{amino acids}}$, and $\delta^2H_{\text{amino acids}}$. Deleting $\delta^{15}N_{\text{amino acids}}$ from the data set, considering that they were not significantly different, did not improve separation of the two countries. In fact, it slightly decreased the separation distance, reflecting that even tough not significantly different, $\delta^{15}N_{\text{amino acids}}$ contributed to the LDA. All the LDA results (shown in density plots) were given in the Supporting document, Figure S4.3, except for the one with the most utility displayed below (Figure 4.2).

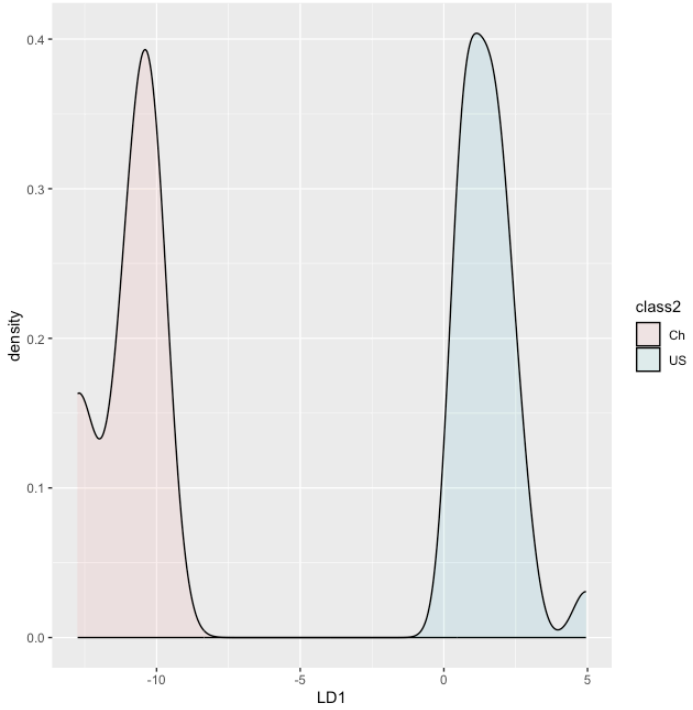

Figure 4.2. Density plot of the $\delta^{13}C_{amino\ acids}$, $\delta^{15}N_{amino\ acids}$, and $\delta^{2}H_{amino\ acids}$ of samples from China and the USA

Our findings suggested that CSIA, either fatty acids ($\delta^{2}H_{fatty\ acids}$ combined with $\delta^{13}C_{fatty\ acids}$) or amino acids ($\delta^{2}H_{amino\ acids}$ combined with $\delta^{13}C_{amino\ acids}$) had utility in separating samples from China and the USA. This confirmed previous reports that carbon and hydrogen stable isotope fractionation was sensitive to variations in geographical features, i.e., distance to coast, elevation, and humidity. The LDA results demonstrated that CSIA-amino acid yielded a larger contrast between the two countries than CSIA-fatty acid. When time and funding were limited, $\delta^{2}H_{amino\ acids}$ combined with $\delta^{13}C_{amino\ acids}$ or $\delta^{2}H_{amino\ acids}$ alone were likely sufficient for discriminating the samples from the two countries. $\delta^{15}N$, however, was not useful, most likely because the two countries applied similar fertilization strategy.

Abbreviations

BSIA: bulk stable isotope analysis; CSIA: compound-specific stable isotope analysis; Ala: alanine; Asx: asparagine or aspartic acid; Glx: Glutamate or glutamine; His: Histidine; Hyp: Hydroxyproline; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Met: Methionine; Phe: Phenylalanine; Pro: Proline; Ser: Serine; Thr: Threonine; Tyr: Tyrosine; Val: Valine.

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For Table of Contents Only

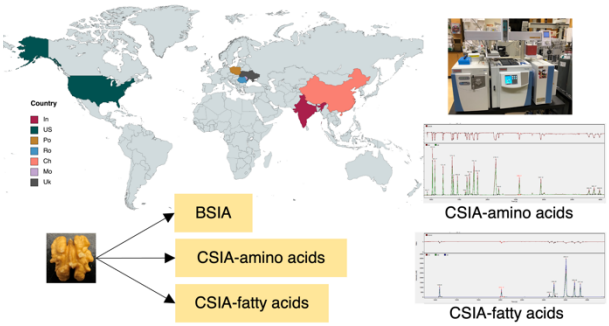


Figure 4.3. Table of content figure depicting the countries where the samples were sourced from, analytical methods, an analytical instrument, and chromatograms of CSIA-amino acids and CSIA-fatty acids

Supporting Information

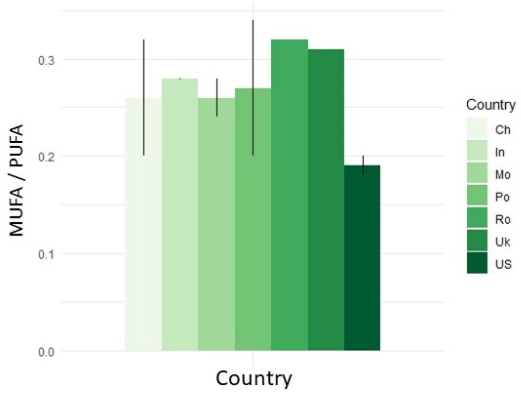
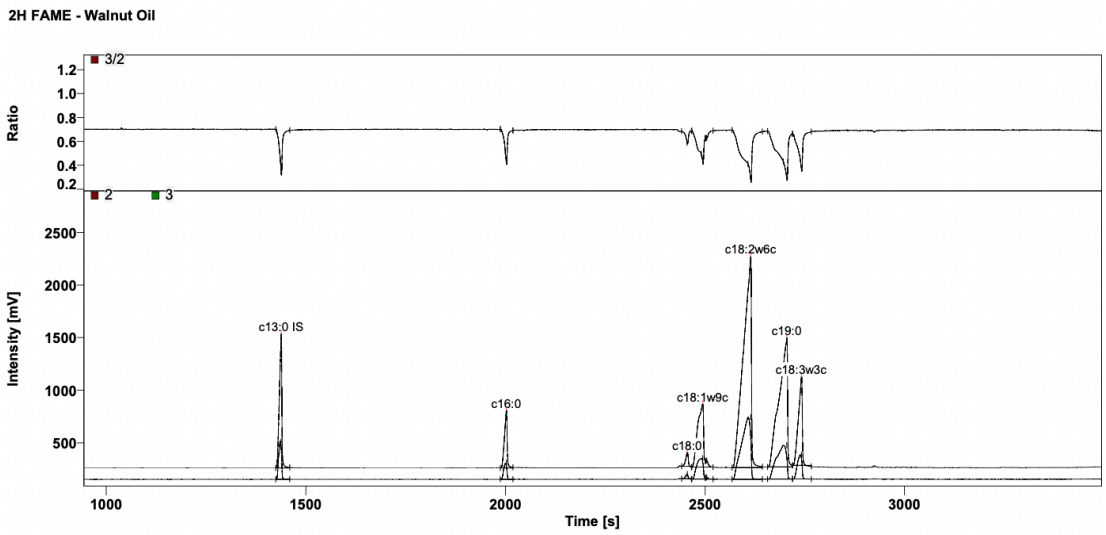
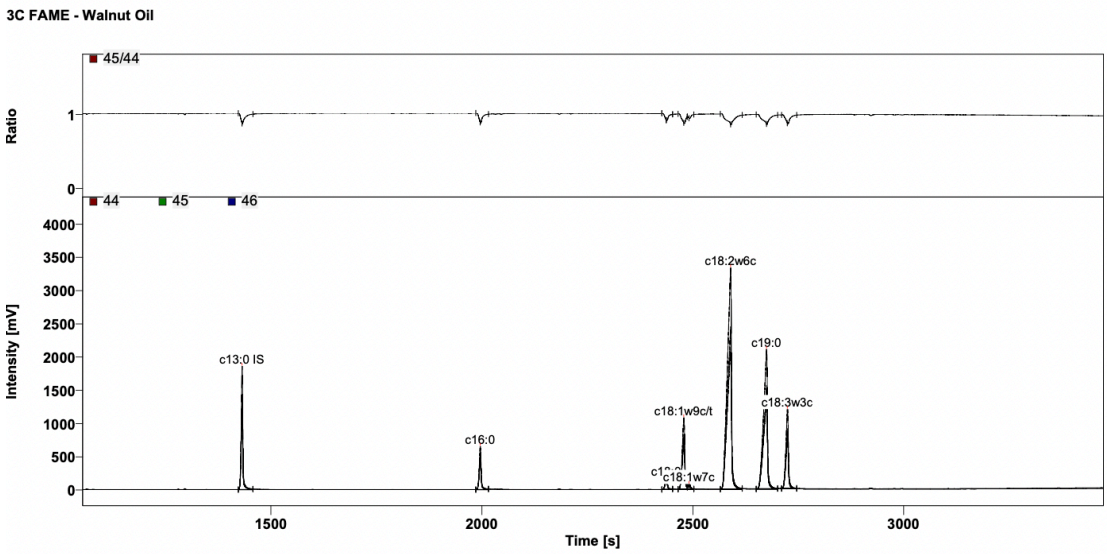


Figure S4.1. Bar plot of the ratio of monounsaturated fatty acid (particularly oleic acid) to polyunsaturated fatty acids (linoleic and linolenic acids) of samples sourced from China (Ch), India (In), Moldova (Mo), Poland (Po), Romania (Ro), Ukraine (Uk), and the USA (US)



a) ^{2}H of fatty acids



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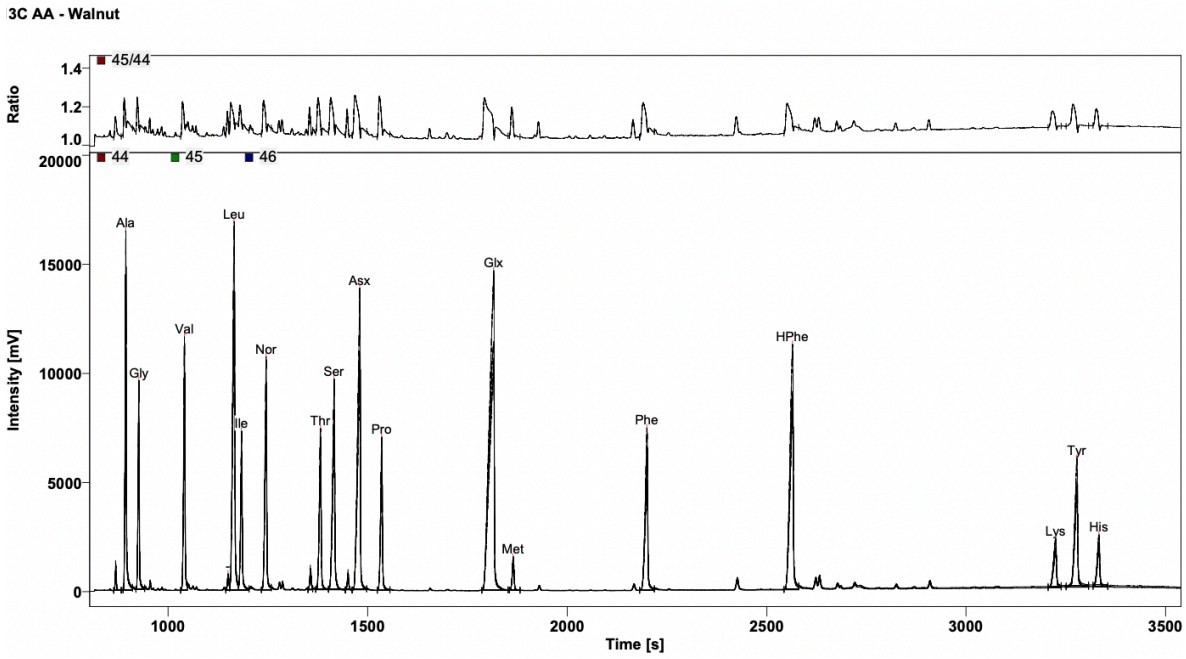
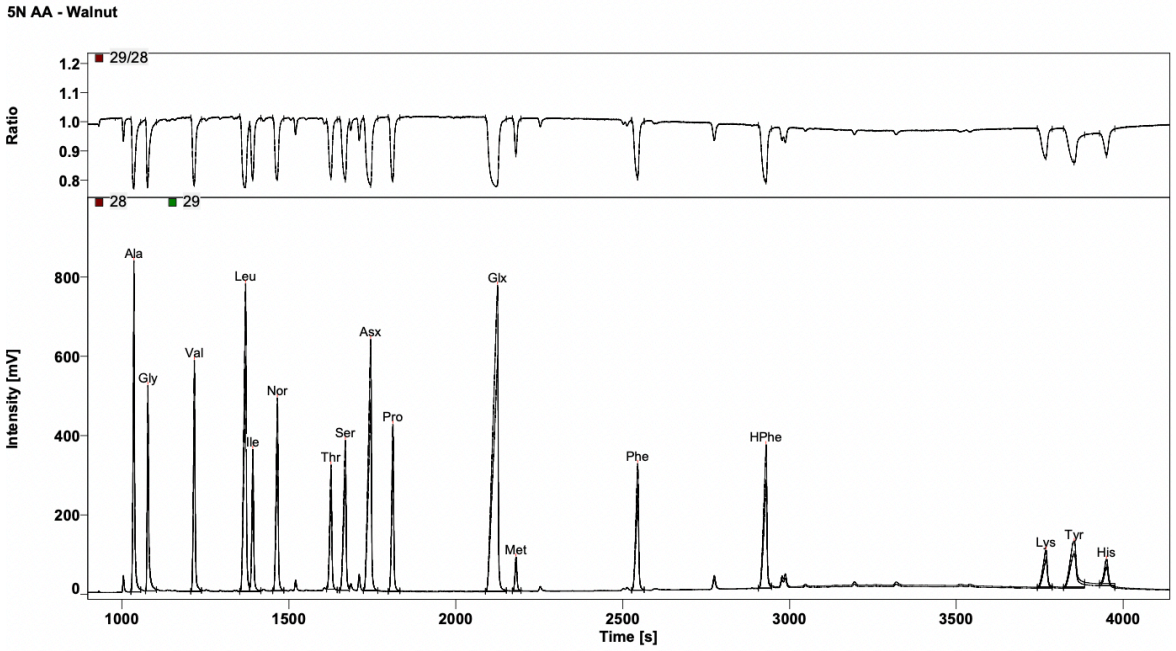
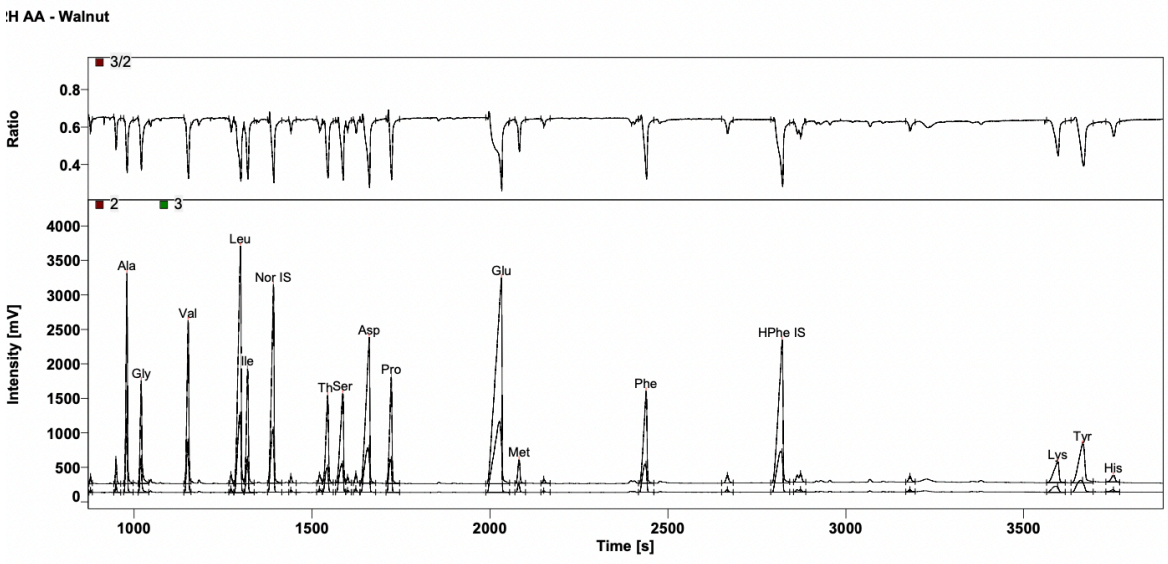


c) ^{13}C of amino acids

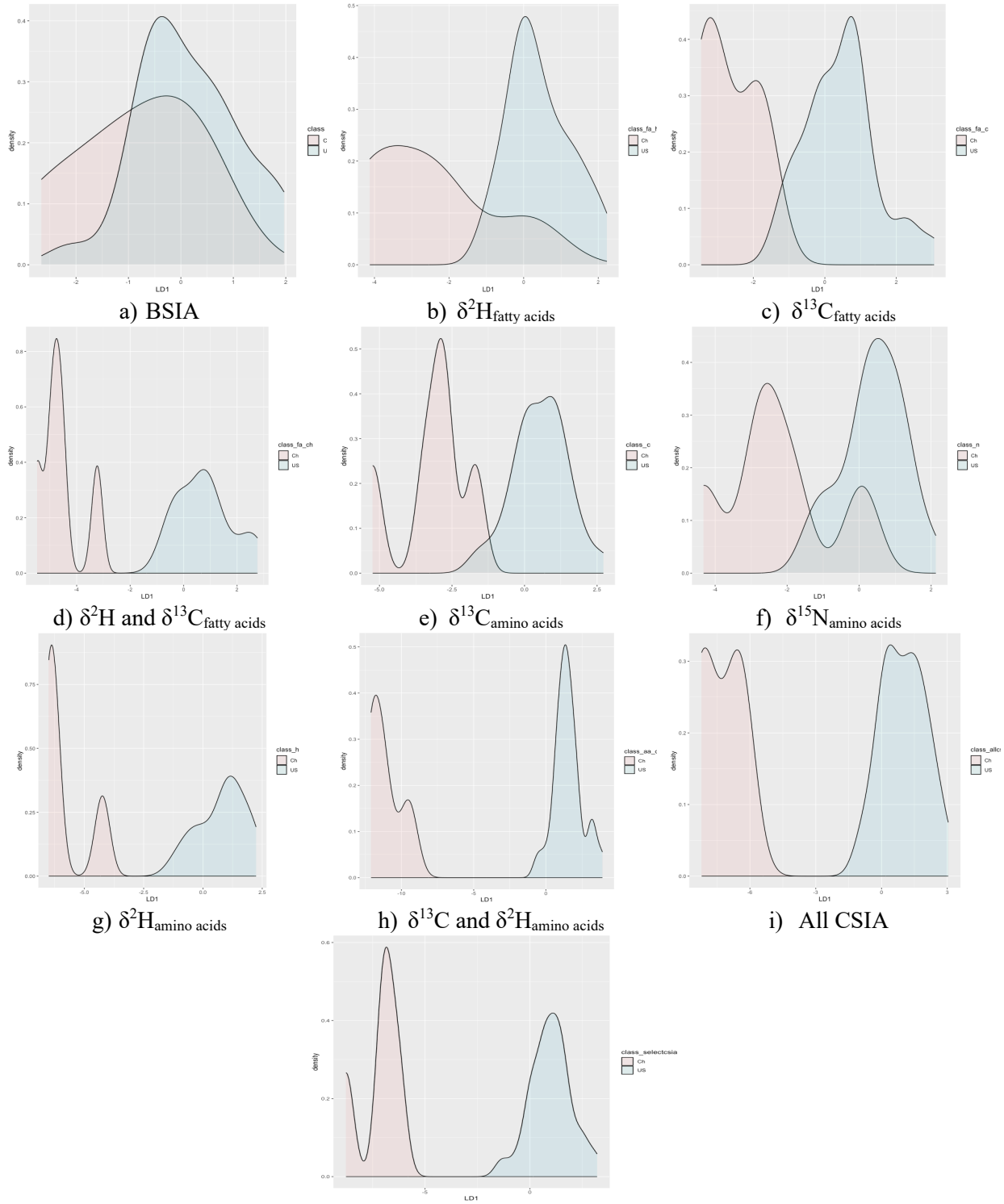


d) ^{15}N of amino acids



e) ^{2}H of amino acids

Figure S4.2. Typical chromatograms of a) ^{2}H of fatty acids, b) ^{13}C of fatty acids, c) ^{13}C of amino acids, d) ^{15}N of amino acids, and e) ^{2}H of amino acids



a) BSIA b) $\delta^2H_{fatty\ acids}$ c) $\delta^{13}C_{fatty\ acids}$

d) δ^2H and $\delta^{13}C_{fatty\ acids}$ e) $\delta^{13}C_{amino\ acids}$ f) $\delta^{15}N_{amino\ acids}$

g) $\delta^2H_{amino\ acids}$ h) $\delta^{13}C$ and $\delta^2H_{amino\ acids}$ i) All CSIA

j) $\delta^2H_{C18:1n\text{-}9}$, $\delta^{13}C_{C18:2n\text{-}6}$, $\delta^{13}C_{C18:3n\text{-}3}$, $\delta^{13}C_{Gly}$, $\delta^{13}C_{Leu}$, $\delta^{13}C_{Val}$, δ^2H_{Glu}, δ^2H_{Ile}, δ^2H_{Leu}, and δ^2H_{Thr}

Figure S4.3. LDA density plots of multiple parameters: a) BSIA, b) $\delta^2H_{fatty\ acids}$, c) $\delta^{13}C_{fatty\ acids}$, d) δ^2H and $\delta^{13}C_{fatty\ acids}$, e) $\delta^{13}C_{amino\ acids}$, f) $\delta^{15}N_{amino\ acids}$, g) $\delta^2H_{amino\ acids}$, h) $\delta^{13}C$ and $\delta^2H_{amino\ acids}$, i) all CSIA, and j) $^2H_{C18:1n\text{-}9}$, $\delta^{13}C_{C18:2n\text{-}6}$, $\delta^{13}C_{C18:3n\text{-}3}$, $\delta^{13}C_{Gly}$, $\delta^{13}C_{Leu}$, $\delta^{13}C_{Val}$, δ^2H_{Glu}, δ^2H_{Ile}, δ^2H_{Leu}, and δ^2H_{Thr} of samples sourced from China and the USA (US)

CHAPTER 5

Comparing Chemical Profiles of Walnuts from Various Provenance

Abstract

Walnuts from various origins were analyzed for their chemical properties to obtain an overview of their quality and to seek potential origin markers. Walnuts were sourced from multiple countries, and the focus was the US and China. The samples were analyzed for the overall quality index (free fatty acidity (FFA), peroxide value (PV), oxidative stability), metabolites (total fat, triacylglycerols, fatty acid composition, volatiles, beta-sitosterol, tocopherols, total phenol, and phenolic compounds), compound-specific stable isotope analysis (CSIA) of amino acids and fatty acids, and NMR profile. Our findings suggested that the US and China samples shared similarities in FFA, PV, total fat, all volatiles (except for 2-butanone and p-xylene), and total phenol. Significant differences were found in oxidative stability, triacylglycerols (TAGs), fatty acid composition, beta-sitosterol, tocopherols, phenolic compounds, $\delta^{13}C_{Gly}$, $\delta^{13}C_{His}$, $\delta^{13}C_{Met}$, $\delta^{2}H_{C16:0}$, $\delta^{2}H_{C18:1w9c}$, $\delta^{2}H_{C18:2w6}$, $\delta^{2}H_{C18:3w3}$, $\delta^{13}C_{C18:1w9c}$, and $\delta^{13}C_{C18:2w6}$. The data demonstrated that the US and China were mostly similar in quality. They can be differentiated with an application of linear discriminant analysis on TAG, volatile, phenolics, and CSIA-fatty acids.

Keywords: walnut, quality, metabolite, authenticity, origin

Introduction

Authentic foods are “free from adulteration, especially with regard to composition, nature and varietal purity, geographical origin, and manufacturing method”(Eurofins Scientific, 2023). Authenticity is essential for the food system because it corresponds to improved consumer perception and experience. Information on the origin of food commodities helps form consumers’ choices because many perceive that origin is linked with quality. A study shows that country or region of origin is among the critical factors that panelists consider when they evaluate foods for purchase(Chousou et al., 2018; Sidali et al., 2021). The origin of walnuts is also linked to some consumer perceptions and walnuts from certain regions are sold at higher prices than those from other places in the same market. However, limited data is showing if the variation in origin is associated with differences in their chemical profiles, particularly those related to quality. Evaluation of their chemical profiles should help provide an overview of their quality. In addition, they also could provide markers for authenticating provenance.

English or Persian walnuts were originally from Central Asia and are now cultivated in various countries in temperate climate zones. China dominates world production, and the US is the second biggest producer. Even though the US produces less walnuts than China, it is the largest walnut exporter in the world, so they can be found in various countries(Agricultural Marketing Resource Center, 2006).

The US walnut production is concentrated in California. California’s walnuts comprised 99% of the country’s total production. The remaining 1% is from some other states like Oregon(Olsen, 2006). On the other hand, walnut-growing regions in China are more widespread. It covers the northwest (e.g., Xinjiang and Shaanxi); southwest (e.g., Yunnan and Sichuan); the lower Yellow River and the Bohai Bay (e.g., Shanxi and Shandong); and the central (e.g., Hubei and Anhui)

areas. Among these regions, Xinjiang and Yunnan are very important as together they contribute to around 55% of the country's production(Chen & Qi, 2022).

An overview of walnuts from multiple countries is provided. To compare the profiles of the two biggest walnut producers, China and the US, the collection year was included in some parameters as a variable because seasonal differences might reflect variations in climate and agricultural practices. In addition, the chemical profiles of walnuts sourced from the two main regions in China (Xinjiang and Yunnan) were also compared to evaluate the similarity of samples from the country.

Various chemical parameters were selected for this study. They comprised overall quality indicators, lipid and phenol metabolites, compound-specific stable isotope analysis (CSIA) of amino acids and fatty acids, and nuclear magnetic resonance (NMR) profile. The overall quality measurement included free fatty acidity (FFA), peroxide value (PV), and oxidative stability. Free fatty acids are produced from the hydrolysis of triacylglycerols, mainly with the activity of enzyme lipase. A higher concentration of free fatty acids is commonly associated with poorer quality. Free fatty acids can be oxidized in the presence of oxygen and lipoxygenases (LOXs)(Stolterfoht et al., 2019), generating hydroperoxides that can exert some toxic effects. Linoleic acid hydroperoxide, for example, can induce toxicity in endothelial cells(Kaneko & Baba, 1999). Hydroperoxides are not stable(Frankel, 1983). They can break down into small compounds that are volatiles, with the activity of hydroperoxide lyases (HPLs). These volatiles, consisting of various compound groups like aldehydes and ketones, are involved in forming the aroma and flavor profiles of walnuts, including rancidity. Intense rancid flavor is typically associated with low quality and disliked by many consumers.

Walnuts contain high concentrations of lipids (±65%), and most of them are triacylglycerols (TAGs). Their composition, including fatty acid position and structure, affects their oxidative stability. TAGs with oleic acid at any position are stable(Neff et al., 1994), while those with linolenic acids at 1st and 3rd position or linoleic acid at the 2nd position are less stable(Neff et al., 1992). TAG profile can vary with walnut varieties(Amaral et al., 2004) and changes in environmental conditions, i.e., drought(Ferreira et al., 2021). The composition of fatty acids, the building blocks of triacylglycerols, is an important parameter that links to the oxidative stability of free fatty acids, TAGs, and foods that contain them. In addition, it also links to health benefits and variations in geographical properties. The unsaturated degree, indicated by the iodine value, correlates positively with the overall oxidative stability index(Kerrihard et al., 2015). Polyunsaturated fatty acids have lower oxidative stability while saturated or monounsaturated fatty acids are more stable. However, walnuts with high concentrations of polyunsaturated fatty acids have more health benefits because their intakes are linked to reduced risks of cardiovascular diseases(Marangoni et al., 2020), diabetes, and DNA damage. Besides indicating oxidative stability and health effects, fatty acid composition is also linked to changes in the geographical properties of the locations where walnuts are cultivated. The concentration of oleic acid, for example, is higher in walnuts from areas with more intense precipitation(Wu et al., 2020).

Walnuts contain minor lipid components like tocopherols and sterols. Tocopherols in walnuts are gamma-, alpha-, delta-, and, less frequently reported, beta-tocopherol. Tocopherols can limit lipid oxidation. Walnut oil that is stripped of tocopherols has more volatile aldehydes than non-stripped walnut oil. Additionally, tocopherols relate to walnuts' nutritional properties. They have been shown to reduce the risk of heart disease, cancer, and Alzheimer’s(Tucker & Townsend,

2005). Sterols in walnuts comprise beta-sitosterol, stigmasterol, campesterol, clesroesterol, Δ^5-avenasterol, Δ^7-stigmasterol, and Δ^7-avenasterol. Among these compounds, beta-sitosterol has the highest concentration (100 – 180 mg/100 g oil)(Amaral et al., 2003). Sterol consumption is associated with lower concentrations of serum TAGs and low-density lipoprotein (LDL)-C(Plat et al., 2015).

Besides lipids, walnuts have high concentrations of phenols. The phenol content in walnuts is higher than that of many other nuts, such as almond, Brazil nut, chasew, hazelnut, macadamia, peanut, pine nut, and pistachio(Bolling et al., 2010). Phenolic compounds in walnuts are mainly of the hydrolyzable tannin group. One of them, ellagic acid, can act as an antiproliferative agent, gut microbiome modulator, and epigenetic effector. It also helps in ameliorating obesity-related complications(Kang et al., 2016). Phenolic compounds in walnuts may act as antioxidants to inhibit quality degradation based on a study using walnut phenolics to improve the stability of walnut oil(Grosso et al., 2018). Geographical conditions may affect the concentrations of phenolic compounds(Amaral et al., 2008) because biotic and abiotic stressors that can affect phenol metabolism in plants vary with location.

The efficacy of compound-specific stable isotope analysis for origin authentication was assessed. Stable isotope ratios, alone or combined with other chemical parameters, have been helpful in discriminating the geographical provenance of walnuts(Athaillah et al., 2023) and a confirmation study on walnuts collected from a different year was needed. Geographical attributes, for instance, distance from coast, altitude, and latitude affect hydrogen isotope fractionation. Greater distance from the nearest coast is correlated with depletion in the hydrogen stable isotope ratio of the precipitation, the so-called ‘continental effect’(Gori et al., 2015). Latitude also influences hydrogen isotopes, with higher values in water around tropical areas(Bowen, 2010). Sources of

water impact hydrogen isotope ratios in plants, with hydrogen isotope ratios of plants receiving groundwater being lower than those plants with primarily precipitation or river water sources(Meng et al., 2023; Bowen, 2010). Nitrogen isotopic variation in plants is influenced by the nitrogen sources available to plants, where plants receiving animal manure have a higher nitrogen isotope ratio than those receiving synthetic fertilizer(Paolini et al., 2015). Carbon isotope fractionation may also vary with nitrogen sources(Paolini et al., 2015). In the current study, compound-specific analysis was preferred over bulk analysis because many previous studies demonstrated that the latter was less effective for discrimination (Athaillah et al., 2023; Paolini et al., 2016).

Materials and Methods

Sampling

Walnuts (0.5– 2 kg) were sourced from eleven countries: the US, China, India, Poland, Moldova, Turkey, Chile, Uzbekistan, Greece, Romania, and Ukraine. Sampling was done in 2020 and 2022. Samples were collected from different manufacturing companies, orchards, or handlers. Mostly, they were received as in-shell or shelled, but one sample was received as in-hull. The samples from the US were all from California except for one sample from Oregon. The Californian samples were sourced from various parts of Central Valley. A sample from China had no information on its growing location while the remaining seven samples were from Xinjiang, Yunnan, or Shaanxi provinces.

All the Californian samples were from Chandler, Howard, or Tulare varieties except for one sample that had no cultivar information. Cultivars of two China samples were known (185 and Yunnan 103), while cultivar information of the other six samples was not available. We also did

not have information on the varieties of the samples from India, Poland, Moldova, Turkey, Chile, Uzbekistan, Greece, Romania, and Ukraine (Table 5.1). The samples were stored at 0-4 °C until analysis. Subsets of samples were selected randomly for analysis.

Table 5.1. Sample codes, countries of origin, state/province of origin, collection year, cultivar, and condition upon received

No	Code	Country	State or Province	Collection year	Cultivar	Condition
1	US-a-1	USA	California	2020	Chandler	In-shell
2	US-a-2	USA	California	2020	Chandler	In-shell
3	US-a-3	USA	California	2020	Chandler	In-shell
4	US-a-4	USA	California	2020	Chandler	In-shell
5	US-a-5	USA	California	2020	Chandler	In-shell
6	US-a-6	USA	California	2020	Chandler	In-shell
7	US-a-7	USA	California	2020	Chandler	In-shell
8	US-a-8	USA	California	2020	Chandler	In-shell
9	US-a-9	USA	California	2020	Chandler	In-shell
10	US-a-10	USA	California	2020	Howard	In-shell
11	US-a-11	USA	California	2020	Howard	In-shell
12	US-a-12	USA	California	2020	Howard	In-shell
13	US-a-13	USA	California	2020	Howard	In-shell
14	US-a-14	USA	California	2020	Howard	In-shell
15	US-a-15	USA	California	2020	Howard	In-shell
16	US-a-16	USA	California	2020	Howard	In-shell
17	US-a-17	USA	California	2020	Howard	In-shell
18	US-a-18	USA	California	2020	Howard	In-shell
19	US-a-19	USA	California	2020	Howard	In-shell
20	US-a-20	USA	California	2020	Tulare	In-shell
21	US-a-21	USA	California	2020	Tulare	In-shell
22	US-a-22	USA	California	2020	Tulare	In-shell

23	US-a-23	USA	California	2020	Tulare	In-shell
24	US-a-24	USA	California	2020	Tulare	In-shell
25	US-a-25	USA	California	2020	Tulare	In-shell
26	US-a-26	USA	California	2020	Tulare	In-shell
27	US-a-27	USA	California	2020	Tulare	In-shell
28	US-a-28	USA	California	2020	Tulare	In-shell
29	US-a-29	USA	California	2020	Tulare	In-shell
30	US-b-1	USA	California	2022	na	In-shell
31	US-b-2	USA	California	2022	Chandler	Shelled
32	US-b-3	USA	California	2022	Chandler	In-shell
33	US-b-4	USA	California	2022	Chandler	In-hull
34	US-b-5	USA	California	2022	Chandler	In-shell
35	US-b-6	USA	California	2022	Chandler	In-shell
36	US-b-7	USA	California	2022	Chandler	In-shell
37	US-b-8	USA	California	2022	Chandler	Shelled
38	US-b-9	USA	California	2022	Chandler	In-shell
39	US-b-10	USA	California	2022	Howard	In-shell
40	US-b-11	USA	California	2022	Howard	Shelled
41	US-b-12	USA	California	2022	Howard	In-shell
42	US-b-13	USA	California	2022	Tulare	In-shell
43	US-b-14	USA	California	2022	Tulare	In-shell
44	US-b-15	USA	California	2022	Tulare	Shelled
45	US-b-16	USA	Oregon	2022	na	Shelled
46	Cn-a-1	China	Xinjiang	2020	na	Shelled
47	Cn-a-2	China	Xinjiang	2020	na	Shelled
48	Cn-a-3	China	Yunnan	2020	na	Shelled
49	Cn-a-4	China	Shaanxi	2020	na	Shelled
50	Cn-a-5	China	na	2020	na	Shelled
51	Cn-b-1	China	Yunnan	2022	Yunnan 103	In-shell
52	Cn-b-2	China	Yunnan	2022	na	In-shell
53	Cn-b-3	China	Xinjiang	2022	185	In-shell

54	In-a-1	India	na	2020	na	Shelled
55	In-b-1	India	na	2022	na	In-shell
56	In-b-2	India	Kashmir	2020	na	Shelled
57	Po-a-1	Poland	na	2020	na	In-shell
58	Po-a-2	Poland	na	2020	na	Shelled
59	Mo-a-1	Moldova	na	2020	na	Shelled
60	Mo-a-2	Moldova	na	2020	na	Shelled
61	Tr-b-1	Turkey	na	2022	na	Shelled
62	Tr-b-2	Turkey	na	2022	na	In-shell
63	Cl-b-1	Chile	na	2022	na	In-shell
64	Cl-b-2	Chile	na	2022	na	In-shell
65	Uz-b-1	Uzbekistan	na	2022	na	Shelled
66	Gr-b-1	Greece	na	2022	na	Shelled
67	Ro-a-1	Romania	na	2020	na	In-shell
68	Uk-a-1	Ukraine	na	2020	na	Shelled

*na: not available

Oil extraction

Oil was extracted following a published method(Athaillah et al., 2023).

Quality analysis

Free fatty acidity (FFA)

Free fatty acid analysis was conducted on 10 g of oil samples following European Union Commission Regulation (EEC) no 2568/91. Three replicates were analyzed for each sample.

Peroxide value (PV)

The peroxide value analysis was performed as illustrated in the AOCS official method Cd 8b-90. Approximately 5 g of oil was used for the analysis, and two or three replicates were analyzed for each sample.

Oxidative stability

Oxidative stability of the kernels (0.5 g) was measured following a published study on walnut(Grilo & Wang, 2021). The analysis was done in triplicates.

Lipid analysis

Total fat content

Fat content was analyzed using a published method(Athaillah & Wang, 2024). The analysis was done in triplicates.

Triacylglycerols (TAG) and beta-sitosterol

Oil samples (±80 mg) were mixed thoroughly with 10 mL of chloroform: methanol (1:1, v/v). One μL of the solution was then injected into a liquid chromatography (LC) system (Agilent 1260 Infinity II, Agilent Technologies Inc., California, USA) connected to tandem mass spectrometry (6470 triple quadrupoles, Agilent Technologies Inc., California, USA). Separation was done on a Thermo Scientific™ Accucore™ C18 column (100 mm x 2.1 mm, 2.6 μm) with acetonitrile: water: ammonium acetate 1 M: formic acid (200:800:10:1, v/v) as the mobile phase A and acetonitrile: isopropanol: ammonium acetate 1 M: formic acid (100: 900: 10:1, v/v) as the mobile phase B. The flow rate was 0.20 mL/min and the column temperature was 55 °C. At the start, the mobile phase B was 35%, then changed to 70% at 3 min, 85% at 7 min, and 90% at 10 min. The 90% proportion was held for 2 min, then increased to 97% at 12.5 min. Later, it was decreased to 35% at 18 min, and that ratio remained constant until 23 min. The mass spectrometry (MS) was operated at MS2 scan mode and positive ionization. The scan mode searched for ions from 100 to 1600 m/z with a scan time of 200 ms and a fragmentor energy of

200 V. The cell accelerator voltage was set at 5 V. Compound identification was done by comparing the retention time and mass/charge (m/z) to those of standards. TAGs were identified using $[M+NH_4]^+$ ions, whereas sterols were identified using $[M+1-H_2O]^+$ ions. TAG quantitation was done by calculating the percentage of the peak area of a TAG compound to the total peak area of TAGs studied. For free sterol, the actual concentration was quantified. The analysis was conducted in triplicates.

Fatty acid profile

Sample preparation for this analysis followed a previous study(Athaillah & Wang, 2024) and the analysis was done following this method(Green & Wang, 2020). Triplicates were used for each sample.

Volatile organic compounds

Walnut kernels (10-15 kernel halves) were ground using a food processor, and 3 ± 0.1 g of them was placed in a 20-ml vial. Volatile organic compounds were detected and quantified using headspace-solid phase microextraction (HS-SPME) connected to a gas chromatography-mass spectrometry (GC-MS), following a published method(Grilo & Wang, 2021). Three replicates were analyzed for each sample.

Tocopherols

The analysis was performed following a previous study(Athaillah & Wang, 2024). Triplicates were used for each sample.

Phenol analysis

Total phenol

Sample preparation for this analysis followed a previous study(Athaillah & Wang, 2024). Total phenol was analyzed using Folin Ciocalteu method(Polari & Wang, 2020) with few modifications. An aliquot of the extract (0.2 mL) was diluted with water to 5 mL. Folin Ciocalteu reagent (0.5 mL) was added, followed by 3 mL of 17.5% (w/v) sodium carbonate. After the addition of 1.5 mL of water, the mixture was mixed and then incubated for 30 min at dark. Later, the absorbance at 725 nm was measured. The concentration of total phenolic compounds was calculated using a calibration curve prepared with gallic acid.

Individual free phenols

Phenol extracts were prepared in the same way used for total phenol assay. The extract was filtered using a nylon filter (45 μm) and 2 μL of it was injected into a liquid chromatography coupled to a tandem mass spectrometry (LC-MS/MS). The LC-MS/MS analysis was done following a previous report(Athaillah & Wang, 2024). This analysis was done in triplicates.

CSIA of amino acids and fatty acids

The sample preparation and analytical procedure were similar to those in a previous study(Athaillah et al., 2023).

Statistical analysis

Statistical analysis was conducted to determine if the parameters varied with origins. For some parameters, i.e., total fat, fatty acid profile, volatiles, total phenol, phenolics, and NMR, the statistical analysis involved the significant difference of varying sample collection years, but for

the other parameters, i.e., FFA, PV, oxidative stability, TAGs, beta-sitosterol, tocopherol, and CSIA-amino acids and fatty acids, the collection year was not considered. An analysis of variance (ANOVA) followed by the Tukey HSD test was conducted. Chemometric linear discriminant analysis (LDA) was used to reduce the dimensionality of some complex data, e.g., triacylglycerols and phenols. The statistical analysis was conducted using R Studio 2022.07.01 (Posit software, PBC, Boston, MA, USA) and R 4.2.2. (The R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

Overall Quality

Free fatty acidity

FFA values of samples from the US and China were lower than those of other countries: Chile, Greece, India, Turkey, and Uzbekistan. No significant difference between samples from the US and China was shown (p-value 0.946) (Table 5.2). The single Oregon sample, sold in small broken kernel pieces, was an outlier with a considerably high FFA value. Normally, triacylglycerols are confined in oil bodies (oleosomes) inside kernel cells(Nikiforidis, 2019). During kernel breaking, it is possible that the oil bodies rupture and expose triacylglycerols to lipase, and this process releases free fatty acids. In addition, kernel breaking also means that the pellicle (kernel skin) integrity is compromised. Normally, this layer helps protect kernels from moisture and oxygen that affect triacylglycerol degradation(Ortiz et al., 2019). Increased moisture content (from 6 to 15% or 6 to 20%) has been linked with more intense lipase activity(Pournik et al., 2019). Therefore, it was expected that the FFA value was high in that sample. Meanwhile, only a small difference was observed between the FFA values of the

Chinese samples from Xinjiang and Yunnan. They were all received as in-shell walnuts. Therefore, minimum moisture was expected to diffuse into the kernels and trigger lipase activity.

High FFA value was also noted in one of the two India samples for a similar reason as noted above because one of them was also sold as halves and pieces. Meanwhile, the relatively high value of the Uzbekistan sample was accompanied by the very dark skin of the kernel. The dark skin may be due to several factors, including genetics, late harvest, and prolonged storage after harvest(Adkison et al., 2021; Grilo & Wang, 2021) and they can lead to higher FFA.

Our findings suggested that the US and China samples had better quality, in regard to lower FFA values, than those of the other countries. This could be greatly affected by better postharvest practices, e.g., storing in-shell walnuts at low temperatures. Walnut shell is quite effective in blocking oxygen and moisture from diffusing into kernels. Moisture can facilitate lipid degradation, demonstrated by lower FFA values of samples stored in-shell than those stored as shelled kernels. Moreover, temperature during storage significantly affects the parameter. Storing samples at 5 °C effectively lowered FFA values, as compared to at 15 and 25 °C. Lastly, harvest time also has some effects on FFA. Walnuts harvested one month later than the control developed more free fatty acids(Adkison et al., 2021).

Peroxide values

The peroxide values (PV) of the China and US samples were not significantly different (Table 5.2). The non-significant difference could be due to the short time between sample harvest and analysis.

The high PV value of the Oregon sample was related to its condition when sold, which was broken pieces; therefore, it had a larger surface area for oxygen exposure that could induce auto-

or enzymatic oxidation of lipids. Multiple reports suggest this(Adkison et al., 2021; Ortiz et al., 2019).

Similar to FFA, low PV values benefit from storing as in-shell walnuts at low temperatures (5 °C). Shells are able to suppress oxygen diffusivity into kernels, while low temperature slows down peroxide formation and is not an optimum condition for lipoxygenase enzymes(Adkison et al., 2021).

Identical to the FFA observation, the samples from the US and China had lower PV values than those of the other countries, whereas the Uzbekistan sample had the highest value. The low values of the US and China might indicate early harvest and proper storage (in-shell and at low temperatures). The opposite might be true for those of other countries.

Oxidative stability

Oxidative stability is expressed as induction time or time at which volatile acids, the tertiary products of oxidation, are generated rapidly. The China samples had the highest average induction time (18.03 hr), followed by the samples from India (16.41 hr), and then Uzbekistan (13.20 hr). The lowest mean of induction time was observed in the sample from Chile (10.92 hr). The samples from the US, Greece, and Turkey had similar average values (12-13 hr).

The induction time of the China samples was significantly higher than those of the US (p-value 0.0001) (Table 5.2). The higher oxidative stability of the samples from China, India, and Uzbekistan coincided with their relatively larger concentration of beta-sitosterol (Figure 5.4).

Table 5.2. FFA (g/100 g), PV (meq O_2/kg), and induction time (hr) of walnuts from the US (US), China (Cn), Chile (Cl), Greece (Gre), India (In), Turkey (Trk), and Uzbekistan (Uzb)*

Country	FFA	PV	Induction time (hr)
US	0.09 ± 0.17a	0.51 ± 0.41a	12.56 ± 1.68b
Cn	0.09 ± 0.02a	0.20 ± 0.15a	18.03 ± 1.51a
Cl	0.27	1.38	10.92
Gre	0.22 ± 0.02	1.55 ± 0.15	12.78 ± 0.12
In	0.28	2.16	16.41
Trk	0.18	1.35	12.89
Uzb	0.30 ± 0.05	3.12 ± 0.32	13.20 ± 1.27

*Significant difference (p-value < 0.05) between the US and China samples for each parameter was marked as different letters.

Lipids

Total fat

The Chinese samples had significantly larger fat content than those from India, while the values of the samples from the USA were not significantly different from those of China or India. There was no significant difference between the US and China samples for the two collection years. The fat content of the US or China samples collected in 2022 was comparable to those collected in 2020.

Some studies suggest that the total fat content of tree nuts is affected by altitude and microclimate. It has a negative correlation with altitude(Zeneli et al., 2005). Walnut orchards in Kashmir, the main walnut cultivation area in India, are located between 1580 – 5236 amsl(Lone et al., 2023). It is difficult to compare the altitude of the walnut-growing regions in China because of the large diversity. However, based on the comparison to Central Valley, CA – USA, where most of the US samples were cultivated and the average elevation of 91 amsl, altitude is

unlikely a dominant contributor to fat content. Other factors like cultivar and microclimate may affect the parameter more. Fat content varies significantly among some varieties of walnuts like Alvand, Chaldoran, Caspian, Persia, Chandler, C-25, and 35t(Goodarzi et al., 2023). Microclimate is shown to have a significant impact on the total fat content of chestnuts with higher light intensity, higher temperature, and lower humidity corresponding to larger fat contents(Wen et al., 2020). A combination of these factors might explain our findings.

The values of the other countries were quite similar to those of the USA and in between those of the averages of India and China samples (Figure 5.1). Overall, the values reported in the current study were similar to those reported previously on walnuts grown in Albania(Zeneli et al., 2005), Portugal(Amaral et al., 2003), and China(Ma et al., 2023). The values were, however, slightly lower than those reported for some Argentinian walnuts (74-79 g/100 g)(Cittadini et al., 2020) and this discrepancy could be due to variations in the cultivar, pre- and post-harvest conditions, and analytical technique. Our values were slightly higher than those of walnuts grown in Kashmir, India (58.3 g/100g)(Amin et al., 2017). The data on the Kashmir-India walnuts confirmed our finding that the samples from that region had relatively lower fat content that could be attributed to genetics and geographical parameters.

Among the China samples, a significant difference was observed between those from Xinjiang and Yunnan. The samples from Yunnan had a considerably higher fat content (Figure 5.1). Higher fat content can be both advantageous and disadvantageous. It is preferred for higher yield for walnut oil production. Walnut oil can be used in foods and supplements. However, it likely develops rancidity earlier than the low-fat walnuts, assuming that the other factors affecting lipid oxidation are similar.

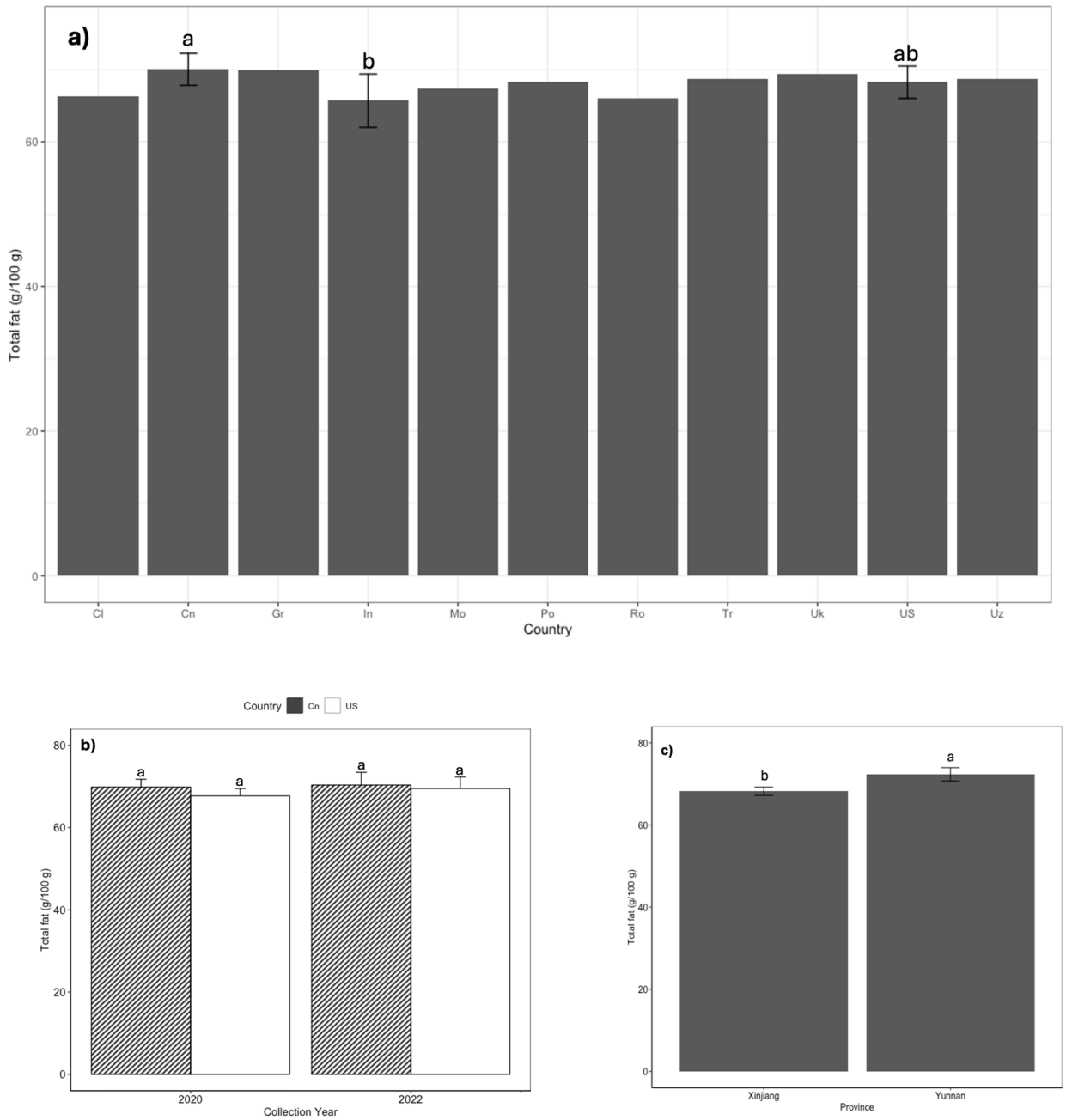


Figure 5.1. The total fat values of the samples from various countries of origin (a); from China and the US collected in 2020 and 2022 (b); or from Xinjiang and Yunnan provinces in China (c)

*Cl: Chile, Cn: China, Gr: Greece, In: India, Mo: Moldova, Po: Poland, Ro: Romania, Tr: Turkey, Uk: Ukraine, US: The United States, and Uz: Uzbekistan

Triacylglycerols

Triacylglycerols (TAGs) accounted as the major sub-class of lipids present in walnuts. Fifteen TAG compounds were analyzed in our current study. Out of the fifteen TAGs, twelve had been identified in the previous studies(Bouabdallah et al., 2014; Wang et al., 2022; Yan et al., 2021) and this was the first time identifying the other three TAGs in walnuts. The newly found TAGs were tricaprylin, 1-palmitin-2-stearin-3-olein, and 1,2-laurin-3-myristin. The relative concentrations of these TAGs were less than 1% (Table 5.3). Tricaprylin contained caprylic (C8:0) acid that is rarely found in walnuts. It has only been once documented, similar to lauric (C12:0) and myristic (C14:0) acids(Yan et al., 2021). Caprylic (or octanoic) acid has nematicidal(Wang et al., 2023) and bactericidal(Venkitanarayanan et al., 2013) activities. Lauric acid is known to be able to exert antimicrobial and antioxidant activities(Hoa et al., 2022). Whereas the myristic acid effect may be less clear. A study found that the concentration was reduced in the leaves of tomato plants after they were infested with root-knot nematodes but did not elaborate on whether it was released into the environment to fight off the infestation(Eloh et al., 2016). Another study suggested an opposite effect of the fatty acid. It was stipulated to act as a chemoattractant and even stimulated the growth of a microbial pathogen *Ralstonia solanacearum* that infested tobacco roots(Li et al., 2017).

TAGs play some crucial roles in plant development and stress adaptation. TAGs provide energy resources, either for seeds before they could become photoautotrophs or during post-germinative growth. Abiotic stress can induce *de novo* TAG synthesis, accumulation, and degradation. TAGs can undergo changes during post-harvest handling and storage. They can degrade into diacylglycerols (DAGs) and free fatty acids. Storing pecan at 90% relative humidity and 25 °C for up to 90 days led to a decrease in triacylglycerols and an increase in diacylglycerols and free

fatty acids(Jia et al., 2023). TAGs also can undergo oxidation, particularly those containing polyunsaturated fats, and the oxidation products include epoxy hydroperoxides, epoxy epidioxides, and mono-epoxides(Zeb, 2012).

The US samples had a similar relative amount of trilinolein and trilinolenin as those of China. However, they had significantly more 1,2-linolein-3-linolenin (p-value 0.0051), 1,2-linolein-3-stearin (p-value 0.025), and tripalmitin (p-value 0.016), but less 1-olein-2,3-linolein (p-value 0.0004), trilaurin (p-value 0.0312), tristearin (p-value 0.0312), and 1,2 laurin-3-miristin (p-value 0.0004) than the Chinese samples. The concentration of linoleic acid-containing TAGs, particularly those with linoleic acids in 1st and 2nd positions, were higher in the samples from the US than those from China. The oxidation rate of TAGs with linoleic acids attached to the 1st and 2nd glycerol carbons tends to be relatively high(Mateos et al., 2005).

Table 5.3. Percentage of peak area of triacylglycerols in walnuts from the US (US), China (Cn), Chile (Cl), Greece (Gre), India (In), Turkey (Trk), and Uzbekistan (Uzb)*

TAG	US	Cn	Cl	Gre	In	Trk	Uzb
Trilaurin	0.01 ± 0.00b	0.02 ± 0.00a	0.02	0.02	0.02	0.02	0.02
1-Olein-2,3-linolein	15.17 ± 0.73b	17.76 ± 1.87a	16.06	16.51	16.04	15.96	16.45
Tricaprylin	0.01 ± 0.00a	0.01 ± 0.00a	0.01	0.01	0.01	0.01	0.01
Triolein	8.59 ± 0.69a	9.20 ± 0.80a	7.86	7.65	7.69	8.33	9.93
Tristearin	0.01 ± 0.00b	0.02 ± 0.00a	0.01	0.02	0.02	0.02	0.01
Tripalmitin	0.01 ± 0.00a	0.01 ± 0.00b	0.01	0.01	0.01	0.01	0.01
Trilinolein	29.11 ± 0.87a	28.95 ± 0.68a	28.91	29.77	30.13	28.23	30.06
1,2-Linolein-3-linolenin	24.88 ± 0.64a	22.98 ± 2.10b	25.70	25.09	25.66	24.73	23.02
1,2-Palmitin-3-linolein	0.53 ± 0.08a	0.60 ± 0.12a	0.56	0.65	0.48	0.62	0.44
1,2-Olein-3-stearin	3.38 ± 0.56a	4.13 ± 0.79a	2.89	2.29	3.23	4.01	4.19
1-Palmitin-2-olein-3-stearin	0.03 ± 0.00a	0.04 ± 0.00a	0.04	0.03	0.03	0.04	0.03
1-Palmitin-2-stearin-3-olein	0.72 ± 0.14a	0.74 ± 0.02a	0.60	0.81	0.54	0.83	0.66

1,2-Laurin-3-myristin	0.01 ± 0.00b	0.02 ± 0.00a	0.01	0.02	0.01	0.01	0.01
1,2-Linolein-3-stearin	14.21 ± 0.74a	13.04 ± 0.53b	13.12	13.41	11.88	13.51	12.68
Trilinolenin	3.32 ± 0.57a	2.48 ± 1.11a	4.21	3.71	4.27	3.69	2.48

*Significant difference (p-value < 0.05) between the US and China samples for each parameter was marked as difference letters.

Linear discriminant analysis (LDA) analysis could distinguish samples from the USA and China, shown as a complete separation of their peaks in the LDA density plot (Figure 5.2). Only one linear discriminant (LD 1) was observed because only two countries were used for the statistical analysis. LD 1 was dominated by 1,2-laurin-3-myristin, 1-palmitin-2-olein-3-stearin, and tristearin (Figure 5.2).

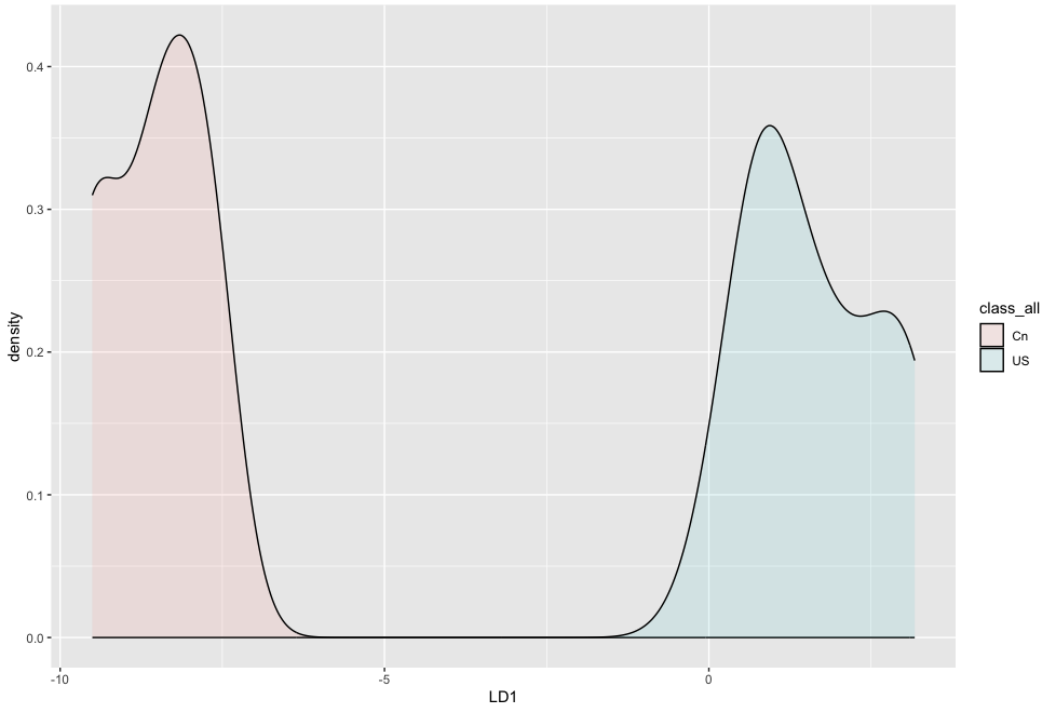


Figure 5.2. LDA density plot of triacylglycerols in walnuts from the US (US) and China (Cn)

Fatty acid profile

The fatty acid profile of walnut samples from various origins has been documented. Significant difference (p-value < 0.05) between the samples from the US, China, and India was observed in palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids but not in palmitic (16:0), margaric (17:0), arachidic (20:0) and gondoic (20:1) acids. The palmitoleic acid content of the China samples was significantly higher than the US and India samples. The US samples had higher stearic acid than those from India, but the values were similar to those from China. The oleic acid concentration in the US samples was significantly lower than those of China and India and also lower than the samples from all the other countries investigated in this study: Poland, Moldova, Turkey, Chile, Uzbekistan, Greece, Romania, and Ukraine. For linoleic acid, the US samples had significantly higher values than those of India but similar to those from China. Whereas linolenic acid in the China sample was significantly lower than those of the US and India (Table 5.4).

The higher content of oleic acid, a monounsaturated fatty acid (MUFA), in the China and India samples might be associated with a higher degree of precipitation during the growing season, as shown in a previous study comparing multiple growing regions of walnuts in China. The concentrations of polyunsaturated fatty acids (PUFA) did not vary as much as MUFA across the growing regions with varying climates, particularly precipitation and temperature(Wu et al., 2020). However, another study suggested that linoleic acid concentration had a medium to strong positive correlation with average relative humidity, minimum relative humidity, minimum temperature, and precipitation. It had a medium to strong negative correlation with sunshine duration. Linolenic acid concentration had a similar correlation to the parameters, except that it did not correlate with precipitation and sunshine duration(Yang et al., 2022).

Table 5.4. Percentages of peak areas of fatty acids of the samples from various countries of origin; from China and the US collected in 2020 and 2022; or from two provinces in China: Xinjiang and Yunnan

Country	CY	Pro-vince	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1
Country comparison (focus on US, Cn, In)											
US	-	-	6.59 ± 0.52a	0.06 ± 0.01b	0.04 ± 0.01a	2.62 ± 0.26a	13.79 ± 1.04b	62.35 ± 1.32a	14.35 ± 1.50a	0.07 ± 0.02a	0.13 ± 0.07a
Cn	-	-	6.79 ± 0.51a	0.11 ± 0.07a	0.04 ± 0.01a	2.46 ± 0.36ab	17.91 ± 2.95a	61.31 ± 2.66ab	11.23 ± 2.68b	0.05 ± 0.02a	0.09 ± 0.04a
In	-	-	5.95 ± 0.10a	0.06 ± 0.01b	0.04 ± 0.00a	2.18 ± 0.53b	16.92 ± 4.65a	59.62 ± 2.75b	15.01 ± 2.51a	0.06 ± 0.02a	0.16 ± 0.08a
Po	-	-	6.56	0.11	0.04	2.23	18.38	61.58	10.99	0.03	0.07
Mo	-	-	6.51	0.07	0.04	2.30	17.22	62.22	11.50	0.03	0.07
Tr	-	-	6.55	0.07	0.04	2.67	15.18	59.94	15.24	0.09	0.19
Cl	-	-	6.10	0.05	0.04	2.27	14.74	60.19	16.29	0.08	0.22
Uz	-	-	6.62	0.08	0.05	2.64	18.48	60.60	11.28	0.08	0.17
Gr	-	-	5.83	0.06	0.04	2.19	15.16	60.84	15.58	0.08	0.21
Ro	-	-	6.53	0.08	0.04	2.15	21.43	59.30	10.36	0.03	0.08
Uk	-	-	6.57	0.08	0.04	2.43	21.39	58.33	11.05	0.03	0.08
Comparison of country (US and Cn) and collection years											
US	2020	-	6.67 ± 0.48a	0.06 ± 0.01b	0.04 ± 0.00a	2.58 ± 0.24ab	13.54 ± 0.90b	62.71 ± 1.17a	14.26 ± 1.64a	0.05 ± 0.01b	0.08 ± 0.01c
US	2022	-	6.44 ± 0.58a	0.06 ± 0.01b	0.03 ± 0.02a	2.70 ± 0.28a	14.28 ± 1.15b	61.64 ± 1.35a	14.52 ± 1.21a	0.09 ± 0.01a	0.23 ± 0.01a
Cn	2020	-	6.54 ± 0.30a	0.08 ± 0.03b	0.04 ± 0.00a	2.59 ± 0.38ab	18.08 ± 3.19a	60.96 ± 3.42a	11.60 ± 2.61b	0.03 ± 0.01c	0.07 ± 0.00c
Cn	2022	-	7.20 ± 0.59a	0.16 ± 0.09a	0.04 ± 0.01a	2.25 ± 0.23b	17.64 ± 3.16a	61.89 ± 0.80a	10.61 ± 3.25b	0.08 ± 0.01a	0.14 ± 0.03b
Comparison of two provinces in Cn											
Cn	-	Xin-jiang	6.75 ± 0.07a	0.07 ± 0.01b	0.04 ± 0.01a	2.49 ± 0.19a	16.96 ± 4.58a	59.41 ± 3.73a	14.12 ± 0.68a	0.06 ± 0.03a	0.10 ± 0.06a
Cn	-	Yun-nan	7.15 ± 0.64a	0.18 ± 0.06a	0.04 ± 0.01a	2.20 ± 0.17a	19.67 ± 0.68a	61.78 ± 0.77a	8.83 ± 0.96b	0.06 ± 0.03a	0.10 ± 0.04a

*Cl: Chile, Cn: China, Gr: Greece, In: India, Mo: Moldova, Po: Poland, Ro: Romania, Tr: Turkey, Uk: Ukraine, US: The United States, and Uz: Uzbekistan

Focusing on the comparison between the samples from the US and China collected in 2020 and 2022, some significant difference was found. Lower concentrations of oleic acid in the samples from the US than those from China were consistent, as it occurred in 2020 and 2022. Higher concentrations of linolenic acid in the US samples, compared to those of the China samples, were also consistent in the two collection years. Oleic acid concentration might correspond to the climatic properties of the growing regions as demonstrated in a previous study on walnuts(Wu et al., 2020). Variation in linolenic acid concentrations of iron walnuts and a hybrid (iron walnut x common walnut) was observed between two cultivation sites (Jiulong and Leibo in China), and it may be influenced by altitude and minimum relative humidity(Yang et al., 2022).

Among the Chinese samples, a significant difference was found in palmitoleic and linolenic acids. The Xinjiang samples had a lower concentration of palmitoleic acid and a higher concentration of linolenic acid. The latter trend was also demonstrated in an earlier study comparing the chemical profiles of walnuts grown in southwest, northwest, and northern China, at which linolenic acid concentration was higher in the sample from northwest China, including Xinjiang region(Liu et al., 2023) and it could have resulted from variation in genetics and geographical attributes(Yang et al., 2022).

Volatiles

Volatiles in walnuts were dominated by aldehydes, ketones, alcohols, esters, and carboxylic acid which is consistent with previous reports on raw walnuts(Pei et al., 2023). Some significant difference in volatile profile was observed among the countries of origin. Concentrations of hexanal; 2-butenal, 3-methyl; 2-heptenal (E); 2-pentenal (E); 2,4-heptadienal; 2-heptanone; 1-octen-3-ol; 1-penten-3-ol; and furan-2-ethyl were significantly higher in the samples from India than those from the US and China. China samples had higher concentrations of 2-butanone and

p-xylene than those from the US. They also had higher concentrations of 1-penten-3-one and ethylbenzene than the US samples, but the concentrations were similar to those from India (Table 5.5). The fact that the India samples had larger concentrations of many volatiles than the China and the US samples indicated that they might have been stored at higher temperatures or for longer time than the samples from the other countries(Jensen et al., 2001). The higher volatile concentration in the India sample was accompanied by lower linoleic acid concentration (Table 5.4), which is likely because it had been depleted due to oxidation reactions. In fact, many volatile compounds, namely hexanal, heptenal and 1-octen-3-ol, are derived from linoleic acid(Cossignani et al., 2014; Wurzenberger & Grosch, 1984). Hexanal is a common indicator of rancidity, and it has a moderate positive correlation with free fatty acid and peroxide values(Sharma et al., 2022). A study compared the effect of temperature (21 and 5 °C) and light exposure (light vs dark) on hexanal development during storage of walnuts. The largest increase in hexanal concentration during storage was noted when walnuts were stored at 21 °C with exposure to light, and the smallest increase was found on walnuts stored at 5 °C in the dark(Jensen et al., 2001).

There was no variation between the samples from the US and China that was consistent in the two collection years (2020 and 2022), except for 2-butanone the concentration was higher in the China samples. A larger concentration of 2-butanone in the China samples was linked with a lower content of linolenic acid (Table 5.4), which is likely the substrate for 2-butanone synthesis(Gómez-Cortés & Camiña, 2019). 2-Butanone was also detected in pecans and shelled pecans have higher concentrations of this compound, compared to unshelled pecans(Thewes et al., 2021). This coincided with the larger proportions of unshelled samples in walnuts from China than those from the US.

Even though the US samples sourced in 2022 had higher concentrations of propanal, nonanal, 2,4-heptadienal, and acetone than the China samples sourced in the same year, the trend was not shown in samples collected in 2020. The higher concentrations of these compounds were likely linked with extreme heat occurring during the maturation period of most of the US walnuts sourced in 2022(Athaillah & Wang, 2024).

Comparing the US samples collected from 2020 and 2022, propanal, pentanal, nonanal, 2,4-heptadienal, acetone, 2-butanone, 5-hepten-2-one-6-methyl, and 3-buten-2-ol-methyl were found at higher concentrations in 2022. Less variation was noted between the China samples collected in 2020 and 2022 (Table 5.6). The larger variation between collection years in the samples from the US might be related to a larger proportion of walnuts with dark skin in 2022. Dark skin walnuts are associated with the degradation of some metabolites, resulting in more volatiles that are associated with the lipoxygenase pathway(Athaillah & Wang, 2024; Lee et al., 2011).

Different regions within the same country were associated with only few changes in volatile compounds. The Xinjiang samples had higher concentrations of 3,5-octadien-2-one and 3-(1-methyl ethyl) cyclohexene and lower concentrations of furan, 2-ethyl, than the samples from Yunnan. The other volatiles had similar concentrations between the samples from Xinjiang and Yunnan (Table 5.6). The high similarity between the two locations might correspond to identical genetics, agricultural practices, and postharvest handling.

Table 5.5. Peak areas of volatiles of the samples from various countries of origin

Compound	US	Cn	In	Po	Mo	Tr	Cl	Uz	Gr	Ro	Uk
1	624.14 ± 940.41a	71.37 ± 59.51a	15.25 ± 4.69a	19.33	14.09	92.33	21.94	87.9	424.1	0.76	73.09
2	11.09 ± 14.76a	1.66 ± 0.67a	23.79 ± 10.62a	141.67	232.31	44.08	76.1	284.39	400.82	3.96	304.20
3	159.87 ± 189.58b	21.75 ± 20.28b	430.89 ± 92.34a	2968.53	4247.38	897.29	1715.49	6569.3	13957.72	40.29	5201.84
4	0.58 ± 0.56a	0.19 ± 0.11a	1.05 ± 0.81a	11.12	16.23	2.69	8.56	9.64	43.14	0.57	19.47
5	1.16 ± 1.23a	0.80 ± 0.52a	1.63 ± 0.44a	2.73	4.11	2.66	3.15	3.71	11.05	0.18	6.10
6	7.44 ± 12.84a	2.17 ± 3.41a	3.26 ± 4.36a	14.78	23.49	36.18	66.11	14.13	52.06	6.27	13.28
7	0.86 ± 0.97ab	0.29 ± 0.15b	2.31 ± 3.14a	0.58	0.70	1.36	133.73	0.24	0.85	0.08	0.27
8	1.50 ± 1.83a	1.70 ± 2.07a	2.06 ± 0.62a	12.56	15.72	4.61	121.6	33.53	93.03	1.05	22.99
9	1.41 ± 1.04b	0.60 ± 0.52b	3.69 ± 1.02a	33.48	27.83	10.51	10.93	94.14	371.86	0.96	43.5
10	0.39 ± 0.44b	0.12 ± 0.08b	1.09 ± 0.73a	2.38	3.41	2.46	1.47	15.43	50.26	0.04	6.38
11	0.29 ± 0.25b	0.13 ± 0.07b	0.86 ± 0.33a	1.40	1.32	1.48	1.01	6.35	36.11	0.10	3.11
12	634.23 ± 932.13a	72.49 ± 57.48a	36.42 ± 19.37a	17.67	21.34	241.41	49.04	207.76	319.57	9.56	23.83
13	2.25 ± 1.87b	1.08 ± 0.40b	5.71 ± 2.64a	47.04	106.28	32.51	53.58	57.62	135.36	0.93	63.23
14	25.39 ± 21.49b	109.45 ± 103.97a	22.59 ± 13.75b	33.58	7.63	112.44	87.09	16.78	60.63	3.55	8.11
15	1.82 ± 1.50b	3.42 ± 2.45a	2.69 ± 0.78ab	4.56	3.04	6.73	1.2	20.8	86.04	0.22	10.76
16	0.95 ± 0.54a	1.07 ± 0.77a	1.43 ± 0.35a	1.20	2.26	5.56	2.89	2.81	2.23	0.32	2.14
17	1.49 ± 1.85a	0.81 ± 0.40a	2.64 ± 1.14a	5.46	3.88	8.46	7.83	38.57	20.38	0.76	8.39
18	7.99 ± 13.49a	2.72 ± 2.01a	1.53 ± 0.46a	3.00	5.59	5.95	21.00	0.69	73.98	1.58	12.04
19	3.85 ± 3.54b	2.60 ± 1.68b	18.41 ± 13.87a	69.12	171.55	31.87	60.14	231.41	867.78	0.67	122.19
20	23.21 ± 27.67b	8.65 ± 5.14b	84.84 ± 59.08a	118.44	205.95	74.41	99.69	333.78	681.36	5.56	243.41
21	32.27 ± 17.49a	47.62 ± 13.86a	39.28 ± 4.33a	15.88	23.07	57.94	29.14	54.69	4.38	46.37	5.49
22	309.74 ± 355.92a	240.95 ± 313.10a	61.08 ± 48.14a	8.10	46.86	1157.86	91.99	267.58	362.51	278.38	8.44

23	7.25 ± 10.83a	2.45 ± 3.82a	12.26 ± 7.66a	172.11	324.86	25.75	123.71	466.68	842.64	1.25	263.69
24	601.85 ± 752.68a	271.16 ± 387.53a	86.10 ± 125.05a	363.67	645.88	120.17	64.99	20.55	18.77	327.63	2807.97
25	4.01 ± 3.80b	7.03 ± 6.49b	18.65 ± 11.61a	40.7	44.48	14.17	44.67	91.13	79.93	1.27	49.89
26	6.57 ± 11.66b	28.55 ± 46.89a	32.83 ± 28.81ab	6.68	16.57	813.57	127.27	10.35	9.2	4.89	10.35
27	2.49 ± 5.16b	26.60 ± 54.48a	13.67 ± 10.89ab	4.39	11.13	398.79	57.35	3.74	6.1	5.91	7.89
28	0.19 ± 0.35a	0.09 ± 0.09a	0.64 ± 0.58a	2.09	0.93	0.55	2.48	7.89	2.29	0.13	1.28

*1: propanal, 2: pentanal, 3: hexanal, 4: octanal, 5: nonanal, 6: 2-butenal, 7: 2-butenal-3-methyl, 8: 2-hexenal (E), 9: 2-Heptenal, (E)-, 10: 2-Pentenal, (E)-, 11: 2,4-Heptadienal, (E,E)-, 12: Acetone, 13: 2-Heptanone, 14: 2-Butanone, 15: 1-Penten-3-one, 16: 5-Hepten-2-one, 6-methyl-, 17: 5-Hepten-2-one, 6-methyl-, 18: 3-Buten-2-ol-2-methyl, 19: 1-Octen-3-ol, 20: 1-Penten-3-ol, 21: 2-Pentanol-4-methyl, 22: Isopropyl alcohol, 23: Pentanoic acid, 24: Ethyl acetate, 25: Furan,2-ethyl, 26: Ethylbenzene, 27: p-Xylene, 28: Cyclohexene, 3-(1-methylethyl)-

**Cl: Chile, Cn: China, Gr: Greece, In: India, Mo: Moldova, Po: Poland, Ro: Romania, Tr: Turkey, Uk: Ukraine, US: The United States, and Uz: Uzbekista

Table 5.6. The peak areas of volatiles of the samples from China and the US collected in 2020 and 2022 or from two provinces in China: Xinjiang and Yunnan

Compound	Comparison between China and the US collected in 2020 and 2022				Comparison of two provinces in Cn	
	US		Cn		Xinjiang	Yunnan
	2020	2022	2020	2022		
1	74.83 ± 90.32b	1703.88 ± 940.17a	30.73 ± 19.51b	139.10 ± 24.97b	76.26 ± 46.90a	103.07 ± 74.78a
2	15.20 ± 16.95a	3.01 ± 2.16b	1.62 ± 0.64ab	1.74 ± 0.85ab	1.67 ± 0.74a	1.87 ± 0.89a
3	189.71 ± 221.90a	85.61 ± 48.08a	20.23 ± 15.68a	24.29 ± 30.53a	37.96 ± 25.21a	6.72 ± 0.11a
4	0.48 ± 0.30a	0.71 ± 0.81a	0.19 ± 0.13a	0.18 ± 0.08a	0.21 ± 0.10a	0.10 ± 0.06a
5	0.63 ± 0.34b	2.09 ± 1.69a	0.48 ± 0.32b	1.34 ± 0.20ab	0.70 ± 0.41a	1.01 ± 0.78a
6	6.07 ± 9.91a	10.50 ± 17.45a	1.28 ± 1.11a	3.64 ± 5.75a	0.56 ± 0.31a	3.99 ± 5.46a
7	0.78 ± 0.59a	1.02 ± 1.49a	0.23 ± 0.03a	0.39 ± 0.24a	0.19 ± 0.05a	0.41 ± 0.20a

8	1.46 ± 1.14a	1.60 ± 2.81a	1.20 ± 0.88a	2.54 ± 3.44a	0.90 ± 0.67a	2.98 ± 3.18a
9	1.34 ± 1.07a	1.39 ± 0.88a	0.57 ± 0.23a	0.66 ± 0.92a	0.99 ± 0.68a	0.23 ± 0.18a
10	0.46 ± 0.43a	0.28 ± 0.46a	0.12 ± 0.07a	0.13 ± 0.12a	0.11 ± 0.04a	0.17 ± 0.11a
11	0.18 ± 0.15b	0.51 ± 0.26a	0.34 ± 0.24b	0.18 ± 0.10b	0.14 ± 0.05a	0.15 ± 0.09a
12	88.36 ± 86.55b	1708.13 ± 925.94a	33.30 ± 17.44b	137.80 ± 26.83b	76.24 ± 45.63a	103.51 ± 72.72a
13	2.17 ± 1.88a	2.03 ± 1.33a	0.94 ± 0.45a	1.31 ± 0.11a	1.14 ± 0.58a	1.13 ± 0.20a
14	15.18 ± 9.16c	45.66 ± 24.96b	56.34 ± 47.24b	197.98 ± 120.67a	133.25 ± 172.69a	113.12 ± 45.74a
15	1.64 ± 1.43b	2.13 ± 1.67b	2.42 ± 2.47ab	5.09 ± 1.47a	2.91 ± 3.35a	4.89 ± 1.13a
16	0.70 ± 0.29b	1.40 ± 0.60a	0.90 ± 0.56ab	0.82 ± 0.34ab	1.81 ± 0.83a	0.53 ± 0.08a
17	1.41 ± 0.97a	1.62 ± 2.87a	1.52 ± 1.21a	0.92 ± 0.85a	1.11 ± 0.26a	0.49 ± 0.19b
18	2.78 ± 1.53b	17.88 ± 20.15a	2.66 ± 2.51ab	2.83 ± 1.27ab	2.48 ± 1.53a	1.87 ± 0.53a
19	3.60 ± 3.29a	3.47 ± 2.25a	3.13 ± 1.65a	1.71 ± 1.57a	3.21 ± 0.78a	2.42 ± 2.80a
20	29.96 ± 31.79a	8.49 ± 3.66a	9.54 ± 6.36a	7.18 ± 2.53a	13.18 ± 5.10a	5.16 ± 0.96a
21	36.92 ± 19.35ab	22.69 ± 8.02b	48.20 ± 17.89a	46.65 ± 5.49ab	54.12 ± 2.51a	39.39 ± 9.21a
22	358.02 ± 285.92a	235.34 ± 467.02a	167.00 ± 152.94a	364.20 ± 509.78a	162.69 ± 136.53a	354.25 ± 517.41a
23	9.60 ± 12.45a	2.43 ± 4.59a	0.82 ± 0.20a	5.17 ± 5.76a	4.42 ± 6.23a	1.55 ± 1.62a
24	788.94 ± 808.31a	278.32 ± 515.50a	253.57 ± 417.25a	300.47 ± 418.79a	281.82 ± 434.51a	95.21 ± 66.62a
25	4.58 ± 4.30b	2.96 ± 2.47b	3.05 ± 2.96ab	2.48 ± 1.17a	2.48 ± 0.54b	14.54 ± 3.42a
26	3.53 ± 3.30b	12.09 ± 18.12b	3.58 ± 2.01b	6.30 ± 5.72a	54.01 ± 75.99a	19.50 ± 14.81a
27	1.92 ± 1.54b	3.52 ± 8.48b	1.37 ± 0.81b	2.65 ± 3.23a	59.57 ± 87.75a	9.48 ± 7.43a
28	0.20 ± 0.17a	0.17 ± 0.55a	0.18 ± 0.25a	0.10 ± 0.13a	0.11 ± 0.00a	0.03 ± 0.02b

*1: propanal, 2: pentanal, 3: hexanal, 4: octanal, 5: nonanal, 6: 2-butenal, 7: 2-butenal-3-methyl, 8: 2-hexenal (E), 9: 2-Heptenal, (E)-, 10: 2-Pentenal, (E)-, 11: 2,4-Heptadienal, (E,E)-, 12: Acetone, 13: 2-Heptanone, 14: 2-Butanone, 15: 1-Penten-3-one, 16: 5-Hepten-2-one, 6-methyl-, 17: 5-Hepten-2-one, 6-methyl-, 18: 3-Buten-2-ol-2-methyl, 19: 1-Octen-3-ol, 20: 1-Penten-3-ol, 21: 2-Pentanol-4-methyl, 22: Isopropyl alcohol, 23: Pentanoic acid, 24: Ethyl acetate, 25: Furan,2-ethyl, 26: Ethylbenzene, 27: p-Xylene, 28: Cyclohexene, 3-(1-methylethyl)-

**Cl: Chile, Cn: China, Gr: Greece, In: India, Mo: Moldova, Po: Poland, Ro: Romania, Tr: Turkey, Uk: Ukraine, US: The United States, and Uz: Uzbekistan

Our data showed that the US, India, and China samples were distinct regarding their volatiles, based on the separation of their ellipses in the LDA plot (Figure 5.3). In the LDA plot, the India samples were located far from both the US and China samples, but the US and China ellipses were close. It was likely that the China and the US samples were more similar in their postharvest handling (e.g., storage condition and period and kernel integrity) than the India samples.

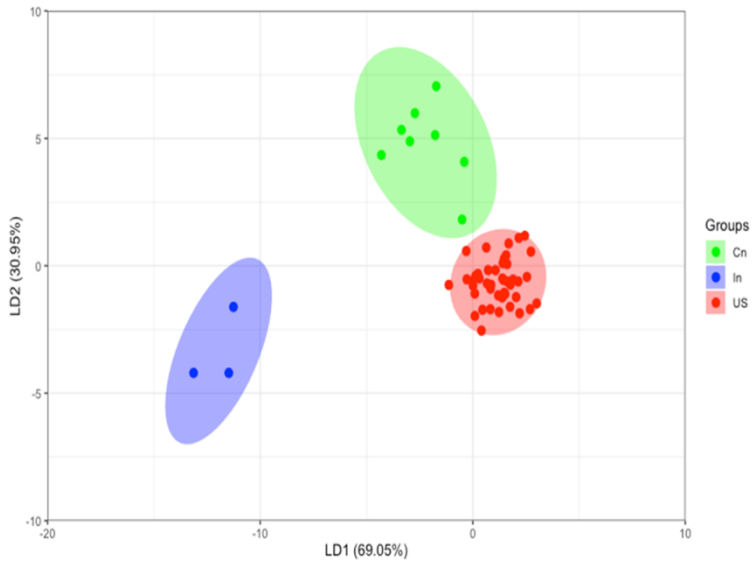


Figure 5.3. LDA plots of the volatile profile of walnuts from the USA (US), China (Cn), and India (In)

Beta-sitosterol

Beta-sitosterol is the most prevalent sterol in walnuts(Crews et al., 2005). The values varied across the countries. The lowest value was in the Turkey sample, followed by that from Greece. The highest was found in China, followed by India and Uzbekistan. The values of samples from the US and Chile were similar and in the middle of the range (Figure 5.4). Another study also

determined the quantity of beta-sitosterol in walnuts from various countries. The maximum value (252.0 mg/100 g), which exceeds the values in the current study, was found in a sample from France, while the minimum value was found in some US samples(Crews et al., 2005). The value of the US sample in that report was lower than what we found in the current study, probably due to different cultivars, sample collection area, analytical procedure, environmental factors, and sample storage and handling. Beta-sitosterol concentrations of our samples were in the range of those previously reported on walnuts grown in Portugal(Amaral et al., 2003), Tunisia(Abdallah et al., 2015), and Italy(Verardo et al., 2013). The value of the Chandler variety of our California samples was similar to that of Chandler grown in Italy(Verardo et al., 2013).

The China samples had a higher concentration of beta-sitosterol than the US samples, which coincided with its higher oxidative stability, as determined by the Rancimat test that uses a temperature of 110 °C to accelerate oxidation reactions. The sterol may still be effective at high temperatures, based on a heat-stability study showing that only a minor reduction of beta-sitosterol concentration is observed after heating(Kmiecik et al., 2011). A study shows that beta-sitosterol can inhibit methyl linoleate oxidation(Yoshida & Niki, 2003).

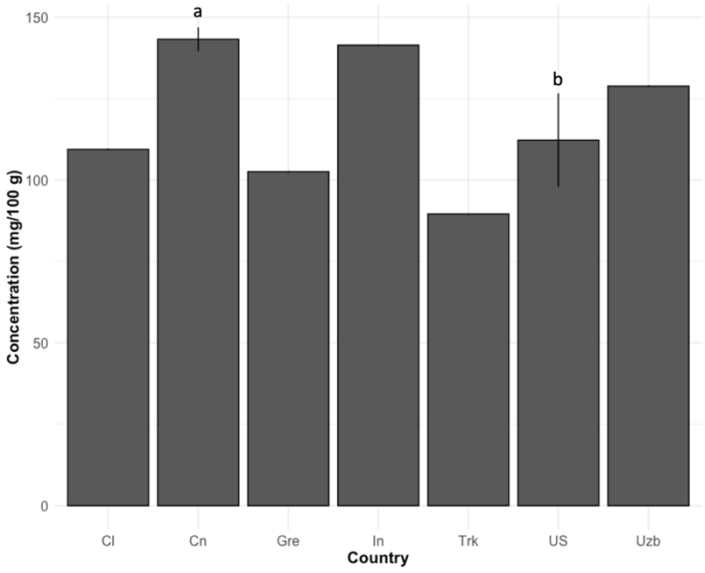


Figure 5.4. Concentration of beta sitosterol (mg/100 g oil) in walnuts from the US (US), China (Cn), Chile (Cl), Greece (Gre), India (In), Turkey (Trk), and Uzbekistan (Uzb)*

*Significant difference (p-value < 0.05) between the US and China samples was marked as difference letters.

Tocopherols

Tocopherols in our samples were similar to those previously reported in walnuts(Yang et al., 2022). Some variations in the concentrations of tocopherols were demonstrated. Delta-tocopherol of the US samples was lower than that of the China samples. Alpha-tocopherol of the US samples was significantly higher than those of China. No significant difference was observed for gamma- and total tocopherol values between the US and China. Compared with the samples from the other countries, the China samples had the highest average concentration of delta-tocopherol, except for the sample from Uzbekistan (Table 5.7).

Delta tocopherol concentration had a moderate to strong positive correlation with precipitation level and a moderate to strong negative correlation with sunshine duration(Yang et al., 2022), therefore it was understandable that the concentration in the US samples was less than those of

Yunnan in China, considering that the total annual precipitation in the main walnut growing location (Central Valley in California) in 2020 or 2022 was less than 305 mm while that of Yunnan is around 1200 mm(Gao et al., 2023). However, precipitation alone was not able to explain the higher value of the Xinjiang samples because the average precipitation in Xinjiang is around 200 mm(Zhou et al., 2023). It was likely that the varieties influenced the values as well, as shown in some earlier studies(Wu et al., 2020).

The higher concentration of alpha-tocopherol in the US samples might be associated with preharvest extreme heat occurring a few weeks before their harvest time (September and October 2022). Synthesis of alpha-tocopherol is known to increase during heat stress while concentrations of delta and gamma tocopherols decreased(Chennupati et al., 2011).

Table 5.7. Tocopherol concentrations (mg/kg) of the samples from various countries of origin

Country	Delta	Alpha	Gamma	Total
US	18.51 ± 3.32b	28.91 ± 8.95a	403.56 ± 48.88a	450.98 ± 54.34a
Cn	47.70 ± 18.96a	6.22 ± 2.66b	390.59 ± 46.32a	444.51 ± 25.26a
In	29.43	5.74	491.52	526.69
Tr	22.39	7.68	443.53	473.60
Cl	22.55	4.62	451.67	478.83
Uz	54.56	7.25	305.49	367.30
Gr	21.99	4.49	473.63	500.11

*Cl: Chile, Cn: China, Gr: Greece, In: India, Tr: Turkey, US: The United States, and Uz: Uzbekistan

Phenols

Total Phenols

The Moldova samples had the highest total phenols, followed by the sample from Ukraine; the Greece sample had the lowest value. No significant difference between the samples from the US, China, and India. When comparing the samples from the US and China across the two collection years, no significant difference was found between the two countries and the two collection years. In addition, there was no interaction effect between country and collection year (Figure 5.5). Values of all the samples exceeded previously reported values for walnuts grown in the US but were similar or higher to those grown in Turkey and lower than those grown in Romania(Trandafir & Cosmulescu, 2020). It was important to note that the variation could be because of different extraction techniques employed(Trandafir et al., 2017), besides the nature of the samples.

Different regions within countries were also investigated on its effect on total phenol. There was a significant difference in total phenol between samples from Xinjiang and Yunnan. The Xinjiang samples had a higher total phenol than the Yunnan samples; this trend was opposite to that of total fat. The higher content in the Xinjiang sample was in agreement with a previous study concluding that the total phenol content of walnuts cultivated in northwest China, i.e., Xinjiang, was higher than those grown in southwest China, i.e., Yunnan(Liu et al., 2023). The variation could originate from different varieties, geographical attributes, and pre- and post-harvest parameters. Differences in total phenols among multiple walnut cultivars planted in China have been demonstrated before(Wu et al., 2020). Additionally, harvest time also may affect the values. Walnuts harvested on the 121st day after full bloom had higher total phenol than those picked a week later. However, the values were not significantly different from the same walnut variety harvested on day 135th after full bloom(Wei et al., 2022). Overall, the total phenol contents of dried walnuts decrease upon storage. Storing them at ambient temperature

results in a larger decline in total phenol content than storing them under refrigeration(Ahad et al., 2020).

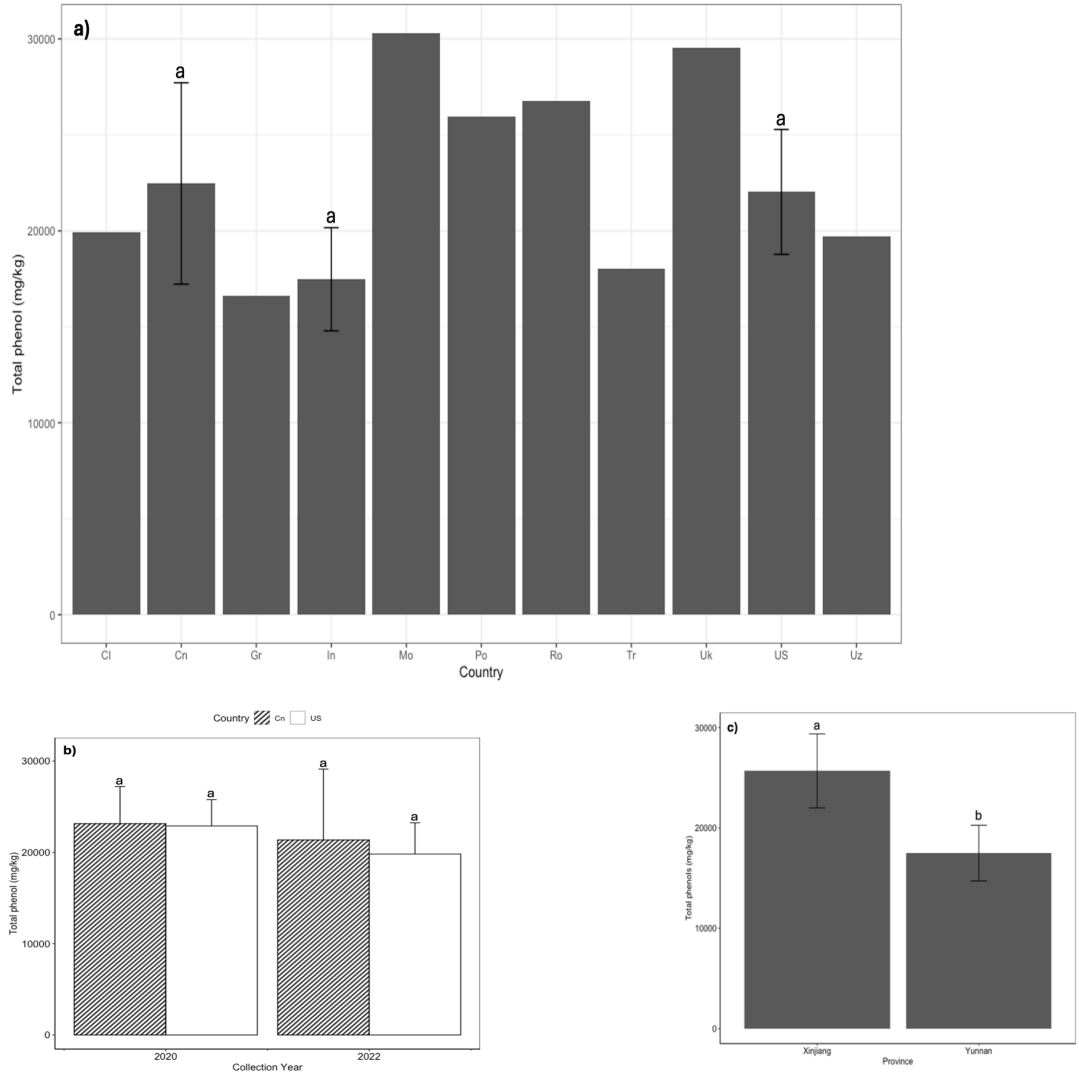


Figure 5.5. The total phenol values of the samples from various countries of origin (a); from China and the US collected in 2020 and 2022 (b); or from Xinjiang and Yunnan provinces in China (c)

*Cl: Chile, Cn: China, Gr: Greece, In: India, Mo: Moldova, Po: Poland, Ro: Romania, Tr: Turkey, Uk: Ukraine, US: The United States, and Uz: Uzbekistan

Individual Free Phenols

Fourteen phenolic compounds were detected and quantified in walnuts (with skin on). Among the US, China, and India samples, there was no significant difference for 3,4 dihydroxybenzoic acid, caffeic acid, epicatechin gallate, p-coumaric acid, sinapic acid, ellagic acid, quercetin, and naringenin concentrations. Concentrations of gallic acid, catechin, and procyanidin B1 in the US samples were higher than those in the samples originating from China and India. For chlorogenic acid and ferulic acid, the concentrations of the China samples were higher than those of the US and India. Whereas vanillic acid concentration was significantly higher in the India sample than in the US and China samples (Table 5.8).

Many of these phenolics possess biological functions, particularly antioxidant capacity, as shown in some *in vitro* antioxidant assays(Apak et al., 2007; Csepregi et al., 2016). Biotic stress can affect their concentrations. Catechin and procyanidin concentrations increased during the infection of some poplar trees by the fungus *Plectosphaerella populi*, indicating their roles in the plant defense system(Ullah et al., 2019). Geographical factors may also influence it. A study on *Medicago minima* showed that those grown in lower semi-arid zones with a cold-steppe climate had more flavonoids than the same species but cultivated in higher arid regions with the same climate(Kabtni et al., 2020). Central Valley in California, where most of the US samples were grown, had a lower altitude than Xinjiang and Yunnan, where most of the China samples were sourced from. Therefore, it agreed with the finding in *Medicago minima*.

Data from the other countries presented some interesting findings. The samples from Moldova had higher concentrations of gallic acid; 3,4-dihydroxybenzoic acid, and ellagic acid, compared to the other countries. The samples from Poland had higher contents of chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, and ferulic acid. The Ukraine sample had higher epicatechin gallate and quercetin. Catechin concentrations of all the other countries were lower than those of

the US samples. For procyanidin B1, the average concentration of the US samples was higher than the other countries, except for Ukraine's which is a comparable concentration to that of the US.

Focusing on a comparison between the US and China samples, we found consistently higher catechin concentrations in the US walnuts, after considering the data from both 2020 and 2022 samples. Some variations in the values of phenolic compounds, i.e., gallic acid, vanillic acid, ferulic acid, sinapic acid, ellagic acid, and naringenin, were noted between the US samples collected in 2020 and 2022, indicating some climatic effects. When comparing samples originating from China, those collected in 2020 differed from those sourced in 2022 in the concentrations of ferulic acid and naringenin. The variations of the samples from the same country but different collection times could be due to genetics (different cultivars or different distribution of samples from varying cultivars), climate, and pre- and post-harvest handling.

These findings suggested that catechin had the potential to be used as a marker to differentiate the US walnuts from those of the other countries. The reason for the high concentration of catechin in the US walnuts could be genetic, rootstock, and agroclimatic conditions, as shown in a study on almonds(Čolić et al., 2021).

The concentrations of catechin, procyanidin B1, and naringenin in the samples from Xinjiang were significantly higher than those of Yunnan, but for sinapic acid, the reverse trend was observed. Similar values were demonstrated for the other phenolic compounds between walnuts from the two regions. The variation was likely due to genetic diversity because a study on Chinese walnuts demonstrated total flavonoids differed among varying cultivars(Wu et al., 2020) and the two regions have varying cultivars that are commonly planted. The difference between the values of some phenolics in the Xinjiang samples (33.54 ± 6.86 mg/kg) to those from the US

(65.00 ± 17.38 mg/kg), was not as large as the difference between the Yunnan samples (6.88 ± 2.46 mg/kg) to the US. The reason could be because Xinjiang and Central Valley were more similar in precipitation levels(Li et al., 2015; NOAA, 2022; Zhou et al., 2023) and lower precipitations lead to accumulation in catechin(Čolić et al., 2021). A higher concentration of catechin and other flavonoids might correspond to more health benefits. However, it might also link to bitterness and astringent taste(Lesschaeve & Noble, 2005).

Table 5.8. Concentrations (mg/kg) of individual free phenol of the samples from various countries of origin; from China and the US collected in 2020 and 2022; or from two provinces in China: Xinjiang and Yunnan

Country	CY	Province	Gallic acid	3,4 Di-hydroxy benzoic acid	Catechin	Chlorogenic acid	Procyanidin B1	Vanillic acid	Caffeic acid	Epi catechin gallate	p-Coumaric acid	Ferulic acid	Sinapic acid	Ellagic acid	Quercetin	Naringenin
Country comparison (focus on US, Cn, In)																
US	-	-	12.78 ± 5.42a	0.08 ± 0.03a	65.00 ± 17.38a	0.92 ± 1.58b	26.07 ± 9.20a	0.61 ± 0.55b	0.06 ± 0.03a	0.58 ± 0.28a	0.12 ± 0.05a	0.16 ± 0.11b	0.29 ± 0.12a	448.03 ± 193.50a	0.45 ± 0.14a	0.26 ± 0.10a
Cn	-	-	6.52 ± 3.58b	0.08 ± 0.05a	22.74 ± 13.77b	4.01 ± 6.95a	9.80 ± 6.50b	0.37 ± 0.50b	0.05 ± 0.03a	0.38 ± 0.28a	0.15 ± 0.09a	0.38 ± 0.24a	0.41 ± 0.21a	405.42 ± 187.17a	0.42 ± 0.19a	0.32 ± 0.30a
In	-	-	10.25 ± 5.62ab	0.09 ± 0.01a	14.78 ± 12.73b	0.22 ± 0.23ab	8.32 ± 1.57b	1.49 ± 1.09a	0.09 ± 0.07a	0.35 ± 0.08a	0.16 ± 0.07a	0.37 ± 0.22ab	0.22 ± 0.11a	434.15 ± 83.60a	0.26 ± 0.08a	0.14 ± 0.14a
Po	-	-	9.06	0.10	11.35	7.67	7.30	3.52	0.16	0.28	0.42	0.84	0.63	349.61	0.52	0.36
Mo	-	-	18.52	0.20	20.15	nd	13.56	nd	0.12	0.65	0.31	0.05	0.36	726.24	0.47	0.54
Tr	-	-	6.08	0.07	30.79	nd	14.77	0.93	0.06	0.36	0.11	0.22	0.27	393.79	0.28	0.16
Cl	-	-	14.27	0.17	15.33	nd	9.45	1.63	0.09	0.37	0.20	0.73	0.87	550.52	0.34	0.09
Uz	-	-	7.00	0.14	21.99	nd	10.30	1.47	0.05	0.33	0.10	0.48	0.39	506.33	0.51	0.51
Gr	-	-	8.01	0.08	19.05	nd	10.30	0.70	0.05	0.35	0.10	0.24	0.22	282.59	0.14	0.02
Ro	-	-	11.62	0.09	33.52	nd	9.08	1.75	0.19	0.38	0.24	0.67	0.25	494.85	0.25	0.33
Uk	-	-	15.98	0.15	38.87	0.47	25.86	nd	0.08	0.79	0.23	0.05	0.23	481.92	1.18	0.46
Comparison of country (US and Cn) and collection years																
US	2020	-	14.50 ± 5.31a	0.07 ± 0.04a	67.04 ± 13.39a	1.04 ± 1.77a	26.90 ± 7.83a	0.45 ± 0.54b	0.06 ± 0.03a	0.64 ± 0.30a	0.11 ± 0.03a	0.10 ± 0.06c	0.25 ± 0.08b	370.43 ± 90.96b	0.47 ± 0.15a	0.29 ± 0.09ab

US	2022	-	9.11 ± 3.43b	0.07 ± 0.03a	55.83 ± 27.12a	0.55 ± 0.92a	23.61 ± 11.72ab	1.26 ± 0.78a	0.08 ± 0.02a	0.44 ± 0.12a	0.14 ± 0.08a	0.29 ± 0.08b	0.38 ± 0.15a	642.11 ± 233.75a	0.38 ± 0.09a	0.15 ± 0.06c
Cn	2020	-	8.10 ± 3.40b	0.06 ± 0.04a	26.13 ± 9.57b	4.52 ± 8.94a	12.89 ± 5.45bc	0.19 ± 0.42b	0.05 ± 0.02a	0.46 ± 0.33a	0.14 ± 0.06a	0.28 ± 0.21b	0.36 ± 0.18ab	343.83 ± 131.91b	0.46 ± 0.17a	0.45 ± 0.30a
Cn	2022	-	3.91 ± 2.31b	0.11 ± 0.06a	17.08 ± 20.08b	3.16 ± 2.74a	4.65 ± 4.971.26c	0.68 ± 0.52ab	0.06 ± 0.03a	0.24 ± 0.12a	0.18 ± 0.14a	0.55 ± 0.20a	0.49 ± 0.28a	508.08 ± 250.04ab	0.35 ± 0.23a	0.11 ± 0.18bc
Comparison of two provinces in Cn																
Cn	-	Xinjiang	5.14 ± 3.52a	0.04 ± 0.04a	33.54 ± 6.86a	nd a	10.35 ± 1.25a	0.69 ± 0.60a	0.03 ± 0.02a	0.38 ± 0.23a	0.09 ± 0.01a	0.43 ± 0.09a	0.23 ± 0.10b	328.58 ± 160.40a	0.34 ± 0.10a	0.39 ± 0.07a
Cn	-	Yunnan	6.02 ± 2.79a	0.12 ± 0.04a	6.88 ± 2.46b	9.97 ± 9.06a	3.42 ± 2.85b	0.31 ± 0.45a	0.07 ± 0.03a	0.20 ± 0.09a	0.23 ± 0.11a	0.45 ± 0.36a	0.64 ± 0.04a	447.72 ± 286.90a	0.36 ± 0.23a	0.01 ± 0.01b

*Cl: Chile, Cn: China, Gr: Greece, In: India, Mo: Moldova, Po: Poland, Ro: Romania, Tr: Turkey, Uk: Ukraine, US: The United States, and Uz: Uzbekistan
**nd: not detected

Linear discriminant analysis results suggested that the phenolic profile successfully separated walnut samples originating from three countries (the USA, China, and India) (Figure 5.3). The success of chemometrics of phenolic data in clustering samples based on origins is also shown in other matrices, like olive(Japón-Lujan et al., 2006) and saffron(Senizza et al., 2019). The success might be due to variations in geographical properties, cultivars, and agroclimatic conditions.

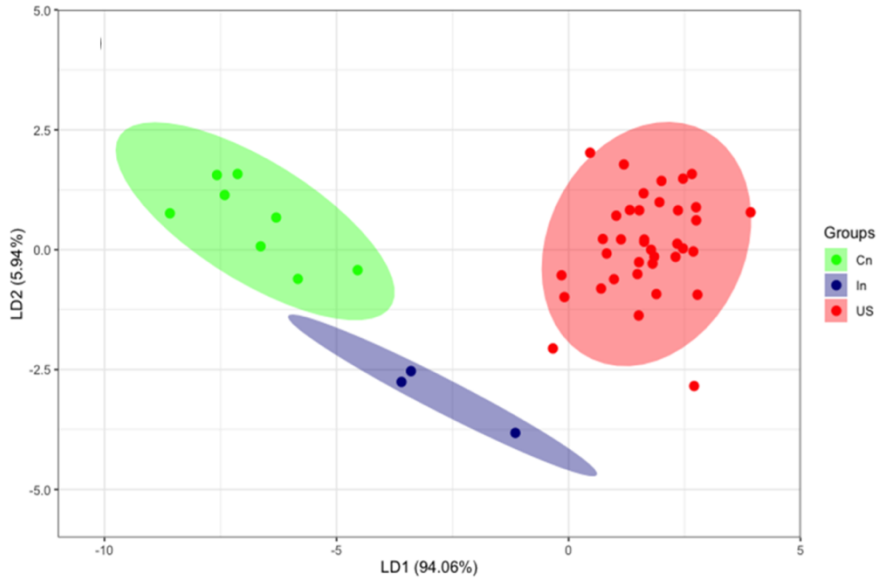


Figure 5.6. LDA plots of the phenolic profile of walnuts from the USA (US), China (Cn), and India (In)

Compound-Specific Stable Isotope

Compound-specific stable isotope analysis (CSIA) was performed on both fatty acids and amino acids of the walnut samples sourced in 2022. CSIA of fatty acids was conducted on all samples, but four samples from the US were not analyzed for CSIA of amino acids because of insufficient quantity. Across all amino acids, India had higher $\delta^{15}N_{\text{amino acids}}$ relative to the other countries investigated (Table 5.9),) while Chile, Greece, and Uzbekistan had the lowest values of $\delta^{15}N_{\text{amino-}}$

$_{acids}$. Higher $\delta^{15}N_{amino\ acids}$ values generally correspond to organic nitrogen sources, especially animal manure, whereas decreased $\delta^{15}N_{amino\ acids}$ suggest the use of synthetic fertilizer(Kim et al., 2022).

The most enriched amino acid was proline, and it indicated a kinetic isotope effect during its synthesis that was mediated by several enzymes, which were γ-glutamyl phosphate, γ-glutamyl semialdehyde, and Δ^1-pyrrolidine-5-carboxylate(Csonka & Leisinger, 2007). Proline plays an important role in plants' osmolyte regulation. During water stress, plants accumulate this amino acid and the concentration decreases after plants are rehydrated because proline is converted into glutamate by proline dehydrogenase or Δ^1-pyrrolidine-5-carboxylate dehydrogenase(Kiyosue et al., 1996). The large enrichment of this amino acid potentially occurred because of intense catabolism or thermodynamic isotope effect during its biosynthesis(Styring et al., 2014). Besides rehydration, increased activity of the proline dehydrogenase enzyme may also occur during defense responses against pathogens(Cecchini et al., 2011). Meanwhile, $\delta^{15}N_{Glx}$ values were in the middle range, confirming glutamate's central role in the synthesis of other amino acids(Styring et al., 2014).

Based on a similar study in wheat(Paolini et al., 2015), we predict that most samples in the current study received their N source from either synthetic fertilizer or green manure. Considering that many orchards in the US and China apply conventional farming techniques, it was likely that the samples from these countries receive synthetic fertilizer(Bai et al., 2020; OPN, 2021). Synthetic fertilizer has minimal or no isotope fractionation during its production via the Haber-Bosch process(van Leeuwen et al., 2014).

Significant differences in the $\delta^{13}C_{amino\ acids}$ between the samples from the US and China were observed for $\delta^{13}C_{Gly}$, $\delta^{13}C_{His}$, and $\delta^{13}C_{Met}$. For the $\delta^{13}C_{Gly}$ and $\delta^{13}C_{His}$, the US samples were more

enriched but for $\delta^{13}C_{Met}$, it was the opposite. There has been very limited information on $\delta^{13}C_{amino\ acids}$ from origin studies, so comparison with previous findings was difficult. Other studies using this parameter mainly focused on discriminating plants grown using conventional and organic farming. A previous study demonstrated that $\delta^{13}C_{Glx}$ of tomatoes grown using the organic method was different from that grown using the conventional method, but no difference was observed for other amino acids(Bontempo et al., 2020). Based on previous studies of $\delta^{13}C_{bulk}$ in plants, we predicted that the difference in several $\delta^{13}C_{amino\ acids}$ could be due to variability in the water regime (control, mild water stress, and moderate water stress). Plants that are exposed to mild or moderate water stress have more enriched $\delta^{13}C$(Peuke et al., 2006; Serret et al., 2017). Besides the water regime, genotypes have also been shown to affect $\delta^{13}C_{bulk}$ values(Serret et al., 2017).

Fatty acid hydrogen isotope values of $\delta^{2}H_{C16:0}$, $\delta^{2}H_{C18:1w9c}$, $\delta^{2}H_{C18:2w6}$, and $\delta^{2}H_{C18:3w3}$ from the US samples were significantly higher than those of China. Previously, we found that only $\delta^{2}H_{C18:1w9c}$ values were contrasted between these two countries(Athaillah et al., 2023). The variability of $\delta^{2}H_{fatty\ acids}$ among the countries could be due to different types of water sources or different levels of irrigation. Groundwater is typically more depleted than precipitation. Similarly, deep soil water is more depleted as compared to surface soil water. Higher levels of irrigation tend to increase $\delta^{2}H$ values although the effect is not uniform across cultivars, parts of plants, and irrigation levels(Meng et al., 2023). Based on this information, it was likely that samples from the US mainly obtained water through irrigation. The China samples, on the other hand, might receive groundwater or irrigation water but at a smaller amount than those from the US, Chile, Greece, India, Turkey, and Uzbekistan. Another possible reason for the lower $\delta^{2}H_{fatty\ acids}$ values of the China sample was the continental effect. Precipitation that occurs at locations

further from the coast is more depleted in ^{2}H because of Rayleigh distillation(Gori et al., 2015). Xinjiang and Yunnan are, in fact, at greater distances from the nearest coast than California's Central Valley, where most of the US samples were grown.

For samples from all the countries, oleic acid was the most enriched fatty acid, and linolenic acid was the most depleted. The high enrichment of oleic acid could be because it is the substrate for linoleic acid through desaturation reaction and the reaction occurs intensively because linoleic acid is the most dominant fatty acid in walnuts(Chikaraishi et al., 2004; Verardo et al., 2013). The lowest value of $\delta^{2}H_{C18:3w3}$ should be because linolenic acid is not used to generate any other fatty acids. Whereas linoleic acid fell isotopically between oleic and linolenic acid because it acts as both product (during desaturation of oleic acid) and substrate (for linolenic acid production)(Chikaraishi et al., 2004).

$\delta^{13}C_{C18:1w9c}$ of the US samples was significantly higher than the China counterpart, but the opposite trend was noticed for $\delta^{13}C_{C18:2w6}$. Variation in $\delta^{13}C_{fatty\ acids}$ might be due to water stress. Different cultivars have varying patterns of $\delta^{13}C$ changes as an effect of water stress. In a study on grapes, Pinot Noir that was exposed to water stress had ^{13}C enrichment in C_{18}-C_{22} fatty acids, whereas in the Chasselas variety, enrichment occurred in C_{24}-C_{32}, but a depletion was noticed for $C_{18:2}$(Spangenberg et al., 2020). Based on this study, we assumed that the variation that we observed was caused by both environmental factors (water stress) and genotype.

For the classification of origin between the US and China, using either the combination of amino acid and fatty acid data or solely using fatty acid data was successful (Figure 5.7). The latter resulted in a larger separation shown in the density plot.

Table 5.9. $\delta^{15}N_{amino\ acids}$, $\delta^{13}C_{amino\ acids}$, $\delta^{2}H_{fatty\ acids}$, and $\delta^{13}C_{fatty\ acids}$ (‰) in walnuts from the US (US), China (Cn), Chile (Cl), Greece (Gre), India (In), Turkey (Trk), and Uzbekistan (Uzb)*

Parameter	US	Cn	Cl	Gre	In	Trk	Uzb
Amino acids							
$\delta^{15}N_{Ala}$	1.44 ± 1.17a	0.51 ± 1.58a	0.47	-0.21	2.91	1.73	0.46
$\delta^{15}N_{Asx}$	2.90 ± 1.31a	2.46 ± 1.37a	1.73	2.25	4.20	2.42	2.35
$\delta^{15}N_{Glx}$	2.05 ± 1.14a	2.02 ± 1.40a	1.65	1.64	3.96	2.00	1.47
$\delta^{15}N_{Gly}$	2.06 ± 1.62a	1.61 ± 1.37a	1.84	1.42	3.49	2.34	2.44
$\delta^{15}N_{His}$	-1.30 ± 1.65a	-0.87 ± 0.37a	-2.37	-2.88	0.14	-1.77	-1.00
$\delta^{15}N_{Ile}$	1.52 ± 1.19a	0.60 ± 0.89a	-0.01	0.08	3.46	0.65	0.49
$\delta^{15}N_{Leu}$	0.42 ± 1.13a	-0.82 ± 1.34a	-1.07	-1.29	1.86	-0.30	-0.43
$\delta^{15}N_{Lys}$	2.43 ± 1.84a	2.81 ± 1.66a	1.54	1.19	4.29	2.14	1.92
$\delta^{15}N_{Met}$	0.79 ± 1.53a	0.78 ± 1.46a	-0.32	1.81	3.70	1.34	0.06
$\delta^{15}N_{Phe}$	-0.09 ± 1.43a	-0.37 ± 0.93a	-0.82	-2.78	2.70	-0.77	0.13
$\delta^{15}N_{Pro}$	4.35 ± 1.31a	3.88 ± 0.90a	3.77	2.50	6.38	3.80	3.17
$\delta^{15}N_{Ser}$	1.66 ± 1.80a	0.86 ± 0.93a	1.19	0.20	3.58	1.32	1.34
$\delta^{15}N_{Thr}$	0.44 ± 1.41a	2.26 ± 1.27a	0.32	3.87	3.71	0.65	0.02
$\delta^{15}N_{Tyr}$	2.64 ± 1.88a	2.58 ± 0.56a	0.18	-1.08	5.20	1.54	3.53
$\delta^{15}N_{Val}$	3.68 ± 1.39a	3.26 ± 1.49a	2.85	2.71	5.03	3.39	2.77
$\delta^{13}C_{Ala}$	-19.66 ± 0.84a	-20.08 ± 0.37a	-21.49	-21.45	-19.90	-17.95	-20.10
$\delta^{13}C_{Asx}$	-20.64 ± 0.71a	-19.61 ± 0.18a	-20.97	-20.78	-19.74	-18.49	-19.25
$\delta^{13}C_{Glx}$	-22.09 ± 0.44a	-22.25 ± 0.27a	-22.74	-22.64	-22.34	-20.08	-22.41
$\delta^{13}C_{Gly}$	-19.81 ± 1.04a	-22.30 ± 0.27b	-21.82	-22.55	-21.23	-18.54	-21.81
$\delta^{13}C_{His}$	-21.82 ± 1.45a	-24.46 ± 0.89b	-23.61	-24.05	-21.86	-23.61	-19.12
$\delta^{13}C_{Ile}$	-22.68 ± 0.54a	-23.19 ± 0.82a	-22.72	-24.03	-23.28	-20.70	-21.86
$\delta^{13}C_{Leu}$	-27.56 ± 0.59a	-27.52 ± 0.18a	-28.51	-28.17	-27.99	-25.85	-27.04
$\delta^{13}C_{Lys}$	-19.68 ± 0.64a	-18.93 ± 0.76a	-20.38	-21.26	-19.88	-17.79	-19.17
$\delta^{13}C_{Met}$	-33.75 ± 1.31b	-31.38 ± 0.57a	-32.74	-31.38	-30.38	-30.80	-30.64
$\delta^{13}C_{Phe}$	-30.71 ± 0.59a	-30.57 ± 0.06a	-31.17	-30.85	-30.32	-28.42	-30.59
$\delta^{13}C_{Pro}$	-23.99 ± 0.68a	-23.88 ± 0.37a	-24.55	-25.11	-24.20	-22.23	-23.62
$\delta^{13}C_{Ser}$	-15.63 ± 0.71a	-16.14 ± 0.60a	-15.06	-16.57	-13.98	-13.24	-14.80

$\delta^{13}C_{Thr}$	-14.96 ± 0.40a	-15.70 ± 0.75a	-15.11	-17.73	-15.33	-13.05	-15.47
$\delta^{13}C_{Tyr}$	-28.75 ± 0.55a	-28.49 ± 0.72a	-29.09	-29.16	-28.49	-26.58	-28.65
$\delta^{13}C_{Val}$	-28.31 ± 0.71a	-28.57 ± 0.68a	-29.51	-29.36	-28.85	-26.99	-27.90
Fatty acids							
$\delta^{2}H_{C16:0}$	-193.70 ± 7.43a	-215.69 ± 28.53b	-180.55	-198.32	-197.41	-203.09	-187.44
$\delta^{2}H_{C18:1w9c}$	-122.86 ± 8.18a	-154.87 ± 43.19b	-110.74	-121.24	-126.14	-132.15	-132.01
$\delta^{2}H_{C18:2w6}$	-207.90 ± 7.99a	-232.48 ± 27.75b	-203.03	-212.06	-209.43	-218.28	-206.88
$\delta^{2}H_{C18:3w3}$	-215.31 ± 8.36a	-237.12 ± 25.18b	-208.07	-219.36	-216.96	-222.85	-211.41
$\delta^{13}C_{C16:0}$	-30.60 ± 0.71a	-30.82 ± 0.45a	-30.53	-30.73	-30.23	-29.24	-29.97
$\delta^{13}C_{C18:1w9c}$	-29.01 ± 0.65a	-30.80 ± 1.52b	-29.22	-29.54	-29.17	-27.56	-29.09
$\delta^{13}C_{C18:2w6}$	-29.90 ± 0.65b	-28.82 ± 1.44a	-30.25	-30.57	-30.28	-28.80	-30.24
$\delta^{13}C_{C18:3w3}$	-30.02 ± 0.65a	-30.47 ± 0.18a	-30.68	-31.06	-30.77	-29.16	-30.69

*Significant difference (p-value < 0.05) between the US and China samples for each parameter was marked as difference letters.

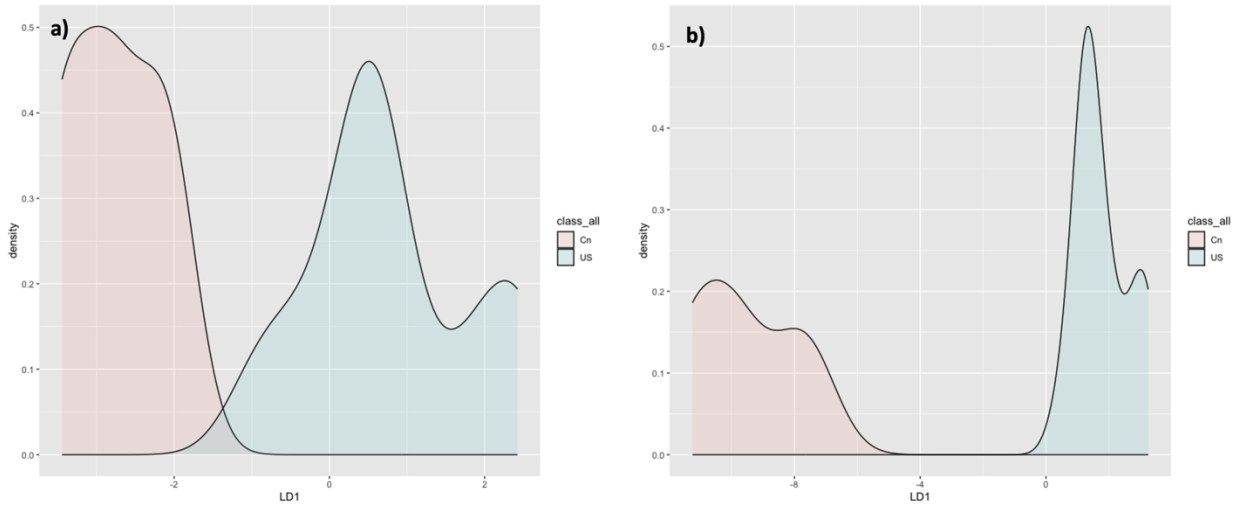


Figure 5.7. LDA density plot of combination of fatty acid and amino acid stable isotope (a) and fatty acid stable isotope only (b) in walnuts from the US (US) and China (Cn)

Conclusion

Walnuts from the US and China had similar concentrations of free fatty acids, peroxides, total fat, all volatiles except 2-butanone, and total phenol. The India samples had a lower total fat than the China samples but similar to those from the US. The oxidative stability and concentrations of beta-sitosterol and delta-tocopherol of the China samples were higher than those of the US. However, the US samples had a larger concentration of alpha-tocopherol. Significant differences in seven TAGs (1,2-linolein-3-linolenin, 1,2-linolein-3-stearin, tripalmitin, 1-olein-2,3-linolein, trilaurin, tristearin, and 1,2-laurin-3-miristin) between the samples from the US and China were found. Some differences in the fatty acid profile of samples from the US, China, and India were observed: the US samples had a lower concentration of oleic acid than those from China and India; the China samples had a higher concentration of palmitoleic acid and a lower content of linolenic acid than the samples from the US and India; and the India samples had less stearic and linoleic acids than the US samples but not different from those of China. The India samples had higher concentrations of many volatiles, i.e., hexanal, 3-methyl-2-butenal, 2-heptenal, 2-pentenal, 2,4-heptadienal, 2-heptanone, 1-octen-3-ol, 1-penten-3-ol, and furan-2-ethyl, compared to those from the US and China. The China, India, and the US samples were similar in their total phenol. They, however, differed regarding the concentrations of gallic acid, catechin, chlorogenic acid, procyanidin B1, vanillic acid, and ferulic acid. Linear discriminant analysis (LDA) on TAG, volatile, phenol, CSIA-amino acids ($\delta^{13}C_{amino\ acid}$ and $\delta^{15}N_{amino\ acid}$) and fatty acids ($\delta^{13}C_{fatty\ acid}$ and $\delta^{2}H_{fatty\ acid}$) or CSIA-fatty acids only was effective in discriminating walnut origins. The data suggested that the quality of the US and China samples, in regard to the primary (peroxides) and secondary oxidation (volatile) products, were largely similar. Based on

the volatile data, the India samples were more oxidized than those from the US and China.

TAGs, volatiles, phenols, and CSIA-fatty acids could be markers for walnut origins.

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CHAPTER 6

Physical and chemical characteristics of walnut (*Juglans regia* L.) kernels with different skin lightness

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Running head: Comparison of light vs dark walnuts

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Abstract

Walnuts undergo rigorous grading before being sold to customers. There are multiple parameters used for the grading, including skin lightness. Walnuts with light skin receive superior grades while walnuts with dark skin are given poor grades or even rejected. However, information on the quality and physicochemical properties of walnuts with varying skin lightness levels is minimal. Therefore, we studied the quality of kernels of varying skin lightness from three common cultivars grown in California, the USA (Chandler, Howard, and Tulare). The samples were subjected to size and weight, fat content, free fatty acid, peroxide value, oxidative stability, volatiles, tocopherols, fatty acid profile, and phenol measurements. The dark kernels had significantly lower weight and fat content, higher oxidative stability, and more volatiles than their light counterparts. The dark kernels had higher concentrations of some phenolics but low procyanidin B1 and non-existent epicatechin gallate, compared to the light kernels, indicating that these two phenolics were likely involved in antioxidant mechanism. Oxidation and depletion of epicatechin gallate likely contributed to the darkening of walnut color.

Introduction

Walnut is an important crop for many producers and handlers in various countries, including China, the United States, and India. Walnut fruits consist of husk, shell, kernel skin, and kernel (without skin). After harvest, walnuts are typically dehusked, dried, and sorted based on in-shell size. The dried walnuts are later shelled, followed by separation of kernels from the broken shells (Liu et al., 2021). Subsequently, they are graded using multiple factors, including skin lightness, size, cleanliness, proper drying, and appearance (free of shell, foreign material, insect injury, shriveling, mold, and kernel discoloration)(USDA, 2017). Walnuts with light skin are commonly preferred by consumers, therefore they are given superior grades and priced higher. On the other hand, walnuts with darker skin are less preferred, therefore they are given poor grades or sometimes rejected. The consequence of having a large amount of walnuts with dark skin includes profit reduction or even loss and increased waste, in the case the dark walnuts are rejected by consumers. California walnut industry experienced this issue in the harvest season in 2022 and the California Walnut Commission estimated the subsequent loss to be one billion USD(California Farm Bureau, 2022).

There are multiple reasons for this skin darkening. It can be induced by pre-harvest and post-harvest conditions. The pre-harvest condition includes genetics; climate, namely excessive sun exposure and heat stress; and late harvest. Genetics have some role in kernel appearance, including skin lightness. A study shows that Chandler has lighter skin than variety Jamal(Pakrah et al., 2021). Late harvest also led to kernel skin browning, as evidenced in both Chandler and Jamal walnut varieties harvested at three different time points relative to packing tissue browning(Pakrah et al., 2021). Walnuts that were directly exposed to sunlight had darker skin than walnuts that were covered with kaolin, a material than can reflects sunlight(Mahmoudian et

al., 2021). High fruit surface temperature can also lead to sunburn browning, as in the case in apples(Trandafir et al., 2017).

After harvest, kernel skin darkening can occur during extended storage. Kernel skin contains various phenolic compounds, and they can undergo oxidation when oxygen is present. The reaction is accelerated by polyphenol oxidase (PPO) enzyme that is regulated by jrPPO1 gene(Escobar et al., 2008). Polyphenol oxidase catalyzes the oxidation of phenols into quinones. These quinones are highly reactive and later transform into phytomelanins(Araji et al., 2014). The oxidation reaction is favored at high oxygen pressure(Adiletta et al., 2020), high humidity(Yuan et al., 2019) and moderate temperatures (25 – 50 °C)(Taranto et al., 2017), even though it can also occurs at cold temperature when accompanied with high oxygen pressure and high humidity(Yuan et al., 2019).

Pre- or post-harvest insect/pest infestation and pathogen infection can also trigger activity of PPO enzymes and this process is one of plant adaptation strategies against biotic stress, for instance in defense against walnut blight inducing bacteria(Jiang et al., 2019). In this study, the skin darkening was likely a consequence of preharvest heat stress because a few weeks prior to the harvest time, extreme heat occurred in California – USA, where all the samples were sourced from. Multiple walnut growing locations in the region experienced increased temperature up to 46 °C. The darkening was noted in all cultivars commonly grown in California; the fruits were harvested at their normal harvest time; the darkening was noticed right after harvest so it was not developed during storage; and samples that had signs of pest/microbial attack were excluded from the study.

To understand if the kernel skin darkening is linked with changes in quality and physicochemical characteristics of walnut kernels, multiple parameters were assessed, and the values were compared between the light and dark walnuts. Size and weight were evaluated to understand whether disruption occurred during the filling phase of the kernels. Whereas total fat is an indicator whether the skin darkening is linked to changes in lipid metabolism and production.

Free fatty acidity and peroxide value are often used to predict the susceptibility or extent of lipid oxidation in various matrices because free fatty acids are substrates whereas hydroxy peroxides are primary products of oxidation reaction. Oxidative stability is an accelerated aging method to predict shelf-life of a sample by exposing it to high temperature and high oxygen flow. Volatile compounds can be used to understand walnut oxidation because many of these compounds are generated through the process(Adkison et al., 2021; Frankel, 1980). Fatty acid profile was analyzed to determine if fatty acid metabolism is altered. Tocopherols are antioxidants that have been known to mitigate oxidation in foods(Kumar et al., 2013) and evaluating the concentrations can help understand if the skin darkening is associated with oxidation. Lastly, another potential alteration is modified phenol metabolism. The skin darkening is likely associated with phenol oxidation, so various phenolic compounds would be determined.

Various cultivars may have different degrees of skin darkening, as indicated in the earlier study(Mahmoudian et al., 2021). However, very little information on other cultivars or other physical and chemical features are available. Cultivar effect was investigated in this study to help determine future cultivar selection for the growers. The three commonly grown cultivars in California, USA: Chandler, Howard, and Tulare, were included in this study.

Materials and Methods

Sampling

Walnuts (±2 kg) were obtained from multiple locations in California during September and October 2022. Completely randomized design was selected for the sampling. Chandler, Howard, and Tulare were sourced from eight, three, and three orchards or handlers, respectively. The locations of the orchards where the samples were sourced from were provided in Table 6.1, along with the positions of the nearest weather stations. Temperature and precipitation were recorded for each weather station from The US National Weather Service website. Samples were stored at ±5 °C prior to analysis.

Table 6.1. Location of orchards where the samples were grown and locations of their nearest weather stations

No	Sample code	Orchard location	Location of the nearest weather station	Maximum temperature in September 2022 (ºC)	Mean of maximum temperature in September 2003-2021 (ºC)
1	Chandler 1	Visalia, CA	Visalia, CA	36	37.16
2	Chandler 2	Colusa, CA	Marysville AP, CA	46	39.26
3	Chandler 3	Glenn, CA	Red Bluff, CA	46	40.84
4	Chandler 4	Lemoore/Greater Hanford, CA	Hanford area, CA	46	39.32
5	Chandler 5	Lemoore/Greater Hanford, CA	Hanford area, CA	46	39.32
6	Chandler 6	Lemoore/Greater Hanford, CA	Hanford area, CA	46	39.32
7	Chandler 7	Hanford, CA	Hanford 1S, CA	44	38.95
8	Chandler 8	Los Malinos, CA	Red Bluff, CA	46	40.84
9	Tulare 1	Woodlake, CA	Visalia, CA	36	37.16
10	Tulare 2	Tehama, CA	Red Bluff, CA	46	40.84
11	Tulare 3	Hanford, CA	Hanford 1S, CA	44	38.95
12	Howard 1	na*	na		
13	Howard 2	Colusa, CA	Marysville AP, CA	46	39.26
14	Howard 3	Yuba City, CA	Marysville AP, CA	46	39.26

*na: not available

The lightness (L*) values of the kernel skin were measured using a colorimeter (ColorReader Pro, Data Color, Lawrenceville, NJ, USA). Values from 40 to 59.9 were defined as "light" whereas values from 20 to 39.9 were grouped into "dark". The light group covered colors extra light and light, while the dark group corresponded to light amber and amber. These four colors are widely used for walnut grading(USDA, 2000). Completely randomized design was used when subsets of samples were selected for each analysis.

Oil Extraction

Walnut kernels (±400 g) were fed into an expeller/cold-press machine (KK Oil Prince F Universal, Reut, Germany). The expeller screw was rotated at 22 rpm and a die number 7 was used. The resultant slurry was centrifuged at 10,000 g for 15 min to separate the oil from solid particles. The oil samples were kept at -20 °C until analysis.

Weight and Size

Ten half kernels of each sample were subjected to this analysis. Each of them was measured for their weight, width, and length.

Fat Content

Approximately 15 walnut halves were ground using a food processor and 3 ± 0.1 g of them was added into a cellulose thimble (33 × 150 mm, Whatman 603 Cellulose thimbles). The thimbles were placed in a Buchi Universal Extractor E-800 (Buchi Corporation, New Castle, United States). The fat was extracted 30 times using n-hexane. The residual hexane was evaporated to obtain the fat mass. Three replicates were analyzed for each sample.

Free Fatty Acid Content

Free fatty acid analysis was conducted following European Union Commission regulation (EEC) no 2568/91. Approximately 10 g of oil was used for the analysis. Three replicates were measured.

Peroxide Value

The peroxide value analysis was performed as illustrated in AOCS official method Cd 8b-90. Approximately 5 g of oil was used for the analysis. Duplicates were analyzed for each sample.

Oxidative Stability

Oxidative stability was measured following a published study on walnuts(Grilo & Wang, 2021) using a Rancimat apparatus 617 (Metrohm AG, Herisau, Switzerland). Five kernel halves were randomly selected and then ground. Ground samples (0.5 g) was used for the analysis. Three replicates were analyzed.

Volatile Organic Compounds

Volatile organic compounds were detected and quantified using a headspace - solid phase microextraction (HS-SPME) connected to a gas chromatography-mass spectrometry (GC-MS), following a published method(Grilo & Wang, 2021). Around fifteen kernel halves were randomly selected and ground. A subset (±3 g) was used for the analysis. Three replicates were measured.

Tocopherols

The cold-pressed oil from each sample (0.2 g) was added with 0.9 mL of isopropanol ad 0.1 mL of internal standard (α-tocopherol acetate), then mixed thoroughly using a vortex. The mixture

was then centrifuged at 10,000 rpm for 10 min. The supernatant was collected and filtered using a nylon filter (0.45 µm). The samples were then analyzed using a high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD) (Agilent 1290 Infinity II LC System, Agilent Technologies, California, United States). Reverse phase LC followed a published method (Górnaś, 2015) with modifications in the dilution factor, HPLC detector type, and quantification method. Injection volume was 5 µL. Separation was done using a PFP column (150 mm x 4.6 mm, 3 µm) with methanol: water (93:7, v/v) as the mobile phase). The flow followed isocratic mode at 1 mL/min. The DAD wavelength was set at 290 nm. This analysis was done in triplicates.

Fatty Acid Profile

Walnut oil (±20 µL) was placed in a tube. Hexane (3 mL) was added to dissolve the oil sample. Methanolic potassium hydroxide (0.2 mL, 2 M) was added to derivatize the fatty acids in the oil to fatty acid methyl esters (FAMEs). The mixture was vortexed and then left to stratify until the upper solution became clear. The upper phase was then subjected into a gas chromatography-flame ionization detector, GC-FID (Agilent 7890A GC, Agilent Technologies, California, USA). The GC-FID analysis was done following this method(Green & Wang, 2020). Peak identification was performed using a 37-FAME mix, certified reference material (TraceCERT, Millipore Sigma, USA). This analysis was done in triplicates.

Phenols

Fifteen kernel halves were randomly selected and ground. Ground walnut kernel (±3 g) was placed in a centrifuge tube. First, lipid portion was removed through hexane extraction. Hexane (25 mL) was added, and the mixture was vortexed for 5 s, and sonicated for 5 min at room

temperature. The mixture was centrifuged at 2,000 g for 5 min and later, the hexane phase was removed. This lipid extraction was done one more time with 15 ml of hexane. Afterward, 5 mL of aqueous acetone (acetone: water, 7:3, v/v) was added to the defatted sample for phenol extraction. The mixture was vortexed for 2 min and sonicated for 30 min. The mixture was centrifuged at 10,000 g for 5 min. The aqueous acetone phase was transferred into a centrifuge tube. Phenol extraction was repeated, and the aqueous acetone layer was combined with that of the first extraction. The solution was then filtered using a nylon filter (45 μm) and 2 μL of it was injected into a liquid chromatography coupled to a tandem mass spectrometry (LC-MS/MS).

Compound separation was done in the LC (Agilent 1260 Infinity II, Agilent Technologies Inc., California, USA) using a Poroshell 120 EC-C18 column (100 x 2.1 mm, 1.9 μm). The flow rate was 0.30 mL/min. Mobile phase A was 0.5% formic acid in water and mobile phase B was a mixture of acetonitrile and methanol (1:1, v/v). The gradient conditions were as followed: 0-4 min / 15% B; 8 min / 17% B; 10 min / 25% B; 15 min / 50% B; 17 min / 75% B; 20 min / 95% B; and 23 min / 15% B. The column temperature was 40 °C. The ion source of the MS was Agilent Jet Stream electrospray ionization (AJS ESI). The mass spectrometer (6470 triple quadrupole, Agilent Technologies Inc., California, USA) parameters included gas temperature 350 °C; gas flow 10 L/min; nebulizer pressure 275.79 kPa; sheath gas temperature 350 °C; sheath gas flow 8.5 L/min; capillary voltage 3500 V (positive) and 2500 V (negative); and nozzle voltage 500 V (positive) and 1000 V (negative). Additional information on MS/MS setting for compound identification was provided in the Supporting document (Table S6.1). This analysis was done in duplicate.

Statistical Analysis

Analysis of variance (ANOVA) was conducted to detect if there was any significant difference between the two skin lightness levels of the whole dataset (data from the three cultivars combined), the three varieties (Chandler, Howard, and Tulare), and if the interaction between lightness and cultivar was significant. Tukey HSD was used as the pairwise test. Statistical significance was set at $p < 0.05$. All the analysis was performed using R Studio 2022.07.01 (Posit software, PBC, Boston, MA, USA) and R 4.2.2. (The R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

Weight, Length, and Width

The light kernels of Tulare had significantly higher weight than the dark kernels of Chandler and Howard (p-value < 0.05). When comparing the weight of the dark kernels of Chandler, Howard, and Tulare, however, they were not significantly different from the weight of their respective light kernels (p-values were > 0.05) (Table 6.2 and 6.3). Because of no significant interaction between skin lightness and cultivar, there was not enough evidence suggesting that the three cultivars had varying reduction in weight that could be linked to the skin darkening.

When we considered data of the three cultivars combined, the light kernels had significantly larger value for weight than the dark kernels (p-value 0.0002). Generally, only a small portion of recently harvested walnuts had dark kernel skin. Because maximum temperature in September 2022 was higher than those of previous years (NOAA, 2022) (Table 6.1 and Figure S6.1), it was reasonable to link the weather event to the skin darkening. Kernel weight reduction due to heat stress was shown earlier in olives (Benlloch-González et al., 2019) and lentils (Sita et al., 2018).

Olive trees planted inside a temperature-controlled open-top-chamber set at 4 °C higher than the ambient temperature had smaller fruits, compared to the plants grown at ambient temperature. In lentils, heat stress reduced the seed growth rate by 30-44% and shortened the seed filling period, decreasing the seed weight and ultimately the yield (Sita et al., 2018). This reduction could be due to several reasons: severe transpiration; impaired photosynthesis (Benlloch-González et al., 2019); and decreased sucrose metabolism and transport (Kaushal et al., 2013).

Even though zero precipitation (Supporting Information, Figure S6.2) was recorded during the heat wave event occurred in early September 2022 or around 122th day after walnuts were pollinated (Supporting Information, Figure S6.3), the water content of the light and dark kernels were not significantly different (Supporting Information, Figure S6.4). It was likely because the orchards were irrigated to minimize the damage. Another possibility was rehydration of the kernels because approximately two weeks after the maximum temperature was recorded, the areas near Marysville, Red Bluff, and Hanford, where some samples were sourced from, experienced a spike in precipitation level (Supporting Information, Figure S6.2).

Statistical analysis using ANOVA demonstrated significant difference on weight for either lightness levels of the kernel skin or cultivar and no interaction between them was observed (Table 6.2 and Table 6.3). Considering both light and dark kernels together, Tulare had significantly higher value for weight (p-value 0.021), which is consistent with previous finding of Tulare kernels being heavier than Chandler and Howard kernels(UC, 2020).

Size reduction as an effect of heat stress is less studied than weight decrease, though it has been observed in wheat(Ullah et al., 2022) and rice(Cao et al., 2008). In our study, there was no significant difference in the length and width between the two groups of varying lightness, either when cultivar was considered or not (p-value > 0.05) (Table 6.2 and Table 6.3).

Table 6.2. P-values of the ANOVA results for the effects of skin lightness for the whole dataset, cultivar, or interaction between lightness and cultivar for various quality and metabolite parameters

Parameter	Lightness	Cultivar	Lightness * Cultivar
Weight	**0.0002**	**0.0208**	0.858
Length	0.222	0.851	0.807
Width	0.6685	0.2823	**0.0449**
Fat content	**<0.0001**	**<0.0001**	**0.0001**
FFA	0.275	0.445	0.724
PV	0.656	0.530	0.841
Oxidative stability	**0.0174**	**0.0028**	0.9979
1-Octen-3-ol	0.1160	**0.0126**	**0.0430**
1-Hexanol	**0.0247**	0.0629	0.3751
3-Buten-2-ol-2-methyl	0.627	0.114	0.204
1-Penten-3-ol	**0.0101**	**0.0134**	0.0660
1-Pentanol	**0.0001**	**0.0003**	**0.0007**
Hexanal	0.150	0.103	0.116
Pentanal	0.111	0.116	0.116
Butanal 2-methyl	**0.0020**	0.2656	0.1914
Butanal, 3-methyl	**0.0028**	0.1443	0.4996
Ethyl acetate	0.9520	0.0542	0.9477
Acetic acid methyl ester	0.0594	0.4150	0.9784
Butyrolactone	0.3439	**0.0027**	0.7611
Acetone	**0.0058**	0.9266	0.7036
2-Heptanone	**0.0376**	0.0991	0.4415
Delta tocopherol	0.263	0.618	0.368
Gamma tocopherol	0.773	**0.027**	0.631
Alpha tocopherol	0.1614	0.0343	0.3621
Total tocopherol	0.5243	**0.0131**	0.6668
C16.0	0.573	**<0.0001**	0.887
C16.1	0.2785	0.3132	0.0637
C17.0	0.925	0.691	0.642
C18.0	0.3436	**0.0024**	0.7829

C18.1	0.737	0.590	0.998
C18.2	0.818	0.463	0.900
C18.3	0.330	**<0.0001**	0.539
C20.0	0.831	**<0.0001**	0.781
C20.1	0.2518	**0.0001**	0.1339
SFA	0.796	**<0.0001**	0.963
MUFA	0.736	0.555	0.998
PUFA	0.8404	**0.0434**	0.9957
MUFA to PUFA	0.905	0.725	0.918
C18.1 / C18.2	0.640	0.690	0.963
C18.2 / C18.3	0.461	**<0.0001**	0.546
Gallic acid	**0.0107**	0.2984	0.5599
3,4 Dihydroxybenzoic acid	0.0998	0.5718	**0.0407**
Catechin	0.177	0.819	0.837
Chlorogenic acid	0.856	**<0.0001**	0.516
Procyanidin B1	**0.0153**	0.2069	0.9499
Vanillic acid	**0.0054**	0.0964	0.3490
Caffeic acid	0.798	0.617	0.563
Epicatechin	0.085	0.608	0.983
Epicatechin gallate	**<0.0001**	0.168	0.247
p-Coumaric acid	0.126	0.915	0.464
Ferulic acid	0.105	0.096	0.516
Sinapic acid	0.845	0.217	0.239
Ellagic acid	**0.0494**	0.1754	0.3024
Quercetin	**0.0001**	**0.0233**	**0.0350**
Naringenin	**0.0002**	**0.0021**	0.2624

Table 6.3. Weight, length, and width of half kernels of light and dark walnuts (cv. Chandler, Howard, Tulare, and the whole dataset). The values were expressed as average ± standard deviation*

Cultivar	Lightness	Weight (g)	Length (cm)	Width (cm)
Chandler	Light	2.75 ± 0.21^{ab}	2.83 ± 0.10^{a}	2.55 ± 0.15^{a}
	Dark	2.32 ± 0.29^{b}	2.79 ± 0.12^{a}	2.51 ± 0.14^{a}
Howard	Light	2.78 ± 0.36^{ab}	2.83 ± 0.06^{a}	2.67 ± 0.15^{a}
	Dark	2.19 ± 0.33^{b}	2.73 ± 0.15^{a}	2.47 ± 0.06^{a}
Tulare	Light	3.16 ± 0.42^{a}	2.83 ± 0.06^{a}	2.53 ± 0.06^{a}
	Dark	2.67 ± 0.12^{ab}	2.80 ± 0.00^{a}	2.73 ± 0.12^{a}
Whole dataset	Light	2.84 ± 0.32^{A}	2.83 ± 0.08^{A}	2.57 ± 0.14^{A}
	Dark	2.37 ± 0.31^{B}	2.78 ± 0.11^{A}	2.36 ± 0.31^{A}

*Different lowercase letters indicate significant difference ($p < 0.05$) between the combination of the skin lightness and cultivar. Different uppercase letters showed significant difference ($p < 0.05$) between the light and dark groups for the whole dataset. Whole dataset means that the data from the three cultivars were combined, and they were only grouped based on the lightness index.

Fat Content

Fat is the main component of walnut kernels that it generally accounts for 64-72% by weight(Yi et al., 2017). There was an interaction between cultivar and lightness index (p-value of 0.0002). When the total fat content of the light and dark kernels of each cultivar was examined, we found a significant reduction for Howard, but not for Chandler and Tulare (Table 6.2 and Table 6.4). When cultivar variation was excluded and the whole dataset was used for comparison of the light and dark groups, a significant reduction in the fat content of the dark kernels was observed (p-value < 0.0001). Higher fat content in extra light walnuts than the light, amber, and dark amber walnuts was found in a previous study but the reason behind it was not discussed(Li et al., 2022). In the current study, the reason for the darkening was highly likely pre-harvest high temperature.

Reduction of fat content because of heat stress has been found in olives(Benlloch-González et al., 2019; García-Inza et al., 2014), canolas(Rivelli et al., 2023), and lentils(Sita et al., 2018). Increase of temperature by 4 °C led to a significant decrease in oil content in olives because fruit maturation was forwarded(Benlloch-González et al., 2019). Another study in olive demonstrated that oil concentration decreased linearly by 1.1% when temperature was increased within the range of 16 – 32 °C for four months(García-Inza et al., 2014). The same study also showed that increasing temperature by 7 °C for 1 month resulted in a significantly lower oil content(García-Inza et al., 2014). A similar trend was observed in canola; in which heat stress up to 35 °C resulted in a reduction of seed oil, from 42.9% to 39.3%(Rivelli et al., 2023). These studies were all conducted in a controlled setting and comparison was made between heat-treated plants to control. Ours was an observational study and the comparison was made between the light and dark kernels. The light kernels likely had less heat radiation, e.g., from shaded branches, but were from the same plants or orchards with the dark kernels. This may be a source of non-significant difference between the light and dark groups for Chandler and Tulare, nonetheless, a decrease in fat content in the dark kernels of Howard or all samples from the three cultivars was observed in our study, suggesting some changes in lipid metabolism.

Table 6.4. Fat content of light and dark walnuts (cv. Chandler, Howard, Tulare, and the whole dataset). The values were expressed as average ± standard deviation*

Cultivar	Lightness	Fat content (g/100 g)
Chandler	Light	68.31 ± 2.62^{ab}
	Dark	65.81 ± 2.78^{b}
Howard	Light	67.01 ± 1.20^{ab}
	Dark	52.22 ± 0.78^{c}
Tulare	Light	71.22 ± 2.15^{a}
	Dark	66.13 ± 1.85^{ab}
Whole dataset	Light	68.71 ± 2.61^{A}
	Dark	62.49 ± 6.53^{B}

*Different lowercase letters indicate significant difference ($p < 0.05$) between the combination of the skin lightness and cultivar. Different uppercase letters showed significant difference ($p < 0.05$) between the light and dark groups for the whole dataset. Whole dataset means that the data from the three cultivars were combined, and they were only grouped based on the lightness index.

Another factor to consider was the timing of the extreme heat in relation to walnut maturation periods. A previous study showed that lipid synthesis in walnut occurred rapidly between 74 – 88 days after pollination. From 88 days to 144 days (full maturation), the process occurred at a steadier rate(Jin et al., 2023). The heat wave incidence in the present study occurred during the later stage of lipogenesis (around 122 days after pollination) (Supporting Information, Figure S6.3) at which the lipid formation likely occurred steadily; therefore, it was reasonable that when the process was impaired, only a slight reduction in the total fat content was observed in Chandler and Tulare. It is proposed that had the heat event occurred between 74-88 days after pollination, the fat reduction could have been more severe.

Free Fatty Acid (FFA)

Free fatty acids are products of triacylglycerol hydrolysis, and this process is influenced by moisture content, water activity, temperature, and enzyme activity(Yang et al., 2019). FFA

values of our kernels were like those reported previously in recently harvested walnuts(Ortiz et al., 2019; Wang et al., 2006).

No significant difference of free fatty acid values between the samples of the two lightness indexes for each cultivar or for the whole dataset (all the samples from the three cultivars combined) (p-value > 0.05). Interaction effect between lightness and cultivar was also not detected (p-value > 0.05) (Table 6.2 and Table 6.5). There was no evidence of the link between kernel skin darkening and lipid degradation, particularly fatty acid release.

Table 6.5. Free fatty acid (FFA, % oleic acid), peroxide value (PV, mEq O_2/kg), and induction time (hr) of light and dark walnuts (cv. Chandler, Howard, Tulare, and the whole dataset). The values were expressed as average ± standard deviation*

Cultivar	Lightness	FFA (% oleic acid)	PV (mEq O_2/kg)	Induction time (hr)
Chandler	Light	0.04 ± 0.01^{a}	0.33 ± 0.05^{a}	11.49 ± 1.07^{b}
	Dark	0.05 ± 0.02^{a}	0.35 ± 0.05^{a}	12.85 ± 1.43^{ab}
Howard	Light	0.05 ± 0.01^{a}	0.28 ± 0.04^{a}	13.70 ± 2.41^{ab}
	Dark	0.05 ± 0.00^{a}	0.30 ± 0.18^{a}	15.10 ± 1.34^{a}
Tulare	Light	0.04 ± 0.01^{a}	0.32 ± 0.19^{a}	13.47 ± 0.47^{ab}
	Dark	0.05 ± 0.01^{a}	0.26 ± 0.09^{a}	14.77 ± 0.62^{a}
Whole dataset	Light	0.04 ± 0.01^{A}	0.32 ± 0.10^{A}	12.46 ± 1.66^{B}
	Dark	0.05 ± 0.01^{A}	0.31 ± 0.09^{A}	13.81 ± 1.61^{A}

*Different lowercase letters indicate significant difference ($p < 0.05$) between the combination of the skin lightness and cultivar. Different uppercase letters showed significant difference ($p < 0.05$) between the light and dark groups for the whole dataset. Whole dataset means that the data from the three cultivars were combined, and they were only grouped based on the lightness index.

Peroxide Value (PV)

Hydroperoxides are primary products of oxidation and generated through auto-oxidation or enzymatic oxidation. Auto-oxidation generally occurs at a slow rate. Meanwhile, enzymatic oxidation is more rapid and mediated by lipoxygenases (LOXs)(Buranasompob et al., 2007;

Salcedo & Nazareno, 2015). For both mechanisms, polyunsaturated fatty acids, that are abundant in walnuts(Salcedo & Nazareno, 2015), act as the main substrate. Hydroperoxides are later transformed into volatiles and this process is assisted by hydroperoxide lyases(Piccirillo et al., 2005).

The concentrations of PVs were not significantly different among Chandler, Howard, and Tulare or between the light and dark kernels, either for each cultivar or for the whole dataset (p-value > 0.05) (Table 6.2 and Table 6.5). The interaction between lightness of the kernel skin and cultivar was also not found (p-value > 0.05). Similar to FFA, previous studied on this topic is limited. It seems that the kernel skin darkening was not linked to considerable alterations in the primary lipid oxidation products.

Oxidative Stability

Oxidative stability measurement using a Rancimat apparatus is a rapid technique to understand stability of products that are prone to lipid oxidation. The measurement output is commonly expressed as induction time, which is the time when samples start to produce volatiles, i.e., volatile acids, when exposed to high temperature and oxygen pressure.

The light kernels of Chandler had significantly lower induction time than the dark kernels of Howard and Tulare. However, there was no interaction effect between kernel skin lightness and cultivar (p-value > 0.05), shown as no considerable variation of the induction time changes among Chandler, Howard, and Tulare. This demonstrated that the change in oxidative stability was not cultivar specific.

Surprisingly, the induction time was significantly higher in the dark kernels when the data from all cultivars were compared (p-value 0.0174) (Table 6.5). This might be because the dark kernels

had lower fat content, thus less substrates for the oxidation reaction. Another possible reason for the larger induction time of the dark kernels is that they had higher concentration of some phenols (Table 6.8) that might generate some antioxidant capacity during the accelerated aging condition.

While this accelerated aging technique tremendously shortens analysis time comparing to real-time shelf stability test, it has limitations and should be used with cautions(Mancebo-Campos et al., 2007). A real-time shelf-life testing for light and dark kernels would be necessary to confirm how the induction time translates into shelf life.

Volatile Organic Compounds

To assess the extent of lipid oxidation, amino acid degradation, and carbohydrate catabolism(Schwab et al., 2008), HS-SPME combined with GC-MS was used to detect multiple groups of volatile compounds, including alcohols, aldehydes, esters, furans, and ketones. The list of the compounds, along with their sensory descriptors(Acree, 2004) and peak areas, is shown in Table 6.6.

Table 6.6. Volatile organic compounds of light and dark walnuts (cv. Chandler, Howard, Tulare, and the whole dataset). The values were expressed as average ± standard deviation

Group	Compound	Sensory descriptor	Peak area (10*3)							
			Chandler		Howard		Tulare		Whole dataset	
			Light	Dark	Light	Dark	Light	Dark	Light	Dark
Alcohol	1-Octen-3-ol	Mushroom	2.81 ± 1.43^{a}	2.56 ± 0.32^{a}	4.20 ± 0.82^{a}	5.58 ± 0.19^{a}	1.83 ± 0.14^{a}	4.63 ± 2.30^{a}	2.62 ± 1.24^{A}	7.12 ± 12.11^{A}
	1-Hexanol	Resin, flower, green	4.75 ± 1.94^{a}	8.27 ± 5.75^{a}	9.88 ± 7.08^{b}	17.59 ± 8.08^{a}	7.20 ± 4.23^{a}	20.91 ± 15.14^{a}	6.65 ± 4.36^{B}	13.76 ± 10.15^{A}
	3-Buten-2-ol	Sweet	10.33 ± 3.38^{a}	10.70 ± 3.64^{a}	30.21 ± 30.22^{a}	14.35 ± 5.90^{a}	8.93 ± 4.11^{b}	15.29 ± 5.80^{a}	10.75 ± 13.93^{A}	15.75 ± 11.57^{A}
	1-Penten-3-ol	Banana, cucumber, melon	6.59 ± 2.37^{a}	7.79 ± 4.03^{a}	8.50 ± 1.90^{a}	14.47 ± 3.67^{a}	7.83 ± 2.05^{b}	18.27 ± 6.43^{a}	7.35 ± 2.08^{B}	28.51 ± 58.00^{A}
	1-Pentanol	Fruit, balsamic	2.10 ± 1.04^{a}	3.00 ± 1.43^{a}	4.43 ± 2.19^{b}	10.49 ± 3.49^{a}	1.84 ± 0.75^{a}	12.89 ± 4.37^{a}	2.54 ± 1.45^{B}	8.15 ± 5.58^{A}
Aldehyde	Hexanal	Grass, tallow, fat	58.14 ± 20.10^{a}	84.99 ± 48.40^{a}	94.12 ± 33.76^{b}	165.82 ± 23.68^{a}	84.58 ± 32.36^{b}	259.83 ± 97.79^{a}	72.34 ± 26.62^{A}	149.57 ± 95.95^{A}
	Pentanal	Almond, malt, pungent	nd	nd	nd	6.83 ± 4.69	nd	12.85 ± 6.25	nd	4.79 ± 6.40
	Butanal, 2-methyl	Cocoa, nut	10.68 ± 5.49^{b}	16.13 ± 8.66^{a}	9.90 ± 2.60^{b}	27.91 ± 11.41^{a}	7.40 ± 2.26^{a}	19.20 ± 7.26^{a}	9.81 ± 4.46^{B}	19.31 ± 9.58^{A}
	Butanal, 3-methyl	Fruit, cocoa, nut, leaf	2.09 ± 2.07^{a}	4.68 ± 2.89^{a}	3.71 ± 1.55^{a}	8.97 ± 3.30^{a}	1.56 ± 1.37^{b}	7.10 ± 4.42^{a}	2.39 ± 1.83^{B}	6.51 ± 3.61^{A}
Ester	Ethyl acetate	Pineapple	74.29 ± 43.05^{a}	101.04 ± 33.48^{a}	256.34 ± 145.27^{a}	200.41 ± 94.77^{a}	78.42 ± 15.48^{b}	140.43 ± 23.80^{a}	164.30 ± 188.58^{A}	156.43 ± 106.40^{A}
	Acetic acid methyl ester	Fruit, wine, green, ether	31.59 ± 17.48^{a}	52.20 ± 38.90^{a}	38.74 ± 19.16^{b}	54.55 ± 11.10^{a}	49.37 ± 6.75^{a}	67.12 ± 10.34^{a}	36.93 ± 16.79^{A}	55.90 ± 29.80^{A}

Furan	Butyrolactone	Caramel, sweet	1.31 ± 0.48[a]	1.50 ± 0.65[a]	4.31 ± 3.57[a]	5.71 ± 1.74[a]	1.90 ± 1.05[b]	2.89 ± 1.38[a]	2.33 ± 2.23[A]	3.04 ± 1.15[A]
Ketone	Acetone	Ether, apple, pear	1833.98 ± 925.16[b]	2607.78 ± 674.06[a]	1893.97 ± 1078.07[a]	2660.34 ± 79.15[a]	1676.17 ± 792.08[a]	3056.01 ± 573.15[a]	1813.01 ± 861.57[A]	2715.09 ± 575.12[B]
	2-Heptanone	Fruit, banana	1.29 ± 0.34[a]	2.15 ± 1.95[a]	2.24 ± 1.16[a]	5.56 ± 4.97[a]	1.63 ± 0.53[b]	3.82 ± 1.14[a]	1.59 ± 0.70[A]	3.32 ± 2.89[B]

*Different lowercase letters indicate significant difference ($p < 0.05$) between the combination of the skin lightness and cultivar. Different uppercase letters showed significant difference ($p < 0.05$) between the light and dark groups for the whole dataset. Whole dataset means that the data from the three cultivars were combined, and they were only grouped based on the lightness index.

**nd, not detected.

1-Octen-3-ol, 1-hexanol, 3-buten-2-ol, 1-penten-3-ol, and 1-pentanol were amongst the alcohols that were identified. The light Tulare kernels had less 1-octen-3-ol than the dark kernels of Howard. The light Chandler samples had lower concentration of 1-hexanol than the dark Tulare. Concentration of 3-buten-2-ol-2-methyl was similar across the skin lightness levels and cultivars. The light and dark kernels of Chandler and the light kernels of Tulare had significantly lower concentration of 1-penten-3-ol than the dark kernels of Tulare. The light and dark kernels of Chandler, the light kernel of Howard, and the light kernel of Tulare had lower concentration of 1-pentanol than the dark kernel of Tulare. The light kernels of Chandler and Tulare also had less amount of 1-pentanol than the dark Howard sample. These data suggested that Tulare dark kernels were more often to contain higher concentration of alcoholic volatiles. Higher concentration of alcohols is associated with increased ripeness level(Tietel et al., 2010). Significant difference between the dark and light kernels of the whole dataset was observed for 1-hexanol, 1-penten-3-ol, and 1-pentanol. Significant difference among Chandler, Howard, and Tulare was noted for 1-octen-3-ol, 1-penten-3-ol, and 1-pentanol. Significant interaction effect between the two parameters was shown for 1-octen-3-ol and 1-pentanol (Table 6.2).

The light kernels of Chandler and Tulare had the lowest concentrations of butanal, 2-methyl and butanal, 3-methyl, whereas the dark kernels of Howard had the highest. Significant differences between the dark and light kernels of the whole dataset were noted for the two compounds. Differences between the cultivars was not found for any aldehydes and no interaction effect between skin lightness and cultivar was apparent (p-value > 0.05).

There was no significant difference for the two esters that were identified (ethyl acetate and acetic acid methyl ester) among the varying skin lightness and cultivar. There was also no interaction effect between the two parameters. In addition to alcohols, aldehydes, and esters,

butyrolactone (a furan) and two ketones were also found. The Howard dark kernels had higher concentration of butyrolactone than the other combinations of variety and skin lightness level. Butyrolactone concentration was only significantly different among the cultivars but not between the light and dark kernels, including for the whole dataset.

Two ketones, acetone and 2-heptanone, were identified. Our data showed no significant difference for all pairwise comparison for combination of varying cultivars and skin lightness for both acetone and 2-heptanone. However, significant variation was observed when the whole dataset was used to compare the light and dark groups.

Methyl branched alcohols, aldehydes, ketones, and esters are generated through degradation of amino acids. Whereas aliphatic alcohols, aldehydes, acids, lactones, and esters are produced from lipoxygenase oxidation pathway of fatty acids. Pentanal is likely produced from oxidation of linoleic acid as well(Cao et al., 2014). It was not clear how butanal, 2-methyl and butanal, 3-methyl are produced in plants. Several studies suggested that they were produced by fungus to promote plant growth(Jiang et al., 2021) or released during heating of protein and fat(Bhatti et al., 2014). The higher concentration of pentanal in the Howard and Tulare dark kernels, compared to their respective light kernels, and butanal, 2-methyl and butanal, 3-methyl in all the dark kernels, indicated oxidation of lipid and possibly protein as well. It was not clear how butyrolactone was synthesized. However, furanones are most likely synthesized from carbohydrates(Schwab et al., 2008).

Acetone, a ketone that was present at very large amount in our samples, may be synthesized from oxidation of unsaturated fatty acids, likely linolenic acid(Cao et al., 2014), to peroxyl radicals followed by cyclization to form epidioxide radicals. Epidioxide radicals were converted to acetone through fragmentation(Fruehwirth et al., 2021). 2-Heptanone is likely produced through

oxidation of linoleic acid(Li et al., 2020). The higher concentration of acetone and 2-heptanone in the dark kernels suggested oxidation of polyunsaturated fatty acids.

Tocopherols

Tocopherols, mostly in forms of alpha, gamma, and delta, are bioactives in walnuts and help prolong shelf life of food by donating hydrogen to suppress oxidation(Balakrishnan & Schneider, 2023). They are also important antioxidants for biological systems by preventing various diseases in vitro or in mice(Gysin et al., 2002; Juretić et al., 2021).

Tocopherol concentration is often represented per oil mass as tocopherols are present in the oil phase or oil characteristics is the focus of the studies. Some studies do not clearly describe whether the values are based on oil or kernel mass(Abdallah et al., 2015; Amaral et al., 2005). In this study, the data were presented as tocopherol concentration in the kernels as the focus was the effects on kernels and to account for variability in oil content between samples.

Beta tocopherol was not discussed because it was lower than the quantification limit. Among the three types of tocopherols, gamma was the most abundant (Figure 6.1), similar to previous studies(Abdallah et al., 2015; Amaral et al., 2005). Delta and gamma tocopherol concentrations in our samples were comparable to a previous study except for alpha isomer, which may attribute to cultivar variation.

Concentrations of delta, gamma, alpha, and total tocopherols were not affected by the skin lightness level or the interaction between skin lightness and cultivar (p-value > 0.05). The comparison between the light and dark kernels were made with two scenarios: cultivar parameter included and excluded. Except for delta tocopherol, they were affected by cultivar. Chandler had

significantly higher gamma, alpha, and total tocopherol than Tulare but neither Chandler nor Tulare was significantly different from Howard, in that regards (Figure 6.1).

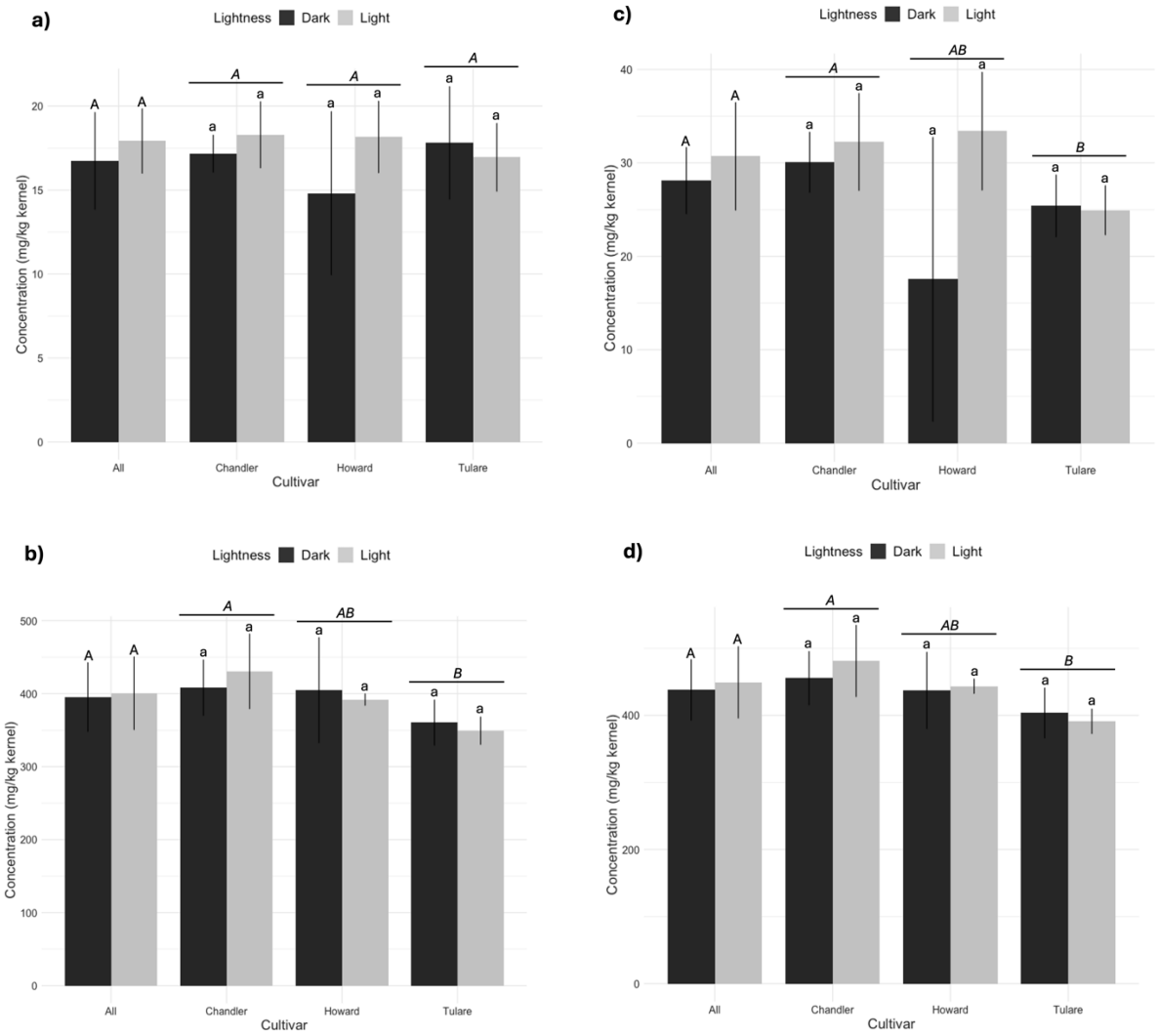

Figure 6.1. Concentration of tocopherol: a) delta, b) gamma, c) alpha, and d) total in the light and dark walnuts (cv. Chandler, Howard, Tulare, and the whole dataset). The values were expressed as average ± standard deviation.

Note: Different lowercase letters indicate significant difference ($p < 0.05$) between the combination of the skin lightness and cultivar. Different uppercase letters showed significant difference ($p < 0.05$) between the light and dark groups for the whole dataset. Whole dataset means that the data from the three cultivars were combined, and they were only grouped based on the lightness index. Different uppercase and italic letters showed significant difference ($p < 0.05$) among Chandler, Howard, and Tulare.

Our finding suggested that the different kernel skin lightness was not associated with significant changes in tocopherol concentrations in walnuts. This disagreed with a similar study that showed that amber walnuts had significantly less gamma tocopherol than extra light, light, and light amber walnuts grown in China. The discrepancy could be resulted from variation in cultivar, agricultural practices, geographical factors, and more importantly the reason underlying the variation in skin color. It was not clear what factors caused the kernel skin darkness in that study(Li et al., 2022).

Fatty Acid Profile

Fatty acids in walnut were dominated by linoleic acid, followed by linolenic or oleic acid, palmitic acid, and stearic acid. Some minor components included palmitoleic, margaric, eicosanoic, and eicosenoic acids. This profile was similar to those reported previously(Gecgel et al., 2011; Verardo et al., 2013). Our data showed that the kernel skin lightness was not associated with any changes in fatty acid profile of walnuts (p-value > 0.05), either when cultivar was included or excluded from the statistical analysis (Table 6.2 and Table 6.7). This finding was in contrast with those of Li et al. and the disagreement could be due to variation in genetics, environment, agricultural practices, and post-harvest handling(Li et al., 2022).

Cultivar affected some fatty acids. The significant difference in the fatty acid profile due to cultivar variation was noted for palmitic acid (p-value < 0.0001), stearic acid (p-value 0.0024), linolenic acid (p-value < 0.0001), eicosanoic acid (p-value < 0.0001), and eicosenoic acid (p-value 0.0001). Concentrations of palmitic, stearic, and eicosanoic acids were higher in Tulare than in Howard and Chandler. Meanwhile, Tulare had lower concentration of linolenic and eicosenoic acids than Howard and Chandler. There was no significant difference of the fatty acid

profile between Chandler and Howard. In addition, there was no significant interaction between cultivar and lightness (p-value > 0.05).

Table 6.7. Fatty acid profile of the light and dark walnuts (cv. Chandler, Howard, Tulare, and the whole dataset). The values were expressed as average ± standard deviation.

Compound	Fatty acid (%)							
	Chandler		Howard		Tulare		Whole dataset	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
C16:0	6.06 ± 0.19^{b}	6.14 ± 0.33^{b}	6.25 ± 0.23^{b}	6.39 ± 0.22^{b}	7.41 ± 0.24^{a}	7.39 ± 0.31^{a}	6.43 ± 0.62^{A}	6.49 ± 0.62^{A}
C16:1	0.06 ± 0.00^{a}	0.06 ± 0.01^{a}	0.04 ± 0.02^{a}	0.07 ± 0.00^{a}	0.06 ± 0.01^{a}	0.05 ± 0.01^{a}	0.06 ± 0.01^{A}	0.06 ± 0.01^{A}
C17:0	0.03 ± 0.02^{a}	0.03 ± 0.02^{a}	0.02 ± 0.03^{a}	0.04 ± 0.02^{a}	0.04 ± 0.02^{a}	0.04 ± 0.01^{a}	0.03 ± 0.02^{A}	0.03 ± 0.02^{A}
C18:0	2.62 ± 0.32^{a}	2.54 ± 0.23^{a}	2.60 ± 0.08^{a}	2.37 ± 0.22^{a}	3.03 ± 0.05^{a}	2.99 ± 0.06^{a}	2.72 ± 0.30^{A}	2.63 ± 0.30^{A}
C18:1	14.1 ± 0.71^{a}	14.3 ± 0.53^{a}	13.7 ± 1.33^{a}	13.7 ± 1.57^{a}	13.8 ± 1.22^{a}	13.9 ± 1.77^{a}	13.97 ± 0.87^{A}	14.11 ± 1.00^{A}
C18:2	61.7 ± 0.78a	62.0 ± 1.00^{a}	61.4 ± 2.26^{a}	61.7 ± 3.10^{a}	62.8 ± 1.37^{a}	62.5 ± 1.96a	61.94 ± 1.18^{A}	62.08 ± 1.47^{A}
C18:3	15.0 ± 0.58^{a}	14.6 ± 0.56^{a}	15.7 ± 0.70^{a}	15.4 ± 1.05^{a}	12.6 ± 0.19^{b}	12.8 ± 0.26^{b}	14.51 ± 1.28A	14.28 ± 1.07A
C20:0	0.09 ± 0.00^{bc}	0.09 ± 0.00^{c}	0.08 ± 0.01^{c}	0.08 ± 0.04^{c}	0.11 ± 0.00^{ab}	0.11 ± 0.01^{a}	0.09 ± 0.01A	0.09 ± 0.02A
C20:1	0.24 ± 0.01^{a}	0.23 ± 0.01^{a}	0.24 ± 0.00^{a}	0.22 ± 0.04^{ab}	0.21 ± 0.01^{b}	0.21 ± 0.01^{b}	0.23 ± 0.01^{A}	0.22 ± 0.02^{A}
SFA	8.81 ± 0.32^{b}	8.80 ± 0.21^{b}	8.96 ± 0.26^{b}	8.86 ± 0.49^{b}	10.59 ± 0.30^{a}	10.53 ± 0.38^{a}	9.28 ± 0.84^{A}	9.24 ± 0.82^{A}
MUFA	14.44 ± 0.71^{a}	14.58 ± 0.53^{a}	13.94 ± 1.30^{a}	14.03 ± 1.53^{a}	14.03 ± 1.22^{a}	14.20 ± 1.77^{a}	14.25 ± 0.87^{A}	14.39 ± 1.00^{A}
PUFA	76.74 ± 0.52^{a}	76.62 ± 0.65^{a}	77.10 ± 1.56^{a}	77.10 ± 2.05^{a}	75.37 ± 1.47^{a}	75.27 ± 2.13^{a}	76.46 ± 1.10^{A}	76.36 ± 1.38^{A}
MUFA / PUFA	0.19 ± 0.01^{a}	0.19 ± 0.01^{a}	0.19 ± 0.02^{a}	0.18 ± 0.03^{a}	0.19 ± 0.02^{a}	0.19 ± 0.03^{a}	0.19 ± 0.01^{A}	0.19 ± 0.02^{A}
C18:1 / C18:2	0.23 ± 0.02^{a}	0.23 ± 0.01^{a}	0.22 ± 0.03^{a}	0.23 ± 0.04^{a}	0.22 ± 0.03^{a}	0.22 ± 0.04^{a}	0.22 ± 0.02^{A}	0.23 ± 0.02^{A}

C18:2 / C18:3	4.12 ± 0.20[b]	4.25 ± 0.22[b]	3.92 ± 0.32[b]	4.04 ± 0.48[b]	4.98 ± 0.10[a]	4.87 ± 0.12[a]	4.30 ± 0.46[A]	4.37 ± 0.38[A]

*Different lowercase letters indicate significant difference ($p < 0.05$) between the combination of the skin lightness and cultivar. Different uppercase letters showed significant difference ($p < 0.05$) between the light and dark groups for the whole dataset. Whole dataset means that the data from the three cultivars were combined, and they were only grouped based on the lightness index. MUFA / PUFA: ratio of MUFA to PUFA; C18:1 / C18:2: ratio of oleic to linoleic acid; C18:2 / C18:3: ratio of linoleic to linolenic acid.

**SFA, saturated fatty acids; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Phenols

Twenty-four free phenolic compounds were analyzed in walnut kernels. Epigallocatechin gallate, vanillin, rutin, myricetin, trans cinnamic acid, procyanidin B2, syringic acid, luteolin, and kaempferol were below their limit of detection in all the samples. Other compounds are shown in Table 6.8. Our data demonstrated that the dark and light kernels differed in concentrations of several phenols. Chandler, Howard, and Tulare similarly had no detectable epicatechin gallate in the dark kernels. Significant interaction between skin lightness index and cultivar was only found on 3,4 dihydroxybenzoic acid and quercetin. Both Chandler and Tulare, but not Howard, had higher quercetin in their dark kernels than their respective light kernels. Significant change in 3,4 dihydroxybenzoic acid was only found on Tulare, whilst significant change in naringenin content was only shown in Chandler. Chandler was more adaptive, and Howard was the least adaptive among them.

When the whole dataset (cultivar variation was not considered) was used for comparison, the concentrations of gallic acid, procyanidin B1, vanillic acid, epicatechin gallate, ellagic acid, quercetin, and naringenin were different between the light and dark kernels. The dark kernels had significantly higher concentrations of gallic acid, vanillic acid, ellagic acid, quercetin, and naringenin, but less procyanidin B1 and epicatechin gallate (Table 6.8).

Table 6.8. Phenol profile of the light and dark walnuts (cv. Chandler, Howard, Tulare, and the whole dataset). The values were expressed as average ± standard deviation*

Phenol	Concentration (μg/g) kernel							
	Chandler		Howard		Tulare		Whole dataset	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Ellagic acid	731.88 ± 236.48^{a}	739.47 ± 218.90^{a}	552.14 ± 95.01^{b}	852.72 ± 67.12^{a}	729.11 ± 231.97^{a}	1254.54 ± 480.42^{a}	671.35 ± 194.80	960.93 ± 376.28
Catechin	63.90 ± 32.84^{a}	43.80 ± 13.34^{a}	65.71 ± 7.53^{a}	37.19 ± 13.86^{a}	45.63 ± 40.34^{a}	39.09 ± 41.82^{a}	60.44 ± 26.33^{A}	40.38 ± 24.22^{A}
Procyanidin B1	30.39 ± 16.27^{a}	18.82 ± 5.17^{a}	25.08 ± 1.00^{a}	11.12 ± 2.49^{b}	19.31 ± 2.14^{a}	9.13 ± 6.42^{a}	26.16 ± 11.01^{A}	13.26 ± 6.49^{B}
Gallic acid	8.70 ± 3.20^{b}	19.90 ± 6.00^{a}	9.08 ± 1.39^{b}	18.52 ± 2.62^{a}	11.34 ± 8.17^{a}	34.15 ± 22.41^{a}	9.41 ± 3.73^{B}	24.90 ± 14.62^{A}
Epicatechin	8.31 ± 7.50^{a}	4.31 ± 1.90^{a}	8.72 ± 4.59^{a}	4.64 ± 0.18^{a}	5.52 ± 1.37^{a}	2.39 ± 1.48^{a}	7.82 ± 5.32^{A}	3.67 ± 1.68^{A}
Epicatechin gallate	0.43 ± 0.12^{a}	nd^{b}	0.56 ± 0.03^{a}	nd^{b}	0.42 ± 0.07^{a}	nd^{b}	0.47 ± 0.10^{A}	nd^{B}
3,4 Dihydroxybenzoic acid	0.09 ± 0.02^{a}	0.08 ± 0.03^{a}	0.09 ± 0.02^{a}	0.10 ± 0.02^{a}	0.03 ± 0.03^{a}	0.11 ± 0.03^{a}	0.07 ± 0.03^{A}	0.10 ± 0.03^{A}
Vanillic acid	1.08 ± 0.35^{b}	2.53 ± 0.86^{a}	1.04 ± 0.35^{a}	2.04 ± 0.72^{a}	2.45 ± 1.41^{a}	2.70 ± 0.27^{a}	1.37 ± 0.83^{B}	2.47 ± 0.62^{A}
Chlorogenic acid	nd	nd	1.84 ± 0.83^{a}	2.32 ± 0.39^{a}	nd	nd	0.61 ± 1.01^{A}	0.58 ± 1.08^{A}
Caffeic acid	0.09 ± 0.01^{a}	0.07 ± 0.06^{a}	0.07 ± 0.01^{a}	0.10 ± 0.01^{a}	0.10 ± 0.04^{a}	0.10 ± 0.03^{a}	0.08 ± 0.02^{A}	0.09 ± 0.04^{A}
p-Coumaric acid	0.12 ± 0.02^{b}	0.22 ± 0.07^{a}	0.19 ± 0.16^{a}	0.18 ± 0.05^{a}	0.13 ± 0.01^{a}	0.23 ± 0.06^{a}	0.15 ± 0.09^{A}	0.21 ± 0.06^{A}
Ferulic acid	0.28 ± 0.04^{a}	0.34 ± 0.09^{a}	0.31 ± 0.10^{a}	0.30 ± 0.10^{a}	0.36 ± 0.18^{a}	0.49 ± 0.07^{a}	0.31 ± 0.09^{A}	0.39 ± 0.11^{A}
Sinapic acid	0.33 ± 0.08^{a}	0.36 ± 0.02^{a}	0.53 ± 0.14^{a}	0.37 ± 0.08^{a}	0.36 ± 0.24^{a}	0.44 ± 0.06^{a}	0.40 ± 0.15^{A}	0.39 ± 0.06^{A}
Quercetin	0.37 ± 0.06^{b}	0.65 ± 0.06^{a}	0.38 ± 0.03^{a}	0.45 ± 0.05^{a}	0.32 ± 0.11^{a}	0.47 ± 0.05^{a}	0.36 ± 0.06^{B}	0.53 ± 0.11^{A}
Naringenin	0.22 ± 0.04^{b}	0.47 ± 0.11^{a}	0.12 ± 0.03^{a}	0.25 ± 0.08^{a}	0.12 ± 0.02^{b}	0.26 ± 0.06^{a}	0.16 ± 0.06^{B}	0.34 ± 0.13^{A}

*Different lowercase letters indicate significant difference ($p < 0.05$) between the combination of the skin lightness and cultivar. Different uppercase letters showed significant difference ($p < 0.05$) between the light and dark groups for the whole dataset. Whole dataset means that the data from the three cultivars were combined, and they were only grouped based on the lightness index.

Increased concentrations of some free phenols in the dark-colored walnuts had been demonstrated in an earlier study(Wang et al., 2022) and it agreed with our findings. Because the mechanism was not explained in that study, we looked for a plausible reason in studies on other matrices. The mechanism could be similar to that occurred in tomato exposed to heat stress at which the phenol level increase was accompanied by higher activity of phenylalanine ammonia-lyase, an enzyme involved in phenol synthesis(Rivero et al., 2001). The increase in soluble phenolics likely prevented extensive oxidative damage and maintained osmotic balance(Wahid, 2007). Walnut plants responded to stress by synthesizing more phenolics. While the increased concentrations were apparent in some phenols, level of some others (i.e., procyanidin B1 and epicatechin gallate) reduced, likely because they performed their antioxidant function.

Lower amount of procyanidin B1, a catechin oligomer, in the dark kernels could be attributed to oxidation and formation of procyanidin A(Kondo et al., 2000). Another possibility for reduction of procyanidin B1 was oxidation and through that process the compound was converted into phlobaphenes or quinones. These two compounds were likely involved in the browning of pecan skin(Zhang et al., 2024). There were several possibilities of the oxidation types: autooxidation, enzymatic oxidation, and quinone-mediated oxidation(Liu et al., 2022). Procyanidin B1 had high values for all of the following antioxidants assays: Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), and deoxyribose assay that could be attributed to its multiple hydroxyl groups that were potent hydrogen donors and its conjugated double bonds that could delocalize electrons to stabilize phenoxy radical(Soobrattee et al., 2005).

Epicatechin gallate was only found in the light kernels of Chandler, Howard, and Tulare, but not in their dark kernels. Higher concentration of epicatechin gallate in walnut kernels with lighter skin was also noted by Wang et al.(Wang et al., 2022). The reduction of its concentration as

walnut skin became darker was likely due to auto-oxidation of that compound, resulting in δ- and γ- dehydrodicatechins that gave brown color(Tan et al., 2023).

Conclusion

Overall, the quality, in regards of FFA and PV, of dark and light kernels was similar. There were no changes in the fatty acid profile and tocopherols between the two groups on Chandler, Howard, and Tulare. However, several physical and chemical attributes varied between the two skin lightness groups. The dark kernels had less weight and fat content but higher oxidative stability. Concentration of eight volatile compounds (i.e., 1-octen-3-ol; 1-hexanol; 1-penten-3-ol; 1-pentanol; butanal, 2-methyl; butanal, 3-methyl; acetone; and 2-heptanone) were higher in the dark kernels. There were no significant changes in the concentration of tocopherols and fatty acid profile. On the other hand, concentrations of several phenols increased while epicatechin gallate and procyanidin B2 decreased likely because they were transformed into other compounds that gave brown color of the kernel skin. Our data suggested that the skin lightness was linked to some changes in the physical (weight) and chemical (fat content, oxidative stability, volatiles, and phenols) properties of the kernels.

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Conflict of Interest

The authors declare no conflict of interest in this study.

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Supporting Information

Table S6.1. Precursor ion (m/z), product ion (m/z), dwell time (ms), fragmentor voltage (V), collision energy (V), cell accelerator voltage (V), and polarity for each phenolic compound analyzed.

Compound	Precursor ion	Product ion	Dwell time (ms)	Fragmentor (V)	Collision energy (V)	Cell accelerator voltage (V)	Polarity
Rutin	611.2	303	10	122	13	4	Positive
Procyanidin B2	579	325	10	140	40	4	Positive
Procyanidin B1	579	321	10	140	32	4	Positive
	579	127	10	140	32	4	Positive
Epigallocatechin gallate	459.1	289	10	109	8	4	Positive
	459.1	139	10	109	20	4	Positive
Epicatechin gallate	443	139	10	100	25	4	Positive
	443	123	10	100	13	4	Positive
Chlorogenic acid	355.1	163	10	104	13	4	Positive
Myricetin	319.1	245	10	148	32	4	Negative
	319.1	153	10	148	36	4	Negative
Ellagic acid	301	271	10	155	25	4	Negative
	301	257	10	155	25	4	Negative
Quercetin	301	151	10	131	23	4	Negative
Epicatechin	291.1	139	10	99	16	4	Positive
	291.1	123	10	99	32	4	Positive
Catechin	291	139	10	89	12	4	Positive
	291	123	10	89	36	4	Positive
Luteolin	287	153	10	170	25	4	Positive
	287	135	10	170	25	4	Positive
Kaempferol	287	121	10	165	25	4	Positive
Naringenin	273	153	10	132	25	4	Positive

	273	147	10	132	21	4	Positive
Sinapic acid	223	208	10	97	13	4	Negative
	223	192.9	10	97	21	4	Negative
Syringic acid	197	182	10	89	13	4	Negative
	197	123	10	89	25	4	Negative
Ferulic acid	193	178	10	95	9	4	Negative
	193	134	10	95	17	4	Negative
Caffeic acid	179	135	10	92	17	4	Negative
Gallic acid	169	125	10	94	13	4	Negative
Vanillic acid	169	125	10	89	9	4	Positive
	169	65	10	89	25	4	Positive
p-Coumaric acid	163	119	10	86	17	4	Negative
3,4 Dihydroxybenzoic acid	153	109	10	89	13	4	Negative
Vanillin	151	136	10	79	13	4	Negative
	151	92	10	79	21	4	Negative
Trans cinnamic acid	147	103	10	74	9	4	Negative

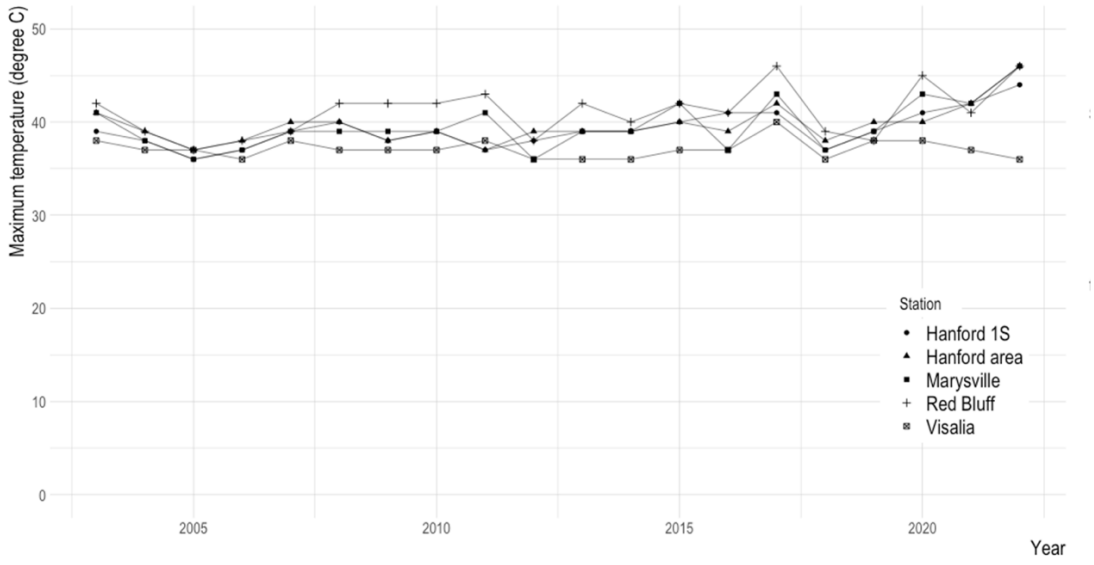

Figure S6.1. Maximum temperature (°C) recorded for September of 2003-2022 in weather stations located in Hanford 1S, Hanford area, Marysville, Red Bluff, and Visalia in California, USA

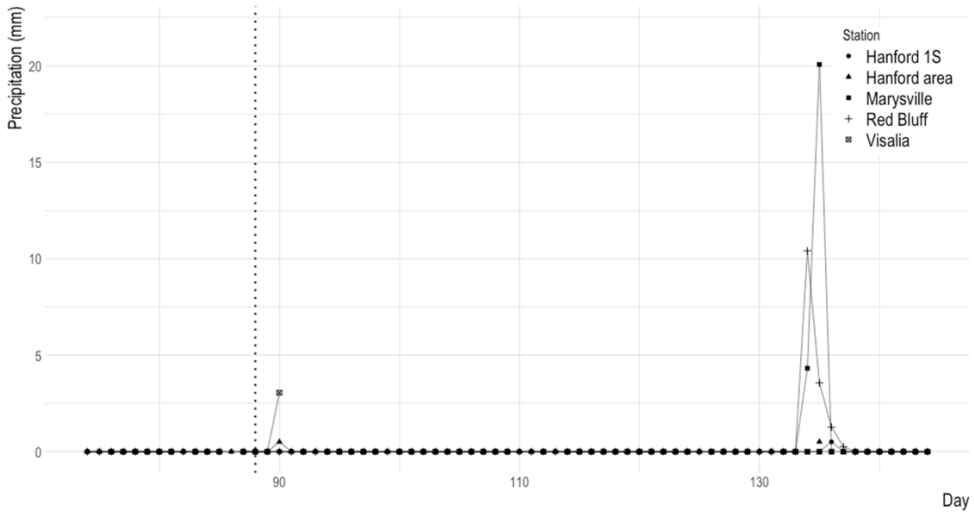

Figure S6.2. Precipitation (mm) recorded during 74-144 days after walnuts were generally pollinated (20th July – 28th September 2022) in weather stations located in Hanford 1S, Hanford area, Marysville, Red Bluff, and Visalia in California, USA

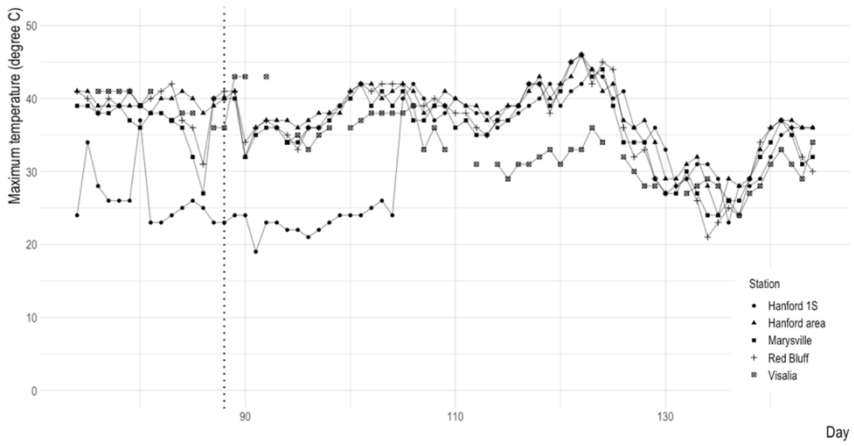

Figure S6.3. Maximum temperature (°C) recorded during 74-144 days after walnuts were generally pollinated (20th July – 28th September 2022) in weather stations located in Hanford 1S, Hanford area, Marysville, Red Bluff, and Visalia in California, USA

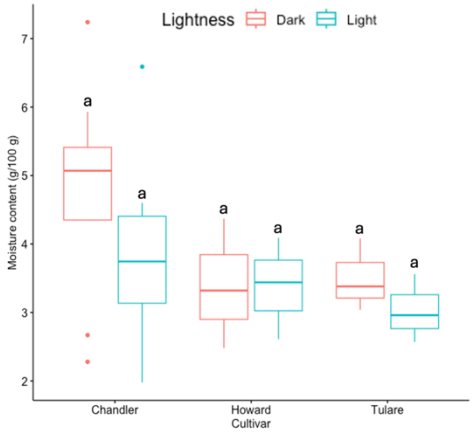

Figure S6.4. Moisture content of light and dark walnuts (cv. Chandler, Howard, and Tulare). The values were expressed as average ± standard deviation.

Note: Different lowercase letters indicate significant difference ($p < 0.05$) between the combination of the skin lightness and cultivar

CHAPTER 7

ANALYSIS OF JUGLONE IN WALNUT SHELLS AND WOODS

Abstract

Juglone is a quinone known for its environmental effects and health benefits. Juglone has long been associated with walnut plants and deleterious environmental and health effects. Recent studies, however, suggest that juglone is beneficial at low concentrations and toxic at high concentrations. Therefore, knowing the concentration of juglone in walnut industry byproducts, particularly shells and wood chips, will help determine their safety and potential to be used as value-added products. The extraction procedure was optimized, and an analysis method was developed. Three solvents (methanol, aqueous acetone, and chloroform) were compared. Methanol performed effectively for wood chips, and aqueous acetone was the optimum solvent for shells. Our data showed that juglone concentration in black walnut shells (0.45 ± 0.12 µg/g db) was similar to that in English walnut shells (0.74 – 1.70 µg/g db). Juglone concentrations in wood chips were 28.84 ± 1.54 and 65.50 ± 2.13 µg/g db for English and black walnuts, respectively. Shells and wood chips are considered safe because their juglone concentrations were lower than the level that adversely affects some animals and plants.

Keywords: juglone, walnut, shells, wood chips, byproducts

Introduction

Juglone is a quinone found in several plants, including black walnut (*Juglans nigra*)(von Kiparski et al., 2007), English walnut (*Juglans regia*)(Matławska et al., 2015), Japanese walnut (*Juglans sieboldiana*), pecan (*Carya illinoensis*)(Borazjani et al., 1985), butternut (*Juglans cinerea*), and tigernut (*Juglans mandshurica*). It is grouped into quinones because its aromatic rings have two carbonyl groups. Like other quinones, it may be formed through the oxidation of phenols, either through enzymatic(Brütsch et al., 2018; Moon et al., 2020) or non-enzymatic pathway(Jongberg et al., 2011). Another possible mechanism of juglone synthesis is phylloquinone metabolism(McCoy et al., 2018).

Juglone has abundant potential health benefits at low concentrations. Juglone suppressed the growth of multiple cancer cells, including pancreatic cells at 5 μM(Karki et al., 2020); human papillomavirus (HPV) HeLA cells MIA PaCa-2 at concentrations of 10 – 100 μM(Zhao et al., 2019); lung cancer cells C33A at concentrations of 20-100 μM(Zhang et al., 2015); and gastric cancer cells SGC-7901 at concentration of 36.51 μM(Ji et al., 2011). In addition, juglone possesses bacteriostatic, antitumor(Bhargava & Westfall, 1968), antiinflammation(Chen et al., 2021), and antioxidant activities. The antioxidant mechanisms of this compound can be through hydrogen atom donation, electron transfer(R. Jin, 2010), and metal chelation(Tamafo Fouegue et al., 2016).

Some reports demonstrate the toxic effects of juglone when the concentrations are high. Dogs that were given juglone intravenously at 5 mg/kg body weight showed hemorrhage in the lungs and necrosis and inflammation in the liver portal area. When the concentration was increased to 10 mg/kg, the effect was mortality. The results opposed the no adverse effects found when

juglone was administered at 3 mg/kg(Boelkins et al., 1968). In mice, mortality was observed when the juglone was given at 40 mg/kg of body weight.

Juglone is found to demonstrate allelopathic effects at relatively high concentrations. At 250 μM, it showed phototoxic activity against lettuce roots(Babula et al. 2014). At 1 mM, juglone suppressed shoot and root elongation of wheat, barley, corn, cucumber, watermelon, tomato, garden cress, radish, bean, and alfalfa(Kocacë Aliskan & Terzi, 2001).

Agricultural byproduct management and valorization are essential parts of overall strategies to address climate change and reduce the carbon footprint from agricultural production. Walnut shells can be utilized in various products, including fuel, furniture, and skin care, while the wood chips can be used as mulch. Information on juglone concentration in these byproducts would help determine their utilization and safety. There has not been any information on juglone concentration in walnut shells. Juglone presence in walnut woods has been reported, but the concentration is lacking(Coldea et al., 2020).

Material and Methods

Materials

Juglone or 5-hydroxy-1,4-naphtoquinone (CAS #481-39-0) was sourced from Sigma Aldrich (St. Louis, MO, USA). Water, acetonitrile, methanol, acetone, formic acid, acetic acid, and chloroform were HPLC grade or higher and obtained from Fisher Scientific (Waltham, MA, USA).

Shells were collected from English and black walnuts (Figure 7.1). The English walnut shells were of Chandler, Howard, and Hartley cultivars. No cultivar information was available for the black walnut sample. Wood chips, sized approximately 2 cm, were from unknown English and

black walnut cultivars. All the samples were sourced by the California Walnut Commission and received in our facility in December 2021. Upon receipt, the samples were stored at -20° C. Shells were separated manually from the kernels using a nut-cracking tool. Samples were analyzed not later than two months after they were received.

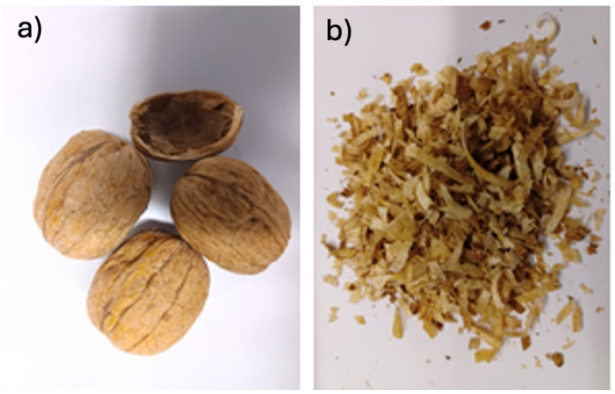

Figure 7.1. Shells (a) and wood chips (b) of walnut plants

LC-MS/MS Analysis

The method was modified from Shen et al.(Shen et al., 2021). An Agilent 1260 Infinity II LC was coupled with a 6470 triple quadrupole mass spectrometer that was equipped with Agilent Jet Stream (AJS) electrospray ionization (ESI) (Agilent Technologies, Santa Clara, CA, USA). The separation was done on a Poroshell 120 EC-C18 column (100 x 2.1 mm, 1.9 µm) heated at 40 °C. The flow rate of the mobile phase was 0.3 mL/min. Mobile phase A was water with 0.5% formic acid, and mobile phase B was a combination of acetonitrile and methanol (1:1, v/v). The auxiliary gas temperature was 350 °C with a flow rate of 12 L/min. The nebulizer pressure was set at 20 psi. The sheath gas temperature was 120 °C with a gas flow of 3 L/min. The voltages were 3000 and 500 V, for the capillary and nozzle, respectively.

Multiple reaction monitoring (MRM) was performed. Two pairs of precursor and product ions were tried on juglone in methanol (5 µg/mL). The limit of detection (LOD) and quantification (LOQ) of these two settings were determined and compared.

LC-DAD Analysis

The analysis was done using an LC-DAD (Agilent 1290 Infinity II LC system, Agilent Technologies, Santa Clara, CA, USA), and a published method for phenols(Crawford et al., 2018) was adopted with modifications. The injection volume was 20 µL, and the flow rate was 1 mL/min. Separation was done on an Agilent Zorbax Eclipse Plus C18 column (4.6 x 250 mm, 5 µm), using water: acetic acid (97:3, v/v) as mobile phase A and acetonitrile: methanol (1:1, v/v) as mobile phase B. At the start of the analysis, the mobile phase comprised 65% A and 35% B. The composition was changed to 60% A and 40% B at 5 min, 30% A and 70% B at 10 min, 0% A and 100% B at 15 min, and back to 65% A and 35% B at 20 min. Multiple wavelengths were used in the initial study (280, 320, 340, 365, 400, and 440 nm) using a juglone standard diluted in MeOH containing 1% formic acid to make a final concentration of 0.25 µg/mL. The trial employed 10 measurements at each wavelength. The wavelength that generated higher sensitivity was then selected for routine analysis.

Limit of Detection and Quantitation

Limit of detection (*LOD*) was determined using Eq.1.

$$\text{LOD} = t_{(n-1,0.99)} \; x \; \frac{RSD}{100\%} \; x \; amount \; measured$$ (Parra and Taylor 2014)

with $t_{(n-1,}$ 0.99) indicated one-tailed Student t-distribution with a 99% confidence interval, RSD was defined as relative standard distribution, and the amount measured referred to the

concentration of juglone in the injected solution. The limit of quantitation (*LOQ*) was set as 3 x *LOD*.

Solvent Optimization

Various solvents and solvent combinations were evaluated for their efficacy in extracting juglone from the walnut industry's byproducts. Wood chips were ground using a food chopper (Cuisinart, Connecticut, USA) until the particle size was reduced to ± 1 mm. The shell samples were ground using an electric grain/herb grinder (Newtry, China).

One gram of sample was added with 25 mL of the solvent (acetone:water (7:3, v/v); methanol; and chloroform) and then mixed thoroughly for 2 min using a vortex apparatus (Fisher Vortex Genie 2, Fisher Scientific, MA, USA). The mixtures were then placed in an ultrasound bath for 5 min (Turbo®sonic 6000, Raytech Industries, CT, USA). Later, they were centrifuged, and the resultant supernatant was dried (45 °C) to concentrate the extract. The drying was done using a rotavapor (Rotavapor R-300, Buchi, New Castle, USA). The dried aliquot was reconstituted with methanol containing 1% formic acid. The extract was then filtered with 0.45 µm nylon filters and subjected to HPLC analysis.

Sample Analysis

After determining the optimum solvent for each matrix, the samples were analyzed using a similar protocol as mentioned in the Solvent Optimization section, except for a few modifications. The sample amount was increased to 2.5 g and the sonication time was extended to 30 min to increase the amount of juglone detected. Three replicates were prepared for each sample.

Moisture Content

Moisture content analysis was done using an oven set at 105 °C. Approximately, 3 g of samples was dried until constant weight was reached. The analysis was done in triplicate.

Statistical Analysis

Statistical data analysis was conducted using R Studio and R 4.1.2 (The R Foundation, Boston, MA, USA). Significant difference among treatments was assessed using analysis of variance (ANOVA). When significant difference was observed, Tukey test, with $p < 0.05$, was applied to compare the means.

Results and Discussion

LC-MS/MS Analysis

Multiple reaction monitoring (MRM) mode requires selecting ions for precursor and product ions. Pairs of 173 m/z (as the precursor ion) and 145 (as the product ion) for negative electrospray ionization (ESI) and 175 m/z (as the precursor ion) and 119 m/z (as the product ion) for positive ESI were selected based on previous work(Shen et al., 2021) and in-house optimization step. Ion 145 m/z could result from the loss of CO from ion 173 m/z (Figure 7.2).

The production of ion 119 m/z from ion 175 m/z resulted from the loss of two CO groups (Figure 7.3).

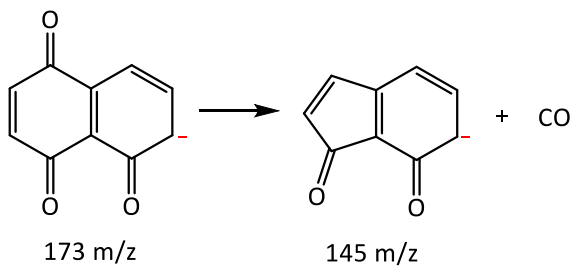

Figure 7.2. Fragmentation of deprotonated juglone

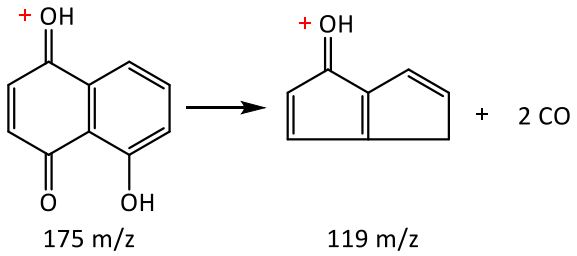

Figure 7.3. Fragmentation of protonated juglone

LOD and LOQ of these two MRM settings were later determined. The values were slightly smaller using 175 → 119 m/z pair at positive ESI than when 173 → 145 m/z (negative ESI) was applied (Table 1). The values of LOD and LOQ of the first setting (175 → 119 m/z) were identical to those reported in a similar study(Shen et al., 2021). Slightly decreased sensitivity when using the 173 → 145 m/z was likely because not all juglone molecules were converted into $[M-H]^-$ at negative ionization. Some might become $M^{\cdot -}$, as for a hydrophilic naphtoquinone (Elkin et al., 2013). It was supported by the observation of ion 174 m/z in the mass spectrum of juglone for negative ESI (Figure 7.4). This incidence might not happen at a constant ratio for all replicates used in the LOD determination. Therefore, the relative standard deviation used in quantifying LOD was slightly higher than that for positive ESI.

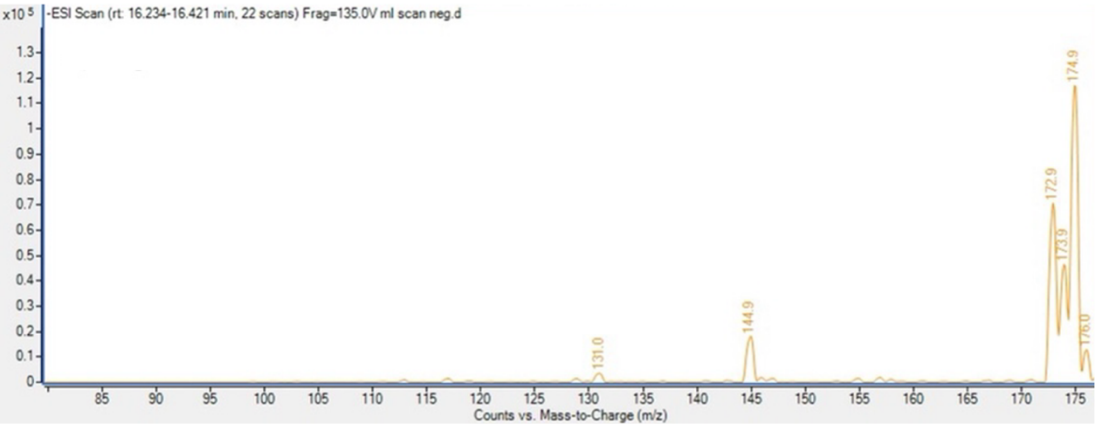

Figure 7.4. A mass spectrum of juglone measured using negative electrospray ionization (ESI) and scan mode

Table 7.1. LOD and LOQ of juglone analysis using LC-MS/MS, described as concentrations in the extracts injected to the LC and in the walnut samples

Parameters	173 → 145 at negative ESI		175 → 119 at positive ESI	
	Extract (µg/mL)	Sample (µg/g wb)	Extract (µg/mL)	Sample (µg/g wb)
LOD	0.11	0.09	0.09	0.07
LOQ	0.33	0.27	0.27	0.21

HPLC-DAD Analysis

Several wavelengths were used to determine the optimum setting for juglone detection. Reading at 400 nm yielded the highest peak areas (4.90 ± 0.19), while the minimum value was observed at 320 nm (1.36 ± 0.10) (Table 7.2). The highest standard deviation occurred at 280 nm, while the lowest was at 440 nm. Analysis conditions with the lowest RSD will benefit from lower limits of detection and quantitation and hence improved sensitivity. From the seven wavelengths above, 280 and 320 nm gave the highest RSDs. Therefore, they were avoided because high precision and sensitivity were the targets. Thus, the wavelength of 440 nm with the lowest RSD was selected for routine analysis. LOD and LOQ values were determined. The LOD and LOQ

values for the extracts were 0.02 and 0.06 µg/mL, respectively, equivalent to LOD and LOQ of 0.01 and 0.03 µg/g samples, respectively. They were 10 times lower than those reported in a previous study(Sharma et al., 2009). Because of the lower detection limits than those of LC-MS/MS, HPLC-DAD was used to analyze juglone in walnut shells and wood chips.

Table 7.2. Averages, standard deviations, and relative standard deviations (RSD)s of peak areas of juglone solution (0.25 µg/mL) at different wavelengths in HPLC-DAD

Peak area	Peak area					
	280	320	340	365	400	440
Average	1.97	1.36	2.47	1.81	4.90	4.62
Standard deviation	0.15	0.10	0.12	0.11	0.19	0.12
RSD (%)	7.86	7.64	4.91	6.22	3.97	2.53

Table 7.3. LOD and LOQ values for juglone measurement using LC-DAD, described as concentrations in the extracts injected into the LC and in the walnut samples

Parameters	Value	
	Extract (µg/mL)	Sample (µg/g wb)
LOD	0.02	0.01
LOQ	0.06	0.03

Solvent Optimization

Several solvents were assessed for their effectiveness in extracting juglone from shells and wood chips. Aqueous acetone (acetone: water 7:3, v/v) released more juglone from walnut shells than methanol and chloroform (Table 7.4). In the case of wood chips, juglone was extracted most effectively using methanol, followed by aqueous acetone, and then chloroform (Table 7.4).

Table 7.4. Concentrations of juglone extracted from shells, wood chips, and husks of walnuts using methanol, acetone: water (7:3), and chloroform

Matrix	Juglone concentration (µg/g)		
	Methanol	Acetone:water (7:3)	Chloroform
Shells	<LOD	2.17 ± 0.90^{b}	0.48 ± 0.04^{a}
Wood chips	64.35 ± 4.89^{c}	56.87 ± 7.62^{b}	2.30 ± 0.12^{a}

*Results are average and standard deviations of 7 replicates
** For the same matrix, values with different superscript letters are significantly different ($p \leq 0.05$)

The different effectiveness of the solvents in shells and wood chips is likely due to the different chemical composition, structure, rheology (i.e., hardness), moisture content, and particle size of the materials(Shirsath et al., 2012). Shells are more rigid and less porous than wood chips. Walnut shells comprise ethanol/water extractives (10.6%), lipophilic dichloromethane extractives (0.6%), lignin (29.9-49.1%), polysaccharide (49.7%), ash (0.7-3.6%), and phenols(Han et al., 2018; Pirayesh et al., 2012; Queirós et al., 2019; Wei et al., 2010). The shell's polysaccharide consists of holocellulose and α-cellulose(Pirayesh et al., 2012). Pyroligneous acid from this material contains some organic acids and phenols with antioxidant capacities(Jahanban-Esfahlan & Amarowicz, 2018; Wei et al., 2010). Walnut woods contain holocellulose, cellulose, lignin(Waliszewska et al., 2015), and phenols(Costa et al., 2021).

The effectivity of methanol in extracting juglone from wood chips could be due to better interaction with the ultrasound energy used to help release juglone from the plant cells. Solvents have varying capacities for absorbing and transmitting the ultrasound energy. Solvents/solvent mixtures with lower viscosity, lower surface tension, and medium vapor pressure increase extraction effectiveness because of intense cavitation during the ultrasound treatment(Hemwimol et al., 2006). Methanol has lower viscosity and surface tension than aqueous acetone (7:3,

v/v)(Machado et al., 1999; Song & Peng, 2008), and these two factors might explain the increased efficacy of methanol for wood chips.

It was unclear why acetone was more effective than the other solvents on shells, but our finding agreed with previous reports on similar topics. A previous study on pistachio byproducts containing shells showed that aqueous acetone also performed better than aqueous methanol, aqueous ethanol, and water in extracting phenols. Acetone or aqueous acetone also extracts higher concentrations of phenols than some other solvents from cashew nut shells (Senthil Kumar et al., 2009) and hazelnut shells(Yuan et al., 2018).

Even though juglone has been detected in walnut woods, the concentration information is lacking. Previous studies demonstrate that juglone is present in walnut heartwoods(Hosseinihashemi, 2012) and apple brandy aged with walnut wood chips (Coldea et al., 2020), but the concentrations are unavailable. In addition, there have not been any reports comparing the effectiveness of multiple solvents for extracting juglone from walnut woods.

Linearity

Good method linearity was achieved by sufficiently high r^2 values (> 0.995) (Table 7.5), making it suitable for the analytical purpose. We used different ranges of peak areas to draw the correlation between peak area and concentration for each matrix because there is considerable variation in peak area values among them.

Table 7.5. Extraction solvent, calibration equation, concentration range, and r^2 values for each matrix analyzed (shells and wood chips)

Matrix	Extraction solvent	Calibration equation	Range (μg/mL)	r^2
Shells	Acetone:water (7:3)	y = -1.36 + 24109.00x	0.12 – 1.00	0.9953
Wood chips	Methanol	y = 16.76 + 19064.00x	2.00 – 125.00	0.9994

*x: juglone concentration (μg/mL)
**y: peak area

Juglone Concentration in Samples

Shells

To our understanding, juglone presence in walnut shells had not been reported previously. Even though the composition of walnut shell pyroligneous acid had been elucidated, juglone was not mentioned(Jahanban-Esfahlan & Amarowicz, 2018; Wei et al., 2010). Juglone concentration in the shell samples ranged from 0.36 to 1.39 μg/g wet-based (wb) or from 0.45 to 1.70 μg/g dry-based (db) (Table 7.6). The concentration was lower than in the wood chips (Table 7.6) or other parts of walnut plants, i.e., leaves(Cosmulescu et al. 2014), bark, and roots(McCoy et al. 2018). The concentration of juglone in shells was comparable to or lower than in walnut husks, pellicles, and peeled kernels(Shen et al. 2021; Sheng et al. 2021). There was no significant difference in juglone concentrations among the shells of the three cultivars (Chandler, Hartley, and Howard).

Table 7.6. Juglone concentration in different parts of walnut plants from species *J. regia* and *J. nigra*. The values are the mean ± standard deviation of three replicates.

Matrix			Concentration in each matrix	
Material	Species	Cultivar	µg/g, wb	µg/g, db
Shells	*J. regia*	Chandler	1.39 ± 0.07^{c}	1.70 ± 0.08^{c}
		Hartley	0.64 ± 0.08^{c}	0.74 ± 0.09^{c}
		Howard	1.13 ± 0.15^{c}	1.34 ± 0.17^{c}
	J. nigra	n.a	0.36 ± 0.10^{c}	0.45 ± 0.12^{c}
Wood chips	*J. regia*	n.a	16.39 ± 0.88^{b}	28.84 ± 1.54^{b}
	J. nigra	n.a	36.39 ± 1.18^{a}	65.50 ± 2.13^{a}

* n.a: not available
** Values with different superscript letters, for the same material and column, are significantly different ($p \leq 0.05$)

Based on the maximum concentration of juglone in walnut shells found in the present study (1.70 µg/g) and the concentration at which juglone can give adverse acute effects on dogs (5 mg/kg body weight)(Boelkins et al., 1968), dogs need to consume approximately 3 kg shell/kg body weight or about 45 kg of shells for a 15-kg dog to get the adverse effects. Consumption of shells at that very high concentration is very unlikely. Therefore, walnut shells seem safe for dogs, considering their low juglone content. It should also be safe for mice because the mortality-induced level of juglone in mice is 40 mg/kg body weight(Jin et al., 2016), and it is equivalent to consuming 600 g of shells by a 25-g mouse to obtain the fatal effect. Similar to dogs, it is very unlikely that mice will eat that many walnut shells. Additionally, the concentration of juglone in walnut shells does not seem to impose an allelopathy effect on field poppy. At 1.15 mM, juglone inhibits field poppy growth completely(Islam & Widhalm, 2020; Topal et al., 2007). To achieve that molarity, 30 kg of shells per 0.25 L of water is needed. The high ratio of shells to water used by the plant is unlikely to occur. A similar concentration is needed for the allelopathy effect of

juglone on wheat, barley, corn, cucumber, watermelon, tomato, garden cress, radish, bean, and alfalfa(Kocacë Aliskan and Terzi 2001).

Wood chips

The wood chips of walnut plants contained juglone. Hashemi found juglone in the heartwood extract of English walnuts, but only % of the area of gas chromatography peaks, not the absolute concentration value, was reported(Hosseinihashemi, 2012). The presence of juglone in English walnut wood chips has also been reported by Coldea et al., as evidenced by the transfer of juglone from the chips to traditional Romanian apple brandy during a rapid aging process, but the concentration of juglone in the chips was not provided(Coldea et al., 2020).

In black walnut samples, juglone concentration was 65.50 ± 2.13 µg/g db (36.39 ± 1.18 µg/g wb). The concentrations were significantly higher than in the English walnut woods (28.84 ± 1.54 µg/g db) (Table 7.6). Juglone concentration in wood chips was lower than in English walnut barks(Medic et al., 2021) and leaves(Cosmulescu et al., 2014; Gîrzu et al., 1998), and *Juglans mandshurica* branch(Li et al., 2022) and higher than in English walnut pellicle and kernels(Shen et al., 2021). It showed that juglone distribution was not uniform across walnut species and plant parts.

The concentration of juglone in the wood chips did not warrant safety concerns, based on the reported toxic level for dogs, mice, and field poppy. At 5 mg juglone/kg body weight, dogs develop lung hemorrhage, necrosis, and inflammation in the liver portal area(Boelkins et al., 1968). That concentration is obtained when dogs eat about 71 g wood chips/kg body weight or about 1.07 kg wood chips for a 15-kg dog. Since dogs are carnivores, intentional consumption of wood chips is uncommon. Contamination might happen, but it is unlikely at a high level, i.e., 1.07 kg. Mortality in mice due to juglone intake is achieved when the concentration is 40 mg/kg

body weight(Jin et al., 2016). That level is equivalent to 571 g wood chips/kg body weight, which is very unlikely to happen. For field poppy, complete growth inhibition occurs when the concentration of juglone is 0.20 g/L of water(Topal et al., 2007) or equal to 2.86 kg wood chip/L of water. For other plants like tomato, garden cress, and corn, the inhibition concentration of juglone is 1mM or equal to 2.49 kg of wood chips/L of water, a high concentration that does not seem to be expected in the environment.

Conclusions

Shells and wood chips are byproducts of the walnut kernel industry that can be valorized into value-added products. Since juglone is a compound with toxic effects at high concentrations, it is vital to understand the concentrations in each part of the walnut byproducts for safe utilization. A procedure for juglone extraction and analysis from shells and wood chips has been developed. Three solvents were evaluated for extraction. Using methanol led to more juglone extracted from the wood chips than chloroform and aqueous acetone. Aqueous acetone was more efficient for shell samples than the two other solvents. The difference in solvent effectiveness between wood chips and shells is likely due to the matrices' different structures, composition, rheology, moisture content, and particle size. Our data suggested that wood chips contained more juglone than shells. The level of juglone in wood chips and shells did not warrant any safety concerns, based on the reported toxic level in some animals and plants.

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CHAPTER 8

CONCLUSIONS

Walnut is a nutritious food and an important crop for California. Numerous pre- and post-harvest factors potentially affect walnut quality and chemical characteristics. The lengthy distribution process, from handlers in California to consumers throughout the US or foreign countries, mainly resulted in quality degradation. Many California walnuts sold at retail, either in the US or overseas, showed signs of lipid and phenol oxidation, evidenced by higher K_{232} and K_{268} and lower antioxidant capacity than reference (samples sourced directly from handlers). However, several samples showed minimum oxidation, indicated by similar K_{232}, K_{268}, and antioxidant capacity to the reference. This indicates that minimizing oxidation during distribution to consumers is possible, and better distribution practices and guidelines are needed.

Variations in California walnuts' cultivars (Chandler, Howard, and Tulare) did not affect quality significantly. Similar findings were found for alpha- and delta-tocopherols and the stable isotope ratios of some elements ($\delta^{15}N_{\text{amino acids}}$, $\delta^{13}C_{\text{amino acids}}$, $\delta^{2}H_{\text{fatty acids}}$, and $\delta^{13}C_{\text{fatty acids}}$). Some significant differences were observed for TAG, fatty acid, beta-sitosterol, and gamma-tocopherol. These differences did not manifest in varying lipid oxidation levels (peroxide value and volatile oxidation markers). However, it should be noted that the measurement was done not long after the samples were collected. Some significant changes in the oxidation products might be apparent if the samples were subjected to long-term storage.

Some similarities and differences in the quality factors and metabolites of walnuts of varying countries of origin were observed. Based on the FFA, PV, and volatile data, the China samples seemed slightly more oxidized than the US samples while the India samples were much more

oxidized than either the China or the US samples. The higher quality of the US samples coincided with the higher concentration of alpha-tocopherol and catechin, which are both known as powerful antioxidants. The higher concentration of many volatiles in the India samples was accompanied by a lower proportion of linoleic acid. Linoleic acid was likely depleted because it has been converted into hydroperoxides and then volatiles during oxidation. The China, India, and the US samples were similar in their total phenol but differed in the concentrations of gallic acid, catechin, chlorogenic acid, procyanidin B1, vanillic acid, and ferulic acid. Differentiation of geographical origins of walnuts could be achieved when linear discriminant analysis was done on triacylglycerol, volatile, phenol, and CSIA-amino acids ($\delta^{13}C_{amino\ acid}$ and $\delta^{15}N_{amino\ acid}$) and fatty acids ($\delta^{13}C_{fatty\ acid}$ and $\delta^{2}H_{fatty\ acid}$) or CSIA-fatty acids only.

In the harvest year of 2022, many walnuts that were recently harvested had darker kernel skin than usual, and it was linked to heat wave occurring at the final kernel maturation period. The skin darkening was accompanied by a reduction of kernel weight and fat content, an increase in volatile concentrations, and an increase in the concentrations of some phenols, and a decrease in the concentrations of epicatechin gallate and procyanidin B2. The changes are parts of plant adaptation strategy. Our data showed that the process involved the oxidation of lipids (indicated by the increased levels of some volatiles) and enhanced synthesis of phenols.

Byproduct utilization has multiple benefits, including waste reduction and increased economic gain. Walnut farming generates various byproducts, including shells and wood chips that can be valorized into various products. However, concern over juglone, that is toxic at high concentrations, might reduce the byproduct utilization. Therefore, its concentration in shells and wood chips should be determined. Our data suggested that the level of juglone in wood chips and

shells did not warrant any safety concerns, based on the published toxic level of juglone for some animals and plants.