

NOTE

Comparative Evaluation of Fatty Acid Composition, Tocopherols, and Volatile Compounds of Walnut Oil between *Juglans mandshurica* Maxim. var. *sachalinensis* (Komatsu) Kitam and *J. regia* L.

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Abstract: We investigated the fatty acid composition and regiospecific distribution of triacylglycerol in *Juglans mandshurica* Maxim. var. *sachalinensis* (Komatsu) Kitam and *Juglans regia* L. oils. Significant differences are observed in the fatty acid compositions and regiospecific distribution of triacylglycerol in both oils. In addition, we measured volatile compounds and tocopherol content in two walnut oils. In results of volatile compound analysis, vanillin is specifically detected from *J. mandshurica* var. *sachalinensis* oil, and was not detected in *J. regia* L. oil. Notably, γ -tocopherol content in the *J. mandshurica* var. *sachalinensis* oil was significantly higher than *J. regia* L. oil.

Key words: walnut oil, *Juglans mandshurica* var. *sachalinensis*, *Juglans regia* L., fatty acid composition, tocopherol content, volatile compound

1 Introduction

Juglans regia L. is one of the most popular walnuts all over the world, and it has well been investigated its function and nutrition^{1,2)}. In particular, unsaturated fatty acids (UFAs), including oleic acid, linoleic acid, and α -linolenic acid, are abundant in *J. regia* L. oil³⁻⁹⁾. The intake of UFAs including α -linolenic acid from *J. regia* L. oil can decrease total plasma cholesterol and low-density-lipoprotein (LDL) cholesterol, thereby reducing the risk of coronary heart disease¹⁰⁻¹²⁾.

Triacylglycerol (TAG) is a major component of edible oils and consists of three fatty acids and one glycerol moiety. The binding positions of fatty acids on TAG are the *sn*-1,3 and *sn*-2, according to the primary and secondary alcohol groups on glycerol, respectively. The TAG molecular species from *J. regia* L. oil are mainly composed of trilinolein (LLL), followed by dilinoleoyl-linolenoyl-glycerol (LLLn) and dilinoleoyl-oleoyl-glycerol (OLL)^{7,9,13,14)}. Recent studies have revealed that the binding position of fatty acids on TAG affects the functions of fats and oils, such as

absorption and oxidative stability. Martin *et al.* revealed that the TAG containing UFAs at the *sn*-2 position was less oxidized than that at the *sn*-1,3 position¹⁵⁾. Therefore, the effect of the regiospecific distribution of fatty acids on oxidative stability needs to be assessed in order to estimate the quality of walnut oils. In addition to UFAs, walnut oil contains tocopherols, which inhibit the oxidation of UFAs. Tocopherol isomers of *J. regia* L. oil is mainly composed of α -, γ -, and δ -tocopherol^{3,6,8,9,16-18)}. Among them, α - and γ -tocopherol are the most abundant natural antioxidants in vegetable fats¹⁹⁾. Tocopherols have numerous beneficial properties, such as anti-inflammatory and antiproliferative effects in human cancers²⁰⁾. In this way, walnut oils display many benefits to human health and are good sources of UFAs and tocopherols. To present, few studies have investigated tocopherol content in walnut species other than *J. regia* L.

J. mandshurica Maxim. var. *sachalinensis* (Komatsu) Kitam (Onigurumi in Japanese) is endemic to Japan, with only a few studies reporting on the species. Chaudhary *et*

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al. reported that extracts from leaves and immature fruits of *J. mandshurica* var. *sachalinensis* and *J. mandshurica* var. *cordiformis* have a transthyretin amyloid fibril disruption ability²¹⁾. Machida *et al.* isolated four enantiomerically pure α -tetralones and three phenolic glycoside syringates from the fruits and bark of *J. mandshurica* var. *sachalinensis*^{22, 23)}. However, fatty acid composition and regiospecific distribution of fatty acids in Japanese walnut oil are not well understood.

This study aimed at comparing the composition and regiospecific distribution of fatty acids between *J. mandshurica* var. *sachalinensis* and *J. regia* L. oils. In addition, volatile compounds and tocopherol content were also investigated between two walnuts oils.

2 Materials and Methods

2.1 Chemicals and materials

J. mandshurica var. *sachalinensis* were collected from Aizu-Wakamatsu City, Fukushima, Japan. *J. regia* L., imported from the USA, were purchased from Assist Food Inc. (Gunma, Japan). CHIRAZYME L-2 C4 and other reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

2.2 Preparation of walnut oil

Walnut oil from *J. mandshurica* var. *sachalinensis* and *J. regia* L. were extracted respectively, according to the method of Folch *et al.* with slightly modification²⁴⁾. In brief, the walnut sample (100 g) was homogenized in 10 volumes of a mixture of chloroform/methanol (2/1, v/v). The homogenate was vacuum filtered through a filter paper using a Buchner funnel. The filtrates were evaporated in vacuo to dryness and used as walnut oil for analysis.

2.3 Enzymatic reaction of TAG in walnut oil

The regiospecific distribution of fatty acids in walnut oil was evaluated using CHIRAZYME L-2 C4²⁵⁾. Walnut oil (0.5 g) was mixed with 5 g of 99.5% ethanol and 0.33 g of CHIRAZYME L-2 C4. The mixture was incubated in a 30°C water bath for 3 h with shaking at 150 rpm and dried in a rotary evaporator. The resulting oil (0.1 mL) was applied to a Sep-Pak Silica cartridge (0.65 g, Waters Co., Milford, MA, USA), which was pre-equilibrated with a solvent mixture of hexane and diethyl ether (8:2, v/v), and eluted with *n*-hexane and diethyl ether to remove the free fatty acid and diacylglycerol fractions. Then, 2-monoacylglycerol (2-MAG) was eluted with diethyl ether and dried in a rotary evaporator.

2.4 Analysis of fatty acid composition in walnut oil and 2-MAG fraction

The walnut oil and 2-MAG fractions prepared enzymati-

cally were methyl esterified according to a modified version of the American Oil Chemist's Society (AOCS) official method, Ce 1b-89²⁶⁾. Walnut oil (50 mg) was mixed with 1.0 mL of 0.5 N sodium hydroxide in methanol and heated at 100°C for 5 min. The resulting solution was added to 1.5 mL of 14% boron trifluoride solution in methanol and reheated at 100°C for 5 min. After cooling the solution to room temperature, 1.5 mL of hexane and 3.0 mL of 20% saline solution were added and mixed. The supernatant was transferred to a new screw-capped sample tube and used as a sample for analysis of fatty acid composition.

The fatty acid methyl esters were subjected to a GC-flame ionization detector (GC-FID) system (Nexis GC-2030, Shimadzu Corporation, Kyoto, Japan) equipped with an InertCap Pure-WAX capillary column (30 m $\times$ 0.25 mm ID, 0.25 μ m, GL Sciences Ltd., Tokyo, Japan). The temperature of the injection port and detector was set at 250°C. The column temperature, was initially set at 40°C for 5 min and increased to 250°C at a rate of 10°C/min and held for 15 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. The split ratio was 50/1 (v/v), and the injection volume was 1 μ L. The fatty acid species were identified using the retention time of a standard fatty acid methyl ester (Supelco 37 Component FAME Mix, Merck, Darmstadt, Germany). The content of each fatty acid was calculated using the GC-FID chromatogram.

2.5 Quantifications of tocopherols in walnut oil

Tocopherol content was quantified using an HPLC system that included a PU-2080 plus pump (JASCO Corporation, Tokyo, Japan), a CO-2065 Plus column oven at 40°C (JASCO), and an FP-2020 Plus fluorescence detector (JASCO). The mobile phase was hexane/acetic acid (85:15, v/v) and the flow rate was 1.0 mL/min with monitoring at 298 nm and 325 nm for excitation (Ex) and emission (Em), respectively. One gram of walnut oil was dissolved in 10 mL of hexane and add 2,2,5,7,8-pentamethyl-6-hydroxychroman as an internal standard, and 10 μ L of the prepared samples were injected into an Inertsil NH₂ column (250 mm $\times$ 4.6 mm, 5 μ m particle size, GL Sciences Ltd.). The amounts of each tocopherol were calculated using Vitamin E Reference Standard (FUJIFILM Wako Pure Chemical Corporation).

2.6 Analysis of volatile compounds in walnut oil

A solid phase microextraction (SPME) fiber (length 10 mm) coated with 50/30 mm divinylbenzene/carbon WR/polydimethylsiloxane (DVB/CAR/PDMS) phase (Restek, Tokyo, Japan) was used to extract volatile compounds. The fibers were conditioned before use and thermally cleaned between analyses by inserting them into the injector port of the gas chromatography system set at 270°C for 30 min in a stream of helium.

Headspace SPME was used to extract the headspace

volatiles from the samples. The walnut oils (500 mg) were placed in a 20 mL headspace vial fitted with a silicone septum. As an internal standard, 10 μ L of cyclohexanone (1,000 μ g/mL in methanol) was added. After an equilibration time of at least 10 min, SPME sampling was performed by exposing the fiber for 60 min in the headspace of the sample at 50°C. After sampling, the SPME device was placed into a GC system equipped with a mass spectrometer (GC: Nexis GC-2030, MS: TQ8050NX, Shimadzu Corporation) and a capillary column (InertCap Pure-WAX, 30 m $\times$ 0.25 mm ID, 0.25 μ m thickness, GL Sciences Ltd.). The column temperature was set at 40°C for 5 min, then raised to 250°C at 5°C/min, and held for 15 min. The injector temperature was set to 250°C. The flow rate of the carrier gas (helium) was 1.0 mL/min. The mass spectrometer detector was operated in the electron impact ionization mode at 70 eV. The analysis was performed in SCAN mode in the 30–550 m/z range. Tentative identification of constituents was based on comparison of the retention time and mass fragmentation with pure standards, on computer matching with commercial mass spectra libraries (NIST & WILEY) and a home-made library built from pure substances, all analyzed under identical conditions.

2.7 Statistical analysis

Across all the experiments, our analyses considered the average of triplicate measurements from two species of walnut oils. A student's *t*-test was used to estimate differences between a 33.3 mol/100 mol as a theoretical value with the experimental regiospecific value of each fatty acid at the *sn*-2 position. To determine the tocopherol content, student's *t*-test was also performed to assess differences between oils. Statistical significance was set at $p < 0.05$.

3 Results and Discussion

This is the first report to investigate the composition and regiospecific distribution of fatty acids in oil extracted from *J. mandshurica* var. *sachalinensis*. *J. mandshurica* var. *sachalinensis* and *J. regia* L. contained 41.7 and 46.5 g of oil per 100 g of kernels, respectively. Table 1 shows the total fatty acid composition and fatty acid composition at the *sn*-2 position of TAG in *J. mandshurica* var. *sachalinensis* and *J. regia* L. Total fatty acid composition was similar between the *J. mandshurica* var. *sachalinensis* and *J. regia* L. oils. Linoleic acid was the most abundant (71.7 and 60.8 g/100 g oil, respectively), followed by oleic acid (13.0 and 15.0 g/100 g oil, respectively), and α -linolenic acid (11.1 and 15.2 g/100 g oil, respectively). In contrast, palmitic acid was a major component of the saturated fatty acids in both *J. mandshurica* var. *sachalinensis* and *J. regia* L. oils, with values of 2.8 and 6.1 g/100 g oil, respectively. Li *et al.* investigated the fatty acid composition of *J. mandshurica* var. *cordiformis*, a close relative of *J. mandshurica* var. *sachalinensis*, and found that the major fatty acids consisted of linoleic acid (69.0–72.1%), oleic acid (13.0–13.9%), α -linolenic acid (8.0–12.0%), and palmitic acid (2.4–2.7%)²⁷. These findings are consistent with those of *J. mandshurica* var. *sachalinensis* in the present study, although Li *et al.* used walnuts from Canada²⁷. This suggests that fatty acid compositions among a close relative of walnut is closely similar, irrespective of the place and environment.

The majority of the fatty acids at the *sn*-2 position of TAG in *J. mandshurica* var. *sachalinensis* and *J. regia* L. was linoleic acid (75.3 and 69.9 g/100 g oil), followed by oleic acid (16.8 and 15.6 g/100 g oil, respectively), and α -linolenic acid (7.5 and 13.9 g/100 g oil, respectively). The regiospecificity of each fatty acid at the *sn*-2 position of TAG in the *J. mandshurica* var. *sachalinensis* and *J. regia* L. oil is shown in Fig. 1. In the regiospecific distribu-

Table 1 Regiospecific analysis of fatty acid composition (g/100 g oil) in *J. mandshurica* var. *sachalinensis* and *J. regia*.

Fatty acid	Total (g/100 g oil)			<i>sn</i> -2 (g/100 g oil)		
	<i>J. mandshurica</i> var. <i>sachalinensis</i>	<i>J. regia</i> L.	<i>p</i> -value	<i>J. mandshurica</i> var. <i>sachalinensis</i>	<i>J. regia</i> L.	<i>p</i> -value
Palmitic acid (C16:0)	2.8 $\pm$ 0.0	6.1 $\pm$ 0.0	< 0.001	0.25 $\pm$ 0.0	0.33 $\pm$ 0.0	< 0.001
Stearic acid (C18:0)	0.8 $\pm$ 0.0	2.4 $\pm$ 0.0	< 0.001	N.D.	N.D.	
Oleic acid (C18:1)	13.0 $\pm$ 0.0	15.0 $\pm$ 0.0	< 0.001	16.8 $\pm$ 0.0	15.6 $\pm$ 0.0	< 0.001
Linoleic acid (C18:2)	71.7 $\pm$ 0.0	60.8 $\pm$ 0.0	< 0.001	75.3 $\pm$ 0.0	69.9 $\pm$ 0.0	< 0.001
α -linolenic acid (C18:3n3)	11.1 $\pm$ 0.0	15.2 $\pm$ 0.0	< 0.001	7.5 $\pm$ 0.0	13.9 $\pm$ 0.0	< 0.001
Eicosenoic acid (C20:1n9)	0.2 $\pm$ 0.0	0.2 $\pm$ 0.0	0.01	N.D.	N.D.	

The data are represented as mean $\pm$ SD (n = 3).

N.D.: Not detected (< 0.1).

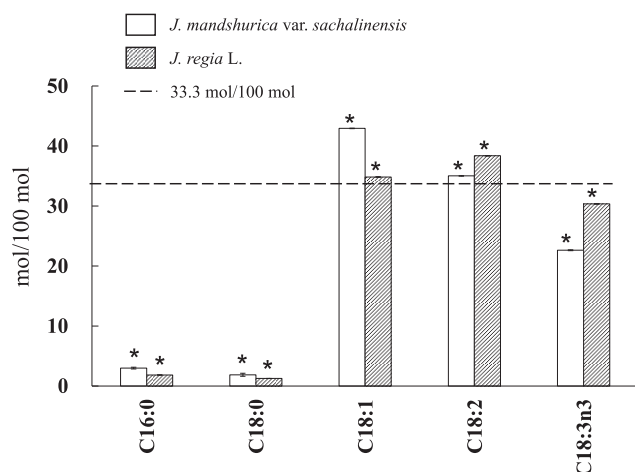


Fig. 1 Regiospecific analysis of fatty acids at the *sn*-2 positions of TAG (mean $\pm$ SD) in *J. mandshurica* var. *sachalinensis* and *J. regia* L. oils. The dotted line at 33.3 mol/100 mol indicates the regiospecific value at which the fatty acid is equally distributed. Single asterisks are shown a significantly differences from 33.3 mol/100 mol ($p < 0.05$).

tion of fatty acids, palmitic, stearic, and α -linolenic acids were selectively esterified at the *sn*-1,3 position in both *J. mandshurica* var. *sachalinensis* and *J. regia* L. oil. Meanwhile, oleic and linoleic acids tended to bind to the *sn*-2 position. Previous studies have suggested that differences in fatty acid and TAG composition among walnut varieties may be attributed to genetic factors^{7, 8, 15}. However, Amaral *et al.* investigated the TAG composition of 26 walnut (*J. regia* L.) samples of different geographical origins and suggested that the TAG composition can be strongly influenced by not only genetic factors but also environmental factors such as growth conditions¹³. Further research is required to elucidate the effects of genetic and environmental factors on the fatty acid composition in *J. mandshurica* var. *sachalinensis* oil. According to previous reports, palmitic, stearic, and α -linolenic acids were selectively esterified at the *sn*-1,3 position, and oleic and linoleic acids were selectively esterified at the *sn*-2 position of TAG in *J. regia* L. oil^{7, 8, 13, 14}. This tendency supports our results for the regiospecific distribution of fatty acids in *J. regia* L. oil (Fig. 1). We previously investigated the effect of the binding position of fatty acids in TAG on the catabolic rates of fatty acids, and observed that the catabolic rate of palmitic, oleic, and α -linolenic acids bound to the *sn*-2 position of TAG is slowly catabolized for long duration compared with each was bound to the *sn*-1,3 position^{28, 29}. We proposed that saturated fatty acids bound to the *sn*-1,3 position in walnut oils can more rapidly be catabolized and converted to energy. In addition, high concentrations of UFAs in *J. regia* L. oils reduce LDL concentrations and enhance the level of high-density lipoprotein

Table 2 Volatile composition (mg/kg) of *J. mandshurica* var. *sachalinensis* and *J. regia* L. oil.

	<i>J. mandshurica</i> var. <i>sachalinensis</i>	<i>J. regia</i> L.
Aldehyde		
Hexanal	1.38 $\pm$ 0.02	0.24 $\pm$ 0.01
2,4-Heptadienal	2.49 $\pm$ 0.06	N.D.
Nonanal	0.19 $\pm$ 0.00	0.15 $\pm$ 0.00
2,4-Decadienal	1.85 $\pm$ 0.27	N.D.
Benzaldehyde	1.00 $\pm$ 0.03	N.D.
Alcohol		
1-Pentanol	0.30 $\pm$ 0.01	N.D.
1-Hexanol	0.96 $\pm$ 0.00	0.49 $\pm$ 0.02
Ketone		
3,5-Octadien-2-one	5.49 $\pm$ 0.08	0.80 $\pm$ 0.05
Others		
Vanillin	1.47 $\pm$ 0.32	N.D.

The data are represented as mean $\pm$ SD (n = 3).

N.D.: Not detected.

(HDL) and are associated with a decreased risk of cardiovascular diseases^{2, 15}. In particular, α -linolenic acid in the walnut oil has been reported to decrease the amount of LDL cholesterol^{12, 30}. Accordingly, *J. mandshurica* var. *sachalinensis* oil is a good source of α -linolenic acid, similar to that of *J. regia* L. oils.

The volatile components were detected in the *J. mandshurica* var. *sachalinensis* and *J. regia* L. oil and are detailed in Table 2. 1-Hexanol, heptanal, hexanal, and nonanal were detected in *J. regia* L. oil (Table 2), which is consistent with previous reports^{4, 6, 7, 31}. On the other hand, in *J. mandshurica* var. *sachalinensis*, the major aldehydes were hexanal, 2,4-heptadienal, nonanal, 2,4-decadienal, and benzaldehyde. Other volatile components were 1-pentanol, 1-hexanol, 3,5-octadien-2-one, and vanillin. In this study, the secondary oxidation products such as 2,4-heptadienal, 2,4-decadienal, and benzaldehyde were only detected in *J. mandshurica* var. *sachalinensis* oil (Table 2). The secondary oxidation products, such as aldehyde, alcohol, and ketone, are typical lipid hydroperoxide derivatives derived from oleic acid, linoleic acid, and α -linolenic acid^{4, 8, 16}. More interestingly, vanillin, which contributes to sweet and vanilla aromas, was only detected in *J. mandshurica* var. *sachalinensis* oil. This suggests that vanillin is specific to *J. mandshurica* var. *sachalinensis* oil, and was not detected in *J. regia* L. oil in this study and previous studies^{4, 6, 7, 31}.

Table 3 shows the tocopherol content of the *J. mandshurica* var. *sachalinensis* and *J. regia* L. oils. There were no significant differences in δ -tocopherol content between

Table 3 Tocopherol content ($\mu\text{g/g}$) of *J. mandshurica* var. *sachalinensis* and *J. regia* L. oil.

	<i>J. mandshurica</i> var. <i>sachalinensis</i>	<i>J. regia</i> L.	<i>p</i> -value
γ -tocopherol	745 ± 22	374 ± 22	<0.001
δ -tocopherol	30 ± 9	15 ± 2	0.056

The data are represented as mean $\pm$ SD (n = 3).

J. mandshurica var. *sachalinensis* and *J. regia* L oil (30 and 15 $\mu\text{g/g}$, respectively). No α - or β -tocopherol was detected in both oils ($<4.73 \mu\text{g/g}$). In this study, *J. regia* L. oil consisted of γ -tocopherol (374 $\mu\text{g/g}$) and δ -tocopherol (15 $\mu\text{g/g}$), which are consistent with previous reports where γ -tocopherol was the main component (197-375 $\mu\text{g/g}$), followed by α - (12.3-38.0 $\mu\text{g/g}$) and δ -tocopherol (5.38-62.1 $\mu\text{g/g}$)^{3,8,9,13}. On the other hand, the γ -tocopherol content in *J. mandshurica* var. *sachalinensis* oil (745 $\mu\text{g/g}$) was significantly higher than that of *J. regia* L. oil (Table 3). Tocopherols are important antioxidant components of *J. regia* L. oil^{8,16}. The distribution of tocopherol isomers can also affect the formation and composition of volatile oxidation compounds³². Based on this, it is assumed that content of tocopherols plays a key role as antioxidation agent in walnut oils. Our results suggest that the abundant γ -tocopherol in *J. mandshurica* var. *sachalinensis* suppresses UFA oxidation, when the walnut oil stored at long term. Additionally, γ -tocopherol can effectively reduce platelet aggregation, LDL oxidation, and delay intra-arterial thrombus formation³³. We therefore propose that the high γ -tocopherol content in *J. mandshurica* var. *sachalinensis* identifies it as a potentially important source of natural antioxidants in the maintenance of healthy bodily function.

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