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ORIGINAL PAPER

Lipid Classes, Fatty Acid Distributions and Triacylglycerol Molecular Species of Broad Beans (*Vicia faba*)

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Abstract Seed oils from four legume cultivars of *Vicia faba*, grown in Japan, were extracted and classified by thin-layer chromatography (TLC) into eight fractions. The major lipid components were triacylglycerols (TAG: 48.8–50.1%) and phospholipids (PL: 47.5–50.5%), while hydrocarbons (HC), steryl esters (SE), free fatty acids (FFA), diacylglycerols (1,3- and 1,2-DAG) and monoacylglycerols (MAG) were present in minor proportions (1.8–2.4%). All lipid samples had high amounts of total unsaturated FA, representing 79.7–82.8% and 77.6–79.7% for TAG and PL, respectively. Molecular species and FA distributions of TAG, isolated from the total lipids in the broad beans, were analyzed by a combination of argentation-TLC and GC. Fourteen different molecular species were detected. With a few exceptions, the main TAG components were S_2D (6.1–8.9%), SD_2 (7.8–10.5%), SMT (6.3–8.5%), M_2D (4.5–6.2%), MD_2 (18.9–21.8%), D_3 (21.0–23.9%) and MDT (8.1–10.2%) (where S, M, D, and T denote a saturated fatty acid, a monoene, a diene, and a triene, respectively). These results suggest that the lipid classes, FA distributions and TAG molecular species of broad beans are not dependent on the cultivation areas during the growing season.

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Introduction

Among the legume seeds some are used as vegetables and others as supplementary sources of protein in animal diets [1]. The widespread use of legumes makes this food group an important source of lipid and fatty acids (FA) in animal and human nutrition. Some publications dealing with the total lipid and FA composition have been reviewed by a few researchers [2, 3]. *Vicia faba* beans (*VFB*) are consumed by man and domestic animals as an important source of protein, especially in countries with a shortage of high-quality protein sources. Several reports [4, 5] suggest that *VFB* have a deleterious effect on rats, whereas others report good results, with no apparent ill effects. With chicks receiving a high proportion of *VFB* in their diets, Bletner et al. [6] obtained poor growth, feathering and feather pigmentation, but this was improved considerably by addition of a source of good quality protein. Some beans are used as staple foods in many countries and are receiving increasing attention as preventive products against coronary heart disease [7, 8]. *V. faba* beans are a rich source of carbohydrate, protein, fiber, vitamins and minerals [3]; however, they also contain antinutritional factors. α-Galactosides, phytates and trypsin inhibitors are among these factors, and their concentrations differ widely among different cultivars of *V. faba* beans [9].

Poor growth after receiving diets containing *V. faba* beans is attributed, by some investigators, to a deficiency in, or an imbalance of, some of the essential amino acids, and to the presence of growth inhibitors associated with the

protein [4, 5]. The negative effect of *VFB* has also been attributed to contamination of the beans with poisonous substances, such as castor seeds or croton seed residues. The contradictory results described in the literature may also be due to differences in the nutritive value as a result of variability in the composition of different cultivars of the bean. To the best of our knowledge, however, a literature search revealed that there is limited information on the lipid component and FA distribution of broad beans [3]. In the present study, broad bean samples obtained from four cultivars were determined with respect to their lipid class composition, their FA profiles and, most importantly, their molecular species of triacylglycerols (TAG), in an attempt to evaluate the composition and quality characteristics of the oils. The data obtained would be useful to both producers and consumers.

Materials and Methods

Samples

The commercially available mature broad beans (*V. faba*) used in this study were from four different cultivars-Minpo, Sanuki, Nintoku and Sanren-grown in different districts of Japan during the summer of 2006. These cultivars (Takii Seed Co. Kyoto, Japan) were selected for uniformity based on seed weights of 1.06–1.10 g for Minpo, 1.31–1.35 g for Sanuki, 2.21–2.30 g for Nintoku and 2.31–2.35 g for Sanren. All the beans were divided into groups for storage in separate stainless-steel containers at 4 °C prior to the experiments.

Reagents and Standards

All solvents and chemicals used were of analytical grade (Nacalai Tesque, Kyoto, Japan). However, diethyl ether was further purified to remove peroxides. Thin-layer chromatography (TLC) precoated silica gel 60 plates (10 × 20 or 20 × 20 cm, 0.25 mm layer thickness) were purchased from Merck (Darmstadt, Germany). The TLC standard mixture, containing monoacylglycerols (MAG), diacylglycerols (DAG), free fatty acids (FFA), triacylglycerols (TAG), steryl esters (SE) and hydrocarbons (HC), was purchased from Nacalai Tesque. Standard TAG (glyceryl trimyristate, glyceryl tripalmitate, glyceryl tristearate, glyceryl trioleate, glyceryl trilinoleate and glyceryl trilinolenate) were procured from Sigma Chemical Co. (St. Louis, MO, USA). Methyl pentadecanoate (C15:0, 100 mg; Merck) was dissolved in *n*-hexane (20 mL) and used as the internal standard for quantitative analyses. Boron trifluoride (BF_3) in methanol (14%; Wako Pure Chemical Inc., Osaka, Japan) was used to prepare fatty acid methyl esters (FAME).

Chemical Analysis

The AOAC [10] methods were used to determine the chemical composition of the broad beans. Samples were analyzed in triplicate for fat, protein and moisture contents according to the standard methods. Fat content was determined by solvent extraction (Method 991.36), protein content by the Kjeldahl method (Method 981.10) and moisture content by oven-drying to constant weight at 105 °C (Method 925.40).

Extraction of Lipids

In order to obtain fine flour, beans (50 seeds) were ground to pass through a 0.5 mm sieve, using a Maxim homogenizer (Nihonseiki Kaisha Ltd Tokyo, Japan) at high speed for 10 min at 0 °C and extracted with 300 mL of chloroform/methanol (2:1, vol/vol). The lipids were further extracted by vigorous shaking of triplicates samples. These solvents contained 0.01% butylated hydroxytoluene (BHT) to inhibit the oxidative degradation of lipids during analysis. The homogenate was vacuum filtered through defatted filter paper on a Buchner funnel, and the filter residue was rehomogenized with a second volume of chloroform/methanol. The filtrates were combined and dried in a rotary vacuum evaporator at 35 °C. The residue was dissolved in 100 mL of chloroform/methanol (2:1, vol/vol); then, 20 mL aqueous potassium chloride (0.75%) was added [11], and the phases were mixed vigorously. After phase separation, the chloroform layer was withdrawn, dried over anhydrous sodium sulfate, filtered, and the filtrate was concentrated under vacuum. The extracted lipids were weighed to determine the lipid content of the beans and then transferred to a 25-mL brown glass volumetric flask with chloroform/methanol (2:1, vol/vol).

Lipid Analysis and TAG Composition

Using previously described methods [12], the total lipids were fractionated by TLC into eight fractions. Bands corresponding to HC, SE, TAG, FFA, 1,3-DAG, 1,2-DAG, MAG and PL were scraped into test-tubes [105 × 16 mm, poly (tetrafluoroethylene)-coated screw caps]. Methyl pentadecanoate (2 or 20 μL) from a standard solution (5 mg/mL) was added to each tube as the internal standard with a microsyringe (Hamilton Co., Reno, NV, USA). With the exception of HC, FAME were prepared from the isolated lipids by heating with silica-gel for 30 min at 80 °C in BF_3/methanol on an aluminium block bath [13]. After cooling, 5 mL *n*-hexane was added to each tube and washed several times with deionized water to remove BF_3 and silica gel.

The *n*-hexane layer containing the FAME was recovered and dried over anhydrous sodium sulfate. The solvent was then vaporized under a gentle stream of nitrogen, and the residue was quantified on a Shimadzu Model-14B GC (Shimadzu, Kyoto, Japan) equipped with a hydrogen flame ionization detector and a capillary column (ULBO HE-SS-10 for Fames fused silica WCOT, cyanopropyl silicone, 30 m × 0.32 mm i.d.; Shinwa Chem. Ind., Ltd., Kyoto, Japan) at a column temperature of 180 °C. The injection and detector temperatures were held at 220 and 250 °C, respectively. The initial oven temperature was 180 °C. This temperature was maintained for 5 min and then increased at a rate of 2 °C/min to 200 °C, which was held for 15 min. Helium was used as the carrier gas, at a flow rate of 1.5 mL/min, and the GC was operated under a constant pressure of 180 kPa. All samples were dissolved in *n*-hexane for injection. The component peaks were identified and calibrated by comparison with standard FAME (F & OR mixtures no. 3, Altech-Applied Science, State College, PA, USA), using an electronic integrator (Shimadzu C-R4A). The detection limit was 0.05% of total FA for each FAME in the FAME mixture used, and the results are expressed as weight percentage of total FAME.

The TAG removed from silica gel was directly analyzed by GC following the method of Matsui et al. [14], using a Shimadzu Model-14A GC equipped with a hydrogen flame ionization detector [15]. A glass column (500 × 3.0 mm i.d.) was packed with 2 wt% OV 17 (phenyl methyl silicone; Nishio Co., Tokyo, Japan) supported on 80/100 mesh silanized Shimalite W. Helium was used as the carrier gas at a flow rate of 50 mL/min. The column temperature was programmed from 280–330 °C at 2 °C/min, and the injection and detector temperatures were 350 °C. TAG peaks were identified by co-chromatography with known standards. Peak areas were calculated by addition of a known weight (50 mg) of glyceryl trimyristate (trimyristin) as the internal standard, using an electronic integrator (Shimadzu C-R6A).

TAG Species Analysis

Molecular species separation from total TAG was performed by $AgNO_3$–TLC according to the method of Bilyk et al. [16]. Briefly, plates were coated to 0.25 mm thickness with a slurry of 42 g silica gel 60 G (Merck) and 8.0 g $AgNO_3$ dissolved in 100 mL deionized water. Freshly prepared plates were activated at 120–125 °C for 10 h, then stored before use in a desiccator in the dark.

TAG classes differing in unsaturation were separated by $AgNO_3$–TLC using 1.0–2.5% (vol/vol) methanol in chloroform, depending on differences in their degree of unsaturation [17]. In the case of broad TAG, three solvents of increasing polarity were required to separate the lipid classes. The plates (20 cm × 20 cm) were streaked with 10–15 mg TAG using a microsyringe (Hamilton Co., Reno, NV, USA), developed with 1.0% (vol/vol) methanol in chloroform, and S_3, S_2M, SM_2, S_2D, M_3 and SMD (where S, M, and D denote a saturated fatty acid, a monoene, and a diene, respectively) were easily separated. The second TAG molecular species such as SMT, M_2D, SD_2, and MD_2 (where T denotes a triene) were separated by developing the plate with 2.0% (vol/vol) methanol in chloroform. Finally, the most difficult to separate were D_3, MDT, D_2T and DT_2, and these were separated by developing the plate using 2.5% (vol/vol) methanol in chloroform. To obtain a good separation, it was very important to use a small sample size (15 mg/TLC plate). This system was varied according to the humidity and temperature conditions.

Individual TAG bands were visualized by spraying with 0.1% 2′,7′-dichlorofluorescein (Nacalai Tesque; Kyoto, Japan) in methanol and viewed under ultraviolet (254 or 365 mm) radiation. Each TAG subfraction was identified by comparison with the R_f values of a TAG standard. Bands were recovered from the plates by extraction with 3.5% aqueous HCl in the purified diethyl ether. The combined extracts with the diethyl ether were purified by alumina column chromatography (30 cm × 5.0 mm i.d. alumina column; Biomedicals, Eschwege, Germany) to remove the 2′,7′-difluorofluorescein. In preliminary experiments, it was confirmed that each TAG class was fully recovered (>99.5%) by passing TAG standards through the alumina column using this procedure.

The solvent was then vaporized in small glass containers under a gentle stream of nitrogen. Methyl pentadecanoate of the standard solution (5–10 μL) was added to each tube as the internal standard. Relative amounts of each TAG subfraction were quantified by GC described in the preceding paragraphs and then by comparison of the FAME within each TAG fraction containing the internal standard.

Statistical Treatment

All preparation and measurements were carried out in triplicates, and the results were subjected to one-way analysis of variance (ANOVA) [18]. Multiple comparison tests were performed to determine any significant differences ($P < 0.05$) among treatments [19].

Results and Discussion

Lipid Components in the Beans

The weight of broad beans decreased in the order Sanren > Nintoku > Sanuki > Minpo (data not shown). The major chemical components were as follows: moisture

Table 1 Lipid components in the oils obtained from broad beans

Cultivar	Total lipids (mg/100 g beans)	Triacylglycerols (mg/100 g beans)	Phospholipids (mg/100 g beans)	Others (mg/100 g beans)
Minpo	$2366.7^{a} \pm 110.8$	$1181.0^{a} \pm 56.8$ (49.9)	$1140.8^{a} \pm 53.4$ (48.2)	$45.0^{b} \pm 2.0$ (1.9)
Sanuki	$2312.5^{a} \pm 105.6$	$1128.5^{a} \pm 53.0$ (48.8)	$1147.0^{a} \pm 54.6$ (49.6)	$37.0^{a} \pm 1.6$ (1.6)
Nintoku	$2925.3^{c} \pm 126.3$	$1465.6^{c} \pm 70.1$ (50.1)	$1389.5^{c} \pm 67.3$ (47.5)	$70.2^{c} \pm 3.2$ (2.4)
Sanren	$2595.3^{b} \pm 118.7$	$1238.0^{b} \pm 58.5$ (47.7)	$1310.6^{b} \pm 60.5$ (50.5)	$46.7^{b} \pm 2.2$ (1.8)

Mean values ± standard error. Each value represents the average of three replicates, and expressed as milligrams of lipid per 100 g beans. Values in parentheses are relative wt% content of individual lipids in total lipids. "Others" include minor components such as HC, SE, FFA, DAG (1,3- and 1,2-DAG) and MAG. Values in the same column with different superscripts are significantly different from those of individual cultivars ($p < 0.05$)

3.5–3.7%, fat 2.3–2.9% and protein 24.8–25.6%. There were no significant differences ($P > 0.05$) in these contents among the four cultivars. Broad beans are high in complex carbohydrates, protein, and fiber, yet are extremely low in fat [4]. Profiles of the different lipid classes in the beans were compared among the four cultivars (Table 1). Predominant components were TAG (47.7–50.1%) and PL (47.5–50.5%), accompanied by very small amounts (less than 2.4%) of other lipid components. These minor components are designated as 'others' in Table 1. The broad beans are not oilseeds but typical vegetable seeds [20]. Therefore, the PL content is quite significant, while glycolipids are present only in trace amounts, indicating that PL form the essential components of the cell membranes in the beans. There were no significant differences ($P > 0.05$) in the lipid components among the four cultivars. However, the amounts of TAG were higher in the broad beans than in peas reported previously [21].

FA Composition of TAG and PL

FA compositions (expressed in terms of the esters by weight) of TAG and PL in the beans were compared among the four cultivars (Fig. 1). The principal FA components of legumes are generally palmitic (16:0), oleic (18:1n-9) and linoleic (18:2n-6) acids, the distribution of which varies according to these lipid classes. However, these FA distribution patterns were very similar to each other among the four cultivars. The samples had high amounts of total unsaturated FA (which consisted mainly of linoleic (18:2n-6) acid, followed by oleic (18:1n-9) acids), representing 79.7–82.8 and 77.6–79.7% for TAG and PL, respectively. Some differences ($P < 0.05$) in fatty acid composition were found when comparing the two lipid classes. With a few exceptions, the percentage of palmitic (16:0) and oleic (18:1n-9) acids was higher ($P < 0.05$) in the PL (Fig. 1, right) whereas that of linoleic (18:2n-6) and α-linolenic (18:3n-3) acids was higher ($P < 0.05$) in the TAG (Fig. 1, left). However, the percentage of stearic (18:0) acid was almost the same value between the TAG and PL. These FA profiles are not similar to the results observed in typical vegetable seeds such as peas [21] or kidney beans [22]. The oils from legumes could be a potential source of tocopherols (unpublished work). The activity of vitamin E in broad bean, field pea and lentil was almost two times higher than that in soybean oil [3].

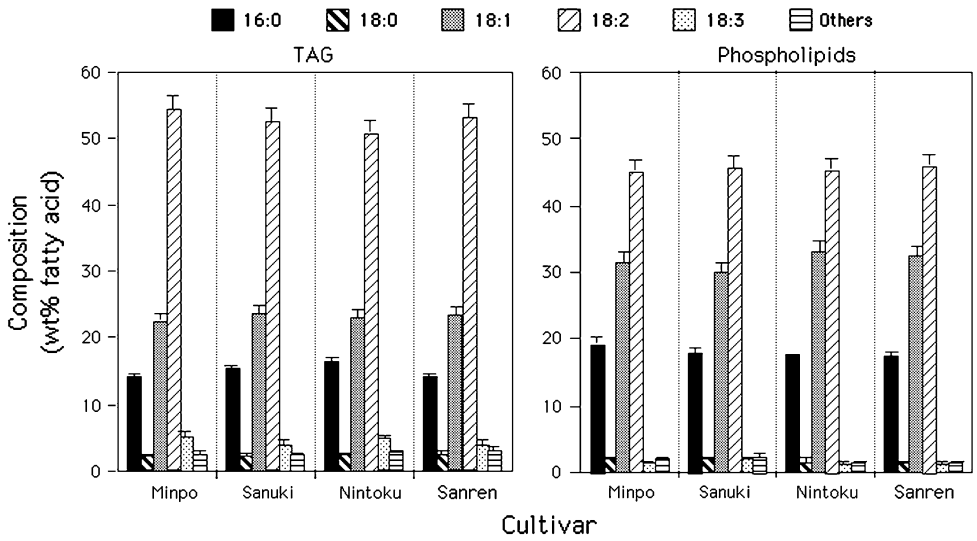

Fig. 1 FA distribution of TAG and PL isolated from broad beans. *Each value* represents the average of three replicates, and *vertical bars* depict the mean SD. Other minor FA include C14:0, C16:1, C20:0 and C22:0

Table 2 TAG content in the oils prepared from broad beans

Acyl chain	Cultivar			
	Minpo	Sanuki	Nintoku	Sanren
48	$2.4^{a} \pm 0.1$ (0.2)	$2.3^{a} \pm 0.1$ (0.2)	$2.9^{a} \pm 0.1$ (0.2)	$2.5^{a} \pm 0.1$ (0.2)
50	$51.9^{a} \pm 2.3$ (4.4)	$51.9^{a} \pm 2.3$ (4.6)	$70.3^{c} \pm 3.0$ (4.8)	$64.3^{b} \pm 3.0$ (5.2)
52	$377.9^{a,b} \pm 16.5$ (32.0)	$354.3^{a} \pm 15.7$ (31.4)	$474.8^{c} \pm 21.3$ (32.4)	$402.3^{b} \pm 18.1$ (32.5)
54	$745.2^{a,b} \pm 32.6$ (63.1)	$716.5^{a} \pm 31.8$ (63.5)	$913.1^{c} \pm 41.3$ (62.3)	$765.1^{b} \pm 35.2$ (61.8)
56	$3.5^{a,b} \pm 0.1$ (0.3)	$3.4^{a} \pm 0.1$ (0.3)	$4.4^{c} \pm 0.2$ (0.3)	$3.7^{b} \pm 0.1$ (0.3)

Mean values ± standard error. Each value represents the average of the replicates, and expressed as milligrams of lipid per 100 g beans. Values in a row with different superscripts are significantly different at $p < 0.05$. Values in parentheses are relative wt% of the individual TAG within total TAG

Distribution of TAG Molecular Species

The total carbon number (TCN) of FA in the TAG of the four broad cultivars ranged from 48 to 56 as listed in Table 2. For example, in the case of tristearin, the TCN is 54. Each value is an average of triplicate measurements and is expressed as mg lipid within total TAG. Dominant components consisted of 52 (31.4–32.5%) and 54 (61.8–63.5%) TCN, followed by small amounts of 50 (4.4–5.2%) TCN, with very small amounts (0.2–0.3%) of 48 and 56 TCN. These distribution patterns are in close agreement with the results reported previously for peas [21]. These results would reflect the differences in fatty acid content (Fig. 1, left) and molecular species of TAG (Fig. 2) among the four cultivars. This is supported by the fact that TAG composed of monoene (M), diene (D) and triene (T) moieties (MD_2, D_3 and MDT) were detected in greater amounts than other TAG species (Fig. 2).

The FA compositions of the individual bands isolated by $AgNO_3$–TLC were determined by GC. According to these results, the distribution patterns of the individual TAG molecular species were illustrated graphically in Fig. 2. Fourteen different molecular species were detected in the oils extracted from these broad beans. The three-letter designation does not suggest fatty acyl positional isomers in the TAG: P, palmitic (16:0); St, stearic (18:0); O, oleic (18:1n-9); L, linoleic (18:2n-6); Ln, α-linolenic (18:3n-3) FA moieties. These molecular species were arranged according to the degree of unsaturation on the acyl chain length of TAG (from left to right in Fig. 2). With a few exceptions, such as S_3 for Nintoku and M_3 for Sanren, the major TAG species were S_2D (PPL or PStL or StStL), SD_2 (PLL or StLL), SMT (POLn or StOLn), M_2D (OOL), MD_2 (OLL), D_3 (LLL) and MDT (OLLn) in all four cultivars. On the other hand, the other species such as S_3 (PPP or PPSt or PStSt or StStSt), S_2M (PPO or PStO or StStO), SM_2 (POO or StOO), M_3 (OOO), SMD (POL or StOL), D_2T (LLLn) and DT_2 (LLnLn) were minor components (less than ca. 5.0 wt%). However, those from SD_2 to DT_2 were basically similar among the four cultivars. With a few exceptions, the distribution patterns in the molecular species of TAG were very similar to each other among the four cultivars.

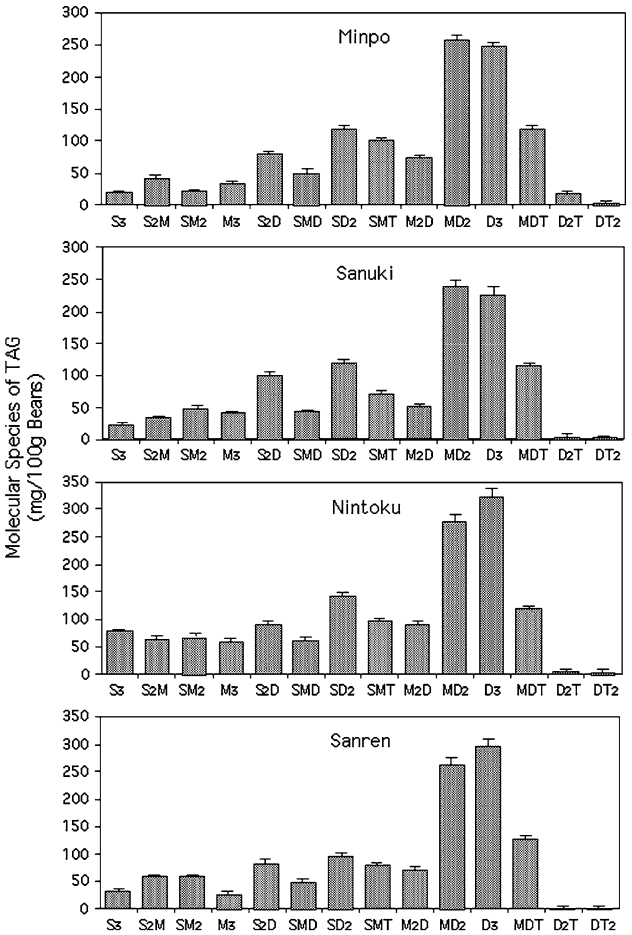

Fig. 2 Characteristics of the major molecular species of TAG isolated from broad beans. Saturated FA (S) consist of myristic (14:0), palmitic (16:0), stearic (18:0), arachidic (20:0) and behenic (22:0) acids. Unsaturated FA, palmitoleic (16:1), oleic (18:1), linoleic (18:2) and linolenic (18:3), are denoted as monoene (M), diene (D) and triene (T), respectively. *Vertical bars* depict the mean standard deviation

Table 3 Content of FA in the TAG isolated from broad beans

	FA	Cultivar			
		Minpo	Sanuki	Nintoku	Sanren
Experimental	S	$206.7^{a} \pm 9.8$ (17.4)	$212.2^{a} \pm 10.3$ (18.8)	$297.3^{c} \pm 12.0$ (20.3)	$225.3^{a} \pm 11.2$ (18.2)
	M	$276.4^{a} \pm 12.5$ (23.3)	$278.7^{a} \pm 12.6$ (24.7)	$354.7^{c} \pm 15.6$ (24.2)	$307.0^{b} \pm 13.7$ (24.8)
	D	$641.3^{b} \pm 30.0$ (54.2)	$592.5^{a} \pm 26.3$ (52.5)	$744.5^{c} \pm 35.2$ (50.8)	$658.6^{b} \pm 31.5$ (53.2)
	T	$60.2^{b} \pm 2.8$ (5.1)	$45.1^{a} \pm 2.0$ (4.0)	$68.9^{c} \pm 2.4$ (4.7)	$47.6^{a} \pm 2.1$ (3.8)

Mean values ± standard error. Each value represents the average of the replicates, and expressed as milligrams FA per 100 g beans. Values in a row with different superscripts are significantly different at $p < 0.05$. Experiemental values are obtained by GC in comparison with a known amount of methyl pentadecanoate as internal standard using TAG isolated from broad beans. Values in parentheses are shown as total relative wt% of individual S, M, D and T, respectively. Abbreviations are shown in Fig. 2

Table 3 presents the FA contents (S, M, D, and T) in the TAG isolated from the broad beans, expressed as milligrams FA within the TAG in 100 g beans according to their degree of unsaturation on the acyl chain lengths of the FA moieties. Briefly, the amounts of individual FA were summed up as S (16:0, 17:0, 18:0, 20:0, and 22:0), M (16:1 and 18:1), D (18:2) and T (18:3) from the results obtained by GC using methyl pentadecanoate as the internal standard. The theoretical contents of FA were calculated from the relative percentages of each TAG species based on the data in Fig. 2 and their distribution of each FA, which comprised the experimental value (Table 3). There were no quantitative or qualitative differences ($P < 0.05$) in the distribution between the found and calculated (theoretical) values.

Major lipid components in broad beans were PL and TAG, while HC, SE, FFA, DAG and MAG were also present in minor proportions. The main TAG species were dipalmitolinolein, palmitostearolinolein, distearolinolein, palmitodilonlein, palmitostearolinolein, palmitoleolinolenin, stearoleolinolenin, dioleolinolein, oleodilinolein, trilinolein and oleolinoleolinolenin among the four cultivars. With a few exceptions, however, these distribution profiles were not significantly different ($P > 0.05$) among the four cultivars. The lipid components and FA distributions were almost the same as well as the molecular species of TAG in the four cultivars, and it was not influenced by genetic variability and planting location.

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