# Tocopherol Distribution and Molecular Species of Triacylglycerols in Soybean Embryonic Axes

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**ABSTRACT:** Soybean embryonic axes were separated from other tissues, i.e., the cotyledons and seed coat. The molecular species and FA distribution of TAG isolated from total lipids in the embryonic axes were analyzed by a combination of argentation-TLC and GC, and were investigated in relation to their tocopherol distribution, which was determined by HPLC. The dominant components were γ-tocopherols, with much smaller amounts of α-, β-, and δ-tocopherols. A modified argentation-TLC procedure, developed to optimize the separation of the complex mixture of total TAG, provided 16 different groups of TAG, based on both the degree of unsaturation and the total acyl-chain length of FA groups. With a few exceptions, the major TAG components were $S_2D$ (6.8–10.3%), SMD (6.9–11.2%), $SD_2$ (7.2–9.8%), SMT (3.2–7.4%), SDT (11.5–19.5%), D3 (3.5–8.3%), MDT (4.5–7.7%), $D_2T$ (11.1–20.6%), and $DT_2$ (8.2–15.7%) (where S denotes saturated FA, M denotes monoenes, D denotes dienes, and T denotes trienes). These results indicate that there were significant differences ($P < 0.05$) not only in tocopherol distribution but also in the molecular species of TAG among the four cultivars. Therefore, these tissues should be made available as raw materials for soybean-germ oil or soy milk, based on the differences in the distributions of tocopherol homologs and the molecular species of TAG within the embryonic axes.

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**KEY WORDS:** $AgNO_3$-TLC, embryonic axis, molecular species, soybeans, triacylglycerols, vitamin E homologs.

Soybeans are the world's single most important source of edible oil and vegetable protein. The nutritional value of soybeans is determined not only by the quantity but also by the quality of its oil and protein. One quality factor that has recently gained much attention is the FA composition of soybean oil. There is increasing evidence of a relationship between the consumption of saturated fat and elevated serum cholesterol levels (1), as well as between the linolenic acid content in a food system and oxidative stability and loss of flavor (2). However, the normal FA composition of soybean oil is often not considered ideal in terms of oxidative stability. In addition, soybeans and their products are relatively good sources of vitamin E. Tocopherols are important biological and nutritive components of our food. In addition to their function as cholesterol synthesis inhibitors in animals and plants (3,4), tocopherols are essential for protecting PUFA against oxidative deterioration.

Tocopherol homologs exert their antioxidant effect through numerous biochemical and biophysical mechanisms, including scavenging active oxygen species and free radicals and through action as efficient chain terminators in lipid autoxidation reactions. Generally, antioxidative and biological activities of the isomers increase in the following order: α,β,γ, and δ (5), but this order of antioxidant potency in vegetable oils may be influenced significantly by experimental conditions, such as temperature or light (6). Information on the FA distribution in soybean seeds is limited (7). In an oilseed plant such as the soybean, there are two types of lipids: membrane and storage lipids. Storage lipids are mainly TAG, which are localized in the seed oil bodies of cotyledon tissues. Membrane lipids are mainly phospholipids (PL), which are low in quantity and located in all cells of living tissues, including the embryonic axis. Soybean-germ oil promotes the excretion of cholesterol into feces (8) and is effective for people with a high risk of lifestyle-related diseases (9). However, few reports are available concerning the various lipids in soybeans within each tissue. The objective of the present study was to determine not only the distribution of tocopherols but also the different molecular species of TAG in the embryonic axes of soybeans.

## EXPERIMENTAL PROCEDURES

*Materials.* Commercially available soybeans (*Glycine max* L.) used for this study were from four different cultivars (Nouhime, Fukunari, Tsurunoko, and Okuhara) grown in Japan during the summer of 2004. Mature seeds (Takii Seed Co., Kyoto, Japan) were selected for uniformity based on a seed weight of 270–319 mg for Nouhime and Fukunari and 320–369 mg for Tsurnoko and Okuhara. The seeds were hand-selected to eliminate those with cracked or otherwise damaged seed coats. All the beans were cleaned and divided into groups for storage in stainless-steel containers at 4°C until analysis.

*Reagents and standards.* All chemicals and solvents used were of analytical grade (Nacalai Tesque, Kyoto, Japan). Vitamin E homologs (α, β, γ, and δ) were procured from Eisai Co. (Tokyo, Japan). All tocopherols were the D-form (*RRR*-), and the purity of each tocopherol was better than 98.5% as determined by HPLC, as described in a later section. Precoated silica-gel 60 TLC plates ($10 \times 20$ cm$^2$ or $20 \times 20$ cm$^2$, 0.25 mm

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layer thickness) were purchased from Merck (Darmstadt, Germany). The TLC standard mixture, containing hydrocarbons (HC), steryl esters (SE), TAG, FFA, and DAG, was from Nacalai Tesque. Standard TAG (trimyristin, tripalmitin, tristearin, triolein, trilinolein, and trilinolenin) were obtained from Sigma Chemical Co. (St. Louis, MO). Methyl pentadecanoate (15:0, 100 mg; Merck) was dissolved in *n*-hexane (20 mL) and used as the internal standard. Boron trifluoride ($BF_3$) in methanol (14%; Wako Pure Chemical Inc, Osaka, Japan) was used to prepare FAME.

*Seed sectioning and lipid extraction.* The soybeans were first dissected into three structural parts (the seed coat, embryonic axis, and cotyledons) with a razor blade. One thousand beans were separated and sectioned for each genotype. These embryonic axes were crushed using a Maxim homogenizer (Nihonseiki Kaisha Ltd., Tokyo, Japan) at high speed for 10 min at 0°C with 100 mL of chloroform/methanol (2:1, vol/vol) fortified with 0.01% BHT, which was added to inhibit the oxidative degradation of lipids during analysis. The lipids were extracted according to the procedure described by Takagi and Yoshida (10). The homogenate was vacuum filtered through defatted filter paper on a Büchner 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 of aqueous potassium chloride (0.75%) was added (11) and the phases were vigorously mixed. After phase separation, the chloroform layer was withdrawn, dried with anhydrous sodium sulfate ($Na_2SO_4$), and filtered, and the organic phase was concentrated under vacuum. The extracted lipids were weighed to determine the lipid content of the axes and then transferred to a 25-mL brown glass volumetric flask with chloroform/methanol (2:1, vol/vol) (10).

*Tocopherol analysis*. The lipids (200 mg) were carefully transferred to a 2-mL brown volumetric flask, and the solvents were removed under nitrogen gas at ambient temperature in a draft chamber. The residue was dissolved in the mobile phase used for HPLC analysis (*n*-hexane/1,4-dioxane/ethanol, 490:10:1, by vol). The chromatographic system was the same as previously described (12) and operated at a flow rate of 2.0 mL/min. An aliquot (5–10 μL) of these solutions was injected with a fully loaded 20-μL loop onto the column. Each tocopherol was monitored with a fluorescence detector (Shimadzu RF-10 AxL; Shimadzu Instrument Inc., Kyoto, Japan) set at 295 nm excitation wavelength and 320 nm emission wavelength, and was quantified as previously described (13).

*Lipid class analysis and FA compositions.* The total lipids were fractionated by TLC into seven fractions using previously described methods (12). The crude lipid extracts were applied on TLC plates as 7-cm bands (*c* 20 mg per plate) with a microsyringe (Hamilton Co, Reno, NV). The TLC standard mixture was applied as a reference on one side of each plate, and the plates were developed in *n*-hexane/diethyl ether/acetic acid (80:30:1 or 60:40:1, by vol). The plate was covered with another glass plate, leaving the reference zone exposed to be visualized by exposure to iodine vapor. Bands corresponding to HC, SE, TAG, FFA, 1,3-DAG, 1,2-DAG, and PL were scraped into test tubes [105 × 16 mm; poly(tetrafluoroethylene)-coated screw caps]. Methyl pentadecaonate (15:0, 25 or 100 μg) was added to each tube as an internal standard. FAME were prepared from the isolated lipids by heating for 30 min at 80°C in $BF_3$/methanol under nitrogen gas on an aluminum block bath (14). After cooling, 5 mL of *n*-hexane was added to this solution. The organic layer containing the FAME was recovered. The solvent was then vaporized under a gentle stream of nitrogen, and the residue was quantified on a Shimadzu Model-14B gas chromatograph equipped with an FID and a capillary column (ULBO HE-SS-10, 30 m × 0.32 mm i.d.; Sginwa Chem. Ind., Ltd., Kyoto, Japan) at a column temperature of 180°C. The injection and detection temperatures were held at 220 and 250°C, respectively. Helium was used as the carrier gas at a flow rate of 1.5 mL/min, and the gas chromatograph was operated under a constant pressure of 180 kPa. The component peaks were identified and calibrated by comparison with standard FAME mixtures (F & OR mixtures No. 3; Applied Science, State College, PA), using an electronic integrator (Shimadzu C-R6A). The other GC conditions were the same as described previously (12). The detection limit was 0.05% of total FA for each FAME in a FAME mixture, and results are expressed as the weight percentage (%) of total FAME.

*TAG composition*. TAG isolated by TLC were further analyzed by GC following the method of Matsui *et al.* (15), using a Shimadzu Model-14B gas chromatograph equipped with an FID. A glass column (500 × 3.0 mm i.d.; Shimadzu) was packed with 2.0% OV 17 (phenyl methyl silicone) supported on silanized Shimalite (80–100 mesh). Helium was used as the carrier gas at a flow rate of 50 mL/min. The column temperature was programmed from 250 to 340°C at 4°C/min, and the detector and injection port temperatures were set at 350°C. TAG peaks were identified by co-chromatography with known standards. Peak areas were calculated by addition of a known weight (50 μg) of trimyristin as the internal standard using an electronic integrator (Shimadzu C-R6A).

*TAG species analysis*. Molecular species separation of total TAG was carried out 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 45 g of silica gel 60 G (Merck) and 6.0 g of silver nitrate dissolved in 90 mL of 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 argentation-TLC using 0.8 to 5.0% (vol/vol) methanol in chloroform, depending on their degree of unsaturation (12). For the quantitation of species containing the linolenic acid, a trienoic acid, plates (20 × 20 $cm^2$) were streaked with 10–15 mg TAG by a microsyringe (Hamilton Co., Reno, NV) and developed with 5.0% methanol in chloroform. The remaining species were separated by streaking 8–10 mg TAG on the plates and by developing these plates using 0.8–2.8% methanol in chloroform. This system was varied with regard to humidity and temperature conditions. Individual bands were visualized by spraying with 2′,7′-dichlorofluorescein

(Nacalai Tesque; 0.1% in methanol) and viewed under UV (254 or 365 nm) irradiation. Each TAG subfraction was identified by comparison with $R_f$ values of a TAG standard. Bands were recovered from the plates by extraction with 10% aqueous HCl in diethyl ether. The combined extracts were purified by alumina column chromatography (5.0 × 30 mm alumina column; Biomedicals, Eschwege, Germany) to remove the 2′,7′-dichlorofluorescein. In the previous study, we confirmed that TAG were fully recovered (>99.2%) after passing the above-mentioned solvents through an alumina column. The identity and purity of each band was verified by analytical-TLC after co-chromatography with the reference TAG mixture. Relative amounts of each TAG subfraction were determined by comparison of FAME with a known amount (25 μg) of methyl pentadecanoate as the internal standard. Each subfraction was converted into FAME and then quantified by GC as described in the preceding paragraphs.

*Statistical analysis*. All experiments were carried out in triplicate and the results were analyzed by one-way of ANOVA (17). Multiple comparison tests were performed to determine any significant differences ($P < 0.05$) among treatments (18).

## RESULTS AND DISCUSSION

*Structural part and sections of soybeans*. Soybean seed (cv. Nouhime) was composed of typical percentages of the embryonic axis, seed coat, and cotyledons. Cotyledons comprised the predominant fraction, at 45.8 to 46.5% of the seed mass, whereas the seed coat accounted for 5.2 to 6.7% and the axis was a very small part, at 1.5 to 2.3%. No significant differences ($P > 0.05$) in these percentages were observed among the four cultivars.

*Tocopherol distributions and lipid components in the soybean embryonic axis*. The amount of total tocopherols per 100 grams of oil was highest in the axis, followed by cotyledons and the seed coat (data not shown). Tocopherol contents varied significantly ($P < 0.05$), not only among the four cultivars but also within the embryonic axis (Fig. 1). The greatest amount of total tocopherols ($P < 0.05$) was observed in Nouhime (274.7 mg), followed by Fukunari (243.4 mg), Tsurunoko (228.6 mg), and Okuhara (199.9 mg). Figure 1 shows the distribution of individual tocopherols in the embryonic axis of soybeans. In general, the dominant component in the four cultivars was γ-tocopherol (74.2–80.0%), with much smaller amounts of δ- (12.4–15.1%), α- (6.8–12.0%), and β-tocopherols (0.4–0.7%). Almonor *et al.* (19) suggested that the level of γ-tocopherol appeared to be directly associated with changes in oil quality that were mediated by genetic and environmental influences on the concentration of linolenic acid. Therefore, α-tocopherol was higher in the embryonic axis for all cultivars than in other tissues, as reported in a previous paper (20). Although α-tocopherol is a less potent antioxidant than other tocopherols in biological systems, it has great value in the cells of all living tissues (21). Regardless of the soybean cultivar (22), PC, PE, and PI were the major PL found in the embryonic axis. The highest concentration was observed for PC (46.5–51.1%), followed by PE (25.0–26.4%) and PI (14.9–18.4%). Since membrane lipids are involved in such fundamental cell processes as ion transport, energy generation, and biological reactions, they are highly conserved in terms of both quantity and quality.

Comparisons were made of the amounts of SE, TAG, FFA, 1,3-DAG, 1,2-DAG, and polar lipids in the axis of soybean repetitions (Fig. 2). A similar pattern was observed in the lipid

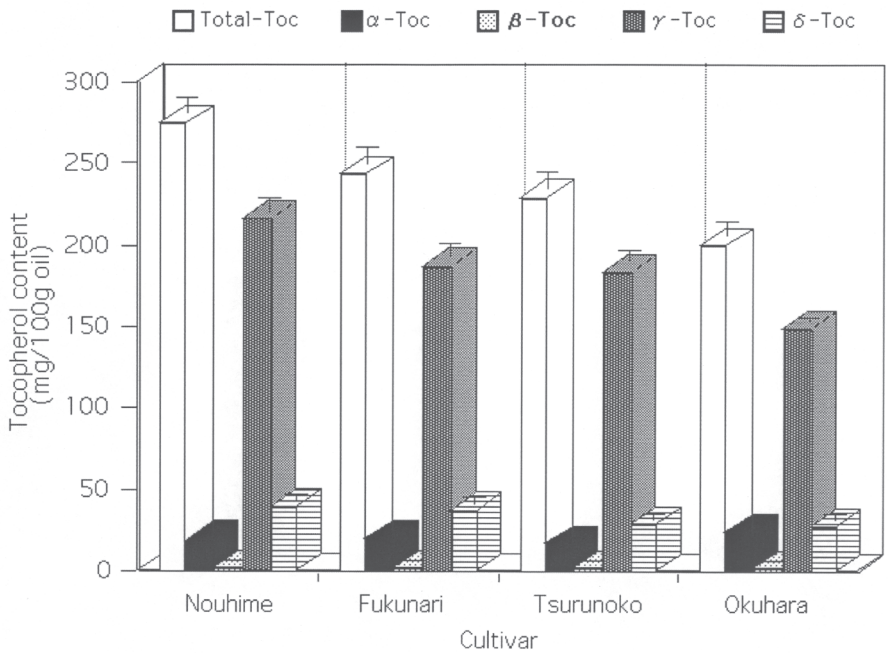


**FIG. 1.** Tocopherol (Toc) distributions in the oils prepared from the soybean embryonic axis. Each value represents the average of three replicates, and horizontal bars depict the mean SD.

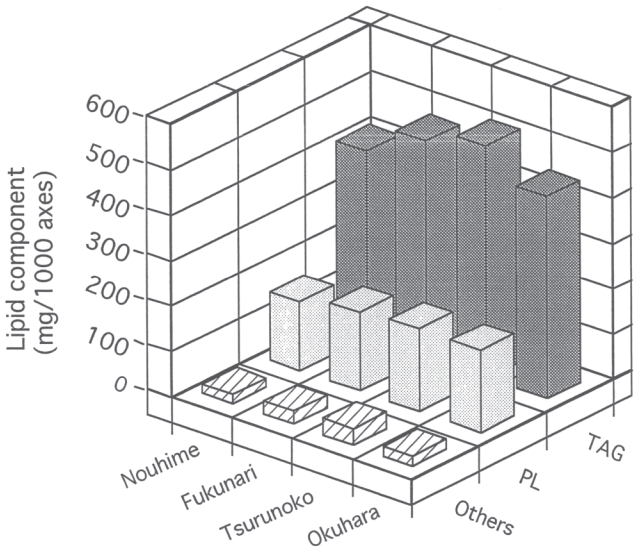


**FIG. 2.** The content of lipid components in the oils obtained from the embryonic axis of soybeans. Each value represents the average of three replicates, and horizontal bars depict the mean SD. PL, phospholipids. Others include minor lipid components such as hydrocarbons, steryl esters, FFA, 1,3-DAG, and 1,2-DAG.

components among the four cultivars. The major fraction in all cultivars was TAG, constituting 70.0% in the axis. The other acyl lipids, such as 1,3-DAG, 1,2-DAG, SE, and FFA, are minor components and are designated as "others" in Figure 2. On a percentage basis (data not shown), the greatest amount of PL was found in the axis (24.8 to 27.7%), followed by the seed coat (20.0 to 23.3%) and cotyledons (5.7 to 6.8%). Membrane lipids are mainly PL, which are low in quantity and located in the cells of all living tissues.

*Major TAG content and total FA composition.* The embryonic axis contained even carbon-numbered TAG for $C_{42}$ to $C_{56}$ in the four cultivars. Dominant components consisted of $C_{52}$ (27.0–30.7%) and $C_{54}$ (53.1–59.1%) TAG in the four cultivars. Minor components (<5.0 mg) such as $C_{42}$, $C_{44}$, and $C_{56}$ TAG were omitted from Figure 3. These results would depend on differences in the amounts of TAG composed of oleic, linoleic, and linolenic acids. This is supported by the fact that TAG composed of one diene (D) and two triene (T) moieties ($DT_2$) were detected to be more than 1.3- to 1.5-fold greater in the Nouhime and Tsurunoko cultivars than in the Fukunari or Okuhara cultivars (Fig. 4). The FA compositions (expressed in terms of the esters by weight) of TAG were compared among the four cultivars as shown in Figure 4. A small difference ($P < 0.05$) occurred in the FA composition of TAG between the Nouhime or Tsurunoko and Fukunari or Okuhara cultivars. The former were higher (42.4 to 43.6%) in linoleic (24.3 to 26.0%) or linolenic acid and lower (8.5 to 10.5%) in oleic acid than the latter. In general, the percentages of saturated FA such as palmitic and stearic were higher ($P < 0.05$) in Fukunari or Okuhara than in Nouhime or Tsurunoko.

*Distribution of TAG species.* The patterns of the TAG molecular species obtained from the embryonic axis of soybeans were illustrated graphically according to the GC data (Fig. 5). Sixteen different molecular species were detected in the oils extracted from these axes. The three-letter designation does not suggest fatty acyl positional isomers in the TAG: P, palmitic; St, stearic; O, oleic; L, linoleic; Ln, linolenic FA moieties. The major TAG species were $D_2T$ (LLLn), SDT (PLLn or StLLn), $DT_2$ (LLnLn), $SD_2$ (PLL or StLL), SMD (POL or StOL), $S_2D$ (PPL, PStL, or StStL), $D_3$ (LLL), MDT (OLLn), and SMT (POLn or StOLn) in all four cultivars. The other species ($S_3$: PPP, PPSt, PStSt, or StStSt; $S_2M$: PPO, PStO, or StStO; $SM_2$: POO or StOO; $M_3$: OOO; $M_2D$: OOL; $MD_2$: OLL; and $M_2T$: OOLn) were minor components (less than *ca.* 10 mg: 2.0%). These results would be due to the differences in the amounts of molecular species of TAG for the four cultivars. In general, between Nouhime and

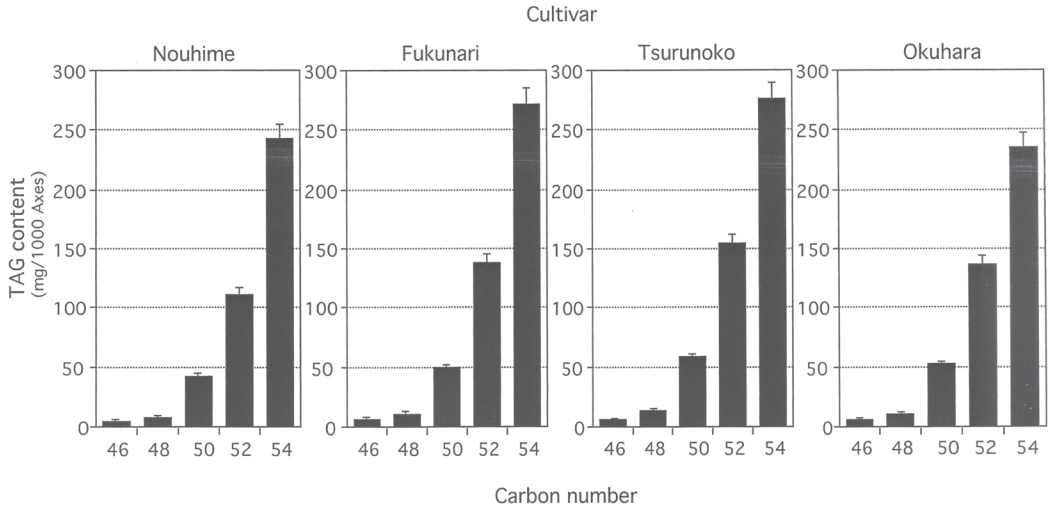


**FIG. 3.** TAG content in the embryonic axis of soybeans. The carbon number denotes the total length of the three acyl chains present in a TAG. For example, $C_{54}$ is predominantly composed of 18:0, 18:1, 18:2, and 18:3. Horizontal bars represent the mean SD.

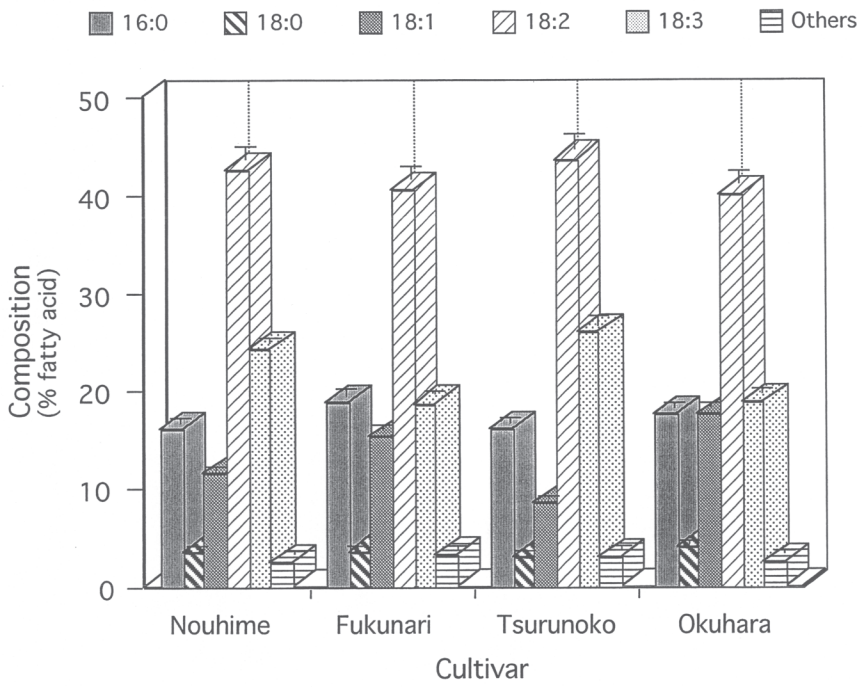


**FIG. 4.** FA distribution of TAG isolated from the embryonic axis of soybeans. Each value represents the average of three replicates, and vertical bars show the mean SD. Other minor FA include 14:0, 16:1, 17:0, and 20:0.

Tsurunoko or Fukunari and Okuhara, the distribution patterns of the molecular species of TAG were very similar to each other. Table 1 presents the FA contents (S, M, D, and T) in the TAG isolated from the embryonic axis of soybeans, expressed as milligrams in 1,000 axes according to their degree of unsaturation. Briefly, the amounts of FA were summed up as S (16:0, 17:0, 18:0, and 20:0), 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 Figure 3 and their distribution of each FA, which comprised the experimental value (Table 1). There were no quantitative or qualitative differences ($P > 0.05$) in distribution between the found and calculated (theoretical) values.

Snyder and Kwon (23) reported that the embryonic axes are the source of beany off-flavors, and some processors of soy milk have tried to remove the germ to avoid off-flavors in soy milk. This knowledge of the lipid class composition, tocopherol profile, and distribution of TAG molecular species in the embryonic axes should provide a good base for further examination of the effect of lipids on the quality of soybean products. Therefore, further studies are necessary to demonstrate what roles the tocopherols play in relation to various acyl lipids in the embryonic axes.

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**TABLE 1**
**Content of FA in the TAG Isolated from the Embryonic Axis of Soybeans[a]**

| | | Cultivar | | | |
|---|---|---|---|---|---|
| | FA[b] | Nouhime | Fukunari | Tsurunoko | Okuhara |
| Experimental[c] | S | 84.13$^{d}$ | 111.52$^{g}$ | 101.65$^{f}$ | 99.37$^{e}$ |
| | M | 51.30$^{d}$ | 82.45$^{e}$ | 51.08$^{d}$ | 81.62$^{e}$ |
| | D | 175.24$^{d}$ | 193.98$^{f}$ | 225.26$^{g}$ | 179.21$^{d,e}$ |
| | T | 99.73$^{f}$ | 88.65$^{d,e}$ | 132.81$^{g}$ | 83.40$^{d}$ |

[a]Each value is an average of three determinations and is expressed as milligrams lipid per 1000 axes.
[b]Saturated FA (S) consisting of myristic (14:0), palmitic (16:0), margaric (17:0), stearic (18:0), and arachidic 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.
[c]Values obtained by GC in comparison with a known amount of methyl pentadecanoate as an internal standard using TAG isolated from embryonic axes. Values in the same row with different superscript letters are significantly different from those among the 4 cultivars ($P < 0.05$).

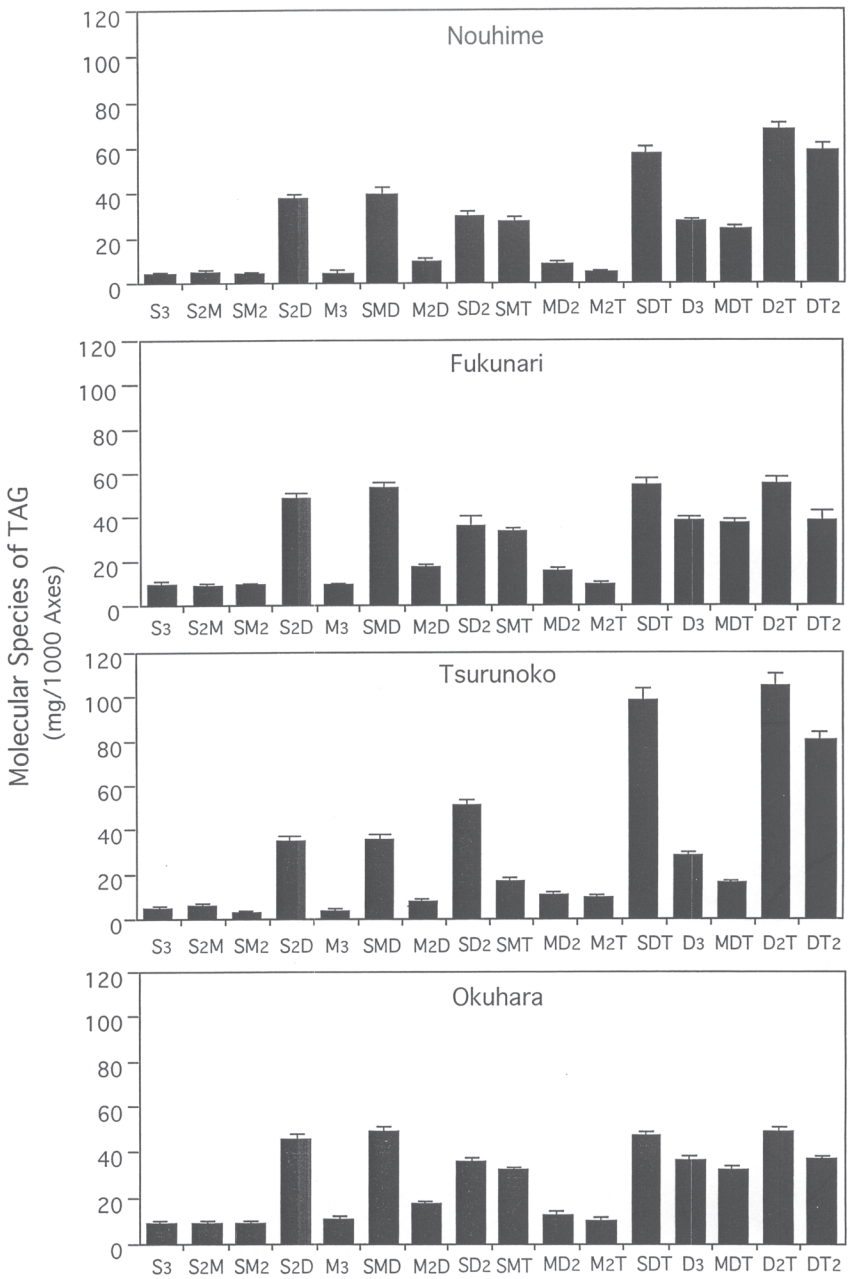


**FIG. 5.** Characteristics of the major molecular species of TAG obtained from the embryonic axis of soybeans. The saturated FA (S) consisted of myristic (14:0), palmitic (16:0), margaric (17:0), stearic (18:0), and arachidic (20:0) acids. The unsaturated FA, palmitoleic (16:1), oleic (18:1), linoleic (18:2), and linolenic (18:3), are denoted as monoenes (M), dienes (D), and trienes (T), respectively. Vertical bars show the mean SD.

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