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Lipid classes, fatty acid composition and triacylglycerol molecular species in the kernels of pumpkin (*Cucurbita* spp) seeds

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Abstract: The lipids extracted from the kernels of pumpkin (*Cucurbita* spp) seeds of three cultivars were classified by thin layer chromatography into six fractions: steryl esters (SEs, 0.5–1.2%), triacylglycerols (TAGs, 92.7–93.4%), free fatty acids (FFAs, 2.9–3.5%), *sn*-1,3-diacylglycerols (1,3-DAGs, 0.4–0.9%), *sn*-1,2-diacylglycerols (1,2-DAGs, 0.7–0.9%) and phospholipids (PLs, 1.5%). Fatty acids derivatised as methyl esters were analysed by gas chromatography with flame ionisation detection. Molecular species and fatty acid distributions of TAGs, isolated from the total lipids in the kernels, were analysed by a combination of argentation thin layer chromatography (TLC) and gas chromatography. A modified argentation TLC procedure, developed to optimise the separation of the complex mixture of total TAGs, provided 11 different groups of TAGs, based on both the degree of unsaturation and the total chain length of fatty acid groups. With a few exceptions, SM_2 (5.8–20.1%), S_2D (8.8–11.2%), M_3 (6.7–24.8%), SMD (6.8–16.7%), M_2D (16.7–23.6%), SD_2 (4.6–15.1%) and MD_2 (4.9–18.6%) were the main TAG components. These results suggest that there are significant differences ($P < 0.05$) not only in fatty acid distributions of acyl lipids but also in molecular species of TAGs among the three cultivars. The differences in pumpkin cultivars could be appreciable, based on the distribution of molecular species in TAGs. However, pumpkin seed kernels could be utilised successfully as a source of edible oils for human consumption.

Keywords: acyl lipids; $AgNO_3$ TLC; free fatty acids; kernels; molecular species; pumpkin (*Cucurbita* spp) seeds; triacylglycerols

INTRODUCTION

Recently, much attention has been focused on the utilisation of food-processing by-products and wastes as well as underutilised agricultural products. Obviously, such utilisation would contribute to maximising the available resources and result in the production of various new products for food. At the same time a major contribution to avoiding waste disposal problems could be made. The problems of industrial waste are becoming harder to solve, and much effort will be needed to develop the nutritional and industrial potential of by-products, waste and underutilised agricultural products. Only a small portion of plant material is utilised directly for human consumption.[1] The remaining portion of this material, or part of it, may be converted into nutrients for either food or feed or into fertiliser; thus an important contribution to food resources or industrial products could be made.[2] The search for lesser-known crops, many of which are potentially valuable as human and animal foods, has been intensified to maintain a balance between population growth and agricultural productivity, particularly in tropical and subtropical areas of the world. In this respect, pumpkin (*Cucurbita* spp) seeds, which remain in large quantities as waste products after the removal of the peel, could be used. Pumpkin seeds are utilised directly for human consumption as a snack after salting and roasting, because these seeds are an excellent source of oil (37.8–45.4%) and protein (25.2–37.0%).[3]

Pumpkin seeds possess valuable dietetic and medicinal advantages besides being a source of edible oils, proteins and minerals of good quality. After extracting the oil from the ground seeds, the residual cake contains about 73% crude protein.[4] The value of pumpkin seeds as a useful source of proteins and oils has been reviewed by several workers.[5–7] However, these studies have been conducted on

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the fatty acid level of total lipids in the seeds, but triacylglycerols, the main component of the seeds, have not been investigated at the molecular species level. The composition of total fatty acids is often the only information provided in studies on seed lipids. Until now there has been no information on the functional properties of pumpkin seed products.

In the present study, seed samples obtained from three cultivars were analysed with respect to the fatty acid composition of the separated lipid classes—triacylglycerols (TAGs), free fatty acids (FFAs) and phospholipids (PLs)—in an attempt to evaluate the composition and quality characteristics of the oils.

MATERIALS AND METHODS

The commercially available pumpkin (*Cucurbita* spp) seeds used in this study were from three Japanese cultivars, *Rikyu*, *Kuriebisu* and *Yatsuko*, grown in Japan during the summer of 2002. These cultivars (Takii Seed Co, Kyoto, Japan) were selected for uniformity based on seed weights of 120–150 mg for *Yatsuko*, 150–200 mg for *Kuriebisu* and 170–250 mg for *Rikyu*. The seeds were hand selected to eliminate those that were cracked or otherwise damaged. All the seeds were divided into groups for storage in stainless steel containers at 4 °C until needed.

Reagents and standards

All chemicals and solvents used were of analytical grade (Nacalai Tesque, Kyoto, Japan). Thin layer chromatography (TLC) pre-coated silica gel 60 plates (10 × 20 or 20 cm × 20 cm, 0.25 mm layer thickness) were purchased from Merck (Darmstadt, Germany). The TLC standard mixture, containing diacylglycerols (DAGs), FFAs, TAGs and steryl esters (SEs), was from Nacalai Tesque. Standard TAGs (trimyristin, tripalmitin, tristearin, triolein, trilinolein and trilinolenin) were obtained 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 internal standard. Boron trifluoride (BF_3) in methanol (14%, Wako Pure Chemical Inc, Osaka, Japan) was used to prepare fatty acid methyl esters (FAMEs).

Extraction of lipids

The kernels were peeled from the whole pumpkin seeds with a razor blade. The kernels (200 seeds) were extracted using a Maxim homogeniser (Nihonseiki Kaisha Ltd, Tokyo, Japan) at high speed for 10 min at 0 °C with 150 ml of chloroform/methanol (2:1 v/v) fortified with butylated hydroxytoluene (BHT, 0.1 g kg^{-1}), which was added to inhibit the oxidative degradation of lipids during analysis. The homogenate was vacuum filtered through defatted filter paper in a Buchner funnel, and the filter residue was rehomogenised 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 v/v), then 20 ml of aqueous potassium chloride (7.5 g kg^{-1}) was added[8] and the phases were vigorously mixed. After phase separation the chloroform layer was withdrawn, dried with anhydrous sodium sulphate and filtered, and the organic phase was concentrated under vacuum. The extracted lipids were weighed to determine the lipid content of the kernels and then transferred to a 25 ml brown glass volumetric flask with chloroform/methanol (2:1 v/v).[9]

Lipid class analysis and TAG composition

Using previously described methods,[10] the total lipids were fractionated by TLC into six fractions. Bands corresponding to SEs, TAGs, FFAs, 1,3-DAGs, 1,2-DAGs and PLs were scraped into test tubes (105 mm × 16 mm, poly(tetrafluoroethylene)-coated screw caps). Methyl pentadecanoate solution (C15:0, 25 or 100 μg) was added as internal standard to each tube. FAMEs were prepared from the isolated lipids by heating for 90 min at 80 °C in BF_3/methanol on an aluminium block bath.[11] After cooling, 5 ml of *n*-hexane was added. The organic layer containing the FAMEs was recovered. The solvent was removed under a stream of nitrogen and the residue was quantitated in a Shimadzu GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) as described previously.[8] The detection limit was 0.05 wt% of total fatty acids for each FAME in a FAME mixture, and results are expressed as wt% of total FAMEs.

On the other hand, TAGs isolated by TLC were analysed by GC following the method of Matsui *et al*,[12] using a Shimadzu GC-14A gas chromatograph equipped with a hydrogen flame ionisation detector.[13] TAG peaks were identified by co-chromatography with standards. Peak areas were calculated by addition of a known weight (50 μg) of trimyristin internal standard using an electronic integrator (Shimadzu C-R4A).

TAG species analysis

Molecular species separation of total TAGs was carried out by silver nitrate/silica gel TLC according to the method of Bilyk *et al*.[14] Briefly, TAG classes differing in unsaturation were separated by argentation TLC using 1.8% (v/v) methanol in chloroform, depending on their degree of unsaturation.[15] This system was varied according to temperature and humidity conditions. Individual bands were visualised by spraying with 2′,7′-dichlorofluorescein (Nacalai Tesque, 0.1% in methanol) and viewed under ultraviolet radiation. 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 mm × 30 mm, alumina column) to remove the 2′, 7′-dichlorofluorescein.

The identity and purity of each band were verified by analytical silver nitrate/silica gel TLC after co-chromatography with the reference TAG mixture. Determination of the relative amounts of each TAG subfraction was made by comparison of FAMEs with a known amount (25 μg) of methyl pentadecanoate as internal standard. Each subfraction was converted into FAMEs and quantitated by GC as described above.

Statistical analysis

All experiments were done in triplicate and the results were analysed by one-way analysis of variance.[16] Multiple comparison tests were performed to determine any significant differences ($P < 0.05$) among treatments.[17]

RESULTS AND DISCUSSION

The weights of whole pumpkin seeds and their component parts are shown in Fig 1. The weight of whole seeds decreased in the order *Rikyu* > *Kuriebisu* > *Yatsuko*. Profiles of the different acyl lipid classes in the kernels of pumpkin seeds are presented in Fig 2. Each value is an average weight (mg per 200 seeds) of three determinations within each structural part of pumpkin seeds. In all three cultivars the predominant components were TAGs, with smaller amounts of FFAs and PLs. The amounts of these three components were highest in *Kuriebisu*, followed by *Rikyu* and *Yatsuko*. Other components such as SEs, 1,3- and 1,2-DAGs were also detected in very small amounts in the kernels of the three cultivars. There were significant differences ($P < 0.05$) in these values among the three cultivars of pumpkin seeds. As shown in Fig 2, the amounts of lipid components differed ($P < 0.05$) among the kernels of the three cultivars. However, the relative percentages of the lipid classes were very similar among the three cultivars; namely, TAGs dominated (92.7–93.4%), followed by FFAs (2.9–3.5%) and phospholipids (1.5%), with smaller amounts of the other lipids, ie SEs, 1,3- and 1,2-DAGs (<1.2%). The results are similar to previous findings.[18] The presence of FFAs and DAGs in lipid samples may be due to the partial enzymatic hydrolysis of reserve TAGs during storage of the seeds. Aboul-Nasr *et al*[19] reported that TAGs were the major lipid fraction in pumpkin seed oil. Normally, the presence of these compounds in plants is considered to be a result of degradation of TAGs caused by different reasons,

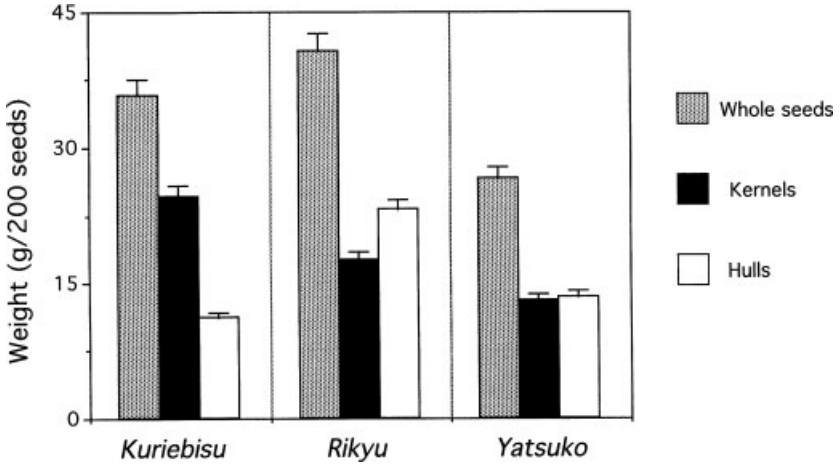

Figure 1. Weights of pumpkin seeds and their component parts. Each value represents the average of three replicates, and vertical bars show the standard error of the replicates.

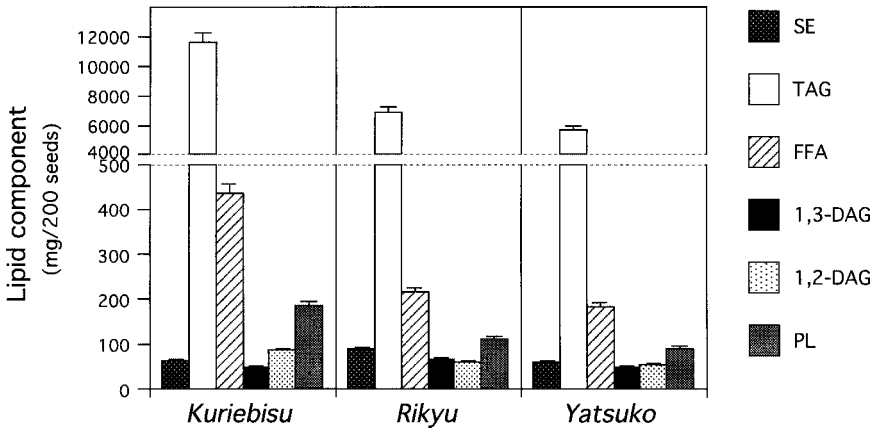

Figure 2. Lipid components in oils prepared from the kernels of pumpkin seeds. Each value represents the average of three replicates, and vertical bars show the standard error of the replicates. SE, steryl esters; TAG, triacylglycerols; FFA, free fatty acids; DAG, diacylglycerols; PL, phospholipids.

eg inappropriate storage, fungal contamination, etc. The presence of 0.5–2.5% FFAs in different coffee species has been reported.[20]

Fatty acid distributions (expressed in terms of the esters by weight) of TAGs, FFAs and PLs in the kernels were compared among the three cultivars (Fig 3). The fatty acid compositions of the cultivars *Rikyu* and *Yatsuko* were essentially the same, not only overall but also with respect to individual lipid types. The principal fatty acids for each genotype were oleic, linoleic, palmitic and stearic acids. All lipid samples had high amounts of total unsaturated fatty acids (which consisted mainly of linoleic followed by oleic acid), representing 78.5–81.2% for TAGs, 70.6–76.3% for FFAs and 68.5–70.2% for PLs. The fatty acid distribution patterns were very similar, not only between TAGs and FFAs but also among the three cultivars. However, a small difference ($P < 0.05$) in fatty acid composition was found when comparing *Rikyu* and *Yatsuko* with *Kuriebisu*. The percentage of oleic acid was lower ($P < 0.05$) in *Kuriebisu* than in the other cultivars (*Rikyu* and *Yatsuko*), and the value was compensated by an increase ($P < 0.05$) in linoleic acid. Low percentages were obtained for myristic, palmitoleic, linolenic and arachidic acids, denoted 'others' in Fig 3. The data showed that the percentage composition of oleic acid was higher than that of linoleic acid in the TAGs and FFAs, while the reverse was true in the fatty acid distribution of PLs of *Kuriebisu*. The fatty acid profiles of TAGs and FFAs were generally characterised by relatively higher percentages of unsaturated acids, linoleic and oleic, and relatively lower amounts of saturated acids, palmitic or stearic, compared with those of PLs.[21] *Rikyu* and *Yatsuko* had similar patterns of fatty acid distribution of TAGs, FFAs and PLs (Fig 3). The presence of high amounts of the essential fatty acid linoleic acid suggests that these oils are highly nutritious owing to the ability of unsaturated vegetable oils to reduce serum cholesterol.[22,23] The fatty acid profiles of total lipids were very similar to those of TAGs (Fig 3), because TAGs accounted for more than 92% of the total lipids in the pumpkin kernel oils (Fig 2). Therefore the fatty acid compositions of total lipids were omitted from Fig 3.

Pumpkin kernels contained even carbon-numbered (50–56) TAGs for all three cultivars as shown in Fig 4. Dominant components consisted of 52 and 54 TAGs, with much smaller amounts of 50 and 56 TAGs. The greatest amount of these TAGs was observed in *Kuriebisu,* followed by *Rikyu* and *Yatsuko*. These values reflect the differences in fatty acid content among the three cultivars (Fig 2). Figure 5 shows the patterns of the TAG molecular species isolated from the kernels of pumpkin seeds. Eleven different molecular species were detected in the oils extracted from the kernels. The three-letter designation does not suggest fatty acyl positional isomers in the TAGs. With a few exceptions, major TAG species were M_2D (OOL), MD_2 (OLL), SMD (POL or StOL), SD_2 (PLL or StLL), S_2D (PPL, PStL or StLL), SM_2 (POO or StOO) and M_3 (OOO) for *Kuriebisu*, M_3, SM_2, M_2D, SMD, S_2D and MD_2 for *Rikyu* and M_3, M_2D, SM_2, S_2D and SMD for *Yatsuko*. The other species (S_3, S_2M, D_3 and D_2T) were minor components (less than *ca* 5.0%). These results are not necessarily in agreement with the findings for other seeds such as sunflower and soybean seeds.[24] As shown in Fig 3, the fatty acid profiles of TAGs were very similar in the cultivars *Rikyu* and *Yatsuko*. However, the molecular species of TAGs differed ($P < 0.05$) among the three cultivars, and these values would reflect the differences in the cultivars.

Fig 6 summarises the fatty acid contents (S, M and D) for the TAGs isolated from the kernels of pumpkin seeds, expressed as mg per 200 seeds. Briefly, the amounts of fatty acids were summed up as S (16:0, 18:0 and 20:0), M (16:1 and 18:1) and D (18:2) from the results obtained by GC using pentadecanoate

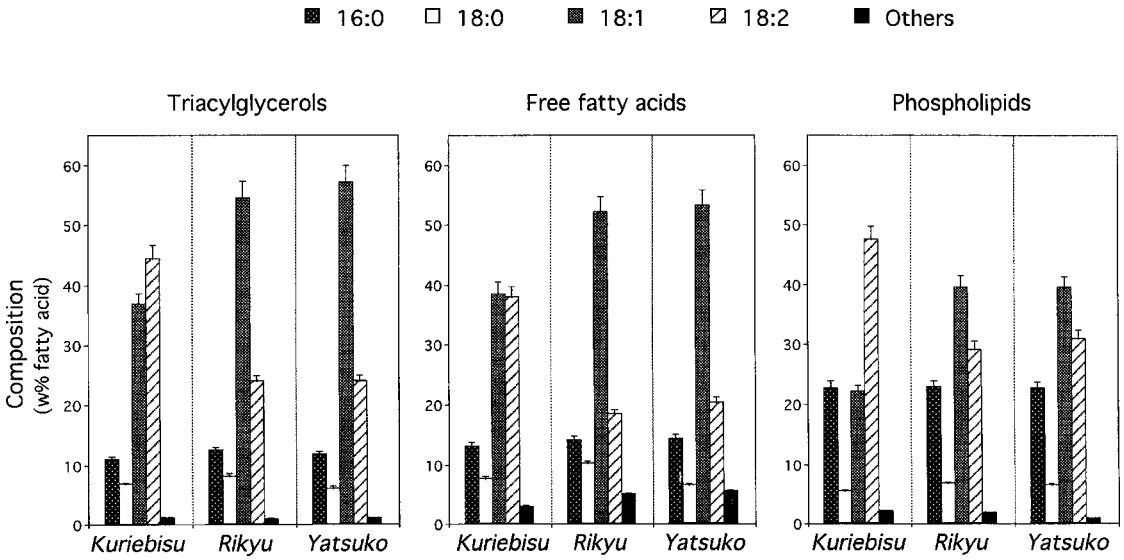

Figure 3. Fatty acid distributions of triacylglycerols, free fatty acids and phospholipids in kernels of pumpkin seeds. Each value represents the average of three replicates, and vertical bars show the standard error of the replicates. Other minor fatty acids include 14:6, 16:1, 18:3 and 20:0.

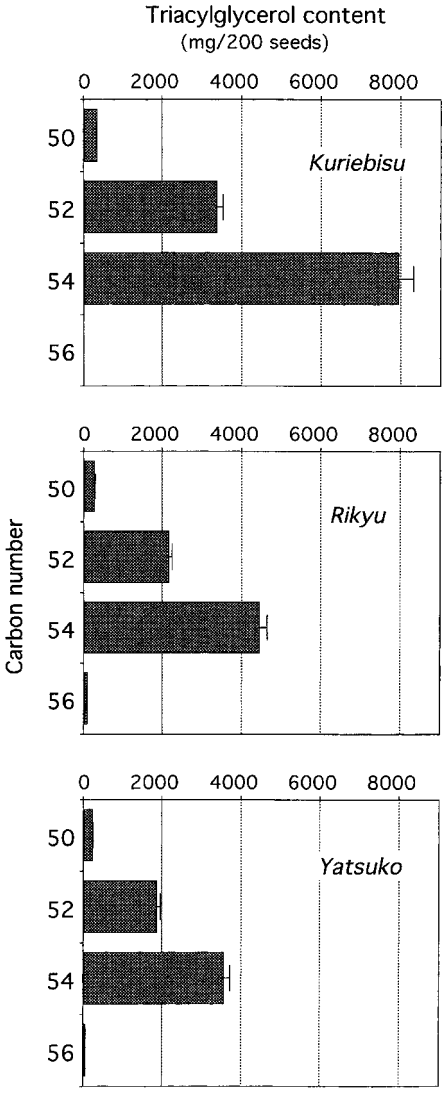

Figure 4. Triacylglycerol content in the kernels of pumpkin seeds. Carbon number denotes the total length of three acyl chains present in a triacylglycerol. Horizontal bars represent the standard error of the replicates.

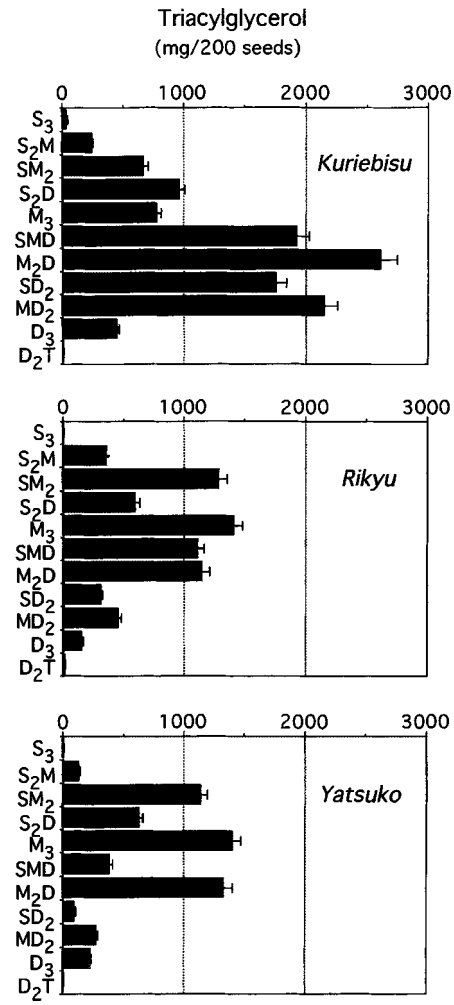

Figure 5. Characteristics of molecular species of triacylglycerols obtained from kernels of pumpkin seeds. Saturated fatty acids (S) consist of myristic (14:0), palmitic (16:0) and stearic (20:0) acids. Unsaturated fatty acids, oleic (18:1), linoleic (18:2) and linolenic (18:3), are denoted as monoene (M), diene (D) and triene (T), respectively. Horizontal bars represent the standard error of the replicates.

as internal standard. There were no quantitative or qualitative differences ($P < 0.05$) in the distribution between the experimental and calculated (data not shown) values.

CONCLUSIONS

Fatty acid distributions of major acyl lipids in the kernels of pumpkin seeds were observed using three cultivars. Furthermore, characteristics of molecular species of their TAGs were investigated. Pumpkin seed kernels could be utilised successfully as a source of edible oils for human consumption. Because of their high content of unsaturated fatty acids, pumpkin oils might be acceptable substitutes for highly unsaturated oils. We believe that the lipid class composition, the fatty acid profiles and the TAG composition of these samples of pumpkin seeds provide a good base for future examination of the quality of oils in the kernels of pumpkin seeds. To the best of the

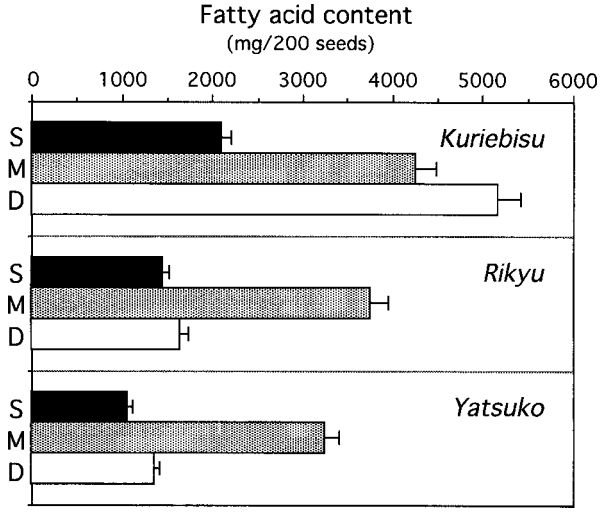

Figure 6. Content of fatty acids in triacylglycerols isolated from kernels of pumpkin seeds. Each value represents the average of three replicates, and vertical bars show the standard error of the replicates. Values obtained by GC in comparison with a known amount of methyl pentadecanoate as an internal standard using triacylglycerols isolated from the kernels. See figure 5 for abbreviations.

authors' knowledge, this is the first report on the TAG composition of pumpkin kernels. These results indicate that the differences in pumpkin cultivars could be appreciable, based on the distribution of molecular species of TAGs in the kernels. However, pumpkin seed flour has great potential for addition to food systems, not only as a nutrient supplement but also as a functional agent.

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