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Roasting effects on fatty acid distribution of triacylglycerols and phospholipids in the kernels of pumpkin (*Cucurbita* spp) seeds

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Abstract: In this study, changes in fatty acid distributions of pumpkin seeds (*Cucurbita* spp) in the course of the roasting process were investigated. Whole pumpkin seeds were exposed to microwaves for 6, 12, 20 or 30 min at a frequency of 2450 MHz. The kernels were separated from the seeds, and the lipid components and the fatty acid distributions of triacylglycerols (TAGs) and phospholipids (PLs) were analysed by a combination of thin layer chromatography and gas chromatography. Major lipid components were TAGs, free fatty acids (FFAs) and PLs, while steryl esters and diacylglycerols were also present in minor proportions. The greatest PL losses ($p < 0.05$) were observed in phosphatidyl ethanolamine, followed by phosphatidyl choline or phosphatidyl inositol. With a few exceptions, significant differences ($p < 0.05$) in fatty acid distributions occurred when the seeds were microwaved for 20 min or more. Nevertheless, the positional characteristics of fatty acid distributions in the TAGs were still retained after 20 min of roasting: unsaturated fatty acids were predominantly concentrated in the *sn*-2-position, and saturated fatty acids primarly occupied the *sn*-1- or *sn*-3-position. These results suggest that no significant changes in fatty acid distribution of TAGs and PLs would occur within 12 min of microwave roasting, thus ensuring that a good-quality product would be obtained.

Keywords: enzymatic hydrolysis; fatty acid distributions; kernels; microwave roasting; phospholipids; pumpkin (*Cucurbita* spp) seeds; triacylglycerols

INTRODUCTION

Microwave ovens are found in the majority of homes in Japan. Today more people use microwaves for cooking and reheating than ever before. Microwaves are used in the food industry not only for warming, drying thawing, baking and roasting, but also for other applications such as sterilising and pasteurising many types of foods.[1,2] Microwave penetration depths within a product are determined by the electrical and physical properties, and can vary significantly with chemical composition, product temperature and the frequency at which the microwave operates.[3] Microwave energy effects on various food components could differ significantly from those of conventional cooking. For example, it has been speculated that reactive free radicals may be formed from exposure to microwave energy,[4] especially in those applications that results in abnormally high temperatures, as with frying and toasting.

Increased attention has recently been focused on the utilisation of food-processing byproducts and wastes, as well as under-utilised 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 search for lesser-known crops, many of which are potentially valuable as human and animal foods, has intensified to maintain a balance between population growth and agricultural productivity, particularly in the tropical and subtropical areas of the world.

The value of pumpkin (*Cucurbita* spp) seeds as useful sources of proteins and oils has been reviewed by a few workers.[5,6] Although pumpkin seeds are utilised directly in several countries for human consumption as snacks after roasting and salting,[7] there are no publications concerned with the compounds responsible for the aroma of microwave-roasted pumpkin seeds. Furthermore, there has been no information on how microwave energy affects not only the lipid components in the kernels of

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pumpkin seeds but also the fatty acid distribution of triacylglycerols (TAGs) and phospholipids (PLs).

The primary purpose of this research was to examine the influence of microwave roasting on the composition and distribution of fatty acids in TAGs as well as PLs in the kernels of pumpkin seeds roasted in a microwave oven. A secondary objective was to compare the results among two cultivars with those obtained from unroasted pumpkin seeds.

MATERIALS AND METHODS

Sample seeds

Commercially available pumpkin seeds (*Cucurbita* spp) used in this study were from two Japanese cultivars, *Yatsuko* and *Kuriebisu*, that were grown in Japan during the summer of 2003. Cultivars were purchased from Takii Seed Co (Kyoto, Japan) and selected for uniformity based on seed weight of 120–150 mg for *Yatsuko* and 150–200 mg for *Kuriebisu*. The seeds were hand-selected to eliminate those with cracked and/or otherwise damaged seed hulls. 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 G 60 plates (10×20 or $20 \times 20\,cm^2$, 0.25 mm layer thickness) were purchased from Merck (Darmstadt, Germany). The TLC standard mixture, containing diacylglycerols (DAGs), free fatty acids (FFAs), triacylglycerols (TAGs) and steryl esters (SEs), was from Nacalai Tesque (Kyoto, Japan). Lipase (porcine pancreas) was purchased from Sigma Chemical Co (St Louis, MO) and was used after purification as previously described.[8] Standard PLs and fatty acid methyl esters (FAMEs) were the same as previously described.[9] A total of 100 mg of methyl pentadecanoate (C15:0, Merck) were dissolved in *n*-hexane (20 ml) and stored in a 10-ml glass volumetric flask until needed as an internal standard. Boron trifluoride (BF_3) in methanol (14%, Wako Pure Chemical Inc, Osaka, Japan) was used to prepare the FAMEs.

Microwave roasting of pumpkin seeds

A Sharp microwave oven (Model R-5550, Osaka, Japan) capable of generating 0.5 kW power at a frequency of 2450 MHz was used. Whole pumpkin seeds were arranged in a single layer in Pyrex Petri dishes (12.0 cm diameter). Three dishes, containing 100 seeds each, were placed on the turntable plate in the oven. The total weight of the 100 seeds thus treated was 13.2 (±0.3) g for *Yatsuko* and 17.8 (±0.4) g for *Kuriebisu*, respectively. After covering the dishes, the seeds were microwaved for 6, 12, 20 or 30 min.[10] The temperatures of the oven during microwave treatments were recorded automatically along with heating time. As soon as they were removed from the oven after each treatment, the internal temperature of the treated seeds was determined with a chromel–alumel thermocouple as previously described.[11]

Lipid extraction from pumpkin kernels

The roasted whole pumpkin seeds were allowed to cool to ambient temperature before homogenisation for lipid extraction. After microwave roasting the seeds were peeled with a razor blade to yield the kernels. The kernels (200 seeds) were extracted using a Maxim homogeniser (Nihonseiki Kaisha Ltd, Tokyo, Japan) at high speed for 10 min (0 °C, under ice) 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 on a Büchner 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,[12] and the phases were vigorously mixed. After phase separation, the chloroform layer was withdrawn, dried with anhydrous sodium sulfate and filtered. 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] and stored under nitrogen in the dark at −25 °C until analysed. Samples of unroasted pumpkin seeds were extracted by the same procedures and used as a control.

Lipid class analysis

Using previous methods,[13] the total lipid extracts 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 (105×16 mm; poly(tetrafluoroethylene)-coated screw caps). Methyl pentadecanoate solution (C15:0, 25 or 100 μg) was added as an internal standard to each tube.

Part of the PLs was further separated on TLC with chloroform/methanol/acetic acid/deionised water (170:30:20:7, v/v/v/v) into several bands. After development, each band was located by exposure to iodine vapour, and PL classes were identified not only by comparison with R_f-values of standard PLs similarly chromatographed, but also by the specific spray reagents: Dragendorff reagent for choline lipids,[14] 0.25% ninhydrin in acetone for amino-containing lipids, and molybdate reagent for PLs.[15] Bands corresponding to phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) and phosphatidyl inositol (PI) were carefully scraped into test tubes, respectively, and methyl pentadecanoate (25 μg) was added as described above.

Fatty acid methyl esters were prepared from the isolated lipids by heating the samples at 80 °C for 90 min in BF_3–methanol on an aluminium block bath.[16] To this solution after cooling, 5 ml of *n*-hexane was added. The organic layer containing the FAMEs was recovered. The solvent was then vaporised under a gentle stream of nitrogen and the residue was quantified on a Shimadzu Model-14A GC (Shimadzu, Kyoto, Japan) equipped with a hydrogen ionisation detector as described.[9] A glass column, 200 cm × 3 mm id, was packed with 15% EGSS-X on a 100–200 mesh Gaschrom Q (Nishio Co Inc Ltd.). The column oven temperature was run isothermally at 180 °C, and the injection port and detector temperatures were both 220 °C. Helium carrier gas flow rate was 40 ml min^{-1}. All samples were dissolved in *n*-hexane for injection. The detection limit was 0.05 wt% of total fatty acids for each FAME in a FAME mixture, and the results were expressed as wt% of total FAMEs.

Enzymatic hydrolysis of lipids

Triacylglycerol hydrolysis *in vitro* was carried out according to the method previously described.[8] In the previous work, we confirmed by TLC and GC that no fatty acids in the *sn*-2-position of TAGs are transferred to the *sn*-1- or *sn*-3-position within 60% hydrolysis (for 20 min). The reaction products were separated by TLC as already described.[8] The FFAs and *sn*-2-monoacylglycerol bands were scraped off the plate and transmethylated. The procedure was checked by comparing the fatty acid composition of the original TAGs and those remaining after the partial hydrolysis.

Statistical analysis

All experiments were done in triplicate, and results were subjected to an analysis of variance for a completely random design as described by Steel *et al*[17] to examine the least significant difference at the level of 0.05.

RESULTS AND DISCUSSION

Microwave roasting and lipid components

The internal temperature of the pumpkin seeds at the end of each roasting time was compared (data not shown). Briefly, the temperature of the seed sample was 25 °C before microwave roasting and was increased to 92, 116, 123 and 130 °C, at 6, 12, 20 and 30 min of microwave roasting, respectively. Profiles of the different lipids in the kernels of pumpkin seeds before and after microwave roasting were compared among two cultivars (Table 1).

The major lipid component, TAGs, still represented >90 wt% of the total lipids after 30 min of roasting, while FFAs and PLs were also present in minor proportions. However, SEs, 1,3- and 1,2-DAGs were designated as 'others' in Table 1 because these lipids were detected in very small amounts. FFA level was higher in *Kuriebisu* than in *Yatsuko*. The presence of FFAs in oil samples may be due to the partial enzymatic hydrolysis of reserve TAGs during storage of the seeds.[18]

As microwave roasting proceeded, a significant change in FFAs, 1,3- and 1,2-DAGs was observed at 12 min, and even more pronounced differences ($p < 0.05$) were observed at 30 min. Conversely, the amount of PLs gradually decreased by 14.1–21.4 mg for 12 min, 27.4–44.7 mg for 20 min, and 40.7–66.3 mg for 30 min, respectively. Cossignani *et al*[19] reported that there was a significant decrease in the TAG fraction and increased DAG and monoacylglycerol fractions in olive oil following microwave treatment. Although Abou-Gharbia *et al*[20] observed that TAGs of sesame seeds were gradually hydrolysed randomly by microwaves to produce DAGs and FFAs, the present work showed that there was an increase in 'others' fractions, primarily with increased FFAs and browning substances.[21] A partial hydrolysis of the ester bond in TAGs would be caused by molecular friction of electrical dipoles under an oscillating electric field of specific frequency.

Table 1. Lipid components in the oils prepared from the kernels of pumpkin seeds roasted at different times in a microwave oven (at a frequency of 2450 MHz)[a]

		Total lipids	Triacylglycerols	Free fatty acids	Phospholipids	Others
Cultivar	Roasting time (min)			(mg/200 seeds)		
Kuriebisu	Unroasted	12 431.0a	11 610.6a (93.4)	435.0d (3.5)	186.5a (1.5)	198.9a (1.6)
	6	12 065.2b	11 231.2b (93.0)	453.4d (3.8)	175.3b (1.5)	205.3ab (1.7)
	12	11 784.6c	10 890.8c (92.4)	505.2e (4.3)	165.1c (1.4)	223.5c (1.9)
	20	11 374.4d	10 417.3c (91.5)	575.3f (5.1)	143.8d (1.3)	238.0de (2.1)
	30	11 013.9e	10 019.9e (91.0)	631.9g (5.7)	120.2e (1.1)	241.9e (2.2)
Yatsuko	Unroasted	6117.4f	5670.8f (92.7)	177.4a (2.9)	97.9f (1.6)	171.3a (2.8)
	6	6051.0g	5597.2g (92.5)	187.5ab (3.1)	90.8g (1.5)	175.5ab (2.9)
	12	5984.6h	5511.8h (92.1)	197.5b (3.3)	83.8h (1.4)	191.5cd (3.2)
	20	5873.7i	5362.7i (91.3)	234.9c (4.0)	70.5i (1.2)	205.6d (3.5)
	30	5720.6j	5171.4j (90.4)	274.6c (4.8)	57.2j (1.0)	217.4e (3.8)

[a] Each value is the average of three determinations and expressed as mg of lipid in 200 kernels. Values in parentheses are relative percentage content of the individual lipids in total lipids. Others include minor lipid components such as steryl esters, diacylglycerols, browning substances and unknown. Values in the same column with different letters are significantly different from those for unroasted seeds ($p < 0.05$).

Effect of microwave roasting on fatty acid distributions of TAGs

The changing profiles of composition and positional distribution of fatty acids of TAGs isolated from the kernels of pumpkin seeds roasted for different time periods in a microwave oven were compared for the two cultivars (Fig 1). The principal fatty acids for each genotype were oleic, linoleic, palmitic and stearic. However, significant differences ($p < 0.05$) occurred in the fatty acid distributions between *Yatsuko* and *Kuriebisu*. The percentage of oleic acid was lower ($p < 0.05$) in *Kuriebisu* than in *Yatsuko*, and the value was compensated by an increase ($p < 0.05$) in linoleic acid (Fig 1).

Nevertheless, the principal characteristics for the positional distribution of fatty acids still remained during microwave roasting; unsaturated fatty acids, especially linoleic and/or oleic, were predominantly concentrated in the *sn*-2-position among both cultivars. However, saturated fatty acids, especially palmitic and stearic, were mostly located in the *sn*-1,3-position. Therefore, oleic acid was almost evenly distributed in the *sn*-1-, *sn*-2- or *sn*-3-position, and the results were in agreement with those reported by other researchers.[22] These trends were more pronounced in *Kuriebisu* than in *Yatsuko*.

Effect of microwave roasting on major phospholipid components

To clarify the effects of microwave roasting on PLs, further separation of the PL fraction into three fractions (PE, PC and PI) was carried out on TLC in the presence of authentic samples. Table 2 presents the changing pattern in the PL fractions of the pumpkin kernels before and after microwave roasting. Dominant components in the kernels were PC, followed by PE and/or PI among the two cultivars. With longer roasting time, the rate of PL losses ($p < 0.05$) increased in the following order, PI, PC and

Table 2. Major phospholipid contents in the oils obtained from the kernels of pumpkin seeds roasted at different times in a microwave oven (at a frequency of 2450 MHz)[a]

Cultivar	Roasting time (min)	Phosphatidyl-ethanolamine	Phos-phatidyl-choline	Phos-phatidyl-inositol
Kuriebisu	Unroasted	35.4a (19.0)	102.8a (55.1)	48.3a (25.9)
	6	32.8b (18.7)	96.0b (54.8)	46.5a (26.5)
	12	30.6c (18.5)	90.7c (54.9)	43.8b (26.5)
	20	27.0d (18.8)	75.3d (52.2)	41.5c (28.9)
	30	15.3i (12.7)	70.9e (59.0)	34.0d (28.3)
Yatsuko	Unroasted	22.0e (22.5)	52.8f (53.9)	23.1e (23.6)
	6	20.1f (22.1)	47.7g (52.5)	23.0e (25.4)
	12	17.5g (20.9)	43.5h (51.9)	22.8e (27.2)
	20	12.2h (17.3)	36.8i (52.2)	21.5e (30.5)
	30	7.8j (13.7)	29.6j (51.7)	19.8f (34.6)

[a] Each value is the average of three determinations and expressed as mg lipid per 200 kernels. Values in parentheses are relative percentage content of the individual lipids in total lipids. Values in the same column with different letters are significantly different from those for unroasted pumpkin seeds ($p < 0.05$).

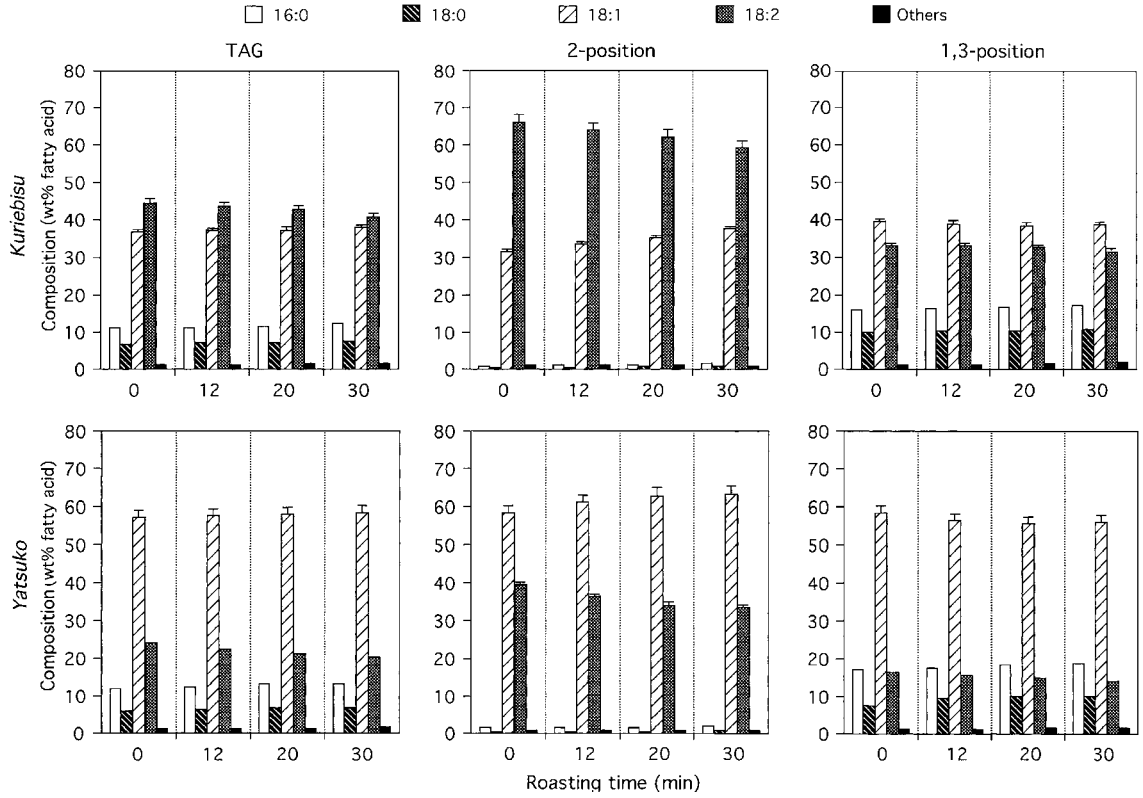

Figure 1. Changes in composition and positional distribution of fatty acids of triacylglycerols prepared from the kernels of pumpkin seeds roasted at different times in a microwave oven (at a frequency of 2450 MHz). Each value represents the average of three replicates, and vertical bars show the standard error of the replicates. Others include minor fatty acids of C14:0, C16:1, C18:3 and C20:0.

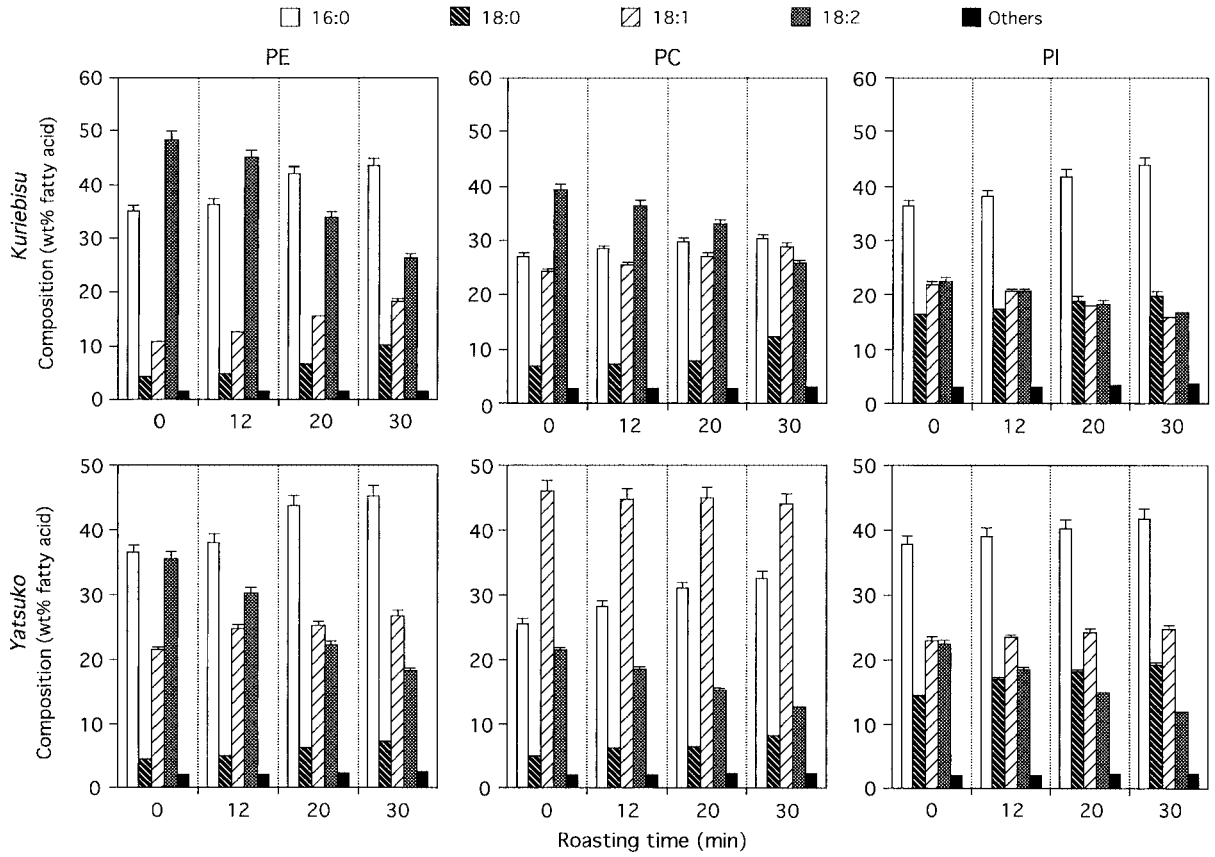

Figure 2. Changes in fatty acid distributions of major phospholipids prepared from the kernels of pumpkin seeds roasted at different times in a microwave oven (at a frequency of 2450 MHz). Each value represents the average of three replicates, and vertical bars show the standard error of the replicates. Others include minor fatty acids of C14:0, C16:1, C18:3 and C20:0.

PE. The trends became more pronounced in *Kuriebisu* than in *Yatsuko* following microwave roasting. The results may reflect the differences in the composition and distribution of fatty acids (Fig 2).

Effect of microwave roasting on fatty acid distributions in major phospholipids

The changing profiles of composition and distribution of fatty acids of PE, PC and PI in the pumpkin kernels were compared before and after microwave roasting among two cultivars (Fig 2). The major fatty acids in three PLs were linoleic, oleic, palmitic and stearic acids. With a few exceptions, these fatty acid distributions differed ($p < 0.05$) not only among the individual PLs but also between the two cultivars. However, PI was unique in that it had the highest saturated fatty acid content among the three PLs, although their distribution patterns were similar between *kuriebisu* and *Yatsuko.*

However, fatty acid distribution patterns in the PC varied significantly ($p < 0.05$) not only among other two PLs but also between *Kuriebisu* and *Yatsuko*. The percentage of oleic acid was lower ($p < 0.05$) in *Kuriebius* than in *Yatsuko,* and the value was compensated by an increase ($p < 0.05$) in linoleic acid. The results were in agreement with those for TAGs as shown in Fig 1. Although small significant differences ($p < 0.05$) were observed in the percentage of each fatty acid, these profiles in the PE were essentially the same between the two cultivars.

The percentage of linoleic acid showed significant decrease ($p < 0.05$) in three PLs after 20 min of microwave roasting (with a few exceptions). The percentage composition and distribution of linoleic acid was more significantly decreased ($p < 0.05$) in the PLs (Fig 2) than in TAGs (Fig 1) as well as in the two cultivars. The amino group of PE can apparently facilitate hydrogen or electron donation to tocopherols in the seeds.[23] At elevated temperatures, some of the naturally occurring classes of PLs, and in particular PE, greatly enhance the activity of primary antioxidants in edible oils, but PI is without synergistic activity. Furthermore, PLs are reported to cause a browning phenomenon during roasting.[24] The data for 6 min of roasting were omitted from Figs 1 and 2 because they were essentially the same as those before microwave roasting. These results may be due to the high penetration power of microwaves and also the differences in the composition and distribution of fatty acids between the two cultivars.

CONCLUSIONS

When the increase in the temperature was a consequence of the microwave energy, the loss of the TAGs and PLs such as PE was more generalized, suggesting a synergistic effect between temperature

and microwave energy. This results in a lower percentage of linoleic acid and a greater percentage of oleic, palmitic and stearic acids in the pumpkin kernels. These results suggest that the differences in pumpkin cultivars could be appreciable, based on the fatty acid distribution of TAGs as well as PLs in the kernels. Because microwave roasting does not have any adverse effects on seed or oil quality,[25] the use of short-term microwave roasting to reduce seed moisture and retard seed deterioration is technically feasible and should be examined for economic feasibility by oilseed processing mills. Moreover, 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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