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Profiles of Fatty Acid Distribution of Different Acyl Lipids from Red and Black Rices

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Abstract

The present study is focused on the variation in fatty acid (FA) composition of the different acyl lipids in red and black rices. Total lipids were fractionated by TLC into eight subfractions. Significant differences ($P < 0.05$) in FA distribution were found among the different acyl lipids when the two cultivars were compared. There were no significant differences ($P > 0.05$) in the positional distribution of FA in triacylglycerols; unsaturated FA was predominantly concentrated in the *sn*-2 position while saturated FA primarily occupied the *sn*-1 position or *sn*-3 position in the oils. Significant differences ($P < 0.05$) in FA distribution existed when the individual phospholipids were compared. Based on the FA composition of the different acyl lipids, it seems that red and black rices are very similar to each other with a few exceptions. The results suggest that these rice lipids may be well incorporated into our daily diet to improve value of the Japanese diet.

Keywords: diacylglycerols, fatty acid distributions, free fatty acids, phospholipids, rice seeds, steryl esters, triacylglycerols

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops cultivated in the world. Rice is consumed throughout the world and is stable in many cultures (Xue et al., 2008). It is consumed as a staple by over one-half of the world's population with approximately 95% of production in Asia (Bahattacharjee, Singhal & Kulkarni, 2002). Red and black rices, both found in Japan, are classified as *Oryza sativa* L. The colored grains are cause by anthocyanin pigments that gives the dulled rice a red and brownish red color in red rice or a dark purple color in black rice. Nowadays those two types of rice were gaining popularity in Japan as functional food and often mixed with rice to enhance the flavor, color, and nutritional value (Itani & Ogawa, 2004).

Black rice is popular in Asian countries where it is often mixed with white rice before cooking to enhance the flavor, color, and nutritional value (Bouis et al., 2003). Black rice is broadly known as enriched rice with medicinal effects. It is superior to robust nourishment and acts enhancer for some functions of spleen, liver, stomach, and intestine, and as a hematopoietic agent in pharmaceuticals (Lui, 2007; Yawadio, Tanimori, & Morita, 2007). Anthocyanin pigments, cyanidin 3-glucoside and peonidin 3-glucoside, were isolated from these plants (Ryu, Park, & Ho, 1998; Abdel-Aal, Young & Rabalask, 2006). Various compounds, such as phenolic acids, vanillin acid, ferulic acid, protocatechuic acids, phenolic acid esters, phytic acid, γ-oryzanol, and inositol were found from rice bran (Hudson et al., 2000). Black rice has a number of nutritional advantages over common rice, such as higher protein, total essential amino acids, vitamin B_1, and minerals (which vary with cultivars and production location) (Suzuki et al., 2004). Black rice has a relatively intense flavor that is distinctly different from other types of aromatic rice. Flavor is considered the single most critical quality trait in rice affecting consumer preference (Suwanstri et al., 2002; Shen et al., 2009).

The composition of total fatty acids (FA) is often the only information provided in studies on rice lipids. Therefore, in this study, the red and black rices were analyzed with respect to FA composition of the separated lipid classes-triacylglycerols (TAG), diacylglycerols (DAG), free FA (FFA), phospholipids (PL), steryl esters (SE)-in an attempt to investigate the variability in their relatively proportions and the FA percentages in each lipid class.

2. Materials and Methods

2.1 Materials

Commercially available mature rice used in this study was from red and black rices grown in different districts of Japan during the summer of 2010. These rices were selected for uniformity based on seed weight of 63.8-64.9 mg for red and 54.5-55.0 mg for black. The rices were hand-selected to eliminate those that were cracked or otherwise damaged. Rices of each cultivar were stealed in a polyethylene bag under a nitrogen gas and stored in a stainless steel container at -20℃ until analysis.

2.2 Reagents and Standards

All solvents and chemicals used were of analytical grade (Nacalai Tesque, Kyoto, Japan), but diethyl ether was further purified to remove peroxides. TLC plates (silica gel 60 G, 20 x 20 cm, 0.25 mm thickness) were procured from Merck (Darmstadt, Germany). A 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 (Kyoto, Japan). A phospholipid kit from Serdary Research Laboratory (Missisauga, ON, Canada) was used as phospholipid (PL) standard. Lipase from porcine pancreas was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), and used after purification with acetone and then diethyl ether according to the previously mentioned (Yoshida & Alexander, 1983). Glyceryl-*sn*-1,3-myristate-*sn*-2-oleate (Sigma Chemical Co.) was used as TAG standard for enzymatic hydrolysis. FA methyl ester (FAME) standards (F & OR mixture #3) were procured from Altech-Applied Science (State College, PA, USA). The internal standards, pentedecane and methyl pentadecanoate, were purchased from Merck, and then 100 mg of each was dissolved in *n*-hexane (20 ml). Boron trifluoride (BF_3) in methanol (14%; Wako Pure Chemical Inc., Osaka, Japan) was used to prepare the FAME.

2.3 *Methods*

2.3.1 Chemical Analysis

AOAC (1997) methods were used to determine the chemical composition of these rices. 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 a Kjeldahl method (Method 981.10) and moisture content by oven-drying to constant weight at 105˚C (Method 925.40).

2.3.2 Extraction of Lipids

Rices (100g) 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 beforte extraction. Total lipids were extracted from 50 g of rice flour in 300 ml chloroform/methanol (2:1, v/v) with vigorous shaking for 15 min at 0°C three times, following the Folch procedure (Folch, Lee, & Sloane-Stanley, 1957). These solvents contained 0.01% butylated hydroxytoluene (BHT; Wako Pure Chemical Inc.) to inhibit oxidative degradation of lipids during analysis. Namely, the extraction was repeated thrice, and the individual extracts were vacuum-filtered through defatted filter paper on a Buchner funnel. These filterates were combined and dried in a rotary evaporator at 35°C. The residue was dissolved in 100 ml of chloroform/methanol (2:1, v/v). Then, 20 ml aqueous KCl (0.75%) were added (Folch, Lee & Sloane-Stanley, 1957), and mixed vigorously. After phase separation, the chloroform layer was removed, dried over anhydrous Na_2SO_4, filtered, and the filtrate was concentrated under vacuum in a rotary evaporator at 35°C. The extracted lipids were weighed to determine the lipid content of the rice and then transferred to a 25-ml brown glass volumetric flask with chloroform/methanol (2:1, v/v) and kept under nitrogen at -35°C until further use.

2.3.3 Lipid Analysis

According to the previously described procedure (Yoshida, Tomiyama, & Mizushina, 2010), total lipids were separated by TLC into eight sufractions with a solvent system of *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Bands corresponding to HC, SE, TAG, FFA, 1,3-DAG, 1,2-DAG, unknown and PL were scraped into separate test-tubes [105 x 16 mm; poly (tetrafluoroethylene)-coated screw caps]. Methyl pentadecanoate (10-100 g) from a standard solution (5 mg/ml) was added to each tube as the internal standard with a microsyringe (Hamilton Co., Reno, NV, USA), except that pentadecane (10 g) was used as the internal standard for HC analysis. 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 (Kitts et al., 2004). After cooling, 5 ml of *n*-hexane was added to each tube and washed several times with deionized water to remove the BF_3 and silica gel. The *n*-hexane layer containing the FAME was recovered and dried over anhydrous Na_2SO_4. The solvent was then vaporized under a gentle stream of nitrogen, and the residue (FAME) was quantified by gas chromatography (GC, Shimadzu model-14B,

Kyoto, Japan) equipped with a hydrogen flame ionization detector (FID) at 250°C and a polar capillary column (ULBO HE-SS-10 for FAME fused silica WCOT [serial no. PSC5481], cyanopropyl silicone, 30 m x 0.32 mm i. d.; Shinwa Chem. Ind., Ltd., Kyoto, Japan).

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 180kPa. The oven temperature was programmed from an initial temperature of 180°C (2 min held), and increased to 200°C at a rate of 2°C/min, and then held isothermally (200°C) for 15 min. Both injection and detector temperatures were set at 250°C, respectively. All samples were dissolved in *n*-hexane and the aliquot (2-5 μl) was injected with a microsyringe. The component peaks were identified and compared against that of the standard FAME using an electronic integrator (Shimadzu C-R4A). The detection limit was 0.05 wt-% for each FAME in the FAME mixture, and the results are expressed as wt-% of total FAME. The other GC conditions were as previously reported method (Yoshida et al., 2008).

Samples of the extracted polar lipids, obtained as described above, were further separated by TLC into several fractions with chloroform/methanol/acetic acid/deionized water (170:30:20:7, by volume) as the mobile phase. PL classes were detected iodine vapor and were consistent with authentic standards. Bands corresponding to phosphatidyl ethanolamine (PE), phoshatidyl choline (PC), phosphatidyl inositol (PI) and others were carefully scraped into separate test-tube. Methyl pentadecanoate (10-25 g) of a standard solution (5 mg/ml) was added to each tube as the internal standard with a microsyringe. Their FAME were prepared by the same method as described above and quantified by GC.

2.3.4 Enzymatic Hydrolysis of TAG

TAG hydrolysis was carried out *in vitro* according to the previously described method (Yoshida & Alexander, 1983). A 30 min reaction was selected based on the preliminary results using the standard TAG (glyceryl-*sn*-1,3-myristate-*sn*-2-oleate: Sigma Chemical Co.). After approximately 60% of the TAG was hydrolyzed, 0.5 ml of 6 M HCl and 1 ml ethanol were added to stop the reaction. In the preliminary experiments, no FA (oleic acid) at the *sn*-2 position of standard TAG was transferred to the *sn*-1 or *sn*-3 position at 60% hydrolysis for 30 min. The reaction products were separated by TLC with *n*-hexane/diethyl ether/acetic acid (60:40:1, v/v/v) as previously described (Yoshida & Alexander, 1983). The FFA and *sn*-2 MAG bands were carefully scraped into test-tubes, respectively and then methylated (Kitts et al, 2004). The procedure was checked by comparing the FA compositions of the original TAG and the TAG remaining after partial hydrolysis. The constituent FA were analyzed by GC described above.

2.4 Statistical Analyses

All experiments were repeated at three times. The results were subjected to one-way analysis of variance (ANOVA) (Steel, Torrie, & Duckey, 1995). Significant differences ($P < 0.05$) were identified using multiple comparison tests, following a previously described method (Baker, 1980).

3. Results and Discussion

3.1 Lipid Compositions in the Rices

The lipid content of the rice samples analyzed was 2.2-2.7% (data not shown). The percentage of lipid contents was higher in black rice than in red rice. As shown in Table 1, these different lipid classes were compared between the two cultivars.

Table 1. Lipid components obtained from red and black rices

Lipid class	Cultivar (mg/100g rice)	
	Red	Blank
Hydrocarbons	3 ± 0.1^{a} (0.1)	14.6 ± 0.6^{b} (0.4)
Steryl esters	62.1 ± 2.5^{a} (2.1)	65.8 ± 2.8^{a} (1.8)
Triacylglycerols	2260 ± 30^{a} (76.4)	2945 ± 38^{b} (80.5)
Unknown	38.5 ± 1.2^{b} (1.3)	15.3 ± 0.7^{a} (0.4)
Free fatty acids	289 ± 18^{b} (9.8)	263 ± 12^{a} (7.2)
1,3-Diacylglycerols	121 ± 6^{a} (4.1)	138 ± 8^{b} (3.8)
1,2-Diacylglycerols	79.9 ± 2.6^{a} (2.7)	84.1 ± 2.9^{a} (2.3)
Phospholipids	104 ± 5^{a} (3.5)	132 ± 7^{b} (3.6)

Mean of three analyses ± standard deviation. Values in parentheses are relative wt-% contents of individual lipids in total lipids. Values in a row with different superscript are significantly different between individual cultivars ($P<0.05$).

Predominant components were TAG (76.4-80.5%), FFA (7.2-9.8%), 1,3-DAG (3.8-4.1%) and PL (3.5-3.6%), with very small amounts (0.1-2.7%) of other lipid components. With a few exceptions, these distribution patterns were very similar to each other when the two cultivars were compared. No substantial differences ($P > 0.05$) in the lipid components could be observed using values estimated by combining TLC and GC, using the internal standard (C15:0). These results are similar to those reported by other researchers previously (Pham et al, 1998). Presumably, the minor components, such as FFA, 1,3- and 1,2-DAG, may be due to the partial enzymatic hydrolysis of reserve TAG during storage of the rice seeds after harvesting (Okunishi & Ohtsubo, 2008). The lipid components resulting from 'fat by hydrolysis' in starch granules were examined, showing the presence of FFA with lysolecithin and lysoglycolipids (Hirayama & Matsuda, 1973).

3.2 FA Compositions of the Different Acyl Lipids in the Rices

FA compositions (expressed in terms of the esters by weight) of individual lipid classes in rice seeds were compared between the two cultivars (Figures 1 and 2). The principal FA components are generally palmitic (16:0), stearic (18:0), oleic (18:1*n*-9) and linoleic (18:2*n*-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 two cultivars in the different acyl lipids. Palmitic (16:0) acid showed the following percentages in the different lipid classes: 1,3-DAG (13%), SE, total lipids, TAG and 1,2-DAG (22-25%), PL (30%) and FFA (35%). Stearic (18:0; 13%) acid in the SE fraction showed higher ($P < 0.05$) than that in other fractions. Oleic (18:1*n*-9) acid was in a range of 27.6-28.4% (PL), 32-33.2% (FFA), 34.0-34.8% (total lipids), 36.6-37.5% (1,2-DAG and SE) and 41.8-45.% (1,3-DAG and TAG). On the other hand, linoleic (18:2*n*-6) acid was in a range of 25.5-29.5% (SE, FFA and TAG), 33.5-36.3% (1,3-DAG, 1,2-DAG and PL) and 39.2-40.3% (total lipids), respectively. Therefore, the samples presented significant amounts of total unsaturated FA, which consisted mainly of oleic (18:1*n*-9) and linoleic (18:2*n*-6) acids, representing 77.0-77.3% for 1,3-DAG, 74.6-74.8% for total lipids or TAG, 71.7-72.3% for 1,2-DAG, 66-66.6% for PL, 63.5-63.7% for SE and 60.9-61.5% for FFA, respectively.

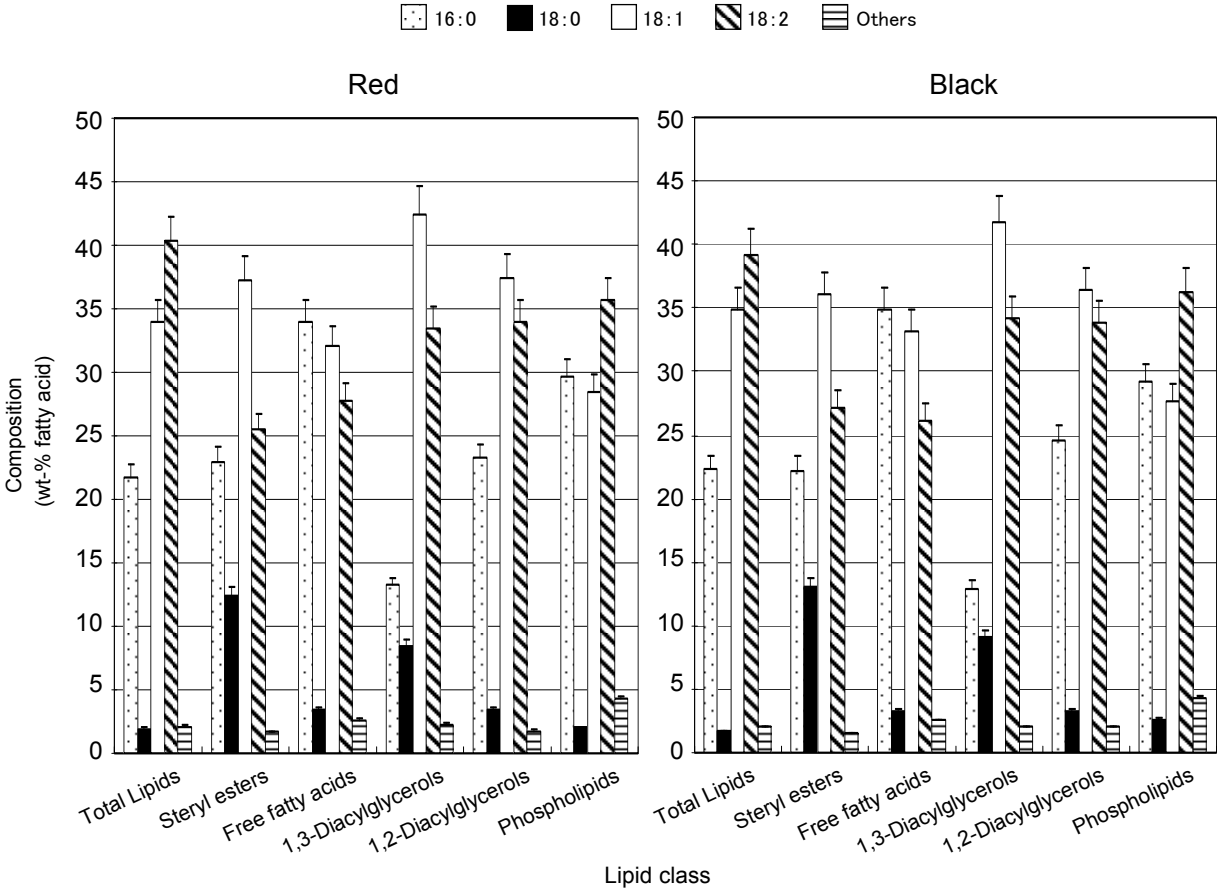

Figure 1. Fatty acid distribution of differente lipids obtained from red and black rices. Each value is the average of three replicates, and vertical bars depict the mean and standard deviation of the replicates. Others minor fatty acids include 14:0, 16:1 and 20:0

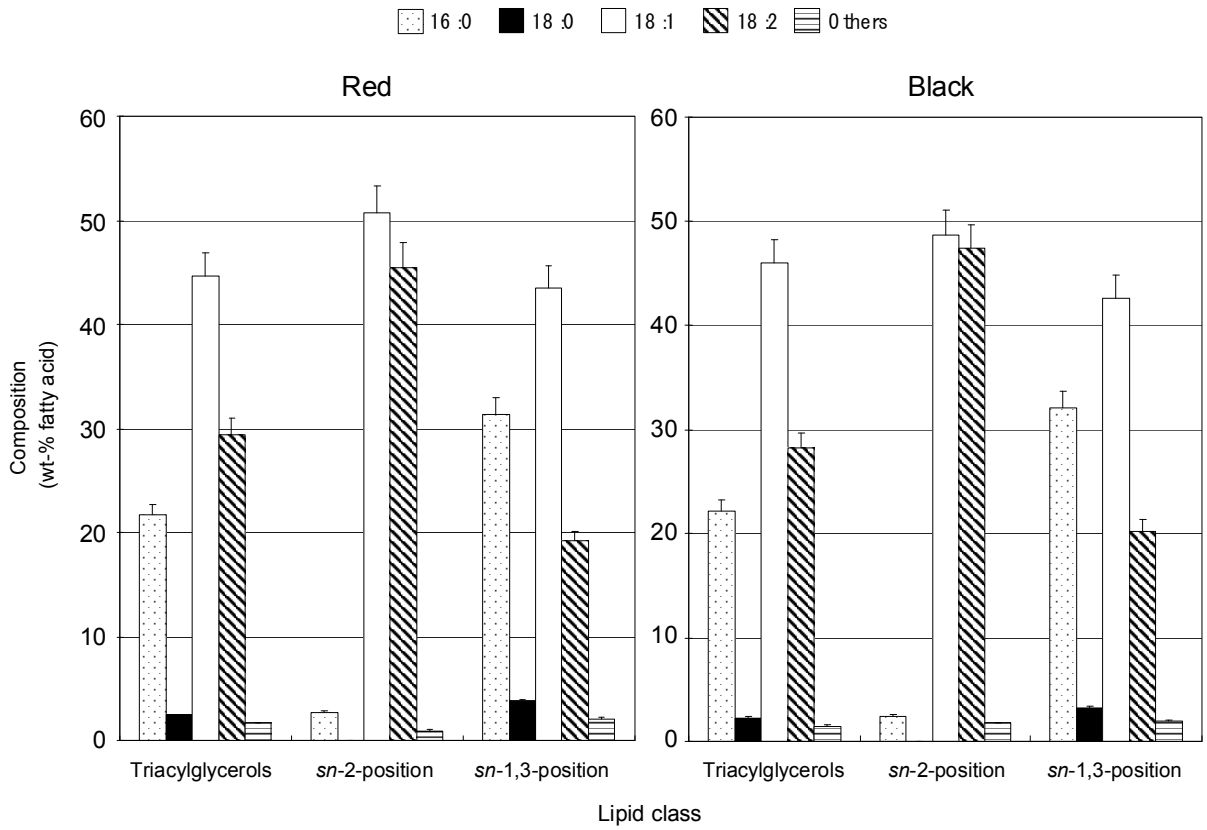

Figure 2. Composition and positional distribution of fatty acids of triacylglycerols obtained from red and black rices. Each value is the average of three replicates, and vertical bars depict the mean and standard deviation of the replicates. Others minor fatty acids include 14:0, 16:1 and 20:0

3.3 Positional Distribution of FA within TAG in the Rices

Our preliminary work on the positional distribution of FA on the glycerol backbone was done with pancreatic lipase (Yoshida & Alexander, 1983). The profiles of composition and distribution of FA in TAG were compared between the two cultivars (Figure 2). The major component was palmitic (16:0; 21.7-22.1%), oleic (18:1*n*-9; 44.6-45.9%) and linoleic (18:2*n*-6; 28.2-29.5%) acids, followed by stearic (18:0; 2.3-2.5%) and α–linolenic (18:3*n*-3; 1.0-1.2%) acids. Linoleic (18:2*n*-6) acid was predominantly (45.0-47.3%) concentrated in the *sn*-2 position of TAG molecules, while saturated FA such as palmitic (16:0) and stearic (18:0) acids primarily located in the *sn*-1 or the *sn*-3 position (35.2%). With a few exceptions, however, oleic (18:1*n*-9) acid was almost evenly distributed at high levels (42.6-50.8%) in the *sn*-1, 2, or 3 molecules, corroborating results of previous researchers (Reske, Siebrech & Hazebroek, 1997). No significant difference ($P > 0.05$) occurred in the FA distributions among the two cultivar. Taken together, the positional distributions of FA in the TAG were very similar to the results obtained from other plant seed lipids such as kidney beans (Mabaleha & Yeboa, 2004) and peas (Yoshida et al., 2007).

3.4 FA Distribution of Major PL in the Rices

To clarity the distribution of individual PL in these rices, further separation of the PL fraction into several fractions (PE, PC, PI, and others) was done on TLC in the presence of authentic standards. Comparison were made of the two cultivars for the profiles of PE, PC, PI, and others (Table 2).

Regardless of cultivars, PC, PE, and PI were the principal PL in the these rices, and the highest content was observed for PC (52.3-53.7%), PE (22.3-23.1%), and PI (20.6-21.3%), and accompanied by very small amounts (3.3-3.4%) of other PL.

Table 2. Content of Major Phospholipids Obtained from Red and Black Rices

Phospholipid class	Cultivar (mg/100g rices)	
	Red	Blank
Phosphatidyl ethanolamine	30.5 ± 1.1^{b} (23.1%)	23.3 ± 0.6^{a} (22.3%)
Phosphatidyl choline	69.0 ± 1.8^{b} (52.3%)	56.2 ± 1.3^{a} (53.7%)
Phosphatidyl inositol	18.2 ± 0.8^{a} (21.3%)	21.5 ± 0.6^{b} (20.6%)
Others	4.4 ± 0.2^{a} (3.3%)	3.6 ± 0.1^{a} (3.4%)

Mean of three analyses ± standard deviation. Values in parentheses are the relative wt-% contents of individual lipids in PL. "Others" include minor PL components such as diphosphatidylglycerol, phosphatidic acid, phosphatidylglycerol and lysophospholipid. Values in the same column with different superscripts are significantly different from those of individual cultivars (P<0.05).

Table 3. Fatty Acids Distribution of Major Phospholipids Obtained from Red and Black Rices

Phospholipid	Cultivar	Fatty acid (wt-%)				
		16:0	18:0	18:1	18:2	Others
Phosphatidyl ethanolamine	Red	17.8 ± 0.8^{a}	1.9 ± 0.1^{a}	36.2 ± 1.7^{b}	42.8 ± 2.0^{c}	1.3 ± 0.1^{a}
	Blank	18.7 ± 0.9^{a}	2.7 ± 0.1^{b}	34.5 ± 1.6^{b}	41.8 ± 1.9^{c}	2.3 ± 0.1^{b}
Phosphatidyl choline	Red	25.8 ± 1.0^{b}	2.9 ± 0.1^{b}	43.7 ± 2.1^{c}	26.4 ± 1.2^{b}	1.2 ± 0.1^{a}
	Blank	25.6 ± 1.2^{b}	2.6 ± 0.1^{b}	42.3 ± 2.1^{c}	27.8 ± 1.9^{b}	1.7 ± 0.1^{a}
Phosphatidyl inositol	Red	45.7 ± 1.0^{b}	2.9 ± 0.1^{b}	26.5 ± 2.1^{a}	23.7 ± 1.2^{a}	1.2 ± 0.1^{a}
	Blank	45.6 ± 1.0^{b}	3.2 ± 0.1^{c}	25.7 ± 2.1^{a}	24.3 ± 1.2^{a}	1.2 ± 0.1^{a}

Mean of three analyses ± standard deviation. Each value is expressed relative wt-% contents of individual FA in each lipid class. "Others" include minor FA such as 14:0, 16:1 and 20:0. Values in the same column with different superscripts are significantly different between individual cultivars (P<0.05).

Table 3 shows the distributions of FA in the PE, PC, and PI between the two cultivars. The major FA in the three PL were commonly palmitic (16:0), stearic (18:0), oleic (18:1n-9) and linoleic (18:2n-6) acids. With a few exceptions, these FA profiles were very similar to each other in the major individual PL between the two cultivars. When comparing FA profiles in the three PL between the two cultivars, the percentage of linoleic (18:2n-6) acid was significantly (P < 0.05) higher in PE (41.8-42.8%) than in PC (26.4-27.8%), while the percentage of oleic (18:1n-9) was significantly (P < 0.05) higher in PC (42.3-43.7%) than in PE (34.5-36.2%). Furthermore, PI was very unique in that it had the highest saturated FA contents (49.3-49.6%) among the three PL, although their patterns were very similar between the two cultivars. On the other hand, the percentage of oleic (18:1n-9) and linoleic (18:2n-6) acids were almost at the same levels (25.7-26.5% and 23.7-24.3%, respectively) between the two cultivars. The percentage of palmitic (16:0) acid was significantly (P < 0.05) higher in PI (45.6-45.7%) than in PE (17.8-18.7%) or PC (25.6-25.8%) between the two cultivars. The FA distribution in PI differed significantly (P < 0.05) from that of PE or PC among the two cultivars, presumably owing to differences in their biosynthetic pathway (Vogel & Browse, 1996).

The data for FA distributions of minor lipid components (Table 2), such as diphosphatidylglycerol, phosphatidic acid, phosphatidylglycerol and lysophospholipid were omitted from this paper as the samples were too small to obtain reliable results for these individual PL.

4. Conclusions

This study has shown that major lipid components in red and black rices are TAG, FFA and PL, while other components are also presented in minor proportions. The distribution patterns in the FA within the individual acyl lipids are very similar to each other between the two cultivars. To the best of the authors' knowledge this is the first report of the FA distribution patterns in the different lipids of red and black rices. Therefore, these colored rices may be well incorporated into our daily Japanese diets to improve nutritional value. The data obtained from this study would provide valuable information to both producers and consumers during

manufacture of traditional rice foods in Japan.

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