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Optimization of One-step Extraction/Methylation Method for Analysis of Fatty Acid Composition in Brown Rice

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ABSTRACT: Traditionally fatty acid composition used to be analysed by a GC and the sample preparation process includes lipid extraction from sample and subsequent methyl esters preparation, which are time-consuming and cumbersome. As an alternative, simultaneous extraction/methylation methods are being developed for rapid and simplified sample preparation. To optimize one-step extraction/methylation method for analysis of fatty acid composition in brown rice, various reaction factors such as sample to reaction solution ratio, reaction time and temperature, shaking intensity were changed and resultant fatty acid composition data were evaluated in comparison with previous reports. The ratio of sample weight to reaction solution volume was the most critical factor in that higher sample to reaction solution ratio caused overestimation of palmitic acid and linoleic acid composition, resulting in underestimation of oleic acid. Lower reaction temperature also induced overestimation of linoleic acid and underestimation of oleic acid. Reaction duration and the intensity of shaking prior to and during the reaction, however, caused no significant changes in analysis results. In conclusion, the optimum condition was mixing 5 grains (about 0.2 g) of brown rice with 680 μL of extraction/methylation mixture and 400 μL of heptane, followed by reaction at 80 °C for 2 hours.

Keywords: fatty acid composition, brown rice, methylation, one-step extraction/methylation

The fatty acid, a group of monocarboxylic acids with various length of hydrocarbon chain, is a major component of lipid, which in turn is an important constituent of living organisms. Fatty acid is classified according to the length of hydrocarbon as well as to the presence and location of carbon-carbon double bonds in hydrocarbon chain. Most of naturally found double bonds in fatty acids are in cis- form, which results in structural kink and subsequent lowered melting point of fatty acid. Consequently, the fatty acid composition has been an important research topic for cold-stress physiologists because any plant species or varieties with higher degree of fatty acid unsaturation could maintain higher membrane fluidity and function even at low temperature conditions (Ariizumi *et al.*, 2002; Jung *et al.*, 1983). Recently, more focus on fatty acid has been given to its human physiological activity (Kayama, 1996 and references there in), and there have been many reports on seed fatty acid composition of oil crops as well as cereal crops including rice (Kitta *et al.*, 2005; Hong *et al.*, 2003; Khaton and Gopalakrishna, 2004; Seo *et al.*, 1999). Also to meet consumers' preference on high edible quality rice, researches on fatty acid in rice have been intensively conducted because fatty acid readily suffers oxidation during milling process and storage (Kang *et al.*, 1996; Kim and Chun, 1996; Son *et al.*, 1996; Zhou *et al.*, 2003; Lam and Proctor, 2002; So *et al.*, 1999).

Traditionally fatty acid composition has been analysed by using a gas chromatography (GC) as its corresponding fatty acid methyl ester (FAME) (Son and Han, 2004; Seppanen-Laakso, 2002). However, its sample preparation prior to GC analysis is rather a time- and labor- costing process consisting of lipid extraction followed by breaking the ester bonds, and subsequent methyl esters formation. As an alternative, one-step procedure simultaneously conducting both extraction and methylation processes to prepare FAME samples are recently being developed and validated for various plant tissues (Garces and Mancha, 1993; Meier *et al.*, 2006; Kim *et al.*, 2000 and references there in). Such a simplified method is especially useful in case of dealing with many number of samples such as genetic variation screening or breeding programs.

Rice is consumed generally in white rice form after milling brown rice. Rice bran, the removed fraction during milling process, contains about 12 to 15% total lipids and its fatty acid composition is quite different from those in white rice, and consequently different milling degree results in different fatty acid composition of rice (Kim and Chun, 1996). These facts indicate the necessity of more precise application of one-step extraction/methylation process for brown or white rice fatty acid analysis because the reaction efficiency of one-step process might be affected by the relative compositional ratio of polyunsaturated fatty acids (Meier *et al.*, 2005). The objective of this research was to develop an opti-

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mal condition for fatty acid composition analysis of brown rice by changing various reaction conditions and comparing the results with previously reported values.

MATERIALS AND METHODS

Brown rice materials

Rice plants (cv. Chucheongbyeo) were cultivated and harvested in 2004 according to the standard field management practices of Korea. Harvested seeds were dehulled manually by using a TR-120 (KT Engineering, South Korea) to collect brown rice. Dehulled brown rice were stored at room temperature prior to analysis in order to avoid any possible changes in fatty acid composition which usually occur under low temperature conditions, and the storage duration was not longer than 2 weeks. The objective material for this experiment was brown rice, and consequently brown rice sample were not powdered but directly used for analysis to keep constant weight ratio of bran to white rice. The average weight of 25 grains of brown rice was 0.56 g.

Basic and modified fatty acid analysis condition

As a basic control method, fatty acid analysis samples were prepared according to Kim *et al.* (2000) as followings: 25 grains of brown rice were added into a 1.5 mL clear glass vial containing 680 μL of methylation mixture (MeOH : benzene : DMP : H_2SO_4 = 39 : 20 : 5 : 2, where MeOH and DMP stands for methanol and 2,2-dimethoxypropane, respectively). After adding 400 μL of heptane, the final solution was mixed vigorously, and placed in a water bath at 80 °C for 2 hours. After cooling down to room temperature, the reaction solution except for brown rice seeds was transferred into a 1.5 mL centrifuge tube. After centrifugation at 1,000 rpm for 1 min, upper heptane layers were carefully transferred into a vial and used for GC analysis.

Modified fatty acid analysis condition

To find out optimal condition, each analysis factors were changed from above-mentioned basic conditions. To establish optimal sample weight to reaction solution volume ratio two sets of experiments were conducted. As the first set of experiment 2, 3, 5, 6, 8, 9, 10, 13, 14, 16, 18, 20, and 25 grains of brown rice were added with 680 μL of methylation solution and 400 μL of heptane. Based upon the results of this first set of experiment, further lower sample weight to reaction volume conditions were tested in that 5 grains of brown rice were added with 680 μL / 400 μL, 1,020 μL / 600

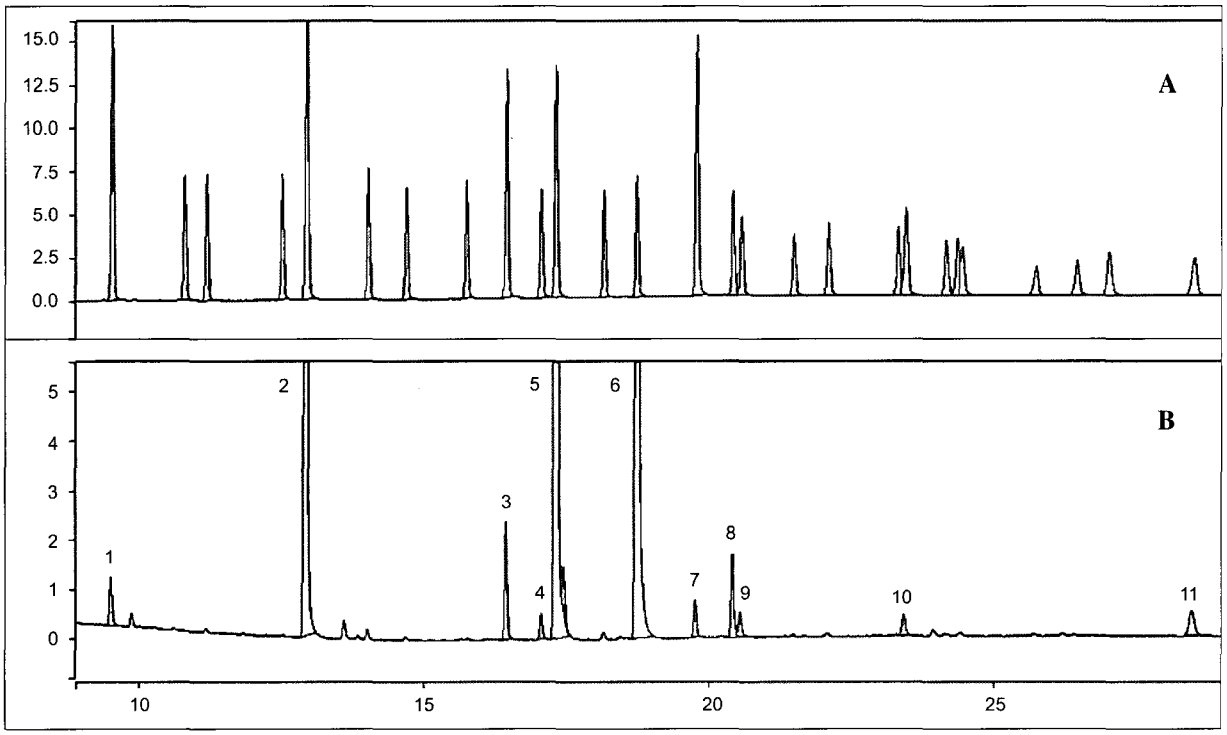

Fig. 1. Chromatogram of authentic FAME standard (A) and brown rice sample (B). Numbers in chromatogram (B) represent fatty acid methyl esters as followings: 1, myristic acid (14:0); 2, palmitic acid (16:0); 3, stearic acid (18:0); 4, elaidic acid (18:1n9t); 5, oleic acid (18:1n9c); 6, linoleic acid (18:2); 7, arachidic acid (20:0); 8, linolenic acid (18:3); 9, cis-11-eicosenoic acid (20:1); 10, behenic acid (22:0); 11, lignoceric acid (24:0).

μL, and 1,360 μL / 800 μL of methylation solution / heptane, respectively. To clarify the impacts of shaking on fatty acid composition, the reaction mixture containing 5 grains of brown rice in 1,020 μL methylation solution and 600 μL heptane as well as the reaction mixture containing 10 grains of brown rice in 1,360 μL methylation solution and 800 μL heptane were kept in 80 °C water bath for 2 hours under shaking or non-shaking conditions. Three levels of reaction temperature: 65 °C, 80 °C, and 90 °C, as well as 4 levels of reaction duration: 1, 2, 3, and 4 hours were also tested to elucidate the impacts of reaction temperature and duration on analysis results of brown rice fatty acid composition.

GC analysis condition

For fatty acid composition analysis Gas chromatography (CP-3800, Varian, Australia) equipped with CP-SIL 88 TAILOR MADE FAME column (50 m × 0.25 mm) was used. The injector and FID detector temperatures were both 210 °C. The carrier gas was He, and the split ratio was 1 : 20. The time programming of oven temperature was as followings: initially 110 °C was maintained for 5 min, and increased up to 210 °C by 5 °C min^{-1}, and maintained at 210 °C for 40 more minutes. Each fatty acid methyl esters were identified by comparing the retention time of authentic FAME standard (Sigma, USA) consisting of 37 kinds of FAME. Under our experimental conditions, total 11 kinds of FAME were detected and out of those 11 FAME, 5 major fatty acids: palmitic, stearic, oleic, linoleic, and linolenic acids were considered in calculating FAME composition. The composition of each fatty acid was expressed in percentage value corresponding to the peak area of each FAME relative to total peak area calculated by the summation of 5 major FAME peaks areas. Typical chromatograms of authentic standard and brown rice sample are shown in Fig. 1.

RESULTS AND DISCUSSIONS

Effects of sample weight to reaction solution volume ratio

Generally the sample to solvent volume ratio significantly affects the efficiency of extraction. To clarify the optimal condition for one-step extraction/methylation, the volume of reaction solution was fixed as 680 μL methylation solution and 400 μL heptane and the brown rice sample weight were changed by adding 2 to 25 grains of brown rice. The ratio of sample weight to reaction mixture volume proved itself a very critical factor in analysis results (Fig. 2). The higher sample to reaction solution ratio, i.e., higher number of grains per unit reaction solution volume, significantly increased the relative composition of palmitic (16:0) and

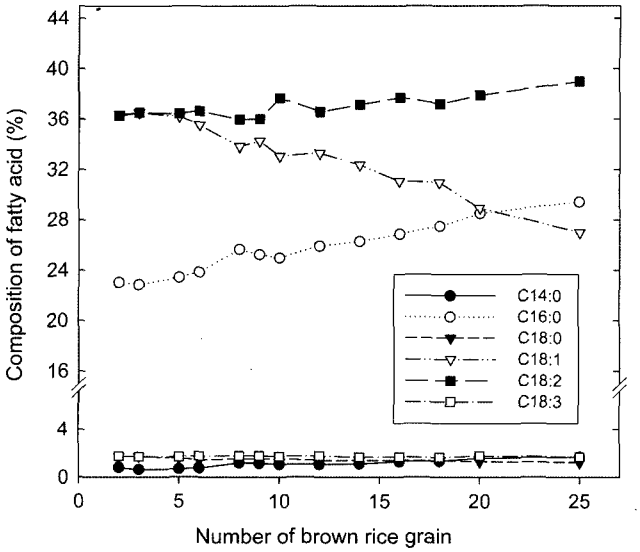

Fig. 2. Effects of sample weight to one-step extraction/methylation reaction solution volume ratio on results of fatty acid composition of brown rice. Different number of brown rice grains were added into reaction solution consisting of 680 μL of methylation mixture and 400 μL of heptane.

linoleic (18:2) acid, but decreased oleic acid (18:1) (Fig. 2). The palmitic acid composition which was as low as 23.4 % at 5 grain increased up to 25.0 % and 29.4 % when 10 and 25 grains of brown rice were added, respectively. The linoleic acid composition (18:2) also showed similar trends of increasing under higher sample weight to reaction volume ratio in that only 36.4 % detected at 5 grains increased up to 39.0 % when 25 grains were used. Such increases in palmitic and linoleic acid composition resulted in consequently decreased composition of another major fatty acid, e.g., oleic acid (18:1), which decreased from 36.2 % at 5 grains down to 27.0 % at 25 grains. Other minor fatty acids composition exhibited relatively slight changes compared to changes in major fatty acids. Such relatively slight changes in minor fatty acids, however, might be the results not from ineffectiveness of altered sample to reaction solution ratio but from relatively small composition of minor fatty acids compared to major three fatty acids (palmitic, oleic, and linoleic acids) consisting over 95 % of total fatty acids in brown rice.

No significant composition changes in all fatty acids could be detected when brown rice sample weight were reduced below 5 grains, indicating that 5 grains of brown rice seems to be the maximum sample weight acceptable for 680 μL / 400 μL of methylation solution / heptane conditions to ensure constant analysis results. Additional detail studies on low sample weight to reaction solution volume ratio were conducted by increasing the volume of methylation solution/heptane (in μL) up to 680/400, 1,020/600, and 1,360/800 μL, while maintaining brown rice sample weight as 5

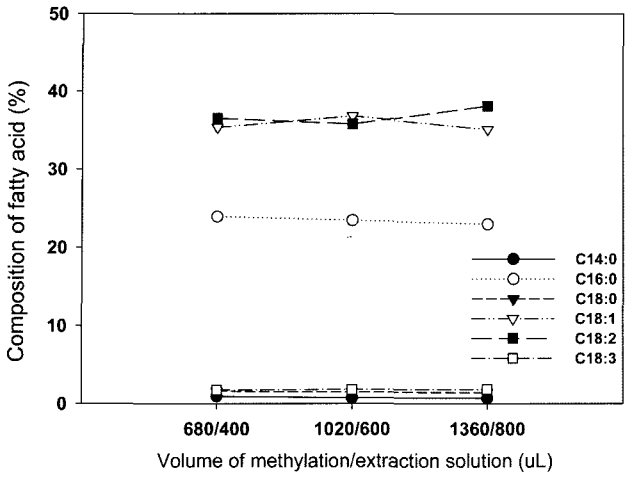

Fig. 3. Effects of sample weight to one-step extraction/methylation reaction solution volume ratio on results of fatty acid composition of brown rice. Five brown rice grains were added into different volume of reaction solutions consisting of methylation solution and heptane.

grains. No significant fatty acid composition could be observed among tested combinations (Fig. 3), indicating that sample weight to reaction solution volume ratio lower than 5 grains per 680 μL methylation solution and 400 μL heptane may not affect the fatty acid composition results. Based upon these results it could be concluded that adding 5 grains of brown rice into 680 μL of methylation solution and 400 μL heptane was the optimal condition. When fatty acid composition results obtained under this condition were compared to previous reports, the palmitic (24.0 %), oleic (35.4 %), and linoleic acid composition (36.5 %) observed in this experiment were similar to Son *et al.* (1996) who reported 20.3 %, 35.0 %, and 41.1 %, respectively, for each fatty acid in brown rice of the same Chucheongbyeo cultivar, as well as Kitta *et al.* (2005) who reported 24.6 %, 36.2 %, and 32.5 %, respectively, for brown rice of cv. Koshihidari cultivated in Niigata, Japan.

Effects of vortex intensity mixing reaction

In general the shaking provides higher chance of mixing and homogeneity of the ingredients in mixture solution during the reaction process. One-step extraction/methylation method consists of two mixing or shaking processes; the first is mixing brown rice with methylation and heptane solution prior to the initiation of reaction in a water bath, and the second is the shaking above-mentioned mixture solution during the reaction process. When the intensity effects of mixing brown rice with methylation solution and heptane prior to the initiation reaction were tested by hand-shaking or vortexing mixture solution for 5, 10, and 20 sec, no significant variations in analysis results of fatty acid composition could be found (data not shown). Similarly, when analysis data were compared between shaking and non-shaking mixture solution during 2 hours of reaction, no significant variations could be found (Table 1). These results suggested the fact that mixing or shaking intensity prior to or during one-step extraction/methylation has rare impacts on fatty acid analysis data. Consequently, it could be concluded that simple hand-shaking or brief vortexing the mixture solution prior to the reaction process in a water bath might be enough, and that no additional shaking during the one-step reaction in a water bath is necessary.

Effects of reaction temperature and duration

To find out optimal reaction temperature and duration for one-step extraction/methylation, reaction mixtures containing 5 grains of brown rice, 680 μL methylation solution, and 400 μL heptane were placed in a water bath of different temperatures for different durations. When the effects of extended reaction duration up to 4 hours were tested, no changes could be observed in oleic acid composition, while slightly decreasing and increasing tendency could be found in linoleic and palmitic acid, respectively (Fig. 4A). However, the actual differences between 1 and 4 hours were negligible in that composition of palmitic acid which showed 22.6 % at 1 hour of reaction duration increased only by 0.9 %, resulting in 23.5 % after 4 hours of reaction duration. In contrast to reaction duration, the reaction temperature caused significant increase in oleic and decrease in linoleic acid, especially between 65 °C and 80 °C, while no changes

Table 1. Fatty acid composition of brown rice as affected by shaking the reaction mixture solution during one-step extraction/methylation reaction in an 80 °C water bath.

Reaction solution (μL)	Heptane (μL)	Shaking intensity	Fatty acid composition (%)					
			14:0	16:0	18:0	18:1	18:2	18:3
1,020	600	No shaking	0.7	23.4	1.5	36.8	35.8	1.8
		Shaking	0.7	23.3	1.7	35.1	37.4	1.8
1,360	800	No shaking	0.8	22.4	1.4	37.1	36.6	1.8
		Shaking	0.7	23.4	1.6	36.0	36.6	1.7

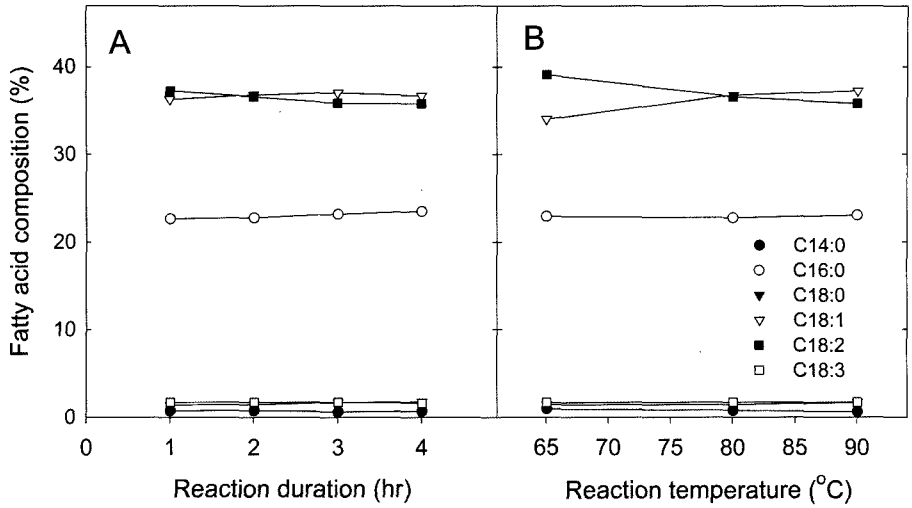

Fig. 4. Effects of reaction duration (A) and temperature (B) of one-step extraction/methylation on brown rice fatty acid composition analysis results.

in palmitic acid could be observed (Fig. 4B). This temperature-dependent changes, however, were no longer effective when reaction temperature was over 80 °C. These data indicated the necessity of keeping reaction temperature at least 80 °C.

CONCLUSIONS

Based upon our experimental data as described above, adding 5 grains of brown rice to 680 μL of methylation solutions (MeOH : benzene : DMP : H_2SO_4 = 39 : 20 : 5 : 2) and 400 μL heptane and subsequent keeping this reaction mixture at 80 °C for 2 hours seems to be an optimal condition for one-step extraction/methylation method to analyse fatty acid composition of brown rice.

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