Effect of Urea-to-Fatty Acid Ratio and Crystallization Temperature on the Fatty Acid Composition of Rice Bran Oil Concentrate by Urea Complexation

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Abstract

Processes for the production of polyunsaturated fatty acid (PUFA) concentrates have important because of the beneficial properties of these substances to human health and in functional foods. This study investigated the changes in different urea-to-fatty acid (U/FA) ratios (1:1, 2:1 and 3:1, w/w) and crystallization temperatures (-10, 0 and 10°C) on the fatty acid composition of rice bran oil concentrate, compared with native rice bran oil (control). Overall, the amount of PUFA in the rice bran oil concentrate increased as U/FA ratios increased and crystallization temperature decreased, while saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) decreased. The results showed that the U/FA ratio at 3:1 had the highest PUFA (56%) among complexation treatments. The higher PUFA concentrations in low-temperature crystallization were obtained at -10°C, with PUFA rising to 60.6%.

Keywords: Polyunsaturated fatty acids, Rice bran oil concentrate, Urea inclusion, Crystallization temperature

Introduction

Rice bran oil is extracted from rice bran during the milling of rice, and has gained popularity and commercial importance because of its health benefits. Rice bran contains unsaturated fatty acids (38-42% oleic acid, 32-35% linoleic acid) and valuable fractions such as neutral detergent fiber, hemicellulose and unsaponifiable matter (Nicolosi, Ausman & Hegsted, 1991; Visser, Zock, Meijer & Katan, 2000). Compared to other vegetable oils, rice bran oil contains high (4%) unsaponifiable matter levels, including phytosterols, triterpene, alcohols, tocols and γ-oryzanol (Nicolosi et al., 1991; Raghuram & Rukmini, 1995). Linoleic acid is widely recognized as an essential fatty acid, decreasing blood cholesterol, preventing atherosclerosis and providing other health effects; so rice bran oil is seen as an ideal edible vegetable oil (Marlene, Richard, Silvia & Michael, 2005). Rice bran oil has been in high demand for pharmaceutical and dietetic purposes. However, rice bran oil is unattractive because it contains substantial amounts of undesirable saturated fatty acids (SFA). Some studies indicated that the polyunsaturated fatty acids (PUFA) concentrates, devoid of more SFA, are much better than rice bran oil itself since they allow the daily intake of total lipid to be kept as low as possible (Haagsma, Gent, Luten, Jong & Doorn, 1982; Sharma, Srivastava & Saxena, 2015). Rice bran oil has been preferentially used as a raw material to prepare PUFA concentrates due to its potential in the food and pharmaceutical industries' applications.

The PUFA concentrates can be produced by several methods, including supercritical fluid extraction, freezing crystallization, urea complexation, molecule distillation, silver ion complexation, lipase concentration and high-performance liquid chromatography (Medina, Grima, Giménez & González, 1998; Liu, Zhang, Hong & Ji, 2006; Corrêa, Peixoto, Gonçalves & Cabral, 2008; Chakraborty & Raj, 2009). However, the simplest and

most efficient technique for obtaining PUFA concentrates in the form of free fatty acids is the urea complexation method. The main application of the urea complexation method is the separation of saturated and mono-unsaturated fatty acids (MUFA) from PUFA (Medina et al., 1998; Wanasundara & Shahidi, 1999; Liu et al., 2006).

Urea complexation has the advantage of having highly stable complexed crystals. As a result, the filtration does not necessarily have to be carried out at the very low temperatures required by the solvent crystallization of fatty acids (Wanasundara & Shahidi, 1999; Liu et al., 2006). In addition, it is simple to operate, the equipment involves little investment, the reagent is cheap, urea can be recycled, the production cost is low and it is easy to expand the scale in production for industrialization. This technique is also favored by many researchers and commercial oil factories because the complexation depends upon the shape, size, geometry and configuration of the fatty acid moieties due to the presence of multiple double bonds, rather than pure physical properties such as melting point or solubility (Medina et al., 1998; Wanasundara & Shahidi 1999; Liu et al., 2006). However, a literature search revealed no information on the fatty acid composition of rice bran oil purified by urea complexation. Therefore, changes in fatty acid composition as affected by the urea complexation method of glutinous rice bran oil concentrate were investigated in the present study, with our goal being to use rice by-products to produce rice bran oil concentrate.

Methods and Materials

Materials

Rice bran of *Oryza sativa* L., cultivar RD-6 (a popular glutinous rice cultivar for consumption in the North and Northeast of Thailand) was collected from the Roi-Et Agricultural and Food Products, Co., Ltd., Roi-Et Province, Thailand. Prior to conducting the experiment, the rice bran was stored at -20°C. The extraction, refining (R) and bleaching (B) of the oil was carried out according to recommended procedures for commercial oil (Sunarya, Hole & Taylor, 1996). The rice bran oil was stored under nitrogen at -25°C in an amber glass container until used. Fatty acid methyl esters were purchased from either Fluka (Buchs, Switzerland) or Sigma (St. Louis, USA). The acetic acid, methanol, acetonitrile and other solvents and reagents used in the HPLC analysis were purchased from Merck (Darmstadt, Germany). All other chemicals used in this study were analytical grade.

Preparation of Free Fatty Acids from Rice Bran Oil

The preparation of free fatty acids from rice bran oil took place according to the following procedure. Rice bran oil (175 g) was treated with 200 ppm butylated hydroxytoluene (BHT) before saponification with a mixture of KOH (40.25 g), distilled water (77 ml), and 95% aqueous ethanol (462 ml). The saponification was operated at $62\pm2^{\circ}$C for 1 hr under nitrogen. Distilled water (350 ml) was added to the saponified mixture. The aqueous layer was acidified to a pH of 1.0 with 3 N HCl. The mixture was transferred to a separating funnel and the liberated fatty acids were extracted into 350 ml hexane. The hexane layer containing free fatty acids was then dried over anhydrous sodium sulfate, and the solvent was removed in a rotator evaporator at 40°C under vacuum to recover free fatty acids which were then stored under nitrogen at -25°C in dark amber glass containers until used in the urea complexation (Wanasundara & Shahidi, 1999).

Preparation of Rice Bran Oil Concentrates by Urea Complexation

The separation of rice bran oil concentrates from the hydrolyzed fatty acid mixture of parboiled rice bran oil was carried out by urea-fatty acid adduct formation according to the following procedure. Free fatty acid (300 g) was mixed with 20% (w/v) urea in 95% aqueous ethanol and heated at 60-70°C, with stirring, until the whole mixture turned into a clear homogeneous solution. To study the effect of the urea-to-fatty acid (U/FA) ratio on the fatty acid composition of glutinous rice bran oil, the U/FA ratio was changed by using different amounts of urea (1:1, 2:1 and 3:1 w/w) at 4°C for 8 hr. To study the effect of crystallization temperature on the fatty acid composition of glutinous rice bran oil, the urea-fatty acid (3:1) adduct was allowed to crystallize at -10°C, 0°C and 10°C for 8 hr. The crystals formed were separated from the liquid by filtration under suction using a Buchner funnel lined with a No.1 Whatman filter paper. The filtrate was diluted with an equal volume of water and was acidified to pH 4-5 with 6 N HCl; an equal volume of hexane was subsequently added. The mixture was stirred thoroughly for 1 hr and then transferred to a separating funnel. The hexane layer, containing liberated fatty acids, was separated from the aqueous phase. The hexane phase was washed out with distilled water (2×150 ml) to remove any remaining urea and then dried over anhydrous sodium sulfate, and the solvent was removed in a rotator evaporator at 40°C under vacuum (Thammapat, Siriamornpun & Raviyan, 2016).

Gas Chromatography (GC) Analysis

Free fatty acids were transformed into the corresponding methyl esters. In detail, 3 ml of HCl- methanol reagent and 1 ml of toluene reagent were added to the 100 mg of extracted lipid samples and they were then heated at 70°C for 2 hr (Thammapat, Raviyan & Siriamornpun, 2010). Fatty acid methyl esters were extracted in 2 ml of hexane, and stored at -25°C before chemical analysis. The fatty acid methyl esters were analyzed by gas chromatography, using a Shimadzu (GC-2014) device with a flame ionization detector (FID). The esters were separated on a 60 m × 0.25 mm i.d. wall-coated open tubular fused silica capillary column coated with DB-WAX. Column injector temperature was 250°C and detector temperature was 270°C. The carrier gas was nitrogen flowing at 1.27 ml/min. The temperature program was 150°C -180°C at 20°C/min, then from 180°C to 220°C at 2.5°C/min, held at 220°C for 3 min, then from 220°C to 230°C at 10°C/min, held at 230°C for 3 min and from 230°C to 235°C at 5.0°C/ min, held at 235°C for 10 min (Thammapat, Raviyan & Siriamornpun, 2010). Individual methyl esters were identified against the retention time of standard methyl esters. The fatty acid composition was calculated by the following formula.

Fatty acid composition = area under each peak / total areas of all fatty acids appearing in the chromatogram x 100

Statistical analysis

A completely randomized design was used in the experiment. Statistical analysis was conducted using SPSS (Statistical Program for Social Sciences, SPSS Corporation, Chicago, IL). The results are presented as mean ± standard deviation of determinations for triplicate samples. Differences were considered statistically significant at $p<0.05$. Data were analyzed by Duncan's post hoc test where differences were detected for homogenous subsets.

Results

The changes in U/FA ratios and crystallization temperatures on the fatty acid composition of the rice bran oil concentrate, compared with native rice bran oil (control) are reported as follows;

Effect of U/FA Ratio on Fatty Acids Composition

The effect of the U/FA ratio on the fatty acid composition of the rice bran oil is shown in Table 1. The most abundant fatty acids found in all samples were linoleic acid (C18:2), oleic acid (C18:1) and palmitic acid (C16:0). After urea inclusion complexing with different U/FA ratios, the PUFA of the rice bran oil concentrate was significantly higher ($p<0.05$) than that of the control (natural rice bran oil), while SFA and MUFA decreased. In general, the amounts of myristic acid (14:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1), and oleic acid (C18:1), decreased significantly ($p < 0.05$) when the U/FA ratio increased from 1:1 to 3:1. PUFA were the major fatty acids, present in concentrates after urea complexation. The amounts of linoleic acid (18:2), linolenic acid (18:3), dihomo-γ-linolenic acid (C20:3) and arachidonic acid (C20:4) increased respectively from 44.4% to 50%, 1.1% to 3.2%, 0.45% to 1.80% and 0.43% to 1.35% of the total fatty acids when the U/FA ratio increased from 1:1 to 3:1.

Table 1 Influence of urea to fatty acid ratio on the fatty acid composition of urea concentrates obtained at 4°C from rice bran oil

Fatty acids (%)	Rice bran oil	Urea: Fatty acid ratio		
		1:1	2:1	3:1
C14:0	0.77±0.16[a]	0.33±0.02[b]	0.24±0.02[bc]	0.15±0.04[c]
C16:0	15.62±0.35[a]	13.78±0.17[b]	10.12±0.43[c]	7.75±0.85[d]
C16:1 n-9	3.50±0.19[a]	3.26±0.08[a]	2.46±0.11[b]	1.39±0.16[c]
C18:0	0.34±0.07[a]	0.22±0.06[b]	0.06±0.02[c]	0.04±0.02[c]
C18:1n-9	39.41±0.52[a]	36.01±0.12[b]	35.02±0.63[b]	34.33±0.36[c]
C18:2n-6	38.63±0.41[d]	44.42±0.20[c]	48.91±0.19[b]	49.97±0.40[a]
C18:3 n-3	1.01±0.03[b]	1.09±0.04[b]	1.48±0.42[b]	3.21±0.24[a]
C20:3 n-6	0.43±0.06[c]	0.45±0.03[c]	0.95±0.25[b]	1.80±0.14[a]
C20:4 n-6	0.29±0.03[b]	0.43±0.05[b]	0.59±0.16[b]	1.35±0.32[a]
$\sum$n-3 PUFA	1.01±0.03[b]	1.09±0.04[b]	1.48±0.42[b]	3.21±0.24[a]
$\sum$n-6 PUFA	39.35±0.32[d]	45.30±0.25[c]	50.44±0.56[b]	53.12±0.76[a]
Concentration factor (f) [a]	–	1.15	1.29	1.40

Mean values ± standard deviation of determinations for triplicate samples.

Values with the different superscripts in each row are significantly different ($p < 0.05$).

[a] Concentration factor ($f= X_u/X_E$).

X_u = PUFA concentration in urea concentrate (% of total fatty acids).

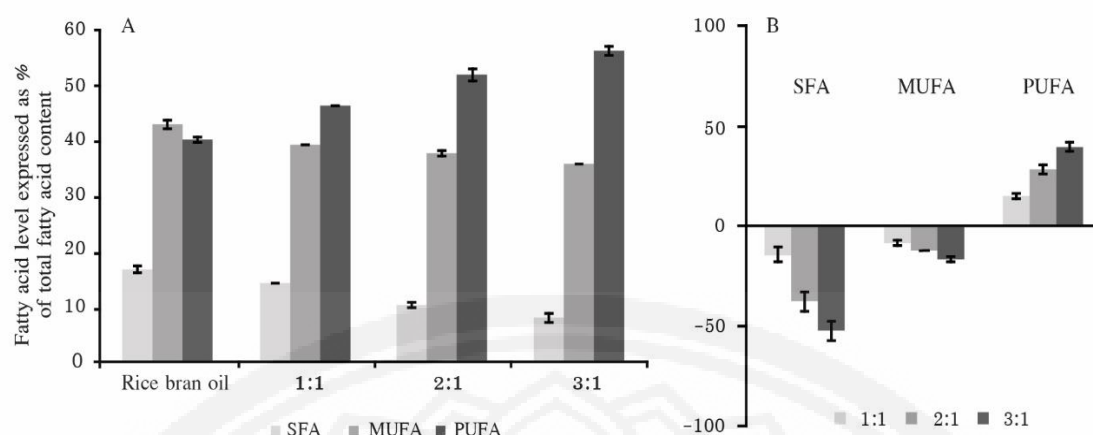X_E = PUFA concentration in rice bran oil extract (% of total fatty acids).

Figure 1 Influence of urea to fatty acid ratio on the fatty acid composition of urea concentrates from the rice bran oil. (A) Effect of urea to fatty acid ratio on the fatty acid composition of the rice bran oil and (B) Percentage increases or decreases of fatty acid composition in the rice bran oil concentrated by different U/FA ratios

Changes in total SFA, MUFA and PUFA were observed in Figure 1(A), PUFA increased, whereas SFA and MUFA decreased with an increase in U/FA ratio. Percentage increases or decreases for the fatty acid content of the rice bran oil concentrate by different ratios of U/FA are presented in Figure 1(B). Interestingly, there were increases in PUFA of 15.0%, 28.7% and 39.6% when the U/FA ratio increased to 1:1, 2:1 and 3:1, respectively. On the other hand, SFA and MUFA decreased after increasing the U/FA ratio.

Effect of Crystallization Temperature of Urea Complexation on Fatty Acid Composition

The fatty acid composition of the rice bran oil showed some variations when the crystallization temperature of urea complexation changed (Table 2). The amounts of linoleic acid (18:2), linolenic acid (18:3), dihomo-γ-linolenic acid (C20:3) and arachidonic acid (C20:4) increased respectively from 47.9% to 51.8%, 1.3% to 4.5%, 1.0% to 2.6% and 0.6% to 1.8% of the total fatty acids when the crystallization temperature decreased from 10°C to -10°C. After urea inclusion complexing with different crystallization temperatures, PUFA of the rice bran oil concentrate was significantly higher ($p<0.05$) than that of the control (natural rice bran oil). PUFA of the rice bran oil concentrate showed an increasing trend as crystallization temperature decreased (Figure 2A).

Our finding showed that at -10°C the highest PUFA among crystallization temperature treatments was achieved. Percentage increases or decreases in the fatty acid content of the rice bran oil concentrate by crystallization temperature compared to the rice bran oil are shown in Figure 2B. The amounts of PUFA increased by 28.9%, 40.7% and 50.2% of the total fatty acids when the crystallization temperature decreased from 10°C to -10°C. However, SFA and MUFA decreased after decreasing crystallization temperature.

Table 2 Influence of crystallization temperature on fatty acid composition of urea concentrates obtained with a 3:1 urea to fatty acid ratio from the rice bran oil

Fatty acids (%)	Rice bran oil	Crystallization temperature (°C)		
		10	0	−10
C14:0	0.77±0.16[a]	0.30±0.05[b]	0.14±0.08[bc]	0.05±0.02[c]
C16:0	15.62±0.35[a]	11.70±0.41[b]	7.57±0.72[c]	4.74±0.54[d]
C16:1 n−9	3.50±0.19[a]	2.42±0.18[b]	1.27±0.09[c]	0.95±0.14[d]
C18:0	0.34±0.07[a]	0.07±0.02[b]	0.04±0.02[b]	0.02±0.01[b]
C18:1n−9	39.41±0.52[a]	34.73±0.37[b]	34.21±0.17[bc]	33.64±0.37[c]
C18:2n−6	38.63±0.41[d]	47.87±0.64[c]	49.94±0.29[b]	51.82±0.27[a]
C18:3 n−3	1.01±0.03[b]	1.33±0.26[c]	3.24±0.25[b]	4.47±0.44[a]
C20:3 n−6	0.43±0.06[d]	0.98±0.11[c]	2.03±0.20[b]	2.55±0.23[a]
C20:4 n−6	0.29±0.03[b]	0.60±0.17[b]	1.55±0.27[a]	1.76±0.10[a]
$\sum$n−3 PUFA	1.01±0.03[c]	1.33±0.26[c]	3.24±0.25[b]	4.47±0.44[a]
$\sum$n−6 PUFA	39.35±0.32[d]	49.46±0.39[c]	53.52±0.60[b]	56.13±0.53[a]
Concentration factor (f) [a]	–	1.26	1.41	1.50

Mean values ± standard deviation of determinations for triplicate samples.

Values with the different superscripts in each row are significantly different ($p < 0.05$).

[a] Concentration factor ($f = X_u/X_E$).

X_u = PUFA concentration in urea concentrate (% of total fatty acids).

X_E = PUFA concentration in rice bran oil extract (% of total fatty acids).
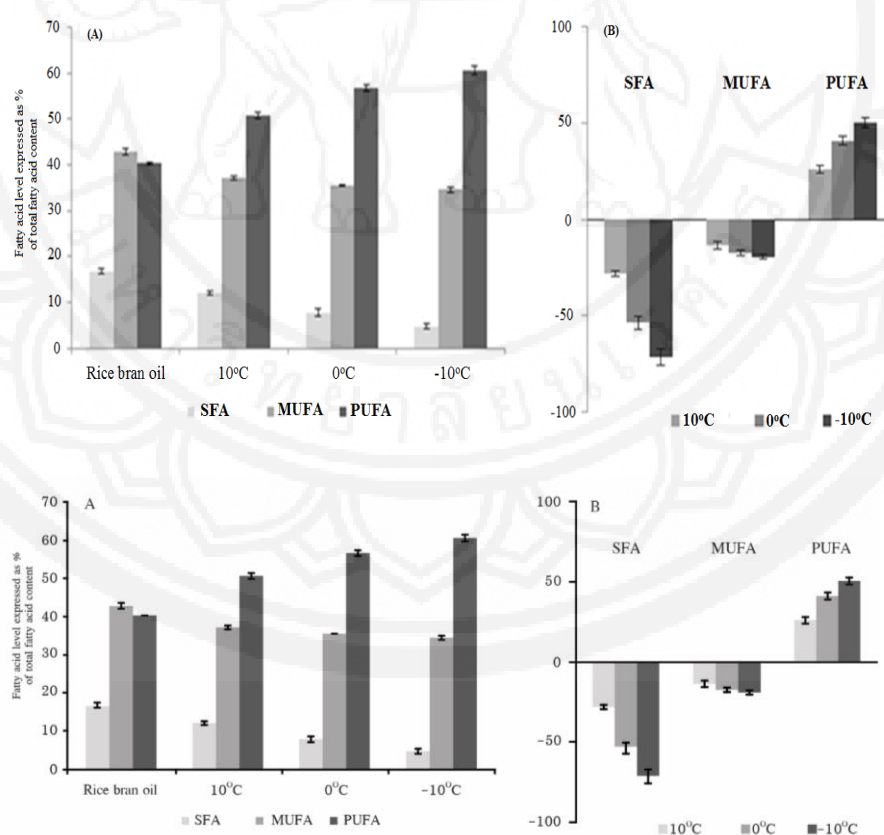

Figure 2 Influence of crystallization temperature on the fatty acid composition of urea concentrates from the rice bran oil. (A) Effect of crystallization temperature on the fatty acid composition of the rice bran oil and (B) Percentage increases or decreases of fatty acid composition in the rice bran oil concentrated by different crystallization temperatures.

Discussion

After urea inclusion complexing with different U/FA ratios, the PUFA level of the rice bran oil concentrate showed an increasing trend as the U/FA ratio increased. When using higher amounts of urea, there was a tendency for fatty acids to form urea compounds and therefore the PUFA concentration factors are higher (Wanasundara & Shahidi, 1999). The U/FA ratio may be used to segregate the fatty acids by their degree of unsaturation. When insufficient amounts of urea are used, fatty acids compete among themselves for complexing with urea; hence fractionation may occur according to the different competing tendencies of the different fatty acids (Medina et al., 1998; Thammapat & Siriamornpun, 2017). As shown in Table 1 with a U/FA ratio of 1:1 to 3:1, the SFA and MUFA are eliminated while the PUFA remains in solution.

The results showed that the U/FA ratio at 3:1 had the highest PUFA among complexation treatments (Figure 1A). Wanasundara & Shahidi (1999) have reported that the content of total ω-3 fatty acids was highest at a U/FA ratio of 4.5. While Hai-bo, Xue-yi, Jing-bo, Qi, Wen-bing & Yi-ping (2009) have also reported that the content of α-linoleic acid was highest at a U/FA ratio of 3. Generally, enrichment of PUFA in the concentrate varied inversely with increasing U/FA ratio. When urea crystallizes from a solution of fatty acids of varying degrees of unsaturation, the saturated and monounsaturated fatty acids (long and straight-chain molecules) are first included in crystals while the PUFA remained in solution. Therefore, the urea technique fractionates fatty acids mainly according to the degree of their unsaturation (Medina et al., 1998; Shahidi & Wanasundara, 1998).

Among the crystallization temperatures, -10°C had the highest PUFA content. At low temperatures, fatty acids have a greater tendency to form urea compounds than at high temperatures, which could be used to separate fatty acids by their degree of unsaturation. Fatty acids are crystallized at a suitable temperature depending on the concentration of complexes desired (Zhang, Hong & Ji, 2006).

Hai-bo et al. (2009) have reported that more production of α-linoleic acid was concentrated from crude perilla oil by gradient cooling urea complexation. A simple method of gradient cooling urea complexation was used to purify α-linolenic acid by using urea to form inclusion complexes with the saturated and the less unsaturated fatty acids, which enhanced the purity of α-linoleic acid by above 90%. The tendency to form urea compounds increases with decreasing crystallization temperature; the optimum temperature depends on the particular PUFA (Traitler, Wille & Studer, 1988). However, the higher the crystallization temperature, the lower the tendency of fatty acids to form urea compounds (Medina et al., 1998), and therefore the PUFA concentration factors are lower (Table 2).

In our study, we observed that the U/FA ratio and temperature, both of which are strongly related, are the most influential variables affecting the degree of PUFA concentration. The variation in PUFA concentration with the U/FA ratio is different when the crystallization temperature is low than when it is high (Medina et al., 1998). Complete removal of saturated fatty acids by urea complexation may be impossible since some of the saturated fatty acids do not complex with urea during crystallization (Ratnayake, Olsson, Matthews & Ackman, 1988).

Conclusion and Suggestions

Rice bran oil as a good component in pharmaceutical, food and nutrition has become widely acknowledged owing to the wide coverage of rice bran oil's health benefits. This study reveals that the U/FA ratio and crystallization temperature were the most important factors of rice bran oil concentrate by urea complexation. In addition, we have demonstrated that significant differences were found in fatty acids of rice bran oil concentrate compared to natural rice bran oil. The amounts of PUFA in the rice bran oil increased as the U/FA ratio increased and crystallization temperature decreased, while the amounts of SFA and MUFA in the concentrated samples decreased. The highest PUFA concentrations in low-temperature crystallization were obtained at $-10^{\circ}C$ and the U/FA ratio at 3:1. These findings suggest that urea complexation should be considered a very effective method for concentrating PUFA from rice bran oil leading to its potential beneficial use in the food and pharmaceutical industries.

Acknowledgements

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