

## Concentration of Polyunsaturated Fatty Acid of Rice Bran Oil by Urea Complexation–A Response Surface Approach

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### Abstract

The processes for the concentration of fatty acids in the production of polyunsaturated fatty acids (PUFA) have become very important because of the beneficial properties of these substances to human health and in functional foods. Optimization of the concentrating conditions of Polyunsaturated fatty acid (PUFA) extracted from rice bran oil was studied to obtain maximum concentration. The crude rice bran oil was extracted from the KDM105 rice bran variety, and the extracted oil was used as fresh crude oil. The PUFA was concentrated by the urea complexation method. A hexagonal rotatable design was applied to examine the effects of crystallization temperature and urea-to-fatty acid ratio on the total content of PUFA (Y). We investigated the changes of different crystallization temperatures (-10, 0 and 10°C) and urea-to-fatty acid ratios (2:1, 3:1 and 4:1, w/w) on the total PUFA content. The second order polynomial regression model for response was employed to generate the response surfaces. Under the optimum conditions of -7.2°C and a urea-to-fatty acid ratio of 2.91 (w/w), the total concentration of PUFA could be increased by up to 77.48% was obtained.

**Keywords :** Polyunsaturated Fatty Acids, Rice Bran Oil Concentrate and Urea Inclusion

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## Introduction

Rice is the most important cereal product in Asia and is the most overwhelming staple food in most populations of this region (Wadsworth, 1992; Van Hoed *et al.*, 2006). It is grown in more than 100 countries, and there are around 18,000 varieties that count for about 25% of the world's food grain production (Hernandez *et al.*, 2000). Rice processing or milling produces several streams of material, including husks, milled rice, and bran. The bran is the hard outer layer of rice consisting of aleurone and pericarp. Rice bran, a by-product of rice milling industry is an indispensable, less expensive abundantly available as soft and fluffy off-white powdery material, during the milling period (Satter *et al.*, 2014). The utilization of commercial rice bran and its potential food values are recognized, and it has recently been increased with the development of highly promising processes and products (Rosniyana *et al.*, 2007). Recently, the use of rice bran is gaining importance in many studies due to the fact that, during the processing of whole rice, large amounts of the grain's outer layers are removed, raising the concentration of nutrients in the bran and rendering it into an important source of nutrients for the food industry and human consumption (Imsanguan *et al.*, 2008; Lilitchan *et al.*, 2008).

Rice bran is an incredible source of vitamins, minerals, amino acids, essential fatty acids, dietary fiber and more than 100 antioxidant nutrients that helps to fight against disease and promote to good health (Malekean *et al.*, 2000). Rice bran products have been widely used in food, agricultural and cosmetic industries. With the advent of biomedical research, scientists have unlocked important pharmacological and physiological properties within constituents of

rice bran, supporting a role for these products in health maintenance and disease prevention (Jariwalla, 2001). In the earlier times, rice bran was used as either fertilizer or animal feed. But these days, it is used for extraction of oil namely rice bran oil (Hammond, 1994; Van Hoed *et al.*, 2006). The most successful countries producing rice bran oil are in India and Thailand (Sharma *et al.*, 2015).

Rice bran oil is composed of 4% unsaponifiables, 2-4% free fatty acids and 88-89% neutral lipids. The unsaponifiable fraction is a complex mixture of naturally occurring antioxidant compounds such as vitamin E and  $\gamma$ -oryzanol. Rice bran oil has several unique properties that render its suitability for niche markets like nutraceutical and pharmaceutical industries. 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). It has been reported that polyunsaturated fatty acids (PUFA) concentrates devoid of more saturated fatty acids are preferred to rice bran oil. They, at the lowest possible amount, supply the daily intake of total lipid (Haagsma *et al.*, 1982). Therefore, consumption of appropriate amounts of PUFA needs to be considered. In production of highly concentrated PUFA components, separation of essential fatty acids is difficult. Rice bran oil is a complex mixture of fatty acids with varying chain lengths and instauration degrees. Therefore, commercial production of rice bran oil concentrates with high percentages of PUFA is now a main concern for researchers in this area

The available methods to produce PUFA concentrate includes supercritical fluid extraction, urea complexation, molecule distillation, freezing crystallization, lipase concentration, silver ion

complexation and high performance liquid chromatography (Medina *et al.* 1998; Liu *et al.*, 2006; Corrêa *et al.*, 2008; Chakraborty and Raj, 2009). However, Urea complexation is the most simple and the most efficient technique for obtaining PUFA concentration in the form of free fatty acids. For elimination of fatty acids, urea complexation is a good established technique (Homayooni *et al.*, 2014). The main application of the urea complexation method is the separation of SFA and mono-unsaturated fatty acids (MUFA) from PUFA (Medina *et al.*, 1998; Wanasundara and Shahidi, 1999; Liu *et al.*, 2006).

Urea complexation is favoured because the complexation depends upon the configuration due to presence of multiple bonds rather than the physical properties of the molecules, such as melting point or solubility. Pure urea crystallizes in a tightly packed tetragonal structure with channels of 5.67 Å in diameter. SFA, whose straight chain molecules, form hexagonal crystal with urea in 8-12 Å channel diameter. However, monoenes are more readily complexed as compared to dienes, which in turn, are more readily complexed than trienes. Urea complexes formation depends on the degree of unsaturation of the fatty acids (Medina *et al.*, 1998; Wanasundara and 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. This research has contributed to advances in the implementation of the technological processing of PUFA concentrate. Urea complexation of rice bran oil was carried out to concentrate PUFA of oil. Variables such as crystallization temperature ( $X_1$ , °C) and urea-to-fatty acid ratio ( $X_2$ , w/w) were studied collectively in order to optimize the conditions to obtain a maximum concentration of PUFA. Moreover, this study shows model

equations that predict the PUFA concentrate of rice bran oil based on crystallization temperature and urea-to-fatty acid ratio.

## Materials and Methods

### 1. Materials

Rice bran of *Oryza sativa* L., cultivar KDML105 was collected from the Roi-Et Agricultural and Food Products, Co, Ltd., Roi-Et Province, Thailand. Prior to conducting the experiment, rice bran was stored at -20°C. The extraction, refining (R) and bleaching (B) of the oil were carried out according to the recommended procedures for commercial oil (Sunarya *et al.*, 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 other solvents and reagents used in the gas chromatography analysis were purchased from Merck (Darmstadt, Germany). All other chemicals used in this study were of analytical grade.

### 2. 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  $62\pm2$  °C for 1 h under nitrogen. Distilled water (350 ml) was added to the soap and then acidified with 6 N hydrochloric acid to release the free fatty acids. The mixture was

transferred to a separating funnel and the liberated fatty acids were extracted into 350 ml hexane. The hexane layer was washed with distilled water and dried over anhydrous sodium sulphate. 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.

### 3. 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 then heated at 60-70°C, with stirring, until the whole mixture turned into a clear homogeneous solution. Two variables we evaluated were crystallization temperature ( $X_1$ ), and urea-to-fatty acid ratio ( $X_2$ ). The urea-to-fatty acid ratio was changed by using different amounts of urea (2:1, 3:1 and 4:1 w/w). Initially, the urea-fatty acid adduct was allowed to crystallize at -10°C, -5°C, 0°C, 5°C and 10°C for 8 hrs. The crystals formed (urea-fatty acid adducts) are also referred to as the urea complexing fraction) were separated from the liquid (non-urea complexing fraction) 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 acidified to pH 4-5 with 6 N HCl; an equal volume of hexane

was subsequently added. The mixture was stirred thoroughly for 1 h and then transferred to a separating funnel. The hexane layer, containing liberated fatty acids, was separated from the aqueous layer containing urea. The hexane phase was washed out with distilled water (2x150 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.

### 4. 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 h. 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 a Shimadzu (GC-2014) gas chromatography 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 and detector temperatures were 250 and 270°C, respectively. The carrier gas was nitrogen flowing at 1.27 ml/min. The temperature program was 150-180°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 *et al.*, 2010). Individual methyl esters were identified against the retention time of standard methyl

esters. Fatty acid composition was calculated by the following formulae.

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

### 5. Optimization procedure for production of PUFA concentration by urea complexation of rice bran oil

In this study, the hexagonal rotatable design (Gacula and Singh, 1984) was employed to study the total content of PUFA (Y variable) by urea complexation of rice bran oil. The crystallization temperature ( $X_1$ ) and urea-to-fatty acid ratio ( $X_2$ ) were independent variables employed to optimize Y variable. The level of variables for the development of the model is represented in Table 1. Triplicate reactions were carried out at all designed points except at the central point (0,0) where four replications were performed to allow the estimation of the 'pure error'. All experiments were carried out in a randomized order to minimize the effect of unexplained variability in the observed responses due to extraneous factors. A quadratic polynomial regression model was assumed for predicting individual Y variable. The model proposed for each response of Y is:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \sum \beta_{ij} X_i X_j$$

In this model,  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  were intercept, linear, quadratic and interaction regression coefficient terms, respectively, and  $X_i$  and  $X_j$  were independent variables. The Design-

Expert was used to analyze the data of urea complexation by fitting all the data to suitable regression models. Response surfaces were developed using the fitted quadratic polynomial equations obtained from the response surface regression (RSREG) analysis by holding the independent variables with the least effect on the response at a constant value and by changing the levels of the other variables.

## Results and Discussion

### 1. Fatty acid composition of rice bran oil

The compositions of fatty acids of rice bran oil are shown in Table 2 and Figure 1. The predominant fatty acids of rice bran were MUFA (59.75%), followed by PUFA (38.35%) and SFA (1.89%). The stearic acid (C18:0) was the major SFA, while oleic acid (C18:1) and linoleic acid (18:2) were the major MUFA and PUFA, respectively. These results are in agreement with previous studies on fatty acids of RD-6 rice, a popular glutinous rice cultivar for consumption in the North and Northeast of Thailand (Thammapat *et al.*, 2016). The major contributors to n-3 PUFA was 18:3 while 18:2 was found to be the major n-6 PUFA. Similar results were observed in other rice, such as RD-6 (Thammapat *et al.*, 2015; Thammapat *et al.*, 2016), Koshihikari, Kyeema and Doongara (Zhou *et al.*, 2002).

Table 1 Variables used for hexagonal rotatable design

| Coded-variable levels |       | Natural-variable levels                                  |                                       |
|-----------------------|-------|--|---------------------------------------|
| $Z_1$                 | $Z_2$ | $X_1$ (Crystallization Temperature, $^{\circ}\text{C}$ ) | $X_2$ (Urea-to-fatty acid ratio, w/w) |
| 1.00                  | 0.00  | 10.0   | 3.00                                  |
| 0.50                  | 0.87  | 5.0  | 3.87                                  |
| 0.50                  | -0.87 | 5.0  | 2.13                                  |
| -0.50                 | 0.87  | -5.0   | 3.87                                  |
| -0.50                 | -0.87 | -5.0   | 2.13                                  |
| -1.00                 | 0.00  | -10.0  | 3.00                                  |
| 0.00                  | 0.00  | 0.0  | 3.00                                  |

Table 2 Fatty acid compositions (% total fatty acids) of rice bran oil

| Fatty acids | Fatty acid content (%) |
|-------------|------------------------|
| C14:0       | 0.36 $\pm$ 0.09        |
| C16:0       | 0.51 $\pm$ 0.11        |
| C16:1 n-9   | 25.21 $\pm$ 0.59       |
| C18:0       | 1.02 $\pm$ 0.18        |
| C18:1 n-9   | 34.54 $\pm$ 0.52       |
| C18:2 n-6   | 35.10 $\pm$ 1.67       |
| C18:3 n-3   | 2.07 $\pm$ 0.16        |
| C20:3 n-6   | 0.41 $\pm$ 0.13        |
| C20:4 n-6   | 0.77 $\pm$ 0.13        |

Mean values  $\pm$  standard deviation of determinations for triplicate samples.

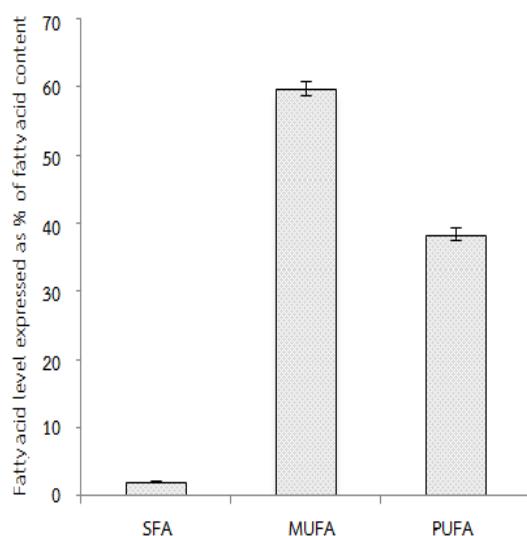


Figure 1 Fatty acid level of total fatty acid of rice bran oil

## 2. Concentration of PUFA of rice bran oil

Experimental values obtained for responses: The total content of PUFA of rice bran oil for ten design points are given in Table 3. The maximum increase of PUFA content was 73.56% in the non-urea complexed fraction, which was obtained at crystallization temperature of  $-5^{\circ}\text{C}$  and urea-to-fatty acid ratio of 3.87. Fractionation results showed a total reduction in SFA and MUFA. The highest reduction was observed at the  $-5^{\circ}\text{C}$ . Complete removal of SFA and MUFA by urea complexation may be impossible since some of the SFA and MUFA do not

complex with urea during crystallization (Homayooni *et al.*, 2014).

The urea-to-fatty acid ratio and crystallization temperature were the most influential variables affecting the degree of PUFA concentration. Generally, enrichment of PUFA in concentrate and liquid recovery yield varied inversely with the decreasing crystallization temperature as well as with the increasing urea-to-fatty acid ratio (Liu *et al.*, 2006). At low temperatures, fatty acids had a greater tendency to form urea compounds than at high temperatures, and the urea-to-fatty acid ratio could be used to segregate or isolate the fatty acids by their degree of unsaturation. Hasnisa and Jumat (2014) have reported similar results for the urea complexation experiment conducted on soybean oil. Crystallization of fatty acids in the presence of urea occurs at lower temperatures depending on the degree of the unsaturated fatty acids. The complexation limit depends on the urea-to-fatty acid ratio and crystallization temperature. It can be concluded that lower crystallization temperature derived low percentage of PUFA. This is related to the crystallization of fatty acids at a suitable temperature depending on the required degree of complex concentration.

In our present study, it was observed that the urea-to-fatty acid ratio and crystallization temperature, both of which are strongly related, are the most influential variables affecting the degree of PUFA concentration. The variation in PUFA content with the urea-to-fatty acid ratio is different when

the crystallization temperature is low, then when it is high (Medina *et al.*, 1998).

### 3. Analysis of model

Optimization of processing conditions, such as urea-to-fatty acid ratio ( $X_1$ ) and crystallization ( $X_2$ ), to maximize the PUFA content in rice bran oil by urea complexation was determined. The multiple regression coefficients obtained by employing a least square technique to predict a quadratic polynomial model for the PUFA content ( $Y$ ) is summarized in Table 4. Multiple regression equations were generated relating the response variable to coded levels of the independent variables. Multiple regression coefficients were determined to predict quadratic polynomial models for PUFA content of rice bran oil. Analysis of variance (ANOVA) showed that the selected quadratic models adequately represented the data obtained for PUFA content. Examination of these coefficients with the t-test, for the PUFA content, indicated that linear and quadratic term of urea-to-fatty acid and crystallization temperature were highly significant ( $P<0.01$ ), whereas the interaction term of quadratic term of urea-to-fatty acid and crystallization temperature was also significant ( $P<0.05$ ). These results suggested that the linear and quadratic terms of urea-to-fatty acid and crystallization temperature may be the primarily determining factors for PUFA content. The coefficients of independent variables determined for the quadratic polynomial models (Table 4) for the PUFA content ( $Y$ ) of rice bran oil is given below:

$$Y = 56.06 - 10.25Z$$

These results show that the models predicted for Y were adequate as indicated by error analysis that showed non-significant lack-

of-fit ( $P > 0.05$ ). The adjusted correlation coefficient for the determination ( $R^2$ ) of the PUFA content was 0.96.

Table 3 Hexagonal rotatable design arrangement and response for PUFA of rice bran oil

| Variable levels                         |                                       | Response (Y) |
|---|---------------------------------------|--------------|
| $X_1$ (Crystallization Temperature, °C) | $X_2$ (Urea-to-fatty acid ratio, w/w) | PUFA (%)     |
| 10.0                                    | 3.00                                  | 52.14±0.35   |
| 5.0                                     | 3.87                                  | 60.71±0.18   |
| 5.0                                     | 2.13                                  | 59.94±0.12   |
| -5.0                                    | 3.87                                  | 73.56±0.42   |
| -5.0                                    | 2.13                                  | 68.35±0.15   |
| -10.0                                   | 3.00                                  | 72.25±0.30   |
| 0.0                                     | 3.00                                  | 56.80±0.09   |
| 0.0                                     | 3.00                                  | 56.32±0.46   |
| 0.0                                     | 3.00                                  | 55.75±0.24   |
| 0.0                                     | 3.00                                  | 55.35±0.40   |

Table 4 Regression coefficients of predicted quadratic polynomial model for response variable

| Variables          | Coefficients ( $\beta$ ) |  |
|--------------------|--------------------------|--|
|                    | Y (%)                    |  |
| Intercept          | 56.06                    |  |
| <i>Linear</i>      |                          |  |
| $Z_1$              | -10.25**                 |  |
| $Z_2$              | 1.73**                   |  |
| <i>Quadratic</i>   |                          |  |
| $Z_{11}$           | 6.14**                   |  |
| $Z_{22}$           | 10.73**                  |  |
| <i>Interaction</i> |                          |  |
| $Z_{12}$           | -2.56*                   |  |
| $R^2$              | 0.99                     |  |

Y: the PUFA content

\*\* $P < 0.01$  highly significant, and \* $P < 0.05$  significant

#### 4. Optimization of process conditions to maximize content of the PUFA of rice bran oil

Two variables were given the fitted model were we chose it as the axis for the response surface plots. The linear, quadratic and interaction terms in second order polynomial were used to generate a three-dimensional response surface graph. The response surface for the PUFA content is given in Figure 2. The results of canonical response surfaces were given in Table 5. The stationary points for the PUFA content of rice bran oil by urea complexation predicted a maximum increase of 78.90% at the urea-to-fatty acid ratio of 2.91 and crystallization temperature of -7.2°C. The result of the three-dimensional response surface indicated that the PUFA content would be increased with low temperature and high urea-to-fatty acid ratio.

Previous studies have reported different variable processing conditions for producing cereal oil concentrates. For example, Hasnisa and Jumat (2014) have reported that the content of PUFA was highest at the crystallization temperature of 4°C, urea-to-fatty acid ratio of 3 and crystallization time of 24 h. Our study used a lower crystallization temperature than those used in these studies, because of the higher long chain MUFA content in rice bran oil. It would be difficult to remove those fatty acids from the mixture as they were included in the urea crystals (Medina *et al.*, 1998). Accordingly, the specific crystallization temperature of any individual cereal oil should be determined. The

crystallization temperature also depends on the degree of concentration desired (Shahidi and Wanasundara, 1998).

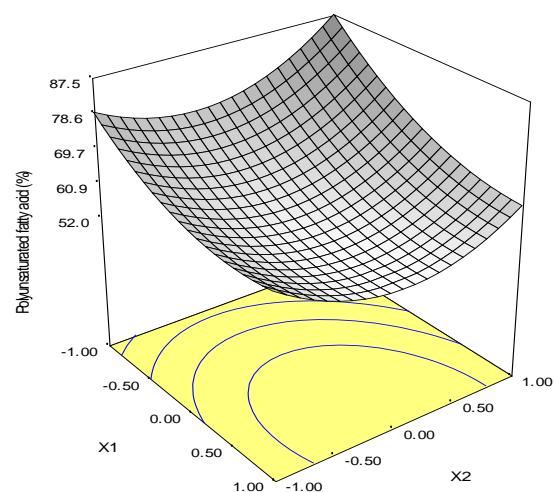


Figure 2 Response surface for the effect of crystallization temperature and urea-to-fatty acid ratio on the content of PUFA of rice bran oil concentrates.

The adequacy of the models predicted was examined by performing independent experiments at the optimal conditions for increased PUFA content by urea complexation conditions. Verification results revealed that the predicted values from this model was reasonably close to the observed one (Table 5), indicating that the model is suitable for the prediction of the study response. Our developed process has added nutritional value to rice bran oil with respect to bioactive substance.

#### Conclusion

The processing parameters were found to be: a crystallization temperature of -7.2°C and urea-to-fatty acid ratio of 2.91. We selected

Table 5 Predicted and observed values for response variable in urea complexation experiment of rice bran oil

| Response variable      | Critical values of independent variables |                                | Stationary point | Predicted value (%) | Observed value <sup>a</sup> (%) |
|------------------------|--|--------------------------------|------------------|---------------------|---------------------------------|
|                        | Crystallization temperature (°C)         | Urea-to-fatty acid ratio (w/w) |                  |                     |                                 |
| Total PUFA content (%) | -7.20                                    | 2.91                           | Maximum          | 78.90               | 77.48±1.14                      |

<sup>a</sup> Mean values ± standard deviation of determinations for triplicate samples

these conditions in order to achieve the highest levels of the PUFA content of rice bran oil. Under these conditions, the PUFA content could be increased up to 77.48%. The urea complexation process at low temperature and high urea-to-fatty acid ratio was demonstrated to be a feasible method for the increase of PUFA content in rice bran oil concentrates. Our finding suggests that urea complexation could be considered as a very effective method for concentrating PUFA from rice bran oil leading to a potential use for the application in the food and pharmaceutical industries.

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