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Optimisation of enzymatic hydrolysis for concentration of vitamin E in palm fatty acid distillate

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Abstract

Vitamin E was concentrated from palm fatty acid distillate (PFAD) in this study using a commercial immobilised *Candida antarctica* lipase (Novozyme 435). The PFAD and water mixture was hydrolysed at 65±1 °C. Free fatty acids (FFA) liberated were neutralised using an alkali and the salts formed were then washed off. Factors affecting the enzymatic hydrolysis and vitamin E concentration of extracted fraction were optimised using response surface methodology (RSM). A central composite design was employed to study the responses, namely percentage of FFA (Y_1) and vitamin E concentration (Y_2), while lipase concentration (X_1), reaction time (X_2) and water content of the reactant (X_3) were the independent variables. Results showed that the regression models generated adequately explained the data variation and significantly represented the actual relationships between the reaction parameters and the responses. For both regression models of FFA and vitamin E, the first- and second-order variable of water content was the most significant factor. The optimum reaction parameters for maximum yield in FFA and vitamin E concentrations were identified from their respective contour plots. It was suggested that for maximum vitamin E concentration, the hydrolysis should be carried out with 2.5% (w/w) of lipase and 45.2–47.3% (v/w) of water for 5.5–5.7 h.

Keywords: Enzymatic hydrolysis; Free fatty acids; Palm fatty acid distillate; Response surface methodology; Vitamin E

1. Introduction

Vitamin E (tocopherols and tocotrienols) is a natural antioxidant that plays significant roles in food preservation and disease prevention. Tocopherols and tocotrienols inhibit acylglycerol peroxidation by accepting free radicals (Olcott & Emerson, 1973; Sherwin, 1974). Findings show that tocopherols and tocotrienols suppress cholesterol production in liver (Qureshi, Burger, Peterson, & Elson, 1986), give protection against certain types of experimental cancers (Kato, Yamoka, Tanaka, Komiyama & Umezewa, 1985; Tengerdy, 1980), enhance the body's immune system (Corwin & Gordon, 1982) and reduce cellular aging (Walton & Packer, 1980). One of the major sources of natural vitamin E is fatty acid distillate (FAD). Palm fatty acid distillate (PFAD), for instance, contains about 0.4% of vitamin E, which is mainly represented by tocotrienols (about 70%; Ab Gapor, 1990). There are a number of methods developed to separate vitamin E from FAD. These include saponification (Fizet, 1993), transesterification to form methyl esters, followed by distillation (Ab Gapor, et al., 1993; Hunt, Jeromin, & Johannisbauer, 2000), solvent extraction or partitioning and crystallisation (Eastman Kodak Co., 1996; Goh, Lai, & Kam, 1992; Shibue & Tamura, 1984; Willging, 1985), column chromatography (TechniKrom Inc., 2001). Normally these methods are used in combinations to increase the purity of tocopherols and tocotrienols (Goh et al., 1992; Ong, Choo, Goh, & Kam, 1994).

Several researchers (Ghosh & Bhattacharyya, 1996; Ramamurthi & McCurdy, 1993) have reported extrac-

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tions of vitamin E from FAD using lipases. Lipases were applied to transesterify free fatty acids (FFA) in FAD into methyl esters. In this study, vitamin E in PFAD was concentrated by enzymatic hydrolysis, followed by alkali neutralisation. The water-soluble FFA salts formed were then removed by washing, using water, to give a vitamin E-concentrated fraction. The more the acylglycerols in PFAD are hydrolysed, the higher will the concentration of vitamin E in the remaining fraction be, after neutralisation and washing.

There are many factors affecting the degree of hydrolysis, such as substrate and enzyme concentrations, reaction time, reaction temperature and water content of the reaction mixture. The results of one-factor-at-a-time experiments do not reflect the actual changes under the reaction conditions as they ignore interactions between factors, which are present simultaneously (Wanasundara & Shahidi, 1998). To obtain maximum recovery of vitamin E, the hydrolysis process must be optimised and response surface methodology (RSM) is an effective tool for this purpose. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. It is a faster and less expensive method for gathering data compared to full-factorial experimentation.

In this study, reaction variables i.e. enzyme concentration (X_1), reaction time (X_2) and water content of the reaction mixture (X_3), were selected for optimisation of the acylglycerol hydrolysis in PFAD using a commercial immobilised thermally stable *Candida antarctica* lipase (Novozyme 435). Enzyme concentration and reaction time were the major factors that affect the economy of the separation of vitamin E. Large amounts of water in the reaction mixture shift the equilibrium of the reaction to hydrolysis. However, too much water may decrease the degree of hydrolysis. The reaction temperature was set at the optimum temperature (65 °C) for lipase hydrolytic activity in this study and was excluded from the RSM as described by Shankar, Arrawal, Sarkar, and Singh, (1997). The objective of this study was to understand the relationships between these variables and the responses (i.e. the percentage of FFA and total tocopherols and tocotrienols expressed as vitamin E concentration). Another objective of this study was to determine the optimal conditions for the hydrolysis process that determine the maximum recovery of vitamin E.

Table 1
Independent variables and their levels for central composite design in optimisation of enzymatic hydrolysis of palm fatty acid distillate

Independent variables	Symbol	Coded variable levels				
		−1.68 (−α)	−1	0	1	1.68 (α)
Enzyme concentration,% (w/w)	X_1	0.63	2.50	5.25	8.00	9.87
Reaction time, h	X_2	0.64	2.00	4.00	6.00	7.36
Water content,% (v/w)	X_3	24.77	35.00	50.00	65.00	75.23

2. Materials and methods

2.1. Materials

PFAD (iodine value, 56.8 g of I_2/100 g of oil; slip point, 48.5 °C; vitamin E concentration, 0.37%) was provided by Jomalina Pte. Ltd., Tanjung Panglima Garang, Selangor, Malaysia. The commercial immobilised thermally stable *Candida antarctica* lipase (Novozyme 435) was a gift from Novo Nordisk, Bagsvaerd, Denmark. All chemicals used were either of high-performance liquid chromatography (HPLC) or analytical grades.

2.2. Experimental design

A central composite design was employed to study the responses, namely percentage of FFA, Y_1 and vitamin E concentration, Y_2. The independent variables were X_1,X_2 and X_3 representing enzyme concentration, reaction time and water content, respectively. The settings for the independent variables were as follows (low/high value): enzyme concentration [% (w/w)], 2.5/8.0; reaction time (h), 2/6; and water content [%(v/w)], 35/65. Each variable to be optimised was coded at five levels: −1.68, −1, 0, +1, and +1.68. This gives a range of these variables of 0.63–9.87% (w/w), 0.64–7.36 h and 24.77–75.23% (v/w), respectively, including the star points (Table 1). The star points were added to the design to provide for estimation of curvature of the model (Joglekar & May, 1987). Six replicate runs at the centre (0,0,0) of the design were performed to allow the estimation of the pure error. All experiments were carried out in a randomised order to minimise the effect of unexplained variability in the observed responses due to extraneous factors (Wanasundara & Shahidi, 1998).

2.3. Enzymatic hydrolysis of PFAD

A total of 100 g of melted PFAD and water mixture was placed in a 250-ml conical flask. Novozyme 435 lipase was added to the reaction mixture and the flask was flushed with nitrogen and sealed with a rubber cap. The flask was then placed in a water bath at 65±1 °C and hydrolysis was started by stirring the mixture using a magnetic stirrer. The reaction was stopped by filtering out the lipase using a two-layer cheese cloth. Several millilitres of the reaction mixture were then withdrawn for determination of FFA content and the remaining amount was used for extraction of tocopherols and tocotrienols.

Table 2
Central composite design and experiment data for enzymatic hydrolysis of palm fatty acid distillate

Run	Independent variables[a]			Responses	
	X_1	X_2	X_3	Y_1	Y_2
1	−1	−1	−1	90.9	3.06
2	1	−1	−1	89.2	2.64
3	−1	1	−1	92.0	3.64
4	1	1	−1	90.1	2.92
5	−1	−1	1	92.6	3.78
6	1	−1	1	90.3	2.93
7	−1	1	1	93.0	3.98
8	1	1	1	93.1	4.24
9	−1.68	0	0	90.5	2.88
10	1.68	0	0	90.5	3.09
11	0	−1.68	0	90.1	2.88
12	0	1.68	0	94.1	5.18
13	0	0	−1.68	89.2	2.73
14	0	0	1.68	93.8	4.62
15	0	0	0	93.1	4.36
16	0	0	0	93.1	4.19
17	0	0	0	93.5	4.66
18	0	0	0	93.1	3.94
19	0	0	0	93.0	4.05
20	0	0	0	93.1	4.24

[a] Independent variables X_1, X_2 and X_3 represent enzyme concentration [% (w/w)], reaction time (h) and water content [% (v/w)], respectively, while responses Y_1 and Y_2 represent percentage of free fatty acid (%) in palm fatty acid distillate and concentration of vitamin E (%) extracted, respectively.

2.4. Determination of FFA

The melted reaction mixture withdrawn was centrifuged at 1400 ***g*** for a few seconds to break the emulsion. PFAD (0.500 g) was then weighed into a 250-ml conical flask and 50 ml of neutralised isopropanol was then added to it in order to dissolve the sample. The sample was titrated with 0.05 N NaOH to the phenolphthalein end-point (PORIM, 1995).

2.5. Extraction and determination of vitamin E

Melted sample was centrifuged at 1400 g to separate water from PFAD. PFAD (15 g) was removed from the centrifuge tube and dissolved in 30 ml ethanol. The sample was then neutralised with 0.5 N NaOH to the phenolphthalein end-point and 100 ml hexane were added. The mixture was then transferred into a separatory funnel, shaken for 1 min and left to stand until two distinct layers were formed. The bottom layer (aqueous phase), containing FFA salt, was discarded. The top layer containing vitamin E was centrifuged at 2500 ***g*** for 30 s to remove water residues. The hexane layer was finally transferred to a 250-ml round-bottom flask and evaporated at 60 °C under vacuum. Vitamin E (total tocopherols and tocotrienols) of the sample was then determined using HPLC. The sample (0.05 g) was dissolved in 2 ml of hexane and 5 μl of it were injected into a HPLC system (Waters Model 600 Controller, Milford, MA) equipped with a UV detector at 280 nm (Waters Model 486 UV Detector, Milford, MA). The column used was a Purospher STAR RP-18 endcapped column (55 mm in length, i.d. 4 mm, particle size 3 μm; Merck, Darmstadt, Germany), while the mobile phase was methanol:water (95:5 v/v). The flow rate was maintained at 1.0 ml/min and the oven temperature was set at 40 °C. By comparing with published chromatograms and natural occurrence (Tan & Brzuskiewicz, 1989; Wang, Ning, Krishnan, & Matthees, 1998), α-tocopherol, α-, γ- and δ-tocotrienols were identified. Peak area was used for quantification of tocols. Due to the unavailability of α-, γ- and δ-tocotrienol standards, D-α-tocopherol (Sigma Chemical Co., St. Louis, MO) was used as external standard for quantification for all four tocols (Wang et al., 1998), based on the assumption that they have the same UV intensity. Total concentration of α-tocopherol, α-, δ- and γ-tocotrienols was expressed as vitamin E concentration.

2.6. Statistical analysis

The responses were analysed using Minitab release 11.12 software (Minitab Inc.). A quadratic polynomial regression model was assumed for predicting both Y responses, namely Y_1 and Y_2. The model proposed for each response of Y was:

$$Y = \beta_o + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij}x_i x_j \quad (1)$$

where β_o is an intercept; β_i, first-order model coefficient, β_{ii}, quadratic coefficient for the ith variable, β_{ij}, interaction coefficient for the interaction of variables i and j; X_i and X_j are independent variables. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Contour plots were developed using the fitted full quadratic polynomial equations obtained by holding one of the independent variables at a constant value and changing the levels of the other two variables.

3. Results and discussion

3.1. Model fitting

Table 2 shows the responses, i.e. the percentage of FFA in PFAD and concentration of vitamin E after neutralisation and washing. It was found that, in general, for every increase of 1% in FFA, the concentration of vitamin E extracted increased by about 0.45%. The

responses and variables were fitted to each other by multiple regressions. A good fit was obtained and there were no outliers observed for either regression:

$$\%\text{FFA} = 80.738 + 0.333X_1 + 1.180X_2 + 0.319X_3 - 0.044X_1^2 - 0.136X_2^2 - 0.004X_3^2 + 0.028X_1X_2 + 0.007X_1X_3 + 0.006X_2X_3 \quad (2)$$

$$\%\text{vitamin E} = -2.274 + 0.126X_1 + 0.457X_2 + 0.181X_3 - 0.013X_1^2 - 0.056X_2^2 - 0.002X_3^2 + 0.017X_1X_2 + 0.002X_1X_3 + 0.002X_2X_3 \quad (3)$$

The R^2 value and standard error (S.E.) of Eqs. (2) and (3) were 0.909 and 0.673; 0.861 and 0.391, respectively. The probability (P) values of regression models (2) and (3) was less than 0.000 and 0.003, respectively, with no significant lack-of-fit ($P > 0.001$ and $P > 0.05$ for Eqs. (2) and (3), respectively). Also, the predicted results, according to the models for FFA value and vitamin E concentration, were close to the observed experimental responses. The R and S.E. values between the experimental and predicted results for FFA value and vitamin E concentration were 0.953 and 0.504; and 0.921 and 0.271, respectively. These indicated that the generated models adequately explained the data variation and significantly represented the actual relationships between the reaction parameters.

3.2. Effects of parameters

ANOVA results showed that the FFA value was significantly affected by the first-order (linear) ($P = 0.030$) and second-order ($P = 0.001$) of the variables. It was found that all first-order parameters had a positive effect on the FFA, with water content (X_3) being the most significant factor ($P = 0.006$). At the same time, all the second-order variables had a negative effect, in which both reaction time (X_2) and water content were of high significance ($P = 0.012$ and 0.000, respectively). Interaction between the variables had a very minor influence on FFA value with $P > 0.05$. These results suggested that the linear and/or second-order effects of the variables were the primary determining factors for the FFA produced by the lipase.

As in the regression model of FFA, the regression model of vitamin E [Eq. (3)] showed that all first-order variables had positive effects on the vitamin E concentration, while the second-order variables exerted negative effects. The first- and second-order of water content were the most significant ($P = 0.006$ and $P = 0.000$, respectively) factors affecting the vitamin E concentration. Interactions among the variables did not influence the vitamin E recovery, as shown by relatively small intercepts with P values of larger than 0.520.

3.3. Optimisation of enzymatic hydrolysis

Eqs. (2) and (3) show that FFA value and vitamin E concentration have a complex relationship with the independent variables that encompass both first- and second-order polynomials and may have more than one maximum point. Analysing the contour plots for FFA and vitamin E concentration is the best way to evaluate the relationships between responses and variables and interactions that existed herein (Xu, Fomuso, & Akoh, 2000). Figs. 1 and 2 show the contour plots of FFA and vitamin E concentration, respectively, as a function of the interactions of any two of the variables by holding the other one at low value. All three plots in Fig. 1 show similar relationships with respect to the effects of each variable. High levels of enzyme concentration and water content in the reaction mixture increased the FFA value after a sufficient reaction time. However, when water content was more than 55% (v/w), FFA value started to decrease even at high levels of enzyme concentration and long reaction time (Fig. 1A and B). This was in accordance with Shankar and co-workers' findings (1997). High concentration and long reaction time yielded high levels of FFA. However, if enzyme concentrations greater than 10% (w/w), a long reaction time (> 7 h) would not further increase the FFA levels (Fig. 1C). For vitamin E concentration, it was found that a water content of 45–55% (v/w) resulted in the highest level of vitamin E as shown in Fig. 2A and B. Increase in lipase concentration in the reaction mixture might increase vitamin E concentration (Fig. 2A); however, a long reaction time (more than 6 h) decreased the concentration (Fig. 2B and C).

Tables 3 and 4 show the optimum conditions of the hydrolysis reaction to yield maximum levels of FFA and vitamin E, respectively, by holding one variable at a time at low, middle and high values. It was noted that the optimum conditions for producing FFA and vitamin E were slightly different. There are a number of combinations of the variables that could give maximum levels of FFA and vitamin E, respectively. The most efficient condition would use the lowest amount of enzyme to achieve full hydrolysis of PFAD in the minimal time and would afford the maximum vitamin E concentration. Since enzyme concentration was the most important factor in reducing the production cost,

it is suggested that the hydrolysis should be carried out with 2.5% (w/w) of lipase and 45.2–47.3% (v/w) of water for 5.5–5.7 h. The other viable option was hydrolysing the PFAD with 45.6–50.0% (v/w) of water and 5.3% (w/w) of lipase for about 5.9 h. Even though other combinations might slightly increase the degree of hydrolysis, much more lipase and/or longer reaction time are required.

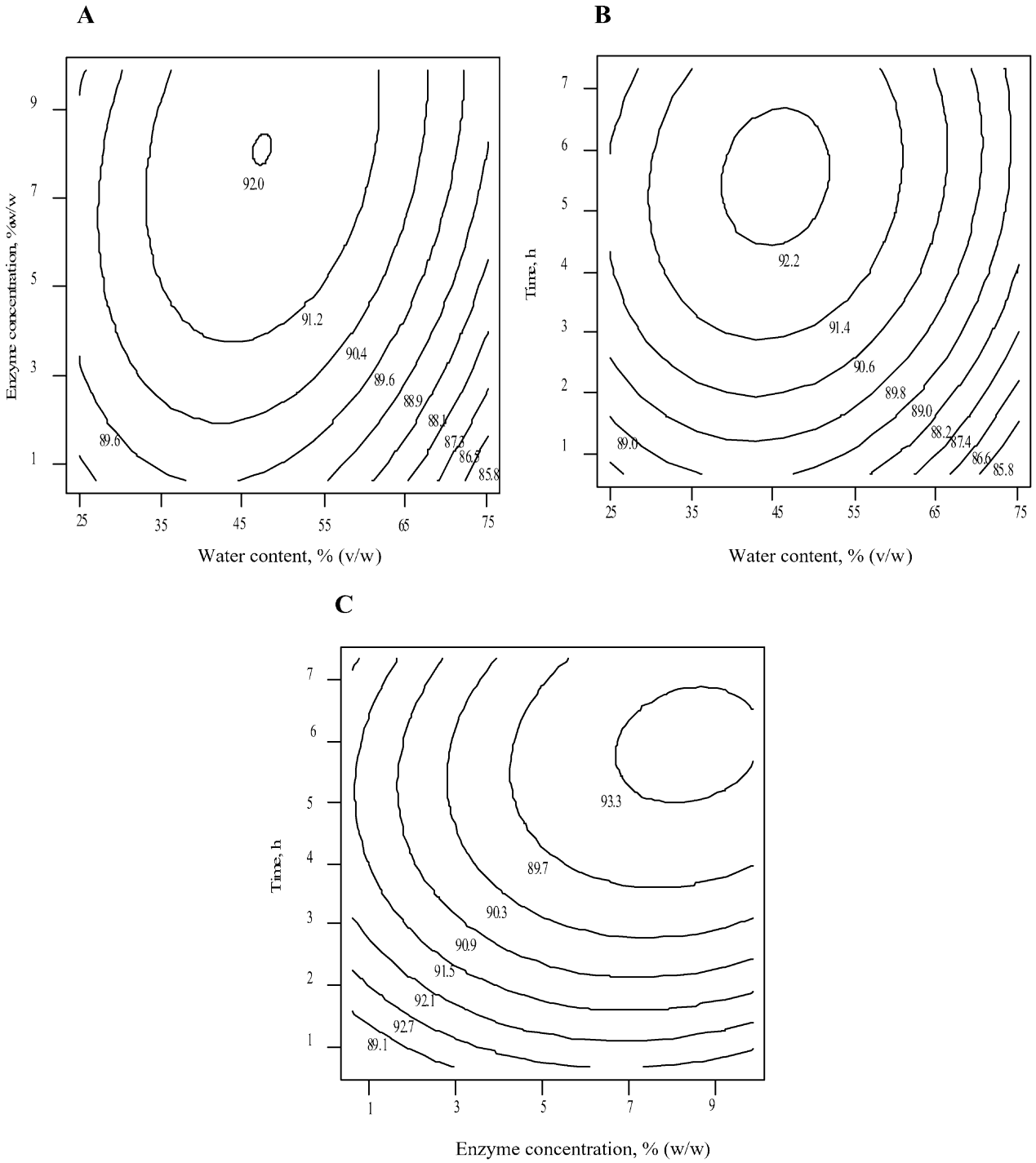

Fig. 1. Contour plots of FFA levels as a function of the interactions of two variables by holding the other one at low value: interactions of enzyme concentration and water content when reaction time was held at 2 h (A), interactions of reaction time and water content when enzyme concentration was held at 2.5% (w/w) (B), and interactions of reaction time and enzyme concentration when water content was held at 35% (v/w) (C). Figures in the plots are FEA levels (%).

3.4. Verification of the regression models

Independent experiments were carried out for all 18 optimal hydrolysis conditions (Tables 3 and 4) to examine the adequacy of the predicted values by the models. The predicted FFA levels were close to the experimental data as shown in Table 3. The verification results showed a close correspondence of predicted and experimental FFA values. Mean differences (MD) and standard deviations of differences (SDD) between the

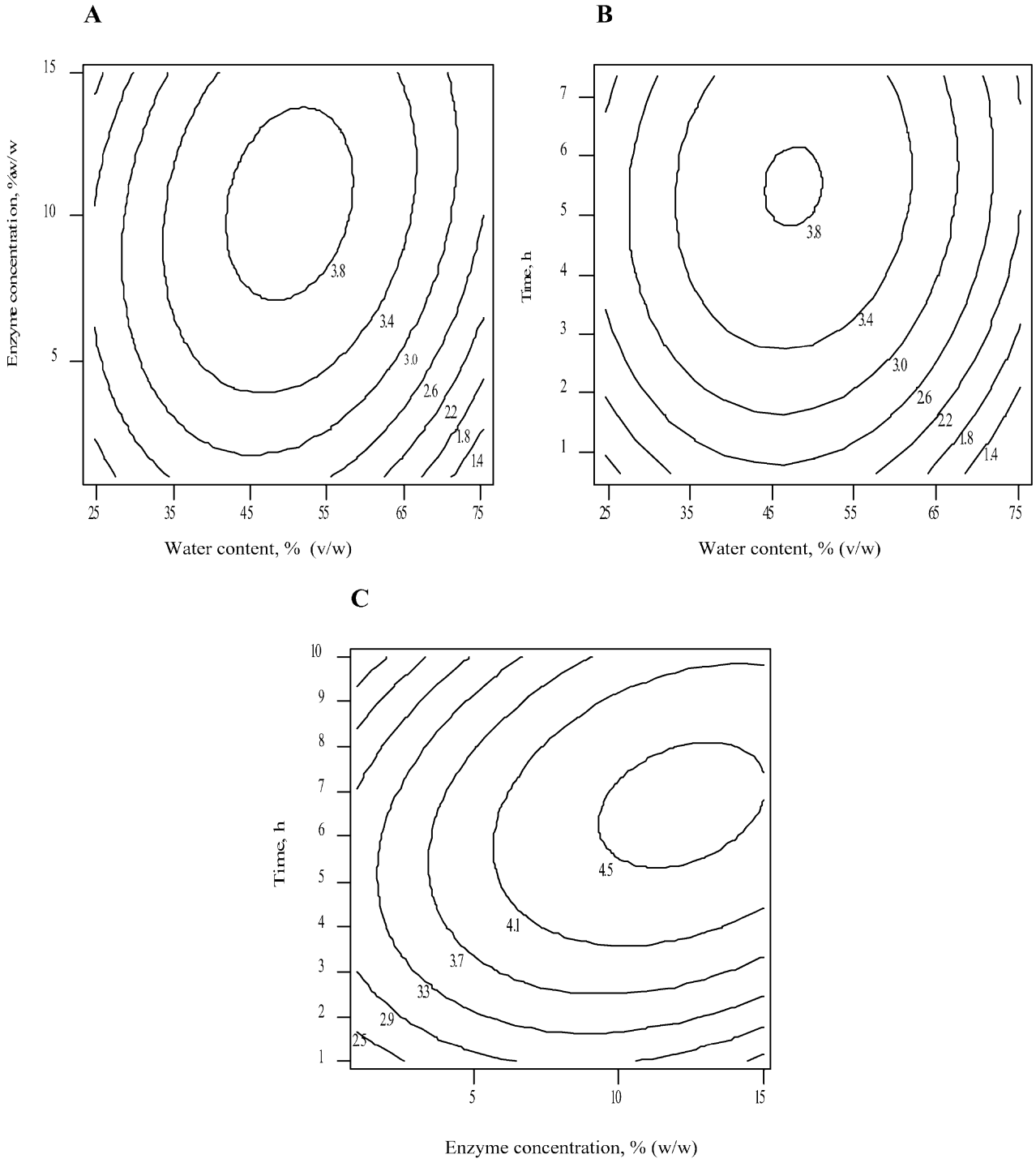

Fig. 2. Contour plots of vitamin E as a function of the interactions of two variables by holding the other one at low value: interactions of enzyme concentration and water content when reaction time was held at 2 h (A), interactions of reaction time and water content when enzyme concentration was held at 2.5% (W/N) (B), and interactions of reaction time and enzyme concentration when water content was held at 35% (v/w) (C). Figures in the plots are Vitamin E concentration (%).

Table 3
Predicted and experimental free fatty acid values of enzymatic hydrolyate of palm fatty acid distillate by holding an independent variable at low, middle and high levels

Hold levels	Hold variable	Critical variables		Stationary	Response Y_1	
					Predicted	Experimental
	X_1	X_2	X_3			
Low	2.50	5.56	45.2	Maximum	92.3	91.9±0.91
Middle	5.25	5.90	45.6	Maximum	93.6	94.1±0.63
High	8.00	6.24	50.5	Maximum	94.2	93.8±0.46
	X_2	X_1	X_3			
Low	2.00	8.11	47.4	Maximum	91.9	92.4±0.53
Middle	4.00	8.86	49.6	Maximum	93.6	93.2±0.19
High	6.00	9.66	51.7	Maximum	94.4	94.7±0.26
	X_3	X_1	X_2			
Low	35.0	8.38	5.97	Maximum	93.4	92.9±0.27
Middle	50.0	9.74	6.39	Maximum	94.4	93.6±0.37
High	65.0	11.00	6.80	Maximum	93.7	94.9±0.12

Units for X_1, X_2, X_3 and Y_1 are% (w/w), h,% (v/w) and%, respectively. For abbreviations, see Table 2.

Table 4
Predicted and experimental vitamin E concentration of enzymatic hydrolysate of palm fatty acid distillate by holding an independent variable at low, middle and high levels

Hold levels	Hold variable	Critical variables		Stationary	Response Y_2	
					Predicted	Experimental
	X_1	X_2	X_3			
Low	2.50	5.46	47.4	Maximum	3.96	3.60±0.11
Middle	5.25	5.85	50.0	Maximum	4.57	4.23±0.13
High	8.00	6.41	50.9	Maximum	5.02	4.82±0.09
	X_2	X_1	X_3			
Low	2.00	9.87	49.6	Maximum	3.99	3.69±0.15
Middle	4.00	11.8	52.6	Maximum	4.86	4.59±0.20
High	6.00	12.8	54.1	Maximum	5.26	4.91±0.18
	X_3	X_1	X_2			
Low	35.0	10.5	6.27	Maximum	4.57	4.02±0.05
Middle	50.0	13.9	7.15	Maximum	5.34	4.75±0.07
High	65.0	16.0	7.53	Maximum	5.24	4.68±0.14

Units for X_1, X_2, X_3 and Y_2 are% (w/w), h,% (v/w) and%, respectively. For abbreviations, see Table 2.

experimental and predicted results for FFA levels were −0.102 and 0.488, respectively, indicating that the models were reasonable and of high accuracy to predict FFA level. A high chi-square result ($P = 0.9999$) further confirmed the finding. For the regression model of vitamin E, the MD and SDD results of the predicted and experimental data were also small (−0.392 and 0.277, respectively), with a chi-square result of $P = 0.9999$. The generated regression model of vitamin E was capable of predicting vitamin E concentration with an appreciably high accuracy.

In this study, RSM successfully optimised the conditions used in the hydrolysis of PFAD and subsequently maximised the levels of vitamin E after extraction. Various operating conditions that could be applied to preconcentrate vitamin E were proposed. The generated regression models could be used to predict the degree of hydrolysis of PFAD and concentration of vitamin E. Results of this study might serve as a guideline for a larger scale hydrolysis, such as in pilot plant, for concentration of vitamin E from PFAD.

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