### Acid and Alkaline Hydrolysis Extraction of Non-Extractable Polyphenols in Blueberries: Optimisation by Response Surface Methodology

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#### **Abstract**

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Polyphenols, including extractable polyphenols (EPP) and non-extractable polyphenols (NEPP), are natural and secondary metabolic substances in plants that have beneficial properties to human health. However, NEPP associated with dietary fiber and protein are not taken into account in most literature data. In this paper, NEPP were released from blueberries with acid or alkaline hydrolysis methods, and the related extraction conditions were determined and optimised by response surface methodology (RSM). The results showed that NEPP yield obtained with alkaline hydrolysis was much higher than that obtained with acid treatment. The NEPP yield in alkaline hydrolysis process was significantly affected by the NaOH concentration and liquid/solid ratio, while in the acid hydrolysis process, the NEPP yield was significantly affected by the temperature, time and liquid/solid ratio. The second order polynomial models were developed for predicting NEPP content in blueberries. The optimisation of the extraction process of NEPP in blueberries would provide a good idea and basis for the application of non-extractable fractions.

Keywords: blueberry polyphenols; acid hydrolysis process; alkaline hydrolysis process; response surface analysis

Vaccinium is the family of all blueberries which includes more than 450 plants. Those plants grow widely all around the world. Blueberry is an increasingly important commercial crop in many parts of the world. Many reports have indicated that blueberry fruits have a wide range of properties beneficial to human health, including antimicrobial (SINGH et al. 2010), antiallergenic (Chung & Champagne 2008), antidiabetic (Vuong et al. 2009), anticancer (Seeram 2008), and antioxidant (Castreón et al. 2008) activities.

Blueberries contain many bioactive compounds, such as polyphenols, terpenes, or dietary fibre. Espe-

cially, polyphenols, which are natural and secondary metabolic substances in plants, have been widely studied by researchers for their strong antioxidant health benefits. Most of the studies on polyphenols in plants referred to the compounds determined in aqueous-organic extracts. However, an appreciable amount of polyphenols trapped in cores or bound to cell wall constituents may remain insoluble after the aqueous-organic extraction treatment (Arranz et al. 2009). Insoluble (non-extractable) polyphenols are the components of cell walls, while soluble (extractable) polyphenols are compartmentalised within the plant cell vacuoles (Beckman 2000). Non-

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extractable polyphenols (NEPP) are mainly hydrolysable tannins and proanthocyanidins associated with dietary fiber and/or protein (ARRANZ et al. 2009). The determination of NEPP requires chemical or enzymatic treatment to release polyphenols from the food matrix prior to the chromatographic or spectrophotometric analysis of the corresponding solutions or hydrolysates (Guyot et al. 2001; Hellström & MATTILA 2008). Acid and alkaline hydrolysis which are the main procedures usually performed in the aqueous-organic extracts and powdered samples for ester bond cleavage, may degrade hydroxycinammic and benzoic acids (KRYGIER et al. 1982), At present, the literature data may be of limited use for these studies because most reports on the concentrations and composition of food polyphenols deal with extractable polyphenols (EPP) analysed in aqueous organic extracts, whereas significant amounts of bioactive NEPP that remain in the extraction residues are not taken into account (SAURA-CALIXTO et al. 1991; Huemme & Schereier 2008). Saura-Calixto et al. (2007) pointed out that most data from the literature on "total polyphenol content" (TPP) refer only to EPP and ignore NEPP. The TPP in foods is actually made up of EPP plus NEPP.

As we know, the extraction methods of EPP in plants have been widely studied, and some of them have been taken as the classical and traditional methods in extracting the bioactive compounds from plants (Pinelo et al. 2005; Silva et al. 2007). Few researches have been carried out into the extraction methods of NEPP from plants due to their characteristics of NEPP associated with the cell walls. However, the acid or alkali extraction methods were mentioned in a few reports (Saura-Calixto et al. 2007; Arranz et al. 2009), the detailed extraction processes and relative extraction parameters having not been shown. The effects of acid and alkaline hydrolysis methods on the yield of NEPP have not been compared in the respective literature, either.

The aim of the present paper was to choose a suitable raw material for extracting NEPP in view of the chemical components of blueberries. Moreover, the response surface methodology (RSM) was employed to optimise the extraction conditions, such as the solvent type, liquid/solid ratio, extraction time and temperature, which could maximise the yield of NEPP. The further purpose was to compare the difference between NEPPs obtained with alkaline and acid hydrolyses, and to determine the optimal extraction method of NEPP and provide the technical reference for the NEPP in other plants.

#### MATERIAL AND METHODS

*Plant materials*. Fresh blueberries (*Vaccinium angustifolium* L.) were cultivated and collected from Jinan, China, in July. The blueberries were pulped and freeze-dried, pulverised, and stored at −20°C until further use.

 $\it Reagents$ . All chemical reagents (methanol, ethanol,  $\rm H_2SO_4$ ,  $\rm Na_2CO_3$ , NaOH) were bought from Fisher Scientific Co., Ltd. (Shanghai, China). The standard sample of gallic acid and Folin-Ciocalteu phenol reagent (2 mol/l) were purchased from Sigma-Aldrich Co. (Shanghai, China).

**Determination of polyphenols content.** Polyphenols content in the extracts was determined by Folin-Ciocalteu colorimetric method (SLINKARD & SINGLETON 1977). Briefly, the extracts (100 μl) was mixed with 2 ml HPLC grade water, then Folin-Ciocalteu's phenol reagent (200 μl) was added and the solution was mixed. After 3 min, 900 μl of 20% (w/v)  $Na_2CO_3$  solution were added, and the mixture was incubated for 2 h in the dark at room temperature. The absorbance of each sample was measured at 765 nm. Polyphenol content was expressed as mg gallic acid equivalent in dried blueberries.

*Extraction procedure.* Dried blueberry powder was dispersed in methanol/water (v/v, 50:50) at liquid to solid ratio (ml/g) of 40:1. The mixture was sonicated at 300 W for 1 h and subsequently centrifuged at 4000 rpm for 10 minutes. The extraction was performed twice and the recollected residues were combined. The rcoímbined residues were vacuumdried at 60°C for 12 hours.

Selection of related variables with alkaline hydrolysis treatment. A first set of tests were performed to select the related factors in NEPP extraction with alkali hydrolysis treatment as well as the experimental ranges for the independent variables. Firstly, the influence of different NaOH concentrations (0.1, 0.5, 1.0, 2.0, and 4.0 mol/l) on the NEPP extraction was investigated under the following fixed conditions: liquid/solid ratio 10:1, extraction time 1 hour. Secondly, we studied the impact of the liquid/solid ratio (5:1, 10:1, 15:1, 20:1, 25:1, 30:1 ml/g) on the NEPP content under the following fixed conditions: NaOH 1.0 mol/l, extraction time 1 hour. Finally, the impact of the extraction time (0.5, 1.5, 2.5, 3.5, 4.5 h) on the NEPP content was determined under the fixed conditions of NaOH 1.0 mol/l, liquid/solid ratio 10:1.

Selection of related variables with acid hydrolysis treatment. We also studied and selected the related factors in NEPP extraction with acid hydrolysis treat-

Table 1. Independent variables and their levels in the response surface design

T	To donor donk or with the	Factor level				
Treatment	Independent variables	-1	0	1		
Alkaline hydrolysis	$X_1$ : NaOH (mol/l)	1	2	4		
	$X_2$ : time (h)	4	4.5	5		
	$X_3$ : liquid/solid ratio (ml/g)	10:1	15:1	20:1		
	$X_1$ : temperature (°C)	70	80	90		
Acid hydrolysis	$X_2$ : time (h)	15	20	25		
	$X_3$ : liquid/solid ratio (ml/g)	15:1	20:1	25:1		

ment. Firstly, the influence of the extraction solvent type (methanol/H<sub>2</sub>SO<sub>4</sub>, ethanol/H<sub>2</sub>SO<sub>4</sub>) on the NEPP yield was investigated by considering the methanol/  $H_2SO_4$  or ethanol/ $H_2SO_4$  ratios (90:10, 95:5, 97:3) under the following fixed conditions: liquid/solid ratio 20:1, extraction time 20 h, temperature 70°C. Secondly, we studied the impact of the liquid/solid ratio (5:1, 10:1, 15:1, 20:1, 25:1 ml/g) on the NEPP content under the following fixed conditions: methanol/H2SO4 (90:10), extraction time 20 h, temperature 70°C. Thirdly, the impact of the extraction time (10, 15, 20, 25 h) on the NEPP content was determined under the following fixed conditions: methanol/H<sub>2</sub>SO<sub>4</sub> (90:10), liquid/solid ratio 20:1, extraction temperature 70°C. Finally, we studied the effect of temperature (50, 60, 70, 80, 90°C) on the NEPP yield under the following fixed conditions: methanol/H<sub>2</sub>SO<sub>4</sub> (90:10), liquid/ solid ratio 20:1, extraction time 20 hours.

Experimental design for the response surface procedure. After determining the preliminary range

of extraction variables through the single-factor test, a three-level-three-factor, Box-Behnken factorial design was employed in this optimisation study. In the alkaline hydrolysis treatment, the independent variables of NaOH concentration  $(X_1)$ , ultrasonic time  $(X_2)$  and liquid/solid ratio  $(X_3)$  were selected to optimise the extraction of NEPP. In the acid hydrolysis treatment, the independent variables of extraction temperature  $(X_1)$ , time  $(X_2)$  and liquid/solid ratio  $(X_3)$  were selected to optimise the extraction of NEPP. The ranges of the independent variables and their levels are presented in Table 1. NEPP yield (Y) was taken as the response for the combination of the independent variables (Table 2). All the experiments were carried out at random in order to minimise the effect of the unexplained variability in the observed responses due to systematic errors. The data from the central composite design were analysed by multiple regressions to fit the following quadratic polynomial model:

Table 2. Box-Behnken experimental design and results for yield of NEPP extracted with alkaline hydrolysis and acid hydrolysis methods

No.	,	Alkaline hydrolysis				Acid hydrolysis				
	$X_1$	$X_2$	$X_3$	yield (mg/g)	$X_1$	$X_2$	$X_3$	yield (mg/g)		
1	1	1	0	48.68	1	1	0	19.12		
2	0	0	0	50.86	0	0	0	24.37		
3	0	1	1	54.96	0	1	1	20.72		
4	1	0	1	52.03	1	0	1	16.70		
5	0	0	0	47.74	0	0	0	25.42		
6	0	-1	1	52.28	0	-1	1	19.73		
7	1	-1	0	41.99	1	-1	0	18.00		
8	0	0	0	48.17	0	0	0	25.13		
9	0	1	-1	48.77	0	1	-1	20.68		
10	-1	0	-1	42.88	-1	0	-1	18.63		
11	0	-1	-1	43.57	0	-1	-1	14.90		
12	-1	-1	0	40.09	-1	-1	0	21.59		
13	-1	0	1	45.73	-1	0	1	22.78		
14	-1	1	0	38.10	-1	1	0	25.36		
15	1	0	-1	51.97	1	0	-1	14.97		

$$y_k = b_{ko} + \sum_{i=1}^{3} b_{ki} \chi_i + \sum_{i=1}^{3} b_{kii} \chi_i^2 + \sum_{i=1}^{3} b_{kij} \chi_i \chi_j \quad (1)$$

where:  $b_{k0_i}$ ,  $b_{ki'}$ ,  $b_{kij}$  – constant regression coefficients of the model;  $\chi_i$ ,  $\chi_i$  – independent variables

*Statistical analysis*. The data analyses were performed using the Statistical Analysis System (SAS 9.0; SAS Inst., Cary, USA). The analyses of variance were performed by ANOVA procedure. The mean values were considered significantly different when P < 0.05.

#### **RESULTS AND DISCUSSION**

## Single extraction factors of NEPP in blueberries

Alkaline hydrolysis treatment. NEPP in plants may be released by mean of acid or alkaline hydrolysis methods. At the beginning of this study, single factors of alkali hydrolysis treatment of NEPP were determined. The factors of liquid/solid ratio, NaOH concentration, and extraction time were investigated to determine the appropriate experimental ranges to be considered in the optimisation process. As shown in Figure 1, the extraction factors of NEPP in blueberries, such as NaOH concentration, extraction time, and liquid/solid ratio, were determined in this experiment. A significant effect was observed of NaOH concentration on the NEPP yield, and the NEPP yield was the highest at the treatment with 0.1 mol/l NaOH (Figure 1a). The NEPP yield increased with the prolonged extraction time (Figure 1b). As shown in Figure 1c, the effect of the liquid/solid ratio on the NEPP yield was significant. According to the central point, we selected the following conditions used in RSM: the NaOH concentration 1.0-4.0 mol/l, extraction time 4.0-5.0 h, liquid/solid ratio 10:1-20:1, respectively.

*Acid hydrolysis treatment*. In the acid hydrolysis treatment, the factors of the extraction solution, liquid/solid ratio, methanol/ $H_2SO_4$  ratio, extraction

temperature and time were detected to determine the appropriate experimental ranges. As shown in Figure 2a, the NEPP yield obtained in the methanol/ H<sub>2</sub>SO<sub>4</sub> treatment was significantly higher than that in the ethanol/H<sub>2</sub>SO<sub>4</sub> treatment. The methanol/H<sub>2</sub>SO<sub>4</sub> ratio (90:10, 95:5, 97:3) did not have any significant effect on the NEPP yield. Based on the related reports (Arranz et al. 2009), the methanol/H2SO4 ratio of 95:5 was fixed in the following experiments, and it was not considered in the optimisation process, either. In the following treatments, methanol/H2SO4 (95:5) was chosen as the extraction solvent. As shown in Figure 2b, the liquid/solid ratio (5:1, 10:1, 15:1, 20:1, 25:1) affects the NEPP yield. The NEPP yield increased with the increasing temperature in the range from 50°C to 90°C (Figure 2c). The effect of temperature on the polyphenols extraction level was widely researched. The results from the present study are in accordance with the literature (CACACE & MAZZA 2003; LIYANA-PATHIRANA & SHAHIDI 2005; PINELO et al. 2005). There was no significant effect of the extraction time (10, 15, 20, 25 h) on the NEPP yield (Figure 2d). Based on the above results, three factors, the liquid/solid ratio, extraction temperature and time were chosen in the following optimisation process. According to the central point, we selected the following conditions used in RSM: the fixed extraction solution, methanol/H<sub>2</sub>SO<sub>4</sub> (95:5, v/v); liquid/solid ratio 15:1-25:1; extraction temperature 70–90°C; time 15–25 h, respectively.

#### Modelling of NEPP extraction

Alkaline hydrolysis treatment. The RSM consists of an empirical modelling technique, which has been used to evaluate the relationship between the experimental and predicted results. To obtain the proper model for the optimisation of the extraction process, the Box-Behnken analysis, which is generally the best design for the response surface optimisation, was selected with three process variables (liquid/solid ratio,

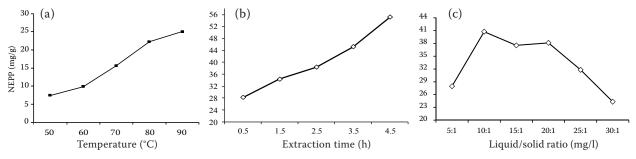


Figure 1. The independent variables experiment of NEPP obtained from alkali hydrolysis treatment: NaOH concentration (a), extraction time (b), and liquid/solid ratio (c)

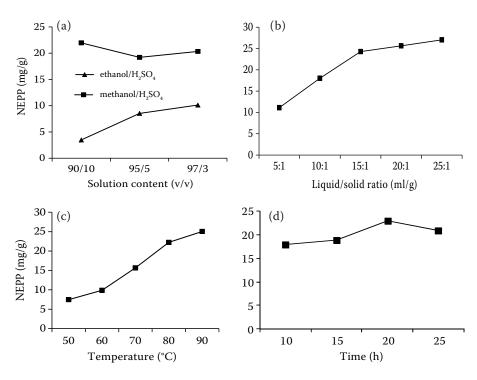


Figure 2. The independent variables experiment of NEPP obtained from acid hydrolysis treatment: solution content (a), liquid/solid ratio (b), temperature (c), time (d)

NaOH concentration, and extraction time) at three levels. The experiments were performed according to the experimental design in order to research the optimum conditions, and to study the effect of the process variables on the extraction of NEPP from blueberries. The predicted values were obtained by a model fitting technique using the Design Expert Software Version 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA) which proved to be sufficiently correlated with the observed values. Table 2 shows the process variables and the exxperimental data. The results of the analysis of variance, goodness-of-fit and the adequacy of the models are summarised in Table 3. The data showed a good fit with Eq. (2), being were statistically acceptable at P < 0.05 level and adequate with a satisfactory  $R^2$  value ( $R^2 = 0.9197$ ). The full model filled Eq. (2) was made in three-dimensional and contour plots to predict the relationships between the independent and dependent variables. Eq. (2) being developed to present the relationships between NEPP and alkaline extraction variables.

$$Y = 49.35 + 3.50X_1 + 1.58X_2 + 2.22X_3 + 2.19X_1X_2 - 0.70X_1X_3 - 0.63X_2X_3 - 4.45X_1^2 - 2.67X_2^2 + 3.26X_3^2$$
 (2)

Regression analysis was performed on the experimental data and the coefficients of the model were evaluated for significance. The effects of NaOH concentration and liquid/solid ratio on the extraction of NEPP were significant. However, the effect of the extraction time on the yield of NEPP was insignifi-

cant. The interaction values in three variables were not significant either (P > 0.05)

Acid hydrolysis treatment. Three process variables (liquid/solid ratio, extraction temperature and time) at three levels (Table 1) were selected in the response surface optimisation to determine a proper mode in the acid hydrolysis treatment of NEPP. Table 2 shows the process variables and experimental data, while Table 3 summarises the results of the analysis of variance, goodness-of-fit and the adequacy of the models. The data showed a good fit with Eq. (3), which were statistically acceptable at P < 0.05 level and adequate with a satisfactory  $R^2$  value ( $R^2 = 0.9935$ ). Eq. (3) was explained to present the relationship between NEPP and acid extraction variables.

$$Y = 24.97 - 2.45X_1 + 1.46X_2 + 1.34X_3 - 0.66X_1X_2 - 0.61X_1X_3 - 1.20X_2X_3 - 2.35X_1^2 - 1.61X_2^2 - 4.36X_3^2$$
 (3)

As shown in Table 3, except for  $X_1$  (time) ×  $X_3$  (liquid/solid ratio), the coefficients of others variables significantly affected the NEPP yield (P < 0.05).

According to the above results, the NEPP yield from the alkaline hydrolysis method was much higher than that from the acid hydrolysis method.

# Influence of process variables on the yield of NEPP

Response surface methodology (RSM) plays a key role in efficiently identification of the optimum values

Coefficient	DF ·	SS		MS		F-v	<i>F</i> -value		<i>P</i> -value	
		alkaline	acid	alkaline	acid	alkaline	acid	alkaline	acid	
$\overline{X_1}$	1	97.14	47.87	97.14	47.87	23.48	206.64	0.0019	< 0.0001	
$X_2$	1	19.75	16.99	19.75	16.99	4.77	73.35	0.0652	0.0004	
$X_3$	1	39.66	14.45	39.66	14.45	9.59	62.35	0.0174	0.0005	
$X_1X_2$	1	18.86	1.76	18.86	1.76	4.56	7.58	0.0701	0.0402	
$X_1X_3$	1	1.95	1.46	1.95	1.46	0.47	6.32	0.5148	0.0536	
$X_2X_3$	1	1.58	5.74	1.58	5.74	0.38	24.76	0.5559	0.0042	
$X_1^2$	1	82.41	20.33	82.41	20.33	19.92	87.76	0.0029	0.0002	
$X_2^2$	1	30.19	9.56	30.19	9.56	7.30	41.27	0.0306	0.0014	
$X_3^2$	1	44.67	70.08	44.67	70.08	10.79	302.50	0.0134	< 0.0001	
Model	9	331.82	178.15	36.87	19.79	8.91	85.44	0.0044	< 0.0001	
Residual error	5	28.97	1.16	4.14	0.23					
Lack of fit	3	20.29	0.20	6.76	0.068	3.12	3.12	0.124	0.154	
Pure error	2	8.68	0.59	2.17	0.29					

DF- degree of freedom; SS - sum of squares; MS - mean squares

of the independent variables. In the response surface plot and contour plot, the extraction yield of NEPP was obtained along with two continuous variables, while the other one variable was fixed constant at its zero level (the centre value of the test ranges). The maximum predicted value from the surface was confined in the smallest ellipse in the contour diagram. Elliptical contours are obtained when perfect interaction exists between the both independent variables. The independent variables and the maximum predicted values from the figures corresponded with the optimum values of the dependent variables (responses) obtained by the equations.

*Alkaline hydrolysis treatment*. NEPP from blueberries were extracted by the alkaline hydrolysis method and the extraction process was carried out at different extraction times, NaOH concentrations, and liquid/

solid ratios, to determine the optimum extraction conditions. 3D response surface and contour plots were used to present the effects of the process variables on the NEPP extraction. The response surface and contour plots showed the relative effects of two variables when the remaining variable was kept constant. The response surface plots estimating the specific surface area of NEPP extraction versus independent variables are presented in Table 3 and Figure 3.

As shown in Figure 3 and Table 3, the yield of NEPP substantially changed with NaOH concentration and liquid/solid ratio, while the extraction time had only a slight effect on the NEPP yield. As Fick's second law of diffusion predicts a final equilibrium between the solution concentration in the solid matrix and in the bulk solution after a certain time, an excessive time is not useful to extract more phenolics (SILVA et al. 2007).

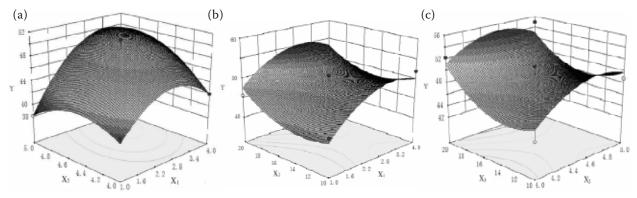


Figure 3. The surface plots of the yield of NEPP extracted with alkali hydrolysis method as affected by NaOH concentration  $(X_1)$ , time  $(X_2)$ , and liquid/solid ratio  $(X_3)$ : (a) NaOH concentration  $(X_1)$  and time  $(X_2)$ ; (b) NaOH concentration  $(X_1)$  and liquid/solid ratio  $(X_2)$ ; (c) time  $(X_3)$  and liquid/solid ratio  $(X_3)$ 

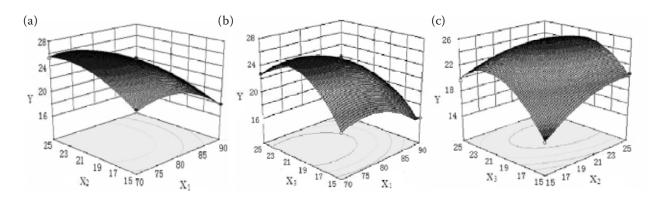


Figure 4. The surface plots of the yield of NEPP extracted with acid hydrolysis method as affected by temperature  $(X_1)$ , time  $(X_2)$ , and liquid/solid ratio  $(X_3)$ : (a) temperature  $(X_1)$  and time  $(X_2)$ ; (b) temperature  $(X_1)$  and liquid/solid ratio  $(X_3)$ ; (c) time  $(X_2)$  and liquid/solid ratio  $(X_3)$ 

The 3D plot in Figure 3a, the plot of NEPP yield against the extraction time and NaOH concentration showed significant changes with the time and NaOH concentration. It may be attributed to the degradation of NEPP upon a longer extraction time and a higher water/solid ratio. The obtained results indicated that there is a maximum in the NEPP extraction at a certain NaOH concentration and extraction time. At a constant NaOH concentration and liquid/solid ratio, the yield changed slightly with the changes in water/solid ratio, the highest yield could be obtained at a water/solid ratio of 20:1 (Figure 3b). Figure 3c showed that the interaction effect of the liquid/solid ratio and extraction time on the NEPP yield, which indicated the effect of liquid/solid ratio on NEPP yield, was higher than that of the extraction time.

According to the RSM, the obtained optimum conditions in alkaline hydrolysis were as follows: NaOH concentration 3.1 mol/l, liquid/solid ratio 20:1, extraction time 4.67 h, the predicted value of NEPP was 55.51 mg/g. In the real experiment, we used the extraction conditions: NaOH concentration 3.0 mol/l, liquid/solid ratio 20:1, extraction time 4.5 h, the observed value of NEPP was 54.33 mg/g. The experimental results were close to the predicted values. Thus, these results confirm the predictability of the model for the alkaline hydrolysis extraction of NEPP from blueberries in the experimental condition used.

Acid hydrolysis treatment. To determine the optimum extraction conditions for NEPP in the acid hydrolysis method, the extraction process was carried out at warying extraction time, temperature, and liquid/solid ratio. The response surface plots estimating the specific surface area of NEPP extraction by the acid hydrolysis versus independent variables are presented in Table 3 and Figure 4. The yield of NEPP changed

substantially with the extraction time, temperature, and liquid/solid ratio (P < 0.01). As shown in Figure 4a, it may be due to the degradation of NEPP upon higher extraction temperatures. A similar tendency is shown in Figure 4b, the results revealing that the NEPP yield may be decreased upon a higher temperature and a higher liquid/solid ratio. Figure 4c indicates that a higher liquid/solid ratio may reduce the NEPP yield, while a longer extraction time has only a slight effect on the NEPP yield.

According to the RSM, the obtained optimum conditions in acid hydrolysis were as follows: liquid/solid ratio 20.7:1, extraction temperature 73.9°C, extraction time 22.7 h, methanol/ $H_2SO_4$  (90:10, v/v), the predicted value of NEPP being 26.19 mg/g. In the real experiment, we chose closely optimised conditions of liquid/solid ratio 21:1, extraction temperature 75°C, extraction time 23 h, methanol/ $H_2SO_4$  (90:10, v/v), and the NEPP yield of 27.25 mg/g having been observed. The experimental result was very close to the predicted value. Thus, these results confirm the predictability of the model for the acid hydrolysis extraction of NEPP from blueberries in the experimental condition used.

#### **CONCLUSIONS**

In this study, the statistical methodology, Box-Behnken Response Surface design, has been demonstrated to be effective and reliable in finding the optimal conditions for NEPP extraction from blueberries.

- (1) In alkaline hydrolysis process, the NEPP yield was significantly affected by NaOH concentration and liquid/solid ratio, and the effect of the extraction time was insignificant.
- (2) In acid hydrolysis process, the NEPP yield obtained in the methanol/ $\rm H_2SO_4$  treatment was sig-

- nificantly higher than that obtained in the ethanol/  $\rm H_2SO_4$  treatment. The NEPP yield was significantly affected by the extraction temperature, time and liquid/solid ratio.
- (3) The NEPP yield obtained from alkaline hydrolysis method was much higher than that from acid hydrolysis method.
- (4) Under the optimised conditions of alkali and acid hydrolysis processes, the experimental NEPP yield agreed closely with the predicted value, which indicated the validity of the second order polynomial models developed for predicting NEPP content in blueberries.

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