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Optimisation of extraction conditions for phenolic compounds from limau purut (*Citrus hystrix*) peels

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Abstract: The objective of this study was to optimise the extraction conditions for phenolic compounds from limau purut (*Citrus hystrix*) peels using response surface methodology (RSM). A central composite rotatable design (CCRD) was applied to determine the effects of ethanol concentration (%), extraction temperature (°C), and extraction time (min) on total phenolic content (TPC) from limau purut (*Citrus hystrix*) peels. The independent variables were coded at five levels and their actual values were selected based on the results of single factor experiments. Results showed that ethanol concentration was the most significant ($p<0.001$) factor affecting the TPC. The optimum extraction conditions were found to be ethanol concentration of 52.9%, extraction temperature of 48.3°C, and extraction time of 126.5 min. Under the optimised conditions, the experimental value for TPC was 1291.8 mg GAE/100g DW, which reasonably close to the predicted value (1268.8 mg GAE/100g DW).

Keywords: Limau purut (*Citrus hystrix*) peels, Phenolic compounds, Antioxidants, Total phenolic content (TPC); Response surface methodology (RSM)

Introduction

Free radicals are highly reactive and unstable molecules that likely to react with food lipids, causing the lipid oxidation (Kormin, 2005). Lipid oxidation is one of the major concerns in food industry as they may contributed to the losses in fatty food quality by formation of products having negative effect on taste, flavour, colour, nutritional value, and storage stability (Juntachote *et al.*, 2006). In order to suppress the oxidative deterioration, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are usually added to the fresh or processed foods (Ekanayake *et al.*, 2004). However, there has been growing concern over the use of synthetic antioxidants due to their possible toxic and carcinogenic effects (Arabshashi-Delouee and Urooj, 2006). Thus, the search of natural and safe antioxidants, especially of plant origin, has increased considerably in these recent years.

Limau purut (*Citrus hystrix*) or wild lime is a tropical fruit that commonly cultivated in Malaysia and many other Southeast Asia countries (Cyber Plant Conservation Project, 2006). The juice and leaves of limau purut are popularly used as food condiments in the ASEAN region, however, the peels are often wasted. These waste materials contain principally biodegradable organic matter and their disposal may create serious environmental problems. Interestingly, the peels of limau purut have been reported to contain a variety of phenolic compounds, mainly flavanone, flavone and flavonol (Fadlinizal and Amin, 2006). These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants (Balasundram *et al.*, 2006). Phenolic compounds have attracted the attention of food and medical scientists due to their strong in vitro and in vivo antioxidant activities and their ability to scavenge the free radicals (Pinelo *et al.*, 2005; Li *et al.*, 2006; Silva *et al.*, 2007). However, up to now, limau purut peels are mostly used in some industries for their essential oil (FRIM, 2006). The research that focused on the recovery of limau purut peels as a source of phenolic antioxidants is still in its infancy.

The availability of phenolic compounds in limau purut peels as antioxidant source is ensured. However, the economical feasibility of an industrial process also requires working in such a way that high extraction efficiency is attained. Many factors have been established to influence the extraction efficacy, such as extraction methods, particle size,

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solvent type, solvent concentration, solvent-to-solid ratio, extraction temperature, extraction time and pH (Pinelo *et al.*, 2005; Banik and Pandey, 2007; Silva *et al.*, 2007).

In general, process optimisation could be achieved by either empirical or statistical methods (Liyana-Pathirana and Shahidi, 2005; Juntachote *et al.*, 2006). Empirical method adopted one-factor-at-a-time approach, in which one factor is varying at a time while all others are kept constant (Juntachote *et al.*, 2006; Banik and Padney, 2007). The main drawbacks of this method are including the inability to determine interaction between the variables, time-consuming, costly and less effective (Sin *et al.*, 2006).

Response surface methodology (RSM), originally described by Box and Wilson (1951), has been introduced to overcome the weakness and limitations of classical method (Liyana-Pathirana and Shahidi, 2005). Unlike the conventional empirical method, RSM can take into account the possible interrelationship among the test variables while minimizing the number of experiments (Silva *et al.*, 2007) Thus, it is a powerful tool that can provide a complete optimal condition to improve a process. In particular, Rodrigues and Pinto (2007) applied RSM in optimising the extraction of phenolic compounds from coconut shell. Likewise, Juntachote *et al.* (2006) optimised the production of phenolic extracts from various herbs and spices. But, optimisation of extraction of phenolic antioxidants from limau purut peels has not been reported yet. Thus, this study was aimed to investigate the effects of solvents (acetone, ethanol, and methanol), solvent concentration (20-100%), extraction time (60-420 min), and extraction temperature (25-60°C) on the extraction of phenolic compounds from limau purut peels using single factor experiment. Secondly, to optimise the extraction conditions for phenolic compounds from limau purut peels using RSM.

Materials and Methods

Plant material

Fresh limau purut (~3.5 kg) was purchased from a wet market in Mentakab, Pahang, Malaysia. The fruits of uniform shape and colour were selected whereas blemished and diseased fruits were eliminated. The chosen fruits had an average length of 4.0-5.6 cm, width of 3.8-5.8 cm and weight of 34-90 g.

Chemical reagents

All the solvents and chemicals used were of analytical grade. Deionized water used for the preparation of all the solutions was purified by Milli-Q purification system (Millipore) (Massachusetts, USA).

Sample preparation

Upon arrival at the laboratory, samples were thoroughly washed with tap water, manually peeled and the peels were cut into small pieces of about 1.0 $cm^{2.}$ The fresh peels were dried at 45°C for 24 h in a convection oven (Memmert, Germany). After drying, the dried peels were milled to fine powder (0.5 mm) with a miller (Model MF 10 basic; IKA®WERKE, Germany) at 4000 rpm. Ground powders were vacuum-packaged into a nylon-linear low-density polyethylene film (Flexoprint, Malaysia) by using Vacuum Packager (Model DZQ400/500) (Zhejiang, China). The well-packaged samples were stored at room temperature until use.

Solvent extraction

Approximately 2 g of dried sample was weighed and extracted with 20 mL of the extracting solvent in a conical flask. Conical flask was covered with parafilm (Pechiney plastic packaging) and aluminium foil to prevent light exposure. The mixture was shaken at constant rate using a water bath shaker (Memmert, Germany) for different times at required temperature. After the extraction, the limau purut peels extract was then filtered through a Whatman No. 1 filter paper, and the clear solution was collected in an amber reagent bottle. The filtrate was subsequently used for the determination of TPC. All the extractions were replicated once.

Experimental design

The experimental design for this study was divided into two major parts. Firstly, single factor experiments were performed to determine the appropriate range of conditions for limau purut peels phenolics extraction, namely, solvent type, solvent concentration, extraction time, and extraction temperature by varying one independent variable at a time while keeping the others constant. Secondly, the optimisation of phenolic compounds extraction was carried out using RSM and a second order polynomial model was developed.

Single factor experiments

(a) Selection of solvent type

By fixing extraction time (180 min) and extraction temperature (25°C), samples were extracted with 60% (v/v) acetone, 60% (v/v) ethanol, and 60% (v/v) methanol respectively. The extraction procedures were described in solvent extraction section. The best solvent type was selected according to the value of TPC (mg GAE/100g DW).

(b) Effect of solvent concentration on extraction of phenolic compounds

Using the best solvent type selected in single factor experiments section (a), samples were extracted with solvent ranging from 20% (v/v) to 100% (v/v) by fixing the extraction time and extraction temperature at 180 min and 25°C, respectively. The best solvent concentration was selected according to the value of TPC (mg GAE/100g DW).

(c) Effect of extraction time on extraction of phenolic compounds

Samples were extracted using the best solvent type and the best solvent concentration selected in single factor experiments sections (a) and (b), respectively. The extraction procedures were repeated as described in section of single factor experiments by varying the extraction time from 60 to 420 min while fixing the extraction temperature constant at 25°C. The best extraction time was selected according to the value of TPC (mg GAE/100g DW).

(d) Effect of extraction temperature on extraction of phenolic compounds

Using the best solvent type and the best solvent concentration selected in single factor experiments sections (a) and (b), samples were extracted at various extraction temperature ranged from 25 to 60°C at the optimum time determined in single factor experiments section (c). The extraction procedures were repeated as described in solvent extraction section. The best extraction temperature was selected according to the value of TPC (mg GAE/100g DW).

Based on the results of single factor experiment, the ranges of three factors (solvent concentration, extraction temperature and extraction time) were determined for RSM.

Experiment of RSM

A three-factor (X_1, X_2 and X_3) and five level (-α, -1, 0, 1, and + α) central composite rotatable design (CCRD) was applied to optimise the phenolics extraction from limau purut. The complete CCRD design comprised of twenty experiments with eight factorial points, six axial points and six center points (Table 1). Six replicate runs at the centre of the design were performed to allow a good estimation of pure error (Sin *et al.*, 2006). The independent variables studied were ethanol concentration (X_1, %), extraction temperature (X_2, °C) and extraction time (X_3, min) while the dependent variable (response variable) measured was TPC (Y, mg GAE/100g dry weight, DW). Each experiment was performed in replicate and the average values were taken as the response, Y.

Determination of total phenolic content (TPC)

TPC was determined using Folin-Ciocalteu reagent according to the method described by Lim *et al.* (2007) with slight modifications. Crude extracts obtained from extraction were diluted 15 times before use. Approximately 0.3 mL of diluted samples was added into aluminium foil-wrapped test tubes followed by 1.5 mL of Folin-Ciocalteu's reagent (10 folds dilution) and 1.2 mL of 7.5% (w/v) sodium carbonate. The blank sample was prepared by replacing 0.3 mL of sample with 0.3 mL of deionised water. The test tubes were covered with parafilm, vortexed for 10 s and allowed to stand in the dark environment at room temperature for 30 min. Absorbance was measured against the blank sample at 765 nm using UV light spectrophotometer (Model XTD 5; Secomam) (Ales Cedex, France). Each extract was analyzed in triplicate. A calibration curve of gallic acid was plotted by plotting absorbance vs concentrations of gallic acid (mg/L).

Statistical analysis

The experimental results in single factor experiments were analyzed using Minitab software (Minitab Version 15.1.1.0.). All data were expressed as means ± standard deviations of triplicate measurements. One-way analysis of variance (ANOVA) with Tukey's test was used to determine the significant differences (p<0.05) between the means.

The Design Expert (Version 6.0.10, Stat-Ease Inc., Minneapolis) statistical software was employed to design the CCRD and to analyze the experimental data in RSM. Experimental data were fitted to the following second order polynomial model and regression coefficients were obtained. The generalized second-order polynomial model proposed for the response surface analysis was given as follows:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_i X_j$$

Equation (1)

where β_0, β_i, β_{ii}, β_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively. Xi and Xj are coded value of the independent variables while k equals to the number of the tested factors (k=3). The ANOVA tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were analyzed statistically by computing the F-value at a probability (p) of 0.001, 0.01 or 0.05.

Table 1. Three factors and five levels CCRD together with the experimental and predicted values under different extraction conditions

Run	Independent variables			Dependent variable	
	X_1, Ethanol concentration (%)	X_2, Time (min)	X_3, Temperature (°C)	Total phenolic content (mg GAE/100g DW)	
				Experimental	Predicted
1	42.2	31.1	126.5	1207.7	1213.6
2	77.8	31.1	126.5	1169.2	1169.6
3	42.1	48.9	126.5	1252.4	1254.8
4	77.8	48.9	126.5	1189.2	1187.7
5	42.1	31.1	233.5	1186.3	1184.3
6	77.8	31.1	233.5	1180.9	1175.1
7	42.2	48.9	233.5	1215.0	1211.2
8	77.8	48.9	233.5	1188.2	1178.9
9	60.0	40.0	180.0	1241.2	1240.6
10	60.0	40.0	180.0	1232.5	1240.6
11	60.0	40.0	180.0	1224.2	1240.6
12	60.0	40.0	180.0	1250.5	1240.6
13	30.0	40.0	180.0	1152.7	1149.5
14	90.0	40.0	180.0	1077.3	1085.4
15	60.0	25.0	180.0	1192.1	1191.4
16	60.0	55.0	180.0	1223.7	1229.3
17	60.0	40.0	90.0	1282.6	1276.7
18	60.0	40.0	270.0	1234.0	1244.7
19	60.0	40.0	180.0	1248.5	1237.3
20	60.0	40.0	180.0	1240.8	1237.3

Verification of model

Optimal conditions for the extraction of phenolic compounds from limau purut peels were obtained using the second-order polynomial model of RSM. The suitability of the model equation for predicting the response values was verified by conducting the extractions under the recommended optimal conditions. In this study, a numerical optimisation method was adopted to find a point that maximizes the response. A series of solutions was generated and the solution to be employed for the verification would be selected based on its desirability and suitability. The experimental and predicted values of TPC were compared in order to determine the validity of the model. To confirm the results, runs were carried out in replicate under the selected optimised conditions.

Results and Discussion

A calibration curve of gallic acid was constructed to measure the amount of phenolic compounds in the limau purut peels. The calibration equation for gallic acid was $y = 10.278x + 0.0085$ ($R^2 = 0.997$). All the results in this study were computed from the above calibration curve and expressed as gallic acid equivalent (GAE) in mg per 100 g dry weight (DW).

Single factor experiments

Effect of solvent type on extraction of phenolic compounds

The selection of extraction solvents is critical for the complex food samples as it will determine the amount and type of phenolic compounds being extracted. Aqueous alcohols particularly acetone, ethanol and methanol are most commonly employed in phenolics extraction from botanical materials (Naczk and Shahidi, 2004; Hayouni *et al.*, 2007). Figure 1(a) showed that aqueous acetone was slightly better than aqueous methanol and aqueous ethanol in extracting phenolics from limau purut peels under the same extraction conditions (60%, 25°C, and 180 min). However, the differences in TPC among all

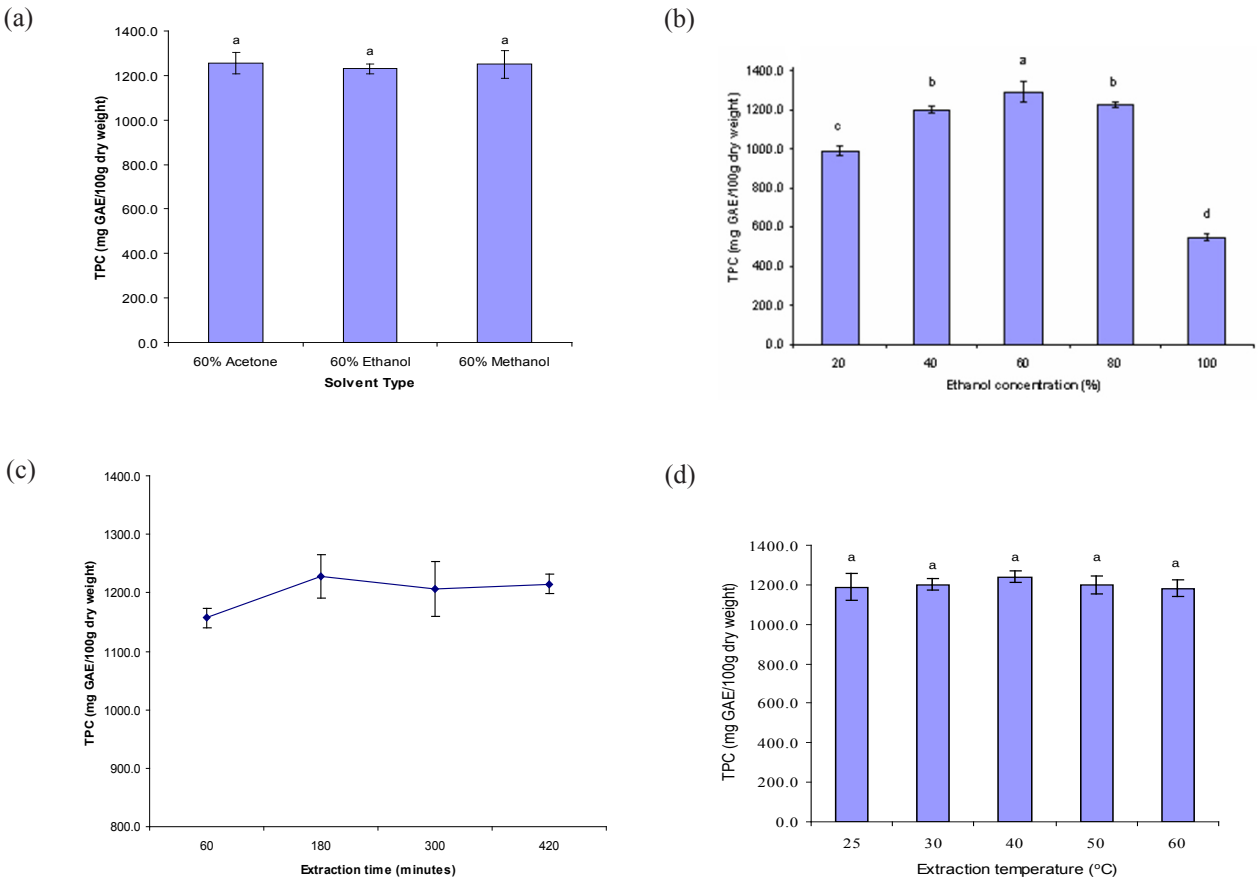

Figure 1. Effect of (a) solvent type; (b) ethanol concentration; (c) extraction time; and (d) extraction temperature on total phenolic content from limau purut peels. Values marked by different letters are significantly different (p<0.05).

the solvent extracts were not significant (p>0.05), indicating that phenolic compounds from limau purut peels might present a wide coverage of polarity. So ethanol which is categorized under GRAS (Generally Recognized as Safe) would be preferable in view of the application in food system. Ethanol was chosen as the extraction solvent for the next experiments.

Effect of ethanol concentration on extraction of phenolic compounds

The effects of ethanol concentration on extraction of phenolic compounds from limau purut peels were shown in Figure 1(b). TPC increased with the increment of the ethanol concentration up to 60% (1290.8 mg GAE/100g dry weight, DW) followed by a reduction until reaching a minimum of 547.8 mg GAE/100g DW at 100%. Similarly, Cacace and Mazza (2003) revealed that maximum total phenolics in black currants extracts was obtained at about 60% ethanol followed by a decrease with further increase in concentration. Nepote *et al.* (2005) also found that increased the ethanol concentration beyond 70% will dramatically reduced the amount of phenolics extracted from peanut skins. A remarkable drop in TPC at 100% ethanol revealed that absolute solvent do not ensure a good recovery of phenolic compounds as compared to aqueous ethanol. Thus, moderate ethanol concentration of 30, 60% and 90% were selected as the lower, middle and upper levels, respectively, to be employed in RSM optimisation.

Effect of extraction time on extraction of phenolic compounds

Extraction time was another main parameter in the extraction procedure. The extraction time can either be as short as few minutes or very long up to 24 hours (Laponik *et al.*, 2005; Lee *et al.*, 2005). In this study, the range of extraction time was designed based on the practical and economical aspects. Figure 1(c) showed that an increase in extraction time increased from 60 to 180 min was accompanied by a small increment in TPC from 1156.8 to 1227.2 mg GAE/100g DW. After 180 min, further increase in process duration did not significantly (p>0.05) improve the recovery

Table 2. Analysis of variance (ANOVA) of the second-order polynomial model for total phenolic content of *citrus hystrix* peels

Regression	Degree of freedom	Sum of squares	Mean square	***F* Value**	**Prob > *F***
Model	9	37673.89	4185.99	37.43	< 0.0001
Linear	3	7943.44	2647.81	1.29	0.3134
Quadratic	3	28755.56	9585.19	85.70	< 0.0001
Cross-product	3	974.89	324.96	0.13	0.9398
Lack of fit	5	592.73	118.55	1.15	0.4605
Pure error	4	413.92	103.48		
Residual error	9	1006.64	111.85		

Coefficient of determination, R2 = 0.9740
Adjusted R^2 = 0.9480.
Coefficient of variation, CV = 0.87.

of phenolics. This observation was well explained by Fick's second law of diffusion, which stated that final equilibrium will be achieved between the solute concentrations in the solid matrix (plant matrix) and in the bulk solution (solvent) after a certain time, hence, an excessive extraction time was not useful to extract more phenolic antioxidants (Silva *et al.*, 2007). Furthermore, prolonged extraction process might lead to phenolics oxidation due to light or oxygen exposure. Taking into account of these facts, an extraction time of 90–270 min was selected for RSM optimisation.

Therefore, moderate extraction temperature of 25, 40 and 55°C were chosen as the lower, middle and upper levels, respectively, to be applied in RSM optimisation.

Effect of extraction temperature on extraction of phenolic compounds

The selection of an appropriate extraction temperature was the final step in a series of single factor experiments. The extraction of phenolic compounds was increased slightly when extraction temperature increased from 25 to 40 °C as reflected in Figure 1(d). This result was in accordance with the study of Juntachote et al. (2006), which reported that TPC for holy basil and rosemary increased at elevated temperature due to enhanced phenolics solubility, faster diffusion rate, and increased mass transfer. However, it should be noted that increasing the temperature beyond certain values may promoting possible concurrent decomposition of phenolic compounds which were already mobilized at lower temperature or even the break down of phenolics that are still remained in the plant matrix. Additionally, high temperature may encourage solvent loss through vaporization and increase the cost for extraction process from the industrialization point of view. Therefore, moderate extraction temperature of 25, 40 and 55°C were chosen as the lower, middle and upper levels, respectively, to be applied in RSM optimisation.

Reproducibility

The reproducibility of the extraction method and TPC assay was estimated using percentage relative standard deviation (% RSD). The overall % RSD was small and fall between 1.1-5.5% (Data not shown) indicated the high reproducibility of both the extraction method and TPC assay.

Response surface methodology (RSM) experiments

Fitting the model

Based on the observations from single factor experiments, the ranges of each independent variable (ethanol concentration, extraction temperature, and extraction time) that influence TPC were selected. In this study, the lower and upper values for the factors were set at +alpha (+α=1.682) and -alpha (-α=1.682) and thus all the factor levels was chosen within the limits that were desirable and practical. In RSM, natural variables are transformed into coded variables that have been defined as dimensionless with a mean zero and the same standard deviation (Liyana-Pathirana and Shahidi, 2005). The experimental and predicted values for response (TPC) under different combination of extraction conditions were given in Table 1. The results showed that TPC of limau purut peels ranged from 1077.3 to 1282.6 mg GAE/100g DW. By applying multiple regression analysis,

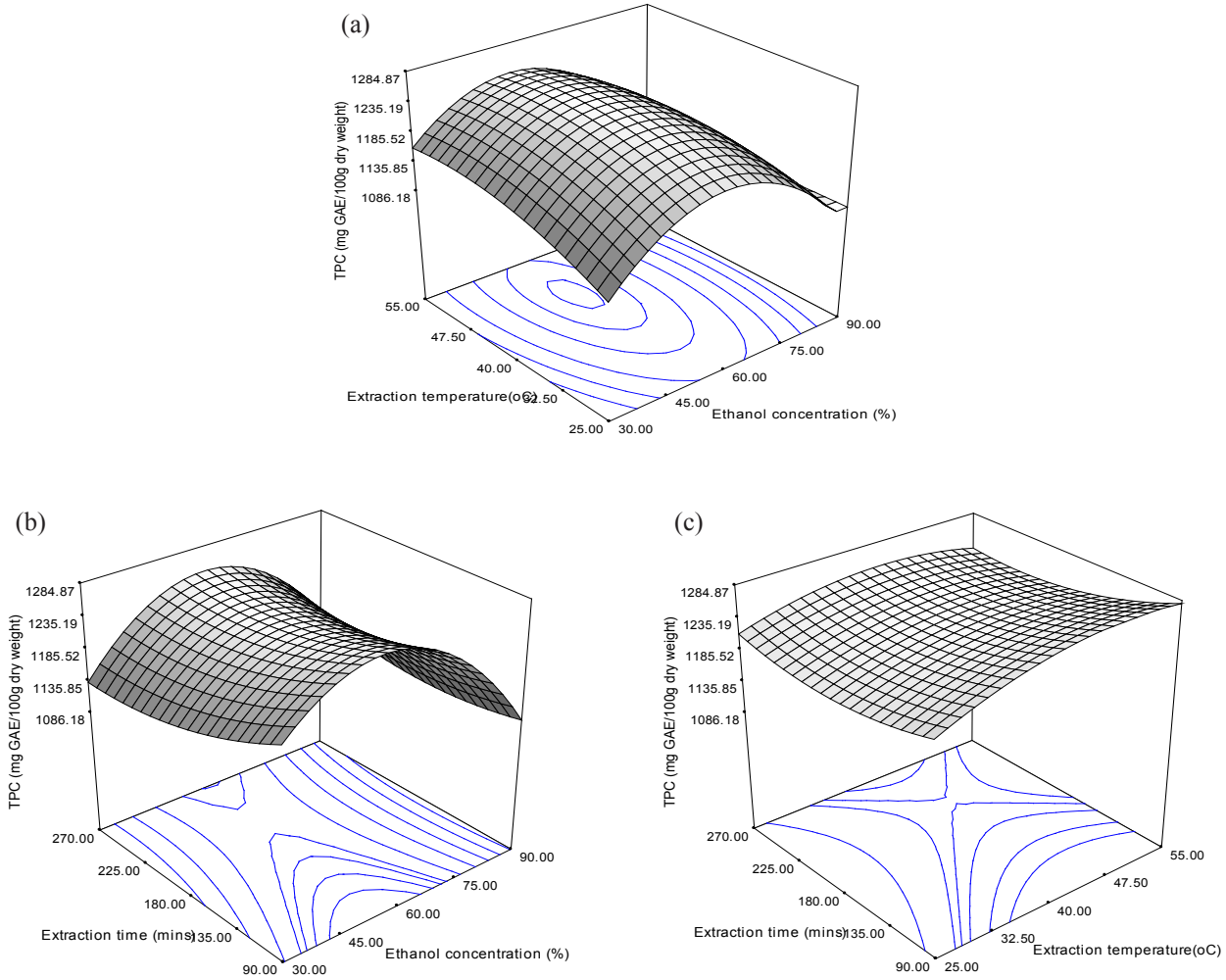

Figure 2. Response surface plot corresponding to total phenolic content (TPC) of limau purut peels as a function of (a) ethanol concentration and extraction temperature; (b) ethanol concentration and extraction time; and (c) extraction temperature and extraction time. The value of the missing independent variable in each plot was kept at the middle level.

relationship between the tested independent variables and the response was explained in Equation (2):

$$Y = 1238.95 - 32.09X_1 + 11.27X_2 - 9.52X_3 - 119.89X_1^2 - 9.54X_2^2 + 8.26X_3^2 - 9.72X_1X_2 + 14.62X_1X_3 - 3.59X_2X_3$$

Equation (2)

To fit the response function and experimental data, the linearity and quadratic effect of the independent variables, their interactions and regression coefficients on the response variables were evaluated by analysis of variance (ANOVA) (Table 2). The ANOVA of the regression model showed that the model was highly significant due to a very low probability value (p<0.0001). The fitness and adequacy of the model was judged by the coefficient of determination (R^2) and the significance of lack-of-fit. R^2 which was defined as the ratio of the explained variation to the total variation, was a measure of the degree of fit (Wang *et al.*, 2008). The closer the R^2 value to unity, the better the empirical model fits the actual data (Fan *et al.*, 2007). By referring to Table 2, R^2 value for the regression model of TPC was 0.9740, which was closed to 1. This suggested that the predicted second order polynomial models defined well the real behaviour of the system. In addition, the value of adjusted R^2 (0.9480) was also very high to advocate for a high significance of the model. The adjusted R^2 was a corrected value for R^2 after the elimination of the unnecessary model terms. If there were many non-significant terms have been included in the model, the adjusted R^2 would be remarkably smaller than the R^2 (Myers and Montgometry, 2002). In this study, the adjusted R^2 was very close to the R^2 value. Besides, the absence of any lack of fit (p>0.05) also strengthened the reliability of the models. A small coefficient of variation (0.87) revealed that the experimental results

Table 3. Estimated regression coefficients of the second-order polynomial model for total phenolic content of *citrus hystrix* peels

Regression coefficients	Total phenolic content (mg GAE/100g DW)
Intercept	
X_0	1238.95
Linear	
X_1, Ethanol concentration	-32.09***
X_2, Extraction temperature	11.27**
X_3, Extraction time	-9.52**
Quadratic	
X_1^2	-119.89***
X_2^2	-9.54**
X_3^2	8.26*
Interaction	
*X*12	-9.72
*X*13	14.62*
*X*23	-3.59

were precise and reliable.

The multiple regression results and the significance of regression coefficients for the TPC model were tabulated in Table 3. The *P*-values were used as a tool for checking the significance of each coefficient, which in turn might indicated the interaction patterns between the variables (Hou and Chen, 2008). The smaller the *P*-value, the more significant was the corresponding coefficient. It could be observed from Table 3 that both the linear and quadratic term of all parameters (ethanol concentration, X_1; extraction temperature, X_2; and extraction time, X_3) had significant (at least at $p<0.05$) effect on TPC. In addition, TPC was also significantly influenced by the interactions between ethanol concentration and extraction time, X_{13} ($p<0.05$). Among all the three extraction parameters studied, ethanol concentration had the most critical role in the extraction of phenolic compounds from limau purut peels followed by extraction temperature and extraction time.

Analysis of response surface plot

Figure 2 illustrated three-dimensional response surfaces plots by presenting the response in function of two factors and keeping the other constant at its middle level. Each figure revealed the effects of the selected parameters on TPC.

The predicted response surface showing the effect of ethanol concentration and extraction temperature on TPC at constant time (180 min) appeared as a saddled shape (Figure 2a). Figure 2(a) depicted a higher amount of phenolic content yielded in the region at ethanol concentration between 50 and 60% and extraction temperature between 43 and 50°C. Both ethanol concentration and extraction temperature showed significant negative quadratic effects on TPC at $p<0.001$ and $p<0.01$, respectively (Table 3). Therefore, TPC gradually mounted up with the increase of ethanol concentration and extraction temperature, and achieved optimum value at about 55% and 45°C, before it began to decrease. However, the contour gradient in extraction temperature coordinate direction was less than that in ethanol concentration coordinate direction, namely ethanol concentration is more important than extraction temperature as reflected by its higher negative quadratic coefficient (β_{11} = -119.89) compared to latter (β_{22} = -9.54). In general, the polarity of ethanol-water mixture would increase continuously with the addition of water to ethanol. More polar phenolic compounds such as aglycones and glycosides of flavonoids, didymin, eriocitrin, neohesperidin, and neoeriocitrin that found mainly in limau purut peels may be extracted according to "like dissolves like" principle. Thus, it could be seen that phenolics extracted using 60% ethanol was higher than that of 90% ethanol (Figure 2a).

Figure 2(b) denoted the effects of ethanol concentration and extraction time on total phenolic content (TPC) at fixed extraction temperature of 40°C. Ethanol concentration demonstrated a pronounced influence on TPC in linear and quadratic manner

Table 4. Optimum conditions, predicted and experimental value of response under those condition

Optimum conditions			TPC (mg GAE/100gDW)	
Ethanol concentration (%, v/v)	Extraction temperature (°C)	Extraction time (min)	Experimental[a]	Predicted
52.9	48.3	126.5	1291.8 ± 67.0	1268.8

[a] Mean ± standard deviation (n = 2).

(p<0.001) (Table 3). Its linear and quadratic effects on TPC were both negative, which explained the nature of the curve as shown in Figure 2(b). At lower and upper levels of time, TPC went up corresponsive with the increase of ethanol concentration up to 55% and further increase in ethanol concentration leads to deceleration of phenolics extraction. Furthermore, extraction of phenolic compounds was observed to be positively influenced by the synergism between ethanol concentration and extraction time (p<0.05). This implicated that the extraction was largely favoured in two cases: short extraction time in the presence of low ethanol concentration or long extraction time in the presence of high ethanol concentration. From the industrialization point of view, low ethanol concentration with shorter extraction time would be more adequate as long extraction period rendered the extraction procedure time consuming and uneconomical.

The relationship of extraction temperature and extraction time with TPC was shown in Figure 2(c). Both of the factors displayed significant linear and quadratic effect (at least at p<0.05) on TPC (Table 3). With regard to extraction temperature, TPC of limau purut peels extracts increased readily with increasing temperature up to 47.5°C and followed by a slight decrease afterwards. This suggested that incubation in warm water did improve phenolics extraction, yet was gentle enough to avoid heat degradation of the target phenolic antioxidants. Mild heating might soften the plant tissue, weaken the cell wall integrity, hydrolyze the bonds of bound phenolic compounds (phenol-protein or phenol-polysaccharide) as well as enhance phenolics solubility, thus more phenolics would distribute to the solvent (Juntachote *et al.*, 2006; Li *et al.*, 2006; Spigno *et al.*, 2007). At optimum extraction temperature (about 47.5°C), higher amounts of phenolic contents were obtained with short extraction time. In other words, long extraction time may compensate the beneficial effects of moderate temperature by inducing oxidation or degradation of phenolic compounds, yielding low TPC.

By combining all the results presented in Figure 2, the following conclusions can be drawn. It was clear that ethanol concentration had the most critical role in the the extraction of phenolic compounds from limau purut peels followed by extraction temperature and extraction time. Solubility of phenolic compounds could be enchanced using an aqueous ethanol over a limited compositional range. In general, it was found that ethanol that ranged from 40-80% had greater efficiency in the extraction of polyphenol compounds compared to pure ethanol (Jayaprakasha *et al.*, 2007). This seems to be agreed with 50-60% ethanol reported in the present study. On the other hand, time and temperature of extraction were important variable to be optimised in order to minimize the energy cost of the process. The results revealed that extraction carried out at moderate temperature (40-50°C) for shorter time (90-120 min) was enough to saturate the solutions with phenolic compounds. In the meantime, this condition was able to minimize the possible impact on plant phenolics which might heat and light sensible.

Verification of predictive model

Table 4 showed that the experimental results were very close to the predicted one. This implied that there was a high fit degree between the values observed in experiment and the value predicted from the regression model. Hence, the response surface modeling could be applied effectively to predict extraction of phenolic compounds from limau purut peels.

Conclusion

The present study confirmed the advantages of RSM over classical method in optimising the extraction conditions for phenolic antioxidants from limau purut peels. The results from RSM showed that TPC of limau purut peels were most affected by ethanol concentration followed by extraction temperature and extraction time. Using the numerical optimisation

method, the optimum conditions for maximum TPC were as follows: ethanol concentration, 52.9%; extraction temperature, 48.3°C; and extraction time, 126.5 min. Under the mentioned conditions, 1291.8 mg GAE/100g DW of phenolics were extracted from the limau purut peels, which well agreed with the predicted value (1268.8 mg GAE/100g DW). The second-order polynomial models developed were satisfactory in describing and predicting the phenolics extraction from limau purut peels. With the application of RSM, the interaction effects among the extraction factors can be accessed as well the solvent usage and extraction time can be reduced as compared to single factor experiment. Further works may carry out under the optimum conditions to elucidate the identity of phenolic compounds responsible for the antioxidant properties of limau purut peels.

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