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Optimization of multifaceted factorials for maximum extraction of polyphenols, phenolic acids and flavonoids from lemon peel (*Citrus limon*) using response surface methodology

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ABSTRACT

One of the most important Citrus species is *Citrus limon* (Rutaceae), also known as lemon. Phenolic acids, flavonoids, amino acids, carbs, and vitamins could be found throughout the entire lemon fruit, including the peel, seed, and pulp. However, lemon peel, a by-product of the lemon, is never fully utilised and is constantly discarded. The lemon peel was employed in this work to optimise the quantity of polyphenols, phenolic acids, and flavonoids utilising response surface methodology (RSM). A central composite design (CCD) was used in this study, and factors including ethanol concentration (70–90%), extraction temperature (45–65 °C), and extraction duration (80–120 minutes) were examined. Based on the results obtained, ethanol concentrations and extraction temperatures were significantly affected the total polyphenols, phenolic acids and flavonoids content. However, the experimental data was adequately fitted with the second-order polynomial models. Based on the multi-responses optimized extraction conditions were as follows: 85.77%, 65 °C and 120 minutes for ethanol concentration, extraction temperature and extraction time, respectively. This optimized condition could be useful for phenolics extraction from lemon peel.

Keywords: *Citrus limon*, response surface methodology, polyphenol, phenolic acid, flavonoid

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1 Introduction

Rutaceae is one of the diverse plant family which consist of several important genus including *Citrus*. The genus *Citrus* consists of several important species which produce *Citrus* fruits that are known for its nutritional value and medicinal properties. *Citrus limon* or commonly known as lemon, third rank of the most important *Citrus* species after oranges and mandarin. *C. limon* is a woody plant with evergreen leaves and can grows up to three meters tall. It produces yellow edible fruits and classified as a main raw materials of *C. limon* (Mabberley, 2004). The lemon fruit is an oval, elongated and the color is green at immature stage and turn yellow during ripening. The pericarp is a thin and wax-covered layer which act as a skin of the fruit. Underneath the pericarp, there is a mesocarp layer which can be divided into flavedo and albedo. The flavedo is the outer part of mesocarp contain carotenoids dyes and oil vesicles, meanwhile, albedo which is the inner part of mesocarp is made of spongy white parenchyma tissue. The most inner part of the fruit which is endocarp or fruit flesh is filled with juicy pulp and divided into segments by the white spongy tissue of mesocarp (Mabberley, 2004; Klimek-Szczykutowicz et al., 2020). The

whole fruit of lemon including peel, seed and pulp contains flavonoids, vitamins, limonoids, carboxylic acids, courmarins, amino acids, carbohydrates and phenolic acids (Ledesma-Escobar et al., 2015; Czech et al., 2019). In the studies conducted on the isolation of bioactive compounds from the essential oils of lemon fruits pericarp, the results showed that the main chemical compounds are limonene, ρ-mentha-3,8-diene, β-pinene, γ-terpinene and myrcene with a percentage of 69.9, 18.0, 11.2, 8.21 and 4.4%, respectively (Russo et al., 2015; Kaskoos, 2019). Based on the pharmacological studies conducted, the bioactive compounds of lemon fruit were found to possess several pharmacological properties including antioxidant, anti-microbials, anti-cancer, anti-inflammatory, anti-diabetic and organs protective potential (Kim et al., 2015; Otang and Afolayan, 2016).

Recently, nutraceutical and pharmaceutical industries have set a new trend in extracting the bioactive compounds from the agro-industrial by-products. The by-product which commonly be discarded is contained a huge amount and wide ranges of bioactive compounds which can be studied and used for the production of drugs and supplements (Pinto et al., 2021). The first step in isolating bioactive compound from plant sample is extraction stage. Extraction stage is the most important step in obtaining maximum amounts of bioactive compounds from the plant sample (Kamarudin et al., 2020). However, conventional extraction procedure such as one-factor-at-time method is labor intensive process due to various extraction parameters need to be optimized and this method is unable to analyze the interaction effect between the parameters (Ibrahim and Elkhidir, 2011). According to Aybastıer et al. (2013), various factors can directly affect the efficiency of extraction procedure including extraction time, temperature, types of solvents and solid-to-liquid ratios. Thus, in order to optimize multiple extraction parameters efficiently, a suitable methodology such as response surface methodology could be introduced for conducting and analyzing multiple factorials in one experimental design (Azahar et al., 2017).

To date, the extraction of phenolic compounds such as polyphenols, phenolic acids and flavonoids from lemon peel by implementing different ethanol concentration, extraction temperature and extraction time has not been conducted. Hence, this study was conducted to maximize the phenolic compounds extraction from lemon peel by using response surface methodology.

2 Materials and Methods

2.1 Plant materials

The ripe lemon fruits which were originated from South Africa were acquired from a local fruit store in Selangor, Malaysia. The fruits were cleaned with tap water and the peel were removed from the flesh manually prior to the drying process. The lemon peel was wrapped in paper and dried in a 50°C oven until it was completely dry. A commercial blender was used to crush the dried lemon peel, and the powder was kept in an airtight container.

2.2 Extraction procedure

The dried powder of lemon peel at the weight of 0.5 g was placed in the vials. Into each vial, a total of 25 mL of different concentrations of ethanol (70-90%) was added. The mixtures were placed in the water bath at different temperatures (45-65 °C) and incubated at the time ranges between 80 to 120 minutes as indicated in the design matrix in Table 1. The design matrix used in this study was generated using Design Expert software version 11.0 (Stat-Ease Inc., Minneapolis, USA). After the incubation, the mixtures were filtered using filter paper No. 1 and the extracts obtained were used for further experiment.

2.3 Experimental design, modelling and chemical extraction optimization

This study was conducted using Central Composite Design (CCD) by analyzing three factorials including ethanol concentration (X_1, %), extraction temperature (X_2, °C) and extraction time (X_3, minutes). A total of 20 experimental runs were generated (Table 2) with each variable were coded with $-\alpha$, -1, 0, 1 and α (Table 1) and performed in a randomized order. The responses recorded in this experiment were total polyphenols content (Y_1, mg GAE/g of dry weight (DW)), total phenolic acids content (Y_2, mg GAE/g DW) and total flavonoids content (Y_3, mg RE/g DW). The second-order polynomial was used to fit the optimal values of responses (Y) and regression coefficient (β) was generated. A second-order polynomial equation was postulated for each response (Y) following the equation (1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_1 x_1 + \sum_{i=1}^{k=1} \sum_{j=2}^{k} \beta_{ij} x_i x_j + \sum_{i=2}^{k} \beta_{ii} {x_i}^2 \quad (1)$$

Where various x_i values are independent variables parameters which affecting the dependent responses Y. Meanwhile, β_0, β_i, β_{ii}, β_{ij} and k are regression coefficient for intercept, linear, quadratic, interaction terms and number of parameters, respectively.

2.4 Phytochemical analysis

The total polyphenols and phenolic acids content were conducted using Folin-Ciocalteu method and total flavonoids content was analyzed using Aluminum

Table 1. Independent variables levels for central composite design

Independent variable	Levels				
	$-\alpha$	-1	0	1	α
Ethanol concentration (X_1) (%)	63.18	70	80	90	96.82
Extraction temperature (X_2) (°C)	38.18	45	55	65	71.82
Extraction time (X_3) (minutes)	66.36	80	100	120	133.64

Chloride Colorimetric method. All the procedures were followed according to the methods explained by Haida et al. (2022).

2.5 Model validation

The extraction conditions of lemon peel were optimized to extract maximum phenolics content by using response surface methodology. The generated optimum extraction conditions for all responses were used for model validation. The obtained experimental values were compared with the predicted values and the variation between the experimental and predicted values were based on paired t-test.

2.6 Statistical analysis

The experiment was conducted by repeating each experimental run for three times and the data was expressed as mean. All the data were statistically analyzed using analysis of variance (ANOVA). The significant level for parameters, interaction between variables, regression equation and 3D surface graph were generated by Design Expert software version 11.0.

3 Results

3.1 Model fitting

This study was aimed to optimize the three extraction variables with five coded levels for each variable in one design namely as central composite design. The observed values of 20 experimental runs were tabulated in Table 2 and the results of analysis of variance (ANOVA) were presented in Table 3. In the ANOVA analysis, the level of significance (p-value) was expressed as <0.001, <0.05 and >0.05 which indicated the model was highly significant, significant and not significant, respectively. This indicated that the actual relationship between model responses and process parameter were adequate for all model terms. In addition, a strong correlation between predicted and experimental values were obtained for all model responses as the coefficient of determination (R^2) and adjusted coefficient of determination (R^2 Adjusted) obtained were more than 0.8.

3.2 Influence of process parameters on total polyphenols content

Based on the results obtained in Table 2, the lemon peel extract was recorded the total polyphenols content between 7.01 to 14.72 mg GAE/g DW. The highest polyphenols content recorded was 14.72 mg GAE/g DW from the treatment of 80% ethanol concentration at a extraction temperature of 71.82°C for 100 minutes. In addition, ANOVA analysis in Table 3 showed that the lack of fit value displayed a non-significant value with p>0.05 which indicated that the proposed model is well-fitted. Besides that, the results also showed high coefficient of determination for both R^2 and R^2 Adjusted with the values are 0.95 and 0.90, respectively (Fig. 1a). The equation for polyphenols content was generated by fitting the second-order polynomial equation, as follows:

$$\begin{aligned} TPP = & -38.08 + 1.43x_1 - 0.11x_2 - 0.03x_3 - \\ & 0.009{x_1}^2 + 0.003{x_2}^2 + 0.0004{x_3}^2 + \\ & 0.003x_1x_2 + 0.001x_1x_3 - 0.0005x_2x_3 \end{aligned}$$

Based on the ANOVA in Table 3, the results showed that ethanol concentration and extraction temperature exhibited a highly significant linear effect on total polyphenols content. In contrast, extraction time exhibited a non-significant linear effect on total polyphenols content. In the quadratic model, it showed that ethanol concentration significantly yielded a negative effect and extraction temperature significantly yielded a positive effect towards total polyphenols content (p<0.001 for ethanol concentration and p<0.05 for extraction temperature; Table 3). In term of interaction effect, there was no significant interaction between ethanol concentration, extraction temperature and extraction time.

3.3 Influence of process parameters on total phenolic acids content

The total phenolic acids of lemon peel extracts produced were ranged between 24.79 to 46.44 mg GAE/g DW. The lowest was produced by using 96.82% (v/v) ethanol at a temperature of 55 °C for 100 minutes (Table 2). Meanwhile, the highest was produced by using 80% (v/v) ethanol at a temperature of 71.82 °C for 100 minutes. Based on the ANOVA analysis,

Table 2. Central composite design (CCD) with responses of the dependent variables to extraction conditions

Run	Independent variables			Dependent variables		
	X_1	X_2	X_3	Y_1	Y_2	Y_3
1	90	65	80	9.6	35.4	87.02
2	70	65	120	12.48	40.87	59.52
3	80	55	100	11.5	37.6	72.29
4	80	55	133.64	11.33	37.7	69.85
5	70	45	120	10.58	39.68	53.02
6	70	65	80	12.9	42.8	56.52
7	80	55	100	11.51	37.64	73.68
8	90	45	80	9.36	29.42	73.57
9	63.18	55	100	11.31	38.24	49.29
10	80	55	100	11.44	37.7	74.52
11	80	71.82	100	14.72	46.44	91.63
12	90	65	120	9.83	36.6	87.63
13	80	55	66.36	11.24	40.74	78.85
14	96.82	55	100	7.01	24.79	79.91
15	80	38.18	100	10.82	39	65.18
16	70	45	80	11.63	39.36	61.52
17	80	55	100	12.24	39.51	81.85
18	80	55	100	11.48	37.81	73.96
19	80	55	100	11.43	37.78	72.91
20	90	45	120	9.35	33.07	75.91

Y_1: total polyphenols content; Y_2: total phenolic acids content; Y_3: total flavonoids content

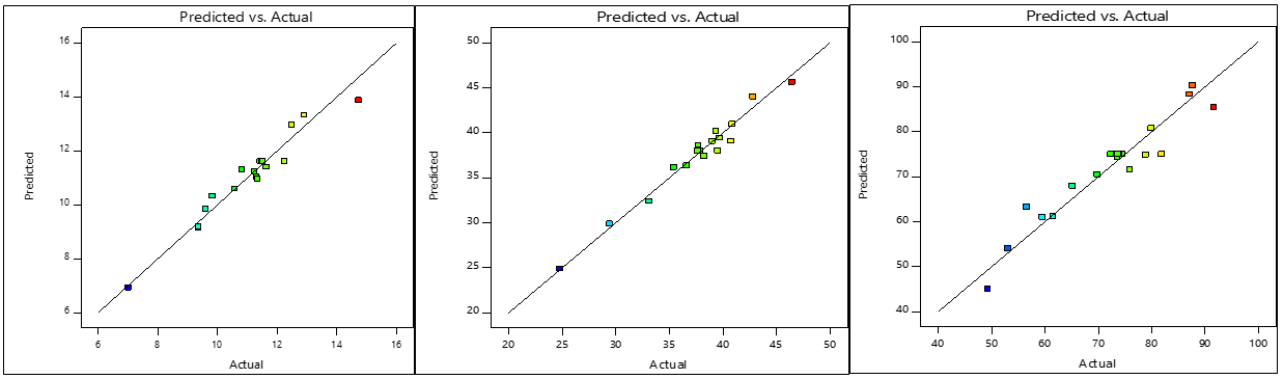

Figure 1. Predicted vs actual values of (a) total polyphenols content, (b) total phenolic acids content, and (d) total flavonoids content

Table 3. Regression coefficient (β), coefficient of determination (R^2 and Adj. R^2) and F-test value of the predicted second order polynomial models for the total polyphenols, phenolic acids and flavonoids content

Source of variation	Y_1			Y_2			Y_3		
	SS	F-value	p-value	SS	F-value	p-value	SS	F-value	p-value
Model	45.18	20.13	< 0.0001	385.45	38.26	< 0.0001	2305.46	11.63	0.0003
Linear									
X_1	20.38	81.71	< 0.0001	189.26	169.08	< 0.0001	1540.51	69.96	< 0.0001
X_2	7.99	32.06	0.0002	52.01	46.47	< 0.0001	370.72	16.83	0.0021
X_3	0.0884	0.3544	0.5649	0.2568	0.2294	0.6423	22.9	1.04	0.3318
Interaction									
X_1X_2	0.7503	3.01	0.1135	2.98	2.66	0.134	70.03	3.18	0.1049
X_1X_3	0.357	1.43	0.2591	5.22	4.66	0.0562	8.93	0.4053	0.5387
X_2X_3	0.0946	0.3794	0.5517	2.76	2.47	0.1473	11.93	0.5418	0.4786
Quadratic									
${X_1}^2$	12.49	50.08	< 0.0001	85.36	76.26	< 0.0001	261.08	11.86	0.0063
${X_2}^2$	1.72	6.9	0.0253	33.64	30.05	0.0003	5.62	0.2552	0.6244
${X_3}^2$	0.4649	1.86	0.2021	1.21	1.09	0.322	9.44	0.4286	0.5275
Residual	2.49			11.19			220.21		
Lack of fit	2	4.02	0.0764	8.45	3.08	0.1213	158.62	2.58	0.1612
Pure error	0.4966			2.74			61.59		
Cor total	47.67			396.65			2525.66		
Parameters used for the adequacy check of the model									
C.V. (%)	4.5			2.81			6.52		
R^2	0.95			0.97			0.91		
R^2 Adjusted	0.9			0.95			0.83		
Adequate precision	19.69			27.73			13.63		

Y_1: total polyphenols content; Y_2: total phenolic acids content; Y_3: total flavonoids content; SS: sum of square

Table 4. The optimum conditions for extraction and the results of the predicted and validated values of each response

Optimized condition	Predicted	Validated
X_1 = 85.77%	Y_1 = 11.50	Y_1 = 12.41
X_2 = 65 °C	Y_2 = 38.93	Y_2 = 39.94
X_3 = 120 minutes	Y_3 = 86.94	Y_3 = 86.35

the F-value of lack of fit recorded was 3.08 and non-significant which indicated the model is well-fitted (Table 3). Besides, the analysis showed that the coefficient of determination recorded for both R^2 and R^2 Adjusted were very high with 0.97 and 0.95 (Fig. 1b) and the adequate precision was greater than 4. By fitting the second-order polynomial, the equation for total phenolic acids content could be expresses as follows:

$$TPC = -1.54 + 2.78x_1 - 1.68x_2 - 0.31x_3 - 0.02{x_1}^2 + 0.0007{x_2}^2 + 0.007{x_3}^2 + 0.006x_1x_2 + 0.004x_1x_3 - 0.003x_2x_3$$

Based on Table 3, highly significant effect ($p<0.001$) was shown for the linear model of ethanol concentration and extraction temperature. In contrast, a non-significant effect was obtained for the linear model of extraction time. The quadratic model showed that ethanol concentration showed a negative significant effect and extraction temperature showed a positive significant effect against total phenolic acids content. The ANOVA results also revealed that the interaction model of ethanol concentration, extraction temperature and extraction time were not significantly affected the total phenolic acids content.

3.4 Influence of process parameters on total flavonoids content

The total flavonoids content of lemon peel extracts ranged from 53.02 to 91.63 mg RE/g DW in this experiment. The maximum total flavonoids concentration was obtained after 100 minutes of treatment with 80% (v/v) ethanol at 71.82 °C (Table 2). Meanwhile, the treatment with 70% (v/v) ethanol at 45 °C for 120 minutes resulted in the lowest total flavonoids concentration. The model was very significant with $p<0.001$ according to the ANOVA analysis in Table 3. The lack of fit F-value was found to be non-significant, indicating that the suggested model is well-fitted. Both the R^2 and R^2 Adjusted coefficients of determination were above 0.91 and 0.83, respectively (Fig. 1c). The equation for total is obtained by fitting the second-order polynomial.

$$TFC = -99.91 + 5.71x_1 - 3.14x_2 - 0.42x_3 - 0.04{x_1}^2 + 0.006{x_2}^2 + 0.0002{x_3}^2 + 0.03x_1x_2 + 0.005x_1x_3 - 0.006x_2x_3$$

The linear model revealed that ethanol concentration and extraction temperature had a significant influence on total flavonoids content ($p<0.05$) (Table 3). However, extraction time had no significant influence on total flavonoids content ($p>0.05$). In the quadratic model, ethanol concentration had a negative significant effect on total flavonoids content, while extraction temperature and duration had a non-significant quadratic model. The ANOVA revealed that non-significant interaction effects between ethanol concentration, extraction temperature, and extraction duration in the interaction model.

3.5 Optimization of process parameters and model validation

This study was carried out to optimize the extraction conditions of phenolic content specifically polyphenols, phenolic acids and flavonoids content by implementing response surface methodology. Based on the results obtained in Table 4, the optimal extraction conditions for maximum phenolics extraction from lemon peel was 85.77% of ethanol concentration, 65 °C of extraction temperature and 120 minutes of extraction time, respectively. The results in Table 4 also showed that the experimental values obtained were in agreement and reliable for the extraction process of lemon peel.

4 Discussion

The results of response surface methodology were further analyzed and interpreted using 3D surface graph (Figs. 2 to 4). Based on these figures, it showed that as the concentration of ethanol were increased, the accumulation of polyphenols and phenolic acids were decreased. This could be caused by the investigated ethanol concentration were not an optimal concentration for the high phenolics extraction of lemon peel. In the previous study on different *Citrus* species, it was found that the optimal ethanol concentration was between 50 to 70% (Assefa et al., 2016; Živković et al., 2018; Hosseini et al., 2018). The increment of ethanol concentration may improve the extraction efficiency by disrupt the interaction between cell wall structural components and phenolic compounds, however, high percentage of ethanol concentration also could induce protein denaturation, causing the increment of plant matrix viscosity and preventing the release of phenolics (Mudrić et al., 2020). In addition, a study by Sharmila et al. (2016) found that the extraction of phenolic compounds from *Cassia auriculata* leaves was improved as the water to methanol ratio was increased. In the high polarity of solvent, the process to break the hydrogen bonds is easier and resulted to high accumulation of phenolic compounds (Jovanović et al., 2017). In addition, increment of water to solvent also will enhances the contact area between solid materials and solvent. Besides, the extraction of highly hydrophilic fraction of the glycosylated phenolic compounds (Arruda et al., 2016).

From the 3D surface graph, a significant increment of polyphenols, phenolic acids and flavonoids accumulation was observed as the extraction temperature was increased from 45 to 65 °C (Figs. 2 to 4).

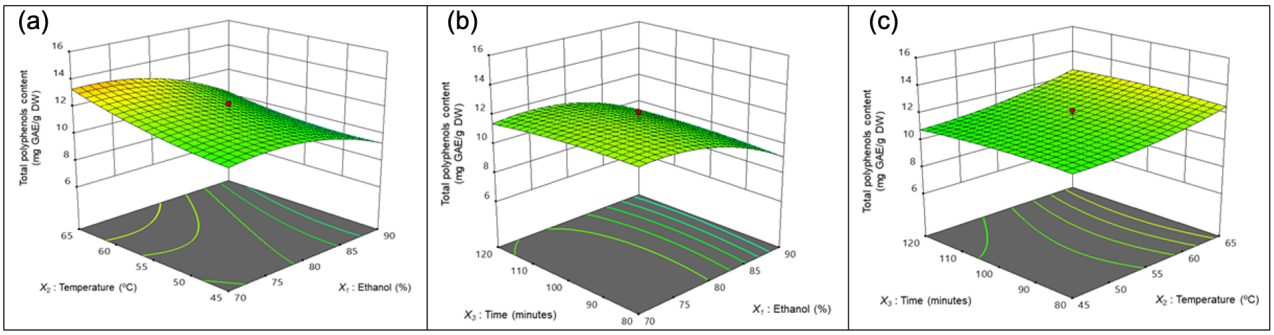

Figure 2. 3D surface graphs for the interaction effects between (a) ethanol concentrations and extraction temperatures, (b) ethanol concentrations and extraction times, (c) extraction temperatures and extractions times on total polyphenols content

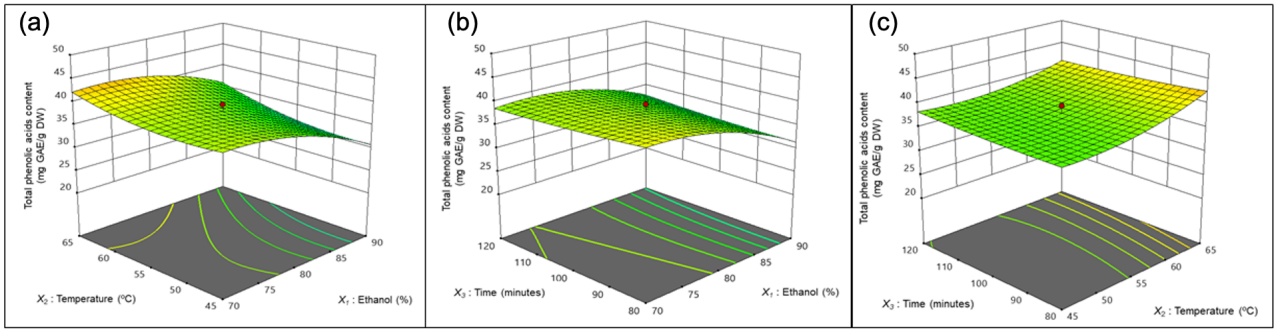

Figure 3. 3D surface graphs for the interaction effects between (a) ethanol concentrations and extraction temperatures, (b) ethanol concentrations and extraction times, (c) extraction temperatures and extractions times on total phenolic acids content

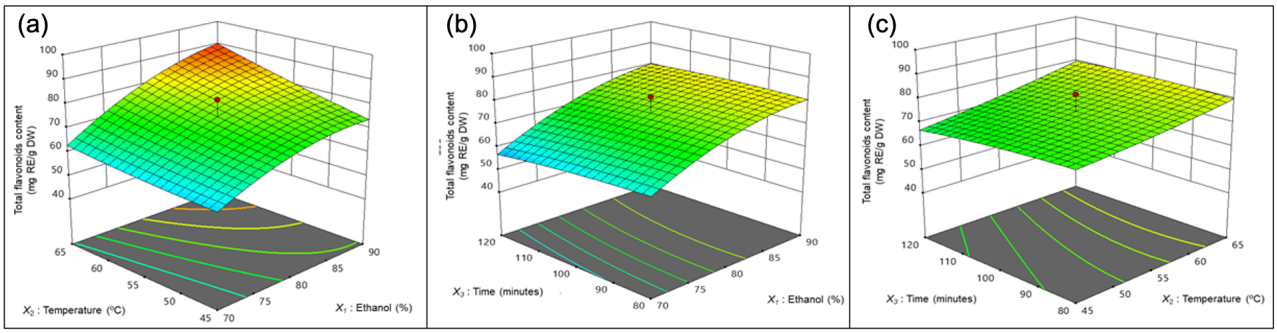

Figure 4. 3D surface graphs for the interaction effects between (a) ethanol concentrations and extraction temperatures, (b) ethanol concentrations and extraction times, (c) extraction temperatures and extractions times on total flavonoids content

According to Stokes-Einstein equation, the increment of temperature will significantly increase the diffusion rate (Pinelo et al., 2006). By increasing the extraction temperature, it will improve the extraction condition by influencing the solubility, vaporpressure, surface tension system and viscosity of the sample. The high amount of phenolics extracted at high temperature also could be due to thermal destruction of sub-cellular compartments and cell wall of the sample which lead to higher release of phenolics (Mokrani and Madani, 2016; Nwozo et al., 2016; Bunea et al., 2008). The increment of phenolic extraction as influenced by extraction temperature were also reported by several studies on various plant samples (Zhou et al., 2018; Deng et al., 2017; Thoo et al., 2010).

A non-significant effect was observed on all parameters recorded as affected by extraction time. Based on the 3D surface graph in Figs. 2 to 4, the amounts of total polyphenols, phenolic acids and flavonoids content showed a flat curve despite increment of extraction time from 80 to 120 minutes. This finding was in contrast with the previous research conducted on other species of Citrus which higher phenolics were obtained at 80 to 120 minutes of incubation time (Dao et al., 2020; Hien et al., 2018; Colodel et al., 2018; Tran et al., 2019). Based on Fick's second law of diffusion which explained that the final equilibrium between the solute concentrations in the plant matrix and in the solvent might be reached at a certain time (Silva et al., 2007). The prolongation of extraction time can increase the chance of sample to denature which contribute to high amount of phenolics obtain. However, prolongation of extraction time also can cause decrement amount of phenolics accumulation due to excessive exposure of plant sample to heat can cause fluctuation. Hence, the suitable extraction time for the maximum phenolics content extracted from lemon peel might not be in the ranged tested in this study.

5 Conclusion

In this study, the extraction of polyphenols, phenolic acids and flavonoids content of lemon peel were successfully maximized by implemented response surface methodology. The results obtained showed that ethanol concentration and extraction temperatures were significantly affected the extraction of polyphenols, phenolic acids and flavonoids. In addition, coefficient correlations showed good correlations between independent variables and responses and the experimental data were adequately fitted the second-order polynomial models. The optimized extraction conditions obtained for ethanol concentration, extraction temperature and extraction time were 85.77%, 65 °C and 120 minutes. Under this optimized extraction conditions, the predicted values were in accordance with experimental values indicating that the model was suitable. This optimized condition could be useful for phenolics extraction from lemon peel.

Acknowledgments

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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