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RESEARCH ARTICLE

Assessment of fatty acid composition and response surface optimization of ultrasonic-assisted extraction of phenolic compounds from *Pouteria campechiana* pulp

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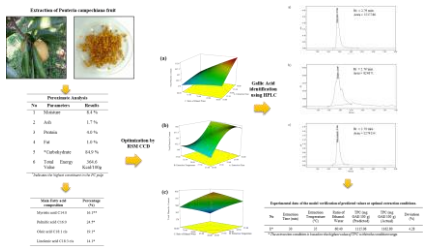
Graphical abstract



Abstract

Pouteria campechiana (PC) pulp was analyzed for its fatty acid composition. Fourteen different kinds of fatty acids were found with the major fatty acids *viz*. palmitic acid (C16:0), oleic acid (C18:1), myristic acid (C14:0) and linolenic acid (C18:2), making up approximately 73.8 % of the total fatty acid content (TPC). An ultrasonic-assisted extraction (UAE) of the polyphenolic bioactive components in PC pulp powder were statistically optimized using the Central Composite Design (CCD). Conditions that maximized TPC in the crude extract of PC pulp powder were assessed for factors, ratio of ethanol:water, extraction temperature and extraction time. The established optimum conditions of the CCD model with a value of R^2 = 0.8833, were within the studied range and agreed well with the predicted values. Under an optimized condition [30 min, 35 °C and ratio of ethanol:water, 60:40 (%, v/v)], the highest TPC was 1162.80 mg GAE/100 g in comparison to the predicted 1115.06 mg GAE/100 g. High Performance Liquid Chromatography confirmed that gallic acid and its derivatives were the major components, comprising a ~0.03 % (w/w) of the PC pulp crude extract. Pertinently, a high recovery value in the HPLC validation data (109.84 %, R^2 = 0.9995) suggests that the method was accurate.

Keywords*: *Pouteria campechiana, fatty acid composition, response surface methodology, total phenolic content, ultrasound-assisted extraction.

INTRODUCTION

Malaysia is one of few Southeast Asian countries that has several underutilized fruits and as such, they are rarely eaten (Ikram et al., 2009). Many of the nutritional or phytochemical components prevailing in these native fruit species are yet to be discovered. *Pouteria campechiana* (PC), belonging to the family Sapotaceae is among such fruit. PC is commonly called 'Buah Kuning Telur' in Malay while it bears a simpler English name "canistel'. Historically, PC is native to Central America but the fruit is widely distributed around the tropical and subtropical regions in South America and parts of Asia such as Sri Lanka, Indonesia and south Asian countries (de Lanerolle et al., 2009; Silva et al., 2009). The fruit of PC is ovoid-shaped measuring between 7.5−12.5 cm in length and 5−7.5 cm in breadth (Ma et al., 2004).When its skin is peeled away, it reveals a yellow pulp which centre may consists one to four hard seeds (Balerdi and Shaw, 1998). The richly textured PC pulp consists of a myriad of nutrients *viz.* carbohydrates, vitamin A and minerals (Coronel et al., 1986). The pulp can be eaten fresh and its rich texture makes it a popular ingredient in custards, ice creams, milkshakes, jam as well as marmalade. Claustro et al. (1997) reported that the seeds of PC can be made into a precooked drink that is comparable to coffee. Despite the various interesting uses, consumption of the PC fruit is unheard of among Malaysians.

The phytochemical composition of PC pulp mainly has triterpenes and flavonoids (Ma et al., 2004), in which the former may be found as long chains of acetate esters (Hernández et al., 2006; Lott and Jackes, 2001; Solís et al., 2004) Pertinently, bioactivity seen in extracts of PC pulp is associated with the plethora of polyphenolic antioxidants *viz.* gallic acid, myricitrin, (+)-gallocatechin, (+)-catechin, dihydromyricetin, (+)-catechin-3-O-gallate and (−)-epicatechin (Ma et al., 2004). Natural fatty acids are also found in other Pouteria sp. i.e. the nuts of *P. lucuma* and are known for promoting skin regeneration (Rojo et al., 2010), Whereas the extracts of *P. ramiflora* have neuroprotective effects against oxidative damage. Other roles of these fatty acids also include restoring levels of myosin-Va protein in the brain and preventing hippocampal neuronal loss (Da Costa et al., 2013). In view of such interesting reports, the study believes the polyphenolic rich extract of PC pulp (Ma et al., 2004) may be suited as a bioactive ingredient in formulating an anti-ageing nanoemulsion for use on human skin.

To prevent the loss or destruction of potential bioactive components in the PC pulp during extraction, appropriate steps must, therefore, be taken. Selection of the extraction method for obtaining plant-based bioactive components usually depends on factors such as the nature of plant material and types of compounds present (Sasidharan et al., 2011). In this regard, the study resorted to using ultrasonic-assisted extraction (UAE) to extract the bioactive compounds from the PC pulp. The method was chosen as the sonication process would promote the rapid dispersion of solids into the surrounding medium (Cintas and Luche, 1999).

Efficiency of UAE of bioactive compounds from plant materials lies in the cavitation phenomena produced by the ultrasonic energy, which is said to increase both the permeability of plant cell walls (Handa, 2008) and the stabilities of target compounds to be extracted (Mason and Peters, 1999). The application of UAE for isolating target compounds from plant materials typically involves the use of liquid

solvents i.e. applied in solid/fluid media (Esclapez et al., 2011). This extraction process is faster and more efficient as compared to conventional methods *viz*. maceration/stirring. This is due to the higher surface contact area between the solid and liquid phases induced by multiple particle disruption processes in UAE (Herrera and Luque de Castro, 2004). One main advantage of UAE is that additional modifications on the extracts are not required, except for filtration of the extracts before chromatographic analysis (Esclapez et al., 2011).

Conversely, UAE applications in solid/gas systems, however, are uncommon due to impedance mismatch and the poor transmission of ultrasound through air (Esclapez et al., 2011). Most importantly, the use of UAE is considerably greener for harvesting bioactive compounds in plant materials (Dal Pra et al., 2017). The operating process of UAE is simple, requiring the sample to be mixed with a suitable solvent(s) before placing into an ultrasonic bath/ultrasonic probe (Hromadkova and Ebringerová, 2003). One marked different between an ultrasonic bath and probe is that the former only allows the optimization of extraction time and temperature. Conversely, the ultrasonic probe is more versatile where factors including extraction time and temperature, output of the ultrasonic source (amplitude) and energy pulsation possibility can be optimized (Adam et al., 2009).

Nonetheless, the different combination of UAE process parameters could have significant effects on the extraction yield of bioactive compounds from the PC pulp. To overcome this issue, the study employed a statistical approach called response surface methodology (RSM) to identify the optimum combination of extraction parameters that guarantees maximum extraction effectiveness. This second order polynomial prediction model has been commonly used to establish the best experimental process parameters. Unlike other statistical methods such as that of single factor experiment and orthogonal design method, RSM allows the observation of the interactive effects between process parameters while reducing the number of experiments (Isah et al., 2017; Manan et al., 2018; Manan et al., 2016; Marzuki et al., 2015). Herein, the study focused on developing a highly efficient protocol for UAE of PC pulp bioactive compounds. To date, there are no reports detailing the optimization of extraction of polyphenolic compounds from PC pulp by UAE. By this virtue, there is a need to develop a suitable UAE method with favors maximum extraction of polyphenolic compounds with high antioxidant activity. In this study, significant extraction parameters were identified and the optimum conditions of UAE were predicted using the generated Central Composite Design (CCD) model.

EXPERIMENTAL

Reagents

Folin-Ciocalteu's phenol reagent was purchased from Sigma-Aldrich (St. Louis, USA). The grapeseed oil was purchased from Borges (Catalonia, Spain). Absolute ethanol, methanol and anhydrous gallic acid standard were purchased from Merck (Darmstadt, Germany). Other chemicals such as potassium hydroxide, sodium carbonate, hydrochloric acid and boron trifluoride-methanol solution were also acquired from Sigma-Aldrich (St. Louis, USA). Nylon microfilter with a pore size of 0.45 µm was purchased from Phenomenex.

Sample preparation

The mature ripened fruits of *P. campechiana* (PC) were collected from Nasuha Herbs and Spices (2° 2' 21.38" N latitude, 102° 34' 8.7" E longitude) in the Pagoh area of Johor, Malaysia. The fruits were cleaned under running tap water and only the pulp portion was collected for the subsequent drying process (Fig. 1). A 300 g of the pulp was chopped up into fine pieces and dried at 50 °C in an oven for 24 h before grinding into powder using a standard blender. The powdered sample was packed in a polyethylene bag and stored in a freezer at −10 °C during the experimental period. Extraction of the PC pulp was carried out for 24 h using a Soxhlet apparatus that used petroleum ether (30-60 %). The obtained crude extract, in a form of a relatively viscous yellow liquid with an orangy resin-like precipitate was vacuum evaporated prior to fatty acid analysis.

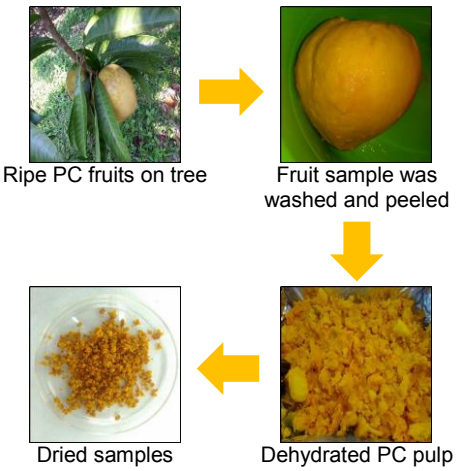


Fig. 1 PC fruits drying process.

Proximate analysis

The moisture content of the PC pulp was analyzed according to the protocol set out by the Association of Analytical Communities based on the AOAC 934.01-Moisture (Loss on Drying) (AOAC 934.01, 1934). The PC pulp was dried in a vacuum oven at 70 °C to a constant weight before the measurement was carried out. Ash content of the PC pulp was determined according to the AOAC 923.03-Ash (Direct Method) whereas the Kjeldahl method was used to estimate the protein content, was done as described by the AOAC 988.05 and 981.10. Estimation of the protein content was calculated by multiplying the total nitrogen content (%) by a factor of 6.25. Lipids in the PC pulp were extracted using a Soxhlet apparatus maintained at temperatures between 40–60 °C using petroleum ether as the solvent (BS ISO 8262-3:2005)–Fat Gravimetric Method). Total carbohydrate (TC) content including the fiber content in the PC pulp was estimated using the following equation: [TC = 100 − (Moisture + Total ash + protein + fat)]. Finally, calculation of total energy value, expressed in Kcal/100 g (wet basis), was based on Methods of Analysis for Nutrition Labelling (Nutrition Labelling and Education Act, 1991). All measurements were performed in triplicates.

Fatty acid (FA) composition analysis

Fatty acid methyl esters (FAME) were prepared as described by the following IUPAC methodology without heating. A 100 g sample of PC pulp extract was saponified with 1.2 mL of methanolic potassium hydroxide (0.5 M) at 60 °C for 10 min, neutralized with hydrochloric acid (0.7 M). The mixture was methylated in a 3.0 mL solution of boron trifluoride-methanol for approximately 10 mins in a 60 °C water bath. The fatty acids were extracted with petroleum ether under varying temperatures that ranged between 40−60 °C. The obtained FAME was separated using a GCMS-QP2010 PLUS Shimadzu, Japan. Also, the FAME fractions were separated on a VARIAN Saturn 2100D GC-MS equipped with a CP7420 (100 m × 0.25 mm i.d.) column and helium (1 mL/min) was used as the carrier gas. The oven temperature was programmed as follows: initial oven temperature of 80 °C held for 1 min, increased to 160 °C at 20 °C/min, increased to 198 °C at 1 °C/min, and finally increased to 250 °C at 5 °C/min, held for 5 min. Injector and detector temperatures were 250 °C and 180 °C, respectively (Villa-Rodríguez et al., 2011). The retention times of the fatty acids were compared with those of standards for their identification and the composition of each fatty acid was reported as relative percent.

Ultrasound-assisted extraction (UAE) process of PC

The ultrasound-assisted extraction (UAE) of PC pulp powder was carried out using an ultrasonic processor (VCX 130, Sonics, UK). The power and frequency of the ultrasonic processor was set at 130 watts and 20 kHz, respectively, using a variable power output controller. The probe used in this process was a 6 mm in diameter titanium horn and the sample was irradiated directly from the horn. Sonication was carried out in a stainless steel beaker that consisted of a 10 g of fresh PC pulp mixed in variable ratio of ethanol:water (v/v) as the solvent (working volume 100 mL). The mixture was sonicated for durations ranging from

5−30 min under continuous mode. The resulting suspension was centrifuged at 6 000 rpm for 20 min and filtered through a filter paper Whattman No. 1. Ethanol was removed by vacuum evaporation under 40 °C on a rotary evaporator. The samples were frozen, lyophilized and stored at 4 °C.

Experimental design and optimization of process parameter

Optimization experiment was carried out using response surface methodology (RSM) to extract polyphenols in the PC pulp powder while maximizing the response i.e. antioxidant activity. A full-factorial three-level-three factor, Central Composite Design (CCD) required 20 experimental runs that comprised of six axial points, eight factorial points and six central points, were used in the experiment. The relevant extraction factors assessed were: extraction time (A: 10, 20 and 30 min), extraction temperature (B: 25, 30, 35 °C) and ratio of ethanol:water (C: 60, 70 and 80 %, v/v) to affect the response i.e. total phenolic content (TPC). Table 1 shows the independent variables and their levels, as well as the actual and coded values for the process optimization. Each factor was coded at three levels (−2, −1, 0, +1, +2).

Table 1 The actual and coded independent variables for the Central Composite design for the extraction of total phenolic content of *Pouteria Campechiana* fruit.

Variables	Coded Levels				
	−2	−1	0	+1	+2
A: Extraction time (min)	3.18	10	20	30	36.82
B: Extraction temperature (°C)	21.59	25	30	35	38.41
C: Ratio of ethanol (% v/v)	53.18	60	70	80	86.82

All experiments were performed in triplicates. For clarity, the range of values for the three independent factors were determined by an earlier screening study. Experimental data were fitted to a second-order polynomial model to obtain the regression coefficients (β). A mathematical regression model for the response of Y (the predicted response) was fitted in Equation 1.

$$Y = \beta_0 + \sum_{i=1}^{a} \beta_i X_i + \sum_{i=1}^{a} \beta_{ii} X_i^2 + \sum_{ij=1(i\neq j)}^{ka} \beta_{ij} X_i X_j$$

where Y is response (TPC, [mg GAE/100 g dw], β_0 is the coefficient constant, β_i is the linear coefficient, β_{ii} is the coefficient of squared effect, β_{ji} is the coefficient of interaction effect, and X_i and X_j are the coded values of variables i and j, respectively (extraction time [$X1$], extraction temperature [$X2$], and ethanol concentration [$X3$]).

Total phenolic content (TPC)

TPC was determined using Folin–Ciocalteu method described by (Aseervatham et al., 2014). Briefly, 100 µL of Folin–Ciocalteu reagent and 200 µL of Na_2CO_3 (2 %, w/v) were transferred into a test tube containing 100 µL of the PC extract (1 mg/mL). The mixture was incubated for 15 min at 45 °C with shaking at 200 rpm before the absorbance was read at 765 nm using a UV–visible spectrophotometer (SHIMADZU, 1800 UV-VIS, JAPAN). TPC was expressed as milligrams per gram of gallic acid equivalents (mg/g GAE). Different concentrations of gallic acid (GA) (0–10 µg/mL) were prepared by dissolving GA in methanol. A linear standard graph was obtained by plotting the various concentrations of GA along the x-axis and absorbance along the y-axis. All experiments were performed in triplicates.

High performance liquid chromatography (HPLC) analysis

PC crude extract was dissolved in HPLC grade methanol to make up a 20 mg/mL sample solution before filtering through a 0.45 µm nylon microfilter (Phenomenex). The extracts were analyzed using a HPLC (Agilent 1200 series, Agilent Technologies, USA) equipped with a UV detector (G1314B, Agilent) and an Eclipse XDB-C18 column (5 µm, 250 mm×4.6 mm, Agilent). The mobile phase consisted of 3 % aqueous acetic acid (A) and methanol (B) set at a flow rate of 1 mL/min. The mobile phase composition began with 100 % of A that was maintained for 1 min, followed by a linear increase to 63 % of B in 27 min, and then the composition returned to the initial condition within 5 min for the next run. Comparison of retention times and spectral data of the samples with those of the standards were used to identify and assignment the peak of the phenolic compounds. Quantification of phenolic compounds was estimated using the respective calibration curves for gallic acid.

RESULTS AND DISCUSSION

Proximate analysis

Table 2 presents the results for the proximate analysis of PC pulp (dry weight). Results revealed that the contents for moisture, ash and protein in PC pulp powder are very low, amounting to approximately 8.4, 1.7 and 4.0 %, respectively. The low moisture content (8.4 %) seen here indicates the PC pulp can be sufficiently dehydrated for storage over an extended period of time without the loss of quality. This is because a high moisture content can subsequently lead to a reduced keeping quality and shortened shelf life (Jaafar et al., 2009). The low ash content (1.7 %) infers a low quantity of total inorganic minerals whereas the high percentage of carbohydrates (84.9 %) agreed well with the high total energy value (364.6 kcal/100 g) of the PC pulp powder. These results are consistent with the richly textured fresh PC pulp, suggesting it is categorically a high calorie food, similar to an earlier description by Berto et al. (2015).

Table 2 Results of proximate analysis for the pulp of PC.

No	Parameters	Test Method	Results
1	Moisture	In-house STP/FL313/002/07 (based on AOAC 934.01)-Moisture (Loss On Drying)	8.4 %
2	Ash	In-house STP/FL313/001/07 (based on AOAC 923.03)-Ash (Direct Method)	1.7 %
3	Protein	In-house STP/FL313/005/07 (based on AOAC 988.05 & 981.10) – Protein (Kjeldahl Method)	4.0 %
4	Fat	In-house STP/FL313/003/07 (based on BS ISO 8262-3:2005)–Fat (Gravimetric Method)	1.0 %
5	*Carbohydrate	In-house STP/FL313/007/07 based on Methods of Analysis for Nutrition Labelling	84.9 %
6	Total Energy Value	In-house STP/FL313/007/07 based on Methods of Analysis for Nutrition Labelling	364.6 Kcal/100g

Fatty acid (FA) composition analysis

It is worth mentioning here that reports on the fatty acid composition of PC pulp remains limited (Silva et al., 2009). Table 3 illustrates the fatty acid composition in PC pulp, expressed as the percentage of total fatty acid (TFA) content of the PC crude extract. A total of 14 different types of fatty acids was identified with palmitic acid (C16:0, 24.5 %), oleic acid (C18:1, 19.1 %), myristic acid (C14:0, 16.1 %) and linolenic acid (C18:2, 14.1 %) being the major types of fatty acids that cumulatively amounted to 73.8 % of the TFA content in the PC pulp. The present study confirmed that the PC pulp is comprised of a substantially high percentage of saturated fatty acid (SFA), suggesting that the fruit is a good source of SFA. SFA (55.9 %) formed a considerable proportion of the TFA composition in PC pulp, in which myristic acid (C14:0) and palmitic acid (16:0) were the dominant fatty acids, constituting 40.6 % and 24.5 % of the TFA content, respectively. An approximate 23.4 % of monounsaturated fatty acids (MUFA) was present whereas polyunsaturated fatty acids (PUFA) contributed 16.5 % of TFA. The order of abundance in terms of unsaturation of the fatty acids are as follows:

SFA > MUFA > PUFA

Table 3 Fatty acid content of PC pulp (dry weight).

Fatty acid composition	Percentage (%)
Hexanoic acid C6:0	0.1
Caprylic acid C8:0	3.1
Capric acid C10:0	1.2
Lauric acid C12:0	3.9
Myristic acid C14:0	**16.1****
Palmitic acid C16:0	**24.5***
Palmitoleic acid C16:1	4.4
Stearic acid C18:0	3.3
Oleic acid C18:1 cis	**19.1***
Oleic acid C18:1 trans	< 0.1
Linoleic acid C18:2 cis	2.4
Linoleic acid C18:2 trans	< 0.1
Linolenic acid C18:3 cis	**14.1***
Linolenic acid C18:3 trans	< 0.1
Arachidic acid C20:0	0.6
Behenic acid C22:0	< 0.1
Lignoceric acid C24:0	3.0
Others	4.2

Fatty acids found in natural oils have several beneficial properties suitable for application in cosmetic and personal care products. In cosmeceutical emulsions, plant oils can be constituents of the oily phase due to their low molecular weights and low viscosities. These qualities are the reasons for plant oils being preferred over mineral oils to prepare emulsions (Bwai et al., 2013). Interestingly, the fatty acid content in the PC pulp was found comparable to the pulp of the more widely known fruit, the Hass avocado (*Persea americana*) (Villa-Rodríguez et al., 2011). The findings are consistent with our observation on the pulps of ripened PC and Hass avocado being similarly textured, having a rich and yet unsweetened taste. The relatively high contents of oleic and palmitic acid imply their potential application for topical cosmetic uses. Various studies have shown that oleic acid exhibits the best permeation enhancing effect among unsaturated fatty acids (Kim et al., 2008; Rabasco Álvarez and González Rodríguez, 2000), whereas for the SFAs, palmitic acid is the most potent (Kim et al., 2008; Vermaak et al., 2011). Skin permeation enhancement effects were also reported for linoleic, lauric, myristic and stearic acids (Vermaak et al., 2011). Based on the findings, the PC pulp has a fatty acid profile that can act as an enhancer for drug permeation for transdermal and topical drug delivery. Moreover, the aforementioned fatty acids are natural and edible, which increases their applicability as an additive or component in cosmetics (Kanikkannan and Singh, 2002; Tanojo et al., 1997).

Optimization of the ultrasound-assisted extraction condition for improving phenolic contents using response surface methodology (RSM)

RSM is a statistical method to analyse and estimate the optimum levels of selected factors within a design range (Sharmila et al., 2016). The use of RSM has been found practical for various optimization experiments to extract polyphenolic compounds from several types of fruits *viz*. Citrus sinensis L. peel (Khan et al., 2010), *Mangifera pajang* peels (MPP) (Prasad et al., 2011) and *Malus domestica* (Alberti et al., 2014). In this study, the effects of process parameters *viz*. solvent ratio (ethanol:water, % v/v), extraction temperature and extraction time on the yield and TPC in the PC pulp extract were investigated using a full factorial three-factor-three-level Central Composite Design (CCD).

RSM experiments and fitting the models

The present study attempted to identify the best factors for extracting phenolic compounds. Subsequently, the optimized conditions were validated for the highest TPC in the UAE of PC pulp. The interaction was represented as a response surface plot, plotted as a function of two targeted variables, while the other variable was held constant. The CCD model used various statistical analysis parameters viz. P–value, F–value, adjusted determination of coefficient (Adj. R^2) and coefficient of determination (R^2) to represent the statistical significance of the developed quadratic model. The value of adequate precision was used as a measure of the signal to noise ratio. Adequacy of the generated model was assessed using analysis of variance (ANOVA) to describe the data and to express the quality of the fitted model. A model that is significant can be represented by a P-value < 0.05 while a P-value < 0.0001 suggests the term is highly significant. P-value describes the significance and interaction capability of each variable, in which variables showing lower P-values exhibit greater significance. In this study, regression analysis was used to determine the best fitting model whereas the best combination of factors was established using the optimization function based on the ridge maximum and canonical analyses. Table 4A represents the generated equation in terms of coded factors. Fitting of the data to various models (linear, two factorial, quadratic and cubic), the corresponding ANOVA revealed that the TPC was well described by a quadratic polynomial model. The values of R^2 and Adj. R^2 were 0.8833 and 0.7536, respectively, indicating acceptable accuracy and general availability of the polynomial model. A $R^2 > 0.80$ usually implies a satisfactory model (Poojary and Mugeraya, 2012; Mohamad et al., 2015) while a value of adequate precision, 8.7733 (measures the ratio signal to noise) higher than 4, suggests sufficient signals were attained (Table 4A).

The positive sign in front of terms imply synergistic effects, whereas the negative sign infers an antagonistic effect influencing the independent variables affecting the TPC in the UAE of PC pulp. The antagonistic interaction between extraction time *versus* extraction temperature (−5.07AB) (Table 4A) and extraction time *versus* ratio of EtOH:water (−137.14AC) (Table 4A), as well as their corresponding linear terms were also significant (P-value 0.0297) (Table 5). The data also indicate that simultaneously increasing both factors will not improve the value of TPC. Conversely, a positive term for the effect of temperature *versus* ratio of ethanol:water, (+ 0.97BC) (Table 4A) strongly implies their synergistic interaction would improve the TPC.

Likewise, a positive term for the effect of temperature *versus* ratio of ethanol:water, (+ 0.97BC) (Table 4A) strongly implies their synergistic interaction would improve the TPC, too. The relatively high computed F-value of 6.81 (> 4.0), a small P-value (0.0040) and the insignificant lack-of-fit (*P*-value 0.1073) obtained for the model further affirmed its suitability to predict the experiment (Table 4B). This was supported by the computed F-value of the model (6.81) being higher than the tabular $F_{0.05\,(9,10)} = 3.02$, implying that the degree of freedom relative to the residual was significant at the 5% confidence level. The lack of fit (F-value 3.37) that was lower than the tabular $F_{0.05\,(4,5)} = 5.19$,

Table 4 (A) Quadratic polynomial equations for the estimated coded and processed factors for TPC in the PC pulp crude extract and (B) ANOVA for the second-order polynomial model of the CCD.

(A) Response	Quadratic polynomial model equations	R^2	Adj. R^2	Adequate precision
Total Phenolic Content (mg GAE/100 g dw)	601.97 + 190.85A − 154.74B − 231.94C − 5.07AB − 137.14AC + 0.97BC − 19.89A2 – 16.56B2 – 26.42C2	0.8833	0.7536	8.7733

Note : A : Extraction time (min), B: Extraction temperature (°C), C: Ratio of ethanol: water (% v/v)

(B) ANOVA	Source of Variation	Sum of Squares	Degree of freedom	Mean Square	*F*-value	*P*-value
TPC (mg GAE/100 g dw)	Model	1538784.95	10	153878.49	6.81	0.0040*
	Residual	203292.28	9	22588.03		
	Lack of Fit	148328.67	4	34020.05	3.37	0.1073^{ns}
	Pure Error	54963.61	5	12702.01		
	Corr Total	1742077.22	19			

* *Significant at P < 0.05.*
^{ns} *Not significant at P > 0.05.*

suggests that the lack of fit was insignificant relative to the pure error (Table 4B). The ANOVA for the linear terms for extraction time, (A) (P-value < 0.0011) and ratio of ethanol:water, (C) (P-value < 0.0003) were found highly significant, while the extraction temperature (B) was significant.

Table 5 ANOVA for the second order polynomial model and coefficient values for TPC in PC pulp extract.

Source	Degrees of Freedom	*F*-value	*P*-value
Linear			
A	1	22.02	0.0011**
B	1	6.00	0.0368*
C	1	32.52	0.0003**
Interactions			
AB	1	0.01	0.9261^{ns}
AC	1	7.01	0.0297*
BC	1	0.00	0.9858^{ns}
Quadratic			
A^2	1	0.46	0.6274^{ns}
B^2	1	1.94	0.6855^{ns}
C^2	1	0.71	0.5214^{ns}

Note: A: Extraction time (min), B: Extraction temperature (°C), C: Ratio of ethanol: water (% v/v).
** *Significant at P < 0.01.*
* *Significant at P < 0.05.*
^{ns} *Not significant at P > 0.05.*

Pertinently, the data evidently showed that the ratio of ethanol:water, (C) had the largest impact on the TPC in the UAE process (Table 5).

Correspondingly, the experimentally obtained values (Table 6) for the response variables agreed well with the predicted values (R^2 = 0.8833), indicative of a satisfactory model. The relationship between the predicted and experimental percentage conversions is shown in Figure 2a. For this study, TPC values from the UAE of PC crude extract was within 136.07−1047.54 mg GAE/100 g dry weight.

Effect of process variables on TPC

The effects of process variables, extraction time (A), extraction temperature (B) and the ratio ethanol:water (C) on the UAE of PC pulp is depicted in Figure 2b. The data clearly indicate the TPC increases linearly with increasing extraction time (A). Conversely, the TPC improved when the extraction temperature (B) was increased up to − 0.500 before a decline was seen. As the temperature increased, solvent density and viscosity are also reduced, resulting in higher rates of mass transfer. The number of cavitation bubbles within the sonication mixture are also increased, producing a cohesive force that decreased the tensile strength following a reduction in solvent viscosity (Kong et al., 2015). This behaviour is also consistent with reports describing higher extraction temperatures that incite the breaking of phenolic matrix bonds that ultimately affect the membrane structure of plant cells and increase coagulation of lipoproteins (Prasad et al., 2011). The data also demonstrate that the use of a 60:40 (v/v) of ethanol:water can enhance extraction efficiency of the UAE to yield a maximum TPC. This might be due to the intensified swelling which increased disruption of plant cells due to presence of higher amounts water. Also, the contact surface area between the solvent and plant matrix are inadvertently elevated (Addai et al., 2013) and more phenolic compounds are solubilized into the surrounding medium, hence the TPC is increased.

Table 6 Experimental design and results of the CCD.

	A:	B:	C:		
Run No.	Extraction time (min)	Extraction temperature (°C)	Ratio of ethanol: water (% v/v)	Actual TPC (mg GAE/100 g dw)	Predicted TPC (mg GAE/100 g dw)
1	30	25	80	240.98	342.89
2	10	25	60	406.15	416.86
3	36.82	30	70	1045.08	866.67
4	20	30	70	524.59	601.97
5	30	25	60	971.31	1082.99
6	10	25	80	136.07	225.32
7	10	35	60	414.34	469.22
8	30	35	60	1047.54	1115.06
9	20	30	70	469.26	601.97
10	3.18	30	70	268.03	224.73
11	20	30	70	589.34	601.97
12	20	21.59	70	926.23	815.37
13	10	35	80	236.48	281.57
14	20	38.41	70	405.74	294.88
15	30	35	80	232.79	378.85
16	20	30	70	615.57	601.97
17	20	30	86.82	288.93	137.19
18	20	30	53.18	987.30	917.33
19	20	30	70	778.69	601.97
20	20	30	70	596.31	601.97

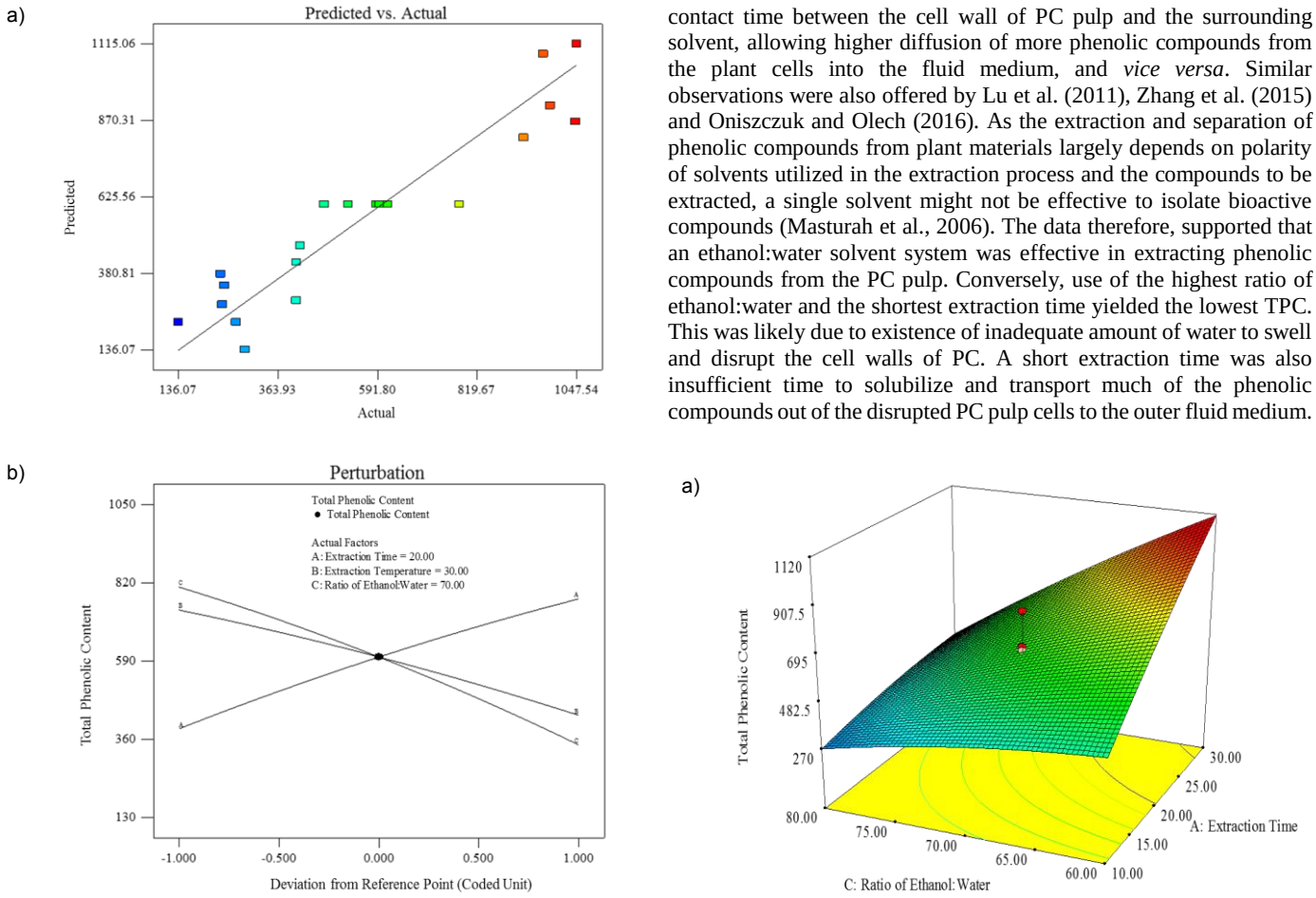


Fig. 2 a) Comparison between the predicted and the actual values for the TPC (mg GAE/100 g dw) in PC extracts. b) The deviation of the reference point for TPC value for the effect of (A) extraction time, (B) extraction temperature and (C) solvent ratio.

Mutual effect of experimental factors on the extraction of PC fruits

Effect of extraction time and ratio of ethanol:water

When compared to the conventional Soxhlet extraction method, UAE works more rapidly and is more convenient. The generated ultrasound transfers energy while concurrently accelerating movement in a material to produce the cavitation effect that disrupts plant cell walls and liberates the compounds into the surrounding fluid. The sonication process also creates heat which further speeds up the extraction process (Teng et al., 2016), and promotes mass transfer.

Figure 3 illustrates the (a) response surface and (b) contour plots for the effect of extraction time (A) and ratio of ethanol:water (C), and their mutual interaction on the TPC in the UAE of PC pulp powder. In this assessment, the temperature was held at 30 °C. The data revealed the interactions between the reaction parameters were significant because of a small P-value (0.0297). The effect of ratio of ethanol:water (F-value 32.52) was more significant as compared to extraction time (F-value 22.02) to affect the TPC (Table 4). UAE of PC pulp yielded a maximum TPC of 1056.01 mg GAE/100 g when the ratio of ethanol:water and sonication time were maintained at any values between 60 – 63% (v/v) and 26 – 30 min, respectively. The result corroborates an earlier study that described the extraction of polyphenols from the root bark of *Wikstroemia indica* was optimum when using a 63 % ratio of ethanol:water (Lu et al., 2011). Similarly, Prasad and others (2011) achieved maximum TPC when they extracted phenolics from *Mangifera pajang* Kosterm peel, using a 68 % ratio of acidified aqueous methanol.

The data evidently show that a maximum TPC was achieved when the lowest ratio of ethanol:water and the longest extraction time were used. This is because a longer extraction time increases duration of contact time between the cell wall of PC pulp and the surrounding solvent, allowing higher diffusion of more phenolic compounds from the plant cells into the fluid medium, and *vice versa*. Similar observations were also offered by Lu et al. (2011), Zhang et al. (2015) and Oniszczuk and Olech (2016). As the extraction and separation of phenolic compounds from plant materials largely depends on polarity of solvents utilized in the extraction process and the compounds to be extracted, a single solvent might not be effective to isolate bioactive compounds (Masturah et al., 2006). The data therefore, supported that an ethanol:water solvent system was effective in extracting phenolic compounds from the PC pulp. Conversely, use of the highest ratio of ethanol:water and the shortest extraction time yielded the lowest TPC. This was likely due to existence of inadequate amount of water to swell and disrupt the cell walls of PC. A short extraction time was also insufficient time to solubilize and transport much of the phenolic compounds out of the disrupted PC pulp cells to the outer fluid medium.

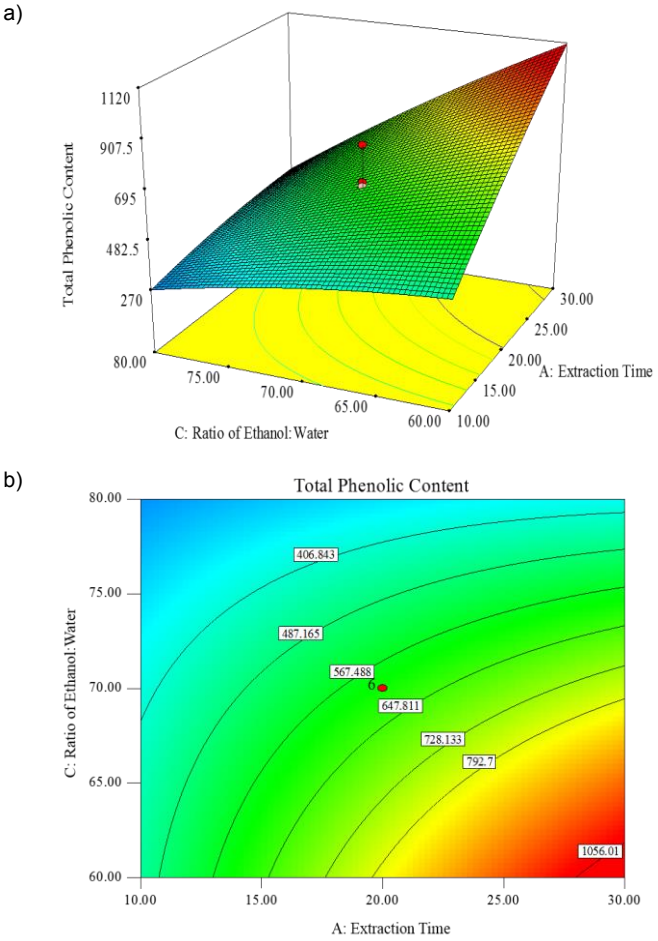


Fig. 3 Response a) surface plot and b) contour plot showing the effect of extraction time (A) and ratio of solvent (C), and their mutual interaction to affect the TPC in the UAE of PC pulp [Extraction temperature held constant at 30 °C].

Effect of extraction time and extraction temperature

The (a) response surface curve and (b) contour plot for the effect of extraction time (A) and extraction temperature (B), and their mutual interaction on the TPC in the UAE of PC pulp powder is represented in Figure 4. In this evaluation, the ratio of ethanol:water was maintained at 70 % (v/v). The F-value indicated that the effect of extraction time (22.02) was more significant than extraction temperature (6.00), implying that the former has a greater influence in improving extracted TPC in the UAE process. The outcome seen here generally agrees with the use of a prolonged extraction time that tended to improve extraction efficiency. It promotes the complete cracking the PC pulp cell through acoustic cavitation, permitting higher diffusion of solvent molecules to dissolve the phenolic compounds (Sharmila et al., 2016).

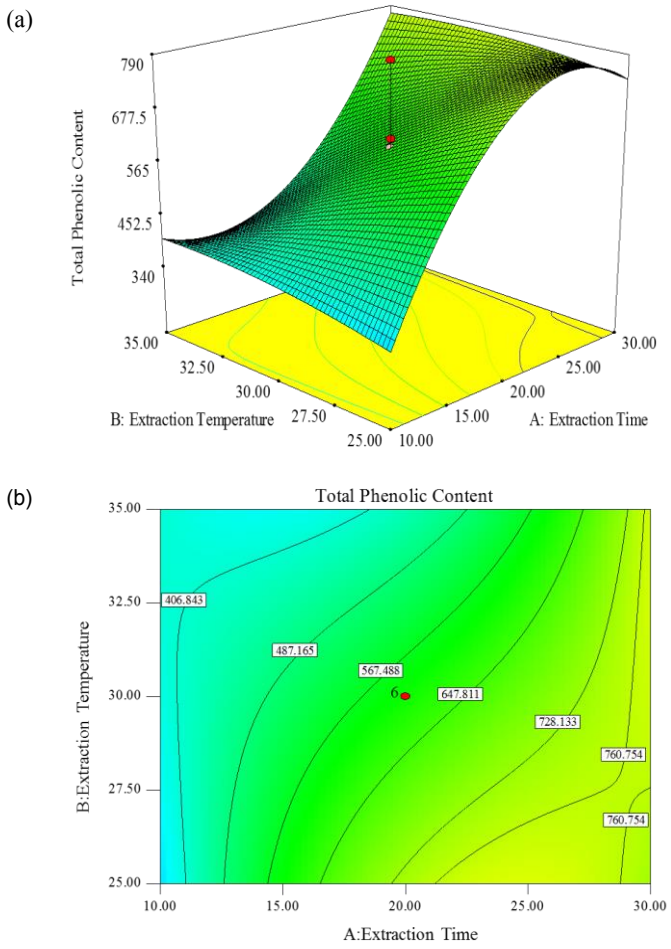


Fig. 4 Response a) surface plot and b) contour plot showing the effect of extraction time (A) and extraction temperature (B), and their mutual interaction to affect the TPC in the UAE of PC pulp [Ratio of ethanol:water held constant at 70 % (v/v)].

The data indicate the extraction process carried out between 27−30 min and extraction temperatures between 27.5−30 °C gave the highest TPC at 760.55 mg GAE/100 g. TPC was increased with increasing extraction time due to the higher accessibility of solvent molecules and more phenolic compounds that could permeate into the ethanol:water mixture, given enough time. Also, the result may have something to do with the longer extraction time that tends to increase the temperature of the ethanol:water mixture. This decreases the dielectric constant of water along with increasing solubility of the phenolic compounds, enhancing extraction rate, diffusion rate and reducing both solvent viscosity and surface tension (Prasad et al., 2011). These changes synergistically support the higher TPC in the UAE process of PC. However, the study was mindful that the use of higher extraction temperatures can also adversely affect stability of the extracted phenolic compounds. This is clearly demonstrated when further increment in the reaction temperature was counterproductive to the response of this study and yielded lower TPC likely due to degradation of phenolic compounds. Under elevated temperatures, higher interferences on compound stability can occur, invoked by enzymatic and chemical degradation or reaction with other plant components. These circumstances would normally reduce efficiency of the UAE process (Durling et al., 2007).

Effect of extraction temperature and ratio of ethanol:water

Figure 5 depicts the (a) response surface curve and (b) contour plot for the effect of extraction temperature (B) and ratio ethanol:water (C), and their mutual interaction on the TPC in the UAE of PC pulp powder. Based on the contour plot, it can be seen that a maximum TPC of 929.23 mg GAE/100 g was attained using a solvent ratio of ~ 60% (v/v) and any values of extraction temperatures between 25−27 °C. As indicated by the F-value, the effect solvent ratio (32.52) was more significant in relevance to the extraction temperature (6.00). Such outcome can be correlated to the similar polarity between ethanol:water used in the extraction fluid with that of phenolic compounds during extraction, as reported by (Sharmila et al., 2016). Moreover, Naczk and Shahidi (2004) reported that several phenolic compounds can occur naturally as glycosides.

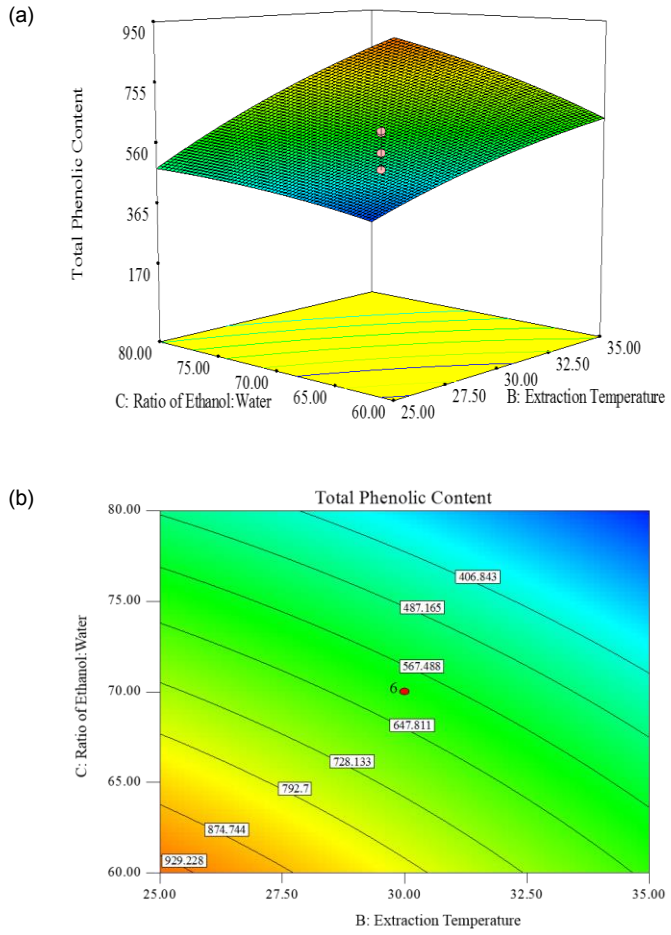


Fig. 5 Response surface plot and contour plot showing the effect of ratio of ethanol:water (C) and extraction temperature (B), and their mutual interaction affect the TPC in the UAE of PC pulp [Extraction time held constant at 20 min].

It is possible that the presence sugar in PC pulp contributed to improved solubility of the phenolic compounds in the extraction fluid. Alberti et al. (2014) also described a similar observation. The study noted that the lowest TPC was obtained when using 80 % (v/v) of ethanol:water. Conversely, high concentrations of ethanol to water have been known to promote protein denaturation, and consequently prevent the dissolution of phenolic compounds into the fluid medium (Odabaş and Koca, 2016), hence consistent with the obtained lower TPC values. Moreover, an elevated extraction temperature naturally increases decomposition of phenolics (Naczk and Shahidi, 2004) and intensifies solvent loss through vaporization. Combination of such changes would generally reduce mass transfer during extraction (Tan et al., 2013). So, lower concentrations of phenolic compounds are transferred out of the PC cells into the outer fluid, to yield lower TPCs.

Model verification

Verification of the model equation to predict the optimum response values was carried out using the optimization function in the Design Expert 7.1.6 software. The optimized condition was established as follows: extraction time (A) of 30 min, extraction temperature (35 °C) and solvent ratio (ethanol:water, 60:40, % v/v) to obtain a predicted

yield of 1132.74 mg GAE/100 g. The experimental values for the highest TPC (1162.80 mg GAE/100 g) accorded quite well with the predicted TPC (1115.06 mg GAE/100 mg). Since the corresponding percentage deviation was an acceptable 4.28 % (deviation < 5 %), optimization by the CCD model can be deemed accurate and therefore, applicable. The highest TPC in the crude extract of PC in this study was similar to that result obtained by Aseervatham et al. (2014), with the former obtaining a slightly lower TPC of 1150±1.23 mg GAE/100 g.

Analysis of PC crude extract using High Performance Liquid Chromatography (HPLC)

Figure 6 illustrates the HPLC chromatogram of PC pulp crude extract, spiked sample and standard GA. Linearity of the method was assessed by diluting the GA standard solution into a series of concentrations. All calibration curves were constructed by plotting the peak areas of the standard solutions versus their corresponding concentrations.

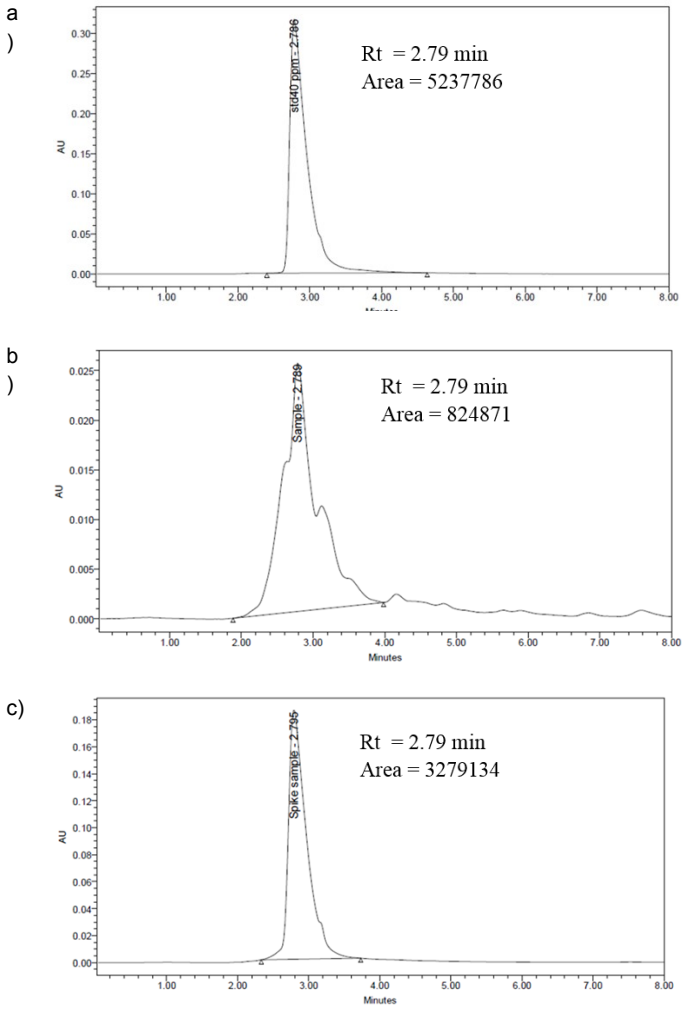


Fig. 6 HPLC chromatograms read at 280 nm for the a) GA standard (a), crude PC pulp extract (b) and spiked PC pulp extract (c).

The PC crude extract was spiked with standard GA (20 mg/ml in methanol). As seen in the graph (Figure 6), the retention times for peaks of the GA spiked sample from the PC crude extract (2.79 min, area = 3279134) (Figure 6c) and crude PC (2.79 min, Area = 824871) (Figure 6b) coincided with a peak for the GA standard (2.79 min, Area = 5237786) (Figure 6a). The results strongly suggest GA or its derivatives were present in the crude extract of PC pulp. Based on the chromatogram, it was determined that the crude extract contained ~ 0.03 % (w/w) in GA, with a good percentage recovery of 104.95 % in the HPLC validation data. The value of the regression coefficient (R^2) of GA standard was higher than 0.98, indicating a good linearity of the HPLC method.

CONCLUSION

The results of the present study indicate that each factor, for the exception of extraction temperature, in the UAE of PC pulp powder has a significant effect on the TPC. The study successfully optimized the extraction of the phenolic compounds from the pulp of PC using the CCD. Under an optimized condition, the experimental values obtained for a maximum TPC was 1162.80 mg GAE/100 g (dw), fitted well with the predicted result (1115.06 mg GAE/100 g) with only a 4.28 % deviation. Hence, the protocol for an UAE of PC pulp powder proposed in this study permits a rapid and maximum extraction of phenolic compounds.

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