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PROCESS CONDITION OPTIMIZATION FOR SOLVENT EXTRACTION OF COCOA POD BY POLYPHENOLS ANTIOXIDANT LEVEL AND ACTIVITIES

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ABSTRACT –*This study was carried out to obtain the optimum condition of solvent extraction of cocoa pod extract using aqueous ethanol by measuring its polyphenols antioxidant levels and activities. Parameters of interest are solvent percentage (75-95%), time of extraction (15-45 min) and temperature of extraction solvent (30-40^{o}C). The extraction was carried out using a water bath shaker at 120 rpm. The dependent variables are total phenolic content (TPC; mg GAE/g), total flavonoids content (TFC; mg RE/g), DPPH scavenging effect (DPPH %), effective concentration of DPPH (EC_{50D}), FRAP value (µmol Fe^{2+}/L), effective concentration of FRAP (EC_{50F}), β-carotene bleaching (BC %) and effective concentration of β-carotene bleaching assay (EC_{50B}). The results showed that varying the condition of extraction influence significantly, only onto two dependent variables, namely TPC and EC_{50D}. Interaction between solvent percentage and temperature significantly affected EC_{50D}, at p-value 0.003, while solvent percentage and time affected TPC (p=0.002). Optimum extraction condition was achieved at solvent percentage 80.94%, extraction time 17.48 minutes and 35.29^{o}C resulting TPC value of 153.30 mg GAE/g and EC_{50D} at 0.086, which was validated. In addition, significant strong positive correlation was observed between TPC and TFC (r=0.985), as well as EC_{50D} versus TPC (r=0.670) and TFC (r=0.785). These results showed scavenging activity was contributed by the TPC and TFC, while ion reducer and inhibition of lipid peroxidation was contributed by probably other groups of bioactive compounds in cocoa pod extract. The model can be used to extract the polyphenols antioxidant compounds from cocoa pods at optimal operating condition.*

Keywords: Optimization, cocoa pod, solvent extraction, polyphenols

INTRODUCTION

Bulk of cocoa pod husks are discarded onto the plantation floor during extraction of the cocoa beans for processing. The cocoa pod husks are used as a source of fertilizer (Agbeniyi *et al.*, 2011), potash for soap-making, scrub particles in body wash (Karim *et al*., 2012) and particle board making (Hii *et al*., 2006). Cocoa pods come in various colours depending on the clones and become yellowish when ripe. The various colours of pods could resemble the antioxidant compound that becomes the shield of protection for the cocoa fruits from pests (Chuang, et al. 2016).

Response surface methodology has been used to optimize the extraction condition to achieve research objectives. In further, Box Behken Design (BBD) was used to develop experimental models within the factor level given by the researcher. This method was seldom used when the researcher is focusing to optimize the condition within their predicted level.

The objective of this experiment is to obtain the optimal solvent extraction condition of cocoa pod husks, where the extract should contain high antioxidant level and activity but using less solvent percentage. The extract will be used in cosmetic formulation.

MATERIALS AND METHOD

Sample Preparation **–** The cocoa pod husks were collected in bulk from the cocoa fermentation facility in Cocoa Research and Development Centre, Malaysian Cocoa Board, Hilir Perak, Selangor. The husks were washed with tap water thoroughly to remove debris and chopped into slices using Mechanical Chopper (FC-312, Zhaoqing Fengxiang Food Machinery, China)

prior to drying (High Performance Dryer, FD-825, Protech, Malaysia). The temperature of the dryer was set at 50ºC for 2 days to ensure the removal of unnecessary moisture from the cocoa pods. Dried cocoa pods were ground into powder at 1 mm using a grinding machine (Automatic Hammer Mill Grinder, China) and kept in a tight container for storage. One gram of cocoa pod powder was soaked with 100 ml of aqueous ethanol (GmbH, Germany) in a conical flask with cover and shaked at 120 rpm in the water bath shaker (BS-21, Lab Companion, Korea). Distilled water was used as a heating medium. The decanted portion of the extract was filtered using filter paper (Whatmann filter No.1) and subjected to solvent removal using rotary vacuum to dryness. Then, the extract was freeze-dried (Freeze Dryer, FDA8512, Labconco, USA). The sample was stored in a tight container at -20ºC until being used for analysis.

Total Phenolic Content (TPC) – Folin-Ciocalteau's method was used to determine the total phenolic content. Gallic acid standard (Sigma-Aldrich, UK) was used to obtain the calibration curve. The measurement was carried out using UV-Visible Spectrophotometer (Cary 60, Agilent, USA) at 765 nm.

Total Flavonoid Content (TFC) – The measurement of total flavonoids content (TFC) was carried out using the microplate reader (ThermoFischer, USA) at wavelength of 405 nm using aluminium chloride colorimetric assay. The method suggested by Chang (2002) was adapted. The samples expressed in milligram rutin equivalent per gram sample (mg RE/g).

Scavenging Effect – Stock solution of 0.2 M DPPH (MW 394.23 g/mol; 1,2-diphenyl-2-picrylhydrazyl; Aldrich, UK) was prepared in ethanol (Kollin Chemicals, Germany). Next, 1.2 mL of 0.2 M DPPH was diluted to 0.078 M of DPPH with 3 mL ethanol (GmbH) and 0.5 mL DMSO (Merck, USA). Extract samples were pipetted at 30 µL into microplate well, and 270 µL of DPPH solution was added. The assay was left for at least 10 min before the measurement at absorbance 550 nm using microplate reader (ThermoFischer, USA).

Ion Reducing Ability – Ferric Reducing Antioxidant Power or FRAP was depending upon color transformation of ferric tripyridyltriazine Fe(III)-TPTZ complex to the ferrous tripyridyltriazine Fe(II)-TPTZ by reductant (or extract) at low pH. Fe(II)-TPTZ has intensive blue color and can be monitored at maximum wavelength of 593 nm or 620 nm (Arnous, 2002; Rodrigues, 2011) in acidic condition. Methods by Arnous (2002) and Pulido (2000) were adapted to determine the FRAP values. Initially, ferric chloride and TPTZ solutions were prepared. Ferric chloride solution was made by dissolving 3 mM of Ferric chloride ($FeCl_3$, MW 198.83 g/mol, QRec) in 5 mM citric acid (monohydrate; MW 210.14 g/mol, Mallinckrodt) with distilled water. The TPTZ solution was made by dissolving 1 mM of TPTZ (2,4,6-tripyridyl-s-triazine, MW 312.33 g/mol, Fluka) in 0.05 M hydrochloric acid (Merck, 1.825 mL conc. HCL diluted with distilled water up to 1 L). The cocoa pod extract at 15 µL were added to 270 µL of TPTZ solution and were measured at 620 nm for initial reading using the microplate reader. Next, 15 µL of ferric chloride solution was added to each well and measured directly. Then, the mixture was incubated for 30 min at 30-37ºC with interval measurement of 2-3 min. The change in the absorbance ($\Delta A = A_{tmin} - A_{0min}$) was calculated and related to ΔA of Fe^{2+} standard solution, which was determined by the standard calibration curve.

Inhibition of Lipid Peroxidation – The methods proposed by Mariod (2010) and Othman (2007) with some modifications were used to measure the antioxidant activity of the extract by measuring the absorbance of mixture at 450 nm wavelength using microplate reader. The β-carotene solution was made by dissolving 2 mg of β-Carotene (MP Biomedicals) in 100 µL of chloroform (Merck). Linoleic acid (Sigma-Aldrich) at 0.2 mL and 2 mL of Tween20 (Merck) was added to the solution and mixed thoroughly. Immediately, 10 mL of distilled water was added to make a transparent emulsion. Cocoa pod extract at 20 µL and 200 µL of β-carotene solution was added into a microplate well. Absorbance was recorded at zero time and subsequent measurement for every 20 min for 2 h at 50ºC. Negative control was β-carotene in linoleic acid solution without any samples. Butylated hydroxytoluene (BHT, Sigma-

Aldrich) was used as a positive control. A calculation of antioxidant activity using β-carotene bleaching assay was obtained by measuring the degradation rate of the tested samples in the assay (Othman, 2007). Equation for calculation of degradation rate is;

$$\text{Degradation rate } (D_R) = \ln [a/b] \times 1/t \quad (4)$$

where; a = initial absorbance (time 0), b = absorbance at t and t = absorbance time (min). The degradation rate value was used to calculate the antioxidant activity by the following equation (5). Antioxidant activity(AC) by BCB is;

$$AC\ (\%) = (D_{Rcontrol} - D_{Rsample})/D_{Rcontrol} \times 100 \quad (5)$$

Serial dilution (at 7.8-1000 µg/mL) of cocoa pod extract was used in determination of antioxidant activity to obtain the effective concentration value. Effective concentration, EC_{50}, indicates the concentration of the sample required to scavenge the free radical at 50%. The lower the concentration obtained at EC_{50}, the higher the scavenging activity of an extract. Effective concentration at 50% is the concentration needed to exhibit antioxidant activity at 50%, with D or B in the subscript referring to DPPH and β-carotene bleaching assays, respectively.

Box Behnken Design of Experiment **–**Three levels of factors was adopted in this study with total runs of 15. The input variables or factors to be considered were solvent percentage (75-95%), time of extraction (15-45 min) and temperature of extraction (30-40^{o}C). The Box Bohnken design of experiment was summarized in Table 1 with the factors and dependent variables in run order. MINITAB Software version 14.12 was used to design the experiment and find the interaction of the variables.

Statistical of Analysis **–** All experiments were performed in triplicates dan data was tabulated by mean values. Validation of the optimized condition was performed in triplicate experiments. The average value of the experiments was compared with the predicted values of the optimized conditions and the significant difference was measured using t-test. In addition, Pearson correlation was used to investigate the relationship among the dependent variables. Correlation value is considered significant when its *p*-value is less than 0.05.

Table 1 Experimental design for solvent extraction of cocoa pod extract with antioxidant level and antioxidant activity

Run Order	Factors			Dependant variables							
				Antioxidant level		Antioxidant activity at 250 ppm			Effective concentration		
	solvent percentage (X_1)	temperature (X_2)	time of extraction (X_3, min)	total phenolic content (Y_1, mg GAE/g)	total flavonoid content (Y_2, mg RE/g)	Scavenging activity (Y_3, DPPH %)	Ion reducing ability (Y_4, µmol Fe^{2+}/L)	Inhibition of lipid peroxidation (Y_5, %)	EC_{50D} (Y_6)	EC_{50F} (Y_7)	EC_{50B} (Y_8)
1	85	40	45	80.93	77.52	97.19	653.68	49.19	0.005	73.15	3.21
2	95	40	30	364.35	178.17	91.25	168.07	61.05	0.178	97.01	1.85
3	85	30	15	329.52	160.26	87.50	547.62	64.17	-0.240	67.02	2.01
4	75	35	45	29.53	26.40	85.31	573.38	38.41	0.066	261.73	3.50
5	75	35	15	23.94	27.56	90.63	593.07	49.19	0.344	35.77	2.91
6	85	40	15	93.69	64.53	90.63	538.91	47.25	0.034	23.89	2.83
7	85	35	30	147.21	79.23	90.63	535.88	63.95	-0.043	45.70	1.73
8	85	30	45	115.83	70.51	89.38	526.22	61.14	0.034	31.99	5.61
9	95	30	30	499.06	550.15	81.88	398.38	65.29	0.871	50.33	-0.57
10	75	40	30	219.05	284.67	93.75	596.10	70.96	0.217	58.01	0.54
11	95	35	45	359.89	336.94	94.38	220.72	16.90	0.485	23.70	9.23
12	85	35	30	229.54	255.62	94.69	551.03	28.81	0.256	128.86	4.92
13	95	35	15	681.13	803.53	86.56	611.25	9.67	0.796	32.14	9.78
14	75	30	30	226.05	282.98	93.75	759.36	43.79	-0.056	72.58	4.69
15	85	35	30	228.77	248.90	94.69	503.30	51.47	0.1997	33.73	1.14

Data is tabulated by mean value of triplicates; Unit min is minutes; mg GAE/g is milligram gallic acid equivalent per gram; mg RE/g is milligram routine equivalent per gram; µmol Fe2+/L is micromole ferric; DPPH is 1,2-diphenyl-2-picrylhydrazyl; EC_{50} is effective concentration of the extract to inhibit oxidation process at 50 percent; where D for scavenging activity, F for ion reducing ability and B is inhibition of lipid peroxidation. The value can be negative as it was plotted from the graph of concentration of extract versus antioxidant activity (data not shown in this paper).

RESULTS AND DISCUSSIONS

The obtained experimental results of 15 runs are summarized in Table 1. Two dependent variables (TPC and EC_{50D}) were significantly affected by the independent variables (solvent percentage, temperature and time of extraction) in this experimental study. The TPC was represented by the linear regression equation (equation 1) affected by the solvent percentage and time of extraction. The equation 1 means that the level of TPC can be increased with longer extraction of time but on the other hand, increasing the amount of solvent percentage will result in vice versa. The EC_{50D} was predicted by the second order polynomial and interaction equation (equation 2) of solvent percentage and temperature of extraction.

$$Y_1 = 352.2 - 0.306\ X_1 + 0.088\ X_3 \quad \text{(Equation 1)}$$

$$Y_6 = -0.347605X_1 + 0.818505X_2 + 0.003168X_1^{\,2} - 0.00483X_1X_2 \quad \text{(Equation 2)}$$

$R^2 = 0.825$,
Lack of Fit, LoF = 0.603,
Y_1 = Total Phenolic Content
$Y_6 = EC_{50D}$; Effective Concentration to inhibit oxidant activity by 50%
X_1 = solvent percentage,
X_2 = temperature
X_3 = time of extraction

The contour of TPC (Y_1) is illustrated in Figure 1. It demonstrated that increasing the solvent percentage can increase the TPC value. This indicated that cocoa pod extract has more phenolic compounds with high polarity as in accordance to Chew (2011) that the polarity of the phenolic compound extracted was based on the polarity of the solvent concentration. In accordance with Chew (2011), there is no single certain solvent give the highest level of antioxidant compounds extracted. Longer time of extraction did not apparently the TPC value probably the compound was saturated in the extracting solvent.
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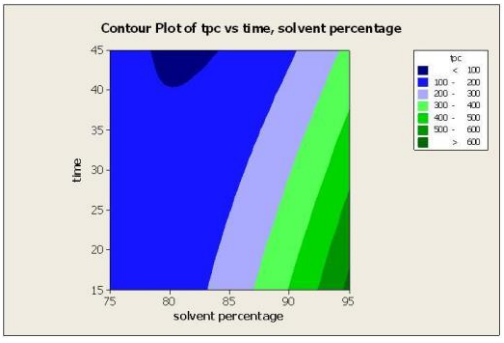

Figure 1: Contour Plot of Total Phenolic Content versus Time and Solvent Percentage

Figure 2 illustrated the effect of temperature and solvent percentage onto the EC_{50D} where targeted value (0.050) can be obtained at temperature of between 32ºC to 39ºC and solvent percentage of between 77% to 87%. The EC_{50D} value must be the lowest as possible (targeted at 0.050) to show that the extract exerted the antioxidant activity by scavenging the oxidizing ion at low concentration. At high temperature, the effectiveness was too low, while at high solvent percentage, high EC_{50D} value was obtained. When high EC_{50D} was obtained, it showed that a high amount of extract was needed to exert antioxidant activity which is uneconomical.

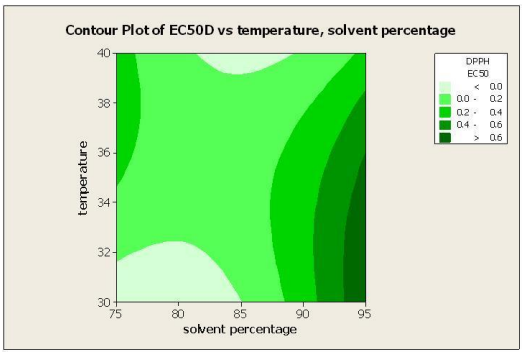

Figure 2: Contour Plot of EC_{50D} by Temperature and Solvent Percentage

To evaluate the model obtained from the experiment carried out, the optimization graph in Figure 3 was used to give us the solvent percentage, time and temperature of extraction based on the model of equation 1 and equation 2. We used the targeted value of TPC at 100 and EC_{50D} at 0.05 to get the better results. Based on the graph, the solvent percentage of 80.94% at 35.29ºC for 17.48 minutes was the optimum operating condition suggested by the model to obtain the high TPC level and effectiveness.

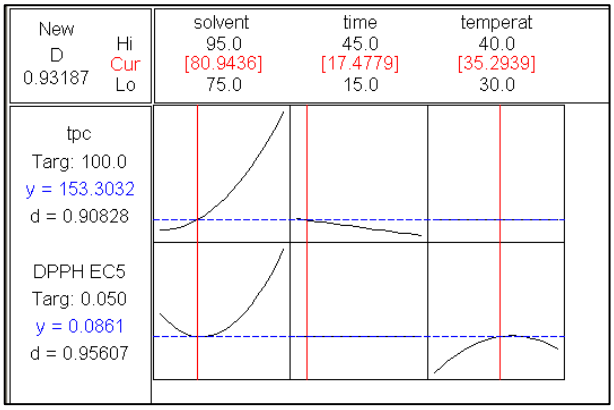

Figure 3: Optimization Graph for Total Phenolic Content and EC_{50D} by Solvent Percentage, Time and Temperature

The result show insignificant value of TPC and EC_{50D} was obtained when the process condition (solvent 80.94%, time of extraction 17.48 minutes and temperature 35.29°C) in Figure 3 were applied in the laboratory. Summary of the validation for data was in Table 2.

Table 2: Validation by Experimental Results

Variables	Optimization value	Experimental value	p-value
TPC	153.3032	132.79 ± 20.77	0.229
EC_{50D}	0.0861	0.1001 ± 0.0677	0.754

Table 3 summarized the significant correlation value of TPC and TFC indicating that the antioxidant level of cocoa pod extract was highly contributed by the phenolic and flavonoid compound. These compounds also contributed to the scavenging activity (EC_{50D}) of cocoa pod extract as high correlation values were obtained significantly. The other antioxidant activity measurements (ion reducing ability and inhibition of lipid peroxidation) were contributed by other antioxidant compounds that existed in cocoa pod extract.

Table 3: Correlation of the Dependent Variables

Dependent variables	Pearson correlation	P-Value
TPC *vs* TFC	0.925	0.000
TPC *vs* EC_{50D}	0.670	0.006
TFC *vs* EC_{50D}	0.785	0.001

TPC is Total Phenolic Content
TFC is Total Flavonoid Content
EC_{50D} is Effective Concentration to inhibition oxidation at 50% using DPPH

CONCLUSION

Utilizing a response surface design to optimize the operating condition revealed that although high TPC value can be achieved by increasing the solvent percentage, the effectiveness of the extract is also reducing.

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REFERENCES

Arnous, A.; Makris, D.P.; Kefalas, P. (2002). Correlation of pigment and flavonoid content with antioxidant properties in selected aged regional wines from Greek. *J. Food Comp. Anal.. 15*; 655-665.

Chang, C. C.; Yang, M. H.; Wen, H. M.; Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal. 10*(3), 178-182.

Mariod, A.A.; Ibrahim, R.M.; Ismail, M.; Ismail, N. (2010). Antioxidant activity of the phenolic leaf extracts from *Monechma ciliatum* in stabilization of corn oil. *J. Am. Oil Chem. Soc. 87*, 35-43.

Rodrigues, E.; Poerner, N.; Rockenbach, I.I.; Gonzaga, L.V.; Mendes, C.R.; Fett, R. (2011). Phenolic compounds and antioxidant activity of blueberry cultivars grown in Brazil. *Cienc. Tecnol. Aliment. Campinas. 31*(4), 911-917.

Othman, A.; Ismail, A.; Ghani, N.A.; Adenan I. (2007). Antioxidant capacity and phenolic content of cocoa beans. *Food Chem*., *100*: 1523-1530.

Chew, K.K.; Ng, S.Y.; Thoo, Y.Y.; Khoo, M.Z.; Wan Aida, W.M.; Ho, C.W. (2011). Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of *Centella asiatica* extracts. *Int. Food Res. J.*, *18*: 571-578.

Hii, C.L., Chiow, F.S. (2006). Development of Particle Board from Cocoa Shells. Malaysian Cocoa Journal, 2:22-27.

Karim, A.A.; Abdullah, N. A. (2012). Stability study of cocoa butter body scrub formulated with ground cocoa pod husk. *Malaysian Cocoa J. 7*: 42-47.

Agbeniyi, S.O., Oluyole, K.A. and Ogunlade, M.O. (2011). Impact of cocoa pod husk fertilizer on cocoa production in Nigeria. *World J. Agric. Sci. 7*(2), 113-116.

Chuang, K.J.; Chen, Z.J.; Cheng, C.L.; Hong, G.B. (2018). Investigation of the antioxidant capacity, insecticidal ability and oxidation stabilty of *Chenopodium formosanum* seed extract. *Int. J. Mol. Sci*. *19*: 2726.