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Modeling and Optimization of *Mitragyna speciosa* Extraction using Box Behnken Design

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ABSTRACT

The extraction of kratom (*M. speciosa*) leaf powder was optimized with preliminary extraction to be further optimized with the Box-Behnken experimental design. The individual and interactive effects of process variables (sample-to-solvent ratio, extraction time, solvent concentration) were assessed. The preliminary extraction results showed that ultrasound-assisted extraction (UAE) and methanol were chosen for further optimization. The experimental data were analyzed by Pareto analysis of variance (ANOVA) and second-order polynomial models were developed using multiple regression analysis. The model developed showed a good fit with the experimental data with a high coefficient of correlation (R^2) and predictive ability (predicted R^2). An optimization study was performed and the optimal extraction conditions were sample-to-solvent ratio value 1.5:10; extraction time of 10 minutes, and methanol concentration of 100%.

Keywords: Kratom; Mitragyna speciosa; mitragynine; Ultrasound-assisted extraction; extraction optimization

INTRODUCTION

Mitragyna speciosa (Korth.) Havil., also known as kratom, is an endogenous plant from Southeast Asia (Rech *et al*., 2015). The species *M. speciosa* is a member of the Rubiaceae (coffee) family, which is very diverse, consisting of small shrubs to colossal trees (Razafimandimbison and Bremer, 2001; Raffa *et al*., 2013). The Genus of Mitragyna is known to be a tree or shrubs with opposite leaves, the flowers are sessile and arranged in compact globose heads, properties which *Kratom* inherit. On average, *Kratom* could grow to a height of 3-4 m, although it is capable to grow up to 25 m tall, with its stem diameter up to 1 m. The trunk is usually straight, with smooth outer bark and gray. Its leaves are ovate-acuminate in shape, with glossy dark green color and could grow to over 14-20 cm in length, and 7-12 cm wide (Raffa, 2014; Raffa *et al*., 2013; Ratsch, 2005). Kratom has been used traditionally for various treatments, including fatigue, cough, pain, colds, diarrhea, diabetes, hypertension, increased stamina and sexual prowess, and opium withdrawal (Suwanlert, 1975; Chua and Schmelzer, 2001; Ratsch, 2005; Assanangkornchai *et al*., 2007; Tanguay, 2011; Singh *et al*., 2019). Due to its opioid activity, kratom gaining popularity as a recreational psychoactive drug (Prozialeck *et al*., 2012; Cinosi *et al*., 2015). Due to the opioid activity and increased use of Kratom, the plant is deemed dangerous in some countries, including Indonesia (EMCDDA, 2012; Great Britain, 2016; Byrne, 2017). In 2008 kratom was banned for processed food, followed by restriction for sale as traditional medicine and health supplement in 2016 and expected to be deemed illegal in 2022 (Badan Pengawas Obat dan Makanan, 2016; Andilala, 2019; Rokib, 2019). In 2020 the plant was enlisted in medicinal plant classification by Ministry of Agriculture of Indonesia (Menteri Pertanian Republik Indonesia, 2020).

Recently ultrasound-assisted extraction (UAE) has gained favor, due to the common extraction method having a prolonged extraction time, requiring a huge amount of solvent, and low efficiency. This could be overcome with the use of the hot extraction method, but its incompatibility with thermolabile or volatile compounds in the raw material is a major drawback (Mandal *et al*., 2015). UAE method reduced the extraction time, solvents used to gain higher extraction content. UAE has a lower operating temperature when compared with the hot method, which means less degradation due to high-temperature exposure, making it very useful for thermolabile compounds (Ahmad *et al*., 2015; Chemat and Strube, 2015).

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Table I. Mobile phase gradient used for kratom analysis

Minute	% A	% B
0	95%	5%
3-9	25%	75%
12-end	80%	20%

Table II. Independent variable level description for extraction optimization

Level	-1	0	+1
Sample-to-solvent ratio(X1)	0.5: 10	1: 10	1.5: 10
Extraction time(X2)	1 hour	2 hours	3 hours
Methanol concentration(X3)	Methanol 70%	Methanol 80%	Methanol 90%

METHODOLOGY

Materials

Kratom powder was purchased from an online vendor. Other materials used include aqua dest, 70% ethanol, 96% ethanol, acetic acid, methanol p.a., chloroform, ethyl acetate, ammonia solution, methanol pro-HPLC, formic acid pro HPLC, and acetonitrile pro HPLC.

Methods

Preliminary extraction is conducted by comparing two different methods: Macerationand Ultrasound-assisted extraction based on research using five different solvents: methanol 70%, 0.5M acetic acid in methanol 70%, chloroform, ethanol, and ethyl acetate. Preliminary extraction will be carried out with 2 g of sample with 20 g solvent (1:10 sample-to-solvent ratio) and 30 minutes extraction time for ultrasound-assisted extraction, and 24 hours for maceration. The acquired extract is then filtered using a membrane filter and analyzed using HPLC and TLC-densitometry. After analysis, Box Behnken experimental design was used for further extraction optimization.

The extract obtained is developed in a TLC system based on research conducted by Kowalczuk *et al*. (2013). The mobile phase used is hexane: ethyl acetate: 25% ammonia solution (30:15:1 v/v/v) with TLC Silica Gel Plates 60 F_{254} as stationary phase. Developed chromatograms then dried and examined with CAMAG® TLC Scanner 3 at λ 254nm and sprayed with Dragendorf reagent. The HPLC analysis is based on a method by Fu *et al*. (2015). The analysis is carried out using Hitachi L-2130 coupled with Hitachi L-2420 UV-Vis Detector in conjunction with Merck LiChrosphere RP-18 (5 μm) 4.0 x 125 mm as stationary phase and gradient of formic acid 0,1% solution (A) and acetonitrile (B) at 0,7 mL/minutes for 20 minutes with a reading by UV detector at 247 nm. The gradient system is shown in Table I.

RESULT AND DISCUSSION

Preliminary extraction

The preliminary extraction is conducted in search of the method and solvent used for further optimization. Two methods of extraction that have been examined were maceration for 24 hours and ultrasound-assisted extraction (UAE) for 30 minutes, and the five solvent types examined were methanol, 0.5M acetic acid in methanol, chloroform, ethyl acetate, and ethanol. The extract obtained was then analyzed with TLC-densitometry and HPLC. ANOVA analysis (α=0,05) was done towards the data obtained. The result showed that for the HPLC method, methanol, acetic acid 0,5M in methanol, and ethanol have no significant statistical difference between them (p-value = 0,527). For TLC densitometry, only methanol and acetic acid 0.5M in methanol showed no significant statistical difference (p-value = 0,207). Maceration and UAE do not show significant statistical difference with methanol as the solvent in both method (TLC p-value = 0,802; HPLC p-value = 0,482). Methanol is selected as the solvent of choice due to easier procurement with no additional preparation step needed, better safety factor, and cheaper with no statistical difference. While UAE is selected as the method of choice due to its ability to extract *M.speciosa* in a short time.

Analysis and verification

Optimization by Box-Behnken experimental design

Optimization is carried out using 15 points Box-Behnken experimental design for three factors with three levels each. Box Behnken design was selected since it requires fewer runs than the central composite design for three variables. The design is shown in Table II. While the design matrix and observed results are shown in Table III.

Table IIIa. Box Behnken design matrix and observed results

RunOrder	X_1	X_2	X_3	Observed value (HPLC)	Predicted Value (HPLC)	Observed value (TLC)	Predicted Value (TLC)
1	0	1	-1	91789	85249.44	9791.5	10526.3
2	0	1	-1	82648	85249.44	11266.0	10526.3
3	0	1	1	103814	106424.4	13421.7	15998.8
4	0	0	0	92707	89627.69	12552.6	13468.1
5	1	1	0	135392	135254.2	15855.4	17867.7
6	0	-1	-1	76762	80961.44	10778.7	11624.3
7	0	-1	-1	82081	80961.44	12417.7	11624.3
8	-1	0	-1	34065	40059.21	6866.7	6810.8
9	0	0	0	83281	89627.69	15560.7	13468.1
10	0	-1	-1	84988	80961.44	11661.0	11624.3
11	0	-1	1	124418	106436.4	13109.8	15050.8
12	0	1	-1	88282	85249.44	11430.5	10526.3
13	0	-1	1	109344	106436.4	14707.8	15050.8
14	1	0	-1	113790	107607.7	11787.2	13463.7
15	0	-1	-1	74174	80961.44	11367.0	11624.3
16	-1	0	1	51422	45571.71	8763.8	8602.8
17	-1	1	0	42319	52331.21	8164.7	8165.3
18	1	0	1	147420	148745.2	17113.5	20570.7
19	1	0	-1	101323	107607.7	13325.4	13463.7
20	1	0	-1	112435	107607.7	14774.9	13463.7
21	-1	0	1	43920	45571.71	9042.3	8602.8
22	1	1	0	140860	135254.2	19132.5	17867.7
23	-1	1	0	47479	52331.21	8398.0	8165.3
24	1	0	1	130066	148745.2	21875.0	20570.7
25	0	0	0	93391	89627.69	13428.7	13468.1
26	-1	0	-1	43711	40059.21	6812.5	6810.8
27	0	0	0	85401	89627.69	12628.0	13468.1
28	1	-1	0	133805	135554.2	15834.8	17550.7
29	-1	-1	0	39133	47755.21	8955.8	8632.3
30	0	0	0	89103	89627.69	12163.3	13468.1
31	-1	-1	0	54595	47755.21	8744.4	8632.3
32	-1	1	0	57967	52331.21	7901.6	8165.3
33	1	1	0	127361	135254.2	19050.6	17867.7
34	1	1	0	141953	135254.2	17695.8	17867.7
35	0	0	0	88339	89627.69	14414.5	13468.1
36	0	0	0	84687	89627.69	14758.9	13468.1
37	-1	0	-1	59229	40059.21	6964.6	6810.8
38	1	-1	0	145507	135554.2	18135.5	17550.7
39	0	1	1	120668	106424.4	19059.6	15998.8
40	0	0	0	88233	89627.69	13647.1	13468.1
41	0	0	0	85212	89627.69	12176.1	13468.1
42	-1	1	0	53432	52331.21	7683.6	8165.3
43	-1	0	-1	33610	40059.21	7081.5	6810.8
44	0	0	0	99851	89627.69	15159.7	13468.1
45	-1	-1	0	57130	47755.21	8682.2	8632.3
46	-1	0	1	50846	45571.71	9234.6	8602.8
47	0	0	0	97520	89627.69	14033.2	13468.1
48	1	0	1	153461	148745.2	22659.1	20570.7
49	1	0	1	116082	148745.2	21143.1	20570.7
50	0	1	1	100235	106424.4	16268.4	15998.8
51	0	-1	1	93680	106436.4	16727.6	15050.8
52	0	1	1	106619	106424.4	16507.2	15998.8

Table IIIb. Box Behnken design matrix and observed results

RunOrder	X_1	X_2	X_3	Observed value (HPLC)	Predicted Value (HPLC)	Observed value (TLC)	Predicted Value (TLC)
53	0	-1	1	100538	106436.4	15780.8	15050.8
54	0	0	0	87525	89627.69	12431.7	13468.1
55	0	1	-1	75879	85249.44	10483.2	10526.3
56	1	0	-1	100567	107607.7	13686.3	13463.7
57	1	-1	0	133805	135554.2	19624.7	17550.7
58	1	-1	0	137043	135554.2	18008.2	17550.7
59	-1	-1	0	35433	47755.21	8776.5	8632.3
60	-1	0	1	38230	45571.71	8640.7	8602.8

The second-order polynomial equation expresses the relationship of Box–Behnken experimental design model and the input variables with interaction terms was fitted between obtained experimental results, as shown here:

$$HPLC\ peak = 4787 + 602844\,X_1 - 591\,X_2 + 69\,X_3 - 1235196\,X_1^2 = +15{,}46\,X_2^2 - 1{,}67\,X_3^2 - 1219\,X_1 * X_2 + 7125\,X_1 * X_3 = -2{,}15\,X_2 * X_3$$

$$TLC\ area = 2417 + 61588\,X_1 - 137{,}5\,X_2 + 55{,}2\,X_3 - 270446\,X_1^2 = +0{,}655\,X_2^2 - 0{,}668\,X_3^2 + 196\,X_1 * X_2 + 1063\,X_1 * X_2 + 1{,}023\,X_2 * X_3$$

The experimental data were evaluated by ANOVA and the significance of the variables were evaluated. Given the p-values of each variable, it could be determined that for both HPLC and TLC analysis that two linear coefficients (X_1, X_3) and one interactive coefficient (X_1X_3) were significant. There is a difference in quadratic coefficient between the two methods, X_2^2 for HPLC and X_1^2 for TLC. The P-value of the HPLC model (0,000) indicates that the model can explain the variation in response. The R^2 of 95,30 % and P-value of lack-of-fit (0,612) indicate that the HPLC model fits with the data without significance lack-of-fit. For TLC, P-value of model (0,000), R^2 of 91.79% and P-value of lack-of-fit (0,904) indicate the same. The predicted R^2 value of 90.84% (HPLC) and 88.09% (TLC), shows that both of the models have a good predictive ability.

Accuracy of the model

The residual plot for the experimental data is presented in Figure 1 and Figure 2. The residuals normal probability plot and residuals histogram for both HPLC was normally distributed. The residuals versus fits are used to verify the assumption that the residuals are randomly distributed and have constant variance, while residual versus plots are to verify the independence of each residual. No recognizable patterns are present in both verses fit and versus order. For TLC, a slight long tail is detected in the normal probability plot and a fanning pattern is also detected in residual versus fits, indicating a difference in variance between data. However, as the histogram is not skewed, Box-Cox Transformation is not needed. The residual versus order plot shows no trends or patterns.

Effect of process

The mathematical model created showed that the sample-to-solvent ratio has a positive value towards peak height response. This indicates that the higher the sample-to-solvent ratio is, the higher response will be achieved. This happens due to the higher concentration gradient in a higher sample-to-solvent ratio raising the rate of mass transfer of soluble active compounds. The rate of mass transfer gradually decreases as the concentration of active principle in the solvent increases, until equilibrium is reached (Handa *et al*., 2008). It should be noted that a higher gradient concentration does not mean the extraction will be optimum, as a higher sample amount could result in solubility saturation.

Extraction time is one of the most influencing factors to extraction, including UAE. Longer extraction time leads to more comprehensive extraction but risks exposure to a higher temperature that can evaporate the solvent, degrade the active compounds and reduce the intensity and effectivity of cavitation (Mandal *et al*., 2015). The extraction time has a negative effect on the response. This suggests that only a short period is needed for optimal extraction, and further extraction is not effective while also bearing the risk of high-temperature exposure. It should be noted that the study is done using an ultrasound bath, so alteration and evaluation of energy output

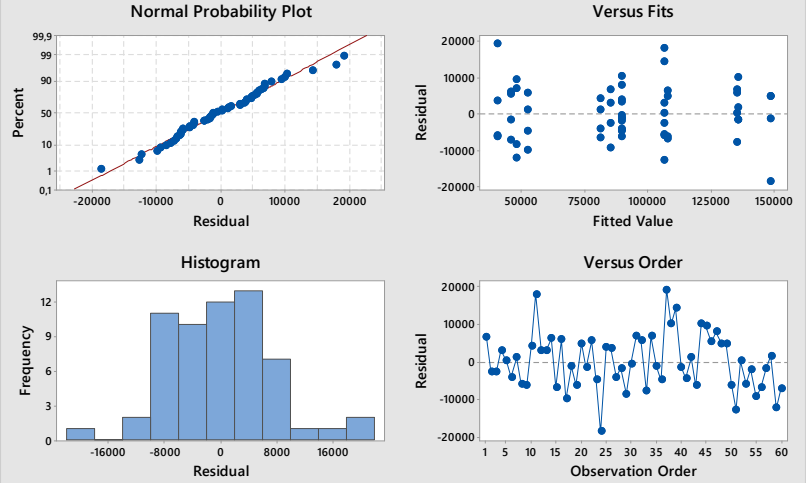


Figure 1. Residual Plot for HPLC analysis

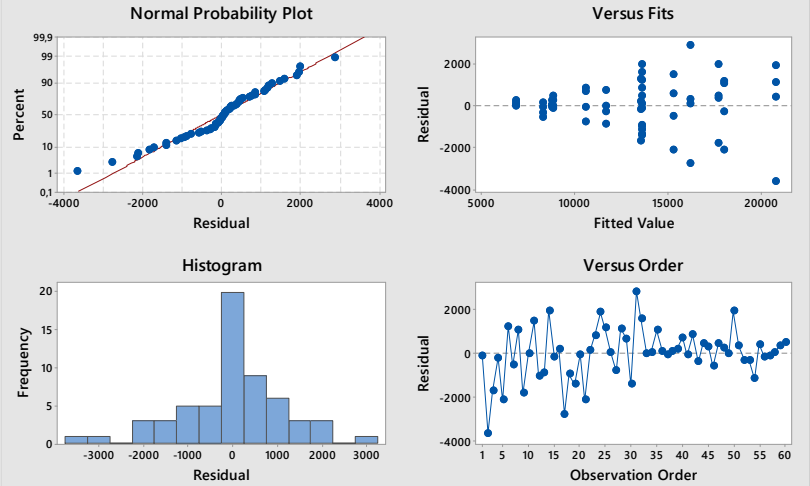


Figure 2. Residual plot for TLC analysis

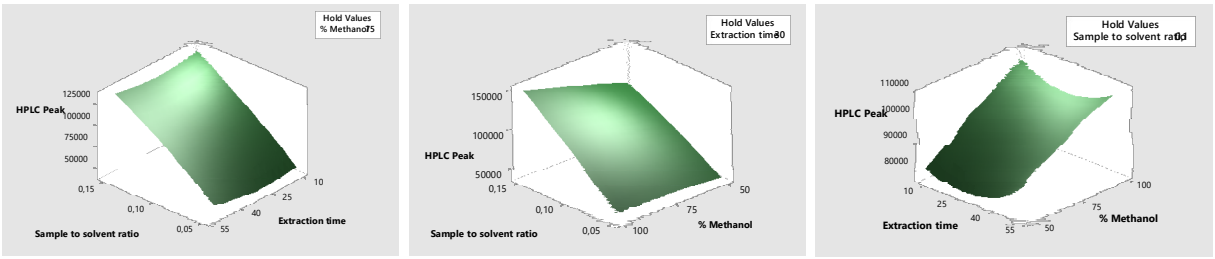
might become a challenge. Rest time between cycle, change in medium, or method for controlling medium temperature is needed for consistent extraction.

Methanol concentration has a positive effect on the response, it means that the higher the methanol concentration, the more effective the extraction takes place. This could be related to a change in polarity, where the more dilute methanol will increase its polarity, and alter its ability to dissolve the desired compounds. The optimum methanol concentration is 100%, this concentration is conducted using pro analysis grade methanol. The effects of variables can be seen as response surface models in Figure 3 and Figure 4.

Optimization and confirmation

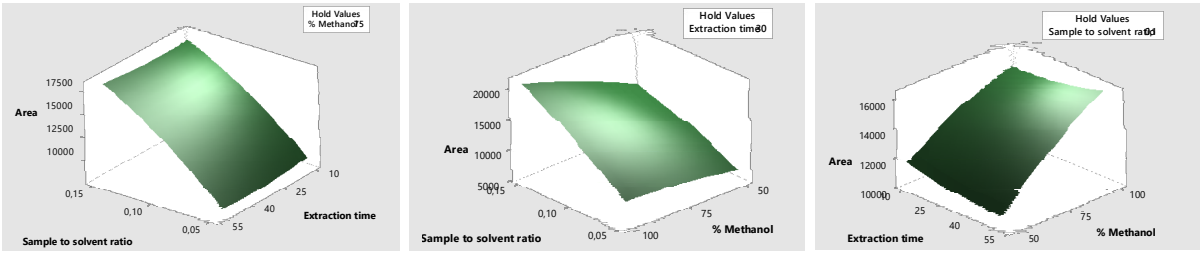
The mathematical model created for HPLC response showed that the optimized extraction system is as follows: sample-to-solvent ratio value 1.5:10; extraction time of 10 minutes, and methanol concentration of 100%, with predicted mean of peak height value range of 146637 to 165598 ($\alpha = 0.05$), and predicted standard error of 4750 (RSD 3,04%). Verification of the optimized system resulted in a mean peak height value of 158536. The mathematical model for the TLC method suggests that the optimized extraction system is as follows: sample-to-solvent ratio value 1.5:10; extraction time of 50 minutes, and methanol concentration of 100%. with predicted mean of peak height value range of 405,8 to 451,0 ($\alpha = 0.05$), and predicted standard error of 11,3 (RSD 2,64%). Verification of the optimized system resulted in a mean peak height value of 19719,9.

The difference in extraction time between the HPLC model and TLC model might happen due to the difference in the separation ability of the systems. Confirmation of the extraction time using



(a) (b) (c)

Figure 3. Response-surface 3D graph of (a.) the effect of sample-to-solvent ratio and extraction time; (b.) the effect of sample-to-solvent ratio and methanol concentration; (c.) the effect of extraction time and methanol concentration on HPLC method



(a) (b) (c)

Figure 4. Response-surface 3D graph of (a.) the effect of sample-to-solvent ratio and extraction time; (b.) the effect of sample-to-solvent ratio and methanol concentration; (c.) the effect of extraction time and methanol concentration on TLC method

ANOVA for HPLC (p-value = 0,054; α = 0,05) and TLC (p-value = 0,269; α = 0,05) suggest that there is no statistical difference between both times. Thus, 10 minutes is selected to be the preferred extraction time using ultrasound-assisted extraction due to the shorter extraction time.

CONCLUSION

In the present study, Box-Behnken response surface design was carried out to optimize and study the effects of the process variable (sample-to-solvent ratio, time, solvent concentration) on kratom extraction using UAE. Preliminary extraction is conducted beforehand to determine the method and the solvent to be optimized further. Statistical analysis and mathematical optimization were performed using Minitab® 18.1. The second-order polynomial models were developed for predicting response for both HPLC and TLC. The response surface plots were built for estimating the interaction between the process variable on the responses. The results showed that the sample-to-solvent ratio was the most responsible variable compared to the other two variables. This indicates that a higher ratio might be applicable, although it might lead to solubility saturation and inhibit the extraction performance. The optimized condition for response maximization is sample-to-solvent ratio value 1.5:10; extraction time of 10 minutes, and methanol concentration of 100%. Verification under the optimized condition the response agreed closely with the predicted response. This study can be useful for the development of analytical methods, including detection, and quantification for characterization and forensic use.

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