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Application of fractional factorial design to optimize extraction method of artemisinin from *Artemisia annua*

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ABSTRACT: Fractional factorial design was applied to optimize the extraction process of artemisinin from *Artemisia annua* L. Three different techniques i.e., Soxhlet, maceration, and microwave-assisted extraction were optimized to determine the optimum extraction conditions. Thin layer chromatography and high performance liquid chromatography were used to determine the yield of artemisinin in the sample. The optimum condition was obtained by immersing 0.5 g of sample in 3 ml of chloroform for 2 min. The amount of artemisinin extracted from the sample was $47.9 \pm 0.6 \times 10^{-3}$% w/w. In addition, the predicted values calculated by the model were in good agreement with the experimental values.

KEYWORDS: optimization, dummy variables, ANOVA, TLC, HPLC, antimalarial

INTRODUCTION

Artemisinin (Fig. 1) is an endoperoxide sesquiterpene lactone that has an antimalarial activity, even to the *Plasmadium falciparum* strains resistant to conventional drugs[1]. This compound is isolated from *Artemisia annua* L. which is mostly grown in Asia. Artemisinin mainly accumulates in the leaves and flower buds in concentrations in the range 0.01–1% w/w, depending on the plant origin, stage of development, and cultivation conditions[1–5]

Due to its antimalarial activity, artemisinin has become available commercially as antimalarial drug[6–9]. Although this compound has been successfully synthesized, the synthesis is as expensive as the extraction of artemisinin from natural products. Thus extraction of artemisinin from *A. annua* is still the most preferable technique. In past decades, even though such extraction techniques of artemisinin have been widely reported, yet no study has been conducted to provide a better understanding on how the compound behaves as different extraction techniques of artemisinin from *A. annua* are combined. Previous studies are based mainly on a single extraction technique[4,5]. Microwave-assisted extraction[5] or super critical CO_2 extraction procedures[4] have been used without comparing the results with other extraction methods.

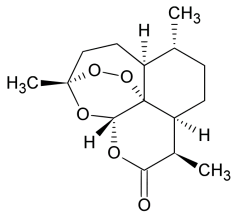

Fig. 1 Structure of artemisinin.

Currently, there are no optimization studies that focus on using different types of extraction procedures as one of the independent variables that could have a significant effect on artemisinin yield as dependent variable. Since extraction method is crucial to get the maximum amount of artemisinin from the plant, this paper presents a study on the optimization of extraction methods and some other important parameters on the extraction procedure of artemisinin with the use of fractional factorial design (FFD). Both categorical and numerical variables were used to study the effect of such extraction procedure in extracting artemisinin

from *A. annua*. In this study, three parameters, extraction methods (categorical variable), the ratio between sample and solvent (numerical variable), and extraction time (numerical variable) were optimized to get the highest yield of artemisinin as its response. The total artemisinin was analysed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). From the optimization process, a mathematical model was generated to predict the amount of artemisinin extracted from the sample for different values of the variables, and to describe the significance and the effect of such important variables to the artemisinin yield; in this case, extraction time was also included as a variable.

EXPERIMENTS

Materials

A. annua samples were provided by Medicinal Crops Research Institute, Ministry of Health, Indonesia. As extraction solvent, analytical reagent grade of hexane, chloroform, and methanol were purchased from Merck (purity 98%). A GF254 silica gel plate was used in thin layer chromatography analysis. An HPLC grade of methanol, acetonitrile, and ethyl acetate (Merck, purity 99%) were used as mobile phase in HPLC analysis. Pure artemisinin from Sigma Aldrich (purity 98%) was used as a standard.

Extraction Method

In this study, three different extraction methods, namely Soxhlet, maceration, and microwave-assisted extraction (MAE), were used to extract artemisinin from the plant sample. Two independent numerical variables, namely the sample/solvent ratio and the extraction time, were varied to optimize the extraction of artemisinin. For each type of categorical variable (extraction methods), the range of numerical variables (sample/solvent ratio and extraction time) are different. All three independent variables (extraction method, sample/solvent ratio, and extraction time) were combined randomly by the software based on fractional factorial design (FFD) rules to optimize the effect of those three variables to the yield of artemisinin. A series of trials were conducted based on this experimental design randomly generated by FFD. The extraction methods used in this study were the following:

Soxhlet extraction

In the Soxhlet extraction method, the samples were extracted with hexane using three different sample/-solvent ratios (25/250, 50/250, and 100/250 g/ml) during three different times (90, 180, and 360 min). Following the extraction process, the filtrates were collected and then concentrated using rotary evaporator until a waxy paste was obtained. These paste filtrates were then diluted in hexane (with the ratio 12 ml of hexane for each gram of concentrated filtrate) and left overnight. This hexane-filtrate solution was then filtered and the hexane-soluble phase was partitioned using liquid-liquid extraction with acetonitrile. The acetonitrile phase was then used in further quantifications in TLC and HPLC analysis.

Maceration extraction

In the maceration technique, samples were immersed in chloroform at three different sample/solvent ratios (0.5/3, 1/6, and 2/12 g/ml) for three different extraction times (0.5, 1, and 2 min). After the extraction, the solutions were filtrated and the filtrates used in further quantifications in TLC and HPLC analysis.

Microwave-assisted extraction (MAE)

In MAE, samples were extracted with hexane at three different sample/solvent ratios (0.5/30, 1/60, and 2/120 g/ml) for three different extraction times (7.5, 15, and 30 min) using microwave extraction apparatus. After the extraction, the solutions were then filtrated to separate the raw plant sample and the extract filtrate. These filtrates were then partitioned using liquid-liquid extraction technique with acetonitrile. The acetonitrile phase was then used in further quantifications in TLC and HPLC analyses.

Quantification of artemisinin

The existences of artemisinin in the sample were confirmed both qualitatively and quantitatively using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). In the qualitative analysis using TLC, artemisinin standard and extract solutions obtained from the extraction process were spotted onto the silica gel GF254 TLC plate. This TLC system was then developed using 7.5% of ethyl acetate in chloroform as its mobile phase. To detect the presence of artemisinin in the sample, the plate was sprayed with 50% of sulphuric acid and then heated at 100 °C for an hour. Comparison of retention factor (R_f) of standard and sample was used to determine which spot belongs to artemisinin.

For quantitative analysis, HPLC analysis was carried out to identify and quantify the presence and the amount of artemisinin in the sample using a Hitachi HPLC instrument (pump model L-2130, column oven model L-2300) equipped with UV-Vis Detector (model L-2420). The analysis was conducted with

pre-column method, in which all extract solutions were evaporated from their solvent, and then diluted with 200 µl of methanol and 800 µl of NaOH 0.2%. The solutions were then heated at 50 °C for 30 min, and then another 200 µl of methanol and 800 µl of acetic acid 0.05 M were added to the solution. The final solutions were then injected to the HPLC instrument based on a described method[7], using a C-18 column (Li-Chrospher, diameter: 4.6 mm, length: 125 mm). The mobile phase used was methanol/acetonitrile/buffer, 0.9 mM Na_2HPO_4, and 3.6 mM NaH_2PO_4 (pH: 7.76) in a ratio 45/10/45. Flow rate was maintained at 0.3 ml/min, injection volume was 20 µl, and detector wavelength was at 260 nm.

Experimental design and optimization

To investigate the effect of tested independent variables to the response within the investigation range, a fractional factorial design (FFD) analysis was performed. In FFD, the number of experimental points is expressed as j^{k-1}, where j is the number of factors tested and k is the number of levels. Since there were three factors and three levels, this results in nine experimental points that were carried out in random order. In addition, to model the relationship between the responses and the variables tested, the data were subjected to a multiple linear regression analysis.

Since the extraction method is a categorical data, to use it in the regression analysis it needs to be converted to a "dummy" variable. A dummy variable is a numerical variable that is used to represent a binary categorical variable[8]. The number of dummy variables required to represent categorical data with multiple levels (n) is expressed as ($n-1$). Thus since there are three different extraction methods to be optimized ($n = 3$), two dummy variables are required to represent them (assigned as $D1$ and $D2$). Table 1 presents the two dummy variables used to represent the extraction method. In addition, all the numeric variables (the ratio of sample/solvent and extraction time) are assigned three successive coded levels. The high, middle, and low range are assigned the coded levels 2, 1, and 0.5, respectively. Table 2 presents the range of numeric independent variables and their coded level. In this study, each point was carried out in two repetitions. Those experimental designs (in coded level) are presented in Table 3.

Statistical analysis

To investigate the success of the optimization process, ANOVA was used for graphical analysis of the data. The coefficient of determination, R^2, was used to check on the quality of model generated, and the Fisher's F-test to test its statistical significance, using the same program, with P-value (probability of error) evaluated at 95% confidence level.

Table 1 Dummy variables used for representing extraction method.

Extraction Method	$D1$	$D2$
Soxhlet	0	1
Maceration	1	0
Microwave-Assisted Extraction (MAE)	0	0

Table 2 The two numeric independent variables and their corresponding coded level.

Independent variables	Coded factor level		
	0.5	1	2
Sample/solvent ratio (g/ml)			
Soxhlet	25/250	50/250	100/250
Maceration	0.5/3	1/6	2/12
MAE	0.5/30	1/60	2/120
Extraction time (min)			
Soxhlet	90	180	360
Maceration	0.5	1	2
MAE	7.5	15	30

Validation of experiment

The mathematical model generated by the software during FFD implementation was validated by conducting an experiment on the given optimal setting.

RESULTS AND DISCUSSION

Extraction and quantification of artemisinin

Based on the experimental design generated by FFD in Table 3, nine extraction processes were conducted to extract artemisinin from the sample. Quantitative and

Table 3 3^{k-1} Fractional factorial experimental design for extraction of artemisinin (in coded level).

Run	Extraction method	$D1$	$D2$	Sample/solvent ratio	Extraction time
1	Soxhlet	0	1	0.5	0.5
2	Soxhlet	0	1	1	2
3	Soxhlet	0	1	2	1
4	Maceration	1	0	0.5	1
5	Maceration	1	0	1	0.5
6	Maceration	1	0	2	2
7	MAE	0	0	0.5	2
8	MAE	0	0	1	1
9	MAE	0	0	2	0.5

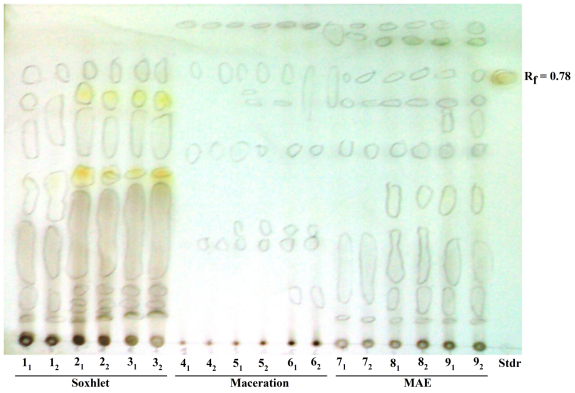

Fig. 2 TLC chromatogram for extraction of *A. annua* and standard of artemisinin (k_1: k run, first repetition; k_2: k run, second repetition; $k = 1, 2, \ldots, 9$).

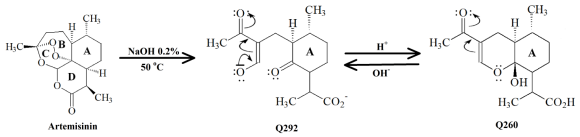

Fig. 3 Chemical reaction pathways for artemisinin derivatization to Q260[7].

qualitative analysis were then conducted to quantify the response.

The chromatogram shows a spot at $R_f = 0.78$ showing that all extraction procedures had successfully extracted artemisinin from the sample (Fig. 2). In addition, it can also be inferred that both Soxhlet and MAE extracted more substances than maceration, as evidenced by the larger number of spots. A possible explanation is that these techniques use thermal (Soxhlet) and microwave (MAE) energy to break cell membrane of the plant.

In the quantitative analysis conducted with the pre-column technique, artemisinin is previously converted into Q260 before injection into the HPLC instrument. Q260 is an artemisinin derivative compound that can strongly absorb UV light at 260 nm. Many studies have been conducted to determine the chemical derivation pathways of artemisinin to Q260[7,9,10]. These chemical reaction pathways are presented in Fig. 3.

Based on the given chemical reaction pathways, artemisinin was hydrolysed using base and acid to form Q260. At first, artemisinin was hydrolysed using NaOH in order to break the ring B, C, and D (lactone) on the artemisinin structure to form Q292. Afterwards, Q292 was hydrolysed again using acetic acid. In this process, a proton (H^+) activates a pair

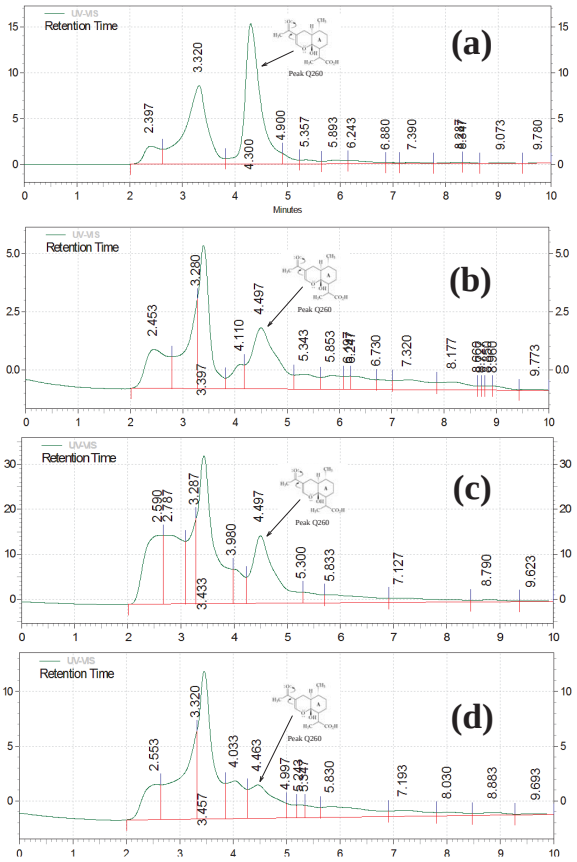

Fig. 4 HPLC chromatograms of (a) standard artemisinin 20 ppm; (b) extract from 360 min of Soxhlet extraction at the ratio sample/solvent of 50/250 g/ml (Run 2); (c) extract from 2 min of maceration extraction at the ratio sample/solvent of 2/12 g/ml (Run 6); (d) extract from 30 min of MAE method at the ratio sample/solvent of 0.5/30 g/ml (Run 7).

of free electrons in the oxygen to form a heterocyclic piran ring that integrates to ring A to form Q260. In the HPLC chromatogram (Fig. 4) the peak of Q260 was observed at a retention time of approximately 4.3 min. From the calculated yields (Table 4), it is obvious that maceration techniques extracted more artemisinin than Soxhlet and MAE. The peak area for Q260 for maceration (Fig. 4c) was larger than both in the Soxhlet (Fig. 4b) and MAE (Fig. 4d).

Apparently, Soxhlet and MAE procedures extract most of the plant soluble components. But they also extract massive amounts of large compounds (e.g., chlorophyll) that may interfere with HPLC (clogging) or MS (matrix). Even though additional sample preparations can be conducted, such as filtering, evaporating, or specific solid phase extraction, to reduce the amount of these interferences, the procedures are

Table 4 Yield of artemisinin in the sample based on HPLC analysis.

Run	$D1$	$D2$	X_1	X_2	Yield of artemisinin $\pm$ SD[a] ($\times 10^{-3}$% w/w)
1	0	1	0.5	0.5	3.04 ± 0.05
2	0	1	1	2	8.23 ± 0.03
3	0	1	2	1	3.22 ± 0.06
4	1	0	0.5	1	34.3 ± 0.2
5	1	0	1	0.5	42.7 ± 0.3
6	1	0	2	2	46.05 ± 0.04
7	0	0	0.5	2	38.92 ± 0.09
8	0	0	1	1	35.21 ± 0.08
9	0	0	2	0.5	19.34 ± 0.04

X_1: Sample/solvent ratio.
X_2: Extraction time.
[a] SD: standard deviation

Table 5 Numerical optimization for fractional factorial design, in terms of coded level.

No.	$D1$	$D2$	X_1	X_2	Predicted yield $\pm$ SD[a] ($\times 10^{-3}$% w/w)	d
1	1	0	0.5	2	47.9 ± 0.6	0.985*
2	1	0	1	2	46.7 ± 0.6	0.937
3	1	0	1	1	40.4 ± 0.5	0.913
4	1	0	0.5	0.5	38.4 ± 0.5	0.853
5	1	0	2	1	38.1 ± 0.5	0.711
6	0	0	1	2	36.9 ± 0.5	0.731
7	1	0	2	0.5	34.9 ± 0.4	0.634
8	0	0	2	2	34.6 ± 0.4	0.592
9	0	0	0.5	1	31.7 ± 0.4	0.534
10	0	0	0.5	0.5	28.5 ± 0.4	0.422

X_1: Sample/solvent ratio; X_2: Extraction time.
d: Desirability.
[a] SD: standard deviation.
* selected.

not only time consuming and may reduce the recovery. In the case of artemisinin extraction, interference by other compounds can be avoided by maintaining the integrity of the cell membrane, as artemisinin is localized entirely in the subcuticular space of the *glandular trichome*[11]. During maceration, as the cell membrane is not disrupted, unwanted massive compounds like chlorophyll do not appear in the extract.

Statistical analysis

To optimize the extraction method with a highest yield, a fractional factorial design (FFD) optimization was conducted. The independent variables and response values shown in Table 4 were used to generate a mathematical model that expresses the behaviour of the experiment. This model allows the assessment of predicted response (Y) as a function of the independent variables and their interactions. The calculated model in terms of coded factors generated by FFD for the responses in this study is:

$$Y = 26.5 - 2.30\,X_1 + 6.33\,X_2 + 9.87\,D1 - 26.3\,D2, \quad (1)$$

where Y is yield of artemisinin; X_1 (sample/solvent ratio), X_2 (extraction time), $D1$, and $D2$ (dummy variables) are the independent variables.

To assess the goodness of the fit of the model that has been generated by the FFD, the ANOVA was conducted. The probability of error P was 0.011, R^2 was 0.938, adjusted R^2 0.877, adequate precision (AP) 10.35, standard deviation (SD) 0.10, and coefficient of variation (CV) 1.25%. Based on the result, it is shown that the model is good enough to express the interaction of the independent variables and their responses. The calculated R^2 values are desirably high (close to 1). The predicted R^2 value is also in agreement with the adjusted R^2 value. In addition, the AP value for the response is greater than 4, which is desirable. It implies that all of the predicted models could be used to navigate the design space defined by the FFD[12–14]. Furthermore, the result indicated that the model is considered reproducible. This is due to the value of CV, which is the error expressed as a percentage of the mean, being less than 10%.

Process optimization

In order to optimize the extraction condition, a desirability function (d) for multiple responses was used[15]. This function can be described as follows:

$$d = \left(\prod_{i=1}^{N} m_i^{r_i} \right)^{1/\sum r_i},$$

where N represents the number of responses, r_i refers to the importance of a particular response and m_i is the partial desirability function for specific responses. In this study, the ultimate goal was to extract the highest amount of artemisinin from the sample. Table 5 presents the numerical result of optimization using FFD. Table 5 shows that the optimum condition is whenever $D1$ is 1 and $D2$ is 0, the ratio of sample/solvent is at 0.5, and the extraction time is equal to 2. In other words, the best condition to extract artemisinin from *A. annua* is by using maceration method ($D1 = 1$, $D2 = 0$), and to immerse the sample for about 2 min with a ratio of sample/solvent of 0.5/3 g/ml. This optimum formulation condition carries a desirability value of 0.985.

Validation of experiment

To validate the optimum process condition generated by FFD, a laboratory test was conducted at the selected optimum condition (see Table 5). The extraction procedure was conducted with maceration technique, 2 min of extraction time, and with the ratio of sample/solvent of 0.5/3 g/ml. An accuracy value between predicted and experimental values was calculated to validate the optimization process. Based on the result, the experimental yield of artemisinin, 49.1×10^{-3}% w/w, was found to be in good agreement with the predicted value using the model, $47.9 \pm 0.6 \times 10^{-3}$% w/w. The calculated accuracy for predicted and experimental values was 97.4%, indicating that the process optimization in FFD is reliable to predict the rheological properties of the fluid.

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