The Application of Response Surface Methodology for Optimization of Tetracycline Determination Using Natural Reagent from *Diplazium esculentum (Retz.) Sw.*

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***Abstract*—This research aims to optimize tetracycline determination for pharmaceutical samples using iron(III) contained in *Diplazium esculentum (Retz.) Sw. (D. esculentum)* extraction. The method was based on the complex formed between tetracycline and iron(III), characterized by an absorption maximum at 430 nm using UV-Visible spectrophotometry. From the statistical data analysis by Response Surface Methodology (RMS), The Box-Behnken design was used for optimizing pH, volume of natural reagents and reaction time that affected the tetracycline determination. The results show that, the model fitted well (R^2 = 99.79%). The optimum condition obtained from the equation were as follows: pH, 5; volume of natural reagent, 2.00 mL at any time. In addition, reaction time was not statistically significant. Comparing the sensitivity of the complex formed between tetracycline and iron(III) from the equation to the real experiment, it was found that they were nearly equal which indicate that RSM is an appropriate method to optimize tetracycline determination. Under the optimum conditions, a linear calibration curve was obtained over the range of 1.00–20.00 mg L^{-1}. Limit of detection (defined as 3σ) was 0.22 mg L^{-1} and limit of quantification (defined as 10σ) was 2.15 mg L^{-1}. The relative standard deviation of 3.43% for determining 10.00 mg L^{-1} of tetracycline (n=11) are obtained. The recommended method has been applied to the quantitation of tetracycline in pharmaceutical samples.**

***Index Terms*—tetracycline, Diplazium esculentum (Retz.) Sw., Response Surface Methodology**

I. Introduction

Tetracycline antibiotics (TCs), important broad-spectrum antibiotics [1], which are frequently applied for producing medicine because of their excellent performance for antimicrobial properties and cost-effective [2]. However, TCs can be easily accumulated into human body by food chain, which lead to critical effect to human health even in small quantity, such as organ damage and deafness [3].

UV–visible (UV-Vis) spectrophotometry is one of the most popular method applied for tetracycline assay because of its simplicity, rapidity, low costs and broad applications. Several UV-Vis spectrophotometric methods were developed for tetracycline assay by using many complexing reagents such as diazotized 4-aminoantipyrine [4], chloramine-T [5], ammonium vanadate [6] and iron (III) [7]. The mentioned methods are based on cost effectiveness but they used expensive chemicals and hazardous reagents, resulting in an environmental and human impact.

Recent researches have been focused on developing methods that are human and the environmental friendly [8]. Green chemistry is one of the trends aimed at reducing the use of toxic chemicals reagents. This leads to a new concept that uses green analytical techniques to reduce toxic chemicals [9].

There are few literatures used green reagents for assaying pharmaceutical compounds; Hassan *et al.* [10] analyzed cefadroxil determination by using *Lawsonia inermis* extraction as natural reagents. Palamy *et al.* [11] assayed doxycycline using natural reagents containing iron (III) extracted from *Senna alata (L.) Roxb. (S. alata)*, *Polygonum hydropiper L. (P. hydropiper)* or *Diplazium esculentum (Retz.) Sw. (D. esculentum)*. Hence, using natural reagent extractions along with a spectrophotometric system could give a high performance for assaying samples. Moreover, it will be an interesting method because there has been not much research about it.

By the fact that common active pharmaceutical compounds can be chelated with metal ions. Thus, plants

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that contain iron(III) may be used as pharmaceutical compounds.

D. esculentum is an edible rhizomatous fern usually consumed in Asia and Oceania [12]. In Thailand, it is known as Phak khut. It is a good source of iron, which contained 44.6 mg kg^{-1} [13]. Thus, *D. esculentum* extraction as an alternative natural reagent may be used as a source for pharmaceutical determination. Experimental condition optimization is an important step to develop a successful extraction process. Variables such as ratio of acid, concentration of acid and extraction time will affect the efficiency of extraction.

To optimize the experimental conditions, Response Surface Methodology (RSM) was applied to develop a statistical model based on the interaction among the parameters [14]. RSM has lesser trials than other methods such as one factor at a time approach [15]. It also a better method to locate a region of the optimized parameters [16]. RSM has two type of design; Box-Behnken design (BBD) and Central Composite Design (CCD). BBD has lesser replications than CCD.

Therefore, the aim of this research is to optimize variables for the tetracycline assay, including pH, volume of natural reagents and reaction time using RSM, by employing BBD to maximize the absorption at 430 nm using UV-Vis spectrophotometry as complex formed between tetracycline and iron(III) extract from *D. esculentum*.

II. Experimental Methodology

A. Chemicals Preparation

All chemicals used in this work were analytical reagent grade and all solutions were prepared with double-distilled deionized water. Tetracycline standard was purchased from Fluka (Switzerland). Nitric acid (HNO_3) and hydrochloric acid (HCl) were purchased from Merck (Germany).

Standard solution of tetracycline (10 mg L^{-1}) was prepared by 0.0100 g of tetracycline and the volume was adjusted to 1000 mL double-distilled deionized water 1.0 mol L^{-1} of HNO_3.

B. Extraction of Iron(III) from Plants as Natural Reagent

D. esculentum were bought from the market in Rayong, Thailand. They were washed with tap water. Next step was cut them into small pieces. Then dried them in an oven at 60 °C for 12 hours. The plant powder was contained in a receptacle and heated in a furnace at 400 °C for 4 hours. After that, clean ashes were stored in sealed containers at room temperature.

The extraction process starting with 5 g of ashes were extracts in a mixture of 25 mL 1.00 M HNO_3 and 75 mL 1.00 M HCl on a hot plate for 60 min. After that, waited the mixture to cool down to room temperature and filtered them by Whatman No 42 filter paper. Finally, the extracts were adjusted with double-distilled deionized water to a volume of 100 mL and to get a solution of natural reagents in this experimental.

C. Preparation of Pharmaceutical Samples

Tetracycline (in both tablets and capsules) were bought from a store in Rayong Province, Thailand. The powder from them was collected in amount of 100 mg power using a balance analyzer. Then, it was dissolved with 250 mL of double-distilled deionized water and filtered by Whatman No 42 filter paper. It was diluted again with the same substance to obtain the appropriate concentrations for the measurement.

D. Procedure for Tetracycline Assay

A 1.00-5.00 mL of tetracycline (10 mg L^{-1}) was placed into a 10 mL volumetric flask and mixed with 2.0 mL of natural reagent. Then acetate buffer solution (pH 5.0) were adjusted to 10.0 mL and reacting for 10 min at room temperature. Finally, the sensitivity was measured using cuvette at 430 nm against a reagent blank using UV-Vis spectrophotometry.

E. Optimization of Iron(III) Extraction by RSM

The optimization process can be divided into 2 parts. First, a single-factor experiment was conducted to select an appropriate range of each parameters such as pH, volume of natural reagent and reaction time. The independent variables and their levels are shown in Table I.

Second, 3 levels and 3 factors BBD was used to optimize the parameters by using Minitab Software. The BBD consists of 15 replications as shown in Table II. This design was used to find the optimal conditions for tetracycline determination by using natural as iron(III) extract from *D. esculentum.*

F. Statistical Analysis

RSM and Analysis of Variance (ANOVA) were employed to determine the coefficient and statistical significance of each parameter and optimize the region of response variable [17]. The model that used to predict the response is shown in Eq. (1)

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=0}^{2} \sum_{j=i+2}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y denotes as the predicted response; β_0 is the interception which is constant ; β_1, β_2, and β_3 are the regression coefficients for each parameter; β_{11}, β_{22}, β_{33} are the quadratic effect for each parameter; and β_{12}, β_{13}, β_{23} are the interaction effect for each parameter [18]. The acceptability of the model was determined by coefficient of determination (P-value) obtained from ANOVA. Statistical significance of the model was set at the 5% [19].

TABLE I. Independent Variables Used in RSM Design

Independent variables	Code	Coded levels		
	Units	-1	0	+1
pH	X_1	4.00	5.00	6.00
Volume of natural reagent (mL)	X_2	1.00	2.00	3.00
Reaction time (min)	X_3	0	10	20

TABLE II. EXPERIMENTAL VALUES OF DROPLET SIZE OBTAINED FROM BBD

Run	X_1	X_2	X_3	Sensitivity (A.U.)
1	4.00	2.00	0	0.0346
2	6.00	2.00	0	0.0469
3	5.00	1.00	20	0.0714
4	4.00	2.00	20	0.0339
5	5.00	3.00	0	0.0781
6	6.00	1.00	10	0.0439
7	5.00	2.00	10	0.0799
8	5.00	1.00	0	0.0712
9	6.00	2.00	20	0.0457
10	6.00	3.00	10	0.0446
11	5.00	3.00	20	0.0764
12	5.00	2.00	10	0.0788
13	4.00	3.00	10	0.0332
14	4.00	1.00	10	0.0314
15	5.00	2.00	10	0.0784

III. RESULTS AND DISCUSSION

A. Statistical Analysis and Model Fitting

The model is shown in Table III. It showed that pH and volume of natural reagents have positive ($p < 0.05$) effect on the sensitivity which dominate factors in optimizing the tetracycline determination while reaction time has negative effect ($p > 0.05$). The predicted response variable can be expressed as Eq. (2).

$$Sensitivity = -0.9082 + 0.38072x_1 + 0.01699x_2 + 0.000373x_3 - 0.037404x_1^2 - 0.003354x_2^2 - 0.000014x_3^2 + 0.000275x_1x_2 - 0.000012x_1x_3 - 0.000042x_2x_3 \quad (2)$$

Sensitivity is calculated in the absorbance unit. X_1, X_2 and X_3 represent the variables of pH, volume of natural reagents and reaction time respectively.

TABLE III. ANOVA FOR THE FITTED QUADRATIC POLYNOMIAL

Source	DF[a]	SS[b]	MS[c]	F-value	P-value
Model	9	0.0054	0.0006	213.03	0.000
X_1	1	0.0002	0.0003	91.67	0.000
X_2	1	0.0000	0.0000	4.23	0.095
X_3	1	0.0000	0.0000	0.11	0.751
X_1^2	1	0.0051	0.0051	1817.07	0.000
X_2^2	1	0.0000	0.0000	10.24	0.024
X_3^2	1	0.0000	0.0000	2.57	0.170
X_1X_2	1	0.0000	0.0000	0.15	0.715
X_1X_3	1	0.0000	0.0000	0.02	0.888
X_2X_3	1	0.0000	0.0000	1.21	0.332
Error	5	0.0004	0.0003		
Lack of fit	3	0.0006	0.0002	44.48	0.022
Pure error	2	0.0000	0.0000		
Total	14	0.0055			

[a] Degree of freedom, [b] Sum of squares, [c] Mean squares, $R^2 = 99.79\%$, $R^2_{adj} = 99.40\%$

From Table III, the value of R^2 was 99.79%, which means 99.79% of the variation could be explained by the fitted model. The R^2_{adj} obtained was 99.40% which is very close to R^2. Therefore, the model is significant. The P-value of the model is 0.000 which showed that that the model is significant and proper for predicting the sensitivity [20].

B. Response Surface

The response surface plots for each interaction are shown in Fig. 1. It presented the effect and interaction of independent variables on the signal of iron(III)-tetracycline complex.

Fig. 1A, 1B and 1C shows the effect of pH and volume, pH and time, volume and time on the sensitivity respectively. The plots showed that pH and volume of natural reagents affect of iron(III)-tetracycline complex. Extremely high or low value resulted in a loss of sensitivity. On the other hand, reaction time has no effect on sensitivity.

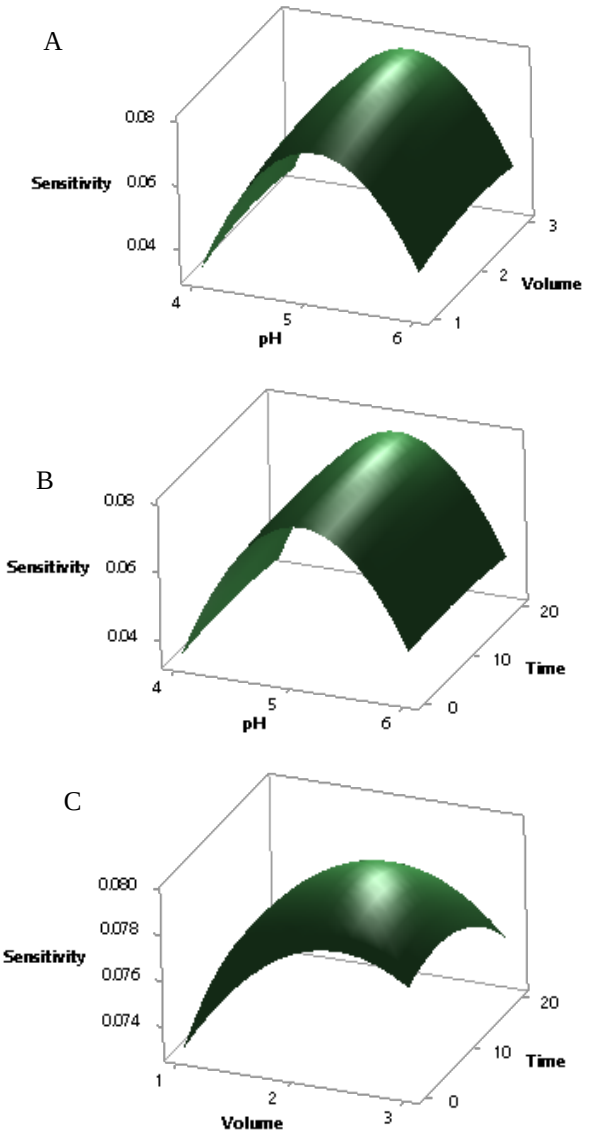

Figure 1. Response surface plot showing the effect of variables on the sensitivity (absorbance unit).

C. Optimization of Condition Parameters and the Model Validation

The optimal conditions for tetracycline determination were ascertained in order to simultaneously maximize the absorbance of iron(III)-tetracycline complex. It was found that the optimal conditions are: pH of 5.00, volume of natural reagents of 2.00 mL at any time. The experimental and predicted values for the sensitivity of iron(III)-tetracycline complex from *D. esculentum* were 0.0799 and 0.0845, respectively. These results confirmed the predictability of the model to give the maximum sensitivity of iron(III)-tetracycline complex for the experimental process used.

D. Analytical Characteristics

The optimized conditions were used to obtain the analytical figures of merit of the proposed method. A linear calibration curve was obtained over the range of 1.00–20.00 mg L^{-1}. The regression model is $y = 0.0911x + 0.0076$ ($R^2 = 0.9979$) where y represents absorbance and x is tetracycline concentration in mg L^{-1}. The limit of detection (defined as 3σ) was 0.22 mg L^{-1} and limit of quantification (defined as 10σ) was 2.15 mg L^{-1}. The relative standard deviation of 3.43% for determining 10.00 mg L^{-1} of tetracycline (n=11) are obtained.

E. Applications

The recommended method has been applied to the determination of tetracycline in pharmaceutical samples (250 mg per tablets or capsules). As shown in Table IV, the tetracycline values for pharmaceutical samples obtained from the experiments were not significantly different from confidence level at 95%. The results show that, the proposed method provided accurate and precision results, indicating that this method can be applied for the tetracycline determination for pharmaceutical samples.

TABLE IV. ACCURACY OF THE PROPOSED METHOD FOR TETRACYCLINE DETERMINATION

Pharmaceutical sample	Tetracycline found (mg)	
	Label value	Proposed method[a]
TA-1	250.00	243.58 ± 0.08
TA-2	250.00	238.21 ± 0.06
TA-3	250.00	247.12 ± 0.09
TA-4	250.00	241.58 ± 0.11
TA-5	500.00	489.96 ± 0.09

[a] Average from three determinations
P-value = 0.939

IV. CONCLUSION

In this study, RSM was used to optimize the experimental variables for tetracycline determination. The optimal conditions to obtain the highest sensitivity of complex between tetracycline and iron(III) were determined as follows: pH of 5.00, volume of natural reagent of 2.00 mL and reaction time at any value. Under the optimum conditions, A linear calibration curve was obtained over the range of 1.00–20.00 mg L^{-1}. Limit of detection (defined as 3σ) was 0.22 mg L^{-1} and limit of quantification (defined as 10σ) was 2.15 mg L^{-1}. The relative standard deviation of 3.43% for determining 10.00 mg L^{-1} of tetracycline (n=11) are obtained. The recommended method has been applied to the quantitation of tetracycline for pharmaceutical preparation. Moreover, natural reagents used for this research are green chemistry, which have less toxic. Therefore, they can be used as an alternative for green chemistry analysis.

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