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Synthesis of molecularly imprinted polymers from AnAc for the separation of γ-oryzanol

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Abstract−The selectivity of gamma-oryzanol (γ-oryzanol) was recognized by molecularly imprinted polymer (MIP). Polymeric materials were successfully synthesized via thermal polymerization method using γ-oryzanol as template, anacardic acid (AnAc) as functional monomer, toluene as porogen, benzoyl peroxide (BPO) as initiator and divinylbenzene as crosslinker. Binding performance of MIPs was evaluated by MINITAP 14 for variance of analysis, linear regression analysis and adequating model through full factorial experimental technique in terms of adsorption capacity. Analysis of variance with 95% confidence level suggested significant interaction effect (amounts of template, porogen, crosslinker) on adsorption capacity of MIPs. The strongest interaction is between the amount of porogen and the amount of crosslinker. It was also found that a linear regression model for adsorption capacity represents the experimental data with the correlation coefficients (R^2) greater than 0.9. The MIP synthesis with 0.8 mmol of template, 6 ml of porogen and 10 ml of crosslinker provided the highest adsorption capacity of MIP (1.14 mg/g-adsorbent). The proposed method is relatively rapid and easy to perform for the separation of γ-oryzanol in non-aqueous systems.

Key words: Analysis of Variance, Molecularly Imprinted Polymer, Molecular Recognition, Gamma-oryzanol, Anacardic Acid

INTRODUCTION

γ-Oryzanol has been used in various fields for several decades. It is a naturally occurring mixture of ferulate, esterified with sterols or triterpene alcohols. The most important properties of γ-oryzanol have been shown to be able to reduce cholesterol absorption [1]. It is appropriate for the treatment of the inflammatory process and it can inhibit linoleic acid and cholesterol oxidation. In addition, it is insoluble in water and a potential antioxidant for the food, pharmaceutical and cosmetic industries [2]. Due to its antioxidant effects, γ-oryzanol may also be effective at inhibiting certain cancers. Several preliminary studies on animals have shown that γ-oryzanol may help inhibit tumor cell growth. However, more tests on humans are needed to study the effect of γ-oryzanol as an anticancer agent [3,4].

γ-Oryzanol can be found in rice bran [5], wheat bran, rye bran [6], and rice bran oil [7]. Rice bran oil is one of the richest sources of γ-oryzanol. The beneficial effects of γ-oryzanol on human health have generated global interest in developing facile methods for its separation from natural sources such as rice bran oil [8]. Various extraction techniques have been applied to γ-oryzanol in rice bran oil such as liquid-liquid extraction, solid phase extraction, supercritical fluid extraction and direct solvent extraction [9-12].

Molecularly imprinting polymer (MIP), an alternative extraction method, is simple, rapid and economical [13]. It has been successfully demonstrated in many applications such as chiral molecule isolation, biosensor and biochemical isolation [14-16]. A molecularly imprinted polymer can be obtained by radical polymerization of a mixture of functional monomers, porogen and crosslinker in the presence of a template. The imprinted polymer is a rigid polymer with the print molecule embedded in it. The removal of the template results in binding sites with complementary size, shape and functionality to the template [17]. MIP is a tool for the preparation of polymeric materials with high selectivity of specificity interaction. The synthesis of MIP involves several steps as follows: (1) self organizing between template and monomer; (2) polymerization by adding crosslinker to form a polymer network; (3) removal of the template via extraction with appropriate method. The resulting polymer, therefore, holds molecular recognition capability due to the specific imprinted sites. It will selectively adsorb molecules that resemble the template molecule. The specific interactions between the functional monomer and the template can be either covalent or non-covalent. The former requires chemical extraction in order to remove template molecules, while physical extraction is sufficient for the latter [18]. There are many types of interactions such as electrostatic interaction, π-π bonding, hydrophobic interaction and hydrogen bonding [18]. In general, hydrogen bonding is a strong interaction of non-covalent type playing a significant role on adsorption of MIP's.

Anacardic acid (AnAc) is a major constituent (about 90%) of cashew nut shell liquid (CNSL), a by-product from the cashew industry [19,20]. CNSL can be extracted from cashew nut shell by different methods such as roasting, solvent extraction, or mechanical extraction. It is often considered as one of the natural sources of phenolic compounds used as raw materials to produce friction linings, paints, vanishing paints, laminating resins, rubber compounding resins, polyurethane based polymers, surfactants, epoxy resins, etc [21]. In nature, CNSL consists mainly of AnAc, cardanol, cardol and 2-methyl cardol. Different extraction and purification techniques of AnAc have been proposed [22-25]. Due to the molecular struc-

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ture and functional groups, AnAc is considered a cheap and renewable monomer potentially useful for polymer production [26,27].

Amongst various applications of MIP reported in literature, statistical analysis has not been used to analyze the interaction effects of synthesis conditions such as template, porogen, crosslinker, etc. In the present work, we synthesized MIP in a particle format using γ-oryzanol as a template and studied the binding characteristics towards γ-oryzanol with AnAc as the functional monomer. Full factorial technique was used in experimental design. Analysis of variance was used to determine the effect of different parameters on adsorption capacity. Regression analysis was used to estimate the adsorption capacity. In addition, it can simultaneously determine an adequating model of the MIP. Parameters of this work are amount of template (γ-oryzanol), amount of porogen (toluene) and amount of crosslinker (divinylbenzene). The ability of the imprinting polymer was compared to a non-imprinted material (NIP).

EXPERIMENTAL SECTION

1. Materials

γ-Oryzanol (98% purity) was obtained from Connell Bros. Co., Ltd. (Bangkok, Thailand). Divinylbenzene (DVB) was purchased from Sigma-Aldrich. Benzoyl Peroxide (BPO) was purchased from Sandreac. Toluene was purchased from Merck. Acetronitrile was purchased from LabScan (Bangkok, Thailand). Ethanol was purchased from Merck. All chemical reagents were analytical grade or better and were used without further purification. The structure of γ-oryzanol and AnAc is illustrated in Fig. 1 and Fig. 2, respectively.

2. Preparation of Anacardic Acid

The method for synthesizing of AnAc was adapted from [27]. Fresh and dry cashew nut shells were obtained from Methee Phuket Co., Ltd. (Bangkok, Thailand). Pretreatment processes including washing and sun drying were performed to reduce field contaminants. Size reduction of the shells was carried out to make the extraction process more efficient. While kept in the dark, the shell pieces (125 g) were dissolved in ethanol (1,000 ml) at room temperature for 1 h; thereafter the solution was filtered. The clear solution was reacted with $Ca(OH)_2$ (31.25 g) at 50 °C for 3 h. The calcium anacardate cake obtained was vacuum filtered and washed thoroughly with ethanol. The wet cake (62.5 g) was dried in an oven at 50 °C for 2 h. After that, the cake was added into a beaker containing a mixture of excess 37%HCl (68.125 ml), ethylacetate (350 ml) and deionized water (250 ml) followed by stirring at room temperature for 1 h. The organic phase was dried over sodiumsulfate anhydrous and concentrated using a rotary evaporator at 40 °C to obtain AnAc.

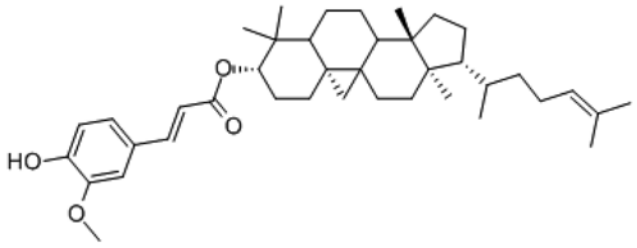

Fig. 1. Structure of γ-oryzanol.

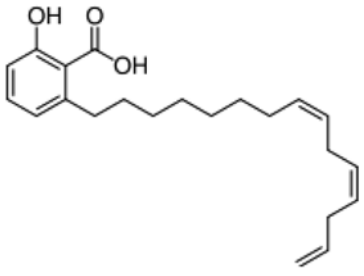

Fig. 2. Structure of major component in anacardic acid.

Table 1. The amount of functional monomer, template molecule, crosslinker, porogen and initiator used for the preparation of M1-12 and N

Entry	Functional monomer (mmol)	Template molecule (mmol)	Crosslinker (ml)	Porogen (ml)	Initiator (mmol)
M1	4	0.8	5	3	2
M2	4	0.8	5	6	2
M3	4	0.8	10	3	2
M4	4	0.8	10	6	2
M5	4	0.6	5	3	2
M6	4	0.6	5	6	2
M7	4	0.6	10	3	2
M8	4	0.6	10	6	2
M9	4	0.4	5	3	2
M10	4	0.4	5	6	2
M11	4	0.4	10	3	2
M12	4	0.4	10	6	2
N	4	-	5	3	2

3. Preparation of the MIP

The method for synthesizing MIP particles was adapted from [28]. Table 1 shows the amounts of the template molecule, functional monomer, porogen, crosslinker and initiator used for the preparation of MIP1-12 and non-imprinted polymer (NIP). γ-Oryzanol was used as the template with AnAc as the functional monomer and DVB as crosslinker. The porogen that was tested was toluene. BPO served as the radical initiator for the polymerization. The composition of the imprinted polymer was as follows 0.4 mmol, 0.6 mmol or 0.8 mmol of γ-oryzanol; 4 mmol of AnAc; 5 ml or 10 ml of DVB, 3 ml or 6 ml of the toluene; and 2 mmol of BPO. After mixing the template, monomers, initiator, crosslinker and porogen in a 50 ml round-bottom flask, the pre-polymerization solutions were stirred with a magnetic stirrer for 5 min. Then, the solution was purged with nitrogen gas for 8 min. The container was sealed and placed in a water bath at 60 °C for 48 h. NIP was also prepared the same way without the addition of the template to the polymer mixture. After polymerization, the rigid polymer was crushed into powder and screened with mesh size 140-200, which corresponds to 75-106 μm. The particles were washed by acetonitrile five times. The template was extracted by soxhlet extraction at a temperature of 115 °C with acetonitrile as the extraction solvent. The removal process was performed until γ-oryzanol could no longer be detected in extracted samples of the MIP.

4. Binding Test

250 mg of the dried imprinted or the dried non-imprinted particles was incubated with 10 ml of 0.05 mg/ml γ-oryzanol in acetonitrile and placed in a sonicator at 25 °C for a period of time ranging from 4 h to 24 h. The samples were analyzed by UV-VIS spectrophotometer at 315 nm. The amount of γ-oryzanol bound to MIP, n, was calculated by subtracting the amount in the solution after each period of time from the initial γ-oryzanol concentration. The binding process was repeated one time.

5. Quantitative Analysis of γ-Oryzanol Content

γ-Oryzanol content in the sample was determined spectrophotometrically (2100 series UV-VIS spectrophotometer, UNICO, USA). The calibration curve was obtained with pure γ-oryzanol in the concentration range 0.01-0.05 mg/ml [29]. In this range, absorption obeys Beer's law and the calibration curve obtained at 315 nm [11] is a straight line passing through the origin (R^2=0.9997), and the slope represents the specific coefficient ($E_{(315\ nm)}$=28.16 g^{-1} L cm^{-1}).

6. Experimental Design and Analysis

A series of experimental conditions for synthesis of MIP was designed in order to determine the effect of different parameters on adsorption capacity. A design of full factorial experiments was used to study the effects of the amount of template (t), the amount of porogen (p) and the amount of crosslinker (c) on adsorption capacity towards γ-oryzanol as shown in Table 1. Analysis of variance was used to interpret results of adsorption capacity for MIPs in order to categorize main and interaction effects. In general, either too much of template molecules or porogen or too little of initiator can negatively affect the formation of MIP, resulting in gel or liquid phase. Preliminary experiments (with 0.8 mmol of template, 3-6 ml of porogen, and 5 ml of crosslinker) showed that the polymerization of MIP was incomplete for the amount of initiator of less than 2 mmol. After that, we varied the amount of template from 0.4-0.8 mmol while other variables were fixed (3-6 ml of porogen, 5 ml of crosslinker, 2 mmol of initiator). It was found that 0.8 mmol of template was the maximum amount for successful synthesis of MIP. Therefore, the limits of different parameters were chosen as shown in Table 1.

This work employed MINITAP 14, which is the statistical software for analyzing data to check the effect and interaction of several parameters in the response of an experimental system. Statistical analysis of our experimental results involved hypothesis testing, which can be performed by analyzing the value of F-score for a certain significance level (α). If the value of the test statistic is unlikely, then the null hypothesis is rejected. More details on hypothesis testing can be found elsewhere [30,31]. MINITAP software also provides the p-value, another test statistic, which can be used to determine whether the main hypothesis is accepted. If the p-value is less than the significance level (α), then we reject the null hypothesis. On the other hand, if the p-value is larger than the significance level (α), then the null hypothesis is accepted.

RESULTS AND DISCUSSION

1. Evaluation of the Specificity of MIP

The specific binding of the MIPs and NIP was evaluated in terms of its ability towards γ-oryzanol. Specific interactions of γ-oryzanol were considered from the imprinting capacity [13]. Non-specific interactions can be estimated by measuring the binding to non-imprinted polymer, which is also present in the NIP [32]. MIPs and NIP were incubated with γ-oryzanol solution for different periods of time up to 13 h. The percentage by mole of γ-oryzanol bound to the imprinted polymer was compared to a non-imprinted material as a function of time, as shown in Fig. 3.

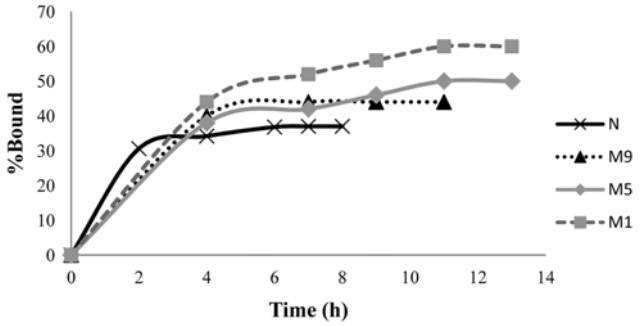

Fig. 3. Percentage of γ-oryzanol bound to polymeric material as a function of incubation time of the polymeric material with γ-oryzanol in acetonitrile.

Fig. 3. shows that γ-oryzanol is bound to both imprinted and non-imprinted polymer. However, the percentage of γ-oryzanol bound to imprinted polymer is greater than that bound to γ-oryzanol. Adsorption rates were fast in the first 4 h and they later slowed down. One can speculate that γ-oryzanol binds to polymeric material in a period of time 0-4 h due to the effect of pores from porogen and eluting template at the polymer surface. After that, MIPs tend to increase while NIP does not change with time, because MIPs have active sites from eluting template, but NIP does not have any active sites.

The amount of template for each experiment was varied while the other experimental parameters, e.g., amount of divinylbenzene, toluene, were fixed. The percentage of γ-oryzanol bound to MIPs was plotted against incubation time; additionally, the results obtained using MIPs were compared with those using NIP. The percentage deviations (based on NIP) were 18.9%, 35.1% and 62.2%, when the amount of template was 0.4, 0.6 and 0.8 mmol (M5, M9 and

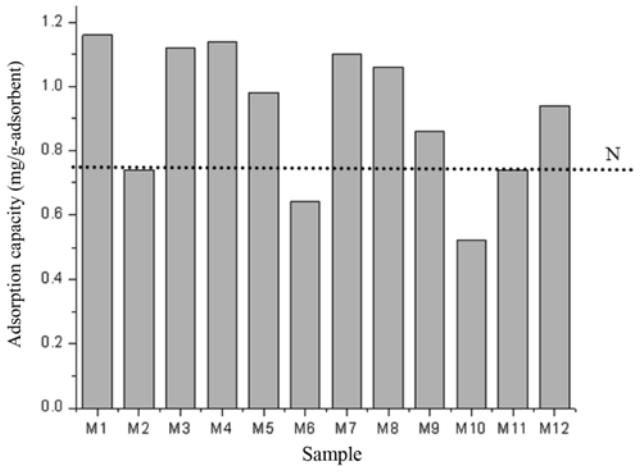

Fig. 4. Adsorption capacity of γ-oryzanol from the imprinted polymer test (M) and the non-imprinted polymer test (N); for sample codes in the x-axis refer to table 1.

M1), respectively. This may indicate that MIP particles show a certain degree of specificity for γ-oryzanol.

2. MIP as Adsorbent for Soxhlet Extraction Clean-up

The adsorption capacity of γ-oryzanol in each sample is found in Fig. 4. In the figure, all conditions are saturated adsorption. MIPs synthesized with 10 ml of crosslinker required much time to achieve equilibrium. Due to the excess amount of crosslinker, the MIP becomes more and more rigid, and MIP pores are not flexible [33]; all of these factors result in an increased MIP equilibration time.

In addition to certain amounts of monomer and initiator, the amount of template, porogen and crosslinker used will affect the concentration prior to the formation of MIP. The morphology of MIP synthesized with different formulae directly affects the adsorption performance. The adsorption capacity of the obtained MIP for γ-oryzanol was found to increase with increasing the amount of template. MIP for γ-oryzanol was prepared for many cases, e.g., different amount of porogen and crosslinker. For 0.8 mmol of amount of template, it appears that 3 ml of porogen (M1) provided better adsorption performance compared to 6 ml of porogen (M2). However, for M3 and M4, 6 ml of porogen for MIP synthesis yielded slightly better adsorption capacity compared to that of 3 ml of porogen (M3). It can be speculated that there is a strong interaction effect between the amount of porogen and crosslinker, which can be proved by an analysis of variance. However, when the used volume of DVB is below 5 ml, the volume of toluene can have a significant effect on the adsorption capacity. Furthermore, when the used amount of crosslinker is above 10 ml, the amount of porogen has little effect on the adsorption capacity. It can be seen that when the amount of porogen is 6 ml and the amount of crosslinker is 5 ml, the adsorption capacity will be less than that of N with 3 ml of porogen (and same amount of crosslinker) since a higher porogen volume would dilute the solution and thus cause imprinted sites to have more defects [34].

To interpret the experimental results, MINITAP 14 was used to calculate an adequating model, variance of analysis and linear regression analysis in amount of template, amount of porogen and amount of crosslinker. A full factorial experiment technique was performed.

3. The Normality Assumption

A check of the normality assumption could be made via a normal probability plot. When the histogram of the residuals lies on a straight line, this data will have a normal distribution [30]. In visualizing the straight line, we place more emphasis on the central values of the plot than on the extremes.

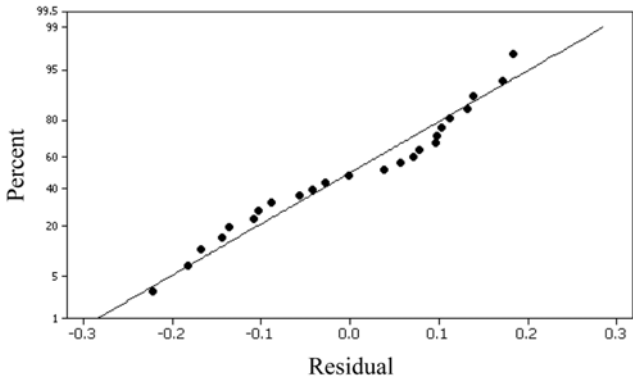

Fig. 5. Normal probability plot of residuals (response is adsorption capacity).

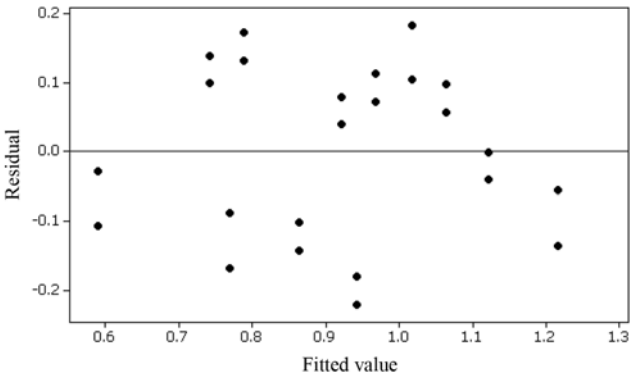

Fig. 6. Plot of residuals versus fitted value (response is adsorption capacity).

The normal probability plot is shown in Fig. 5. The general impression from examining the data distribution is that it is approximately normal. The tendency of the normal probability plot looks fairly straight, which implies that the largest residuals are not very large. In addition, a straight line can be fit to the points and added as a reference line. The further the points vary from this line, the greater the indication of departures from normality.

4. Plot of Residuals Versus Fitted Values

Residuals are estimated of experimental error and examination of the residuals is a key part of all statistical modeling. When the model is adequate, the residuals should be structureless [30]. In visualizing a structureless distribution, data is distributed with a mean of zero.

The relative of residuals versus fitted value is shown in Fig. 6. It shows that the usual structure is apparent because the mean of error is zero and the variance of error is constant. Specifically, these assumptions are that the observations are adequately described by independent errors. In any case, transformation brings the residuals distribution closer to normal.

5. Analysis of Variance of Adsorption Capacity

Statistical tests for equality of variance on full factorial technique have also been proposed to study the main effect and interaction effect of parameters in the system. These tests may be viewed as formal tests of the following hypotheses [30]:

H_0: the average adsorption capacity (μ) for all level are same ($\mu_1=\mu_2=\mu_3=\mu_4$; μ_i)

H_1: the above hypothesis is not true for at least one μ_i

The adsorption capacities for twelve separate experiments of MIP adsorption were analyzed by analysis of variance (ANOVA) to investigate the variances of response (adsorption capacity, y). Each experiment was repeated one time.

The variance analysis in Table 2 looks at the factors affecting adsorption capacity. It must analyze the interaction effect before the main effect. On a 95% confidence interval, ANOVA finds that the F-values of t*p, t*c and p*c are 6.54, 4.87 and 160.67, respectively, and the p-value of less than significance level (0.05) indicates that we can reject the null hypothesis (H_0, a particular factor has no effect on adsorption capacity of MIP); in other words, the interaction effects of t*p, t*c and p*c are highly significant [35].

Table 2. Variance analysis of adsorption capacity

Source	Degree of freedom	Sum of squares	Mean square	F-value	Prob>F (p-value)
Amount of template (t), mmol	2	0.312133	0.156067	97.54	0.000
Amount of porogen (p), ml	1	0.141067	0.141067	88.17	0.000
Amount of crosslink (c), ml	1	0.240000	0.240000	150.00	0.000
t*p	2	0.020933	0.010467	6.54	0.012
t*c	2	0.015600	0.007800	4.87	0.028
p*c	1	0.273067	0.273067	160.67	0.000
t*p*c	2	0.014533	0.007267	4.54	0.034
Error	12	0.019200	0.001600		
Total	23	1.036533			

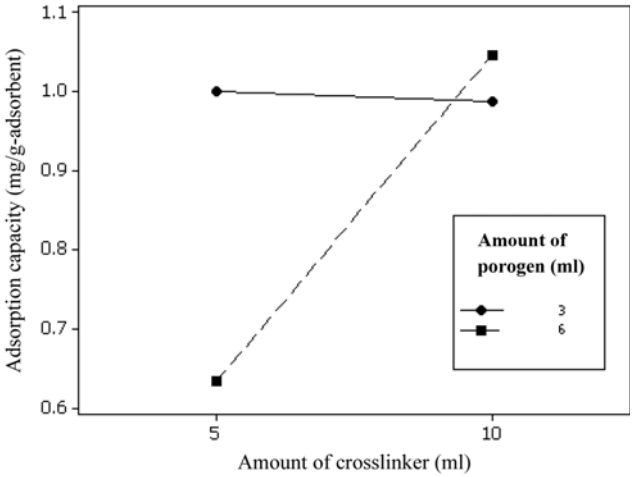

Fig. 7. Interaction plot (data means) for adsorption capacity at difference amount of crosslinker and porogen.

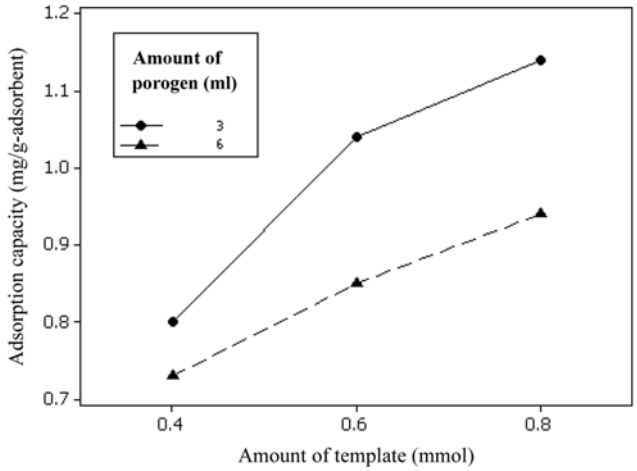

Fig. 8. Interaction plot (data means) for adsorption capacity with different amounts of template and porogen.

The impact of experimental parameters on adsorption capacity can be identified by the p-value; a smaller p-value means a stronger impact on the response.

6. Interaction Effect on Adsorption Capacity

The equilibrium adsorption capacity was determined at 25 °C for each synthesized MIP. Results were translated into variance of analysis (Table 2). The interaction effect was then analyzed according to p-values. Fig. 7 shows the adsorption capacity as a function of amount of crosslinker for different amounts of porogen. It can be seen that when the amount of porogen is increased, the adsorption capacity will increase through appropriate with amount of crosslinker. Adsorption capacity will decrease when the amount of crosslinker is increased beyond a certain point [33]. For 3 ml of porogen, the adsorption capacity decreased if more than 5 ml of crosslinker was added. For 6 ml of porogen, the adsorption capacity increased very sharply as the amount of crosslinker was increased from 5 to 10 ml. A high porogen volume (with 5 ml of crosslinker) resulted in the dilution of solution and caused imprinted sites to have more defects [34]. Therefore, the adsorption effectiveness of the obtained MIP for γ-oryzanol increased with optimal amount of porogen and crosslinker. The interaction effect for different amounts of template and porogen is shown in Fig. 8. The adsorption capacity increased with increasing amount of template as the AnAc-to-γ-oryzanol molar ratio was increased from 4 : 0.4 to 4 : 0.8 at the amount of porogen 3-6 ml. This is because the amount of template molecules is directly related to the amount of imprinted sites. The adsorption capacity of porogen at 6 ml is less than that at 3 ml because the amount of porogen of 6 ml with 5 ml of crosslinker will dilute the solution. In Fig. 8, for 3 ml of porogen, adsorption capacity increased sharply for 0.4 to 0.6 mmol template molecule and then increased slightly from 0.6 to 0.8 mmol. This indicates that less amount of template at low porogen volume has a greater impact on adsorption capacity than a high porogen volume. The effect of interaction factors between the amount of template and the crosslinker is neglected in this paper, since adsorption capacity is minimally affected in that regard compared with the aforementioned factors.

7. Linear Regression Analysis

Regression model fitting was performed, and the statistical model of full factorial design is linear. Data were analyzed by multiple linear regression analysis. The relationship between the adsorption capacity and the test variables in coded factor was calculated according to Eq. (1).

$$Y = 1.51 + 0.500\,t - 0.274\,p - 0.142\,c + 0.0900\,t*c + 0.0384\,p*c + 0.017\,p*t - 0.0167\,p*t*c \quad (1)$$

where Y (mg/g-adsorbent) represents the adsorption capacity, t (mmol) is the amount of template, p (ml) is the amount of porogen, and c (ml) is the amount of crosslinker. Variance analysis was used to es-

timate the regression coefficient. The correlation between adsorption capacity and experimental parameters has an F-value of 37.47, implying that the model is significant. R^2 of this model is 91.7%; in this case the model can be considered accurate. Therefore, it is reasonable to apply this model for the prediction of adsorption capacity.

All of coefficients have been estimated using data from twenty-four experiments. As shown in the proposed model, the term t has the largest coefficient; in other words, t has the strongest effect on adsorption capacity. Increasing the amount of template results in the higher adsorption capacity of obtained MIP. It is noted that the values of p and c must also be optimized in order to obtain high adsorption capacity.

According to response optimization using Eq. (1), the optimal amounts of template, porogen and crosslinker are 0.8 mmol, 3 ml and 10 ml (M3), respectively. However, experimental results showed that M4 (0.8 mmol of template, 6 ml of porogen, 10 ml of crosslinker) has the highest adsorption capacity. This slight contrast in terms of the amount of porogen at the optimum condition is because these two preparation conditions yielded MIPs of comparable adsorption capacity with the difference of only 1.7%.

CONCLUSION

The experimental results in this work have shown that MIP particles prepared by the thermal polymerization method using toluene as porogen, AnAc as functional monomer, BPO as initiator and DVB as crosslinker clearly recognize property for γ-oryzanol. The binding characteristic shows a certain degree of specificity for γ-oryzanol. However, an analysis of variance with a 95% confidence level suggests that the interaction effect of the amount of porogen and crosslinker, the amount of template and porogen and the amount of template and crosslinker have great influence on the adsorption capacity for γ-oryzanol. The highest adsorption capacity of MIP was 1.14 mg/g-adsorbent, prepared by using 0.8 mmol of template, 6 ml of porogen and 10 ml of crosslinker. Full factorial design, including the three main factors (t, p and c), was used to predict the adsorption capacity. The linear regression coefficient was as high as 0.917 in the MIP sorbent prepared by γ-oryzanol. The selectivity of molecularly imprinted polymer prepared in this work is an attractive feature for further investigation.

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