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Effect of Porogenic Solvent on Selective Performance of Molecularly Imprinted Polymer for Quercetin

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Molecularly imprinted polymers (MIP's) for quercetin were successfully synthesized by a thermal polymerization method using quercetin as template molecule, acrylamide as functional monomer and ethylene glycol dimethacrylate as cross-linker in the presence of four different porogenic solvents: 1,4-dioxane, tetrahydrofuran (THF), acetone, and acetonitrile. The selective performance of obtained MIP's was evaluated through HPLC analysis. The results indicated that the MIP obtained in THF showed the highest capacity and selectivity. The Scatchard method also supported HPLC results. The results were interpreted by computational quantum chemical analysis through Onsager self-consistent reaction field (SCRF) technique in term of stabilization energy. It was also found that the amount of porogenic solvent used had impact on the adsorption effectiveness of MIP's.

Keywords: *quercetin, molecularly imprinted polymer, porogenic solvent, computational quantum chemical analysis*

1. Introduction

Recently, molecular imprinting technique (MIT) becomes one of most powerful methods to determine and separate natural products. The principle of MIT is to utilize the functional groups on a target molecule (also called template molecule) interacting with complementary functional groups of appropriate functional monomers to assemble its own recognition site, and then this site is "frozen" by polymerizing functional monomers with a high concentration of cross-linker. Subsequent removal of the template molecule by extraction creates a binding site with the precise spatial arrangement of the functional groups[1]. This polymeric material will re-bind with template molecule at high selectivity and affinity constants. The obtained polymer is called molecularly imprinted polymer (MIP). Since it was invented by Wuff et al. in 1972[2], MIT has been potentially used in many applications such as chiral molecule separation[3], biosensor[4], biochemical separation[5,6], antibody simulation[7], enzyme catalysis simulation[8], and so on. Quercetin (3,3', 4, 5, 7- penta-hydroxy flavone) is a member of the flavonols subclass and is considered one of the most eminent flavonol compounds occurring in wine (see Figure 1). It is the most active compound in flavone class and widely exists in leaves, fruits, and flowers of many plants[9]. Quercetin is a natural antioxidant because its hydroxyl groups attached to the aromatic rings can quench the active free radical[10]. Since it has low concentration and structural similarity to other flavonols in natural states, it is very difficult to determine and separate. There were some reports on quercetin determination and separation by MIT. Weiss et al. reported that MIP for quercetin was prepared by using quecertin as template molecule, 4-vinylpyridine as functional monomer and ethylene glycol dimethacrylate as cross-linker[1]. The prepared MIP showed high effectiveness on quercetin separation. Yan et al. also prepared an effective MIP for quercetin by using acrylamide as a functional monomer[11]. He et al. reported the effectiveness of MIP's prepared by using different functional monomers such as methacrylic acid, acrylamide, and 2-vinylpridine and found the effectiveness of MIP's for quercetin was varying with different functional monomers[12]. The dispersion solvent (porogenic solvent) was also important for making effective MIP's because the solvent has different interactions with template molecule and with functional monomers. These interactions will strongly impact on the structure formation of MIP's and further affect the recognition and separation of the MIP's for template molecules.

In this paper, we will report the effect of porogenic solvents on MIP separation for quercetin and interpret the results through computational quantum chemical analysis. MIP's for quercetin will be prepared using quercetin as template molecule, acrylamide as functional monomer and ethylene glycol dimethacrylate as cross-linker in the presence of four different porogenic solvents: 1,4-dioxane, tetrahydrofuran, acetone, and acetonitrile. HPLC and Scatchard method will be used to evaluate the effectiveness of MIP's for quercetin. Onsager self-consistent reaction field (SCRF) technique will be used to calculate the stabilization energy of the porogenic solvents on template molecule, functional monomer and obtained polymer.

2. Experimental

2.1. Chemicals

Quercetin was purchased from Shanghai Sinopharm Chemical Reagent Co., Ltd. Acrylamide (AA) was supplied from Tianjin Chemical Research Institute. Ethylene glycol dimethacrylate (EGDMA) was purchased from Sigma–Aldrich (Taufkirchen, Germany). 2,2'-Azo-bisisobutyronitrile (AIBN) was obtained from Shanghai Chemical Reagent Company. Tetrahydrofura, acetone, acetonitrile, 1,4-dioxane, methanol and anhydrous acetic acid were all from Merck (Darmstadt, Germany). The control solution (20 $\mu g \cdot mL^{-1}$) of quercetin in methanol was prepared in the laboratory.

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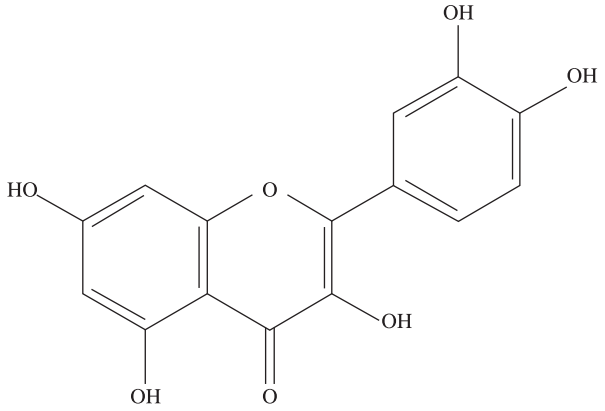

Figure 1. Chemical structure of quercetin.

2.2. HPLC analysis

The HPLC-analysis was performed using EasySep™-1010 HPLC (UnimicroTechnologies Co., Ltd., Shanghai) with a 1010LC liquid delivery pump and a 1010UV ultraviolet/visible variable wavelength detector with a spectral range from 190 to 700 nm. Column was C-18 column (2.1 × 250 mm). Mobile phase was acetonitrile – 0.1 wt. (%) H_3PO_4 water solution (36:64, V/V), and the column was rinsed with it until a stable baseline was obtained. The detection was carried out with UV at 365 nm. Column oven temperature was controlled at 25 °C. The total flow-rate was maintained at 0.5mL/min and 20 μL sample was injected.

2.3. Preparation of molecularly imprinted polymer[11]

In four corked test tubes were respectively added 5 mL porogenic solvents of 1,4-dioxane, THF, acetone and acetonitrile labeled with sample number of P1, P2, P3 and P4. In above four test tubes were added 0.13 g template molecule quercetin (0.4 μmol) and 0.2 g functional monomer acrylamide (2.8 μmol). After the test tubes were shaked in SHA-B Water Bath Isothermal Shaker for half hour, 3.5 mL cross-linker EGDMA (18.5 μmol) and 0.01 g initiator AIBN were added. After degassed for 15 minutes, the test tubes were filled with nitrogen and sealed. The test tubes were immersed into isothermal water bath and the mixtures were thermally polymerized at 60 °C for 24 hours The resulted polymers were then grinted and sieved through 105 μm sieve. To remove template molecule quercetin, the polymer particles were extracted with the mixture of methanol and acetic acid (9:1, V/V) in Soxhlex extractor for 24hours. The extracted polymer particles were then extracted with methanol to remove acetic acid. Finally, the polymer particles (MIP) was dried at 60 °C under vacuum for 24 hours and stored in a desiccator for further use. Four MIP's were obtained with sample number of P1 (1,4-dioxane), P2 (THF), P3 (acetone) and P4 (acetonitrile). As references, non-imprinted molecular polymers (NMIP's) were prepared following the same procedure but without addition of template molecule. The corresponding NMIP's were labeled with number of P5 (1,4-dioxane), P6 (THF), P7 (acetone) and P8 (acetonitrile). The compositions of MIP's and NMIP's were shown in Table 1.

2.4. Adsorption determination of MIP's and NMIP's for quercetin

In eight 50 mL Erlenmeyer flasks were respectively added 100 mg of sample P1, P2, P3, P4, P5, P6, P7, and P8. They were then added 25 mL quercetin-methanol solution with concentration of 20 $\mu g.mL^{-1}$. The mixtures were shaked in SHA-B Water Bath Isothermal Shaker. Every 2 hours, 20 μL solution was taken out for HPLC analysis to measure the concentration of quercetin and determine the adsorption of quercetin in different times.

2.5. Computational quantum chemical analysis

According to calculation method of Gaussian 98® B3LYP/6 -31+ G**, the stabilization energy of quercetin and acrylamide with different solvents was obtained through calculating the interaction energy in gas and solvent. The simulation of solvent effect is based on the Onsager self-consistent reaction field (SCRF) technique[13], which supposes that the solute molecule is embedded into a spherical cavity with radius α_0 surrounded and the solvent is represented by a continuous dielectric, characterized by a given dielectric constant. The gas-phase geometry of molecule quercetin and functional monomer is used as its initial guess to start the full optimizations at the same level of B3LYP/6-31+ G** with different dielectric constants of porogenic solvents 2.25, 7.58, 20.7 and 37.5. A radius α_0 automatically resorted from quantum mechanical procedures. After stationary points were located, vibrational frequencies were calculated in order to ascertain that each structure was characterized to be the stable structure (no imaginary frequencies). The stabilization energy by solvents is calculated as $E_{solvation} = E_{in\ solvent}$–Ein gas. The $E_{solvation}$ is the stabilization energy by solvents, the relative energy of a complex in a solvent to that in the gas phase. The stabilization energy by solvent was used to evaluate the interaction strength of quercetin with solvent, and acrylamide with solvent. All calculations were carried out with the Gaussian98® program on a Pentium® computer using the default convergence criteria.

3. Results and Discussion

3.1. Preparation of MIP's

MIP's for quercetin were prepared according to literature[11], which reported a successful MIP obtained by using acrylamide as functional monomer, ethylene glycol dimethacrylate (EGDMA) as cross-linker and THF as porogenic solvent through thermal polymerization method. Acrylamide was selected as functional monomer because its MIP showed more adsorption effectiveness for quercetin than other functional monomers such as methacrylic acid[11]. The ratio of quercetin and acrlyamide also affected the effectiveness of adsorption. The MIP obtained at ratio of 1:7 showed the best adsorption for quercetin. 2,2'-Azo-bisisobutyronitrile (AIBN) was used as free radical initiator because its half-life time at 60 °C is around 10 hours[14] and was proper for polymerization time. In order to evaluate the effect of porogenic solvent on the formation of MIP, four porogenic solvents were selected with different polarity. The dielectric constants of 1,4-dioxane, THF, acetone, and acetonitrile are 2.25, 7.58, 20.7 and 37.5, respectively. The generated imprinted polymers and non-imprinted polymers were summarized in Table 1. The synthesis process of MIP can be described in Figure 2. In quercein molecule, there are five hydroxyl groups and one carbonyl group. These groups can form hydrogen bonds with functional groups such as hydroxyl, amino and carbonyl group. In this study, acrylamide was used as functional monomer. During the formation of MIP, amide group of acrylamide will combine with template molecule (quercetin) through hydrogen bonds. This spatial structure will be "frozen" after acrylamide polymerizing and cross-linked by EGDMA. Since the hydroxyl groups on carbon number 3 and 5 on quercetin molecule have strong intramolecular hydrogen bonds with carbonyl group on carbon number 4. Only three hydroxyl groups have intermolecular hydrogen bonds with polymer and control the precise imprinting sites. After removal of template molecule, the specific imprinting sites will be maintained. These sites will selectively adsorb quercetin molecule.

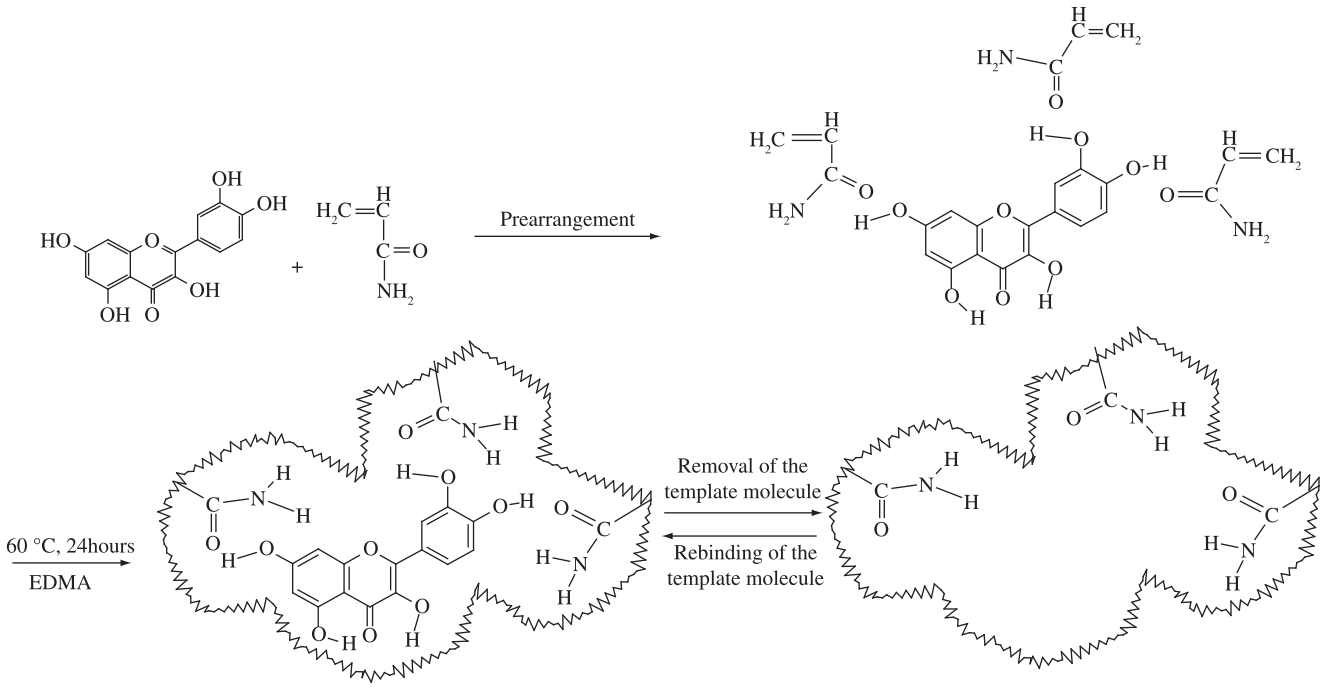

Figure 2. The synthesis process of MIP for quercetin.

Table 1. Feed composition of MIP's and NMIP's.

Sample#	Quercetin/g	Acrylamide/g	EGDMA/mL	(AIBN)/g	Porogenic solvents
P1	0.13	0.2	3.5	0.01	1,4-Dioxane
P2	0.13	0.2	3.5	0.01	THF
P3	0.13	0.2	3.5	0.01	Acetone
P4	0.13	0.2	3.5	0.01	Acetonitrile
P5	-	0.2	3.5	0.01	1,4-Dioxane
P6	-	0.2	3.5	0.01	THF
P7	-	0.2	3.5	0.01	Acetone
P8	-	0.2	3.5	0.01	Acetonitrile

3.2. Adsorption of MIP's and NMIP's for quercetin

Among the falconoid, quercetin has five hydroxyl groups attached to the aromatic rings, along with the electronic delocalization throughout the whole system, which results from the formation of stable radicals[10]. As commonly known, hydrophobic interactions are one of the binding mechanisms in order to efficiently recognize the template molecules during MIP selection process. Since the solubility of quercetin in methanol was superior to most of other solvents, a certain concentration of quercetin solution in methanol was used. Quercetin removed MIP's and NMIP were added into quercetin-methanol solution with the concentration of 20 $\mu g \cdot mL^{-1}$ for adsorption testing. After every two hours, 20 µL solution was taken out and run HPLC analysis. The adsorption of quercetin would reduce the concentration of quercetin in methanol solution. As to HPLC analysis, acetonitrile –0.1 wt.(%) H_3PO_4 water solution (36:64, V/V) was chosen as aqueous mobile phase. Methanol was eluted first and did not have effect on quercetin separation.

The adsorption of quercetin onto MIP's was due to precise site recognition and strong interaction between quercetin and functional group on the imprinted sites. The interaction included van der Waals force and hydrogen bonds. The adsorption results were shown in Figure 3. It was found that the adsorption amount (Q) of quercetin on either MIP's or NMIP's was increasing with increasing adsorption time. The adsorptions were fast in the first 6 hours and then slow down after 10 hours. After 24 hours, assumed adsorption equilibrium was achieved. The fast adsorption in the first few hours was due to rapid recognition of quercetin onto imprinted sites on the surface. When these imprinted sites were occupied, it was becoming difficult for quercetin to penetrate into the polymer and find imprinted sites inside. This would cause the adsorption to slow down.

Although both MIP and NMIP showed similar trend on quercetin adsorption, the adsorption amount was very different. MIP adsorbed much more quercetin than NMIP. This was because MIP had a lot of spatial imprinted sites, which selectively adsorbed quercetin and NMIP has no imprinted sites. This result confirmed the effectiveness of MIP for quercetin. The morphology of MIP and NMIP can be measured by scanning electronic microscopy (SEM) or other methods to prove this observation and will be reported later. The imprinting

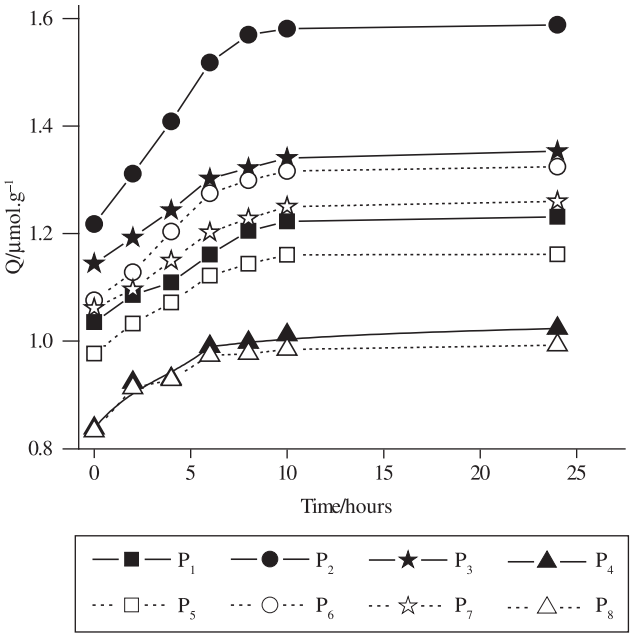

Figure 3. Adsorption of MIP's and NMIP's for quercetin with time.

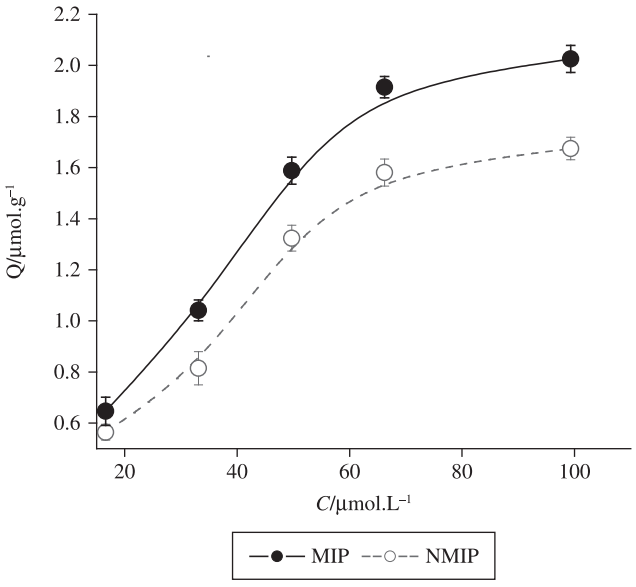

Figure 4. Adsorption isotherm of polymers using THF as disperse medium.

factor (Q_{MIP} / Q_{NMIP}) can be used to evaluate the efficiency of MIP made in different porogenic solvents. Q_{MIP} is the saturated amount of quercetin adsorption for MIP and Q_{NMIP} is the saturated amount of quercetin adsorption for NMIP prepared in the same porogenic solvent. The imprinting factors were shown in Table 2. It was found that THF as porogenic solvent demonstrated the highest imprinting factor. The order of porogenic solvent on adsorption efficiency was THF > acetone > 1,4-dioxane > acetonitrile.

In order to evaluate the quercetin adsorption of MIP, isothermal adsorption curve was also determined. MIP and NMIP were both prepared in THF. The test was performed at 20 °C. The saturated adsorption (Q) on quercetin was each measured by using various initial concentrations (C) of quercetin-methanol solutions from 10 to 100 μmol.L^{-1}. The saturated adsorption was considered to achieve after 24 hours adsorption. The isothermal adsorption curve was obtained by plotting each saturated adsorption with various concentrations of quercetin-methanol solution and shown in Figure 4. The results in Figure 4 indicated that the amount of saturated adsorption quickly increased with increasing initial concentration of quercetin-methanol solution. When the initial concentration increased to 70 μmol.L^{-1}, the amount of saturated adsorption almost reached maximum amount and did not increase much with increasing the initial concentration. NMIP had the same trend as MIP, but its amount of saturated adsorption was much lower.

Scatchard model is a common method to evaluate the adsorption property of MIP. The equation of Scatchard[15] can be expressed as follow:

$$Q / C = (Q_{\max} \quad Q) / K_d \tag{1}$$

where C (μmol.mL^{-1}) is the equilibrium concentration of quercetin; Q (μmol.g^{-1}) is the equilibrium adsorption amount of each concentration; Q_{Max}(μmol.g^{-1}) is the maximum amount of adsorption; and K_d(μmol.mL^{-1}) is dissociation equilibrium constant at imprinted sites. Both MIP and NMIP were prepared in THF. The Scatchard plots (Q/C vs. Q) were shown in Figure 5. From the plots in Figure 5, it can be found that it was linear for MIP and nonlinear for NMIP. The linear response reflected the uniform imprinted sites that solely adsorb quercetin. The linear equation for MIP can be expressed as follow:

$$Q / C = \quad 32.32424Q + 98.30464, R^2 = 0.9903 \tag{2}$$

where the slope is –32.32424 (1/K_d) and the intercept is 98.30464 (Q_{max}/K_d).K_d and Q_{max} can be calculated as 0.03094 μmol.mL^{-1} and 3.0414 μmol.g^{-1}, respectively. The nonlinear response for NMIP indicated that there were no imprinted sites for quercetin.

Table 2. Imprinting factors of different MIP's.

Porogenic solvents	Q_{MIP}/μg.g^{-1}	$Q_{Control}$/μg.g^{-1}	Imprinting factor
1,4-Dioxane	372	353	1.05
THF	480	400	1.20
Acetone	409	381	1.07
Acetonitrile	309	300	1.03

3.3. Effect of polarity of porogenic solvent on MIP

The origin of molecular imprinting technique was from the model of "donor-receptor" and "antibody-antigen" in Biology. The dispersion solvent provides the environment for MIP formation. The polarity of solvent will affect its interaction with template molecule and functional monomer. This interaction will strongly impact on the structure of imprinting sites in MIP. Figure 6 showed the relationship between imprinting factors of obtained MIP's and dielectric constant of porogenic solvents. It clearly indicated that the medium polar solvent gave better imprinting factor of MIP's. It means that medium polar solvent such as THF is good porogenic solvent for preparing MIP's for quercetin.

In order to interpret the experimental results, computional quantum chemical analysis was used to calculate stabilization energy of quercetin and acrylamide in various porogenic solvents. The Onsager self-consistent reaction field (SCRF) technique was performed. The stabilization energy of quercetin and acrylamide in different solvents was calculated and shown in Table 3. $E_{quercetin}$ and E_{AA} respectively represented the stabilization energy of quercetin and acrylamide in

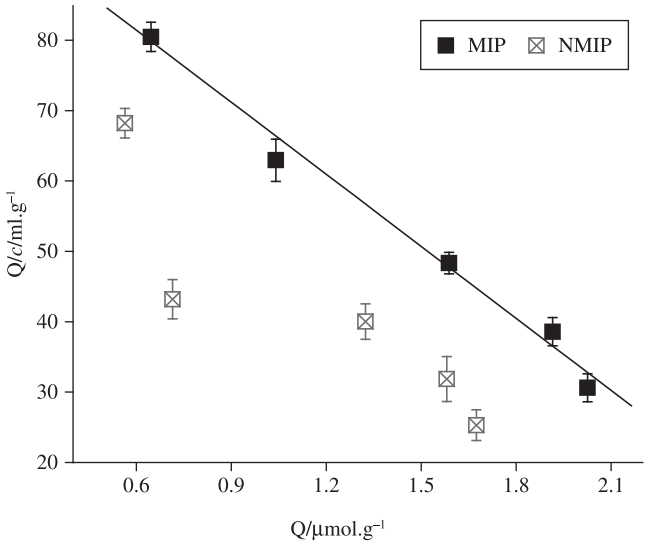

Figure 5. Scatchard plots of MIP and NMIP for quercetin.

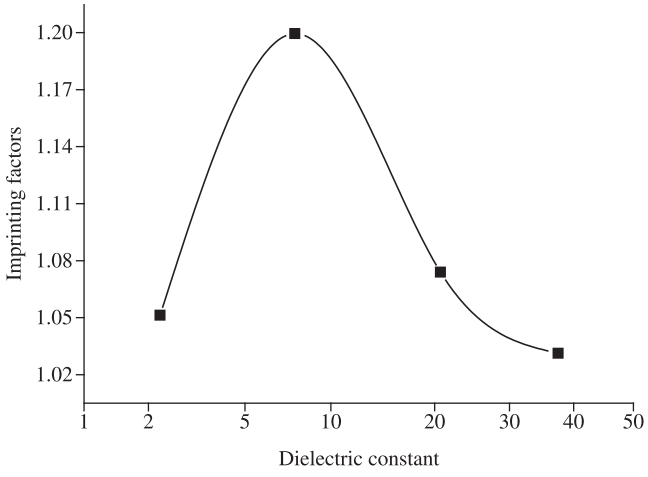

Figure 6. Imprinting factors of MIP's for quercetin and dielectric constants of porogenic solvents.

solvent. From the results in Table 3, it was found that the stabilization energy for both quercetin and acrylamide increased with increasing the polarity of solvent. It means that interaction between solvent and quercetin or acrylamide increases with increasing polarity of solvent. In Figure 6, we knew that the MIP with the highest imprinting factor was prepared in a medium polar solvent. High polarity of solvent did not produce high imprinting factor. The reason is probably because there is a competition between interaction of quercetin and acrylamide, and their interaction with porogenic solvent. The interaction between quercetin and acrylamide is reduced when the polarity of porogenic solvent increases. The polar solvent will have strong interaction with both quercetin and acrylamide, and cause them less chance to interact with each other. This leads to forming fewer imprinting sites in MIP and reduces its adsorption effectiveness for quercetin. When the solvent is less polar it has less interaction with either template molecule or functional monomer. The interaction of template molecule and functional monomer is strong and the formed MIP will quickly precipitate due to less solubility in less polar solvent. Only when the solvent has medium polarity does it have proper

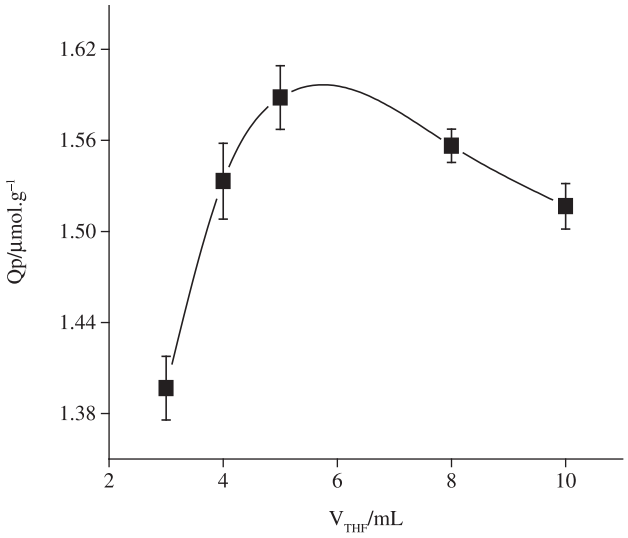

Figure 7. Effect of THF volume used in preparing MIP on quercetin adsorption.

Table 3. Stabilization energy of solvent with quercetin and acrylamide.

Porogenic Solvents	Dielectric constant/	E_{AA}/kJ.mol^{-1}	$E_{quercetin}$/kJ.mol^{-1}
1,4-Dioxane	2.25	3.232	4.782
THF	7.58	4.003	6.898
Acetone	20.7	7.251	9.784
Acetonitrile	37.5	9.177	11.241

interaction with quercetin and acrylamide. This helps to form uniform imprinting sites in MIP. This was why THF used as porogenic solvent produced better MIP's than other solvents.

3.4. Effect of amount of porogenic solvent on quercetin adsorption of obtained MIP

At certain amount of functional monomer, template molecule, and cross-linker, the amount of porogenic solvent used will affect the concentrations and gel point of formed polymer. The morphology of formed MIP will be changed and this will further affect the adsorption performance of MIP for template molecule. MIP for quercetin prepared in various amounts of THF was evaluated and shown in Figure 7. The results indicated that there was an optimal amount of THF used to obtain best MIP. The optimal THF volume was around 5 mL if there were 0.2 g acrylamide, 0.13 g queretin and 3.5 mL EGDMA in reaction mixture. It means that at low volume of THF below 5 mL, the adsorption effectiveness of resulted MIP for quercetin increased with increasing the volume of THF, and at high volume of THF above 5mL the adsorption effectiveness decreased with increasing the volume of THF. Low volume of THF caused formed polymer to precipitate early and would not form good MIP. High THF volume, however, would dilute the solution and cause imprinted sites more defects.

4. Conclusions

MIP for quercetin was prepared by thermal polymerization method using quercetin as template molecule, acrylamide as functional monomer and ethylene glycol dimethacrylate as cross-linker

in the presence of four different porogenic solvents of 1,4-dioxane, tetrahydrofuran, acetone, and acetonitrile. The results indicated that the type of porogenic solvent had big impact on formation of MIP and its quercetin adsorption. The best solvent was THF and the worst solvent was acetonitrile. According to computational quantum chemical analysis in term of the stabilization energy. The MIP obtained in medium polar solvent would demonstrate better molecular recognition ability. The amount of ponogenic solvent used also had impact on adsorption effectiveness of MIP for quercetin. There was an optimal amount of solvent when the amount of template molecule and functional monomer was kept the same.

Acknowledgements

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