SHORT COMMUNICATION

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STUDY TOWARD THE PREPARATION OF AQUEOUS COMPATIBLE SHIKIMIC ACID IMPRINTED POLYMER

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ABSTRACT

Shikimic acid is an important component in the production of several important drugs particularly the anti-influenza drug, Oseltamivir. Commercially, shikimic acid is extracted from the Chinese star anise or produced through the fermentation process by modified strain of E. coli. *Regardless of the method of production, shikimic acid needs to be purified before it can be used for the intended purpose. Conventional method in the purification of shikimic acid usually involves the use of non-specific adsorbents that are less effective in isolating the shikimic acid. Molecularly imprinted polymer, being one of the latest adsorbents in separation science, offers an alternative technique that is more selective and specific than the conventional adsorption methods. This article is the exploratory work done to optimise the formulation for the preparation of molecular imprinted polymer for shikimic acid that is 100% compatible with aqueous systems. It emphasises particularly on the effects of various template: monomer: cross-linker ratios (TMX) on the performance of final polymer, including the preliminary evaluation results of the polymer performance.*

Keywords: shikimic acid, imprinted polymer, non-covalent imprinting, 4-vinylpyridine, aqueous phase.

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INTRODUCTION

Shikimic acid (3,4,5-trihydroxy-1-cyclohexene-1-carboxylic acid) (SA), is an important organic acid intermediate for the biosynthesis of lignin, aromatic amino acids and most alkaloids of plants and microorganisms (Davis, 1955; Sprinson, 1961).

Besides its established use as the raw material for the industrial synthesis of the anti-influenza drug Oseltamivir, it has also been reported that SA is used in the synthesis of (−)-zeylenone, the preparation used for chemotherapy of cancerous diseases (Liu *et al*., 2004; Zhang *et al*., 2006). In addition, SA also holds a high value in the agriculture sector, being the raw material used for the synthesis of derivatives of safer herbicides (Amrhein *et al*., 1980) and anti-bacterial agents, compared to other conventional products because of its capability to block the shikimate pathway in plants and bacteria without any negative effects in mammals.

There are three methods available for the production of SA. The first method, being the most popular one, is the extraction and purification of SA from plant tissues. In this method, polar solvent such as water, methanol and ethanol are used to extract the SA from plants such as Chinese star anise (Edmonds and Payne, 2005), certain varieties of pine, fir and spruce (Sui, 2008) and leaves of several

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varieties of sweetgum tree (Enrich *et al*., 2008; Martin *et al*., 2010). Although the production of SA via this extraction method seems simple, the separation and purification of SA from the crude extract is tedious and involve several steps before a product with acceptable quality is produced.

The second method involves the use of engineered microorganisms from various strains of *E. coli* in the biosynthesis of SA or the biotransformation of quinic acid to SA. The production of SA via the microbial route has been reviewed by Kramer's group (Krämer *et al*., 2003).

Another method for producing SA is by chemical synthesis and this has been reviewed extensively (Campbell *et al*., 1993; Jiang and Singh, 1998).

Recently, it was reported that esterified SA was found in the palm oil mill effluent (POME) by Sambanthamurthi *et al*. (2011). Although the reported amount of SA (in esterified form) was low, this could still be potential alternative source for SA.

Regardless of the method of production, crude products of SA always contain impurities that need to be removed or the quality of final product will be adversely affected (Ize-Ludlow *et al*., 2004). Therefore, the separation and purification of SA from its crude form is an essential and challenging step.

Molecular imprinted polymer (MIP) is a custom designed polymeric material that acts as artificial receptor and is able to specifically bind to specific template molecules or group of molecules. This special feature of MIP makes it a potentially alternative solution for the simple and effective purification of SA from its crude form. Although a large scale production and utilisation of MIP for the enrichment of targeted analyte is yet to be implemented at this point of time, exploratory investigation on the preparation of effective SA imprinted polymer is still worth to carry out.

This article reports the preliminary work done to optimize the formulation for the preparation of MIP for SA that is 100% compatible with aqueous systems. The effects of various template: monomer: cross-linker (TMX) ratios on the performance of final polymer, including the preliminary evaluation results of the polymer are also presented.

MATERIALS AND METHODS

Materials

Shikimic acid [SA, Purity 98% by high performance liquid chromatography (HPLC)] was purchased from Hangzhou APIChem Technology Co., Ltd., China and methyl shikimate (SE, Purity >95% HPLC) was purchased from Santa Cruz Biotechnology. All solvents used were from Fisher Chemicals with quality of at least A.R. Grade. The 4-vinyl pyridine (4VP, 95% purity), acrylamide (ACM, ≥ 98% purity), methacrylic acid (MAA, 99% purity) and 4,4-Azobis(4-cyanovaleric acid) (ACVA, ≥75% purity) were from Sigma Aldrich.

MIP Preparation

Preparation of imprinted polymer involves the mixing of SA (the template) and a monomer in an appropriate porogen according to the quantity stated in *Table 1*. After 12 hr of standing in the refrigerator at a temperature 2°C -6°C, EGDMA and 22.0 mg of ACVA were added to the mixture which was then sonicated for 10 min with a gentle nitrogen gas bubbling through the mixture. Following this, polymerisation was initiated by placing the mixtures in a water bath at 60°C for 24 hr.

After 24 hr of polymerisation, the glass container was broken and the polymer obtained was crushed, ground and sieved to obtain polymer particles with the size of approximately 125 μm.

The fine polymer particles were then subjected to 24 hr soxhlet extraction using 10% acetic acid in methanol solution followed by extraction with methanol for another 24 hr to wash off remaining residual acetic acid in the polymer.

The polymer particles were then vacuum dried at room temperature for 24 hr before further evaluations. The above procedure was used for the preparation of each of the polymer listed in *Table 1*.

For the polymer formulations in *Table 1*, polymers which contain no templates are termed as non-imprinted polymers (NIP) and they act as control polymers for the individual formulation. The sample codes used in this article should be read in such a way that the first alphabet describes the type of polymer (M - imprinted polymer, N - non-imprinted polymer). First digit after the alphabet represents the molar ratio of template in the formulation, second digit represents the molar ratio of functional monomer and last two digits represent the molar ratio of cross-linker in the formulation followed by the codes for functional monomer used in the formulation.

Fourier Transformed Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) Analysis

FTIR measurements for all polymers were performed using KBr pellet method. SEM micrographs were captured using Hitachi VP-SEM S-3400N.

Batch Rebinding Experiments

For each processed polymer, 20 mg was mixed with 1.5 ml of 100 μg ml^{-1} of SA solution in a 2 ml Eppendorf tube. The mixture was then shaken on the shaker for a period of 4 hr followed by

TABLE 1. TEMPLATE, MONOMER AND CROSS-LINKERS RATIOS FOR THE PREPARATION OF POLYMERS IN THIS STUDY

Entry	Sample code	Type	Polymer composition			
			Template (mmol)	Monomer	Monomer (mmol)	Cross-linker (mmol)
1	M1420 MAA	MIP	1	MAA	4	20
2	N0420 MAA	NIP	0	MAA	4	20
3	M1420 4VP	MIP	1	4VP	4	20
4	N0420 4VP	NIP	0	4VP	4	20
5	M1420 AAM	MIP	1	AAM	4	20
6	N0420 AAM	NIP	0	AAM	4	20
7	M1620 4VP	MIP	1	4VP	6	20
8	N0620 4VP	NIP	0	4VP	6	20
9	M1820 4VP	MIP	1	4VP	8	20
10	N0820 4VP	NIP	0	4VP	8	20
11	M1210 4VP	MIP	1	4VP	2	10
12	N0210 4VP	NIP	0	4VP	2	10
13	M1410 4VP	MIP	1	4VP	4	10
14	N0410 4VP	NIP	0	4VP	4	10
15	M1610 4VP	MIP	1	4VP	6	10
16	N0610 4VP	NIP	0	4VP	6	10
17	M1810 4VP	MIP	1	4VP	8	10
18	N0810 4VP	NIP	0	4VP	8	10

Note: Porogen used in the polymer preparation was 10 ml water/methanol (1:4) mixture.
MIP – molecular imprinted polymer. MAA – methacrylic acid. 4VP -4-vinyl pyridine. NIP – non-imprinted polymer. AAM – acrylamide.

centrifugation at 3000 rpm (955 g) for 5 min to enable phase separation. The top layer was taken for HPLC analysis to determine the amount of SA extracted.

HPLC Analysis

Quantification of SA was conducted using an Agilent 1100 series HPLC with UV detection at 213 nm. The column used was Rezex™ ROA-Organic Acid H^+ (8%), 150 x 7.8 mm from Phenomenex Inc. at room temperature. The mobile phase was 0.005N sulphuric acid solution at a flow rate of 0.6 ml min^{-1}.

Cross-reactivity Study

In a typical cross-reactivity test, bi-components solution of SA/SE at the concentration of 100 μg g^{-1} in water was first prepared.

A 20 mg sample of each processed polymer particle was mixed with 1.5 ml of the solution in a 2 ml Eppendorf tube. The mixture was then shaken on the shaker for a period of 24 hr followed by centrifuging at 3000 rpm (955 g) for 5 min to enable phase separation. The top layer was taken for HPLC analysis to determine the concentration of individual component.

RESULTS AND DISCUSSION

FTIR and SEM Analysis of Imprinted Polymer

FTIR measurements are presented in *Figure 1*. The absence of adsorption peak at 1948 cm^{-1} assigned to the vinyl bending vibration of 4VP in IR spectrum 1(c) for MIP as compared to the IR spectrum 1(a) for 4VP indicates the participation of 4VP in polymerisation. The same goes to the weakening of adsorption peak at 1637 cm^{-1} and the persistence of strong adsorption peak at 1731 cm^{-1} in spectrum 1(c)

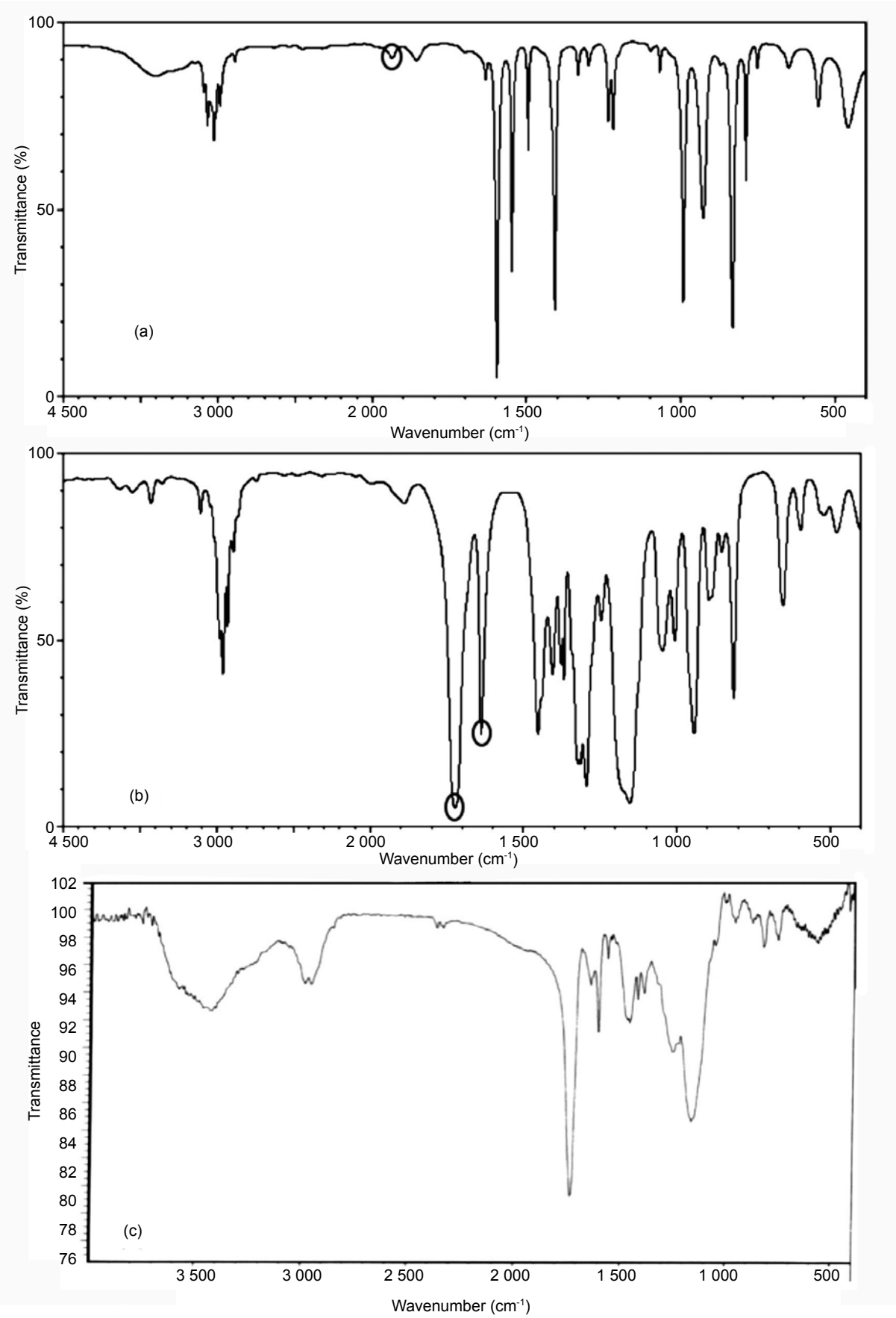

Figure 1. Fourier transformed infrared spectroscopy (FTIR) spectrum of 4VP 1 (a), EDGMA 1 (b) and MIP 1(c).

compared to IR spectrum of cross-linker EGDMA [1(b)] assigned to the stretching vibration peak of C=C and C=O groups respectively, suggesting the cross linking of the MIP.

From the SEM micrographs (*Figures 2a* and *2b*), it can be seen that the surface morphology of MIP is more porous compared to NIP that is more dense in nature, and this probably explains the difference in their binding behaviour toward SA.

Batch Rebinding

The important factor in determining the success of a non-covalent imprinted polymer for specific adsorption of template as described in this article is the amount of effective pre-polymerisation complex present during polymerisation. This is an equilibrium controlled (following the Le Chatelier's principle) process and a proper design of TMX ratio will have a direct effect on the performance of the final polymer.

To quantify the effectiveness of an imprinted polymer, a parameter called imprinting factor (IF) was used. The IF is defined as the absorption ratio

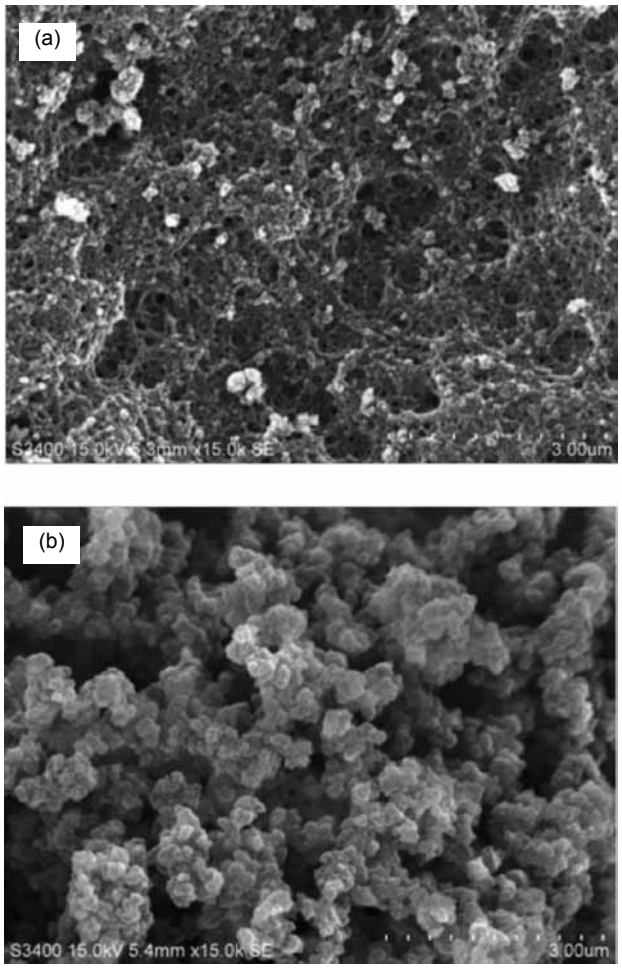

Figure 2. Scanning electron microscopy (SEM) images of MIP (a) and NIP (b).

of imprinted polymer and control polymer. An imprinted polymer is said to be effective when an IF value >1.0 is obtained. The higher the IF value, the more significant is the imprinting effect. The rebinding results of various polymers described in this article are shown in *Table 2*.

The preparation of the SA imprinted polymer was first carried out with a typical TMX ratio of 1:4:20 (row 1-6, *Tables 1* and *2).* Ratio commonly used by most researchers in the MIP field (Andersson *et al*., 1999). Three types of functional monomers with different pH characteristics are usually used. It can be seen (*Table 2*) that under this TMX ratio, all three imprinted polymers showed some selectivity for SA as compared to the controls, and 4VPl-based polymer was more selective compared to other polymers based on MAA and AAM.

This result is expected as an acidic template such as SA usually imprints well with a basic functional monomer (Kempe *et al*., 1993) such as 4VP.

After determination of the best functional monomer to be used, subsequent experiments were to identify the best TMX ratio with optimum performance. Ratios of the functional monomer were increased to 1:6:20 and 1:8:20 respectively, and the batch rebinding results (row 7 and 9, *Table 2*) indicated that both imprinted polymers have better selectivity when compared to the polymer prepared under general TMX ratio of 1:4:20, and the polymer prepared using TMX ratio of 1:6:20 produced the best imprinting factor of 3.98. This could be attributed to the increase in the functional monomer ratio that shifted the pre-polymerisation equilibrium step to the right, thereby generating more effective binding sites in the final polymer. Although this is generally true for all the non-covalent imprinting systems, too much of the functional monomer may cause the increase in non-selective binding sites (Tom *et al*., 2012) as shown by polymer prepared using the TMX ratio of 0:8:20 with higher adsorption of NIP compared to 0:6:20 counterpart (row 8 and 10, *Table 2*). It is important to highlight that a balance between effective binding sites and non-selective binding sites should be taken into consideration during the formulation of imprinting polymers.

After the ratio of functional monomer has been decided, optimisation of the cross-linker was carried out using the cross-linker ratios of 10 (row 11-18, *Table 1*).

Taking into consideration that the intended use of final polymer would be in a highly aqueous medium, too much of hydrophobic EGDMA use in polymer preparation may affect the wettability of the polymer surface and a high ratio of cross-linker is known to hinder the template molecules from moving in and out of the effective binding sites (Cormack and Elorza, 2004), a lower ratio of cross-linker was tested. The rebinding results (row 15-16, *Table 2*) showed that the IF was increased when a

TABLE 2. BATCH REBINDING EXPERIMENT RESULTS

Entry	Polymer code	Binding (%)	Imprinting factor (α)	Rebinding solvent
1	M1420 MAA	1.4	1.56	H_2O
2	N0420 MAA	0.9		
3	M1420 4VP	8.7	2.12	
4	N0420 4VP	4.1		
5	M1420 AAM	3.1	1.29	
6	N0420 AAM	2.4		
7	M1620 4VP	7.70	3.98	
8	N0620 4VP	1.94		
9	M1820 4VP	12.72	2.20	
10	N0820 4VP	5.79		
11	M1210 4VP	2.13	∞	
12	N0210 4VP	Nil*		
13	M1410 4VP	12.62	0.87	
14	N0410 4VP	14.50		
15	M1610 4VP	26.85	4.18	
16	N0610 4VP	6.43		
17	M1810 4VP	33.03	1.41	
18	N0810 4VP	23.50		

Note: * Quantity of SA determined in this sample is higher than the amount spiked. This may be due to the interference from the very fine particles formed during polymerisation at low monomer content solution, therefore the α value cannot be calculated.

lower ratio of the cross-linker was used to prepare the SA imprinted polymer.

To further examine the best formulation for the SA imprinted polymer, a series of polymers with different 4VP ratios (2, 4 and 8) and fixed cross-linker ratio (10) were prepared and tested. Results from the rebinding experiments confirmed that the SA imprinted polymer prepared using TMX ratio of 1:6:10 was the best with an IF of 4.18 compared to the other formulations that gave IF ranging from 0.87 to 1.41. The test functional monomer ratio was stopped at 8 because the IF of TMX at 1:8:10 started to decrease indicating an increase of the non-selective binding sites, *i.e.*, too much of the functional monomer.

Cross-reactivity Study

Specificity is a key parameter in describing the effectiveness of an imprinted polymer. To quantitatively measure the degree of selectivity of an imprinted polymer, cross-reactivity test is usually performed. A good imprinted polymer should show higher IF value for the template than interference analyte.

Figure 3 is the HPLC chromatograms for the mixture of SA and SE at 100 ppm each in water before (blue) and after the adsorption by imprinted polymer (red) and the control polymer (orange) respectively.

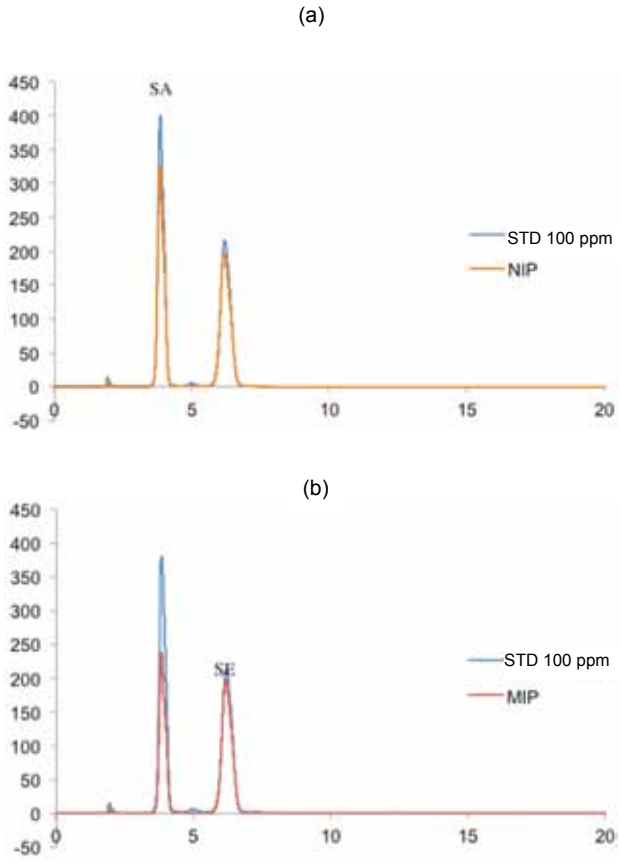

Figure 3. Chromatograms of SA and SE mixture before (blue) and after adsorption by SA imprinted polymer (red) and control polymer (orange).

It is noted that the adsorption of SA onto imprinted polymer is higher than those in the control polymer. Whereas, the adsorption of SE onto both SA imprinted polymer and control polymer remain unchanged. This suggested that the SA imprinted polymer prepared is selective toward SA.

Another important message from this result is related to the binding sites characteristic of the SA imprinted polymer, the only different between SA and SE is at the carboxylic acid functional group of SA, with the substitution of the -COOH group by-$COOCH_3$ group.

In the esterified form, no acidic hydrogen is available in SE to establish the ionic interaction as what had happened between SA and SA imprinted polymer. With this difference in the interaction, selective binding of SA over SE by SA imprinted polymer is achieved.

CONCLUSION

The best formulation for the preparation of the SA imprinted polymer was investigated and identified. It was found that 4VP was the best functional monomer producing an imprinted polymer that showed a high affinity toward SA. A careful examination of other TMX ratios indicated that 1:6:10 was the best ratio.

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