CHAPTER II

SYNTHESIS OF MOLECULARLY IMPRINTED POLYMER SELECTIVE TO GENISTEIN ISOFLAVONE USING FRAGMENT IMPRINTING TECHNIQUE

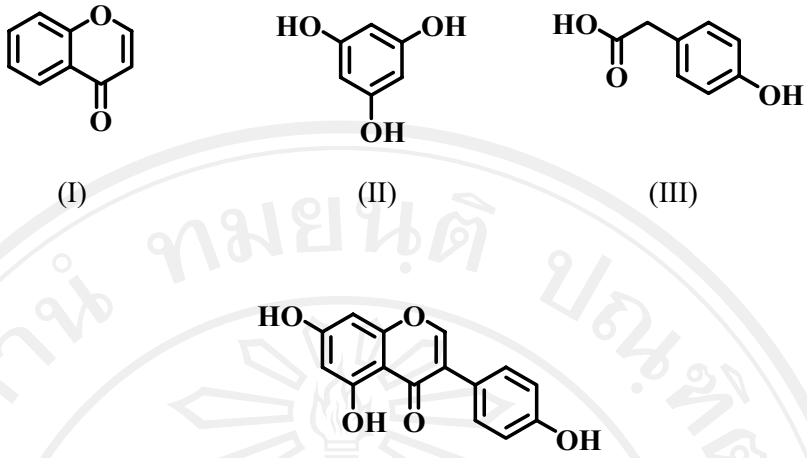
2.1 Introduction

Molecularly imprinted polymer becomes one of the most promising separation or isolation techniques in the last decade according to its high selectivity to the target molecule. It has been largely demonstrated that the MIPs offer more benefits than conventional separation or isolation techniques. Their inherent advantages include reusability, simplicity, low cost, high affinity, physical and chemical stability over a wide range of experimental conditions and solvents.

Molecularly imprinted polymers have been applied to selectively separation of many groups of natural products. Among flavonoids, quercetin is the most popular template used in this effective technique. Many researches had been successful in the imprinting of flavonoid molecules such as catechin,[32] flavonol[33] and diadzein[34]. However, there has been no references reported the use of genistein as a template in MIP, this flavonoid is of interest among many researchers in the study of its pharmaceutical effect, synthesis, extraction and purification.[35-38] Although genistein is commercially available, it is difficult to obtain enough material from the extraction and purification in natural sources. To prepare MIP by the imprinting process, the target compound is generally required in slightly large scale. Therefore, it is almost impossible to use this approach using genistein.

Nevertheless, there is a novel alternative imprinting techniques that produces the imprinting materials using fragment template molecules which have some part of functional group of the targeting compound. It is called “Fragment Imprinting Technique” (FIT). The FIT has been studied in the imprinting of many toxic compounds and rare compounds such as halogenated aromatic compounds.[39-42] Moreover, FIT has been applied for the use to imprint the water-soluble toxic compounds such as cylindrospermopsin and domoic acid which are hardly to imprint in organic solvent as commonly.[43] The results from these studies suggested that there is a possibility to apply the FIT technique for the selective separation of a lot of toxic compounds and rare natural compounds.

In this study, the FIT technique was used for synthesis of imprinted polymer selective to genistein. Chromone, phoroglucinol and 4-hydroxyphenyl acetic acid that are commonly used as the starting materials in the synthesis of isoflavones were used as fragment templates to synthesize the MIPs using bulk polymerization technique. The chemical structures of genistein and fragment templates are shown in **Figure 2.1**.



(I) (II) (III)

Genistein

Figure 2.1 Chemical structures of genistein, chromone (I), phloroglucinol (II) and 4-hydroxyphenylacetic acid (III)

2.2 Experimental section

2.2.1Chemicals and Reagents

Genistein, $C_{15}H_{10}O_5$, assay 98%, Fluka, China.

Chromone (I), $C_9H_6O_2$, assay 99%, Sigma-Aldrich, China.

Phloroglucinol (II), $C_6H_6O_3$, assay 99%, Fluka, China.

4-Hydroxyphenylacetic acid (III), $C_8H_8O_3$, assay 98%, Fluka, France.

Methacrylic acid (MAA), $C_4H_6O_2$, assay 98%, Fluka, Switzerland.

Acrylamide (AA), C_3H_5ON, Fluka, Switzerland.

4-Vinylpyridine (4-VP), C_7H_7N, Aldrich, Germany.

Ethyleneglycol dimethacrylate (EGDMA), $C_{10}H_{14}O_4$, Fluka, Germany.

Azobisisobutyronitrile (AIBN), $C_8H_{12}N_4$, BDH, England.

Benzoyl peroxide (BZP), $C_{14}H_{10}O_4$, Janssaen Chemical, Belgium.

Acetonitrile (ACN), C_4H_3N, RCI Lab scan, Thailand.

Dichloromethane (DCM), $C_2H_2Cl_2$, RCI Lab scan, Thailand.

Methanol (MeOH), CH_4O, RCI Lab scan, Thailand.

Acetic acid (AcOH), $C_2H_4O_2$, Carlo Erba, Italy.

Potassium bromine (KBr), KBr, Merck, Germany.

2.2.2 Instruments

UV-VIS spectromemter (Thermo spectronic, Geneysy 10-S), WI, USA.

SPE manifold (Restek, Resprep^{TM} 24-port manifold), USA.

Scanning electron microscope (JEOL, 633F), Japan.

Fourier-transform infra-red spectroscopy (Bruker, Tenser 27), Switzerland.

2.2.3 Synthesis of imprinted and non-imprinted polymers

The imprinted and non-imprinted polymers were synthesized following this process. The template (1.0 mmol) was dissolved in 0.5 ml porogen (ACN). Then, the functional monomer (4.0 mmol) was added into the solution and allowed to self-assembly for 15 mins. After that, the cross-linker (20.0 mmol) and initiator (0.50 mg) were added into the solution. The solution was cooled in ice bath and then purged with nitrogen for 15 min. After that, the solution was polymerized at 60°C for 12 hrs. The non-imprinted polymer was synthesized under the same method in the absence of the template. The resulted polymer was ground in mortar, sieved using 75 μm pore size sieve and sorted by acetone to get rid of the fine particle. The template was removed from the polymer by extraction with 20% AcOH in MeOH. The polymers were washed with ACN followed by MeOH to remove residual acid. The pure polymers, that have no template and un-reacted starting materials, were dried in oven for overnight before use.

2.2.4 Binding studies of the polymers with their corresponding templates

After the template was removed, the binding affinity of the polymer towards their template molecules was evaluated by binding studies using adsorption experiment. The procedure for the adsorption activity was as follows: An aliquot of fifty milligrams of each polymer was packed into a 3 ml SPE-cartridge. The cartridge was fritted with polyethylene disks at the top and the bottom. The cartridge was then preconditioned with 1.0 ml of MeOH 3 times and 1.0 ml of ACN 3 times, successively, before sample loading. The template solution in ACN (0.30 mM, 1 ml) was loaded into the cartridge and the collected volume was adjusted to 1.0 ml with

ACN. The amount of unbound template was determined using UV-Vis spectrophotometer at λ_{max} 294 nm and 277 nm for (I) and (III), respectively. The same procedure was followed for every template solutions.

The binding performances of MIPs were determined by the percentage bound (*% Bound*) and imprinting factors (α) which were calculated according to equations. **(2.1)** and **(2.2)**, respectively.

$$\% Bound = \frac{Q_{bound}}{Q_{initial}} \times 100 \qquad \textbf{(2.1)}$$

Where Q_{bound} is the amount of analyte bound to the polymer after loading and $Q_{initial}$ is the initial amount of analyte before loading to the polymer.

$$\alpha = \frac{\% Bound_{MIP}}{\% Bound_{NIP}} \qquad \textbf{(2.2)}$$

Where $\% Bound_{MIP}$ and $\% Bound_{NIP}$ represent the percentages of bound analyte by MIP and NIP, respectively.

2.2.5 Binding studies of the polymers with genistein

The affinity of the polymers towards genistein was investigated using the same way with the previous binding studies **(2.2.4)**. The standard genistein 0.1 mM in ACN was used for instant. Additionally, DCM was used as another solvent in the binding study of the polymers to genistein to study the effect of polarity of solvent. The appropriated polymers among each template used were selected for further evaluation in the next experiment.

2.2.6 Characterization of the polymers

The morphology of the selected polymers was examined in both imprinted and non-imprinted forms by scanning electron microscopy (SEM) after the template molecule has been removal (for MIP). The surface of the polymers was then covered with a thin, electrically conductive layer by gold sputtering prior to the observation of the samples, because polymers were insufficiently conductive on their own. After that, the SEM micrographs were taken.

FT-IR (fourier-transform infra-red spectroscopy) spectra were recorded using FT-IR spectrometer with a spectral range of 4000-400 cm^{-1}. The selected polymers were mixed and ground with KBr to make KBr disc for FT-IR spectra collection.

2.3 Results and Discussion

2.3.1 Synthesis of imprinted and non-imprinted polymers

Molecularly imprinted polymers and control polymers were synthesized with a molar ratio of the template to the functional monomer and the cross-linker as 1:4:20. **Table 2.1** shows the composition of the synthesized polymers. In this study, Chromone (I), phloroglucinol (II) and 4-hydroxyphenylacetic acid (III) which are the commercial available and commonly used in synthesis of isoflavones were used as templates to imprint the soybean isoflavone molecules. The bulk polymerization, a simple effective procedure that is usually used to synthesize molecularly imprinted polymers, was chosen in this study. The selection of functional monomer was based on hydrogen bond with the templates that will occur during imprinting process. Three types of functional monomers were chosen, such as methacrylic acid (MAA), 4-vinylpyridine (4-VP) and acrylamide (AA), which were acid, base and neutral

functional monomer, respectively. The compositions of the polymer are showed in **Table 2.1**. Benzoyl peroxide (BZP) was used as initiator except in the case of PII-MAA, PII-VP, PII-AA and PIII-MAA, that azobisisobutyronitrile (AIBN) was used as initiator due to the reactivity of template to initiator. The same cross-linker, ethylene glycol dimethacrylate (EGDMA), was employed for all polymers.

Table 2.1 Composition of the imprinted polymers

Polymer	**Template (1 mmol)**	**Monomer (4 mmol)**	**Cross-linker (20 mmol)**	**Initiator**	**Porogen**
PI-MAA	I	MAA	EGDMA	BZP	ACN
PI-VP		VP	EGDMA	BZP	ACN
PI-AA		AA	EGDMA	BZP	ACN
PII-MAA	II	MAA	EGDMA	AIBN	ACN
PII-VP		VP	EGDMA	AIBN	ACN
PII-AA		AA	EGDMA	AIBN	ACN
PIII-MAA	III	MAA	EGDMA	AIBN	ACN
PIII-VP		VP	EGDMA	BZP	ACN
PIII-AA		AA	EGDMA	BZP	ACN

2.3.2 Binding of the polymers with their corresponding templates

Binding performance of the synthesized polymers was investigated by binding their template to the corresponding polymers in the same solvent used during MIP

synthesis. Indeed, it has often been reported that the recognition of template molecules by imprinted polymer was better in the porogenic solvent.[44,45] **Figure 2.2** shows the binding performance of the polymer which used chromone as a template. All of the polymers using chromone template obtained from different monomer types had low % bound (15-18%) and there was no difference in % bound between the MIPs and their NIPs. So, their imprinting factor (α) was certainly low. It is suggested that the chromone molecules bound to the polymer by non-specific interaction. The imprinting process of this molecule may not succeed even in any types of selected functional monomers. It can be explained that the chemical structure with only carbonyl and ether functionality is very hard to imprint using non-covalent imprinting techniques.

The binding performances of the synthesized polymers using 4-hydroxyphenylacetic acid as template are shown in **Figure 2.3**. The results show that the polymer obtained from 4-VP (PIII-VP) had the highest % bound especially in its MIP (65.9%). The value of imprinting factor obtained from PIII-VP (1.9) was also high. The difference in the amount of 4-hydroxyphenylacetic acid bound to MIP and NIP indicated that 4-hydroxyphenylacetic acid has highest potential to imprint among all the templates used in this technique. It has been reported that the difference of pKa between template and functional monomer can affect the imprinting ability of the imprinted polymer.[46-48] It is implied that, beside the hydrogen bond, the electrostatic interactions between acidic template and basic functional monomer was occurred. According to this reason, among the monomers used, 4-VP exhibited the best recognition ability. In other words, the PIII-VP MIP can recognize its template, whereas PIII-MAA and PIII-AA have no recognition ability because the lack of this

kind of interaction. Unfortunately, the polymers obtained from phloroglucinol template (PII-MAA, PII-AA and PII-VP) were not investigated in this experiment because of the non-linearlity of UV absorbance obtained over the concentration range.

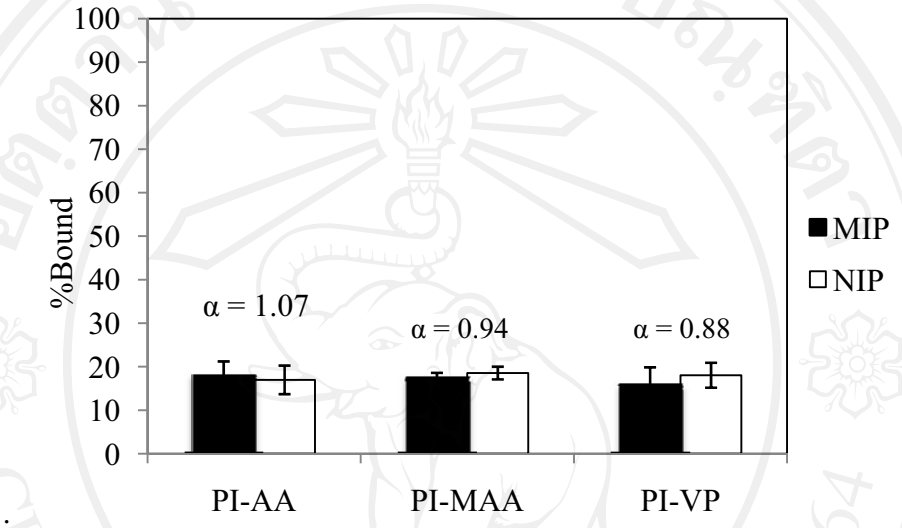


Figure 2.2 Percentage bound obtained from MIPs from chromone template and their corresponding NIPs

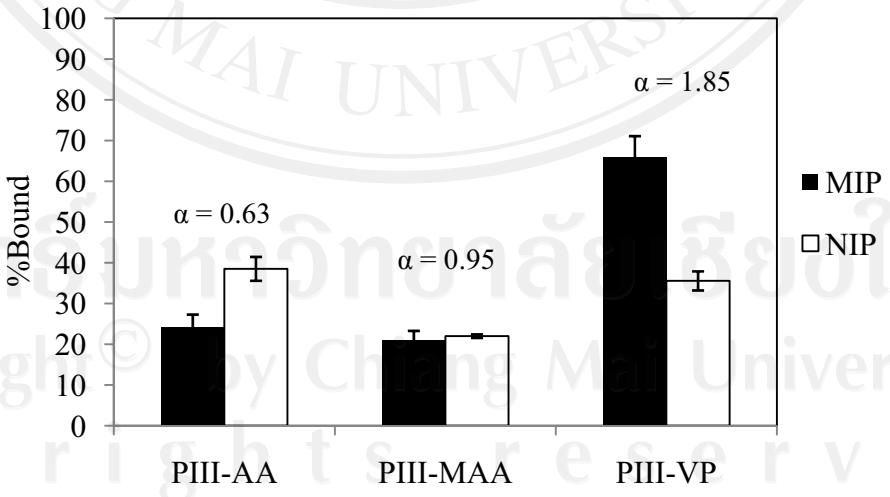


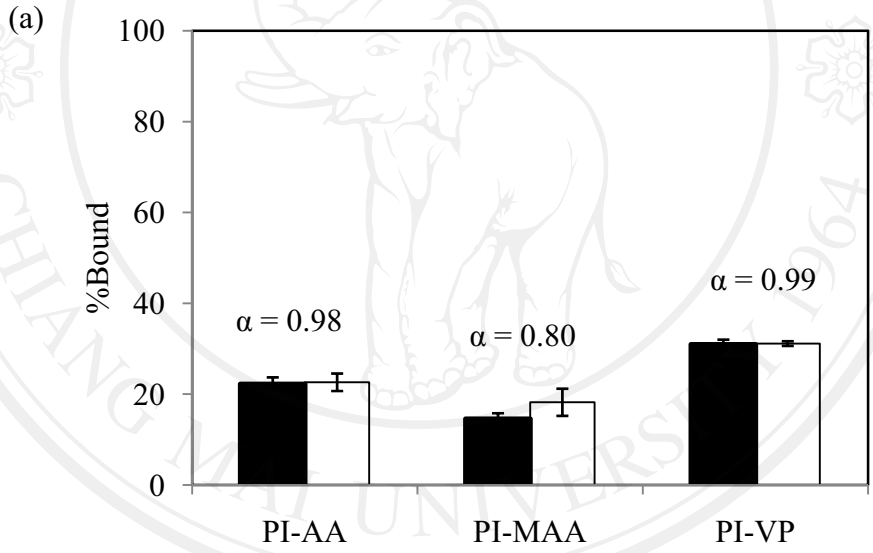
Figure 2.3 Percentage bound obtained from MIPs from 4-hydroxyphenylacetic acid and their corresponding NIPs

2.3.3 Binding of the polymers with genistein

To verify the affinity of the synthesized polymer to genistein, the molecular recognition of polymeric particles was estimated with respect to their abilities to adsorb genistein from standard solutions in ACN. Measurement of the unbound substrate concentration in the eluted solution after loading was carried out as previously described (**2.2.5**). As shown in **Figure 2.4 (a)**, all of the polymers obtained from chromone template, had no differences in % bound of genistein between MIPs and their NIPs. This can be explained in the same way as previous study in **2.3.2** that there was no recognition ability in polymer obtained from chromone template. Similar to MIPs from chromone template, the polymers obtained from phloroglucinol template gave the low imprinting factors in binding study with genistein. The percentage bounds and imprinting factors of MIPs from phloroglucinol template are shown in **Figure 2.4 (b)**. Nevertheless, among the MIPs obtained using 4-hydroxyphenylacetic acid as a template, the PIII-VP showed the highest imprinting factor (1.19), **Figure 2.4 (c)**. It demonstrated that the specific binding sites of the PIII-VP were not only bound to their template but also bound to genistein.

The affinity of polymer in DCM was investigated to study the effect of polarity of solvent. From the concept of non-covalent polymerization technique, the binding of target analyte to the MIP is normally occurred by specific interaction such as hydrogen bonding. Therefore, the polarity of solvent is very important. The percentage bound can be increased when the polarity of solvent decreases. As expected, the results showed that the % bound of genistein was increased over 2-3 times when DCM was used in all polymers **(Figure 2.5)**. Therefore, the polarity of solvent had effect on the % bound of the polymers because the H-bonding between

genistein and the polymers was destroyed by the high polarity of solvent. Moreover, the decrease in polarity of solvent can increase recognition ability of the polymers especially PI-VP, PII-AA and PIII-VP. The polymer which had the highest imprinting factor in each of the same template molecule in imprinting process, which were PI-VP, PII-AA and PIII-VP, was chosen to be a representative and used in further study on extraction efficiency towards genistein.



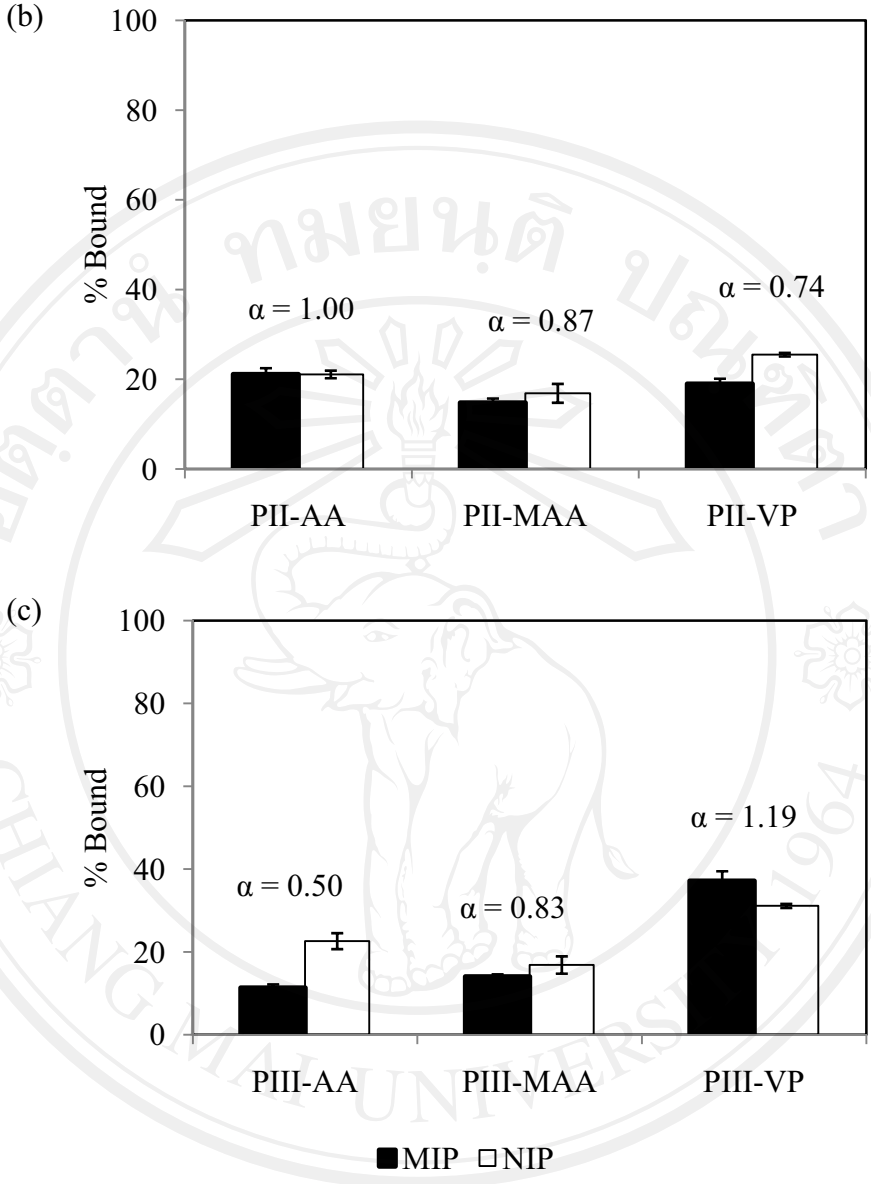
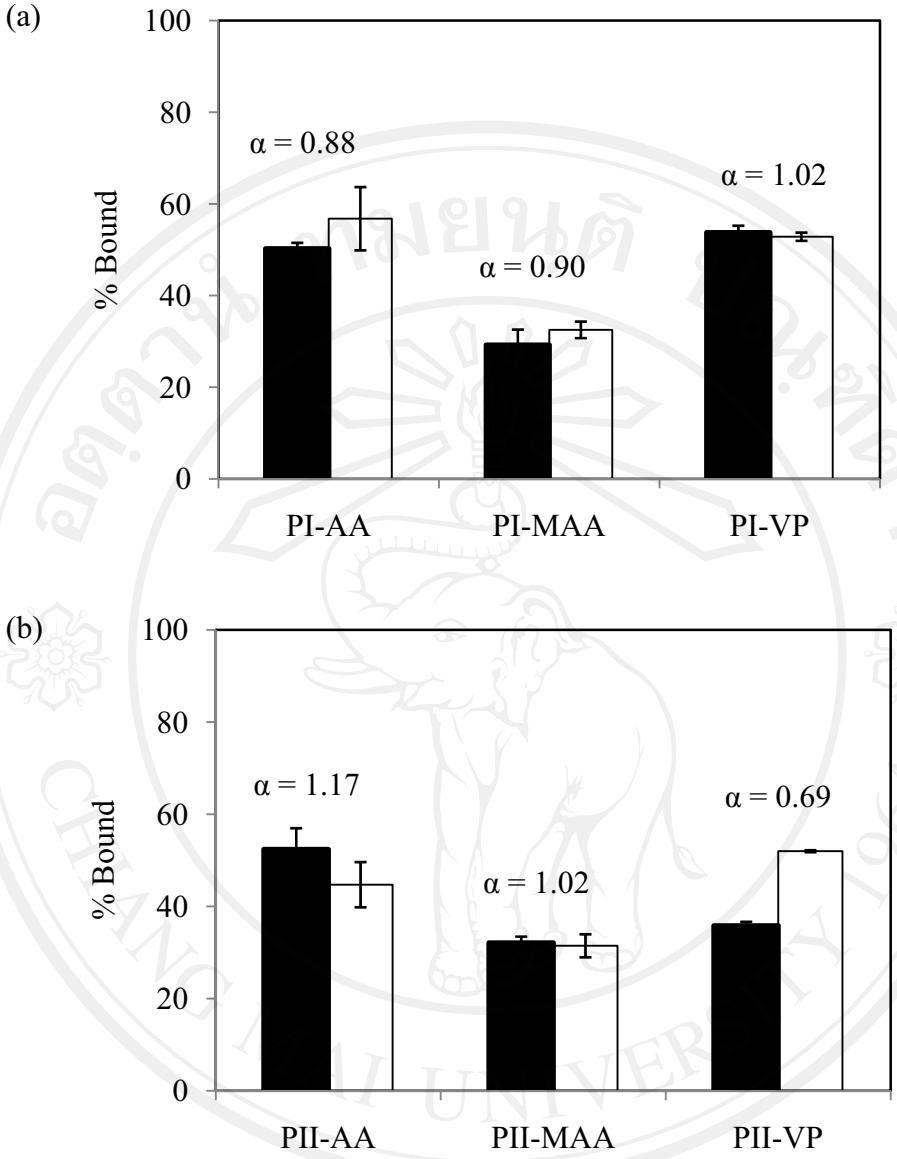


Figure 2.4 Imprinting factor and % bound to genistein in ACN of the synthesized polymers obtained by using chromone (a), phloroglucinol (b) and 4-hydroxyphenylacetic acid (c) as templates.



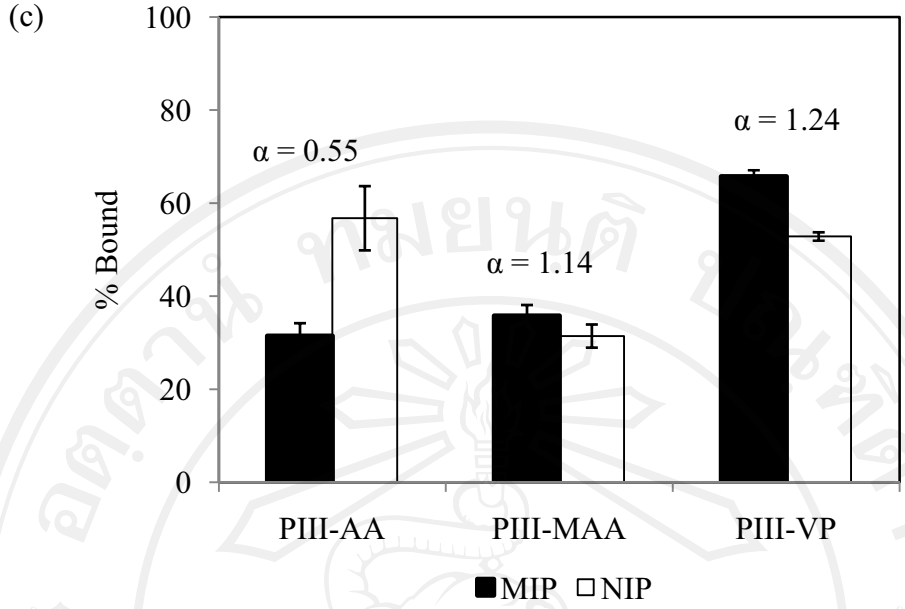


Figure 2.5 Imprinting factor and % bound to genistein in DCM of the synthesized polymers obtained by using chromone (a), phloroglucinol (b) and 4-hydroxyphenylacetic acid (c) as templates.

2.3.4 Characterization of the synthesized polymers

Scanning electron microscopy is a suitable method used to observe the structures of MIPs. The images of morphology of the selected imprinted polymers and their non-imprinted counterparts are shown in **Figure 2.6**. These evidences revealed that the PI-VP and PII-AA polymer had moderately rough structure but there were no differences in the morphology compared with their corresponding NIPs. However, the structure of PIII-VP showed a rougher surface than its NIP.

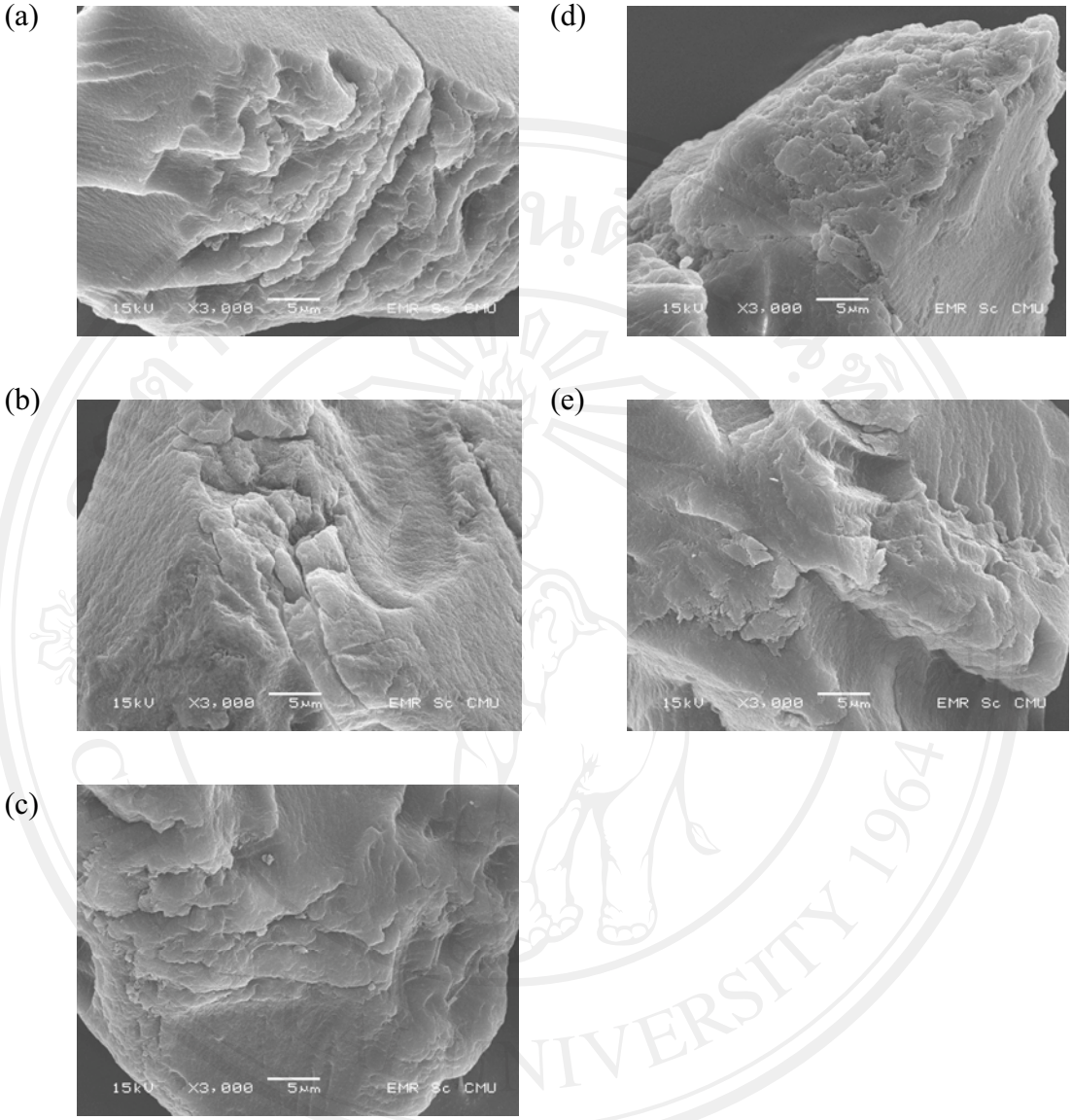


Figure 2.6 SEM images of MIPs and NIPs obtained: (a) PI-VP MIP, (b) PII-AA MIP, (c) PIII-VP MIP, (d) NIPs of PI-VP and PIII-VP and (e) PII-AA NIP

The FT-IR was used in chemical characterization of the selected polymers. **Figure 2.7** shows the FT-IR spectra of the selected polymers. It was found that all of the polymers using 4-VP as functional monomer show the two bands of C=C and C=N stretching of aromatic amine at 1598 and 1335 cm^{-1}, respectively, and the polymers using AA as functional monomer show the two bands of N-H stretching at

3460 and 3464 $cm^{-1}$and the band of C=O stretching at 1650 cm^{-1} of primary amide. This indicated that the selected polymers contain their corresponding function as the used functional monomers.

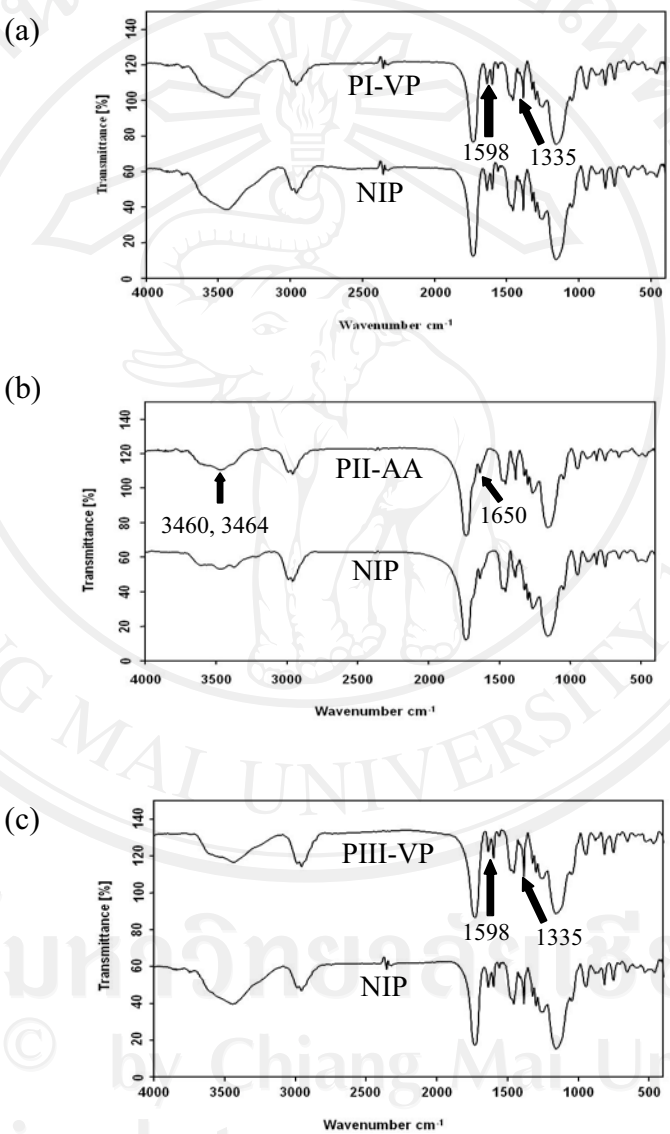


Figure 2.7 FT-IR spectra of (a) PI-VP, (b) PII-AA and (c) PIII-VP and their corresponding NIPs