CHAPTER III

OPTIMIZATION OF MOLECULARLY IMPRINTED SOLID PHASE EXTRACTION FOR GENISTEIN

3.1.Introduction

MIPs are synthetic polymeric materials that have specific recognition to the template used in their synthesis which are easy to prepared and contain chemical and physical stability. Up to now, MIPs have been successfully used in several fields including bioassay, biochemical sensors, affinity separation, organic synthesis and catalysis.[37] Due to the specific recognition offered by MIPs, they are often applied in fields where binding with high selectivity and affinity is required. Among the application on affinity based separation, the use of MIP materials as sorbents for solid phase extraction (SPE), called molecularly imprinted solid-phase extraction (MISPE), is the most widely investigated.[49] The first application was performed by Sellergen et al in 1994 for the extraction of pentamidine from urine.[50]

Nowadays, MISPE is applied for the selective extraction or clean-up of target analytes from various complex matrices. Many molecules in the pharmaceutical such as antibiotics and steroids were extracted from biofluids using this technique.[51,52] In the environmental fields, MIPs were mainly developed for the selective extraction of a particular class of pollutants including phenolics compounds, triazines and phenylureas from various matrices.[53] For agricultural and food sector, several herbicides, pesticides, food additives and components were investigated for the

selective extraction. It has already been used to extract analytes from complex samples like plant extracts and good results were obtained from the selective recognition by the MIP of specific analytes and related compounds.[54-58]

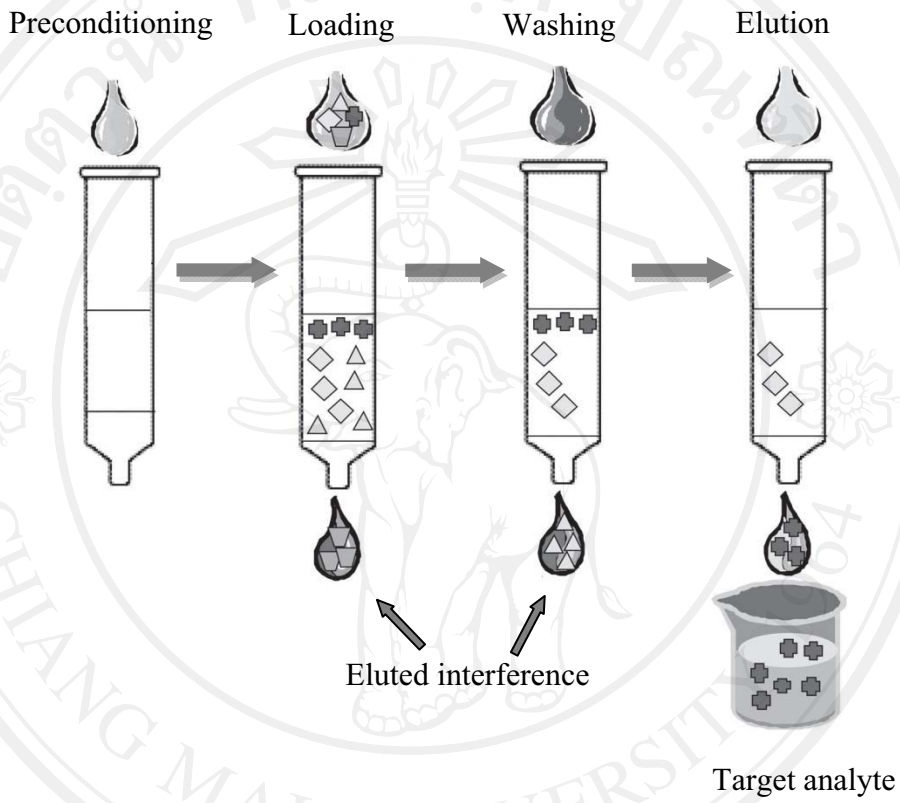


Figure 3.1 General procedure of SPE process

The general SPE procedure is presented in **Figure 3.1**. After a conditioning step, the sample was applied through the MIP cartridge then a washing step removes interfering compounds that were partially retained from the loading step. The analytes desorption is achieved by elution with solvent able to disrupt the selective interactions involved between the MIP and the target analyte to achieve the effective extraction. In this study, the MISPE protocol of PIII-VP will be optimized to obtain suitable conditions for genistein extraction. After the optimized MISPE protocol was

achieved, the PIII-VP polymer will be applied in the extraction of genistein from soybean extracts.

3.2.Experimental section

3.2.1Chemicals and Reagents

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

Quercetin, $C_{15}H_{10}O_7$, assay 95%, Sigma-Aldich, Germany.

2-Napthol, $C_{10}H_8O_1$, assay 99.99%, Fluka, China.

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

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

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

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

Formic acid (FA), CH_2O_2, Carlo Erba, Italy.

Triethylamine (TEA), $C_6H_{15}N$, Fluka, Switzerland.

3.2.2 Instruments

High performance liquid chromatograph (Agilent, HP1100), Germany.

Mass spectrometer (Agilent, HP1100), Germany.

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

Freezer dryer (Snijders, type 2040), Holland.

HPLC column (Agilent, Eclipse XDB-C18, 250 x 4.6 mm and 5 μm), USA.

HPLC column (Thermo, Hypersil Keystone, 150 x 4.6 mm and 5 μm), USA.

3.2.3 Optimization of MISPE protocol

The polymer (200 mg) was packed into the commercial SPE cartridge and capped with polyethylene frits on top and bottom side. The SPE was performed using 24-port SPE manifold combined with vacuum pump. The cartridge packed with the polymer was washed with MeOH 1.0 ml for 3 times then ACN 1.0 ml for 3 times before conditioning with the solvent since to solvent using in the loading step.

3.2.3.1 Optimization of loading conditions

After precondition step, the cartridge was dried in vacuum for 1-2 min. Then the one milliliter of standard 20 ppm genistein in MeOH aqueous solution was loaded into the cartridge and reloaded twice. The difference composition of MeOH in water was varied as loading solution. **Table 3.1** shows the loading condition used in the optimization. The solution after loading was collected and evaporated until no MeOH left. The fraction was then freeze-dried and re-dissolved to 1.0 ml with ACN containing 20 ppm of internal standard (2-napthol) before being subjected to analysis by HPLC. The HPLC condition is shown in chromatographic analysis **(3.3.2)**. The loading condition which can retain most of genistein was selected as loading solution. The amount of genistein released from the cartridge was determined and calculated into percentage retained by comparison with initial loading amount according to the equation **(3.1)**.

$$\%\ Retained = \frac{A_{load}}{A_{initial}} \times 100 \qquad \textbf{(3.1)}$$

Where A_{load} is the loaded amount of genistein on the cartridge after loading step and $A_{initial}$ is the initial amount of genistein before loading into the cartridge.

Table 3.1 Loading conditions for optimization of MISPE procedure

Entry	Loading conditions
1	20% MeOH in water
2	40% MeOH in water
3	80% MeOH in water

3.2.3.2 Optimization of washing conditions

After the standard genistein 20 ppm (1.0 ml) was loaded into the cartridge. The cartridge was dried under vacuum. Then the genistein retained on the cartridge was washed with 1.0 ml of washing solution for 5 times. The washing conditions are shown in **Table 3.2**. The genistein on the cartridge was eluted from the cartridge with 1.0 ml of 1% FA in ACN for 5 times. The solution was collected and then evaporated to dryness under vacuum at 40°C using rotary evaporator, then re-dissolved into 1.0 ml with ACN containing 20 ppm 2-napthol internal standard before being subjected to analysis by HPLC. The amount of genistein eluted from the cartridge was reported by % recovery where it was compared with the initial amount of genistein before loading, according to the equation **(3.2)**. The condition which gave the highest % recovery was selected for extraction of genistein.

$$\%\ Recovery = \frac{A_{elute}}{A_{initial}} \times 100 \qquad \textbf{(3.2)}$$

Where A_{elute} is the amount of genistein after being eluted from the cartridge and $A_{initial}$ is the initial amount of genistein before being loaded into the cartridge.

Table 3.2 Washing conditions for optimization of MISPE procedure

Entry	Washing conditions
1	40% MeOH in water
2	20% ACN in water
3	20% ACN in water with 0.1% FA
4	30% ACN in water with 0.1% FA

3.2.3.3 Optimization of elution conditions

After the cartridge was washed in washing step, the genistein was eluted from the cartridge by 1.0 ml of eluting solvent for 5 times. The elution systems used in the optimization was based on ACN due to its eluotropic strength. The elution conditions are shown in **Table 3.3**. The fractions were collected and evaporated to dryness by rotary evaporator at 40°C. The fraction was then re-dissolved in ACN containing 20 ppm 2-napthol before being subjected to analysis by HPLC. The amount of genistein eluted from the cartridge was reported by % recovery where it was compared with the initial amount of genistein before loading, according to the equation **(3.2)**. The condition which gave the highest % recovery was selected for extraction of genistein.

Table 3.3 Elution conditions for optimization of MISPE procedure

Entry	Elution conditions
1	ACN
2	1% FA in ACN
3	1% TEA in ACN

3.2.4 Chromatographic analysis

The HPLC was done following the method from literature.[59] The high-pressure liquid chromatograph equipped with a dual pump and a UV-DAD detector was used to separate, identify, and quantify isoflavone. Separation of isoflavones from soybean sample was achieved by Agilent Eclipse XDB-C18 C18 reversed phase HPLC column (250 x 4.6 mm and 5 μm internal diameter), and the samples were injected using an automatic injector. A linear HPLC gradient was used with solvent A (0.1% formic acid in distilled water) and solvent B (0.1% formic acid in acetonitrile). Following the injection of 20 μL of the sample, solvent B was increased from 15 to 35% for 50 min and then held at 35% for 10 min. The solvent flow rate was 1.0 ml/min. The wavelength of the UV detector was set at 260 nm. Determination of genistein from standard application was performed by a Thermo Hypersil Keystone C-18 reversed phase HPLC column (150 x 4.6 mm, 5 μm and internal diameter). The gradient was started with 10% B and then increased to 90% B in 20 min. The solvent rate was 1.0 ml/min.

3.2.5 MISPE Selectivity

The selectivity of the optimized protocol for extraction of genistein was investigated. Genistein was substituted by quercetin in the loading solution. The chemical structures of genistein and quercetin are shown in **Figure 3.2**. Quercetin, 20 ppm in 40%MeOH/water, was subjected to the optimized MISPE procedure. The eluent collected from the MISPE process was dried using rotary evaporator under 40°C, re-dissolved in 1.0 ml ACN containing 20 ppm of 2-napthol as internal standard

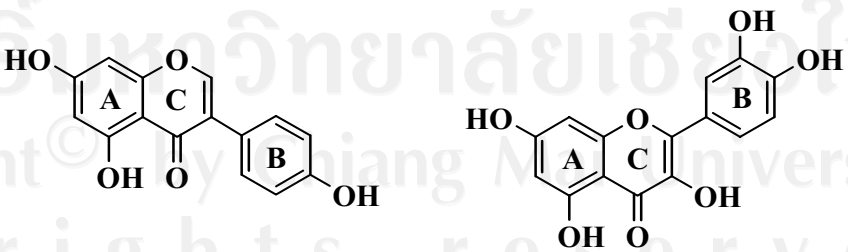
prior to HPLC analysis. Competitive binding between genistein and quercetin was studied using the mixture solution of genistein and quercetin in MISPE process. The eluted amounts of genistein and quercetin from the cartridge were determined by HPLC under the same condition with the determination of standard genistein and calculated into % recovery according to equation **(3.2)**. The factors which indicated the specificity and the selectivity of the polymer in the MISPE process are the imprinting factor (α') and the selectivity factor (ε) which were calculated according to the equation **(3.3)** and **(3.4)** respectively.

$$\alpha' = \frac{R_{MISPE}}{R_{NISPE}} \qquad \textbf{(3.3)}$$

Where R_{MISPE} and R_{NISPE} are the genistein recoveries from MISPE and NISPE, respectively.

$$\varepsilon = \frac{R_{genistein}}{R_{quercetin}} \qquad \textbf{(3.4)}$$

Where $R_{genistein}$ and $R_{quercetin}$ are the percentage recoveries of genistein and quercetin from MISPE process, respectively.



Genistein Quercetin

Figure 3.2 The chemical structures of genistein and quercetin

3.2.6 Application of the PIII-VP polymer to the extraction of genistein from soybean extracts

3.2.6.1 Preparation of soybean extracts

The extraction of soybean isoflavone was performed according to the reported literature.[60-62] The soybean seeds were ground and sieved. Two grams of ground soybean seed were added with 10 ml of ACN. Two milliliters of 0.1 N HCl in water was added. The mixture was shaken for 1 hr at room temperature. The mixture was filtered through a Whatman no.1 filter paper. The filtrate was dried in a vacuum rotary evaporator at a temperature below 30°C and re-dissolved in 10 ml of 40% HPLC grade methanol in distilled water before being applied to MISPE. The re-dissolved sample was filtered through a 0.45 μm filter unit (VertiPureTM NYLON Syringe Filter, 13 mm) before being subjected to analysis using LC-ESI-MS for identification of genistein in the sample extracts.

3.2.6.2 MISPE of soybean extract

The SPE cartridge containing 200 mg of PIII-VP was conditioned with the described process. The sample extracts was then loaded onto the polymer cartridge, then washed and eluted with the developed conditions stated above. The extract obtained was evaporated to dryness and re-dissolved in 1 ml of ACN containing 20 ppm 2-napthol internal standard before HPLC analysis. The percentage of genistein recovery from the MISPE process was calculated comparing with the amount of genistein presented in soybean extracts following to the equation **(3.2)**.

3.2.6.3 LC-ESI-MS

The confirmation with mass-spectroscopy was performed using a method with a Agilent Eclipse XDB-C18 HPLC column (4.6 mm x 150 mm, 5 μm) column, injection volumes of 20 μl and a gradient elution program with 0.1% FA in water (A) and 0.1% FA in ACN (B), 15-35% B in 50 min maintained 10 min with 1.0 ml/min flow rate. The wavelength of UV detection was 260 nm. The electrospray ion mass spectrometer (ESI-MS) was operated in the negative ionization mode and optimized fragmentor of 70, scanned from *m/z* 120 to 600. ESI was conducted using a needle voltage of 4.0 kV, capillary temperature at 330°C. High-purity nitrogen was used as drying gas at flow rate of 10 L/min and nebulizer gas at 45 psi.

3.3 Results and Discussion

3.3.1 Optimization of MISPE conditions

MISPE is based on conventional SPE procedure, therefore conditioning, loading, washing and elution steps are performed as a matter of routine. In this study, the MISPE conditions for extraction of genistein were optimized. Among the selected polymer (PI-VP, PII-AA and PIII-VP), PIII-VP was used in the optimization experiment because of its high imprinting factor.

3.3.1.1 Optimization of loading conditions

To achieve maximum loading of genistein, the loading condition for MISPE was optimized using the mixture of methanol and water, the solvent commonly used in the extraction of genistein from soybean samples.[63] The different standard genistein

solutions were loaded on the SPE cartridges. The amount of genistein bound to the polymers was determined and presented in **Figure 3.1**. The result shows that all of genistein was retained on the cartridge when using 20% and 40% MeOH whereas almost 20% of genistein was not retained in MISPE cartridge when 80% MeOH. It is noted that there was no difference in the genistein adsorption between MIP and NIP. This data suggested that genistein probably binds to the polymer mainly via hydrophobic adsorption, and the high MeOH content in the loading solution can reduce such interaction. Moreover, the use of high MeOH content is benefit to dissolve all components from the soybean extract. Since the content of MeOH at 40% gave the highest % retained containing highest content of MeOH, this condition was selected as the loading condition.

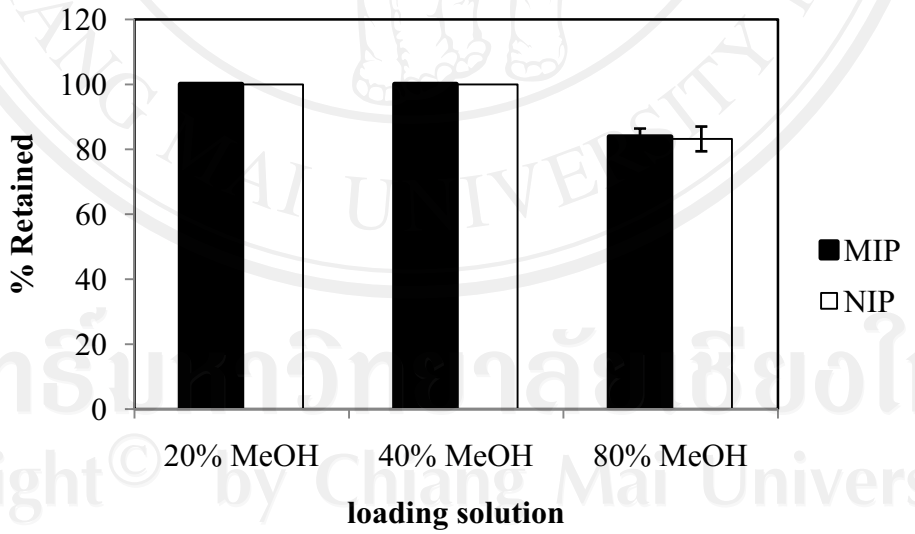


Figure 3.3 The percentage of genistein retained on the cartridge using different loading conditions

3.3.1.2 Optimization of washing conditions

After the analytes were adsorbed on the cartridge, the washing step was then optimized to remove interferences remained on the cartridge without the target analyte loss. In this step, the interfering species were eliminated by disruption the non-specific interaction. Four conditions were applied and the results were shown in **Figure 3.2**. It was found that for both MIP and NIP, low percentage genistein recovery was obtained when 40% MeOH was used as washing solution. However, the highest % recovery of genistein (86.0%) was obtained when the cartridge was washed with 20% ACN containing 0.1% FA. It is suggested that the addition of small amount of acid can improve the retention of genistein on the cartridge at 20% ACN content. However, the percentage recovery decreased when increasing the ACN content. Therefore, the 20% ACN containing 0.1% FA which gave the highest % recovery of genistein was chosen as the washing condition.

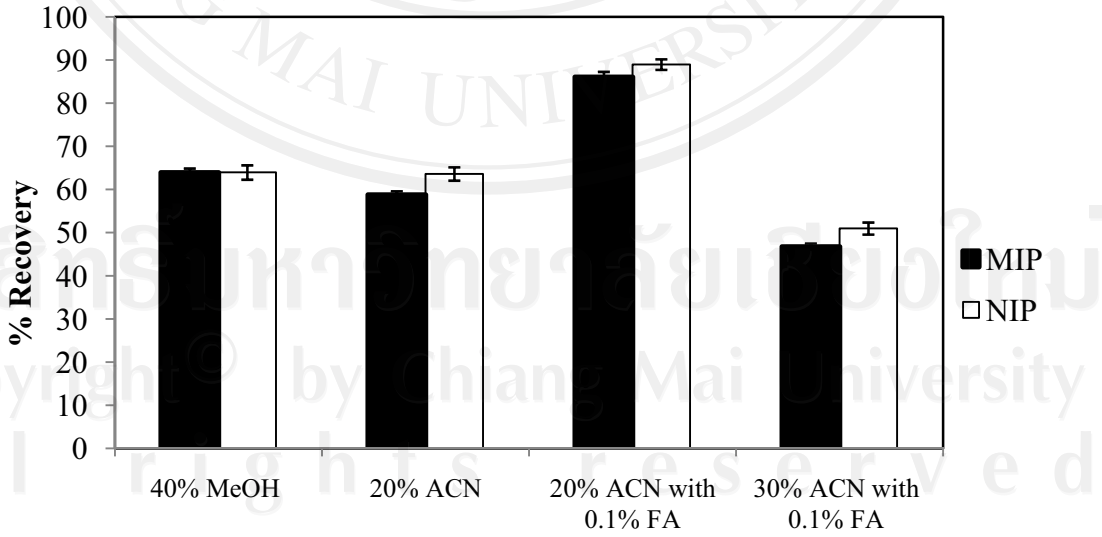


Figure 3.4 The percentage of genistein recoveries from washing step with different washing solvents

3.3.1.3 Optimization of elution conditions

Once the washing solution has been optimized, it is also very important that the analyte can be efficiently desorbed in elution step with the high recovery. For this reason, the eluent to be used must be also optimized. All of the most eluents that are often used is ACN.[3] In addition, it has been reported that acid or base was generally used to enhance the efficiency of elulent because they can destroy the specific interaction in MIP.[22] Therefore, ACN solutions were chosen as elution solvent to be optimized in this study. The results are shown in **Figure 3.3**. It was found that among the optimized conditions, the highest % recovery of genistein obtained when using 1% FA in ACN as an eluent (84.3%). The non-imprinted cartridge showed the same results except in ACN. It was also observed that the % recovery from MIP using ACN eluent was lower than that of NIP causing from the strong specific interaction between MIP and genistein.

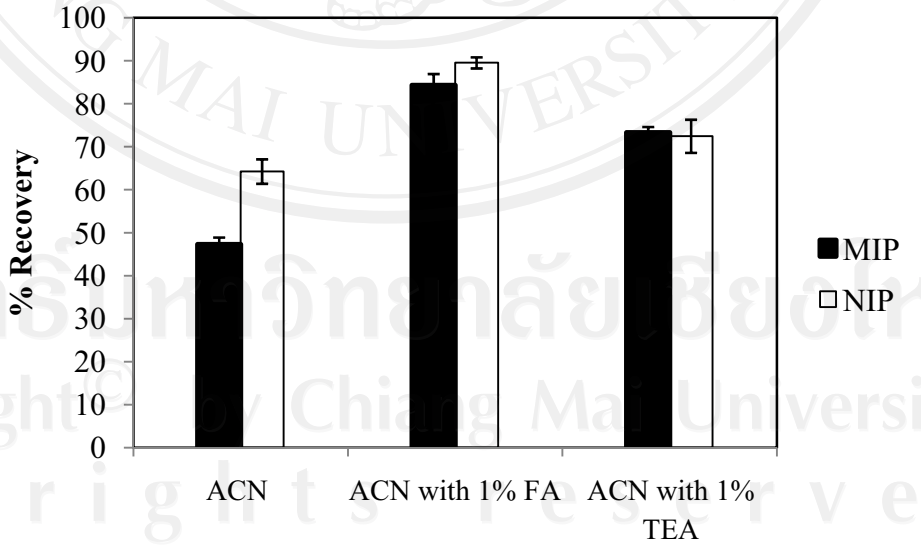


Figure 3.5 The percentage of genistein recoveries from eluting step with different eluting solvents

From the optimization of MISPE conditions, the genistein was loaded into the cartridge with 40% MeOH in water. The highest genistein recovery was obtained by using 20% ACN in water containing 0.1% FA as a washing solution and 1% FA in ACN as an eluting solution. Nevertheless, there was no significant difference in the genistein recoveries from MIP and NIP cartridges. This suggested that the non-specific hydrophobic interaction was the main factor that governed the binding of genistein to both MIP and NIP. Previous study have also found that the specific analyte-polymer interaction was hindered by water and the good recognition ability can generally reached when non-polar aprotic solvent was applied.[64] Unfortunately, this is not possible in our case since the solubility of genistein in non-polar aprotic solvent is limited.

3.3.2 MISPE Selectivity

Genistein and quercertin are in the same group of flavonoids which are structurally related. The main difference of quercetin from genistein is the position of phenyl substituent (aromatic ring B) connected to ring C on the chromone-type skeleton (**Figure 3.2**).[30] To investigate the selectivity of MISPE protocol, quercetin was, therefore, selected for selectivity study.

The previously described MISPE procedure was applied for extraction of both genistein and quercetin using PI-VP, PII-AA and PIII-VP cartridges in comparison with their corresponding NIP cartridges. From **Table 3.4**, the quercetin recoveries from PI-VP and PII-AA are higher than genistein recovery. However, this was lower in case of PIII-VP. It is suggested that PIII-VP has selectivity toward genistein than quercetin ($\varepsilon > 1$) while the others (PI-VP and PII-AA) are more selective toward

quercetin ($\varepsilon < 1$). Higher number of hydroxyl group in quercetin may lead to stronger interaction with the PI-VP and PII-AA. However, the template effect seems to play important role in controlly polymer selectivity (PIII-VP). Therefore, it is possible to recover quercetin from the cartridges due to their cross-reactivity which can be seen when structurally related compounds were applied in MISPE.[65]

Table 3.4 Percentage recoveries, imprinting factor and selectivity factors from MISPE process of PI-VP, PII-AA and PIII-VP

Entry	Polymer	Compound	% Recovery	α′	ε
1	PI-VP	genistein	44.5 ± 1.7	0.93	0.65
2	PI-VP	quercetin	68.3 ± 0.7	1.07	
3	PII-AA	genistein	54.8 ± 2.4	1.04	0.63
4	PII-AA	quercetin	86.7 ± 0.9	1.04	
5	PIII-VP	genistein	84.3 ± 2.7	0.94	1.53
6	PIII-VP	quercetin	55.2 ± 1.1	0.97	
7	PIII-VP	genistein*	84.5 ± 1.6	1.17	1.50
8	PIII-VP	quercetin*	56.3 ± 1.3	0.99	

* The compound was loaded as the mixture of genistein and quercetin solution

The competitive extraction of genistein and quercetin in MISPE process was investigated on PIII-VP polymer. **Entry 7 and 8** in **Table 3.4** show the quercetin and genistein recoveries from the mixture solution. The percentage recoveries of both

flavonoids from the mixture were similar to those obtained from their individual solutions. The selectivity factor of 1.50 suggested that the presence of quercetin in the mixture solution had no effect on % recovery of genistein on the MISPE process. The slightly higher α′ value (1.17) when loaded with the mixture indicated the enhanced binding interaction of genistein with polymer in a presence of quercetin.

It is also noted that there was no significant difference in % recoveries between all MIPs and their NIPs suggesting that the recoveries of genistein and quercetin from the optimized MISPE process were not obtained by specific interaction. The log K_{ow} values of genistein (2.840)[66] and quercetin (1.480)[67] indicated that genistein was less polar than quercetin. Consequently, the more retention of genistein on PIII-VP may be due to hydrophobic interaction.

3.3.3 Extraction of genistein from soybean extract

The PIII-VP was applied to the purification of genistein in soybean extracts. The extraction of genistein from soybean extracts was performed under the developed MISPE procedure. After MISPE process, the sample extract was analysed by HPLC and the recovery was calculated. **Figure 3.6(a)** and **(b)** show the HPLC chromatogram from soybean extracts before and after MISPE process, respectively. There are eight isoflavones found in the soybean extract obtained from the LC-MS analysis. Three isoflavones can be identified as malonyl diadzin (**a**), daidzein (**g**) and genistein (**h**) (**Appendix B, Figure B.4**). It can be seen that the MISPE procedure can percolate interferences. The soybean extract can be cleaned up when the PIII-VP was used as a sorbent. The genistein relative content was doubly increased after the MISPE process (5.51% to 10.98%).The extraction of genistein from soybean using

the polymer on MISPE yielded 73.1% recovery. It has been reported from Tekel J. *et al*[68] that genistein can be recovered at 63.5 to 89.6% when using octadecyl (C18) SPE cartridge for sample clean up. This suggested that the similar extraction efficiency of genistein was obtained from this MISPE in comparison to the commercial SPE sorbent.

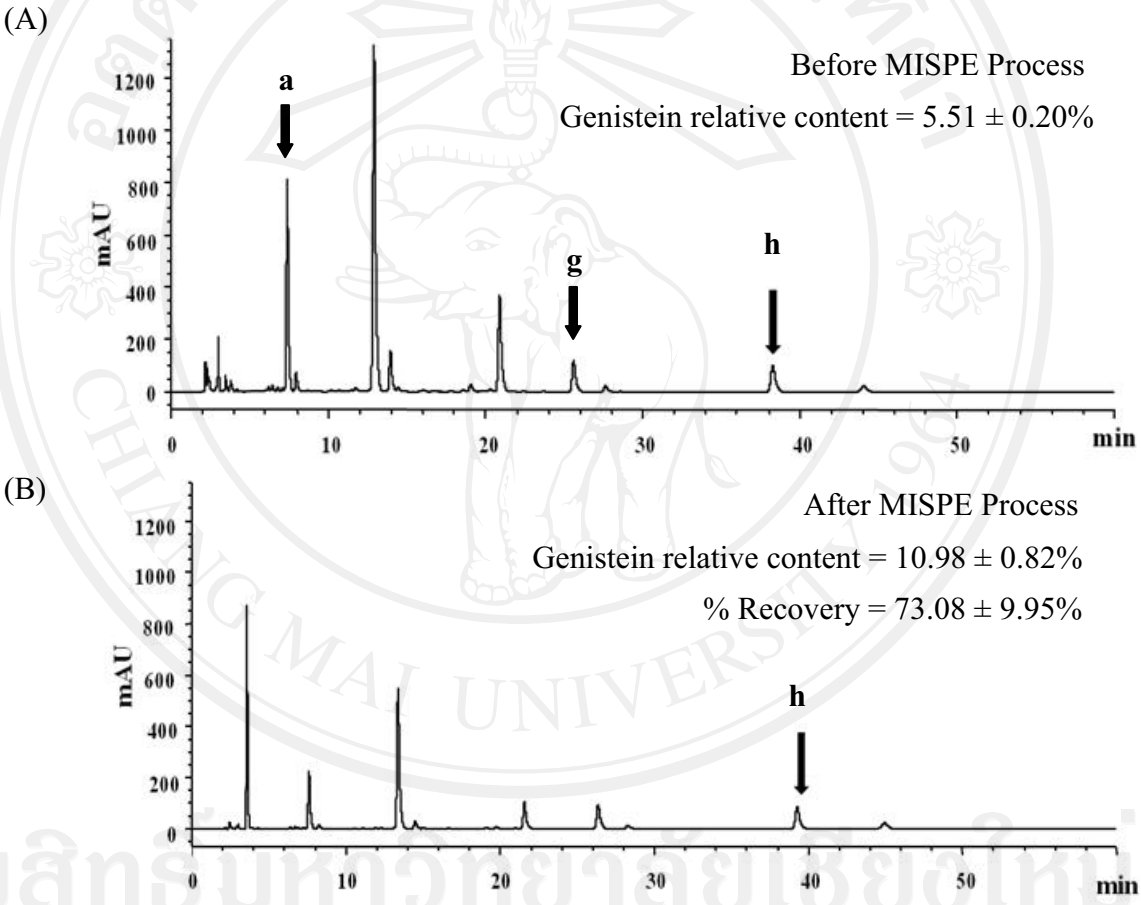


Figure 3.6 The HPLC chromatograms of soybean sample before (A) and after (B) MISPE process; (a) malonyl daidzin, (g) diadzein and (h) genistein