

## CHAPTER IV

### STUDY OF MIXED-MODE MISPE FOR SELECTIVE EXTRACTION OF GENISTEIN

#### 4.1 Introduction

The mixed-mode SPE is designed by the combination of two or more types of sorbents to extract several analytes containing various types of chemistry from sample within one step. It offered a possibility to reduce the multiple step purification to one step and to increase retention of a wide spectrum of analytes and to improve sample recoveries. Mixed-mode SPE significantly improves sample clean-up compared to SPE based on a single retention mechanism. In the cartridge combination, the unique advantage of each cartridge was encouraged for each others to improve the selectivity and efficiency of the extraction. For example, the combination of MIP and commercial SPE was applied to purify alkyl methylphosphonic acids from the extraction process. The crude sample was cleaned-up using commercial SPE further applied on MISPE to purify the target analytes before analysis by LC-MS technique.

The obtained chromatogram showed the good result using this approach.<sup>69</sup>

Moreover, the propazine MIP and its NIP were combined to improve the purity of triazines extracted from vegetable extracts as a new clean-up protocol.<sup>70</sup> Some interferences were removed by the use of NIP prior to the MISPE step. According to this new mixed-mode SPE procedure, the interferences were almost completely removed allowing the determination of triazines to be done in low concentration level.

For identification of isoflavone conjugates in red clover, the combination of two types of sorbent was investigated.<sup>63</sup> Co-polymer sorbents on the basis of N-vinylpyrrolidone and divinylbenzene are selected for extraction procedures. The mixed-mode SPE was applied to simplify the sample matrix and separate the flavones from the sample. The results confirmed the separation efficiency of the mixed-mode SPE procedure in terms of the purity of the samples. The combination of unique cartridge was applied to enhance the selectivity of the extraction process.

In this study, the new MISPE strategy was also developed to enhance the selectivity of genistein extraction by the combination of three template-based MIPs obtaining by fragment imprinting approach. From the chapter II and III, the MIPs from several genistein fragment templates and functional monomers were synthesized, characterized and optimized for using as the sorbent on MISPE process for the extraction of genistein. The selected MIPs from each fragment template which are PI-VP, PII-AA and PIII-VP were connected to perform the mixed-mode MISPE under the hypothesis that, the combination of MIPs which can partially recognized genistein structure should be able to enhance binding selectivity of MISPE process.

## 4.2 Experimental section

### 4.2.1 Chemicals 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-Naphthol,  $C_{10}H_8O$ , 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.

### 4.2.2 Instruments

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

Mass spectrometer (Agilent, HP1100), Germany.

SPE manifold (Restek, Resprep<sup>TM</sup> 24-port manifold), USA.

Freezer dryer (Snijders, type 2040), Holland.

HPLC column (Agilent, Eclipse XDB-C18, 250 x 4.6 mm and 5  $\mu$ m), USA.

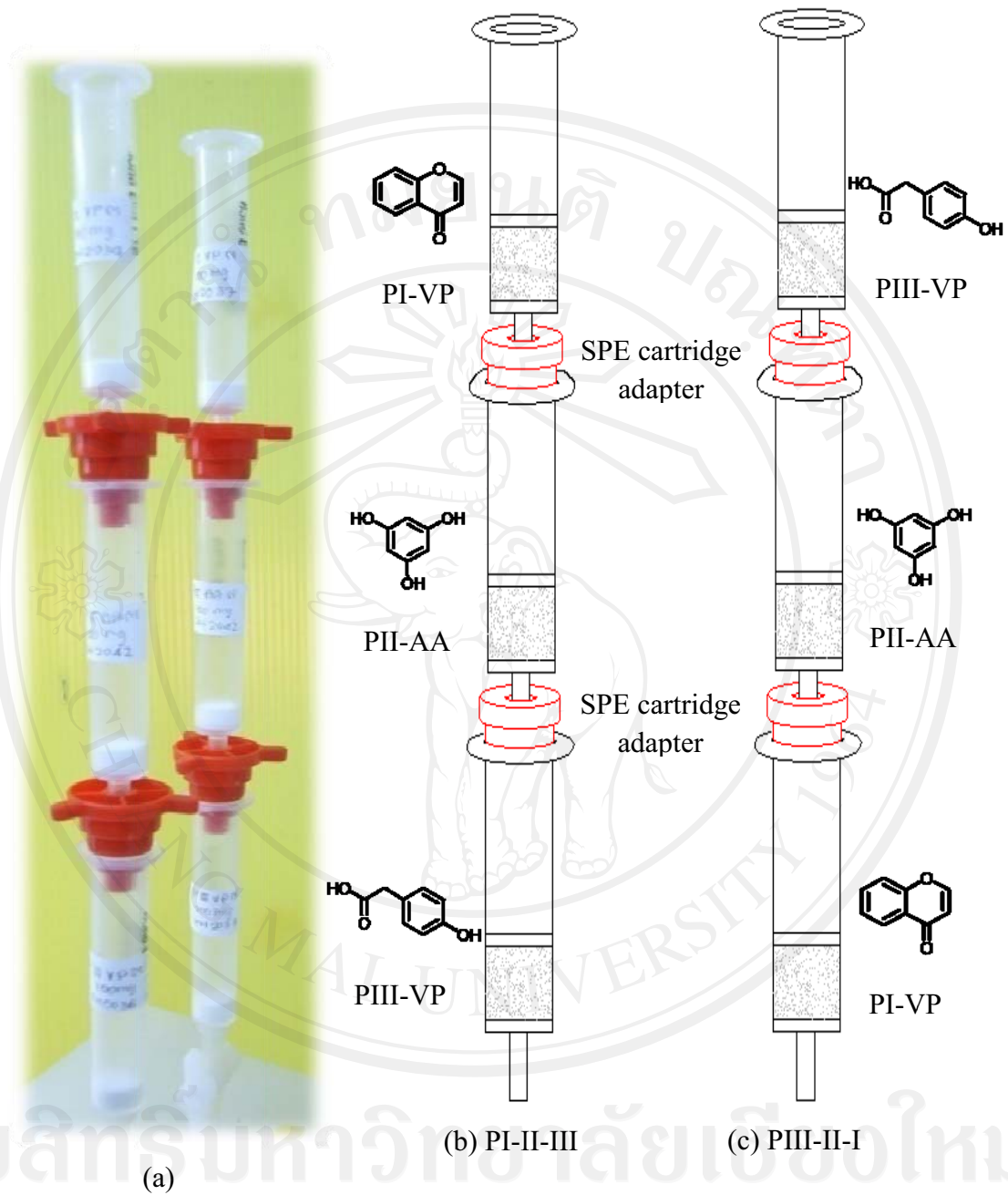
HPLC column (Thermo, Hypersil Keystone, 150 x 4.6 mm and 5  $\mu$ m), USA.

### 4.2.3 Mixed-mode MISPE of PI-II-III and PIII-II-I

In each cartridge, one hundred milligrams of MIP were packed. The selected three cartridges were connected by SPE cartridge adapter. Two methods of cartridges arrangement were examined. According to **Table 4.1**, the PI-VP was connected to PII-AA then PIII-VP cartridge called PI-II-III. In another arrangement, PIII-VP was connected to PII-AA then called PIII-II-I. The instrumental set up of mixed-mode MISPE cartridges is demonstrated in **Figure 4.1**. After the connected cartridges were successively preconditioned with 1 ml MeOH for 3 times, then 1 ml ACN for 3 times and 1 ml 40% MeOH in water for 3 times, the genistein 20 ppm in 40% MeOH in water was applied into the connected cartridge. Next, the cartridges were washed and eluted with the optimized MISPE procedure obtained from MISPE optimization in Chapter III. The solution eluted from the cartridge was then dried and re-dissolved before being subjected to analysis by HPLC using as the same method as used in MISPE process from Chapter III.

**Table 4.1** MIP cartridge order for mixed-mode MISPE

Name	MIP cartridge order		
	1 <sup>st</sup> cartridge	2 <sup>nd</sup> cartridge	3 <sup>rd</sup> cartridge
PI-II-III	PI-VP	PII-AA	PIII-VP
PIII-II-I	PIII-VP	PII-AA	PI-VP



**Figure 4.1** The instrumental set up of MIP cartridges for mixed-mode MISPE

#### 4.2.4 Selectivity of PI-II-III and PIII-II-I

The mixed-mode MISPE selectivity was determined using quercetin, a structurally related compound, similar to the MISPE selectivity test in Chapter III. The one ml of quercetin 20 ppm in 40% MeOH in water was subjected to the mixed-mode MISPE procedure. The eluent collected from the mixed-mode MISPE extraction process was dried using rotary evaporator under 40°C and then re-dissolved with 1 ml ACN containing 20 ppm of 2-naphthol internal standard prior to HPLC analysis. The eluted amounts of quercetin from the connected cartridge were determined by HPLC under the same condition with the determination of genistein from standard application and calculated for % recovery as mentioned earlier.

#### 4.2.5 Extraction of genistein from soybean extracts using PI-II-III

The soybean extracts were prepared using the method as mention previously in Chapter III. The mixed-mode cartridge was conditioned with the described process. Next, the sample extract was loaded onto the polymer cartridge, then washed and eluted with the developed condition stated above. The extract obtained was evaporated to dryness and re-dissolved in 1 ml of ACN containing 20 ppm 2-naphthol 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 equation (3.2) from Chapter III.

### 4.3 Results and discussion

#### 4.3.1 MISPE of PI-II-III and PIII-II-I

The mixed-mode MISPE was performed using the previously describe MISPE procedure to improve the selectivity of the extraction. **Table 4.2** shows the genistein recovery from PI-II-III and PIII-II-I MISPE in comparison with one cartridge SPE. The percentage recoveries of these polymers were shown again in **Table 4.3**. Both mixed-mode set up gave the similar genistein recovery at 64.5 and 62.9% respectively. The genistein recovery obtained from the mixed-mode MISPE was lower than the MISPE from PIII-VP (84.3%) but higher than PI-VP (44.5%) and PII-AA (54.8%). The results show that the combination of PI-VP and PII-AA with PIII-VP decreased the genistein recovery. However, the selectivity was slightly better when the mixed-mode MISPE was performed on PI-II-III connected cartridge.

**Table 4.2** Percentage of genistein recoveries from mixed-mode MISPE process of PI-VP, PII-AA, PIII-VP, PI-II-III, PIII-II-I and their NIPs

Polymer	% Recovery		$\alpha'$
	MISPE	NISPE	
PI-VP	44.5 ± 1.7	47.6 ± 0.7	0.93
PII-AA	54.8 ± 2.4	52.8 ± 2.3	1.04
PIII-VP	84.3 ± 2.7	89.5 ± 1.3	0.94
PI-II-III	64.5 ± 2.0	59.2 ± 1.8	1.09
PIII-II-I	62.9 ± 2.6	68.5 ± 2.4	0.92

### 4.3.2 Selectivity of PI-II-III and PIII-II-I

The selectivity of mixed-mode MISPE protocol was investigated in the same way as Chapter III. The previously described MISPE procedure was applied to the extraction of quercetin on PI-II-III and PIII-II-I MISPE. In addition the extractions on their corresponding NIPs were performed for comparison. From **Table 4.3**, the quercetin recoveries obtained from PI-II-III and PIII-II-I were 62.4% and 47.1% respectively. PIII-II-I gave the highest selectivity factor (1.33). It can be seen that not only for the genistein, the specificity toward quercetin was also observed.

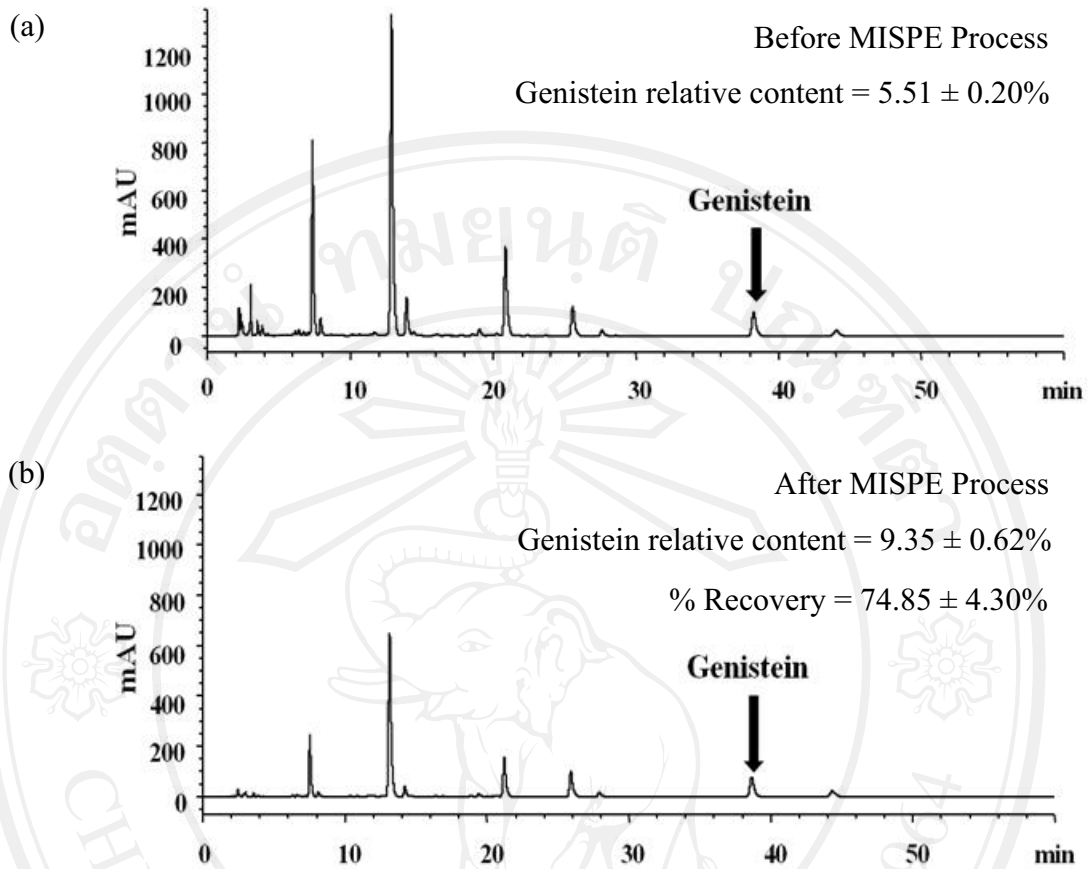
**Table 4.3** Percentage recoveries, imprinting factor and selectivity factors from mixed-mode MISPE process of PI-II-III and PIII-II-I

Polymer	Compound	% Recovery	$\alpha'$	$\epsilon$
PI-II-III	genistein	64.5 ± 2.0	1.09	1.03
	quercetin	62.4 ± 1.5	1.40	
PIII-II-I	genistein	62.9 ± 2.6	0.92	1.33
	quercetin	47.1 ± 0.5	0.91	



### 4.3.3 Extraction of genistein from soybean extracts using PI-II-III

The PI-VP, PII-AA and PIII-VP were applied in mixed-mode MISPE for 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 recoveries was calculated. The **Figure 4.2** show the HPLC chromatogram from soybean extracts before **(a)** and after **(b)** MISPE process. It can be seen that the mixed-mode MISPE procedure can also percolated interferences. The soybean extract was cleaned up when the combination of MIP from fragment imprinting PI-II-III was applied. The genistein relative content was increased from 5.51% to 9.35% after the MISPE process. The extraction of genistein from soybean using the mixed-mode MISPE yielded recovery at 74.85%. It was found that the others soybean isoflavones and their derivatives can be seen in the chromatogram after mixed-mode MISPE process due to their cross-reactivity.



**Figure 4.2** The HPLC chromatograms of soybean sample before (a) and after (b) mixed-mode MISPE process