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Research Article

Synthesis quercetin imprinted polymer by precipitation polymerization method, directed to apply as sorbent for selective solid-phase extraction

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Quercetin; Quercetin imprinted polymer; Precipitation polymerization method; Selective solid-phase extraction;

ABSTRACT

Quercetin, a plant flavonoid with potent antioxidant action, has observed numerous health benefits. However, it is difficult to determine quercetin in natural products because of its complexity. Besides, molecular imprinted polymer (MIP) exhibits high selective, affinity and stability. To simplify the procedure of sample treatment, the quercetin imprinted polymer was synthesized by precipitation polymerization method, then applied as sorbent for selective solid-phase extraction. The aim of our study was to determine the suitable conditions of polymer polymerization reaction for high selective absorption of quercetin imprinted polymer in nano-size, directed to apply as sorbent for selective solid-phase extraction. MIP was synthesized by mixing Acrylic acid (AA), ethylene glycol dimethacrylate (EGDMA), 2,2'-azobisisobutyronitril (AIBN), quercetin in a large amount of porogen at 70ºC in 8 hours. The prepared quercetin-MIP was employed as the sorbent for solid extraction phase column (Quercetin-MISPE). The results of IR, UV-Vis confirmed the presence of template molecules only in MIP. The MIP targeting quercetin could absorb and rebind quercetin. The productivity of this method was 10%. The selective absorption of quercetin-MIP was demonstrated by comparing quercetin with rutine, kaempferol and isorhamnetin via HPLC analysis. We have successfully synthesized the molecular imprinted polymer for quercetin by using precipitation polymerization method, prepared and employed the quercetin-MIP as the sorbent for solid extraction phase column and investigate its characteristics. Quercetin absorption of MIP is 9.7% higher (2.85×) compared to rutine of 3.4%, while there is no absorption in case of NIP (0%) and MIP with kaempferol (0%) and isorhamnetin (0 %).

1. INTRODUCTION

Quercetin, a plant flavonoid with potent antioxidant action, has observed numerous health benefits: anti-inflammatory, anti-allergy, anti-cancer and many more[1-2]. Various techniques for the extraction of flavonoids from food and herbal materials have been reported all over the world from pressurized fluid extraction, soxhlet extraction, microwave assisted extraction to

ultrasonication[3]. However, it is difficult to determine quercetin in a natural product because of its complex component. Generally, to selectively extract a nature substance, solid-phase extraction (SPE) was widely used to partly eliminate the impurities. The target compounds or the impurities are separated from other compounds in the mixture according to their physical and chemical properties. Increasing the selectivity of SPE could be the modern challenge for herbal sample. Molecularly imprinted polymer (MIP), on the other hand, is a promising technique to create artificial receptors with high selectivity as well as affinity and long-term stability, which can be used as alternative materials in different application fields [4-5]. For this purpose, the analyte used as a template in a polymerization reaction forming a high crosslinked matrix. After the template compound is removed by suitable way, the polymer would reveal interaction sites in that are ideally suited to reincorporate the template species as a result of steric adaptation and the formation of an optimized interaction network between template and functional groups of the polymer (Figure 1)[6]. In recent years, SPE based on MIP has been a novel approach for sample preparation to enhance extraction productivity prior to using other analytical methods[7]. According to many studies, regarding the ability of selectivity, MIP outweighed conventional stationery phase and therefore was a promising material for SPE, so MIP could cater for selective extraction at a high level[8-10].

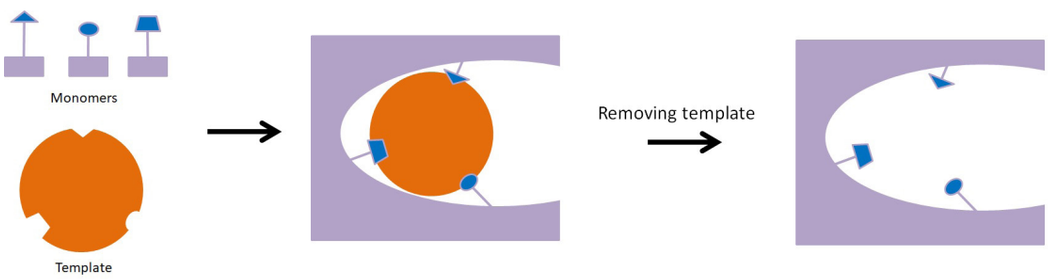

Figure 1. Principles of molecular imprinting in bulk. Adapted from ref.[11].

The aim of our study, therefore, was to determine the suitable polymerization conditions for high selective absorption of quercetin-imprinted polymer in nano-size, used as a sorbent for selective solid-phase extraction, directed to apply for a determination of quercetin in the finished product. A modified bulk imprinting approach was used for quercetin imprinting and the resulting MIP was tested via IR, UV-Vis spectrum and HPLC. The successfully synthesized the quercetin-MIP by using a precipitation polymerization method, was prepared and employed as the sorbent for solid extraction phase column and investigated its characteristics. So far, a sample from Vietnamese drug market was analyzed by HPLC to examine the effectiveness of pre-treatment via that selective solid-phase extraction system.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Quercetin (>98%) was supplied by National Institute of Drug Quality Control at Ho Chi Minh city. Acrylic acid (AA), methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), 2,2' -azobisisobutyronitril (AIBN), 4-vinyl pyrrolidone (4-VP) were purchased from Sigma-Aldrich. All solvents were of HPLC or analytical grade: HCl, methanol, ethanol, acetone, phosphoric acid, n-buthanol, distilled water. Appliances: HPLC system (Perkin Elmer), UV-Vis spectrophotometer (Hitachi), IR spectrometry (Bruker), Particle size distribution measurement (Malvern).

2.2. Methods

2.2.1. Preparation of quercetin-imprinted polymer (QIP) and optimization of the polymerization reaction

Quercetin-imprinted polymer (QIP) or quercetin molecularly imprinted polymer (quercetin-MIP) was synthesized by mixing cross linker, functional monomer(s) and the radical starter template quercetin and porogenic solvent under the condition of 70°C for 8 hours. The initial formula

of polymer showed in table 1. After being formed, polymer particles were washed by washing and elution solvents in order to eliminate all the redundant reagents. The same polymer without template in its recipe (quercetin-NIP) was parallel prepared and served as a non-imprinted reference in order to compensate for physical and non-specific effects.

Table 1. Predicted formula for QIP (or quercetin-MIP)

	Components	Reagents	Amount
1	Template molecule	Quercetin	750mg
2	Monomer	AA	1000µL
3	Cross - linker	EGDMA	4000µL
4	Polymerization initiator	AIBN	500µL
5	Porogenic solvent	Methanol	500mL

2.2.2. Preparation of MISPE column and optimization of elution solvent

The SPE system was connected to a vacuum pump. A SPE cartridge volume was 10 ml and its diameter was 13 mm. Before loading the quercetin-MIP, the cartridge was washed several times by MeOH - acetic acid (9:1) until no traces of quercetin could be detected by UV-Vis spectrophotometer at 370 nm. Then, 1 g of quercetin-MIP was packed into the cartridge by wet packing method and a filter paper was fitted on top of the quercetin-MIP head. This SPE column (MISPE) was used for all experiments afterwards.

2.2.3. Examination of rebinding ability of QIP

In order to find out the suitable process of removing and rebinding quercetin, quercetin was determined in eluted solution by UV-Vis after loading into MISPE and NISPE. The present of template on MIP, NIP, washed-MIP and washed-NIP was confirmed via IR spectrum. Different types of monomer were investigated to improve the rebinding effect of MIP. In order to find out the homogeneous and stable material, we changed the amount of crosslinker and checked the size and the morphology of polymer particle via microscope and particle size distribution measurement. Next, quercetin was loaded into and washed out the SPE column 10 times for testing the reusable capacity of MISPE.

2.2.4.Examination of Selectivity ability of QIP

Quercetin has an extreme similar structure to rutine. Thus, rutine was used to investigate the selectivity of QIP by using NIP and QIP as sorbents for solid-phase extraction system. Next, HPLC was used to analyze two samples containing both quercetin and rutine: one had been handled by NISPE and the other one MISPE.

The HPLC analysis was conducted with a Perkin Elmer HPLC system (USA). Active compounds were separated with a Nucleosil C18 column (250 x 4 mm, 10 µm) which was kept in the external thermostat at a constant temperature of 25 °C. The injection volume was 25 µL, and the elution flow rate was 1.0 mL/min. Eluents A and B were used for the gradient elution. Solution A was MeOH and solution B was phosphoric buffer solution pH 2.5. The following gradient was used: 0 min to 1.5 min with 30% A and 70% B; 1.5 min to 3.0 min from 30% to 50% A and from 70% to 30% B; 3.0 min to 8.0 remain 50% A and B each; 8.0 min to 10 min from 50 to 30%A and from 50 to 70% B. Chromatographic peaks were identified according to the concurrence of analytes and standard compound retention times and UV absorption spectrum within 210–500 nm. The separated active compounds were analyzed at a 254 nm wavelength to ensure their maximum absorption.

2.2.5. Application of the MISPE technique

We quantified the amount of quercetin in film-coated tablet "Cao Bach Qua 40 mg" of TV Pharm Pharmaceutical Joint Stock Company in Vietnam (SOP: IV-266NL; lot number: 004; manufacture date: 31.12.2013; expiry date: 31.12.2016). Its analytical process had been validated

before, thus this study only evaluated system suitability. Total flavonoid (Quercetin, Kaempferol, Isorhamnetin) content in one tablet was 9.6 mg (approximately 40 mg *Ginkgo biloba* extract) and have to comply the requirement: not less than 3,733% of total flavonoid content based on dry powder.

The analysis was conducted with same HPLC system to the former one. However, HPLC conditions was differently changed by using mixture of phosphoric acid solution pH 2.0 and MeOH at 50:50 ratio as isocratic mobile phase. Chromatographic peaks were identified according to the concurrence of analytes and standard compound retention times. The separated active compounds were analysed at a 370 nm wavelength to ensure their maximum absorption.

Once being removed the film layer, tablets were well ground into powder, which was used to prepare test samples below:

+ Test sample 1: 100 mg of powder was dissolved into a one-neck round-bottomed flask containing 30 mL MeOH: 25% HCl (4:1) and stirred well. A reflux apparatus on top of a steam bath was set up and the flask was heated for 135 minutes. After that, it was cooled, and the solution inside was poured into a 50mL volumetric flask. The round-bottomed flask was carefully rinsed many times by distilled water and that washing solution was also poured into the volumetric flask. Then, added distilled water to the calibration mark and mixed thoroughly. Finally, this solution was filtered through filter paper (0.45 µm of pore size) before being analyzed by HPLC system to determine total quercetin content.

+ Test sample 2: the process of sample preparation was similar to test sample 1. However, after being hydrolysed, 10 mL and 50 mL acid-hydrolyzed solution above was loaded through MISPE, and quercetin content in the elution solution of MeOH - acetic acid (9:1) was calculated according to a linear regression equation.

+ Quercetin standard curve: A six-point curve was desired with concentrations of 10, 20, 30, 40 and 50 ppm.

Table 2. Results of washing MIP and NIP by different solvents

Tubes	Solvents	Results	
		MIP	NIP
1	H_2O	(-)	(-)
2	Methanol	(+)	(+)
3	Ethanol	(+)	(+)
4	n-Buthanol	(+)	(+)

(-): negative result; (+): positive result

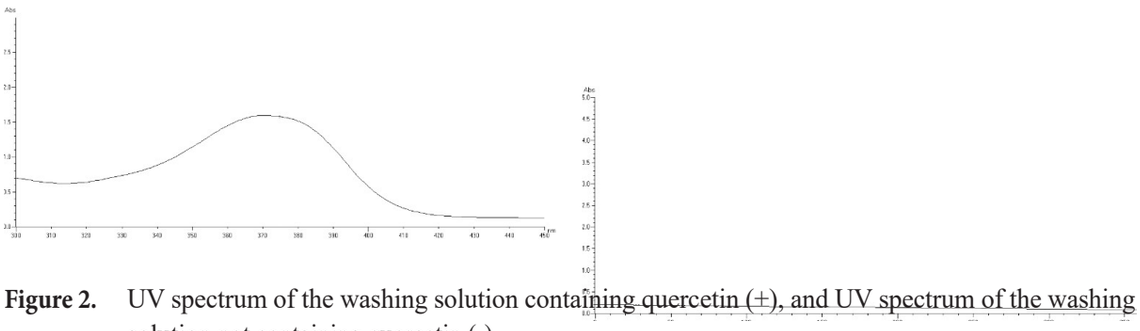

Figure 2. UV spectrum of the washing solution containing quercetin (+), and UV spectrum of the washing solution not containing quercetin (-)

3. RESULTS AND DISCUSSION

3.1. Washing solvents

3.1.1. Elimination of rebundant reagents

As regards the table 2, the reagents in the synthetic reaction are almost insoluble in water, so when being washed with water, the residue could not be thoroughly eliminated (in solution and on the surface of the particles, mainly residual quercetin). The remaining solvents (ethanol, methanol and n-buthanol) can all wash this excess, but it appeared that methanol and n-buthanol work more effectively than ethanol. As such, methanol was chosen as the original clean-up solvent since it was less expensive and more common in laboratories than n-buthanol. Figure 2 showed the positive and negative results respectively.

Table 3. Continuation of washing process using elution solvents to remove quercetin

Tubes	Solvents	Results	
		MIP	NIP
5	10% HCl solution	(-)	(-)
6	0.01% SDS solution	(-)	(-)
7	Tween 80	(-)	(-)
8	Methanol: Acetic acid (9:1)	(+)	(-)

(-) : no quercetin - (+) : quercetin

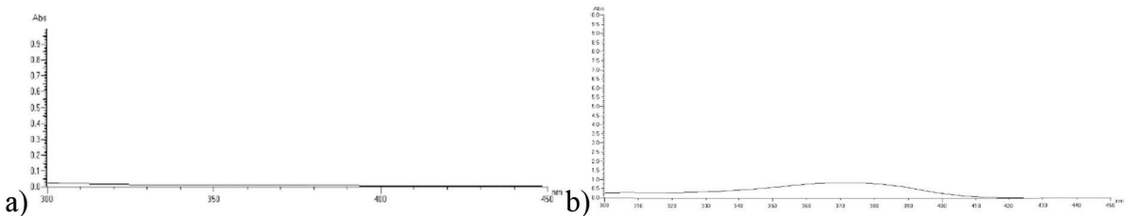

Figure 3. UV spectrum of the elution solution when using 10% HCl solution (-) (a) and UV spectrum of the elution solution when using MeOH - acetic acid (+) (b)

3.1.2. Elimination of quercetin

According to table 3 (tubes 5, 6, 7, 8), only MeOH - acetic acid (9:1) was able to completely remove quercetin from the structure of MIP. MeOH - acetic acid (9:1) was a polar solvent which can dissolve quercetin more than other solvents, so it could break hydrogen bonds between quercetin molecules and polymer, substituting solvent into holes. As such MeOH - acetic acid (9:1) could push the quercetin molecule out of the polymer particle structure.

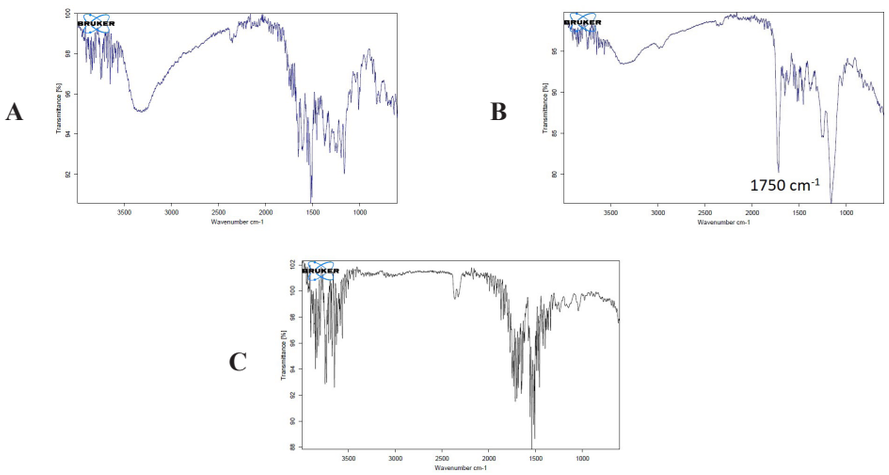

Figure 4. IR spectrum of NIP (a), MIP (b) and washed-MIP (9:1) (c)

There were many similarities between NIP and MIP spectra that observed in figure 4, but only MIP spectrum had a peak at 1750cm^{-1} which meant the presence of C=O functional group[12]. As seen on quercetin molecule, there are five hydroxyl groups and one carbonyl group which is no presence in polymer structure. This 1750 cm^{-1} peak could be the indicator for quercetin in polymer structure. That could be explained by the forming of hydrogen bonds between quercetin and monomer. After being washed with MeOH - acetic acid (9:1), the area of this peak reduced significantly. Thus, it is clear that there are hydrogen bonds in the structure of the polymer, and MeOH - acetic acid (9:1) could break these links.

Table 4. Quercetin content in washing and elution solutions, sampled from three kinds of SPE columns: acrylic acid (1st formula), methacrylic acid (2nd formula) and N-vinyl pyrrolidone (3rd formula)

Formula	Type	Times	Quercetin content (%)					
			Non-loading solution	Washing solution of MeOH	Elution solution of MeOH - acetic acid (9:1)	(a+b+c)	Mean content of (c)	Mean content of (d)
			(a)	(b)	(c)	(d)		
1	MIP	1	22.59	74.49	11.61	108.69	10.92	99.45
		2	18.15	65.97	11.03	95.15		
		3	21.14	63.25	10.12	94.51		
	NIP	1	24.31	74.78	0.00	99.09	0.00	100.06
		2	22.06	77.51	0.00	99.57		
		3	23.34	78.17	0.00	101.51		
2	MIP	1	18.52	75.49	4.52	98.53	4.80	98.78
		2	20.22	73.07	5.64	98.93		
		3	21.68	72.98	4.23	98.89		
	NIP	1	22.87	77.72	0.00	100.59	0.00	99.91
		2	18.16	80.41	0.00	98.57		
		3	19.32	81.25	0.00	100.57		
3	MIP	1	14.82	78.69	7.14	100.65	6.65	100.16
		2	14.52	77.87	7.62	100.01		
		3	15.35	79.28	5.18	99.81		
	NIP	1	17.27	73.72	0.00	90.99	0.00	96.35
		2	18.36	81.41	0.00	99.77		
		3	18.03	80.25	0.00	98.28		

3.2. Monomers

It is clear that the 1st formula was optimum as MeOH–acetic acid (9:1) could completely eliminate quercetin from MIP and MeOH could completely eliminate quercetin from NIP. Choosing suitable monomer is of extreme importance to build interactions between the templates and substrates[13]. Due to polyphenol structure of quercetin, there was a need to use a monomer which could synthesize a polymer having as many carboxyl group as possible. Thus, hydrogen bonds could easily be formed and enhance the quantity of template-substrate bonding. That is why acrylic acid was more appropriate among the three monomers investigated in this case. In fact, there was no obstacle to interrupt the forming of hydrogen bonds compared to the case of methacrylic acid with the presence of many methyl groups. As opposed to methacrylic

acid, N-vinyl pyrrolidone which has a nitrogen in additional group could form N-H hydrogen bond with –OH of quercetin. Nonetheless, perhaps as N-H bonds were stronger than H-H ones, it was far more difficult to break N-H bonds resulting in the unavailability of quercetin in elution solution, which also means MeOH - acetic acid (9:1) could not break N-H bonds.

3.3. Crosslinker - the amount of AIBN

The cross-linker is important element forming rigidity of the material because of its role in building up the polymer network, the optimal concentration of cross-linkers (EGDMA) should be 60-70% (w/w) and this ratio was also the same with several studies[14]. At this concentration, the amount of cross-linker were high enough to maintain the stability of the polymer matrix and recognition sites so far. Possibly, high concentration of cross-linkers enabled the sustainability of three-dimensional structure of polymers. Nevertheless, the higher the concentration of EGDMA was, the harder the ability of template washing and rebinding was owing to the incapability of squeezing out of and into recognition sites[15].

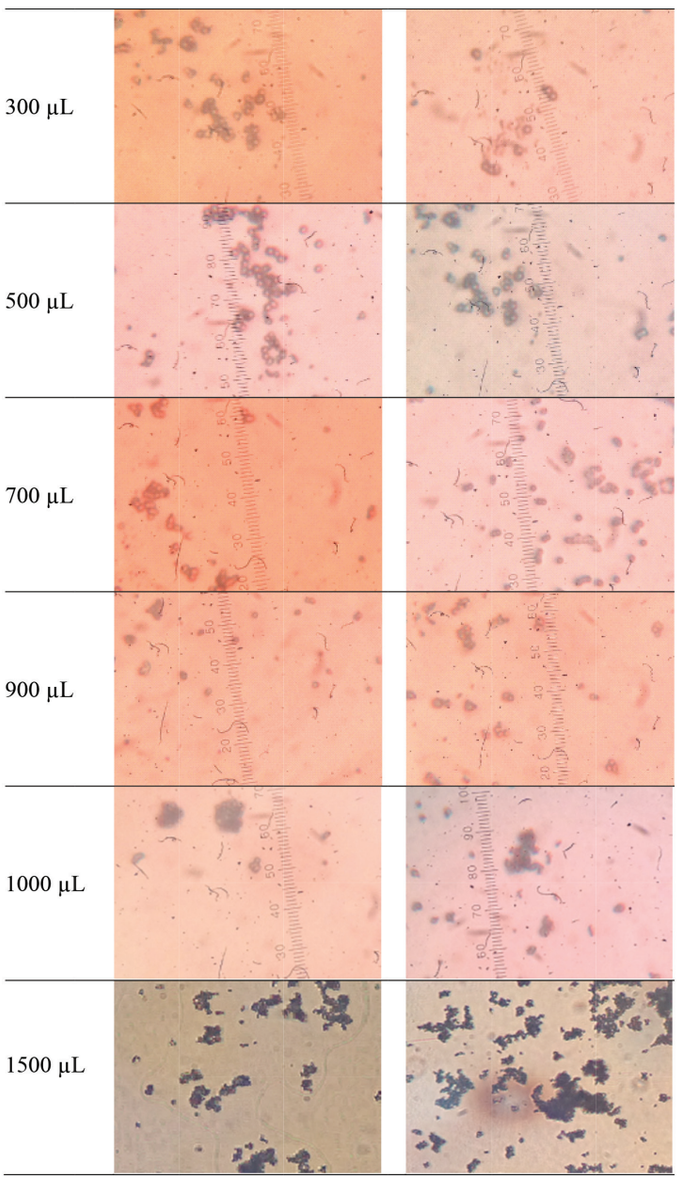

Figure 5. Different particle forms and sizes corresponding to different amounts of AIBN from 300 to 1500 µL seen under microscope (x100)

AIBN was the initiator which creates free radicals in polymerization reaction, and those radicals in turn would react with monomer. If the amount of AIBN is too high, the monomer chain formed could be too short, and so far, the particle size could be too small that leads to some severe problems, especially SPE column congestion. In contrast, if the amount of AIBN is too low, polymerization reaction rate would be longer.

In this experiment, the amount of AIBN was:

+ 300 - 700µL: particles were nearly equal (800-1200nm) in size and loose

+ 900 - 1500**µ**L: particles were too small (<600nm) and had a tendency to stick together

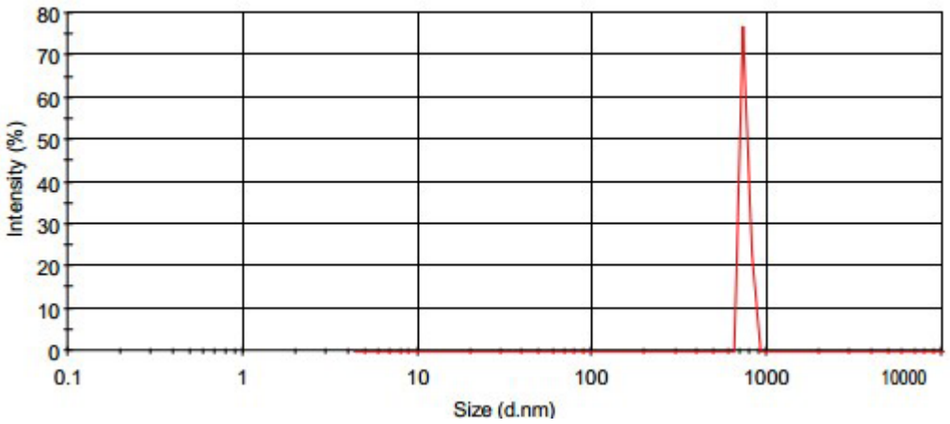

Figure 6. The result of particle size distribution (the polymer's recipe with 500 µL AIBN).

Therefore, 500µL was the suitable amount of AIBN for the formula investigated. The particle size distribution was confirmed by the Zetasizer Nano ZS system (Malvern) (Figure 6). All things considered, we had the final formula for QIP synthesis showed in table 5.

Table 5. The optimum formula for QIP synthesis

	Components	Reagents	Amount
1	Template molecule	Quercetin	750 mg
2	Monomer	AA	1000 **µL**
3	Cross - linker	EGDMA	4000 **µL**
4	Polymerization initiator	AIBN	500 **µL**
5	Porogenic solvent	Methanol	500 mL

3.4. Rebinding ability of MIP

As can be seen in the table 6, MIP showed a sustainable rebinding ability in the first six times.

Table 6. Reusable ability of MIP on rebinding quercetin effect:

Times	Quercetin content compared to total loading content (%)		
	Non-loading solution (%)	Washing solution of MeOH (%)	Elution solution of MeOH - acetic acid (9:1) (%)
	(a)	(b)	(c)
1	18.27	71.97	9.13
2	15.76	75.75	8.99
3	22.21	68.12	9.16
4	25.52	66.44	8.57
5	25.54	67.49	6.98
6	27.48	61.93	3.02
7	29.25	63.14	0.00
8	32.20	67.74	0.00
9	30.21	68.25	0.00
10	34.20	66.91	0.00

Table 7. HPLC figures in examination of QIP selectivity ability

		Non-loading solution (%)	Washing solution of MeOH (%)	Elution solution of MeOH: acetic acid (9:1) (%)	Total flavonoid collected (%)
MIP	Rutin	9.20	98.25	3.40	100.85
	Quercetin	7.76	83.40	9.70	100.86
NIP	Rutin	17.80	82.70	0.00	100.50
	Quercetin	15.10	85.50	0.00	100.06

Note: The results were based on the total original content.

3.5. Selectivity ability

We see retention capacity of MIP is 9.70% of the quercetin retaining on loaded column, and the whole quercetin was recovered after the methanol was eluted (100.06 %) in case NIP. Compared to rutin, quercetin is preserved more, 9.7% compared to 3.4% (table 7). Therefore, MIP is capable of selectively storing quercetin in a mixture of quercetin and rutin.

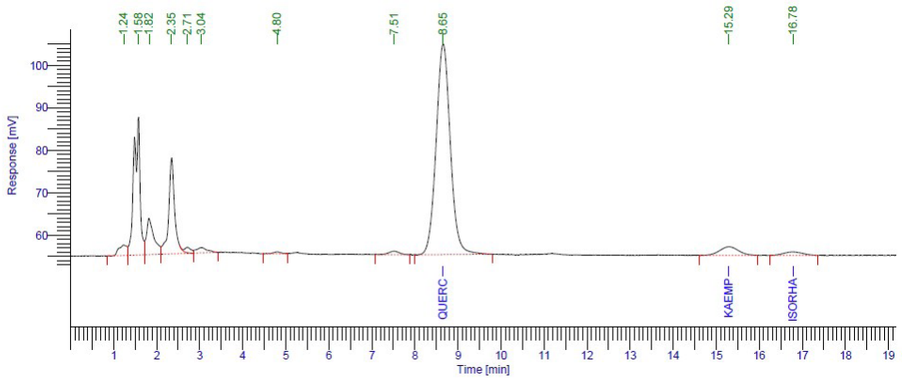

Figure 7. HPLC chromatogram of product without hydrolysis

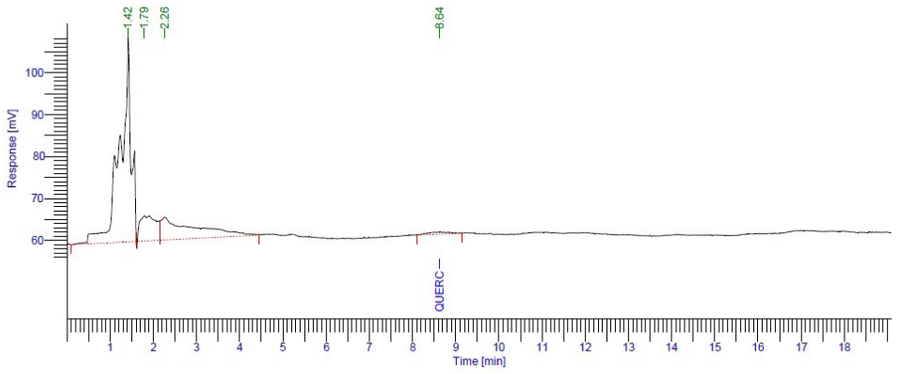

Figure 8. HPLC chromatogram of 10mL acid-hydrolyzed solution

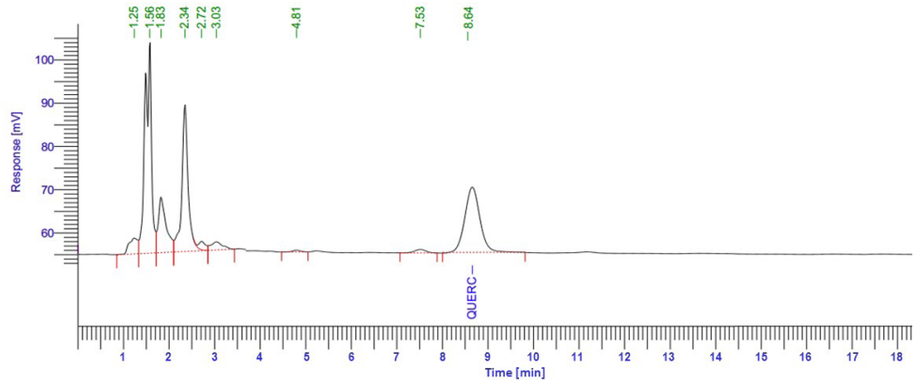

Figure 9. HPLC chromatogram of 50mL acid-hydrolyzed solution

3.6. Application of MISPE technique

Obviously, the quercetin content could easily be determined as its peak was separated remarkably in all the chromatograms. However, to determine kaempferol and isorhamnetin content, the non-hydrolysed sample was recommended (test sample 1).

The aforementioned results show that quercetin-MIP (QIP) was not suitable for quantifying quercetin in quercetin-containing products. Although the MIP selectively captured quercetin, figure 7 showed that we could directly quantify quercetin via HPLC without using the MISPE column. Figure 8 and figure 9 showed the chromatogram of test sample 2 treated by quercetin-MISPE without kaempferol and isorhamnetin peaks. Thus, the best application of quercetin-MIP is to capture and enrich quercetin content in herbal extracts of medicinal plants such as ginkgo. In addition, it is also essential to refine or separate quercetin from impurities owing to the selective quercetin selectivity on the SPE column.

4. CONCLUSION

The precipitated polymerization in large solvent was first used to synthesize quercetin-MIP for SPE, which was extremely different from other methods used before. Another outstanding result was nano-sized particles first synthesized based on this new method and therefore the particle size was homogeneous compared to particles formed by ground the bulk polymer[12].

We have successfully synthesized the molecular imprinted polymer for quercetin by using precipitation polymerization method, prepared and employed the quercetin-MIP as the sorbent for solid extraction phase column and investigate its characteristics. Quercetin-MIP rebinding and selectivity abilities were also examined and there were positive figures found (the ability of re-absorption 6 times). Quercetin absorption of MIP is 9.7% higher (2.85×) compared to rutin of 3.4%, while there is no absorption in the case of NIP (0%) and MIP with kaempferol (0%) and isorhamnetin (0 %).

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Conflict of interest

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