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Synthesis of Molecular Imprinting Solid Phase Extraction (MI-SPE) for Quercetin Purification as Active Compound in Plant Extract

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Abstract: Quercetin is natural flavonoid that has been widely studied and is known to have a biological activity such as antitumor, anti-inflammatoryandanti-cancer. The process of purifying active compounds from plants requires long stages and the uses of copious amounts of solvent. To reduce the solvent requirement as a form of green chemistry, a Solid Phase Extraction (SPE) isolation method was developed using molecularly imprinted polymers (MIP) technique. This study aimed to synthesize and characterized molecularly imprinted polymer for recognition of quercetine in Plant extract using acrylamide as the functional monomer in acetonitrile: methanol as a porogenic solvent. The research method used include synthesis of MIP sorbent using bulk polymerization method continue with characterization of MIP sorbent and finally applying the sorbent on purification of quercetin from plant extract. The result showed that MI-SPE had an adsorption capacity of 190.37 mg/g and selective to quercetin against another flavonoid compound such as luteolin and genistein by comparing its imprinting factor. FTIR spectra revealed that polymerization process has completed by absences of the vinyl peak. Based on that, MI-SPE made from acrylamide potential to be used as extraction material for quercetin purificationfrom a plant extract of Plectranthus scutellarioides.

Keywords: Quercetin, MI-SPE, Purification of active compound from natural product

INTRODUCTION

Indonesia is known to have natural wealth with various types of plants that are efficacious as a medicine. These plants have active compounds that are used as a treatment fordisease and to maintain health. One of the active compounds of plants reported to have biological effects is quercetin. Quercetin is a group of flavonoids that are widely studied and have biological activities such as antioxidants, antitumor, antibacterial, antiviral, anti-inflammatory and anticancer (1). Currently, the isolation of active compounds from a plant is mostly done by liquid-liquid extraction method and chromatography where this method requiredmanysolvents and done with long stages. Solid phase extraction (SPE) with molecular imprinting technique is an isolation method that develops as an effort to reduce solvent usage(2).

Conventional SPE sorbent that synthesizes without molecular imprinting technique has a disadvantage regarding selectivity. To enhance that selectivity of SPE sorbents, molecular imprinting polymer (MIP) technique in the making of SPE was then developed. SPE sorbents produced from MIP techniques contain specific and selective binding sites that can be formed through polymerization between templates, functional monomers, crosslinkers, and initiators in a porogen solvent (3,4,5). Combination of the mixture will result in a high sensitivity receptor polymer in binding a target molecule

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that has a similar characteristic to the target compound (6). MIP also has advantages that are very easy and fast to be prepared, resistant to high conditions such as high concentrations of organic solvents, temperature, pressure, strong acids and bases (1,4,5).MI-SPE that apply to the sample cleaning procedure of a matrix or impurity in the determination of target molecules in the fields of biology, food, pharmaceuticals and environmental analysis. Also, MI-SPE may be used for the isolation of active compounds from natural ingredients such as flavonoids, polyphenols, alkaloids(1,7).

Currently, several MI-SPE synthesis studies for natural product isolation have been performed, one of which is the synthesis of MI-SPE quercetin using methacrylic acid as a monomer. Methacrylic acid monomer produces poor adsorption ability in research preceding a low adsorption capacity on a single quercetin compound (8). The use of the number of monomers affects maximizing the formation of the polymer complex and the imprinting effect so that it is necessary to adjust the template function with the monomer complementarily (such as the H bond-donor with the H bond -acceptor)(9). Also, the number of crosslinkers used also affects the polymer pore size distribution, which is very important for structuring the site of binding sites(10). In the quercetin molecule, there are five hydroxyl groups and one carbonyl group. The groups may form hydrogen bonds with functional groups such as hydroxyl, amino and carbonyl groups(11).

Acrylamide is a hydrophilic functional monomer that can interact actively with template molecules through hydrogen bonds (12). According to the research from Kartasasmita et.al(9), the use of acrylamide may yield better adsorption capacity than other monomers such as methacrylic acid. Bulk polymerization method is an easy method, requires no significant amount of solvent and does not require a stirring process such as polymerization precipitation method(13).

Based on the above background, we will synthesize a material for solid phase extraction of quercetin by molecular imprinting technique. The composition of the material will consist of monomer :template :crosslinker with ratio 1:4:20. Sorbent obtained is expected to be used as an alternative method of extraction of quercetin compound after evaluation oftheir adsorption characteristics.

EXPERIMENTAL

Materials

The instruments used in this research are mechanical agitator (IKA HS 260 Basic), Fourier Transform Infrared (FTIR) (Shimadzu, IR Prestige-21), oven (Haraeus), UV-Vis spectrophotometer (Analytik Jena, Specord 2000) water bath (Memmert), digital scales (Mettler Toledo), ultrasonic (NEY 1510) and glass tools commonly used in laboratories.

The materials used in this study are acrylamide (Fluka), 2-2-Azobis-isobutyro-nitrile (AIBN) (Aldrich), ethylene glycol dimethacrylate (EDGMA) (Aldrich), acetonitrile Pro HPLC (Fisher), quercetin (Sigma Aldrich), ethanol (Merck), methanol pro HPLC (Fisher), genistein (Solistree), luteolin (Solistree), potassium bromide (Pike Technologies). Materials usedunless otherwise stated is pro analysis.

METHODS

Synthesis of Quercetin MI-SPE using Bulk Polymerization Method

The imprinted and corresponding non-imprinted polymer used in this study were prepared by thermally initiated free radical polymerisation. Briefly, quercetin (200 mg, 0.66 mmol), acrylamide (187.65 mg, 2.64 mmol) were transferred into a glass vial and mixed with 10 mL of methanol and 2.5 mL of acetonitrile.

Upon complete dissolution, 2.48 mL (13.2 mmol) of EDGMA were added followed by 1.4 mL of AIBN. The pre-polymerisation solutions were ultra-sonicated for 30 min and then hermetically sealed. The vials were placed in a water-bath at 60^{o}C for 24 h. The corresponding non-imprinted polymer was prepared in a similar fashion but without addition of the template in the pre-polymerisation mixture. Ratio of template: monomer: crosslinker can be seen in Table 1.

After the polymer was formed, the template was then extracted usingsoxhletation extraction. The extraction was performed using methanol solvent for 24 h. The procedure was repeated until MI-SPE did not contain the template when monitored using an HPLC(14).

Table 1: Ratio of Template: Monomer: Crosslinker on MIP and NIP Synthesis

	Quercetin (Template)	Acrylamide (monomer)	EDGMA (Crosslinker)
MIP	0.66 mmol	2.64 mmol	13.2 mmol
NIP	-	2.64 mmol	13.2 mmol

Evaluation of Adsorption Capacity

Evaluation of adsorption capacity is done by varying the concentration of quercetin solution that is 1, 2.5, 5, 10, and 15 mgL^{-1}. A total of 5 ml of quercetin solution was introduced into a vial containing 20 mg of MIP sorbent, then shaken using agitator at 120 rpm for 3 h at room temperature. After 3 h, the mixture was filtered,and the filtrate was measured in absorbance using a UV spectrophotometer. NIP sorbents were also treated in the same way as MIP. The evaluation of the adsorption capacity of MI-SPE is plot on the Freundlich isotherm adsorption curve (14,15,16).

Determination of MI-SPE Quercetin Sorbent Selectivity

A solution of quercetin, luteolin, and genistein with a concentration of 5 mgL^{-1} each were prepared. A total of 5 ml of the solution was introduced into a different vial containing 20 mg of MIP sorbent. The solution mixture was shaken using agitator at 120 rpm for 3 h at room temperature, then filtered. The obtained filtrate was measured by UV spectrophotometer at 374 nm. The NIP sorbent is performed in the same way. Selectivity can be known by calculating the imprinting factor value. Imprinting factor value was determined from ratio of distribution coefficient of MIP and NIP (17)

MIP and NIP Characterization via FTIR Instruments

A total of 10 mg of MIP sorbent was crushed together with 200 mg of potassium bromide (KBr) then molded into pellets. The infrared spectrum of MIP sorbents was observed using FTIR instruments. Transmission is measured at wave numbers 4000 - 400 cm-1. Determination of MIP sorbent functional groups is performed before and after extraction with the same method. NIP sorbents are also presented in the same way (14).

Application of MI-SPE Quercetin for Purification of Quercetin from Plectranthus scutellarioides Plant Extract

MI-SPE Quercetin that already made and characterized then applied to purify quercetin from *Plectranthus* scutellarioides extract. 2 g of *Plectranthus scutellarioides*extract was dissolved in 10 mL acetonitrile and then was added 10 mL HCl 1.2 M for 2 h to made all quercetin glycoside turn into aglycone form. After 2 h, the extract was filtered, filtrate then was loaded into 500 mg MI-SPE quercetine that already put into cartridges. Washing was done using chloroform and eluted with 5 mL of methanol. Solution from elution step then was injected into HPLC system with acetonitrile and 2% v/v acetic acid (40% : 60% v/v) (pH 2.6) at a flow rate of 1.3 mL/minutes, and ultraviolet (UV) detection at 370 nm (17). The limit of detection (LOD) of the chromatographic system was 0.022 μg/mL. All the extraction was done in a solvent based on optimation.

RESULT AND DISCUSSION

Synthesis of Quercetine MI-SPE using Bulk Polymerization Method

In this study, the sorbent synthesis of MI-SPE quercetin was performed by the non-covalent approach. The making of MIP can be done with two approaches method; they are a covalent and non-covalent method. In the covalent process, before polymerization, functional monomers and templates are bound to each other through covalent bonds. After polymerization, the covalent bonds are broken down, and the templateis removed from the polymer. In non-covalent techniques, non-covalent interactions are used to link functional monomers with templates. This can be easily obtained by mixing the monomers, templates, and cross-linkers directly into the polymerization process. Non-covalent methods are chosen since the method is capable of producing non-covalent interactions among functional monomers and a template molecule through ionic interactions, hydrogen bonding, and hydrophobic interactions. Also, this non-covalent approach is chosen because of its flexibility for various shapes, sizes, molecular template functions, and shorter synthesizing times, and can produce recognition sides with higher affinity than covalent approaches (18).

The polymerization process in this study is carried out by thermolysis method by placing the vial inside the water bath at 60°C for 24 h. This temperature is chosen because the AIBN initiator decomposes at that temperature. When the initiator is decomposed by heat, free radicals are formed,and unpaired electrons react with the monomers to form long polymer chains. The polymerization process stops when two free radicals react to each other. During polymerization, the complex formed between template molecules and functional monomers will be stabilized by cross-linkers into rigid forms (9).

The method chosen in MI-SPE quercetin synthesis is bulk polymerization method. The advantages of this method include the conventional method with natural and universal preparation procedures, and does not require unique skills and advanced tools in its use. Also, this method does not require solvents

(porogenic) in large quantities (9). MIP and NIP sorbent mass obtained in this synthesis process can be seen in Table 2.

After the polymer is formed, extraction of the polymer is performed. Extraction aims to eliminate the template quercetin from the MIP polymer matrix so that the space in the polymer initially occupied by the quercetin template will eventually leave the cavity. Under the right conditions, this cavity can re-bind the template molecule or its specific structural analog. Quercetin template extraction was done by soxhletation method using 200 ml of methanol solvent.

Table 2: MIP and NIP Mass Resulted from Polymer Synthesis

Polymer	Mass (gram)
MIP	11.04
NIP	10.46

The synthesis of sorbent MI-SPE quercetin by using acrylamide monomer has been done in previous research by comparison of the template: functional monomer: cross-linker is 1: 7:45 and using various porogenic solvent such as acetone, 1,4-dioxane, and THF. In the previous study, MI-SPE sorbent produced low IF (Imprinting Factor) (11). In this study we used different comparison of template: monomer: cross-linker that is 1: 4: 20 and used distinct porogen that is acetonitrile mixture: methanol (1: 4). Acrylamide was chosen as a functional monomer because in previous studies acrylamide gave a better percent of adsorption compared to other monomers such as methacrylic acid (9). Based on research results (8), a low adsorption capacity of quercetin was prepared using methacrylic acid. Acrylamide has an amino group (NH_2) that can act as a hydrogen bond donor, while carboxyl (OH) groups play a role in receiving hydrogen bonds. Acrylamide monomers can form hydrogen bonds with quercetin template molecules because of the presence of 5 hydroxyl groups on quercetin which act as a hydrogen bond donor. High monomer concentrations in MIP preparations can increase the polymer adsorption capability of molecular targets because it can increase the number of non-covalent interactions during the polymerization process (13).

For optimal printing, the template should be completely soluble in the porogen. Therefore methanol is used in MI- SPE quercetin synthesis because quercetin is soluble in methanol. Acetonitrile as an aprotic polar porogen may produce polymers of larger pore size and surface area so that the analyte is readyIn the polymer. Research Qi *et al.* (14) showed that polymers synthesized with acetonitrile porogen yield are better retention capacity than non-polar chloroform. A high amount of porogen can increase the surface area and pore volume so that it can form well-distributed pores and has a high capacity (19).

Ilustration on the schematic synthesis of the MI-SPE quercetin can be seen on Figure 1.

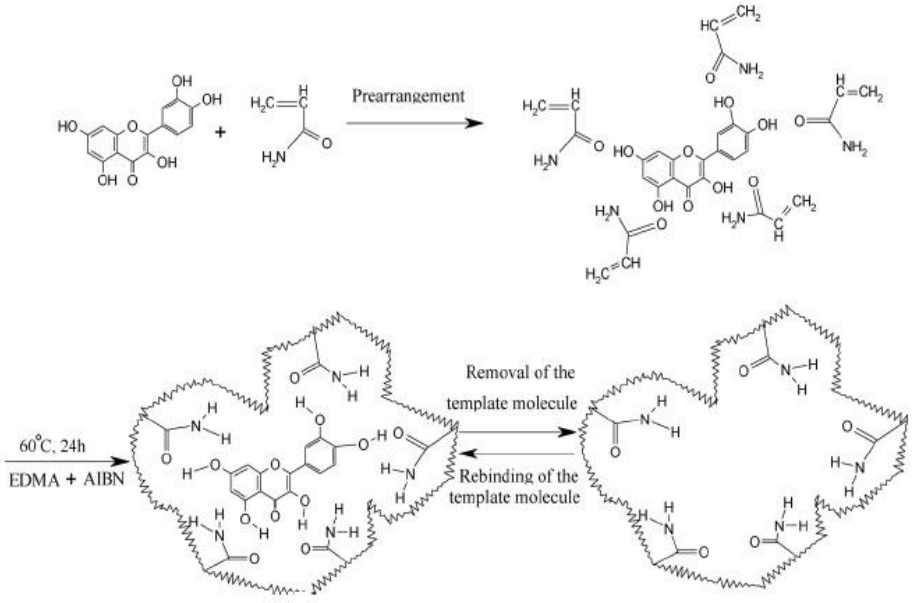


Figure 1: Ilustration on the schematic synthesis of the MI-SPE quercetin

MI-SPE Adsorption Capacity Determination

Based on the determination of adsorption ability, acetonitrile was found as the best solvent to support MIP sorbent adsorption (data not shown). Based on that, adsorption capacity was done using acetonitrile

solvent with various variations of quercetin concentration in acetonitrile solvent, ie, 1, 2.5, 5, 10, and 15 mgL^{-1}. Adsorption capacity result of MIP and NIP can be seen at Table 3.

The MIP sorption adsorption capacity evaluation process is carried out by introducing 5 ml of quercetin solution with various concentrations into the vial containing 20 mg of MIP sorbent. After that, the solution mixture was shaken using a mechanical agitator for 3 h at a rate of 120 rpm at room temperature, then the solution mixture was filtered, the filtrate was taken,and the absorbance was measured using a UV spectrophotometer. This test is done in the triple.

The m values vary from zero to one, where if m is close to 1 then the system is more homogeneous and if the value m equals one means the system is homogeneous. If m is 0, it indicates a heterogeneous system. From the Freundlich isotherm linear curve, the MIP and NIP parameters can be seen in Table 3.

Table 3: Adsorption capacity result of MIP and NIP

Sorbent	m	Adsorption capacity (a) (mg/g)	Imprinting Factor
MIP	0.412	190.37	6.127
NIP	0.965	48.32	

The MI-SPE sorbent sorption adsorption capacity was evaluated to determine the mechanism of adsorption interaction occurring between target molecules and MIP sorbent surfaces. Freundlich isotherm adsorption method is used in assessing MI-SPE sorbent adsorption capacity because this method is accurate to know the heterogeneity of MIP sorbents exponentially and is suitable for MIP sorbents synthesized by non-covalent approach (20). Also, another Freundlich isotherm excess is a logarithmic form that can be easily calculated since it can be converted into linear functions.

Adsorption of Freundlich isotherms is an adsorption process that occurs by adsorption of physics (physisorption) which involves only intramolecular forces and weakly bound. According to Freundlich, the amount of adsorbed substances will be proportional to the added pressure or concentration. In Table 3, it is seen that NIP sorbent has a more linear Freundlich isotherm adsorption curve than the MIP sorbent which can be seen from the value of R. The parameter value m for MIP shows an amount not approaching 1 which means the distribution of binding region to the MIP sorbent is heterogeneous. While the parameter value m for NIP shows a value close to 1, show the distribution of binding region in NIP sorbent more homogeneous than MIP. Unequal distribution of binding regions may result in the adsorption capacity of each molecule in different sorbents. This also affects the plot result of the MIP curve is less linear compared to the NIP curve. In addition to the parameter m, we can see also the value of parameter an (adsorption capacity) for MIP sorbent (190.37) larger than NIP (48.32). This is means that MIP sorbent has adsorption capacity and binding affinity to quercetin that is greater than NIP sorbent. MIP sorbents have a specific binding site for quercetin resulting in a molecular interaction between the quercetin target molecule and the MIP sorbent. The adsorption capacity of (a) NIP sorbents is less than that of the MIP sorbent because NIP sorbents have no specific binding sites for quercetin so quercetin is not bound to the NIP sorbent.

Distribution Coefficient Determination and Selectivity Test

Determination of distribution coefficient value is done on MIP and NIP sorbent. This was done to test the selectivity of the sorption of MI-SPE quercetin produced by comparing the MI-SPE sorbent distribution coefficient value to the NIP sorbent.

Determination of MI-SPE sorbent selectivity was done by comparing the sorbent distribution coefficient value of MI-SPE to other flavonoid group compounds, i.e., genistein, and luteolin. The sorbent selectivity of MI-SPE is obtained from the measurement of imprinting factor (IF) which is the ratio between distribution coefficient (Kd) MIP and NIP. The calculation of imprinting factor values can be seen in Table 4.

Table 4: Imprinting factor (IF)

Compound	Kd		IF
	MIP	NIP	
Quercetin	88.352	14.419	6.127
Luteolin	24.976	28.528	0.8755
Genistein	7.725	51.216	0.1508

Based on Table 4 it can be seen that the MIP sorbent has the most significant distribution coefficient value of the quercetin compound, the second against luteolin, and the smallest, i.e.,to genistein. This

shows that the amount of quercetin bound to MIP sorbents is higher than the amount of luteolin and bound genistein. At IF values can be seen that the largest IF value is on the compound quercetin, second luteolin, and the smallest, i.e., genistein. The IF values represent the selectivity of the resulting sorbent. If the IF value is high, then the sorbent is increasingly selective towards the target molecule. From this, it can be said that the sorbent yield of MI-SPE quercetin is selective against the quercetin compound. The largest IF value after quercetin is luteolin. Thissuggests that there is a tendency of sorbents in the binding of luteolin because luteolin has a structure similar to quercetin. The similarities of the quercetin and luteolin structures can be seen in the number of benzene rings, both of which have benzopyran and carboxyl groups. Also, quercetin and luteolin have the same hydroxyl group position that is bonded to ring A at position 5.7 and bound to ring B at position 3 and 4positions. The difference is only in the number of free hydroxyl groups in which there are 5 hydroxyl groups and 4 of hydroxyl groups (7,8,21). Comparison of quercetin, luteolin and genistein structure can be seen in Figure 2.

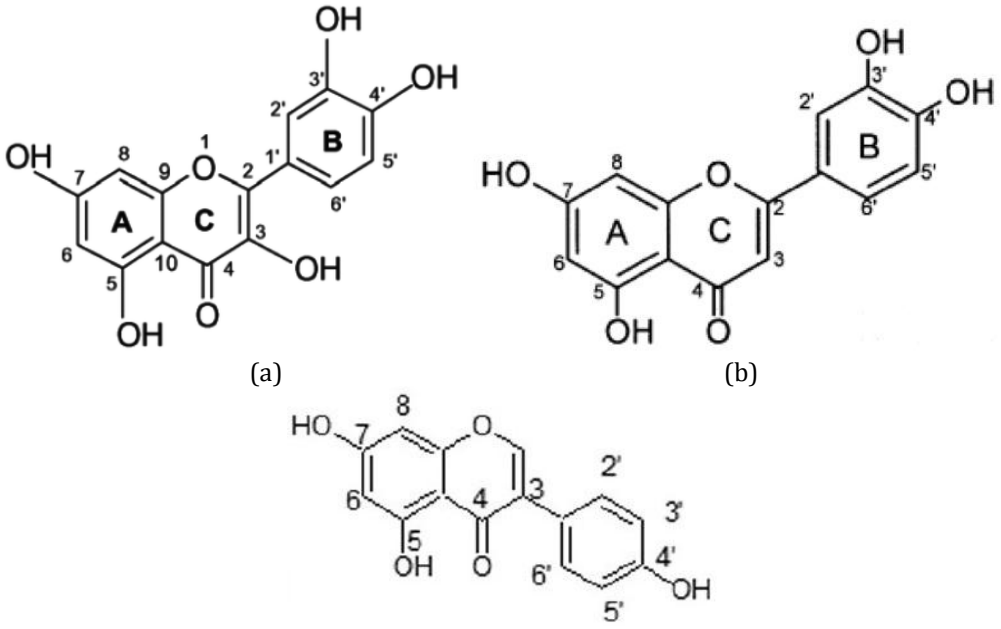


Figure 2: Structure of (a) Quercetine, (b) Luteolin, and (c) Genistein [7, 8]

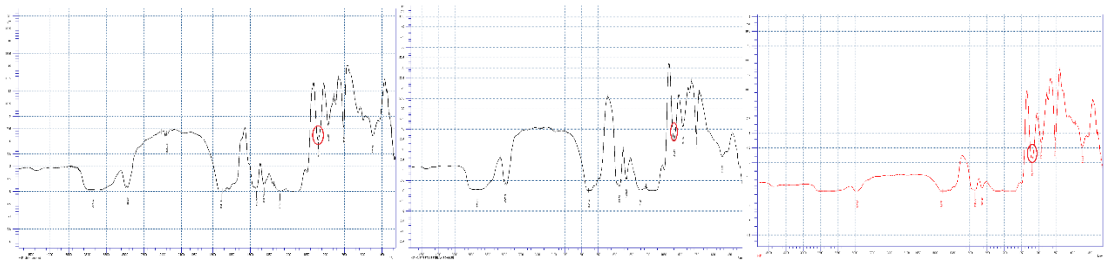
MIP and NIP Characterization via FTIR Instruments

The Fourier Transform Infrared (FTIR) instrument is used to identify the functional groups of the generated MIP and NIP sorbents, IR spectra can also be used to see if the polymerization reaction has been completed which is indicated by the absence of vinyl group absorption of the doublet peak at 1000 cm^{-1} and 900 cm^{-1}.Also, IR spectra can also be used to show changes in composition that occur between MIP sorbents containing templates with no template (22). The peaks obtained from the measurement with FTIR instrument show a particular functional group as seen in Table 5.

Table 5: Results of FTIR Sorben MIP and NIP Analysis

Wavenumber (cm^{-1})			Functional Groups
MIP Sorbent Before Extraction	**MIP Sorbent After Extraction**	**NIP Sorbent**	
3515.33	3494.11	3600-3300	-NH *stretching*
2955.00	2988.75	2955.00	C-H *stretching*
1732.11	1730.18	1746.57	C=O *stretching*
1452.42	1454.35	1455.32	CH_2*bending*
1264.36	1262.43	1391.86	C-O *stretching*

Based on Table 5. it can be said that the polymerization reaction has run correctly which is indicated by the absence of vinyl group absorption on the FTIR spectrum of MIP or NIP. The vinyl group will give a typical uptake of doublet peaks at 1000 cm-1 and 900 cm-1 wavenumbers. The absence of vinyl group uptake signifies that the polymerization reaction has been completed (23).



(a) (b) (c)

Figure 3: FTIR spectra of (a)MIP before template extraction (b) MIP after template extraction (c) NIP

Application of MI-SPE Quercetine for Purification of Quercetin from Plectranthus Scutellarioides Plant Extract

From chromatogram of quercetine before and after SPE processes (Fig.4), it indicates that MIP process produced a better and higher peak of Quercetine. This kind of height showed that MI-SPE process on the extraction of quercetine from *Plectranthus scutellarioides* was better than usual liquid-liquid extraction in extracted quercetine. The process has an efficient time compared to the conventional method because bysingle solid phase extraction already have a higher peak compared to singleliquid-liquid extraction. Treatment with single liquid-liquid extraction could not resulted a peak of quercetine that can be seen using our HPLC method with LOD 0.022 µg/mL and still have higher unwanted matrices peak before 5 min. This means MI-SPE quercetine that resulted from this research can washed out unwanted matrices and resulted on shortcut the process of isolation to have pure quercetin.

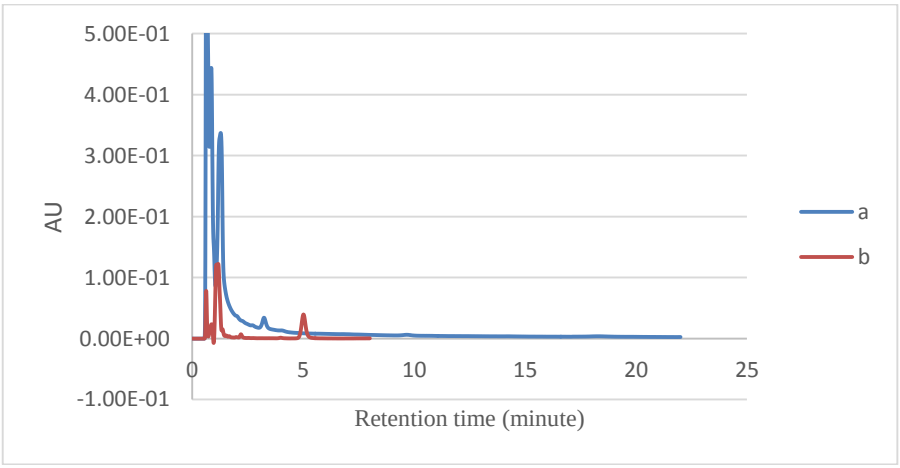


Figure 4: Chromatogram of Quercetin in Plectranthus scutellarioides extract (a) treatment with liquid liquid extraction, (b) treatment with MI-SPE quercetin

CONCLUSION

MI-SPE quercetin made from acrylamide monomer with acetonitrile:methanol as porogenic solvent has an adsorption capacity 190.37 mg/g and IF value 6.127 with the absence of vinyl group on FTIR spectra indicated completed polymerization synthesis. MI-SPE quercetin can be used to purified quercetin from *Plectranthus scutellarioides.*

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