PALACKÝ UNIVERSITY OLOMOUC

Faculty of science

Department of physical chemistry

BACHELOR THESIS

Lund 2011 **Jan Václavík**

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ADSORPTION OF QUERCETIN INTO MOLECULAR IMPRINTED POLYMERS

BACHELOR THESIS

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Abstract

The aim of this work was to investigate the adsorption of the antioxidant quercetin into molecular imprinted polymers, which were formed in presence of quercetin as a template molecule. In the first introductions parts, background of this thesis is described as well as a knowledge of the quercetin, molecular imprinted polymers and high performance liquid chromatography as a separation method with ultraviolet detector, to understand all parts of the study. The thesis contains brief survey of previous publications regarding molecular imprinted polymers where quercetin was used as a template molecule and in the main part of the thesis, a detailed description of the sample collection and the procedure to investigate the adsorption of quercetin can be found.

Souhrn

Cílem této práce bylo prozkoumat adsorpci antioxidantu quercetinu molekulárně otištěného do polymeru. V úvodní části je popsáno pozadí dané práce. Pro porozumění všech části studie je dále předložena znalost o quercetinu, o metodě molekulárně vtištěných polymerů a o vysoce účinné kapalinové chromatografii využité jako separační metoda s UV detekcí. Práce zahrnuje stručnou zprávu o předešlých publikacích zabývajících se molekulárně vtištěnými polymery, ve kterých vzorovou molekulou byl quercetin. V hlavní části práce je vylíčena metoda odebíráni vzorků a postupný vývoj při testování adsorpce quercetinu.

I want to thank my supervisor PhD student Sofia Lindahl as well as all members of Green Technology Group for their interest, valuable advices and their time they dedicated to me and my thesis. Thanks belong also to my family for their support throughout my studies.

I hereby clarify the thesis was developed independently and all literature sources and information used for the thesis are mentioned in the list of references. I agree the thesis is available at library of Physical chemistry, Faculty of science, Palacký University Olomouc.

In Lund …….

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Abbreviations

2-VP	2-vinyl pyridine
4-VP	4-vinyl pyridine
AA	Acrylamide
ABCN	1,1′-azobiscyclohexanecarbonitrile
ACN	Acetonitrile
AIBN	2,2´-azobisisobutyronitrile
DMSO	Dimethylsulfoxide
EGDMA	Ethylene glycol dimethacrylate
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IR	Infrared spectrometry
IS	Internal standard
LC	Liquid chromatography
MAA	Methacrylic acid
MIP	Molecular imprinted polymer
NIP	Molecular non-imprinted polymer
NPLC	Normal-phase liquid chromatography
Q	Quercetin
RPLC	Reversed-phase liquid chromatography
SPE	Solid-phase extraction
Theo-Cl	8-chlorotheophylline
THF	Tetrahydrofuran
UV	Ultraviolet spectrometry

1. Introduction

1.1 Quercetin

Quercetin (3,3´,4´,5,7-pentahydroxy flavone) and its glucosides belong to a wide group called flavonoids, which possess antioxidative and anti-inflammatory properties, which means they may have a positive effect on human body. It happens due to hydroxyl groups attached to the flavonol backbone that are able to eliminate active free radicals[1]. Flavonoids are present in most edible fruits, vegetables and plants such as apples, grapefruits, yellow onion, tea and red wine[2]. In case of quercetin one of the highest aglycone (quercetin with no glucose bonded) amounts are found in yellow onion as well as the two most common glucosides quercetin-4´-glucoside and quercetin-3,4´-diglucoside that occurs at even higher concentrations than the aglycone quercetin[2]. The two most common sugar groups usually attached to quercetin are d-glucose and l-rhamnose[3]. Quercetin is just one compound among many of similar flavonols occurring in nature and this is the reason why it is demanding to separate and determine the aglycon quercetin occurring in plans, see Table 1 and Fig. 1.

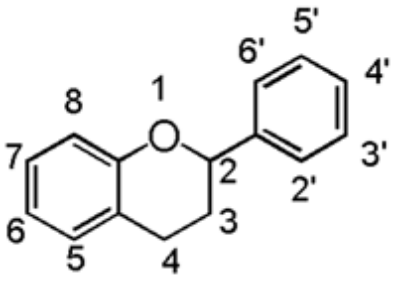


Figure 1. Basic structure and nomenclature of flavonoids [Source: http://metabolomics.jp/wiki/Category:FL 14 October, 13:35]

Table 1. Structural variations of common flavonoids with represented positions of substitutions that can be seen in Fig. 1. [Source: Taken over citation 4]

Flavonoid	Total No. of OH groups	Position of OH groups	Substitutions on the generic structure	Position of the substitutions
Myricetin	6	3,5,7,3′,4′,5′.		
Gossypetin	6	3,5,7,8,3′,4′.		
Quercetagen	6	3,5,6,7,3′,4′.		
Quercetin	5	3,5,7,3′,4′.		
Morin	5	3,5,7,2′,4′.		
Robinetin	5	3,7,3′,4′,5′.		
Myricetrin	5	5,7,3′,4′,5′.	O-Rh	3
Rutin	4	5,7,3′,4′.	O-Ru	3
Kaempferol	4	3,5,7,4′.		
Quercetrin	4	5,7,3′,4′.	O-Rh	3
Fisetin	4	3,7,3′,4′.		
Datiscetin	4	3,5,7,2′.		
Rhamnetin	4	3,5,3′,4′.	O-Me	7
Tamarixetin	4	3,5,7,3′	O-Me	4′
Silybin	3	3,5,7.	O-Lig-O	4′
Galangin	3	3,5,7.		
Kaempferide	3	3,5,7.	O-Me	4′
Diosmin	2	3,3′.	O-Ru, O-Me	5,4′
Robinin	2	5,4′.	O-Gal-Rh, Rh	3,7′
Troxerutin	1	5	O-Ru, O-He, O-He, O-He	3,7,3′,4′.
3-OH-Flavone	1	3		

Rh = rhamnose = 6-deoxy-L-mannose ($C_6H_{12}O_5$); Lig = lignin; Ru = rutinose = 6-O-D-glucose ($C_{12}H_{12}O_{10}$); He = hydroxyethyl (CH_2CH_2OH); Me = methyl (CH_3); Gal = galactose ($C_6H_{12}O_6$).

1.2 Onion waste

Every year agricultural and food industries produce within European Union approximately 450 000 tons[5] of onion waste as a by-product, that is collected and later used for animal feed, incineration or composting. However, this onion waste contains highly valuable compounds such as quercetin, quercetin glucosides (Fig. 2) and other flavonoids and these can be extracted and for example might be utilized as additives in food, cosmetics and pharmaceuticals. The most valuable compound that can be found in onion waste is quercetin. Unfortunately, the amount of aglycon quercetin in onion waste is rather low, because quercetin is present as glucosides such as quercetin-3,4´-diglucoside and quercetin-4´-glucoside[6]. These sugar groups can be removed by hydrolysis catalyzed by high concentration of HCl or enzyme[7], but before the hydrolysis the extraction of quercetin and its glucosides from onion waste must be performed. One of the reasons why hydrolysis of glucoside groups is prefered is because quercetin has higher antioxidative strength in comparison to quercetin glucosides. According to common procedure solid-liquid extraction with high concentration of HCl in aqueous methanol is used to hydrolyze the glucosides groups[8].

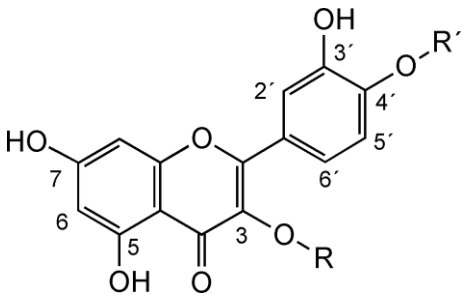


Figure 2. Chemical structures of quercetin (R, R´ = H), quercetin-3-glucoside (R = glucose and R´= H), quercetin-4´-glucoside (R = H and R´ = glucose) and quercetin-3,4´-diglucoside (R, R´ = glucose). [Source: taken over citation 9]

1.3 Extraction and hydrolysis of quercetin from onion waste

Turner *et al.*[10] has developed a more sustainable method than methanol-HCl extraction and hydrolysis, where pressurized hot water extraction is combined with an enzyme catalyzed hydrolysis, which has unquestionable less harmful impact on the environment than organic solvent methanol and hydrochloric acid[9]. The hydrolysis is catalyzed by a thermostable enzyme β-glucosidase, which is also more sustainable than hydrochloric acid[9]. After extraction and hydrolysis the isolation of quercetin from the raw extract is needed. The most common method to isolate quercetin is solid-phase extraction (SPE) with C_{18} bonded silica used as a sorbent[11]. Apart from traditional methods, there is a promising technique called molecular imprinted polymers (MIPs) that could pertinently be used to isolate quercetin with higher selectivity and efficiency.

1.4 Molecular imprinted polymers

Molecular imprinted polymers, also called synthetic antibodies, are produced in the presence of a template molecule which imprints its structure into the polymer matrix, see Fig. 3. Beside the template molecule, a monomer, a cross-linker, an initiator and a solvent are needed to produce MIPs and all of them must be soluble in the used solvent. Furthermore, the solvent must not interfere too much with interactions between the template molecule and the monomer[11]. The template molecule has certain property restrictions such as necessary

occurrence of functional groups on its skeleton or it must be ligand (ion, atom or molecule which can donate free electron pair). Furthermore, the template molecule should not be polymerisable and it should influence the polymerization process as little as possible. The desired influence is imprinting process of template molecule into the polymer structure.

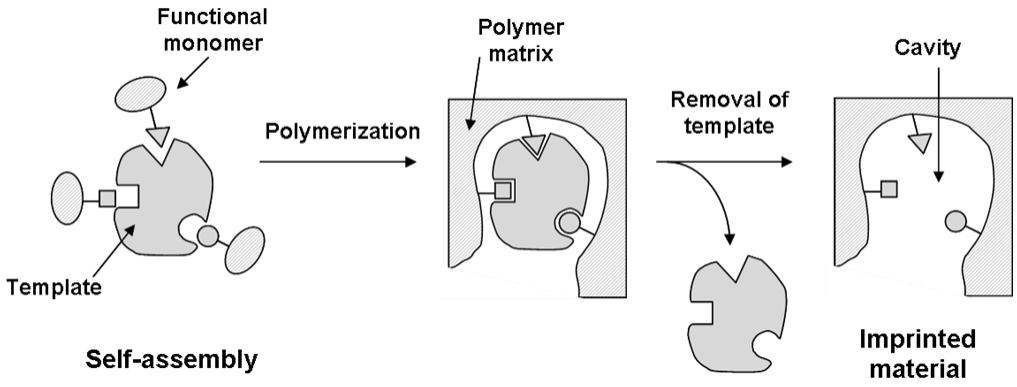


Figure 3. Scheme of molecular imprinting process. [Source: http://en.wikipedia.org/wiki/Molecular_imprinting 9 October 2010 11:38]

The imprinting process is based on interactions between functional groups of the template molecule and complementary functional groups of the selected monomer or mixture of monomers. The chemical structures of the most common monomers such as methacrylic acid (MAA)[12], 4-vinyl pyridine (4-VP)[12-16] and acrylamide (AA)[14, 17, 18] used for quercetin are shown in Fig. 4 as well as 2-vinyl pyridine (2-VP) which was tested in this work. According to the type of the interaction between functional groups there are different categories of imprinting processes. When the interactions are covalent, it is called covalent imprinting and if the interactions are non-covalent such as hydrogen bonds, intermolecular forces, ion-pairs or electrostatic interactions, it falls into the category of non-covalent imprinting. In metal ion imprinting the metal itself function as a template or is part of the polymer-template interaction[19]. Since four of quercetin´s five hydroxyl groups on carbon number 3´, 4´, 5 and 7 (Fig. 2) are suited for intermolecular hydrogen bonding, MIPs where quercetin is used as template molecule belong to non-covalent imprinting category. The fifth hydroxyl group on carbon number 3 has strong intramolecular hydrogen bonds with carbonyl group on carbon number 4, therefore it cannot participate in any other interactions[17].

A B C D

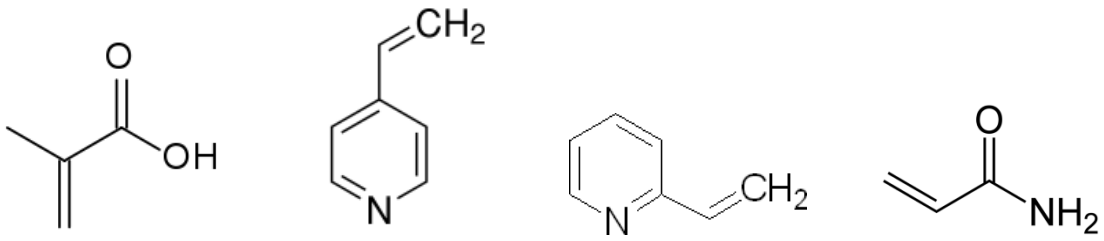


Figure 4. The chemical structure of (**A**) methacrylic acid, (**B**) 4-vinyl pyridine, (**C**) 2-vinyl pyridine, (**D**) acrylamide.

When the polymerisable monomer has formed interactions to the template molecule in a specific way, formed complex is for instance copolymerize by thermal polymerization in the presence of a high concentration of cross-linker such as most common ethylene glycol dimethacrylate (EGDMA) and the polymerization can be initiated by a free radical initiator for example 2,2´-azobisisobutyronitrile (AIBN)[12-18] (Fig. 5) and heat.

A B

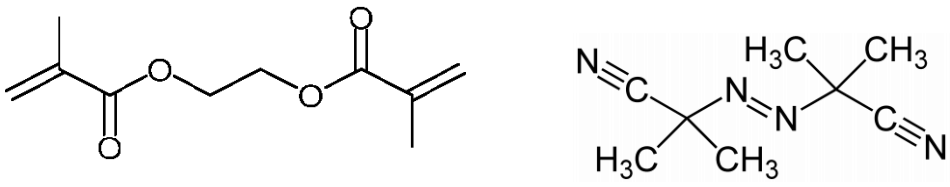


Figure 5. Chemical structure of across-linker (**A**) ethylene glycol dimethacrylate and a thermal initiator (**B**) 2,2´-azobisisobutyronitrile.

The next step is to remove the template molecule by extraction to create cavities in the polymer matrix with certain arrangement of functional groups that are complementary to the template molecule in terms of shape, size and functionality. Thus, the obtained polymer should have high selective recognition and affinity to the template molecule[18]. In the case of non-covalent imprinting, it may happen that not all template molecules are sufficiently removed which may result in template molecule bleeding during tests and applications[19]. In

addition to MIPs there are molecularly non-imprinted polymers (NIPs) produced following the same procedure as MIPs but without any template molecule present during the polymerization process. The difference between specific and non-specific binding of quercetin to the MIPs is observed by comparing the binding capacity of MIPs and NIPs. It is expected that NIPs will have only non-specific binding while MIPs will have both specific and non-specific binding and hence the higher adsorption efficiency than NIPs.

There are several reports[12-18] on effective MIPs, where quercetin is used as a template molecule and it has been proven that it is important to use suitable monomer and solvent to prepare effective MIPs for quercetin. Theodoridis *et al.*[12] has reported succesful MIPs for quercetin using 4-VP respectively MAA as functional monomers, and dimethylsulfoxide (DMSO) respectively acetonitrile (ACN) as porogenic solvents. There are some more publications based on 4-VP and aceton[13, 15, 16]. Song *et al.*[17, 18] and Yan *et al.*[14] prepared effective MIPs with AA and tetrahydrofuran (THF) or aceton as porogenic solvents. In all cases, authors used EGDMA as a cross-linker and either 1,1′-azobiscyclohexanecarbonitrile (ABCN, see Fig 6) or AIBN as thermal initiator for the polymerization process.

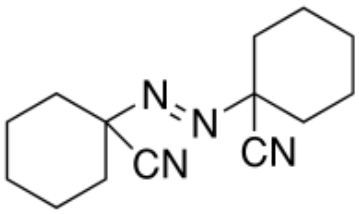


Figure 6. Chemical structure of thermal initiator 1,1′-azobiscyclohexanecarbonitrile.

Although monomers such as 2-VP and 4-VP can be toxic throughout longer exposure, we can actually work with them since MIPs and NIPs can be used more than once. The washing out the target molecule out of the MIPs is simply performed again and again for hundreds of uses so the sustainable ideology is kept.

1.5 Liquid Chromatography

Compounds that are not sufficiently volatile for gas chromatography such as quercetin can be analyzed by liquid chromatography (LC)[20]. LC is a separation technique in which a liquid sample, containing a mixture of analytes, is injected between two phases in a column. The stationary phase consists of permeable same-sized particles which interact with molecules of analyte according to the affinity of the analyte to the stationary phase. The mobile phase, into which the mixture of samples is dissolved, is liquid and drifts molecule of analyte by the flow and by interactions between mobile phase and analyte through the stationary phase, see Fig. 7. Separation of mixtures of analytes is based on differential partitioning of analytes between the stationary and mobile phase. Even really small difference between two very similar compounds such as quercetin and morin (differ in the position of one hydroxyl group, see Fig. 8) results in different retention time and thus the separation of these very similar compounds is possible. The time at which a specific analyte elute out of the end of the column is called retention time. Nowadays, the most common method in LC is High Performance Liquid Chromatography.

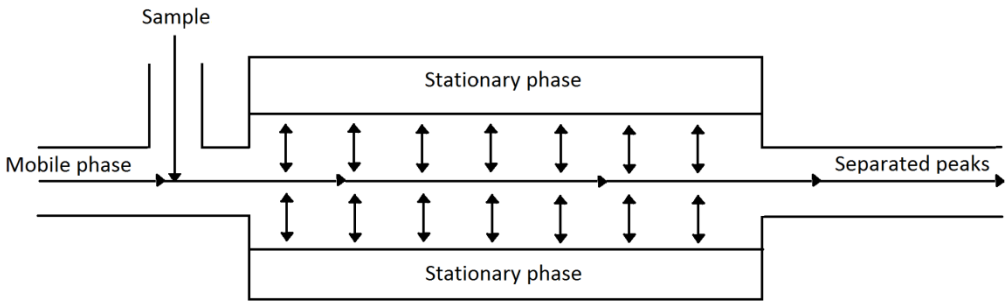
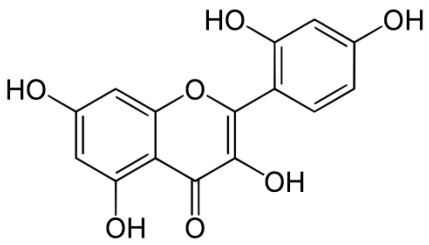


Figure 7. Scheme of liquid chromatography separation. The mobile phase including the sample mixture passes through the column and interacts with stationary phase.

A



B

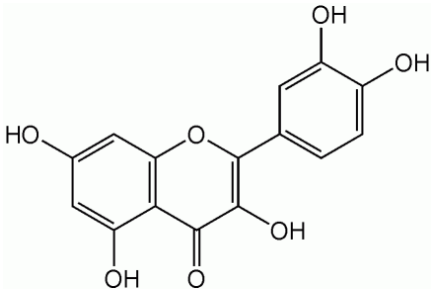


Figure 8. (**A**) Morin 2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one (**B**) Quercetin 2-(3,4-dihydroxyphenyl)- 3,5,7- trihydroxy- 4*H*- chromen- 4-one

1.5.1 High performance liquid chromatography

High performance liquid chromatography (HPLC) utilizes high pressure to force molecules of analyte through a column which is packed with small particles of sorbent giving high-resolution separation[20]. Commonly, HPLC systems consists of solvent reservoirs, a solvent degasser, a gradient valve, a mixing vessel for delivery of the mobile phase, a high-pressure pump, a switching valve, a guard column, an analytical column, a detector (i.e. UV) and a computer to control the instrument and interpret results, see Fig. 9.

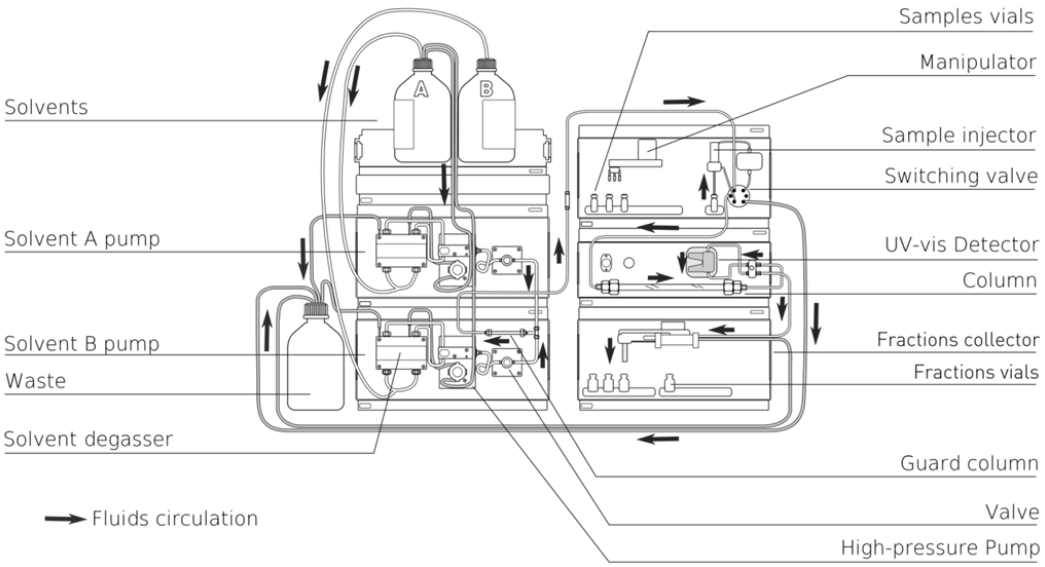


Figure 9. A common HPLC system with fraction collector, which is necessary for preparative HPLC. [Source: http://en.wikipedia.org/wiki/File:Preperative_HPLC.svg 24 September 2010, 19:25]

1.5.1.1 The HPLC column and its properties

Liquid chromatography is performed with particle packed steel or plastic columns. If open tubular columns would be used like in gas chromatography (GC), solute molecules would not have enough time to travel in solvent channel due to slow diffusion in liquids[21]. The most common size of particles is 3-10 μm, which requires pressure of several tens bars depending on the diameter of the column, the length of the column, used solvents, solvent viscosity and how well packed the column is, to achieve flow rates of 0,5-5 ml/min. As the size of inner particles of column decrease, the efficiency of chromatography increases. The reason why small particles are used is shorter optimum run time and better resolution of the separation. Disadvantage of miniaturization to less than 3 μm particles is the need of pressure reaching to several hundred bars to attain flow rates of 0,5-5 ml/min. Usage of particles less than 1,5 μm requires even higher pressure, however increasing of temperature can be used to decrease viscosity of solvent, and thereby also the pressure[22]. Analytical columns are usually 5-30 cm long and have an inner diameter of 1-5 mm[(20)]. Irreversible adsorbed impurities in sample or solvents can cause considerable damage to the analytical column. Therefore, to protect the analytical column, a short guard column possessing the same properties such as inner diameter and particle size of the stationary phase is used. The guard column is situated before the analytical column, is regularly replaced and is not as expensive as the analytical column. An example of an analytical column is shown in Fig. 10.

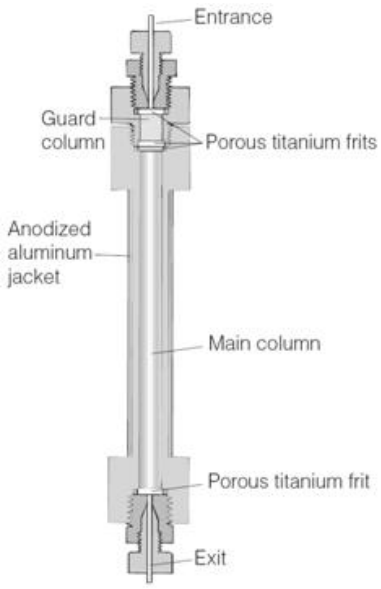


Figure 10. An analytical column with a replaceable guard column to collect irreversibly adsorbed impurities [Course: taken over citation 21]

1.5.1.2 The stationary phase

Small spherical, micro porous silica particles are commonly used as inner filling of the column and are usually stable up to pH 8. A silica surface has silanol groups (Si-OH) that are fully protonated when the pH is below 3. When the pH is above 3, silanol groups dissociate to negative $Si\text{-}O^-$ which cause binding of protonated bases and tailing as a result. Liquid-liquid partition chromatography utilize bonded stationary phase covalently attached to the silica surface. The most common bonded non-polar stationary phase is octadecyl (C_{18}). If the siloxane bond (Si-O-SiR) is not protected by a bulky alkyl group, than it is easily attacked by H_3O^+ cations, which leads to hydrolysis of stationary phase. When there are for example isobutyl groups attached to siloxane bond, the stationary phase is stable at low pH and even at elevated temperatures (e.g., pH 0,9 at 90°C)[21].

1.5.1.3 Principle of elution

When the sample, containing the analytes, is injected into the analytic column, the analytes more or less interact with the stationary phase. The eluent strength (ε°) is a physical quantity describing the ability of specific solvent to elute molecules of analyte out of the given adsorbent of the stationary phase. In fact solvent molecules displace analyte molecules. Table 2 sorts out solvents according to their eluent strength on bare silica where pentane is defined as 0 value. Chromatography where bare silica is used as an adsorbent is typical normal-phase liquid chromatography (NPLC). In NPLC, stationary phase is more polar than the mobile phase. As the polarity of the used solvent increases, the eluent strength increase as well. It means that molecules of analyte are eluted more rapidly than if less polar solvent would be used. However, the more common technique is reverse-phase liquid chromatography (RPLC) in which the stationary phase is less polar than the solvent. In RPLC, a less polar solvent elutes molecules of analyte more rapidly than more polar solvent[20, 21, 23]. An advantage of RPLC over NPLC is the use of less organic solvents.

Table 2. Eluotropic series and ultraviolet cutoff wavelengths of solvents for adsorption chromatography on silica. [Course: taken over citation 23]

Solvent	Eluent strength (ε°)	Ultraviolet cutoff (nm)
Pentane	0.00	190
Hexane	0.01	195
Heptane	0.01	200
Trichlorotrifluoroethane	0.02	231
Toluene	0.22	284
Chloroform	0.26	245
Dichloromethane	0.30	233
Diethyl ether	0.43	215
Ethyl acetate	0.48	256
Methyl *t*-butyl ether	0.48	210
Dioxane	0.51	215
Acetonitrile	0.52	190
Acetone	0.53	330
Tetrahydrofuran	0.53	212
2-Propanol	0.60	205
Methanol	0.70	205

1.5.1.4 Isocratic and gradient elution

Isocratic elution means the ratio of solvents is not changing throughout the whole analysis, but if isocratic elution does not provide desired separation during a short run time, gradient elution can be used, see Fig. 11. When gradient elution is used, it means, that at the beginning of the analysis there is different composition of the mobile phase than at the end. The composition of the mobile phase is changed in order to increase the elution strength of the mobile phase to speed up the elution of the compounds that have stronger interactions with the stationary phase[21, 23].

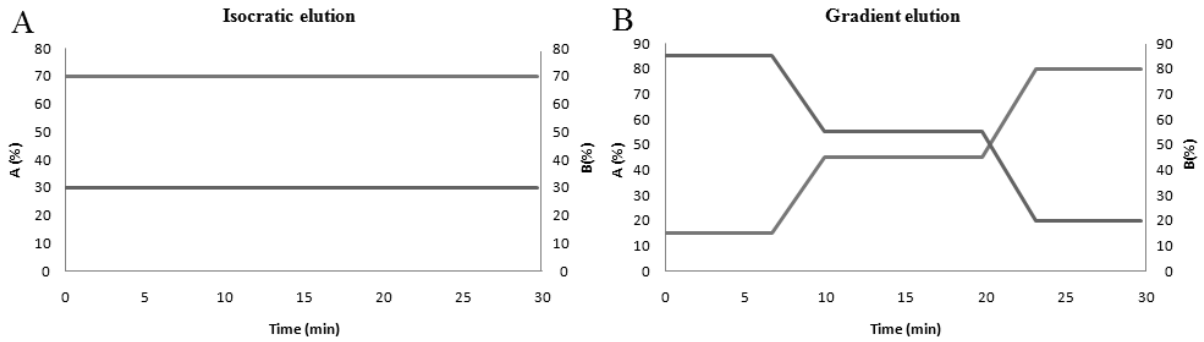


Figure 11. (A) Isocratic elution with 70 % of solvent A and 30 % of solvent B throughout the whole analysis. **(B)** Gradient elution with 15 % of solvent A for the first 7 min, than eluent strength is increased rapidly over 3 min to 45 % of solvent A and held for 10 min and finally the solvent was changed to 80 % B over 2 min and held to the end of the separation.

2. Aim of study

The aim of this bachelor thesis was to evaluate the efficiency of adsorption of quercetin into the MIPs and NIPs previously prepared by Lindahl S. and Pakade V. as well as to investigate different conditions and method for adsorption and analysis. These MIPs and NIPs were formed with three different functional monomers MAA, 2-VP, 4-VP and the focus was aimed at methanol used as an organic solvent in which the adsoption ability of MIPs was investigated. Analyzed MIPs might later be used in on-line extraction/hydrolysis/isolation flow system for onion waste to isolate the aglycone quercetin.

3. Materials and Methods

3.1 Chemicals

Standard quercetin and internal standard 8-chlorotheophylline were purchased from Sigma-Aldrich (Germany). Gradient Grade B&J Brand methanol for chromatography was supplied from Germany Honeywell and 98-100% formic acid was obtained from Merck (Germany). Ultra pure water (Milli Q, Millipore, USA) was used for all solutions. The MIPs and NIPs were kindly provided by Lindahl S. and Pakade V.

3.2 Liquid chromatography

The quantification of quercetin and 8-chlorotheophylline was carried out with isocratic elution at flow of 150 μL/min at temperature of 25 °C on a HPLC-UV system UltiMate 3000 from Dionex (Germering, Germany) using an Agilent Zorbax SB-C18 column, 100 x 2,1 mm, 3,5 μm (Agilent Technologies, USA). The injection volume was 5 μL. The column was equilibrated with mobile phase for 55 min before use. The mobile phase A was composed of 99,5 % methanol and 0,5 % formic acid and mobile phase B was composed of 99,5 % water and 0,5 % formic acid. Both solvents A and B were kept in the sonication bath for 30 minutes before use. The UV/VIS detector was operating at two different wavelengths of 275 nm and 350/370 nm due to difference between amplitudes of adsorption spectrums of quercetin and 8-chlorotheophylline. Data were collected by Chromeleon software on a desktop computer. The standard calibration solutions used in LC were prepared in the same solvent as samples were analyzed in. A stock solution for quercetin of concentration 0,3 g/L in methanol was prepared in the laboratory and stored at - 18 °C as well as a stock solution for 8-chlorotheophylline of concentration 0,3 g/L in methanol and water in ratio 1:1.

3.3 Sample collection

50/150 mg of particular MIPs were weighed into a 25 mL Erlenmeyer flask where a stirring bar was subsequently added. Depending on desired concentration, a certain volume of quercetin was added into a 25 mL volumetric flask from the stock solution together with a certain volume of internal standard 8-chlorotheophylline and replenished with solvent. After

homogenization of the solution, the initial reference concentration of quercetin was transferred into a 1,5 mL dark vial containing the same volume of water and 1 % of formic acid in order to dilute the sample to half. Then, the solution with quercetin and internal standard was added into the Erlenmeyer flask with MIPs in order to start the reaction and placed on the stirrer. After each hour the stirring was turned off and MIPs were settled for 5 minutes and after that sample was transferred into the eppendorf tube with water containing 1 % of formic acid. Then, the eppendorf tube was put into the centrifuge for another 5 min in order to spin down the rest of MIPs and sample was transferred into the 1,5 mL dark vial afterwards. The procedure of sample collection is shown in Fig. 12. If much of the MIPs were taken out during sampling they were returned back and samples containing high concentrations of quercetin were diluted more times before analysis with HPLC system. The sampling method went through some modifications that are described below and a summary of all experiments are shown in Table 3.

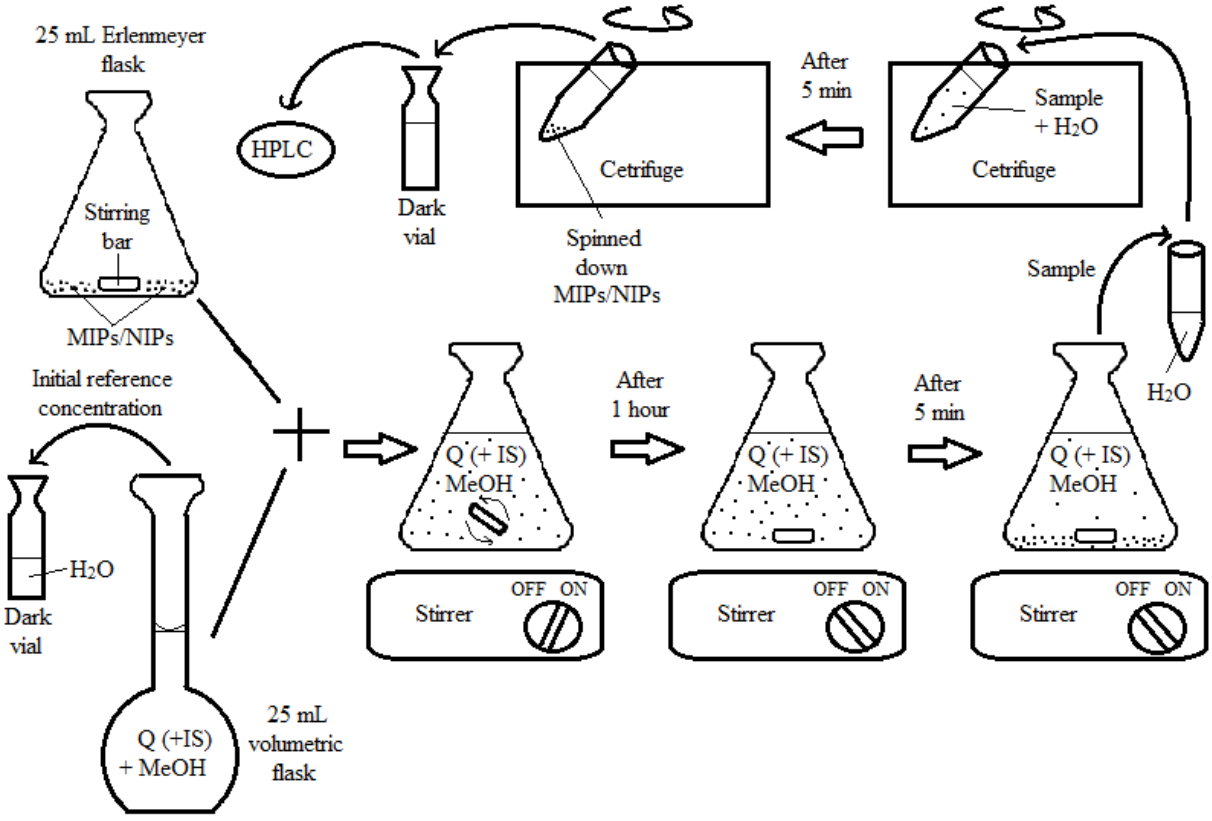


Figure 12. Scheme of sample collection. Q = quercetin, IS = internal standard, MeOH = methanol, HPLC = high performance liquid chromatography.

Table 3. Summary of all adsorption experiments of quercetin (Q). IS stand for internal standard.

Experiment	Polymer	Amount of polymer (mg)	Conc. of Q (µM)	Conc. of IS (µM)	Number of replicates	Amount of sample (µL)	When samples were taken (h)	Water present (%)
1st	MAA NIP	50	10	0	2	500	0-8,24	0
		50	87	0	2	500	0-8,24	0
		50	190	0	2	500	0-8,24	0
2nd	2VP MIP/NIP	50	3,3	0	1	500	0-6	0
		50	16,5	0	1	500	0-6	0
		50	66	0	1	500	0-6	0
3rd	2VP MIP	50	66	20	3	500	0,0.5,1,1.5,2-6,24	0
		150	66	20	3	500	0,0.5,1,1.5,2-6,24	0
4th	2VP MIP	50	66	20	3	350	0-4,6,7,24	0
	4VP MIP	50	20	20	1	350	0-4,6,7,24	0
		50	30	20	1	350	0-4,6,7,24	0
		50	40	20	1	350	0-4,6,7,24	0
5th	MAA MIP	50	66	40	1	350	0,24	10
	MAA NIP	50	66	40	1	350	0,24	10
	MAA MIP	50	330	40	1	350	0,24	0
	MAA NIP	50	330	40	1	350	0,24	0
6th	MAA MIP	50	300	40	2	100	0-7,24	10
		50	300	40	2	100	0-7,24	30
7th	4VP MIP	50	200	100	3	350	0,1,3,5,7,24	10
	4VP MIP	8	200	100	3	350	0,1,3,5,7,24	10

4. Results and Discussion

4.1 Absorption spectrum of quercetin and internal standard

The absorption spectrum of quercetin and 8-chlorotheophylline were obtained on double beam UV/VIS spectrophotometer Cary 1E (Varian, Australia) in order to know what wavelengths is necessary to set the UV/VIS detector, that belongs to HPLC system, to collect data, see Fig 13. Absorption was measured from 800 nm to 200 nm and 275 nm for internal standard and 370 nm for quercetin were selected. As a blank methanol was used for quercetin and for internal standard water and methanol in ratio 1:1 was used.

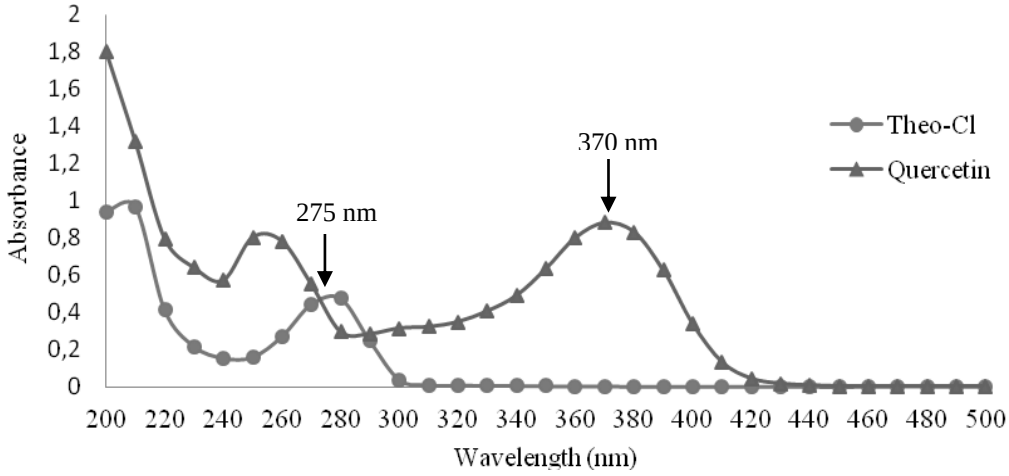


Figure 13. Absorption spectrum of quercetin and 8-chlorotheophylline (Theo-Cl) measured from 200 nm to 800 nm.

4.2 Adsorption of quercetin into MIPs and NIPs

The first experiment was done to evaluate adsorption of 3 different concentration of quercetin 16,5 μM, 66 μM and 330 μM to 50 mg of NIPs formed with MAA as a functional monomer. Duplicate samples were taken every hour for only 8 hours because after 8 hours the adsorption is slow due to the fact that all imprinted sites on the surface of polymer are probably already taken, so the rest of the quercetin molecules have to penetrate with difficulty inside the polymer structure to find vacant imprinted sites. The last sample was taken after 24 hours. An insignificant decrease of concentration with time is shown in Fig. 14. It is difficult to say, why the greater concentration provide greater decrease of concentration of quercetin. Plot of samples containing 16,5 μM of quercetin is not shown due to its even lesser decrease of concentration compared to 66 μM samples. Apparently some calculation errors occurred during preparation of concentrations of quercetin, because approximately 200 μM quercetin instead of 330 μM is observed as well as 86 μM quercetin instead of 66 μM.

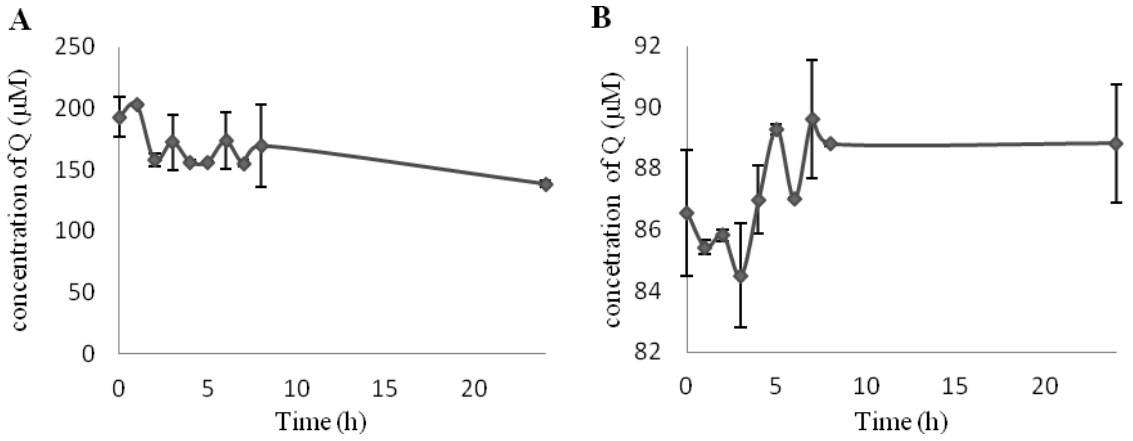


Figure 14. Adsorption of quercetin into MAA NIPs during 24 hours, n = 2, error bars correspond to standard deviation. (**A**) Adsorption of 200 μM quercetin. (**B**) Adsorption of 86 μM quercetin.

Because NIPs with MAA did not provide desired decrease of concentration of quercetin, in the next experiment 2-VP MIPs and NIPs were used. After some reading of publications[17, 18], regardless the first experiment, the focus was changed to lower concentration of quercetin. Specifically 3,3 μM, 16,5 μM and 66 μM of quercetin was tested with 50 mg of MIPs and NIPs as well, but no replicates were done just to see if the MIPs with 2-VP absorb better and also the experiment time was decreased due to assumption that the

greatest difference between concentrations is at first hours during the reaction. Comparison between adsorption plots of MIPs and NIPs are shown in Fig. 15, and the in case of 3,3 μM quercetin, there most like happened some kind of pipetting error, because from the plot can be observed that the initial area representing concentration is close to zero.

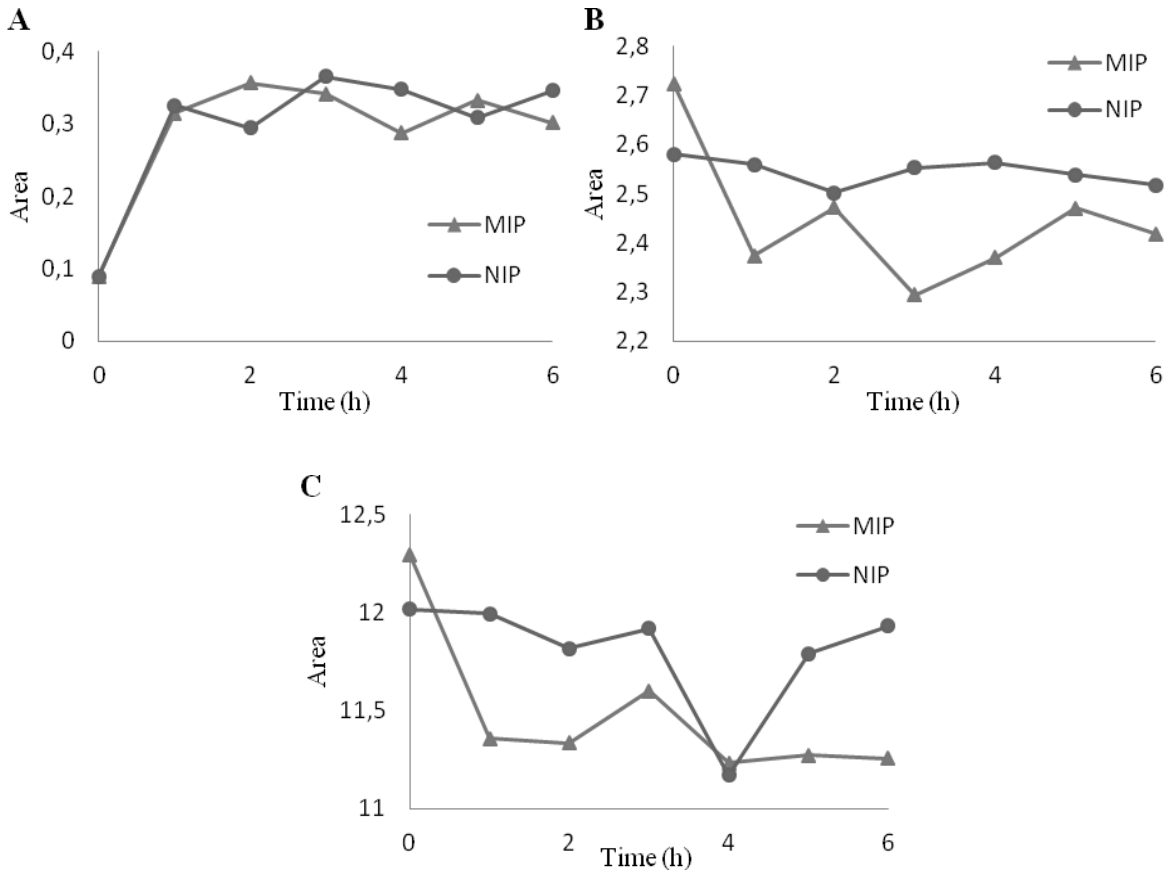


Figure 15. Adsorption of quercetin into 2-VP MIPs and NIPs during 6 hours, n = 1. (**A**) Adsorption of 3,3 μM quercetin. (**B**) Adsorption of 16,5 μM quercetin. (**C**) Adsorption of 66 μM quercetin.

It was decided to integrate an internal standard[21] since by now all adsorption plots show unstable adsorption curves probably due to deviations at pipetting and other errors occurred during sample collection. 8-chlorotheophylline was chosen because its non-hydroxyl groups chemical structure, see Fig. 16, does not allow to bind specifically with MIPs/NIPs and non-specific binding is as little as possible. Furthermore, the difference in retention times

between quercetin and 8-chlorotheophylline is low, therefore they can be separated and quantified during short run time.

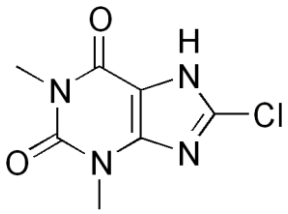
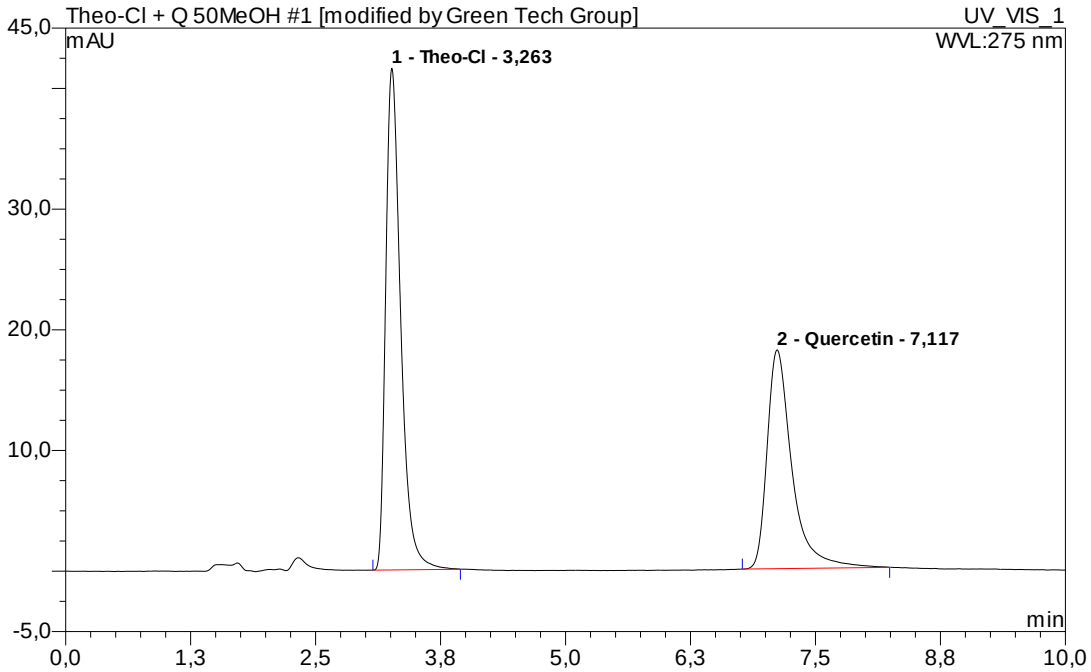


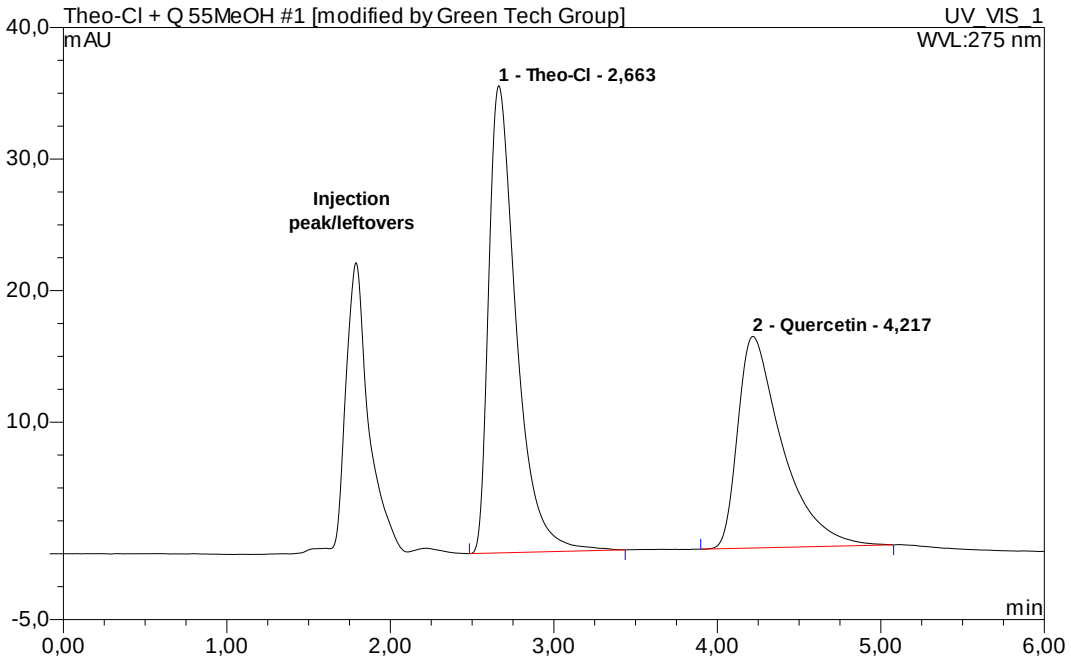
Figure 16. Chemical structure of 8-chlorotheophylline used as a internal standard.

Four different ratios of mobile phase consisted of methanol, water and 0,5% of formic acid were tested to optimize quality of the separation and duration of run time. A ratio of 60 % methanol and 40 % water seemed to be ideal, but mobile phase consisted of 55 % methanol and 45 % water was used instead because when high concentration of quercetin would be used, the peaks might overlap as well as in case of unexpected tailing or fronting. Chromatograms of different ratios of mobile phase are shown in Fig. 17.

A



B



C

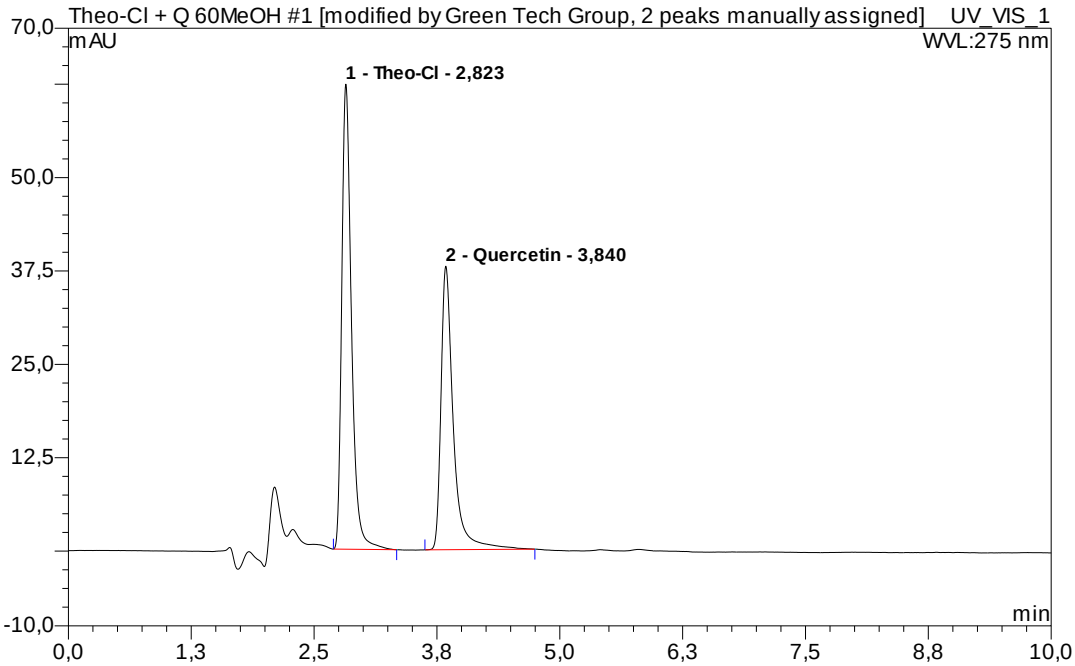


Figure 17. Chromatograms of optimization of ratio of mobile phase for separation of quercetin and internal standard 8-chlorotheophylline (Theo-Cl) detected at wavelength of 275 nm. As the amount of methanol increase, the eluent strength increase as well, which results in separation during shorter time. (**A**) Separation in 50 % of methanol and 50 % of water. (**B**) Separation in 55 % of methanol and 45 % of water. (**C**) Separation in 60 % of methanol and 40 % of water. In all cases 0,5 % of formic acid is present in mobile phase too.

To evaluate whether there is any adsorption of internal standard to MIPs, 20 μM internal standard in methanol was added into the 25 mL Erlenmeyer flask together with 50 mg of MIPs for 24 hours to see if the concentration of internal standard changes with time. If the concentration would change with time it would mean that the internal standard binds too much either specifically or non-specifically to MIPs and that the use of 8-chlorotheophylline as the internal standard would not be correct. From Fig. 18 can be observed that the concentration of internal standard in presence of MIPs is almost constant, therefore the use of this internal standard is appropriate.

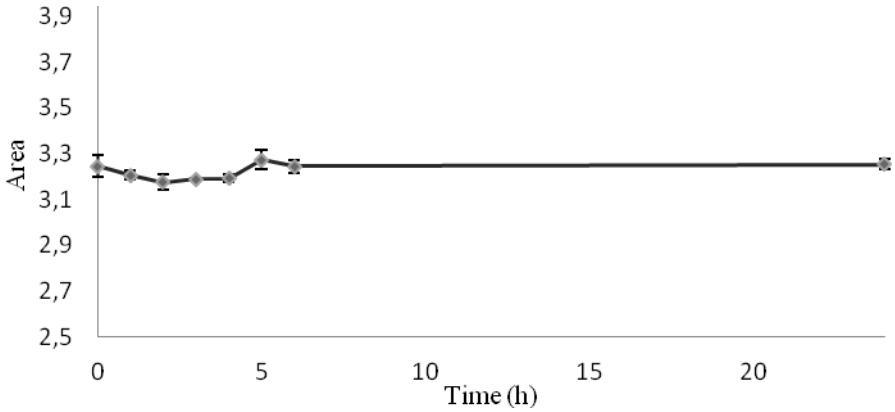


Figure 18. Plotting of change of concentration of internal standard 8-chlorotheophylline in presence of MIPs during 24 hours, n = 3, error bars correspond to standard deviation.

In the next experiment, mixture of 20 μM internal standard and 66 μM quercetin was added to both 150 mg and 50 mg of 2-VP MIPs to see some possible difference of adsorption of quercetin to different amounts of MIPs. Also the number of collected samples during first two hours was increased at one sample each half a hour. This particular experiment was done to find out if the problem with very low adsorption is due to insufficient amount of MIPs and to inspect the adsorption more properly during first hours when the adsorption is assumed to be the fastest. From Fig. 19 it can be deduced that the increased amount of 2-VP MIPs does not provide any significant increase of adsorption of quercetin. There was one more thought that led to modifying the sampling method in following experiments. In both cases the initial

reference concentration seems to increase very quickly right from the beginning due to the fact, that each time the sample is taken, the total reactional volume decrease of 500 μL which is 2 % error and since the total volume of reaction is indirectly proportional to concentration, the concentration increase each time the sample is taken. To minimize this effect 1 mL of sample was taken to eppendorf tube but without any water present and placed to the centrifuge in order to spin down remaining MIPs. When the MIPs were sediment only 350 μL of sample was taken from the eppendorf tube into the vial where had been added 350 μL of water with 1 % of formic acid. The rest volume of 650 μL was after homogenization returned back to the corresponding 25 mL Erlenmeyer flask.

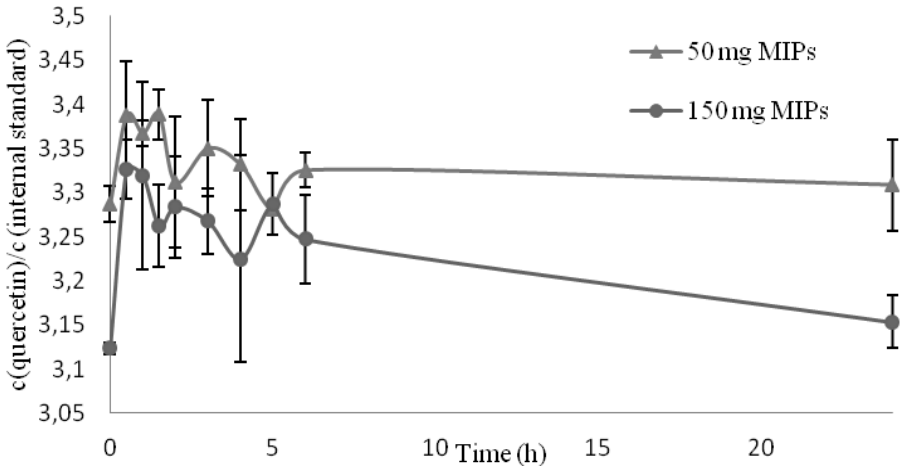


Figure 19. Adsorption of 66 μM quercetin into different amounts of 2-VP MIPs, n = 3, error bars correspond to standard deviation.

In the next experiment it was considered that quercetin maybe prefers to stay in solvent methanol rather than bind into 2-VP MIPs and also that MIPs used so far might had been prepared in an incorrect way or the process of washing out the template molecule was not done properly. It would mean that there would not be enough vacant cavities in polymer structure for quercetin to bind in so the concentration would not change with time. To evaluate these possible influences 66 μM quercetin was tested with previous used 2-VP MIPs and 20 μM, 30 μM, 40 μM quercetin and a 4-VP MIPs together with internal standard, all dissolved in 90 % of methanol and 10 % of water. The concentration of internal standard was doubled to achieve more accurate comparison between peaks of quercetin and 8-

chlorotheophylline. Graphs of results from described experiment are shown in Fig. 20 and easy conclusion can be made that neither addition of 10% of water to methanol used as a solvent in adsorption reaction, nor 4-VP MIPs did not improve the adsorption of quercetin.

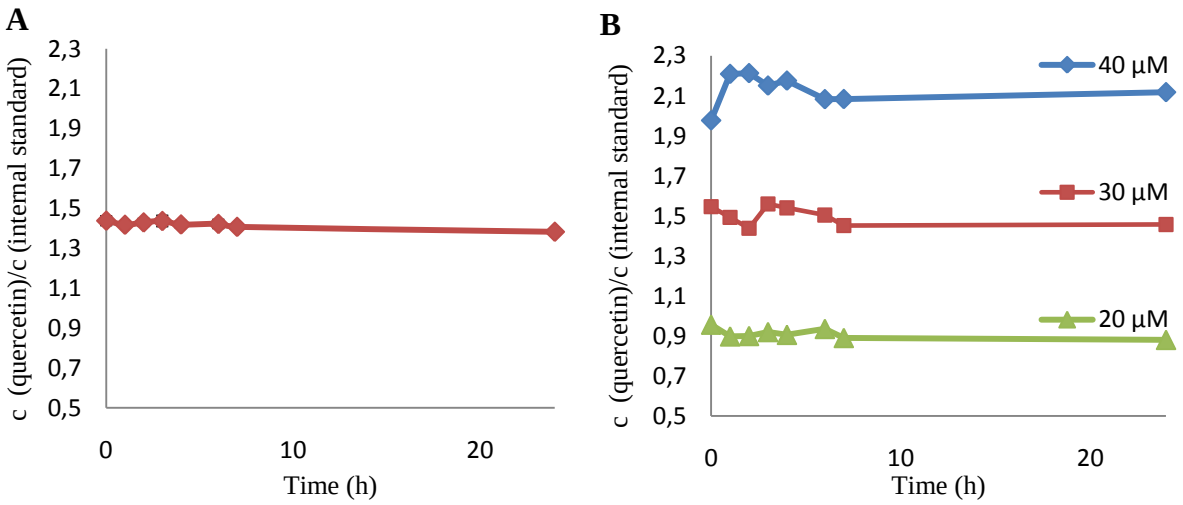


Figure 20. Adsorption of quercetin (Q) into MIPs dissolved in 90 % methanol and 10 % water. (**A**) Adsorption of 66 µM quercetin into 2-VP MIPs, n = 3, error bars correspond to standard deviation and IS stand for internal standard. (**B**) Adsorption of 3 different concentration of quercetin into 4-VP MIPs, n = 1

Since 2-VP and 4-VP MIPs used so far showed worse adsorption ability than NIPs with MAA as a functional monomer, following test were done to evaluate if water present in solution improves adsorption ability of MAA MIPs and whether high concentrations of quercetin really absorb better than lower concentrations. Fig. 21 shows difference between concentrations of quercetin at the beginning of a reaction and after 24 hours when the equilibrium is assumed to be reached and small trend can be noticed that higher concentrations of quercetin show better adsorption ability than low concentrations as well as water present improves the adsorption ability. However we cannot be sure in these conclusions since only one replicate was done.

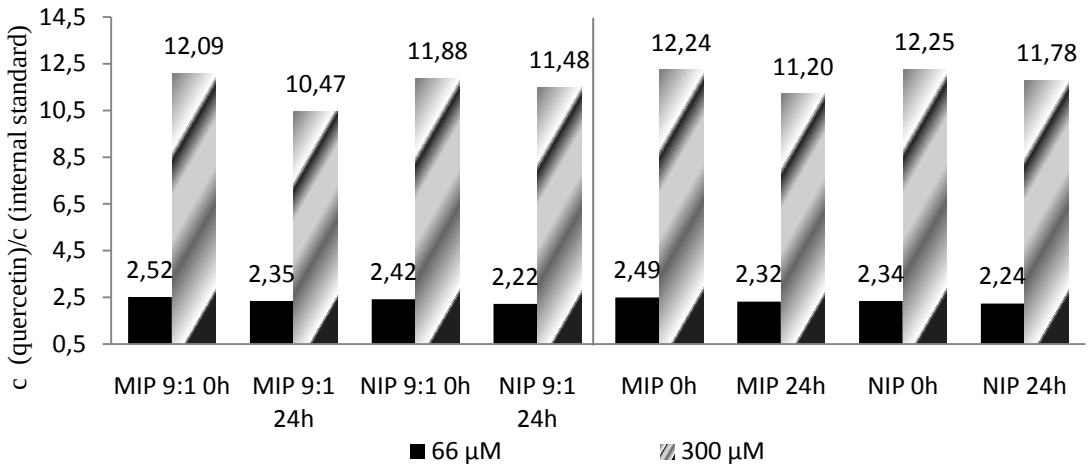


Figure 21. Adsorption of 66/300 µM quercetin into the MIPs and NIPs with MAA used as a monomer in presence of 10 % water (left side of the graph) and without presence of water in solvent (right side of the graph), n = 1.

Next experiment was done to investigate spotted trend from previous test during 24 hours. Amount of water present during reaction was increased to 30 % and compared to adsorption plot of quercetin in presence of 10 % water. Concentration of 300 µM quercetin and 50 mg of MAA MIPs was used. Fig. 22 shows that increased amount of water does not provide higher adsorption capacity, however there are some differences in first 5 hours that cannot be explained.

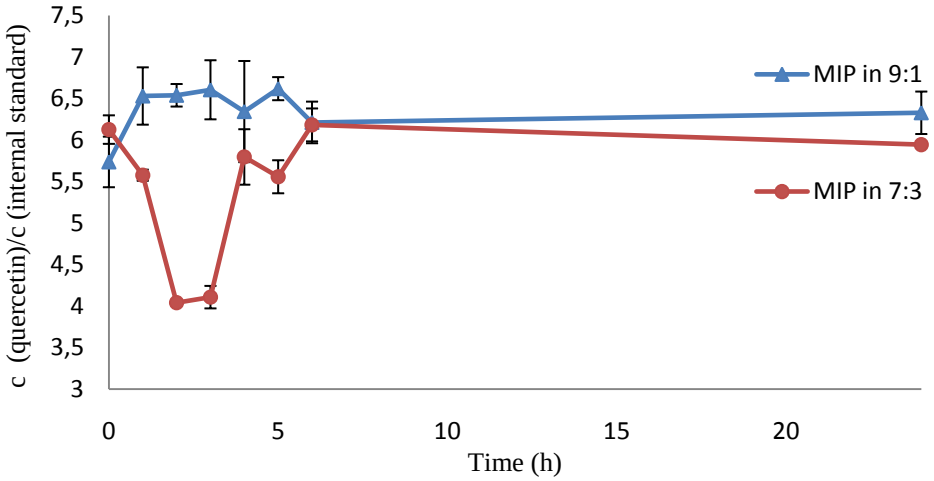


Figure 22. Adsorption of 300 µM quercetin (Q) into MAA MIPs in presence of 10 % of water (9:1) and 30 % of water (7:3), n = 2, error bars correspond to standard deviation and IS stand for internal standard.

In the last experiment two different scales of the volume of reaction, in which the adsorption of quercetin was tested, were compared to each other. The larger scale refers to 25 mL of solvent containing 50 mg of 4-VP MIPs, 200 μM quercetin and 100 μM internal standard. The smaller scale refers to 4 mL of solvent containing 8 mg of 4-VP MIPs, 200 μM quercetin and 100 μM internal standard. In case of smaller scale samples were taken every time from different 4 mL vial to prevent decrease of volume of reaction. From Fig. 23 it can be observed that the decrease of the volume of reaction, caused by taking samples each time from the same Erlenmeyer flask, does not effect much the adsorption of quercetin into MIPs, however this conclusion was tested only for 4-VP MIPs and for other MIPs and NIPs it might not be correct statement.

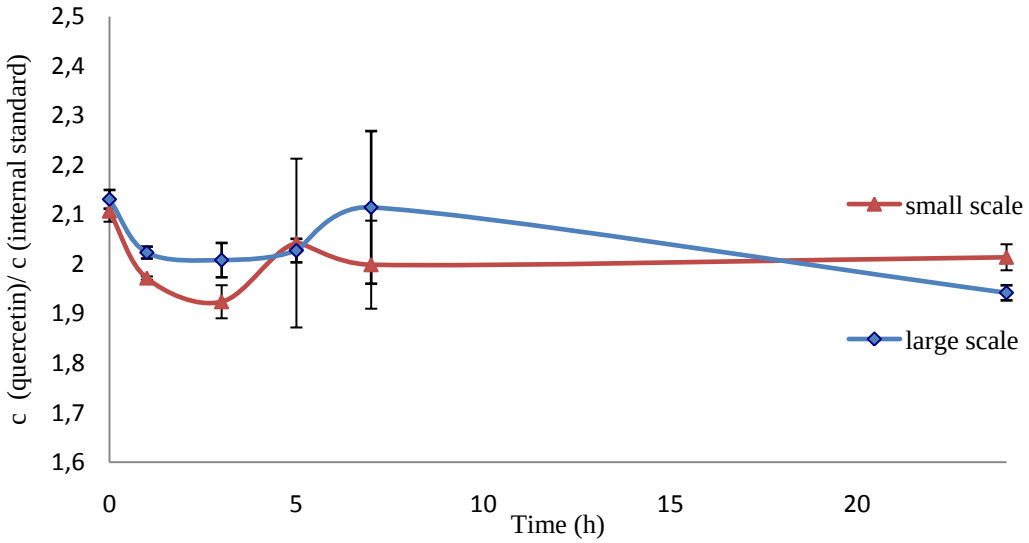


Figure 23. Comparison between adsorption of 200 μM quercetin into 4-VP MIPs in smaller (4 mL of solution containing 8 mg of MIP) and larger (25 mL of solution containing 50 mg of MIPs) scale of the volume of reaction, n = 3 and error bars correspond to standard deviation.

5. Conclusions and Future aspects

Adsorption of different concentrations of quercetin into MIPs and NIPs, prepared in presence of quercetin as a template molecule, was investigated in solvent methanol as well as in methanol with 10 % or 30 % of water. Used MIPs and NIPs were prepared with three different monomers such as 2-VP, 4-VP and MAA.

According to all results none of these MIPs and NIPs provided sufficient adsorption of target molecule quercetin. Although most MIPs and NIP provided some adsorption in presence of high concentrations of quercetin, it was still not significant for any useful purposes.

It seems that in some experiments concentration of quercetin decreased quickly at first three hours of reaction and then increased back. The concentration of quercetin might increase back due to long ongoing stirring, which might force the molecules of quercetin leave the cavities in polymer structure.

In other experiments the reference concentration of quercetin seemed to be rather low compared to the following samples. This phenomenon could be caused by insufficient dissolution of quercetin during sampling of reference concentration. When the reactional Erlenmeyer flask is placed on the stirrer, the quercetin is dissolved completely and so the concentration of subsequent samples seemed to be higher than the sample of reference concentration of quercetin. This problem should be easily solved in future by placing the 25 mL volumetric flask containing the quercetin into the sonication bath before taking the reference sample. However, this phenomenon could be caused by bleeding of MIPs as well.

The easiest explanation of the low adsorption ability of used MIPs and NIPs is that the methanol either with or without water present in reaction is not a good solvent for evaluation of the adsorption of quercetin into used MIPs and NIPs. The quercetin maybe prefers to stay in methanol rather than bind into the cavities in polymer structure. So other solvents for example acetone, THF or ACN should be used to investigate the binding capacities of MIPs and NIPs in the future.

6. References

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