Journal of Chromatography A, 1230 (2012) 15–23

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Molecularly imprinted polymers targeting quercetin in high-temperature aqueous solutions

Vusumzi Pakade[a], Sofia Lindahl[b], Luke Chimuka[a], Charlotta Turner[b,*]

[a] School of Chemistry, University of the Witwatersrand, P/Bag 3, WITS 2050, Johannesburg, South Africa
[b] Department of Chemistry, Centre for Analysis and Synthesis, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

ARTICLE INFO

Article history:
Received 16 August 2011
Received in revised form 14 January 2012
Accepted 19 January 2012
Available online 25 January 2012

Keywords:
Extraction
High temperature
Molecular imprinting
Quercetin
Water
Yellow onion

ABSTRACT

Molecularly imprinted polymers (MIPs) targeting quercetin were prepared from 4-vinylpyridine and ethylene dimethacrylate (EDMA) under various solvent systems with the aim to form MIPs with high recognition for the quercetin molecule in aqueous systems at high temperature. A MIP prepared from the three-component solvent mixture of THF/H_2O/MeOH showed potential in its application for the determination of quercetin in plants (onion). The polymer particles before and after washing were characterized by infrared spectroscopy and thermogravimetric analysis. Surface morphology was recorded by scanning electron microscopy. The binding capacity of the MIPs was investigated at 25 and 84 °C, respectively, in batch mode. Parameters, including the influence of pH, extraction time and binding capacity, were evaluated. The slopes for the effect of extraction time revealed that the mass transfer of the analytes was higher at 84 °C than at 25 °C. Also, the binding capacity for the most promising MIP and its corresponding NIP was higher at 84 °C. The binding capacity for the MIP was ~30 μmol g^{-1} at 25 °C and ~120 μmol g^{-1} at 84 °C, while for the corresponding NIP, it was ~15 and ~90 μmol g^{-1}, at 25 and 84 °C, respectively. A demonstration of MIP selectivity at higher temperature using standard solutions of selected flavonols showed that the MIP still retained its selectivity for quercetin. Similar selectivity was observed when preliminary application studies on aqueous yellow onion extracts were investigated.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

The technique of molecular imprinting has, over the years, gained a lot of applications in biology and in chemistry, and it continues to attract interest in various fields. These include, but are not limited to, biosensors [1], antibody simulation [2], chiral resolution [3], enzyme catalysis simulation [4], and biochemical separation [5]. Molecular imprinting technique mimics natural molecular recognition [6–8], in that, macromolecules possessing high affinity and selectivity for the imprint/target compounds are produced through formation of predefined interaction between the template molecule and the ligand system. The most widely used technique for preparing molecularly imprinted polymers (MIPs) is the non-covalent imprinting approach, which is based on physical interaction of the template molecule and the functional monomer through, for example, hydrogen bonding, hydrophobic, electrostatic bonding and/or dispersion interactions [9,10]. The less usage of the covalent imprinting approach is due to the bottleneck of polymerizable functional units that possess reversible covalent bonds [11]. Mostly, MIPs are used as sorbents for the solid-phase extraction (MISPE) technique, as this offers both pre-concentration and removal of interferences [12]. However, Nemulenzi et al. [13] also demonstrated the potential of combining MIPs with liquid membrane for the extraction of β-estradiol from aqueous samples.

Although MIPs offer several advantages (high thermal stability and mechanical strength) over other polymeric materials there has been a general concern on the applicability of MIPs in polar solvents such as water [14]. In the non-covalent imprinting approach, the presence of polar solvents such as water disrupts the interaction of the template and the monomer resulting in polymers with poor level of recognition [15]. Several studies about preparation of MIPs in aqueous environments have been reported [16–23]. The successful imprinting in these studies was achieved by using hydrophilic monomers [18,20], a two-step extraction method [21], and surface imprinting [19] and it has also been noted that the template molecule can offer specific solutions [22]. Templates that can form ionic interactions are much better to be extracted by MIPs in aqueous media than those possessing hydrogen bonding. Recently, Shen and Ye [23] reported a new technique for producing water-compatible MIPs for propranolol using Pickering emulsion

* Corresponding author. Tel.: +46 46 222 8125; fax: +46 46 222 8209.
E-mail address: charlotta.turner@organic.lu.se (C. Turner).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2012.01.051

polymerization in which a combination of hydrophobic and electrostatic interactions was the mode of recognition. However, in the above examples the MIPs were not used at high temperature.

In this study, the aim was to prepare quercetin MIPs in aqueous environments for selective recovery of quercetin from aqueous yellow onion extracts at high temperature. To our knowledge, this is the first time MIPs have been prepared in aqueous medium and used at higher temperature. The selectivity of the prepared MIPs was investigated in adsorption studies with structurally related compounds, and evaluated by high performance liquid chromatography (HPLC).

However, MIPs are not immune to drawbacks as the issue of batch-to-batch reproducibility in surface area and particle size distribution is yet to be satisfactorily resolved [24]. Also, the post-treatment of crushing and grinding has a potential of increasing non-specific binding due to the possibility of destroying the binding active sites thereby reducing batch-to-batch reproducibility. Although there have been substantial efforts in using other polymerization techniques such as emulsion polymerization [25] and precipitation polymerization [26] instead of bulk polymerization, bulk polymerization is still being widely used owing to its simplicity, robustness, compatibility with a variety of templates and because it requires limited organic chemistry [27–29]. Hence, in this study, bulk polymerization was used.

Quercetin, the most active antioxidant of the flavonol family, is present in vegetables and fruits, such as onions, apples and grapes in low amounts [30]. Besides antioxidant properties, quercetin possesses antitumor and antiviral properties as well as aiding in adjusting the immune system [31]. In fruits and vegetables, quercetin is present as glycosides, where glucose and rhamnose are the two most common sugar groups [32]. In yellow onion, quercetin-3,4′-diglucoside and quercetin-4′-glucoside are the main abundant glucosides [32]. Nonetheless, the aglycone and other quercetin glucosides are also present but in lower amounts. Quercetin species can be extracted from onion using solid–liquid extraction with aqueous methanol, which normally involves the use of high concentrations of HCl to catalyze the hydrolysis of glucosides to aglycones [33,34]. However, a more sustainable alternative method to methanol–HCl extraction/hydrolysis has been proposed and it involves the use of pressurized hot water as extraction solvent and enzyme-catalyzed hydrolysis [35,36]. Chromatographic methods have been widely used for the isolation of quercetin from plants employing a variety of solvent combinations [37–39]. However, MIPs offer much improved selectivity for a particular solute group than the mentioned chromatographic methods [40–42].

2. Materials and methods

2.1. Chemicals

Quercetin, kaempferol, disodium hydrogen phosphate, triethylamine and 1,1′-azobis(cyclohexanecarbonitrile) (ACCN) were purchased from Sigma–Aldrich (Steinheim, Germany). Quercetin-3,4′-diglucoside and quercetin-4′-glucoside were purchased from Polyphenols Laboratory AB (Sandnes, Norway) and morin was purchased from Extrasynthese (Genay, France). Methanol (Gradient grade) was purchased from B&J Brand (Honeywell, Germany), formic acid and citric acid monohydrate were purchased from Merck (Darmstadt, Germany). 4-Vinylpyridine (4-VP) and ethylene dimethacrylate (EDMA) were purchased from Acros Organics (Geel, Belgium). A stock solution of quercetin (0.3 g L^{-1}) in methanol and 0.5% formic acid was prepared in the laboratory and kept at −18 °C when not in use. Ultrapure water (MilliQ) was used in all experiments and chemicals were used as received. The pH was adjusted with citric acid monohydrate (0.1 M) and disodium hydrogen phosphate (0.2 M). The structurally related compounds tested are given in Fig. 1.

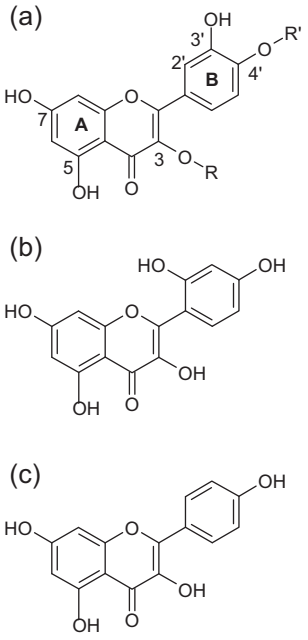

Fig. 1. Chemical structures of (a) quercetin (R, R′ = H), quercetin-4′-glucoside (R = H, R′ = glucose), quercetin-3,4′-diglucoside (R, R′ = glucose), (b) morin and (c) kaempferol.

2.2. Monomer–template interaction as studied by FTIR and UV/vis

Quercetin and 4-VP were stirred in THF/H_2O/MeOH (6:3:1, v/v/v) for 2 h at room temperature. Thereafter, this complex solution was passed through a solid-phase extraction (SPE) cartridge containing anhydrous $MgSO_4$ for drying out water, and then the eluate was bubbled with nitrogen to remove the organic solvent. The remains of this were then placed in the FTIR on a diamond plate using a liquid dispenser to acquire the spectrum. Fourier transform infrared spectroscopy (FTIR) spectra were recorded in the frequency range of 4000–400 cm^{-1} using a Bruker Alpha FTIR spectrometer (Ettlingen, Germany) with Attenuated Total Reflectance (ATR) solid state method with 24 scans. UV–vis absorption spectra were obtained using a Varian Cary 1E double beam spectrophotometer (Palo Alto, CA, USA) scanning from 190 to 600 nm.

2.3. MIP preparation

The quercetin imprinted polymers were prepared according to published procedures but with slight modifications [41]. The amounts of reagents used for each polymer are summarized in Table 1. In Table 1, M1 and M2 refer to MIPs, and N1 and N2 refer to corresponding NIPs for the MIPs, respectively. M3 and M4 are MIPs that were used as references in order to see how MIPs prepared in pure MeOH and THF perform in the recognition of quercetin compared to those prepared in aqueous medium, M1 and M2. In brief, for M2 and N2, a mixture of quercetin (0.4 mmol) and 4-VP (4 mmol) was stirred at room temperature in a 50 mL round bottomed flask containing 10 mL THF/H_2O/MeOH (6:3:1, v/v/v) porogenic mixture for 30 min to establish monomer–template interactions. After which, the polymerization reaction vessels were placed on ice to prevent unwanted polymerization. EDMA (18.5 mmol) and ACCN initiator (100 mg) were added. The solution mixture was purged with nitrogen for 10 min to remove dissolved oxygen, sealed and stirred in an oil bath at 60 °C (12 h) to initiate polymerization. After

Table 1
Composition of MIPs and NIPs.

Polymer	Quercetin (mmol)	4-VP (mmol)	EGDMA (mmol)	ACCN (mg)	Solvent	Solvent volume (mL)
M1	1.0	10.0	40.0	100	MeOH/H_2O (9:1, v/v)	10
N1	–	10.0	40.0	100	MeOH/H_2O (9:1, v/v)	10
M2	0.4	4.0	18.5	100	THF/H_2O/MeOH (6:3:1, v/v/v)	10
N2	–	4.0	18.5	100	THF/H_2O/MeOH (6:3:1, v/v/v)	10
M3	0.4	4.0	18.5	50	MeOH	5
M4	0.4	4.0	18.5	50	THF	5

12 h of polymerization, the temperature was increased to 80 °C for 3 h to achieve a solid monolith polymer. After polymerization, the polymers were crushed, ground and sieved through 88 μm sieves and finer particles were removed by sedimentation over acetone, decanting the solution in hourly intervals for 3 cycles. The resulting polymer particles were dried in an oven at 60 °C overnight, thereafter, the template was washed off by Soxhlet extraction for 24 h with MeOH/acetic acid (9:1, v/v). Following which, the particles were in addition washed with MeOH, MeOH/triethylamine (9:1, v/v) and MeOH in that order, and finally the polymers were dried in an oven at 55 °C overnight to give the final MIPs. Non-imprinted polymer (NIP) was prepared and washed in the same manner but quercetin was omitted. MIPs and NIPs prepared in other porogens used a similar preparation and washing procedure, and they were also subjected to the same treatment process in terms of template removal.

2.4. Characterization of MIPs and NIPs

Surface morphological information of MIP and NIP was obtained using a scanning electron microscope (SEM) JOEL Model JSM 6700F (Tokyo, Japan). Brunauer–Emmett–Teller (BET) instrument (MicromeriticsTristar) was used for the surface area determinations. Thermal gravimetric analysis (TGA) was performed using a TA Instruments Q500 TGA in high-resolution dynamic heating mode (New Castle, US) at a heating rate of 10 °C min^{-1} up to 500 °C under air atmosphere at 60 mL min^{-1}.

2.5. Determination of binding capacity

Several experiments were conducted at 25 °C and 84 °C in order to evaluate the binding capacity of the MIPs and NIPs. These experiments include adsorption as a function of time and concentration of quercetin, effect of pH, effect of initial temperature, and the selectivity of MIP for quercetin from structurally related compounds. All experiments were conducted in triplicates unless otherwise stated. Experiments on adsorption as a function of quercetin concentration for the MIP and NIP were investigated in static adsorption mode where 8 mg polymer particles were stirred in 4 mL MeOH/H_2O (7:3, v/v) solutions containing five different concentrations of quercetin at room temperature or 84 °C for pre-determined time intervals. Adsorption rate studies were conducted at pH 5.5 both at 25 °C and 84 °C and samples were drawn at 0.5; 1.0; 2.0; 4.0; 6.0; 10.0 h intervals. For experiments conducted at 25 °C, 4 mL of 218 μmol L^{-1} quercetin solution and 8 mg of polymer were used and for experiments conducted at 84 °C, 4 mL of 1.0 mmol L^{-1} quercetin solution and 4 mg of polymers were used. The influence of pH was studied at pH 4, 5.5 and 7, although the desired pH in a combined extraction/biocatalysis/MIP-based clean-up targeting quercetin in onion is pH 5.5, because this is the pH at which the enzyme hydrolysis of quercetin glucosides is performed. For the influence of pH, 8 mg of MIPs was stirred in 4 mL MeOH/H_2O (7:3, v/v) mixture containing quercetin (initial concentration 66 μmol L^{-1}) at 25 °C for 24 h. In all experiments, after the reaction time, samples were withdrawn from the reaction vials at suitable time intervals and placed in 1.5-mL centrifuge tubes and the solid material was spin down in a bench-top centrifuge. The supernatant was transferred into an HPLC vial containing MilliQ water with 0.5% formic acid for the determination of un-extracted concentration of quercetin. The quercetin concentration was measured as described in Section 2.6.

For investigations on binding as a function of quercetin concentration, initial concentrations in the range of 30–400 μmol L^{-1} were used. The experiments were conducted at 25 °C and stirred for 24 h whereas experiments at 84 °C were stirred only for 2 h. The amount adsorbed (Q, μmol g^{-1}) was calculated from Eq. (1):

$$Q = (C_0 - C_e)\frac{V}{W} \quad (1)$$

where C_0 and C_e (μmol mL^{-1}) are the initial and final concentrations respectively, V (mL) is the volume of the solution used for the extraction and W (g) is the mass of the polymer used for extraction.

2.6. HPLC analysis

HPLC–UV analysis was performed using a chromatographic system, UltiMate-3000® from Thermo Fisher (former Dionex, Germering, Germany). An Agilent Zorbax SB-C_{18} column (100 mm × 2.1 mm, 3.5 μm) with an Agilent Zorbax SB-C_8 pre-column (12.5 mm × 2.1 mm, 5 μm) was used for isocratic separation with a methanol/water (50:50, v/v) mobile phase containing formic acid (0.13 M), at a flow rate of 0.15 mL min^{-1}. The injection volume was 5 μL and detection was accomplished at 350 and 370 nm, respectively. Quantification of quercetin and all other compounds tested was performed using a five-point calibration curve of standards at concentrations between 1 and 75 μmol L^{-1}. Each vial taken to analysis had a total volume of 1.00 mL.

2.7. Selectivity studies

In order to examine quercetin selectivity by M2 and N2, a solution containing morin, quercetin, quercetin-4′-glucoside, quercetin-3,4′-diglucoside and kaempferol was used. The concentration of each compound was 66 μmol L^{-1} prepared in MeOH/H_2O (7:3, v/v). The pH of the solution was adjusted to 5.5 with a citric acid-phosphate buffer. Experiment was done in batch mode at 25 °C and 84 °C in triplicates. In short, 8 mg of the polymers were stirred in reaction vials containing 4 mL of the solution for 2 h (at 84 °C) and 6 h (at 25 °C), and non-adsorbed concentration of each substance was measured as previously described (see Section 2.6) after the binding reached equilibrium.

The distribution constant (k_d, mL g^{-1}) for each substance was calculated using Eq. (2):

$$k_d = \frac{Q}{C_e} \quad (2)$$

where Q (μmol g^{-1}) and C_e (μmol L^{-1}) are as described previously.

The selectivity coefficient (k) of MIP for quercetin with respect to the competitor species (quercetin-4′-glucoside, quercetin-3,

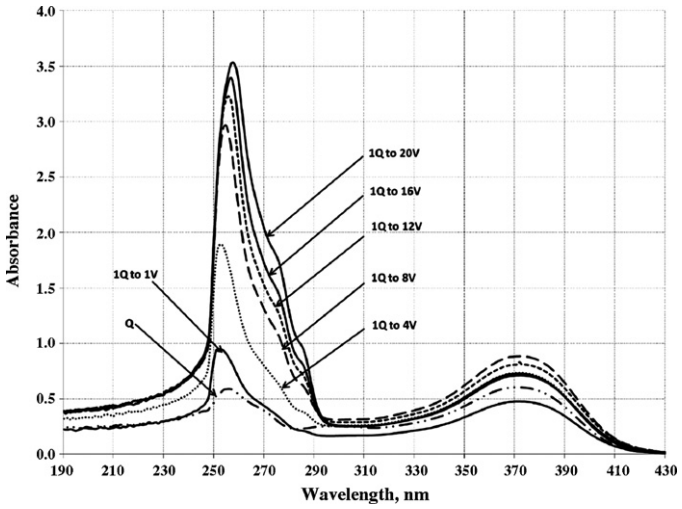

Fig. 2. The UV/vis spectra of different ratios of quercetin:4-VP (Q:V) in THF/H_2O/MeOH (6:3:1, v/v/v), three times scan.

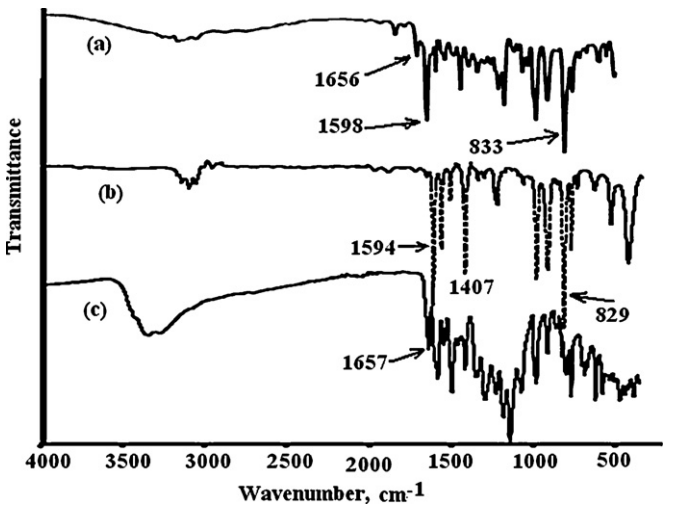

Fig. 3. FTIR spectra of quercetin-4–vinylpyridine complexes (a), 4-vinylpyridine (b) and quercetin (c).

4′-diglucoside, kaempferol or morin), referred to as B in Eq. (3) was calculated from the distribution constant as indicated in Eq. (3):

$$k = \frac{k_{d,(quercetin)}}{k_{d,B}} \quad (3)$$

The value of k gives an indication on the recognition ability and selectiveness of the MIP for quercetin with respect to other similar compounds. Similarly, a relative selectivity coefficient k' can be calculated as illustrated in Eq. (4), where the value of k' shows the imprinting effect on binding affinity and selectivity of MIP for quercetin over NIP [43].

$$k' = \frac{k_{MIP}}{k_{NIP}} \quad (4)$$

2.8. Application to aqueous yellow onion extract

The potential of future application of the prepared MIPs was tested on aqueous yellow onion extracts in batch mode. Yellow onion extract was prepared using pressurized hot water extraction as described by Turner et al. [35]. The pH of the aqueous onion extract solution was adjusted to pH 5.5 with the citric acid/phosphate buffer. In triplicates, 4 mL of pH 5.5 buffered yellow onion extract solution were extracted with 8 mg MIPs at 25 and 84 °C for 24 and 2 h, respectively. Concentration of quercetin before and after application of MIPs was quantified as described in Section 2.6. MIPs were first washed with 2 mL of MeOH in two portions of 1 mL and finally washed with 2 mL of MeOH/acetic acid (9:1, v/v) in one portion by stirring the MIPs at room temperature for 20 min. To calculate the recovery, quercetin in the methanol extracts (washing solution) and in the methanol/acetic acid extracts were combined.

3. Results and discussion

3.1. UV/vis studies of monomer–template interactions

The effect of the solvent on the monomer–template strength was investigated by stirring the following ratios: 1:1, 1:4, 1:8, 1:12, 1:16 and 1:20 of quercetin-to-4-VP (Q:V) in THF/H_2O/MeOH (6:3:1, v/v/v) for 2 h at room temperature. The resulting solution mixtures were scanned using the UV/vis spectrophotometer from 190 to 600 nm and the results are depicted in Fig. 2. It can be observed that quercetin absorbs light at two different wavelengths, i.e. at ~250 and 370 nm, where the band at 250 nm is attributed to the benzoyl chromophore and the band at 370 nm is assigned to the cinnamoyl chromophore of the quercetin molecule [44]. The benzoyl and cinnamoyl chromophores are the rings labeled A and B in the quercetin molecule in Fig. 1. There is no significant shift of the wavelength for any of the quercetin:4-VP (Q:V) ratios at 370 nm, except that the ratio of 1:8 and 1:12 (Q:V) have the highest absorbencies in that order. However, there is a noticeable change for the bands at ~250 nm wavelength. With the increase in the ratio of 4-VP, lambda max (λ_{max}) shifts to the longer wavelength gradually. This shift can be said to obey Einstein shift phenomenon, which is defined as a shift toward longer wavelengths of spectral lines emitted by atoms in strong gravitational field resulting in molecules with less energy and lower frequency. In this case the Einstein shifts are a result of the formation of hydrogen bonding and π–π interactions between the template molecule quercetin and the 4-VP resulting in the formation of a self-assembled complex of quercetin:4-VP.

Based on these observations, it can be said that strong monomer–template for the pre-polymerization complex can be achieved under the porogen conditions used. A 1:10 (quercetin:4-VP) ratio which is a compromise for the benzoyl and cinnamoyl chromophore intensity and shifts was regarded as the optimum ratio, hence, this ratio was used for polymer fabrication (Table 1). This is slightly different from the 1:8 ratio previously used by Molinelli et al. [41] for the preparation of quercetin MIPs with 4-VP in acetone. Excess of monomer is needed to create strong interactions between monomer and template but one has to be careful as this can lead to the creation of single-point attachments, which then lead to non-specific binding. Therefore, a compromise was made in order to form the desired multipoint interaction, i.e. a 1:10 ratio was chosen. Similar results in which an excess of monomers was used was reported by Sun and Qiao [17] in their study on methacrylic acid:ofloxacin imprinted polymers under MeOH/water (9:1, v/v) conditions. The optimum ratio of ofloxacin-to-methacrylic acid was 1:10 [17].

3.2. FTIR studies of monomer–template interaction

The spectra for quercetin:4-VP complex, 4-VP, and quercetin are shown in Fig. 3. Vibrational bands corresponding to both quercetin and 4-VP are present in Fig. 3(a) (although the frequency was shifted from 1594 to 1598 cm^{-1}, and from 829 to 833 cm^{-1}) implying their involvement in complex formation. No real shift of the quercetin C=O band (1657–1656 cm^{-1}) was observed because this functional group is not involved in hydrogen bonding with 4-VP. Furthermore, quercetin hydroxyl characteristic

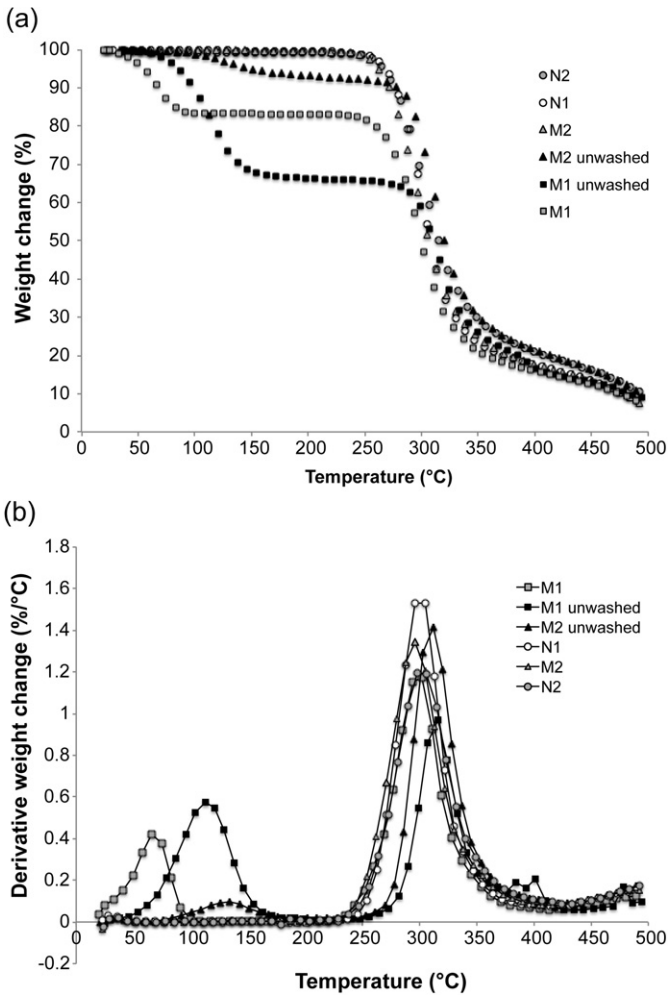

Fig. 4. TGA curves of M1 (washed and unwashed), M2 (washed and unwashed), N1 and N2. (a) The weight change in % as function of temperature (°C), and (b) the derivative of the weight change as function of temperature (°C).

bands C—OH stretch bend at 1160–1030 cm^{-1}, C—OH in-plane bend at 1440–1260 cm^{-1} as well as C—OH wag at 700–600 cm^{-1} (Fig. 3(b)) have also disappeared. Also, the hydroxyl broad band of quercetin (Fig. 3(c)) is slightly flattened out on the complex (Fig. 3(a)). Both these changes are attributed to the involvement of quercetin to complexation with 4-VP molecules. Furthermore, complexation might result in molecules of lower energy due to limited movement of the resulting bonds, which is supported by the redshifts observed in UV–vis spectra.

3.3. Characterization of MIPs and NIPs

The FTIR spectra of unwashed MIPs and washed MIPs and NIPs showed similar backbone structure indicative of the high degree of cross-linking (EDMA) agent used (data not shown), characterized by strong vibration bands at 1720 and 1140 cm^{-1} attributed to the C=O and C—O functional groups of EDMA, respectively. All polymers exhibited an almost diminished vibration band at 1736 cm^{-1}, indicating that almost all the monomer 4-VP was polymerized as this peak is normally attributed to unpolymerized double bonds of the monomer [45]. The presence of quercetin in unwashed MIPs was characterized by a broad band at around 3300 cm^{-1}, whose intensity diminished in washed MIPs.

TGA plots of M1 (washed and unwashed), M2 (washed and unwashed), N1 and N2 are presented in Fig. 4. It can be observed

Table 2
Pore size diameter and surface area summary results for the studied polymers ($n = 1$).

Polymer	Average pore diameter (nm)	Surface area ($m^2\ g^{-1}$)
M1	10.2	222
N1	19.7	71
M2	6.7	334
N2	5.3	69
M3	17.6	6
M4	12.9	2

that there are differences in decomposition patterns between washed and unwashed MIPs, and between MIPs and NIPs. There are two decomposition steps for washed and unwashed M1 and unwashed M2, but in the case of N1, N2 and washed M2, there seem to be only one decomposition step. However, the experiment was only allowed to run up to 500 °C and at this temperature the polymers have not completely decomposed. For washed M1, almost 15% weight loss is seen up to 90–100 °C and for unwashed M1 there is almost 30% weight loss up to 150 °C. For unwashed M2 the first weight loss up to 150 °C is approximately 10%. Weight losses at temperatures below 150 °C are normally attributed to water loss and decomposition of free monomer, cross-linker and monomer–template complex [46–48]. Larger weight loss for M1 suggests that more of free monomer and cross-linker were remaining in this polymer. Most importantly, all the polymers appear to be rigid up to 250 °C, and beyond that temperature they started to disintegrate.

SEM analysis was performed to observe the morphology of the particles. Although no pores were observed under the detector resolution used in SEM, however, the particles exhibited irregular shape due to crushing and the particle size distribution look similar (images not shown). Further, it was proved by BET that M2 particles exhibited higher surface area (334 $m^2\ g^{-1}$) than N2 (69 $m^2\ g^{-1}$) as well as higher pore size diameter. Results are summarized in Table 2.

3.4. Polymerization of quercetin water-compatible MIPs

MIPs were prepared in two different aqueous environments, that is, using MeOH/H_2O (9:1, v/v) (M1) and THF/H_2O/MeOH (6:3:1, v/v/v) (M2) as porogens, respectively. As references, two other polymers were made in pure methanol and pure THF solvents (M3 and M4, respectively, see Table 1). It is well known that MIPs perform better if the same solvent for preparation is used for re-binding [49]. Therefore, solvent for re-binding experiments containing maximum amount of water was investigated, as this would mimic the aqueous environments of the extracts for the ultimate application. MIPs and NIPs were stirred in different proportions of methanol/water mixtures (9:1, 7:3 and 5:5) and adsorption of quercetin was investigated (Fig. 5). Control samples were also included, labeled “Stds”, which refers to quercetin standard solutions used in each adsorption experiment, but without the addition of MIPs/NIPs. Maximum re-binding solvent was found to be methanol/water (7:3) for M2, hence this was used for all subsequent experiments. Monomer–template binding strength can be affected by the relative permittivity of the solvent as well as other solvent parameters, which could result in MIPs with poor recognition capabilities [49].

In the case of M2, the porogenic solvent and the re-binding solution have the same water content. Quercetin possesses phenolic hydroxyl groups that behave as weak acids, as such strong hydrogen-bonding interaction can be established with the vinylpyridine. Therefore, these interactions can be said to be stronger in the case of M2 compared to other polymers under the tested conditions when the water content was 30% (Fig. 5). Chen et al. [50] reported hydrophobic interactions as a mode of

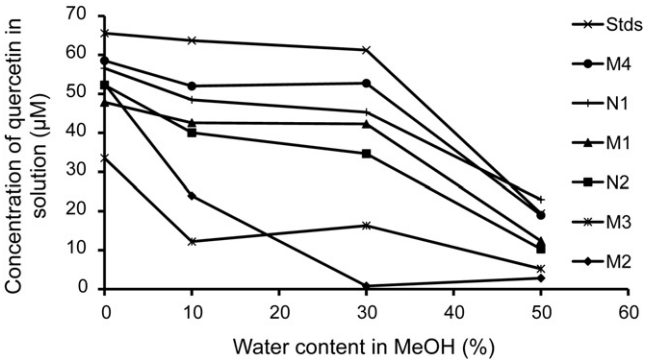

Fig. 5. Effect of water content on quercetin removal by MIPs and NIPs. Amount polymer 8 mg, solution volume 4 mL, contact time 24 h, temperature 25 °C, initial concentration 66 μM, pH not controlled (MeOH/H_2O), $n = 2$. See Table 1 for explanations on MIPs and NIPs.

recognition when the water content was greater than 65%. When water content was lower than 10%, hydrogen bonding was predicted as the main mode of recognition [17,50].

It has been previously demonstrated that a medium polar aprotic solvent such as THF gives better imprinting factor for quercetin MIPs involving hydrogen bonding [42]. However, the authors reported lower imprinting factor when more polar solvents than THF were used. In our studies we increased the polarity by adding water and methanol to THF to form a ternary solvent mixture with a ratio of 6:3:1 (THF/H_2O/MeOH, the porogen used to make M2 and N2) due to the miscibility of the three. MeOH is expected to impart the porogenic effect while higher volumes of THF will balance the disturbing effect of water while providing higher chances of hydrogen bond formation.

3.5. The effect of pH

The performance of the prepared MIPs at three different pHs (pH 4, 5.5 and 7) was investigated (Fig. 6). The aim here was to see if the binding capacity was better or worse at pH 5.5, which is the optimum pH for enzyme-catalyzed hydrolysis of quercetin glucosides in onion extract.

Obviously, the pH plays a crucial role in the affinity between quercetin and the MIP. This is the same pH effect that is used to washout quercetin template molecules from the MIP, i.e. an acidified methanol solution is commonly used. A low pH is believed to charge the amino groups of the MIP resulting in the release of quercetin molecules to the solution. In re-binding, one has to avoid low pH as it is shown that both MIP and NIP exhibited lower binding capacities at pH 4, see Fig. 6. M2 seems to work well at the desired

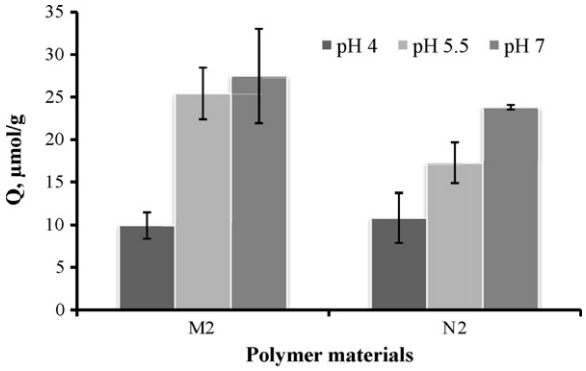

Fig. 6. Effect of pH on binding capacity. Initial concentration of quercetin 66 μmol L^{-1}, amount of polymer 8 mg, solution volume 4 mL, contact time 90 min, temperature 25 °C.

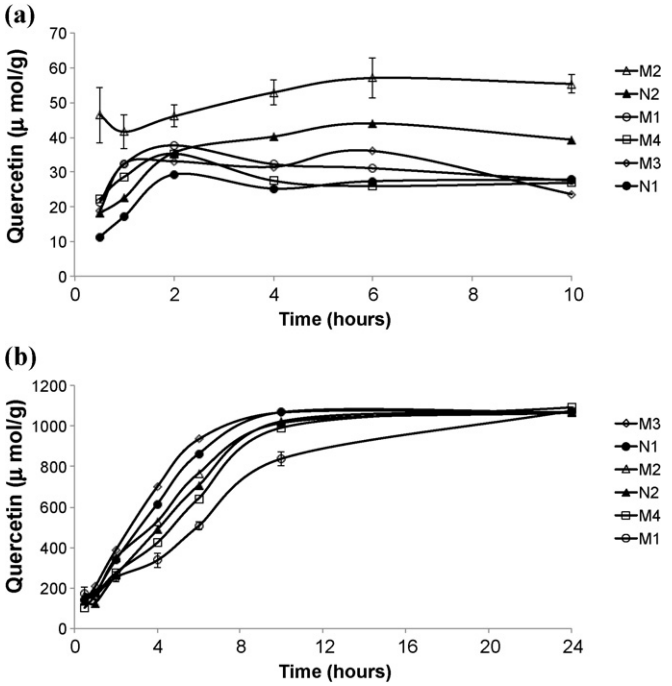

Fig. 7. Effect of contact time on binding capacity of polymers at 25 °C (a) and 84 °C (b). pH was 5.5 and solvent was MeOH/H_2O, 7:3 (v/v). Error bars showing standard deviation are only displayed for one of the curves in each figure, due to limited space. $n = 3$.

pH 5.5 (Fig. 6) and this is a good sign that these MIPs can be used for selective extraction of quercetin from yellow onion extracts.

3.6. Adsorption as a function of time

The influence of contact time on the adsorption of quercetin to the polymers was investigated at 25 °C and 84 °C, and the results of these investigations are depicted in Fig. 7(a) and (b), respectively. For the studies at 25 °C, an initial concentration of 218 μmol L^{-1} was used whereas 1000 μmol L^{-1} initial concentration was used for the studies at 84 °C. All experiments were conducted in triplicates but due to overlap of the adsorption curves, error bars were included only for one of the MIPs in Fig. 7. Notably in this figure is that M2 exhibited a higher binding capacity at about 55 μmol g^{-1} after 6 h closely followed by its corresponding NIP, N2, at 42 μmol g^{-1} after 6 h. All the other polymers were either below or in level with N2. These results reveal that M2 had better imprinting capabilities compared to other polymers. The graphs of the tested MIPs and NIPs in Fig. 7(b) reach a plateau after 24 h, possibly with the exception of M1. This plateau was not reached because the polymers had reached saturation but rather because all the quercetin present in the initial solution was adsorbed to the polymers. Hence, since all quercetin in the solution was adsorbed it is not possible to evaluate if there is any difference in binding capacity between the different MIPs and NIPs. The fastest adsorption at 84 °C (Fig. 7(b)) is seen for M3 and N1, then followed by M2, N2 and M4 and the slowest adsorption was seen for M1. When the slopes of Fig. 7(a) and (b) for the first 5 h are compared, it reveals that the influence of higher temperature (84 °C) leads to faster mass transfer. It is also clear that a higher temperature leads to a larger magnitude of binding capacity (Fig. 7(a)). The effect of higher binding capacity at higher temperature can be explained by the lower viscosity and surface tension of the solvent, enabling improved wetting of the MIPs and NIPs. A higher temperature also leads to lower relative permittivity, i.e. weakened hydrogen bonding, which could also result in higher binding capacity. A follow up detailed study

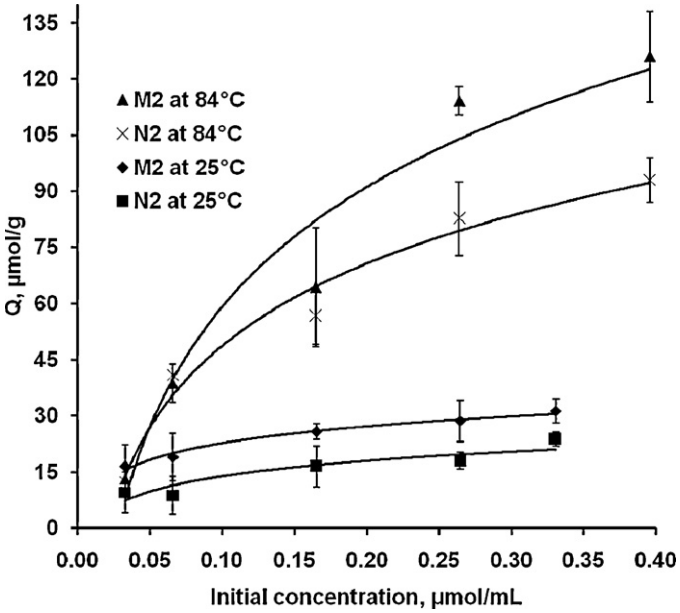

Fig. 8. Effect of initial quercetin concentration on binding capacity of polymers M2 and N2. Amount of polymer 8 mg, solution volume 4 mL, contact time 24 h at 25 °C, or 2 h at 84 °C, pH 5.5, solvent MeOH/H_2O (7:3, v/v).

on the effects of temperature on binding capacity is planned in the nearest future.

3.7. Binding capacity of the MIPs at different temperatures

Binding capacity was evaluated at 25 °C and 84 °C using M2 and N2. The initial concentration was varied between 10 and 400 μM and the results are depicted in Fig. 8. When the experiments were done at room temperature, 25 °C, it can be observed that M2 is approaching steady state at about 270 μmol L^{-1} of about 28 μmol g^{-1} whereas N2 at about the same concentration had a binding capacity of 16 μmol g^{-1}. This difference in binding capacity is attributed to predefined imprinting cavities present in the MIP and absent in the NIP in addition to shape and size, and unspecific binding. Therefore, the MIP (M2) has slightly higher degree of quercetin recognition than its corresponding NIP (N2). However, for the same initial concentration range the binding capacity of both M2 and N2 increased by 4-folds when the experiments were conducted at higher temperature, 84 °C, confirming the results in Fig. 7 as discussed above. To test whether the quercetin was actually adsorbed on the polymer particles or it was just an effect of degradation, reaction vials containing the different initial concentrations of quercetin solution were also subjected to the same temperature for the duration of the experiments. No MIPs/NIPs were added in these solutions. Indeed it showed that there was no degradation involved as concentrations before and after exposure were similar (data not shown). The observed results are in agreement with the theory that MIPs prepared at higher temperature tend to work better at higher temperatures [22,51], and our MIP was prepared at 80 °C due to the use of the ACCN initiator which requires higher temperature to form radicals.

3.8. Selectivity

The selectivity of the MIP, M2, to quercetin amongst some of its structural related compounds, morin and kampferol (Fig. 1(b) and (c)), was evaluated at the selected processing temperature of 84 °C in triplicates. Distribution constant (k_d), selectivity coefficient (k) and the relative selectivity coefficient (k') were calculated as described in Eqs. (2)–(4), respectively. The results are illustrated in Table 3. As shown in Table 3, M2 and N2 have the same binding capacity as demonstrated by the Q and k_d values tabulated. Although the capacity of the MIP is expected to be higher than that of the NIP, this is not demonstrated in Table 3 because all the dissolved quercetin was adsorbed, even though the solution was nearly saturated. Another explanation to the similar binding capacity is that MIPs are less selective in binding target compounds when a polar solvent is used. Instead, the selectivity of the MIPs is demonstrated from washing and elution steps, as described further below for a real sample. However, as discussed above, the imprinting effect of M2 prevailed over N2 when the concentration of the analyte was higher (Fig. 8). Because of the high cost of the other compounds tested for selectivity we have only been able to work with relatively low concentrations as mentioned already.

Table 3
Distribution coefficient (k_d), selectivity coefficient (k) and relative selectivity coefficient (k') of the MIP (M2) and NIP (N2) taken from results conducted at 84 °C.

Polymer	Analyte	k_d (mL g^{-1})	k	k'
M2	Quercetin	14,260	–	–
	Kaempferol	388	37	1.19
	Morin	148	97	1.07
N2	Quercetin	14,260	–	–
	Kaempferol	452	31	–
	Morin	156	91	–

Fig. 9(a) shows a typical chromatogram of the selectivity standard solution mixture before the application of MIP (M2) and Fig. 9(b) is a typical chromatogram obtained after MIP application as described above. It can be seen that the MIP was able to selectively remove all the quercetin from the mixture of compounds containing kaempferol, morin, quercetin-4′-glucoside and quercetin-3,4′-diglucoside. Both quercetin-4′-glucoside and quercetin-3,4′-diglucoside are relatively "bulky" molecules due to attached glucose moieties and hence unable to fit in the quercetin cavities created by imprinting. Therefore, almost none of these were selectively adsorbed, particularly not the diglucoside,

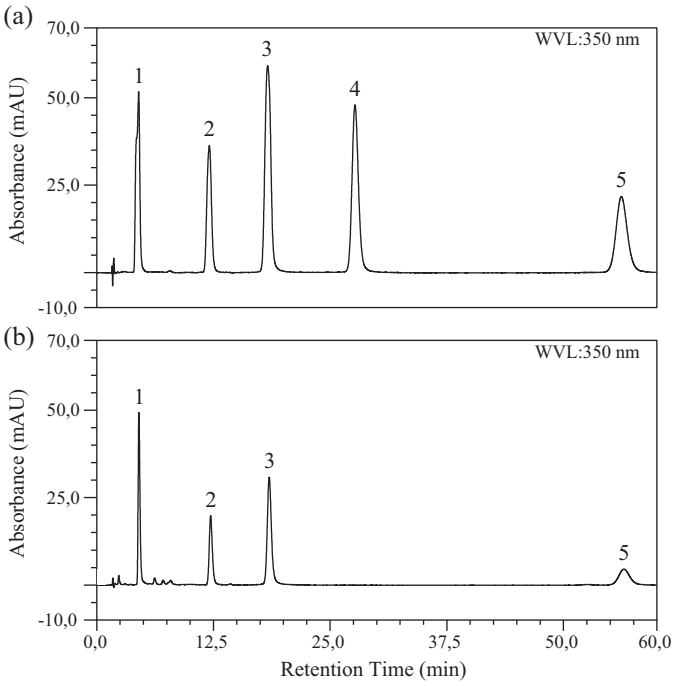

Fig. 9. HPLC chromatograms of a standard solution of Q-3,4′ (1), Q-4′ (2), morin (3), quercetin (4) and kaempferol (5), before (a) and after (b) the addition of M2 MIPs.

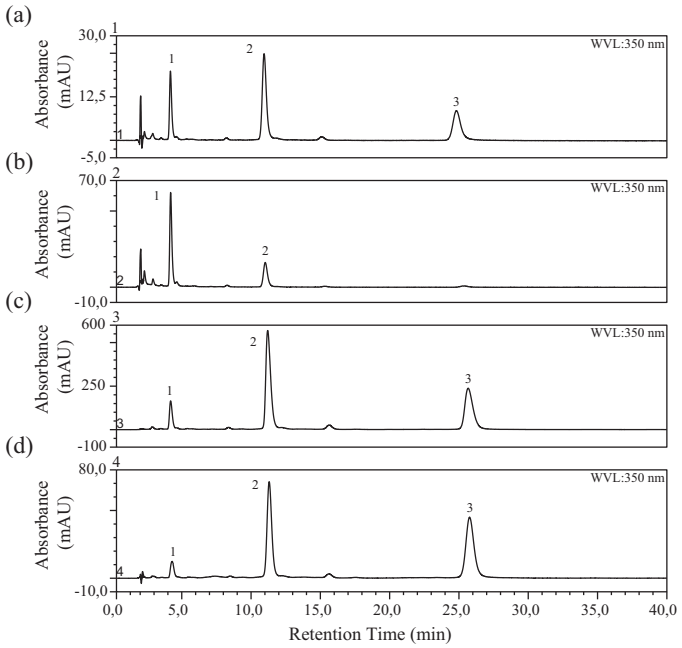

Fig. 10. HPLC chromatograms of (a) onion extract before addition of M2 MIP, (b) onion extract after addition of M2 MIP, (c) collected MeOH washing solution and (d) collected MeOH/acetic acid washing solution. 1 = Q-3,4′, 2 = Q-4′, 3 = Q.

whereas to some extent morin and kaempferol were adsorbed on the polymer (Fig. 9). This shows that MIPs can be selective but they still suffer from unspecific binding.

As mentioned earlier, quercetin-4′-glucoside and quercetin-3,4′-diglucoside are commonly hydrolyzed to quercetin aglycone, therefore these are not included in Table 3 for calculations of selectivity and distribution factors. The selectivity factor and distribution constant values summarized in Table 3 further emphasize that both M2 and N2 can bind the quercetin substantially well. The imprinting effect of M2 over N2 is demonstrated by the k and k' values, which show that M2 is better than N2. However, k' is just above 1, implying closeness in binding the quercetin molecule by M2 and N2. Hydrogen bonding and π–π interactions are most likely the main recognition sites, as explained by the structure of the quercetin molecule. The presence of water in these experiments might disrupt hydrogen bonding resulting in more π–π interactions (aromaticity of quercetin and 4-VP monomer). Studies are underway to further improve the selectivity of quercetin MIPs in pure aqueous environments.

3.9. Application to a yellow onion extract

Fig. 10(a) shows an aqueous yellow onion extract (ten times dilution) obtained from pressurized hot water extraction of yellow onion. Fig. 10(b) shows the chromatogram of the onion extract after MIP application, Fig. 10(c) shows a chromatogram of M2 particles washed with pure methanol solution (2 mL) for 75 min and Fig. 10(d) shows M2 particles washed with MeOH/acetic acid (9:1, v/v) after the MeOH wash. The prepared polymer seemed to work well under the tested conditions, in that almost no quercetin peak was observed in Fig. 10(b) compared to in Fig. 10(a), implying a possible adsorption to the polymer. A similar trend was observed when N2 was applied to the aqueous onion extract (data not shown). However, since quercetin was found both in the MeOH and MeOH/acetic acid wash solutions, and this trend was the same for N2, it is hard to draw any conclusions on the ratio between unspecifically and specifically bound quercetin. For future studies, it needs to be investigated further the choice of washing solution for unspecifically bound quercetin.

4. Conclusion

Novel quercetin MIPs that can be used at high temperatures under aqueous conditions were produced using a THF/H_2O/MeOH porogenic mixture. All MIPs and NIPs produced were intact at temperatures of up to 250 °C as determined by TGA. The most promising MIP worked well at pH 5.5 and with the re-binding solution methanol/water (7:3), demonstrating a significantly higher binding capacity of the MIP compared to its corresponding NIP. Furthermore, our results demonstrate that a higher temperature (84 °C compared to 25 °C) can beneficially be used to obtain faster adsorption kinetics as well as higher binding capacity and more research in this direction is planned. Finally, the MIP demonstrated good affinity and selectivity to the quercetin from yellow onion extract solution. No conclusive remark on the type of bonding (unspecific or specific) could be made on experiments conducted at 84 °C for 24 h as all the quercetin was adsorbed on both MIP and NIP. However, the influence of the imprinting effect under these conditions was observed when adsorption of structurally related analogs was tested. Other monomers that can have adequate hydrogen bonding under aqueous conditions are currently being explored.

Acknowledgments

The financial support from National Research Foundation (NRF) of South Africa, the Swedish Research Council Formas (209-2006-1346, 229-2009-1527), the Swedish Foundation for International Cooperation in Research and Higher Education (STINT, YR2009-7015) and the Swedish Research Council (VR, 2010-333) is gratefully appreciated. Prof. Patric Jannasch and Carlos Rodriguez Arza (Centre for Analysis and Synthesis, Department of Chemistry, Lund University) are thanked for help with TGA instrument, Prof. Reine Wallenberg (*ditto*) is thanked for SEM analysis and Prof. Ola Wendt (*ditto*) is acknowledged for usage of FTIR instrumentation.

References

[1] B.S. Ebarvia, C.A. Binag, F. Sevilla, Anal. Bioanal. Chem. 378 (2004) 1331.
[2] L. Ye, K. Mosbach, React. Funct. Polym. 48 (2001) 149.
[3] M. Quaglia, L.E. De, C. Sulitzky, G. Massolini, B. Sellergren, Analyst 126 (2001) 1495.
[4] G. Wulff, Chem. Rev. 102 (2002) 1.
[5] M.L. Zhang, J.P. Me, Q. Zhou, G.Q. Chen, Z. Liu, J. Chromatogr. A 984 (2003) 173.
[6] K.J. Shea, Trends Polym. Sci. 2 (1994) 166.
[7] K. Mosbach, O. Ramstrom, Biotechnology 14 (1996) 163.
[8] H. Zhang, L. Ye, K. Mosbach, J. Mol. Recogn. 19 (2006) 248.
[9] B. Sellergren, M. Lepisto, K. Mosbach, J. Am. Chem. Soc. 110 (1988) 5853.
[10] R. Arshady, K. Mosbach, Makromol. Chem. 182 (1981) 687.
[11] J. O'Mahony, A. Molinelli, K. Nolan, M.R. Smyth, B. Mizaikoff, Biosens. Bioelectron. 21 (2006) 1383.
[12] E. Caro, N. Masque, R.M. Marce, F. Borrull, P.A.G. Cormack, D.C. Sherrington, J. Chromatogr. A 963 (2002) 169.
[13] O. Nemulenzi, B. Mhaka, E. Cukrowska, O. Ramström, H. Tutu, L. Chimuka, J. Sep. Sci. 32 (2009) 1941.
[14] B. Dirion, Z. Cobb, E. Schillinger, L.I. Andersson, B. Sellergren, J. Am. Chem. Soc. 125 (2003) 15101.
[15] P.A.G. Cormack, A.Z. Elorza, J. Chromatogr. B 804 (2004) 173.
[16] J.L. Urraca, M.C. Moreno-Bondi, A.J. Hall, B. Sellergren, Anal. Chem. 79 (2007) 695.
[17] H.W. Sun, F.X. Qiao, J. Chromatogr. A 1212 (2008) 1.
[18] F. Qiao, H. Sun, J. Pharmaceut. Biomed. 53 (2010) 795.
[19] G. Pan, Y. Zhang, X. Guo, C. Li, H. Zhang, Biosens. Bioelectron. 26 (2010) 976.
[20] H. Yan, K.H. Row, G. Yang, Talanta 75 (2008) 227.
[21] Y. Hu, R. Liu, Y. Zhang, G. Li, Talanta 79 (2009) 576.
[22] E.V. Piletska, A.R. Guerreiro, M.J. Whitcombe, S.A. Piletsky, Macromolecules 42 (2009) 4921.
[23] X. Shen, L. Ye, Chem. Commun. 47 (2011) 10359.
[24] K. Booker, M.C. Bowyer, C.I. Holdsworth, A. McCluskey, Chem. Commun. (2006) 1730.
[25] N. Perez-Moral, A.G. Mayes, Anal. Chim. Acta 504 (2004) 15.

[26] A. Beltran, R.M. Marce, P.A.G. Comack, F. Borrull, J. Chromatogr. A 1216 (2009) 2248.
[27] M. Gallego-Gallegos, R. Muñoz-Olivas, C. Cámara, J. Environ. Manage. 90 (2009) S69.
[28] L. Chen, S. Xu, J. Li, J. Chem. Soc. Rev. 40 (2011) 2922.
[29] A. Beltran, F. Borrull, P.A.G. Cormack, R.M. Marcé, Trends Anal. Chem. 29 (2010) 1363.
[30] P.C.H. Hollman, I.C.W. Arts, J. Sci. Food Agric. 80 (2000) 1081.
[31] N. Russo, M. Toscano, N. Uccella, J. Agric. Food Chem. 48 (2000) 3232.
[32] M. Biesaga, K. Pyrzynska, Crit. Rev. Anal. Chem. 39 (2009) 95.
[33] K.R. Price, M.J.C. Rhodes, J. Sci. Food Agric. 74 (1997) 331.
[34] A.M. Nuutila, K. Kammiovirta, K.M. Oksman-Caldentey, Food Chem. 76 (2002) 519.
[35] C. Turner, P. Turner, G. Jacobson, K. Almgren, M. Waldebäck, P. Sjöberg, E. Nordberg-Karlsson, K.E. Markides, Green Chem. 8 (2006) 949.
[36] S. Lindahl, A. Ekman, S. Khan, C. Wennerberg, P. Börjesson, P.J.R. Sjöberg, E. Nordberg-Karlsson, C. Turner, Green Chem. 12 (2010) 159.
[37] Y. Wei, Q. Xie, D. Fisher, I.A. Sutherland, J. Chromatogr. A 1218 (2011) 6206.
[38] E. Gikas, F.N. Bazoti, N. Papadopoulos, A. Alesta, G. Economou, A. Tsarbopoulos, Anal. Lett. 44 (2011) 1463.
[39] S.M. Tzouwara-Karayanni', S.M. Philianos, Microchem. J. 27 (1982) 155.
[40] L. Zhu, X. Xu, J. Chromatogr. A 991 (2003) 151.
[41] A. Molinelli, R. Weiss, B. Mizaikoff, J. Agric. Food Chem. 50 (2002) 1804.
[42] X. Song, J. Li, J. Wang, L. Chen, Talanta 80 (2009) 694.
[43] Z. Yuan, Y. Liu, F. An, Microchim. Acta 172 (2011) 89.
[44] J. Shan, B. Wang, Sep. Sci. Technol. 46 (2011) 164.
[45] Z. Li, M. Day, J.F. Ding, K. Faid, Macromolecules 38 (2005) 2620.
[46] S. Azodi-Deilami, M. Abdouss, S.R. Seyedi, Cent. Eur. J. Chem. 8 (2010) 687.
[47] M. Khajeh, Y. Yamini, E. Ghasemi, J. Fasihia, M. Shamsipur, Anal. Chim. Acta 581 (2007) 208.
[48] N. Arabzadeh, M. Abdouss, Colloid J. 72 (2010) 446.
[49] L.Q. Wu, Y.Z. Li, J. Mol. Recogn. 17 (2004) 567.
[50] Z.Y. Chen, R. Zhao, D. Shangguan, G.Q. Liu, Biomed. Chromatogr. 19 (2005) 533.
[51] S. Li, X. Huang, M. Zheng, W. Li, K. Tong, Sensors 8 (2008) 2854.