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A Molecularly Imprinted Polymer as Artificial Receptor for the Detection of Indole-3-carbinol

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ABSTRACT: Molecularly imprinting polymer technology is used to prepare a molecularly imprinted polymer (MIP) for the selective recognition of indole-3-carbinol (I3C), a chemopreventive and chemotherapeutic phytochemical associated with the anticancer activities of cruciferous vegetables. Prepolymerization study via nuclear magnetic resonance technique is done to choose the best functional monomer that establishes more interaction with the template. The prepared MIP is tested before in batch experiments and subsequently used as solid-phase extraction sorbent for the selective detection of I3C from standard solutions. In order to verify the selectivity of the MIP, the binding of structurally related compounds, such as indole-3-acetonitrile, teophylline, and tryptophan, on the polymer is investigated. The experiments indicate that the MIP is highly selective for I3C with an association constant of $K_a = (1.37 \pm 0.07) \times 10^3\ M^{-1}$. Standard mixture solution loaded on MIP-SPE cartridge give a recovery of 95% for I3C, while the other compounds are totally eluted during washing step. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40819.

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INTRODUCTION

Molecular imprinting is a very versatile synthetic approach for the synthesis of highly selective polymeric receptors having artificial generated recognition sites which are able to specifically rebind a target molecule in preference to other closely related compounds.[1–4] Molecularly imprinted polymers (MIPs) are created by a process where a target molecule acts as template, around which interacting and crosslinking monomers are arranged and copolymerized to form a cast-like shell complementary to that of the template. Removing the template from the polymer, a molecular memory is imprinted in the polymer that retains selective molecular information regarding both morphological and functional properties of the template.[5–7]

One of the attractive features of the MIPs is their use in different scientific fields[8–14] and a wide range of target molecules[15–20] have been successfully used.

In this work, the synthesis of a MIP used as indole-3-carbinol (I3C) receptor is described. I3C is produced endogenously from naturally occurring glucosinolates which can be found in a wide variety of plant food substances including members of the cruciferous vegetables such as broccoli, cabbage, cauliflower, brussels sprouts, collard greens. Recent dietary and epidemiological studies have suggested the benefit of dietary intake of fruit and vegetables, which provide phytochemicals, particularly I3C, that plays an important role in the prevention of many types of cancer, in particular hormone-related cancer.[21–23] I3C treatment of human reproductive cancer cells activates or dampens specific trascriptional, signal transduction and metabolic cascades that lead to a cell cycle arrest, apoptosis, down-regulation of cancer cell migration and modulation of hormone receptor signaling.[24–27]

Many analytical attempts such as gas chromatography,[28] GC/mass spectrometry,[29] and high-performance liquid chromatography (HPLC) have been used to analyze the breakdown products of indolic glucosinolates in cruciferous vegetables. However, these approaches require arduous derivatization step that does not provide accurate results due in part to its insensitivity. More specifically, an analytical procedure was developed for detecting I3C in plasma with a HPLC and UV detection combination[30] but this method is time-consuming or uses laborious extraction protocols to detect the trace amounts of the I3C present in cruciferous vegetable samples.

This article presents preliminary results for the preparation and evaluation of the effectiveness of a novel MIP that could be used for the specific extraction of I3C from cruciferous vegetables. This simple and cheap method could be an alternative to the expensive and non-specific methods that are in use.

Additional Supporting Information may be found in the online version of this article.

Usually reports for I3C detection deal with its quantification along with many other compounds. The novelty in the methodology proposed in this article is in the preparation of a sorbent capable of retaining specifically I3C molecule that can be considered a feasible system for clean-up, pre-concentration, and an easier I3C quantification.

To the best of our knowledge no other works using I3C as template for MIP system were made. Recently, only some reports explained the preparation of MIP for other indole derivatives such as indole-alkaloids[31] and indole-3-acetic acid.[32,33]

To verify the creation of interactions between functional monomer and the I3C and to develop the best procedure of synthesis, prepolymerization studies via NMR technique were done testing different monomers. For this reason, two functional monomers were used: a typical acidic monomer such as methacrylic acid (MAA) and a basic monomer such as 4-vinylpyridine (4VP).

The synthesized MIP was characterized with scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). After characterization, the binding affinity of the synthesized MIP was tested in batch experiments by UV–vis spectroscopy and the binding characteristics of the polymer were examined by Scatchard and Langmuir analysis. Moreover, in order to verify the selectivity of the MIP, the binding of structurally related compounds, such as indole-3-acetonitrile, theophylline, and tryptophan, on the polymer was investigated. Finally, the polymer was used as solid-phase extraction (SPE) sorbent for the selective detection of I3C from standard solutions. Corresponding unimprinted blank polymer (NIP) was prepared using the same procedure in the absence of I3C and the polymer obtained was tested to demonstrate the absence of binding affinity.

EXPERIMENTAL

Materials

I3C (98%), teophylline (99%), tryptophan (98%), indole-3-acetonitrile (98%), ethylene glycol dimethacrylate (EGDMA, 98%), 4VP (95%), α-α′-azoisobutyronitrile (AIBN, 98%) and MAA (99%), acetic acid (99.7%) sodium acetate (99%), and methanol-d_4 (99.8%) were purchased from Sigma-Aldrich (Steinheim, Germany, www.sigmaaldrich.com). Ethanol, methanol, and HPLC-grade acetonitrile were supplied from J.T. Baker (Deventer, Holland, www.vwr.com). All buffer solutions were prepared with ultrapure water obtained with a water purification system (Human Corporation, Korea, www.humancorp.co.kr). Chromabond empty SPE cartridges (3 mL) were supplied by Macherey-Nagel (Düren, Germany, www.mn-net.com). C18 Sep-Pak cartridges (500 mg) were purchased from Waters Corporation (Milford, MA, www.waters.com).

Apparatus

FTIR analysis was carried out on a JASCO 660 plus infrared spectrometer (Jasco Analytical Instruments, Easton, MD, www.jascoinc.com). Dry polymers (MIP-4VP and MIP-MAA) were dispersed in a matrix of KBr, followed by a compression at 10 tons to form pellets and 4VP was spread directly on an ATR ZnSe crystal. Proton nuclear magnetic resonance (^{1}H-NMR) experiments were performed on an Advance 400 spectrometer (Bruker, Billerica, MA, www.bruker.com) operating at 400 MHz with TMS as internal standard. Sample temperature was stabilized at 25°C. SEM images were recorded with a JSM 6500 F microscope (JEOL, Tokyo, Japan, www.jeol.com), equipped with a field emission source. UV–vis spectra were obtained with a Cary 100 Scan UV–vis spectrophotometer (Varian, Palo Alto, CA, www.chem.agilent.com). Sonication was carried out using a Sonorex RK 102H ultrasonic water bath (Bandelin Electronic, Berlin, Germany, Europe, www.bandelin.com). Centrifugation was achieved with a PK121 multispeed centrifuge (Thermo Electron Corporation, Waltham, MA, www.thermoscientific.com). For pH measurements, pHmeter Basic 20, (Crison, Alella, Barcelona, Spain, Europe, www.crisoninstruments.com) was used. HPLC analysis was performed using an Agilent 1100 Series Liquid Chromatography system coupled to a diode array detector (DAD). Chromatography separation was carried out on a 250 × 4.6 mm i.d., 5 μm Phenomenex Gemini-NX C18 column (Phenomenex, Torrance, CA, www.phenomenex.com) thermostated at 25°C. The mobile phase was composed of water (Solvent A) and acetonitrile (Solvent B) at a flow rate of 0.8 mL min^{-1}. The following gradient was utilized: 0 min, 2% B; 5 min, 5% B; 10 min, 10% B; 15 min, 20% B; 30 min, STOP. The UV absorbance of I3C was monitored with a DAD at 278 nm.

Pre-polymerization Study

^{1}H-NMR was used to study the interaction between I3C and the functional monomer (MAA or 4VP). A series of measurements was performed at variable concentration of MAA and at a fixed concentration of the template, using a decreasing amount of MAA titrated into a constant amount of I3C. The template concentration was 0.0468*M* and the concentrations of added MAA were 0.468*M*, 0.1872*M*, 0.0936*M*, 0.0468*M*, in methanol-d_4. Instead, a fixed concentration of 4VP equal to 0.468*M* was used.

Polymers Preparation

The preparation of MIP for I3C was carried out as follows: to a solution of I3C (0.0936 mmol, as template) in methanol (2 mL) was added 0.936 mmol of functional monomer (4VP) in a glass tube. After adding of EGDMA (5.616 mmol, as crosslinker) and AIBN (0.0844 mmol, as initiator), the mixture was purged with nitrogen gas in a sonicating bath for 5 min to remove oxygen which could inhibit the polymerization. After that, the glass tube was sealed and thermal polymerization was performed by heating at 60°C for 20 h. The resulting polymer was ground in a mortar and sieved in the range 20–70 μm. Successively the polymer was washed with ethanol/acetic acid (7/3 vol/vol), kept in a ultrasonic bath for 20 min and then centrifuged at 8000 rpm for 10 min until no template molecules were detected from the recovered solutions with a UV–vis spectrophotometer at 278 nm. Finally, other washings steps with ethanol were done in order to remove the residual acetic acid present. We quantified the I3C percentage released in each washing step by using an external calibration curve of I3C and a recovery higher than 95% was obtained.

The resulting solution dried under vacuum was used for rebinding studies and to prepare SPE cartridges. A corresponding blank polymer (NIP) was prepared in the absence of template

and treated identically as for the corresponding imprinted polymer.

Finally, for FTIR control purposes another polymer was prepared in the same manner of the above MIP except for using as functional monomer MAA instead of 4VP.

Batch Rebinding Experiments and Adsorption Studies

The polymer (20 mg) was added to a solution (3.5 mL) of I3C in methanol at known concentrations (0 to 4.0 $\times$ $10^{-3}M$) in vials. The resulting suspension was shaken for 16 h at room temperature, then the polymer was rapidly removed by filtration and the resulting solution was analyzed by UV–vis spectrophotometer at 278 nm. The amount of I3C bound to the polymer, $[I3C]_b$, was calculated by subtraction of the concentration of free I3C, $[I3C]_e$, from the initial I3C concentration $[I3C]_i$. I3C concentrations in solution were determined as an average value of three measurements. Scatchard analysis was provided by the Scatchard equation [eq. (1)]:

$$B/[I3C]_e = (B_{max} - B)K_a \quad (1)$$

where B represents the amount of I3C bound per gram of polymer, K_a is the association constant and B_{max} is the apparent maximum number of binding sites. Therefore, K_a and B_{max} of the polymer were determined from the slope and the intercept, respectively, by plotting of $B/[I3C]_e$ versus B. Batch rebinding experiments and Scatchard analysis were performed in a similar manner with the corresponding blank polymer.

Langmuir model was also used to evaluate the adsorption process.

The Langmuir isotherm is represented by:

$$[I3C]/B = [I3C]/Q_{max} + 1/K_L Q_{max} \quad (2)$$

where Q_{max} (mg g^{-1}) represents the maximum adsorption capacity, K_L (L mg^{-1}) is the Langmuir constant that represents the affinity between solute and adsorbent.

Similarly, rebinding tests were also carried out incubating the polymer with a methanol solution of theophylline, tryptophan, or indole-3-acetonitrile 9 $\times$ $10^{-4}M$ in order to verify its binding selectivity.

SPE Cartridge Experiments

A 180 mg amount of dry polymer (MIP and NIP, respectively) was packed in an empty SPE cartridge between two polyethylene frits. The polymer cartridges were conditioned using 5 mL of methanol and then 5 mL of water. About 1 mL of a water solution containing a mixture of four structurally similar compounds (0.20 mM), I3C, indole-3-acetonitrle, theophylline and tryptophan, was loaded onto the cartridge. Afterwards, a series of selective washing with Na/acetate buffer solution 50 mM (pH 7) were successively loaded in order to elute the unbound compounds. Then, I3C was extracted three times with 3 mL of methanol. All fractions eluted were evaporated to dryness under vacuum at 40°C. The residue was dissolved in 1 mL of distilled water and injected into the HPLC system. Chromatograms were acquired using different wavelengths (278 nm, 274 nm, 280 nm). For quantitative determinations, reference standard solutions of I3C, indole-3-acetonitrile, theophylline, or tryptophan in the concentration range of 5–100 μg mL^{-1}, were analyzed and the peak areas were plotted versus the concentration. The same experiment was performed using both a cartridge packed with the corresponding blank polymer (NIP) and a commercial C18-SPE cartridge.

RESULTS AND DISCUSSION

It is very difficult to foresee *a priori* what is the preferential interaction of a template molecule with a functional monomer and the molar ratio in the polymerization mixture. In this work, a synthesis protocol that was a right agreement between previous works[34,35] was found and tested. A thermal-initiate bulk polymerization was used in the preparation of MIP for I3C using methanol as porogen and EGDMA and 2,2′-azobisisobutyronitrile as crosslinker and initiator, respectively.

Since crosslinker and initiator would be much less important than functional monomer for the interaction of template and functional monomers, an ^{1}H-NMR study was performed to study the interaction between I3C and two typical functional monomers, 4VP and MAA, with basic and acid properties, respectively. The main object of this study was to choose the proper functional monomer. To this aim, we evaluated what happens during prepolymerization step, by using template and monomer in the same solvent and at the same concentration of the synthesis. Even if a detailed NMR study, such as titration curves, by using different template–monomer ratio would be fundamental for the evaluation of complex stoichiometry and stability and also for understanding the nature of the interaction we believe that this is beyond the scope of this work. Thus 0.468M of 4VP or MAA and 0.0468M of I3C in methanol-d_4 were used. The choice of a ratio between template and monomer of 1 to 10 was mainly driven from our experience in MIP field and a synthesis protocol as a right agreement between previous works[31,32] was used.

4VP alone (Supporting Information Figure S1) shows two signals ascribable to the pyridinic ring protons at 8.5 ppm for the two protons nearest to the nitrogen and at 7.43 ppm for the remaining two aromatic protons. The three vinylic protons give three different signals at 6.7 ppm, 6.1 ppm and 5.5 ppm. MAA alone (Supporting Information Figure S2) gives a simple NMR spectrum with two vinylic signals at 6.1 ppm and 5.6 ppm and the methyl protons at 1.9 ppm. Finally, the template I3C alone [Figure 1(a)] gives a signal for each aromatic ring protons as a multiplet at 7.7 ppm, 7.4 ppm, 7.1 ppm and 7.0 ppm for the benzylic protons and as a singlet at 7.2 ppm for the pyrrolic proton in addition to a methylene protons signal at 4.8 ppm that is not included in the figure. The protic solvent used for NMR analysis does not allow us to follow the signals of OH and NH protons of I3C or MAA due to very fast proton exchange rates. There are not significant differences in NMR monomer signals when the template is added in solution. On the other hand, useful information were obtained in the aromatic region of I3C as emphasized in Figures 1 and 2 where only the enlarged ^{1}H-NMR spectra in the region between 6.9 ppm and 7.9 ppm were shown. When 4VP was used, the proton chemical shifts of I3C and 4VP almost did not change after mixing both compounds, as can be seen in Figure 1. But the

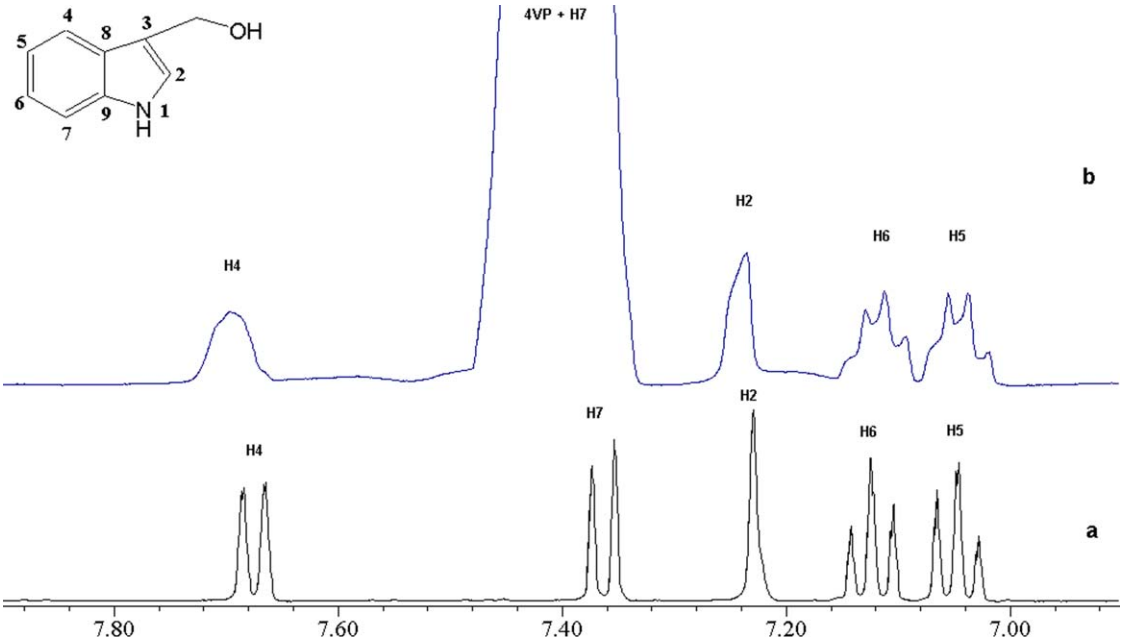

Figure 1. Part of enlarged ^{1}H-NMR spectra of I3C alone (a) and mixed with 4VP in a ratio 1 to 10. Both spectra are registered in methanol-d_4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

NMR signals shapes of the aromatic protons of I3C (H2, H4, H5, H6) have changed with a broadening of all signals when 4VP is added to the I3C solution [Figure 1(b)]. This indicates that a possible interaction between template and 4VP occurred with a modification of the chemical environment around the aromatic rings of the I3C[36] which is fundamental for a feasible formation of holes into the polymer matrix and a good rebinding capacity.

When MAA was tested in the same conditions used for 4VP, different results were found (Figure 2). After mixing MAA with I3C, a complicated spectrum was obtained [Figure 2(d)]. Further studies were made to understand these results. Thus different I3C/MAA ratios were prepared and analyzed and the formation of the dimer of I3C in the prepolymerization condition was hypothesized. In fact the decreasing of I3C signals and the simultaneous increasing of the signals ascribable to the

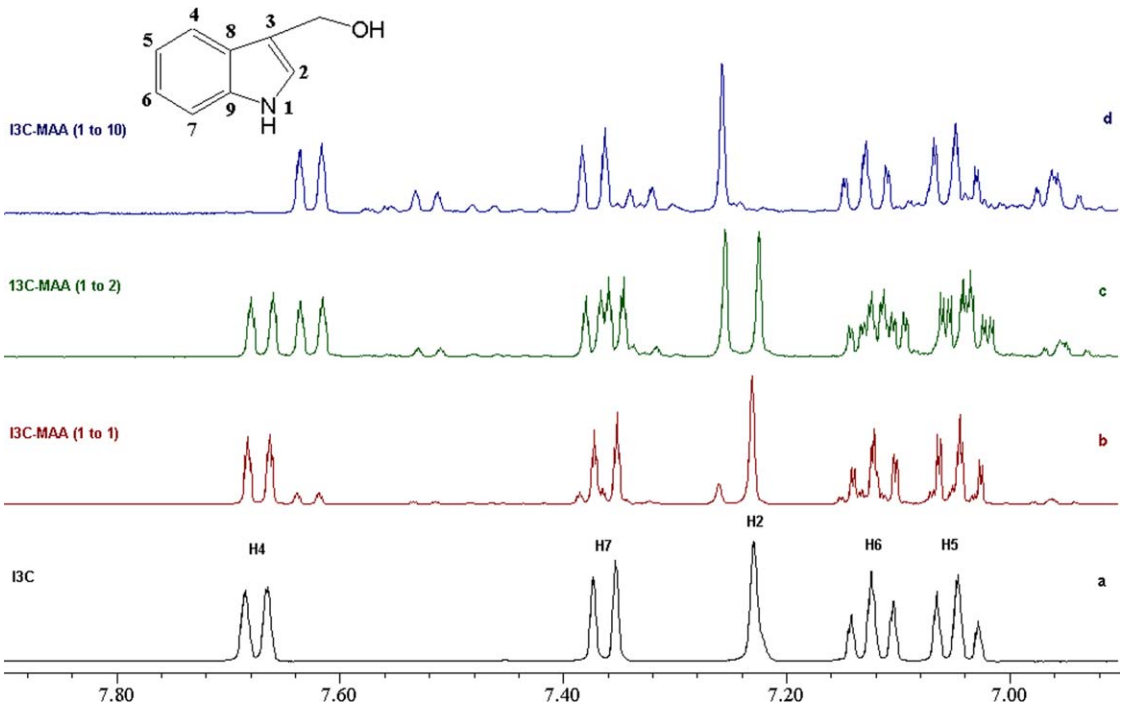

Figure 2. Part of enlarged ^{1}H-NMR spectra of I3C alone (a); and mixed with MAA in a ratio 1 to 1(b); 1 to 2 (c); and 1 to 10 (d). All spectra are registered in methanol-d_4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

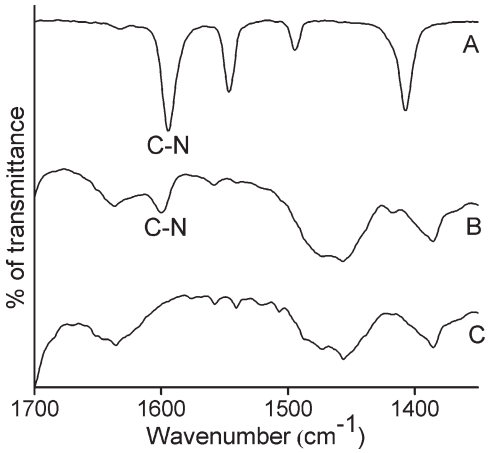

Figure 3. FTIR spectra of 4VP (A), MIP-4VP (B), and MIP prepared with MAA instead of 4VP (C).

dimer[37] were found when the equivalents of MAA raise from 1 [Figure 2(b)] to 2 [Figure 2(c)], to 10 [Figure 2(d)]. This is reasonable due to the effect of the acidic environment used.[24,38] For this reason we decided to not use MAA for polymer synthesis of I3C-imprinted and unimprinted polymers.

A chemical characterization of I3C-imprinted polymer was made by FTIR analysis. Figure 3 shows the enlarged FTIR spectra in the region 1700 cm^{-1}−1350 cm^{-1} of 4VP (A), MIP-4VP (B), and MIP-MAA (C). Characteristic bands of 4VP [Figure 3(A)] can be observed at 1595 cm^{-1} and 1495 cm^{-1} typical of pyridine ring stretching and corresponding to the stretching vibration absorption of C-N and C=C bonds, respectively.[39,40] In the polymer prepared with 4VP, the signal typical of pyridine ring stretching at around 1600 cm^{-1} appears in the IR spectrum [Figure 3(B)]. A comparison with the IR spectrum of a polymer prepared by using MAA instead of 4VP [Figure 3(C)] allows to confirm this result since it is noticeable the absence of signals in the region at around 1600 cm^{-1}. FTIR spectrum of NIP was also registered but not reported in Figure 3 because it was identical to MIP-4VP spectrum.

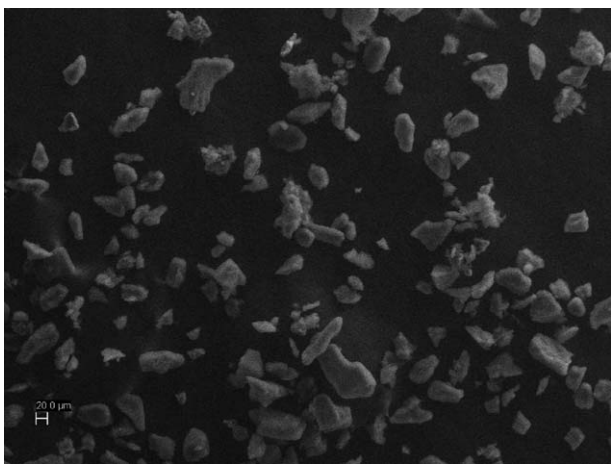

Figure 4. SEM image of the MIP.

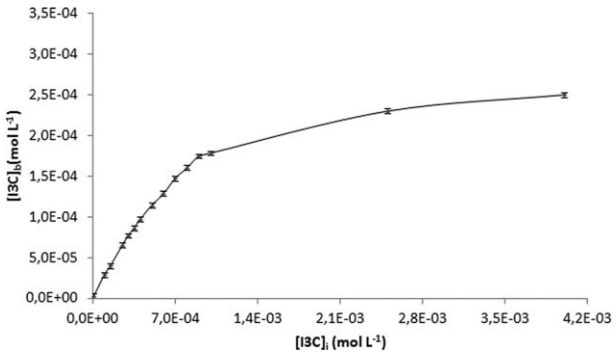

Figure 5. Binding isotherm for the MIP.

As it can be seen in SEM image (Figure 4), depending on the bulk polymerization method used, MIPs have various physical shapes and a wide size distribution.

The SEM results confirm that a bulk polymer was obtained, similarly to other SEM images reported from previous works on MIP synthesized with the same technique.[41–43]

The binding behavior of the prepared MIP was evaluated by batch rebinding tests and the binding data were processed with Scatchard equation in order to estimate the binding properties of the polymers. The binding curve reported in Figure 5 shows the amount of I3C bound $[I3C]_b$ in 16 h as a function of the initial concentration of I3C ranging from 0 to 4.0 × $10^{-3}M$. As it can be seen, the initial increase in binding is followed by a saturation at the concentration value of 3.0 × $10^{-3}M$, indicating that the available receptor sites have been saturated with I3C.

Figure 6 shows the Scatchard plot for I3C polymer. Usually, a nonlinear profile was commonly observed in the Scatchard assessment of MIP indicating the presence of binding sites that exhibit various affinities to the ligand.[35,44] However, there are some studies in literature that showed a linear profile of binding sites for imprinted polymers.[16,45–47]

In this work, a single straight line was obtained, which indicates that there exists one kind of binding sites in the MIP with a single association constant of $K_a = (1.37 \pm 0.07) \times 10^3 M^{-1}$. The apparent maximum number of binding sites for the MIP was 56.99 ± 0.08 $\mu M\ g^{-1}$. The K_a value obtained appears to be in agreement with other works present in literature.[35,48,49]

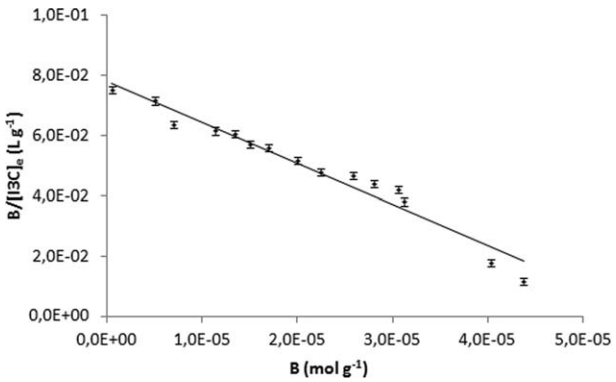

Figure 6. Scatchard plot for the MIP.

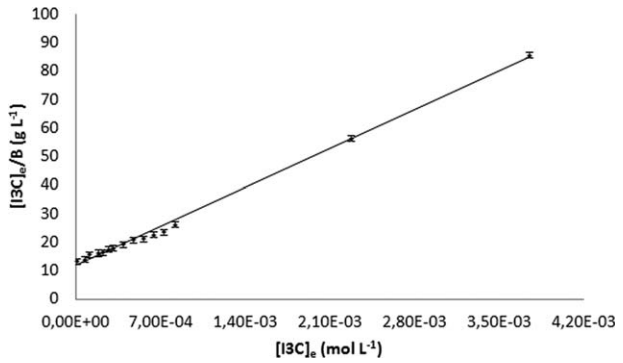

Figure 7. Langmuir isotherm of MIP.

In order to confirm the homogeneity of binding sites distribution, a Langmuir model was also employed to fit the adsorption process. The calculated values of Q_{max} was 51.4 μM g^{-1} with a Langmuir constant K_L of 1.64 × 10^3 mol^{-1} (Figure 7).

According to the values of correlation coefficients (R^2) of the Langmuir isotherm equal to 0.996, this model is suitable to describe the binding affinity of the MIP system, indicating that the adsorption of I3C on the polymer is homogenous. Thus, the results obtained from Langmuir model confirmed the conclusions of Scatchard analysis.

The binding behavior of the polymer depends on several factors: synthesis conditions, polymerization techniques, template molecules and monomers used. Moreover, optimizing the amount of crosslinker and reducing the concentration of the template, the polymer binding properties are enhanced and the level of low affinities interactions is decreased, improving the homogeneous sites formation.

The corresponding NIP showed no binding affinities for I3C confirming that the selectivity was due to the imprinting of the polymer matrix and not to the intrinsic affinity of the template to the functional monomers alone.

Batch rebinding experiments with other similar structurally compounds, were also conducted to evaluate the selectivity of the MIP. As it can be seen in Figure 8, the polymer did not show any binding capacity for teophylline and tryptophan. It

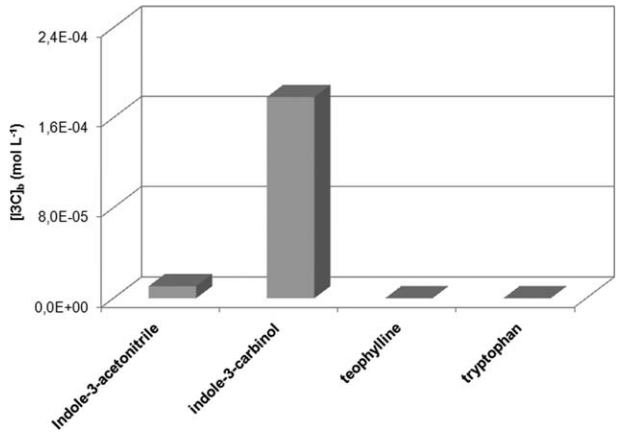

Figure 8. Binding affinity of the MIP for similar structural compounds.

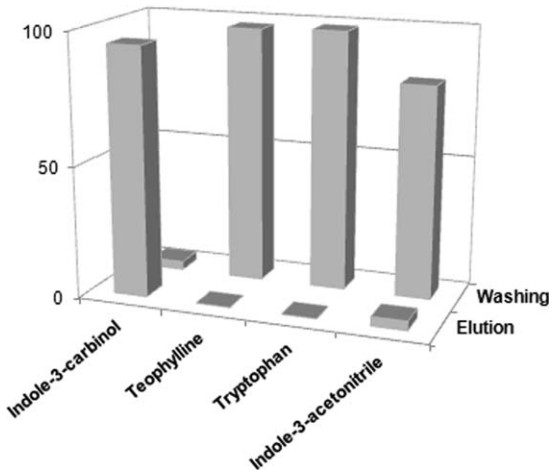

Figure 9. Recoveries yield (%) for I3C and the other compounds in the washing and elution steps of MIP-SPE experiment.

can be noted that only a slight retention for indole-3-acetonitrile was found. It is probably due to the very high structural affinity of this compound with I3C. However, there is a considerably higher adsorption capacity of the MIP for the template if compared to the adsorption of indole-3-acetonitrile.

Also the corresponding blank did not show any binding capacity toward the other compounds except for indole-3-acetonitrile. In this case, a slight retention was observed similarly to the results obtained for the corresponding MIP. This means that the synthesized polymer exhibited high recognition property only against I3C.

The selectivity of the MIP was also investigated using it as sorbent for solid-phase extraction of I3C and comparing its performance with that of both the corresponding NIP and conventional C18 cartridge. About 1 mL of a 0.20 m*M* water solution of four compounds with similar structures, which were indole-3-acetonitrile, I3C, theophylline, tryptophan was loaded on the MIP-SPE cartridge. Then, the cartridge was washed with 4 mL of Na/acetate buffer solution 50 m*M* (pH 7) in order to elute the unbound compounds and to obtain a high degree of selectivity. Finally, I3C was eluted with 3 mL of methanol. At each step, the collected solutions were carefully analyzed by HPLC.

Figure 9 shows the elution profile obtained with the MIP-SPE cartridge when the mixture was loaded. As it can be seen, a high extraction recovery (95%) in the elution step was obtained for I3C and a small amount (3%) was found in the washing step, while the other compounds, in particular theophylline and tryptophan, were eluted only in the washing step with recoveries ranging from near 98%. Indole-3-acetonitrile was also eluted in the washing step but with a recovery near to 90%. A small amount (3%) of this compound was eluted with I3C in the elution step, probably for the high structural affinities. However, a high retention and a good specificity for I3C was showed by the MIP-SPE cartridge. These data confirmed the possibility of washing interfering compounds from the MIP while retaining the analyte and emphasized the selectivity of the MIP for I3C.

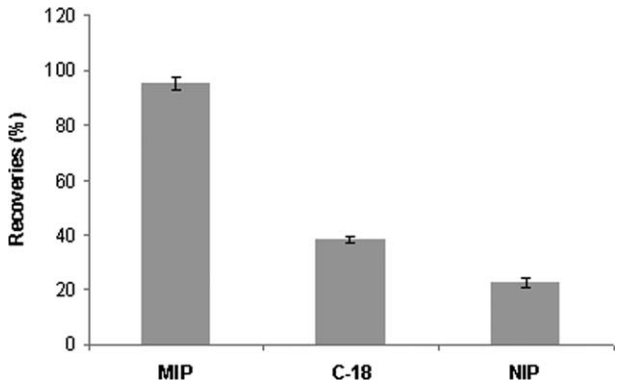

Figure 10. Recovery yield (%) for I3C in the elution solution after SPE using MIP-, NIP-, and a commercial C18-SPE cartridges.

The same experiment was carried out using both the NIP–SPE and the commercial C18-SPE cartridges in order to control and evaluate the risk of the development of the non-specific interactions in the retention process. Figure 10 shows the recovery yields in the elution solution after the extraction of the I3C using MIP-, NIP- and C18-SPE cartridges. The extraction recovery yields with relative standard deviation (RSD) were 95% (RSD 2%) for the MIP, 40% (RSD 1%) for the C18 sorbent and only 25% (RSD 1.5%) for the NIP. These data indicated a real improvement of the extraction recovery of I3C using MIP instead of a commercial C18 sorbent material in SPE experiments. On the other hand, the blank polymer (NIP) under the same conditions allowed most the I3C to elute in the loading and washing steps.

CONCLUSIONS

A highly specific and selective I3C-imprinted polymer was prepared using 4VP as functional monomer. The effects of the use of a basic functional monomer that established most interaction with I3C were examined. The results obtained suggest that I3C can be specifically bound to the synthesized polymer. Moreover, the use of the polymer as solid-phase extraction sorbent was demonstrated and the promising results demonstrate the feasibility of using this MIP for extraction of this compound from cruciferous vegetables.

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