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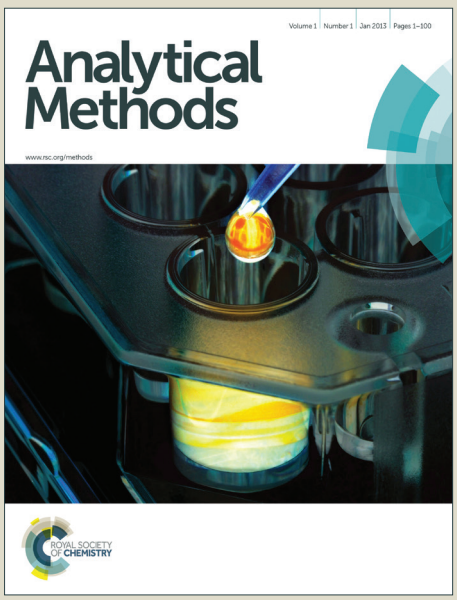

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Molecularly imprinted hydrophobic polymers as a tool for separation in capillary electrochromatography

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Abstract

The use of molecular imprinted polymers (MIPs) which provides a means for preparing stationary phases of predetermined selectivity for target molecule in capillary electrochromatography (CEC) is attractive in that it combines selectivity, higher separation efficiency and shorter analysis time. Bisphenol A (BPA)-imprinted monolithic capillary BPA/PMAPA column was synthesized for selective separation of bisphenol A (BPA) from aqueous solutions containing competitor molecule phenol (PH) which resembles in size and shape to the template molecule. BPA-imprinted monolithic column was prepared in the presence of template molecule BPA, which results in the formation of recognition cavities complementary to the template molecule, after removal of template molecule. Aminoacid based monomer, N-methacryloyl-L-phenyl alanine (MAPA) was used as the functional monomer. The new stationary phase contains both charged and hydrophobic groups originates from MAPA monomer, which behaves both electroosmotic flow (EOF) supplier and hydrophobic matrix. The MAPA containing BPA imprinted column behaved as a mixed mode stationary phase, as ion exchanger and hydrophobic matrix depending on pH of medium.

Scanning electron microscope was used to identify structural features of the molecular imprinted column**.** MIP Column performance was evaluated by using electrochromatographic separation of alkylbenzenes. The novelty of this work originated from dual separation mechanism shown by MAPA which has ability to form both hydrophobic and electrostatic interactions by charged and hydrophobic groups of phenylalanine aminoacid. This new column with mixed-mode characteristic was then used as the stationary phase in CEC for the selective separation of BPA in MIP system successfully.

1. Introduction

Molecularly imprinted polymers (MIPs) are being used as selective materials in a wide scope of applications such as chromatography, catalyst, sensor and drug delivery studies [1]. MIP is an artificial receptor made by imprinting target molecule in a polymer network, which act as a template, followed by removing the template via washing to give the permanent template cavities that are complementary in size and shape to the template. Polymerization around a templating

ligand, using monomers which have ability to participate in non-covalent interactions, constitute permanent memory for the imprint species are formed enabling the polymer selectively rebind the imprint molecule from a mixture or closely related compounds [2]. MIPs show high affinity to the template molecule compared to other molecules and this property is the basic driving force for different applications [3]. MIPs offer distinct advantages compared to natural/proteinaceuous receptors such as ease in preparation, low cost, tolerance to extreme chemical and thermal conditions, long shelf life, and enhanced versatility in experimental design [4].

Capillary electrochromatography (CEC) is an electrophoretic method that utilizes best features of both capillary electrophoresis (CE) and high performance liquid chromatography (HPLC) [5]. High separation efficiency, low solvent and sample consumption, increased mass sensitivity, and low operational costs have made CEC an attractive separation technique [6]. The combination of capillary electrochromatography which provides a high degree of separation efficiency and short separation times, with molecular imprinting, which provides a means for preparing stationary phases of predetermined selectivity and reduces band broadening associated with pressure driven parabolic-flow profiles [7,8]. The capillary column is the heart of a CEC system because it serves not only as a separation channel but also as a pumping device to transport the mobile phase through the system. Therefore the techniques for column preparation are the key to the development of CEC. According to the existing state of stationary phase, the CEC columns used so far can be classified into three main types; packed column (PC), Open tubular (OT) and monolithic column. In packed column CEC, there are some major practical problems such as difficulties of packing the small LC stationary particles with 1.5-5 μm diameters in narrow bore capillary and frit [9]. In OT-columns, the stationary phases are covalently attached, coated or adsorbed onto the inner wall of the capillary column. One advantage of OT column is that it completely eliminates the retaining frits [10,11]. As the surface of the open tube column is very limited, this column can only afford a low phase ratio and low sample capacity. In contrast, monolithic columns can avoid the troubles resulted from the frits in packed column and possess much higher surface areas and adsorption capacities over OT column. Furthermore, the inner diameter used for monolithic column can be as large as for packed column, thus the detection sensitivity is higher relative to OT column [12–16].

To generate electo-osmotic flow (EOF), charged groups should be coupled to the surface of organic polymer monoliths. Generally, three types of organic monoliths based on

polyacrylamides [17,18], polymethacrylate esters [19,20] and polystyrenes [21] have been prepared as the stationary phases for CEC. Due to simple procedures of their preparation and chemical stability in a broad pH range, monolithic columns containing a wall-supported continuous porous bed have shown a great potential for CEC [22]. The use of monolithic column seems a new trend in CEC.

The presence of compounds in the environment with estrogenic activity has become a subject of major concern worldwide. Endocrine disruptors can bind to cellular receptors for estrogens and interfere with steroid-mediated regulatory functions in living organisms, humans included, exerting estrogen-like effects. Bisphenol A (2,2-bis(4-hydroxyphenyl) propane; BPA) is known to be one such compound. BPA is used mainly as a material for the production of epoxy resins and polycarbonate plastics. Because of an increase in products based on epoxy resins and polycarbonate plastics, human exposure to BPA has increased. Canale et al prepared molecularly imprinted polymer (poly-4-vinylpyridine-co-trimethylolpropane-trimethacrylate) for selective separation of BPA from water, by LC method [23]. Lee et al synthesized narrowly dispersible BPA-imprinted polymeric microspheres which were used as selective solid phase extraction (SPE) sorbents for BPA from different sample matrice and analyzed by CE [24].

In the present study, BPA-imprinted methacrylate-based monolithic column **was** synthesized to separate BPA selectively from aqueous solution containing competitor reagent PH. Preorganization monomer MAPA shows both ion exchanger and hydrophobic character depending on pH of medium. Polymer solution **was** prepared by mixing methacryloyl phenylalanine (MAPA) as electro-osmotic flow (EOF) supplier, ethylene dimethacrylate (EDMA) as crosslinker, template molecule bisphenol A (BPA) and pore maker ethanol (EtOH). The nonpolar monomer MAPA has two pKa values (pKa_1 2.58, pKa_2 9.24). The charge of column can be arranged with changing the pH of running buffer. Denizli et al synthesized aminoacid based polymers and seperated some biomolecules by using these polymers. They prepared glutamic acid based poly(BMA-EDMA-MAGA) and used for the separation of hydrophobic aminoacids in CEC system. BMA was used as hydrophobic part and MAGA was used as EOF supplier [7]. Denizli et al also used same polymer system as a chiral monolithic column for the enantioseparation of hydrophobic D,L-amino acids. [6], aniline and acids [25]. In all studies, two different monomers were used as EOF supplier and hydrophobic matrix separately. The novelty of this work is, hydrophobic monomer MAPA was used as both EOF

supplier and hydrophobic matrix because it has multiplicity character due to charged and hydrophobic groups of phenylalanine aminoacid group. This new column with mixed-mode characteristic of reversed-phase (RP) and strong ion exchange functionalities was then applied as the stationary phase in CEC for the selective separation of BPA in MIP system. An important advantage of this approach is that the monolithic column does not require further functionalization. Our study is the first report using aminoacid based monolith which contains just one monomer type which is used as both EOF supplier and hydrophophic matrix for the separation of BPA.

The performance of monolithic column was investigated by means of electrochromatographic separation of alkyl benzenes. Finally the optimized MIP column with multiplicity character was used for the separation of BPA as selectively by using capillary electrochromatography method **at** different separation conditions i.e. pH, electrical field and buffer content. Imprinting effect was evaluated on the separation performance of monolithic MIP column by comparing with nonimprinted NIP column as electrochromatographically.

2. Materials and Methods

2.1 Instrumentation

Separation performance of BPA imprinted monolithic column is investigated by using a Prince CEC-760 equipped with a photodiode array detector (Prince Technologies B.V. Cornelis Houtmanstraat 267825 VG Emmen The Netherlands). A μHPLC (micro-high performance liquid chromatography) pump is used to flush and condition of the monolithic columns.

2.2 Reagents and Materials

Ethylene dimethacrylate (EDMA), 3-trimethoxysilylpropyl methacrylate (TMSPM) and bisphenol A are purchased from Sigma-Aldrich (Milwaukee, WI, USA). Fused-silica capillaries (i.d. 100 mm and o.d. 375 mm) are supplied by Polymicro Technologies (Phoenix, AZ, USA). Alkyl benzenes are obtained from Merck AG while, 2,2-bis(4-hydroxyphenyl) propane (bisphenol A) and phenol are supplied by Sigma-Aldrich. Methanol and acetonitrile (ACN) (HPLC grade) are obtained from Merck A.G (Darmstadt, Germany). N-methacryloyl-L-

phenylalanine (MAPA) is supplied from Nanoreg (Ankara, Turkey). All the test compounds are of analytical grade.

2.3 Preparation of BPA imprinted monolithic column

The inner surface of fused silica capillary was modified for silanization to ensure anchoring of the monolithic polymer matrix to the wall of capillary column [25]. The procedure involves the following steps; to clean and activate the inner surface of the capillary, the fused silica capillary was first etched by flushing the capillary with a solution of 0.2 M NaOH for 3 h at 750 mbar pressure and then with water, methanol and nitrogen for 15 min respectively at the same pressure. TMSPM–methanol (50/50 v/v) solution was filled by applying same external pressure in CEC. The capillary monolithic column was plugged at both ends with GC septa and submerged into a thermostatic bath at 35^{o}C for 15 h. TMSPM provides to ensure covalent bonding of the monolith to the capillary inner wall. The capillary column was washed with methanol for 15 min and dried by flushing N_2 for 30 min at room temperature. BPA-imprinted monolithic column (MIP) was prepared by using EDMA as a crosslinker, MAPA as a functional monomer, BPA as a template, ethanol as a porogen and AIBN as a initiator. The polymerization mixture was sucked into the pretreated silica capillary columns of 27 cm in effective length and 36 cm in total length by applying an external pressure of 750 mbar in CEC. After plugging of the column at both ends with GC septa, the column was put into a thermostatic bath at 70^{o}C for 2 h. The monolithic columns were washed with methanol for 2 h by using µHPLC to remove the unreacted monomer and then washed with 0.1 M NaOH for template removal then equilibrated with water. The NIP matrix (without addition of template molecule) was prepared in an identical manner without BPA. The monomer solution was introduced through the column with an CEC instrument at 2000 mbar. To obtain optimized monolithic capillary column we evaluated different parameters including polymerization time, crosslinker ratio and functional monomer ratio. Except than MIP1 column, back pressure values too high and there was no permability. For further studies MIP1 column was selected for evaluation of electrochromatographic studies and named as MIP in the continuous figures. The polymerization content was given in Table 1.

The chemical structure of the MIP column was shown in Figure 1. Following polymerization, the continuous bed column was ready for use. Since the bed is attached covalently to the tubing wall,

no frit is required to support the bed, which simplifies the preparation of the column. A detection window was made at the end of the polymer bed using a microtorch. Polymerization recipes used for the preparation of BPA-imprinted and non-imprinted (without template, BPA) monolithic columns was shown in Table 1.

Table 1. Polymerization recipes for the preparation of monolithic columns.

Figure 1. The molecular formula of MIP monolithic column.

2.4. Electrophoresis conditions of capillary electrochromatography

BPA-imprinted monolithic column is equilibrated with ACN/phosphate buffer by using HPLC pump for 2 h then the column is connected to CEC system. Mobile phases are prepared by mixing appropriate volumes of acetonitrile (ACN) and phosphate buffer (PB) solution. The column is equilibrated with a PB containing ACN in CEC system for 30 min then the separation procedure is carried out following application of the sample with buffer containing pH 7.0 (10 mM, PB) and ACN as organic phase. Mobile phase content ratios (ACN/PB), mobile phase pH (5.5, 7.0, 11) and applied voltages (5 kV-30 kV) is changed for optimization of separation. Thiourea is used as a marker. The applied voltage is changed between 5 kV and 30 kV. The column performance of the MIP based column is estimated by using alkyl benzenes with unretained marker, thiourea (THA). Alkyl benzenes are liquid, whereas the other samples used in this study are solid. Sample mixture is prepared by dissolving each compound in the mobile phase. The concentration of alkyl benzenes and THA is 0.5 mg/ml in the sample mixture. The concentrations of BPA and phenol are 1.5 mg/ml respectively. All samples are injected electrokinetically at 5 kV for 3 s. The columns are kept at room temperature (25 ^{o}C).

The binding characteristic of the imprinted polymer was evaluated to determine binding strength of BPA by calculating imprinting factors using Equation 1;

$$IF = k_{MIP}/k_{NIP} \quad (1)$$

Where IF is imprinting factor, $k_{MIP\ and}$ k_{NIP} are retention factors of MIP and NIP columns calculated by using retention times ($k = (t_R - t_0)/t_0$).

The monolithic column permeability K, is measured by flowing methanol solution through the column. Permeability is calculated using Equation 2;

$$K = (F\eta L) / (\pi r^2 \Delta P) \quad (2)$$

where, F is flow rate, η is viscosity, L is length of the column, r is internal radius of the column and ΔP is pressure drop. The electro-osmotic mobility, μ_{eof}, is calculated by the following equation:

$$\mu_{eof} = L_e L_t / V t_R \quad (3)$$

where L_e is the length of the column from the inlet to the detection window, L_t is the total length of the column, V is the applied voltage and t_R is the elution time of unretained compound, EOF marker, THA. The theoretical plate number (N) is calculated using Equation 4 for a column with a length of L_e:

$$N = 5.54\ (t_r / W_{0.5})^2 \quad (4)$$

in which t_r and $W_{0.5}$ are the retention time and peak width at half-height, respectively. The plate height (H) is found by using Equation 5:

$$H = L/N \quad (5)$$

3. Results and Discussion

3.1. Preparation and characterization of the BPA imprinted monolithic column

Recently, polymer-based stationary phases are used as an alternative to conventional modified silica based polymers. Problems, encountered with packed bed capillaries such as packing of beads into a tube with very small diameter, formation of bubbles within the capillary during runs are the main driving forces for the development of the polymer-based stationary phases. The

polymer-based phases are prepared by in situ polymerization procedures, yielding polymer monoliths [26–31]. In general, the polymerization of monomers into the monoliths is less laborious than the packing of particles into capillary columns. Furthermore since the polymer is covalently attached to the inner surface of the capillary, frits can be avoided completely.

To prepare methacrylate-based BPA imprinted monolithic columns for CEC, MAPA was used as a functional monomer, EDMA as a crosslinker and ethanol as a porogenic solvent. MAPA provides hydrophobicity and affords negatively charged functionalities at pH > pI (pI 5.48) to generate cathodic EOF. Phenylalanine and charged carboxyl groups in MAPA structure behave as a mixed mode stationary phase.

The pressure drop across the MIP and NIP columns was measured as a function of linear velocity using methanol. Results showed a good permeability for the MIP column with lower back pressure values. An advantage of the in situ technique of a column with MIPs without any tedious steps is its high reproducibility and rapid mass transport. Furthermore, the preparation of this type of MIP is more cost-efficient because it requires much smaller amounts of template molecules. However, the MIP column often suffers from high back pressures and low efficiency that result in poor application and practical separation. In order to acquire a monolith with a high selectivity and low back pressure, a MIP is prepared in a chromatographic column using a non-covalent imprinting technique. At a flow rate of 5.0 μL/min, the pressure drop of the MIP and NIP column (id. 100 μm; effective length: 27 cm; total length: 36 cm) is 14 bar and 20 bar respectively at this flow rate. The MIP column prepared with lower back pressure values has cavities to allow the mobile phase to flow through the column. Pressure drop versus the velocity of the fluid for MIP column shows a linear relationship, this indicates that permeability and mechanical stability of the prepared monolithic stationary phase are excellent. Pressure drop increased with increasing flow rate from 1.0 μL/min to 5.0 μL/min of methanol as linearly for MIP (R^2: 0.959) column. Linearity deviation of NIP column (R^2: 0.7639) may be originate from not existed cavities to facilitate hydrodynamic flushing. Figure 2 shows the backpressure values of the each monolithic columns.

A very important characteristic of a column is its permeability which represents the resistance to mobile phase flow through the monolithic column. Permeability can be determined by pumping different solvents through the column at different linear flow rates. The permeability is

calculated as $3.7.10^{-11}$ m^2 for MIP, $2.9.10^{-11}$ m^2 for NIP column. The permeability of the NIP monolith is found as slightly lower with respect to the MIP column at constant flow rate.
Figure 2. Back pressure values of MIP and NIP columns.

The scanning electron microscope (SEM) images of the empty silica, MIP and NIP monolithic columns (E) are shown in Figure 3. As shown in SEM images, the monolithic columns are well attached to the capillary wall. SEM images show that MIP and NIP monolithic columns were composed of spherical micro-globules (2 μm) agglomerated into larger clusters inter dispersed by large pore channels, which are characteristic structure of monolithic columns. The SEM images of the MIP and NIP columns show that macroporous structure didn't change dramatically with the imprinting of column. In Figure 3, [A-1], [A-2] refers to empty and [B-1] [B-2] refers to MIP column, [C-1] [C-2] refers to NIP column.

Figure 3. SEM images of columns; empty column [A], MIP column [B] and (NIP) column [C] with different magnifications; 600x, 1850x for [A-1], [A-2] and 600x, 2000x for [B-1] [B-2] MIP column and [C-1] [C-2] NIP column.

The column performance of the MIP column was estimated by using alkyl benzenes with unretained marker THA. Electro-chromatographic behaviour of MIP column was evaluated using a mobile phase containing pH 7.0, 10 mM PB and ACN with different voltages. Figure 4 shows evaluation of column performance for alkyl benzene separation by CEC. The applied voltage is 15 kV for [A] and 20 kV for [B] for the analysis and 5 kV for the injection of 0.25 min sample loading time. Separation of alkyl benzenes namely, ethyl benzene (EB), propyl benzene (PB) and butyl benzene (BB) was performed by using ACN/PB (pH 7.0 10 mM) as a mobile phase. THA was used as unretained marker. The ACN concentration was chosen as 50% (v/v) in experiments. Elution order of the alkyl benzenes are in the range; ethyl benzene > propyl benzene > butyl benzene, respectively. These results can be explained on the basis of hydrophobic interactions between aromatic groups of MAPA and alkyl benzenes. Butyl benzene eluted lately because it has more hydrophobic methylene groups existed in the structure. Increasing of electrical field from 15 kV to 20 kV resulted in decrement of elution times.

Figure 4. Column performance estimation with separation of alkyl benzenes. conditions; buffer: 50% pH 7.0 15 mM PB; 50% ACN; sample: 0.5 mg/ml for THA, 0.2 µl/ml for alkyl benzenes; sample injection: 5 kV, 0.25 min; UV wavelength: 200 nm; applied voltage; [A] 15 kV; [B] 20 kV.

As shown here, a mixture of alkyl benzenes was succesfully performed and the electro-chromatographic separation was completed in 15 min with the applied voltage of 15 kV and 20 kV. The theoretical plate numbers up to 45876 plates/m for the separation of alkyl benzenes was obtained with MIP column using THA [32]. Plate height values were given for PB/ACN mixture (i.e. 50:50%, 10 mM, pH 7.0) used for the separations of homolog series of alkyl benzenes. Relatively low plate numbers explains that both electro-phoretic and strong hydrophobic interactions governs the separation. Table 2 shows plate numbers and heights.

Table 2. Plate number and heights for the separation of alkyl benzenes

3.2. Electrochromatography Separations

3.2.1. Effect of Electric Field

Electroosmotic flow (EOF) is a very important factor in electromigration technique such as CE and CEC for the understanding of the separation behavior and mechanism in CE and CEC [33]. In order to generate EOF in monoliths, incorporated ionizable functional group into monomers such as AMPS, 2-acrylamido-2-methylpropane sulfonic acid, is generally used to generate EOF in a poly(BMA-EDMA) monolithic matrix for CEC [34]. In this study, amino acid based (MAPA) monolithic MIP column is used as a novel weak cation exchange monomer, which also has the capability to produce EOF. THA is used as an EOF marker for the EOF measurements. The direction of EOF is determined by the sign of the net surface charge. Monolithic columns with charged groups generates anodic or cathodic EOF depending on different pH values of the mobile phase. The EOF in the continuous beds derivatized with MAPA originates from the carboxylic acid (-COOH) and amino ($-NH_2$) functional groups of the phenylalanine part of MAPA monomer. Phenylalanine has a pI of 5.48 (pKa_1 2.58, pKa_2 9.24) but the actual pI value in the monolithic column is unknown. Deprotonation of the acid functionalities increases with

the increasing pH of the mobile phase. Separation of BPA is performed with mobile phase containing ACN as organic phase and pH 7.0 PB. Carboxyl groups at pH 7.0 appear to be ionized (negatively charged) resulting in a stable EOF. The column exhibited cathodic EOF in different pH values of the mobile phase higher than pI of phenylalanine (pI: 5.48). In this respect, the continuous beds have advantage over beds of packed silica beads, which have a very small EOF at low pH and poor chemical stability at a pH above 8 [35]. The direction of EOF at different pH values is shown in Figure 5. As shown at pH values higher than pI of MAPA shows cathodic EOF because of deprotonation.

Figure 5. A schematic representation for the electro-osmotic flow formation in the MIP column operated in electro-chromatography mode with cathodic electro-osmotic flow.

The continuous beds can be used over a broad pH range, which is important, since one has the freedom to choose the pH that affords optimum resolution. At pH 7.0 separation of BPA is performed selectively. Figure 6 shows separation of BPA from phenol at pH 7.0 with different applied electrical field (5 kV, 10 kV, 15 kV, 20 kV). Retention time of BPA is shortened with the increment of applied voltage. BPA eluted lately in each time because of strong interaction between BPA and MIP column which has specific cavity for imprinted BPA. The elution order of BPA is according to hydrophobicity as well EOF at neutral pH. Selective separation of BPA, its pKa (pKa 9.9-11.3) is higher than the pH of running buffer (pH 7.0) could be performed by hydrophobic interactions and electro-osmotic flow because there is no electrostatic interactions such as repulsive or attractive forces between charged groups of MAPA amino acid used as chromatographic surface and neutral molecule BPA at this pH. Analysis of BPA is difficult in CEC because of its relative acidity. Tendency of migration is generally against EOF especially at higher pH values because of deprotonation of phenolic groups. Ion-supressed mode using low pH mobile phase is recommended to separate charged molecules. Monolithic surface is weakly negative charged at pH 7.0, which results in the direction of EOF from anode to cathode. Therefore, in addition to cathodic EOF, separation mechanism is governed mainly both by electrophoretic mobility and chromatographic partition between the stationary phase and the mobile phase.

Figure 6. Separation of BPA selectively from aqueous media containing competitor reagent phenol by MIP column. conditions; buffer: 50% pH 7.0 10 mM PB; 50% ACN; sample: 1.5 mg/ml phenol and BPA, 0.5 mg/ml THA; sample injection: 5 kV, 0.15 min; UV wavelength: 200 nm ; applied electrical field A) 5 kV; B)10 kV C)15 kV D) 20 kV.

3.2.2 Effect of pH

Buffer pH effect on electro-chromatographic separation was also investigated. The charge of all ionizable groups of the monolithic column is controlled by the buffer pH of the mobile phase. Net surface charge density of charged groups such as carboxylic acid and secondary amino groups of the MAPA affords charged functionalities to generate EOF and provides ion-exchange interactions. In this structure, MAPA provides a hydrophobic surface also. The use of amino acid based monomer for the synthesis of monolithic column which has dual character is one of the originalities of this novel approach. Magnitude of ionization depends on pI of phenylalanine and pH of buffer. Figure 7 shows the electro-chromatographic separation of BPA from phenol at different pH values with ACN/PB buffer ratio of 50/50 v/v determined as the most appropriate value by the experiments. There are several reports in the literature on polymethacrylate-based monoliths most of them need post-functionalization with different chromatographic ligands. The original side of the present study is monolithic column can be utilized without modification both in anodic and cathodic flow modules. As seen in Figure 8 when a buffer pH is 5.5, the surface charge of monolithic stationary phase **is** almost zero and weakly cathodic EOF is observed (phenylalanine pI: 5.48). pKa value of BPA is between 9.9 and 11.3 so at wide range pH values dissociation of BPA is suppressed. Elution of molecules to be separated is based on mainly hydrophobic interactions between phenyl side chain of column and phenolic groups of molecules, i.e. separating BPA and phenol at low pH value. Longer retention time of BPA may be due to longer residence time in template-shaped cavities created in polymer matrices memory with template. It is confirmed that hydrophobic interactions are mainly responsible for the separation of the BPA by the MIP column. When buffer pH is increased to 7.0 elution times decreased. Because of dissociation of carboxyl groups increment and protonation decrement of secondary amine groups results in negatively charged surface of monolithic column and cathodic EOF (pKa_1 1.83 and pKa_2 9.13). As pH is increased further to pH 11, ionization of carboxylic

acid group and deprotonation of amine groups results in negatively charged column and cathodic EOF also. At pH 11, the monolithic surface is negatively charged totally. Under these conditions, the direction of EOF is from anode to cathode. Therefore, cathodic EOF is observed. But retention times of molecules didn't decrease so much. The effective separation is observed by means of the hydrophobic interaction between the surface of the column and the analytes and reverse migration of negatively charged molecules as well. With increasing pH of the buffer, the dissociation of carboxyl groups becomes stronger, the ionization of secondary amine groups becomes weaker thus cathodic EOF is observed. In spite of the EOF increment, retention times again increased when compared to pH 7.0. At this pH, phenolic groups of BPA remains slightly negatively charged. At pH 11 both electro-osmotic flow and hydrophobic interactions play combined role on separation so multivariate parameters affect the analysis. At higher pH values, number of negatively charged groups of MAPA increase so EOF is expected to increase but slightly negatively charged BPA molecules tend to migrate against EOF. Hydrophobic interactions weakens at high pH values because of hydrophobic interactions between aromatic groups of phenylalanine and molecules to be separated weakens. As a result with the increasing pH values molecules are eluted much later than pH 7.0. Accordingly, monolithic surface is negatively charged, which results in the direction of EOF from anode to cathode. Therefore, in addition to cathodic EOF, BPA separation mechanism in this case is governed mainly by both electrophoretic mobility and chromatographic partition between the stationary phase and the mobile phase. At pH 11, retention times of molecules take shorter time than at pH 5.5 and take longer time than at pH 7.0. Electrophoretic mobility of moecules decreased from $1.8.10^{-4}$ to $9.0.10^{-5}$ for pH 7.0 and pH 5.5 respectively and $1.4.10^{-4}$ $m^2.v^{-1}.s^{-1}$ for pH 11 at 15 kV. Figure 8 shows the EOF for marker molecule by MIP column at different pH and electrical field values. From these results, we can suggest that the electro-chromatographic separation mechanism of the BPA based on hydrophobic interactions at pH 5.5, both EOF and hydrophobic at pH 7.0 and pH 11. In other words, hydrophobic interaction between MIP matrix and BPA molecules gets weaker when pH is increased. So main factor on separation at pH 11 is both EOF and hydrophobic interactions. EOF measurements with different pH values are successfully achieved and shown in Figure 8.

Figure 7. Separation of BPA selectively from aqueous media containing competitor reagent by MIP column. conditions; buffer: 50% 10 mM PB; 50% ACN; sample: 1.5 mg/ml phenol and BPA, 0.5 mg/ml THA; sample injection: 5 kV, 0.15 min; UV wavelength: 200 nm; applied electrical field : 15 kV A) pH 5.5 B) pH 7.0 C) pH 11.

Figure 8. EOF of MIP column for thiourea at different pH and electrical field; applied voltage: 5, 10, 15, 20 kV and mobile phase pH; 5.5, 7.0, 11; buffer: 50% 10 mM PB; 50% ACN; sample: 1.5 mg/ml THA; sample injection: 5 kV, 0.15 min; UV wavelength: 200 nm; applied electrical field : 15 kV A) pH 5.5 B) pH 7.0 C) pH 11.

3.2.3 Effect of Organic Solvent content on separation

Effect of organic solvent content is also investigated on the separation of BPA selectively at different percentage of ACN and pH 7.0 PB. Acetonitrile content in the mobile phase affects the magnification of EOF. The level of separation capacity depends on EOF. For this reason, the effect of mobile phase compositions on the separation mechanism was also investigated. Figure 9 shows the effect of ACN concentration on EOF. With the increase of ACN content in mobile phase, EOF decreases. In this study, the optimized mobile phase adjusted to pH 7.0 is used for the separation of the BPA from competitor agent phenol with different ACN content. The surface of the monolithic column is also negatively charged at pH 7.0 but degree of ionization depends on pH of buffer. Thus, the column exhibited cathodic EOF, which results in the direction of EOF from anode to cathode.

When buffer ratio (pH 7.0) is increased from 50% to 55%, decreased elution times is expected. It can be explained that the deprotonation of carboxyl group increase and carboxyl groups appear to be ionized more than 50% at 55% PB ratio. Accordingly, more negatively charged surface of monolithic column, more EOF and shorter elution times could be observed. But in our study increment of PB ratio resulted in longer retention times. A decrease in the concentration of acetonitrile increased the interaction between the nonpolar group of solutes and the stationary phase. Further increment of BP ratio to 60% results in negligible changing of retention times. This may be due to all chargable groups of column was negatively charged at 60% and further increment didn’t increase the number of negatively charged groups. Interaction of nonpolar

groups remained optimal and same with 55% due to hydrophobic interactions. Migration time effect with a lowering of the concentration of acetonitrile verified by experiments is shown in Figure 9. So separation of the solutes at lower ACN ratio is governed mainly by hydrophobic interaction chromatography.

Figure 9. Separation of BPA selectively from aqueous media containing competitor reagent by MIP at different buffer content ratios. conditions; sample: 1.5 mg/ml phenol and BPA, 0.5 mg/ml THA; sample injection: 5 kV, 0.15 min; UV wavelength: 200 nm ; applied electrical field : 15 kV (A) buffer: 50% pH 7.0 10 mM PB; 50% ACN; (B) buffer: 55% pH 7.0 10 mM PB; 45% ACN (C) buffer: 60% pH 7.0 10 mM PB; 40% ACN.

3.2.4. Separation performance

NIP colum used as reference, which did not contain the template BPA, is also prepared in parallel with the MIP by using the same process is used for comparative study of BPA separation from phenol. As shown in Figure 10, NIP column couldn't separate BPA and phenol effectively. MIPs possess a three-dimensional memory cavity for the template molecule. The cavity is complementary in shape, size and functional group orientation with respect to the template molecule, so MIPs can specially recognize the template molecule from mixtures. But NIP columns don't have memory to the molecules to be separated from each other. To compare separation performance of MIP and NIP columns, experiments are performed at pH 11 phosphate buffer and ACN (50/50%).

Figure 10. Separation performance of MIP and NIP columns for BPA selectively from aqueous media containing competitor molecule phenol. conditions; buffer: 50% pH 11, 10 mM PB; 50% ACN; sample: 1.5 and 3.0 mg/ml of phenol and BPA and 0.5 mg/ml THA; sample injection: 5 kV, 0.1 min; UV wavelength: 200 nm; applied electrical field: 15 kV, A) MIP B) NIP column.

According to the CEC studies, MIP column could separate BPA from aqueous solutions of BPA in the presence of competitor molecule, phenol successfully while NIP column couldn't separate BPA from phenol. This may be there is not specific cavity memories of BPA molecules for the NIP columns. Analysis results indicated that there are more recognition sites and stronger

interaction between BPA and MIP recognition sites in the imprinted monolithic columns than that in the non-imprinted ones. On the other hand, the column indicates that the BPA show higher elution times than phenol molecules. From these results, we can suggest that the electro-chromatographic separation mechanism between BPA and BPA imprinted matrix **is** based on interactions of the cavities remaining throughout the monolithic column after polymerization as well as EOF. In other words, the interaction between phenol and matrix is weak. However, the interaction between BPA and matrix is strong. For this reason, phenol molecules migrate faster than BPA on the amino acid based, BPA imprinted monolithic column.

Separation performance of MIP column for BPA from aqueous media containing competitor molecule is evaluated by using competitor molecule phenol or not. In Figure 11A, separation is performed by using phenol molecule. To check the last migrated compound whether BPA or not sample preparation is performed without competitor phenol molecule. As seen in Figure 11B, BPA eluted with the same retention time of Figure 11A. The retention factors for MIP (k_{MIP}) and NIP (k_{NIP}) columns was calculated as 0.35 and 0.16 respectively. The imprinting factor, calculated by the ratio of k_{MIP}/k_{NIP} is 2.18 supports the design and choice of BPA as a template.

In literature, Lili Zhu et al [36] developed electrochemical sensor based on magnetic molecularly imprinted nanoparticles for determination of BPA. They prepared nanoparticles having regular morphology, high saturation magnetization and good monodispersity. They demonstrated that the response of BPA on imprinted electrode was 2.6 times as much as that on non-imprinted sensor.

The superparamagnetic surface molecularly imprinted Fe3O4@MIP nanoparticles for bisphenol A (BPA) were prepared by Jizhong Liu et al via surface initiated atom transfer radical polymerization (si-ATRP). The Fe3O4 core was compactly encapsulated with a polychloromethylstyrene (PCMS) layer via mini-emulsion polymerization. The BPA imprinted Fe3O4@MIP revealed specific selectivity and high affinity to the template BPA over structural analogues. Moreover, the surface-imprinted MIP nanoparticles showed good site accessibility for BPA. The imprinting factors for BP is 1.48 [37]. Naoko Inoue et al prepared a hydrophilic molecularly imprinted polymer (MIP) for the hydrophobic compound bisphenol A (BPA) in aqueous solution using 3-acrylamidoN,N,N-trimethylpropan-1-aminium chloride (AMTC) as the functional monomer, The MIP showed the highest activity among the three polymers, and the imprinting factor as

calculated from the amount of BPA bound to the MIP divided by the amounts bound to NIP is 1.8 [38]. Magnetic molecularly imprinted polymers (MMIPs) for bisphenol A (BPA) were firstly prepared by miniemulsion polymerization method and were used as selective adsorbents of BPA by Zhu li-li and coworkers. The MMIPs NPs exhibited a regular morphology and good monodispersion and the size was 123 nm. The imprinting factor was 3.5 [39]. In spite of the reduced retentive characteristics for monolithic columns [40]. In this study, BPA imprinted monolithic capillary MIP column was used successfully to determine BPA from aqueous water with satisfactory imprinting factors which was estimated as 2.18.

Figure 11. Separation performance of MIP column for BPA from aqueous media containing competitor molecule phenol [A] with BPA and without BPA [B]. conditions; buffer: 50% pH 11, 10 mM PB; 50% ACN; sample: 1.5 and 3.0 mg/ml of phenol and BPA and 0.5 mg/ml THA; sample injection: 5 kV, 0.1 min; UV wavelength: 200 nm; applied electrical field: 15 kV.

4. Conclusion

A monolithic molecularly imprinted polymers (MIPs) with specific recognition ability for BPA are prepared by in-situ polymerization, using MAPA as amino acid based functional monomer, ethylene glycol dimethacrylate as crosslinking agent, ethanol as porogenic solvents and 2,2'-azobisisobutyronile as initiator in a single step. Structural features of MIP and NIP column are identified by SEM. The results show that the large through-pore allows mobile phase to flow through the MIP and NIP columns with a low back pressure. The other pores lead to the molecular recognition. Preparation of these monoliths are performed by a simple, one step, in-situ, free-radical polymerization process directly within the chromatographic column, without the tedious procedures of the grinding, sieving, and column packing. Some chromatographic conditions such as pH, the composition of the mobile phase, applied electrical field are used to separate BPA and effects of molecular recognition are discussed. As a result, separation conditions such as pH, electric field and buffer composition are studied. The optimized monolithic column resulted in excellent separation of a BPA from structurally related phenol molecules within 20 min in isocratic elution condition. These experiments are performed in capillaries with 100-μm i.d. A further reduction in analysis time (about 5 min) is achieved with

increasing EOF and decreasing hydrophobic interactions (Figure 7) and at the same time increasing the field strength 4-fold (from 5 kV to 20 kV) decreases elution time 10 fold (from 50 min to 5 min). It is confirmed that both the hydrophobic interactions and Electrophoretic separation are responsible for the separation of the BPA by the MIP monolithic column. The technique of molecular imprinting creates specific recognition sites in polymers by using template molecules. It has been shown that the MIPs possess high selectivity and sensitivity for template molecules. Possible recognition mechanisms between the monolithic column and molecules to be separated are both hydrophobic interactions and EOF depending on pH of the buffer molecule and the MIP column. Selective recognition for BPA is also achieved in the cavities remaining throughout the monolithic column after polymerization. An important advantage of this study is that the surface of monolith does not require functionalization. These molecularly imprinted polymers demonstrate very good thermal and mechanical stability and can be used in aggressive media. MIPs possess several advantages over their biological counterparts including low cost and easy preparation, besides the good physical and chemical stability. An approach have been made in combining the advantages of the dual separation mode of a reversed phase and ion exchange column with high selectivity and the separation efficiency of MIP column.

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FIGURE LEGEND

Figure 1. The molecular formula of MIP monolithic column.

Figure 2. Back pressure values of MIP and NIP columns.

Figure 3. SEM images of columns; empty column [A], MIP column [B] and (NIP) column [C] with different magnifications; 600x, 1850x for [A-1], [A-2] and 600x, 2000x for [B-1] [B-2] MIP column and [C-1] [C-2] NIP column.

Figure 4. Column performance estimation with separation of alkyl benzenes. conditions; buffer: 50% pH 7.0 15 mM PB; 50% ACN; sample: 0.5 mg/ml for THA, 0.2 µl/ml for alkyl benzenes; sample injection: 5 kV, 0.25 min; UV wavelength: 200 nm; applied voltage; [A] 15 kV; [B] 20 kV.

Figure 5. A schematic representation for the electro-osmotic flow formation in the MIP column operated in electro-chromatography mode with cathodic electro-osmotic flow.

Figure 6. Separation of BPA selectively from aqueous media containing competitor reagent phenol by MIP column. conditions; buffer: 50% pH 7.0 10 mM PB; 50% ACN; sample: 1.5 mg/ml phenol and BPA, 0.5 mg/ml THA; sample injection: 5 kV, 0.15 min; UV wavelength: 200 nm ; applied electrical field A) 5 kV; B)10 kV C)15 kV D) 20 kV.

Figure 7. Separation of BPA selectively from aqueous media containing competitor reagent by MIP column. conditions; buffer: 50% 10 mM PB; 50% ACN; sample: 1.5 mg/ml phenol and BPA, 0.5 mg/ml THA; sample injection: 5 kV, 0.15 min; UV wavelength: 200 nm; applied electrical field : 15 kV A) pH 5.5 B) pH 7.0 C) pH 11.

Figure 8. EOF of MIP column for thiourea at different pH and electrical field; applied voltage: 5, 10, 15, 20 kV and mobile phase pH; 5.5, 7.0, 11; buffer: 50% 10 mM PB; 50% ACN; sample: 1.5 mg/ml THA; sample injection: 5 kV, 0.15 min; UV wavelength: 200 nm; applied electrical field : 15 kV A) pH 5.5 B) pH 7.0 C) pH 11.

Figure 9. Separation of BPA selectively from aqueous media containing competitor reagent by MIP at different buffer content ratios. conditions; sample: 1.5 mg/ml phenol and BPA, 0.5 mg/ml THA; sample injection: 5 kV, 0.15 min; UV wavelength: 200 nm ; applied electrical field : 15 kV (A) buffer: 50% pH 7.0 10 mM PB; 50% ACN; (B) buffer: 55% pH 7.0 10 mM PB; 45% ACN (C) buffer: 60% pH 7.0 10 mM PB; 40% ACN.

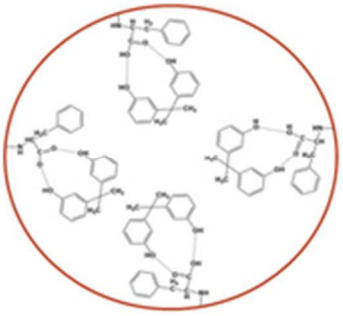
Figure 10. Separation performance of MIP and NIP columns for BPA selectively from aqueous media containing competitor molecule phenol. conditions; buffer: 50% pH 11, 10 mM PB; 50% ACN; sample: 1.5 and 3.0 mg/ml of phenol and BPA and 0.5 mg/ml THA; sample injection: 5 kV, 0.1 min; UV wavelength: 200 nm; applied electrical field: 15 kV, A) MIP B) NIP column.

Figure 11. Separation performance of MIP column for BPA from aqueous media containing competitor molecule phenol [A] with BPA and without BPA [B]. conditions; buffer: 50% pH 11, 10 mM PB; 50% ACN; sample: 1.5 and 3.0 mg/ml of phenol and BPA and 0.5 mg/ml THA; sample injection: 5 kV, 0.1 min; UV wavelength: 200 nm; applied electrical field: 15 kV.

TABLE LIST

Table 1. Polymerization recipes for the preparation of monolithic columns.

Table 2. Plate number and heights for the separation of alkyl benzenes.



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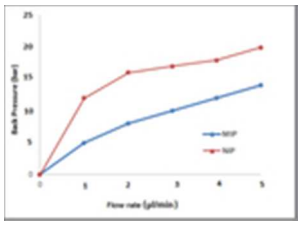

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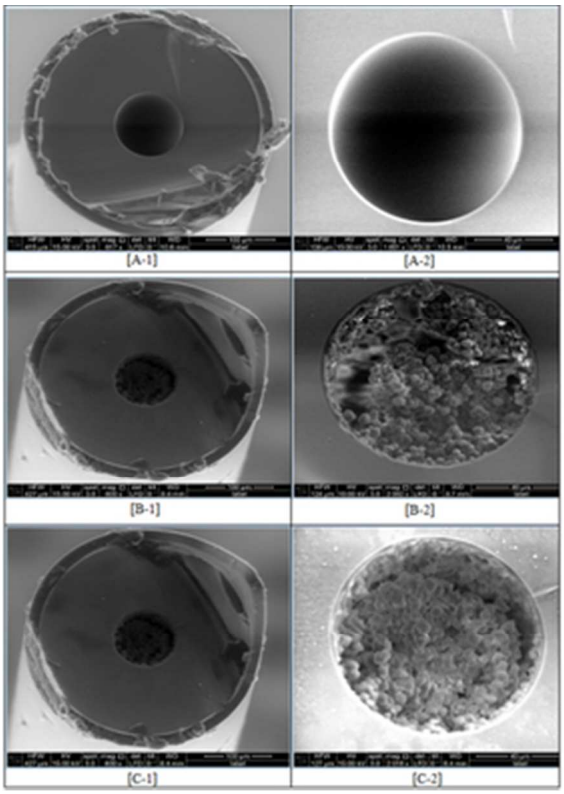

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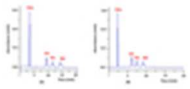

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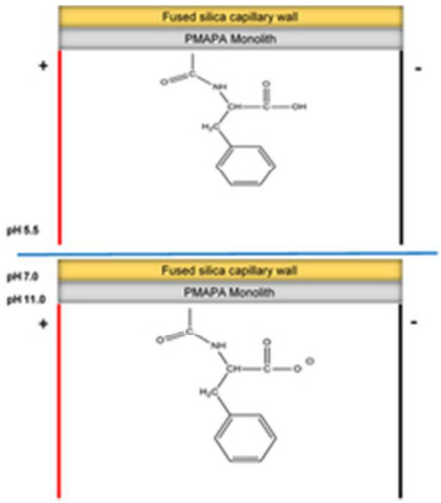

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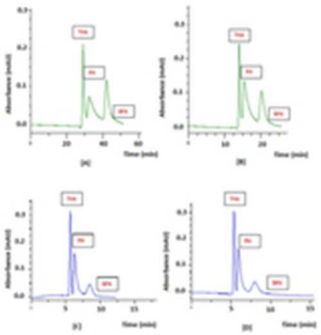

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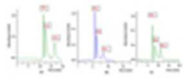

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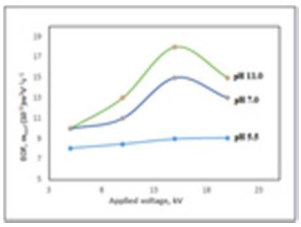

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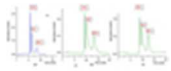

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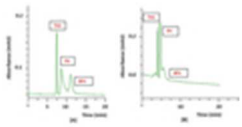

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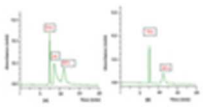

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Table 1. Polymerization recipes for the preparation of monolithic columns.

	EtOH mL	EDMA mL	MAPA mL	BPA mg	AIBN mg	T °C	Time h	permeability
MIP1	2.6	0.9	1.075	90	22.5	70	2	yes
MIP2	2.6	0.9	1.075	90	22.5	70	4	no
MIP3	2.6	1.8	1.075	90	22.5	70	2	no
MIP4	2.6	0.9	2.150	90	22.5	70	2	no
NIP	2.6	0.9	1.075	-	22.5	70	2	yes

Table 2. Plate number and heights for the separation of alkyl benzenes

	pH	THA	EB	PB	BB
N (plates/m)	5.5	45876	12499	6404	4343
	7.0	35456	12133	3462	1246
h (m)	5.5	$5.8.10^{-6}$	21.10^{-6}	42.10^{-6}	62.10^{-6}
	7.0	$7.6.10^{-6}$	22.10^{-6}	77.10^{-6}	216.10^{-6}

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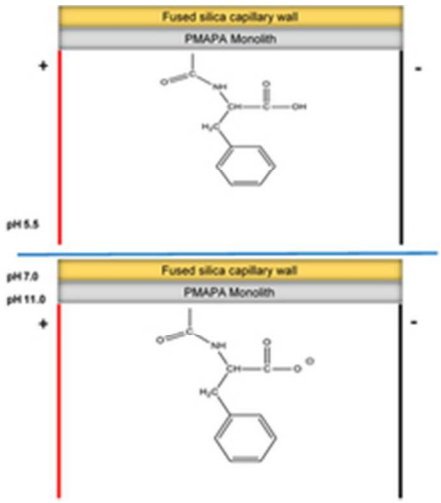
5x1mm (300 x 300 DPI)



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