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Development and characterization of an immunoaffinity monolith for selective on-line extraction of bisphenol A from environmental water samples

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ABSTRACT

Matrix interference and contamination from analytical procedures are two major issues in the detection of trace level of bisphenol A (BPA) in environmental water. In this paper, we report a highly selective and efficient method for on-line extraction of BPA from water samples followed by quantification with liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI/MS/MS). Poly(ethylene dimethacrylate-glycidyl methacrylate) monolith was synthesized in 50 mm × 4.6 mm i.d. stainless steel cartridges and the epoxy-groups on the surface of the monolith were hydrolyzed and oxidized to aldehyde functions. Antibodies against BPA were covalently immobilized onto the monolithic column via Schiff base reaction. The optimum application buffer and elution buffer were found to be pH 5.5 phosphate buffered saline (PBS) and methanol–water (70:30, v/v), respectively. The obtained immunoaffinity monolithic columns were on-line coupled to LC–MS/MS using column-switching valves and the system was applied to analyze BPA in real environmental water samples. The method achieved a detection limit of 0.3 ng L^{-1} using a sample volume of 100 mL. The linear calibration range was 1.0–160 ng L^{-1}. Samples including tap water, lake water and effluent from municipal sewage treatment plant were all measured with satisfactory results.

1. Introduction

Bisphenol A (BPA) is an estrogenic high-production chemical used as monomer component of polycarbonate plastics and epoxy resins [1–3]. Due to the extensive use of these two products, BPA has been frequently detected in environmental water samples [4–6]. With the increasing concern of the toxicity and estrogenic activity of BPA at very low concentration [7–10], highly sensitive and reliable detection methods are demanded for routine analysis of BPA in environmental water samples to reduce the potential human exposure risk.

For preconcentration of trace BPA in water samples, many approaches have been developed, such as polarity-based extraction [11,12], molecularly imprinted polymer [13] and continuous flow liquid membrane extraction [6,14]. But most of them showed limited selectivity and enrichment capacity. Another important issue in BPA detection is to avoid the contamination introduced by the sample pretreatment or analytical procedures. Watabe et al. [6] found that manual preparation and injection of water samples might introduce considerable BPA levels during the analysis and overestimate the BPA concentration.

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In our previous studies, we had developed an immunoaffinity column for the determination of BPA in serum [15]. The columns had been prepared by immobilization of BPA antibodies on CNBr-activated Sepharose 4B, which showed excellent selectivity for BPA with little non-specific adsorption. However, the soft mechanical property of the support prevented its on-line application in high-performance liquid chromatographic instruments, resulting in low-throughput and relatively high risk of contamination from manual operation. Silica-based immunoaffinity materials have been successfully developed and applied for on-line extraction of a number of real systems [16–18]. In our preliminary study, however, the silica support with immobilized irrespective proteins to BPA showed notable non-specific adsorption to this compound [19]. Therefore, we moved to a new type of stationary phase, the monolithic material, which has shown great potential in application in affinity chromatography [20,21]. Since the poly(ethylene dimethacrylate-glycidyl methacrylate) (EDMA-GMA) monolith was first reported by Svec and Huber [22], it has been widely used as a support for immobilization of biological macromolecules [23–27]. In this study, we attempted to synthesize this material in a 50 mm × 4.6 mm i.d. stainless steel cartridge and immobilize anti-BPA antibodies on the surface of the monolith. The binding performance of the generated immunoaffinity monolith was fully optimized and characterized. The established method offers a highly efficient and reliable approach for quantitative analysis of trace level of BPA in various environmental water samples.

2. Experimental

2.1. Chemicals and reagents

Bisphenol A (BPA, >99% pure) standard, glycidyl methacrylate (GMA, 97% pure), ethylene dimethacrylate (EDMA, 98% pure), bovine serum albumin (BSA), ovalbumin (OVA), sodium borohydride (98% pure), sodium cyanoborohydride (94% pure) and azobisisobutyronitrile (AIBN, 98% pure) were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). 1-Dodecanol (DoOH, 98% pure), cyclohexanol (CyOH, 98% pure) and periodic acid were all obtained from Beijing Chemical Co. (Beijing, China). All other chemicals were of the highest purity commercially available and were used without further purification.

Precautionary measures have been taken to reduce BPA contamination from the analytical procedure. BPA-free water was obtained by passing distilled demineralized water through Supelco C18-SPE column (Bellefonte, PA, USA) precleaned with methanol. The quality was controlled by liquid chromatography–tandem mass spectrometry (LC–MS/MS) detection. In addition, all of the glassware used to prepare the standards and collect the samples for analysis was treated at 300 °C to eliminate residual BPA contamination [6].

Three types of environmental water samples were collected and measured, including tap water, lake water and effluent from municipal sewage treatment plant (MSTP). All samples were filtered with 0.45 μm membranes pre-cleaned with BPA-free water and stored at 4 °C before analysis.

2.2. Instrumentation

A 2PB00C peristaltic pump from Beijing Satellite Factory (Beijing, China) was used to deliver the solvents (or antibody solutions) through the monolith column during the preparation. A Waters Lambda-Max 481 LC Spectrophotometer from Waters (Milford, MA, USA) was used to monitor the on-line UV absorption signals of proteins flowing out of the column.

An Agilent 1100 HPLC with an ultraviolet detector (UVD) was used to optimize and evaluate the performance of the obtained immunoaffinity monolithic column (IAMC). The IAMC was connected to a C18 column (Dikma Techonologies Diamonsil, 5 μ, 150 mm × 4.6 mm) using a Rheodyne 7725 manual injector (Cotati, CA, USA) as the switching-valve. The flow-rates of the mobile phases were all kept at 0.5 mL min^{-1} and the detection wavelength was set at 227 nm.

2.3. Synthesis of the poly(EDMA-GMA) monoliths

The poly(EDMA-GMA) monolith was synthesized by an in situ bulk polymerization using a modification of previously reported methods [23,29]. Two empty home-made stainless steel cartridges (50 mm × 4.6 mm i.d.), with both ends open and all the frits and outside wall wrapped with polytetrafluoroethylene (PTFE) film, were vertically put into a 50 mL centrifuge tube. In a clean and dry flask, GMA, EDMA, CyOH and DoOH at a volume ratio of 12:8:21:9 were added and mixed thoroughly. To per millilitre of the mixture, 4.7 mg of AIBN was added as the initiator. The solution was ultra-sonicated for 3 min and then purged with nitrogen for 3 min before slowly pouring into the centrifuge tube until the two cartridges were totally immersed in the mixture solution. The tube was sealed and put in a water bath. The temperature of the water bath was increased from room temperature to 80 °C in an hour and then held at 80 °C for 23 h. After the polymerization was completed, the cartridges were taken out of the tube and the PTFE film was removed with the outside polymer. The monolithic column was attached to the peristaltic pump and 100 mL of acetonitrile was pumped through the column at a flow-rate of 0.5 mL min^{-1} to remove the unreacted monomers and porogenic diluents.

2.4. Immobilization of BPA antibodies onto the monolith

Antibodies to BPA were prepared and purified following procedures described previously [28]. In brief, 4,4-bis(4-hydroxy-phenyl)valeric acid was coupled to BSA to prepare the complete antigen, which was used to immunize rabbits. The obtained antiserum was purified using a saturated ammonium sulfate method. The affinity constant of the antibody was found to be 1.1×10^8 L mol^{-1}. The antibodies were covalently attached on the monolith via the Schiff base reaction between the amino groups on the antibody and the aldehyde groups on the support. First, the above obtained monolith was flushed with 0.5 mol L^{-1} H_2SO_4 for 1 h at a flow-rate of 0.5 mL min^{-1}. Then the two ends of the cartridge were sealed and the column was incubated in a water bath at 60 °C for

3 h. After that, the column was washed successively with water (100 mL), 50 mg mL^{-1} periodic acid in 90% acetic acid solution (90 mL) and pH 6.0, 0.50 mol L^{-1} phosphate buffer (90 mL) to oxidize the diol groups on the support to aldehyde groups. Next, 20 mg of BPA antibody in 20–30 mL of pH 6.0, 0.50 mol L^{-1} phosphate buffer, containing 1 mg mL^{-1} $NaBH_3CN$ was pumped through the column under recirculation at room temperature. The immobilization process was monitored using an on-line UV detector and the reaction was allowed to continue until the UV absorbance at 280 nm no longer decreased. After the coupling reaction, 60 mL of 1.5 mg mL^{-1} $NaBH_4$ in 0.1 mol L^{-1} phosphate buffer (pH 8.0) was allowed to flow through the column to block the remaining unreacted aldehyde groups. Finally, the obtained IAMC was flushed with 0.01 mol L^{-1} PBS (pH 7.4) and stored at 4 °C in the same buffer until further use.

A reference column was prepared in the same way as described above but in the absence of the anti-BPA antibody.

2.5. Optimization of the operating conditions and characterization of the binding performance of the obtained IAMC

For optimization of the application buffer, BPA in different solutions were tested. The detailed operating procedure is described in the Supplementary Information. To determine the effect of pH on the adsorption, pH of the solution was changed between 4.0 and 8.0. To observe the effects of ionic strength, pure water and 0.01 mol L^{-1} PBS were compared. The bound BPA on the IAMC was eluted with 70% methanol in water and the IAMC was regenerated with 0.01 mol L^{-1} PBS (pH 7.4) after each test.

To assess the selectivity of the IAMC, 20 μL of the mixture of standard solutions of BPA, phenol and hydroquinone in the optimum application buffer at different concentrations were measured and compared.

The binding capacity of the IAMC was measured by applying 20 μL of BPA solution of different concentrations until the elution peak area no longer increase with the increase of BPA concentration. The maximum adsorption capacity was determined based on the elution peak area and corresponding calibration curve of BPA concentration versus the peak area which was directly obtained on the C18 analytical column using BPA standard solutions. The non-specific adsorption of the support was estimated by performing the same experiments as above using the reference columns.

2.6. On-line coupling of the IAMC with LC–MS/MS and application to real water samples

The IAMC-LC–MS/MS analysis was performed on HP 1100 system (Agilent Techonologies) connected to an MS^n Trap SL System with electrospray ionization (ESI) interface. BPA standard solutions or filtered environmental water samples were applied to the system under above optimum conditions. Determination of BPA was carried out in the negative-ion mode with the capillary potential of 3500 V. The drying nitrogen gas was introduced into the capillary region at a flow-rate of 8.0 L min^{-1} with the temperature set at 330 °C. The gas pressure and collision potential were set at 35 psi (2.4×10^5 Pa) and 1.05 V, respectively. Quantitative analysis was performed using selected ion *m*/*z* 212.

2.7. Comparison with the SPE offline extraction followed by LC–MS/MS quantification

For validation of the established method, the tested water samples were also extracted using off-line solid-phase extraction (SPE) cartridges (Supelco, Bellefonte, PA, USA) and analyzed using the LC–MS/MS. Before extraction, the C18-SPE columns (3 mL) were pre-conditioned by washing with 5.0 mL of methanol followed by 5.0 mL of pure water. Water samples (100–200 mL) were passed through the SPE cartridges which were then washed with 5.0 mL of pure water. Then 5.0 mL of 70% methanol in water was used to elute the retained compounds. The eluent was dried under a stream of nitrogen at 40 °C and reconstituted in the mobile phase (200–500 μL). Then 10 μL of the reconstituted sample was injected into the LC–MS/MS system for quantification.

3. Results and discussion

3.1. Preparation of the monolithic supports

Due to the slight shrinkage of the EDMA-GMA material during polymerization, voids may be formed at the monolith–column-wall interface and cause mobile phase flowing through the gap and not completely interacting with the adsorbents [21,24]. Mallik et al. managed to avoid this by using a specially designed piston frits and PEEK lined columns [24]. In our experiments, we tried to address the problem by immersing the empty cartridges with two ends open in the polymerization solutions instead of sealing the ends of the column. Besides, we compared the influences of two different positions of the cartridges (vertical vs. horizontal) and the polymerization temperature on the property of the resultant polymer. It was found that a vertical position of the cartridge in the polymerization solution and a programmatic temperature increase from room temperature to 80 °C are essential factors for obtaining a monolithic column with reproducible back pressures and uniform morphology. Characterization results of the poly(EDMA-GMA) monolith were shown in the Supplementary Information.

According to previous study [23], the immobilization method had a substantial influence on the amount of IgG immobilized on the monolith. In this study, Epoxy and Schiff base methods were both tested. Though the epoxy groups on the monolith could be used directly to react with amino groups on the proteins, in our experiment, it was found that the reaction rate of this route was only about 20% of the Schiff base method. So Schiff base method was used for all the latter experiments. The amount of immobilized antibody on the monolith was determined by comparing the antibody concentration in the circulating solution before and after the coupling reaction, and was found to be 0.21 ± 0.02 μmol IgG per gram of the dry polymer monolith ($n = 4$).

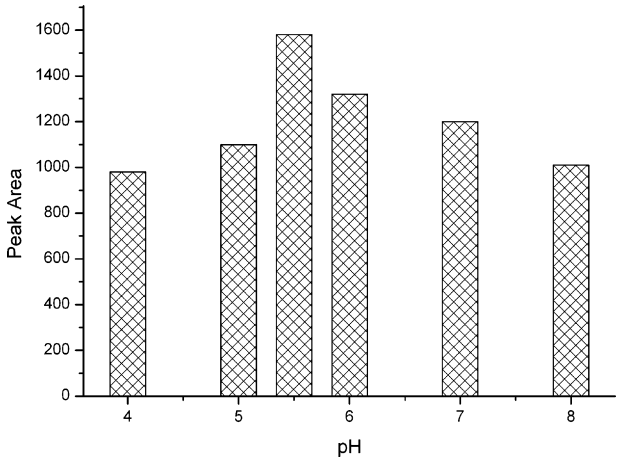

Fig. 1 – Effect of pH on the binding amount of BPA on the IAMC. BPA concentration: 20 μg mL^{-1}; detector: UVD at 227 nm; injection volume: 20 μL.

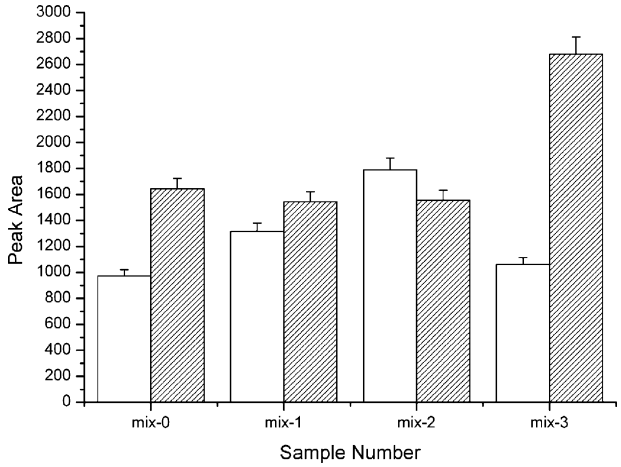

Fig. 2 – Selectivity of the obtained IAMC. Flowing-out peak (empty bar); elution peak (shadowed bar). Mix-0 (BPA 47 ng μL^{-1}, hydroquinone 17 ng μL^{-1} and phenol 47 ng μL^{-1}) is used as a reference. In mix-1, the concentration of phenol was doubled and the other two remained the same as mix-0. In mix-2 and mix-3, the concentrations of hydroquinone and BPA were doubled, respectively and the other two remained the same as mix-0. The injection volume is 20 μL.

3.2. *Binding performance of the obtained IAMC*

Since the poly(EDMA-GMA) monolith was first introduced by Svec and Huber [22], it has been synthesized in different molds such as glass tubes and stainless steel cartridges and used for immobilization of a variety of ligands such as protein G [26], antibodies [23], dyes [29], human serum albumin [24], lectin Con A [27], etc. In the existing studies, however, limited information can be found concerning the application of poly(EDMA-GMA)-based immunoaffinity monolith to extract small and relatively hydrophobic molecules from real sample systems. In this study, we first investigated the effects of different buffer conditions on the binding performance of the obtained IAMC.

The effect of medium pH on the binding ability of the IAMC to BPA was measured and the maximum binding amount was achieved at pH 5.5 in both pure water and 0.01 mol L^{-1} PBS (the data for pure water was shown in Fig. 1). This is most likely because the poly(EDMA-GMA) material is more hydrophilic at this pH which enables the antibody achieve its optimum conformation to bind with the target antigen. It was also found that the retention percent of BPA in pH 5.5, 0.01 mol L^{-1} PBS was close to 100%, while that in pure water was about 90 ± 2%, indicating that a proper ionic strength is also important for enhancing the antibody–antigen combination. The bound BPA on the IAMC was eluted with mixture solutions of methanol/water at different volume ratios and 70% methanol in water allowed nearly 100% recovery of the bound BPA (data not shown).

The selectivity of the IAMC was tested and Fig. 2 shows the observed peak area data of the flowing-out peaks and elution peaks when applying 20 μL of the mixture standard solutions containing different amount of BPA, hydroquinone and phenol. It can be seen that the flowing-out peak area increased with the increase of the amount of phenol and hydroquinone, but remained the same when the BPA amount increased. While looking at the elution peak area, it showed proportional increase with the increase of BPA amount, but remained stable with the increase of the other two compounds.

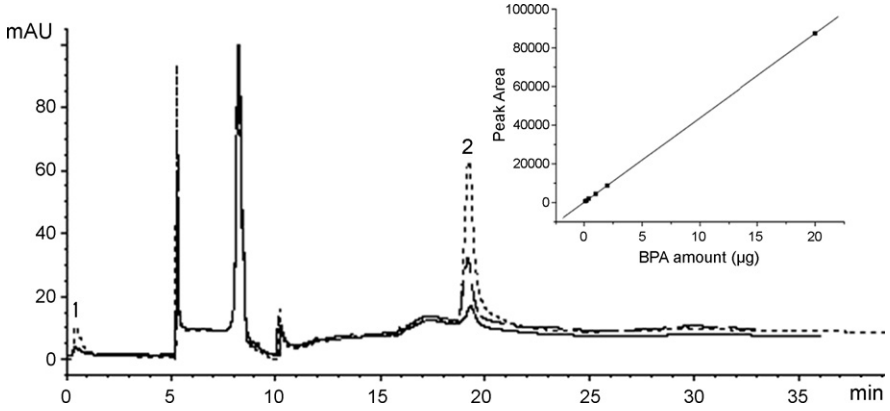

Fig. 3 – Maximum loading capacity test results of the IAMC. Peak 1: flowing-out peak; peak 2: eluted peak.

Table 1 – Results for quantitative analysis of BPA in different water samples

Samples	BPA[a]	Recovery (%)	R.S.D. (%)	BPA[b] (ng L^{-1})	Recovery (%)	R.S.D. (%)
Tap water	1.0	–	2.4	n.d.[c]	–	–
Tap water + 4.0 ng L^{-1}	4.5	87	1.5	3.1	78	2.8
Lake water	3.5	–	2.8	3.0	–	3.9
Lake water + 6.0 ng L^{-1}	10.3	110	3.2	8.0	83	4.6
MSTP effluent	70	–	4.7	28	–	5.6
MSTP effluent + 40 ng L^{-1}	115	113	4.3	49	53	6.3

[a] BPA concentration (ng L^{-1}) in the original water sample measured by IAMC extraction ($n = 3$); sample volume: tap water (150 mL), lake water (150 mL) and MSTP effluent (30 mL).
[b] BPA concentration (ng L^{-1}) in the original water sample measured by C18-SPE extraction ($n = 3$); sample volume: tap water (200 mL), lake water (200 mL) and MSTP effluent (100 mL).
[c] n.d.: not detected.

To assess the maximum binding capacity of the IAMC, BPA standard solutions at different concentrations were measured by the system and the chromatograms were recorded and shown in Fig. 3. It can be seen that with the increase of the loaded amount of BPA, the eluted peak area of BPA increased. The maximum binding capacity of the IAMC with an injection volume of 20 μL was found to be 32 μg per column. For comparison, the reference column showed a maximum binding capacity of 8.3 μg per column, about 26% of that of the IAMC. On the reference column, the predominant groups are alcohols, which may account for the above non-specific adsorption. After the immobilization of BPA antibodies, most of the alcohol groups on the IAMC are replaced by the IgG molecules and the amount of remaining alcohol groups is much smaller than that on the reference column. So the contribution of non-specific adsorption to the binding capacity of the IAMC should be insignificant. The total binding capacity of the IAMC (0.3 μmol g^{-1}) was not as large as reported BPA-imprinted polymeric microspheres column (13 μmol g^{-1}) [30], but the selectivity was greatly improved since the latter showed serious non-specific binding with phenol and other structural-related compounds. The molar binding capacity of the immobilized anti-BPA IgG on the IAMC (1.1 μmol BPA μmol^{-1} of IgG) is much larger than that of a previously reported sol–gel immunoaffinity chromatography for determination of BPA (0.14 μmol BPA μmol^{-1} of IgG) [31].

3.3. *Determination results of the real environmental water samples*

Since the pH and ionic strength of the real environmental water samples were normally between those of the pure water

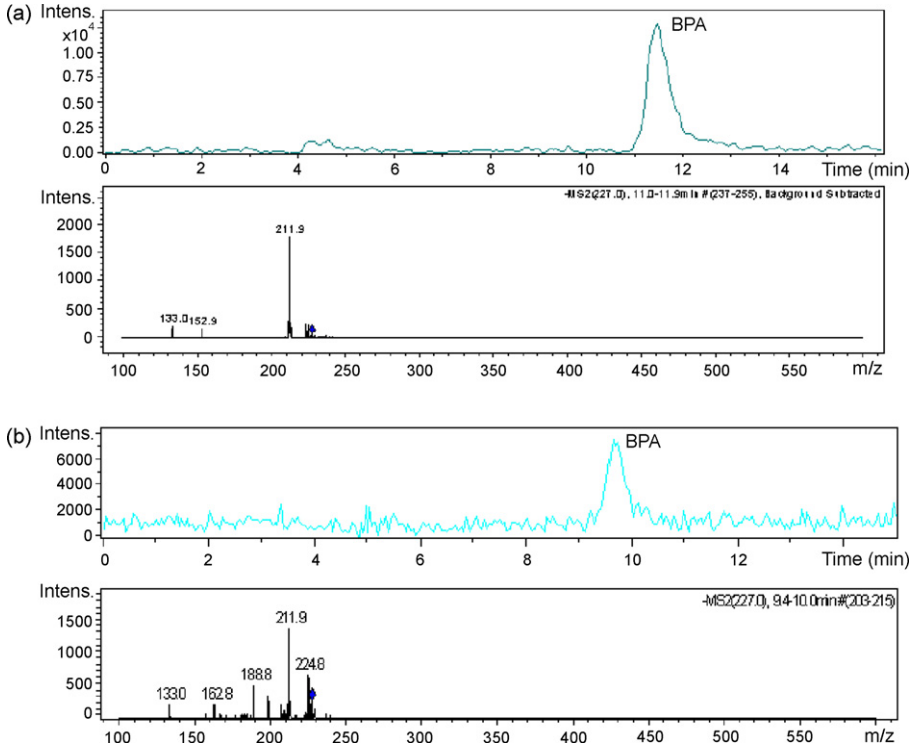

Fig. 4 – Comparison of the chromatograms of lake water sample obtained with different extraction methods. (a) 150 mL of lake water preconcentrated by IAMC; (b) 200 mL of lake water preconcentrated by C18-SPE and reconstituted in 200 μL of the mobile phase. The injection volume is 10 μL.

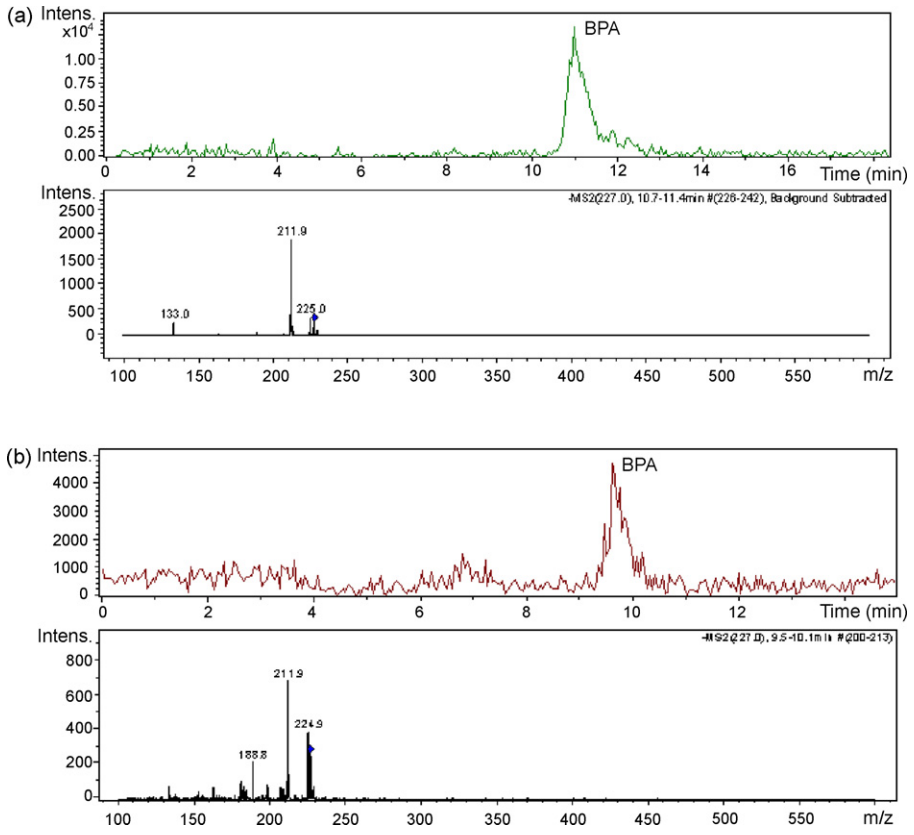

Fig. 5 – Comparison of the chromatograms of MSTP effluents obtained with different extraction methods. (a) 30 mL of MSTP effluent preconcentrated by IAMC; (b) 100 mL of MSTP effluent preconcentrated by C18-SPE and reconstituted in 500 μL of the mobile phase. The injection volume is 10 μL.

and 0.01 mol L^{-1} PBS, and higher salt concentration was disadvantageous to the MS/MS detector, the real water samples were directly loaded onto the IAMC without further adjusting the pH and ionic strength. To confirm the recovery of the method, BPA solutions at low concentration levels (4 ng L^{-1}) were applied to the column at large loading volumes (100 mL). The eluted peak area was found to be >91% of that obtained by injecting 20 μL of 20 ng mL^{-1} BPA standard solution ($n = 3$). The overall method managed to achieve a detection limit of 0.3 ng L^{-1} with an enrichment of 100 mL sample. The linear calibration range was found to be 1.0–160 ng L^{-1}. Based on above results, spiked and non-spiked environmental water samples were analyzed with the established method and the results were given in Table 1. The data obtained by offline C18-SPE extraction followed by LC–MS/MS quantification was also shown for comparison. Representative chromatograms of the lake water and MSTP effluent samples were shown in Figs. 4 and 5, respectively.

As can be seen, the BPA concentration data obtained by the two extraction methods were generally in good agreement for the lake water samples. For the tap water, BPA was not detected by the C18-SPE method because the concentration level of the reconstituted sample was below the detection limit. For 200 mL of tap water that was concentrated by the SPE column, the dried extract needs to be redissolved in no more than 70 μL of the mobile phase for quantification of <1.0 ng L^{-1} BPA in the original sample. Otherwise, larger volume of water sample should be collected and concentrated, which also increases the time cost and the amount of matrix interferences. Actually this is one of the inherent drawbacks of the offline extraction method. For the MSTP effluent samples (100 mL) purified by C18-SPE method, the reconstituted sample (500 μL) appeared yellow and turbid, indicating insufficient removal of the matrix components. The recovery was less than 60%. While with the IAMC extraction, clean chromatograms and reliable quantification results were obtained.

From above comparison, we concluded that the developed IAMC extraction method had distinct advantages for eliminating complex matrix interferences during sample extraction. It also allows fast, selective and contamination-free enrichment of aqueous BPA without having undergone other pretreatments. The overall method offers a highly efficient and reliable approach for quantitative analysis of trace level of BPA in various environmental water samples.

4. Conclusion

A rigid anti-BPA immunoaffinity column was successfully prepared by using poly(EDMA-GMA) monolith as the support and immobilization of BPA antibodies via Schiff base reaction. With the obtained immunoaffinity monolith as an on-line extraction column, a highly selective and effi-

cient approach for extraction and quantification of BPA in environmental water samples was developed. Little interfering peaks were observed in the chromatograms when the system was applied to real environmental water samples. The risk of sample contamination was also reduced to the minimum since the water samples could be directly applied to the system without the need of any other pretreatment. The method offers a promising approach for routine analysis of BPA in various environmental water samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2008.05.036.

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