Indian Journal of Chemistry
Vol. 54A, August 2015, pp. 1051-1056

Molecularly imprinted polymer for detection of endocrine disrupting chemical epinephrine in drinking water and biological buffers

Darshana G Gour & Reddithota J Krupadam*

CSIR-National Environmental Engineering Research Institute, Nehru Marg, Nagpur 400 020, India

Email: rj_krupadam@neeri.res.in

Received 14 May 2015; revised and accepted 24 July 2015

A polymer for selective extraction of epinephrine has been prepared using molecular imprinting technique with two functional monomers, viz., vinylbenzyl chloride and divinylbenzene and a simple and sensitive analytical procedure has been developed for detection of epinephrine, a neurotransmitter and an emerging pollutant, in drinking water resources.. The imprinted polymer shows selective extraction of epinephrine from water with a capacity of 9.82 mg g^{-1}, which is reduced to 9.45 mg g^{-1} in biological buffer (***p***H 7.0). The polymer shows better selectivity for epinephrine in water in the presence of bisphenol-A and nicotine (93%) as compared to the commercial polymer, XAD. The selective adsorption mechanism of the polymer has been investigated using infrared spectroscopy.

Keywords: Analytical chemistry, Solid phase extraction, Molecularly imprinted polymers, Imprinted polymers, Epinephrine, Water pollution

The scale of complexity associated with analysis of water contaminants shows the levels of various pollutants detected in samples. Some of the pollutants monitored at parts per billion/trillion in water resources include endocrine disruptors (EDCs), phenols, pesticides, pharmaceuticals, dyes and sulfonates. It is therefore necessary to develop appropriate monitoring tools that are capable of identifying and analyzing pollutants even in the presence of a large number of other chemicals. Epinephrine, 1- (3, 4- dihyroxyphenyl)-2-methylaminoethanol, is a derivative of amino acid tyrosine secreted from adrenal gland of mammals and this hormone has a significant role in the nervous system acting as a neurotransmitter in the mammalian central nervous system. High levels of epinephrine are associated with stress and extremely high levels with trauma, and neuroendocrine tumors[1, 2]. These characteristics also make adrenaline a potent doping agent and therefore the World Anti-Doping Agency tests epinephrine during competitive games/sports[3]. It is also used in medicine in the treatment of heart attack, bronchial asthma and cardiac surgery[4]. The wastes originating from hospitals and pharmaceutical industries contain epinephrine and contaminate nearby water resources.

The detection of epinephrine in drinking water and biological fluids such as blood and serum needs complicated and time consuming sample preparation procedures to achieve low detection limits, since these chemical as occur in low concentrations (nmol L^{-1} or μg L^{-1}). The detection methods reported in literature are based on fluorimetry[5], spectrophotometry[6], chromatography[7] and voltammetry[8]. However, these methods are complicated because they need derivatization steps or combination with various hyphenated detection methods. Due to this, there is a growing demand for simple, effective and reliable analytical method for monitoring of epinephrine in pharmaceutical wastes contaminated waters.

Molecularly imprinted polymers (MIPs) were used as an effective solid phase extraction materials for low concentration water pollutants such as polycyclic aromatic hydrocarbons[9], cyanotoxins[10], and pharmaceutical residues[11]. The MIP prepared with multi-walled carbon nanotubes (MWCNTs) on silica particles showed low detection of epinephrine[11], i.e., $3.0\times10^{-}8$ ***M*** while the epinephrine MIP hyphenated with flow injection chemiluminescence (CL) method achieved the highest sensitivity reported till date[12] (3.0×10^{-9} ***M***). In the present study, a nanopattern of epinephrine molecules was created in the polymer matrix using molecular imprinting, with the aim of selectively separating epinephrine from water followed by high performance liquid chromatography (HPLC). The analytical procedure showed better sensitivity compared with conventional methods for the detection of epinephrine in water resources.

Experimental

Epinephrine, the target molecule of analysis, was purchased from Acros Organics (Geel, Belgium). The functional monomers for polymer preparation, viz., vinylbenzyl chloride (VBC) and divinyl benzene (DVB) were obtained from Sigma-Aldrich (Buchs, Switzerland). The functional monomers VBC

and DVB were distilled to remove inhibitors under vacuum before use. The polymerization was initiated using 2, 2'-azobisisobutyronitrile (AIBN, Sigma-Aldrich) and crystallized with ethanol. The polymerization was carried out in acetonitrile, procured from Merck (Darmstadt, Germany).

The imprinted polymer was prepared as follows: In a 50 mL glass vial, 5 mL of acetonitrile was taken and to this epinephrine (0.91 mg) was added. The solution shaken for a few minutes and then the functional monomer VBC (3.26 mL) was added, followed by the addition of DVB (11.9 mL) to the pre-polymer complex after 5 min. The polymer reaction mixture was sonicated for about 5 min and then purged with nitrogen gas for 5 min. The polymerization reaction was initiated using 10 mg of AIBN. The sealed glass vial containing reaction mixture was freeze thaw-degassed by submerging the vial in liquid nitrogen and holding the frozen tube under vacuum of 100 mTorr for 15 min. The vial was sonicated for 5 min and placed in water bath at 60 °C for 16-18 h. After completion of polymerization, the vial was taken out of the water bath and the polymer monolith was removed from the vial and then crushed in a ball mill and sieved to a size between 72 and 500 mesh. The polymer particles of size <100 μm were washed several times using dichloromethane (DCM) to remove the targeted epinephrine from the polymer. After DCM washings, methanol was used to wash the polymer particles untill there was no epinephrine in the washing solutions. Finally, the polymer particles were dried at 108 °C for 24 h, before being used for analysis of epinephrine. The non-imprinted polymer (NIP) was prepared by following the above procedure without epinephrine.

The prime function of the polymer is selective extraction of targeted epinephrine in trace concentrations from the solutions. The extraction capacity of the polymer was determined using adsorption assay as follows: In a 20 mL scintillation vial, 10 mL of epinephrine solution (1 μg L^{-1}) was taken and to this solution, 50 mg of dry polymer (MIP) was added and kept in a water bath shaker for 30 min. The temperature of the water bath was kept at 20 °C. After 30 min, the solution was centrifuged at 15000 rpm for about 5 min. The supernatant of the solution was analyzed for epinephrine using HPLC. The quantity of epinephrine extracted onto MIP was calculated by subtraction using a calibrating curve obtained from the same experiment without MIP. The extraction kinetics experiments were extended to different time intervals (30, 60, 120, 180, 240 min) of MIP contact with epinephrine. Each experiment was repeated at least twice for each time intervals. The same experiment was conducted using epinephrine spiked biological buffer (***p***H 7.0) and ground water samples to study the effect of matrix on extraction of epinephrine. The extraction experiments were conducted on non-imprinted polymer and also the conventional polymers used currently for trace pollutants analysis.

The standard stock solution (1.0 μg L^{-1}) of epinephrine was prepared in acetonitrile diluted with Milli-Q water (1:99, ***v/v***) and the solution was filtered through a Whatmann No. 41 filter. Standard solutions of epinephrine was prepared in the range of 0.1–100 μg L^{-1} to prepare the calibration curve. The calibration was done with a six-point curve for epinephrine with $\boldsymbol{R}^2 > 0.996$. The calibration standards were constructed and then analyzed with an internal standard. Recovery of epinephrine from MIP was determined by spiking standard epinephrine after extraction from pharmaceutical waste samples. After addition of four internal standards (0.1, 1, 10, and 100 μg L^{-1}) to each extracted sample, a standard addition plot was drawn and linear regression was carried out on the data points. The slope of the regression line corresponds to the recovery value. For each sample (water, buffer ***p***H 7.0 and ground water), the quality assurance and quality control consisted of one blank, one spiked-blank, and one triplicate sample. Triplicate samples agreed within 10%, and epinephrine was absent from all water blanks studied.

The concentration of epinephrine in the solutions was measured using HPLC (Knauer, Smartline 5000) equipped with C18 column (μBondapack column, particle size: 3.9×300 mm) and UV detector and fitted with a Eurochrom data acquisition and analysis unit. The mobile phase used was a mixture of water: methanol (***p***H adjusted to 3.1 with ammonium acetate). The flux was maintained at 1.0 mL min^{-1} and the UV detection of epinephrine was monitored at 280 nm.

The surface morphology of epinephrine MIP was recorded a on scanning electron microscope (Jeol-JSM-6380, 15 kV; current 10^{-12} to 10^{-9} A; magnification 10,000X. Before analysis, the polymer was platinum sputtered using ion sputter fine coater under vacuum (~10^{-3} Torr). The surface functionality of the polymer was characterized using a FTIR

spectrometer (Perkin Elmer, model Spectrum 1) with KBr as the matrix.

Results and discussion

To study the extraction capacity, epinephrine extraction was carried out at saturation levels of epinephrine in water. The nanopatterns in the polymer formed due to molecular imprinting afforded maximum epinephrine uptake under saturation conditions. The extraction experiments were carried out using 10 μg L^{-1} aqueous solution. The standard deviations were calculated for experiments performed in three different times. The extraction experiments were initially carried out in aqueous solution and the same conditions were applied for buffer ***p***H 7.0 and groundwater samples. The extraction of epinephrine was quite fastr and within 60 min. the polymer was saturated with epinephrine (Fig. 1) . It was found that the extraction of epinephrine was less efficient at concentrations below 1 μg L^{-1}. MIP contains populations of binding sites with different affinities; the concentration of high affinity binding sites is relatively low in the polymer and this could be the reason for a less efficient binding of epinephrine at concentrations below 1 μg L^{-1}.

The cross-selectivity results obtained by extraction experiments using equimolar concentrations of epinephrine and two other compounds (bisphenol-A and nicotine) are reported in Table 1 along with the values obtained under the same conditions by competitive extraction carried out on the commercially used adsorbent resin, XAD. It is clear from these data that MIP is capable of recognizing epinephrine from structurally similar compounds, i. e., bisphenol A and nicotine. The normalized cross-selectivity of bisphenol A and nicotine were 6.29% and 7.02% for nicotine and bisphenol, respectively. This may be explained by the fact that in MIP, the non-specific absorption contributing to the extraction is quite low, while in competitive assay the response is mainly due to contribution of interaction within specific binding sites of MIP formed during molecular imprinting. The extraction of epinephrine from ground water and buffer (***p***H 7.0) samples, spiked with the toxin was slightly lower (96% for ground water and 93% for buffer ***p***H 7.0 at epinephrine conc. of 10 μg L^{-1}) (Fig. 2). The commercially used polymer XAD showed poor extraction for epinephrine (5.11 mg g^{-1}), which is about 52% lower than with MIP; also there is

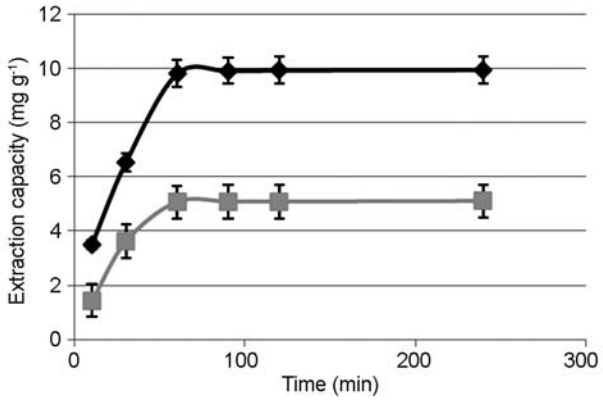

Fig. 1 – Extraction kinetics of epinephrine onto MIP and XAD. [Initial conc. of epinephrine: 10 μg L^{-1}; amt of MIP or XAD: 50 mg; vol. of sample: 10 mL].

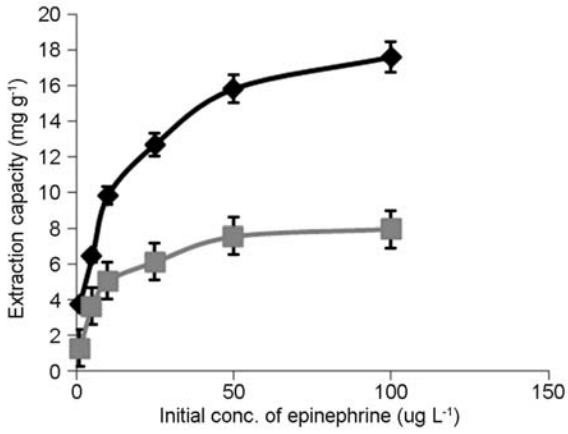

Fig. 2 – Effect of initial concentration of epinephrine on MIP/XAD extraction. [Contact time: 60 min; vol. of sample: 10 mL; amt of MIP/ XAD: 50 mg].

Table 1 – Extraction capacities of MIP[a] and XAD[b] for epinephrine from different aqueous solutions[c]

Medium of extraction	Epinephrine		Nicotine		Bisphenol-A	
	MIP	XAD	MIP	XAD	MIP	XAD
Water	9.82±0.13	5.07±0.12	0.61±0.11	2.39±0.16	0.69±0.11	2.56±0.13
Buffer (***p***H 7.0)	9.45±0.11	1.83±0.09	0.76±0.12	1.75±0.13	0.83±0.13	1.89±0.11
Ground water	9.56±0.15	1.97±0.11	0.65±0.11	1.83±0.14	0.76±0.12	1.68±0.09

[a]MIP, molecularly imprinted polymer; [b]XAD, commercial resin with polystyrene divinylbenzene matrix. [c]The extraction data was obtained using analogues of epinephrine (nicotine and bisphenol-A) with initial concentration of 1.0 μg/L. The selectivity values obtained using the same conditions by competitive assay are 6.29 and 7.02% for nicotine and bisphenol with the normalized epinephrine value of 100%.

no specific extraction of epinephrine from aqueous solutions. The non-specific extraction was responsible for binding of epinephrine and its structural analogues onto the commercial adsorbent XAD resin in almost equal quantities. The extraction studies shows that the nanopatterns formed during molecular imprinting improve both the extraction capacity as well as the selectivity of the polymer for epinephrine. A very small amount of epinephrine analogues (<4%) was found during the epineprine extraction experiments. The MIP reported in this study is a very sensitive material for selective extraction of epinephrine from water and would be useful in monitoring of water resources.

A combination of electrostatic binding and hydrogen bonding provided the necessary conditions for successful recognition of epinephrine in aqueous solutions. The slightly lower extraction of epinephrine from buffer ***p***H 7.0 solutions would be due to the inability of OH^- present in the solution to compete effectively with the strong NH- group present in the polymer for binding with the epinephrine. It is evident that the structure of epinephrine analogoues differs in the functionality and size and this difference is sufficient for prevention of efficient interaction of epinephrine analogues with the polymer binding sites. The functional monomer with amide and carboxylic acids functionality are conducive for the formation of hydrogen-bonding complexes. FTIR spectra shows the possible bonds between O−H···O and N−H···O hydrogen bonds in the pre-polymer complex. The FTIR spectra of MIP before and after adsorption of epinephrine shows the formation of hydrogen bonding as the primary recognition event. Imprinted polymer after adsorption of epinephrine shows strong peaks at 3663 cm^{-1} and 3359 cm^{-1}, which are characteristic peaks of alcoholic –OH and secondary amine –NH group. These peaks are absent in the polymer before adsorption (Fig. 3). Although it can form strong ionic interactions with basic functional groups, the hydrogen bonding ability of this functional group is not very strong in polar solvents such as water. For templates having both hydrogen bonding and acidic functional groups, the combination of methacrylic acid and a basic functional monomer (vinylpyridine) has previously been shown to impart MIPs with improved enantiomeric recognition[13]. Unlike the carboxyl group, the amide group is not ionizable, which could be advantageous for molecular recognition in water. Even more intriguing is the fact that template binding by polymer shows a decline in selective extraction when water is replaced by buffer of ***p***H 7.0. Similar performance was observed for blank polymers as well, although the magnitude of template binding was smaller for these polymers as compared with the MIP. The electron micrographs shows that the MIP has cavities while the reference polymer (NIP) has no such defined cavities (Fig. 4). The roughness of the particle

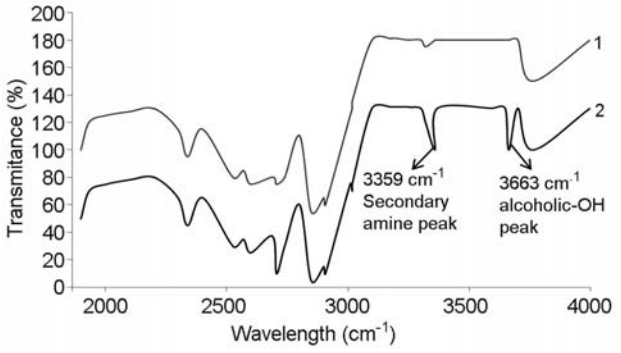

Fig. 3 – FTIR spectra of before (1) and after (2) extraction of epinephrine onto MIP

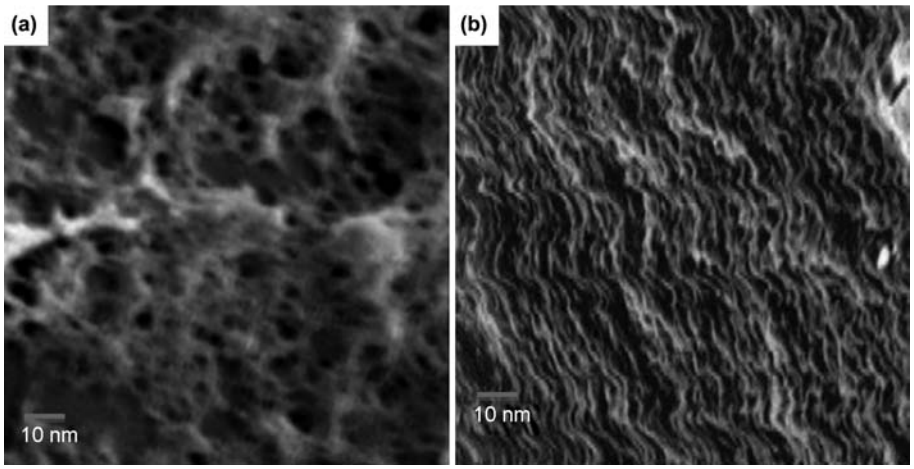

Fig. 4 – Scanning electron micrographs of (a) epinephrine imprinted polymer and (b) reference polymer (prepared without epinephrine).

surface should be considered as a factor increasing the surface area for high extraction of epinephrine from water.

The lower detection limit achieved with MIP extraction followed by HPLC analysis was 0.27 μg L^{-1}; the value is about 10-folds better than the methods described in the literature[9-11]. An important factor for such high sensitivity of the method is elimination of matrix complexity and selective extraction of epinephrine in pre-designed nanocavities in the polymer. To know the contribution of nonspecific interactions with the MIP, the same extraction procedure was followed with the reference polymer (NIP); which was prepared with the same composition as the MIP except for the absence of the template. This comparison of extraction capacities shows that the decrease in binding of epinephrine on to the MIP is due to non-specific interactions. The chromatograms of epinephrine cleaned with MIP are very clear and the selective pre-concentration produced a clean extract for HPLC analysis as compared to the polymer resin XAD (Fig. 5). As shown, the MIP extract of biological buffers produced better quality chromatograms.

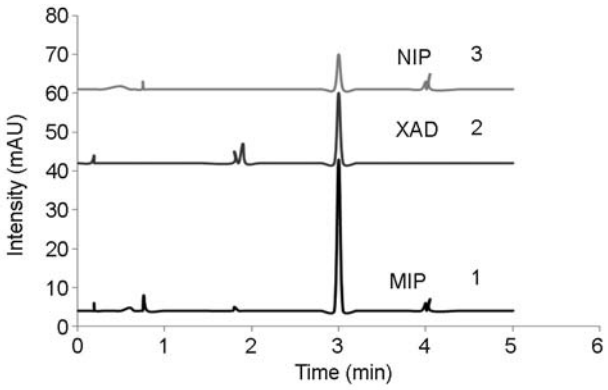

Fig. 5 – Chromatograms of epinephrine (1.0 μg L^{-1}) after extraction using (1) MIP, (2) XAD and (3) reference polymer (NIP).

Sensitivity, linearity, recoveries, precision and the study of matrix effects were considered as the criteria for the validation of the analytical methodology developed herein. Validation of data for each matrix studied is presented in Table 2. Calibration curves were generated using linear regression analysis and over the studied concentration range (1–100 μg L^{-1}) gave good fits (r^2 = 0.996). Five-point calibration curves were generated daily, and the possible fluctuation in signal intensity was checked by injecting a standard solution at two concentration levels after each 8–10 injections.

Recoveries achieved for all target compounds ranged from 60% to 102% and from 50% to 116% for aqueous solutions and acetonitrile, respectively. Nevertheless, as other performance data, such as repeatability and sensitivity, were good, the low recovery was not considered to be an obstacle for reliable determination. The limits of detection calculated for biological buffer was between 2.76 and 3.54 μg L^{-1} for epinephrine, which is lower than the stipulated standard set by biomedical diagnostics (10 μg L^{-1}). The repeatability of epinephrine determination was evaluated by spiking epinephrine stock solution into biological buffer samples to make a final concentration of 10 μg L^{-1} (n = 5). The repeatability was 3.1% RSD. The repeatability of epinephrine at 10 μg L^{-1} in buffer7.0 was 3.4% (n = 5). LOD and LOQ in the biological buffers were estimated similarly based on the chromatogram of the 10 μg L^{-1} spiked buffer samples (pH 7.0) and were calculated as 2.35 and 2.13 μg L^{-1}, respectively. The method developed for epinephrine quantification in complex biological fluids was three times higher

Table 2 – Analytical performance of the MIP extraction followed by HPLC analysis for epinephrine

Sample	Added (μg L^{-1})	Conc. of epinephrine						LOD (μg L^{-1})
		Intra-day (n=3)			Inter-day (n=3)			
		Found (μg L^{-1})	CV (%)	Bias (%)	Found (μg L^{-1})	CV (%)	Bias (%)	
Buffer (pH 7.0)	1.0±0.03	1.36±0.03	6	-3	1.31±0.03	5	1	0.27±0.03
Ground water	1.0±0.05	1.38±0.03	8	1	1.32±0.03	7	-2	0.16±0.02
Milli-Q water	1.0±0.03	1.39±0.02	5	2	1.35±0.02	5	1	0.11±0.02

Data collected after MIP extraction of epinephrine was spiked with standard epinephrine solution of concentration (1.0 g L^{-1}). Recoveries of epinephrine were determined by spiking standard after eluting MIP. A standard addition plot was constructed and the linear regression was performed on the data points. The slope is the regression corrections to the recovery value.

CV (%) = (SD/mean)×100

Bias (%) = [(measured concentration – spiked concentration)/spiked concentration]×100.

LOD: Three times the standard deviation calculated at the spiked level considered (RSD, n = 3).

when compared with the peak-to-peak noise of a series of standard chromatographic peaks measured with the consideration of LOD (S/N=3). Consequently, the analysis suggests that the level of epinephrine could be negligible in the deionized water used for this experiment. Considering these values for recovery and repeatability from complex biological fluids, it is implicit that results were reproducible.

The proposed analytical method using HPLC analysis with MIP extraction is highly efficient method for trace quantification of epinephrine in aqueous solutions. The experimental variables were optimized for selective separation of epinephrine from water using MIP adsorbent. The epinephrine extraction data suggests that the MIP provided a reliable and effective recovery of epinephrine (i.e., about 97%) in the concentration range 1.0-10 μg L^{-1} from water. The results obtained for calibration linearity, precision, accuracy and matrix effect show that the proposed method is efficient for epinephrine analysis. The reference material, XAD, showed 45% lower extraction capacity compared with the MIP and this may be responsible for achieving the LOD value of 10 μg L^{-1} for epinephrine in water. The proposed method is quite simple and sensitive for detection of epinephrine in water and other environmental and biomedical matrices.

Acknowledgment

The authors acknowledge the financial support of Council of Scientific and Industrial Research, New Delhi, India under XII Five Year Plan projects under the topic “Metrology in Chemistry” (Grant No. MIST-PSC 111).

References

1 Holger J S, Engebresten K M, Fritzlar S J, Patten L C, Harris C R & Flottemesch T J, ***Clin Chem***, 45 (2007) 396.

2 Behonick G S, Novak M J, Neally E W & Baskin S T, ***J Appl Toxicol***, 21 (2001) 15.

3 Taylor D W, Petrera M, Hendry M & Theodoropoulos J**,** ***Clin J Sport Med,*** 21 (2011) 344.

4 Stiell I G, Hébert P C, Wells G A, Vandemheen K L, Tang A S L, Higginson L A J, Dreyer J F, Clement C, Battram E, Watpool I, Mason S, Klassen T & Weitzman B N, ***Lancet,*** 358 (2001) 105.

5 Jinghe Y , Guiling Z, Xia W, Fang H, Cunguo L, Xihui C, Limei S & Yuanju D, ***Anal Chim Acta,*** 363 (1998) 105.

6 De Jong W H A, de Vries E G E, Wolffenbuttel B H R & Kema I P, ***J Chromatogr B***, 878 (2010) 1506.

7 Sabbioni C, Saracino M A, Mandrioli R, Pinzauti S, Furlanetto S, Gerra G & Raggi M A, ***J Chromatogr A***, 1032 (2004) 65.

8 Sharath Shankar S & Kumara Swamy B E, ***J Electrochem Sci,*** 9 (2014) 1321.

9 Krupadam R J, Khan M S & Wate S R, ***Water Res,*** 44 (2010) 681.

10 Krupadam R J, Patel G P & Balasubramanian R, ***Environ Sci Pollut Res,*** 19 (2012) 1841.

11 Dai C-M, Zhang Y-L & Chen L, ***Anal Chim Acta,*** 758 (2013) 93.

12 Jianxiu D, Lihua S & Jiuru L, ***Anal Chim Acta,*** 489 (2003) 183.

13 Piletsky S A, Karim K, Piletska E V, Day C J, Freebairn K W, Legge C & Turner A P F, ***Analyst,*** 126 (2001) 1826.