Molecularly imprinted polymer / cryogel composites for solid phase extraction of bisphenol A from river water and wine

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Abstract

Superporous monolithic hydrogels (cryogel monoliths) are elastic, sponge-like materials that can be prepared in an aqueous medium through a cryotropic gelation technique. These monoliths show interesting properties for the development of high throughput solid phase extraction supports to treat large volumes of aqueous samples. In this work a cryogel-supported molecularly imprinted solid-phase extraction approach for the endocrine disruptor bisphenol A (BPA) from river water and wine samples is presented. An imprinted polymer with molecular recognition properties for BPA was prepared in acetonitrile by thermal polymerization of a mixture of 4,4'-dihydroxy-2,2-diphenyl-1,1,1,3,3,3-trifluoropropane as a mimic template of BPA, 4-vinylpyridine and trimethylolpropane trimethacrylate in molar ratio 1+6+6. Fine imprinted particles (<10 μm) were embedded in a *poly*-acrylamide-*co*-N,N'-methylenbisacrylamide cryogel obtained by ammonium persulphate-induced cryopolymerization at -18 °C. The resulting monolithic gel was evaluated for its use as a sorbent support in an off-line solid phase extraction approach to recover BPA from dilute aqueous samples with minimum pre-loading work-up. The optimised extraction protocol resulted in a reliable MISPE method suitable to selectively extract and preconcentrate BPA from river water and red wine samples, demonstrating the practical feasibility of cryogel-trapped imprinted polymers as solid phase extraction materials

Keywords

Bisphenol A, molecularly imprinted polymer, cryogel, solid phase extraction, water analysis, wine analysis

1 - Introduction

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 [1,2]. Bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA, **1**) is known to be one such compound [3]. This substance is an important intermediate, used in the manufacture of epoxy, phenolic and polycarbonate resins, as an antioxidant and polymerization inhibitor in poly(vinylchloride). As a consequence of production, processing and disposal of plastic materials and composites, it can be released into the environment and subjected to bioaccumulation processes [4,5]. Thus, there is a significant demand for sensitive and selective analytical methods for its determination in environmental and food samples.

As the concentration of BPA is usually very low, sample pretreatment steps are mandatory. For this purpose, solid-phase extraction on packed columns has been widely applied [6,7]. The main drawbacks associated with the use of standard extraction cartridges are the low selectivity of the retention mechanism and the column clogging when large amounts of samples are loaded. The problem of low selectivity for traditional sorbents used in solid phase extraction can be addressed by using packing materials based on molecularly imprinted polymers (MIPs) in the so-called MISPE (Molecularly Imprinted Solid Phase Extraction) approach. In fact, these materials, acting as artificial receptors for a given target molecule, are selective for a particular analyte or group of related analytes [8,9]. In the last years, many successful applications of MIPs in solid phase extraction have been reported in literature for environmental, food or clinical matrices [10-13].

Very recently, the problem of cartridge clogging has been approached by the use of cryogels as chromatographic supports for MISPE. Cryogels are extremely porous media prepared by polymerization at temperatures below –18 °C from polar monomers dissolved in water. This solution is frozen and ice crystals are formed. Consequently, the original solution is segregated into two phases, one enriched with polymerizing monomers and one containing growing ice crystals. After the polymerization is terminated, the ice is allowed to thaw, leaving behind an organic monolith containing continuous interconnected micron-sized supermacropores [14,15]. It has been shown that micron-size MIP particles can be introduced in the cryopolymerization mixtures, remaining permanently trapped in the walls of the resulting cryogels, and that such composites are suitable for the MISPE of analytes in turbid samples, such as estradiol from sewage treatment plants [16] and bilirubin from untreated human blood [17,18].

As some preliminary studies to extract BPA from large volumes (up to 100 ml) of environmental (river water) and beverage (wine) samples using a MISPE approach failed because of very frequent cartridge clogging that produced unacceptable long loading times (up to 8 hours), we considered the use of cryogle-embedded MIPs to solve this problem. Thus, in this work we describe the preparation of a molecularly imprinted polymer for BPA and its use to obtain MIP/cryogel composites with flow properties suitable for the selective extraction and preconcentration of this endocrine disruptor from large volumes of aqueous samples such as river water and red wine.

2 – Experimental

2.1 – Materials

Acrylamide (AM), 2,2-bis-(4-hydroxy-3-methylphenyl)-propane (bisphenol C, BPC), bisphenol A (BPA), bis-(4-chlorophenyl)-methane (BCMe), 4,4'-dichlorobenzhydrol (DCBzH), 17β-estradiol (E2), estrone (E1), 16β,17β-estriol (E3), 17α-ethynyl-17β-estradiol (EE2), 4,4'-(hexafluoroisopropylidene)-diphenol (FBPA), trimethylolpropan trimethacrylate (TRIM) and 4-vinylpyridine (4-VP) were from Sigma–Aldrich–Fluka (Milan, Italy). Acetic acid, acetone, acetonitrile (LC gradient grade), ammonium persulphate (APS), 2,2'-azo-bis-(2-methylpropionitrile) (AIBN), isopropanol, methanol, N,N'-methylen-*bis*-acrylamide (MBAM), poly(ethylene glycol) 8000, propylene glycole, N,N,N',N'-tetramethylethylendiamine (TEMED) and triethylamine were from VWR International (Milan, Italy).

Polymerization inhibitors in TRIM and 4-VP were removed by cleanup on activated alumina columns. Analytes stock solutions were prepared by dissolving 20.0 mg of substance in 4.00 ml of acetonitrile and stored in the dark at -20 °C. All the solvents were of HPLC quality, other chemicals were of analytical grade.

The water of the River Po was taken in March 2008 in Torino in a single sampling, filtered on 0.22-μm cellulose acetate filters, and stored in silanized glass bottles in the dark at 4 °C. Wine samples (commercial mixture of several Italian and Spanish red wines) was purchased in a local supermarket in October 2008. To precipitate tannins in accordance with the literature [19], red wine was diluted 1:1 (v/v) with an 1% (v/v) aqueous solution of poly(ethylene glycol) 8000, incubated at 4 ◦C overnight, centrifuged at 8000 rpm for 15 min, filtered on 0.22-μm polypropylene membranes, transferred in glass bottles and stored in the dark at 4 ◦C.

All the glassware, SPE cartridges and tubing used in BPA extraction and analysis were rinsed in methanol and acetone immediately before of use.

The high-performance liquid chromatography apparatus (L-6200 constant-flow binary pump, L-4250 UV-Vis detector, Rheodyne 7100 six-port injection valve provided with 5ml injection loop, D7000 data acquisition system) was from Merck-Hitachi (Darmstadt, Germany).

2.2 – Synthesis of molecularly imprinted polymer

The imprinted polymer was prepared in according to a method reported in literature by Navarro-Villoslada et al. [20], with minor modifications. In a 25 ml thick wall borosilicate glass vial, a solution was prepared by dissolving 60.0 mg (0.178 mmoles) of FBPA in 1.50 ml of anhydrous acetonitrile. Then, 142 μl of 4-VP (1.07 mmoles), 456 μl of TRIM (1.07 mmoles) and 5 mg of AIBN were added. The polymerisation mixture was degassed in an ultrasonic bath for 10 minutes under moderate vacuum, and purged with nitrogen. The vial was sealed, and the mixture was photopolymerised at 4 °C overnight using a 200 W medium-pressure mercury lamp. The bulk polymer obtained was broken with a steel spatula and ground in a mechanical mortar. Then, the template was extracted in a Soxhlet apparatus by means of methanol. No efforts were made to measure the amount of template molecule recovered. The polymer was mechanically wet-sieved to three different particle sizes (90-38 μm, 15–38 μm, <15 μm) and dried under vacuum at 70 °C for 2 h. A blank polymer was prepared in the same experimental conditions by omitting the template.

2.3 - Column packing and liquid chromatography

An adequate amount of polymer (particle size 15–38 μm) was suspended in a 1+1 (v/v) methanol–water mixture and the slurry packed in a 100 mm stainless-steel HPLC column (i.d. 3.9 mm, geometrical volume 1.19 cm^3) at constant pressure of 10 MPa. The packed column was washed at 1 ml/min with 9+1 (v/v) methanol–acetic acid until a stable baseline was reached (254 nm). After equilibration, the pressure in the column was of 4 MPa using acetonitrile-acetic acid 99+1 (v/v) as a mobile phase and at a flow rate of 1 ml/min.

To perform the liquid chromatography experiments columns were equilibrated at a flow rate of 1 ml/min with 40 ml of acetonitrile-acetic acid 98+2 (v/v). Then, 10 μl of stock solution of analyte diluted 1+9 (v/v) with mobile phase were injected and eluted at 1 ml/min, and the absorbance recorded at 254 nm. Each elution was repeated three times to assure the chromatogram reproducibility. Column void volumes were measured by eluting 10 μl of acetone 0.1% (v/v) in acetonitrile-acetic acid 98+2 (v/v), and the absorbance recorded at 276 nm.

The retention factor, k, was calculated as $(t-t_0)/t_0$, where t is the retention time of the analyte, and t_0 the retention time corresponding to the column void volume. The selectivity factor (α) is defined as an index of the imprinted polymer selectivity toward analogs of bisphenol A. It was calculated as k_{analog}/k_{BPA}. The imprinting factor (IF) is defined as an index of the imprinting efficacy respect to a NIP prepared in the same conditions. It was calculated as k_{MIP}/k_{NIP}, where k_{MIP} is the capacity factor of BPA eluted on the imprinted column and k_{NIP} is the capacity factor of BPA eluted on the NIP.

2.4 – Preparation of MIP / cryogel composites

The MIP / cryogel composite monoliths were prepared by radical cryopolymerization of aqueous solutions of acrylamide and N,N'-methylen-bis-acrylamide in the presence of suspended MIP powders. Table 1 shows the amount of acrylic monomers and MIPs used to prepare the monoliths. Proper amounts of AM and MBAM were dissolved in 1.9 ml of ultrapure water containing 0.1% (v/v) of propylene glycole. MIP powders were added and mixtures were homogenized and degassed by ultrasonication under vacuum for about 10 minutes. Suspensions were transferred in 10 ml PTFE solid phase extraction cartridges provided with air-tight plugs and outlet stopcocks. Then, the cartridges were saturated with nitrogen, cooled to 4 °C, and 2 µl of TEMED and 100 µl of ultrapure water containing 1.5 mg of APS were rapidly added by vortexing under nitrogen. The cryopolymerization mixtures were frozen overnight at -18 °C, thawed at room temperature and washed with 100 ml of deionised water, discarding those cartridges apparently clogged or showing bubbles and discontinuities in the gel when examined against a light source. When not used, working cartridges were stored at 4 °C in the presence of water containing 0.1% (w/v) of sodium azide and 20% (v/v) of isopropanol.

2.5 - Flux properties of cryogels

Cartridges containing cryogels were equilibrated with 20 ml of deionised water and connected with a peristaltic pump previously calibrated to work with fluxes ranging from 0.25 to 10 ml/min. Then, cartridges were eluted with deionised water for a fixed amount of time, collecting water in calibrated flasks. From the mass of the water contained in the flasks, the flux was calculated as w/(d*t), where w is the mass of the water (g), d is the water density at 25 °C (0.9998 g/ml), and t is the sampling time (minutes). Each measure was repeated three times to assure reproducibility.

2.6 - MISPE of BPA in aqueous samples

The cartridge containing the cryogel composite Cryo33 was connected to a vacuum manifold, washed with 3x1 ml of acetonitrile–triethylamine 95+5 (v/v) and equilibrated with 5x1 ml of ultrapure water. 100 ml of river water or red wine samples were fortified

with 1.0, 10.0 and 100 μg/l of BPA and loaded on the cartridge, applying a vacuum to facilitate the passage of the sample through the cryogel. After sample loading, the cartridge was washed with 3x1 ml of acetonitrile. BPA was recovered by eluting the cartridge with 3x1 ml of acetonitrile–triethylamine 95+5 (v/v). To evaluate the reproducibility of the extraction protocol, each extraction was repeated five times and BPA recovery was evaluated as the average of the single values measured.

Reverse phase HPLC analysis was used for quantification of BPA after MISPE. chromatographic separation was performed on a monolithic octadecyl-silica column (Chromolith Performance RP-18, 100 mm x 4.6 mm, VWR International). The detection wavelength was 220 nm. The mobile phase consisted of acetonitrile–water 8+2 (v/v). The mobile phase flow-rate was set to 1.00 ml/min. Reference standard solutions of BPA of concentration 0.05, 0.1, 0.25, 0.5, 1, 2.5 and 5 μg/ml were analysed three times consecutively and peak areas were plotted against concentration. A calibration curve was drawn using weighted linear regression (weight = 1/conc.).

3 – Results and discussion

3.1 – BPA-imprinted polymer

Usually, as imprinted sites can be formed deep in the bulk of a polymer, it is not possible to quantitatively remove template molecules from an imprinted polymer, even after repeated and harsh washing procedures [21]. So, it is not uncommon that a mobile phase change during a solid phase extraction process based on a BPA-imprinted polymer causes a slow leach of some residual template molecules out of the polymeric matrix, thus contaminating the extracted samples up to trace level. The preferred solution to this drawback consists of the application of the mimic template approach. In this case a substance with a molecular structure similar to BPA is used as a putative template, producing imprinted polymers able to well recognize both the mimic molecule and BPA. Several mimic templates have been reported in literature, based on deuterated BPA [22,23], 4,4'-methylen-bis-phenol [24] or *p*-tertbutylphenol [25,26].

In this paper we choose a fluorinated derivative of BPA, namely 4,4'-(hexafluoroisopropylidene)-diphenol (FBPA, **2**), as a mimic template. This molecule can be considered very similar to BPA in terms of stiffness, 3D-structure and spatial orientation of the substituents on the aromatic rings, as the trifluoromethyl group can be considered an isosteric substituent for the methyl group [27]. On the other hand, six fluorine atoms noticeably increase the molecule hydrophobicity for FBPA (logP 4.23) compared to BPA (logP 3.32), making the separation of these molecules by reverse

phase-HPLC a simple task.

In order to evaluate the effectiveness of FBPA as a mimic template, the retention times of this molecule, of BPA, and other structurally related compounds — which are reported in figure 1 — they were measured on a HPLC column packed with the imprinted polymer and compared with the corresponding data measured on a blank column. The results, listed in table 2, show that the imprinted polymer strongly retains the mimic template FBPA and, to a lesser extent, the target molecule BPA, while the strictly related BPC — that only differs from BPA for the presence of a methyl group in ortho-position to the phenolic hydroxyls — is poorly recognized. The same happens for related compounds that lack the hydroxyls, substituted with the isosteric chlorine (DCBzH and BCMe) [27], and for some estrogenic substances not related to BPA but known to be endocrine disruptors (E1, E2, E3 and EE2) [28]. To sum up, the FBPA-imprinted polymer shows a good selectivity for the target molecule, resulting in a material suitable for MISPE of BPA.

3.2 – MIP / cryogel composites

The main drawback encountered during the preparation of cryogels with the composition reported in table 1 is related to the large number of failed cartridges (about 2/3 of the prepared cartridges), which resulted in extremely low water flux (usually below 1 ml/h) or the presence of bubbles, density discontinuities and fractures in the gel matrix. It is clear that the main experimental difficulty related to this approach is the correct formation of a homogenous superporous gel in sub-zero thermal conditions. Despite this, when “working” homogenous and porous cryogel were obtained, their flux performances showed themselves to be reproducible when several cartridges were prepared.

It is well known that the flux properties of cryogels are related to the composition of the pre-polymerization mixture. In fact, increasing the relative amount of the cross-linker MBAM in *poly*-acrylamide cryogels causes a decrease of the velocity of the mobile phase flow, due to the reduced mean size of the interconnected macropores [15]. This is confirmed by the mobile phase velocities, measured in this work, for cryogels with increasing AM : MBAM molar ratios, as reported in figure 2, where it is possible to see that for a cryogel prepared in the absence of MIP particles the mobile phase velocity doubles when the molar ratio passes from 3:1 to 12:1.

The effect of varying amounts of particulate embedded in the cryogels is less known, but it is reasonable that the inclusion of objects with mean dimensions comparable to cryogel macropores (10-100 μm) should further reduce the mobile phase flow by the occlusion of many of the flowpaths through the column. In fact, cryogels prepared in

presence of MIP particles show different flow velocities when eluted with water. From figures ~~3a-3c~~ 3, 5 and 6 it is possible to observe that increasing amounts of these particles decrease the water flux significantly — in some cases of one order or more of magnitude — compared to the related cryogel prepared in the absence of any MIP particle. Moreover, the flow properties seem to be influenced by the size of the embedded particles. In fact, big particles seems to be able to more efficiently occlude the flow path compared to small particles. This can be easily explained considering that small particles are better embedded in the pore walls that larger particles [16,17], leaving more room in the intercommunicating pores, exerting less resistance in this manner to the mobile phase flow.

To sum up, considering the combined effect of the different experimental variables on the flux properties of the prepared cryogels, Cryo33 (molar ratio AM : MBAM 1+12, containing 200 mg of MIP particles with particle size <15 μm, measured flow velocity 4.3 ml/min) was retained, as the composite, with flux properties more suitable for the solid phase extraction of water samples.

3.3 - MISPE of BPA in aqueous samples

Concerning the BPA extraction from aqueous samples, river water and red wine tested as matrices caused no difficulty and satisfactory sample clean-up and preconcentration was achieved by the MISPE protocol, as can be seen in figures 6 and 7, where the chromatograms of river water (fig. 6) and red wine (fig. 7) spiked with 100 μg/l of BPA are reported. It can be seen that BPA cannot be detected when samples are directly separated by RP-HPLC without preliminary solid-phase extraction, while the same samples analysed after solid-phase extraction and preconcentration show very clean chromatographic traces, where peaks corresponding to BPA can be easily detected and, as a consequence, quantified. In the same chromatograms it is possible to observe a very small peak corresponding to the retention time of FBPA, indicating the presence of a bleeding effect (i.e., release of the residual template from the imprinted polymeric matrix). Anyway, such bleeding does not hamper the use of this MISPE method to detect BPA, as the two peaks are very well resolved.

The recovery of the MISPE extraction was determined by comparing the detector response of extracted aqueous samples with that of directly injected standards prepared in deionised water. Recoveries were determined at three concentration levels and came out at between 94 and 102% (94±9% at 1 μg/l, 102±3% at 10 μg/l and 97±3% at 100 μg/l) for river water and between 90 and 98% (90±11% at 1 μg/l, 95±5% at 10 μg/l and 98±2%

at 100 μg/l) for red wine. Thus, they were reproducible and in good accordance with the recoveries performed on deionised water. It was observed, however, that the recovery diminished if more than 10-20 consecutive extractions were performed on both river water and red wine in the same cartridge. This was attributed to the accumulation of particulate matter and matrix components retained by the composite. In several cases, long term sonication (>30 min) and extensive washing of the cryogel was not sufficient for its complete regeneration.

The possibility of detecting and quantifying very small amounts of BPA in river water samples was investigated by extracting increasing volumes of water (100, 250, 500, 800 and 1000 ml) spiked with decreasing amounts of BPA (100, 40, 20, 12.5 and 10 ng) . As can be seen from figure 8, in the dilution range considered, the analyte recovery was good, with values between 75 and 125% (3σ). Thus, with this extraction technique it is possible to measure, without difficulty, highly diluted amounts of BPA down to 10 ng/l in water samples.

4 - Conclusions

Cartridge clogging due to particulate obstruction of the stationary phase is a frequently encountered drawback when large volumes of sample are loaded onto a MISPE column. In this work, we show that the use of superporous poly-acrylamide-*co*-N,N'-methylen-bis-acrylamide cryogels embedding micron-sized imprinted particles is useful to avoid column clogging. The experimental results indicate that a careful choice of the cryogel composition makes it possible to set up a method for molecularly imprinted solid-phase extraction of the endocrine disruptor bisphenol A from diluted river water and wine samples with minimum pre-loading work-up. As a consequence, a reliable off-line MISPE method was obtained, with near quantitative recoveries when the extraction protocol was performed on real samples. Moreover, preconcentration and quantitative extraction of bisphenol A from river samples was shown to be feasible down to 10 ng/l, with analyte recovery values between 75 and 125% (3σ). At the present studies are ongoing to evalue the feasibility of a cryogel-based on-line MISPE method suitable for complex matrices.

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Tables

Table 1: composition of cryopolymerization mixtures

cryogel	AM : MBAM, molar ratio	AM, mg (mmoles)	MBAM, mg (mmoles)	MIP, mg
Cryo10	3 + 1	51.5 (0.725)	37 (0.24)	0
Cryo11	3 + 1	51.5 (0.725)	37 (0.24)	50[a]
Cryo12	3 + 1	51.5 (0.725)	37 (0.24)	100[b]
Cryo13	3 + 1	51.5 (0.725)	37 (0.24)	200[c]
Cryo20	6 + 1	103 (1.45)	37 (0.24)	0
Cryo21	6 + 1	103 (1.45)	37 (0.24)	50[a]
Cryo22	6 + 1	103 (1.45)	37 (0.24)	100[b]
Cryo23	6 + 1	103 (1.45)	37 (0.24)	200[c]
Cryo30	12 + 1	206 (2.90)	37 (0.24)	0
Cryo31	12 + 1	206 (2.90)	37 (0.24)	50[a]
Cryo32	12 + 1	206 (2.90)	37 (0.24)	100[b]
Cryo33	12 + 1	206 (2.90)	37 (0.24)	200[c]

a: particle sizes 90-38 μm; b: particle sizes 15–38 μm; c: particle sizes <15 μm

Table 2: retention data for FBPA, BPA and related compounds applied to MIP and NIP columns. Mobile phase: acetonitrile–acetic acid 98+2 (v/v). Retention factors (k), selectivity factors (α) and imprinting factors (IF) were calculated as reported in section 2.3

	k_{MIP}	k_{NIP}	α	IF
BPA	3.95	1.69	1.00	2.33
FBPA	6.55	1.62	1.66	4.05
BPC	1.86	1.18	0.47	1.57
E1	1.33	0.85	0.34	1.57
E2	1.93	1.28	0.49	1.51
E3	0.51	0.85	0.13	0.60
EE2	1.92	1.17	0.49	1.64
DCBzH	1.33	0.85	0.34	1.57
BCMe	0.75	0.54	0.19	1.40

Figures

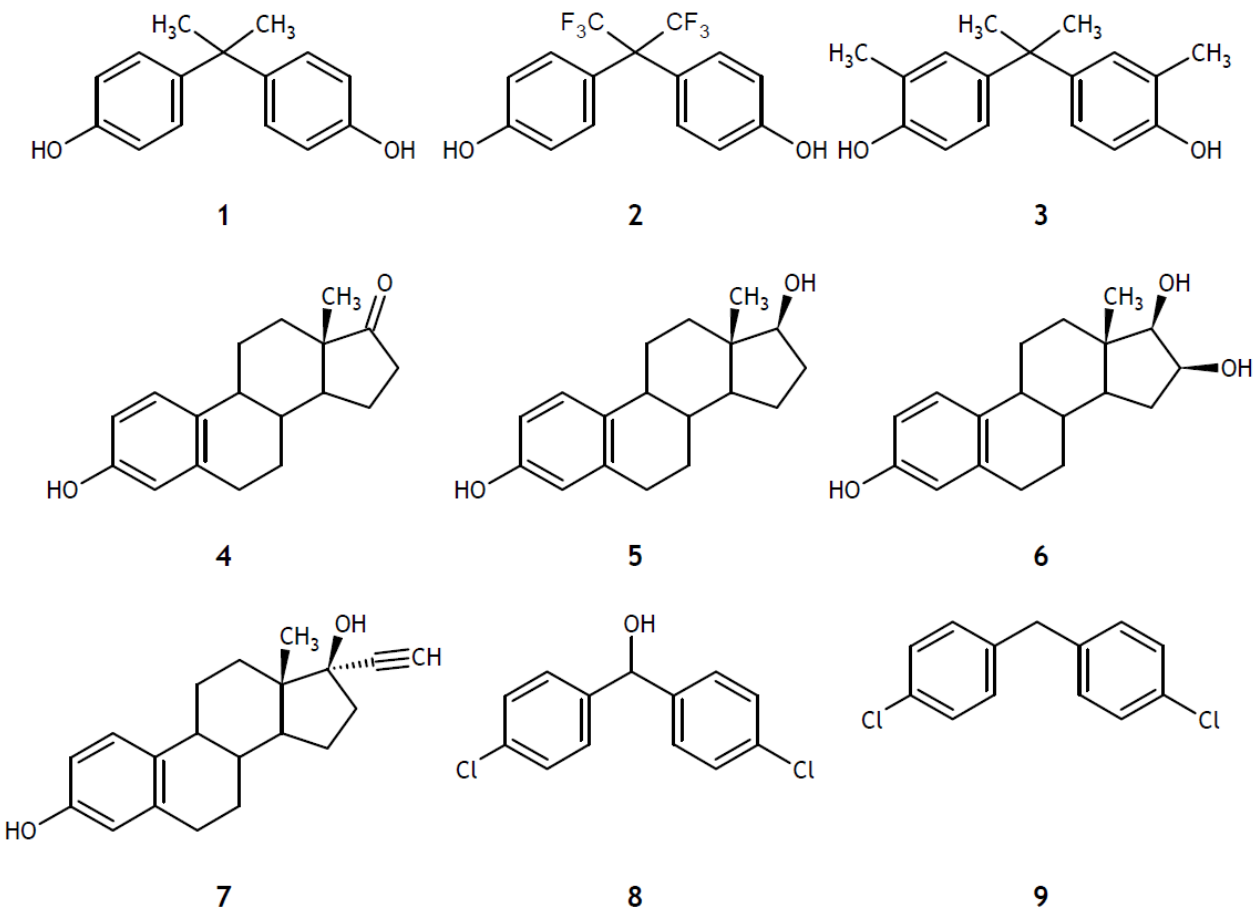

Figure 1: chemical structures of bisphenol A and related compounds used in this work. **1**, bisphenol A (BPA); **2**, 4,4'-(hexafluoroisopropylidene)-diphenol (FBPA); **3**, 2,2-bis-(4-hydroxy-3-methylphenyl)-propane (bisphenol C, BPC); **4**, estrone (E1); **5**, 17β-estradiol (E2); **6**, 16β,17β-estriol (E3); **7**, 17α-ethynyl-17β-estradiol (EE2); **8**, 4,4'-dichlorobenzhydrol (DCBzH); **9**, bis-(4-chlorophenyl)-methane (BCMe)

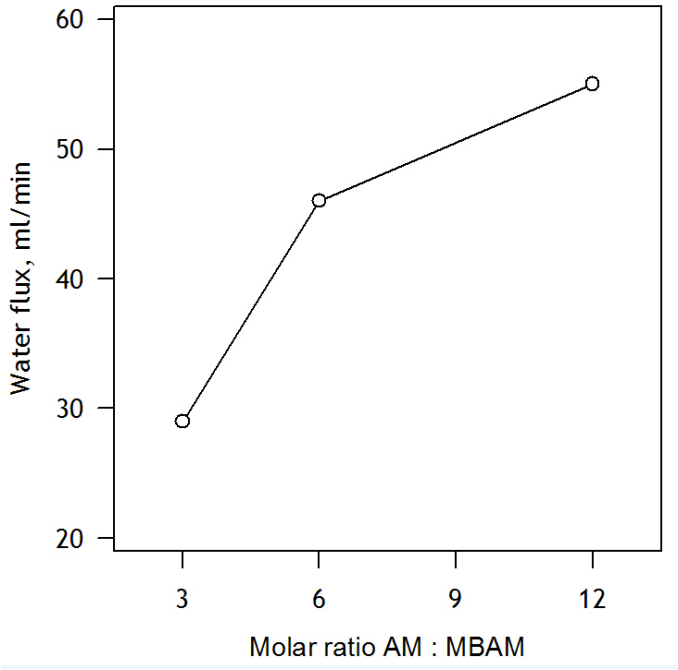

Figure 2: effect of molar ratio AM : MBAM on the water flux for cryogel columns without MIP particles

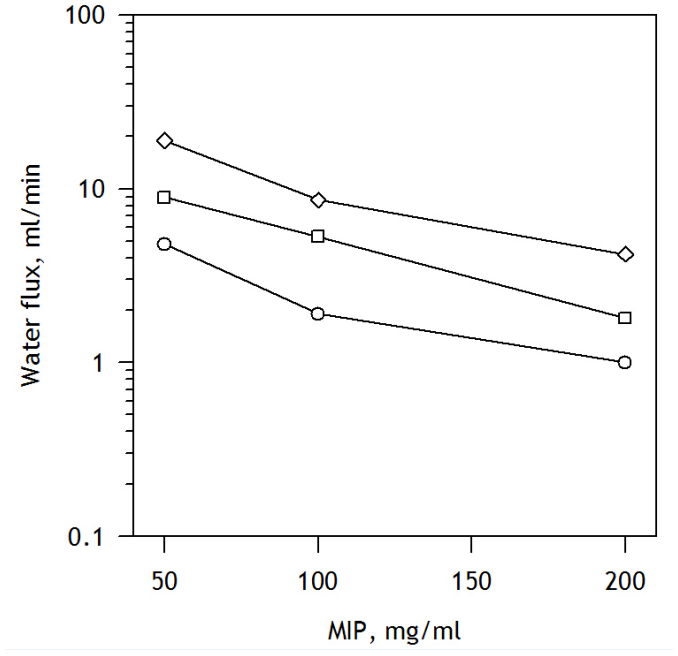

Figure 3: effect of the amount of MIP particles embedded on the water flux for a cryogel column prepared with a molar ratio AM : MBAM 12+1 (cryo31-33). Open circles: MIP particle size 90-38 μm. Open squares: MIP particle size 15–38 μm. Open diamond: MIP particle size <15 μm.

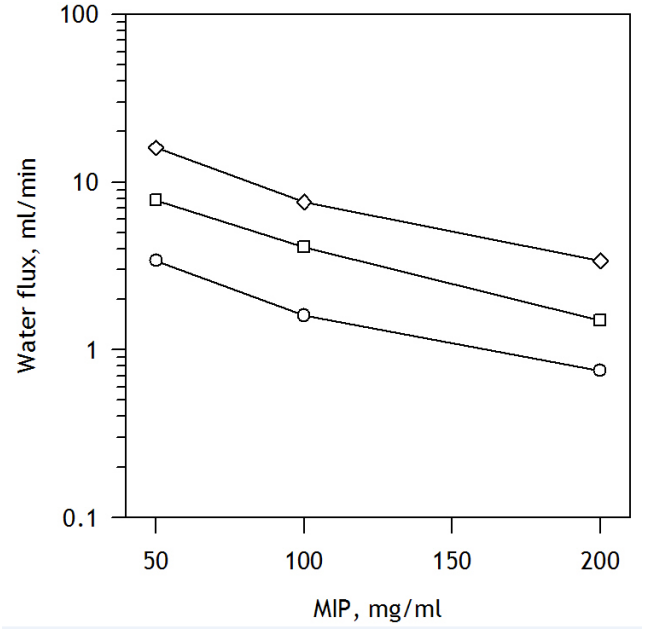

Figure 4: effect of the amount of MIP particles embedded on the water flux for a cryogel column prepared with a molar ratio AM : MBAM 6+1 (cryo21-23). Open circles: MIP particle size 90-38 μm. Open squares: MIP particle size 15-38 μm. Open diamond: MIP particle size <15 μm.

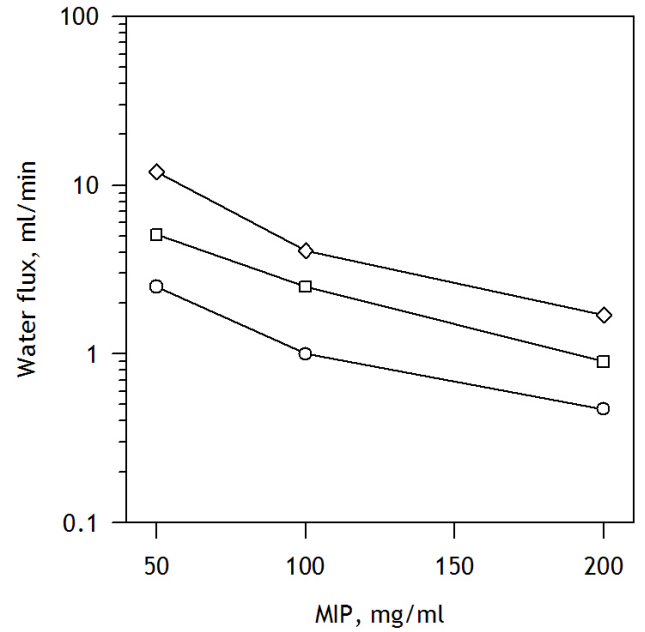

Figure 5: effect of the amount of MIP particles embedded on the water flux for a cryogel column prepared with a molar ratio AM : MBAM 3+1 (cryo11-13). Open circles: MIP particle size 90-38 μm. Open squares: MIP particle size 15-38 μm. Open diamond: MIP particle size <15 μm.

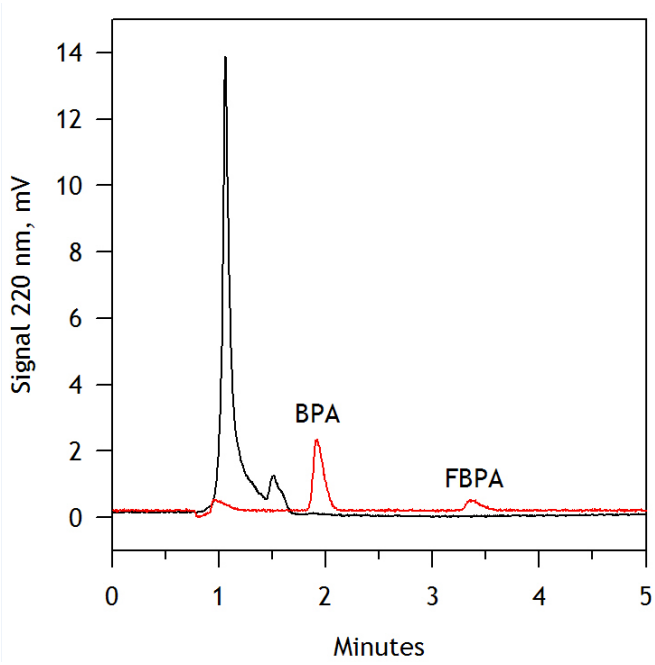

Figure 6: RP-HPLC of river water added with 100 μg/l of BPA and extracted (red) or not extracted (black line) with MISPE protocol. For experimental details, see text.

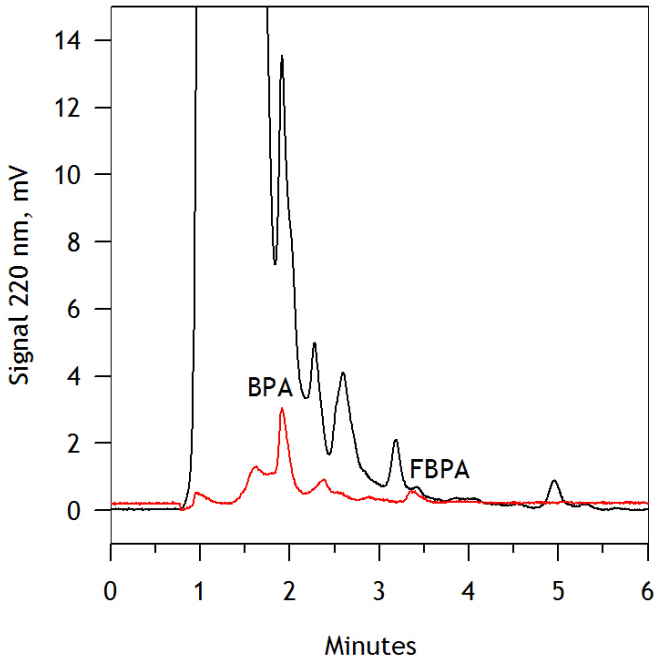

Figure 7: RP-HPLC of red wine added with 100 μg/l of BPA and extracted (red line) or not extracted (black line) with MISPE protocol. For experimental details, see text.

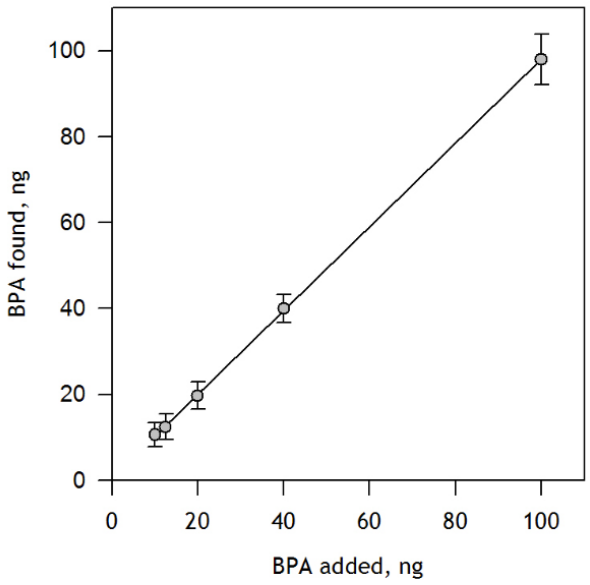

Figure 8: preconcentration of river water samples containing BPA in the range 10 – 100 ng (1 μg/l). Data expressed as the mean of three separate samplings ±3 standard deviation