Flow injection fluorimetric determination of β-estradiol using a molecularly imprinted polymer

Juan C. Bravo,[ab] Pilar Fernández*[a] and Jesús S. Durand[a]

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The steroidal compound 17β-estradiol (E2) is a major estrogen now widely studied because of its potential endocrine disruption effects. The present paper describes a simple, rapid, flow injection (FI) method for the fluorimetric determination of β-estradiol involving a molecularly imprinted polymer (MIP) packed into a microcolumn. Microwave-assisted extraction, a relatively new technique, was used to help remove the template from the MIP; this improved extraction efficiency. The optimization of the FI parameters and the intrinsic fluorescence of β-estradiol allowed its direct determination without a derivatization step. When the possible interference of other estrogens was examined, the system displayed excellent specificity for β-estradiol; no response to the natural estrogens estrone and estriol and the xenoestrogen bisphenol A was recorded. Under optimum experimental conditions, linear calibration graphs were obtained from 4 to 80 μg L^{-1} with a detection limit of 1.12 μg L^{-1}.

Introduction

In recent years, environmental estrogens have received increasing attention given their potential to alter the normal endocrine function of animals. Along with other categories of substances they have been referred to as endocrine disruptors or endocrine disrupting chemicals (EDCs). This term describes both the synthetic chemicals and natural compounds that may affect the endocrine system.

The steroidal hormone 17β-estradiol (E2) is present in the environment and is believed to enter water systems *via* human and animal waste products. Low levels of 17β-estradiol in the aquatic environment can cause the same endocrine disruption effects in animals as EDCs, thus, it is of prime concern to understand the fate and behavior of this compound and the removal efficiency of different methods. As suggested in a recent critical review of the fate of EDCs in wastewater as a research priority[1] in recent years a number of laboratory degradation studies have been conducted contributing greatly to a better understanding of these points. Concentrations of E2 used in this type of studies are orders of magnitude higher than those occurring naturally. For instance, Ternes *et al.*[2] carried out studies of the transformation of natural estrogens in sewage treatment plants using aerobic batch experiments, with estradiol concentrations of 1 μg L^{-1} and 1 mg L^{-1}. In both cases the analytical method for the determination of estradiol consisted of solid phase extraction (SPE) followed by a silica gel clean-up and derivatization for detection by GC-MS/MS. The detection limits were in the low ng range and the retention time for E2 was 22 min. Coleman *et al.*[3] described a method for the study of the photocatalytic degradation of 17β-estradiol on immobilized TiO_2 under laboratory controlled conditions with a range of E2 initial concentrations from 13 to 817 μg L^{-1}. The concentration of E2 was determined using HPLC with fluorescence detection with a retention time of 17 min. In the same way Ohko *et al.*[4] evaluated the degradation of E2 by TiO_2 photocatalysis in Pyrex reaction vessels with 272 μg L^{-1} E2 solutions. Degradation of E2 was monitored by HPLC-fluorescence analysis. Jürgens *et al.*[5] studied the biodegradation and photodegradation of estradiol and ethynylestradiol (EE2) in English rivers dispensing water samples into PTFE conical flasks and spiking the water with stock solutions of E2 and EE2 to give nominal concentrations from 20 to 100 ng L^{-1} and 100 μg L^{-1} E2; the analysis was carried out by HPLC-MS or HPLC-UV respectively giving this last method a quantification limit of 10 μg L^{-1} in the original samples. Yoon *et al.*[6] conducted a series of batch absorption experiments employing powdered activated carbon in model (distilled) water and two raw drinking waters spiked with bisphenol A (BPA), β-estradiol and ethynylestradiol at an initial concentration of 27 μg L^{-1}. HPLC with fluorescence detection was used to determine E2 showing a retention time of 17 min and a detection limit of 313 ng L^{-1}. Li *et al.*[7] studied the aerobic batch degradation of 17β-estradiol spiked into the activated liquor from a sewage treatment plant at initial concentrations of 10, 30 and 50 μg L^{-1}. They observed spiked E2 (30 μg L^{-1}) decreased to 12.2 and 6.3 μg L^{-1} after 30 min at 5 and 20 °C with high microbial population density. A continuous-flow aerobic degradation study is to be undertaken by the same authors.

On the other hand, molecular imprinting has become a promising technique that provides polymers with specific recognition properties. The process involves the formation of a molecular complex between functional monomers and a molecule that acts as a template (imprinting molecule) in the presence of an appropriate solvent, followed by polymerization in

[a] *Departamento de Ciencias Analíticas, Facultad de Ciencias, Universidad Nacional de Educación a Distancia, 28040, Madrid, Spain. E-mail: pfhernando@ccia.uned.es*
[b] *Departamento de Medio Ambiente, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Carretera de A Coruña Km 7.5, 28040, Madrid, Spain*

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the presence of a crosslinker. Removal of the template from the polymer leaves specific sites complementary in shape and functionality to the molecule that was imprinted. These sites provide the capacity for specific rebinding with the template. Molecularly imprinted polymers (MIPs) have been used in a wide range of applications such as solid-phase extraction,[8] chemical sensors,[9] and chromatographic separations,[10,11] *etc.*; several reports have been published in which β-estradiol acts as a template and in which different methods of polymerization have been used. Ye *et al.*[12] prepared MIPs using various steroid compounds as the template for screening a model steroid library following a previous protocol[11] with methacrylic acid (MAA) as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as the crosslinker to form a polymer monolith. Sanbe and Haginaka[13] utilized a multi-step swelling and polymerization method with 4-vinylpyridine (4-VPY) as a functional monomer, EGDMA as a crosslinker and water as the suspension medium to obtain uniformly sized MIPs for bisphenol A and β-estradiol and evaluated the retention and recognition properties. Kugimiya *et al.*[14] employed 2-(methacryloyloxy)ethyl phosphate as functional monomer and EGDMA as crosslinker to prepare a polymer block and evaluated the polymer affinity for E2. Ye *et al.*[15] developed a novel method for the preparation of molecularly imprinted microspheres using several template molecules to demonstrate the general applicability of the methodology. E2 imprinted microspheres were prepared using precipitation polymerization with MAA and EGDMA as functional monomer and crosslinker respectively; the binding capacity of the imprinted polymers was estimated from saturation studies. Dong *et al.*[16] synthesized MIPs for the recognition of five steroid compounds. When β-estradiol was used as the template molecule, MAA and EGDMA were utilized as functional monomer and crosslinker respectively to form a hard block polymer. Imprinting effect and binding properties were evaluated. To our knowledge only one published article focuses on the analytical determination of 17β-estradiol using a method based on MIP technology; Rachkov *et al.*[17] developed a fluorescence sensing system for the determination of E2 using a MIP and liquid chromatography.

A critical step in the molecular imprinting technique is the extraction of the template from the imprinted polymer. It is known that a small portion of the template remains unextracted even after extensive washing, and this can cause problems since it might bleed from the polymer during the elution step. Therefore, methods that can reduce this bleeding to acceptable levels are being sought. Usually, the extraction of the template from the MIP is undertaken by washing several times with a solvent (incubated) or by soxhlet extraction until the analyte is undetectable in the washing solution. Despite its good results, the soxhlet extraction method has the drawback that up to 24 h are necessary for the complete removal of the imprinted molecule. In the present work, the template underwent microwave-assisted extraction with the aim of reducing this time and increasing extraction efficiency.[18]

The major objective of this work was to develop an analytical method for routine analysis of β-estradiol in waters at low ppb levels. The proposed method for the determination of β-estradiol combines the advantages of flow injection analysis (FIA), such as small sample volumes, short analysis times, high sample throughput, simple instrumentation and low cost, with the high selectivity and sensitivity provided by the use of a MIP and the measurement of fluorescence.

Experimental

Chemicals and reagents

Methacrylic acid (MAA), ethyleneglycol dimethacrylate (EGDMA), β-estradiol, estrone, estriol and bisphenol A (BPA) were purchased from Sigma-Aldrich (Steinheim, Germany). Azobisisobutyronitrile (AIBN) was obtained from Fluka (Buchs, Switzerland). HPLC grade methanol and acetonitrile (ACN) were purchased from Sigma-Aldrich; analytical grade acetic acid was supplied by Merck (Darmstadt, Germany). All water used was Milli-Q grade (Millipore, Bedford, MA, USA). Stock solutions of each of the analytes were prepared at 100 mg L^{-1} in acetonitrile and stored at −20 °C in the dark. Working solutions were prepared at various concentrations by the appropriate dilution of the stock solutions in water and acetonitrile.

Instrumentation

A model CN-6T UV lamp (Vilber Lourmat, Marne La Vallée, France) was used to initiate the polymerization process. Fluorescence intensity was measured using a model LS-50B luminescence spectrometer (Perkin-Elmer, Beaconsfield, UK). Instrumental parameters and processing data were controlled by FL Winlab software. The excitation and emission wavelengths were set at 281 and 305 nm respectively. A Perimax peristaltic pump (Spetec, Erding, Germany), a six-way injection valve (Omnifit, Cambridge, UK) with a 500 µL sampling loop, and a 100 µL quartz flow-through cell (Hellma 176.051-QS) in the sample compartment of the spectrofluorimeter, were used to set-up the FIA manifold (Fig. 1). A 1.2 cm long, 0.8 mm i.d. PTFE column filled with the MIP was connected to the FI system. An ETHOS SEL microwave oven (Milestone, Sorisole, Italy) was used to remove the template from the imprinted polymer. Instrumental parameters were controlled by MLS-easyWAVE-combiCHEM 3.5.2.0 software.

Preparation of the MIP

The polymerization procedure was based on a typical protocol for preparing methacrylic polymers:[11,12,16] 27.4 mg of template molecule (β-estradiol), 0.254 mL of functional monomer (methacrylic acid), 2.832 mL of crosslinker monomer (ethyleneglycol dimethacrylate) and 62.5 mg of initiator

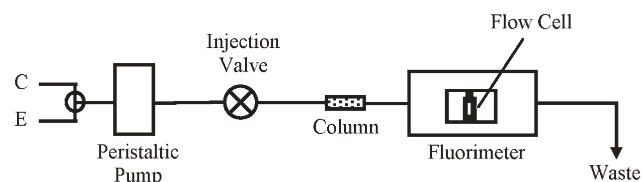

Fig. 1 Flow injection system for determining β-estradiol. C carrier (H_2O : ACN 97.5 : 2.5% [v/v]); E eluent (H_2O : ACN 50% [v/v]).

(azobisisobutyronitrile) were dissolved in acetonitrile in a test tube. This mixture was sonicated for 2 min and then purged with argon for 5 min. The tube was sealed with parafilm and polymerization performed using UV irradiation at 365 nm and 4 °C for 2 h followed by heating at 60 °C in an oven for 16 h. The template : functional monome r: crosslinker (T : M : C) ratio chosen was 1 : 30 : 150. This provided a large number of high affinity sites.[19] The polymer obtained was ground in a mortar and sieved to a range of particle sizes from >32 μm to >355 μm. Non-imprinted polymer (NIP) was prepared following the same procedure without adding the β-estradiol.

Removal of the template

The imprinted β-estradiol was removed by microwave-assisted extraction (MAE). Standard MAE conditions were as follows: 30 mg of MIP and 20 ml of washing solution (methanol containing 10% [v/v] acetic acid[16]),were placed in the vessels exposed to MAE over a 5 min heating ramp period up to 100 °C. This temperature was then maintained for 20 min. After extraction, the reaction vessels were allowed to reach room temperature, opened, and their contents transferred to a vacuum filtration system for removal of the solvent. The MIP particles were then collected and replaced in the vessels for a new round of extraction. The washing solution was kept for extraction efficiency studies. This process was performed three times for different particle sizes in order to ensure the total extraction of the template.

Batch rebinding studies

The binding behavior of the imprinted polymer was initially determined by batch adsorption. Imprinted polymer (2 mg) was incubated and shaken in 15 mL screw cap vials for 24 h at room temperature with 4 mL of ligand solution (500 μg L^{-1} β-estradiol), varying the pH and the H_2O : ACN ratio. The concentration of free (unbound) estradiol in the supernatant was analyzed by direct measurement of fluorescence. The binding percentage was then calculated.

Column preparation

After the extraction of the template, the polymer particles were dried and 1 mg of the sieved, imprinted polymer was slurry-packed into the capillary column with the aid of a peristaltic pump. The column was then connected to the flow system.

Flow injection procedure

Fig. 1 shows the flow injection system used. The procedure was started by flowing the carrier solution at a flow rate of 0.35 mL min^{-1} through the microcolumn until a stable baseline fluorescence signal was achieved. At this point, 500 μL of sample were injected into the capillary column through the injection valve; the estradiol is adsorbed onto the imprinted polymer at this moment. The eluent solution was then passed through the column to remove the adsorbed estradiol. The relative intensity of fluorescence at 305 nm (employing an excitation wavelength of 281 nm) was measured continuously using slit widths of 5 and 10 nm for excitation and emission respectively. The resulting peak due to the passage of the estradiol through the flow cell was recorded and the peak height used for quantification. The signal value then returned to the baseline and the system was ready for the next determination.

Results and discussion

Extraction of the template from the MIP

As mentioned earlier, template extraction is a crucial step for removing the imprinted molecule, leaving selective cavities ready for analyte recognition. The effectiveness of the extraction was evaluated by fluorimetric analysis of the washing solutions containing the extracted template (collected after the successive microwave-assisted extractions). Fig. 2 shows the extraction results. Comparison of the fluorescence intensity of the imprinted and non-imprinted polymers showed the complete removal of the template from the MIP to occur during the first extraction. Successive extractions are therefore unnecessary. Compared to the most common extraction methods (incubation and soxhlet), this procedure reduces the time required by several hours. In addition, less solvent is used.

No differences in extraction behavior were observed for different particle sizes. Thus, microwave-assisted extraction is effective for achieving efficient extraction of the template and should be used as a routine post-treatment step with imprinted polymers.[18]

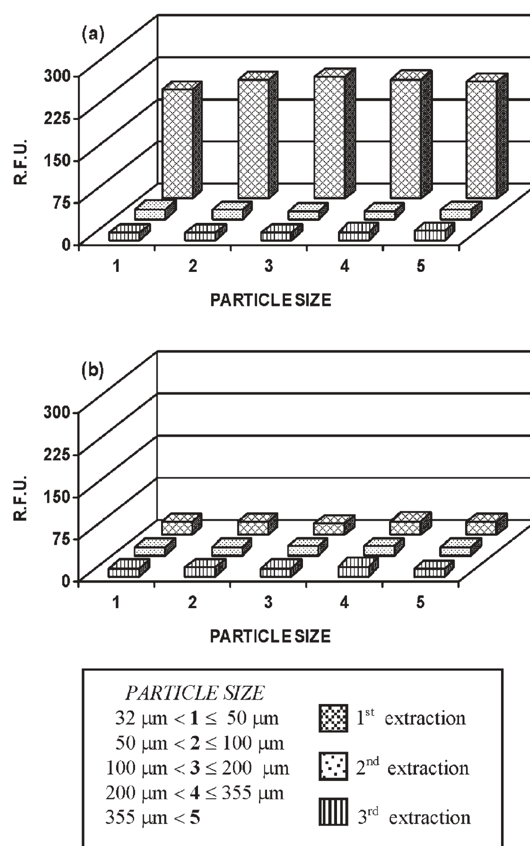

Fig. 2 Microwave-assisted extraction of E2 from the imprinted (a) and non-imprinted (b) polymer.

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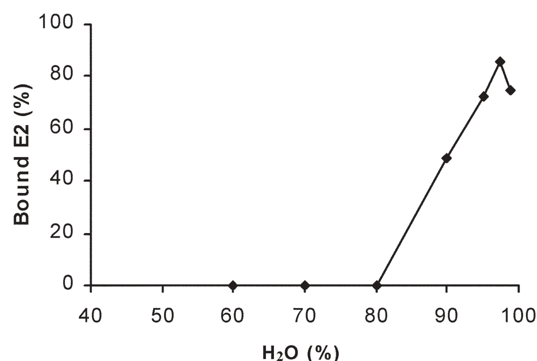

Fig. 3 Effects of H_2O percentage on the binding capacity of the imprinted polymer.

Batch rebinding experiments

Following the same procedure described above in the experimental section, the ability of the polymer to rebind β-estradiol was evaluated by batch adsorption assays using solutions of E2 in H_2O : ACN as a solvent.

Different H_2O : ACN ratios (range 60–100% of H_2O) were tested as the binding solvent. Fig. 3 shows the effects of the H_2O percentage on the binding capacity of the imprinted polymer.

The binding of E2 increased as a function of H_2O percentage, with a maximum at 97.5% H_2O. This increase in the percentage of water leads to a strong hydrophobic interaction.[20,21] Since water interacts strongly with hydrogen bonds, thus reducing those between the analyte and the imprinted polymer, hydrogen bonding is probably of less importance than hydrophobic interaction for retaining the analyte on the MIP.

The effects of pH on the binding capacity were also evaluated. The pH of the E2 solutions were first adjusted to 2, 4, 6, 8, 10 and 12 with either HCl or NaOH and then treated according to the procedure described above. Fig. 4 shows the results obtained. Clearly, under these experimental conditions, the binding behavior of E2 was not greatly affected by pH.

Optimization of FI system variables

A number of variables influencing the system were optimized. In order to choose the particle size that allowed the best use of the FI system, several microcolumns filled with imprinted polymer were prepared. Particles <50 μm produced an overpressure on the system, while those >100 μm led to broad fluorescence peaks. The optimum particle size was therefore between 50 and 100 μm. The eluent solution was studied using the flow injection apparatus shown in Fig. 1. Increasing the acetonitrile content of the solvent decreased the strength of the hydrophobic interaction and the analyte was eluted. 50% H_2O : ACN was selected for the complete elution of the analyte. The flow injection variables studied were the sample volume (which influenced the sensitivity of the method and the sample frequency), and the flow rate (which determines the rate at which the analyte can be eluted). Values of 500 μL and 0.35 mL min^{-1} were chosen, respectively, as a compromise between sensitivity and good sample throughput.

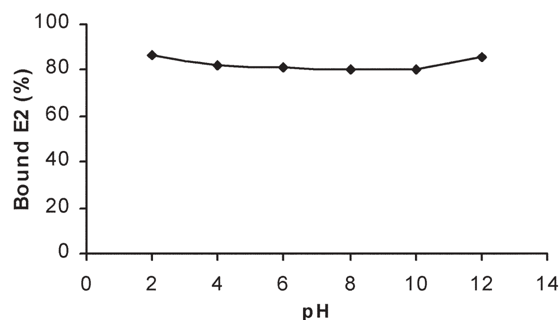

Fig. 4 Effects of pH on the binding capacity of the imprinted polymer.

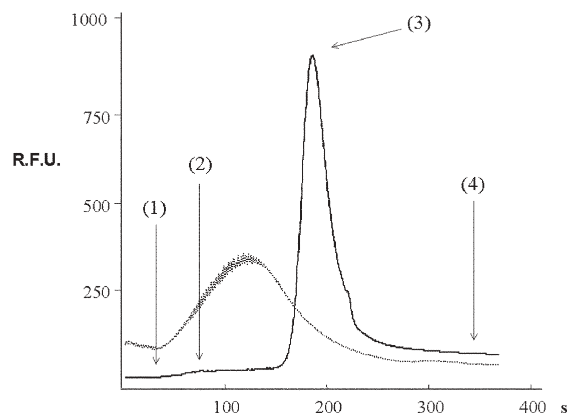

Fig. 5 Response curve of the FI system for β-estradiol injection. Solid line: MIP; dotted line: NIP.

Response curve

Experiments using imprinted and non-imprinted polymer were carried out following the flow injection procedure described above. Fig. 5 shows the response curve. The continuous line represents the MIP system signal; the NIP system signal is represented by the dotted line. In the imprinted polymer system it can be appreciated how an initial fluorescence signal was obtained corresponding to the carrier solution (baseline) (1). After injection of the sample, the β-estradiol was totally retained by the MIP and no increase in fluorescence intensity was observed (2). When the eluent passed through the system a rapid increase in the fluorescence signal was produced, which declined after reaching a maximum (3). Finally, the carrier solution was passed through again to recover the baseline and regenerate the system for the next test (4). In the NIP system, the β-estradiol was not retained by the MIP, flowing with the carrier and producing a small increment in the fluorescence intensity that immediately returned to baseline.

Table 1 Analytical figures of merit

Linear range	4–80 μg L^{-1}
Detection limit[a] (DL)	1.12 μg L^{-1}
Quantification limit[a] (QL)	3.72 μg L^{-1}
Relative standard deviation	1% (n = 10; 40 μg L^{-1})
Sampling throughput	10 h^{-1}

[a] Detection limit ($3\sigma_B$). Quantification limit ($10\sigma_B$).

Table 2 Analytical characteristics of MIP-based systems for β-estradiol determination

Determination system	Linear range	R^2	Detection limit	Response time	Reference
Chromatographic	0.1–4 μM	0.998	100 nM	15 min	17
Spectrofluorimetric	0.01–0.3 μM	0.997	4 nM	6 min	This work

Analytical characteristics

With the FI system shown in Fig. 1 and under the optimum conditions described above, a linear calibration curve was obtained in the range 4–80 μg L^{-1} described by the equation $y = 10.019x + 4.3627$ ($R^2 = 0.9966$).

Table 1 shows the analytical figures of merit for the proposed system. The detection limit using the $3\sigma_B$ criterion[22] (σ_B being the standard deviation of the blank) and the relative standard deviation were evaluated. Compared to results in the literature, this method has a lower detection limit and shorter response time than other MIP-based systems for the determination of β-estradiol (Table 2).

Selectivity

The interference effects of some steroidal and non-steroidal estrogens were examined. Fig. 6 shows the structures of the selected estrogens. The criterion for interference was fixed at an e_r of $< \pm 5\%$ of the average fluorescence signal calculated for the established level of β-estradiol.

The natural estrogens estrone and estriol and the xenoestrogen bisphenol A showed no interference with estradiol in this system, which confirms the presence of highly specific binding sites on the imprinted polymer. This was achieved by the T : M : C ratio used in the polymerization process. The highest ratio of E2 to possible interferent tested was 1 : 100.

Applications

In order to validate the performance of the proposed system it has been used in the determination of β-estradiol in spiked natural water samples. Samples were collected in Madrid and filtered prior to adding E2. The concentration examined was in the typical range used in degradation studies. As the results in Table 3 show, the measured values were in excellent agreement with the value of the amount of E2 actually added to the real water samples.

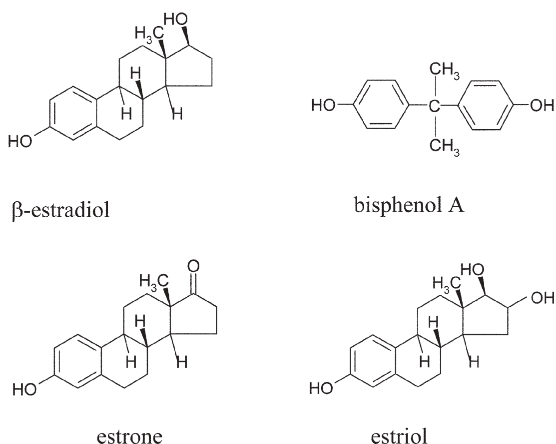

Fig. 6 Chemical structures of the selected estrogens.

Table 3 Analytical applications

Sample	E2 added/μg L^{-1}	E2 recovered/μg L^{-1} [a]
Raw drinking water	50	52.1 ± 0.2
Pond water	50	51.3 ± 0.2
Well water	50	52.0 ± 0.1

[a] Mean of three determinations.

Conclusions

This flow injection system with fluorimetric transduction, proposed for the determination of β-estradiol, shows good sensitivity and selectivity. These qualities are consequences of the selective adsorption and preconcentration of the analyte by the MIP. Detection limit and response time of the system are lower than other MIP-based procedures; reproducibility is excellent, and the procedure is inexpensive and does not require qualified personnel, which are important advantages with respect to other analytical techniques.

The described method has proved to be effective for the determination of β-estradiol in waters at μg L^{-1} levels and it has been demonstrated that this approach could be a useful tool for analytical purposes, especially for determining estradiol in degradation studies.

Acknowledgements

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