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Modified paramagnetic beads in a microfluidic system for the determination of ethinylestradiol (EE2) in river water samples

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ABSTRACT

In this work, we have developed and characterized a novel microfluidic immunoassay methodology for rapid and sensitive quantification of ethinylestradiol (EE2) in river water samples. The detection of EE2 was carried out using a competitive direct immunoassay method based on the use of anti-EE2 polyclonal antibodies immobilized on magnetic microspheres 3-aminopropyl-modified manipulated for an external removable magnet. The EE2 present in the water sample was allowed to compete with EE2-horseradish peroxidase (HPR) conjugated for the immobilized anti-EE2 antibody. The HPR, in the presence of hydrogen peroxide (H_2O_2) catalyzes the oxidation of catechol (Q) whose back electrochemical reduction was detected on gold electrode at 0.0 V. The response current obtained from the product of enzymatic reaction is inversely proportional to the amount of EE2 in the water sample. The electrochemical detection can be done within 1 min and total assay time was 30 min. The calculated detection limits for electrochemical detection and the ELISA procedure are 0.09 and 0.32 ng L^{-1} respectively and the intra- and inter-assay coefficients of variation were below 5.8%. Our electrochemical immunosensor showed higher sensitivity and lower time consumed than the standard spectrophotometric detection ELISA method, which shows the potential for assessment of EE2 in river water samples.

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1. Introduction

Emerging contaminants are previously unknown or unrecognized pollutants. Most of them have been present in the environment for a long time, but their significance and presence are only now being elucidated and, therefore, they are generally not included in the legislation. There is not a clear agreement about which compounds can be considered as emerging pollutants. They can be classified in various categories according to their chemical class (chemicals of totally new structure), type of use (new uses in industry or in consumer realms), type of effect (new discovered effects), source (new or previously unknown origins for existing chemicals), and exposure (pathways that had not been anticipated or had been previously discounted as not possible) (Daughton, 2004). Taking into account these criteria, compounds that can be considered as emerging contaminants are the so-called pharmaceuticals and personal care products (PPCPs), steroids, xenoestrogens, endocrine disrupting compounds (EDCs), and others (Lopez de Alda et al., 2003).

Between these chemical substances we can find: ethinylestradiol (EE2, 17-ethinyl-13-metil-7,8,9,11,12,13,14,15, 16,17-decahidro-6H-ciclopenta[a] fenantrene-3,17-diol) this is a synthetic estrogen, estradiol derivative for oral administration and it is largely utilized as part of oral contraceptive. On the other hand, EE2 was incorporated at environment and its considered an EDCs. Endocrine disrupting effects observed in the aquatic ecosystem have stimulated broad scientific and public interest. First studies already began in the 1970s when adverse effects of synthetic estrogens were discussed for the first time (Tabak and Bunch, 1970). Research was intensified in the early 1990s with the advent of reproductive problems in some freshwater fish populations (Sumpter et al., 1994; Bern, 1991).

Steroid hormones, both natural and synthetic, can be found in the environment as a result of human or animal excretion due to growing population concentration and intensive farming. Hormones, such as estradiol, estrone and EE2 have been found in water at ng L^{-1} levels (Larsson et al., 1999; Ternes et al., 1999; Belfroid et al., 1999) but, even at these low concentrations, some of them may induce estrogenic responses and cause adverse effects on aquatic and terrestrial organisms and on humans (Daughton, 2004; Lopez de Alda et al., 2001).

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The EE2 and other estrogenic hormones are usually quantified in aqueous matrices using standard instrumental methods such as gas chromatography–mass spectrometry (GC–MS/MS) or high performance liquid chromatography–mass spectrometry (LC–MS/MS). Current GC–MS/MS and LC–MS/MS methods achieve detection limits below 1 ng L^{-1} after sample enrichment. This enrichment might cause problems with recovery. In addition chromatographic methods require an extensive clean-up sample due to ionization suppression. Enrichment and clean-up steps are by themselves error-prone and can lead to decreased precision and accuracy of results (Zühlke et al., 2004; Aerni et al., 2004; Hohenblum et al., 2004; Tacey et al., 1994; Warren and Fotherby, 1974; Fotherby et al., 1981; Morvay et al., 1980; De la Pena et al., 1975; Dyas et al., 1981). Alternatively immunoassays can be used for monitoring of EE2 in environmental samples (Schneider et al., 2004, 2005; Coille et al., 2002; Goda et al., 2003). Immunoassay methods are rapid, sensitive, and cost effective analyses for a variety of environmental contaminants and they are a very efficient screening tool. These methods are also highly selective due to the extraordinary discriminatory capabilities of antibodies. Immunoassay methods are fast and relatively easy compared to conventional GC/MS and HPLC.

Microfluidic devices consist of microchannels for transporting fluids, with part or all of the necessary components to an immunoassay procedure integrated (Becker and Locascio, 2002; Sia and Whitesides, 2003; Erickson and Li, 2004). These devices offer many potential advantages including reduced reagent consumption, smaller analysis volumes, faster analysis times, and increased instrument portability (Park et al., 2006). Microfluidic biosensors based on electrochemical detection have also been investigated using amperometric (Kwakye et al., 2006; Lammertyn et al., 2006) and potentiometric (Suzuki and Matsugi, 2005) measurement. Conductometric biosensors are a very promising class of analytical devices characterized by their high sensitivity (Watson et al., 1987; Hnaiein et al., 2008).

The use of magnetic nanoparticles as labels in biosensing has become a very interesting topic in research (Jaffrezic-Renault et al., 2007), they are particularly suitable for integration in microfluidic devices. Magnetic nanoparticles are designed for concentration, separation, purification and identification of molecules and specific cells (Pankhurst et al., 2003; Gijs, 2004; Shinkai, 2002). Bead-based microfluidic immunoassays have an edge over normal fluidic systems, as it employs microbeads as a solid support. There are three main advantages in the use of these microbeads. Firstly, the surface to volume ratio is greatly increased even in a microfluidic device (Verpoorte, 2003). As a result, the sensitivity of assays is increased due to higher efficiency of interactions between samples and reagents. Secondly, the analytes attached onto the beads can be easily transported in a fluidic system using pressure-driven flow. Finally, there are a variety of surface modifications available on these microbeads, which will introduce multiple functionalities to a single microfluidic design. Therefore, antibodies, antigens, DNA, RNA and a vast number of other biological molecules can be easily attached to the microbeads for transport and analysis in a fluidic system. The benefit of incorporating microbeads in microfluidics systems has led researchers to seek different strategies to immobilize microbeads in channels of detection and reaction (Peterson, 2005).

In this article we developed a microfludic system with magnetic nanoparticles incorporated into the central microchannel, which were retained there for the action of the external magnet. This device was coupled with flow injection system and electrochemical detection for sentitive quantification of EE2 present in environmental samples. EE2 detection in these samples was carried out using a competitive immunoassay. The EE2 in the water sample is allowed to compete immunologically with EE2-HRP for the immobilized anti-EE2 antibodies. HRP in the presence of hydrogen peroxide (H_2O_2) catalyzed the oxidation of catechol (Q) to o-benzoquinone (P). The electrochemical reduction back to Q was detected on gold electrode at 0.0 V. The response current obtained from the product of enzymatic reaction is proportional to the activity of the enzyme and, consequently, to the amount of the EE2 in the water sample. Compared with the conventional immunoassay techniques, our microfluidic immunosensor showed enough sensitivity to determine very low levels of EE2 in unknown samples, with the advantage of the use of smaller volumes of reagents and samples and the improvement efficiency with regard to time analysis.

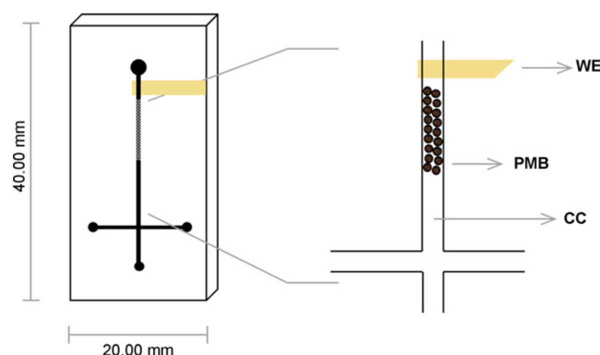

Fig. 1. Schematic representation of microfluidic immunosensor. WE: gold working electrode; PMB: paramagnetic beads; CC: central channel. All measurements are given in millimeters.

2. Materials and methods

2.1. Reagents and solutions

All reagents were of analytical or biochemical grade. Polyclonal rabbit anti-EE2 serum label (SA 2150) and EE2-HRP conjugate were supplied by Dr. Rudolf J. Schneider BAM Federal Institute for Materials Research and Testing Department I Analytical Chemistry, Reference Materials Division I.5 BioAnalytics Working Group I.51 Immunochemical Methods Berlin, Germany. For the ELISA assays, reagents were used in according with Hintemann et al. (2006). The development of the polyclonal antibody has been described in Schneider et al. (2004) and the preparation of enzyme conjugate has been described in Schneider et al. (2005). Glutaraldehyde (25% aqueous solution) and H_2O_2 were purchased from Merck, Darmstadt. Micro particles, magnetic, amino funtionalized (53572) were purchased by Fluka, Buchs/Schweiz, USA. Ethinylestradiol and Catechol was purchased from Sigma Chemical Co., St. Louis. Aqueous solutions were prepared using purified water from a Milli-Q system. The river water samples were collected from rivers of San Luis State, Argentina.

2.2. Sample preparation

Environmental water samples were collected from six rivers of San Luis State, Argentina. Tap water and ultrapure water were used as controls. For the electrochemical measurements the water samples (500 ml) were passed subsequently through folded filter papers (Whatman) and by vacuum through 934-AH^{TM} RTU Glass Microfiber Filters glass (Whatman), in according with Schneider et al. (2005) and adjusted to pH 7.0 using 0.1 M phosphate buffer.

2.3. Flow-through reactor/detector unit

The main body of the sensor was made of Plexiglas. Fig. 1 illustrates the design of the flow-through chamber containing the microfluidic immunosensor and the detector system. The gold layer

electrode of 80 nm thickness was deposited at central channel (CC) by sputtering (SPI-Module Sputter Coater with Etch mode, Structure Probe Inc., West Chester, PA) and the gold thickness electrode was measured using a Quartz Crystal Thickness Monitor model 12161 (Structure probe Inc., West Chester, PA). The diameter of the CC and the accessory channels was 100 μm. The electrode was cleaned and preconditioned using cyclic voltammetry in 0.5 M sulphuric acid by 3-fold cycling in the potential range between −300 and 1300 mV at 100 mV s^{-1} scan rate. Temperatures of solutions and reagents were conditioned before the experiment using a Vicking Masson II Laboratory water bath (Vicking SRL, Buenos Aires, Argentina).

Amperometric detection was performed using an electrochemical analyzer (Model LC-4, Bioanalytical System). The BAS 100B electrochemical analyzer Bioanalytical Systems was used for cyclic voltammetric analysis. The potential applied to the gold electrode was 0.0 V versus the Ag/AgCl wire pseudo-reference electrode and a Pt wire was used as a counter-electrode. At this potential, a catalytic current was well established. Pumps (Baby Bee Syringe Pump, Bioanalytical Systems) were used for pumping, simple introduction, and stopping flow. The pseudo-reference and counter electrode were located in the outlet, at the end of the CC.

All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.).

2.4. Immobilization of anti-EE2 antibodies on paramagnetic beads

Rabbit polyclonal anti-EE2 antibody was immobilized on magnetic microbeads modified with amino groups in an Eppendorf tube. 100 μL of magnetic beads amino funtionalized were washed with 1.0 mL of PBS buffer pH 7.2 for three times. The pellet was suspended in 1.0 mL of an aqueous solution of 5% (w/w) glutaraldehyde at pH 10.00 (0.20 M sodium carbonate buffer, pH 10) with continuous mixing for 2 h at room temperature. After three washes with PBS buffer pH 7.2 to remove the excess of glutaraldehyde, 250 μL of antibody preparation (dilution 1:2500 in 0.01 M PBS, pH 7.2) was coupled to the residual aldehyde groups with continuous mixing for 12 h at 4 °C. The immobilized antibodies preparation was finally washed with PBS (pH 7.2) and resuspended in 250 μL of the same buffer at 5 °C. Immobilized antibody preparations were perfectly stable for at least 1 month.

2.5. Amperometric analysis of EE2 in river water samples

This method was applied to determine EE2 in 6 river water samples. Initially, the immobilized antibodies preparations on magnetic microbeads were condicionated with desorption buffer (0.1 M citrate buffer pH 2.00) 1 mL, mixing at room temperature for 5 min. After that, they were rinsed with 0.01 M PBS (pH 7.2). The nonspecific binding was blocked by 10 min treatment at 37 °C with 3% skim milk in a 0.01 M phosphate buffer saline (PBS), pH 7.2 and finally washed with 0.01 M PBS buffer (pH 7.2) and stored in 250 μL of the same buffer. Then, 25 μL of modified magnetic microbeads were mixed with 100 mL of sample filtrate and shaker for 10 min at room temperature. EE2 present in the sample was allowed to react immunological with the modified magnetic beads, which have a large binding surface area per volume and consequently a large number of analytes molecules can be bound in a small final volume, allowing a sensitive detection (Pamme, 2006; Verpoorte, 2003). Then, the magnetic microbeads were recovered using an external magnet and washed three times with 0.01 M PBS buffer (pH 7.2) to remove the excess of sample and resuspended in 250 μL of 0.01 M PBS (pH 7.2). The microfluidic device was prepared by injection of 20 μL magnetic microbeads in the flow system by a micro pump at

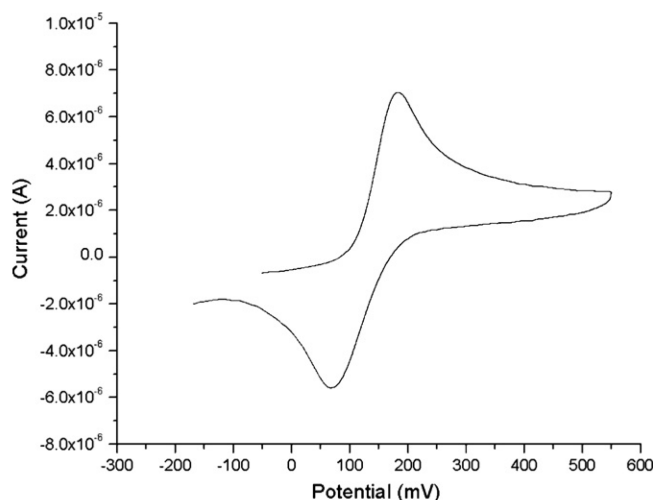

Fig. 2. Cyclic voltammogram of catechol in aqueous solution containing 1 mM of Q in 0.10 M phosphate/citrate buffer, pH 5.0 with a Gold electrode. Scan rate: 100 mV s^{-1}.

a flow rate of 5 μL min^{-1} for 4 min. A permanent magnet was used to attract the beads at specific area of the channel, near of the gold electrode plate. The magnet was not moved during the experiment to keep the beads into the channel and they were not carried away by the continuing flow. As the carrier buffer, 0.01 M PBS (pH 7.2) was used. This was followed by the injection 25 μL of the EE2-HRP conjugate (dilution 1:10,000, in 0.01 M PBS, pH 7.2) into the PBS carrier stream at a flow rate of 5 μL min^{-1} for 5 min at 37 °C. The flow line of the immunosensor was washed with 0.1 M sodium citrate buffer pH 5, and 5 μL of substrate solution (1 mM H_2O_2 and 1 mM Q in 0.1 M citrate buffer, pH 5) was injected into the carrier stream at 5 μL min^{-1} for 1 min and the enzymatic product (P) was measured on the surface of a gold electrode at 0.0 V.

A standard curve for the electrochemical procedure was produced following our protocol with a series of standards that covered the relevant range (0.01–60 ng L^{-1}). Amperometric measurements were performed at 0.0 V at room temperature in 0.1 M phosphate-citrate buffer, pH 5, and the resulting anodic current was displayed on the *x*–*y* digital recorder. The stock solution of Q was prepared freshly before the experiment and stored in the dark for the duration of the experiment.

3. Results and discussion

3.1. Electrochemical study of catechol with the gold electrode

The electrochemical behaviour of Q was examined by cyclic voltammetry at the gold electrode (Fig. 2). A cyclic voltammetric study of 1 mM in an aqueous solution containing 0.1 M sodium citrate buffer pH 5 was performed by scanning the potential from −200 to 550 mV versus Ag/AgCl. The cyclic voltammogram showed a well-defined anodic peak and a corresponding cathodic peak, which corresponds to the transformation of Q to P and vice versa in a quasi-reversible two-electron process. A peak current ratio (I_{C1}/I_{A1}) of nearly unity, particularly during the recycling of potential, can be considered a criterion for the stability of P produced at the surface of electrode under experimental conditions. In Fig. 2, a well-defined cathodic peak was observed at 69 mV using a bar gold electrode, although the 0.0 V was the potential applied for the amperometric detection in our system, in that way we can be sure that all the oxidized P has been reduced to Q. This point is important to be consider because the gold electrode in the microfluidic immunosensor is different in relation with a bar gold electrode, and the peak potential could be displaced.

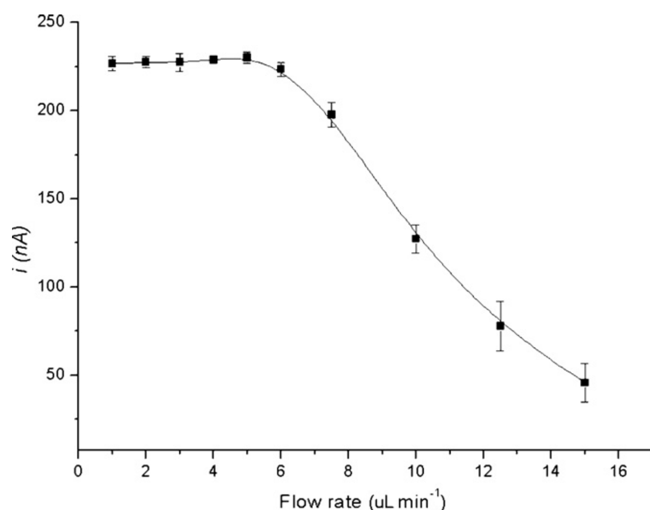

Fig. 3. Effect of flow rate analyzing a EE2 standard of 10 ng L^{-1} at different flow rates from 1 to 15 μL min^{-1}.

3.2. Optimum conditions for the immune reactions and the determination of enzymatic products

Microparticles are often used as solid supports for immunoassay reactions. They feature a large binding surface area per volume and hence a large number of analyte molecules are bound within a small volume, allowing for sensitive detection (Pamme, 2006; Verpoorte, 2003) use in conjunction with microchips, provide several advantages over conventional techniques, such as easy of handing and high reaction efficiencies (Bienvenue et al., 2006; Choi et al., 2002; Lim and Zhang, 2007; Sato et al., 2002). However various factors that affect the biochemical reaction must be considered because the reaction conditions in the microbiochip are different than those of conventional microtubes or well plates. Microfluidic control systems are essential for the control of a minute volume of fluid because of their rapid and precise control features, therefore, in our microbiochip, all reactions and washing procedures were performed using a syringe pump. The flow rates of the sample and reagent have an effect on the reaction efficiencies of the antigen–antibody interactions and unlike conventional immunoassays, samples and reagents in our system are continuously flowing through the microbiochips. Therefore, it is very important to consider flow rate when designing microfluidic biosensors (Maeng et al., 2008). In a flow system, the flow rate of the solution passing through the microfluidic device channel is the main factor, affecting the dispersion of the analytes, yield of the reaction between the antibodies immobilized on microbeads and the antigens present in the sample (one of most critical process for the determination) and response of the electrochemical detector.

The optimal flow rate was determined by analyzing a standard of 10 ng L^{-1} EE2 at different flow rates and evaluating the current generated during the immune reaction. As shown in Fig. 3, flow rates from 1 to 5 μL min^{-1} had little effect on antigen–antibody reaction. Inversely, when the flow rate exceeded 7.5 μL min^{-1}, the signal was dramatically reduced. Therefore, a flow rate of 5 μL min^{-1} was used for injections of reagents and washing buffer.

The response current obtained from the product of the enzymatic reaction is proportional to the activity of the enzyme conjugated and, consequently, inversely proportional to the amount of EE2 in the river samples water. The volume of magnetic beads injected after the pretreatment sample procedure was studied in the range of 5–25 μL. Sensitivity was almost tripled in the range 5–20 μL. Over 20 μL of magnetic beads dilutions, the central channel was obstructed. Finally a sample volume of 20 μL was used to evaluate other parameters. The rate of enzymatic response under flow conditions was studied in the pH range 4–7 and reached a maximum at pH 5.0. The pH value used was 5.0 in phosphate-citrate buffer. The effect of varying Q concentration from 0.1 to 5 mM on the enzymatic response was evaluated. The optimum Q concentration determined, 1 mM, was then used.

Table 1
Sequences required for the EE2 immunoassay.

Sequence	Condition	Time
Pretreatment of samples	Capture antibody coated microbeads + sample	10 min
Washing step	PBS buffer, pH 7.2	3 min
Injection of magnetic beads	20 μL of modified magnetic beads at 5 μL min^{-1}	4 min
Washing buffer	Flow rate: 5 μL min^{-1} (PBS, pH 7.2)	3 min
Enzyme conjugate	25 μL EE2-HRP at 5 μL min^{-1}	5 min
Washing buffer	Flow rate: 5 μL min^{-1} (PBS, pH 7.2)	3 min
Substrate	5 μL of 0.1 M phosphate/citrate buffer, pH 5.0 containing 1 mM of H_2O_2 and 1 mM of Q	1 min
Signal analysis	LC-4C amperometric detector, 0.10 V	1 min

3.3. Quantitative test for the detection of EE2 in the microfluidic immunosensor

Under the selected conditions described above, the electrochemical response of the enzymatic product is inversely proportional to the concentration of EE2 in the river water sample. Table 1 summarizes the complete analytical procedure required for the EE2 immunoassay using our system. A linear calibration curve to predict the concentration of EE2 in river water sample was produced within 0.01–60 ng L^{-1}. The linear regression equation was $i = 282.11 - 4908 \times C_{EE2}$ with the linear regression coefficient $r = 0.998$. The coefficient of variation (CV) for the determination of 5 ng L^{-1} of EE2 solution was below 4.1% (six replicates).

These values demonstrate that our microfluidic immunosensor can be used to quantify the amount of EE2 in unknown samples. An immunoassay was also carried out as described (Hintemann et al., 2006), in which the absorbance changes were plotted against the corresponding EE2 concentration, with the analytical working range for EE2 between 0.1 and 1500 ng L^{-1}. The limit of detection (LOD) was considered as the concentration that gives a signal three times the standard deviation (SD) of the blank. For electrochemical detection and ELISA, the LODs were 0.006 and 0.052 ng L^{-1} EE2, respectively.

The specificity of the antiserum was previously tested in the EE2 CLEIA (Schneider et al., 2005) by determination of the molar cross-reactivities for estradiol and estrone, steroids frequently occurring in STP effluents. The values for estradiol were 0.2% and for estrone were 0.1%, respectively. The antiserum shown cross reactivity only for EE2 metabolites conjugated at ring position 3; EE2-3-sulphate sodium salt and EE2-3-glucuronide sodium salt exhibiting cross reactivity around 30 and 15%, respectively (Schneider et al., 2004).

Table 2
Within-assay precision (five measurements in the same run for each control sample) and between-assay precision (five measurements for each control sample, repeated for 3 consecutive days).

[a]Control	Within-assay		Between-assay	
	Mean	CV (%)	Mean	CV (%)
0.1	0.098	2.61	0,106	3.91
5	5.07	3.94	5.24	5.72
60	61.25	4.06	62.11	5.41

[a] ng L^{-1} EE2.

Table 3
Determination of EE2 in original and spiked water sample aliquots.

Sample N°	EE2 ($ng\,L^{-1}$)	Spiking level ($ng\,L^{-1}$)			
		1	Recovery (%)	5	Recovery (%)
SW1	4.60 ± 0.09	5.66 ± 0.14	106	9.67 ± 0.31	101.4
SW2	5.91 ± 0.13	6.87 ± 0.10	96	11.13 ± 0.23	104.4
SW3	2.75 ± 0.08	3.73 ± 0.06	98	7.68 ± 0.12	98.6
SW4	3.77 ± 0.08	4.81 ± 0.09	104	8.88 ± 0.29	102.2
SW5	8.92 ± 0.15	9.98 ± 0.21	106	13.79 ± 0.42	97.4
SW6	12.47 ± 0.26	13.59 ± 0.37	112	17.62 ± 0.37	103
TW	2.17 ± 0.05	3.20 ± 0.11	103	7.24 ± 0.23	101.4
UW	1.65 ± 0.03	2.69 ± 0.05	104	6.59 ± 0.14	98.8

Representative samples of tap water, ultrapure water and surface water were spiked separately with 1.0 and 5.0 $ng\,L^{-1}$ of EE2. SW: surface water; TW: tap water; UW: ultrapure water.

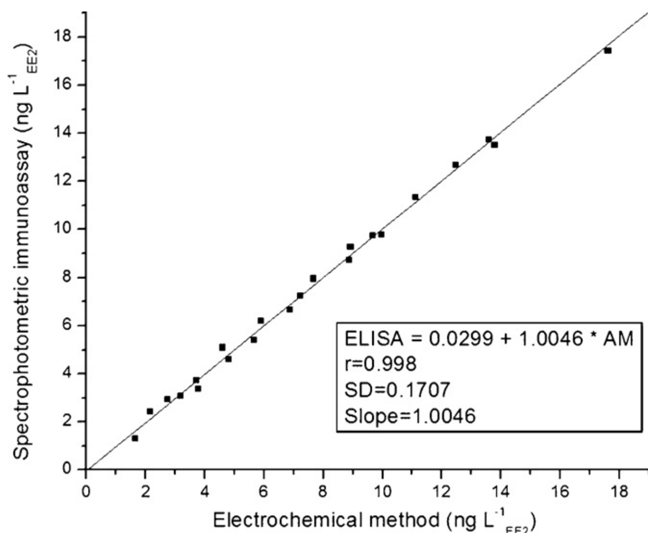

Fig. 4. Correlation between proposed method and immunophotometric assays.

The precision of the electrochemical assay was checked with control EE2 solution at concentrations of 0.1, 5, and 60 $ng\,L^{-1}$. The within-assay precision was tested with five measurements in the same run for each sample. These series of analyses were repeated for 3 consecutive days to estimate between-assay precision. The results obtained are summarized in Table 2. The EE2 assay showed good precision; the CV within-assay values were below 4.1% and the between-assay values below 5.8%.

This method was applied to determine EE2 in six river water samples. Concentration results were plotted against spike levels and interpolated using weighted linear regression (Table 3).

The electrochemical system was compared with an spectrophotometric immunoassay (Hintemann et al., 2006) for the quantification of EE2 in water samples and spiked water samples (see Table 3). The slopes obtained were reasonably close to 1, indicating good correspondence between the two methods (Fig. 4). Compared with the spectrophotometric immunoassay, our method shows similar sensitivity and its sensitivity is high enough to determine EE2 in unknown samples with very low levels, with the advantage of the use of smaller volumes of reagents and samples and the improvement efficiency with regard to time analysis.

4. Conclusions

In this work, we have developed a microbiochip microfluidic immunosensor coupled with flow injection (FIA) system that can be used for the rapid sensitive and selective quantification of EE2 in water samples using electrochemical detection. The integration of a microfluidic device based on the use of modified paramagnetic microbeads, with a gold electrode to measure an electrical signal, increased the capability to determine low levels of EE2 with high sensibility. The increased reactive surface area and the reduced diffusion distances in our immunoaffinity microfluidics device permitted a faster time of analysis (30 min) and a less sample consumed than conventional immunoassay techniques. Owing to the wider applications in many fields, miniaturized magnetic immunosensors will make a significant contribution to faster, direct, and secure analysis in many fields as clinical, environmental and food determinations.

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