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A novel amperometric enzyme inhibition biosensor based on xanthine oxidase immobilised onto glassy carbon electrodes for bisphenol A determination

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ABSTRACT

A novel and simple biosensor for the determination of bisphenol A (BPA) based on xanthine oxidase (XOD) enzymatic inhibition has been developed. The biosensor was prepared from xanthine oxidase immobilised by crosslinking with glutaraldehyde, with hypoxanthine as enzyme substrate, and was successfully applied to the determination of BPA using fixed potential amperometry. Biosensor performance was optimised with respect to the applied potential, influence of pH of the electrolyte solution, XOD loading and the substrate concentration. The enzyme inhibition mechanism was evaluated from Cornish-Bowden plus Dixon plots and was found to be reversible and competitive with an apparent inhibition constant of 8.15 nM. Under optimised conditions, the determination of BPA can be achieved in the linear range up to 41 nM with a detection limit of 1.0 nM, which is equal to the lowest reported in the literature, with very good repeatability and reproducibility. The selectivity of the biosensor was evaluated by performing an interference study and found to be excellent; and stability was investigated. It was successfully applied to the detection of BPA in mineral water and in river water.

1. Introduction

Concerns are growing among the public and the scientific community with respect to chemicals, known collectively as endocrine disrupting compounds (EDCs), and which have been extensively studied in relation to malformation, cancers, sexual precocity, neural and behavioral changes in infants and children [1,2].

Bisphenol A (BPA), one of the typical endocrine-disrupting chemicals, is suspected of interfering with the normal function of the endocrine system causing adverse effects for humans and wildlife [3]; it can mimic the body's own hormones and lead to an increased risk of cancer [4,5]. However, BPA is important as a major component in the production of polycarbonate (PC) and epoxy resin (EP), which are extensively used to make plastic food and beverage containers, water bottles, baby bottles, and some dental sealants. Moreover, what is more serious is that the migration of BPA from these materials into food has been reported [6–9], and which can lead to acute toxicity to aquatic organisms and has also been shown to occur in human cultured cells [10–12]. Additionally, BPA may even induce obesity, diabetes, infertility, birth defects, prostate cancer and breast cancer [13,14]. During the past few years, the frequent environmental accidents resulting from BPA have been considered as one of the most fundamental concerns at a global level. Bisphenol A is present in raw sewage, in river water and sediments, and can also migrate into drinking water. It is released into the environment due to domestic and industrial activities. Thus, it is very important to remove BPA from the environment and to determine its presence in trace quantities. In this context, a rapid, simple, sensitive and high-selective electroanalytical method for the determination of bisphenol A would be highly desirable.

Until now, the analytical techniques for determination of BPA are mainly high performance liquid chromatography (HPLC) [15], gas chromatography (GC) [16], mass spectrometry [17], immunochemical methods [6], immunochromatographic lateral flow assay [18], and enzyme-linked immunosorbent assay [19]. However, all these technologies are time-consuming, use expensive instrumentation and require large sample volumes [20]. Electrochemical biosensors, on the contrary, have shown advantages like simplicity, rapidity and low cost and have the benefit of high sensitivity, potential for miniaturization, speci-

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ficity, the possibility of in situ analysis and allowing the determination of environmental pollutants. Nowadays, the various biosensors that have been fabricated for bisphenol A analysis involve incorporation of different biomolecules such as peptides [21–23], antibodies [24–26], aptamers [27–29] and enzymes [30–34]. Most of the enzyme biosensors for BPA are based on tyrosinase [30,33,34], or laccase [35]. The major drawbacks of most of these is that some chemical species can be interferents and behave like the enzyme substrate, interact specifically with the immobilized enzyme or can bind to the immobilized enzyme, causing changes in its active site. Enzyme inhibition is the result of a reduction in observed enzymatic activity caused by the presence of an inhibitor in the system [36]. Thus, biosensors based on enzyme inhibition are reliable tools for the detection of many toxic compounds and are extremely useful for application in clinical, food and environmental samples [37].

In the present work, xanthine oxidase (XOD) enzyme-based electrodes are proposed for the first time for inhibitive determination of bisphenol A. The experimental conditions influencing the analytical performance of the XOD biosensor were optimised. The response of the sensor to bisphenol A was evaluated by measuring the activity of xanthine oxidase after adding different concentrations of BPA. The biosensor exhibited an attractive performance for determination of BPA, with good selectivity and stability. Practical application of the developed modified electrode to the determination of BPA was successfully carried out.

2. Experimental

2.1. Reagents and solutions

Xanthine oxidase (E.C. 1.1.3.22, from buttermilk 0.068 U/mg) and glutaraldehyde (GA) (25% v/v) aqueous solution were acquired from Fluka, bovine serum albumin (BSA) and hypoxanthine were from Aldrich. BPA was obtained from Sigma–Aldrich.

All solutions were prepared using Millipore Milli-Q nanopure water (resistivity > 18 MΩ cm). The phosphate buffer solution (PB), 0.1 M was prepared over the pH range of 6.0–8.0 and was used as supporting electrolyte for biosensor evaluation and for all the electrochemical measurements. A stock solution of 0.2 M BPA was prepared in methanol and stored in a refrigerator at 4 °C. For BPA detection, the stock solution was diluted with buffer to 1.0 μM and from this solution small aliquots (2–10 μL) were added to the cell. Experiments were performed at room temperature, 25 ± 1 °C.

2.2. Electrochemical instrumentation and measurements

All electrochemical measurements were performed with a conventional three-electrode system in an electrochemical cell of 2 mL volume, containing the glassy carbon electrode (GCE) as working electrode with 0.00785 cm^2 surface area, a platinum electrode as counter electrode and an Ag/AgCl electrode as reference electrode.

The amperometric measurements were carried out at room temperature using a computer controlled IviumStat electrochemical analyser with IviumSoft software (version 2.024, Ivium Technologies, The Netherlands).

The pH measurements were carried out with a Crison 2001 micro pH-meter (Spain) at room temperature.

2.3. Enzyme immobilization

The immobilization of xanthine oxidase (XOD) onto the surface of the GCE was done by cross-linking with glutaraldehyde and bovine serum albumin (BSA) as previously described for several enzyme biosensors [38–40] in order to increase the attachment of the enzyme to the transducer and facilitate electron exchange between substrate and enzyme active centre. A mixture of 3 mg of XOD and 3 mg of BSA in 30 μL phosphate buffer (pH = 7) was prepared. From this solution, 1 μL (which is equivalent to 0.0068 U XOD) was placed over the GCE, and immediately 1 μL of the cross-linking agent glutaraldehyde (2.5% v/v in Milli-Q water) was added and then dried at room temperature for 1–2 h. The electrodes were kept at 4 °C in phosphate buffer, pH 7.0 until needed.

2.4. Enzyme inhibition measurements

In order to evaluate the inhibition of the activity of xanthine oxidase, the XOD/GCE modified electrode was dipped into a stirred phosphate buffer solution at pH 7.5, and a fixed potential of −0.5 V vs. SCE was applied. After stabilisation of the baseline current, a fixed amount of hypoxanthine (substrate) was added to record a steady-state current (I_0) before adding the inhibitor. The concentration of added bisphenol A was increased to inhibit the xanthine oxidase activity, and the current decrease (I_1), which is proportional to the final concentration of the inhibitor in solution, was recorded. In order to evaluate the percentage of inhibition (I (%)) due to the bisphenol A inhibitor, the following expression was used:

$$I\,(\%) = \frac{I_0 - I_1}{I_0} \times 100$$

3. Results and discussion

3.1. Biosensor for bisphenol A development and optimization

Experimental variables that can affect the performance of the amperometric inhibition biosensors, namely the pH of the supporting electrolyte, the applied potential, the substrate concentration, and the enzyme loading, were studied in order to optimise response to bisphenol A by inhibition.

3.1.1. Effect of pH

Taking into account previous work based on XOD [40] initial measurements were performed at an applied potential of −0.3 V. The effect of the pH value of the 0.1 M phosphate buffer solution was examined in the range from 6.0 to 8.0. The response of the biosensor to 10 nM bisphenol A, in the presence of 0.3 mM hypoxanthine varied significantly with pH. The dependence of the current response on pH is illustrated in Fig. 1. As can be seen, the amperometric signal rises with increase in pH from 6.0 to 7.5, where a maximum is exhibited, and then decreases. Thus, phosphate buffer solution pH = 7.5 was chosen as the most suitable for amperometric measurements.

3.1.2. Effect of the applied potential

In order to optimise the response of the XOD/GCE biosensor towards bisphenol A, the amperometric response to 10 nM bisphenol A was tested in the presence of 0.3 mM hypoxanthine at fixed potentials from −0.5 V to −0.1 V vs Ag/AgCl (Fig. 2). As can be seen, the response to BPA decreases with a less negative potential value. The higher current response is exhibited at −0.5 V vs. Ag/AgCl, which was chosen for further studies.

3.1.3. Effect of substrate concentration

The substrate concentration can influence the monitoring of enzymatic activity under inhibition conditions. The effect of substrate con-

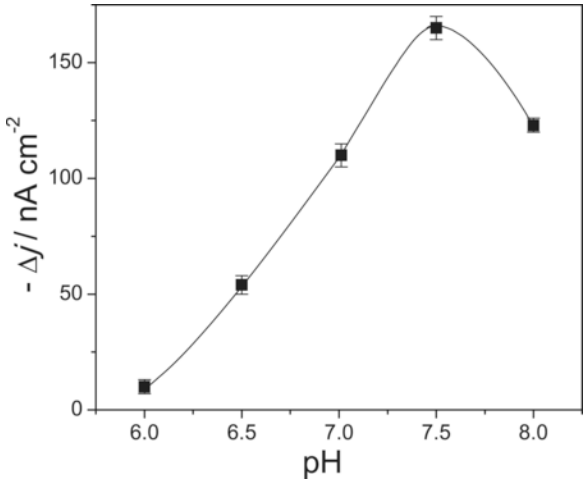

Fig. 1. Effect of pH on the amperometric response to 10 nM BPA in 0.1 M PB at XOD/GCE biosensor in the presence of 0.3 mM hypoxanthine. Applied potential −0.3 V vs. Ag/AgCl.

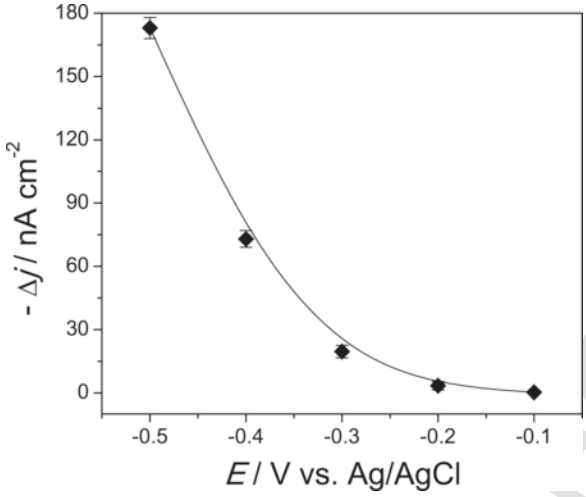

Fig. 2. Effect of the applied potential on the amperometric response to 10 nM BPA at XOD/GCE biosensor in 0.1 M PB pH 7.5 in the presence of 0.3 mM hypoxanthine.

centration on inhibition can be tested by measuring the activity of XOD with varying concentrations of hypoxanthine substrate and injecting the same concentration of BPA inhibitor. Thus, the sensitivity of the biosensor to bisphenol A as a function of three hypoxanthine concentrations 0.3, 0.5 and 1.0 mM was examined (not shown). The sensitivity to bisphenol A increases with decrease in hypoxanthine concentration. The highest sensitivity for BPA determination was achieved in the presence of the lowest concentration of hypoxanthine tested, namely 0.3 mM, the response decrease being 89% of this for 0.5 mM and 53% for 1.0 mM. In order to achieve high sensitivity of the response to BPA, a concentration of 0.3 mM of hypoxanthine was selected for further experiments.

3.1.4. Effect of enzyme concentration

The amount of enzyme was varied in order to optimise the response, by keeping the concentration of BSA (10%) constant and varying the concentration of XOD. Fig. 3 shows the response of bisphenol A for various enzyme concentrations: 0.0034, 0.0068 or 0.0136 U immobilised on the GCE. An increase of the response toward bisphenol A by 48% is clearly observed when increasing the enzyme concentration from 0.0034 to 0.0068 U, but a decrease of 40% for 0.0136 U compared with 0.0034 U. Thus a XOD concentration of 0.0068 U immobilised on the GCE was chosen for future experiments.

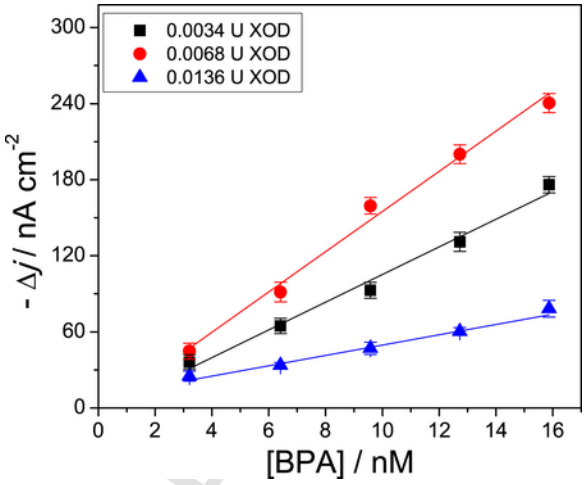

Fig. 3. Calibration curves for the determination of BPA in 0.1 M PB pH 7.5 for three different enzyme concentrations in the presence of 0.3 mM hypoxanthine. Applied potential −0.5 V vs Ag/AgCl.

3.2. Analytical performance of the biosensor for bisphenol A

The amperometric measurement of bisphenol A at the XOD/GCE biosensor was carried out in 0.1 M phosphate buffer solution, pH 7.5, at an applied potential of −0.5 V vs. Ag/AgCl and in the presence of 0.3 mM hypoxanthine. Aliquots of stock solution of bisphenol A were added, leading to a decrease in the hypoxanthine response, that can be deduced to be due to inhibition of XOD activity, as illustrated in Fig. 4(a). Although in other work [41], performed under different experimental conditions, it was found that BPA can activate XOD, here there is inhibition. Their matrix (animal models and cell cultures) is significantly more complex than that used by us and XOD is in solution rather than immobilised. Immobilization often leads to restrictions in the enzyme conformations, which can greatly influence the apparent enzyme activity. In [41] it was found that BPA induces secondary structure alterations of XOD and this probably forms the basis of the explanation for the different observed behaviour.

In Fig. 4(b), the calibration curve for bisphenol A gives a linear response between 1.0 and 41 nM of bisphenol A following the equation $-\Delta j$ (μA cm^{-2}) = 0.0245c (nM) + 0.00065 (R^2 = 0.9998), and a detection limit calculated as (3xSD_{blank})/slope was 1.0 nM. A comparison of the performance achieved by the new biosensor and other BPA biosensors in the literature is shown in Table 1. The detection limit for bisphenol A achieved here is significantly lower than most of the biosensors and is only equalled by two tyrosinase-based biosensors [2,3].

The inhibition constant, K_i, can be determined from the Dixon plot [47], Fig. 5(a), in which the inverse of the enzyme activity is plotted vs. inhibitor concentration for at least two different enzyme-substrate concentrations. For the evaluation of this constant, the Dixon plot was made for three different substrate (hypoxanthine) concentrations: 0.3, 0.5 and 1.0 mM. Following this method, the extrapolated abscissa intercept of each linear plot corresponds to the inhibition constant, which was determined to be K_i = 8.15 nM.

3.3. Determination of the type of inhibition

For the evaluation of the type of enzyme inhibition, the data were analysed using Dixon and Cornish-Bowden plots. The Dixon plot by it-

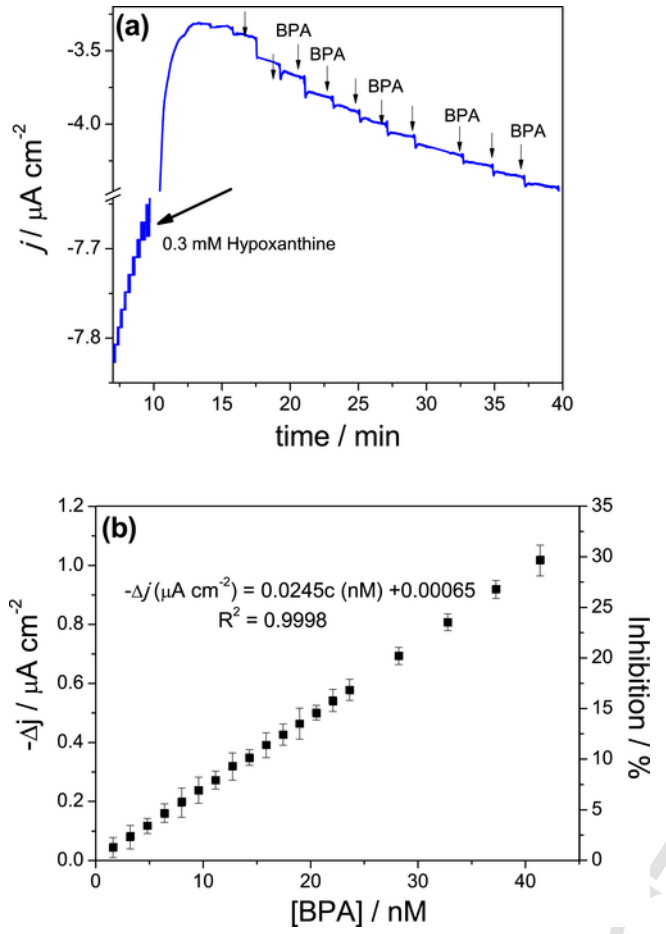

Fig. 4. (a) Amperometric response at XOD/GCE in the presence of 0.3 mM hypoxanthine and (b) corresponding calibration curve and degree of inhibition for different concentrations of BPA in 0.1 M PBS pH 7.5. Applied potential −0.5 V vs Ag/AgCl.

self cannot clearly distinguish between competitive and mixed reversible inhibition. In the Cornish-Bowden plot [48], the ratio of substrate concentration over enzyme activity is plotted vs. inhibitor concentration, but cannot always distinguish between mixed and uncompetitive inhibition. By using both Dixon and Cornish-Bowden plots, it is possible to elucidate the type of inhibition. Fig. 5(a) and (b) show Dixon and Cornish-Bowden plots using three different concentrations of hypoxanthine, 0.3, 0.5 and 1.0 mM. From the Dixon plot, the crossing lines enable it to be deduced that the inhibition is mixed or competitive. The same inhibition data in the Cornish-Bowden plot show that the inhibition is competitive because the straight lines drawn through the experimental points are parallel. Further evidence that the mechanism of inhibition by bisphenol A is competitive is the fact that inhibition is decreased by increasing the amount of substrate, from 48.4% in the presence of 0.3 mM hypoxanthine to 22.1% in the presence of 1.0 mM hypoxanthine.

3.4. Interference studies

Several phenolic compounds and inorganic ions were measured to examine whether they interfere with the determination of bisphenol A. Under the optimal conditions, the influence of some potential interferent species on the current response of 30 nM BPA was studied. The results indicated that the presence of 100-fold concentration of interfering substances had no significant influence on the signals of BPA with the deviation below ±7%. The results are shown in Table 2. The proposed sensor is thus demonstrated to show good selectivity for the determination of BPA in real samples.

Table 1
Performance comparison of the developed biosensor for BPA detection with other biosensors in the literature.

Electrode configuration	Linear range / μM	Detection limit /nM	References
Tyr/SWCNT-PolyLys/GCE	0.004–11.5	0.97	[2]
Tyr-GR-Au-CS/GCE	0.0025–3.0	1.0	[3]
Tyr/TiO_2-MWCNT-PDDA-Nafion/GCE	0.28–45.05	66	[30]
CYP2C9–PAM/GCE	1.25–10	580	[31]
ctDNA-SWNT/GCE	0.01–20	5.0	[32]
Tyr-rGO-CS/ITO	0.01–50	0.74	[33]
Tyr/nano-Au/T-NH_2/AuE	0.399–234	133	[34]
Lacc–TH–CB/SPE	0.5–50	200	[35]
CuMOF-Tyr-CS/GCE	0.05–3.0	13	[42]
Tyr-APTES-nTiO_2/Ti	0.01–1.0	10	[43]
Tyr-DAPPT-rGO/GCE	0.001–38	0.35	[44]
PLT-Tyr-GOx/AuE	0.05–1.0	16	[45]
Tyr/Au@PDA-rGO-CS/GCE	0.012–3.68	0.1	[46]
XOD/GCE	0.001–0.041	1.0	This work

Tyr: tyrosinase; Lacc: laccase; XOD: xanthine oxidase; CYP2C9: human cytochrome P450 2C9; ctDNA: calf thymus DNA; SWCNT: single-walled carbon nanotubes; MWCNTs: multi-walled carbon nanotubes; GR: graphene; rGO: reduced graphene oxide; PolyLys: poly(lysine); PDDA: poly(diallyldi-methylammonium chloride); PAM: polyacrylamide; T-NH_2: thioctic acid amide; TH: thionine; CuMOF: copper centered metal-organic framework; APTES: 3-aminopropyltriethoxysilane; nTiO_2: nanostructured titanium dioxide; DAPPT: 1,3-di(4-amino-1-pyridinium) propane tetrafluoroborate ionic liquid; PDA: polydopamine; PLT: L-tyrosine polymer; CS: chitosan; GCE: glassy carbon electrode; SPE: screen printed electrode; ITO: indium tin oxide.

3.5. Reproducibility, repeatability and lifetime

Reproducibility, tested by measuring the same concentration, 30 nM, of BPA at five different electrodes prepared in the same way, gave a relative standard deviation (RSD) of 3.2% (Fig. 6a). The repeatability of the modified electrode was investigated by recording the response at a fixed concentration, 30 nM BPA five successive times (Fig. 6b). The RSD of these five measurements was 2.4%.

The lifetime of the biosensor was monitored daily by measuring the response to BPA in the presence of hypoxanthine during 15 days. After this period, the response to BPA dropped to 75% of the initial value (Fig. 6c). This is a bit less than for other biosensors for BPA [2,22], but is similar to that obtained in [30] with tyrosinase, and can be ascribed to a decrease in xanthine oxidase activity, as previously observed [40]. However, such a loss of activity does not compromise use as an effective inhibition biosensor.

3.6. Application

To evaluate the fabricated enzyme inhibition biosensor for bisphenol A determination in environmental samples, application of the biosensor was examined by recovery measurements in mineral water and in river water in which no BPA was detected previously. The results are presented in Table 3. The average recovery was in the range of 97.9–103%, which indicates the efficacy of the biosensor for practical analysis.

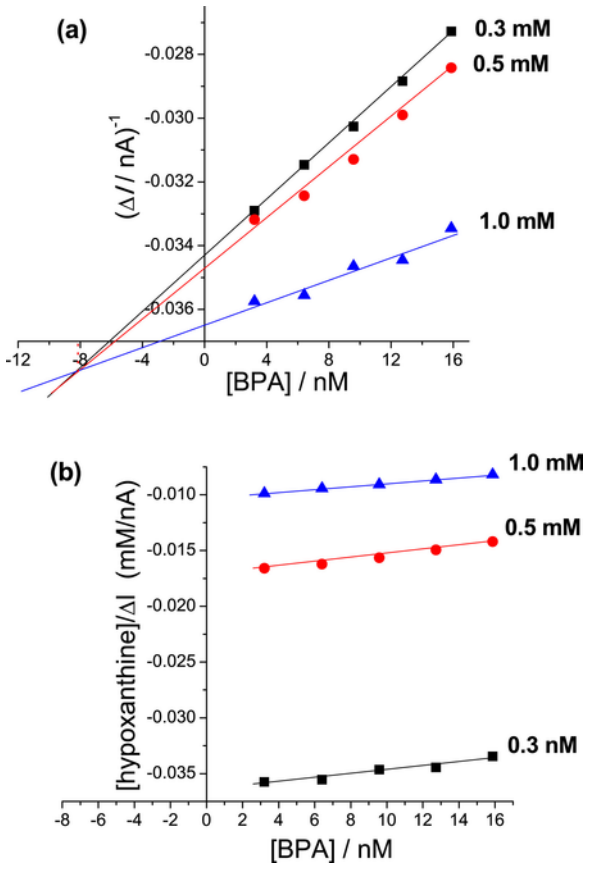

Fig. 5. (a) Dixon plots and (b) Cornish-Bowden plots for BPA for three different hypoxanthine concentrations.

Table 2
Interferences from other species on the inhibition response to 30 nM BPA, concentration ratio 100:1 (3000 nM).

Interferent	*I* change (%)
K^+	−6.8
Na^+	3.4
Mg^{2+}	−2.8
Zn^{2+}	−1.3
Ethanol	2.4
Methanol	2.9
Hydroquinone	−5.0
Phenol	−3.6

4. Conclusions

A novel, easy to prepare electrochemical enzyme biosensor for inhibition assays, based on xanthine oxidase immobilised on glassy carbon electrode has been developed for bisphenol A. The optimised biosensor exhibited high sensitivity and low detection limit, with good selectivity and stability, and enabled successful detection in water samples. The inhibition constant was determined by using a Dixon plot together with the Cornish-Bowden plot, and the enzyme inhibition mechanism of BPA on the XOD activity the inhibition was shown to be competitive. The biosensor described here has a simpler architecture and showed much better performance than other biosensors reported in the literature for BPA and is a new and promising approach for its detection.

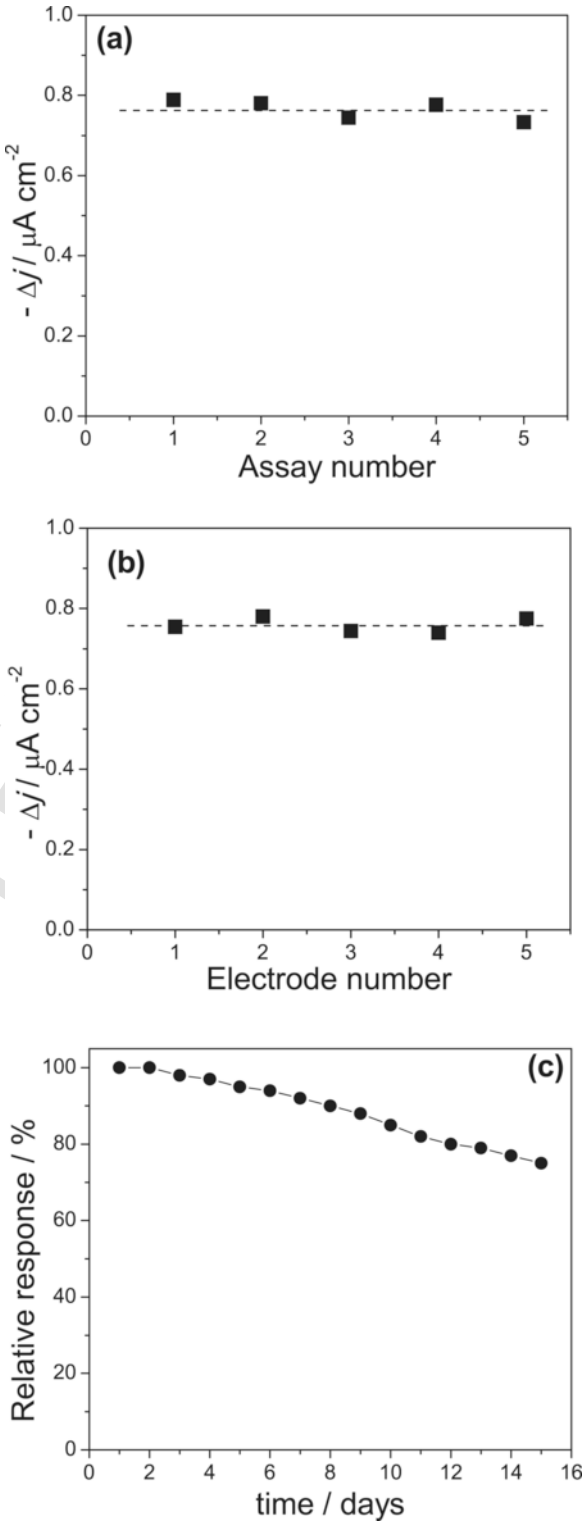

Fig. 6. Graphical representation of (a) repeatability; (b) reproducibility and (c) stability of the XOD/GCE biosensor.

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Table 3
Determination of bisphenol A in water samples.

Sample	Added (nM)	Found (nM)	Recovery (%)
Mineral Water	4.80	4.73	98.5
	8.00	8.15	101.8
River Water	4.80	4.70	97.9
	8.00	8.24	103.0

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