Talanta 106 (2013) 249–254

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Molinate quantification in environmental water by a glutathione-S-transferase based biosensor

Túlio I.S. Oliveira [a,b,1], Marcela Oliveira [a,c,1], Subramanian Viswanathan [a], M. Fátima Barroso [a,*], Luísa Barreiros [d], Olga C. Nunes [d], José A. Rodrigues [c], Pedro de Lima-Neto [b], Selma E. Mazzetto [b], Simone Morais [a], Cristina Delerue-Matos [a]

[a] REQUIMTE, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal
[b] Departamento de Química Orgânica e Inorgânica, Centro de Ciências, Universidade Federal do Ceará, Bloco 940 Campus do Pici, 60455-970, Fortaleza, Ceara, Brazil
[c] REQUIMTE, Chemistry and Biochemistry Department, Faculty of Sciences, University of Porto, Rua do Campo Alegre 687, 4169-007 Porto, Portugal
[d] LEPAE, Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

ARTICLE INFO

Article history:
Received 5 June 2012
Received in revised form
19 October 2012
Accepted 24 October 2012
Available online 9 November 2012

Keywords:
Glutathione-S-transferase
Enzymatic biosensor
Voltammetry
Molinate
Rice paddy field floodwater

ABSTRACT

A glutathione-S-transferase (GST) based biosensor was developed to quantify the thiocarbamate herbicide molinate in environmental water. The biosensor construction was based on GST immobilization onto a glassy carbon electrode via aminosilane–glutaraldehyde covalent attachment. The principle supporting the use of this biosensor consists of the GST inhibition process promoted by molinate. Differential pulse voltammetry was used to obtain a calibration curve for molinate concentration, ranging from 0.19 to 7.9 mg L^{-1} and presenting a detection limit of 0.064 mg L^{-1}. The developed biosensor is stable, and reusable during 15 days. The GST-based biosensor was successfully applied to quantify molinate in rice paddy field floodwater samples. The results achieved with the developed biosensor were in accordance with those obtained by high performance liquid chromatography. The proposed device is suitable for screening environmental water analysis and, since no sample preparation is required, it can be used *in situ* and in real-time measurements.

1. Introduction

Molinate (azepan-1-yl-ethylsulfanyl-methanone) is a pre-emergent thiocarbamate herbicide used worldwide in flooded fields during rice seeding to control the overgrowth of weeds [1]. After being applied to the field, this selective herbicide can be dissipated by physical, chemical and biological phenomena. Molinate and its degradation products have been detected in natural environments such as soil, rivers, lakes, underground streams, supply waters and in laboratory-scale decontamination systems [2].

High-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) or photodiode-array (PDA) detectors is frequently described as the most useful technique to assess and monitor molinate in real samples (e.g. river water, paddy field floodwater and soil) [2–5]. Although chromatographic techniques are accurate, precise and robust, they are not resourceful for routine control since they require tedious sample pre-treatments, highly qualified technicians and sophisticated instruments, being costly and time consuming [6]. Moreover, HPLC originates large quantities of organic effluents (usually toxic solvents).

Electroanalytical approaches are of special interest in environmental monitoring due to the high sensitivity inherent to the electrochemical detection and the possibility to miniaturize the required instrumentation, thereby making the construction of compact and portable analysis devices possible [7]. An alternative methodology based on electrochemical procedures, using a hanging mercury drop electrode (HMDE), has been used to quantify xenobiotics in real samples [6]. A previously developed HMDE method to quantify molinate in river water showed high sensitivity (detection limit of 3.5×10^{-8} mol L^{-1}) [8]. However, the utilization of elemental mercury, which is toxic and promotes severe health effects [9], constitutes a major drawback. Thus, researchers have been investigating more environmental friendly, cheaper and rapid alternative methods for the detection and screening of xenobiotics. Electrochemical enzymatic biosensors have filled the gap in this regard and have been intensely developed for pesticide quantification. Taking into account that many pesticides are designed to inhibit various enzymes, it seemed logical to use these enzymes for detection purposes. Therefore, for the construction of an electrochemical enzymatic biosensor, these enzymes are often used, incorporated and

* Corresponding author. Tel.: +351 228340500; fax: +351 228321159.
E-mail address: mfb@isep.ipp.pt (M. Fátima Barroso).
[1] Túlio I.S. Oliveira and M.J.C. Oliveira equally contributed to this work.

0039-9140/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2012.10.074

immobilized into a sensor [10]. The majority of the previously developed biosensors are based on the use of acetylcholinesterase or butyrylcholinesterase enzymes [11,12]. Furthermore, some publications describe the use of the alkaline phosphatase, tyrosinase and laccase enzymes [13–15] being scarce those based on glutathione-S-transferase (GST, EC.2.5.1.18). This enzyme, a cytosolic multicomponent protein, catalyzes the nucleophilic addition of the thiol of reduced glutathione (GSH) to a wide range of electrophilic compounds. This conjugation reaction functions has a detoxification mechanism of endogenous compounds (peroxidized lipids) and exogenous compounds such as xenobiotics (X) as it is represented in the following equation [16,17]:

$$GSH + X \xrightarrow{GST} GSX \quad (1)$$

Andreou and Clonis [18] reported the development of a fiber-optic biosensor based on the GST immobilization for the detection of atrazine in real water samples. In this study, the GST catalyzed nucleophilic attack of GSH on atrazine released H^+ which could be detected as a pH variation; this change was directly correlated with the analyte concentration. Some optical and electrochemical GST based biosensors have been developed for the detection and quantification of the fungicide captan in contaminated samples. The detection mechanisms of these biosensors were based on the inhibition process promoted by captan on the GST enzyme [19–21]. No study was found concerning the development of a GST based biosensor for molinate detection.

The purpose of this work was to develop an alternative approach for the quantification of molinate in environmental waters. Biosensor advantages include low cost, reliability, no or little sample preparation, disposability, as well as the possibility to perform *in situ* and real-time measurements [22]. Thus, the alternative methodology was based on the development of a GST based electrochemical biosensor. The principle of this enzymatic biosensor consisted on the GST inhibition process promoted by molinate in the presence of the substrates GSH and 1-chloro-2, 4-dinitrobenzene (CDNB). The construction of the biosensor was based on the immobilization of GST onto a glassy carbon electrode (GCE) via an aminosilane–glutaraldehyde covalent attachment. Considering the potential future application of the developed biosensor, it was used to quantify the thiocarbamate molinate in real rice paddy floodwater samples. The results achieved with the GST-biosensor were compared with those obtained by HPLC–UV.

2. Experimental

2.1. Chemicals and solutions

Glutathione-S-transferase (GST) (from equine liver), substrates 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione-S-reduced (GSH), molinate (certified purity higher than 99.6%), 3-aminopropyltriethoxysilane (APTES), (Ref. no. A3648), $K_4[Fe(CN)_6]$ and Glutaraldehyde (GA) were purchased from Sigma (Madrid, Spain). Other chemicals were Merck (Darmstadt, Germany) pro-analysis grade and were used as received.

GST enzyme solution (0.5 mg mL^{-1}) and the substrate GSH (0.1 mol L^{-1}) were freshly prepared and stored at +4 °C. The substrate CDNB (0.1 mol L^{-1}) was prepared in absolute ethanol and frozen. GA (grade I, 2% aqueous solution) was freshly prepared.

All the electrochemical measurements were performed in 0.1 mol L^{-1} phosphate buffered saline (PBS, pH 7.0), at 25 °C. All solutions were freshly prepared with Type I deionised water (18.0 MΩ cm) obtained from a Simplicity 185 (Millipore, Darmstadt, Germany) purification system.

2.2. Apparatus

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed with an Autolab PGSTAT 10 (EcoChemie, Utrecht, The Netherlands) potentiostat controlled by the GPES software (EcoChemie, Utrecht, The Netherlands). A conventional three-electrode cell was used, which included a glassy carbon electrode (GCE) (0.07 cm^2; Metrohm, Herisau, Switzerland) as the working electrode, a platinum wire as counter-electrode and an Ag/AgCl, KCl (sat'd) Ag|AgCl|KClsat reference electrode (Metrohm, Herisau, Switzerland) to which all potentials were referred to.

The GCE was mechanically cleaned by polishing with γ-Al_2O_3 (0.3 μm and 0.05 μm) until a shining surface was obtained and then rinsed with water. Next, the GCE was immersed in piranha solution (H_2O_2:H_2SO_4, 1:3, v/v) for 10 min. For the electrochemical activation of the surface (in order to generate –COOH and –OH functional groups on top of GCE surface), the GCE was cycled from −0.2 to +1.6 V in 0.5 mol L^{-1} H_2SO_4 solution at 50 mV s^{-1}.

Molinate quantification by a comparative method was carried out using a high performance liquid chromatograph (Knauer, Berlin, Germany) equipped with an UV/vis detector K-2501 (Knauer, Berlin, Germany) operating at 220 nm and a LiChroCART® 250-4 Lichrospher® 100 RP-18 (5 μm) column (Merck, Darmstadt, Germany) protected by a LiChroCART® 4-4 guard column (Merck, Darmstadt, Germany). The mobile phase was a mixture of methanol:water (80:20, v/v) at a flow rate of 0.8 mL min^{-1} [5].

The determination of GST activity was carried out using a Shimadzu 160-A spectrophotometer with a quartz cell (optical path of 1.00 cm).

Ultra-pure water (18.2 MΩ cm) was produced by a Simplicity 185 apparatus (Millipore, Molsheim, France).

2.3. Standard enzyme assays

One unit of the GST enzyme is defined as the amount of enzyme that will conjugate 1.0 μmol of CDNB with reduced GSH per minute at pH 6.5 and 25 °C [18].

The GST activity was spectrophotometrically measured according to the procedure described by [18]. In a suitable quartz cuvette, 1500 μL of 0.1 mol L^{-1} PBS solution (pH 6.5), 15 μL of GSH solution (1 mmol L^{-1}) and 15 μL of ethanolic CDNB solution (1 mmol L^{-1}) were mixed. Next, the reaction was initiated by the addition of 20 μL of GST solution. The GST activity was monitored at 340 nm with a scan time of 10 s during 3 min.

2.4. GST based biosensor preparation

The schematic representation of the GST based biosensor construction is shown in Fig. 1. A procedure similar to the one reported by Singh et al. [21] was used. To perform the chemical modification of the GCE surface, the cleaned GCE was firstly rinsed with dichloromethane and dried under a nitrogen stream. Next, the GCE was immersed in APTES solution (10% in CH_2Cl_2) for 12 h. The aminosilane–GCE was then rinsed with dichloromethane and the modified electrode was immersed in a GA solution (2% aqueous solution) for 30 min. After that, the chemically modified electrode was washed with PBS (pH 7.0) and immersed in PBS (pH 8.0) just for a few minutes to block the GA that did not react. Finally, it was dried under a nitrogen stream.

Following the preparation of the GCE electrode, the enzyme immobilization was carried out. The GST immobilization was

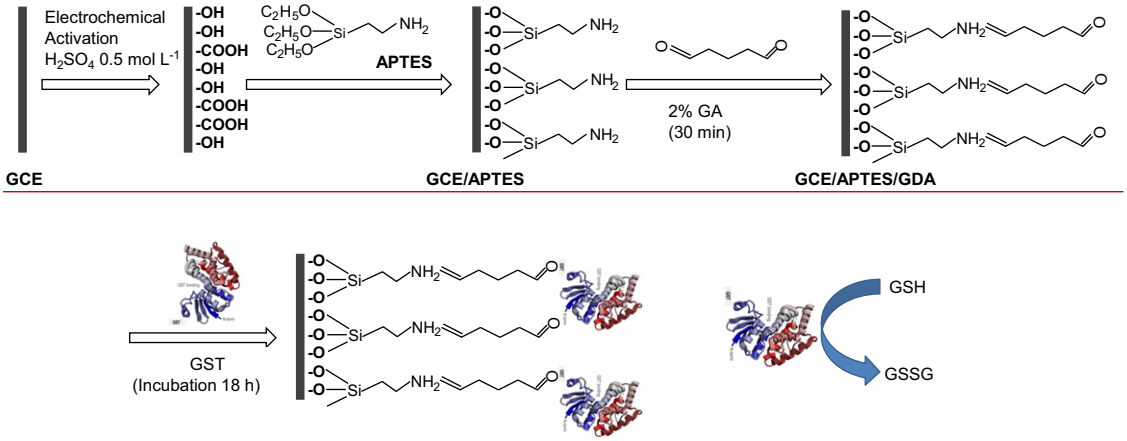

Fig. 1. Schematic representation of the GST based biosensor preparation.

performed by placing 40 μL of a GST solution (0.5 mg mL^{-1}) onto GCE/APTES/GA for 18 h. The GCE/APTES/GA/GST was stored in PBS solution (pH 7.0) at +4 °C.

2.5. Electrochemical measurements

All CV and DPV measurements were performed in a glass electrochemical cell containing 0.1 mol L^{-1} of PBS (pH 7.0).

The cyclic voltammograms were recorded by cycling the potential between −0.2 and +0.6 V at a scan rate of 50 mV s^{-1}. DPV measurements were carried out applying a potential range between −0.2 and +0.6 V. All electrochemical assays were performed using $[Fe(CN)_6]^{4-}$ as an electroactive indicator.

2.6. Rice paddy floodwater analysis

Floodwater samples were collected from a rice paddy field in the experimental farm "Bico da Barca", from Direção Regional de Agricultura e Pescas do Centro (DRAP Centro), located in the Mondego river valley, Montemor-o-Velho, central Portugal. This rice paddy field is used in conventional agriculture and, at the time of sample collection, had a background of molinate application of six years. Samples collection was performed 5 h after Ordram (commercial formulation containing 7.5% w/w of molinate) application at a rate of 50 kg ha^{-1} [4] and were stored at −20 °C until analysis.

Molinate quantification by HPLC–UV was performed as previously described [4,5]. Briefly, 20 mL aliquots of the floodwater sample were extracted twice with the same volume of n-hexane (20 mL). The resulting hexane extracts were pooled dried under vacuum, reconstituted in 1 mL of methanol and subsequently analyzed by HPLC–UV. Cycloate (5 mg L^{-1}) (Riedel-de Haën, Seelze, Germany) was used as internal standard for molinate extraction and quantification.

The electroanalytical determination of molinate in rice paddy floodwater, by means of the GST based biosensor, was carried out using the method of standard addition. A volume of environmental water sample was transferred to the electrochemical cell containing the supporting electrolyte (0.1 mol L^{-1} PBS pH 7.0), the substrate (1 mmol L^{-1} of GSH and 1 mmol L^{-1} of CDNB), the electron mediator (1 mmol L^{-1} $[Fe(CN)_6]^{4-}$) and quantified after three successive additions of molinate standard stock solution with 10 s incubation time. After each addition, DPV measurements were recorded from −0.2 to +0.6 V.

3. Results and discussion

Considering that GST plays an important role on molinate biodegradation [23,24], this enzyme was selected to construct a biosensor to quantify this herbicide in real environmental samples.

The susceptibility of biological material to environmental factors, external to the electrode, was circumvented by employing an immobilization method. Indeed, the physical adsorption method is of great scientific interest because it permits the use of biomolecules protected from physical damage by matrix immobilization, which in turn provides greater stability when applied in technological processes [25]. In this work, different strategies were used to perform the GST immobilization onto the electrode surface. The first one was carried out by using a gold electrode (instead of a GCE) and the immobilization step was performed via self-assembled monolayer (SAM). The alkanethiol film was formed by incubating a solution of cystamine followed by glutaraldehyde. However, using this methodology, the immobilized GST was not stable, since it did not show reproducibility on the scanned voltammograms (data not shown). Therefore, the strategy was modified and a procedure similar to the one used by [21] was applied.

Fig. 1 shows several analytical steps applied for the preparation of the enzymatic based biosensor. After cleaning and electroactivating the GCE on sulfuric acid, an aminosilane–SAM covalently attached on the GCE surface was performed by using APTES–glutaraldehyde. The sylane group of APTES was anchored at the GCE either by bonding the oxygen atom or by very scarce amount of surface water [21]. Glutaraldehyde is a bifunctional compound that binds proteins through their free amino groups, thus forming a three-dimensional network [18]. As a result, the aldehyde group, at one end from the glutaraldehyde molecule, binds with the amino group of APTES and the other aldehyde group (from the other end) binds with the amino group of GST.

3.1. Electrochemical characterization of GCE/APTES/GA/GST

The electrochemical behavior of the enzymatic modified electrode was investigated by CV. In order to monitor the characteristics of the modified biosensor surface, cyclic voltammograms were performed between −0.2 V and +0.6 V vs. AgCl/Ag, in 0.1 mol L^{-1} PBS solution using the $[Fe(CN)_6]^{4-}$ as an electron mediator in the presence of 1 mmol L^{-1} of GSH and 1 mmol L^{-1} of CDNB. As it can be observed in Fig. 2(a–c), the modification of the carbon surface, by the application of the APTES/GA on the surface, promoted a decrease on the peak intensity (i_p) due to the

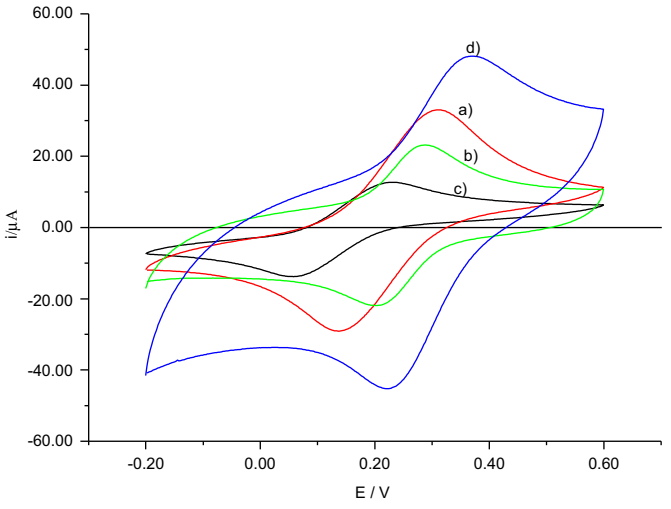

Fig. 2. Cyclic voltammograms obtained in PBS containing substrate (1 mmol L^{-1} GSH and 1 mmol L^{-1} CDNB) and electron mediator $[Fe(CN)_6]^{4-}$ at: (a) bare GCE; (b) GCE/APTES; (c) GCE/APTES/GA and (d) GCE/APTES/GA/GST.

resistance to the electron transport generated by the compounds covalently bonded to the GCE surface. As it was expected, when the enzymatic biosensor (GCE/APTES/GA/GST) was cycled, an increase of the i_p was observed and the electrocatalytic activity was found to be stable.

The electrocatalytic activity exhibited by this biosensor was tested by modification of some analytical parameters such as the scan rate (ν) and the pH of the electrolyte.

The effect of ν on the biosensor activity was assessed between 0.01 and 0.2 V s^{-1} in PBS containing 1 mmol L^{-1} of $[Fe(CN)_6]^{4-}$, 1 mmol L^{-1} of molinate, 1 mmol L^{-1} of GSH and 1 mmol L^{-1} of CDNB. The peak intensity i_p increased with the increase of v. The electrochemical mass transport process was analyzed by studying the mathematical relationship between the (i) i_p vs. v; (ii) i_p vs. $v^{1/2}$ and (iii) peak potential (E_p) vs. logν. The analysis of the mathematical equations obtained indicated that the electrocatalytic activity of the GST based biosensor is mainly controlled by a diffusion process [26].

(i) (i_p vs. ν): i_p (A) $= 1.13 \times 10^{-4}\,\nu + 1.54 \times 10^{-5}$, $r = 0.87$;
(ii) (i_p vs. $(\nu)^{1/2}$): i_p (A) $= 6.82 \times 10^{-5}\,(\nu)^{1/2} + 7.22 \times 10^{-6}$, $r = 0.944$;
(iii) (E_p vs. logν): E_p (V) $= -0.043\,\log(\nu) + 0.089$; $r = 0.999$.

The effect of pH on the performance of the enzymatic biosensor was evaluated within the range of 5–8 in buffer solutions containing molinate at a fixed concentration (5.0 mg L^{-1}). According to [21], enzymes are amphoteric molecules containing a large number of acid and basic groups mainly situated on their surface. The charges of these groups change, according to their acid dissociation constants and to the environmental pH, thus affecting the electroactivity of the enzymatic biosensor. In the studied pH range, the biosensor displayed an optimum electrochemical response at pH 7.0. Therefore, this pH value was selected for the subsequent studies.

3.2. Rice paddy field floodwater analysis

Considering that DPV is a more sensitive technique than CV, it was used to study the relationship between molinate concentration and the percentage of inhibition of the peak current. DPV techniques were performed in the potential range of −0.2 V to +0.6 V using 0.1 mol L^{-1} PBS at pH 7.0 in the presence of 1 mmol L^{-1} GSH, 1 mmol L^{-1} CDNB and 1 mmol L^{-1} $[Fe(CN)_6]^{4-}$. The detection of molinate by the developed enzymatic biosensor was based on the inhibition of the catalysis reaction performed by GST. The inhibition percentage (*IR*, %) was calculated using the following equation [6,15,16]:

$$IR(\%) = \left[1 - \left(\frac{I}{I_0}\right)\right] \times 100 \qquad (2)$$

where I_0 is the baseline current measured on a solution containing the substrate, at a fixed concentration and in the absence of the inhibitor; I is the current achieved in the presence of the inhibitor (molinate). The current signal obtained in the inhibition process is smaller than I_0, thus, the concentration of molinate, which varied during the quantification analysis, can be related to the *IR* obtained. An increased amount of inhibitor in the reaction causes an increase in the rate of decline in enzyme activity. Fig. 3a shows the DPV voltammograms recorded using the GST based biosensor and an increasing molinate concentration. As expected, increasing the amount of inhibitor produced a decrease on the current signal. The relationship between the current signal and the molinate concentration is demonstrated in Fig. 3b and c. Using a reaction time of 10 s, the rate of inhibition of GST enzyme increased linearly with the logarithm of molinate concentration in the range of 0.19–7.9 mg L^{-1} [% *IR* = 20.53 log molinate concentration (mg L^{-1}) + 30.00; $r = 0.99$; $n = 6$]. The maximum GST inhibition rate obtained was 53.2% (Fig. 3c). The limits of detection (LOD) and limits of quantification (LOQ) were defined and determined (according to Eq. (3) where σ is the standard deviation and s is the slope of the calibration curve) as the minimum detectable amount of analyte with a signal-to-noise ratio of 3:1 and 10:1, respectively [27]. A LOD of 0.064 mg L^{-1} was determined, with a corresponding LOQ of 0.094 mg L^{-1}.

$$\mathrm{LOD} = 3\frac{\sigma}{s}$$
$$\mathrm{LOQ} = 10\frac{\sigma}{s} \qquad (3)$$

In order to assess the performance and accuracy of the biosensor, a molinate standard solution was added to the electrochemical cell containing environmental water samples, using the multiple standard addition method to reduce the matrix effects. Three molinate standard additions (0.20, 0.50, 1.00 mg L^{-1}) were added to samples to provide an increased inhibition rate response. Recoveries ranged from 90.0% to 105.0% indicating that this methodology is suitable for the quantification of molinate in real environmental water samples.

The developed biosensor was used to detect and quantify molinate in rice paddy field floodwater samples. Molinate was detected in the floodwater samples at a concentration of 1.48 ± 0.11 mg L^{-1}. To validate the methodology, the results achieved with the GST-biosensor were compared with those obtained by HPLC–UV (1.66 ± 0.20 mg L^{-1}) and no significant differences were observed proving that the developed methodology is accurate at the studied concentration level.

The stability of the GST-based biosensor was also characterized. When exposed to a concentration of 2.00 mg L^{-1} of molinate, the biosensor response was reproducible (RSD 8.72%) during 15 days, when stored at +4 °C.

The sensor performance was also compared with other results previously reported in the literature. The developed GST-based biosensor presents the widest concentration range for the quantification of molinate in environmental samples (Table 1). The working concentration of the bare GCE [28] range is about 10^{-4} mol L^{-1} (93–170 mg L^{-1}) and can be successfully applied for the quantification of phytopharmaceutical products. However, it is unsuitable for the detection and quantification of molinate in environmental samples. Although the lowest LOD was reported

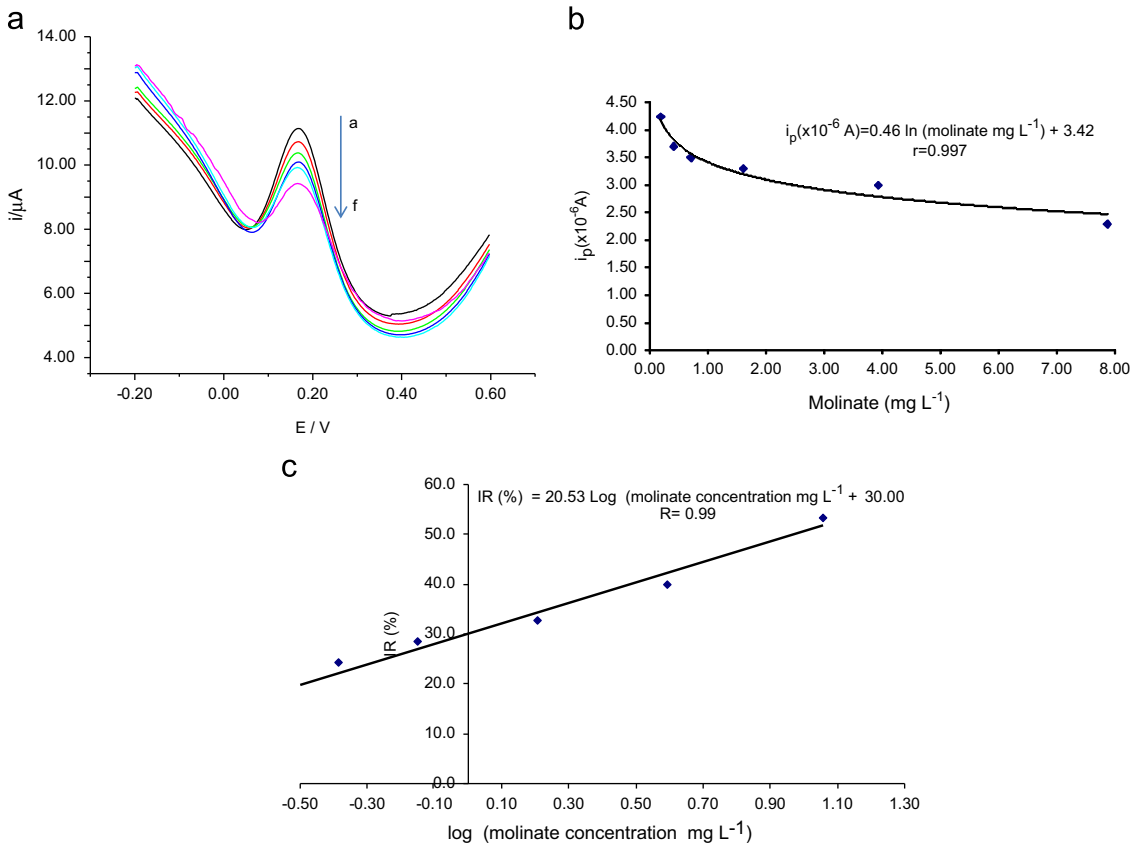

Fig. 3. (a) Differential pulse voltammograms obtained in a analytical buffer containing substrate (1 mmol L^{-1} GSH and 1 mmol L^{-1} CDNB), electron mediator $[Fe(CN)_6]^{4-}$ and increasing molinate concentrations: (a) 0.19; (b) 0.41; (c) 0.71; (d) 1.61; (e) 3.93 and (f) 7.87 mg L^{-1}. (b) Relationship obtained between peak current and molinate concentration. (c) Logarithm calibration plot of *IR* (%) of GST activity vs. log (molinate concentration, mg L^{-1}).

Table 1
Comparison of different analytical methods for the quantification of molinate.

Analytical method	Linear range (mg L^{-1})	Detection limit (mg L^{-1})	Reference
HPLC	0.9–37.5	0.9	[4]
Voltammetric-GCE	92.75–168.7	[a]	[28]
Voltammetric-HMDE	0.94–1.69	6.6×10^{-3}	[8]
GST-based biosensor	0.19–7.9	0.064	Present paper

GCE—Glassy carbon electrode; HMDE—Hanging mercury drop electrode; GST—Glutathione-S-transferase

[a] Not indicated.

for the HMDE-based procedure, mercury is toxic and it should be clearly avoided for analytical purposes.

HPLC–UV technique is the mostly used analytical technique for the quantification of molinate in real environmental water samples. Although it is a reliable and robust technique, it is not appropriate for field analysis or for rapid and cost-effective monitoring while biosensors-based methodologies are [6].

4. Conclusions

An enzymatic biosensor was constructed and employed for molinate determination in environmental waters. The transducer was carried out by immobilizing the GST enzyme onto GCE via aminosilane–glutaraldehyde covalent attachment. The proposed concept is based on the electrochemical detection of the pesticide, using the inhibition of GST which leads to a decrease in current peak measurement by DPV. The developed biosensor presents good analytical features that enable the quantification of molinate in aqueous environmental samples. The main advantages of this sensor include the simplicity in designing and preparation, reduced measurement time, good precision and high accuracy, good selectivity and low limit of detection. Moreover, this alternative method is less expensive, namely in what concerns to reagent consumption and equipment involved.

The GST-based biosensor was successfully applied in the quantification of molinate in rice paddy floodwater. The device produced accurate results and faster responses than those provided by HPLC–UV used in this work as a comparative method.

The use of biosensors is the new trend in the future of environmental monitoring as they are suitable for real-time measurement with screening purposes.

Novelty statement

As far as we know, no previous study exists concerning the development of a glutathione-S-transferase based biosensor for molinate detection. The developed enzymatic biosensor presents several advantages, such as, reliability, no sample preparation, low cost and the possibility to perform *in situ* and in real time molinate environmental water analysis.

Acknowledgments

The authors wish to thank the Brazilian and Portuguese research funding institutions CAPES and FCT, respectively, for the financial support through the project No. 313/11 (Proc.

4.4.1.00) "Determinação eletroanalítica de pesticidas em amostras de águas naturais e de alimentos utilizando ultramicroelectrodos/biossensores". M. F. Barroso is grateful for the post-doc fellowship (SFRH/BPD/78845/2011) financed by POPH–QREN–Tipologia 4.1–Formação Avançada, subsidized by Fundo Social Europeu and Ministério da Ciência, Tecnologia e Ensino Superior. This work has been also supported by Fundação para a Ciência e a Tecnologia (FCT) through Grant no. PEst-C/EQB/LA0006/2011.

References

[1] A. Campbell, Regul. Toxicol. Pharmacol. 53 (2009) 195–204.
[2] M. Castro, A.C. Silva-Ferreira, C.M. Manaia, O.C. Nunes, Chemosphere 59 (2005) 1059–1065.
[3] C. Coelho, A.S. Oliveira, M.F.R. Pereira, O.C. Nunes, J. Hazard. Mater. 138 (2006) 343–349.
[4] L. Barreiros, C.M. Manaia, O.C. Nunes, Biodegradation 22 (2011) 445–461.
[5] L. Barreiros, B. Nogales, C.M. Manaia, A.C. Silva-Ferreira, D.H. Pieper, M.A. Reis, O.C. Nunes, Environ. Microbiol. 5 (2003) 944–953.
[6] M. LeDoux, J. Chromatogr. A 1218 (2011) 1021–1036.
[7] V. Somerset, P. Baker, E. Iwuoha, Mercaptobenzothiazole-on-gold organic phase biosensor systems: 3. Thick-film biosensors for organophosphate and carbamate pesticide determination, in: V.S. Somerset (Ed.), Intelligent and Biosensors, InTech, Croatia, 2010, pp. 185–204.
[8] M.F. Barroso, O.C. Nunes, M.C. Vaz, C. Deleure-Matos, Anal. Bioanal. Chem. 381 (2005) (2005) 879–883.
[9] EPA, EPA Fact Sheet EPA-823-F-01-011, Office of Water, Washington, DC, 2001.
[10] J.S. Dyk, B. Pletschke, Chemosphere 82 (2001) 291–307.
[11] A.N. Ivanov, R.R. Younusov, G.A. Evtugyn, F. Arduini, D. Moscone, G. Palleschi, Talanta 85 (2011) 216–221.
[12] D. Du, S. Chen, J. Cai, A. Zhang, Talanta 74 (2008) 766–772.
[13] S.K. Moccelini, I.C. Vieira, F. Lima, B.G. Lucca, A.M.J. Barbosa, V.S. Ferreira, Talanta 82 (2010) 164–170.
[14] G.Y. Kim, J. Shim, M.S. Kang, S.H. Moon, J. Hazard. Mater. 156 (2008) 141–147.
[15] S.C. Fernandes, I.C. Vieira, A.M.J. Barbosa, V.S. Ferreira, Electroanalysis 23 (2011) 1623–1630.
[16] P. Kapoli, I.A. Axarli, D. Platis, M. Fragoulaki, M. Paine, J. Hemingway, J. Vontas, N.E. Labrou, Biosens. Bioelectron. 24 (2008) 498–503.
[17] D.M. Townsend, K.D. Tew, Oncogene 22 (2003) 7369–7375.
[18] V.G. Andreous, Y.D. Clonis, Anal. Chim. Acta 460 (2002) 151–161.
[19] J.W. Choi, Y.K. Kim, B.K. Oh, S.Y. Song, W.H. Lee, Biosens. Bioelectron. 18 (2003) 591–597.
[20] J.W. Choi, Y.K. Kim, S.Y. Song, I.H. Lee, W.H. Lee, Biosens. Bioelectron. 18 (2003) 1461–1466.
[21] R.P. Singh, Y.J. Kim, B.K. Oh, J.W. Choi, Electrochem. Commun. 11 (2009) 181–185.
[22] T. Kuila, S. Bose, P. Khanra, A.K. Mishra, N.H. Kim, J.H. Lee, Biosens. Bioelectron. 26 (2011) 4637–4648.
[23] K. Kreuz, R. Tommasini, E. Martinoia, Plant Physiol. 111 (1996) 349–353.
[24] A. Campbell, D. Holstege, R. Swezey, D. Medina-Cleghorn, J. Environ. Sci. Health A 71 (2008) 1338–1347.
[25] M. Ghiaci, H. Aghaei, S. Soleimanian, M.E. Sedaghat, Appl. Clay Sci. 43 (2009) 289–295.
[26] J. Wang, Analytical Electrochemistry, Second ed., Wiley-VCH, New York, 2000.
[27] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, Fifth ed., Pearson Education Limited, Harlow, 2005.
[28] E.M. Garrido, J.C. Lima, C.M. Delerue-Matos, A.M.O. Brett, Int. J. Environ. Anal. Chem. 75 (1999) 149–157.