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Screen-printed biosensor based on the inhibition of the acetylcholinesterase activity for the determination of codeine

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ABSTRACT

The current paper presents the chronoamperometric determination of codeine using screen-printed carbon electrodes that incorporate tetrathiafulvalene in the matrix of the working electrode, as mediator, and cross-linked acetylcholinesterase. Applying a potential of +250 mV, a 1 mM solution of acetylthiocholine in electrolyte solution pH 7 gives an oxidation signal due to the dimerization of its metabolite after the reaction with the enzyme. This electrochemical signal is decreased by consecutive additions of a solution of codeine, which allows the performance of curves of calibration for the validation of this electrochemical method, giving a reproducibility of 3.31% ($n=6$) and a capability of detection of 20 μM. This type of inhibition has been studied by means of a Lineweaver–Burk plot. Additionally, the developed biosensor was used for the determination of the quantity of codeine in pharmaceutical commercial tablets and urine samples.

1. Introduction

Codeine, or methylmorphine, is an alkaloid that is extracted from opium and one of the most common used antitussive drugs, being also used as analgesic and antidiarrheal [1,2]. Although it belongs to the same opium family as morphine and heroin, codeine is used for the treatment of moderated pain and induces less euphoria and sedation than the first two, but it can also produce addiction [3]. Even though codeine does not produce as strong effects as heroin, it is also considered as a drug of abuse.

This drug has been determined in diverse matrixes by different methodologies such as gas chromatography [4,5], liquid chromatography [5], electrophoresis [6], chemiluminescence [7] or electrochemistry [3,8–19]. Although electrochemical methods have given good results, rather high potentials must be applied for the direct oxidation of this analyte, which carries possible interferences. This problem can be sometimes solved by the incorporation of a biorecognition element, giving a specific biosensor. In this way, an enzymatic procedure based on the immobilization of morphine dehydrogenase and salicylate hydroxylase onto a Clark-type oxygen electrode [20] and an aptamer based method [21] were developed to quantify codeine.

In this work, the detection of codeine has been attempted using acetylcholinesterase (AChE) based screen-printed carbon electrodes (SPCEs), taking into account the reversible inhibition of the enzyme activity in the presence of this drug [22]. Acetylthiocholine iodide (ATI) acts as a substrate for AChE [23,24], generating thiocholine and acetic acid (Fig. 1). The enzymatically-generated thiocholine can be electrochemically detected at slightly high potentials using SPCEs [25,26], which could provoke the interference of other substances in the media or even the direct oxidation of the analyte [3]. In order to reduce this potential, chemical mediators such as cobalt phtalocyanine (CoPC) [27–32], Prussian Blue [33,34], 7,7,8,8-tetracyanoquinodimethane [35–37] or poly(3,4-ethylenedioxythiophene) (PEDOT) [38] have been added in solution. Moreover, the use of SPCEs allows incorporating the mediator into the working electrode in the screen-printed fabrication, which undoubtedly simplifies the chronoamperometric measurement procedure. In this way, CoPC [39] and PEDOT [40] have been screen-printed for the detection of the enzymatically-generated thiocholine. Tetrathiafulvalene (TTF) seems an optimum mediator in order to be screen-printed, since its non-solubility in water avoids risks of dissolution when working in aqueous solutions. Thus, TTF based SPCEs have been built by screen-printing a mixture of mediator and carbon paste in this work. In the next step, AChE have been cross-linked to TTF modified SPCEs (TTF-SPCEs) with glutaraldehyde (GA) and bovine serum albumin (BSA) to obtain a biosensor for codeine detection. According to Fig. 1, two molecules of the enzymatically-generated thiocholine dimerize thanks to the mediator which is converted in its reduced form. The mediator is then reoxidized on the electrode giving an oxidation signal, which can be related to the substrate. When codeine is added to the solution, the transformation of acetylthiocholine in thiocholine is avoided and consequently, the

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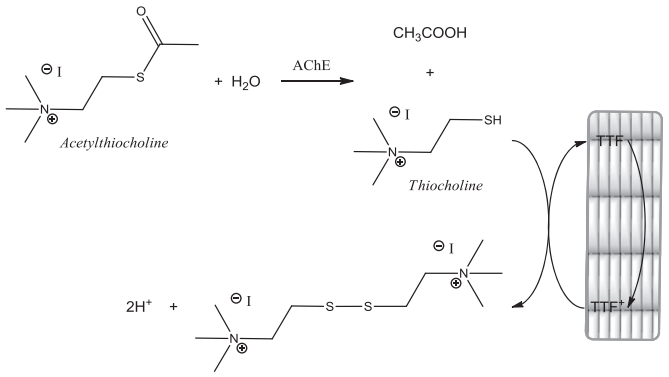

Fig. 1. Enzymatic reaction involved in the codeine chronoamperometric determination.

decreasing in the oxidation signal can be related to the concentration of the drug in solution. Immobilization and operational conditions that affect the chronoamperometric response of codeine have been optimized. The performance of the developed biosensor has been checked in terms of precision, capability of detection and application to pharmaceutical samples.

2. Experimental

2.1. Reagents

Screen-printed transducers were fabricated by sequential deposition of different commercial inks that define the different parts of the three-electrode system, namely carbon ink (C10903P14, Gwent Electronic Materials, Torfaen, UK), dielectric ink (D2071120D1, Gwent Electronic Materials, Torfaen, UK), Ag/AgCl ink (Electrodag 6037 SS, Acheson Colloiden, Scheemda, The Netherlands) and Ag ink (Electrodag 418, Acheson Colloiden, Scheemda, The Netherlands).

Analytical grade chemicals with no further purification were used. All solutions were prepared in Milli-Q water.

TTF was purchased from Acros Organics (Geel, Belgium).

GA and BSA were obtained from Sigma (Steinheim, Germany) as well as the enzyme AChE from *Electrophorus electricus* (Type IV-S, 410 units/mg protein).

100 mM NaH_2PO_4·(Merck, Darmstadt, Germany) and NaOH (J.T. Baker, Deventer, The Netherlands) buffer containing 100 mM of KCl (Merck, Darmstadt, Germany) was used as supporting electrolyte.

ATI was supplied by Fluka Analytical (Steinheim, Germany). Stock solutions were daily made in supporting electrolyte and stored at 4 °C when not in use.

Stock standard solution of codeine was prepared by dissolving the adequate amount of codeine hydrochloride (Alcaliber S.A., Madrid, Spain) in supporting electrolyte.

2.2. Apparatus

A DEK 248 printing machine (DEK, Weymouth, UK) was used for the manufacture of the SPEs.

A μAutolab electrochemical system with GPES software (Eco Chemie, Utrecht, The Netherlands) was utilized to carry out electrochemical measurements.

pH of the solutions was adjusted with a pH meter from HANNA instruments Model HI221 (USA).

3. Methods

3.1. Fabrication of TTF-SPCEs

Each different component from the electrochemical transducer, that is conductive silver tracks, Ag/AgCl reference electrode, carbon counter electrode and dielectric layer, was defined by sequential deposition of the different inks on polyester films (HiFi Industrial Film, Dardilly, France) and its subsequent curing, according to the manufacturer's specifications.

The working electrode ink was prepared by thoroughly mixing the carbon ink with TTF (5% w/w) and immediately screen-printed.

3.2. Immobilization of the enzyme AChE onto TTF-SPCEs (AChE-TTF-SPCEs)

Cross-linking immobilization through GA and BSA was used in order to attach the enzyme to the carbon surface of the working electrode. Volume and concentration of AChE, GA and BSA were optimized to obtain the highest analytical response for the inhibition with codeine (Table 1). The optimum conditions of immobilization obtained for the development of this biosensor were 2.5 μL of a 0.1%w/v AChE solution in 10 mM phosphate pH 6, 1.25 μL of a 6% w/v BSA solution in 10 mM phosphate pH 6 and 1.25 μL of a 2.5% v/v GA solution in water. These quantities were mixed and deposited onto the working electrode. After 120 min under 4 °C, the biosensor was ready to be used.

3.3. Measuring procedure

Cyclic voltammetric measurements were executed at room temperature in a 100 μL drop of KCl 100 mM, scanning the potential between 100 mV and 600 mV vs. screen-printed Ag/AgCl electrode, at a scan rate of 50 mV s^{-1}.

Chronoamperometric measurements were made at room temperature in a cell containing 5 mL of supporting electrolyte, of the desired pH, under constant mechanical stirring. Experimental measurements were performed applying a fixed potential of +250 mV vs. screen-printed Ag/AgCl electrode. After reaching a stable baseline, a volume of a solution of ATI was added into the electrochemical system to a final concentration of 1 mM, except for the optimization process, and after the stabilization of the signal, successive additions of 10 μL of a 10 mM codeine solution were made.

4. Results and discussion

The use of screen-printed technology allows the fabrication of electrodes with different characteristics. In this case, the screen-printed mediator leads to the reduction of the usual high applied potential needed in chronoamperometric measurements using AChE

Table 1
Influence of the amount of the cross-linking reagents used for AChE immobilization onto TTF-SPCEs in the chronoamperometric current registered for codeine (pH 7, ATI concentration = 1 mM, applied potential = 250 mV vs. screen-printed Ag/AgCl).

μL AChE 0.1%	μL BSA 6%	μL GA 2.5%	Current (nA)
2.5	0	2	59
2.5	0.5	1.5	86
2.5	1	1	89
2.5	1.5	1.5	105
2.5	1.5	0.5	81
2.5	2	0	46

based SPCEs [25,26]. TTF incorporation into the carbon matrix of the working electrode was checked by cyclic voltammetry. As it can been observed in Fig. 2, TTF is oxidized to TTF^+ at a potential of +250 mV vs. screen-printed Ag/AgCl electrode, which was the chosen potential for the chronoamperometric measurements since the oxidized specie is required for the development of the enzymatic system (Fig. 1) [41].

After the immobilization of AChE by cross-linking on the TTF-SPCEs, the developed biosensors were used for the determination of codeine by chronoamperometry. For the study of this system, experimental factors such as pH of the medium (from 3 to 11) and the concentration of ATI in solution (from 0.6 to 3.2 mM) were optimized, applying a potential of +250 mV vs. screen-printed Ag/AgCl electrode, to obtain the best current response related to a 0.2 mM of codeine solution. The optimum values for these experimental variables were pH 7 for the supporting electrolyte and 1 mM for concentration of ATI.

TTF-SPCEs without further modification and TTF-SPCEs modified with BSA and GA (See Section 3.2.) were used to carry out control experiments under these optimum conditions. The addition of codeine to the electrochemical cell, after the addition of ATI, did not alter the registered chronoamperometric current in any case, which points out the successful interaction between AChE and codeine that allows the quantification of this drug.

The type of inhibition between AChE and codeine was also studied. Chronoamperometric measurements were carried out, in presence and absence of codeine, by subsequent additions of a 50 mM ATI solution into 5 mL of supporting electrolyte pH 7 [25]. The inhibitory effect of codeine was studied by means of the Lineweaver–Burk plot (Fig. 3), which in the presence of codeine shows a constant maximum rate of the reaction, V_{max}, and an increase in the apparent Michaelis–Menten constant, K_M^{app}, suggesting a competitive inhibition [42]. In addition, it was observed that the excess of substrate also inhibits the activity of this enzyme [22].

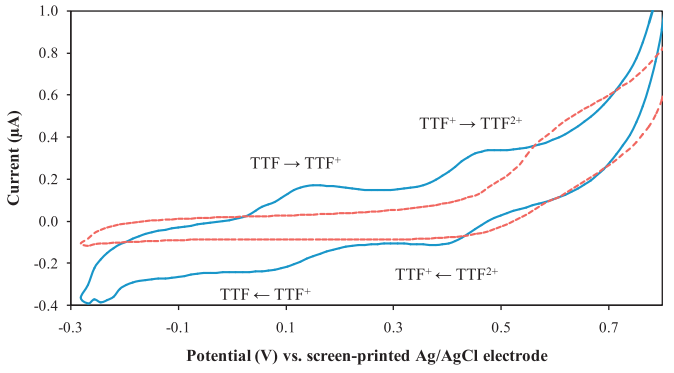

Fig. 2. Cyclic voltammogram of a SPCE-TTF (solid line) in a 100 μL drop of a 100 mM KCl solution compared to a SPCE (dashed line). Scan rate, 100 mV s^{-1} vs. screen-printed Ag/AgCl electrode.

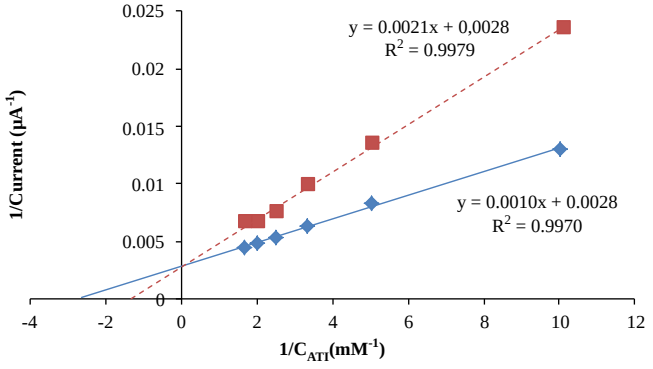

Fig. 3. Lineweaver–Burk plot for the study of the type of inhibition between AChE and codeine. Calibration curves recorded at pH 7 and +250 mV vs. screen-printed Ag/AgCl electrode by consecutive additions of ATI in presence of 0.20 mM of codeine (dashed line) and absence (solid line) of it.

4.1. Validation of the method

The validation of an analytical method is performed to guarantee its quality. This justification is established in terms of precision, studying the reproducibility (inter-biosensors) and the repeatability (intra-biosensor), as well as its capability of detection.

Calibration curves were performed with different biosensors in order to obtain the reproducibility (inter-biosensors) of the method in terms of relative standard deviation (RSD) associated to the slopes of these curves. Each biosensor was used to carry out one curve at the optimum conditions in the range from 20 to 200 μM of codeine. After recording the data, the anomalous points of these calibration curves were detected and eliminated using PROGRESS program [43]. Fig. 4 shows the validated data of these curves. The reproducibility of the method was estimated to be 3.31% ($n=6$).

The same procedure was carried out to calculate the repeatability (intra-biosensor) of this method. However, it was observed that it was not possible to perform more than one calibration curve with the same electrode. This fact is not really significant due to the disposable nature of the biosensor and its good reproducibility.

The calculation of the capability of detection of the electroanalytical method was obtained from different calibration curves. The validated parameters of these linear regressions, in the same conditions as for the precision, were used to estimate the capability of detection of these biosensors for a given probability of false positive (α) and negative (β) [44,45]. The minimum detectable net concentration was found to be lower than the one of the first addition of the standards used to build the calibration curves, 20 μM. Therefore, from an analytical point of view, the first point of the calibration curve has been taken as the capability of detection of this method [46].

These values confirm the good performance of the developed procedure for the chronoamperometric determination of codeine, in relation with the previous chronoamperometric biosensor reported (Table 2).

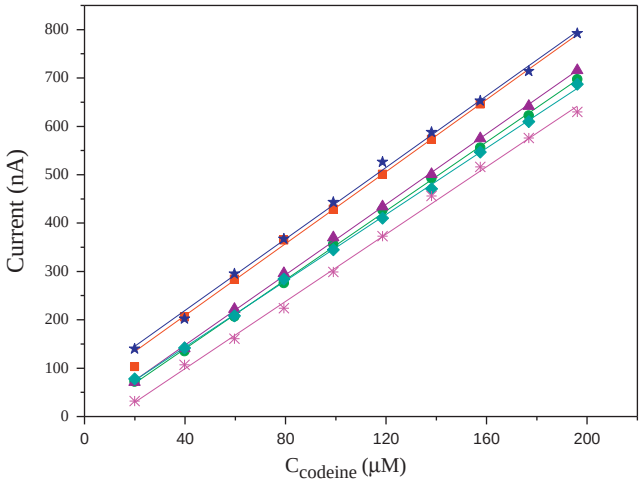

Fig. 4. Experimental points and OLS regressions for the different calibration curves carried out to evaluate the reproducibility of AChE-TTF-SPCEs in the determination of codeine.

Table 2
Analytical characteristics of chronoamperometric biosensors for codeine detection.

Electrode	Bioelement	Mediator	Supporting electrolyte pH	Potential	Reproducibility	Capability of detection	Sample	Ref
Sintered planar Pt	Morphine dehydrogenase	Phenazine methosulphate	8	0.1 V	Not shown	9 μM	Not shown	[47]
SPCEs	AChE	TTF	7	+0.25 V	3.31% ($n=6$)	20 μM ($\alpha=\beta=0.05$)	Codeisan® urine samples	This work

4.2. Application in pharmaceutical drugs and urine samples

The developed biosensor was used for the determination of codeine in complex matrixes, such as Codeisan®, with codeine phosphate ½H_2O as active principle (28.7 mg per tablet), and urine samples.

A tablet of Codeisan® was homogeneously crushed and an aliquot was weight and dissolved in 1 mL of a 100 mM phosphate buffer solution, pH 7. After centrifuging this suspension at 4 °C for 1 min at 13,000 rpm, the supernatant was pipetted and used as studied solution. Urine samples, collected from a healthy person, were spiked with codeine to a final concentration of 10.7 mM. Standard addition methodology was used to minimize the possible sample matrix effects.

Chronoamperometric measurements were performed at the optimized experimental conditions. After adding the substrate of the AChE and obtaining a stable baseline, a volume of 10 μL of the studied solution was added into the electrochemical cell and successive additions of 10 μL from the 10 mM codeine solution were made. The values of the concentration of codeine found in the tablet and in the urine samples were [29.37 ± 3.13] mg/tablet ($n=5$, $\alpha=0.05$) and [10.8 ± 1.15] ($n=3$, $\alpha=0.05$), respectively. These results agree with the values shown in the patient information leaflet (28.7 mg) and with the spiked one (10.7 mM).

5. Conclusions

AChE-TTF-SPCEs have been developed in order to determine codeine. Taking advantage of the screen-printed technology, TTF-SPCEs have been fabricated. The use of TTF as a mediator, in this case, allows the application of a lower potential and, consequently, the avoidance of possible influences from different substances and the direct electrochemical oxidation of codeine in the carbon electrode.

The interaction between codeine and AChE allows the determination of this drug through the monitoring of the chronoamperometric signal of the dimer formed from the thiocoline, and the metabolite obtained from the reaction between AChE and ATI. The capability of the enzyme to act on the substrate is competitively inhibited by codeine and thus, the ATI oxidation chronoamperometric signal decreases. Taking this into account, curves of calibration have been carried out to estimate the precision of the method (Reproducibility, 3.31%, $n=6$), as well as its capability of detection (20 μM, $\alpha=\beta=0.05$) under the optimum conditions (supporting electrolyte pH 7 and applied potential of +250 mV vs. screen-printed Ag/AgCl electrode). Moreover, this biosensor has been successfully used in the quantification of codeine in pharmaceutical tablets and urine samples, which highlights their good performance in relation with the previously reported chronoamperometric based biosensor.

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References

[1] B.L. Velázquez, P. Lorenzo, A. Moreno, I. Lizasoain, J.C. Leza, M.A. Moro, A. Portolés, Farmacología Básica y Clínica, 18th ed., Médica Panamericana, Madrid, 2008.
[2] A.C. López Castellano, L. Moreno Royo, V. Villagrasa Sebastián, Manual de Farmacología: Guía para el uso racional del medicamento, Elsevier, Madrid, 2006.
[3] J.M.P.J. Garrido, C. Delerue-Matos, F. Borges, T.R.A. Macedo, A.M. Oliveira-Brett, Electroanalysis 16 (2004) 1427–1433.
[4] R. Wasels, F. Belleville, J. Chromatogr. A 674 (1994) 225–234.
[5] M.E. Bosch, A.R. Sánchez, F.S. Rojas, C.B. Ojeda, J. Pharm. Biomed. Anal. 43 (2007) 799–815.
[6] C. Cruces-Blanco, A.M. García-Campaña, Trends Anal. Chem. 31 (2012) 85–95.
[7] P.S. Francis, J.L. Adcock, J.W. Costin, S.D. Purcell, F.M. Pfeffer, N.W. Barnett, J. Pharm. Biomed. Anal. 48 (2008) 508–518.
[8] A. Campean, M. Tertis, R. Sandulescu, Cent. Eur. J. Chem. 9 (2011) 688–700.
[9] M.H. Pournaghi-Azar, S. Kheradmandi, A. Saadatirad, J. Solid State Electrochem. 14 (2010) 1689–1695.
[10] E. Bishop, W. Hussein, Analyst 109 (1984) 143–145.
[11] J.M.P.J. Garrido, C. Delerue-Matos, F. Borges, T.R.A. Macedo, A.M. Oliveira-Brett, Anal. Lett. 35 (2002) 2487–2498.
[12] J.M. Kauffmann, M.P. Prete, J.C. Vire, G.J. Patriarche, Z. Fresenius, Anal. Chem. 321 (1985) 172–176.
[13] S. Komorsky-Lovric, N. Vukasinovic, R. Penovski, Electroanalysis 15 (2003) 544–547.
[14] F. Li, J. Song, D. Gao, Q. Zhang, D. Han, L. Niu, Talanta 79 (2009) 845–850.
[15] M.H. Pournaghi-Azar, A. Saadatirad, J. Electroanal. Chem. 624 (2008) 293–298.
[16] M.H. Pournaghi-Azar, A. Saadatirad, Electroanalysis 22 (2010) 1592–1598.
[17] Y. Shih, J.M. Zen, H.H. Yang, J. Pharm. Biomed. Anal. 29 (2002) 827–833.
[18] J.M. Zen, M.R. Chang, H.H. Chung, Y. Shih, Electroanalysis 10 (1998) 536–540.
[19] J.M.P.J. Garrido, C. Delerue-Matos, F. Borges, T.R.A. Macedo, A.M. Oliveira-Brett, Anal. Lett. 37 (2004) 831–844.
[20] C.G. Bauer, A. Kühn, N. Gajovic, O. Skorobogatko, P.J. Holt, N.C. Bruce, A. Makower, C.R. Lowe, F.W. Scheller, Fresenius J. Anal. Chem. 364 (1999) 179–183.
[21] M. Saberian, H. Hamzeiy, A. Aghanejad, D. Asgari, BioImpacts 1 (2011) 31–36.
[22] M. Repetto, Toxicología avanzada, Diaz de Santos, Madrid, 1995.
[23] H.M.A. Hassanein, Acetylcholinesterase Inhibition, in: M. Parveen, S. Kumar (Eds.), Recent Trends in the Acetylcholinesterase System, IOS Press, Amsterdam, The Netherlands, 2005.
[24] P.C. Pandey, S. Upadhyay, H.C. Pathak, C.M.D. Pandey, I. Tiwari, Sensor Actuat. B—Chem. 62 (2000) 109–116.
[25] O. Domínguez-Renedo, M.A. Alonso-Lomillo, P. Recio-Cebrián, M.J. Arcos-Martínez, Sci. Total Environ. 426 (2012) 346–350.
[26] S. Sanllorente-Méndez, O. Domínguez-Renedo, M.J. Arcos-Martínez, Sensors 10 (2010) 2119–2128.
[27] N. Ben Oujji, I. Bakas, G. Istamboulié, I. Ait-Ichou, E. Ait-Addi, R. Rouillon, T. Noguer, Food Control 30 (2013) 657–661.
[28] G.A. Evtugyn, R.R. Younusov, A.N. Ivanov, R.R. Sitdikov, A.V. Galuchin, H.C. Budnikov, I.I. Stoikov, I.S. Antipin, Electroanalysis 24 (2011) 554–562.
[29] A.N. Ivanov, R.R. Younusov, G.A. Evtugyn, F. Arduini, D. Moscone, G. Palleschi, Talanta 85 (2011) 216–221.
[30] R.K. Mishra, R.B. Dominguez, S. Bhand, R. Muñoz, J.L. Marty, Biosens. Bioelectron. 32 (2012) 56–61.
[31] N.A. Pchelintsev, P.A. Millner, Anal. Chim. Acta 612 (2008) 190–197.
[32] G. Valdés-Ramírez, D. Fournier, M.T. Ramírez-Silva, J.L. Marty, Talanta 74 (2008) 741–746.
[33] I. Ben Rejeb, F. Arduini, A. Arvinte, A. Amine, M. Gargouri, L. Micheli, C. Bala, D. Moscone, G. Palleschi, Biosens. Bioelectron. 24 (2009) 1962–1968.
[34] N. Gan, X. Yang, D. Xie, Y. Wu, W. Wen, Sensors 10 (2010) 625–638.
[35] A. Hildebrandt, R. Bragós, S. Lacorte, J.L. Marty, Sensor Actuat. B—Chem. 133 (2008) 195–201.
[36] A. Hildebrandt, J. Ribas, R. Bragós, J.L. Marty, M. Tresànchez, S. Lacorte, Talanta 75 (2008) 1208–1213.
[37] L. Rotariu, L.G. Zamfir, C. Bala, Anal. Chim. Acta 748 (2012) 81–88.

[38] T. Noguer, T. Sikora, G. Istamboulie, E. Jubete, E. Ochoteco, J.L. Marty, J. Sensors 2011 (2011).
[39] A. Crew, D. Lonsdale, N. Byrd, R. Pittson, J.P. Hart, Biosens. Bioelectron. 26 (2011) 2847–2851.
[40] G. Istamboulie, T. Sikora, E. Jubete, E. Ochoteco, J.L. Marty, T. Noguer, Talanta 82 (2010) 957–961.
[41] I. Kaminska, M.R. Das, Y. Coffinier, J. Niedziolka-Jonsson, P. Woisel, M. Opallo, S. Szunerits, R. Boukherroub, Chem. Commun. 48 (2012) 1221–1223.
[42] G.L. Turdean, Int. J. Electrochem. 2011 (2011) 1–15.
[43] P.J. Rousseeuw, A.M. Leroy, Robust Regression and Outlier Detection, Wiley, New York, 1989.
[44] ISO11843, Capability of detection, Genève, Switzerland, Part I 1997 and Part II 2000.
[45] J. Inczédy, T. Lengyel, A.M. Ure, A. Gelencsér, A. Hulanicki, Compendium of Analytical Nomenclature, 3rd ed., Port City Press Inc., Baltimore, 2000.
[46] M.A. Alonso-Lomillo, O. Domínguez-Renedo, L. Ferreira-Gonçalves, M.J. Arcos-Martínez, Biosens. Bioelectron. 25 (2010) 1333–1337.
[47] P.J. Holt, L.D. Gray Stephens, N.C. Bruce, C.R. Lowe, Biosens. Bioelectron. 10 (1995) 517–526.