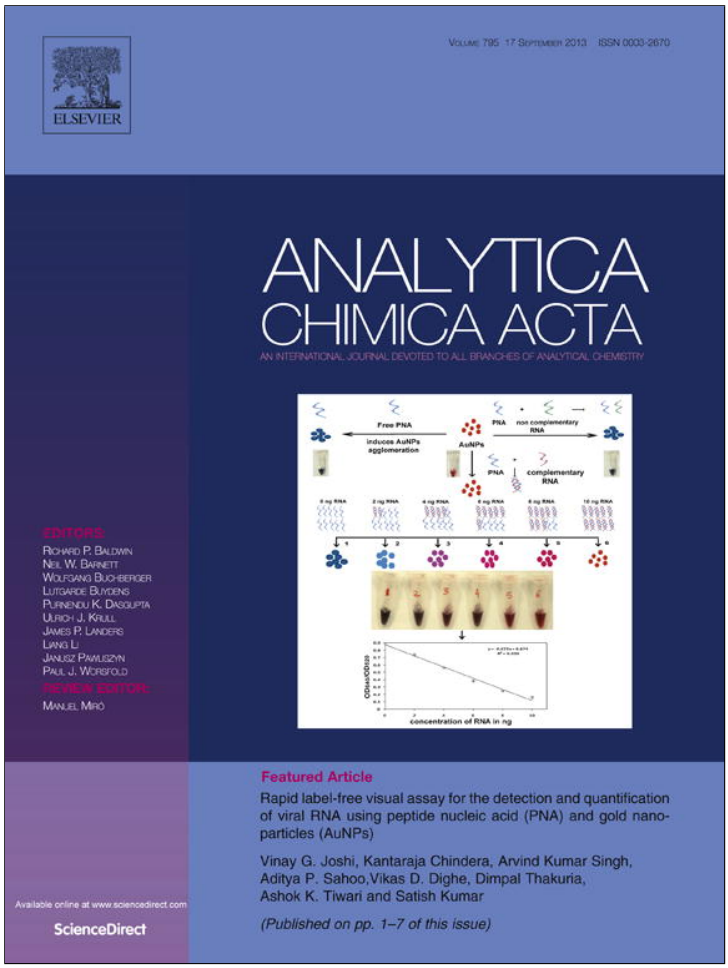


Analytica Chimica Acta 795 (2013) 52–59

Contents lists available at SciVerse ScienceDirect

Analytica Chimica Acta

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

A novel amperometric biosensor based on a co-crosslinked L-lysine-α-oxidase/overoxidized polypyrrole bilayer for the highly selective determination of L-lysine

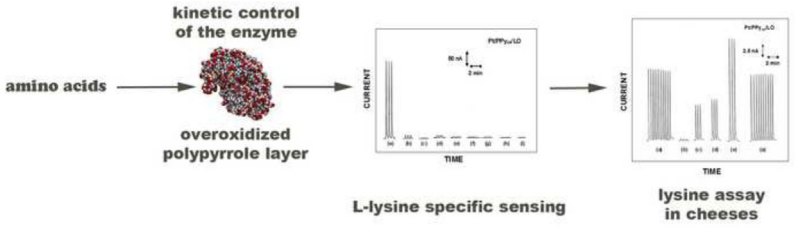
Antonio Guerrieri[a,*], Rosanna Ciriello[a], Tommaso R.I. Cataldi[b]

[a] Dipartimento di Scienze, Università della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy
[b] Dipartimento di Chimica, Università di Bari "Aldo Moro", Via E. Orabona 4, Bari, Italy

HIGHLIGHTS

- We report a novel, highly selective, L-lysine amperometric biosensor.
- Kinetic control of L-lysine-α-oxidase increased enzyme specificity.
- Overoxidized polypyrrole membrane allowed interferent rejection.
- The biosensor proved successfully for preliminary lysine analysis of pharmaceutical and food samples.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 20 February 2013
Received in revised form 11 July 2013
Accepted 15 July 2013
Available online 18 July 2013

Keywords:
Biosensor
L-lysine determination
L-lysine-α-oxidase
Overoxidized polypyrrole
Flow injection analysis
Food analysis

ABSTRACT

An amperometric biosensor for the determination of L-lysine based on L-lysine-α-oxidase immobilized by co-crosslinking on a platinum electrode previously modified by an overoxidized polypyrrole film is described. The optimization of experimental parameters, such as pH and flow rate, permitted to minimize significantly substrate interferences even using a low specific, commercial enzyme. The relevant biases introduced in the measurement of lysine were just about 1% for L-arginine, L-histidine and L-ornithine, roughly 4% for L-phenylalanine and L-tyrosine. The developed approach allowed linear lysine responses from 0.02 mM up to 2 mM with a sensitivity of 41 nA/(mM × mm^2) and a detection limit of 4 μM (S/N = 3). No appreciable loss in lysine sensitivity was observed up to about 40 days. Allowing polypyrrole layer to remove interference from electroactive compounds, the present method revealed suitable to detect L-lysine in a pharmaceutical and cheese sample, showing a good agreement with the expected values.

1. Introduction

L-lysine is an essential amino acid of great significance in clinical, nutritional and biotechnological fields: its level in foodstuffs or body fluids, for example, may indicate the nutritional quality or certain diseases, respectively. Owing to this, many efforts have been devoted to devise simple and sensitive analytical methods to detect lysine in real matrices. Since scarcely revealable, classical lysine analysis requires chemical derivatization followed by a chromatographic step but, in recent years, analytical systems based on immobilized enzymes have been increasingly used for detection of many compounds, including amino acids.

Most successful enzymatic methods for lysine analysis involve the use of L-lysineα-oxidase (LO) as a reagent according to the following scheme:

$$\text{L-Lysine} + O_2 \rightarrow \alpha\text{-keto-}\varepsilon\text{-aminocaproate} + H_2O_2 + NH_3$$

where cyclization of α-keto-ε-aminocaproate to the intramolecular dehydrated form Δ^1-piperidine-2-carboxylate proceeds

* Corresponding author at: Dipartimento di Scienze, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy. Tel.: +39 0971 205460; fax: +39 0971 205678.
E-mail address: antonio.guerrieri@unibas.it (A. Guerrieri).

0003-2670/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aca.2013.07.036

spontaneously. This reaction is usually monitored amperometrically by measuring either the consumption of oxygen with Clark-type electrodes [1–4] or the formation of hydrogen peroxide mainly with platinum electrodes [5–7]. Anyway, these enzyme-based analytical approaches are not without drawbacks. The electrochemical oxidation of hydrogen peroxide at such electrodes takes place at a potential of about +650 mV *versus* Ag/AgCl: unfortunately, at this working potential, endogenous electroactive compounds (*e.g.* ascorbate) are also oxidized, thus interfering with lysine determination in real sample analysis. To improve the specificity of the amperometric detection, the lowering of the detection potential is certainly a useful analytical technique but require mediation by ruthenium/rhodium [8] and Prussian Blue [9] glassy carbon modified electrodes. Alternatively, when using noble metal electrodes, electrochemical interference can be effectively diminished by electrosynthesizing a permselective polymer directly on the electrode surface. As it is well known, non conducting polymers grow until electrode surface is completely covered, thus forming uniform, very thin films free from defects or pinholes which allow small molecules like hydrogen peroxide to permeate through to the film while hindering larger ones [10–13]. Such an approach is surely advantageous when compared with the employment of a sequence of three different membranes (a cellulose acetate membrane to eliminate faradic interferences, the biocatalytic layer and a dialysis membrane to prevent microbial attack of LO) which resulted in a high response time [14].

Permselective, electrosynthesized films from diaminobenzene monomers (DAB) were till now the more investigated in developing interference-free lysine biosensors [15–18], in some cases even in conjunction with the above cited mediators [8,18] but their effectiveness appears questionable if this double approach was pursued. Conducting polymers, like polypyrrole (PPy), owing to their structure (a charged backbone filled with solvated counterions) have poor permselective behaviours, despite their interesting technological properties. Anyway, among them, PPy can acquire successful permselective characteristics simply by discharging for a few hours at +0.7 V *versus* SCE the pristine PPy modified electrode (usually Pt), an overoxidation process [19] which render it not conducting (PPy_{ox}). While non-conducting polymers like those derived by DAB grow already as insulating, thin (nearly monolayer) film, nicely pristine, conducting PPy can further grow assuming the desired thickness: this permits a further control and modulation of molecular diffusion through the polymeric membrane and hence fine permselective characteristics of the resulting overoxidized polymer, PPy_{ox}. Indeed PPy_{ox} films have been successfully employed by the authors, alone [20] or in conjunction with an insulating film [21] for the realization of interference and fouling free amperometric biosensors. The enzyme of interest was immobilized on the PPy_{ox} platinum modified electrode by glutaraldehyde co-crosslinking with bovine serum albumin, preserving the permselective properties of the underlying PPy_{ox} layer while assuring an high loading and long term stability of the enzyme.

In order to extend the above successful and well-tested electrode modification technique to lysine biosensing, in our laboratory an immobilization protocol of LO by co-crosslinking onto bare Pt electrode have been preliminary developed [7]. Particularly, a thoroughly optimization of the immobilization procedure permitted to achieve a fast response biosensor with improved long term stability which resulted very promising for flow-injection applications. The aim of the present work was therefore to combine the bilayer PPy_{ox}/co-crosslinked LO for the realization of a lysine biosensor free from faradic interference or fouling problems.

Nevertheless, a flow-through application of such a sensor in real matrices like pharmaceutical and food samples, containing free amino acid at appreciable contents, could be possible only after improving the enzyme specificity. Indeed, L-lysine-α-oxidase is known to catalyze in solution the oxidation of other L-amino acids, besides L-lysine, to varying extents [22]. Furthermore, the selectivity of the enzyme of interest towards each substrate can vary with different batches of enzyme [6] and the entity of this change may be different depending on the particular method of immobilization adopted [5,23]. In order to maximize the sensor response towards L-lysine, a careful control of the kinetic behaviour of the device was here attempted by optimizing experimental variables such as pH and flow rate. This simple approach revealed successful allowing to notably minimize the oxidation of other substrates by LO even in the case of ornithine and arginine whose interference on lysine response has not always been solved [17] or required more complex strategies like a proper choice of the enzyme source [8]. It is important to remark that a low specific, commercial L-lysine-α-oxidase enzyme was employed throughout the entire experimental work, thus showing the effectiveness of the approach.

2. Experimental

2.1. Reagents

L-lysine, L-tyrosine, L-cysteine, L-tryptophan, L-phenylalanine, L-histidine, L-arginine, L-ornithine, L-aspartic acid, L-glutamic acid and pyrrole were purchased from Aldrich (Aldrich-Chemie, Germany). Sigma Chemical Co. (USA) provided L-Lysine-α-oxidase (EC 1.4.3.14, from *Trichoderma viride*, 20–40 units per mg protein), glutaraldehyde (grade II, 25% aqueous solution) and bovine albumin (fraction V). Pyrrole was purified by distillation under vacuum at 52 °C. All the other chemicals were of analytical reagent grade. The pyrrole and interferent solutions were prepared just before their use. L-lysine stock solutions were stored at 4 °C. The pharmaceutical sample, Aminozim, was from Searle Farmaceutici, division of Monsanto Italiana S. p. A., Rozzano (MI). Cheese samples were obtained from a local grocery store.

2.2. Apparatus

A Gilson (Gilson Medical Electronics, Villiers-Le-Bel, France) Minipuls 3 peristaltic pump and a seven port injection valve (Rheodyne mod. 7725, Cotati, CA, USA) equipped with a 20 μL sample loop were used for the flow-injection experiments. A PEEK tubing (0.25 mm ID, 150 cm length) was used to connect the sample injection valve to the electrochemical cell. An EG&G (Princeton Applied Research, Princenton, N.J.) Model 400 electrochemical detector was used. The detector included a thin-layer electrochemical cell with a Pt disc (3 mm diameter) working electrode and an Ag/AgCl, 3 M NaCl reference electrode. Two thin layer flow cell dual gaskets (Bioanalytical Systems, Inc., USA) of 0.004 in. thickness were used. To record flow injection signals a Kipp & Zonen (Delft BV, Holland) mod. BD 11 E Flatbed Yt recorder was used.

Controlled electrochemical deposition of polypyrrole film was carried out using an EG&G model 263A potentiostat/galvanostat equipped with a M270 electrochemical research software (EG&G) version 4.23 for data control and acquisition.

2.3. Biosensor preparation

Each electrode modification has been preceded by a cleaning procedure consisting in dipping the Pt working electrode surface with hot nitric acid and then in polishing it by alumina (0.05 μm particles) mechanical abrasion, extensive washing and sonication in bidistilled water. The electrode was afterwards immersed in a 0.5 M sulphuric acid solution and its potential cycled between −0.255 and +1.225 V *versus* SCE at 100 mV s until a steady-state cyclic voltammogram was obtained.

The electropolymerization of polypyrrole films (PPy) was performed at a constant potential of +0.7 V *versus* SCE, until a deposition charge of typically 300 mC cm^{-2} was achieved, by using a solution of 0.4 M pyrrole in 0.1 M KCl supporting electrolyte. The Pt/PPy modified electrode was overoxidized at +0.7 V *versus* SCE in a phosphate buffer (pH = 7.0, I = 0.1 M) until a steady-state background current was obtained (which requires at least 7 h). Overoxidised Pt/PPy_{ox} electrodes were then washed and air-dried at room temperature.

Lysine biosensors were prepared by following the procedure elsewhere developed by the authors [7]. 25 units of lysine oxidase (approximately 1 mg) were dissolved into 250 μL of phosphate buffer, pH 7.4, I 0.1 M: 50 μL of the enzymatic solution were used to dissolve 2.6 mg of BSA, and then carefully mixed with 5 μL of 5% glutaraldehyde solution (25% glutaraldehyde solution diluted 1:5 with phosphate buffer). Four to five μL of the resulting solution, depending on the electrode area, were carefully pipetted onto the Pt/PPy_{ox} working electrode surface, avoiding air bubble formation, and carefully spread out to cover the electrode surface completely. The modified electrode was then air-dried at room temperature for few minutes. To realize Pt/LO modified electrodes, the reaction solution was pipetted directly onto the already polished platinum electrode surface. Pt/PPy_{ox}/LO or Pt/LO modified electrodes were preliminarily soaked in the background electrolyte for a few minutes just to removing weakly bound or adsorbed enzyme and to permit swelling of the enzyme layer. When not in use, the enzyme electrode was stored in phosphate buffer, pH 7.4, I 0.1 M, at 4 °C in the dark.

2.4. Electrochemical measurements

All the electrochemical measurements were performed using a detection potential of +0.7 V *versus* Ag/AgCl/NaCl (3 M) in phosphate or acetate buffer (I 0.1 M). Solutions and carrier stream were air saturated and the temperature was ambient. Unless otherwise stated, the flow rate in flow-injection experiments was 0.6 mL min^{-1}.

2.5. Samples preparation

Aminozim, an oral solution based on the association of various L-amino acids and used as a tonic, was diluted 1:1000 with acetate buffer, pH 5, I 0.1 M, before injection. Cheese samples (Mozzarella, Emmenthal, Provolone and *Parmigiano Reggiano*) were grated, dissolved in hot distilled water (2 g of cheese in 20 mL of water) and sonicated for half an hour. The suspension was filtered by passage through a filter paper, diluted 1:100 with the supporting electrolyte and then injected.

3. Results and discussion

The specificity of a biosensor depends on that of both the enzyme and the hydrogen peroxide amperometric detection. As it was previously discussed, faradic interference, produced by endogenous species electroactive at the detection potential, can be powerfully overcome by electrosynthesizing a permselective film onto the electrode surface as will be demonstrated, once more, later in the paper. As far as enzyme selectivity, a careful study of several parameters affecting enzymatic catalysis is necessary in order to minimize the interference effects of L-amino acids on lysine. Enzyme selectivity studies have been carried out at Pt/LO modified electrodes on changing flow rate and pH by measuring the interference effect on L-lysine response by L-arginine, L-ornithine, L-histidine, L-tyrosine and L-phenylalanine, which are known as substrate for LO from *T. viride* [22], *i.e.* the enzyme herewith used.

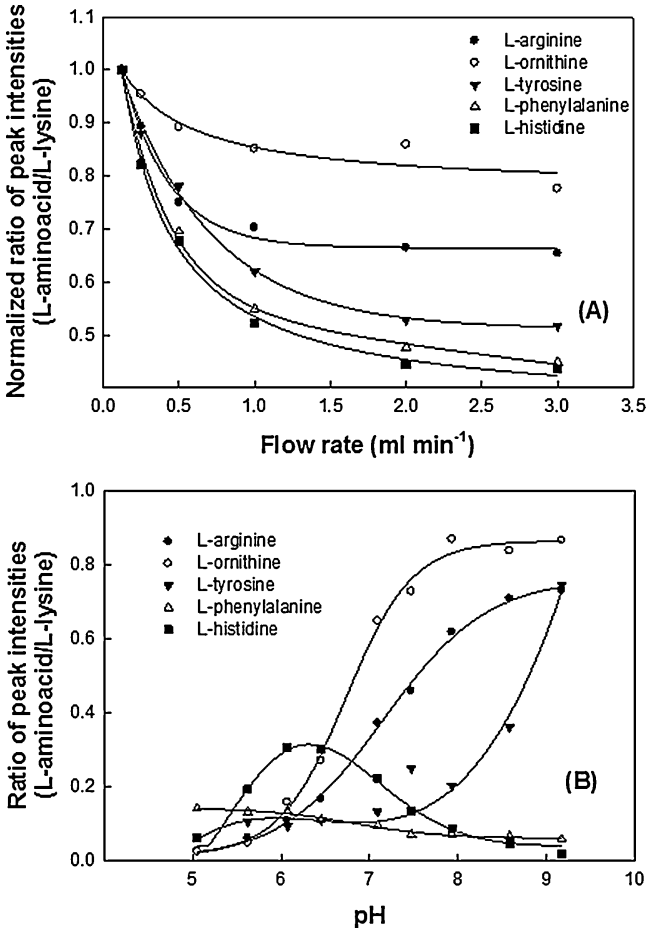


Fig. 1. Normalized ratio (a) and ratio (b) between each L-aminoacid (L-arginine, L-ornitine, L-histidine, L-tyrosine, L-phenylalanine 1 mM) and L-lysine peak intensities as a function of flow rate (a) and pH (b) at a typical Pt/LO biosensor. Substrate and amino acids concentrations: 1 mM. Supporting electrolyte: phosphate buffer pH = 7.5, I = 0.1 M (a); acetate/phosphate/borate buffer I = 0.1 M (b). Flow rate: 0.6 mL min^{-1} (b). Injection volume: 20 μL.

3.1. Influence of flow rate

It is common knowledge that flow rate can significantly influence the kinetic behaviour of an enzyme-based amperometric sensor as it affects the permanence time of the substrate as well as diffusion of substrate/product at the enzyme in membrane. Flow rate experiments were preliminary performed at pH 7.5, *i.e.* the pH of maximal activity of LO immobilized by co-crosslinking [7], very close to that reported for the enzyme free in solution [22].

Flow rate studies evidenced opposite behaviours between lysine and the other amino acids here investigated. In fact, while lysine responses increased with flow rate (as will be shown and discussed later), the relevant responses to the other amino acids decreased. As a consequence, the normalized ratios between the flow-injection responses of each amino acid and that of lysine in equimolar concentrations decreased on increasing the flow rate for almost each amino acid investigated (see Fig. 1a). This behaviour seems to suggest a different rate for the catalytic oxidation of the various substrates here considered: other amino acids react with LO slower than lysine owing to their lower affinities, thus a relatively low flow rate is required to observe an appreciable substrate oxidation. Indeed, these experimental results agree with the values of Michaelis constants reported for L-lysine (0.04 mM), L-ornithine (0.44 mM) and L-phenylalanine (14 mM) [22], showing an higher affinity of the enzyme for its natural substrate. In agreement with

these findings, Marconi et al. [6] observed that the current change, due to injection in batch of standard solutions containing equal concentrations of lysine and phenylalanine, was almost totally due to the lysine at least for the first 30 s, while Saurina et al. [24] reported fastest response for lysine.

Never before fully exploited, the findings from Fig. 1a involve that, tuning favourably flow rate, it is possible to minimize the interfering amino acids responses with respect to lysine one. While minimizing enzymatic interference required high flow rates (see Fig. 1a), interference effects coming from the oxidation of most electroactive species as *e.g.* ascorbate and tryptophan (see next section) unfortunately increase with flow rate since diffusion controlled. In the present study, the optimal flow rate able to minimize both interference effects was found at 0.6 mL min^{-1} and consequently mainly adopted in the present work.

3.2. Influence of pH

Amino acids, including L-lysine, are differently charged with pH, depending on their isoelectric points (p*I*), and enzyme affinity for a certain substrate is obviously affected by its charge state. Hence it follows that a comparative study of the influence of pH on the enzymatic catalysis of the interferent amino acids besides lysine must be carried out in order to establish the optimal pH value for an interferent-free lysine detection. The influence of pH on the response of the biosensor was here studied in the range 5–9 using a universal buffer at a fixed ionic strength so to avoid any change in the ionic composition of the supporting electrolyte.

Fig. 1b displays the peak current ratio between each L-amino acid and L-lysine flow-injection responses, in equimolar concentrations, as a function of pH. As can be seen, the major contribution to interference, particularly on increasing pH, comes from arginine and ornithine (less from tyrosine) which have notably similar structures and very close pK_a values to lysine: this behaviour thus is due since these amino acids showed an enhancement of their current response with pH quite similar to that observed for lysine [7]. Considering that the charge state modification with pH is almost the same for all these substrates, the above behaviour could be ascribed to the formation of improper ionic forms of the active site of enzyme and/or of the substrates, *i.e.* to a true pH effect [7]. Particularly, taking into account the p*I* values of the enzyme and the herewith investigated amino acids as well as their pK_a values, the behaviour depicted in Fig. 1b confirms that the active site of the enzyme must be in its negative charged form to bind the relevant substrate while the diprotic, positively charged amino acid form should be the main active specie able to bind to the enzyme in the pH range here studied [7]. In agreement with this view, an opposite behaviour was observed for the remaining amino acids histidine and phenylalanine (see Fig. 1b), since in this case both substrates come (or are) in the neutral, inactive form, on increasing the pH (p*I* 7.6 and 5.5, respectively).

Accordingly to this study, the interference in lysine detection, strongest at higher pH values, was minimal at pH around 5, *i.e.* in the pH region where the catalytic conversion of the natural substrate, L-lysine, is significantly higher than that of the other, interfering, amino acids. At higher pH, the difference between lysine and other amino acids responses is lower due to the improved catalytic activity of LO and thus the interfering effect is more pronounced. A pH 5 was thus chosen as optimal value being the interference of all the tested substrates negligible with respect to the response of lysine.

The strong impact of pH on the enzymatic response and on the kinetic behaviour of the proposed biosensor has been further evidenced by studying its hydrodynamic behaviour at two different pH. Fig. 2 shows the normalized responses of a typical LOD biosensor for injection of lysine 1 mM as a function of flow rate at pH 5 (acetate buffer, *I* 0.1 M) and pH 7.5 (phosphate buffer, *I* 0.1 M). It

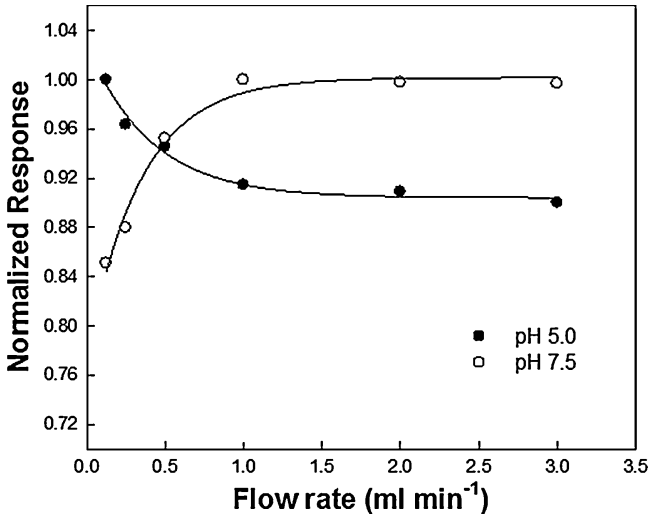


Fig. 2. Normalized responses of a typical Pt/LO biosensor for injection of lysine 1 mM as a function of flow rate at two different pH values. Supporting electrolyte: phosphate buffer pH = 7.5, *I* = 0.1 M; acetate buffer pH 5.0, *I* = 0.1 M.

is interesting to observe that curve profile is reversed by passing from pH 5 to pH 7.5. Since lysine oxidation increases with pH [7], at pH 7.5 the enzyme catalysis in membrane is surely sufficiently fast to render the diffusion of substrate and/or of the relevant product, at the immobilized enzyme layer, the rate determining step. As a consequence, the diffusion-controlled current produced at the electrode surface by the oxidation of hydrogen peroxide due to the enzyme catalysis increases as flow rate is increased in a Levich-like fashion, reaching a limit value at the membrane thickness [7]. On the contrary, at pH 5, since lysine catalysis proceeds at a slower rate, an opposite behaviour was observed (see Fig. 2), as it could be expected when enzymatic catalysis become the rate determining step: the current decrease with flow rate has been rationalized due to either hydrogen peroxide build-up in membrane and to an increased residence-time of substrate in membrane [7].

Therefore, by switching pH from weakly acidic to weakly alkaline values, it is possible to change the kinetic control of the system from merely enzymatic to diffusive. The above experimental findings hence suggest that, properly fine-tuning flow rate and pH, a significant lowering of the response of interfering amino acids relative lo lysine can be achieved, therefore improving the specificity of the enzymatic catalysis towards the target analyte. Notably, an optimal flow rate of 0.6 mL min^{-1} (see above) was observed even at pH 5, showing that the behaviour here observed and discussed for lysine is the same for the other studied amino acids.

3.3. Interference studies

Once optimized enzyme specificity, the anti-interferential properties of overoxidized polypyrrole film PPy_{ox} were investigated in order to assure the production of a lysine biosensor free from faradic interference besides from enzymatic ones. For this, the permselective behaviour of platinum electrodes modified by a non conducting film of PPy_{ox} was investigated by comparing, before and after polymer electrosynthesis, the flow-injection amperometric responses of common electrochemical probes such as ascorbic acid and cysteine, typically found in natural samples. In agreement with previous findings [20], the electrodic modification reduced the ascorbic acid response to about 3% of the initial value while cysteine responses were not detected at all (data here not shown). Anyway, amino acids themselves can be electroactive at the applied potential thus representing possible faradic interferents. As an example, tryptophan is known to be electrochemical oxidable even at the

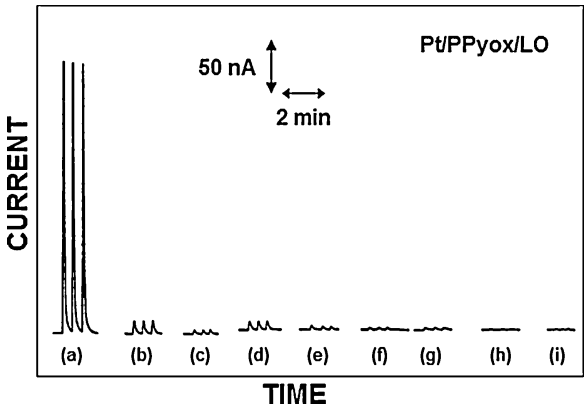


Fig. 3. Typical flow injection peaks for triplicate injections respectively of L-lysine (a), L-phenylalanine (b), L-ornitine (c), L-tyrosine (d), L-histidine (e), L-arginine (f), L-tryptophan (g), L-aspartic acid (h) and L-glutamic acid (i) standard solutions (1 mM) at a Pt/PPy_{ox}/LO biosensor. Flow rate: 0.6 mL min^{-1}. Supporting electrolyte: acetate buffer pH = 5.0, I = 0.1 M; injection volume: 20 μL.

detection potential used in the present work: being an essential amino acid for humans and herbivores, it is sometimes added to dietary and feed products as food fortifier, therefore representing a possible faradic interferent for lysine detection. Notably, as it will be demonstrated by discussing later Fig. 3, the PPy_{ox} electrode modification produced a so drastic attenuation of diffusion through the film of this as well other amino acids (see below) that it was impossible to evaluate their current signals for some cases. Thus, the double and concomitant approach coming from the use of a PPy_{ox} permselective film and the selection of pH and flow rate able to minimizing of enzymatic interference effects, allowed us to minimize current signals deriving from the oxidation of electroactive species, as well as those coming from the interference amino acids, respectively.

Fig. 3 shows the flow-injection responses for L-lysine (a), L-phenylalanine (b), L-ornithine (c), L-tyrosine (d), L-histidine (e), L-arginine (f), L-tryptophan (g), L-aspartic acid (h) and L-glutamic acid (i) at a Pt/PPy_{ox}/LO modified electrode, all at a concentration value of 1 mM. As can be seen, interferences due to various amino acids, even the electroactive ones, were powerfully reduced and practically minimized compared with lysine signal. While the relevant biases, measured at equimolar concentrations of lysine and amino acid interferent and expressed as percentage, are listed in Table 1, it is worth of note that the selectivity of the proposed biosensor is quite appealing and somewhat superior if compared with the interferences figures elsewhere reported.

Indeed, the interfering effect of ornithine and arginine is surely the basic problem of the selectivity of most L-lysine biosensing systems based on L-lysine-α-oxidase from *T. viride* [17]. For L-ornithine, for example, typical interference bias ranges from 28% up to 90% [4,23,25] whereas interference from L-arginine on L-lysine response was of 15% [23]. To overcome the poor enzyme specificity, other approaches were therefore proposed. The lowering of the temperature reaction down to 10 °C, for example, resulted in an ornithine interference effect nearly negligible but, unfortunately, caused also a significantly loss in lysine response [26]. Even a thoughtful selection of the enzyme source [8] was attempted to minimize the amino acid interference: indeed, LO from *Sebastes schlegeli*, used in an optical enzyme sensor, permitted an interference less than 3% for arginine [27].

The present work, as we demonstrate in Fig. 3 and Table 1, outlines the possibility of achieving optimal and better specificity values, using a common and low cost commercial enzyme like L-lysine-α-oxidase from *T. viride*, simply by fine-tuning easily controlled experimental parameters as pH and flow rate and

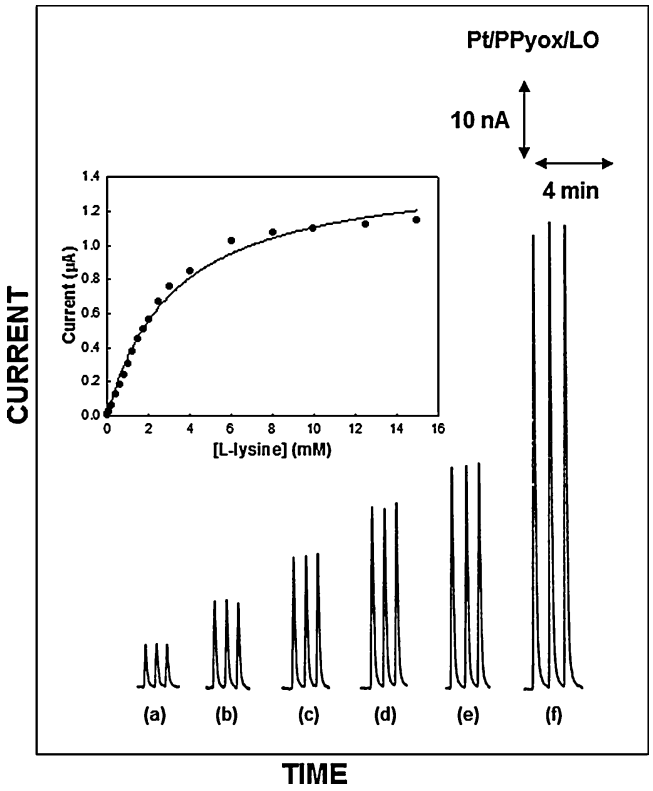


Fig. 4. Typical flow injection peaks for triplicate injections respectively of (a) 0.02, (b) 0.04, (c) 0.06, (d) 0.08, (e) 0.1, (f) 0.2 mM lysine standard solutions at Pt/PPy_{ox}/LO biosensor. Flow rate: 0.6 mL min^{-1}. Supporting electrolyte: acetate buffer pH = 5.0, I = 0.1 M; injection volume: 20 μL. Inset: relevant calibration curve.

thus without enzyme source selection [*e.g.* 8, 27] or thermosetting the experimental set up [26]. Not less important, the use of the PPy_{ox} electrode modification, never explored before, assured also an increased selectivity towards main electroactive interfering compounds (*e.g.* ascorbate and tryptophan), a feature not always reported elsewhere even using DAB and/or PANI films [8,9,15–18,28,29].

3.4. Analytical performances of the biosensor

In Fig. 4, the flow injection peaks relevant to repeated injections of increasing concentrations of standard lysine solutions at a Pt/PPy_{ox}/LO electrode are depicted along with a typical calibration curve. As can be seen, the low response time of the biosensor (less than 6 s when measured as $t_{0.95}$ in batch solution [7]) gave nicely responsive peaks permitting high throughput for sample injections. The plot of the calibration curve shows the well-known behaviour expected for an enzyme-catalyzed reaction with linear and saturated response at low and high substrate concentration, respectively. Fitting of the linear part of the calibration graph gave a regression line with a slope of (0.291 ± 0.003) μA mM^{-1} and an intercept of $(4.5 \pm 3.4)\ 10^{-3}$ μA (correlation coefficient better than 0.99911) and hence typical sensitivities of 41 nA mM^{-1} mm^{-2} were achieved: please note that this value has been obtained at pH 5 and in the presence of the PPy_{ox} underlying film (which typically halved the current response just due to the enzymatic membrane) while, in most cases, relevant sensitivities, elsewhere reported, refer to pH 7.5. The proposed Pt/PPy_{ox}/LO biosensor permitted also linear lysine responses from 0.02 mM up to 2 mM, which seems better than many sensing devices using LO and amperometric detection (see Table 1).

Table 1
Comparison of L-lysine biosensors.

Sensor	Interference Bias (%)								Linear Range (upper limit)	LOD	Response time	Operational stability[a]	Reference
	Phe	*Orn*	*Tyr*	*His*	*Arg*	*Trp*	*Asp*	*Glu*					
dioxygen sensing biosensors													
LO in gelatin	0	–	0	7.0	2.5	Trace	0	–	2 mM	10 μM	40 s[b]	20 days	[2]
LO/catalase	–	16	–	–	11	–	–	–	0.67 mM	6.7 μM	5 s[b]	1 month	[3]
hydrogen peroxide sensing biosensors													
Polycarbonate/LO/cellulose	Low	Yes	Low	Low	Yes	–	–	–	1 mM	0.5 μM	120 s	Less than 10 days	[5]
1,2-DAB/LO crosslinking	–	–	–	–	–	–	–	–	2 mM	0.2 μM	12–16 s	–	[15]
Ruthenium/Rodium 1,2-DAB LO co-crosslinking	0.7	3.4	–	–	1.1	–	–	–	0.125 mM	2 μM	–	–	[8]
1,2-DAB on Au/LO crosslinking	12	6	56	31	–	–	–	–	10 μM	0.1 μM	–	–	[16]
1,3-DAB on Au/LO crosslinking	31	48	–	0.1	31	–	–	–	1 mM	50 μM	–	20 days	[17]
Prussian Blue/nafion/LO co-crosslinking	–	–	–	–	–	–	–	–	0.7 mM	5 μM	5–15 s[b]	3 days	[9]
Prussian Blue/1,2-DAB/LO co-crosslinking	–	–	–	–	–	–	–	–	1 mM	50 μM	15 s	60 h	[18]
AuNP + cMWCNT/PANI or 1,2-DAB/LO[c]	5–6	3	1–7.4	3–13	−4–8	−3–6	–	3–6	600 μM	5–20 μM	2–4 s	20 days	[28]
Diamond paste with LO	–	–	–	–	–	–	0.98	0.07	0.1 μM	4 pM	–	2 months	[29]
PPy_{ox}/LO co-crosslinking	4.5	1.4	3.7	1.2	0.90	0.67	n.d.	n.d.	2 mM	4 μM	Less than 6 s[d]	40 days	This paper
optical biosensors													
ECL by ruthenium complex[e]	–	–	–	–	Low	–	–	Low	0.68 mM	9.8 nM	At least 18 s	–	[30]

–, data unavailable since or not reported, tested or not compared to lysine response; n.d., not detected (S/N equal or lower than 1).
[a] Time required for a sensitivity loss of 10%.
[b] Requires minutes for a successive analysis.
[c] AuNP: gold nanoparticles; cMWCNT: carboxylated multiwalled carbon nanotubes; PANI: polyaniline.
[d] From reference [7].
[e] ECL: electrochemiluminescence.

The estimated detection limit at a signal-to-noise ratio of 3 was found to be 4 μM, corresponding to an absolute amount of 80 pmol of lysine for a 20 μL sample injection. Such a low value is surely notable if compared with the detection limits reported for a lysine biosensor based on polyaniline and poly 1,2-DAB modified Au electrodes (5 μM and 20 μM respectively) as achieved by combining the synergistic effect of gold nanoparticles and multiwalled carbon nanotubes [28]. Indeed, as Table 1 shows, other sensing devices permitted lower detection limits as *e.g.* in the significant case of a diamond paste-based LO amperometric biosensor [29] but in all these cases the relevant interference biases were higher and/or, among the various substrates there tested, the enzymatic interfering problem, like that caused by ornithine and arginine, was not treated at all.

The operational stability of Pt/PPy_{ox}/LO biosensor was tested by repeated injections of lysine samples under the continuous run of the flow injection system: no significant differences on lysine responses was observed during all the working day (typically 10–12 h). Storage stability (see Table 1) was investigated by discontinuously monitoring lysine responses of a typical biosensor stored in a phosphate buffer pH 7.5 at 4 °C in the dark when not in use (please note that no particular effort was made to avoid bacterial growth in the storage buffer). No appreciable loss in lysine sensitivity was observed up to one month: just after 40 days and more, a loss of sensitivity was observed but still was the 90% of its initial value. From Table 1, only the diamond paste-based LO biosensor [29] appears the more stable in storage, but, unfortunately, with the limitations already described above.

While this paper is focused mainly on the enzymatic, electrochemical approaches for L-lysine detection, some other non-enzymatic but optical methods [30 and references therein cited] have been proposed for the same purpose. Indeed, electrochemiluminescence (ECL) approaches, to cite the more sensitive, yield so low detection limits to permit trace analysis of lysine (see Table 1) but its application to biological sample would require [30] a separation approach (*e.g.* HPLC or CE) which suggest a poor specificity of the method.

3.5. Application to real samples

The performances of the herewith proposed biosensor, both in terms of sensitivity, stability and selectivity, proved so promising to encourage us to experiment L-lysine detection in real samples. To test the effective selectivity of the developed biosensor, the content of L-lysine has been determined in a pharmaceutical sample, Aminozim, a tonic drug containing many amino acids in relevant amounts (a 10 mL of Aminozim sample is certified to contains, between other amino acids, 66.4 mg of L-aspartic acid, 234.4 mg of L-glutamic acid, 3.8 mg of L-tyrosine, 47.4 mg of L-phenylalanine, 122.8 mg of L-lysine, 40.4 mg of L-histidine and 46.4 mg of L-arginine). The flow injection peaks relevant to the repetitive injections of an Aminozim solution diluted 1:1000 with acetate buffer (pH 5, *I* 0.1 M) at a Pt/PPy_{ox}/LO electrode (see Fig. S1 in Supplementary Data), gave, after calibration, a lysine concentration of 0.091 ± 0.014 mM: accordingly to a paired Student's *t*-test, evaluated for $1-\alpha = 0.95$, the found lysine concentration

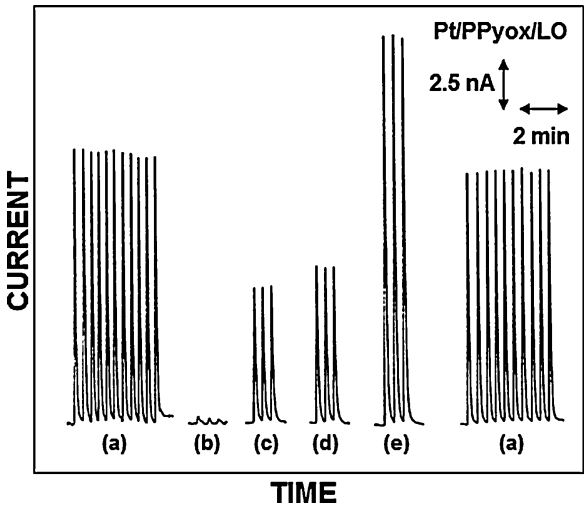


Fig. 5. Typical flow injection peaks for triplicate injections respectively of L-lysine 0.04 mM standard solution (a) and aqueous extracts from *Mozzarella* (b), *Emmenthal* (c), *Provolone* (d) and *P. Reggiano* (e) cheeses at a Pt/PPy_{ox}/LO biosensor. Flow rate: 0.6 mL min^{-1}. Supporting electrolyte: acetate buffer pH = 5.0, *I* = 0.1 M; injection volume: 20 μL.

resulted not significantly different from the declared drug composition (0.084 mM).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.07.036.

Once checked the selectivity of the proposed biosensor, food samples were also analyzed in the attempt to test the biosensor ability to monitoring their nutritional quality, which, as above described, is associated to the content of free available lysine. Indeed, L-lysine is so easily damaged by heat treatment and storage conditions of foods to constitute an important index for the assessment of food processing techniques [31].

While in many foods lysine is found bound forming proteins, cheeses contain such a amino acid in its free form in notable quantity. The release of free amino acids in cheeses occurs during ripening and is essentially caused by a proteolysis process. Rennet, plasmin, starter proteinases and peptidases, as enzymes from the non-starter microflora (particularly lactobacilli), all contribute to hydrolyse proteins to small peptides and/or free amino acids, all molecules acting as precursors for flavour forming reactions [32]. L-Lysine is one of the most abundant amino acids at the end of ripening for different kinds of cheese so the determination of free lysine can provide an estimate of the ripening time and therefore of the nutritional value of a cheese. Notably, the lysine detection in cheese does not require any sample pre-treatment (acidic hydrolysis) which unavoidably increases the complexity of the analytical procedure: in fact, the analyte can be easily sampled by merely water extraction of the given cheese (see Section 2). In this respect, preliminary studies performed on spiked samples with lysine showed recovery values ranging from 84 to 99%, depending on the sampled cheese.

The proposed biosensor was hence tested for the determination of L-lysine content in several cheeses which differ for both the ripening time and the manufacturing technology. Fig. 5 shows the current peaks recorded after triplicate injections of a 0.04 mM lysine standard solution and aqueous extracted from some cheese varieties while the lysine concentration values found for each cheese are listed in Table 2. *Mozzarella* cheese production is based on the employment of *Str. Thermophilus* as starter which has a poor proteolytic activity; moreover the ripening period is completely absent. Lysine content in such cheese is therefore negligible as it is possible to see from the relevant current signals reported in Fig. 5. For *Emmenthal*, instead, the proteolytic activity of the relevant starters is enough to release of an amount of free aminoacids equal to 25 g/Kg of cheese while the ripening time, non less of 6 months, justifies a relatively high lysine content in the case of *Provolone*. The use of *Pediococchi* microorganisms, with strong proteolytic activity, justifies moreover the high lysine content found in the case of *P. Reggiano*: it is worth to note that the ripening time for this last cheese is the longest one. The concentration values found for each cheese (see Table 2) appear quite similar with data elsewhere reported. Casella et al. [33], for example, by using liquid chromatography with electrochemical detection, found a lysine concentration of 29 μmol g^{-1} in for *Emmenthal*; in the case of *P. Reggiano* cheese, a free lysine content of 3 g for 100 g of total proteins (corresponding to 67 μmol g^{-1} of cheese) was found by reversed phase liquid chromatography with a fluorescence detector [34].

Table 2
L-lysine content as evaluated in cheese samples.

Cheese	L-Lysine content	
	mM	μmol g^{-1} [a]
Mozzarella	–	–
Emmenthal	2.1	21
Provolone	2.4	24
P. Reggiano	6.1	61

[a] μmol of lysine g^{-1} of cheese.

4. Conclusions

An amperometric L-lysine biosensor based on L-lysine-α-oxidase from *T. viride* (a simple commercial enzyme) immobilized by co-crosslinking and an underlying electrosynthesised, overoxidized polypyrrole film has been developed. Even if the used enzyme is known to be poorly specific, a proper kinetic control of the sensor, achieved by simply adjusting pH and flow rate as here studied and optimized, allowed high selectivity of the enzyme catalysis towards lysine with no need of selecting the source of the enzyme or other approaches lowering the sensitivity towards the target analyte. Notably, the use of the overoxidized polypyrrole film assured excellent interferent rejection characteristics towards major electroactive compounds. All these features combined with its worthy analytical performances resulted in a lysine biosensor which appears promising for real sample analysis with low risk of interferences from sample.

Indeed, preliminary results showed that the proposed biosensor might be successful for lysine analysis of pharmaceutical formulations rich in amino acids as well as much complex samples such as cheese. In this last case, the use of this biosensor could permit to verify the importance of the technology of cheese processing and production on the free lysine content and so to establish the incidence of the times and the conditions of maturation. Not less important, the evaluation of free lysine content in cheese could assume a particular relevance for the individualization and/or confirmation of possible frauds in cheese-based foods: in fact, as lysine can be damaged upon thermal treatments, its detection allows to underline the use of prohibited raw materials in the production of cheeses, such as powdered milk, casein, caseinate and melted cheeses, that is semi-manufactured that have suffered of a particularly hard thermal treatment. Accordingly, much work in this direction is in progress in our laboratory and the relevant results will be the topic of a future paper.

Acknowledgements

Work carried out with financial support provided by MIUR – PRIN 2009 Project. This work comes from Honours Degree

Thesis (2000) of Dr. Stefania De Biasi which is fully acknowledged for her experimental help. Part of this work was already presented as poster communication at XVI Congresso Nazionale di Chimica Analitica, 24–28 September 2001, Portonovo (Ancona), Italy.

References

[1] J.L. Romette, J.S. Yang, H. Kusakabe, D. Thomas, Biotechnol. Bioeng. 25 (1983) 2557–2566.
[2] D. Pfeiffer, U. Wollenberger, A. Makower, F. Scheller, L. Risinger, G. Johansson, Electroanalysis 2 (1990) 517–523.
[3] E. Vrbova, M. Marek, E. Ralys, Anal. Chim. Acta 270 (1992) 131–136.
[4] K. Siegler, B. Weber, E. Weber, K. Tonder, E. Klingner, F. Riechert, J. Chem. Technol. Biotechnol. 59 (1994) 279–287.
[5] M.G. Lavagnini, D. Moscone, G. Palleschi, D. Compagnone, C. Cremisini, Talanta 40 (1993) 1301–1306.
[6] E. Marconi, G. Panfili, M.C. Messia, R. Cubadda, D. Compagnone, G. Palleschi, Anal. Lett. 29 (1996) 1125–1137.
[7] A. Guerrieri, T.R.I. Cataldi, R. Ciriello, Sens. Actuators B 126 (2007) 424–430.
[8] S.C. Kelly, P.J. O'Connell, C.K. O'Sullivan, G.G. Guilbault, Anal. Chim. Acta 412 (2000) 111–119.
[9] F. Ricci, A. Amine, C.S. Tuta, A.A. Ciucu, F. Lucarelli, G. Palleschi, Anal. Chim. Acta 485 (2003) 111–120.
[10] D. Centonze, A. Guerrieri, C. Malitesta, F. Palmisano, P.G. Zambonin, Ann. Chim. (Rome) 82 (5/6) (1992) 219–234.
[11] R. Ciriello, T.R.I. Cataldi, D. Centonze, A. Guerrieri, Electroanalysis 12 (11) (2000) 825–830.
[12] R. Ciriello, A. Guerrieri, F. Pavese, A.M. Salvi, Anal. Bioanal. Chem. 392 (2008) 913–926.
[13] A. Guerrieri, R. Ciriello, D. Centonze, Biosens. Bioelectron. 24 (2009) 1550–1556.
[14] F. Mazzei, F. Botrè, G. Favero, E. Podestà, C. Botrè, Microchem. J. 91 (2009) 209–213.
[15] A. Curulli, S. Kelly, C. O'Sullivan, G.G. Guilbault, G. Palleschi, Biosens. Bioelectron. 13 (1998) 1245–1250.
[16] I.D. Karalemas, C.A. Georgiu, D.S. Papastathopoulos, Talanta 53 (2000) 391–402.
[17] M.H. Divritsioti, I.D. Karalemas, C.A. Georgiu, D.S. Papastathopoulos, Anal. Lett. 36 (2003) 1939–1963.
[18] A. Curulli, F. Valentini, S. Orlanduci, M.L. Terranova, G. Palleschi, Biosens. Bioelectron. 20 (2004) 1223–1232.
[19] D. Centonze, A. Guerrieri, C. Malitesta, F. Palmisano, P.G. Zambonin, Fresenius J. Anal. Chem. 342 (1992) 729–733.
[20] A. Guerrieri, G.E. De Benedetto, F. Palmisano, P.G. Zambonin, Biosens. Bioelectron. 13 (1) (1998) 103–112.
[21] A. Guerrieri, V. Lattanzio, F. Palmisano, P.G. Zambonin, Biosens. Bioelectron. 21 (9) (2006) 1710–1718.
[22] H. Kusakabe, K. Kodama, A. Kuninaka, H. Yoshino, H. Misono, K. Soda, J. Biol. Chem. 255 (1980) 976–981.
[23] A.L. Simonian, I.E. Badalian, T.T. Berezov, I.P. Smirnova, S.H. Khaduev, Anal. Lett. 27 (1994) 2849–2860.
[24] J. Saurina, S. Hernandez-Cassou, E. Fabregas, S. Alegret, Anal. Chim. Acta 371 (1998) 49–56.
[25] F. Preuschoff, U. Spohn, E. Weber, K. Unverhau, K.H. Mohr, Anal. Chim. Acta 280 (1993) 185–189.
[26] R.L.C. Chen, M.H. Lee, K. Matsumoto, Anal. Sci. 12 (1996) 87–90.
[27] H. Hendo, Y. Hayashi, Y. Kitani, H. Ren, T. Hayashi, Y. Nagashima, Anal. Bioanal. Chem. 391 (2008) 1255–1261.
[28] N. Chauhan, A. Singh, J. Narang, S. Dahiya, C.S. Pundir, Analyst 137 (2012) 5113–5122.
[29] R.I. Stefan-van Staden, R.M. Nejem, J.F. van Staden, H.Y. Aboul-Enein, Biosens. Bioelectron. 35 (2012) 439–442.
[30] S. Wang, J. Yu, F. Wan, S. Ge, M. Yan, M. Zhang, Anal. Methods 3 (2011) 1163–1167.
[31] K.J. Carpenter, V.H. Booth, Nutr. Abstr. Rev. 43 (1973) 423–451.
[32] F.A. Exterkate, A.C. Alting, Int. Dairy J. 5 (1995) 15–28.
[33] I.G. Casella, M. Gatta, T.R.I. Cataldi, J. Chromatogr. A 878 (2000) 57–67.
[34] M. Careri, S. Spagnoli, G. Panari, M. Zanoni, G. Barbieri, Int. Dairy J. 6 (1996) 147–155.