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Biosensor for Aluminum(III) Based on α-Chymotrypsin Inhibition using a Disposable Screen-Printed Carbon Electrode and Acetyl-Tyrosine Ethyl Ester as Substrate

Miriam Barquero-Quirós[1]*, Olga Domínguez-Renedo[2], Maria Asunción Alonso-Lomillo[2] and María Julia Arcos-Martínez[2]

[1]University of Costa Rica, CELEQ, San Pedro de Montes de Oca, 11500-2060 San José, Costa Rica
[2]Department of Chemistry, Faculty of Sciences, University of Burgos, Plaza Misael Bañuelos s/n,09001 Burgos, Spain

Abstract

We report on a new amperometric assay for Al(III) ions based on the inhibition of α-chymotrypsin enzyme. The immobilization of the enzyme was performed on screen-printed carbon electrodes modified with gold nanoparticles and polyvinyl alcohol. N-acetyl-tyrosine ethyl ester, used as enzyme substrate, produces an oxidation amperometric signal, which at increasing aluminum concentration is diminished. The developed system has a detection limit of 1.5 ± 0.1 μM (n=7) for Al(III). The reproducibility of the method is 6.6% (n=5) and its repeatability is 8.2% (n=3). Main interferences at low concentration, include As(V), Cd(II), and Mo(VI) ions. The developed method was successfully applied to the determination of Al(III) in spiked white wine sample. Results agree with the certified value giving a value of percent recovery of 103 ± 4% (n=4).

Keywords: α-chymotrypsin; Acetyl-L-tyrosine ethyl ester; Biosensor; Aluminum; Screen-printed electrodes; Gold nanoparticles.

Introduction

Aluminum is an element of ubiquitous presence being linked to several neurological disorders, such as Alzheimer's disease [1-3]. Moreover, increasing aluminum contamination due to industrial uses and clarification of tap water has augmented the interest on the development of methods for the analysis of this element at trace levels.

The determination of aluminum in aqueous solution has been traditionally performed by electrothermal atomic absorption spectrometry methods using complexing agents, long preconcentration time and sophisticated sample pretreatment. Another technique frequently used in the determination of Al(III) is fluorescence spectroscopy [4], which involves a high cost of analytical instrumentation. Electro analytical techniques allow to obtain low detection limits, however the high reduction potential of Al(III) limits their applicability. Ion complexation with chelating agents [5-11] has been presented as an interesting alternative to solve this problem. In this way, good results have been obtained with Hg electrodes, however their use is beginning to be reduced because of their toxicity and environmental issues. Amperometric biosensors constitute a good alternative based on their sensitivity, selectivity and environmental friendliness, accounting for their increasing analytical application in many fields [12,13]. The amperometric biosensor response is influenced by pH, applied potential and substrate concentration, thus the selection of proper conditions may considerably improves its performance and stability.

Enzymatic electrodes performance is linked to an immobilization enzyme method [14] that should not denature the protein nor modify its active site. An immobilization method that achieves this goal is polyvinyl alcohol (PVA) encapsulation-polymerization, which has led to the development of biosensors with high activity and stability [15-17]. On the other hand, the performance of enzymatic electrodes is improved with electrodeposited nanoparticles taking into account its increased use [18-22].

Aluminum accelerates proteolysis of a β-amyloid peptide that initiates neuritic plates in Alzheimer's disease [23]. The different effects of aluminum on the binding of synthetic substrates and macromolecular inhibitors to α-chymotrypsin suggest the occurrence of an aluminum-linked conformational change in the enzyme and pH dependence [24]. Other enzymes as calpains, which are Ca dependent, and cerebral cortex catepsins are also inhibited by Al(III) [25]. Aluminum induces Tau protein phosphorilation and aggregation through a phosphate bridge [26] and also promotes aggregation of β-amyloid protein both present in neuritic plates [27-31]. Their inhibitor effect over neurofilament proteins suggests accumulation inside neuronal space after aluminum administration [32]. Therefore, aluminum could affect proteases enzymatic activity, modifying their enzymatic structure, interfering in the recognition process of macromolecular inhibitors and altering the biochemical processes lead by proteases. Thus, the study Al(IIII) inhibitory effect on α-chymotrypsin using acetyl tyrosine ethyl ester (ATEE) as enzymatic substrate is of great importance. In this way, aluminum behaves as an inhibitor for chymotrypsin enzyme, which is a serine endopeptidase and hydrolyzes ATEE producing acetyl tyrosine which can be oxidized at an electrode surface [33].

Determination of Al(III) through this method offers several advantages, such as high sensitivity and low cost equipment. These advantages can be improved by using screen-printed electrodes (SPEs) due to their disposable character and great versatility. To the best of author's knowledge, there is no report in the literature of a screen-printed carbon electrodes (SPCEs) based biosensors for aluminum determination using ATEE as a substrate. This kind of biosensors offer low detection limits, narrower calibration range, and uses a lower

***Corresponding author:** Miriam Barquero-Quirós, University of Costa Rica, CELEQ, San Pedro de Montes de Oca, 11500-2060 San José, Costa Rica, Tel: 001-506-2511-2442; Fax: 001-506-2511-3217; E-mail: miriam.barquero@ucr.ac.cr

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applied potential [34,35], being their disposability character, low cost and environmental friendliness very significant. Moreover, this work reports for the first time the Km(app) (Michaelis Menten apparent constant) value of ATEE with and without Al(IIII) by means of SPCEs. Finally, the influence of NaCl concentration over noise and signal stability of biosensor electrode was determined.

Experimental Section

Reagents

In order to prepare hand-made SPCE several inks were used including Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink) and Electrodag 452 SS (dielectric ink) from Acheson Colloiden (Scheemda, The Netherlands), in a DEK 248 printing machine (DEK, Weymouth, UK, http://www.dek.com) using polyester screens with appropriate stencil designs mounted at 45° to the printer stroke.

TKA Purification System, inverse osmosis, with a UV lamp irradiation system was used to supply purified water needed to prepare all solutions. α-chymotrypsin enzyme (57.24 U/mg) and ATEE were purchased from Sigma, (Steinheim, Germany, http://www.sigmaaldrich.com), bovine serum albumine (BSA) and PVA fully hydrolyzed were obtained also from Sigma (Steinheim, Germany, http://www.sigmaaldrich.com), Hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4$) was purchased from Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany, http://www.sigmaaldrich.com).

Titrisol solutions were used to prepare stock standard solutions of Al, Fe, Cu, Sn, Zn, Co, Ni, Se Cr, Cd, Pb and Se and were from (Merck, Darmstad, Germany http://www.merck.com). Solutions of V, Mo, W, and Mg were acquired from High Purity Standard (Charleston SC, USA, http://www.highpuritystandards.com). Ca solution used was obtained from Inorganic Ventures (Lakewood, New Jersey USA, http://www.inorganicventures.com). As and Hg solutions were prepared from Atomic Spectroscopy Standards solutions (Perkin Elmer Co, Norwalk, USA http://www.perkinelmer.com).

Al solutions used for spike and wine enrichment were from High Purity Standard (Charleston, SC, USA, http://www.highpuritystandards.com) confirmed against standard reference material SRM 3101. 0.1 M acetate buffer was prepared from acetic acid and sodium acetate both Suprapur, adjusted at pH 7.8 (Suprapur, Merck, Darmstadt, Germany http://www.merck.com). 10 mM $CaCl_2$ from Merck (Darmstad, Germany http://www.merck.com) and 40 mM NaCl from J.T. Baker. (Deventer, The Netherlands, http://www.jtbaker.nl), were used as support electrolyte.

Equipment

Autolab PGSTAT Echochemie 128 N with GPS software was used to record electrochemical measurements from (Echochemie, Utrech, Netherlands http://www.echochemie,nl). pH values were adjusted with a pH meter Mettler Toledo Seven Multi, (Schwerzenbach, Switzerland http://www.mt.com)

Scanning Electronic Microscope S-3700 N Hitachi, http://www.was used to obtain picture of deposited Au nanoparticles.

Screen printed electrodes preparation

Inks used to construct hand-made SPCEs used in the determination of aluminum were placed as successive layers on a polyester substrate. Four different screens with appropriate stencils were used to transfer the required design following the printing procedure described in previous works [36,37]. A picture of dimensions of the SPCE used is shown in Figure 1a.

Modification of SPCEs with gold nanoparticles

Metallic gold nanoparticles (AuNPs) deposits were obtained by direct electrochemical deposition on the SPCE surface using a 0.1 mM solution of $HAuCl_4$ in 0.5 M H_2SO_4. The deposition was performed by applying a potential of +0.18 V during 15 seconds under stirring conditions [38,39]. Figure 1b shows the described formation of AuNPs on SPCEs.

Signal stability of enzyme α-chymotrypsin electrode was improved with AuNPs. In this way, stability is better compared with enzyme electrode without gold nanoparticles as it is shown in Figure 2.

α-chymotrypsin immobilization in AuNPs/SPCEs

The enzyme was immobilized by entrapment with PVA on the surface of AuNPs/SPCEs. The optimum immobilization procedure was reached by mixing 40 µl of a 30 mg/ml of enzyme solution, 20 µl of 1.7% (w/v) BSA solution, 24 µl of a 5% (w/v) PVA and 20 µl Britton-Robinson buffer solution at pH 7.8 [35]. Then, 10 µl of this mixture were placed on the working electrode surface. Immediately afterwards 3 µl of enzyme solution were placed over the mixture. Once the aliquot of the mixture used to immobilize the enzyme was deposited on the electrode surface it was kept under laboratory ceiling fluorescent lamp (λ 350-750) nm at 9×10^2 lux for 4 h at 24°C in air conditioned system and then stored for 24 hours at 4°C before using and between measurements. These storage conditions allowed biosensors good performance.

Amperometric determination of aluminum

The α- chymotrypsin biosensor was placed in an electrochemical cell containing 5 mL of acetate buffer solution pH 7.8 with 40 mM NaCl and 10 mM $CaCl_2$ [40-42]. An adequate potential was then applied and once a steady-state current was established, a defined amount of ATEE substrate was added to the cell. A large oxidation current was observed

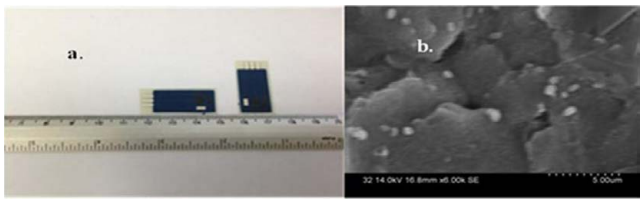

Figure 1a: Dimensions of screen printed electrode system used; **b:** Scanning electronic microscopy (SEM) picture of gold nanoparticles deposited on SPCE.

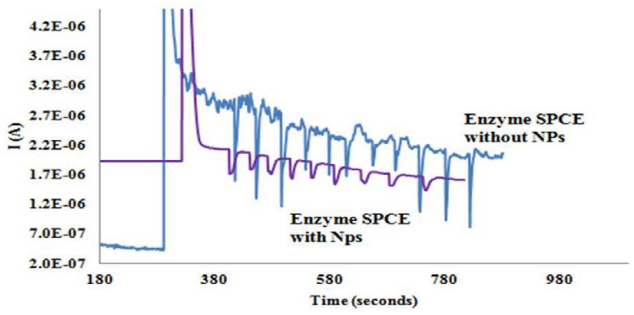

Figure 2: Aluminun α-chymotrypsin inhibition curves without and with gold nanoparticles, NaOAc pH 7.8, 10 mM $CaCl_2$, 40 mM NaCl; Eap, +0.7 V vs. Ag/AgCl).

due to the oxidation of the enzymatic reaction product after the addition of ATEE substrate. Once a steady-state current was set again, a selected volume of aluminum stock solution was consecutively added and a calibration curve was constructed. The addition of aluminum resulted in a decrease of the amperometric response proportional to the amount of metal added. Enzyme electrodes were conditioned in the acetate buffer solution pH 7.8 between each calibration set.

Results and Discussion

The α-chymotrypsin/AuNPs/SPCE biosensor produces an amperometric signal, which is quantitatively related to the concentration of ATEE as shown in Figure 3. The ATEE oxidation signal is considerably affected by addition of Al(III) producing a decrease in its amperometric response which relates to Al(III) concentration. In this way, Al(III) inhibition action was quantitatively evaluated by determining the difference between the steady-stated current obtained for ATEE in absence of Al(III) (I_0) and the steady-state current in the presence of Al(III) (I). The parameter ΔI (I_0-I) presents a linear dependence in the Al(III) concentration range from 3.6 μM to 36 μM.

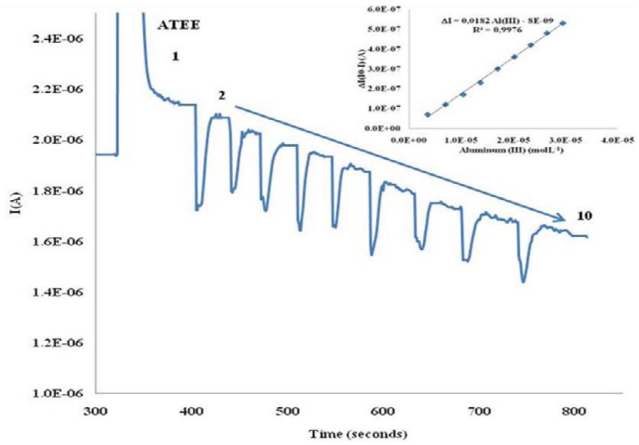

Figure 3: Amperometric recording for ATEE addition 100 μl ATEE 0,010 M (1) and consecutive additions of aliquots 50 μl 3.70 × 10^{-4} M Al (III) (2–10) to 5,00 mL buffer solution, under the optimal conditions (supporting electrolyte NaOAc pH 7.8, 10 mM $CaCl_2$, 40 mM NaCl; Eap, +0.7 V vs. Ag/AgCl). Insert figure, a calibration curve for aluminum additions.

Selection of experimental conditions

Aluminum inhibition effect was quantitatively evaluated by determining the difference between the ATEE steady-state current in the absence of aluminum (I_0) and the steady-state current in the presence of aluminum (I). The parameter ΔI (I_0- I) depends on ATEE concentration, applied potential (Eap) and pH of the buffer solution. Different experiments were carried out in order to optimize these variables.

First, the effect of applied potential in the chronoamperometric response of the developed biosensor was studied. The inhibitive signal of Al(III) was analyzed using operational potentials ranging from +0.3 to +0.8 V at pH 6.8 and 7.8, recommended for the α-chymotrypsin enzyme, as it is shown in Figure 4, parts **a** and **b**. A high quality amperometric signal at +0.7 V was obtained, thus, this potential was selected as the best value for the determination of aluminum.

The influence of pH was also studied in the range from 5 to 8 values. The results obtained are shown in Figure 4, part **c,** obtaining a value of 7.8 as the most adequate for Al(III) determination, taking into account the better stability conditions for the enzymatic electrode. Higher pH values produce enzyme instability and aluminum precipitation.

Finally, the influence of the concentration of ATEE was also studied in the range from 0.10 mM up to 0.30 mM. As it is shown in Figure 4 part **d**, the current increases in linear dependence with ATEE concentration

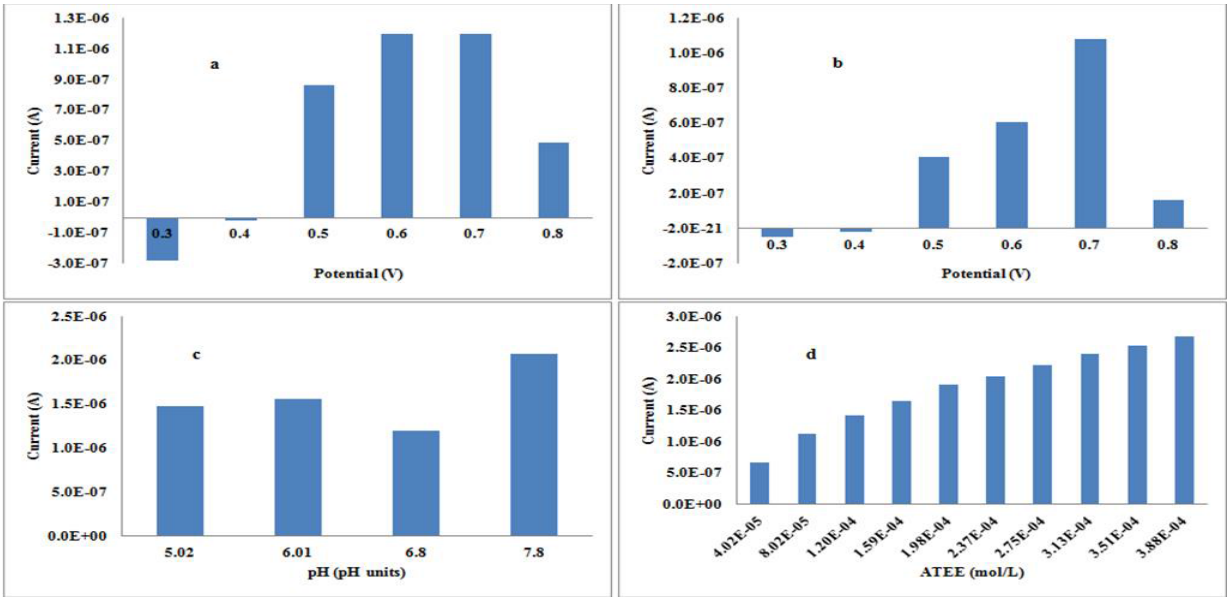

Figure 4a: Current response of ATEE with potential, pH 6.8 NaOAc, 10 mM $CaCl_2$, 40 mM NaCl; **b:** Current response of ATEE with potential, pH 7.8 NaOAc, 10 mM $CaCl_2$, 40 mM NaCl; **c:** Current response of ATEE with pH in 10 mM of $CaCl_2$ and 40 mM NaCl, Eap, +0.7 V vs. Ag/AgCl; **d:** Current response of ATEE concentration pH 7.8 NaOAc, 10 mM $CaCl_2$, 40 mM NaCl; Eap, +0.7 V vs. Ag/AgCl.

showing a tendency to substrate saturation. A concentration of 0.20 mM ATEE in cell was then found to give a high inhibition response of aluminum, while upper concentrations were noisy.

Aluminum inhibition behavior

Experiments performed with this biosensor showed that addition of $CaCl_2$ and NaCl improved stability of calibration curve. Therefore, their effect on the stability of the biosensor was investigated following the method of Lineweaver-Burk. Measurements were performed using NaCl concentrations ranging from 10 mM to 80 mM in $CaCl_2$ 10 mM. Considering apparent Michaelis Menten (Km app) values, signal stability, linearity and sensitivity of plots, concentrations of 10 mM $CaCl_2$ and 40 mM NaCl were selected as the most suitable for the inhibitive determination of Al(III). Figure 5 shows Lineweaver-Burk plots for NaCl increasing concentrations. The results obtained indicate that slope and therefore Km app values increase with NaCl concentration.

Once NaCl concentration was selected, at 40 mM, the inhibitory effect of Al(III) ions on the response of the α-chymotrypsin biosensor was investigated following the method of Lineweaver-Burk in presence of increasing amounts of inhibitor. The Km value $(1.17 \pm 0.06) \times 10^{-4}$ found without Al(III) was lower than the one obtained in presence of 100 µl of aluminum 3.70×10^{-4} M $(2.41 \pm 0.07) \times 10^{-4}$ and 400 µl of aluminum 3.70×10^{-4} M $(4.52 \pm 0.31) \times 10^{-4}$. Al(III) presence increases slope and Km app value and diminishes enzyme–substrate affinity. As it is shown in Figure 6, the studied process resembles a competitive inhibition.

Calibration and detection limit

As it has been mentioned above, Figure 3 showed a linear

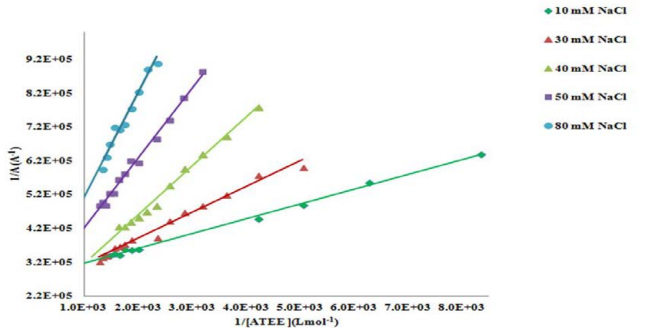

Figure 5: Lineweaver- Burk plot for NaCl increasing concentration, pH 7.8 NaOAc, 10 mM $CaCl_2$, Eap, +0.7 V vs. Ag/AgCl.

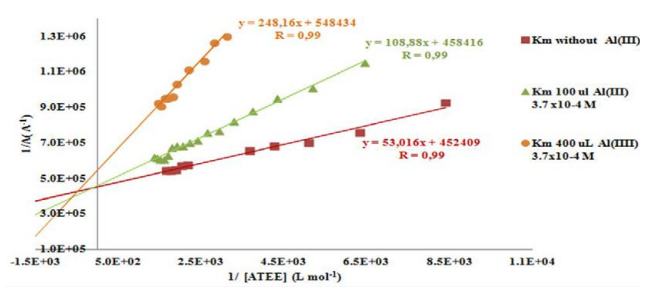

Figure 6: Lineweaver- Burk plot showing competitive inhibition for α-chymotrypsin AuNPs/SPCE biosensor without and with Al(III), pH 7.8 NaOAc, 10 mM $CaCl_2$, 40 mM NaCl; Eap, +0.7 V vs. Ag/AgCl.

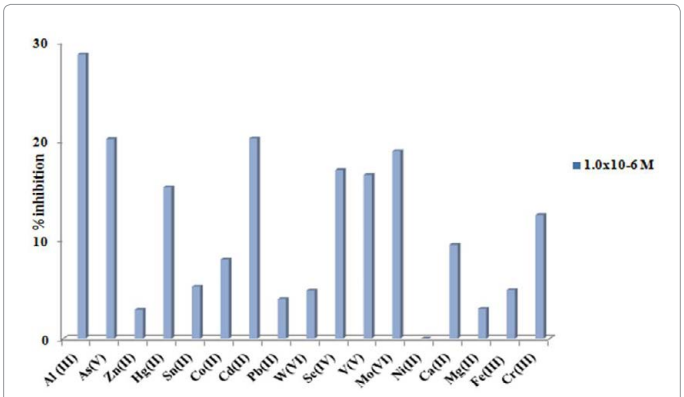

Figure 7: Inhibition percentage of current of α-chymotrypsin AuNPs/SPCE in presence of: Al(III); As(V); Zn(II); Hg(II); Sn(II); Co(II); Cd(II); Pb(II); W(VI); Se(IV); V(V); Mo(VI); Ni(II); Ca(II); Mg(II); Fe(III); Cr(III); at 10^{-6} M ; [ATEE] 0.2 mM; pH 7.8 NaOAc, 10 mM $CaCl_2$, 40 mM NaCl, Eap, +0.7 V vs. Ag/AgCl.

dependence between ΔI and Al(III) concentration in a concentration range from 3.6 µM to 36 µM. The regression parameters obtained for the calibration curve were $\Delta I = 0.0182\ [Al(III)] - 8.0 \times 10^{-9}$, ($R^2 = 0.9976$). Figures of merit such as precision and detection limit were evaluated by means of calibration curves obtained at optimum conditions. Standard deviation (Sy/x) of seven calibration curves was used to calculate limit of detection, giving a value of (1.5 ± 0.1) µM for this parameter under the optimum working conditions, using 3Sy/x criteria.

Precision

This parameter was calculated in terms of repeatability and reproducibility. Repeatability was carried out using the same electrode surface. In this way, several successive calibrations for Al(III) were tested. The electrodes were conditioned for 5 min in a buffer solution, pH 7.8, between experiments. The relative standard deviation (RSD) obtained for the slopes of the three curves with the same electrode was 8.2%. Likewise, the reproducibility of the amperometric signal was checked using the slopes of five regressions carried out with different electrode surfaces. The RSD reproducibility value obtained was 6.6%. These results suggest that the fabrication procedure of the chymotrypsin/AuNPs/SPCEs biosensors is reliable and allows reproducible electroanalytical responses to be obtained with different electrodes constructed using the method described in this work as it is shown in Table 1.

Accuracy

The accuracy of the developed method was evaluated by means of the analysis of a standard reference material (SRM) High Purity Standards solution (Lot Number 1121015, (1000 ± 3) mg/L, confirmed against reference material SRM 3101 (lot number 060502), spiked in white wine sample, using the standard addition method. Results are shown in Table 2. The aluminum average concentration quantified by the developed procedure, (1025 ± 30) mg/L (n=4; α=0.05), matches the certified value of the standard reference material in agreement with aluminum stock concentration, and mean deviation. These results suggest that the developed biosensor method with α-chymotrypsin enzyme is accurate and reliable for aluminum determination in white wine sample.

Interferences

Interference study was performed comparing the percentage of inhibition showed by the developed α-chymotripsine based biosensor in

Analytical Characterization	Mean Value	RSD
Limit of detection (M) n=7	1.5×10^{-6}	11.7%
Limit of quantification (M) n=7	5.0×10^{-6}	10.5%
Repeatability n=3	1.552×10^{-2}	8.2%
Reproducibility n=5	2.282×10^{-2}	6.6%
Accuracy n=4	103%	4 .4%
Linear range (M)	$(3.6\text{-}36.0) \times 10^{-6}$	

Table 1: Performance analytical parameters of biosensor α-quimotripsina/ATEE.

Replicates	[Al(III)] added (mol/L)	[Al(III)] found (mol/L)	Al(III) found (mg/L)	% Recovery
1	3.56×10^{-6}	3.84×10^{-6}	1079	108
2	3.56×10^{-6}	3.45×10^{-6}	970	97
3	3.56×10^{-6}	3.67×10^{-6}	1031	103
4	3.56×10^{-6}	3.64×10^{-6}	1021	102
% Mean Recovery				103
% RSD				4

Table 2: Recovery of certified Standard Reference Material SRM (1000 mg/L ± 3 mg/L) Al(III) spiked to white wine sample replicates.

the presence of aluminum and other foreign ions. Three concentration levels were tested, namely 1 mM; 0.1 mM and 1 μM. As it can be seen in (Figure 7), the highest interference effect was found for As(V); Cd(II) and Mo(VI) for the lowest level of concentration tested, but toxic ions should not be naturally present in wine. Due to some proteases enzymes are activated by Ca(II), this one is not a real interference, neither common ions as Mg(II) and Fe(III).

Conclusions

The development of a novel biosensor based on the inhibition of α-chymotripsin enzyme using AuNPs/SPCEs allows the amperometric determination of aluminum. The biosensor precision was studied in terms of the RSD of the slopes of several calibrations resulting in a reproducibility value of 6.6%. The method developed in this work presents several advantages, including lower detection limit, 1.5 μM, than other previous described ones [34,35].

The cross linking and entrapment method with PVA allowed immobilizing the enzyme over the AuNPsSPCE electrode surface. The optimum conditions for the best amperometric response of ATEE were at working potential of +0.7 V and a pH value of 7.8. In addition, the presence of Ca(II) and NaCl produce a stabilizing effect over the amperometric signal; in this way concentrations of 10 mM of Ca(II) and 40 mM of NaCl resulted to be optimal considering sensitivity, stability and Km(app) values. The Lineweaver-Burk plots obtained in the presence of Al(III) showed competitive inhibition and the performance of the developed biosensor was evaluated through its figures of merit that were adequate to quantify Al(III) at low concentration level. Moreover, the analytical performance of the developed biosensor was demonstrated by its application to the analysis of spiked white wine samples with a High-Purity Standards stock solution. The recovery value (1025 ± 30) mg/L obtained agreed with the certified reported aluminum SRM value of 1000 mg/L ± 3 mg/L. Finally, the main interferences at low level concentration included As(V), Cd(II) and Mo(VI).

Overall, the ATEE substrate was suitable for quantitative determination of aluminum using a SPE modified with AuNPs and α-chymotripsin enzyme. The developed method presents a lower detection limit and uses a lower potential than other enzymatic methods for the analysis of Al(III).

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The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

References

1. Zatta P, Drago D, Bolognin S, Sensi SL (2009) Alzheimer's disease, metal ions and metal homeostatic therapy. Trends Pharmacol Sci 30: 346-355.
2. Exley C (2012) The coordination chemistry of aluminum in neurodegenerative disease. Coordination Chemistry Reviews 256: 2142-2146.
3. Percy ME, Kruck TP, Pogue AI, Lukiw WJ (2011) Towards the prevention of potential aluminum toxic effects and an effective treatment for Alzheimer's disease. J Inorg Biochem 105: 1505-1512.
4. Weijun L, Shouzhi P, Duohua J, Shiqiang C, Gang L, et al. (2011) Fluorescent probes for Al(III) and Cr(III) based on a photochromic diarylethene bearing a fluorescent rhodamine unit. Microchimica Acta 174: 329-336.
5. Romero R, Tahan J, Moronta A (1992) Two alternative sample mineralization procedures to permit subsequent polarographic determination of total soluble aluminum in hemodialysis water. Analytica Chimica Acta 257: 147-154.
6. Camara M, Domínguez O, Arcos M (2003) Simultaneous determination of chromium(VI) and aluminum(III) by adsorptive stripping voltammetry with Pyrocatechol Violet. Helvetica Chimica Acta 86: 2434-2440.
7. Wang X, Lei J, Bi S, Gan N, Wei Z (2001) Determination of the speciation of aluminum(III) in natural waters by adsorption stripping voltammetry and complexation with Al(III)-solochrome violet RS. Analytica Chimica Acta 449: 35-44.
8. Arancibia V, Muñoz C (2007) Determination of aluminium in water samples by adsorptive cathodic stripping voltammetry in the presence of pyrogallol red and a quaternary ammonium salt. Talanta 73: 546-552.
9. Qiong L, Lirong W, Danli X, Guanghan L (2006) Determination of trace aluminum in foods by stripping voltammetry. Food Chemistry 97: 176-180.
10. Shahrya A, Atusa B, Abbas F (2010) Quantification of subnanomolar levels of aluminum by adsorptive stripping voltammetry using rubeanic acid as a selective chelating agent. Electroanalysis 22: 1889-1893.
11. Beltagi A, Ghoneim M (2009) Simultaneous determination of trace aluminum (III), copper (II) and cadmium(II) in water samples by square-wave adsorptive cathodic stripping voltammetry in the presence of oxine. Journal of Applied Electrochemistry 39: 627-636.
12. Renedo OD, Alonso-Lomillo MA, Martínez MJ (2007) Recent developments in the field of screen-printed electrodes and their related applications. Talanta 73: 202-219.
13. Albareda M, Merkoçi, A, Alegret S (2000) Configurations used in the design of screen-printed enzymatic biosensors. A review. Sensors and Actuators B: Chemical 69: 153-163.
14. Sassolas A, Blum LJ, Leca-Bouvier BD (2012) Immobilization strategies to develop enzymatic biosensors. Biotechnol Adv 30: 489-511.
15. Ulich T, Ulbricht M, Tomaschewski G (1996) Immobilization of enzymes in photochemically cross-linked polyvinyl alcohol. Enzyme and Microbial Technology 19: 124-131.
16. Gohil J, Bhattacharya A, Ray P (2006) Studies on the crosslinking of Poly(Vinyl Alcohol). Journal of Polymer Research 13: 161-169.
17. Wang P, Sergeeva MV, Lim L, Dordick JS (1997) Biocatalytic plastics as active and stable materials for biotransformations. Nat Biotechnol 15: 789-793.
18. Du D, Ding J, Cai J, Zhang J, Liu L (2008) In situ electrodeposited nanoparticles for facilitating electron transfer across self-assembled monolayers in biosensor design. Talanta 74: 1337-1343.
19. Wang Z, Zhao J, Li F, Gao D, Xing B (2009) Adsorption and inhibition of acetylcholinesterase by different nanoparticles. Chemosphere 77: 67-73.
20. Shulga O, Kirchhoff J (2007) An acetylcholinesterase enzyme electrode

stabilized by an electrodeposited gold nanoparticle layer. Electrochemistry Communications 9: 935-940.

21. Alonso-Lomillo MA, Yardimci C, Domínguez-Renedo O, Arcos-Martínez MJ (2009) CYP450 2B4 covalently attached to carbon and gold screen printed electrodes by diazonium salt and thiols monolayers. Anal Chim Acta 633: 51-56.
22. Xu Z, Chen X, Qu X, Jia J, Dong S (2004) Single-wall carbon nanotube-based voltammetric sensor and biosensor. Biosens Bioelectron 20: 579-584.
23. Yumoto S, Kakimi S, Ohsaki A, Ishikawa A (2009) Demonstration of aluminum in amyloid fibers in the cores of senile plaques in the brains of patients with Alzheimer's disease. J Inorg Biochem 103: 1579-1584.
24. Angeletti M, Lupidi G, Eleuteri AM, Tacconi R, Fioretti E, et al. (1997) Effect of aluminum on the binding properties of α-chymotrypsin. Journal of Biological Inorganic Chemistry 2: 320-326.
25. Lupidi G, Angeletti M; Eleuteri A, Fioretti E, Marini S, et al. (2002) Aluminum modulation of proteolytic activities. Coordination chemistry reviews 228: 263-269.
26. Zatta PF1 (1995) Aluminum binds to the hyperphosphorylated tau in Alzheimer's disease: a hypothesis. Med Hypotheses 44: 169-172.
27. Sakamoto T, Saito H, Ishii K, Takahashi H, Tanabe S, et al. (2006) Aluminum inhibits proteolytic degradation of amyloid beta peptide by cathepsin D: a potential link between aluminum accumulation and neuritic plaque deposition. FEBS Lett 580: 6543-6549.
28. Bolognin S, Zatta P, Lorenzetto E, Valenti MT, Buffelli M (2013) β-Amyloid-aluminum complex alters cytoskeletal stability and increases ROS production in cortical neurons. Neurochem Int 62: 566-574.
29. Ricchelli F, Drago D, Filippi B, Tognon G, Zatta P (2005) Aluminum-triggered structural modifications and aggregation of beta-amyloids. Cell Mol Life Sci 62: 1724-1733.
30. Bolognin S, Messori L, Drago D, Gabbiani C, Cendron L, et al. (2011) Aluminum, copper, iron and zinc differentially alter amyloid-Aβ(1-42) aggregation and toxicity. Int J Biochem Cell Biol 43: 877-885.
31. Drago D, Bettella M, Bolognin S, Cendron L, Scancar J, et al. (2008) Potential pathogenic role of beta-amyloid(1-42)-aluminum complex in Alzheimer's disease. Int J Biochem Cell Biol 40: 731-746.
32. Kushkuley J, Metkar S, Chan WK, Lee S, Shea TB (2010) Aluminum induces neurofilament aggregation by stabilizing cross-bridging of phosphorylated c-terminal sidearms. Brain Res 1322: 118-123.
33. Kozlova NO, Bruskovskaya IB, Melik-Nubarov NS, Yaroslavov AA, Kabanov VA (1999) Catalytic properties and conformation of hydrophobized alpha-chymotrypsin incorporated into a bilayer lipid membrane. FEBS Lett 461: 141-144.
34. Barquero-Quirós M, Domínguez-Renedo O, Alonso-Lomillo MA, Arcos-Martínez MJ (2014) Acetylcholinesterase inhibition-based biosensor for aluminum(III) chronoamperometric determination in aqueous media. Sensors (Basel) 14: 8203-8216.
35. Barquero M, Domínguez O, Alonso M, Arcos M (2012) Biosensor for aluminium(III) Based on Its Inhibition of α-Chymotrypsin Immobilized on a Screen-Printed Carbon Electrode Modified with Gold Nanoparticles. Microchimica Acta 179: 65-70.
36. Domínguez O, Arcos M (2007) A novel method for the anodic stripping voltammetry determination of Sb(III) using silver nanoparticle-modified screen-printed electrodes. Electrochem Communications 9: 820-826.
37. Gonzalo-Ruiz J, Asunción Alonso-Lomillo M, Javier Muñoz F (2007) Screen-printed biosensors for glucose determination in grape juice. Biosens Bioelectron 22: 1517-1521.
38. Dai X, Nekrassova O, Hyde ME, Compton RG (2004) Anodic stripping voltammetry of arsenic(III) using gold nanoparticle-modified electrodes. Anal Chem 76: 5924-5929.
39. Domínguez Renedo O, Arcos Martínez MJ (2007) Anodic stripping voltammetry of antimony using gold nanoparticle-modified carbon screen-printed electrodes. Anal Chim Acta 589: 255-260.
40. Sousa F, Jus S, Erbel A, Kokol V, Cavaco A, et al. (2007) A novel metalloprotease from Bacillus cereus for protein fibre processing. Enzyme and Microbial Technology 40: 1772-1781.
41. Castillo FJ, Pacheco RA, García FL, Navarrete MA, Félix ML (2006) Purification and biochemical characterization of chymotrypsin from the viscera of Monterey sardine (Sardinops sagax caeruleus). Food Chemistry 99: 252-259.
42. Hong J, Gong P, Xu D, Dong L, Yao S (2007) Stabilization of alpha-chymotrypsin by covalent immobilization on amine-functionalized superparamagnetic nanogel. J Biotechnol 128: 597-605.