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Development and Selection of Aluminum Screen Printed Amperometric Biosensor Modified with Nanoparticles and Catalase Enzyme

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Abstract: Two biosensors were developed based in aluminum quantitative inhibition on gluthathione peroxidase and catalase enzymes. Screen printed electrode of gold was modified with gold nanoparticles and catalase enzyme. Screen printed carbon electrode was modified with gold nanoparticles; and glutathione peroxidase enzyme. Biosensors experimental conditions were optimized and characterized trough Michaelis Menten apparent constant. Inhibitory aluminum effect was confirmed by increasing constant apparent values with Al(III) concentration. Kinetic study for glutathione peroxidase resembles a competitive inhibition, while catalase showed competitive inhibition. Michaelis Menten apparent inhibition constant of catalase was lower than glutathione peroxidase biosensor and also its slope inhibition calibration curve was higher, therefore catalase biosensor was validated by its performance parameters.

Catalase biosensor showed a detection limit of (1.0 ± 0.1) μM and quantification limit of (3.4 ± 0.3) μM. The repeatability was 8.3 % (n=3) and reproducibility was 10.7 % (n=5). Accuracy was tried spiking two concentrations of Al(III) standard reference material traceable to NIST. Recovery spiked concentrations of aluminum certified reference standard was 103.1± 5.3 (n=4) and 105.9 ± 4.4 (n=4).

Keywords: Aluminum, Screen printed carbon electrodes, Gold screen printed electrode, Catalase glutathione peroxidase inhibition, Validation.

1. Introduction

Aluminum is the third element present on earth considering soil, oceans and atmosphere. Soil acidification (pH < 5) allows solubility of toxic forms of Al into the soil, where its low concentration inhibit root growth and cause impairment of metabolic functions [1]. Its toxicity on living organisms had been showed on fishes, larvae fishes and laboratory rats [2].

In humans, aluminum had been named as participant in development of multiple neurological diseases by oxidative stress, cell mediated toxicity, apoptosis, inflammatory events in the brain, glutamate toxicity, effects on calcium homeostasis, gene expression and Al induced neurofibrillary tangle (NFT) formation [3]. The generation of oxidative stress may be attributed to its oxidant action on animals and humans. The oxidative stress has been

implicated in pathogenesis of various neurodegenerative conditions including Alzheimer's (AD) and Parkinson's diseases [4]. Considerable effort had been made with the goal to ameliorate its toxic effects using minerals [5] and natural extracts of plants containing flavonoids [6], impact of Al-stress in Al-resistant and Al-sensitive blueberries genotypes [7] and amino acids [8] had been evaluated with these purpose.

Catalase enzyme (Cat) catalyzes hydrogen peroxide (H_2O_2) oxidation [9] in aerobic organisms playing fundamental role of protection against oxidative stress. Cat activity is well characterized [10] and is found available in commercial preparations. Had been used in peroxide, glucose, short chain aliphatic alcohols and glutamine detection. Cyanide ion and flavonoids inhibit its activity [11-12]. A significant decrease in the catalase activity was observed after aluminum exposure, the low levels of Cat activity following its exposure may be due to the utilization of the enzyme in converting the H_2O_2 to H_2O [2, 7].

Aluminum also caused a significant reduction in activity of glutathione peroxidase (GPer) and Cat besides decreases glutathione concentration [2], increases lipid peroxidation and produces activity enzymes diminution It is considered an inductor of reactive oxygen species (ROS) and decreases activity of free radicals enzymes scavengers [13]. GPer enzyme function is to remove hydroperoxides formed *in vivo* and is inhibited by metals [4, 14], in cells aluminum diminishes gluthathione reduced in mitochondria [15]. Mostly of GPer biosensors has been devoted to gluthathione determination in biological matrixes [15-17] not regarding inhibition effect of aluminum on this enzyme. By other hand considering that Cat and GPer are natural antioxidant enzymes in the body and present the first line of defense against free radical damage [18], aluminum effect on these enzymes activity should be considered [19, 21]. Aluminum inhibitory effect on superoxide dismutase enzyme (SOD) had been showed and it is influenced by metallic nanoparticles [22]. A distinct feature of aluminum toxicity is its ability to create pro-oxidant environment [23]. Aluminum induced effects on enzymatic and non-enzymatic antioxidants and lipid peroxidation, are expressed on oxidative stress-related genes [24], increasing erythrocytes fragility and lipid peroxidation level [25]. In plants root apex is the major perception site of Al toxicity, producing a rise of H_2O_2 as response. Also SOD, Cat activities were changed [26]. Some toxic effects can be ameliorate with antioxidants present in plants extracts that profile as anti-AD preparations [27-28]. Other hand screen printed electrodes (SPEs) and screen printed carbon electrodes (SPCEs) had been showed versatility through its surface modification with nanoparticles (NPs), biomolecules and enzymes [29-30]. Therefore development of biosensors using Cat and GPer based in quantitative aluminum inhibition on these enzymes [31-32] profiles as an interesting alternative to the others biosensors developed for aluminum determination and confirms its inhibitory role on enzymes scavengers of free radicals [33-36].

2. Materials and Methods

2.1. Reagents

Solutions were prepared with purified water supplied by TKA Purification System, inverse osmosis, with a UV lamp irradiation system. Catalase enzyme (2000-5000 units/mg protein) and glutathione peroxidase (200UN) from bovine erythrocytes were purchased from (Sigma Aldrich Steinheim, Germany), bovine serum albumine (BSA), glutaraldehide and hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4$), DL cysteine hidrocloride were obtained from (Sigma Aldrich, Steinheim, Germany). Britton Robinson supporting electrolyte solutions were prepared as usual with boric, phosphoric and acetic acids (Merck, Darmstadt, Germany), pH values were obtained adjusting with NaOH Suprapur solution, (Merck, Darmstadt, Germany). Suprapur H_2O_2 30 % used also was obtained from (Merck, Darmstadt, Germany). Titrisol solutions were used to prepare stock standard solutions of Al, Fe, Cu, Sn, Zn, Co, Ni, Cr, Cd, Pb and Se were from (Merck, Darmstad, Germany). Solutions of V, Mo, W, Mg were acquired from High Purity Standard (Charleston SC, USA). Ca solution used was obtained from Inorganic Ventures (Lakewood, New Jersey USA). As and Hg solutions were prepared from Atomic Spectroscopy Standards solutions (Perkin Elmer Co, Norwalk, USA). polyvinyl alcohol (PVA) fully hydrolyzed were obtained also from (Sigma Aldrich Steinheim, Germany). Aluminum solutions used for spike were prepared from High Purity Standard (Charleston, SC, USA) confirmed against standard reference material SRM 3101. Several inks were used in the fabrication of SPCEs, namely Electrodag PF-407 A (carbon ink), Electrodag 6037 SS (silver/silver chloride ink) and Electrodag 452 SS (dielectric ink) supplied by Acheson Colloiden (Scheemda, The Netherlands). Gold Polymer Paste C2041206D2 of Gwent Group was used for fabrication of gold screen printed electrodes (AuSPEs).

2.2. Equipment and Software

An electrochemical system Autolab PGSTAT Echo Chemie128 N with GPS software was used to record electrochemical measurements (Echo Chemie, Utrech, Netherlands). All pH values were adjusted with a pHmeter (Mettler Toledo Schwerzenbach, Suitzerland).

Hand-made SPCEs and AuSPEs were produced on a DEK 248 printing machine (DEK, Weymouth, UK) using polyester screens with appropriate stencil designs mounted at 45º to the printer stroke and were

used with glutaraldehyde aluminum biosensor. Hand-made AuSPEs were used in catalase aluminum biosensor.

3. Methodology

3.1. Modification of SPCES and AuSPEs with Gold Nanoparticles

Metallic gold nanoparticles (AuNPs) were obtained by direct electrochemical deposition on the AuSPEs 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 (AuNPs/AuSPE) [37]. Same procedure was used to deposit AuNPs on SPCEs (AuNPs/AuSPEs).

3.2. Enzymes Immobilization Procedure

3.2.1. Glutathione Peroxidase Enzyme

The AuNPs/AuSPCE was washed thoroughly with purified water and wiped. Glutathione peroxidase enzyme was immobilized on its surface using (PVA). 10 µL of mix prepared mixing 20 µL gluthathione peroxidase enzyme 3.41 mg/mL, 1 µL of BSA 1.68 % w/v and 12 µL of PVA 5 % w/v were dropped on AuNPs/SPCE surface and GPer/AuNPs/SPCE was obtained. Immediately it was stored at 4^0C until its use and between measurements. GPer/AuNPs/SPCE showed very low repeatability and reproducibility compared with Cat/AuNPs/AuSPEs.

3.2.2. Catalase Enzyme

The AuNPs/AuSPE was washed thoroughly with purified water, wiped and an aliquot of 10 µL of mix prepared mixing 40 µL of catalase enzyme 10 mg/mL, 20 µL o BSA 1.68 % w/v and 20 µL of glutaraldehyde 2.5 % v/v was dropped on AuNPs/AuSPE surface and was obtained Cat/AuNPs/AuSPE. Immediately it was stored at 4^0C until its use and between measurements.

4. Results and Discussion

As it is well known from literature Gper is a selenium-containing antioxidant enzyme that effectively reduces H_2O_2 and lipid peroxides to water and lipid alcohols, respectively, and in turn oxidizes glutathione to glutathione disulfide. Other hand Cat is an oxide reductase that catalizes conversion of H_2O_2 to O_2. These enzymes not had been reported for aluminum determination, although previous experiments had shown that aluminum inhibits oxide reductases [22], this fact constitutes the base of biosensors developed in this work (Fig. 1).

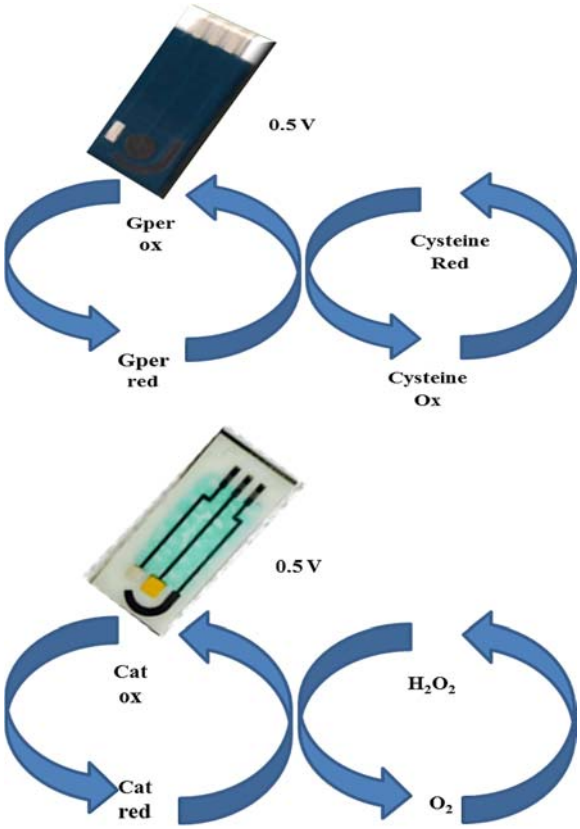

Fig. 1. Schemes of possible oxidation mechanism of cysteine on GPer/AuNPs/SPCE and H_2O_2 on Cat/AuNPs/AuSPE.

4.1. Optimization of Experimental Conditions for GPer/AuNPs/SPCE

The parameters steady state current of substrate, namely (I_0) and steady state current in Al(III) presence and its difference ($\Delta(I_0$-I) depends on substrates concentration, applied potential (Eapp) and pH solution. Therefore, an optimization of these variables was performed for biosensor in order to ensure the quality of the results.

Slopes of Al(III) inhibition calibration curves were performed at different pH and potentials. High linearity and signal stability was obtained at 0.5 V, at higher potential signal was too noise. Therefore slopes were obtained with pH at 0.5 V. Different cysteine concentrations were tested from 2.0×10^{-4} M to 2.5×10^{-3} M. The best slope value was obtained to pH 7.0; Eapp of 0.5 V and cysteine cell concentration of 5.94×10^{-4} M in cell; results showed in Fig. 2.

4.2. Kinetics and Gluthathione Peroxidase Inhibition

Michaelis Menten Km apparent values estimated by Lineweaver-Burk plot were obtained in presence and absence of Al(III) with GPer/AuNPs/SPCEs. Inhibitory aluminum effect was tried performing successive additions of cysteine after five minutes of

contact between GPer/AuNPs/SPCEs and Al(III) solution. Inhibitory aluminum effect was confirmed by increasing Kmapp value at Al(III) presence. Aluminum inhibition effect on gluthathion peroxidase enzyme looks like acompetitive mode in (Fig. 3). Lineweaver–Burk plot values GPer/AuNPs/SPCEs in presence and absence of Al(III) are showed in Table 1. Inhibitory effect of Al(III) on Gper and corresponding calibration curve are showed in Fig. 4.

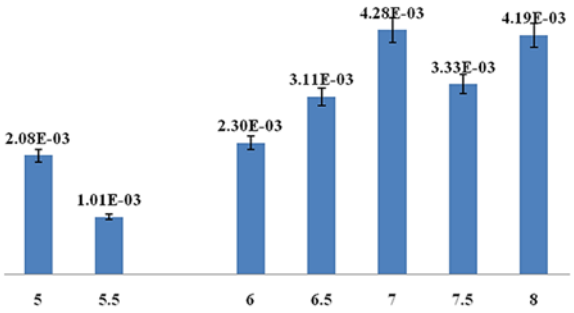

Fig. 2. Slopes of Al(III) inhibition calibration curves at 0.5 V vs. Ag/AgCl SPE with different pH values.

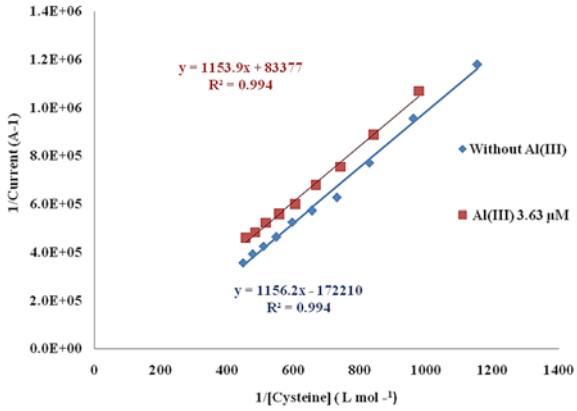

Fig. 3. Line weaver–Burk plot GPer/AuNPs/SPCEs in presence and absence of Al(III), Britton Robinson buffer pH 7.0, Eapp= +0.5 V *vs.* Ag/AgCl SPE.

Table 1. Kmapp and Line weaver–Burk plot values GPer/AuNPs/SPCEs in presence and absence of Al(III).

Al[III]	Km/Vm (Slope)	1/Vm (Intercept)	Km app, M
0	1156±31	$(1.7\pm0.2)\times10^{5}$	$(6.7\pm0.9)\times10^{-3}$
3.63 µM	1153.9±33	$(8.3\pm2)\times10^{-4}$	$(1.4\pm0.3)\times10^{-2}$

4.3. Optimization of Experimental Conditions for Cat/AuNPs/AuSPEs Biosensor

Three concentrations of catalase (15.0 mg/mL; 10.0 mg/mL and 5.0 mg/mL) were used in the mix and tested through their slopes in Al(III) inhibition calibration curves. Catalase concentration of 15 mg/mL show low adherence to electrode surface and AlIII) inhibition calibration curves were not performed.

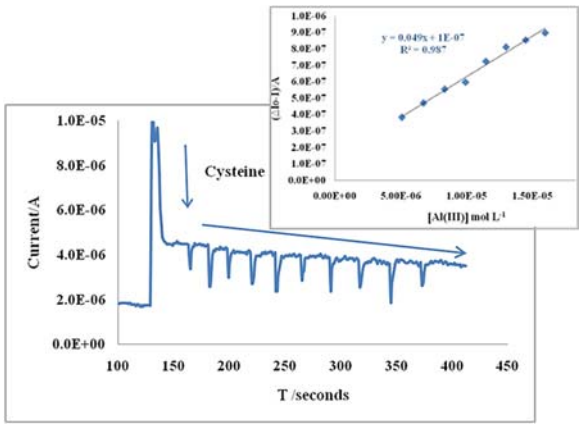

Fig. 4. Inhibitory aluminum effect on cysteine with Gper/AuNPs/SPCEs under optimized conditions, pH 7.0 Britton Robinson buffer, Eapp = 0.5 V *vs.* Ag/AgCl SPE. Upper-calibration curve corresponding to this cronoamperogram.

At pH 7.0 recommend for enzyme; applied potential was changed and slopes were obtained. H_2O_2 concentration was changed from 1.0×10^{4} M to 6×10^{-3} M. Selected conditions were pH 7.0 and 0.5 V and catalase concentration of 5.0 mg/mL in mix with H_2O_2 9.64 $\times10^{4}$ M in cell regarding signal stability. Selected conditions are showed in Fig. 5 for Cat/AuNPs/AuSPE.

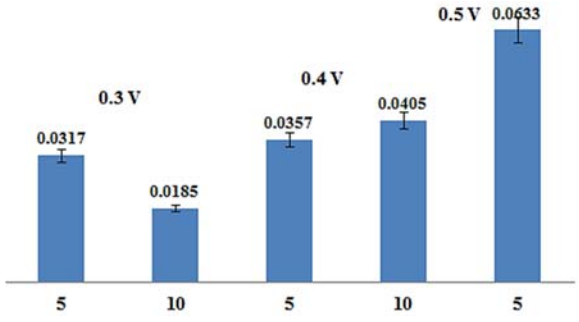

Fig. 5. Slopes of Al(III) inhibition calibration curves with E app and pH 7.0 with two enzyme concentration in the mix.

4.4. Kinetics and Catalase Inhibition

Michaelis Menten Km apparent values estimated by Lineweaver-Burk plot were obtained in presence and absence of Al(III) with AuNps/Cat/AuSPEs. Inhibitory aluminum effect was tried performing successive additions of H_2O_2 after five minutes of contact between AuNps/Cat/AuSPCs and Al(III) solution. Inhibitory aluminum effect was confirmed by increasing Kmapp value at Al(III) presence.

Aluminum inhibition effect on catalase enzyme looks like competitive mode. Lineweaver–Burk plot is showed in Fig. 6 and in Table 2 are showed corresponding plot values in presence and absence of Al(III).

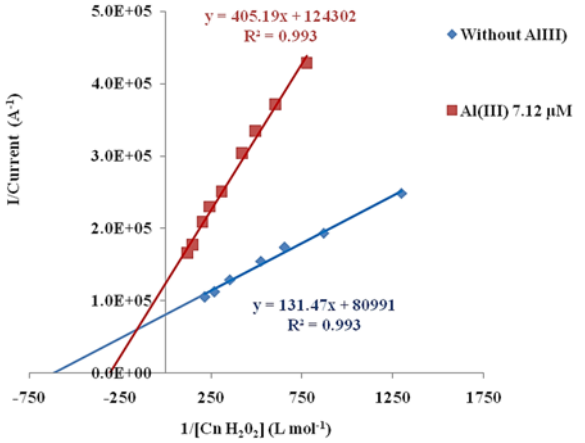

Fig. 6. Lineweaver–Burk plot Cat/AuNPsAuSPEs in presence and absence of Al(III), Britton Robinson buffer pH 7.0, Eapp= 0.5 V *vs*. Ag/AgCl.

Table 2. Kmaap and Line weaver–Burk plot values Cat/AuNPsAuSPs in presence and absence of Al(III).

Al(III)	Km/Vm (Slope)	1/Vm (Intercep)t	Km app, M
0	131±5	$(8.1\pm0.4)\times10^{4}$	$(1.63\pm0.09)\times10^{-3}$
7.12 µM	405±13	$(1.24\pm0.05)\times10^{5}$	$(3.3\pm0.2)\times10^{-3}$

Due Kmapp inhibition value of Cat/AuNps/AuSPCs was lower than GPer/AuNPs/SPCEs, and slope of its inhibition calibration curve was higher; the former biosensor was selected to determine validation parameters.

4.5. Aluminum Determination Procedure

Cat/AuNps /AuSPCs biosensor was collocated in the electrochemical cell containing 5.00 mL of Britton Robinson buffer pH 7.0. A potential of 0.5 V was applied, and once a stable current was set, an aliquot of H_2O_2 was added to cell. It was observed a large oxidation current due to H_2O_2 addition namely I_0. Once a plateau corresponding with steady state response was reached; aliquots of aluminum stock solution were added successively.

Presence of Al(III) ions produces catalase enzyme inhibition which causes a decrease in the H_2O_2 amperometric signal. Al(III) concentration influence in inhibition process, can be quantitatively evaluated determining the difference between the steady state current in absence of Al(III) (I_0) and the steady state current(I) in the presence of Al(III). Plotting $\Delta(I_0\text{-}I)$ *vs*. Al(III) concentration a linear regression is obtained The inhibition process and corresponding linear regression are shown in Fig. 7.

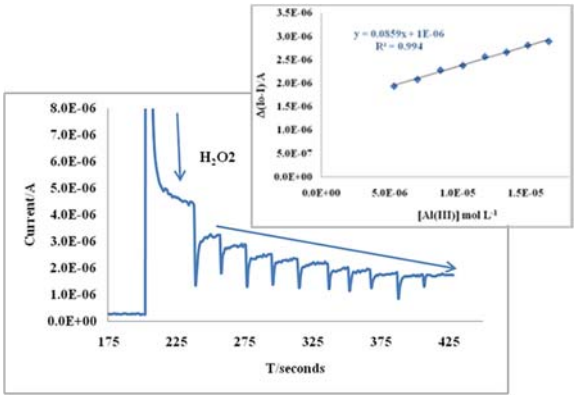

Fig. 7. Inhibitory aluminum effect on H_2O_2 with Cat/AuNPs/SPEs under optimized conditions, pH 7.0 Britton Robinson buffer, Eapp = 0.5 V *vs*. Ag/AgCl SPE. Upper-calibration curve corresponding to this cronoamperogram.

4.5.1. Validation of Cat/AuNps/AuSPE Biosensor

Cat/AuNps/AuSPE was selected to perform validation of the developed biosensor through its performance parameters.

Limit of detection (LOD) is the least amount of analyte that can be determinate with a level of certainty previously established. LOD is usually estimated from calibration curves. The limit of detection under the optimum working conditions (1.0 ± 0.1) µM was calculated from the standard deviation (Sy/x) of five Al(III) inhibition calibration curves accordingly with the criteria 3Sy/x, [38], its RSD was 7.8 %. Analogous to LOD, quantification limit (LOQ) was estimated under optimal conditions from the standard deviation of five Al(III) inhibition calibration curves using the criteria 10Sy/x, its value was (3.4± 0.3) µM, with a RSD of 7.8 %.

Precision is usually calculated in terms of reproducibility and repeatability. Repeatability was assessed using the same electrode surface. In this way, three successive calibrations for Al(III) were performed with Cat/AuNps/AuSPEs prepared under described experimental conditions. The electrodes were conditioned in a Britton Robinson buffer solution, pH 7.0, stirring for 5 min, between experiments. Repeatability slopes value had a RSD of 8.3 %. Likewise, the reproducibility of the amperometric signal was checked using the slopes of four regression lines carried out with different electrode surfaces. The RSD slopes value was 10.7 %.

These results suggest that the fabrication procedure of the Cat/AuNps/AuSPCs based biosensors is reliable and allows reproducible amperometric responses.

The accuracy of the developed method was tested by a recovery study in which two near known amounts of Al(III) standard reference material (SRM), SRM High Purity Standards solution (Lot Number 1121015, (1000 ±3) mg L^{-1}) was spiked to a buffer solution. Recovery concentration values are shown in Table 3.

Table 3. Recovery of Al(III) SRM (1000 ± 3) mg/L spiked to buffer solution, pH 7.0; Eapp = 0.5 V vs. Ag/AgCl SPE with Cat/AuNPs/AuSPEs.

Added SRM (M)	Found SRM (M)	SRM (mg/L)	Recovery %
9.569×10^{-6}	9.483×10^{-6}	990.0	99.0
	1.003×10^{-5}	1047.4	104.9
	1.054×10^{-5}	1100.3	110.0
	9.431×10^{-6}	984.5	98.5
Mean		**1030.6**	**103.1**
SD		**54.5**	**5.5**
RSD%		**5.3**	**5.3**
D.F. dilution factors 5.70 mL/0.200 mL and 200 mL/1.00 mL			
Added SRM (M)	**Found SRM(M)**	**SRM (mg/L)**	**Recovery %**
9.408×10^{-6}	9.443×10^{-6}	1002.8	100.3
	1.022×10^{-5}	1085.6	108.6
	1.042×10^{-5}	1106.9	110.7
	9.782×10^{-6}	1038.8	103.9
Mean		**1058.5**	**105.9**
SD		**46.7**	**4.7**
RSD%		**4.4**	**4.4**
D.F. dilution factors 5.70 mL/0.300 mL and 200 mL/1.00 mL			

The aluminum average concentrations quantified by the developed procedure, (1031 ± 55) mg/L (n =4; α = 0.05) and (1059 ± 47) mg/L (n =4; α = 0.05) match the certified value of the sample considering the associated uncertainty. Results of reproducibility of recovery show that developed Cat/AuNPs/AuSPEs biosensors can be applied to Al(III) determination in aqueous solutions.

4.5.2. Study of Interferences on Catalase/AuNps/AuSPE Biosensor

Interference study was performed comparing the percentage of inhibition of substrate current showed by Catalase/AuNps/AuSPE based biosensor in the presence of aluminum and other foreign ions. It was accomplished recording inhibition current for every metal and expressing it as percentage of substrate current corrected by electrode residual current. Three concentration were tested, namely1×10^{-3} M, 1×10^{-4} M and 1×10^{-6} M, in cell. As it can be seen in Fig. 8, the highest interference effect was found for Sn(II) and Ni(II) for 1×10^{-6} M, but these toxic ions should not be naturally present in water. Ca(II), Mg(II) usually found in water are low interferences at three tested concentrations and Fe(III) shows interference at 1×10^{-4} M and 1×10^{-3} M. At level of 10^{-4} M and 10^{-3} M, most interfering ions are Sn(II), Cd(II) and As(V). Al(III) showed inhibition on catalase enzyme at three tested concentrations as it can be seen in Fig. 8.

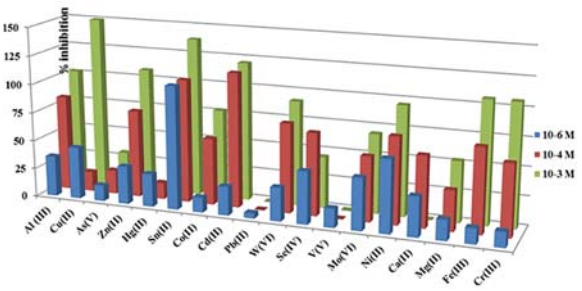

Fig. 8. Inhibition percentage of current at Catalase/AuNps/AuSPE, in presence of: Al(III), Cu(II), As(V), Zn(II), Hg(II), Sn(II), Co(II), Cd(II), Pb(II), W(VI), Se(IV), V(V), Mo(VI), Ni(II), Ca(II), Mg(II), Fe(III) and Cr(III) at three concentration levels; Britton Robinson buffer pH 7.0, Eapp = +0.5 V *vs.* Ag/AgCl SPE.

5. Conclusions

Two procedures used to immobilize catalase and glutathione peroxidase on surface screen printed electrodes were developed, optimized and characterized.

Km app inhibition constants value and slope of aluminum inhibition calibration curve were used as criteria to select developed biosensors. Due aluminum inhibition constants estimated with catalase and H_2O_2 showed lower values; catalase enzyme had a higher affinity for H_2O_2 substrate than glutathione peroxidase for cysteine. According to Lineweaver plots Al[III] inhibition on gluthathione looks like acompetitive for GPer/AuNPs/SPCEs. Likewise its inhibition on Cat/AuNPs/AuSPEs resembles competitive inhibition.

Aluminum exerts its inhibition effect on these enzymes at very low concentrations and it was estimated first time through their Kmapp determinations.

Electrodeposition is a fast and clean method to deposit AuNps on SPCEs and AuSPEs increasing conductivity and loading of enzyme.

Validated biosensor shows good repeatability and reproducibility and very good accuracy. LOD value obtained could be applied to determination of low aluminum concentration in aqueous matrix

Although metals Sn(II) and Ni(II) for 1×10^{-6} M and Fe(III) and Cr(III) shows interference 1×10^{-3} M, if they are present in aqueous samples, there are methods to remove them.

Development of biosensors using Cat and GPer based in quantitative aluminum inhibition confirm its inhibitory role on enzymes scavengers of free radicals.

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