COMPARATIVE STUDY OF DIFFERENT ALCOHOL SENSORS BASED ON SCREEN-PRINTED CARBON ELECTRODES

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Abstract

Different very simple single-use alcohol enzyme sensors were developed using Alcohol Oxidase (AOX) from three different yeast, Hansenula sp., Pichia pastoris and Candida boidinii, and employing three different commercial mediator-based Screen-Printed Carbon Electrodes as transducers. The mediators tested, Prussian Blue, Ferrocyanide and Co-Phthalocyanine were included into the ink of the working electrode. The procedure to obtain these sensors consists of the immobilization of the enzyme on the electrode surface by adsorption. For the immobilization, an AOX solution is deposited on the working electrode and left until dried (1 h) at room temperature. The best results were obtained with the biosensor using Screen-Printed Co-Phthalocyanine/Carbon Electrode and AOX from Hansenula sp. The reduced Cobalt-

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AOX: Alcohol Oxidase
SPPBCEs: Screen-Printed Prussian Blue/Carbon Electrodes
SPFCEs: Screen-Printed Ferrocyanide/Carbon Electrodes
SPCPCEs: Screen-Printed Co-Phthalocyanine/Carbon Electrodes

phthalocyanine form is amperometrically detected at + 0.4 V (vs. Ag pseudo reference electrode). This sensor shows good sensitivity (1211 nA mM^{-1}), high precision (2.1 % RSD value for the slope value of the calibration plot) and wide linear response (0.05-1.00 mM) for ethanol determination. The sensor provides also accurate results for ethanol quantification in alcoholic drinks.

Keywords: Ferrocyanide, Prussian Blue, Co-phthalocyanine, Screen-Printed Carbon Electrode, Alcohol oxidase.

Introduction

The determination of ethanol is of great importance in food industry, medicine and biotechnology because of its toxicological and psychological effects [1]. The food, beverage and pulp industries need fast, simple and economic analytical methods to control fermentation processes and product quality [2]. Several methods and strategies have been reported for the determination of ethanol, e.g. gas chromatography, liquid chromatography, refractometry and spectrophotometry, among other [2].

The use of enzymes for the detection and quantification of ethanol in complex samples offers a better specificity and therefore, a simpler sample treatment. Alcohol oxidase (AOX) [3,4], NAD^+-dependent alcohol dehydrogenase (ADH) [1,5,6] and PQQ-dependent alcohol dehydrogenases [7,8] have been used as bioselective elements in ethanol biosensors. In this work, AOX produced by three methylotrophic yeasts [9], *Hansenula sp, Pichia pastoris*, *Candida boidinii*, have been used.

Alcohol oxidase oxidizes low molecular weight alcohols to the corresponding aldehyde, using molecular oxygen (O_2) as the electron acceptor, according to the following reaction:

$$RCH_2OH + O_2 \xrightarrow{AOX} RCHO + H_2O_2$$

The oxidation of alcohol by AOX is irreversible due to the strong oxidising character of O_2 and can be monitored by measuring either the decrease in O_2 concentration or the increase in H_2O_2 concentration [9].

The electrochemical determination of ethanol is based on the oxidation or reduction of H_2O_2 generated by the enzyme-catalyzed reaction. In order to shuttle electrons involved in the electrochemical oxidation or reduction of H_2O_2 at low potential values, the use of mediators such as Meldola blue [10-12], ferrocene [13-15], ferrocyanide [10,16,17], Prussian Blue [18,19] or Co-phthalocyanine [20,21], is a well-known strategy. There are several ways to incorporate a mediator in an enzymatic biosensor e.g., by a membrane [22,23], into a Nafion gel [24], by cross-linking [25], by electrodeposition [26] or inclusion in the working electrode [16,27].

Screen-printing is a well-established technique to fabricate electrochemical biosensors because of inherent advantages such as miniaturisation, versatility, low cost and the possibility of mass production [28]. All these advantages make these devices interesting tools in biosensors design [28]. Moreover, Screen-Printed Carbon Electrodes (SPCEs) with mediators incorporated in the carbon ink are commercially available.

In this paper, three commercial SPCEs modified with different mediators included in the carbon ink, Prussian Blue, Ferrocyanide and Co-phthalocyanine, and AOX from three different sources, Hansenula sp., Pichia pastorisis and Candida boidinii, were evaluated and compared in order to design simple, disposable and reliable alcohol sensors.

2. Experimental

2.1 Chemicals

Alcohol Oxidase from Hansenula polymorpha (AOX; ref. A0438), Alcohol Oxidase solution from Pichia pastoris (AOX; ref. A2404), Alcohol Oxidase from Candida boidinii (AOX; ref. A6941), Horseradish Peroxidase, Type VI-A (HRP; ref. 6782), ascorbic acid (ref. A5960), gallic acid (ref. G7384) and L-cysteine (ref. W326305) were purchased from SIGMA (Spain). Ethanol absolute, methanol, ortho-Phosphoric acid 85 % and Sodium hydroxide (pellets) were delivered by MERCK (Spain). All chemicals were of analytical reagent grade. The Milli-Q water used was obtained from a Millipore Direct-Q^{TM} 5 purification system. Stock solutions of ethanol, AOX and AOX/HRP were prepared daily in 0.1 M phosphate buffer of suitable pH and stored at 4ºC in refrigerator. Britton-Robinson buffer solutions of pH 3 and 9, and phosphate buffer 0.1 M solutions of pH values between 4 and 8 were used for pH studies.

2.2 Apparatus and measurements

Chronoamperometric measurements were carried out using an ECO Chemie μAutolab type II potentiostat interfaced with a Pentium 166 computer system and controlled by the Autolab GPES software version 4.8 for Windows 98. All measurements were performed at room temperature. Screen-Printed Prussian Blue/Carbon Electrodes (SPPBCEs; DRP-710), Screen-Printed Ferrocyanide/Carbon Electrodes (SPFCEs; ref DRP-F10), Screen-Printed Co-Phthalocyanine/Carbon Electrodes (SPCPCEs; ref DRP-410), and an edge connector (ref. DRP-DSC) were purchased from DropSens, S.L. (Oviedo, Spain). These devices consist of a working electrode (4mm diameter), a carbon auxiliary electrode and a silver pseudo-reference

electrode printed on an alumina substrate. The working electrode is made of Prussian Blue/Carbon, Ferrocyanide/Carbon or Co-Phthalocyanine/Carbon in each case. An insulating layer delimits the electrochemical cell and the electric contacts.

For comparison purposes, real samples were also analyzed by gas chromatography with HP6890 chromatograph composed of an injector, a 2 m long packed column and a flame ionization detector (FID).

2.3 Electrode modification with enzymes

The used procedure is the same in all cases. After a first washing step with Milli-Q water, 10 μL of a mixture of AOX/HRP solution was deposited on the SPCE and left to dry 1 h onto the electrode. The mixture of enzymes was prepared in phosphate buffer 0.1 M pH 6 in an adequate concentration. For the Screen-Printed Co-Phthalocyanine/Carbon Electrodes HRP was not included in the mixture.

2.4 Analytical signal recording

A 40 μL aliquot of the ethanol solution was deposited on the sensor and chronoamperometry by applying a potential of -0.1 V during 100 s for SPPBCEs, -0.1 V during 170 s for SPFCEs and +0.4 V during 170 s for SPCPCEs was employed to record the analytical signals. A different sensor was used for each measurement.

2.5 Interferences measurement

Methanol, gallic acid, cysteine and ascorbic acid were checked as potential interferences for the amperometric response of the biosensor. For all of them, an adequate dilution in phosphate buffer 0.1 M pH 6 was the only sample treatment needed. 40 μL of each solution were dropped on different sensors and the

chronoamperogram was recorded using the experimental conditions mentioned in Section 2.4.

2.6 Real samples measurement

The developed SPCPCEs, biosensor was used to analyze different alcoholic beverages (Rioja wine, hazelnut liqueur and tequila). In all cases, a 1:10000 dilution in phosphate buffer 0.1 M pH 6 was the only sample treatment needed. Then, the chronoamperogram was recorded upon deposition of 40 μL of each sample solution on the sensor surface using the experimental conditions mentioned in Section 2.4.

2.7 Gas Chromatographic measurements

In order to compare the results obtained with the enzymatic sensor, the alcoholic beverages were also analyzed by GC using an internal standard method. A calibration plot for ethanol in concentrations ranging between 0 and 10 % (v/v) was constructed using 5 % of propanol as internal standard. The samples were previously diluted to obtain an adequate ethanol concentration.

3. Results and Discussion

In the case of the SPPBCEs and SPFCEs, the enzymatic reaction is monitored by the electrochemical reduction of the $Fe(CN)_6^{3-}$ (Figure 1.A) generated in the enzyme reaction with HRP, while in the case of the SPCPCEs, the reaction is monitored by the electrochemical oxidation of Co^{2+} (Figure 1.B).

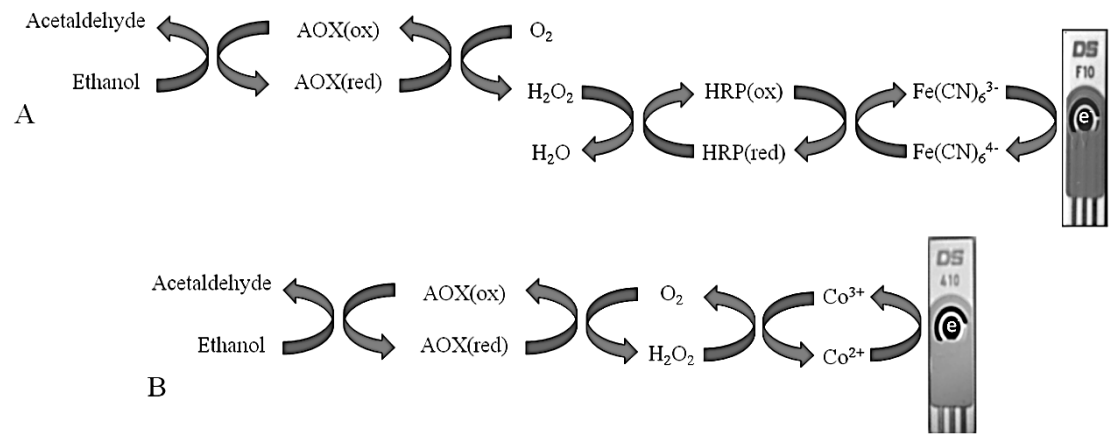

Figure 1. Enzymatic reactions at the surface of SPPBCEs and SPFCEs (A) and SPCPCEs (B).

3.1 Comparison of enzymes using the different Screen-Printed Electrodes

Following the methodology described in Section 2.3, different SPFCEs and SPPBCEs biosensors were prepared with 0.05 U/μL of each AOX and HRP. Regarding SPCPCEs, the same methodology was used but without HRP. The three enzymes sources, Hansenula sp., Pichia pastoris and Candida Boidinii, were tested in all cases.

Calibration plots for ethanol were constructed with each enzymatic sensor. Figure 2 shows the comparison of these calibrations using the three AOX enzymes as recognition element in SPPBCEs (Figure 2.A), SPFCEs (Figure 2.B) and SPCPCEs (Figure 2.C). The analytical parameters for the calibration plots are summarized in **Table 1**.

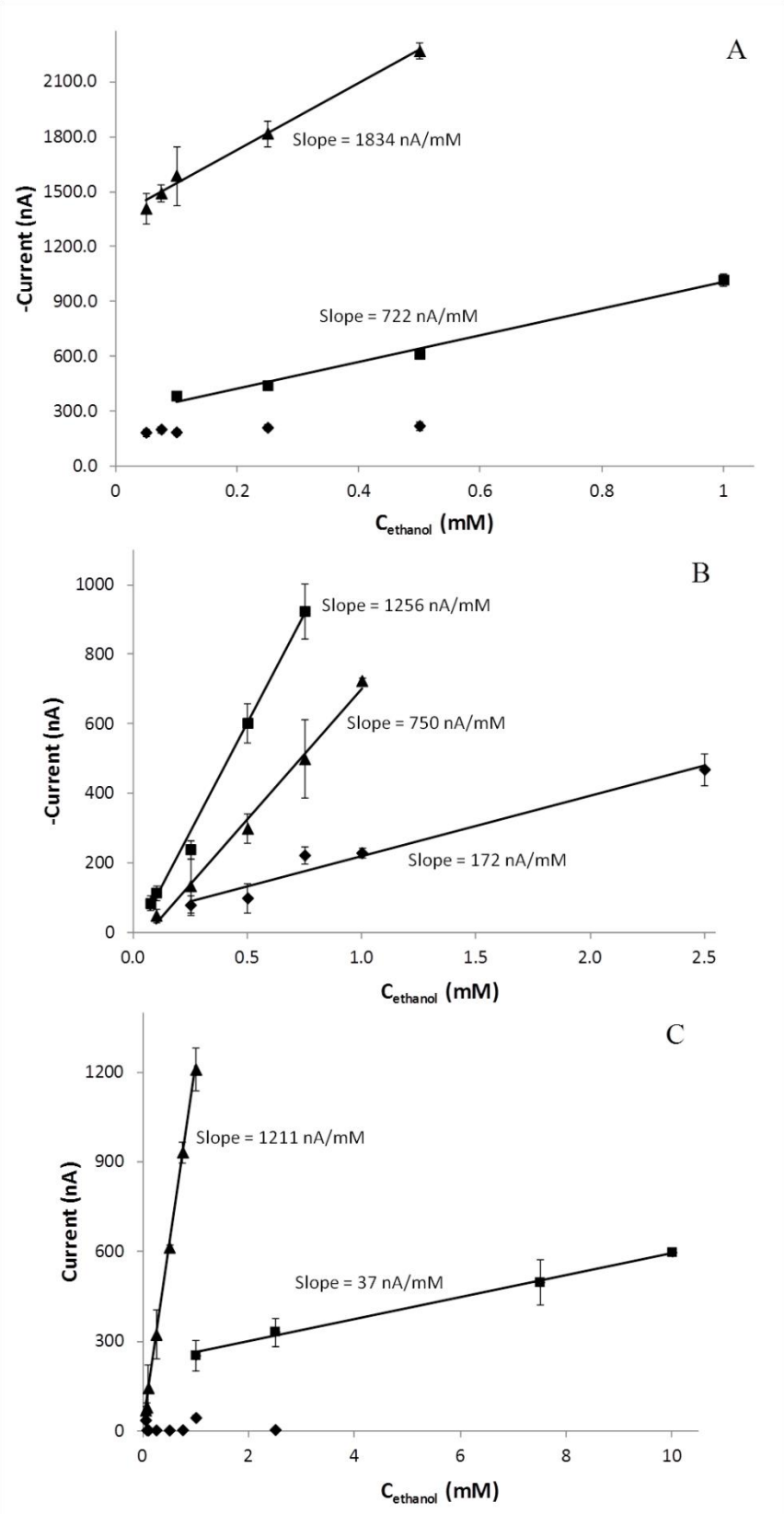

Figure 2. Calibration plots for ethanol obtained with SPPBCEs (A), SPFCEs (B) and SPCPCEs (C)-based biosensors. AOX from Hansenula (▲), Pichia Pastoris (■) and Candida Boidinii (♦). Ethanol diluted in phosphate buffer 0.1 M pH 6. Data are given as average ±SD (n=3).

Although the highest slope value was obtained with SPPBCEs and AOX from Hansenula, the blank responses were very high and affected seriously to the reproducibility of the slope values obtained with different biosensors. However, the slope values found in the case of SPFCEs and AOX from Pichia Pastoris were lower but more reproducible. Nevertheless, the SPCPCEs modified with AOX from Hansenula showed the best results with a good correlation coefficient and a wide linear range. Moreover, in this case, HRP is not necessary to achieve an adequate performance of the sensor with the subsequent lower cost. The reason why different sources of enzymes gave rise to so different results is not clear at present.

Table 1. Characteristics of the calibration plots for ethanol obtained with SPPBCEs, SPFCEs and SPCPCEs-based biosensors.using AOX from Hansenula, Pichia Pastoris and Candida Boidinii.

		Equation	R^2	Linear Range (mM)
SPPBCEs	Hansenula	-i (nA) = 1834 $C_{ethanol}$ (mM) + 1359	0.992	0.05 – 0.50
	Pichia Pastoris	-i (nA) = 722 $C_{ethanol}$ (mM) + 282	0.990	0.1 – 1.0
	Candida Boidinii	——	—	—
SPFCEs	Hansenula	-i (nA) = 750 $C_{ethanol}$ (mM) - 48	0.993	0.1 – 1.0
	Pichia Pastoris	-i (nA) = 1256 $C_{ethanol}$ (mM) - 27	0.994	0.075 – 0.750
	Candida Boidinii	-i (nA) = 172 $C_{ethanol}$ (mM) + 48	0.96	0.25 – 2.50
SPCPCEs	Hansenula	i (nA) = 1211 $C_{ethanol}$ (mM) + 11	0.9991	0.05 – 1.00
	Pichia Pastoris	i (nA) = 37 $C_{ethanol}$ (mM) + 226	0.995	1 – 10
	Candida Boidinii	——	—	—

3.2 Optimization of parameters for the biosensor design

3.2.1 Enzymes concentration

In order to evaluate this experimental variable, different biosensors were prepared by depositing, on SPCPEs, solutions of AOX from Hansenula with different concentrations. For each concentration, chronoamperograms for 1 mM ethanol solution, prepared in phosphate buffer 0.1 M pH 6, were recorded. The obtained results are summarized in Figure 3. The biosensor response increased with the AOX concentration until a plateau was reached at 0.15 U/µL, value that was chosen for further studies.

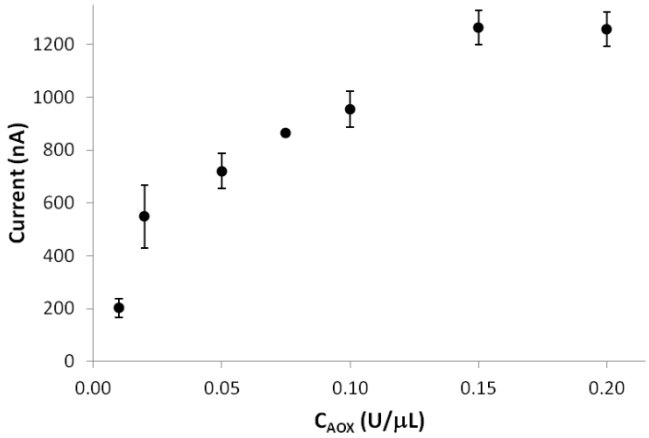

Figure 3. Effect of the enzyme concentration on the responses for 1 mM ethanol of different biosensors prepared with SPCPEs and AOX from Hansenula. Data are given as average ±SD (n=3).

3.2.2 Effect of pH

The pH effect on the analytical signal was checked by recording chronoamperograms upon deposition of 40 µL of 1 mM ethanol solution and by applying a potential of +0.4 V during 170 s. The ethanol stock solution was diluted using buffers with pH values between 3 and 9 and the results are displayed in Figure 4.

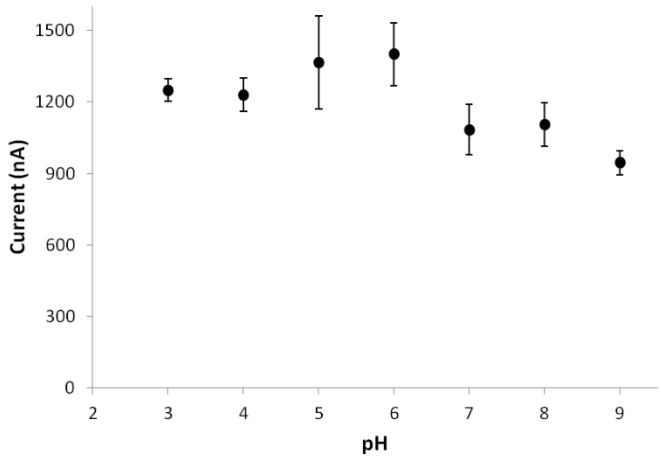

Figure 4. Effect of the pH value on the response of the biosensor constructed with SPCPEs and AOX from Hansenula (0.15 U μL^{-1}). Ethanol concentration, 1 mM. Data are given as average ±SD (n=3).

As it can be seen, the higher responses were obtained at pH values between 5 and 6. However, considering the better reproducibility achieved at pH 6, this pH value was chosen for further studies.

3.3. Analytical characteristics of the biosensor

Chronoamperometry at +0.4 V during 170 s allowed a calibration plot for ethanol to be obtained with the equation, i (nA) = 1211 $C_{ethanol}$ (mM) + 11, and the linear range, 0.05 mM – 1.00 mM, given in **Table 1**. The detection limit (LOD) was calculated according to the $3s_b/m$ criteria, where m is the slope of the linear range of the corresponding calibration plot, and s_b was estimated as the standard deviation of the intercept. The LOD value thus obtained was 0.02 mM. It is interesting to remark that this simple biosensor design show sensitivity, detection limit and range of linearity values comparable, or in some cases better, than those for other alcohol sensors developed in the last years which need the use of polymers, membranes or cross-linkers

(**Table 2**). Moreover, the time of fabrication of this sensor is one of the shortest, in spite of some of the fabrication time estimated are shorter than they really are because these fabrication times were estimated adding the times indicated in the articles for some of the manufacturing stages for each sensor.

Table 2. Analytical characteristics of some alcohol sensors.

Analytical performance		Sensitivity	Detection limit	Linear range	K_M	Reproducibility (RSD %)	Stability	Fabrication time	Reference
AOX immobilization by adsorption on SPCPCEs		1211 nA mM^{-1}	0.02 mM	(0.05 – 1.00) mM	2.4 mM	2.1 %	No loss after 2 months	≈ 1 h	Present work
AOX and bovine liver catalase immobilization in a photoreticulated poly(vinyl alcohol) membrane at surface of interdigitated microelectrodes		363 µS mM-1	0.001 mM	Up to 0.07 mM	——	1.5 – 4 %	5 % signal decrease after 4 months	≈ 50 min	[29]
Conducting polymers to AOX immobilization	Polypyrrole	21.4 nA mM^{-1} cm^{-2}	170 mM	——	12300 mM	——	50 % activity lost after 28 days	——	[30]
	Poly(3,4-ethylenedioxythiophene)	21.4 nA mM^{-1} cm^{-2}	170 mM	——	7800 mM	——	20 % activity lost after 28 days		
	Poly(3,4-ethylenedioxypyrrole)	22.2 nA mM^{-1} cm^{-2}	170 mM	——	6000 mM	——	23 % activity lost after 28 days		
AOX inmobilization by electrochemical polymerization in poly(3,4-ethylenedioxythiphene) on platinum printed electrodes		——	0.3 mM	(0.3 – 20) mM	——	4.2 %	No loss after 2 months	≈ 15 min	[31]
AOX immobilization by cross-linking with glutaraldehyde using bovine serum albumin and poly(neutral red) as redox mediator		171.8 nA mM^{-1}	0.03 mM	Up to 0.7 mM	1.96 mM	8.6 %	12 % of sensitivity lost after 6 weeks	≈ 1 h 10 min	[32]
AOX/chitosan immobilization by eggshell membrane		——	0.03 mM	(0.06 – 0.8) mM	3.89 mM	3.4 %	13.4 % of sensitivity lost after 3 months	≈ 6 h 40 min	[33]
AOX immobilized by glutaraldehyde co-crosslinking with bovine serum albumin on a electropolymerized film modified gold electrode		4100 nA mM^{-1} cm^{-2}	0.0023 mM	Up to 0.75 mM	5.30 mM	——	——	≈ 1 h 20 min	[34]
Os complex-modified redox hydrogel to crosslinking peroxidase and electrodeposition paint entrapping the AOX	Royal palm tree peroxidase	98000 nA mM^{-1}	0.02 mM	——	9.5 mM	——	——	≈ 20 h	[35]
	HRP	60000 nA mM^{-1}	0.02 mM	——	9.6 mM	——			
Graphite-Teflon-alcohol oxidase-HRP-ferrocene electrode		551 nA mM^{-1}	0.0053 mM	(0.02 – 2) mM	0.0138 s^{-1}	6.5 %	No loss of enzymes activity after 3 months	≈ 3 h 25 min	[13]
Alcohol dehydrogenase with 4-ferrocenylphenol and 2,4,7-trinitro-9-fluorenone electrochemically polymerised on Screen-Printed		——	——	(0 – 1) mM	1.05 mM	——	——	≈ 34 min	[8]
Poly(carbamoyl)sulfonate hydrogel to AOX immobilization on Screen-Printed	Hansenula sp.	30.2 nA mM^{-1}	——	(0.01 – 3.0) mM	——	——	40 – 50 % signal decrease after 18 days	≈ 22 h	[2]
	P. pastoris	10.6 nA mM^{-1}	——	(0.02 – 3.75) mM	——	——			
	C. boidinii	6.2 nA mM^{-1}	——	(0.04 – 3.75) mM	——	——			
Enzymes immobilized by entrapment in a photocrosslinkable polyvinyl alcohol containing stilbazolium groups on Screen-Printed	Meldola blue and ADH	5.282 nA mM^{-1}	0.1 mM	(0.3 – 8) mM	7 mM	47 %	1 month	≈ 4 h	[36]
	Co-phthalocyanine and AOX	1.01 nA mM^{-1}	10 mM	(15 – 110) mM	80 mM	41 %			
Quinohemoprotein ADH (QH-ADH), Poly(ethylene glycol) diglycidyl ether (PEGDGE) and Os-complexed poly(1-vinylimidazole) redox polymer ($PVI_{13}dmeOs$)	QH-ADH/PEGDGE/graphite	1570 nA mM^{-1} cm^{-2}	0.01 mM	(0.01 – 0.2) mM	0.45 mM	——	——	≈ 20 h	[37]
	QH-ADH/$PVI_{13}dmeOs$/ PEGDGE/graphite	7280 nA mM^{-1} cm^{-2}	0.001 mM	(0.005 – 1) mM	1.27 mM	——			
	QH-ADH/PEGDGE/ Screen-Printed	50000 nA mM^{-1} cm^{-2}	0.005 mM	(0.005 – 0.1) mM	62 mM	——			
	QH-ADH/$PVI_{13}dmeOs$/ PEGDGE/Screen-Printed	336000 nA mM^{-1} cm^{-2}	0.001 mM	(0.001 – 0.25) mM	183 mM	——			
PQQ-dependent alcohol dehydrogenase	Carbon Electrodes	4630 nA mM^{-1} cm^{-2}	0.0018 mM	——	4.75 mM	——	——	≈ 20 h	[38]
	Graphite Electrodes	6830 nA mM^{-1} cm^{-2}	0.005 mM	——	0.71 mM	——	——		
	Screen-Printed Electrodes	179000 nA mM^{-1} cm^{-2}	0.001 mM	——	1.19 mM	——	10 % signal decrease after 5 days. Stable for the next 6 days		

Furthermore, the sensor showed a Michaelis-Menten kinetics behaviour. The apparent Michaelis-Menten constant (K_M) was calculated using the Lineweaver-Burk linearization, and the value obtained was 2.4 ± 0.7 mM. This K_M value resulted to be lower than those calculated with other enzyme sensors using polymers, membranes, or cross-linkers, as well as with many sensors based on Screen-Printed Electrodes (see **Table 2**) indicating a high bioaffinity to ethanol with the developed design as a consequence of the simplicity of the AOX immobilization strategy.

Table 3. Calibration plot equations for five different alcohol sensors. (n = 7 in all calibration plots). Each point was measured three times.

	Equation	R^2
Calibration plot 1	i (nA) = 1211 $C_{ethanol}$ (mM) + 11	0.9991
Calibration plot 2	i (nA) = 1180 $C_{ethanol}$ (mM) + 40	0.993
Calibration plot 3	i (nA) = 1210 $C_{ethanol}$ (mM) - 11	0.990
Calibration plot 4	i (nA) = 1242 $C_{ethanol}$ (mM) + 2	0.9997
Calibration plot 5	i (nA) = 1182 $C_{ethanol}$ (mM) + 30	0.998
Mean slope	(1205 ± 26) nA/mM	

In order to evaluate the reproducibility of the sensors, several sensors were prepared in different days to carry out four different calibration plots for ethanol. Each sensor was used for only one measurement (single-use). Each calibration plot was constructed using ethanol solutions prepared the same day of the measurement. **Table 3** shows the equations calculated for each calibration plot and the reproducibility was estimated in terms of the RSD value calculated from the corresponding slope values. The biosensor exhibited an excellent reproducibility with a mean slope value of (1205 ± 26) nA mM^{-1} and a RSD of 2.1 % (n=5). The achieved reproducibility can be advantageously compared with other alcohol sensors previously reported involving

many more steps in their fabrication (**Table 2**). This low RSD value is very important taking into account that the biosensor construction relies on the use of a commercial Screen-Printed Electrode and is a single-use sensor. Therefore, the high reproducibility achieved allows ethanol determination to be carried out with a very simple and rapid procedure (just one standard and the sample).

3.4. Specificity of the sensor

The specificity of the sensor was checked under the experimental conditions explained in the section 2.4. The potential interfering agents tested were methanol, ascorbic acid, gallic acid and cysteine. The concentration of these interferents in red wine is: methanol 38-200 mg/L [13,33], ascorbic acid 20 mg/L [39,40], polyphenols like gallic acid 2000 mg/L [41,42] and aminoacids like cysteine 2000 mg/L [43]. Agree with this, to evaluate those interferences, solutions of 5 x 10^{-4} M of ethanol, 10^{-6} M of methanol, 10^{-7} M of acid ascorbic, 10^{-5} M of gallic acid and 10^{-5} M of cysteine were prepared. 40 μL of those solutions were dropped on the sensor and chronoamperograms were recorded as explained in the section 2.4. The different results obtained are resumed in the **Table 4**. The present sensor shows a good specificity for the ethanol. The signals recorded for the methanol, ascorbic acid, gallic acid and cysteine measurements are equal as the recorded for the background.

Table 4. Study of the interferences caused by methanol, ascorbic acid, gallic acid and cysteine. $C_{ethanol} = 5 \cdot 10^{-4}$ M, $C_{methanol} = 10^{-6}$ M, $C_{ascorbic\ acid} = 10^{-7}$ M, $C_{gallic\ acid} = 10^{-5}$ M and $C_{cysteine} = 10^{-5}$ M. Each point was measured three times. Data are given as average ± SD (n=3).

Background	Ethanol	Methanol	Ascorbic acid	Gallic acid	Cysteine
(155 ± 7) nA	(805 ± 42) nA	(135 ± 11) nA	(175 ± 7) nA	(185 ± 9) nA	(125 ± 8) nA

3.5. Storage ability

In order to evaluate the storage stability of the ethanol biosensor, two sets of sensors were prepared, and light protected stored at -20ºC during 30 days and 60 days respectively. In the calibration plot of **Figure 5** each concentration was measured using 9 sensors: 3 sensors prepared and used in the same day, 3 sensors stored 30 days at -20ºC and 3 sensors stored 60 days at – 20ºC. The slope and the correlation coefficient obtained was 1219 ± 160 nA mM^{-1}, and 0.997 respectively. These data are better than those reported for other sensors that need the use of polymers, membranes or cross-linkers (**Table 2**), including the alcohol sensor based on Screen-Printed Electrodes modified with alcohol dehydrogenase by physical adsorption [38] where a sensitivity loss of 10% after 5 days is observed.

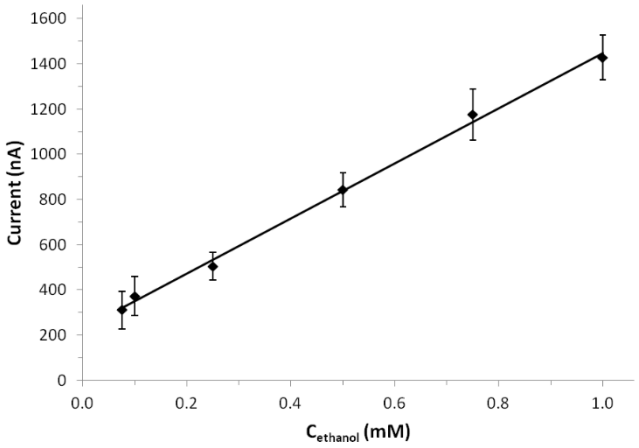

Figure 5. Calibration plot using 9 sensors for each concentration: 3 prepared and used the same day, 3 stored 30 days at -20ºC and 3 stored 60 days at -20ºC.

3.6. Application to real samples

Different alcoholic drinks were analyzed using the developed alcohol sensor. The samples were treated as described in the Section 2.6 and the analytical signal was

recorded according to Section 2.4. The samples were also analyzed by GC to compare the results, which are summarized in **Table 5**.

Table 5. Determination of ethanol in real samples with the developed biosensor and by applying GC. Data are given as average ±SD (n=3).

	$C_{ethanol}$ labeled (%)	GC			Sensor of this work		
		$C_{ethanol}$ (%)	SD	RSD (%)	$C_{ethanol}$ (%)	SD	RSD (%)
Rioja wine	12.5	13.9	0.7	4.7	12.2	0.4	2.8
Hazelnut liqueur	20	20.2	0.5	2.4	20.1	0.8	3.9
Tequila	38	38	1	2.8	38.0	0.7	1.8

The application of the Student's *t*-test demonstrated that there were no significant differences between the values labeled, those obtained with the biosensor and those obtained with GC, at a 0.05 significance level, thus demonstrating the good accuracy and precision achieved.

4. Conclusions

From all assayed alcohol sensors, the better results obtained was with the sensor developed using a commercial Screen-Printed Carbon Electrode containing Co-phthalocyanine as redox mediator into the working electrode. This sensor does not need a pretreatment step to be used as transducer in this sensor. Moreover, the sensor fabrication was extremely simple consisting on the immobilization by adsorption on the SPCPCE of only one enzyme, AOX from Hansenula. Therefore, the use of other reagents such as cross-linkers or polymers or the need for covalent bindings, are avoided. The developed biosensor shows high sensitivity (1211 nA mM^{-1}), low detection limit (0.02 mM), high reproducibility (2.1 %) and a wide linear response

(0.05-1.00 mM), characteristics that can be advantageously compared with others alcohol sensors previously reported. Furthermore, this enzymatic alcohol sensor is able to determine ethanol in alcoholic drinks with just a dilution with Mili-Q water as sample treatment.

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