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Ethanol Content in Must Grape by Alcohol Dehydrogenase Biosensors Based on Doped - Polyaniline Modified Screen Printed Electrodes

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The presence of ethanol in wine grapes is an important parameter for the evaluation of their quality in winemaking. In this study we report the development of an amperometric biosensor for the determination of ethanol in must grape based on the alcohol dehydrogenase enzyme immobilized on polyaniline doped with poly(2-acrylamido-2-methyl-1-propane) sulfonic acid polymer. The doped polymer was able to detect β-nicotinamide-adenine dinucleotide, reduced at low potential with a linear range from 0.005-2 mM and a sensitivity of 653.15 μA M^{-1}. Under optimized conditions, the alcohol dehydrogenase biosensor showed a limit of detection of 0.001 %vol ethanol and a sensitivity of 3551 nA %vol^{-1}. Finally, the ethanol biosensor was used for ethanol determination in must from red and white wine grapes and matrix effect was evaluated.

Introduction

The control of specific parameters during food manufacturing process is a very important task to ensure high quality and safety of final food products. Ethanol is one of the most important analyte associated to the state of the health of the grapes; its presence reveals the starting of premature alcoholic fermentations by indigenous yeasts that can interfere during winemaking process. Therefore, an accurate and fast measurement of ethanol concentration during delivery of grapes is highly needed in the wine industry. The use of biosensors as an analytical method to determine the presence of ethanol represents an attractive alternative for the food industry because of their high sensitivity, selectiveness, ease and rapidity of use (Siontorou 2014; dos Santos et al., 2013).
Previous studies (Tsai et al., 2007)) reported the development of screen-printed amperometric ethanol biosensors using alcohol dehydrogenase (ADH) and β-nicotinamide-adenine dinucleotide (NAD^+) cofactor. The enzymatic reaction produces acetaldehyde and β-nicotinamide-adenine dinucleotide reduced (NADH). The direct electrochemical oxidation of NADH at bare electrodes only proceeds with high overpotentials at 1 V vs. silver/silver chloride reference electrode (Ag/AgCl) and leads to fouling of the electrode surface. In fact, the overpotential higher than 0.8 V vs. Ag/AgCl leads to the oxidation of other electroactive species present in the food samples that interfere with the accurate determination of ethanol (Bartlett et al., 2002). To avoid this problem, redox mediators (Jiang et al., 2009) and conducting polymers (Prieto-Simòn et al., 2004) have been employed for NADH oxidation at lower potentials. In particular, over the last few years, special attention has been given to the use of polyaniline (PANI) conducting polymer for the production of sensor devices (Mano et al., 2007). The problem, which must be overcome in using PANI for the development of biosensors, is to ensure that the polymer preserves the electrical conductivity at neutral pH. In fact, the conducting behaviour emerladine form of PANI exists in acidic media (pH 2.5-3), which are not favorable for the activity of

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dehydrogenase enzymes and the stability of NADH cofactor. Bartlett et al. (2002) showed that emeraldine can be shifted to neutral or basic pH, by incorporation into the polymer of some counter anions, such as poly(vinylsulfonate) or poly(acrylate). So, conducting polymeric composites have been fabricated by doping PANI with poly(2-acrylamido-2-methyl-1-propane sulfonic acid) (PAAMPSA) (Albanese et al., 2014), poly(acrylic acid) (Hu et al., 2013), poly(styrene sulfonate) (PSS) (Mazeiko et al., 2013) and poly(vinylsulfonate) (PVS) (Prakash et al., 2013) showing an increase of the the electrical, electrochemical and optical properties of the sensors developed.
The aim of this study was to develop a cheap, stable and fast amperometric ethanol biosensor based on ADH immobilized on a conducting polymer modified electrodes, for the detection of low ethanol concentration in grape musts.

1. Experimental Section

1.1 Reagents

Aniline (C_6H_7N), PAAMPSA, (Mw = 2000kDa), hydrochloric acid (HCl), β-nicotinamide–adenine dinucleotide hydrate (NAD^+, >99 %), β-nicotinamide-adenine dinucleotide, reduced dipotassium salt (NADH, >95 %), Ethanol (99,8 %), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H2O$), potassium chloride (KCl), Nafion®, and alcohol dehydrogenase (ADH-EC 1.1.1.1, 285 $U \cdot mg^{-1}$) have been purchased from Sigma Aldrich (St. Louis, MO, USA).

1.2 Synthesis of PANI – PAAMPSA

Screen – printed carbon electrodes (SPCEs) are based on a three electrode layout, produced in three steps printing different consecutive ink layers on transparent polyester films, as described by Albanese et al. (2011). After an electrochemical electrode treatment (6 min) at 1.7 V vs. Ag/AgCl as reference electrode in a 0.05 M phosphate buffer solution (PB 0.1 M KCl, pH 6.8), the conductive PANI-PAAMPSA polymer was electrochemically synthesized by Cyclic Voltammetry (CV), according to Albanese et al. (2014). The capability of PANI-PAAMPSA film as conducting polymer for NADH oxidation was investigated by Cyclic Voltammetry of PANI-PAAMPSA/SPCE with and without 0.4 mM NADH.

1.3 Ethanol Biosensor Manufacturing

The immobilization of the enzyme was carried out by dropping 22U (enzymatic unit) of ADH on PANI-PAAMPSA modified electrode and left to get dried up. Finally, few microliters of Nafion solution (1 %) was dropped on the enzymatic layer. All the biosensors produced were stored overnight at 4 °C when not in use.

1.4 Electrochemical Measurements

All electrochemical experiments were carried out with a PalmSens potentiostat/galvanostat connected to a personal computer for data recording and visualization. The amperometric measurements were performed in a Flow Injection Analysis (FIA) apparatus as described by Albanese et al. (2010). The biosensors were placed in a handmade electrochemical cell at room temperature while a constant potential of 0.4 V vs. Ag/AgCl was applied. Carrier solution PB at pH 9.0 were pumped by a peristaltic pump at 0.5 $mL \cdot min^{-1}$ flow rate to the injection valve with a 500 µL sample loop. The analysis requires a minimum sample dilution with PB, in order to have the optimum pH condition.

2. Results and Discussions

2.1 PANI – PAAMPSA Screen Printed Electrodes

The working principle of ethanol biosensor based on the alcohol dehydrogenase (ADH) enzyme immobilized on a PANI-PAAMPSA modified screen-printed electrode was described in Figure 1.

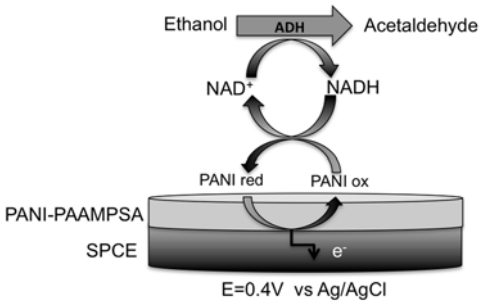

Figure 1: Enzymatic reaction involved for ethanol determination by ADH on a PANI-PAAMPSA/SPCE

As reported by Tarver et al. (2009) the doping with PAAMPSA during the polymerization of aniline gives good levels of solubility and electrical conductivity at neutral and basic pH to the resulting polymer. Thus, the capability of PANI-PAAMPSA film as conducting polymer for NADH oxidation was investigated by Cyclic Voltammetry of PANI-PAAMPSA/SPCE with and without NADH solutions at pH 7.0 and pH 9.0 (Figure 2).

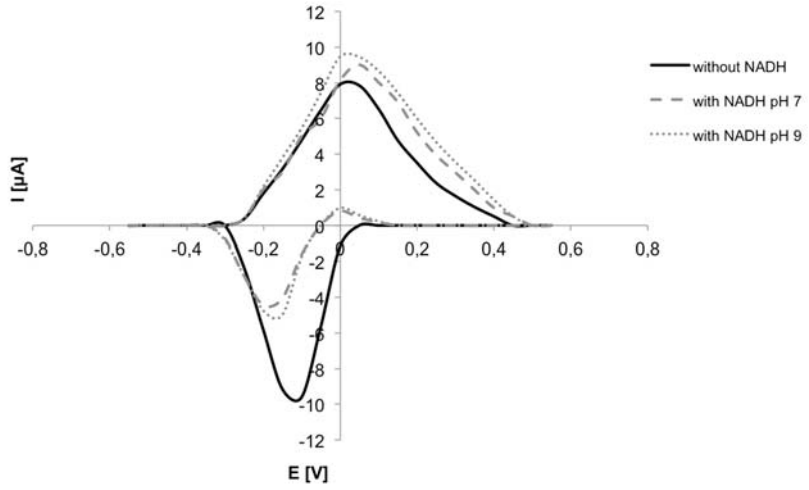

Figure 2: Cyclic voltammetry of PANI-PAAMPSA/SPCE in PB 0.1 M pH 7.0 without NADH (a) PB 0.1 M pH 7.0 with 0.4 mM NADH (b) and PB 0.1 M pH 9.0 with 0.4 mM NADH (c)

According to Albanese et al. (2014), who studied the capability of PANI-PAAMPSA modified screen-printed carbon electrode to detect β-nicotinamide-adenine dinucleotide phosphate (NADPH) at low potential, the Cyclic Voltammetry recorded in the presence of NADH displayed an increasing anodic peak at 0.1 V due to the electrocatalytic oxidation of NADH by the conducting emeraldine form of PANI and a significant reduction of the cathodic peak. Moreover, no differences in PANI-PAAMPSA cyclic voltammograms were found in presence of NADH solutions at pH 7.0 and pH 9.0 (Figure 2). These results confirm the activity and the stability of PANI-PAAMPSA polymer at basic conditions.
Thus the linear response of PANI-PAAMPSA modified electrode towards different concentrations of NADH solutions at 0.1 V vs. Ag/AgCl, pH 9.0 was evaluated. The linear range 0.005-2 mM, the sensitivity 653.15±11.43 μA M^{-1} and the Relative Standard Deviation (RSD of 1.2 %), calculated on three injections of NADH 0.04 mM, highlighted the capability of PANI-PAAMPSA modified SPE as conducting polymer in the development of dehydrogenase biosensors.

2.2 Optimization of Ethanol Biosensor

Enzymatic activity is strongly influenced by buffer pH values. For this reason the influence of the pH on the ADH biosensor response was verified by injections of ethanol standard solutions at different pH (ranging from 7.0 to 9.5). The optimum pH values that led to the maximum chronoamperometric current were for pH 8.5 and 9.0 (Figure 3). In these trials the stoichiometric ratio between ethanol and NAD^+ was used.

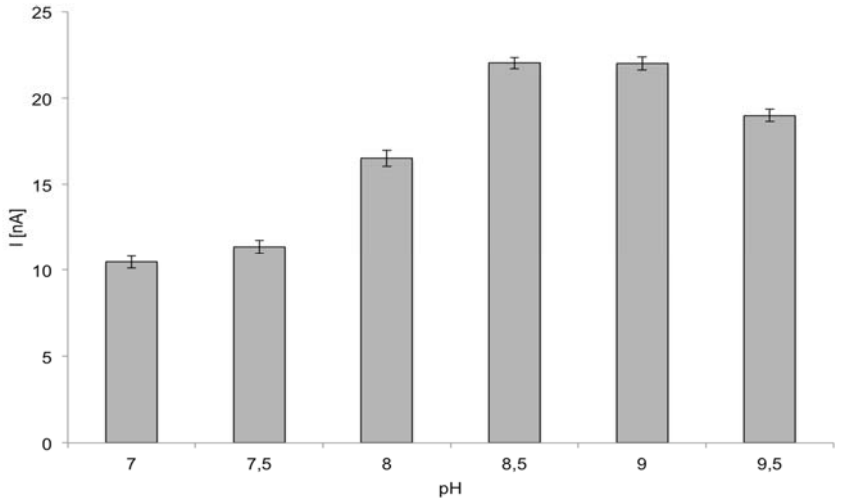

Figure 3: Effect of pH on the response of ADH biosensor in the presence of 0.007 %vol ethanol solution

Next, in order to obtain high performances for ADH biosensor, different work potentials were investigated. The optimum applied potential was selected by carrying out different chronoamperograms with ethanol solutions ranging from 0.01 to 0.025 %vol. The highest current response for all ethanol concentrations was obtained at 0.4 V, so it was taken as optimum potential.

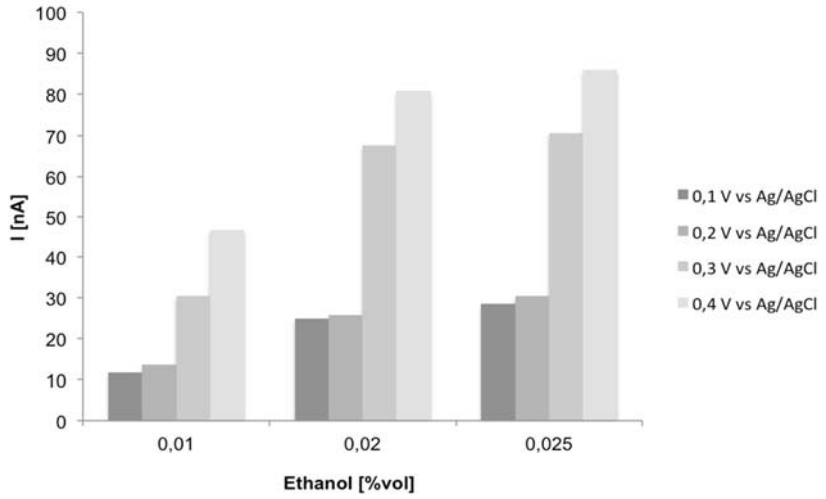

Figure 4: Effect of working potential on the response of ADH biosensor in the presence of ethanol solutions ranging from 0.01 to 0.025 %vol.

The calibration curve of the ADH biosensor, with optimized conditions, was carried out by systematic injections of ethanol solutions ranging from 0.001 to 0.03 %vol (Figure 5).

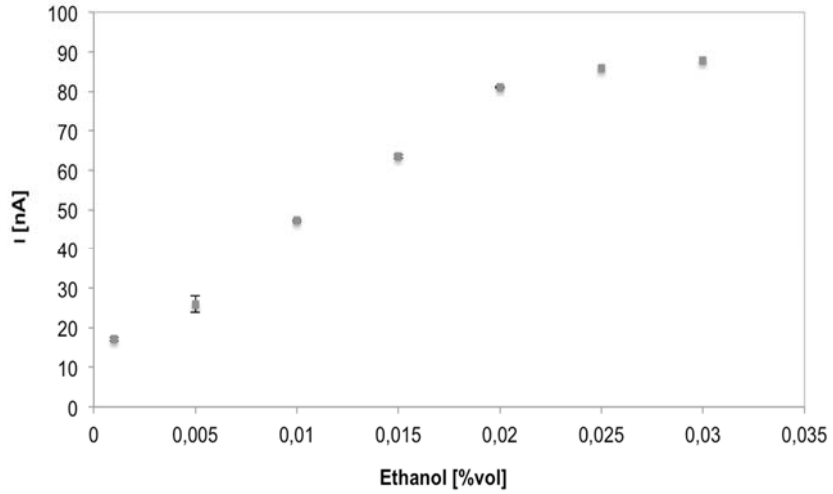

Figure 5: calibration curve of ADH biosensor in optimized condition

The analytical parameters of the ADH biosensors developed in this work showed a linear range from 0.001 to 0.02 %vol, a limit of detection (defined as the ethanol concentration that yields a signal-to-noise ratio S/N=3) of 0.001 %vol and a sensitivity of 3,551±20 nA $\%vol^{-1}$. Finally, ADH biosensor showed good reproducibility, with a RSD of 3.04 % calculated on five different biosensors.

2.3 Operational and Storage Stability

An important parameter that affects the use of biosensors as analytical device for analysis of real samples is the operational stability, defined as the retention of enzyme activity when it is in use. This parameter was evaluated by 70 injections (ethanol 0.008 %vol) during 7 h, with a high repeatability of current during the experiment and a sensitivity loss of 1.84 % during the test (data not shown). Next, storage stability was also evaluated to estimate the potential commercialization of the developed ethanol biosensor. For this purpose different ethanol biosensors were stored at 4 °C without any chemical preservative and characterized at regular interval times. After two months 85 % sensitivity was retained (data not shown).

2.4 Analysis of real samples

The capability of the ADH biosensor developed in this work to measure ethanol content in grape must samples was investigated. At first, the matrix effect on the ADH biosensor was evaluated by the comparison of calibration curves obtained with ethanol standard solutions and with red and white must samples spiked with ethanol. The matrix effect, was calculated by Eq(1):

$$Recovery\ \% = 100 \cdot S_m / S_b \quad (1)$$

where S_m and S_b represent the sensitivity of biosensors measured with spiked ethanol juice samples and ethanol standard solutions respectively. The results in Table 1 showed that a minimum matrix effect exist only for red grape, characterised by higher flavonoids content. For this reason the percentage recovery was approached to 100% by increasing the dilution factor of the sample undergoing measurement. These results highlighted that a lower potential can be used with PANI-PAAMPSA modified SPE reducing electrochemical interferences due to real matrix in white and red grape juices.

Table 1: Matrix effect of grape juice samples on the response of ethanol biosensor

Sample	Dilution Factor	Sensitivity nA $\%vol^{-1}$	Recovery %
Standard Solutions		3,551±17	
Red Grape	1:10	3,451±12	97.2
	1:15	3,548±10	99.9
White Grape	1:10	3,527±9	99.3

Finally, the alcohol content in different red and white must samples was compared with those obtained using pycnometer method. Good agreement between the two data set was observed with a percentage error lower than 4 %.

3. Conclusions

A screen-printed amperometric biosensor, based on the immobilization of ADH on the surface of PANI-PAAMPSA modified electrode, has been presented. The PANI/PAAMPSA modified electrode was able to detect the direct oxidation of NADH at a lower potential than that one commonly required for carbon electrodes (1 V). The ADH biosensor showed a linear range from 0.001 to 0.02 %vol and a good sensitivity of 3551 nA $\%vol^{-1}$, enough to allow the detection of very low ethanol levels in must grapes, in order to evaluate grapes quality in winemaking. The analytical performances, together with the capability to reduce the electrochemical interferences present in food matrices showed the potential of ADH biosensor as a highly capable analytical device for a fast ethanol measurement in must and grape real samples.

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