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A disposable, simple, fast and low-cost paper-based biosensor and its application to the determination of glucose in commercial orange juices.

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Abstract

A new biosensor for monitoring glucose levels in beverages is presented. The measurements are performed using potentiometric detection. Working electrodes are made using platinised paper as support and a biocompatible polymeric membrane made of a mixture of polyvinyl alcohol and chitosan containing glucose oxidase as the recognition layer. The system is based on the detection of the hydrogen peroxide generated by an enzymatic reaction performed in a highly sensitive, selective and simple way. The biosensors display suitable analytical performance (sensitivity -119.6 ± 6.4 mV/dec in the 0.03-1.0 mM range with a limit of detection of 0.02 mM). Determination of glucose in commercial orange juices is presented. These results were validated against conventional standard methods, showing good accuracy and fast analytical response. The methodology presented

herein does not require complex samples treatment, offering an alternative to conventional methods, particularly for determinations performed with minimal expertise and without a laboratory infrastructure.

Highlights

- A paper-based biosensor for monitoring the levels of glucose in beverages is introduced.
- The system shows high sensitivity, selectivity and fast response.
- GOx entrapped in a biocompatible polymeric membrane enhances the sensor long term stability.
- The system allows the accurate, fast and ultra-low cost determination of glucose without the need of an analytical laboratory.

1. Introduction

Glucose is one of the most important monosaccharides found in nature, either as a monomer or as a part of more complex structures that serve as energy reserves in animals and plants. From a metabolic perspective, glucose is involved in some of the most fundamental processes, such as the photosynthesis and respiration. In the food industry, glucose is employed in a wide range of applications, such as a substrate for yeast in the fermentation process, as a flavour enhancer, etc. (Galant, Kaufman, & Wilson, 2015). In fermentation processes such as winemaking, monitoring the concentration of compounds such as glucose in the ferment broth is important to control the evolution of the process. Fluctuations of the glucose concentration are closely related to contamination of microorganisms as well as to

the quality of the final product (Mao, Wu, & Ying, 2008). For the food industry, the quality of the products must be periodically evaluated and controlled in order to preserve the product properties along the production and supply chain as well as to optimize economic aspects, etc. Besides, the importance of monitoring the process comes from consumer demanding safety products as well as the regulations from the government. Conventional methods for quality control and food safety involve analytical techniques that are expensive, time consuming, and require specific equipment and trained people. Official methods for measuring glucose in different products such as fruit juices, syrups, honey and non-alcoholic beverages include chemical, volumetric and polarimetric methods, and gas and anion exchange chromatographic methods (Official Methods of Analysis of AOAC INTERNATIONAL, 2005). For this reason, developing simpler, faster and cheaper methods for detecting and quantifying glucose has gained great importance in food analysis (Monosik, Stredansky, Tkac, & Sturdik, 2012; Neethirajan & Jayas, 2010; Walker & Lupien, 2000).

Electrochemical biosensors could have a significant impact on food quality control by providing devices for faster monitoring during food production, packing, storage and even transport (Galant et al., 2015; Mannino & Wang, 1992). The most popular electrochemical biosensors make use of an enzymatic reaction coupled to amperometric detection. This technique is highly sensitive, although it usually requires the use of more than one enzyme and additional chemical compounds. For this reason, alternative approaches that combine good analytical performance, simplicity and low-cost are sought after. Potentiometric methods, for example, show

significant advantages. Potentiometry, based on the measurement of the difference of electrical potential between a working and a reference electrode under almost zero current conditions, is relatively fast, label free and has a wide linear range. With an instrumentation that is fairly inexpensive, with very low power consumption and simple to operate, potentiometric methods are robust, simple and compact. Furthermore, recent advances in printed electronics and nanotechnology have allowed the development of paper-based, ultra low-cost sensors (Novell, Parrilla, Crespo, Rius, & Andrade, 2012). Indeed, potentiometry is an ideal tool for performing field, on-site and out of the lab chemical measurements (Bakker & Bu, 2000; Bakker & Pretsch, 2007; Janata, 2009).

Direct potentiometric measurements are ubiquitously used to monitor pH (glass electrode), ions (ion-selective electrodes) and the redox potential of a system (Oxidation reduction potential – ORP electrodes - which are often as simple as a platinum probe). Indirect potentiometric measurements monitor a change produced as a result of a specific reaction with the analyte. The use of enzymes as a highly specific recognition event has been proposed decades ago. Potentiometric biosensors based on monitoring the changes in pH (Timur & Telefoncu, 2004) or in the redox potential (Tasca et al., 2007) have been proposed, but their impact has been scarce. In the case of pH, the buffer capacity of a sample limits the applicability of the technique. For the redox sensors, the interference produced by the presence of redox-active substances has traditionally been considered a serious obstacle. Nevertheless, with the increasing need for simpler, cheaper and robust sensors, the interest in potentiometric tools has revived. Potentiometric biosensors are scarcely

used in food analysis. Some examples, such as the use of an ISE for the detection of NH_4^+ to measure urea in milk (Trivedi et al., 2009) was reported. A promising approach using enzyme-based potentiometric sensors immobilized on ZnO nanostructures have been proposed, although no validation with real samples has been yet reported (Usman Ali, Nur, Willander, & Danielsson, 2010). Very recently, our group has proposed an alternative approach by monitoring the change in the redox potential produced by the use of an oxidase enzyme (Parrilla, Cánovas, & Andrade, 2017). Since this type of enzymes generates hydrogen peroxide, the change on the redox potential was monitored by a Pt electrode coated with a Nafion membrane. Nafion is a sulfonated fluoropolymer that acts as a permselective barrier and thus minimizes interferences produced by redox active anions (Romero, Ahumada, Garay, & Baruzzi, 2010), such as ascorbate, a substance commonly used as a food preservative (Sung & Bae, 2006). Also, it has been reported that the generation of a Donnan potential due to the ion-exchange capacity of Nafion leads to an enhancement of the sensitivity of the detection, which is increased more than 5 times when compared to the bare Pt electrodes (Parrilla, Cánovas, & Andrade, 2016). Thus, in the simplest form, an enzyme trapped on the Nafion coating is used as a potentiometric biosensor.

Potentiometric electrodes with immobilized enzymes are a promising tool to address the growing interest in the food industry for the development of robust, fast, simple and affordable methods to generate biochemical information without the need of a laboratory. For this reason, this work presents for the first time a paper-based potentiometric biosensor for monitoring glucose in drinks and beverages. The

biosensor is based on the use of platinized paper as substrate in order to reduce the manufacturing cost, a Nafion coating to increase the sensitivity and minimizing the interference on glucose measurements, and a layer of chitosan/PVA blend for the immobilization of the enzyme, which enhances the enzyme activity and improves the stability of the sensor. As a proof of concept, orange juice has been selected, since the bulk of organic citrus juice consists of orange juice and is one of the most popular among consumers due to its organoleptic properties (Liu, 2003; Spreen, 2001).

This work present the construction and analytical optimization of this sensor for the direct determination of glucose in orange juice. The results show that the device is robust, simple and fast, opening a new avenue for the use of potentiometric tools in the food industry.

2. Materials and methods

2.1. Reagents

Glucose oxidase from *Aspergillus niger* with activity of 138 U/mg solid (EC 1.1.3.4), Nafion 5 wt. % (in mixture of lower aliphatic alcohols and water contains 45 % water), glucose, methanol, acetic acid (99-100 %), calcium carbonate, polyvinyl alcohol (PVA) and chitosan with 75-85 % of deacetylation were purchased from Sigma-Aldrich, Spain. Analytical grade salts of dibasic sodium (Na_2HPO_4) monobasic potassium (KH_2PO_4) phosphate, potassium chloride (KCl) and sodium chloride (NaCl) were purchased from Sigma-Aldrich. All solutions were prepared using double distilled deionized water (18.1 $M\Omega \cdot cm^{-1}$) produced by Milli-Q water system (Millipore Corporation, Bedford, MA). Phosphate buffer saline (PBS) was prepared by dissolving 0.100 M of Na_2HPO_4; 0.018 M of KH_2PO_4; 0.14 M of NaCl and 0.003

M of KCl in double distilled deionized water and adjusting the pH to 7.4.

2.2. Sensor preparation

2.2.1. Enzymatic membrane cocktail preparation.

Three different solutions with variable amount of enzyme were prepared in order to have final concentrations of 0.14, 0.41 and 0.69 Units/µL of the enzymatic cocktail. First, glucose oxidase was dissolved in 1.0 mL of a solution of 1 % wt of PVA in water. Thereafter, 2.0 mL of a solution containing 1 % wt chitosan in 1 % wt acetic acid were added. The solution was thoroughly mixed with a vortex mixer until a homogenous solution was obtained. The mixture was always freshly prepared before it was drop casted onto the platinised paper.

2.2.2. Sensor construction.

To build the redox-sensitive substrate, a 100 nm layer of platinum was sputtered onto one side of a conventional filter paper (Whatman number 5) using a radiofrequency sputtering source (ATC Orion 8-HV, AJA International) operated at 3 mTorr, for 65 s at 200 W.

The conductive paper was then cut into strips of 0.5 cm x 2.0 cm and then sandwiched between two 1.0 cm x 1.5 cm plastic masks (ARcare 8565, Adhesives Research Inc., Limerick, Ireland) as shown in Figure 1. The top mask has an orifice of 0.3 cm in diameter that leaves exposed the electroactive platinized window to cast the membrane, as described elsewhere (Novell, Guinovart, Blondeau, Rius, & Andrade, 2014; Novell et al., 2012). Thereafter, two aliquots of 5 µL of Nafion solution at 2.5 % in methanol were subsequently drop casted onto the electroactive window and dried at room temperature for at least 3 hours. Finally, 8 µL of the

enzymatic membrane cocktail was drop casted. The sensor was placed in an oven at 37 °C for 2 hours. Figure 1 shows a scheme of the sensor with the different layers. The sensor is stored at 4 °C in a desiccator with $CaCO_3$ until use.

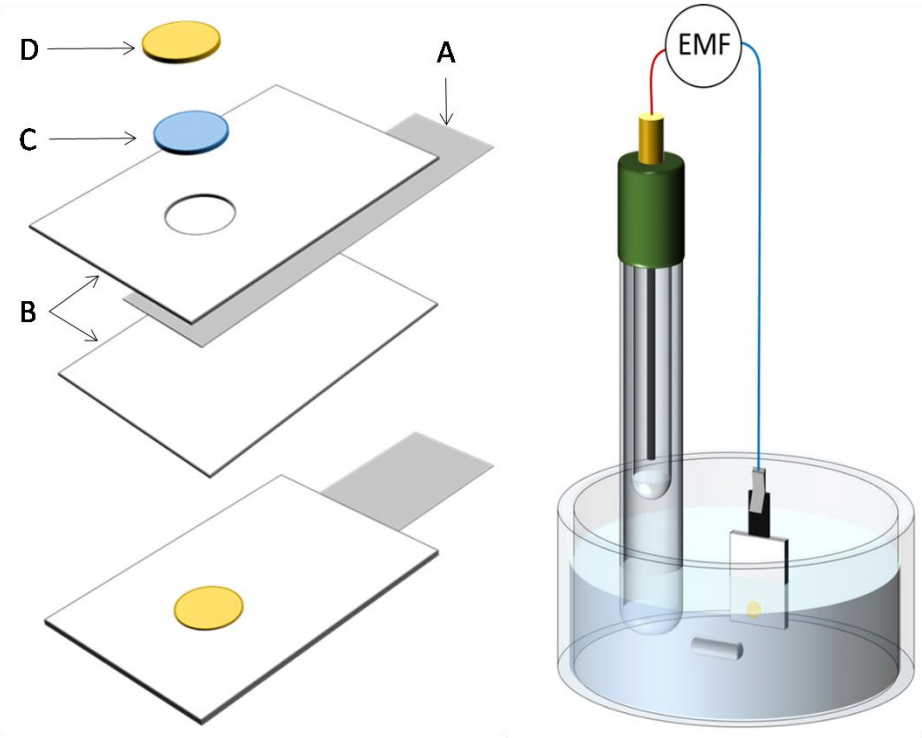

Figure 1 Schematic representation of glucose sensor construction (left): strip of platinised paper (A) sandwiched between two plastic masks (B), with a Nafion layer (C), and a layer of enzymatic membrane made with GOx/Chitosan/PVA (D). Illustration of the measuring setup (right).

2.3. Electrochemical measurements

Electromotive force (EMF) was measured with a high input impedance (10^{15} Ω) EMF16 multichannel data acquisition device (Lawson Laboratories, Inc. Malvern) at room temperature in a stirred 100 mM phosphate buffer solution (PBS at pH 7.4). A double junction Ag/AgCl/KCl 3 M (type 6.0726.200 Methrom AG) containing 1 M of lithium acetate was used as the reference electrode. The paper electrode was connected to the measuring instrument with a small clamp that makes contact with

the exposed platinised end of the paper. The electrode was immersed until the membrane was fully covered by the solution. All the experiments were conducted at room temperature (approximately 23ºC) Measurements were performed by adding a suitable amount of sample (or standard), and the reading was performed once a stable signal was obtained.

2.4. Enzymatic assay

As a reference method, a commercial glucose assay kit (Sigma-Aldrich, GAGO20-1KT) was used. The analytical procedure was performed according to the manufacturer's instructions. Absorbance measurements were carried out in an 8453 UV-Vis spectrophotometer from Agilent Technologies (Barcelona, Spain) with a 10 mm light path glass cuvette (Hellma Analytics, Germany).

2.5. Analysis of real samples

To validate the sensor response, the proposed potentiometric method was applied to the determination of glucose in 10 different brands of orange juice. The samples were shaken before opening and used without any pre-treatment. The sensors were first calibrated and rinsed 3 times with PBS, and finally applied to the measurement of the samples. Samples were diluted 1:1000, and 1:500 with buffer solution (100 mM PBS, pH 7.4) depending on the concentration of glucose stated on the packaging; some samples were low-carbohydrate thus requiring less dilution to fall within the linear range. A proper amount of juice was added to the cell containing PBS to achieve the dilutions. The values reported are the average of the measurements performed using 3 different sensors.

3. Results and discussion

3.1. Principle of detection of glucose

The oxidation of β-D-glucose to gluconic acid catalysed by the enzyme glucose oxidase (GOx) uses oxygen as an electron acceptor and generates hydrogen peroxide (H_2O_2) as a reaction by-product:

$$glucose + H_2O + O_2 \xrightarrow{GOx} gluconolactone + H_2O_2$$

A plethora of approaches using this reaction for the determination of glucose have been proposed and are now in use. Most of the current methods make use of a dual enzymatic system, where the hydrogen peroxide generated is used as a substrate of a second reaction. In the spectrophotometric techniques, for example, the H_2O_2 generated is used to oxidize a chromophore in a secondary reaction with horseradish peroxidase, with a resulting change of colour that can be measured (Johnson, Lambert, Johnson, & Sunderwirth, 1964; Trinder, 1969). The same is true for the amperometric techniques, where the peroxide cannot be detected directly because of the interference of oxygen. Evidently, the need to incorporate an additional enzymatic reaction to detect peroxide adds complexity to the system. In this work, on the other hand, the hydrogen peroxide can be directly detected by the change produced on the redox potential of the solution. Indeed, as a generic ORP detector, Pt probes are sensitive to changes on the redox potential. In the case of the peroxide, the reaction

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$$

produces a change that can be followed by the Pt electrode (Kumar, Kulkarni, Dhaneshwar, & D'Souza, 1994). Thus, the concentration of glucose can be

calculated directly from the change in the redox potential produced by the hydrogen peroxide generated (Wingard, Castner, Shang, Wolfson, Drash & Liu, 1984). Nevertheless, because of the interferences produced by any other redox-active species in the sample, this potentiometric approach has very limited applications.

In a recent work we have proposed a solution to this problem by using a Pt electrode coated with a Nafion membrane. Nafion is a polyelectrolyte with negatively charged sulfonate groups that act as a permselective barrier towards large anions. We have previously demonstrated that this approach minimizes the interference of typical redox-active anions, such as ascorbate (Parrilla et al., 2016) while it also significantly enhances the sensitivity for the detection of peroxide (and therefore, for glucose). This approach has shown optimum results for the determination of glucose in serum and whole blood. Therefore, after proper optimization, it should be expected that it could also be applied to the analysis of beverages.

3.2. Sensor construction

The immobilization of the receptor is a crucial step for the construction and performance of the sensor. In particular, enzyme immobilization has been largely studied (Brady & Jordaan, 2009; Brena & Batista-Viera, 2013). One of the approaches often used is the entrapment or encapsulation via inclusion of the enzyme in a polymer lattice such as chitosan, carboxymethyl cellulose, agarose or starch (Sheldon, 2007). Chitosan and polyvinyl alcohol (PVA) are very attractive materials for immobilization of enzymes due to their high affinity for proteins, easy preparation and biodegradability. Moreover, chitosan is a natural polyaminosaccharide soluble in aqueous acidic media at pH lower than 6.5, which

is commonly used for the immobilization of enzymes using the solvent evaporation method. On the other hand, PVA is a synthetic polymer that has been widely used in biochemical and biomedical applications. The high compatibility between PVA and chitosan caused by intermolecular hydrogen bonding interactions allows a membrane to be obtained with good characteristics for the entrapment of the GOx (Batista, Marques, Yamashita, & Flávia, 2013; Kumar et al., 2010; Ming, Yu, Lang, & Chien, 2004; Srinivasa, Ramesh, Kumar, & Tharanathan, 2003).

We therefore introduce here a sensor based on two layers: first, the Nafion layer that provides sensitivity enhancement and selectivity and second, the recognition layer based on the GOx entrapped in the biocompatible chitosan/PVA matrix that enhances the stability on time. The optimization of the sensor response was studied as a function of the GOx load, then the analytical parameters were characterized and the sensor was validated with real samples, and stability over time.

3.3. Optimization of the response

For the optimization of the GOx load, three different enzymatic solutions were prepared in order to afford 1.1, 3.3 and 5.5 Units of GOx per sensor. Figure 1S (see supplementary information) shows the behaviour of the sensor in terms of sensitivity when increasing the GOx amount on the surface of the sensor. As it can be observed, as the enzyme load was increased (measured in units of enzymatic activity), no significant improvement was detected. For the highest amount of GOx, a slight sensitivity decrease was observed showing a sensitivity of -110.3 ± 10.3 mV/dec, and the linear range was narrower (from 0.03 to 0.3 mM), which may be related to a decrease in the enzyme activity due to active site blocking (Bankar, Bule,

Singhal, & Ananthanarayan, 2009). Therefore, the subsequent experiments were performed using 0.14 U/µL of GOx i.e. 1.1 Units of GOx per sensor. However, the standard deviation of the sensitivity did not follow a clear trend and it could not be assumed that it came from the enzyme amount. It could probably be more related to the sensor construction process that involves several manual steps. Moreover, the storage time between the construction and the use of the sensors maybe a relevant parameter for this issue. This parameter will be indeed evaluated in the following sections.

3.4. Analytical performance

The performance of the sensors was assessed by monitoring the change in electrochemical potential produced when increasing the concentration of glucose. Figure 2A shows the potentiometric time trace of a glucose sensor that shows that the EMF decreases as the concentration of glucose increases, as it could be expected from the reaction of oxidation of peroxide. The EMF was measured in a range from $10^{-5.5}$ to 10^{-2} M of glucose. Variation in concentrations above 10^{-2} M did not produce any significant change. Figure 2B displays the calibration curve for the glucose sensor and two control experiments (blank electrodes): (a) an electrode made only with a layer of Nafion over the platinised paper and (b) an electrode made with the layer of Nafion and the immobilization membrane (Chitosan/PVA) without the enzyme. None of the blank electrodes shows a response to the addition of glucose due to the absence of enzymatic activity, i.e., since there is no generation of H_2O_2, no change in response of the platinised paper occur.

Optimized figures of merit for the determination of glucose are summarized in Table

1. The sensor shows a sensitivity of -119.6 ± 6.4 mV/dec in the 0.03 to 1 mM linear range with a limit of detection of 0.02 ± 0.01 mM, in agreement with what has been already reported (Parrilla et al., 2017). It should be stressed that the relatively high sensitivity obtained is the result of the use of Nafion membrane. Indeed, bare Pt electrodes show responses in the order of 20-40 mV/decade (depending on the condition of the Pt surface) (Parrilla et al., 2016). This enhanced sensitivity is the result of the Donnan potential created by the polymeric membrane, which enhances the sensitivity for the detection of Pt. Therefore, this coated platinised paper-based sensor presents better figures of merit than some other amperometric sensors (Mignani, Scavetta, & Tonelli, 2006), and clearly a much simpler construction and operation.

In terms of the limit of detection, the concentration of glucose in many beverages is well above the limit of detection, thus encouraging further development of the sensor. The response time of the sensor is between 20 to 30 seconds, much faster than the conventional enzymatic assay that usually requires as much as 30 minutes for the development of the colorimetric reaction. In addition, the potentiometric paper-based sensor requires less reagents and equipment compared to the standard methods, such as the enzymatic assay or chromatography, making the new sensor a simple, faster and low cost method.

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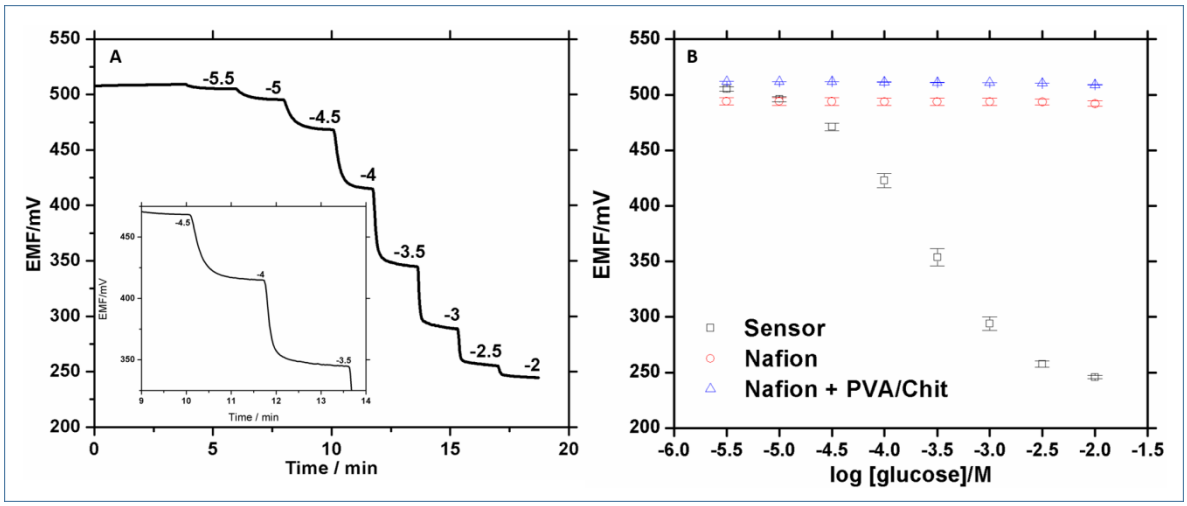

Figure 2 Potentiometric response for the glucose sensor. (A) Time trace for the sensor upon increasing glucose concentration and (B) calibration plot for the sensor and blank electrodes (mean ± S.D., N=3).

Table 1 Analytical performance of glucose sensor (N=3).

	Glucose sensor (N=3)
Sensitivity (mV/dec)	-119.6 ± 6.4
Linear Range (mM)	0.03 to 1.0
LOD (mM)	0.02 ± 0.01
Response time (s)	20-30

These analytical parameters are compared with the ones reported for other potentiometric glucose biosensors in Table 1S. The developed sensor has the highest sensitivity, which is crucial for the detection of glucose in real samples. The linear ranges are comparable in most of the examples reported although some previous works reports much lower limit of detection. Nevertheless, the usefulness of both the linear range and limit of detection should be focused on the detection in real samples, i.e. a low limit of detection would be relevant for determination of glucose in saliva for instance. In the selected reports, only half of the works displayed detection in real samples. For the detection of glucose in beverages, the performance of the proposed sensor meets the required analytical standards. Lastly,

325 if we compare the response time, there are sensors with response time lower than
326 10s, our sensor response is 20-30s. In addition, traditional methods used in the food
327 and beverage fields typically require in the order of 30 minutes only for the enzymatic
328 reaction to be completed. Therefore, a sensor with a response time below the single
329 minute is very promising.

330 3.5. Interferences

331 Reducing agents -such as ascorbic acid (AA)- might interfere with the sensor
332 response. Ascorbic acid is often employed in food industry as a preservative. In
333 drinks and beverages it can be found in a range from 0.14 to 2.44 mM of fruit juice
334 (Kabasakalis, Siopidou, & Moshatou, 2000). This is a very high concentration that
335 may have a significantly negative effect on the measurements. However, since
336 samples have to be diluted to fit within the linear range of the sensors, the effect of
337 the ascorbic acid is also minimized. The concentration of ascorbic acid used is 0.01
338 mM, which corresponds to the highest amount of AA found in a diluted sample of
339 fruit juice according to the values reported. This issue is demonstrated in Figure 3
340 where calibration curves with and without AA background are reported. There is a
341 decrease in the initial potential from 491.8 ± 4.0 to 457.6 ± 8.3 mV and the sensitivity
342 of the electrodes decreased from -116.2 ± 2.9 down to -100.9 ±3.8 mV/dec when AA
343 is added as background. However, the linear range as well as the limit of detection
344 remain the same (Table 2S, supplementary information).

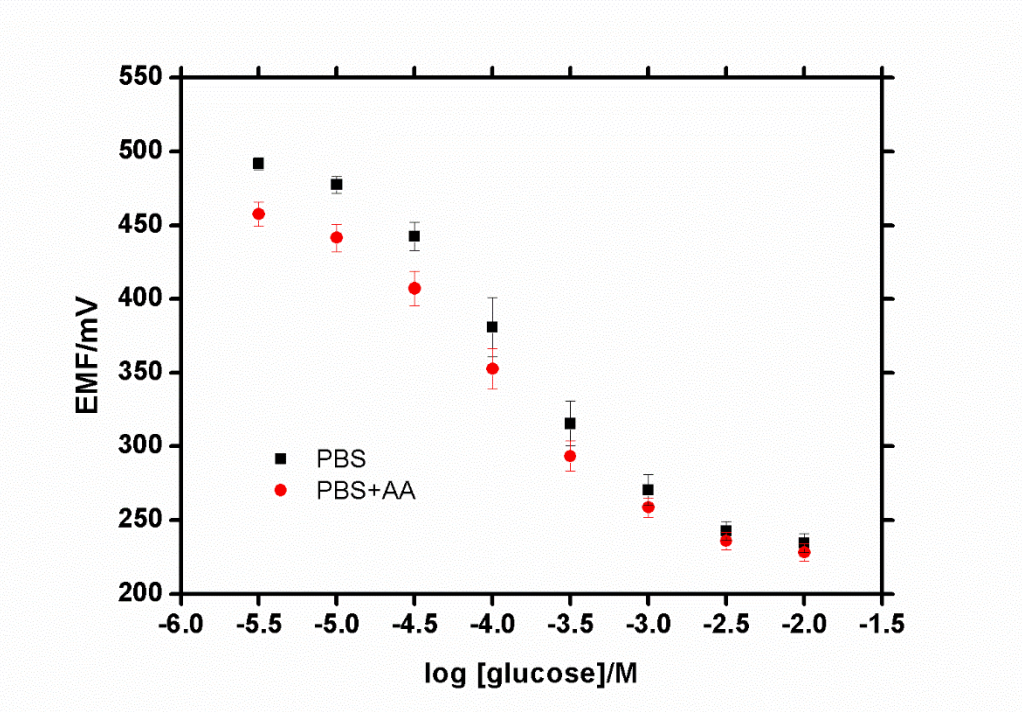

Figure 3 Calibration plot for sensors using PBS and PBS with ascorbic acid (0.01 mM) used as a background (mean ± S.D., N=3).

3.6. Analysis of real samples and validation

Figure 4 shows the comparison between the values obtained using two different methods: the potentiometric sensor and the commercial enzymatic assay. Nine different samples of commercial orange juices (3 of them sold as reduced sugar content) were measured by both methods and compared. As it can be seen, the correlation between the methods was linear, with a slope close to 1 and an intersection close to 0, which confirms the good correlation between the two methods. The slight difference between the values could be related to the standard solutions of glucose used (see values in the supplementary information Table 3S). The solution provided with the enzymatic kit contains benzoic acid as a preservative, which was not added to the potentiometric standard solutions. In order to confirm

this issue, solutions of 0.3 mM were prepared from the enzymatic kit and together with the potentiometric standard solutions were measured with the paper-based sensors. The response obtained for the standard solution of the kit was higher by 3.3% (in mV) than the response for the standard solution used for potentiometry. When the response in potential was converted into glucose concentration (M) the overestimation for the standard solution for potentiometry was 19 % higher than the one calculated for the standard solution of the kit. Further studies are in process to overcome this issue. The supplementary information contains the summarized values and the values with a preliminary correction made taking into consideration the possible effect caused by the benzoic acid (Figure 3S). The difference between the measurement made by our sensor and the reference method is indeed caused for the benzoic acid, which was already reported in the past (Hall & Keuler, 2009).The concentration of glucose measured with the sensors is in agreement with the concentration obtained with the reference method, making the proposed sensor useful to measure the glucose concentration in real samples without the interference of AA.

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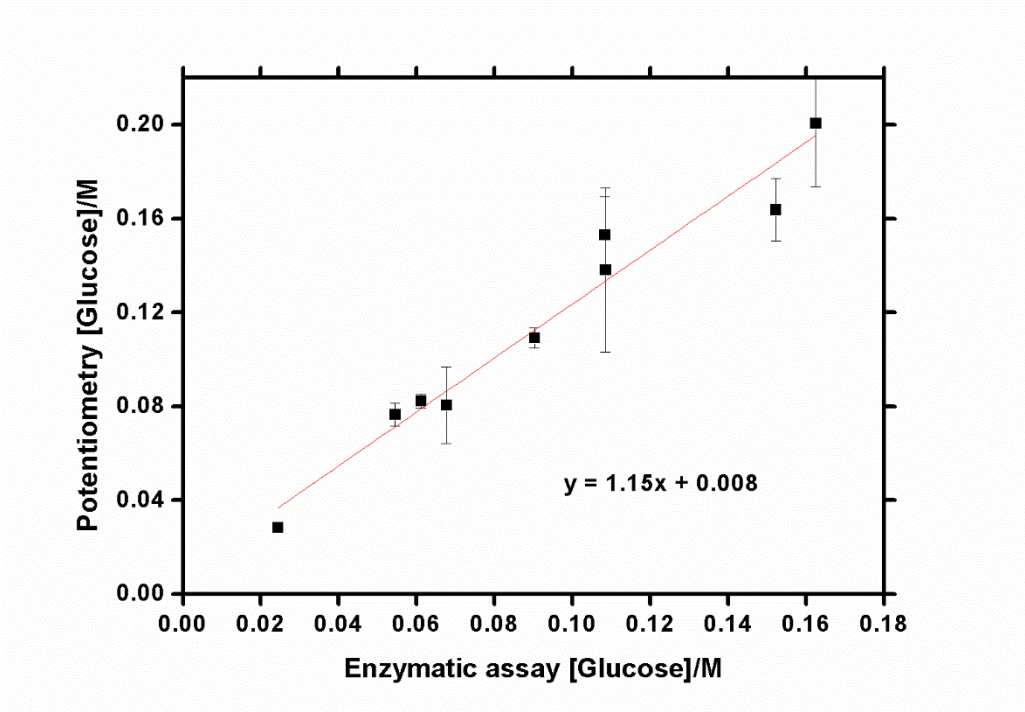


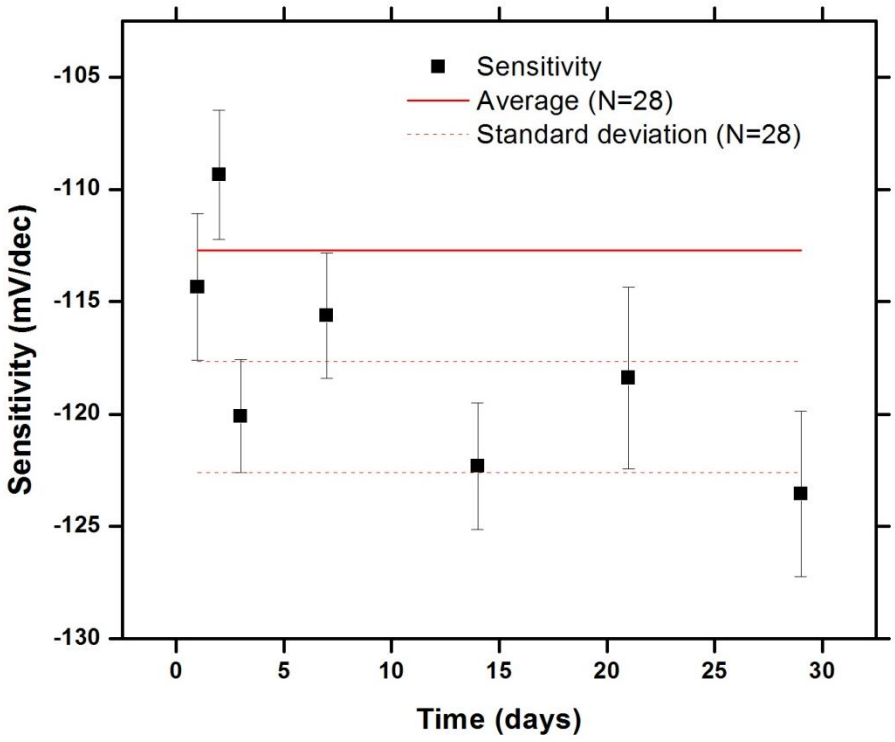
Figure 4 Prediction of glucose (M) in real samples by the sensor and enzymatic assay.

3.7. Shelf life of sensors.

The shelf life of the sensor was assessed by evaluating the performance after 1, 2, 3, 7, 14, 21 and 28 days. A batch of 28 electrodes was used for this study. All of them were prepared on the same day and under the same conditions. A set of four electrodes was evaluated (sensitivity, LR and LOD) every day (storing the others in the fridge). Figure 5 shows the average of the sensitivity obtained during a month with a standard deviation as low as 4.9 mV/dec. Therefore, the performance of the sensors does not deteriorate over time. The linear range (from 0.03 to 1 mM) as well as the limit of detection (LOD) remains in the same interval during the whole study. Sensitivity and LOD values for each day of evaluation, with the standard deviation corresponding to each set of 4 electrodes, are shown in supplementary information

(Table 4S). The shelf life of the sensor depends on the conservation of the enzymatic activity. Using biocompatible polymeric membranes to immobilize the enzyme may enhance its stability in the sensor. The value of sensitivity on day 28 (-123.5 ± 3.7) confirms that the enzyme activity has remained constant. This improvement could be related to the structure of the chitosan/PVA matrix because the structure of the polymeric lattice protects the enzyme from variations of the chemical surrounding during the storage time such as pH and temperature that may denature it (Batista et al., 2013; Brena & Batista-Viera, 2013; Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007; Sheldon, 2007). The sensor can be used for at least one month after its construction if stored at 4 °C under minimal controlled humidity conditions. Moreover, the concentration of glucose of one selected brand of orange juice previously evaluated (sample 9, supplementary information) was measured every day with the sensors previously calibrated. Even on the final days of measurements, that is when the paper-based sensors present higher standard deviation, the glucose concentration value obtained for the tested juice is in accordance with the value obtained by the reference method (Table 4S,

406 supplementary information).



407

408 **Figure 5** Sensitivity (mV/dec) average values with its standard deviation over time. The average value
409 of all the sensors with the standard deviation are represented by the horizontal lines (N=28).

410

411 ## 4. Conclusion

412 The development of a low cost potentiometric enzyme-based electrode for the
413 determination of glucose in fruit juices has been described. Using a low amount of
414 enzyme, we have achieved the development of a sensor with high sensitivity for the
415 analyte. This sensor also reports sufficient selectivity to perform the measurements
416 in real samples without any complex pre-treatment of the sample. What is more, no
417 special reagents nor equipment is required. The combination of the potentiometric
418 detection with paper-based sensors and the use of a Nafion membrane which allows
419 direct detection of hydrogen peroxide makes this system a low-cost alternative for

conventional methods. For example, from an instrumental point of view, an existing pH-meter device or a simple voltmeter could be used to monitor the signal."This work provided the basis for taking the analysis out of the laboratory, which can be an improvement for the food industry as well as for the wine industry. We are currently working on the enzyme immobilization method to reach direct potentiometric measurements (without any treatment, dilution etc.). Eventually, the versatility of the approach could be demonstrated by the incorporation of other enzymes of great interest for the agro-food field.

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References

Bakker, E., & Bu, P. (2000). Selectivity of potentiometric ion sensors. *Analytical Chemistry*, *72*(6), 1127–1133.

Bakker, E., & Pretsch, E. (2007). Modern potentiometry. *Angewandte Chemie - International Edition*, *46*(30), 5660–5668.

Bankar, S. B., Bule, M. V., Singhal, R. S., & Ananthanarayan, L. (2009). Glucose oxidase - An overview. *Biotechnology Advances*, *27*(4), 489–501.

Batista, K. A., Marques, F., Yamashita, F., & Flávia, K. (2013). Lipase entrapment in PVA / Chitosan biodegradable fi lm for reactor coatings. *Materials Science &*

Engineering C, *33*, 1696–1701.

Brady, D., & Jordaan, J. (2009). Advances in enzyme immobilisation. *Biotechnology Letters*, *31*, 1639–1650.

Brena, B., & Batista-Viera, F. (2013). Immobilization of enzymes: a literature survey. In J. M. Guisan (Ed.), *Methods in Biotechnology: Immobilization of Enzymes and Cells* (Vol. 1051, pp. 15–31). Springer.

Galant, A. L., Kaufman, R. C., & Wilson, J. D. (2015). Glucose : Detection and analysis. *Food Chemistry*, *188*, 149–160.

Hall, M. B., & Keuler, N. S. (2009). Factors affecting accuracy and time requirements of a glucose oxidase-peroxidase assay for determination of glucose. *Journal of AOAC International*, *92*(1), 50–60.

Janata, J. (2009). *Principles of chemical sensors*. *Principles of Chemical Sensors*.

Johnson, G., Lambert, C., Johnson, D. K., & Sunderwirth, S. G. (1964). Colorimetric determination of glucose, fructose and sucrose in plant materials using a combination of enzymatic and chemical methods. *Agricultural and Food Chemistry*, *12*(3), 216–219.

Kabasakalis, V., Siopidou, D., & Moshatou, E. (2000). Ascorbic acid content of commercial fruit juices and its rate of loss upon storage. *Food Chemistry*, *70*(3), 325–328.

Kumar, H. M. P. N., Prabhakar, M. N., Prasad, C. V., Rao, K. M., Kumar, T. V. A., Rao, K. C., & Subha, M. C. S. (2010). Compatibility studies of chitosan / PVA

blend in 2 % aqueous acetic acid solution at 30 ◦ C. *Carbohydrate Polymers*, *82*(2), 251–255.

Kumar, S. D., Kulkarni, A. V, Dhaneshwar, R. G., & D'Souza, S. F. D. (1994). Potentiometric studies at the glucose oxidase enzyme electrode. *Bioelectrochemistry and Bioenergetics*, *34*, 195–198.

Liu, P. (2003). *World markets for organic citrus and citrus juices*. *FAO Commodity and trade policy research*.

Mannino, S., & Wang, J. (1992). Electrochemical methods for food and drink analysis. *Electroanalysis*, *4*, 835–840.

Mao, X.-L., Wu, J., & Ying, Y.-B. (2008). Application of electrochemical biosensors in fermentation. *Chinese Journal of Analytical Chemistry*, *36*(12), 1749–1755.

Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., & Fernandez-Lafuente, R. (2007). Improvement of enzyme activity, stability and selectivity via immobilization techniques. *Enzyme and Microbial Technology*, *40*(6), 1451–1463.

Mignani, A., Scavetta, E., & Tonelli, D. (2006). Electrodeposited glucose oxidase/anionic clay for glucose biosensors design. *Analytica Chimica Acta*, *577*(1), 98–106.

Ming, J., Yu, W., Lang, T., & Chien, M. (2004). Evaluation of chitosan / PVA blended hydrogel membranes. *Journal of Membrane Science*, *236*, 39–51.

Monosik, R., Stredansky, M., Tkac, J., & Sturdik, E. (2012). Application of Enzyme

484 Biosensors in Analysis of Food and Beverages. *Food Analytical Methods*, *5*(1),
485 40–53.

486 Neethirajan, S., & Jayas, D. S. (2010). Nanotechnology for the food and
487 bioprocessing industries. *Food and Bioprocess Technology*, *4*(1), 39–47.

488 Novell, M., Guinovart, T., Blondeau, P., Rius, F. X., & Andrade, F. J. (2014). A
489 paper-based potentiometric cell for decentralized monitoring of Li levels in
490 whole blood. *Lab on a Chip*, *14*(7), 1308.

491 Novell, M., Parrilla, M., Crespo, G. A., Rius, F. X., & Andrade, F. J. (2012). Paper-
492 based ion-selective potentiometric sensors. *Analytical Chemistry*, *84*(11),
493 4695–702.

494 Parrilla, M., Cánovas, R., & Andrade, F. J. (2016). Enhanced potentiometric
495 detection of hydrogen peroxide using a platinum electrode coated with Nafion.
496 *Electroanalysis*, pp. 1–9.

497 Parrilla, M., Cánovas, R., & Andrade, F. J. (2017). Paper-based enzymatic
498 electrode with enhanced potentiometric response for monitoring glucose in
499 biological fluids. *Biosensors and Bioelectronics*, *90*, 110–116.

500 Romero, M. R., Ahumada, F., Garay, F., & Baruzzi, A. M. (2010). Amperometric
501 biosensor for direct blood lactate detection. *Analytical Chemistry*, *82*(13),
502 5568–5572.

503 Sheldon, R. A. (2007). Enzyme immobilization : the quest for optimum
504 performance. *Advanced Synthesis & Catalysis*, *349*, 1289–1307.

505 Spreen, T. H. (2001). Proyecciones de la producción y consumo mundial de los
506 cítricos para el 2010. In *2001 China/FAO Simposio sobre cítricos* (pp. 5–12).

507 Srinivasa, P. C., Ramesh, M. N., Kumar, K. R., & Tharanathan, R. N. (2003).
508 Properties and sorption studies of chitosan – polyvinyl alcohol blend films.
509 *Carbohydrate Polymers*, *53*, 431–438.

510 Sung, W. J., & Bae, Y. H. (2006). Glucose oxidase, lactate oxidase, and galactose
511 oxidase enzyme electrode based on polypyrrole with polyanion/PEG/enzyme
512 conjugate dopant. *Sensors and Actuators, B: Chemical*, *114*(1), 164–169.

513 Tasca, F., Timur, S., Ludwig, R., Haltrich, D., Volc, J., Antiochia, R., & Gorton, L.
514 (2007). Amperometric biosensors for detection of sugars based on the
515 electrical wiring of different pyranose oxidases and pyranose dehydrogenases
516 with osmium redox polymer on graphite electrodes. *Electroanalysis*, *19*(2–3),
517 294–302.

518 Timur, S., & Telefoncu, A. (2004). Acetylcholinesterase (AChE) electrodes based
519 on gelatin and chitosan matrices for the pesticide detection. *Artificial Cells,*
520 *Blood Substitutes, and Immobilization Biotechnology*, *32*(3), 427–42.

521 Trinder, P. (1969). Determination of blood glucose using an oxidase-peroxidase
522 system with a non-carcinogenic chromogen. *Journal of Clinical Pathology*,
523 *22*(2), 158–61.

524 Trivedi, U. B., Lakshminarayana, D., Kothari, I. L., Patel, N. G., Kapse, H. N.,
525 Makhija, K. K., … Panchal, C. J. (2009). Potentiometric biosensor for urea

526 determination in milk. *Sensors and Actuators, B: Chemical*, *140*(1), 260–266.

527 Usman Ali, S. M., Nur, O., Willander, M., & Danielsson, B. (2010). A fast and
528 sensitive potentiometric glucose microsensor based on glucose oxidase
529 coated ZnO nanowires grown on a thin silver wire. *Sensors and Actuators, B:*
530 *Chemical*, *145*(2), 869–874.

531 Walker, R., & Lupien, J. R. (2000). Glutamate safety in the food supply the safety
532 evaluation of monosodium glutamate. *The Journal of Nutrition*, 1049–1052.

533 Wingard, L B; Castner, J F; Shang, J Y; Wolfson, S K; Drash, A L; Liu, C. C.
534 (1984). Immobilized Glucose Oxidase in the Potentiometric Detection of
535 Glucose. *Applied Biochemistry and Biotechnology*, *9*, 95–104.

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