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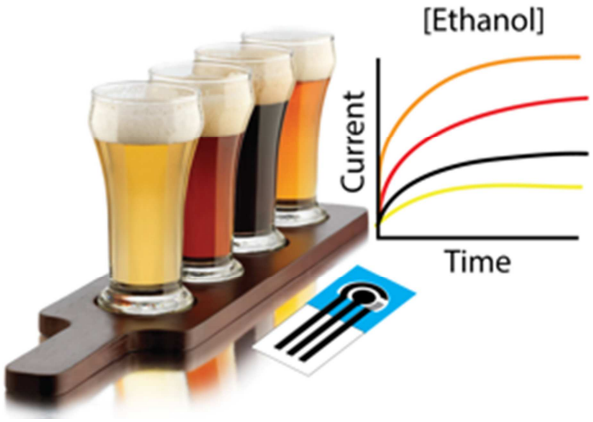
Graphical abstract



A paper-based nanomodified electrochemical biosensor for ethanol detection in beers

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Abstract

Herein, we report the first example of a paper-based screen-printed biosensor for the detection of ethanol in beer samples. Common office paper was adopted to fabricate the analytical device. The properties of this paper-based screen-printed electrode (SPE) were investigated by cyclic voltammetry, electrochemical impedance spectroscopy, and scanning electron microscopy and compared with the well-established polyester-based SPEs as well. Paper demonstrated similar properties when compared with polyester, highlighting suitability towards its utilization in sensor development, with the advantages of low cost and simple disposal by incineration. A nanocomposite formed by Carbon Black (CB) and Prussian Blue nanoparticles (PBNPs), namely CB/PBNPs, was utilized as an electrocatalyst to detect the hydrogen peroxide generated by the enzymatic reaction between alcohol oxidase (AOx) and ethanol. After optimizing the analytical parameters, such as pH, enzyme, concentration, and working potential, the developed biosensor allowed a facile quantification of ethanol up to 10 mM (0.058 $\%_{vol}$), with a sensitivity of 16.2 $\mu A/mM\ cm^2$ (2793 $\mu A/\%_{vol}\ cm^2$) and a detection limit equal to 0.52 mM (0.003$\%_{vol}$). These

satisfactory performances rendered the realized paper-based biosensor reliable over the analysis of ethanol contained in four different types of beers, including Pilsner, Weiss, Lager, and alcohol-free. The proposed manufacturing approach offers an affordable and sustainable tool for food quality control and for realization of different electrochemical sensors and biosensors as well.

Keywords: Paper-based-screen-printed electrode; wax-printing; Carbon black; Prussian blue nanoparticles; Ethanol oxidase.

Introduction

Food process monitoring and quality control of food products are huge issues in the agri-food sector, as highlighted by Hamburg in her editorial: "ensuring the safety and quality of food and medical products has never been more complicated" [1]. Thus, methods capable to provide rapid, specific, and easy responses are required. Besides the traditional methods such as chromatography, immunochromatography, mass spectrometry, NMR, PCR, and light-dependent approaches (UV, IR, CD, fluorescence) [2,3], several research activities in analytical chemistry have recently focused, on facile, cost-effective, miniaturized, and sustainable approaches, particularly toward the analysis of the most consumed products such as food and beverages [4,5]. In a report conducted in 2013, it was estimated that the global beer consumption reached 188.81 billion liters in 2013, up 0.5% from 2012, representing its 28th consecutive year of growth [6]. After water and tea, respectively being the 1st and the 2nd, beer is the 3rd most drunk beverage in the world, the 1st when just considering the alcoholic ones [7]. Ethanol content is one of the most important elements of beer and it is used to ensure the authenticity of the beer, thus the development of analytical methods which allow rapid and affordable detection of ethanol in the beer is highly required. A reliable detection can improve not only the confidence of consumers, but can also be a useful tool for process control. Indeed, in the case of beer production, companies need methodologies to classify beers in their categories (Pilsner, Lager, Weiss, etc.), depending on several properties, i.e. alcoholic strength, color, flavor,

sugars content, bitterness, origin, etc. Furthermore, food authorities ask for sustainable analytical methods capable to confirm the quality of the labeled foodstuffs and beverages [8-10]. Among the ingredients that characterize a beer, ethanol is a strategic compound in revealing the storing condition of the beer and in developing new-product such as low-alcoholic beers. Electroanalysis is well established as a technique for facile detection of ethanol. In spite of the well-known reference methods, the electroanalytical methods coupled with the breakthrough in nanomaterials are living a *Electronalysis Renaissance* as claimed by Escarpa [11]. Definitely, the synthesis and application of nanosized materials have boosted electrochemical devices' miniaturization, avoiding performance losses. Nanomaterials and nanoscale systems have unquestionably impacted the sensing and diagnostic platform field, as well as other areas of electrochemistry such as batteries, fuel cells, and solar cells. [12-14]. Literature is highly oriented towards the finding of easy procedures to obtain carbonaceous, metal, and polymeric micro, nano, and metamaterials capable to provide a cornucopia of electroanalytical devices. Brett's research group utilized a carbon film electrode (CFE) modified with multiwalled carbon nanotubes (MWCNTs) to detect ethanol by using an alcohol oxidase (AOx)-based amperometric biosensor, highlighting the importance in using MWCNTs able to enhance the sensitivity 20-fold with respect to the biosensor without the nanomodifier [15]. The presence of MWCNTs allowed them to detect the enzymatic by-product (hydrogen peroxide, H_2O_2) at a less negative potential with respect to the unmodified CFE. Pingarrón and his group also exploited the unique properties of MWCNTs, by fabricating a bulky working electrode constituted by gold nanoparticles, MWCNTs and Teflon as the binder. In this case, ethanol dehydrogenase (ADH) in presence of its cofactor (NAD^+) was used as sensing bioelement which produced the electroactive NADH. They applied this biosensor to detect the ethanol up to 1 mM, in commercial beers by utilizing a 10 mL-stirred batch amperometry [16]. Mentana and colleagues immobilized AOx onto a gold electrode modified electropolymerized polypirrole for simultaneous monitoring of ethanol and glucose in alcoholic drinks during fermentation. By optimizing the thickness of the PPY film, they were able to obtain a perm-

selective membrane capable to "reject" the interfering species during measurements, detecting selectively ethanol with a linear range up to 1.5 M and a detection limit of 5 mM [17]. Recently, Sharifi et al. [18] developed an ADH-based stirred amperometric biosensor by modifying a glassy carbon electrode with nickel oxide nanoparticles (NiOxNPs) and Nafion. The electrocatalytic task of these NPs allowed the detection of reduced enzymatic cofactor (NADH) detection at 0.45 V (vs. Ag/AgCl). This nanomodified biosensor showed an accurate quantification of ethanol in several beverages such as fruit juice and non-alcoholic beer. Despite these achievements, the scientific community is currently involved in a continued attempt to satisfy the cost-lowering demand for rapid, disposable, user-friendly, and sustainable sensing platforms.

Although the use of the above-cited novel (nano)materials represents a big deal in electroanalysis, their marriage with easy manufacturing techniques and affordable substrates is highly commendable. In the last decade, paper has been involved in the electroanalytical field [19,20]: it represents an ideal platform for sensing devices in analytical chemistry, being light, biocompatible, and suitable for most of the (bio)assays. Furthermore, it is easy to use, store, transport, modify and safely dispose of by incineration [21,22]. A major advantage of paper consists of tackling the rise of costs especially in developing countries with limited resources. Requirements in analysis, however, are wide: paper has shown to bridge diverse analytical techniques to self-testing procedures depending on the specific need. Due to its white color, paper is highly amenable to colorimetric [24,25], fluorimetric [26], or chemiluminescent [27] assays which can be detected by the naked eye or just by involving LED as well as a cellular phone [28,29]. Switching to electrochemical tests, Whitesides's group convincingly demonstrated the possibility to combine an electrochemical paper-based strip with a commercially available glucometer, i.e. True Track blood™ (CVS/Pharmacy), adaptable to many analytes other than glucose, e.g. cholesterol, lactate, ethanol [30]. Low cost manufacturing approaches such as wax printing and screen-printing technologies perfectly matched chromatographic Whatman® paper utilized in Whitesides's research, allowing an easy production almost everywhere. Paper allowed the storage of all the chemical reagents in the testing area,

avoiding chemical management at end-user stage. As shown, this procedure has provided a concrete opportunity in lowering the price from about $ 0.5-1 to $ 0.014 per strip, by just using paper instead of a plastic substrate, without thinking of the waste removal requirements. However, paper based material includes a plethora of cellulose-based substrates, depending on the purity, chemical treatment, structure, fibers density, porosity, and obviously, on the cost [31-33]. In 2012, it was reported that an approach combining screen-printing, wax printing, and office paper, to fabricate silver ink electrochemical devices, made fabrication costs fall by 96.7% whether compared to that employed by using chromatography paper [34]. Encouraged by this promising data, we have chosen to join the well-owned manufacturing facilities of screen-printing and wax-printing technologies to office paper. Here, we developed the first paper-based electrochemical biosensor to detect ethanol in commercial beers. AOx was chosen as the bio-recognition element able to oxidize ethanol, giving hydrogen peroxide (H_2O_2) as one of the by-products. Ethanol was indirectly quantified by monitoring the concentration of the produced H_2O_2. In order to sensitively detect H_2O_2, we exploited the use of graphite-based screen-printed electrodes (SPEs) modified with a nanocomposite formed by Carbon Black and Prussian Blue nanoparticles (CB/PBNPs). The CB/PBNPs nanocomposite was previously studied by our group highlighting its extremely advantageous properties due to the nano-sized PBNPs, grown onto CB, towards the H_2O_2 electrochemical determination [35]. The device properties were carefully investigated through the use of scanning electron microscopy (SEM), bending tests, cyclic voltammetry (CV), chronoamperometry, and electrochemical impedance spectroscopy (EIS). This paper-based electrochemical biosensor was successfully challenged towards detection of ethanol in standards and real samples, demonstrating a high accuracy level in the measurements. The effectiveness of this office-paper biosensor perfectly satisfied the requirements of a quick, affordable, accurate, disposable, and sustainable platform, particularly required in resource-limited environments.

2. Materials and methods

2.1 Reagents and equipments

Potassium ferricyanide ($K_3[Fe(CN)_6]$), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) potassium dihydrogen phosphate, alcohol oxidase (AOx) from *Pichia pastoris* (1470 U/mL), Nafion®, glutaraldehyde, bovine serum albumin (BSA), acetic acid, glucose, ascorbic acid, and uric acid were purchased from Sigma-Aldrich (USA). Commercial beers were bought from the local supermarket. All the solutions were prepared in distilled water. Voltammetric, chronoamperometric and impedimetric measurements were performed using a portable PalmSens Instrument (PalmSens, Netherlands) in connection with a laptop. In impedimetric measurements, a sinusoidal voltage perturbation of 10 mV amplitude was applied over the frequency range 100 kHz to 0.1 Hz with 10 measurement points per frequency decade. For the fitting of the data obtained by EIS, Z-views software (Scribner Associates, Inc.) was used directly connected with the PS3 software.

The morphology was investigated by observation at field emission scanning electron microscopy (FEG-SEM, Leo Supra 35, UK).

2.2 Fabrication of paper-based SPEs

Two types of office paper were used as a substrate for the fabrication of the paper-based SPEs: Fabriano Copy 2 (80 g/m^2) and Fabriano Multip@per (100 g/m^2). Firstly, a drawing software (Adobe Illustrator) was used to design the pattern on the white background (Fig.1A). Then, the specific pattern was printed onto paper by means of ColorQube 8580 office wax-printer from Xerox (USA) (Fig.1B). Subsequently, the wax-printed paper was placed in an oven for 4 min at 100 °C (Fig.1C), allowing the wax to diffuse through the paper, producing a hydrophobic confinement (sky blue) around the hydrophilic semicircle (white). After the hydrophilic area was defined, the three-electrode system was manually screen-printed (Fig.1D) using an Ag/AgCl ink (Electrodag 477 SS) to print the pseudo-reference electrode, and a graphite-based ink (Electrodag 421) to print the

working and counter electrodes. All the conductive-inks were purchased from Acheson (Italy). The performance of the paper-based SPEs were compared with "classic" polyester-based SPEs that were fabricated by using a 245 DEK (UK) semi-automatic screen-printing machine.

(Figure 1)

2.3 CB/PBNPs nanocomposite synthesis

Briefly, 1 g of CB powder was suspended in 10 mL of a solution containing 0.1 M $K_3[Fe(CN)_6]$ 0.1 mol/L in 0.01 M HCl. This suspension was stirred for 5 min in order to promote the adsorption onto CB. Then, to form PB, 10 mL of 0.1 M $FeCl_3 \cdot 6H_2O$ in 10 mmol/L HCl were added, and the mixture was stirred for 10 min. CB with adsorbed PB, referred as CB/PBNPs, was collected by centrifugation and washed with 0.01 M HCl until the washing solution became colorless (5 cycles were necessary to obtain a colorless supernatant). The precipitate was collected and dried in oven at 100 °C for 1.5 h. 10 mg of CB/PBNPs dry powder were dispersed into 10 mL of a 50% N,N-Dimethylformamide (DMF) aqueous solution, and then sonicated for 60 min at 59 kHz. The dispersion was stored in the dark at room temperature and remained stable for more than 1 month.

2.4 Ethanol paper-based biosensor

A drop casting approach was utilized in order to modify the bare paper-based SPEs (Fig. 1E). Firstly, SPEs were modified with 2 μL of CB/PBNP dispersion. After the solvent vaporization, the enzyme was immobilized by using the procedure optimized by Piermarini et al. [36] with a slight modification. Briefly, the enzymatic mixture was composed by 25 μL of commercial AOx solution, already containing 4% (w/v) BSA, 8 μL of 5% (v/v) Nafion® and 5 μL of 2.5% (v/v) glutaraldehyde. In order to select the best amount for the modification, 1, 2, and 4 μL of the resulting mixture were drop cast onto the working electrode and allowed to dry for 1 h.

3. Results and discussion

3.1 Comparison among electrodes screen-printed on polyester, 80 g/m^2 office-paper and 100 g/m^2 office-paper

Prior to beginning the development of the biosensor, office paper SPEs (manufactured using 80 and 100 g/m^2 office papers) were finely characterized in order to understand their electrochemical behavior when compared with the widely utilized polyester-based SPE. Cyclic voltammetry experiments were carried out in order to characterize the electrochemical properties of the different SPEs. The measurements were performed by covering the SPEs with a 100 μL-drop of solution. 50 and 200 μL were also used as working volume, but the former amount was not enough to entirely cover the testing area and the latter did not produce a signal difference when compared with 100 μL. $[Fe(CN)_6]^{4-/3-}$ couple was chosen as the redox-probe to perform this study. The experiments were carried out by varying the scan rate from 20 to 1000 mV/s for each kind of electrode. As displayed in Fig. 2 there is no difference in term of peak-to-peak separation (around 500 mV) when paper substrates were used.

(Figure 2)

By plotting the height of peaks versus the square root of the scan rate (i_P vs (scan rate, v)$^{0.5}$), information related to the diffusion mechanism at surface was obtained [37]. The working electrode diameter of the SPEs fabricated on paper is 0.4 cm, while the diameter of the working electrode utilized for the polyester-based is equal to 0.3 cm with geometric area of 0.126 and 0.071 cm^2, respectively. Taking into account the different geometric areas, in Fig. 2 the behavior of current density *vs* potential of paper-based SPE and polyester-based SPEs was reported. The normalized

peak intensities were linear towards the square root of the scan rate, indicating a diffusion-controlled mechanism in the electrochemical process at the electrode (inset i of Fig. 2). The diffusional mechanism was also confirmed by plotting the log (i_P) vs. log (scan rate,*v*), (inset ii of Fig. 2). Slopes equal to 0.4, 0.4 and 0.45 were obtained for paper-, 80 and 100 g/m^2, and polyester-based SPE, which are close to the theoretical value of 0.5 [38].

Furthermore, the morphology of the SPEs was examined via SEM analysis.

(Figure 3)

As shown in Fig. 3A-F, micrographs at different magnifications did not reveal significant differences between the three SPE typologies. The electrochemical impedance spectroscopy was also adopted to evaluate the feasibility to exploit office paper as an effective substrate. Using $[Fe(CN)_6]^{4-/3-}$ couple as redox probe, the electron transfer resistance (R_{ct}) was determined for the different sensing platforms. Fitting of spectra was done using the equivalent electrical circuit showed in the inset of Fig. 3G which comprises the electrolyte resistance (Re) in series with a parallel combination of the interfacial charge transfer resistance (R_{ct}), the diffusion of the analytes in solution, corresponding to Warburg impedance (Zw), and a constant phase element (CPE). The office-paper 80 g/cm^2 SPE was characterized by R_{ct} equal to 17061 ± 98 Ω and the 100 g/m^2 was consistent with a R_{ct} equal to 16000 ± 90 Ω, which were significantly higher than the value found (8597 ± 41 Ω) using a polyester-based SPE (Fig.3C), demonstrating the hindering of electron-transfer of the electrochemical probe (ferro/ferricyanide) using a paper based sensor. The characterization experiments carried out confirmed however the possibility to use office paper to build up the electrochemical platform. In terms of electrochemical and morphologic features, office paper behaved roughly similar to the better-known polyester, and the differences observed by EIS experiments, due to its fine sensitivity (higher than voltammetric ones), could be the result of ink adhesion to the particular substrate. The 80 g/m^2 is commonly utilized office paper, and it is

cheaper than the 100 g/m^2 one; it was identified as a suitable substrate towards the development of an electrochemical sensor, and it was interrogated by performing mechanical stress experiments. After 50 bending cycles, the paper-based sensor did not exhibit a decrease in the recorded voltammetric peak current and no change in the oxidative peak potential when $[Fe(CN)_6]^{4-/3-}$ couple was used as a redox probe (Fig.3H). This achievement highlights the relevance in using this kind of flexible sensor especially towards their potential application in those environments where mechanical stress could be encountered, i.e. wearable items.

3.2 Detection of H_2O_2 by means of CB/PBNPs modified 80 g/m^2 paper-based SPE

After 80 g/m^2 office paper was selected as the support to develop the ethanol biosensor, the working electrode was finely modified in order to obtain a suitable platform for the enzymatic by-product detection. Ethanol was electrochemically detected by monitoring H_2O_2 produced during the reaction between ethanol and molecular oxygen (O_2) catalyzed by ethanol oxidase (AOx), Eq. 3.

$$\text{Ethanol} + O_2 \xrightarrow{\text{AOx}} \text{Acetaldehyde} + H_2O_2 \quad (3)$$

As widely reported in literature, ferric hexacyanoferrate, or Prussian Blue (PB), is undoubtedly recognized as the best low-potential mediator for H_2O_2 detection [39,40]. In our recent work, we tailored the dimension of PBNPs by using CB [31]. We observed that the smallest PBNPs were obtained by involving CB as nucleation site and, furthermore, the lowest-diameter NPs (around 20 nm) allowed to reach the lowest limit of detection related to H_2O_2. Herein, we modified the working electrode surface with the above-mentioned nanocomposite, namely CB/PBNPs, in order to enhance the performance of the paper-based SPE. The electrode was modified by an easy drop-casting procedure using just 2 μL of the dispersion.

(Figure 4)

As clearly visible in Fig.4, this modification allowed to sensitively detect hydrogen peroxide, which was impossible using an unmodified SPE. The CB/PBNPs-paper-SPE was capable to detect H_2O_2 linearly up to 10 mM with a sensitivity of 51.9 μA/mM cm^2, using data obtained from a calibration curve equal to $y = (3.63 \pm 0.19)\ x + (6.29 \pm 1.18)$, where y and x indicate, respectively, the current expressed in μA and the H_2O_2 concentration in mM. It was possible to calculate the detection limit by using the definition of $3\sigma_b$/slope, where σ_b represents the standard deviation calculate on blank measurements and the slope is taken from the calibration curve. The detection limit was found to be equal to 0.60 mM, and it is almost comparable with a paper-based PB sensor previously reported by Dungchai and colleagues [19]. These satisfactory results indicate the possibility to exploit this platform as an effective tool towards the realization of an ethanol biosensor employing H_2O_2 as the electroactive readout.

3.3 Development of the paper-based ethanol biosensor

The promising electrochemical reduction performance of CB/PBNPs nanocomposite toward hydrogen peroxide was used to develop an amperometric biosensor based on the alcohol oxidase enzyme. As previously written in the “Materials and methods” section, the enzyme was immobilized onto the working electrode surface via a cross-linking procedure. Parameters such as the pH, enzymatic units, and working potential were sequentially optimized in order to achieve the best operative conditions. A concentration of 4 mM (0.023 $\%_{vol}$) ethanol was chosen for these studies, and each value represents the mean of three different electrodes as shown in Fig. 5.

(Figure 5)

The first optimization regarded the selection of the optimum working pH. Measurements were carried out in phosphate buffered solutions varying the pH in a range comprised between 6 and 8.

As shown in Fig. 5, the best sensitivity was achieved at pH 7.4. These findings were in good agreement both with other papers [41,42] and with the specification sheet released from the enzyme manufacturer (Sigma Aldrich) [43]. A value of pH equal to 7.4 was selected in the successive experiments.

Subsequently, the amount of alcohol oxidase enzyme to be immobilized onto the working electrode was optimized. To carry out this study, paper-based electrodes were modified with different amounts of the enzymatic mixture: the electrodes were modified with 1, 2, and 4 U of enzyme, respectively 1, 2, and 4 μL of the enzymatic mixture reported in section 2.4. As illustrated in Fig. 5, the response reached its maximum value when the electrode was drop casted with 2 μL (2 U), and it was selected as the optimal amount. Experimentally, an excessively thick layer of enzymatic membrane hindered the diffusion of the produced H_2O_2 at the electrode.

The last optimization involved the study of the applied potential. The working potential, versus an Ag/AgCl pseudo-reference, was varied between 0 and -0.2 V. The current reached its maximum value by applying a potential of -0.1 V (vs. Ag/AgCl) which was selected as the working potential. Following these optimizations, ethanol was rapidly (40 s) detected in standard solution.

Ethanol concentration was linearly measured up to 10 mM (0.058 $\%_{vol}$) in 100 μL of 0.05 M phosphate-buffered solution containing 0.1 M KCl at pH 7.4 deposited on the hydrophilic area of the sensor. The chronoamperometric records are displayed in Fig. 6.

(Figure 6)

The cathodic current increased linearly with ethanol concentration from 0 to 10 mM with a sensitivity of 16.2 μA/mM cm^2. Although these measurements were taken without stirring the solution, thanks to the outstanding features of the CB/PBNPs nanocomposite, the sensitivity achieved compared positively with other ethanol biosensors previously reported [17,18,44,45]. The limit of detection calculated as LOD = $3\sigma_b$/slope was found to be 0.52 mM (0.003 $\%_{vol}$) and the

repeatability, evaluated using ethanol 4 mM (0.023 $\%_{vol}$), was 7% (n=3). The shelf-life was evaluated after the biosensor was stored in the dark at 4°C. The biosensor showed no significant decrease of initial activity after a storage of 3 weeks, but the response decreased of 50% after the fourth week, Figure 7.

(Figure 7)

The working stability and the reusability of biosensor were also evaluated. After the paper-based biosensor was rinsed and dried, it resulted twisted, and in presence of 4 mM (0.023 $\%_{vol}$) no change of signal was recorded (Supporting Information, Figures S1 and S2). This could be ascribable to the rinsing procedure and the loss of the enzyme from the electrode surface. However, the biosensor was conceived as a one-shot device due to its low cost.

3.4 Interference study

In order to investigate the selectivity of the developed biosensor toward the detection of ethanol, different substances were utilized to perform the selectivity tests. The biosensor was interrogated in presence of methanol, acetic acid, glucose, and ascorbic acid. These compounds may be present in commercial beers due to many reasons: methanol, glucose, and acetic acid could be present as a consequence of the fermentation process, while ascorbic acid, because of its antioxidant capacity, is usually used in the foodstuff industry in order to diminish residual oxygen after packaging. As shown in Fig.8 only methanol gave an interfering response but it was not effective as much as ethanol.

(Figure 8)

3.5 Real sample measurements

The developed paper-based biosensor was interrogated towards the detection of ethanol in commercial beers. To assure the quality of a final product, it is important to evaluate if the content of ethanol reported onto the label correspond to the effective amount. In this study four different kinds of beers were analyzed, a “Lager” (Best Bräu, Poland), a “Pilsner” (Ceres Top Pilsner, Denmark), a “Weiss” (Franziskaner, Germany) and an “alcohol-free” (Tourtel, Italy) beer, whose ethanol content reported on the labels were respectively, 4.7 $\%_{vol}$ (0.805 M), 4.6 $\%_{vol}$ (0.787 M), 5 $\%_{vol}$ (0.856 M) and < 0.5 $\%_{vol}$ (0.086 M). Lager, Pilsner and Weiss beers were diluted approximately to a final concentration of 0.2 M in water, while the alcohol-free one was not diluted. To perform the quantification of ethanol in the real samples, a 100 µL-drop of sample, containing roughly 4 mM (0.023 $\%_{vol}$) of ethanol, was analyzed with the same procedure followed for standard solutions. As displayed in Table 1, the ethanol found in the three “alcoholic” beers showed very good agreement with the ethanol content declared by the producer. Regarding the “non-alcoholic” beer, the signal recorded resulted in a concentration of 0.059 M (0.322 $\%_{vol}$), relative standard deviation (RSD) = 7%. For each beer analysis, one individual paper-based biosensor was utilized using the standard addition method. In Table 1, obtained values are reported, demonstrating the good accuracy of the proposed method.

(Table 1)

4. Conclusions

This work reports the development of a paper-based electrochemical ethanol biosensor firstly applied to commercial beer products. By following facile and affordable approaches, it has been possible to realize a disposable device capable to accurately detect ethanol in different beer

typologies. The content of ethanol in commercial samples of Pilsner, Lager, Weiss, and alcohol-free beers, was analyzed with the developed biosensor, achieving a 100% correlation with the alcoholic grade reported on the label of these beers. The paper electrode was modified with the CB/PBNPs-nanocomposite and alcohol oxidase enzyme (immobilized via cross-linking) displaying a very quick and sensitive response after just 40 s. The biosensor was obtained by coupling screen-printing and wax-printing facilities, which are characterized by an extreme operative simplicity and high-customizable settings, allowing to create the most-effective experimental set-up. Furthermore, the use of common office paper as substrate for fabrication resulted in a simple, sustainable, mechanically robust, and easily waste disposable device, fundamental in the management of waste. Common office paper demonstrated its effective reliability towards the development of affordable and robust platforms, that could reveal satisfactory applications in other (bio)systems. This paper strongly established office paper as a valuable substrate in (bio)sensor development, suitable for performing analyses “on-site” and/or to be integrated into portable devices that could be subjected to mechanical stress, such as the wearable ones.

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References

[1] M.A. Hamburg, Advancing regulatory science, Science 331 (2011) 987.

391 [2] V. García-Cañas, C. Simó, M. Herrero, E. Ibáñez, A. Cifuentes, Present and future challenges in
392 food analysis: Foodomics, Anal. Chem. 84 (2012) 10150-10159.
393
394 [3] B.B. Dzantiev, N.A. Byzova, A.E. Urusov, A.V. Zherdev, Immunochromatographic methods in
395 food analysis, Trac- Trend. Anal. Chem. 55 (2014) 81-93.
396
397 [4] A.W. Martinez, S.T. Phillips, G.M. Whitesides, E. Carrilho, Diagnostics for the developing
398 world: microfluidic paper-based analytical devices, Anal. Chem. 82 (2009) 3-10.
399
400 [5] A. Escarpa, Lights and shadows on Food Microfluidics, Lab Chip 14 (2014) 3213-3224.
401
402 [6] Kirin Beer University Report Global Beer Consumption by Country in 2013
403 http://www.kirinholdings.co.jp/english/news/2014/1224_01.html.
404
405 [7] M. Nelson, The barbarian's beverage: A history of beer in ancient Europe, Routledge, 2005.
406
407 [8] M.J. Dennis, Recent developments in food authentication, Analyst 123 (1998) 151-156.
408
409 [9] A.G. Mignani, L. Ciaccheri, A.A. Mencaglia, H. Ottevaere, E.E.S. Baca, H. Thienpont, Optical
410 measurements and pattern recognition techniques for autheticating top-fermented and bottom-
411 fermented beers and predicting the alcoholic strength, Sensors 2012 IEEE 1-4.
412
413 [10] C. O'Neill, D. Houtman, S. Aupers, Advertising real beer: Authenticity claims beyond truth
414 and falsity, European Journal of Cultural Studies 17 (2014) 585-601.
415
416 [11] A. Escarpa, Food electroanalysis: sense and simplicity, The Chemical Record 12 (2012) 72-91.

[12] A. Chen, S. Chatterjee, Nanomaterials based electrochemical sensors for biomedical applications, Chem. Soc. Rev. 42 (2013) 5425-5438.

[13] S. Guo, E. Wang, Noble metal nanomaterials: controllable synthesis and application in fuel cells and analytical sensors, Nano Today 6 (2011) 240-264.

[14] L. Ma, K.E. Hendrickson, S. Wei, L.A. Archer, Nanomaterials: Science and applications in the lithium–sulfur battery, Nano Today 10 (2015) 315-338.

[15] C. Gouveia-Caridade, R. Pauliukaite, C.M. Brett, Development of electrochemical oxidase biosensors based on carbon nanotube-modified carbon film electrodes for glucose and ethanol, Electrochim. Acta 53 (2008) 6732-6739.

[16] J. Manso, M.L. Mena, P. Yáñez-Sedeño, J.M. Pingarrón, Alcohol dehydrogenase amperometric biosensor based on a colloidal gold–carbon nanotubes composite electrode, Electrochim. Acta 53 (2008) 4007-4012.

[17] A. Mentana, C. Palermo, D. Nardiello, M. Quinto, D. Centonze, Simultaneous and accurate real-time monitoring of glucose and ethanol in alcoholic drinks, must, and biomass by a dual-amperometric biosensor, J. Agric. Food. Chem. 61 (2012) 61-68.

[18] E. Sharifi, A. Salimi, E. Shams, Electrocatalytic activity of nickel oxide nanoparticles as mediatorless system for NADH and ethanol sensing at physiological pH solution, Biosens. Bioelectron. 45 (2013) 260-266.

443 [19] W. Dungchai, O. Chailapakul, C.S. Henry, Electrochemical detection for paper-based
444 microfluidics, Anal. Chem. 81 (2009) 5821-5826.
445
446 [20] A.W. Martinez, S.T. Phillips, G.M. Whitesides, E. Carrilho, Diagnostics for the developing
447 world: microfluidic paper-based analytical devices, Anal. Chem. 82 (2009) 3-10.
448
449 [21] X. Li, D.R. Ballerini, W. Shen, A perspective on paper-based microfluidics: current status and
450 future trends, Biomicrofluidics 6 (2012) 1-13.
451
452 [22] C. Parolo, A. Merkoçi, Paper-based nanobiosensors for diagnostics, Chem. Soc. Rev. 42
453 (2013) 450-457.
454
455 [23] http://bccresearch.blogspot.it/2012/04/global-point-of-care-diagnostics-
456 market.html#.VysRNGPhrR0.
457
458 [24] A.W. Martinez, S.T. Phillips, E. Carrilho, S.W. Thomas III, H. Sindi, G.M. Whitesides, Simple
459 telemedicine for developing regions: camera phones and paper-based microfluidic devices for real-
460 time, off-site diagnosis, Anal. Chem. 80 (2008) 3699-3707.
461
462 [25] R.C. Murdock, L. Shen, D.K. Griffin, N. Kelley-Loughnane, I. Papautsky, J.A. Hagen,
463 Optimization of a paper-based ELISA for a human performance biomarker, Anal. Chem. 85(2013),
464 11634-11642.
465
466 [26] Y. Ma, H. Li, S. Peng, L. Wang, Highly selective and sensitive fluorescent paper sensor for
467 nitroaromatic explosive detection, Anal. Chem. 84 (2012) 8415-8421.
468

[27] J.L. Delaney, C.F. Hogan, J. Tian, W. Shen, W, Electrogenerated chemiluminescence detection in paper-based microfluidic sensors, Anal. Chem. 83 (2011) 1300-1306.

[28] A.W. Martinez, S.T. Phillips, E. Carrilho, S.W. Thomas III, H. Sindi, G.M. Whitesides, Simple telemedicine for developing regions: camera phones and paper-based microfluidic devices for real-time, off-site diagnosis, Anal. Chem. 80 (2008) 3699-3707.

[29] A. Ozcan, Mobile phones democratize and cultivate next-generation imaging, diagnostics and measurement tools, Lab Chip 14 (2014) 3187-3194.

[30] Z. Nie, F. Deiss, X. Liu, O. Akbulut, G.M. Whitesides, Integration of paper-based microfluidic devices with commercial electrochemical readers, Lab Chip 10 (2010) 3163-3169.

[31] A.K. Yetisen, M.S. Akram, C.R. Lowe, Paper-based microfluidic point-of-care diagnostic devices, Lab Chip 13 (2013) 2210-2251.

[32] S. Cinti, D. Talarico, G. Palleschi, D. Moscone, F. Arduini, Novel reagentless paper-based screen-printed electrochemical sensor to detect phosphate, Anal. Chim. Acta 919 (2016) 78-84.

[33] S. Cinti, C. Minotti, D. Moscone, G. Palleschi, F. Arduini, Fully integrated ready-to-use paper-based electrochemical biosensor to detect nerve agents, Biosens. Bioelectron. DOI 10.1016/j.bios.2016.10.091.

[34] W.R. De Araujo, T.R.L.C. Paixao, Fabrication of disposable electrochemical devices using silver ink and office paper, Analyst 139 (2014) 2742-2747.

[35] S. Cinti, F. Arduini, G. Vellucci, I. Cacciotti, F. Nanni, D. Moscone, Carbon black assisted tailoring of Prussian Blue nanoparticles to tune sensitivity and detection limit towards H_2O_2 by using screen-printed electrode, Electrochem. Commun. 47 (2014) 63-66.

[36] S. Piermarini, G. Volpe, M. Esti, M. Simonetti, G. Palleschi, Real time monitoring of alcoholic fermentation with low-cost amperometric biosensors, Food Chem. 127 (2011) 749-754.

[37] A.J. Bard, L.R. Faulkner, Electrochemical Methods. Fundamentals and Applications (2^{nd} ed.) John Wiley & Sons, New York (2001).

[38] P.M. Hallam, C.E. Banks, Quantifying the electron transfer sites of graphene, Electrochem. Commun. 13 (2011) 8-11.

[39] A.A. Karyakin, E.E. Karyakina, L. Gorton, Amperometric biosensor for glutamate using Prussian blue-based “artificial peroxidase” as a transducer for hydrogen peroxide, Anal. Chem. 72 (2000) 1720-1723.

[40] F. Ricci, G. Palleschi, Sensor and biosensor preparation, optimisation and applications of Prussian Blue modified electrodes, Biosens. Bioelectron. 21 (2005) 389-407.

[41] M.M. Barsan, C.M. Brett, An alcohol oxidase biosensor using PNR redox mediator at carbon film electrodes, Talanta 74 (2008) 1505-1510.

[42] S.R. Chinnadayyala, A. Kakoti, M. Santhosh, P. Goswami, A novel amperometric alcohol biosensor developed in a 3rd generation bioelectrode platform using peroxidase coupled ferrocene activated alcohol oxidase as biorecognition system, Biosens. Bioelectron. 55 (2014) 120-126.

[43] http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Datasheet/6/a2404dat.pdf.

[44] M. Boujtita, J.P. Hart, R. Pittson, Development of a disposable ethanol biosensor based on a chemically modified screen-printed electrode coated with alcohol oxidase for the analysis of beer, Biosens. Bioelectron. 15 (2000) 257-263.

[45] C. Shan, H. Yang, D. Han, Q. Zhang, A. Ivaska, L. Niu, Electrochemical determination of NADH and ethanol based on ionic liquid-functionalized graphene, Biosens. Bioelectron. 25 (2010) 1504-1508.

546 **Figures**

547

548 **Figure 1**

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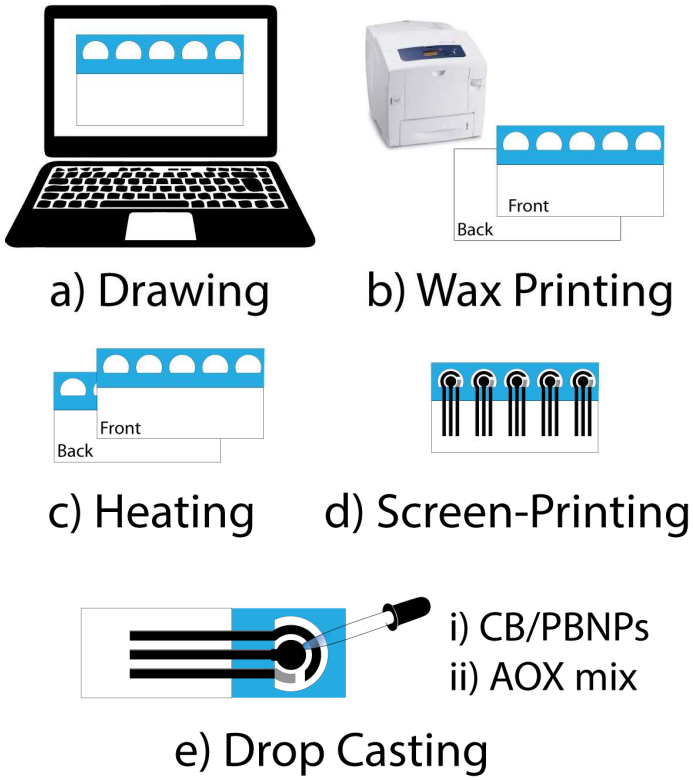

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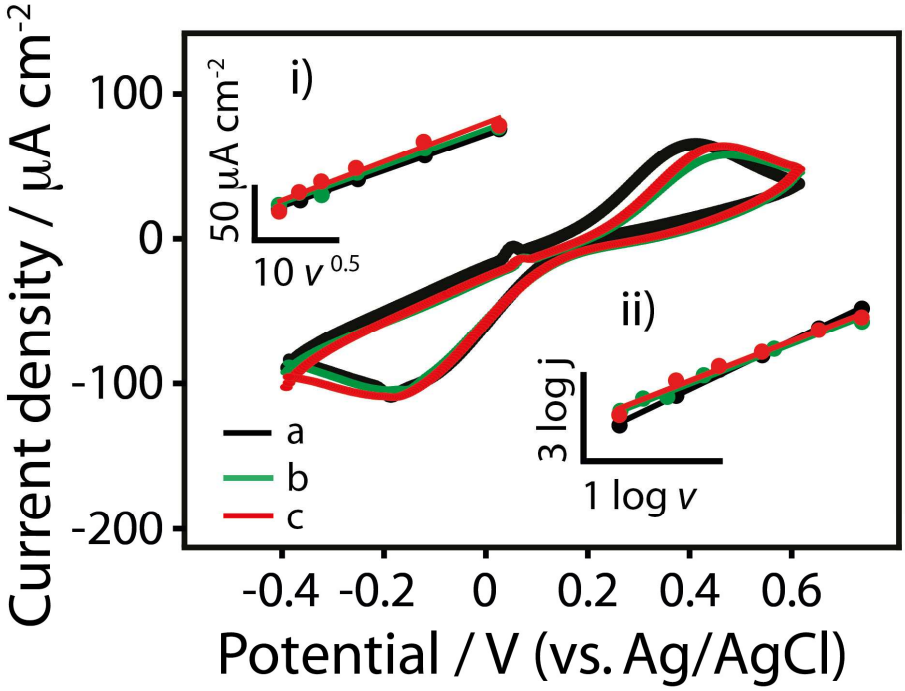
Figure 2



578 **Figure 3**

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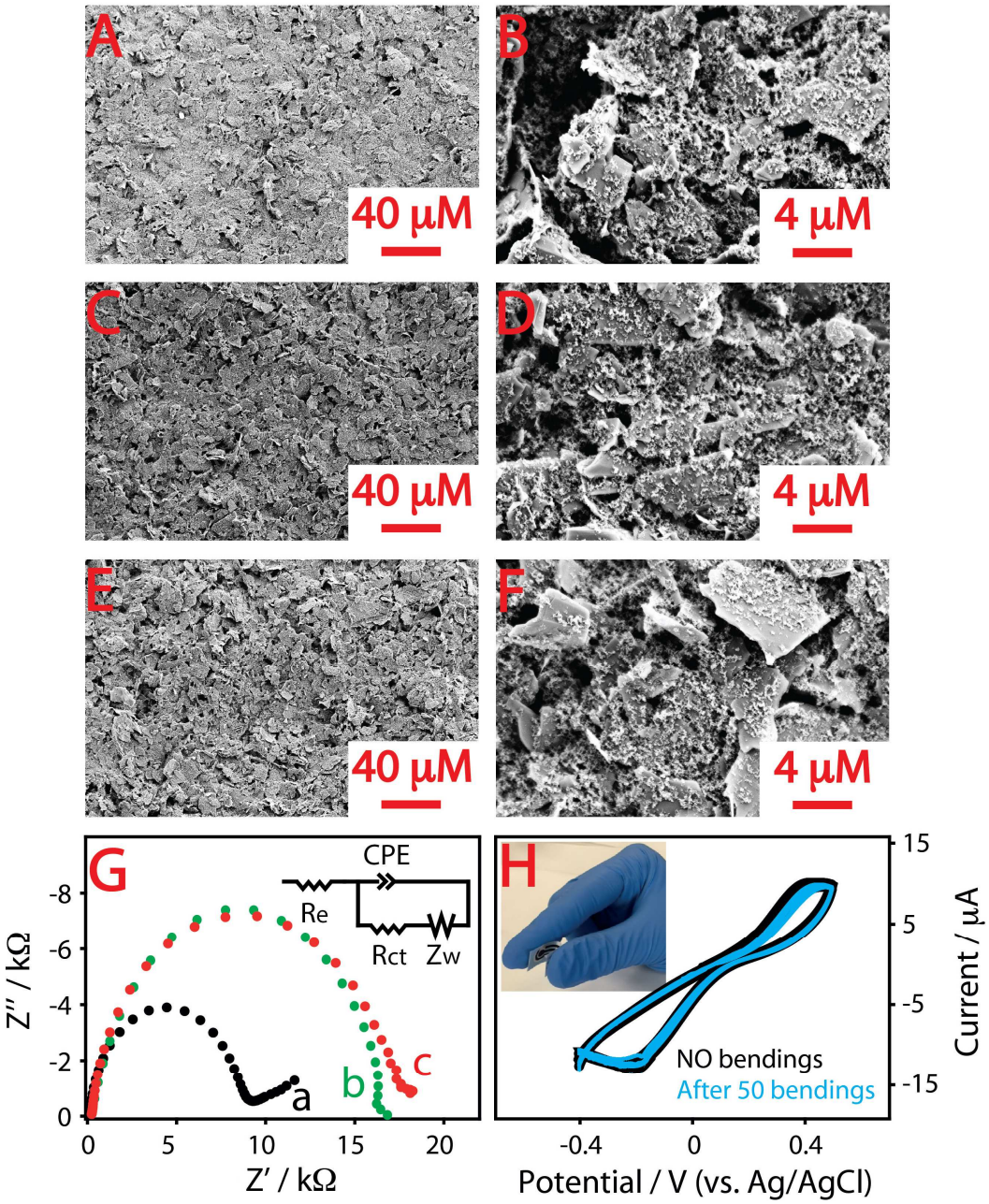

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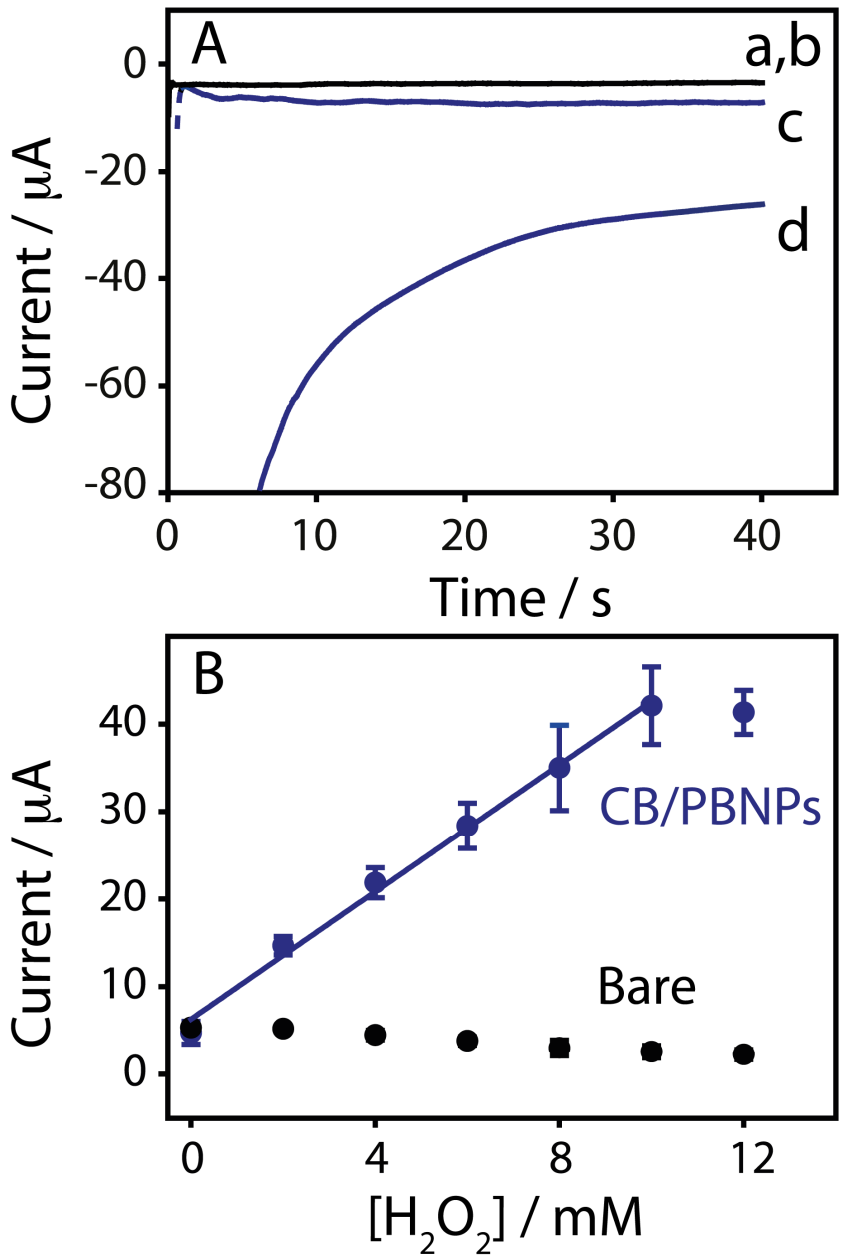
Figure 4


A
a,b
c
d
0
-20
-40
-60
-80
Current / µA
0
10
20
30
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Time / s
B
CB/PBNPs
Bare
40
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10
0
Current / µA
0
4
8
12
[H2O2] / mM

596 **Figure 5**

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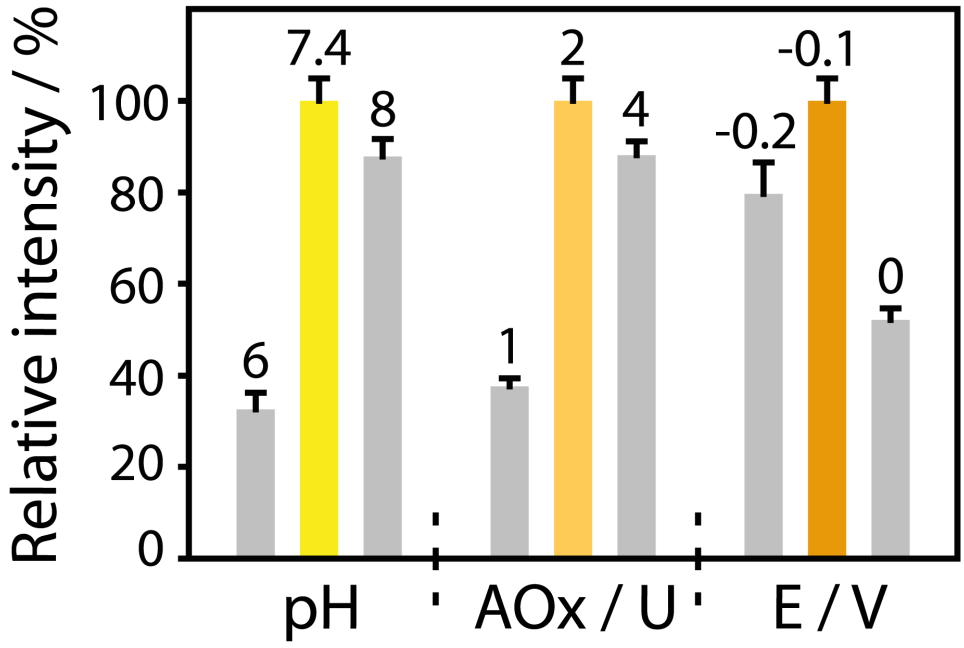

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613 **Figure 6**

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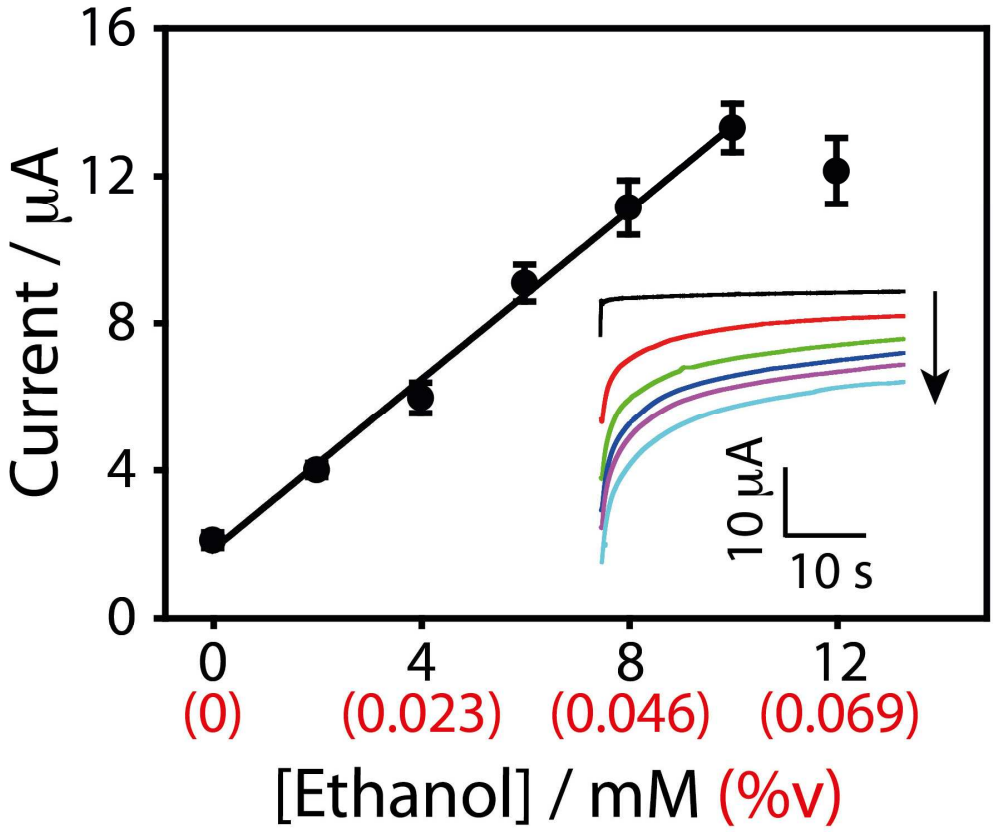

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628 **Figure 7**

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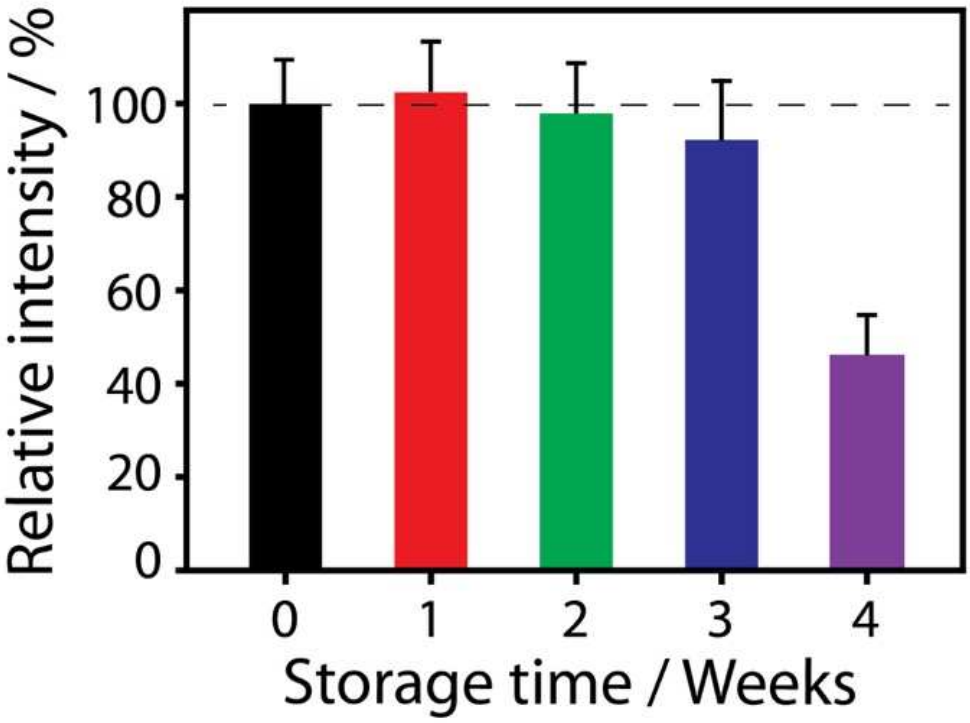

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Figure 8

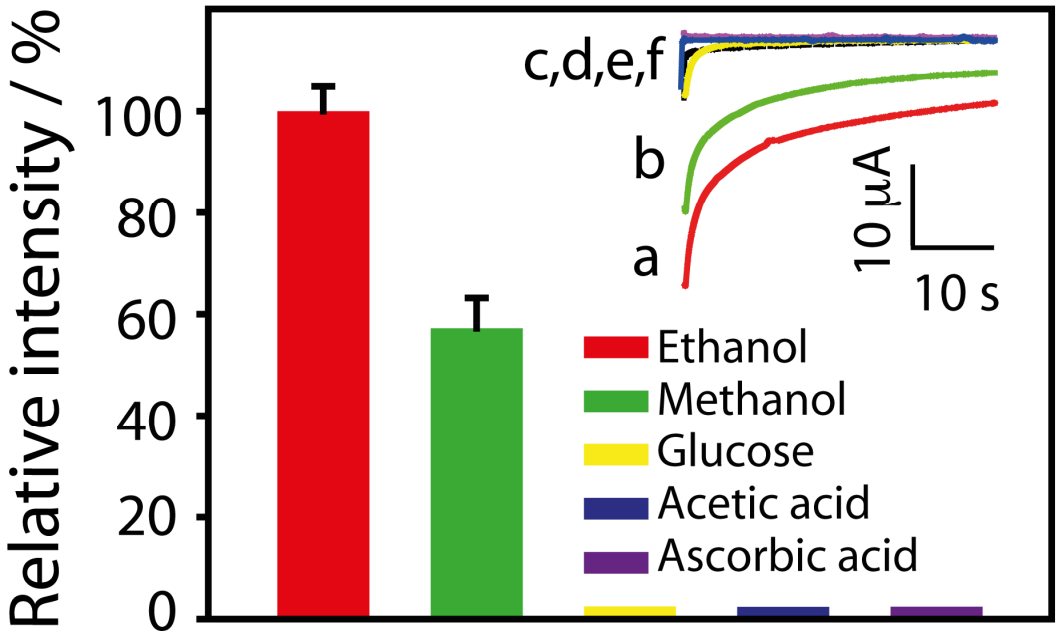

Table 1: Detection of ethanol in commercial beers.

Beer	**Lager** Best Bräu, Poland	**Weiss** Franziskaner, Germany	**Pilsner** Ceres, Denmark	**Alcohol free** Tourtel, Italy
				
Label [ethanol] / $\%_{vol}$ (M)	4.7% (0.805 M)	5% (0.856 M)	4.6% (0.787 M)	<0.5% (0.086 M)
Found [ethanol] / $\%_{vol}$ (M)	4.7 ± 0.4 (0.803 ± 0.075)	5 ± 0.4 (0.858 ± 0.067)	4.4 ± 0.24 (0.756 ± 0.039)	0.34 ± 0.03 (0.059 ± 0.004)
RSD / %	9.3	7.9	5.3	7.5

675 **Figures caption**

676

677 Figure 1: Paper-based ethanol biosensor fabrication route.

678

679 Figure 2: Cyclic voltammetry experiments performed in 1 mM $[Fe(CN)_6]^{4-/3-}$ prepared in 0.05 M
680 phosphate buffer containing 0.1 M KCl (pH=7.4) with a scan rate of 50 mV/s using (a, black line)
681 polyester-based, (b, green line) 100 g/m^2 paper-based and (c, red line) 80 g/m^2 paper-based SPE.
682 Inset i) Current intensities vs. (scan rate)$^{0.5}$ acquired in 1 mM $[Fe(CN)_6]^{4-/3-}$ varying the scan rate
683 from 20 to 1000 mV/s using (black line) polyester-based, (green line) 100 g/m^2 paper-based and
684 (red line) 80 g/m^2 paper-based SPE. Inset ii) log (current intensities) vs. log (scan rate) acquired in 1
685 mM $[Fe(CN)_6]^{4-/3-}$ varying the scan rate from 20 to 1000 mV/s using (black line) polyester-based,
686 (green line) 100 g/m^2 paper-based and (red line) 80 g/m^2 paper-based SPE.

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688 Figure 3: SEM micrographs of (A,B) polyester-based, (C,D) 100 g/m^2 paper-based and (E,F) 80
689 g/m^2 paper-based SPE. (G). Complex plane impedance plots at open circuit potential carried out in
690 5 mM $[Fe(CN)_6]^{4-/3-}$ prepared in 0.1 M KCl. using (a, black) polyester-based, (b, green line) 100
691 g/m^2 paper-based and (c, red line) 80 g/m^2 paper-based SPE (Inset: Randles circuit). (H) Cyclic
692 voltammetry experiments performed in 1 mM $[Fe(CN)_6]^{4-/3-}$ prepared in 0.05 M phosphate buffer
693 containing 0.1 M KCl (pH=7.4) with a scan rate of 100 mV/s using (black line) non-bended, n=3,
694 and (cyan line) 50-fold bended 80 g/m^2 paper-based SPE, n=3.

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696 Figure 4: (A) Chronoamperograms using a bare paper-based SPE (a, black line) in absence and (b,
697 black line) in presence of 6 mM H_2O_2, and using a CB/PBNPs-modified paper-based SPE (c, blue
698 line) in absence and (d, blue line) in presence of 6 mM H_2O_2. (B) Calibration plot comparison
699 toward H_2O_2 obtained without (black line) and with (blue line) CB/PBNPs modification onto paper-

based SPE. Current sampled at 40 s in a 0.05 M phosphate buffer containing 0.1 M KCl (pH=7.4) with an applied potential of -0.1 V (vs. Ag/AgCl). Error bars refer to standard deviation, n=3.

Figure 5: Optimization of the experimental conditions for the chronoamperometric detection of ethanol. Current response was recorded after 40 s. pH optimization: 2 U of AOx immobilized, -0.1 V (vs. Ag/AgCl), 0.05 M phosphate buffer containing 0.1 M KCl at pH 6, 7.4 and 8. AOx units: -0.1 V (vs. Ag/AgCl), 0.05 M phosphate buffer containing 0.1 M KCl at pH 7.4 immobilizing 1, 2 and 4 U of AOx. Potential optimization: 2 U of AOx immobilized, 0.05 M phosphate buffer containing 0.1 M KCl at pH 7.4 applying a potential of -0.2, -0.1 and 0 V (vs. Ag/AgCl). All the bars are the results of three replicates and report their standard deviation.

Figure 6: Calibration plot of ethanol in 0.05 M phosphate buffer containing 0.1 M KCl at pH 7.4. Inset: Chronoamperograms relative to the calibration plot in a 0-12 mM (0-0.069 $\%_{vol}$) range of ethanol. Measurements carried out applying a potential of -0.1 V (vs. Ag/AgCl), immobilizing 2 U of AOx onto the CB/PBNPs-modified paper based SPE, and recording the current at 40 s. Error bars refer to standard deviation, n=3.

Figure 7: The shelf-life of the biosensor was evaluated by testing a 4 mM (0.023 %vol) ethanol solution prepared in 0.05 M phosphate buffer containing 0.1 M KCl at pH 7.4, applying -0.1 V (vs. Ag/AgCl) and immobilizing 2 U of AOx onto the CB/PBNPs-modified paper-based SPE. The measurements were carried out using a fresh biosensor (black bar) and biosensors stored at 4°C in the dark per 1 week (red bar), 2 weeks (green bar), 3 weeks (blue bar) and 4 weeks (violet bar).

Figure 8: Selectivity of the ethanol biosensor in presence of (a, red bar) 4 mM ethanol, (b, green bar) 4 mM (0.023 $\%_{vol}$) methanol, (c, yellow bar) 0.5 mM glucose, (d, blue bar) 0.5 mM acetic acid

726 and (e, violet bar) 0.5 mM ascorbic acid. Inset: Chronoamperograms relative in presence of all the
727 species described above. Experiments carried out in 0.05 M phosphate buffer containing 0.1 M KCl
728 at pH 7.4, applying -0.1 V (vs. Ag/AgCl) and immobilizing 2 U of AOx onto the CB/PBNPs-
729 modified paper-based SPE. Error bars refer to standard deviation, n=3.

730

731 Table 1: Detection of ethanol in commercial beers.

732

Highlights

- ✓ Novel ethanol biosensor fabricated onto office paper
- ✓ Enhanced hydrogen peroxide detection using Carbon black/Prussian blue nanoparticles
- ✓ Only 100 µL required to perform measurements
- ✓ Paper-based electrochemical device coupled to a portable potentiostat
- ✓ Rapid quantification of ethanol in beer samples