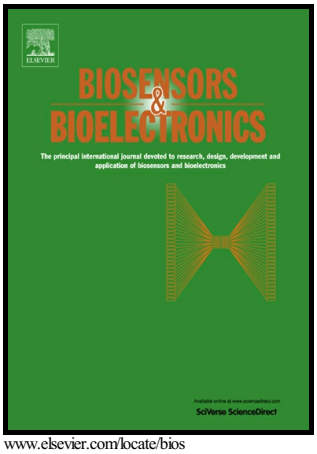

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Specific bioanalytical optical and photoelectrochemical assays for detection of methanol in alcoholic beverages

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Abstract

Methanol is a poison which is frequently discovered in alcoholic beverages. Innovative methods to detect methanol in alcoholic beverages are being constantly developed. We report for the first time a new strategy for the detection of methanol using fluorescence spectroscopy and photoelectrochemical (PEC) analysis. The analytical system is based on the oxidation of cysteine (CSH) with hydrogen peroxide (H_2O_2) enzymatically generated by alcohol oxidase (AOx). H_2O_2 oxidizes capping agent CSH, modulating the growth of CSH-stabilized cadmium sulphide quantum dots (CdS QDs). Disposable screen-printed carbon electrodes (SPCEs) modified with a conductive osmium polymer (Os-PVP) complex were employed to quantify resulting CdS QDs. This polymer facilitates the "wiring" of *in situ* enzymatically generated CdS QDs, which photocatalyze oxidation of 1-thioglycerol (TG), generating photocurrent as the readout signal. Likewise, we proved that our systems did not suffer from interference by ethanol. The PEC assays showed better sensitivity than conventional methods, covering a wide range of potential applications for methanol quantification.

Keywords: Quantum dots, methanol, fluorescence, photoelectrochemistry, cysteine.

1. Introduction

The uncontrollable use of hazardous materials in food and beverages instigates the development of sensitive, affordable and simple assays and sensors for industrial laboratories, distributors and end users. One of the most toxic compounds is methanol. It can be found mainly in alcoholic beverages generated by natural fermentation or distillation. Humans do not tolerate methanol because of its conversion to formate which inhibits mitochondrial cytochrome C oxidase, causing hypoxia at the cellular level, and acidosis (Skrzydlewska, 2003). It is well-known that the ingestion of methanol provokes disturbances in the central nervous system, affecting to the optical system and even death. The lethal dose of methanol lies between 30 and 120 mL (i.e. 1–2 mL kg^{-1} body weight of pure methanol). Usually, humans are exposed to methanol by oral ingestion of alcoholic beverages containing this simplest alcohol. Nowadays, strict regulations contribute to avoiding the deceptive practices of adulteration of alcoholic beverages. Fermentation of alcoholic beverages under standard conditions usually yields aqueous ethanol solutions with negligible concentration of methanol. Nevertheless, incidence of methanol contamination of traditionally fermented beverages is increasing globally due to activities of contaminating pectinase producing yeasts, fungi and bacteria (Ohimain, 2016). In addition the alcoholic strength is altered using “extra” methanol as illicit alcohol (Paine and Dayan, 2001).

Since the beginning of the last century, the development of methods for methanol detection in alcoholic beverages gained in importance (Georgia and Morales, 1926). The standard detection methods are based on gas chromatography as the standard method (Wang et al., 2004a, b). Among other physical techniques for methanol detection are Raman spectroscopy (Boyaci et al., 2012; de Goes et al., 2016), Fourier transform infrared spectrometry (Bangalore et al., 1994; Garrigues et al., 1997; Yang et al., 2016), flow tube mass spectrometry (Chambers-Bedard and Ross, 2016), surface plasmon resonance (Manera et al., 2004), measurement of refractive index and

evaporation rate (Tai et al., 2016), hybrid capillary electrophoresis (Santos et al., 2017) evanescent wave optical sensor (Okuda et al., 2017), quartz tuning forks (Sampson et al., 2017). Those published physical techniques require expensive devices to read out the signal and cannot be readily miniaturized. Electrochemical biosensors and analytical assays employing enzymes as recognition elements for selective determination of methanol in ethanol are not very common, and usually need at least two enzymes. For instance, a bi-enzymatic analytical system was published, which consists of two biosensors, one based on alcohol dehydrogenase (ADH) that responds only to the ethanol and the second one based on alcohol oxidase (AOX) that responds to both methanol and ethanol. Bi-enzymatic biosensor responsive to both ethanol and methanol employing alcohol oxidase and horseradish peroxidase was reported (Hasunuma et al., 2004).

The development of new nanomaterials opens up new opportunities for detection of analytes using enzymes as biorecognition elements. Several enzymes can catalyze biocatalytic generation *in situ* of metallic nanoparticles (NPs) (Fanjul-Bolado et al., 2007; Shlyahovsky et al., 2005; Xiao et al., 2005). However, the reported use of metallic NPs for detection of enzymatic activities is restricted due to their low photocatalytic activities and fluorescent properties. As opposed, semiconductor nanoparticles (SNPs) exhibit quantum effects during a photoexcitation process, hence those particles are referred to as quantum dots (QDs) and are exploited extensively in bioanalytical applications. Combining electrochemistry with the light, photoelectrochemical (PEC) assays are an emergent technique for innovative detection (Huang and Zhu, 2013; Zhao et al., 2016; Zhou et al., 2015). The PEC process transforms luminous energy directly into electrochemical energy. The presence of capping agent as stabilizer favors the quantum confinement effect. One of the efficient stabilizers is cysteine (CSH) that owing to its thiol functional group easily binds to SNPs (Chatterjee et al., 2006; Huang and Lan, 2015). However, CSH is easily oxidized to

cystine (CSSC) in the presence of radix mediator such as hydrogen peroxide (H_2O_2) produced during oxidation of methanol by alcohol oxidase from *Hansenula sp*. specific to only to methanol. The modulation of *in situ* growth of CSH-stabilized SNPs could be followed by fluorescence spectroscopy and PEC assays. The fluorescence readout signal is related to the rate of *in situ* formation of CSH-stabilized CdS QDs inhibited by H_2O_2. Likewise, we carried out PEC studies to evaluate the quantification of CdS QDs grown *in situ*. The amount of formed CdS QDs is related with the photocurrent generated in the system. Other factors defining the electrochemical response are applied potential, energy of photons, intensity of excitation light and rate of electron transfer between the electrode surface and QDs. Previous works based on PEC technology employed electrocatalysts deposited onto expensive electrodes (Devadoss et al., 2015). Nonetheless, PEC is a powerful tool that allows manufactured low-cost devices. In the present work, we employ disposable screen-printed carbon electrodes (SPCEs) as simple and inexpensive disposable devices. To facilitate the electron transfer, a conductive polymer was employed to "wire" CdS QDs to the electrode surface. Previously, poly(vinylpyridine) osmium bipyridine (Os-PVP) conductive polymer was used for "wiring" redox enzymes (Katakis et al., 1994; Vreeke et al., 1992; Yang et al., 1995). Here, we modified disposable SPCEs with Os-PVP complex to validate our fluorogenic and PEC approaches to the methanol quantification in real alcoholic beverages such as vodka and cider opening up a new opportunity for the manufacture of inexpensive and easy-handle mobile analytical systems.

2. Material and methods

2.1. Chemicals

Methanol specific alcohol oxidase from *Hansenula sp*., sodium sulfide (Na_2S), cadmium nitrate $Cd(NO_3)_2$, 1-thioglycerol, methanol, ethanol and another chemicals were purchased from Sigma Aldrich. Different alcoholic beverages such as ciders and vodka were purchased in local market in San Sebastián (Spain).

2.2. Characterization

2.2.1. Spectroscopy and optical methods

Transmission electron miscroscopy images were collected with a JEOL JEM 2100F operating at 120 kV. Axio Observer Microscope (Zeiss) controlled with Axiovision software was employed to take fluorescence images of CdS QDs on SPCEs modified with Os-PVP complex. Fluorescence measurements were performed on a Varioskan Flash microplate reader (Thermo Scientific) using black microwell plates at room temperature. The system was controlled by SkanIt Software 2.4.3. RE for Varioskan Flash.

2.2.2. Photoelectrochemistry

Every electrochemical tests were led in a Autolab Electrochemical Workstation (Model: PGSTAT302N, Metrohm Autolab, The Netherlands) furnished with NOVA 1.10 software. Disposable screen-printed carbon electrodes (SCPEs) were purchased from DropSens (model DRP-110). Electrical contact to workstation was finished with a special boxed connector provided by DropSens. The light source was a compact UV illuminator (UVP, Analytik Jena AG). All PEC measurements were performed at room temperature. All the potentials reported in our work were measured against Ag/AgCl. Unless otherwise specified, all experimental outcomes described here are averaged from three autonomous estimations (n = 3).

2.3. Methods

2.3.1. AOx assay

Varying amounts of methanol or ethanol were incubated with different amounts of AOx in citrate-phosphate buffer (pH 7.5) for 40 min at room temperature, in the presence of CSH (7.5 µL, 1 mM). After that, Na_2S (10 µL, 1 mM) and $Cd(NO_3)_2$ (2.5 µL, 50 mM)

were added to the samples (87.5 µL). The emission spectra of the resulting CdS QDs were recorded after 5 min at λ_{exc} = 300 nm.

2.3.2. CdS QD-mediated determination of alcohol mixtures

Different mixtures of methanol and ethanol were incubated with CSH (7.5 µL, 1 mM) in citrate-phosphate buffer (pH 7.5) for 40 min at room temperature (taking into account that two alcoholic solutions with ethanol content of 40% and 6% were prepared). After that, Na_2S (10 µL , 1 mM) and $Cd(NO_3)_2$ (2.5 µL, 50 mM) were added to the samples (87.5 µL). The emission spectra of the resulting suspensions were recorded after 5 min at λ_{exc} = 300 nm.

2.3.3. Quantification of methanol in real samples

Quantification of methanol in cider and vodka was performed by the standard addition method. Samples of alcoholic beverages were spiked with known different concentrations of methanol. Thus, the corresponding final concentration of methanol in mixtures was determined. The dilution factor of samples in the assay was 1:10000.

2.3.4. Photoelectrochemical detection

Before beginning the PEC assays, the SPCEs were at first pretreated electrochemically by cyclic voltammetry (CV) at a potential range of 0 – 0.6 V in citrate-phosphate buffer (pH 7.5). Subsequently, a 40 µL drop of Os-PVP complex (1.375 mg mL^{-1}) was dropcasted on the SPCEs and electrodeposited by CV scanning (2 cycles at scan rate of 50 mV s^{-1}). Afterwards, SPCEs were flushed out with ultrapure water and dried under argon atmosphere. At last, 40 µL of sample were dropped on the SPCE and PEC measurements were carried out with an UV-illuminator at 302 nm and a controlled potential of 0.3 V vs. Ag/AgCl. The reliance of photocurrent on time was measured at 5 minutes during 10 seconds. It is important to point out that it is necessary to add the reducing agent 1-thioglycerol (20 mM) to amplify the photocurrent response.

3. Results and discussion

3.1. Fluorogenic assays

We developed the fluorogenic assay for methanol using evaluation assessment of the enzymatic activity of AOx as represented in Scheme S1 and Scheme 1. First, the enzyme AOx catalyzes the oxidation of methanol with oxygen to yield hydrogen peroxide (H_2O_2). Second, hydrogen peroxide, resulting from the enzymatic oxidation of the target analyte, converts two molecules of cysteine to one molecule of cysteine. This process leads to conversion of two active thiol groups (-SH) into one inactive disulfide bridge (-SS-) which does not stabilizes the growth of CdS QDs. Third, cysteine carrying one thiol group is a very efficient stabilizer for CdS QDs formed in situ from Cd^{2+} and S^{2-} ions. Thus, the bio-catalytic process ending up in formation H_2O_2, decreases concentration of the stabilizing agent cysteine, and consequently, the amount of CdS QDs formed in situ. In previous works, it was demonstrated that CSH is able to stabilize CdS QDs under harsh experimental conditions that include high reaction temperatures (over 80 °C) (Kumar et al., 2013) or irradiation with γ-rays (Chatterjee et al., 2006). Under our experimental conditions the growth of CdS QDs occurs under mild physiological conditions in aqueous buffer solutions. CdS QDs growth is time dependent as it can be seen in Fig. S1. The fluorescence intensity increases up to 30 min and then it levels off. This increase could be due to the high amount of reagents in the media that provides a constant source of reagents up to their complete consumption. After 5 min of incubation more than 60% of maximum fluorescence intensity was observed. After 60 min the signal achieved 100% of intensity, but this long incubation time will not be suitable for a point of care device or its use in clinical diagnosis that requires short procedure times. That is why we chose 5 min as the best time in relation with the signal obtained and the total time consumed.

In order to verify the operating mechanism of described system, various control experiments were carried out as shown in Fig. S2. The influence of oxygen on non

specific oxidation of cysteine was evaluated with the control experiment in which ions of Cd^{2+} and S^{2-} were mixed in the presence of cysteine in citrate-phosphate buffer (pH 7.5). Fluorescent QDs were formed as one can see in curve 1. The reaction mixture containing CSH, AOx, Cd^{2+}, S^{2-} ions demonstrated emission peak characteristic of CdS QDs (curve 2). The mixture composed of CSH, methanol, Cd^{2+}, S^{2-} ions exhibited high emission peak too (curve 3). Curves 1, 2 and 3 are identical. According to Fig. S1 the fluorescence of CdS QDs kept in open air doesn't decrease with time. Thus, under our experimental conditions oxygen is not able to oxidase cysteine non specifically. The presence of oxygen in the media is mandatory since it allows AOx to transform methanol into formaldehyde. In the absence of oxygen the enzyme would not be able to catalyze the oxidation of methanol.

The emission peaks of similar mixtures prepared without CSH, containing methanol, Cd^{2+}, S^{2-} ions (curve 5) or AOx, Cd^{2+}, S^{2-} ions (curve 6) were much lower. Fluorescence decreases only in the absence of the capping agent (cysteine) in open air (curve 5 and 6). The reaction mixture containing all components (AOx, methanol, CSH, Cd^{2+}, S^{2-} ions) exhibited low emission peak (curve 4). This can be explained by the decrease in CSH concentration caused by its oxidation with H_2O_2, produced in the course of enzymatic redox process according to Scheme S1. An extra control was also conducted to rule out the influence of AOx on the fluorescence signal in absence of alcohol, CSH, $Cd(NO_3)_2$ and Na_2S. Under these conditions no fluorescence was observed (curve 7).

Time of incubation and CSH concentration were optimized. Different incubation times were tried in the presence and absence of methanol. The greatest difference between fluorescence signals registered in reaction mixtures with and without methanol was achieved when the incubation time was 40 minutes (Fig. S3A). Hence incubation time of AOx with cysteine and methanol was employed in the following experiments. Published protocols for the synthesis of CSH-stabilized NPs require much longer

incubation time and harsh experimental conditions (Chatterjee et al., 2006; Huang and Lan, 2015; Kumar et al., 2013). Incubation was performed at varying concentrations of CSH. It was found that the optimum CSH concentration was 0.075 mM (Fig. S3B). At this concentration, the fluorescence readout signal showed the highest intensity, indicating the presence of higher amount of CSH-stabilized CdS QDs. The amount of AOx was also optimized using different concentrations of the enzyme in the presence of a fixed alcohol (methanol or ethanol) concentration (0.03 g L^{-1}) as shown in Fig. 1A and 1B. In the presence of methanol fluorescence intensity was inversely related to the amount of AOx in the assay mixture Fig. 1B (dark line) reaching saturation at 5 µg $L^{-1}$of this enzyme. According to the calibration plot this assay for enzymatic activity of AOx has a limit of detection (LOD) equal to 0.14 µg L^{-1} at a signal-to-noise ratio of 3 (S/N=3). The average relative standard deviation (RSD) calculated from the calibration plot was 8% (unless otherwise specified, RSD was always acquired utilizing no less than three independent measurements). When ethanol was used instead of methanol no response to increasing concentrations of AOx was observed (Fig. 1B dashed line). Transmission electron miscroscopy was used to determine the morphological characteristics of obtained CdS QDs (Fig. S4). TEM images confirmed the presence of spheroidal QDs with an average diameter of 2.03 ± 0.86 nm. CdS QDs could not be detected by TEM at saturating methanol concentration of 0.03 g L^{-1} (Fig. S4C).

The emission spectra of CdS QDs recorded at varying concentrations of a methanol or ethanol in the presence of a fixed AOx concentration of 5 µg L^{-1} are depicted in Fig. 1C. In the case of methanol, decrease in the fluorescence signal is inversely proportional to methanol concentration as one can observe in Fig. 1D (bold line). The response to increasing concentrations of methanol is typical for an enzymatic system governed by the Michaelis-Menten kinetic model considering affinity of the enzyme to its substrate. Lower Michaelis-Menten constant (K_M) value means higher affinity of an enzyme to its substrate. The assay demonstrated a linear response up to 1.5 mg L^{-1} and

asymptotically approaches its maximum starting from 5 mg L^{-1}. The apparent Michaelis-Menten constant (K_M) was calculated by fitting the experimental results to the equation $I=I_{max}[Methanol]/(K_M+[Methanol])$. The value of 1.66 mg L^{-1} correlates well with the literature data (Barsan and Brett, 2008; Gulce et al., 2002; Yildiz and Toppare, 2006). Furthermore, the LOD was found equal to 0.21 mg L^{-1} (6.8 µM) at S/N=3. The RSD calculated from the alcohol calibration plot was 10%. The fluorogenic assay showed a better sensitivity than classical chromatography tests (Plotka-Wasylka et al., 2016; Wang et al., 2004a, b; Zhang et al., 2015) and other fluorometric assays (Kucera and Sedlacek, 2017) as can be seen in Table S1. The influence of ethanol on the performance of this fluorogenic assay was studied. In the presence of increasing amounts of ethanol (Fig. 1D dashed line) the fluorescence signal did not decrease, remaining constant within the experimental error.

Additional calibration was performed using aqueous 40% (v/v%) alcohol mixtures with different methanol/ethanol molar ratios ranging between 0.0 and 1.0. According to the experimental calibration plot (Fig. S5A) LOD was equal to 0.047 (molar fraction) with RSD of 8.15 % (S/N=3). Thus, this simple fluorogenic assay based on enzymatic modulation of the growth of CdS QDs is able to determine methanol in the presence of high amounts of ethanol. Other previously published enzymatic optical assays based on AOx either are not able to detect methanol in the presence of ethanol (Azevedo et al., 2005; Verduyn et al., 1984) or employ rare reagents which are not commercially available (Anthon and Barrett, 2004) (Table S1).

3.2. PEC assays

3.2.1 Optimization of photoelectrochemical response

Several parameters were optimized to obtain maximum performance of PEC measurements. We used Os-PVP conductive polymer complex immobilized on the

surface of SPCE to “wire” CSH-stabilized CdS QDs generated during the assay. The efficient anchoring of osmium polymer on the electrode surface was achieved through electrostatic adsorption during CV. We optimized the protocol for the deposition of Os-PVP complex controlling the number of scans of CV. This methodology consists of ramping the potential linearly versus time in cyclical phases in the range between 0 and 0.6 V vs. Ag/AgCl. Final CV revealed redox reversible waves related to the central osmium atom (Barroso et al., 2016). Another aspect of PEC assays is the employment of a reducing agent to neutralize holes generated in CdS QDs upon excitation of electrons by photons. We selected TG because it has high affinity to CdS QDs due to thiol functional group. Therefore, the electronic transfer rate between the surface of CdS QDs and TG is very high (Yang, 2006). On the other hand, TG can be used as a capping agent (Ben Brahim et al., 2015; Deepika et al., 2015; Silva et al., 2014).

Fig. S6 shows the effect of TG concentration on photocurrent observed at SPCE modified with Os-PVP complex in the presence of fixed amounts of CdS QDs. According to this plot, registered photocurrent achieved a plateau in the presence of 20 mM TG. This concentration of TG was selected for subsequent PEC measurements.

Finally, the effect of applied potential on the anodic photocurrents was studied. The applied potential was optimized in order to minimize the small background photocurrent originated from nonspecific photoelectrochemical oxidation of TG on the electrode surface modified with Os-PVP complex in the absence of CdS QDs. Photocurrents were registered in the presence ($\Delta I_{Os\text{-}PVP/TG}$) and absence ($\Delta I_{Os\text{-}PVP}$) of TG using SPCEs modified with the redox polymer. The lowest ratio between both responses ($\Delta I_{Os\text{-}PVP/TG}/\Delta I_{Os\text{-}PVP}$) was achieved at 0.3 V vs. Ag/AgCl as it can be noticed in Fig. S7. At this applied potential the ratio $\Delta I_{Os\text{-}PVP/TG}/\Delta I_{Os\text{-}PVP}$ was close to 1.0 indicating that nonspecific photoxidation of TG does not influence the response of the PEC assay. Hence the potential of 0.3 V vs. Ag/AgCl was used in the following experiments.

3.2.2. Photocurrent response

The operation of the developed PEC assay for methanol is based on the interaction between SPCE, osmium polymer and CSH-stabilized CdS QDs whose quantity depends on the enzymatic oxidation of methanol as illustrated in Scheme 1. Biocatalytic oxidation of MeOH catalyzed by AOx ends up in formation of hydrogen peroxide which oxidizes CSH. Concentration of the latter influences the rate of formation of CSH-stabilized CdS QDs *in situ*. A droplet of the assay mixture is placed on the surface of a SPCE (without drying) modified with Os-PVP complex and irradiated with a UV lamp. CSH-stabilized QDs absorb photons with energies upper than that of their band gaps, producing the excitation of electrons from occupied valance band (VB) to the empty conduction band (CB). Electron holes-pairs with enough long life are generated due to the charge separation. Holes on VB are neutralized by electrons originating from TG. Free electrons are transferred from CB to Os-PVP complex, and finally to the electrode surface. This flow of electrons is driven by the UV light with an emission peak at 300 nm and an applied potential of 0.3 V vs. Ag/AgCl. The influence on photocurrent of varying concentrations of AOx in the presence of a fixed alcohol (methanol or ethanol) concentration (0.03 g L^{-1}) is shown in Fig. 2A and 2B. The determination of AOx in using methanol as a substrate demonstrated a LOD of 0.01 µg L^{-1} (S/N=3) based on the calibration plot showed in Fig. 2B. The RSD calculated from calibration plot was 6.3%. As before, the photocurrent was not affected by ethanol (Fig. 2B dashed line).

The presence CSH-stabilized CdS QDs in droplets of assay mixture placed on SPCEs was corroborated by fluorescence spectroscopy as depicted in Fig. S8. In the absence of methanol, the fluorescence is maintained (Fig. S8A). When methanol was present in the assay mixture the registered fluorescence was significantly lower (Fig. S8B). It should be noted that the osmium polymer is not fluorescent.

The photocurrent response to varying concentrations of alcohol (methanol or ethanol) using a fixed amount of AOx (5 μg L^{-1}) are depicted in Fig. 2A. The observed behavior of PEC assay is similar to that of the fluorogenic assay. The photocurrent intensities decreased in the presence of increasing methanol amounts, remaining stable in the presence of ethanol (Fig. 2B bold and dashed line respectively). The PEC assay demonstrated a consistent K_M value of 1.88 mg L^{-1} similar to one obtained in fluorogenic assay. The LOD of PEC assay was 0.16 mg L^{-1} (5 μM) at S/N=3. The average relative standard (RSD) deviation was 5.8%. The PEC methodology proved to be more sensitive by two times in comparison with previously published amperometric biosensor for methanol which is not able to detect methanol in the presence of ethanol (Chinnadayyala et al., 2014; Chinnadayyala et al., 2015; Du et al., 1996; Hasunuma et al., 2004; Wen et al., 2014). The previously published bi-enzymatic biosensor for determination of methanol in the presence of ethanol has worse detection limit (5 mM) 1000 time higher than the LOD of the present PEC assay (Bucur et al., 2008) (Table S1). It should be noted that the fluorometric assay is ten times less sensitive than the novel PEC method. It provides a simple and reliable method to determine methanol content in real samples. To best our knowledge this is the first PEC assay for methanol suitable for its determination in the presence of ethanol.

PEC assay was also applied to detection of methanol in aqueous 40% (v/v%) alcohol mixtures with different methanol/ethanol molar ratios ranging between 0.0 and 1.0. According to the calibration plot (Fig. S5B) LOD was equal to 0.017 (molar fraction) with RSD of 5.80%. This detection limit is less than that of the fluorogenic assay for methanol.

In Table S2 there are some examples of the main reported techniques utilized to detect methanol. These procedures present some disadvantages like the need of standards or internal/external references, sophisticated or expensive apparatus or reagents, expert

operators, long measuring times and sample pre-treatment. On the other hand, in our case both the optical and photoelectrochemical procedure are simpler and easier handling, they do not need expensive reagents or apparatus and can be easily miniaturized. Moreover, no pre-treatment nor standards or references are required. All these advantages make our procedures more suitable for methanol detection and its application in analytical laboratories.

3.3. Validation with alcoholic beverages

3.3.1. Simulation of alcoholic strength

It is well known that methanol is not suitable for humans. Thus, its content must be controlled. Alcoholic beverages are classified by the regulation 110/2008 of the European Parliament and of the Council (15 January 2008). It describes the definition, description, presentation, labeling and the protection of geographical indications of spirit drinks. We simulated two types of alcoholic beverages: cider and vodka. Our aim was to validate our fluorogenic and PEC assays using two aqueous solutions of different alcoholic strengths (6% and 40%, v/v%). In order to simulate cider, mixtures containing 6% of total alcohol were spiked with different concentrations (μg L^{-1}) of methanol (Fig. 3A and 3C). The protocol was slightly modified for alcoholic beverages with the higher alcoholic strength of 40% such as vodka. Fig. 4A and 4C represents the effect of varying concentrations of methanol spiked into 40% ethanol solution. As one can see the increase in the methanol concentration is linearly related with the decrease in the readout signal.

3.3.2 Study of real samples

The methanol content for different alcoholic beverages is described by regulation (EC) No 110/2008. In case of vodka, the value should not exceed 100 mg L^{-1} (3.12 mM). For ciders, we take as the reference the royal decree 72/2017 by Spanish Government

stating that the maximum content of methanol should be not higher than 200 mg L^{-1} (6.24 mM). We applied the standard addition method in order to validate the fluorogenic and PEC methods.

3.3.2.1 Cider

Three local ciders (Etxeberria, Gurutzeta and Izeta) were selected for this study. Different known methanol concentrations close to maximum allowed were added to ciders (added concentrations of methanol were 0, 1, 2, 3, 4 and 5 µg L^{-1}). Thereafter, the fluorescence signal and PEC response were evaluated, plotting the concentrations of added standards on the x-axis against their corresponding readout signal on the y-axis. The linear regression for each dilution was performed to calculate the intercept of the calibration lines with the x-axis, which represents the content of methanol in the dilute real samples (Fig. 3B and 3D). Taking into consideration dilution factors of the ciders samples, we found out that the methanol concentration was under the limit established by law as depicted in Table 1. Both methods corroborated well the content of methanol. PEC method demonstrated better sensitivity.

3.3.2.2 Vodka

Vodka was chosen because it is the most popular alcoholic beverage in the Eastern European countries and Russia (Wisniewska et al., 2015). So, a large number of potential consumers could be intoxicated by the adulterated vodka. The same procedure of added standards described in the above section was applied. Known quantities of methanol were added to vodka samples (Fig. 4B and 4D). By linear regression the amount of methanol was computed (Table 1) which was within the methanol levels specified by the corresponding legislation.

Table 1
Content of methanol in different alcoholic beverages quantified by fluorogenic and PEC methods.

Methanol / mg L^{-1}	Fluorescence / au	PEC / nA
Etxeberria	134 ± 21	111 ± 25
Izeta	124 ± 33	121 ± 30
Guruzeta	145 ± 25	137 ± 20
Vodka	18 ± 3	24 ± 5

4. Conclusions

Adulterated alcoholic beverages with extra methanol drive the searching of new detection methods. This work opens a new concept based on the specificity of AOx to methanol producing H_2O_2, which influences in the concentration of the capping agent CSH, ending up in the modulation of in situ generated CdS QDs. The quantification of methanol was followed by classical fluorescence spectroscopy and emerging PEC process. The latter facilities the "wiring" of CdS QDs with disposable SPCEs sensitized with Os-PVP complex. This strategy allows the reproducible fabrication of a very simple device. Both methods are more sensitive and more selective than the previously reported bioassays and biosensors. The present approach proved to be efficient for the fast monitoring of methanol in any alcoholic beverages.

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Figures Captions

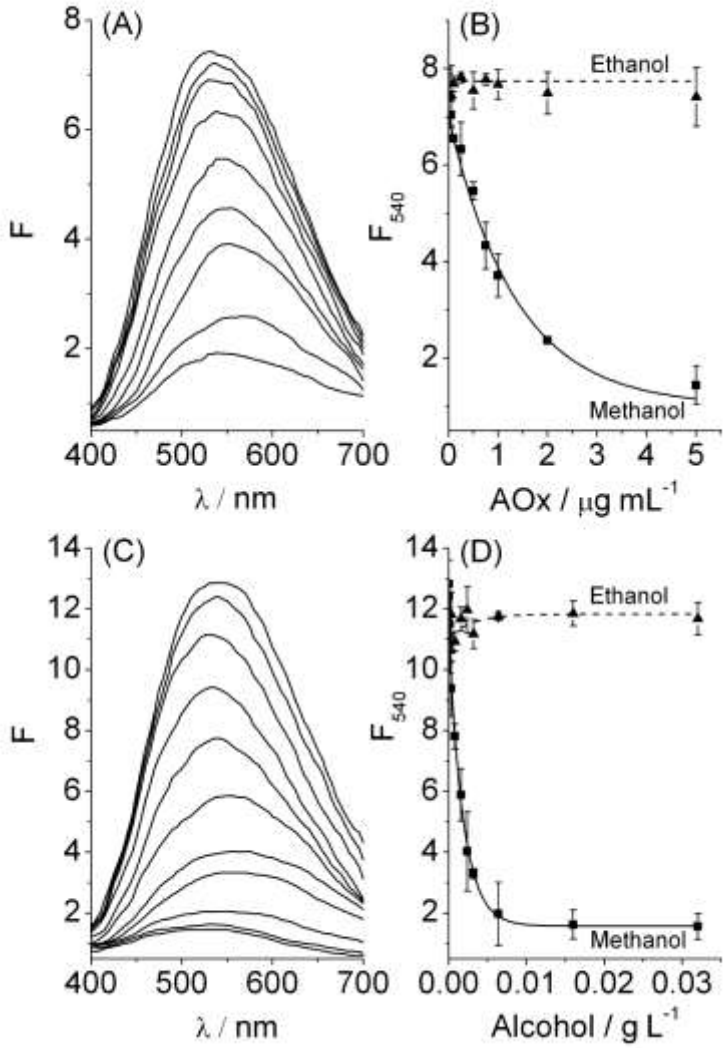


Fig. 1. (A) Fluorescence emission spectra of the system containing methanol (0.03 g L^{-1}), cysteine (CSH, 0.075 mM), Na_2S (0.1 mM), $Cd(NO_3)_2$ (1.25 mM) and different concentrations of AOx (0 to 5 µg mL^{-1}). (B) Calibration curves of AOx for methanol (dark line) and ethanol (dashed line) obtained using F_{540}. (C) Fluorescence emission spectra of the system containing alcohol oxidase (AOx, 5 µg mL^{-1}), CSH (0.075 mM), Na_2S (0.1 mM), $Cd(NO_3)_2$ (1.25 mM) and different concentrations of methanol (0 to 0.03 g L^{-1}). (D) Calibration curves of methanol (dark line) and ethanol (dashed line) obtained using F_{540}.

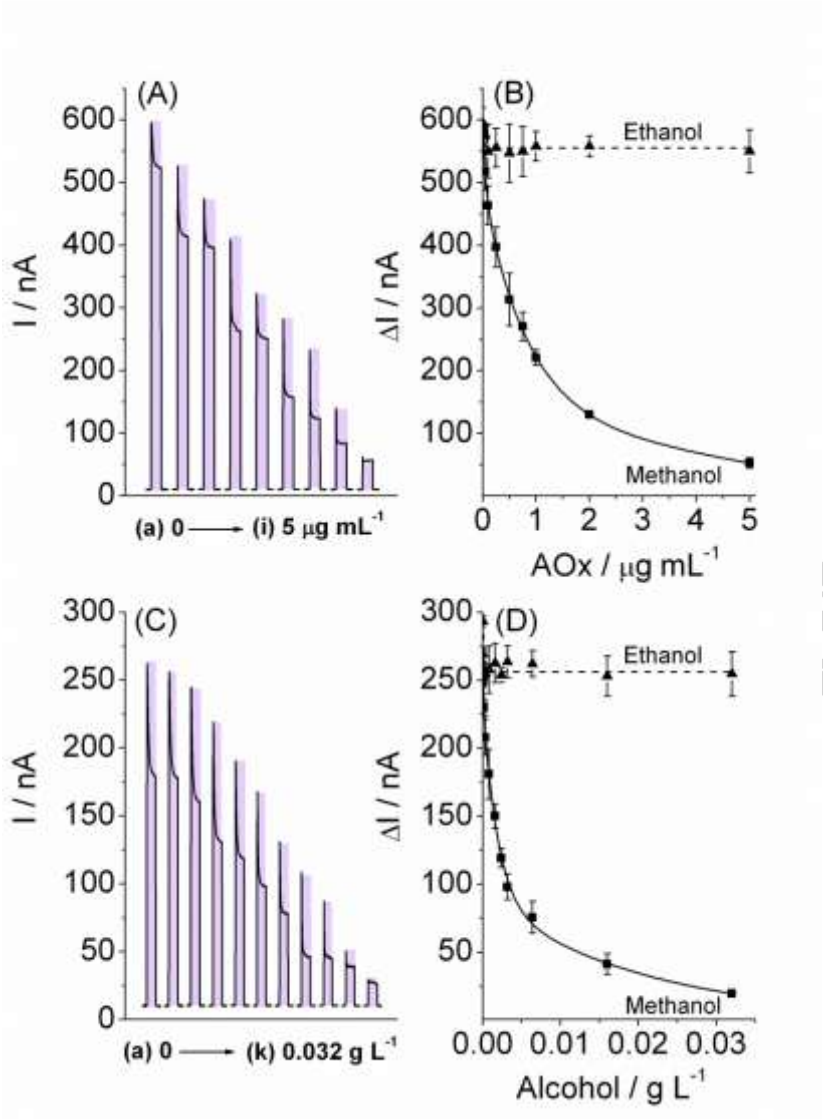


Fig. 2. (A) Photocurrent response of the system containing methanol (0.03 g L^{-1}), cysteine (CSH, 0.075 mM), 1-thioglycerol (TG, 20 mM), Na_2S (0.1 mM), $Cd(NO_3)_2$ (1.25 mM) and different concentrations of AOx (0 to 5 µg mL^{-1}). (B) Calibration curves of AOx for methanol (dark line) and ethanol (dashed line) obtained at 0.3 V (*vs.* Ag/AgCl) and 300 nm excitation light. (C) Photocurrent response of the system containing alcohol oxidase (AOx, 5 µg mL^{-1}), CSH (0.075 mM), TG (20 mM), Na_2S (0.1 mM), $Cd(NO_3)_2$ (1.25 mM) and different concentrations of methanol (0 to 0.03 g L^{-1}). (D) Calibration curves of methanol (dark line) and ethanol (dashed line) obtained at 0.3 V (*vs.* Ag/AgCl) and 300 nm excitation light.

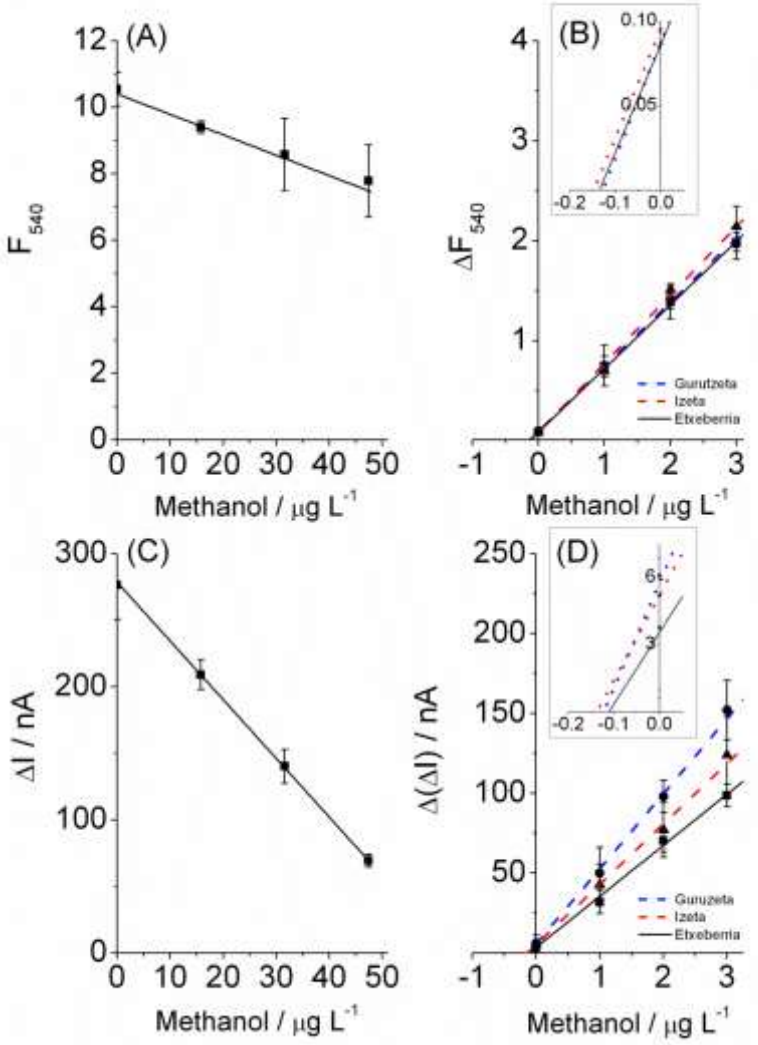


Fig. 3. Calibration curve of methanol obtained by (A) fluorescence and (C) photoelectrochemical (PEC) assays. Quantification of methanol in three different ciders with the method of standard addition for (B) fluorescence and (D) PEC methods. The system contained different known amounts of added methanol standards. Insets are amplified areas of added standard curves.

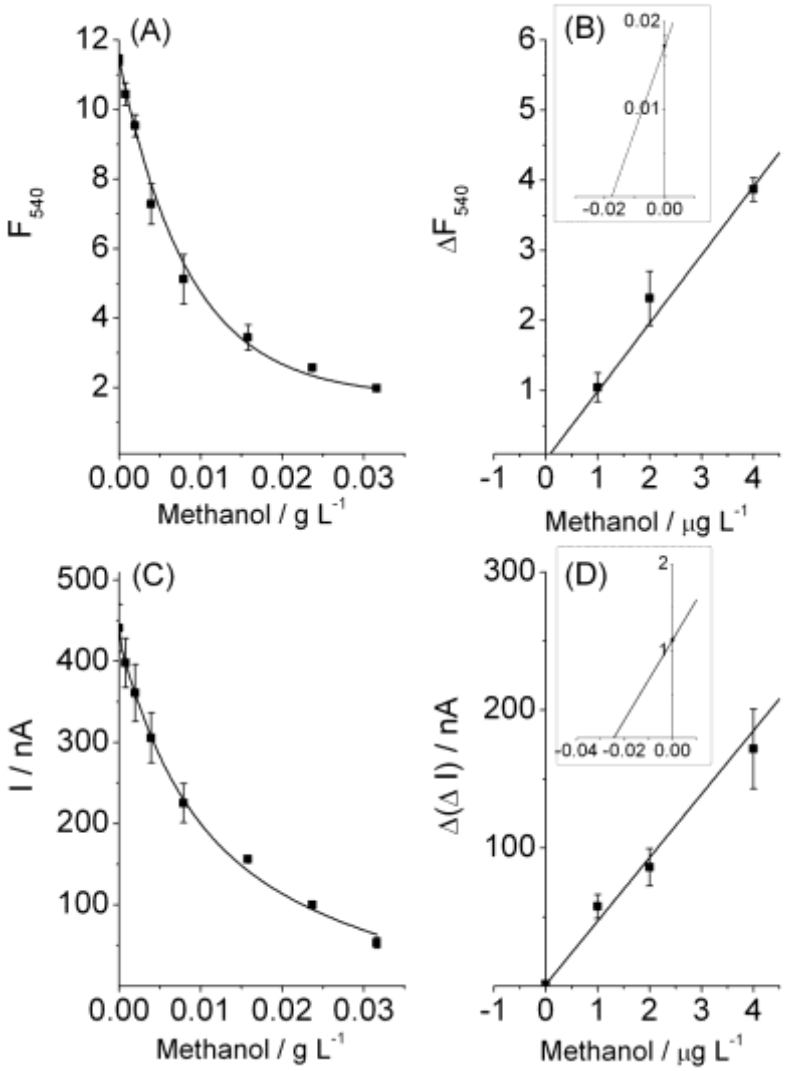
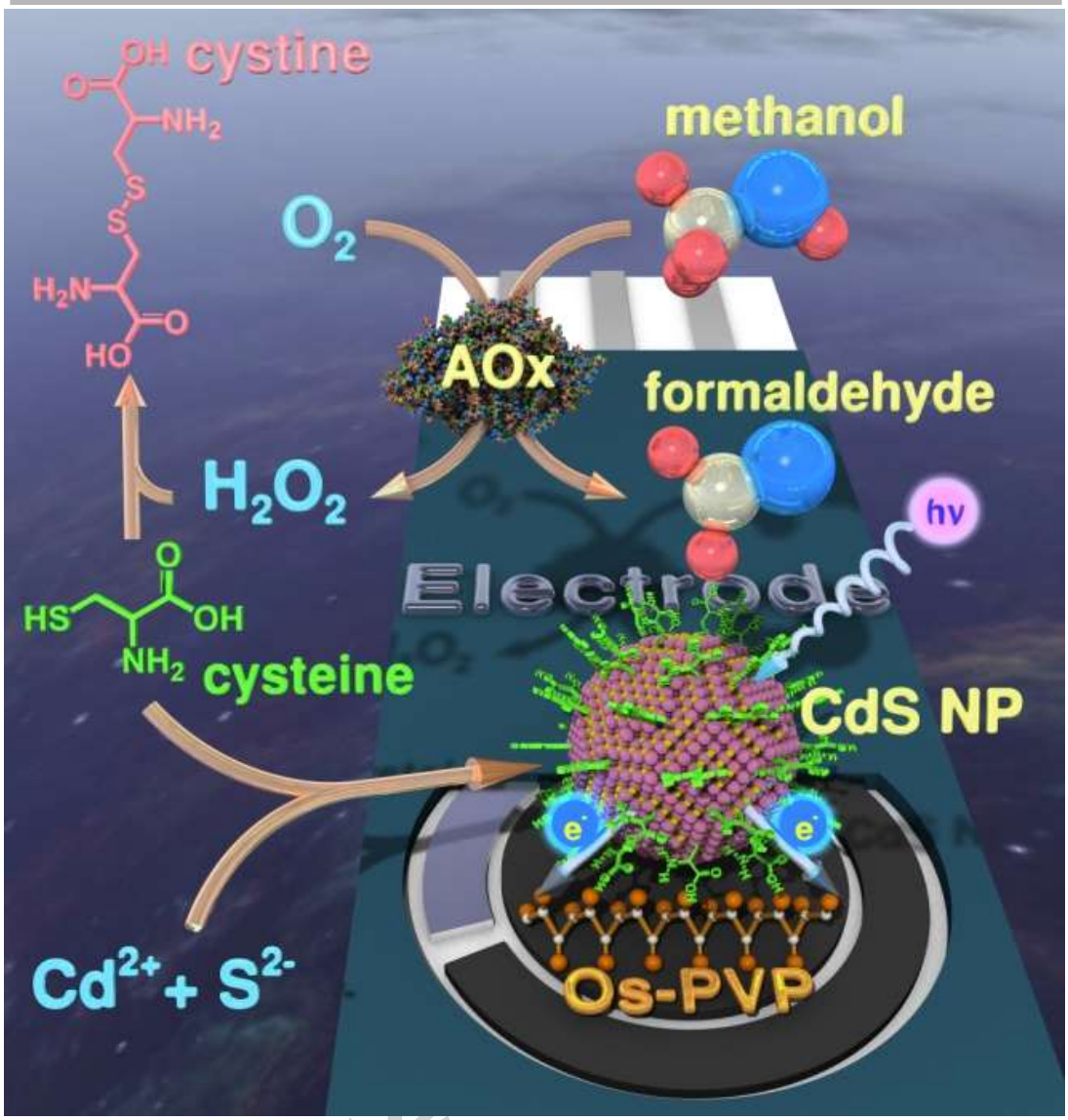


Fig. 4. Calibration curve of methanol obtained by (A) fluorescence and (C) photoelectrochemical (PEC) assays. Quantification of methanol in vodka with the method of standard addition for (B) fluorescence and (D) PEC methods. The system contained different known amounts of added methanol standards. Insets are amplified areas of added standard curves.



Scheme 1. Photoelectrochemical assay for methanol through detection of CdS QDs "wired" by an Os-PVP complex to the surface of a screen-printed carbon electrode.

Graphical abstract

Highlights

- Cysteine (CSH) as stabilizer of CdS QDs growing in aqueous buffered solutions.
- Alcohol oxidase (AOx) modulates the growth of CSH-capped CdS QDs.
- Quantification of methanol in alcoholic beverages: cider and vodka.
- SPCEs modified with Os-PVP polymer for photoelectrochemical (PEC) analysis.
- PEC methodology showed lower detection limit than the standard methods.