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Extraction-less, rapid assay for the direct detection of 2,4,6-trichloroanisole (TCA) in cork samples

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ABSTRACT

2,4,6-trichloroanisole (TCA), the cork taint molecule, has been the target of several analytical approaches over the few past years. In spite of the development of highly efficient and sensitive tools for its detection, ranging from advanced chromatography to biosensor-based techniques, a practical breakthrough for routine cork screening purposes has not yet been realized, in part due to the requirement of a lengthy extraction of TCA in organic solvents, mostly 12% ethanol and the high detectability required. In the present report, we present a modification of a previously reported biosensor system based on the measurement of the electric response of cultured fibroblast cells membrane-engineered with the pAb_{78} TCA-specific antibody. Samples were prepared by macerating cork tissue and mixing it directly with the cellular biorecognition elements, without any intervening extraction process. By using this novel approach, we were able to detect TCA in just five minutes at extremely low concentrations (down to 0.2 ppt). The novel biosensor offers a number of practical benefits, including a very considerable reduction in the total assay time by one day, and a full portability, enabling its direct employment for *on-site*, high throughput screening of cork in the field and production facilities, without requiring any type of supporting infrastructure.

1. Introduction

Over the last 10 years, 2,4,6-trichloroanisole (2,4,6-TCA or TCA) has become one of the most notorious threats to the sustainability of the cork industry. Its presence, even at the trace concentration of 2–4 parts per trillion (ppts), is enough to give a musty, mold and/or earthy off-odor to affected wines, therefore masking the natural wine aroma and diminishing the quality of the final product. Taking into account reports claiming that the portion of tainted bottles can be higher than 30% [1], the presence of TCA can (and actually does) result into an economic loss and occasional damage of the profile and the reputation of the wine industry. This is particularly important in Europe, leading worldwide wine and cork production (about 2.7 million hectares of cork forests growing in Portugal and Spain). The availability of rapid, cost-efficient and high throughput screening tests for TCA is an imminent requirement of the cork and wine industry. In particular, performance characteristics such as high sensitivity, speed and low cost are highly desired. Despite the prevalence of cork taint, the wine industry widely uses cork stoppers because some enologists believe that they have a positive contribution to wine ageing and excellent sealing properties [2], and because of the positive image they create for the consumer. Therefore, the analytical need for TCA detection created a dedicated market exceeding one billion € per annum.

Because of its volatility, cork and wine matrix complexity and the low human taste threshold, TCA detection is especially challenging and beyond the sensitivity of most analytical systems [3]. In response to this challenge, the analytical community has developed powerful techniques for the detection of TCA in a variety of samples (cork, wine and even water) based on either crhromatographic or bioanalytical techniques. Among the first, gas chromatography/mass spectrometry (GC–MS) [4] has been mainly used and more recently combined with solid phase microextraction coupled with gas chromatography–ion trap mass spectrometry (GC–ITMS) [5]. Current instrumentation based on these methods allows for a satisfactory sensitivity (down to less than 1 ppt of TCA) but is associated with a very low capacity (low throughput) in terms of number of samples/test cycle, speed and

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cost. These features are increasingly satisfied by emerging rapid assays, such as immunoanalytical techniques making use of a specific antibody raised against TCA. Thus, efficient microplate-based enzyme-linked immunosorbent assays (ELISAs) [6,7] and immunoamperometric [8] techniques for TCA analysis have been developed. Also the potential of ion mobility spectrometry (IMS) for detection of 2,4,6-trichloroanisole (2,4,6-TCA) in wine has been reported. The observed limits of detection depend on the presence of ethanol in the samples [8]. In a similar application, among the reported strategies for the detection of TCA in wine, electrochemical immunosensing provides the most rapid response times since chromatography and ELISA methods often require extraction, preconcentration and sample preparation steps, increasing the analysis time.

In a previous article in *Talanta*, we have reported the development of a Bioelectric Recognition Assay (BERA) biosensor system based on the measurement of the electric response of cultured membrane-engineered fibroblast cells suspended in an alginate gel matrix due to the change of their membrane potential in the presence of the TCA. Membrane-engineered cells were prepared by osmotic insertion of specific TCA antibodies into the membrane of the cells. The biosensor was able to detect TCA in few minutes (3–5 min) at extremely low concentrations (0.1 ppt), thus demonstrating higher sensitivity than the human sensory threshold. In addition, the assay was quite selective against other haloanisoles and halophenols structurally related to or co-occurring with TCA [9].

In the present study, we report a considerably modified version of the previous cell-based biosensor. By changing both, the way of inserting TCA-specific antibodies (pAb_{78}) into the cells (electro-insertion vs. osmotic insertion) as well as the interface between the recording unit and the cellular biorecognition elements (in suspension and in direct contact with the electrode strip vs. immobilization), we managed to detect TCA directly in macerated cork tissue without any intervening extraction process, yet rapidly and at extremely low concentrations (down to 0.2 ppt). The practical significance of these results for the *on-site*, high throughput screening of TCA in cork in the field and production facilities is discussed.

2. Experimental setup

2.1. Materials

Monkey African green kidney (Vero) cells cultures were originally provided from LGC Promochem (Teddington, UK). 2,4,6-trichloroanisole was purchased from Sigma-Aldrich (St. Louis, USA). Polyclonal antibodies against TCA (pAb_{78}) were obtained from the Nanobiotechnology for Diagnostics(Nb4D) group (IQAC-CSIC, Barcelona, Spain). Cork samples were originally provided from Amorim SA (Portugal). All other reagents were purchased from Fluka (Buchs, Switzerland).

2.2. Cell culture

Vero cells were cultured in Dulbecco's medium with 10% fetal bovine serum (FBS), 10% antibiotics (streptomycin–penicillin) and 10% l-glutamine and l-alanine. After cell detachment from the culture vessel by adding trypsine/EDTA for 10 min at 37 °C cells were concentrated by centrifugation (2 min, 1200 rpm, 25 °C), at a density of 2.5×10^6 ml^{-1}.

2.3. Electroinsertion of antibodies into the cell membrane

Membrane-engineered cells were created by electroinserting pAb_{78} polyclonal antibodies into the membrane of Vero cells, following a modified protocol of Zeira et al. [10]. Cells were centrifuged at 1000 rpm for 6 min and then resuspended in 40 μl of Dulbecco's medium provided with 10% fetal bovine serum (FBS). Subsequently, cells were incubated together with the antibodies (0.4 μg ml^{-1}) for 20 min on ice. Then the cell-antibody mixture was transferred to appropriate electroporator cuvettes. Electro-insertion was performed by applying two square electric pulses at 1800 V/cm. After electroinsertion, the mixture was transferred on small Petri dishes (60×15 mm^2) which contained 3 ml of Dulbecco's medium provided with 10% fetal bovine serum (FBS) and incubated at 37 °C and 5% CO_2 for 1 day. On the day of measurement, the medium was removed from the Petri dishes and cells detachment from the culture was achieved by adding 2 ml of nutrient medium and collecting the cells in Eppendorf tubes.

2.4. Biosensor device

A customized device was developed in order to measure electric signals from the cellular biorecognition elements and allowing for high throughput screening and high speed of assay. The device is a portable potentiometer, having a replaceable guide bearing eight pairs of electrodes connecting on the underside (Fig. 1). The system is based on a modification of a design previously reported by our group [11].This configuration provides a connection interface to insert electrode strips directly into the instrument. The counter-electrode canceled out by the measuring system. Each electrode strip was comprised of a 0.5 mm thick ceramic substrate with three screen printed electrodes (working electrode – WE, reference electrode – RE and counter-electrode – CE). In order to facilitate high throughput screening, DRP-8 × 110 disposable sensor strips (WE: carbon, RE: Ag/AgCl) bearing eight electrode pairs (corresponding to eight measurement channels) were purchased from DropSens (Asturias, Spain). The counter-electrode was canceled out by the measuring system. Thus, the potentiometer, through its array of eight electrode pairs, received measurements from corresponding eight units of cellular biorecognition elements interacting with the assayed sample(s).

2.5. Sample preparation

Assay samples were prepared by macerating a small amount (2 g) of each cork sample to a detritus-like form. A total of 20 cork

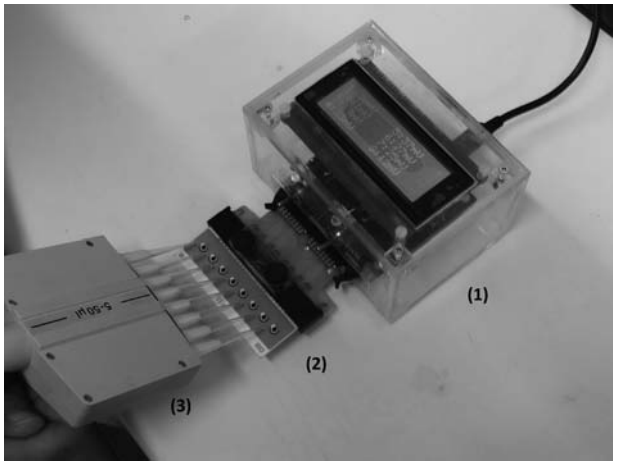

Fig. 1. The biosensor device based on membrane-engineered cells. The portable potentiometer (1) is connected to the eight-channel disposable sensor (2) through a customized interface. The mixture of cells (the biorecognition elements) and cork detritus in suspension are placed on the top of each of the eight carbon screen-printed electrodes contained in each disposable sensor strip with the aid of a multichannel pipette (3).

samples were used (10 TCA-free/10 TCA-positive). No further sample handling took place regarding the biosensor-based test. For validation purposes, the rest (non-macerated) of each cork sample was extracted with 12% ethanol (v/v) and submitted to GC–MS analysis for TCA according to a standard protocol [12]. Solutions of increasing TCA concentrations (from 0 to 10 ppt) were prepared in order to calibrate the system before the measurements.

2.6. Assay procedure

Cell suspensions (containing the membrane-engineered biorecognition elements) were distributed in Eppendorf tubes (200 μl per tube) and mixed with the detritus-like cork sample (0,01 g/tube) (each tube contained a different cork sample). After gentle agitation for about 1 min, a drop of cell-sample mixture (40 μl ≈ 40×10^3 cells) was placed on the top of each of the eight carbon screen-printed electrodes contained in each disposable sensor strip with the help of an automatic pipette (Fig. 1).

The response of the cells to the different samples (control and positive sample sets) was recorded as a time-series of potentiometric measurements (in Volts). The duration of each measurement was 180 s and 360 values/sample were recorded at a sampling rate of 2 Hz.

2.7. Biosensor validation

TCA solutions from cork samples were analyzed by GC/MS at an independent laboratory certified from the Portuguese Association for Certification (APCER), according to ISO Standard 20752:2007 and OIV's Resolution 296/2009 for determination of TCA in wine from cork stoppers.

2.8. Data analysis and experimental design

Both biosensor and chromatographic sample analyses were conducted according to a double-blind protocol. Experiments were set up in a completely randomized design and each experiment was repeated three times. In each application, a set of eight biosensors was tested against each individual sample.

3. Results and discussion

The biosensor was calibrated against increasing TCA concentrations. The biosensor responded in a linear pattern against increasing TCA concentrations (from 0 to 10 ppt) which indicated considerable hyperpolarization of the membranes of the Vero cells (associated with a numerical decrease of the cell membrane potential) (Fig. 2). It is worth mentioning that no response was observed on cell-free electrodes, indicating that the working principle of the biosensor is indeed based on the membrane-engineered biorecognition elements.

The results of the assay performed with the novel biosensor configuration, based on membrane-engineered cells as the biorecognition elements, are shown in Fig. 3. Vero cells membrane-engineered with the pAb_{78} polyclonal antibodies responded to the presence of TCA in cork detritus by considerable membrane hyperpolarization, as indicated by a net increase of the sensor potential by almost 28 mV and a shift from positive (TCA-free control samples) to negative values (TCA-tainted samples). The response was not dependent on the actual TCA concentration (as determined by the parallel validation studies described under 2.7). On the other hand, we observed a variation of response due to different testing dates, corresponding to different replications of the experiment and also to cells of different ages. Our

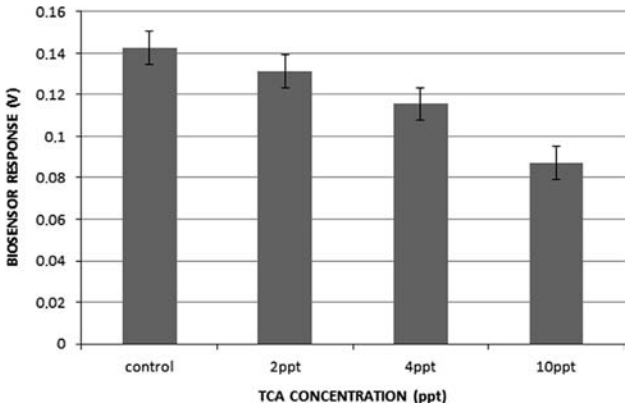

Fig. 2. Results of the biosensor calibration: increasing membrane hyperpolarization (decreasing membrane potential values) was associated with increasing TCA concentrations ($n=24$ replications for each concentration and error bars represent standard errors of the average value of all replications with each range of concentration).

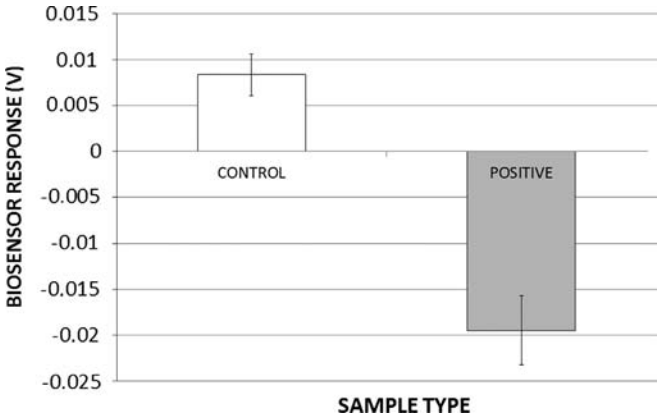

Fig. 3. Biosensor response against control (open column) and positive (gray column) samples. The sensor responded to the presence of TCA with considerable membrane hyperpolarization (negative change of the biosensor's signal). A steady-state membrane potential of 4 ± 2 mV was observed against control (TCA-free) samples ($n=24$ replications for each concentration and error bars represent standard errors of the average value of all replications with each range of concentration).

methodological approach allowed for testing the consistency of each measurement channel, by (a) assaying each sample simultaneously on all eight different measurement channels and (b) repeating the measurements at three different time periods. The response from each channel against TCA calibration standards was quite reproducible (variation 7–11%). The observed variation was higher against control samples (27%) than against positive ones (19%), possibly due to the chemical modification of extract constituents between the different assay periods. However, variation in response against a sample could not interfere with this sample's classification as either control or positive, due to the considerably different magnitude and sign (±) of response between the two sample sets. In addition, due to the fact that, according to the chromatographic analysis performed in an independent laboratory, the TCA concentration in the positive set of cork samples ranged from 0.2 to 14.7 ppt, we can assume a detection limit close to the lowest determined value (0.2 ppt). In addition, results obtained with the biosensor were highly correlated ($r^2=0.9864$) with those derived with chromatographic analysis (Fig. 4).

A key novelty in the assay developed in the present study, compared with the previously reported use of membrane-engineered cells for the detection of TCA, is the use of the cellular

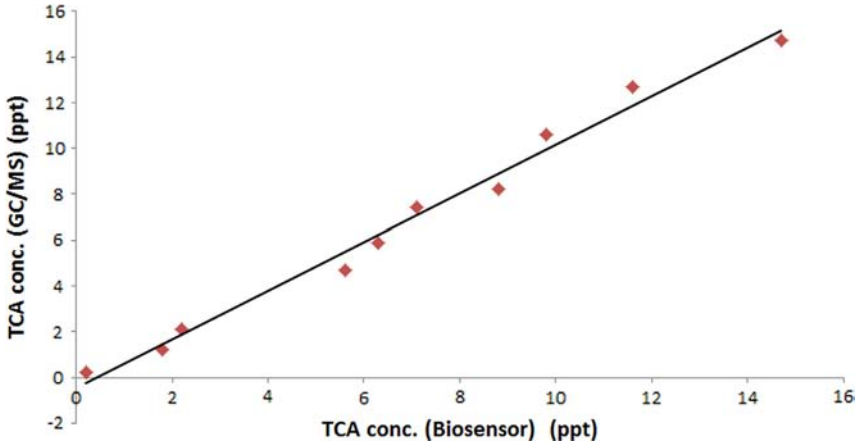

Fig. 4. Correlation of the TCA concentration determined with either the biosensor or GC/MS in the TCA-positive samples ($n=10$) (average values are shown).

biorecognition elements in suspension, instead of immobilized in gel. It is possible that the direct contact between the working electrode and the cell-sample mixture, as provided by the new biosensor configuration, resulted to a higher sensitivity of the assay compared to the previous biosensor setup: in the latter case, the vast majority of the immobilized biorecognition elements were separated from the measuring electrode by the gel, therefore any changes in the cell membrane potential could be detected only in an indirect manner (through the change of electrolyte concentration in the surrounding medium). This difference in the mode of measurement may also be responsible for the observed hyperpolarization as part of the cellular response to TCA, whereas immobilized cells in the previous version responded by membrane *depolarization*, i.e. by the opposite reaction [13]. Cell membrane hyperpolarization is the dominant change in the engineered cell membrane electric properties following a mechanical distortion of the membrane (according to the novel assay principle described in the invention) as also expected by the concurrent change in the actin cytoskeleton structure, in particular the actin cytoskeleton network adjunct to the sites of the interaction, including the circumferential actin belt and changes in the propagation of electric signals along actin filaments [14].

Another essential difference is the use of electroinsertion, contrary to osmotic insertion in the previous study, for the engineering of cell membranes with the pAb_{78} TCA-specific antibody. As such, electroinsertion may be a more efficient process leading the incorporation of a large number of antibody molecules per cell membrane surface unit, compared to the milder osmotic insertion method [15]. The exact number of pAb_{78} antibody molecules incorporated into the cell membrane has not been measured in the present study; previous reports estimate it at $8–10 \times 10^3$ per cell [10]. It might be worth mentioning that it has been recently shown that membrane-engineered cells carry antibodies inserted at the correct orientation, i.e. pointing their antigen-binding sites outwards, in their major percentage (over 90%); due to their appropriate orientation, the antibodies retain their normal antigen-recognition properties [16].

Although one of the possible disadvantages of the novel approach compared to other analytical technologies for TCA detection is the fact that it cannot offer reliable quantitative results, in the present context the possibility to rapidly select cork stoppers contaminated with TCA before delivering them to the wine factories would be desirable for most of the cork stopper and wine producers. An important drawback for the determination of TCA using the present approach is the extremely low solubility of TCA in water (log *P*, 4.11), which allows only for a minor fraction of its total concentration in cork to be detected by the otherwise ultra-sensitive system. It is well-known that lipophilic substances in aqueous media tend to adsorb to surfaces, which produces a decrease in the concentration of analyte that can readily interact with the antibody. It is very much possible that the amount of TCA determined by the cellular biorecognition elements is nevertheless correlated with the actual concentration in the cork samples. This is a target of future experiments.

Solvent-free analytical methods are gaining rapid importance. These methods seem that they are here to stay. Mostly, analytical samples are generally unsuitable for direct analysis due to their complexity and they necessitate the introduction of appropriate sample preparation methods before the analysis step. To the problems of these sample-preparation methods belong their low efficiency, large solvent consumption, more waste and high cost, as well as the fact that they are time- and energy-consuming. These shortcomings have led to consideration of innovative techniques in the so-called green analytical chemistry, which typically use less solvent and energy [17–19]. Today more and more studies use solvent-free techniques at the sample preparation stage, which are consistent with Green Chemistry principles [20].

4. Conclusion

The essential improvement over the previous biosensor is the absolute lack of a sample extraction step, which is associated with a number of practical benefits, including: (a) a very considerable reduction in the total assay time by one day (b) elimination of requirement for laboratory space for sample preparation (c) elimination of the solvent cost and (d) non-requirement for the disposal of waste. In addition, the device is characterized by a full portability and ease of use, requiring minimum effort by the end operator. This is further facilitated by the simple use of cells in suspension, which can be stored for up to four weeks at room temperature without any considerable lose in viability. Therefore, the novel biosensor system satisfies at least some of the conditions that could render it suitable for routine applications as an efficient high throughput, point-of-care analytical tool.

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