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Development of a cellular biosensor for the detection of 2,4,6-trichloroanisole (TCA)

Vassileios Varelas[a], Nuria Sanvicens[d,b], M-Pilar-Marco[b,d], Spiridon Kintzios[a,c,*]

[a] Laboratory of Plant Physiology, Faculty of Biotechnology, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
[b] Applied Molecular Receptors Group, IQAC-CSIC, Jordi Girona, 18-26, 08034- Barcelona, Spain
[c] The EMBIO Diagnostics Project, Nicosia, Cyprus
[d] CIBER de Bioingeniería, Biomateriales y Nanomedicina, (CIBER-BBN), Spain

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ABSTRACT

2,4,6-trichloroanisole (TCA) is a microbial metabolite formed from chlorophenols through the activity of several natural fungal strains present on the cork oak bark. TCA is the primary compound responsible for the mousty/mould off-odour known as "cork taint" present in cork stoppers, wine, water and alcoholic beverages. Chromatographic and electrochemical methods are currently used for the determination of TCA, however its detection at low concentrations remains a technical challenge. The aim of this study was the development of a rapid novel biosensor system based on the Bioelectric Recognition Assay (BERA). The sensor measured 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 analyte. Membrane-engineered cells were prepared by osmotic insertion of 0.5 μg/l of specific TCA antibodies into the membrane of the cells. The BERA-based sensor was able to detect TCA in a few minutes (3–5 min) at extremely low concentrations (10^{-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. Finally the sensor was tested against real white wine samples from cork soaks. At this real test, the BERA sensor was able to detect TCA from cork soaks rapidly (3–5 min) at very low concentrations (1.02–12 ng/l), covering the whole range for the detection threshold for wines (1.4–10 ng/l). Therefore, this novel biosensor offers new perspectives for ultra-rapid, ultra-sensitive and low-cost monitoring of TCA presence in cork and wine and possibly also other food commodities.

1. Introduction

2,4,6-trichloroanisole (TCA) is associated with the cork and wine industry as it is the main contaminant causing cork taint in bottled wines. TCA gives a musty, mould and/or earthy off-odour to affected wines, therefore masking the natural wine aroma and diminishing the quality of the final product. Consequently, its presence results into an economic loss and occasional damage of the profile and the reputation of the wine industry. It has been estimated that the portion of tainted bottles can be higher than 30% [1]. Apart from TCA, several other volatile compounds have been reported to contribute to cork taint, including chloroanisoles, guaiacol, geosmine, 2-methylisoborneol, pyrazines, 1-octen-3-ol and 1-octen-3-one. Of these, chloroanisoles and especially TCA and at a lesser extent 2,3,4,6-tetrachloroanisol (TeCA) and pentachloroanisole (PCA) are responsible for at least 80% of the cases of cork taint reported in wines [2]. On top of that, TCA has been shown to have a very low detection sensory threshold (1.4–10.0 ng/l) [3]. In this same context, TCA-related earthy and musty odours have also been detected in drinking water [4] and can be sensed by consumers at a 10 ppt level or less.

Traditionally, TCA determination has relied on chromatographic techniques such as gas chromatography/mass spectrometry (GC–MS) [5] and more recently solid phase microextraction coupled with gas chromatography–ion trap mass spectrometry (GC–ITMS) [6]. However, these analytical methods have failed to meet the demand for high throughput TCA analysis and to achieve a detection limit close to the human sensory threshold (a few ppts) [3]. Immunoanalytical techniques involving a specific antibody raised against TCA offer a far more promising alternative. Sanvicens et al. [7,8] were the first to report an immunoassay technique for TCA using an enzyme-linked immunosorbent assay (ELISA) while Moore et al. [9] described the development of an immunoamperometric technique. The authors reported a detection limit in the

* Corresponding author at: Laboratory of Plant Physiology, Faculty of Biotechnology, Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece.
Tel.: +30 210 5294292; fax: +30 210 5294286.
E-mail address: skin@aua.gr (S. Kintzios).

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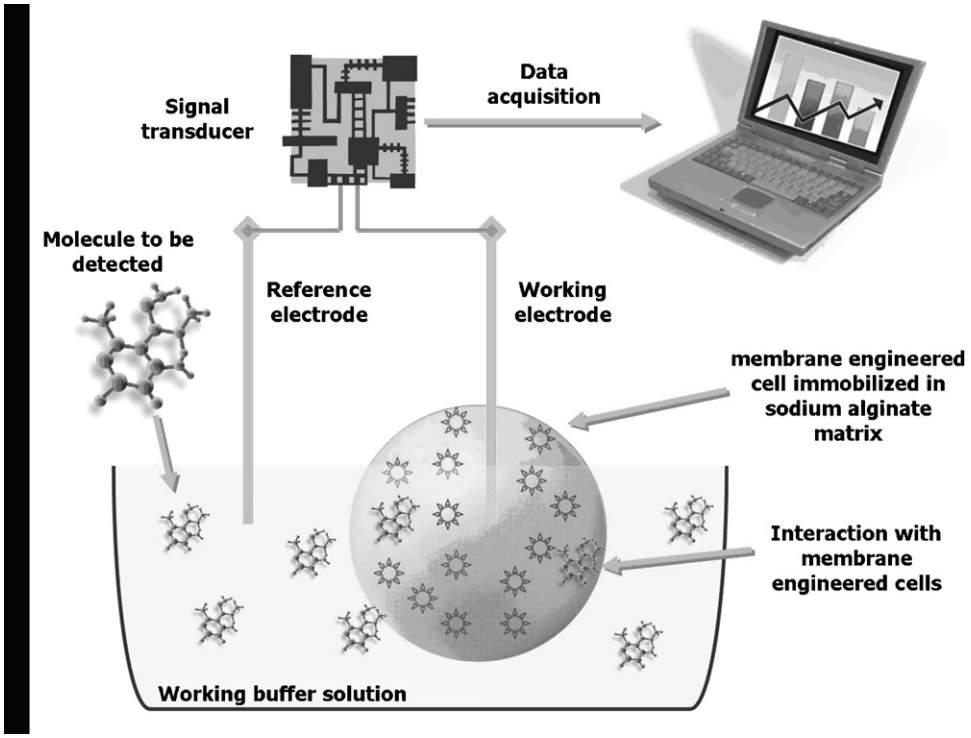

Fig. 1. Schematic representation of the immobilized cell biosensor. The reference electrode is inserted directly in the assayed sample, while the measuring electrode is inserted in the immobilized cell-gel bead, which has an approximate diameter of two mm. Measurement and reference electrodes are connected via wiring to the PMD 1608-FS data converter. The membrane-engineered cell is not shown to scale.

range of 29–87 ppt, although the duration of the assay was lengthy and extensive sample pre-treatment was required for the analysis of TCA in actual wine samples. Sanvicens et al. [10] developed an immunosorbent solid-phase extraction followed by ELISA with a detectability for TCA of 440 ng/l in white wine. More recently, Duarte et al. [11] reported the development of an electrochemical displacement immunosensor with a limit of detection of 200 ppb, i.e. well above the human sensory threshold.

In an attempt to increase the sensitivity of TCA detection, we herewith report the development of a novel biosensor based on mammalian cells. Cells were membrane-engineered by the artificial insertion of TCA-specific antibodies. Consequently, binding of TCA on the homologous, antibody-bearing sites on the cell membrane caused considerable changes of the cell membrane potential, according to the principles of the molecular identification through membrane-engineering [12–14]. Engineered cells were immobilized in calcium alginate gel and their electrophysiological responses against TCA and the structurally related and/or co-occuring 2,4,6-trichlorophenol (TCP) (a precursor of TCA and its main competitor in real samples), 2,4,6-tribromoanisole (TBA), 2,6-dichloroanisole (2,6-DCA), 2,3-DCA, 3,5-DCA and 2,3,4,5-tetrachloroanisole (TeCA) were measured according to the principles of the Bioelectric Recognition Assay [15].

2. Experimental

2.1. Materials

HaK (golden Syrian hamster adult kidney) cell 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_{76}), as well as standard solutions of 2,4,6-TCP, 2,4,6-TBA, 2,6-DCA, 2,3-DCA, 3,5-DCA and 2,3,4,5-TeCA were obtained from the Department of Chemical and Biomolecular Nanotechnology IQAC-CSIC, Barcelona, Spain (Sanvicens et al., [7,8]). Cork samples were originally provided from Amorim SA (Portugal) and Portugal Cork SA (Greece). All other reagents were purchased from Fluka (Buchs, Switzerland).

2.2. Cell Culture

HaK hamster cells were cultured in Dulbecco's medium with 10% heat-inactivated foetal calf serum (FCS), 10% antibiotics (streptomycin) and 10% l-glutamine.

2.3. Sensor fabrication from "membrane-engineered" cells: anti-TCA antibodies osmotic insertion in HaK cells.

Membrane-engineered cells (5×10^6/ml) were created by insetting via osmosis pAb_{76} into the membrane of HaK cells, following a modified protocol from Okada and Rechsteiner [16]. Cells were centrifuged at 1000 rpm for 10 min at 4 °C and then resuspended in 500 μl of a hypertonic solution (sucrose 0.5 M, 10% PEG 1500, PBS) containing 1.0 μg/ml pAb_{76} for 10 min at 25 °C. Subsequently, the cells-antibody mixture was centrifuged at 1000 rpm for 10 min at 4 °C and then resuspended and incubated for 2 min at 25 °C in 300 μl of a hypotonic solution (DMEM:H_2O = 6:4). Subsequently, the cells were centrifuged at 1000 rpm for 10 min at 4 °C and then resuspended in 500 μl DMEM. Finally, cells were centrifuged at 1000 rpm for 2 min at 4 °C (this was repeated for three times) and resuspended in 1 ml cell culture medium containing 20% heat-inactivated foetal calf serum (FCS).

Subsequently, cells were used as biorecogniton elements in manufacturing sensors based on the Bioelectric Recognition Assay. BERA is based on measuring changes of the membrane potential of immobilized cells, affected by their interaction with target analytes, including low molecular weight compounds such as pesticides [15,17]. One ml of membrane-engineered cells (at a density of 2.5×10^6/ml) were mixed with 2 ml of 2% (w/v) sodium alginate solution and then the mixture was added drop wise, by means of a

22G syringe, in 0.2 M $CaCl_2$. Each of the resulting calcium alginate beads had an approximate diameter of 2 mm and contained approximately 5×10^4 cells. Each bead was designated as a consumable BERA-TCA sensor. In a separate experiment, osmotic-treated with PBS but not membrane-engineered cells were also used for sensor fabrication in order to assay their response against TCA.

2.4. Recording and data processing

Each cell-bearing bead (BERA-TCA sensor) was connected to an electrode made from 80% Cu, electrochemically coated with an Ag/AgCl layer and having a diameter of 0.75 mm. A cell-free bead was attached to the reference electrode, as described previously [13,14] (Fig. 1). Electrodes were connected to the recording device, which comprised the PMD-1608FS A/D card (Measurement Computing, Middleboro, MA). The software responsible for the recording of the signal and processing of data was InstaCal (Measurement Computing).

For each assay, the sensor system, comprising of beads attached to the working and the reference electrode, was immersed into the sample solution (200 μl). The response of each sensor was estimated by recording the absolute maximum change of the sensor potential from 0 s to 300 s (achievement of stable response of the biosensor).

2.5. Detection assay

Standard solutions of TCA were prepared by dissolving the compound in PBS (pH 7.4) at concentrations 0.0 (control), 10^{-1}, 10^1, 10^3, 10^5 and 10^7 ppt. In order to test the selectivity of the assay, the response of the biosensor against 10^4 ppb (10^7 ppt) solutions of either 2,4,6-TCP, 2,4,6-TBA, 2,6-DCA, 2,3-DCA, 3,5-DCA or 2,3,4,5-TeCA was also investigated. Finally BERA-TCA sensors were also tested for assaying TCA preparations in white wine, from TCA extracted from cork soaks in white wine according to ISO standard 20752:2007 [18] and OIV's Resolution 296/2009 for determination of TCA in wine from cork stoppers [19] at concentrations 0.0 ng/l (control: white wine without TCA used for cork soaks), 1.02 ng/l, 2.3 ng/l, 5.1 ng/l, 6.0 ng/l, 11.5 ng/l and 12.0 ng/l.

2.6. Fluorescent microscopy assay of TCA-membrane engineered cell interaction

Cell membrane potential changes in membrane-engineered HaK-TCA cells at different TCA concentrations (10^3 and 10^7 ppt) were also monitored as changes in cytoplasmic Ca^{2+} concentration by the uptake of the acetomethyl ester of Fluo3 [13]. After application of 5 μl of the dye, the fluorescence of the specimens was recorded for 5 min at 10 s intervals. Slides with stained cells were mounted on a Zeiss Axiolab fluorescent microscope equipped with a BP-546 excitation filter and an FT-580 chromatic beam splitter. A digital camera (SONY S75 digital still camera) was attached to the microscope with adjustable BP-546/FT-580 or G365/FT 395 excitation filter/chromatic beam splitter combinations. In order to control photobleaching, we kept specimen exposure times at a minimum level. No significant alteration of the intensity of the fluorescence was observed during the observation of the specimens.

2.7. BERA Sensor validation

The sensor was tested against standard solutions of TCA, 2,4,6-TCP, 2,4,6-TBA, 2,6-DCA, 2,3-DCA, 3,5-DCA and 2,3,4,5-TeCA. TCA solutions from cork soaks were analyzed conventionally with GC/MS at the Laboratório Investigação E Desenvolvimento, LDA (Portugal) 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.

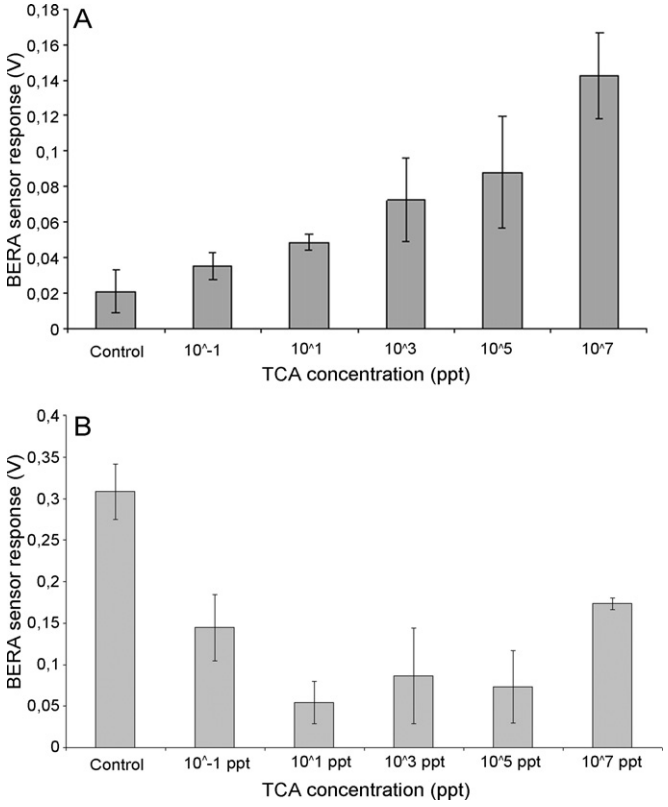

Fig. 2. (A) Differential responses of the biosensor based on immobilized HaK-TCA cells to different TCA concentrations (expressed as ng/l or parts per trillion – ppts). The sensor responded with considerable membrane hyperpolarization in a concentration depended pattern. A steady-state membrane potential of 20 ± 1 mV was observed against control solutions (PBS). (B) Responses of the biosensors based on osmotic-treated but not membrane-engineered cells, i.e. non-bearing pAb_{76}. The steady-state potential in this case was 300 ± 3 mV ($n = 5$ replications for each concentration and error bars represent standard errors of the average value of all replications with each range of concentration).

2.8. Data analysis and experimental design

Both biosensor and conventional sample analysis were conducted according to a double-blind protocol. Experiments were set up in a completely randomized design and each experiment was repeated three times. Results were assessed by a standard analysis of variance. In each application, a set of five biosensors was tested against each individual sample.

3. Results and discussion

3.1. Biosensor response to different TCA concentrations.

Results of the assay performed with the BERA-TCA biosensors with standard TCA solutions at concentrations varying from 10^{-1} ppt to 10^7 ppt are shown in Fig. 2A. Sensors containing HaK cells engineered with the pAb_{76} polyclonal antibodies responded to the presence of TCA by considerable membrane hyperpolarization, as indicated by the net increase of the sensor potential compared to the control. The response was concentration-depended (in the range of assayed concentrations). The limit of detection was 0.1 ppt TCA, thus demonstrating that the sensitivity of the novel biosensor is considerably higher than the human sensory threshold (5–20 ppt) [3] or the best analytical system reported so far for TCA

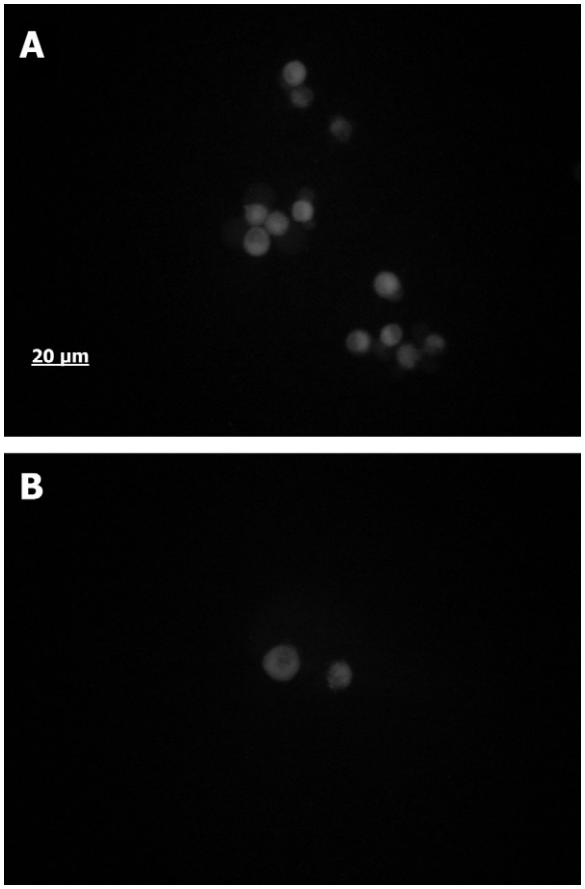

Fig. 3. Changes (expressed as differences in fluorescence intensity) of the cytoplasmic calcium ion concentration in HaK-TCA cells, membrane-engineered with pAb_{76} at different TCA concentrations (A: 10^3 ppt, B: 10^7 ppt).

[7,8]. On the contrary, a totally different pattern of response against TCA was observed for BERA sensors based on osmotic-treated but not membrane-engineered cells, i.e. non-bearing the TCA-specific pAb_{76} (Fig. 2B). More specifically, considerable membrane depolarization (lowering of the sensor potential) was observed in the presence of TCA (e.g. opposite to the hyperpolarization effect observed with the membrane-engineered cells). This non-concentration depended response to TCA (especially in the range 10^1–10^5 ppt) is possibly due to the direct action of the haloanisole molecules on the cell membrane, a hypothesis which merits further investigation.

Fluorescence microscopy revealed that HaK-TCA cells membrane-engineered with pAb_{76} responded to increasing TCA concentrations by a depletion of their intracellular Ca^{2+} stores (Fig. 3A and B). Similar responses were observed in earlier reports for cells membrane-engineered with virus-specific antibodies after treating them with the homologous viral antigens [13,14], indicating a possible contribution of the efflux of Ca^{2+} ions to the sensor response.

3.2. Biosensor response to possible interfering compounds.

The biosensor responded selectively to 10^7 ppt TCA than to other haloanisols/halophenols (at the same concentration) structurally related to or co-occurring in samples with TCA (Fig. 4), even though a signal was also detected against them. However, the sensor's response to TCA was several times higher than to other haloanisols/halophenols. Moore et al. [9] have reported that 2,4,6-TCP is a potent cross-reactant with the pAb_{76} antibody

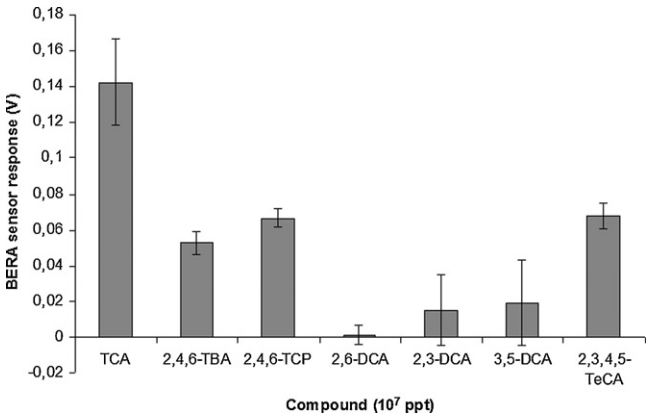

Fig. 4. Differential responses of the biosensor to analytes structurally related to or co-occurring in samples with TCA at a concentration of 10^7 ppt ($n = 5$ replications for each concentration and error bars represent standard errors of the average value of all replications with each range of concentration).

and a very hazardous substance [20]. TBA and TeCA are common contributors to cork taint [2,21,22]. The selectivity of the novel cell-based assay could be further improved either by using cells membrane-engineered with antibodies against interfering haloanisoles/halophenols (in order to screen them out) or by modifying the conditions affecting the binding efficiency between TCA molecules and electroinserted antibodies, such as pH and/or the ionic strength of the assay solution.

3.3. Biosensor response to different TCA concentrations from cork soaks

The results of the response of BERA-TCA sensor containing HaK cells engineered with the pAb_{76} polyclonal antibodies against cork soaks in white wine are shown in Fig. 5. The sensor responded linearly at the TCA concentration range of 1.02–12.0 ppt with 2.81–17.31% variation. The limit of detection for TCA (1.02 ppt) is demonstrating that the sensitivity of the novel biosensor is higher than the human sensory threshold (1.4 ppt) [3]. Furthermore, the range of the sensor response is covering the regulatory detection threshold for wines (1.4–10.0 ppt) [3]. The absolute value of the response of the sensor against various concentrations of TCA in wine were different than the response against TCA in PBS (standard solutions–Fig. 2A) demonstrating a considerable matrix effect.

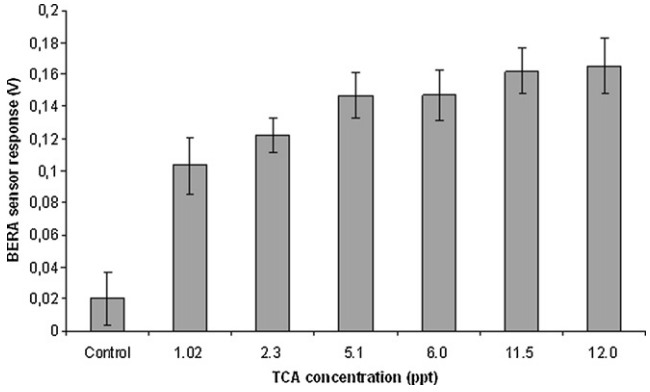

Fig. 5. Differential responses of the biosensor on immobilized HaK-TCA cells to different TCA concentrations extracted from cork soaks. The sensor responded selectively at a range between 1.02 ppt and 12.0 ppt (ng/l) covering the range for the detection threshold for TCA in wines (1.4–10 ppt). Control is white wine without TCA ($n = 5$ replications for each concentration and error bars represent standard errors of the average value of all replications with each range of concentration).

4. Conclusion

The availability of new, rapid assays 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. A few promising biosensor technologies for TCA detection have emerged during the last years. However, they were characterised by less than satisfactory sensitivity and rather long assay times. The novel BERA-based biosensor could represent a new generation of analytical tools for TCA determination, since it enables ultra-sensitive and rapid testing. Using the membrane-engineering approach described in the present study and due to the availability of antibodies against a variety of halophenols and haloanisols, it is now possible to construct biosensors able to selectively detect different environmentally important contaminants at trace concentrations [7,8,20]. In addition, it is possible to adapt the assay system for high throughput TCA analysis. Using a multiple cell-electrode interface array (currently under testing by our research group), it is possible to conduct more than 1150 individual tests/h, a capacity considerably larger than with conventional immunoassay systems. Finally, the preliminary tests with real cork and wine samples have demonstrated the practical applicability of the system, without any elaborate sample pretreatment.

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References

[1] M.A. Sefton, R.F. Simpson, Aust. J. Grape Wine Res. 11 (2005) 226–240.
[2] D.L. Capone, G.K. Skouroumounis, D.A. Barker, H.J. McLean, A.P. Pollnitz, M.A. Sefton, Aust. J. Grape Wine Res. 5 (1999) 91–98.
[3] S. Prak, Z. Gunata, J.P. Guiraud, S. Schorr-Galindo, Food Microbiol. 24 (2007) 271–280.
[4] L. Zhang, R. Hu, Z. Yang, Water Sci. Technol. 54 (2006) 335–344.
[5] A. Salemi, S. Lacorte, H. Bagheri, D. Barceló, J. Chromatogr. A 1136 (2006) 170–175.
[6] Y.H. Sung, T.Y. Li, S.D. Hyang, Talanta 65 (2005) 518–524.
[7] N. Sanvicens, F. Sánchez-Baeza, M.P. Marco, J. Food Agr. Chem. 51 (2003) 3924–3931.
[8] N. Sanvicens, B. Valera, M.P. Marco, J. Food Agr. Chem. 51 (2003) 3932–3939.
[9] E. Moore, M. Pravda, G. Guilbault, Analytica Chimica Acta 484 (2003) 15–24.
[10] N. Sanvicens, E.J. Moore, G. Guilbault, M.P. Marco, J. Agric. Food Chem. 54 (2006) 9176–9183.
[11] M.V. Duarte, P. Lozano-Sanchez, I. Katakis, Biosens. Bioelectron. 24 (2009) 2205–2210.
[12] G. Moschopoulou, S. Kintzios, Anal. Chim. Acta 573–574 (2006) 90–96.
[13] G. Moschopoulou, K. Vitsa, F. Bem, N. Vassilakos, A. Perdikaris, P. Blouhos, C. Yialouris, D. Frossiniotis, I. Anthopoulos, O. Maggana, K. Nomikou, V. Rodeva, D. Kostova, S. Grozeva, A. Michaelides, A. Simonian, S. Kintzios, Biosens. Bioelectron. 24 (2008) 1033–1036.
[14] S. Perdikaris, N. Alexandropoulos, S. Kintzios, Sensors 9 (2009) 2176–2186.
[15] E. Flampouri, S. Mavrikou, S. Kintzios, G. Miliaids, P. Aplada-Sarli, Talanta 80 (2010) 1799–1804.
[16] C.Y. Okada, M. Rechsteiner, Cell 29 (1982) 33–41.
[17] S. Mavrikou, E. Flampouri, G. Moschopoulou, O. Mangana, A. Michaelides, S. Kintzios, Sensors 8 (2008) 2818–2832.
[18] Cork stoppers-Determination of releasable 2,4,6-trichloroanisol (TCA), International Standard Organisation, Switzerland, Geneva, 2007. http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=39703.
[19] Determination of releasable 2,4,6-trichloroanisole in wine by cork stoppers, International Organisation of Vine and Wine, Paris, 2009. http://news.reseau-concept.net/images/oiv_uk/Client/OIV-Oeno_296-2009_EN.pdf.
[20] R. Galve, F. Sanchez-Baeza, F. Camps, M.P. Marco, Anal. Chim. Acta 452 (2002) 191–206.
[21] P. Chatonnet, G. Guimberteau, D. Dobourdieu, J.N. Boidron, J. Int. Sci. Vigne Vin. 28 (1994) 131–138.
[22] C. Pizarro, N. Pérez-del-Notario, J.M. González-Sáiz, J. Chromatogr. A 1149 (2007) 138–144.