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Development of a biosensor for the quantitative detection of 2,4,6-trichloroanisole using screen printed electrodes

Eric Moore, Miloslav Pravda, George G. Guilbault*

Laboratory of Sensor Development, Chemistry Department, University College Cork, Cork, Ireland

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Abstract

Immunoassay techniques can provide a simple, practical and inexpensive method for analysis of 2,4,6-trichloroanisole (TCA). Enzyme linked immunosorbent assay (ELISA) and electrochemical techniques were investigated with the purpose of attaining high selectivity and sensitivity. Both assays incorporated a direct format for analysis of TCA using alkaline phosphatase (AP), as the labeling species. TCA has no functional groups through which linkage of enzymes or proteins can be achieved so alternative hapten molecules were developed. Molecular recognition between TCA and the antibodies that were raised against haptens whose chemical structures were similar to the target analyte was good. This was especially true for the competition between free analyte and hapten A-AP conjugate for the protein G purified polyclonal antibodies (pAb_{76}). ELISA results using the direct format of analysis were poor when compared to the achieved results for the electrochemical sensor. A limit of detection of 1 ng/ml (1 ppb) was achieved for the best ELISA system while a limit of detection of 29 pg/ml (29 ppt) was obtained for the electrochemical sensor.

Keywords: Immunosensor; Trichloroanisole; Screen printed electrode; IgG; Biosensors; Alkaline phosphatase; *p*-Aminophenyl phosphate

1. Introduction

Cork taint, a musty/mouldy scent in affected bottles, is one of the most serious problems affecting the wine industry. Previous research work has identified 2,4,6-trichloroanisole (TCA) as one of the most important contaminants that give this musty/mouldy taste to wine [1]. There are other contaminates that can also be present in wine such as 2-acetylpyrroline [3] and 2-acetyltetrahydropyridines [4], but TCA is the main contributor. Such contamination is responsible for returned and sub-standard merchandise leading to roughly 1000 million Euro losses annually in the wine sector alone. Estimates of the incidence of contamination in corked bottles range from 2.5 to 5% [5–7].

In a recent study of wines [8], it was observed that all of the Australian wines that were tainted had TCA present at or above the sensory threshold value. Although the presence of TCA is due to the microbial transformation of trichlorophenol (TCP) and its derivatives [7,9–13], the source of the contamination has not yet been clearly identified. In addition, various penta-, tetra-, tri- and di-chloroanisole isomers

* Corresponding author. Tel.: +353-21-490-2208; fax: +353-21-490-3103.
E-mail address: g.guilbault@ucc.ie (G.G. Guilbault).

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or derivatives have been identified in other food sectors not related with the cork industry (vegetable oils, raisins, water reservoirs, chicken meat, etc.) [14,15]. Some studies have focused on the prevention of these contaminants before exposure to the food industry [16].

In the 1980s, a musty taint in packaged dried vine fruit was found. The taint was sporadic and appeared only in fruit transported in freight containers with general-purpose use. This compound was eventually identified as 2,4,6-trichloroanisole. It was found to have formed from the corresponding chlorophenol in the packaging material by fungi [17]. Not less than 17 species of fungi isolated from the packaging were shown to produce the 2,4,6-trichloroanisole. Species of fungi identified included *Aspergillus* [2], *Eurotium* [2] and *Penicillium* [7], together with a single species each of *Paecilomytes*, *Merimbla* and *Fusarium*. The species responsible for the highest concentration of methylation of 2,4,6-trichlorophenol were *Paecilomyces variotti*, *Fusarium oxysporum* and *Aspergillus flavus* [18].

The sources of the microorganisms of biomethylation were the fibreboard packaging materials and also the timber floors of the freight containers were a major cause of contamination of the fruit. Again 2,4,6-trichloroanisole was found to be the main offender, along with its precursor 2,4,6-trichlorophenol, together with various species of fungi with known ability to biomethylate chlorophenols [19].

Chloroanisoles have been known to cause food taint in eggs [20], cocoa powder [21], coffee [22] and dried fruit [8], beverages [23] and in many more foods and beverages [24–27]. The presence of TCA in natural water is also of great concern because of higher contamination risks to the food industry [28,29]. Until the source is identified and the problem solved, the only solution is to detect the contamination and eliminate contaminated material. This should dramatically help to reduce the loss of both capital and foodstuffs attributed to this form of contaminated waste. However, the low sensory perception threshold and the complexity of the food analytical matrices require laborious extraction and clean-up techniques, expert personnel, and expensive analytical equipment for the detection and quantification of the compound [30].

The bark of the cork oak *Quarks suber* that grows mainly in countries near the Mediterranean Sea is used in the production of corks for the wine industry [31]. World production is about 300,000 metric tonnes per year: Portugal 55%, Spain 28%, Algeria 6%, Morocco 4%, Italy 3%, Tunisia 3% and France 1%. About 60% of the cork harvest is used for the production of single piece stoppers [32,33]. The original bark of the tree is stripped off and the bark that has regrown, reproduction bark, is used to make the cork stoppers. Reproduction *bark* is uniform in texture and density, is impermeable to liquids and air (preventing oxidation of the wine), has the ability to adhere to a glass surface, is compressible, resilient and chemically inert.

A simple and efficient biosensor with a limit of detection of sub ppb concentration would provide a tool for monitoring TCA formation at an early stage, preventing contamination of wine, food products and water supplies. The human sensory threshold value for 2,4,6-TCA in wine is in the range 5–20 ppt [1], the variability being due to the type of wine used, the sensitivity of the judge and the training of the judge. These concentrations are beyond the sensitivity of most analytical techniques without a preconcentration step. 2,4,6-Trichloroanisole has been determined by many methods over time such as GC-multiple ion monitoring (MIM)-mass spectroscopy (MS) [34], distillation or headspace gas chromatography (GC) [35]. More recently thermal desorption of 2,4,6-trichloroanisole from a cork sample has been introduced into a special injector [36].

The analytical determination of 2,4,6-trichloroanisole has traditionally relied on labor-intensive procedures such as Soxhlet; liquid–liquid or solid phase extraction and subsequent concentration, [2], that uses hazardous solvents. Dynamic headspace sampling of cork material combined with cyro-focusing has used also been used, [12]. Identification and determination of such low concentrations have proved to be very challenging and requires sophisticated instrumental techniques. Extraction of the compound from food by solid phase micro extraction [37], separation and identification by gas chromatography coupled to mass spectroscopy (MS) [29,38], for the sensory evaluation of components as eluted from the GC column is essential for the initial determination of the offending compound responsible for the taint [2]. Therefore, many techniques are available for TCA analysis but low detection has not been achieved and extraction of TCA from solid samples still remains a problem.

2. Experimental

2.1. Reagents

The substrate for the electrochemical detection of alkaline phosphatase (AP, EC 3.1.3.1, type VII-S, Sigma), was 4-aminophenyl phosphate (*p*-APP) (Universal Sensors). The DEA buffer (pH 9.5) contained 0.1 M diethanolamine, 50 mM KCl (Sigma) and 1 mM $MgCl_2$ (Sigma). The blocking buffer (pH 7.4) contained 50 mM Tris (Sigma), 1 mM $MgCl_2$ and 1% (w/v) bovine serum albumin (BSA, Sigma). Immobilisation buffer was 50 mM sodium carbonate buffer (1% NaCl) (Sigma). The washing buffer (pH 7.4) used contained 0.05% Tween in 50 mM Tris (Sigma), 1 mM $MgCl_2$. Dialysis buffer used for purification of enzyme conjugates was 0.1 M PBS at pH 7.4. The analyte was 2,4,6-trichloroanisole (Aldrich) and the polyclonal antibodies raised against hapten A and B, pAb_{76} and pAb_{79} were obtained from the Department of Biological Organic Chemistry, IIQAB-CSIC, Barcelona, Spain, [39]. Both hapten A and B are derivatives of TCA. They were synthesised and conjugated to AP. All other chemicals were of analytical grade or better, all solutions were prepared daily with doubly distilled water.

2.2. Apparatus

All amperometric experiments were performed at room temperature using a 5 ml stirred batch glass cell. Electrochemical workstation BAS 100B/W (Bioanalytical Systems, BAS, USA) was used to control the three electrode set-up consisting of a carbon screen printed strip working electrode, a Ag/AgCl (3 M NaCl, BAS) reference electrode and a platinum wire auxiliary electrode.

2.3. Procedures

2.3.1. Conjugation protocol for AP tracers

AP conjugates were prepared by the *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)cabodiimide (EDC) conjugation protocol. A NHS ester is perhaps the most common activation agent for creating acylating agents. Formation of such esters may be achieved by the reaction of a carboxylate with NHS in the presence of a carbodiimide [40] (Fig. 1).

A molar ratio of 10:1 was used in the conjugation of hapten to enzyme. In forming the active intermediate a 2.3 M excess of NHS and EDC reagents was used. The hapten was dissolved in an aqueous solution of 20% tetrahydrofuran (THF). All three reagents were reacted and left to shake for 4 h at room temperature. The enzyme was dissolved in a 0.1 M PBS at pH 7.4 and it was then added to the reaction mixture as the second step. The reaction was allowed to proceed for at least another 18 h after which it was purified. Purification was achieved by dialysis. The AP conjugate was diluted to a total volume of 500 μl using an alkaline phosphatase stabilising buffer, which contained some sodium azide. Aliquots of 100 μl volumes were made of the conjugate and they were then stored in the freezer at −18 °C.

2.3.2. Characterisation of conjugates

It is important to characterise conjugates in terms of concentration. With every conjugate that is prepared the optimum dilution of the conjugate is calculated, but this value can vary depending on such parameters as reaction yield, technician, etc. Correlating a dilution to a concentration value gives a basis for comparison of prepared conjugates.

A fresh set of protein standards in a range of 200–0.5 μg/ml and also the working reagent as outlined in the Pierce manual [41] were prepared. Enzyme linked immunosorbent assay (ELISA) plate was blocked for 2 h with 1% BSA block buffer and then washed with washing buffer. A volume of 150 μl of each standard or unknown sample was pipetted into the appropriate microtiter plate wells. The plate was covered and incubated it at 37 °C for 2 h. After incubation, the plate was allowed to cool to room temperature. The absorbance was measured at a wavelength of 562 nm for each assay. If using a plate reader blanks were subtracted from signals. A standard curve was prepared by plotting the absorbance obtained for each BSA standard against its concentration in μg/ml. The protein concentration for each unknown sample was determined using the standard curve.

2.3.3. ELISA protocol

The antibody was diluted to optimum concentration (0.75 μg/ml for pAb_{76}) in an immobilisation buffer and 50 μl of it was added to each of the wells marked for assay. The antibodies were pipetted on the ELISA

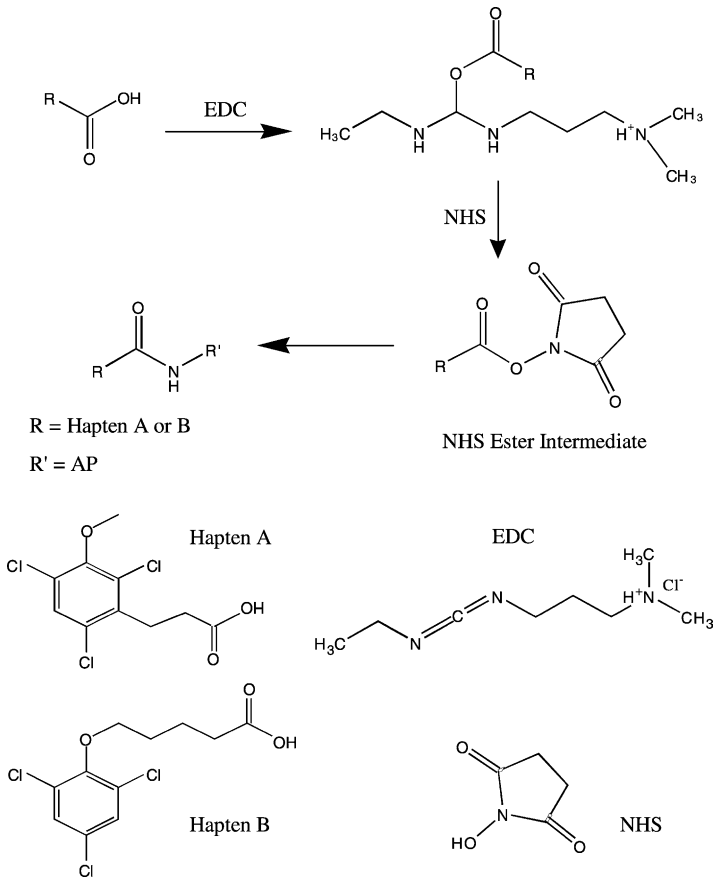


Fig. 1. Reaction profile for the NHS/EDC conjugation protocol.

plate and allowed to bind for 1 h at 37 °C. After primary immobilisation, the excess antibody that had not bound was removed by washing three times with the wash buffer. Two hundred microlitres of the blocking solution was added to each well and this solution was then left to block for 1 h at 37 °C. The ELISA plate was again washed three times to remove the excess block buffer. Blocking prevents non-specific binding at later stages in the ELISA.

A 10-fold serial dilution of TCA was done using the block buffer as the dilution buffer. The stock solution contained a 1 mg/ml of TCA prepared in THF solvent. This solution was always prepared fresh, prior to analysis. All handling of TCA was done in glass as TCA is a volatile substance that adsorbs to plastic and sample loss can be significant. The optimum dilution of the conjugate, which was found to give a high response, was also prepared. The dilution series of TCA and the optimum dilution/concentration was then applied to the specific wells on the plate with subsequent incubation for 1 h at 37 °C. Twenty-five microlitres of TCA solution and conjugate were added to each well.

The plate was washed to remove the unbound TCA solution and conjugate. Fifty microlitres of *p*NPP substrate (1 mg/ml) was added to each well, the absorbance was read at 405 nm and results were plotted.

2.3.4. Matrix preparation

As TCA is present in cork used in the wine industry, it is necessary to extract it from the cork and into an organic solvent. Preparation of a suitable extract matrix for analysis of TCA is described. Cork extract

was prepared using the following solvents, ethanol, methanol and THF. Cork samples were crushed and mixed with the solvents (ca. 1 g/10 ml). The samples were soaked in these solvents for 48 h at room temperature. The extracts were diluted with buffer until a 10% solvent content was achieved. This solution was spiked with TCA and used in competitive assay. Two controls were used in the experimental design. The first control involved the solvents diluted to 10% concentration with buffer as like the extracts, spiked with TCA, but these having no contact with any cork. The second control was the normal assay, with the buffer spiked with TCA.

2.3.5. Screen printed electrode (SPE) manufacture

The SPE were prepared using Electrodag 423 SS carbon ink, Electrodag 477 SS silver ink for conductor paths, and a Matt Vinyl White MV27 (Apollo, London, UK) for insulation layers. Screen-printer DEK 247 was used for printing. The squeegee velocity was set to 4. Each layer was left for 1 h to evaporate the solvent to obtain a dry path. The electrode area was 4 mm × 4 mm, i.e. 16 mm^2. After printing the last path, the electrodes were cured at 80 °C overnight. Fig. 2 illustrates the SPE design and layer development.

2.3.6. Electrochemical protocol

A scheme illustrating the experimental design of the biosensor is shown in Fig. 3. The immunoelectrodes were prepared as follows: A drop of 5 μl of 7.5 μg/ml pAb_{76} in the immobilisation buffer was spread onto the surface of the working electrode and the electrodes were incubated for 1 h at room temperature. The unbound IgG was washed away with water and the electrode surface was then blocked by incubating in 200 μl of blocking buffer for 1 h at 37 °C. The excess BSA block buffer was washed away with water. For the direct capture assay, the electrodes were immersed in 150 μl of hapten A-AP conjugate (3.5 μg/ml). The antigen–antibody capture was allowed to proceed for 1 h at 37 °C. For a direct competitive assay the electrodes were immersed in 150 μl of a 1:1 mixture of hapten A-AP (3.5 μg/ml) and the TCA analyte. A serial dilution of the TCA was done in order to establish a calibration plot. The electrodes were washed for a final time with water and tested amperometrically by adding *p*APP at +300 mV in a stirred DEA buffer cell. The preparation for "blank" electrodes was identical, except that there was no primary immobilisation step.

All electrochemical experiments were performed at room temperature (22 °C) in a stirred batch cell at +300 mV containing DEA buffer at pH 9.5. DEA solution containing 1 mM *p*APP (final concentration) was injected for amperometry of immunoelectrodes. Spectrophotometric detection was done at a wavelength of 405 nm.

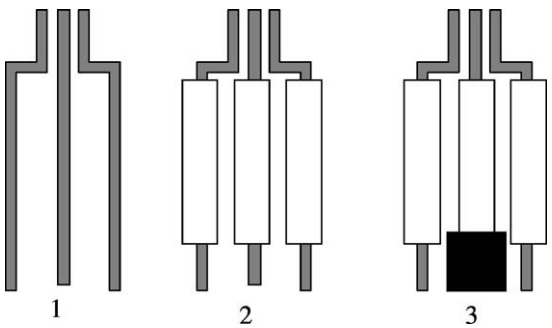


Fig. 2. SPE design: (1) Ag conducting layer, (2) printing of insulation layer, (3) final modification with printing of carbon working area.

3. Results

3.1. Characterisation of conjugates

The Pierce Micro BCA protein assay reagent was used for the quantitative colorimetric determination of total protein in diluted aqueous solutions. Table 1 correlates the optimum assay dilution of the enzyme conjugates to a concentration value.

3.2. TCA competition assay

The combination of hapten A-AP with the protein G purified pAb_{76} gave the best response that is

Table 1
Characterisation of conjugates

Enzyme conjugate	Optimum dilution ELISA	Correlated concentration (μg/ml)
Hapten A-AP	1/500	34.66
Hapten B-AP	1/500	35.10

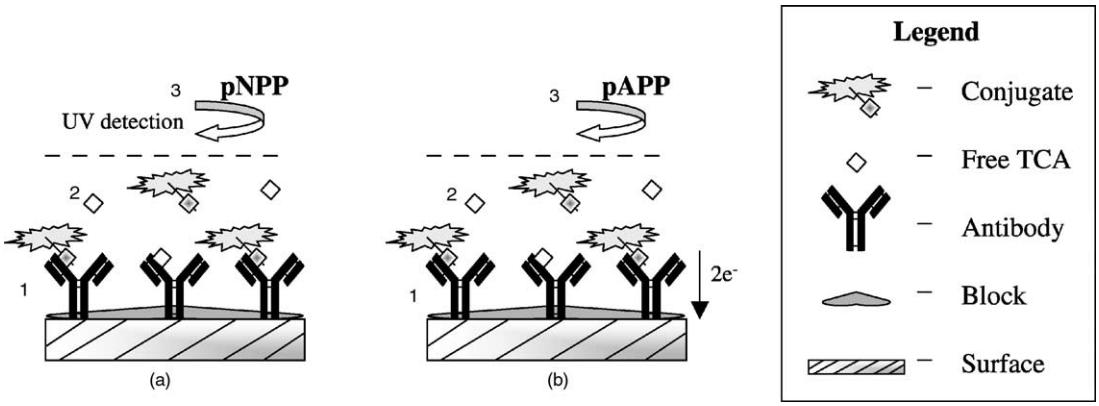


Fig. 3. Schematic illustrating biodesign of the sensor: (1) primary immobilisation of pAbs, (2) competition between free TCA and enzyme conjugate for pAb, (3) addition of substrate; (a) ELISA and (b) amperometry.

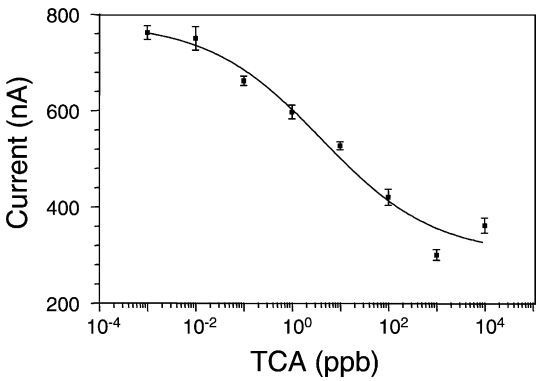


Fig. 4. Competition trend observed between free TCA and hapten A-AP for pAb_{76} with amperometric detection.

illustrated in Fig. 4. The electrochemical response from the conjugate depicts the expected sigmoidal curve, which was also obtained in the ELISA studies. A high concentration of antibody was immobilised onto the surface of the electrode. Five microlitres of a 7.5 μg/ml pAbs was used in the initial immobilisation step onto the working area of the electrode. This concentration was 10 times as much as was required with the ELISA studies.

The best competition results obtained for the analysis of TCA in buffer are illustrated in Table 2 for antibodies raised against A and B, pAb_{76} and pAb_{79}, respectively. The pAb_{76}, which was purified by protein G, gave the better LOD of 29 ppt and a linear range from 0.45 ppm to 34 ppt. In comparison with the ELISA study the LOD and linear range are both significantly better.

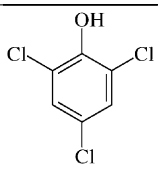
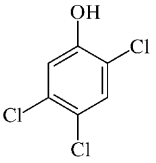
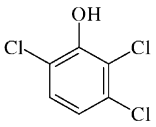
3.3. Cross-reactivity

Cross-reactivity studies were performed on the purified antibodies raised against hapten A and B for TCP isomers. Table 3 outlines the extent of cross-reactivity. The purified antibodies, $pAb_{74–79}$ all exhibited the same percentage cross-reactivity trend when exposed to 2,4,6-TCP and its isomers.

Table 2
Analysis of competition assays with pAb_{76} and pAb_{79}

Mode of detection	IC_{50}	Linear range	LOD	R.S.D. (%)	R^2
ELISA–TCA competition with hapten A-AP	3.66 ppm	80 ppm to 65 ppb	34 ppb	<5	0.991
ELISA–TCA competition with hapten B-AP	0.82 ppm	10 ppm to 45 ppb	22 ppb	<6	0.995
Electrochemical–TCA competition with hapten A-AP	4.05 ppb	450 ppb to 34 ppt	29 ppt	<4	0.967
Electrochemical–TCA competition with hapten B-AP	5.27 ppm	10 ppm to 53 ppb	12 ppb	<5	0.973

Table 3
Relative cross-reactivity for each of the TCP isomers

%	TCP isomers
48	 2,4,6-TCP
16.4	 2,4,5-TCP
0	 2,3,6-TCP

3.4. Matrix effects

There are various matrix considerations which need to be clarified in order for the biosensor to be of any use in real sample analysis. This is especially true in the case of direct analysis of wine and cork for TCA contamination. Therefore the compatability and function of the biosensor in wine, cork extract and solvent matrix is very important.

3.4.1. Interference of the wine matrix

Matrix effects on the biosensor system were investigated. Initial studies of preparing the TCA standards in both red and white wine and analysis using the SPE were unsuccessful. The wine matrix inhibited the competition reaction as can be seen in Fig. 5. Removal of the TCA from the wine matrix by extraction into a suitable solvent would allow real samples analysis and would provide a means for sample pre-treatment.

3.4.2. Interference to due extract matrix

The series of plots shown below in Fig. 6 indicates that the solvent of choice did not interfere with the competition trend in ELISA. Up to a maximum 10% solvent concentration was sufficient for analysis of the extract in the direct ELISA format.

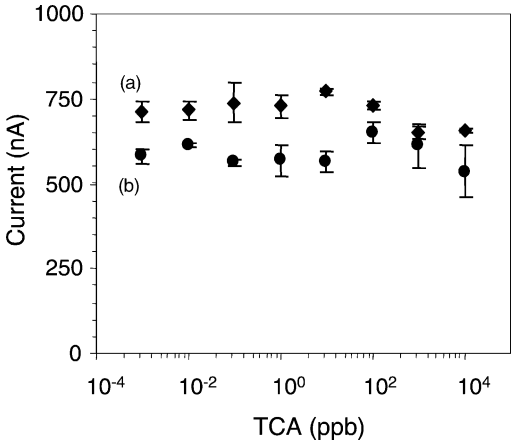


Fig. 5. Wine matrix effect on competition trend with amperometric detection: (a) and (b) represent experiments in two different days.

3.5. Solvent effects

3.5.1. Impact on SPE composition

Solvent effects for the electrodes were also investigated, as a suitable matrix was required for the extraction of TCA from cork. This matrix would contain the solvent that would be used for the extraction of TCA from the cork and would not interfere with the SPE or the competition curve. The results of the solvent studies show that either ethanol or methanol can be used in the matrix, as they have no physical effects on the stability of the SPE. Other solvents corroded the ink. These solvents are obviously not suitable for use with the biosensor.

3.5.2. Impact on competition trend

Addition of EtOH to the buffer matrix increases the LOD from 29 to 87 ppt. Although EtOH has little apparent effect on the physical characterisation of the SPE, it does however affect the competition trend for the TCA assay. There is a shift in both the IC_{50} and LOD values. Fig. 7 represents a calibration plot done in 7% EtOH. This amount is sufficient for analysis of TCA. As the EtOH amount is increased the LOD increases further and the sensor sensitivity loss is significant. The linear range of the calibration plot shown in Fig. 7 extends from 1000 to 0.1 ppb.

No assay sensitivity are observed when standards are prepared in buffered and non-buffered wine and

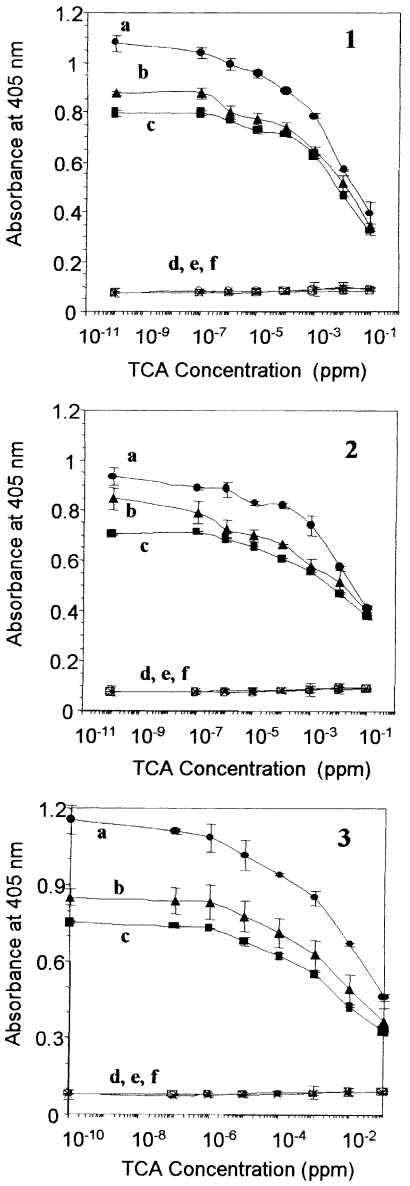


Fig. 6. Solvent effect for ELISA competition trends using (1) THF, (2) EtOH and (3) MeOH as solvents for extraction of TCA from cork. Each graph has six plots illustrating comparison between competition buffers. (a) Tris–BSA, (b) Tris–solvent–BSA, (c) Tris–extract–BSA and the respective blank response, (d) Tris–BSA, (e) Tris–solvent–BSA, (f) Tris–extract–BSA.

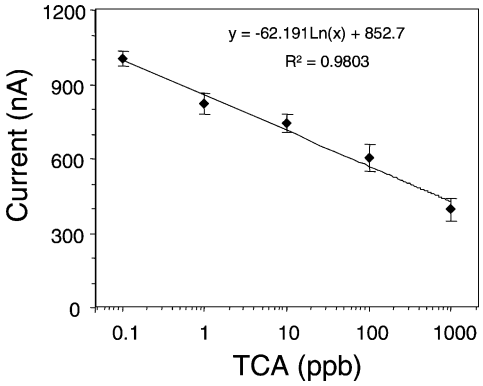


Fig. 7. Calibration plot achieved with a 7% EtOH buffer matrix for the detection of TCA in real sample analysis.

cork extract matrix, Fig. 5. Spiked sample analysis show consistent results. Real sample analysis in both the wine and cork extract matrix are unobtainable at present until a sample pre-treatment step is introduced which would convert the sample matrix to the buffered matrix. It should be noted that real samples in this study are essentially both wine and cork extract spiked with TCA.

4. Discussion

The fundamental part of any biodetection system is to produce excellent antibodies, which are directed against the analyte of interest. The most crucial phase in the development of the immunochemical technique is the design of the chemical structure of the immunising hapten. The characteristics of the immunising hapten directly influence the specificity and selectivity of the antibodies produced. It is thus vital that the hapten represents a mimic of the TCA analyte regarding its chemical structure, spatial conformation, electronic distribution and hydrophobic properties. It is also advisable to avoid modification to the immunogenic groups or to introduce new ones as this leads to complications later on in the biodetection system, i.e. antibody recognition of the target analyte TCA. Theoretical calculations and computer modeling studies were used to obtain optimal structures of the haptens and were performed by IIQAB-CSIC, Barcelona, [39].

Carbon screen printed electrodes were used for all the electrochemical experiments. All the assays that

were done were of the direct format. The best results for the biosensor were achieved with the pAb_{76} antibodies raised against hapten A. The competition assay between hapten A-AP and free TCA gave a LOD of 29 ppt. This was three orders of magnitude better than competition results observed for the same system with ELISA. The competition assay was done in a 50 mM Tris–HCl buffer matrix.

The wine matrix inhibited analysis of the TCA for both the ELISA and electrochemical systems. The response was erratic and no calibration trend was achieved. This problem may be overcome by the extraction of the TCA from wine and hence changing to a more suitable matrix that the sensor can analyse, e.g. 7% EtOH. Specific capture of TCA utilising immunoaffinity columns could eliminate the matrix phenomenon. TCP was shown to cross-react with the polyclonal antibodies raised against both haptens. The 2,4,6-TCP isomer exhibited the highest interference. The use of an anion exchanger can be used to remove the TCP molecule from the matrix where specific analysis of TCA is vital. TCP is a charged species and when passed through the anion exchange resin, it is retained, while neutral species like TCA pass freely through in the column.

Sample pre-treatment is an important part in the development of a biosensor for the analysis of TCA in wine. Coupling an anion exchanger column with an immunoaffinity column could create an efficient sample clean-up system. It would eliminate matrix and cross-reactivity interferences and provide a way for specific assay of TCA. A biosensor has been designed that can detect a concentration of 29 ppt of TCA in a buffer matrix and 87 ppt in a 7% EtOH buffer matrix, but fails to meet real sample analysis in wine until such time as a reliable extraction or sample pre-treatment protocol is established.

5. Conclusions

The biosensor is disposable, cheap to produce and can be used as a screening tool. These are perhaps the greatest advantages of the sensor when compared to already established methods for TCA detection. Although the sensor has difficulties in operating in a wine matrix, it can detect TCA in both a buffer and 7% EtOH matrix. As TCA is formed by the microbial transformation of TCP, it would be practical to be able to screen for both compounds for environmental analysis. It therefore would have environmental applications where water contamination due to TCA or TCP is important to monitor and control.

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References

[1] A.P. Pollnitz, K.H. Pardon, D. Liacopoulos, G.K. Skouroumounis, M.A. Sefton, Aust. J. Grape Wine Res. 2 (1996) 184–190.
[2] A. Rapp, Nahrung-Food 42 (1998) 351–363.
[3] M. Hederich, P.J. Costello, P.J. Grbin, P.A. Henschke, Nat. Prod. Lett. 7 (1995) 129–132.
[4] C.R. Struss, T. Hereszytn, Chem. Ind. (1984) 109–110.
[5] P. Fuller, Aust. New Zealand Wine Ind. J. 10 (1995) 58.
[6] C. Smith, Pract. Vineyard Winery, November/December (1992) 49.
[7] T.H. Lee, R.F. Simpson, in: G.N. Fleet (Ed.), Wine Microbiology and Biotechnology, Harwood, Philadelphia, 1993, p. 353.
[8] M.A. Sefton, A.P. Pollnitz, K.H. Pardon, D. Liacopoulos, G.K. Skouroumounis, Aust. J. Grape Wine Res. 2 (1996) 184.
[9] L.H. Aung, J.L. Smilanick, P.V. Vail, P.L. Hartsell, E. Gomez, J. Agric. Food Chem. 44 (1996) 3294–3296.
[10] A.J. Cserjesi, E.L. Johnson, Can. J. Micro. 18 (1972) 45–49.
[11] J.M. Gee, J.L. Peel, J. Gen. Micro. 85 (1974) 237–243.
[12] J. Jäger, J. Diekmann, D. Lorenz, L. Jakob, Aust. J. Grape Wine Res. 2 (1996) 35–41.
[13] F.B. Whitfield, T.H.L. Nguyen, J.H. Last, J. Sci. Food Agric. 54 (1991) 595–604.
[14] J. Stoffelsma, K.B. de Roos, J. Agric. Food Chem. 21 (1973) 738.
[15] F.R. Cutis, C. Dennis, J.M. Gee, M.G. Gee, N.M. Griffiths, D.G. Peel, J.L. Land, D. Robinson, J. Sci. Food Agric. 25 (1974) 811.
[16] H. Maarse, L.M. Nijssen, S.A.G.F. Angelino, in: Proceedings of the 2nd Wartburg Aroma Symposium, Wartburg, Akademie Verlag, 16–19 November 1987, pp. 43–61.
[17] F.B. Whitfield, T.H.L. Nguyen, K.J. Shaw, J.H. Last, C.R. Tindale, G. Stanley, Chem. Ind. (1985) 661–663.

[18] C.R. Tindale, F.B. Whitfield, S.D. Levingston, T.H.L. Nguyen, J. Food Sci. Agric. 49 (1989) 437–447.
[19] J.L. Hill, A.D. Hocking, F.B. Whitfield, Food Chem. 54 (1995) 161–166.
[20] J.E.R. Frijters, J.M.H. Bemelmans, J. Food Sci. 42 (1977) 1122–1123.
[21] F.B. Whitfield, C.R. Tindale, K.J. Shaw, G. Stanley, Chem. Ind. (1984) 772–774.
[22] J.C. Spadone, G. Takeoka, R.J. Liardon, Agric. Food Chem. 38 (1990) 226–233.
[23] H.T. Badings, in: H. Maarse (Ed.), Volatile Compounds in Foods and Beverages, Marcel Dekker, New York, 1991.
[24] D.S. Motrtram, Int. J. Food Sci. Tech. 33 (1998) 19–29.
[25] G. Reineccius, Crit. Rev. Food Sci. Nutr. 29 (1991) 381–402.
[26] F.B. Whitfield, Int. J. Food Sci. Tech. 33 (1998) 31–51.
[27] F.B. Whitfield, L.H. Last, in: G. Charalambous (Ed.), The Shelf Life of Foods and Beverage, Elsevier, Amsterdam, 1986, pp. 483–499.
[28] K. Aoyama, J. Appl. Bacteriol. 68 (1990) 405–410.
[29] B.G. Brownlee, G.A. MacInnis, L.R. Noton, Environ. Sci. Technol. 27 (1993) 2450–2455.
[30] R.F. Curtis, D.G. Land, N.M. Griffiths, M. Gee, D. Robinson, J.L. Peel, et al., Nature 235 (1972) 223–224.
[31] P. Danesh, F.M. Velez Caldas, J.J. Figueiredo Marques, M.V. San Romã, J. Appl. Micro. 82 (1996) 689–694.
[32] V. Mazzoleni, P. Caldentey, M. Careri, A.M. Mangia, O. Colagrande, Am. J. Enol. Viticulture 45 (1994) 401–406.
[33] A. Peña-Neira, T. Hernández, M.C. García-Vallejo, E. Cadahia, F. de Simón, Am. J. Enol. Viticulture 50 (1999) 285–290.
[34] F.B. Whitfield, K.J. Shaw, T.H.L. Nguyen, J. Sci. Food Agric. 37 (1986) 85–96.
[35] T. Ramsted, J.S. Walker, Analyst 117 (1992) 1361–1366.
[36] A. Hoffmann, W.R. Sponholz, in: P. Sandra, G. Devos (Eds.), Proceedings of the 16th International Symposium on Capillary Chromatography, Publication 25, Gerstel, Mühlheim/Ruhr, 1994, pp. 27–30.
[37] C. Fischer, U. Fischer, J. Agric. Food Chem. 45 (1997) 6.
[38] T.J. Evans, C.E. Butzke, S.E. Ebeler, J. Chromatogr. A 786 (1997) 293–298.
[39] N. Sanvicens, M.P. Marco, J. Agric. Food Chem., in press.
[40] G.T. Hermanson, Bioconjugation Techniques, Academic Press, London, 1996, Chapter 9, p. 436.
[41] Pierce Micro BCA Protein Assay Instruction Manual, Pierce, Rock ford, IL, March 1999.