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A screen-printed, amperometric biosensor array incorporated into a novel automated system for the simultaneous determination of organophosphate pesticides☆

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ABSTRACT

Organophosphate pesticides present serious risks to human and environmental health. A rapid reliable, economical and portable analytical system will be of great benefit in the detection and prevention of contamination. A biosensor array based on six acetylcholinesterase enzymes for use in a novel automated instrument incorporating a neural network program is described. Electrochemical analysis was carried out using chronoamperometry and the measurement was taken 10 s after applying a potential of 0 V vs. Ag/AgCl. The total analysis time for the complete assay was less than 6 min. The array was used to produce calibration data with six organophosphate pesticides (OPs) in the concentration range of 10^{-5} M to 10^{-9} M to train a neural network. The output of the neural network was subsequently evaluated using different sample matrices. There were no detrimental matrix effects observed from water, phosphate buffer, food or vegetable extracts. Furthermore, the sensor system was not detrimentally affected by the contents of water samples taken from each stage of the water treatment process. The biosensor system successfully identified and quantified all samples where an OP was present in water, food and vegetable extracts containing different OPs. There were no false positives or false negatives observed during the evaluation of the analytical system. The biosensor arrays and automated instrument were evaluated *in situ* in field experiments where the instrument was successfully applied to the analysis of a range of environmental samples. It is envisaged that the analytical system could provide a rapid detection system for the early warning of contamination in water and food.

1. Introduction

Organophosphate pesticides (OPs) are widely used in agriculture due to their high efficiency as insecticides (Gogol et al., 2000). OPs have been shown to result in high levels of acute neurotoxicity (Kamanyire and Karalliedde, 2004) and carcinogenicity (Alavanja et al., 2004), with the majority being hazardous to both human health and to the wider environment (Jaga and Dharmani, 2003). Indeed, the inhibition of the regulatory activity of the enzyme acetylcholinesterase (AChE) by OPs in humans can lead to a serious disturbance of normal neuronal function which can be fatal (Basanta et al., 1995). Despite a gradual decline in long-term use in agriculture, the amount of the "active ingredient" applied in the UK has increased in other sectors (CSL, 2010). As a consequence of this, there has been increased concern related to possible contamination of the environment and food by these compounds and a corresponding increased regulation for their control (CEC, 2006). An essential component in the legislatory mechanism is the rapid detection of contamination to allow for remediation measures to be effectively implemented, potentially by a portable and inexpensive sensing device to allow rapid assessment *in situ*. Developments in biosensor technology using disposable screen-printed carbon electrodes (SPCEs) for electrochemical analysis have provided a cost-effective and reliable array format that is suitable for OP analysis (Hart et al., 2004, 2007).

Enzyme-based biosensors that have been previously reported for the measurement of OPs are based on either AChE or organophosphate hydrolase (OPH). OPH biosensors have been developed for the analysis of individual OPs (Wanekaya et al., 2008) and more recent developments include the use of mutant forms of OPH (Mulchandani et al., 2001). AChE-based biosensors have been in development for over 20 years and have more recently been incorporated in very sensitive and selective OP biosensors systems

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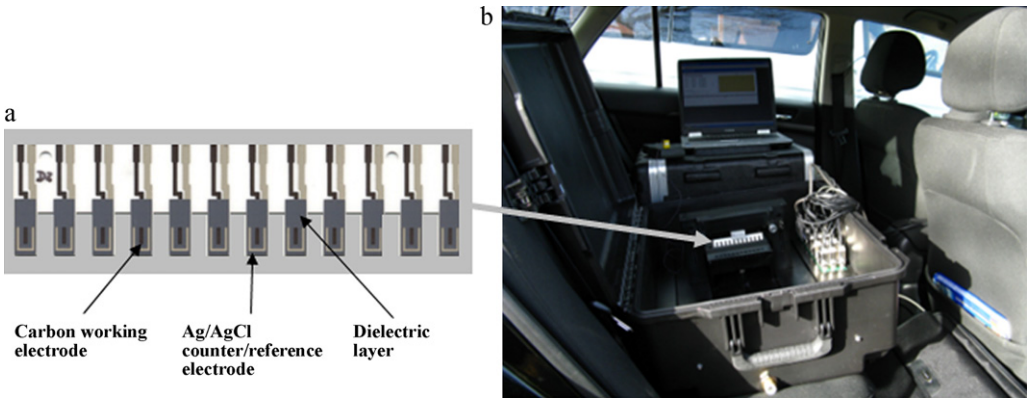

Fig. 1. (a) Electrode array comprising 12 screen-printed carbon electrodes modified with CoPC and an Ag/AgCl counter/reference electrode printed on an alumina substrate; (b) array in the prototype biosensor system operating in the field powered from a car battery via the lighter socket.

(Andreescu and Marty, 2006). Developments in the engineering of enzymes based on *Drosophila melanogaster* AChE (*Dm*AChE) have provided a large resource of enzymes with differing specificities to OPs (Schulze et al., 2007). Indeed, studies using arrays of biosensors with different AChE enzymes have successfully differentiated between two different OPs using a neural network (Bachmann et al., 2000). Presently, however, there are no reported automated biosensor systems, incorporating a neural network, designed for the differentiation of more than two OPs, or an instrument specifically designed for rapid OP detection that is suitable for commercial or regulatory applications.

The purpose of the present study was to develop such a system and evaluate it for the rapid identification and quantification of specific OP residues in environmental and food samples based on the requirements of end-users. The proposed prototype instrument incorporates an electrochemical biosensor array based on screen-printing technology allowing mass production at low cost (Wring and Hart, 1990). Following electrochemical characterisation of the array, studies were carried out to integrate the array into the automated analytical system and to evaluate it with a variety of sample types with selected OPs of importance in environmental and food monitoring. The inclusion of a neural network program allowed the modelling of the responses of the AChE enzymes to the OPs using the large volumes of calibration data that was generated; this facilitated the interpretation of the analytical results efficiently and accurately. The pattern recognition capabilities allowed for the identification and quantification of OPs in the various sample matrices, which would not be possible without the neural network program. This paper presents the results of these studies.

2. Material and methods

2.1. Reagents

Deionised water was obtained from a Purite Select Analyst 80 System (Purite, Oxfordshire, UK). All reagents were of analytical or higher grade. The OP pesticides were obtained from QMX Laboratories (Thaxted, UK) and stock standards of 2×10^{-3} M were prepared in methanol (VWR International, Lutterworth, UK) and stored at 4 °C. For the calibration studies, OP solutions were produced by diluting the stock standard with 0.05 M phosphate buffer pH 8.0. The 0.05 M pH 8.0 phosphate buffer was produced by titrating sodium dihydrogen orthophosphate dihydrate with tri-sodium orthophosphate (VWR International, Lutterworth, UK) to pH 8.0. Acetylthiocholine chloride (Sigma, Dorset, UK) was used to produce 50 mM solutions by dilution using 0.5 M phosphate buffer pH 8.

2.2. Biosensor preparation

The amperometric biosensor arrays were based on 12 screen-printed sensors, deposited side by side onto a ceramic substrate, and designed to be compatible with commercially available 96-well microtitre plates. The working electrode (width 1 mm, height 3 mm) contained 5% (m/m) CoPC in a carbon ink (C2030408P3) developed jointly by UWE and Gwent Electronic Materials. An Ag/AgCl electrode served as a reference/counter electrode and a dielectric layer was printed over the two electrodes to define their areas (Fig. 1a). To convert these CoPC-SPCEs into biosensors a fixed concentration of the appropriate enzyme (supplied by Groupe de Biochimie des Protéines, Universite Paul Sabatier, Toulouse, France) was immobilised onto the surface of the working electrode using glutaraldehyde. In the present study the biosensor arrays were used within 24 h of fabrication, although they have been shown to be stable for a minimum of 48 days stored at room temperature.

2.3. Instrumentation

Electrochemical measurements were carried out with a prototype automated instrument developed by Uniscan Instruments Ltd. (Buxton, UK) and the University of the West of England (Bristol, UK), which comprised 12 Uniscan PG580RM potentiostats integrated with an automated sensor and substrate development system in two protective plastic boxes (Uniscan TST-RM™) for simultaneous chronoamperometric measurement using modified UiEChem™ software (Fig. 1b). Reagents were arranged in a 96-well microtitre plate and the measurements were made at a temperature controlled at 37 °C.

2.4. Determination of acetylcholinesterase activity

Before preparation of the amperometric biosensors, AChE activity was measured using the method described by Ellman et al. (1961). Using spectrophotometry at a wavelength of 412 nm, the thionitrobenzoate resulting from the reaction of DTNB with thiocholine (the product of the enzymatic hydrolysis of acetylthiocholine substrate) can be measured.

2.5. OP biosensor operation

Chronoamperometry was used as the measurement technique for biosensor operation as described previously by the authors (Crew et al., 2004). Briefly, biosensors were exposed to OP solutions of varying concentrations for 3 min prior to their introduction into wells containing acetylthiocholine chloride. The acetylthiocholine chloride was present in excess, thereby avoiding any effects on the biosensor responses from a limited substrate concentra-

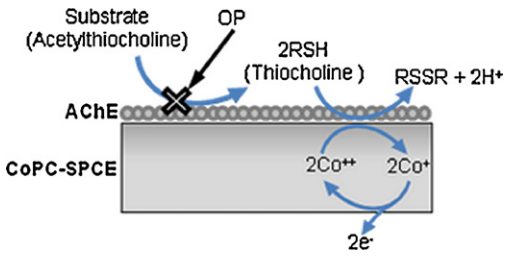

Fig. 2. Schematic diagram showing the reactions taking place during the operation of the proposed amperometric biosensor.

tion. After a short incubation time (60 s), the potential was applied (0 V vs. Ag/AgCl) with current measurements taken 10 s later. The total analysis time was approximately 300 s. Solutions containing the OPs dichlorvos, malaoxon, chlorpyrifos-oxon, chlorpyrifos-methyl-oxon, chlorfenvinphos and pirimiphos-methyl-oxon were prepared at concentrations between 10^{-5} and 10^{-9} M in 0.05 M phosphate buffer pH 8.0. These solutions were then used in calibration studies using the biosensors prepared with the following variants of AChE: WT (B131), B02, B04, B65, B394 and B421. The calibration data generated was used to train the neural network, which will be able to interpret the complex patterns present within the data sets. The prior calibration of the responses from the six enzymes to the various analytes and concentration ranges allowed the generation of look-up tables for random test data production. C++ code was used to generate 10,000 simulated events varying between the extremes of the concentration ranges, 20% of which having zero-concentration of any organophosphates. This data was then used to train, test and validate a neural network designed in NeuroSolutions™ (NeuroDimension, Inc., Gainesville, USA). The chronoamperometric data was then fed directly to the neural network to give both analogue (quantity) and digital (presence/absence) responses. The analytical system was used to examine the effects of food extracts and environmental samples in the presence or absence of OPs with the biosensors. Food extracts were prepared as described by Crew et al. (2004) and environmental samples for laboratory analysis were collected and stored at 4 °C until analysed. Environmental samples for field testing were analysed immediately following collection without filtering or pretreatment. During the field testing the analytical device was powered directly from a standard car battery via the cigarette lighter socket.

3. Results and discussion

3.1. Principle of operation of the proposed biosensor array

Fig. 2 shows the operating principle for the measurement of OPs using the proposed screen-printed amperometric biosensor array. The immobilised enzyme converts acetylthiocholine chloride to its electroactive product, thiocholine, which is detected with the CoPC-SPCEs. This occurs by the thiol reducing Co^{2+} to Co^{+}, which can then be re-oxidised back to Co^{2+} at a potential of 0 V vs. Ag/AgCl, the current generated during the re-oxidation step constitutes the analytical response (Hartley and Hart, 1994). In the presence of OPs, AChE is inhibited leading to a reduced production of thiocholine and hence a lower anodic current. This decrease is proportional to the OP concentration (Crew et al., 2004).

3.2. Cyclic voltammetric and chronoamperometric behaviour of thiocholine using the novel CoPC-SPCE array

The response of our new SPCE array to thiocholine was initially examined by cyclic voltammetry with 5 mM thiocholine in 0.5 M phosphate buffer pH 8 (Fig. 3). A single well-defined anodic peak was present at −0.26 V (I_a) on the forward scan and two cathodic peaks on the reverse scan (II_c = −0.47 V and I_c = −0.72 V). Peak I_a is the result from the electrocatalytic oxidation of the thiol moiety producing the corresponding disulfide; peak I_c is the electrocatalytic reduction of the disulfide back to the original thiol species and Peak II_c is probably due to a reduction process occurring in the macrocyclic phthalocyanine molecule (Hart and Hartley, 1994). The oxidation and reduction peaks were not present in the buffer solution in the absence of thiocholine.

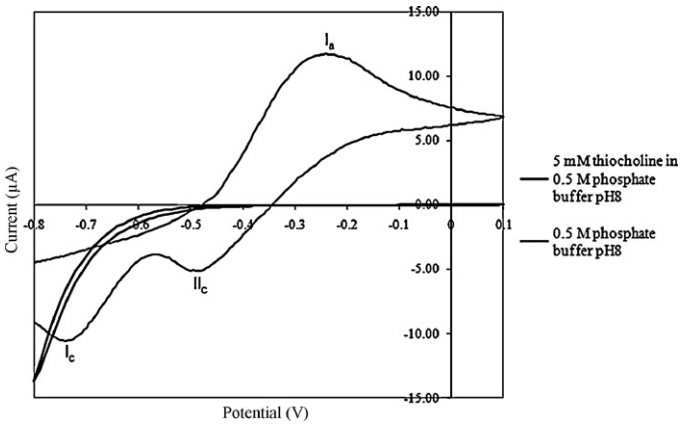

Fig. 3. Cyclic voltammetry of 5 mM thiocholine in 0.5 M phosphate buffer pH 8 compared to 0.5 M phosphate buffer pH 8 only. Electrochemical parameters: initial potential −0.8 V, switch potential +0.1 V, final potential −0.8 V and scan rate 20 mV s^{-1}.

The reproducibility of the CoPC-SPCEs across the 12-sensor arrays was examined by chronoamperometry with 5 mM thiocholine, using the protocol described earlier. The currents produced by nine arrays of the CoPC-SPCEs (108 individual sensors) had a mean current at 10 s of 6.4 μA with a coefficient of variation of 4.8%.

3.3. Calibration studies

The inhibition responses from the calibration of each array of 12 biosensors with each concentration of the 6 OPs was collated and used to train the neural network. Fig. 4 shows a typical range of chronoamperometric responses for the biosensors. In this example, B04-based biosensors were inhibited with chlorfenvinphos at concentrations of 10^{-5} M to 10^{-9} M. The resulting calibration plot (Fig. 4, inset) using the current mea-

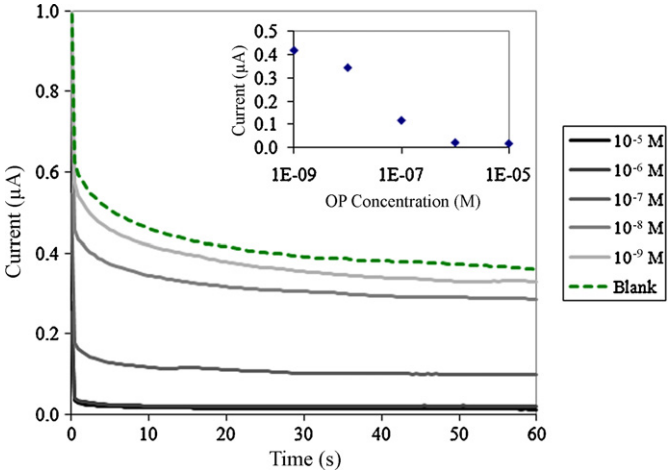

Fig. 4. Chronoamperograms produced from biosensors with the B04 enzyme inhibited with chlorfenvinphos (10^{-5} to 10^{-9} M).

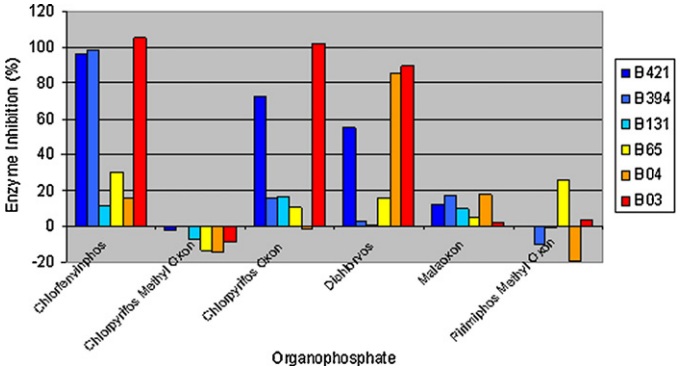

Fig. 5. Inhibition profiles constructed from the inhibition of each of the enzymes by each of the OPs at a concentration of 10^{-6} M.

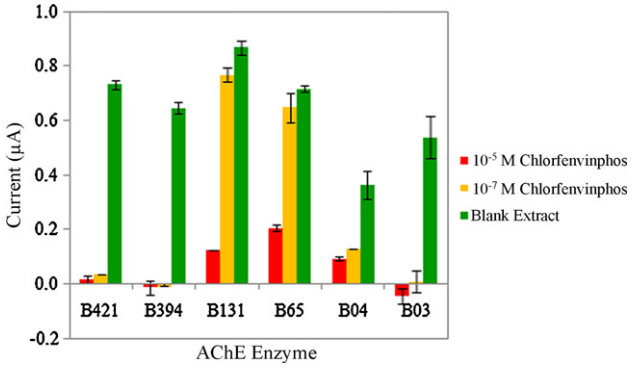

Fig. 6. Chronoamperometric responses taken 10 s after applying a potential of +0.00 V (vs. Ag/AgCl) of wheat extracts spiked with two concentrations of chlorfenvinphos and compared to an unspiked extract using the biosensor array incorporated into the prototype instrument.

surements taken at 10 s illustrate the typical inhibition profile obtained.

The inhibition of the enzymes by each OP was converted to a percentage inhibition by comparing the response to a blank measurement taken in the absence of the OP. The percentage inhibition was subsequently used to standardise the data for input into the neural network. An example of the differential inhibition present is shown in Fig. 5. The inhibition produced by each OP at a concentration of 10^{-6} M with each AChE enzyme, demonstrates clear differences that were utilised by the neural network for analysis. The differences in inhibition occasionally included an increase in the enzyme activity where individual enzymes were positively affected by some OPs at particular concentrations. Enhancement of the enzyme by the OPs was uncommon and resulted in a limited increase of less than 20% of the activity. The patterns obtained were unique to each concentration of OP and sufficiently different for the neural network to differentiate between the OPs. At this initial stage of training, the neural network identified the presence of each OP and concentrations in spiked buffer solutions.

3.4. Analytical applications

3.4.1. Water analysis

Prior to any field testing, the effectiveness of the neural network in identifying the presence of an OP in solution was examined using deionised water spiked with each OP at final concentrations between 10^{-5} M and 10^{-7} M; the neural network identified the presence of an OP in each of the spiked solutions.

Initial evaluation of the prototype biosensing system for environmental sample analysis involved the laboratory-based analysis of water samples from widely different origins. Eight water samples were taken that represented each stage of the water treatment process. These included samples of raw reservoir and river water, water from the various filtration and treatment steps (including ozonation), effluent and drinking water. None of the samples were pretreated or filtered prior to analysis. The sample type did not significantly reduce the chronoamperometric response of the biosensor array compared to a deionised water blank solution (*t*-test, $P = 0.90$). The enzyme responses across all the water samples had a coefficient of variation of 8.6%. Interrogation of the data by the neural network did not indicate any inhibition of the biosensor array. Therefore, the neural network did not identify the presence of OPs in any of these sample types and inferred that the biosensors were not affected by any matrix effects or interfering compounds present in the samples.

The design of this novel analytical system included the capability of the equipment to be transported in a standard road vehicle and operated in the field powered by a conventional car battery via the cigarette lighter socket. To test the ability of the system for this purpose the prototype analytical system was transported and operated by a single person in locations in the Forest of Dean, Gloucestershire, UK. Four different types of environmental samples were analysed immediately after collection, including rainwater, river water adjacent to arable land, river water adjacent to coniferous forest and lake water. Deionised water was also analysed at each location as the reference blank solution for comparison. Following analysis in the field location, it was evident that the output from the biosensor array was not significantly different from the blank solutions (*t*-test, $P = 0.67$) and the maximum current output was achieved in each case with a coefficient of variation for biosensor output of 7.5% across all sample types. Consequently, the interpretation of the data by the neural network indicated that no OPs were present in any of the samples. It should be mentioned that the samples were not treated or filtered and that the biosensors operated effectively without degradation of the enzyme despite the samples being biologically active.

3.4.2. Food analysis

In order to study the effect of food extracts on the biosensor array response, a broad range of raw foods was selected. Extracts of wheat, cabbage, apple, orange and cherry were extracted using a non-toxic solvent based extraction and reconstituted in methanolic 0.05 M phosphate buffer pH 8 as described by Crew et al. (2004). The responses were compared to the those obtained from a blank solution containing 0.05 M phosphate buffer pH 8 only. The chronoamperometric responses obtained in the presence of any of the food extracts were not significantly different from those obtained with buffer solution indicating that no interfering compounds or matrix effects were present. This also demonstrated that direct comparison to the responses obtained during the calibration studies and the water analysis was feasible and that the neural network could be trained using buffer solutions only for food extract analysis. The mean coefficient of variability for biosensor responses in the presence of the extracts was 6.7%.

To validate the biosensor array for the analysis of food sample extracts, a selection of different food types were spiked with known quantities of six different OPs then extracted and interrogated using the biosensor system, followed by interpretation by the neural network. The neural network identified the presence of the inhibiting OP in every spiked extract, and conversely indicated the absence of any OP in every sample where no OP was present. Consequently the system did not produce any false positives or false negatives. Fig. 6 shows a typical example of chronoamperometric responses obtained with the biosensor array using wheat extracts spiked with two different concentrations of chlorfenvinphos. When the known concentration was compared with the concentration calculated by

the neural network, the recovery was 78% and 93% for 10^{-5} M and 10^{-7} M chlorfenvinphos, respectively.

4. Conclusions

This paper describes the development of a novel automated electrochemical instrument for the determination and identification of OPs. The biosensor arrays incorporating six different AChE enzymes were used successfully to provide rapid, accurate and reliable inhibition data on a variety of samples. The time taken for the analysis of each sample was approximately 300 s and this period of time may easily be extended in order to increase the sensitivity of the analytical system. The data was used to train the neural network in order to complete the evaluation of the prototype instrument. During the evaluation stage, the biosensor system was not detrimentally affected by any of the sample matrices. This is despite other biosensor systems designed for OP pesticide detection in environmental samples frequently requiring sample pretreatment and demonstrating a high sensitivity to matrix effects and cross-contamination (Rodriguez-Mozaz et al., 2006). Indeed, the 'blank' food extracts showed very good reproducibility and low variability even between extracts from markedly different food types. Similarly, no detrimental effect was observed with any of the samples taken from the various stages of the water treatment process, or in any of the environmental samples analysed in the field.

The analyses performed on the calibrants and spiked extracts demonstrated that the system could successfully identify the presence of an OP in all the samples. In these circumstances the neural network did not produce any false positives or false negatives; an essential prerequisite for the implementation of a system such as this for water, food or environmental monitoring.

The electrochemical analytical system and biosensor array demonstrated robustness, precision and portability and its capacity to be taken and operated to a field site. The portability of the system was clearly demonstrated in the field trials and the equipment completed all of the analyses and interpretation of the environmental samples without any malfunction or need for repetition. A single person was readily able to transport and operate the equipment in a standard road vehicle at a field site. This study demonstrated that both qualitative and quantitative analysis, using AChE-based electrochemical biosensor arrays, with neural network recognition were readily feasible for the *in situ* analysis of environmental samples and food extracts.

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