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A novel automated flow-based biosensor for the determination of organophosphate pesticides in milk

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ABSTRACT

This work describes the development of an automated flow-based biosensor that employs genetically modified acetylcholinesterase (AChE) enzymes B394, B4 and wild type B131. The biosensor was based on a screen printed carbon electrode (SPE) that was integrated into a flow cell. Enzymes were immobilised on cobalt (II) phthalocyanine (CoPC) modified electrodes by entrapment in a photocrosslinkable polymer (PVA-AWP). The automated flow-based biosensor was successfully used to quantify three organophosphate pesticides (OPs) in milk samples. The OPs used were chlorpyriphos-oxon (CPO), ethyl paraoxon (EPOx) and malaoxon (MOx). The total analysis time for the assay was less than 15 min. Initially, the biosensor performance was tested in phosphate buffer solution (PBS) using B394, B131 and B4 biosensors. The best detection limits were obtained with B394; therefore, this biosensor was used to produce calibration data in milk with three OPs in the concentration range of 5×10^{-6} M to 5×10^{-12} M. The limit of detection (LOD) obtained in milk for CPO, EPOx and MOx were 5×10^{-12} M, 5×10^{-9} M and 5×10^{-10} M, respectively, with a correlation coefficient $R^2 = 0.9910$. The automated flow-based biosensor successfully quantified the OPs in different fat-containing milk samples. There were no false positives or false negatives observed for the analytical figures of merit for the constructed biosensors. This method is inexpensive, sensitive, portable, non-invasive and provides real-time results. This analytical system can provide rapid detection of highly toxic OPs in food matrices such as milk.

1. Introduction

Milk is an essential nutritional food for infants and the young (Padilla et al., 2004; Zhang et al., 2005). Milk contaminated with traces of pesticides is a matter of serious concern. There is an urgent need for highly sensitive techniques to rapidly screen for neurotoxic pesticides in food items such as milk. The most commonly found pesticide group, organophosphorus pesticides (OPs), have been widely used to protect fields and fruit crops in the agricultural sector, and parasite control in domestic animals for veterinary practices (Jeanty et al., 2002; Salas et al., 2003). Nevertheless, the intensive and indiscriminate application of OPs and their high acute toxicity have generated risks to humans and the environment (Andreescu and Marty, 2006). The main action of OPs affects the functioning of the nervous system by binding to the enzyme acetylcholinesterase (AChE). This results in an overstimulation of the nerves with symptoms such as weakness or paralysis of the muscles (Fort, 1999). The resulting public concern created a demand for the development of reliable, sensitive, simple and low-cost methods for their fast determination. OPs' toxicity justifies the need for accurate and reliable methods to monitor pesticide levels.

OPs can appear in milk by several possible routes: insecticides may be used directly on dairy cattle for ectoparasite control; insecticides residues may be present in pasture, forages, or animal feed; and insecticides may be present as pest control in stable or dairy factories. The cows can absorb these compounds by all routes (inhalation, ingestion and dermal absorption) and thus can secrete contaminated milk (Bolles et al., 1999; Gazzotti et al., 2009). There are several reports on the presence of OPs in milk worldwide using various techniques (Juhler, 1997; Mallatou et al., 1997; Salas et al., 2003; Pagliuca et al., 2005; Cardeal and Dias, 2006; Mishra et al., 2010). Maximum residue limits (MRL) and acceptable daily intake (ADI) values for parent insecticides have been set by several organizations, such as the Food and Agriculture Organization of the World Health Organization (FAO/WHO) and the European Union (EU), thus requiring adequate methodology for enforcement. The EU has set a very low limit for pesticides in baby food. According to this regulation, infant formulae must not contain residues of individual pesticides at levels exceeding 10 $\mu g\,kg^{-1}$, which is, in practice, the

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minimum detectable level using the officially accepted detection methods (Commission Directive 1999/50/EC).

Detection methods for monitoring the presence of these neurotoxic compounds require high sensitivity and accuracy because pesticides are most often found at trace levels. Although traditional analytical techniques fulfil these requirements, they are not suitable for direct monitoring of pesticides in real samples. Standard analytical methods based on gas chromatography or high performance liquid chromatography coupled with mass selective detectors are time consuming and expensive to control sufficient amounts of milk samples before they are ingested by the consumer (Martinez et al., 1992; Pylypiw, 1993).

New analytical approaches are oriented to the development of systems that can provide quantitative information about the analyte with high accuracy, low cost, short time response and minimal sample preparations. The reported flow systems are coupled with a wide variety of detectors (Del Valle, 2010); however, electrochemical methods such as potentiometry and amperometry are particularly interesting because of their low cost, small size and the possibility of performing in situ measurements. Several AChE-based biosensors (Istamboulie et al., 2007; Hildebrandt et al., 2008; Valdés-Ramírez et al., 2008, 2009) and flow-based biosensors have been reported for the detection of OPs (Jeanty et al., 2002; Bucur et al., 2005; Prieto-Simón et al., 2006; Shi et al., 2006; Crew et al., 2011). Ours is the first report of a developed automated flow-based biosensor system that enables accurate OPs detection in milk at very low inhibitor concentrations and without the need for any pre-treatment or organic solvents.

The aim of this work was to develop an automated flow-based biosensor system for online detection of OPs in milk. The low cost biosensor utilized three AChE (one wild type and two genetically modified) enzymes immobilised on screen printed electrode (SPE) to quantify ethyl paraoxon (EPOx), chlorpyriphos-oxon (CPO) and malaoxon (MOx) in milk. The measurement of these pesticides in milk is important to evaluate the overall consumer risk, especially relating to the safety of infants and children (Eskenazi et al., 1999).

2. Experimental

2.1. Chemicals, biochemicals and stock solutions

Wild type enzyme acetylcholinesterase (B131) and genetically modified enzymes B394 and B4 from *Drosophila melanogaster* were produced by our laboratory (IMAGES, Perpignan, France). Acetylthiocholine iodide (ATChI), acetylthiocholine chloride (ATChCl), 5,5′-dithio bis (2-nitrobenzoic acid) (DTNB), and phosphate buffer (PBS, 0.1 mol L^{-1} K_2HPO_4/KH_2PO_4 pH 7 containing 0.1 mol L^{-1} KCl) were obtained from Sigma Aldrich (Steinheim, Germany). A 0.1 M ATChCl stock solution was prepared daily and stored at 4 °C. HPLC-grade acetonitrile was supplied by Carlo Erba (Italy) and photocrosslinkable poly (vinyl alcohol) (azide unit pendant water-soluble photopolymer, PVA-AWP) was purchased from Toyo Gosei (Japan). The pesticides ethyl paraoxon and malaoxon were obtained from Fluka, Sigma Aldrich (Germany). Chlorpyriphos-oxon was procured from Dr. Ehrenstorfer (Augsburg, Germany). Stock solutions of pesticides (10^{-3} M) were prepared in acetonitrile and stored at 4 °C. Working solutions of pesticides were prepared daily in distilled water by dilution from the stock. Slide-A-Lyzer Dialysis Cassettes were purchased from Thermo Scientific (Rockford, USA) for purification of enzyme powder in the liquid phase.

The paste used for screen-printing, i.e. Electrodag PE-410, 423SS and 6037SS, were obtained from Acheson (Plymouth, UK) and Timrex T15 graphite was supplied by Timcal (Lonza, Switzerland). Tetracyanoquinodimethane (TCNQ)-modified carbon-paste was prepared as reported previously (Andreescu et al., 2002).

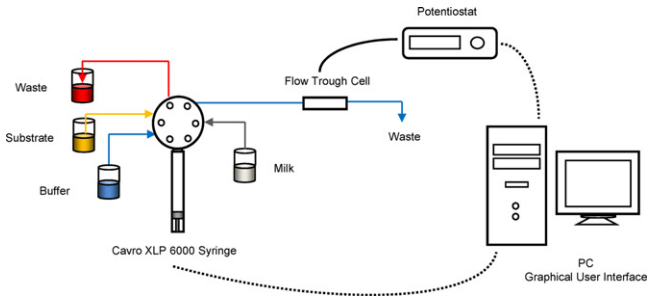

Fig. 1. Schematic of the automated flow-based biosensor for the analysis of OPs in milk samples.

A glycerophthalic paint (Astral, France) was used as an insulating layer. Hydroxyethyl cellulose (HEC) medium-viscosity was purchased from Fluka (France). Transparent polyvinyl chloride (PVC) sheets (200 mm × 100 mm × 0.5 mm) were used as printing supports. Milk samples were bought from local market of Perpignan (France) for analysis.

2.2. Apparatus

Spectrophotometric measurements were performed using a Hewlett-Packard model 8451A diode-array spectrophotometer. Amperometric measurements were carried out with a 641 VA Potentiostat (Metrohm, Switzerland) connected to a BD40 (Kipp and Zonen, The Netherlands) flatbed recorder. SPEs were fabricated using a semi-automatic DEK248 printing machine according to a procedure previously described (Andreescu et al., 2002) in a three-electrode configuration. The working electrode was a 4-mm diameter disk, the auxiliary electrode was a 16 mm × 1.5 mm curved line and the Ag/AgCl pseudo-reference electrode was a 5 mm × 1.5 mm straight line.

2.3. Instrumentation

The Electrochemical measurements were carried out with an automated flow-based system. The flow system is comprised by a modular syringe pump coupled to a multiport valve (Cavro XLP 6000), a custom flow-through cell, a 12 bits data acquisition card (National Instruments) and a 641VA potentiostat (Metrohm, Switzerland). A graphical user interface was developed in Lab-View 8.5 to control the whole system. The schematic of automated flow-based biosensor is presented in Fig. 1. The reagents were arranged in different reservoirs and the measurements were performed at 25 °C.

2.4. Spectrophotometric measurements

To determine both the enzymatic activities and the inhibition constants, spectrophotometric measurements were performed. The Ellman method was used to determine AChE activity. This method is based on the enzymatic reaction product (TCh) that reacts quantitatively and irreversibly with DTNB, producing a yellow compound (5-thio-2-nitrobenzoate) that can be spectrophotometrically detected at 412 nm (Ellman et al., 1961). The assay was performed using PBS (600 μL), 300 μL 1 mg/1 mL DTNB (in PBS), 100 μL 10 mmol L^{-1} ATChI (in 0.9% NaCl) and 10 μL enzyme that was added to a spectrophotometric cell. The kinetic spectrometric measurements were performed within 1 min and the enzymatic activity in mU mL^{-1} was calculated.

2.5. Construction of the biosensors

The biosensors were manufactured in groups of 24 (reference, working, and auxiliary electrodes) on PVC sheets. The following layers were consecutively printed: a silver conducting film, a carbon pad, an Ag/AgCl layer on the reference electrode, a cobalt (II) phthalocyanine (CoPC) layer on the working electrode, and, finally, an insulating layer. After each deposition the electrodes were dried at 60 °C for 40 min. The working electrode was a 4-mm diameter disk, the auxiliary electrode was a 16 mm × 1.5 mm curved line, and the Ag/AgCl pseudo-reference electrode was a 5 mm × 1.5 mm straight line.

Three different kinds of biosensors were prepared using genetically modified AChEs, B394 and B4, and wild type B131. Each enzyme was immobilised on the working electrode surface by entrapment in a polymeric matrix of PVA-AWP. On the working electrode surface, 3 μL of enzymatic solution containing 30% enzyme and 70% PVA-AWP was manually spread in order to immobilise 1 mU enzyme per electrode. Afterwards, the electrodes were exposed to neon light (15 W) for 4 h at 4 °C to promote photopolymerisation between azide groups. After drying for 72 h at 4 °C, the biosensors were ready to use.

2.6. Amperometric measurement in PBS and milk

The activities of the AChEs immobilised on the electrodes were determined by electrochemically monitoring of the thio-choline formed upon enzymatic hydrolysis of ATChCl. Amperometric measurements were carried out using a 641VA potentiostat (Metrohm, Switzerland) connected to a BD40 (Kipp & Zonen, The Netherlands) flatbed recorder. The applied potential was 100 mV to a printed Ag/AgCl reference electrode. In order to perform the amperometric measurements, the biosensor strip was vertically inserted into the flow cell of the automated flow system and integrated into the potentiostat. A working potential of 100 mV was applied to the reference electrode and 500 μL of the substrate was dispensed three times using an automated pump. The current produced (Amplitude) was recorded using the data acquisition card. The pesticide analysis was completed in a three-step procedure as follows: first, the initial response of the biosensor to the substrate ATChCl (1 mM) was recorded three times; then, 500 μL min^{-1} of each pesticide was dispensed through the flow cell for 10 min to inhibit the biosensors. The stock pesticide solution (10^{-3} M) was diluted 1000 times to 10^{-6} M, and then further diluted. Each pesticide was tested against each biosensor. Finally, the residual response of the biosensor was recorded again. The electrodes were cleaned between each measurement. For the calibration curve in milk (without fat), the concentrations were kept the same as the buffer.

2.7. Sample recovery from milk

The accuracy of the biosensor test was checked by spiking milk samples with EPOx, CPO and MOx. The biosensor B394 was used to analyse high-fat containing milk samples. Two different fat-containing milk samples (15.5% and 30%) were examined. Initially, milk samples were analysed directly. There was a reduction in peak height by 20–30% between milk and PBS. Therefore, we filtered the milk samples and passed the filtrate through the biosensor. This was followed by a washing step to clean the tubing with PBS using the automated flow system. The matrix effect was eliminated and the peak heights were quite similar. After elimination of matrix effect, the recovery studies were carried out using CPO, EPOx and MOx. Two different concentrations of each were chosen from the linear range of calibration curve. The first concentration was near the lower concentration of the calibration curve and the other one is the maximum. Milk was spiked with each pesticide

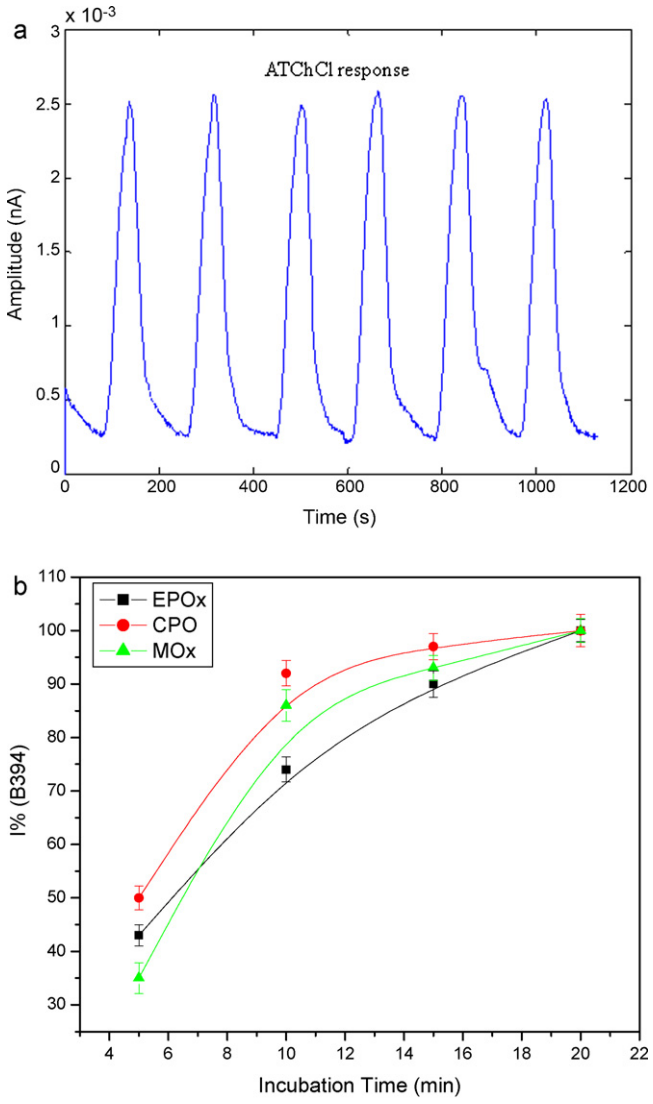

Fig. 2. (a) The stability of the biosensor in peak responsiveness following six consecutive injections of 1 mM ATChCl. (b) The effect of incubation time on the performance of biosensor B394 using EPOx, CPO and MOx (ATCh = 1 mM).

(CPO: 5×10^{-11}, 5×10^{-7} M, EPOx: 5×10^{-9}, 5×10^{-7} M and MOx: 5×10^{-10}, 5×10^{-7} M) and recoveries were calculated.

3. Result and discussion

To characterise the biosensors, the calibration curve, stability, and reproducibility were determined for each. The biosensor response to successive additions of ATChCl substrate and the operational stability were tested. The electrodes showed good amperometric response to the ATChCl substrate. To evaluate the operational stability of the electrode, the response of the biosensor to the additions of 1 mM substrate, while rinsing the cell between measurements, was repeatedly measured. Fig. 2a represents the stability of the biosensor as a peak response in five consecutive injections of 1 mM ATChCl. All electrodes showed good operational stability and had no obvious enzyme leaching. This can be due to the strong electrostatic binding between the enzyme and the electrodes. All biosensors were stable for at least up to $N = 5$ assays, with a response variation of less than 5%. The response time of the biosensors was approximately 2 min. The electrodes retained full enzymatic activity after storage for up to 1 month. For the storage experiments, the electrodes were dipped in PBS and stored at 4 °C

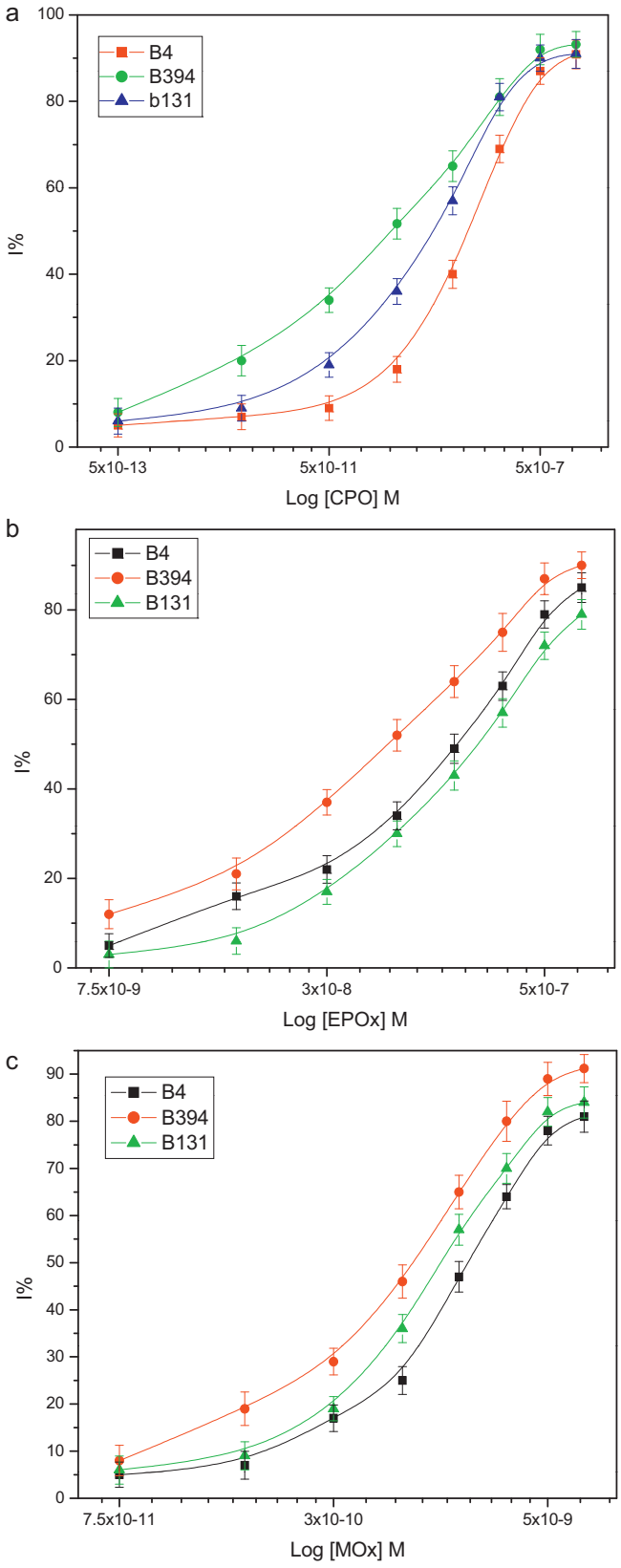

Fig. 3. (a) Calibration curves of CPO in PBS obtained using biosensors B4, B394 and B131 with optimised experimental conditions. (b) Calibration curves of EPOx in PBS obtained using biosensors B4, B394 and B131 with optimised experimental conditions. (c) Calibration curves of MOx in PBS obtained using biosensors B4, B394 and B131 with optimised experimental conditions.

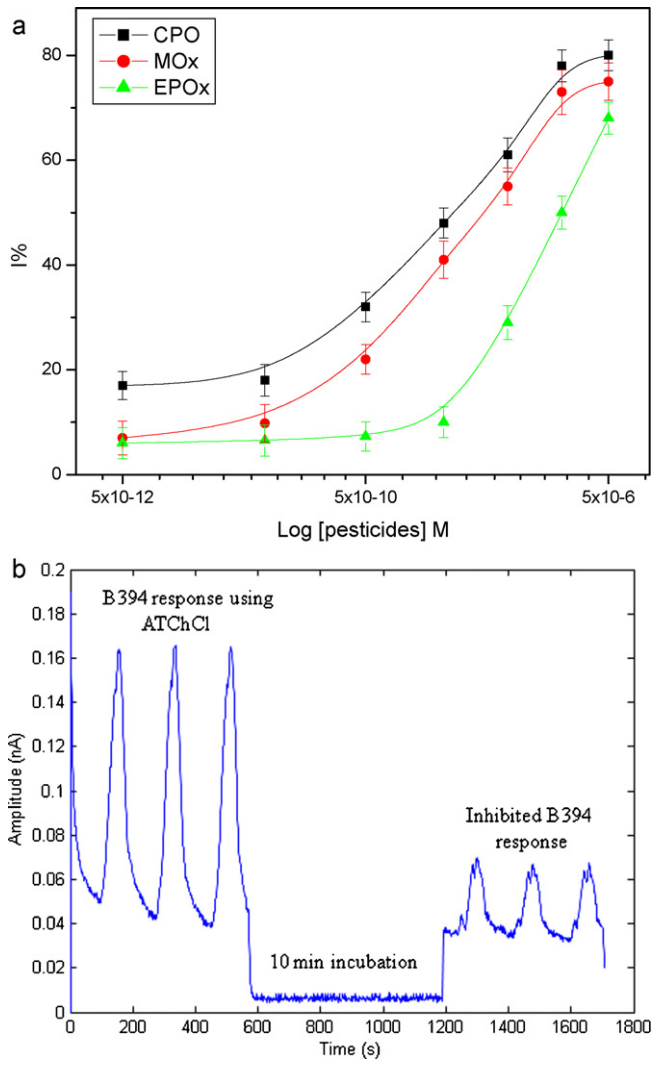

Fig. 4. (a) Calibration curves for CPO, MOx and EPOx in milk samples using biosensor B394 with optimised experimental conditions. (b)The real peaks obtained during the analysis of MOx (5×10^{-9} M) in a milk sample using biosensor B394 with optimised experimental conditions.

in a covered container to prevent PBS evaporation and contamination. These storage conditions are mild and easy to achieve a good response. The electrodes can be re-used if the milk does not contain OPs.

3.1. *Effect of flow rate and incubation time*

The flow rate was the main factor that affected the analytical performance of the automated flow-based biosensor. This study examined the effect of the flow rate on the biosensor response. Flow rates of 500 μL min^{-1} and 1000 μL min^{-1} were tested to achieve the optimum current. The flow rate of 500 μL min^{-1} was optimised as the best compromise between the peak amplitude and speed of the pump used in the automated flow system.

Pesticide detection studies were carried out at saturation substrate concentrations (1 mM). Three pesticides, CPO, EPOx and MOx, were tested at a concentration of 2.5×10^{-7} M to determine their effect on enzyme activity at different incubation times (5, 10, 15 and 20 min). It can be seen in Fig. 2b that the level of inhibition of the enzyme increased with increasing incubation period. There is always a trade off between incubation time and sensitivity. However, many practical analytical applications require rapid

Table 1
Analytical figures of merit for developed biosensors (B394, B131 and B4) in PBS.

Pesticide	Biosensor	Linear range	LOD	R^2	S.D. ($N = 3$)
CPO	B394	5×10^{-7}–5×10^{-11}	5×10^{-12}	0.99767	0.55433
CPO	B131	5×10^{-7}–5×10^{-10}	5×10^{-11}	0.98612	1.63570
CPO	B4	5×10^{-7}–5×10^{-9}	5×10^{-10}	0.99104	1.41620
EPOx	B394	5×10^{-7}–3×10^{-8}	7.5×10^{-9}	0.99835	0.39645
EPOx	B131	5×10^{-7}–6×10^{-8}	3×10^{-8}	0.99953	0.22903
EPOx	B4	5×10^{-7}–6×10^{-8}	1.5×10^{-8}	0.99970	0.18765
MOx	B394	5×10^{-9}–3×10^{-10}	1.5×10^{-10}	0.99387	0.95561
MOx	B131	5×10^{-9}–3×10^{-10}	3×10^{-10}	0.99509	1.02806
MOx	B4	5×10^{-9}–6×10^{-10}	3×10^{-10}	0.99486	0.95414

Table 2
Recovery studies of CPO, EPOx and MOx in two different fat-containing milk samples using biosensor B394 under optimum conditions. The recovery rates for CPO, EPOx and MOx in the milk samples range from 98.2% to 90.8%.

Pesticides added (M)	Found (M)	% of recovery (15% fat milk)	S.D. ($n = 3$)	Found (M)	% of recovery (30% fat milk)	S.D. ($n = 3$)
CPO						
5×10^{-11}	4.92×10^{-11}	98.5	2.70	4.75×10^{-11}	95.0	3.03
5×10^{-7}	4.91×10^{-7}	98.2	2.40	4.50×10^{-7}	90.0	3.25
EPOx						
5×10^{-9}	4.80×10^{-9}	96.0	2.63	4.45×10^{-9}	89.0	3.21
5×10^{-7}	4.75×10^{-7}	95.0	2.82	4.35×10^{-7}	87.0	3.11
MOx						
5×10^{-10}	4.85×10^{-10}	97.0	2.03	4.63×10^{-10}	92.60	3.34
5×10^{-7}	4.82×10^{-7}	96.5	2.32	4.67×10^{-7}	93.40	3.46

analysis. Given that the slope of the sensitivity is less than that of the incubation time, it is useful to choose the lowest incubation time that leads to a reasonable sensitivity. An increase in the inhibition of biosensors was less pronounced beyond 10 min for each pesticide. Therefore, an incubation time of 10 min was used. This value is comparable with most of the data reported in the literature (Istamboulie et al., 2007; Sinha et al., 2010).

3.2. Biosensor performance in PBS

Numerous relationships between the inhibition percentage (*I*%) and the inhibitory concentration and/or inhibition time are described in the literature (Zhang et al., 2001). The inhibitory effect of the three OPs in PBS was evaluated with three constructed biosensors, namely B394, B131 and B4. For each insecticide, three calibration curves corresponding to the biosensors were plotted at different concentration ranges. The inhibition responses of all three biosensors were evaluated in PBS using CPO, EPOx and MOx prior to milk analysis. Each experimental point was the mean of three measurements. The inhibition experiments showed a high level of intra-laboratory reproducibility with a coefficient of variation of 3.43%. The dynamic range for all the pesticides was kept similar to observe the affinity of the biosensors to the pesticides.

The dynamic range for CPO was determined to be 5×10^{-6} to 5×10^{-13} M. The LODs obtained for B394, B4 and B131 biosensors were 5×10^{-12}, 5×10^{-10} and 5×10^{-11} M, respectively, with an average % RSD of 2.82. The calibration curve of CPO is shown in Fig. 3a. For EPOx, the dynamic range was determined to be 5×10^{-6} to 7.5×10^{-9} M for each biosensor. Fig. 3b shows that the LODs obtained for B394, B4 and B131 were 7.5×10^{-9}, 1.5×10^{-8}, 3×10^{-8} M, respectively (% RSD = 2.934).

In the case of the inhibition caused by MOx, the dynamic range was determined to be 7.5×10^{-11} to 1×10^{-8} M. The LOD obtained for B394 was 1.5×10^{-10} M. For B4 and B131, the LODs were similar (3×10^{-10} M) though there were differences in I% (B4 = 17% and B131 = 19%). The inhibition caused by MOx is represented in Fig. 3c. It was observed that biosensor B394 exhibited more affinity towards MOx compared to the other two biosensors. Fig. 3c shows the behaviour of the biosensors to each pesticide. At the lower pesticide concentrations, the *I*% was less; it slowly showed linearity and then reached a plateau. The analytical figure of merits for the developed biosensors is presented in Table 1.

3.3. Calibration curve in milk

During the pesticide analysis in PBS, it was observed that biosensor B394 was the most sensitive among the three biosensors. Therefore, biosensor B394 was used for the pesticide analysis in milk. To produce calibration data, milk without fat was used to determine the biosensor response. The wide dynamic range, from 5×10^{-12} to 5×10^{-6} M, was chosen to analyse spiked milk samples. Using B394, the LODs obtained for EPOx, CPO and MOx were 5×10^{-9}, 5×10^{-12} and 5×10^{-10} M, respectively. It is clearly visible in Fig. 4a that the linear range of the tested pesticides was in a similar range, 3×10^{-10} to 5×10^{-9} M with S.D. of 3.59% and $R^2 = 0.9926$. The detection limits obtained in this study are the most sensitive LODs in milk compared to reported methods (Zhang et al., 2005). Fig. 4b shows the real peaks obtained during the analysis of MOx in milk. It can be seen that there is no significant variation in three peaks before and after incubation but the *I*% can be easily noticed. The flow-based biosensor system exhibited quite stable signals during the analysis of the pesticides. The inhibition values obtained from the milk samples correlated well with the calibration curve in PBS. There was no unspecific inhibition caused by the milk samples. The LODs of the biosensor B394 (signal to noise ratio ≥ 3) were similar in buffer and in milk.

3.4. Applicability of biosensor in different milk samples

Two milk samples of the same brand that differed only in their fat content were analysed after filtration using 5 μm filter paper. The milk samples were spiked with CPO (5×10^{-11} and 5×10^{-7} M), EPOx (5×10^{-9} and 5×10^{-7} M) and MOx (5×10^{-10} and 5×10^{-7} M). The spiked concentrations were chosen from biosensor B394's linear range of the calibration curve constructed for milk. As expected, no significant inhibitions were observed. This finding leads to the conclusion that after filtration, the matrices did not affect the analysis. In each spiked concentration, the

percentage of the recovery obtained was smaller than 100%. The percentage was in expected values as milk is a complex matrix. The recoveries were not less than 95% for lower fat milk (15.5%) samples but were less than 90% in higher fat containing milk (30%) samples. The higher percentage milk could have a small effect on the biosensor.

The obtained recoveries are presented in Table 2. Though direct incubation in the milk samples would be advantageous, the low recovery rates and matrix effects would impede sample analysis. The biosensor food screening test was fast compared to chromatographic methods. The AChE biosensor test is suitable for milk analysis, but more suited to low fat milk samples. The recovery rate of OPs in milk was in the range of the legislative regulations that require a recovery rate between 70% and 110% (European Communities, 1997).

4. Conclusions

The development of an automated flow-based biosensor test for the detection of organophosphate pesticides in milk was described. The biosensor met the requirements set by EU regulations with respect to detection limits for tested milk samples as well as recoveries. The biosensors incorporated three different AChE enzymes, which were used successfully to provide rapid, accurate and reliable inhibition data. Biosensor B394 could determine EPOx, CPO and MOx down to 5×10^{-9}, 5×10^{-12} and 5×10^{-10} M, respectively, in milk samples. The analyses performed on the calibrants and spiked samples demonstrated that the system could successfully determine the presence of OPs. The test could be completed in less than 15 min with good reproducibility. The reliability of the response characteristics of the newly constructed sensor made it suitable to use as a detector in an automated system, such as a flow injection system. The proposed system can be applied successfully in online monitoring of OPs in milk processing units and collection centres.

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