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Quantification of Methomyl Levels in Cabbage, Tomato, and Soya Milk Using a Renewable Amperometric Biosensor

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An enzymatic biosensor based on inhibition of acetylcholinesterase activity was developed to analyze methomyl contamination in different matrices. The detection limits under optimal working conditions were found to be 30.4 μg L^{-1} methomyl using the biosensor and 0.15 μg L^{-1} using HPLC. The biosensor was then applied to analysis of methomyl in fruit and vegetable samples at concentrations in the ppb range in the absence of sample pretreatment. Recovery levels using the biosensor were effective, ranging from 78.0 to 96.5%, while the HPLC method yielded 57.0 to 99.5% recovery. Results for total carbamate concentrations obtained using the acetylcholinesterase biosensor were compared to those obtained using HPLC.

Keywords: Biosensor; Pesticides; Food analysis; Methomyl

1. INTRODUCTION

Methomyl is a carbamate insecticide that is highly toxic and hazardous [1]. It is also used as an acaricide to control ticks and spiders foliar treatment of vegetables, fruits, field crops, cotton, commercial ornamentals, and through treatments in and around poultry houses and dairies [2]. Methomyl kills these pests through two primary mechanisms of action: 1) as a “contact insecticide” that kills target insects by direct contact and b) as a “systemic insecticide” that causes systemic poisoning of insects that absorb the agent by feeding on treated plants. Methomyl can be absorbed by plants in the absence of phytotoxicity, or harm to the plant. The presence of this pesticides residue in fruits, and especially in vegetables, has become a major public health concern in the last decade.

Analytical methods have been developed to determine methomyl levels, including high performance liquid chromatography (HPLC) coupled with UV-visible absorption or fluorescence detection, which provides both selectivity and sensitivity [3-6]. However, the HPLC-fluorescence detection method frequently requires labeling of carbamates with a fluorophore, since not all carbamates are fluorescent. In addition, HPLC-based methods frequently require laborious and time-consuming extraction and clean-up steps that require highly-qualified laboratory teams. These methods also are not environmentally friendly due to the large amount of organic solvent generated by the procedures. These issues can lead to major problems when rapid and sensitive measurements are needed in order to undertake corrective actions in a timely manner.

Development of reliable, fast, and inexpensive analytical systems to monitor pesticides is currently an area of intense investigation. Biosensors based on acetylcholinesterase represent an emerging and promising technique for toxicity analysis, environmental monitoring, and assessment of food quality [7-10]. Acetylcholinesterase-based biosensor models have been shown to be fast, simple, and selective methods for pesticide analysis because they combine the selectivity of an enzymatic reaction with operational simplicity. This type of methodology could serve as an alternative to chromatographic analysis of pesticides and could simplify sample preparation, decreasing cost and analysis time as a result.

The most critical step in the development of biosensors is the immobilization step, in which the biological recognition element is associated with a physico-chemical transducer [11]. The analytical performance of the biosensor can be negatively affected by this process. Therefore, intensive efforts are underway for the development of effective immobilization methods, allowing for improvements in operational and storage stability, response time, linear range, and sensitivity, while preserving the enzyme affinity for the substrates or/and inhibitors. Carbon paste electrodes (CPEs) have previously been utilized for sensor design in electroanalysis. CPEs are composed of a mixture of graphite powder and a water-immiscible pasting liquid. These electrodes offer the advantages of low background current, a renewable surface, and low cost, in addition to providing a highly suitable environment for enzyme immobilization [12].

In general, widespread application of biosensors is still vulnerable to problems due to low stability and insufficient detection limits. For this reason, and considering previous experience from our group in this area [12-17], the primary goal of the present study was to propose a simple, economical use of a carbon paste acetylcholinesterase biosensor with high stability and sufficient detection limits for analysis of methomyl concentrations in foods. We evaluated the levels of methomyl pesticides in tomato, soya milk, and cabbage using a well-established standard procedure for comparison. A practical and applicable biosensor must satisfy the detection limits set by official regulations. To validate this novel methodology, the results obtained were compared to those from high-performance liquid chromatography.

2. EXPERIMENTAL PROCEDURE

2.1. Materials

Acetylcholinesterase (AChE) (E.C. 3.1.1, type XII-S, 0.26 U/mg) from bovine erythrocytes, acetylthiocholine iodide, and glutaraldehyde were purchased from Sigma. Cobalt phtalocyanine (97%) was obtained from Aldrich, and carbon powder (Acheson – 38) was purchased from Fischer, Nujol was obtained from Schering-Plough, and methomyl 99.0% was kindly provided by the Bayer Company. All other reagents used in this study were from Merck (PA, USA). All solutions were prepared with water purified by a Milli-Q ultra-purifier (Millipore, Inc.). When necessary, the solutions were de-aerated with N_2. Materials and parts used for matrix solid phase dispersion (MSPD) and sample preparation were purchased from Varian Sample Prepbelow Products (Harbor City, CA, USA). Chromabond® C18 ec, loaded in a syringe with a coarse frit of 40 μm, was used for MSPD.

2.2. Apparatus

Electrochemical measurements were conducted in a three electrode Pyrex® cell under controlled temperature (30 ^{o}C). The working electrode was the amperometric biosensor, and its preparation is described in detail in a subsequent section. The reference electrode was comprised of an Ag/AgCl (3.0 mol L^{-1}) system, and a Pt wire was used as the auxiliary electrode. Measurements were conducted in a Model 283 EG&G PARC potentiostat/galvanostat linked to a PC computer with M270 software supplied by EG&G PARC. For amperometric experiments, an operating potential of 100 mV versus the integrated in-the-strip reference electrode (Ag/AgCl) was selected.

HPLC experiments were performed in a Shimadzu LC-10AT high performance liquid chromatography instrument linked to a UV-vis detector (Shimadzu SPD-10AV) set to 230 nm. The mobile phase consisted of a 50:50 (v/v) mixture of acetonitrile and water, with flow-rate of 1 mL/min under isocratic conditions. A Merck LiChrosorb RP-18 (5 μm) chromatographic column was used for separations.

2.3. Biosensor construction

Carbon paste electrodes modified with AChE were prepared by thoroughly hand-mixing carbon powder (0.18 g), cobalt phtalocyanine (CoPC; $0.9x10^{-3}$ g), and mineral oil (Nujol®; 70:30 [m/m]) using a mortar and pestle. The addition of CoPC was used to improve the sensitivity due to its catalytic effect [18]. To an aliquot of 8.8×10^{-2} g of the mixture was then added 5 μL of a 1% glutaraldehyde solution to promote cross-linking between the enzyme molecules. A total of 1.7×10^{-2} g of this mixture was then modified by adding 2.4 mg of AChE and mixing until obtaining a uniformly moist paste. This paste was then introduced using a 0.5 mL hypodermic syringe and was connected with a copper wire as the external electric contact. The primary advantage in this type of setup is the possibility of easily renewing the electrode surface prior to each measurement by simply polishing the electrode with a sulphite paper sheet.

Experiments were carried out in a solution containing 2.0 x 10^{-3} mol L^{-1} acetylthiocholine iodide (AcSChI) prepared in 10 mL of 0.1 mol L^{-1} phosphate buffer, pH 7.4, at 30 ^{o}C. Methomyl additions were taken from a 1.0 x 10^{-2} mol L^{-1} stock solution prepared in acetonitrile. Pesticide quantifications in cabbage leaves were performed by immersing the biosensor in liquefied leaf samples (spiked with methomyl or not) for the indicated incubation times, followed by transfer of the biosensor to the cell containing the substrate diluted in buffer solution as described above. The rate of inhibition was determined and plotted against the pesticide concentration. Percentage of inhibition was calculated using the following equation (1), where ΔI_0 and ΔI_1 represent the biosensor responses before and after the incubation time, respectively.

$$\text{Inhibition rate (\%)} = (I_0 - I_1) / I_0 \times 100\% \quad (1)$$

The recovery experiments were carried out by adding a known amount of methomyl to the supporting electrolyte followed by standard additions from the methomyl stock solution and plotting the resulting analytical curve. All measurements were performed in triplicate. The recovery efficiencies (R%) for the different systems under investigation were calculated using Eq. (2) where the value [methomyl] found refers to the concentration obtained by extrapolation of the analytical curve in the corresponding spiked samples.

$$\%R = 100\frac{[pesticide]found}{[pesticide]added} \quad (2)$$

2.4. Sample Extract

Samples were first homogenized. The chopped sample was blended using a blender equipped with a stainless steel cut unit, a glass jar of approximately 50 mL, and pulse options. Approximately 40 g of homogenized sample was placed into a 250 mL beaker. After spiking the solution with an appropriate amount of methomyl, the contents of the beaker were allowed to stand at room temperature for 10 minutes. The contents were mixed with a spatula to obtain a free-flowing powder. To this solution, 2 mL acetonitrile (corresponding to 5% v/m of the electrolyte) was added, and the solution was homogenized by shaking for 20 min to complete evaporation of the solvent. The sample was then centrifuged at 3600 rpm for 15 min, and the liquid supernatant was analyzed by biosensor immersion for the indicated incubation times.

2.5. MSPD Extraction

For MSPD extractions, 0.5 g of the homogenized sample was placed into a glass and gently combined with 0.5 g of C18 sorbent for 5 min using a pestle. This mixture was then introduced into a chromabond® C18 syringe with a coarse frit of 40 μm. The pesticide was eluted with 10 mL of ethyl acetate that was collected dropwise by applying a low vacuum. The eluent was collected and evaporated under a nitrogen stream at room temperature. The extract was then reconstituted with 300 μL of acetonitrile, and 20 μL was subjected to chromatographic determinations.

3. RESULTS AND DISCUSSION

Food monitoring is increasingly required for regulatory purposes, for control of food import and export, and for research to evaluate trends or seasonal variations of contaminants. Environmental monitoring and food control generally require the analysis of large number of samples, promoting the need for low-cost, rapid, and automated methods of analysis. Use of cholinesterase (ChE) enables to the simultaneous detection of a wide variety of related toxic compounds, such as organophosphorus and carbamate pesticides.

Quantification of methomyl was performed using two distinct methodologies: (1) an electrochemical method using an ChE-based enzymatic biosensor and (2) high performance liquid chromatography. The development and optimization of the biosensor has been previously described [12]. In the present study, the only parameter requiring optimization was the incubation time. Adsorption of methomyl to the serine residue in the active site of the enzyme decreases the rate of thiocholine production by ChE, resulting in a decreased oxidation current in chronoamperometric experiments. In order facilitate maximum inhibition, we optimized the time allowed for exposure of the biosensor to the pesticide. Thiocholine can be oxidized at approximately 0.1 V *vs* Ag/AgCl using a carbon paste electrode, forming the corresponding dimmer structure [19]. Figure 1 shows the typical amperometric response of the biosensor to 2.0 x 10^{-3} mol L^{-1} AcSCh before (curve 1) and after addition of 5.0 x 10^{-5} (curve 2) and 1.0 x 10^{-4} mol L^{-1} (curve 3) methomyl for 10 minutes.

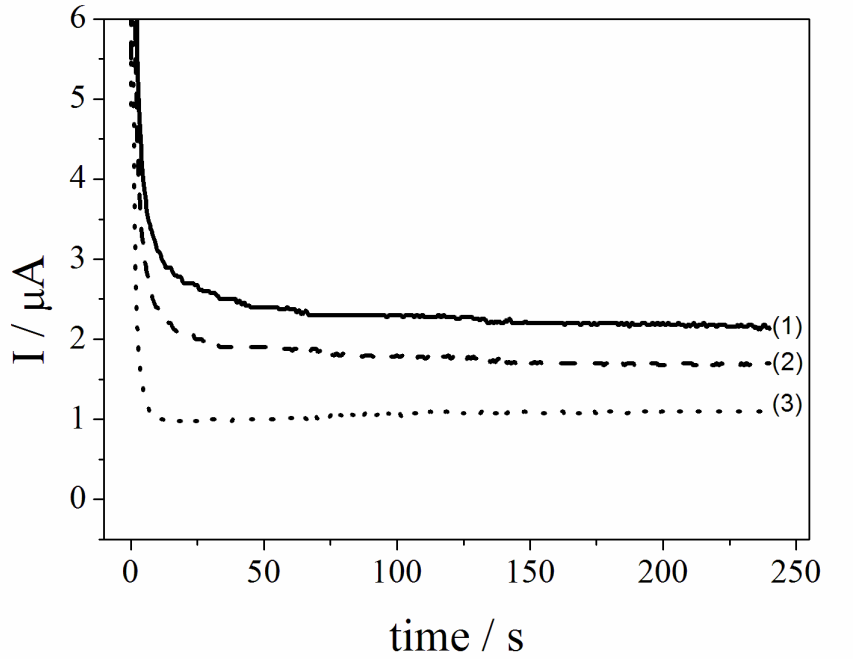

Figure 1. Chronoamperograms for enzymatically generated thiocholine oxidation at 0.1 V vs Ag/AgCl, with the biosensor immersed in (1) phosphate buffer solution, pH 7.4, containing 2.0x10^{-3} mol L^{-1} AcSCh, (2) the same solution after the addition of 0.50x10^{-4} mol L^{-1} and (3) after the addition of 1.0x10^{-5} mol L^{-1} Methomyl to the electrolyte.

Incubation time is defined as the time the biosensor remains immersed in the solution containing the pesticide. To optimize this parameter, the biosensor was immersed in 2.0 x 10^{-3} mol L^{-1} AcSCh containing 1.0 x 10^{-5} mol L^{-1} methomyl. The inhibition rates were then plotted against the incubation time (Figure 2). After 12 minutes of incubation time, the inhibition was found to stabilize. In this manner, 12 minutes was selected as the optimum incubation time, and all inhibition experiments presented in the next sections used this length of immersion.

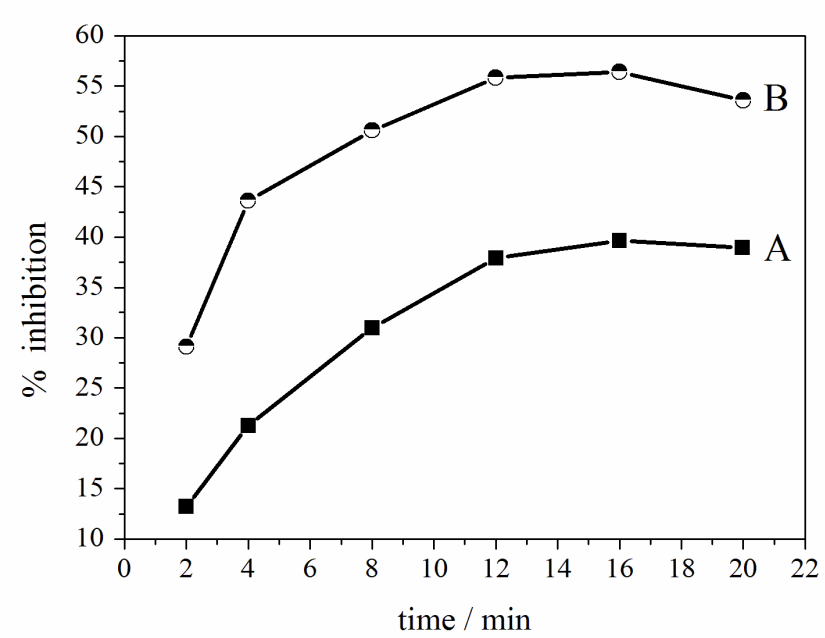

Figure 2. Effect of the incubation time in the percentage of inhibition for thiocholine oxidation in phosphate buffer. Curve (A) refers to incubation in a solution containing 1.0x10^{-5} mol L^{-1} Methomyl while curve (B) to 2.0x10^{-5} mol L^{-1} Lannate®.

Using the optimized conditions established in the studies described above, calibration plots were generated using standard solutions containing methomyl. Figure 3 shows the analytical curves determined by immersing the biosensor in different concentrations of methomyl. Before each experiment, the biosensor surface was renewed by polishing with a sheet of paper to remove the previously inhibited enzyme layer. The analytical curve presented a good linear response in the concentration range from 0.90 to 5.0 x 10^{-6} mol L^{-1} with a correlation coefficient of 0.998. Data from the standards was used to calculate the following straight line equation: I_p = -0.33A – 7.3 x 10^6 A/mol. The detection limit (LD) and quantification limit (LQ) were calculated based on the standard deviation of 10 current-time measurements of the blank solution (without the pesticide) using the equations (3,4) [20,21]:

$$LD = \frac{3S_B}{b} \qquad LQ = \frac{10S_B}{b} \tag{3, 4}$$

where S_B is the standard deviation of the blank solution, and b is the slope of the analytical curve.

The LD value obtained for methomyl was 1.8 x 10^{-7} mol L^{-1} (30.4 μg L^{-1}), and the LQ was 6.2 x 10^{-7} mol L^{-1} (100.0 μg L^{-1}). These values are lower than the value allowed by the Brazilian government of 3.0 mg L^{-1} [22]. The results for 10 successive methomyl determinations under the same conditions revealed a variation coefficient of 5.0% for the current at the peak potential of thiocholine. Thus, electrode renewal was associated with good reproducibility for the surface.

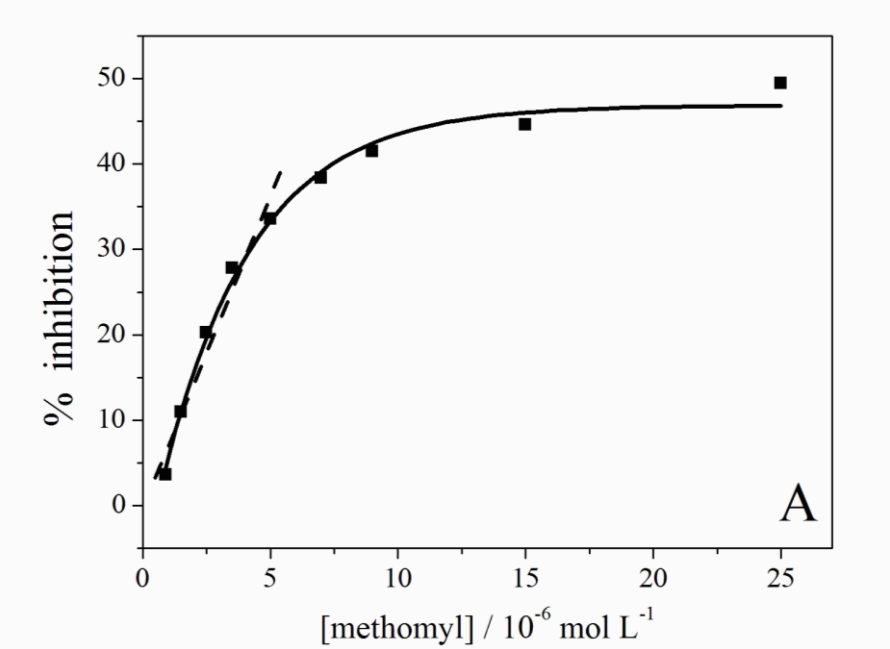

Figure 3. Analytical curve obtained with the biosensor for different concentrations of (A) Methomyl and (B) Lannate® in 0.1 mol L^{-1} phosphate buffer, pH 7.4, and 2.0x10^{-3} mol L^{-1} AcSCh. Cell temperature of 30 ^{o}C.

Figure 4 shows chromatograms for methomyl, with a well-defined peak appearing at a retention time of 3.6 min. The inset in Figure 4 shows the relationship between peak area and pesticide concentration. A defined linear relationship was observed in the concentration range between 0.5 x 10^{-6} and 10.0 x 10^{-5} mol L^{-1}, with a slope of 9.2 x 10^{9}, and a correlation coefficient of 0.999. The LD and LQ were calculated using equations (2) and (3), and the standard deviation of the blank solutions was based on six values of linear coefficients from 6 different analytical curves. The calculated values were 9.0 x 10^{-10} mol L^{-1} (0.15 μg L^{-1}) for LD and 3.0 x 10^{-9} mol L^{-1} (48.6 μg L^{-1}) for LQ, which were considerably lower than those calculated for the biosensor, as expected for a chromatographic technique.

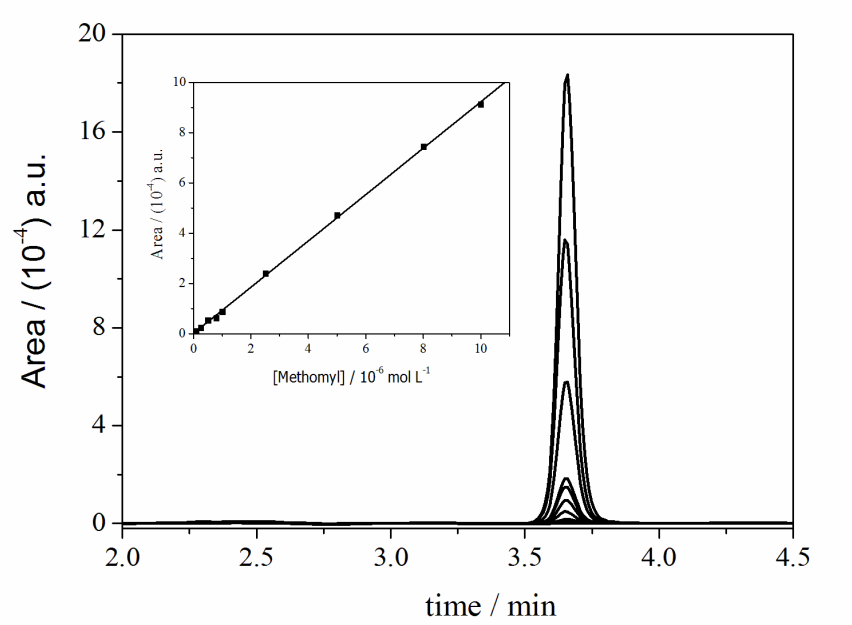

Figure 4. Chromatograms for different concentrations of Methomyl ($0.5x10^{-6}$ to $10.0x10^{-5}$ mol L^{-1}) in 0.1 mol L^{-1} phosphate buffer, pH 7.4. The eluent was a mixture of acetonitrile and water 50:50 v/v, with flow-rate of 1 mL/min under isocratic conditions. The monitored wavelength was 230 nm. Insert: the analytical curve obtained by the areas of the chromatographic peaks.

Previous studies indicate that methomyl is moderately persistent and highly mobile [23,24]. It is susceptible to hydrolysis at lower pH values (neutral to acidic) and degrades slowly in alkaline conditions. Methomyl photolysis occurs more quickly in water, but more slowly in food. To analyze the effectiveness of the biosensor in determining methomyl levels in foodstuffs as cabbage, tomato, and soya milk were spiked with methomyl and analyzed using the biosensor and by HPLC. First, the influence of the matrix concentration was evaluated. Figure 5 shows an amperometric response after immersion of the biosensor in the sample for 10 min, followed by return to the electrochemical cell. The amperogram of the biosensor after the immersion in the sample (line) was compared with that after immersion in pure buffer solution (dotted line). No inhibitory effects were observed in this case, indicating that the matrix had no observable effect on thiocholine oxidation (or on enzymatic activity). These experiments were repeated five times using different samples, and we observed a RSD value of 4%. A small difference in matrix effect was observed for each matrix study. However, the enhancement or decrease in response differed from matrix to matrix by less than 5%. The small matrix influence on the analytical sensitivity has frequently been observed and related to the organic matter dissolved in natural waters, mainly humic and fulvic acids [13].

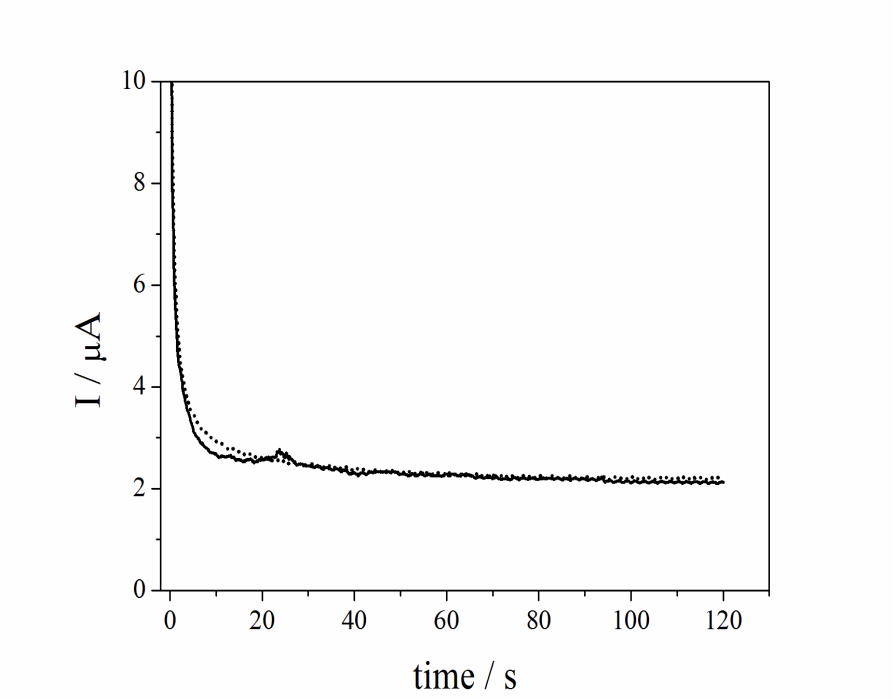

Figure 5. Chronoamperograms obtained with the biosensor in phosphate buffer containing 2.00x10^{-3} mol L^{-1} AcSChI at 30 ^{o}C (dotted line) and in the same electrolyte after incubation in the cabbage sample, without spiking (full line).

Recovery measurements were then performed. The samples were mixed and spiked with 2.0 x 10^{-6} and 5.0 x 10^{-6} mol L^{-1} methomyl. The liquid extraction procedure is detailed in the Materials and Methods section. The values obtained were 96.5–85.0% for tomato, 90.0–78.0% for soya milk, and 95.0–90.2% for cabbage. These values were similar to those obtained using the HPLC procedure. HPLC quantification experiments were carried out using 20 μL aliquots of the appropriate extract obtained from the different samples (see experimental procedure). The experiments were carried out in triplicate, and the resulting values are presented in Table 1. Samples for HPLC experiments were prepared in a similar manner to those used for biosensor experiments, but with the use of an additional extraction step. As expected, the HPLC method provided much lower detection limits, but with a slightly lower recovery, due to the pre-treatment of the samples. The values obtained ranged from 57.0–58.2% (Table 1), revealing a low efficiency for HPLC in analysis of cabbage samples. The primary drawbacks to HPLC observed in this study were the much longer analysis time, as well as the use of toxic organic solvents, which could be avoided by using the electroanalytical procedure. In this analysis, a negative control sample was run with each set of spiked extracts, and the samples with no inhibition were chosen for the recovery study. In contrast, by simply diluting the sample for screening analysis, a good result can be achieved; but in situations when high accuracy is required, the ChE biosensor can efficiently offer a rapid, inexpensive, and reliable alternative, since the technician knows precisely which inhibitor is present in the sample. In other words, although the selectivity of the proposed method could not be compared to that of HPLC, the biosensor allows for the analysis of a large number of samples with no need for clean-up steps, such those required for chromatographic

methods. Frequent routine analyses can be safely carried out using this simpler and less expensive electroanalytical method in the absence of losses in either reliability or precision.

Table 1. Recovery values for methomyl in differents matrices of foods.

Technique	Sample	Concentration		Recovery %
		Added (mol L^{-1})	Recovered (mol L^{-1})	
Biosensor	Tomate	2.0×10^{-6}	1.9×10^{-6}	96.5
		5.0×10^{-6}	4.2×10^{-6}	85.0
	Soya Milk	2.0×10^{-6}	1.8×10^{-6}	90.0
		5.0×10^{-6}	3.9×10^{-6}	78.0
	Cabbage	2.0×10^{-6}	1.9×10^{-6}	95.0
		5.0×10^{-6}	4.5×10^{-6}	90.2
HPLC	Tomate	2.0×10^{-6}	1.9×10^{-6}	99.5
		5.0×10^{-6}	4.1×10^{-6}	83.6
	Soya Milk	2.0×10^{-6}	1.8×10^{-6}	90.0
		5.0×10^{-6}	4.3×10^{-6}	86.0
	Cabbage	2.0×10^{-6}	1.1×10^{-6}	57.0
		5.0×10^{-6}	2.9×10^{-6}	58.2

4. CONCLUSIONS

In conclusion, we have developed a disposable amperometric biosensor based on the enzyme acetylcholinesterase that was successfully used for quantification of methomyl in different samples. This methodology clearly has an advantage over other biological methods, such as ELISA-based techniques, which can only determine one analyte per analysis. Results from this analysis showed that the biosensor had a limit of detection lower than the maximum value allowed by the Brazilian government for contamination in food. The biosensor seems to be promising for direct analysis of methomyl levels. Electroanalytical detection of methomyl can safely be carried out without the use of toxic organic solvents. This methodology also allows for a shorter analysis time, since no pre-treatment procedures are needed, even for real sample analysis. The biosensor was able to successfully analyze methomyl contamination in various foods, and was shown to possess an easily renewable surface. Other advantages of this biosensor include ease of preparation, high stability, and high selectivity.

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