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Effect of chlorpyrifos on the inhibition of the enzyme acetylcholinesterase by cross-linking in water-supply samples and milk from dairy cattle

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ABSTRACT

A methodology for the determination of chlorpyrifos in water-supply samples and in milk from dairy cattle was developed. An amperometric biosensor was used to inhibit the enzyme acetylcholinesterase (AChE), which was immobilized by the cross-linking method (crosslinks between the enzyme and the sensor). The potential applied, the amount of enzyme to be immobilized and the acetylthiocholine (ACTh) concentration were optimized before calibration and analysis of the samples was performed. The concentration of chlorpyrifos was determined in the range of 1.0×10^{-6} M to 5.0×10^{-2} M with a detection limit of 5.0×10^{-6} M. Spiked water samples showed high recoveries (91.32% and 93.98% for low and high chlorpyrifos levels, respectively), while milk samples exhibited a matrix effect with recoveries of 82.81% and 79.77% for high and low chlorpyrifos levels, respectively. The average concentration of chlorpyrifos in the water supply samples (5.11×10^{-6} M), determined using the biosensor, was compared using gas chromatography and gave an average value of 3.04×10^{-6} M. The results allow it to be concluded that although chromatographic methods are still more exact, biosensors are promising tools for the determination of analytes in the field, as they have a low cost, a reduced analysis time and good reproducibility in the data.

1. Introduction

Pesticides are widely used chemicals, especially in agriculture. Their extensive use has led to the contamination of water sources, and they have had a major impact on health and the environment [1]. This is especially true for organophosphorus pesticides which are used for pest control and other domestic purposes [2]. Chlorpyrifos is still one of the most commonly used organophosphorus pesticides. Due to agriculture and point source discharges, this pesticide is principally responsible for the toxicity of a large part of aquatic life [3]. However, in recent years it has been shown that chlorpyrifos may not only be present in water bodies, but also but also in livestock sites, as cattle herds could drink water from contaminated waterways. This leads to the production of milk contaminated with the aforementioned pesticide. Therefore, the quality of milk from these cattle herds must be monitored. Biosensors can be an effective and simple tool for monitoring contaminants such as chlorpyrifos in milk [4] and water.

Biosensors are analytical devices that use the sensitivity and selectivity of a bioreceptor adhered on the surface of a transducer. The transducer is able to respond and transform a biochemical and/or physicochemical property into a measurable signal as a result of recognition between the bioreceptor and target analyte [5]. These are coupled to elements such as biological sensing enzymes, antibodies, microorganisms or DNA, and integrated into transducers that can be electrochemical and optical, among other types [6]. Amperometric biosensors are based on measuring changes in the current of the working electrode due to oxidation or reduction of metabolic products or intermediates generated in biochemical reactions [7,8].

Chlorpyrifos is considered a neurotoxic compound that irreversibly inhibits the enzyme acetylcholinesterase (AChE), essential for the functioning of the central nervous system in humans and insects. This results in the accumulation of the neurotransmitter acetylthiocholine (ATCh), which interferes with muscular responses and vital organs, causing severe symptoms and eventually death [9]. When AChE is immobilized on the surface of the working electrode (SPEs), its interaction with the substrate produces an electroactive species. In this process, the acetylthiocholine (ACTh) can replace the original substrate of AChE, and therefore, the ACTh is hydrolyzed in the same way as the original substrate, producing thiocholine (TCh) and the corresponding carboxylic acid (acetic acid in this case) [5]:

$$\text{acetylcholine} + H_2O \xrightarrow{\text{AChE}} \text{thiocholine} + \text{acetic acid}$$

$$2\ \text{thiocholine} \xrightarrow{\text{oxidation}} \text{dithio–bis–choline} + 2H^+ + 2e^-$$

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In this study, an enzymatic amperometric biosensor was used in order to establish a methodology for the rapid detection of the pesticide chlorpyrifos in water supply samples and milk from dairy herds. The methodology was based on the inhibition of acetylcholinesterase enzyme activity (AChE), which was immobilized using the cross-linking method on the surface of Screen Printed Electrodes (SPEs).

2. Materials and methods

2.1. Reagents

Acetylcholinesterase from Electric eel (AChE, specific activity of 500 U/mg solid), acetylthiocholine chloride (ATCh) with a purity ≥99%, Albumin from bovine serum (BSA, Cohn V Fraction > 96%), glutaraldehyde (GA, grade I, 8%), Nafion® (Perfluorinated membrane 5 wt%), 5,5′-dithio-(2-nitrobenzoic acid) (DTNB) and sodium monohydrogen phosphate (PBS, Na_2HPO_4) were purchased from Sigma–Aldrich Co. (Stein-Heim, Germany). The AChE, ATCh, GA and DTNB were kept at −20 °C. All solutions were prepared with ultrapure water (average conductivity 0.04 μS/cm). Chlorpyrifos standard solution was purchased from Dr. Ehrenstorfer, GmbH (Germany) with a purity ≥99%. Stock solutions were prepared by dissolution of the Chlorpyrifos standard solution in HPLC grade methanol (Merck) and kept at −20 °C for a maximum of 1 week.

2.2. Amperometric biosensor

Amperometric measurements were carried out with a SPE AmpBio device PS, (Biosensor Srl, Formello, Rome, Italy), with dimensions of 22.0 cm wide × 15.0 cm long × 10.0 cm high, and composing of two measuring cells in parallel designed for the measurement of Screen Printed Electrodes (SPEs). The biosensor operated under a flow of 200 μL/min, with a current of 5 μA and a variable voltage of +100 to +400 mV, in agreement with the experimental design, and connected online to a computer (Hewlett Packard) for the acquisition of information (Fig. 1(a). BIOCOM software was used (Biosensor S.r.l, Formello, Rome, Italy).

Screen Printed Electrodes (SPEs) were purchased through the company DropSens, SL (Spain), with dimensions of 3.4 cm long × 1.0 cm wide and 0.05 cm thick. Contacts of the reference electrode and the connection electrodes were silver and both the working electrode and the auxiliary electrode (with a diameter of 4 mm) were made of carbon (Fig. 1(b).

2.3. Free enzyme activity

Determining the activity of the free enzyme AChE was carried out in a Thermo (EVO EVON 600LC-237 001) spectrophotometer, in accordance with the method described by Ellman et al. [10] and modified by Nunes et al. [11], which is based on two coupled reactions [12]:

$$\text{acetylthiocholine (ATCh)} \xrightarrow{\text{Acetylcholinesterase(AChE)}} \text{acetic acid} + \text{thiocholine}$$

$$\text{thiocholine} + 5,5'\text{dithio-bis-2-nitrobenzoic acid(DTNB)} \rightarrow \text{2-nitrobenzoate-5-mercaptothiocholine} + \text{5-thio-2-nitrobenzoate}(TNB^{2-})$$

The final TNB^{2-} product is yellow coloured and can be detected optically at 412 nm.

In accordance with the procedure established by Nunes et al. [11], the following volumes were added sequentially in a quartz cuvette: 200 μL of DTNB (2.5 mmol/L), 350 μL of PBS (2 mmol/L, pH 8.0), 50 μL of the enzyme/pesticide solution previously incubated at 30 °C, and 200 μL of ACTh (2 mmol/L). The volumes were determined spectrophotometrically at 412 nm. Aliquots of the enzyme/pesticide solution were taken at intervals of 2 min over a 20 min period. Assays in the absence of both the pesticide and the enzyme/pesticide solution were used as controls and blanks, respectively. Finally, the biomolecular constant (k_i) was determined.

2.4. Protocol for amperometric analysis

2.4.1. Immobilisation of the enzyme in SPEs

The AChE was immobilized using the cross-linking method, which involves the use of bifunctional reagents such as glutaraldehyde that cause intermolecular links between the enzyme and the transducer [13–15]. To carry out the immobilization, 5 μL of GA (0.25% *w/v*) were placed on the working electrode (SPEs) and allowed to dry at 4 °C, then 3 μL of a solution containing 30 μL of BSA (5% w/v), 30 μL of Nafion (1% w/v) and 30 μL of AChE (units of enzyme used varied depending on the design of the experiments) were added and allowed to dry again at 4 °C. The SPEs were stored in 2 mmol/L PBS at pH 8.0 and 4 °C for at least 3 days before the testing began [9]. This was because according to studies by Dou et al. [16] and Hildebrandt et al. [2], AChE has an optimum working pH in the range of 7.0 to 8.5 with a maximum response at pH 8.0. The SPEs were stored under conditions of hydration since when stored dry, the enzyme is rapidly denatured and loses nearly all its activity, giving very low, almost undetectable, signals. Prior to analysis, the SPEs were regenerated for 10 min with PBS (2 mmol/L, pH 8.0) with stirring [9] at room temperature (22 °C).

2.4.2. Optimization assays

In order to find the best conditions for validation, several experiments were developed in which the voltage, units of the AChE enzyme, and mmol/L of ACTh were varied in different proportions. The first test was performed keeping the ATCh and AChE constant (0.12 U and 3 mmol/L, respectively) and varying the voltage applied at rates of +100 mV, +200 mV, +300 mV and +400 mV. In the second assay the AChE was varied in proportions of 0.06 U, 0.12 U and 0.24 U, but the ACTh remained constant at 3 mmol/L and the voltage used was that which gave the best response in test 1. Finally, in a third experiment the ATCh was varied in proportions of 1 mmol/L, 3 mmol/L, 5 mmol/L, 7 mmol/L and 9 mmol/L but the AChE and voltage remained constant in accordance with the best results from experiments 1 and 2. Assays were performed in triplicate and new biosensors were employed in each case to avoid problems with reproducibility and data response.

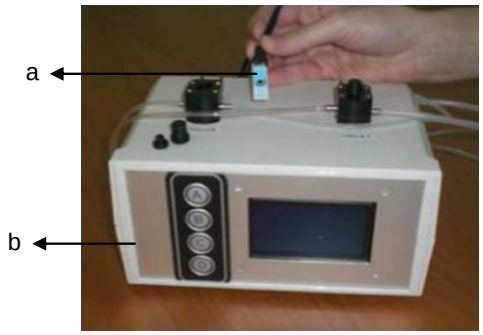

Fig. 1. Amperometric biosensor (SPE AmpBio PS) used for the determination of chlorpyrifos. (a) Amperometric unit. (b) Screen Printed Electrodes (SPEs).

2.4.3. Inhibition assays

The inhibition assays were performed at room temperature using the best conditions for the AChE, ACTh, and voltage obtained in optimizing assays. The SPEs (after immobilisation of the enzyme) were connected to the measuring cells of the equipment, then 2 mmol/L of PBS at pH 8.0 was added using a flow of 200 μL/min until the signal stabilized and was less than 0.1 μA. Subsequently, the ATCh was added and the signal generated by the enzyme/substrate reaction was recorded as Io. The SPEs were washed with the same PBS and then incubated with a chlorpyrifos solution of known concentration for 20 min (The range of the calibration curve was 1.00×10^{-6} M to 5.00×10^{-2} M). All pesticide solutions were prepared in ultrapure water (from a stock solution) and not in PBS, since it is known that chlorpyrifos degrades easily at an alkaline pH. The SPEs were then washed again with PBS and a second signal measurement was carried out after adding the ATCh. This measurement was recorded as *I*. Finally, the inhibition percentage *I* (%) was calculated, which corresponds to a fixed concentration of chlorpyrifos in the sample [11,17]:

$$I(\%) = \left[\frac{(I_0 - I)}{I_0}\right] \times 100$$

where: I_0 and I correspond to the enzyme/substrate reaction before and after incubation of the inhibitor. Inhibition assays were carried out in triplicate.

2.4.4. Repeatability of the method

Repeatability was determined by measuring the inhibition (% *I*) generated by 3 different concentrations of chlorpyrifos, each using 10 new electrodes (SPEs), before immobilization of the enzyme under the optimal conditions established. In accordance with the procedure given by Vandeginste and Quadt, [18], the action and warning limits above and below the mean were calculated in each case.

2.4.5. Analysis of samples of water, milk and matrix effect

The water supply used for the daily activities of a dairy farm located in the municipality of San Pedro de los Milagros (Antioquia, Colombia) was monitored. Samples were collected on different days, taken to the laboratory in amber glass containers at 4 °C and filtered to 0.45 μm prior to analysis in order to remove solids which would cause measurement interference. Subsequently, the matrix effect was evaluated by spiking the samples with low, medium and high levels of chlorpyrifos. The aforementioned values were obtained from the previously standardized calibration curve. In the case of milk, samples were centrifuged at 5000 rpm to remove fat and cream, and then the supernatant was removed and filtered through qualitative filter paper.

2.5. Chromatographic analysis

The data obtained for the amperometrically measured water-supply samples were compared with results obtained using an Agilent Technologies (model 6890) gas chromatograph with a NPD detector and a Rtx-5Sil MS Restek 30 m × 320 μm × 0.25 μm capillary column. The carrier gas was helium flowing at 45 cm/s and measured at 110 °C. The temperature ramp was the following: a rate of 30 °C/min and 10 °C/min until 100 °C and 5.0 °C/min until 270 °C, and the temperature of the injector and detector was 300 °C. The retention time of chlorpyrifos in the chromatographic analysis was 16.55 ± 0.5 min. Prior to chromatographic analysis, an extraction of the analyte was carried out with C18 cartridges.

2.6. Statistical analysis

Multidimensional analysis was performed to find correlations between the different parameters analyzed. Using the Statgraphics plus 5.1 program, the Pearson product-moment correlations were obtained by ANOVA analysis. Results were considered significant at $\rho < 0.05$.

3. Results and discussion

3.1. Optimization of measurement conditions

The stage where the measurements are optimized with the biosensor is one of the most crucial steps for the development of a method of analysis. It ensures appropriate conditions are met and avoids erroneous results due to inhibition of the enzyme as a result of the absence of some condition necessary to carry out the enzymatic reaction. Fig. 2 shows the current response by applying different potentials (mV). The results show that when +200 mV were applied to Screen Printed Electrodes after immobilisation of the enzyme AChE, a response of 4.69 ± 0.14 μA was generated. This current response was the highest obtained with all applied potential values. Higher values (+300 mV, +400 mV) resulted in a much lower response: 3.42 ± 0.43 μA and 2.01 ± 0.25 μA, respectively. A potential of +100 mV generated a response of 4.18 ± 0.21 μA, a value very close to that obtained with +200 mV. Therefore, it can be concluded that a potential range of +100 mV to +200 mV is suitable for carrying out analyzes, with +200 mV being the chosen potential. Studies by Liu et al. [19], found an optimal potential of +150 mV, while Marques et al. reported an optimum value of 180 mV, and others such as Istamboulie et al. [20], Hildebrandt et al. [2], Kulys and D'costa, [21], and Schuelza et al. [22], have reported adequate potentials of +100 mV. All values are similar to those obtained in this study. Theoretically, the ACTh is oxidized at a relatively high potential (+400 mV) on the surface of a conventional electrode [23]. However, according to Gogol et al. [24], the use of nafion during immobilisation of the enzyme AChE helps lower the oxidation potential of ACTh and thus potentials lower than +400 mV can be applied. This was the case when the enzyme AChE was immobilized using GA, nafion and BSA, the latter being a protein essential for generating good responses and avoiding interference with glutaraldehyde.

The amount of enzyme used is a critical step for determining an analyte as it can lead to high currents and greater degrees of inhibition, and thus, low detection limits. The results show a greater response (4.74 ± 0.28 μA and 4.93 ± 0.37 μA) with higher amounts of the enzyme (0.12 U and 0.24 U, respectively) (Fig. 3), compared to lower amounts (0.06 U with a response of

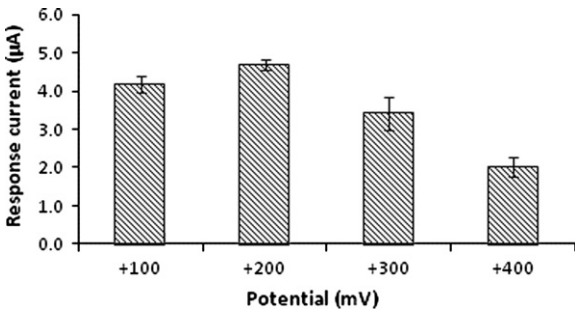

Fig. 2. Current response at different potentials applied, with 0.12 U AChE and 3 mmol/L ATCh. Assays were performed in triplicate and the bars correspond to the deviation of the data.

1.30 ± 0.32 μA). Studies by Dou et al. [16] determined that at 0.15 U of AChE a faster catalytic reaction occurred, than with lower values (0.1 U and 0.08 U). Several authors have reported that values ranging from 0.1–1.82 U [17,25–27], or even up to 134.4 U [17,28], are required to conduct a proper immobilization. The aforementioned values are comparable with the results obtained in this study. Zamfir et al. [23], showed that with 0.13 U and 0.24 U of AChE it was possible to obtain a detection limit of 1.0×10^{-3} M of Chlorpyrifos in both cases, while with a greater amount of enzyme (0.96 U) a higher detection limit was obtained (1.2×10^{-3} M). However, they also demonstrated that using the sol–gel method of immobilization, lower detection limits can be obtained, employing a lesser amount of the enzyme (0.017 U of AChE) than the cross-linking method used in this study. This is because the glutaraldehyde added during the immobilization by cross-linking tends to generate a good reproducibility and stability in the data but requires a larger amount of enzyme to carry out the reaction. Although the results found in this study showed that with 0.24 U of AChE a higher current response was acquired, the difference was not statistically representative of the results obtained with 0.12 U. Furthermore, in terms of cost and this method implies using double the amount of enzyme, and therefore, for further tests 0.12 U of AChE was employed.

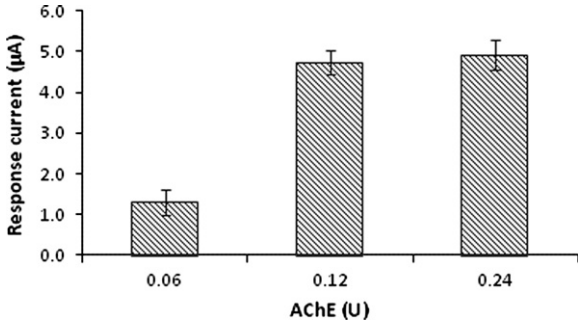

Fig. 3. Current response against different units of the enzyme (AChE) immobilized, with 3 mmol/L ATCh and a potential of +200 mV. Assays were performed in triplicate and the bars correspond to the deviation of the data.

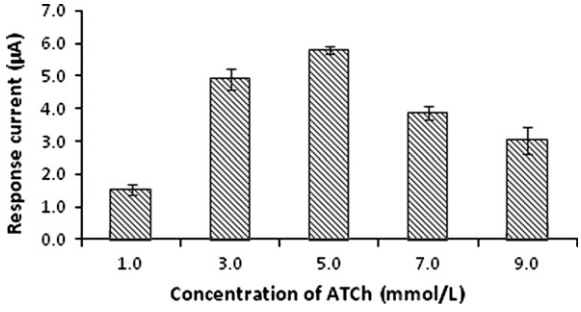

Fig. 4. Current response against different concentrations of ACTh, with 0.12 U AChE and a potential of +200 mV. Assays were performed in triplicate and the bars correspond to the deviation of the data.

In all tests conducted, the ATCh stored at −20 °C was prepared before each analysis was performed. This was done in order to avoid degradation of the ACTh, taking into account that both ACTh degradation and the acetylation of AChE produce the same final product: thiocholine. Therefore, if degradation of ACTh takes place, it will give rise to erroneous results. Fig. 4 shows the variation of the current (μA) against the concentration of ACTh (mmol/L) used to carry out the reaction. The results showed that a concentration of 5.0 mmol/L gives the maximum current response (5.78 ± 0.12 μA), as did the results obtained by Zamfir et al. [23] and Dou et al. [16].

Values above and below 5.0 mmol/L resulted in lower responses (3.87 ± 0.23 μA and 3.01 ± 0.41 μA for 7.0 and 9.0 mmol/L of ACTh, respectively, and 1.52 ± 0.15 μA and 4.91 ± 0.31 μA for 1.0 and 3.0 mmol/L of ACTh, respectively). In the first case (high concentrations), low currents are due to inhibition of the enzyme by an excess of substrate (ACTh). This feature is only attributed to AChE, since according to studies by Gogol et al. [24], Butyrylcholinesterase (BChE), which is the second type of cholinesterase, is not usually inhibited in this way. In the second case (low concentrations), low currents are due to slow diffusion and transfer of ACTh in the enzyme layer. A lack of saturation of the enzymatic catalytic sites leads to reactions only on the electrode surface, thereby giving small currents. In these types of situations, the reactions are helped when the tests are carried out with substrate stirring [9] or higher flow rates (347 μL/min according to Shi et al. [29]) than those employed in this study (200 μL/min), allowing rapid diffusion of ACTh in the enzyme layer.

3.2. Calibration curve of chlorpyrifos and the biomolecular inhibition rate constant (k_i)

With the aim of measuring the inhibition percentage of the enzyme AChE, concentrations of chlorpyrifos in the range of 1.0×10^{-6} M to 5.0×10^{-2} M were employed, under predetermined optimal conditions. Fig. 5a shows the calibration curve obtained and its linearization using a semi-logarithmic representation (Fig. 5b). It yields a first order correlation and a statistically significant relationship ($\rho < 0.05$) between the inhibition percentage ($I\%$) and the logarithm of the chlorpyrifos concentration (log C). Fig. 5a shows that in the last three chlorpyrifos concentrations tested, it was possible to obtain a consistent trend in terms of the inhibition percentage, reaching a maximum of 90.93% at a concentration of 5.00×10^{-2} M. This means that it was not possible to obtain a 100% inhibition of AChE, a phenomenon attributable to

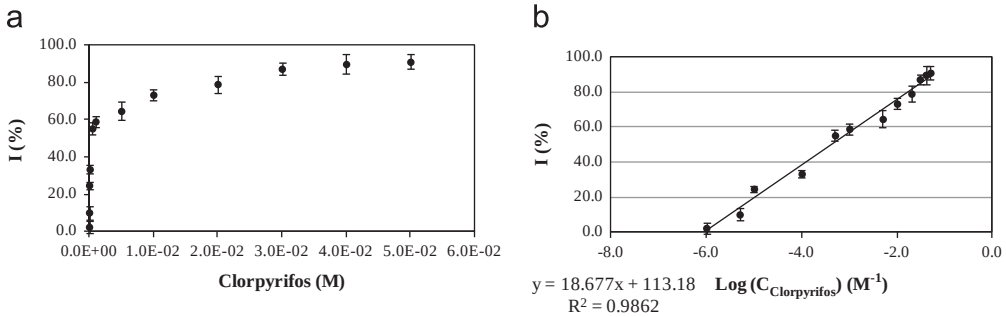

Fig. 5. Chlorpyrifos calibration curve (AChE of 0.12 U, ATCh of 5 mmol/L and +200 mV). (a) Behavior of the inhibition percentage at different concentrations of chlorpyrifos. (b) Linearization of the curve using a semi-logarithmic representation, in accordance with ANOVA, $\rho < 0.05$. A statistically significant relationship between the variables can be seen for a confidence level of 99%.

Table 1
Studies using AChE for the determination of chlorpyrifos in water.

Analyte	Equation	R^2	LOD (M)	Reference
Chlorpyrifos	I (%)=18.68 log (C)+113.18[a]	0.9862	5.00×10^{-6}	**This study**
Chlorpyrifos	I (%)=32.2 log (C)+277[a]	0.9835	4.00×10^{-6}	[23]
	–	0.9992	1.20×10^{-4}	
	–	0.9988	1.00×10^{-4}	
	–	0.9890	8.00×10^{-5}	
	–	0.9735	7.00×10^{-5}	
	–	0.9735	3.00×10^{-5}	
Chlorpyrifos	I (%)=15.8 ln (C)+96.49[a]	0.9946	1.14×10^{-8}	[20]
Chlorpyrifos	I (%)=9.646C+9.576[b]	0.9969	5.70×10^{-6}	[2]
Chlorpyrifos	–	–	5.70×10^{-1}	[12]
Ethyl chlorpyrifos oxon	–	–	2.30×10^{-9}	[17]
	–	–	1.12×10^{-8}	
	–	–	6.00×10^{-11}	

[a] Concentration as *M*.
[b] Concentration as μg/L.

possible saturation of the active sites of the enzyme, such saturation results in enzyme/pesticide equilibrium.

The limit of detection (LOD) was determined as the concentration of chlorpyrifos that would cause 10% inhibition of AChE, which is the minimum accuracy required to avoid erroneous responses. Zamfir et al., [23] (Table 1), evaluated the effect of different enzyme units on the detection limit for chlorpyrifos, achieving an LOD of 4.00×10^{-6} M, while Hildebrandt et al. [2] found a limit of detection (LOD) of 5.70×10^{-6} M. Both values are close to the magnitude of the LOD obtained in this study (5.00×10^{-6} M). In contrast, Istamboulie et al. [20] and Andreescu et al. [17] obtained much lower detection limits, using more robust methods of restraint, such as magnetic nanoparticles and sol–gel, respectively.

It was necessary to evaluate the biomolecular constant (k_i) of the enzyme AChE, whose value was 3.7×10^3 M^{-1} min^{-1}. Although during an immobilization process the stability of the enzyme generally improves, its activity can also be lost because its binding with the SPEs may preclude the passage of the substrate to the active site, leading to inactive forms. As a result, it is possible to find out the degree of coupling of chlorpyrifos to the enzyme. Jeanty et al. [30] found a very similar k_i value (2.4×10^3 M^{-1} min^{-1}) when also using chlorpyrifos. Other studies, such as Nunes et al. [9], yielded higher values in the order of 1.4×10^4 M^{-1} min^{-1}, 5.5×10^4 M^{-1} min^{-1}, 2.5×10^6 M^{-1} min^{-1} and 5.2×10^5 M^{-1} min^{-1} using the pesticides Aldicarb, Carbaryl, Carbofuran and Methomyl, respectively. The above results suggest that the enzyme AChE has a greater sensitivity to carbamates than to organophosphates (in this case chlorpyrifos), possibly because carbamates are better suited to the enzyme active site and cause a greater inhibition. Organophosphate pesticides and carbamates have a similar mechanism to produce toxicity, associated with inhibition of AChE. Jeanty et al. [30] also determined the biomolecular constant for chlorpyrifos-methyl, a metabolite of chlorpyrifos, finding an average value of 3.4×10^2 M^{-1} min^{-1}, indicating a low sensitivity of AChE compared to chlorpyrifos. Marques et al. [31] found that the AChE type of enzyme from Electrophorus electricus (*ee*) used in this study has a greater sensitivity (1.1×10^3 M^{-1} min^{-1}) than AChE from human erythrocytes (he) and AChE from bovine erythrocytes (be). k_i values were about 5.0×10^2 M^{-1} min^{-1} and 7.4×10^1 M^{-1} min^{-1} for he and be, respectively when tested with the same organophosphate pesticide [5,31].

3.3. Repeatability assays

Fig. 6 shows the results found regarding the repeatability of the method, in accordance with the methodology established by Vandeginst and Quadt[18], for three levels of chlorpyrifos: low (1.0×10^{-5} M), medium (1.0×10^{-3} M) and high (4.0×10^{-2} M). Percentages of inhibition (I%) were within the low and high warning ranges, and only for the lowest concentration (Fig. 6a) a measurement slightly higher than the warning limit appears. From a statistical point of view, this measurement is not considered very representative. The relative standard deviation (RSD) for each of the cases (Low, medium and high) was 8.16%, 5.89% and 4.92%, respectively, in accordance with the procedures established by the Environmental Protection Agency (EPA) [30]. For biological assays, a maximum RSD of 25% is set as acceptance criteria, a criterion that the results from this study do meet. According to Vandeginst and Quadt [18], the obtained results were in a range that was statistically acceptable for residue analysis in the laboratory.

3.4. Analysis of real samples

When the matrix effect in the water supply was evaluated, it was found that the same source of water was used for human consumption, for washing the milking parlor and milk storage tank, and for livestock consumption. The presence of chlorpyrifos in this water has a toxic effect associated with the inhibition of AChE, an enzyme essential for the transmission of nerve impulses both in humans and animals. Andresescu and Marty [5] have stated that this enzyme is connected with human memory and Alzheimer's disease. According to the results, the water supply had an average chlorpyrifos concentration of 5.11×10^{-6} M ($n=5$) when determined amperometrically, and 3.04×10^{-6} M ($n=5$) when determined by gas chromatography. These results demonstrate that the amperometric biosensor did operate well as a screening method under the appropriate conditions. However, it might be possible to reach a higher level of accuracy by improving the immobilization conditions of the enzyme AChE or by using a more sensitive AChE (dm), as found by Andresescu and Marty [5] and Marques et al. [31], in their research. Gas chromatography is an accurate and sensitive analytical technique for the determination of analytes. However, it requires a prior extraction and concentration of the analyte in the laboratory, thereby involving longer analysis times and higher costs. Biosensors are a promising analytical technique for the analysis of environmental samples *in situ*.

After determination of the chlorpyrifos concentration in the samples, they were spiked with two levels of concentration from the calibration curve (Table 2). This proved that for the water supply samples spiked with a low level of chlorpyrifos (1.0×10^{-4} M), it was possible to obtain a recovery percentage of 91.32%, and for a high level (4.0×10^{-2} M) a percentage of 93.98%.

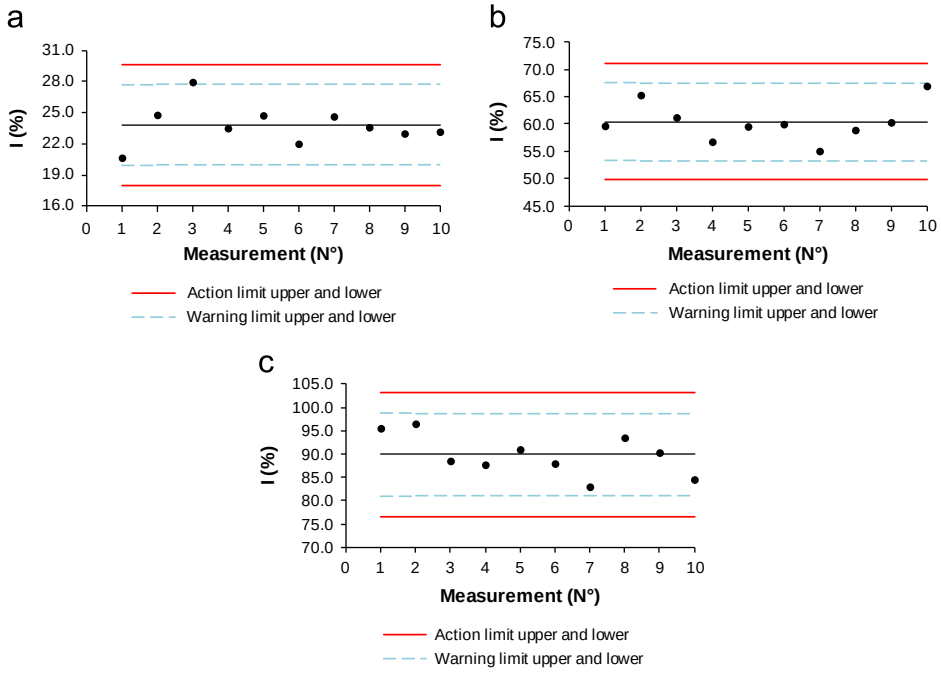

Fig. 6. Repeatability of the method (AChE of 0.12 U, ATCh of 5 mmol/L ,+200 mV). (a) Concentration of chlorpyrifos 1.0×10^{-5} M. (b) Concentration of chlorpyrifos 1.0×10^{-3} M. (c) Concentration of chlorpyrifos 4.0×10^{-2} M.

Table 2
Evaluated matrix effect on water supply and milk samples from a dairy farm.

Matrix	*n*	*I* (%)			Recovery (%)	
		Unspiked mean	Spiked mean (1.0×10^{-4} M)	Spiked mean (4.0×10^{-2} M)	Spiked mean (1.0×10^{-4} M)	Spiked mean (4.0×10^{-2} M)
Water supply	5	14.17 ± 1.87	35.13 ± 3.49	81.83 ± 1.85	91.32 ± 5.07	93.98 ± 3.12
Milk	5	6.94 ± 2.64	31.86 ± 3.01	69.46 ± 4.57	82.81 ± 7.82	79.77 ± 75.25

These were the highest rates of recovery and were where the matrix evaluated generated a certain effect on the determination of the inhibition percentage. Such an effect may be associated with other factors in the sample, such as dissolved solids or chloride content, which interfere with potentiometric measurements, or in this case, amperometric measurements. The matrix effect in milk samples was also evaluated and recoveries were found to be lower (82.81% and 79.77% for low and high chlorpyrifos levels, respectively). However, milk is a more complex matrix due to the presence of minerals, proteins, carbohydrates and lipids. Although the samples were previously centrifuged to avoid interference, an excess in the content of proteins like albumin that are also present in the enzyme immobilisation process, could have led to weaker signals.

4. Conclusion

According to the optimization trials, it was determined that with 0.12 U of AChE, a potential of +200 mV and an ACTh concentration of 5 mmol/L , high responses were generated which favoured the determination of chlorpyrifos amperometrically. The results showed that the method was sensitive (LOD= 5.00×10^{-6} M) for the determination of chlorpyrifos, with good repeatability at different levels of the calibration curve (RSD=8.16%, 5.89% and 4.92% for low, middle and high levels of chlorpyrifos, respectively). Very good recoveries were achieved with water samples (91.32% and 93.98% for low and high chlorpyrifos levels, respectively) and acceptable recoveries were achieved with milk samples (82.81% and 79.77% for low and high levels, respectively).

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