EXTRACTION AND DETECTION OF PESTICIDES BY CHOLINESTERASE INHIBITION IN A TWO-PHASE SYSTEM: A STRATEGY TO AVOID HEAVY METAL INTERFERENCE

2.1 Introduction

Pesticides are among the most important environmental pollutants because of their significant presence in the environment. The use of pesticides in agriculture has progressively increased since World War II with a concomitant increase in world food production. In this context, industrial emission of pesticides during their production, and more importantly the presence of residues of these chemicals and their metabolites in food, water and soil, has become a problem for society at large [1]. Among the many methods reported for pesticide detection, chromatographic methods such as High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) are used as reference methods [2,3]. Thus enzymatic methods have been adopted as an alternative to classical methods (GC, HPLC) for faster and simpler detection of some environmental pollutants [4,5].

The use of cholinesterase enzymes for inhibition-based determination of pollutants has shown great promise for environmental screening analysis [6-8]. Acetylcholinesterase or Butyrylcholinesterase are irreversibly inhibited by organophosphorous and carbamate pesticides and reversibly inhibited by heavy metals [9-11] and a comparison of the activity of Acetylcholinesterase (AChE) before and after exposure to environmental samples can provide an evaluation of the pollution level. The measurement of enzymatic activity can be accomplished by electrochemical [6,7,12], chemiluminescent [13] or spectrophotometric methods [14,15].

In the most commonly used spectrophotometric assay method for pesticide detection, acetylthiocholine is used as substrate of AChE and the production of thiocholine is measured by Ellman's method [15], as shown in eq.1. DTNB (5,5'-dithiobis-2-nitrobenzoic acid), reacts with

thiocholine to give TNB, 2-nitro-5-thiobenzoic acid, a yellow-coloured product with an maximum absorption at 412 nm.

eq. 2.0
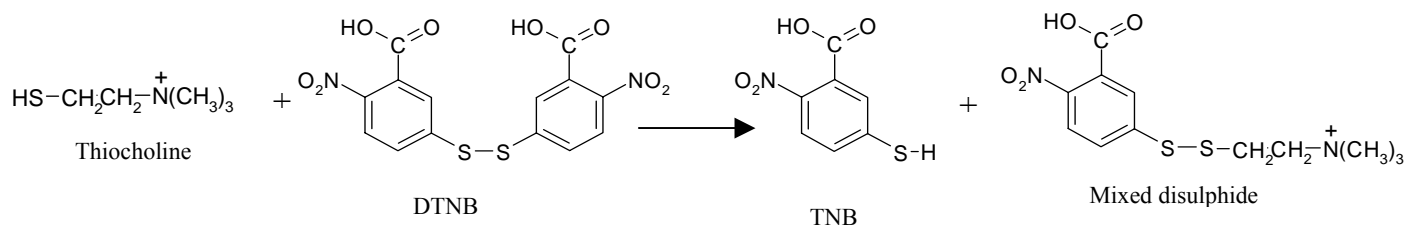
However, it should be pointed out that this method could be negatively affected by the presence of heavy metals in the sample. Given the high affinity of thiols for heavy metals [31-33], the thiocholine produced by the enzymatic reaction could in fact react with heavy metals present in the sample solution. Such metal binding is expected to interfere with the reaction described in eq.2.1 and thus would lead to an overestimation of pesticide level when this spectrophotometric determination of AChE activity is used. To avoid this problem, a separation step would seem to be required in which pesticides are first extracted into an organic phase. This would eliminate the presence in the sample solution of heavy metals and other water soluble enzymatic inhibitors such as fluoride [34] and hyplochlorite [47], given their low solubility in organic solvents.

A major problem relates to the effect of the organic solvents normally used for pesticide extraction on Acetylcholinesterase activity as many of these solvents are known to inhibit the AChE reaction [16,17]. At the same time, liquid extraction with organic solvents [18-20] or Solid Phase Extraction (SPE) [21-23] followed by chromatographic methods (gas chromatography or high-performance liquid chromatography) are the procedures usually adopted for pesticide determination. Extraction steps are routinely employed for the measurement of pesticides from real samples and, in fact, fruit, vegetable and cereal samples are always blended with organic solvents in order to achieve an efficient extraction of pesticides. Organic solvents are also used in the case of liquid samples (i.e. fruit juices, wine, water) in order to achieve a sort of preconcentration of the analyte [24], given that pesticides are usually much more soluble in organic solvents than in water.

Several articles have demonstrated the possibility of circumventing the problem of enzyme inactivation by constructing inhibition-based enzyme biosensors operating in different types of non aqueous solvents [25-28]. However, AChE has demonstrated very low activity in organic solvents [16,17,29]. Successful AChE determination with organic solvents has been demonstrated only by use of an organic solvent saturated with water [17,29], but there was still a considerable loss in enzyme activity.

The aim of our work was to develop a practical assay for pesticides based on the use of Ellman's method to determine their inhibition of Acetylcholinesterase activity. The strategy involved the use of two immiscible phases, an organic one containing the pesticides and an aqueous one containing the enzyme so that the pesticides would be extracted into the aqueous phase by virtue of their binding to the active site of the AChE at the interface between the two phases. In the present paper 1) the direct effect of heavy metals on the reaction in Ellman's method and 2) the effect of organic solvents on enzymatic activity have been fully investigated. Finally, optimised conditions were defined in order to determine the inhibition of AChE activity by pesticides dissolved in the organic phase, thus minimising the potential interference of heavy metals through their reaction with either the enzyme or the thiocholine produced.

The results demonstrated that this approach provides an easy and time-efficient assay which enabled the detection of paraoxon with detection limits that were of the same order of magnitude as those found for the assay in aqueous solutions. Moreover the interference by heavy metals was avoided.

2.2 EXPERIMENTAL

2.2.1 Apparatus and reagents

For spectrophotometric measurements an Unicam 8625 UV/VIS spectrophotometer was used.

All chemicals from commercial sources were of analytical grade.

Thiocholine measurements were carried out using the classical Ellman's method based on the reaction between thiocholine and DTNB (5,5'-dithiobis-(2-nitrobenzoic acid). Acetylcholinesterase (AChE) from electric eel (EC 3.1.1.7, 244 units/mg), acetylthiocholine chloride, and DTNB were obtained from Sigma (St. Louis, MO). The solution of DTNB (1 mM) used in the experiment was prepared in phosphate buffer 10 mM, pH 7.0. For the measurement of reaction between thiocholine and heavy metals, aqueous solutions were prepared with silver, cadmium, lead and nickel nitrates and ferric (II) sulphate from Carlo Erba (Milano); cupric sulphate, mercury chloride, zinc sulphate, aluminium potassium sulphate, manganese chloride, arsenic acid (sodium salt) from Sigma (St. Louis, MO); iron(III) chloride from Fluka. Measurement of the effect of the solvent on AChE was carried out using hexane and toluene from Carlo Erba (Milano); octanol, cyclohexane and chloroform from Sigma (St. Louis, MO), iso-hexane PESTANAL® from Riedel de Haen, dichloromethane from Aldrich, ciclohexanone from Fluka. The measurement of enzyme inhibition was carried out using paraoxon (O,O-diethyl-O-(-4-nitro-phenylphosphate)) obtained from Sigma (St. Louis, MO). Stock solutions (4,5 mM) of paraoxon were prepared in ethanol. Different concentrations of pesticides were obtained by diluting the stock solution in hexane.

2.2.2 Measurement of thiocholine

Thiocholine was produced enzymatically using AChE as enzyme and acetylthiocholine as substrate (since thiocholine is not commercially available). For this purpose 1mL of acetylthiocholine solution 1 M was prepared in phosphate buffer 0.1 M (pH= 8) and 100 units of AChE were added to this solution. After 1 hour, the concentration of thiocholine produced by AChE was estimated spectrophotometrically by Ellman's method. For this purpose 900 μl of phosphate buffer solution (0.1 M, pH=8), 100 μl of 0.1 M DTNB and 5 μl thiocholine solution (diluted 1:100 in water) were put in a cuvette. Absorbance was measured at different times and the real concentration was evaluated using the Lambert–Beer law with the known molar extinction coefficient of TNB ($\varepsilon = 13600$ M^{-1} cm^{-1}) [15].

After 1 hour, the hydrolysis reaction of acetylthiocholine is completed and 1 mL solution of 1 M thiocholine is obtained. The solution is stable for one day at 4° C.

2.2.3 Reaction between thiocholine and heavy metals

To study the reaction between thiocholine and heavy metals, 900 μl of phosphate solution (0.1 M, pH=8), 10 μl of thiocholine solution ($1 \cdot 10^{-3}$ M) and 10 μl of heavy metal solutions of various concentrations were added to the same cuvette. After 10 minutes, 100 μl of 0.1 M DTNB were added and the TNB produced was evaluated at 412 nm. The absorbance decrease, relative to the solution without heavy metal, was then correlated to the percentage of thiocholine that has reacted with heavy metal cations as follows:

$$R\% = (A_0 - A_R) / A_0 \quad \textbf{eq. 2.1}$$

where:

R%= % of thiocholine which reacts with heavy metals

A_0= absorbance obtained with thiocholine solution alone

A_R= absorbance obtained for thiocholine in presence of heavy metals.

2.2.4 Measurement of the Enzymatic Activity in Aqueous Solution

The measurement of AChE activity in aqueous solution was carried out using 5 mL of phosphate solution (0.1 M, pH 8.0) containing a known amount of AChE. 900 μl of this solution and 10 μl of 0.1 M acetylthiocholine were put in a cuvette. The absorbance measurement was performed 20 minutes after the addition of the substrate (acetylthiocholine) adding 100 μl of 0.1 M DTNB in the same cuvette. The enzymatically generated thiocholine reacts with DTNB to give the yellow-coloured TNB.

2.2.5 Measurement of Pesticide in Aqueous Solution

For determination of pesticide inhibition, the same procedure as described above was used, except that the aqueous solution was spiked with different amounts of paraoxon and carbofuran. The degree of inhibition was calculated as follows:

$$I\% = (A_0 - A_i) / A_0 \qquad \textbf{eq. 2.2}$$

where:

$I\%$ = % of inhibition;

A_0 = absorbance obtained in phosphate buffer;

A_i = absorbance obtained for phosphate buffer spiked with pesticide .

2.2.6 Measurement of the effect of organic solvents on enzyme activity

To evaluate the effect of the organic solvent on the enzyme activity, 5 mL of phosphate buffer solution (0.1 M, pH 8.0) containing a known amount of AChE was mixed with 5 mL of an organic solvent. The two immiscible solutions were then gently stirred for ten minutes (incubation time), (see scheme 2.1). 900 μl were then taken from the aqueous phase, which contained the enzyme. The sample was put in a cuvette and the residual enzymatic activity was evaluated using the procedure described above. Inactivation of enzyme by exposure to organic solvent was evaluated using eq. 2.2 where:

$I\%$ = % of enzyme inactivation due to organic solvent;

A_0 = absorbance obtained in aqueous solution;

A_i = absorbance obtained for enzyme in aqueous solution in contact with organic solvent for 10 minutes (scheme 2.1a).

2.2.7 Measurement of inhibition by pesticide present in organic solvent

For pesticide measurements, the same procedure described above was used, except that the organic solvent (hexane) was spiked with different amounts of paraoxon (scheme 2.1b) or carbofuran and the degree of the inhibition was calculated using eq. 2.2 where:

I%= % of inhibition

A_0= absorbance obtained by using hexane without pesticide (scheme 1a)

A_i= absorbance obtained by using hexane spiked with pesticide (scheme 1b).

2.3 Results and discussion

2.3.1 Reaction between heavy metals and thiocholine

The high affinity of thiols for heavy metals has been widely reported in the literature [32,33,35,36]. The complexation between some thiols such as penicillamine and cysteine with heavy metals has been studied for several applications in pharmacological and enviromental areas [37,38]. Despite this, to our best knowledge, a study concerning the reaction between thiocholine and heavy metals has not been reported in the literature. In this work thiocholine complexation with heavy metals has been evaluated. As already pointed out in the introduction, heavy metal cations could represent a major interference in the measurement of thiocholine produced by AChE when enzyme inhibition is used as a means for pesticide detection. Figure 2.1 shows the concentration of different heavy metals which decrease the thiocholine signal by 15%.
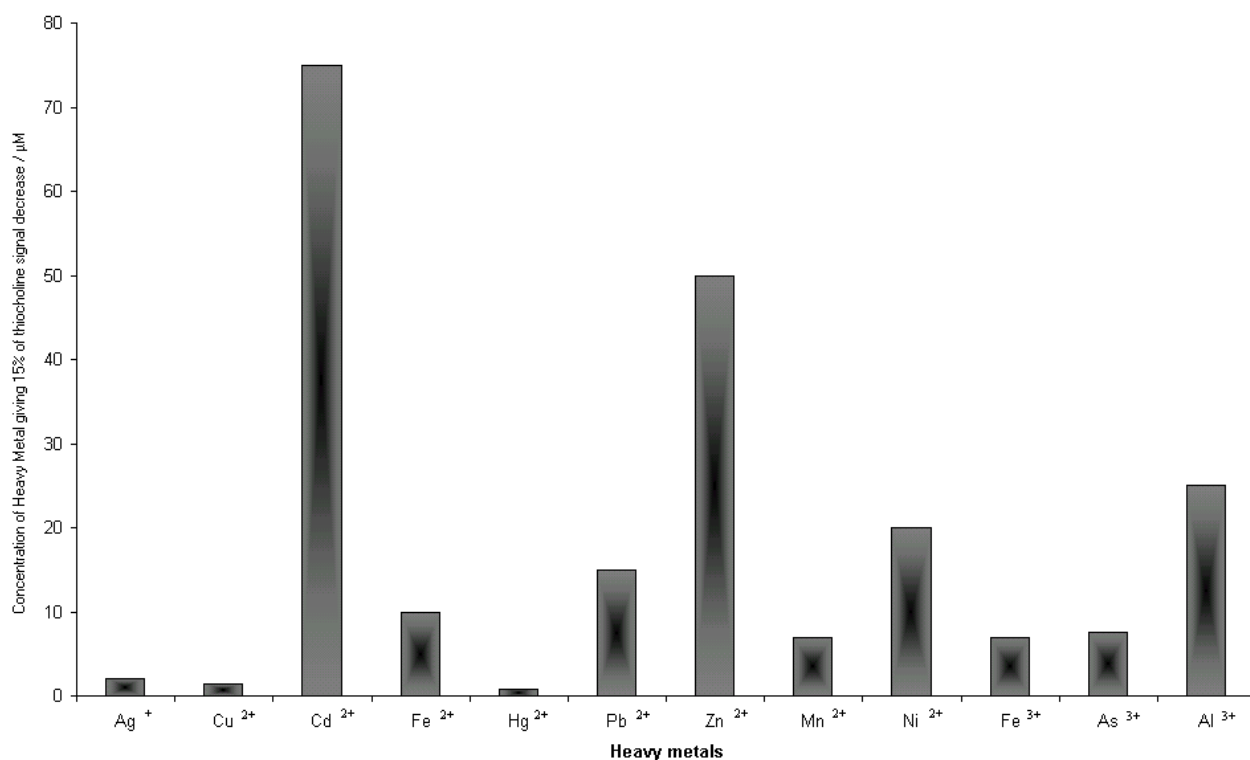
Fig. 2.1: Concentrations of different heavy metals cations giving a decrease of 15% of thiocholine signal. Thiocholine concentration $1\cdot10^{-5}$ M. Phosphate buffer 0.1 M, pH =8.

The choice of heavy metals to analyse was made considering their presence in the environment. This study demonstrates that most of the metal cations, usually present in aqueous samples, react with thiocholine greatly affecting its spectrophotometric measurement. Furthermore, this decrease is inversely proportional to thiocholine concentration (results not shown). The presence of metal ions in the sample could in fact not only act as a inhibitor of acetylcholineterase, but also react with thiocholine enzymatically produced. This could bring to the wrong conclusion that heavy metals act as competitive inhibitors when the Ellman's method is accomplished for acetylcholinestearase inhibition studies [48].

The extent of the reaction between the heavy metals and thiocholine is regulated by the affinity of thiocholine for the heavy metal and the stoichiometry of the thiocholine-heavy metal complex. For example to observe a 15% absorbance decrease, a lower concentration of Hg^{2+} is required relative to

Zn^{2+} or Ni^{2+} which are known to be bound by thiols with a lower affinity. In the case of Fe, with the two different oxidation states, the different concentration for a 15% absorbance decrease is accounted for the ability of Fe^{2+} to react with only two molecules of thiocholine while Fe^{3+} can react with three molecules of thiocholine.

In general thiocholine is more strongly bound by Hg^{2+}, Cu^{2+} and Ag^+. The concentration of Hg^{2+} and Ag^+ necessary for 15% signal decrease is higher than the normal concentrations of these heavy metals in aqueous samples. This demonstrates that Hg^{2+} and Ag^+ could be any source of error for inhibition-based pesticide assay. On the other hand, Cu^{2+} presents the biggest problem in pesticide analysis because of its high concentration in aqueous samples. The maximum admissible concentration of copper in drinking water is in fact 20 μM [39], while a much lower amount, 1.4 μM, is sufficient for a 15% decrease of the thiocholine signal. This result demonstrates that any method based on AChE inhibition, with the measurement of thiocholine, being used to assess the presence of pesticides, requires a separation between pesticide and heavy metals. The method proposed here attempts to overcome this problem by allowing the measurement of pesticide contained in an organic solvent with an enzymatic method.

2.3.2 Principle of the method

The approach is to have the pesticide interact with the enzyme at the interface between an organic and an aqueous phase as illustrated in scheme 2.1. The AChE is solubilised in a phosphate buffer solution that is placed in contact with an equal amount of an organic solvent containing the pesticide to be measured. The solutions are stirred during a fixed incubation time. The stirring facilitates the interaction enzyme-pesticide by increasing the interface and mixing the respective components in solution.

Given that virtually all organophosphate and carbamate pesticides inhibit AChE by binding irreversibly to a hydroxyl group at the catalytic centre, this reaction could have the effect of extracting the pesticide into the aqueous phase. The solubility of pesticides in aqueous solutions is

usually very low and thus the equilibrium between the two phases almost completely favours the organic solvent. However, at the interface, AChE in the aqueous phase can react with the pesticide in organic solvent, thus removing it from the equilibrium. This allows a larger amount of pesticide to be transferred into the aqueous phase through the irreversible binding to the enzyme. For this reason it can be said that the enzyme extracts the pesticide from the organic solvent.

After a defined period of time, the residual enzyme activity in the aqueous phase is measured by Ellman's method [15], after, this phase is separated from the organic one.

SCHEME 2.1

a)

b)
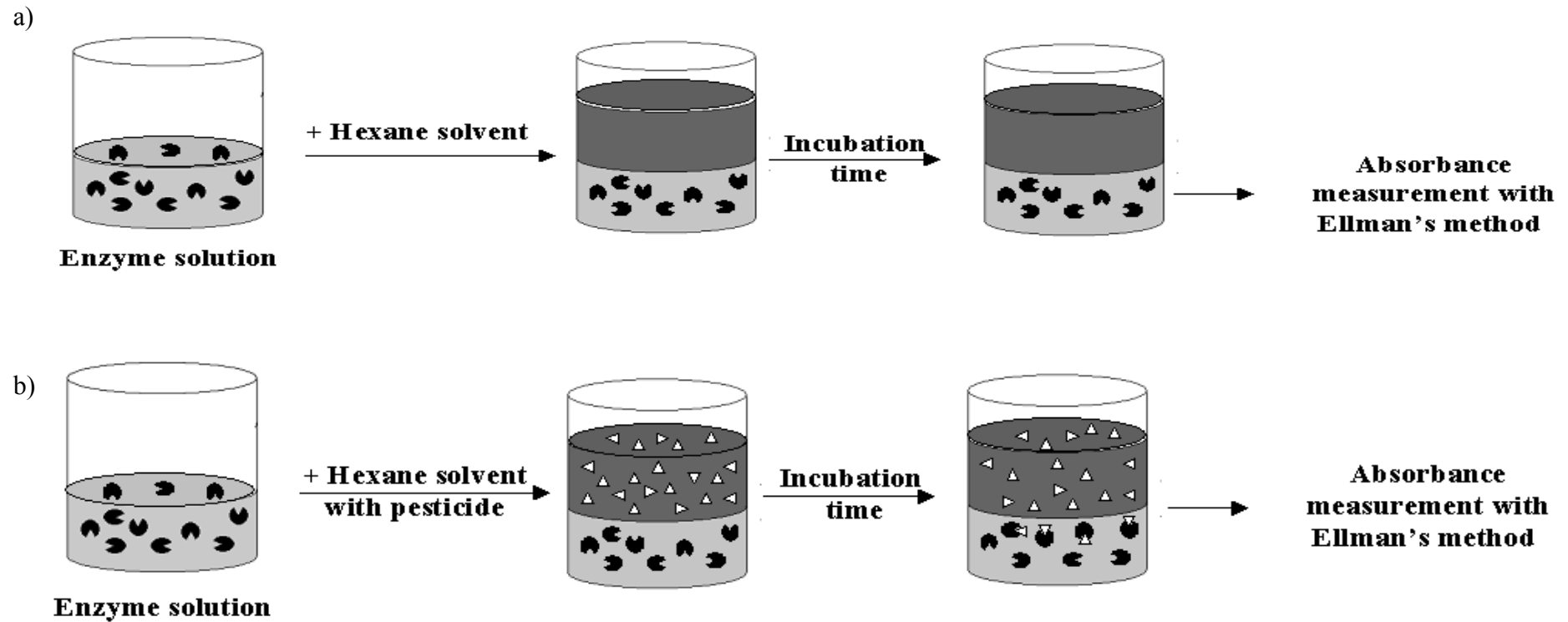
Scheme 2.1: Procedure for the evaluation of the effect of the organic solvent on enzyme activity (a) and for the measurement of pesticide (b)

Enzyme solution (AChE, 7 mU/mL) in phosphate buffer (0.1 M , pH 8.0).

Pesticide solution (organic solvent hexane).

Organic solvent

2.3.3 Characterisation of enzyme activity

In order to achieve the lowest detection limit for pesticide measurements, it was first necessary to determine the concentration of enzyme, amount of substrate, and the incubation time (reaction time between enzyme and pesticide) which gave optimal results for AChE in aqueous solution, without any exposure to organic solvent.

The first parameter to be optimised was the concentration of the enzymatic substrate (i.e. acetylthiocholine). Fig. 2.2 shows the relation between the degree rate of reaction and the concentration of acetylthiocholine. 1 mM is the lowest concentration which gives the rate near the maximum rate observed at saturating substrate. This concentration was selected for the rest of the work. It is also possible from this plot to determine the Michaelis-Menten (K_M) constant for acetylthiocholine which is 0.22±0.05 mM. The K_M for the natural substrate (acetylcholine) is 0.095 mM [40], demonstrating the obviously higher affinity of the enzyme for its natural substrate.
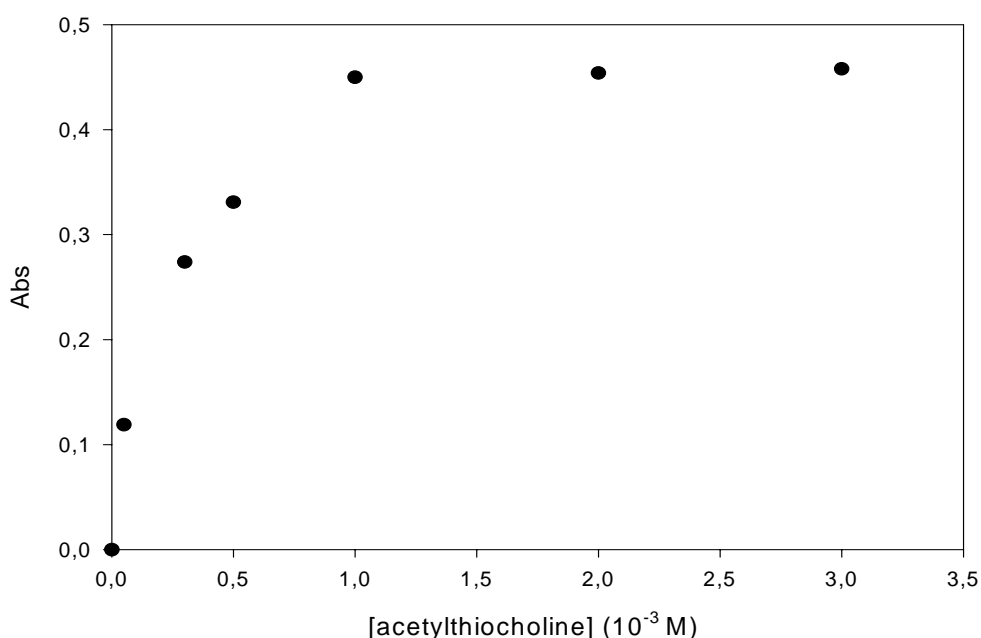

Fig. 2.2: Optimization of substrate concentration. Incubation time=10 min. Reaction time=20 min. Concentration of enzyme=7 mU/mL.

Using the previously chosen substrate concentration (1 mM) with an enzyme concentration of 7 mU/mL, reaction time has been optimised. Under these conditions, the thiocholine production (absorbance) is linearly related to the reaction time, (y=-0.024+0.023x, where y is the absorbance and x are the minutes of the reaction time). The detection limit is strictly related to the reaction time, as longer time of enzymatic reaction results in higher production of thiocholine. On the other hand, for a screening method, the time needed to reach the final result should always be as short as possible, consequently, 20 minutes were chosen as a compromise between a reasonable time of analysis and attainment of a final absorbance value near 0.4.

Finally, the enzyme concentration was optimised. This parameter is also an important factor in determining the final detection limit. The absorbance vs. enzyme concentration obtained with the previously optimised experimental parameters (reaction time 20 min, 1 mM substrate concentration) shows a linear correlation (y=0.041+0.067x, where y is absorbance and x are units of enzyme) in the range studied (3-9 mU/mL). It is desirable to use the lowest concentration of enzyme that gives an appreciable production of thiocholine, because this provides for a higher sensitivity to the inhibitor. With an enzyme concentration of 7 mU/mL it is possible to reach a value of absorbance near 0.4, after 20 minutes from the injection of substrate. This concentration of enzyme, 1mM of substrate and 20 minutes of enzymatic reaction, were then chosen to allow a measurement of the degree of enzyme inhibition with a sufficient precision.

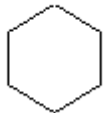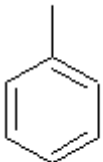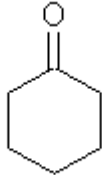## 2.3.4 Effect of different organic solvents on enzyme activity

Finding conditions for an efficient extraction of pesticides from organic solvent by cholinesterase enzyme (see scheme 2.1) was the objective of the next experiments. In this perspective, the choice of the organic solvent has to be made considering two parameters: 1)

the ability of the organic solvent to extract pesticides from real samples and 2) the effect of the organic solvent on enzyme activity.

The organic solvents tested were then chosen because of their different structures and because of their common characteristic of very high insolubility in aqueous solution (tab. 2.1). An extremely low solubility is in fact an essential requirement for the organic solvent; this would prevent the mixing between the enzyme and organic solution which could have a dramatic effect on enzyme activity, and thus interfere with the measurement of the degree of inhibition. In addition the study of organic solvents with different structures could give a clearer evaluation of the mechanism of interaction between organic solvent and enzyme.

For these preliminary experiments, an incubation time of 10 minutes was chosen on the basis of previous studies [41]. The effect of the organic solvent on enzyme activity was evaluated as described in the experimental section (scheme 2.1). Table 2.1 summarises the results obtained for the organic solvents tested. In addition to the residual activity of the esterase, value of the solubility in water is also given. It is evident that there is a relation between solubility in water and residual activity.

Organic solvent	Structure	Solubility in water (g/l)	Residual activity* %
Hexane	$CH_3(CH_2)_4CH_3$	insoluble	100
Octanol	$CH_3(CH_2)_6CH_2OH$	insoluble	95
Iso-hexane PESTANAL®	isomer mixture	insoluble	100

Cyclohexane		0,052	81
Chloroform	$CHCl_3$	7,42	71
Dichloromethane	CH_2Cl_2	20	49
Toluene		0,67	47
Cyclohexanone		100	0

*average RSD %=10%

Table 2.1: Effect on enzyme activity of different organic solvents. Concentration of enzyme=7mU/mL. Incubation time=10 min. Acetylthiocholine concentration =1mM. Reaction time=20 min. See text and scheme 2.1a for details.

The use of organic solvents which have higher solubility, such as cyclohexanone or dichloromethane, resulted in low residual activity of AChE (0% and 49% respectively), thus demonstrating an inhibiting effect on enzymatic activity. With the organic solvents which are completely insoluble, such as hexane or octanol, the residual activity of esterase was not or slightly reduced (100% and 95% respectively). On the basis of these results it appears that the solubility of the organic solvent can affect the optimum condition of the enzyme activity in phosphate buffer, significantly decreasing its ability to hydrolyse substrates. Only toluene

seems to not obey to this behaviour. Despite its very low solubility in water, toluene results in a strong inhibition of AChE (ca. 47% of residual activity). The effect of toluene on enzymatic activity can be probably explained by considering another factor. The active site of AChE is at bottom of a deep and narrow cleft that penetrates halfway into the enzyme structure and become wider at its base. Fourteen aromatic residues line a substantial portion of the cleft surface [42]. The high degree of inhibition observed in the case of toluene is probably due to a direct interaction between the aromatic residues found in the cleft and the aromatic portion of toluene, as already demonstrated for other aromatic compounds (i.e. calixarenes) [30].

The organic solvent which gave the best results in terms of residual activity was hexane (100 % of residual activity). However, further studies demonstrated that inactivation of AChE increases strongly with increase in incubation time. In fact, after 30 and 60 minutes with hexane, the residual activity fell to 25% and 0% respectively. The optimum conditions for pesticide determination were then chosen 10 minutes for incubation time and hexane as organic solvent. This choice also takes into account the wide use of hexane for the extraction of chemical contaminants from real samples [43-46]. Finally paraoxon was selected as standard pesticide for this preliminary study.

2.3.5 Measurement of inhibition by paraoxon

A preliminary study with paraoxon dissolved in organic solvent to demonstrate the enzyme ability to extract the pesticides from non aqueous solvent has been performed. For this purpose an organic solution (hexane) spiked with $1.8 \cdot 10^{-6}$ M of pesticide was used. In the first experiments, the organic solvent containing the pesticides was put in contact for 10 minutes with aqueous solution in which the enzyme was dissolved. Then, the enzyme residual activity was measured as described in the experimental section. In a successive experiment, the organic solution containing the pesticide was put in contact with aqueous solution without enzyme. After 10 minutes, 900 μl of aqueous solution were put in a cuvette and the enzyme

was added. After 10 minutes (incubation time), acetylthiocholine was added and the enzyme residual activity was measured as described in the experimental section. In the first case, the enzyme inhibition obtained was 75% while in the second experiment a 15% of enzyme inactivity, probably due to the pesticide spontaneously passed in the aqueous solution, has been observed. This demonstrate that the enzyme dissolved in aqueous phase markedly increases the extraction of pesticides into organic phase. The calibration curve for paraoxon dissolved in hexane is shown in fig. 2.3 (see experimental section and scheme 2.1 for details).

At a concentration of paraoxon of $1.8 \cdot 10^{-7}$ M, an inhibition of about 30% could be observed. The curve (fig 2.3) seems to be linear up to $2.9 \cdot 10^{-6}$ M which corresponds to 90% of inhibition. 50% of inhibition was obtained with $2 \cdot 10^{-7}$ M and $9 \cdot 10^{-7}$ M of paraoxon present in aqueous solution and aqueous/organic solution, respectively. The different degree of inhibition is due to the different reaction of enzyme at the interface in aqueous/organic solution. The sensitivity of this method observed in this preliminary study is lower than the method based on esterase inhibition in aqueous solution because the reaction between enzyme and pesticide is greatly favoured in aqueous solution than at the interface aqueous/organic solution and in the time allowed for the reaction the enzyme can not extract the total amount of pesticides present in organic solvent. However, we should keep in mind that pesticide can be concentrated using liquid extraction with organic solvent.
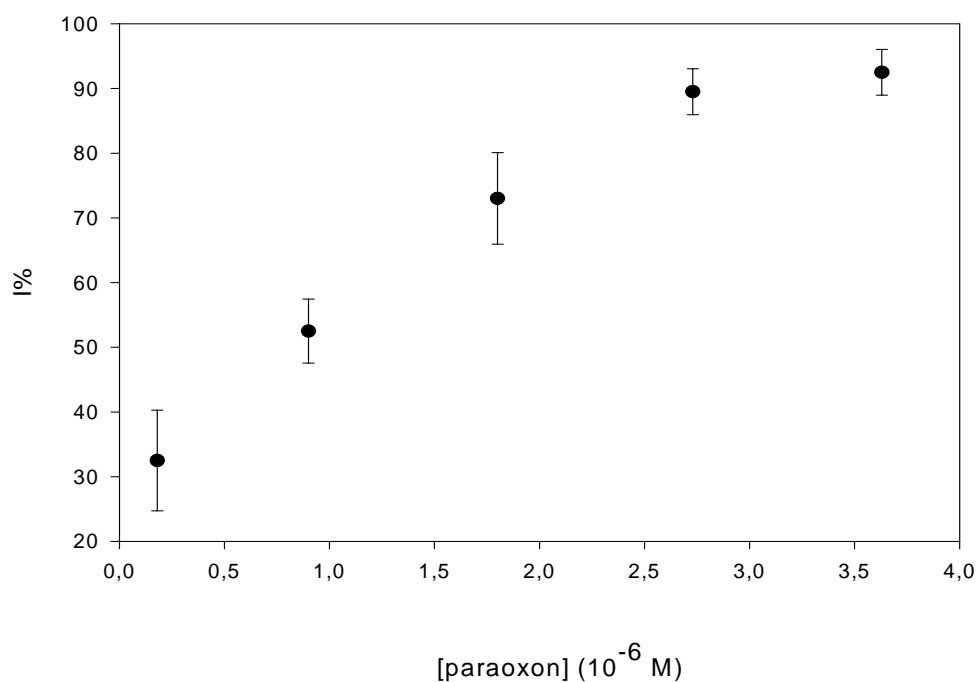
Fig. 2.3: Calibration curve for paraoxon. Incubation time=10 min. Reaction time=20 min. Concentration of enzyme=7 mU/mL. Acetylthiocholine concentration=1mM. See text and scheme 1b for details

2.4 Conclusions

The study of the reaction between thiocholine and heavy metals has been accomplished to demonstrate that some heavy metal cations, usually present in samples, could have a strong influence in the measurement of pesticides, limiting the reaction of thiocholine with DTNB and thus overestimating the pesticide concentration. This demonstrates that in the case of any method based on the use of AChE with final measurement of thiocholine for pesticide detection, a separation between pesticide and heavy metals is necessary.

A novel method for the detection of pesticides by inhibition of AChE has been developed. It makes use of two immiscible phases in which the pesticide (organic phase) and the enzyme (aqueous phase) are solubilised. The enzyme (AChE) is then able to extract the pesticide at

the aqueous/organic interface by an irreversible binding reaction. This results in an inhibition of the enzyme which can then be related to pesticide concentration. An inhibition of 50% has been observed with $9 \cdot 10^{-7}$ M of paraoxon.

This method seems very promising as a biosensing test for screening pesticides in environmental samples. Specifically the use the two immiscible phases with an appropriate choice of organic solvent circumvents the intractable problem of the inactivation of cholinesterase enzymes by organic solvents. At the same time the pesticide analyte is introduced into the assay system in an organic phase like (or compatible) that resulting from the standard extraction methods. In this way the presence of heavy metals, or other water soluble enzyme inhibitors, that could interfere with the inhibition-based assay is largely avoided.

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