A flow-injection conductimetric system procedure is proposed for the determination of paraoxon. The method is based on the inhibition of immobilized acetylcholinesterase in controlled porous glass beads (CPG) with acetylcholine as substrate. The enzymatic reaction is inhibited by a diethyl p-nitrophenylphosphate (paraoxon) solution. A 1,1'-trimethylene-bis(4-formylpyridinium bromide)dioxime solution was used to reactivate the enzyme. The correlation between the peak height, for a given acetylcholine concentration, is linear from $1.0 \times 10^{-7}$ to $5.0 \times 10^{-5}$ mol $l^{-1}$ of paraoxon; therefore, the quantitative limit of detection was about $1.0 \times 10^{-8}$ mol $l^{-1}$ of paraoxon. For semi-quantitative purposes, the limit can be considered to be about $1.0 \times 10^{-7}$ mol $l^{-1}$. The relative estimated standard deviation obtained using a $1.0 \times 10^{-3}$ mol $l^{-1}$ acetylcholine solution and a $5.0 \times 10^{-6}$ mol $l^{-1}$ paraoxon solution was 2.2% ($n=8$).

Keywords Pesticide, acetylcholinesterase, acetylcholine, conductimetry, flow-injection, paraoxon

The enzyme acetylcholinesterase (AchE) has an important physiological role in the nerve function. In blood, the AchE is inhibited in varying degrees by numerous compounds. A large number of AchE irreversible inhibitors are known, such as organophosphates, carbamates, sulfonic acid esters.¹ The reactivation of AchE after the action of various irreversible inhibitors has been examined²–⁵ in analytical chemistry, and clinically used in cases of poisoning.

Organophosphorus pesticides are potent inhibitors of the AchE. Due to their relatively low persistence in the environment⁶–⁷, they are compounds widely applied in agriculture. Considerable interest in the determination of these compounds has resulted in the development of various methods with different systems of detection. Several biosensors based on immobilized enzymes have been recently developed based on the inhibition of enzymatic activity.⁸–¹²

This paper describes a conductimetric flow injection (FI) system for the determination of diethyl-p-nitrophenylphosphate (paraoxon). The method was based on the generation of acetic acid by the reaction of acetylcholine with the enzyme cholinesterase. This enzyme was chemically immobilized on controlled porosity glass beads (CPG). A gas-diffusion membrane¹³ permitted permeation of the acetic acid formed. The relation between the concentration of acetylcholine and the conductimetric response was used to measure the enzymatic activity.¹⁴ The inhibition of the immobilized cholinesterase was done by introducing in the system a given concentration of paraoxon solution and observing the decrease of the reaction product as a decrease in the signal height, as a result of the decrease in the quantity of acetic acid formed. This relationship may be used for the quantitative determination of the pesticide. For reactivation of the immobilized enzyme, 1,1'-trimethylene-bis(4-formylpyridinium bromide) dioxime (TMB-4) was used.²,⁵

This method does not require long periods of waiting between acetylcholinesterase and pesticide introductions in the system. The reactivation of cholinesterase by TMB-4 is rapid and also complete. Gas-diffusion is a very selective technique because only a few species are sufficiently volatile at room temperature to permeate the membrane. The FI system proposed is low in cost, easy to operate and requires only small quantities of reagents. The enzymatic reactor can be used at least for more than one hundred determinations without observable lost of the enzyme activity.

Similar systems have been used previously for the determination of volatile acidity, mainly due to acetic acid, in spoiled beer¹⁵, vinegar¹⁶ and wine.¹⁷

Experimental

Materials

Acetylcholinesterase (E.C.3.1.1.7, type VI-S, from
electric eel, 1000 U mg\(^{-1}\)); acetylcholine chloride; diethyl-p-nitrophenylphosphate (paraoxon), 95%; and 1,1'-trimethylene-bis(4-formylpyridinium bromide) dioxime (TMB-4) were obtained from Sigma (St. Louis, MO). All other reagents were analytical grade chemicals. Acetylcholine and TMB-4 solutions were prepared in 0.1 mol l\(^{-1}\) phosphate buffer (pH=8.0). Acetylcholine solutions were prepared daily. Stock solutions of paraoxon (6.0\(\times\)10\(^{-3}\) mol l\(^{-1}\)) were prepared in acetone. Standard solutions were prepared by dilution of the stock solution with phosphate buffer. All stock solutions were stored in a refrigerator at 4° C. The water used in experiments was first distilled in a glass distillator and then deionized in a Milli-Qplus system. The silanization and the subsequent immobilization of the enzyme on the CPG were performed as described earlier\(^{13}\) and stored in phosphate buffer, at 4° C.

**Apparatus**

**Peristaltic pump:** Ismatec mp13 GJ4.

**Conductimeter:** Micronal, Model B-331, connected to a chart recorder.

**Gas diffusion cell:** This cell has been described previously\(^{18}\) and is similar to that of van der Linden.\(^{13}\)

**Conductimetric cell:** The conductimetric cell, which has a volume of about 42 µl, has been already described.\(^{19}\) The constant of this cell is 0.20 - 0.25 cm\(^{-1}\).

**Sampling valve:** This sampling valve has been described previously.\(^{20}\)

**Method**

The scheme of the flow injection system used is shown in Fig. 1. The sample of acetylcholine (S) is introduced into the carrier stream (A1), a 0.1 mol l\(^{-1}\) phosphate buffer at pH 8.0, which is pumped by the peristaltic pump (P), at a flow rate of 1.19 ml min\(^{-1}\). It passes through the enzymatic reactor (ER) in a polyethylene tube (3.5 cm long and 3.0 mm in internal diameter). In sequence, the solution is mixed with the 1.0 mol l\(^{-1}\) sulfuric acid stream (A2) and passes through the diffusion cell (DC). Then, the acetic acid that diffuses through the PTFE membrane is carried out by the deionized water stream (A3) to the conductimetric cell (C). Paraoxon solutions and 5.0\(\times\)10\(^{-6}\) mol l\(^{-1}\) TMB-4 in phosphate buffer, pH 8.0, are also sampled in S and introduced into flow A1 by the sample valve (V).

**Results and Discussion**

Figure 2 shows an FI curve of the enzyme inhibition by the pesticide paraoxon and of the regeneration of the enzymatic activity; here determinations were done in triplicate, and an injection volume of 100 µl was used. The peaks “a” correspond to triplicate signals of a 1.0\(\times\)10\(^{-3}\) mol l\(^{-1}\) acetylcholine solution. The “b” region is related to the introduction of the 5.0\(\times\)10\(^{-6}\) mol l\(^{-1}\) paraoxon solution, when a signal is not observed; “c” is the peaks obtained with a 1.0\(\times\)10\(^{-3}\) mol l\(^{-1}\) acetylcholine solution after the inhibition of the enzymatic column with the paraoxon solution; “d” is the region that corresponds to the introduction of a 5.0\(\times\)10\(^{-5}\) mol l\(^{-1}\) TMB-4 solution in order to regenerate the enzyme; “e” is the triplicate peaks of the 1.0\(\times\)10\(^{-3}\) mol l\(^{-1}\) acetylcholine solution obtained after the regeneration of the enzyme. It can be easily observed that the reactivation of the enzyme occurs rapidly and completely.

A typical calibration curve for paraoxon with a linear correlation from 1.0\(\times\)10\(^{-3}\) mol l\(^{-1}\) to 1.0\(\times\)10\(^{-1}\) mol l\(^{-1}\) is presented in Fig. 3. The curve follows the equation

\[ h=1.124\times10^{0}C+1.661 \]

with a correlation coefficient of 0.9987. Here, h represents the difference between the peak height (cm) before and after the inhibition of the enzyme by the pesticide and C the acetylcholine concentration (mol l\(^{-1}\)). As can be observed in Fig. 2, the recovery of the enzymatic activity is easily performed with a 5.0\(\times\)10\(^{-5}\) mol l\(^{-1}\) TMB-4 solution. However, for more concentrated paraoxon solutions, more concentrated TMB-4 solutions are necessary.

The relative estimated standard deviation (s=±2.2%) was determined for eight (n=8) determinations for a 1.0\(\times\)10\(^{-3}\) mol l\(^{-1}\) acetylcholine solution with a 5.0\(\times\)10\(^{-4}\)
The quantitative limit of detection can be considered as about $1.0 \times 10^{-8}$ mol l$^{-1}$ of paraoxon. However, for semi-quantitative purposes concentrations about $1.0 \times 10^{-8}$ mol l$^{-1}$ can be detected.

**FIA optimization system**

In order to optimize the method, some important analytical parameters, such as total flow-rate, enzymatic reactor temperature, sample volume and substrate concentration, were studied.

Table 1 shows the effect of the flow rates ($F_{RA1}=F_{RA2}=F_{RA3}$) on the peak heights and on the difference between the peak heights before and after the inhibition of the enzyme with paraoxon. The height (A) (without paraoxon), shows a great increase with the decreasing of the flow-rate. The height (B) (after inhibition with paraoxon) also shows an increasing with the decreasing in the flow rate. These increases can be attributed to two principal causes. One is related with the increasing of the time of contact between the acetylcholinesterase and the substrate. The other cause is the increasing of the proportion of the acetic acid formed that diffuses though the membrane, as diffusion depends on the time that the donor solution remains in the permeation cell.

In the height (B) the factor of increase from the flow 2.79 ml min$^{-1}$ ($h=2.3$ cm) to the flow 1.19 ml min$^{-1}$ ($h=4.1$ cm) is 1.78. Comparatively the factor of increase of the height (A) related to the same flows is $12.1$ cm/4.8 cm = 2.52. The smaller relative increase in the height (B) with respect to the decrease of the flow-rate can be attributed to the fact that the great part of the reaction sites on the enzyme are blocked by the paraoxon. As a consequence, the augmentation of the residence time does not result in much more reaction as the concentrations of the sites are relatively small.

**Fig. 2**  Paraoxon inhibition of cholinesterase and reactivating effect with $5.0 \times 10^{-6}$ mol l$^{-1}$ TMB-4 solution. From left to right: (a) triplicate signals for the $1.0 \times 10^{-4}$ mol l$^{-1}$ acetylcholine solution; (b) introduction of the $5.0 \times 10^{-6}$ mol l$^{-1}$ paraoxon solution (one introduction); (c) triplicate signals for $1.0 \times 10^{-3}$ mol l$^{-1}$ acetylcholine solution after the inhibition of the cholinesterase; (d) introduction of the $5.0 \times 10^{-3}$ mol l$^{-1}$ TMB-4 solution (one introduction); (e) triplicate signals for $1.0 \times 10^{-3}$ mol l$^{-1}$ acetylcholine solution, after the reactivation of the enzyme with TMB-4. Polyethylene sampling loop, 100 µl (3.0 mm i.d.). Conductance: $1$ cm = 8 µS.

**Fig. 3**  Calibration curve for paraoxon in the range of $1.0 \times 10^{-5}$ mol l$^{-1}$ to $1.0 \times 10^{-4}$ mol l$^{-1}$, described by the equation $h=1.124 \times 10^6 C+1.661$ with a correlation coefficient of 0.9987. For this equation, $h =$ difference of peak height (cm) before and after the enzymatic inhibition by the pesticide and $C=$acetylcholine concentration (mol l$^{-1}$). The reactivation of the cholinesterase was made with a $5.0 \times 10^{-5}$ mol l$^{-1}$ TMB-4 solution. Polyethylene sampling loop, 100 µl (1 mm i.d.), in all cases. Conductance: $1$ cm = 8 µS.

![Graph showing calibration curve for paraoxon](image)

**Table 1** Effect of the flow-rates ($F_{RA1}=F_{RA2}=F_{RA3}$) for determination of paraoxon

<table>
<thead>
<tr>
<th>Flow-rate/ ml min$^{-1}$</th>
<th>Height (A)$^a$/ cm</th>
<th>Height (B)$^b$/ cm</th>
<th>(A)=(B)$^c$/ cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.19</td>
<td>12.1</td>
<td>4.1</td>
<td>8.0</td>
</tr>
<tr>
<td>1.70</td>
<td>7.2</td>
<td>2.4</td>
<td>4.8</td>
</tr>
<tr>
<td>2.19</td>
<td>5.9</td>
<td>2.7</td>
<td>3.2</td>
</tr>
<tr>
<td>2.79</td>
<td>4.8</td>
<td>2.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Temperature=25°C. 1 cm = 8 µS.

a. $1.0 \times 10^{-3}$ mol l$^{-1}$ acetylcholine before the enzyme inhibition by paraoxon $5 \times 10^{-6}$ mol l$^{-1}$.

b. $1.0 \times 10^{-3}$ mol l$^{-1}$ acetylcholine after the enzyme inhibition by paraoxon $5 \times 10^{-6}$ mol l$^{-1}$.

c. Height (A)$^a$-Height (B).
with respect of that of acetylcholine. In the case of the height (A), the increase in the signal in function of the decrease of the flow-rate can be easily understood in terms of the increase of the reaction performance as the concentration of the sites of the enzyme is relatively high with respect to the acetycholine concentration. The compromise between (A) and (B), that is (A)-(B), leads to a significant increase at lower flows.

In Table 2 is shown the effect of the temperature of the enzymatic column, from 20 up to 42°C, on the signal height. It can be easily observed that the non-inhibited enzyme is quite influenced by temperature. The height (A) continuously increases until 37°C. At 42°C, a small decreasing is observed, probably indicating thermal denaturation of the enzyme. This supposition was reinforced by the fact that, after the heating at 42°C the lost activity was not recovered at lower temperatures. Contrasting with the non-inhibited enzyme, the activity of the inhibited can be considered temperature independent in the range studied. The association of the two facts leads to an increasing of the difference (A)-(B) with the increase of the temperature, with a maximum at 37°C.

Table 3 shows the effect of the acetylcholine concentration on the signal height in both the non-inhibited (height A) and in the inhibited enzyme (height B). It can be observed that in the two cases a significant increase occurs. Going from $0.2 \times 10^{-4}$ to $8.0 \times 10^{-4}$ mol l$^{-1}$ of acetylcholine the augmentation of the signal is higher in (A) (increases 6.85 times) than in (B) (increases 5.2 times). This leads to an increasing in the difference (A)-(B). This phenomenon can be understood in a similar way as explained in the above comments of Table 1 with the difference that in this case the variation of the concentration occurs and there is variation of the residence time as a consequence of the flow-rate.

In Table 4 are presented the data obtained with the increase of the volume of the sampling loop. The concentrations of the acetylcholine, of the paraoxon and of TMB-4 are constant and are described in the table. The variation of the volume introduced in the system does not change the concentrations, but only the quantity of each compound, i.e., the number of moles. Increasing the volume from 70 µl up to 160 µl the signal height (A) of the non-inhibited enzyme increases continuously. Observing the height (B), it can be seen that the signal does not decrease more after 100 µl of injected solution of paraoxon, i.e., the maximum of inhibition is achieved at this volume. Therefore the difference (A)-(B) is favored with the increasing of the volume of the sampling loop that is the same for all the solutions introduced in the system.

From the results obtained in this work, it can be considered that the method is quite satisfactory for the quantitative analysis of paraoxon in the proposed concentration range. About 10 determinations can be performed per hour. The enzymatic reactor can be at least used for more than one hundred times for concentrations ranging from $1.0 \times 10^{-5}$ to $1.0 \times 10^{-7}$ mol l$^{-1}$ of paraoxon, without decreasing of the enzyme activity.
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References


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