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Flow-Injection Spectrophotometric Determination of Paraoxon by Its Inhibitory Effect on the Enzyme Acetylcholinesterase

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A spectrophotometric enzymatic flow injection (FI) system for the determination of diethyl-*p*-nitrophenylphosphate (paraoxon) is proposed. The method was based on the determination of the acetic acid formed by the enzymatic reaction of the acetylcholinesterase, immobilized on glass beads, with the substrate acetylcholine. The acetic acid formed permeates through a PTFE membrane and is received by a solution (pH 7.0) containing the acid-base indicator Bromocresol Purple (B. C. P.), leading to a pH change and therefore to a color change. The variation of the absorbance of the solution is detected spectrophotometrically at 400 nm. The determination of paraoxon is related to its inhibitory action on the enzyme. Therefore the analytical signal is the difference between the signal that corresponds to the free and the one that corresponds to the inhibited enzyme, considering a fixed acetylcholine concentration. The correlation between the peak height and paraoxon concentration at a given acetylcholine concentration is linear in the range from 5.0 $\times 10^{-7}$ mol L^{-1} to 5.0 $\times 10^{-5}$ mol L^{-1} (r = 0.998) of paraoxon, with a relative estimated standard deviation (R.S.D.) of ±1.7% (n = 10) considering a solution containing 5.0 $\times 10^{-6}$ mol L^{-1} of paraoxon and a solution containing 5.0 $\times 10^{-3}$ mol L^{-1} of acetylcholine. Therefore, the quantitative limit detection is about 2.5 $\times 10^{-7}$ mol L^{-1} of paraoxon (3σ). A 1,1′-trimethylene-bis(4-formylpyridinium bromide)dioxime (TMB-4) solution was used to reactivate the enzyme.

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Insecticides, fungicides, herbicides, *etc*. are compounds employed in the agriculture to control pests. They are applied on foods during the production process, stock, transport and sale, before and/or after the harvest to decrease deterioration. Some of them are employed as regulating agents of the growth of plants and as agents to prevent the premature ripeness of the fruits.[1]

Organophosphorus compounds are a generation of pesticides that are very rapidly decomposed by the action of water and by the sunlight. However, considering that they are very soluble in water, the mechanism of their action, their high level of toxicity and their widespread use in agriculture, they can represent a real danger for the equilibrium of the ecosystems.[2-6] Therefore it is necessary to monitor continuously and carefully such kinds of compounds in the environment. Several analytical procedures have been developed for the determination of the organophosphorus pesticides based on the acetylcholinesterase inhibition, using different methods of detection depending on the substrate.[7-13]

A frequent analytical problem is the irreversible inhibition of an immobilized enzyme and the gradual loss of the activity of the enzymatic reactor that becomes useless after few analyses. Different nucleophilic agents have been used in some procedures for the acetylcholinesterase reactivation.[14-16] TMB-4, 1,1′-trimethylene-bis(4-formylpyridinium bromide)dioxime, was shown to be an excellent regenerator of the enzyme.[17]

This paper describes a spectrophotometric flow injection (FI) system for the determination of diethyl-*p*-nitrophenylphosphate (paraoxon). The principle of the method is based on the irreversible inhibition caused by the pesticide of acetylcholinesterase immobilized on controlled porous glass beads (CPG).[18] The acetic acid generated by the reaction of the substrate acetylcholine with the enzyme permeates through a PTFE gas-diffusion membrane[19-22] into a solution (pH 7.0) of Bromocresol Purple (BCP).[20] The change of the intensity of the color due to the presence of acetic acid was measured spectrophotometrically. The relation between the concentration of the acetic acid formed and the spectrophotometric response was used to measure the enzymatic activity. After the introduction of a given concentration of paraoxon solution in the system, one observes a decrease of the peak height. This means that there has been a decrease of the concentration of the acetic acid formed, a fact that is correlated with the extent of the inhibition of the enzyme that, in turn, is proportional to the paraoxon concentration. The quantitative determination of the pesticide can be done by considering this decrease in the peak height. For the reactivation of the immobilized enzyme, a TMB-4 solution was used.[17]

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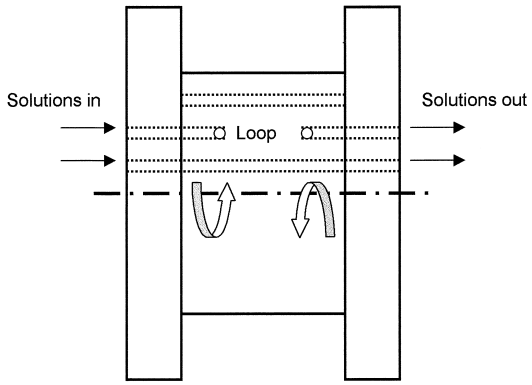

Fig. 1 Schematic design of the sampling valve.

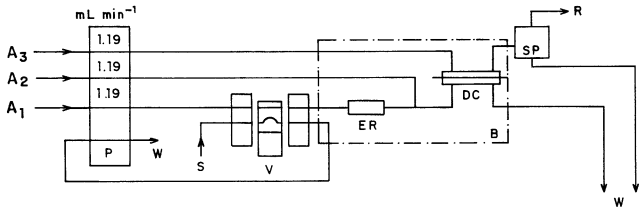

Fig. 2 Flow injection system: A_1, 0.1 mol L^{-1} phosphate buffer stream (pH 8.0); A_2, 1.0 mol L^{-1} sulfuric acid solution; A_3, 1.0×10^{-4} mol L^{-1} BCP solution stream (pH 7,0); S, sample; ER, enzymatic reactor; DC, diffusion cell; V, sampling valve system; B, water-bath; SP, spectrophotometer set at 400 nm; R, recorder.

Experimental

Reagents and solutions

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 were stored in a refrigerator at 4˚C.

Bromocresol Purple (BCP) solution (1.0×10^{-4} mol L^{-1}) was prepared by dissolving 0.27 g of BCP in 10 mL of ethanol, the volume being diluted to 500 mL with the phosphate buffer; a 50.0 mL volume of this solution was diluted to 500 mL with the phosphate buffer to obtain the working solution. The pH was adjusted to 7.0 by dropwise addition of dilute NaOH solution. In order to avoid absorption of CO_2 from the air, the BCP solution was kept in a bottle protected by a tube containing $CaCl_2$–NaOH–$CaCl_2$ (respite). This solution was pumped to the FI system through another tube.

The water used to prepare the solutions was first distilled in a glass distillator and then deionized in a Milli-Qplus system, and always boiled (degassed).

The silanization of the surface of the glass beads and the immobilization of the enzyme on the CPG[18] were performed in the following way: to 0.2 g of CPG were added 10 mL of a solution of 5% nitric acid and the mixture was boiled for 30 min. Then the CPG was separated by filtration on a sintered-glass filter, washed with deionized water and dried in an oven at 95˚C. This dry CPG was added to a solution prepared with 1.0 mL of 3-aminopropyltriethoxysilane and 9.0 mL of water (the pH was adjusted to 3.45 by gently dropping 5 mol L^{-1} of hydrochloric acid). The mixture was heated at 75˚C in a water bath for 150 min. Every 15 min the system was gently swirled. The resulted alkylaminated CPG was filtered off, washed with deionized water and then dried at 95˚C. The alkylamination process was repeated to ensure complete activation of the glass. The cross linking-linking agent, 2.5% glutaraldehyde, was prepared in the following way: a 2.5 mL volume of 50% glutaraldehyde solution was diluted to 50 mL with 0.1 mol L^{-1} phosphate buffer (pH = 7). In a flask 1 mL of this was added to 0.2 g of the alkyamino-CPG. The reaction, under swirling, was allowed to proceed for 1 h at room temperature. The resultant activated-CPG was filtered off and washed three times with deionized water. The acetylcholinesterase (1.0 mg – 1000 U) was dissolved in 1.0 mL of phosphate buffer (0.1 mol L^{-1}, pH = 8.0) at 4˚C and added onto this last CPG. The system was allowed to stay for 150 min, then it was filtered and washed with the phosphate buffer. The immobilized enzyme on CPG was stored in a phosphate buffer solution (0.1 mol L^{-1}; pH = 8.0) in a refrigerator at 4˚C.

Apparatus

Peristaltic pump: Ismatec mp13 GJ4. Spectrophotometer: single-beam Carl Zeiss Model PM2D set at 400 nm, connected to a chart recorder. A quartz flow cuvette with 1.00 cm pathlength was used. Gas diffusion cell: Similar to the cell that has been described by van der Linden.[19] Sampling valve: This sampling valve has been described previously in detail.[20] As can be seen from Fig. 1, it essentially consists of three round pieces of “black PTFE” tightly connected through their centers by a screw. Holes of about 1.8 mm in diameter are the paths for the solutions and also the connections for the polyethylene tubes (2.0 mm o.d.) that conduct these solutions to and from the valve. Two of the pieces of “black PTFE” are fixed on a metallic base and the third (on which is placed a small plastic tube that is the sampling loop of constant volume) is located between the other two as a sandwich. This central piece can be moved in a teeter movement around its axis. This movement allows the introduction of the sample and/or the reagent in an adequate flow. A thermostatic bath Lauda Model RCS RC6 was used to study the influence of the temperature on the enzymatic column.

Flow-injection system

The scheme of the flow injection system used is shown in Fig. 2. The sample of acetylcholine (S) is introduced into the carrier stream (A_1) that is a 1.0×10^{-1} mol L^{-1} phosphate buffer (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 a 1.0 mol L^{-1} sulfuric acid stream (A_2). The acetic acid formed passes through the PTFE membrane in the diffusion cell (DC) and is carried out by BCP solution 5.0×10^{-5} mol L^{-1} in pH = 7.0 (A_3) to the spectrophotometer (SP). Paraoxon solutions and the 5.0×10^{-4} mol L^{-1} TMB-4 solution in phosphate buffer (pH = 8.0) are also sampled in S and introduced into the flow A_1 by the sample valve (V). The sampling loop of 100 μL was made of polyethylene tube (1.0 mm i.d.). The temperature was controlled at 25.0 ± 0.5˚C by the bath B, which is a continuous filled tap water-bath.

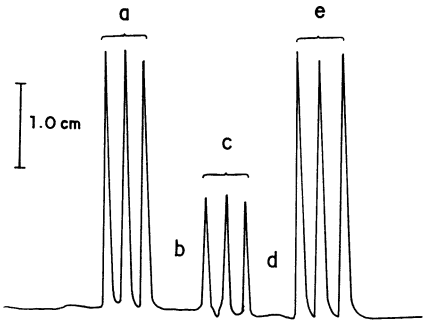

Fig. 3 Profile of the analytical signals for the determination of the pesticide before and after inhibition by paraoxon and the reactivation of the acetylcholinesterase by a TMB-4 solution. From the left to right: (a) triplicate signals of the 1.0×10^{-3} mol L^{-1} acetylcholine before the inhibition by paraoxon; (b) absence of signal after the introduction of 5.0×10^{-6} mol L^{-1} paraoxon solution (triplicate); (c) triplicate signals of the 1.0×10^{-3} mol L^{-1} acetylcholine solutions after the inhibition with paraoxon; (d) introduction of 5.0×10^{-5} mol L^{-1} TMB-4 solution; (e) triplicate signals for 1.0×10^{-3} mol L^{-1} acetylcholine solution after the reactivation of the enzyme with TMB-4. Polyethylene sampling loop, 100 μL (1.0 mm i.d.).

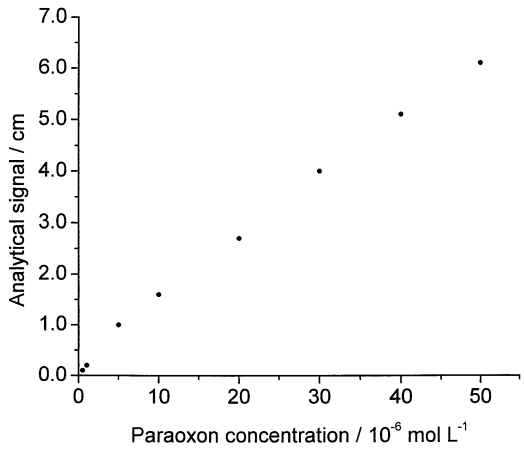

Fig. 4 Calibration curve for the spectrophotometric determination of paraoxon. Polyethylene loop of 100 μL (1.0 mm i.d.). Temperature = 25°C. Flow-rate, (FR): $FR_{A1} = FR_{A2} = FR_{A3} = 1.19$ mL min^{-1}.

Results and Discussion

Figure 3 shows the FI spectrophotometric profile curve of the inhibition of the acetylcholinesterase by 5.0×10^{-6} mol L^{-1} paraoxon solution and of the reactivation of the enzyme by 5.0×10^{-5} mol L^{-1} TMB-4 solution.

The peaks **a** show, in triplicate, the signal correspondent to the 1.0×10^{-3} mol L^{-1} acetylcholine solution before the inhibition by paraoxon, using a sampling loop of 100 μL (1.0 mm i.d.). The region **b** is related to the introduction of 5.0×10^{-6} mol L^{-1} paraoxon solution and indeed no signal was observed. When the paraoxon solution was injected, one found the inhibition of the enzyme due to the interaction of the pesticide with the active centers of the enzyme, as do numerous inhibitors of the acetylcholinesterase that form an enzyme-inhibitor complex. Thus when the 1.0×10^{-3} mol L^{-1} acetylcholine solution is injected again, in region **c** was observed a decrease in the peak height as a consequence of the decreasing of the population of the active sites of the enzyme. The region **d** corresponds to the introduction of a 5.0×10^{-5} mol L^{-1} TMB-4 solution. This stage, consists of the reactivation of the enzyme with the liberation of the active sites from the inhibitor. The pesticide binds more strongly with TMB-4 than with acetylcholinesterase; therefore the enzyme is regenerated. The TMB-4 does not cause any signal when it is injected in the system. Finally stage **e** confirmed the regeneration of acetylcholinesterase, when a 1.0×10^{-3} mol L^{-1} acetylcholine solution was injected in triplicate experiments. The heights of the peaks observed after the reactivation of the enzymatic activity were of the same intensity as before the inhibition of the enzyme. Then the responses obtained show that the regeneration of acetylcholinesterase occurs completely and rapidly for these experimental conditions.

A typical FI calibration curve (Fig. 4) for the spectrophotometric determination of paraoxon, in the range of concentration 5.0×10^{-7} mol L^{-1} to 5.0×10^{-5} mol L^{-1}, using a polyethylene sampling loop of 100 μL (1.0 mm i.d.), can be represented by the equation:

$$h' = 0.24 + 1.21 \times 10^5 C$$

with a correlation coefficient of $r = 0.998$ ($r^2 = 0.996$). In the equation, h' represents the difference between the peaks height ($h_1 - h_2$) in cm (1.0 cm = 8 mV = 0.008 absorbance) before (h_1) and after (h_2) the enzymatic inhibition by the pesticide. C represents the paraoxon concentration (mol L^{-1}). This equation reproduces very well the analytical signal beyond the concentration 5.0×10^{-6} mol L^{-1}. However, below this concentration the predicted signals are a little higher than the experimental ones. Observing attentively the experimental values one will notice that the analytical calibration curve can be dissociated into two curves, one correlating the data above the concentration 5.0×10^{-6} mol L^{-1} ($h' = 0.45 + 1.15 \times 10^5 C$, $r = 0.999$, $r^2 = 0.998$) and the other below ($h' = 2.0 \times 10^5 C$, $r = 1.000$, $r^2 = 1.000$). Mainly in low concentrations it is recommended to consider the calibration curve constructed in each specific range.

The relative standard deviation (R.S.D.) was measured by using a 5.0×10^{-3} mol L^{-1} acetylcholine solution, a 5.0×10^{-6} mol L^{-1} paraoxon solution and a 5.0×10^{-3} mol L^{-1} TMB-4 solution. An R.S.D. of ±1.7% ($n = 10$) was obtained.

The method showed that a quantitative limit of detection of paraoxon of 2.5×10^{-7} mol L^{-1} can be achieved (3σ). About 10 analyses can be performed in 1 h. The same enzymatic reactor can be used to perform more than 100 analyses without loss of activity.

FI system optimization

For the optimum performance of the system, some analytical parameters were studied, using the flow system shown in Fig. 2. The variables studied were the enzyme reactor temperature, the flow rate of the system, the loop volume and the substrate concentration.

The effect of the enzymatic reactor temperature was examined by using a thermostatic water bath. The temperature of the reactor was kept constant within ±0.05°C during the analyses.

In Table 1 is shown the effect of the temperature on the enzyme activity from 20 up to 42°C. Table 1 shows that the height h_1 continuously increases with the increase of the temperature until 37°C and decreases a little after this temperature. The height h_2 slowly decreases with the augmentation of the temperature. As a consequence an

Table 1 Effect of the temperature on the enzymatic column

Temperature/°C	h_1[a]/cm[c]	h_2[b]/cm	$h_1 - h_2$/cm
20	2.8	1.7	1.1
25	3.2	1.7	1.5
30	3.4	1.6	1.8
32	3.6	1.6	2.0
37	3.7	1.6	2.1
42	3.5	1.5	2.0

a. h_1, analytical signal height: 1.0×10^{-3} mol L^{-1} acetylcholine solution before the enzyme inhibition by 5.0×10. mol L^{-1} paraoxon solution.
b. h_2, analytical signal height: 1.0×10^{-3} mol L^{-1} acetylcholine solution after the enzyme inhibition by 5.0×10^{-6} mol L^{-1} paraoxon solution.
c. 1.0 cm = 8 mV = 0.008 (absorbance). Flow-rate (FR): $FR_{A1} = FR_{A2} = FR_{A3} = 1.19$ mL min^{-1}.

Table 2 Effect of the flow-rate (FR) on analytical signal heights

Flow-rate/mL min^{-1}	h_1[a]/cm[c]	h_2[b]/cm	$h_1 - h_2$/cm
1.19	4.2	1.7	2.5
1.70	2.8	1.3	1.5
2.19	2.5	1.2	1.3
2.79	2.2	1.0	1.2

a. h_1, analytical signal height: 1.0×10^{-3} mol L^{-1} acetylcholine solution before the enzyme inhibition by 5.0×10^{-6} mol L^{-1} paraoxon solution.
b. h_2, analytical signal height: 1.0×10^{-3} mol L^{-1} acetylcholine solution after the enzyme inhibition by 5.0×10^{-6} mol L^{-1} paraoxon solution.
c. 1.0 cm = 8 mV = 0.008 (absorbance). Flow rate (FR): $FR_{A1} = FR_{A2} = FR_{A3}$. Temperature = 25°C.

Table 3 Effect of the loop volume on the signal heights

Volume/μL	h_1[a]/cm[c]	h_2[b]/cm	$h_1 - h_2$/cm
70	2.2	1.6	0.6
100	2.8	1.7	1.1
125	3.3	1.8	1.5
160	3.6	1.8	1.8

a. h_1, analytical signal height: 1.0×10^{-3} mol L^{-1} acetylcholine solution before the enzyme inhibition by 5.0×10^{-6} mol L^{-1} paraoxon solution.
b. h_2, analytical signal height: 1.0×10^{-3} mol L^{-1} acetylcholine solution after the enzyme inhibition by 5.0×10^{-6} mol L^{-1} paraoxon solution.
c. 1.0 cm = 8 mV = 0.008 (absorbance). Temperature = 25°C. Flow-rate (FR): $FR_{A1} = FR_{A2} = FR_{A3} = 1.19$ mL min^{-1}.

Table 4 Effect of the concentration of acetylcholine on determination of paraoxon

Substrate/mol L^{-1}	h_1[a]/cm[c]	h_2[b]/cm	$h_1 - h_2$/cm
1.0×10^{-3}	2.3	1.3	1.0
2.0×10^{-3}	2.6	1.4	1.2
4.0×10^{-3}	3.1	1.4	1.5
5.0×10^{-3}	3.3	1.6	1.7
6.0×10^{-3}	3.4	1.6	1.8
8.0×10^{-3}	3.5	1.6	1.9

a. h_1, analytical signal height: after the introduction of the paraoxon solution.
b. h_2, analytical signal height: after the enzyme inhibition by 5.0×10^{-6} mol L^{-1} paraoxon solution.
c. 1.0 cm = 8 mV = 0.008 (absorbance). Temperature = 25°C. Flow-rate (FR): $FR_{A1} = FR_{A2} = FR_{A3} = 1.19$ mL min^{-1}.

increasing of the difference $h_1 - h_2$ is observed; it shows the higher value at 37°C, decreasing a little after this temperature. The decreasing of the signals over 37°C probably indicates that the denaturation of the enzyme is beginning in this temperature. Coherently equivalent results were obtained with conductimetric detection.[13] If one considers the intensity of the signal, the temperature of 37°C is the best for the analytical purposes, but from the point of view of the operational simplicity the ambient temperature, 25°C, was adopted for the work.

The effect of flow-rate on the paraoxon determination was examined in the range from 1.19 to 2.79 mL min^{-1}. Table 2 shows that the heights h_1 and h_2 decrease with the increasing of the flow-rate. The changes in the signal heights due to change in the flow-rate can be understood as results of changes in the contact time of the enzyme with the substrate as well as with the inhibitor and with the reactivator (TMB-4). The decrease in the peaks height observed, related with the augmentation of the flow-rate, can be mainly attributed to the decrease of residence time of the substrate in the enzymatic column. On the contrary, an increase of the contact time between the substrate and the enzyme causes an increase of the quantity of the reaction product and therefore an increase of the signal height. Considering the reactivation of the enzyme where the contact with the reactivator is necessary to remove the inhibition, one concludes that the increase of the contact time with the inhibited enzyme causes a better regeneration of the enzymatic activity. One other important factor, which is dependent on the flow-rate of the system, is the rate of permeation of the acid through the PTFE membrane. The increase of the time of permeation, as a consequence of the decrease of the flow-rate of the system, leads to more acetic acid diffusing through the membrane and therefore to higher signals.

In Table 2 can be observed the h_1, h_2 and $h_1 - h_2$ heights, correspondent respectively to the signals of acetylcholine before the inhibition by paraoxon, after the paraoxon inhibition and to the difference between them in function of the flow-rate of the system. It can be easily observed that the most favorable condition corresponds to the smaller flow-rate where the compromises among the different variables discussed above lead to the best signal. Lower flow-rates could be used, but in this case the number of analyses that could be performed per hour (analytical frequency) will be very low.

The effect of the volume of the sampling loop from 70 to 160 μL on the absorbance signal was also studied. The variation of the volume of the loop implies the variation of the introduced volume of acetylcholine, of pesticide and of TMB-4 as the same introduction valve was always used. Table 3 shows that the signal $h_1 - h_2$ increases with the increase of the volume.

The effect of the concentration of the substrate on the analytical signal was also investigated. As can be observed in Table 4, the absorbance signal increased with the increase of the substrate concentration. The heights of the peaks h_1 increase continuously as a consequence of the concentration of acetylcholine from 1.0×10^{-3} mol L^{-1} to 8.0×10^{-3} mol L^{-1}. The heights of the peaks h_2 increased by the influence of the acetylcholine concentration from 1.0×10^{-3} mol L^{-1} to 5.0×10^{-3}

mol L^{-1}, then it remained constant up to 8.0×10^{-3} mol L^{-1}. Therefore, an increase in the difference $h_1 - h_2$ is observed with the increase in the concentration of acetylcholine.

From the results obtained we can consider that the proposed analytical system for determination of paraoxon is quite satisfactory for the quantitative analysis of paraoxon in the studied concentration range. The method shows good precision; only low-cost apparatus is required; very small volumes of the solutions of the reagents are used. The enzyme reactor can be used for more than 100 analyses without loss of activity and about 10 determinations can be performed per hour.

References

1. L. Larini, "*Toxicologia dos Inseticidas*", **1979**, Sarvier, São Paulo, 5.
2. A. C. Guyton, "*Tratado de Fisiologia Médica*", **1984**, Interamericana, Rio de Janeiro, 118.
3. M. D. Fernandes and M. L. S. Queiroz, *Immunopharm. Immunot*., **1999**, *21*, 621.
4. M. A. Brown and K. A. Brix, *J. Appl. Toxicol*., **1998**, *18*, 393.
5. G. A. Jamal, *Adverse Drug React. T. Reviews*, **1997**, *16*, 133.
6. D. L. Hunter and S. Padilla, *Toxicol. Method*., **1999**, *9*, 189.
7. G. A. Evtugyn, A. N. Ivanov, E. V. Gogol, J. L. Marty, and H. C. Budnikov, *Anal. Chim. Acta*, **1999**, *385*, 13.
8. W. R. Everett and G. A. Rechnitz, *Anal. Lett*., **1999**, *32*, 1.
9. C. Nistor and J. Emneus, *Waste Manage*., **1999**, *19*, 147.
10. D. Barceló and M. C. Hennion, *Anal. Chim. Acta*, **1995**, *318*, 1.
11. P. Mulchandani, A. Mulchandani, I. Kaneva, and W. Chen, *Biosensors Bioeletronics*, **1999**, *14*, 77.
12. N. A. Ivanov, G. A. Evtugyn, R. E. Gyurcsanyi, K. Tóth, and H. C. Budnikov, *Anal. Chim. Acta*, **2000**, *404*, 55.
13. T. C. Rodrigues, M. Tubino, O. E. Godinho, and G. Oliveira Neto, *Anal. Sci*., **1997**, *13*, 423.
14. C. M. Garcia, T. M. Munoz, and A. Townshend, *Anal. Chim. Acta*, **1994**, *295*, 287.
15. E. Hellstrom-Lindahl, H. Moore, and Y. Nordberg, *J. Neurochem*., **2000**, *74*, 777.
16. H. P. M. van Helden, B. Groen, E. Moor, B. H. C. Westerink, and P. L. B. Bruijnzeel, *Drug Chem. Toxicol*., **1998**, *21 (suppl. 1)*, 171.
17. E. J. Poziomek, B. E. Hackley Jr., and G. M. Steinberg, *J. Org. Chem*., **1958**, *23*, 714.
18. M. E. Leon-Gonzalez and A. Townshend, *Anal. Chim. Acta*, **1990**, *236*, 267.
19. W. E. van der Linden, *Anal. Chim. Acta*, **1983**, *151*, 359.
20. M. Tubino and F. G. Barros, *Quim. Nova*, **1991**, *14*, 49.
21. O. E. Godinho, T. C. Rodrigues, M. Tubino, and G. Oliveira Neto, *Anal. Proc*., **1995**, *32*, 333.
22. M. Tubino and F. G. Barros, *J. Assoc. Off. Anal. Chem*., **1991**, *74*, 346.