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Determination of Organophosphorus and Carbamate Pesticides Using a Piezoelectric Biosensor

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A biosensor based on the quartz crystal microbalance has been developed for the determination of organophosphorus and carbamate pesticides. Detection is based on the inhibitory effects of these compounds on the activity of acetylcholinesterase immobilized on one of the faces of the crystal. Exposure of the immobilized enzyme to a solution of the histological substrate, 3-indolyl acetate, gives rise to the formation of an indigo pigment insoluble product that deposits (precipitates) on the crystal surface. The rate and extent of the enzymatic reaction can be followed in real time by measuring the frequency changes associated with the mass changes at the crystal surface induced by the accumulation of the enzymatic reaction product (indigo pigment). The presence of paroxon (organophosphorus pesticide) or carbaryl (carbamate pesticide) is detected by a diminution of the signal (frequency change) arising from their inhibitory effects. Calibration curves were constructed by plotting the percentage of inhibition vs the logarithm of the pesticide concentration. Detection limits of 5.0×10^{-8} and 1.0×10^{-7} M were obtained for paroxon and (after a 5-min preincubation) carbaryl, respectively.

As families, organophosphorus compounds and carbamates represent a large number of pesticides. Because of their low persistence and high effectiveness, they are widely employed in agriculture. Their mode of action has been ascribed to their ability to inhibit acetylcholinesterase activity.[1] These pesticides exhibit fairly high acute toxicity, so that sensitive, rapid, and reliable detection and determination of these toxic substances is very important for the protection of the environment and human health.

The determination of these molecules can be performed by various chemical methods,[2] with the most widely used being gas chromatography, liquid chromatography, and spectroscopy.[3−6] However, methods which can identify simple compounds in complex matrixes and reach detection limits compatible with those imposed by law for pesticide residues in the environment usually require tedious extraction and cleanup procedures prior to instrumental analysis and, thus, are not generally suitable for routine analysis.

Because they represent an alternative to the above-mentioned techniques, enzymatic methods based on the inhibition of acetylcholinesterase (AChE) activity by the action of organophosphorus and carbamate pesticides have been the object of intense investigation due to their sensitivity and specificity. In addition, their use can be coupled to various analytical techniques by the use of different substrates.[7−9] Biosensors based on AChE or cholinesterase (ChE) activities as molecular recognition systems for the detection of these compounds have been designed by combining the biological system with a variety of transducers, including potentiometric,[10−15] spectrophotometric,[16−18] voltammetric,[19] and piezoelectric.[20] In this way, in previous work[21] we demonstrated the determination of organophosphorus and car-

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(1) Corbett, I. R. *The Biochemical Mode of Action of Pesticides*; Academic Press: New York, 1974; p 540.

(2) McCully, K. A. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 401−405.

(3) Sherma, J. S. *Manual of Analytical Quality Control for Pesticides and Related Compounds in Human and Environmental Samples*; EPA-600/2-81-059; U.S. Environmetnal Protection Office, Office of Research and Development, Health Effects Research Laboratory: Research Triangle Park, NC, 1976.

(4) Pieper, G. R. *Bull. Environ. Contamin. Toxicol.* **1979**, *22*, 167−174.

(5) Ambrus, A.; Lantos, J.; Visi, B.; Cslatos, I.; Sarvari, L. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 1095−1099.

(6) Blama, J. J.; Jacksdn, P. J. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 733−768.

(7) Kovac, J.; Markova, V.; Králik, P. *J. Chromatogr.* **1974**, *100*, 171−174.

(8) Baum, G.; Ward, F. *Pestic. Chem. Proc. Int. Congr. Pestic. Chem., 2nd* **1971**, *4*, pp 215−225.

(9) Granmer, M.; Peolpes, A. *Anal. Biochem.* **1973**, *55*, 255−265.

(10) Durand, P.; Mallevialle, J.; Nicand, J. M. *J. Anal. Toxicol.* **1984**, *8*, 112−117.

(11) Tran-Minh, C. *Ion-Sel. Electrodes Rev.* **1985**, *7*, 41−45.

(12) Tran-Minh, C.; Pandey, P. C.; Kumaran, S. *Biosens. Bioelectron.* **1990**, *5*, 461−47.

(13) Kumaran, S.; Meir, H.; Danna A. M.; Tran-Minh, C. *Anal. Chem.* **1991**, *63*, 1914−1918.

(14) Kumaran, S.; Tran-Minh, C. *Anal. Biochem.* **1992**, *200*, 187−194.

(15) Rogers, K. R.; Foley, M.; Alter, S.; Koga, P.; Eldefrawi, M. *Anal. Lett.* **1991**, *24*, 191−198.

(16) Kindervater, R.; Kunnecke, W.; Schmid, R. D. *Anal. Chim. Acta* **1990**, *234*, 113−117.

(17) Leon-Gonzalves, M. E.; Townshend, A. *Anal. Chim. Acta* **1991**, *236*, 267−272.

(18) Rogers, K. R.; Cao, C. J.; Valdes, J. J.; Eldefrawi, A. T.; Eldefrawi, M. *Fundam. Appl. Toxicol.* **1991**, *16*, 810−820.

(19) Medyantseva, E. P.; Budnikov G. K.; Babkina, S. S. *Zh. Anal. Khim.* **1990**, *45*, 1386−1392.

(20) Guilbault, G. G.; Ngeh-Ngwainbi, J. In *Analytical Uses of Immobilized Biological Compounds for Detection, Medical and Industrial Uses*; Guilbault, G. G., Mascini, M., Eds.; NATO ASI Series C, Mathematical and Physical Sciences 226; Reidel: Dordrecht, The Netherlands, 1988; p 187.

(21) Pariente, F.; Hernández, L.; Lorenzo, E. *Anal. Chim. Acta* **1993**, *293*, 399−407.

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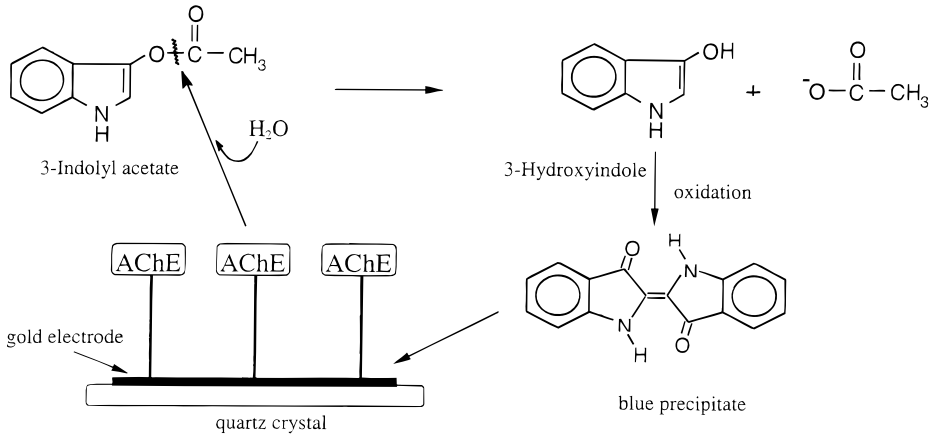
Scheme 1. Depiction of the Operating Principle of the QCM Biosensor for the Determination of Organophosphous and Carbamate Pesticides on the Basis of Their Inhibitory Effects on the Reaction of Immobilized Acetylcholinesterase (AChE) with 3-Indoyl Acetate To Yield the Isoluble Blue Indigo Dye Which Subsequently Precipitates on the Surface of the Quartz Crystal



bamate pesticides with a biosensor based on AChE using 4-aminophenyl acetate as substrate amenable for amperometric detection.

Recently, several sensor applications of the quartz crystal microbalance (QCM) have been reported, including the detection of toxins and bacteria,[22] immunoreactions,[23−28] DNA hybridization,[29−32] microbial growth,[33−35] and detection of herbicides.[36] This type of transducer can be used in the design of biosensors by immobilizing an enzyme such as AChE onto one of the faces of the crystal, which serves as the frequency-controlling element in an oscillator circuit. This device operates on the basis of changes in the resonant frequency of the crystal which can arise as a result of changes in the mass of the oscillator or properties such as thickness or elasticity of films present on the crystal surface.

We report here on the application of this method to follow the enzymatic activity of AChE, immobilized on one of the faces of the crystal and exposing the same to a solution containing a substrate (3-indoyl acetate, 3-IA) that is enzymatically converted to an insoluble product (indigo pigment) that deposits on the crystal surface, giving rise to frequency changes. The rate and extent of the enzymatic reaction can be followed, in real time, by measuring the frequency changes associated with the mass changes at the QCM surface induced by the enzymatic reaction product. Given that organophosphorus as well as carbamate pesticides inhibit AChE activity, the determination of acetylcholinesterase inhibitors such as paroxon or carbaryl can be carried out by following the diminution of the signal from levels found in their absence.

EXPERIMENTAL SECTION

Reagents. Acetylcholinesterase (EC 3.1.1.7, type III) from electric eel was purchased from Sigma as a solution containing 0.8 IU of enzymatic activity per microliter of solution and 5 mg of ammonium sulfate per milligram of protein. This enzyme preparation was stored frozen at −20 °C in small aliquots. The histological substrate, 3-indolyl acetate (3-IA; for chemical structure see Scheme 1), 97%, was purchased from Aldrich and was used as received. Stock solutions of 3-IA (50 mg/mL) were prepared immediately prior to use in dimethylformamide (DMF). Cystamine, bovine serum albumin (BSA, fraction V, 96% purity), and glutaraldehyde (grade I, 50% aqueous solution) were obtained from Sigma Chemical Co. and used as received. Glutaraldehyde was stored at −20 °C to prevent autopolymerization. Carbaryl and paroxon pesticides were obtained from Aragonesas S.A. (Madrid) and Aldrich, respectively, and stock solutions were prepared in ethanol. All other chemicals were of at least reagent grade and were used as received. Deionized water from Milli-Q and Milli-RO systems (Millipore) was used to prepare all solutions.

Apparatus and Procedures. AT-cut quartz crystals (5 MHz) of 24.5 mm diameter from Maxtek Co. were used in the QCM measurements. The crystals have both faces overtone-polished as well as gold electrodes deposited over a Ti adhesion layer. An asymmetric keyhole electrode format was used, with the side having the larger gold pad facing the solution. The area of the working side was 1.33 cm^2, whereas the area on the opposite side was 0.33 cm^2.

The quartz crystal resonator was set in a probe (TPS-550, Maxtek) made of Teflon, in which the oscillator circuit was

(22) Guilbault, G. G., Mascini, M., Eds. *Uses of Immobilized Biological Compounds*; Kluwer Academic Publisher: Dordrecht, The Netherlands, 1993; pp 377−385.
(23) Geddes, N. J.; Paschinger, E. M.; Furlong, D. N.; Ebara, Y.; Okahata, Y.; Than, K. A.; Edgar, J. A. *Sens. Actuators B* **1994**, *17*, 125−131.
(24) Muramatsu, H. J.; Dicks, M.; Tamiya, E.; Karube, I. *Anal. Chem.* **1987**, *59*, 2760−2763.
(25) Koblinger, C.; Drost, S.; Alberl, F.; Wolf, H.; Koch, S.; Woias P. *Biosens. Bioelectron.* **1992**, *7*, 397−404.
(26) Ebersole, R.; Ward, M. D. *J. Am. Chem. Soc.* **1988**, *110*, 8623−8628.
(27) Nakanishi, K.; Karube, I.; Hiroshi, S.; Uchida, A.; Ishida, Y. *Anal. Chim. Acta* **1996**, *325*, 73−80.
(28) Minunni, M.; Mascini, M.; Carter, R. M.; Jacobs, M. B.; Lubrano, G. J.; Guilbault, G. G. *Anal. Chim. Acta* **1996**, *325*, 169−174.
(29) Yamaguchi, S.; Shimomura, T.; Tatsuma, T.; Oyama, N. *Anal. Chem.* **1993**, *65*, 1925−1927.
(30) Su, H.; Kallury, K. M. R.; Thompson, M.; Roach, A. *Anal. Chem.* **1994**, *66*, 769−777.
(31) Su, H.; Thompson, M. *Biosens. Bioelectron.* **1995**, *10*(3/4), 329−340.
(32) Ito, K.; Hashimoto, K.; Ishimori, Y. *Anal. Chim. Acta* **1996**, *327*, 29−35.
(33) Fengjiao, H.; Wenhong, Z.; Quing, G.; Lihua, N.; Shouzhuo, Y. *Anal. Lett.* **1994**, *27*, 655−669.
(34) Nivens, D. E.; Chambers, J. Q.; Anderson, T. R.; White, D. C. *Anal. Chem.* **1993**, *65*, 65−69.
(35) Ebersole, R. C.; Foss, R. P.; Ward, M. D. *Bio/Technology* **1991**, *9*, 450−454.
(36) Guilbault, G. G.; Hock, B.; Schmid, R. *Biosens. Bioelectron.* **1992**, *7*, 411−419.

included. For the experiments, the probe was immersed in 50 mL of 0.1 M phosphate buffer solution (pH 8.0) contained in a water-jacketed cell connected to a thermostated bath (24.0 ± 0.1 °C). Frequencies were monitored with a Maxtek plating monitor (PM-740) that was interfaced to a personal computer.

Immobilization of AChE on the Quartz Crystals. The quartz crystals with deposited gold electrodes were cleaned by exposure to piranha solution (one part 30% H_2O_2 to three parts of concentrated H_2SO_4. ***Caution*****:** Piranha solution can react violently with organics!) for 15 min, rinsed with water, and stored in 1.0 M sulfuric acid. Prior to use, the crystals were washed with water and acetone and dried with a nitrogen stream. Immobilization of AChE was carried out according to two different methods. The first one is based on direct adsorption of the enzyme on the gold surface. For this purpose, 40 μL of 0.1 M phosphate buffer (pH 8.0) containing the desired amount of AChE was spread over the gold electrode surface and allowed to stand for 1 h at 4 °C. Afterward, the crystals were washed three times with phosphate buffer and dried in air at room temperature. In the second procedure, the AChE was covalently immobilized on the gold surface according to a procedure that we have previously described.[37] In this case, the crystals were incubated in an aqueous solution of cystamine for 1 h at room temperature, and thoroughly rinsed with water to remove all the nonchemisorbed material, and dried. The cystamine-functionalized crystals were subsequently immersed in a 10% (v/v) aqueous glutaraldehyde solution for 30 min. After the crystals were rinsed (2×) with buffer solution, 40 μL of AChE-containing buffer was spread on the functionalized gold surface and allowed to react for 2 h at 4 °C. Afterward, the remaining active carboxaldehyde groups were blocked by treating with 0.1 M glycine solution for 30 min at room temperature. The enzyme-modified crystals were rinsed with phosphate buffer and dried under a nitrogen stream.

The AChE-modified crystals were mounted in the QCM probe, and subsequently the assembled biosensor was immersed in buffer solution to prevent protein denaturation.

Determination of AChE Activity Using the QCM. The assembled AChE-QCM biosensor was immersed in 50 mL of 0.1 M pH 8 phosphate buffer and placed in the thermostated cell at 24.1 ± 0.1 °C. The frequency was monitored for a initial period of 10−20 min until the signal stabilized to ±1 Hz.

Once the response was stabilized, the enzymatic reaction was initiated by adding 150 μL of the substrate stock solution (3-IA, 50 mg/mL). Immediately following substrate addition, the solution was gently stirred with a magnetic bar for 1−3 min. As discussed below, the stirring time is important in order to obtain complete dissolution of the substrate in the aqueous reaction media (note that the 3-IA stock solution is in DMF). After stirring, a stabilization period of 10 s was allowed, and afterward the frequency was continuously monitored. Frequency changes (ΔF) were calculated as the difference between the frequency values obtained at any time (F_t) and the initial value (F_o) taken as the value after the 10-s waiting period. Time evolutions were obtained by plotting ΔF values in hertz versus time in minutes. For analytical measurements, experimental data were fitted to a first-order kinetic equation (see text) using a nonlinear regression program.

Determination of Carbaryl and Paroxon at the AChE-QCM Biosensor. Carbaryl and paroxon were selected as representative of carbamate and organophosphorus pesticides, respectively. Determination of carbaryl was carried out according to a preincubation protocol in which the enzyme-modified crystal was in contact with a solution of the inhibitor for 5 min. Afterward, the enzyme-modified crystal was removed from the preincubation solution, washed with phosphate buffer, and placed in the QCM probe, and the frequency response was monitored following the addition of the substrate as described above. In the case of paroxon, the aqueous solution containing pesticide was added simultaneously to the substrate, and the frequency response was determined. The choice of whether the determination was carried out in the presence of pesticide (paroxon) or after a preincubation time period (carbaryl) was based on the specific mechanism of inhibition exhibited by each pesticide, as discussed in more detail below.

The percent inhibition, %I, was calculated according to

$$\%I = \frac{(\Delta F_c - \Delta F_p)}{\Delta F_c} \times 100 \qquad (1)$$

where ΔF_c is the frequency response obtained in the absence of pesticide and ΔF_p is the frequency response obtained after the addition of a determined amount of inhibitor (paroxon) or after the preincubation of the AChE-modified crystal with inhibitor (carbaryl).

RESULTS AND DISCUSSION

Adsorption of AChE onto a Quartz Crystal Resonator and Measurement of Enzyme Activity. In previous work,[21] we described the design and characterization of amperometric biosensors for the determination of organophosphorus and carbamate pesticides on the basis of their inhibitory effects on AChE activity. In those biosensors, the enzyme was immobilized by direct adsorption onto gold-covered nylon meshes which were subsequently placed on the surface of a glassy carbon electrode.[37] The enzymatic activity was followed using 4-aminophenyl acetate which, upon hydrolysis (by the immobilized AChE), gives 4-aminophenol, which is detected amperometrically at +0.2 V. In the present case, we initially employed a similar approach (for enzyme immobilization) for the construction of a AChE-QCM biosensor where the analytical signal was changes in frequency arising from mass changes of the oscillator.

The selection of an appropriate substrate is important in order to enhance the sensitivity of the QCM transducer. For this reason, 3-IA (used in the histological recognition of AChE and esterases, in general) was chosen since it gives rise to water-insoluble indigo pigment products. As is shown in Scheme 1, AChE hydrolyzes the ester bond of 3-IA, giving rise to acetate and 3-hydroxyindole as products. The hydroxyl group tautomerizes, forming a ketone which in neutral or alkaline conditions dimerizes to form a water-

(37) Pariente, F.; La Rosa, C.; Galan, F.; Hernández, L.; Lorenzo, E. *Biosens. Bioelectron.* **1996**, *11*, 1115−1128.

insoluble indigo pigment product.[38,39] This compound is a member of the class of indigo dyes[40] and is characterized by an intense dark blue color and negligible solubility in aqueous media. Thus, the proposed AChE-QCM biosensor is based on the decrease in frequency concomitant with the deposition of the insoluble product generated by the enzymatic hydrolysis of 3-IA. If, after the enzymatic reaction, the crystal surface is observed under a microscope, the presence of a dark blue deposit is clearly evident (not shown), corroborating that the enzymatic hydrolysis of the 3-IA and the formation of the indigo pigment have, indeed, taken place. (Optical microscopic images are presented in ref 37.)

The indigo pigment product is strongly bound to the surface, and no apparent loss of material was observed, even after washing or stirring in aqueous media. The reason for this strong adhesion is not well understood, but as other authors[26] have suggested, it likely involves, at least in part, interactions (hydrophobic, $\pi-\pi$ interactions, or hydrogen bonding) between the indigo pigment product and protein residues on the QCM surface. It is also worth restating the fact that all frequency changes were measured in situ (i.e., in the presence of 3-IA), further precluding any potential loss of material from the surface.

In crystals treated in the same fashion but without the enzyme layer, no change in frequency nor generation of a blue precipitate was evident, even after prolonged exposure to the substrate. This indicates that the enzyme (AChE) layer is necessary for the reaction to take place.

As described in the Experimental Section, frequency changes were measured in situ following the addition of the 3-IA to the solution. Figure 1 shows the frequency decreases obtained for the AChE-QCM biosensor loaded with different amounts of adsorbed AChE (Figure 1, curves b−f). For comparison, a control experiment was carried out with an unmodified crystal (Figure 1, curve a), showing a very small decrease in frequency after the addition of substrate. This indicates that background hydrolysis of 3-IA in the absence of enzyme is essentially negligible.

The slopes of the ΔF/time plots were dependent on the enzyme loading, with steeper slopes observed for higher loadings of enzyme. The shapes of the ΔF/time profiles suggested an exponential decay, and for this reason the data in Figure 1 were fitted to a first-order kinetics equation:

$$\Delta F_t = -\Delta F_{max}\,(1 - e^{-kt}) \qquad (2)$$

where ΔF_t is the frequency change (in hertz) at time t, ΔF_{max} is the frequency change at long times ($t \rightarrow \infty$), and k is the first-order rate constant (min^{-1}). In addition, the initial rate of the decay can be obtained from eq 2 as $t \rightarrow 0$:

$$v_o = -\Delta F_{max} k \qquad (3)$$

Table 1 lists the different kinetic parameters obtained after fitting the experimental values presented in Figure 1 to eq 2. As can be seen, k remains virtually constant (except for the case of

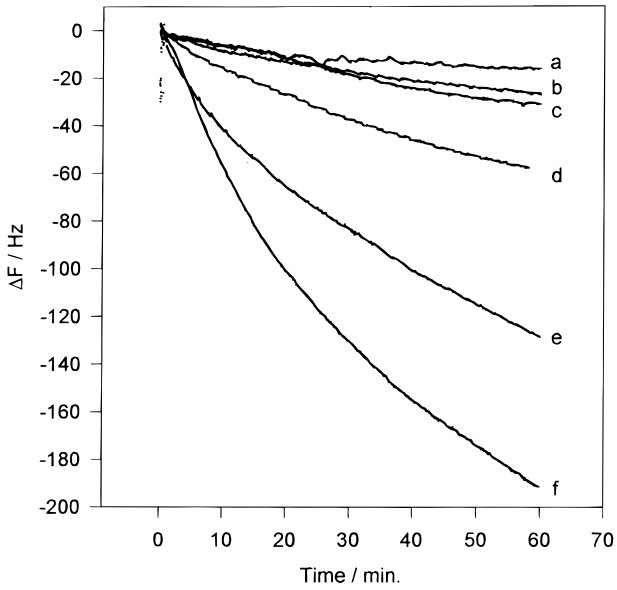


Figure 1. Frequency responses of QCM crystals modified with different amounts (units) of acetylcholinesterase (AChE) upon the addition of 3-indolyl acetate (at a concentration of 0.8 mM): (a) nonmodified, (b−f) modified with (b) 0.4, (c) 0.9, (d) 1.9, (e) 3.7, and (f) 7.4 units of AChE, respectively. The measurements were carried out in 0.08 M phosphate (pH 8.0) buffer at 24 °C. The frequency was monitored after addition of substrate and subsequent stirring (for 1 min) of the solution.

Table 1. First-Order Kinetic Parameters Obtained for an AChE-QCM Biosensor as a Function of AChE Loading in the Presence of 0.8 mM 3-Indolyl Acetate

AChE loading (units)	ΔF_{max} (Hz)	k (min^{-1})	v_o (Hz min^{-1})
0.4	18	0.0264	0.47
0.9	32	0.0269	0.87
1.9	92	0.0170	1.56
3.7	156	0.0230	3.57
7.4	228	0.0290	6.61

AChE loading of 1.9 units), whereas the values of ΔF_{max} and v_o are strongly dependent on the AChE loading. In fact, a plot of v_o vs AChE loading exhibits a linear relationship (Figure 2). Similarly, a linear relationship is also obtained when ΔF_{max} is plotted vs loading. These results indicate that the QCM can be used to follow the activity of enzymes immobilized on the crystal surface.

Although it is also possible to study the effects of the substrate concentration on the AChE-QCM biosensor response, in the present case these studies were precluded by the low solubility of 3-IA under the experimental conditions employed. For this reason, a standard concentration of 3-IA of 0.8 mM was employed in all studies. Because of this solubility limitation, the stirring time (following addition of substrate) plays a key role in the response of the biosensor. Figure 3 shows the frequency changes obtained for an AChE-QCM biosensor loaded with 0.4 U of AChE in the presence of 3-IA (0.8 mM) after 1 (Figure 3a) and 3 (Figure 3b) min of stirring. In both cases, the frequency shows an exponential decay with time, but overall frequency changes and v_o values were greatly enhanced when a 3-min stirring time period was employed. However, stirring times greater than 3 min did not result in significant increases in these values. For these

(38) McGadey, J. *Histochemie* **1970**, *23*, 180−184.
(39) Blake, M. S.; Johnston, K. H.; Russell-Jones, C. J.; Gotschlich, E. C. *Anal. Biochem.* **1984**, *136*, 175−179.
(40) Gordon, P. F.; Gregory, P. *Organic Chemistry in Colour*; Springer-Verlag: New York, 1983; p 82.

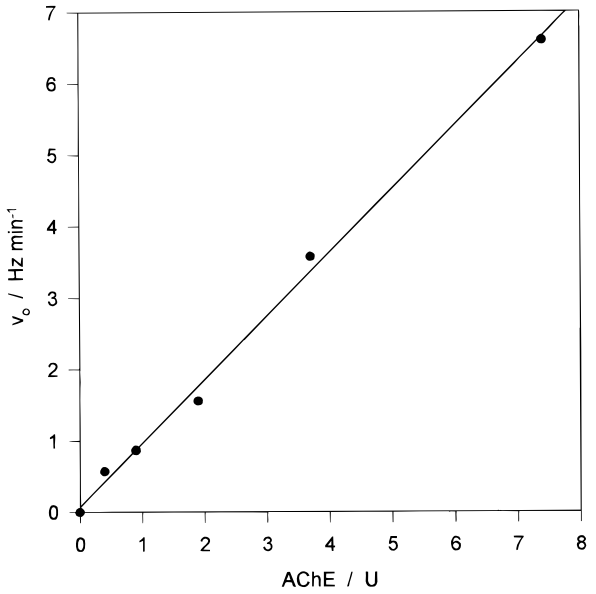


Figure 2. Plot of the calculated initial rates (ν_o) versus enzyme loading on the surface of an AChE-QCM biosensor.

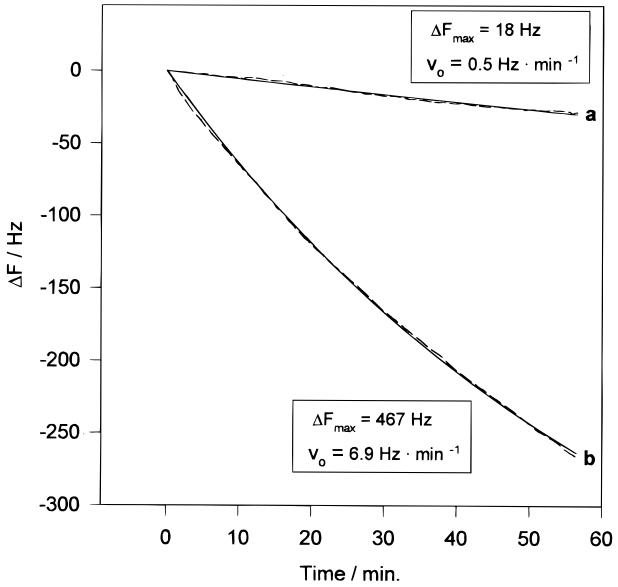


Figure 3. Effect on the response of an AChE-QCM biosensor probe (with 0.4 unit of AChE immobilized on its surface) of the stirring time: 1 min (curve a) or 3 min (curve b) after the addition of 150 μL of a 3-indolyl acetate stock solution (50 mg/mL, in DMF) to 50 mL of 0.08 M phosphate (pH 8.0) buffer. Solid lines correspond to fits of the data to eq 2.

reasons, a stirring time of 3 min (after the addition of substrate) was employed in all subsequent experiments.

The previously discussed results involved the direct adsorption of the enzyme (AChE) onto the gold surface. To test alternative immobilization procedures, cystamine was employed as a bifunctional reagent to modify the gold surface. In this approach, we made use of the well-known propensity of sulfur atoms to adsorb to gold surfaces. The strategy is based on the covalent bonding of the enzyme to the amino headgroups present in the adsorbed layer of cystamine, employing glutaraldehyde as cross-linker. The activity of the immobilized AChE was evaluated using the same procedures as described above. The responses obtained (data not shown) were very similar to those for the case of direct

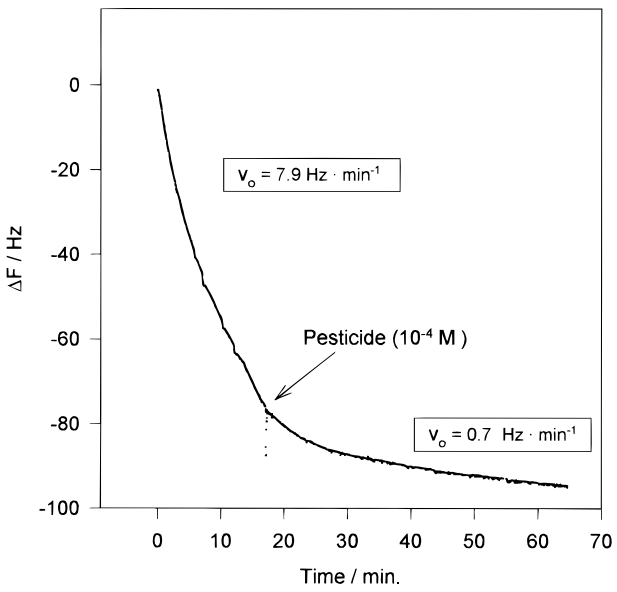


Figure 4. Depiction of the inhibitory effects on an AChE-QCM biosensor loaded with 3.7 units of AChE of the addition of paroxon (0.1 mM) at the time indicated by the arrow. Other conditions were as in Figure 1.

enzyme adsorption. In addition, there was no appreciable difference in the stability of the enzyme immobilized by either procedure. Since enzyme immobilization via coupling to adsorbed cystamine was clearly a more complex procedure, yet it resulted in no appreciable gains relative to direct adsorption, in all further experiments the direct adsorption of the enzyme onto the gold surface was used as the immobilization procedure. A clear advantage of this procedure is the ease with which the QCM probe could be modified with the enzyme.

AChE-QCM Biosensor Response in the Presence of Pesticides. The inhibitory effects of organophosphorus and carbamate pesticides on AChE activity are well know. Such inhibitory effects can be studied, in situ, using an AChE-QCM biosensor from which kinetic parameters can be evaluated. Figure 4 presents the frequency changes obtained for an AChE-QCM biosensor loaded with 3.7 U of AChE in the presence of 3-IA (0.8 mM). After 17 min, paroxon (0.1 mM) was added to the solution (as indicated by the arrow in Figure 4), and the frequency change with time was again monitored. As can be seen, the pesticide acts by decreasing the rate of frequency change. In addition, a relatively rapid stabilization of the frequency also takes place. These observations are consistent with a diminution in the amount of active enzyme present due to the inhibitory effects of paroxon. Similar results were obtained for the carbamate pesticide carbaryl. Thus, the QCM can be used to follow the enzymatic activity of AChE immobilized on the crystal surface and also in the determination of organophosphorus or carbamate pesticides due to their inhibitory effects.

Determination of Pesticides with the AChE-QCM Biosensor. The mechanism of chemical action of organophosphorus and carbamate pesticides on AChE is well established.[41] Hydrolysis of organophosphorus pesticides by the enzyme results in the blocking of the enzyme's active center in a substrate-independent

(41) Eto, M. *Organophosphorous Pesticides: Organic and Biological Chemistry*; CRC Press: Boca Raton, FL, 1974; pp 131−133.

manner. On the other hand, carbamates compete with the substrate for the enzyme's active center, and their inhibitory effect can be prevented in the presence of high concentrations of substrate.[42] That is, organophosphorus pesticides generally exhibit noncompetitive inhibition, whereas carbamates exhibit competitive inhibition. In previous work,[43] we described the type of inhibition that carbaryl (carbamates) and paroxon (organophosphorus) exhibit in an amperometric biosensor based on immobilized AChE. In the case of carbaryl, it exhibited competitive inhibition when the substrate was present and noncompetitive inhibition in its absence (i.e., if the biosensor was preincubated with the pesticide). On the other hand, the inhibitory effects of paroxon were of mixed type and essentially independent of conditions.

Based on these observations, one would anticipate that the determination of these pesticides would be dependent on the specific inhibitory mechanism. Thus, as would be expected, the use of the preincubation method gave rise to the lowest detection limit in the case of carbaryl. On the other hand, for paroxon, the differences in the detection limits reached using one approach vs the other were not so marked, which is in accord with mixed inhibition.

In the preincubation approach, the time of incubation (which is proportional to the extent of enzyme inhibition) is an important factor in order to obtain the best limit of detection. The relationship between percentage of inhibition (%I) and preincubation time (t) has been described by Aldridge[44] as

$$\log\left(\frac{100}{\%I}\right) = k_2[\mathrm{I_o}]t \qquad (4)$$

where k_2 is the bimolecular rate constant for inhibition and [$\mathrm{I_o}$] is the concentration of inhibitor.[45] Clearly, the degree of inhibition is dependent on incubation time, a fact that is often used to enhance detection limits. In our case, at a fixed concentration of carbaryl, the degree of inhibition increased with the incubation time, as predicted by eq 4. However, for incubation times over 5 min, the increase in inhibition became negligible. Thus, a 5-min incubation period was employed.

Figure 5 shows the response to a 0.8 mM concentration of 3-IA of an AChE-QCM biosensor loaded with 0.4 unit of AChE in the absence (control) and in the presence of increasing concentrations of paroxon. We note that a relatively low enzyme loading (0.4 unit) was employed. This is due to the fact that, for sensors based on enzymatic inhibition (as in the present case), maximal changes (and thus better limits of detection) are generally observed for low enzymatic loadings. Figure 6 shows the biosensor response to substrate (0.8 mM 3-IA) after a preincubation period of 5 min in solutions containing different concentrations of carbaryl (in the absence of substrate). In both cases, the frequency changes were plotted versus time, and the experimental data were fitted to eq 2.

As can be ascertained in Figures 5 and 6, significant frequency changes (and thus percentage inhibition) were observed for times

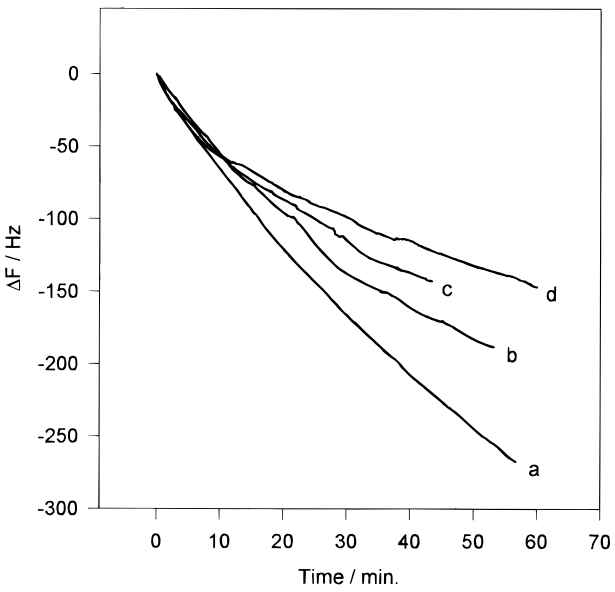


Figure 5. Response of AChE-QCM biosensors (loaded with 0.4 unit of enzyme) upon the addition of 0.8 mM of 3-indolyl acetate in the presence of increasing concentrations of paroxon: (a) 0, (b) 0.1, (c) 0.25, and (d) 1.0 μM. The other experimental conditions were 0.08 M phosphate (pH 8.0) buffer, 3-min stirring time, and a temperature of 24 °C.

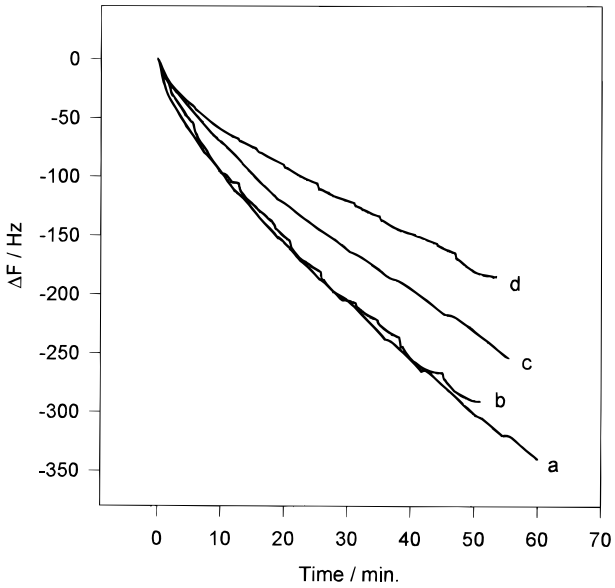


Figure 6. Frequency responses in the presence of 3-indolyl acetate (0.8 mM) of AChE-QCM biosensors (loaded with 0.4 unit of AChE) that had been preincubated in the presence of (a) 0, (b) 0.1, (c) 0.5, or 1.0 μM carbaryl. Other experimental conditions are as in Figure 5.

around 30 min. Thus, in constructing calibration curves (%I vs log [pesticide]), ΔF values at 30 min (ΔF_{30}) were employed according to eq 1. Although the choice of 30 min is clearly arbitrary, it represents a good compromise between low-level detection and time of analysis.

Calibration curves for paroxon and carbaryl are presented in Figure 7a and b, respectively. Linear calibration plots were obtained over the range of 5.0×10^{-8}–1.0×10^{-5} and 1.0×10^{-7}–5.0×10^{-5} M for paroxon ($y = 284.8 + 38.4x$; $r = 0.9997$) and carbaryl ($y = 244.3 + 35.3x$; $r = 0.9916$), respectively. The limit of detection (defined as the concentration of inhibitor required

(42) O'Brien, R. D.; Hilton, B. D.; Gilmour, L. *Mol. Pharmacol.* **1966**, *2*, 593–605.
(43) La Rosa, C.; Pariente, F.; Hernández, L.; Lorenzo, E. *Anal. Chim. Acta* **1994**, *295*, 273–282.
(44) Aldridge, W. N. *Biochem. J.* **1950**, *46*, 451–460.
(45) Skladal, P.; Mascini, M. *Biosens. Bioelectron.* **1992**, *7*, 335–343.

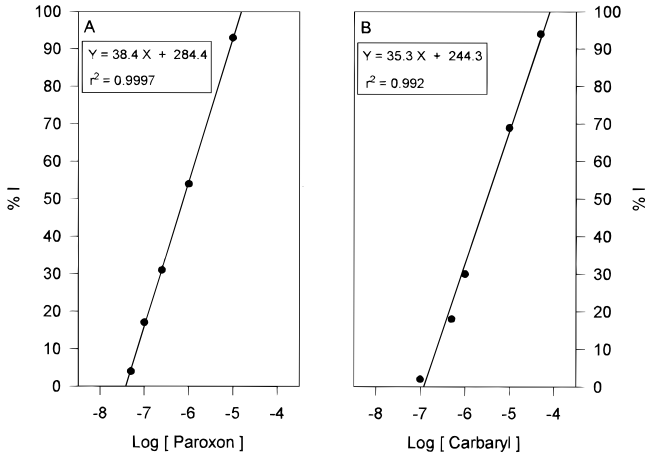


Figure 7. Calibration curves of percentage of inhibition, %*I*, vs log of the concentration of paroxon (a) or carbaryl (b). Calibrations were done in 0.08 M phosphate buffer (pH 8.0) with AChE-QCM biosensors loaded with 0.4 unit of AChE in the presence of 3-indolyl acetate (0.8 mM) under the following conditions: (a) direct measurements in the presence of increasing amounts of paroxon; (b) preincubation with carbaryl (5 min) and subsequent measurement in the presence of substrate.

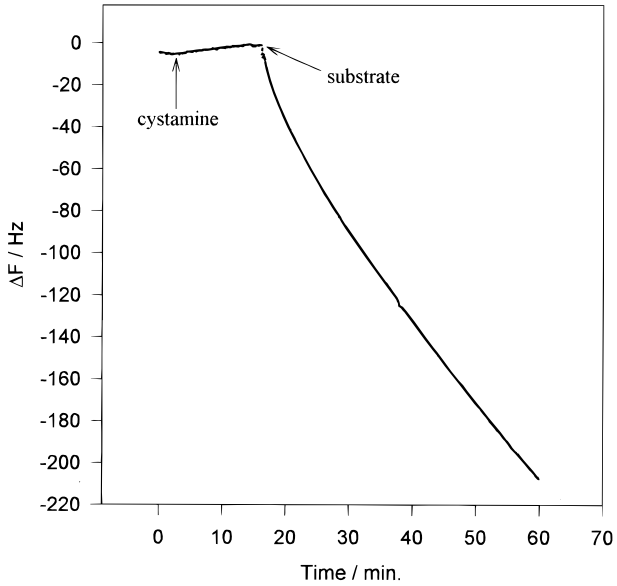


Figure 8. Comparison of the response of an AChE-QCM biosensor upon the addition of 0.4 mM cystamine and 0.8 mM 3-indolyl acetate for a crystal whose remaining exposed gold surface had been blocked with BSA (0.3% v/v) (see text).

to achieve 5% inhibition) was found to be 5.0×10^{-8} and 1.0×10^{-7} M for paroxon and carbaryl, respectively. These detection limits are comparable to, or lower than, literature values for these pesticides.[46−49] However, in our case, we were able to employ significantly shorter incubation times.

The reproducibility of this sensor was determined for three consecutive assays of 5.0×10^{-6} M carbaryl or paroxon using three different AChE crystals, and the relative standard deviation was calculated to be about ±10%. It should be noted that, because of aspects inherent to this approach (and generally to all approaches based on inhibition of enzymatic activity), a modified crystal can only be used for a single determination, thus precluding a more extensive statistical analysis.

Interferences. The QCM method is less susceptible to interference effects when compared to other methods for following enzymatic activity. For example, optical clarity is not required during the measurement step, as is generally the case for optical detection methods. Electrode poisoning or interferences by undesirable electrochemically active substances that can arise during amperometric detection are also avoided. In the QCM method, interferences generally arise as a result of molecules that can bind or adsorb strongly (irreversibly) to the surface of the crystal. In the preincubation method employed (for carbaryl), even if materials were to adsorb, the fact that the relative change in frequency (ΔF) is used as the analytical signal makes this approach virtually insensitive to such interference effects.

However, in cases where preincubation is not employed (i.e., paroxon), nonspecific adsorption of material would clearly represent a significant interference effect, particularly if part of the gold surface were exposed. To minimize such effects, the sensor was immersed in a 0.3% (v/v) solution of BSA so as to block all remaining exposed sites. If such a sensor was then exposed (in the absence of substrate) to a 0.4 mM solution of cystamine, there was virtually no frequency change, as can be seen in Figure 8, indicating that, even at such a high concentration of a species known to absorb strongly to gold surfaces, there is no detectable interference effect. Thus, one can assume that, for the concentration levels that would be typically encountered in an analysis sample, such nonspecific adsorption effects (interference) would be negligible.

Moreover, upon subsequent addition of substrate (3-IA), there was no change in the enzymatic activity relative to that of a sensor that had not been exposed to the cystamine solution. Thus, the enzymatic activity is clearly retained.

CONCLUSIONS

We have shown that the inhibitory effects of paroxon and carbaryl on the enzymatic activity of AChE immobilized on the surface of a quartz crystal oscillator can be used in the low-level and rapid determination of these pesticides when 3-indolyl acetate is used as substrate to follow enzymatic activity. Calibration curves were constructed by plotting the percentage of inhibition vs the logarithm of the pesticide concentration. Detection limits of 5.0×10^{-8} and 1.0×10^{-7} M were obtained for paroxon and (after a 5-min preincubation) for carbaryl, respectively. This AChE-QCM sensor provides a very promising technique for rapid and low-level determination of organophosphorus and carbamate pesticides. It is more sensitive and faster than chromatographic methods. The device may be portable, making possible its use for in-field analysis. The detection is quite selective and can be performed without expensive instrumentation. In addition, the method has the potential to be coupled to a flow injection system to perform “on line” analysis.

(46) Bernabei, M.; Cremisini, C.; Mascini, M.; Palleschi, G. *Anal. Lett.* **1991**, *24*, 1317−1331.
(47) Hartley, I. C.; Hart, J. P. *Anal. Proc. Incl. Anal. Commun.* **1914**, *31*, 333.
(48) Guilbault, G. G.; Jordan, J. M. *CRC Crit. Rev. Anal. Chem.* **1988**, *19*, 1−28.
(49) Heldman, E.; Balan, A.; Horowitz, O.; Ben-Zion, S.; Torten, M. *FEBS* **1985**, *180*, 243.

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