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Electrochemical Bioassay for the Investigation of Chlorpyrifos-methyl in Vine Samples

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This paper reports the optimization of an electrochemical bioassay for the determination of chlorpyrifos-methyl and its application to the analysis of grape and vine leaf samples treated with that pesticide. The analytical method was based on electrochemical determination of the extent of the inhibition exerted by the pesticide on acetylcholinesterase using the substrate acetylthiocholine. Two similar calibration plots were obtained, in the range of 1−300 ng/mL, respectively, for chlorpyrifos-methyl in pure standard form and in the commercial preparation Reldan, with comparable coefficients of variation (CV) in the range of 10% < CV < 20%. After an insecticide treatment, samples were analyzed to evaluate its persistence both in grapes and in vine leaves. Samples were evaluated using different extraction procedures: one based on solvent extraction of pesticide residue from grapes and the other based on aqueous extraction from vine leaves using phosphate buffer. The grape solvent extracts were analyzed using both gas chromatography and electrochemical bioassay, whereas the vine leaf buffer extracts were analyzed using the electrochemical bioassay. Quantitative analysis of chlorpyrifos-methyl determined in the two samples, with the electrochemical bioassay, showed a comparable decrease profile over the experimental period.

KEYWORDS: Bioassay; enzyme inhibition; screen-printed electrodes; acetylcholinesterase; chlorpyrifos-methyl

INTRODUCTION

Pesticides and insecticides are applied worldwide to a broad variety of crops for field and postharvest protection, including vines. Particularly, organophosphate and carbamate pesticides are extensively used due to their high insecticidal activity and relatively low persistence (*1*).

Because their target enzyme, acetylcholinesterase (AChE), is common to neural transmission both in insects and in mammals, including humans, they represent a potential hazard for human health (*2*). Transmittance of insecticides to humans is possible either by exposure during field operations or by the food chain. Acute poisoning on exposure to insecticides is common, especially among agriculture workers. The annual world number of deaths due to this reason was estimated to be 200000 in the 1980s (*3*).

Chlorpyrifos-methyl has been one of the most widely marketed and used insecticides in Italy in the past few years (*4*); its maximum admissible level is 200 ng/g in grapes before harvesting. Chlorpyrifos-methyl toxicity has been recently been reviewed by the U.S. EPA (*5*).

Concern regarding the occurrence of pesticides in the environment as well as in wines remains an important issue (*6*). For this reason a correct pesticide application practice is regarded as an urgent need. The availability of analytical data on the actual level of crop protection is a key feature in planning pesticide treatments.

Sensitive analytical methods for an accurate evaluation of residues are based on liquid chromatography (LC), gas chromatography (GC), mass spectrometry (MS), or hyphenated techniques (e.g., GC-MS and LC-MS/MS), but due to the high cost of instrumentation and complex and time-consuming procedures, these techniques are not suitable as screening methods and for in-field use (*7*, *8*). The availability of rapid analytical screening procedures is a real need for food control laboratories. Moreover, the determination of pesticide levels in plant material directly in the field can influence decision-making in a variety of circumstances (e.g., treatments and harvesting time schedule). Many approaches have been proposed to meet the increasing requirements for fast and reliable analytical methods.

The use of enzyme inhibition as a means for pesticide detection is a well-established procedure. Dating to the original publication by G. Guilbault et al. in 1962, many biosensors and bioassays for the detection of anticholinesterase insecticides have been extensively studied and demonstrated (*9*−*12*).

In the present paper we describe the optimization and application of an electrochemical bioassay based on the chemical modification of screen-printed electrodes (SPEs) to grape and

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vine leaf extracts. SPEs can be massively produced at low cost; therefore, they can be used as single-use sensors suitable for the analysis of complex matrices that can foul the electrode surface or interfere with successive measurements. The use of differential pulse voltammetry combined with the use of disposable electrodes was found to be particularly suitable for the development of a fast screening protocol based on electrochemical measurements. The chemical modifier used was 7,7,8,8-tetracyanoquinodimethane. This electron mediator enhances the feasibility of the electrochemical oxidation of thiol-containing molecules at graphite electrodes. The enzymatic inhibition assay was carried out using acetylthiocholine as the substrate (*13*). The enzyme product, thiocholine, was measured via electrochemical oxidation at the surface of the chemically modified electrode (CME) producing an anodic current proportional to the enzyme activity. Calibration curves were obtained using chlorpyrifos-methyl both in pure standard solution and in the commercial preparation Reldan.

The final aim of the present work was to develop a report laboratory, which included extraction and measurement procedures, that will allow in-field analysis. One of the extraction methods proposed avoided the use of organic solvents and laboratory tools, such as a sonicator, homogenizer, ultraturrax, and centrifuge. In an experimental field we treated grapes with the suggested dose of Reldan (1.5 mL/L). Grape and leaf samples were then collected over a 20 day period and analyzed to assess the decay of the insecticide residues both in grapes and in vine leaves. The proposed method can be applied to in-field monitoring of other organophosphate and carbamate molecules, thus contributing to an improved and safer use of pest control treatments.

MATERIALS AND METHODS

Reagents. Sodium phosphate, potassium chloride, acetylcholinesterase (AChE) from electric eel (EC 3.1.1.7, 1070 units/mg protein), acetylthiocholine (ATCh chloride salt), perfluorinated ion-exchange resin (Nafion), 7,7,8,8-tetracyanoquinodimethane (TCNQ), methanol, acetone, hexane, ethyl acetate, ethyl ether, and acetonitrile were obtained from Sigma. The insecticide Reldan (chlorpyrifos-methyl, 223 g/L) was purchased by Dow AgroSciences, and chlorpyrifos-methyl was from Riedel-de Haen (Sigma, Italy).

Ultrapure water was obtained with a Purelab pro 20 apparatus (USF).

Apparatus. SPEs were produced by the Biosensor Laboratory, University of Florence. They were printed as elsewhere described (*10*).

Electrochemical experiments were carried out using a PGSTAT12 (Autolab Eco Chemie Netherlands) potentiostat. The data current was elaborated by GPES 4.7 software.

The samples were homogenized with an Ultraturrax purchased from Ika-Werke, sonicated with a Star sonic 90 purchased from Liarre, and centrifuged with an ALC centrifuge 4237 R. SPE C18 (3 mL) cartridges were obtained from J. T. Baker. The silica gel desiccator was purchased from Sigma.

The GC analysis was performed by a DANI model 8500 gas chromatograph equipped with a DB-1 capillary column (15 m × 0.25 μm × i.d. 0.25 mm; J&W Scientific, Folsom, CA) and with an NPD flame detector.

Vineyard Treatment, Grapes, and Leaf Sampling. The electrochemical bioassay was used to evaluate the persistence of chlorpyrifos-methyl on vines in a trial performed in a local vineyard.

An insecticidal treatment was carried out in a cv. Trebbiano d'Abruzzo vineyard, 22 years old, located in Giulianova, in the wine-growing area of Teramo, Abruzzo, Italy. The vineyard had 2000 vines in an area of 18000 m^2 and was cultivated with the Tendone system. The trial was done, under controlled conditions, on six vines, randomly located. They were treated using Reldan (1.5 mL/L), the active principle of which is chlorpiryfos-methyl, applied by portable pump at 2 L/vine.

The treatment was performed on August 30, 2001. The first sampling was done on the same day (SO); further samples were collected on September 1, 5, 11, 14, and 21, respectively S1, S5, S11, S14, and S21. Samples consisted of three leaves and three grapes from each vine per sampling day. Within each sampling day, samples obtained from the different plants were mixed and aliquots of controlled size extracted.

During the trial period no rains occurred.

Pesticide Extraction. Samples were extracted using two different procedures: one for grape and the other for vine leaves. These were, respectively, ethyl acetate liquid extraction and phosphate buffer-based liquid extraction.

Extracts obtained using the former were evaluated both by gas chromatography analysis and bioassay, whereas those obtained with the latter procedure were evaluated using the electrochemical bioassay.

Ethyl Acetate Extraction. The ethyl acetate extraction procedure was as follows: 150 g of grape samples was crushed and homogenized. Fifteen milliliters of ethyl acetate was added to 5 g of the homogenized sample and mixed thoroughly for a few minutes; the sample was then sonicated for 15 min, frozen for 20 min, and finally centrifuged for 15 min at 3000 rpm. The supernatant was removed for bioassay analysis.

For GC analysis a further SPE purification of extracts was carried out as described. An SPE C18 column (3 mL) was used for sample cleanup; the cartridge was conditioned with 5 mL of methanol, 5 mL of deionized water, and 5 mL of acetonitrile/water (85% v/v). An aliquot of extract (1.5 mL) was added to the cartridge. The elution was performed using 5 mL of acetonitrile/water (85% v/v). To the eluted extract were added, with mixing, 25 mL of hexane/ethyl ether (1:1), 75 mL of water, and 5 mL of sodium chloride; the mixture was then allowed to separate. The organic layer was removed and evaporated under a cold gentle nitrogen stream. The residue was dissolved in 1 mL of hexane and analyzed by GC.

Phosphate Buffer Extraction. Vine leaves were cut into small pieces (~0.3 cm^2). Ten milliliters of 0.1 M phosphate buffer at pH 7.4 was added to 1 g of vine leaf sample and vigorously shaken for 1 min. After 10 min, the buffer solution was removed for analysis.

The solution pH was checked before the analysis. No pH variations were observed for this matrix. Aliquots were used for the electrochemical bioassay without further treatment.

On the contrary, performing the buffer extraction on grape samples, we observed a dramatic pH reduction despite the high buffer concentration. The pH decrease, due to the high organic acid content, causes a strong diminution of enzyme activity.

For this reason the phosphate buffer extraction was not applied to grape samples.

Gas Chromatographic Measurement. GC-NPD analysis was performed on the grape extracts using the following experimental conditions.

The injection was made using a split 1:100 injector held at 270° C. The oven temperatures were as follows: 2 min at 98 °C, raised from 98 to 145 °C at 20 °C/min, 30 s at 145 °C, raised from 145 to 270 °C at 4.5° C/min, and finally 30 min at 270 °C. The carrier gas was He at 2 mL/min. The temperature of the NPD detector was held at 280°C. Standard chlorpyrifos-methyl (100 ng/mL) was used as calibrating solution.

Quantification was performed with a Chrom-card software integrator.

Electrochemical Procedures. The screen-printed devices consist of a graphite working electrode, a silver pseudo-reference electrode, and a graphite counter electrode. The CMEs were prepared by depositing, on the working electrode surface, 2 μL of a 0.25 mM TCNQ in 5% Nafion solution. Prior to use, sensors were stored overnight at room temperature, in a desiccator. The same conditions were used for weekly storage.

Each electrochemical measurement was performed using a CME as a single-use sensor. The electrochemical procedure consisted of a pretreatment step followed by the electrochemical acetylcholinesterase activity determination. The pretreatment was performed to activate the mediator and to eliminate electrochemically interfering substances that could be present on the electrode surface.

The pretreatment was as follows: 100 μL of phosphate buffer, pH 7.4, and 100 mM KCl, were placed on the electrochemical device, and two cyclic voltammetry (CV) scans were performed in the range −600/+600 mV versus an Ag pseudo-reference electrode, at a scan rate of 100 mV/s, followed by three DPV scans in the potential window

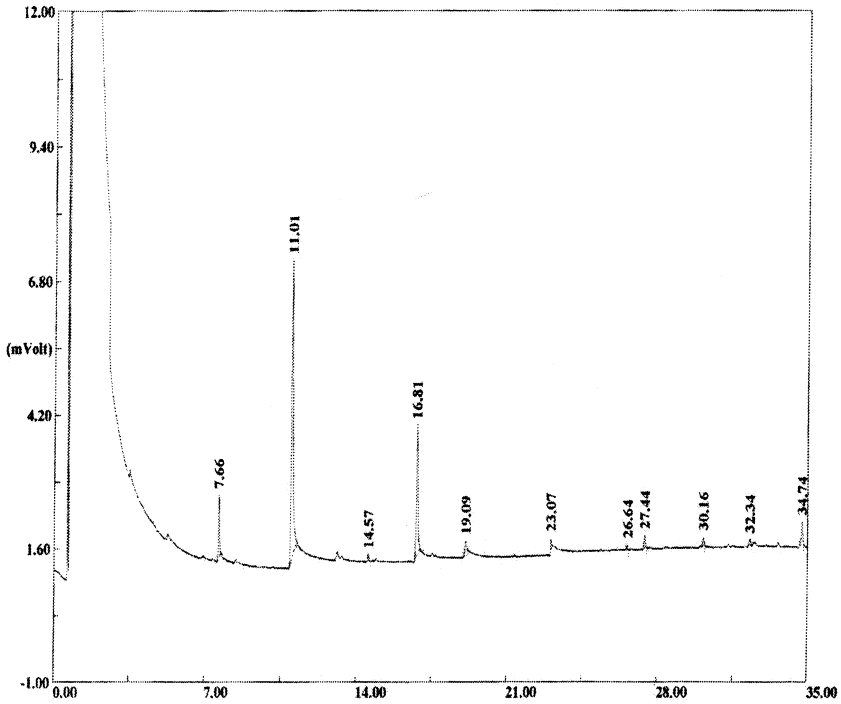

Figure 1. Typical chromatogram obtained for a grape extract, chlorpyrifos-methyl peak position in 16.81 min. No interfering peaks were observed.

+250/+750 mV versus the Ag pseudo-reference electrode, pulse amplitude of 50 mV, pulse width of 60 ms, scan rate of 25 mV/s. The electrochemical determination was performed as described below.

Sensor Preparation and Stability. The optimal TCNQ loading for electrode modification was evaluated in CV experiments.

CMEs were prepared as described using different TCNQ loadings in the range of 0.025−0.50 mM; the measurement of mediator was performed by depositing 100 μL of phosphate buffer, pH 7.4, and 100 mM KCl on the electrochemical device, and two CV scans were performed in the range of −600/+600 mV versus the Ag pseudo-reference electrode, scan rate of 100 mV/s. The charge of the anodic peak of the second scan was taken as the TCNQ loading signal.

To evaluate the stability of the CMEs, 20 electrodes were stored at room temperature in a desiccator to shield them from light until the moment of use. Stability was evaluated by CV over a period of 5 days. The procedure was as follows: 100 μL of 0.1 M phosphate buffer with 0.1 M KCl, pH 7.4, was placed onto the SPEs, and two scans were run in the range of −600/+600 mV versus the Ag pseudo-reference electrode at a scan rate of 100 mV/s. The charge of the anodic peak of the second scan was taken as the TCNQ loading signal; five electrodes were tested on days 0, 2, 3, and 5.

Acetylcholinesterase Determination Using DPV. To optimize the determination of the acetylcholinesterase activity, we developed the following procedure: an enzyme solution, in the range of 0.1−1.2 units/mL, was mixed with acetylthiocholine (0.5 mM) in 1 mL of 0.1 M phosphate buffer with 0.1 M KCl, pH 7.4; the reaction was allowed to proceed for 20 s, and a 100 μL aliquot was deposited onto the planar surface of the CME. After 80 s, the DPV measurement was performed, in the potential range 250−700 mV versus the Ag pseudo-reference electrode, pulse amplitude of 50 mV, scan rate of 25 mV/s, and pulse width of 60 ms. Following this procedure we obtained a current peak at 0.5 V, which was exploited as the analytical signal. Each measurement was performed in triplicate. The concentration of acetylcholinesterase used in the inhibition assays was the lowest concentration (0.4 unit/mL) producing the highest current.

Chlorpyrifos Calibration Curves. Calibration plots were obtained, in the range of 1−300 ng/mL, respectively, for chlorpyrifos-methyl in pure standard form and in commercial preparation Reldan.

Standard solutions were obtained in 0.1 M phosphate buffer with 0.1 M KCl, pH 7.4, with acetylcholinesterase (0.4 unit/mL) and incubated for 10 min.

This solution was used for the enzyme activity measurement as above-described. Each measurement was performed in triplicate.

The oxidation current peak obtained by DPV was measured (I_2) and compared with the oxidation current value obtained without pesticide (I_1).

The percent of inhibition (I%) was obtained according to the following formula:

$$I\% = 100(I_1 - I_2)/I_1$$

Acetylcholinesterase Inhibition Measurements Using Grape and Vine Leaf Samples. Ethyl acetate extracts from grape (1 mL) were evaporated under a cold gentle nitrogen stream and dissolved in 1 mL of 0.1 M phosphate buffer with 0.1 M KCl, pH 7.4. The vine leaf extracts were ready for use without further treatments.

If necessary, samples were further diluted with the same buffer to obtain a current signal within the working assay range. The enzyme inhibition was evaluated as for standard solutions.

A quantitative evaluation of the chlorpyrifos-methyl content was obtained using the calibration curve obtained using Reldan.

RESULTS

Gas Chromatography. The purified grape extracts, S0, S1, S5, S11, S14, and S21, could be analyzed using GC-NPD using the procedure optimized for the standard solution as no interfering substances were found in the extracts. **Figure 1** shows a typical chromatogram for grape extract; the position of the chlorpyrifos-methyl peak was determined using an internal standard on a blank sample, and it was at 16.81 min.

Data obtained in GC analysis are reported in **Table 1**.

The chlorpyrifos-methyl content in grapes showed a fairly stable trend during the first 5 days after the treatment and a mean decrease of 45% with respect to day 0 over the last 3 sampling days. These data were then used to assess the performance of the electrochemical bioassay.

Electrochemical Procedures Evaluation. *Sensor Preparation and Stability.* The charge of the anodic peak obtained in the second scan in CV measurements, using electrodes with different mediator loadings, was used to evaluate the optimal

Table 1. Chlorpyrifos-methyl Content in Grape Samples Collected in a 21 Day Period after Treatment

sample	GC chlorpirifos-methyl (ng/mL)	sample	GC chlorpirifos-methyl (ng/mL)
S0	700	S11	350
S1	800	S14	450
S5	650	S21	400

[a] Data were calculated using a 100 ng/mL chlorpyrifos-methyl standard solution.

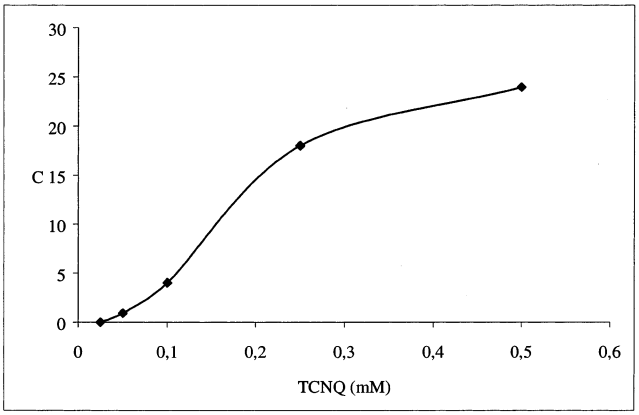

Figure 2. Charge (C) of the anodic peak in cyclic voltammetry experiments used as analytical parameter for TCNQ loading evaluation. 0.25 mM in 5% Nafion solution was found as the optimal mediator concentration.

mediator concentration (**Figure 2**). A 0.25 mM TCNQ in 5% Nafion solution was found to be the optimal TCNQ concentration and used for the preparation of the CMEs.

The stability of the sensors, stored in a desiccator at room temperature in the dark, was assessed on a weekly basis using the CV response as TCNQ loading index. The results showed appreciable stability as the anodic peak charges measured at days 2, 3, and 5 were, respectively, 94, 98, and 101% of the day 0 charge mean value; the coefficient of variation varied from 10 to 8% ($n = 5$) (**Figure 3**).

These results showed an appreciable stability, which allowed the preparation of the electrodes on a work-week basis.

Acetylcholinesterase Activity by DPV. The enzyme acetylcholinesterase catalyzes the cleavage of acetylthiocholine to acetic acid and thiocholine:

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

The thiocholine produced in the enzymatic reaction reduces the electrochemical mediator TCNQ, which is reoxidized at the electrode surface at +0.5 V versus the Ag pseudo-reference electrode, producing a current related to the extent of the enzyme reaction.

The peak current obtained using different concentrations of AChE (0.1−1.2 units/mL) was used to choose the optimal enzyme concentration for the bioassay. In **Figure 4** the current versus enzyme concentration, using two different substrate concentrations (0.25 and 0.50 mM), is reported. The optimal concentration was chosen in the steepest part of the calibration plot corresponding to the lowest concentration (0.40 unit/mL) producing the highest current using 0.50 mM acetylthiocholine. The current mean value was 48.2 μA with a CV = 12%, n = 5. A substrate concentration of 0.25 led to the use of a higher enzyme concentration (0.8 unit/mL) to obtain the maximum signal; this could affect the sensitivity of the assay. Therefore, 0.50 mM acetylthiocholine was used in further experiments.

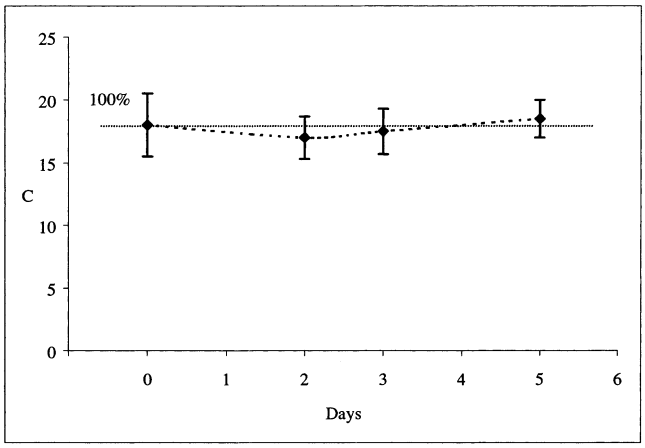

Figure 3. Evaluation of TCNQ-modified SPE stability in a 5 day period. The charges (C) of the anodic peak in cyclic voltammetry experiments were used as analytical parameters for mediator stability evaluation. A stable trend was observed over a 5 day period.

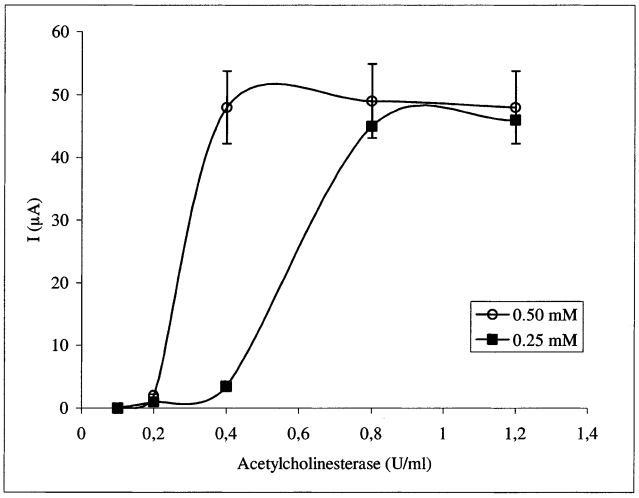

Figure 4. Measurement of the acetylcholinesterase activity by DPV. Optimal enzyme concentration was 0.40 units/mL as this was the lowest concentration producing the highest current. The CV was 12%.

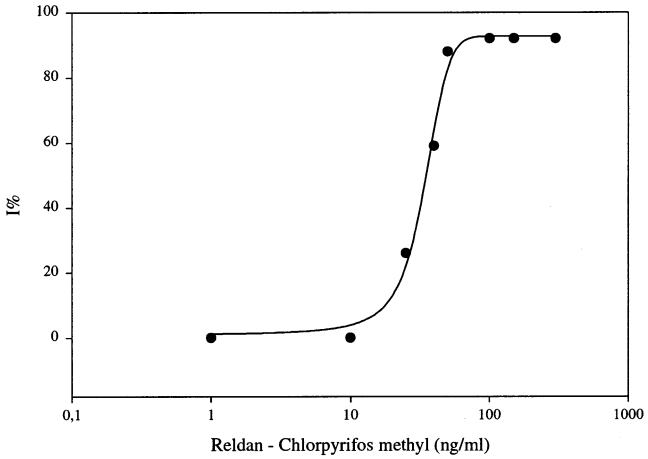

Figure 5. Calibration curve obtained for chlorpyrifos-methyl in Reldan. The curve is described by the equation $y = 92.7/\{1 + e^{[-(x-33.9)/7.7]}\}$. The detection limit was 22 ng/mL, and the calculated I_{50}% was 35 ng/mL.

Chlorpyrifos Calibration Curves. **Figure 5** shows the calibration curve obtained for chlorpyrifos-methyl in Reldan. The sigmoidal curve, described by the equation $y = 92.7/\{1 +$

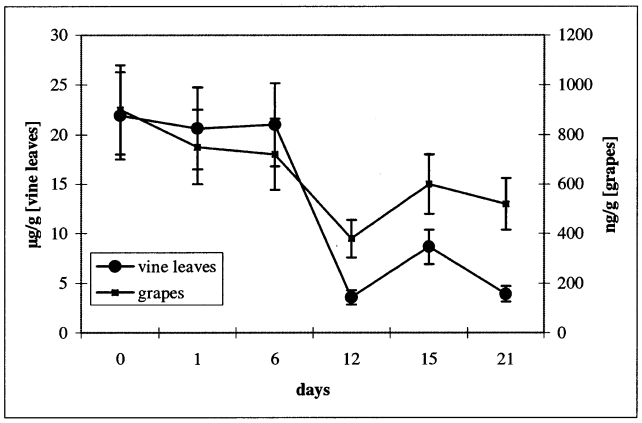

Figure 6. Comparison of chlorpyrifos-methyl content in grapes and vine leaves sampled over a 21 day period analyzed using the electrochemical bioassay.

$e^{[-(x-33.9)/7.7]}\}$, which fits the inhibition data, was used for the calculation of pesticide residue in samples.

The detection limit, 22 ng/mL, was obtained using the $I\%$ value ($I\% = 16\%$) calculated using the formula $I\% = I_0 - (I_0 - 2 \times SDI_0)/I_0$, in the calibration curve equation, and the calculated $I_{50}\%$ was 35 ng/mL. These values indicate an appreciable sensitivity, namely, a mean value of 3.4% inhibition per nanogram per milliliter, in the working range of 22−100 ng/mL. As expected, the curve obtained using chlorpyrifos-methyl pure standard in the same working range ($y = 96.1/\{1 + e^{[-(x-33.0)/13]}\}$) nearly overlaps with the former; the correlation between the two data sets, in the linear range of 22−100 ng/mL, was described by the equation $y = 15.40 + 0.85x$, $r^2 = 0.992$.

Determination of Chlorpyrifos-methyl in Grape and Vine Leaf Samples. Grape ethyl acetate extracts (S0, S1, S5, S11, S14, and S21) were analyzed with the enzyme inhibition assay as described. The chlorpyrifos-methyl content was hence calculated using the calibration curve obtained with Reldan. Data comparison with GC analysis is reported in **Table 1**. Results obtained with the two analytical methods were in agreement and indicated that the bioassay was able to detect chlorpyrifos-methyl with satisfactory accuracy with respect to the analytical purpose of the method.

The vine leaf buffer extracts were ready for bioassay analysis without further treatments.

Comparison of the chlorpyrifos-methyl content in grapes and vine leaves sampled over a 21 day period is reported in **Figure 6**. Although a higher content of chlorpyrifos-methyl residue was detected in grape leaves (5−22 µg/g) with respect to grapes (180−850 ng/mL), comparable residue content profiles in both matrices were obtained. The different insecticide amounts measured in the two matrices were due to the higher surface/weight ratio of the leaves. Also, their position determines a wider exposed surface to pesticide treatments compared to grapes that often are partially covered by leaves.

This feature is also possibly involved in the variation of residue content that occurred in the two matrices during the trial period. The contents of pesticide in grapes and leaves at day 1 were, respectively, 850 ng/g and 22 µg/g (ratio = 1:25); comparable values were found up to day 6. An evident change in the content ratio between the two matrices occurred at day 12 (ratio = 1:12). Comparable ratios were found up to day 21. This phenomenon could be due to the wide surface exposed by leaves to atmospheric agents. Therefore, as expected, we observed a higher pesticide content and a greater degradation rate of chlorpyrifos-methyl in leaf samples than in grape samples.

These results suggest that the quantification of pesticide residue in vine leaves, which can be successfully extracted with phosphate buffer, can provide useful analytical information for the assessment of the status of vineyard protection.

Conclusions. The system presented here serves as an example for an electrochemical bioassay that can be applicable for the quantification of chlorpyrifos-methyl during in-field evaluation. Both the extraction method using phosphate buffer and the measurement protocol are applicable without complicated laboratory tools. The bioassay can measure low amounts of the pesticide in solution (22 ng/mL) and hence can be directly used for the monitoring of insecticide in vine samples well below the maximum acceptable level (200 ng/g). The reproducibility obtained for blank samples (CV = 12%) makes the application of the method for quantitative analysis straightforward.

According to the data obtained with grape samples and vine leaf samples, the latter matrix can be used for an evaluation of the protection level in the vineyard and help agriculture operators in treatment planning and control.

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