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Najwa Ben Oujji, Idriss Bakas, Georges Istamboulie, Ihya Ait-Ichou, Elhabib Ait-Addi, et al.. An easy-to-use colorimetric enzymatic test-system for organophosphorus insecticides detection in olive oil. Science Innovation, 2014, pp.5-9. 10.11648/j.si.s.2014020601.12 . hal-01166296

HAL Id: hal-01166296

<https://hal-univ-perp.archives-ouvertes.fr/hal-01166296>

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An easy-to-use colorimetric enzymatic test-system for organophosphorus insecticides detection in olive oil

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To cite this article:

Najwa Ben Oujji, Idriss Bakas, Georges Istamboulie, Ihya Ait-Ichou, Elhabib Ait-Addi, Régis Rouillon, Thierry Noguer. An Easy-to-Use Colorimetric Enzymatic Test-System for Organophosphorus Insecticides Detection in Olive Oil. *Science Innovation*. Special Issue: Biosensors: Innovative Technique for Food Safety and Environmental Monitoring. Vol. 2, No. 6-1, 2014, pp. 5-9. doi: 10.11648/j.si.s.2014020601.12

Abstract: Immobilization of a low amount of electric eel acetylcholinesterase on microtiter plates by bioencapsulation in a sol-gel composite led to the preparation of a simple colorimetric enzymatic-assay for pesticides detection in olive oil. This bioassay has been used to carry out inhibition studies with three pesticides mainly used in the treatment of olive trees: Malaoxon, Methidathion-oxon and Omethoate, and tested using standard solutions and real samples of olive oil. The developed bio-tools showed a good reproducibility and stability as well as good analytical performances with limits of detection at least 10 times less than the maximum residue limit tolerated by international regulations. The obtained LODs were as low as 10^{-9} M for the widely used pesticide Malaoxon, 10^{-8} M for Methidathion oxon and 10^{-7} M for Omethoate. The developed test was designed for fast detection of the three target insecticides in such a complex matrix as olive oil without a laborious pre-treatment and after a simple liquid-liquid extraction, which demonstrate the potentiality of this technique to be used for commercial purpose.

Keywords: Electric Eel Acetylcholinesterase, Bioassays, Organophosphorus Insecticides, Olive Oil, Colorimetric Test, Sol-Gel

1. Introduction

The cultivation of olive trees to obtain olives and olive oil has been known since antiquity. At the time of the Roman Empire, olive cultivation had spread throughout the Mediterranean basin and today, with 98% of olive trees that exist in the world, the Mediterranean countries present the big part of the world production of this yield. Olive oil is the main ingredient and essential component of the healthy diet for majority of people living in that area, since it possesses highly beneficial nutritional and culinary properties due to its unique composition in containing fatty acids and antioxidants [1]. Considering these beneficial properties and increasing worldwide distribution, there has been an extended demand for efficient cultivation and mass production of olive oil [2]. Beside relevant advances in production, there are some serious problems caused by different insects which attack and damage olive trees. One of them is the common olive oil fruit fly *Bactrocera oleae*, which induces an increase of acidity, affects fruits shelf life and decreases the yield of the olives. In

order to protect trees from the infection and avoid significant losses in olive oil production, trees are usually treated with suitable pesticides which act as repellents against flies or other insects. Despite the advantages which agrochemicals offer for the improvement of the olive's yield, they still remain highly toxic and compromise the quality of olive oil. Therefore, regular monitoring and detection of these chemicals are essential for consumer protection and healthy diet [3].

Pesticide-residue determination in olive oil is becoming a very important and challenging issue. Conventional methods of detection of organophosphorus pesticides rely on an analysis by gas chromatography with specific detection. Although these techniques are very powerful and can detect very low concentrations, they are still very expensive and require highly skilled personnel, expensive purification steps and specialized major equipment. In the last decades, new technologies based on biological detection systems have emerged. Among these techniques, biosensors have been shown to be very promising due to their simplicity and cost effectiveness compared to conventional techniques [4].

Biosensors and bioassays based on the inhibition of acetylcholinesterase (AChE) and other enzymes have been intensively studied in the aim of detecting organophosphorus insecticides in water and other matrix, but in our knowledge, it's the first time a study have interested to the development of enzymatic tools to detect these compound in olive oil.

The present paper describes the development of a Simple Colorimetric Enzymatic-Assay for the rapid and sensitive detection of three organophosphorus insecticides widely used in the treatment of olive trees: Malaoxon, Omethoate and Methidathion-oxon. The systems are based on the immobilization of a low amount of electric eel acetylcholinesterase by bioencapsulation in a sol gel matrix on Elisa microplates. This test presents the advantage of saving time, reagents and solvents as it allows analyzing 96 samples in a single assay in just 2 h, this time including the samples preparation, the incubation with pesticides and the enzymatic measurement, using small volumes of chemicals reagents and solvents, which is not possible using the traditional chromatographic systems.

2. Materials and Methods

2.1. Chemicals and Stock Solutions

AChE (EC 3.1.1.7) from electric eel (EE) (Type V-S, 1,000 U/mg) was purchased from Sigma-Aldrich (St Quentin-Fallavier, France). Acetylthiocholine Iodide (ATChI), and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB-Ellman's reagent) were provided by Sigma. In order to minimize hydrolysis, ATChI solution was prepared daily in 0.9% NaCl (Sigma-Aldrich) solution. Stock solutions of enzymes and DTNB were prepared in 0.1 M phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, Sigma-Aldrich) at pH 7. The organophosphorus insecticides Malaoxon, Omethoate and Methidathion were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Pesticide stock solutions (concentration 10^{-3} M) were prepared in acetonitrile and stored at 4 °C, working pesticide solutions were prepared daily in distilled water by dilution of the stock solution. The oxidation of Methidathion was achieved using N-bromosuccinimide (NBS) provided by Sigma-Aldrich. Hexane, Acetonitrile and Dichloromethane were purchased from Sigma-Aldrich (France). Sol-gel matrices were prepared using tetramethoxysilane (TMOS), poly(ethyleneglycol) 600 (PEG), hydroxyethyl-cellulose (HEC) (Sigma-Aldrich) and hydrochloric acid (HCl) from Carlo Erba, Italy.

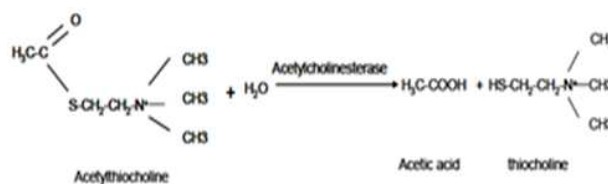
2.2. Apparatus

The determination of AChE activity was carried out with a Hewlett Packard diode array 8451A spectrophotometer. Colorimetric measurements were performed with a Labsystems Multiskan EX 126 microtiter plate reader (Thermo Life Sciences, Cergy Pontoise, France). The enzyme was immobilized on microtiter plates PS, form U, 96 wells (Greiner, Germany) and Chromatographic measurements were performed using an HPLC L-2000 series LaChrom

Elite® system from Merck-Hitachi (VWR, France).

2.3. Determination of Acetylcholinesterase Activity

The activity of AChE was measured spectrophotometrically by monitoring at 412 nm the appearance of thionitrobenzoate resulting from the reaction of DTNB with thiocholine, the product of the enzymatic hydrolysis of acetylthiocholine substrate, according to the procedure described by Ellman *et al.* [5]. This method is based on the use of a synthetic substrate: acetylthiocholine, whose hydrolysis liberates thiocholine and acetic acid according to the reaction:



The thiocholine reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) yielding a yellow complex absorbing at 412 nm ($\epsilon = 1.36 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (Figure1)

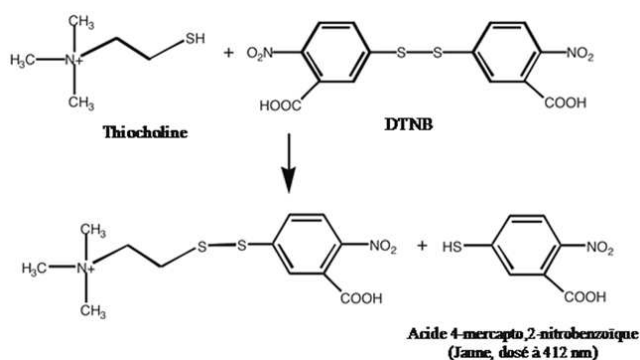


Figure 1. Colorimetric Detection of thiocholine by Ellman method (1961)

2.4. Immobilization Protocol

The Immobilization of the bioreceptor presents the first and the most important step in the development of a bioassay. It can be defined as a technique which limits the free displacement of the biological element. The immobilization method used must retain maximum enzyme activity and preserve the three-dimensional conformation of the enzyme, especially at its active site. In this study, the research has been directed towards the sol-gel technique to attach the enzyme to the microplate:

The sol-gel technique has been known for over than 150 years thanks to Ebelmen, which was the first to describe the synthesis of silica from a silicon alkoxide [6]. Recently, this process has been used to entrap a wide variety of biological species, including enzymes and living microorganisms for various applications [7; 8].

Sol-gel process involves hydrolysis of alkoxide precursors under acidic conditions followed by condensation of the hydroxylated units, which leads to the formation of a porous gel. First, a low-molecular weight metal alkoxide precursor molecule such as tetramethoxysilane (TMOS) or

tetraethoxysilane (TEOS) is hydrolysed in the presence of water at acidic pH, resulting in the formation of (Si-OH) groups. In a second step, the condensation reaction between silanol moieties at alkaline pH results in the formation of siloxane (Si-O-Si) polymers, creating a matrix in which an enzyme can be successfully entrapped [9]. In some cases, the cracking of the sol-gel polymers can be prevented using doping agents such as poly(ethyleneglycol).

In this work the protocol of immobilization was the following: the sol-gel solution was prepared by mixing 150 μL of the precursor tetramethoxysilane (TMOS) with 413 μL of distilled water, 400 μL of HCl 1 mM, and 37 μL of PEG 600. This mixture was sonicated for 15 min and stored for one night at 4°C. The enzymatic solution prepared in phosphate buffer (pH 8) was mixed 1:1 (v/v) with HEC (2%). Then, this solution was mixed with the sol-gel in a ratio 2:1 (v/v) [10]. 20

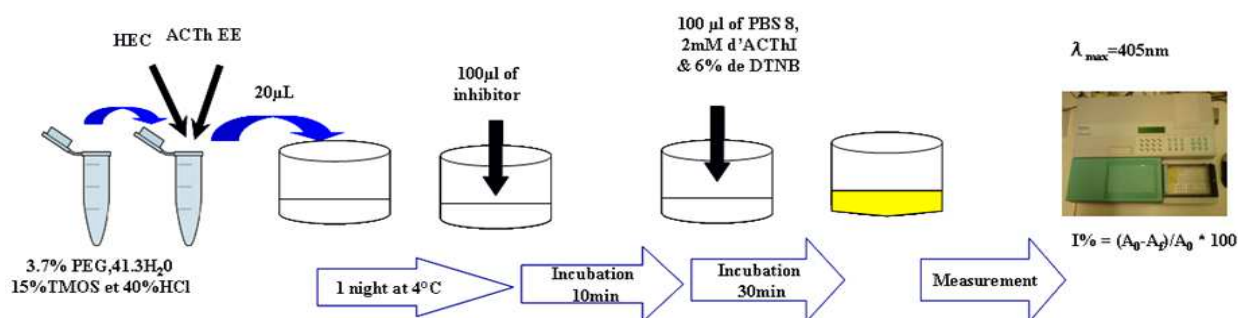


Figure 2. Immobilization and colorimetric measurement protocols.

2.6. Sample Preparation and Pesticides Extraction

Many of the commercially available organophosphorus (OP) insecticides are phosphorothionates (lipophilic), which are characterized by one thione moiety (P=S) and three -OR groups attached to a phosphorus atom, whereas their respective oxidized analogues are more polar, characterized by a double phosphorus oxygen bond (P=O), which actually makes them less stable, more toxic and potent AChE inhibitors [11]. Therefore, this study focuses on the detection of the oxidized forms of three organophosphorus insecticides mainly used in the treatment of olive trees: Malathion, Dimethoate and Methidathion.

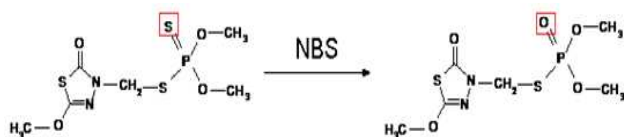


Figure 3. Oxidation of Methidathion using *N*-bromosuccinimide (NBS)

The oxidized forms of Dimethoate and Malathion, respectively called Omethoate and Malaoxon are commercially available, but in the case of methidathion an oxidation step must be carried out (Figure 3) using *N*-bromosuccinimide (NBS) [11]. The efficiency of this oxidation step was controlled using reverse-phase HPLC, it was shown that 3.10^{-5} M NBS was sufficient for the total oxidation of a 10^{-5} M Methidathion solution. The effect of NBS on AChE-EE has been also investigated to ensure that the enzyme was not affected by the

μL of the obtained solution was quickly spread at the bottom of the microtiter plate wells and allowed to dry for one night at 4 °C, so the final concentration of the enzyme immobilized in each well was 29mIU.

2.5. Colorimetric Measurements

To measure the enzymatic activity of the fixed enzyme, 200 μL of phosphate buffer pH 8 containing 2 mM ACTh-I and 6% DTNB were added to the well, and the microplate was incubated for 30 min in room temperature. The absorbance was then measured at 405 nm using the Labsystems Multiskan EX 126 microtiter plate reader. The inhibition measurements were carried out using the same procedure as described above, after incubation of the immobilized enzyme for 10 min with 100 μL of different concentrations of pesticide as shown in Figure 2.

oxidizing agent, it was shown that in assays conditions NBS did not inhibit AChE [12].

The determination of the three target pesticides in natural olive oil samples was performed after a simple liquid-liquid extraction procedure as described previously [12;13]: 10 mL of a commercially available bio extra virgin olive oil purchased in a local supermarket was spiked with a known concentration of pesticide diluted in Hexane. The mixture was heated at 50 °C for 30 min, and 500 μL of this olive oil was added to 400 μL of acetonitrile and 100 μL of dichloromethane. The mixture was centrifuged at 13,400 rpm for 90 s and the resulting supernatant was recovered and used as a pesticide mother solution, other pesticide solutions were prepared by diluting this mother solution.

3. Results and Discussion

3.1. Reaction Time

The incubation time required for the reaction between the immobilized enzyme and the substrate was evaluated by measuring the absorbance continuously for 35 min using the microtiter plate reader. It appears from the obtained kinetics, that the reaction was completed 30 min after the addition of the acetylthiocholine Iodide. This reaction time was therefore applied to all measurements.

3.2. Storage Stability

The storage stability of the developed devices was evaluated

by measuring the enzymatic activity each day for two months. The results showed a good storage stability of the developed Kits, with less than 10% decrease of activity after one month of storage at 4 °C and 21% after 2 months (Figure 4).

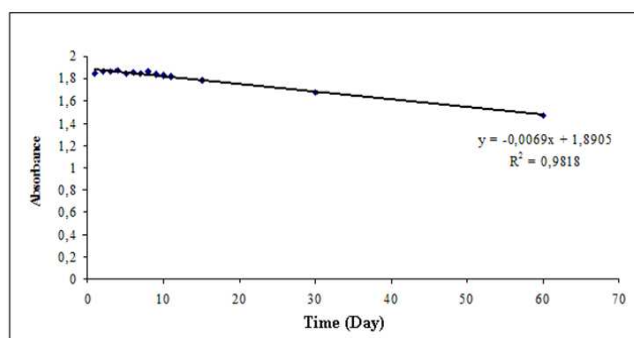


Figure 4. Storage stability of the developed Kits

The developed tests present also a good operational stability, as they maintained their initial analytical response for at least 7 successive determinations as well as a good reproducibility

Table 1. Performances of the developed bioassay for the detection of Omethoate, Methidathion-Oxon and Malaoxon. LOD (IC_{10}), IC_{50} and IC_{100} are the concentrations of pesticides inducing respectively 10%, 50% and 100% decrease of the signal.

Pesticide	Equation	R ²	IC ₁₀ (M)	IC ₅₀ (M)	IC ₁₀₀ (M)	Dynamic Range (M)
Omethoate	$I=7.3113\text{Ln}(C)+129.24$	0.9894	8.2×10^{-8}	1.9×10^{-5}	1.2×10^{-2}	$7.5\times 10^{-8}-5\times 10^{-3}$
Methidathion oxon	$I=25.291\text{Ln}(C)+446.41$	0.9439	3.2×10^{-8}	1.5×10^{-7}	1.1×10^{-6}	$7.5\times 10^{-9}-10^{-6}$
Malaoxon	$I=12.357\text{Ln}(C)+241.17$	0.9776	7.5×10^{-9}	1.9×10^{-7}	1.0×10^{-5}	$5\times 10^{-9}-7.5\times 10^{-5}$

3.4. Real Samples Analysis

Olive oil Samples were analysed before and after spiking with known concentrations of Malaoxon, Methidathion-oxon and Omethoate. The amount of pesticide spiked was 10^{-6} M, 7.5×10^{-8} M and 2.5×10^{-7} M for the Methidathion oxon; 2.5×10^{-5} , 10^{-5} and 10^{-4} M for the Omethoate and 10^{-8} , 5×10^{-7} and 2.5×10^{-7} M for the Malaoxon. The unspiked samples revealed no inhibitory effect, whereas the results obtained with the spiked samples were consistent and in good agreement with the spiked amount of pesticides and present a good correlation with the inhibition rate obtained with standards solutions. Table 2 shows the recovery yields calculated as $(\%R = (P_1/P_0)\times 100)$, where P_0 is the added (real) quantity of pesticide and P_1 is the found quantity.

Table 2. Recovery yields (%) obtained for the determination of Omethoate, Malaoxon and Methidathion-Oxon pesticides in spiked olive oil samples. RSD < 3 %.

	Pesticide Concentration(M)	Recovery (%)
Omethoate	2.5×10^{-5}	102.32
	10^{-5}	111.49
	10^{-4}	114.70
Methidathion Oxon	10^{-6}	117.14
	7.5×10^{-8}	108.37
	2.5×10^{-7}	109.96
Malaoxon	10^{-8}	121.16
	5×10^{-7}	114.30
	2.5×10^{-7}	101.14

A comparison between the limits of detection obtained by

with a Relative Standard Deviation (RSD) of 3.4 %.

3.3. Inhibition Measurements

The insecticides detection was carried out using an incubation time between the enzyme and 100 μ l of the inhibitor for 10 min, the analytical performances of the developed colorimetric test for the detection of the three target pesticides are summarized in Table 1. The limit of detection LOD was defined as the pesticide concentration that produced 10% decrease of the signal (IC_{10}), whereas IC_{50} and IC_{100} are the concentrations of pesticides inducing respectively 50% and 100% decrease of the signal. It was found that the developed systems present good analytical performance with good limits of detection as low as 7×10^{-9} for the Malaoxon, 3×10^{-8} for the Methidathion oxon and 8×10^{-8} for the Omethoate. The obtained LODs were at least 10 time less than the maximum residue limit tolerated by international regulations according to the French Ministry of Agriculture and Food in 2012 (AF920805) (see table 3)

the developed test and the MRLs for these three pesticides according to the French Ministry of Agriculture and Food in 2012 (AF920805) is presented in Table 3. It should be noted that detection limits reported in this table are obtained by application of the devices on natural samples of olive oil. The obtained LODs are much lower than the maximum residue limit tolerated by international regulations.

Table 3. Comparison between the LODs obtained with the developed method and the LMRs establish by the French Department of Agriculture and Agrifood 2012 (AF920805) (ppm: parts-per-million).

	LMR (AF920805)	LOD
Malaoxon	0,5 ppm	0,001 ppm
Methidathion Oxon	1 ppm	0,006 ppm
Omethoate	2 ppm	0,09 ppm

In a previous work [13], we have used the same sol-gel method to immobilize electric eel acetylcholinesterase on screen printed electrodes (SPE), which led to development of an efficient amperometric biosensor able to detect the three target pesticides in olive oil. Thus, the sol-gel method has enabled us the preparation of two analytical devices based on two different detection modes: amperometric and optical. Both tools presented good operational stability, good storage stability and good reproducibility, but when applying on natural samples of olive oil, the amperometric biosensor give better results with recovery percentage of 100% of all the concentrations tested and it proved to be best suited for the determination of insecticides in real samples of olive oil.

4. Conclusions

The immobilization of 28 mIU of electric eel acetylcholinesterase, by sol-gel method, on microtiter plates led to the preparation of a simple, cheap, fast and efficient colorimetric test able to detect and quantify three of the most used pesticides in the treatment of olive trees (Malaoxon, Omethoate and Methidathion oxon), in a such complex matrix as olive oil. The measurement was performed without a laborious pre-treatment and after a simple liquid-liquid extraction. The developed Kits present a good operational and storage stability, a good reproducibility as well as a good analytical performance with limits of detection at least 10 times less than the maximum residue limit tolerated by the international regulations (MRLs).

Acknowledgements

Authors acknowledge with gratitude the Averroes programme (EC programme Erasmus Mundus) for granting this work.

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