Food Control 30 (2013) 657–661

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Sol–gel immobilization of acetylcholinesterase for the determination of organophosphate pesticides in olive oil with biosensors

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ARTICLE INFO

Article history:
Received 7 July 2012
Received in revised form
31 August 2012
Accepted 3 September 2012

Keywords:
Electric eel acetylcholinesterase
Organophosphorus insecticides
Sol–gel
Amperometric biosensors
Olive oil
Screen-printed electrodes

ABSTRACT

This paper presents the construction of amperometric biosensors for the detection of organophosphorus insecticides widely used in the treatment of olive trees. The systems are based on the immobilisation of acetylcholinesterase on screen-printed electrodes by bioencapsulation in a sol–gel composite. The enzyme activity was estimated by measuring the thiocholine produced by the enzymatic hydrolysis of the acetylthiocholine chloride using cobalt phtalocyanine as mediator. The developed devices have been used to carry out inhibition studies with three pesticides: malathion, methidathion and dimethoate (in their oxidized form), and tested using standard solutions and real samples of olive oil. These biosensors showed good operational stability as they maintained their initial analytical signal response during 10 successive measurements, and a good reproducibility with a relative standard deviation of 3%. The limits of detection of the developed devices were very compatible with the maximum residue limit tolerated by international regulations, they were as low as 10^{-9} M for the widely used pesticide malaoxon(oxidized malathion). The developed sensors were successfully used for the determinations of insecticides in real samples of olive oil.

1. Introduction

The extensive use of organophosphorus and carbamate insecticides in modern agriculture has raised serious public concern regarding the environment and food safety. In the field of olive oil, insecticide treatments are applied every year to control the fly population, mainly based on pesticides belonging to the class of organophosphates. These chemicals allow crop protection of olive trees, however their residues detected in the oil and fruits are a major risk for consumer health. Therefore, both European Union and the Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) of the United Nations have established maximum pesticide residues limits (MRLs) for olives and olive oil (Lentza-Rizos & Avramides, 1995; Luchetti, 2002). Concerning the three pesticides used in this study, the MRLs are in order of 0.5 mg/kg for malathion, 2 mg/kg for dimethoate and 1 mg/kg for the methidathion, according to the French Department of Agriculture and Agri-Food (AF920805) (2012). As for olive oil, these MRLs correspond to molar concentrations ranging from $1.7 \cdot 10^{-6}$ M (malathion) to $9.7 \cdot 10^{-6}$ M (dimethoate).

In the last years, growing attention has been paid to the development of reliable, fast and low cost analytical systems to monitor pesticides from environmental and food industry. Chromatographic techniques (GC, HPLC) generally coupled with UV or MS detectors are currently used as reference methods and allow the detection of a wide range of pollutants with a very high sensitivity, reliability and precision. In spite of their advantages, these techniques require expensive instrumentation and highly trained personnel, are time consuming, and are not easily adapted to in field analysis (Andreescu, Barthelmebs, & Marty, 2002). New technologies based on biological detection systems have emerged and can be a good alternative for these classical methods. Among these techniques, biosensors have been shown to be very promising due to their simplicity and cost effectiveness compared to conventional techniques.

Biosensors are analytical devices which tightly combine biorecognition elements and physical transducers for detection of target compounds. In enzyme-based biosensors, the biological element is the enzyme which reacts selectively with its substrate

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http://dx.doi.org/10.1016/j.foodcont.2012.09.005

(Guilbault, Pravda, & Kreuzer, 2004). Amperometric biosensors based on inhibition of acetylcholinesterase (AChE) activity have been extensively studied to detect the presence of organophosphate pesticides in various kinds of samples (Andreescu & Marty, 2006; Istamboulie, Cortina-Puig, Marty, & Noguer, 2009).

This work describes the development of a cheap, fast and simple amperometric biosensor based on the inhibition of AChE for the detection of three organophosphorus insecticides commonly used for the treatment of olive trees: Malathion, Dimethoate and Methidathion in their oxidized forms (Fig. 1). The sensor was designed for the fast detection of insecticides contained in olive oil after a simple liquid–liquid extraction. Previous works have described the development of amperometric and optical biosensors based on the immobilisation of AChE on magnetic microbeads by covalent coupling which had a good analytical performance with reasonable reproducibility and stability (Ben Oujji et al., 2012). This work aims to enhance the performance of the previously described systems by optimizing the immobilisation procedure of AChE on low-cost screen-printed electrodes using sol–gel entrapment. The general principle of the sensing device is shown in Fig. 2. To our knowledge it is the first description of a sol–gel based biosensor for the detection of these pesticides in olive oil.

2. Materials and methods

2.1. Chemicals and stock solutions

AChE (EC 3.1.1.7) from electric eel (EE) (Type V-S, 1000 U/mg) was purchased from Sigma–Aldrich (St Quentin-Fallavier, France). Acetylthiocholine chloride (Sigma–Aldrich) solutions were prepared daily in 0.9% NaCl (Sigma–Aldrich) in order to minimize hydrolysis. Stock solutions of enzymes were prepared in 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_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 (Sigma–Aldrich) and stored at 4 °C, pesticide solutions were prepared daily in distilled water by dilution of the stock solution. The oxidation of methidathion was obtained using N-bromosuccinimide provided by Sigma–Aldrich. Sol–gel matrices were prepared using tetramethoxysilane (TMOS), poly(ethyleneglycol) 600 (PEG), hydroxyethyl-cellulose (HEC) (Sigma–Aldrich) and hydrochloric acid (HCl) (Carlo Erba, Italy). Graphite (Electrodag 423SS) and silver/silver chloride (Electrodag 418SS) inks were obtained from Acheson (Plymouth, UK). Cobalt phtalocyanine-modified carbon paste was purchased from Gwent Electronic Materials, Ltd. (Gwent, UK). Poly(vinyl)chloride (PVC) sheets (200 mm × 100 mm × 0.5 mm), supplied by SKK (Denzlingen, Germany), were used as support for the screen-printed electrodes. A glycerophtalic paint (Astral, France) was used as insulating layer.

2.2. Apparatus

The determination of AChE activity was carried out with a Hewlett Packard diode array 8451A spectrophotometer. Screen-printed electrodes were produced using a semi-automatic DEK248 printing machine according to a procedure previously described (Andreescu et al., 2002), but in a three-electrode configuration. The working electrode was a 4 mm-diameter disk, the auxiliary electrode was a 16 mm × 1.5 mm curved track and the Ag/AgCl pseudo-reference electrode was a 5 mm × 1.5 mm straight track. Amperometric measurements were carried out with a 641VA potentiostat (Metrohm, Switzerland), a constant potential of 100 mV was applied between screen-printed working and reference (Ag/AgCl) electrodes. The current was measured using a BD40 (Kipp & Zonen, The Netherlands) flatbed recorder.

2.3. Encapsulation in sol–gel

Among several industrial applications, sol–gel is a well-know technology for building physical matrices dedicated to enzyme immobilization (Brinker & Sheerer, 1990). Sol–gel has been used to entrap a wide variety of biological species, including enzymes and live microorganisms for different applications (Alvarez, Desimone, & Diaz, 2007; Andreescu et al., 2002). Sol–gel immobilization has been shown to improve the stability and catalytic activity of the biomolecules.

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 (Sassolas, Blum, & Leca-Bouvier, 2012) (Fig. 3). 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 immobilisation 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) (Hayat, Barthelmebs, & Marty, 2012). 2 μL of the obtained solution was quickly deposited on the surface of the working electrode, and allowed to dry for 3 h at room temperature. The amount of enzyme entrapped on each electrode was 3 mIU.

2.4. Determination of acetylcholinesterase activity

Before immobilization, enzymatic activity of AChE was determined using the Ellman's method (Ellman, Courtney, Andres, & Featherstone, 1961), based on the reaction between the enzymatic reaction product thiocholine with DTNB, which leads to the formation of a yellow compound (5-thio-2-nitrobenzoate) that can be spectrophotometrically detected at 412 nm.

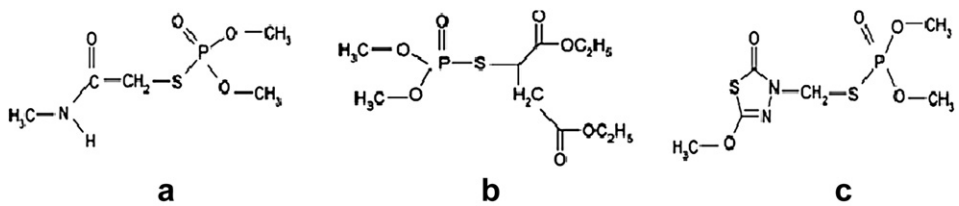

Fig. 1. Structure of organophosphates insecticides used in this study in their oxidized forms: (a) omethoate; (b) malaoxon; (c) methidathion oxide.