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Analytical Methods

Agent orange herbicides, organophosphate and triazinic pesticides analysis in olive oil and industrial oil mill waste effluents using new organic phase immunosensors

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ABSTRACT

New immunosensors working in organic solvent mixtures (OPIEs) for the analysis of traces of different pesticides (triazinic, organophosphates and chlorurates) present in hydrophobic matrices such as olive oil were developed and tested. A Clark electrode was used as transducer and peroxidase enzyme as marker. The competitive process took place in a chloroform–hexane 50% (V/V) mixture, while the subsequent enzymatic final measurement was performed in decane and using tert-butylhydroperoxide as substrate of the enzymatic reaction. A linear response of between about 10 nM and 5.0 μM was usually obtained in the presence of olive oil. Recovery tests were carried out in commercial or artisanal extra virgin olive oil. Traces of pesticides were also checked in the oily matrix, in pomace and mill wastewaters from an industrial oil mill. Immunosensors show good selectivity and satisfactory precision and recovery tests performed in olive oil gave excellent results.

1. Introduction

The speedy determination of any traces of pesticides in food oil has become an increasingly urgent need felt by both the food industries operating in this sector and consumer associations. Atrazine and simazine (triazinic herbicides) are among the most widely used weedkillers (Environmental Protection Agency, 2013; Ackerman, 2007). Although banned in the European Union (Krämer & Schirmer, 2007; Wackett, Sadowsky, Martinez, & Shapir, 2002), they are the most widely used herbicides in the US, while 2,4-D and 2,4,5-T, i.e. respectively dichloro-, or trichloro-phenoxyacetic acid are chlorinated phytopharmaceuticals, used as synthetic defoliants and forming the active principle of the so-called "agent orange" notoriously used in recent conflicts (Quastel, 1950; Freeman et al., 2011). On the other hand, parathion, a typical organophosphate pesticide, is a potent insecticide and acaricide. It was originally developed by IG Farben (Interessen-Gemeinschaft Farbenindustrie AG) in the 1940s. According to the non-governmental organization Pesticide Action Network (or PAN), parathion is one of the most dangerous pesticides. Its use is banned or restricted in 23 countries and its importation is illegal in a total of 50 countries (Fee, Gard, & Yang, 2005; Metcalf, 2002; Environmental Protection Agency, 2007). Nevertheless, due to the widespread use made of these pesticides in the past and unfortunately in certain areas also in recent times the need to detect their presence in different environmental and food matrixes has constantly been felt in the last few years. This has resulted in the development of numerous analytical methods (Font, Manes, Moltó, & Picó, 1993; Holden & Marsden, 1969; Pylypiw, Arsenault, & Thetford, 1997). The emphasis has been laid on methods that may be applied also "in situ" (Hennion & Barcelo, 1998; Hassoon & Schechter, 2000; Henriksen, Svensmark, Lindhardt, & Juhler, 2001). However, it should be stressed that many phytopharmaceutical compounds, as well as the above-mentioned pesticides, are more soluble in organic solvent or solvent mixtures than in aqueous solutions (Conte, Milani, Morali, & Abballe, 1997). This can cause serious problems in chemical analysis, which have only been partially solved by techniques such as gas chromatography (Conte et al., 1997; Hogendoorn & Van Zoonen, 2000; Eisert & Levsen, 1996; Coulson, Cavanagh, & Stuart, 1959) or MS (Wong, Webster, & Halverson, 2003; Kawaguchi, Inoue, Yoshimura, & Sakui, 2004). The difficulties can increase when the low solubility in water solution of the analyte (i.e. several pesticides) is coupled with the very low solubility of the real matrix in which the analyte is contained, for instance, edible oils. Enzymatic electrodes capable of operating in organic solvents, i.e. OPEEs have made a substantial contribution to solving this problem (Saini, Hall, Downs, & Turner, 1991; Campanella, Lelo, Martini, & Tomassetti, 2007; Campanella,

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Bonanni, Martini, Todini, & Tomassetti, 2005; Campanella, Dragone, Lelo, Martini, & Tomassetti, 2006; Sarkar & Gupta, 1989). Sometimes, however, their LOD is not sufficiently low; in addition, since these OPEEs for pesticide analysis are inhibition biosensors, it follows that this kind of device is relatively unselective versus pesticides belonging to different phytopharmaceutical classes. It is a known fact that immunosensors are the most selective biosensors, and our team, as well as other authors (Garcés-García, Morais, González-Martínez, Puchades, & Maquieira, 2004), has recently fabricated several immunosensors for pesticide determination (Tomassetti, Martini, & Campanella, 2012; Raman Suri, Boro, Nangia, & Gandhi, 2009; Rekha, Thakur, & Karanth, 2000). However this kind of immunosensor was able to operate only in aqueous solution and to test pesticides in aqueous matrices (Raman Suri et al., 2009; Tomassetti et al., 2012; Campanella, Eremin, Lelo, Martini, & Tomassetti, 2011). Therefore, when the problem arose of having to determine traces of pesticides in oily matrices, it was necessary to replace OPEEs (Organic Phase Enzyme Electrodes) with OPIEs (Organic Phase Immuno Electrodes). On the other hand, the development of new OPIE devices for pesticide analysis in edible oil matrices raised serious problems, both because of the scant information concerning effective immunocomplex formation in organic solvents available in the literature (Saini et al., 1991), and because the organic solvent used must satisfy several different requirements. These include the fact that the solvent can completely dissolve both the pesticide, the oily matrix and the labelled antibody, as well as not being too volatile and having a suitable log *p* value (Tomassetti et al., 2012). A series of tests were thus carried out in previous work (Tomassetti et al., 2012) by the authors using different solvents, different electrochemical transducers, different immunosensor construction and operating geometries. This series of trials led to the development of an amperometric immunosensor for the analysis of traces of triazinic pesticides in olive oil, working in 50% (V/V) chloroform n-hexane mixture, using a Clark electrode for oxygen made of PTFE as transducer and horseradish peroxidase as marker, as illustrated in previous research. This device was certainly innovative vis-à-vis what has so far been reported in literature, but it was also very suitable, as the k_{aff} value of the immunological method measured using the Langmuir curve was found to be of the order of 10^6 M^{-1} in the presence of the oily phase and about 10^7 M^{-1} in the absence of the oily phase (Tomassetti et al., 2012). These values show that, even when the antibody reaction occurs in organic solvent, antigen–antibody complex formation takes place more than satisfactorily and allows an immunological method to be developed correctly. The presence of an oily matrix does not affect the k_{aff} value more significantly. Finally the developed classical competitive organic phase assay also evidenced the need for a good solubility of the substrate of the final enzymatic reaction (i.e. tert-butylhydroperoxide). The organic solvent found to be best suited for the task was decane, even though the same 50% (V/V) chloroform n-hexane mixture utilized also for the competitive step also works satisfactory to perform the final enzymatic measurement (Tomassetti et al., 2012).

2. Experimental

2.1. Apparatus

The amperometric measurements were performed in a 5 mL thermostated glass cell at 23 °C under constant stirring. The Clark electrode, supplied by Universal Sensor Inc., New Orleans (USA), was connected to an amperometric biosensor detector provided by the same firms and to an analog recorder Amel mod. 868. In all experiments performed in organic phase, the plastic cap of the electrodes was replaced by a PTFE cap.

2.2. Reagents and materials

Anti-atrazine monoclonal antibody, anti-dichloro-phenoxyacetic acid (i.e. 2,4-D) and anti-trichloro-phenoxyacetic acid (i.e. 2,4,5-T) antibodies, as well as atrazine and simazine carboxyderivative, dichloro-phenoxyacetic acid (i.e. 2,4-D) and trichloro-phenoxyacetic acid (i.e. 2,4,5-T), were provided by Dr. S. Eremin (Department of Chemical Enzymology, Faculty of Chemistry, Moscow State University, Russia). Anti-parathion was a commercial antibody and was obtained from Acris (Acris Antibodies, Herford, Germany). 1-Chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine (i.e. Atrazine), 6-chloro-N,N′-diethyl-1,3,5-triazine-2,4-diamine (i.e. simazine), N-tert-butyl-6-chloro-N′-ethyl-1,3,5-triazine-2,4-diamine (i.e. terbuthylazine), diethyl 4-nitrophenyl phosphate (i.e. Parathion) were supplied by Pestanal Sigma–Aldrich (Sigma Aldrich, Milan, Italy). Potassium chloride, dibasic and monobasic anhydrous potassium phosphate RPE, chloroform RPE, dichloromethane RPE and diethyl ether RPE were supplied by Carlo Erba Reagents (Carlo Erba, Milan, Italy). Ny+ Immobilon Affinity membrane (porosity 0.65 μm) was provided by Millipore (Millipore Corporation, Vimodrone, Milan, Italy). The biotinylation kit, supplied by Sigma Immunochemicals (Sigma, Milan, Italy), was composed of biotinylation reagent (BAC-SulfoNHS, namely biotinamido hexanoic acid 3-sulfo-N-hydroxysuccinimide ester), 5 M sodium chloride solution, micro-spin column (2 mL) (in practice, a small empty cylindrical vessel prepackaged with Sephadex G-50), 0.1 M sodium phosphate buffer pH 7.2, 0.01 M phosphate buffer saline (PBS) pH 7.4 (reconstituted with 1 L of deionised water to give 0.01 M phosphate buffer, 0.138 M NaCl, 2.7 mM KCl, pH 7.4); lastly Extravidin® peroxidase (containing 0.2 mL of Extravidin Peroxidase conjugate at 2.0 mg mL^{-1}, with 0.01% thimerosal). Phenol, dialysis membrane (art. D-9777), 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide, albumin (from bovine serum) (BSA) and TRIS (hydroxymethyl-aminomethane), tert-buthylhydroperoxide solutions in decane solvent and TWEEN® 20, provided by Sigma Aldrich (Sigma Aldrich, Milan, Italy).

2.3. Samples

Pomace, olive oil, mill waste water, washing olive waters and olive oil samples were provided by an industrial (three centrifugation type) mill located in Central Italy. Two different analyzed commercial extra virgin olive oil samples, produced by the most important industrial Italian olive oil producer firms, were purchased from a local shop and stored in a sealed dark glass bottle, while two other extra virgin olive oil products, also stored in a sealed dark glass bottle, were supplied directly by a farmer from an area north of Rome (Italy).

3. Methods

3.1. Immunosensor assembly

The type of electrochemical transducer used was an amperometric gaseous diffusion amperometric electrode for O_2 determination (see Supporting Information Fig. A). The transducer consisted of a Clark type electrode. For the immunosensor assembly, in practice, three membranes were mounted on the PTFE cap of the Clark electrode, in the following order: the gas-permeable membrane, the dialysis membrane and the Immobilon membrane with antibody immobilized on it. The membranes were kept in place by a nylon net and a PTFE O-ring. A constant potential of –650 mV with respect to an $Ag/AgCl/Cl^-$ anode was applied to the Pt cathode of the oxygen electrode. Horseradish peroxidase enzyme was used as marker for immunocomplex detection.

3.2. Antibody immobilization on Immobilon membrane

A commercial Immobilon membrane was used for antibody immobilization. It consisted of a positively charged nylon membrane with polyester reinforcement optimized for reliable and reproducible transfer, immobilization, hybridization, and subsequent reprobing. The Immobilon Ny+ Membrane was cut into 1 cm^2 surface area disks and 50 μL of a 0.01 M antibody solution was directly deposited on the membrane surface. The membrane was then dried at room temperature for about 24 h and stored at 4 °C.

3.3. Atrazine pesticide carboxylation

The atrazine carboxylation method (Goodrow, Harrison, & Hammock, 1990) is as follows (see Fig. 1(a)): to a stirred heterogeneous mixture of 1.01 g of atrazine (i.e. 5.01 mM) in 100 mL of absolute ethanol a solution of 0.574 g of 3-mercaptopropanoic acid was added (i.e. 5.40 mM) together with 0.714 g of 85% KOH (i.e. 10.8 mM) in 10 mL of absolute ethanol, under N_2. At reflux the mixture became homogeneous and a precipitate (KCl) soon began to form. Reflux was continued (4 h). The hot mixture was filtered, and the filtrate concentrated to a white solid. The solid was triturated with 25 mL of 5% $NaHCO_3$ and filtered.

3.4. Parathion carboxylation

A solution of 3.0 g of 4-nitrophenol (i.e. 21.6 mM) in 15 mL of acetonitrile was added dropwise to a stirred solution containing 4.59 g (i.e. 27.8 mM) of methyldichlorothiophosphate, 20 g of finely ground K_2CO_3 and 20 mL of acetonitrile. After stirring for 1 h at room temperature the mixture was filtered through Celite and the solvent removed. The following format was applied to obtain the synthesis of the carboxylated parathion (see Fig. 1(b)): to a stirred solution of 500 mg of O-methyl-O-(4-nitrophenyl)phosphorochloridothioate (i.e. 1.87 mM) in 3 mL of MeOH cooled in an ice-water bath, a solution of 274 mg (i.e. 4.88 mM) of KOH and 229 mg of 4 aminobutyric acid (i.e. 2.22 mM) in 1.7 mL of MeOH was added dropwise. After stirring for 5 min, the reaction mixture was filtered and extracted with 1 M HCl-chloroform. The extract was dried over $MgSO_4$, and the solvent evaporated (Kim, Lee, Chung, & Lee, 2003).

3.5. Albumin-pesticide conjugation

The carboxylate pesticide was dissolved in 5 mL of dichloromethane to which 38 mg (i.e. 0.33 mM) of N-hydroxysuccinimide, 68 mg (i.e. 0.33 mM) of N,N-dicyclohexylcarbodiimide and 3.7 mg (i.e. 0.03 mM) of 4-dimethylaminopyridine were added. The mixture was stirred for 3 h and filtered, and the solvent removed. The format for coupling the carboxylate pesticide to the carrier protein is shown in Fig. 1(c), where, for the sake of example, the format used in the case of the atrazine pesticide is illustrated. Briefly, to prepare hapten-BSA conjugates, 20 mg (i.e. 0.3×10^{-3} mM) of BSA were dissolved in 2 mL of borate buffer (0.2 M, pH 8.7) to which 0.4 mL of DMF was added. A solution of an active ester (0.018 mM) in 0.1 mL of DMF was then added to the stirred protein solution, and stirring was continued for one day at 4 °C (Kim et al., 2003; Liu et al., 2007).

3.6. Albumin-pesticide biotinylation and peroxidase conjugation scheme

The avidin–biotin peroxidase technique (illustrated in Fig. 1(d)) is based on the use of a biotinylated antigen and of avidin horseradish peroxidase conjugate as part of the labelling system (Duk, Lisowska, Wu, & Wu, 1994; Rao, Anderson, & Bachas, 1999). The technique exploits the high affinity binding of biotin to avidin. The BiotioTag kit is specially designed for the small scale labelling of antibodies using biotinamido hexanoic acid 3-sulfo-Nhydroxysuccinimide ester (BAC-SulfoNHS) as the labelling reagent. This reagent is particularly useful when mild reaction conditions are required for the biotinylation of sensitive biomolecules such as antibodies, enzymes and surface proteins. After the labelling reaction, the biotinylated protein is separated from the unreacted or hydrolyzed reagent by a fast gel-filtration step using G-50 microspin columns. BAC-SulfoNHS reacts with free amino groups of proteins to form stable amide bonds. Extravidin binds to biotin with a high affinity and

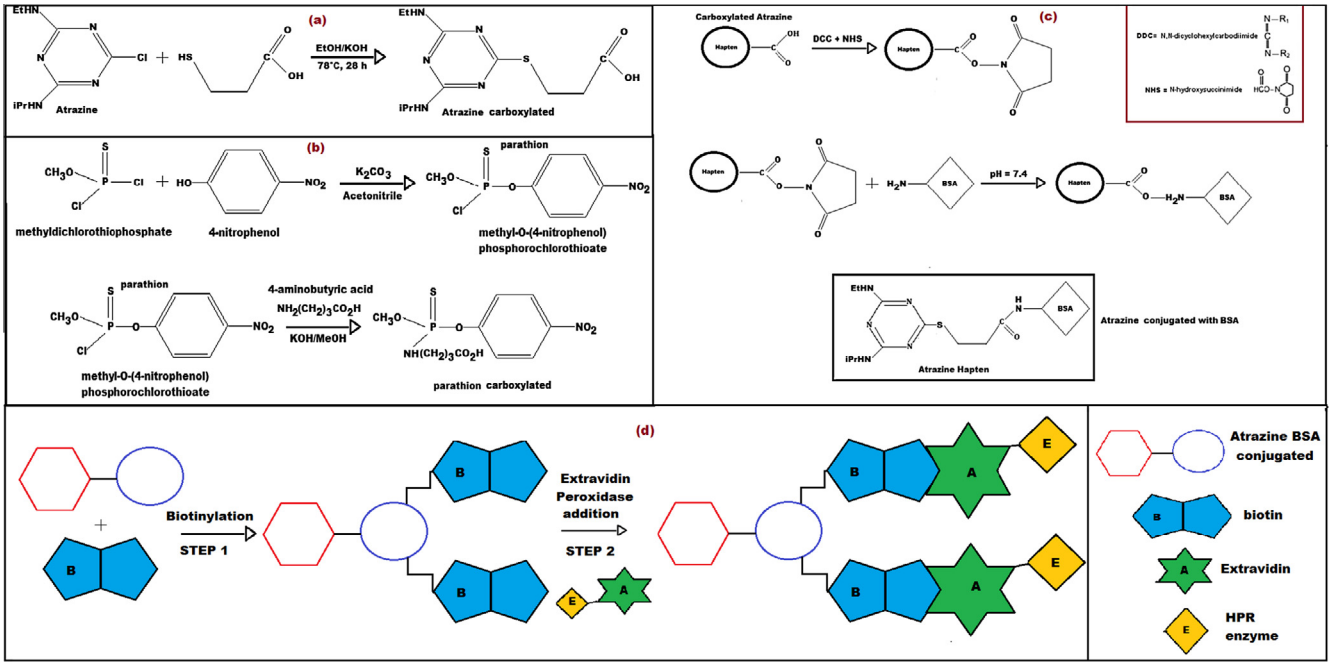

Fig. 1. (a) Atrazine carboxylation; (b) parathion carboxylation; (c) conjugation with BSA; (d) biotinylation and conjugation with Extravidin-peroxidase.

specificity. The high affinity for biotin alleviates non-specific binding interactions commonly associated with the strongly basic avidin protein. The use of the extended spacer arm greatly improves the interaction between Extravidin and the biotinylated macromolecule, thus overcoming the steric hindrance present at the biotin binding sites of Extravidin. In brief: 0.1 mL of 4.5×10^{-4} M pesticide–albumin solution in sodium phosphate buffer (pH 7.2; 0.1 M) was prepared. BAC-SulfoNHS solution 5 mg mL^{-1} was also prepared separately by dissolving 5 mg of biotinamido hexanoic acid 3-sulfo-N-hydroxysuccinimide ester in 30 μL DMSO (dimethylsulphoxide) and adding sodium phosphate buffer (pH 7.2; 0.1 M) for a final volume of 1 mL. 10 μL of BAC-SulfoNHS solution were immediately added to the pesticide–albumin solution with gentle stirring and the mixture incubated under stirring for 30 min at 2–8 °C. Then the resin contained in a micro-spin G-50 column was re-suspended in the column by vortexing and equilibrated with 0.2 mL of PBS, (pH 7.40; 0.01 M); this buffer was needed also for the elution of the labelled protein from the column. The biotinylation reaction mixture was applied to the top-centre of the resin and the column was centrifuged for 5 min at 700×*g*. The purified sample was then collected at the bottom of an Eppendorf test tube. This step was repeated twice more and a total of three fractions were collected. As Extravidin binds to biotin with a high affinity and specificity, an Extravidin peroxidase solution (20 μL, 2.0 mg mL^{-1}), diluted 1:100 in PBS containing 1% BSA solution, was added to the collected fractions and incubated for 1 h at room temperature, then gently rinsed with PBS, (pH 7.4; 0.01 M).

3.7. Determination of pesticide by immunosensor. (Competitive format between pesticide Biotin-Avidin-peroxidase conjugated and non-conjugated, both free in chloroform–hexane mixture, for the antibody immobilized in membrane)

For this purpose, the Immobilon membrane, on which the antibody was immobilized, was fixed to the head of the amperometric Clark type electrode as described in Section 3.1. Before measurement, the immunosensor was dipped into a Tris–HCl buffer solution, (pH 8.0; 0.1 M), containing 0.05% Tween-20 by weight and 2.5% BSA by weight (bovine albumin was used to minimize non specific adsorption on the membrane). The pesticide to be determined was added in 5 mL of chloroform–n-hexane mixture 50% (V/V) contained in the measurement cell, together with a fixed supply of pesticide Biotin-Avidin-peroxidase conjugated, i.e. 20 μL (0.01 M solution) of conjugated pesticide in the same solvent mixture. The peroxidase-conjugated pesticide was allowed to compete with the non-conjugated pesticide, both free in solution, in binding with the antibody immobilized on the Immobilon membrane. After washing with the same solvent mixture to remove all the unbound labelled pesticide, the specific substrate of the enzyme, i.e. 20 μL of tert-buthylhydroperoxide solution 1% V/V was added to 5 mL of the selected organic solvent, i.e. decane, in which the immunosensor was dipped, under stirring. The signal measured (as nA) of the transducer correlated directly with the pesticide concentration to be measured. The higher the pesticide concentration to be measured, the lower the oxygen consumed in the enzymatic reaction reported in Fig. (A) of Supporting Information, the higher the amperometric signal of the O_2 reduced at the Clark electrode. The sequence for measuring the pesticide by the above format is schematized in Fig. 2. Using this format a calibration curve was constructed (by plotting the current variation ΔI (nA) as a function of the log of pesticide concentration (as *M*) and employed to determine the unknown concentration of pesticide contained in the sample. In this way the original data of the immunosensor response to increasing antigen concentration, which always display logarithmic trends, take on a sufficiently linear trend. Moreover, the application of other, even more complex methods, described in the literature to obtain the same result, for instance the so called logit-log method, or equations with three-four parameters (Flynn, 2004) do not provide better results than those described above. The enzymatic reaction response took about 15–20 min. Individual measurements were performed, each time using a new membrane.

3.8. Membrane regeneration

A membrane already used in a previous test can be regenerated by suitably rinsing it in a solution of glycine 0.1 M and $MgCl_2$ 2.5 M, at pH = 2. This solution is apparently capable of splitting

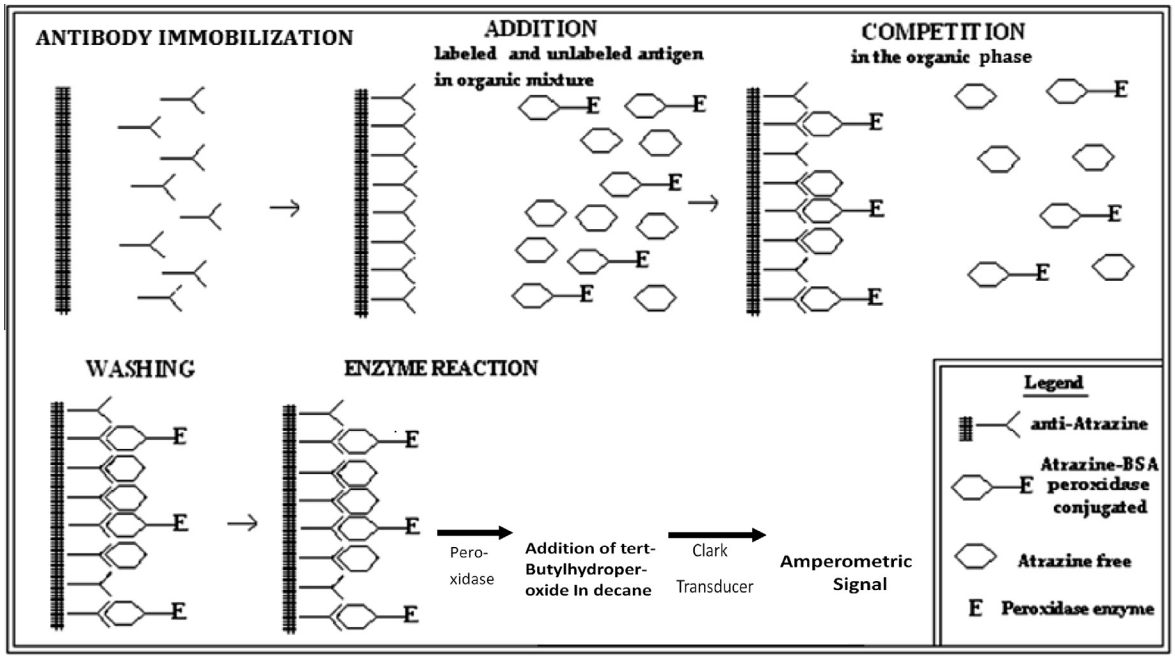

Fig. 2. Measurement: competition between pesticides and a fixed concentration of peroxidase pesticide conjugated, both free in organic phase solution, for the antibody immobilized in membrane.

the antibody complex. However, it was found that the immunosensor response when using a regenerated membrane may be up to 15% lower than in the previous measurement. This would of course affect the repeatability of the measurement and thus the method's precision.

3.9. Pesticides measurement in real oily samples or mill effluents and recovery tests

The immunosensor described in this paper was used first of all to measure the triazinic (atrazine, simazine, tert-buthylazine), organophosphate (i.e. parathion) and chlorurate (i.e. 2,4-D and 2,4,5-T) pesticides possibly present in commercial and artisanal olive oils using the respective calibration curves, then to measure the traces of triazinic pesticides found during the olive oil production process in different waste effluent samples (e.g. olive washing and vegetable waters), as well as in olive oil and pomace, firstly several tests were performed to check for traces of triazinic pesticides in olive oil samples examined 'as is'. To this end, 0.5 mL of the oily sample were added in 4.5 mL of chloroform–n-hexane 50% (V/V) mixture and the measure was performed as described in paragraph 3.7 above using the simazine calibration curve to obtain the final concentration of triazinic pesticides, expressed as simazine concentration. In addition, recovery tests were carried out on the oily samples spiked with known concentrations of each pesticide in order to obtain a final pesticide concentration of about 10^{-8} M. For this purpose 200 μL of a solution of pesticide 10^{-5} M was dissolved in 4.5 mL of chloroform–n-hexane 50% (V/V) mixture, to which 0.5 mL of a commercial or artisanal extra virgin olive oil sample were added. Also in this case the measurements were performed as described in paragraph 3.7 and using the respective calibration curves to obtain the concentrations of spiked solutions.

A second kind of test was run to determine triazinic pesticides concentration in real samples taken during olive oil production process in industrial mills (olive washing waters, olive mill wastewaters (vegetable waters), oil and olive oil). When the olive oil sample was being tested, 0.5 mL of the sample were added directly to the measurement cell, as previously explained for the measures carried out on commercial and artisanal oils, while, in the case of the sample of "olive washing waters" and "olive mill wastewaters" (vegetable waters) 2.0 mL and 1.0 mL samples, respectively, were taken and added to the measurement cell making up to 5.0 mL with chloroform–n-hexane 50% (V/V) mixture. The measurement was then performed as above described in Section 3.7. In the case of the pomace sample, 50 g of the sample were mixed with the chloroform–n-hexane 50% (V/V) mixture, stirred for about 10 min, then centrifuged at 1500 rpm for 8 min. Then 2.5 mL of the supernatant were removed and added to 2.5 mL of chloroform–n-hexane 50% (V/V) mixture in the measurement cell and the measurement performed in the normal way.

4. Results and discussion

4.1. Parameter optimization

The factors such as the concentration of the labelled antigen used free in solution, or the concentration of the substrate used in the final enzymatic reaction, the competitive step time and so on, were optimized in a previous investigation (Tomassetti et al., 2012). The Clark type electrode was used as in practice the gas-permeable membrane protects the working and reference inner electrodes. This ensures that the system is more repeatable and the method 'robust' (Sassolas, Prieto-Simón, & Marty, 2012). As explained in the introduction, the choice of the organic solvent in which to perform the competitive format involved taking multiple factors into consideration. An ad hoc table, as reported in a previous paper (Tomassetti et al., 2012), showed how the chloroform–n-hexane 50% (V/V) mixture, used also in the present work, represents a good compromise between all the various requirements set out in the "Section 1". Indeed, both the checked analyte and the oily samples are completely soluble in this solvent mixture, as is also the marked antigen. The hydrophobicity value of this mixture is still satisfactory ($\log p < 3$, but > 2), and the dielectric constant (DEC = 3.7) is neither too high nor too low. In other words the choice fell on an organic but non alcoholic mixture that had previously proved to be particularly suitable also when enzymatic OPEEs were being developed (Campanella, De Luca, Sammartino, & Tomassetti, 1999). Other tests, performed in the previous research and in the same conditions (Tomassetti et al., 2012), evidenced that also the signal obtained from the final peroxidase enzymatic reaction was higher when performed in organic solvent, particularly in decane, than in aqueous solution, since, as reported in the literature (Saini et al., 1991; Tomassetti et al., 2012), the enzymatic sensor's response is several times better when operating in organic solvent (or solvent mixture) than in aqueous buffer (owing to the lower interference from hydrophilic ionic species, lower microbial contamination, enhanced thermostability, and so on).

4.2. Carboxylation and BSA conjugation formats

With regard to the fabrication of the labelled pesticide required in the functioning of the immunosensors constructed, it should be noted that, in the case of atrazine, as described in the preceding Section 3.3, it had first to be carboxylated (see Fig. 1(a)). The latter could thus be conjugated with the BSA (Fig. 1(c)). As far as the enzymatic labelling is concerned, the classical method was used involving avidin and Extravidin (Duk et al., 1994; Rao et al., 1999) (Fig. 1(d)) as described in Section 3.6. Also in the case of the parathion it was necessary to follow the same reaction format, described for atrazine in Fig. 1(d), to obtain the labelled hapten. Of course the reactions to achieve carboxylation (see Fig. 1(b)) were slightly different from those used to carboxylate the atrazine (Fig. 1(a)). Finally, in the case of the 2,4-D and the 2,4,5-T, it was naturally not necessary to perform pesticide carboxylation as their molecule already possesses a carboxyl group. It was therefore sufficient directly to perform the conjugation with BSA, and thus the customary enzymatic labelling with avidin and Extravidin illustrated respectively in Fig. 1(c) and (d) for the atrazine. Fig. 2 shows the scheme of the measurement and the competitive format between the pesticide and a fixed concentration of peroxidase pesticide conjugated, both free in the organic phase mixture, for the antibody immobilized in the membrane.

4.3. Analytical data

The behaviour of new OPIEs' response as a function of growing 2,4-D, 2,4,5-T, or parathion pesticide concentration, the corresponding calibration curves and the confidence intervals for three pesticide determinations, obtained using a semilogarithmic scale, are shown in Fig. 3, while the analytical data obtained for the respective calibration curves, for the analysis of these pesticides in extra virgin olive oil, are summarized in Table 1. On the other hand, the same data, for the analysis of atrazine and simazine, already found in the previous research (Tomassetti et al., 2012), are, for the reader's convenience, also displayed in the same Table 1 in the present paper. Significantly, the linear range is very similar in all cases (between about 10 nM and 5.0 μM) as is the LOD (between about 4 and 8 nM). The highest calibration sensitivity was found to be that of the immunosensor for atrazine towards atrazine itself, i.e. almost double that of the other immunosensors towards the respective pesticides. However, also in the case of the

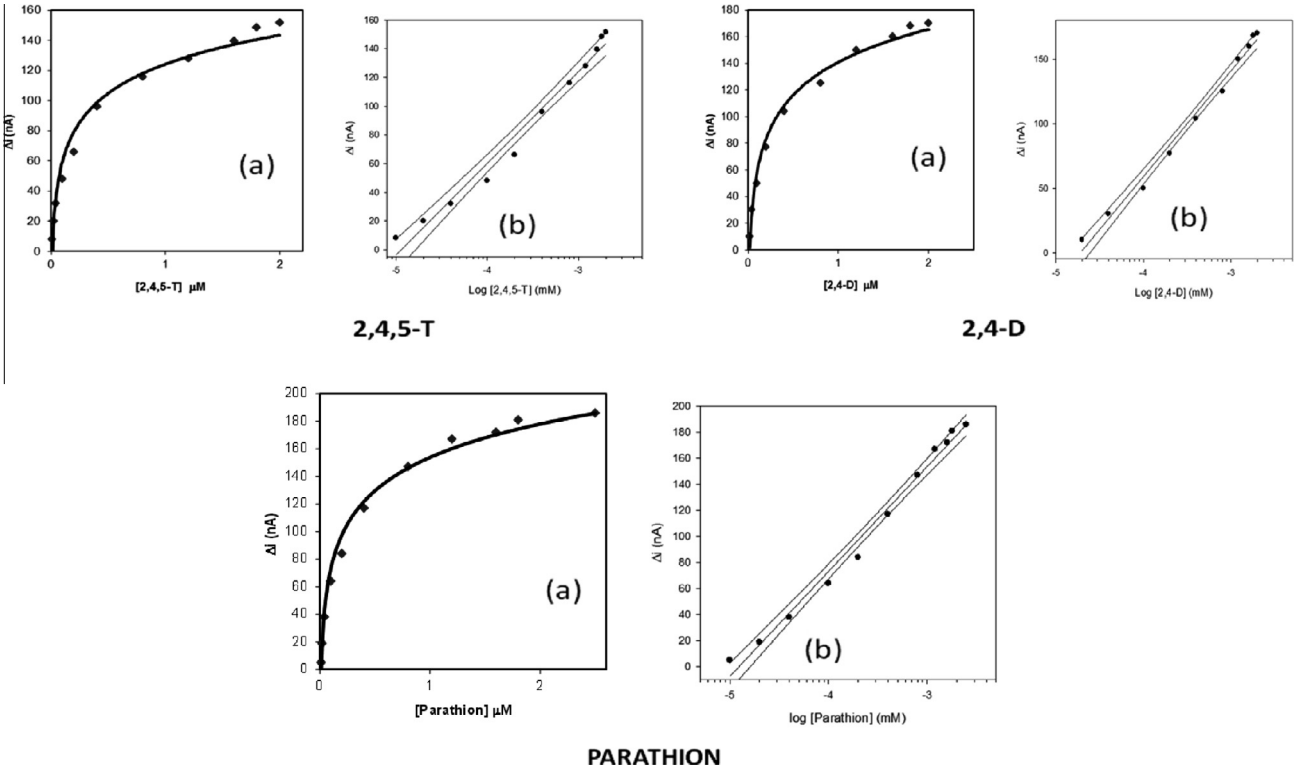

Fig. 3. (a) Behaviour of immunosensors response, respectively to 2,4,5-T, 2,4-D and parathion, as a function of growing pesticide concentration, using the Immobilon membrane for antibody immobilization, an amperometric electrode for O_2 as transducer and the competition in chloroform – n-hexane (50% V/V) in the presence of olive oil, lastly final enzymatic measurement in decane; (b) corresponding calibration curves and confidence intervals for respective pesticides determination obtained using a semilogarithmic scale.

Table 1
Analytical data of new OPIEs, obtain for different pesticides measurement: of 2,4-D, 2,4,5-T, parathion, atrazine and simazine calibration curves in presence of extra virgin olive oil, using Clark electrode for oxygen as transducer. The solvent used for competitive step was chloroform–n-hexane (50% V/V), while in the final enzymatic measurement decane was used as solvent and tert-butylhydroperoxide as substrate.

Pesticide	2,4,5-T	2,4-D	Parathion	Atrazine	Simazine
Regression equation ($y = \Delta i$ (nA), $x = M$)	$y = 72.1$ (±1.2) log$x$ + 143.6 (±10.9)	$y = 98.1$ (±1.9) log$x$ + 468.6 (±14.9)	$y = 80.5$ (±2.1) log$x$ + 395.2 (±17.5)	$y = 177.9$ (±4.2) log$x$ + 881.8 (±34.4)	$y = 98.1$ (±1.9) log$x$ + 468.6 (±14.9)
Linear range (M)	$1.2 \times 10^{-8} - 5.0 \times 10^{-6}$	$1.5 \times 10^{-8} - 5.0 \times 10^{-6}$	$1.0 \times 10^{-8} - 2.5 \times 10^{-6}$	$1.2 \times 10^{-8} - 5.0 \times 10^{-6}$	$1.2 \times 10^{-8} - 5.0 \times 10^{-6}$
Correlation coefficient	0.9869	0.9827	0.9882	0.9895	0.9869
Repeatability of the measurement (as pooled SD%)	7.2	7.3	7.0	6.1	7.2
Low limit of detection (LOD) (M) (RSD% $\leqslant$ 5.0)	0.4×10^{-8}	0.8×10^{-8}	0.5×10^{-8}	0.8×10^{-8}	0.8×10^{-8}

latter, the sensitivity towards the respective pesticides was found to be very good (between about 72 and 98 nA/log$M$). Generally speaking, the precision was of the same order and always acceptable (the pooled SD was found to be $\leqslant$7%, in any case). The Supporting Information (Table (a)) illustrates the selectivity of the immunosensor devices developed for the different pesticides (i.e. triazinic, chlorurate and organophosphate) by comparing the % response of each respective immunosensor to different pesticides. It may be observed that of the immunosensors constructed, the one for parathion analysis is certainly the most selective. Also noteworthy is that, except for the cases in which the structural formula of the pesticide being tested is very similar to that of the specific antigen of the antibody used (for instance, this is the case of simazine versus atrazine, or of 2,4,5-T versus 2.4-D), the antibody response to antigens belonging to different classes of pesticide, (i.e. paraoxon and carbaryl) is either very low or even negligible. This means that each of the immunosensors constructed possesses a good selectivity versus other classes of pesticide that differ from the class of pesticides for which it was constructed.

4.4. k_{aff} value determination

It was decided to test the validity also from the point of view of the immunological methods underlying the immunosensors constructed. For this purpose, a comparison is made in Supporting Information (Table (b)) between the k_{aff} and IC_{50} values obtained for the different pesticides tested using the Langmuir curve for the respective immunosensors. It is noteworthy that the k_{aff} values all lie between about (4×10^6 and 6×10^6) M^{-1}; in the case of simazine, the value is slightly lower (about 2.6×10^6), which is only to be expected as it was calculated using the immunosensor constructed for atrazine. Moreover, this is in agreement with what may be observed in the table of selectivities (see S.I. Table (a)).

4.5. Tests on real samples

A series of tests were run on real samples of extra virgin olive oil. Table 2 shows the triazinic pesticide concentrations (expressed as simazine concentration) found in commercial or artisanal extra

Table 2
Triazinic pesticides concentration (expressed as simazine concentration, both as *M* and as mg kg^{-1} of oil) found in commercial or artisanal extra virgin olive oil samples, using the atrazine OPIE.

Sample	Found concentration of simazine RSD% ⩽ 6.5		Value found in literature (mg kg^{-1})*
	(*M*)	(mg kg^{-1})	
Commercial oil n.1	Lower of the LOD value (0.8×10^{-8})	Lower of the LOD value (0.002)	0.005–0.5
Commercial oil n.2	2.8×10^{-8}	0.007	0.005–0.5
Artisanal oil n.1	6.5×10^{-8}	0.016	0.005–0.5
Artisanal oil n.2	Lower of the LOD value (0.8×10^{-8})	Lower of the LOD value (0.002)	0.005–0.5

* Ferrer et al., 2005.

Table 3
Traces of triazinic pesticides (expressed as simazine concentration) found during olive oil production process in different fluid samples.

Samples	Enzymatic measurement in phosphate buffer Concentration (*M*) In all cases RSD% ⩽ 5.5	Enzymatic measurement in organic solvent (decane) Concentration (*M*) In all cases RSD% ⩽ 5.5
Olive washing waters	1.71×10^{-8}	2.43×10^{-8}
Olive mill wastewaters (vegetable waters)	4.00×10^{-8}	4.05×10^{-8}
Pomace	2.16×10^{-8}	8.25×10^{-8}
Olive oil	2.82×10^{-8}	6.52×10^{-8}

virgin olive oil samples using the new OPIE for atrazine. Values are expressed both in *M* and in (mg kg^{-1} of oil) and referred to simazine, since the latter is the triazinic pesticide most frequently found in olive oil samples (Amvrazi & Albanis, 2006; Garcia-Reyes, Ferrer, Michael Thurman, Fernández-Alba, & Ferrer, 2006; Ferrer et al., 2005). In practice, in only one sample of the various artisanal oil samples tested were pesticide concentrations found that, although very small, proved not to be completely negligible, while the traces found in one of the commercial oils, although detectable, were so low as to be deemed practically negligible. However, also several recovery tests were carried out on spiked extra virgin olive oil samples in which the absence of any traces of triazinic pesticides had been ensured. In this case the tests were performed alternatively using both atrazine and simazine, or else tert-butylazine and, in the Supporting Information (Table (c)), it can be seen how recoveries always lie between about 96% and 104%, and are thus satisfactory. Other recovery tests were carried out also for the other classes of non triazinic pesticides using the respective OPIEs. As shown in the same Supporting Information (Table (c)), also in the case of the recovery tests involving 2,4-D or 2,4,5-T, or the Parathion pesticide, carried out in spiked extra virgin olive oil samples lacking any trace of pesticides, the percentage recoveries were found to be comparable to those found for the triazinic pesticides, and were thus also more than satisfactory. One final test was performed to check for the possible presence of traces of triazinic pesticides (expressed as simazine concentration) found during the olive oil production process in an industrial mill in Central Italy. For this purpose the atrazine OPIE was used to test both the oily products and the wastewater from a "centrifugation type" industrial mill. Samples of olive oil, pomace, olive mill wastewaters (vegetable waters) and olive washing waters were therefore analyzed. Since in this case the various samples were of different types, i.e. oily, mixture of solid-oil phase, water–oil emulsion and, in one case, also essentially aqueous phase, the tests were performed using the atrazine immunosensor both operating entirely in organic solvent mixture, following the format described in the Sections 3.9 and 3.7, or else following the same format but operating in aqueous medium. Table 3 shows how very small traces of pesticide were detected in all types of sample, the larger traces being found in pomace if operating in organic solvent. It should also be noted that the values obtained using the OPIE are always slightly higher than those obtained using the immunosensor operating in aqueous medium. As expected, the greater differences are those obtained for olive oil and for pomace since, as these products are highly hydrophobic, the determination using the OPIE gives much better results than when an immunodevice operating in aqueous medium is used. In the other two cases in which the samples were either water–oil emulsions (vegetable waters), or an almost wholly aqueous sample (olive oil mill washing waters), the results obtained were much more similar but in any case always slightly higher when an OPIE was used than those obtained using the immunosensor operating in aqueous solution. In our view, since the pesticide is much more soluble in organic solvent than in aqueous medium (Conte et al., 1997), this fact favours the distribution of the pesticide in the organic solvent, thus facilitating its determination and providing, also in this case, values that are only slightly higher than those obtained using the immunosensor operating in aqueous medium.

5. Conclusions

The results obtained so far have shown that several immunosensors operating in a 50% (V/V) chloroform–hexane solvent mixture can be developed for the purpose of determining atrazine, simazine, parathion, 2,4-D and 2,4,5-T in olive oil. It was also observed that good results were obtained using a Clark type transducer, performing not only the competitive assay but also the actual final enzymatic reaction in organic solvent instead of in aqueous solution, i.e. in practice carrying out the final electroenzymatic measurement using a classical OPEE. Best results were obtained using as substrate for the peroxidase catalyzed enzymatic reaction a solution of tert-butylhydroperoxide in decane. It was also demonstrated that when using the atrazine OPIE good results were obtained not only in the analysis of extra virgin olive oil or pomace oil, but also in the analysis of partially aqueous effluents which normally originate in ordinary industrial mills used to produce olive oil. The simplicity of the method, the relatively low cost and the ease of using these new immunodevices to detect traces of different types of pesticide in such an important food oil as olive oil means that the method is of great practical interest to the modern food industry. Fresh research is therefore already under way to extend the method also to other types of food oil, such as seed oil, in which for instance the detection of several of the pesticides tested, such as 2,4-D and 2,4,5-T, is generally much more frequent.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.07.137.

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