Food Chemistry 169 (2015) 358–365

Contents lists available at ScienceDirect

Food Chemistry

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

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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ARTICLE INFO

Article history:
Received 18 December 2013
Received in revised form 2 July 2014
Accepted 30 July 2014
Available online 7 August 2014

Keywords:
Immunosensors
Organic phase
Triazinic
Organophosphate and chlorurate pesticides
Analysis
Olive oil
Mill waste effluents

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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http://dx.doi.org/10.1016/j.foodchem.2014.07.137
0308-8146/

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.