DETERMINATION OF CHLORPYRIFOS, IMAZALIL AND THIABENDAZOLE IN WATER, WINE AND GRAPE SAMPLES USING ENZYME-LINKED IMMUNOSORBENT ASSAYS

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ABSTRACT

Pesticides are significant sources of diffuse pollutants that can cause environmental and food contamination, as well as health implications for living organisms. Thus, there is the necessity of simple, fast and cost-effective methods for the quantitative determination of pesticides in both environmental and food samples.

The aim of this work was the development and application of competitive indirect enzyme linked immunosorbent assays for the determination of chlorpyrifos, imazalil and thiabendazole in water, wine and grape samples. For this purpose, microtitration wells were coated with the respective pesticide-bovine serum albumin conjugate and blocked. After washing, 50 μL of calibrators in appropriate matrix and 50 μL of analyte-specific monoclonal antibody solution were added to wells and incubated for 1h. Then, an anti-mouse IgG-peroxidase solution was added in each well, followed by addition of chromogenic substrate (ABTS/H_2O_2) and measurement of the optical density at 405 nm. The assays had dynamic ranges from 0.05 to 2 ng/mL, and were precise (intra- and inter-assay coefficients of variation less than 5 and 8%, respectively). The detection limits were 0.005 ng/mL for chlorpyrifos and imazalil and 0.002 ng/mL for thiabendazole. For the determination of pesticides in wine samples (both white and red) a 30-times dilution was adopted, whereas for determination in grapes a 100-times dilution of grapes juice was necessary to avoid matrix effects. Taken into account the MRLs set by the EU for each particular pesticide in water (0.1 ng/mL for all pesticides), in wine and grapes (0.5 μg/mL for chlorpyrifos, 0.05 μg/mL for imazalil and thiabendazole, respectively), the assays developed meet the requirements for determination of the three pesticides in water, wine or grape samples.

Keywords: pesticide detection, chlorpyrifos, imazalil, thiabendazole, immunoassays, water samples, wine, grapes

1. Introduction

Pesticides have been widely used against pests that can damage crops such as insects, fungi and weeds, in order to prevent or reduce losses and improve product quality, for many years (Ntzani *et al.,* 2013). However, beside the associated benefits from pesticide use, the irrational application of these chemical compounds has led to their accumulation in surface and ground water, soil and food. Throughout years, many studies have been performed regarding the determination of pesticide residues in different matrices, as well as the adverse effects that they can pose to living organisms (Aktar *et al.,* 2009).

Chlorpyrifos, imazalil and thiabendazole are three pesticides that are widely used the first one as insecticide and the other two as fungicides, for the protection of crops both prior to and after harvesting. Competent authorities have performed risk assessment for these substances and acceptable daily intakes (ADI) have been derived based on the toxic effects on humans. In

detail, for chlorpyrifos an ADI of 0.001 mg/kg per body weight per day was set based on neurotoxicity studies on rats (EFSA Journal 2014; **12**:3640), while for imazalil and thiabendazole an allowable daily intake (ADI) of 0.025 mg/kg and 0.1 mg/kg of body weight per day, respectively, were set based on developmental toxicity studies performed in rabbits and rats (EFSA Journal 2014; **12**:3750). Due to the toxic side effects that these pesticides can cause, maximum residue limits (MRLs) are set for many food commodities, amongst them table and wine grapes where chlorpyrifos residue must be lower than 0.5 μg/mg while the MRL for each of imazalil and thiabendazole is set at 0.05 μg/mg (EU Regulation No 396/200). Moreover, according to the E.U. Directive 98/83/EC regarding drinking water, each pesticide must not exceed a concentration of 0.1 ng/mL, whereas the total concentration of all pesticides must be lower than 0.5 ng/mL.

In order to ensure food and water safety, reliable and accurate methods for the determination of pesticide residues in different matrices are of high importance and this is why pesticide residue analysis is becoming one of the most active directions in the field of analytical chemistry. Several chromatographic methods, for the determination of the pesticides of interest in water, wine and grape samples have been developed with high specificity, sensitivity and accuracy (Wu *et al.,* 2009; Dasika *et al.,* 2012; Walorczyk *et al.,* 2010). Nonetheless, most chromatographic methods require a laborious and time consuming sample preparation prior to analysis, in order to remove matrix interferences, while a large volume of toxic organic solvents is used.

Enzyme-linked immunosorbent assays (ELISA) have emerged as alternative methods to chromatographic ones for the determination of environmental contaminants since they are simple, cost effective and permit fast analysis of large samples numbers (Dankwardt *et al.,* 2001). A number of methods based on monoclonal or polyclonal antibodies have been developed for chlorpyrifos, imazalil and thiabendazole determination in various matrices such as water, vegetables, fruits and fruit juices (Brun *et al.,* 2005; Moreno *et al.,* 2007; Bushway *et al.,* 1994). However, to our knowledge, no ELISA method for the determination of pesticides using a simple sample preparation such as the dilution with assay buffer has been reported.

This paper describes the development and optimization of a simple indirect enzyme immunoassay procedure for the determination of chlorpyrifos, imazalil and thiabendazole in water, wine and grape samples, without the need of prior sample extraction.

2. Experimental

2.1. Materials and Instrumentation

The mouse monoclonal antibodies against chlorpyrifos, imazalil and thiabendazole, and the respective pesticides conjugates with bovine serum albumin were purchased from the Grupo de Inmunotecnologia of Universidad Politécnica de Valencia (Valencia, Spain). Goat anti-mouse IgG (Fc specific)-Peroxidase (anti-mouse IgG-HRP) conjugate produced in goat, pesticide analytical standards (PESTANAL®), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was from Acros Organics (Geel, Belgium), absolute ethanol from Carlo Erba SpA (Milano, Italy) and 96-well polystyrene plates were from Greiner Bio-One GmbH (Frickenhausen, Germany). The water that was used throughout the study was doubly distilled. Optical density of the microtitration wells was measured at 405 nm using Victor^3 1420 Multilabel Counter (Perkin Elmer; Milano, Italy).

2.2. Standard and sample preparation

A 1 mg/mL stock pesticide solution was prepared in absolute ethanol and stored at -20ºC. From this stock solution, calibrators with concentrations ranging from 0.05 to 2 ng/mL were prepared by serial dilutions in 50 mM phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 0.2% (w/v) BSA (assay buffer). Tap water was used for developing the assay in water, while wine and grape samples were purchased from local markets. All matrices were analyzed with LC-MS/MS method and found to be free of target pesticides. Water, wine and grape samples containing known concentrations of pesticides were prepared through spiking with the same pesticide

solutions used for preparing calibrators in assay buffer. Prior to analysis, wine samples were passed through a polytetrafluoethylene filter of 0.45 μm pore size, while grapes were homogenized and centrifuged for 5 min to remove suspended particles. Then, grape and wine samples were diluted 100- and 30-times, respectively, with assay buffer.

2.3. Enzyme-Linked Immunosorbent Assays

Microtitration wells were coated overnight with 100 μL per well of pesticide-BSA conjugate (at concentrations 0.25 ng/mL for chlorpyrifos and 0.5 ng/mL for imazalil and thiabendazole) in 50 mM carbonate buffer, pH 9.3 (coating buffer). After washing three times with 300 μL of 50 mM phosphate buffer, pH 7.4, 0.9% (w/v) NaCl, containing 0.05% (v/v) Tween 20 (washing buffer), wells were blocked for 1h with 50 mM phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl and 2% (w/v) BSA. Wells were washed like previously and then, 50 μL of calibrators in appropriate matrix and 50 μL of analyte-specific monoclonal antibody solution (at concentrations 130 ng/mL, 100 ng/mL and 50 ng/mL for chlorpyrifos, imazalil and thiabendazole, respectively) were added and incubated for 1h, while shaking. After another washing step, 100 μL of a 0.5 μg mL^{-1} goat anti-mouse IgG-HRP conjugate solution in assay buffer containing 0.05% (v/v) Tween 20 were added in each well and incubated for 30 minutes, while shaking. Finally, 100 μL of chromogenic peroxidase substrate (H_2O_2/ABTS) were added in each well and the optical density at 405 nm was measured after 30 min. All steps of the ELISA method were performed at room temperature.

3. Results and discussion

Competitive immunoassays were developed for the three pesticides using protein-analyte conjugate for coating the microtitration wells. The immobilized analyte molecules compete with the molecules in the sample for binding to the antibody. An enzyme-labelled secondary antibody was employed for the detection of immunocomplexes formed on the wells after the completion of the assay. The influence of several assay parameters to the assay performance, i.e, the half maximal inhibitory concentration (IC_{50}) and the maximum absorbance signal (A_0), was evaluated. It was found, that the assay signal and sensitivity were mainly affected by the BSA-conjugate used for coating and the analyte-specific monoclonal antibody concentrations. Through a checkerboard experiment, the optimum combination of conjugate/monoclonal antibody concentrations were determined and found to be 250 ng/mL and 130 ng/mL for chlorpyrifos, 500 ng/mL and 100 ng/mL for imazalil, and 500 ng/mL and 50 ng/mL for thiabendazole, respectively.

To eliminate matrix interferences, different sample dilutions with the assay buffer were tested for each one of the matrices tested. As is shown in Figure 1a for thiabendazole, for both white and red wine samples at least 30-time dilution was required so as the zero calibrator signal to match that obtained with assay buffer. Regarding grapes (Figure 1b), it was found that in order to eliminate matrix effects, the homogenized and centrifuged samples should be diluted at least 100 times (Figure 1b). It should be noted that for water samples no dilution was required. Similar results were obtained for the other two pesticides.

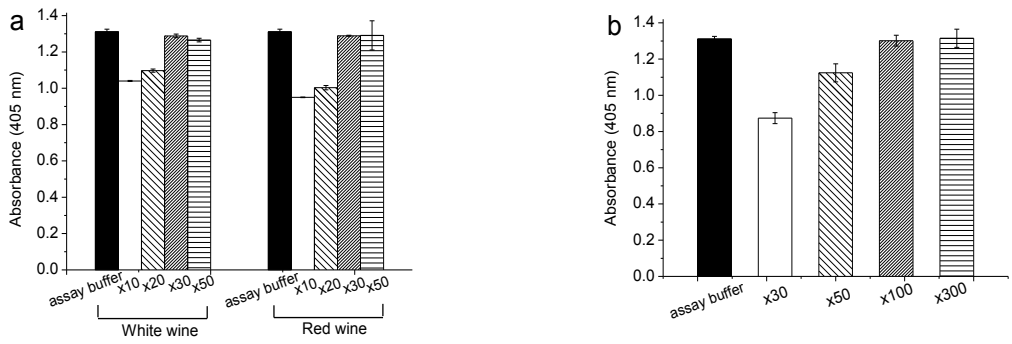

Figure 1: Effect of dilution of white/red wine (a) and grape samples (b) with assay buffer on the zero calibrator signals obtained for the thiabendazole assay.

The calibration curves obtained with the optimized ELISA protocols (Figure 2) had dynamic ranges between 0.02 ng/mL and 2 ng/mL, for chlorpyrifos and imazalil, and between 0.01 ng/mL and 1 ng/mL for thiabendazole. The limits of detection, calculated as the concentration corresponding to 100-3SD of the zero calibrator value, were 0.005 ng/mL for chlorpyrifos and imazalil and 0.002 ng/mL for thiabendazole. The assays for all target analytes were precise with intra- and inter-assay coefficients of variation less than 5% and 8%, respectively, and accurate with recoveries ranging from 83 to 115%.

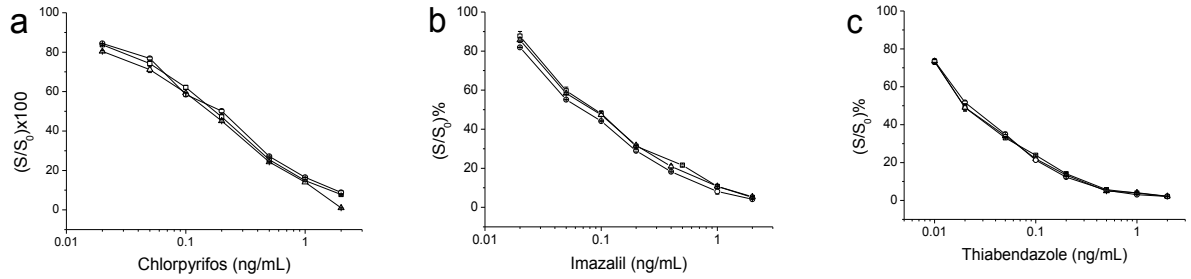

Figure 2: Calibration curves for chlorpyrifos (a), imazalil (b) and thiabendazole (c) obtained with calibrators prepared in water (-□-), wine diluted 30 times with assay buffer (-○-) and grapes diluted 100 times with assay buffer (-Δ-).

4. Conclusions

Competitive enzyme-linked immunosorbent assays for the quantitative detection of chlorpyrifos, imazalil and thiabendazole in water, white and red wine and grapes were developed. The assays were sensitive enabling detection of the three pesticides at concentrations as low as 0.06 and 0.2 ng/g for thiabendazole, and 0.15 ng/g and 0.5 ng/g for chlorpyrifos and imazalil in undiluted wines and grapes, respectively. These values that are at least 100 times lower than the MRLs set by the competent authorities and thus could be used for the determination of chlorpyrifos, imazalil and thiabendazole in water, wine and grape samples.

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