Application of a monoclonal-based immunoassay for the screening of imazalil in fruit juices

María-José Moreno, Emma Plana, Ángel Montoya, Pasquale Caputo, Juan Manclus

► To cite this version:

María-José Moreno, Emma Plana, Ángel Montoya, Pasquale Caputo, Juan Manclus. Application of a monoclonal-based immunoassay for the screening of imazalil in fruit juices. *Food Additives and Contaminants*, 2007, 24 (07), pp.704-712. 10.1080/02652030701243101 . hal-00577551

HAL Id: hal-00577551

<https://hal.science/hal-00577551>

Submitted on 17 Mar 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Application of a monoclonal-based immunoassay for the screening of imazalil in fruit juices

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2006-313.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	16-Jan-2007
Complete List of Authors:	Moreno, María-José; Universidad Politécnica de Valencia, Instituto de Investigación e Innovación en Bioingeniería Plana, Emma; Universidad Politécnica de Valencia, Instituto de Investigación e Innovación en Bioingeniería Montoya, Ángel; Universidad Politécnica de Valencia, Instituto de Investigación e Innovación en Bioingeniería Caputo, Pasquale Manclus, Juan; Universidad Politécnica de Valencia, Instituto de Investigación e Innovación en Bioingeniería
Methods/Techniques:	Immunoassays, Screening - ELISA
Additives/Contaminants:	Pesticide residues
Food Types:	Fruit juices

SCHOLARONE™
Manuscripts

Abstract

A monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) was developed for the quantification of imazalil [(*RS*)-1- β -allyloxy-2,4-dichlorophenylethyl]imidazole] in apple, tomato and orange juice samples. From an imazalil hapten which mimics the analyte structure, several monoclonal antibodies were obtained. An ELISA in the conjugate-coated format was developed and optimized using the antibody showing the highest sensitivity. For standards, the detection limit of the ELISA was 0.2 nM (0.06 ng ml⁻¹), with an I₅₀ value of 1.6 nM (0.5 ng ml⁻¹). The study of the influence of the matrices on the assay reliability indicated that the ELISA could determine imazalil in fruit juices at the low ng ml⁻¹ level simply by diluting the sample, without any clean-up or concentration step. Recovery and precision of the method were evaluated by spiking juice samples with imazalil in the 10–500 ng ml⁻¹ range. The mean recovery from fruit juices was 97% and the mean coefficient of variation was around 20%. In addition to being precise and accurate, the method has proved to be simple and sensitive, with a quantification limit well below the maximum residue limits for imazalil in these matrices.

Keywords: Imazalil; Azole fungicide; Monoclonal antibodies; Immunoassay; ELISA; Fruit juices.

Introduction

Imazalil [(RS)-1- β -allyloxy-2,4-dichlorophenylethyl]imidazole], is a systemic imidazole fungicide used to control a wide range of fungi on fruit, vegetables, and ornamental plants. It is also used for the treatment of barley and wheat seeds prior to planting, and to treat equipment and egg storage areas in chicken hatcheries. Imazalil was initially classified as likely to be carcinogenic in humans, but recent studies have provided sufficient evidence that imazalil is a threshold carcinogen. Exposure studies concluded that there is a reasonable certainty of no harm to any population subgroup from aggregate exposure to imazalil from dietary (food and water) exposure and all other non-occupational sources. On the basis of risk quotients, imazalil used at the recommended application rates should not result in an acute or chronic risk to mammal and avian species. Imazalil is moderately toxic to rats, freshwater fish and invertebrates following acute exposure. Results of imazalil ecological risk assessments show that none of the risk quotient values exceed the level of concerns for either terrestrial or aquatic non-target organisms. Fate studies showed that imazalil degrades slowly in soil and is not mobile, which mitigates the likelihood that this chemical will reach surface water and accumulate in fish (US Environmental Protection Agency 2006; Tomlin 1997). As mentioned above, imazalil is particularly widely used for post-harvest treatment of citrus, banana, and other fruit to control storage decay. Due to this application, imazalil residues can remain in fruit and, obviously, in their derivatives. This fact poses the need to establish legal directives to control their levels through the Maximum Residue Levels (MRLs), which are established by each country. In this sense, in Europe, the current legislation allows imazalil residue levels of up to 5 mg kg⁻¹ in fresh fruit (FAO/WHO 2006). Therefore, it is necessary to develop of appropriate analytical methods for the follow-up of imazalil residues in food.

Imazalil has been analysed by various chromatographic methods. They involve different extraction procedures followed by gas chromatography (Garrido et al. 1997; Matsumoto 2001; Yamazaki and Ninomiya 1996), liquid chromatography (Blasco et al. 2006; Yoshioka et al. 2004; Zamora et al. 2004) or gas-liquid chromatography (López and Riba 1999) analysis, coupled to different detection systems. The limits of detection of these techniques were in the range of 1–100 ng g⁻¹. Recently, a new method for rapid quantification of imazalil in citrus fruit has been developed, which uses flow-injection electrospray ionization tandem mass spectrometry (Ito et al. 2003). To date, these chromatographic methods are the most used, but they generally require a laborious sample pre-treatment and high-cost equipment. Immunochemical techniques, mainly enzyme-linked immunosorbent assays (ELISA), are alternative or complementary analytical tools to conventional methods. Immunochemical methods are based on the interaction of an analyte with an antibody that recognizes it with high affinity and specificity. They are simple, cost-effective, and field-portable, do not require sophisticated instrumentation, and are able to analyse a large number of samples. In practice, one of the most important advantages of immunoassays for pesticide analysis lies on their minimal requirement of sample cleanup. Over the last decade, several ELISAs based on polyclonal antibodies for residue analyses of azole fungicides have been developed (Cairolì et al. 1996; Chen et al. 1996; Forlani et al. 1992). Nevertheless, the amount of antiserum produced by even a group of animals is limited, and its characteristics vary from animal to animal and also between different breedings from the same animal. Monoclonal antibodies (MAb) can be produced in unlimited quantities and offer the possibility to standardize the assay method. Recently, the development of a sensitive ELISA for the detection of imazalil in citrus fruits samples based on monoclonal antibodies has been described (Watanabe et al. 2000). This ELISA required

imazalil extraction from samples, although no further clean-up was needed other than appropriate dilution of filtrated methanolic extracts. The same authors used this MAb in an immunoaffinity column clean-up for imazalil determination in citrus fruits by HPLC (Watanabe et al. 2001).

The analysis of fungicide residue in juice matrices by immunoassays offers considerable advantages over chromatographic techniques, since analyte extraction is not required and samples could be directly analysed by diluting them adequately. Therefore, the aim of this work was to obtain MAbs to imazalil for the development of enzyme-linked immunosorbent assays, whose analytical parameters could allow the simple detection of imazalil in fruit juice samples with a high sample throughput.

Materials and methods

Reagents and instrumentation

Analytical standard of imazalil and azole fungicides were from Riedel-de Haën (Seelze, Germany). Working stock solutions were prepared in N,N-dimethylformamide and stored at -20°C. Analytical grade solvents and silica gel 60, 0.06-0.2 mm, for column chromatography, were from Scharlau (Barcelona, Spain). Imazalil precursor, 1-(2, 4-dichlorophenyl)-2-(1-imidazolyl) ethanol, was from Acros Organic (Geel, Belgium). Potassium hydride 30 % wt dispersion in mineral oil, ethyl 6-bromohexanoate, ovalbumin (OVA), *o*-phenylenediamine (OPD), Sephadex G-50, and Freund's complete and incomplete adjuvants were obtained from Sigma-Aldrich Química (Madrid, Spain). Bovine serum albumin fraction V (BSA), enzyme-immunoassay grade horseradish peroxidase (HRP), hybridoma fusion and cloning supplement (HFCS), and polyethylene glycol (PEG) 1500 were purchased from Roche Diagnostics (Barcelona, Spain). Peroxidase-labelled rabbit anti-mouse immunoglobulins was obtained from Dako

(Glostrup, Denmark). Culture media (high-glucose Dulbecco's Modified Eagle's medium with GLUTAMAX I and sodium pyruvate, DMEM), fetal calf serum (Myoclon Super Plus), and supplements were from Gibco (Paisley, UK). Culture plasticware was from Corning (New York, USA). P3-X63-Ag8.653 mouse plasmacytoma line was from American Tissue Type Culture Collection (Rockville, USA). Polystyrene culture plates (High Binding Plates, cat. no. 3590) were from Costar (Cambridge, USA).

ELISA plates were washed with a 96 PW microplate washer from SLT Labinstrument GmbH (Salzburg, Austria), and absorbances were read in dual-wavelength mode (490-650 nm) with a Emax microplate reader from Molecular Devices (Sunnyvale, USA). Ultraviolet-visible reported spectra were recorded on a UV-160A Shimadzu spectrophotometer (Kyoto, Japan).

Preparation of imazalil protein conjugates

The structure of the imazalil hapten IMH [1-(2, 4-dichlorophenyl)-2-(1-imidazolyl) hexanoic acid] used to prepare imazalil protein conjugates is depicted in Figure 1. IMH was prepared by introduction of an alkyl chain spacer, ending in a carboxylic acid, by O-alkylation of the hydroxyl group of an imazalil precursor (Ito et al. 1998).

[Insert Figure 1 about here]

Preparation of immunizing conjugates. IMH hapten was covalently attached to BSA using the modified active ester method (Langone and van Vunakis 1982) and the mixed-anhydride method (Rajskowsky et al. 1977). According to the first method, 15 μmol of the hapten was reacted with stoichiometric amounts of N-hydroxysuccinimide and dicyclohexylcarbodiimide in 0.1 ml of DMF overnight at room temperature. After centrifuging, 0.083 ml of the clear supernatant containing the active ester was dissolved in 0.317 ml of DMF and slowly added to 0.6 ml of a 25 mg ml^{-1} BSA solution in 0.2 M

borate buffer, pH 9. The initial hapten to protein molar ration in the mixture was 55:1. The mixture was allowed to react at room temperature for 4 h with stirring, and finally the conjugate was purified by gel filtration on Sephadex G-50 using 100 mM sodium phosphate buffer, pH 7.4, as eluant. The extent of coupling of the hapten to BSA was estimated by the determination of the number of protein free amino groups before and after conjugation, using 2,4,6-trinitrobenzenesulfonic acid (TNBS) as the titration reagent (Habeeb 1966). The hapten to protein molar ratio was evaluated as 20.

According to the mixed-anhydride method, 16.70 μmol of the hapten was treated with stoichiometric amounts of tri-butylamine and isobutyl chloroformate in 0.229 ml of DMF for 30 min at room temperature. To the reaction mixture was added DMF up to 0.4 ml, and was slowly added to a solution of 15 mg of BSA in 0.6 ml of 0.2 M borate buffer, pH 9. The initial hapten to protein molar ration in the mixture was 73:1. After stirring for 2 h at room temperature, the conjugate was purified as above. The hapten to protein molar ratio in the conjugate was evaluated as 10.

Preparation of the coating conjugate. IMH hapten was covalently attached to OVA using the mixed-anhydride method. The hapten (11.2 μmol) was treated with stoichiometric amounts of tri-n-butylamine and isobutyl chloroformate in 0.153 ml of DMF for 30 min at room temperature. To the reaction mixture was added DMF up to 0.5 ml, and then was slowly added to 1.5 ml of a 20 mg ml^{-1} OVA solution in 0.2 M borate buffer, pH 9. The initial hapten to protein molar ration in the mixture was 17:1. After stirring for 2 h at room temperature, the conjugate was purified by gel filtration on Sephadex G-50 using 100 mM sodium phosphate buffer, pH 7.4 as eluant. The extent of coupling of the hapten to OVA was determined by the TNBS protocol above described. The hapten to protein molar ratio in the conjugate was evaluated as 5.

Preparation of the enzyme tracer. IMH hapten was covalently attached to HRP using the mixed-anhydride method. 11.2 :mol of the hapten was treated with stoichiometric amounts of tri-n-butylamine and isobutyl chloroformate in 0.153 ml of DMF for 30 min at room temperature. To 0.1 ml of the reaction mixture was added 0.9 ml of DMF, and 0.1 ml of this dilution (0.73 :mol) was slowly added to 1 ml of a 2.2 mg ml⁻¹ HRP solution in 0.2 M borate buffer, pH 9. The initial hapten to protein molar ration in the mixture was 15:1. After stirring for 2 h at room temperature, the conjugate was purified by gel filtration on Sephadex G-50 using 100 mM sodium phosphate buffer, pH 7.4, as eluant.

Production of monoclonal antibodies to imazalil

Four BALB/c female mice (8-10 weeks old) were immunized with the BSA-IMH conjugates. First dose consisted of 100 :g of conjugate intraperitoneally injected as an emulsion of 250 :l of a 1:1 (v/v) mixture of PBS (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and complete Freund's adjuvant. Three subsequent injections were given at three-week intervals using the immunogen emulsified in incomplete Freund's adjuvant. After a resting period of at least three weeks from the last injection in adjuvant, mice received a final soluble intraperitoneal injection of conjugate in PBS, four days prior to cell fusion. Cell fusion and hybridoma selection procedures were carried out essentially as previously described by Manclús et al. (2004).

Antibodies were directly purified from late stationary phase hybridoma culture supernatants by saline precipitation with saturated ammonium sulfate (1:1, v/v), followed by affinity chromatography on a HiTrap Protein G column (Amersham Biosciences, Uppsala, Sweden).

ELISA procedures

ELISA plates were coated overnight with conjugate or antibody solutions in 50 mM carbonate buffer, pH 9.6. A volume of 100 μ l per well was used throughout all assay steps, and all incubations were carried out at room temperature. After each incubation, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Two basic formats were used depending on the assay component immobilized into the ELISA plates. In the conjugate-coated format, an indirect ELISA was used for the screening of culture supernatants, and a competitive indirect ELISA was used for the study of antibody sensitivity and specificity to analytes. In the antibody-coated format, the specific antibody was coated directly or by using a capture auxiliary antibody, and competitive ELISAs were followed to evaluate the assay properties using the HRP enzyme tracer. For competition assays the concentrations of antibodies, hapten conjugates, or enzyme tracers were optimized by checkerboard titration.

Conjugate-coated format. A competitive ELISA, based on an homologous conjugate-coated format, was chosen. Immunoassays were performed as follows: ELISA plates were coated overnight with 0.75 μ g ml⁻¹ of OVA-IMH conjugate. Then, culture supernatant, or antibody dilutions in PBST (PBS containing 0.05% Tween 20) were added and incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labelled rabbit anti-mouse immunoglobulins diluted 1/2000 in PBST. Finally, peroxidase activity bound to the wells was determined by adding the substrate solution (2 mg ml⁻¹ OPD and 0.012% H₂O₂ in 25 mM citrate and 62 mM sodium phosphate, pH 5.35). After 10 min, the reaction was stopped with 2.5 M sulfuric acid, and the absorbance at 490 nm was read and recorded. For competitive assays, the procedure was the same except that after coating a competition step was introduced by adding 50 μ l of

the competitor followed by 50 :1 of the appropriate concentration of antibody (culture supernatant or purified MAb).

Antibody-coated format. In this format, plates were coated overnight with monoclonal antibodies either directly at 1 :g ml⁻¹ or indirectly with goat anti-mouse immunoglobulins at 2 :g ml⁻¹. Next, the competition was established for 1 h between imazalil standards and the selected dilutions of the enzyme tracer. Peroxidase activity was measured as above.

Preparation of imazalil standards and spiked juice samples

From a 1 mM stock solution of imazalil in DMF, serial dilutions in this solvent from 1 mM to 12.8 nM were prepared. From these concentrations, standards from 2 :M to 25.6 pM were prepared by 1/500 dilution in PBS. As the assay procedure involved the addition of the same volume of the appropriate immunoreagent concentration, imazalil standards in the final assay ranged from 1 :M to 12.8 pM (297.17 to 3.8 10⁻³ ng ml⁻¹).

Tomato, orange and apple juices were obtained from a local supermarket and were used without any sample pretreatment. For ELISA determination, spiked juice samples were daily prepared by dilution in PBS, and each dilution was analyzed in triplicate. The imazalil concentration in the samples was calculated by averaging values obtained at each dilution.

Data analysis

Competitive curves were obtained by plotting absorbance values against the logarithm of analyte concentration. Using a commercial software (Sigmaplot, Jandel Scientific), sigmoid curves were fitted to a four-parameter logistic equation:

$$y = \{(A-D)/[1+(x/C)^B]\}+D$$

where A is the asymptotic maximum (maximum absorbance in absence of analyte, $A_{\max}$), B is the curve slope at the inflection point, C is the x value at the inflection point (corresponding to the analyte concentration giving 50% inhibition of $A_{\max}$, I_{50}), and D is the asymptotic minimum. Imazalil concentration of fruit juice samples was determined by interpolation of the mean absorbance values on the standard curve run in the same plate (SOFTmax Pro Software, Molecular Devices).

Results and discussion

Imazalil immunoassay development

Imazalil, like most pesticides, is a small organic molecule which is non-immunogenic by itself and lacks a functional group for coupling to proteins. Therefore, the synthesis of pesticide-like molecules containing a functional group suitable for protein conjugation is a necessary and critical step in the production of high-affinity antibodies. The hapten IMH used to obtain monoclonal antibodies was synthesized by introduction of a spacer arm through the hydroxyl group of 1-(2, 4-dichlorophenyl)-2-(1-imidazolyl) ethanol, an imazalil synthesis precursor. Four cell fusion experiments were performed with each of the BSA conjugates of this hapten. In the screening of culture supernatants only wells showing a 50% signal inhibition in the presence of 1 μ M imazalil, with respect to that obtained in the absence of analyte, were considered as containing high-affinity antibody-secreting clones. From all fusion experiments, seven hybridomas fulfilled the above-mentioned criterion. These hybridomas were subsequently cloned and expanded in culture medium.

For the evaluation of the conjugate-coated format, non competitive experiments were carried out to estimate MAb titer. Thereafter, inhibition curves using imazalil as competitor were performed with the coating conjugate. Under these conditions, I_{50}

values in the nanomolar range were obtained (Table I). With respect to the antibody-coated formats, noncompetitive experiments were carried out prior to inhibition assays. Concentrations of HRP-IMH conjugate from 2 to 0.016 $\mu\text{g ml}^{-1}$ were tested in plates previously coated with the MAbs directly immobilized to the plate. HRP-IMH was only recognized by two MAbs (IMH-41 and IMH-42). Imazalil inhibition curves were performed for these two MAbs. I_{50} values of 118 and 130 nM were obtained for IMH-41 and IMH-42, respectively (Table I). The effect of a pre-coating step with goat anti-mouse Igs (indirect antibody-coated format) was also evaluated. This approach improved the assay sensitivity, as evidenced by a two-fold decrease of I_{50} values. As shown in Table I, among the monoclonal antibodies and ELISA formats evaluated MAb IMH-51 used in the conjugate-coated format provided the highest affinity ELISA to imazalil and, therefore, this combination of antibody and ELISA format was selected to carry out this study. The influence of the nonionic surfactant Tween 20, BSA (commonly used in ELISA to reduce nonspecific interactions), ionic strength, and pH on ELISA characteristics was examined (data not shown). The addition of Tween 20 to the assay buffer of the competitive step slightly improved the assay sensitivity (five-fold decrease of the I_{50} values). The highest assay sensitivity was achieved with a PBS buffer at neutral pH containing only a small amount of Tween 20 (0.0025%).

[Insert Table I about here]

Specificity of the imazalil immunoassay

The specificity of the IMH-51 monoclonal antibody was evaluated by performing competitive assays with other imidazole and triazole pesticides and determining their respective I_{50} values. As shown in Table II, the assay was very specific for imazalil, since the assayed pesticides were not significantly recognized. In detail, the imidazole moiety has an important role in the analyte-antibody interaction since just the change to

1
2
3 a triazole ring, while maintaining a similar exposed structure as represented by
4
5 penconazole, drastically reduces the compound recognition (cross-reactivity < 0.12%).
6
7 Furthermore, the imazalil metabolite, from which the IMH hapten was synthesized, was
8
9 poorly recognized.
10
11

12 [Insert Table II about here]
13

14 [Insert Figure 2 about here]
15
16

17 *Analytical characteristics of the imazalil immunoassay*

18
19 Figure 2 shows the standard curve obtained for the optimized imazalil competitive
20
21 immunoassay with MAb IMH-51. This sigmoid curve was obtained by averaging 8
22
23 individual standard curves performed during the imazalil determination in juice
24
25 samples. Prior to averaging, each individual curve was normalized by expressing the
26
27 absorbance (A_{490}) as the percentage of the maximum response ($100 \times A_{490}/A_{490, \max}$).
28
29 Table III summarizes the variability of the mathematical parameters that define the
30
31 sigmoid equation. Results are the average of 8 independent runs, each one fitted without
32
33 normalization as used for direct interpolation of samples. Coefficients of variation (CV)
34
35 were around 20% for A, B and C. The high CV of parameter D is due to the very low
36
37 values obtained for asymptotic minimum absorbances, normally below the microplate
38
39 reader precision. Referring to assay analytical parameters, the mean imazalil I_{50} value
40
41 was 1.6 nM (0.5 ng ml^{-1}) and the detection limit, estimated as the imazalil concentration
42
43 that reduced absorbance to 90% of the maximum, was 0.2 nM (0.06 ng ml^{-1}). The
44
45 working range was defined as the concentrations giving 20-80% inhibition, i.e., 0.4-8.0
46
47 nM ($0.12 - 2.4 \text{ ng ml}^{-1}$). These parameters indicate that this ELISA can be considered
48
49 sensitive enough for imazalil determination in fruit juices.
50
51
52
53
54
55
56

57 [Insert Table III about here]
58
59

60 *Evaluation of matrix effects*

Food samples contain substances that may interfere in analyte determinations. These interferences are known as matrix effect. In immunoassays, these effects are manifested in different ways, the most common ones being a loss of assay sensitivity (increase of I_{50}) or a decrease of colour development (overestimate), though a combination of both effects is also possible. Thus, the influence of the selected juices on imazalil immunoassays should be determined prior to the application of the method to samples spiked with the analyte. With this idea in mind, two experiments were performed.

In a first experiment, the matrix influence on standard curve parameters was analysed. Imazalil standards were prepared in duplicate in PBS buffer containing a variable proportion of each juice. A decrease of the maximum absorbance and of the assay sensitivity were observed at matrix proportions higher than 1/25, 1/50 and 1/100 for tomato, orange, and apple juice, respectively (data not shown). These results suggested that imazalil could be analysed in juices simply by appropriately diluting samples in PBS, without the need of a cleanup step.

In a second experiment, juice samples were spiked with imazalil at several levels and they were properly diluted to give a concentration close to the midpoint of the working range. The objective of this experience was to determine the matrix dilution that provided accurate and precise determinations of imazalil. Table IV shows the statistical analysis obtained for each fortified juice sample determined four times at the indicated dilutions. Matrix effects were reduced to acceptable levels when tomato, orange and apple juice samples were diluted at least 1/25, 1/50 and 1/100, respectively, as proved by the recovery and the precision data obtained. Mean recoveries were 81.4 %, 100.8 %, and 97.7 % for tomato, orange and apple, respectively, and most interassay CV were below 20 %.

[Insert Table IV about here]

Analysis of spiked juice samples

To evaluate the performance of the imazalil immunoassay, fruit juices were spiked with known concentrations of imazalil in the 10–500 ng ml⁻¹ range, and directly analysed by ELISA without any pre-treatment other than a 1/25, 1/50 and 1/100 dilution for tomato, orange and apple juice, respectively. After dilution, these fortification levels entered directly into the assay working range of the imazalil ELISA, and they are well below the maximum residue limits for imazalil in these crops (2–5 mg kg⁻¹). Spiked samples were analysed four times independently. In order to obtain the imazalil concentrations, absorbances from triplicate wells were interpolated in the standard curve performed in the same plate. Table V summarizes the results obtained for each spiked juice sample. Coefficients of variation can be considered as acceptable, ranging from 8.3 to 30.8, while recoveries were fairly good (mean values of 88%, 106%, and 97% for tomato, orange and apple juices, respectively). Taking all together, linear regression analysis indicated a good correlation between the concentration of imazalil added to juice samples and the concentration determined by ELISA ($y = 0.987x - 2.042$, $r^2 = 0.993$). These results prove that this immunoassay is able to quantitatively detect concentrations of imazalil as low as 10–50 ng ml⁻¹ in these juices.

[Insert Table V about here]

Conclusions

Following general guidelines for hapten design, IMH hapten was synthesized and used to produce monoclonal antibodies to imazalil. One of them, IMH-51, was selected for the development of a homologous indirect ELISA on the basis of its affinity and specificity to imazalil. The sensitivity of this immunoassay, expressed as the I₅₀ value for standards, was 1.6 nM imazalil (0.5 ng ml⁻¹). Most important, the ELISA presented here is able to determine down to 10–50 ng ml⁻¹ of imazalil in tomato, orange, and

apple juices with acceptable accuracy and precision for a residue analysis method. Furthermore, the simplicity of the immunoassay is clearly evidenced by its high sample throughput, since dozens of samples can be simultaneously analyzed simply by dilution.

Acknowledgements

References

Blasco C, Font G, Picó Y. 2006. Evaluation of 10 pesticide residues in oranges and tangerines from Valencia (Spain). *Food Control* 17: 841–846.

Cairolì S, Arnoldi A, Pagani S. 1996. Enzyme-linked immunosorbent assay for the quantitation of the fungicide tetraconazole in fruits and fruit juices. *Journal of Agricultural and Food Chemistry* 44: 3849–3854.

Chen T, Dwyre-Gygac C, Hadfield ST, Willetts C, Breuil C. 1996. Development of an enzyme-linked immunosorbent assay for a broad spectrum triazole fungicide: hexaconazole. *Journal of Agricultural and Food Chemistry* 44: 1352–1356.

FAO/WHO Food Standards [internet] Rome (Italy): Pesticide MRLs, Pesticide Residues in Food. 2006 - [cited 2006 March 2]. Available from: <http://www.codexalimentarius.net>

Garrido J, de Alba M, Jiménez I, Casado E, Folgueiras ML. 1997. Chromatographic analysis of imazalil and carbendazim in fruits: Method validation and residue monitoring program 1995. *Journal of Chromatography A* 765: 91–97.

Forlani F, Arnoldi A, Pagani S. 1992. Development of an enzyme-linked immunosorbent assay for triazole fungicides. *Journal of Agricultural and Food Chemistry* 40: 328–331.

- Habeeb AF. 1966. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Analytical Biochemistry* 14, 328–336.
- Ito S, Hayashi M, Watanabe S, Fujii A, Omoda N. 1998. Hapten compound and antibody of imazalil and measurement. Patent: JP10257886.
- Ito Y, Goto T, Oka H, Matsumoto H, Miyazaki Y, Takahashi N, Nakazawa H. 2003. Simple and rapid determination of thiabendazole, imazalil, and o-phenyl phenol in citrus fruit using flow-injection electrospray ionization tandem mass spectrometry. *Journal of Agricultural and Food Chemistry* 51: 861–866.
- Langone JJ, van Vunakis H. 1982. Radioimmunoassay of nicotine, cotinine, and γ -(3-piridyl)- γ -oxo-N-methylbutyramide. *Methods in Enzymology* 84, 628–640.
- López ML, Riba M. 1999. Residue levels of ethoxyquin, imazalil, and iprodione in pears under cold-storage conditions. *Journal of Agricultural and Food Chemistry* 47: 3228–3236.
- Manclús JJ, Abad A, Lebedev FM, Mojarad F, Micková B, Mercader JV, Primo J, Miranda MA, Montoya A. 2004. Development of a monoclonal immunoassay selective for chlorinated cyclodiene insecticides. *Journal of Agricultural and Food Chemistry* 52: 2776–2784.
- Matsumoto H. 2001. Simultaneous determination of imazalil and its major metabolite in citrus fruit by solid-phase extraction and capillary gas chromatography with electron capture detection. *Journal of AOAC International* 84: 546–550.
- Rajskowsky KM, Cittanova N, Desfosses B, Jayle MF. 1977. The conjugation of testosterone with horseradish peroxidase and a sensitive enzyme assay for the conjugate. *Steroids* 29, 701–713.
- Tomlin C. 1997. *The pesticide manual: A world Compendium*. The British Crop Protection Council: Farnham, Surrey, UK. p 691.

US Environmental Protection Agency [internet]. Available from:

<http://www.epa.gov/oppsrrd1/reregistration/imazalil/>. Accessed 2006 September 25.

Watanabe E, Watanabe S, Ito S, Hayashi M, Watanabe T, Yuasa Y, Nakazawa H. 2000.

Development of an enzyme-linked immunosorbent assay for the fungicide imazalil in citrus fruits. *Journal of Agricultural and Food Chemistry* 48: 5124–5130.

Watanabe E, Yoshimura Y, Yuasa Y, Nakazawa H. 2001. Immunoaffinity column

clean-up for the determination of imazalil in citrus fruits. *Analytica Chimica Acta* 433: 199–206.

Yamazaki Y, Ninomiya T. 1996. Determination of imazalil residues in lemons by gas

chromatography with nitrogen-phosphorus detection. *Journal of AOAC International* 79: 787–790.

Yoshioka N, Akiyama Y, Teranishi K. 2004. Rapid simultaneous determination of o-

phenylphenol, diphenyl, thiabendazole, imazalil and its major metabolite in citrus fruits by liquid chromatography-mass spectrometry using atmospheric pressure photoionization. *Journal of Chromatography A* 1022: 145–150.

Zamora T, Pozo OJ, López FJ, Hernández F. 2004. Determination of tridemorph and

other fungicide residues in fruit samples by liquid chromatography-electrospray tandem mass spectrometry. *Journal of Chromatography A* 1045: 137–143.

Table I. Sensitivity of antibodies estimated as I_{50} values for imazalil (ng ml^{-1}), obtained with different ELISA formats.¹

MAB	Conjugate-coated format	Direct antibody-coated format	Indirect antibody-coated format
IMH-41	61	35	20
IMH-42	180	39	20
IMH-51	2	- ²	-
IMH-52	80	-	-
IMH-53	77	-	-
IMH-54	10	-	-
IMH-55	6	-	-

¹ Competitive ELISAs were performed in optimum conditions, i.e., limiting concentrations of immunoreagents giving maximum absorbance around 1.0.

² No recognition.

Table II. Cross-reactivity¹ of several compounds with LIB-IMH51 monoclonal antibody.

Compound	Chemical structure	Cross-reactivity (%)
Imazalil		100
Climbazole		< 0.1
Prochloraz		< 0.1
Hexaconazole		< 0.1
Myclobutanil		< 0.1
Penconazole		0.12
Propiconazole		< 0.1
Tebuconazole		< 0.1
Tetraconazole		< 0.1
Triadimefon		< 0.1

¹ Cross-reactivity was calculated as (I₅₀ imazalil/I₅₀ compound) x 100.

Table III. Variability of the mathematical parameters of the sigmoid standard curve.¹

	A	B	C (I ₅₀)	D
Mean ²	0.91	1.02	1.61	0.024
SD	0.2	0.21	0.4	0.012
CV (%)	22	21	25	70
Minimum	0.64	0.8	1.03	0.008
Maximum	1.28	1.41	2.11	0.039

¹ Asymptotic maximum absorbance (A), slope at the inflexion point (B), concentration value in nM at the inflexion point (C), and asymptotic minimum absorbance (D) are the parameters which fit the sigmoid equation $y = \{(A-D)/[1+(x/C)^B]\} + D$.

² n = 8.

Table IV. Influence of matrix dilution on the reliability of the imazalil ELISA.

	Spiked levels (ng ml ⁻¹)	Sample dilution factor ¹	Imazalil found ² (ng ml ⁻¹)	Recovery (%)	CV (%)
Tomato juice	10	5	5.1	51.0	33.4
	50	25	41.0	82.0	22.6
	100	50	75.9	75.9	20.4
	200	100	177.3	88.7	11.3
	500	250	394.7	78.9	10.5
Orange juice	10	5	26.2	262.0	21.7
	50	25	39.7	79.4	20.0
	100	50	102.8	102.8	14.5
	200	100	213.0	106.5	24.2
	500	250	465.7	93.1	11.2
Apple juice	10	5	3.3	33.0	73.6
	50	25	35.8	71.6	14.3
	100	50	76.5	76.5	29.6
	200	100	183.7	91.9	9.8
	500	250	516.9	103.4	19.8

¹ Sample dilution before adding to ELISA plate.

² Data obtained from five independent determinations.

Table V. ELISA reproducibility and accuracy in spiked juice samples.

	Imazalil spiked level (ng ml ⁻¹)											
	Tomato juice				Orange juice				Apple juice			
	10	25	50	100	25	50	100	250	50	100	250	500
Mean value found [†]	9.7	22.8	35.0	94.6	33.4	44.1	98.8	262.2	36.2	108.0	273.6	486.7
SD	1.6	5.4	8.5	19.4	10.3	11.1	22.3	39.2	3.5	28.2	44.1	40.2
CV (%)	16.5	23.6	24.3	20.5	30.8	25.2	22.6	14.9	9.7	26.1	16.1	8.3
Minimum	8.7	17.7	26.8	72.2	21.8	33.6	73.9	217.1	32.7	75.7	224.4	440.3
Maximum	11.6	27.8	44.2	107.3	43.5	54.3	116.8	287.8	39.7	127.8	309.6	510.3
Recovery (%)	97.0	91.2	70.0	94.6	133.6	88.2	98.8	104.9	72.4	108.0	109.4	97.3

[†] Data are the average of 4 independent determinations.

Figure 1. Structures of imazalil and of the IMH hapten.
63x67mm (600 x 600 DPI)

Figure 2. Normalized averaged standard curve for imazalil ELISA ($100 \times A_{490}/A_{\max}$). Each point represents the mean of 8 values $\pm$ standard deviation.
339x299mm (150 x 150 DPI)