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Comparative study of monoclonal and recombinant antibody-based immunoassays for fungicide analysis in fruit juices

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Comparative study of monoclonal and recombinant antibody-based immunoassays for fungicide analysis in fruit juices

Running head: Comparative immunoanalysis of fungicides in juices

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Abstract

A comparative study of the analytical performance of enzyme-linked immunosorbent assays (ELISAs), based on monoclonal and recombinant antibodies, for the determination of fungicide residues in fruit juices has been carried out. To this aim, three murine hybridoma cell lines secreting specific monoclonal antibodies against tetraconazole, thiabendazole, and imazalil were used as a source of immunoglobulin gene fragments for the production of single-chain variable fragment (scFv) and fusion scFv-pIII recombinant antibodies in *Escherichia coli*. Selected recombinant antibodies displayed cross-reactivity profiles very similar to those of the parent monoclonal antibodies. Imazalil and tetraconazole recombinant antibodies showed one order of magnitude lower affinity than their respective monoclonal antibodies, whereas the thiabendazole recombinant antibodies showed an affinity similar to that of their parent monoclonal antibody. On the other hand, scFv-pIII fusion fragments showed similar analytical properties as, and occasionally better than, scFv recombinant antibodies. Finally, ELISAs developed from each antibody type showed similar analytical performance when applied to the analysis of the target fungicides in fruit juices.

Keywords: fungicide residues; monoclonal antibodies; recombinant antibodies; immunoanalysis; ELISA; fruit juices

Introduction

Imazalil [(*RS*)-1-(β-allyloxy-2,4-dichlorophenylethyl)imidazole], tetraconazole [(*RS*)-2-(2,4-dichlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propyl-1,1,2,2-tetrafluoroethyl ether], and thiabendazole [2-(4-triazolyl)benzimidazole] are broad spectrum antimicrobial compounds widely used as fungicides in the post harvest treatment of fruit and vegetables. Because of their application, residues of these fungicides can remain in agricultural products and, obviously, in their derivatives. This fact claims the establishment of legal directives worldwide to control their levels through the Maximum Residue Limits (MRLs). To guarantee consumer protection in the EU, MRLs have been harmonized by the European Commission in the Regulation No. 396/2005. According to this regulation, tetraconazole, imazalil, and thiabendazole levels up to 0.5, 5, and 15 mg/kg have been fixed, respectively, in fresh fruit and in their derivatives (EU Pesticide Database 2013). Therefore, appropriate analytical methods for an efficient monitoring of these fungicide residues in food are required.

Nowadays, liquid chromatography coupled with single or tandem mass spectrometry is the most common analytical methodology for these fungicides (Ferrer et al. 2011; Hiemstra and de Kok 2007; Yoshioka et al. 2010). These chromatographic methods require sophisticated and high-cost equipment only available in centralized, well-equipped laboratories. Immunochemical techniques, mainly enzyme-linked immunosorbent assays (ELISA), are alternative or complementary analytical tools to conventional methods. These techniques are simple, cost-effective, and field-portable; moreover, they do not require sophisticated instrumentation, and are able to analyze a large number of samples (Morozova et al. 2005).

Antibodies have been used during the last decades as immunoanalytical tools for the detection of low molecular weight contaminants in food and the environment.

Polyclonal antibodies are easy to obtain but their heterogeneity and limited supply can be a serious drawback for method standardization. The advent of hybridoma technology allowed to produce monoclonal antibodies (MAbs) of invariant specificity and selectivity and to immortalize the cells that produce these antibodies. However, MAb production is expensive, time-consuming, and considered as a rather inefficient procedure. As a third alternative, recombinant technology can be used to generate antibody fragments, as immunoanalytical tools for small analytes, in a more cost-effective and efficient manner than using the hybridoma technology; furthermore, the use of animals for antibody production can be minimized and eventually eliminated. Moreover, recombinant antibody (RAb) properties can be tailored at convenience by a range of DNA mutagenesis and protein engineering techniques (Brichta et al. 2005; Kramer and Hock 2007; Markus et al. 2011; Sheedy et al. 2007).

Recombinant antibody fragments can be isolated from display libraries using *in vitro* selection technologies that essentially mimic the *in vivo* process. The most frequently used are: phage display, ribosome display, or cell surface display in bacteria or yeast systems (Sheedy et al. 2007). Particularly, the advent of phage display technology, which allows the rapid and efficient isolation of RAbs from libraries of antibody fragments displayed on the surface of filamentous bacteriophages, was decisive in this evolution (Brichta et al. 2005). In the last decades, a large number of RAbs against high molecular weight molecules have been obtained to be used as diagnostic or therapeutic tools in health sciences and related areas. More recently, RAb technology has expanded to other applications such as the immunochemical analysis of low molecular weight compounds. Particularly, the production of RAbs to agrochemicals such as paraquat (Graham et al. 1995), diuron (Scholthof et al. 1997), parathion (Garret et al. 1997), cyclohexanedione (Webb et al. 1997), picloram (Yau et

al. 1998), chlorpyrifos (Alcocer et al. 2000), atrazine (Charlton et al. 2001), malathion (Nishi et al. 2003), 2,4−dichlorophenoxyacetic acid (Brichta et al. 2003), simetryn (Nishi et al. 2005), and methamidophos (Li et al. 2006) has been described. Initially, hapten-specific RAbs were obtained from hybridoma cells; subsequently, immunized lymphocytes were the source of genetic material to construct immune phage-display antibody libraries from which hapten-specific RAbs have been selected (Brichta et al. 2005; Markus et al. 2011). Lately, hapten-specific RAbs have also been isolated from non-immune or even synthetic libraries (Brichta et al. 2003; Kramer and Hock 2007; Markus et al. 2011). The most usual recombinant antibody fragment was the single chain variable fragment (scFv), where the V_H and V_L chains are single-domain linked by a 15 amino acids long polypeptide linker (Brichta et al. 2005). Expression of scFvs fused to proteins of interest has also been described. Different fusion protein partners have been evaluated to increase scFv solubility and stability, and to improve the detection, purification or immobilization of scFv (Leong and Chen 2008). Thus, short peptides as c-*myc*, poly-histidine, FLAG or HSV and larger polypeptides as the truncated pIII protein of phage M13 or alkaline phosphatase have been used for detection and purification; on the other hand, engineered single-amino acid residues have been introduced for site-specific chemical coupling of RAbs (Alcocer et al. 2000; Krebber et al. 1997; Markus et al. 2011; Nishi et al. 2005).

In the last decades, numerous studies on the application of MAb-based immunoassays for the determination of agrochemical residues in food and environmental samples have been reported (Morozova et al. 2005). As previously argued, advances in RAb production and selection, the introduction at convenience of functional molecules for detection or immobilization, and the possibility of modifying RAb properties afford a powerful technology to obtain tailor-made binders for

analytical purposes in food (Markus et al. 2011). However, few studies dealing with the application of recombinant-based immunoassays to the analysis of agrochemicals in food and environmental samples have been reported to date (Alcocer et al. 2000; Nishi et al. 2005). Consequently, understanding the potential, limitations and opportunities of RAbs in food analysis as compared to conventional antibodies is an important issue to find out the future role of RAbs as immunochemical tools.

The aim of this work was to carry out a comparative study of the analytical performance of immunoassays, based on MAbs and on their derived RAbs, to imazalil, tetraconazole, and thiabendazole. To this aim, each RAb was cloned from their respective hybridoma cell line and expressed in *E. coli*. Apart from scFv, scFv-pIII antibody fragments were assayed because these fusion fragments may offer production, detection and purification advantages. Finally, a comparison of the analytical properties of the ELISAs developed with monoclonal and recombinant antibodies in the analysis of fruit juices spiked with the target fungicides was performed.

Materials and Methods

Reagents and Instruments

Analytical standards of tetraconazole and 2-(2,4-Dichlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanol (DTP) were from Isagro Ricerca (Galliera, Italy). Hexaconazole standard was from Syngenta (Bracknell, UK). All of the other fungicide standards were from Riedel-de Haën (Sigma-Aldrich S.A., Madrid, Spain). Ovalbumin (OVA), *o*-phenylenediamine (OPD), cloramphenicol, kanamycin, tetracycline, nalidixic acid, isopropyl-β-thiogalactopyranoside (IPTG), and 10× Blocking Buffer (BB) were also obtained from Sigma. Peroxidase-labelled rabbit anti-mouse immunoglobulins were

obtained from Dako (Glostrup, Denmark). Restriction endonuclease *SfiI* was purchased from Roche Diagnostics (Sant Cugat del Vallés, Spain). T4 DNA ligase was from Fermentas (Madrid, Spain). Taq DNA polymerase MasterMix was purchased from Eppendorf (Hamburg, Germany). *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 glnV44 relA1 lac* / F' Tn*10* (Tet^r) $proA^+B^+$ $lacI^q$ Δ*(lacZ)M15*) was from Stratagene (Cedar Creek, TX) and *E. coli* HB2151 (*K12, ara* Δ*(lac-pro) thi* / F' $proA^+B^+$ Δ*(lacZ)M15*) was from Maxim Biotech (South San Francisco, CA). M13KO7 helper phage was purchased from GE Healthcare (Barcelona, Spain). 2×YT medium and agarose for DNA electrophoresis were from Conda Laboratories (Madrid, Spain). Primers were purchased from Integrated DNA Technologies (IDT, Coraville, IA). 9E10 anti-*myc* hybridoma cell line was from American Tissue Type Culture Collection (Rockville, MD). The pAK100 phagemid was kindly provided by Dr. A. Plückthun (Inst. Biochemistry, University of Zürich, Switzerland). This phagemid allows the expression of a *myc* tag between the scFv fragment and the truncated pIII protein (Krebber et al. 1997).

Polymerase chain reactions were carried out in the Mastercycler gradient thermal cycler from Eppendorf. Flat bottom polystyrene ELISA plates (High Binding Plates) were from Costar (Cambridge, MA). ELISA plates were washed with a 96 PW microplate washer from SLT Labinstruments GmbH (Salzburg, Austria). Absorbance was read in dual-wavelength mode (490-650 nm) with an Emax microplate reader from Molecular Devices (Sunnyvale, CA). mRNA concentrations were determined by an UV-160A Shimadzu spectrophotometer (Kyoto, Japan).

Cloning of scFv Antibody Fragments

Messenger RNA isolation and first-strand cDNA synthesis. The production of the parent monoclonal antibodies TN3C-33, IMH-51, and DTPH-41 (raised against thiabendazole, imazalil, and tetraconazole, respectively) has been previously described (Abad et al. 2001; Manclús et al. 2008; Moreno et al. 2007). For each hybridoma, 5×10^6 cells were pelleted by centrifugation at 780*g* for 5 min and resuspended in 400 µl of mRNA extraction buffer. mRNA was isolated and purified using the QuickPrep Micro mRNA kit from GE Healthcare. Purified mRNA was precipitated with ethanol, washed and air-dried. The pellet was resuspended in 50 µl of molecular biology grade water. mRNA purity and concentration were determined by UV spectrophotometry. Next, 0.1 µg of mRNA served as the template for first-strand cDNA synthesis using the corresponding kit from GE Healthcare.

Amplification of immunoglobulin variable regions by PCR. cDNA was used as PCR template for the amplification of V_L and V_H domains using the oligonucleotide primers described by Krebber et al. (1997). These primers are complementary to the 5' and 3' coding sequences of each murine immunoglobulin variable regions, and encode additional sequences for restriction endonucleases and the linker sequence. V_L primers were selected according to the MAb IgG isotype. PCR was carried out under the following conditions: after 3 min denaturation at 92 ºC, Taq DNA polymerase was added, followed by 5 cycles of 1 min at 92 ºC, 1 min at 50 ºC, 1 min at 72 ºC, and 20 cycles of 1 min at 92 ºC, 1 min at 63 ºC, 1 min at 72 ºC. The PCR amplified DNA products were concentrated by Microcon YM30 filter devices from Millipore (Bedford, MA), purified by preparative agarose gel electrophoresis using the MinElute gel

extraction kit from Qiagen (Izasa,Barcelona, Spain), and quantified by analytical gel electrophoresis using DNA molecular weight markers from Fermentas.

Assembly of scFv antibody fragments. Assembly reactions were performed as described by Krebber et al. (1997) with few modifications for imazalil scFv fragments. Briefly, reactions contained equimolecular amounts (10 ng) of V_H and V_L DNA, scfor and scback primers, and the Eppendorf MasterMix (2.5×) containing the Taq DNA polymerase and an appropriate mixture of dNTPs. V_L and V_H products were first assembled into the scFv format by splicing by overlap extension PCR and subsequently scFv was amplified by the scback and scfor primer set. Assembled scFv fragments were purified as above.

The scFv DNA and the phage display vector pAK100 were digested with *SfiI*, purified and ligated. The ligation product was transformed into electrocompetent *E. Coli* XL1-Blue cells. Transformed cells were plated onto 2×YT solid agar medium containing 1 % (w/v) D-glucose and 25 µg/ml chloramphenicol (2×YT-GC) and grown overnight at 37 ºC.

Screening of scFv Colonies

Single chloramphenicol-resistant colonies were picked into 96 × 2.2 ml tube plates (Eppendorf) containing 2×YT-CG medium (400 µl) and incubated overnight at 30 ºC with orbital shaking at 250 rpm. Forty µl of overnight culture was added to 400 µl of 2×YT-GC medium and incubated until the culture reached an optical density at 550 nm (OD_{550}) of 0.4. Cells were pelleted by centrifugation at 1000*g* for 5 min at room temperature and resuspended in 400 µl of 2×YT-C (no glucose) containing 1mM IPTG. The incubation was continued for 20 h. Finally, 100 µl of phosphate-buffered saline (PBS, 10 mM phosphate, 0.15 M NaCl, pH 7.5) containing 0.5 % Tween 20 was added

to each well and plates were centrifuged at 1000*g* for 10 min. Supernatants were then checked for the presence of scFv-pIII fusion proteins that recognized thiabendazole, imazalil, or tetraconazole. The screening consisted of the simultaneous performance of a non-competitive (in the absence of analyte) and a competitive (in the presence of analyte) indirect ELISA. Immunoassay conditions are described in the fungicide immunoassay procedure.

ScFv production was accomplished by transforming HB2151 cells (*E. Coli* non-suppressor strain) with plasmids containing the specific scFv insert. Previously, these plasmids were extracted and purified from positive *E. Coli* XL1-Blue cells with the Perfectprep Plasmid Mini kit purchased from Eppendorf. Transformed cells were plated onto 2×YT-GC solid agar medium containing 25 µg/ml of nalidixic acid. Several nalidixic acid-resistant colonies were picked into 96 × 2.2 ml tube plates containing 2×YT-GC medium (400 µl) to produce scFv as described above. Supernatants were checked for RAb activity with the screening ELISA.

Panning of Phage Displayed scFv Antibodies

Transformed XL1-Blue colonies were scraped off the agar plate into 10 ml 2×YTGC medium and cells were counted. To rescue scFv displaying phages, 7 ml of 2×YTCG medium was inoculated with 10^8 cells and incubated at 37 ºC with shaking. When an OD_{550} of 0.5 was reached, 2.5 µl of 1M IPTG and 10^{10} cfu of M13KO7 helper phage were added to 5 ml of cell culture. After 15 min of infection without shaking, 5 ml of fresh 2xYTCG medium containing 0.5 mM IPTG was added and the incubation was continued for 2 h at 30 ºC with shaking. Then, 30 µg/ml of kanamycin was added and the phage production was carried out overnight. For phage purification, 1.1 ml of phage culture was centrifuged at 1000*g* at 4 ºC for 15 min to sediment cells. Next, 250 µl of

PEG/NaCl solution (2.5 M NaCl containing 20 % w/v PEG 8000, Sigma) was added to 1 ml supernatants. After incubating for 30 min at 4 ºC, precipitated phages were pelleted as above, resuspended in 1 ml of 2×YT medium and sequentially filtered through a 3 and 0.45 µm filters. Finally, recombinant phages were titrated by infecting XL1-Blue cells. Panning was performed at room temperature as follows. The corresponding hapten conjugate, at 10 µg/ml (100 µl per well) in 50 nM carbonate buffer, pH 9.6, was passively absorbed to ELISA plates overnight at room temperature. Unbound coating conjugate was removed and plates were washed 3 times with 300 µl/well of 1×BB. Next, unoccupied sites were blocked with 200 µl/well of 1×BB for 1h. Then, phage preparations were diluted to 10^{10} phages/ml in 1×BB and let stand for 30 min before adding 100 µl per well (10^9 phages) and incubating for 1h. After washing 5 times with 300 µl/well of 1×BB, phages were competitively eluted by incubating for 30 min with 100 µl per well of 100 nM analyte. Eluted phages were used to infect 100 µl of XL1-Blue cells. Finally, infected cells were plated onto solid 2×YTGC medium and grown overnight at 37 ºC. Transformed colonies can be used to prepare a new recombinant phage population for the next panning round as required.

Production of Recombinant Antibodies

Small scale production of RAb fragments, both scFv and scFv-pIII, was carried out using the method described by Kipriyanov et al. (1997) with slight modifications. For each fungicide, 10 ml of 2×YTGC medium was inoculated with the corresponding XL1-Blue or HB2151 cells containing DNA sequences encoding the scFv-pIII or scFv fragments, respectively, and grown overnight with shaking at 37 ºC. Two ml of overnight culture was added to a 2 L flask containing 100 ml of 2×YT-GC medium and cells were incubated at 37 ºC with shaking at 250 rpm until the culture reached an OD_{550}

of 0.8. Cells were then pelleted by centrifugation at 1500*g* for 10 min at room temperature and resuspended in 100 ml of freshly prepared 2×YT-C (containing 0.4 M saccharose and 0.1 mM IPTG), and the incubation was continued overnight at 30 ºC. Cultures were centrifuged at 5000*g* for 10 min at 4 ºC. Next, supernatants were centrifuged again at 30000*g* for 20 min at 4 ºC rendering the initial supernatant (So). Pellets were resuspended in 5 ml of cool periplasmic extraction buffer (50 mM Tris/HCl, 1mM EDTA, and 20 % saccharose) and cooled for 1 h in an ice-water bath with occasional shaking. Finally, supernatants were collected by centrifugation at 30000*g* for 30 min at 4 ºC to render the periplasmic supernatant (Sp). So and Sp supernatants were mixed and concentrated in an ultrafiltration cell (Amicon). ScFv and scFv-pIII fragments were confirmed by ELISA. Supernatants were concentrated and stored at -20ºC. RAb activity was stable throughout this study.

Fungicide Immunoassays

MAb-based ELISAs were performed using specific hapten coating conjugates as previously described (Abad et al. 2001; Manclús et al. 2008; Moreno et al. 2007). RAb-based ELISAs were performed as follows. A volume of 100 µl 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). ELISA plates were coated overnight by adding the specific hapten conjugate in 50 mM carbonate buffer, pH 9.6. Then, 50 µl per well of standards or sample extracts adequately diluted in PBS were added to triplicate wells, followed by 50 µl per well of RAb dilution in PBS containing 0.0025% Tween 20, and plates were incubated for 1h. After washing, plates were incubated for 1 h with anti-*myc* MAb at 1 µg/ml in PBST (PBS containing 0.05% Tween 20). 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 OPD and 0.012% H_2O_2 in 25 mM citrate and 62 mM sodium phosphate, pH 5.35). After 10 min, the reaction was stopped with 2.5 M sulphuric acid and the absorbance at 490 nm was read and recorded.

Preparation of Standards and Samples

Analyte stock solutions in the appropriate range for each immunoassay were prepared by serial dilution in *N*,*N*-dimethylformamide (DMF) and stored at -20 ºC. From these stock solutions, working standards were daily prepared by 1/500 dilution in PBS. Orange and apple juices were obtained from a local supermarket. Twenty five ml of fruit juice samples were spiked with thiabendazole at 50, 100, and 1000 ng/ml by adding 25 µl of 50, 100, or 1000 µg/ml thiabendazole stock solutions in DMF, respectively. For imazalil or tetraconazole, juice samples were spiked at 1000, 2000, and 5000 ng/ml by adding 25 µl of 1000, 2000, or 5000 µg/ml fungicide stock solutions in DMF, respectively. For ELISA determinations, spiked juice samples were appropriately diluted in PBS and analyzed without any further treatment. Each dilution was analyzed in triplicate and the fungicide concentration in samples was calculated by averaging the values obtained at each dilution.

Results and Discussion

Hybridoma cell lines secreting MAbs against fungicides were used as a source of immunoglobulin genes. RAbs were expressed in *E. coli* as single chain variable fragments (scFv) in a non-suppressor strain, and as fusion proteins of scFv and bacteriophage coat pIII truncated protein (scFv-pIII) in a suppressor strain. The major

advantage of the fusion protein over the other scFv format is that no additional transformation is required because *E. coli* XL1-blue is a suppressor strain. Both RAb fragments were produced to study their properties and analytical performance.

Cloning and Expression of scFv and scFv-pIII RAb Fragments

Messenger RNA was isolated from TN3C-33, IMH-51 and DTPH-41 hybridoma cell lines, secreting anti-thiabendazole, anti-imazalil and anti-tetraconazole MAbs, respectively, and then the first strand cDNA synthesis was carried out. V_L and V_H genes were selectively amplified by PCR, using the universal primer sets specific for the variable regions of murine immunoglobulin V genes (Krebber et al. 1997). Next, rounds of PCR were performed to link the V_L and V_H genes via a $(Gly_4Ser)_3$ encoding DNA linker sequence in the V_L-linker-V_H configuration (scFv). ScFv gene fragments were then cloned into the phagemid vector pAK100 and used to transform XL1-Blue cells. These transformations yielded populations of 1.5×10^4 (thiabendazole), 3.5×10^3 (imazalil), and 3.5×10^4 (tetraconazole) colonies. Screening of functional recombinant antibodies was performed by expressing individual transformed XL1-Blue colonies in the presence of 1 mM IPTG. As *E. Coli* XL1-Blue is a suppressor strain, translation proceeds through the amber stop codon producing the scFv-pIII fusion protein. Bacterial supernatants were then checked for the expression of functional fusion proteins. The screening consisted of the simultaneous performance of a non-competitive and a competitive indirect ELISA, to test the ability of antibody fragments to bind the corresponding OVA conjugate of the immunizing hapten and to recognize thiabendazole, imazalil, or tetraconazole in solution, respectively. Colony analysis was carried out by detection of scFv-pIII fusion proteins instead of phage-displayed scFv because the latter can be less efficient than the former, particularly for selecting hapten-

specific scFv. In this sense, phage-displayed scFv analysis is a more laborious procedure because a phage rescue step is required. Moreover, the size and complexity of phage-displayed scFv may contribute to unspecific interactions that would mask the specific ones (Mersmann et al. 1998; Tout et al. 2001).

By analysing a few colonies of each population, 34 of 48 and 40 of 48 tested colonies rendered wells with scFv-pIII antibody fragments recognizing thiabendazole and tetraconazole, respectively, in competitive assays. With regard to imazalil, none of 48 tested colonies expressed scFvs recognizing this fungicide. Therefore, a round of panning was performed searching for functional imazalil-specific scFvs. Panning was performed in ELISA plate wells coated with 10 µg/ml of OVA-IMH (Moreno et al. 2007). After the first round of panning, 6 of 96 randomly selected colonies rendered wells with scFv-pIII antibodies recognizing imazalil.

Plasmids containing the specific scFv insert for thiabendazole, imazalil or tetraconazole from three colonies expressing scFv-pIII antibodies for each fungicide were used to transform non-suppressor *E. coli* HB5151 cells for scFv production. A few HB2151 clones containing the specific scFv insert for imazalil, tetraconazole, or thiabendazole were selected to produce scFv fragments. All assayed clones produced scFv antibodies that recognized the free analyte. Recombinant scFv and scFv-pIII fragments were detected in the culture medium and in the periplasmic space of transformed cells.

Characterization of scFv Recombinant Antibodies

RAbs were used to develop ELISAs in the conjugate-coated format. Immunoassay development and optimization was performed as previously reported for monoclonal antibody-based ELISAs to conazoles (Manclús et al. 2008). Standard curves obtained

under optimum assay conditions, for each fungicide and antibody type, are depicted in Figure 1. Analytical parameters of the competitive curves were estimated and compared to those of the parent MAbs. Results are shown in Table 1. As it can be observed, imazalil and tetraconazole RAbs showed one order of magnitude lower affinity (estimated as lower I_{50} values, concentrations giving 50% inhibition of the maximum absorbance) than their respective MAbs, whereas thiabendazole RAbs showed similar affinity to that of their parent MAb. With regard to the studied recombinant fragments, both of them presented a similar behaviour except for imazalil. In this case, the sensitivity of the ELISA configured with the fusion fragment was higher (lower I_{50} value) than that of the ELISA configured with free scFv. When affinities of RAbs and those of their parental MAbs have been compared in the literature, diverse results have been reported. While some authors reported that RAbs showed lower affinity than their parental MAb, others reported a comparable or even higher affinity of RAbs over their counterpart MAbs (Graham et al. 1995; Kramer and Hock 2007; Nishi et al. 2005; Tout et al. 2001). Results obtained in this study and those reported in the literature point out that the binding site structure of scFvs differs somehow from that of their respective parental antibody molecules (MAb). These differences in the scFv structure could be attributed to the absence of constant domains, the presence of a linker peptide, and the introduction of mutations derived from the use of degenerated primers to amplify antibody genes. To this respect, the cloning and expression of antibody genes as Fab recombinant fragments could mimic better the MAb structure and activity.

The specificity of MAbs and that of their derived RAbs were evaluated by performing competitive assays using several structurally related compounds as competitors. All imazalil antibodies displayed the same reactivity pattern. Immunoassays were very specific for imazalil, because the assayed fungicides

(climbazole, hexaconazole, myclobutanil penconazole, prochloraz, propiconazole, tebuconazole, tetraconazole, and triadimefon) were not recognized (cross-reactivity <0.1 %) (Moreno et al. 2007). With regard to the specificity of thiabendazole antibodies, the cross-reactivity pattern of RAbs was found to be very similar to that of the parent monoclonal antibody **(**Table 2**)**. Only chlorfenazole and fuberidazole were slightly recognized. On the other hand, only minor differences between the cross-reactivity pattern of the MAb to tetraconazole and that of its derived RAbs were observed **(**Table 3**)**. In this case, the immunoassays were not specific to tetraconazole because penconazole, cyproconazole, myclobutanil, and DTP (hydrolysis product of tetraconazole) were recognized at a significant extent: 44-90%, 32-50%, 14-33% and 24-58%, respectively.

Recovery Analysis of Spiked Juice Samples by the Developed ELISAs

The analytical performance of the three antibody types in the analysis of thiabendazole, imazalil, or tetraconazole in orange and apple juices was investigated. Immunoassays are not completely free from interferences caused by unidentified compounds of the food matrix. Therefore, it is advisable to determine the importance of these so-called matrix effects prior to the recovery test. Accordingly, the influence of orange and apple juices on the thiabendazole, imazalil, and tetraconazole ELISA performance was tested by obtaining standard curves in the presence of a variable proportion of each juice. Results proved that thiabendazole, imazalil, and tetraconazole could be analyzed in juices simply by appropriately diluting samples in PBS (data not shown). Thus, thiabendazole could be analyzed in orange and apple juices just by diluting samples 1/10 and 1/100 in PBS, respectively, in monoclonal and recombinant antibody based ELISAs. On the other hand, imazalil could be analyzed in orange and apple juices by

diluting samples 1/50 and 1/100, respectively, in MAb-based ELISA and by diluting both juice samples 1/100 in RAb-based ELISAs. Finally, tetraconazole could be analyzed in these juice samples by diluting both juices 1/50 in both types of ELISA.

Apple and orange juices were spiked with imazalil or tetraconazole at 1000, 2000, and 5000 ng/ml or with thiabendazole at 50, 100, and 1000 ng/ml. Samples were adequately diluted to enter the respective working range and directly analyzed by ELISA. Juice samples without analyte (non-spiked samples) were also included in the analysis as negative controls. No false positive results were obtained. Table 4 shows the reproducibility and recovery values obtained by ELISA for each juice, analyte and antibody type. Reproducibility of both monoclonal and recombinant imazalil ELISAs was acceptable. The highest coefficients of variation observed at 1000 ng/ml corresponded to the determination near the quantification limit. Overall, recoveries were also quite acceptable, although there was a tendency of both recombinant antibody-based ELISAs to overestimate. The tetraconazole analytical data obtained for both fruit juices can be considered as fairly good. In this case, both recombinant antibody ELISAs presented a similar behaviour. With regard to thiabendazole ELISAs, the analytical data obtained for both fruit juices can be also considered as good enough. Recombinant and monoclonal antibody-based ELISAs presented similar behaviour, all of them showing a tendency to analyte overestimation in the apple samples.

In this study the functionality and usefulness of recombinant antibodies as analytical tools for the detection of low molecular weight contaminants in food samples have been proved. Future work will include RAb purification and stability studies, as well as scFv engineering to modify their analytical properties at convenience.

Conclusions

RAbs against three fungicides have been produced by cloning and expression of hybridoma antibody variable regions. RAb-based immunoassays showed higher analyte limits of detection than those based on the parental MAbs. On the other hand, similar analytical parameters were estimated for the two types of RAbs under study, scFv fragments and scFv-pIII fusion fragments.

Analysis of spiked fruit juices revealed that comparable recoveries and coefficient of variations were obtained by using either RAb or MAb-based immunoassays in their respective measuring range.

RAbs can be obtained in a more cost-effective manner than MAbs because the recombinant production in bacteria requires simple inexpensive media for rapid growth and it can easily be scaled-up. Moreover, RAb binding characteristics could be modified as required by diverse antibody engineering techniques. Therefore, RAb-based immunoassays can become a very valuable immunoanalytical tools for the rapid screening of fungicide residues in fruit juices.

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Compliance with Ethics Requirements

María-José Moreno declares that she has no conflict of interest.

Emma Plana declares that she has no conflict of interest.

Juan J. Manclús declares that he has no conflict of interest.

Angel Montoya declares that he has no conflict of interest.

This article does not contain any studies with human or animal subjects.

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Figure legends

Figure 1. Competitive curves of monoclonal (●), scFv (▲) and scFv-pIII (■) antibodies for imazalil, tetraconazole, and thiabendazole in the conjugate-coated ELISA format.

Figure
Click here to download Figure: Figure 1.doc

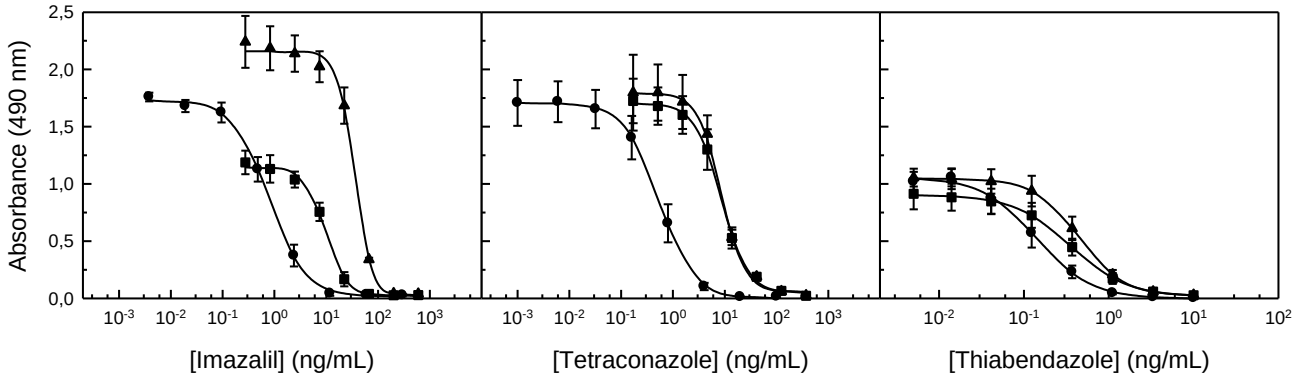

Figure 1 Competitive curves of monoclonal (●), scFv (▲) and scFv-pIII (■) antibodies for imazalil, tetraconazole, and thiabendazole in a conjugate-coated ELISA format.

Table
Click here to download Table: Table 1.doc

Table 1 Summary of the Analytical Characteristics of Imazalil, Tetraconazole, and Thiabendazole Immunoassays Developed with RAbs and MAb[a]

Fungicide	Antibody	I_{50} (ng/mL)	Working range (ng/mL)	LOD (ng/mL)
Imazalil	MAb IMH.51	0.8	0.26−2.4	0.1
	RAb IMH.51	35.0	21.0−59.0	15.0
	RAb IMH.51-pIII	9.6	5.0−19.0	3.4
Tetraconazole	MAb DTPH.41	0.5	0.2−1.4	0.1
	RAb DTPH.41	8.5	4.0−18.5	2.0
	RAb DTPH.41-pIII	8.6	3.5−20.0	2.0
Thiabendazole	MAb TN3C.33	0.14	0.07−0.5	0.02
	RAb TN3C.33	0.44	0.2−1.0	0.1
	RAb TN3C.33-pIII	0.35	0.1−0.9	0.06

[a] Data are the average of 4 independent standard curves

Table
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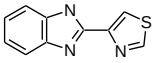
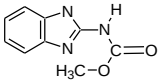
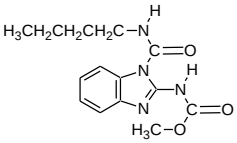
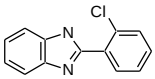
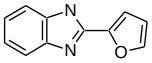
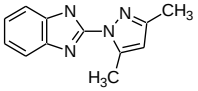
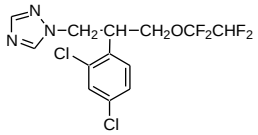
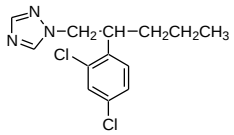
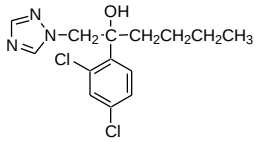
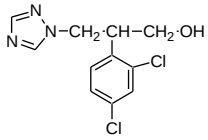
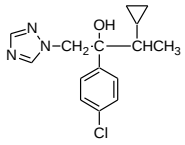
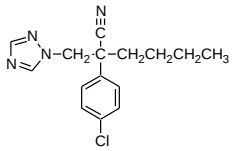
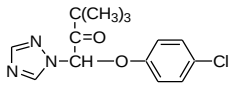
Table 2 Recognition of Structurally Related Compounds by Thiabendazole Antibodies

Chemical structure	Compound	Cross-reactivity[a] (%)		
		MAb TN3C.33	RAb TN3C.33	RAb TN3C.33-pIII
	Thiabendazole	100	100	100
	Carbendazim	< 0.1	< 0.1	< 0.1
	Benomyl	< 0.1	< 0.1	< 0.1
	Chlorfenazole	2	5	4
	Fuberidazole	4	11	8
	Rabenzazole	0.1	0.4	0.3

[a] Cross-reactivity was calculated as (I_{50} thiabendazole/I_{50} compound) x 100

Table
Click here to download Table: Table 3.doc

Table 3 Recognition of Structurally Related Compounds by Tetraconazole Antibodies

Chemical structure	Compound	Cross-reactivity[a] (%)		
		MAb DTPH.41	RAb DTPH.41	RAb DTPH.41-pIII
	Tetraconazole	100	100	100
	Penconazole	44	87	90
	Hexaconazole	< 0.1	0.5	0.7
	DTP	24	58	50
	Cyproconazole	32	63	50
	Myclobutanil	14	33	26
	Triadimefon	1	1	1

[a] Cross-reactivity was calculated as (I_{50} tetraconazole/I_{50} compound) x 100

Table
Click here to download Table: Table 4.doc

Table 4 Analysis of Apple (A) and Orange (O) Juices Spiked with Imazalil, Tetraconazole, or Thiabendazole by Monoclonal- and Recombinant-Based ELISAs

	Imazalil				Tetraconazole				Thiabendazole			
	Spiked level (ng/mL)[a]	Mean value found (ng/mL)[b]	CV (%)	Recovery (%)	Spiked level (ng/mL)[c]	Mean value found (ng/mL)[b]	CV (%)	Recovery (%)	Spiked level (ng/mL)[d]	Mean value found (ng/mL)[b]	CV (%)	Recovery (%)
MAb	A-1000	1360	44	136	A-1000	1190	18	119	A-50	86	16	172
	A-2000	2120	11	106	A-2000	1680	10	84	A-100	145	17	145
	A-5000	3950	18	79	A-5000	3900	14	78	A-1000	1290	14	129
	O-1000	1350	23	135	O-1000	1210	14	121	O-50	44	4	88
	O-2000	2100	22	105	O-2000	1880	3	94	O-100	101	12	101
	O-5000	4200	18	84	O-5000	5000	8	100	O-1000	1180	11	118
RAb	A-1000	-[e]	-	-	A-1000	1090	7	109	A-50	50	9	100
	A-2000	-	-	-	A-2000	2120	5	106	A-100	135	20	135
	A-5000	7600	18	152	A-5000	4401	13	88	A-1000	1060	9	106
	O-1000	-	-	-	O-1000	1320	10	132	O-50	41	19	82
	O-2000	-	-	-	O-2000	2320	7	116	O-100	110	5	110
	O-5000	7800	11	156	O-5000	5300	19	106	O-1000	1030	4	103
RAb-pIII	A-1000	1110	44	111	A-1000	1170	1	117	A-50	71	8	142
	A-2000	2340	14	117	A-2000	2160	12	108	A-100	135	11	135
	A-5000	5950	17	119	A-5000	4750	7	95	A-1000	1220	7	122
	O-1000	1250	17	125	O-1000	1450	15	145	O-50	50	5	100
	O-2000	3000	7	150	O-2000	2640	8	132	O-100	133	8	133
	O-5000	7500	17	150	O-5000	6400	6	128	O-1000	1120	5	112

[a] Imazalil spiked samples at 1000, 2000, and 5000 ng/mL were diluted 1/1000, 1/1000, and 1/2000, respectively, for the MAb-based ELISA; diluted 1/100 for the RAb-based ELISA; and diluted 1/300 for the RAb-pIII-based ELISA. [b] Data are the average of four independent determinations. [c] Tetraconazole spiked samples at 1000, 2000, and 5000 ng/mL were diluted 1/500, 1/1000, and 1/2500, respectively, for the MAb-based ELISA, and diluted 1/100, 1/100, and 1/500, respectively, for the RAb-based ELISAs. [d] Thiabendazole spiked samples at 50, 100, and 1000 ng/ml were diluted 1/100, 1/100, and 1/1000, respectively, for MAb- and RAb-based ELISAs. [e] Samples below the ELISA detection limit.