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Development and application of recombinant antibody-based immunoassays to tetraconazole residue analysis in fruit juices

Running title: Recombinant immunoassays for tetraconazole analysis in juices

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ABSTRACT

Tetraconazole is currently used as a fungicide in fruit and vegetables. The aim of this work was the development of immunochemical techniques based on recombinant antibodies for the screening of tetraconazole residues in fruit juices. Recombinant antibodies were produced from a hybridoma cell line secreting a monoclonal antibody specific for tetraconazole and from lymphocytes of mice hyperimmunized with tetraconazole haptens conjugated to bovine serum albumin. From these antibodies, enzyme-linked immunosorbent assays in the conjugate-coated format were developed, which were able to detect tetraconazole standards down to 1 ng/mL. From recovery studies with spiked samples, these immunoassays determined tetraconazole in orange and apple juices with acceptable reproducibility (coefficients of variation below 25%) and recoveries (ranging from 78−145%) for a screening technique. The analytical performance of RAb-based immunoassays was fairly similar to that of the MAb-based immunoassays. Due to their simplicity and high sample throughput, the developed recombinant-based immunoassays can be valuable analytical tools for the screening of tetraconazole residues in fruit juices at regulatory levels.

KEYWORDS: immunochemical analysis; recombinant antibodies; scFv; ELISA; tetraconazole; fungicide residues; fruit juices

1. Introduction

Tetraconazole [(RS)-2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propyl-1,1,2,2-tetrafluoroethyl ether] is a broad-spectrum systemic fungicide with protective, curative, and eradicant properties (Tomlin, 2009). It belongs to the family of triazole fungicides which exert its antifungal activity by inhibiting the sterol biosynthesis (Stenersen, 2004). Environmental fate studies indicate that tetraconazole is expected to be persistent and moderately to slightly mobile in soil, so it tends to accumulate in soil and has the potential to reach surface water via run-off and spray drift. Tetraconazole is moderately toxic to freshwater and estuarine/marine fish and freshwater invertebrates, but it is highly toxic to estuarine/marine invertebrates. At the recommended application rate, there are no statistically significant toxic effects to terrestrial or aquatic plants. On an oral acute basis, tetraconazole is slightly toxic to mammals and moderately toxic to birds. Reproductive chronic effects were observed in birds and mammals. Moreover, the United States Environmental Protection Agency classifies tetraconazole as a compound likely to be carcinogenic in humans (EPA, 2005).

Tetraconazole is currently an approved active substance in the European Union (EU Pesticide Database, 2012). Maximum residue levels (MRLs) were harmonized by the European Commission in the Regulation No. 396/2005. According to this regulation, tetraconazole levels up to 0.5 mg/kg were fixed in fruit and vegetables. Consequently, tetraconazole residues can be potentially found in fruit and processed fruit. In fact, fungicide residues have been detected at low levels in some orange juices (FDA, 2012). Therefore, the availability of a high throughput screening analytical technique for tetraconazole residues would be very useful for juice producers and regulatory/surveillance agencies.

The most frequent analytical methods for the determination of azole fungicide residues in food are liquid and gas chromatography (Ferrer, Martínez-Bueno, Lozano, & Fernández-Alba, 2011). Immunochemical methods can be useful as complementary analytical tools to conventional methods for the screening of large number of samples. In general, immunoassays are simple to perform, cost-effective, robust, and amenable to on-site monitoring; however, high quality antibodies are required to develop these assays. In past years, ELISAs for the determination of azole fungicides using polyclonal antibodies have been reported. Forlani, Arnoldi, and Pagani (1992) obtained polyclonal antibodies that recognized tetraconazole and penconazole, with which they developed an ELISA that detected tetraconazole between 25-10000 µg/L in fruit (Cairoli, Arnoldi, & Pagani, 1996). With regard to other azole fungicides, Chen, Dwyre-Gygax, Hadfield, Willetts, and Breuil (1996) produced high affinity polyclonal antibodies against hexaconazole with a limit of detection (LOD) of 0.1 µg/L in buffer. Danks, Chaudhry, Parker, and Baker (2001) developed a polyclonal ELISA to tebuconazole with a working range between 0.02-20 mg/L. Szèkács and Hammock (1995) obtained polyclonal antibodies that detected up to 200 µg/L of myclobutanil. However, the development of polyclonal antibodies has several limitations: the quantity of antibodies obtained is limited by the animal size and lifespan, and results vary from one animal to another. Hybridoma technology provides unlimited quantities of monoclonal antibodies (MAbs) and offers the possibility to standardize the assay method. In this sense, we previously reported the development of sensitive immunoassays based on MAbs for conazole fungicides determination in fruit juice. Thus, Manclús, Moreno, Plana, and Montoya (2008) described a hexaconazole-specific immunoassay with LOD of 0.3 µg/L and a conazole-specific immunoassay with a LOD between 0.1−0.7µg/L; and Moreno, Plana, Montoya, and Manclús (2007) developed an imazalil immunoassay with a LOD

of 0.6 µg/L. Moreover, Watanabe et al. (2000) obtained MAbs against imazalil and applied them to the determination of this fungicide in fruit juices with good recoveries. Nevertheless, hybridoma technology is a rather inefficient procedure for selecting high-sensitivity antibodies because only hundreds of clones are normally assayed from a 10^8 lymphocyte population on average. Lately, recombinant antibodies (RAbs) are gaining importance as immunoanalytical tools of agrochemicals and food contaminants because highly diverse antibody libraries and the application of display technologies make the antibody selection procedure much more efficient and versatile (Brichta, Hnilova, & Viskovic, 2005). In comparison to MAbs, RAbs can be obtained in a more rapid and cost-effective process. Moreover, RAb properties can be modified as required (Markus, Janne, & Urpo, 2011; Fitzgerald, Leonard, Darcy, Danaher, & O'Kennedy, 2011).

Functional expression of antibody fragments from bacteria was first reported by Bird et al. (1988). Over the last years, hapten-specific RAbs have been produced from hybridoma cell lines, hyperimmunized animals, and even from non-immunized, generic libraries (Brichta, Hnilova, & Viskovic, 2005; Markus, Janne, & Urpo, 2011). Nevertheless, immunized libraries were a straightforward starting material for obtaining high affinity antibodies to low molecular weight compounds (Tout, Yau, Trevors, Lee, & Hall, 2001; Kramer & Hock, 2003).

In this work, the development and application of a recombinant-based screening immunoassay of tetraconazole is described. To this aim, we report the successful cloning and expression in *E. coli* of RAbs against tetraconazole, starting from the genetic material of both hybridoma and spleen cells. Two types of RAb fragments were expressed: single-chain variable fragment (scFv) proteins by itself and fused to a truncated pIII protein of M13 phage capsid (scFv-pIII fusion proteins) (Mersmann et al., 1998). Their analytical properties were compared with those of their parental MAb.

Finally, monoclonal and recombinant-based ELISAs were developed and applied to the determination of tetraconazole residues in fruit juices.

2. Experimental

2.1. Materials

Tetraconazole and 2-(2,4-dichlorophenyl)-3-(1H-1,2,4-triazol-1-yl)propanol (DTP, a tetraconazole degradation product) analytical standards were from Isagro Ricerca (Galliera, Italy). Hexaconazole analytical standard was from Syngenta Agro (Braknell, UK). Penconazole, cyproconazole, myclobutanil, and triadimefon analytical standards, ovalbumin (OVA), *o*-phenilendiamine (OPD), antibiotics (kanamycin, chloramphenicol and tetracycline), 10× Blocking buffer (BB), isopropyl *β*-D-thiogalacto-pyramoside (IPTG), and polyethylene glycol (PEG 8000) were obtained from Sigma-Aldrich Quimica (Madrid, Spain). Peroxidase-labeled 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 Kit was purchased from Eppendorf (Hamburg, Germany) and PCR master mix and Extensor Hi-fidelity PCR DNA amplification master mix were from Abgene (Epsom, UK). Electrocompetent *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*) cells were from Stratagene (Cedar Creek, TX) and *E. coli* HB2151 (*K12, ara* Δ*(lac-pro) thi* / F‘ $proA^+B^+$ Δ*(lacZ)M15*) cells were from Maxim Biotech (South San Francisco, CA). M13KO7 helper phage and peroxidase-labeled anti-M13 monoclonal antibody were purchased from GE Healthcare (Barcelona, Spain).

pAK100 phagemid was kindly provided by Dr. A. Plückthun (Institute of Biochemistry, University of Zürich, Switzerland). 2×YT medium (1.6% w/v tryptone, 1% w/v yeast extract, and 0.5 % w/v NaCl), SOB medium (2% tryptone w/v, 0.5% w/v yeast extract, 0.05% w/v NaCl, 2.5 mM KCl, and 10 mM $MgCl_2$), and agarose for DNA electrophoresis were from Conda Laboratories (Madrid, Spain). Primers were purchased from Integrated DNA Technologies (IDT) (Coraville, IA). Anti-*myc* 9E10 hybridoma cell line was from American Tissue Type Culture Collection (Rockville, MD).

Hybridoma fusion and cloning supplement (HFCS) was purchased from Roche Diagnostics. Culture media (high-glucose Dulbecco's Modified Eagle's medium with Glutamax I and sodium pyruvate, DMEM), fetal bovine serum (FBS, myoclone Super plus), and hypoxantine-thymidine (HT) and hypoxantine-aminopterine-thymidine (HAT) supplements were from Gibco (Paisley, Scotland).

Autoclavable 96-deepwell plates (2.2 mL/well) were obtained 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), and absorbance was read in dual-wavelength mode (490-650 nm) with an Emax microplate reader from Molecular Devices (Sunnyvale, CA). mRNA concentration was determined by an UV-160A Shimadzu spectrophotometer (Kioto, Japan). The electroporator 2510 and the Mastercycler gradient thermal cycler were from Eppendorf. The Labofuge 400 centrifuge and the microplate rotor for two 96-well standard plates were from Heraeus (Hanau, Germany). The nucleic acid electrophoresis HE 33 unit was purchased from GE Healthcare and the Doc-Print gel electrophoresis documentation system was from Vilber Lourmat (Marne-la-Vallée, France).

Hapten DTPH (Figure 1) and immunoreagents (OVA-DTPH and BSA-DTPH conjugates, DTPH-41 MAb against tetraconazole) were previously obtained as described (Manclús, Moreno, Plana, & Montoya, 2008).

2.2. Immunization procedure

Animal manipulation was carried out in accordance with the Spanish regulation currently in force and under the approval of the local Ethical Committee for Research. BALB/c female mice (8-10 week old) were immunized intraperitoneally with BSA-DTPH conjugate (100 µg) as an emulsion 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 (250 µL). Two secondary immunizations were performed at three-week intervals using the immunogen emulsified in incomplete Freund's adjuvant. Finally, at least 3 weeks after the last injection in adjuvant, an additional boost in PBS was administered 4 days before spleen lymphocyte extraction.

2.3. Preparation of hybridoma and spleen cells

Hybridoma DTPH-41 cells were thawed in a 37 ºC bath and washed with DMEM. After centrifugation at 280*g* for 5 min, cells were resuspended in 10 mL of DMEM containing 1% HT, 1% HFCS, and 10% FBS, and cultured for 3 days. Hybridoma cells were centrifuged, counted and used for mRNA extraction.

On the other hand, spleen was excised and splenocytes released from the sac in DMEM medium. After filtration and centrifugation at 328*g* for 5 min, spleen cells were resuspended in 50 mL of the same medium, counted, and used for mRNA extraction.

2.4. Cloning of scFv fragments and library construction

2.4.1. mRNA isolation and cDNA synthesis

mRNA was purified from hybridomas (5×10^7 cells) and lymphocytes (1×10^7 cells) using the QuickPrep Micro mRNA purification kit (GE Healthcare) and RNeasy kit (Qiagen), respectively. Extracted mRNA was precipitated with ethanol, resuspended in molecular biology grade water and quantified by spectrophotometry. About 100 ng of mRNA was reverse-transcripted using random hexamer polymers and the first strand cDNA synthesis kit from GE Healthcare.

2.4.2. Synthesis of V_H and V_L genes from cDNA

cDNA was then used for the amplification of the variable region of heavy- (V_H) and light-chain ($V_{L\kappa}$) domains using a primer set described by Krebber et al. (1997) with slight modifications. Taq polymerase and Hi-Fidelity polymerase were used for hybridoma and lymphocyte variable chain amplification, respectively. PCR reactions were performed under the following conditions: 5 cycles of 1 min at 92 ºC, 1 min at 50 ºC, and 1 min at 72 ºC; and 20 cycles of 1 min at 92 ºC, 1min at 62 ºC, and 1 min at 72 ºC. PCR products were concentrated by using MicroconYM30 centrifugal filter devices (Millipore, Bedford, MA). V_L and V_H genes were purified by preparative agarose gel electrophoresis, using the MiniElute Gel Extraction Kit from Qiagen (Izasa, Barcelona, Spain), and quantified by analytical gel electrophoresis using DNA molecular weight markers from Fermentas.

2.4.3. Construction of scFv fragments

Equimolecular amounts of both chains were assembled by splicing using overlap extension PCR (SOE-PCR). For scFv synthesis from hybridomas, the experimental procedure described by Krebber et al. (1997) was followed. For scFv synthesis from lymphocytes, some modifications were introduced. An initial denaturation at 92 ºC for 3 min was followed by the addition of the Hi-fidelity polymerase and 7 cycles of 1 min at 92 ºC, 30 s at 63 ºC, 50 s at 58 ºC, and 1 min at 72 ºC in the absence of primers. After adding the outer primers *scback* and *scfor* (Krebber et al., 1997), 23 cycles of 1 min at 92 ºC, 30 s at 63 ºC, and 1 min at 72 ºC were performed, followed by 5 min at 72 ºC. scFv fragments were purified by gel electrophoresis as above.

2.4.4. Cloning into pAK100 and transformation

*S*cFv fragments were digested with *SfiI* for 3 h at 50 ºC. After purification, 20 ng of the fragment were ligated into the *SfiI*-digested pAK100 vector (molar ratio vector:insert, 3:2). The ligated product was purified and concentrated by ethanol precipitation. For each transformation, 1 µL at 5 ng/µL (referred to ng of insert) was used to transform 40 µL of electrocompetent *E.coli* XL1-blue cells prepared as described in the following section (scFv from hybridomas) or 40 µL of electrocompetent *E.coli* XL1-blue cells from Stratagene (scFv from lymphocytes). Electroporated cells were plated into 2×YT-agar plates containing 1% glucose and 25 µg/mL chloramphenicol (2×YT+G+C medium) and incubated overnight at 37 ºC. Several colonies were selected for screening and the rest were scrapped off the plate into 2 mL of 2×YT and stored at -70 ºC after addition of 15% glycerol.

2.4.5. Production of electrocompetent E. coli cells

Ten mL of an *E.coli* overnight culture was inoculated into 200 mL of fresh SOB medium and incubated at 37 ºC until an optical density at 550 nm (OD_{550}) of 0.6 was reached. The culture was chilled on ice for 10 min and centrifuged (3700*g*) for 15 min at 4 ºC. The pellet was suspended in the same volume of MilliQ sterile water. This step was repeated once again. Then, the pellet was suspended in 40 mL of milliQ sterile water with 10% of glycerol and centrifuged for 10 min. The volume of pellet was determined and it was resuspended in the same volume of water with 10% glycerol, frozen in liquid nitrogen and stored at -70 ºC in 100 µL aliquots.

2.5. Screening by phage ELISA

2.5.1. Phage recovery

Single colonies were picked into 96-well culture plate containing 150 µL of 2×YT+G+C medium per well and incubated at 37 ºC with orbital shaking at 250 rpm until an OD_{550} of 0.5 was reached. After adding 50 µL of 2×YT+G+C medium complemented with 2 mM IPTG and 2.5×10^8 cfu of helper phage M13K07, plates were incubated overnight at 30 ºC with shaking. Then, plates were centrifuged at 1500*g* for 5 min and supernatants were recovered.

2.5.2. Phage ELISA

ELISA plates were coated overnight with OVA-DTPH and OVA (1 µg/mL) in coating buffer (50 mM carbonate buffer, pH 9.6). Each well was blocked with 100 µL of 1× blocking buffer (BB) for 1 h at room temperature. After emptying plates, 100 µL of supernatant samples were added and incubated for 1 h. Samples were prepared by diluting supernatants with the same volume of 2× BB and incubating for 30 min. After

washing 5 times with 1× BB, plates were incubated for 1h with 100 µL of peroxidase-labeled anti-M13 monoclonal antibody (1/5000 in 1× BB). Finally, plates were washed and revealed as described in section 2.10.

2.6. Screening by soluble scFv ELISA

Colonies were picked into a 96-deepwell (2.2 mL) culture plate containing 400 µL 2×YT+G+C medium. After incubating overnight at 30 ºC with orbital shaking at 250 rpm, 40 µL were inoculated into 400 µL of fresh medium and incubated at the same temperature until an OD_{550} of 0.4 was reached. After centrifugation at 1000*g* for 5 min, the pellet was resuspended in 400 µL of 2×YT medium containing 1 mM IPTG and 25 µg/mL chloramphenicol, and incubated overnight at 30 ºC with shaking. Then, 100 µL of 0.5% Tween 20 in water was added to each well and the plate was incubated for 3 h at 30 ºC with shaking at 200 rpm. After centrifuging at 1000*g* for 10 min, the supernatant was checked by ELISA.

General ELISA conditions are described in section 2.10. Plates were coated overnight with OVA-DTPH (1µg/mL) in coating buffer. Then, diluted supernatants (1/2 in PBS) were added to each well and incubated for 1 h. After washing, anti-myc MAb at 1 µg/mL in PBS-T (PBS containing 0.05% Tween 20) was added and incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins (1/2000 in PBS-T). Finally, peroxidase activity was revealed as described in section 2.10. Inhibition assays to ascertain binding to free analyte were performed as above with the addition of the fungicide in the supernatant reaction step.

2.7. Panning

2.7.1. Production of recombinant phages

To rescue scFv displaying phages, 5 mL of 2×YT+G+C medium were inoculated with 10^8 cells from the glycerol library stock and incubated at 37 ºC with shaking until a OD_{550} of 0.5 was reached. Next, 10^{10} cfu of helper phage and 2.5 µL of 1 M IPTG were added. After incubating for 15 min at 37 ºC without shaking, 5 mL of 2×YT+G+C medium containing 0.5 mM IPTG was added. The culture was incubated for 2 h at 26 ºC and kanamycin at 30 µg/mL was then added, followed by an overnight incubation. Two aliquots of 1.1 mL from the overnight culture were centrifuged at 1000*g* for 15 min at 4 ºC. To 1 mL of supernatant of each tube, 250 µL of PEG/NaCl (20% w/v Polyethilenglycol 8000 in 2.5 M NaCl) was added, gently mixed by inversion, and kept on ice for 30 min. After centrifugation at 10000*g* for 15 min at 4 ºC, supernatants were removed and pellets were resuspended in 1 mL of 2×YT medium. Finally, phages were sequentially filtered with 3 and 0.45 µm filters, titered by infecting *E. coli* XL1-Blue and plating into 2×YT agar plates. For long time storage, glycerol up to 30% was added and phages were frozen at -70 ºC.

2.7.2. Selection of tetraconazole binding recombinant phages

ELISA plate wells were coated overnight at room temperature with 10 µg/mL OVA-DTPH (100 µL) in sterile coating buffer. Control panning with OVA coated wells at the same concentration was carried out in parallel. Wells were washed 3 times with 300 µL of 1× BB and blocked for 1 h with 200 µL of the same buffer. In parallel, a solution of 10^{10} phages per mL of BB was incubated for 30 min at 37 ºC. After emptying the well, 10^9 phages in 100 µL of BB were added and incubated for 1 h at room temperature.

Unbound phages were removed by washing 5 times with 300 µL of 1× BB, aspirating and dispensing with a pipette 5 times for every wash.

To elute bound phages, 100 µL of 100 nM analyte in 1× BB was added to each well and incubated for 30 min at room temperature with shaking at 100 rpm. Eluted phages were used to infect 100 µL of *E. coli* XL-1blue cells (DO_{550} between 0.4 and 0.8). After 30 min of incubation at 37 ºC, infected cells were plated into 2×YT+G+C agar plates and incubated overnight at 37 ºC. Some colonies were checked by screening ELISA and the rest were scrapped off the plates and stored at -70 ºC with 15% glycerol for subsequent rounds of panning.

2.8. Soluble Expression of scFv Fragments

The expression of scFv gene fragments was carried out following the protocol described by Kipriyanov, Moldenhauer, and Little (1997) with minor modifications.

2.8.1. Expression of scFv-pIII fusion proteins

Single colonies were inoculated into 10 mL of 2×YT+G+C medium and incubated overnight at 37 ºC with shaking at 250 rpm. Two mL of overnight cultures was inoculated into 100 mL of fresh medium and incubated until an OD_{550} of 0.8 was achieved. Cultures were centrifuged at 1500*g* for 10 min and pellets were resuspended in 100 mL of 2×YT containing 0.4 M saccharose, 25 µg/mL chloramphenicol, and 0.1 mM IPTG, and incubated overnight between 24-30ºC. Overnight cultures were centrifuged at 5000*g* for 10 min at 4 ºC. Supernatants were cleaned by centrifugation at 30000*g* for 20 min at 4 ºC. Pellets were resuspended in 5 mL of periplasmic extraction buffer (50 mM tris/HCl, 20% sucrose, and 1 mM EDTA) and incubated for 1 h in ice

with occasional shaking. After centrifugation at 30000*g* for 30 min at 4 ºC, periplasmic supernatants were recovered. Both, culture medium and periplasmic supernatants were analyzed as previously described in section 2.6. Next, supernatants were concentrated using ultra-filtration cells from Amicon (Beverly, CA) and stored at -20ºC.

2.8.2. Expression of soluble scFv

For the expression of soluble scFv antibody fragments, the corresponding pAK100-scFv phagemid was purified using the commercial kit Perfectprep® plasmid mini (Eppendorf). After quantification by electrophoresis, 200 µL of competent, non-suppressor *E. coli* HB2151 cells was transformed with 10 µL of 5 ng/µL pAK100-scFv by the $CaCl_2$ procedure and plated into 2×YT+G+C containing 25 µg/mL nalidixic acid. Transformed colonies were checked by screening ELISA and positive colonies were used to obtain scFv proteins using the same protocol as for scFv-pIII fusion proteins.

2.9. Sequence determination

The ABI PRISM Big Dye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems) was used for cycle sequencing with fluorescently labelled dideoxynucleotides. Cycle sequencing reactions were carried out on an ABI GeneAmp PCR System 9700 thermal cycler. Sequencing products were analysed by capillary electrophoresis on an ABI 3100 Genetic Analyser (Applied Biosystems). The primers used were 5'-CAG GAA ACA GCT ATG ACC-3´ and 5'-CTT ATT AGC GTT TGC CAT-3'. The DNA sequence was translated into aminoacid sequences using the Chromas 2.01 software (www.technelysium.com.au).

2.10. Tetraconazole ELISA based on monoclonal antibody

ELISA was performed by adding a volume of 100 µL per well throughout all assay steps. All the incubations were carried out at room temperature and, after each incubation, plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20). Plates were coated overnight with 1 µg/mL of OVA-HH (Manclús, Moreno, Plana, & Montoya, 2008). After washing, 50 µL of MAb DTPH 4.1 (0.06 µg/mL in PBS 0.0025%) followed by 50 µL of standards or samples were added. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins (1/2000 in PBS-T). Finally, peroxidase activity was revealed with 2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.35. The reaction was stopped 10 minutes later by adding 2.5 M H_2SO_4 and the absorbance was read at 490 nm using 650 nm as the reference wavelength.

2.11. Tetraconazole ELISA based on recombinant antibodies

For recombinant antibody ELISAs, plates were coated with OVA-DTPH at 0.25 µg/mL for hybridoma-derived RAb ELISA or at 0.5 µg/mL for lymphocyte-derived RAb ELISA. At the competition step, 50 µL of supernatant diluted in PBS with 0.0025% Tween 20 was added together with 50 µL of standards or samples. The rest of the assay was performed as described in section 2.6.

2.12. Preparation of tetraconazole standards and spiked juices

Tetraconazole serial dilutions were prepared in DMF from a 1 mM stock stored at -20 ºC. For recombinant assays, eight serial 3-fold dilutions were made from a 372.1 µg/mL concentration. Standards were prepared daily by diluting 1/500 in PBS. As the assay involved the addition of the same volume of the antibody dilution, final standard concentrations were 372.10, 124.03, 41.34, 13.78, 4.59, 1.53, 0.51, and 0.17 ng/mL. For monoclonal assays, eight serial 5-fold dilutions were made from a 100 µg/mL concentration. Final standard concentrations in assay were 100, 20, 4, 0.8, 0.16, 0.03, 0.0064, and 0.00128 ng/mL.

Apple and orange juices were obtained from a local supermarket. We assumed that these juices did not contain tetraconazole residues. The juices were spiked with tetraconazole at 1, 2, and 5 µg/mL by adding adequate analyte concentrations in PBS prepared from stock solutions in DMF. For ELISA determinations, spiked juice samples were directly analyzed just diluting 1/500, 1/1000, and 1/2500 in PBS, respectively, for the MAb-based ELISA, and 1/100, 1/100, and 1/500, respectively, for RAb-based ELISAs. After dilution, all fortification levels entered into their respective assay working ranges. Samples were analyzed four times independently.

2.13. Data analysis

Standards and samples were run in triplicate wells and mean absorbance values were processed. Standard curves were obtained by plotting absorbance values against the logarithm of analyte concentration. Sigmoid curves were fitted to a four-parameter logistic equation, using commercial software (Sigmaplot, Jandel Scientific) as previously reported (Moreno, Plana, Montoya, & Manclús, 2007). Tetraconazole

concentrations in fruit juices were obtained by interpolating mean absorbance values in the standard curve run in the same plate (SOFTmax Pro Software, Molecular Devices).

3. Results and discussion

3.1. Production of tetraconazole scFv RAbs from hybridomas

The hybridoma cell line secreting DTPH-41 MAb specific to tetraconazole was previously obtained and characterized by Manclús, Moreno, Plana, and Montoya (2008). The selected MAb was an IgG_1 *kappa* isotype. mRNA was isolated from hybridoma cells and used for first strand cDNA synthesis. Heavy and *kappa* light variable domains (V_H and $V_{L\kappa}$) were amplified and the predominant PCR products were of the expected sizes (data not shown). These V_H and $V_{L\kappa}$ genes were purified and assembled into a 750 bp scFv gene using an overlap extension of the nucleotide sequence of the flexible linker $(Gly_4\text{-}Ser)_3$. (Krebber et al., 1997).

The purified scFv DNA was cloned into the phagemid vector pAK100. This vector allows the RAb expression as functional scFv-pIII fusion proteins, both as soluble proteins and as recombinant phages presenting the fusion protein on the phage surface. Transformation of *E. coli* XL1-blue cells with the recombinant phagemid yielded a library of 5×10^4 transformed colonies. To detect positive clones, two screening methods were assayed: phage ELISA and soluble scFv-pIII ELISA.

Forty eight colonies were randomly picked and screened by phage ELISA and soluble scFv ELISA, performing non-competitive and competitive (100 nM tetraconazole as competitor) assays simultaneously. In both cases, about 75% of the colonies were screened as positive (A_{490} >1 in non-competitive assays), and 90% of the positive

colonies were screened as competitive (evident signal reduction in the presence of 100 nM tetraconazole). Usually, complete signal inhibition was achieved in the soluble scFv ELISA while just a signal decrease was observed in the phage ELISA. This fact pointed out that recombinant scFv-displaying phages, because of their size and complexity, were prone to unspecific interactions, which may mask analyte recognition in competitive assays. Moreover, Tout, Yau, Trevors, Lee, and Hall (2001) reported that scFv phages competed much more weakly than soluble scFv for binding to the herbicide picloram, which could be attributed to differences in the valency and folding of antigen binding sites of phage-bound (pentavalent) versus soluble scFv antibody fragments. Therefore, we decided to carry out the screening of small ligand-binding RAb libraries by the soluble scFv ELISA.

Three of the competitive colonies showing a high signal in absence of analyte were selected. Their culture supernatants were assayed against several concentrations of tetraconazole and all the colonies showed the same profile. One of them was selected and named as RAb DTPH H-4.1.

3.2. Production of tetraconazole scFv RAbs from lymphocytes

As other authors reported (Tout, Yau, Trevors, Lee & Hall, 2001), V_L amplification and scFv assembly from the genetic material of lymphocytes were problematic. The 750 bp scFv migrating fragment appeared as a wide, undefined smear with fragments of unexpected size in the electrophoresis gel. To solve this problem two additional polymerases from Abgene (PCR Master Mix and Extensor Hi-Fidelity PCR master mix) and different PCR conditions were checked. The highest yield of scFv fragment was achieved using Hi-Fidelity polymerase and the PCR conditions described in Material

and Methods. In addition, as most MAbs to small molecules reported on the literature were IgG1κ isotype (Dübel et al., 1994), only V_Lκ domains were amplified and used to obtain the scFv fragment. This fragment was purified, digested, and cloned into pAK100 in the same way as the fragment coming from hybridomas. Finally, four independent transformations gave 3×10^4 transformed colonies. This library size was considered as adequate taking into account that it came from immunized lymphocytes. Likewise, Tout, Yau, Trevors, Lee, and Hall. (2001) reported the isolation of high-affinity scFv against small molecules from an immunized library of similar size (10^4).

As expected, the initial screening of 96 clones did not render positive colonies. To enrich the library with positive colonies, a round of panning was undertaken. At this point, we took into account the fact that some researchers have proved the usefulness of competitive elution with analytes in the enrichment of clones producing scFv antibodies with high-affinity and specificity for small-ligand targets (Brichta, Hnilova & Viskovic, 2005). Then, plates were coated with 10 µg/mL of OVA-DTPH and bound recombinant phages were competitively eluted with 100 nM tetraconazole. Recovered phages from the first round of affinity selection were used to infect *E. coli* XL1-Blue cells. After plating on agar, 41 colonies were obtained and all of them were analyzed by competitive and non competitive soluble fragment ELISA. Seven colonies were positive and three of them showed clear signal reduction in presence of 100 nM tetraconazole. These three RAbs were studied in depth and only two unique sequences were identified, RAb DTPH L-1.1 and RAb DTPH L-1.2.

Since all the colonies obtained after the first round of panning were checked, it made no sense applying additional rounds of affinity selection. Additional rounds would just increase the proportion of the same positive clones in our population. Therefore, the specific elution procedure followed in this study allowed us to obtain high-affinity RAb

against small ligands in the first round of panning. In contrast, the application of unspecific elution required several rounds of panning to isolated RAb recognizing analytes with high affinity, as reported by other authors (Tout, Yau, Trevors, Lee, & Hall, 2001; Li et al., 2006).

3.3. Sequence analysis

DNA fragments of the three selected scFv to tetraconazole, one derived from a hybridoma (RAb DTPH H-4.1) and two derived from lymphocytes (RAb DTPH L-1.1 and RAb DTPH L-1.2), were sequenced and their respective amino acid sequences were deduced. Segment designation and amino acid numbering were assigned according to Kabat, Wu, Perry, Gottesman, and Foeller (1991). Amino acid sequence alignment is shown in Table 1. As it can be seen, the identities of the variable domains are higher than 90%, except for V_H of RbAb DTPH L-1.2 (identity of 85%). Approximately half of the amino acid substitutions are located in complementary determining regions (CDRs). It is noteworthy that the affinity to tetraconazole of the three scFv was very similar, being their amino acid sequence significantly different. This is especially evident for V_H CDR of RAb DTPH L-1.2.

On the basis of comparative studies of known antibody structures and sequences, it has been argued that there is a small repertoire of main-chain conformations for at least five out of the six hypervariable regions of antibodies, and that the particular conformation adopted is determined by a few key conserved residues. The commonly occurring main-chain conformations of the hypervariable regions are called canonical structures (Clothia & Lesk, 1987). Amino acid sequences of the three RAbs were analyzed using AbCheck and applying auto-generated SDR (specificity-determining

residues) templates (Martin, 1996). RAb DTPH H-4.1 and RAb DTPH L-1.2 showed similar results; CDRs L2 and L3 were identified as canonical class 1/7A and 1/9A, respectively; whereas CDR L1, H1, and H2 were similar, but no identical, to class 4/16A, 1/10A, and 2/10A, respectively. Canonical class assignment for RAb DTPH L1.1 showed almost the same results, except for CDR H1 that matched exactly 1/10A class.

3.4. ScFv expression and characterization

Soluble scFv antibodies were obtained following the protocol described by Kipriyanov, Moldenhauer, and Little (1997). Two *E. coli* strains were used for expression: *E. coli* XL1-Blue suppressor strain was used for expressing scFv-pIII fusion proteins, and *E. coli* HB2151 non suppressor strain was used for expressing soluble scFv proteins. Both types of fragments were produced to study the influence of an additional protein fragment (pIII) on RAb analytical characteristics. The expression of antibody fragments fused to a protein like pIII may increase protein solubility and hence soluble expression yield; moreover, the pIII protein can be used as a tag for rapid detection and purification of scFv (Mersmann et al., 1998). In addition, the production of fusion proteins involves fewer steps than soluble scFv.

Preliminary characterization of the two RAbs derived from lymphocytes showed that both had a very similar affinity to tetraconazole. During the characterization process, RAb DTPH L-1.1 proved to be unstable, as evidenced by a signal decrease over the time (data not shown). Instability of the V_H–V_L interaction that favours aggregation has been hypothesized to be the main cause of scFv inactivation (Leong & Chen, 2008). Therefore, this RAb was discarded for the application study. Competitive ELISAs in the

conjugate-coated format were designed and optimized from each antibody type. Standard curves of optimized tetraconazole ELISAs configured with MAb DTPH 4.1, RAb DTPH H-4.1, and RAb DTPH L1.2 are shown in Figure 2. Only scFv curves have been represented because scFv-pIII curves were almost identical. Analytical parameters of their respective competitive inhibition curve are shown in Table 2. For DTPH 4.1 MAb, the concentration giving 50% inhibition of the maximum absorbance (I_{50} value) was 0.5 ng/mL tetraconazole, while I_{50} values for RAbs were between 5.7 and 8.6 ng/mL. Other parameters, such as LOD and working range, maintained a similar ratio between monoclonal and recombinant antibodies. Comparing RAb data, both of them, hybridoma or lymphocyte derived, showed similar parameters. Moreover, analytical parameters were also fairly the same regardless of the RAb type, soluble scFv or fusion scFv-pIII. In general, all RAbs obtained in this study displayed one order of magnitude lower affinity (higher I_{50} value) to the analyte than MAb DTPH 4.1. When the RAb affinity and that of its parental MAb have been compared in the literature, diverse results have been reported. While some authors reported that RAbs showed lower affinity than its parental MAb (Kramer & Hock, 1996; Graham, Porter, & Harris, 1995; Choi et al., 2004), *i.e.* the same behaviour as we found, others reported a comparable (Tout, Yau, Trevors, Lee, & Hall, 2001; Alcocer, Doyen, Lee, & Morgan, 2000; Nishi, Ishiuchi, Morimune, & Ohkawa, 2005) or even higher (Alcocer, Doyen, Lee, & Morgan, 2000; Strachan, Grant, Learmonth, Longstaff, Porter, & Harris, 1998; Pan et al., 2006) affinity of RAbs over their counterpart MAbs. Results obtained in this study and those reported in the literature suggest that the folding of each scFv and, therefore, their analyte interaction properties depend on its particular amino acid sequence.

3.5. Cross Reactivity

The specificity of each antibody was evaluated by performing competitive ELISAs in the conjugate–coated format with structurally related compounds as competitors and comparing their respective I_{50} values. Cross-reactivity data for the MAb and RbAbs are presented in Table 3. In general, all the antibodies assayed showed a similar cross reactivity profile characterized by a high recognition of penconazole and DTP (tetraconazole degradation product) and a low recognition of hexaconazole and triadimefon. Furthermore, all of them recognized myclobutanil and cyproconazole to different extents. In particular, RAb DTPH H-4.1 cross-reactivity for almost all the compounds was slightly higher than that of the MAb, whereas RAb DTPH L-1.2 cross-reactivity for cyproconazole and triadimefon was lower than that of the other antibodies. Concerning to both types of recombinant fragments, cross-reactivity patterns of soluble scFv and fusion protein scFv-pIII were nearly identical.

3.6. Analysis of spiked juice samples

It has been previously proved that the MAb-based immunoassays can precisely and accurately determine triazole fungicide residues in fruit juices (Manclús, Moreno, Plana, & Montoya, 2008). To test the analytical performance of RAb-based immunoassays, optimized conjugate-coated ELISAs were applied to the determination of tetraconazole in spiked fruit juices. Apple and orange juices were spiked with tetraconazole at 1, 2, and 5 µg/mL, and directly analyzed by these immunoassays without any treatment other than dilution in PBS (at least 1/100) to minimize matrix effects. Taking into account this dilution and the limit of detection range in buffer, the actual LODs of RAb-based immunoassays for tetraconazole in apple and orange juices were in the 0.1−0.2 µg/mL

range. Table 4 summarizes the results obtained for each spiked juice. In general, coefficients of variations can be considered as acceptable, most of them were below 20%. For apple juice, recoveries were fairly good, ranging from 78 to 119 %, regardless of the antibody type used. For orange juice, acceptable recoveries were obtained with the MAb-based assay, while the RAb-based assays tended to overestimate as recoveries ranged from 100 to 145 %. Finally, both scFv and scFv-pIII fragments showed almost identical behavior in tetraconazole determination in fruit juices. On the other hand, non-spiked juice samples were also included in the analysis as negative controls. Tetraconazole concentrations below the detection limit of the assays were found in all negative samples.

4. Conclusion

RAbs for tetraconazole were produced from a hybridoma cell line and from lymphocytes of hyperimmunized mice. They are the first reported for this fungicide. With these RAbs, immunoassays with LOD about 1−2 ng/mL for tetraconazole standards were developed. The sensitivity of these immunoassays is higher than that of the polyclonal antibody-based immunoassay developed by Forlani et al. (1992) but lower than that of the monoclonal antibody-based immunoassay developed by our group (Manclús, Moreno, Plana, & Montoya, 2008). Nevertheless, RAbs can be obtained in a more rapid, cost-effective manner than monoclonal antibodies.

For tetraconazole determination in apple and orange juices, only a 1/100 dilution in buffer is required to minimize matrix effects. Under these conditions, the practicability of this screening technique has been demonstrated since the fungicide residues content

in the fruit juices tested can be measured down to 100 ng/mL with acceptable accuracy and precision for a screening technique.

To the best of our knowledge, this work is the first report on the application of RAbs to pesticide residue analysis in food. The major advantage of this immunochemical technique over existing methods is its high sample throughput because as many as 120 juice samples can be determined daily by an analyst.

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Figure captions:

Figure 1. Structure of tetraconazole and of the immunizing hapten DTPH.

Figure 2. ELISA standard curves for tetraconazole using MAb DTPH 4.1 (■), RAb DTPH H-4.1 (●) and RAb DTPH L-1.2 (▲). Each point represents the mean value of four replicates ± standard deviation.

Table(s)

Table 1

Comparison of deduced aminoacid sequences of RAbs derived from hybridoma and lymphocyte cells.[a]

RAb	Heavy chain (V_H)
	FR-1
DTPH H-4.1	Q V Q L Q QS G AE L AK P GA S VKMS CK A S D Y T F T
DTPH L-1.2	– – – – – – – – – – V T – – – – – – – – – – – – – – G – – – I
DTPH L-1.1	– G – – – –
	CDR-H1
DTPH H-4.1	R Y W M H
DTPH L-1.2	S S – – –
DTPH L-1.1	S – – – –
	FR-2
DTPH H-4.1	W V K Q R P G Q G L E W I G
DTPH L-1.2	– – – E – – – – – – – – – –
DTPH L-1.1	– – – – – – – – – – – – – – –
	CDR-H2
DTPH H-4.1	Y I N P S T G Y T E Y N Q K F K D
DTPH L-1.2	W – Y – R – – H I – – – – – – – –
DTPH L-1.1	– – – – – – – – – – – – – – – – –
	FR-3
DTPH H-4.1	K A T L T A D I S S S T A Y M Q L S S L T S E D S A V Y Y C A R
DTPH L-1.2	– – – – – – – S – – T – – – – – – – – – – – D – – – – – – – – –
DTPH L-1.1	– – – – – – – K – R K – N
	CDR-H3
DTPH H-4.1	A T N Y A M D Y
DTPH L-1.2	– G Y D – L – –
DTPH L-1.1	– D Y D – – – –
	FR4
DTPH 4.1	W G Q G T S V T V S S A
DTPH 1.2	– – – – – – – – – – – –
DTPH 1.1	– – – – – – – – – – – –

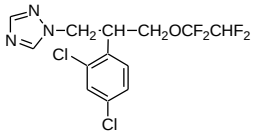
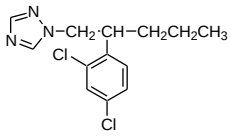
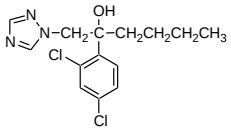
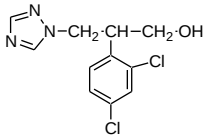
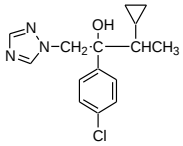
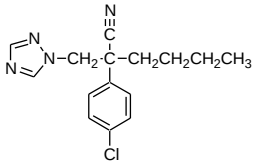
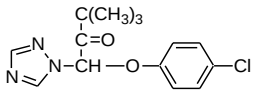
RAb	Light chain (V_L)
	FR-1
DTPH H-4.1	D V V M T Q T P L S L P V S L G D Q A S I S C
DTPH L-1.2	– I – – – – S – – – – – – – – – – – – – – –
DTPH L-1.1	– I – – – – S – – – – – – – – – – – V – – – –
	CDR-L1
DTPH H-4.1	R S S Q S V V H S N G N T Y L Q
DTPH L-1.2	– – – – – I – – – – – – – – – E
DTPH L-1.1	– – – – N I – – – T T – – – – –
	FR-2
DTPH H-4.1	W F L Q K P G Q S P K L L I Y
DTPH L-1.2	– Y – – – – – – – – – – – – –
DTPH L-1.1	– I – – – – S – – – – – – – –
	CDR-L2
DTPH H-4.1	K V S N R F S
DTPH L-1.2	– – – – – – –
DTPH L-1.1	– – – – – – –
	FR-3
DTPH H-4.1	G V P D R F S G S G S G T D F T L K I S R V E A E D L G V Y Y C
DTPH L-1.2	– –
DTPH L-1.1	– – – – – – – S –
	CDR-L3
DTPH H-4.1	F Q G S H V P W T
DTPH L-1.2	– – – – – – – – –
DTPH L-1.1	– – – – – – – – –
	FR-4
DTPH H-4.1	F G G G T K L E L K R
DTPH L-1.2	– – – – – – – – I – –
DTPH L-1.1	– – – – – – – – – – –

[a] Identical aminoacids to RAb DTPH H-4.1 are indicated with a dash (−).

Table 2
Analytical Characteristics of Tetraconazole Immunoassays Based on MAb and RAbs.

Antibody	I_{50} (ng/mL)	Working range (ng/mL)	LOD (ng/mL)
MAb DTPH 4.1	0.5	0.2-1.6	0.1
RAb DTPH H-4.1	8.5	3.8-18.5	2.2
RAb-pIII DTPH H-4.1	8.6	3.4-20.3	1.8
RAb DTPH L1.2	6.4	2.5-15.0	1.3
RAb-pIII DTPH L-1.2	5.7	1.9-15.4	0.9

Table 3
Cross-reactivity[a] of Tetraconazole Antibodies towards Several Triazole Compounds

Chemical structure	Compound	MAb DTPH 4.1	Recombinant antibodies			
			scFv DTPH H-4.1	scFv-pIII DTPH H-4.1	scFv DTPH L-1.2	scFv-pIII DTPH L-1.2
	Tetraconazole	100	100	100	100	100
	Penconazole	44	87	90	64	47
	Hexaconazole	<0.1	0.5	0.7	<0.1	<0.1
	DTP	24	58	50	40	29
	Cyproconazole	32	63	50	7	5
	Myclobutanil	14	33	26	13	11
	Triadimefon	1	1	1	0.2	0.1

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

Table 4
Reproducibility and Accuracy of Tetraconazole Immunoassays in Spiked Fruit Juice Samples

Immunoassay develop with	Apple juice				Orange juice		
	Spiked level (ng/mL)	Found[a] (ng/mL)	CV (%)	Recovery (%)	Found[a] (ng/mL)	CV (%)	Recovery (%)
MAb DTPH 4.1	1000	1190	18	119	1210	14	121
	2000	1681	10	84	1876	3	94
	5000	3922	14	78	4987	8	100
RAb DTPH H-4.1	1000	1091	7	109	1316	10	132
	2000	2116	5	106	2326	7	116
	5000	4401	13	88	5323	19	107
RAb-pIII DTPH H-4.1	1000	1172	1	117	1449	15	145
	2000	2151	12	108	2647	8	132
	5000	4757	7	95	6390	6	128
RAb DTPH L-1.2	1000	875	3	88	1178	7	118
	2000	1823	8	91	2519	12	126
	5000	4051	21	81	5190	10	104
RAb-pIII DTPH L-1.2	1000	1006	24	101	1300	12	130
	2000	2036	7	102	2724	8	136
	5000	3969	13	79	5274	3	106

[a] Data are the average of four independent determinations.

Figure 1

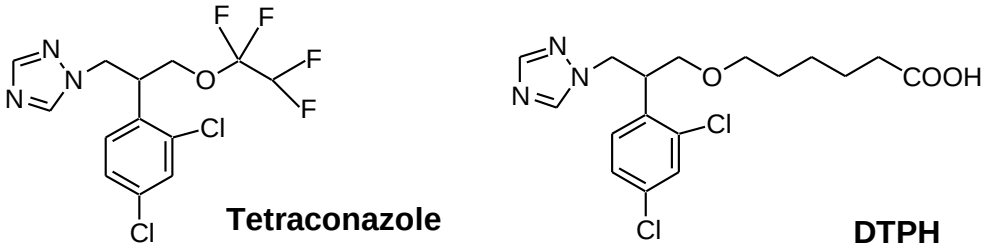


Figure 1. Structure of tetraconazole and of the immunizing hapten DTPH.

Figure 2

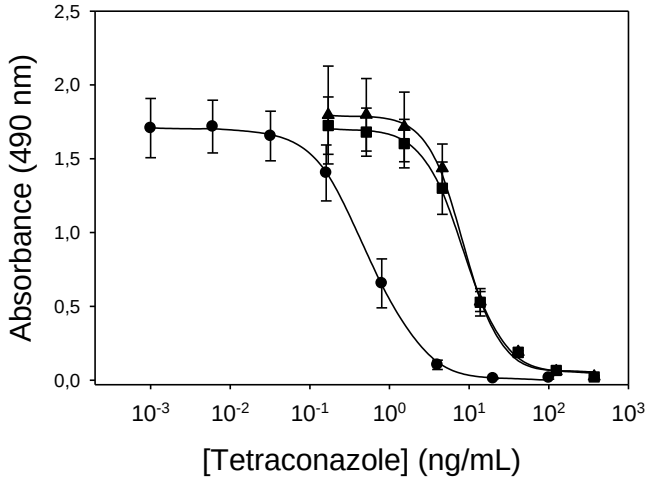


Figure 2. ELISA standard curves for tetraconazole using MAb DTPH 4.1 (■), RAb DTPH H-4.1 (●) and RAb DTPH L-1.2 (▲). Each point represents the mean value of four replicates ± standard deviation.