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Analytical Methods

Development and application of recombinant antibody-based immunoassays to tetraconazole residue analysis in fruit 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 hyperimmunised 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% to 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.

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, 2013). Maximum residue levels (MRLs) were harmonised 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, 2013). 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 recognised tetraconazole and penconazole, with which they developed an ELISA that detected tetraconazole between 25 and 10,000 μg/L in fruit (Cairoli, Arnoldi, & Pagani, 1996). With regard to other azole

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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 and 20 mg/L. Székács & 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 standardise 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 and 0.7 μg/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 (Fitzgerald, Leonard, Darcy, Danaher, & O'Kennedy, 2011; Markus, Janne, & Urpo, 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, hyperimmunised animals, and even from non-immunised, generic libraries (Brichta et al., 2005; Markus et al., 2011). Nevertheless, immunised libraries were a straightforward starting material for obtaining high affinity antibodies to low molecular weight compounds (Kramer & Hock, 2003; Tout, Yau, Trevors, Lee, & Hall, 2001).

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 *Escherichia 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-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 MasterMixKit 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-labelled 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 (Fig. 1) and immunoreagents (OVA-DTPH and BSA-DTPH conjugates, DTPH-41 MAb against tetraconazole) were previously obtained as described (Manclús et al., 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

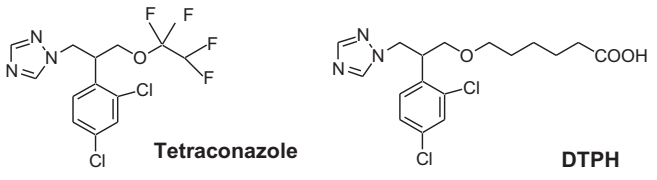

Fig. 1. Structure of tetraconazole and of the immunizing hapten DTPH.