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Analytical Methods

Immunoassays for trifloxystrobin analysis. Part II. Assay development and application to residue determination in food

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ABSTRACT

Immunochemical assays constitute complementary analytical methods for small organic molecule determination. We herein describe the characterisation and optimisation of two competitive enzyme-linked immunosorbent assays in different formats using monoclonal antibodies to the Quinone outside inhibitor (QoI) fungicide trifloxystrobin. Antibody selectivity was evaluated using a variety of agrochemicals and the main trifloxystrobin metabolite. Acceptable tolerance of the immunoassay to methanol, ethanol, and acetonitrile was observed in all cases, whereas a dissimilar influence of buffer pH and ionic strength was found. Moreover, the influence of Tween 20 over the analytical parameters was studied. The limits of detection of the optimised assays were below 0.1 μg L^{-1}. Excellent recoveries, even at 10 μg kg^{-1}, were obtained when strawberry, tomato, and cucumber samples spiked with trifloxystrobin were analysed. Finally, statistical agreement was found between immunoassay and reference chromatographic results using blind-spiked and in-field treated samples.

1. Introduction

Trifloxystrobin is a modern agrochemical exhibiting rapid and potent fungicidal activity and possessing a particular mode of action that confers much lower toxicity to humans and the environment than other traditional antimycotic substances. Due to this fact, its use has rapidly grown after it was introduced in the market in the late 1990s – global sales reached 450 M€ in 2010 (Bayer, 2010). Trifloxystrobin belongs to the strobilurin family of new-generation agrochemicals. It has been registered in more than 80 countries, and nowadays it is commercialised by Bayer CropScience under different trade names (Flint, Delaro, Madison, etc.) as the only active ingredient or formulated together with other fungicides. Although some cases of natural or acquired resistance of fungal pathogens like *Ventura inaequalis* or *Fusarium graminearum* towards trifloxystrobin have been recently reported (Dubos, Pasquali, Pogoda, Hoffmann, & Beyer, 2011; Frederick & Cox, 2012), trifloxystrobin is widely employed to fight fungal diseases in a variety of crops such as cereals, strawberries, oranges, grapes, tomatoes, cucumbers, etc. Metabolism in plants is low but complex, with the demethylated derivative being the most relevant metabolite (Fig. 1), yet trifloxystrobin accounts for more than 95% of the residue. Consequently, most regulatory agencies just include the pesticide itself as the only analytical target in samples from plant origin, even though particular legislations also take into account the demethylated metabolite (Health Canada, 2012). In the EU, trifloxystrobin was approved as low risk active substance for plant protection in 2003. European and US maximum residue limits (MRL) for trifloxystrobin range from 0.05 to 30 mg kg^{-1} in authorised crops. According to the 2010 EU report on pesticide residues in food (EFSA, 2013), this fungicide was one of the most frequently found pesticides – between 2% and 5% of the analysed food samples contained trifloxystrobin residues at or below the MRL – and it was among the most common combinations in samples with multiple residues.

During the last decade, an effort has been put in order to develop analytical methods for trifloxystrobin analysis in foodstuffs. The first approach was described in 2002 and comprised liquid chromatography coupled to tandem mass spectrometry (MS) with electrospray ionisation (Taylor, Hunter, Hunter, Lindsay, & Le Bouhellec, 2002). Recently, multiresidue analysis

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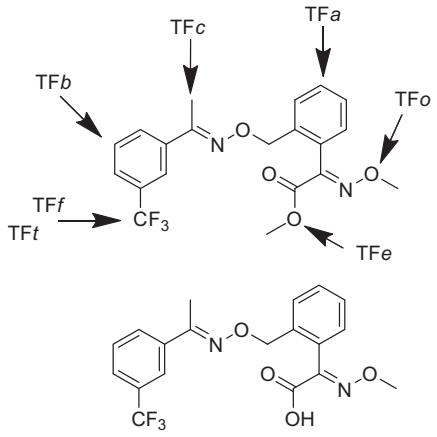

Fig. 1. Top: trifloxystrobin; the arrows point out the attachment sites of the C-6 hydrocarbon spacer arm of haptens. Bottom: main trifloxystrobin metabolite.

including trifloxystrobin was addressed by high-performance liquid chromatography (HPLC) or gas chromatography (GC) with different detection systems (Abreu, Correia, Herbert, Santos, & Alves, 2005; Campillo, Vinas, Aguinaga, Ferez, & Hernandez-Cordoba, 2010; Likas, Tsiropoulos, & Miliadis, 2007; Mastovska, Dorweiler, Lehotay, Wegscheid, & Szpylka, 2010; Sannino, Bolzoni, & Bandini, 2004; Schurek et al., 2008). Alternatively, for low cost, in-situ, and/or rapid applications in samples with limited number of target analytes, immunochemical methods can be an attractive strategy. As the first approach, most research laboratories preferentially use the competitive enzyme-linked immunosorbent assay (cELISA) because of the high analytical capacity and simplicity for standardisation. Depending on the immobilised reagent and on the enzymatic detection step, two main formats are usually configured; that is, the antibody-coated direct cELISA and the conjugate-coated indirect cELISA (d-cELISA and i-cELISA, respectively). Immunochemical methods enabling the sensitive determination of some strobilurin fungicides in foodstuff have been recently reported by our group (Esteve-Turrillas, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011; Esteve-Turrillas et al., 2010; Mercader et al., 2012). In this paper, the development of novel monoclonal antibody-based immunoassays with different selectivity and in different cELISA formats is reported. Furthermore, a validation study was undertaken using blind and in-field treated samples, and statistical agreement with GC–MS was assessed. To the best of our knowledge, the determination of trifloxystrobin in food samples by immunoassay is herein reported for the first time.

2. Experimental

2.1. Reagents and instruments

Trifloxystrobin (CAS Registry No. 141517-21-7, Mw 408.13) analytical standard was kindly provided by Bayer CropScience (Leverkusen, Germany). Other pesticide standards were obtained from BASF (Limburgerhof, Germany), Fluka/Riedel-de-Haën (Seelze, Germany), Dr. Ehrenstorfer (Augsburg, Germany), or Syngenta (Basel, Switzerland). Triphenylphosphate was from Sigma/Aldrich (Madrid, Spain). Primary/secondary amine and solvents were from Scharlab (Barcelona, Spain). Enzyme immunoassays were performed using regular reagents, buffers, and plastic ware as reported in the preceding paper (López-Moreno, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2013). The employed monoclonal antibodies (mAbs) and protein–hapten conjugates were those described in previous articles (López-Moreno et al., 2013; Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008). Peroxidase conjugate to rabbit anti-mouse immunoglobulin polyclonal antibody was from Dako (Glostrup, Denmark). Microplate wells were washed with an ELx405 microplate washer and absorbances were read with a PowerWave HT, both from BioTek Instruments (Winooski, VT, USA).

2.2. Competitive ELISAs

Eight-point standard curves, including a blank, were prepared in borosilicate glass tubes by 10-fold serial dilution in PBS (10 mM phosphate, pH 7.4 containing 140 mM NaCl), starting from a 100 μg L^{-1} trifloxystrobin solution in PBS. Experimental values were fitted using the SigmaPlot software (Systat Software Inc., Chicago, IL, USA) to a four-parameter logistic equation:

$$y = (A_{max} - A_{min}) \Big/ \left[1 + (X/C)^{B}\right] + A_{min}$$

were A_{max} is the absorbance reached in the absence of analyte, A_{min} is the background signal, C is the analyte concentration at the inflexion point of the sigmoidal curve, and B is the slope at the inflexion point.

For assay characterisation, the concentration of trifloxystrobin that was necessary to induce a 50% inhibition of the antibody–conjugate reaction (IC_{50}) was taken as a reference. The analyte concentration that inhibited 10% (IC_{10}) the immunochemical reaction was considered as the limit of detection (LOD) of the assay. Curves were normalised and average values were calculated from independent experiments. Cross-reactivity (CR) was calculated as percentage value from the quotient between the IC_{50} for trifloxystrobin and the IC_{50} for the studied compound, both in molar concentration units.

2.2.1. Antibody-coated direct cELISA

Microwells were coated with 100 μL per well of antibody solution in coating buffer (50 mM carbonate, pH 9.6) by overnight incubation at room temperature. Next day, plates were washed four times with washing solution (150 mM NaCl containing 0.05% (v/v) Tween 20). The competitive step was run with 50 μL per well of analyte or sample solution in PBS and 50 μL per well of enzyme tracer solution in PBST (PBS containing 0.05% (v/v) Tween 20). After 1 h incubation at room temperature, plates were washed again as described before. Finally, signal was generated with 100 μL per well of a 0.012% (v/v) H_2O_2 and 2 mg mL^{-1} of *o*-phenylenediamine solution in 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4. The enzymatic activity was stopped after 10 min with 2.5 M sulfuric acid. Absorbance was spectrophotometrically measured at 492 nm with 650 nm as a reference wavelength.

2.2.2. Conjugate-coated indirect cELISA

Coating was performed overnight at room temperature with 100 μL per well of OVA conjugate solution in coating buffer. Then, coated microwells were washed as previously described. Competition was done with 50 μL per well of analyte or sample solution in PBS and 50 μL per well of antibody solution in PBST by incubation at room temperature during 1 h. After another washing step, 100 μL per well of enzyme-labeled secondary antibody (diluted 1/2000) in PBST was added and incubated during 1 h at room temperature. Finally, plates were washed and signal was generated and read as indicated above.

2.3. Buffer evaluation and solvent tolerance

A biparametric approach with a central composite design was followed using the Minitab software (Minitab Inc., State College, PA, USA). Trifloxystrobin was prepared in Milli-Q water, and