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Analytical Methods

Development of competitive enzyme-linked immunosorbent assays for boscalid determination in fruit juices

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ABSTRACT

Boscalid is a modern, broad-spectrum carboxamide pesticide highly efficient against most fungal diseases affecting valuable crops. In this study, a boscalid-mimicking derivative with a six-carbon spacer arm replacing the chlorine atom at the pyridine ring of the target molecule was synthesized and coupled to carrier proteins. Following rabbit immunization, antibodies against this agrochemical were obtained for the first time, and they were characterised in terms of affinity and specificity, tolerance to solvents, and robustness to changes in buffer pH and ionic strength, using two assay formats. Both of the optimised immunoassays showed limits of detection below 0.1 μg/L. Moreover, matrix effects of grape, peach, apple, and tomato juices were evaluated. Finally, a simple and easy procedure was set up for boscalid determination with spiked samples, affording limits of quantification of 10 μg/L, a value well below the sensitivity levels required for monitoring campaigns of pesticide residue analysis in food.

1. Introduction

Boscalid (BL, see Fig. 1 for the chemical structure) is a broad spectrum fungicide that is being intensively employed throughout the world to fight against highly destructive plant pathogens, such as *Botrytis cinerea*, *Sclerotinia* spp., *Leveillula taurica*, or *Spherotheca macularis*. It is the only member of the pyridine carboxamide group of pesticides and it shows a biological mode of action consisting in the inhibition of the enzyme succinate ubiquinone reductase, also known as complex II, in the mitochondrial electron transport chain (Avenot & Michailides, 2007). BL is a blockbuster fungicide of BASF's crop protection pipeline. In 2003, it was approved in the US and in 2008 it was included into Annex I of the Council Directive 91/414/EEC, that is, the EU positive list of authorised agrochemicals (http://www.pesticideinfo.org; EC Regulation, 2008.). Since then, due to its excellent performance, the actual peak sales potential has increased to more than € 300 million. This fungicide is currently used to treat over 100 crop varieties, including vegetables, fruits, and cereals, across more than 70 countries and has more than 200 indications (http://www.agro.basf.com). For commercial use and to avoid resistance, it is frequently formulated together with an active principle of the strobilurin family such as pyraclostrobin, dimoxystrobin, or kresoxim-methyl. In line with the "Good Agricultural Practices" concept defined by the FAO/WHO aimed at protecting consumers, most countries have approved different legislations stating the maximum residues levels (MRLs) of pesticides in food and feed (http://www.mrldatabase). As indicated by the European Food Safety Authority (EFSA Journal, 2010), the tolerated levels for BL in some relevant food commodities in the EU are: 10 mg/kg for lettuce, spinach, most leafy brassica, and strawberries; 5 mg/kg for kiwifruits and table and wine grapes; 3 mg/kg for stone fruits; 2 mg/kg for pome fruits and most head brassica; and 1 mg/kg for flowering brassica and tomatoes (http://ec.europa.eu/sanco_pesticides/public/index.cfm). Similar values are reported by the Codex Alimentarius Commission (www.codexalimentarius.net) as well as by the US Environmental Protection Agency (http://www.epa.gov/pesticides/PPISdata/index.html).

Current analytical methodologies for the determination of BL in food and feed are based on chromatographic separations by both gas and liquid chromatography coupled to mass spectrometry detectors. BL can be extracted from the sample by alternative methodologies, though they are mostly based on the use of high amounts of organic solvents. Acetone has been employed to

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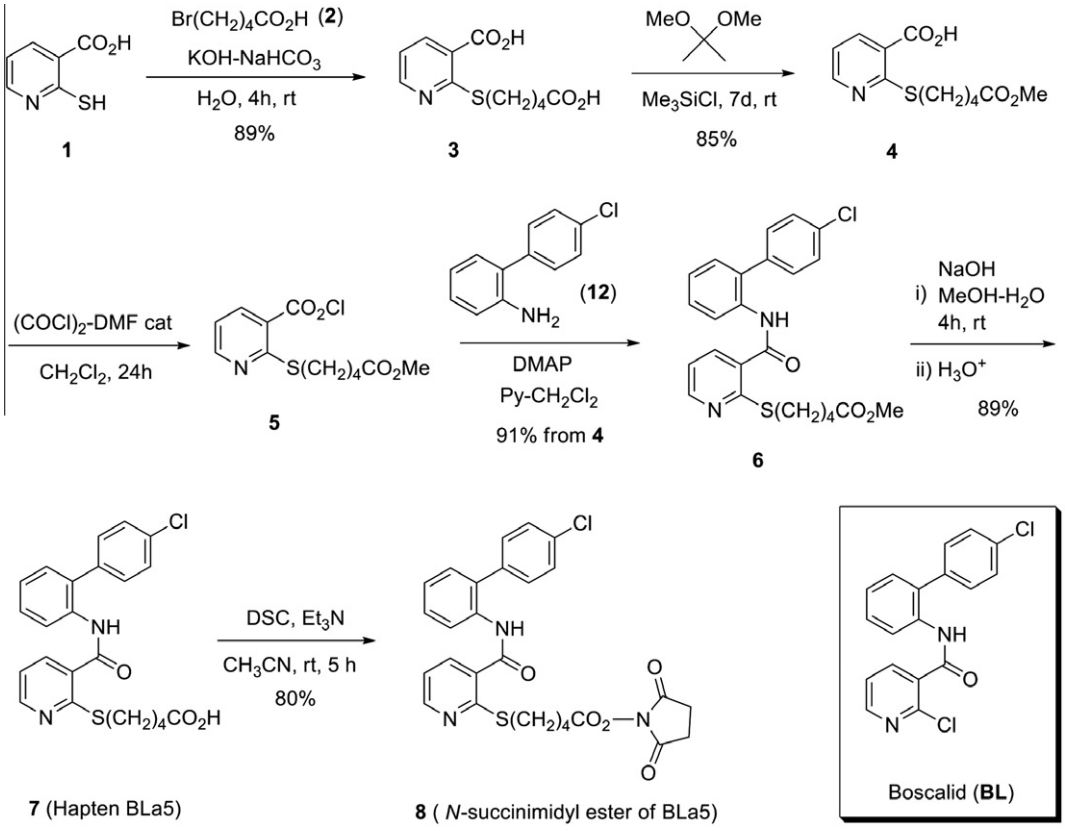

Fig. 1. Synthetic pathway of hapten BLa5, the activation reaction with disuccinimidyl carbonate, and the structure of boscalid.

effectively extract BL and other pesticides from different fruit and vegetable matrices, followed by a clean-up step based on liquid–liquid extraction with dichloromethane (Hiemstra & de Kok, 2007; Liu, Dong, Qin, & Zheng, 2010). For multi-residue analysis, the QuEChERS (Quick Easy, Cheap, Effective, Rugged, and Safe) methodology, which employs acetonitrile to extract pesticides together with a clean-up of the extract based on solid-phase extraction with primary–secondary amine (Lehotay, 2007), or with C_{18} or graphitized carbon black phases (Walorczyk, 2008; Walorczyk & Gnusowski, 2009), is widely used and it has been broadly validated. However, sample extraction and clean-up demand important hand labour, thus decreasing sample throughput and increasing costs. In addition, the use of organic solvents has a negative impact on the environment. During the past decade, many competitive enzyme-linked immunosorbent assays (cELISA) have been published for the analysis of residues of a broad variety of pesticides in different kinds of food samples, and several immunochemical methodologies have been approved to tackle the analysis of chemical residues in food, feed, and environmental samples (http://www.epa.gov/pesticides/methods/index.htm). Competitive immunoassays are required because of the small size of the target molecules. They have been widely used due to their simplicity, high sample throughput, low cost, and minimal environmental impact. Moreover, immunoassays constitute a highly versatile technology, so they can be implemented in many different analytical platforms and adapted to specific applications. Particularly, cELISAs in a microtiter plate can be developed using different formats for the analysis of small chemical molecules, yet the antibody-coated direct competitive assay (d-cELISA) and the conjugate-coated indirect competitive assay (i-cELISA) are the most common formats.

To our knowledge, neither the production of antibodies nor the development of immunoassays for BL residue analysis in food has been published so far. In this article, we report the original synthesis of a functionalised derivative used for the generation of novel antibodies against BL and the development and characterisation of direct and indirect immunoassays for the analysis of this relevant pesticide in foodstuffs. The cross-reactivity of the developed immunoassays towards commonly used fungicides was evaluated and, after optimization of assay parameters, the selected analytical procedures were applied to fortified juices of relevant commodities, such as grapes, peaches, apples, and tomatoes.

2. Experimental

2.1. Reagents and instrumentation

BL (2-chloro-*N*-(4′-chlorobiphenyl-2-yl)nicotinamide, CAS Registry No. 188425-85-6, MW 343.2 g/mol) and other pesticide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Technical grade BL was kindly provided by BASF. Horseradish peroxidase (HRP), ovalbumin (OVA), and *o*-phenylenediamine were purchased from Sigma–Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were used for conjugate purification with an ÄKTA workstation. Polyclonal goat anti-rabbit immunoglobulin peroxidase conjugate (GAR–HRP) was from BioRad (Hercules, CA, USA). Bovine serum albumin (BSA) fraction V was purchased from Roche Applied Science (Mannheim, Germany). Fetal bovine serum (FBS) and Freund's adjuvants were from Sigma–Aldrich (Madrid, Spain). Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Ultraviolet–visible spectra and ELISA absorbances were read with a PowerWave HT from BioTek Instruments