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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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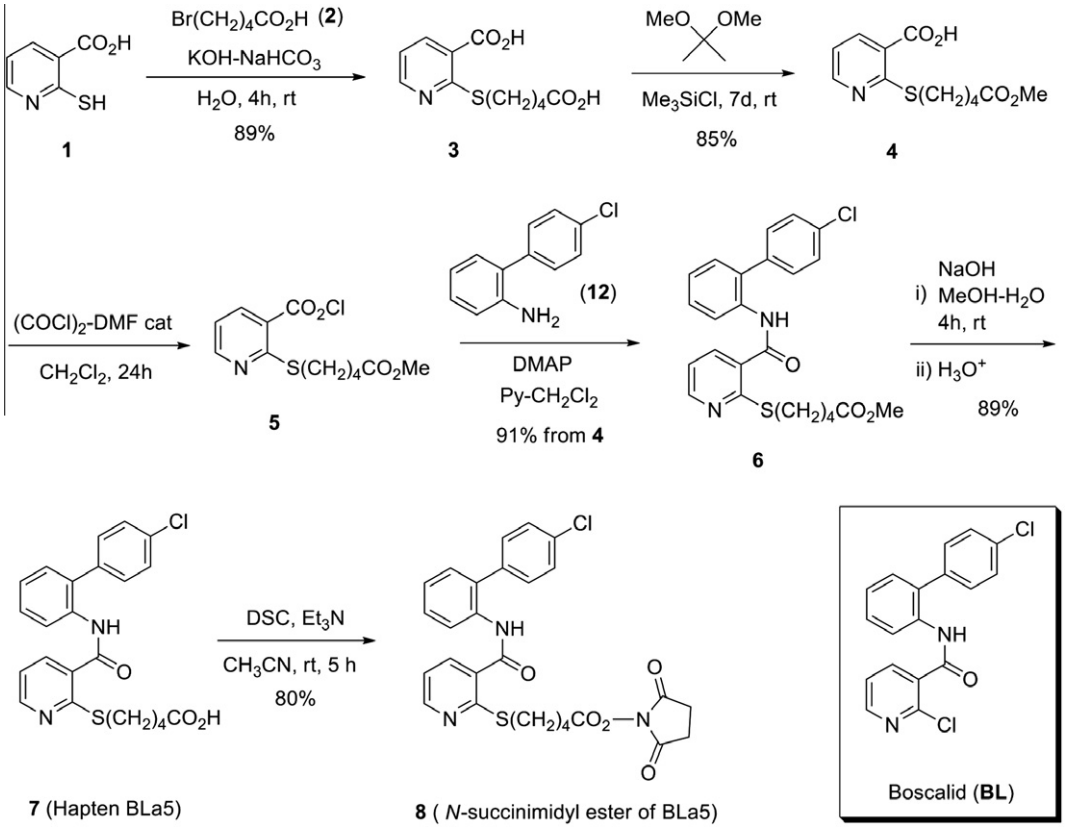

Fig. 1. Synthetic pathway of hapten BLa5, the activation reaction with disuccinimidyl carbonate, and the structure of boscalid.

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

(Winooski, VT, USA). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments.

The composition, concentration, and pH of the employed buffers were as follows: (i) PB, 100 mM sodium phosphate buffer, pH 7.4; (ii) PBS, 10 mM sodium phosphate buffer, pH 7.4 with 140 mM NaCl; (iii) PBST, PBS containing 0.05% (v/v) Tween 20; (iv) 2 × PBST, 20 mM sodium phosphate buffer, pH 7.4 with 280 mM NaCl and 0.05% (v/v) Tween 20; (v) CB, 50 mM sodium carbonate–bicarbonate buffer, pH 9.6; (vi) Washing solution, 150 mM NaCl and 0.05% (v/v) Tween 20; and (vii) Developing buffer, 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4.

2.2. Hapten synthesis

Previously known 4′-chloro-[1,1′-biphenyl]-2-amine (**12**) (Bradsher & Wissow, 1946; Felpin, Fouquet, & Zakri, 2009) was readily prepared in high yield in two steps from 1-iodo-2-nitrobenzene (**9**) and 4-chlorophenylboronic acid (**10**) as described in the Supplementary Data (Fig. S1). 2-Mercaptonicotinic acid (**1**), 5-bromopentanoic acid (**2**) and all the other reagents used for the synthesis of the functionalised hapten were acquired from commercial sources and used without purification. Reactions were performed in oven-dried glassware under a nitrogen atmosphere containing a Teflon-coated stirrer bar and a dry septum. For the exclusion of atmospheric oxygen from the reaction media, three freeze-pump thaw cycles were preformed before the reagents were mixed. All solvents were purified by distillation and, if required, they were dried according to standard methods. The reactions were monitored with the aid of thin-layer chromatography using 0.25 mm pre-coated silica gel plates. Visualisation was carried out with UV light and aqueous ceric ammonium molybdate solution. Chromatography refers to flash column chromatography and it was carried out with the indicated solvents on silica gel 60 (particle size 0.040–0.063 mm). All melting points were determined using a Kofler hot-stage apparatus or a Büchi melting point apparatus and are uncorrected. All NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at room temperature (rt) on a Bruker AC-300 spectrometer (300.13 MHz for ^1H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in the ^1H NMR spectra (7.26 and 2.50 ppm) and to solvent carbons in the ^{13}C NMR spectra (77.0 and 39.43 ppm). Carbon substitution degrees were established by distortionless enhancement by polarisation transfer pulse sequences. A combination of correlation spectroscopy and heteronuclear single quantum coherence experiments was used in most cases for the assignment of ^1H and ^{13}C chemical shifts. Infrared (IR) spectra were measured as thin films between NaCl plates for liquid compounds and as KBr pellets for solids using a Nicolet Avatar 320 spectrometer. Mass spectra (MS) and high-resolution mass spectra (HRMS) were run by the electron impact (EI) at 70 eV in a Micromass VG Autospec spectrometer.

Compounds used in this study present minor safety concerns. However, it is advisable to work in a well-ventilated fume hood during synthesis reactions. A schematic representation of the synthetic procedures is depicted in Fig. 1.

2.2.1. Preparation of 2-(4-carboxybutylthio)nicotinic acid (**3**)

A solution of sodium 5-bromopentanoate in H_2O, prepared from 5-bromopentanoic acid (**2**) (700.1 mg, 3.89 mmol) and $NaHCO_3$ (326.7 mg, 3.89 mmol) in 3 mL of H_2O, was added drop wise to a solution of 2-mercaptonicotinic acid (**1**) (500 mg, 3.23 mmol) in 10% aqueous KOH (5 mL) at rt. The reaction mixture was stirred at 60 °C for 4 h, cooled in an ice-water bath, and acidified with concentrated HCl to pH 2–3. The solid precipitated product was collected, washed with water, and dried under vacuum to give nearly pure diacid **3** (735.4 mg, 89%) as a white solid. Mp 145–148 °C (from C_6H_6–methanol); IR ν_{max}/cm^{-1} (KBr) 3495, 3100–2500, 3071, 2957, 1716, 1682, 1561, 1423, 1389, 1300, 1224, 1076, 763, 718, 558; ^1H NMR (300 MHz, DMSO-d_6) δ: 8.60 (1H, dd, J = 4.6, 1.8 Hz, H-6 Py), 8.18 (1H, dd, J = 7.6, 1.8 Hz, H-4 Py), 7.21 (1H, dd, J = 7.6, 4.6 Hz, H-5 Py), 3.09 (2H, t, J = 6.3 Hz, H-1), 2.26 (2H, t, J = 6.6 Hz, H-4), 1.62 (4H, m, H-2 and H-3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.27 (COOH), 166.30 (Py-COOH), 160.79 (C-2 Py), 151.82 (C-6 Py), 138.88 (C-4 Py), 123.53 (C-3 Py), 118.62 (C-5 Py), 33.18 (C-1), 28.74 (C-4), 27.98 (C-2), 23.91 (C-3); MS (EI) m/z 255 (M^+, 28), 222 (4), 211 (3), 210 (6), 209 (12), 207 (21), 182 (21), 169 (100); HRMS (EI), calculated for $C_{11}H_{13}NO_4S$ 255.05603, found 255.05661.

2.2.2. Preparation of 2-(5-methoxy-5-oxopentylthio)nicotinic acid (**4**)

Trimethylsilyl chloride (*ca.* 20 μL, *ca.* 0.158 mmol) was added to a stirred solution of diacid **3** (376 mg, 1.58 mmol) in 2,2-dimethoxypropane (2.4 mL) and anhydrous methanol (1.6 mL). The mixture was stirred at rt for a week, poured into water, and extracted with ethyl acetate. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and concentrated to give pure methyl ester **4** (338 mg, 85%) as a white solid. Mp 102–103 °C (from C_6H_6); IR ν_{max}/cm^{-1} (KBr) 3300–2500, 3033, 2964, 1728, 1671, 1574, 1554, 1421, 1299, 1388, 1187, 1071, 888, 764, 718, 558; ^1H NMR (300 MHz, $CDCl_3$) δ: 8.59 (1H, dd, J = 4.6, 1.8 Hz, H-6), 8.31 (1H, dd, J = 7.8, 1.8 Hz, H-4), 7.08 (1H, dd, J = 7.8, 4.6 Hz, H-5), 3.67 (3H, s, CO_2Me), 3.21 (2H, t, J = 6.9 Hz, H-1), 2.38 (2H, t, J = 7.2 Hz, H-4), 1.80 (4H, m, H-2 and H-3); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 173.98 (CO_2Me), 169.61 (COOH), 163.21(C-2 Py), 152.62(C-6 Py), 139.85 (C-4 Py), 121.75 (C-3 Py), 118.15 (C-5 Py), 51.55 (CO_2Me), 33.69 (C-1), 29.51 (C-4), 28.44 (C-2), 24.38 (C-3); MS (EI) m/z 269 (M^+, 28), 239 (2), 238 (21), 236 (4), 224 (6), 210 (4), 182 (18), 169 (100); HRMS (EI), calculated for $C_{12}H_{15}NO_4S$ 269.07218, found 269.07342.

2.2.3. Preparation of methyl 5-((3-((4′-chloro-[1,1′-biphenyl]-2-yl)carbamoyl)pyridin-2-yl)thio) pentanoate (**6**)

Oxalyl chloride (35.8 μL, 0.715 mmol) was added drop wise to a stirred solution of acid **4** (175 mg, 0.65 mmol) in anhydrous CH_2Cl_2 (4 mL) followed by a catalytic amount (16 μL) of dry *N*,*N*-dimethylformamide (DMF). The mixture was stirred at rt for 24 h and then the solvent and the excess of reagent were removed under vacuum. Dry benzene (10 mL) was added and the distillation repeated to give crude methyl 5-(3-(chlorocarbonyl)pyridin-2-ylthio)pentanoate (**5**) (186.8 mg) that was used in the next step without further purification.

A mixture of the crude acid chloride obtained above, 4′-chloro-[1,1′-biphenyl]-2-amine (**12**) (100 mg, 0.488 mmol), pyridine (52.6 μL, 0.65 mmol), and a catalytic amount of 4-dimethylaminopyridine (DMAP) (1.61 mg) in anhydrous CH_2Cl_2 (8 mL) was stirred at rt for 3 days. After this time, the reaction mixture was poured into water and extracted with ethyl acetate. The organic extracts were washed with brine and dried over anhydrous Na_2SO_4. The residue left after evaporation of the solvent was purified by flash chromatography eluting with hexane–ethyl acetate mixtures (from 9:1 to 6:4) to afford the amide **6** (202 mg, 91%) as a white solid. Mp 105–107 °C (from hexane–C_6H_6); IR ν_{max}/cm^{-1} (KBr) 3447, 3246, 2945, 1735, 1651, 1514, 1478, 1292, 1173, 1089, 762; ^1H NMR (300 MHz, $CDCl_3$) δ: 8.45 (1H, dd, J = 4.8, 1.8 Hz, H-6 Py), 8.41 (1H, br d, J = 8.1 Hz, H-3 PhPh), 8.12 (1H, br s, NH), 7.79 (1H, dd, J = 7.8, 1.8 Hz, H-4 Py), 7.44–7.22 (7H, m, H-2′/H-6′, H-3′/H-5′, H-4, H-5, and H-6 PhPh), 7.04 (1H, dd, J = 7.8, 4.8 Hz, H-5 Py), 3.66 (3H, s, CO_2Me), 3.14 (2H, t, J = 6.9 Hz, H-5), 2.36 (2H, t, J = 7.2 Hz, H-2), 1.68 (4H, *m*, H-3 and H-4); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 173.80 (CO_2Me), 164.45 (CON), 156.51 (C-2 Py), 150.70 (C-6 Py), 136.73 (C-4 Py), 136.59 (C-2 PhPh), 134.49 and 134.09 (C-1′ and

C-4′ PhPh), 132.01 (C-3 Py), 130.92 (C-2′/C-6′), 130.14 (C-4 PhPh), 129.61 (C-1 PhPh), 129.17 (C-3′/C-5′), 128.81 (C-6 PhPh), 124.99 (C-5 PhPh), 122.23 (C-3 PhPh), 119.05 (C-5 Py), 51.53 (CO_2Me), 33.54 (C-5), 29.81 (C-2), 28.63 (C-4), 24.17 (C-3); MS (EI) *m/z* 454 (M^+, 9), 425 (6), 123 (16), 342 (11), 341 (11), 340 (12), 339 (21), 253 (9), 258 (39), 252 (39), 251 (100); HRMS (EI), calculated for $C_{24}H_{23}{}^{35}ClN_2O_3S$ 454.11179, found 454.11181.

2.2.4. Preparation of 5-((3-((4′-chloro-[1,1′-biphenyl]-2-yl)carbamoyl)pyridin-2-yl)thio)pentanoic acid (7, hapten BLa5)

A solution of methyl ester **6** (112 mg, 0.246 mmol) in methanol (5.1 mL) and aqueous 2 M NaOH (1.23 mL) was stirred at rt for 4 h. Most of the solvent was evaporated under reduced pressure and the resulting residue diluted with water (2 mL) and acidified with formic acid. The white precipitate formed was filtered out, washed with water, and dried under vacuum to give pure hapten BLa5 (**7**) (97 mg, 89%) as a white solid. Mp 172–175 °C (from cold methanol); IR ν_{max}/cm^{-1} (KBr) 3500–2500, 3435, 3305, 3035, 2928, 2853, 1691, 1653, 1513, 1473, 1444, 1388, 757; ^1H NMR (300 MHz, DMSO-d_6) δ: 12.05 (1H, br s, COOH), 10.03 (1H, br s, NH), 8.50 (1H, dd, J = 4.8, 1.8 Hz, H-6 Py), 7.67 (1H, br d, J = 6.9 Hz, H-3 PhPh), 3.39–7.35 (9H, m, H-2′/H-6′, H-3′/H-5′, H-4, H-5 and H-6 PhPh and H-4), 7.19 (1H, dd, J = 7.5, 4.8 Hz, H-5 Py), 3.10 (2H, t, J = 6.0 Hz, H-5), 2.25 (2H, t, J = 6.3 Hz, H-2), 1.61 (4H, m, H-3 and H-4); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 174.17(COOH), 165.29 (CON), 156.94 (C-2 Py), 149.88 (C-6 Py), 135.07 (C-4 Py), 137.83 (C-2 PhPh), 135.07 (C-1′ PhPh), 134.22 (C-4′ PhPh), 131.95 (C-3 Py), 130.62 (C-1 PhPh), 130.48 (C-2′/C-6′), 130.09 (C-4 PhPh), 128.15 (C-3′/C-5′ PhPh and C-6 PhPh), 127.77 and 126.84 (C-3 and C-5 PhPh), 118.60 (C-5 Py), 33.13 (C-5), 28.66 (C-2), 28.32 (C-4), 23.74 (C-3); MS (EI) *m/z* 440 (M^+, 7), 342 (3), 341 (11), 340 (15), 339 (25), 239 (6), 238 (35), 237 (100); HRMS (EI), calculated for $C_{23}H_{21}{}^{35}ClN_2O_3S$ 440.09614, found 440.09463; UV (PB), ε (280 nm) = 5.42 mM^{-1} cm^{-1}, ε (260 nm) = 10.68 mM^{-1} cm^{-1}.

2.2.5. Preparation of N-succinimidyl 5-((3-((4′-chloro-[1,1′-biphenyl]-2-yl)carbamoyl)pyridin-2-yl)thio)pentanoate (*8*)

To activate hapten BLa5 (**7**) for protein conjugation, the *N*-succinimidyl ester was prepared (Fig. 1) according to a previously described procedure (Esteve-Turrillas et al., 2010). Briefly, acid **7** (30 mg, 0.068 mmol) and *N*,*N*′-disuccinimidyl carbonate (DSC) (27.9 mg, 0.109 mmol, 1.6 equiv.) were dissolved in 0.9 mL of dry acetonitrile under nitrogen atmosphere. Then, triethylamine (36 μL, 0.258 mmol, 3.8 equiv.) was added and the reaction mixture was stirred at rt for 5 h. The solution was diluted with chloroform, washed with an aqueous saturated solution of $NaHCO_3$ and brine, and then dried over anhydrous Na_2SO_4. The residue left after evaporation of the solvent was purified by column chromatography, using chloroform as eluent, to give the pure active *N*-succinimidyl ester of hapten BLa5 8 (29.3 mg, 80%) as a viscous oil. ^1H NMR (300 MHz, $CDCl_3$) : δ 8.47 (1H, dd, J = 4., 1.8 Hz, H-6 Py), 8.41 (1H, br d, J = 8.1 Hz, H-3 PhPh), 8.11 (1H, br s, NH), 7.79 (1H, dd, J = 7.8, 1.8 Hz, H-4 Py), 7.46–7.21 (7H, m, H-2′/H-6′, H-3′/H-5′, H-4, H-5 and H-6 PhPh), 7.05 (1H, dd, J = 7.8, 4.8 Hz, H-5 Py), 3.17 (2H, t, J = 6.9 Hz, H-5), 2.83 (4H, br s, $COCH_2CH_2CO$), 2.67 (2H, t, J = 7.2 Hz, H-2), 1.90–1.60 (4H, m, H-3 and H-4).

2.3. Preparation of protein–BLa5 conjugates

Protein conjugates were prepared by reaction of the purified *N*-succinimidyl ester of BLa5 **(8)** with the free amine groups of the carrier protein. The final hapten-to-protein molar ratio (MR) was calculated using the absorbance values of the conjugate by assuming that the molar absorption coefficients of the hapten and the protein were the same for the free and the conjugated forms.

2.3.1. Immunizing conjugate

A conjugate to BSA was prepared using 200 μL of a 50 mM solution of the *N*-succinimidyl ester **8** in anhydrous DMF. Briefly, the active ester solution was added drop wise over a 15 mg/mL BSA solution (2 mL) in CB and the mixture was gently stirred at rt in amber glass vials during 4 h. The conjugate was purified by gel filtration using PB as eluent. The collected volume was brought to 30 mL with PB and the conjugate was stored frozen at −20 °C. The calculated final MR of the immunogen was 11.

2.3.2. Coating conjugate

Several OVA conjugates were prepared with different starting quantities of the *N*-succinimidyl ester **8** in order to afford a collection of coating antigens with a range of hapten-to-protein MRs. A fixed volume (100 μL) of a solution of the *N*-succinimidyl ester **8** in DMF (10, 50, or 100 mM) was added drop wise to 2 mL of a 15 mg/mL OVA solution in CB under stirring. The reaction was gently mixed at rt during 2.5 h in amber glass vials and the conjugate was purified as before. The pooled fractions containing the conjugate were brought to 30 mL using PB and stored frozen at −20 °C.

2.3.3. Enzyme tracer

For the preparation of a reporter conjugate, HRP was used as carrier. One hundred microlitres of activated hapten solution in DMF (10 mM) was added drop wise to a 2.2 mg/mL HRP solution (1 mL) in CB under gentle stirring. The coupling reaction was allowed to proceed during 4 h at rt with moderate stirring in amber glass vials. The conjugate was separated from uncoupled hapten by gel chromatography and it was brought to 1 mg/mL with PB, and then diluted 1/2 with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal. Tracer conjugate aliquots were stored at −20 °C in amber vials except the working solution that was kept at 4 °C.

2.4. Production of rabbit polyclonal antibodies

Two antisera were generated, named rBLa5#1 and rBLa5#2, from two female New Zealand white rabbits weighing 1–2 kg, which had been immunised by subcutaneous injection with 0.3 mg of BSA–BLa5 conjugate in 1 mL of a 1:1 mixture of PB and complete Freund's adjuvant. Animals were boosted at 21-day intervals with the same immunogen suspended in a mixture of 0.5 mL of PB and 0.5 mL of incomplete Freund's adjuvant. Whole blood was collected from the ear vein of the rabbits and by intracardiac puncture 10 days after the fourth injection. Blood samples were allowed to coagulate overnight at 4 °C. Then, the serum was separated by centrifugation and precipitated with a solution of saturated ammonium sulphate. This procedure was repeated again and the precipitates were stored at 4 °C. Animal manipulation was performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Fisheries, and Food.

2.5. Antibody-coated direct competitive ELISA

Ninety-six-well polystyrene ELISA plates were coated with 100 μL of a particular antiserum diluted in CB, and plates were incubated overnight at rt. Coated plates were washed four times with washing solution and received, afterwards, 50 μL per well of analyte standard in PBS plus 50 μL per well of HRP–BLa5 tracer solution in PBST. The immunological reaction took place during 1 h at rt, and then plates were washed as described above. Finally, retained peroxidase activity was determined by addition of 100 μL per well of freshly prepared 2 mg/mL *o*-phenylenediamine solution containing 0.012% (v/v) H_2O_2 in developing buffer. The enzymatic reaction was stopped after 10 min at rt by addition of 100 μL per

well of 2.5 M sulphuric acid. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

2.6. Conjugate-coated indirect competitive ELISA

ELISA plates were coated with 100 μL per well of OVA–BLa5 solution in CB by overnight incubation at rt. Coated plates were washed four times with washing solution and then received 50 μL per well of analyte in PBS plus 50 μL per well of antiserum dilution in PBST. The immunological reaction took place during 1 h at rt and plates were washed again as before. Next, 100 μL per well of a $1/10^4$ dilution of GAR–HRP conjugate in PBST containing 10% FBS was added, and plates were incubated 1 h at rt. After washing the plates, the retained peroxidase activity was determined as aforementioned.

2.7. Standards and data treatment

From a stock solution of BL at 10 g/L in anhydrous DMF, eight-point standard curves were prepared from 10^{-2} to 10^4 μg/L by a 10-fold serial dilution in PBS, plus a blank (no analyte). Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay sensitivity was defined as the concentration of analyte at the inflection point of the fitted curve, typically corresponding to a 50% inhibition (IC_{50}) of the maximum absorbance (A_{max}) if the background signal approaches to zero.

2.8. Influence of buffer composition on assay performance

Two immunoassays were selected using each of the two most common cELISA formats. The first assay used the direct format and plates coated with a $1/6 \times 10^4$ dilution of antibody rBLa5#1. The second ELISA employed the indirect format and plates coated with a 0.1 μg/mL solution of OVA–BLa5 (MR = 2) and antibody rBLa5#2. BL standard solutions were mixed 1:1 with a 20 ng/mL tracer solution for the d-cELISA, or with a $1/2 \times 10^5$ antibody dilution for the i-cELISA. For solvent studies, analyte standard curves were prepared in water containing between 0.5% and 10% of methanol, ethanol, acetonitrile, or acetone, and immunoreagents were prepared in 2 × PBST. The influence of the buffer ionic strength and the pH was evaluated following a central composite design, consisting of a two-level full factorial design ($\alpha = 1.414$) with two factors and three replicates that included 12 cube, 12 axial, and 15 centre points, and involving a total of 39 randomised buffer studies (see Table S1 in the Supplementary Data). The ionic strength values of the evaluated buffers were between 50 and 300 mM, and the studied pH values ranged from 5.5 to 9.5. Initially, a 40 mM trisodium citrate, 40 mM disodium hydrogen phosphate, and 40 mM Tris solution was prepared. To aliquots of that solution, known volumes of 5 M HCl was added in order to reach the required pH in each case. Then, the ionic strength of each solution was calculated considering the initial buffer concentrations and the added HCl, and then an appropriate volume of 2 M NaCl was used to achieve the required ionic strength for every buffer. At last, Tween 20 was added to all buffers to a final concentration of 0.05% (v/v). BL standard curves were prepared in water and they were mixed as described above with tracer or antibody solutions prepared in every of the studied buffers. The A_{max} and IC_{50} values of the inhibition curves were employed as response values and fitted to a multiple regression equation, including curvature and interaction terms, using Minitab 14.1 software (Minitab Inc., State College, PA, USA).

3. Results and discussion

3.1. Synthesis of BLa5 and its N-succinimidyl ester

Antigens should closely resemble the target ligand, though fragments of the molecule have been successfully used as mimics (Connolly, Fodey, Crooks, Delahaut, & Elliott, 2002; Fodey, Delahaut, & Elliott, 2007; Mercader, Primo, & Montoya, 1995). Our approach was to prepare a functionalised hapten (BLa5) containing the three characteristic rings of BL. Moreover, this compound contained a five-carbon atom spacer arm linked to the pyridine ring through a sulphur atom which replaced a chlorine atom present in the molecule. The sulphur atom behaves as a good mimetic of the chlorine atom, both in electronic and steric terms. This strategy has been successfully followed before by our group (Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011) and others (Manclús, Primo, & Montoya, 1996; Matsuo, Nishi, Morimune, Okawa, & Miyake 2005) in the design of haptens for the production of antibodies against chlorine-containing molecules. First attempts to carry out the direct nucleophilic replacement of the chlorine atom of BL by the (4-carboxybutyl)mercaptide group failed, so a straightforward synthetic sequence was designed for the preparation of the desired hapten (Fig. 1).

The synthesis of hapten BLa5 was initiated with the preparation of nicotinic acid derivative **3**. This intermediate was obtained in two steps from commercially available 2-mercaptonicotinic acid (**1**). Thus, reaction of **1** with 5-bromopentanoic acid (**2**) in aqueous potassium hydroxide solution gave de dicarboxylic acid **3** (Da Settimo et al., 2000). The aliphatic carboxylic group of **3** was chemoselectively protected as its methyl ester by treatment with 2,2-dimethoxypropane in the presence of a catalytic amount of anhydrous HCl, which was generated *in situ* from trimethylchlorosilane (Rodriguez, Nomen, & Spur, 1998), to afford the intermediate methyl ester **4** in about 76% overall yield for the two steps. With compound **4** at hand, the synthesis of the hapten BLa5 was readily completed as follows. First, the aromatic carboxylate acid group was transformed into the corresponding acid chloride (**5**), which was subsequently coupled to 4′-chlorobiphenyl-2-amine (**12**) without purification to give the methyl ester of hapten BLa5 (**6**) in an excellent 91% overall yield. Finally, basic-promoted hydrolysis of the methyl ester moiety of **6** afforded the hapten BLa5 (**7**) in 89% yield after its chromatographic purification.

Hapten coupling to proteins is very often performed by covalently linking a carboxylate group of the hapten to the ε-amine residues of the carrier polypeptide. A very common strategy to achieve such conjugates consists in the activation of the carboxylic acid by formation of an active ester using carbodiimide and coupling to the protein, all performed in a one-pot reaction. During that reaction, undesired by-products are formed which reduce the reaction yields and make it difficult to purify the activated hapten. Lately, we have applied in our lab an activation reaction, uncommon for the preparation of hapten bioconjugates, using DSC to obtain the *N*-succinimidyl ester of carboxy-functionalised haptens. This reagent was originally described by Ogura, Kobayashi, Shimizu, Kawabe, and Takeda (1979) for the synthesis of active esters. It is a simple strategy that allows an easy purification of the active ester by chromatography on silicagel, mostly due to the absence of significant by-products (Kang et al., 2009; Parra, Mercader, Agulló, Abad-Fuentes, & Abad-Somovilla, 2011). The active *N*-succinimidyl ester of hapten BLa5 (**8**) was obtained by this procedure in an 80% yield after purification (Fig. 1), and it was stable when stored in solution at −20 °C under dry conditions. The availability of pure active ester 8 facilitated the preparation of immunogen and assay conjugates with tunable and reproducible hapten-to-protein MRs.

Table 1
Assay parameters for the checkerboard competitive assay with antisera rBLa5#1 and rBLa5#2.

Direct format								
1/[pAb]	HRP–BLa5 (μg/L)	rBLa5#1			rBLa5#2		
		A_{max}	Slope	IC_{50} (μg/L)	A_{max}	Slope	IC_{50} (μg/L)
6×10^4	10	1.42	0.57	0.9	2.00	0.82	3.3
	3	0.79	0.52	0.4	1.00	0.61	1.6
3×10^4	10	2.30	0.77	2.7	3.37	0.61	5.6
	3	1.03	0.71	1.5	1.32	0.65	4.8

Indirect format									
OVA–BLa5		rBLa5#1				rBLa5#2			
MR	Conc. (mg/L)	1/[pAb]	A_{max}	Slope	IC_{50} (μg/L)	1/[pAb]	A_{max}	Slope	IC_{50} (μg/L)
8	1.0	10^5	1.49	0.67	48.2	3×10^5	1.54	0.70	75.2
		3×10^5	0.63	0.76	33.5	10^6	0.59	0.82	69.8
	0.1	10^5	1.34	0.66	4.0	3×10^5	1.37	0.56	5.7
		3×10^5	0.56	0.76	3.8	10^6	0.48	0.75	5.0
4	1.0	10^5	2.11	0.57	20.9	3×10^5	1.98	0.71	38.5
		3×10^5	0.89	0.71	17.5	10^6	0.79	0.74	28.7
	0.1	10^5	1.32	0.67	1.8	3×10^5	1.42	0.71	3.9
		3×10^5	0.57	0.66	1.3	10^6	0.45	0.87	4.4
2	1.0	10^5	2.00	0.61	5.6	3×10^5	1.98	0.53	9.1
		3×10^5	0.89	0.64	4.3	10^6	0.79	0.55	7.3
	0.1	3×10^4	1.62	0.80	2.0	10^5	1.68	0.62	1.5
		10^5	0.81	0.65	0.9	3×10^5	0.88	0.57	0.9

3.2. Immunoreagent evaluation

3.2.1. Affinity

Two rabbits were immunised with conjugate BSA–BLa5, and the cleared-out antisera, called rBLa5#1 and rBLa5#2, were obtained and evaluated. In order to find the proper immunoreagent concentration, a checkerboard competitive screening analysis for each antibody was performed in two immunoassay formats (d- and i-cELISA), as previously described (Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008). For direct assays, plates were coated with several dilutions of the antiserum (from $1/10^4$ to $1/10^5$), and next day a range of tracer conjugate concentrations (from 3 to 100 ng/mL) was evaluated under competitive conditions. For indirect assays, plates were coated with different OVA–BLa5 conjugate solutions (0.01, 0.10, or 1.00 μg/mL), and the competitive step was carried out using a range of antibody dilutions (from $1/3 \times 10^3$ to $1/10^6$). As a result, a collection of inhibition curves was obtained – one curve for each pair of immunoreagent concentrations – in each assay format. Fig. S2 of the Supplementary Data section shows one of those series of curves for one antibody (rBLa5#2) in the i-cELISA format using coating conjugates with different MRs. Irrespectively of hapten loading in the bioconjugate, coating plates with OVA–BLa5 at 0.1 μg/mL resulted in the best inhibition curves. A summary of the results of the competitive checkerboard study, containing the respective curve parameters (A_{max}, slope, and IC_{50} values), is listed in Table 1 for direct and indirect assays (only results for the two immunoreagent concentrations giving A_{max} values immediately above and

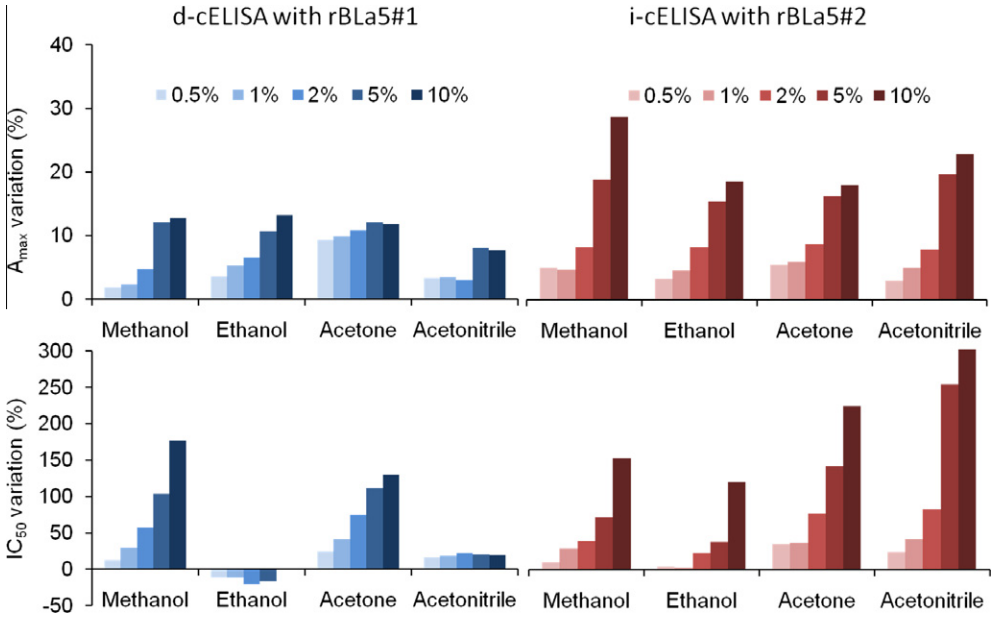

Fig. 2. Effect of organic solvents over the maximum signal and sensitivity of the d-cELISA using antibody rBLa5#1 (blue bars) and the i-cELISA using antibody rBLa5#2 (red bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

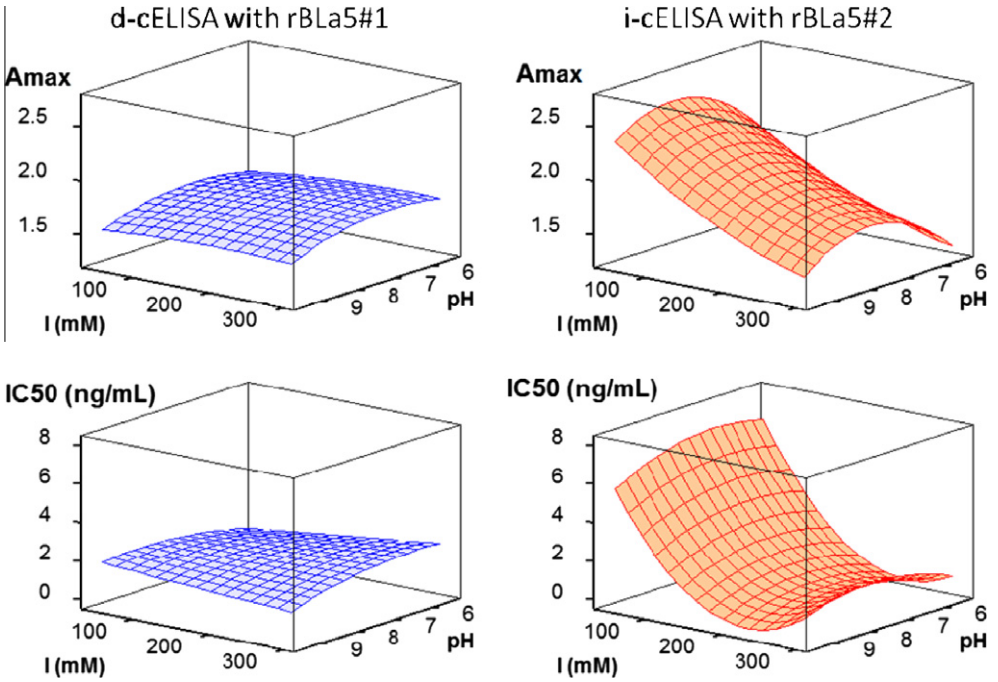

Fig. 3. Influence of buffer conditions over the assay curve parameters of the rBLa5#1-based d-cELISA and the rBLa5#2-based i-cELISA.

below 1.0 are listed; and in the case of the i-cELISAs, data with 0.01 μg/mL conjugate are not included).

For selection of the best reagent combination, the following criteria were used: A_{max} of the inhibition curve between 1.0 and 1.5, inferior asymptote equivalent to the background signal, lowest IC_{50} value, and minimum immunoreagent consumption. The best immunoassay in the d-cELISA format was achieved with antiserum rBLa5#1, whereas in the i-cELISA format it was antiserum rBLa5#2 the finest performing one. For the latter format, the sensitivity could be improved not only with low coating concentrations but also by reducing the hapten molarity of the coating conjugate. Thus, conjugate OVA–BLa5 with a MR of 2 at 0.10 μg/mL was selected for this cELISA.

3.2.2. Selectivity

Cross-reactivity studies were performed in order to find other compounds that could be recognised by the generated antibodies. Twenty-two widely used fungicides were assayed; i.e., kresoxim-methyl, trifloxystrobin, pyraclostrobin, azoxystrobin, dimoxystrobin, fluoxastrobin, metominostrobin, picoxystrobin, fenhexamid, captan, mepanipyrim, pyrimethanil, procimidone, tolylfluanid, cyazofamid, tebuconazole, fenamidone, fludioxonil, vinclozolin, imidacloprid, cyprodinil, and benzanilide. Calibration curves were prepared up to 10 μM for each fungicide in PBS and measured by the selected direct and indirect ELISAs. From this study, it could be concluded that both antisera were highly specific to BL because no inhibition was exerted by any of the studied pesticides.

3.3. Assay characterisation and optimization

3.3.1. Solvent tolerance

The presence of high amounts of solvents in the sample extract may have an effect on some curve parameters of the cELISAs, mostly over the A_{max} and the IC_{50} values. Thus, the influence of different percentages of ethanol, methanol, acetonitrile, and acetone was assessed in order to evaluate the robustness of the proposed immunoassays (Fig. 2). In general, ethanol was the best tolerated solvent in both assays, though the presence of solvents in the competitive step was better accepted by the BLa5#1-based direct assay than by the BLa5#2-based indirect one. Variations of the A_{max} value were below 10% when the solvent concentration in the sample was equal or lower than 10% in the direct assay and 2% in the indirect assay. Regarding sensitivity, the IC_{50} values were also essentially unaffected by a 10% ethanol or acetonitrile in the rBLa5#1-based assay, whereas methanol and ethanol exerted the lowest influence over the rBLa5#2-based ELISA. Thus, the selected d-cELISA could be recommended for the analysis of BL in liquid food samples with high concentrations of ethanol, such as wine or beer, and for solid samples that had been extracted with acetonitrile or ethanol. Finally, acetone extracts should be avoided.

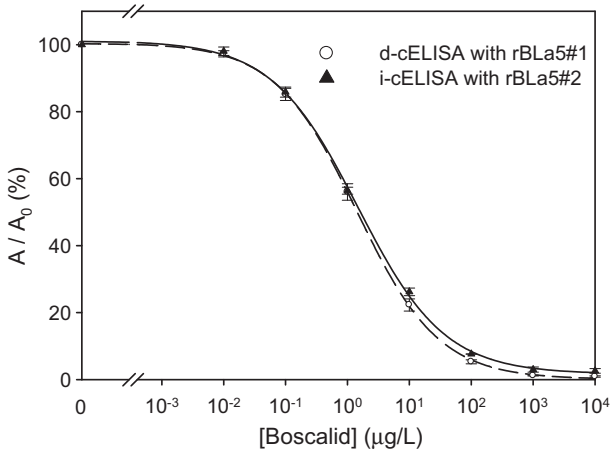

Fig. 4. Standard curves in buffer for the two optimised immunoassays. For the d-cELISA, plates were coated with a $1/6 \times 10^4$ dilution of antiserum rBLa5#1, and assays were run with 10 ng/mL HRP–BLa5 tracer conjugate. For the i-cELISA, microwells were coated with a 0.1 μg/mL OVA–BLa5 (MR = 2) solution, and the dilution of antiserum rBLa5#2 in the immunological reaction was $1/2 \times 10^5$. The A_{max} value was around 1.3. For normalisation, the absorbance value of the blank control that was run in the same microplate was employed. Values are the mean of three independent determinations.

Table 2
Recovery values obtained with the proposed cELISAs from spiked juice samples.

ELISA	Sample dilution	Boscalid (μg/L)	Recovery (% ± s, n = 3)			
			Grape	Peach	Apple	Tomato[a]
rBLa5#1 (d-cELISA)	1/100	10	88 ± 8	130 ± 19	96 ± 1	97 ± 19
		50	106 ± 4	116 ± 4	119 ± 9	96 ± 18
		100	101 ± 6	105 ± 10	100 ± 5	112 ± 12
		500	103 ± 3	108 ± 9	105 ± 6	119 ± 6
		1000	96 ± 6	98 ± 14	113 ± 12	103 ± 7
		5000	110 ± 9	111 ± 13	107 ± 4	117 ± 4
rBLa5#2 (i-cELISA)	1/500	50	112 ± 22	104 ± 5	111 ± 22	115 ± 27
		100	106 ± 8	123 ± 13	117 ± 5	114 ± 23
		500	113 ± 21	96 ± 31	108 ± 8	119 ± 18
		1000	101 ± 2	118 ± 7	112 ± 13	93 ± 4
		5000	93 ± 7	92 ± 12	106 ± 13	116 ± 16
		10,000	91 ± 6	92 ± 13	99 ± 9	107 ± 31

[a] A 1/500 dilution was performed in tomato samples for the rBLa5#1-based d-cELISA.

3.3.2. Buffer conditions

The inhibition curves obtained using buffers with varying pH and ionic strength values were almost equivalent for the BLa5#1-based d-cELISA (Fig. 3). In fact, there was not a significant regression ($P > 0.05$) between the studied factors (pH and ionic strength) and each response value (A_{max} or IC_{50}). In the case of the BLa5#2-based indirect immunoassay, A_{max} values were affected by changes in buffer ionic strength and pH values, whereas the ionic strength had a stronger influence over the IC_{50} values than the pH ($P < 0.05$). A decrease in the buffer ionic strength provided a continuous increase in the A_{max}, whereas the IC_{50} value stayed almost constant at ionic strengths from 150 to 300 mM and it increased quickly at values lower than 150 mM. From this study, we could conclude that the developed direct assay was a more robust immunoassay than the indirect one.

3.4. Determination of boscalid in food samples

Standard curves of the optimised immunoassays in PBS are depicted in Fig. 4. The theoretical limit of detection of those immunoassays, estimated as the IC_{10} value of the inhibition curve (the concentration of BL that provided a 10% inhibition of A_{max}), was 0.05 μg/L for both ELISAs. For the present study, different juices were selected in order to cover a wide range of fruit categories in which this pesticide may be present, such as: berries (grapes), stone fruits (peaches), pome fruits (apples), and fruiting vegetables (tomatoes).

3.4.1. Matrix effects

Commercial unprocessed juices were diluted with deionised water, and a BL standard curve was prepared with every sample dilution and mixed with tracer or antibody solution in 2 × PBST. Fig. S3 in the Supplementary Data shows the resultant inhibition curves with the two developed immunoassays for the four studied food samples. First, it was found that a 1/100 sample dilution was enough to remove the matrix effects in the direct ELISA, except for the tomato juice which required a 1/500 dilution. Second, analysis of BL in fruit juice samples by the i-cELISA demanded a 1/500 dilution for any of the studied food samples.

3.4.2. Recovery studies

Several commercial grape juices from different sources were analysed by the described cELISAs but no boscalid-containing samples were found. Thus, grape, peach, apple, and tomato juice samples were spiked with BL (analytical standard grade) and they were homogenised by vortex mixing. Fortified samples were measured by the developed cELISAs and BL recoveries were calculated. As seen in Table 2, satisfactory recoveries were mostly found and experimental limits of quantification for BL as low as 10 μg/L were obtained. It could also be observed that a better precision was generally noticed with the d-cELISA than with the i-cELISA, and that the tomato juice produced more interferences than other assayed food matrices.

4. Conclusions

The first reported functionalised derivative of the fungicide BL has been synthesized and novel high-affinity and selective antibodies against such a widely used pesticide have been produced. Those antibodies were characterised in two cELISA formats and two assays were selected considering their equivalent sensitivities (IC_{50} values were 0.9 μg/L), each of them using a different antibody and format. The antibody-coated direct ELISA, based on antibody rBLa5#1, was demonstrated to be faster and a more robust assay, showing good tolerance to ethanol, methanol, and acetonitrile, as well as to pH and ionic strength variations. The two optimised cELISAs displayed a limit of detection of 0.05 μg/L for BL standards in buffer. This value is well below the MRLs established for BL by the different international legislations in most commodities, providing a comfortable dilution margin for the analysis of residues of this fungicide at pertinent levels. Finally, both of the proposed immunoassays were employed in the determination of BL residues in foodstuffs, evaluating the matrix effects of grape, apple, peach, and tomato juices and estimating the BL recoveries in fortified samples, with good results. This study provides new evidence about the expediency of the ELISA methodology for the selective and sensitive analysis of pesticide residues in food samples.

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Limited amounts of the immunoreagents described in this paper are available upon request for evaluation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2012.04.090.

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