



Sensitive Monoclonal Antibody-Based Immunoassays for Kresoxim-methyl Analysis in QuEChERS-Based Food Extracts

Josep V. Mercader,^{†,‡} Rosario López-Moreno,^{‡,§} Francesc A. Esteve-Turrillas,[†] Consuelo Agulló,[§] Antonio Abad-Somovilla,^{*,§} and Antonio Abad-Fuentes^{*,†}

[†]Department of Biotechnology, Institute of Agrochemistry and Food Technology, Consejo Superior de Investigaciones Científicas (IATA-CSIC), Agustí Escardino 7, 46980 Paterna, València, Spain

[§]Department of Organic Chemistry, Universitat de València, Doctor Moliner 50, 46100 Burjassot, València, Spain

Supporting Information

ABSTRACT: Kresoxim-methyl is nowadays widely used to combat a diversity of common diseases affecting high-value crops. In this article, we report the development and characterization of two novel immunoassays for the analysis of this pioneer strobilurin fungicide, and for the first time, a validation study with food samples was performed. A direct and an indirect competitive immunoassay based on a new anti-kresoxim-methyl monoclonal antibody were developed for sensitive and specific chemical analysis. Optimized assays showed limits of detection of 0.1 $\mu\text{g/L}$. Fruit and vegetable samples were extracted with acetonitrile by the QuEChERS procedure and analyzed by the developed immunoassays after a simple dilution in buffer, affording limits of quantification below US and European maximum residue limits. Immunochemical results of samples from kresoxim-methyl-sprayed strawberry fields demonstrated good statistical agreement with gas chromatography coupled to mass spectrometry as reference technique.

KEYWORDS: competitive ELISA, rapid methods, strobilurins, residues, QuEChERS, Deming regression

■ INTRODUCTION

Kresoxim-methyl was one of the two first strobilurin pesticides to be registered back in 1992.¹ This fungicide is particularly active against Ascomycetes, such as *Venturia inaequalis*, *Podospheera leucotricha*, *Leveillula taurica*, and *Botrytis cinerea*, which are responsible for a large variety of plant diseases.² Like other strobilurins, kresoxim-methyl inhibits mitochondrial respiration of fungi, which prevents infection and makes it highly active.³ Although it is considered a low-hazard chemical to mammals (oral LD_{50} > 2000 mg/kg in rats) and bees, kresoxim-methyl is very toxic to aquatic organisms including fish (LD_{50} = 150 $\mu\text{g/L}$), plankton, and algae.^{4–6} Nowadays, kresoxim-methyl is being extensively used worldwide, with total global sales reaching 400 M€ in 2010.⁷ Concomitantly, its residues in food have also risen, though to concentrations mainly below the legal maximum residue limits (MRL): in the European monitoring program for 2006, kresoxim-methyl was encountered only in strawberries, whereas just two years later its residues were also found in carrots, cucumbers, and pears; and in 2009, up to six different food commodities contained measurable levels of this chemical (www.efsa.europa.eu/en/topics/topic/pesticides.htm). Kresoxim-methyl displays low metabolism in plants, the original compound being the main residue, and therefore the only target chemical for residue monitoring. European and US MRLs range between 0.05 and 1 mg/kg and between 0.15 and 1.5 mg/kg, respectively, for most fruits and vegetables [www.ec.europa.eu/sanco_pesticides; www.mrlatabase.com].

Current health and ecological concerns about chemical residues in food and the environment have compelled private and public organizations to reinforce pesticide monitoring

programs. Consequently, the diversity of analytical applications has grown, and tools with alternative performing properties are demanded. Since it is highly difficult for a method to be simultaneously sensitive, accurate, high capacitive, rapid, cheap, portable, and user and environmentally friendly, analysts should choose the best strategy for each particular demand. Nowadays, chromatographic methods are the most employed techniques for pesticide residue determination. In 1998, Cabras et al.⁸ described the first applied approach for kresoxim-methyl analysis using gas chromatography (GC) with mass spectrometry (MS) detection. Since then, a diversity of methods for this chemical have been published, mainly as multiresidue strategies.^{9–11} In addition, immunochemical methods have become complementary analytical tools for chemical residue analysis. During the past decade, novel antibody-based kits for pesticide determination have been steadily introduced into the market with different applications, also for strobilurin fungicides [www.abraxiskits.com]. Undoubtedly, the most extended immunoanalytical method for small organic chemicals is the competitive enzyme-linked immunosorbent assay (cELISA).

During recent years, basic studies regarding the relationship between the structure of kresoxim-methyl haptens and the activity of the generated antibodies have been published by our group.^{12,13} Now, we herein report for the first time the application of competitive immunoassays to the analysis of kresoxim-methyl in foodstuffs. Following development and

Received: January 20, 2014

Revised: March 6, 2014

Accepted: March 10, 2014

Published: March 10, 2014

characterization of monoclonal antibody-based direct and indirect cELISAs, samples of strawberries, cucumbers, and tomatoes were fortified with kresoxim-methyl and extracted using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methodology¹⁴ in order to evaluate immunoassay performance. Validation of the newly developed rapid methods was carried out by comparison with GC–MS through Deming regression analysis and Bland–Altman plots using samples from sprayed crops.

MATERIALS AND METHODS

Reagents and Instruments. The employed monoclonal antibody (KM0#117) and the homologous assay conjugates using horseradish peroxidase (HRP–KM0) and ovalbumin (OVA–KM0) were generated in our lab, and their preparation was as described elsewhere.¹³ The affinity to kresoxim-methyl of monoclonal antibody (mAb) KM0#117 clearly improves that of a former antibody that we produced some years ago, at the beginning of this project.¹² Rabbit anti-mouse immunoglobulin polyclonal antibody conjugated to peroxidase (RAM–HRP) was from Dako (Glostrup, Denmark). *o*-Phenylenediamine was purchased from Sigma/Aldrich (Madrid, Spain). 96-well Costar flat-bottom high-binding polystyrene ELISA plates were purchased from Corning (Corning, NY, USA). ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments.

Pestanal-grade kresoxim-methyl (methyl (*E*)-methoxyimino[α -(*o*-tolylxy)-*o*-tolyl]acetate, CAS registry number 143390-89-0, M_w 313.35) was purchased from Fluka/Riedel-de-Haën (Seelze, Germany). Other pesticide standards were also from Fluka/Riedel-de-Haën or from BASF (Limburgerhof, Germany), Bayer CropScience (Frankfurt, Germany), Dr. Ehrenstorfer (Augsburg, Germany), or Syngenta (Basel, Switzerland). Triphenylphosphate (TPP) was from Sigma/Aldrich (Madrid, Spain), and primary–secondary amine (PSA) for dispersive solid phase extraction cleanup was from Scharlab (Barcelona, Spain). Chromatographic determinations were carried out with a 6890N GC apparatus furnished with a 7683 series autosampler, a HP-5MS (30 m \times 0.25 mm \times 0.25 μ m) capillary column, and a quadrupole 5973N mass detector, all from Agilent Technologies (Santa Clara, CA, USA).

Immunoassays. General Procedures. Eight-point standard curves, including a blank, were prepared in borosilicate glass tubes by 10-fold serial dilution in PBS starting from a 100 μ g/L solution also in PBS. Pesticide concentrated stock solutions (100 mg/L) in anhydrous *N,N*-dimethylformamide—kept at -20°C in amber glass vials—were used to prepare the first standard point. Experimental values were fitted using the SigmaPlot software (Systat Software Inc., Chicago, IL, USA) to a four-parameter logistic equation:

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

where A_{\max} is the absorbance that was reached in the absence of analyte, A_{\min} is the background signal, C is the analyte concentration at the inflection point of the sigmoidal curve, and B is the slope at the inflection point. Curves were normalized, and average values were calculated from independent experiments.

The concentration of kresoxim-methyl inducing a 50% inhibition (IC_{50}) of the reaction between antibody and hapten conjugate was taken as the key criterion for assay characterization. The limit of detection (LOD) was calculated as the analyte concentration causing a 10% inhibition (IC_{10}) of the immunochemical reaction. Cross-reactivity (CR) was estimated as a percentage value from the quotient between the IC_{50} value for kresoxim-methyl and the IC_{50} for the studied compound, both in molar concentration units.

Antigen-Coated Indirect Competitive ELISA. Microplates were coated by overnight incubation with 100 μ L per well of OVA–KM0 conjugate solution in 50 mM carbonate buffer, pH 9.6. All incubation steps were performed at room temperature with sealed plates, and after each step, microwells were washed four times with washing

solution (150 mM NaCl with 0.05% (v/v) Tween 20). The competitive step was carried out during 1 h with 50 μ L per well of analyte solution in PBS plus 50 μ L per well of a dilution of antibody KM0#117 in PBST (PBS containing 0.05% (v/v) Tween 20). Retained mAb was amplified by incubation during 1 h with 100 μ L per well of RAM–HRP diluted 1/2000 in PBST. Finally, a signal was generated with 100 μ L per well of 0.012% (v/v) H_2O_2 in 62 mM phosphate and 25 mM citrate, pH 5.4 containing 2 g/L of *o*-phenylenediamine. Ten minutes later, enzymatic activity was stopped with 100 μ L per well of 2.5 M H_2SO_4 . Absorbance was immediately read at 492 nm using 650 nm as reference wavelength.

Antibody-Coated Direct Competitive ELISA. Polystyrene plates were coated by overnight incubation with 100 μ L per well of a solution of antibody KM0#117 in 50 mM carbonate buffer, pH 9.6. All incubation steps were carried out at room temperature, and after each incubation step, microwells were washed as described above. The competitive step was performed during 1 h with sequential addition of 50 μ L per well of analyte solution in PBS and 50 μ L per well of a solution of tracer HRP–KM0 in PBST. Signal was generated and plates were read as before.

Solvent, Detergent, and Buffer Studies. The influence of acetonitrile was evaluated using kresoxim-methyl standard curves in PBS with different solvent contents, whereas antibody or tracer solutions were prepared in PBST. Variation of A_{\max} and IC_{50} values due to Tween 20 concentration was also assessed. Moreover, a central composite design was followed for buffer studies, consisting of a two-level full factorial design ($\alpha = 1.414$) with two factors (pH and ionic strength) and three replicates that included 12 cube, 12 axial, and 15 center points; that is, 39 randomized assays under 9 different buffer conditions (Table S1 in the Supporting Information). The corresponding 9 buffers were set up from a 40 mM citrate, 40 mM phosphate, and 40 mM Tris solution, as described elsewhere.¹⁵ Ionic strength and pH values of each buffer were adjusted using 2 M NaCl and 5 M HCl, respectively. All buffers contained 0.05% (v/v) Tween 20. Kresoxim-methyl was prepared in Milli-Q water, and antibody or tracer solutions were diluted in the studied buffers. A_{\max} and IC_{50} values of the 39 resulting curves were fitted as functions of pH and ionic strength using the Minitab software (Minitab Inc., State College, PA, USA).

Sample Treatment and Analysis. Tomatoes, cucumbers, and strawberries were obtained from local supermarkets and extracted using the QuEChERS method.¹⁴ Briefly, 20 g of homogenate was mixed under vigorous stirring with 2 g of sodium acetate and 8 g of anhydrous magnesium sulfate in 20 mL of acetonitrile containing 1% (v/v) acetic acid. The organic phase was separated by centrifugation at 2200g during 5 min, and a 10 mL aliquot was cleaned up by vortexing with PSA (500 mg) in the presence of 1.5 g of anhydrous magnesium sulfate. After a second centrifugation step, extracts were filtrated with a Teflon filter (0.2 μ m) and stored at -20°C . Extracts were fortified with kresoxim-methyl, diluted 50-fold in PB (100 mM phosphate, pH 7.4), and analyzed by the developed cELISAs using antibody or tracer solutions prepared in PBT (PB containing 0.05% (v/v) Tween 20). A kresoxim-methyl standard curve in PB was run in each plate.

In order to better simulate real-world situations, greenhouse strawberry crops were sprayed with a nebulizer using a commercial kresoxim-methyl formulation from BASF (Stroby), which was prepared as recommended by the manufacturer (100 mg/L kresoxim-methyl in water containing 20% (v/v) alkyl polyglycol ether). Samples were collected at different days to obtain positive samples covering a wide range of kresoxim-methyl concentrations, homogenized, and stored frozen at -20°C . Residues were extracted by the described QuEChERS procedure and analyzed by the optimized cELISAs and GC–MS. Immunochemical determinations were performed as described for spiked samples. For chromatographic analysis, one microliter of clean extract containing 500 μ g/L of TPP as internal standard was injected in splitless mode at 280 $^\circ\text{C}$, employing helium as carrier with a steady flow of 1 mL/min. The temperature of the oven (110 $^\circ\text{C}$) was held during 1 min; then, it was increased at a rate of 15 $^\circ\text{C}/\text{min}$ until 280 $^\circ\text{C}$ and kept constant at the final temperature during 15 min. Electron impact ionization at 70 eV was

used with the ion source at 225 °C. The employed quantification ions were m/z 116 and 131 for kresoxim-methyl and m/z 325 and 326 for TPP. Retention times were 11.0 and 14.0 min for kresoxim-methyl and TPP, respectively. For method validation, Deming regression and Bland–Altman analysis were applied using the SigmaPlot software (version 12.0).

RESULTS AND DISCUSSION

Assay Selectivity. Two mAb-based immunoassays for kresoxim-methyl were studied. Both assays employed mAb KMo#117 and the homologous conjugate but different cELISA formats (for hapten structure, see Figure S1 in the Supporting Information). Immunoassay selectivity with structurally related compounds was assessed toward the main strobilurin fungicides (trifloxystrobin, azoxystrobin, picoxystrobin, dimoxystrobin, metominostrobin, orysastrobin, pyraclostrobin, and fluoxastrobin; see Figure S2 in the Supporting Information). In both cELISA formats, antibody KMo#117 was highly selective to kresoxim-methyl (CR values with other strobilurins were below 1%). On the other hand, recognition toward chemicals potentially present in real samples was verified using active principles that are commonly formulated together with kresoxim-methyl, such as boscalid, fenpropimorph, epoxiconazole, propiconazole, tebuconazole, and pyrimethanil, and none of them was noticeably recognized by mAb KMo#117.

Tolerance to Solvents and Detergents. QuEChERS methodology for pesticide extraction from food matrices consists of a liquid phase extraction of homogenates with acetonitrile. Hence, tolerance of the described cELISAs to that solvent was appraised. The A_{\max} and IC_{50} values of inhibition curves that had been run in the presence of different amounts of acetonitrile (from 0.5% to 10%, v/v) were compared to those obtained in the absence of solvent (see Figure 1). Only a slight influence over the assay signal was observed, and up to 2% acetonitrile was fairly well tolerated by the studied immunoassays.

Immunochemical competitive reactions are usually performed in the presence of different additives. Tween 20 is a common detergent for unspecific binding minimization; however, it has been often shown to exert a negative effect over the analytical parameters of immunoassays for small organic analytes.¹⁶ The relative variation of A_{\max} and IC_{50} values in the presence of different concentrations of Tween 20 (from 0% to 0.1%, v/v), taking 0.025% as a reference detergent concentration, is depicted in Figure 1. A similar behavior was observed with the two studied cELISAs, i.e., Tween 20 increased the A_{\max} of both assays, but it also increased the IC_{50} value.

Influence of pH and Ionic Strength. The analytical influence of physicochemical conditions such as pH and ionic strength was evaluated. Competitive assays were performed following a biparametric study with composite design in which the center point conditions (pH = 7.5 and I = 175 mM at 25 °C) were similar to those of PBS. A series of buffers were prepared with a mixture of citrate, phosphate, and Tris in order to cover a wide effective pH range. The ionic strength of each buffer was fixed with NaCl, and Tween 20 was added. Figure 2 shows the overlaid contour plots for the responses (A_{\max} and IC_{50} values) to pH and ionic strength changes of the two studied immunoassays, taking the results at center point conditions as the reference values. A constricted area for acceptable pH and ionic strength variations (the area stretching changes on A_{\max} and IC_{50} values below $\pm 20\%$; area in white

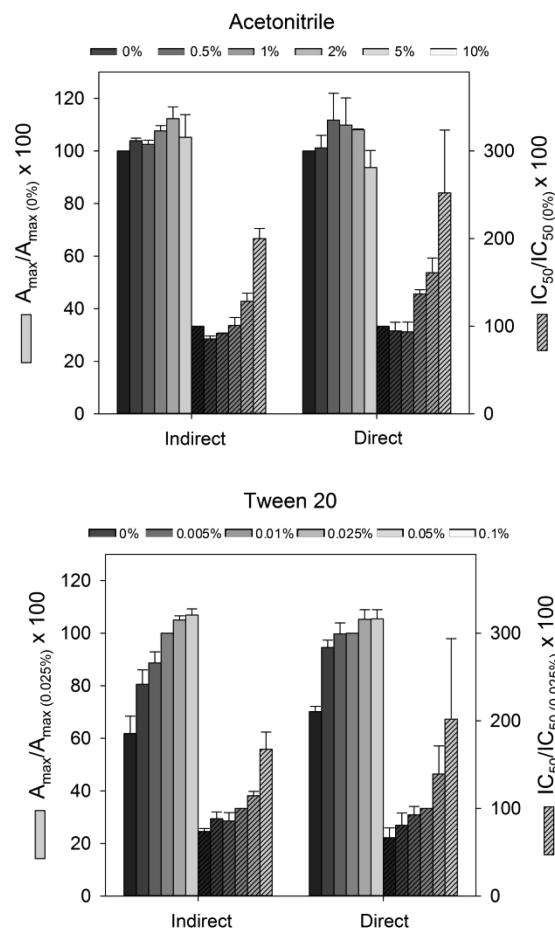


Figure 1. Influence of acetonitrile (upper graph) and Tween 20 (lower graph) contents over A_{\max} and IC_{50} values of the studied immunoassays.

color) was found for the indirect cELISA. With this assay, at pH 7.5, ionic strength values below 150 mM increased both A_{\max} and IC_{50} values above tolerable levels ($>120\%$), whereas values over 200 mM decreased excessively the A_{\max} ($<80\%$). On the contrary, the direct cELISA was very robust— A_{\max} and IC_{50} changes stayed below $\pm 20\%$ —to alterations of either pH or ionic strength conditions (white area in lower graph of Figure 2).

Immunoassay Validation. The assays were validated by investigating the LOD, LOQ, trueness, and repeatability. Final assay conditions of the developed cELISAs and the optimized standard inhibition curves can be found in Table 1. Optimum antibody concentrations were 100 and 300 $\mu\text{g/L}$ for the indirect and direct format, respectively, whereas optimum assay conjugate concentrations were 100 $\mu\text{g/L}$ for the former and 30 $\mu\text{g/L}$ for the latter. Signals in the absence of analyte (A_{\max}) were high (around 2.0), background signals (A_{\min}) were low, and slopes were moderate (between -1.0 and -1.2). Under those conditions, the IC_{50} values for kresoxim-methyl were below 1 $\mu\text{g/L}$ for both cELISAs, and the LODs were around 0.10 $\mu\text{g/L}$. Attempts to reduce these values by decreasing immunoreagent concentrations just resulted in lower A_{\max} without a concomitant effect on assay sensitivity.

To evaluate the trueness and precision, the described immunoassays were applied to the analysis of kresoxim-methyl in diverse fortified foodstuffs. Nowadays, this pesticide is employed against a variety of plant diseases in cucumber,

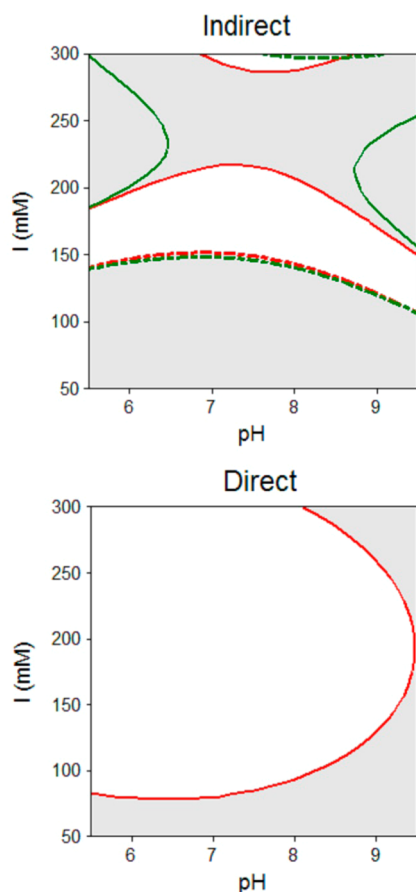
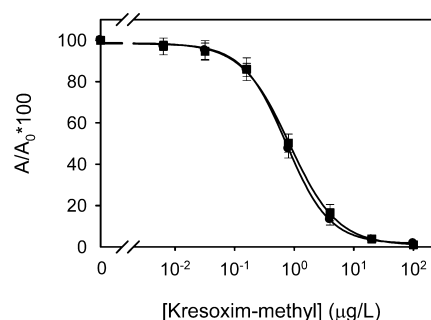


Figure 2. Overlaid contour plots for the A_{\max} and IC_{50} dependence upon pH and ionic strength conditions of the studied immunoassays. White areas set the limits of acceptable pH and I conditions; those with A_{\max} (red) and IC_{50} (green) variations between 80% (solid line) and 120% (dashed line), taking as a reference (100%) the average of the A_{\max} and IC_{50} values of the center point conditions of the composite design.

tomato, and strawberry crops. Extracts of those food samples were prepared following a QuEChERS procedure that was essentially based on the AOAC Official Method 2007.01 for pesticide extraction from food matrices.¹⁷ Homogenized foodstuffs were treated with acetonitrile in the presence of buffered saline and $MgSO_4$, and then a cleanup step using PSA was performed. Extracts from kresoxim-methyl-free samples (as judged by GC–MS) were fortified at four concentration levels and analyzed with the optimized immunoassays after a simple dilution in buffer. In general, quantitative recoveries (between 70% and 120%) and good precision values (relative standard deviation below 20%) were retrieved with each cELISA (Table 2), in accordance with the EU validation guidelines for pesticide residue analysis in food.¹⁸ For both immunoassays, the limit of quantification (LOQ) for the studied foodstuffs was set at 0.01 mg/kg, which is lower than the European MRLs for kresoxim-methyl in these food products (0.05, 0.5, and 1.0 mg/kg for cucumbers, tomatoes, and strawberries, respectively).

As further validation of the developed cELISAs, strawberry samples from crops that we had sprayed with a commercial formulation of kresoxim-methyl were employed as model and relevant commodity in order to evaluate the performance of our novel analytical methods under real-like conditions. Fruits were extracted following a QuEChERS procedure and then analyzed

Table 1. Conditions and Parameters of the Optimized Immunoassays^a



	immunoassay	
	indirect cELISA	direct cELISA
mAb	KMo#117	KMo#117
	100 $\mu\text{g/L}$	300 $\mu\text{g/L}$
conjugate	OVA–KMo	HRP–KMo
	100 $\mu\text{g/L}$	30 $\mu\text{g/L}$
A_{\max}	2.390 ± 0.254	2.037 ± 0.208
A_{\min}	0.039 ± 0.015	0.021 ± 0.009
slope	-1.198 ± 0.059	-1.082 ± 0.056
IC_{50} ($\mu\text{g/L}$)	0.738 ± 0.032	0.838 ± 0.044
LOD ($\mu\text{g/L}$)	0.103	0.097
buffer	PB + 0.025% Tween 20	PB + 0.025% Tween 20
time (h)	2.5	1.5

^aValues are the mean of 16 independent experiments.

by the optimized immunoassays and by GC–MS (Table S2 in the Supporting Information). Trueness of the developed cELISAs was also assessed by statistical method comparison using the Deming regression, which accounts for errors in observations on both methods. Orthogonal regression analysis showed that the results provided by both cELISAs were statistically comparable to those retrieved by the reference method; i.e., the 95% confidence intervals for the intercept and for the slope included 0 and 1, respectively (Table 3). The corresponding regression lines can be seen in Figure S3 in the Supporting Information. Moreover, good correlation between the studied immunochemical technique and the reference chromatography approach was evidenced by the Bland–Altman plot (Figure 3), as the average values of both methods were randomly distributed around the average difference, and they were mostly inside the limits of agreement (average difference ± 1.96 s), meaning that only random deviations occurred.

In summary, two mAb-based immunoassays to kresoxim-methyl—one indirect and one direct cELISA—have been characterized, optimized, and validated using food samples. These assays showed no CR with the most common strobilurin pesticides or a series of fungicides potentially present in relevant foodstuffs. A relative tolerance to acetonitrile contents was observed, and lower influence of pH and ionic strength changes over assay analytical parameters was found with the direct assay. Both immunoassays showed IC_{50} values below 1 $\mu\text{g/L}$ under optimized conditions. The LOQs were fixed at 10 $\mu\text{g/kg}$ from the analysis of fortified tomato, cucumber, and strawberry samples which had been extracted by the QuEChERS method. Overall, good recoveries and precision values were found. Method trueness was also demonstrated by comparison with a reference chromatographic method through statistical analysis using Deming regression and Bland–Altman plot.

Table 2. Recoveries and Precision Values Obtained by Analyzing Replicate Spiked Samples ($n = 4$)

sample	spiked [KM] ^a ($\mu\text{g/kg}$)	indirect			direct		
		found [KM] ($\mu\text{g/kg}$)	RSD (%)	recovery (%)	found [KM] ($\mu\text{g/kg}$)	RSD (%)	recovery (%)
tomato	10	9.1 \pm 0.8	8.8	91.3 \pm 7.7	11.6 \pm 1.4	12.1	75.5 \pm 21.9
	30	29.1 \pm 3.8	13.1	97.0 \pm 12.8	32.8 \pm 4.8	14.6	95.8 \pm 15.2
	100	103.2 \pm 7.5	7.3	103.2 \pm 7.5	110.8 \pm 3.9	3.5	110.8 \pm 3.9
	300	313.2 \pm 28.8	9.2	104.4 \pm 9.6	361.2 \pm 9.3	2.6	120.5 \pm 3.1
cucumber	10	8.1 \pm 0.9	11.1	81.3 \pm 9.1	7.5 \pm 1.1	14.7	75.4 \pm 10.7
	30	30.4 \pm 5.5	18.1	101.2 \pm 18.2	29.5 \pm 3.3	11.2	98.3 \pm 10.9
	100	104.4 \pm 6.4	6.1	104.4 \pm 6.4	116.3 \pm 4.0	3.4	116.3 \pm 4.0
	300	306.0 \pm 12.3	4.0	102.0 \pm 4.1	336.0 \pm 26.4	7.9	112.0 \pm 8.8
strawberry	10	9.7 \pm 1.9	19.6	97.2 \pm 18.7	11.5 \pm 1.6	13.9	115.1 \pm 15.8
	30	29.0 \pm 5.3	18.3	96.7 \pm 17.6	34.4 \pm 5.6	16.3	114.7 \pm 18.7
	100	100.0 \pm 19.2	19.2	100.0 \pm 19.2	120.3 \pm 8.3	6.9	120.3 \pm 8.3
	300	308.4 \pm 60.9	19.7	102.8 \pm 20.3	360.6 \pm 12.0	3.3	120.7 \pm 4.0

^aKM: kresoxim-methyl.

Table 3. Correlation between the Developed Immunoassays and GC–MS by Deming Regression of In-Field Treated Samples

cELISA	intercept	95% confidence interval for intercept	slope	95% confidence interval for slope	r^a	N^b
indirect	0.015 \pm 0.027	[−0.045; 0.074]	1.03 \pm 0.04	[0.94; 1.12]	0.9838	14
direct	0.055 \pm 0.026	[−0.003; 0.112]	0.99 \pm 0.03	[0.91; 1.06]	0.9882	14

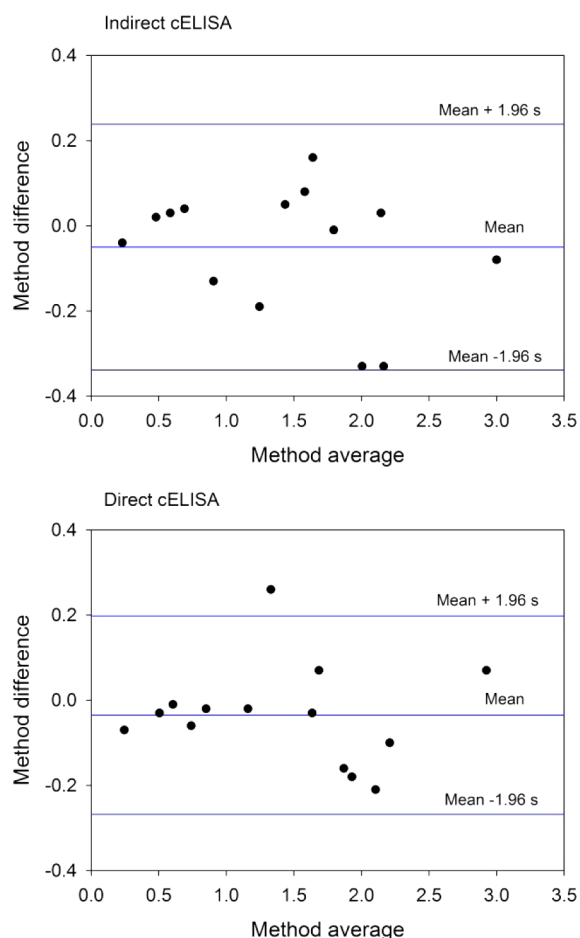
^aCorrelation coefficient. ^bNumber of samples.

Figure 3. Bland–Altman dispersion for comparison of results obtained by the developed cELISAs and by a reference chromatographic method. Samples were analyzed three times by the immunochemical methods and twice by GC–MS.

■ ASSOCIATED CONTENT

§ Supporting Information

Studied buffer conditions, chemical structure of hapten KMo, chemical structures of strobilurins, raw data from the analysis of in-field treated samples, and Deming regression lines. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: antonio.abad@uv.es (A.A.-S.). Tel: +34-963544509. Fax: +34-963544328.

*E-mail: aabad@iata.csic.es (A.A.-F.). Tel: +34-963900022. Fax: +34-963636301.

Author Contributions

†These authors contributed equally to this work

Funding

This work was supported by the Spanish *Ministerio de Ciencia e Innovación* (MICINN) (AGL2006-12750-C02-01/02/ALI and AGL2009-12940-C02-01/02/ALI) and cofinanced by FEDER funds. R.L.-M. was hired by MICINN under a predoctoral FPI grant. F.A.E.-T. and J.V.M. were hired by CSIC with postdoctoral contracts, the former under the JAE-doc program and the latter under the *Ramón y Cajal* program, both cofinanced by MICINN and by the European Social Fund (ESF).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Ana Izquierdo-Gil and Laura López-Sánchez for excellent technical assistance. Limited amounts of the described immunoreagents are available upon request.

■ REFERENCES

- (1) Bartlett, D. W.; Clough, J. M.; Godwin, J. R.; Hall, A. A.; Hamer, M.; Parr-Dobrzanski, B. The strobilurin fungicides. *Pest Manage. Sci.* **2002**, *58*, 649–662, DOI: 10.1002/Ps.520.
- (2) Balba, H. Review of strobilurin fungicide chemicals. *J. Environ. Sci. Health, Part B* **2007**, *42*, 441–451, DOI: 10.1080/03601230701316465.
- (3) Ypema, H. L.; Gold, R. E. Modification of a naturally occurring compound to produce a new fungicide. *Plant Dis.* **1999**, *83*, 4–19, DOI: 10.1094/pdis.1999.83.1.4.
- (4) Environmental Protection Agency (1998). Kresoxim-methyl pesticide fact sheet. http://oaspub.epa.gov/apex/pesticides/f?p=CHEMICALSEARCH:31:17590345896928::NO:1,3,31,7,12,25:P3_XCHEMICAL_ID:1460. Accessed 17.12.13.
- (5) Australian Pesticides and Veterinary Medicines Authority (2000). Evaluation of the new active Kresoxim-methyl in the product Strobly WG fungicide. <https://portal.apvma.gov.au/pubcris>. Accessed 17.12.13.
- (6) European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment of the active substance kresoxim-methyl. *EFSA J.* **2010**, DOI: 10.2903/j.efsa.2010.1891.
- (7) Das Capital Management and Advisors (2011). Rallis India. http://www.dascap.com/house_view_pdf/Rallis.pdf. Accessed 17.12.13.
- (8) Cabras, P.; Angioni, A.; Garau, V. L.; Pirisi, F. M.; Brandolini, V. Gas chromatographic determination of azoxystrobin, fluazinam, kresoxim-methyl, mepanipyrim, and tetraconazole in grapes, must, and wine. *J. AOAC Int.* **1998**, *81*, 1185–1189.
- (9) Christensen, H. B.; Granby, K. Method validation for strobilurin fungicides in cereals and fruit. *Food Addit. Contam.* **2001**, *18*, 866–874, DOI: 10.1080/02652030121435.
- (10) Sannino, A.; Bolzoni, L.; Bandini, M. Application of liquid chromatography with electrospray tandem mass spectrometry to the determination of a new generation of pesticides in processed fruits and vegetables. *J. Chromatogr. A* **2004**, *1036*, 161–169, DOI: 10.1016/j.chroma.2004.02.078.
- (11) Koesukkiwat, U.; Lehotay, S. J.; Miao, S.; Leepipatpiboon, N. High throughput analysis of 150 pesticides in fruits and vegetables using QuEChERS and low-pressure gas chromatography-time-of-flight mass spectrometry. *J. Chromatogr. A* **2010**, *1217*, 6692–6703, DOI: 10.1016/j.chroma.2010.05.012.
- (12) Mercader, J. V.; Suárez-Pantaleón, C.; Agulló, C.; Abad-Somovilla, A.; Abad-Fuentes, A. Hapten synthesis and monoclonal antibody-based immunoassay development for the detection of the fungicide kresoxim-methyl. *J. Agric. Food Chem.* **2008**, *56*, 1545–1552, DOI: 10.1021/jf073039x.
- (13) López-Moreno, R.; Mercader, J. V.; Agulló, C.; Abad-Somovilla, A.; Abad-Fuentes, A. Structure-immunogenicity relationship of kresoxim-methyl regioisomeric haptens. *Org. Biomol. Chem.* **2013**, *11*, 7361–7371, DOI: 10.1039/c3ob41570h.
- (14) Anastassiades, M.; Lehotay, S. J.; Stajnbaher, D.; Schenck, F. J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive solid-phase extraction” for the determination of pesticide residues in produce. *J. AOAC Int.* **2003**, *86*, 412–431.
- (15) Abad-Fuentes, A.; Esteve-Turrillas, F. A.; Agulló, C.; Abad-Somovilla, A.; Mercader, J. V. Development of competitive enzyme-linked immunosorbent assays for boscalid determination in fruit juices. *Food Chem.* **2012**, *135*, 276–284, DOI: 10.1016/j.foodchem.2012.04.090.
- (16) Mercader, J. V.; Suárez-Pantaleón, C.; Agulló, C.; Abad-Somovilla, A.; Abad-Fuentes, A. Hapten synthesis and monoclonal antibody-based immunoassay development for detection of the fungicide trifloxystrobin. *J. Agric. Food Chem.* **2008**, *56*, 2581–2588, DOI: 10.1021/jf800157s.
- (17) Lehotay, S. J. Determination of pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate: Collaborative study. *J. AOAC Int.* **2007**, *90*, 485–520.
- (18) European Commission DG SANCO (2007). Method validation and quality control procedures for pesticide residues analysis in food and feed. http://www.eurl-pesticides.eu/docs/public/tmpl_article.asp?CntID=615&LabID=100&Lang=EN. Accessed 17.12.13.