**Immunoassays for pyraclostrobin analysis in
processed food products using novel monoclonal
antibodies and QuEChERS-based extracts**

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Abstract

Strobilurins are a family of synthetic natural-derived pesticides widely used for fungi control in many crops. As part of a larger research project, a collection of monoclonal antibodies to one of the most relevant strobilurins, *i.e.* pyraclostrobin, has been generated using functionalized haptens with the spacer arm located at different sites. The toxophore aliphatic moiety, as observed with other strobilurins, and the planar aromatic two-ring system characteristic of this molecule were revealed as the best tethering sites for high-affinity monoclonal antibody production. A central derivatization position did not generate a good immune response, but such derivatives were useful as heterologous competitors in order to improve assay sensitivity. Direct and indirect assays were characterized in terms of solvent influence, pH, and ionic strength. Immunoassays with limits of detection below 0.1 µg/L were developed. A QuEChERS-based procedure was applied to the extraction of blueberry, strawberry, raspberry, peach, apricot, and plum jams. Clean acetonitrile extracts were properly diluted in water and analyzed by both optimized immunoassays with good accuracy and precision at levels covering the European maximum residue limits for those fruits.

Highlights:

► Haptens of pyraclostrobin with alternative linker positions have been evaluated.
► A set of high-affinity monoclonal antibodies to this fungicide has been generated.
► Two competitive immunoassays have been optimized in different formats.
► A QuEChERS-based method has been applied for fruit marmalade extraction.
► Quantitative results have been obtained covering the European maximum residue limits.

Keywords:

Hapten tethering site, Antibody, Strobilurin, Pesticide residue, Rapid methods

Abbreviations:

$A_{\max}$: maximum absorbance; BSA: bovine serum albumin; cELISA: competitive enzyme-linked immunosorbent assay; CR: cross-reactivity; d-cELISA: antibody-coated direct cELISA; DMF: *N,N*-dimethylformamide; HRP: horseradish peroxidase; IC_{50} : analyte concentration affording a 50% inhibition of the $A_{\max}$; i-cELISA: conjugate-coated indirect cELISA; mAb: monoclonal antibody; MR: molar ratio; MRL: maximum residue limit; OVA: ovalbumin; PB: 100 mM sodium phosphate buffer, pH 7.4; PBS: 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl; PBST: PBS containing 0.05% (v/v) Tween 20; PSA: primary–secondary amine.

1. Introduction

Strobilurins are a relatively new family of pesticides used to fight major fungal infections in plants. These fungicides possess a toxophore moiety mimicking the natural bioactive methyl (*E*)- β -methoxyacrylate chemical group (Bartlett, Clough, Godwin, Hall, Hamer, & Parr-Dobrzanski, 2002). Pyraclostrobin, which belongs to the second generation of strobilurins developed by BASF, is composed of a novel methoxycarbamate toxophore together with a characteristic planar two-ring aromatic system and the aryl bridge common to all synthetic strobilurins (Fig. 1). The primary markets of this fungicide are North and South America and Europe, with current global annual sales approaching \$ 1 billion, and its residues are among the most frequently found in fruits and vegetables, particularly in blueberries and strawberries (Scientific report of EFSA, 2011; USDA pesticide data program, 2011). The maximum residue limits (MRL) for this strobilurin are, for most fruits, between 0.2 and 3 mg/kg in the EU (http://ec.europa.eu/sanco_pesticides/public/index.cfm) and between 0.6 and 8 mg/kg in the US (www.ecfr.gov). Nevertheless, little information is available other than institutional and official reports regarding the analysis of pyraclostrobin residues in processed food products.

Analytical methods proposed by the manufacturer to the Joint FAO/WHO Meeting on Pesticide Residues were all based on HPLC with either UV or MS detection (Declercq, 2005). Outstandingly, de Melo Abreu and coworkers (De Melo Abreu, Caboni, Cabras, Garau, & Alves, 2006) published a multiresidue method for the analysis of the main strobilurins in grapes and wine, which consists of a liquid-

liquid extraction followed by liquid chromatography coupled to a diode array detector. Continuous efforts have been put during the last decades to develop novel techniques for rapid, cheap, and environmental-friendly chemical residue analysis in food. Recently, a breakthrough has occurred in the field of pesticide residue analysis with the development of the QuEChERS (acronym for Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction methodology. It is a highly beneficial approach that simplifies the analysis of pesticide residues in fruit, vegetables, cereals, and processed products thereof. It was first published by Anastassiades, Lehotay, and coworkers in 2003 ([Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003](#)) and subsequently, many official analytical methods have included QuEChERS-based approaches for pesticide extraction from foods, such as the AOAC International method 2007.01 and the European standard method EN 15662 ([Lehotay, Anastassiades, & Majors, 2010](#)).

Immunochemical techniques constitute complementary analytical methods which can contribute to increase food safety standards by lowering analytical costs and time. Immunoassays can be developed in a variety of economical and user-friendly formats including the classical 96-well microplate and many other innovative configurations, such as the immunofiltration assays ([Sardinha, Gil, Mercader, & Montoya, 2002](#)) or the lateral flow tests ([Blažková, Mičková-Holuvobá, Rauch, & Fukal, 2009](#)). However, availability of high-performance antibodies is the bottle neck for sensitive assay development. Successful generation of specific antibodies to small organic molecules, such as pyraclostrobin, is greatly dependent upon a proper design of immunizing and assay haptens. In previous studies, the importance of the linker length for the generation of anti-pyraclostrobin monoclonal

antibodies (mAbs) was evaluated in our laboratory (Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008). Also, a thorough investigation was performed in rabbits about the influence of the derivatization site on the immunogenicity of conjugates of pyraclostrobin (Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011). Based on those results, we reasoned that mAbs with superior performance might be generated from hapten PYs5, a functionalized pyraclostrobin mimic with the spacer arm at the chlorophenyl moiety of the target molecule. In the present article, we describe the production and characterization of novel mAbs against pyraclostrobin using bioconjugates with alternative linker tethering sites. Also, new 96-well microplate immunoassays based on the competitive enzyme-linked immunosorbent assay (cELISA) method were developed and optimized with homologous and heterologous conjugates using two different assay formats: the conjugate-coated indirect competitive format (i-cELISA) and the antibody-coated direct competitive format (d-cELISA). Our purpose was to develop a rapid and economic procedure, including extraction and analysis, for pyraclostrobin determination in complex processed food products such as marmalades.

2. Materials and methods

2.1. Reagents and instruments

Pyraclostrobin [methyl {2-[1-(4-chlorophenyl)pyrazol-3-ylloxymethyl]phenyl}methoxycarbamate] (CAS number 175013-18-0, MW 387.8 g/mol) and other pesticide standards, Pestanal grade, were purchased from Riedel-de-Haën (Seelze, Germany). Trifloxystrobin was kindly provided by Bayer

CropScience (Frankfurt, Germany), and azoxystrobin and picoxystrobin standards were obtained from Syngenta (Basel, Switzerland). Primary-secondary amine (PSA) was from Scharlab (Barcelona, Spain). Sephadex G-25 HiTrap Desalting columns from General Electric Healthcare (Uppsala, Sweden) were used for conjugate purification in the ÄKTA workstation. Polyclonal rabbit anti-mouse immunoglobulin peroxidase conjugate was from Dako (Glostrup, Denmark). Ovalbumin (OVA), tributylamine, isobutyl chloroformate, and o-phenylenediamine were purchased from Sigma-Aldrich (Madrid, Spain). Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA.). ELISA absorbances were read with a PowerWave HT microplate reader from BioTek Instruments (Winooski, VT, USA). Microplates were washed with an ELx405 microplate washer also from BioTek Instruments. Fruit jam samples were purchased from local markets and stored at 4 °C.

2.2. Conjugate preparation

Syntheses of pyraclostrobin functionalized derivatives with alternative linker tethering sites and equivalent linkers – namely, haptens PYa6, PYo5, and PYS5 (Fig. 1) – and preparation of immunizing and enzyme tracer conjugates using bovine serum albumin (BSA) and horseradish peroxidase (HRP), respectively, were described in previous articles (Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008; Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011). For the present study, novel coating OVA conjugates were prepared with the three pyraclostrobin haptens at different hapten-to-protein molar ratios (MR). Briefly, haptens were activated in DMF with equivalent amounts of

tributylamine and isobutyl chloroformate during 1 h at room temperature. Next, a fraction of that mixture containing 0.9, 4.5, or 9.0 μmol of activated hapten was diluted to 100 μL with DMF and added to 30 mg of OVA in 2 mL of 50 mM carbonate buffer, pH 9.6. Conjugation was kept during 2.5 h at room temperature and the conjugate was purified by gel filtration with Sephadex G-25 using PB (100 mM sodium phosphate, pH 7.4) as eluent. MRs of the purified conjugates were calculated by differential absorbance as previously described (Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008).

2.3. Antibody generation

Novel mAbs for pyraclostrobin were produced from mice immunized with BSA–PYa6 and BSA–PYs5. Eight BALB/c female mice were immunized (four animals with each conjugate) by intraperitoneal injections. Standard immunization, cell fusion, hybridoma cloning, and antibody purification procedures were followed (Mercader & Abad-Fuentes, 2009). Hybridoma screenings were conducted by a two-step strategy for better clone selection as previously described for mAb production with BSA–PYo5 as immunogen (Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008). Briefly, indirect competitive assays using the homologous coating conjugate (carrying the same hapten that was used for immunization) and 100 nM pyraclostrobin in solution were run in parallel with a control assay without competitor. Those supernatants that afforded saturated signals in the blank assay were reevaluated by a checkerboard competitive screening test, in which diverse pyraclostrobin concentrations (50, 10, and 1 nM) were assayed with serial dilutions of culture supernatant and different coating

concentrations of the homologous conjugate. Selected hybridomas were cloned by limiting dilution and expanded in culture plates. Finally, stable antibody-producing clones were cryopreserved in liquid nitrogen. Immunoglobulin isotype was determined using the Mouse MonoAb-ID kit (HRP) from Invitrogen (Carlsbad, CA, USA).

2.4. Antibody-coated direct competitive ELISAs

Antibodies were immobilized onto polystyrene 96-well plates. Coating was performed with 100 μ L per well of a 1 μ g/mL mAb solution in coating buffer (50 mM sodium carbonate–bicarbonate buffer, pH 9.6) and overnight incubation at room temperature. Then, microwells were washed four times with washing solution (150 mM NaCl and 0.05% (v/v) Tween 20), and they received 50 μ L per well of analyte in PBS (10 mM sodium phosphate buffer, pH 7.4 with 140 mM NaCl) plus 50 μ L per well of HRP tracer in PBST (PBS containing 0.05% (v/v) Tween 20). No blocking step was performed. The immunological reaction took place during 1 h at room temperature, and plates were washed again as described above. Finally, signal was generated with 100 μ L per well of a 2 mg/mL o-phenylenediamine solution containing 0.012% (v/v) H₂O₂ in 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4. Absorbance was spectrophotometrically measured at 492 nm.

2.5. Conjugate-coated indirect competitive ELISAs

ELISA plates were coated with 100 μ L per well of OVA conjugate solution in coating buffer by overnight incubation at room temperature. Coated plates were washed four times with washing solution, and then received 50 μ L per well of

analyte in PBS plus 50 μ L per well of anti-pyraclostrobin mAb in PBST. No blocking step was performed. The immunological reaction took place during 1 h at room temperature and plates were washed again as before. Next, 100 μ L per well of a 1/2000 dilution of secondary antibody–HRP conjugate in PBST was added, and plates were incubated 1 h at room temperature. After washing, color was developed and measured as aforementioned.

2.6. Standards and data analysis

Eight-point standard curves, including a blank, were prepared by 5-fold serial dilution in PBS, starting from a 0.1 μ M pyraclostrobin standard. A 0.1 mM pyraclostrobin stock solution in anhydrous *N,N*-dimethylformamide (DMF) was used to prepare the first standard. Experimental values were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Immunoassay sensitivity was estimated as the analyte concentration affording a 50% inhibition (IC_{50}) of the maximum absorbance (A_{max}). Cross-reactivity (CR) was calculated from the quotient between the IC_{50} of pyraclostrobin and the IC_{50} of the evaluated cross-reactant.

2.7. Solvent, ionic strength, and pH studies

For solvent studies, analyte standard curves were prepared in water containing between 0.5 and 10% (v/v) methanol, ethanol, or acetonitrile, and immunoreagents were prepared in 2 $\times$ PBS containing 0.05% (v/v) Tween 20. The influence of buffer ionic strength and pH was assessed with direct and indirect assays following a central composite design, consisting of a two-level full factorial design ($\alpha = 1.414$) with 2 factors and 3 replicates that included 12 cube, 12 axial, and 15 centre

points, and involving a total of 39 randomized buffer studies as previously published (Abad-Fuentes, Esteve-Turrillas, Agulló, Abad-Somovilla, & Mercader, 2012). Ionic strength values of the evaluated buffers were between 50 and 300 mM, and 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. Known volumes of 5 M HCl was added until the required pH for every buffer was reached. The ionic strength of each buffer was calculated considering initial buffer concentrations and added HCl, and then an appropriate volume of 2 M NaCl was used to achieve the established ionic strength for every buffer. Finally, Tween 20 was added to all buffers to a final concentration of 0.05% (v/v). Pyraclostrobin standard curves were prepared in water and 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).

2.8. Extraction procedure

Commercial strawberry, apricot, peach, raspberry, blueberry, and plum marmalades containing 60% fruit were acquired from a local supermarket. A QuEChERS-based method, similar to the EN 15662 European Standard, was adapted for pyraclostrobin extraction from jam samples. Briefly, a 10 g sample was vortexed in a 50 mL polypropylene tube with 10 mL of acetonitrile during 2 min. Then, 1 g of $Na_3Cit \cdot 2H_2O$, 0.5 g of $Na_2Cit \cdot 1.5H_2O$, 1 g NaCl, and 4 g of anhydrous

241 MgSO₄ was added. The mixture was vortexed 2 min and centrifuged 3 min at
242 2200×g. Then, a 3 mL extract was introduced into a 15 mL polypropylene
243 centrifuge tube containing 75 mg of PSA and 450 mg of anhydrous MgSO₄,
244 vortexed 2 min, and centrifuged 3 min at 2200×g. Finally, purified extracts were
245 filtered through a 0.22 µm Teflon filter and analyzed by cELISA after proper dilution
246 in water.

247 **3. Results and discussion**

248 *3.1. Antibody and conjugate selection*

249 Previous studies using three pyraclostrobin mimicking haptens with alternative
250 linker tethering sites identified hapten PYs5 as a superior derivative for the
251 generation of high-affinity anti-pyraclostrobin rabbit polyclonal antibodies, whereas
252 PYa6 was shown to be clearly deficient ([Mercader, Agulló, Abad-Somovilla, &](#)
253 [Abad-Fuentes, 2011](#)). To further confirm this result, mice were immunized with
254 BSA conjugates of haptens PYa6 and PYs5, and cell fusions were carried out with
255 the best responding mouse from each group of immunized animals.

256 Splenocytes from PYa6-immunized mice afforded four hybridomas whose culture
257 supernatants gave positive binding to OVA–PYa6, but only a slight or no
258 recognition of the free analyte was observed as judged by the poor inhibition
259 exhibited in competitive experiments in the presence of 50 nM pyraclostrobin. This
260 result confirms the low capacity of PYa6 to generate high-affinity antibodies to
261 pyraclostrobin, as previously observed with rabbit polyclonals. On the contrary, six
262 PYs5-derived hybridoma culture supernatants displayed almost complete inhibition
263 in the presence of 10 nM pyraclostrobin. Those six promising candidates were

further cloned and expanded, and the mAb was purified from culture supernatants, all of them being of the IgG₁ isotype with κ light chains. Purified antibodies were evaluated by checkerboard cELISA with direct and indirect formats using homologous and the two described site-heterologous conjugates. At this point of the study and in order to develop an immunoassay with a sensitivity as high as possible for pyraclostrobin, a set of four mAbs previously produced in our lab were also incorporated to this study ([Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008](#)). Those were the best antibodies produced from hapten PYo5, which incorporated the spacer arm at the aliphatic toxophore moiety.

For every immunoreagent pair (antibody/coating conjugate or antibody/enzyme tracer), a series of inhibition curves was obtained from checkerboard assays; each curve corresponding to particular antibody and conjugate concentrations. [Fig. 2](#) shows, for every pair of immunoreagents in the indirect format, the $A_{\max}$, IC_{50} , and slope values from the precise concentrations that gave rise to the lowest IC_{50} value and an $A_{\max}$ closest to 1.0. It was observed that most of the antibodies bound OVA-PYa6 conjugate, independently of the origin of the mAb, whereas PYo5-type antibodies could not recognize OVA-PYs5 and PYs5-type mAbs did not bind OVA-PYo5. This result fits well with the Landsteiner principle, saying that the main binding epitopes are formed by those groups that are located distal from the linker tethering site ([Landsteiner, 1962](#)). Assay sensitivities achieved with PYo5-type antibodies and the homologous conjugate could be slightly improved with the heterologous OVA-PYa6 conjugate without significantly changing the slope of the inhibition curves. The lowest IC_{50} values with homologous assays were those obtained with the newly generated mAbs from hapten PYs5. In those cases, the

heterologous conjugate OVA–PYa6 did not generally help to markedly reduce the IC_{50} values, and curves with higher slopes were retrieved under such conditions. In brief, the assays with the lowest IC_{50} values were obtained with mAbs produced using BSA–PYs5 as immunogen.

In the d-cELISA format, fewer antibody/tracer combinations gave adequate A_{max} values (Fig. S1 in the Supplementary Data). In fact, PYs5-type antibodies only bound to homologous tracers (HRP–PYs5). With PYo5-type antibodies, signal was obtained using homologous (HRP–PYo5) and heterologous (HRP–PYa6) tracers. Again, the most sensitive assay was achieved with a PYs5-type antibody.

Overall, these results indicate that both, the planar two-ring system characteristic of pyraclostrobin and the toxophore methoxycarbamate moiety were adequate linker tethering sites for antibody production. However, the slightly better performance of PYs5-type antibodies could be explained by the presence of the linker in the immunizing hapten at the chlorinated position of pyraclostrobin. It is well known that halogens enhance binding to antibodies that had been produced by immunizing haptens with bulky hydrophobic moieties, such as the spacer arm, located at the halogenated position of the target molecule (Shelver, Keum, Kim, Rutherford, Hakk, Bergman, & Li, 2005). Moreover, a sulfur linkage to the spacer arm has been shown to be a good homologue of chlorine atoms of the target molecule, probably because both atoms have nonbonded electron pairs and sulfur resembles halogen electronic properties better than carbon (Matsuo, Nishi, Morimune, Okawa, & Miyake, 2005; Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011). On the contrary, haptens with the spacer arm at the central bridging aromatic ring (PYa6), characteristic of all synthetic strobilurin

fungicides, generated mAbs with lower affinity to pyraclostrobin. However, hapten PYa6 afforded an interesting heterologous assay conjugate for some antibodies. Similar results were previously observed with rabbit polyclonal antibodies (Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011). For immunoanalytical purposes, the best direct and indirect immunoassays were provided by mAb PYs5#11 together with the homologous conjugate.

3.2. Specificity evaluation

Selectivity of the 10 mAbs was assessed by i-cELISA using the homologous conjugate. The main strobilurins and a series of fungicides commonly used in fruit crops were employed as competitors; namely, azoxystrobin, kresoxim-methyl, picoxystrobin, trifloxystrobin, dimoxystrobin, orysastrobin, famoxadone, fenamidone, boscalid, fludioxonil, and cyazofamid. No inhibition was observed up to 1 μ M of competing analyte with any of the mAbs. In addition, competitive immunoassays were performed with 1-(4-chlorophenyl)-1*H*-pyrazol-3-ol, which is a metabolite of pyraclostrobin (Declercq, 2005). This molecule was synthesized in our lab and holds only the planar two-ring aromatic system characteristic of this strobilurin fungicide (Table 1). Interestingly, PYo5-type antibodies recognized the metabolite (CR values were between 0.3 and 1.3%) somewhat better than PYs5-type antibodies (CR values were below 0.04%). Overall, these results show that both groups of antibodies were highly selective of pyraclostrobin, exhibiting low binding to the metabolite, and that the observed CR differences reflect the principle that those moieties located distal from the hapten derivatization site have a greater contribution to the interaction between the antibody and the antigen.

3.3. Immunoassay characterization

Direct and indirect competitive assays were characterized and optimized using mAb PYs5#11 and the homologous conjugate. Immunoreagent concentrations were selected in order to achieve inhibition curves with $A_{\max}$ values around 1.0. First, the optimum MR of OVA–PYs5 conjugate for i-cELISAs was determined. Three conjugates with different MRs (0.4, 3, and 5) were evaluated by checkerboard competitive assay. It was observed that equal antibody concentrations (20 ng/mL) were required in order to achieve an $A_{\max}$ around 1.0 if any of the two conjugates of higher MR was employed for coating. On the contrary, using the conjugate with the lower MR, a 60 ng/mL antibody solution was necessary for an equivalent signal. Regarding sensitivity, non-statistically different values were observed: 1.13 ± 0.04 nM, 1.25 ± 0.07 nM, and 1.36 ± 0.07 nM for conjugates with increasing MR, respectively. Thus, OVA conjugate with an intermediate MR (3 mol of hapten per mol of protein) was chosen for i-cELISA development.

Solvents coming from the extraction procedure may be present during immunoanalysis. Methanol and ethanol are solvents quite commonly used in pesticide residue extraction for food analysis. Moreover, QuEChERS-based methods employ acetonitrile for sample treatment. As depicted in Fig. 3, ethanol and methanol were rather well tolerated up to 10% (v/v) by both cELISAs. Particularly, 20% (v/v) methanol could be present in the i-cELISA without significantly changing the $A_{\max}$ and IC_{50} values. On the contrary, acetonitrile rapidly increased the IC_{50} value of both assays.

Influence of pH and ionic strength over the $A_{\max}$ and IC_{50} values of the selected direct and indirect immunoassays was studied by a multiparametric approach. As seen in [Fig. 4](#), changes of pH and ionic strength modified the IC_{50} value of the d-cELISA. For that reason, a buffer with a higher buffering capacity was chosen for further assay development; that is, buffer PB (100 mM sodium phosphate, pH 7.4; $I = 223$ mM at 25 °C). Under those conditions, $A_{\max}$ and IC_{50} values were maintained, whereas the slope of the curve became more moderate. The same buffer was taken for i-cELISA development because potential pH or ionic strength changes due to matrix effects could also modify the $A_{\max}$ and IC_{50} values of this assay (see [Fig. S2 in the Supplementary Data](#)).

The standard curves of the optimized cELISAs can be seen in the figure inside [Table 2](#), in which the main analytical parameters of both assays are summarized. Finally, these immunoassays were applied to the analysis of extracts of marmalade samples.

3.4. Food analysis

An adaptation of the QuEChERS extraction procedure was applied before the analysis of pyraclostrobin. Fruit jam samples were homogenized, extracted with acetonitrile using citrate as buffer, and cleaned-up with PSA. Then, extracts were diluted in water and analyzed by the developed cELISAs. Fairly low matrix effects were observed for the six studied marmalades in both immunoassays ([Fig. S3](#) and [Fig. S4 in the Supplementary Data](#)). Extracts were fortified at relevant levels attending to legal tolerance values and they were diluted 1/300 in water in order to enter into the working range of the standard curve. Recoveries were evaluated by

three independent determinations of the extracts with the optimized cELISAs using a pyraclostrobin standard curve prepared in acetonitrile and also diluted 1/300 in water. As seen in Table 3, both immunoassays could accurately and precisely measure pyraclostrobin in different fruit marmalades covering regular MRLs for the concerned fruits (200–2000 µg/kg).

In conclusion, a collection of mAbs to pyraclostrobin has been prepared using immunizing haptens with alternative linker positions. The importance of the tethering site was revealed not only for high-affinity antibody production but also for heterologous hapten preparation. Moreover, novel immunoassays have been deeply studied using the two main cELISA formats. The analytical properties of both assays were equivalent, though the direct format is much quicker. Ionic strength, pH, and solvent contents were optimized. For sample extraction, a QuEChERS approach was applied to six different fruit marmalades. The developed mAb-based assays were demonstrated to be suitable for pyraclostrobin quantification in such processed food products. QuEChERS and cELISA together constitute an attractive strategy for rapid, cheap, and clean pyraclostrobin residue analysis in foodstuffs.

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405 **Appendix. Supplementary material**

406 Supplementary data associated to this article can be found, in the online
407 version, at doi:

References

- Abad-Fuentes, A., Esteve-Turrillas, F. A., Agulló, C., Abad-Somovilla, A., Mercader, J. V. (2012). Development of competitive enzyme-linked immunosorbent assays for boscalid determination in fruit juices. *Food Chemistry*, 135, 276–284.
- Anastassiades, M., Lehotay, S. J., Stajnbaher, D., Schenck, F. J. (2003). Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive solid-phase extraction” for the determination of pesticide residues in produce. *Journal of AOAC International*, 86, 412–431.
- Bartlett, D. M., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., Parr-Dobrzanski, B. (2002). Review. The strobilurin fungicides. *Pesticide Management Science*, 58, 649–662.
- Blažková, M., Mičková-Holubová, B., Rauch, P., Fukal, L. (2009). Immunochromatographic colloidal carbon-based assay for detection of methiocarb in surface water. *Biosensors and Bioelectronics*, 25, 753–758.
- Declercq, B. (2005). Pyraclostrobin. In *Pesticide residues in food 2004. Evaluations, part I: residues. FAO plant production and protection paper 182/1* (pp. 1007–1118). Rome: FAO Information Division.
- De Melo Abreu, S., Caboni, P., Cabras, P., Garau, V. L., Alves, A. (2006). Validation and global uncertainty of a liquid chromatographic with diode array detection method for the screening of azoxystrobin, kresoxim-methyl,

429 trifloxystrobin, famoxadone, pyraclostrobin and fenamidone in grapes and
 430 wine. *Analytica Chimica Acta*, 573–574, 291–297.

431 Landsteiner, K. (1962). *The specificity of serological reactions*. (Revised ed.). New
 432 York: Dover Publications.

433 Lehotay, S. J., Anastassiades, M., Majors, R. E. (2010). The QuEChERS
 434 revolution. *LC GC Europe*, 23, 418–429.

435 Matsuo, R., Nishi, K., Morimune, K., Okawa, H., Miyake, S. (2005). Monoclonal
 436 anti-alachlor antibody for immunoassay of herbicidal alachlor analogs in
 437 environment, food and body. Japanese Patent 2005035893.

438 Mercader, J. V., Abad-Fuentes, A. (2009). Monoclonal antibody generation and
 439 direct competitive enzyme-linked immunosorbent assay evaluation for the
 440 analysis of the fungicide fenhexamid in must and wine. *Journal of*
 441 *Agricultural and Food Chemistry*, 57, 5129–5135.

442 Mercader, J. V., Agulló, C., Abad-Somovilla, A., Abad-Fuentes, A. (2011).
 443 Synthesis of site-heterologous hapens for high-affinity anti-pyraclostrobin
 444 antibody generation. *Organic and Biomolecular Chemistry*, 9, 1443–1453.

445 Mercader, J. V., Suárez-Pantaleón, C., Agulló, C., Abad-Somovilla, A., Abad-
 446 Fuentes, A. (2008). Production and characterization of monoclonal
 447 antibodies specific to the strobilurin pesticide pyraclostrobin. *Journal of*
 448 *Agricultural and Food Chemistry*, 56, 7682–7690.

449 Sardinha, J. P. M., Gil, M. H., Mercader, J. V., Montoya, A. (2002) Enzyme-linked
450 immunofiltration assay used in the screening of solid supports and
451 immunoreagents for the development of an azinphos-methyl flow
452 immunosensor. *Journal of Immunological Methods*, 260, 173–182.

453 Scientific reports of EFSA. The 2009 European Union report on pesticide residues
454 in food. (2011). *EFSA Journal*, 9, 2430–2655. Available on-line at:
455 <http://www.efsa.europa.eu/en/publications/efsajournal.htm>. Accessed
456 November 29, 2012.

457 Shelver, W. L., Keum, Y. S., Kim, H. J., Rutherford, D., Hakk, H. H., Bergman, A.,
458 Li, Q. X. (2005). Hapten syntheses and antibody generation for the
459 development of a polybrominated flame retardant ELISA. *Journal of*
460 *Agricultural and Food Chemistry*, 53, 3840–3847.

461 Suárez-Pantaleón, C., Mercader, J. V., Agulló, C., Abad-Somovilla, A., Abad-
462 Fuentes, A. (2008). Production and characterization of monoclonal and
463 polyclonal antibodies to forchlorfenuron. *Journal of Agricultural and Food*
464 *Chemistry*, 56, 11122–11131.

465 Suárez-Pantaleón, C., Mercader, J. V., Agulló, C., Abad-Somovilla, A., Abad-
466 Fuentes, A. (2011). Forchlorfenuron-mimicking haptens: from immunogen
467 design to antibody characterization by hierarchical clustering analysis.
468 *Organic and Biomolecular Chemistry*, 9, 4863–4872.

469 USDA pesticide data program. Annual summary, calendar year 2009. (2011).
470 Available on-line at: <http://www.ams.usda.gov/pdp>. Accessed November 29,
471 2012.

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476 **Figure legends**

477 **Fig. 1.** Structure of pyraclostrobin and the three employed haptens.

478 **Fig. 2.** Parameters of selected inhibition curves with $A_{\max}$ values close to 1.0
479 obtained by competitive checkerboard i-cELISA. Results are the mean of three
480 independent determinations.

481 **Fig. 3.** Influence of solvent contents over the $A_{\max}$ and IC_{50} values of the optimized
482 cELISAs. Results are the mean of three independent determinations.

483 **Fig. 4.** Influence of pH and ionic strength over $A_{\max}$, slope, and IC_{50} values of the
484 homologous d-cELISA using mAb PYs5#11. Areas with darker color indicate
485 higher values of the corresponding assay parameter.

Pyraclostrobin

Hapten PYa6

Hapten PYo5

Hapten PYs5

Figure 1

Figure 2

Figure 3

Figure 4

Table 1

Chemical structure of the studied pyraclostrobin metabolite and cross-reactivity values for the evaluated mAbs.

mAb	CR (%) ^a	mAb	CR (%) ^a
PYs5#11	0.02	PYo5#21	0.25
PYs5#12	0.01	PYo5#31	1.29
PYs5#13	0.02	PYo5#37	0.31
PYs5#14	0.02	PYo5#38	0.71
PYs5#15	0.02		
PYs5#16	0.04		

^a Values are the mean of three independent experiments.

Table 2

Standard curves, assay conditions, and main analytical parameters of the optimized cELISAs^a.

Format	i-cELISA	d-cELISA
Antibody	PYs5#11	PYs5#11
	at 20 ng/mL	at 1.0 $\mu\text{g/mL}$
Conjugate	OVA-PYs5	HRP-PYs5
	at 1.0 $\mu\text{g/mL}$	at 15 ng/mL
A_{max}	1.02 ± 0.07	1.39 ± 0.04
Slope	1.18 ± 0.07	1.18 ± 0.05
IC_{50} ($\mu\text{g/L}$)	0.50 ± 0.03	0.61 ± 0.04
LOD ($\mu\text{g/L}$) ^b	0.08 ± 0.01	0.09 ± 0.02
Assay buffer	PB with	PB with
	0.025% Tween 20	0.025% Tween 20
Assay time (h)	2.5	1.5
Solvent tolerance	Methanol 20%	Methanol 10%

^a Values are the mean of six independent determinations. ^b LOD values were calculated as the analyte concentration affording a 10% inhibition.

Table 3

Recovery values from spiked fruit marmalade extracts using the two developed cELISAs.

Assay	[PY] (µg/kg)	Recovery (% ± s, n = 3)					
		Blueberry	Strawberry	Raspberry	Peach	Apricot	Plum
d-cELISA	2000	81.7 ± 0.6	76.0 ± 3.6	85.3 ± 4.0	85.3 ± 4.2	86.0 ± 5.3	81.3 ± 1.5
	1000	91.0 ± 2.6	81.7 ± 4.2	91.7 ± 4.7	92.3 ± 7.5	98.3 ± 5.0	87.0 ± 9.8
	500	102.0 ± 4.6	85.7 ± 7.2	100.0 ± 2.9	96.7 ± 9.0	99.7 ± 4.5	90.3 ± 11.4
	200	106.3 ± 2.5	94.7 ± 7.6	101.0 ± 8.7	94.7 ± 8.5	100.0 ± 8.9	97.0 ± 11.5
	100	108.7 ± 15.0	99.7 ± 20.4	105.7 ± 7.8	94.7 ± 10.1	96.7 ± 10.4	116.0 ± 13.1
i-cELISA	2000	85.3 ± 4.5	87.3 ± 6.5	82.7 ± 3.1	90.0 ± 7.0	93.0 ± 8.5	90.7 ± 8.2
	1000	90.0 ± 7.5	83.7 ± 1.5	89.0 ± 2.6	97.7 ± 4.9	98.3 ± 6.4	85.7 ± 7.9
	500	96.3 ± 6.0	91.7 ± 7.8	99.0 ± 7.0	101.3 ± 9.5	98.3 ± 10.8	90.0 ± 9.4
	200	96.0 ± 6.9	93.3 ± 5.5	99.7 ± 8.4	114.7 ± 3.5	103.7 ± 8.3	106.3 ± 8.3
	100	101.7 ± 4.2	111.0 ± 3.6	105.0 ± 11.4	118.3 ± 3.8	113.0 ± 6.9	109.3 ± 10.5

SUPPLEMENTARY DATA

Immunoassays for pyraclostrobin analysis in processed food products using novel monoclonal antibodies and QuEChERS-based extracts

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Fig. S1.

Fig. S2.

Fig. S3.

Fig. S4.