Development of Monoclonal Antibody-Based Competitive Immunoassays for the Detection of Picoxystrobin in Cereal and Oilseed Flours

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20 **Abstract**

21 Picoxystrobin is a new generation fungicide primarily developed to be used in cereal
22 crops. In the present study a novel collection of specific monoclonal antibodies has been
23 produced using different immunizing haptens based on a carboxy-functionalized identical
24 spacer arm attached to alternative positions of the target pesticide molecule. Two
25 competitive enzyme-linked immunosorbent assays have been developed employing
26 hapten heterology, one using the antibody-coated direct format and the other in the
27 conjugate-coated indirect format. Both immunoassays have been characterized in terms of
28 selectivity, solvent tolerance, and buffer conditions, affording similar limits of detection at
29 or below 0.1 µg/L. Finally, the optimized assays were applied to the analysis of
30 picoxystrobin in wheat, corn, oat, barley, and soybean flours. Average recovery values
31 from spiked samples were between 84 to 115%.

32

33 **Keywords**

34 ELISA, hapten, spacer arm, strobilurins, pesticide residues, food safety

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1. Introduction

The intensive and occasionally abusive use of agrochemicals for crop yield improvement often leads to the presence of residual amounts of pesticides in cereals, possibly affecting the quality and safety of the final food product ([Ruske, Gooding, & Dobrasczyk, 2004](#); [Dornez et al., 2008](#)). Picoxystrobin (PC) is a new broad-spectrum fungicide that belongs to the strobilurin family of pesticides and whose biological activity derives from the same β -methoxyacrylate toxophore group found in the natural active principle (strobilurin A) produced by the fungus *Strobilurus tenacellus* ([Clough, 1993](#)). The mechanism of action of PC is the inhibition of mitochondrial respiration by binding to the Q₀ site of cytochrome b, thus blocking electron transport between cytochrome b and cytochrome c₁ which eventually leads to disruption of the energy cycle ([Bartlett et al., 2002](#)). PC is effective against highly destructive pests in cereal crops such as *Septoria tritici*, *Leptosphaeria nodorum*, yellow rust, brown rust, ear diseases, and eyespot in wheat crops; net blotch, brown rust, powdery mildew, and *Rhynchosporium* in barley crops; crown rust and powdery mildew in oat crops; and sclerotinia in oilseed rape ([DuPont Global Website, 2011](#)). This strobilurin fungicide shows preventive and curative properties, and it is currently formulated and commercialized under different trademarks by DuPont and Syngenta for cereal and oilseed crop protection ([Bartlett, 2001](#); [Balba, 2007](#)).

According to EC regulation 396/2005, the maximum residue limit (MRL) for PC in most cereals and oilseeds is 50 $\mu\text{g/kg}$, whereas a specific MRL of 200 $\mu\text{g/kg}$ has been established for barley and oat ([European Commission, 2005](#)). At present, diverse analytical methodologies are available for the analysis of PC residues in foodstuffs, most of which are based on liquid or gas chromatography coupled to mass spectrometry detectors. Pesticide extraction is usually carried out with different organic solvents such as acetone ([Hiemstra & de Kok, 2007](#)), ethyl acetate ([Schurek et al., 2008](#); [Taylor, Keenan,](#)

61 [Reid, & Uría-Fernández, 2008](#)), or acetonitrile ([Walorczyk & Gnusowski, 2009](#)). In some
62 cases, extraction was achieved by ultrasonic treatment combined with different clean-up
63 approaches ([Bo, Wang, Guo, Qin, & Lu, 2008](#); [Campillo, Viñas, Aguinaga, Férez, &](#)
64 [Hernández-Córdoba, 2010](#); [Viñas, Martínez-Castillo, Campillo, & Hernández-Córdoba,](#)
65 [2010](#)), whereas the application of solid-phase microextraction resulted in a substantial
66 improvement in sensitivity for the determination of PC in baby foods ([Viñas, Campillo,](#)
67 [Martínez-Castillo, & Hernández-Cordoba, 2009](#)).

68 As complementary analytical tools to chromatographic methods, a large number of
69 studies based on enzyme-linked immunosorbent assay (ELISA) technology have been
70 published for the determination of a wide variety of agrochemical residues in diverse food
71 commodities ([Morozova, Levashova, & Eremin, 2005](#); [Van Emon, Chuang, Dill, & Xiong,](#)
72 [2008](#)). Immunochemical methods are simple, rapid, and sensitive. In addition, intricate
73 sample treatments are often not required, so direct dilution in an aqueous solution usually
74 suffices for the analysis of liquid samples or for extracts of solid samples. Thus, ELISAs
75 constitute not only a very useful routine laboratory approach where a large number of
76 repetitive analyses need to be performed, but also a means for screening for the presence
77 of specific compounds in a considerable assortment of samples. Moreover, since
78 immunoassays can be implemented in many different formats, they can be adapted to a
79 great variety of analytical circumstances. Nevertheless, the technique requires high-quality
80 immunoreagents together with extensive and thorough characterization and validation
81 studies.

82 Whilst ELISA is a well-established methodology for the detection and analysis of
83 certain chemical contaminants in cereals such as mycotoxins ([Skerritt, 1998](#); [Goryacheva,](#)
84 [Rusanova, Burmistrova, & De Saeger, 2009](#)), few studies have been published for the
85 determination of fungicide residues by enzyme immunoassay in those complex food

86 matrices (Danks, Chaudhry, Parker, Barker, & Banks, 2001; Jiang, Shi, Wu, & Wang,
87 2011). In previous publications, we described the syntheses of three PC derivatives
88 functionalized with the same linker at rationally-selected sites of the target molecule (Parra
89 et al., 2011) and the application of selected immunoassays to the analysis of PC residues
90 in beer (Esteve-Turrillas et al., 2010). In the present study, novel monoclonal antibodies
91 (mAb) to PC have been raised using regioisomeric PC haptens as immunogens. The
92 affinity-purified antibodies were characterized in two competitive ELISA (cELISA) formats,
93 the antibody-coated direct (d-cELISA) and the conjugate-coated indirect (i-cELISA)
94 assays. In order to improve the limit of detection (LOD) of the selected immunoassays, the
95 three available PC haptens were also evaluated as antigens in heterologous assays; i.e.,
96 assays employing conjugates carrying a different hapten to that employed for the
97 generation of the antibody. A direct and an indirect cELISA were characterized and
98 optimized for solvent tolerance and buffer conditions. Finally, both immunoassays were
99 validated for the determination of PC residues present in different cereal and oilseed flours
100 at concentration levels in accordance with the European MRLs for those foodstuffs.

101 **2. Materials and methods**

102 *2.1 Reagents and instrumentation*

103 Analytical-grade PC (methyl (E)-3-methoxy-2-{2-[6-(trifluoromethyl)-2-pyridyloxy
104 methyl]phenyl}acrylate, CAS Registry No. 117428-22-5, MW 367.32 g/mol) and other
105 employed pesticides were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or
106 Dr. Ehrenstorfer (Augsburg, Germany). All pesticide standards were prepared as
107 concentrated solutions in *N,N*-dimethylformamide (DMF) and were kept at -20 °C in amber
108 glass vials. Sephadex G-25 HiTrap Desalting columns and HiTrap Protein G HP columns
109 from GE Healthcare (Uppsala, Sweden) were used for conjugate and antibody purification,

110 respectively. Polyclonal rabbit anti-mouse immunoglobulins conjugated to peroxidase
111 (RAM–HRP) was from Dako (Glostrup, Denmark). Bovine serum albumin (BSA) fraction V
112 and Hybridoma Fusion and Cloning Supplement were purchased from Roche Applied
113 Science (Mannheim, Germany). HT (hypoxanthine–thymidine) and HAT (hypoxanthine–
114 aminopterin–thymidine) supplements and gentamicine solution were obtained from Gibco
115 BRL (Paisley, Scotland). Horseradish peroxidase (HRP), ovalbumin (OVA),
116 o-phenylenediamine, cell culture media, fetal bovine serum, polyethylene glycol, amino
117 acid solutions, Red Blood Cell Lysing Buffer Hybri-Max, and Freund’s adjuvants were from
118 Sigma-Aldrich (Madrid, Spain). P3-X63-Ag-8.653 mouse plasmacytoma cell line was from
119 the European Collection of Cell Cultures (Wiltshire, UK). Culture plastic ware and Costar
120 flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY).
121 ELISA plates were washed with an ELx405 microplate washer from BioTek Instruments
122 (Winooski, VT) and the absorbance values were read in dual wavelength mode (492–650
123 nm) with a PowerWave HT device, also from BioTek Instruments.

124 Composition, concentration, and pH of the employed buffers were: (i) PB, 100 mM
125 sodium phosphate buffer, pH 7.4; (ii) PBS, 10 mM sodium phosphate buffer, pH 7.4, with
126 140 mM NaCl; (iii) PBST, PBS containing 0.05% (v/v) Tween 20; (iv) 2×PBST, 20 mM
127 sodium phosphate, pH 7.4, with 280 mM NaCl and 0.05% (v/v) Tween 20; (v) CB, 50 mM
128 sodium carbonate–bicarbonate buffer, pH 9.6; (vi) Washing solution, 150 mM NaCl and
129 0.05% (v/v) Tween 20; (vii) Enzyme substrate buffer, 25 mM sodium citrate and 62 mM
130 sodium phosphate buffer, pH 5.4; and (viii) CitBT, 100 mM citrate buffer, pH 6.0, with 32
131 mM NaCl and 0.05% (v/v) Tween 20.

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134 2.2 *Protein–hapt en conjugates*

135 Three PC haptens (PCa6, PCb6, and PCo6) with the same linker at three
136 alternative tethering sites were employed in the present study ([Fig. 1](#)). Those
137 regioisomeric haptens contained a functional carboxylate group which was activated for
138 coupling to the free amine groups of the carrier proteins. The synthesis of the PC
139 derivatives and the preparation of the different conjugates were previously described
140 ([Parra et al., 2011](#); [Esteve-Turrillas et al, 2010](#)). BSA was used for immunogen
141 preparation, OVA for conjugate-coated indirect assays, and HRP as enzyme tracer in d-
142 cELISAs.

143 2.3 *Monoclonal antibody production*

144 Animal manipulation was performed according to the European Directive
145 2010/63/EU on the protection of animals used for scientific purposes. Animals were
146 immunized with BSA–PCb6 and BSA–PCo6 conjugates following equivalent procedures to
147 those used in a previous study where the immunogen was BSA–PCa6 ([Esteve-Turrillas et](#)
148 [al., 2010](#)). Briefly, sets of four mice each received three 200 µL intraperitoneal injections at
149 three week intervals. Injections consisted of a 1:1 emulsion of a 1 mg/mL conjugate
150 solution in PBS and Freund's adjuvant (complete for the first dose and incomplete for
151 subsequent ones). After a resting period of at least 3 weeks from the last injection with
152 adjuvant and four days before cell fusion, a booster injection in PBS was administered. For
153 hybridoma production, murine myeloma cells and spleenocytes from immunized mice were
154 fused using polyethylene glycol 1500. Next, hybrid cells were discriminated by employing
155 aminopterin-containing supplement. Then, they were cloned by limiting dilution and
156 cultured following published protocols ([Mercader & Abad-Fuentes, 2009](#)).

157 After cell fusion, a sequential double-screening strategy was applied in order to
158 identify hybridomas producing high-quality antibodies. First, culture supernatants from the
159 cell fusion experiment were screened by differential i-cELISA using plates coated with a
160 1000 ng/mL homologous OVA–hapten solution, as previously described ([Abad et al.,](#)
161 [1997](#)). Within each set of mice immunized with a common conjugate, a low selection
162 pressure (1000 nM PC as competitor) was applied in the screening of the first cell fusion,
163 whereas subsequent hybridizations were assayed at this stage with 100 nM PC. Second,
164 an additional screening was performed with the supernatants from those wells that
165 afforded saturated signals in the first step. Checkerboard competitive indirect assays were
166 carried out using microplates coated with 100 and 1000 ng/mL solutions of the
167 homologous conjugate, serial dilutions of the supernatant, and higher selection pressure
168 (lower PC concentrations, typically 5 and 50 nM) ([Mercader, Suárez-Pantaleón; Agulló,](#)
169 [Abad-Somovilla, & Abad-Fuentes, 2008](#)). Following subcloning of the selected cell lines,
170 immunoglobulins were purified from hybridoma culture supernatants by protein G affinity
171 chromatography, and the antibodies were stored at 4 °C as ammonium sulfate
172 precipitates. For daily usage, an aliquot was diluted in PBS containing 0.5% (w/v) BSA and
173 0.005% (w/v) thimerosal, and was kept cold in amber vials. The immunoglobulin isotype
174 was determined using the Mouse MonoAb-ID kit (HRP labeled) from Invitrogen (Carlsbad,
175 CA).

176 2.4 Direct cELISA procedure

177 Ninety-six-well polystyrene ELISA plates were coated with 100 µL per well of a
178 1000 ng/mL mAb solution in CB by overnight incubation at room temperature. Coated
179 plates were washed four times with washing solution. PC standard curves were prepared
180 in PBS as six-fold dilution series from a 0.9 g/L stock solution in DMF. The competitive
181 immunological reaction was performed with 50 µL per well of standard or sample plus 50

182 μL per well of HRP tracer in PBST. After 1 h at room temperature, plates were washed
183 again as described. Then, signal was produced by adding 100 μL per well of freshly
184 prepared 2 mg/mL o-phenylenediamine and 0.012% (v/v) H_2O_2 in enzyme substrate buffer.
185 Finally, the enzymatic reaction was stopped after 10 min at room temperature with 100 μL
186 per well of 2.5 M sulfuric acid. The absorbance was immediately read at 492 nm with a
187 reference wavelength at 650 nm.

188 2.5 *Indirect cELISA procedure*

189 Microplates were coated with 100 μL per well of a 100 or 1000 ng/mL OVA
190 conjugate solution in CB by overnight incubation at room temperature. PC standards were
191 prepared as described for the previous format. Coated plates were washed four times with
192 washing solution, and they received 50 μL per well of standard or sample in PBS plus 50
193 μL per well of mAb in PBST. After 1 h incubation at room temperature, plates were washed
194 again. Next, 100 μL per well of a 1/2000 dilution of RAM–HRP conjugate in PBST was
195 added, and plates were incubated an additional 1 h at room temperature. Finally, after
196 washing, signal was generated as described above for the direct cELISA procedure.

197 2.6 *Buffer studies*

198 Influence of Tween 20 concentration and buffer pH and ionic strength over the
199 curve parameters of the selected assays was evaluated following a multiparametric
200 approach ([Esteve-Turrillas et al., 2010](#)). Twenty buffers of different ionic strength, pH, and
201 detergent concentration were assayed. Briefly, a central composite design was carried out
202 consisting of a two-level full factorial design ($\alpha = 1.414$), with 3 factors and 3 replicates,
203 which involved 8 cube, 6 axial, and 6 center points. Buffer conditions were fixed using
204 NaCl, Tween 20, and three buffering systems: citrate ($\text{pK}_{\text{a}2} = 4.8$, $\text{pK}_{\text{a}3} = 6.4$), phosphate
205 ($\text{pK}_{\text{a}2} = 7.2$), and Tris ($\text{pK}_{\text{a}} = 8.1$). PC standard curves were prepared in water and they

206 were mixed with the tracer (for direct competitive assays) or the mAb (for indirect
207 competitive assays) diluted in every studied buffer. The responses using distinct conditions
208 were fitted by a multiple regression equation, including curvature and interaction terms,
209 using Minitab 14.1 software (Minitab Inc., State College, PA).

210 2.7 *Data treatment*

211 Raw or normalized absorbance values were fitted to a four-parameter logistic
212 equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL). Assay
213 sensitivity was estimated as the concentration value at the inflection point of the sigmoidal
214 curve, typically corresponding to the analyte concentration affording a 50% inhibition (IC_{50})
215 of the maximum absorbance (A_{max}). The LOD was estimated as the concentration of PC
216 that provided a 10% reduction of A_{max} , and cross-reactivity (CR) was calculated as the
217 percentage of the ratio between the IC_{50} value for PC and the IC_{50} value for the
218 corresponding pesticide (Fernández, Pinacho, Sánchez-Baeza, & Marco, 2011; Davies,
219 2005).

220 2.8 *Sample processing and analysis*

221 Wheat, corn, oat, barley, and soybean flours were purchased from local
222 supermarkets. Five grams of flour were weighed in 15-mL centrifuge tubes and extracted
223 with 10 mL of methanol using an ultrasonic bath for 10 minutes at room temperature.
224 Then, samples were centrifuged at 2200×g for 5 min, and 40 µL of methanolic extract was
225 diluted to 1 mL with Milli-Q water. For direct assays the tracer was diluted in CitBT, and for
226 indirect assays the mAb was diluted in 2×PBST.

227

228

229 3. Results and discussion

230 3.1 *Antigen and monoclonal antibody selection*

231 Following standardized procedures for the generation of hybridoma cell lines, a
232 collection of mAbs was eventually produced: four antibodies (PCa6#12, PCa6#13,
233 PCa6#15, and PCa6#21) using the immunogen of PCa6 ([Esteve-Turrillas et al., 2010](#)),
234 three (PCb6#11, PCb6#21, and PCb6#22) from hapten PCb6, and seven (PCo6#11,
235 PCo6#13, PCo6#14, PCo6#16, PCo6#17, PCo6#18, and PCo6#21) from hapten PCo6. All
236 of the available antibodies were of the IgG₁ isotype with κ light chains, except PCo6#14,
237 which was an IgG_{2a}(λ). In order to select the best assay in each ELISA format,
238 checkerboard competitive assays were performed by simultaneous evaluation of the
239 fourteen purified mAbs in combination with the protein conjugates of the three synthetic
240 haptens. The immobilized reagent (antibody or OVA conjugate for the direct or indirect
241 format, respectively) was prepared at 100 and 1000 ng/mL, whereas the assayed
242 concentrations of the reagent in solution (enzyme tracer or antibody for the direct or
243 indirect format, respectively) during the competitive reaction were 10, 30, 100, 300, and
244 1000 ng/mL. Thus, a set of 10 inhibition curves was obtained for every pair of
245 immunoreagents. For each antibody–antigen combination, [Table 1](#) lists the IC₅₀ and A_{max}
246 values from the best inhibition curve in each ELISA format. The lower asymptotes were
247 equivalent to the background in all cases. Remarkably, mAbs showing IC₅₀ values to PC
248 lower than 3 μ g/L were derived from every immunizing hapten, regardless of the linker
249 tethering site. Therefore, no hapten was shown to be inherently superior to others with
250 respect to its suitability for the generation of high-affinity mAbs to PC. It was noticed that in
251 the direct format most antibodies just recognized the homologous conjugate, whereas a
252 more permissive binding to antigen structure variations was observed in the indirect
253 format. For example, mAbs PCa6#12 and PCb6#21 bound all three OVA conjugates but

254 recognition was lost when heterologous tracers were used in the antibody-coated format.
255 Concerning assay antigens, conjugates of PCa6 were bound just by homologous-derived
256 antibodies, independently of the assay format, with the particular exception of mAb
257 PCb6#21 in the i-cELISA format. In contrast, OVA–PCo6 was generally recognized by
258 most of the antibodies, particularly in the i-cELISA format. The same result had been
259 previously observed with an equivalent OVA conjugate of the strobilurins azoxystrobin and
260 pyraclostrobin carrying the linker arm also at the methoxy group of the toxophore moiety
261 (Parra, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, submitted; Mercader, Agulló,
262 Abad-Somovilla, & Abad-Fuentes, 2011). Probably, the many degrees of freedom of the
263 toxophore moiety could explain the universal character as coating antigen of derivatives at
264 this tethering site.

265 The CR of the mAbs towards other strobilurins and relevant pesticides was also
266 assessed. The assayed compounds were: kresoxim-methyl, trifloxystrobin, pyraclostrobin,
267 dimoxystrobin, azoxystrobin, fluoxastrobin, metominostrobin, orysastrobin, procymidone,
268 boscalid, and fenhexamid. Calibration curves were prepared up to 2 μ M in PBS; higher
269 concentrations were not studied because of the poor water solubility of most pesticides.
270 Low CR values was generally found (below 0.1%), and in those cases that some inhibition
271 was observed with non-target compounds, the CR value was just slightly over 1%.

272 Several immunoassays with sensitivities in the low part-per-billion range were
273 identified and re-evaluated (Table 1S in Supplementary Data). For further optimization and
274 sample analysis, two assays were selected in different cELISA formats. First, a direct
275 homologous assay with mAb PCb6#21 was chosen because of its elevated signal and low
276 tracer consumption. Second, the assay with antibody PCa6#15 in combination with OVA–
277 PCb6 was also selected because it afforded a highly specific detection of PC in the i-
278 cELISA format.

279 3.2 Assay characterization

280 3.2.1 Solvent tolerance

281 Organic solvents such as acetone, acetonitrile, and methanol are usually employed
282 for pesticide extraction from food samples. As a consequence, small amounts of solvents
283 may be present during the immunological reaction which may affect the antibody–analyte
284 interaction, thus providing inaccurate results. PC standard curves were prepared in PBS
285 containing increasing amounts of acetone, acetonitrile, methanol, or ethanol.
286 Concentrations of the assayed solvents equal or lower than 5% resulted in variations of
287 the $A_{\max}$ values lower than 10% (results not shown). Concerning sensitivity, most solvents
288 clearly increased IC_{50} values of both assays. The presence of up to 5% methanol in the
289 standard curve resulted in an IC_{50} variation lower than 20%, making it the best tolerated
290 solvent (Fig. 2).

291 3.2.2 Buffer conditions

292 A multiparametric study was performed in order to evaluate the robustness and
293 stability of the selected assays upon changes in buffer properties, *i.e.*, ionic strength (I),
294 pH, and surfactant concentration. Assayed I values ranged from 50 to 300 mM, pH was
295 tested from 5.5 to 9.5, and the Tween 20 concentration was assayed from 0.00 to 0.05%
296 (v/v). Central point conditions were pH 7.5, I = 163 mM, and 0.025% Tween 20, which are
297 those of the assays described in the Materials and Methods section. The $A_{\max}$ and IC_{50}
298 values of each PC inhibition curve that was obtained using every buffer were fitted by a
299 multiple regression equation, including curvature and interaction terms. It was found that
300 the influence of the detergent concentration over the curve parameters was not relevant
301 (results not shown). Thus, a Tween 20 concentration of 0.025% in the assay was selected
302 in order to prevent unspecific interactions with the sample matrix.

303 Variations of pH and ionic strength resulted in dissimilar effects over the inhibition
304 curve parameters of the two studied ELISAs. Fig. 3 shows the surface plots depicting
305 changes of $A_{\max}$ and IC_{50} values as a function of buffer ionic strength and pH. In the case
306 of the d-cELISA, based on mAb PCb6#21, the $A_{\max}$ increased at higher ionic strength and
307 at low pH, whereas the IC_{50} had a maximum at the central point and it decreased at
308 extreme values. Consequently, phosphate buffer conditions were deemed suboptimal for
309 this ELISA, as results indicated that sensitivity could be enhanced using a buffer of higher
310 ionic strength and lower pH. Hence, a 300 mM citrate buffer, pH 6.0, with 0.025% Tween
311 20 (v/v) was proposed for further studies. As expected, when PC standard curves were run
312 in parallel under preliminary (phosphate) and optimized (citrate) conditions, a 10%
313 reduction of the IC_{50} value was achieved. Regarding the i-cELISA with mAb PCa6#15, the
314 $A_{\max}$ showed a maximum value close to the central point, and the IC_{50} was minimal with
315 ionic strengths from 180 to 240 mM at any pH. Therefore, this model predicted that
316 phosphate was an adequate buffer to obtain the best performance in this immunoassay.

317 Fig. 4 shows the inhibition curves obtained for both optimized ELISAs. The
318 d-cELISA was carried out with microplates coated using a 1000 ng/mL PCb6#21 solution
319 and a 50 ng/mL HRP-PCb6 solution in CitBT. The i-cELISA employed plates that had
320 been coated with a 1000 ng/mL OVA-PCb6 solution and 80 ng/mL of mAb PCa6#15 in
321 2xPBST. PC standards were prepared in Milli-Q water by serial dilution in both cases. The
322 theoretical LODs of the developed ELISAs were 0.10 $\mu\text{g/L}$ and 0.07 $\mu\text{g/L}$ for direct and
323 indirect assays, respectively. Low variability was found for both ELISAs; intra-assay
324 coefficients of variation (three replicates) of the analytical standards in the dynamic range
325 (20-80% signal) of the inhibition curves were below 4%, whereas inter-assay coefficients
326 of variation (three replicates) were below 6%.

327

328 3.3 Determination of picoxystrobin in flour samples

329 3.3.1 Matrix effects

330 Wheat, corn, oat, barley, and soybean flours were selected as relevant commodities
331 to evaluate the applicability of the proposed ELISAs. Methanol was chosen for extraction
332 because it was reasonably well-tolerated by the developed cELISAs (see above), and its
333 efficiency for the extraction of pesticides from wheat flour had been previously
334 demonstrated (Skerritt, Guihot, Hill, Desmarchelier, & Gore, 1996). Analytical procedures
335 for the determination of pesticide residues in food by cELISA often require a dilution step
336 in order to avoid solvent interferences and matrix effects. Flour methanolic extracts were
337 diluted with Milli-Q water and employed to prepare PC standard curves. The matrix
338 influence was evaluated independently for each of the five flour samples, and the five
339 curves for each dilution were averaged. Low dilutions (1/10) had variable effects over the
340 curve parameters depending on the flour type so the average curve had a large dispersion
341 (Fig. 1S in Supplementary Data). However, a 1/100 dilution of the flour extracts
342 significantly minimized matrix effects, thus resulting in inhibition curves essentially
343 overlaying that obtained in buffer.

344 3.3.2 Recovery studies

345 Flour samples were spiked with fixed amounts of PC and extracted with methanol,
346 diluted as described with Milli-Q water, and measured by the two developed cELISAs
347 under the optimized conditions. Unspiked flour extracts were also run in every experiment
348 as negative controls. PC determinations in oat and barley flours were accurate with both
349 cELISAs at concentrations covering by far the MRLs (200 µg/kg) for these foodstuffs
350 (Table 2). Regarding wheat, corn, and soybean flours, higher interferences were observed
351 with the direct ELISA at the MRL (50 µg/kg), but the recovery yields achieved with the

indirect assay were acceptable at this concentration. In general, the indirect assay afforded better recovery values at low PC concentrations than the direct assay. Average recovery values ranged from 101 to 115% with the d-cELISA and from 84 to 112% with the i-cELISA.

In summary, novel mAbs to PC were produced using regioisomeric synthetic haptens carrying the same spacer arm tethered to alternative positions of the target molecule. When employed as immunogens, each of those haptens allowed the generation of mAbs with good affinity and selectivity to PC. This collection of mAbs showed a more restrictive binding profile to heterologous conjugates than the polyclonal antibodies obtained in a previous study using the same haptens ([Parra, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2011](#)). In addition, two competitive immunoassays using either the antibody-coated d-cELISA or the conjugate-coated i-cELISA format were developed, with LODs at or below 0.1 µg/L. When the optimized assays were applied to methanolic extracts of wheat, corn, oat, barley, and soybean flours fortified with PC at concentrations in line with the European MRLs for this pesticide in those food commodities, satisfactory recoveries were obtained after a simple dilution in water, particularly with the indirect assay format.

Abbreviations

BSA, bovine serum albumin; cELISA, competitive enzyme-linked immunosorbent assay; CR, cross-reactivity; d-cELISA, direct cELISA; DMF, *N,N*-dimethylformamide; HRP, horseradish peroxidase; i-cELISA, indirect cELISA; LOD, limit of detection; mAb, monoclonal antibody; MRL, maximum residue limit; OVA, ovalbumin; PC, picoxystrobin; RAM–HRP, polyclonal rabbit anti-mouse immunoglobulins conjugated to peroxidase. For

376 buffer abbreviations and composition and for the definitions of $A_{\max}$ and IC_{50} see the
377 Materials and methods section.

378

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387 Limited amounts of the described immunoreagents are available upon request.

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495

496 **Figure captions**

497

498 **Fig. 1.** Structure of picoxystrobin and of the three regioisomeric synthetic haptens
499 employed for mAb production and assay development.

500 **Fig. 2.** Effect of solvent concentration on the sensitivity (IC_{50}) of the two selected ELISAs.
501 Methanol (circles), ethanol (triangles down), acetonitrile (squares), and acetone
502 (diamonds).

503 **Fig. 3.** Surface plots of the A_{max} and IC_{50} values as a function of buffer pH and ionic
504 strength (I) for the d-cELISA and the i-cELISA.

505 **Fig. 4.** Picoxystrobin standard curves obtained with the optimized cELISAs. Values are the
506 mean of three independent experiments. The homologous direct assay with mAb PCb6#21
507 (triangles up) afforded an assay whose slope and IC_{50} values were -1.06 ± 0.02 and 0.86
508 ± 0.03 $\mu\text{g/L}$, respectively, whereas the indirect assay using mAb PCa6#15 and OVA–PCb6
509 (circles) had a slope of -1.08 ± 0.03 and an IC_{50} value of 0.94 ± 0.03 $\mu\text{g/L}$. The A_{max}
510 values were between 1.0 and 1.5.

511

Table 1

Figures of merit from the most sensitive immunoreagent combinations as evaluated by checkerboard direct and indirect competitive experiments.

mAb	Direct assay						Indirect assay					
	HRP-PCa6		HRP-PCb6		HRP-PCo6		OVA-PCa6		OVA-PCb6		OVA-PCo6	
	$A_{\max}$	IC_{50} ($\mu\text{g/L}$)	$A_{\max}$	IC_{50} ($\mu\text{g/L}$)	$A_{\max}$	IC_{50} ($\mu\text{g/L}$)	$A_{\max}$	IC_{50} ($\mu\text{g/L}$)	$A_{\max}$	IC_{50} ($\mu\text{g/L}$)	$A_{\max}$	IC_{50} ($\mu\text{g/L}$)
PCa6#12	2.31	5.15	- ^a	-	-	-	1.20	2.22	0.69	2.28	1.18	3.18
PCa6#13	1.61	2.24	-	-	1.23	3.42	1.78	1.38	-	-	1.55	1.77
PCa6#15	2.05	2.32	1.23	0.89	-	-	1.55	2.62	1.70	1.03	-	-
PCa6#21	1.14	7.86	1.44	0.83	-	-	1.18	0.89	1.94	1.05	1.10	0.94
PCb6#11	-	-	1.64	1.52	-	-	-	-	1.72	3.13	1.14	0.88
PCb6#21	-	-	1.90	0.94	-	-	1.80	3.54	2.11	1.17	1.41	2.62
PCb6#22	-	-	2.02	3.33	-	-	-	-	1.67	1.51	-	-
PCo6#11	-	-	-	-	1.04	2.76	-	-	-	-	0.98	1.41
PCo6#13	-	-	-	-	1.12	2.00	-	-	-	-	0.94	1.13
PCo6#14	-	-	-	-	1.13	3.20	-	-	-	-	1.36	4.25
PCo6#16	-	-	0.59	4.26	-	-	-	-	0.83	2.57	1.52	6.25
PCo6#17	-	-	-	-	-	-	-	-	-	-	2.39	4.80
PCo6#18	-	-	-	-	-	-	-	-	-	-	1.29	10.87
PCo6#21	-	-	1.50	1.08	1.23	2.54	-	-	1.16	1.75	1.59	2.01

^a The highest $A_{\max}$ observed for these combinations was below 0.5.

Table 2

Recovery values obtained with the proposed immunoassays from flour extracts spiked with picoxystrobin.

assay	[PC] in the flour ($\mu\text{g/kg}$)	recovery (% $\pm$ s, n=3)				
		wheat	corn	oat	barley	soybean
d-cELISA using	25	- ^a	-	-	-	-
mAb PCb6#21 and	50	127 $\pm$ 32	139 $\pm$ 10	136 $\pm$ 2	140 $\pm$ 5	139 $\pm$ 6
HRP-PCb6	100	130 $\pm$ 9	126 $\pm$ 6	121 $\pm$ 5	120 $\pm$ 1	105 $\pm$ 3
	250	109 $\pm$ 11	106 $\pm$ 4	98 $\pm$ 6	101 $\pm$ 5	93 $\pm$ 4
	500	104 $\pm$ 3	107 $\pm$ 7	100 $\pm$ 5	95 $\pm$ 3	91 $\pm$ 4
	1000	99 $\pm$ 6	98 $\pm$ 5	85 $\pm$ 6	85 $\pm$ 8	77 $\pm$ 7
	Average	114 $\pm$ 14	115 $\pm$ 17	108 $\pm$ 20	108 $\pm$ 22	101 $\pm$ 23
i-cELISA using	25	101 $\pm$ 9	99 $\pm$ 9	74 $\pm$ 25	120 $\pm$ 35	134 $\pm$ 9
mAb PCa6#15 and	50	127 $\pm$ 15	121 $\pm$ 11	88 $\pm$ 9	106 $\pm$ 26	122 $\pm$ 11
OVA-PCb6	100	110 $\pm$ 4	104 $\pm$ 13	93 $\pm$ 8	107 $\pm$ 13	113 $\pm$ 6
	250	112 $\pm$ 3	110 $\pm$ 8	86 $\pm$ 10	107 $\pm$ 6	92 $\pm$ 10
	500	118 $\pm$ 7	118 $\pm$ 8	80 $\pm$ 3	101 $\pm$ 7	89 $\pm$ 13
	1000	105 $\pm$ 5	98 $\pm$ 6	80 $\pm$ 25	96 $\pm$ 11	73 $\pm$ 9
	Average	112 $\pm$ 9	108 $\pm$ 10	84 $\pm$ 7	106 $\pm$ 8	104 $\pm$ 23

^a Below assay detectability.

Fig. 1. Mercader et al.

Fig. 2. Mercader et al.

d-cELISA with mAb PCb6#21

i-cELISA with mAb PCa6#15

Fig. 3. Mercader et al.

Fig. 4. Mercader et al.