Site-heterologous haptens and competitive monoclonal antibody-based immunoassays for pyrimethanil residue analysis in foodstuffs

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ABSTRACT

Pyrimethanil is a widely used post-harvest fungicide whose residues are commonly found in fruits and vegetables. We herein report the synthesis of two new functionalized derivatives of pyrimethanil carrying an equivalent spacer arm at different positions of the molecule. The influence of the linker tethering site on the affinity of monoclonal antibodies was shown. Moreover, the development of competitive immunoassays in different formats and with high sensitivity to the target analyte – IC₅₀ values below 0.3 µg/L – is described. Optimized assays were characterized by the determination of the limit of quantification, trueness, and precision using water-diluted QuEChERS extracts of fruits and vegetables fortified with pyrimethanil. Finally, in-field treated and blind spiked strawberry, tomato, and cucumber samples were analyzed by the developed immunoassays and a reference chromatographic method. Using Deming regression analysis, we show that results from both methodologies were statistically comparable.

Chemical compounds studied in this article

Pyrimethanil (PubChem CID: 91650); Cyprodinil (PubChem CID: 86367); Mepanipyrim (PubChem CID: 86296).

Keywords: Anilinopyrimidine fungicides; ELISA; Food safety; Pesticide residues; Regioisomeric haptens

1. Introduction

Pesticides are widely used to protect crops against pest infestation. Since residues remain on food products at a major or lesser extent, governmental pesticide control programs are implemented each year, such as those performed by the European Food Safety Authority (EFSA) or the United States Environmental Protection Agency (US EPA). In those programs, it has been observed that consumers are particularly exposed to fungicides, probably due to extensive postharvest or near-harvest application of these compounds. Pyrimethanil (4,6-dimethyl-*N*-phenyl-2-pyrimidinamine) is a systemic anilinopyrimidine fungicide ([Fig. 1a](#)) commonly used in treatments for controlling the grey mold *Botrytis cinerea* on fruits, vegetables, and ornamentals. Pyrimethanil was approved as active substance for crop protection in the EU in 2006 ([European Commission, 2006](#)), and residues of this fungicide have been frequently found in food samples ever since. The 2011 EFSA report pointed out that the percentage of surveillance samples with detectable residues of pyrimethanil was 5.8% for fruits and nuts, whereas a lower incidence (1.1%) was found in vegetables ([EFSA, 2014](#)). The EU maximum residue limits (MRLs) for this fungicide have been set at 1 to 10 mg/kg for most food products ([EU Pesticides Database](#)).

The most frequently employed techniques for pesticide residue determination in food are based on liquid (LC) or gas chromatography (GC) preferentially coupled with tandem mass spectrometry (MS–MS). Nevertheless, these methodologies are not commonly available in resource-limited settings. Immunochemical methods such as the enzyme-linked immunosorbent assay (ELISA) are spreading for rapid, simple, and high-throughput analysis of particular pesticides in complex food matrices ([Morozova, Levashova, & Eremin, 2005](#)). Immunoassays are an efficient tool to rapidly screen hundreds of samples with a simple test. In the last decade, numerous immunoassays have been validated for the analysis of agrochemicals in food, including insecticides ([Cui et al., 2014; Watanabe & Miyake, 2013](#)), herbicides ([Sanchis et al., 2012; Sharma, Kukkar, Ganguli, Bhasin, & Suri, 2013](#)), fungicides ([Esteve-Turrillas, Abad-Fuentes, & Mercader, 2011; Esteve-Turrillas, Agulló, Abad-Fuentes, Abad-Somovilla, & Mercader, 2012](#)), and plant-growth regulators ([Suárez-Pantaleón et al., 2012](#)). Two years ago, a competitive ELISA (cELISA) for pyrimethanil analysis was reported by our group ([Mercader, Esteve-Turrillas, Agulló, Abad-Somovilla, &](#)

Abad-Fuentes, 2012). In that study, a functionalized derivative of pyrimethanil was synthesized and polyclonal antibodies (pAb) to this fungicide were generated. The aim of the present study was to increase the sensitivity and performance of the method by using rationally designed novel haptens in order to produce monoclonal antibodies (mAb) of superior affinity. These immunoreagents were characterized by antibody-coated direct and conjugate-coated indirect cELISA, and a procedure for the determination of pyrimethanil residues in fruit and vegetables has been optimized, characterized, and compared with a reference chromatographic method.

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2. Experimental

2.1. Reagents and instrumentation

Pyrimethanil and the employed pesticide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Pesticide stock solutions were prepared in *N,N*-dimethylformamide and kept at -20°C in amber glass vials. Primary–secondary amine (PSA) for dispersive solid phase extraction clean-up was from Scharlab (Barcelona, Spain). Triphenylphosphate (TPP), horseradish peroxidase (HRP), ovalbumin (OVA), and *o*-phenylenediamine were purchased from Sigma/Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting and HiTrap Protein G HP columns from GE Healthcare (Uppsala, Sweden) were used for conjugate and antibody purification, respectively. HRP-labeled rabbit pAbs against mouse immunoglobulins (RAM–HRP) were from Dako (Glostrup, Denmark). Bovine serum albumin (BSA) fraction V was purchased from Roche Applied Science (Mannheim, Germany). Culture plastic ware and Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Ultraviolet–visible (UV–vis) spectra and ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments.

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The composition, concentration, and pH of the employed buffers were: i) PB, 100 mmol/L sodium phosphate buffer (pH 7.4); ii) PBS, 10 mmol/L sodium phosphate buffer (pH 7.4) with 140 mmol/L NaCl; iii) PBST, PBS containing 0.05% (v/v) Tween 20; iv) CB, 50 mmol/L carbonate–bicarbonate buffer (pH 9.6); v)

Washing solution, 15 mmol/L NaCl and 0.05% (v/v) Tween 20; vi) Enzyme buffer, 25 mmol/L citrate and 62
95 mmol/L sodium phosphate buffer (pH 5.4).

2.2. Hapten synthesis

Hapten PM_n was prepared directly from pyrimethanil and hapten PM_m was synthesized from
1-iodo-3-nitrobenzene and 2-chloro-4,6-dimethylpyrimidine, as detailed in Fig. 2. Carboxylate groups in
100 both haptens were transformed into their corresponding *N*-succinimidyl esters using *N,N'*-disuccinimidyl
carbonate for further coupling to proteins, as previously published for hapten PM_p (Mercader et al., 2012)
(Fig. 1). Complete experimental details for the preparation of these haptens, full spectrometric
characterization data, and copies of the NMR spectra are given in the Supplementary Data file.

105 2.3. Preparation of protein–hapten conjugates

Purified active esters of the newly synthesized haptens (PM_n and PM_m) were conjugated to three
carrier proteins – BSA for immunizing conjugates, HRP for direct assays, and OVA for indirect assays –
following standard procedures commonly used in our laboratory (Parra, Mercader, Agulló, Abad-Fuentes, &
Abad-Somovilla, 2011). Preparation of PM_p conjugates was previously described (Mercader et al., 2012).
110 Conjugates were purified by size exclusion chromatography, and the degree of conjugation was estimated
by differential absorbance measurements at 280 nm. For details see the Supplementary Data file.

2.4. Monoclonal antibody generation

Animal manipulation was approved by the Ethics Committee of the Universitat de València and it
115 was carried out in compliance with Spanish laws and guidelines (RD1201/2005 and law 32/2007) and
according to the European Directive 2010/63/EU concerning the protection of animals used for scientific
purposes

Three BALB/c female mice were immunized with each BSA conjugate (PM_p, PM_m, and PM_n) by
intraperitoneal injections. Monoclonal antibodies were produced from immunized animals using standard
120 hybridoma technology. A double screening analysis of cell culture supernatants was conducted by indirect

cELISA (Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008). The first screening consisted of parallel evaluation of supernatants in microtiter wells containing 100 nmol/L pyrimethanil in PBS (test well) or PBS alone (control well). The second screening was done by checkerboard evaluation of hybridoma cultures, consisting of several supernatant dilutions, several assay conjugate concentrations, and a standard pyrimethanil curve. Detailed procedures for immunization, cell fusion, hybridoma screening and cloning, and antibody purification and isotyping are described in the Supporting Information file. Antibodies were stored at 4 °C as ammonium sulfate precipitates. For daily usage, an aliquot was diluted 1/2 with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal, and kept at 4 °C.

2.5. Competitive ELISA procedures

2.5.1. Antibody-coated direct format

Ninety-six-well polystyrene ELISA plates were coated with 100 µL of antibody solution in CB by overnight incubation at room temperature. Plates were washed four times with washing solution and each coated well received 50 µL of analyte solution in PBS plus 50 µL of HRP tracer solution in PBST. For sample analysis, extracts were conveniently diluted with deionized water and the enzyme tracer solution was prepared in PBST-2x. The competitive reaction was carried out at room temperature for one hour. After washing plates as described above, color was developed by addition of 100 µL per well of freshly prepared 2 mg/mL o-phenylenediamine and 0.012% (v/v) hydrogen peroxide in enzyme buffer. The enzymatic reaction was stopped after 10 min at room temperature with 100 µL per well of 1 mol/L sulfuric acid. Absorbances were immediately read at 492 nm with a reference wavelength at 650 nm.

2.5.2. Conjugate-coated indirect format

Microplates were coated with 100 µL per well of OVA conjugate solution in CB by overnight incubation at room temperature. After plate washing, wells received 50 µL of analyte solution in PBS plus 50 µL of antibody solution in PBST. Sample analysis was performed using diluted extracts in deionized water and the antibody solution in PBST-2x. The competitive reaction was carried out at room temperature for

one hour. Next, plates were washed again and 100 μ L per well of secondary antibody (RAM–HRP conjugate diluted 1/2000 in PBST) was added. Following incubation for 1 h at room temperature and washing, the signal was produced and read as for the antibody-coated direct format.

2.5.3. Data analysis

Eight-point standard curves, including a blank, were prepared from a 2 mg/mL pyrimethanil stock solution by serial dilution in PBS. Experimental values were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay sensitivity was estimated as the concentration of analyte at the inflection point (IC_{50}) of the resulting sigmoidal curve, and A_{max} was the upper asymptote of the curve.

2.6. Food sample treatment and extraction

Strawberry, tomato, and cucumber plants were grown in a greenhouse and they were in-field treated with Scala 400 SC (BASF Crop Protection, Limburgerhof, Germany) at the dose recommended by the manufacturer (2.0 L/ha). In order to obtain samples with different contents of pyrimethanil residues, samples were collected at different times (from 1 to 14 days after the application of the agrochemical) and short-term stored at 4 °C. Samples were chopped, homogenized using a T-25 Ultra-Turrax blender from IKA (Staufen, Germany), and stored in polypropylene tubes at –20 °C until analysis.

The QuEChERS method was employed for extraction and purification of fungicide residues from food samples (Lehotay, 2007). Homogenized samples (5 g), plus 50 μ L of internal standard (TPP, 50 mg/L), were introduced in 50 mL polypropylene centrifuge tubes containing 0.5 g of sodium acetate and 2.0 g of anhydrous magnesium sulfate. Samples were then extracted with 5 mL of 1% (v/v) acetic acid in acetonitrile by vortexing 1 min, and centrifuged for 5 min at 2200 g. The 1ml extracts were then cleaned-up with 50 mg of PSA and 150 mg of anhydrous magnesium sulfate, vortexed 1 min, and centrifuged 5 min at 2200 g. Purified extracts were then filtered through a 0.22- μ m Teflon filter and analyzed by the optimized cELISAs and ultra performance liquid chromatography coupled to a triple quadrupole mass spectrometer (UPLC–MS–MS).

2.7. Chromatographic analysis

175 UPLC–MS–MS was selected as reference methodology in order to obtain maximum sensitivity and selectivity in the analysis of pyrimethanil-treated samples. UPLC was performed on a Waters Acquity UPLC system from Waters (Milford, MA, USA), equipped with a binary solvent delivery system, an auto sampler, and a BEH C₁₈ (1.7 μ m, 2.1 x 50 mm) column. The injection volume was 5 μ L and the mobile phase consisted of 0.5% (v/v) formic acid in water (A) and acetonitrile (B). The elution gradient started at 30% of B at a flow-
180 rate of 350 μ L/min, and then B was linearly increased to 85% in 3.4 min and to 98% in 1.7 min. The obtained retention times, using the aforementioned conditions, were 1.3 and 3.8 min for pyrimethanil and TPP, respectively. Tandem mass acquisitions were performed in a Waters Acquity triple quadrupole mass spectrometry detector, equipped with a Z-spray electrospray ionization source, with 3.5 KV capillary voltage, at 120 °C as source temperature, and 300 °C as desolvation temperature. The employed
185 parameters were: ESI+, parent ion 199.9 m/z, daughter ions 82.1 and 107.1 m/z, 30 eV collision energy, and 20 V cone energy for pyrimethanil; and ESI+, parent ion 328.3 m/z, daughter ions 77.0 and 152.4 m/z, 30 eV collision energy, and 25 V cone energy for TPP.

3. Results and Discussion

190 3.1. Hapten synthesis and conjugation

The previously reported pyrimethanil derivative PM*p* hold the linker at the *para* position of the aniline aromatic moiety. The two new haptens described here could be considered regioisomers of that derivative; they have a similar (PM*n*) or identical (PM*p*) carboxylated spacer arm at different positions of the pyrimethanil framework. In hapten PM*n* a similar C5 carboxylated alkyl chain is bonded at the
195 secondary bridged nitrogen atom, while in hapten PM*m* a C6-length chain is attached to the meta position of the phenyl ring, thus keeping both aromatic rings essentially unmodified (Fig. 1b). The synthesis of both haptens was quite straightforward and efficient. Thus, the synthesis of hapten PM*n* was carried out directly from pyrimethanil in two consecutive steps; first, alkylation reaction with methyl 5-bromopentanoate of the amide anion derived from the reaction of pyrimethanil with NaH, followed by basic methyl ester

200 hydrolysis and acidic workup (Fig. 2, upper). This two-step sequence allowed the preparation of hapten PMn in 60% overall yield (93% based on recovered unreacted pyrimethanil). On the other hand, the preparation of hapten PMm involved a single step – an aromatic nucleophilic substitution reaction between 4,6-dimethylpyrimidine (**1**) and 6-(3-aminophenyl)hexanoic acid (Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2010) – that took place quite efficiently by simple heating of both reactants in
205 dioxane at reflux (Fig. 2, lower).

Conjugates of haptens PMp, PMm, and PMn were prepared by the active ester method using *N,N'*-disuccinimidyl carbonate, which allowed a straightforward purification of the *N*-succinimidyl derivative. Thus, since no undesirable secondary reactions may occur during the conjugation, immunizing and assay conjugates were prepared, with high yields, by the same procedure. The obtained hapten-to-protein molar
210 ratios for the three sets of BSA, OVA, and HRP conjugates were 13, 7, and 3 for PMp; 16, 7, and 3 for PMm; and 8, 4, and 1 for PMn, respectively. These results were as expected and these values were consistent for those carriers across multiple conjugations.

3.2. Production and characterization of monoclonal antibodies

215 Immunogens of all three haptens (PMn, PMm, and PMp) were employed for mice immunization, and three cell fusion experiments were carried out with mice challenged with each BSA-hapten bioconjugate. Among hybridomas secreting antibodies able to recognize the homologous assay conjugate, 6 cell lines that tightly bound pyrimethanil in solution were identified and eventually stabilized; 4 were from mice immunized with BSA–PMm and 2 were from mice immunized with BSA–PMp. Four antibodies
220 were of the IgG₁ isotype, whereas mAbs PMp#22 and PMm#22 were IgG_{2b} and IgG_{2a} immunoglobulins, respectively. All of them contained κ -type light chains.

Remarkably, antibodies able to significantly bind pyrimethanil in solution were not identified from mice immunized with the conjugate BSA–PMn despite animals immunized with this conjugate showed titers equivalent to those exhibited by mice immunized with BSA–PMp and BSA–PMm, and also in spite of
225 numerous hybridomas able to efficiently recognize the coating conjugate OVA–PMn were found from PMn-

immunized mice. This result underlines the importance of the spacer arm location in the generation of high-affinity antibodies to pyrimethanil, and confirms previous findings observed with polyclonal antibodies against the two other fungicides of the anilinopyrimidine family (Fig. 1), *i.e.*, cyprodinil and mepaniprim (Esteve-Turrillas et al., 2012; Esteve-Turrillas, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2013).

230 The affinity of the generated mAbs was characterized by checkerboard competitive analysis. For direct assays, plates were coated with 1000 µg/L mAb solution and a range of tracer conjugate concentrations (from 1 to 300 µg/L) was evaluated under competitive conditions using a pyrimethanil standard curve from 0.002 to 2000 µg/L plus a blank. For indirect assays, plates were coated with the OVA conjugate solution at 10, 100, and 1000 µg/L, and the competitive step was carried out using a range of
235 mAb solutions (concentrations from 1 to 300 µg/L). Assay heterology was also evaluated using either PM_p or PM_m conjugates in both cELISA formats – initial studies showed that mAbs were not able to bind PM_n-based assay conjugates. With this approach, a set of inhibition curves was retrieved for each mAb/assay conjugate pair. Table 1 lists the immunoreagent concentrations and the parameters of the best inhibition curve – the curve simultaneously affording the A_{max} value nearest to 1.0 and the lowest IC₅₀ value – for
240 every antibody/conjugate combination. Almost all of the antibodies provided IC₅₀ values below 1 µg/L. The highest sensitivity in both cELISA formats was afforded by mAb PM_m#31, which showed excellent IC₅₀ values near 0.2 µg/L. Moreover, the A_{max} values in both cELISA formats and the IC₅₀ value in the indirect assay were improved when this antibody was used in combination with the heterologous conjugate (HRP-PM_p or OVA-PM_p).

245 A number of post-harvest or anti-botrytis agrochemicals currently in use in common agricultural practices were tested for recognition by mAbs. Assayed crop protection compounds were: cyprodinil, mepaniprim, kresoxim-methyl, trifloxystrobin, pyraclostrobin, azoxystrobin, dimoxystrobin, fluoxastrobin, metominostrobin, picoxystrobin, fenhexamid, captan, procymidone, tolylfluanid, cyazofamid, tebuconazole, fenamidone, fludioxonil, vinclozolin, imidacloprid, boscalid, and benzanilide. Standard curves
250 were prepared for each fungicide (from 0.01 to 10 µmol/L in PBS) and binding was determined by direct cELISA for every antibody. CR was calculated according to Equation 1.

$$CR (\%) = \frac{IC_{50}^{pyrimethanil}}{IC_{50}^{compound}} \times 100 \quad (\text{Equation 1})$$

As expected, only cyprodinil and mepanipyrim, the two other members of the anilinopyrimidine family of fungicides and therefore closely structurally related to pyrimethanil, were significantly recognized.

CR values ranged from 3% to 12% for mepanipyrim, whereas cyprodinil was mostly recognized similarly to pyrimethanil so, unexpectedly, the rather voluminous cyclopropane group in cyprodinil seems not to greatly interfere with antibody binding. Anyhow, from an analytical point of view, encountering more than one anilinopyrimidine fungicide in the same sample is not likely because they share the same mode of action.

3.3. Assay selection

Assay optimization and validation was carried out with mAb PMm#31 and the heterologous HRP-PMp and OVA-PMp conjugates, which provided the most sensitive assays in direct and indirect cELISA formats, respectively. Immunoreagent concentrations were adjusted in order to obtain an A_{max} value between 1.0 and 1.5. Table 2 shows the inhibition curve parameters of the selected assays – calculated as the average results from three independent experiments. The limit of detection (LOD) – the concentration of pyrimethanil that provided a 10% reduction of A_{max} (IC_{10}) – was 0.05 µg/L and 0.04 µg/L for the direct and the indirect cELISA, respectively. Intra-day and inter-day curve precision values – established as the relative standard deviation (RSD) of A_{max} and IC_{50} values of replicate analyses performed on three consecutive days (3 curves per day and 3 replicates per curve) – were in both cases satisfactory, with RSD values lower than 20%.

3.4. Matrix effects

Strawberries, tomatoes, and cucumbers were selected as representative matrices in order to evaluate possible interferences over the assay parameters. Pyrimethanil-free food samples were extracted by the QuEChERS methodology (AOAC Official Method 2007.01), a widely applied approach for pesticide

residue extraction from fruits and vegetables ([Lehotay, 2007](#)). Acetonitrile extracts were diluted in water as the easiest strategy to reduce matrix interferences. Pyrimethanil standard curves (from 100 µg/L, 6-fold dilution factor) were prepared in QuEChERS extracts diluted 1/5, 1/15, 1/50, 1/150, and 1/500 in deionized water. In addition, a standard curve was prepared with no extract as control. [Fig. 3](#) shows the variation of $A_{\max}$ and IC_{50} values of the resulting inhibition curves for strawberry, tomato, and cucumber samples. Deionized water was also extracted by the QuEChERS procedure and employed as reference. Regarding $A_{\max}$ values, the indirect assay was more robust, showing little variations at lower dilutions, whereas the strongest matrix effect was observed with strawberry extracts in the direct assay. Changes in IC_{50} values were more similar in both assays, and they remained almost constant for extract dilutions higher than 1/50. Thus, matrix effects could be easily avoided with a 50-fold dilution in water for tomatoes and cucumbers, but a higher dilution of strawberry extracts (at least 500-fold) would be required in order to reduce matrix interferences.

3.5. Recovery studies

Fungicide-free strawberry, tomato, and cucumber samples were chopped, homogenized, and fortified with pyrimethanil – at 5, 10, 50, 100, 500, 1000, and 5000 µg/kg – using few microliters of concentrated stock solution in acetonitrile and homogenizing by vortex. Spiked samples were extracted by QuEChERS and analyzed by the developed direct and indirect cELISAs. The limit of quantification (LOQ) for pyrimethanil residues was estimated as the minimum evaluated concentration that afforded recovery values comprised between 80 and 130%. Quantitative recoveries were obtained for tomato and cucumber spiked samples at all of the evaluated levels using a 1/50 or a 1/500 sample dilution, so a LOQ of 5 µg/kg was defined for those samples in both immunoassays ([Table 3](#)). Regarding strawberries, since a 500-fold sample dilution was required, the obtained LOQs were 100 and 50 µg/kg for the direct and the indirect cELISA, respectively. Considering that the MRLs established by the EU legislation are 5000, 1000, and 1000 µg/kg for strawberries, tomatoes, and cucumbers, respectively ([EU Pesticides Database](#)), the proposed immunoassays could be suitable for the determination of pyrimethanil residues in these food commodities.

3.6. Comparison with UPLC–MS–MS

Strawberry, tomato, and cucumber crops were treated with a pyrimethanil-based commercial product as indicated by the manufacturer. In-field treated samples were extracted by the QuEChERS methodology and the final extract was measured by the proposed cELISAs and also by UPLC–MS–MS. As shown in [Table S1](#) in the Supporting Information file, pyrimethanil concentrations ranged from 1100 to 7600 µg/kg for strawberries, from 28 to 1000 µg/kg for tomatoes, and from 8 to 2600 µg/kg for cucumbers.

In order to statistically contrast analytical data from cELISA and chromatography, a higher number of samples and a wider range of analyte concentrations are required. Thus, blind-spiked samples were prepared. A total of eight samples per matrix were spiked by an external operator, extracted by QuEChERS, and measured by UPLC–MS–MS and the two developed immunoassays ([Table S1](#) of the Supplementary Data file). For comparison of results, Deming regression was employed, which appeared a suitable approach for this aim because it takes into account the standard deviations of both sets of measurements.

[Fig. 4](#) shows the obtained Deming regression curves for direct and indirect cELISAs with the corresponding 95% confidence interval (CI) bands. The comparison of the direct immunoassay with UPLC–MS–MS produced a Deming regression slope of 0.99 (CI from 0.96 to 1.03) and an intercept of –0.2 (CI from –1.5 to 1.1). For the indirect assay, a slope of 1.00 (CI from 0.96 to 1.03) and an intercept of –0.8 (CI from –2.9 to 1.3) was found. Therefore, the obtained Deming curves had slopes and intercepts statistically equal to 1 and 0, respectively, so we could conclude that the two developed cELISAs provided fully equivalent results to those obtained by the reference chromatographic procedure.

4. Conclusions

We have designed and synthesized two new functionalized molecules for the generation of antibodies to the fungicide pyrimethanil. Haptens with the linker at *meta* or *para* positions of the aniline ring of pyrimethanil (haptens PM*m* and PM*p*) provided antibodies with higher affinity than those resulting from the derivative with the spacer arm at a central position (hapten PM*n*). Two cELISAs in different

formats have been developed with a significant improvement in assay sensitivity – IC₅₀ values lower than
330 0.3 µg/L –, as compared to previously reported immunoassays. Optimized assays were validated for the
analysis of pyrimethanil residues in fruits and vegetables, affording limits of quantification well below the
corresponding European MRLs. Finally, in-field treated and blind-spiked strawberry, tomato, and cucumber
samples were extracted following the QuEChERS procedure and analyzed by the proposed cELISAs, and
statistically comparable results to those obtained using UPLC–MS–MS were found.

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Immunoreagents described in this article are available for evaluation and research purposes.

Appendix A

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Supplementary data to this article can be found at.....

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Figure 1. Molecular structures of the three anilinopyrimidine fungicides (a) and of the three haptens used for the generation of monoclonal antibodies and assay development (b).

410 **Figure 2.** Synthesis pathway of haptens PM*m* and PM*n*.

Figure 3. Variation of maximum absorbance ($A_{\max}$) and IC_{50} values of the competitive assays caused by strawberry (triangles), tomato (squares), and cucumber (diamonds) QuEChERS extracts. Water (circles) was used as control. Food extracts were diluted in water as indicated in the graphs and the resulting solutions
415 were used to prepare pyrimethanil standard curves. It can be clearly appreciated that the indirect immunoassay is less prone to suffer from matrix interferences than the direct format, in particular concerning variations in the maximum signal.

Figure 4. Comparative study between the developed direct and indirect cELISAs and UPLC–MS–MS using in-
420 field treated and blind-spiked samples. Deming regression (black line) is clearly within boundaries corresponding to 95% confidence interval (blue lines), thus proving that both immunoassays provided analytical results comparable to those afforded by the chromatographic method.

Table 1

Assay parameters obtained from checkerboard characterization of mAbs.

mAb	Direct cELISA						Indirect cELISA					
	[mAb] ($\mu\text{g/L}$)	HRP hapten	[T] ^a ($\mu\text{g/L}$)	A _{max}	Slope	IC ₅₀ ($\mu\text{g/L}$)	[mAb] ($\mu\text{g/L}$)	OVA hapten	[C] ^b ($\mu\text{g/L}$)	A _{max}	Slope	IC ₅₀ ($\mu\text{g/L}$)
PMp#11	1000	PMp	10	1.58	0.93	0.74	100	PMp	100	0.98	1.11	2.21
	1000	PMm	300	— ^c			300	PMm	100	0.80	1.31	0.43
PMp#22	1000	PMp	10	1.88	0.93	1.83	100	PMp	100	0.86	1.17	1.89
	1000	PMm	100	1.14	1.11	2.02	300	PMm	100	1.09	1.02	1.04
PMm#22	1000	PMp	10	1.12	0.99	0.49	30	PMp	100	1.47	1.11	3.71
	1000	PMm	10	1.76	1.13	2.44	30	PMm	100	0.79	1.13	12.34
PMm#31	1000	PMp	10	1.90	1.07	0.24	100	PMp	100	1.65	1.64	0.26
	1000	PMm	10	1.08	1.21	0.22	100	PMm	100	0.79	1.43	0.47
PMm#32	1000	PMp	3	0.77	1.27	0.51	30	PMp	100	0.96	1.05	0.54
	1000	PMm	10	0.97	1.16	0.44	30	PMm	100	1.21	0.97	0.71
PMm#33	1000	PMp	3	0.98	1.03	0.39	100	PMp	100	1.63	1.20	0.77
	1000	PMm	10	0.87	1.24	0.36	100	PMm	100	1.52	1.12	0.76

^a Enzyme tracer concentration. ^b Assay conjugate concentration. ^c No signal.

Table 2

Standard curve parameters of the selected immunoassays.

	Direct	Indirect
Immunoreagents		
Conjugate (µg/L)	HRP-PM _p (6)	OVA-PM _p (100)
Antibody (µg/L)	PMm#31 (1000)	PMm#31 (50)
Curve parameters (n=3)		
A _{max} (a.u.)	1.26 ± 0.03	1.39 ± 0.07
Slope	1.31 ± 0.07	1.18 ± 0.05
IC ₅₀ (µg/L)	0.28 ± 0.01	0.27 ± 0.01
A _{min} (a.u.)	0.01 ± 0.01	0.01 ± 0.01
Limit of detection (µg/L)	0.05	0.04
Intra-day precision (n=3)		
A _{max} (%)	3.1	3.7
IC ₅₀ (%)	4.3	4.7
Inter-day precision (n=3)		
A _{max} (%)	11.7	3.3
IC ₅₀ (%)	8.9	15.6

Circles (direct format). Triangles (indirect format).

Table 3

Immunoassay recoveries from fruit and vegetable spiked samples.

cELISA	[PM] ^a (µg/kg)	Recovery (% ± s, n=3)		
		Strawberry (dilution 1/500)	Tomato (dilution 1/50)	Cucumber (dilution 1/50)
Direct	5	– ^b	103 ± 1	130 ± 25
	10	– ^b	104 ± 3	109 ± 8
	50	– ^b	91 ± 7	98 ± 3
	100	113 ± 18	95 ± 2	94 ± 14
	500	108 ± 9	105 ± 5 ^c	110 ± 9 ^c
	1000	105 ± 9	101 ± 13 ^c	106 ± 5 ^c
	5000	116 ± 10	99 ± 16 ^c	117 ± 19 ^c
Indirect	5	– ^b	120 ± 24	108 ± 11
	10	– ^b	104 ± 11	107 ± 9
	50	102 ± 7	97 ± 3	97 ± 7
	100	101 ± 7	99 ± 4	92 ± 6
	500	103 ± 3	97 ± 4 ^c	102 ± 5 ^c
	1000	104 ± 2	101 ± 4 ^c	104 ± 4 ^c
	5000	104 ± 6	104 ± 1 ^c	105 ± 3 ^c

^a Pyrimethanil spiked concentration. ^b Concentration was below the limit of quantification. ^c Sample was diluted 500-fold.

(a)

Pyrimethanil: $\text{R} = \text{CH}_3$

Cyprodinil: $\text{R} = \text{cyclopropyl}$

Mepanipyrim: $\text{R} = \text{C}\equiv\text{CCH}_3$

(b)

Hapten PMn : $\text{R}^1 = (\text{CH}_2)_4\text{CO}_2\text{H}$; $\text{R}^2 = \text{R}^3 = \text{H}$

Hapten PMm : $\text{R}^1 = \text{R}^3 = \text{H}$; $\text{R}^2 = (\text{CH}_2)_5\text{CO}_2\text{H}$

Hapten PMp : $\text{R}^1 = \text{R}^2 = \text{H}$; $\text{R}^3 = (\text{CH}_2)_5\text{CO}_2\text{H}$

Figure 1

Figure 2

Figure 3

Figure 4

Supporting Information

Site-heterologous haptens and competitive monoclonal antibody-based immunoassays for pyrimethanil residue analysis in foodstuffs

Francesc A. Esteve-Turrillas, Josep V. Mercader, Consuelo Agulló, Antonio Abad-Somovilla, and Antonio Abad-Fuentes

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Hapten synthesis

General information

The progress of reactions was monitored by thin layer chromatography (TLC) performed on F254 silica gel plates. Plates were visualized by immersion with ethanolic ceric ammonium molybdate or phosphomolybdic acid and heating. Column chromatography was performed on Merck silica gel 60, 230-400 mesh. 6-(3-Aminophenyl)hexanoic acid was prepared as previously described.¹ Solvents were purified by standard methods.²

Melting points were determined using a Kofler hot-stage apparatus. IR spectra were measured using a Nicolet Avatar 320 spectrometer. High-resolution mass spectra (HRMS) were obtained with a Micromass VG Autospec spectrometer. ¹H NMR spectra were recorded on Bruker spectrometers at 300 MHz, and ¹³C NMR spectra at 75 MHz. Proton and carbon spectra were referenced to residual solvent (¹H NMR: 7.26/77.00 ppm and 2.50/39.52 ppm for CDCl₃ and DMSO-d₆, respectively). Carbon substitution degrees were established by DEPT pulse sequences.

Preparation of 6-(3-(4,6-dimethylpyrimidin-2-ylamino)phenyl)hexanoic acid (hapten PMm).

A mixture of 2-chloro-4,6-dichloropyrimidine (**1**, 71 mg, 0.5 mmol) and 6-(3-aminophenyl)hexanoic acid (103 mg, 0.5 mmol) in dry 1,4-dioxane (2.5 mL) was stirred and heated at reflux under nitrogen for 24 hours and then cooled to room temperature. The residue left after evaporation of the solvent under reduced pressure was dissolved in formic acid (0.3 mL) and the product precipitated by slow addition of water (approximately 3 mL). The precipitate was filtered off, washed several times with cold water and dried under vacuum to afford hapten PMm as a white solid (109 mg, 70%). Mp 199-202 °C (crystallized from water); ¹H RMN (300 MHz, DMSO-d₆) δ (ppm) 11.97 (1H, br s, COOH), 9.33 (1H, s, N-H), 7.64 (1H, br d, *J* = 8.0 Hz, H-6 Ph), 7.60 (2H, br s, H-2 Ph), 7.13 (1H, br t, *J* = 7.7 Hz, H-5 Ph), 6.73 (1H, br d, *J* = 7.6 Hz, H-4 Ph), 6.60 (1H, s, H-5 Pym), 2.50 (2H, t overlapped with solvent signal, *J* = 7.3 Hz, H-6), 2.30 (6H, s, Mex₂),

(1) C. Suarez-Pantaleón, J. V. Mercader, C. Agulló, A. Abad-Somovilla, A. Abad-Fuentes. Hapten Synthesis and Polyclonal Antibody-Based Immunoassay Development for the Analysis of Forchlorfenuron in Kiwifruit. *J. Agric. Food Chem.* **2010**, 58, 8502–8511.

(2) Armarego, W. L. F.; Perrin, D. D. Purification of Laboratory Chemicals; 4th ed.; Oxford: Butterworth-Heinemann, 1996.

2.19 (2H, t, $J = 7.3$ Hz, H-2), 1.54 (4H, m, H-3 and H-5), 1.30 (2H, m, H-4); ^{13}C RMN (75 MHz, DMSO- d_6) δ (ppm) 174.4 (C-1), 166.7 (C-4/C-6 Pym), 159.7 (C-2 Pym), 142.1 (C-1 Ph), 140.7 (C-3 Ph), 128.1 (C-5 Ph), 120.9 (C-4 Ph), 118.5 (C-6 Ph), 115.9 (C-2 Ph), 110.8 (C-5 Pym), 35.2 (C-6), 33.5 (C-2), 30.5 (C-5), 28.1 (C-4), 24.3 (C-3), 23.4 (Mex2); IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$ 3284, 3207, 3142, 3104, 2945, 2918, 2858, 2503, 1689, 1618, 1596, 1558, 1492, 880, 776, 744, 552; EM (EI, 70 Ev) m/z (%) 313 (M^+ , 77), 312 (20), 254 (12), 226 (81), 213 (80), 212 (20), 199 (12), 198 (4), 73 (100); EMAR, m/z (M^+) calculated for $\text{C}_{18}\text{H}_{23}\text{N}_3\text{O}_2$ 313.17903, found 313.17882.

Preparation of 5-((4,6-dimethylpyrimidin-2-yl)(phenyl)amino)pentanoic acid (hapten PMn).

(i) A solution of pyrimethanil (100 mg, 0.5 mmol) in anhydrous DMF (1.5 mL) was added dropwise to a suspension of prewashed (pentane) NaH (60% in mineral oil, 24.1 mg, 0.60 mmol) in DMF (1.5 mL) at room temperature under nitrogen atmosphere. After the evolution of hydrogen had ceased, methyl 5-bromopentanoate (0.148 mL, 1 mmol, 2.0 equiv.) was added followed by stirring during 72 hours. The reaction was then diluted with water and extracted with EtOAc. The combined organic extracts were washed successively with water, 20% aqueous solution of LiCl, and brine, and dried over anhydrous Na_2SO_4 . The solvent was removed and the residue purified by flash column chromatography, using hexane/EtOAc 9:1 as eluent, to afford an approximately 1:2 mixture of unreacted pyrimethanil and methyl ester **3** (145 mg).

(ii) $\text{LiOH}\cdot\text{H}_2\text{O}$ (81.6 mg, 1.94 mmol) was added to a solution of the above obtained mixture in THF (1.7 mL) and water (0.85 mL). The mixture was stirred overnight at room temperature and then concentrated to dryness under reduced pressure. The residue obtained was dissolved in water (5 mL), carefully acidified with solid KHSO_4 to pH 3-4 and then extracted with EtOAc. The combined organic extracts were dried over anhydrous MgSO_4 and the residue obtained after evaporation of the solvent was purified by flash chromatography, using CHCl_3 -MeOH 9:1 as eluent, to obtain unreacted pyrimethanil (36 mg) followed by hapten PMn (89.8 mg, 60% from pyrimethanil) as a colorless oil. ^1H RMN (300 MHz, CDCl_3) δ (ppm) 7.36 (2H, m, H-3/H-5 Ph), 7.27 (2H, m, H-2/H-6 Ph), 7.18 (1H, tt, $J = 7.2, 1.5$ Hz, H-4 Ph), 6.32 (1H, s, H-5 Pym), 4.04 (2H, t, $J = 7.0$ Hz, H-5), 2.37 (2H, t, $J = 7.0$ Hz, H-2), 2.24 (6H, s, Mex2), 1.66 (4H, m, H-3 and H-4); ^{13}C RMN (75 MHz, CDCl_3) δ (ppm) 179.0 (C-1), 166.9 (C-4/C-6 Pym), 161.7 (C-2 Pym), 144.5 (C-1 Ph),

128.8 (C-3/C-5 Ph), 127.5 (C-2/C-6 Ph), 125.3 (C-4 Ph), 109.9 (C-5 Pym), 49.5 (C-5), 33.7 (C-2), 27.2 (C-4), 24.0 (Mex2), 21.8 (C-3); IR (NaCl) $\nu_{\text{max}}/\text{cm}^{-1}$ 3044, 2928, 2864, 1708, 1578, 1563, 1498, 1475, 1376, 1339, 1219, 694; EM (EI, 70 Ev) m/z (%) 299 (M^+ , 21), 298 (10), 226 (24), 224 (5), 213 (16), 212 (100), 209 (11), 208 (17), 207 (84), 200 (2), 199 (15), 198 (25); EMAR, m/z (M^+) calculated for $C_{17}H_{21}N_3O_2$ 299.16338, found 299.16426.

Preparation of hapten active esters

Anhydrous Et_3N (53 μL , 0.387 mmol, 3.8 equiv.) was added to a solution of hapten PMm o PMn (0.10 mmol) and N,N' -disuccinimidyl carbonate (33 mg, 0.13 mmol, 1.3 equiv.) in anhydrous CH_3CN (1 mL) under nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature. Then, the solution was diluted with chloroform, washed with an aqueous saturated solution of NaHCO_3 and brine and then dried over anhydrous Na_2SO_4 . Flash column chromatography of the residue that was left after evaporation of the solvent, using chloroform as the eluent, afforded the corresponding N -succinimidyl ester.

N -Succinimidyl ester of hapten PMm: 80% yield. ^1H RMN (300 MHz, C_6D_6) δ (ppm) 7.87 (1H, dd, $J = 8.1, 1.3$ Hz, H-6 Ph), 7.39 (1H, br s, N-H), 7.30 (2H, br s, H-2 Ph), 7.23 (1H, t, $J = 7.8$ Hz, H-5 Ph), 6.75 (1H, br d, $J = 7.6$ Hz, H-4 Ph), 5.95 (1H, s, H-5 Pym), 2.41 (2H, t, $J = 7.6$ Hz, H-6), 2.13 (6H, s, Mex2), 2.11 (2H, t overlapped with signal at 2.13, $J = 7.6$ Hz, H-2), 1.70-1.50 (4H, m, COCH_2CH_2), 1.49-1.34 (4H, H-5 and H-3), 1.13 (2H, m, H-4).

N -Succinimidyl ester of hapten PMn: 94% yield. ^1H RMN (300 MHz, CDCl_3) δ (ppm) 7.36 (2H, m, H-3/H-5 Ph), 7.27 (2H, m, H-2/H-6 Ph), 7.19 (1H, tt, $J = 7.2, 1.5$ Hz, H-4 Ph), 6.32 (1H, s, H-5 Pym), 4.08 (2H, t, $J = 6.7$ Hz, H-5), 2.81 (4H, br s, $\text{COCH}_2\text{CH}_2\text{CO}$), 2.71 (2H, t, $J = 7.3$ Hz, H-2), 2.24 (6H, s, Mex2), 1.85-1.69 (4H, m, H-3 and H-4).

Preparation of protein-hapten conjugates

i) Immunizing BSA-hapten conjugates. Two hundred microliters of N -succinimidyl ester hapten solution (50 mM) in DMF was added drop wise to 2 mL of a 15 mg/mL BSA solution in CB and the mixture was gently stirred in an amber glass vial at room temperature for 4 h. The conjugate was purified from uncoupled hapten by size exclusion chromatography using 3 serially connected HiTrap columns and PB as the eluent. Conjugates were stored frozen at -20°C .

ii) Coating OVA-hapten conjugates. Two hundred microliters of *N*-succinimidyl ester hapten solution (50 mM) in DMF was slowly added over a 2 mL OVA solution (15 mg/mL) in CB under gently stirring. The mixture was incubated during 2.5 h at room temperature and the conjugate was purified as described above. The OVA conjugates were stored at -20 °C in amber glass vials.

iii) Tracer HRP-hapten conjugates. One hundred microliters of *N*-succinimidyl ester hapten solution (5 mM) in DMF was slowly added over a 1 mL HRP solution (2.2 mg/mL) in CB under gently stirring. The mixture was reacted for 2.5 h at room temperature and the conjugate was purified using 2 serially connected HiTrap columns and PB as the eluent. The conjugate solution pool was divided into aliquots and stored at -20 °C in amber vials.

Monoclonal antibody production.

i) *Immunization.* BALB/c female mice (8–10 weeks old) were immunized with the BSA conjugates by intraperitoneal injections. Doses consisted of an emulsion of 100 µL of PB containing 100 µg of protein conjugate and 100 µL of Freund's adjuvant. The first dose contained complete Freund's adjuvant, and subsequent doses were given at weeks 3 and 6 using incomplete Freund's adjuvant. After a resting period of at least 3 weeks from the last injection with adjuvant and four days before cell fusion, mice received a booster intraperitoneal injection of 200 µL of a solution containing 100 µg of BSA-hapten conjugate in PB.

ii) *Cell fusion and culture.* P3-X63/Ag 8.653 murine myeloma cells were cultured in high-glucose DMEM supplemented with 2 mM alanylglutamine, 1 mM MEM nonessential amino acids, and 25 µg/mL gentamycin (referred to as s-DMEM) and containing 10% (v/v) fetal bovine serum (FBS). Just before spleen extraction, mouse blood was collected by heart puncture and the serum was diluted 1/10 with storage buffer and kept at 4 °C. After cytolysis of red blood cells by osmotic shock, mouse spleen lymphocytes were fused with myeloma cells at a 4:1 ratio using 1 mL of PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at a density of 1.5×10^5 cells per well in 100 µL of s-DMEM with 15% FBS. Sixteen hours after plating, 100 µL of HAT selection medium was added to each well.

iii) *Hybridoma selection and cloning.* Twelve days after fusion, hybridoma culture supernatants were screened by cELISA in microtiter plates coated with 1.0 µg/mL homologous

conjugate. The signal of each blank assay (without pyrimethanil) was compared with the corresponding competitive result when 0.1 μ M pyrimethanil was used as competitor. Supernatants affording a high ratio between the absorbance of both assays or with saturated signals were rescreened by checkerboard cELISA. Homologous conjugate-coated plates (at 0.1 and 1.0 μ g/mL) were employed and serial supernatant and pyrimethanil dilutions were combined and assayed. Detected high-affinity antibody-producing hybridomas were cloned by limiting dilution in HT medium containing 20% FBS and 1% High Fusion and Cloning Supplement (Roche Applied Science, Mannheim, Germany). Stable clones were expanded, and finally cryopreserved in liquid nitrogen.

iv) *Purification and isotyping of monoclonal antibodies.* Immunoglobulins were purified from late stationary phase culture supernatants by ammonium sulfate precipitation and protein G affinity chromatography. The immunoglobulin isotype was determined using the ImmunoPure Monoclonal Antibody Isotyping kit I (HRP/ABTS) from Pierce (Rockford, IL).

Table S1

Determination of pyrimethanil residues in blind spiked and in-field strawberry (S), tomato (T), and cucumber (C) samples by UPLC-MS-MS and cELISAs.

Sample type	Sample name	[Pyrimethanil] ($\mu\text{g/kg} \pm \text{s}$, $n=2$)		
		UPLC-MS-MS	Direct cELISA	Indirect cELISA
In-field treated	S-01	3700 $\pm$ 400	3300 $\pm$ 200	3300 $\pm$ 400
	S-02	2100 $\pm$ 200	1800 $\pm$ 100	2000 $\pm$ 200
	S-03	1500 $\pm$ 100	1070 $\pm$ 80	1100 $\pm$ 100
	S-04	4700 $\pm$ 500	5150 $\pm$ 80	4500 $\pm$ 100
	S-05	5000 $\pm$ 500	5300 $\pm$ 600	4800 $\pm$ 200
	S-06	5100 $\pm$ 500	5700 $\pm$ 300	5200 $\pm$ 400
	S-07	1100 $\pm$ 100	1200 $\pm$ 60	1000 $\pm$ 100
	S-08	7600 $\pm$ 800	7570 $\pm$ 70	7200 $\pm$ 200
	S-09	5000 $\pm$ 500	4500 $\pm$ 300	5150 $\pm$ 80
	S-10	6000 $\pm$ 600	5300 $\pm$ 400	6500 $\pm$ 300
	S-11	5400 $\pm$ 500	5300 $\pm$ 100	5700 $\pm$ 100
	S-12	5900 $\pm$ 600	6200 $\pm$ 200	5600 $\pm$ 400
	S-13	4600 $\pm$ 500	4400 $\pm$ 400	4800 $\pm$ 400
	T-01	1000 $\pm$ 100	890 $\pm$ 20	960 $\pm$ 30
Blind spiked	T-02	280 $\pm$ 30	280 $\pm$ 8	320 $\pm$ 20
	T-03	43 $\pm$ 7	80 $\pm$ 2	80 $\pm$ 20
	T-04	28 $\pm$ 3	36 $\pm$ 3	46 $\pm$ 4
	C-01	2600 $\pm$ 300	2200 $\pm$ 200	2690 $\pm$ 60
	C-02	1400 $\pm$ 100	1360 $\pm$ 60	1490 $\pm$ 80
	C-03	590 $\pm$ 60	570 $\pm$ 50	600 $\pm$ 50
	C-04	410 $\pm$ 40	430 $\pm$ 10	420 $\pm$ 50
	C-05	8 $\pm$ 1	7.2 $\pm$ 0.1	6 $\pm$ 1
	C-06	11 $\pm$ 1	10.3 $\pm$ 0.1	9 $\pm$ 2
	S-14	170 $\pm$ 10	158 $\pm$ 8	144 $\pm$ 8
	S-15	220 $\pm$ 10	230 $\pm$ 30	230 $\pm$ 20
	S-16	530 $\pm$ 30	530 $\pm$ 50	550 $\pm$ 30
	S-17	700 $\pm$ 40	670 $\pm$ 40	720 $\pm$ 30
	S-18	950 $\pm$ 60	900 $\pm$ 100	940 $\pm$ 10
	S-19	1420 $\pm$ 90	1300 $\pm$ 100	1400 $\pm$ 200
	S-20	1900 $\pm$ 100	2000 $\pm$ 100	1900 $\pm$ 300
	S-21	2400 $\pm$ 100	2300 $\pm$ 100	2300 $\pm$ 400
	T-05	110 $\pm$ 4	120 $\pm$ 20	119 $\pm$ 9
	T-06	270 $\pm$ 10	290 $\pm$ 80	270 $\pm$ 20
	T-07	450 $\pm$ 20	400 $\pm$ 100	450 $\pm$ 60
	T-08	1040 $\pm$ 60	1050 $\pm$ 20	880 $\pm$ 60
	T-09	1600 $\pm$ 100	1290 $\pm$ 20	1400 $\pm$ 200
	T-10	1700 $\pm$ 100	1600 $\pm$ 100	1700 $\pm$ 100
	T-11	2100 $\pm$ 100	2000 $\pm$ 200	2000 $\pm$ 200
	T-12	2400 $\pm$ 200	2300 $\pm$ 200	2600 $\pm$ 300
	C-07	190 $\pm$ 8	190 $\pm$ 20	195 $\pm$ 8
	C-08	330 $\pm$ 20	300 $\pm$ 60	330 $\pm$ 20
	C-09	630 $\pm$ 40	600 $\pm$ 200	630 $\pm$ 50
	C-10	1150 $\pm$ 70	1100 $\pm$ 200	1100 $\pm$ 90
	C-11	1600 $\pm$ 100	1400 $\pm$ 200	1400 $\pm$ 100
	C-12	2200 $\pm$ 100	1900 $\pm$ 200	2100 $\pm$ 200
	C-13	2200 $\pm$ 100	2100 $\pm$ 400	2100 $\pm$ 200
	C-14	2700 $\pm$ 200	2600 $\pm$ 100	2800 $\pm$ 100

Copies of NMR spectra of haptens and *N*-succinimidyl esters

^1H NMR spectrum of hapten PMm (300 MHz, DMSO- d_6)

¹³C NMR and DEPT spectra of hapten PMm (75 MHz, DMSO-d₆)

^1H NMR spectrum of hapten PMn (300 MHz, CDCl_3)

^{13}C NMR and DEPT spectra of hapten PMn (75 MHz, CDCl_3)

^1H NMR spectrum of the active ester of hapten PMm (300 MHz, C_6D_6)

¹H NMR spectrum of the active ester of hapten PMn (300 MHz, CDCl₃)

Journal: LWT – Food Science and Technology

Manuscript Title: Site-heterologous haptens and competitive monoclonal antibody-based immunoassays for pyrimethanil residue analysis in foodstuffs

Dear Editor,

We have modified our manuscript according to your suggestions, and a new file has been resubmitted to the journal.

1) Please provide a maximum of 5 keywords in the manuscript file.

5 keywords are now provided in the manuscript file.

2) All figures placed at the end of the manuscript should be labelled with their names (e.g., Figure 1, Figure 2 etc.).

All figures at the end of the manuscript are now labelled with their names.

We look forward to your positive response.

Yours sincerely,
Antonio Abad-Fuentes