Monoclonal antibody-based immunoassays for cyprodinil residue analysis in QuEChERS-based fruit extracts

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Abstract

Cyprodinil is among the most common agrochemical residues found in highly perishable fruits, such as strawberries. In the present study, high-affinity monoclonal antibodies to this anilinopyrimidine fungicide were raised for the first time with the aim to produce valuable immunochemical analytical assays. Cyprodinil bioconjugates and the generated novel monoclonal antibodies were employed for sensitive competitive immunoassay development in two different formats. The limits of detection of the optimized assays were 20 and 30 ng L^{-1} for the indirect and direct assay, respectively. Influence over assay parameters of different physicochemical factors was studied. Strawberry samples were extracted following the recommended QuEChERS procedure for pesticide residues in food, and analyzed by the optimized immunoassays. Recoveries and coefficients of variation from fortified samples were within standard values. In addition, the obtained immunochemical results with naturally contaminated samples were statistically comparable, according to Deming regression analysis, to those of a reference chromatographic method.

Keywords

ELISA, Anilinopyrimidine, Fungicide residues, Rapid methods, Food safety, Hapten.

Chemical compounds studied in this article:

Cyprodinil (PubChem CID: 86367); Pyrimethanil (PubChem CID: 91650); Mepanipyrim (PubChem CID: 86296); Fluodioxonil (PubChem CID: 86398); Picoxystrobin (PubChem CID: 11285653).

1. Introduction

Cyprodinil is a systemic novel-generation fungicide of the anilinopyrimidine family that was introduced by Ciba-Geigy in the mid-1990s. Its use was first approved by US authorities in June 1998 and it was included in Annex I of Directive 91/414/EEC of the EU in May 2007. Nowadays, cyprodinil is employed by foliar application in a variety of cereal, fruit, and vegetable crops world-wide. It shows protective and curative effects against highly aggressive pathogens such as *E. graminis*, *D. teres*, *V. inaequalis*, and *B. cinerea* (Knauf-Beiter, Dahmen, Heye, & Staub, 1995). Unlike many fungicides that inhibit spore germination, anilinopyrimidines show a different mode of action that consists of inhibiting germ-tube elongation and initial mycelial growth (Rosslenbroich & Stuebler, 2000). Moreover, several studies suggest that anilinopyrimidines also inhibit the secretion of extracellular proteins, including fungal hydrolases associated with pathogenesis, and the biosynthesis of methionine in *Botrytis* spp. (Masner, Muster, & Schmid, 1994; Milling & Richardson, 1995). Such distinct biocide mechanisms make anilinopyrimidines very attractive plant protection products for combined applications aiming to increase the pesticide efficiency and to prevent fungi resistance. Thus, dual fungicide formulations containing cyprodinil have experienced great success in the present decade, and due to extensive use in particular crops, residues are commonly found in food samples. According to the 2010 EU Report on Pesticide Residues, cyprodinil contents above the MRLs were found mainly in leek but also in apple, head cabbage, lettuce, oat, peach, rye, strawberry, and tomato samples, and it was the most frequently found pesticide in strawberries – almost 32% of the analyzed samples contained residues above the detection limit (Scientific Report of EFSA, 2013). In 2012, cyprodinil residues were mainly found in baby food, cherry tomatoes, plums, and winter squash in US food commodities (USDA Pesticide Data Program, 2014). Despite of being moderately toxic to mammals, there is some concern about a potential cyprodinil bioaccumulation effect – the FAO acceptable daily intake (ADI) is 0.03 mg kg^{-1} of body weight (DG SANCO Review Report, 2010).

Classical analytical methods for pesticide residue determination were based on gas chromatography (GC) with electron capture or nitrogen–phosphorous detection, as well as high-performance liquid chromatography (HPLC) with UV detectors. Nowadays, identification and quantification is mainly performed with mass spectrometry (MS) or tandem MS detection. Since 2007, the AOAC International and the European Committee for Standardization have adopted the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methodology (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003) for pesticide residue extraction from food matrices (Lehotay, 2007; European Committee for Standardisation, 2008). These processing and measurement methods provide highly sensitive and robust analysis, and possess the capacity to determine simultaneously a variety of residues. Recently, several studies have been published describing chromatographic multiresidue analytical approaches comprising cyprodinil analysis in wine (Rial-Otero, Yagüe-Ruiz, Cancho-Grande, & Simal-Gándara, 2002; Vaquero-Fernández et al., 2008; Zhang et al., 2009; Fontana, Rodriguez, Ramil, Altamirano, & Cela, 2011; Moeder, Bauer, Popp, van Pinxteren, & Reemtsma, 2012), vegetable (Melo, Aguiar, Mansilha, Pinho, & Ferreira, 2012), and strawberry (Fernandes, Domingues, Mateus, & Delerue-Matos, 2012) samples. Additionally, contemporary analysts requiring sensitive, simple, rapid, specific, and high sample throughput approaches may recourse to molecular receptor-based techniques, like the competitive enzyme-linked immunosorbent assay (cELISA). Essentially, this method employs either rabbit polyclonal or mouse monoclonal antibodies (mAbs) as receptors, together with a protein conjugate of a chemical derivative mimicking the target compound. Both types of immunoglobulins are usually highly specific and can reach very high affinities to pesticides – K_D values lower than 10^{-9} M. However, only monoclonal antibodies guarantee a constant supply of well-defined and homogeneous reagent for full immunoassay characterization, development, and validation, as well as wide implementation in analytical laboratories through readily available commercial sources.

Structurally, cyprodinil is constituted by an anilinopyrimidine backbone with a methyl and a cyclopropyl substituent at the *meta* positions of the pyrimidine ring. As prediction of the optimum linker tethering site for immunizing haptens was not obvious, four cyprodinil derivatives were prepared holding the spacer arm at alternative sites (Fig. 1). Previously, the immunogenicity of the different haptens was assessed by raising rabbit polyclonal antibodies (Esteve-Turrillas, Agulló, Abad-Fuentes, Abad-Somovilla, & Mercader, 2012; Esteve-Turrillas, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2015). The aim of the present study was to generate high-affinity mAbs to cyprodinil and to develop and validate immunoassays for rapid and sensitive analysis of residues of this fungicide in QuEChERS-based fruit extracts. Strawberries are highly perishable fruits heavily consumed by children; moreover, they cannot be pealed easily, and due to their rough surface, residues are hardly washed out, which makes strawberries a complex and relevant commodity to evaluate the performance of the novel methods. As far as we know, these are the first reported mAbs and immunoassays for the determination of cyprodinil levels in this added-value foodstuff.

2. Materials and methods

2.1. Reagents and instruments

Cyprodinil (4-cyclopropyl-6-methyl-*N*-phenylpyrimidin-2-amine, CAS registry number 121552-61-2, Mw 225.29 g mol^{-1}) standard, Pestanal® grade, and other pesticide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Hybridoma fusion and cloning supplement (HFCS) was obtained from Roche Applied Science (Mannheim, Germany). P3-X63-Ag 8.653 mouse plasmacytoma cell line was acquired from the European Collection of Cell Cultures (Salisbury, UK). Cell culture media (high-glucose Dulbecco's modified Eagle's medium, DMEM), gentamicin solution, and hypoxanthine–thymidine (HT) and hypoxanthine–aminopterine–thymidine (HAT) supplements were purchased from Gibco BRL (Paisley, UK). Poly(ethylene glycol) (PEG1500), fetal bovine

serum, 200 mM alanyl–glutamine solution, red blood cell lysing buffer Hybri-Max®, MEM non-essential amino acid solution, Freund's adjuvants, triphenylphosphate (TPP), and *o*-phenylenediamine were obtained from Sigma/Aldrich (Madrid, Spain). HiTrap™ protein G HP columns for mouse IgG purification were procured from General Electric Healthcare (Uppsala, Sweden). Polyclonal rabbit anti-mouse immunoglobulins peroxidase conjugate (RAM–HRP) was acquired from Dako (Glostrup, Denmark). Primary/secondary amine and organic solvents were from Scharlab (Barcelona, Spain). Culture plastic ware and Costar® flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA).

An ELx405 washer and a PowerWave HT reader, both from BioTek Instruments (Winooski, VE, USA), were employed for microtiter analysis. Absorbance was read in dual wavelength mode using 650 nm as reference. Hapten conjugation was verified with a 5800 matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF/TOF) apparatus from AB Sciex (Framingham, MA, USA). Ultra-performance liquid chromatography (UPLC) was performed on a Waters Acquity UPLC system from Waters (Milford, MA, USA), equipped with a binary solvent delivery system, an autosampler, and a BEH C18 (1.7 µm, 2.1 × 50 mm) column. Tandem mass acquisitions were carried out in an Acquity triple quadrupole MS detector, also from Waters, equipped with a Z-spray electrospray ionization source, with 3.5 kV capillary voltage, 120 °C source temperature and 300 °C desolvation temperature.

2.2. Protein conjugates

Bovine serum albumin (BSA), ovalbumin (OVA), and horseradish peroxidase (HRP) conjugates from previous studies were employed for immunization, plate coating, and direct assays, respectively (Esteve-Turrillas et al., 2012; Esteve-Turrillas et al., 2015). The hapten-to-protein molar ratio (MR) of each conjugate was determined by MALDI–TOF. For this purpose, conjugate samples were extensively dialyzed in Milli-Q water and 1 µL at ca 0.3 µg μL^{-1} of every sample solution was spotted onto the MALDI plate. After the droplets were air-dried at

room temperature, 0.75 µL of matrix – 5 mg mL^{-1} sinapinic acid (Bruker) in 0.1% TFA–CH_3CN/H_2O (7:3, v/v) – was added and allowed to air-dry at room temperature. The resulting mixtures were analyzed in positive linear mode – 1500 shots every position – with a mass range of 10000–120000 *m*/*z*. Previously, the plate was calibrated with 1 µL of the TOF/TOF calibration mixture (AB Sciex) in 13 positions. Every sample was calibrated by close external calibration method with a BSA, OVA, or HRP spectrum acquired in a nearby position.

2.3. Monoclonal antibodies

Animal manipulation was carried out in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Food, and Environment. Mice were immunized with BSA conjugates of haptens CD*b* and CD*m* (Fig. 1), following previously published schedules (Suárez-Pantaleón, Mercader, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008). Briefly, each mouse received an intraperitoneal injection with 200 µL of a 1:1 emulsion between complete Freund's adjuvant and 100 mM phosphate buffer, pH 7.4, containing 100 µg of BSA–hapten conjugate. Next, two additional boots were given at 21-day intervals with an equivalent immunogen prepared with incomplete Freund's adjuvant. After a resting period of at least 21 days, and four days before cell fusion, a fourth injection was applied without adjuvant using the same immunizing conjugate dose as before. Cell fusion and hybridoma cloning were performed as described elsewhere (Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008a). High-affinity antibody producing hybridomas were selected following a double-screening technique using culture supernatants as published before [Mercader, Suárez-Pantaleón, Agulló, Abad-Somovilla, & Abad-Fuentes, 2008b]. In brief, parallel and simultaneous assays were run in a first screening procedure with 50 µL per well of culture supernatant plus 50 µL per well of 10 mM phosphate buffered saline, pH 7.4, containing 140 mM NaCl (PBS) with 100 nM cyprodinil or without cyprodinil. Second, supernatants affording saturated signals or noticeable inhibition in the first screening assay were reevaluated by

checkerboard indirect cELISA. Hybridomas were cloned twice by limiting dilution; then, they were stabilized and expanded in culture plates. Finally, antibodies were purified from late stationary-phase culture supernatants by salting out with saturated ammonium sulfate solution and by affinity chromatography. Citrate buffer 100 mM, pH 2.5, was used for antibody elution and fractions were immediately neutralized with Tris-HCl 1 M, pH 9.5.

2.4. Competitive immunoassays

Monoclonal antibody-coated direct cELISAs were carried out with plates coated by overnight incubation at room temperature with 100 µL per well of a 1 µg mL^{-1} antibody solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. After each incubation step, plates were washed four times with 150 mM NaCl containing 0.05% (v/v) Tween 20. Each washed microwell received 50 µL of cyprodinil standard solution in PBS plus 50 µL of enzyme tracer solution in PBST (PBS containing 0.05% (v/v) Tween 20). The competitive reaction was carried out during 1 h at room temperature. Retained peroxidase activity was revealed with 100 µL per well of a 2 mg mL^{-1} *o*-phenylendiamine freshly prepared solution in 25 mM citrate and 62 mM phosphate buffer, pH 5.4, containing 0.012% H_2O_2. Signal generation was stopped after 10 min at room temperature with 100 µL per well of 1 M H_2SO_4. Absorbance was immediately read at 492 nm.

Conjugate-coated indirect cELISAs were performed with plates coated by overnight incubation at room temperature with 100 µL per well of assay conjugate solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. Between incubation steps, plates were washed as described above. The competitive reaction was done during 1 h at room temperature with 50 µL per well of cyprodinil standard solution in PBS plus 50 µL per well of monoclonal antibody solution in PBST. The immunochemical reaction was detected by incubation at room temperature during 1 h with 100 µL per well of RAM–HRP solution (1/2000 dilution) in PBST. Finally, signals were generated and plates were read as indicated for direct assays.

Absorbance values were plotted versus the logarithm of standard concentration and were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from Systat Software Inc. (Chicago, IL, USA). Sigmoidal curves were normalized and average parameters were calculated from independent experiments. Assay sensitivity was estimated as the analyte concentration reducing 50% (IC_{50}) the maximum absorbance (A_{max}), and the limit of detection (LOD) was calculated as the IC_{10} value. Percentage cross-reactivity (CR) was assessed from the quotient between the IC_{50} for cyprodinil and the IC_{50} for the evaluated molecule, both in molar concentration units.

2.5. Ionic strength, pH, and solvent tolerance

To evaluate ionic strength and pH influence, the competitive reaction was carried out with cyprodinil standards in Milli-Q water and tracer or antibody solutions, for direct and indirect cELISAs, respectively, in the studied buffer. Twenty millimolar phosphate buffers, pH 7.4, were prepared containing 0.05% (v/v) Tween 20 and increasing concentrations of NaCl. Regarding pH, a series of 20 mM phosphate buffers were arranged containing 0.05% (v/v) Tween 20 and the adequate NaCl concentration so to keep the ionic strength constant (I = 332 mM). Solvent tolerance was assessed with cyprodinil standard curves prepared in Milli-Q water containing increasing amounts of acetonitrile. The competitive reaction mixture was accomplished with enzyme tracer or monoclonal antibody solutions prepared in 20 mM phosphate, pH 7.4, containing 0.05% (v/v) Tween 20 and 280 mM NaCl.

2.6. Sample extraction and analysis

Strawberries were acquired from different local markets along a 1 month period, and residues were extracted following the QuEChERS method (Lehotay, 2007). Briefly, 15 g of sample homogenate was mixed with 1.5 g of sodium acetate and 6 g of anhydrous magnesium sulfate in 15 mL of acetonitrile containing 1% (v/v) acetic acid. Fifty microliters of TPP was added as internal control. The mixture was vigorously stirred and the organic phase was

separated by centrifugation at 2200×g during 5 min. Primary/secondary amine (50 mg) was used to cleanup 1 mL of extract in the presence of 150 mg of anhydrous magnesium sulfate by vortexing. After a second centrifugation step, clean extracts were filtrated through a Teflon filter (0.2 µm) and stored at −20 °C in amber glass vials.

Extracts of cyprodinil-free strawberry samples, as checked by UPLC, were employed for recovery studies. Moreover, samples were analyzed by the developed cELISAs and by a reference chromatographic method. Deming regression analysis was applied for method comparison using the SigmaPlot software (version 12.0). Immunochemical analysis was carried out by direct and indirect cELISA. Strawberry extracts were diluted with Milli-Q water and enzyme tracer or antibody solutions were prepared in 20 mM phosphate, pH 7.4, containing 0.05% (v/v) Tween 20 and 280 mM NaCl. A cyprodinil standard curve was run in each plate under the same conditions. Standards were prepared in borosilicate glass tubes by 4-fold serial dilution in Milli-Q water starting from a 10 µg L^{-1} cyprodinil solution. UPLC with a triple quadrupole MS detector was selected as reference methodology for cyprodinil sensitive analysis. The injection volume was set at 5 µL and the mobile phase consisted of a mixture between 0.5% (v/v) formic acid in water and acetonitrile. The gradient started at 50% of the acidic aqueous solution, it was linearly increased to 95% in 4 min, and then to 98% in 2 min at a flow rate of 400 µL min^{-1}. The retention times obtained under the aforementioned conditions were 0.7 and 2.2 min for cyprodinil and TPP, respectively. The employed parameters were: ESI+, parent ion 226.2 *m*/*z*, daughter ions 93.1 and 108.1 *m*/*z*, 33 eV collision energy and 45 V cone energy for cyprodinil; and ESI+, parent ion 328.3 *m*/*z*, daughter ions 77.0 and 152.4 *m*/*z*, 30 eV collision energy and 30 V cone energy for TPP.

3. Results and discussion

3.1. Immunoreagent evaluation

Hapten-to-protein MR values of BSA, OVA, and HRP conjugates with cyprodinil derivatives were determined by MALDI–TOF, and results were compared with those obtained previously by UV determination (Table S1). For all conjugates, similar MRs were retrieved by both methods, except for BSA and OVA conjugates with hapten CD*b*. In these cases, the differential absorbance method seemed to underestimate the hapten density. On the contrary, the UV method slightly overestimated the MRs of most HRP conjugates. In general, it is considered that BSA contains 30 lysine ε-amine groups accessible for conjugation (Hermanson, 1996). Thus, the prepared BSA conjugates had about half of the available lysine residues coupled to a hapten molecule as expected, with coupling reaction yields between 54% for hapten CD*m* and 67% for haptens CD*b* and CD*n*. In the case of OVA conjugates, coupling yields were slightly higher than those of BSA, reaching 69% for haptens CD*m* and CD*p* and 77% for haptens CD*b* and CD*n*, based on 20 lysine residues in OVA. Regarding HRP conjugates, all of the available ε-amine groups seemed to hold a cyprodinil hapten.

A battery of mAbs to cyprodinil has been raised in mice by immunization with BSA conjugates of haptens CD*b* and CD*m*. These haptens were selected for immunization considering the good immune responses obtained previously in rabbits [Esteve-Turrillas et al., 2012; Esteve-Turrillas et al., 2015]. Moreover, these synthetic compounds hold the linker at opposite sites of the cyprodinil molecule, thus displaying different immunodeterminant chemical moieties at the distal end for antibody formation. Each novel monoclonal binder was evaluated using conjugates of homologous and heterologous haptens (same or different hapten compared to the hapten of the immunizing conjugate, respectively) by checkerboard direct and indirect cELISA. Not surprisingly, none of the antibodies recognized the corresponding conjugate of hapten CD*n* – holding the linker at a central position of the cyprodinil molecule – in either of the two cELISA formats (results not shown). Regarding the direct format (Table 1), most CD*b*-type antibodies – obtained from mice immunized with a conjugate of hapten CD*b* – only bound the homologous tracer, and only one mAb (CD*b*#33)

could bind haptens CD*m* and CD*p* – with the linker at the aniline ring. On the contrary, a significantly high number of CD*m*-type antibodies – generated from mice immunized with a conjugate of hapten CD*m* – bound not only hapten CD*p* – with the linker at a contiguous position in the same ring – but also hapten CD*b* – holding the spacer at the opposite ring. This result indicates that the methyl moiety at the pyrimidine ring in the immunizing hapten CD*m* has probably increased the probability to find antibodies recognizing the heterologous hapten CD*b*, which holds the spacer in the same position of the methyl substituent of cyprodinil. Concerning the indirect format (Table S2), most antibodies bound the two heterologous assay haptens, independently of the aromatic ring at which the linker was located, thus showing a more versatile behavior of this cELISA format to spacer tethering site heterology. Regarding affinity, mAbs reaching IC_{50} values to cyprodinil as low as 0.2 nM were obtained. Furthermore, the sensitivity of homologous assays could be improved with most mAbs in both cELISA formats using linker-site heterologous haptens.

Antibody specificity was studied by homologous indirect cELISA. The anilinopyrimidine family of fungicides is composed nowadays by three members: cyprodinil, pyrimethanil, and mepanipyrim. Remarkably, CD*m*-type antibodies, derived from a hapten mainly exposing to the immune system the unique moieties in cyprodinil, displayed a higher specificity than antibodies from hapten CD*b* (Table S3). Moreover, mepanipyrim was better recognized than pyrimethanil by most binders. The most specific mAb was CD*m*#12, with CR values below 5%, and the most generic mAb for this group of pesticides was CD*b*#31, with CR values around 60%. Anyhow, no interferences should occur with pyrimethanil or mepanipyrim in food analysis because cyprodinil is usually employed in combination with other fungicides with a different mode of action, but not with another anilinopyrimidine, in order to avoid fungi resistance and to increase biocide efficiency. Common active ingredients that are formulated together with cyprodinil are picoxystrobin and fludioxonil. Studies with these compounds for

295 all of the generated mAbs did not cause signal inhibition when assayed at 10 µM. As a result,
296 no interferences can be expected from other pesticides when food samples will be analyzed.

297 *3.2. Immunoassay characterization*

298 Further studies were carried out with particular mAb and assay conjugate pairs
299 affording inhibition curves with A_{max} values higher than 1.0, low IC_{50} values, and moderate
300 slopes. Higher sensitivity was attained in both formats when a slight linker site heterology
301 (from *meta* to *para* position) was introduced. Thus, mAb CD*m*#31 by direct cELISA and mAb
302 CD*m*#21 by indirect cELISA together with the corresponding assay conjugate of hapten CD*p*
303 seemed to be the most suitable immunoreagent combinations (Tables 1 and S2). Assay
304 conditions and analytical features of the optimized immunoassays are listed in Table 2. In both
305 cELISA formats, the inhibition curves showed very low IC_{50} – between 0.1 and 0.2 µg L^{-1} – and
306 LOD values – at or below 0.03 µg L^{-1}. Background signals were negligible for the two assays.
307 Moreover, inter and intra-day precision of A_{max} and IC_{50} values were satisfactory.

308 Influence of pH and ionic strength as well as solvent concentration over the two
309 selected immunoassays was examined (Fig. 2). Interestingly, for both assays, acidic pH values
310 decreased the maximum signal of the assay, probably due to protonation of the pyrimidine
311 moiety, whereas no substantial changes in A_{max} were observed at basic pHs. Besides, sensitivity
312 of the two immunoassays remained almost constant at any of the studied pH values.
313 Regarding ionic strength, high tolerance to salt concentration was found with the direct cELISA.
314 On the other hand, the indirect assay was more robust at low than at high ionic strength
315 values. Since the QuEChERS procedure employs acetonitrile for pesticide residue extraction,
316 the effect of that solvent over the developed cELISAs was assessed. With the indirect assay, a
317 decrease in A_{max} was found at low acetonitrile concentration and an increase of IC_{50} value was
318 seen particularly at solvent concentrations higher than 2% (Fig. 2). On the contrary, only a
319 slight decrease of maximum signal and sensitivity was observed with the direct assay.

3.3. Strawberry sample analysis

In order to evaluate the trueness and precision of the developed cELISAs, fortified cyprodinil-free strawberry extracts were diluted at least 100 times in water. The higher matrix effect over the indirect assay – required dilution was 300 fold – was probably due to a lower tolerance to acetonitrile (Fig. 2). From this study, recoveries between 81% and 114% for the direct assay and between 82% and 110% for the indirect format were obtained when a cyprodinil concentration range from 10 to 5000 µg L^{-1} was evaluated (Table 3). Relative standard deviations (RSD) were mostly below 20%, except with the direct immunoassay at the highest fungicide levels. Considering the assayed lowest fortified level that met the EU method performance acceptability criteria – mean recovery value in the 70–120% range and a RSD ≤ 20% (DG SANCO, 2007) –, the limit of quantification of both cELISAs could be set at 10 µg L^{-1}, 500 times lower than the EU and the US MRL for cyprodinil in strawberry samples (5 mg kg^{-1}).

3.4. Method assessment

Incidence of cyprodinil residues in 50 commercial strawberry samples was determined by the optimized direct and indirect cELISA, and the positive samples were analyzed by UPLC–MS/MS as a reference chromatographic method. No samples with cyprodinil levels above the MRLs were encountered, yet 9 samples contained residue contents that were detectable by the three applied analytical procedures. According to UPLC–MS/MS analysis, the highest cyprodinil concentration was 907 ng mL^{-1}, the lowest was 41 ng mL^{-1}, and the average content of the positive samples was 274 ng mL^{-1} (Table S4). Statistical data analysis was carried out by Deming regression which considers the standard deviation values of positive samples in both methods (Fig. 3). Comparison of the direct cELISA with the chromatographic method afforded a regression with a slope of 0.90 (95% confidence interval was from 0.68 to 1.13) and an intercept of 16 (95% confidence interval was from −25 to 57). The corresponding Deming regression of the indirect cELISA showed a slope of 0.90 (95% confidence interval was from

345 0.78 to 1.03) and an intercept of −8 (95% confidence interval was from −26 to 11), indicating
346 that agreement of both immunoassays with the reference chromatographic method exists.

347 **4. Conclusions**

348 The first reported mAbs to cyprodinil were generated within the present study. High-
349 affinity antibodies were obtained from both of the immunizing haptens; however, the lowest
350 IC_{50} values and the highest specificity were observed with CD*m*-type mAbs. Regarding linker
351 site heterology, sensitivity improvements were commonly achieved, though haptens with a
352 contiguous tethering site compared to the homologous hapten were better recognized than
353 those with opposite linker sites. Two immunoassays were selected with IC_{50} values below 0.2
354 μg L^{-1}, and LODs of 0.02 and 0.03 μg L^{-1} for the indirect and direct assay, respectively. The
355 direct assay was slightly more robust to physicochemical changes than the indirect format.
356 A_{max} of both cELISAs was sensitive to acidic pH values, whereas high salt concentrations could
357 increase the IC_{50} value of the indirect assay. In order to extract strawberry samples, the
358 QuEChERS methodology using acetonitrile was applied. Recoveries of cyprodinil-fortified
359 samples were acceptable between 10 and 5000 μg L^{-1} for both immunoassays at different
360 dilutions. When strawberry samples from local markets were studied, no violative samples
361 (over 5 mg L^{-1}) were found, although 18% contained detectable levels below 1 mg L^{-1}. The
362 results obtained by the developed cELISAs were in good agreement with those observed by
363 UPLC–MS/MS.

364

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at

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453

454

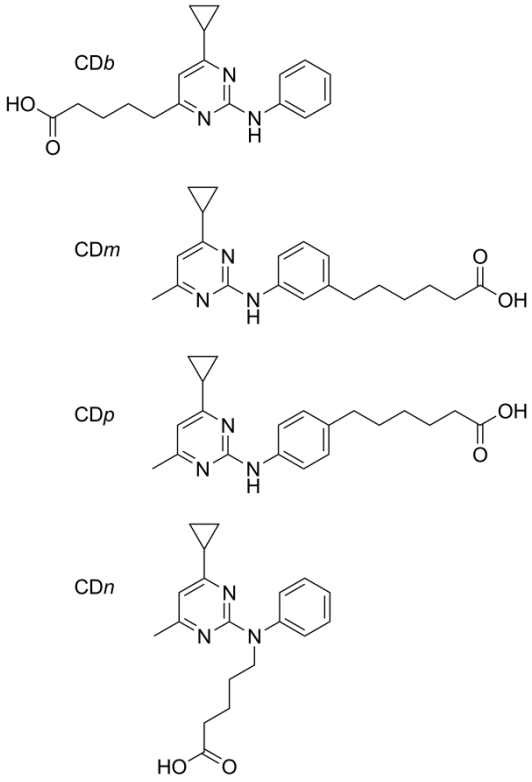
455 **Figure legends**

456 **Fig. 1.** Chemical structure of cyprodinil haptens.

457 **Fig. 2.** Influence of pH, ionic strength, and acetonitrile over A_{max} and IC_{50} values of a direct
458 cELISA with mAb CD*m*#31 and an indirect cELISA with mAb CD*m*#21.

459 **Fig. 3.** Deming regression between the developed cELISAs and UPLC–MS/MS with a 95%
460 confidence interval.

461



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464 Figure 1

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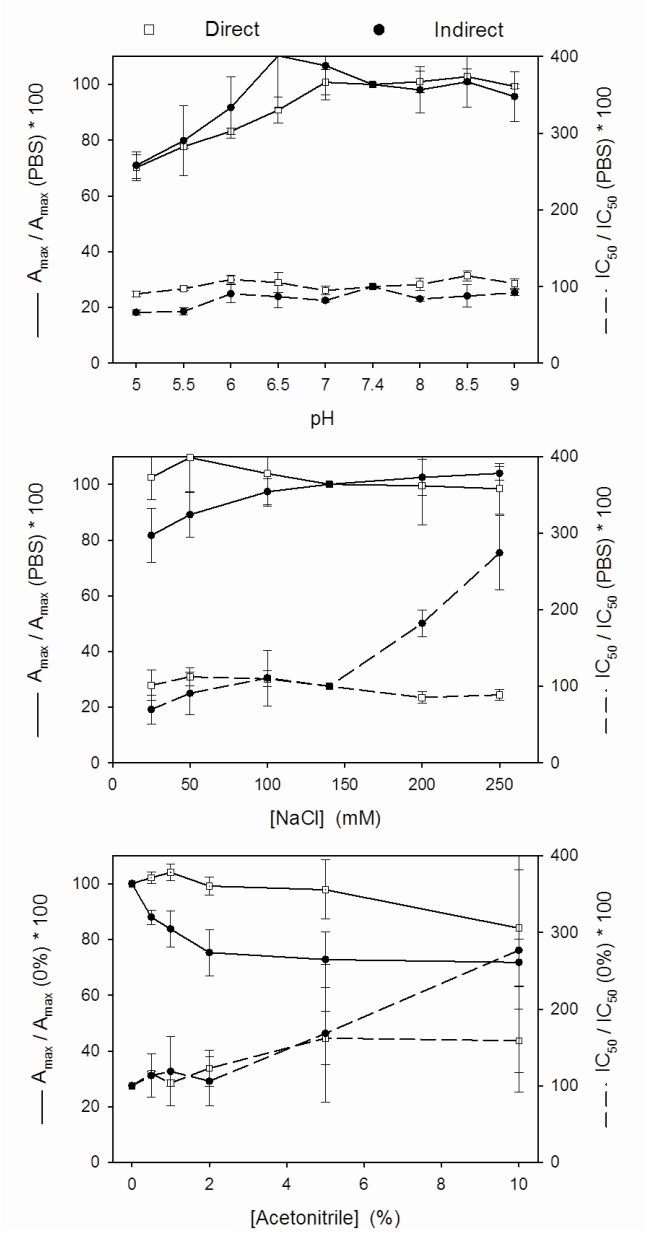
Direct
Indirect
A_max / A_max (PBS) * 100
IC_50 / IC_50 (PBS) * 100
pH
[NaCl] (mM)
A_max / A_max (0%) * 100
IC_50 / IC_50 (0%) * 100
[Acetonitrile] (%)

Figure 2

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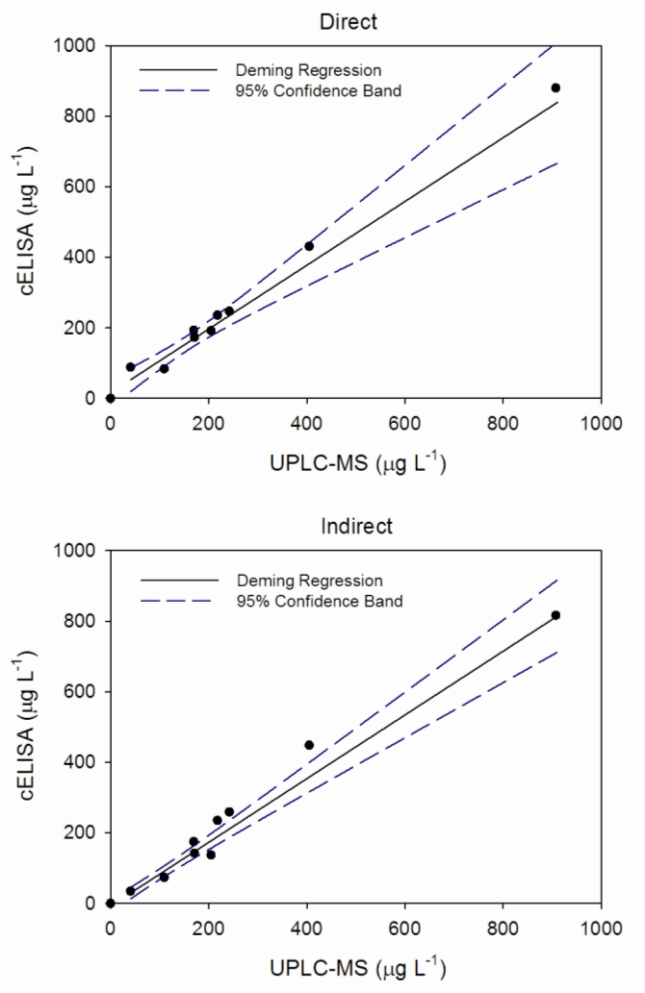

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473 Figure 3

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Table 1
Checkerboard assays by direct cELISA.

	HRP–CD*b*					HRP–CD*m*					HRP–CD*p*				
mAb	[mAb][a]	[HRP][b]	A_{max}	Slope	IC_{50}[c]	[mAb]	[HRP]	A_{max}	Slope	IC_{50}	[mAb]	[HRP]	A_{max}	Slope	IC_{50}
CD*b*#21	1000	100	-[d]	-	-	1000	100	-	-	-	1000	100	-	-	-
CD*b*#31	1000	30	0.85	1.04	0.4	1000	100	-	-	-	1000	100	-	-	-
CD*b*#32	1000	10	0.85	0.88	2.0	1000	100	-	-	-	1000	100	-	-	-
CD*b*#33	1000	10	0.96	0.88	3.1	1000	10	1.31	0.87	3.4	1000	3	0.75	0.80	3.0
CD*m*#11	1000	100	-	-	-	1000	100	1.02	0.98	4.5	1000	100	-	-	-
CD*m*#12	1000	100	-	-	-	1000	100	1.17	1.30	5.7	1000	100	-	-	-
CD*m*#13	1000	100	-	-	-	1000	10	1.01	1.14	1.9	1000	100	1.70	1.04	1.9
CD*m*#14	1000	100	-	-	-	1000	100	0.85	0.96	2.1	1000	100	-	-	-
CD*m*#21	1000	100	-	-	-	1000	100	-	-	-	1000	100	-	-	-
CD*m*#22	1000	3	0.87	1.09	1.5	1000	3	1.24	1.06	6.4	1000	3	1.28	1.06	6.4
CD*m*#23	1000	100	-	-	-	1000	30	1.05	0.94	1.2	1000	100	1.31	1.10	1.2
CD*m*#31	1000	10	0.93	1.49	0.2	1000	3	1.07	1.61	0.7	1000	3	1.21	1.02	0.3
CD*m*#32	1000	10	0.72	1.24	0.3	1000	3	1.40	1.10	0.8	1000	3	1.29	0.91	0.8
CD*m*#33	1000	10	1.00	1.08	0.3	1000	3	1.16	1.24	0.7	1000	3	1.22	0.91	0.3
CD*m*#34	1000	30	1.15	1.08	0.4	1000	3	1.20	0.92	1.3	1000	3	1.28	0.72	1.8
CD*m*#35	1000	10	0.76	1.23	0.3	1000	3	1.31	1.13	0.9	1000	3	1.27	0.95	1.0
CD*m*#36	1000	10	0.75	0.75	0.4	1000	3	1.03	0.98	1.1	1000	3	1.04	0.73	1.5

[a] Antibody concentration in ng mL^{-1}.
[b] Tracer concentration in ng mL^{-1}.
[c] Values are in nM.
[d] A_{max} values were lower than 0.7.

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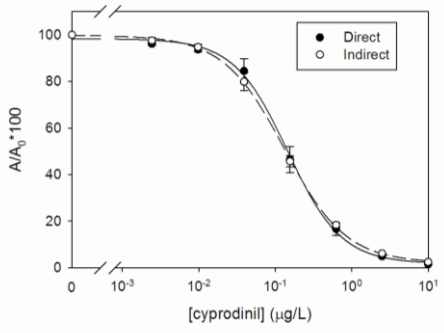
Table 2
Optimized assay conditions and analytical parameters of cyprodinil standard curves.



	Immunoassay	
	Direct	Indirect
Immunoreagents		
mAb	CD*m*#31	CD*m*#21
[mAb] (µg L^{-1})	500	100
Conjugate	HRP–CD*p*	OVA–CD*p*
[Conjugate] (µg L^{-1})	2.5	100
Curve parameters		
A_{max}	1.23 ± 0.07	1.34 ± 0.16
A_{min}	0.02 ± 0.01	0.03 ± 0.01
Slope	−1.25 ± 0.21	−1.12 ± 0.05
IC_{50} (µg L^{-1})	0.17 ± 0.02	0.14 ± 0.01
R^2	0.998	0.999
LOD (µg L^{-1})	0.03	0.02
Intra-day precision (%, n=5)		
A_{max}	6	3
IC_{50}	12	9
Inter-day precision (%, n=5)		
A_{max}	6	14
IC_{50}	10	20

Table 3
Recoveries from spiked strawberry samples measured by the two developed monoclonal cELISAs.[a]

	Direct		Indirect	
Spiked ($\mu g\ L^{-1}$)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
10	98	12	95	12
50	81	6	85	4
100	91	8	90	4
500	113	14	82	10
1000[b]	114[b]	22	92	5
5000[b]	112[b]	26	110	19

[a] Results are the average of 4 independent measurements. Extracts were diluted 100 fold for the direct cELISA and 300 fold for the indirect assay.
[b] Extracts were diluted 1000 fold for both assays.

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Figure(s)

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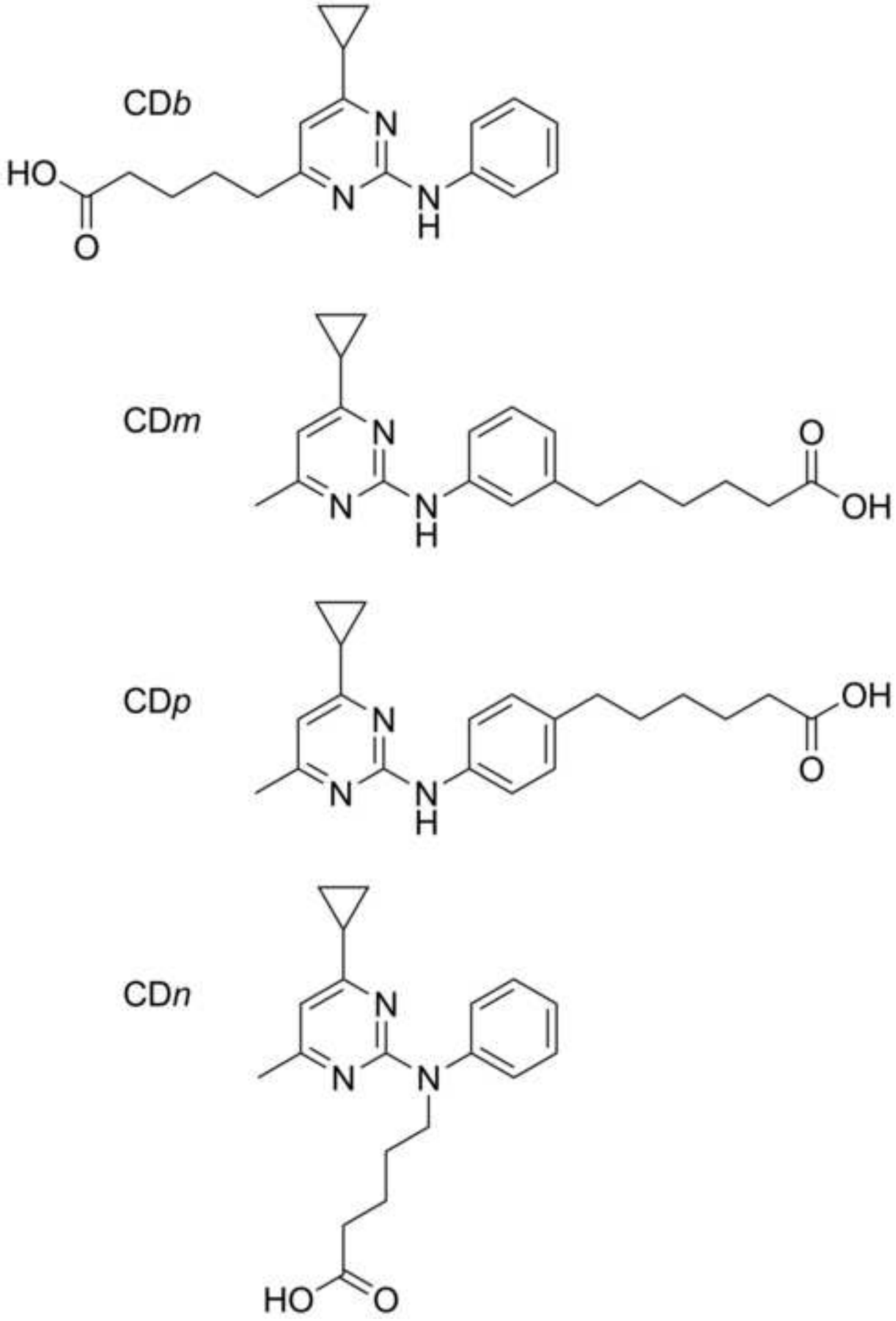

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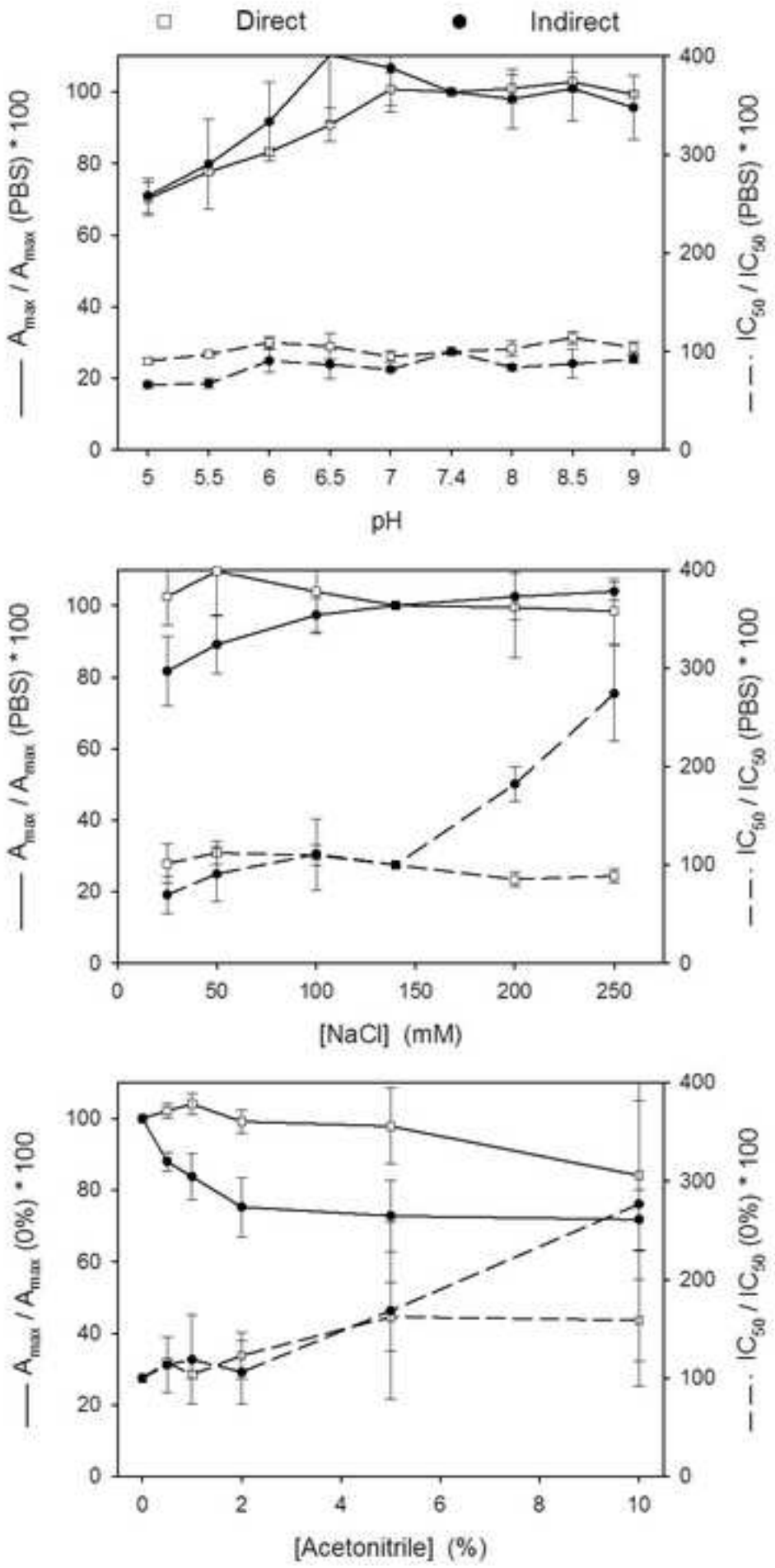

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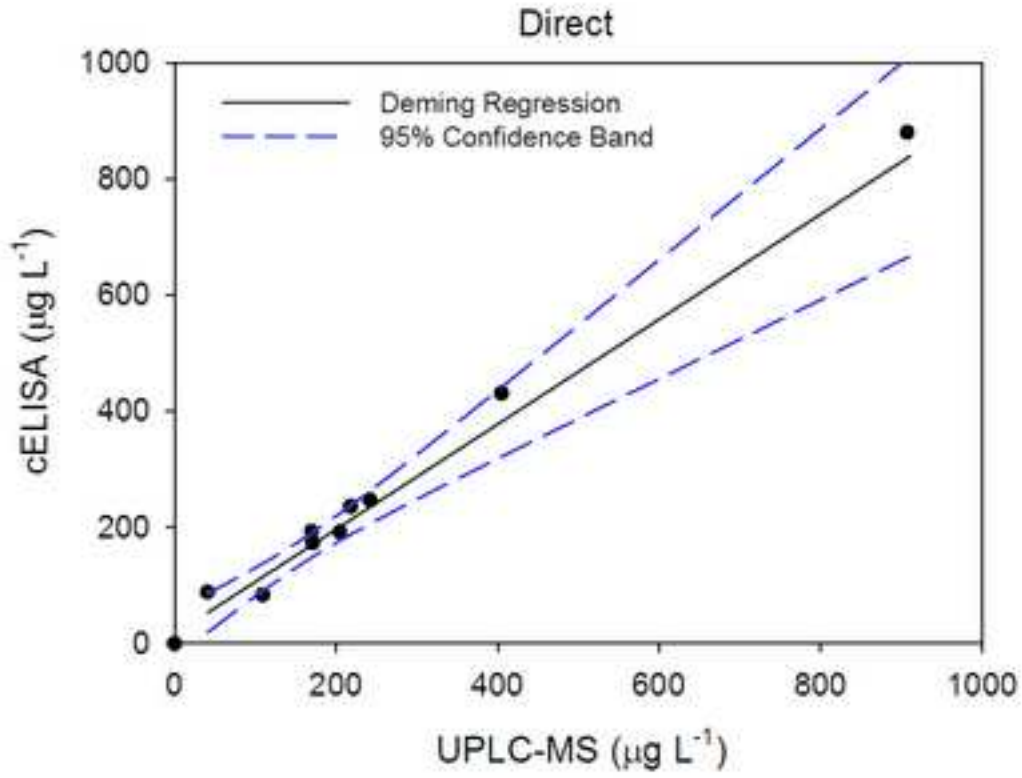

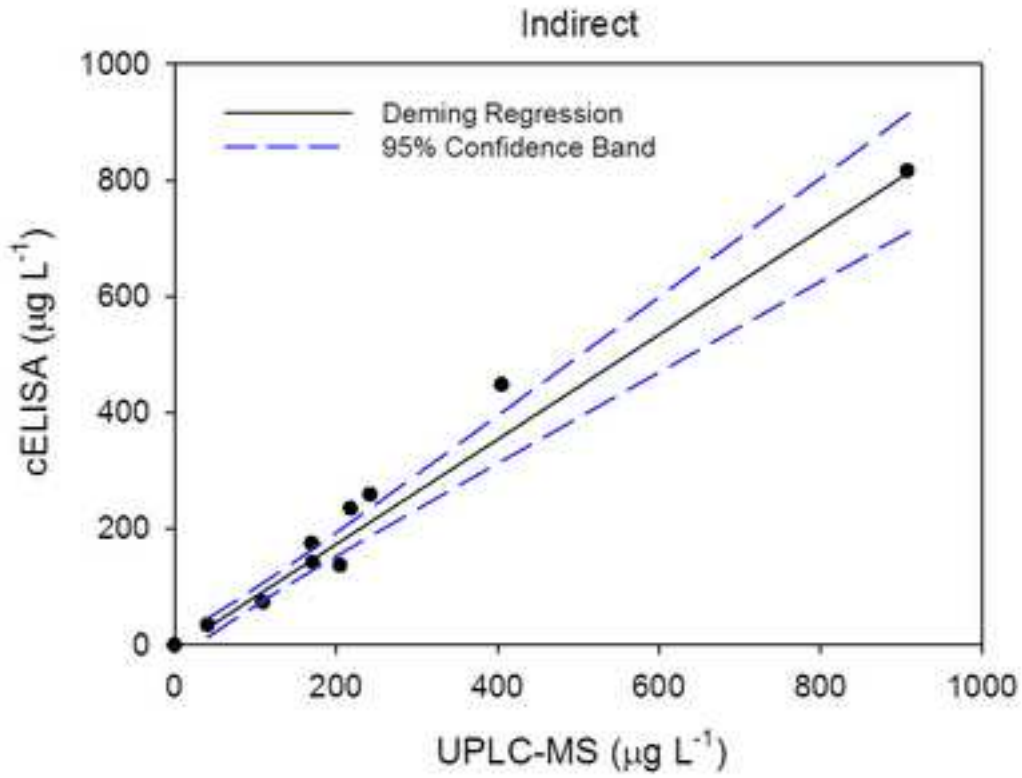

Supplementary Material

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