Immunoreagent Generation and Competitive Assay Development for Cyprodinil Analysis

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Ⓢ Supporting Information

ABSTRACT: Cyprodinil is an anilinopyrimidine fungicide applied worldwide for the prevention and treatment of highly destructive plant diseases in a large variety of crops, including cereals, fruits, and vegetables. This paper describes the development of the first reported immunoassays for cyprodinil. Two original haptens have been synthesized and conjugated to different carrier proteins, and polyclonal antibodies have been produced. Moreover, competitive enzyme-linked immunosorbent assays have been developed and characterized for the analysis of this widely used pesticide. The influence of organic solvents and buffer conditions over the assay analytical parameters was studied. The IC_{50} values of the optimized immunoassays were 1.6 and 2.8 ng/mL for the direct and indirect formats, respectively. Quantitative recoveries were found using spiked apple and grape juice samples after a simple direct dilution, and a limit of quantification of 20 ng/mL for both fruit matrices was achieved. These immunoreagents could be very valuable for the sensitive, straightforward, and rapid monitoring of cyprodinil residues in foodstuffs.

KEYWORDS: anilinopyrimidine pesticides, site heterology, competitive ELISA, immunoassay, fungicide residues, food analysis, hapten

■ INTRODUCTION

Cyprodinil is a broad-spectrum fungicide that belongs to the anilinopyrimidine family of pesticides. This compound and its two congeners (pyrimethanil and mepanipyrim) inhibit the biosynthesis of methionine and possibly act on the secretion of hydrolytic enzymes.[1] Cyprodinil is particularly active against fungi such as *Venturia inaequalis*, *Tapesia yallundae*, *Botrytis* spp., *Alternaria* spp., and *Rhynchospium secalis*.[2,3] The use of this agrochemical has been registered worldwide for a variety of crops, including cereals, pome and stone fruits, vines, strawberries, ornamental crops, and vegetables. Nowadays, several formulations are commercialized by Syngenta and DuPont under different trade names (Kayak, Unix, Switch, Acanto Prima) containing cyprodinil as the single active ingredient or combined with other fungicides such as fludioxonil or picoxystrobin. These formulations are especially recommended for foliar treatments against highly destructive pests such as botrytis in grapes and gray mold in apples.[4,5] As a result of its intensive use, cyprodinil residues are found with 5−10% frequencies in a selection of commodities, according to the 2009 EU Report on Pesticide Residues in Food recently published by the European Food Safety Authority. As a matter of fact, cyprodinil was the second most important residue in table grapes, whereas apples were shown to be one of the major contributors to cyprodinil exposure in children.[6] The maximum residue limits (MRLs) for cyprodinil established by the FAO/WHO, EU, and U.S. regulations range from 2 to 5 mg/kg in grapes and from 0.05 to 4.6 mg/kg in apples.[7−9]

Chromatographic separation techniques are commonly used to control agrochemical residues in food commodities. Although these methodologies require significant investments in equipment and human resources, they are often preferred in surveillance food safety programs because of their capability to simultaneously determine a great variety of different residues in a single run.[10,11] Accordingly, multiresidue chromatographic methods comprising cyprodinil have been reported.[12−14] Nevertheless, for certain particular analytical demands such as on-site analysis or screening of large batches of samples for focused targets, antibody-based detection methodologies can effectively provide rapid, simple, and cost-effective determination of chemicals in food samples.[15−17] The competitive enzyme-linked immunosorbent assay (cELISA) is by far the most employed immunochemical method due to its high selectivity, sensitivity, simplicity, and large sample throughput. In fact, enzyme immunoassays have been validated for the determination of many different agrochemicals in foods with excellent results—comparable to those of reference methodologies.[18−20] cELISA development requires the production of high-affinity antibodies with a particular selectivity against the target analyte, which generally demands the intricate synthesis of haptens containing a spacer arm and a functional chemical moiety. To produce antibodies of outstanding quality, haptens to be used for immunogen preparation should preserve the conformation and electronic properties of the target compound. Previous studies have demonstrated that immunizing

haptens with a functional carboxylate group and a linear six-carbon spacer can provide high-affinity and stereoselective antibodies.[21,22] However, the most advantageous linker tethering site is usually uncertain.

The aim of the present study was to synthesize different immunizing haptens and to produce high-affinity and selective antibodies suitable for immunochemical analysis of cyprodinil in foodstuffs. Two alternative linker tethering sites were evaluated for immunogen and assay conjugate preparation. To our knowledge, these are the first reported immunoreagents (haptens and antibodies) for ELISA development to cyprodinil. The generated polyclonal antibodies (pAb) were characterized, and two cELISAs in different formats—a direct antibody-coated competitive assay (d-cELISA) and an indirect conjugate-coated competitive assay (i-cELISA)—were developed for the determination of cyprodinil residues. Finally, immunoassay performance was evaluated in relevant foodstuffs for this compound such as grape and apple juices.

■ MATERIALS AND METHODS

Reagents and Instrumentation. Cyprodinil (4-cyclopropyl-6-methyl-N-phenyl-2-pyrimidinamine, CAS Registry No. 121552-61-2, MW 225.29 g/mol) and other employed pesticides were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) and Dr. Ehrenstorfer (Augsburg, Germany). Pesticide standards were prepared as concentrated solutions in anhydrous N,N-dimethylformamide (DMF) and were kept at −20 °C in amber glass vials. Methyl 6-(4-aminophenyl)hexanoate (**5**) was prepared by esterification of 6-(4-aminophenyl)hexanoic acid with MeOH under standard conditions.[23] Other reagents were acquired from commercial sources and used without purification. Reactions were monitored with the aid of thin-layer chromatography using 0.25 mm precoated silica gel plates. Visualization was carried out with UV light and a 50% (v/v) aqueous ceric or ammonium molybdate solution. Chromatography refers to flash column chromatography, and it was carried out with the indicated solvents on silica gel 60 (particle size = 0.040−0.063 mm). All melting points were determined using a Kofler hot-stage apparatus and are uncorrected. NMR spectra were recorded on a Bruker AC-300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C), and they were referenced to residual solvent protons in the ^{1}H NMR spectra (7.26 and 2.50 ppm for CDCl$_3$ or DMSO-d_6, respectively) and to solvent carbons in the ^{13}C NMR spectra (77.0 and 39.43 ppm for CDCl$_3$ or DMSO-d_6, respectively). Carbon substitution degrees were established by distortionless enhancement by polarization transfer (DEPT) pulse sequences. Infrared (IR) spectra were measured using a Nicolet Avatar 320 spectrometer. Mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded in the electron impact mode (EI, 70 eV) using a Micromass VG Autospec spectrometer.

Horseradish peroxidase (HRP), ovalbumin (OVA), and o-phenylenediamine (OPD) were purchased from Sigma-Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were used for conjugate purification. Polyclonal goat anti-rabbit immunoglobulins conjugated to peroxidase (GAR−HRP) were from Bio-Rad (Hercules, CA, USA). Bovine serum albumin (BSA) fraction V was purchased from Roche Applied Science (Mannheim, Germany). Fetal bovine serum (FBS) and Freund's adjuvants were from Sigma-Aldrich. Costar flat-bottom high-binding 96-well polystyrene ELISA plates were from Corning (Corning, NY, USA). Triphenylphosphate was from Sigma-Aldrich, and primary−secondary amine was from Scharlab (Barcelona, Spain). Ultraviolet−visible spectra and ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments. An Agilent Technologies (Santa Clara, CA, USA) 6890N GC network system, equipped with a 7683 Series autosampler, a HP-5MS (30 m × 0.25 mm × 0.25 μm) capillary column, and a 5973

mass selective detector, was employed for chromatographic separations.

Different buffers were employed in this study. Composition, concentration, and pH of the employed buffers were as follows: (i) CB, 50 mM sodium carbonate−bicarbonate buffer, pH 9.6; (ii) enzyme substrate buffer, 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4; (iii) PB, 100 mM sodium phosphate buffer, pH 7.4; (iv) PBS, 10 mM sodium phosphate buffer, pH 7.4, with 140 mM NaCl; (v) PBST, PBS containing 0.05% (v/v) Tween 20; (vi) PBST-FBS, PBST containing 10% (v/v) FBS; (vii) PBT-6.0, 200 mM sodium phosphate buffer, pH 6.0, containing 0.05% (v/v) Tween 20; (viii) PBT-7.4, 200 mM sodium phosphate buffer, pH 7.4, containing 0.05% (v/v) Tween 20; and (ix) washing solution, 150 mM NaCl containing 0.05% (v/v) Tween 20.

**Scheme 1. Synthetic Pathway To Prepare Hapten CDn5**
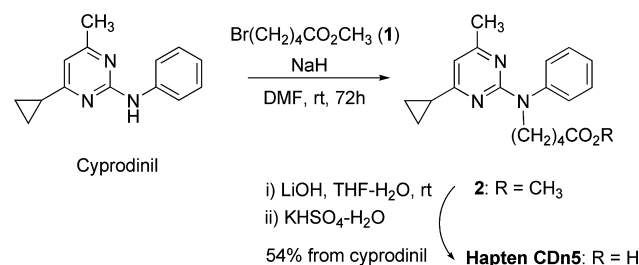
Synthesis of Hapten CDn5 (Scheme 1). *Preparation of Methyl 5-((4-Cyclopropyl-6-methylpyrimidin-2-yl)(phenyl)amino)-pentanoate (2).* A solution of cyprodinil (170 mg, 0,754 mmol) in dry DMF (3 mL) was added dropwise to a stirred suspension of NaH (60% dispersion in mineral oil, 36.9 mg, 0.925 mmol, 1.2 equiv, prewashed with dry pentane) in DMF (2 mL) at 0 °C under nitrogen. After 30 min of stirring at this temperature, the reaction mixture was allowed to warm to room temperature and stirred for 15 min. Then, methyl 5-bromovalerate (**1**, 0.224 mL, 1.51 mmol, 2 equiv) was added, and the mixture was stirred at room temperature for 72 h and then poured into water and extracted with EtOAc. The combined organic layers were washed with aqueous saturated solutions of LiCl and brine and then dried over anhydrous sodium sulfate. The residue remaining after solvent evaporation was chromatographed over silica gel, using hexane/EtOAc 9:1 as eluent, to give a ca. 7:3 mixture (based on ^{1}H NMR spectra) of ester **2** and unreacted cyprodinil (203.4 mg).

Preparation of 5-((4-Cyclopropyl-6-methylpyrimidin-2-yl)-(phenyl)amino)pentanoic Acid (Hapten CDn5) (Scheme 1). LiOH·H$_2$O (158.4 mg, 3.80 mmol) was added to a solution of the previously obtained mixture of ester **2** and cyprodinil (200 mg, ca. 0.41 mmol of **2**) in THF (3.3 mL) and water (1.5 mL). The reaction mixture was stirred at room temperature for 24 h, and then the THF was eliminated in the rotavapor. The residue was diluted with water (4 mL), acidified to pH 3−4 by the addition of solid KHSO$_4$, and extracted with EtOAc. The combined organic layers were dried over Na$_2$SO$_4$, and the solvent was removed under vacuum to afford the crude product that was purified by column chromatography, using CHCl$_3$/MeOH 9:1 as eluent, to give, in order of elution, starting cyprodinil (53 mg) and the desired hapten CDn5 (132.6 mg, 54% from cyprodinil) as a solid: mp 118−121 °C (crystallized from hexane/benzene); ^{1}H NMR (300 MHz, CDCl$_3$) δ 10.0 (1H, br s, COOH), 7.37−7.17 (5H, m, Ph), 6.37 (1H, s, H-5 Pym), 3.99 (2H, t, J = 6.9 Hz, H-5), 2.37 (2H, t, J = 6.9 Hz, H-2), 1.67 (4H, m, H-3 and H-4), 1.75 (1H, m, CH-Cy), 0.96 and 0.87 (2H each, each m, CH$_2$CH$_2$−Cy); ^{13}C NMR (75 MHz, CDCl$_3$) δ 179.2 (C-1), 171.4 (C-2 Pym), 166.2 (C-4 Pym), 161.6 (C-6 Pym), 144.6 (C-1 Ph), 128.6 (C-3/C-5 Ph), 127.4 (C-2/C-6 Ph), 125.1 (C-4 Ph), 108.5 (C-5 Pym), 49.6 (C-5), 33.7 (C-2), 27.4 (C-4), 23.9 (Me), 21.9 (C-3), 16.5 (CH-Cy), 9.8 (CH$_2$CH$_2$−Cy); IR (KBr) v_{max}/cm^{-1} 3400−2600, 3009, 2940, 2861, 1698, 1574, 1558, 1473, 1398, 1307, 1236, 1100, 959, 819, 702; MS (EI) m/z (%) 325 (M$^+$, 25), 324 (11), 310 (2), 253 (6), 252

(33), 250 (2.5), 239 (19), 238 (100), 225 (19), 224 (21), 222 (3), 210 (2), 163 (6); HRMS m/z calculated for $C_{19}H_{23}N_3O_2$ 325.17903, found 325.17857.

Scheme 2. Synthetic Pathway To Prepare Hapten CDp6
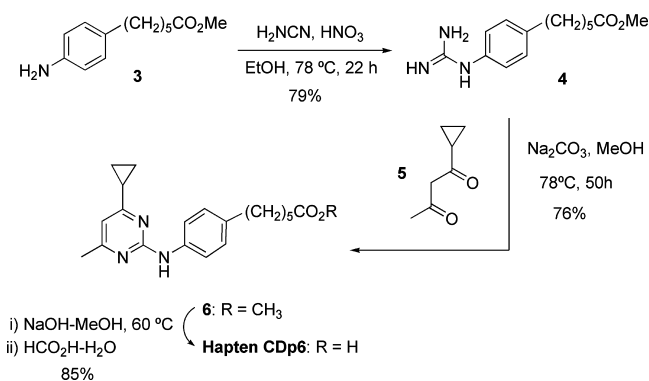
Synthesis of Hapten CDp6 (Scheme 2). *Preparation of Methyl 6-(4-Guanidinophenyl)hexanoate (4).* A mixture of methyl 6-(4-aminophenyl)hexanoate (**3**, 115 mg, 0.515 mmol), 50% aqueous cyanamide solution (75 μL, 0.780 mmol, 1.5 equiv), and concentrated HNO3 (70% w/v, 40 μL, 0.517 mmol, 1 equiv) in EtOH (1 mL) was placed in a dark glass ampule under nitrogen. The ampule was sealed under vacuum and then heated with stirring at 78 °C for 22 h. The content of the ampule was concentrated to dryness under vacuum, and the obtained dark orange oily residue was purified by chromatography, using CHCl3/MeOH 9:1 as eluent, to give the aryl guanidine **4** (134.5 mg, 79%) as a white solid: mp 119−121 °C (crystallized from CHCl3/EtOAc); ^1H NMR (300 MHz, CDCl3) δ 9.54 (1H, s, NH-C(*NH*)−*NH2*), 7.80 (3H, br s, *NH*−C(NH)−*NH2*), 7.20 (2H, apparent d, part AA′ of an AA′BB′ system, J = 8.1 Hz, H-2/H-6 Ph), 7.13 (2H, apparent d, part BB′ of an AA′BB′ system, J = 8.1 Hz, H-3/H-5 Ph), 3.64 (3H, s, CO2Me), 2.60 (2H, t, J = 7.5 Hz, H-6), 2.28 (2H, t, J = 7.5 Hz, H-2), 1.61 (4H, m, H-3 and H-5), 1.34 (2H, m, H-4); ^{13}C NMR (75 MHz, CDCl3) δ 174.1 (CO2Me), 156.6 (NH−C(NH)−NH2), 142.7 (C-1 Ph), 131.6 (C-4 Ph), 130.1 (C-2/C-6 Ph), 125.5 (C-3/C-5 Ph), 51.5 (CO2*Me*), 35.1 (C-6), 33.8 (C-2), 30.8 (C-3), 28.5 (C-5), 24.6 (C-4); MS (EI) m/z (%) 263 (M+, 8), 247 (1), 246 (7), 221 (10), 170 (25), 148 (15), 132 (10), 131 (59), 106 (26), 106 (100); HRMS m/z calculated for $C_{14}H_{21}N_3O_2$ 263.16338, found 263.16446.

Preparation of Methyl 6-(4-(4-Cyclopropyl-6-methylpyrimidin-2-ylamino)phenyl)hexanoate (6). A mixture of aryl guanidine **4** (116 mg, 0.355 mmol), 1-cyclopropylbutane-1,3-dione (**5**, 134.5 mg, 1.066 mmol, 3 equiv), Na2CO3 (18.8 mg, 0.177 mmol, 0.5 equiv), and MeOH (1 mL) was prepared inside a glass ampule, which was evacuated, sealed, and heated at 78 °C with stirring for 50 h. The cooled ampule was opened and the solvent removed under vacuum. The residue was chromatographed on silica gel, using CHCl3 as eluent, to afford the pyrimidyl derivative **6** (95 mg, 76%) as a viscous oil: ^1H NMR (300 MHz, CDCl3) δ 7.51 (2H, apparent d, part AA′ of an AA′BB′ system, J = 8.4 Hz, H-3/H-5 Ph), 7.09 (2H, apparent d, part BB′ of an AA′BB′ system, J = 8.4 Hz, H-2/H-6 Ph), 6.94 (1H, br s, H-NH), 6.48 (1H, s, H-5 Pym), 3.66 (3H, s, CO2Me), 2.56 (2H, t, J = 7.5 Hz, H-6), 2.30 (2H, t, J = 7.6 Hz, H-2), 1.84 (1H, m, CH−Cy), 1.62 (4H, m, H-3 and H-5), 1.36 (2H, m, H-4), 1.13 and 0.98 (2H each, each m, CH2CH2−Cy); ^{13}C NMR (75 MHz, CDCl3) δ 174.2 (CO2Me), 172.4 (C-2 Pym), 166.5 (C-6 Pym), 159.9 (C-4 Pym), 137.8 (C-1 Ph), 135.8 (C-4 Ph), 128.6 (C-2/C-6 Ph), 118.6 (C-3/C-5 Ph), 109.7 (C-5 Pym), 51.4 (CO2*Me*), 35.0 (C-6), 34.0 (C-2), 31.1 (C-3), 28.7 (C-5), 24.8 (C-4), 23.8 (Me), 16.8 (CH-Cy), 10.2 (CH2CH2−Cy); IR (KBr) v_{max}/cm^{-1} 3361, 3273, 3192, 3008, 2930, 2855, 1736, 1595, 1530, 1463, 1405, 1364, 1290, 1248, 959, 818, 757; MS (EI) m/z (%) 353 (M+, 81), 352 (12), 323 (2), 322 (7), 252 (5), 239 (21), 238 (100), 221 (6), 187 (6), 106 (58); HRMS m/z calculated for $C_{21}H_{27}N_3O_2$ 353.21033, found 353.21057.

Preparation of 6-(4-(4-Cyclopropyl-6-methylpyrimidin-2-ylamino)phenyl)hexanoic Acid (Hapten CDp6). A solution of methyl ester **6** (128.5 mg, 0.364 mmol) in a mixture of MeOH (4 mL) and 2 M aqueous NaOH (0.73 mL, 1.46 mmol, 4 equiv) was stirred at 60 °C for 2.5 h. The formed yellowish reaction mixture was transferred into a flask and most of the solvent eliminated at reduced pressure. The brownish semisolid residue obtained was dissolved in formic acid (ca. 0.5 mL), diluted with water, and extracted with CHCl3. The combined organic extracts were washed with brine, dried over anhydrous MgSO4, and concentrated to dryness under vacuum to give hapten CDp6 (104.5 mg, 85%) as a practically white solid, which was shown to be nearly pure by NMR and TLC analysis: mp 146−147 °C (crystallized from cold MeOH); ^1H NMR (300 MHz, DMSO-d_6) δ 11.97 (1H, s, COOH), 9.22 (1H, s, NH), 7.62 (2H, apparent d, part AA′ of an AA′BB′ system, J = 8.5 Hz, H-3/H-5 Ph), 7.04 (2H, apparent d, part BB′ of an AA′BB′ system, J = 8.5 Hz, H-2/H-6 Ph), 6.62 (1H, s, H-5 Pym), 2.48 (2H, t, J = 7.5 Hz, H-6), 2.19 (2H, t, J = 7.6 Hz, H-2), 1.92 (1H, m, CH−Cy), 1.52 (4H, m, H-3 and H-5), 1.27 (2H, m, H-4), 0.99 (4H, m, CH2CH2−Cy); ^{13}C NMR (75 MHz, DMSO-d_6) δ 174.4 (CO2Me), 171.4 (C-2 Pym), 166.1 (C-6 Pym), 159.8 (C-4 Pym), 138.6 (C-1 Ph), 134.4 (C-4 Ph), 128.0 (C-2/C-6 Ph), 118.4 (C-3/C-5 Ph), 109.0 (C-5 Pym), 34.3 (C-6), 33.5 (C-2), 30.8 (C-5), 28.1 (C-4), 24.3 (C-3), 23.3 (Me), 16.3 (CH−Cy), 9.8 (CH2CH2−Cy); IR (KBr) v_{max}/cm^{-1} 3306, 3209, 3006, 2968, 2928, 2848, 1684, 1592, 1559, 1517, 1406, 964, 807; MS (EI) m/z (%) 339 (M+, 75), 338 (12), 252 (6), 250 (2), 240 (2), 239 (20), 238 (100), 237 (2), 236 (2), 224 (2), 222 (2), 131 (4); HRMS m/z calculated for $C_{20}H_{25}N_3O_2$ 339.19468, found 339.19430.

Synthesis of Protein−Hapten Conjugates. BSA, HRP, and OVA conjugates were prepared by activation of the free carboxylate group of synthetic haptens (CDn5 and CDp6) with N,N'-disuccinimidyl carbonate (DSC), and purification of the active ester was done as described by Esteve-Turrillas et al.[24] Briefly, 0.082 mmol of hapten and 0.14 mmol of DSC were dissolved in 0.8 mL of dry acetonitrile under nitrogen atmosphere. Next, 0.31 mmol of triethylamine was added, and the mixture was stirred at room temperature until complete consumption of starting material (as observed by thin-layer chromatography). The solution was diluted with CHCl3, washed with saturated NaHCO3 and brine, and dried over anhydrous Na2SO4. After evaporation of the solvent, the remaining residue was purified by column chromatography, using CHCl3 as eluent, affording the N-succinimidyl ester of the hapten (CDn5-NHS or CDp6-NHS) in a pure form.

Immunizing BSA−Hapten Conjugates. A 50 mM solution (200 μL) of CDn5-NHS or CDp6-NHS in DMF was added dropwise over 2 mL of a 15 mg/mL BSA solution in CB. The conjugation reaction was carried out during 4 h at room temperature with moderate stirring. Next, the conjugate was separated from uncoupled hapten by gel filtration on Sephadex G-25, using PB as eluent. Hapten-to-protein molar ratios (MR) of the purified conjugates were calculated using the absorbance values of the conjugate at 260 and 280 nm, by assuming that the molar absorptions of the hapten and the protein were the same for the free and conjugated forms. MRs of the obtained conjugates were 15 and 14 for BSA−CDn5 and BSA−CDp6, respectively. Finally, both purified conjugates were diluted to 1 mg/mL with PB and stored at −20 °C.

Tracer HRP−Hapten Conjugates. A 10 mM solution (100 μL) of CDn5-NHS or CDp6-NHS in DMF was added dropwise over 1 mL of a 2.2 mg/mL HRP solution in CB. Reagents were allowed to couple during 4 h at room temperature with gentle stirring. The prepared conjugate was separated from uncoupled hapten by gel chromatography as described before. Hapten-to-protein MRs were approximately 3 and 5 for HRP−CDn5 and HRP−CDp6, respectively. Purified conjugates were diluted 1:1 (v/v) with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal and stored at −20 °C in amber vials. For daily use, a working aliquot was kept at 4 °C.

Coating OVA−Hapten Conjugates. A 100 mM solution (100 μL) of CDn5-NHS or CDp6-NHS in DMF was added dropwise over 2 mL of a 15 mg/mL OVA solution in CB. The reaction was carried out during 4 h at room temperature with moderate stirring. Then,

conjugates were purified by gel chromatography as described. Determined MRs were 7 and 10 for OVA−CDn5 and OVA−CDp6, respectively. Finally, conjugates were stored at −20 °C in amber vials at 1 mg/mL in PB containing 0.01% (w/v) thimerosal. Additionally, two coating conjugates were prepared with lower MRs by addition of 10 or 50 μL of activated hapten solution over 2 mL of 15 mg/mL OVA solution in CB. The MRs of these new conjugates were 1 and 3 for OVA−CDn5 and 0.8 and 3 for OVA−CDp6.

Antibody Production. Animal manipulation was performed in compliance with the 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. Two female New Zealand white rabbits weighing 1−2 kg were immunized by subcutaneous injection with 0.3 mg of BSA conjugates in 1 mL of a 1:1 mixture of PB and complete Freund's adjuvant. Animals were boosted at 21 day intervals with the same immunogen suspended in a mixture of 0.5 mL of PB and 0.5 mL of incomplete Freund's adjuvant. Whole blood was collected from the ear vein of the rabbits and by intracardiac puncture 10 days after the fourth injection. Blood samples were allowed to coagulate overnight at 4 °C. Sera were obtained by centrifugation and diluted 1:4 with PBS containing 0.01% thimerosal and stored at 4 °C in amber vials for daily use. For long-term storage, the generated pAbs were precipitated twice with ammonium sulfate.

Direct Competitive ELISA. Microwells were coated with 100 μL of a pAb dilution in CB by overnight incubation at room temperature. Coated plates were washed four times with washing solution and then received 50 μL per well of analyte in PBS plus 50 μL per well of HRP tracer solution in PBST. After 1 h of incubation at room temperature, plates were washed as before, and signal was produced by adding 100 μL per well of freshly prepared 2 mg/mL OPD and 0.012% (v/v) H_2O_2 in enzyme substrate buffer. The enzymatic reaction was stopped after 10 min at room temperature with the addition of 100 μL per well of 2.5 M sulfuric acid, and absorbances were immediately read at 492 nm with a reference wavelength at 650 nm. For sample analysis, fruit juices were diluted with 20% ethanol in deionized water (v/v), and HRP tracer solutions were prepared in PBT-6.0.

Indirect Competitive ELISA. Coating was performed with 100 μL per well of an OVA conjugate solution in CB by overnight incubation at room temperature. Coated plates were washed four times with washing solution after each incubation step. The competitive reaction was performed with 50 μL per well of analyte solution in PBS plus 50 μL per well of pAb dilution in PBST. The immunological reaction took place during 1 h at room temperature, and plates were washed again. Next, 100 μL per well of a 10000-fold dilution of GAR−HRP conjugate in PBST-FBS was added and incubated for 1 h at room temperature. Finally, plates were washed and the signal was generated as aforementioned. For sample analysis, fruit juices were diluted with deionized water, and the pAb solution was prepared in PBT-7.4.

Reference Procedure. QuEChERS method (AOAC International official method)[25] was employed for the extraction of cyprodinil from spiked juice samples and the later purification of extracts. A 5 mL sample, plus 50 μL of internal standard (triphenylphosphate 50 mg/L), was introduced in a 15 mL polypropylene centrifuge tube containing 0.5 g of sodium acetate and 2 g of anhydrous magnesium sulfate. The sample was then extracted with 5 mL of 1% (v/v) acetic acid in acetonitrile by vortexing for 1 min and centrifuged for 5 min at 2200g. Then, a 1 mL extract was introduced to a 2 mL polypropylene centrifuge tube containing 50 mg of primary−secondary amine and 150 mg of magnesium sulfate anhydrous, vortexed during 1 min, and centrifuged for 5 min at 2200g. Finally, the purified extract was filtered through a 0.22 μm Teflon filter and analyzed by GC-MS as follows. One microliter of purified extract was injected in splitless mode at 300 °C by employing helium as carrier with a constant flow of 1 mL/min. The oven temperature program (150 °C) was held for 1 min, increased at a rate of 10 °C/min to 280 °C, and held constant at that temperature during 5 min. The transfer line and source temperatures were 280 and 250 °C, respectively. Electron impact ionization at 70 eV was used, and the employed quantification ions were m/z 224 and 225 for cyprodinil and m/z 325 and 326 for triphenylphosphate.

Data Analysis. Eight-point standard curves, including a blank, were prepared by 10-fold serial dilutions in PBS from a 5 mg/mL cyprodinil stock solution. Sigmoidal curves were fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay sensitivity was estimated as the analyte concentration affording a 50% inhibition (IC_{50}) of the maximum absorbance (A_{max}).

■ RESULTS AND DISCUSSION

Synthesis of Haptens and Molecular Modeling. The synthesis of hapten CDn5, which holds the spacer arm at the amine nitrogen atom and keeps both aromatic rings unmodified, was prepared in a simple way from the cyprodinil molecule itself (Scheme 1). First, the aryl amide anion derived from the reaction of cyprodinil and NaH was alkylated with the alkyl bromide **1**. That alkylation reaction took place very smoothly to afford a mixture of alkylated product **2** and unreacted starting material in a ratio of 70:30, which could not be separated by conventional chromatographic procedures and was therefore directly submitted to basic methyl ester hydrolysis with acid workup to give hapten CDn5, which was then easily separated from cyprodinil by column chromatography. From a practical point of view, the synthesis of CDn5 can be considered satisfactory because it allowed the preparation of this hapten in only two synthetic steps with an overall yield of 54%. On the other hand, the synthesis of hapten CDp6 was somewhat more laborious and required the previous preparation of the arylguanidine **4**, which holds, at the required position of the phenyl ring, a hydrocarbon chain equivalent to that of hapten CDn5 (Scheme 2). This arylguanidine was prepared in good yield by reaction of the 4-alkyl-substituted aniline **3** with excess cyanamide in ethanolic acidic medium. Formation of the pyrimidine ring was then achieved by base-promoted condensation between the arylguanidine **4** and 1-cyclopropylbutane-1,3-dione (**5**). The reaction proceeded very slowly, so >2 days was needed for completion, providing the desired intermediate **6** in a 76% yield. Finally, the synthesis of hapten CDp6 was readily achieved by basic hydrolysis of the methyl ester moiety of **6** using methanolic NaOH. The overall yield for the transformation of aniline **3** into hapten CDp6 through this three-step sequence was ca. 51%.

A detailed conformational analysis of cyprodinil showed that this molecule exists in a rapid equilibrium between several energetically similar extended conformations, all of them having in common a nearly coplanar arrangement of the phenyl-aminopyrimidine moiety. The 3D spatial representation of the most stable conformation (Figure 1) shows that the aminic nitrogen atom and the p-phenyl position could be, in principle, appropriate derivatization sites for the attachment of the hydrocarbon spacer arm. The corresponding haptens (CDn5 and CDp6) preserved the complete skeleton of cyprodinil, and no or few alterations of spatial and/or electronic conformations could be reasonably expected.

Both haptens were activated using DSC, which allowed us to easily purify the active ester, to finely modulate the MR of the conjugates, and to avoid undesired coupling reactions of secondary byproduct.[24] Moreover, the same conjugation procedure could be followed both for immunizing and for assay conjugates.

Immunoreagent Evaluation. *Affinity.* Two rabbits were immunized with each BSA conjugate, and the immunoglobulins were partially purified from the animal crude serum by ammonium sulfate precipitation, affording four different pAbs,
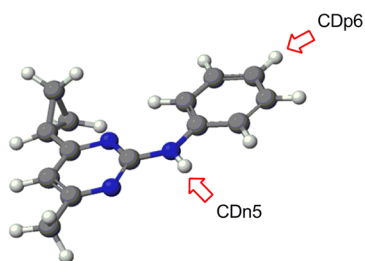
Figure 1. Most stable conformation of cyprodinil, calculated using CONFLEX with MM3 molecular mechanics to systematically search for low-energy conformers, followed by geometry optimization in MO-G using AM1 parameters and including aqueous solvation effects simulated by COSMO (CAChe WorkSystem Pro, version 7.5.0.85). The elements are represented in the following manner: carbon, gray; hydrogen, white; nitrogen, blue. The arrows point out the tethering sites of the hydrocarbon spacer arm of haptens CDn5 and CDp6.

that is, two from BSA−CDp6 (rCDp6#1 and rCDp6#2) and two from BSA−CDn5 (rCDn5#1 and rCDn5#2). A checkerboard competitive screening analysis was carried out for every antibody in two different immunoassay formats (direct and indirect cELISA) using a cyprodinil standard curve from 0.005 to 5000 ng/mL plus a blank. For direct assays, plates were coated with three dilutions of the corresponding antibody (3000-, 10000-, and 30000-fold), and four tracer conjugate concentrations (from 3 to 100 ng/mL) were evaluated under competitive conditions. For indirect assays, plates were coated with OVA−CDp6 or OVA−CDn5 conjugates at different concentrations (10, 100, and 1000 ng/mL), and the competitive step was carried out using a range of six antibody dilutions (from 3000- to 10^6-fold). As a summary, the representative parameters of selected curves for homologous direct and indirect cELISAs are listed in Table 1. CDp6-type antibodies clearly exhibited a higher affinity to cyprodinil than CDn5-type antibodies, particularly in the i-cELISA format. The location of the spacer arm in hapten CDp6 seems to favor the presentation of the cyprodinil mimic to the antigen receptor molecules of the immune system, which eventually results in antibody binding sites that are better shaped to accommodate the free analyte. Immunization with haptens holding the linker at a lateral position of the target molecule, like in CDp6, has been previously reported to produce antibodies with higher affinity than those from haptens with a central tethering site, like in CDn5.[22,26,27] Assay heterology was also evaluated using conjugates of CDp6 with anti-CDn5 pAbs, and vice versa, but no binding was obtained in any of the studied formats,

supporting the idea that the area of the hapten that is free to interact with antibodies largely differs in both derivatives.

Selectivity. Cross-reactivity (CR) studies were carried out with the four pAbs in the direct and indirect cELISA formats. First, recognition of the two other anilinopyrimidine fungicides (mepanipyrim and pyrimethanil) was evaluated (Table 2). Assays were run with immunoreagent concentrations affording both an A_{max} value close to 1.0 and the lowest IC_{50} value for cyprodinil (Table 1). Standard curves were prepared up to 10 μM with every fungicide in PBS, and the IC_{50} values from competitive assays were compared. From this study, it was observed that CR values for a particular antibody were not dependent on the assay format. In addition, very similar values were obtained for the two antibodies produced with the same immunizing conjugate. A slight recognition of the cyprodinil congeners (CR values were between 1.3 and 7.6%) was found by pAbs obtained with the BSA−CDp6 immunogen, whereas antibodies from rabbits immunized with BSA−CDn5 were able to bind those two compounds with equal or even higher affinity than cyprodinil (CR values from 81 to 163%). Selectivity of the produced pAbs was in accordance with Landsteiner's principle; that is, antibodies mainly interact with those moieties of the hapten molecule located distally from the linker arm.[28] Therefore, the higher selectivity of CDp6-type pAbs was probably due to a better exposure of the substituted pyrimidine ring of the analyte (the differing moiety among anilinopyrimidine fungicides) in hapten CDp6 than in hapten CDn5 (R^1 in Table 2). Additionally, 20 widely used fungicides in grape and apple crops (kresoxim-methyl, trifloxystrobin, pyraclostrobin, azoxystrobin, dimoxystrobin, fluoxastrobin, metominostrobin, picoxystrobin, fenhexamid, captan, procimidone, tolylfluanid, cyazofamid, tebuconazole, fenamidone, fludioxonil, vinclozolin, imidacloprid, boscalid, and benzanilide) were also assayed up to 10 μM, but in this case no recognition was observed by any of the produced pAbs.
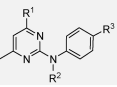
Assay Optimization. *MR of the Coating Conjugate.* We had previously observed that, in the case of i-cELISAs, assay sensitivity can be improved by reducing the hapten molarity of the coating conjugate.[29] Thus, two additional OVA conjugates were prepared with lower MRs. Table S1 of the Supporting Information summarizes a checkerboard competitive screening study for pAb rCDp6#2 with coating conjugates at different MRs and concentrations. It was found that at high coating concentrations, such as 1000 ng/mL, the IC_{50} value improved when the MR of the conjugate was reduced 10-fold. However, the use of the conjugate with the highest hapten loading at a low concentration resulted in a slightly better sensitivity, so the
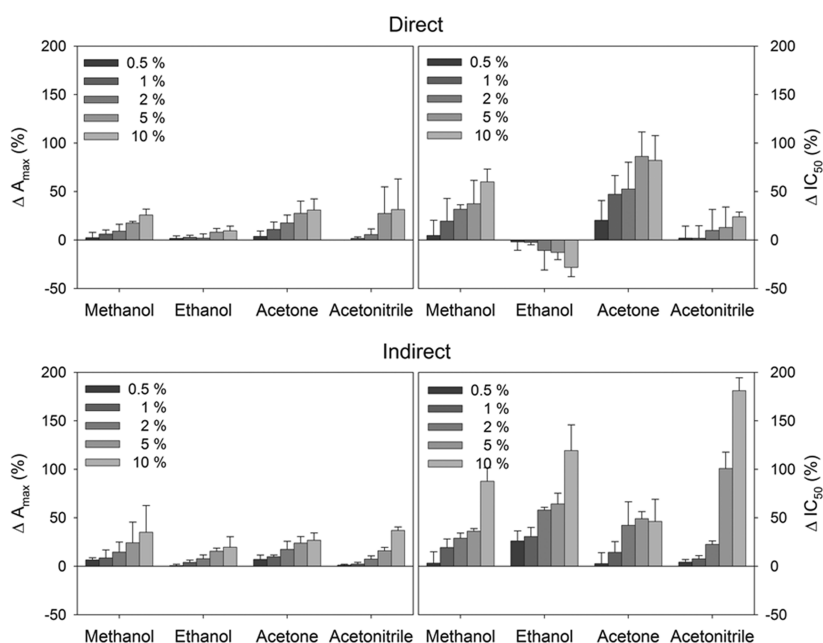
Table 1. Checkerboard Competitive Characterization of the Produced pAbs Using Homologous Conjugates

	direct cELISA				indirect cELISA			
pAb	pAb dil ($\times10^3$)	[HRP−hapten] (ng/mL)	A_{max}	slope	IC_{50} (ng/mL)	pAb dil ($\times10^3$)	[OVA−hapten][a] (ng/mL)	A_{max}	slope	IC_{50} (ng/mL)
rCDp6#1	30	3	0.74	0.55	2.3	3	10	1.42	0.57	8.8
		10	1.68	0.55	4.1	10	10	0.93	0.58	3.8
rCDp6#2	30	3	0.92	0.64	1.8	10	10	1.10	0.64	3.3
		10	2.21	0.64	2.0	30	10	0.83	0.62	1.8
rCDn5#1	30	3	0.50	0.55	45.5	30	10	1.32	0.39	1400.2
		10	1.30	0.59	59.0	100	10	0.97	0.39	756.3
rCDn5#2	30	3	0.48	0.68	59.3	30	10	1.48	0.88	566.4
		10	1.23	0.69	62.9	100	10	0.95	3.46	273.7

[a]The MRs of OVA−CDp6 and OVA−CDn5 were 10 and 7, respectively.

Table 2. Cross-Reactivity Values (% ± s, n = 2) of the Produced Antibodies with the Other Anilinopyrimidine Fungicides

	pAb	cELISA	R¹ = cyclopropyl R² = H R³ = H (cyprodinil)	R¹ = CH₃ R² = H R³ = H (pyrimethanil)	R¹ = CCCH₃ R² = H R³ = H (mepanipyrim)
hapten CDp6	rCDp6#1	direct	100	5.1 ± 0.4	1.3 ± 0.2
R¹ = cyclopropyl		indirect	100	7.6 ± 0.5	1.5 ± 0.3
R² = H	rCDp6#2	direct	100	4.3 ± 0.2	2.2 ± 0.3
R³ = (CH₂)₅CO₂H		indirect	100	4.5 ± 1.3	2.5 ± 0.6
hapten CDn5	rCDn5#1	direct	100	118.9 ± 5.1	94.2 ± 3.4
R¹ = cyclopropyl		indirect	100	144.5 ± 29.2	163.3 ± 15.8
R² = (CH₂)₄CO₂H	rCDn5#2	direct	100	128.0 ± 7.0	81.3 ± 4.5
R³ = H		indirect	100	145.2 ± 22.4	110.2 ± 0.3

Figure 2. Percentage of variation of A_{max} and IC_{50} values as a function of the concentration of different organic solvents for direct and indirect cELISAs using pAb rCDp6#2. For direct assays, plates were coated with a 30000-fold antibody dilution, and the competitive step was performed with a 3 ng/mL homologous tracer solution. For indirect assays, OVA−CDp6 (MR = 10) coating concentration was 10 ng/mL, and a 20000-fold antibody dilution was employed. Results are the mean of three independent determinations.

coating immunoreagent with MR = 10 was selected for further studies.

Solvent Tolerance. In this study, the capacity of the direct and indirect immunoassays to withstand moderate concentrations of common solvents that are widely employed for pesticide extraction from foods was evaluated. To this purpose, cyprodinil standard curves were prepared in PBS containing different proportions (from 0.5 to 10%) of methanol, ethanol, acetone, or acetonitrile, whereas tracer or antibody solutions were prepared in PBST. As seen in Figure 2, higher concentrations of any solvent increased the A_{max} and IC_{50} values in both cELISAs. The only exception to this behavior was with ethanol in the direct format, when an increase in solvent concentration gave rise to a higher A_{max} and, unusually, to a lower IC_{50} value. Accordingly, 20% ethanol was employed to prepare standard curves and samples for food analysis using this immunoassay.

Buffer Studies. The influence of buffer ionic strength and pH was evaluated at room temperature following a central composite design, consisting of a two-level full factorial design ($\alpha = 1.414$) with 2 factors and 3 replicates, which included 12 cube, 12 axial, and 15 center points, and involving a total of 39 randomized buffer assays. The evaluated buffers had ionic strength values between 50 and 300 mM and pH values from 5.5 to 9.5. Those buffers were prepared as described by Parra et

al.[27] For the competitive step, cyprodinil standard curves were run in water, and tracer or antibody solutions were prepared in each of the studied buffers. A_{max} and IC_{50} values of the resulting curves were fitted, by a multiple regression equation, with the assayed pH and ionic strength values of the buffer using Minitab 14.1 software (Minitab Inc., State College, PA, USA).

Concerning the d-cELISA, both an increase of A_{max} and a decrease of the IC_{50} value were observed at lower pH values (Figure 3). Therefore, for further experiments, an alternative buffer system was chosen with a higher buffering capacity (100 mM phosphate) and an optimal pH of 6.0. On the other hand, the sensitivity of the indirect assay was strongly influenced by the ionic strength (Figure S1 of the Supporting Information) of the reaction medium, whereas differences in pH had little effect. In this case, it was important to keep the ionic strength of the assay between 180 and 250 mM. Thus, 100 mM phosphate and pH 7.4 were chosen as the optimum conditions for this assay (I = 220 mM at 25 °C).

Figure 4 shows the inhibition curves of the optimized immunoassays for cyprodinil with pAb rCDp6#2. Assay sensitivity values were 1.6 and 2.8 ng/mL for the direct and indirect cELISAs, respectively. Low slopes (around 0.6) and low background signals were obtained for the studied assays. As a consequence, wide working ranges (defined as IC_{10}−IC_{90}) spanning 2 orders of magnitude were derived: from 0.03 to 74.6
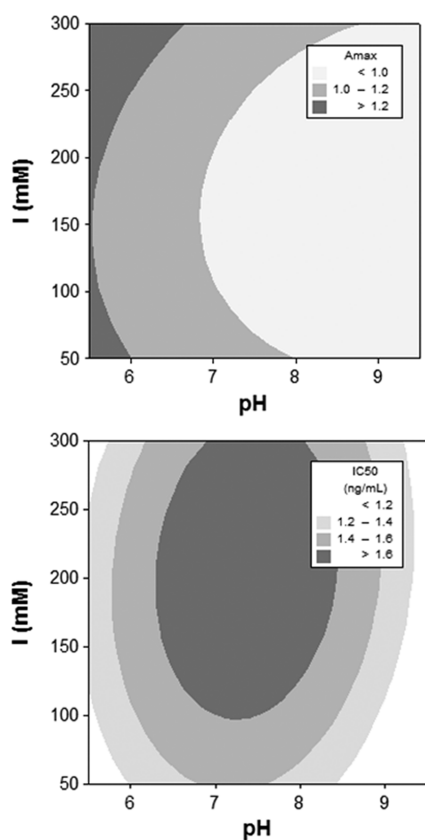
Figure 3. Influence of pH and ionic strength (I) over the A_{max} and IC_{50} values of the selected d-cELISA using pAb rCDp6#2.
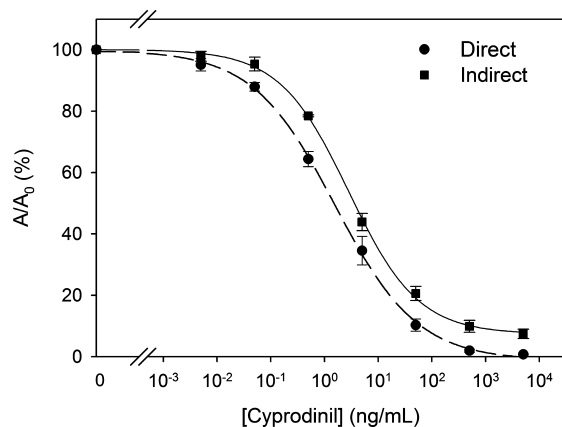
Figure 4. Inhibition curves of the optimized direct and indirect cELISAs for cyprodinil using pAb rCDp6#2. Direct assays were performed with plates coated with a 30000-fold pAb dilution. Competitive reactions were run with HRP−CDp6 at 3 ng/mL in PBT-6.0 and with a cyprodinil standard curve in 20% (v/v) ethanol in water ($A_{max} = 1.0 \pm 0.2$, $r^2 = 0.9995$). For indirect assays, plates were coated with a 10 ng/mL OVA−CDp6 (MR = 10) solution and a 20000-fold pAb dilution in PBT-7.4 was used for the competitive step with cyprodinil standards in water ($A_{max} = 1.3 \pm 0.2$, $r^2 = 0.9995$). Results are the mean of three independent determinations.

ng/mL for the d-cELISA and from 0.11 to 75.2 ng/mL for the indirect assay.

Cyprodinil Determination in Fruit Juices. *Matrix Effects.* Apple and red grape juices were selected as model commodities to evaluate the applicability of the optimized immunoassays. Commercial fruit juices were obtained from

local supermarkets and were fortified with different amounts of cyprodinil. Previously, it was checked by GC-MS that juices did not contain detectable residues of cyprodinil. The most straightforward strategy to remove possible matrix interferences in immunoanalysis is by direct dilution of the samples, a feasible approach for immunoassays endowed with high sensitivity. The minimum dilution factor to obtain reliable results was determined using cyprodinil standard curves prepared in several dilutions of grape and apple juice (10-, 50-, 100-, and 250-fold). Each standard curve was evaluated with the optimized direct and indirect assays. The resulting inhibition curves for each studied juice in every assay format can be seen in Figure S2 of the Supporting Information. From these results, it was established that a sample dilution of at least 250-fold was required to efficiently remove matrix effects in both immuno-assays.

Recovery Studies. Apple and red grape juices were fortified with cyprodinil at 10, 20, 50, 100, 500, 1000, and 5000 ng/mL, using small volumes of 1, 10, or 100 μg/mL cyprodinil stock solutions in DMF. Spiked samples were diluted before analysis as previously indicated. For both food samples, the best reproducibility was achieved with the d-cELISA (Table 3).

Table 3. Recovery Values from Cyprodinil-Spiked Fruit Juice Samples Using the Developed Immunoassays

| cELISA	[CD][b] (ng/mL)	apple juice[a] recovery (%)	apple juice[a] RSD (%)	red grape juice[a] recovery (%)	red grape juice[a] RSD (%)
direct	20	115	8	102	6
	50	110	13	94	10
	100	104	2	95	30
	500	102	4	103	7
	1000	114	13	100	14
	5000	102	17	99	11
indirect	20	−[c]	−	−	−
	50	100	3	90	24
	100	90	22	91	14
	500	124	20	113	9
	1000	125	26	104	9
	5000	103	20	110	18

[a]Samples were diluted 250-fold before being assayed. [b]Cyprodinil. [c]Lower than LOQ.

From this study, the limits of quantification (LOQ) for cyprodinil residues in both fruit juices could be set at 20 ng/mL for the d-cELISA and at 50 ng/mL for the indirect assay, estimated as the minimum evaluated concentration that afforded satisfactory recovery values (between 80 and 130%). These results demonstrate the suitability of the developed immunoassays for the analysis of cyprodinil in apple and grape juices at concentrations comparable to those attained by chromatographic methods[30,31] or to those expected to be found in juices that are elaborated from raw fruits containing cyprodinil residues near MRL values.[32−34] Finally, a limited-sized survey on the presence of cyprodinil residues in commercial bottled Spanish juices was undertaken. Twelve apple juices, 5 white grape juices, 3 red grape juices, and 3 juices containing both apple and grape were assayed by the developed cELISAs, and no samples with incurred residues were found.

Comparison with GC-MS. To validate the developed immunoassays for the analysis of cyprodinil residues in fruit

4809

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juices, juice samples were measured by the two developed cELISAs and results were compared with those obtained by GC-MS, which was considered here as the reference method. The LOQ of the chromatographic procedure (70 ng/mL) was of the same order as those of the developed cELISAs. A total of 16 juice samples (8 apple and 8 red grape) were blind spiked by an external operator and measured. No sample treatment other than dilution in deionized water was carried out for immunochemical determinations, whereas a QuEChERS extraction was required for GC-MS analysis. Equivalent results were obtained by cELISA and by GC-MS; obtained cyprodinil concentrations in juice samples ranged from 149 to 3400 μg/L (Table S2 in the Supporting Information). Deming regression analysis, which takes into account the errors of both sets of measurements for a wide range of concentration values, was used for testing the equivalency of results attained by both techniques.[35−37] Figure 5 shows the obtained Deming
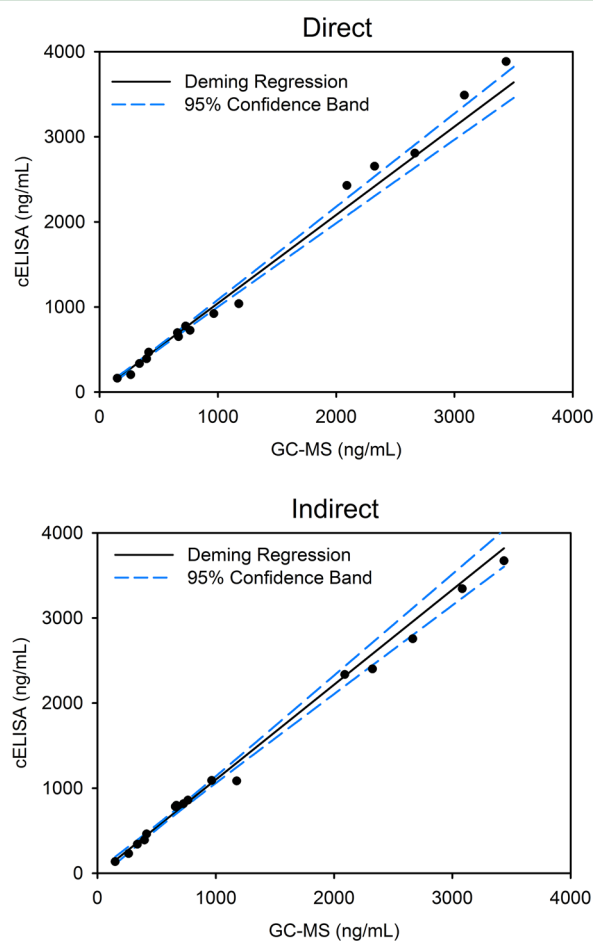
Figure 5. Deming regression between cELISA and GC-MS with a 95% confidence interval, determined using Sigma Plot (version 12.0) software from Systat Software Inc. (Chicago, IL, USA).

regression for direct and indirect cELISAs with 95% confidence interval bands. The comparison of d-cELISA with GC-MS afforded a Deming regression with a slope of 1.04 (confidence interval from 0.98 to 1.09) and an intercept of 6 (confidence interval from −20 to 31), indicating that results were statistically comparable. The Deming regression for i-cELISA had a slope of 1.11 (confidence interval from 1.04 to 1.19) and an intercept of −16 (confidence interval from −68 to 35), showing a slight overestimation of the indirect assay.

Two original cyprodinil-derived haptens have been designed and synthesized using equivalent spacer arms located at different sites, either at the phenyl ring or at the aminic nitrogen of the molecular framework. A single activation reaction with DSC was performed, and the purified N-succinimidyl ester of each hapten was conjugated to different carrier proteins. Bioconjugates were employed to produce pAbs against cyprodinil and to develop competitive immunoassays. Linker tethering site was shown to be a determinant factor to produce high-quality antibodies, in terms of affinity and selectivity. For selection of the best immunoreagent combination, a competitive checkerboard approach was followed using direct and indirect cELISAs. Hapten heterology can usually improve immunoassay sensitivity; however, site heterologies between haptens CDn5 and CDp6 were probably too severe, so only homologous assays could be developed. Two immunoassays were optimized and characterized, reaching IC_{50} values for cyprodinil in the low nanogram per milliliter range. Interestingly, a higher sensitivity was achieved with the direct competitive assay when 20% ethanol was employed for standard or sample preparation. The LOQs of the developed immunoassays were well below the international MRLs for cyprodinil in grapes and apples. Therefore, the produced immunoreagents could be suitable tools for cyprodinil residue analysis in foodstuffs using a reliable, rapid, and low-cost methodology.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Influence of the MR of the coating conjugate, pH, and ionic strength study with the selected i-cELISA, and fruit juice matrix effects over the optimized immunoassays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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