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ARTICLE

Hapten Synthesis and Polyclonal Antibody-Based Immunoassay Development for the Analysis of Forchlorfenuron in Kiwifruit

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High-affinity polyclonal antibodies directed against the synthetic cytokinin forchlorfenuron (CPPU) were produced from three immunizing haptens with equivalent spacer arms located at different positions. A competitive immunoassay was developed with a limit of detection in buffer of 12.42 ± 3.06 ng/L. In addition, the ability of the produced antibodies to recognize a set of synthetic CPPU analogues was studied. It was evidenced that the linker position had a strong impact on the specificity of the generated polyclonals, which were more sensitive to changes at moieties of the target analyte located furthest from the derivatization site of the immunogen. Finally, matrix effects of gold and green kiwifruit over assay parameters were evaluated. Excellent recoveries and low coefficients of variation were found with just a 100-fold dilution of the sample in buffer, hence indicating the effectiveness of the developed immunoassay as an analytical tool for monitoring this agrochemical in kiwifruit samples.

KEYWORDS: CPPU; cytokinin; phenylurea derivative; immunoassay; Landsteiner; antibody specificity; spacer arm; functionalization site; hapten heterology; matrix effect; kiwifruit

INTRODUCTION

Diphenylurea derivatives have been proven to exert many different biological functions in the plant kingdom. Since it was discovered by serendipity that 1,3-diphenylurea (dPU) displayed cytokinin activity (*1*), a great effort was made to elucidate the chemical modifications in the molecule that afforded a significant increase of its effect in plants. Following the studies of Bruce and Zwar (*2*, *3*) on the physiological action of 500 urea derivatives, Takahashi et al. (*4*) synthesized a battery of *N*-phenyl-*N*′-(4-pyridyl)urea compounds and evaluated the influence of the introduced modifications over the cytokinin function. Among these chemicals, forchlorfenuron (**Table 1**), also named CPPU (1-(2-chloro-4-pyridyl)-3-phenylurea), displayed 10 times higher activity than 6-benzylaminopurine, a well-known synthetic adenine-substituted cytokinin that shares the characteristic chemical structure of naturally occurring hormones. The remarkable biological activity of CPPU in different vegetal species has been extensively studied (*5*), and it has been mainly attributed to the 4-pyridyl ring of the molecule. Particularly, the distance between the nitrogen of the pyridine ring and the urea bridge (*6*), and the *meta* position of the chlorine atom in the heterocycle seem to play a major role (*4*, *7*). Despite the many functions that CPPU seems to display, the agrochemical interest has been mainly focused on its capacity to increase fruit size, especially in kiwifruit and table grape (*8*−*10*). In 2006, CPPU received approval for inclusion into Annex I of Directive 91/414/EEC of the European Union as a phytoregulator in the kiwifruit production (*11*), and a maximum residue limit (MRL) of 50 μg/kg was established for this commodity.

Nowadays, there is an increasing demand for high-quality fruits in the international market, particularly regarding the fruit size. This fact has hugely contributed to the commercial success of CPPU all around the world, thus making it feasible that residues of this agrochemical could eventually reach the consumer. In this respect, analytical tools able to identify CPPU-positive samples in kiwifruit, and also in other sorts of fruits because of illicit use, are required. In the food safety field, the control of chemical contaminants and residues, such as pesticides, toxins, hormones, antibiotics, and drugs, has been traditionally carried out by chromatographic techniques. Different methods based on liquid chromatography coupled to diverse detection systems, such as UV and MS, have been described for monitoring CPPU in different vegetal products (*12*−*14*). In the past 3 decades, immunochemical methods, mainly the enzyme-linked immunosorbent assay (ELISA), have gained an important role as analytical tools in the agrochemical area, especially because of their simplicity and high cost-effectiveness and because a simpler sample pretreatment is demanded. Several antibody-based immunoassays (IAs) have been developed for the analysis of urea-derived pesticides belonging to the family of arylureas (*15*), sulfonylureas (*16*), and benzoylphenylureas (*17*). In a previous work (*18*), the production of monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) with high affinity and specificity against CPPU

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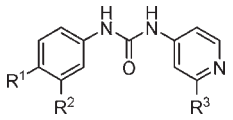
Table 1. Chemical Structure of Forchlorfenuron and Haptens Used To Prepare the Immunogens and Assay Conjugates



compd	R^1	R^2	R^3
forchlorfenuron	H	H	Cl
hapten p6	$(CH_2)_5CO_2H$	H	Cl
hapten m6	H	$(CH_2)_5CO_2H$	Cl
hapten s5	H	H	$S(CH_2)_4CO_2H$

was published by our group. In that article, the importance of the length of the spacer arm was studied at a single derivatization position. In the present study, two additional haptens functionalized at opposite positions of the molecule were prepared with the aim of studying the significance of the derivatization site. Rabbit antisera were produced as an unbiased reagent representing the whole response of the animal immune system. The obtained pAbs were characterized in the antibody-coated direct competitive ELISA (dc-ELISA) and the conjugate-coated indirect competitive ELISA (ic-ELISA) formats in order to evaluate the correlation between the structure of the immunogenic hapten and the affinity and specificity of the produced antibodies. In addition, the influence of hapten heterology on assay sensitivity was assessed. The optimized ELISA was finally applied to the determination of CPPU in kiwifruit samples.

MATERIALS AND METHODS

Chemicals and Instrumentation. Analytical standards of forchlorfenuron [1-(2-chloro-4-pyridyl)-3-phenylurea] (CAS Registry No. 68157-60-8, MW 247.7 g/mol), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (thidiazuron), dPU, 1-(3-chlorophenyl)-3-phenylurea (CdPU), 1-(3-nitrophenyl)-3-phenylurea (NdPU), 1-(4-pyridyl)-3-phenylurea (PPU), *N*-phenylurea, *N*,*N*′-dimethylurea, 6-benzylaminopurine, 6-furfurylaminopurine (kinetin), and 6-(4-hydroxy-3-methylbut-2-enylamino)purine (*trans*-zeatin) were obtained from Sigma-Aldrich-Fluka (Madrid, Spain). 4-Amino-2-chloropyridine was synthesized from 2-chloro-4-nitropyridin *N*-oxide following a literature procedure (*19*). The synthesis of 1-(2-chloro-4-pyridyl)-3-(4-fluorophenyl)urea (4F-CPPU), 1-(2-chloro-4-pyridyl)3-(2,3,4,5,6-pentafluorophenyl)urea (PF-CPPU), 1-(2,6-dichloro-4-pyridyl)-3-phenylurea (dCPPU), 1-(2-chloropyridin-4-yl)-3-(pyridine-4-yl)urea (CdPyU), 1-(2-chloropyridin-4-yl)3-*p*-tolylurea (4M-CPPU), and 1-(2-bromopyridin-4-yl)-3-phenylurea (BPPU) was previously reported (*18*). Stock solutions of all chemicals were prepared in anhydrous *N*,*N*-dimethylformamide (DMF) and stored at −20 °C. All reagents and solvents used for the synthesis of haptens and for the activation of their functional groups were supplied by commercial sources. The chemical reactions were monitored by thin-layer chromatography with 0.25 mm precoated silica gel plates. The purification of the synthesized chemicals was carried out by flash column chromatography on silica gel 60 (particle size of 40−63 μm). Preparative reverse-phase HPLC separations were performed with a Waters chromatograph equipped with a Tracer Excel 120 ODS-B C18 column (15 cm × 1 cm, 5 μm; Teknokroma, Barcelona, Spain), using the indicated eluent. Although most of the compounds used in this work present minor or usual safety concerns, it is recommended that special precautions be taken for the handling of acyl azides and isocyanates. Melting points were obtained in a Kofler hot-stage apparatus or a Büchi melting point apparatus and are uncorrected. NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at room temperature on a Bruker AC-300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in the ^{1}H NMR spectra (7.26 and 2.50 ppm) and to solvent carbons in the ^{13}C NMR spectra (77.00 and 39.43 ppm). Carbon substitution degrees were established by distortionless enhancement by polarization transfer pulse sequences. A combination of correlation spectroscopy and heteronuclear single quantum coherence experiments was used for the assignment of ^{1}H and ^{13}C chemical shifts. Infrared spectra (IR) were measured as thin films between NaCl plates and KBr pellets for liquid and solid compounds, respectively, in a Nicolet Avatar 320 spectrometer. Electron-impact (EI) and fast atom bombardment (FAB) mass spectra (MS and HRMS) were carried out in a Micromass VG Autospec spectrometer. When required, an inert dry argon atmosphere was used for the synthetical operations.

Ovalbumin (OVA, grade V), *o*-phenylenediamine, horseradish peroxidase (HRP, type VI-A), and complete and incomplete Freund's adjuvants were acquired from Sigma-Aldrich (Madrid, Spain). Albumin from bovine serum (BSA, fraction V) was from Roche Applied Science (Mannheim, Germany). Sephadex G-25 HiTrap desalting columns used for the purification of protein−hapten conjugates were from General Electric Healthcare (Uppsala, Sweden). New Zealand female white rabbits were supplied by the University of Valencia, Spain. Polyclonal goat anti-rabbit immunoglobulin peroxidase conjugate (GAR−HRP) was purchased from Bio-Rad (Madrid, Spain). Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY). Plate washing was carried out in an ELx405 microplate washer from BioTek Instruments (Winooski, VT). Ultraviolet−visible (UV−vis) spectra and ELISA absorbances were read with a PowerWave HT, also purchased from BioTek Instruments.

Buffers and Solutions. These were as follows: (1) PB, 100 mM sodium phosphate buffer, pH 7.4; (2) PBS, 10 mM sodium phosphate buffer, pH 7.4, with 140 mM NaCl; (3) PBST, PBS containing 0.05% (v/v) Tween 20; (4) PBT 2×, 200 mM sodium phosphate buffer, pH 7.4, containing 0.05% (v/v) Tween 20; (5) CB, 50 mM carbonate−bicarbonate buffer, pH 9.6; (6) washing solution, 150 mM NaCl containing 0.05% (v/v) Tween 20; (7) developing solution, 2 mg/mL of OPD and 0.012% (v/v) H_2O_2 in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4; (8) stop solution, 2.5 M H_2SO_4.

Hapten Synthesis. The synthesis of 6-(4-(3-(2-chloropyridin-4-yl)ureido)phenyl)hexanoic acid, hapten p6 (**Table 1**), was described in our previous work (*18*). The synthetic sequences that were followed for the preparation of haptens m6 and s5 are shown in **Figure 1**. The synthesis of hapten m6 (**7**) involved the initial preparation of 6-(3-aminophenyl)hexanoic acid (**4**) which was further reacted with 2-chloro-4-isocyanatopyridine (**6**), while the synthesis of hapten s5 (**15**) entailed the preliminary preparation of methyl 5-(4-aminopyridin-2-ylthio)pentanoate (**12**), followed by its reaction with phenylisocyanate (**13**) and hydrolysis of the methyl ester moiety. The characterization data of the intermediate compounds are supplied in the Supporting Information.

Preparation of Hapten m6. *6-(3-Nitrophenyl)hex-5-ynoic Acid* (*3*). A solution of 1-iodo-3-nitrobenzene (**1**) (940 mg, 3.77 mmol) in pyrrolidine (1.4 mL) and $PdCl_2$ (6.7 mg, 0.04 mmol) was stirred at 50 °C for 10 min. Hex-5-ynoic acid (**2**, 343 μL, 339 mg, 3.03 mmol) was added, and the resulting mixture was stirred at 50 °C for 15 h, then poured into water and extracted with EtOAc. The aqueous phase was cooled in an ice bath and carefully acidified with 1 M hydrochloric acid to pH 2−3 and then extracted with EtOAc. The combined organic layers were washed with water and brine, dried over $MgSO_4$, and concentrated. Column chromatography, using CH_2Cl_2 as eluent, afforded the acetylenic acid **3** (556 mg, 79%) as an oil that solidified on standing.

6-(3-Aminophenyl)hexanoic Acid (*4*). A solution of the above obtained acetylenic acid **3** (642 mg, 2.75 mmol) in EtOAc (20 mL) and 10% Pd/C (146 mg) was hydrogenated at room temperature under a hydrogen pressure of 64 psi for 3−4 h. The reaction mixture was filtered through a short Celite pad, and the filter cake was washed with EtOAc. The filtrate and the washings were combined and concentrated under vacuum to afford almost pure amino acid **4** (556 mg, 97%) as a white solid, which was used without further purification for the preparation of the hapten.

6-(3-(3-(2-Chloropyridin-4-yl)ureido)phenyl)hexanoic Acid (*7, Hapten m6*). A solution of the 2-chloro-4-isocyanatopyridine (**6**) prepared as described previously (*18*) from 2-chloroisonicotinoyl azide (**5**, 500 mg, 2.74 mmol) in toluene (0.8 mL) was added dropwise to a stirring solution of the above purified 6-(3-aminophenyl)hexanoic acid (**4**, 539 mg, 2.60 mmol) in anhydrous acetone (2.5 mL). The mixture was kept at room temperature for 3 h. The crude product was purified by silica gel chromatography, using $CHCl_3$/EtOAc 1:1 as eluent, to afford hapten m6 (**7**, 706 mg, 75%) as a white solid. An analytical sample was obtained by preparative reverse

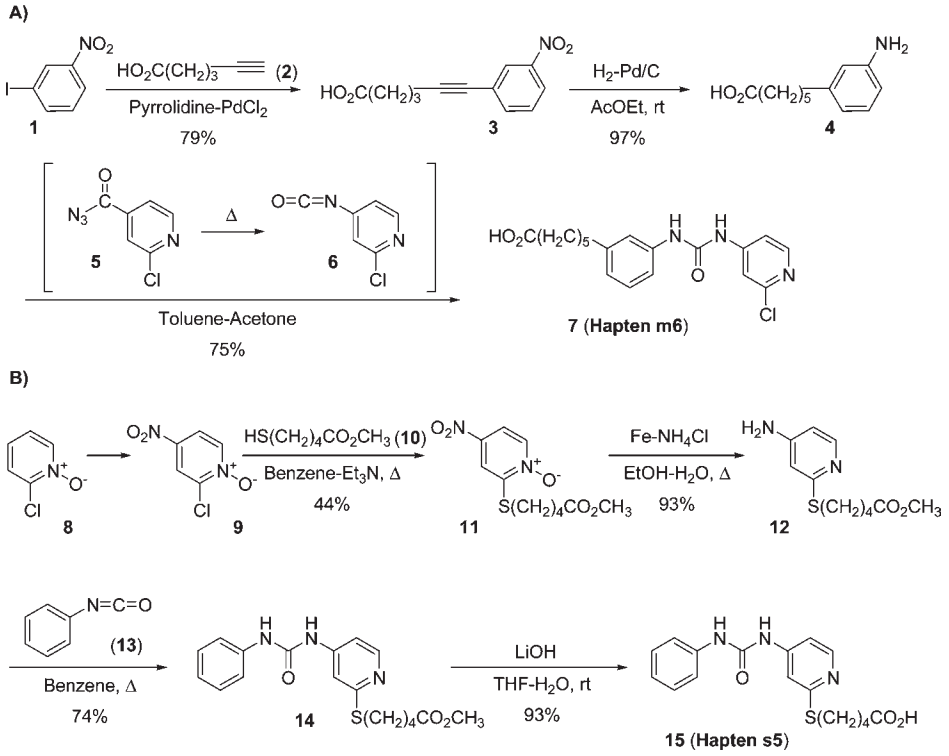


Figure 1. Synthetic routes for the preparation of haptens m6 and s5.

phase HPLC using MeOH/H_2O 8:2 as eluent: mp 196−198 °C (from MeOH−H_2O). ^{1}H NMR (DMSO-d_6), δ: 11.93 (1H, br s, OH), 9.28 and 8.85 (1H each, each s, two NH), 8.14 (1H, d, J = 5.6 Hz, H-6 Py), 7.65 (1H, d, J = 1.9 Hz, H-3 Py), 7.28−7.32 (2H, m, H-2 Ph, H-5 Py), 7.24 (1H, br d, J = 8.0 Hz, H-4), 7.17 (1H, t, J = 7.6 Hz, H-5 Ph), 6.83 (1H, br d, J = 7.3 Hz, H-6 Ph), 2.54 (2H, t, J = 7.5 Hz, H-6), 2.18 (2H, t, J = 7.4 Hz, H-2), 1.54 (4H, m, H-3 and H-5), 1.30 (2H, m, H-4). ^{13}C NMR (DMSO-d_6), δ: 174.32 (C-1), 151.67 (NCON), 150.88 (C-4 Py), 149.66 (C-6 Py), 149.01 (C-2 Py), 142.78 (C-1 Ph), 138.57 (C-3 Ph), 128.49 (C-5 Ph), 122.58 (C-6 Ph), 118.45 (C-2 Ph), 116.04 (C-4 Ph), 111.68 (C-3 Py), 111.12 (C-5 Py), 35.06 (C-6), 33.52 (C-2), 30.57 (C-5), 28.12 (C-4), 24.24 (C-3). IR (KBr): 3375, 3930, 2859, 1721, 1679, 1588, 1193, 1005, 697 cm^{-1}. MS (EI) m/z: 343 ($M^+ - 18$, 2), 319 (3), 317 (11), 233 (7), 208 (8), 207 (60), 155 (18), 156 (18), 154 (57), 107 (100). HRMS (FAB): calcd for $C_{18}H_{21}ClN_3O_3$ [$M^+ + 1$] 362.127 14, found 362.125 36. UV (PB), ε (280 nm) = 11.09 mM^{-1} cm^{-1}, ε (260 nm) = 26.48 mM^{-1} cm^{-1}.

Preparation of Hapten s5. *2-(5-Methoxy-5-oxopentylthio)-4-nitropyridine 1-Oxide* (***11***). A solution of methyl 5-mercaptopentanoate (**10**, 1.01 g, 6.82 mmol) obtained as described by Tanaka et al. (*20*) Et_3N (0.95 mL, 6.82 mmol) in anhydrous benzene (10 mL) was dropwise added during 30 min to a stirring suspension of 2-chloro-4-nitropyridine 1-oxide (**9**, 1.18 g, 6.43 mmol) in benzene (23 mL). Compound **9** was synthesized from **8** following a previously described procedure (*21*). The mixture was kept at reflux overnight under an argon atmosphere, and then it was concentrated. The crude product was purified by column chromatography, using $CHCl_3$/EtOAc 95:5 as eluent, to give methyl ester **11** (850 mg, 44%) as a yellow solid.

Methyl 5-(4-Aminopyridin-2-ylthio)pentanoate (***12***). Iron powder (417 mg, 2.33 mmol) was added to a solution of compound **11** (310 mg, 1.08 mmol) and NH_4Cl (185 mg, 3.43 mmol) in a 4:1 mixture of EtOH/H_2O (10 mL) at reflux. The mixture was stirred vigorously at reflux for 30−40 min, then cooled down to room temperature and filtered through a short pad of Celite, using MeOH to wash. The filtrate was concentrated under reduced pressure, diluted with $CHCl_3$, washed with saturated aqueous solution of Na_2CO_3 and brine, and dried over Na_2SO_4. After removal of solvent under reduced pressure, the residue was purified by chromatography, using $CHCl_3$/MeOH 9:1 as eluent, affording the amino ester **12** (242 mg, 93%) as a slightly colored viscous oil.

Methyl 5-(4-(3-Phenylureido)pyridine-2-ylthio)pentanoate (***14***). Phenyl isocyanate (**13**, 153 μL, 1.40 mmol) was added dropwise to a solution of methyl 5-(4-aminopyridin-2-ylthio)pentanoate (**12**, 270 mg, 1.12 mmol) in anhydrous benzene (5 mL) at reflux under argon, and the mixture was stirred at reflux for 3 h. The residue left after evaporation of the solvent under vacuum was purified by column chromatography, using $CHCl_3$/MeOH 98:2 as eluent, to give the urea methyl ester **14** (300 mg, 74%) as a white solid.

5-(4-(3-Phenylureido)pyridin-2-ylthio)pentatonic Acid (***15, Hapten s5***). A solution of the urea methyl ester **14** (256 mg, 0.71 mmol) in a mixture 3:1 of THF/H_2O (8 mL) was treated with LiOH·H_2O (299 mg, 7.12 mmol) and stirred at room temperature for 3 h. The reaction mixture was diluted with H_2O (20 mL) and extracted with ethyl ether. The organic layer was discarded, and the aqueous layer was evaporated under reduced pressure to eliminate the remains of organic solvent, then cooled in ice and acidified with solid $KHSO_4$ to approximately pH 3. The precipitated formed was filtered off, washed with water, and dried to give the crude product that was purified by column chromatography, using CH_2Cl_2/MeOH 9:1 as eluent, affording the hapten s5 (**15**, 228 mg, 93%) as a white solid: mp 205−210 °C (from DMSO−H_2O). ^{1}H NMR (DMSO-d_6), δ: 11.98 (1H, br s, OH), 9.08 and 8.88 (1H each, each br s, two NH), 8.20 (1H, d, J = 5.7 Hz, H-6 Py), 7.50−7.40 (3H, m, H-2/H-6 Ph and H-3 Py), 7.29 (2H, t, J = 7.8 Hz, H-3/H-5 Ph), 7.06 (1H, dd, J = 5.7, 1.8 Hz, H-5 Py), 7.01 (1H, tt, J = 7.5, 1.1 Hz, H-4 Ph), 3.08 (2H, t, J = 6.5 Hz, H-5), 2.24 (2H, t, J = 6.6 Hz, H-2), 1.63 (4H, m, H-3 and H-4). ^{13}C NMR (DMSO-d_6), δ: 174.23 (C-1), 157.73 (C-2 Py), 151.76 (NCON), 148.30 (C-6 Py), 147.80 (C-4 Py), 138.78 (C-1 Ph), 128.78 (C-3/C-5 Ph), 122.61 (C-4 Ph), 118.66 (C-2/C-6 Ph), 109.36 (C-3 Py), 108.95 (C-5 Py), 33.06 (C-2), 29.30 (C-5), 28.19 (C-4), 23.59 (C-3). IR (KBr): 2500−3500, 3276, 3138, 2954, 1720, 1581, 1557, 1493, 1190, 1041, 833, 749 cm^{-1}. MS (EI) m/z: 301 (1), 265 (6), 252 (7), 226 (11), 219 (8), 193 (6), 791 (21), 166 (20), 153 (44), 140 (32), 126 (35), 119 (38), 93 (100). HRMS (FAB): calcd for $C_{17}H_{20}N_3O_3S$ [$M^+ + 1$] 346.12254, found 346.12388. UV (PB), ε (280 nm) = 12.69 mM^{-1} cm^{-1}, ε (260 nm) = 34.78 mM^{-1} cm^{-1}.

Protein−Hapten Conjugates. Protein conjugates with haptens m6 and s5 were prepared following the same procedure that was used for the previously obtained conjugates with hapten p6 (*18*). BSA was selected for the preparation of the immunogens, whereas assay conjugates used in the ic-ELISAs and the dc-ELISAs were attained by coupling the haptens to OVA and HRP, respectively. Hence, the haptens were linked to the corresponding proteins by activation of their carboxylic acid groups and formation of amide bonds with the free amino groups of the carriers. See the Supporting Information for further details.

Antibody Production. Four female New Zealand white rabbits weighing 1−2 kg were immunized with the immunogens BSA−m6 and BSA−s5 (two rabbits per immunogen). Antibodies against the immunogen BSA−p6 had been obtained before (*18*). The first dose consisted of the administration, by subcutaneous injection in the dorsal region, of 300 μg of conjugate in 1 mL of a 1:1 (v/v) mixture of PB and complete Freund's adjuvant. The rabbits received three additional immunizations at 3-week intervals containing the same amount of conjugate emulsified, in this case, with incomplete Freund's adjuvant. Ten days after the fourth boost, the rabbits were exsanguinated by intracardiac puncture. The collected blood was stored at 4 °C during 24 h to allow coagulation, and then it was centrifuged and the supernatant containing the pAbs was collected. A fraction of the rabbit antisera was used to prepare a working solution by making a 1:5 (v/v) dilution with PBS containing 0.01% (w/v) thimerosal, and it was kept at 4 °C for daily usage. The remainder of the sera was precipitated for its long-term conservation by using a solution of saturated ammonium sulfate (1:1, v/v), and it was stored at 4 °C.

Competitive ELISAs. *Conjugate-Coated Indirect Assays*. The 96-well polystyrene ELISA plates were coated with 100 μL per well of OVA conjugates (0.1 and 1.0 μg/mL) in CB. After overnight incubation, plates were washed four times with washing solution. The competitive step was carried out by the addition of 50 μL per well of CPPU solution serially diluted in PBS (standard curve) and 50 μL of pAb diluted in PBST. The competition took place during 1 h, and then the plates were washed again. The amplification step was performed by the addition of 100 μL per well of a 1/10000 dilution of GAR−HRP in PBST. After 1 h of incubation, plates were washed and the signal was generated by adding 100 μL per well of developing solution. The enzymatic reaction was stopped 10 min later by the addition of 100 μL of stop solution. All of the steps were carried out at room temperature.

Antibody-Coated Direct Assays. Plates were coated by overnight incubation with 100 μL per well of pAb solution in CB. The evaluated coating sera dilutions were 1/5000, 1/15000, and 1/45000. Then the plates were washed and 50 μL of CPPU standards in PBS was added plus 50 μL of enzyme tracer solution in PBST. After 1 h of competition, plates were washed and the signal was directly generated as described before for the ic-ELISA format.

Signal Processing and Standard Curves. Absorbance was monitored in dual wavelength mode (492−650 nm). The signal intensity was plotted against the analyte concentration in logarithmic scale, and the generated sigmoidal curve was mathematically fitted to the following four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL):

$$y = \frac{A - D}{1 + (x/C)^B} + C$$

where A is the maximum absorbance, B is the slope at the inflection point of the curve, C is the concentration of analyte at the inflection point, and D is the minimum signal. The parameter C, typically corresponding to the analyte concentration that reduces 50% the A_{max} (IC_{50}) if D approaches to zero, was adopted as the estimation of the assay detectability. The limit of detection (LOD) of the assay was defined as the concentration of analyte that provided a 10% reduction of the A_{max} (IC_{10}). The dynamic range of the assay was established between the values of IC_{80} and IC_{20}, and they were considered as the upper and the lower limits of quantification (LOQ), respectively.

CPPU and Analogous Compounds. Competitors were prepared as concentrated stock solutions in anhydrous DMF and stored at −20 °C in amber glass vials. These solutions were used to prepare the standard curves employed in the competitive assays (maximum DMF content in standard solutions was 0.05%). Recognition of every competitor by each pAb was calculated using the ic-ELISA format with the homologous coating conjugate. Cross-reactivity (CR) was expressed as the percentage of recognition of the studied compound with respect to CPPU, according to the following formula:

$$CR = \frac{IC_{50(CPPU)}}{IC_{50(chemical)}} \times 100$$

Physicochemical Characterization of the Assay. The influence of ionic strength, pH, and Tween 20 concentration was evaluated following a multiparametric approach. The selected model was based on a central composite design. Briefly, a full factorial design was performed with the 3 factors which included 8 cube, 6 axial, and 6 central points, with 3 replicates each located at random positions, involving a total of 15 buffers. Ionic strength values ranging from 50 to 300 mM, pH values from 5.5 to 9.5, and Tween 20 concentrations from 0.00 to 0.05% were used as axial points. Characteristics of buffers used in this study are shown in Table 1SI in the Supporting Information. The buffers were prepared as follows: (i) a 40 mM citrate, 40 mM disodium hydrogen phosphate, and 40 mM Tris solution (pH 9.9) was prepared, and known volumes of 5 N HCl were added in order to reach the required pH in each case; (ii) the ionic strength of each solution was then calculated, considering the initial solution and the added volume of HCl, and then the appropriate volume of a 2 M NaCl solution was added to every buffer aliquot in order to achieve the required ionic strength; and finally, (iii) Tween 20 was added at the corresponding concentration before the final volume was achieved by addition of water. The concentration of Tween 20 and the ionic strength of the buffers were twice the final values in the assays. For the evaluation of the influence of these parameters, the IAs were carried out as described before, with the particularity that CPPU standard curves were prepared in water and the solutions of the immunoreagents included in the competitive step were prepared in every different assay buffer. A_{max} and IC_{50} values from CPPU inhibition curves obtained with every buffer were fitted by a multiple regression equation, including curvature and interaction terms, using the Minitab 14.1 software (Minitab Inc., State College, PA).

Sample Analysis. *Matrix Effects*. The possible interference of different food matrixes on the analytical features of the best-performing polyclonal IA, using the dc-ELISA format, was evaluated. This study was extended to an IA for CPPU analysis previously developed by our group (*18*), that was based on mAb p6#42 in the ic-ELISA format. The examined matrixes were gold and green kiwifruits purchased in local markets. After homogenization of the whole fruit with an Ultra-Turrax apparatus, the blended mixture was centrifuged during 5 min at 3500 rpm. The supernatant was further diluted in water to achieve final matrix dilutions in the assay of 1/10, 1/30, 1/100, 1/300, and 1/1000. CPPU standard curves were prepared in every diluted matrix and in water as control, and competitive assays were run with both the polyclonal and the monoclonal ELISAs. The solutions of the enzyme tracer and the mAb used in the competitive step were prepared in PBT 2×. The estimation of the minimum required sample dilution to obtain a reliable determination of CPPU was accomplished by comparison of the analytical parameters of the standard curves prepared in the diluted matrices with those obtained in water.

Recovery Analysis. To examine the accuracy and precision of the developed IAs, recovery studies were performed with fortified fruit samples. Kiwifruits were processed as described above, and the supernatants, after the centrifugation step, were spiked with CPPU at a final concentration of 5, 10, 20, 50, and 100 μg/L. Spiked kiwifruit samples were adequately diluted in water to avoid matrix interferences and to allow samples to enter into the working range of the assay. Each sample was determined in duplicate with both IAs, and average absorbance values were interpolated into a standard curve in water run in triplicate in each plate. A CPPU-negative fruit sample was also included as control of absence of matrix interference.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugate Preparation. For the generation of high-quality antibodies against low-molecular-weight compounds, it is well established that the design and synthesis of the immunizing hapten is quite a determinant step that will mainly rule the success of the immune response. An appropriate hapten should mimic the target molecule as much as possible in terms of structure resemblance, size, steric conformation, electronic configuration, and hydrophobic properties (*22*). With respect to the spacer arm, it is generally accepted that medium-sized aliphatic linkers (three to six carbon atoms) are appropriate for an optimum exposition of the conjugated hapten to the immune system (*23*).

In forchlorfenuron, the chloropyridine ring makes the difference between this compound and other urea derivatives. In a

previous work (*18*), the synthetic strategy that was followed for the preparation of CPPU haptens consisted of the introduction of linear hydrocarbon spacer arms at the *para* position of the phenyl ring, that is, at a site distal from the characteristic chloropyridyl moiety. By immunization of mice and rabbits with two CPPU derivatives with linkers of different length, high-affinity mAbs and pAbs for CPPU were generated. In the present work, we have explored the influence of the derivatization site on antibody properties, mainly affinity and specificity. Accordingly, two additional haptens with the spacer arm attached at opposite positions of the CPPU structure were prepared (**Table 1**).

Hapten m6, with a six carbon atom spacer arm at the *meta* position of the phenyl ring, was synthesized following a procedure similar to the one used for the preparation of hapten p6. In these derivatives, the characteristic chloropyridyl ring of CPPU remained accessible to the immune system, and the rotational flexibility of the molecule was preserved. In hapten s5, the 5-methoxy-5-oxopentylmercaptol group was introduced at the C-2 position of the pyridine ring by the nucleophilic substitution of the chlorine atom, followed by hydrolysis of the ester moiety. The choice of a sulfur atom to join the spacer arm to the pyridine ring was based on the similarity between the sulfur and chlorine atoms in terms of their atomic size and their limited capacity of resonance interaction with the aromatic ring, which should produce a minimal change in the electronic distribution of the pyridine ring in the hapten. Both haptens (m6 and s5) retain unchanged the ureido group, a chemical moiety that usually plays a fundamental role in the molecular recognition of this kind of compound because of its ability to participate in the formation of strong hydrogen bonds (*24*). The synthesis of haptens m6 and s5, outlined in **Figure 1**, involved the reaction between a conveniently functionalized aryl- or pyridylamine with the appropriate isocyanate as the key step for the elaboration of the ureido moiety of both haptens.

The preparation of hapten m6 (**7**, **Figure 1A**) started with a two-step sequence that transformed 3-nitroiodobenzene (**1**) into 6-(3-aminophenyl)hexanoic acid (**4**), involving a copper-free $PdCl_2$ catalyzed Sonogashira coupling reaction with 5-hexynoic acid (**2**) to give the acetylenic derivative **3**, followed by catalytic hydrogenation of the triple bond. This strategy, which allowed the efficient incorporation of the saturated alkyl chain that constitutes the spacer arm of the hapten, has been demonstrated to be particularly useful in the synthesis of haptens functionalized at aromatic positions. With aminophenylhexanoic acid **4** at hand, the synthesis of hapten m6 (**7**) was completed by reaction of the amino group of **4** with 2-chloro-4-isocyanatopyridine (**6**), previously prepared immediately before use by Curtius rearrangement of 2-chloroisonicotinoyl azide (**5**), which in turn was prepared from commercially available 4-carboxypyridine 1-oxide (*25*).

The synthesis of hapten s5 (**15**, **Figure 1B**) started with the nucleophilic aromatic substitution of the chlorine atom of the known 2-chloro-4-nitropyridine *N*-oxide (**9** (*21*)) by methyl 5-mercaptopentanoate (**10**) following a literature procedure (*26*). The latter compound was readily prepared from commercial methyl 5-bromopentanoate by substitution of bromine by a mercaptan group using thiourea as the source of sulfur (*20*). It is noted that the nucleophilic aromatic substitution reaction of chlorine by the alkylthio group to give **11** took place with moderate yield (44%), due at least in part to competing substitution reaction of the C-4 nitro group. Once the thioalkyl chain had been incorporated at the C-2 position of the pyridine ring, the synthesis of the key aminopyridine intermediate **12** was completed very efficiently by simultaneous reduction of the nitro and *N*-oxide moieties of **11** with iron under very smooth conditions. The synthesis of hapten s5 (**15**) was finally completed by reaction of aminopyridine **12** with phenyl isocyanate (**13**) to give the pyridylphenylurea derivative **14**, followed by basic hydrolysis of the methyl ester moiety.

Haptens were coupled to BSA by the active ester method for the generation of the immunogens. The hapten-to-protein molar ratios (MR) in the conjugates, estimated by UV spectroscopy, were 34 and 18 for BSA−m6 and BSA−s5, respectively. Assay conjugates were prepared by coupling both derivatives to OVA and HRP, thus obtaining coating conjugates and enzyme tracers to be used in the indirect and direct competitive ELISA formats, respectively. The estimated MRs were 3 and 6 for OVA−m6 and OVA−s5 and was 3 for both HRP−m6 and HRP−s5 conjugates. All of the obtained values were in the normal ranges given the 30−35 ε-amine residues accessible in BSA, 20 in OVA, and 2 in HRP.

Polyclonal Antibody Characterization. The six anti-CPPU pAbs that were produced using haptens p6, m6, and s5 were extensively characterized in both ELISA formats with homologous and heterologous assay conjugates by bidimensional competitive experiments (*23*). This strategy allows the simultaneous evaluation of the influence of several combinations of immunoreagents and their concentrations on both signal intensity and assay sensitivity.

ic-ELISA. Inhibition assays with six different dilutions of each antiserum were carried out in plates coated with every OVA conjugate at two concentrations. **Table 2** shows the results obtained from all immunoreagent combinations affording maximum signals immediately above and below 1.0. All antisera were able to recognize each of the three conjugates at both assayed coating concentrations, even though homologous and pseudo-homologous (haptens with the linker at the same ring) conjugates were generally better bound than conjugates comprising haptens functionalized at opposite positions. Antisera from all three immunizing haptens allowed the development of homologous IAs with excellent sensitivities (IC_{50} values below 1 nM). Generally, the IC_{50} values were better at 0.1 μg/mL than at 1.0 μg/mL. Lower coating concentrations did not further improve the assay sensitivity. The lowest IC_{50} values for homologous combinations providing $A_{max} \geq 0.8$ were between 0.4 and 0.5 nM. According to these results, in terms of antibody affinity, it could be concluded that the three explored derivatization sites at the analyte were appropriate to generate high-quality antibodies against CPPU.

Hapten heterology is a strategy often harnessed to improve assay sensitivity of IAs, especially with pAbs (*27*−*29*), probably because the apparent affinity toward the analyte in solution can be increased because of the modulation of the participation of different antibody populations present in the antiserum during the competition step. In a previous paper (*18*), several strategies of hapten heterology were studied, such as the use of haptens with different spacer arm lengths or haptens with structural modifications with respect to the immunizing hapten. It is noteworthy that any of these heterologies only generated a slight improvement on assay sensitivity in both pAb and mAb-based assays. In the present work, we have explored the concept of heterology regarding to the site of hapten derivatization. This kind of heterology has been widely explored before with good results (*28*, *30*, *31*). For the particular set of immunoreagents described in this work, modest improvements on assay sensitivity were found by the employment of derivatization site heterology, probably because of the already very low IC_{50} values actually displayed by most homologous combinations. Anyhow, in terms of global performance, the best assay used antiserum Rp6#1 at a $1/10^5$ dilution in combination with the heterologous conjugate OVA−s5 at 0.1 μg/mL. The IC_{50} value of this assay was 0.30 ± 0.05 nM, with a slope of −1.01.

Table 2. Characterization of the pAbs with the ic-ELISA Format[a]

		antiserum																	
		Rp6#1			Rp6#2			Rm6#1			Rm6#2			Rs5#1			Rs5#2		
OVA conjugate	concn (μg/mL)	dil[b]	A_{max}	IC_{50}[c]	dil[b]	A_{max}	IC_{50}[c]	dil[b]	A_{max}	IC_{50}[c]	dil[b]	A_{max}	IC_{50}[c]	dil[b]	A_{max}	IC_{50}[c]	dil[b]	A_{max}	IC_{50}[c]
p6	0.1	300	0.98	0.5	300	0.71	0.8	100	0.85	0.3	100	0.63	0.5	30	0.40	0.6	30	0.51	0.5
		100	1.91	1.2	100	1.40	1.4	30	1.73	0.6	30	1.31	1.0	10	1.08	1.8	10	1.02	1.7
	1	2000	0.72	5.0	2000	0.67	10.0	300	0.75	1.6	300	0.71	3.1	100	0.38	0.5	100	0.72	0.3
		1000	1.40	5.1	1000	1.25	10.1	100	1.65	2.4	100	1.60	5.2	30	1.61	0.7	30	2.38	0.9
m6	0.1	300	0.74	0.6	300	0.84	2.3	100	0.94	0.5	100	0.90	1.2	30	0.58	1.4	30	0.65	0.6
		100	1.60	0.8	100	1.86	3.3	30	1.96	0.9	30	1.82	2.3	10	1.21	2.2	10	1.31	1.8
	1	1000	0.53	4.3	1000	na[d]		300	0.73	1.9	300	0.88	7.7	100	0.86	0.3	100	0.94	0.5
		300	1.51	4.7	300	1.46	8.3	100	1.77	3.2	100	2.09	7.9	30	1.85	1.0	30	2.67	1.1
s5	0.1	100	0.97	0.3	100	0.54	0.9	100	0.49	0.3	100	0.58	0.4	1000	0.52	0.2	1000	0.49	0.2
		30	2.32	0.8	30	1.58	1.2	30	1.50	0.6	30	1.41	0.9	300	1.22	0.4	300	1.17	0.4
	1	300	0.58	2.7	300	0.58	2.9	300	0.41	3.3	1000	0.45	1.7	1000	0.52	2.7	1000	0.51	3.0
		100	1.78	1.9	100	1.45	2.9	100	1.06	3.1	300	1.17	2.1	300	1.40	4.2	300	1.46	4.0

[a] Assay parameters represent the average of three independent assays. [b] Dilution factor of antisera referred to as the value $\times 10^3$. [c] Sensitivity using CPPU as competitor expressed in nM. [d] Not assayed.

Table 3. Characterization of the pAbs with the dc-ELISA Format[a]

	enzyme tracer											
	HRP−p6				HRP−m6				HRP−s5			
antiserum	dil[b]	concn[c]	A_{max}	IC_{50}[d]	dil[b]	concn[c]	A_{max}	IC_{50}[d]	dil[b]	concn[c]	A_{max}	IC_{50}[d]
Rp6#1	45	3	0.72	0.9	45	3	0.45	0.6	15	10	0.70	0.4
		10	1.54	0.9		10	1.15	0.7		30	1.08	0.4
Rp6#2	15	3	0.95	2.1	15	3	0.69	1.6	15	100	0.99	0.7
		10	2.44	2.1		10	1.99	1.6		300	1.15	1.3
Rm6#1	15	3	0.59	0.5	15	3	0.65	0.7	5	3	0.56	0.6
		10	1.46	0.5		10	1.81	0.7		10	1.29	0.6
Rm6#2	15	3	0.58	0.6	15	3	0.66	1.3	5	10	0.99	0.8
		10	1.41	0.6		10	1.74	1.4		30	1.58	1.1
Rs5#1	15	10	0.70	0.5	15	10	0.58	0.8	45	3	0.96	0.3
		30	1.65	0.5		30	1.22	0.8		10	2.03	0.4
Rs5#2	15	10	0.75	0.4	15	10	0.89	0.4	15	1	0.39	1.3
		30	1.43	0.4		30	1.74	0.6		3	1.16	1.4

[a] Assay parameters represent the average of three independent assays. [b] Coating dilution factor of antisera referred as the value $\times 10^3$. [c] Concentration of the enzyme tracer in ng/mL. [d] Sensitivity using CPPU as competitor expressed in nM.

dc-ELISA. Antisera were also evaluated in the antibody-coated ELISA format. Three different antiserum dilutions were used for coating the plates, and competitive assays were run with six concentrations of the three tracers. All of the pAbs allowed the development of IAs in this format (**Table 3**). Although the dc-ELISA format is often more restrictive than the ic-ELISA format with respect to conjugate recognition, all six tested antisera were able to recognize all of the haptens as enzymatic conjugates. Generally, the optimum antiserum coating dilution for the development of competitive assays was 1/15000, being possible to use higher dilutions and/or lower concentrations of the enzyme tracers with the homologous or the pseudohomologous conjugates. Again, IAs with sensitivities below 1 nM were generated with all of the polyclonals, with very similar IC_{50} values for the best antisera in both ELISA formats (0.4−0.5 nM). Unlike the ic-ELISA format, in which higher antibody concentrations resulted in sensitivity losses, the dc-format provided almost identical IC_{50} values for the different assayed enzyme tracer concentrations. The highest reductions of the IC_{50} values were obtained with heterologous haptens using opposite derivatization sites, i.e., the two immunoreagent combinations represented by antiserum Rp6#1 with HRP−s5 tracer and antiserum Rs5#2 with HRP−p6 tracer, which provided a 2- and 3-fold decrease in IC_{50} values, respectively, compared with their corresponding homologous tracers. Nevertheless, the best IA in the dc-ELISA format was achieved by antiserum Rs5#1 at a 1/45000 coating dilution with its homologous enzyme tracer, HRP−s5, at a concentration of 3 ng/mL. This assay presented a slope of −1.10 and an IC_{50} value of 0.30 ± 0.02 nM.

Cross-Reactivity and Binding Studies. Several compounds with cytokinin activity of biological origin, such as *trans*-zeatin and kinetin, or of synthetic origin, like 6-benzylaminopurine, were evaluated for antibody binding, and no recognition was observed by any pAb. This finding is of outstanding practical relevance for the application of the developed IAs to the monitoring of CPPU in horticultural samples in which these hormones are potentially present. The ability of the produced antibodies to recognize fragments of CPPU was also studied. CR values for *N*,*N*′-dimethylurea, *N*-phenylurea, and 4-amine-2-chloropyridine were below 0.01% for any of the antisera, indicating that the presence of both rings is necessary for binding. The CR of the defoliant compound thidiazuron, which has also been found to possess cytokinin activity (*32*), was also evaluated. Replacement of the chloropyridyl ring of CPPU by the thiadiazoyl ring present in thidiazuron prevented recognition of this molecule by p6-type and m6-type antisera. On the contrary, medium-to-high CR values were observed with antisera Rs5#1 and Rs5#2 (14% and 43%, respectively). To better map out the main antigenic determinants that are involved in the antibody−analyte interaction, a battery of compounds structurally related to CPPU were tested. Our ultimate goal was to establish a correlation between the position of the functional group of the immunizing hapten and the selectivity of the antibody. With this aim, several chemicals with modifications at both phenyl and chloropyridyl rings were synthesized (*18*) and evaluated in binding studies. The recognition profile of each antiserum against this group of competitors is depicted in **Figure 2**. It can be clearly observed that the specificity profiles found for the six pAbs could be divided into two groups. The first one would be made up of antisera obtained from immunogens BSA−p6 and BSA−m6, while the other group would comprise antisera coming from BSA−s5. According to these profiles, the behavior of antisera obeyed Landsteiner's principle, which establishes that antibody specificity is directed primarily at the portion of the hapten located furthest from the position used for linking it to the carrier protein. That region of the molecule would be most accessible during the immune response, whereas the part of the hapten close to the site of attachment would be sterically hindered by the carrier protein, thus preventing its specific recognition (*33*). Accordingly, those compounds with modifications at the phenyl ring were always

much better recognized by p6-derived and m6-derived antisera than by s5-derived polyclonals. The inclusion of five fluorine atoms at the phenyl ring (PF-CPPU) was the modification causing the maximum disturbance to recognition, due to the significant effect of this alteration over the electronic configuration of CPPU. The influence of this change and that of the replacement of the phenyl ring by a pyridyl ring (CdPyU) was obviously much more important in s5-type antisera because of the distal nature of these modifications with respect to the carboxylate group of hapten s5. Interestingly, the affinity of antisera from immunogen BSA−p6 was almost double for 4M-CPPU than for CPPU, probably because this compound mimics better the immunizing hapten, as it presents a methyl group at the same site where the spacer arm was placed (**Figures 1** and **2**). Regarding compounds with modifications at the pyridyl ring, the results were the opposite; that is, s5-type polyclonals recognized better that sort of analogue than p6-type and m6-type antisera. The greatest difference was observed with CdPU and NdPU, two compounds mainly characterized by the presence of a phenyl ring instead of the pyridyl ring characteristic of CPPU. Noticeably, BPPU, which contained a bromine instead of the chlorine atom

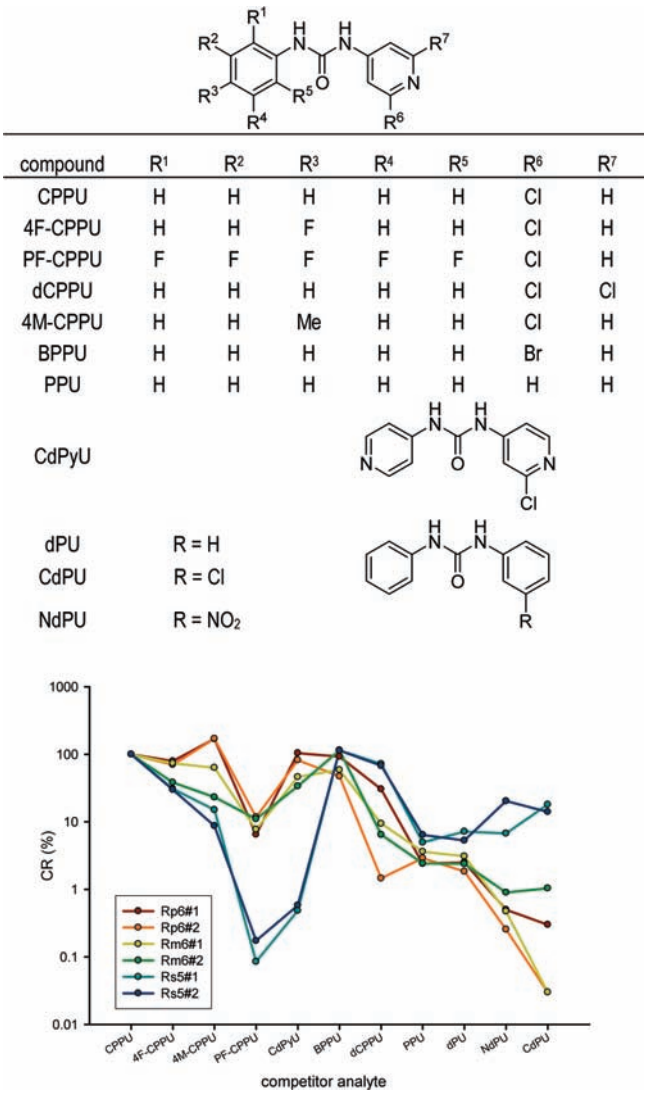


Figure 2. Chemical structure of the competitor analytes and the binding profiles of the pAbs in the ic-ELISA format using the homologous hapten as coating conjugate.

Table 4. Conditions and Parameters of the Developed Assays[a]

	pAb-based dc-ELISA	mAb-based ic-ELISA
antibody	Rs5#1 dil 1/45000	p6#42 at 30 ng/mL
conjugate	HRP−s5 at 5 ng/mL	OVA−p2 at 0.1 μg/mL
A_{max}	1.79 ± 0.19	1.12 ± 0.12
slope	−1.19 ± 0.09	−1.58 ± 0.13
IC_{50} (ng/L)	77 ± 12	50 ± 3
LOD (ng/L)	12 ± 3	13 ± 2
LOQ (ng/L)	25 ± 5 to 276 ± 35	22 ± 2 to 156 ± 28

[a] Average of 10 independent experiments.

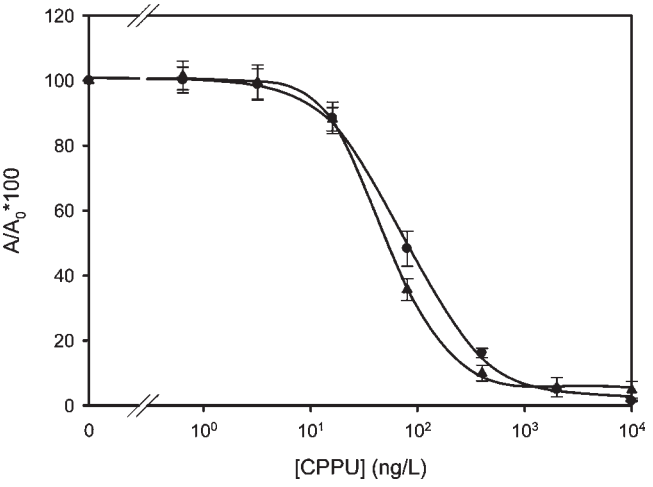


Figure 4. Standard curves for CPPU with the developed IAs with pAb Rs5#1 in the dc-ELISA (circles) and mAb p6#42 in the ic-ELISA (triangles). The curves were run in triplicate in 10 different days.

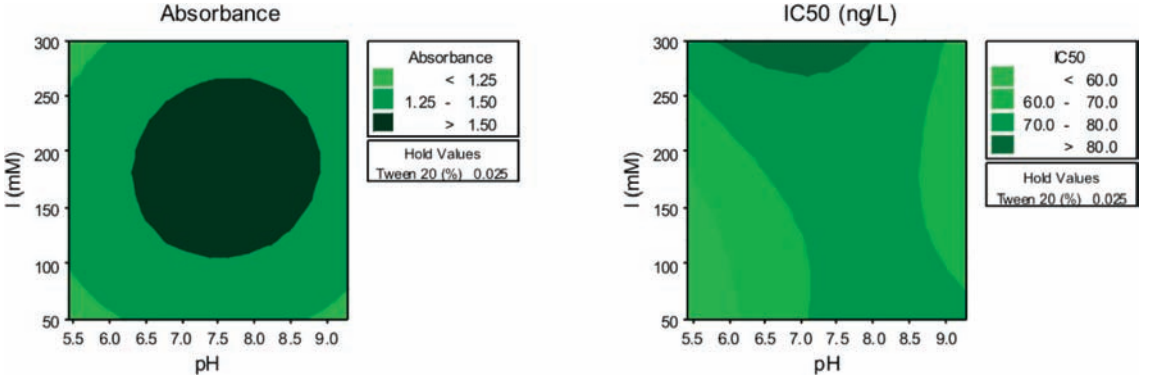


Figure 3. Influence of ionic strength (I) and pH over the signal intensity and the sensitivity of the developed dc-ELISA with pAb Rs5#1 and tracer HRP−s5.

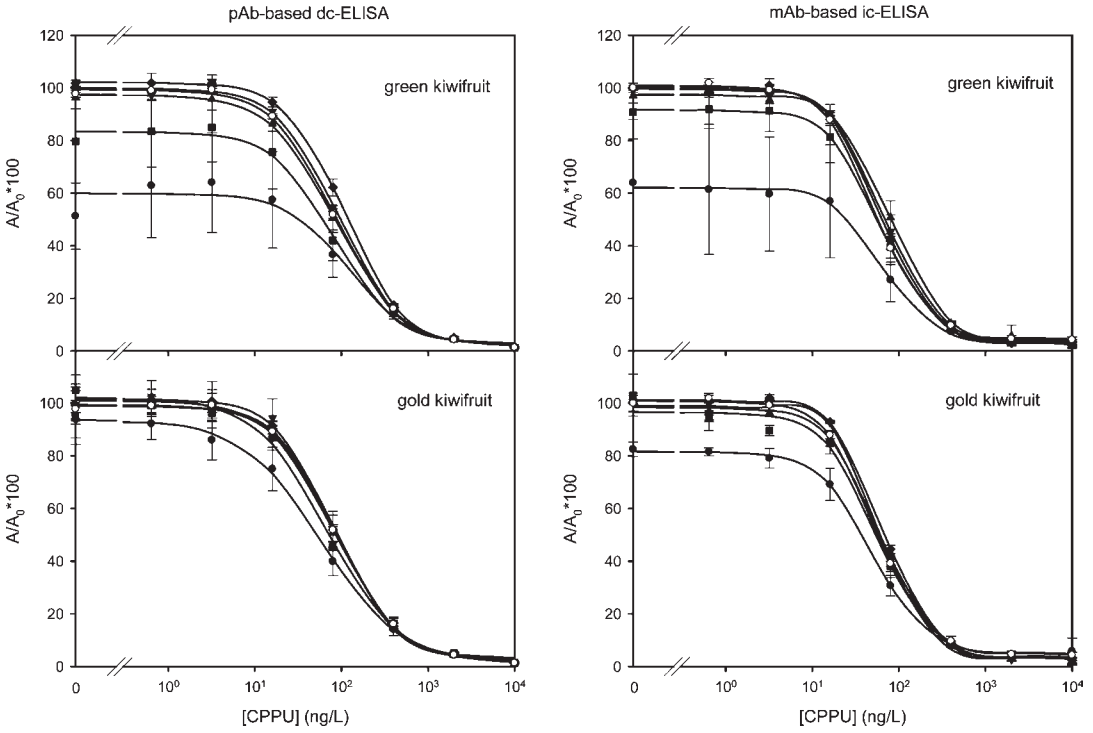


Figure 5. Matrix effects of different kiwifruit juices on assay parameters of the developed IAs: buffer (○), 1/10 (●), 1/30 (■), 1/100 (▲), 1/300 (▼), and 1/1000 (◆). Each value represents the average of three independent replicates.

present in CPPU, was perfectly bound by all antisera. Similar results concerning the exchange of these two halogen atoms in other analytes had been described before by other authors (*34*). However, if the chlorine atom is simply removed (PPU), the recognition by all antisera was severely compromised, thus highlighting the importance of the presence of an electron-withdrawing element at the pyridyl ring for antibody binding.

Physicochemical Characterization of the Assay. The dc-ELISA based on pAb Rs5#1 and its homologous enzyme tracer HRP−s5 was finally selected for the development of an IA to detect CPPU in fruit samples because of the simplicity of this format and especially because of the low immunoreagent concentrations (antiserum and enzyme tracer) required for this assay. Accordingly, the influence of the physicochemical parameters of the buffer more often affecting the IA performance was studied. A multiparametric model was chosen to assess the effect of pH, ionic strength, and Tween 20 concentration on the assay. In this way, the possible interdependence of the different factors on IA performance was considered. The information extracted from this study could be very valuable for the analysis of some particular matrixes, i.e., samples with low pH values typically occurring in fruits.

In **Figure 3**, the simultaneous effects of ionic strength and pH variations on A_{max} and IC_{50} values for a fixed concentration of Tween 20 are depicted (graphs showing the effects of other combinations are included in the Supporting Information). With respect to the A_{max}, an optimum combination of factors was observed at the midpoint of every studied parameter (ionic strength 175 mM, pH 7.5, and 0.025% of Tween 20). The maximum signal intensity was observed at pH values between 6.5 and 8.5 and in the range of ionic strength values between 100 and 250 mM. On the other hand, assay sensitivity seemed to be more dependent on buffer modifications. A decrease in the IC_{50} value was generated at low salt concentrations and acid pH, but the use of higher salt concentrations minimized the effect on assay sensitivity caused by the pH. Likewise, at pH values above 7.5, changes in the ionic strength had almost negligible influence on the IC_{50} value. The effects of both pH and ionic strength were more evident at low concentrations of Tween 20 (see **Figure 1SI** in the Supporting Information). After the evaluation of the influence of buffer composition on the selected assay, we decided to use a buffer with high buffering capability in order to keep a constant pH value, due to the predictable variation of this parameter in fruit samples and the consequent effect on the robustness of the assay. Also, we decided to maintain the assay concentration of surfactant at 0.025%. Therefore, standard curves or samples were run in water and the dilution of the enzyme tracer was carried out in PBT 2× (ionic strength 223 mM and pH 7.4).

The final assay conditions for the proposed polyclonal dc-ELISA are shown in **Table 4**, and the corresponding inhibition curve obtained for CPPU is displayed in **Figure 4**. The standard curve of this assay showed a slope of −1.19, a LOD of 12.42 ± 3.06 ng/L, and a quantitation range between 24.66 ± 5.04 and 275.64 ± 34.67 ng/L. For comparative purposes, **Table 4** and **Figure 4** also include the assay conditions and the standard inhibition curve, respectively, of a previously developed indirect ELISA that uses mAb p6#42 (*18*). The performance of this mAb was also evaluated with the heterologous haptens m6 and s5 both in the indirect and in the direct formats (results not shown), but no improvement on sensitivity was noticed, so the original coating antigen and format were selected for the analysis of food samples.

Sample Analysis. In order to evaluate possible interferences from food matrixes in the polyclonal dc-ELISA and the monoclonal ic-ELISA under study, standard curves of CPPU were run in diluted juices obtained after homogenization of kiwifruits. The matrix effects on the inhibition curves of two kiwifruit varieties (gold and green) are shown in **Figure 5**. Green kiwifruit caused the most noticeable influence on both IAs, mainly on signal intensity, as evidenced by a 40% reduction of the A_{max} value when a 1/10 dilution of the sample juice was assayed. Nevertheless, a 100-fold

Table 5. Recovery Study of CPPU with Fortified Fruits[a]

	pAb-based dc-ELISA						mAb-based ic-ELISA					
	green kiwifruit			gold kiwifruit			green kiwifruit			gold kiwifruit		
spiked concn (μg/L)	recovered concn (μg/L)	recovery (%)	CV (%)	recovered concn (μg/L)	recovery (%)	CV (%)	recovered concn (μg/L)	recovery (%)	CV (%)	recovered concn (μg/L)	recovery (%)	CV (%)
5	3.9 ± 0.5	78	12	4.9 ± 0.8	99	15	5.2 ± 0.7	105	13	4.6 ± 0.7	91	16
10	8.3 ± 1.1	83	14	10.2 ± 2.1	102	20	12.5 ± 0.4	125	4	10.5 ± 1.8	104	18
20	16.4 ± 3.1	82	19	17.8 ± 2.8	89	15	23.1 ± 3.2	116	14	19.1 ± 1.3	96	7
50	45.6 ± 4.7	91	10	40.2 ± 5.3	80	13	52.8 ± 1.3	106	2	49.5 ± 4.5	99	10
100	88.9 ± 13.3	89	15	85.7 ± 14.6	86	17	102.3 ± 2.2	102	2	96.8 ± 9.7	97	10
	mean	84.6	14.0	mean	91.2	16.0	mean	110.8	7.0	mean	97.4	12.2

[a] Each value represents the average of three independent experiments.

dilution in buffer was enough to avoid any negative influence caused by the two matrixes under study.

Finally, the accuracy and precision of the proposed assays were evaluated with fortified kiwifruit samples. Juices obtained from whole kiwifruits were spiked at five CPPU concentrations and analyzed using the developed ELISAs after proper sample dilution (typically 1/100 or 1/500). In the case of the polyclonal dc-ELISA that was developed with antiserum Rs5#1, recovery values in the 78−102% range were obtained, with coefficients of variation (CVs) always below 20% (**Table 5**). For the monoclonal assay in the ic-ELISA format with mAb p6#42, the recovery values ranged between 91% and 125%, with CVs lower than 18%. Mean global recoveries in kiwifruit were 87.9% for the polyclonal assay and 104.1% for the monoclonal one. Regarding precision, mean CVs of 14.9% and 9.6% were found for the polyclonal and the monoclonal ELISAs, respectively. Overall, both IAs have demonstrated their usefulness as analytical tools for the determination of forchlorfenuron residues in kiwifruit at such demanding levels as 5 μg/L.

In summary, antisera with excellent affinities to CPPU (IC_{50} < 1 nM) were obtained from three different immunizing haptens, two of them functionalized at the phenyl ring (m6 and p6) and the other one with the spacer arm at the pyridyl ring (s5) of the target analyte. In addition, all three haptens worked perfectly as assay conjugates in two ELISA formats with the complete set of antisera. The main difference among the antisera, derived from haptens functionalized at opposite positions, was on specificity, thus showing the relevance of the derivatization site in immunizing haptens. The performance of all polyclonals perfectly fitted the rules stated by Karl Landsteiner decades ago. Antibodies from a particular hapten bound worse to chemicals with structural modifications at the side of the analyte furthest from the position that was chosen to link the hapten to the carrier protein. Accordingly, antisera from haptens with the spacer arm attached to the pyridyl ring of the CPPU molecule recognized more weakly those analogues with modifications at the phenyl ring and vice versa. This sort of fine specificity study was only possible because of the synthesized library of chemical analogues of the analyte and the high affinity of the antibodies. Finally, immunoassays developed in this study were able to quantify CPPU in kiwifruit, after a simple 100-fold dilution in buffer, at concentrations 10 times below the MRL.

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Supporting Information Available: Full characterization data of all intermediate compounds in the syntheses of haptens m6 and s5 and an additional table and figure for immunoassay development. This material is available free of charge via the Internet at http://pubs.acs.org.

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