Development and validation of a direct competitive monoclonal antibody-based immunoassay for the sensitive and selective analysis of the phytoregulator forchlorfenuron

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Run-in heading

Development and validation of a direct ELISA to CPPU

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Abstract

Forchlorfenuron is a synthetic phytohormone with cytokinin-like activity used worldwide as a plant growth regulator to increase fruit size in a number of crops, mostly in kiwifruit and grape vines. A monoclonal antibody-based enzyme-linked immunosorbent assay (ELISA) for the determination of forchlorfenuron has been characterized and optimized. The selected immunoreagents afforded a highly selective assay with a limit of detection of 10 ng L^{-1} in buffer. This direct competitive ELISA was validated in terms of trueness, precision, and robustness using both commercial juice and whole fruit samples. Recoveries from fortified kiwifruit juices and white and red musts were between 97 and 131%, with relative standard deviations below 16%. When homogenized whole fruits were analysed after acetonitrile extraction, recoveries between 96 and 113% were found, with a limit of quantification of 5 µg kg^{-1}. The proposed immunoassay was validated by comparison with a reference chromatographic method using fruits from in-field treated grape and kiwifruit vines. Linear regression analysis of ELISA and HPLC-UV determinations showed an excellent correlation (r^2 = 0.998), whereas analysis of the slope (0.99 ± 0.01) and of the intercept (−1 ± 3) clearly proved that the developed competitive immunoassay provided results that were statistically comparable to those obtained by the instrumental method for the analysis of CPPU in fruits at trace levels.

Keywords

CPPU, cytokinin, hapten, ELISA, food safety, HPLC.

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Introduction

Forchlorfenuron, commonly named CPPU, is a synthetic phenylurea-derived cytokinin widely employed as plant growth regulator to increase berry size mainly in kiwifruit and grape vines, including some varieties that are non-tolerant to gibberelic acid [1]. The enhanced fruit enlargement due to CPPU treatments is thought to be derived from a direct stimulation of cell division and/or activation of plant responses typically induced by naturally occurring adenine-substituted cytokinins [2], combined with an inhibition of cytokinin oxidase/dehydrogenase enzymes that are responsible of cell homeostasis, thus increasing the effective concentration of the endogenous phytohormones [3]. CPPU is commercially available as 1% or 0.1% emulsifiable concentrates under different trade names, *i.e.* KT-30 or Sitofex. The use of CPPU as plant growth regulator was approved by the European Commission in 2006 only for kiwifruit crops [4], whereas in the USA it was authorized in 2004 for kiwifruit and grape cultivars [1]. The maximum residue limit (MRL) defined by European Regulation 396/2005 for CPPU is 0.05 mg kg^{-1} in all food commodities [5]. On the other hand, according to the US Code of Federal Regulations, the tolerance levels for CPPU were established at 0.04 mg kg^{-1} for kiwifruits, 0.03 mg kg^{-1} for table grapes, and 0.06 mg kg^{-1} for raisin grapes [6]. Nowadays, the recommended methodologies for the determination of CPPU residues are mainly based on QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction and high-performance liquid chromatography (HPLC) analysis. QuEChERS methodology is a commonly used multiresidue procedure that employs an acetonitrile extraction step coupled to a dispersive solid phase clean-up with a primary–secondary amine (PSA) support [7]. A summary of the up-to-date published strategies for the analysis of CPPU residues in food can be found in the Electronic Supplementary Material file (Table S1).

Consumer protection from exposure to agrochemical residues is strongly dependent on the development and implementation of extensive food control programs. In this regard, the traditionally employed analytical technologies, despite being highly sensitive and selective, present certain drawbacks; namely, the sophistication and cost of the instrumentation, the requirement of highly qualified personnel, and the low-to-medium analytical throughput, together with demanding

sample pretreatments, generally consisting in successive extraction, clean-up, and enrichment steps. Alternatively, immunoanalytical tools, based on analyte detection by specific antibodies in aqueous solutions, usually allow a simplification of sample preparation prior to analysis, representing a rapid, cost-effective, and simple methodology. However, for a major acceptance and implementation of immunoassays, adequate immunoreagents covering relevant chemicals that can potentially be present in food commodities are required. During the last decades, many applications in the food safety area describing the development of immunoassays for the determination of pesticides, hormones, antibiotics, toxins, additives, or industrial residues have been reported [8–12].

In previous studies, a set of functionalized haptens mimicking the molecular structure of CPPU was synthesized and used for animal immunization [13–15]. Afterwards, a large and diverse collection of high affinity monoclonal and polyclonal antibodies was produced and characterized by enzyme-linked immunosorbent assay (ELISA), and the influence of distinct hapten heterology strategies on assay detectability was evaluated. In the current paper, the development of a highly sensitive monoclonal antibody-coated direct competitive ELISA is described for the determination of CPPU in kiwifruit, white grape, and red grape, both as liquid (juice) and solid (fruit) samples. The proposed immunoassay was validated in terms of detectability, selectivity, trueness, precision, and robustness. To this purpose, kiwifruit and grape vines were treated with CPPU in order to obtain fruit samples with incurred hormone residues. Different procedures were evaluated for quantitative analyte extraction, and finally the results obtained by ELISA were validated using a reference method based on HPLC–UV.

Materials and methods

Instruments and reagents

Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Microplate washing was carried out in an ELx405 96-channel microplate washer from BioTek Instruments (Winooski, VT, USA). ELISA absorbances were read with a PowerWave HT, also purchased from BioTek Instruments. A Hitachi (Tokyo, Japan) L-2130 HPLC system,

equipped with a Hitachi L-4500 diode array detector and a Merck KGaA (Darmstadt, Germany) LiChroCART RP-18 column (250 mm × 4 mm, 5 µm) was employed for chromatographic separations. For food sample homogenization and extraction, a T-25 ultra-turrax blender, a vortex mixer MS2 from IKA (Staufen, Germany), and an Eppendorf 5804 centrifuge (Hamburg, Germany) were employed.

The affinity purified monoclonal antibody (s5#34) and the horseradish peroxidase–hapten conjugate tracer (HRP–p6) were obtained in our laboratory as previously published [13, 15]. Analytical grade CPPU (1-(2-chloro-4-pyridyl)-3-phenylurea, CAS Registry No 68157-60-8, MW 247.7 g mol^{-1}), 1-(4-pyridyl)-3-phenylurea, thidiazuron, *trans*-zeatin, kinetin, 6-benzylaminopurine, and *o*-phenylenediamine were obtained from Sigma-Aldrich-Fluka (Madrid, Spain). Pesticide standards were from Fluka/Riedel-de-Haën (Seelze, Germany) or Dr. Ehrenstorfer (Augsburg, Germany). Organic solvents and anhydrous magnesium sulfate were from Scharlau (Barcelona, Spain). Hydrogen peroxide (30%), sulfuric acid (95%), and buffer constituents were purchased from Prolabo-VWR International Eurolab S.L. (Barcelona, Spain). PSA was obtained from Varian (Lake Forest, CA, USA). Sitofex KT-30 emulsion was purchased from Degussa AG (Trostberg, Germany).

ELISA procedure and data treatment

Microplates were coated by overnight incubation at room temperature with 100 µL per well of a 1 µg mL^{-1} monoclonal antibody s5#34 solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. Then, they were washed four times with washing solution [150 mM NaCl and 0.05% (v/v) Tween 20] and each microwell received 50 µL of analyte/sample solution in Milli-Q water plus 50 µL of a 60 ng mL^{-1} HRP−p6 enzyme tracer solution in buffer. After a 1 h incubation at room temperature, plates were washed as before and the response signal was obtained by supplying 100 µL per well of freshly prepared 2 mg mL^{-1} *o*-phenylenediamine solution containing 0.012% (v/v) H_2O_2 in 25 mM sodium citrate and 62 mM sodium phosphate buffer, pH 5.4. The enzymatic reaction was stopped after 10 min at room temperature by adding 100 µL per well of 2.5 M sulfuric acid. The absorbance was immediately read at 492 nm, using 650 nm as reference wavelength.

Standard curves were prepared from 0.6 to 10000 ng L^{-1} in water using a 10 mg L^{-1} CPPU stock solution in anhydrous *N*,*N*-dimethylformamide. Experimental points were fitted to a sigmoidal curve with a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay detectability was estimated as the inflection point of the sigmoidal curve, typically corresponding to the concentration of analyte generating a 50% inhibition (IC_{50}) of the maximum absorbance reached at the zero dose of analyte (A_{max}) if the background signal approaches to zero. The limit of detection (LOD) was established as the concentration of CPPU that provided a 10% inhibition of A_{max}.

Buffer studies

A multiparametric approach was employed as previously described [16]. A central composite design was followed according to the Minitab 14.1 software (Minitab Inc., State College, PA, USA). This strategy consisted in a two-level full factorial design (α=1.414) with 3 factors and 3 replicates, that included 8 cube, 6 axial, and 1 centre points, involving a total of 60 randomized inhibition curves under 15 different assay conditions (see Table S2 in the Electronic Supplementary Material file). The evaluated assay pHs ranged from 5.5 to 9.5, the ionic strengths from 50 to 300 mM, and the Tween 20 percentages from 0 to 0.05% (v/v); taking pH 7.5, ionic strength 175 mM, and 0.025% (v/v) Tween 20 as the centre point of the composite design.

The necessary buffers were obtained as follows. First, a 40 mM trisodium citrate, 40 mM disodium hydrogen phosphate, and 40 mM Tris solution (pH 9.9) was prepared and known volumes of 5 M HCl were added in order to reach the required pH in each case. Then, the ionic strength of all buffers was adjusted with the appropriate volume of a 2 M NaCl solution, taking into account the initial salt solution and the employed HCl. Finally, Tween 20 was added at the corresponding concentration before the final volume was achieved with Milli-Q water. The responses using distinct assay conditions were fitted by a multiple regression equation, including curvature and interaction terms using the Minitab software.

In-field CPPU application

Kiwifruit (Hayward and Bruno varieties), white grape (Macabeo variety), and red grape (Bobal variety) vines were sprayed with the commercial CPPU emulsion concentrate product Sitofex at 10 mg L^{-1}. After application of the agrochemical, fruits were collected and short-term stored at 4 ºC. Samples were chopped, homogenized using an ultra-turrax, and stored in polypropylene tubes at −20 ºC until analysis.

Sample preparation for ELISA

For CPPU determination by ELISA from whole fruits, three different sample preparation procedures were employed:

i) *Liquid fraction separation*: samples were directly obtained from homogenized whole fruits by centrifugation at 2200×g for 5 min.

ii) *Single extraction with solvent*: 5 g of homogenized sample and 1 g of NaCl were thoroughly mixed in a polypropylene tube followed by extraction with 5 mL of HCl 0.1 M in acetonitrile with vortex shaking during 1 min. The acetonitrile extract was collected after centrifugation at 2200×g for 5 min.

ii) *Triple extraction with solvent*: Three consecutive extraction steps were performed with 5 mL of HCl 0.1 M in acetonitrile as before, and the obtained extracts were combined.

All samples were diluted in Milli-Q water at least 100-fold prior to ELISA analysis.

HPLC–UV reference procedure

The extraction procedure of CPPU from fruit samples was adapted from Hu and Li [17]. Briefly, 20 g of homogenized sample, containing 4 g NaCl and 40 µL of internal standard solution (100 mg L^{-1} of 1-(4-pyridyl)-3-phenylurea), were extracted three times with 15 mL each of HCl 0.1 M in acetonitrile by vortex mixing during 1 min. After centrifugation for 5 min at 2200×g, the organic extracts were collected, dried with anhydrous magnesium sulfate, and concentrated until dryness with a rotary evaporator. The remaining residue was reconstituted in 400 µL methanol. Red grape extracts were subjected to further clean-up by dispersive solid-phase extraction with PSA/$MgSO_4$. Finally, they were filtered through a 0.22 µm Teflon filter and analyzed by HPLC–UV. At the end of this extraction procedure, samples had undergone a 50-fold concentration ratio. HPLC

determinations were conducted using a 1 mL min^{-1} methanol:water flow with a gradient from 40% to 90% (v/v) methanol in 15 min, and then 90% (v/v) methanol was run during 5 min. A 20 µL injection volume was employed. Measurement wavelengths were 266 and 256 nm for CPPU and the internal standard, respectively.

Results and discussion

Immunoassay characterization

Hapten synthesis and monoclonal antibody production were published in previous articles [13–15]. Different monoclonal and polyclonal antibodies were characterized, in the aforementioned studies, in two competitive ELISA formats (antigen or antibody coated immunoassays), and diverse hapten heterology strategies were explored. For the present study, an antibody-coated direct competitive ELISA based on monoclonal antibody s5#34 and the heterologous enzyme tracer HRP–p6 was selected because of its higher detectability, moderate slope of the inhibition curve, and shorter analysis time (see Fig S1 for the structure of the immunizing and of the assay hapten). A thorough optimization of this immunoassay was conducted in order to assess the robustness of the assay and to identify possible interferences before it was applied to the analysis of CPPU in food samples.

Assay conditions

The influence of different physicochemical factors (ionic strength, pH, and surfactant concentration) over the ELISA competitive step was evaluated. The A_{max} and IC_{50} values of the inhibition curves obtained with every studied condition (see Table S2 in the Electronic Supplementary Material file) were employed as response values and fitted by a multiple regression equation using the Minitab software. A significant correlation (P>0.05) was not found between the Tween 20 percentage and the obtained responses, indicating that assay signal and detectability were not notably affected by surfactant concentrations lower than 0.05% (v/v). On the contrary, the contour plots displaying the influence of pH and salt concentration over the A_{max} and IC_{50} values of the ELISA inhibition curves (Fig. S2) revealed slight modifications of both parameters if the assay was run under conditions different to those of the centre point of the composite design (pH 7.5,

ionic strength 175 mM, and 0.025% (v/v) Tween 20). Thus, at ionic strength and pH values below those of the reference conditions, lower maximum signals and assay detectabilities were found. By overlaying the A_{max} and IC_{50} contour plots (Fig. 1), the robustness of the studied immunoassay was evidenced by the tolerable variation of the two parameters (below 20%) over a wide range of assay conditions (white area). For further studies, buffer PBT [200 mM sodium phosphate buffer, pH 7.4, containing 0.05% (v/v) Tween 20] was chosen since it afforded a strong buffering capacity under similar assay conditions (almost same pH, *I* = 223 mM, and same detergent concentration) to those of the centre point of the employed multiparametric approach. Fig. 2 shows the inhibition curve of the optimized ELISA for CPPU in Milli-Q water, employing PBT for enzyme tracer preparation. Under these conditions, the calculated LOD for CPPU was 10 ng L^{-1}.

Solvent tolerance

Organic solvents are usually employed for the extraction of agrochemical residues from food commodities. However, the presence of solvents in the immunoreaction medium can often influence the analyte–antibody interaction or the enzyme tracer activity, generating a considerable interference in the immunoassay performance. Thus, the solvent effect over the optimized competitive ELISA was evaluated using CPPU standard curves prepared in water containing different amounts of methanol, ethanol, acetonitrile, or acetone, ranging from 0.5 to 10% (v/v). Fig. 3 shows the variation of A_{max} and IC_{50} values caused by the presence of organic solvent during the immunochemical reaction. As it can be observed, all of the evaluated solvents were quite well tolerated up to 2%. Higher ethanol or acetonitrile contents reduced significantly the detectability, whereas the presence of acetone and methanol during the competitive step was better accepted.

Assay selectivity

The cross-reactivity of the optimized competitive ELISA was determined using a large number of chemicals as competitors. The compounds included in this study were: i) substances with a CPPU-like molecular structure like thidiazuron and benzanilide; ii) adenine-substituted cytokinins, such as trans-zeatin, kinetin, and 6-benzylaminopurine; and iii) a list of pesticides commonly applied to kiwifruit and grape crops, including fenhexamid, boscalid, azoxystrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin, picoxystrobin, dimoxystrobin, fluoxastrobin,

metominostrobin, captan, mepanipyrim, pyrimethanil, procimidone, tolylfluanid, cyazofamid, tebuconazole, fenamidone, fludioxonil, vinclozolin, imidacloprid, and cyprodinil.

Cross-reactivity values, which are inversely correlated to the specificity of the antibody, were expressed as the percentage of recognition to every particular competitor with respect to the IC_{50} value for CPPU. From this study, no relevant binding to any of the tested agrochemicals was observed, with cross-reactivity values below 0.1%. Only the herbicide thidiazuron displayed an evident cross-reactivity of 71%. As described by Moreira-Lima and Barreiro [18], this result could be attributed to the bioisosterism that it seems to exist between the 2-chloropyridyl ring of CPPU and the thiadiazole ring of thidiazuron (Fig. S3 in the Electronic Supplementary Material file). In spite of the high recognition showed towards this compound, it cannot be considered a potential interference because it is not authorized for any crop commodity in the EU [5] and it is only registered for use as pre-harvest cotton defoliant in the USA [19].

Determination of CPPU in fruit juices

Matrix effects

Food matrices can modify the ELISA response, eventually hindering an adequate quantification of the target analyte. This interference is basically dependent on the employed immunoreagents and the commodity under consideration. Matrix influence over competitive immunoassays for small organic compounds can commonly be avoided by simple dilution with working buffer or water prior to analysis [20, 21]. Due to the outstandingly low IC_{50} value (63 ng L^{-1}) offered by the optimized competitive ELISA, a dilution step did not compromise the high detectability levels required for CPPU monitoring campaigns. Commercial bottled juices of kiwifruit and white and red grapes were diluted several times (1/10, 1/30, 1/100, 1/300, and 1/1000; v/v) with Milli-Q water. Those diluted samples were used to prepare CPPU standard curves, from 0.6 to 10000 ng L^{-1}, in order to determine the extent of matrix interference over the immunoassay. From this study, it was found that any negative effects could be reversed for the three juices after a simple 100-fold dilution in water (Fig. S4).

Analysis of spiked juices

The trueness of the optimized immunoanalytical assay was first assessed by measuring spiked fruit juice samples and estimating the overall recovery. Thus, commercial kiwifruit and grape juices, both white and red, were fortified at different CPPU concentrations, ranging from 5 to 100 μg L^{-1}. Three independent determinations of the target analyte from conveniently diluted juices (100-fold at least) were conducted using the proposed competitive immunoassay. Recoveries from 100 to 131% for kiwifruit juice, from 97 to 118% for white grape must, and from 105 to 119% for red grape must were obtained, with relative standard deviations lower than 18% in all cases (Table 1). The limit of quantification (LOQ) for CPPU residues, estimated as the minimum evaluated concentration that afforded recovery values comprised between 80 and 120%, was established at 5 and 10 μg L^{-1} for both grape must samples and for kiwifruit juice, respectively.

Determination of CPPU in whole fruits

CPPU extraction

Different methodologies were evaluated in order to establish the quickest and simplest procedure to allow a quantitative determination of the target analyte in whole fruits. Two CPPU-containing fruit samples from in-field treated grape and kiwifruit vines were homogenized and analyzed by a reference procedure (HPLC–UV) and also by the developed competitive assay. First, ELISA analyses were carried out on the liquid phase obtained after centrifugation of homogenized kiwifruits, white grapes, and red grapes. Recoveries achieved by immunochemical analysis were calculated taking the results afforded by HPLC–UV after a triple extraction with acetonitrile as reference values (100%). It could be observed (Fig. 4) that a direct determination of CPPU in the liquid fraction was not representative of its concentration in the complete sample (with recoveries close to 20%). Nevertheless, it must be noticed that this approach, although insufficient for a true estimation of CPPU contents, could be a solvent-saving, rapid, and cost-effective methodology for the screening of huge numbers of samples. Thereafter, ELISA determinations were performed after a single extraction with solvent or, like for HPLC analyses, after a triple extraction. Acetonitrile was chosen as solvent because it is widely employed for CPPU analysis by instrumental methods [22–25]. As shown in Fig. 4, quantitative results were obtained using a unique extraction step for kiwifruit, but in the case of grape samples a triple extraction was

required. Accordingly, further studies of CPPU determinations by the competitive ELISA were conducted in all samples using a single analytical procedure based on the more exhaustive extraction protocol.

Recoveries

In first instance, spiked samples were chosen to evaluate the efficiency of competitive ELISA determinations following triple extraction with acetonitrile. Kiwifruit, white grape, and red grape untreated fruits (with zero CPPU content) were homogenized, fortified at different CPPU concentrations ranging from 5 to 100 μg kg^{-1}, and subjected to solvent extraction as described. Three independent determinations were conducted by the optimized immunoassay (Table 2). In this study, recoveries ranged from 98 to 110% for kiwifruit, from 96 to 113% for white grape, and from 96 to 108% for red grape, demonstrating the trueness of the proposed analytical strategy. The LOQ for the analysis of CPPU from whole kiwifruit and grape samples with the developed procedure could be established at 5 μg L^{-1}. The relative standard deviation values were below 16% in all cases, a precision in line with that of common immunochemical methods for haptens.

Comparative study with HPLC–UV

Finally, the developed immunoassay was validated using incurred samples by comparison of the analytical determinations of CPPU retrieved by ELISA and HPLC–UV. Most of the assayed samples exceeded the MRLs established for CPPU because the preharvest interval was not strictly observed, a deliberate choice to obtain positive real samples (see Table S3 in the Supplementary Data file). The results obtained by both methodologies were in very good agreement, with a bias ranging from 88 to 111%. A linear regression analysis was performed showing an excellent correlation for the whole set of analyzed samples (Fig. 5), and also for every individual matrix: $[CPPU]_{ELISA}$ (μg kg^{-1}) = (−2 ± 7) + (1.02 ± 0.06) $[CPPU]_{HPLC}$ (μg kg^{-1}), n = 11, r^2 = 0.970, for kiwifruit; $[CPPU]_{ELISA}$ (μg kg^{-1}) = (−3 ± 6) + (1.01 ± 0.02) $[CPPU]_{HPLC}$ (μg kg^{-1}), n = 8, r^2 = 0.997, for white grape; and $[CPPU]_{ELISA}$ (μg kg^{-1}) = (−11 ± 14) + (1.01 ± 0.03) $[CPPU]_{HPLC}$ (μg kg^{-1}), n = 8, r^2 = 0.995, for red grape. Accordingly, the developed competitive ELISA provided results that were statistically comparable to those obtained by the reference method (HPLC–UV) for the analysis of CPPU in fruits at trace levels.

Conclusions

A monoclonal antibody-based immunoassay for the determination of the plant growth regulator forchlofenuron, also named CPPU, employing the antibody-coated direct competitive ELISA format has been optimized and validated. CPPU could be analyzed in commercial juices by the developed immunoassay with excellent performance. Also, different strategies for the preparation of kiwifruit, white grape, and red grape whole fruit samples were evaluated. A qualitative determination of CPPU could be achieved by the direct analysis of the liquid fraction separated from the fruit after a simple centrifugation step, whereas for quantitative measurements a triple extraction procedure with acetonitrile of the homogenized samples was required. The comparison of the developed competitive ELISA with a reference chromatographic method based on HPLC–UV showed an excellent correlation when CPPU was analyzed in fruits from in-field treated kiwifruit and grape vines. This immunoassay provides a versatile alternative tool with high sample throughput to efficiently monitoring CPPU in horticultural commodities with an easy sample pretreatment.

Acknowledgements

This work was supported by *Ministerio de Educación y Ciencia* (PET2006_0423_00 and AGL2009-12940-C02/01/02/ALI), *Ecología y Protección Agricola S.L.*, and FEDER Funds. C.S.-P., F.A.E.-T., and J.V.M. were hired by the CSIC under a predoctoral *I3P* contract, a postdoctoral *JAE* contract, and a *Ramón y Cajal* contract, respectively. All contracts were cofinanced by *Ministerio de Ciencia e Innovación* and the European Social Fund.

We thank Laura López-Sánchez and Ana Izquierdo-Gil for excellent technical assistance.

Electronic Supplementary Material

The online version of this article (doi:XXXX) contains supplementary material, which is available to authorized users.

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Figure Legends

Fig. 1. Overlaid contour plots for the influence of buffer composition (pH and ionic strength) over the immunoassay parameters. Lines indicate the assay conditions affording A_{max} (blue) and IC_{50} (red) values (%) with a 120% (dashed lines) or 80% (solid lines) deviation from the values reached at the centre-point assay conditions. The white area indicates tolerable (< 20%) maximum signal and detectability changes.

Fig. 2. Forchlorfenuron molecular structure and standard curve obtained with the optimized competitive ELISA. Values are the mean of ten independent experiments. The assay parameters were: A_{max} = 1.56 ± 0.13, slope = −1.23 ± 0.09, IC_{50} = 63 ± 6 ng L^{-1}, and A_{min} = 0.008 ± 0.004.

Fig. 3. Effect of different solvents over the A_{max} and IC_{50} values of the ELISA inhibition curve. Values (n=3) are referred to those found in the standard curve prepared in water.

Fig. 4. Recovery values from two CPPU-positive kiwifruit (white bars), white grape (grey bars), and red grape (black bars) samples. Whole fruits were homogenized and CPPU was measured by ELISA after dilution in Milli-Q water using: i) liquid fraction separated by centrifugation; ii) single extract with acetonitrile; iii) triple extract with acetonitrile.

Fig. 5. Comparative study between the optimized competitive ELISA and HPLC–UV results obtained for CPPU analysis in extracts of whole fruit samples from in-field treated vines. The equation of the depicted regression line was: $[CPPU]_{ELISA}$ (µg kg^{-1}) = (−1 ± 3) + (0.99 ± 0.01) $[CPPU]_{HPLC}$ (µg kg^{-1}), n = 27, r^2 = 0.998.

Table 1
ELISA recoveries using fruit juice samples spiked with forchlorfenuron.

Spiked amount ($\mu g\ L^{-1}$)	[CPPU] ($\mu g\ L^{-1} \pm s$, n=3)		
	Kiwifruit juice	White grape must	Red grape must
5	6.5 ± 0.1	5.3 ± 0.8	5.8 ± 0.1
10	11.9 ± 0.1	9.7 ± 0.2	11.9 ± 0.3
20	21.0 ± 0.3	22.0 ± 0.3	22.6 ± 1.4
50	55.0 ± 7.8	54.5 ± 1.4	54.2 ± 1.5
100	100.5 ± 4.1	117.9 ± 19.4	105.0 ± 1.8

Table 2
ELISA recoveries using whole fruit sample extracts spiked with forchlorfenuron.

Spiked amount ($\mu g\ L^{-1}$)	[CPPU] ($\mu g\ L^{-1} \pm s$, n=3)		
	Kiwifruit	White grape	Red grape
5	5.5 ± 0.5	5.3 ± 0.6	5.4 ± 0.4
10	10.3 ± 0.3	9.7 ± 0.7	9.6 ± 1.1
20	19.5 ± 3.0	22.6 ± 3.6	21.3 ± 2.2
50	53.5 ± 6.8	47.8 ± 3.2	49.2 ± 4.0
100	106.9 ± 12.7	96.6 ± 10.1	99.4 ± 9.6

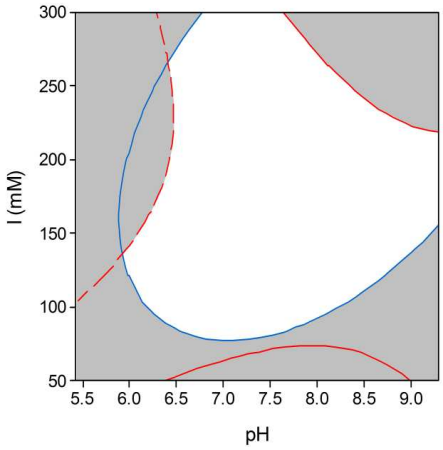

Fig. 1

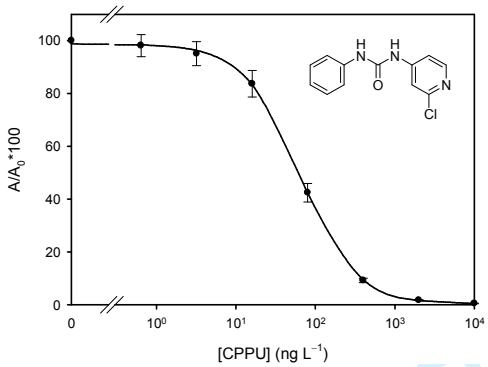

Fig. 2

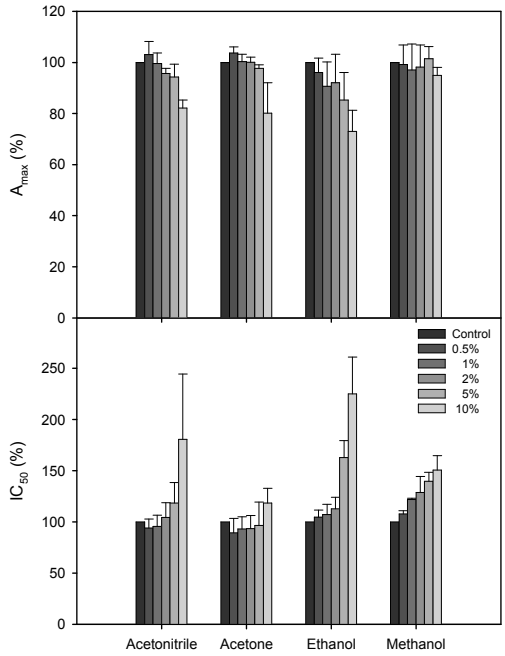

Fig. 3

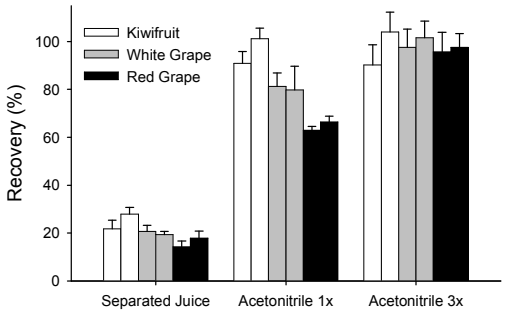

Fig. 4

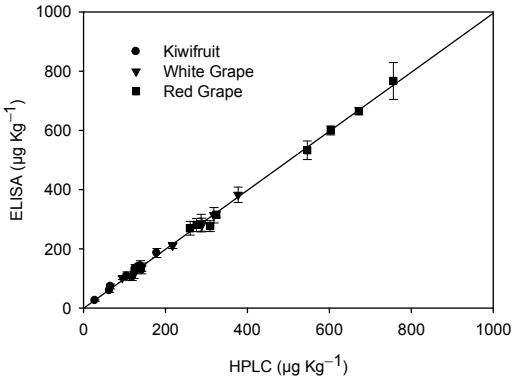

Fig. 5

Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Development and validation of a direct competitive monoclonal antibody-based immunoassay for the sensitive and selective analysis of the phytoregulator forchlorfenuron

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

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Table S1
Summary of the published procedures for the determination of CPPU residues in food[a].

Sample	Extraction	Clean-up	Analysis	Recovery (%)	CV (%)	LOD (μg kg^{-1})	Ref.
Grape	LLE (acetonitrile-hexane)	LLE (dichloromethane)	HPLC–UV	78–81	-[b]	1.0	[1]
Watermelon	QuEChERS	SPE (PSA)	HPLC–UV	95–101	2–10	0.4	[2]
Brown rice, soybean, cabbage, green pepper, Japanese radish, onion, potato, pumpkin, spinach, apple, grape, kiwifruit, melon, orange, pear, peach	LLE (acetone)	SPE	HPLC–UV	88–100	2–7	5.0	[3]
Watermelon	QuEChERS	SPE (PSA)	HPLC–MS	82–106	5–18	1.0	[4]
Cooked wheat flour, polished rice	USE (acetonitrile)	-	HPLC–MS	73–131	2–17	1.5–2.1	[5]
Watermelon, zucchini, tomato	QuEChERS	SPE (PSA)	HPLC–MS	65–87	3–10	0.5	[6]
Kiwifruit juice	Dilution in buffer	-	ELISA	78–104	2–20	1.2–1.3	[7]

[a] *Abbreviations*: CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; LLE, liquid–liquid extraction; LOD, limit of detection; MS, mass spectrometry; PSA, primary–secondary amine; QuEChERS, quick, easy, cheap, effective, rugged, and safe extraction; SPE, solid-phase extraction; USE, ultrasonic extraction; UV, ultraviolet detector. [b] Information not available.

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Table S2

Assay ionic strength (*I*), pH, and Tween 20 concentration and the obtained curve parameters in the central composite design study.

Run order	pH	*I* (mM)	Tween 20 (%, v/v)	A_{max}	IC_{50} (ng L^{-1})
1	7.5	175	0.025	1.67	77.0
2	7.5	175	0.025	1.78	79.3
3	7.5	175	0.000	1.15	40.1
4	7.5	175	0.000	1.09	49.3
5	6.3	250	0.010	0.81	95.4
6	7.5	175	0.025	1.70	71.6
7	7.5	50	0.025	0.81	58.7
8	5.5	175	0.025	1.21	121.1
9	8.7	250	0.010	1.59	62.7
10	7.5	175	0.025	1.69	78.8
11	7.5	175	0.025	1.70	80.2
12	8.7	100	0.040	1.45	84.2
13	6.3	100	0.010	1.41	52.5
14	7.5	50	0.025	0.74	42.8
15	6.3	250	0.010	0.48	84.0
16	7.5	175	0.025	1.89	52.5
17	6.3	100	0.040	1.51	54.0
18	7.5	300	0.025	1.76	47.8
19	9.5	175	0.025	1.26	44.1
20	7.5	175	0.025	1.72	58.2
21	7.5	300	0.025	1.70	53.5
22	7.5	175	0.025	1.76	59.4
23	7.5	175	0.025	1.78	71.6
24	6.3	100	0.010	1.43	70.6
25	8.7	250	0.040	1.74	73.1
26	6.3	100	0.010	1.42	47.3
27	7.5	175	0.000	1.12	36.7
28	7.5	175	0.025	1.70	54.2
29	6.3	100	0.040	1.52	50.0
30	8.7	100	0.040	1.54	45.8
31	5.5	175	0.025	1.19	86.2
32	7.5	300	0.025	1.73	48.8
33	9.5	175	0.025	1.31	39.4
34	8.7	250	0.010	1.74	42.4
35	8.7	100	0.010	1.52	50.3
36	7.5	175	0.025	1.66	81.2
37	8.7	100	0.040	1.55	66.1
38	6.3	100	0.040	1.48	65.6
39	7.5	175	0.050	1.74	60.7
40	8.7	250	0.040	1.74	57.5
41	7.5	175	0.025	1.72	59.9
42	8.7	250	0.010	1.62	44.6
43	7.5	175	0.050	1.69	58.9
44	8.7	100	0.010	1.43	44.1
45	7.5	175	0.025	1.67	57.2
46	8.7	100	0.010	1.43	45.1
47	7.5	50	0.025	0.73	68.9
48	7.5	175	0.025	1.68	76.8
49	5.5	175	0.025	1.21	111.0
50	7.5	175	0.050	1.64	73.1
51	7.5	175	0.025	1.63	70.3
52	7.5	175	0.025	1.61	62.4
53	7.5	175	0.025	1.62	68.9
54	8.7	250	0.040	1.66	65.9
55	6.3	250	0.010	0.73	94.9
56	7.5	175	0.025	1.65	63.9
57	6.3	250	0.040	1.53	75.8
58	9.5	175	0.025	1.17	56.7
59	6.3	250	0.040	1.57	77.3
60	6.3	250	0.040	1.58	80.7

Table S3
Forchlorfenuron contents in whole fruit from in-field treated kiwifruit and white and red grape vines.

		[CPPU] (μg kg^{-1} ± s)		
Sample type	Sample code	HPLC–UV (n=2)	ELISA (n=3)	Bias (%)
Kiwifruit	K1	28 ± 10	26 ± 3	93
	K2	106 ± 6	108 ± 14	102
	K3	139 ± 1	143 ± 17	103
	K4	142 ± 10	128 ± 12	90
	K5	136 ± 10	137 ± 13	101
	K6	66 ± 5	73 ± 8	111
	K7	179 ± 7	186 ± 15	104
	K8	135 ± 17	140 ± 12	104
	K9	126 ± 11	133 ± 14	106
	K10	119 ± 16	105 ± 12	88
	K11	63 ± 9	59 ± 6	94
White grape	WG1	318 ± 6	314 ± 26	98
	WG2	289 ± 13	282 ± 22	98
	WG3	218 ± 31	211 ± 10	97
	WG4	124 ± 5	115 ± 14	93
	WG5	287 ± 23	287 ± 30	100
	WG6	95 ± 2	102 ± 5	108
	WG7	377 ± 3	383 ± 26	102
	WG8	142 ± 9	139 ±14	98
Red grape	RG1	756 ± 37	767 ± 62	101
	RG2	672 ± 28	665 ± 12	99
	RG3	603 ± 25	600 ± 15	100
	RG4	324 ± 32	315 ± 10	97
	RG5	260 ± 2	270 ± 23	104
	RG6	546 ± 69	533 ± 31	98
	RG7	276 ± 3	281 ± 22	102
	RG8	309 ± 35	277 ± 18	90

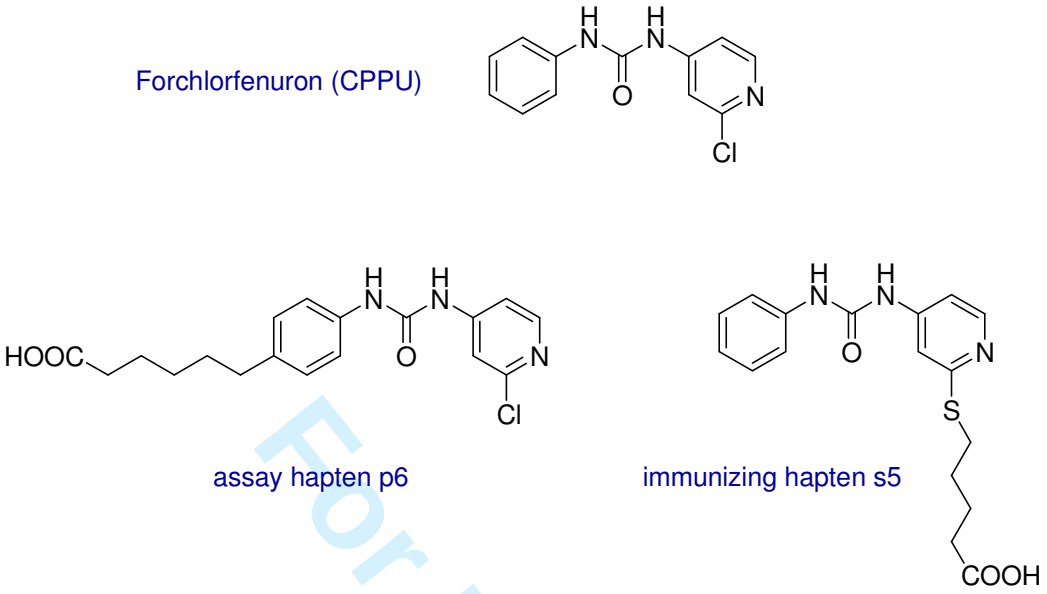


Fig. S1. Structures of the target analyte (forchlorfenuron), the immunizing hapten (s5), and the assay hapten (p6).

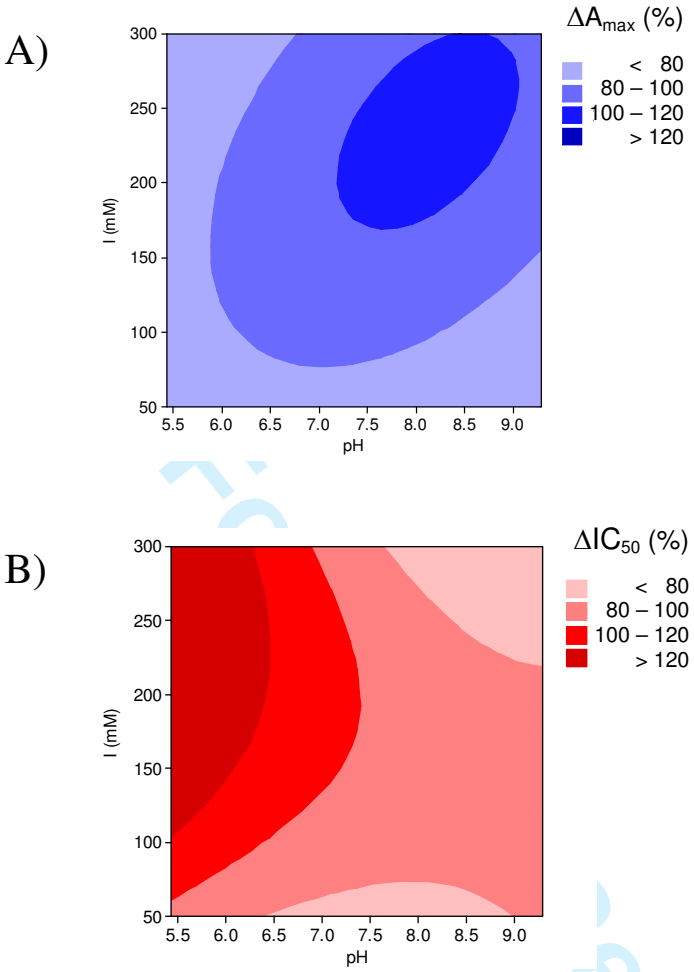


Fig. S2. Contour plots for the variation of A_{max} and IC_{50} values (%) as functions of buffer pH and ionic strength (*I*) at a fixed Tween 20 concentration of 0.025% (v/v). The assay parameters taken as references were those obtained at the centre point of the composite design.

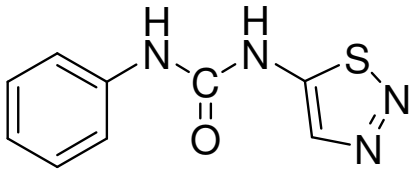


Fig. S3. Thidiazuron

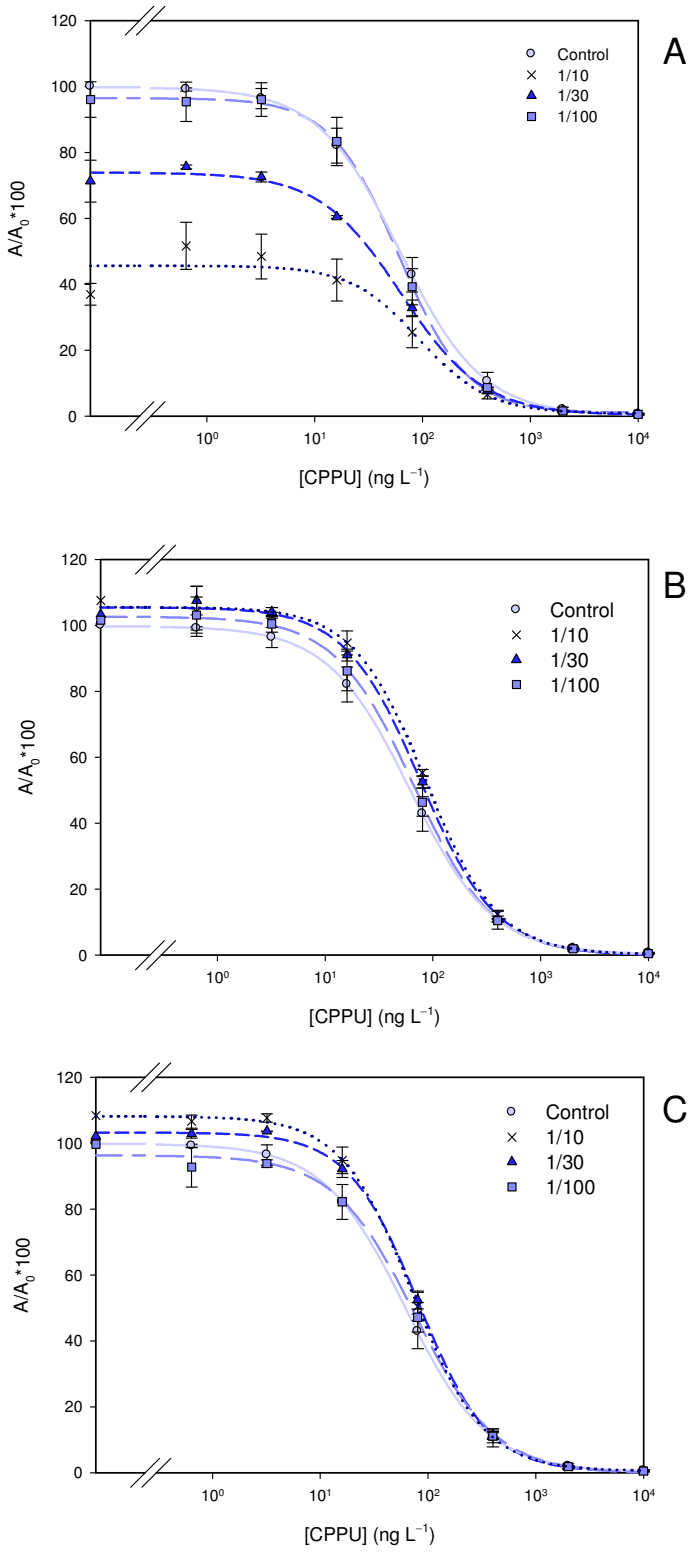
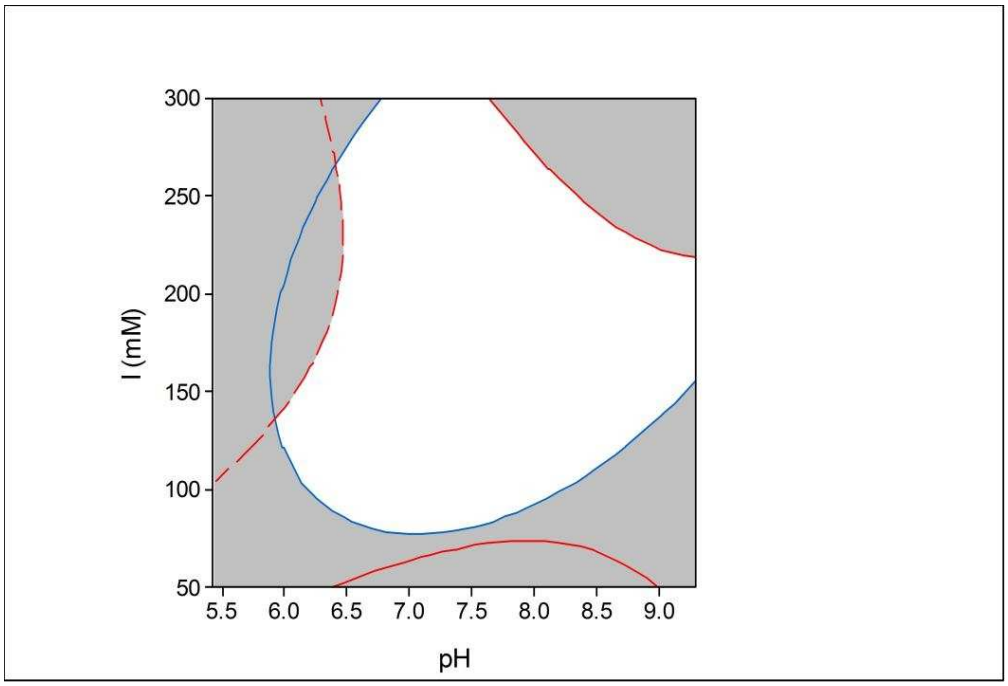


Fig. S4. Matrix interferences produced by kiwifruit juice (A) and white (B) and red grape (C) must over the ELISA inhibition curve.



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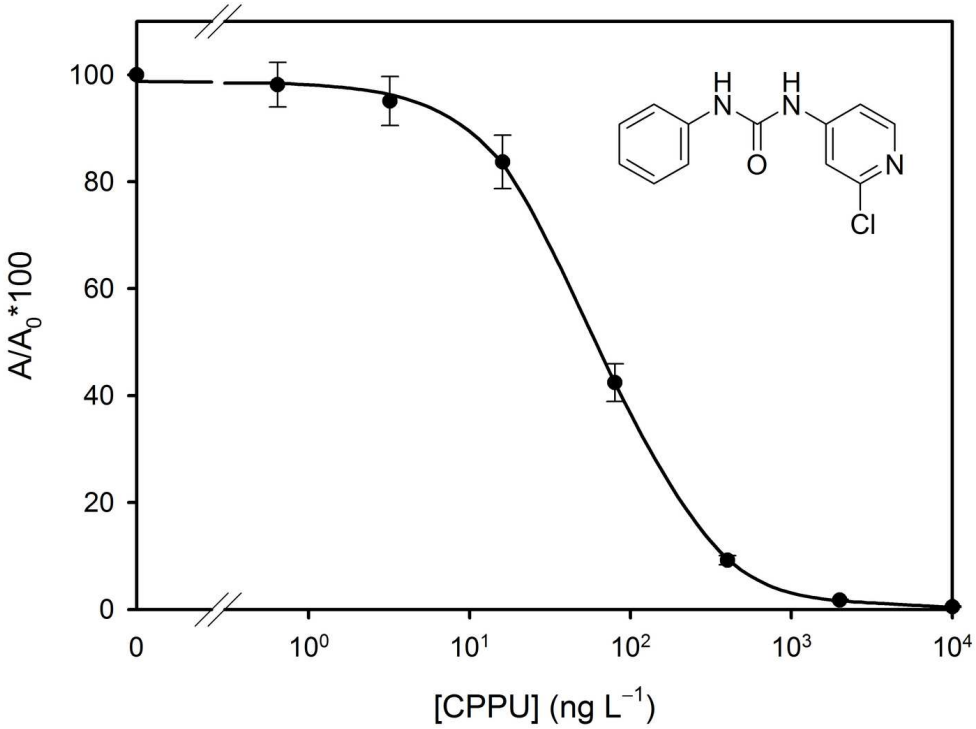

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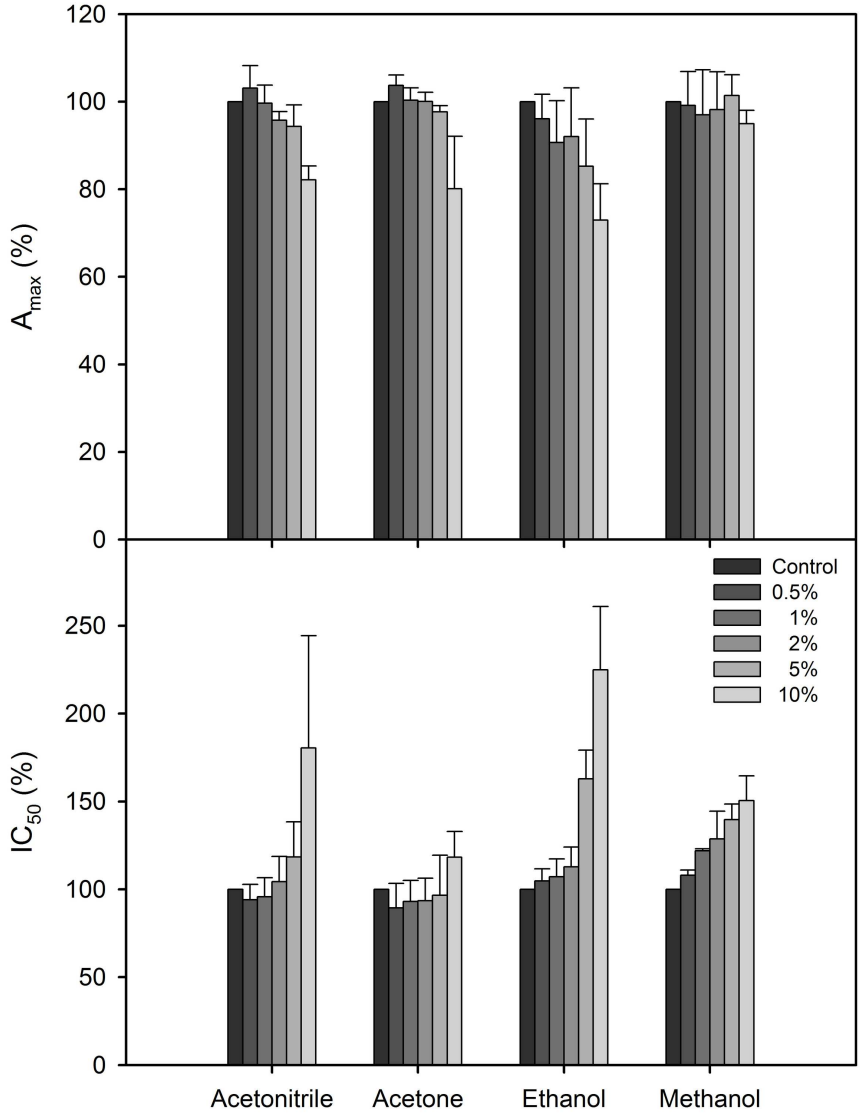

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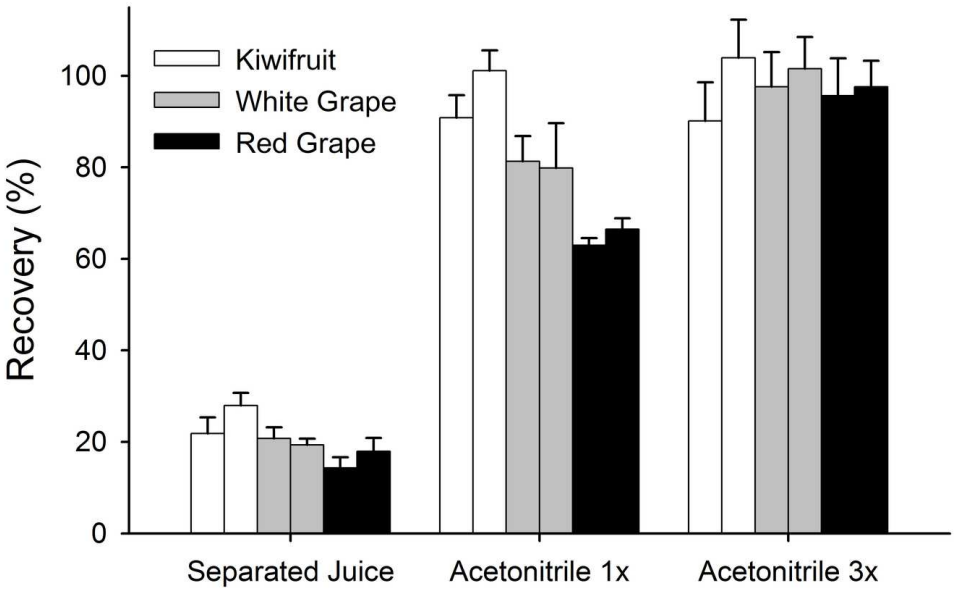

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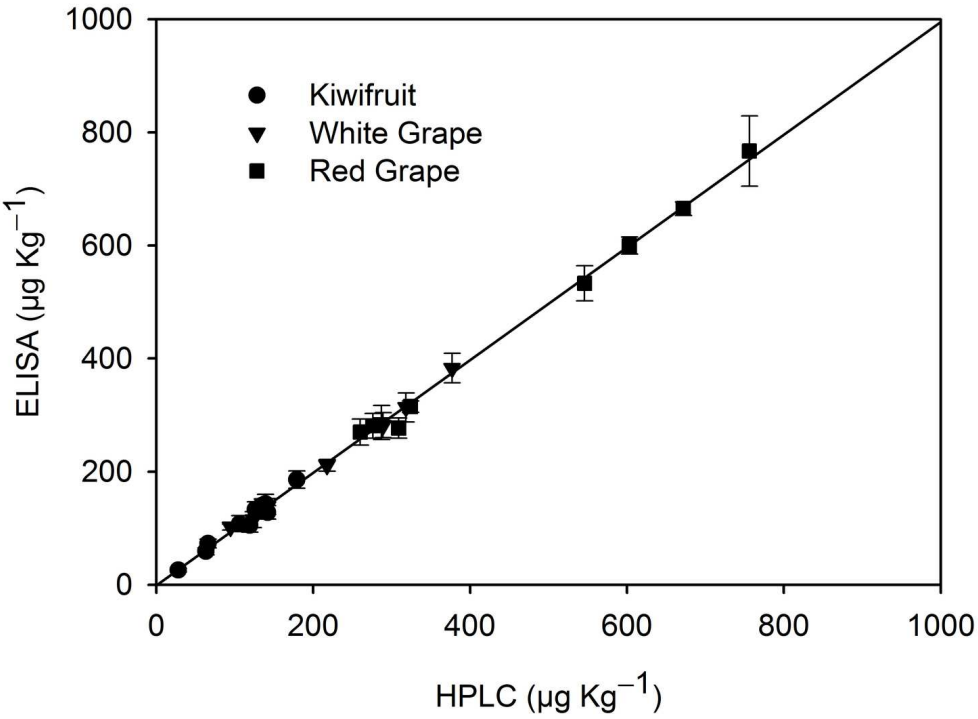

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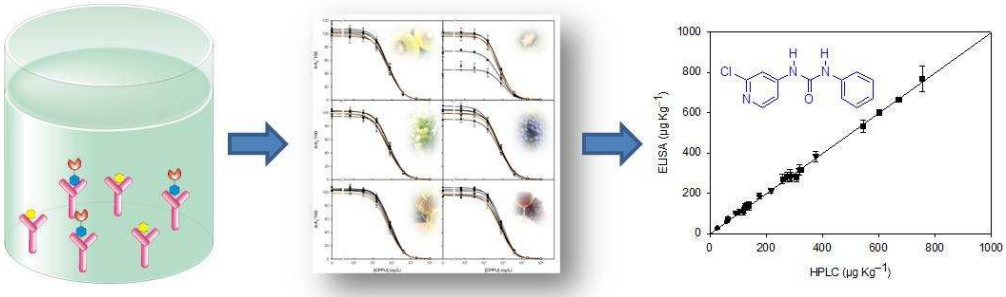

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