Development of an immunochromatographic assay based on carbon nanoparticles for the determination of the phytoregulator forchlorfenuron

Celia Suárez-Pantaleón[a], *Jan Wichers*[b], *Antonio Abad-Somovilla*[c], *Aart van Amerongen*[b,]*, *Antonio Abad-Fuentes*[a,]*

[a] *Department of Biotechnology, IATA−CSIC, Agustí Escardino 7, 46980 Paterna, València, Spain.*

[b] *Biomolecular Sensing & Diagnostics, Wageningen UR Food & Biobased Research, BornseWeilanden 9, P.O. Box 17, 6700 AA Wageningen, The Netherlands.*

[c]*Department of Organic Chemistry, Universitat de València, Doctor Moliner 50, 46100 Burjassot, València, Spain.*

* Authors to whom correspondence should be addressed

A. Abad-Fuentes: Tel.: (+34) 963900022; fax (+34) 963636301; E-mail address: aabad@iata.csic.es

A. van Amerongen: Tel.: (+31)317480164; fax: (+31)317483011; E-mail address: aart.vanamerongen@wur.nl.

Abstract

Rapid analytical methods enabling the determination of diverse targets are essential in a number of research areas, from clinical diagnostics to feed and food quality and safety. Herein, the development of a quantitative immunochromatographic assay for the detection of the synthetic phytoregulator forchlorfenuron (CPPU) is described. The competitive Lateral Flow Immunoassay (LFIA) was based on the immobilization onto a nitrocellulose membrane of an ovalbumin–CPPU conjugate (test line) and on the use of an immunodetection ligand consisting of carbon nanoparticles labelled with an anti-CPPU monoclonal antibody through interaction with a secondary antibody. The presence of CPPU in horticultural samples was visually interpreted by the decrease in the black signal intensity of the test line, according to the competitive character of the format. The quantitative determination of the analyte was easily performed by a two-step procedure consisting in flatbed scanning of the strips followed by computer-based image analysis of the pixel grey volumes of the test lines. Under optimized conditions, the immunochromatographic test afforded a limit of quantification in buffer of 89 ng/L. The accuracy of the strip test was assessed by the analysis of fruit samples with incurred residues, and the obtained results were compared with those derived from two reference methods, ELISA and HPLC. The LOQ of the CPPU-specific LFIA in kiwifruits and grapes was established at 33.4 µg/kg. The excellent analytical performance of the developed strip test demonstrates the potential of immunochromatographic assays for the quantitative monitoring of small organic molecules in complex matrices.

Keywords:

CPPU, hapten, immunoreactive strips, lateral flow immunoassay, carbon nanoparticles, immunosensing.

1. Introduction

Forchlorfenuron (Figure 1S), also referred to as CPPU (1-(2-chloro-4-pyridyl)-3-phenylurea), is a highly effective synthetic plant growth regulator (Takahashi et al., 1978) applied worldwide to increase fruit size, especially of kiwifruits and table grapes (Iwahori et al., 1988; Nickell, 1986). The intensive use of agrochemicals may lead to the presence of residues in foodstuffs when commodities reach the market, an issue of concern and high priority to both public authorities and the general population. Accordingly, the use of agrochemicals in different crops is strictly regulated by international organizations and national governments. With the aim of protecting public health and the environment, Maximum Residue Limits (MRLs) fixing the highest acceptable amount of a particular chemical in a certain crop have been established. For CPPU in particular, MRLs have been set up from 10 to 100 μg/kg, depending on the country and the target horticultural commodity. To ensure the effectiveness of the monitoring systems in place for risk management in food, the implementation of reliable, fast, and user-friendly analytical methods for the traceability of xenobiotics is highly desirable.

The analysis of chemical residues and contaminants has been traditionally dominated by instrumental techniques. Chromatographic methods for CPPU determination based on high-performance liquid chromatography (HPLC) coupled to different detection systems have been reported (Hu and Li, 2006; Valverde et al., 2010). Although highly accurate, reproducible, and sensitive, chromatographic methods are often time-consuming, expensive, and laboratory-oriented as they require well-trained personnel and sophisticated instrumentation (Lee and Kennedy, 2001). Immunoanalytical tools are deemed highly sensitive, selective, rapid, and cost-effective methods, thus complementing chromatographic analysis. In addition, they are especially well-suited for high-throughput screening in difficult matrices without extensive sample pre-treatment (Knopp, 2006). Moreover, antibodies can be easily integrated into a variety of automated systems, from liquid handling workstations to immunosensor-based analytical platforms (Bange et al., 2005). In previous papers, the production of monoclonal and polyclonal antibodies (mAb and pAb, respectively) for

CPPU was described (Suárez-Pantaleón et al., 2008, 2010, 2011), and Enzyme-Linked ImmunoSorbent Assays (ELISAs) were developed and successfully applied to the detection of CPPU in fruit samples. Even though ELISA certainly is one of the most widespread and popular kind of immunoassays, immunochromatographic assays are better suited for on-site rapid applications. The large number of scientific publications in the diagnostic (Andreo et al., 2006; Mens et al., 2008), medical (Lin et al., 2008; Omidfar et al., 2012; Yang et al., 2011), veterinary (Noguera et al. 2011), drug of abuse (Gandhi et al., 2009), environmental (Blažková et al., 2009; Kim et al., 2003; Zhou et al. 2010), and food safety areas (Anfossi et al., 2011; Aldus et al., 2003; Tang et al., 2009; Zhang et al., 2011; Zhou et al., 2009), well illustrates the prominent position that immunochromatographic methods have attained in the analytical field as portable point-of-care devices in recent years (Posthuma-Trumpie et al., 2008).

Strip-based tests were originally designed for sample screening, thus efficiently reducing analytical costs because only non-compliant samples would be submitted to further determination by confirmatory methods. At present, there is an urgent need for strip-based tests meeting requirements of robustness and accuracy while keeping simplicity and affordability. Accordingly, the development of quantitative immunochromatographic tests is gaining increasing attention. Successful examples have been described for the analysis of proteins (Kim et al., 2006; van Amerongen et al., 1994; Wei et al., 2011), nucleic acids (Blažková et al., 2011; He et al., 2011; Noguera et al., 2011; van Amerongen and Koets, 2005), and small organic molecules (Blažková et al., 2010; Campbell et al., 2007; Hua et al., 2010; Kaur et al., 2007; Omidfar et al., 2010; Mirasoli et al., 2012; O'Keeffe et al., 2003; Wang et al., 2011; Xie et al., 2009).

Based on the characterization by ELISA of previously produced immunoreagents, the mAb p6#42, with a remarkable affinity and specificity to the synthetic cytokinin CPPU, was selected for the development of a rapid strip test. In the present work, a mAb-based LFIA using carbon nanoparticles as label is presented for the quantitative and rapid detection of CPPU. The analytical performance of the one-step strip test was assessed by determining CPPU in incurred kiwifruit and grape samples, using ELISA and HPLC as reference methods.

2. Materials and methods

2.1. Reagents

Forchlorfenuron [1-(2-chloro-4-pyridyl)-3-phenylurea, CPPU] (CAS Registry No. 68157-60-8, MW 247.7 g/mol) and 1-(4-pyridyl)-3-phenylurea (PPU) were from Sigma-Aldrich (Madrid, Spain). Stock solutions of CPPU (100 mM) and PPU (100 mM) were prepared in anhydrous *N,N*-dimethylformamide and methanol, respectively, and they were stored at –20 ºC. *Ortho*-phenylenediamine (OPD) was from Sigma-Aldrich (Madrid, Spain). Spezial Schwarz 4 carbon nanoparticles were acquired from Degussa AG (Frankfurt, Germany). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Nitrocellulose Hi-Flow Plus membrane HF090 (capillary flow time of 90 sec/4 cm) was from Millipore (Molsheim, France). Absorption pads (filter paper 2886) were purchased from Schleicher & Schuell (Middlesex, UK).
The synthesis of haptens p6 and p2, the preparation of capture conjugates OVA–p6 and OVA–p2, and the production of mAb p6#42 have been previously described (Suárez-Pantaleón et al., 2008). Cross-reactivity studies with the mAb p6#42 showing the high specificity of the anti-CPPU antibody have been previously reported (Suárez-Pantaleón et al., 2011). The chemical structure of CPPU and both haptens is shown in Figure 1S. Polyclonal goat anti-mouse IgGFcγ fragment specific immunoglobulins (GAM) and polyclonal donkey anti-goat IgG (H+L) immunoglobulins (DAG) were from Jackson Immunoresearch Europe (Sanbio, Uden, The Netherlands). Polyclonal rabbit anti-mouse immunoglobulins labelled with peroxidase (RAM–HRP) were from Dako (Glostrup, Denmark). 96-well Costar flat-bottom high-binding polystyrene microplates were from Corning (Corning, NY, USA).

2.2. Instrumentation

Capture conjugates (CC) were immobilized onto the membranes using a TLC-spotter Linomat IV (Camag, Muttenz, Switzerland). Membranes were cut into strips using a Bio-Dot CM4000 cutter (Biodot Inc., Irvine, CA, USA). Scanning of the strips was carried out with an Epson 3200 Photo

scanner (Seiko Epson, Nagano, Japan). Measurements of the pixel grey volume of the test and control lines were performed using TotalLab image analysis software (Nonlinear Dynamics, Newcastle, UK).

2.3. Buffers

(1) *Coupling buffer*: 5 mM sodium borate buffer, pH 8.8; (2) *Washing solution*: 5 mM sodium borate buffer, pH 8.8, 1% (w/v) BSA, 0.02% (w/v) NaN_3; (3) *Storage buffer*: 100 mM sodium borate buffer, pH 8.8, 1% (w/v) BSA, 0.02% (w/v) NaN_3; (4) *Spraying buffer*: 5 mM sodium borate buffer, pH 8.8; (5) *Running buffer*: 100 mM sodium borate buffer, pH 8.8, 1% (w/v) BSA, 0.05% (v/v) Tween 20, 0.02% (w/v) NaN_3.

2.4. Development of the LFIA

2.4.1. Preparation of carbon nanoparticles–secondary antibody Detection Conjugate (DC)

For the preparation of the DC, carbon nanoparticles were labeled with GAM immunoglobulins (O'Keeffe et al., 2003). A 1% (w/v) suspension of carbon was prepared in demineralized water by sonication. Then, 100 µL of coupling buffer containing 175 µg of GAM were added dropwise to 500 µL of a 5–fold dilution of carbon (0.2%, w/v) in coupling buffer. After overnight incubation at 4 ºC under gentle stirring, the solution was washed four times with washing solution by centrifugation (13600×*g*; 15 minutes). Then, the DC was reconstituted in storage buffer at a final concentration of carbon of 0.2%, and stored at 4 ºC. Before running experiments, the working dilution of the GAM–carbon conjugate was sonicated for 10 seconds.

2.4.2. Immobilization of reagents onto nitrocellulose membranes

Capture conjugates OVA–p6 and OVA–p2 diluted in spraying buffer were immobilized onto plastic-backed membranes at 3 cm from the origin forming the test line (TL). The final concentration of CC on the TL was 62.5, 250 or 1000 ng/strip. DAG immunoglobulins (80 ng/strip), also diluted in spraying buffer, were immobilized onto the nitrocellulose at 3 mm above the TL to form the control

line (CL). After the spraying, the membranes were dried overnight at 37 ºC. Then, the nitrocellulose membranes were placed on a second plastic backing and an absorbance pad was applied at the upper part of the membranes. Subsequently, the membranes were cut into 0.5 × 5 cm strips and they were stored in sealed plastic laminated aluminum bags with a desiccation pad at room temperature until use.

2.4.3. LFIA procedure and signal processing

For convenience, microtiter plate wells instead of plastic housings or tubes were used as containers for running the immunochromatographic assays. Samples or CPPU standard solutions (80 µL) were added to the well followed by 10 µL of DC suspension (typically a 10 or 20–fold dilution) and 10 µL of mAb p6#42. All solutions were prepared in running buffer. Strips were used following opening of the aluminum storage bags and placed vertically into the wells. The fluid was allowed to run through the strip for a maximum of 30 minutes at room temperature. Then the strips were dried at room temperature.

For quantitative purposes the strips were scanned at 1200 dpi resolution in 16-bit grayscale, and the scanned images were saved as 16-bit TIFF files. The image analysis was carried out by measuring the pixel grey volume (PGV) corresponding to a slot placed over the TL (dimensions 65×15 pixels), and the PGV of a slot with the same dimensions placed below the TL was subtracted for background correction. Measured PGVs were plotted against CPPU concentration on a logarithmic scale, and the resulting sigmoidal curve was fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). Assay sensitivity was defined as the concentration of analyte at the inflection point of the fitted curve, typically producing a 50% inhibition (IC_{50}) of the maximum signal (PGV_{max}). The limit of detection (LOD) and the limit of quantification (LOQ) of the assay were defined as the concentration of analyte generating a 10% and a 20% inhibition of the PGV_{max} (IC_{10} and IC_{20}, respectively). The dynamic range (DR) for the quantification of CPPU was established between the values of the IC_{20} and the IC_{80} (concentrations of analyte causing a 20 and 80% inhibition of PGV_{max}, respectively). Normalization of signal

intensity was conducted using the experimental PGV reached at the zero dose of analyte (PGV_0). All the experiments were run in three independent replicates.

2.5. Analysis of food samples

Kiwifruit and grape vines were treated with CPPU at the dose recommended by the manufacturer (10 mg/kg). After the application of the agrochemical, fruits were collected at different times in order to obtain real samples containing diverse levels of CPPU. Fruits obtained from non-treated vines were included in the analysis as negative controls. The QuEChERS-based extraction procedure of CPPU from horticultural samples was based on the methodology described by Hu and Li (2006). CPPU residues were extracted from kiwifruits and grapes following two different approaches for the analysis by HPLC/UV and the immunoassays (LFIA and ELISA) as recently reported (Suárez-Pantaleón et al., 2012). Detailed information is included in the Supplementary data.

2.5.1. Assessment of matrix effects

After LFIA optimization under standard conditions in assay buffer, possible matrix interferences in the assay from fruit samples were studied. In order to determine the influence of the matrix on assay performance, four conditions were evaluated, i.e., absence of analyte and the three concentrations of CPPU generating a 30, 50 and 70% inhibition of PGV_{max} (IC_{30}, IC_{50}, and IC_{70}, respectively). Strips were run under these four conditions in buffer as control, and in extracts prepared from negative fruit samples (as determined by HPLC/UV) at three different dilutions in buffer (1/10, 1/50, and 1/250). Signals achieved at the TL with the diluted extracts were compared with those obtained in assays run in buffer.

2.5.2. Determination of CPPU by LFIA, ELISA and HPLC/UV

Kiwifruit and grape extracts were prepared following the corresponding procedure for their analysis by LFIA and ELISA or HPLC/UV. The CPPU concentration in fruit extracts was determined by all three analytical methods on three different days. For the analysis with the immunoanalytical techniques, samples had to be properly diluted in buffer, either to enter into the

dynamic range of the standard curve or to avoid matrix and solvent effects (300–fold final sample dilution). For the quantitative determination of the cytokinin by LFIA, a standard curve with three CPPU concentrations covering the dynamic range of the assay was performed in parallel to the analysis of the samples, and used for the interpolation of the PGV obtained for every diluted sample. In the case of the ELISA, CPPU concentration was determined by the interpolation of the signal intensity obtained for each diluted sample from an 8–point standard curve run in triplicate in every plate. Particular conditions for CPPU determination by HPLC/UV are described in Supplementary Information. The accuracy and trueness of the developed LFIA test were determined by comparing the quantification of CPPU in fruit extracts provided by the immunostrips with those obtained with the reference methods (ELISA and HPLC/UV) by linear regression analysis. The confidence intervals of the slope and the *y*-intercept were calculated as $a \pm t\ (n–2,\ 95\%) * \sigma$ and $y_0 \pm t\ (n–2,\ 95\%) * \sigma$, respectively. The analytical methods were considered statistically comparable when a = 1 and $y_0 = 0$. Inter-assay relative standard deviation (RSD), calculated as $\sigma/\bar{x} * 100$, was used for the estimation of the precision of the developed LFIA test (n = 3).

3. Results and discussion

3.1. Immunochromatographic assay principle

In one-step LFIAs a number of different labels are being applied for the generation of a visible signal. Gold nanoparticles are often preferred (Seydack, 2005; Anfossi et al., 2011), but other materials such as colloidal carbon (Blažková et al., 2009, 2010 and 2011; Koets et al., 2006; Lönnberg et al., 2008; Noguera et al., 2011; O´Keeffe et al., 2003), latex particles (Campbell et al., 2007) or quantum dots (Zou et al., 2010) and enzymes (Mirasoli et al., 2012) are also employed. In a survey performed by Gordon and Mitchel for sensitivities of labels that are used in lateral flow assays, carbon nanoparticles were ranked above gold and latex nanoparticles (Gordon and Mitchel, 2008). The carbon black-on-white signal allows for sensitivities down to the low picomolar range. Other advantages attributed to carbon nanoparticles are the very low cost of the starting material, the

ease of preparation, and the stability of the conjugates (Posthuma-Trumpie et al., 2012; van Amerongen et al., 1993).

Depending on the detection system used, immunoassays can be classified into two main categories. Large molecules are often detected following an immunometric approach where the assay signal is directly proportional to the amount of target molecule (sandwich format). On the contrary, hapten-like molecules (molecular weight below 5000 dalton), such as CPPU, cannot be simultaneously bound by two antibody molecules. As a consequence, immunoassays directed to haptens usually rely on a competitive approach, wherein the signal intensity is inversely proportional to the analyte concentration in the sample. In the selected LFIA format, an OVA–hapten conjugate was immobilized onto nitrocellulose membranes as capture reagent in the test line. The employed detection conjugate was an immuno-complex consisting of goat anti-mouse immunoglobulins immobilized onto carbon nanoparticles and a specific anti-CPPU mAb that was titrated to yield an optimal maximum signal. By using goat immunoglobulins specific to the Fcγ-fragment of mouse antibodies, a favorable orientation of the mAb on the surface of the complex is guaranteed. Moreover, by changing the specific antibody, this arrangement enables the use of the carbon conjugate as a universal label suitable for the development of LFIA against a wide range of analytes, or even for the development of multiresidue tests. To monitor the correct performance of the immunochromatographic assay, anti-goat immunoglobulins were immobilized at the control line above the test line. The schematic representation of the assay is shown in Figure 1.

Figure 1

The principle of the competitive test is as follows: the immunoreactive strip is dipped into a test tube or a microplate well containing the sample and the test reagents (the immuno-complex detection conjugate and the mAb), so the solution moves through the membrane by capillary force. When reaching the TL, the antigen binding sites of the mAb not occupied by CPPU can bind to the capture conjugate. Hence, the higher the analyte concentration in the sample, the lower the resulting

243 black/grey intensity of the TL in the strip. The appearance of signal at the control line ensures that
244 the membrane flow has occurred properly; otherwise, the test is considered invalid.

245 **3.2. Optimization of the immunochromatographic test**

246 The influence of the immunoreagent concentrations on the assay signal and detectability was
247 evaluated in order to determine the most suitable conditions for the development of an
248 immunodipstick for CPPU detection. Strips were sprayed at three different concentrations with
249 OVA–p6 or OVA–p2 (62.5, 250 and 1000 ng/strip). Two GAM–carbon conjugate solutions [0.01
250 and 0.02% (w/v) of carbon] and five concentrations of the mAb p6#42 (0.125, 0.25, 0.5, 1 and 2
251 µg/mL) were evaluated. The strips were first assayed in buffer without CPPU. The results from these
252 experiments are shown in Figure 2S. Thereafter, immunochromatographic assays based on all those
253 immunoreagent combinations able to afford a signal intensity of 200×10^3 PGV in the absence of
254 analyte were run in buffer with (0.5 µg/L) and without CPPU. These conditions were chosen because
255 a PGV of 200×10^3 was perfectly visible by the naked eye, and a 0.5 µg/L CPPU concentration was
256 able to generate a decrease in the signal intensity clearly distinguishable from the blank.

257 Table 1

258 The ratio between both signals was used to calculate the inhibition provided by each
259 immunoreagent combination (Table 1). As previously observed with the ELISA format using the
260 same mAb (Suárez-Pantaleón et al., 2008), the heterologous CC OVA–p2, with the shortest linker,
261 was better recognized than the homologous CC OVA–p6. Accordingly, OVA–p2 at 1000 ng/strip in
262 combination with the highest concentration of GAM–carbon conjugate (0.02%, w/v) was selected
263 for further work because a lower mAb p6#42 concentration was required (0.2 µg/mL) to afford the
264 maximum inhibition percentage.

265 Figure 2

266 The LOQ of the LFIA with CPPU standards in buffer was 89 ng/L, a value 500 times below the
267 MRL set by the European Commission for CPPU in kiwifruits (European Commission, 2006).

Accordingly, this rapid test could be potentially used for CPPU residue monitoring in horticultural samples, provided that matrix effects do not substantially interfere with the assay performance. Furthermore, this LOQ was just 4 times higher than that of the ELISA (22 ng/L) (Suárez-Pantaleón, 2008). The visual detection limit, defined as the minimum concentration of CPPU causing a clearly visible decrease in the TL signal intensity in the strip, was set at 250 ng/L. This visual detectability is comparable to other immunochromatographic assays for the analysis of low molecular weight compounds such as methiocarb, thiabenzole, indomethacin and aflatoxins (Blažková et al., 2009 and 2010; Li et al., 2009; Liao and Li, 2010; Zhang et al. 2011).

Finally, different concentrations of DAG immunoglobulins were tested for the preparation of the CL in order to obtain a signal intensity similar to that reached in the TL at the zero dose of analyte (Figure 2d).

3.3. Matrix influence on the assay performance

CPPU is registered in the European Union for application to kiwifruit crops. In other countries such as the United States, Japan, South Africa, Chile, and Mexico, this agrochemical is also used as growth promoter in table grape production. Therefore, the strip assay performance was evaluated in both commodities. Since a triple extraction protocol with acetonitrile was proven to be necessary in a previous study (Suárez-Pantaleón et al., 2012) for the quantitative recovery of CPPU residues from fruit samples, the potential interferences on the strip test analytical parameters caused by the matrix and the solvent were simultaneously studied. The signal intensity of the TL in buffer and in diluted extracts prepared from control (non-treated) fruits were compared under four conditions: absence of analyte and those CPPU concentrations producing a 30, 50 and 70% inhibition of PGV_{max} (IC_{30}, IC_{50}, and IC_{70} values, respectively) (Figure 3).

Figure 3

For both matrices, a minimum 100–fold dilution of the extracts in assay buffer was estimated to be required to suppress interferences from the matrix and/or the solvent. When similar experiments

were carried out in the ELISA format, a 100–fold extract dilution was also found to be optimal (results not shown). Accordingly, considering the whole sample preparation protocol (triple solvent extraction and extract dilution), the resulting estimated LOQ in kiwifruit and grape was 33.4 µg/kg and 6.6 µg/kg for LFIA and ELISA, respectively.

3.4. Determination of CPPU in fruits from in-field treated crops

Fruit samples from grape and kiwifruit vines treated in the field with CPPU were processed for analysis by HPLC/UV, ELISA, and LFIA. In the case of HPLC/UV, a pre-concentration step was necessary in order to determine CPPU concentrations at levels below the MRL. On the contrary, for determinations with LFIA and ELISA the extracts were directly analyzed after being properly diluted. The results of the determination of CPPU in the samples carried out by the three technologies are listed in Table 1S. For the evaluation of the analytical performance of the immunochromatographic assay, the results were compared by linear regression analysis with those obtained by ELISA and HPLC/UV (Figure 4).

Figure 4

Excellent correlation coefficients were observed between the quantification of CPPU by LFIA and both ELISA (r^2 = 0.996) and HPLC/UV (r^2 = 0.994), with a slight overestimation of the CPPU fruit content by the immunochromatographic assay [(y = (1.11 ± 0.06) x – (12 ± 11) for LFIA *vs* ELISA and y = (1.08 ± 0.06) x – (12 ± 12) for LFIA *vs* HPLC/UV]. Concerning LFIA precision, the inter-assay relative standard deviation (RSD) values were mostly below 20%.

Finally, the strips were visually evaluated by five different people (Figure 5). All the samples were correctly scored as positive, with the exception of the negative controls (extracts obtained from CPPU-free fruits) and the sample K1, which was identified as positive only by two people.

According to the results herein presented, the CPPU-LFIA could be an effective tool for on-site applications with the objective of conducting a preliminary rapid high-throughput screening of samples. Furthermore, the excellent analytical performance of the developed test supports the

potential application of immunochromatographic assays with semi-quantitative or quantitative purposes.

Figure 5

4. Conclusions

A simple and rapid immunochromatographic test has been developed and optimized for the analysis of CPPU in kiwifruit and grape fruits. Quantitative determinations by the CPPU-LFIA were proved to be feasible by using a flatbed scanner and image analysis software to record and process signal intensities in the immunoreactive strips. The optimized test showed an estimated LOQ in fruit samples of 33.4 μg/kg, in line with the MRL established for CPPU in horticultural commodities. According to the satisfactory correlation between the LFIA, ELISA, and HPLC in the analysis of samples with incurred residues, it can be concluded that the developed immunochromatographic assay meets the specifications typically demanded to analytical methods in terms of detectability, accuracy and precision. This strip test would therefore be very valuable for on-site screening of CPPU residues in crop samples by non-skilled personnel because of its simplicity and independence from sophisticated instrumentation.

Acknowledgements

This work was supported by Ministerio de Educación y Ciencia (AGL2009-12940-C02/01/02/ALI), and cofinanced by FEDER Funds. Celia Suárez Pantaleón was hired by the CSIC under a predoctoral I3P contract. We thank Dr. Geertruida A. Posthuma-Trumpie for excellent technical assistance in software usage.

Appendix A. Supplementary data

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

342 **References**

343 Aldus, C. F., van Amerongen, A., Ariens, R. M., Peck, M. W., Wichers, J. H., Wyatt, G. M., 2003. J.
344 Appl. Microbiol. 95, 380–389.

345 Andreo, F., Domínguez, J., Ruiz-Manzano, J., Prat, C., Blanco, S., Lores, L., Sánchez, M. D.,
346 Latorre, I., Giménez, M., Ausina, V., 2006. Clin. Microbiol. Infect. 12, 682–684.

347 Anfossi, L., D'Arco, G., Baggiani, C., Giovannoli, C., Giraudi, G., 2011. Food Control 22, 1965–
348 1970.

349 Bange, A., Halsall, H. B., Heineman, W. R., 2005. Biosens. Bioelectron. 20, 2488–2503.

350 Blažková, M., Mičková-Holubová, B., Rauch, P., Fukal, L., 2009. Biosens. Bioelectron. 25, 753–
351 758.

352 Blažková, M., Rauch, P., Fukal, L., 2010. Biosens. Bioelectron. 25, 2122–2128.

353 Blažková, M., Javůrková, B., Fukal, L., Rauch, P., 2011. Biosens. Bioelectron. 26, 2828–2834.

354 Campbell, K., Fodey, T., Flint, J., Danks, C., Danaher, M., O'Keeffe, M., Kennedy, D. G., Elliott, C.,
355 2007. J. Agric. Food Chem. 55, 2497–2503.

356 European Commission, 2006, Commission Directive 2006/10/EC, Off. J. Eur. Community, L25, 24–
357 27.

358 Gandhi, S., Caplash, N., Sharma, P., Raman, S. C., 2009. Biosens. Bioelectron. 25, 502–505.

359 Gordon, J., Michel, G. 2008. Clin. Chem. 54, 1250–1251.

360 He, Y., Zhang, S., Zhang, X., Baloda, M., Gurung, A. S., Xu, H., Zhang, X., Liu, G., 2011. Biosens.
361 Bioelectron. 26, 2018–2024.

362 Hu, J. Y., Li, J. Z., 2006. J. AOAC Int. 89, 1635–1640.

363 Hua, X., Qian, G., Yang, J., Hu, B., Fan, J., Qin, N., Li, G., Wang, Y., Liu, F., 2010. Biosens.
364 Bioelectron. 26, 189–194.

365 Iwahori, S., Tominaga, S., Yamasaki, T., 1988. Sci. Hortic.-Amsterdam 35, 109–115.

366 Kaur, J., Singh, K. V., Boro, R., Thampi, K. R., Raje, M., Varshney, G. C., Suri, C. R., 2007.
367 Environ. Sci. Technol. 41, 5028–5036.

Kim, Y. M., Oh, S. W., Jeong, S. Y., Pyo, D. J., Choi, E. Y., 2003. Environ. Sci. Technol. 37, 1899–1904.

Kim M. J., Shim J. Y., Lee Y. T., Lee H. S., 2006. Int. J. Food Sci. Tech. 41, 927–932.

Knopp, D., 2006. Anal. Bioanal. Chem. 385, 425–427.

Koets, M., Sander, I., Bogdanovic, J., Doekes, G., van Amerongen, A., 2006. J. Environ. Monit. 8, 942–946.

Lee, N. A., Kennedy, I. R., 2001. J. AOAC Int. 84, 1393–1406.

Li, D., Wei, S., Yang, H., Li, Y., Deng, A., 2009. Biosens. Bioelectron. 24, 2277–2280.

Liao, J-Y., Li, H., 2010. Microchim. Acta 171, 289–295.

Lin, Y. Y., Wang, J., Liu, G., Wu, H., Wai, C. M., Lin, Y., 2008. Biosens. Bioelectron. 23, 1659–1665.

Lönnberg, M., Drevin, M., Carlsson, J.,2008. J. Immunol. Methods 339, 236–244.

Mirasoli, M., Buragina, A., Dolci, L. S., Simoni, P., Anfossi, L., Giraudi, G., Roda, A. 2012. Biosens. Bioelectron. 32, 283–287.

Mens, P. F., van Amerongen, A., Sawa, P., Kager, P. A., Schallig, H. D., 2008. Diagn. Microbiol. Infect. Dis. 61, 421–427.

Nickell, L. G., 1986. Proc. Plant Growth Regul. Soc. Am. 13, 236–241.

Noguera, P., Posthuma-Trumpie, G. A., van Tuil M., van der Wal, F. J., de Boer. A., Moers, A. P., van Amerongen. A., 2011. Anal. Bioanal. Chem. 399, 831–838.

O'Keeffe, M., Crabbe, P., Salden, M., Wichers, J., Van Peteghem. C., Kohen, F., Pieraccini, G., Moneti, G., 2003. J. Immunol. Methods 278, 117–126.

Omidfar, K., Kia, S., Kashanian, S., Paknejad, M., Besharatie, A., Kashanian, S., Larijani, B., 2010. Appl. Biochem. Biotechnol. 160, 843–855.

Omidfar, K., Khorsand, B., Larijani, B., 2012. Mol. Biol. Rep. 39, 1253–1259.

Posthuma-Trumpie, G. A., Korf, J., van Amerongen, A., 2008. Anal. Bioanal. Chem. 392, 1215–1223.

394 Posthuma-Trumpie, G. A., Wichers, J.H., Koets, M., Berendsen L.B.,van Amerongen, A., 2012.
395 Anal. Bioanal. Chem. 402, 593–600.
396 Seydack, M., 2005. Biosens. Bioelectron. 20, 2454–2469.
397 Suárez-Pantaleón, C., Mercader, J. V., Agulló, C., Abad-Somovilla, A., Abad-Fuentes, A., 2008. J.
398 Agric. Food Chem. 56, 11122–11131.
399 Suárez-Pantaleón, C., Mercader, J. V., Agulló, C., Abad-Somovilla, A., Abad-Fuentes, A., 2010. J.
400 Agric. Food Chem. 58, 8502–8511.
401 Suárez-Pantaleón, C., Mercader, J. V., Agulló, C., Abad-Somovilla, A., Abad-Fuentes, A., 2011.
402 Org. Biomol. Chem. 9, 4863–4872.
403 Suárez-Pantaleón, C., Esteve-Turrillas, F. A., Mercader, J. V., Agulló, C., Abad-Somovilla, A.,
404 Abad-Fuentes, A., 2012. Anal. Bioanal. Chem. 403, 2019–2026.
405 Takahashi, S., Shudo, K., Okamoto, T., Yamada, K., Isogai, Y., 1978. Phytochemistry 17, 1201–
406 1207.
407 Tang, D., Sauceda, J. C., Lin, Z., Ott, S., Basova, E., Goryacheva, I., Biselli, S., Lin, J., Niessner, R.,
408 Knopp, D., 2009. Biosens. Bioelectron. 25, 514–518.
409 Valverde, A., Aguilera, A., Ferrer, C., Camacho, F., Cammarano, A., 2010. J. Agric. Food Chem.
410 58, 2818–2823.
411 van Amerongen, A., Wichers, J. H., Berendsen, L. B., Timmermans, A. J., Keizer, G. D., van Doorn,
412 A. W., Bantjes, A., van Gelder, W. M., 1993. J. Biotechnol. 30, 185–195.
413 van Amerongen. A., van Loon. D., Berendsen, L. B., Wichers, J. H., 1994. Clin. Chim. Acta 229,
414 67–75.
415 van Amerongen, A., Koets, M., 2005. Simple and rapid bacterial protein and DNA diagnostic
416 methods based on signal generation with colloidal carbon particles, in: van Amerongen, A., Barug,
417 D., Lauwaars, M. (Eds), Rapid methods for biological and chemical contaminants in food and feed.
418 Wageningen Academic Publishers, Wageningen, The Netherlands, pp. 105-126.
419 Wang, L., Lu, D., Wang, J., Du, D., Zou, Z., Wang, H., Smith, J. N., Timchalk, C., Liu, F., Lin, Y.,
420 2011. Biosens. Bioelectron. 26, 2835–2840.

Wei, Y., Xiao-bing, L., Guo-wen, L., Bing-bing, Z., Yi, Z., Tao, K., Jia-jia, T., Dong-na, L., Zhe, W., 2011. Biosens. Bioelectron. 26, 3710–3713.

Xie, H., Ma, W., Liu, L., Chen, W., Peng, C., Xu, C., Wang, L., 2009. Anal. Chim. Acta 634, 129–133.

Yang, Q., Gong, X., Song, T., Yang, J., Zhu, S., Li, Y., Cui, Y., Li, Y., Zhang, B., Chang, J., 2011. Biosens. Bioelectron. 30, 145–150.

Zhang, D., Li, P., Zhang, Q., Zhang, W., 2011. Biosens. Bioelectron. 26, 2877–2882.

Zhou, Y., Pan, F. G., Li, Y. S., Zhang, Y. Y., Zhang, J. H., Lu, S. Y., Ren, H. L., Liu, Z. S., 2009. Biosens. Bioelectron. 24, 2744–2747.

Zhou, Y., Zhang, Y., Pan, F., Li, Y., Lu, S., Ren, H., Shen, Q., Li, Z., Zhang, J., Chen, Q., Liu, Z., 2010. Biosens. Bioelectron. 25, 2534–2538.

Zou, Z., Du, D., Wang, J., Smith, J. N., Timchalk, C., Li, Y., Lin, Y., 2010. Anal. Chem. 82, 5125–5133.

435 **Figure legends**

436 **Figure 1.** Schematic representation of the competitive mAb-based LFIA for the detection of
437 forchlorfenuron. Drawings are not to scale.

438 **Figure 2.** Immunoassays developed with the mAb p6#42 for the detection of forchlorfenuron. a)
439 Final assay conditions and analytical parameters of the optimized immunoassays. b) Inhibition
440 curves for CPPU in both immunoassay formats. c) Representative example of results obtained with
441 the quantitative LFIA. d) Representative immunochromatographic strips including the TL and the
442 CL.

443 **Figure 3.** Matrix effect of fruit extracts on LFIA parameters. Four CPPU concentrations were
444 evaluated in buffer (control) and in kiwifruit extract (left graph) and grape extract (right graph)
445 diluted in buffer (1/10, 1/50, 1/250). The signal intensity reached at the TL for the strips run in every
446 diluted extract was normalized with respect to that obtained in buffer in absence of analyte (PGV_0).
447 Each value represents the average of three independent experiments.

448 **Figure 4.** Linear correlation of the data obtained in the quantification of CPPU in kiwifruit (circles)
449 and grape samples (triangles) by LFIA and HPLC (red line) and by LFIA and ELISA (black line).
450 The samples were analyzed in triplicate in different days.

451 **Figure 5.** Representative experiment of the analysis of kiwifruit and grape samples with incurred
452 CPPU residues by immunochromatographic strips. G and K refer to grape and kiwifruit
453 commodities, respectively. The signal intensity of the strips was visually interpreted by 5 people as
454 CPPU positive (+) or CPPU negative (−).

455

Table 1. Influence of immunoreagent concentrations and hapten heterology on LFIA performance

capture conjugate immobilized onto the test line							
OVA–p2				OVA–p6			
[CC][a] (ng/strip)	[DC][b] (%)	[mAb p6#42] (µg/mL)	% inhibition (0.5 µg/L CPPU)[c]	[CC] (ng/strip)	[DC] (%)	[mAb p6#42] (µg/mL)	% inhibition (0.5 µg/L CPPU)
62.5	0.01	n.a.[d]	n.a.	62.5	0.01	n.a.	n.a.
	0.02	1	49.92 ± 7.09		0.02	n.a.	n.a.
250	0.01	1	54.99 ± 9.38	250	0.01	n.a.	n.a.
	0.02	0.5	59.40 ± 2.33		0.02	1	45.47 ± 2.82
1000	0.01	0.5	59.43 ± 2.85	1000	0.01	1	58.04 ± 2.14
	0.02	0.25	66.94 ± 1.73		0.02	0.5	61.95 ± 2.64

[a] Capture conjugate. [b] Detection conjugate (GAM–carbon conjugate). [c] The inhibition percentage has been calculated using the signal intensity obtained in absence of analyte as reference value (n=3). [d] Not applicable because signals below 200×10^3 were obtained with these immunoreagent combinations.

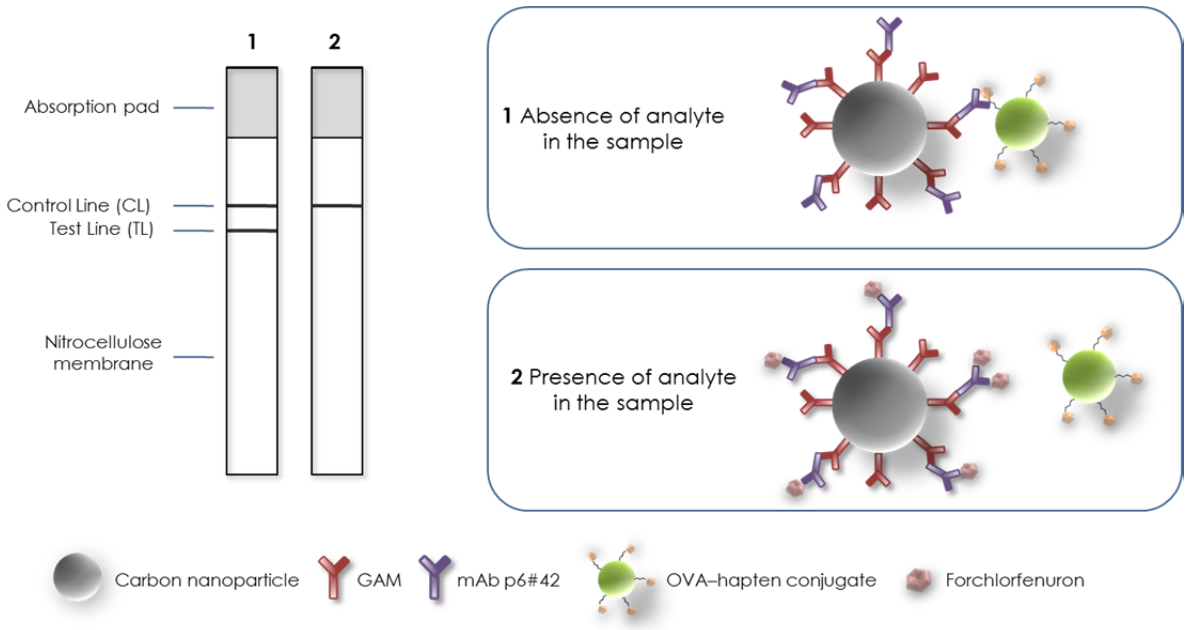

1
2
Absorption pad
Control Line (CL)
Test Line (TL)
Nitrocellulose membrane
1 Absence of analyte in the sample
2 Presence of analyte in the sample
Carbon nanoparticle
GAM
mAb p6#42
OVA–hapten conjugate
Forchlorfenuron

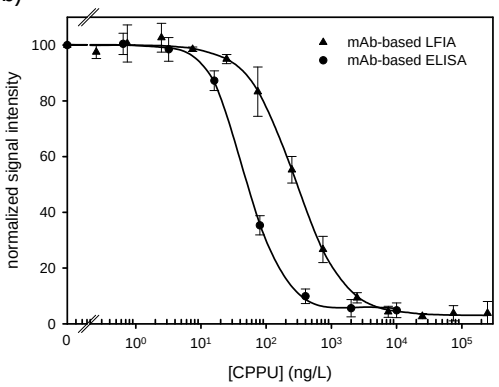
Figure 1

a)

Immunoanalytical tool			
LFIA[a]		ELISA[b]	
[capture conjugate]	OVA–p2 1 µg/strip	[coating conjugate]	OVA–p2 0.01 µg/well
detection conjugate	GAM–carbon 0.02%	RAM–HRP dilution	1/2000
[mAb p6#42][c]	20 ng/mL	[mAb p6#42][c]	30 ng/mL
PGV_{max}	$177\times10^3 \pm 12\times10^3$	A_{max}	1.12 ± 0.12
slope	−1.20 ± 0.06	slope	−1.58 ± 0.13
IC_{50} (ng/L)	286 ± 61	IC_{50} (ng/L)	50 ± 3
LOD (ng/L)[d]	45 ± 9	LOD (ng/L)[d]	13 ± 2
DR (ng/L)[e]	89 ± 9 to 916 ± 22	DR (ng/L)[e]	22 ± 2 to 156 ± 28
assay time	30 min	assay time	2h 10 min

[a] Average of 3 independent experiments. [b] Average of 10 independent experiments. [c] mAb concentration in assay. [d] Limit of detection (IC_{10}). [e] Dynamic range of quantification (IC_{20} – IC_{80}).

b)



c)

0 0.25 0.75 2.5 7.5 25 75 250 750 2500 7500 25000 75000 250000 [CPPU] (ng/L)

d)

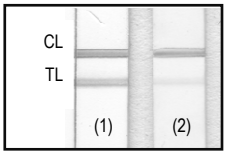


Figure 2

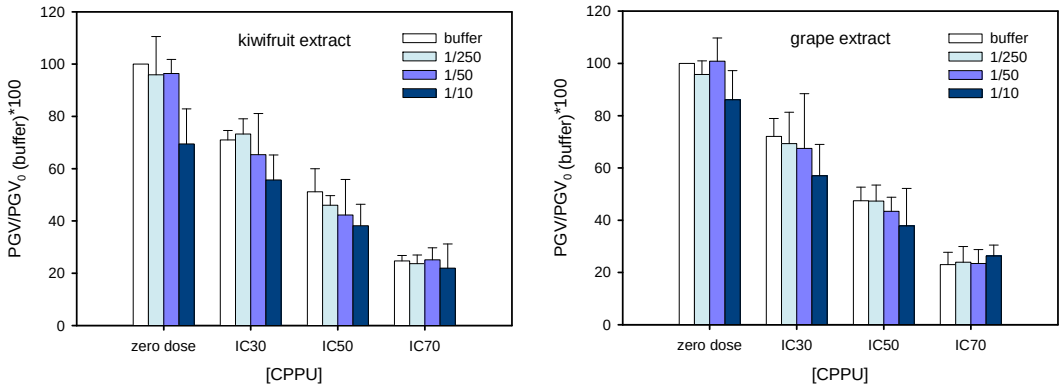

kiwifruit extract
buffer
1/250
1/50
1/10
PGV/PGV0 (buffer)*100
120
100
80
60
40
20
0
zero dose
IC30
IC50
IC70
[CPPU]
grape extract
buffer
1/250
1/50
1/10
PGV/PGV0 (buffer)*100
120
100
80
60
40
20
0
zero dose
IC30
IC50
IC70
[CPPU]

Figure 3

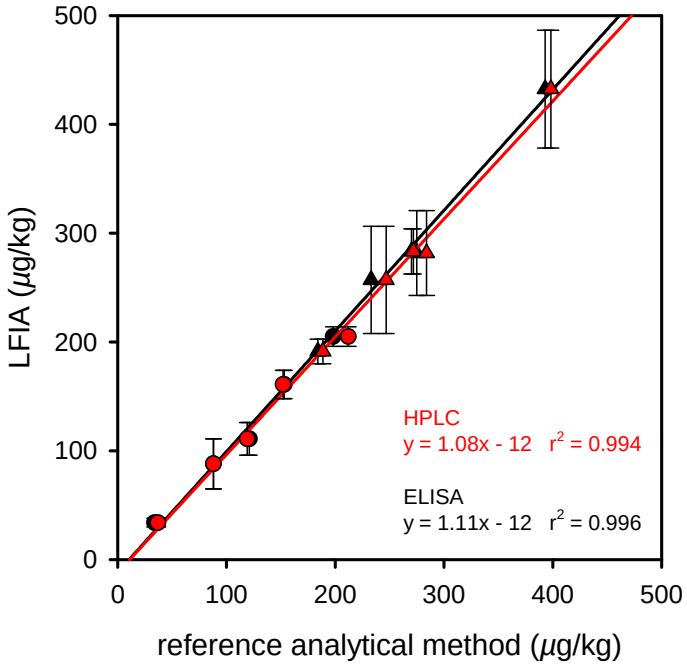

500
400
300
200
100
0
LFIA (µg/kg)
0
100
200
300
400
500
reference analytical method (µg/kg)
HPLC
y = 1.08x - 12 r2 = 0.994
ELISA
y = 1.11x - 12 r2 = 0.996

Figure 4

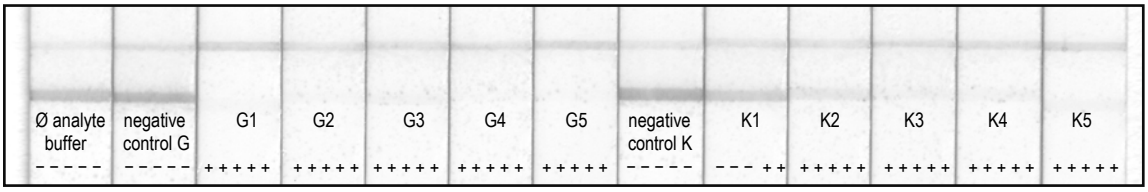

Ø analyte buffer
negative control G
G1
G2
G3
G4
G5
negative control K
K1
K2
K3
K4
K5
– – – – –
– – – – –
+ + + + +
+ + + + +
+ + + + +
+ + + + +
+ + + + +
– – – – –
– – – ++
+ + + + +
+ + + + +
+ + + + +
+ + + + +

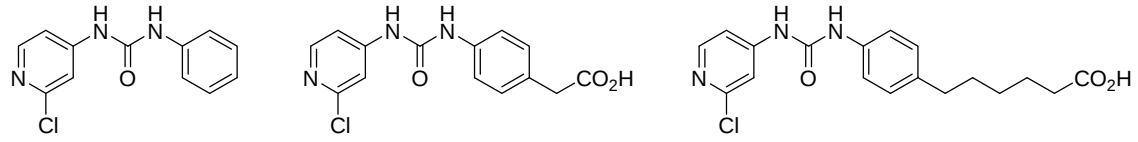
Figure 5

Supplementary data

Development of an immunochromatographic assay based on carbon nanoparticles for the determination of the phytoregulator forchlorfenuron

Celia Suárez-Pantaleón, Jan Wichers, Antonio Abad-Somovilla,

Aart van Amerongen, Antonio Abad-Fuentes



Forchlorfenuron (CPPU) Hapten p2 Hapten p6

Figure 1S. Chemical structure of forchlorfenuron and CPPU derivatives.

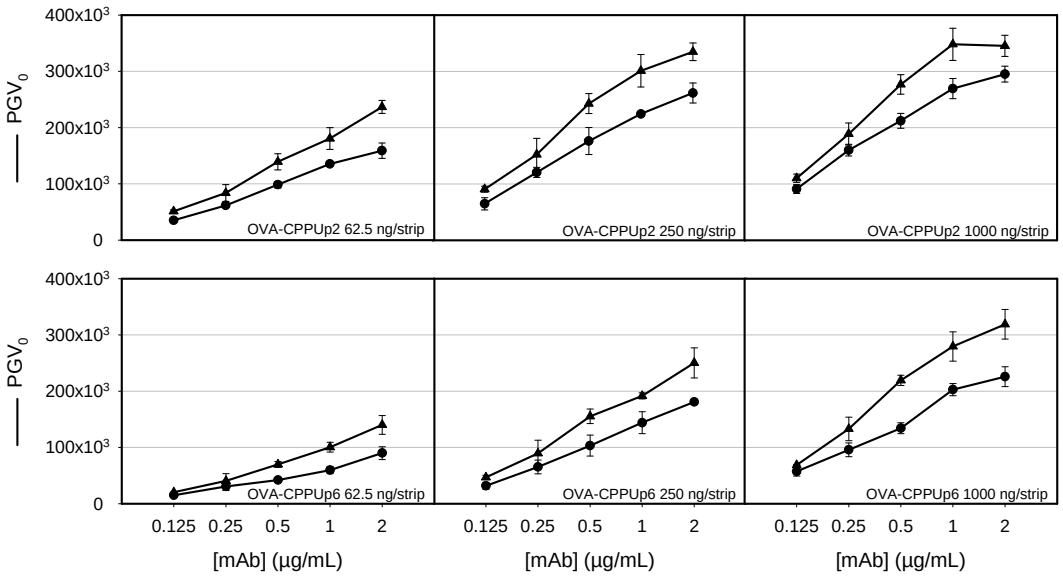


Figure 2S. Influence of immunoreagent concentrations on signal intensity in buffer samples not containing CPPU.

Table 1S. Determination of CPPU in kiwifruit and grape samples with incurred residues by LFIA, ELISA and HPLC-UV

	analytical method[a]					
	LFIA		ELISA		HPLC-UV	
sample	µg/kg	RSD (%)	µg/kg	RSD (%)	µg/kg	RSD (%)
G1[b]	191 ± 11	6	184 ± 7	4	189 ± 4	2
G2	257 ± 49	19	233 ± 10	4	247 ± 9	4
G3	283 ± 21	7	270 ± 2	1	272 ± 39	14
G4	282 ± 39	14	275 ± 6	2	284 ± 17	6
G5	432 ± 54	13	393 ± 7	2	398 ± 7	2
K1[c]	34 ± 4	12	34 ± 1	3	37 ± 9	24
K2	88 ± 23	26	88 ± 5	6	88 ± 2	2
K3	111 ± 15	14	121 ± 4	4	119 ± 16	13
K4	161 ± 13	8	153 ± 7	5	152 ± 5	3
K5	205 ± 9	5	198 ± 4	2	212 ± 5	2

[a] Each value represents the average and the standard deviation of 3 independent determinations. [b] Grape. [c] Kiwifruit.