Development of an immunochromatographic assay based on carbon nanoparticles for the determination of the phytohormone 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,
Bornse Weiland 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.

17 **Abstract**

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

36 *Keywords:*

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

39

40 1. Introduction

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

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

66 CPPU was described (Suárez-Pantaleón et al., 2008, 2010, 2011), and Enzyme-Linked
67 ImmunoSorbent Assays (ELISAs) were developed and successfully applied to the detection of
68 CPPU in fruit samples. Even though ELISA certainly is one of the most widespread and popular
69 kind of immunoassays, immunochromatographic assays are better suited for on-site rapid
70 applications. The large number of scientific publications in the diagnostic (Andreo et al., 2006; Mens
71 et al., 2008), medical (Lin et al., 2008; Omidfar et al., 2012; Yang et al., 2011), veterinary (Noguera
72 et al. 2011), drug of abuse (Gandhi et al., 2009), environmental (Blažková et al., 2009; Kim et al.,
73 2003; Zhou et al. 2010), and food safety areas (Anfossi et al., 2011; Aldus et al., 2003; Tang et al.,
74 2009; Zhang et al., 2011; Zhou et al., 2009), well illustrates the prominent position that
75 immunochromatographic methods have attained in the analytical field as portable point-of-care
76 devices in recent years (Posthuma-Trumpie et al., 2008).

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

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

93 **2. Materials and methods**

94 **2.1. Reagents**

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

114 **2.2. Instrumentation**

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

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

121 **2.3. Buffers**

122 (1) *Coupling buffer*: 5 mM sodium borate buffer, pH 8.8; (2) *Washing solution*: 5 mM sodium
123 borate buffer, pH 8.8, 1% (w/v) BSA, 0.02% (w/v) NaN₃; (3) *Storage buffer*: 100 mM sodium borate
124 buffer, pH 8.8, 1% (w/v) BSA, 0.02% (w/v) NaN₃; (4) *Spraying buffer*: 5 mM sodium borate buffer,
125 pH 8.8; (5) *Running buffer*: 100 mM sodium borate buffer, pH 8.8, 1% (w/v) BSA, 0.05% (v/v)
126 Tween 20, 0.02% (w/v) NaN₃.

127 **2.4. Development of the LFIA**

128 **2.4.1. Preparation of carbon nanoparticles–secondary antibody Detection Conjugate (DC)**

129 For the preparation of the DC, carbon nanoparticles were labeled with GAM immunoglobulins
130 (O’Keeffe et al., 2003). A 1% (w/v) suspension of carbon was prepared in demineralized water by
131 sonication. Then, 100 µL of coupling buffer containing 175 µg of GAM were added dropwise to 500
132 µL of a 5–fold dilution of carbon (0.2%, w/v) in coupling buffer. After overnight incubation at 4 °C
133 under gentle stirring, the solution was washed four times with washing solution by centrifugation
134 (13600×g; 15 minutes). Then, the DC was reconstituted in storage buffer at a final concentration of
135 carbon of 0.2%, and stored at 4 °C. Before running experiments, the working dilution of the GAM–
136 carbon conjugate was sonicated for 10 seconds.

137 **2.4.2. Immobilization of reagents onto nitrocellulose membranes**

138 Capture conjugates OVA–p6 and OVA–p2 diluted in spraying buffer were immobilized onto
139 plastic-backed membranes at 3 cm from the origin forming the test line (TL). The final concentration
140 of CC on the TL was 62.5, 250 or 1000 ng/strip. DAG immunoglobulins (80 ng/strip), also diluted in
141 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 °C. 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 (IC₅₀) 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} (IC₁₀ and IC₂₀, respectively). The dynamic range (DR) for the quantification of CPPU was established between the values of the IC₂₀ and the IC₈₀ (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 (PGV₀). 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} (IC₃₀, IC₅₀, and IC₇₀, 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

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

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

237 Figure 1

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

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

3.2. Optimization of the immunochromatographic test

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

Table 1

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

Figure 2

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

268 Accordingly, this rapid test could be potentially used for CPPU residue monitoring in horticultural
269 samples, provided that matrix effects do not substantially interfere with the assay performance.
270 Furthermore, this LOQ was just 4 times higher than that of the ELISA (22 ng/L) (Suárez-Pantaleón,
271 2008). The visual detection limit, defined as the minimum concentration of CPPU causing a clearly
272 visible decrease in the TL signal intensity in the strip, was set at 250 ng/L. This visual detectability is
273 comparable to other immunochromatographic assays for the analysis of low molecular weight
274 compounds such as methiocarb, thiabenzole, indomethacin and aflatoxins (Blažková et al., 2009 and
275 2010; Li et al., 2009; Liao and Li, 2010; Zhang et al. 2011).

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

279 3.3. Matrix influence on the assay performance

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

290 Figure 3

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

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

297 **3.4. Determination of CPPU in fruits from in-field treated crops**

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

306 Figure 4

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

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

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

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

320 Figure 5

321 **4. Conclusions**

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

333

334 **Acknowledgements**

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

339 **Appendix A. Supplementary data**

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

341

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.

368 Kim, Y. M., Oh, S. W., Jeong, S. Y., Pyo, D. J., Choi, E. Y., 2003. *Environ. Sci. Technol.* 37, 1899–
369 1904.

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

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

372 Koets, M., Sander, I., Bogdanovic, J., Doekes, G., van Amerongen, A., 2006. *J. Environ. Monit.* 8,
373 942–946.

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

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

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

377 Lin, Y. Y., Wang, J., Liu, G., Wu, H., Wai, C. M., Lin, Y., 2008. *Biosens. Bioelectron.* 23, 1659–
378 1665.

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

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

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

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

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

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

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

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

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

421 Wei, Y., Xiao-bing, L., Guo-wen, L., Bing-bing, Z., Yi, Z., Tao, K., Jia-jia, T., Dong-na, L., Zhe,
 422 W., 2011. *Biosens. Bioelectron.* 26, 3710–3713.
 423 Xie, H., Ma, W., Liu, L., Chen, W., Peng, C., Xu, C., Wang, L., 2009. *Anal. Chim. Acta* 634, 129–
 424 133.
 425 Yang, Q., Gong, X., Song, T., Yang, J., Zhu, S., Li, Y., Cui, Y., Li, Y., Zhang, B., Chang, J., 2011.
 426 *Biosens. Bioelectron.* 30, 145–150.
 427 Zhang, D., Li, P., Zhang, Q., Zhang, W., 2011. *Biosens. Bioelectron.* 26, 2877–2882.
 428 Zhou, Y., Pan, F. G., Li, Y. S., Zhang, Y. Y., Zhang, J. H., Lu, S. Y., Ren, H. L., Liu, Z. S., 2009.
 429 *Biosens. Bioelectron.* 24, 2744–2747.
 430 Zhou, Y., Zhang, Y., Pan, F., Li, Y., Lu, S., Ren, H., Shen, Q., Li, Z., Zhang, J., Chen, Q., Liu, Z.,
 431 2010. *Biosens. Bioelectron.* 25, 2534–2538.
 432 Zou, Z., Du, D., Wang, J., Smith, J. N., Timchalk, C., Li, Y., Lin, Y., 2010. *Anal. Chem.* 82, 5125–
 433 5133.
 434

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 (PGV₀).
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.

Figure 1

Figure 2

Figure 3

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 phytohormone forchlorfenuron

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

Aart van Amerongen, Antonio Abad-Fuentes

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

sample	analytical method ^a					
	LFIA		ELISA		HPLC-UV	
	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.