Determination of fenhexamid residues in grape must, kiwifruit, and strawberry samples by enzyme-linked immunosorbent assay

Francesc A. Esteve-Turrillas, Antonio Abad-Fuentes, and Josep V. Mercader*

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Institute of Agrochemistry and Food Technology, Spanish National Research Council, P.O. Box 73, 46100 Burjassot, València, Spain.

* Corresponding author. Tel. +34-963900022 ext. 2316, Fax. +34-963636301, e-mail: jvmmercader@iata.csic.es

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Running title: Determination of fenhexamid residues in fruit samples by ELISA

Abstract

Fenhexamid is a last-generation agrochemical extensively used nowadays to protect high
15 valuable crops and fruits from fungus attacks. In this paper, the development of an indirect competitive immunoassay for the determination of fenhexamid residues in fruit samples is described. After optimization of immunoreagent concentrations, the assay, based on monoclonal antibody FHo4#27, showed high sensitivity and selectivity, with a limit of detection for fenhexamid standards in buffer of 0.04 µg/L. The influence on the analytical parameters of the standard curve of
20 several physico-chemical factors, including pH, ionic strength, presence of solvents, and matrix interferences was studied. For spiked liquid food samples like grape must, just a direct 1:50 dilution in water was sufficient to attain recoveries between 100 and 110%. For kiwifruit and strawberry samples, a double extraction procedure with methanol is proposed in this study, which afforded recovery values that ranged between 103 and 116%. The limit of quantification of the developed
25 assay was 5 µg/kg for grape must, 10 µg/kg for kiwifruits, and 25 µg/kg for strawberries. The

optimized assay was compared with a reference procedure based on liquid chromatography using incurred samples from the market, and an excellent correlation between both methods was found.

Keywords: fenhexamid, fungicide, pesticide residues, monoclonal antibody, immunoassay, ELISA, haptens, validation, rapid methods, food safety.

1. Introduction

Fenhexamid (FH) was introduced in 1999 as a fungicide to control *Botrytis cinerea* and related pathogens in fruit, vegetables, and ornamental plants. This disease, so-called grey mould, considerably affects crops in the field and during fruit storage, even at refrigeration temperatures. FH formulations, containing 50% (w/w) active ingredient, are commercialized by Bayer CropScience AG (Manheim am Rhein, Germany) under different trade names such as Teldor® and Password®, and also by Arysta LifeScience (Cary, NC, USA) as Elevate® and Decree®. This ultimate generation pesticide inhibits the biosynthesis of ergosterol by a particular and new mode of action, which makes it particularly valuable for combined programs aimed to avoid the sprout of resistance (Suty, Pontzen & Stenzel, 1999; Roßlenbroich, 1999). According to the last EU Annual Report on Pesticide Residues (EFSA, 2009) published by the European Food Safety Authority (EFSA) for the year 2007, FH was the 11th most frequently found pesticide in fruits and vegetables, whereas it ranked 6th in 2006. Čuš and coworkers (Čuš, Česnik, Bolta & Gergorčič, 2010) have recently found that 44% of the analyzed wine samples contained FH. Residues of this fungicide are considered as very persistent due to their stability and resistance to hydrolysis. However, its toxicity to mammals is low, with oral and dermal LD₅₀ in rats of 5000 mg/kg, so it does not represent acute hazard under normal uses (FAO/WHO, 2005). The EU maximum residue limits (MRLs) for FH in fruits are for example: 10 mg/kg for kiwifruits and canefruits, and 5 mg/kg for grapes, strawberries, apricots, cherries, and peaches (EC Regulation, 2008).

During the present decade, some papers have been published describing different analytical methodologies for the analysis of FH residues in fruits and vegetables (Table 1). Most of these studies used either gas chromatography (GC) or high performance liquid chromatography (HPLC).

55 In those studies, samples were usually extracted by means of organic solvents, mostly with the aid of a homogenizer, although ultrasonic extraction with methanol has also been employed for FH recovery from strawberry samples (see Table 1 for references). More recent studies used QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology (Anastassiades, Lehotay, Štajnbaher & Schenck, 2003) for the multiresidue analysis of FH and other pesticides in
60 grapes, lemons, onions, tomatoes, pears, and oranges (Lesueur, Knittl, Gartner, Mentler & Fuerhacker, 2008; Kmellár et al., 2008). However, the aforementioned methodologies have a low sample throughput, require expensive equipment, and high amounts of used solvents ought to be processed, which considerably increase the cost of assays in routine analysis.

Recently, a FH derivative was synthesized and high-affinity and selective monoclonal
65 antibodies (mAbs) against FH were produced in our lab (Mercader & Abad-Fuentes, 2009) and they were characterized by enzyme-linked immunosorbent assay (ELISA). Now, in this study, we describe the development of an immunoassay using the conjugate-coated indirect competitive ELISA format for the easy determination of FH residues in liquid foodstuffs and fruits. Because solid food samples require a previous extraction step, different alternatives were evaluated for the
70 quantitative recovery of fungicide residues from kiwifruits and strawberries. Finally, the proposed methodology was validated in terms of sensitivity, selectivity, and accuracy, and it was compared with a reference procedure based on HPLC measurements using incurred fruit samples from local markets.

75 2. Experimental

2.1. Reagents and instruments

Sephadex G-25 HiTrap desalting columns from General Electric Healthcare (Uppsala, Sweden) were used to purify protein conjugates. Ultraviolet–visible spectra and ELISA absorbances were read with a PowerWave HT microplate reader from BioTek Instruments (Winooski, VT, USA). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments. A Hitachi (Tokyo, Japan) L-2130 high performance liquid chromatography 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. Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). An Eppendorf 5804 centrifuge (Hamburg, Germany) was used for solid-particle separation from samples. Polyclonal rabbit antimouse-immunoglobulin peroxidase conjugate (RAM-HRP) was from Dako (Glostrup, Denmark), and ovalbumin (OVA), 1-(4-pyridyl)-3-phenylurea (PPU), and *o*-phenylenediamine (OPD) were from Sigma-Aldrich (Madrid, Spain). Methanol, ethanol, acetonitrile, dimethylformamide (DMF), and anhydrous magnesium sulphate were obtained from Scharlau (Barcelona, Spain). Hydrogen peroxide (30%), sulfuric acid (95%), hydrochloric acid (37%), and sodium chloride were purchased from Prolabo-VWR International Eurolab S.L (Barcelona, Spain). Analytical grade FH (2',3'-dichloro-4'-hydroxy-1-methylcyclohexanecarboxanilide) and other pesticide standards were acquired from Fluka/Riedel-de-Haën (Seelze, Germany). Concentrated working solutions were prepared in DMF and methanol for ELISA and HPLC analysis, respectively, and kept at –20 °C in amber glass vials. Fruit and must samples were directly purchased from local markets and stored at 4 °C. Solid samples were chopped and homogenized using a T-25 ultra-turrax blender from IKA (Staufen, Germany).

2.2. Preparation of protein–hapten conjugates

In this study, a synthetic FH derivative [4-(2',3'-dichloro-4'-(1-methylcyclohexanecarboxamido)phenoxy)butanoic acid] (FHo4, see Fig. 1) was used, which had been obtained by reaction of FH with ethyl 4-bromobutyrate and subsequent hydrolysis of the ester,

as described in a previous article (Mercader et al., 2009). Using this hapten, protein conjugates were prepared by activation of the free carboxylic group of FHo4 and subsequent reaction with the free amine groups of the OVA protein. Two conjugates with different hapten-to-protein molar ratios (MR) were prepared. Briefly, 18 μ mol of hapten was dissolved in 180 μ L of DMF and mixed with 18 μ mol of tributylamine and 18 μ mol of isobutyl chloroformate also in DMF. The concentration of all reagents in the mixture was brought to 90 mM with additional DMF and the activation reaction was let to proceed during 1 h at room temperature. Next, 10 or 50 μ L of activated hapten solution was added drop wise to two vials, each one containing 1 mL of a 15 g/L OVA solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. Therefore, the initial hapten-to-protein ratios in the reaction mixture were 2.5:1 and 12.5:1. The coupling reaction was allowed during 2.5 h at room temperature with moderate stirring. Finally, the conjugate was separated from uncoupled hapten by gel filtration on Sephadex G-25, using 100 mM sodium phosphate buffer, pH 7.4 as eluent. The degree of hapten-to-protein conjugation was measured spectrophotometrically from the absorbance value at 280 nm by assuming that the molar absorption coefficients of the hapten and the protein were the same for the free and the conjugated forms. Spectroscopic characterization indicated that the achieved MRs of the two available conjugates were around 5 and 1. The purified conjugate was diluted to 1 μ g/L with 100 mM sodium phosphate buffer, pH 7.4 and stored at -20°C in amber vials.

2.4. ELISA procedure

Immunoassays were performed in the conjugate-coated indirect competitive ELISA format (ic-ELISA). Usually, ninety-six-well polystyrene ELISA plates were coated with 100 μ L per well of OVA–conjugate solution in 50 mM carbonate–bicarbonate buffer, pH 9.6 by overnight incubation at room temperature. Coated plates were washed four times with washing solution (0.15 M NaCl containing 0.05% Tween 20) and then they received 50 μ L per well of analyte solution plus 50 μ L per well of mAb solution. From a 30 mg/L FH stock solution in DMF, calibration curves in the 60

130 to 0.004 $\mu\text{g/L}$ range were prepared by 5-fold serial dilution in water or PBS (10 mM sodium phosphate buffer with 137 mM NaCl, 2.7 mM KCl, pH 7.4). A blank was included in each curve. The immunological reaction took place for 1 h at room temperature, and plates were washed again as described. Next, 100 μL per well of a 1:2000 dilution of RAM–HRP conjugate in PBS containing 0.05% Tween 20 (PBST) was added, and plates were incubated 1 h at room temperature. 135 After the plates had been washed as described, the retained peroxidase activity was determined by addition of 100 μL per well of freshly prepared developing solution containing 2 g/L OPD and 0.012% (v/v) H_2O_2 in 25 mM citrate and 62 mM phosphate buffer, pH 5.4. The enzymatic reaction was stopped after 10 min at room temperature by addition of 100 μL per well of 2.5 M sulfuric acid. The absorbance was immediately read at 492 nm using 650 nm as reference wavelength. The 140 resulting sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA), and from these curves, assay sensitivity was estimated as the concentration of analyte that reduced 50% (IC_{50}) the maximum absorbance signal (A_{max}) reached at the zero dose of analyte. For immunoreagent evaluation, competitive assays were performed in a bidimensional mode, as previously described (Mercader, 145 Suárez-Pantaleón, Agulló, Abad-Somovilla & Abad-Fuentes, 2008). Briefly, in ELISA plates coated with two concentrations of the OVA-FHo4 conjugate, competitive assays were carried out with FH standard curves in PBS and several mAb concentrations in PBST.

For kiwifruit and strawberry sample analysis, whole fruits were blended and five grams of the homogenate were accurately weighted in a 15 mL centrifuge tube and extracted twice by 1 min 150 vortex mixing of the homogenate using 5.0 mL methanol. The extracts were centrifuged at 3500 rpm for 5 min, the pooled volume of supernatant was measured, and an aliquot was diluted 1:250 with deionized water. Grape must was directly diluted 1:50 with deionized water without solvent extraction. Immunoassays were performed under the following ic-ELISA conditions. Plates were coated with 0.1 mg/L OVA–FHo4 ($\text{MR} = 5$) and 50 μL of sample dilution or diluted sample extract 155 was mixed with 50 μL of FHo4#27 antibody solution (25 $\mu\text{g/L}$) in 200 mM sodium phosphate

buffer, pH 7.4 with 0.05% Tween 20. Thus, an additional one-half dilution of the sample extracts occurred upon assaying. Samples were run in triplicate wells. All other conditions were as previously described.

160 2.5. HPLC reference procedure

Twenty grams of homogenized sample (or 20 mL of grape must), 4 g of sodium chloride, 0.4 mL of 5 N hydrochloric acid solution, and 40 μ L internal standard (100 mg/L PPU in methanol) were mixed in a 50 mL centrifuge tube. Samples were extracted three times with 15 mL acetonitrile by 1 min vortex mixing and centrifuged at 3500 rpm for 5 min. The three acetonitrile extracts were
165 pooled in a round-bottom flask, dried with anhydrous magnesium sulphate, evaporated until dryness in a rotary evaporator, and finally reconstituted in 0.4 mL methanol and filtered into a 2 mL amber glass vial through a 0.22 μ m glass-nylon syringe filter, so that a 50-fold final concentration factor was achieved. Chromatographic separation was accomplished with a methanol:water mobile phase gradient from 50% to 90% methanol in 10 minutes and then 90% methanol during 5 min, at a flow
170 rate of 1 mL/min. An injection loop of 20 μ L was employed and measurement wavelengths were 210 and 255 nm for FH and the internal standard, respectively.

3. Results and discussion

175 3.1. Monoclonal antibody selection

Animal immunization, cell fusion, hybridoma selection and cloning, and protein purification procedures to generate mAbs against FH were published in a previous paper (Mercader et al., 2009). For the present study, seven mAbs (FHo4#21, FHo4#22, FHo4#23, FHo4#24, FHo4#26, FHo4#27, and FHo4#42) were affinity purified and bidimensional competitive assays were
180 performed with 0.1 and 1.0 mg/L of OVA conjugate (MR = 5) solution for plate coating. The competitive step was run with each antibody using serial dilutions of FH and 10, 30, 100, or 300

185 $\mu\text{g/L}$ of mAb. Therefore, one inhibition curve was obtained for every combination of reagents, so a global view of the analyte binding properties of all antibodies could be observed. Table 2 lists the curve parameters of the assay at two mAb concentrations, one of them affording the A_{max} value immediately above 1.0 and the other one giving the first-found A_{max} value below 1.0. The lower asymptotes of the curves were always equivalent to the background. For all of the mAbs, IC_{50} values below 1.0 $\mu\text{g/mL}$ were attained, with values as low as 0.12 $\mu\text{g/L}$ for mAb FHo4#27. Lower IC_{50} values for FH were accomplished when the OVA-FHo4 coating conjugate was used at 0.1 mg/L. The only exception was mAb FHo4#42 because it afforded comparable IC_{50} values at the two
190 assayed coating-conjugate concentrations. Also, regarding the slopes of the inhibition curves, it was seen that they were all close to 1.00 and no generic dependence could be assigned, either to the coating-conjugate concentration or to the antibody concentration. Interestingly, the required concentrations of antibodies FHo4#21, FHo4#22, and FHo4#27 were almost equivalent at the two assayed coating-conjugate concentrations. From this study, it could be concluded that the best assay
195 (lowest IC_{50} value and slope nearest to 1.0) was achieved with mAb FHo4#27 and OVA-FHo4 conjugate at a coating concentration of 0.1 mg/L. These conditions were used for further assay characterization.

3.2. Antibody characterization

200 3.2.1. Selectivity

A cross-reactivity study was performed in order to find other pesticides that could be recognized by antibody FHo4#27. Since there are no commercial fungicides with a molecular structure similar to that of FH, the studied compounds were fungicides which may be encountered together with FH in fruit samples, such as kresoxim-methyl, trifloxystrobin, pyraclostrobin,
205 procymidone, azoxystrobin, picoxystrobin, boscalid, dimoxystrobin, and fluoxastrobin. Calibration curves were prepared with every compound in PBS but no inhibition was observed up to 1 μM with

any of them. Consequently, interferences from other fungicides are not expected in the application of the studied immunoassay to the determination of FH in food samples.

210 3.2.2. Tolerance to solvents

Organic solvents are required to extract pesticide residues from solid foodstuffs. However, the presence of small amounts of solvents in the final extract may have a considerable effect over the results attained by competitive immunoassays. Thus, the influence of most relevant organic solvents over the parameters of the selected immunoassay was studied. Methanol, ethanol, 215 acetonitrile, and DMF were evaluated at concentrations ranging from 0.5 to 10.0%. FH standard curves were prepared in PBS containing different concentrations of solvent and they were mixed in the plate wells with a solution of mAb FHo4#27 at 60 $\mu\text{g/L}$ in PBST. This study showed that the A_{max} value was not affected by the presence of any of the assayed solvents at the assayed concentrations (A_{max} values around 1.4 were obtained in all cases; data not shown). On the contrary, 220 the IC_{50} value rose with increasing solvent concentrations. As depicted in Fig. 2, methanol, acetonitrile, and DMF could be used up to 2% with a little decrease in sensitivity, but in the case of ethanol the assay tolerance was very low and the IC_{50} value increased quickly. Because concentrated stock solutions of the pesticides in DMF were employed to prepare the analyte standard solutions in buffer, a certain concentration ($\leq 0.1\%$) of this solvent was always present in 225 all assays. The obtained results demonstrated that such a small amount of DMF did not affect the sensitivity of our ic-ELISA and methanol and acetonitrile were shown to be convenient extraction solvents for ELISA analysis.

3.2.3. Effect of pH and ionic strength

230 The influence of physicochemical parameters such as pH and ionic strength was evaluated following a monoparametric strategy. FH standard curves were prepared in water and solutions of FHo4#27 antibody were prepared at 60 $\mu\text{g/L}$ in buffers of different ionic strengths or pH. In order

to prepare different ionic strength buffered solutions, a 40 mM sodium phosphate buffer, pH 7.4 containing 2 M NaCl was serially diluted with the corresponding volume of the same buffer but
235 without NaCl. The influence of pH was also checked using several buffers with constant ionic strength but different pH values. For this purpose, a solution of 40 mM citrate, 40 mM disodium hydrogen phosphate, and 40 mM Tris, pH 9.9, was prepared. This buffered solution (pH 9.9) was used to prepared buffers at different pH values using HCl, and different volumes of 2M NaCl were added to keep the ionic strength constant at 175 mM. All these buffers also contained 0.05% Tween
240 20. Fig. 3 shows the observed changes in the $A_{\max}$ and the IC_{50} values when the different studied buffers were used. As it can be seen, ionic strength values lower than that of PBS significantly increased the IC_{50} value, whereas the $A_{\max}$ value rose smoothly. On the contrary, buffers with ionic strength values higher than that of PBS did not modify the IC_{50} value, whereas the $A_{\max}$ was only slightly reduced, meaning that the sensitivity of the assay was maintained even at ionic strength
245 values higher than 500 mM. Also, pH values between 6.5 and 8.5 were quite well tolerated by the developed ic-ELISA. However, a considerable decrease of the absorbance signal was found at pH values lower than 6.5. Accordingly, the use of a strong buffer system enabling neutralization of acidic fruit samples by dilution would be advisable. Thus, for food sample analysis, the assay buffer (PBS) was substituted by sodium phosphate buffer, pH 7.4 at an assay concentration of 100 mM,
250 thus keeping a similar ionic strength.

3.3. ELISA analytical features

For further ic-ELISA development, two OVA-FHo4 conjugates were evaluated with different hapten-to-protein ratios (MR = 5 or 1). As shown above (Table 2), the best performance of
255 the conjugate with a MR of 5 was achieved at 0.1 mg/L coating concentration, whereas for the low MR conjugate, the coating concentration had to be raised to 1.0 mg/L in order to get the equivalent $A_{\max}$ value. The optimum concentration of mAb FHo4#27 was also determined in each case (22 and 25 μ g/L of mAb if the conjugate with MR of 1 and 5 was used, respectively) to reach an $A_{\max}$ value

between 1.0 and 1.5 in the absence of fungicide. Analyte solutions were prepared in water using analytical standards, whereas antibody solutions were prepared in 200 mM sodium phosphate buffer, pH 7.4 containing 0.05% Tween 20. Fig. 4 shows the inhibition curves obtained with each conjugate. No significant differences were observed between the assays performed using an OVA conjugate prepared at a low MR or using an OVA conjugate prepared at a higher MR. The assay using a conjugate with a lower MR afforded an inhibition curve with a slope of 1.03 and an IC_{50} value of 0.31 $\mu\text{g/L}$, whereas if the coating conjugate with a higher MR was used, the curve parameters were 1.09 and 0.27 $\mu\text{g/L}$ for the slope and the IC_{50} , respectively. The lower asymptote of the inhibition curves were 4.8 and 6.8 % of the A_{max} for the assays using coating conjugates with low and high MRs, respectively. Finally, the limit of detection (LOD) was estimated as the concentration of FH that provided a 10% reduction of the A_{max} . The calculated LOD for the developed ic-ELISA, using mAb FHo4#27, was 0.04 $\mu\text{g/L}$ for FH determinations in buffer, independently of the MR of the OVA-FHo4 conjugate that was employed.

3.4. Evaluation of matrix effects

Pesticide determinations in fruit and vegetable samples by immunoassay often require a previous dilution step to avoid matrix interferences. Fruit samples were first measured by the described HPLC procedure in order to check for the absence of FH residues. Whole kiwifruit and strawberry samples were chopped and homogenized, centrifuged at 3500 rpm for 5 min, and the juices were collected for their analysis. Grape must and the obtained kiwifruit and strawberry juices were diluted (1:5, 1:25, 1:50, 1:250, and 1:500) with deionized water and a FH standard curve was prepared with each sample dilution. From this study, it was observed that complete inhibition curves without interferences were reached when a 1:50 or higher dilution of grape must was carried out, whereas for kiwifruit and strawberry juices at least a 1:250 dilution in water was required (results not shown). Given the high affinity of the produced mAb, the applicability of the ic-ELISA was not compromised by the dilution factors that were required to eliminate these matrix effects.

3.5. Recovery studies

Chromatographic analysis of pesticides in food often needs a previous extraction of the residues using an organic solvent for quantitative recovery of the analyte, particularly from solid fruit samples when no juice can easily be pressed out. For this reason, a study was undertaken using
290 different buffers and organic solvents to extract the homogenized samples. Two FH-containing kiwifruit samples (0.3 and 0.7 mg/kg, previously measured by HPLC) and two FH-spiked strawberry samples (0.5 and 1.0 mg/kg) were employed for this purpose. Homogenated samples were accurately weighted and extracted in triplicate experiments using 5.0 mL of deionized water, 200 mM sodium phosphate buffer (pH 7.4), 50 mM carbonate buffer (pH 9.6), acetonitrile, or
295 methanol. Samples were centrifuged and a fraction of the supernatant was diluted 1:500 with deionized water to completely avoid the observed matrix interferences. Fig. 5 shows the FH recoveries obtained for the studied buffers and solvents. As it was expected, organic solvents provided higher extraction yields than aqueous buffers. Among the assayed solvents, acetonitrile and methanol afforded the best extraction yields. When just one extraction with methanol was done,
300 the recoveries ranged from 53 to 81%. However, a second extraction with methanol afforded quantitative recoveries (84-104%; Fig. 5). Hence, methanol was selected for further studies because of its lower influence on the FH inhibition curve. To verify matrix effects of fruit extracts obtained by the optimized extraction procedure, FH-free fruit samples were extracted twice with methanol, diluted with deionized water (1:5, 1:50, and 1:250), and FH standard curves were run with the
305 diluted fruit extracts. Fig. 6 shows that no matrix effects were observed if a 1:250 dilution of the extracts in water was done, as it was also found in the preliminary study of the matrix effects using directly diluted fruit juices.

To determine the limit of quantification (LOQ), the direct dilution procedure was applied to the analysis of grape must samples, whereas kiwifruits and strawberries were processed by double
310 extraction with methanol. Grape must and homogenized kiwifruit and strawberry samples were

spiked with FH at 5, 10, 25, 100, 500, and 2000 µg/kg and measured by the developed ic-ELISA. Unspiked samples were also included in every experiment as negative controls. The calculated recoveries of these assays are listed in Table 3. As it can be seen, quantitative results were achieved, with recovery values ranging from 100 to 110% for grape must, from 103 to 115% for kiwifruit, and from 108 to 111% for strawberry samples. From these results, the LOQ – considered as the lowest assayed FH concentrations that afforded recovery values between 80 and 120% – for grape must, kiwifruits, and strawberries was set at 5, 10, and 25 µg/kg, respectively, around 1000 times lower than the European MRLs.

3.6. HPLC analytical features

A calibration curve was established from the peak area values of external standards with concentrations ranging from 0.5 to 100.0 mg/L in methanol. The detector response was linear over the considered concentration range (the correlation coefficient was 0.999), and the LOQ, set at the concentration of FH that provided a signal-to-noise ratio of 10, was 20 µg/kg for grape must and kiwifruits, and 50 µg/kg for strawberry samples. Recovery studies of the HPLC procedure were performed with grape must, kiwifruit, and strawberry fungicide-free samples spiked with FH at 50, 100, and 1000 µg/kg. In this case, the recoveries ranged from 90 to 106 % (results not shown).

3.7. Validation of the developed ic-ELISA

FH residues in fruit samples acquired in a public market were measured by our ic-ELISA and the reference HPLC procedure. For comparison, the sample set consisted of 10 kiwifruit and 7 strawberry samples in which residues of FH had been previously detected. Whole fruit homogenates were extracted twice with methanol and diluted 1:250 with deionized water for ic-ELISA determinations, whereas for HPLC measurements samples were extracted as described in the Materials and Methods section. Table 4 lists the contents of FH found with each method, ranging from 0.1 to 5.0 mg/kg. Although some of the observed concentrations were actually high, no

samples exceeding the MRLs established by the European Commission were found (EC Regulation, 2008). The contents of FH measured by ic-ELISA highly corresponded to those found by HPLC. In fact, the linear regression analysis between ELISA and HPLC data, shown in Fig. 7, followed the equation: $C_{ELISA} = (0.00 \pm 0.11) + (0.99 \pm 0.05) * C_{HPLC}$ ($r^2 = 0.990$, $n = 17$), where C_{ELISA} is the FH concentration (in mg/kg) attained by our ic-ELISA and C_{HPLC} is the corresponding concentration obtained by HPLC. Concerning each of the two fruit commodities, excellent correlation between both methods was also found: i) $C_{ELISA} = (0.05 \pm 0.16) + (0.98 \pm 0.06) * C_{HPLC}$, ($r^2 = 0.991$, $n = 10$) for kiwifruits and ii) $C_{ELISA} = (-0.08 \pm 0.17) + (1.01 \pm 0.09) * C_{HPLC}$, ($r^2 = 0.990$, $n = 7$) for strawberries. The equivalence of the two applied methodologies demonstrated the suitability of the developed immunoassay for the analysis of FH residues in fruits.

To further prove the sensitivity and applicability of the immunochemical method, the degree of penetration of FH into the edible portion of kiwifruits was studied. To this purpose, four individual pieces of fruit with incurred residues were sliced into two halves and separately extracted and analyzed by ELISA, one of them including the skin, as officially indicated, and the other half after being peeled. Determinations by immunoassay showed that the FH concentration in the edible part of kiwifruit represents $4.7 \pm 1.7\%$ of the amount found in the whole fruit.

4. Conclusions

In a previous article, a simple strategy to synthesize a FH derivative was described. This hapten was successfully used to generate a collection of high-affinity mAbs (Mercader et al., 2009). In the present study, one of these antibodies (FHo4#27) was used to develop an ELISA in the conjugate-coated indirect competitive format, which was applied to the analysis of FH residues in liquid and solid fruit samples. This assay was highly selective and it was shown to tolerate neutral and moderately alkaline pH values, as well as elevated salt concentrations. Matrix effects caused by these two physicochemical parameters could easily be avoided by sample dilution in the appropriate

assay buffer. Although the sensitivity of the assay notably decreased by the presence of ethanol, other solvents such as methanol, acetonitrile, and DMF were well tolerated up to 2%. For liquid samples like grape must, the LOQ of the assay for FH after a simple dilution in water was 5 µg/kg. For solid samples such as kiwifruits and strawberries, the LOQ for FH was 10 and 25 µg/kg, respectively, when a double extraction procedure with methanol was done. Also, the accuracy of the optimized ic-ELISA was demonstrated by comparison with a reference procedure based on HPLC using incurred fruit samples. A summary of the advantages and drawbacks of both methodologies is listed in Table 5, highlighting the developed ic-ELISA as a simple, cheap, reliable, and rapid method for the analysis of FH in food samples.

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Limited amounts of the described immunoreagents are available upon request.

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Figure captions

450 **Fig. 1.** Molecular structures of FH (upper) and hapten FHo4 (lower).

Fig. 2. Influence of organic solvents over the ic-ELISA inhibition curve.

Fig. 3. Effect of the buffer ionic strength and pH on the ic-ELISA inhibition curve.

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Fig. 4. ic-ELISA inhibition curves for FH using OVA–conjugates with different hapten-to-protein MR.

460 **Fig. 5.** Recovery values of FH in kiwifruit and strawberry samples extracted with several buffers or organic solvents and measured by ic-ELISA.

Fig. 6. Interferences produced by kiwifruit and strawberry methanolic extracts diluted in water over the developed ic-ELISA.

465 **Fig. 7.** Comparative study between ic-ELISA and HPLC results obtained for FH determination in kiwifruit (●) and strawberry (▲) samples.

Figure 1.

Esteve-Turrillas, Abad-Fuentes & Mercader

Figure 2.

Esteve-Turrillas, Abad-Fuentes & Mercader

Figure 3.

Esteve-Turrillas, Abad-Fuentes & Mercader

Figure 4

Esteve-Turrillas, Abad-Fuentes & Mercader

Figure 5.

Esteve-Turrillas, Abad-Fuentes & Mercader

Figure 6.

Esteve-Turrillas, Abad-Fuentes & Mercader

Figure 7.

Esteve-Turrillas, Abad-Fuentes & Mercader

Table 1

Published procedures for the analysis of FH in fruits and vegetables.

Sample	Sample treatment	Determination	Recovery (%)	LOQ (µg/kg)	Ref.
Vegetables	SPE	HPLC-UV	69–110	20	Nüßlein, 1999
Grapes, must, wine	LLE	GC-NPD	82–111	100	Cabras et al., 2001
Strawberry	USE	HPLC-MS-MS	94–99	10	Christensen et al., 2003
Grapes	LLE + SPE	HPLC-DAD	41	50	Rial Otero et al., 2003
Caneberry, blueberry, pomegranate	UTH + SPE + LLE	HPLC-MS-MS	72–105	20	Hengel et al., 2003
Apple, lemon, tomato	UTH + LLE	HPLC-MS-MS	82–92	10	Sannino et al., 2004
Strawberry	UTH	HPLC-MS	86–95	1	Rabolle et al., 2006
Tomato, grapes, wine	LLE	GC-ECD	81–102	20	Likas et al., 2007
Panax ginseng	UTH + SPE	GC-ECD	99–112	3	Park et al., 2007
Grapes, lemon, onion, tomato	QuEChERS	GC-MS	83–119	1	Lesueur et al., 2008
Tomato, pear, orange	QuEChERS	HPLC-MS-MS	80–110	5	Kmellár et al., 2008
Grape juice, wine	Dilution	ELISA	95–118	30	Mercader et al., 2009

Abbreviations: ECD, electron capture detector; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high-performance liquid chromatography; LLE, liquid-liquid extraction; MS, mass spectrometry; MS-MS, tandem mass spectrometry; NPD, nitrogen-phosphorus detector; QuEChERS, quick, easy, cheap, effective, rugged, and safe method; SPE, solid-phase extraction; USE, ultrasonic extraction; UTH, Ultra-turrax homogenization; UV, ultra violet detector.

Table 2

Assay parameters obtained from the bidimensional competitive analysis.

mAb	OVA-FHo4 at 0.1 mg/L				OVA-FHo4 at 1.0 mg/L			
	mAb conc. ($\mu\text{g/L}$)	A_{max}	Slope	IC_{50} ($\mu\text{g/L}$)	mAb conc. ($\mu\text{g/L}$)	A_{max}	Slope	IC_{50} ($\mu\text{g/L}$)
FHo4#21	10	0.57	1.08	0.50	10	0.76	1.28	3.04
	30	1.18	1.09	0.60	30	1.68	1.12	3.67
FHo4#22	10	0.70	0.92	0.26	10	0.88	1.10	1.75
	30	1.65	0.89	0.37	30	2.08	0.94	2.71
FHo4#23	30	0.68	1.00	0.50	10	0.73	0.74	1.70
	100	1.45	0.94	0.64	30	1.84	0.78	1.90
FHo4#24	30	0.68	1.00	0.53	10	0.36	1.14	3.92
	100	1.42	0.91	0.63	30	1.04	1.08	4.76
FHo4#26	30	0.63	0.99	0.25	10	0.48	1.10	0.88
	100	1.36	0.95	0.39	30	1.26	0.83	1.12
FHo4#27	10	0.60	0.88	0.12	10	0.48	1.33	1.35
	30	1.40	0.96	0.19	30	1.30	1.14	1.28
FHo4#42	100	0.89	1.02	0.50	10	0.44	0.90	0.88
	300	1.84	1.25	1.04	30	1.20	0.86	0.97

Table 3

Recoveries by ic-ELISA using liquid and solid fruit samples spiked with FH at different levels.

Spiked ($\mu\text{g/kg}$)	Recovery (% $\pm$ s, n=4)		
	Must (1:50)	Kiwifruit (1:250)	Strawberry (1:250)
5	107 $\pm$ 12	n.a. ^a	n.a.
10	104 $\pm$ 2	113 $\pm$ 14	- ^b
25	100 $\pm$ 5	104 $\pm$ 6	108 $\pm$ 13
100	104 $\pm$ 8	105 $\pm$ 6	109 $\pm$ 11
500	110 $\pm$ 20	115 $\pm$ 1	116 $\pm$ 3
2000	n.a.	103 $\pm$ 3	111 $\pm$ 16

^a Values not assayed. ^b Below the quantitation range.

Table 4

Fenhexamid contents found in kiwifruit and strawberry samples obtained from local markets.

Sample	Fenhexamid concentration (mg/kg $\pm$ s)		Bias (%)
	HPLC (n=2)	ELISA (n=3)	
K ^a 1	2.58 $\pm$ 0.25	2.81 $\pm$ 0.18	9.07
K 2	2.73 $\pm$ 0.14	2.74 $\pm$ 0.16	0.54
K 3	3.54 $\pm$ 0.35	3.30 $\pm$ 0.28	-6.94
K 4	0.39 $\pm$ 0.03	0.49 $\pm$ 0.04	23.96
K 5	3.34 $\pm$ 0.06	3.03 $\pm$ 0.02	-9.31
K 6	0.14 $\pm$ 0.00	0.18 $\pm$ 0.05	23.74
K 7	2.91 $\pm$ 0.18	2.83 $\pm$ 0.21	-2.91
K 8	5.00 $\pm$ 0.16	4.93 $\pm$ 0.49	2.91
K 9	0.34 $\pm$ 0.02	0.41 $\pm$ 0.05	19.79
K10	0.34 $\pm$ 0.03	0.35 $\pm$ 0.03	2.65
S ^b 1	3.00 $\pm$ 0.12	2.97 $\pm$ 0.09	-1.20
S 2	2.67 $\pm$ 0.13	2.75 $\pm$ 0.03	3.08
S 3	0.64 $\pm$ 0.00	0.66 $\pm$ 0.05	4.17
S 4	0.48 $\pm$ 0.01	0.48 $\pm$ 0.03	0.37
S 5	1.63 $\pm$ 0.04	1.45 $\pm$ 0.12	-11.26
S 6	2.20 $\pm$ 0.09	2.12 $\pm$ 0.16	-3.69
S 7	1.32 $\pm$ 0.05	1.09 $\pm$ 0.09	-17.31

^a Kiwifruit. ^b Strawberry.

Table 5

Comparison of the analytical features of the developed ic-ELISA and the reference HPLC procedure.

Parameter	ic-ELISA	HPLC
Time (h):		
Pre-treatment	0.25	0.50
Analysis	2.25	0.25
Sample throughput (per hour)	38 per plate	1.3
Solvent consumption (mL per sample)	10 ^a	45
Dilution/concentration	1:50 ^b , 1:250	50:1
LOQ (µg/kg):		
Must	5	20
Kiwifruit	10	20
Strawberry	25	50

^a No solvent was required for the extraction of FH from grape must by the proposed ELISA. ^b A 50 times dilution was used for the determination of fenhexamid in grape must.