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High-affinity antibodies from a full penthiopyrad-mimicking hapten and heterologous immunoassay development for fruit juice analysis --Manuscript Draft--

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Abstract:	<p>New-generation succinate dehydrogenase inhibitors with increased antifungal performance have been developed during the last years. Particularly, penthiopyrad has been recently introduced in the agrochemical market as a complementary fungicide for combined treatments with other active principles displaying a different mode of action. In the present study, rapid and high-throughput analytical methods for penthiopyrad residue determination in foodstuffs were developed. A novel functionalized hapten that completely mimics the molecule of penthiopyrad was designed for high-affinity and specific antibody generation. Moreover, heterologous haptens were synthesized and the influence of their respective molecular modifications over antibody binding was evaluated by competitive enzyme-linked immunosorbent assay. Two immunoassays were optimized using alternative assay formats, affording limits of detection for penthiopyrad in buffer around 70 pg/mL in both cases. Performance, in terms of accuracy and precision, of the developed immunochemical methods was studied using apple juice and red and white grape juices. The obtained results showed that</p>	

	penthiopyrad could be determined in those food samples in compliance with European maximum residue limits.
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RESPONSE TO REVIEWER COMMENTS

We thank the reviewer for his/her kind comments about our manuscript.

As suggested, the following new references have been added to support our comments.

- Ahn et al. 2011
- Li et al. 2017
- Van Emon et al. 2008
- Suri et al. 2009
- Xu et al. 2009

All concentration units given in pg/mL have been changed to ng/mL as properly indicated by the referee (pages 2 and 19).

We agree with the referee that information about the enantioselectivity of the immunoassay would be interesting. Unfortunately, we do not have data on this regard. Commercial formulations of penthiopyrad include a mixture of the enantiomers so we considered that, for practical applications, it was not necessary to determine the assay enantioselectivity. Nevertheless, from the scientific point of view, it is undoubtedly a stimulating question so we will take this idea into account for further studies on this compound.

High-affinity antibodies from a full penthiopyrad-mimicking hapten and heterologous immunoassay development for fruit juice analysis

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Abstract

New-generation succinate dehydrogenase inhibitors with increased antifungal performance have been developed during the last years. Particularly, penthiopyrad has been recently introduced in the agrochemical market as a complementary fungicide for combined treatments with other active principles displaying a different mode of action. In the present study, rapid and high-throughput analytical methods for penthiopyrad residue determination in foodstuffs were developed. A novel functionalized hapten that completely mimics the molecule of penthiopyrad was designed for high-affinity and specific antibody generation. Moreover, heterologous haptens were synthesized and the influence of their respective molecular modifications over antibody binding was evaluated by competitive enzyme-linked immunosorbent assay. Two immunoassays were optimized using alternative assay formats, affording limits of detection for penthiopyrad in buffer around 0.07 ng/mL in both cases. Performance, in terms of accuracy and precision, of the developed immunochemical methods was studied using apple juice and red and white grape juices. The obtained results showed that penthiopyrad could be determined in those food samples in compliance with European maximum residue limits.

Keywords

Target mimicking; Antibodies; Fungicide residues; ELISA; Immunoassay.

Introduction

Succinate dehydrogenase inhibitors (SDHI) are a four-decade old class of fungicides but it was not until the last decade when the new-generation compounds of this family have gained prominence in the agrochemical sector for their higher efficiency and potency against a wider number of plant pathogenic fungi (Avenot and Michailides 2010; www.agropages.com). These active principles inhibit fungal respiration by a unique mode of specifically blocking the ubiquinone-binding site (Q-site) of the mitochondrial complex II, which makes them ideal candidates for combined disease control programs (Gudmestad et al. 2013). Penthiopyrad (Table 1) was developed during the last decade, being one of the latest SDHI fungicides to be commercialized; in 2012 in the US and in 2014 in the EU (Yoshikawa et al. 2011; Proffer et al. 2013; DG SANCO 2013). This pyrazole carboxamide fungicide is a racemic mixture of *R* and *S* enantiomers. It shows great potential for the management of diseases caused by ascomycetes, basidiomycetes, and deuteromycetes, such as *Botrytis cinerea*, *Fusarium oxysporum*, and *Rhizoctonia solani* (Culbreath et al. 2009; Sakurai et al. 2011). It is to be used as a foliar spray in berries, pome fruit, stone fruits, tree nuts, bulb vegetables, brassica vegetables, cucurbit vegetables, fruiting vegetables, leafy vegetables, legumes, peanuts, and root and tuber vegetables (APVMA 2012). Although penthiopyrad displays low toxicity to humans – the median LC₅₀ in rats was shown to be higher than 2 g/kg of body weight –, it may cause reproductive and developmental negative effects. Moreover, moderate-to-high toxicity has been observed to aquatic invertebrates and algae (Pesticides Properties Database). Accordingly, FAO has established an acceptable daily intake of 0–0.1 mg/kg of body weight and an acute reference dose of 1 mg/kg of body weight (FAO 2011).

The reference analytical method for the analysis of penthiopyrad residues involves acetonitrile/water extraction and liquid chromatography in combination with tandem mass spectrometry (HPLC–MS/MS) (EFSA 2013; Gulkowska et al. 2014). Recently, a chromatographic method for the analysis of penthiopyrad in fruits and vegetables, showing a limit of

quantification of 0.01 mg/kg, has been published by our research group (Abad-Fuentes et al. 2015). Nowadays, those instrumental methodologies are the preferred approach for large monitoring programs and multiresidue analysis. For other analytical applications, immunochemical methods constitute potent complementary tools for rapid, sensitive, and high throughput analysis (Dankwardt 2006; Ahn et al. 2011; Li et al. 2017). Antibody-based procedures can be developed using alternative methods, such as the enzyme-linked immunosorbent assay (ELISA), affinity columns, and lateral flow immunoassays. In any case, high-affinity and specific antibodies and bioconjugates are required (Van Emon et al. 2008). Haptens that are employed to prepare immunizing conjugates should mimic as much as possible the target compound. Moreover, a linker arm needs to be introduced in order to separate the hapten core structure from the carrier protein. Linear aliphatic hydrocarbon chains of 4 to 6 carbon length are preferably used. The position of this spacer should be carefully chosen in order to better display the main and characteristic immunodeterminant moieties and to keep unchanged as much as possible the electronic distribution and spatial conformation of the molecule. As a general rule, the linker should be preferably introduced as an elongation of aliphatic substituents, such as n-alkyl groups (Suri et al. 2009; Xu et al. 2009).

In a previous study, the generation of antibodies to penthiopyrad using immunizing conjugates of two haptens with opposite linker tethering sites was demonstrated (Ceballos-Alcantarilla et al. 2014). In those two haptens, named PP α and PP β , equivalent linear linkers were introduced in substitution of any of the two aliphatic groups of the molecule (Table 1), and both immunogens afforded antibodies with similar affinities to penthiopyrad. In the present study, a much more mimetic hapten of penthiopyrad was designed in order to raise antibodies with improved affinity to the target compound. Moreover, alternative heterologous haptens (different to the hapten that was employed for immunization) were prepared and evaluated for sensitivity enhancement. The aim was to develop highly sensitive immunoassays

in different formats using the competitive ELISA method for the analysis of penthiopyrad residues in foodstuffs.

Materials and Methods

Reagents and instrumentation

Pestanal grade penthiopyrad [(RS)-*N*-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide, CAS registry number 131341-86-1, Mw 359.41] was purchased from DuPont (Nambesheim, France). Fungicide concentrated stock solutions were prepared with anhydrous *N,N'*-dimethylformamide (DMF) in amber glass vials and stored at -20 °C. All solvents were purified by distillation and, if required, they were dried according to standard methods (Armarego and Perrin 1997). Reagents used for hapten synthesis were acquired from commercial sources and used without purification.

Laboratory animals were provided by Granja San Bernardo (Navarra, Spain). Goat anti-rabbit immunoglobulin polyclonal antibody conjugated to horseradish peroxidase (GAR-HRP) was obtained from BioRad (Madrid, Spain). Bovine serum albumin (BSA) fraction V was from Roche Applied Science (Mannheim, Germany). Adult bovine serum (ABS), ovalbumin (OVA), horseradish peroxidase (HRP), Freund's adjuvants, Tween 20, and *o*-phenylenediamine were purchased from Sigma/Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were utilized for protein-hapten conjugate purification. Costar flat-bottom high-binding 96-well polystyrene microtiter plates were from Corning (Corning, NY, USA). Hapten ultraviolet spectra and immunoassay absorbances were recorded with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments.

Synthesis of functionalized haptens

Chemical structures of all of the employed haptens are shown in Table 1. Preparation of haptens PPa and PPb has been previously published (Ceballos-Alcantarilla et al. 2014). Preparation of haptens PPd, PPO, and PPM is outlined in Fig. 1. The synthesis of thiophene **3** was accomplished by reaction of freshly prepared 1-methyl-3-(fluoromethyl)-1H-pyrazole-4-carbonyl chloride (**1**) and methyl 3-aminothiophene-2-carboxylate (**2**), following previously published procedures (Ceballos-Alcantarilla et al. 2014).

*Preparation of 5-methyl-5-(4-(1-methyl-3-(trifluoromethyl)-1H-pyrazole-4-carboxamido)thiophen-2-yl) hexanoic acid (Hapten PPd, **4**)*

A mixture of thiophene **3** (78.7 mg, 0.29 mmol), 6,6-dimethyltetrahydro-2H-pyran-2-one (75 mg, 0.59 mmol) and AlCl₃ (267 mg, 2 mmol) in anhydrous dichloroethane (2 mL) was stirred under N₂ at room temperature for 4 h (reaction monitored by TLC, CHCl₃/MeOH 19:1). After this time, the mixture was diluted in ice-water, acidified with concentrated HCl to pH 1, and extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under vacuum. The residue was purified by flash column chromatography (silica gel, CHCl₃/MeOH 49:1) to give hapten PPd (**4**, 84.2 mg, 73%) as a pale brown solid. Mp 106.5–108.5 °C (from CHCl₃/MeOH); IR (neat) $\nu_{\max}$ (cm⁻¹) 3289w, 2961w, 1706s, 1651s, 1582m, 1542s, 1302m, 1132s, 1053s, 830m; ¹H-NMR (300 MHz, CDCl₃) δ 10.27 (br s, 1H, CO₂H), 8.00 (br s, 2H, H₅ Pz and CONH), 7.41 (d, *J* = 1.5 Hz, 1H, H₃ Th), 6.73 (d, *J* = 1.5 Hz, 1H, H₅ Th), 3.96 (s, 3H, NCH₃), 2.29 (t, *J* = 7.1 Hz, 2H, H₂), 1.72–1.58 (m, 2H, H₄), 1.58–1.41 (m, 2H, H₃), 1.34 (s, 6H, H-6 and CH₃-5); ¹³C-NMR (75 MHz, CDCl₃) δ 179.2 (s, C₁), 158.0 (s, CONH), 154.7 (s, C₄ Th), 138.5 (q, *J* = 37.3 Hz, C₃ Pz), 135.6 (s, C₅ Pz), 133.9 (s, C₂ Th), 121.1 (q, *J* = 273.1 Hz, CF₃), 117.1 (s, C₄ Pz), 117.0 (s, C₅ Th), 108.7 (s, C₃ Th), 44.6 (s, C₄), 40.0 (s, NCH₃), 37.6 (s, C₅), 34.3 (s, C₂), 29.8 (s, C₆ and CH₃-5), 20.2 (s, C₃); ¹⁹F-NMR (282 MHz, CDCl₃) δ -59.98 (s, CF₃); HRMS (TOF, ESI+) *m/z* calculated for C₁₇H₂₁F₃N₃O₃S [M+H]⁺ 404.1250, found 404.1248;

UV (PB) ϵ (290 nm) = 2.45 mM⁻¹ cm⁻¹, ϵ (280 nm) = 3.47 mM⁻¹ cm⁻¹, ϵ (270 nm) = 4.15 mM⁻¹ cm⁻¹, ϵ (260 nm) = 4.19 mM⁻¹ cm⁻¹, ϵ (250 nm) = 3.82 mM⁻¹ cm⁻¹.

Preparation of 3-methyl-5-(3-(1-methyl-3-(trifluoromethyl)-1H-pyrazole-4-carboxamido)thiophen-2-yl)-5-oxopentanoic acid (Hapten PPO, 5)

A mixture of thiophene **3** (150 mg, 0.54 mmol), 3-methylglutaric anhydride (104.3 mg, 0.81 mmol), and AlCl₃ (361.9 mg, 2.72 mmol) in anhydrous dichloroethane (2 mL) was stirred under N₂ at room temperature for 5 h (reaction monitored by TLC, CHCl₃/MeOH 19:1). After this time, the mixture was diluted in ice-water, acidified with concentrated HCl to pH 2, and extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under vacuum. The residue was purified by flash column chromatography (silica gel, CHCl₃/MeOH 99:1) to give hapten PPO (**5**, 163.1 mg, 74%) as a pale yellow solid. Mp 179.4–180.8 °C (from EtOAc); IR (KBr) ν_{max} (cm⁻¹) 3275m, 3081m, 2962m, 2926m, 1709s, 1686s, 1625s, 1567s, 1430s, 1293s, 1165s, 1067m; ¹H-NMR (300 MHz, CD₃OD) δ 8.35 (q, J = 0.5 Hz, 1H, H_5 Pz), 8.13 (d, J = 5.4 Hz, 1H, H_5 Th), 7.78 (d, J = 5.4 Hz, 1H, H_4 Th), 4.03 (s, 3H, NCH₃), 2.94 (dd, J = 15.7, 6.2 Hz, 1H, H_4), 2.78 (dd, J = 15.7, 7.3 Hz, 1H, H'_4), 2.72–2.55 (m, 1H, H_3), 2.44 (dd, J = 15.4, 6.1 Hz, 1H, H_2), 2.27 (dd, J = 15.4, 7.6 Hz, 1H, H'_2), 1.06 (d, J = 6.6 Hz, 3H, CH₃-3); ¹³C-NMR (75 MHz, CD₃OD) δ 196.7 (s, C₅), 176.2 (s, C₁), 159.5 (s, CONH), 145.5 (s, C₃ Th), 142.1 (q, J = 38.0 Hz, C₃ Pz), 135.2 (s, C₅ Pz), 134.1 (s, C₅ Th), 123.4 (s, C₄ Th), 122.1 (q, J = 268.2 Hz, CF₃), 121.7 (s, C₂ Th), 117.2 (s, C₄ Pz), 48.5 (s, C₄), 41.7 (s, C₂), 40.1 (s, NCH₃), 28.5 (s, C₃), 20.3 (s, CH₃-3); ¹⁹F-NMR (282 MHz, CD₃OD) δ -63.07 (s, CF₃); HRMS (TOF, ESI+) m/z calculated for C₁₆H₁₇F₃N₃O₄S [M+H]⁺ 404.0886, found 404.0893; UV (PB) ϵ (290 nm) = 5.67 mM⁻¹ cm⁻¹, ϵ (280 nm) = 4.27 mM⁻¹ cm⁻¹, ϵ (270 nm) = 3.02 mM⁻¹ cm⁻¹, ϵ (260 nm) = 4.27 mM⁻¹ cm⁻¹, ϵ (250 nm) = 5.87 mM⁻¹ cm⁻¹.

Preparation of N-(2-(2,4-Dimethyl-6-oxotetrahydro-2H-pyran-2-yl)thiophen-3-yl)-1-methyl-3-(trifluoromethyl)-1H-pyrazole-4-carboxamide (6).

A 3 M solution of MeLi in diethoxymethane (0.68 mL, 2.02 mmol) was added dropwise under N₂ atmosphere to a solution of keto acid **5** (163.1 mg, 0.40 mmol) in anhydrous THF (16.3 mL) at –60 °C. The resulting mixture was stirred at room temperature for 5.5 h (reaction monitored by TLC, CHCl₃/MeOH 19:1). After this time, the mixture was diluted in ice-water, acidified with 1 M aqueous HCl to pH 2, and extracted with EtOAc. The combined organic extracts were washed with brine, dried over anhydrous MgSO₄, filtered, and concentrated under vacuum. The residue, containing a mixture of diastereomers of δ-lactone **6** and the corresponding opened δ-hydroxy acid, as shown by ¹H-NMR, was dissolved in anhydrous CH₂Cl₂ (1.5 mL) in a microwave tube, then PPTS was added (2.0 mg) and the mixture was stirred and heated in microwave oven at 50 °C (300 W) for 10 min. Next, the solvent was evaporated under vacuum and the crude product was purified by flash column chromatography (silica gel, CHCl₃/MeOH 99:1). Lactone **6** (66.5 mg, 41%) was thus obtained as a white solid, whose ¹H-NMR spectrum revealed to be a 3:1 mixture of 2,4-*syn/anti* diastereomers. Mp 136.4–137.9 °C (CHCl₃); IR (KBr) ν_{max} (cm^{–1}) 3401w, 2962w, 1737s, 1683s, 1574m, 1399m, 1296m, 1245m, 1173s, 1136s, 1061s, 883m; ¹H-NMR (300 MHz, CDCl₃), only signals of the major diastereomer are given, δ 9.18 (br s, 1H, CONH), 8.36 (s, 1H, C₅ Pz), 8.05 (d, *J* = 5.5 Hz, 1H, H₄ Th), 7.18 (d, *J* = 5.5 Hz, 1H, H₅ Th), 4.00 (s, 3H, NCH₃), 2.61 (ddd, *J* = 17.2, 4.9, 1.9 Hz, 1H, H₅), 2.40 (ddd, *J* = 14.5, 3.1, 2.1 Hz, 1H, H₃), 2.34–2.25 (m, 1H, H₄), 2.08 (dd, *J* = 17.2, 11.8 Hz, 1H, H'₅), 1.73 (dd, *J* = 14.5, 12.1 Hz, 1H, H'₃), 1.72 (s, 3H, CH₃-2), 1.07 (d, *J* = 6.4 Hz, 3H, CH₃-4); ¹³C-NMR (75 MHz, CDCl₃), only signals of the major diastereomer are given, δ 171.1 (s, C₁), 157.4 (s, CONH), 141.6 (q, *J* = 38.1 Hz, C₃ Pz), 134.8 (s, C₃ Th), 133.4 (s, C₅ Pz), 125.6 (s, C₂ Th), 124.5 (s, C₄ Th), 122.6 (s, C₅ Th), 120.8 (q, *J* = 269.8 Hz, CF₃), 117.2 (s, C₄ Pz), 86.3 (s, C₂), 44.0 (s, C₃), 40.0 (s, NCH₃), 37.8 (s, C₅), 32.0 (s, CH₃-2), 25.1 (s, H₄), 21.2 (s, CH₃-4); ¹⁹F-NMR (282 MHz, CDCl₃) δ –61.71 (s, CF₃); HRMS (TOF, ESI+) *m/z* calculated for C₁₇H₁₉F₃N₃O₃S [M+H]⁺ 402.1094, found 402.1076.

Preparation of 3-methyl-5-(3-(1-methyl-3-(trifluoromethyl)-1H-pyrazole-4-carboxamido)thiophen-2-yl)hexanoic acid (Hapten PPm, 7)

Triethylsilane (1.7 mL, 10.61 mmol) was added under a N₂ atmosphere to a solution of lactone **6** (64.6 mg, 0.16 mmol) and trifluoroacetic acid (0.82 mL, 10.61 mmol) in anhydrous CH₂Cl₂ (1 mL). The resulting mixture was stirred at room temperature for 1.5 h (reaction monitored by CCF, CHCl₃/MeOH 19:1). After this time, solvent was evaporated and the residue was purified by flash column chromatography (silica gel, CHCl₃/MeOH 49:1). Hapten PPm (**7**, 55.2 mg, 85%) was thus obtained as a white solid, whose ¹H-NMR spectrum shown to be a 1:1 mixture of 3,5-*syn/anti* diastereomers. Mp 91.9–92.1 °C (CH₂Cl₂); IR (neat) $\nu_{\max}$ (cm⁻¹) 3126w, 2962w, 2930w, 1705s, 1652s, 1547m, 1486w, 1301m, 1175s, 1137s, 1057s, 833m; ¹H-NMR (300 MHz, CDCl₃) only signals of one of the diastereomers are given, δ 10.72 (br s, 1H, CO₂H), 8.05 (s, 1H, H₅ Pz), 7.71 (br s, 1H, CONH), 7.33 (d, *J* = 5.4 Hz, 1H, H₄ Th), 7.12 (d, *J* = 5.4 Hz, 1H, H₅ Th), 3.93 (s, 3H, NCH₃), 3.12 (ddq, *J* = 7.0, 7.0, 7.0 Hz, 1H, H₅), 2.28 (dd, *J* = 14.9, 6.1 Hz, 1H, H₂), 2.17 (dd, *J* = 14.9, 7.1 Hz, 1H, H'₂), 2.02–1.87 (m, 1H, H₃), 1.76–1.64 (m, 1H, H₄), 1.55–1.43 (m, 1H, H'₄), 1.26 (d, *J* = 7.0 Hz, 3H, H₆), 0.94 (d, *J* = 6.6 Hz, 3H, CH₃-3); ¹³C-NMR (75 MHz, CDCl₃), only signals of one of the diastereomers are given, δ 178.2 (s, C₁), 159.2 (s, CONH), 139.2 (s, C₃ Th), 138.5 (q, *J* = 37.0 Hz, C₃ Pz), 136.0 (s, C₅ Pz), 129.8 (s, C₂ Th), 124.8 (s, C₄ Th), 121.6 (s, C₅ Th), 121.2 (q, *J* = 269.5 Hz, CF₃), 116.9 (s, C₄ Pz), 45.6 (s, C₄), 41.1 (s, C₂), 39.9 (s, NCH₃), 30.4 (s, C₅), 28.2 (s, C₃), 23.1 (s, C₆), 20.0 (s, CH₃-3); ¹⁹F-NMR (282 MHz, DMSO-*d*₆) δ -60.13 (s, CF₃); HRMS (TOF, ESI+) *m/z* calculated for C₁₇H₂₁F₃N₃O₃S [M+H]⁺ 404.1250, found 404.1256; UV (PB) ϵ (290 nm) = 1.34 mM⁻¹ cm⁻¹, ϵ (280 nm) = 2.38 mM⁻¹ cm⁻¹, ϵ (270 nm) = 3.86 mM⁻¹ cm⁻¹, ϵ (260 nm) = 6.05 mM⁻¹ cm⁻¹, ϵ (250 nm) = 9.16 mM⁻¹ cm⁻¹.

Activation of haptens and coupling to proteins

Activation of hapten PPd (PPd-NHS, **8**)

Anhydrous triethylamine (30 μ L, 0.22 mmol) was added to a stirred solution of hapten PPd (**4**, 22.9 mg, 57 μ mol) and *N,N'*-disuccinimidyl carbonate (18.9 mg, 74 μ mol) in anhydrous CH₃CN (520 μ L) at 0 °C under N₂. The reaction mixture was stirred at room temperature for 2 h

(reaction monitored by TLC, CHCl₃-MeOH 19:1) and then diluted with water and extracted with Et₂O. The combined organic layers extracts were washed with a 5% aqueous solution of NaHCO₃ and dried over anhydrous MgSO₄, then filtered and concentrated under vacuum. PPd-NHS ester (**8**, 24.4 mg, 86%) was thus obtained as a colorless oil which required no further purification. ¹H-NMR (300 MHz, CDCl₃) δ 8.13 (s, 1H, CONH), 7.98 (s, 1H, H₅ Pz), 7.45 (d, *J* = 1.5 Hz, 1H, H₄ Th), 6.74 (d, *J* = 1.5 Hz, 1H, H₂ Th), 3.95 (s, 3H, NCH₃), 2.82 (s, 4H, COCH₂CH₂CO), 2.54 (t, *J* = 6.8 Hz, 2H, H₂), 1.79–1.53 (m, 4H, H₃ and H₄), 1.34 (s, 6H, H-6 and CH₃-5).

Activation of hapten PPO (PPO-NHS, **9**)

Anhydrous triethylamine (26 μL, 0.19 mmol) was added to a stirred solution of hapten PPO (**5**, 20 mg, 49 μmol) and *N,N'*-disuccinimidyl carbonate (16.5 mg, 64 μmol) in anhydrous CH₃CN (440 μL) at 0 °C under N₂. The reaction mixture was stirred at room temperature for 1.5 h (reaction monitored by TLC, CHCl₃-MeOH 19:1) and then diluted with water and extracted with Et₂O. The combined organic layers were washed with a 5% aqueous solution of NaHCO₃ and dried over anhydrous MgSO₄, then filtered and concentrated under vacuum. The crude product was purified by flash column chromatography (silica gel, CHCl₃) to give PPO-NHS ester (**9**, 15 mg, 60%) as a colorless oil. ¹H-NMR (300 MHz, CDCl₃) δ 11.68 (br s, 1H, CONH), 8.27 (d, *J* = 5.4 Hz, 1H, H₅ Th), 8.04 (s, 1H, H₅ Pz), 7.54 (d, *J* = 5.4 Hz, 1H, H₄ Th), 4.04 (s, 3H, NCH₃), 2.96 (dd, *J* = 15.6, 6.4 Hz, 1H, H₄), 2.83 (s, 4H, COCH₂CH₂CO), 2.89–2.71 (m, 3H, H₂, H₃ and H'₄), 2.62 (dd, *J* = 16.8, 8.7 Hz, 1H, H'₂), 1.17 (d, *J* = 6.3 Hz, 3H, CH₃-3).

Activation of hapten PPM (PPM-NHS, **10**)

Anhydrous triethylamine (15 μL, 0.11 mmol) was added to a stirred solution of hapten PPM (**7**, 11.7 mg, 29 μmol) and *N,N'*-disuccinimidyl carbonate (9.7 mg, 38 μmol) in anhydrous CH₃CN (260 μL) at 0 °C under N₂. The reaction mixture was stirred at room temperature for 2.5 h (reaction monitored by TLC, CHCl₃-MeOH 19:1) and then diluted with water and extracted with Et₂O. The combined organic phases were washed with a 5% aqueous solution of NaHCO₃

and dried over anhydrous MgSO_4 , then filtered and concentrated under vacuum. The crude product was purified by flash column chromatography (silica gel, CHCl_3) to give P*Pm*-NHS ester (**10**, 8.4 mg, 58%, a 1:1 mixture of diastereomers as shown by ^1H -NMR) as a yellowish oil. ^1H -NMR (300 MHz, CDCl_3) δ 8.02 (s, 1H, H_5 Pz), 7.63 (s, 1H, CONH), 7.34 (d, J = 5.4 Hz, 1H, H_4 Th), 7.13 (d, J = 5.4 Hz, 1H, H_5 Th), 3.98 (s, 3H, NCH_3), 3.14 (ddq, J = 6.9, 6.9, 6.9 Hz, 1H, H_5), 2.81 (s, 4H, $\text{COCH}_2\text{CH}_2\text{CO}$), 2.57 (dd, J = 15.1, 5.8 Hz, 1H, H_2), 2.43 (dd, J = 15.1, 7.6 Hz, 1H, H'_2), 2.15–2.01 (m, 1H, H_3), 1.86–1.50 (m, 2H, H_4), 1.29 (d, J = 6.9 Hz, 3H, H_6), 1.04 (d, J = 6.7 Hz, 3H, CH_3 -3).

Protein conjugation

The succinimidyl ester of the hapten (P*Pd*-NHS, P*Po*-NHS, or P*Pm*-NHS) was dissolved in DMF and added drop wise to a protein solution in 50 mM carbonate buffer, pH 9.6, under gentle stirring, without exceeding a 10% (v/v) DMF final concentration. The coupling reaction was carried out during 2 h at room temperature in amber glass vials. As carrier proteins, BSA, OVA, and HRP for immunogen, coating antigen, and enzyme tracer preparation were employed, respectively. Haptens were employed at 24, 8, and 11 molar excess for BSA (only with hapten P*Pm*), OVA, and HRP, respectively. Next, bioconjugates were purified by size-exclusion chromatography in Sephadex G-25 using 100 mM phosphate buffer, pH 7.4 (PB), as eluent. The immunizing BSA conjugate was filter sterilized and stored frozen at -20°C . The number of hapten molecules that were conjugated to the carrier protein was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in a 5800 MALDI TOF/TOF (ABSciex) in positive linear mode (1500 shots every position) in a mass range of 10000-100000 m/z . Previously, the plate was calibrated with 1 μL of the TOF/TOF calibration mixture (ABSciex), in 13 positions. Every sample was calibrated by 'close external calibration' method with a BSA, OVA or HRP spectrum acquired in a close position.

Antibody generation

Animal manipulation was performed in compliance with Spanish laws and guidelines (RD 1201/2005 and Law 32/2007) and according to the European directive 2010/63/EU concerning the protection of animals used for scientific purposes. Animal protocols were approved by the Ethics Committee for Animal Experimentation and Welfare of the Ethics Commission at the University of Valencia. Two white female rabbits, weighing around 2 kg, were subcutaneously immunized with 300 μ g of BSA-PPm conjugate in a 1:1 emulsion between phosphate buffer solution and Freund's adjuvant (complete for the first injection and incomplete for subsequent boosts). The immunogen was administered 4 times with 21-day intervals. The immunization process was verified with a serum sample collected from the ear vein 10 days after the third injection. The whole blood was sampled ten days after the fourth injection and allowed to coagulate overnight at 4 °C. The serum was separated by double centrifugation and antibodies were precipitated twice with 1 volume of saturated ammonium sulfate solution. Precipitates were stored at 4 °C. A working antibody solution was prepared by 4-fold dilution with PBS (10 mM phosphate buffer, pH 7.4, with 140 mM NaCl) containing 1% (w/v) BSA and kept at 4 °C.

Antibody-coated direct competitive ELISA

Microplate wells were coated with 100 μ L per well of antibody dilution in 50 mM carbonate buffer, pH 9.6, by overnight incubation at room temperature. The day after, plates were rinsed four times with washing solution (150 mM NaCl with 0.05% Tween 20, v/v). The competitive reaction was carried out at room temperature during 1 h by mixing 50 μ L per well of penthiopyrad solution in PBS and 50 μ L per well of peroxidase tracer in PBST (PBS containing 0.05% Tween 20, v/v). After rinsing the plate as before, the immunochemical reaction was revealed at room temperature with 100 μ L per well of enzyme substrate solution (2 mg/mL o-phenylenediamine and 0.012% (v/v) H₂O₂ in 25 mM citrate and 62 mM phosphate buffer, pH 5.4). The enzymatic reaction was stopped after 10 min with 100 μ L per well of 1 M H₂SO₄.

Absorbance was read immediately after assays at 492 nm with a reference wavelength at 650 nm.

Conjugate-coated indirect competitive ELISA

For microwell coating, 100 μ L per well of OVA conjugate solution in 50 mM carbonate buffer, pH 9.6, was applied and incubated overnight at room temperature. Then, plates were washed as indicated above. The competitive reaction step was performed during 1 h at room temperature by mixing 50 μ L per well of penthiopyrad standard solution in PBS and 50 μ L per well of antibody dilution in PBST. After washing, 100 μ L per well of GAR–HRP solution (diluted 1/10000) in PBST was applied and plates were incubated 1 h at room temperature. Plates were washed again as before, and the presence of enzyme activity was revealed and the absorbance was read as described for the previous ELISA format.

Sample and data analysis

Commercial fruit juices were acquired from local supermarkets. Samples were diluted in MilliQ water before assaying and the soluble immunoreagent (tracer or antibody for the direct and indirect assay, respectively) for the competitive step was prepared in 2 $\times$ PBS containing 0.05% (v/v) Tween 20. Eight-point standard curves, including a blank, were prepared in PBS by serial dilution in the same buffer. Experimental values were 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 corresponding to a 50% reduction (IC_{50}) of the maximum absorbance (A_{max}). The linear range and the limit of detection (LOD) were estimated as the concentration of analyte providing a decrease of A_{max} between 20% and 90% for the former and 10% for the latter. Cross-reactivity was determined as the percentage of the quotient between the IC_{50} value for penthiopyrad and the IC_{50} value for the evaluated compound.

Results and Discussion

Hapten synthesis

The molecular structure of the immunizing hapten used in this work (hapten *PPm*) has a very high degree of structural similitude with the parent analyte (Table 1). Specifically, this hapten keeps integrally the skeleton and all functional groups of penthiopyrad, including the branched 4-methylpentan-2-yl aliphatic group at the C-2 position of the thiophene ring that in fact forms part of the carboxylated spacer arm used for its conjugation to the carrier protein. It was expected that this high structural similarity between analyte and hapten should lead to the generation of antibodies of very high affinity and specificity against penthiopyrad. On the other hand, two structural analogues of the immunizing hapten, *PPd* and *PPo* have also been prepared to be used as heterologous haptens for enhancing immunoassay sensitivity. The former hapten has a carboxylated alkyl branched chain located at a different position of the thiophene ring which fundamentally affects the conformational arrangement that this hapten can adopt in relation to the immunogenic hapten *PPm*. The second hapten incorporates a carbonyl group in the spacer arm conjugated to the thiophene ring, which produces an important electronic modification.

The preparation of haptens *PPd*, *PPo*, and *PPm* is schematized in Fig. 1. Their synthesis is based on the initial preparation of the common intermediate pyrazole-4-carboxamide **3**, readily obtained from carbonyl chloride **1** and the aminothiophene derivative **2**, whose thiophene aromatic moiety can be regioselectively functionalized at the C-2 or C-5 positions via a Friedel-Craft acylation reaction with an appropriately functionalized acylating agent. Thus, reaction of thiophene **3** with the sterically more demanding acylating reagent 3,3-dimethyl-tetrahydro-pyran-2-one led to regioselective acylation of the C-5 position of the thiophene ring to afford hapten *PPd* (**4**) in an excellent 73% yield. On the other hand, reaction of thiophene **3** with 3-methylglutaric anhydride led to the regioselective acylation of the C-2

position to give keto acid **5** (hapten PPO), from which the synthesis of hapten PPM was completed by nucleophilic addition of methyl lithium to the ketone carbonyl group, followed by reduction of the benzylic-like C–O bond of intermediate δ -lactone **6** with triethylsilane under relatively strong acidic conditions (trifluoroacetic acid). The hapten PPM (**7**) was thus obtained from **3**, in three synthetic steps with an overall yield of 26%, as a 1:1 mixture of 3,5-*syn/anti* diastereomers.

Immunoreagent preparation

Haptens were covalently coupled to proteins by the active ester method as previously described for other molecules (Esteve-Turrillas et al. 2010). Carboxylated haptens were activated by reaction with DSC and EtN₃ under smooth conditions, and the corresponding active esters were purified by silica gel chromatography and characterized by ¹H NMR spectroscopy. The purified *N*-hydroxysuccinimidyl esters of the synthetic haptens were used for coupling to BSA (hapten PPM only), OVA, and HRP. Conjugation to these three proteins was performed in carbonate buffer, pH 9.6, at room temperature in 2 h, and the obtained conjugates were purified by size-exclusion chromatography. This conjugation strategy afforded high coupling yields, with the adequate hapten-to-protein molar ratios (MR); *i.e.*, 18 for the immunizing conjugate, 4–8 for the coating conjugates, and 1–3 for the enzyme conjugates, as determined by MALDI-TOF-MS (Fig. 2). Moreover, since the purified active ester was used, no undesired secondary reactions could take place; consequently, the same coupling reaction could be applied for preparing the immunizing and the assay conjugates.

Immunizing haptens should resemble as much as possible the target compound, being the linker tethering site one of the major determinants for a correct display of the characteristic chemical moieties of the molecule (López-Moreno et al. 2013). Conjugate BSA–PPM, in which the aliphatic moiety of the molecule was used as linker, was chosen for antibody generation since it carried the hapten that was the best mimic of penthiopyrad. Two polyclonal antibodies

1 were obtained from immunized rabbits, namely antibodies PPm#1 and PPm#2.
2 Immunoglobulins were partially purified by salting out twice, and an aliquot was diluted as
3 described with a BSA containing buffer in order to preserve antibody activity.
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7 **Antibody and bioconjugate characterization**

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10 Antibodies were characterized by direct and indirect competitive ELISA using homologous
11 and heterologous bioconjugates; that is, the hapten of the conjugate was the same as, or
12 different to, that of the immunizing conjugate, respectively (Table 2). Binding was observed for
13 both antibodies with tracer HRP-PPb which, as in the immunizing conjugate, has a linear linker
14 chain attached at the C2-thiophene position. If the linker tethering site was the same as in the
15 immunizing conjugate but the electronic characteristics of the linker were modified, as in
16 hapten PPO, binding was observed, but only with one of the two antibodies. Interestingly, no
17 signal was obtained with any of the two antibodies in combination with tracer HRP-PPd,
18 which, in comparison with the homologous hapten (PPm), has the spacer arm at a
19 regioisomeric position of the thiophene ring. This result is probably due to a significant
20 modification of the spatial conformation of the molecule. However, signal was obtained with
21 tracer HRP-PPa which holds a linear spacer arm at the distal N1-pyrazole nitrogen atom (Table
22 1). The lowest IC₅₀ value, in the direct assay format, was obtained with antibody PPm#2
23 combined with tracer HRP-PPb. In the indirect format, all of the assayed conjugates were
24 bound by both antibodies. The highest sensitivity in this assay format was achieved with
25 antibody PPm#2 and conjugate OVA-PPa. Therefore, IC₅₀ values could be improved in both
26 formats with most of the heterologous conjugates that were recognized by the antibody.
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51 Antibody specificity was evaluated by indirect competitive ELISA using the homologous
52 conjugate. Cross-reactivity with fluxapyroxad was 2% and 1% for antibodies PPm#1 and
53 PPm#2, respectively. No inhibition was observed with other succinate dehydrogenase
54 inhibitors, such as fluopyram and boscalid or by other commonly used fungicides like
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1 fluopicolide, fenhexamid, cyprodinil, pyrimethanil, fludioxonil, azoxystrobin, pyraclostrobin,
2 and tebuconazole.
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5 **Immunoassay development**

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8 For further immunoassay development, antibody PPm#2 was selected together with
9 conjugates HRP-PPb and OVA-PPa for the direct and indirect assay formats, respectively.
10 Antibody and conjugate concentrations were optimized and the main analytical parameters
11 were determined (Table 3). High $A_{\max}$ values with negligible background signals, and IC_{50} values
12 below 0.5 ng/mL for penthiopyrad were obtained. The theoretical LOD was around 0.07
13 ng/mL, and the linear working range was estimated as 0.12–0.98 ng/mL and 0.13–1.41 ng/mL
14 for the direct and indirect assays, respectively. The intra- and inter-day precision of $A_{\max}$ and
15 IC_{50} values were satisfactory for both immunoassays. These results indicate the capability of
16 the developed immunoassays for the analysis of penthiopyrad in samples.
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31 Food samples may induce pH changes and increase the ionic strength of the assay buffer;
32 thus, tolerance to pH and ionic strength changes was assessed for the selected immunoassays
33 (Fig. 3). At basic pH values, the $A_{\max}$ and IC_{50} values remained unchanged in both formats. At
34 acidic pHs, the $A_{\max}$ value slightly decreased for both assays whereas the IC_{50} value for
35 penthiopyrad was unaffected for the indirect immunoassay and it was slightly increased for
36 the direct format. Thus, both assays were quite tolerant to pH variations between 5.5 and 8.0.
37 On the other hand, at ionic strength values higher than that of PBS ($I = 166$ mM) lower $A_{\max}$
38 values were found for the indirect immunoassay, whereas the absorbance of the direct assay
39 and the IC_{50} values of both formats remained unchanged. Buffers with low salt contents are
40 not recommendable because the $A_{\max}$ values of both immunoassays and the IC_{50} value of the
41 indirect assay would increase.
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58 Organic solvents such as methanol and acetonitrile are commonly employed for pesticide
59 residue extraction from food samples (Fig. 4). Particularly, the two selected immunoassays
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1 tolerated quite well increasing methanol contents up to 5%; only the $A_{\max}$ value of the indirect
2 assay increased slightly. The presence of acetonitrile showed dissimilar effects on both
3 immunoassays. For the direct assay, the $A_{\max}$ value decreased whereas the IC_{50} value for
4 penthiopyrad remained almost constant with increasing acetonitrile contents. For the indirect
5 assay, the $A_{\max}$ value was not significantly altered but the IC_{50} value increased almost three
6 times at high concentrations of this solvent. Thus, if solvents need to be employed, methanol
7 would be more recommendable than acetonitrile.
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10 11 12 13 14 15 16 17 **Food sample analysis**

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20 Matrix effects were studied for both of the developed immunoassays. Apple juice and white
21 and red grape juices were selected as representative food samples. Penthiopyrad standard
22 curves, including a blank, were prepared in MilliQ water containing different amounts of fruit
23 juice. It was seen that the matrix effects were minimized with increasing juice dilutions, as
24 expected. Slightly higher matrix effects were generally observed with red grape juice.
25 Interferences could be minimized when the dilution of the juice was at least 1/50 (results not
26 shown).
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31 Fruit juices were fortified with penthiopyrad and analyzed by the two studied
32 immunoassays. Samples were diluted in MilliQ water before assaying. As seen in Table 4,
33 excellent accuracy was found for the direct immunoassay – recoveries were between 81% and
34 116% –, though somewhat high coefficients of variation – mainly below 30% – were observed.
35 The lowest assayed penthiopyrad concentration with acceptable recovery values (limit of
36 quantification) for the three fruit juices was 5 ng/mL. Concerning the indirect assay, recovery
37 values were between 88% and 124%, and good precision values were obtained – coefficients
38 of variation were mostly below 20%. In this case, the limit of quantification was found to be 30
39 ng/mL. No European maximum residue limit (MRL) has been set up for penthiopyrad in fruit
40 juices. Concerning raw commodities, the MRLs are 500 ng/g and 10 ng/g in apples and grapes,
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1 respectively (EU Pesticides Database). Thus, the limits of quantification of the developed
2 immunoassays for penthiopyrad in the studied fruit juice samples are much lower or similar to
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4 the MRLs for the corresponding raw foodstuff.
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7 **Conclusions**

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10 High-affinity and specific antibodies to penthiopyrad have been prepared with a novel
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12 hapten that highly mimics the target molecule by reproducing the characteristic branched
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14 aliphatic moiety of penthiopyrad in the spacer arm of the hapten. Moreover, heterologous
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16 haptens were prepared with important electronic modifications and with different linker
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18 tethering sites. A proximal linker tethering site can hinder antibody binding to a larger extent
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20 than a distal position of the spacer if additional modifications are induced such as
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22 conformational or electronic changes. For small molecules like penthiopyrad, proximal
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24 moieties with respect to the linker tethering site, and even the linker itself, may significantly
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26 participate in antibody binding. Two immunoassays in different competitive ELISA formats
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28 were optimized affording LODs around 0.07 ng/mL. Performance of the developed
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30 immunoassays was verified using three fruit juices as model foodstuff. The experimental limits
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32 of quantification were sufficient for screening penthiopyrad at the European MRLs.
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Compliance with Ethical Standards

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of Interest Eric Ceballos-Alcantarilla declares that he has no conflict of interest. Consuelo Agulló declares that she has no conflict of interest. Antonio Abad-Fuentes declares that he has no conflict of interest. Mónica Escamilla-Aguilar declares that she has no conflict of interest. Antonio Abad-Somovilla declares that he has no conflict of interest. Josep V. Mercader declares that he has no conflict of interest.

Informed Consent Not applicable

Abbreviations

ABS: adult bovine serum; BSA: bovine serum albumin; DMF: N,N'-dimethylformamide; DSC: N,N'-disuccinimidyl carbonate; ELISA: enzyme-linked immunosorbent assay; ESI: electrospray ionization; GAR-HRP: goat anti-rabbit immunoglobulins conjugated to peroxidase; HRMS: high resolution mass spectrometry; HRP: horseradish peroxidase; IR: infrared; LOD: limit of detection; MR: molar ratio; MRL: maximum residue limit; OVA: ovalbumin; PB: phosphate buffer; PBS: phosphate buffered saline; PBST: PBS containing Tween 20; SDHI: succinate dehydrogenase inhibitor; TLC: thin-layer chromatography; UV: ultraviolet.

Figure legends

Fig. 1 Schematic representation of the synthesis of haptens PPd, PPO, and PPM.

Fig. 2 MALDI-TOF-MS spectra of bioconjugates: a) BSA (blue) and BSA-PPm (green); b) OVA (blue) and conjugates OVA-PPd (light blue), OVA-PPO (green) and OVA-PPm (orange); c) HRP (blue) and conjugates HRP-PPd (orange), HRP-PPO (light blue) and HRP-PPm (green).

Fig. 3 Influence of pH and ionic strength (*I*) over the $A_{\max}$ and IC_{50} values of the penthiopyrad immunoassays.

Fig. 4 Tolerance to organic solvents. Values were normalized with respect to the corresponding value at 0% solvent contents.

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Table 1 Chemical structures of penthiopyrad and the synthetic haptens

	R ¹	R ²	R ³
Penthiopyrad	CH ₃	H	CH(Me)CH ₂ CHMe ₂
Hapten PPa	(CH ₂) ₄ CO ₂ H	H	CH(Me)CH ₂ CHMe ₂
Hapten PPb	CH ₃	H	(CH ₂) ₅ CO ₂ H
Hapten PPd	CH ₃	C(Me) ₂ (CH ₂) ₃ CO ₂ H	H
Hapten PPo	CH ₃	H	COCH ₂ CH(Me)CH ₂ CO ₂ H
Hapten PPM	CH ₃	H	CH(Me)CH ₂ CH(Me)CH ₂ CO ₂ H

Table 2 Immunoreagent selection by checkerboard competitive ELISA (n = 3)^a

Direct assay										
pAb	HRP-PPa		HRP-PPb		HRP-PPd		HRP-PPm		HRP-PPo	
	[HRP] ^b	IC ₅₀ ^c	[HRP]	IC ₅₀	[HRP]	IC ₅₀	[HRP]	IC ₅₀	[HRP]	IC ₅₀
PPm#1	10	2.39	100	0.83	300	- ^d	30	1.37	300	-
PPm#2	10	1.97	100	0.80	300	-	30	1.33	100	1.06
Indirect assay										
pAb	OVA-PPa		OVA-PPb		OVA-PPd		OVA-PPm		OVA-PPo	
	[pAb] ^e	IC ₅₀	[pAb]	IC ₅₀	[pAb]	IC ₅₀	[pAb]	IC ₅₀	[pAb]	IC ₅₀
PPm#1	30	1.85	100	1.81	10	1.47	100	6.68	10	2.23
PPm#2	100	0.53	100	0.64	30	0.62	100	1.69	30	0.49

^a A_{max} values were between 0.8 and 1.5. ^b Values are in ng/mL. ^c Values are in nM. ^d No signal was observed. ^e Antibody dilution ×10³.

Table 3 Normalized standard curves, assay conditions, and analytical parameters of the developed immunoassays

	Direct assay	Indirect assay
Antibody	PPm#2 ($1/10^4$)	PPm#2 ($1/3 \times 10^4$)
Conjugate	HRP-PPb (30 ng/mL)	OVA-PPa (100 ng/mL)
A_{max}	1.784 ± 0.177	0.887 ± 0.173
A_{min}	0.015 ± 0.016	0.015 ± 0.005
Slope	-1.372 ± 0.185	-1.176 ± 0.080
LOD (ng/mL)	0.068 ± 0.018	0.067 ± 0.009
IC_{20} (ng/mL)	0.123 ± 0.028	0.132 ± 0.013
IC_{50} (ng/mL)	0.343 ± 0.069	0.430 ± 0.020
IC_{80} (ng/mL)	0.975 ± 0.264	1.406 ± 0.108
Intraday precision (% , n = 3)		
A_{max}	7	7
IC_{50}	11	4
Interday precision (% , n = 3)		
A_{max}	5	18
IC_{50}	28	6

^a Values are the mean of 12 independent replicates.

Table 4 Recovery values obtained with juice samples fortified with penthiopyrad^a

	[Penthiopyrad] (ng/mL)	Recovery (% $\pm$ s, n = 3)		
		Apple juice	White juice	Red juice
Direct assay	5	104 $\pm$ 28	116 $\pm$ 22	103 $\pm$ 11
	10	92 $\pm$ 26	91 $\pm$ 16	92 $\pm$ 25
	30	88 $\pm$ 29	101 $\pm$ 24	97 $\pm$ 24
	50	98 $\pm$ 27	108 $\pm$ 16	94 $\pm$ 15
	100	115 $\pm$ 34	113 $\pm$ 28	112 $\pm$ 14
	300	109 $\pm$ 14	101 $\pm$ 24	89 $\pm$ 13
	500	102 $\pm$ 24	105 $\pm$ 20	97 $\pm$ 22
Indirect assay	30	106 $\pm$ 12	105 $\pm$ 22	124 $\pm$ 25
	50	112 $\pm$ 12	101 $\pm$ 10	119 $\pm$ 13
	100	104 $\pm$ 5	101 $\pm$ 7	115 $\pm$ 6
	300	97 $\pm$ 12	98 $\pm$ 6	96 $\pm$ 15
	500	88 $\pm$ 2	103 $\pm$ 8	90 $\pm$ 15
	1000	95 $\pm$ 13	107 $\pm$ 16	90 $\pm$ 15

^a Juice samples were diluted 1/50 with MilliQ water before assaying.