Analyst

Combined heterologies for monoclonal antibody-based immunoanalysis of fluxapyroxad

Journal:	*Analyst*
Manuscript ID	AN-ART-09-2018-001771.R1
Article Type:	Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Ceballos-Alcantarilla, Eric; Universitat de València, Departamento de Química Orgánica; Instituto de Agroquímica y Tecnología de Alimentos, Biotecnología Lopez-Puertollano, Daniel; Universitat de Valencia Facultat de Quimica; Instituto de Agroquimica y Tecnologia de Alimentos Agulló, Consuelo; Universitat de València, Química Orgánica Abad-Fuentes, Antonio; IATA-CSIC, Abad-Somovilla, Antonio; Universitat de València, Química Orgánica Mercader, Josep; IATA-CSIC,

Journal Name

ARTICLE

Combined heterologies for monoclonal antibody-based immunoanalysis of fluxapyroxad

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Eric Ceballos-Alcantarilla,[a,b] Daniel López-Puertollano,[a,b] Consuelo Agulló,[b] Antonio Abad-Fuentes,[a] Antonio Abad-Somovilla,[b] Josep V. Mercader[a,*]

Nowadays, instrumental methodologies and rapid bioanalytical techniques complement each other for the analysis of chemical toxic compounds. Fluxapyroxad was commercialized a few years ago as fungicide and today it is being used worldwide to control a variety of pests. In the present study, the development of monoclonal antibody-based immunochemical methods for the analysis of this chemical in food samples was evaluated for the first time. Novel haptens were synthesized and protein bioconjugates were prepared. High-affinity and specific monoclonal antibodies to fluxapyroxad were generated from two haptens with alternative linker tethering sites. Haptens with linker site heterology and a structural heterologous hapten with a minor modification of the molecule conformation and volume but with a significant alteration of the electronic density of the pyrazole moiety were confronted for immunoassay development. A direct and an indirect competitive immunoassay were characterized and optimized, showing IC_{50} values for fluxapyroxad of 0.14 and 0.05 ng mL^{-1}, respectively. The combination of two heterologies was particularly adequate in the indirect format. The two developed immunoassays showed excellent recoveries and coefficients of variation in fluxapyroxad-fortified plums and four varieties of grapes. Finally, a good correlation was found between the indirect immunoassay and UPLC–MS/MS when fruit samples with incurred residues of fluxapyroxad were analyzed. These monoclonal antibody-based immunochemical methods hold great promise for fluxapyroxad monitoring.

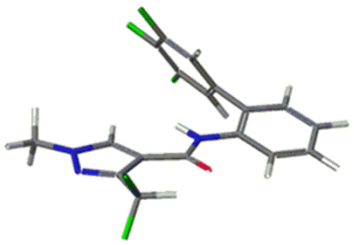
Introduction

Fluxapyroxad (Xemium) inhibits the succinate dehydrogenase (SDH) activity of complex II, in fungal mitochondria, by blocking the ubiquinone binding site (Q-site), thus stopping the electron transport chain.[1-3] The chemical structure of this biocide consists of a biphenyl moiety that is linked to a difluoromethyl pyrazole ring through an amide bridge (Fig. 1). The carboxamide group at the C–4 position of the pyrazole ring seems to be essential for the antifungal activity.[4] Fluxapyroxad is commercialized worldwide by BASF under different trademarks, such as Sercadis, Priaxor, and Merivon (www.agro.basf.com). Shortly after entering the market, residues of this fungicide began to be detected in food samples. In the 2013 and successive European and US pesticide monitoring programs, fluxapyroxad was found in a variety of commodities, even though the maximum residue limits (MRL) were not exceeded in those samples.[5,6] Fluxapyroxad residue in vegetable foodstuffs is defined by the European Commission and the US Environmental Protection Agency as the parent compound because it is largely the principal residue.[7] According to FAO, and in line with most modern fungicides, fluxapyroxad entails low genotoxic, reproductive, immunotoxic, and neurotoxic risks in humans. From a two-year toxicity study in animals, the acceptable daily intake and the acute reference dose for humans were estimated at 0–0.02 mg kg^{-1} and 0.3 mg kg^{-1} of body weight, respectively.[8]

Currently, the reference analytical method for the determination of fluxapyroxad residues in food samples is ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS). This method involves extraction with methanol/water or acetonitrile/water mixtures and can achieve limits of quantification (LOQ) as low as 10 µg kg^{-1}.[7] Lately, a few multiresidue methods that include the analysis of fluxapyroxad in foodstuffs have been developed,[9-12] and alternative approaches using gas-chromatography have also been reported.[13] Moreover, our research group recently reported the generation of polyclonal antibodies specific of fluxapyroxad.[14] Chromatographic and immunochemical methods constitute nowadays complementary strategies for different analytical applications.

[a] Institute of Agrochemistry and Food Technology (IATA), Spanish Council for Scientific Research (CSIC), Agustí Escardino 7, 46980 Paterna, Valencia, Spain. Email: jvmercader@iata.csic.es.
[b] Department of Organic Chemistry, University of Valencia, Doctor Moliner 50, 46100 Burjassot, Valencia, Spain.

† Electronic Supplementary Information (ESI) available: general experimental procedures, reagents, and instruments; spectrometric data of intermediates of the synthesis of hapten FX*b*; synthesis of hapten FX*h* (Fig. S1); preparation of *N*-hydroxysuccinimidyl esters; MALDI mass spectrometry analysis of bioconjugates (Fig. S2); antibody generation; specificity of mAbs (Table S1); influence of pH and ionic strength (Fig. S3); influence of organic solvents (Fig. S4); cELISA and UPLC–MS/MS results (Table S2); ^{1}H NMR spectra of haptens FX*b* and FX*h*. See DOI: 10.1039/x0xx00000x



Fluxapyroxad: R^1 = H; R^2 = CHF_2; R^3 = H
Hapten FXn: R^1 = $(CH_2)_3CO_2H$; R^2 = CHF_2; R^3 = H
Hapten FX*b*: R^1 = H; R^2 = CHF_2; R^3 = $(CH_2)_5CO_2H$
Hapten FX*h*: R^1 = H; R^2 = CH_2OH; R^3 = $(CH_2)_5CO_2H$

Fig. 1. Molecular structures of fluxapyroxad and haptens. Global minimum energy conformation of the *N*-([1,1'-biphenyl]-2-yl)-1*H*-pyrazole-4-carboxamide skeleton of fluxapyroxad. Calculations were performed using Molecular Mechanics (MM3) as implemented in the CAChe program [CAChe WorkSystem Pro software, version 7.5.0.85 (Fujitsu Ltd, Tokyo, Japan)]. A systematic conformational search was performed (all rotatable bonds were rotated by 24 degree steps) and the geometry of the lower energy conformer generated was refined by performing an optimize geometry calculation in MOPAC using AM1 parameters.

Chromatography-based methodologies are robust, precise, and can carry out multiresidue determinations. On the other hand, antibody-based techniques can be rapid, economic, and portable. However, high-quality immunoreagents are required and validation of immunoassays is mandatory. For low-molecular weight compounds like fluxapyroxad, the competitive enzyme-linked immunosorbent assay (cELISA) is probably the preferred immunochemical method because of its large throughput. Two types of immunoreagents are needed for this analytical approach, that is, the antibody and a conjugate that holds a derivative of the target compound, called hapten. Concerning antibodies, the monoclonal option is a limit-less and reproducible reagent, so it is the most accepted molecular binder. About bioconjugates, research is required to elucidate the optimum molecular analogue in terms of carrier, hapten density, spacer arm (composition, position, and length), functional group for coupling, heterologies, etc.

The object of the present study was to develop and validate immunochemical methods to fluxapyroxad using monoclonal antibodies (mAbs). With this aim, novel fluxapyroxad haptens with alternative linker tethering sites and structural heterologies were prepared, and high-affinity and specific mAbs to this fungicide were generated for the first time. The developed immunoassays were characterized and optimized, and the immunochemical results were compared with UPLC–MS/MS using fluxapyroxad contaminated fruit samples.

Experimental

Reagents and instrumentation

Pestanal grade fluxapyroxad (Mw 381.3; IUPAC name 3-(difluoromethyl)-1-methyl-*N*-(3',4',5'-trifluoro[1,1'-biphenyl]-2-yl)-1*H*-pyrazole-4-carboxamide) was acquired from Merck (Madrid, Spain) and technical fluxapyroxad was kindly supplied by BASF (Ludwigshafen, Germany). Other reagents for organic synthesis were obtained from regular suppliers. Solvents and reagents were purified by standard methods.[15] Further details about general experimental procedures and techniques can be found in the ESI‡. Bovine serum albumin (BSA) fraction V for immunogen preparation was purchased to Roche Applied Science (Mannheim, Germany). Ovalbumin (OVA) and horseradish peroxidase (HRP) were acquired to Merck (Madrid, Spain). Other biochemical reagents and instrumentation used for antibody generation and immunoassay development are described in the ESI‡.

Hapten synthesis

Despite the slight safety concerns of the compounds that were employed in this study, it is advisable to work in a well-ventilated fume hood. The synthesis of hapten FX*n* was previously published.[14] Detailed description of the synthesis of hapten FX*h* is provided as ESI‡ (Fig. S1‡). The synthesis of the immunizing hapten FX*b* was performed in four synthetic steps (Fig. 2), as described below. The spectrometric characterization data of all of the intermediate compounds are listed in the ESI‡.

Synthesis of 3-(difluoromethyl)-1-methyl-*N*-(3',4',5'-trifluoro-5-iodo-[1,1'-biphenyl]-2-yl)-1*H*-pyrazole-4-carboxamide (1). Iodine (148 mg, 0.578 mmol, 1.1 equiv) and Ag_2SO_4 (167 mg, 0.525 mmol, 1 equiv) were added to a solution of fluxapyroxad (200 mg, 0.525 mmol) in anhydrous CH_2Cl_2 (1.4

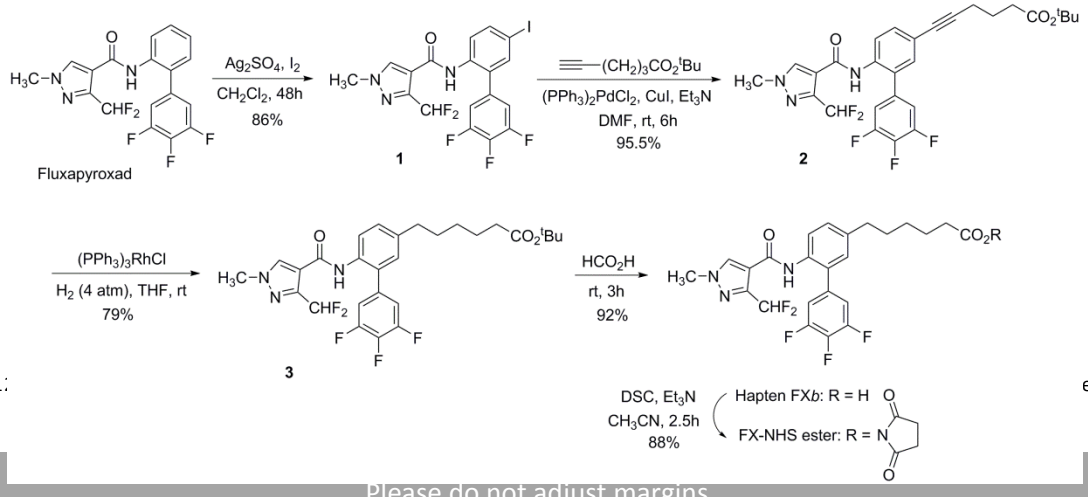


Fig. 2. Synthesis of immunizing hapten FX*b*.

mL) at room temperature. The mixture was stirred in the dark for 24 h, then filtered by suction through celite and washed with CH_2Cl_2. The brown-purple solution was then washed with an aqueous solution of sodium bisulphite and brine, dried over anhydrous $MgSO_4$, and the solvent was evaporated under reduced pressure to give an oily residue that was purified by chromatography, using hexane–EtOAc 8:2 as eluent, to afford iodide **1** (225.5 mg, 86%) as a solid.

Synthesis of *tert*-butyl 6-(6-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hex-5-ynoate (2). To a suspension of iodide **1** (294.5 mg, 0.581 mmol), CuI (10.5 mg, 0.055 mmol, 0.09 equiv) and $(PPh_3)_2PdCl_2$ (16.1 mg, 0.023 mmol, 0.04 equiv) in dry *N*,*N*'-dimethylformamide (DMF) (0.750 mL) under nitrogen was added *tert*-butyl hex-5-ynoate (117.5 mg, 0.698 mmol, 1.2 equiv) and anhydrous Et_3N (0.750 mL). The mixture was stirred at room temperature for 6 h (reaction monitored by TLC, hexane–EtOAc 1:1) and then quenched with H_2O and extracted with EtOAc. The combined organic layers were washed with 1.5% (w/v) aqueous LiCl solution and brine, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The crude product was purified by chromatography, using hexane–EtOAc mixtures from 9:1 to 7:3 as eluent, to afford acetylenic compound **2** (303.5 mg, 95.5%) as a slightly yellowish solid.

Synthesis of *tert*-butyl 6-(6-(3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hexanoate (3). A solution of alkyne **2** (248.2 mg, 0.4533 mmol) and $(Ph)_3P_3RhCl$ (12.6 mg, 0.0136 mmol, 0.03 equiv) in anhydrous THF (2.8 mL) was evacuated and purged under an atmosphere of hydrogen gas. Then, the hydrogen pressure was regulated to 4 atm and the reaction mixture was stirred at room temperature for 22 h, the solvent was removed under reduced pressure, and the residue was purified by chromatography, using hexane–ethyl acetate mixtures from 9:1 to 6:4 as eluent, to afford compound **3** (197.8 mg 79%) as a slightly yellowish, amorphous solid.

Synthesis of 6-(6-(3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hexanoic acid (Hapten FX*b*). A solution of *tert*-butyl ester **3** (60.3 mg, 0.109 mmol) in HCO_2H (1.5 mL) was stirred at room temperature for 3 h. The reaction mixture was diluted with benzene and washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated to dryness, to give hapten FX*b* (49.9 mg, 92%) as a solid. Mp 152–153 °C (from benzene–hexane); IR (neat) ν_{max} (cm^{-1}) 3237 (m), 3132 (w), 2921 (m), 2850 (m), 1715 (m), 1655 (m), 1631 (w), 1523 (s), 1430 (m), 1286 (m), 1196 (m), 1045 (s), 767 (m); ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.01 (1H, d, *J* = 8.3 Hz, H-5 BiPh), 7.96 (1H, s, H-5 Pz), 7.77 (1H, t, *J* = 4.2 Hz, NH), 7.23 (1H, dd, *J* = 8.3, 2.0 Hz, H-4 BiPh), 7.03 (1H, d, *J* = 2.0 Hz, H-2 BiPh), 7.00 (2H, dd, *J* = 8.2, 6.5 Hz, H-2' and H-6' BiPh), 6.65 (1H, t, *J* = 54.2 Hz, CHF_2), 3.92 (3H, s, NMe), 2.63 (2H, t, *J* = 7.5 Hz, H-6), 2.35 (2H, t, *J* = 7.4 Hz, H-2), 1.66 (4H, m, H-3 and H-5), 1.41 (2H, m, H-4); ^{19}F NMR (282 MHz, CD_3OD) δ (ppm) −114.6 (2F, s, CHF_2), −135.8 (2F, d, *J* = 20.0 Hz, F-3' and F-5' BiPh), −164.2 (1F, t, *J* = 20.0 Hz, F-4' BiPh); ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 177.6 (CO_2), 163.2 (CONH), 152.3 (ddd, *J* = 248.2, 9.7, 4.1 Hz, C-3' and C-5' BiPh), 147.0 (t, *J* = 24.7 Hz, C-3 Pz), 143.4 (C-6 BiPh), 140.3 (dt, *J* = 249.6, 15.4 Hz, C-4' BiPh), 137.4 (dt, *J* = 8.1, 4.8 Hz, C-1' BiPh), 137.0 (C-3 BiPh), 134.1 (C-5 Pz), 132.7 (C-1 BiPh), 131.3 (C-2 BiPh), 130.2 (C-4 BiPh), 128.9 (C-5 BiPh), 117.0 (t, *J* = 3.2 Hz, C-4 Pz), 114.3 (dd, *J* = 15.0, 6.7 Hz, C-2' and C-6' BiPh), 111.1 (t, *J* = 235.0 Hz, CHF_2), 39.8 (NMe), 36.2 (C-6), 34.9 (C-2), 32.2 (C-5), 29.8 (C-4), 25.9 (C-3); HRMS (TOF MS ES+) *m/z* calcd for $C_{24}H_{23}F_5N_3O_3$ $[M+H]^+$ 496.1654, found 496.1665.

Hapten activation

The carboxyl group of haptens FX*b* and FX*h* was activated by incubation with *N,N'*-disuccinimidyl carbonate and Et_3N in acetonitrile at 0 °C. The mixture was stirred until complete consumption of the starting material (as observed by thin-layer chromatography). The corresponding active *N*-hydroxysuccinimide esters were purified and characterized by ^1H NMR spectrometry. The detailed activation procedures and the respective spectra are provided as ESI‡.

Bioconjugate preparation

Covalent coupling between haptens and proteins was carried out in 50 mM carbonate buffer, pH 9.6, during 2 h under moderate stirring at room temperature. Immunizing conjugates were prepared by reaction of 10 μmol of purified activated hapten in DMF (200 μL) with 27 mg of BSA in carbonate buffer (1.8 mL). For coating conjugates, 5 μmol of activated hapten in 100 μL of DMF was conjugated to 28.5 mg of OVA in carbonate buffer (1.9 mL), whereas for enzyme assay conjugates, 0.5 μmol of active ester solution in DMF (100 μL) was reacted with 2 mg of HRP in the described coupling buffer (0.9 mL). Conjugates were purified by gel filtration chromatography using 100 mM phosphate buffer, pH 7.4 as eluent. BSA and OVA conjugates were stored frozen at −20 °C, and HRP conjugates were kept at 4 °C. The obtained bioconjugates were characterized by MALDI-TOF–MS in order to determine the hapten-to-protein molar ratio (MR).

Monoclonal antibody generation

Two groups of four mice were immunized by intraperitoneal injections with 100 μg of BSA–FX*n* or BSA–FX*b* conjugate in an oil-to-water emulsion between PBS (10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl) and Freund's adjuvant. The immunization process was verified by characterization of mouse antisera collected 10 days after the third injection. Monoclonals were generated by standard hybridoma technology.[16] Briefly, lymphocytes from two immunized mice were fused to myeloma cells with polyethylene glycol and the obtained hybridoma cells were grown in selective medium. The screening of cell cultures for antibody-producing hybridomas was carried out by a double assay process using the indirect cELISA format with homologous conjugate-coated plates, as previously published.[17] The selected

cells were cloned by limiting dilution, and they were stabilized and expanded, in culture plates, by successive divisions in high-glucose Dulbecco's modified Eagle's medium containing hypoxanthine and thymidine as well as hybridoma fusion and cloning supplement. Antibodies were purified by Protein G affinity chromatography from cell culture supernatants and they were stored as ammonium sulphate precipitates at 4 °C. More information can be found in the ESI‡.

Antibody-coated direct competitive ELISA

Microplates were coated by overnight incubation with 100 µL per well of antibody solution in 50 mM carbonate–bicarbonate buffer, pH 9.6 (coating buffer), at 4 °C. Plates were washed four times with a 150 mM NaCl solution in water (washing solution). The competitive reaction was carried out with 50 µL per well of fluxapyroxad solution in PBS or MilliQ water and 50 µL per well of HRP tracer solution in PBS-T (PBS containing 0.05% (v/v) Tween 20) or 2xPBS-T (20 mM phosphate buffer, pH 7.4, containing 280 mM NaCl and 0.05% (v/v) Tween 20) by incubation at room temperature during 1 h. After washing the plates as before, the retained peroxidase activity was revealed by adding 100 µL per well of a 2 mg mL^{-1} *o*-phenylendiamine solution in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4, containing 0.012% (v/v) H_2O_2, and it was stopped after 10 min at room temperature, with 100 µL per well of H_2SO_4 1 M. The absorbance was read at 492 nm with a reference wavelength of 650 nm.

Conjugate-coated indirect competitive ELISA

Plates were coated by overnight incubation at room temperature with 100 µL per well of OVA conjugate solution in coating buffer. Microwells were washed four times, after each incubation step, with washing solution. The competitive immunochemical reaction was performed by mixing 50 µL per well of fluxapyroxad solution in PBS or MilliQ water and 50 µL per well of antibody solution in PBS-T or 2xPBS-T, and incubation for 1 h at room temperature. The retained mAb was indirectly detect with 100 µL per well of a HRP-labelled secondary antibody dilution (1/2000) in PBS-T and incubation at room temperature during 1 h. Finally, colour signal was obtained and the absorbance was read as described for the previous assay format.

Data analysis

Fluxapyroxad standard curves were prepared by five-fold serial dilutions in PBS or MilliQ water. Absorbance values were fitted to a four-parameter logistic equation using the SigmaPlot software (Chicago, IL). A_{max} is the absorbance that was obtained without analyte. The fluxapyroxad concentration affording a 50% reduction (IC_{50}) of the A_{max} was considered as the reference value for comparison of antibody affinity and immunoassay sensitivity. The LOD was defined as the fluxapyroxad concentration that reduced the A_{max} by 10% (IC_{10}). The lowest fluxapyroxad concentration, experimentally obtained, that provided accurate and precise results in fortified samples was established as the LOQ of the immunoassay.

Sample preparation and extraction

Plum trees and grapevines of four varieties (Bobal, Garnacha, Macabeo, and Tempranillo) from the Utiel-Requena region of Spain were manually sprayed, before harvest, with a technical mixture containing fluxapyroxad (approximately 30%). Suspensions in water were prepared following the manufacturer's instructions (treatment T1) or at a double concentration (treatment T2). Plums and grapes were harvested before the treatment for blank samples and for fortification experiments. After the treatments, two plum samples (S1 and S2) were collected at days D1, D3, D5, and D7, and one grape sample at days D1 and D3. The stones of plums and the stems of grapes were discarded, and then the fruits were homogenized with an Ultra-Turrax blender from IKA (Staufen, Germany).

Fluxapyroxad residues were extracted from fruit samples by the QuEChERS method.[18] Briefly, 5 g of homogenized fruit samples were weighted in a 50-mL polypropylene tube and mixed by vigorous stirring with 0.5 g of sodium acetate, 2 g of anhydrous $MgSO_4$, and 5 mL of acetonitrile containing 1% (v/v) acetic acid and, as internal standard, 500 µg L^{-1} of triphenylphosphate. Next, the tubes were centrifuged at 2000×g during 5 min and the organic phase was collected and added over 50 mg of primary/secondary amine and 150 mg of anhydrous $MgSO_4$ in an Eppendorf tube. The mixture was vigorously mixed for 1 min and centrifuged at 6700×g during 5 min. Then, the cleaned-up organic phase was filtered with a 0.2 µm PTFE filter device (Fisher Scientific, Madrid, Spain) and stored at −20 °C.

UPLC–MS/MS analysis

Determinations were carried out with a multicomponent calibration curve of 7 standards (0, 3, 10, 30, 100, 300, and 1000 µg L^{-1}) prepared by serial dilution of fluxapyroxad in acetonitrile containing 500 µg L^{-1} of triphenylphosphate as internal standard. A five microliter sample was used and a binary mobile phase was applied at 400 µL min^{-1}, consisting of 0.5% (v/v) formic acid in MilliQ water (eluent A) and acetonitrile (eluent B). Starting from a 50% (v/v) mixture of both eluents, elution was carried out by linearly increasing eluent B, during 4 min, until a 95% (v/v) proportion was reached, and then the mobile phase was maintained isocratic during 2 min. The obtained retention times under the aforementioned conditions were 1.1 and 2.1 min for fluxapyroxad and triphenylphosphate, respectively. Signal response was determined from the quotient between the analyte peak area and that of the internal standard multiplied by the concentration of the latter. The retention times were 1.36 and 2.35 min, and the monitored ions were m/z 382 and 328, for fluxapyroxad and triphenylphosphate, respectively. Weighted (1/x) least squares calibration curves were established by linear

regression of the signal and the concentration values of fluxapyroxad.

Results and discussion

Hapten preparation and conjugation

In order to achieve the objectives of this study, two novel functionalized haptens that mimic fluxapyroxad were prepared – named haptens FX*b* and FX*h* (Fig. 1). The former incorporated a linear carboxylated spacer arm at the C-5 position of the biphenyl ring system for its conjugation to the carrier proteins. In the most stable conformation, the fluxapyroxad molecular skeleton adopts an extended conformation with a nearly coplanar disposition between the pyrazole-4-carboxamido and the proximal phenyl group of the biphenyl moiety. The incorporation of such a type of linker at this position caused a minimal modification of the electronic and conformational characteristics of the *N*-([1,1'-biphenyl]-2-yl)-1*H*-pyrazole-4-carboxamide skeleton in relation to the parent analyte. It was expected that this hapten would adequately mimic the molecule of fluxapyroxad during the immunization process, thus leading to the generation of antibodies with high affinity and specificity for this analyte. The synthesis of hapten FX*b* started from fluxapyroxad and it was based on the incorporation of a C-6 hydrocarbon chain at the required position of the biphenyl ring through a Sonogashira-type cross-coupling reaction (Fig. 2). In order to accomplish this goal, the biphenyl ring was initially functionalized via an aromatic electrophilic iodination reaction of the more reactive C-5 position to obtain the iodine derivative **1**, which was then cross-coupled with *tert*-butyl hex-5-ynoate using the conventional Pd/Cu catalytic system. Catalytic hydrogenation of the triple bond of the coupled-alkyne product **2**, followed by mild acid removal of the *tert*-butyl ester moiety to a carboxylic acid group, completed the synthesis of hapten FX*b*. The overall route proved to be highly efficient, affording the target FX*b* hapten from fluxapyroxad in four synthetic steps and nearly 60% overall yield.

The second synthetized hapten (FX*h*) was a functional heterologous molecule of hapten FX*b*, in which the difluoromethyl group (CHF_2) at the C-3 position of the pyrazole ring was replaced by a hydroxymethyl group (CH_2OH) (Fig. 1). This apparently simple functional exchange represents, given the different electronic nature of the groups involved, an important modification not only at the directly modified position but also at the distal pyrazole-4-carboxamido moiety. We expected that anti-fluxapyroxad antibodies would recognize FX*h*-based bioconjugates worse than FX*b*-based bioconjugates, thus increasing their apparent affinity to fluxapyroxad. The synthesis of hapten FX*h* was also initiated from fluxapyroxad and it involved an initial transformation of the difluoromethyl group to a dibromomethyl group (Fig. S1‡) which, under the iodination conditions that were used for the functionalization of the biphenyl ring and the subsequent aqueous workup, underwent hydrolysis to a formyl group to give iodo-aldehyde **5**. The rest of the steps that were used to complete the synthesis of hapten FX*h* were similar to those employed in the synthesis of hapten FX*b*. The synthesis of hapten FX*h* from fluxapyroxad was carried out in five synthetic steps that took place in 19% overall yield.

BSA was chosen to prepare the immunizing conjugate, and OVA and HRP were employed for assay conjugate preparation. A novel BSA conjugate was prepared with hapten FX*b*, whereas assay conjugates were obtained with haptens FX*b* and FX*h*. The achieved hapten-to-protein MR of each conjugate, determined by MALDI-TOF–MS, was 15.8 for the BSA–FX*b* conjugate, and those of OVA and HRP conjugates were 4.6 and 1.4 for hapten FX*b*, and 5.5 and 1.6 for hapten FX*h*, respectively (Fig. S2‡). These results mean that, for the BSA conjugate, about half of the 32 available lysine residues[19] had been modified by a hapten molecule, with a coupling yield of 67%. In the case of OVA and HRP conjugates, lower MR values were achieved, as preferred for assay conjugates.

Monoclonal antibody characterization

Table 1 Checkerboard assay with fluxapyroxad mAbs using the antibody-coated direct cELISA

	HRP–FX*n*				HRP–FX*b*				HRP–FX*h*			
mAb	[mAb][a]	[T][b]	A_{max}	IC_{50}[c]	[mAb]	[T]	A_{max}	IC_{50}	[mAb]	[T]	A_{max}	IC_{50}
FX*n*#11	1000	30	1.08	0.4	1000	300	---[d]		1000	300	---	
FX*n*#18	1000	100	1.43	1.1	1000	300	---		1000	300	---	
FX*n*#111	1000	30	1.55	1.3	1000	300	---		1000	300	---	
FX*n*#218	1000	10	1.23	3.8	1000	300	---		1000	300	---	
FX*n*#222	1000	100	1.07	1.0	1000	300	---		1000	300	---	
FX*n*#226	1000	30	1.55	1.0	1000	300	---		1000	300	---	
FX*n*#233	1000	30	1.01	0.6	1000	300	---		1000	300	---	
FX*n*#313	1000	10	1.11	0.6	1000	300	---		1000	300	---	
FX*n*#356	1000	300	---		1000	300	---		1000	300	---	
FX*n*#362	1000	10	0.95	0.7	1000	30	1.16	0.4	1000	300	---	
FX*n*#368	1000	10	1.13	1.9	1000	300	---		1000	300	---	
FX*b*#21	1000	300	---		1000	30	1.07	7.9	1000	300	---	
FX*b*#113	1000	300	---		1000	300	0.83	47.0	1000	300	---	
FX*b*#115	1000	300	---		1000	10	1.07	17.5	1000	30	1.96	8.3
FX*b*#119	1000	300	---		1000	30	1.11	2.4	1000	300	---	
FX*b*#120	1000	300	---		1000	30	2.28	6.5	1000	300	---	

[a] Antibody concentration ng mL^{-1}. [b] Tracer concentration in ng mL^{-1}. [c] Values are expressed in nM units. [d] Signal was lower than 0.8.

Table 2 Checkerboard assay with fluxapyroxad mAbs using the conjugate-coated indirect cELISA

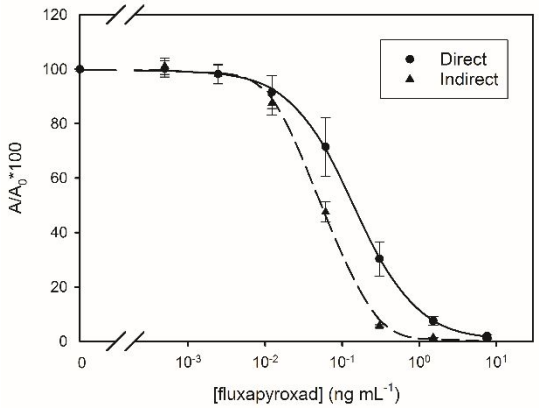
	OVA–FX*n*				OVA–FX*b*				OVA–FX*h*			
mAb	[mAb][a]	[C][b]	A_{max}	IC_{50}[c]	[mAb]	[C]	A_{max}	IC_{50}	[mAb]	[C]	A_{max}	IC_{50}
FX*n*#11	30	100	0.87	0.7	300	1000	---[d]		300	1000	---	
FX*n*#18	100	100	0.82	2.0	300	1000	---		300	1000	---	
FX*n*#111	100	100	1.59	1.3	300	1000	---		300	1000	---	
FX*n*#218	30	100	0.99	2.6	300	1000	---		300	1000	---	
FX*n*#222	100	100	1.26	1.3	300	1000	---		300	1000	---	
FX*n*#226	30	100	0.93	2.1	300	1000	---		300	1000	---	
FX*n*#233	30	100	0.90	0.6	300	1000	---		300	1000	---	
FX*n*#313	30	100	0.85	1.1	300	1000	---		300	1000	---	
FX*n*#356	100	100	1.01	0.3	300	100	1.35	0.8	100	1000	1.69	0.2
FX*n*#362	30	100	1.06	0.6	30	100	0.88	0.2	100	1000	2.93	0.5
FX*n*#368	100	100	1.58	0.4	300	1000	---		300	1000	---	
FX*b*#21	300	1000	---		30	100	1.31	20.6	100	1000	0.86	2.6
FX*b*#113	300	1000	---		300	100	1.93	30.9	300	1000	0.95	3.0
FX*b*#115	300	1000	---		30	100	1.11	38.1	30	1000	0.85	13.8
FX*b*#119	300	1000	---		30	100	1.22	7.6	100	1000	0.90	1.1
FX*b*#120	300	1000	---		30	100	1.25	4.9	300	1000	0.81	2.3

[a] Antibody concentration ng mL^{-1}. [b] Coating-conjugate concentration in ng mL^{-1}. [c] Values are expressed in nM units. [d] Signal was lower than 0.8.

In previous studies, hapten FX*n* afforded excellent polyclonal antibodies to fluxapyroxad.[14] Therefore, in the present study this hapten was also employed, in parallel with the novel hapten FX*b*, for the generation of mAbs. A collection of 11 and 5 mAbs was obtained from hapten FX*n* and hapten FX*b*, respectively, which were named FX*n*-type or FX*b*-type, after the corresponding immunizing hapten. Firstly, specificity of all of the antibodies was assessed by indirect cELISA using the homologous coating conjugate (same hapten as that of the immunizing conjugate) and analyte standard curves prepared in PBS starting at 10 µM. Under these conditions, none of the mAbs recognized other fungicides potentially present in fruit samples, such as azoxystrobin, picoxystrobin, kresoxim-methyl, pyraclostrobin, fenhexamid, fluopicolide, pyrimethanil, and cyprodinil. Other SDH fungicides, like penthiopyrad and fluopyram, were slightly bound by some antibodies (Table S1‡). However, boscalid was unexpectedly recognized by several mAbs, independently of the hapten from which they come from. In fact, antibodies FX*b*#119 and FX*b*#120 bound boscalid even better than fluxapyroxad. Although boscalid and fluxapyroxad share a three aromatic-ring system and an amide bridge, this finding was actually surprising, and we do not have a definite explanation for the unusual binding behaviour of these two antibodies. Nevertheless, several mAbs, mainly those obtained from hapten FX*n*, showed very low or no cross-reactivity with other compounds, particularly pyraclostrobin, a fungicide often mixed with fluxapyroxad in registered formulations, so they were deemed good candidates for the development of selective immunochemical tests.

All of the antibodies were evaluated by checkerboard cELISA using homologous and heterologous (the hapten was different to that used in the immunizing conjugate) conjugates in the antibody-coated direct format and the conjugate-coated indirect format. Fluxapyroxad standard samples were prepared in PBS. Overall, high-affinity mAbs to fluxapyroxad were generated, with five mAbs from hapten FX*n* (FX*n*#11, FX*n*#233, FX*n*#356, FX*n*#362, and FX*n*#368) exhibiting IC_{50} values below 1 nM in either or both formats (Tables 1 and 2), and some of them showing IC_{50} values lower than those previously reported with polyclonal antibodies.[14] With regard to the direct competitive assay format, heterologous enzyme tracers were not recognized by most of the mAbs independently of the linker tethering site, as commonly occurs with mAbs,[20-22] so the lowest IC_{50} values were observed with the homologous enzyme tracers (Table 1). Concerning the indirect format, haptens with the linker at an opposite site were not generally recognized, i.e., the OVA conjugates of haptens FX*b* and FX*h* were not bound by most of the FX*n*-type antibodies, and the OVA conjugate of hapten FX*n* was not recognized by FX*b*-type antibodies (Table 2). The only exceptions to this behaviour were two FX*n*-type mAbs (FX*n*#356 and FX*n*#362), which did bind the heterologous conjugate OVA–FX*b*, one of them experiencing a three-fold improvement in its apparent affinity to fluxapyroxad. On the other hand, all of the FX*b*-type mAbs were able to bind the heterologous OVA–FX*h* conjugate. Under these conditions, affinity improvements of around ten times were observed (mAbs FX*b*#21 and FX*b*#113). As with other mAbs, linker-site heterologous haptens have demonstrated to be an excellent approach in order to enhance assay sensitivity in indirect assays.[23-25] In summary, the antibodies obtained with hapten FX*n* showed superior affinity and specificity to fluxapyroxad than those derived from hapten FX*b*, thus evidencing the relevance of the linker position in the binding properties of the generated antibodies, as also demonstrated for other low molecular weight compounds.[26-28]

Table 3 Standard curve parameters of the selected immunoassays in two different formats (n = 4)



	Direct assay	Indirect assay
mAb	FX*n*#11	FX*n*#356
	1000 ng mL^{-1}	100 ng mL^{-1}
Conjugate	HRP–FX*n*	OVA–FX*h*
	30 ng mL^{-1}	1000 ng mL^{-1}
Buffer	PBS-T	PBS-T
A_{max}	1.99 ± 0.34	1.28 ± 0.07
IC_{50} (ng mL^{-1})	0.14 ± 0.04	0.05 ± 0.01
Slope	1.09 ± 0.11	1.42 ± 0.14
A_{min}	0.010 ± 0.020	−0.002 ± 0.005
LOD (ng mL^{-1})	0.020 ± 0.009	0.012 ± 0.003
Dynamic range (ng mL^{-1})		
(IC_{20}–IC_{90})	0.038–1.147	0.022–0.257
Inter-day precision		
A_{max} (%)	17.0	5.7
IC_{50} (%)	29.9	8.9
Intra-day precision		
A_{max} (%)	4.95	6.34
IC_{50} (%)	6.29	8.79

For further cELISA development, two combinations of immunoreagents were selected according to the lowest IC_{50} value and moderate slope of the inhibition curve, one for the direct format and the other for the indirect format. The optimum concentration of each immunoreagent was determined in order to obtain A_{max} values between 1 and 2 absorbance units. The background signal was always near zero in both assays. The normalized standard curves of the selected immunoassays are shown in Table 3. For the direct assay, mAb FX*n*#11 was chosen in combination with the homologous enzyme tracer. For the indirect format, mAb FX*n*#356 together with the heterologous coating conjugate OVA–FX*h* were selected. In this case, a hapten with a combination of two heterologies (linker site and moiety modifications) was shown to be the best approach. These immunoassays displayed high sensitivity to fluxapyroxad, with LOD values in the low nanogram per litre range, and they are comparable or better than the previously reported immunochemical methods for the analysis of this fungicide.[14] In inter-day precision studies, the direct assay showed higher variability than the indirect assay, which was highly precise both with inter and intra-day determinations.

Immunoassay characterization

The influence of pH and ionic strength over the A_{max} and IC_{50} values of the selected cELISAs was studied (Fig. S3‡). The assay parameters under each of the studied conditions were compared to those obtained under standard conditions (pH 7.4 and 140 mM NaCl concentration). Fluxapyroxad samples were prepared in MilliQ water. We observed that the direct assay was sensitive to pH variations whereas the indirect assay was shown to be highly stable upon pH changes between 5 and 9. Concerning the ionic strength, both immunoassays were quite robust to lower and to higher NaCl concentrations (from 25 to 250 mM). Additionally, the tolerance to methanol, ethanol, acetonitrile, and DMF was evaluated with the two assays (Fig. S4‡). The direct immunoassay was shown to be tolerant to methanol and ethanol up to 10% (v/v), whereas acetonitrile and DMF were less tolerated. On the other hand, the indirect assay was particularly tolerant to these four solvents – the assay parameters remained very stable in the studied concentration range.

Analysis of food samples

Immunoassay performance was evaluated in plums and four varieties of wine grapes (Bobal, Macabeo, Garnacha, and Tempranillo). Samples were homogenized and extracted by the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method, and the acetonitrile extracts were analysed by the two developed cELISAs. Fluxapyroxad-fortified extracts were diluted in MilliQ water before the analysis. Determination of fluxapyroxad by the optimized immunoassays provided excellent recovery values, with coefficients of variation (CV) mostly below 20% (Table 4). This study revealed that the LOQ of fluxapyroxad with the direct assay was 30 ng mL^{-1} for the studied food samples. The experimental LOQ value with the indirect immunoassay was 30 ng mL^{-1} for plums and even lower (10 ng mL^{-1}) for grapes. These low LOQ values are well below the US and European MRLs for fluxapyroxad in grapes (2000 and 3000 ng mL^{-1}, respectively) and plums (3000 and 1500 ng mL^{-1}, respectively).

Fruit samples – including plums and the previous four varieties of grapes – from cultivars that had been treated with a commercial formulation containing fluxapyroxad were collected and the fungicide residues were extracted. Then, extracts were analysed by the developed indirect cELISA – due to its superior performance – and by UPLC–MS/MS as a reference chromatographic technique. The comparison of the analytical results afforded a good correlation between both data sets (r^2 = 0.976; Table S2‡). The regression line had an intercept value of −11.33 and a slope of 1.08 (Fig. 3). Thus, the developed indirect cELISA is an excellent strategy for fluxapyroxad residue monitoring in plums and grapes.

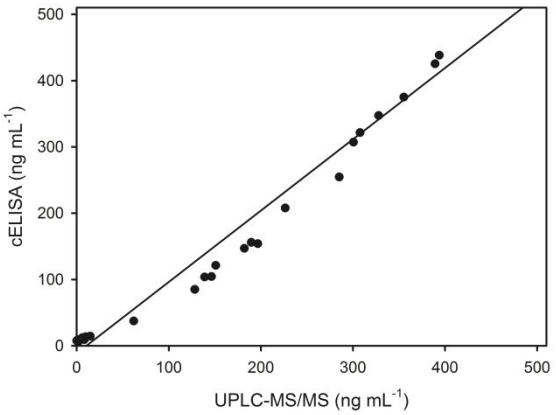


Fig. 3. Regression analysis of results obtained by the developed indirect cELISA and a reference chromatographic method.

Conclusions

High-affinity and specific mAbs to fluxapyroxad have been generated for the first time. The linker position of the immunizing haptens was shown to determine the affinity and specificity of the generated antibodies. These antibodies were evaluated by cELISA using homologous and two types of heterologous conjugates. Competitive immunoassays were developed by alternative direct and indirect procedures. The antibody-coated direct assay showed low binding to heterologous tracers, as usually occurs with mAbs. Concerning the indirect cELISA format, linker-site heterologous haptens were scarcely recognized whereas modification of the molecular structure at a distal site of the molecule was shown to be more appropriate. Finally, the best results, in this format, were achieved with a combination of both types of heterologies. The conjugate-coated indirect assay was validated by determining fluxapyroxad residues extracted from in-field treated fruit samples, showing good correlation with UPLC–MS/MS.

Conflicts of interest

There are no conflicts to declare.

Table 4 Recovery values (%) from plum and grape samples fortified with fluxapyroxad (n = 4)

		Direct				Indirect			
		1/50		1/250		1/250		1/500	
Sample	Fortified (ng/mL)	Rec.[a] (%)	CV (%)	Rec. (%)	CV (%)	Rec. (%)	CV (%)	Rec. (%)	CV (%)
Plums	500	-[b]	-	101	7	105	15	115	14
	300	-	-	104	9	113	7	117	12
	100	86	5	115	9	108	9	83	8
	50	82	13	-	-	94	17	86	15
	30	82	21	-	-	104	14	107	22
	10	-	-	-	-	-	-	-	-
Grapes var. Bobal	500	-	-	95	12	114	13	120	13
	300	-	-	102	14	114	8	105	9
	100	85	9	99	14	114	6	86	12
	50	89	10	97	17	88	14	81	19
	30	81	13	-	-	84	13	92	20
	10	83	11	-	-	98	9	-	-
Grapes var. Macabeo	500	-	-	106	13	104	14	117	7
	300	81	7	103	16	115	6	107	6
	100	95	13	-	-	109	7	83	9
	50	93	11	-	-	89	13	85	12
	30	95	19	-	-	87	15	98	13
	10	-	-	-	-	100	5	-	-
Grapes var. Garnacha	500	-	-	113	21	110	8	120	11
	300	84	19	-	-	117	8	117	9
	100	96	16	-	-	115	10	85	7
	50	94	16	-	-	88	7	83	6
	30	93	22	-	-	88	14	98	12
	10	-	-	-	-	118	6	-	-
Grapes var. Tempranillo	500	-	-	113	16	104	8	116	6
	300	-	-	103	11	119	6	120	14
	100	89	18	-	-	121	6	89	9
	50	85	18	-	-	89	13	85	12
	30	99	14	-	-	89	8	101	13
	10	-	-	-	-	92	15	-	-

[a] Recovery values. [b] Out of range.

Acknowledgements

This work was supported by the Spanish *Ministerio de Economía y Competitividad* (AGL2012-39965-C02 and AGL2015-64488-C2) and cofinanced by European Regional Development Funds. E.C.-A. was recipient of a predoctoral fellowship from the "Atracció de Talent, VLC-CAMPUS" program of the University of Valencia. D.L.-P. was supported by a FPU predoctoral grant (FPU14/05266) from the *Ministerio de Educación, Cultura y Deporte*, Spain. Conjugate analysis was carried out at the Proteomics Section which belongs to ProteoRed, PRB2-3, and was supported by grant PT17/0019, of the PE I+D+i 2013–2016, and funded by ISCIII and ERDF, and animal manipulation was performed at the Animal Production Section, both services belonging to the SCSIE of the University of Valencia.

We acknowledge the excellent technical assistance of Paula Peña-Murgui and Javier Marzo. Limited amounts of the monoclonal antibodies and bioconjugates reported herein are available upon request for evaluation purposes.

Notes and references

1 H. F. Avenot and T. J. Michailides, Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi, *Crop Prot.*, 2010, **29**, 643–651.

2 M.-J. Hu, C.-X. Luo, A. Grabke and G. Schnabel, Selection of a suitable medium to determine sensitivity of *Monilia fructicola* mycelium to SDHI fungicides, *J. Phytopathol.*, 2011, **159**, 616–620.

3 S. Strathmann, S. Walker and J. Barnes, Fluxapyroxad: A new broad-spectrum fungicide, *Phytopathology*, 2011, **101**, S172.

4 Y.-H. Ye, L. Ma, Z.-C. Dai, Y. Xiao, Y.-Y. Zhang, D.-D. Li, J.-X. Wang and H.-L. Zhu, Synthesis and antifungal activity of nicotinamide derivatives as succinate dehydrogenase inhibitors, *J. Agric. Food Chem.*, 2014, **62**, 4063–4071.

5 EFSA Scientific Report. The 2013 European Union report on pesticide residues in food, *EFSA J.,* 2015, **13**(3), 4038.

6 USDA, Pesticide Data Program (2014). Annual Summary, Calendar Year 2013, https://www.ams.usda.gov/datasets/pdp, (accessed 11 September 2018).

7 Pesticide residues in food 2012. FAO Plant Production and Protection Paper 215. Joint FAO/WHO meeting on pesticide residues, Rome, WHO/FAO, 2013, www.fao.org/docrep/017/i3111e/i3111e.pdf, (accessed 11 September 2018).

8 EPA Pesticide Fact Sheet. Fluxapyroxad (2012), https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-138009_02-May-12.pdf, (accessed 11 September 2018).

9 A. Abad-Fuentes, E. Ceballos-Alcantarilla, J. V. Mercader, C. Agulló, A. Abad-Somovilla, and F. A. Esteve-Turrillas, Determination of succinate-dehydrogenase-inhibitor fungicide residues in fruits and vegetables by liquid chromatography–tandem mass spectrometry, *Anal. Bioanal. Chem.*, 2015, **407**, 4207–4211.

10 H. Yang, W. H. Sun, Z. Y. Cao, Y. N. Ma, S. S. Chai, M. L. Qin and M. X. Chen, Determination of eight novel succinate dehydrogenase inhibitor fungicides in vegetables and fruits by modified QuEChERS, *Chin. J. Chromatogr.*, 2016, **34**(11), 1070–1076.

11 X. Chen, F. Dong, J. Xu, X. Liu, X. Wu and Y. Zheng, Effective monitoring of fluxapyroxad and its three biologically active metabolites in vegetables, fruits, and cereals by optimized QuEChERS treatment based on UPLC-MS/MS, *J. Agric. Food Chem.*, 2016, **64**, 8935−8943.

12 J. Gan, L. Lv, J. Peng, J. Li, Z. Xiong, D. Chen and L. He, Multi-residue method for the determination of organofluorine pesticides in fish tissue by liquid chromatography triple quadrupole tandem mass spectrometry, *Food Chem.*, 2016, **207**, 195–204.

13 Y. Shen, Z. Li, Q. Ma, C. Wang, X. Chen, Q. Miao and C. Han, Determination of six pyrazole fungicides in grape wine by solid-phase extraction and gas chromatography−tandem mass spectrometry, *J. Agric. Food Chem.*, 2016, **64**, 3901−3907.

14 J. V. Mercader, A. Abad-Somovilla, C. Agulló and A. Abad-Fuentes, Fluxapyroxad haptens and antibodies for highly sensitive immunoanalysis of food samples, *J. Agric. Food Chem.*, 2017, **65**, 9333–9341.

15 W. L. F. Armarego and D .D. Perrin, *Purification of laboratory chemicals*, Butterworth-Heinemann Ltd, Oxford, 1997

16 J. V. Mercader, C. Suárez-Pantaleón, C. Agulló, A. Abad-Somovilla and A. Abad-Fuentes, Hapten synthesis and monoclonal antibody-based immunoassay development for detection of the fungicide trifloxystrobin, *J. Agric. Food Chem.*, 2008, **56**(8), 2581–2588.

17 J. V. Mercader, C. Suárez-Pantaleón, C. Agulló, A. Abad-Somovilla and A. Abad-Fuentes, Production and characterization of monoclonal antibodies specific to the strobilurin pesticide pyraclostrobin, *J. Agric. Food Chem.*, 2008, **56**(17), 7682–7690.

18 M. Anastassiades, S. J. Lehotay, D. Stajnbaher and F. J. Schenck, Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce, *J. AOAC Int.*, 2003, **86**, 412–431.

19 G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, CA, 1996.

20 J. E. Butler, Solid supports in enzyme-linked immunosorbent assay and other solid-phase immunoassays, *Methods*, 2000, **22**, 4–23.

21 R. López-Moreno, J. V. Mercader, C. Agulló, A. Abad-Somovilla and A. Abad-Fuentes, Immunoassays for trifloxystrobin analysis. Part I. Rational design of regioisomeric haptens and production of monoclonal antibodies, *Food Chem.*, 2014, **152**, 230–236.

22 F. A. Esteve-Turrillas, J. V. Mercader, C. Agulló, J. Marzo, A. Abad-Somovilla, A. Abad-Fuentes, Design and development of heterologous competitive immunoassays for the determination of boscalid residues, *Analyst*, 2014, **139**, 3636–3644.

23 F. A. Esteve-Turrillas, A. Abad-Somovilla, G. Quiñones-Reyes, C. Agulló, J. V. Mercader, A. Abad-Fuentes, Monoclonal antibody-based immunoassays for cyprodinil residue analysis in QuEChERS-based extracts, *Food Chem.*, 2015, **187**, 530–536.

24 F. A. Esteve-Turrillas, J. V. Mercader, C. Agulló, A. Abad-Somovilla, A. Abad-Fuentes, Site-heterologous haptens and competitive monoclonal antibody-based immunoassays for pyrimethanil residue analysis in foodstuffs, *LWT−Food Sci. Technol.*, 2015, **63**, 604−611.

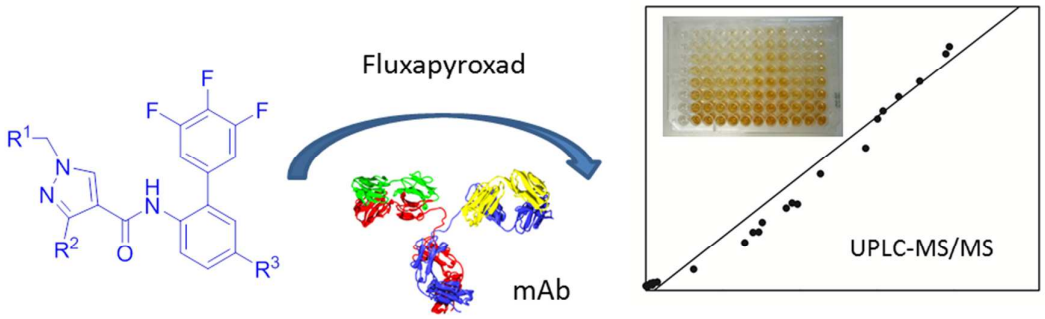
25 J. V. Mercader, J. Parra, F. A. Esteve-Turrillas, C. Agulló, A. Abad-Somovilla and A. Abad-Fuentes, Development of monoclonal antibody-based competitive immunoassays for the

detection of picoxystrobin in cereal and oilseed flours, *Food Control*, 2012, **26**, 162–168.

26 F. A. Esteve-Turrillas, J. V. Mercader, C. Agulló, A. Abad-Somovilla, A. Abad-Fuentes, Mepanipyrim haptens and antibodies with nanomolar affinity, *Analyst*, 2013, **138**, 3360–3364.

27 R. López-Moreno, J. V. Mercader, C. Agulló, A. Abad-Somovilla and A. Abad-Fuentes, Structure–immunogenicity relationship of kresoxim-methyl regioisomeric haptens, *Org. Biomolec. Chem.,* 2013, **11**, 7361–7371.

28 F. A. Esteve-Turrillas, C. Agulló, J. V. Mercader, A. Abad-Somovilla, A. Abad-Fuentes, Rationally designed haptens for highly sensitive monoclonal antibody-based immunoanalysis of fenhexamid, *Analyst*, 2018, **143**, 4057–4066.



Fluxapyroxad high-affinity antibody generation and sensitive immunoassay development from proper hapten design.

SUPPLEMENTARY INFORMATION

Combined heterologies for monoclonal antibody-based immunoanalysis of fluxapyroxad

Eric Ceballos-Alcantarilla, Daniel López-Puertollano, Consuelo Agulló, Antonio Abad-Fuentes, Antonio Abad-Somovilla, Josep V. Mercader

Contents **Page**

General experimental procedures, reagents, and instruments	2
Spectrometric data of intermediates of the synthesis of hapten FXb	3
Synthesis of hapten FX*h* (Figure S1)	6
Hapten activation: preparation of *N*-hydroxysuccinimidyl esters	10
MALDI mass spectrometry analysis of bioconjugates (Figure S2)	11
Antibody generation	12
Specificity of mAbs (Table S1)	13
Influence of pH and ionic strength (Figure S3)	14
Influence of organic solvents (Figure S4)	15
cELISa and UPLC–MS/MS results (Table S2)	16
^{1}H NMR spectra of haptens FX*b* and FX*h*	17

General experimental procedures, reagents, and instruments

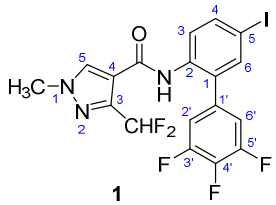
All operations involving air-sensitive reagents were performed under an inert atmosphere of dry nitrogen using syringe and cannula techniques, oven-dried glassware, and freshly distilled and dried solvents. The progress of reactions was monitored by thin layer chromatography (TLC) performed on F_{254} silica gel plates. The plates were visualized at 254 nm by immersion with aqueous ceric ammonium molybdate and heating. Column chromatography refers to flash chromatography and was performed on Merck silica gel 60, 230–400 mesh. All melting points were determined using a Kofler hot-stage apparatus and are uncorrected. IR spectra were recorded using a Nicolet Avatar 320 FT-IR spectrophotometer using liquid films or ATR for solids (IR band intensities: w = weak, m = medium, s = strong). ^{1}H NMR spectra were recorded on Bruker spectrometers, in the solvent indicated, at 300 MHz and ^{13}C NMR spectra at 75 MHz. ^{19}F NMR spectra were acquired at 282 MHz with high power proton decoupling. All proton and carbon spectra were referenced to residual solvent (^{1}H NMR: 7.26 ppm for $CDCl_3$ and 3.31 ppm for CD_3OD); ^{13}C NMR: 77.00 ppm for $CDCl_3$ and 49.00 ppm for CD_3OD). ^{19}F spectra were referenced to $CFCl_3$ as the internal reference which was set at δ 0.00 ppm. Carbon substitution degrees were established by DEPT pulse sequences. Abbreviations used for NMR signals are as follows: s =singlet, d = doublet, dd = double doublet, ddd = doublet of double doublet, t = triplet, dt = double triplet, ddt = double doublet of triplets, q = quadruplet, br = broad, quint = quintuplet, m = multiplet, BiPh = Biphenyl ring, Pz = Pyrazol ring. High-resolution mass spectra (HRMS) were run by the electrospray (ES) mode, which was obtained with a Q-TOF premier mass spectrometer with an electrospray source (Waters, Manchester, UK).

Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were utilized for protein–hapten conjugate purification. Hybridoma fusion and cloning supplement was obtained from Roche Applied Science (Mannheim, Germany). P3-X63-Ag 8.653 mouse plasmacytoma cell line was acquired from the European Collection of Cell Cultures (Salisbury, UK). Cell culture media (high-glucose Dulbecco's modified Eagle's medium), gentamicin solution, and hypoxanthine–thymidine and hypoxanthine–aminopterine–thymidine supplements were purchased from Gibco BRL (Paisley, UK). Poly(ethylene glycol) (PEG1500), fetal bovine serum, 200 mM alanyl–glutamine solution, red blood cell lysing buffer Hybri-Max, MEM non-essential amino acid solution, Freund's adjuvants, and *o*-phenylenediamine, and triphenylphosphate were obtained from Merck (Madrid, Spain). HiTrap protein G HP columns for mouse IgG purification were procured from General Electric Healthcare (Uppsala, Sweden). Rabbit anti-mouse immunoglobulin polyclonal antibody conjugated to peroxidase was from Dako (Glostrup, Denmark). Primary/secondary amine from Varian (Palo Alto, CA) and organic solvents from Scharlab (Barcelona, Spain) were used for sample preparation. Hapten density of protein conjugates was determined with a 5800 matrix-assisted laser desorption ionization time-

of-flight (MALDI-TOF/TOF) mass spectrometry apparatus from ABSciex (Framingham, MA). Costar flat-bottom high-binding 96-well polystyrene ELISA plates were from Corning (Corning, NY). ELISA absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments. Fluxapyroxad residues were determined by HPLC using a UPLC Acquity system from Waters (Milford, MA) furnished with a binary solvent delivery system, an autosampler, and a BEH C18 (1.7 µm, 2.1 × 50 mm) column also from Waters. An Acquity triple quadrupole MS detector, also from Waters, with a Z-spray electrospray ionization source (3.5 kV capillary voltage, and 120 °C and 300 °C source and desolvation temperature, respectively) were employed for tandem mass acquisitions.

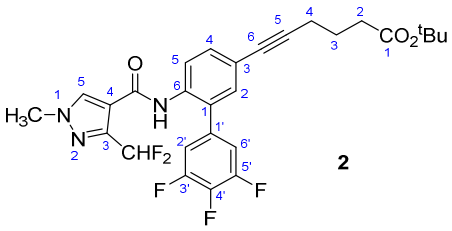
Synthesis of haptens

Spectrometric data of intermediates of the synthesis of hapten FX*b*



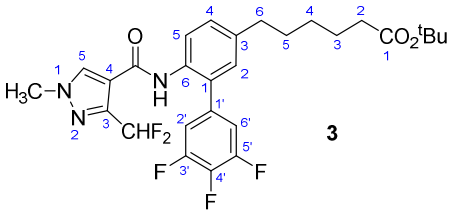
1

3-(Difluoromethyl)-1-methyl-N-(3',4',5'-trifluoro-5-iodo-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**1**). Mp 181.1-182.6 °C (from hexane-Et_2O); IR (neat) ν_{max} (cm^{-1}) 3416 (m), 3139 (w), 3063 (w), 1663 (s), 1542 (s), 1519 (s), 1393 (s), 1038 (s), 763 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.06 (1H, d, *J* = 8.7 Hz, H-3 BiPh), 7.97 (1H, s, H-5 Pz), 7.78 (1H, br s, NH), 7.72 (1H, dd, *J* = 8.7, 2.0 Hz, H-4 BiPh), 7.54 (1H, d, *J* = 2.0 Hz, H-6 BiPh), 6.97 (2H, m, H-2' and H-6' BiPh), 6.60 (1H, t, *J* = 54.2 Hz, CHF_2), 3.92 (3H, s, NMe); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 159.2 (CONH), 151.3 (ddd, *J* = 252.1, 10.0, 4.2 Hz, C-3' and C-5' BiPh), 142.0 (t, *J* = 29.6 Hz, C-3 Pz), 139.8 (dt, *J* = 253.6, 15.1 Hz, C-4' BiPh), 138.4 (C-4 BiPh), 138.1 (C-5 Pz), 136.6 (C-6 BiPh), 134.7 (C-2 BiPh), 132.6 (C-1 BiPh), 132.3 (td, *J* = 8.1, 5.0 Hz, C-1' BiPh), 124.6 (C-3 BiPh), 116.4 (C-4 Pz), 113.8 (dd, *J* = 14.6, 7.2 Hz, C-2' and C-6' BiPh), 111.7 (t, *J* = 232.7 Hz, CHF_2), 88.3 (C-5 BiPh), 39.5 (NMe); HRMS (TOF MS ES+) *m/z* calcd for $C_{18}H_{12}F_5IN_3O$ $[M+H]^+$ 507.9940, found 507.9933.



2

tert-Butyl 6-(6-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hex-5-ynoate (**2**). Mp 104-105.5 °C (from hexane-Et_2O); IR (neat) ν_{max} (cm^{-1}) 3421 (m), 3283 (s), 3124 (m), 2979 (s), 2233 (w), 1724 (s), 1660 (s) 1532 (s), 1149 (s), 1044 (s), 861 (m), 653 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.18 (1H, d, *J* = 8.6 Hz, H-5 BiPh), 7.93 (1H, s, H-5 Pz), 7.82 (1H, t, *J* = 4.0 Hz, NH), 7.42 (1H, dd, *J* = 8.5, 1.9 Hz, H-4 BiPh), 7.23 (1H, d, *J* = 1.9 Hz, H-2 BiPh), 6.97 (2H, m, H-2' and H-6' BiPh), 6.63 (1H, t, *J* = 54.2 Hz, CHF_2), 3.89 (3H, s, NMe), 2.45 (2H, t, *J* = 7.0 Hz, H-4), 2.38 (2H, t, *J* = 7.4 Hz, H-2), 1.87 (4H, quint, *J* = 7.3 Hz, H-3 and H-4), 1.44 (9H, s, CMe_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -109.4 (2F, s, CHF_2), -133.9 (2F, d, *J* = 20.5 Hz, F-3' and F-5' BiPh), -161.5 (1F, t, *J* = 20.5, Hz, F-4' BiPh); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 172.4 (CO_2), 159.2 (CONH), 151.4 (ddd, *J* = 251.6, 10.0, 4.0 Hz, C-3' and C-5' BiPh), 142.1 (t, *J* = 29.7 Hz, C-3 Pz), 139.6 (dt, *J* = 253.0, 15.1 Hz, C-4' BiPh), 136.2 (C-5 Pz), 134.0 (C-6 BiPh), 133.2 (td, *J* = 8.1, 4.9 Hz, C-1' BiPh), 133.0 (C-2 BiPh), 132.2 (C-4 BiPh), 130.6 (C-1 BiPh), 122.6 (C-5 BiPh), 120.5 (C-3 BiPh), 116.4 (C-4 Pz), 113.7 (dd, *J* = 14.6, 7.0 Hz, C-2' and C-6' BiPh), 111.5 (t, *J* = 232.4 Hz, CHF_2), 89.8 (C-5), 80.3 (CMe_3), 80.2 (C-6), 39.5 (NMe), 34.4 (C-2), 28.1 (CMe_3), 24.0 (C-3), 18.8 (C-4); HRMS (TOF MS ES+) *m/z* calcd for $C_{28}H_{27}F_5N_3O_3$ $[M+H]^+$ 548.1967, found 548.1943.



3

tert-Butyl 6-(6-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hexanoate (**3**). IR (neat) ν_{max} (cm^{-1}) 3432 (w), 3293 (m), 3119 (w), 2970 (w), 2930 (s), 1721 (s), 1634 (s), 1531 (s), 1368 (s), 1158 (s), 1044 (s), 856 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.02 (1H, d, *J* = 8.3 Hz, H-5 BiPh), 7.94 (1H, s, H-5 Pz), 7.76 (1H, t, *J* = 4.1 Hz, NH), 7.23 (1H, dd, *J* = 8.4, 2.1 Hz, H-4 BiPh), 7.02 (1H, d, *J* = 2.1 Hz, H-2 BiPh), 6.99 (2H, m, H-2' and H-6' BiPh), 6.65 (1H, t, *J* = 54.2 Hz, CHF_2), 3.91 (3H, s, NMe), 2.61 (2H, t, *J* = 7.5 Hz, H-6), 2.21 (2H, t, *J* = 7.4 Hz, H-2), 1.61 (4H, m, H-3 and H-5), 1.43 (9H, s, CMe_3), 1.36 (2H, m, H-4); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -109.4 (2F, s, CHF_2), -134.4 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -162.3 (1F, t, *J* = 20.6 Hz, F-4' BiPh); ^{13}C NMR (75

MHz, $CDCl_3$) δ (ppm) 173.1 (CO_2), 159.4 (CONH), 151.3 (ddd, *J* = 250.9, 9.7, 4.1 Hz, C-3' and C-5' BiPh), 142.3 (t, *J* = 29.1 Hz, C-3 Pz), 139.9 (C-6 BiPh), 139.4 (dt, *J* = 252.3, 15.1 Hz, C-4' BiPh), 136.0 (C-5 Pz), 134.3 (td, *J* = 7.9, 4.9 Hz, C-1' BiPh), 132.0 (C-3 BiPh), 131.4 (C-1 BiPh),129.8 (C-2 BiPh), 129.1 (C-4 BiPh), 123.7(C-5 BiPh), 116.6 (C-4 Pz), 113.6 (dd, *J* = 14.6, 6.9 Hz, C-2' and C-6' BiPh), 111.6 (t, *J* = 232.9 Hz, CHF_2), 80.0 (CMe_3), 39.5 (NMe), 35.4 (C-6), 35.1 (C-2), 31.0 (C-3), 28.6 (C-5), 28.1 (CMe_3), 24.84 (C-4); HRMS (TOF MS ES+) *m/z* calcd for $C_{28}H_{31}F_5N_3O_3$ $[M+H]^+$ 552.2280, found 552.2253.

Synthesis of hapten FX*h*

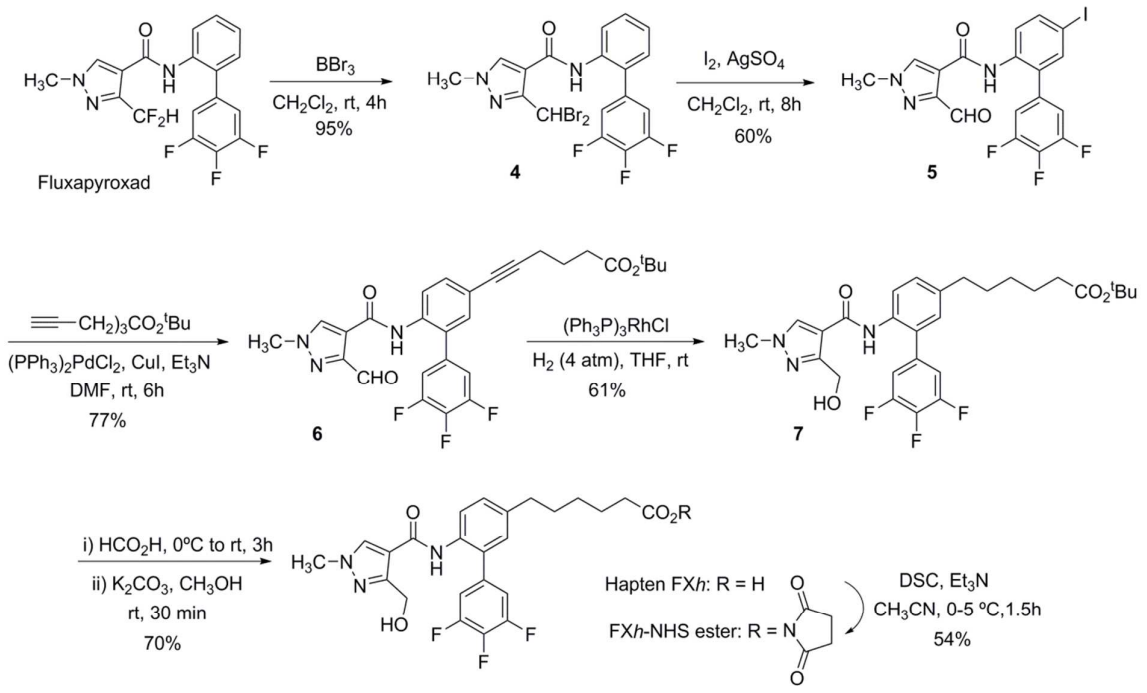
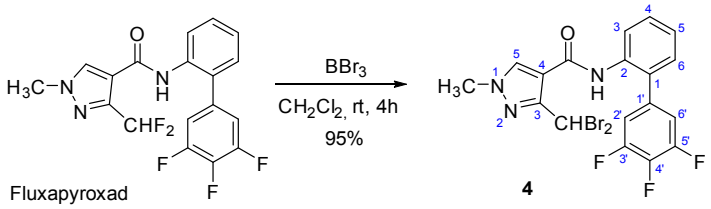
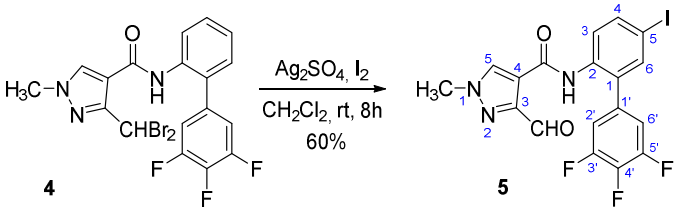


Figure S1. Schematic representation of the synthesis and activation of hapten FX*h*.



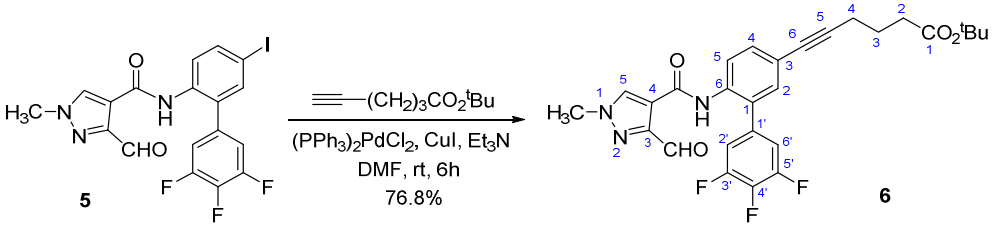
Synthesis of 3-(dibromomethyl)-1-methyl-N-(3',4',5'-trifluoro-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**4**). A 1M solution of BBr_3 in CH_2Cl_2 (3.15 mL, 3.15 mmol, 6 equiv) was dropwise added to a solution of fluoxapyroxad (200 mg, 0.525 mmol) in anhydrous CH_2Cl_2 (8 mL) at -78 °C under nitrogen. The reaction mixture was allowed to warm slowly to room temperature and was stirred for

4 h. The mixture was then cooled to 0 °C, then carefully quenched with water and extracted with CH_2Cl_2. The combined organic extracts were washed with brine, dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give dibromide **4** (251 mg, 95.1%) as a solid, which was deemed sufficiently pure to be used in the next step without any further purification. Mp 198.6-199.4 °C (crystals obtained from slow evaporation from a CH_2Cl_2 solution); IR (neat) ν_{max} (cm^{-1}) 3213 (s), 3117 (w), 3039 (w), 1640 (s), 1541 (s), 1532 (s), 1516 (s), 1492 (s), 1042 (s), 760 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.09 (1H, br d, *J* = 7.8 Hz, H-3 BiPh), 7.54 (1H, br s, NH), 7.52 (1H, s, H-5 Pz), 7.43 (1H, ddd, *J* = 8.1, 6.3, 2.9 Hz, H-4 BiPh), 7.29-7.23 (2H, m, H-5 and H-6 BiPh), 7.19 (1H, s, $CHBr_2$), 7.10-6.99 (2H, m, H-2' and H-6' BiPh), 3.93 (3H, s, NMe); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -133.2 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -161.1 (1F, t, *J* = 20.6 Hz, F-4' BiPh);^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 160.3 (CONH), 151.4 (ddd, *J* = 251.7, 10.0, 4.1 Hz, C-3' and C-5' BiPh), 151.3 (C-3 Pz), 139.5 (dt, *J* = 253.5, 15.1 Hz, C-4' BiPh), 134.4 (td, *J* = 7.8, 5.0 Hz, C-1' BiPh), 133.8 (C-2 BiPh), 131.7 (C-5 Pz), 131.1 (C-1 BiPh), 130.1 (C-6 BiPh), 129.4 (C-4 BiPh), 125.7 (C-5 BiPh), 123.9 (C-3 BiPh), 113.6 (C-4 Pz), 113.6 (dd, *J* = 14.5, 7.0 Hz, C-2' and C-6' BiPh), 39.9 (NMe), 30.1 ($CHBr_2$); HRMS (TOF MS ES+) *m/z* calcd for $C_{18}H_{13}{}^{79}Br_2F_3N_3O$ $[M+H]^+$ 501.9372, found 501.9377.

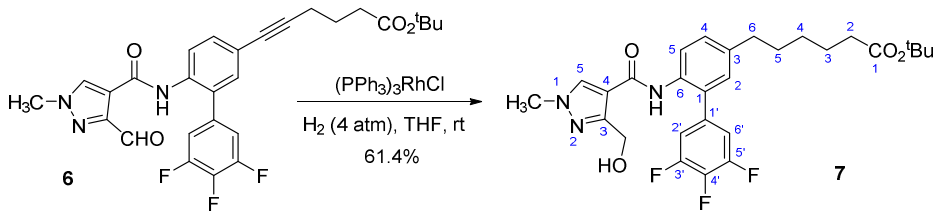


Synthesis of 3-formyl-1-methyl-N-(3',4',5'-trifluoro-5-iodo-[1,1'-biphenyl]-2-yl)-1H-pyrazole-4-carboxamide (**5**). Ag_2SO_4 (104.6 mg, 0.335 mmol, 1.5 equiv) was added in portion-wise to a stirred solution of dibromide **4** (112.6 mg, 0.224 mmol) and iodine (94.8 mg, 0.375 mmol, 1.7 equiv) in CH_2Cl_2 (1.5 mL). The reaction mixture was stirred in the dark at room temperature for 22 hours, diluted with CH_2Cl_2 and filtered through cotton wool plug to separate the yellow precipitate formed. The filtrate was washed with an aqueous solution of sodium bisulfite and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to give a residue that was chromatographed on silica gel to obtain pure iodo-aldehyde **5** (65.6 mg, 60.1%) as an amorphous solid. IR (neat) ν_{max} (cm^{-1}) 3251 (w), 3124 (w), 3070 (w), 2915 (w), 2851 (w), 1688 (s), 1654 (s), 1611 (m), 1585 (m), 1555 (m), 1533 (s), 1035 (s), 783 (m), 764 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 10.65 (1H, br s, NH), 9.69 (1H, d, *J* = 0.6, CHO), 8.15 (1H, s, H-5 Pz), 7.89 (1H, d, *J* = 8.7 Hz, H-3 BiPh), 7.71 (1H, dd, *J* = 8.7, 2.1 Hz, H-4 BiPh), 7.59 (1H, d, *J* = 2.1 Hz, H-6 BiPh), 7.02 (2H, m, H-2' and H-6' BiPh), 4.04 (3H, s, NMe); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -134.4 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -161.7 (1F, t, *J* = 20.6 Hz, F-4'

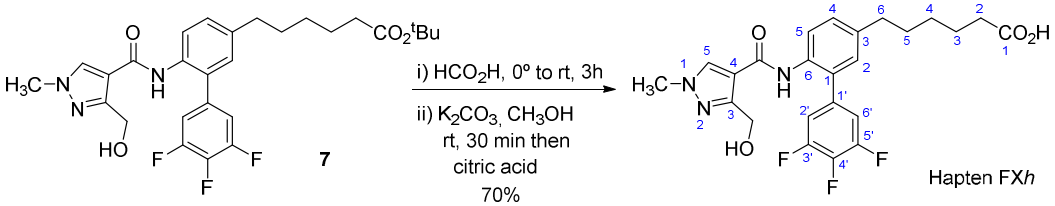
BiPh); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 188.5 (CHO), 159.0 (CONH), 151.2 (ddd, *J* = 250.3, 10.3, 4.3 Hz, C-3' and C-5' BiPh), 145.7 (C-3 Pz), 132.3 (td, *J* = 8.1, 5.0 Hz, C-1' BiPh), 126.2(C-3 BiPh), 113.8 (dd, *J* = 14.7, 6.9 Hz, C-2' and C-6' BiPh), 120.4 (C-4 Pz), 89.0(C-5 BiPh), 40.2 (NMe); HRMS (TOF MS ES+) *m/z* calcd for $C_{18}H_{12}F_3IN_3O_2$ $[M+H]^+$ 485.9921, found 485.9917.



Synthesis of tert-butyl 6-(3',4',5'-trifluoro-6-(3-formyl-1-methyl-1H-pyrazole-4-carboxamido)-[1,1'-biphenyl]-3-yl)hex-5-ynoate (**6**). Et_3N (0.150 mL) was added to a mixture of iodide **5** (59.6 mg, 0.123 mmol), CuI (1.6 mg, 0.008 mmol, 0.07 equiv), $(PPh_3)_2PdCl_2$ (2.6 mg, 0.004 mmol, 0.03 equiv) and *tert*-butyl hex-5-ynoate (25 mg, 0.148 mmol, 1.2 equiv) in dry DMF (0.150 mL) under nitrogen at room temperature. The reaction mixture was stirred at room temperature for 6 hours, quenched with water and extracted with EtOAc. The extracts were washed with an aqueous LiCl solution and brine and dried over anhydrous $MgSO_4$. Chromatographic purification, using hexane-EtOAc mixtures from 9:1 to 1:1 as eluent, afforded acetylenic compound **6** (49.7 mg, 76.8%) as an amorphous solid. IR (neat) ν_{max} (cm^{-1}) 3125 (m), 2977 (w), 2929 (w), 1732 (s), 1678 (s), 1655 (s), 1615 (m), 1589 (s), 1541 (s), 1301 (m), 1149 (s), 1041 (s), 189 (m); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 10.65 (1H, br s, NH), 9.69 (1H, d, *J* = 0.7 Hz, CHO), 8.15 (1H, d, *J* = 0.6 Hz, H-5 Pz), 8.07 (1H, d, *J* = 8.5 Hz, H-5 BiPh), 7.42 (1H, dd, *J* = 8.5, 2.0 Hz, H-4 BiPh), 7.29 (1H, d, *J* = 2.0 Hz, H-2 BiPh), 7.02 (2H, m, H-2' and H-6' BiPh), 4.03 (3H, s, NMe), 2.46 (2H, t, *J* = 7.0 Hz, H-4), 2.40 (2H, t, *J* = 7.3 Hz, H-2), 1.88 (4H, quint, *J* = 7.4 Hz, H-3 and H-4), 1.45 (9H, s, CMe_3); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -134.8 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -162.3 (1F, t, *J* = 20.6, Hz, F-4' BiPh); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 188.4 (CHO), 172.4 (CO_2), 158.9 (CONH), 151.1 (ddd, *J* = 251.1, 10.0, 4.4 Hz, C-3' and C-5' BiPh), 145.6 (C-3 Pz),138.1 (C-5 Pz), 139.5 (dt, *J* = 252.0, 15.2 Hz, C-4' BiPh), 134.3 (C-6 BiPh), 134.1 (td, *J* = 8.1, 5.0 Hz, C-1' BiPh), 133.1 (C-2 BiPh), 132.0 (C-4 BiPh), 131.8 (C-1 BiPh), 124.1 (C-5 BiPh), 120.7 (C-3 BiPh), 120.4 (C-4 Pz), 113.8 (dd, *J* = 14.7, 6.8 Hz, C-2' and C-6' BiPh), 89.7 (C-5), 80.4 (CMe_3), 80.3 (C-6), 40.1 (NMe), 34.4 (C-2), 28.1 (CMe_3), 24.0 (C-3), 18.8 (C-4); HRMS (TOF MS ES+) *m/z* calcd for $C_{28}H_{27}F_3N_3O_4$ $[M+H]^+$ 526.1948, found 526.1944.



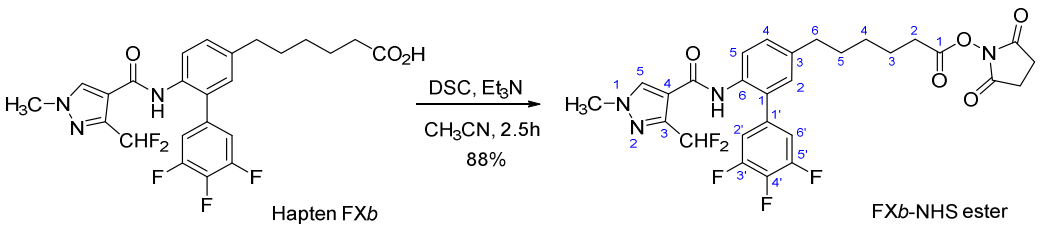
Synthesis of tert-butyl 6-(3',4',5'-trifluoro-6-(3-(hydroxymethyl)-1-methyl-1H-pyrazole-4-carboxamido)-[1,1'-biphenyl]-3-yl)hexanoate (**7**). A solution of alkyne **6** (45.1 mg, 0.086 mmol) and $(Ph_3P)_3RhCl$ (5 mg, 0.005 mmol, 0.06 equiv) in anhydrous THF (500 µL) was stirred under a hydrogen atmosphere of 60 psi at room temperature for 3 days. Chromatographic purification of the residue obtained after evaporation of the solvent, using hexane-EtOAc mixtures from 7:3 to 3:7 as eluent, gave, in order of elution, the aldehyde resulting from the reduction of only the triple bond (17 mg) followed by the product of reduction of both the carbonyl and triple bonds, compound **7** (27.9 mg, 61.4%). Mp 125.8-126.4 °C (from hexane-EtOAc-$CHCl_3$); IR (neat) ν_{max} (cm^{-1}) 3277 (br, m), 3237 (m), 3133 (m), 2923 (s), 2857 (m), 1734 (s), 1656 (s), 1615 (m), 1593 (m), 1547 (s), 1559 (s), 1425 (m), 1164 (s), 1042 (s); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.81 (1H, br s, NH), 7.86 (1H, d, *J* = 8.4 Hz, H-5 BiPh), 7.75 (1H, s, H-5 Pz), 7.22 (1H, dd, *J* = 8.4, 2.1 Hz, H-4 BiPh), 7.03 (3H, m, H-2, H-2' and H-6' BiPh), 4.56 (2H. d, *J* = 6.0 Hz, CH_2O), 3.82 (3H, s, NMe), 3.41 (1H, d, *J* = 6.0 Hz, OH), 2.61 (2H, t, *J* = 7.5 Hz, H-6), 2.20 (2H, t, *J* = 7.2 Hz, H-2), 1.68-1.56 (4H, m, H-3 and H-5), 1.43 (9H, s, CMe_3), 1.36 (2H, m, H-4); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) -134.4 (2F, d, *J* = 20.6 Hz, F-3' and F-5' BiPh), -162.2 (1F, t, *J* = 20.6 Hz, F-4' BiPh); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm) 173.3 (CO_2), 161.9 (CONH), 151.0 (ddd, *J* = 251.0, 10.0, 4.0 Hz, C-3' and C-5' BiPh), 150.4 (C-3 Pz), 140.2 (C-6 BiPh), 135.2 (td, *J* = 8.0, 5.0 Hz, C-1' BiPh), 134.5 (C-5 Pz), 132.3 (C-3 BiPh), 132.2 (C-1 BiPh), 130.0 (C-2 BiPh), 129.2 (C-4 BiPh), 125.0 (C-5 BiPh), 117.4 (C-4 Pz), 113.7 (dd, *J* = 14.8, 6.7 Hz, C-2' and C-6' BiPh), 80.2 (*C*Me_3), 58.1 (CH_2OH), 39.1 (NMe), 35.6 (C-6), 35.3 (C-2), 31.2 (C-3), 28.8 (C-5), 28.2 (C*Me*$_3$), 25.0 (C-4); HRMS (TOF MS ES+) *m/z* calcd for $C_{28}H_{33}F_3N_3O_4$ $[M+H]^+$ 532.2418, found 532.2409.



Synthesis of 6-(3',4',5'-trifluoro-6-(3-(hydroxymethyl)-1-methyl-1H-pyrazole-4-carboxamido)-[1,1'-biphenyl]-3-yl)hexanoic acid (Hapten FX*h*). A solution of *tert*-butyl ester **7** (46.5 mg, 0.087 mmol) in HCO_2H (1 mL) was stirred under anhydrous conditions at 0 °C for 1 hour and then at room

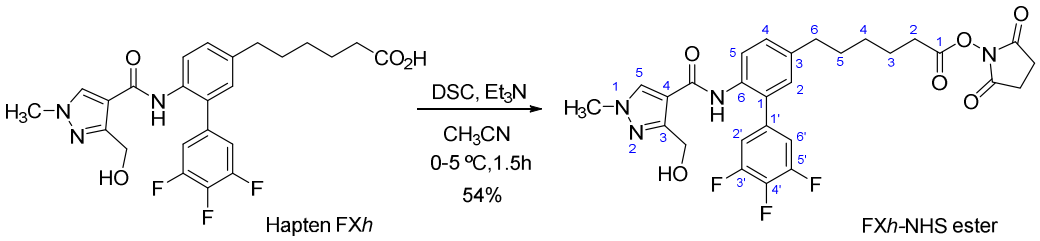
temperature for an additional 2 hours. The mixture was concentrated under vacuum and stripped with toluene to removal residual formic acid. The obtained residue (as shown by ^{1}H NMR spectroscopy, a mixture of hapten FX and the corresponding *O*-formylated derivative) was dissolved in a solution of K_2CO_3 (13.5 mg, 0.098 mmol) in MeOH (2 mL) and the mixture was stirred at room temperature for 30 minutes, then cooled in an ice-water bath and acidified with citric acid. The residue left after evaporation of the solvent was dissolved in EtOAc, washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum. Chromatographic purification of the crude product obtained, using $CHCl_3$-MeOH from 100:0 to 95:5 as eluent, afforded hapten FX*h* (29.1 mg, 70%). Mp 168.6-169.3 °C (from $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm), 8.91 (1H, br s, NH), 7.81 (1H, d, *J* = 8.3 Hz, H-5 BiPh), 7.77 (1H, s, H-5 Pz), 7.21 (1H, dd, *J* = 8.3, 1.8 Hz, H-4 BiPh), 7.05-6.97 (3H, m, H-2, H-2' and H-6' BiPh), 4.56 (2H, s, CH_2O), 3.82 (3H, s, NMe), 2.62 (2H, t, *J* = 7.5 Hz, H-6), 2.33 (2H, t, *J* = 7.3 Hz, H-2), 1.65 (4H, m, H-3 and H-5), 1.41 (2H, m, H-4); ^{19}F NMR (282 MHz, $CDCl_3$) δ (ppm) –134.5(2F, d, *J* = 20.0 Hz, F-3' and F-5' BiPh), -162.3 (1F, t, *J* = 20.0 Hz, F-4' BiPh); ^{13}C NMR (75 MHz, CD_3OD) δ (ppm) 177.6 (CO_2), 164.5 (CONH), 152.3 (ddd, *J* = 248.4, 9.9, 4.1 Hz, C-3' and C-5' BiPh), 152.1 (C-3 Pz), 142.5 (C-6 BiPh), 140.3 (dt, *J* = 250.1, 14.9 Hz, C-4' BiPh), 137.4 (dt, *J* = (8.3, 5.3 Hz, C-1' BiPh), 136.2 (C-5 Pz), 135.7 (C-3 BiPh), 133.2 (C-1 BiPh), 131.2 (C-2 BiPh), 130.1 (C-4 BiPh), 127.8 (C-5 BiPh), 117.5 (C-4 Pz), 114.5 (dd, *J* = 14.8, 6.6 Hz, C-2' and C-6' BiPh), 58.3 (CH_2O), 39.1 (NMe), 36.1 (C-6), 34.9 (C-2), 32.2 (C-5), 29.8 (C-4), 25.9 (C-3); HRMS (TOF MS ES+) *m/z* calcd for $C_{24}H_{25}F_3N_3O_4$ $[M+H]^+$ 476.1792, found 476.1809.

Hapten activation: preparation of *N*-hydroxysuccinimidyl esters



Synthesis of 2,5-dioxopyrrolidin-1-yl 6-(6-(3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamido)-3',4',5'-trifluoro-[1,1'-biphenyl]-3-yl)hexanoate (FX*b*-NHS ester). Hapten FX*b* (23.5 mg, 0.047 mmol) and *N,N'*-disuccinimidyl carbonate (15.65 mg, 0.0611 mmol, 1.3 equiv) were dissolved in anhydrous acetonitrile (0.6 mL) under nitrogen in an ice-water bath. Et_3N (25 µL, 0.179 mmol, 3.8 equiv) was them added and the resulting mixture was stirred at room temperature until complete consumption of starting material (as observed by thin-layer chromatography using $CHCl_3$:EtOH 95:5 as eluent, about 2.5 hours). The reaction mixture was diluted with $CHCl_3$, washed with a 10%

aqueous solution of $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum to give an oily residue that was filtered over a short pad of silica gel, eluting with $CHCl_3$, to afford nearly pure FX*b*-NHS *e*ster (24.6 mg, 87.7%), as determined by the 'H NMR spectra. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.01 (1H, d, *J* = 8.4 Hz, H-5 BiPh), 7.94 (1H, s, H-5 Pz), 7.77 (1H, br t, *J* = 4.2 Hz, NH), 7.23 (1H, dd, *J* = 8.4, 2.4 Hz, H-4 BiPh), 7.03 (1H, d, *J* = 2.4 Hz, H-2 BiPh), 7.00 (2H, m, H-2' and H-6' BiPh), 6.66 (1H, t, *J* = 54.3 Hz, CHF_2), 3.91 (3H, s, NMe), 2.83 (4H, br s, $COCH_2CH_2CO$), 2.63 (2H, t, *J* = 7.5 Hz, H-6), 2.60 (2H, t, *J* = 7.5 Hz, H-2), 1.78 (2H, quint, *J* = 7.5 Hz, H-5), 1.66 (2H, quint, *J* = 7.5 Hz, H-3), 1.47 (2H, m, H-4).



Synthesis of 2,5-dioxopyrrolidin-1-yl 6-(3',4',5'-trifluoro-6-(3-(hydroxymethyl)-1-methyl-1H-pyrazole-4-carboxamido)-[1,1'-biphenyl]-3-yl)hexanoate (FX*h*-NHS ester). Et_3N (14 µL, 0.098 mmol, 3.8 equiv) was added to an ice-water bath cooled solution of hapten FX*h* (12.3 mg, 0.026 mmol) and *N,N'*-disuccinimidyl carbonate (8.0 mg, 0.031 mmol, 1.2 equiv) in anhydrous acetonitrile (500 µL) under nitrogen. The mixture was stirred at the same temperature for 1.5 hours and then diluted with EtOAc and washed with a 10% aqueous solution of $NaHCO_3$ and brine and dried over anhydrous Na_2SO_4. Purification of the residue left after evaporation of the solvent by preparative thin layer chromatography (PTLC), using $CHCl_3$-MeOH 95:5 as eluent, afforded FX*h*-NHS ester (8.0 mg, 54%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 8.76 (1H, br s, NH), 7.89 (1H, d, *J* = 8.3 Hz, H-5 BiPh), 7.77 (1H, s, H-5 Pz), 7.23 (1H, dd, *J* = 8.3, 2.1 Hz, H-4 BiPh), 7.07-7.01 (3H, m, H-2, H-2' and H-6' BiPh), 6.60 (2H, s, CH_2O), 3.85 (3H, s, NMe), 2.83 (4H, br s, $COCH_2CH_2CO$), 2.63 (2H, t, *J* = 7.5 Hz, H-6), 2.61 (2H, t, *J* = 7.4 Hz, H-2), 1.78 (2H, quint, *J* = 7.5 Hz, H-5), 1.67 (2H, m, H-3), 1.47 (2H, m, H-4).

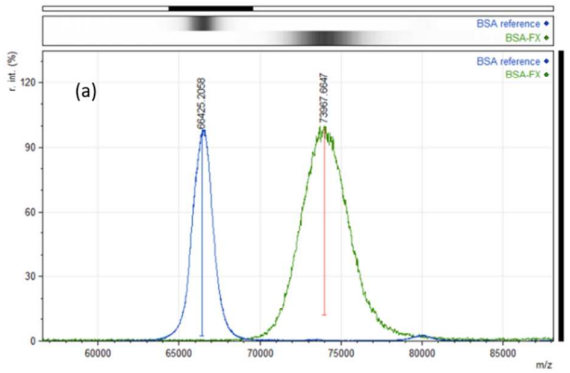
MALDI mass spectrometry analysis of immunizing and assay bioconjugates

Sample preparation. 100 µL of each of the protein conjugate solutions (0.5-1 mg/mL) were dialyzed against milliQ water and then freeze-dried and lyophilized. The samples were dissolved in MilliQ H_2O to theoretical final concentration 1 µg/µL. Then, 1 µL of every sample solution was spotted onto the MALDI plate. After the droplets were air dried at room temperature, 1 µL of matrix

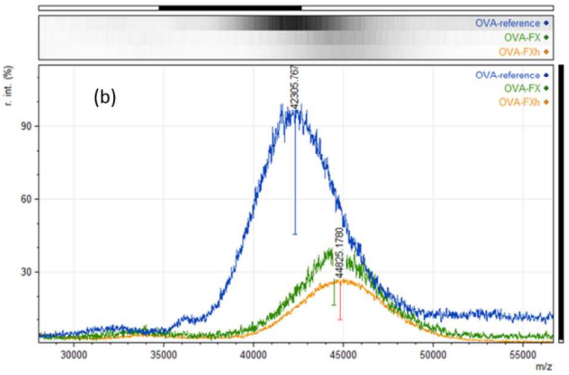
(10 mg/mL sinapinic acid (Bruker) in 0.1% trifluoroacetic acid-CH_3CN/H_2O (7:3 v/v) was added and allowed to air-dry at room temperature.

Mass spectrometry analysis. The resulting mixtures were analyzed in a MALDI-TOF/TOF apparatus 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.

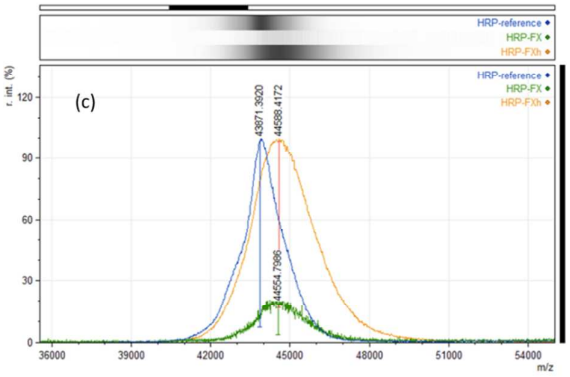
As determined by MALDI-TOF, the bioconjugates prepared showed the final molar ratios (MR) showed in Figure S2.



	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
BSA	66425	-	-	-
BSA-FX*b*	73968	477.4	7543	15.8



	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
OVA	42306	-	-	-
OVA-FX*b*	44494	477.4	2188	4.6
OVA-FX*h*	44825	457.5	2519	5.5



	m/z	Δ*m*/hapten	Δ(*m/z*)	MR
HRP	43871	-	-	-
HRP-FX*b*	44556	477.4	685	1.4
HRP-FX*h*	44588	457.5	717	1.6

Figure S2. MALDI-TOF-MS spectra of proteins (blue) and the corresponding conjugates with haptens FX*b* (green) and FX*h* (orange): (a) BSA conjugates; (b) OVA conjugates; (c) HRP conjugates

Antibody generation

Two groups of four BALB/c female mice each (8–10 weeks old) were immunized by intraperitoneal injections; one group with BSA–FX*n* and the other group with BSA–FX*b*. Each BSA-hapten conjugate solution was emulsified with one volume of Freund's adjuvant. The first dose contained complete Freund's adjuvant, and subsequent doses were given at weeks 3 and 6 using incomplete Freund's adjuvant. Each mouse received 100 μg per boost of immunizing conjugate in 200 μL of emulsion. After a resting period of at least three weeks, mice received an intraperitoneal booster injection of 100 μg of immunogen in 200 μL of sterile PBS four days before cell fusion.

After cell fusion, a two-step screening procedure was followed in order to identify those hybridomas that produced high-affinity binders. Twelve days after fusion, hybridoma culture supernatants were first screened by differential competitive ELISA on microtiter plates coated with 0.1 μg/mL (100 μL per well) of the homologous OVA–hapten conjugate. 50 μL of each supernatant was added to two adjacent wells of an ELISA plate, one containing 50 μL of PBS (blank) and the other containing 50 μL of 200 nM fluxapyroxad in PBS. The signal ratio in both wells was used as the criterion for selecting the antibodies with the highest affinity. Hybridoma supernatants affording signals higher than 3.0 in the absence of fungicide and those already showing high-affinity to fluxapyroxad received fresh culture medium and they were reevaluated on next day by checkerboard indirect competitive ELISA. Each supernatant was assayed at four dilutions (1/8, 1/32, 1/128, and 1/512) in ELISA plates coated with two coating concentrations of the homologous OVA–hapten conjugate (0.01 and 0.1 μg/mL) and using three fluxapyroxad levels (0, 10, and 100 nM).

Specificity of mAbs

Table S1. Cross-reactivity values (%).

mAb	BL[a]	PY[b]	PP[c]	FP[d]
FX*n*#11	---	---[e]	---	---
FX*n*#18	---	---	---	---
FX*n*#111	---	---	---	---
FX*n*#218	0.20	---	---	---
FX*n*#222	---	---	---	---
FX*n*#226	---	---	---	---
FX*n*#233	2.10	---	---	---
FX*n*#313	5.60	---	0.40	---
FX*n*#356	---	---	0.30	---
FX*n*#362	---	---	---	---
FX*n*#368	7.60	---	---	---
FX*b*#21	---	---	0.30	---
FX*b*#113	---	---	0.40	---
FX*b*#115	---	---	---	---
FX*b*#119	566	---	5.10	0.80
FX*b*#120	132	---	1.20	0.20

[a] Boscalid. [b] Pyraclostrobin. [c] Penthiopyrad. [d] Fluopyram. [e] Cross-reactivity was lower than 0.1%.

Influence of pH and ionic strength

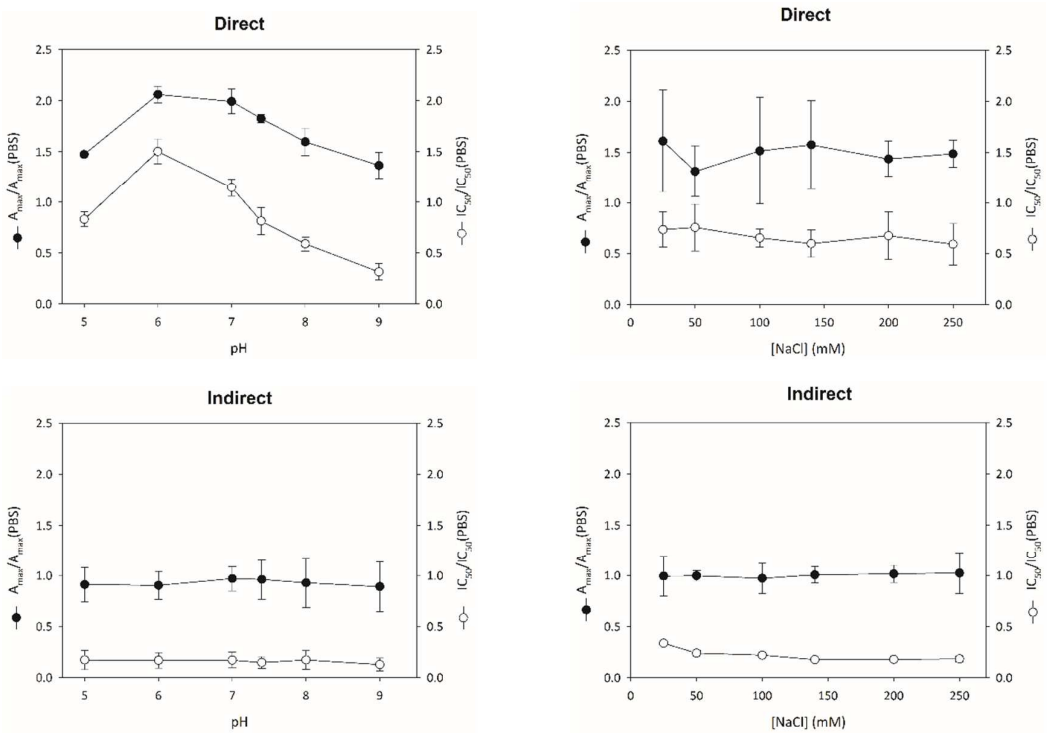


Figure S3. Influence of pH and NaCl concentration over the A_{max} and IC_{50} values of the studied immunoassays to fluxapyroxad.

Influence of organic solvents

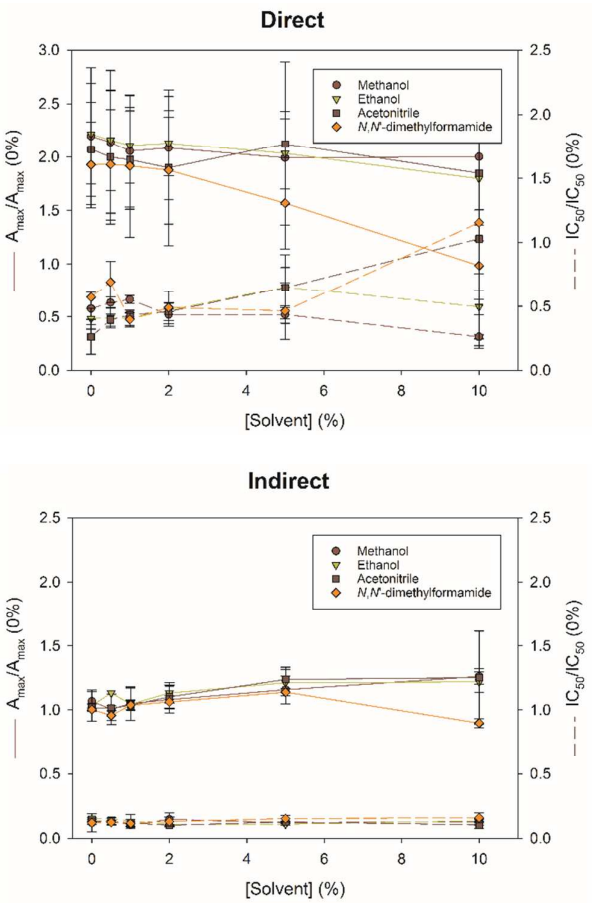


Figure S4. Influence of methanol, ethanol, acetonitrile, and *N*,*N*'-dimethylformamide over the A_{max} and IC_{50} values of the studied immunoassays to fluxapyroxad.

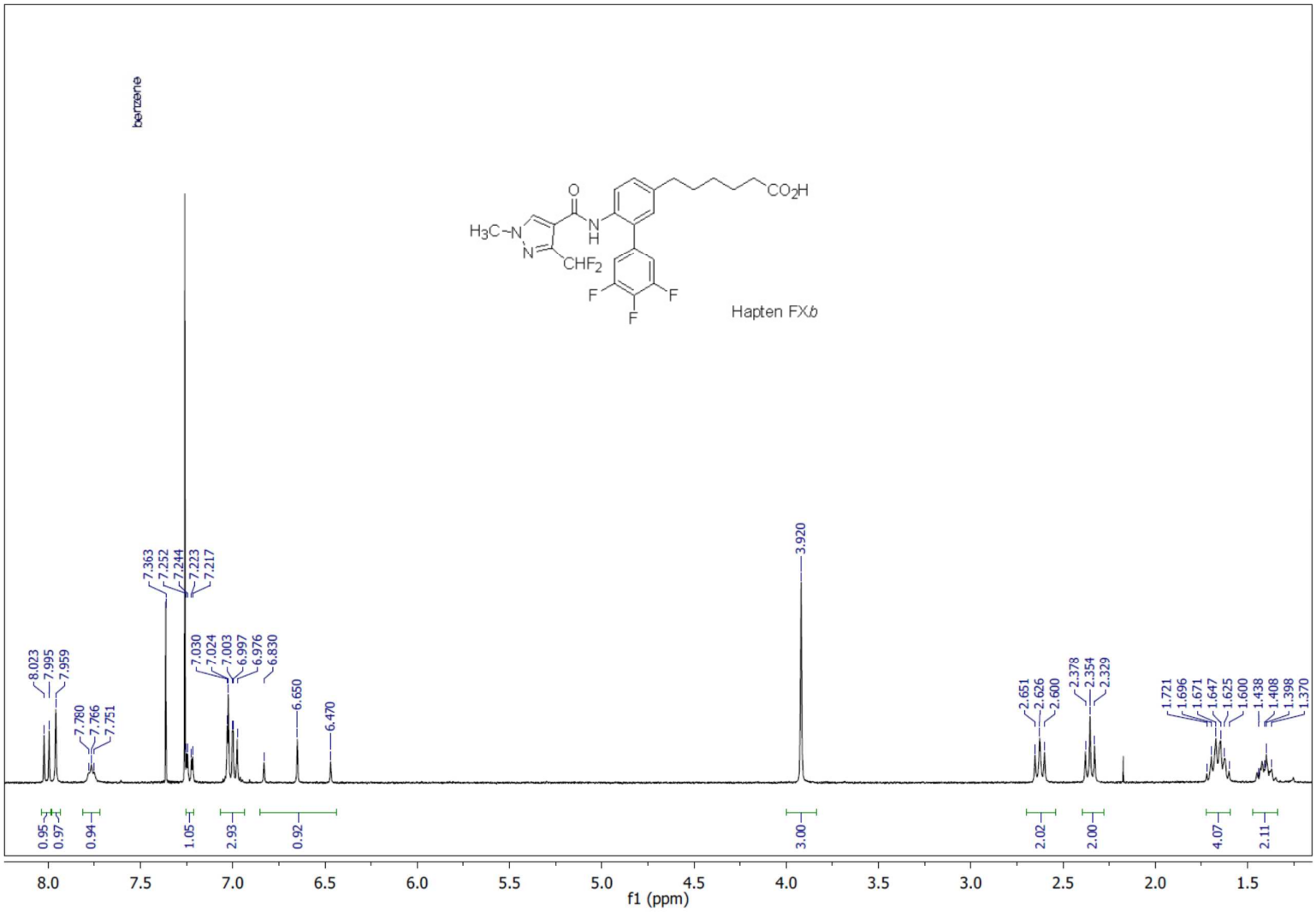
Analysis of in-field treated fruit samples by cELISA and UPLC–MS/MS.

Table S2. Analysis of fluxapyroxad-contaminated fruit extracts by the optimized indirect cELISA and a reference chromatographic method.

Sample code[a]	UPLC-MS/MS (ng/mL)	cELISA (ng/mL)	Sample code[b]	UPLC-MS/MS (ng/mL)	cELISA (ng/mL)
P-T1D1S1	1.24	8.86	GB-T1D1	189.58	156.01
P-T1D1S2	7.32	11.37	GB-T1D3	328.00	347.31
P-T1D3S1	0.28	7.13	GB-T2D1	226.38	207.75
P-T1D3S2	14.78	14.37	GB-T2D3	196.70	153.96
P-T1D5S1	0.10	7.59	GG-T1D1	393.75	438.28
P-T1D5S2	8.13	12.12	GG-T1D3	285.19	254.55
P-T1D7S1	2.35	8.74	GG-T2D1	389.18	425.25
P-T1D7S2	2.06	6.46	GG-T2D3	300.55	307.10
P-T2D1S1	61.96	37.25	GM-T1D1	128.20	85.07
P-T2D1S2	8.29	9.09	GM-T1D3	150.91	121.24
P-T2D3S1	6.07	12.03	GM-T2D1	611.73	713.91
P-T2D3S2	9.20	11.35	GM-T2D3	146.38	104.37
P-T2D5S1	1.99	8.83	GT-T1D1	307.74	321.55
P-T2D5S2	7.25	11.76	GT-T1D3	355.31	375.06
P-T2D7S1	9.84	13.45	GT-T2D1	138.91	103.68
P-T2D7S2	6.23	12.04	GT-T2D3	182.02	146.89

[a] P stands for plums, T for the type of treatment, D for day of sample collection, and S for the different samples that were collected. [b] GB stands for grapes var. Bobal, GG for grapes var. Garnacha, GM for grapes var. Macabeo, and GT for grapes var. Tempranillo.

^{1}H NMR spectrum of hapten FX*b* ($CDCl_3$, 300 MHz)



^{1}H NMR spectrum of hapten FX*h* ($CDCl_3$, 300 MHz)