Abstract

Bioconjugate preparation is a fundamental step for antibody generation and immunoassay development to small chemical compounds. For analytical targets holding in their structure an aryl halogen atom, cross-coupling reactions may be a simple and efficient way to obtain functionalized derivatives; thus offering great potential to elicit robust and selective immune responses after being coupled to immunogenic carrier proteins. However, substitution of the halogen atom by an aliphatic chain might eventually compromise the affinity and specificity of the resulting antibodies. In order to address this issue, proquinazid, a new-generation fungicide with outstanding performance, was chosen as model analyte. Two functionalized derivatives differing in spacer arm rigidity were synthesized by Sonogashira cross-coupling chemistry. These haptens were covalently coupled to bovine serum albumin and the resulting immunoconjugates were employed for rabbit vaccination. Antibodies were tested for proquinazid recognition by direct and indirect competitive immunoassay, and IC50 values in the low nanomolar range were found, thus demonstrating the suitability of this straightforward synthetic strategy for the generation of immunoreagents to compounds bearing an aryl halide. Following antibody characterization, competitive immunoassays were developed and employed to determine proquinazid residues in grape musts, and their analytical performance was satisfactorily validated by comparison with GC–MS. Besides having described the development of the first immunochemical method for proquinazid analysis, an efficient functionalization approach for analytes comprising aryl halides is reported.

Introduction

Antibody-based detection techniques are currently invaluable analytical tools in numerous disciplines, including basic biochemical and biomedical research, forensic toxicology, clinical diagnostics, food safety, and environmental monitoring. The huge success of these
methodologies relies on the exquisite affinity and specificity that antibodies commonly exhibit towards the molecular target. While generation of this sort of binding molecules is most often straightforward for large entities, like proteins and microorganisms, production of antibodies to small organic chemicals, so-called haptens, can frequently be a challenging task. Haptens cannot elicit an immune response by themselves, so coupling to a carrier protein is mandatory in order to trigger an efficient good-quality response. Unfortunately, most interesting targets do not possess ready-to-activate chemical groups for protein conjugation, so adequate derivatives must be prepared, often by total synthesis comprising complex multi-step synthetic approaches. Moreover, even if suitable functional groups are already present in the analyte, such as -COOH, -NH₂, -OH, and -SH, coupling through these positions may not be advisable because potentially strong antigenic determinants—immunodominant epitopes—involved in high-affinity and specific binding (hydrogen bonding, ionic, or electrostatic interactions, etc.) would be concealed.

Since the discovery and development of palladium-catalyzed cross-coupling reactions revolutionized organic chemistry in the early 1970s, utilization of such chemical strategies has increased exponentially both in academia and in industry. These synthetic procedures provide convenient and highly practical methods for the construction of previously difficult or impossible to generate carbon—carbon bonds [1]. Nowadays, one of the most important and widely used palladium-catalyzed cross-coupling reactions is the Sonogashira reaction, which permits the coupling of aryl or vinyl halides to terminal acetylenes under mild conditions [2]. Despite the vast potential of these reactions for introducing new functionalities at desired positions of the molecular framework of the target compound, they have hardly been exploited in immunochemistry for the synthesis of haptens ready to be used for bioconjugate preparation. In the last few years, we have reported the application of the Sonogashira cross-coupling reaction to the successful preparation of conveniently functionalized derivatives of various modern agrochemicals [3–11]. In all these cases and as a key step in each particular synthetic strategy, previous preparation of a halogenated intermediate was required because all of the concerned analytes were lacking from the proper halogen moiety for further introduction of the corresponding spacer arm via cross-coupling chemistry.

While it early became apparent to us that cross-coupling chemistry could be a valuable tool for easily affording functionalized derivatives of compounds already bearing aromatic halogen atoms—particularly iodine, bromine, and chlorine—the question arising was whether the substitution of the halogen atom—a potentially strong antigenic determinant—by an alkynyl chain or the corresponding saturated one, could compromise the affinity and specificity of the eventually derived biomolecular binders.

The past 30 years have witnessed a period of significant expansion in the use of halogenated compounds in several fields, including agrochemical research and development. A recent survey about modern agrochemicals shows that around 79% of the new active ingredients used nowadays are halogen substituted, and 12 of those products are among the 20 best-sold compounds, accounting for sales of US$ 6430 million [12].

Proquinazid is a novel quinazolinone fungicide developed by DuPont, and one of the newest incorporations to the available arsenal of active ingredients enabling to efficiently combat fungal pathogens that damage valuable crops (http://www2.dupond.com). Proquinazid was particularly effective for powdery mildew control in cereals, cucurbits, and grapevines, and it was first registered in several European countries in 2004, even though full approval and inclusion in Annex I of the European Union occurred in 2009 [13], and more recently (2012) in Australia [14]. Proquinazid is thought to act by interfering signal transduction pathways, even though the exact molecular target is unknown [15]. A remarkable chemical feature of proquinazid is the presence of an aryl iodine atom in its structure, which contributes to its excellent field
performance and increased stability to sunlight, allowing field application at low rates [16]. This fact, together with its novelty and efficacy as fungicide, makes proquinazid an outstanding candidate to study the adequacy of cross-coupling reactions to the synthesis of adequate functionalized mimics that enable to trigger specific and strong immune responses and eventually afford high-affinity and specific antibodies. Additionally, to the best of our knowledge, no previous studies dealing with the generation of immunoreagents to proquinazid have been reported. Accordingly, we herein describe the synthesis of two proquinazid haptens via Sonogashira cross-coupling reaction and the evaluation of their suitability for antibody generation. In these haptens, the iodine atom of proquinazid was replaced by an alkynyl carboxylate six-carbon linker in order to examine both the effect of a rigid triple C-C bond directly linked to the derivatization site, and that of the corresponding saturated alkyl chain, which resulted in a more flexible spacer arm. The described haptens were coupled to the appropriate proteins, and the so-obtained bioconjugates were used to immunize rabbits. Finally, performance of the resulting antisera in terms of affinity, specificity, and bioanalytical capability was evaluated by competitive enzyme-linked immunosorbent assay (cELISA).

Materials and Methods

Reagents and instrumentation

Proquinazid (6-i 1odo-2-propoxy-3-propylquinazolin-4(3H)-one, CAS Registry number 189278-12-4, Mw 372.2) and other fungicide standards were purchased from Fluka/Riedel-de-Haën (Seelze, Germany) and Dr. Ehrenstorfer (Augsburg, Germany). Working solutions were prepared as concentrated solutions in N,N-dimethylformamide (DMF) and kept at ~20°C in amber glass vials. Horseradish peroxidase (HRP), ovalbumin (OVA), o-phenylenediamine (OPD) and triphenyl phosphate (TPP) were purchased from Sigma/Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns from GE Healthcare (Uppsala, Sweden) were used for conjugate purification. Polyclonal goat anti-rabbit immunoglobulin peroxidase conjugate (GAR-HRP) was from Biorad (Hercules, CA, USA). Bovine serum albumin (BSA) fraction V was purchased from Roche Applied Science (Mannheim, Germany). Adult bovine serum and Freund’s adjuvants were from Sigma/Aldrich (Madrid, Spain). Ninety-six well Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). Ultraviolet-visible spectra and assay absorbances were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). ELISA plates were washed with an ELx405 microplate washer also from BioTek Instruments.

Composition, concentration, and pH of the employed buffers were: i) PB, 100 mM sodium phosphate buffer (pH 7.4); ii) PBS, 10 mM sodium phosphate buffer (pH 7.4) with 140 mM NaCl; iii) PBS-T, PBS containing 0.05% (v/v) Tween 20; iv) CB, 50mM carbonate-bicarbonate buffer (pH 9.6); v) Washing solution, 150 mM NaCl and 0.05% (v/v) Tween 20; vi) Enzyme substrate buffer, 25 mM citrate and 62 mM sodium phosphate buffer (pH 5.4); and vii) 2xPBS-T, 20 mM sodium phosphate buffer (pH 7.4) with 280 mM NaCl and 0.05% (v/v) Tween 20.

Hapten synthesis

1 General Procedures. Anhydrous solvents were freshly distilled under nitrogen from Na or Na/benzophenone (THF, pentane) or dried over a bed of activated molecular sieves (DMF) or KOH (Et3N). Other solvents and reagents were obtained from commercial sources and used without purification. Reactions were monitored by thin-layer chromatography on 0.25 mm pre-coated silica gel plates. Visualization was carried out with UV light and ethanolic phosphomolybdic acid or aqueous ceric ammonium molybdate solutions. Products were purified by flash column chromatography on silica gel 60 (particle size 0.043–0.063 mm). Melting points
were determined on a Büchi M-560 apparatus and are uncorrected. 1H/13C NMR spectra were recorded at 298 K in the indicated solvent on a Bruker DRX-300 (300/75 MHz) or Bruker Advance-400 (400/100 MHz) spectrometers. The chemical shifts are expressed in ppm (δ scale) relative to the residual solvent for 1H (CDCl₃ at 7.26 ppm) or to the central peak of solvent 13C signal (CDCl₃ at 77.0 ppm). Carbon substitution degrees were established by DEPT pulse sequences. The abbreviation used for NMR data are as follows: s, singlet; d, doublet; dd, double doublet; t, triplet; quint, quintuplet; sext, sextuplet; br, broad; m, multiplet; Qz, quinazoline ring. Infrared (IR) spectra were obtained using a Nicolet Avatar 320 FT-IR spectrometer. High resolution mass spectra (HRMS) were recorded by the electrospray (ES) ionization mode using a Micromass VG Autospec spectrometer.

The following is a detailed description of the synthetic procedures involved in hapten preparation, as schematized in Fig 1, as well as complete spectroscopic data thereof and of the intermediates of their synthesis.

2 Synthesis of hapten PQ(t).

2.1 Preparation of tert-buty1 6-(4-oxo-2-propoxy-3-propyl-3,4-dihydroquinazolin-6-yl)hex-5-ynoate (2): Dry DMF (2 mL) was added to a mixture of proquinazid (203.8 mg, 0.548 mmol), tert-butyl hex-5-ynoate (1) (138.2 mg, 0.822 mmol, 1.5 eqs), Cl₂Pd(PPh₃)₂ (11.6 mg, 0.016 mmol, 0.03 eqs) and CuI (1.05 mg, 0.0054 mmol, 0.01 eqs) under nitrogen. Proquinazid was synthesized in three steps from commercially available 2-amino-5-iodobenzoic acid following a previously described procedure [17], whereas tert-butyl hex-5-ynoate was prepared from commercial hex-5-ynoic acid as reported by Mercader et al.[5]. The mixture was degassed through several freeze—thaw cycles, then anhydrous Et₃N (1.6 mL) was added and the degassing-purging process was repeated twice. After stirring at room temperature for 6 h, the resulting reaction mixture was cooled down, filtered through cotton and most of the Et₃N was removed under vacuum. The residue was diluted with ethyl acetate and washed with a 1.5% aqueous solution of LiCl and brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to produce a dark orange, viscous oil. Purification by silica gel chromatography, using hexane—CHCl₃ 3:2 as eluent, afforded compound 2 (200 mg, 88.5%) as a brownish amorphous solid. 1H NMR (CDCl₃, 300 MHz) δ 8.19 (1H, d, J = 1.9 Hz, H-5 Qz), 7.60 (1H, dd, J = 8.5 and 1.9 Hz, H-7 Qz), 7.34 (1H, d, J = 8.5 Hz, H-8 Qz), 4.42 (2H, t, J = 6.5 Hz, OCH₂CH₂CH₃), 4.04 (2H, m, NCH₂CH₂CH₃), 2.47 (2H, t, J = 7 Hz, H-4), 2.42 (2H, t, J = 7.6 Hz, H-2), 1.93–1.63 (6H, m, H-3, OCH₂CH₂CH₃ and NCH₂CH₂CH₃), 1.45 (9H, s, OCMe₃), 1.05 (3H, t, J = 7.5 Hz, OCH₂CH₂CH₃), 0.95 (3H, t, J = 7.5 Hz, NCH₂CH₂CH₃); 13C NMR (CDCl₃) δ 172.5 (C-1), 162.1 (C-4 Qz), 152.9 (C-2 Qz), 146.5 (C-8a Qz), 137.0 (C-7 Qz), 130.3 (C-5 Qz), 125.4 (C-8 Qz), 119.7 (C-6 Qz), 118.6 (C-4a Qz), 89.4 (C-5), 80.5 and 80.3 (C-6 and O/CMe₃), 70.0 (OCH₂CH₂CH₃), 43.2 (NCH₂CH₂CH₃), 34.4 (C-2), 28.1 (OCMe₃), 24.0 (C-3), 22.0 (OCH₂CH₂CH₃), 21.6 (NCH₂CH₂CH₃), 11.3 (NCH₂CH₂CH₃), 10.5 (OCH₂CH₂CH₃); HRMS (TOF MS ES+) m/z calcd for C₂₄H₃₃N₂O₄ [M+H]+ 413.2440, found 413.2441.

2.2 Preparation of 6-(4-oxo-2-propoxy-3-propyl-3,4-dihydroquinazolin-6-yl)hex-5-ynoic acid (hapten PQ(t)): A solution of tert-buty1 ester 2 (64 mg, 0.155 mmol) in formic acid (1 mL) was stirred at room temperature until TLC [developed with CHCl₃] showed completion of the reaction (about 2.5 h). Benzene was added and the mixture was concentrated under reduced pressure, the residue was dissolved in benzene and concentrated again to obtain a brownish solid. Purification by chromatography, using CHCl₃—MeOH 96:4, afforded pure hapten PQ(t) (49.2 mg, 89%) as a white solid. Mp 113.7–114.0°C (from benzene—hexane). 1H NMR (CDCl₃, 300 MHz) δ 10.47 (1H, br s, OH), 8.11 (1H, d, J = 1.9 Hz, H-5 Qz), 7.52 (1H, d, J = 8.6 and 1.9 Hz, H-7 Qz), 7.27 (1H, d, J = 8.6 Hz, H-8 Qz), 4.34 (2H, t, J = 6.6 Hz, OCH₂CH₂CH₃), 3.96 (2H, m, NCH₂CH₂CH₃), 2.49 (2H, t, J = 7.4 Hz, H-4), 2.44 (2H, t, J = 7 Hz, H-2), 1.86 (2H, quint, J = 7.2 Hz, H-3), 1.75 (2H, sext, J = 7.4 Hz, OCH₂CH₂CH₃), 1.62 (2H, sext, J = 7.5 Hz,
NCH$_2$CH$_2$CH$_3$), 0.97 (3H, t, $J = 7.4$ Hz, OCH$_2$CH$_2$CH$_3$), 0.87 (3H, t, $J = 7.5$ Hz, OCH$_2$CH$_2$CH$_3$); $^{13}$C NMR (CDCl$_3$) $\delta$ 178.8 (C-1), 162.2 (C-4 Qz), 152.9 (C-2 Qz), 146.5 (C-9 Qz), 137.0 (C-7 Qz), 130.3 (C-5 Qz), 125.4 (C-8 Qz), 119.6 (C-6 Qz), 118.6 (C-10 Qz), 89.0 (C-5), 80.9 (C-6), 70.1 (OCH$_2$CH$_2$CH$_3$), 43.2 (NCH$_2$CH$_2$CH$_3$), 32.8 (C-2), 23.6 (C-3), 22.0 (OCH$_2$CH$_2$CH$_3$), 21.6 (NCH$_2$CH$_2$CH$_3$), 18.8 (C-3), 11.3 (NCH$_2$CH$_2$CH$_3$), 10.5 (OCH$_2$CH$_2$CH$_3$); IR (KBr) $\nu_{max}$/cm$^{-1}$ 3400–2400, 2958, 2925, 2854, 2230, 1707, 1701, 1596; HRMS (TOF MS ES+) m/z calcd for C$_{20}$H$_{25}$N$_2$O$_4$ [M+H]$^+$ 357.1814, found 357.1817.

3 Synthesis of hapten PQs. 3.1 Preparation of tert-butyl 6-[(4-oxo-2-propoxy-3-propyl-3,4-dihydroquinazolin-6-yl)hexanoate (3): A solution of alkyne 2 (81.5 mg, 0.197 mmol) and Wilkinson’s catalyst (5.5 mg, 0.006 mmol, 3%) in anhydrous THF (2 mL) was evacuated and purged under an atmosphere of hydrogen gas. The hydrogen pressure was regulated to 3.5 atmospheres and the reaction mixture was stirred at room temperature overnight. The mixture was concentrated to dryness under reduced pressure to afford a brownish viscous oil that was purified by chromatography, using hexane—EtOAc 9:1 as eluent, to furnish compound 3 (72.5...
mg, 88%) as a colorless oil. ¹H NMR (CDCl₃) δ 7.96 (1H, dd, J = 2 and 0.4 Hz, H-5 Qz), 7.45 (1H, dd, J = 8.4 and 2 Hz, H-7 Qz), 7.36 (1H, dd J = 8.4 and 0.4 Hz, H-8 Qz), 4.41 (2H, t, J = 6.5 Hz, OCH₂CH₂CH₂), 4.06 (2H, m, NCH₂CH₂CH₂), 2.68 (2H, t, J = 7.5 Hz, H-6), 2.19 (2H, t, J = 7.4 Hz, H-2), 1.84 (2H, quint, J = 7.4 Hz, H-3), 1.75-1.55 (6H, m, H-5, OCH₂CH₂CH₃ and NCH₂CH₂CH₃), 1.42 (9H, s, OMe), 1.35 (2H, m, H-4), 1.05 (3H, t, J = 7.4 Hz, OCH₂CH₂CH₃), 0.96 (3H, t, J = 7.5 Hz, NCH₂CH₂CH₃); ¹³C NMR (CDCl₃) δ 173.1 (C-1), 163.9 (C-4 Qz), 152.3 (C-2 Qz), 145.3 (C-8a Qz), 138.8 (C-6 Qz), 134.9 (C-7 Qz), 126.0 (C-5 Qz), 125.3 (C-8 Qz), 118.5 (C-4a Qz), 79.9 (OCMe₃), 69.8 (OCH₂CH₂CH₃), 43.1 (NCH₂CH₂CH₃), 35.5 (C-6), 35.3 (C-2), 31.0 (C-3), 29.6 (C-4), 28.1 (OCMe₃), 24.9 (C-5), 22.0 (OCH₂CH₂CH₃), 21.7 (NCH₂CH₂CH₃), 11.3 (NCH₂CH₂CH₃), 10.5 (OCH₂CH₂CH₃); IR (NaCl) νₘₐₓ/cm⁻¹ 2959, 2874, 1736, 1705, 1654, 1597, 1485, 1456, 1351, 1350, 1179, 811, 754; HRMS (TOF MS ES⁺) m/z calcd for C₂₄H₃₇N₂O₄ [M+H]+ 417.2753, found 417.2747.

3.2 Preparation of 6-(4-oxo-2-propoxy-3-propyl-3,4-dihydroquinazolin-6-yl)hexanoic acid (hapten PQs): A solution of the tert-butyl ester 3 (72.1 mg, 0.173 mmol) in formic acid (1.2 mL) was stirred at room temperature for 2.5 h. The reaction mixture was diluted with benzene and concentrated under vacuum to give hapten PQₜ (59 mg, 95%) as a white solid. Mp 124.7–125.7°C (from EtOAc). ¹H NMR (CDCl₃) δ 9.80 (1H, br s, OH), 7.97 (1H, d, J = 2 Hz, H-5 Qz), 7.45 (1H, dd, J = 2, 8.4 Hz, H-7 Qz), 7.37 (1H, d, J = 8.4 Hz, H-8 Qz), 4.42 (2H, t, J = 6.5 Hz, OCH₂CH₂CH₃), 4.06 (2H, m, NCH₂CH₂CH₃), 2.69 (2H, t, J = 7.5 Hz, H-6), 2.34 (2H, t, J = 7.7 Hz, H-2), 1.84 (2H, sext, J = 7.4 Hz, H-5), 1.76-1.60 (6H, m, H-3, OCH₂CH₂CH₉ and NCH₂CH₂CH₃), 1.42 (2H, m, H-4), 1.06 (3H, t, J = 7.4 Hz, OCH₂CH₂CH₃), 0.97 (3H, t, J = 7.4 Hz, OCH₂CH₂CH₃), ¹³C NMR (CDCl₃) δ 163.0 (C-4 Qz), 152.3 (C-2 Qz), 145.4 (C-8a Qz), 138.6 (C-6 Qz), 135.0 (C-7 Qz), 126.0 (C-5 Qz), 125.3 (C-8 Qz), 118.5 (C-4a Qz), 69.8 (OCH₂CH₂CH₃), 43.2 (NCH₂CH₂CH₃), 35.1 (C-6), 30.9 (C-2), 28.5 (C-5), 28.1 (C-4), 24.5 (C-3), 22.0 (OCH₂CH₂CH₃), 21.7 (NCH₂CH₂CH₃), 11.3 (NCH₂CH₂CH₃), 10.5 (OCH₂CH₂CH₃); IR (KBr) νₘₐₓ/cm⁻¹ 3300–2400, 2966, 2933, 2873, 1713, 1683, 1602, 1492, 1191, 936; HRMS (TOF MS ES⁺) m/z calcd for C₂₆H₃₈N₂O₄ [M+H]+ 361.2127, found 361.2130.

Synthesis of the N-succinimidyl esters of haptenes PQₜ and PQs

Activation of the free carboxylate group of haptenes PQₜ and PQs with N,N’-disuccinimidyl carbonate (DSC) and purification of the corresponding succinimidyl active ester was done as follows: the hapten (ca. 21 mg, 0.058 mmol) and DSC (19.3 mg, 0.075 mmol, 1.3 eqs.) were dissolved in dry acetonitrile (1.0 mL) under nitrogen atmosphere at 0°C and treated with anhydrous Et₃N (23 mg, 0.227 mmol, 3.8 eqs.). The reaction mixture was stirred at 0°C until complete consumption of starting material (as observed by TLC, about 1.5 to 2 hours). The reaction mixture was concentrated under reduced pressure without heating to give an orange oily residue that was purified by column chromatography, using CHCl₃–EtOAc 9:1 as eluent, to afford the N-succinimidyl ester of the hapten (PQₜ-NHS or PQs-NHS) in good yield (75–85%) and purity, as evidenced from the corresponding ¹H NMR spectrum.

¹H NMR of activated hapten PQₜ-NHS (CDCl₃, 400 MHz): δ 8.20 (1H, d, J = 2 and 0.5 Hz, H-5 Qz), 7.62 (1H, dd, J = 8.4 and 2 Hz, H-7 Qz), 7.36 (1H, dd J = 8.4 and 0.5 Hz, H-8 Qz), 4.43 (2H, t, J = 6.6 Hz, OCH₂CH₂CH₃), 4.05 (2H, m, NCH₂CH₂CH₃), 2.85 (4H, br s, OCCH₂CH₂CO), 2.83 (2H, t, J = 7.4 Hz, H-4), 2.59 (2H, t, J = 6.9 Hz, H-2), 2.06 (2H, quint, J = 7 Hz, H-3), 1.84 (2H, sext, J = 7 Hz, OCH₂CH₂CH₃), 1.70 (2H, sext, J = 7 Hz, NCH₂CH₂CH₃), 1.06 (3H, t, J = 7.4 Hz, OCH₂CH₂CH₃), 0.96 (3H, t, J = 7.5 Hz, NCH₂CH₂CH₃).

¹H NMR of activated hapten PQs-NHS (CDCl₃, 300 MHz): ¹H NMR (CDCl₃) δ 7.96 (1H, d, J = 2 Hz, H-5 Qz), 7.46 (1H, dd, J = 8.4 and 2 Hz, H-7 Qz), 7.37 (1H, d, J = 8.4 Hz, H-8 Qz),
4.42 (2H, t, J = 6.6 Hz, OCH₂CH₂CH₃), 4.06 (2H, m, NCH₂CH₂CH₃), 2.83 (4H, br s, OCCH₂CH₂CO), 2.71 (2H, t, J = 7.4 Hz, H-6), 2.59 (2H, t, J = 7.4 Hz, H-2), 1.9–1.6 (8H, m, H-3, H-5, OCH₂H₂CH₃ and NCH₂H₂CH₃), 1.45 (2H, m, H-4), 1.06 (3H, t, J = 7.4 Hz, OCH₂CH₂CH₃), 0.96 (3H, t, J = 7.5 Hz, NCH₂CH₂CH₃).

Preparation of hapten protein conjugates

Different conjugates were prepared by reaction of the purified N-succinimidyl ester of each hapten (PQt and PQs) with the free amine groups of three carrier proteins: BSA, HRP, and OVA. The final hapten-to-protein molar ratio (MR) was calculated using the absorbance values of the conjugate by assuming that the molar absorption coefficients of the hapten and the protein were the same for both the free and the conjugated forms.

1. Immunizing conjugates. BSA—hapten conjugates were prepared using 200μL of a 50 mM solution of the N-succinimidyl ester of the hapten (PQt or PQs) in anhydrous DMF. Briefly, the active ester solution was added drop wise over a 15 mg/mL BSA solution (2 mL) in CB and the mixture was stirred at room temperature in amber glass vials during 4 h. Conjugates were purified by Sephadex G-25 gel filtration using PB as eluent. The collected volume was brought to 30 mL with PB and the conjugate was stored at −20°C. The calculated final MR of both immunizing conjugates was 12.

2. Coating conjugates. OVA—hapten conjugates were prepared by adding drop wise 200μL of a 50 mM solution of the N-succinimidyl ester of the hapten (PQt or PQs) to 2 mL of a 15 mg/mL OVA solution in CB under stirring. The reaction was mixed at room temperature during 2.5 h in amber glass vials and the conjugates were purified as before and stored at −20°C. The final MR was 7 and 10 for PQt and PQs hapten, respectively.

3. Enzyme tracers. HRP—hapten conjugates were prepared by adding drop wise 100μL of a 5 mM solution of the N-succinimidyl ester of the hapten (PQt or PQs) to 1mL of a 2.2 mg/mL HRP solution in CB under stirring. The coupling reaction was allowed to proceed during 4 h at room temperature with stirring in amber glass vials. Then, the conjugates were purified by gel chromatography and the pooled fractions were brought to 1 mg/mL with PB, and then diluted 1/2 with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal. Several aliquots of the enzyme tracer conjugate were stored at −20°C in amber vials, except the working solution which was kept at 4°C. The final MR was 2.5 for both HRP conjugates.

Production of antibodies

Two female New Zealand white rabbits were independently immunized by subcutaneous injection with 0.3 mg of immunizing conjugate (BSA—PQt or BSA—PQs) in 1 mL of a 1:1 mixture of sterile PB and complete Freund’s adjuvant. Animals were boosted at 21-day intervals with the same immunogen suspended in a mixture of 0.5 mL of sterile PB and 0.5 mL of incomplete Freund’s adjuvant. Ten days after the fourth injection, rabbits were anaesthetised with xylazine/ketamine and euthanized by intracardiac puncture followed by an overdose of pentobarbital. All efforts were made to minimize suffering. Blood samples were allowed to coagulate overnight at 4°C. Then, the serum was separated by centrifugation and precipitated with a solution of saturated ammonium sulphate. This procedure was repeated again and the precipitates were stored at 4°C. This study was carried out in strict accordance with the recommendations in the European Directive 2010/63/EU concerning the protection of animals used for scientific purposes. The protocol was approved by the Ethics Committee of the Universitat de València (permit number: A1329731961154).
Antibody-coated direct cELISA

ELISA plates were coated with 100 μL of antiserum diluted in CB, and plates were incubated overnight at room temperature. Coated plates were washed four times with washing solution and they received 50 μL per well of analyte standard in PBS plus 50 μL per well of enzyme tracer solution in PBS-T. The competitive reaction was carried out at room temperature for 1 h, and then plates were washed as described above. Finally, signal was produced by addition of 100 μL per well of freshly prepared 2 g/L OPD solution containing 0.012% (v/v) H₂O₂ in enzyme substrate buffer. The enzymatic reaction was stopped after 10 min at room temperature by adding 100 μL per well of 1 M sulphuric acid. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

Conjugate-coated indirect cELISA

Microplates were coated with 100 μL per well of coating conjugate solution in CB by overnight incubation at room temperature. Coated microwells were washed as described and then received 50 μL per well of analyte in PBS plus 50 μL per well of antiserum diluted in PBS-T. The competitive reaction was carried out at room temperature for 1 h, and plates were washed again. Next, 100 μL per well of a 1/10⁴ dilution of GAR—HRP conjugate in PBS-T containing 10% adult bovine serum was added, and plates were incubated 1 h at room temperature. After washing the plates as before, signal was generated and the plates were read as described for the direct cELISA.

Data treatment

Eight-point standard curves, including a blank, were prepared by five-fold serial dilution in PBS from a 1 g/L proquinazid stock solution. 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% inhibition (IC₅₀) of the maximum absorbance (Aₘₐₓ) provided that the background signal approaches to zero.

Buffer composition studies

Two immunoassays were selected using each of the two evaluated cELISA formats. In the direct assay, plates were coated with 1/10⁴ dilution of antibody PQt#2, and a 15 ng/mL solution of tracer HRP—PQt was used in the competitive step. In the indirect cELISA, plates were coated with 0.1 μg/mL OVA—PQs conjugate solution and the antibody PQt#2 was diluted 1/6x10⁴ for competition.

For solvent studies, analyte standard curves were prepared in water containing between 0.5 and 10% of methanol, ethanol, acetonitrile, or acetone, and immunoreagents were prepared in 2xPBS-T. The influence of the buffer ionic strength and the pH was evaluated following a central composite design, consisting of a two-level full factorial design (α = 1.414) with 2 factors and 3 replicates that included 12 cube, 12 axial, and 15 centre points, and involving a total of 39 randomized buffer studies [18]. Proquinazid standard curves were prepared in water and they were mixed as described above with tracer or antibody solutions prepared in every of the studied buffers. The Aₘₐₓ and IC₅₀ values of the inhibition curves were employed as response values and fitted to a multiple regression equation, including curvature and interaction terms, using Minitab 14.1 software (Minitab Inc., State College, PA, USA).
Reference procedure

A QuEChERS dispersive kit (Agilent Technologies, Santa Clara, CA, USA) was employed for the extraction and purification of proquinazid residues from must samples (European Committee for Standardization Standard Method EN 15662) [19]. Purified extracts were filtered through 0.22 μm Teflon filters and analyzed by gas chromatography–mass spectrometry (GC–MS) using an Agilent 6890N GC network system, equipped with a 7683 series autosampler, a HP-5MS (30 m × 0.25 mm × 0.25 μm) capillary column, and a 5973 mass selective detector. GC–MS measurement conditions were as follow: one microliter extract was injected in splitless mode at 300°C by employing helium as carrier with a constant flow of 1 mL/min. The oven temperature program (150°C) was held for 1 min, increased at a rate of 10°C/min up to 280°C and held at this temperature for 2 min. The transfer line and source temperatures were 280°C and 250°C, respectively. Electron impact ionization at 70 eV was used, and the employed quantification ions were 330 and 288 m/z for proquinazid and 325 and 326 m/z for TPP, the internal standard.

Results and Discussion

Synthesis of haptens and preparation of bioconjugates

Hapten synthesis is usually deemed the key step in the generation of antibodies to small organic chemicals, so easy access to suitable functionalized derivatives of target compounds greatly facilitates the production of anti-hapten antibodies and the development of immunochemical methods. If available, functional groups already present in the target molecule, commonly consisting in—SH, —OH, —NH2, and—COOH, constitute a simple and attractive approach to prepare functionalized derivatives, even though coupling through these moieties may sometimes be unwise in terms of antibody affinity and/or specificity. In 2010, the Nobel Prize in Chemistry was awarded jointly to Richard F. Heck, Ei-ichi Negishi, and Akira Suzuki for their roles in discovering and developing highly practical methodologies for C–C bond construction. A particular type of these palladium-catalyzed cross couplings is the so-called Sonogashira reaction, which allows formation under mild conditions of a C–C bond between a terminal alkyne and an aryl or vinyl halide by making use of both palladium and copper catalysts. Accordingly, this sort of reaction enables the facile introduction of a spacer arm in compounds that, like proquinazid, bear an aryl halide in their structure.

Following this approach (Fig 1), an acetylenic derivative of proquinazid (2) was prepared directly from the agrochemical molecule by easy substitution of the iodine atom at the C-6 position of the 4(3H)-quinazolinone ring for the tert-butyl ester of hex-5-ynoic acid (1) in the presence of catalytic amounts of dichloro-bis(triphenylphosphine)palladium (II) and copper (I) iodide at room temperature. The synthesis of hapten PQt was readily completed by acid hydrolysis of the tert-butyl ester moiety of 2 using formic acid at room temperature. The overall yield for the transformation of proquinazid into hapten PQt through this two-step sequence was ca. 78%. Concerning hapten PQs, which has a completely saturated hydrocarbon spacer arm at the same position of the quinazolinone ring, it was straightforward prepared from intermediate 2 in two steps. First, homogeneous catalytic hydrogenation of the triple bond using Wilkinson’s catalyst produced compound 3, which was followed by mild acid hydrolysis of the tert-butyl ester moiety to a carboxylic acid group. The global yield for proquinazid transformation into hapten PQs via this three-step reaction sequence was around 74%.

Activation of the carboxyl moiety of both haptens was easily accomplished using DSC, a procedure that allows obtaining the N-succinimidyl ester derivative of the hapten, i.e. compounds PQt-NHS and PQs-NHS (Fig 1), in high yield. Following purification by conventional column chromatography, the activated haptens were coupled to BSA (immunizing conjugates)
and to OVA and HRP (assay conjugates) with precise hapten-to-protein ratios and avoiding the formation of undesired secondary by-products [4].

Evaluation of the immune response

**Antibody affinity.** The purpose of preparing two haptens—one with a spacer arm containing a triple blond (hapten PQt) and the other one with a fully saturated hydrocarbon chain (hapten PQs)—was to study the effect of linker flexibility on the binding properties of the so-derived antibodies. Two antisera were obtained from each immunizing hapten; those raised from the conjugate of hapten PQs were named PQs#1 and PQs#2, and those generated from hapten PQt were PQt#1 and PQt#2. The ability of each antibody to recognize bioconjugates (OVA—hapten and HRP—hapten) and the free analyte was evaluated by checkerboard competitive screening analysis using both direct and indirect cELISAs. For the direct format, plates were coated with $1/10^4$ and $1/3\times10^4$ dilutions of the antiserum, and next day a range of enzyme tracer concentrations (from 3 to 100 μg/L) was assayed under competitive conditions, i.e., in the presence of a series of proquinazid standard solutions. For the indirect format, plates were coated with 0.1 and 1.0 mg/L OVA—hapten conjugate, and a range of antiserum dilutions (from $1/10^4$ to $1/10^6$) were employed in the competitive step. The performance of each antibody was assessed using both homologous—the hapten in the assay conjugate was the same used to generate the antibody—and heterologous conjugates [20]. The outcome of this study was a collection of inhibition curves for each pair of immunoreagent combinations, and the parameters ($A_{\text{max}}$, slope, and IC$_{50}$ value) from the best inhibition curve ($A_{\text{max}}$ over 0.8 and lower IC$_{50}$ value) for each combination are shown in Table 1 for the direct assay and in Table 2 for the indirect assay.

In the direct format, coating plates with antibodies at a $1/3\times10^4$ dilution resulted in satisfactory inhibition curves, whereas a higher antibody dilution brought about very low signals—with the only exception of antibody PQt#1 which recognized both the homologous and the heterologous enzyme tracers, even though not significant differences in IC$_{50}$ values were observed by changing the assay hapten. In the indirect format, antibodies recognized both coating conjugates, and inhibition curves with sufficient signal were obtained with all immunoreagent combinations.

These results provided compelling evidences of the usefulness of cross-coupling reactions to synthesize proquinazid haptens adequately mimicking the analyte structure, and therefore being appropriate for the production of valuable high-affinity anti-proquinazid antibodies. Regarding the nature of the hydrocarbon spacer arm, results were not clearly conclusive. Globally, the antibodies with the highest affinity to proquinazid were elicited by the hapten bearing

### Table 1. Assay parameters from checkerboard direct cELISA.

| Antibody | HRP—hapten | [Tracer](μg/L) | Antibody dilution | $A_{\text{max}}$ | Slope | IC$_{50}$(μg/L) |
|----------|------------|---------------|------------------|-----------------|-------|----------------|
| PQs#1    | PQs        | 10            | $10^4$           | 1.37            | 0.75  | 33             |
|           | PQt        | 30            | $10^4$           | 1.35            | 0.87  | 38             |
| PQs#2    | PQs        | 30            | $10^4$           | 1.22            | 0.81  | 47             |
|           | PQt        | 100           | $10^4$           | 1.08            | 0.77  | 21             |
| PQt#1    | PQs        | 100           | $10^4$           | _a_             | _a_   | _a_            |
|           | PQt        | 100           | $10^4$           | 0.81            | 0.88  | 20             |
| PQt#2    | PQs        | 10            | $10^4$           | 1.13            | 0.83  | 7              |
|           | PQt        | 10            | $10^4$           | 1.02            | 0.81  | 4              |

*a This tracer was insufficiently recognized by the antibody ($A_{\text{max}}$<0.5)
the more rigid unsaturated linker chain (hapten PQₜ), despite the fact that the replacement of the iodine atom by the triple bond produced a greater modification of the electronic distribution of the dihydroquinazolinone ring atoms—particularly on atom C-5 where the linker was attached—due to resonance effects (Fig 2). The most sensitive combinations were those based on antisera PQ#2, either with the homologous enzyme tracer HRP—PQₜ in the direct format (IC₅₀ = 4 μg/L) or with the heterologous coating antigen OVA—PQₜ in the indirect assay (IC₅₀ = 10 μg/L).

Table 2. Assay parameters from checkerboard indirect cELISA.

| Antibody | OVA—hapten [Conjugate] (μg/L) | Antibody dilution | Aₘₐₓ | Slope | IC₅₀ (μg/L) |
|----------|--------------------------------|-------------------|------|-------|------------|
| PQₕ#1    | PQₕ 100                        | 3×10⁻⁴            | 0.97 | 0.80  | 75         |
|          | PQₜ 1000                       | 10⁵               | 0.78 | 0.63  | 89         |
| PQₕ#2    | PQₕ 100                        | 3×10⁻⁴            | 1.11 | 0.88  | 81         |
|          | PQₜ 1000                       | 3×10⁻⁴            | 0.76 | 0.71  | 43         |
| PQₜ#1    | PQₕ 100                        | 10⁴               | 1.06 | 0.60  | 41         |
|          | PQₜ 100                        | 10⁴               | 1.17 | 0.66  | 50         |
| PQₜ#2    | PQₕ 100                        | 10⁵               | 0.95 | 0.71  | 10         |
|          | PQₜ 100                        | 10⁵               | 1.12 | 0.76  | 13         |

Antibody specificity. Cross-reactivity studies were performed in order to find whether other related compounds could be recognized by the anti-proquinazid antibodies. With this

Fig 2. Partial charges on the atoms in proquinazid and haptons PQₕ and PQₜ. All semi-empirical calculations were performed using the WinMopac 2007 parametric method 3 (PM3) with the MO-G for SCIGRESS program [MO-G Version 1.1, Fujitsu Limited, Tokyo, Japan (2008)]. All initial structures were optimized first by molecular mechanics method (MM3).

![Partial charges on the atoms in proquinazid and haptons PQₕ and PQₜ.](https://doi.org/10.1371/journal.pone.0134042.g002)
aim, concentrations in the $10^{-2}$ nM to $10^4$ nM range of 22 widely used fungicides were assayed: azoxystrobin, pyraclostrobin, dimoxystrobin, picoxystrobin, kresoxim-methyl, trifloxystrobin, fluoxastrobin, fenhexamid, tebuconazole, procimidone, cyazofamid, fenamidone, imidacloprid, tolyfluanid, fludioxonil, boscalid, pyrimethanil, mepanipyrim, cyprodinil, fenpropimorph, propiconazol, and epoxiconazol. It could be concluded that the antibodies were highly specific to proquinazid because no inhibition was observed by any of the evaluated pesticides at the studied concentrations.

**Immunooassay characterization**

Once antibodies were evaluated in terms of affinity and specificity, competitive immunoassays were developed with the most sensitive antiserum (PQ2#2). The influence of different contents of common organic solvents (methanol, ethanol, acetone, and acetonitrile) over the inhibition curve parameters of the proposed immunoassays (see above) was studied (Fig 3).

Variations in $A_{\text{max}}$ and IC$_{50}$ values lower than 20% were deemed as acceptable. Accordingly, satisfactory $A_{\text{max}}$ values in the direct cELISA were found for all evaluated solvents at concentrations up to 10%, whereas IC$_{50}$ values significantly increased at concentrations higher than 2%, with acetonitrile arising as the most tolerated solvent. Concerning the indirect assay, methanol was the solvent with a lower influence over calibration curve parameters—concentrations lower than 10% had essentially no effect. Ethanol and acetone can be employed up to 5% and 2%, respectively, while acetonitrile strongly affects the indirect assay, so its use should be kept to a minimum or even avoided. All together, these results show that the direct assay is more robust and tolerates better the presence of organic solvents than the indirect assay. The direct

![Fig 3. Variation (%) of $A_{\text{max}}$ and IC$_{50}$ values with the contents of organic solvents in the assay buffer. The white area sets the limits of acceptable working concentrations.](https://doi.org/10.1371/journal.pone.0134042.g003)
cELISA seems to be particularly adequate for the analysis of solid samples, which usually require acetonitrile extraction prior to analysis, whereas the indirect assay might be more suitable for the analysis of alcohol-containing food matrices, such as beer, wine, or spirit samples.

The inhibition curves obtained using buffers at different pH and ionic strength ($I$) values perfectly fitted to a sigmoidal equation. However, in the case of extreme pH and $I$ conditions, analytical parameters deviated from those found at neutral conditions (Fig 4). Thus, in the case of the direct cELISA, $A_{\text{max}}$ decreased at basic pH, while $IC_{50}$ increased using buffers with low pH and high $I$ values. Regarding the indirect format, $A_{\text{max}}$ decreased under any condition slightly differing from PBS-T buffer, and $IC_{50}$ was strongly influenced by the concentration of salts in the assay buffer. As it was also observed when studying the influence of organic solvents, it can be concluded that the proquinazid direct cELISA was more tolerant to changes in the buffer composition than the indirect assay.

Analysis of samples

Final assay conditions, parameters of the inhibition curves, and other analytical properties of the proposed immunoassays are shown in Fig 5. The limit of detection (LOD) was estimated as the $IC_{10}$ value of the inhibition curve (analyte concentration that provided a 10% inhibition of $A_{\text{max}}$), with values of 0.2 and 0.7 $\mu$g/L for the direct and the indirect cELISA, respectively.

Since proquinazid is a highly recommended product against powdery mildew infestation in vineyards, white and red grape musts were selected as model commodities in order to evaluate the analytical performance of the proposed antibody-based assays. With this aim, commercial must samples were firstly diluted with deionized water (1/5, 1/25, 1/50, 1/250, and 1/500), and...
Proquinazid standard curves were prepared in every diluted matrix and run in the optimized direct and indirect immunoassays. In both formats, inhibition curves made in must diluted 1/25 and those prepared in buffer were indistinguishable (results not shown), so this minimum dilution factor was chosen for further work in order to diminish matrix effects.

Recovery studies with the optimized cELISAs were performed using red and white must samples which had been spiked with proquinazid at 10, 50, 100, and 1000 μg/L. Adequate recovery values were found for grape musts down to 10 and 50 μg/L for direct and indirect cELISAs, respectively (Table 3). Concerning precision, relative standard deviations (RSDs) lower than 20% were obtained with both immunoassays.

Finally, blind-spiked grape musts were prepared and determined by the proposed direct and indirect cELISAs, as well as by a reference procedure based in QuEChERS extraction and GC-MS analysis (Table 4). Comparison of the results obtained by each cELISA with those obtained...
by the instrumental method was performed using Deming method, which takes into account the experimental errors of every method (MedCalc Software bvba, Ostend, Belgium). The obtained regression curves were: 

\[
[PQ]_{\text{Direct cELISA}} = (2 \pm 5) + (1.00 \pm 0.05) [PQ]_{\text{GC-MS}} \quad (n = 16),
\]

and 

\[
[PQ]_{\text{Indirect cELISA}} = (-5 \pm 10) + (0.98 \pm 0.04) [PQ]_{\text{GC-MS}} \quad (n = 12).
\]

In both cases, results showed that there were not significant differences between the slope and 1 and between the

### Table 3. Recovery values and relative standard deviations (RSD) obtained in the analysis of spiked grape must samples by direct and indirect cELISAs with antibody PQ\#2.

| Sample   | Spiked PQ\(^a\) (µg/L) | Direct cELISA | Indirect cELISA |
|----------|-------------------------|---------------|-----------------|
|          | [PQ] (µg/L ± s, n = 3) | Recovery (%)  | RSD (%)         | [PQ] (µg/L ± s, n = 3) | Recovery (%) | RSD (%) |
| White    |                         |               |                 |                        |              |         |
| must     | 10                      | 8 ± 2         | 84              | 19.9                   |               |         |
|          | 50                      | 47 ± 3        | 94              | 7.1                    | 49 ± 3        | 97       | 6.0     |
|          | 100                     | 94 ± 12       | 94              | 12.6                   | 106 ± 10      | 106      | 10.7    |
|          | 1000                    | 940 ± 80      | 94              | 8.1                    | 862 ± 90      | 86       | 10.1    |
| Red      |                         |               |                 |                        |              |         |
| must     | 10                      | 10 ± 2        | 104             | 17.5                   |               |         |
|          | 50                      | 49 ± 1        | 98              | 2.6                    | 50 ± 8        | 101      | 16.8    |
|          | 100                     | 104 ± 4       | 104             | 3.6                    | 88 ± 9        | 88       | 9.7     |
|          | 1000                    | 960 ± 60      | 96              | 6.4                    | 820 ± 60      | 82       | 7.4     |

\(^a\) PQ stands for proquinazid.

\(^b\) Below assay detectability

do:i:10.1371/journal.pone.0134042.t003

### Table 4. Analysis of proquinazid contents in blind-spiked must samples by chromatographic and immunochemical methods.

| Sample \(^a\) | [proquinazid] (µg L\(^{-1}\) ± s, n = 3) |
|---------------|------------------------------------------|
|               | GC—MS | Indirect cELISA | Direct cELISA |
| W1            | 12 ± 1 | 14 ± 3           | — \(^b\)     |
| W2            | 18 ± 1 | 27 ± 9           | —             |
| W3            | 22 ± 2 | 28 ± 4           | —             |
| W4            | 53 ± 4 | 60 ± 10          | 70 ± 20       |
| W5            | 80 ± 4 | 84 ± 8           | 100 ± 20      |
| W6            | 200 ± 20 | 211 ± 8        | 200 ± 20      |
| W7            | 420 ± 20 | 390 ± 70       | 450 ± 50      |
| W8            | 530 ± 30 | 470 ± 70       | 500 ± 40      |
| R1            | 47 ± 2 | 46 ± 7           | —             |
| R2            | 51 ± 3 | 55 ± 6           | 50 ± 10       |
| R3            | 82 ± 5 | 83 ± 9           | 60 ± 10       |
| R4            | 200 ± 10 | 200 ± 9        | 120 ± 30      |
| R5            | 240 ± 20 | 223 ± 8        | 190 ± 20      |
| R6            | 410 ± 30 | 430 ± 60       | 340 ± 60      |
| R7            | 610 ± 30 | 620 ± 20       | 610 ± 40      |
| R8            | 570 ± 40 | 620 ± 90       | 540 ± 30      |

\(^a\) The letter “W” refers to must samples from white grapes, whereas the letter “R” refers to must samples from red grapes.

\(^b\) Below LOD.

do:i:10.1371/journal.pone.0134042.t004
intercept and 0, at a 95% confidence level, so we could conclude that cELISAs results were statistically comparable to those of the GC—MS method.

Conclusions
Taking advantage of the Sonogashira cross-coupling reaction, two functionalized derivatives of proquinazid were synthesized by replacing the aryl iodine atom by an alkynyl carboxylate hydrocarbon chain and the corresponding saturated one—haptens PQt and PQs, respectively—in order to figure out whether these mimics were suitable haptens for specific and high-affinity antibody generation to this model compound. Following rabbit immunization, antibodies able to efficiently bind proquinazid were elicited from both haptens, thus demonstrating the suitability of this simple and efficient synthetic strategy for immunoreagent preparation to compounds bearing an aryl halide. Importantly, the haptens and antibodies herein described are the first ever reported immunoreagents for proquinazid analysis. Following antibody characterization and assay development, competitive immunoassays were employed to determine proquinazid residues in grape musts, and their analytical performance was satisfactorily validated by comparison with GC—MS.

Acknowledgments
The assistance of Rosemary Travers in hapten synthesis is greatly acknowledged.

Author Contributions
Conceived and designed the experiments: AAS AAF. Performed the experiments: FAET JVM JP CA. Analyzed the data: FAET JVM AAS AAF. Wrote the paper: FAET AAS AAF. Supervised the project: AAS AAF.

References
1. Johansson Seechurn CC, Kitching MO, Colacot TJ, Snieckus V. Palladium-catalyzed cross-coupling: a historical contextual perspective to the 2010 Nobel Prize. Angew Chem Int Ed. 2012; 51(21): 5062–5085. doi:10.1002/anie.201107017
2. Chinchilla R, Nájera C. Recent advances in Sonogashira reactions. Chem Soc Rev. 2011; 40(10): 5084–5121. doi: 10.1039/c1cs15071e PMID: 21655588
3. Suárez-Pantaleón C, Mercader JV, Agulló C, Abad-Somovilla A, Abad-Fuentes A. Hapten synthesis and polyclonal antibody-based immunoassay development for the analysis of forchlorfenuron in kiwi-fruit. J Agric. Food Chem. 2010; 58(15): 8502–8511. doi: 10.1021/jf101660z PMID: 20681638
4. Esteve-Turrillas FA, Parra J, Abad-Fuentes A, Agulló C, Abad-Somovilla A, Mercader JV. Hapten synthesis, monoclonal antibody generation, and development of competitive immunoassays for the analysis of picoxystrobin in beer. Anal Chim Acta. 2010; 682(1–2): 93–103. doi: 10.1016/j.aca.2010.09.042 PMID: 21056720
5. Mercader JV, Agulló C, Abad-Somovilla A, Abad-Fuentes A. Synthesis of site-heterologous haptens for high-affinity anti-pyraclostrobin antibody generation. Org Biomol Chem. 2011; 9(5): 1443–1453. doi: 10.1039/c0ob00688f PMID: 21223057
6. Parra J, Mercader JV, Agulló C, Abad-Fuentes A, Abad-Somovilla A. Concise and modular synthesis of regioisomeric haptens for the production of high-affinity and stereoselective antibodies to the strobilurin azoxystrobin. Tetrahedron. 2011; 67(3): 624–635. doi: 10.1016/j.tet.2010.11.054
7. Esteve-Turrillas FA, Mercader JV, Agulló C, Abad-Somovilla A, Abad-Fuentes A. Mepanipyrim haptens and antibodies with nanomolar affinity. Analyst. 2013; 138(12): 3360–3364. doi: 10.1039/c3an00228d PMID: 23666476
8. López-Moreno R, Mercader JV, Agulló C, Abad-Somovilla A, Abad-Fuentes A. Structure-immunogenicity relationship of kresoxim-methyl regioisomeric haptens. Org Biomol Chem. 2013; 11: 7361–7371. doi: 10.1039/C3OB41570H PMID: 24061390
9. López-Moreno R, Mercader JV, Agulló C, Abad-Somovilla A, Abad-Fuentes A. Immunoassays for trifloxystrobin analysis. Part I. Rational design of regioisomeric haptens and production of monoclonal antibodies. Food Chem. 2014; 152: 230–236. doi:10.1016/j.foodchem.2013.11.150 PMID: 24444931

10. Esteve-Turrillas FA, Mercader JV, Agulló C, Marzo J, Abad-Somovilla A, Abad-Fuentes A. Design and development of heterologous competitive immunoassays for the determination of boscalid residues. Analyst. 2014; 139(14): 3636–3644. doi:10.1039/c3an01104f PMID: 24893153

11. Ceballos-Alcantarilla E, Abad-Fuentes A, Aloisio V, Agulló C, Abad-Somovilla A, Mercader JV. Haptns, bioconjugates, and antibodies for penthiopyrad immunosensing. Analyst. 2014; 139(21): 5358–5361. doi:10.1039/C4an00828f PMID: 25197742

12. Jeschke P. The Unique Role of Halogen Substituents in the Design of Modern Crop Protection Compounds. In: Jeschke P, Krämer W, Schirmer U, Witschel M, editors. Modern methods in crop protection Research. Weinheim: Wiley-VCH Verlag & Co. KGaA; 2013. pp. 73–128. doi:10.1002/9783527655908.ch4

13. EFSA. Conclusion on the peer review of the pesticide risk assessment of the active substance proquinazid on request from the European Commission. EFSA J. 2009; 7(10). doi:10.2903/j.efsa.2009.1350

14. APVMA. Public release summary on the evaluation of the new active proquinazid in the product Dupont Talendo fungicide. Available: http://archive.apvma.gov.au/registration/assessment/docs/prs_proquinazid.pdf. Accessed 4 March 2015.

15. Genet JL, Jaworska G. Baseline sensitivity to proquinazid in Blumeria graminis f. sp. tritici and Erysiphe necator and cross-resistance with other fungicides. Pest Manag Sci. 2009; 65(8): 878–884. doi: 10.1002/ps.1768 PMID: 19418441

16. Selby TP, Sternberg CG, Bereznak JF, Coats RA, Marshall EA. The discovery of proquinazid: A new and potent powdery mildew control agent. In: Lyga JW, Theodoridis G, editors. Synthesis and chemistry of agrochemicals VII. Washington: American Chemical Society; 2007. pp. 209–222.

17. Bereznak JF, Marshall EA, Sternberg CG, Sternberg JA, Sun KM. Preparation of fungicidal quinazolines and useful intermediates. US Pat 6166208A; 1997.

18. Abad-Fuentes A, Esteve-Turrillas FA, Agulló C, Abad-Somovilla A, Mercader JV. Development of competitive enzyme-linked immunosorbent assays for boscalid determination in fruit juices. Food Chem. 2012; 135(1): 276–284. doi:10.1016/j.foodchem.2012.04.090

19. Lehotay SJ, Son KA, Kwon H, Koessukwiwat U, Fu W, Mastovska K, et al. Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. J Chromatogr A. 2010; 1217(16): 2548–2560. doi:10.1016/j.chroma.2010.01.044 PMID: 20144460

20. Mercader JV, Parra J, Esteve-Turrillas FA, Agulló C, Abad-Somovilla A, Abad-Fuentes A. Development of monoclonal antibody-based competitive immunoassays for the detection of picoxystrobin in cereal and oilseed flours. Food Control. 2012; 26(1): 162–168. doi:10.1016/j.foodcont.2012.01.021