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COMMUNICATION

Rapid immunochemical methods for the analysis of proquinazid in strawberry QuEChERS extracts

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Abstract

Proquinazid is a new-generation fungicide authorized in the EU for combating powdery mildew infections in high-value crops. Due to the perishable nature of fruits, alternative analytical methods are necessary to protect consumer's health from pesticide residues. Currently, immunoassays are a well-established approach for rapidly monitoring chemical contaminants. However, the production of high-quality immunoreagents, such as antibodies and bioconjugates, is essential. This study presents a newly designed hapten that maintains the characteristic moieties of proquinazid unmodified. The linear aliphatic substituents of this molecule were used to introduce the spacer arm. A three-step synthesis strategy was optimized to prepare a hapten that displays the entire 6-iodoquinazolin-4(3H)-one moiety with excellent yields. The *N*-hydroxysuccimidyl ester of the hapten was activated and purified to prepare a protein conjugate with high hapten density, which was used as an immunogen. Antibodies were raised and competitive enzyme-linked immunosorbent assays were developed. To enhance the assay's sensitivity, two additional heterologous haptens were prepared by modifying the halogenated substituent at C-6. The optimized assays demonstrated low limits of detection in buffer, approximately 0.05 µg/L. When applied to the analysis of proquinazid in QuEChERS extracts of strawberry samples, the immunoassays produced precise and accurate results, particularly in the 10–1000 µg/kg range.

Keywords Hapten design · ELISA · Residue analysis · Powdery mildew · Strawberry

Introduction

Proquinazid is an effective fungicide utilized for controlling powdery mildew in strawberry fields, primarily caused by *Podosphaera aphanis* and *Podosphaera macularis* [1, 2]. This plague is particularly severe in greenhouse crops, where a hot and humid environment favors the spread of the fungal infection [3]. Developed by DuPont in 1993, proquinazid is still the only member of the quinazolinone family. This compound is highly lipophilic, allowing it to easily penetrate the cuticle of leaves and fruits, thus increasing its resistance to washing. Although the precise target site of this agrochemical is not fully known, it appears to interfere with the signal transduction between fungal and plant cells [4]. This unique mode of action makes proquinazid an ideal candidate for combined pest management strategies aimed at controlling pests and preventing fungal resistance. While proquinazid exhibits low toxicity to mammals, it has been reported to be highly toxic to aquatic organisms [5]. Nowadays, the European maximum residue limits (MRLs) for this fungicide in

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In honor of Professor María Cruz Moreno Bondi.

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strawberry, blueberry, and grape samples are 2.0, 1.5, and 0.5 mg/kg, respectively (https://food.ec.europa.eu/plants/pesticides/eu-pesticides-database_en).

Immunochemical methods currently constitute valuable analytical approaches for chemical contaminant and residue analysis, providing rapid, sensitive, and reliable results. These methods can be used for screening and in situ determination of positive samples at low cost. Presently, the food industry employs immunoassays as complementary techniques to instrumental chromatographic methods to comply with legislation and extend the shelf life of its products. Moreover, both immunochemical and chromatographic analytical methods are useful for official quality control laboratories to ensure consumer's health. To our knowledge, the only reported study concerning immunoassay development for proquinazid analysis was published by our research group [6]. In that study, two haptens were prepared through a straightforward synthesis using the Sonogashira cross-coupling reaction. However, replacing the iodine atom with a hydrocarbon spacer arm significantly modified the electronic density of the aromatic moiety, resulting in the production of antibodies with moderate affinity. It is worth noting that the proquinazid molecule contains two aliphatic, *n*-propyl moieties (Fig. 1) that could serve as ideal positions for the spacer arm. A linear aliphatic linker would perfectly mimic such chemical moieties without significantly altering the electronic and conformational properties of the proquinazid aromatic nucleus.

In the present study, a novel, rationally designed synthetic strategy was conceived to prepare the immunizing hapten. The propoxy group at C-2 of proquinazid was replaced with a linear, six-atom-long hydrocarbon spacer functionalized with an activatable carboxyl group. This substitution effectively incorporates a carboxyethyl moiety ($CH_2CH_2CO_2H$) at the end of the propoxy group via a C–C single bond. In this way, the highly immunodeterminant and characteristic 6-iodoquinazolin-4-one moiety is positioned distally to the hapten-to-protein tethering site, which theoretically represents the optimal design for an immunizing hapten. The aims of the present study were to evaluate the immunogenicity of this novel hapten and to develop sensitive immunoassays for proquinazid using heterologous assay haptens (structurally different from the immunizing hapten) in which the iodine atom was replaced by a fluorine or hydrogen atom. Two competitive enzyme-linked immunosorbent assays (cELISA) were characterized and optimized. Additionally, the applicability of these novel immunoassays was demonstrated through the analysis of proquinazid in a relevant food product.

Materials and methods

General procedures, reagents, and equipment

Organic solvents were dried and distilled prior to use following standard techniques. THF was distilled over Na and benzophenone under N_2 atmosphere just before use. CH_2Cl_2 and CH_3CN were distilled from CaH_2 in the same way. The remaining solvents and commercial reagents were used without prior purification. The operations with air and/or moisture-sensitive reagents were carried out under an inert atmosphere of dry N_2, using syringes and/or cannulas, oven-dried (130 °C) glass material, and freshly distilled and dried solvents. Deuterated solvents for NMR experiments were purchased from Merck (Darmstadt, Germany) and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on precoated silica plates (0.25 mm layer thickness, Silica Gel 60 F254) using UV light as the visualizing agent and ethanolic phosphomolybdic acid or aqueous ceric ammonium molybdate solutions and heat as developing agents. The synthesized compounds, excluding those showing a purity above 95% by NMR, were purified by flash column chromatography using silica gel 60 (particle size 0.043–0.063 mm). $^{1}H/^{13}C$ NMR spectra were recorded at 298 °K in the indicated solvent

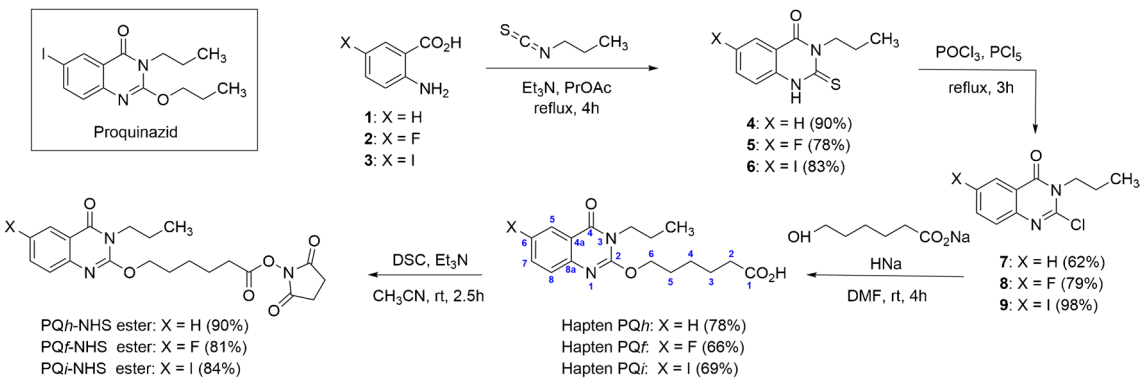

Fig. 1 Synthesis of haptens PQ*h*, PQ*f*, and PQ*i*, and the corresponding *N*-hydroxysuccinimidyl esters (PQ*h*-NHS, PQ*f*-NHS, and PQ*i*-NHS)

on a Bruker DRX-300 (300/75 MHz) or Bruker Avance DRX500 (500/126 MHz) spectrometer. The chemical shifts are expressed in ppm (δ scale) relative to the residual solvent for ^{1}H ($CHCl_3$ at 7.26 ppm and CD_3SOCD_3 at 2.50 ppm) or to the central peak of solvent ^{13}C signal ($CDCl_3$ at 77.0 ppm and CD_3SOCD_3 at 39.52). Carbon substitution degrees were established by DEPT pulse sequences. Assignment of ^{1}H and ^{13}C chemical shifts was determined by COSY and HSQC experiments in most cases. The abbreviations used for NMR data are as follows: s, singlet; d, doublet; dd, double doublet; ddd, double doublet of doublets; t, triplet; dt, double triplet; m, multiplet; br, broad; Qz, quinazolinone ring. High-resolution mass spectra (HRMS) were recorded by the electrospray (ES) ionization mode using a Micromass VG Autospec spectrometer.

Standard Pestanal-grade proquinazid (IUPAC 6-iodo-2-propoxy-3-propylquinazolin-4(3H)-one; CAS number 189278–12-4; Mw 372.2 g/mol) was purchased from Merck (Darmstadt, Germany). Proteins and other biochemical reagents for immunochemical studies were as described in previous studies [6]. Horseradish peroxidase conjugate with goat anti-rabbit immunoglobulins polyclonal antibody (HRP–GAR) was obtained from BioRad (Hercules, CA, USA). Costar flat-bottom high-binding 96-well polystyrene ELISA plates from Corning (Corning, NY, USA) were used. Sephadex G-25 HiTrap® Desalting columns for protein–hapten conjugate purification and Sepharose HiTrap® Protein G HP columns for antibody purification were obtained from GE Healthcare (Uppsala, Sweeden) and operated under an ÄKTA Purifier workstation also from GE Healthcare. Absorbances from ELISA microplates were read with a PowerWave HT from BioTek Instruments (Winooski, VT, USA). Microwells were washed with an ELx405 microplate washer also from BioTek Instruments.

Synthesis of haptens

The synthesis of haptens PQ*h*, PQ*f*, and PQ*i* is schematized in Fig. 1. Details about chemicals, general experimental techniques, and equipment are provided in the Electronic Supplementary Material. Intermediates **4**–**9** were prepared according to similar procedures used in previous studies [7–9]. Details of their syntheses and structural characterization data are given in the Electronic Supplementary Material. Additionally, the structural characterization data of the haptens and the corresponding active esters, as well as copies of the NMR spectra, are included in the Electronic Supplementary Material.

Preparation of 6-((4-oxo-3-propyl-3,4-dihydroquinazolin-2-yl)oxy)hexanoic acid (Hapten PQh) A mixture of sodium 6-hydroxyhexanoate (261.5 mg, 1.696 mmol, 3 equiv) and NaH (60% dispersion in mineral oil, 90.5 mg, 2.242 mmol, 4 equiv) contained in a 10-mL round-bottom flask was washed with anhydrous pentane under nitrogen. The flask was fluxed with nitrogen for a few minutes, cooled in an ice bath, and anhydrous DMF (4 mL) was added while it was being stirred under nitrogen. After the evolution of hydrogen ceased, a solution of 2-chloro-quinazolinone **7** (125.9 mg, 0.565) in DMF (2.5 mL) was added dropwise and the mixture was allowed to warm up to room temperature and stirred for 4 h. Then, the reaction mixture was cooled in an ice-water bath and quenched by the addition of a 5% aqueous solution of $KHSO_4$ and extracted with Et_2O. The combined organic layers were successively washed with H_2O, a 5% aqueous solution of LiCl and brine, and dried over anhydrous $MgSO_4$. Chromatographic purification on silica gel of the residue obtained after evaporation of the solvent, using $CHCl_3$–MeOH 95:5 as eluent, afforded hapten PQ*h* (140.1 mg, 78%).

Preparation of 6-((6-fluoro-4-oxo-3-propyl-3,4-dihydroquinazolin-2-yl)oxy)hexanoic acid (Hapten PQf) The hapten PQ*f* was prepared following the same procedure described above for the preparation of hapten PQ*h*, using sodium 6-hydroxyhexanoate (224.8 mg, 1.458 mmol, 2.8 equiv) and NaH (60% dispersion in mineral oil, 77.8 mg, 1.945 mmol, 3.7 equiv) in anhydrous DMF (3.5 mL) and 2-chloro-quinazolinone **8** (125 mg, 0.519 mmol) in the same DMF (2.3 mL). In this case, the reaction was conducted at room temperature for 5 h and the crude product obtained after the work up of the reaction mixture was purified by chromatography, using $CHCl_3$ and $CHCl_3$–MeOH 98:2 as eluent, to afford hapten PQ*f* (115.5 mg, 66%).

Preparation of 6-((6-iodo-4-oxo-3-propyl-3,4-dihydroquinazolin-2-yl)oxy)hexanoic acid (Hapten PQi) The hapten PQ*f* was also prepared following the same procedure described above for the preparation of hapten PQ*h*, using sodium 6-hydroxyhexanoate (115.6 mg, 0.750 mmol, 3 equiv) and NaH (60% dispersion in mineral oil, 40 mg, 1.00 mmol, 4 equiv) in anhydrous DMF (3 mL) and 2-chloro-quinazolinone **9** (86.0 mg, 0.247 mmol) in DMF (1.5 mL). The reaction mixture was stirred at room temperature for 4 h and worked up using the procedure described for the analogous reaction of **7**. Chromatographic purification of *the crude* product, using $CHCl_3$ as eluent, gave hapten PQ*i* (75.4 mg, 69%).

Synthesis of the *N*-hydroxysuccinimidyl esters

The free carboxylic group of haptens PQ*h*, PQ*f*, and PQ*i* was activated by its transformation into the corresponding *N*-hydroxysuccinimidyl ester as described below (Fig. 1).

Preparation of the N-hydroxysuccinimidyl ester of hapten PQh (PQh-NHS) The hapten PQ*h* (25.6 mg, 0.080 mmol) and *N,N′*-disuccinimidyl carbonate (DSC, 28 mg, 0.109 mmol, 1.3 equiv) were dissolved in anhydrous CH_3CN (1 mL) at 0 °C under nitrogen. Anhydrous Et_3N (32.7 mg, 45 µL, 0.322 mmol, 4 equiv) was added and the reaction mixture was stirred at 0 °C for 2.5 h. Then, the reaction mixture was concentrated under reduced pressure without heating to give an oily residue that was purified by column chromatography, using $CHCl_3$ as eluent, to afford the *N*-hydroxysuccinimidyl ester of the hapten (PQ*h*-NHS, 30.1 mg, 90%).

Preparation of the N-hydroxysuccinimidyl ester of hapten PQf (PQf-NHS) This active ester was prepared (21 mg, 81%) from hapten PQ*f* (20 mg, 0.059 mmol), DSC (23 mg, 0.089 mmol, 1.5 equiv), and Et_3N (24.1 mg, 33 µL, 0.238 mmol, 4 equiv) in anhydrous CH_3CN (1 mL), as described above for the active ester of hapten PQ*h*.

Preparation of the N-hydroxysuccinimidyl ester of hapten PQi (PQi-NHS) This active ester was prepared (29.5 mg, 84%) from hapten PQ*i* (28.9 mg, 0.065 mmol), DSC (22.3 mg, 0.087 mmol, 1.3 equiv), and Et_3N (26.3 mg, 36 µL, 0.195 mmol, 4 equiv) in anhydrous CH_3CN (1 mL), as described above for the active ester of hapten PQ*h*.

Bioconjugate preparation

The immunizing conjugate was prepared by adding dropwise the purified activated hapten PQ*i* (PQ*i*-NHS) in DMF (50 mM) over a 15 mg/mL bovine serum albumin (BSA) solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. A 22-fold molar excess of hapten was used. The reaction mixture was gently stirred for 2 h at room temperature, and the resulting bioconjugate was purified by size exclusion chromatography using 100 mM phosphate buffer, pH 7.4, as the eluent. The fractions containing the BSA conjugate were collected, pooled, diluted with elution buffer, and filter sterilized. The conjugate was stored frozen at − 20 °C. The hapten-to-protein molar ratio (MR) of the bioconjugate obtained was determined by MALDI-ToF–MS (see the Electronic Supplementary Material Fig. S1).

To prepare the coating conjugates, the corresponding purified activated hapten in DMF (50 mM) was added dropwise to a 15 mg/mL ovalbumin (OVA) solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. The conjugation was carried out with 2 µmol of hapten and 0.68 µmol of protein. The reaction mixture was gently stirred for 2 h at room temperature, and the resulting bioconjugates were purified by size exclusion chromatography using 100 mM phosphate buffer, pH 7.4, as the eluent. The fractions comprising the OVA conjugate were collected, pooled, and diluted with elution buffer containing 0.01% (w/v) thimerosal. Subsequently, the conjugate was stored frozen at − 20 °C. The hapten-to-protein MR of these conjugates was determined as before (see the Electronic Supplementary Material Fig. S2).

Enzyme tracer conjugates were prepared by adding dropwise the corresponding purified activated hapten in DMF (5 mM) to a 2.2 mg/mL horseradish peroxidase (HRP) solution in 50 mM carbonate–bicarbonate buffer, pH 9.6. A tenfold molar excess of hapten was used for conjugation. The reaction mixture was gently stirred for 2 h at room temperature, and the resulting bioconjugate was purified by size exclusion chromatography using 100 mM phosphate buffer, pH 7.4, as the eluent. The fractions comprising the enzyme tracer were collected, pooled, and twofold diluted with elution buffer containing 2% (w/v) BSA and 0.02% (w/v) thimerosal. The conjugate was stored at 4 °C, and the hapten-to-protein MR was calculated as described above (see the Electronic Supplementary Material Fig. S3).

MALDI mass spectrometry analysis of bioconjugates

For sample preparation, 100 µL of bioconjugates (0.5–1 mg/mL) was dialyzed against Milli-Q water and lyophilized. The samples were dissolved in Milli-Q water to a theoretical final concentration of 1 mg/mL, and 0.8 µL was spotted onto a Ground-Steel plate. After the droplet was air-dried at room temperature, ca. 0.8 µL of matrix (10 mg/mL sinapinic acid in TA30) was added and allowed to air-dry at room temperature. Then, samples were analyzed in a UltrafleXtreme apparatus of Bruker using a linear method between 20 and 150 kDa in positive mode with a laser repetition rate of 1000 Hz for the generation of MS spectra. Every sample was calibrated by "close external calibration" method with a BSA, OVA, or HRP spectrum acquired in a close position. The analysis of the results was performed using the mMass program (http://www.mmass.org/).

Antibody generation

Antisera were obtained from the blood of rabbits that were immunized with conjugate BSA–PQ*i*, following the protocol described in previous studies [6]. All experiments involving laboratory animals were conducted in accordance with procedure number A1329731961154, approved by the Bioethics Committee of the University of Valencia, and adhered to guidelines established by the Spanish Ministerio de Agricultura, Pesca y Alimentación and European Directive 2010/63/EU concerning the handling and the protection of animals used for scientific purposes.

Antibody-coated direct assays

Microplates were coated by overnight incubation at room temperature with 100 µL per well of antibody solution

in 50 mM carbonate–bicarbonate buffer, pH 9.6 (coating buffer). Following each incubation step, plates were washed four times with washing solution (150 mM NaCl with 0.05% (v/v) Tween 20). The competitive reaction was initiated by adding 50 µL per well of proquinazid solution in PBS (10 mM phosphate buffer, pH 7.4, containing 140 mM NaCl) and 50 µL per well of enzyme tracer solution in PBS-T (PBS containing 0.05% (v/v) Tween-20). After incubation at room temperature for 1 h, the plates were washed again, and the retained peroxidase activity was revealed by adding 100 µL per well of a 2 mg/mL *o*-phenylendiamine solution in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4, containing 0.012% (v/v) H_2O_2. After a 10-min incubation at room temperature, the enzymatic reaction was stopped with 100 µL per well of 1 M H_2SO_4. The absorbance was read at 492 nm, with a reference wavelength of 650 nm.

Conjugate-coated indirect assays

Plates were coated by overnight incubation at room temperature with 100 µL per well of OVA conjugate solution (100 µg/L) in coating buffer. After each incubation step, microwells were washed four times with washing solution. The competitive immunochemical reaction was initiated by mixing 50 µL per well of proquinazid solution in PBS and 50 µL per well of antibody solution in PBS-T, followed by incubation of the plates during 1 h at room temperature. The retained antibody was indirectly detected by adding 100 µL per well of an HRP–GAR solution ($1/10^4$) in PBS-T and incubating at room temperature for 1 h. Finally, the signal was developed and the absorbance was measured as described for the direct assay format.

Sample preparation and data analysis

Eight-point calibration curves, including a blank, were built using the corresponding analyte solutions prepared by serial dilution in buffer. The most concentrated standard solution was derived from a proquinazid stock solution at 1 mg/mL in DMF. A four-parameter logistic equation was fitted to the experimental values to obtain calibration curves. Data processing and graphical representation were carried out using SigmaPlot v14.5 software from SPSS Inc. (Chicago, IL, USA). Strawberry samples were ground and homogenized with a blender. Proquinazid was extracted from strawberry samples using a QuEChERS dispersive kit from Agilent Technologies (Santa Clara, CA, USA), following European guidelines for the analysis of pesticide residues in food (European Committee for Standardization, Standard Method EN 15662) [10]. The half-maximal inhibitory concentration (IC_{50}) of proquinazid was used to estimate immunoassay sensitivity. A maximum absorbance (A_{max}) around 1.0 was considered optimal for immunoassay development. The limit of quantification (LOQ) was defined as the lowest concentration at which the recovery rates were between 80 and 120% and the coefficient of variation (CV) was below 20% [11].

Sample analysis by GC–MS

QuEChERs extracts were filtered using Teflon filters (0.22 µm) and subsequently analyzed by gas chromatography–mass spectrometry (GC–MS) following previously established methods [6]. Briefly, 1 µL of the extract was injected in splitless mode at 300 °C. The oven temperature was kept at 150 °C during 1 min, then ramped up to 280 °C at a rate of 10 °C/min and held at this temperature during 2 min. Electron impact ionization was carried out at 70 eV. Quantification ions were monitored at 330 and 288 m/z for proquinazid and 325 and 326 m/z for the internal standard (TPP).

Results and discussion

Hapten synthesis

The synthesis of the three haptens (PQ*h*, PQ*f*, and PQ*i*) was achieved in just three straightforward steps using a common strategy based on the initial preparation of the 2-chloroquinazolinone nucleus with the appropriate substitution at the C-6 position, *i.e.*, H, F, or I, respectively (Fig. 1). The key intermediates in the synthesis of the haptens, the 2-chloro-quinazolinones **7**, **8**, or **9**, were prepared from the appropriate commercial anthranilic acids (**1**, **2**, or **3**, respectively). The first step involved forming the quinazoline ring through a condensation reaction between the appropriate anthranilic acid and n-propyl isothiocyanate, yielding the corresponding 2-thioxo-2,3-dihydroquinazolinone (**4**, **5**, or **6**). Subsequently, this intermediate underwent nucleophilic aromatic substitution of the sulfur atom at C-2 with a chlorine atom through reaction with phosphorus oxychloride.

Finally, the synthesis of each hapten was completed by incorporating the spacer arm at position C-2. This was achieved by introducing a 5-(carboxypentyl)oxy moiety through another nucleophilic aromatic substitution of the chlorine atom of **7**, **8**, or **9** with the sodium alkoxide derived from the reaction of commercial sodium 6-hydroxyhexanoate with NaH in DMF. The three steps proceed with good yields, resulting in haptens PQ*h*, PQ*f*, and PQ*i* with overall yields of 44%, 41%, and 56%, respectively. NMR spectroscopy and high-resolution mass spectrometry were employed to structurally characterize all of the haptens and intermediates of their synthesis (see the "Experimental" section and the Electronic Supplementary Material). As expected, the partial charges of all equivalent atoms in hapten PQ*i* were essentially identical to those of proquinazid (Fig. 2),

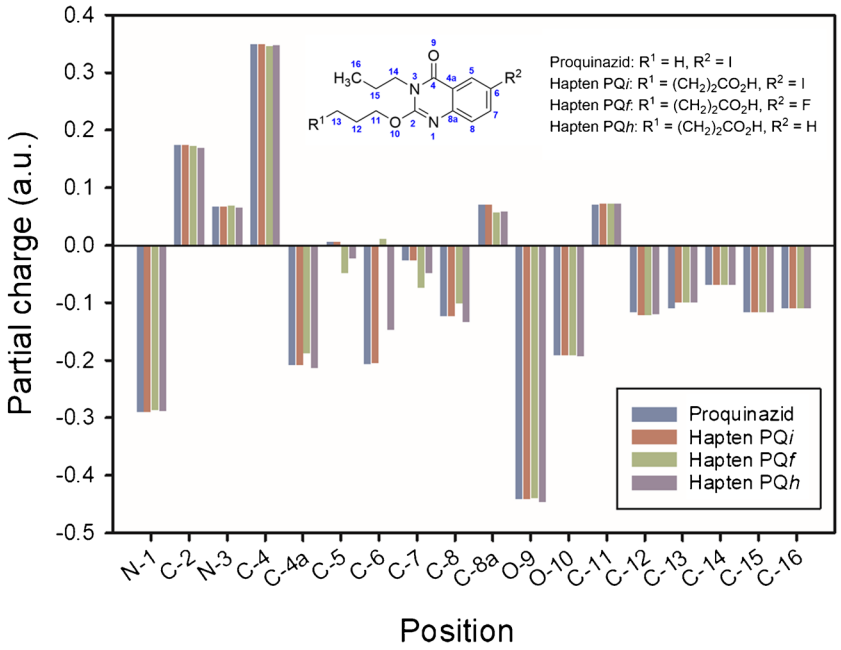

Fig. 2 Partial charges on the equivalent atoms (except hydrogen atoms) of proquinazid and haptens PQ*i*, PQ*f*, and PQ*h*. Calculations were performed using Molecular Mechanics (MM3) as implemented in the SCIGRESS program (MO-G Version 1.1, Fujitsu Limited, Tokyo, Japan (2008)). A systematic conformational search was performed (all rotatable bonds were rotated by 15-degree steps) and the geometry of the generated most stable conformation was refined by performing an optimized geometry calculation in MOPAC using the semi-empirical method PM3, including the solvent effects of water as simulated by the COSMO solvation model

Therefore, this hapten is a perfect mimic of the target molecule and constitutes an adequate candidate for antibody generation. In contrast, the partial charges of the C-5, C-6, and C-7 atoms in haptens PQ*f* and PQ*h* differed slightly from those of proquinazid. Due to the high electronegativity of the fluorine atom, hapten PQ*f* exhibited greater divergences in electronic density at C-6 and surrounding atoms compared to hapten PQ*i*. These differences were more pronounced than those observed in hapten PQ*h*.

Prior to conjugation to the carrier proteins, the carboxylic group of each hapten was activated by transforming it into the corresponding *N*-hydroxysuccinimidyl ester. This transformation was achieved in high yield using DSC and Et_3N as base catalyst in acetonitrile. The resulting active esters, PQ*h*-NHS, PQ*f*-NHS, and PQ*i*-NHS (Fig. 1), were chromatographically purified and structurally characterized by ^{1}H NMR spectroscopy (see the "Experimental" section and the Electronic Supplementary Material).

Bioconjugate and antibody characterization

As determined by MALDI-ToF–MS analysis, the BSA conjugate of hapten PQ*i* exhibited a high hapten density (MR = 18), which is optimal for the immunizing conjugate. On the other hand, the estimated hapten-to-protein MR of the OVA conjugates of haptens PQ*i*, PQ*f*, and PQ*h* were 1.4, 1.5, and 1.7, respectively. Thus, all three coating conjugates had equivalent MR values, indicating their suitability for competitive immunoassay development. Furthermore, the MR values of the three enzyme tracers were between 0.5 and 1.2, indicating a low labelling ratio suitable for the d-cELISA format.

Two antibodies, named PQ*i*#1 and PQ*i*#2, were obtained from rabbits immunized with BSA–PQ*i*. These immunoreagents were characterized using direct and indirect cELISA formats with homologous and heterologous antigens, *i.e.*, conjugates containing the same or different hapten, respectively, compared to the immunizing conjugate. When the homologous conjugate was employed (HRP–PQ*i* or OVA–PQ*i*), the IC_{50} values obtained for antibodies PQ*i*#1 and PQ*i*#2 were in the low part per billion range (Tables 1 and 2). These values are approximately one order of magnitude lower than those obtained in a previous study using haptens with the linker attached to the aromatic ring [6]. This result demonstrates the superior performance of hapten PQ*i* — in which the spacer arm is incorporated through positions that do not substantially modify the electronic and steric characteristics of the common skeleton with the analyte — to generate high-affinity antibodies. This observation is in accordance with findings from previous studies involving different target molecules, where the linker of the immunizing hapten was also formally an elongation of a linear aliphatic substituent of the analyte molecular framework [12, 13].

Concerning the heterologous conjugates with haptens PQ*f* and PQ*h*, little or no sensitivity improvements were observed. Checkerboard titration studies in the direct assay format were carried out by combining diverse antibody solutions (10^4- and 3×10^4-fold dilutions) with enzyme tracer solutions at 3, 10, 30, and 100 µg/L. As listed in Table 1, both heterologous tracers (HRP–PQ*f* and HRP–PQ*h*) were recognized by the antibodies, but no improvement in the IC_{50} value compared to the homologous tracer was achieved. Overall, antibody PQ*i*#1

Table 1 Checkerboard assays by d-cELISA ($n=3$)

	Antibody[a]							
	PQ*i*#1				PQ*i*#2			
Enzyme tracer	[Tracer] (µg/L)	A_{max}	Slope	IC_{50} (µg/L)	[Tracer] (µg/L)	A_{max}	Slope	IC_{50} (µg/L)
HRP–PQ*i*	3	1.09	0.81	0.9	3	1.31	0.86	1.3
HRP–PQ*f*	100	1.10	0.84	1.9	10	0.92	0.79	3.9
HRP–PQ*h*	100	0.38	0.69	1.2	30	0.86	1.10	2.7

[a]Antibody was diluted 10^4-fold

Table 2 Checkerboard assays by i-cELISA ($n=3$)

	Antibody							
	PQ*i*#1				PQ*i*#2			
Coating conjugate[a]	1/[Ab][b]	A_{max}	Slope	IC_{50} (µg/L)	1/[Ab]	A_{max}	Slope	IC_{50} (µg/L)
OVA–PQ*i*	10^4	1.24	0.91	2.6	3×10^4	1.03	0.71	3.5
OVA–PQ*f*	10^4	0.99	0.96	1.8	10^4	1.67	0.64	5.4
OVA–PQ*h*	10^4	0.83	0.73	1.5	10^4	1.74	0.72	4.6

[a]Plates were coated at 100 µg/L. [b]Antibody dilution

showed slightly lower IC_{50} values than antibody PQ*i*#2 by d-cELISA. On the other hand, checkerboard titration assays by i-cELISA were performed using heterologous coating conjugates at 100 µg/L and antibody solutions diluted at 10^4-, 3×10^4-, and 10^5-fold. Table 2 presents the parameters of the best calibration curve obtained for each antibody and coating conjugate pair. As expected, both heterologous conjugates (OVA–PQ*f* and OVA–PQ*h*) were bound by both antibodies, but only a slight or no improvement of the IC_{50} value was observed. The substitution of the iodine atom with a fluorine or hydrogen atom slightly reduced the IC_{50} value for proquinazid obtained with antibody PQ*i*#1, but not with PQ*i*#2. These results indicate that substituting the iodine atom at a distal position of the linker in the proquinazid hapten with fluorine or hydrogen does not significantly disturb antibody binding. This observation is consistent with the high similarity that exists in the partial charges at the equivalent atoms of the three haptens (Fig. 2). Improvement of immunoassay sensitivity using heterologous conjugates is a common strategy in this field. However, this aim is not always achieved, particularly when the d-cELISA format is used [14–16]. The result is determined by the binding properties of the antibody and, very importantly, by the type of heterologies that are introduced (linker position, linker length, changes on relevant moieties, etc.).

Concerning specificity, none of the two antibodies recognized other commonly used fungicides when they were assayed at 10 µM concentration, such as mepanipyrim, cyprodinil, and pyrimethanil. As expected, both antibodies were highly specific of proquinazid.

Immunoassay development

Two immunoassays were developed for the analysis proquinazid in different cELISA formats. In the direct assay, antibody PQ*i*#1 and the homologous tracer were employed. Conversely, in the indirect assay, antibody PQ*i*#1 was paired with the heterologous conjugate OVA–PQ*h*. The influence of pH, ionic strength, and tolerance to organic solvents was evaluated for both immunoassays. With this aim, proquinazid standard solutions were prepared in Milli-Q water o water-diluted organic solvent, whereas the enzyme tracer or the antibody solutions were prepared in 20 mM phosphate buffer, pH 7.4, containing 280 mM NaCl and 0.05% (v/v) Tween-20.

As illustrated in Fig. S4, both the direct and indirect assays exhibited high tolerance to changes in pH between 5.5 and 9.5 and ionic strength values in the 50 to 300 mM range. Only the most extreme values of the assayed ranges of these parameters caused more than ± 20% modification in the A_{max} and IC_{50} values of both assays. Moreover, the tolerance of the two studied immunoassays to methanol and acetonitrile was evaluated. Concerning the direct assay, little amounts of either solvent increased A_{max}, whereas the IC_{50} value decreased with methanol and increased with acetonitrile (Fig. S5). On the other hand, in the i-cELISA, the A_{max} value increased in the presence of methanol but decreased with acetonitrile. However, the IC_{50} value of this assay was increased by the addition of methanol or acetonitrile to the reaction mixture. The standard curves of the optimized immunoassays are depicted in Fig. 3. Both immunoassays demonstrated similar responses to proquinazid. The IC_{50}

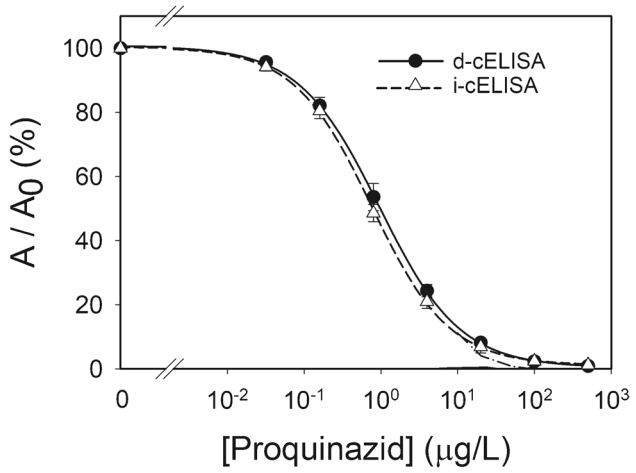

Fig. 3 Standard calibration curves of the two developed immunoassays to proquinazid using antibody PQ*i*#1. The direct assay was performed with antibody-coated plates (10^4-fold dilution) and 3 µg/L of the homologous tracer solution. For the indirect assay, plates were coated with OVA–PQ*h* at 1 mg/L and the antibody dilution in the competitive reaction was 10.[4]

values for proquinazid of the d-cELISA and the i-cELISA were 0.96 and 0.76, respectively. Hence, the developed immunoassays exhibit significantly improved sensitivity compared to previously published immunochemical methods [6], underscoring the superior performance of the novel PQ*i* hapten for antibody generation.

Sample analysis

Strawberry samples were purchased in a local supermarket and homogenized, and the absence of proquinazid was verified by GC–MS. QuEChERS extracts of strawberry samples were then directly diluted in PBS, and the potential matrix effects on both the d-cELISA and i-cELISA methods were evaluated. It was observed that the A_{max} values decreased in both assays at low dilution factors, particularly noticeable in the d-cELISA (Fig. S6). The lower tolerance of the direct assay to acetonitrile likely contributed to the higher matrix effect observed in the extracts on this immunoassay. Next, strawberry extracts were spiked with proquinazid at concentrations ranging from 10 to 1000 µg/kg. These fortified samples were diluted 1000- and 100-fold for the d-cELISA and i-cELISA, respectively. The recovery values obtained are summarized in Table 3. Notably, the lowest assayed concentration (10 µg/kg) was only measurable by the indirect assay. According to these results, the experimental LOQ values were determined to be 50 and 10 µg/kg for the d-cELISA and the i-cELISA, respectively. These values are significantly lower than the European MRL for proquinazid in strawberries (2000 µg/kg). Both immunoassays showed excellent precision and accuracy. The recovery values were between 108 and 120%, and the CV values were below 10% when sample extracts were analyzed by d-cELISA. On the other hand, strawberry extracts provided recovery values between 85 and 109%, and CV values below 15% when the developed i-cELISA was used.

Table 3 Analysis of spiked strawberry samples by direct and indirect cELISA after QuEChERS extraction

Spiked (µg/kg)	d-cELISA (% ± s, *n* = 3)		i-cELISA (% ± s, *n* = 3)	
	Recovery[a] (%)	CV (%)	Recovery[b] (%)	CV (%)
10	-[c]	-	95 ± 14	14.7
50	108 ± 6	5.6	106 ± 8	7.6
100	106 ± 1	0.9	109 ± 13	11.9
500	120 ± 4	3.3	95 ± 7	7.4
1000	110 ± 10	9.1	85 ± 5	5.9

[a]Samples were 1000-fold diluted in PBS. [b]Samples were 100-fold diluted in PBS. [c]Out of range

Validation with GC–MS

Sixteen strawberry samples were blindly spiked with proquinazid and subsequently analyzed using both GC–MS and the developed d-cELISA and i-cELISA methods. The concentrations of proquinazid obtained from these analyses are listed in Table S1. This study revealed excellent correlation between the data obtained from the reference chromatographic method and the newly developed immunoassays. The lowest and the highest concentrations of proquinazid detected were in samples #8 (42 µg/kg) and #7 (3900 µg/kg), respectively. The regression analysis (Fig. 4) yielded a *y*-intercept and a slope of 39.95 ± 26.37 and

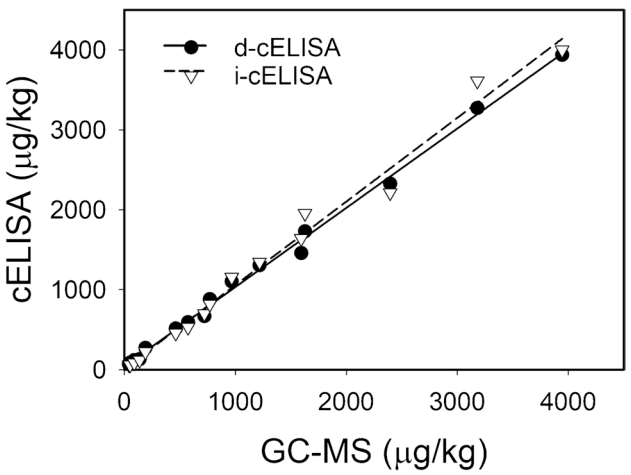

Fig. 4 Regression lines obtained from the analysis of strawberry blind samples by a reference chromatographic technique and the two developed immunoassays

0.99 ± 0.02, respectively, for the d-cELISA ($R^2 = 0.9961$), and 9.39 ± 49.90 and 1.05 ± 0.03, respectively, for the i-cELISA ($R^2 = 0.9877$). These results clearly demonstrate the suitability of the developed immunoassays for the rapid analysis of proquinazid residues in strawberries using QuEChERs extracts.

Conclusions

A novel hapten was designed that kept unmodified the characteristic 6-iodoquinazolin-4(3H)-one moiety of proquinazid. This strategy used one of the two aliphatic moieties of proquinazid to introduce a linear hydrocarbon spacer arm, so the synthetic hapten PQ*i* perfectly mimics the conformation and electronic distribution of the target molecule. As a result, antibodies with significantly higher affinity — more than one order of magnitude greater than previously reported binders — were successfully generated. Two cELISA tests have been thoroughly characterized and optimized. One assay utilized the direct format with the homologous enzyme tracer, whereas the other used the indirect format with a heterologous conjugate. This heterology implies the substitution of the iodine atom by a hydrogen atom, thus making the synthesis of the assay hapten readily accessible. The developed immunoassays were shown to measure proquinazid precisely and accurately in strawberry samples. This highlights their potential utility in food safety and quality control applications.

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Declarations

Ethics approval The study has been performed according to the ethical standards approved by the University of Valencia bioethic committee.

Conflict of interest The authors declare no competing interests.

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