Generation of anti-azoxystrobin monoclonal antibodies from regioisomeric haptens functionalized at selected sites and development of indirect competitive immunoassays

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Abstract

Azoxystrobin is a modern strobilurin fungicide used around the world to combat prime diseases affecting highly valuable crops. Accordingly, residues of this chemical are frequently found in food, even though mostly under maximum tolerated levels. We herein describe the development of an indirect competitive immunoassay for the determination of azoxystrobin residues. A panel of monoclonal antibodies displaying subnanomolar affinity to azoxystrobin was generated using, as immunizing haptens in mice, four functionalized derivatives carrying the same spacer arm located at different rationally-chosen positions. This collection of antibodies was thoroughly characterized with homologous and heterologous antigens, and the immunoassay consisting of monoclonal antibody AZo6#49 and the coating conjugate OVA–AZb6, which displayed an IC_{50} value of 0.102 μg L^{-1} and a LOD of 0.017 μg L^{-1}, was eventually optimized. The response to different pH and ionic strength conditions of the specific assay was studied using a biparametric approach. In addition, the influence of Tween 20 and organic solvents over the assay parameters was also evaluated. After optimization, the developed immunochemical assay was applied to the analysis of azoxystrobin in spiked juices of relevant fruits and vegetables, showing excellent recoveries between 2 and 500 μg L^{-1}.

Keywords

competitive ELISA; site heterology; Landsteiner's principle; fungicide; strobilurins; food safety

Abbreviations

AZ, azoxystrobin; BSA, bovine serum albumin; CR, cross-reactivity; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HAT, hipoxanthine–aminopterine–thimidine; HFCS, hybridoma fusion and cloning supplement; HT, hipoxanthine–thimidine; i-cELISA; indirect competitive ELISA; LOD, limit of detection; LOQ, limit of quantification; mAb, monoclonal antibody; MRL, maximum residue limit; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween 20; RAM–HRP, polyclonal rabbit anti-mouse immunoglobulin peroxidase conjugate.

1. Introduction

Strobilurins comprise a family of powerful fungicides. Their discovery was inspired by the identification of a group of active natural products displaying a potent antifungal activity in the mushrooms *Strobilurus tenacellus* and *Oudemansiella mucida* [1,2]. These agrochemicals exert their fungicide action through binding to the Q_o site of cytochrome b in the mitochondrial electron transport chain [3]. Although strobilurins show much lower toxicity to humans and non-target organisms than the traditional anti-mycotic substances, some of them are toxic to aquatics organisms [4]. Since their commercialization in the late 1990s, the strobilurin family has been steadily growing. Currently, 17 active compounds have been developed, although just 9 of them have received an ISO name and are therefore of global commercial relevance [5,6]. One of the two first strobilurins to be patented was azoxystrobin (AZ, Fig. 1), which was soon after approved worldwide for disease control in most cereals, fruits, and vegetables, being currently registered for use in nearly 120 crops in around 100 countries. At present, this active principle is being massively sold in a variety of formulations under different trade names. As a matter of fact, nearly 4000 tones of AZ was used worldwide in 2009, with global annual sales over $1 billion, which makes it the world's leading proprietary fungicide [7].

Nowadays, the monitoring of pesticide residues is compulsory for governments and private corporations involved in food processing. The European maximum residue limits (MRLs) for AZ in most foodstuffs range between 0.05 and 5 mg kg^{-1} [8]. According to the European Pesticide Monitoring Programs [9], AZ was among the most frequently found pesticides – around 5% of the food samples being analyzed contained residues at or below the MRL. Residues of this fungicide were mainly found in grapes, strawberries, peaches, tomatoes, leeks, head cabbages, peppers, peas, and lettuces. In addition, AZ residues have been found in locally purchased mangoes at levels between 12.7 and 55.8 µg kg^{-1} (16 samples, 100%) [10], and in commercial white and red wines at levels between 0.2 and 2.9 µg L^{-1} (11 samples, 82%) [11]. The analysis of AZ in foods is usually carried out by gas chromatography coupled to mass spectrometry detection (GC–MS) [12–14]. Other approaches have also

been published using fluorescence detection [15], high-performance liquid chromatography with diode array detection [16], desorption electrospray ionization mass spectrometry [17], ultrahigh-performance liquid chromatography tandem mass spectrometry [18], direct analysis in real time coupled to time-of-flight mass spectrometry [19], or liquid chromatography with electrospray tandem mass spectrometry [20].

For many applications, antibody-based bioanalytical techniques represent an attractive alternative to classical chromatographic methods. Immunoassays are generally considered as easy-to-use, affordable, rapid, sensitive, specific, and environmentally friendly analytical approaches, especially indicated for laboratories involved in environmental or food quality and safety programs [21–23]. Furthermore, due to their high versatility, immunochemical methods can easily be adapted to different particular analytical needs. However, immunoassay development demands raising suitable antibodies against the target analyte and evaluation of the produced immunoreagents into appropriate assay formats. The successful generation of specific and sensitive antibodies against small organic molecules, such as AZ, is greatly dependent upon the proper design of the immunizing and assay haptens, which must be coupled to a carrier protein. Following Landsteiner's seminal works [24], the idea that any modification of the target molecule should be introduced at a distal site from unique determinant moieties has been accepted as a general rule to prepare immunogens. However, this so-called "distal position" is sometimes uncertain.

In 2006, Furzer et al. [25] described the production of polyclonal antibodies against AZ using the acidic form of the molecule for direct conjugation. However, monoclonal antibodies (mAbs) are more attractive immunoreagents to industry because they constitute a defined reagent and a constant supply can be guaranteed. Up to now, we have reported the synthesis of haptens, the production of mAbs, and the development of immunoassays for different strobilurin pesticides, *i.e.* kresoxim-methyl, trifloxystrobin, pyraclostrobin, and picoxystrobin [26–29], and also a comprehensive strategy for the synthesis of four derivatives of AZ functionalized at different positions with the same spacer arm has recently been reported by our group [30]. For the present study, mice were immunized with

bioconjugates of those regioisomeric synthetic haptens, and a collection of mAbs against AZ was generated. These mAbs were exhaustively characterized and an enzyme-linked immunosorbent assay (ELISA) was developed and optimized in the conjugate-coated indirect competitive format (i-cELISA). Finally, evaluation of the selected immunoassay was undertaken by determining AZ recoveries in fortified juice samples of relevant commodities.

2. Materials and methods

2.1. Chemicals and instrumentation

Analytical-grade AZ (methyl (*E*)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate) (CAS number 131860-33-8, MW 403.4 g mol^{-1}) was kindly provided by Syngenta AG (Basel, Switzerland). Sepharose HiTrap Protein G HP columns used for antibody purification were purchased from General Electric Healthcare (Uppsala, Sweden). Polyclonal rabbit anti-mouse immunoglobulin peroxidase conjugate (RAM–HRP) was from Dako (Glostrup, Denmark). The immunoglobulin isotype was determined using the Mouse MonoAb-ID kit from Invitrogen (Carlsbad, CA, USA). Freund's adjuvants, thimerosal (sodium ethylmercurithiosalicylate), ovalbumin (OVA), and *o*-phenylenediamine were purchased from Sigma-Aldrich (Madrid, Spain). Bovine serum albumin (BSA) and Hybridoma Fusion and Cloning Supplement (HFCS) were from Roche Applied Science (Mannheim, Germany). P3-X63-Ag8.653 mouse plasmacytoma cell line was from the European Collection of Cell Cultures (Wiltshire, UK). Gentamicine, hipoxanthine–thimidine (HT) and hipoxanthine–aminopterine–thimidine (HAT) solutions were obtained from Gibco BRL (Paisley, Scotland). Cell culture media (high-glucose Dulbecco's Modified Eagle's Medium, DMEM), polyethylene glycol Hybri-Max (PEG 1500), fetal bovine serum (FBS), 200 mM alanyl-glutamine solution, red blood cell lysing buffer Hybri-Max, and MEM non-essential amino acid solution were from Sigma-Aldrich (Madrid, Spain). Culture plasticware and Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY, USA). ELISA absorbances were read with

a PowerWave HT microplate reader and microplates were washed with an ELx405 microplate washer, both from BioTek Instruments (Winooski, VT, USA).

The synthesis of the four functionalized derivatives of AZ used in this work (haptens AZa6, AZb6, AZc6, and AZo6, Fig. 1), as well as the preparation of the immunizing and coating conjugates, has been previously described [30].

2.2. Monoclonal antibody production

Animal manipulation was performed in compliance with the Spanish laws and guidelines (RD 1201/2005 and law 32/2007) and according to the European Directive 2003/65/EC concerning the protection of animals used for experimental and other scientific purposes. Four groups of four BALB/cByJ female mice (8–10 weeks old) were immunized by intraperitoneal injections with BSA–AZa6, BSA–AZb6, BSA–AZc6, or BSA–AZo6 conjugates. Doses consisted of 100 µg of protein conjugate in Freund's adjuvant (complete for the first injection and incomplete for the second and the third ones). The antiserum from each mouse was obtained by submandibular bleeding 9–10 days after the third injection. Sera were diluted 1/5 with PBS (10 mM sodium phosphate buffer, 140 mM NaCl, pH 7.4) containing 0.01% (w/v) thimerosal and stored at 4 ºC in amber glass vials. The antiserum titer was calculated as the required dilution to obtain an absorbance of 1.0 under standard ELISA conditions using the homologous conjugate (a conjugate with the same hapten that was used for immunization) at 1.0 µg mL^{-1}. After a resting period of at least 3 weeks from the last injection with adjuvant and four days before cell fusion, mice received a booster intraperitoneal injection of 100 µg of protein conjugate in 200 µL of PBS.

2.2.1. Cell fusion and culture

P3-X63/Ag 8.653 murine myeloma cells were cultured in high-glucose DMEM supplemented with 2 mM alanyl-glutamine, 1 mM MEM non-essential amino acids, and 25 µg mL^{-1} gentamicin (referred to as s-DMEM) and containing 10% (v/v) FBS. Mouse spleen lymphocytes were fused with myeloma

cells at a 4:1 ratio using 1 mL of PEG 1500 as the fusing agent. The fused cells were distributed in 96-well culture plates at a density of 1.5×10^5 lymphocytes per well in 100 µL s-DMEM with 15% FBS. Twenty-four hours after plating, 100 µL of selection medium (s-DMEM supplemented with HAT) with 20% FBS and 1% (v/v) HFCS was added to each well.

2.2.2. Hybridoma selection and cloning

Twelve days after fusion, hybridoma culture supernatants were assayed following a sequential double-screening process. First, each culture supernatant was analyzed in parallel with and without competitor by i-cELISA as described by Abad et al. [31]. The concentration of AZ in solution was 500 nM in the screening of the first cell fusion of each set of mice, and 100 nM in successive fusion experiments. The signal in non-competitive conditions (absence of analyte) was compared with the competitive one when AZ was used as competitor, and the ratio of both absorbances was used as the criterion to identify good antibody-secreting clones. In addition, competitive ELISAs were performed using serial dilutions of the culture supernatant from those wells that afforded saturated signals in the first screening experiment. The selective pressure was increased in the second assay using lower AZ concentrations, and ELISA plates were coated with antigens at 0.1 µg mL^{-1} to favor competition. The selected hybridomas were cloned by limiting dilution in cloning medium (s-DMEM containing 20% FBS and supplemented with HT and 1% HFCS). Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

2.2.3. Purification of monoclonal antibodies

Immunoglobulins were purified from late stationary-phase culture supernatants by ammonium sulfate precipitation and protein G affinity chromatography following column manufacturer's instructions. A fraction of the purified mAb was diluted 1:1 with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal and stored at 4 ºC in amber glass vials for daily usage. The remaining mAb solution was stored at 4 ºC as ammonium sulfate precipitate.

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170 *2.3. Enzyme-linked immunosorbent assays*

171 Ninety-six-well polystyrene ELISA plates were coated with 100 μL per well of OVA conjugate
172 solution in 50 mM carbonate–bicarbonate buffer, pH 9.6, by overnight incubation at room temperature.
173 Next day, coated plates were washed four times with 0.15 M NaCl containing 0.05% (v/v) Tween 20,
174 and received 50 μL per well of AZ standard solutions plus 50 μL per well of antibody diluted in PBST
175 (PBS containing 0.05% (v/v) Tween 20). AZ standard curves were prepared by serial dilution in PBS
176 using borosilicate glass tubes from a concentrated AZ stock solution in anhydrous *N,N*-
177 dimethylformamide. A blank was included in each curve. The immunological reaction took place during
178 1 h at room temperature. After washing as above, plates received 100 μL per well of a 1/2000 dilution
179 of RAM–HRP conjugate in PBST, and they were incubated 1 h at room temperature. Plates were
180 washed again and the signal was generated by adding 100 μL per well of freshly prepared enzyme
181 substrate solution (2 mg mL^{-1} of *o*-phenylenediamine and 0.012% (v/v) H_2O_2 in 25 mM sodium citrate
182 and 62 mM sodium phosphate buffer, pH 5.4). The enzymatic reaction was stopped after 10 min at room
183 temperature by addition of 100 μL per well of 2.5 M sulfuric acid. The absorbance was immediately
184 read at 492 nm using 650 nm as reference wavelength.

185 Competitive curves were obtained by plotting mean absorbance values versus the logarithm of analyte
186 concentration. The resulting experimental values were fitted to a four-parameter logistic equation using
187 the SigmaPlot software package from SPSS Inc. (Chicago, IL, USA). From those curves, assay
188 sensitivity was estimated as the concentration of analyte at the inflection point of the fitted curve,
189 typically corresponding to a 50% inhibition (IC_{50}) of the maximum absorbance (A_{max}) if the background
190 signal approaches to zero. The limit of detection (LOD) was estimated as the concentration of AZ that
191 provided a 10% inhibition of the A_{max} (IC_{10}), whereas the limit of quantification (LOQ) was established
192 at the IC_{20} and the working range between the IC_{20} and IC_{80} values. Cross-reactivity (CR) was
193 calculated according to the formula:

$$CR = [IC_{50}\ (AZ) / IC_{50}\ (compound)] \times 100$$

2.4. Buffer ionic strength and pH studies

Biparametric analysis of assay response to diverse pH and ionic strength conditions was performed using statistical software from Minitab Inc (State College, PA, USA). Axial points were fixed at 5.5 and 9.5 for pH, and at 50 and 300 mM for the ionic strength. The number and composition of buffers indicated by the software were prepared as follows. A 40 mM trisodium citrate, 40 mM disodium hydrogen phosphate, 40 mM Tris solution was made (pH 9.9, I = 360 mM), and different volumes of 5 M HCl and 2 M NaCl were added to aliquots of that solution in order to achieve buffers with different pH and ionic strength values. The ionic strength of each solution was determined according to the formula

$$I = \tfrac{1}{2} \sum c_i z_i^2$$

where I is the ionic strength, c is the concentration of each ion at equilibrium, and z is its charge. After pH and I adjustment at each desired value, Tween 20 and deionized water were added, so the final composition of every buffer was 20 mM citrate, 20 mM phosphate, and 20 mM Tris containing 0.05% Tween 20 and different amounts of NaCl. Competitive assays were performed with AZ standards in deionized water.

2.5. Sample analysis

Commercial juices from tomatoes, grapes, peaches, and bananas were acquired from a local supermarket. Analysis of those samples by GC–MS (see the Supplementary Data File for chromatographic conditions) proved the absence of AZ residues at levels higher than 20 μg kg^{-1} (LOD of the employed experimental procedure). Competitive assays were performed in plates coated with a solution of OVA–AZb6 at 1 μg mL^{-1}. Each coated well received 50 μL of AZ standard solutions in PBS or 50 μL of sample fortified with AZ and diluted in PBS (1/25, 1/100, and 1/500), plus 50 μL of a 60 ng mL^{-1} solution of mAb AZo6#49 prepared in PBST. Each sample was analyzed in triplicate wells, and

the mean absorbance values were interpolated into the standard curve run in the same plate, also in triplicate wells. Unfortified samples were also diluted in PBS as mentioned above and included as controls.

3. Results and discussion

3.1. Immunological response

In order to elicit antibodies suitable for the development of sensitive immunochemical assays for small organic molecules, the immunizing haptens must mimic as much as possible the structure and electronic distribution of the parent analyte. AZ is a very flexible compound, so it is rather uncertain to predict the linker position that would limit to a lesser extent the conformational freedom of the molecular framework, and simultaneously afford a maximum exposure of the target to the immune system. In this study, four rationally-designed haptens, namely AZa6, AZb6, AZc6, and AZo6 (Fig. 1), which incorporated the same saturated hydrocarbon spacer arm at different positions of the skeleton through C–C single bonds, were evaluated for the production of mAbs. The syntheses of those regioisomeric functionalized haptens of AZ and their conjugation to carrier proteins have been described elsewhere [30].

The immunization process was confirmed and the performance of the immunogens was assessed using antisera collected after the third boost. At this point, all of the animals showed an outstanding response, with antisera showing high titers (~ 10^5) when they were evaluated by ELISA using the homologous coating conjugate. In addition, all antisera bound all heterologous coating antigens (conjugates bearing a hapten different to that of the immunogen) with good titers despite the diversity in spacer arm attachment sites, even at low conjugate concentrations (0.1 μg mL^{-1}). Concerning affinity, the obtained IC_{50} values for AZ were also remarkably low for mouse antisera, particularly those of mice immunized with haptens AZa6 and AZb6. Moreover, some heterologous combinations resulted in up to 6-fold reduction of the IC_{50} values. As listed in Table S1 (Supplementary Data File), the lowest IC_{50}

values were achieved with AZa6-type antisera using OVA–AZb6 for coating. In general, OVA–AZb6 conjugate was the best heterologous coating antigen for all antisera, probably because of the central position of the spacer arm. Anyhow, the four evaluated haptens gave rise to an excellent response in mice, so all of them were deemed appropriate candidates to address hybridoma technology.

3.2. Generation of monoclonal antibodies

At least three cell fusions were performed with splenocytes from mice immunized with each AZ derivative. A sequential double-screening procedure was carried out after the cell fusion for hybridoma selection, as described in the section 2.2.2. In the first screening, two simultaneous assays – one with and one without free analyte – were performed, which helped to identify those antibody-producing clones that bound not only the coating conjugate but also the free analyte. The observed high number of positive clones (wells containing antibodies that recognized the coating conjugate) that were usually found after each cell fusion could already be envisaged from the high titers exhibited by mouse antisera. In most cases, also a high number of competitive clones (wells containing antibodies that recognized the free analyte) were retrieved in this first screening assay (Table S2). With the aim of efficiently ranking hybridomas for further cloning and expansion according to the affinity of the secreted antibodies, a second screening was routinely carried out following a competitive checkerboard assay. Remarkably, high-affinity antibody-producing hybridomas were rather homogeneously derived from all of the immunizing haptens, in keeping with the results observed in competitive experiments with the mouse antisera. In total, 37 hybridoma cell lines were cloned and stabilized; they all produced antibodies of the IgG_1 isotype with κ light chains, with the only exception of mAb AZb6#43 (IgG_{2a}, κ).

3.3. Evaluation of antibody affinity

A straightforward strategy for immunoreagent assessment and affinity determination was followed. Each mAb, at six different concentrations (30–1000 ng mL^{-1}), was tested against the four available coating conjugates at two concentrations (0.1 and 1.0 μg mL^{-1}) under competitive conditions using

8-point AZ standard curves (10^{-4}–10^{2} nM), including a blank. All combinations were tested at least three times. This study provided, for each pair of immunoreagents, a set of 12 inhibition curves displaying different A_{max} values, which allowed a well-founded selection of the best combinations of mAb and assay conjugate for further ELISA development. Concerning antigen recognition, all of the mAbs were able to bind the homologous conjugate with high avidity. In fact, coating antigens had to be used at or below 0.1 μg mL^{-1} in homologous combinations to afford optimum competitive curves. With regard to heterologous coating conjugates, unexpected results were found in some cases. Thus, most AZa6-type mAbs were unable to bind OVA–AZc6, whereas the opposite was not true, *i.e.* AZc6-derived mAbs recognized pretty well OVA–AZa6 as coating antigen. A similar finding was observed with haptens AZb6 and AZa6; whereas most AZb6-type mAbs did not recognize OVA–AZa6, 7 out of 11 AZa6-type mAbs did bind OVA–AZb6. Finally, we found that conjugate OVA–AZo6 was strongly recognized by all of the antibodies, even by those generated from immunizing haptens with the linker at a very different site. Most likely, the many degrees of freedom of the toxophore moiety in AZo6 could probably explain its universal character as coating antigen.

Table 1 summarizes the antibody and coating conjugate concentrations, as well as the curve parameters, for a short selection of mAbs at those particular immunoreagent concentrations that afforded the lowest IC_{50} with an A_{max} value between 0.8 and 1.5 (see Table S3 for the rest of mAbs). Overall, antibodies displaying outstanding affinity to AZ (IC_{50} values in the subnanomolar range) were obtained from any of the four immunizing conjugates. The antigen OVA–AZb6, with the spacer arm located at the central ring of the AZ skeleton, emerged as a very convenient heterologous coating conjugate for the development of sensitive assays, as it was already observed with mouse antisera. Slopes were an issue of concern from this study because very steep inhibition curves were observed with many immunoreagent combinations, which would result in assays with rather narrow working ranges. Accordingly, this curve parameter received special attention in the further selection of immunoreagent combinations.

3.4. Assessment of antibody specificity

The binding properties of each mAb towards relevant members of the strobilurin family (kresoxim-methyl, trifloxystrobin, picoxystrobin, pyraclostrobin, dimoxystrobin, orysastrobin, and metominostrobin) were studied using the homologous OVA conjugate as coating antigen. No inhibition was observed up to 1 μM in any case, so all mAbs, irrespective of their origin, were highly specific to AZ. This finding was not actually unexpected because strobilurin fungicides, although belonging to the same agrochemical group, barely share a minimum part of the whole structure (Fig. S1), and it is well-known that mAbs are biomolecules most often displaying exquisite specificity to their target, being able to discriminate between chiral molecules and geometric isomers [32,33].

The fungicide activity of AZ strongly relies on the *E*-isomer, which in fact makes up for more than 98% of the technical product, whereas the *Z*-isomer, which is mainly generated by photochemical transformation, accounts for up to 25% of the observed degradation products [34,35]. During hapten synthesis, especial precautions were taken to preserve the *E* conformation of the toxophore in all derivatives. Accordingly, we thought it was worthwhile to challenge our large collection of mAbs with the *Z*-isomer in order to further explore the fine specificity of their binding sites and to study the relationship between their stereoselectivity and the derivatization position in the parental hapten. The *Z*-isomer of AZ was prepared as described by Clough et al. [36], and competitive curves with both isomers were simultaneously run in the homologous format with the whole collection of mAbs. The most stereoselective mAbs were those coming from immunizing haptens AZo6 and AZb6, showing mean CR values for each group of mAbs of 3.8% (n = 12) and 3.5% (n = 8), respectively (Fig. 2). On the contrary, mAbs derived from the immunizing hapten AZa6 were less able to discriminate between both isomers, with a mean CR value for the *Z*-isomer of 40.3% (n = 11). Interestingly, this group included two of the antibodies that displayed extreme and opposite selectivity patterns, *i.e.* mAb AZa6#21, whose CR value for the *Z*-isomer was 1.0%, and mAb AZa6#210, with a CR value of 96.7% (Fig. S2). Regarding antibodies that came from the immunizing hapten AZc6, a mean CR value for the

316 *Z*-isomer of 19.0% was found. In brief, stereoselective antibodies (CR < 2%) were generated from all
317 immunizing haptens, but thanks to the large set of available mAbs, we could observe that haptens AZo6
318 and AZb6 were more adequate for this purpose. Otherwise, hapten AZa6 emerged as a very well suited
319 derivative for the production of mAbs displaying equivalent affinities for both geometric isomers.

320 *3.5. ELISA development and characterization*

321 Considering that all of the produced antibodies displayed high affinity and specificity to AZ, some
322 immunoreagents were reevaluated in order to ensure the selection of the best assays. Thus, those
323 combinations that provided IC_{50} values below 0.5 nM and slopes higher than −1.4 in the initial
324 evaluation were tested again using adjusted mAb and antigen concentrations to afford A_{max} values close
325 to 1.0. Table 2 lists the main curve parameters that were reached with the best antibody that was derived
326 from each type of immunogen. The most sensitive immunoassays were achieved with mAbs AZa6#11
327 and AZo6#49, using in both cases OVA–AZb6 as coating conjugate. Taking into account IC_{50} values,
328 slopes, robustness, and cell growth and antibody-producing capacity in culture of the parental
329 hybridoma cell line, mAb AZo6#49 together with conjugate OVA–AZb6 were selected for further assay
330 characterization.

331 The influence of buffer pH and ionic strength was investigated following a biparametric approach. AZ
332 concentrations from 100 nM to 1 pM were prepared in deionized water and the mAb was diluted in 20
333 mM buffers of varying pH and *I* values. Fig. 3 shows the variation of the A_{max} and IC_{50} values of the
334 resulting inhibition curves with regard to these two physicochemical parameters. High pH and *I* values
335 decreased the A_{max}, but they did not significantly modify the IC_{50} inside the assayed range, even though
336 slightly better IC_{50} values were obtained in buffers with extreme pH and ionic strength. Therefore, the
337 best compromise was to keep the ELISA conditions at pH and *I* values equivalent to those of the PBS
338 (pH 7.4 and I = 162 mM at 25 ºC). The moderate dependence of the assay IC_{50} over the pH and salt
339 concentration, together with the particularly high sensitivity of the described ELISA, should permit the

neutralization of the acidic character of most fruits and vegetables by simple dilution with a regular assay buffer.

Tween 20 is a common additive used in ELISA to avoid unspecific interactions between proteins and with the solid support. However, it is known that this detergent may exert a negative influence over the signal and sensitivity of the immunoassay [26]. As a further step in the optimization of the selected i-cELISA for AZ, competitive assays were run in PBS containing different amounts of Tween 20. From this study, it was observed that the usual detergent concentration (0.05%) was optimum for this assay (Fig. S3). Finally, the effect of some commonly employed organic solvents in pesticide residue extraction over the parameters of the inhibition curve was evaluated (Fig. S4). Solvent concentrations at or above 10% severely compromised the performance of the assay, in particular with acetonitrile and acetone. The best tolerated solvent was methanol; concentrations below 5% did not significantly change the curve parameters, and even acceptable A_{max} and IC_{50} values were obtained with 10% methanol. Table 3 shows the main analytical features of the optimized competitive ELISA.

3.6. Food analysis

AZ is extensively used to fight botrytis in high-valuable crops, including tomatoes, peaches, grapes, and bananas, so the performance of the developed i-cELISA was evaluated in juices from those food commodities. Firstly, a study of the matrix effects over the developed immunoassay was undertaken by running AZ standard curves prepared in juice diluted in PBS. It was observed that a direct 1/50 dilution of the juice in assay buffer practically reduced the interferences down to negligible levels (Fig. 4). Next, the four juices were fortified with varying amounts of AZ and measured with the optimized i-cELISA after a simple dilution in buffer; three dilution factors were tested to better evaluate assay performance. As shown in Table 4, good to excellent recoveries over more than two orders of magnitude of analyte concentration could be obtained even from just a single dilution (factor 1/100). In the four analyzed commodities, 2 μg L^{-1} of AZ could be adequately measured by direct dilution in PBS, which is a concentration 10 times below the lowest MRL that has been laid down in the international legislation.

Actually, the newly developed ELISA for AZ is currently being applied as a screening procedure in real samples within the framework of an ongoing project aimed at analyzing the presence of fungicide residues in market commodities by immunochemical methods. So far, two strawberry samples out of 16 bought at local groceries have been found positive by the developed immunoassay. One of those samples contained AZ residues at a concentration of 620 μg kg^{-1}. This sample was further analyzed by GC–MS, which provided a value of 630 μg kg^{-1} (see Fig. S5 in the Supplementary Data File for the GC–MS chromatogram of that sample). The other positive sample contained traces of AZ according to both ELISA and the reference method (15–20 μg kg^{-1}).

4. Conclusions

A collection of mAbs to AZ was generated from four haptens functionalized with the same linker length at different sites. Interestingly, antibodies showing high affinity to the target analyte were commonly found independently of the derivatization site in the immunizing hapten. However, a dependence of the antibody stereoselectivity upon the tethering site of the immunogen was envisaged. A detailed study of the antibody capacity to bind site-heterologous haptens revealed that OVA–AZo6 was recognized by all of the antibodies. Also, AZo6-derived immunoglobulins were the group of antibodies that better bound heterologous conjugates. In addition, the OVA–AZb6 conjugate emerged as the most appropriate antigen for the development of heterologous assays. Little influence of pH, Tween 20, and salt concentration was noticed over the optimized assay using mAb AZo6#49 and OVA–AZb6. Finally, the developed immunoassay showed an excellent performance in recovery studies with spiked juices, allowing the simple and rapid analysis of numerous samples in just an 8-hour working day. Particularly, this AZ ELISA could determine the target fungicide in fruit juices at levels as low as 2 μg L^{-1}, which satisfactorily compares with instrumental chromatographic techniques.

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Limited amounts of the immunoreagents described in this paper are available upon request for evaluation.

Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:

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463 **Figure legends**

464 **Fig. 1.** Chemical structures of AZ and haptens used for immunization and assay development.

465 **Fig. 2.** Minimum, mean, and maximum CR values for the *Z*-isomer of AZ exhibited by the four
466 different mAb populations.

467 **Fig. 3.** Influence of buffer pH and ionic strength on the A_{max} and IC_{50} values of the selected assay.
468 Antibody AZo6#49 was used at 60 ng mL^{-1} and the OVA–AZb6 conjugate was used at 1.0 µg mL^{-1}.
469 Values are the mean of three independent experiments.

470 **Fig. 4**. Matrix effects for tomato, peach, grape, and banana juices. Food samples were diluted in buffer
471 1/5 (●), 1/25 (○), 1/50 (♦), 1/250 (Δ), 1/500 (■). A control was also run with no juice (□).

472

Table 1
Antibody evaluation and assay selection using AZ as competitor in the i-cELISA format

	Coating conjugate															
	OVA–AZa6				OVA–AZb6				OVA–AZc6				OVA–AZo6			
mAb	[mAb][a]	[C][b]	slope	IC_{50}[c]	[mAb]	[C]	slope	IC_{50}	[mAb]	[C]	slope	IC_{50}	[mAb]	[C]	slope	IC_{50}
AZa6#11	30	1.0	−1.23	0.25	20	1.0	−1.28	0.14	1000	1.0	[d]		30	0.1	−1.83	0.30
AZa6#21	100	0.1	−1.59	0.87	25	1.0	−1.24	0.65	300	1.0	−1.73	1.66	30	0.1	−1.34	0.36
AZa6#210	100	0.1	−1.35	0.44	1000	1.0			1000	1.0			30	0.1	−1.11	0.42
AZa6#33	30	0.1	−1.76	0.23	30	1.0	−1.51	0.21	1000	1.0			20	0.1	−1.81	0.15
AZb6#22	1000	1.0			100	0.1	−1.34	0.35	100	1.0	−1.49	0.96	100	0.1	−1.36	0.42
AZb6#23	1000	1.0			100	0.1	−1.43	0.79	100	1.0	−1.17	1.27	100	0.1	−1.31	0.91
AZb6#24	1000	1.0			100	0.1	−1.34	0.29	100	1.0	−1.37	0.88	100	0.1	−1.40	0.36
AZb6#38	300	0.1	−1.17	6.97	100	0.1	−1.26	1.99	1000	1.0			30	0.1	−0.98	3.94
AZc6#22	100	1.0	−1.63	0.39	100	0.1	−1.81	0.47	100	0.1	−1.59	0.40	100	0.1	−1.47	0.39
AZc6#24	100	1.0	−1.42	0.53	300	0.1	−1.64	0.31	100	0.1	−1.64	0.30	100	0.1	−1.43	0.31
AZc6#27	100	0.1	−1.39	0.54	300	1.0	−1.61	1.70	100	0.1	−1.47	0.62	30	0.1	−1.13	0.46
AZc6#31	1000	1.0			1000	1.0			100	0.1	−0.95	0.68	100	0.1	−1.08	0.49
AZo6#31	100	0.1	−1.87	1.28	15	1.0	−1.57	0.20	1000	1.0			30	0.1	−1.89	0.38
AZo6#44	100	0.1	−1.95	0.45	30	1.0	−1.24	0.21	300	1.0	−1.62	0.79	50	0.1	−1.50	0.25
AZo6#45	100	0.1	−1.93	0.67	1000	1.0			30	1.0	−1.59	0.18	30	0.1	−1.40	0.45
AZo6#49	100	0.1	−1.58	0.60	30	1.0	−1.23	0.29	300	1.0	−1.57	0.95	30	0.1	−1.45	0.29

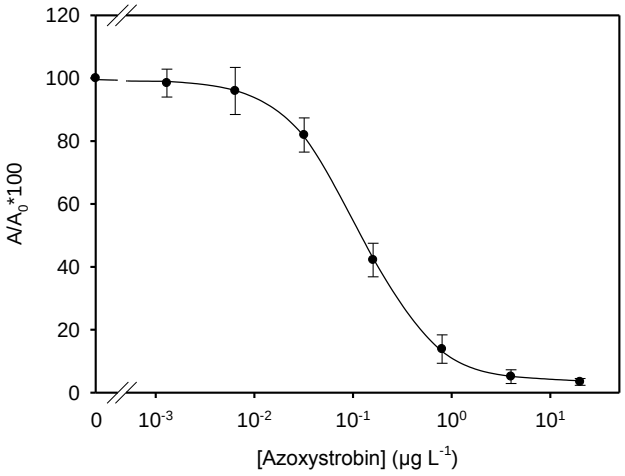
[a] Antibody concentration in ng mL^{-1}.
[b] Coating conjugate concentration in µg mL^{-1}.
[c] Values in nM.
[d] Empty cells indicate that no signal was obtained for this particular combination.

Table 2
Curve parameters of the selected assays

mAb	Coating hapten	Parameters[a]		
		A_{max}	Slope	IC_{50} (nM)
AZa6#11	AZa6	1.3 ± 0.3	−1.2 ± 0.2	0.23 ± 0.04
	AZb6	1.3 ± 0.3	−1.3 ± 0.2	0.15 ± 0.06
AZb6#24	AZb6	0.8 ± 0.2	−1.3 ± 0.2	0.33 ± 0.03
AZc6#24	AZo6	1.3 ± 0.3	−1.3 ± 0.2	0.40 ± 0.05
AZo6#49	AZo6	1.3 ± 0.3	−1.2 ± 0.3	0.29 ± 0.05
	AZb6	1.2 ± 0.2	−1.3 ± 0.1	0.23 ± 0.03

[a] Mean values of five independent experiments. The lower asymptotes (A_{min}) were always below 0.06.

Table 3
Standard curve, conditions, and parameters of the developed assay for AZ[a]



Antibody	mAb AZo6#49 at 60 ng mL^{-1}
Conjugate	OVA–AZb6 at 1.0 µg mL^{-1}
A_{max}	1.0 ± 0.1
A_{min}	0.026 ± 0.007
Slope	−1.15 ± 0.08
IC_{50} (µg L^{-1})	0.10 ± 0.01
LOD (µg L^{-1})	0.017 ± 0.002
Working range (µg L^{-1})	0.035 ± 0.004 to 0.46 ± 0.05
Assay buffer	PBST
Assay time (h)	2.5
Solvent tolerance	methanol 10%

[a] Values are the means of 16 independent determinations.

Table 4
Recovery values (%) from spiked samples as determined by i-cELISA[a]

Sample	Spiked ($\mu g\ L^{-1}$)	Dilution factor 1/25	1/100	1/500
Tomato juice	2	115 ±14	101 ± 8	---[b]
	5	107 ± 5	102 ± 21	---
	10	123 ± 15	117 ± 11	98 ± 10
	50	67 ± 25	105 ± 12	97 ± 8
	200	---	104 ± 13	109 ± 16
	500	---	101 ± 11	106 ± 15
Peach juice	2	103 ± 26	127 ± 22	---
	5	105 ± 18	106 ± 20	---
	10	119 ± 16	118 ± 16	105 ± 18
	50	91 ± 12	111 ± 12	104 ± 13
	200	---	103 ± 15	106 ± 6
	500	---	88 ± 11	103 ± 7
Grape must	2	113 ± 16	113 ± 22	
	5	113 ± 13	99 ± 9	
	10	121 ± 18	112 ± 16	98 ± 16
	50	84 ± 23	105 ± 13	97 ± 17
	200		101 ± 10	99 ± 7
	500		97 ± 13	102 ± 9
Banana juice	2	131 ± 23		
	5	114 ± 18	118 ± 19	
	10	133 ± 20	119 ± 21	122 ± 3
	50	83 ± 13	107 ± 8	102 ± 7
	200		93 ± 19	104 ± 6
	500		96 ± 27	98 ± 6

[a] Values are the mean of six independent determinations.
[b] Concentration out of range.

Figure 1

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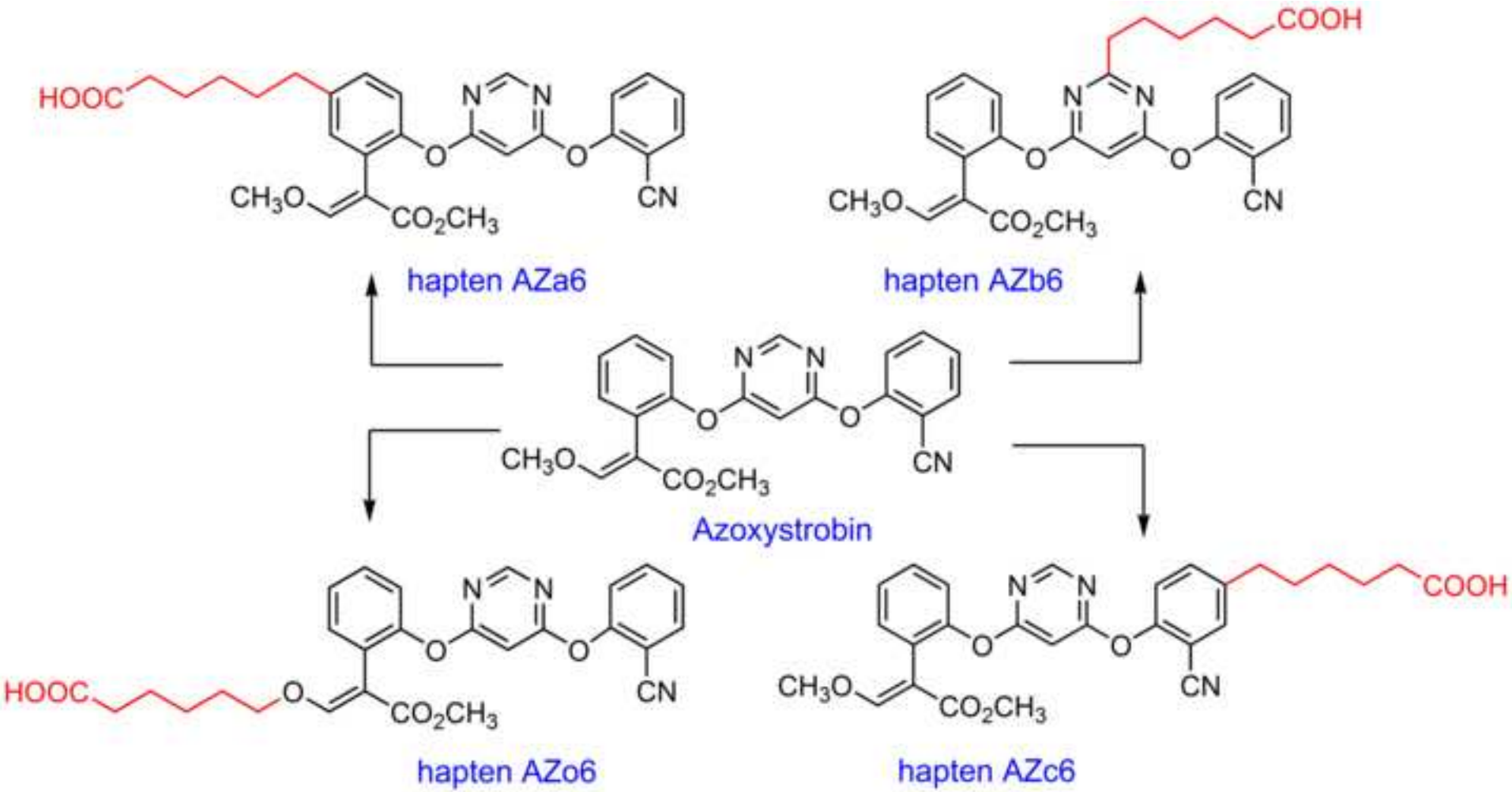

Figure 2

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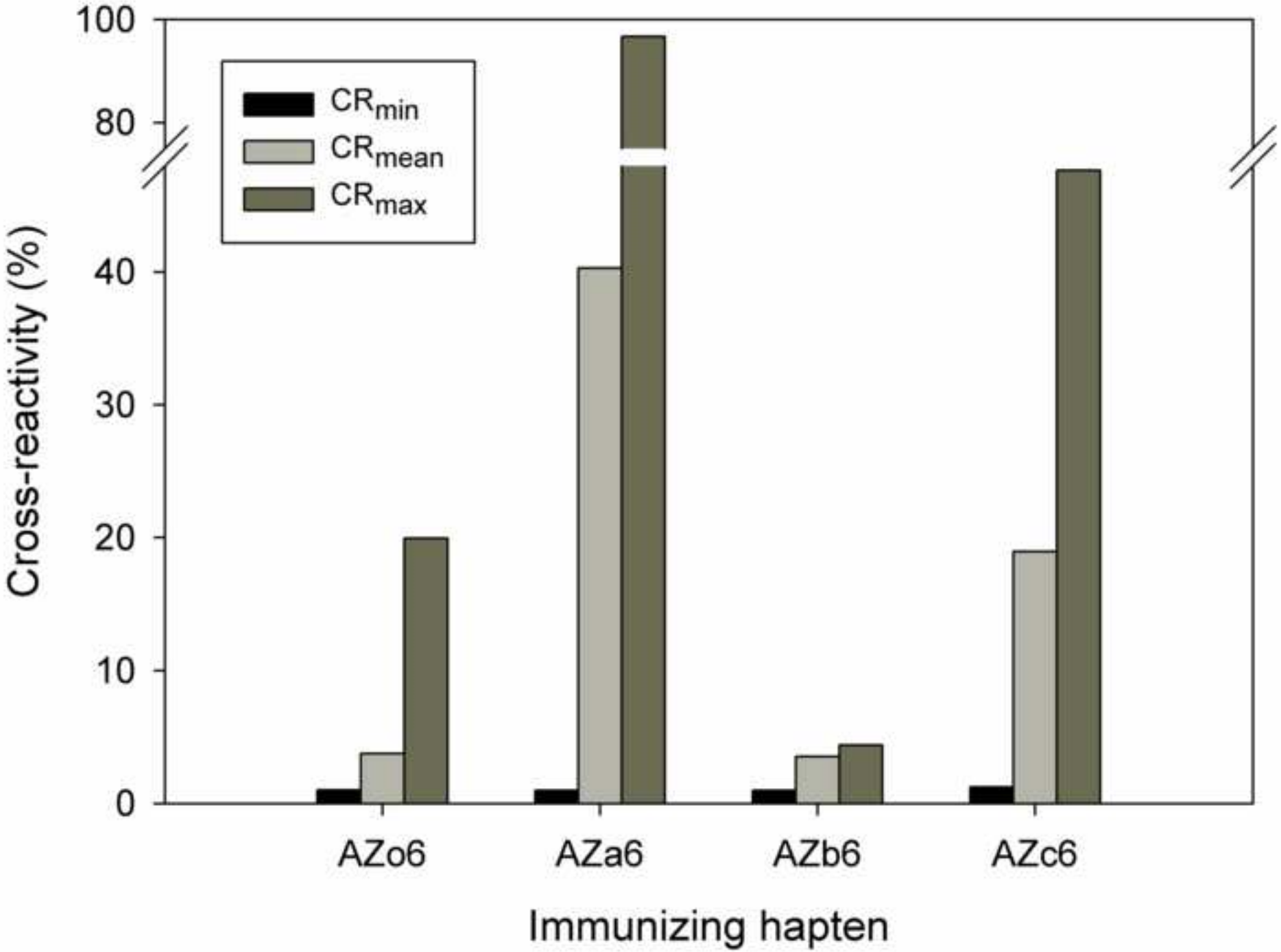

Figure 3

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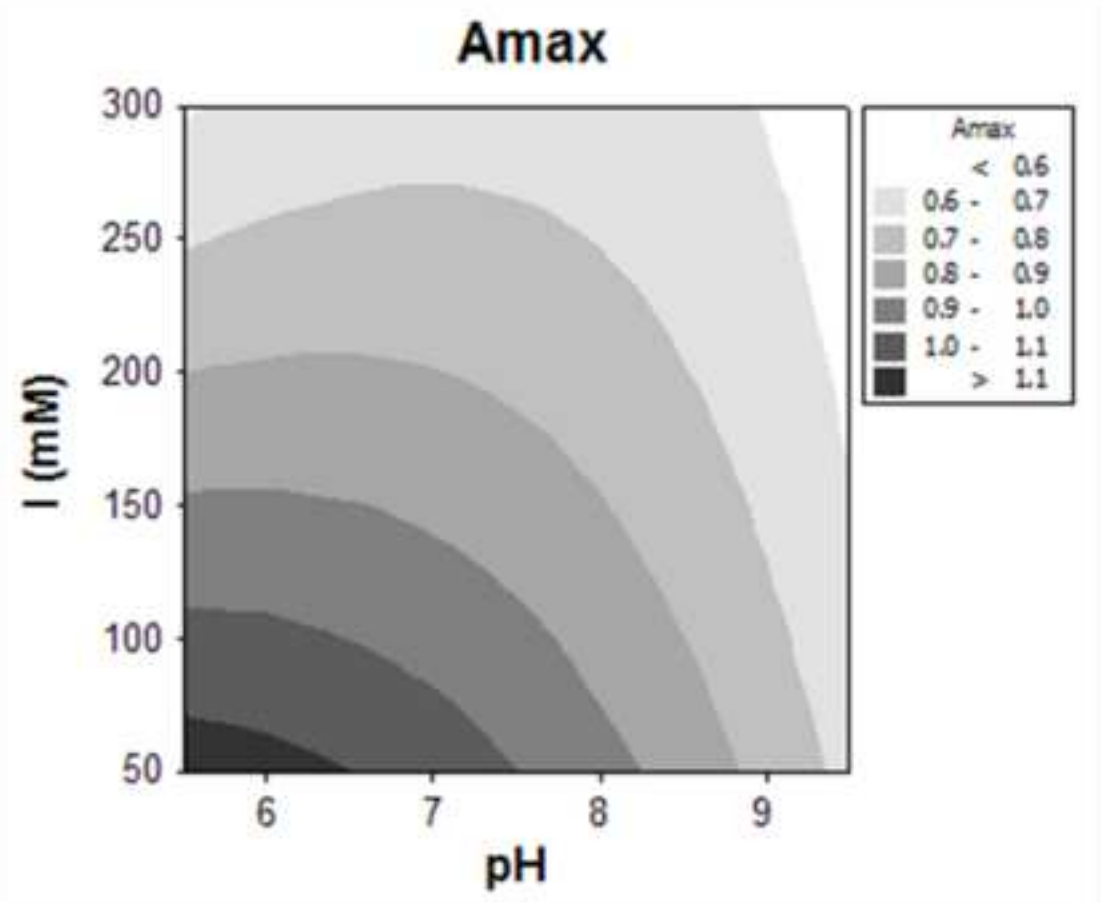

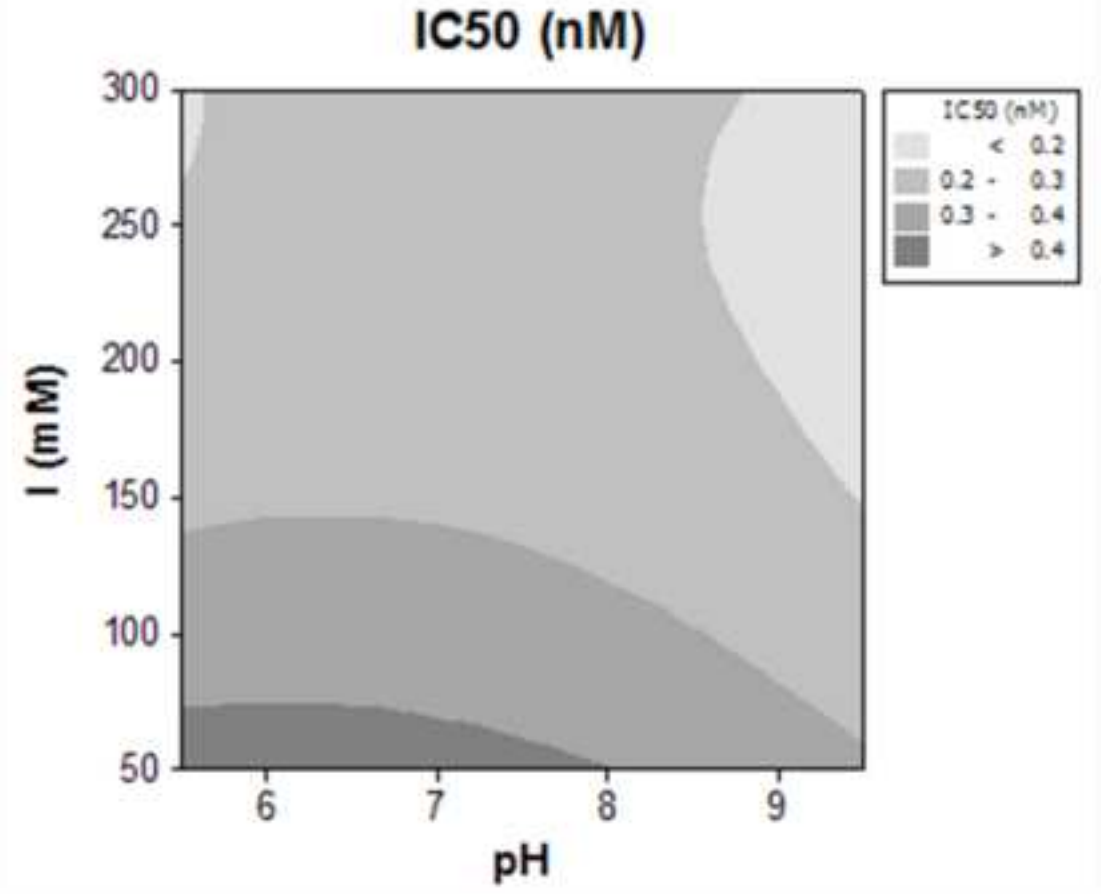

Figure 4

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