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Development of Monoclonal Immunoassays for the Determination of Triazole Fungicides in Fruit Juices

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Enzyme-linked immunosorbent assays (ELISAs) based on monoclonal antibodies for the detection of triazole fungicides have been developed. With this aim, hapten−protein conjugates, containing the common triazole and chlorinated aromatic moieties, were prepared. From mice immunized with these conjugates, several monoclonal antibodies (MAbs) with the ability to sensitively bind several triazoles with different specificity were obtained. Both analyte- and class-specific ELISAs were developed. The hexaconazole-specific immunoassay can determine this fungicide with a limit of detection of 0.3 μg/L in standard buffer. The so-called triazole-specific immunoassay allowed for the detection of tetraconazole, penconazole, cyproconazole, and myclobutanil, with limits of detection in the 0.1−0.7 μg/L range. These immunoassays were applied to the determination of triazoles in spiked fruit juices. Samples were adequately diluted to minimize the matrix effects. Coefficients of variation were below 30%, and recoveries ranged from 62 to 135%. Therefore, the developed immunoassays can determine triazole fungicides in fruit juices down to the maximum residue limits currently legislated, without any sample treatment other than dilution.

KEYWORDS: Triazole fungicides; tetraconazole; penconazole; hexaconazole; monoclonal antibodies; ELISA; immunoanalysis; fruit juices

INTRODUCTION

Triazoles are a class of systemic fungicides that contain the 1,2,4-triazole moiety and are used to control a variety of fungal diseases on fruit, vegetable, legume, and grain crops, both as pre- and postharvest applications (*1*). The biochemical mechanism of their antifungal effect is based on the inhibition of the ergosterol biosynthesis, thereby interfering with fungal cell-wall formation. Specifically, triazoles inhibit sterol 14α-demethylase, and, for that reason, they are classified, among the sterol biosynthesis inhibitors, as steroid demethylation inhibitors (DMI fungicides) (*2*). Their mammalian toxicology, ecotoxicology, and environmental fate have been extensively reviewed (*1, 3*). From the toxicological point of view and as a consequence of the ability to inhibit enzymes involved in the biosynthesis of steroid hormones, the triazole fungicides can potentially produce endocrine-related side effects on humans and wildlife (*4*). Accordingly, approximately half of the triazole fungicides are included in the priority list of chemicals developed within the EU Strategy for Endocrine Disrupters (*5*).

Considering the wide use of triazole fungicides and their toxicological aspects, it is evident that the presence of triazole residues has to be regulated by international and national organizations. Maximum residue levels (MRLs) have been established and are continuously revised (*6*−*8*). There is a range of MRLs depending upon the different commodity−pesticide combinations, but values as low as 20 mg/kg have been fixed. In the case of processed fruits and vegetables, such as juices, MRLs corresponding to the original matrix are normally considered.

Therefore, there is a need of adequate analytical methodology to carry out comprehensive monitoring programs of triazole residues in agricultural products. Triazole residue analysis in fresh or processed fruit and vegetables has been performed by chromatographic methods: gas chromatography (*9, 10*) or liquid chromatography (*11*−*13*). Chromatographic techniques generally require laborious sample pretreatment and high-cost complex equipment; therefore, they are not suitable for rapid analysis of a large number of samples or on-site determinations. Immunochemical techniques are increasingly considered as alternative and/or complementary methods for residue analysis because of their simplicity, cost-effectiveness, portability, and high sample throughput. Nowadays, immunoassays are very valuable analytical techniques for large monitoring or screening analysis (*14, 15*).

There are a few papers reporting on the development of triazole immunoassays. Forlani et al. (*16*) obtained polyclonal antibodies that recognized tetraconazole and penconazole by immunizing with succinylated DTP, a degradation product of tetraconazole. They developed an enzyme-linked immunosorbent assay (ELISA), in the enzyme-tracer format, that detected tetraconazole in the 25−10 000 μg/L range in fruit, after a

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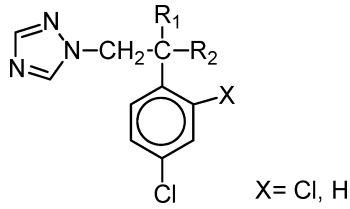


Figure 1. Common structure to several triazole fungicides: bromuconazole, cyproconazole, hexaconazole, myclobutanil, penconazole, propiconazole, and tetraconazole.

cleanup step, and fruit juices with good recoveries and reproducibility (*17*). Szèkács and Hammock (*18*), using a myclobutanil-derived hapten, developed a polyclonal-based ELISA that recognized mainly myclobutanil, with a limit of detection (LOD) of 200 μg/L in buffer. Chen et al. (*19*) obtained polyclonal antibodies, derived from an hexaconazole-derived hapten, that recognized hexaconazole (LOD of 0.1 μg/L in buffer) and displayed cross-reactivity (CR) toward penconazole and propiconazole (CR around 50%). Finally, Danks et al. (*20*) developed a polyclonal-based ELISA to tebuconazole that showed a 0.02−20 mg/L detection range for this triazole and no CR to other triazoles. All of these works have been carried out with polyclonal antibodies.

It is well-know that the generation of sensitive and specific antibodies to small molecules is greatly dependent upon a proper design of immunizing haptens. Particularly, the detection of a group of compounds of similar structure can be often accomplished by judicious synthesis of hapten−protein immunogens containing a common moiety to these compounds (*21, 22*). In this respect, 7 (bromuconazole, cyproconazole, hexaconazole, myclobutanil, penconazole, propiconazole, and tetraconazole) of the 24 triazole fungicides share a common structure, which is depicted in **Figure 1**. This structure is characterized by a central chiral carbon with four distinct substituents: a 1,2,4-triazolyl group, a mono- or dichlorinated phenyl group, and R_1 and R_2 as specific groups for each triazole. It seemed reasonable to hypothesize that immunization with haptens linked to carrier proteins through one of the R groups would raise antibodies to this group of triazole fungicides because the common structure would be adequately exposed to the immunosystem.

Therefore, our main goal was to produce high-sensitivity monoclonal antibodies displaying a broad specificity toward triazole fungicides. This work comprised the synthesis of several haptens presenting the basic structure of this group of triazoles, the design of an appropriate screening protocol to identify antibodies with the required specificity and sensitivity, the development and optimization of ELISAs based on selected monoclonal antibodies, and finally, the study of the analytical performance of the immunoassays in the determination of triazole fungicides in fruit juices.

MATERIALS AND METHODS

Reagents and Instruments. 2-(2,4-Dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol (DTP) was generously provided by Isagro Ricerca Srl (Novara, Italy). Riedel-de Haën and Sigma products were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). Fungicide standards were from Riedel-de Haën. Ovalbumin (OVA) was obtained from Sigma. Bovine serum albumin (BSA) fraction V and enzyme-immunoassay-grade horseradish peroxidase (HRP) were from Roche Diagnostics S.L. (Barcelona, Spain). Peroxidase-labeled rabbit anti-mouse immunoglobulins and goat anti-mouse immunoglobulins were obtained from Dako (Glostrup, Denmark). Flat bottom polystyrene ELISA plates (High Binding Plates) were from Costar (Cambridge, MA).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Varian Gemini 330 spectrometer (Sunnyvale, CA), operating at

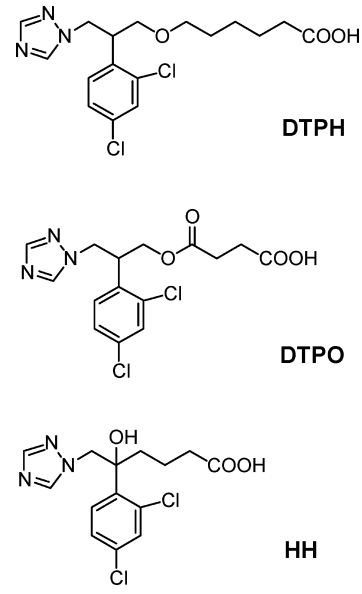


Figure 2. Structure of the triazole haptens used in this study.

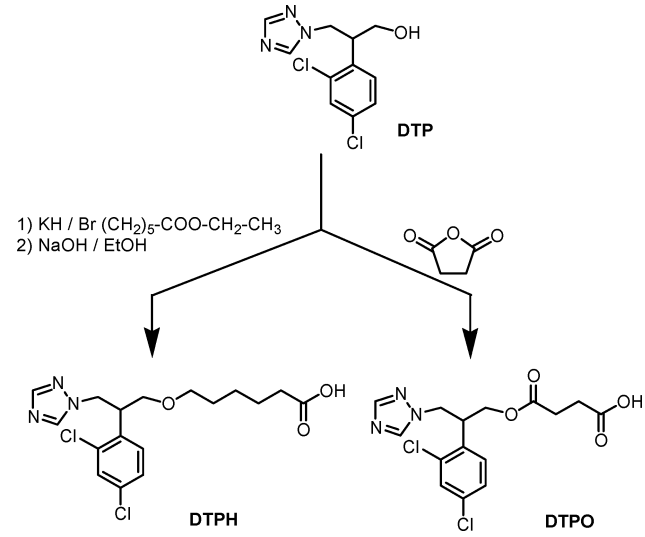


Figure 3. Scheme of the synthesis of the DTP-derived triazole haptens: DTPH and DTPO.

300 and 75 MHz, respectively. Chemical shifts are given relative to tetramethylsilane. ELISA plates were washed with a 96PW microplate washer from SLT Labinstruments GmbH (Salzburg, Austria), and absorbances were read in dual-wavelength mode (490−650 nm) with a Emax microplate reader from Molecular Devices (Sunnyvale, CA).

Hapten Synthesis. Most of the compounds used in this study present only minor safety concerns. However, it is advisable to work in a well-ventilated fume hood during synthesis work. The triazole haptens synthesized in this study are depicted in **Figure 2**. The hexaconazole hapten HH [5-(2,4-dichlorophenyl)-5-hydroxy-6-(1*H*-1,2,4-triazol-1-yl)hexanoic acid] was generously supplied by Syngenta (U.K.). DTPH and DTPO haptens were synthesized from DTP, a degradation product of penconazole and tetraconazole, by O-alkylation and O-succinylation of the hydroxyl group, respectively, using suitable spacer arms to render haptens with a terminal carboxyl group for covalent linking to proteins (**Figure 3**).

6-[2-(2,4-Dichloro-phenyl)-3-[1,2,4]triazol-1-yl-propoxy]-hexanoic Acid (DTPH). KH (0.67 g, 5 mmol), 30 wt % dispersion in mineral oil, was added to a round-bottom flask containing dried tetrahydrofuran (8 mL) and kept on an ice bath with vigorous stirring. DTP (1.088 g, 4 mmol) dissolved in dried *N*,*N*-dimethylformamide (3 mL, DMF) was added drop by drop. After 15 min, ethyl 6-bromohexanoate (1.34 g, 6 mmol) was gradually added. After reflux for 4 h, solvents were

evaporated under reduced pressure. The residue was dissolved in ethyl acetate (15 mL) and washed twice with distilled water (10 mL). The organic phase was dried over anhydrous sodium sulfate and concentrated, and the residue was subjected to column chromatography (92.5:7.5 dichloromethane/methanol) to render DTPH ethyl ester (500 mg, 35%) as an oil. To a solution of the ester (500 mg, 1.4 mmol) in ethanol (18 mL) was added 5 M NaOH (1.52 mL). After reflux for 1 h with stirring, the solvent was evaporated. Then, distilled water (15 mL) was added to the residue, and the mixture was washed twice with ethyl acetate (15 mL). The aqueous phase was acidified to pH 6.5 with 5 M HCl. White crystals appeared while standing at 4 °C, which were collected by filtration and dried to render DTPH (210 mg, 39%). ^{1}H NMR (acetone-d_6) δ: 1.3−1.65 (m, 6, $CH_2CH_2CH_2$), 2.27 (t, 2, CH_2COOH), 3.42 (m, 2, OCH_2), 3.62 (qd, 2, $CHCH_2O$), 4.1 (m, 1, CHaromatic), 4.63 (qd, 2, NCH_2), 7.32−7.57 (m, 3, aromatic), 7.81 and 8.16 (2s, 2, triazole). ^{13}C NMR (acetone-d_6) δ: 174.9, 152.4, 145, 137, 135.7, 133.7, 131.3, 129.8, 128.2, 71.6, 71.1, 50.6, 42.6, 34.3, 30.6, 26.4, 25.4.

Succinic Acid Mono-[2-(2,4-dichloro-phenyl)-3-[1,2,4]triazol-1-yl-propyl] Ester (DTPO). This hapten was synthesized essentially as described by Forlani et al. (*16*). Briefly, the alcohol DTP (1 g, 3.9 mmol) in anhydrous pyridine was treated with succinic anhydride (0.47 g, 4.7 mmol) in the presence of 4-dimethylaminopyridine (24.4 mg, 0.2 mmol). The concentrated residue was dissolved in dichloromethane and washed with 1 M HCl and distilled water. Finally, the oily crude product was crystalized from ethyl ether to give DTPO (510 mg, 37%) as a white solid. ^{1}H NMR (acetone-d_6) δ: 2.58 (s, 4, $COCH_2CH_2CO$), 4.22 (m, 1, CHaromatic), 4.36 (m, 2, NCH_2), 4.68 (m, 2, CH_2OOC), 7.36−7.53 (m, 3, aromatic), 7.82 and 8.21 (2s, 2, triazole). ^{13}C NMR (acetone-d_6) δ: 173.7, 172.5, 152.5, 145.1, 135.9, 135.8, 134.1, 131, 130, 128.4, 64.5, 50.3, 30.6, 29.1, 29.

Preparation of Protein−Hapten Conjugates. *Preparation of Immunizing Conjugates.* Haptens DTPH and HH were covalently attached to BSA using the modified active ester method (*23*). Briefly, 30 μmol of hapten was reacted with stoichiometric amounts of *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in 0.2 mL of DMF overnight at room temperature. After centrifuging, the appropriate volume of activation mixture containing the active ester was dissolved in DMF, up to 0.4 mL, and slowly added to 0.6 mL of a 15 mg/mL BSA solution in 0.2 M borate buffer at pH 9. The initial hapten/protein molar ratio in the mixture was 60:1. The mixture was allowed to react at room temperature for 3 h with stirring, and finally, the conjugate was purified by gel filtration on Sephadex G-50 using 100 mM sodium phosphate buffer at pH 7.4. Because of the low molar extinction coefficient of haptens, the extent of coupling of the hapten to BSA was estimated by the determination of the number of protein free amino groups before and after conjugation, using 2,4,6-trinitrobenzenesulfonic acid (TNBS) as the titration reagent (*24*). The hapten/protein molar ratio was evaluated as 20 and 19, for BSA−DTPH and BSA−HH, respectively.

Preparation of Coating Conjugates. Haptens DTPH, DTPO, and HH were covalently attached to OVA using the mixed-anhydride method (*25*). Briefly, 15 μmol of the hapten was treated with a 20% molar excess of tri-*n*-butylamine and isobutyl chloroformate in a total volume of 0.1 mL of DMF for 30 min at room temperature. To a volume of the reaction mixture containing 7 μmol of activated hapten was added DMF up to 0.25 mL and then was slowly added to 0.75 mL of a 15 mg/mL OVA solution in 0.2 M borate buffer at pH 9. The initial hapten/protein molar ratio in the mixture was 20:1. After stirring for 2 h at room temperature, the conjugate was purified by gel filtration. The extent of coupling of the hapten to OVA was determined by the TNBS protocol, described above. The hapten/protein molar ratio of the conjugates was evaluated as 6, 7, and 9, for OVA−DTPH, OVA−DTPO, and OVA−HH, respectively.

Enzyme Conjugates. Following the same procedure as before, triazole haptens were first activated and then conjugated to HRP (5 mg/mL) using a hapten/HRP molar ratio of 20:1. Enzyme tracers were purified by gel filtration and stored at 4 °C in a 1:1 mixture of saturated ammonium sulfate and PBS containing 0.1% BSA. HRP conjugate concentrations were estimated spectrophotometrically.

Production of Monoclonal Antibodies. Animal manipulation was carried out in accordance with the Spanish regulation currently in force and under the control of the local Ethical Committee for Research. BALB/c female mice (8−10 weeks old) were immunized with BSA−HH and BSA−DTPH conjugates. The first dose consisted of 100 μg of conjugate intraperitoneally injected as an emulsion of PBS and complete Freund's adjuvant. After 2 and 4 weeks of the initial dose, mice received booster injections with the same amount of immunogen emulsified in incomplete Freund's adjuvant. After a resting period of at least 3 weeks from the last injection in adjuvant, mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection of 100 μg of conjugate in PBS, 4 days prior to cell fusion. Selection was based on the ELISA determination of mouse serum titers against the homologous coating conjugates.

The cell fusion procedure was carried out as previously described (*22*). After 8−10 days of cell fusion, culture supernatants were screened for the presence of antibodies that recognized selected triazole fungicides. Screenings consisted of the simultaneous performance of a noncompetitive and competitive indirect ELISA, to test the ability of antibodies to bind the OVA conjugate of the immunizing hapten and recognize the analyte, respectively. The first screening was performed using 1 μM hexaconazole or 1 μM tetraconazole as a competitor for fusions derived from BSA−HH or BSA−DTPH, respectively. Next, wells showing antibodies that sensitively recognized these triazoles (signal inhibition > 50%) were further characterized, this time including three triazoles as competitors (hexaconazole, tetraconazole, and penconazole) at 100 nM, with each one of them in different ELISA wells.

Selected hybridomas were cloned by limiting dilution. Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen. Monoclonal antibodies (MAbs), all being of IgG class, were purified on a small scale directly from late stationary-phase culture supernatants by saline precipitation with saturated ammonium sulfate followed by affinity chromatography on protein G-Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences, Uppsala, Sweden). Purified MAbs were stored at 4 °C as ammonium sulfate precipitates.

Enzyme-Linked Immunosorbent Assays. ELISA plates were coated overnight with assay conjugates in 50 mM carbonate buffer at pH 9.6. A volume of 100 μL per well was used throughout all assay steps, and all incubations were carried out at room temperature. After each incubation, plates were washed 4 times with washing solution (0.15 M NaCl containing 0.05% Tween 20).

Conjugate-Coated Format. Plates were coated with OVA−hapten conjugates. Then, serum, culture supernatant, or antibody dilutions in PBS were added and incubated for 1 h. Next, plates were incubated for 1 h with peroxidase-labeled rabbit anti-mouse immunoglobulins diluted 1/2000 in PBST (PBS containing 0.05% Tween 20). Finally, peroxidase activity bound to the wells was determined by adding the substrate solution (2 mg/mL OPD and 0.012% H_2O_2 in 25 mM citrate and 62 mM sodium phosphate at pH 5.35). After 10 min, the reaction was stopped with 2.5 M sulfuric acid and the absorbance was read at 490 nm with a reference wavelength at 650 nm. For competitive assays, the procedure was the same, except that after coating a competition step was introduced by adding 50 μL of triazole standards or sample dilutions followed by 50 μL of the appropriate concentration of antibody (serum, culture supernatant, or purified mAb).

Antibody-Coated Format. Plates were coated with antibodies at 1 μg/mL in coating buffer. Next, the competition was established between analyte standards and selected dilutions of enzyme tracers (hapten−HRP conjugates) for 1 h. Finally, peroxidase activity was measured as above.

Preparation of Standards and Spiked Juice Samples. From a 1 mM stock solution of each triazole fungicide in DMF, a $^1/_5$ serial dilution from 1 mM to 12.8 nM was prepared in the same solvent. From these intermediate solutions, standards from 2 μM to 25.6 pM were prepared by diluting $^1/_{500}$ in PBS each of the eight concentrations. Because the assay procedure involved the addition of the same volume of the appropriate immunoreagent concentration, fungicide standards in the final assay ranged from 1 μM to 12.8 pM.

Tomato, orange, and apple juices were obtained from local supermarkets. Samples (25 mL) of fruit juices were spiked with a given triazole at 10, 20, 50, and 100 μg/L by adding 25 μL of 10, 20, 50, and 100 mg/L triazole stock solutions in DMF, respectively. For ELISA

Table 1. Summary of the Results of Cell Fusions and Hybridoma Selection

immunizing hapten	fusion number	number of wells seeded	positive[a] (hapten)	competitive[b] (analyte)	number of cloned hybridomas[c]
DTPH	1	384	11	10	1
	2	768	5	3	2
	3	480	4	3	
	4	384	7	7	1
	5	576	17	11	2
HH	1	672	90	48	5
	2	480	5	1	

[a] Wells with antibodies that recognized the OVA−hapten conjugates (1 μg/mL, homologous assays) by indirect ELISA (absorbance > 0.5). [b] Wells with antibodies that recognized hexaconazole or tetraconazole for HH or DTPH haptens, respectively (inhibition > 50% by 1 μM). Culture supernatants giving absorbances out of range were diluted until absorbance < 2.0. [c] Hybridomas secreting antibodies that sensitively recognized at least one triazole (tetraconazole, penconazole, and hexaconazole; inhibition < 50% by 100 nM triazole) were stabilized and cloned.

Table 2. Sensitivity of the Monoclonal Antibodies Derived from Triazole Immunogens[a]

monoclonal antibody	I_{50} values (nM) tetraconazole	penconazole	hexaconazole
DTPH-11	5	30	>1000
DTPH-21	44	113	>1000
DTPH-22	14	26	>1000
DTPH-41	6	13	>1000
DTPH-51	70	890	>1000
DTPH-52	68	126	>1000
HH-11	>1000	>1000	122
HH-12	>1000	>1000	265
HH-13	>1000	>1000	112
HH-14	>1000	>1000	10
HH-15	252	32	170

[a] Data were obtained using homologous ELISAs in the conjugate-coated format.

determination, spiked juice samples were appropriately diluted in PBS and analyzed without any sample pretreatment. Each dilution was analyzed in triplicate, and the triazole concentration in the samples was calculated by averaging values obtained at each dilution.

Data Analysis. Competitive curves were obtained by plotting absorbance values against the logarithm of the analyte concentration. Sigmoid curves were fitted to a four-parameter logistic equation (*26*) using commercial software (Sigmaplot, Jandel Scientific). The fungicide concentration in fruit juice samples was determined by interpolation of the mean absorbance values on the standard curve run in the same plate (SOFTmax Pro Software, Molecular Devices).

RESULTS AND DISCUSSION

Production of Antibodies to Triazole Fungicides. A hapten design strategy aimed at including the triazole structure depicted in **Figure 1** was followed. Three haptens were prepared containing this structure and suitable reactive groups for covalent linking to proteins (**Figure 2**). DTP, the major degradation product of tetraconazole, was an appropriate initial compound for the synthesis of haptens DTPH and DTPO by O-alkylation and O-succinylation, respectively (**Figure 3**). Hapten HH is a hexaconazole derivative characterized by a terminal carboxylic acid.

Mice were immunized with BSA conjugates of haptens DTPH and HH. After the third injection, mouse sera showed a high level of polyclonal antibodies recognizing each respective homologous hapten conjugate, with titers (serum dilution giving 3 times the background absorbance) ranging from $1/10^4$ to $1/10^5$. Polyclonal antibodies from mice immunized with BSA−DTPH and BSA−HH bound competitively tetraconazole and hexaconazole, respectively, in homologous conjugate-coated ELISAs, with I_{50} (concentration giving 50% inhibition of maximum signal) values in the 500−2000 μg/L range. Chen et al. (*19*) obtained a high-titer polyclonal response in rabbits by immunizing with BSA−HH. These antibodies recognized hexaconazole in the same ELISA format with an I_{50} value of 8 μg/L. Not surprisingly, a notorious difference between rabbit and mouse polyclonal response to the same immunogen has been previously found (*22, 27*). In terms of affinity, rabbit polyclonal antibodies are undoubtedly better than those from mice, but mice are usually immunized for applying the monoclonal antibody technology, as in this work.

Table 3. I_{50} Values (nM) for Tetraconazole or Hexaconazole Obtained with Different Haptens and Formats[a]

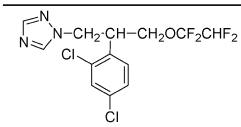
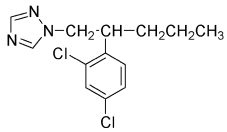
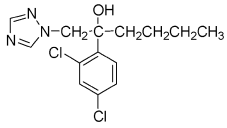
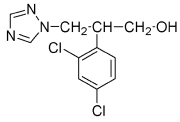
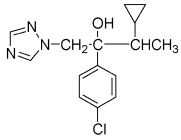
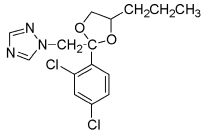
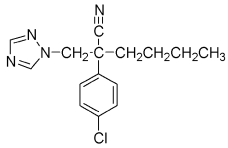
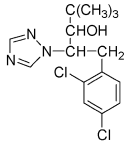
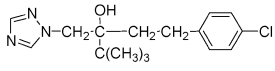
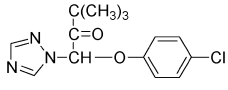
analyte	mAb	conjugate-coated ELISA (OVA−hapten) DTPH	DTPO	HH	antibody-coated ELISA (HRP−hapten) DTPH	DTPO	HH
tetraconazole	DTPH-11	5	5	3	4	4	nr[b]
	DTPH-22	14	25	12	11	10	nr
	DTPH-41	6	10	4	5	5	pr[c]
hexaconazole	HH-14	6	pr	10	nr	nr	nr
	HH-15	110	250	170	98	pr	nr

[a] Competitive ELISAs were performed in optimum conditions, i.e., limiting concentrations of immunoreagents giving maximum absorbance around 1.0. [b] No recognition. [c] Poor recognition.

Production of MAbs. Screening of hybridoma cultures was carried out with simultaneous noncompetitive and competitive ELISAs, using hexaconazole, penconazole, and tetraconazole as competitors, to identify both analyte- and class-specific antibodies. For the initial screening of a given fusion, only one competitor was used. Thus, hybridomas producing antibodies that competitively recognized a triazole of interest were identified. Next, hybridomas were further characterized using screening ELISAs with all three triazoles as competitors. From mice immunized with BSA−DTPH and BSA−HH, five and two fusions, respectively, were undertaken. The results of hybridoma production and selection are showed in **Table 1**. As stated in the Materials and Methods, the design of the screening procedure was very important to identify analyte- and class-specific antibodies that recognized the triazole fungicides studied with high affinity. In the first screening of each fusion, wells containing antibodies able to competitively recognize tetraconazole or hexaconazole (signal inhibition > 50% by 1 μM competitor) were identified. Subsequent hybridoma supernatant characterization allowed for the selection of those showing different recognition patterns for tetraconazole, hexaconazole, and penconazole and the highest sensitivity for these triazoles. Finally, 11 hybridomas were cloned and stabilized, with 6 derived from BSA−DTPH and 5 derived from BSA−HH.

Monoclonal Antibody Characterization. *Affinity.* MAbs produced by each of the 11 selected hybridomas were purified on a small scale from culture supernatants and were subsequently characterized in terms of affinity to triazole fungicides using homologous and heterologous haptens in different ELISA formats. The ability to recognize tetraconazole, penconazole, and hexaconazole was first evaluated using homologous conjugate-coated ELISA format. After selecting the adequate assay concentrations, I_{50} values for these triazole were estimated (**Table 2**). On the one hand, all of the MAbs derived from hapten DTPH displayed a common pattern, which is characterized by the recognition of penconazole and tetraconazole and the absence of recognition of hexaconazole. On the other hand, two

Table 4. Recognition of Several Triazole Fungicides by Selected Monoclonal Antibodies[a]

chemical structure	compound	monoclonal antibody cross-reactivity				
		HH-14	HH-15	DTPH-11	DTPH-22	DTPH-41
	tetraconazole	< 0.1	65[b] (169)[c]	660 (3)	185 (14)	225 (4)
	penconazole	< 0.1	507 (22)	100 (20)	100 (26)	100 (9)
	hexaconazole	100 (6)	100 (110)	0.1	1.4	0.2
	DTP	0.2	51 (215)	500 (4)	15 (173)	54 (17)
	cyproconazole	< 0.1	61 (180)	9.3 (215)	1.6	73 (12)
	propiconazole	< 0.1	1.6	0.5	118 (22)	0.6
	myclobutanil	< 0.1	2	38 (52)	0.2	31 (29)
	diclobutrazol	< 0.1	< 0.1	0.4	0.3	0.2
	tebuconazole	< 0.1	< 0.1	0.2	< 0.1	0.5
	triadimefon	< 0.1	< 0.1	0.5	1	2

[a] The heterologous coating conjugate providing the most sensitive immunoassay was used for each mAb: OVA−DTPH for HH MAbs and OVA−HH for DTPH MAbs. [b] Cross-reactivity was calculated as (I_{50} penconazole or hexaconazole/I_{50} compound) × 100. [c] I_{50} value (nM).

types of MAbs were found to be raised from hapten HH. The first type of MAbs (MAbs HH-11, -12, -13, and -14) is characterized by the recognition of hexaconazole only. The second type (mAb HH-15) seemed to be a class-specific mAb because all three triazoles were recognized.

MAbs showing the lowest I_{50} values, that is, DTPH-11, DTPH-22, DTPH-41, HH-14, and HH-15, were selected for further characterization. Thus, these antibodies were assayed in heterologous ELISAs using all of the haptens synthesized in this study. Specifically, heterology based on different spacer arms was examined (**Figure 2**). As expected, all of the haptens were recognized in the conjugate-coated format (**Table 3**). A

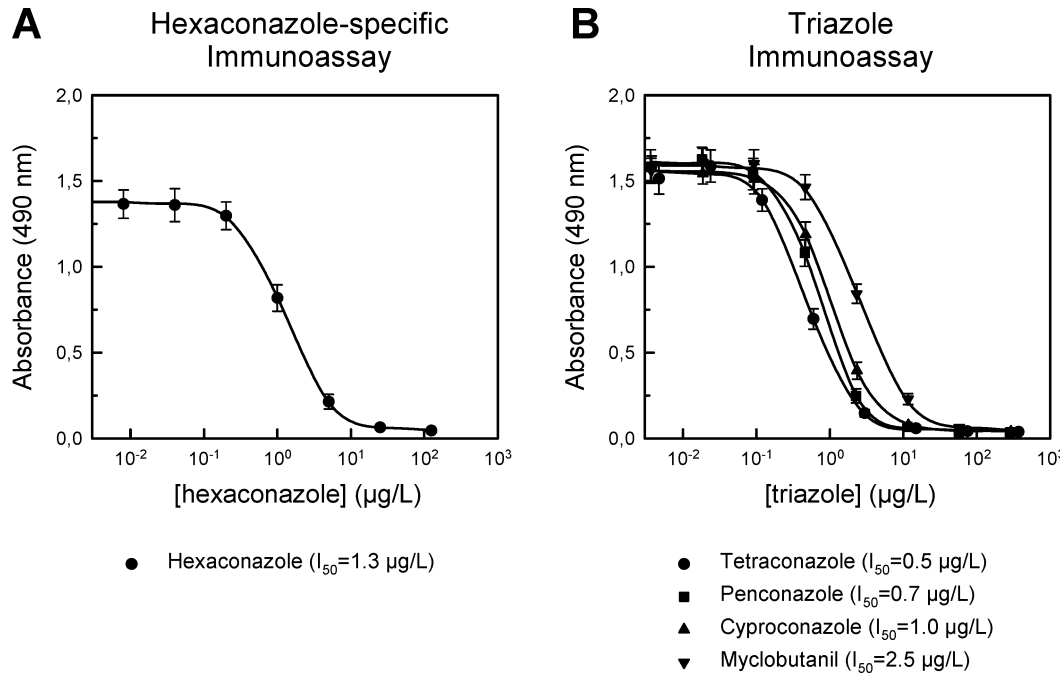


Figure 4. (A) Representative standard curve for hexaconazole obtained under optimized conditions: assay hapten, OVA−DTPH (2 μg/mL) and HH-14 mAb (60 ng/mL); assay buffer, PBS containing 0.0025% Tween 20 and 0.25% DMF. (B) Representative standard curve for detected triazoles: assay hapten, OVA−HH (1 μg/mL) and DTPH-41 mAb (30 ng/mL); the same assay buffer as above. Each point represents the mean value ± standard deviation (SD) of three replicates.

slight improvement of triazole affinity (lower I_{50} value) was achieved by using HH and DTPH as assay haptens for DTPH and HH MAbs, respectively. MAbs were also evaluated in the antibody-coated format. As shown in **Table 3**, the recognition pattern of immobilized antibodies (antibody-coated format) is clearly different from that of antibodies in solution (conjugate-coated format) and is characterized by a much less degree of HRP−hapten recognition. This is particularly remarkable for HH MAbs, because only mAb HH-15 in combination with the HRP−DTPH tracer provided a workable competitive assay. With regard to DTPH MAbs, HRP conjugates of haptens DTPH and DTPO were adequately recognized, whereas the HRP−HH conjugate was almost unrecognized. Such a different behavior of monoclonal antibodies depending upon conjugate or antibody immobilization has been previously reported (*22, 27*). Considering the sensitivity attained by each combination, both formats provided immunoassays of similar affinity, but the use of heterologous assays in the conjugate-coated format provided the most sensitive ELISA to the triazoles under study.

Specificity. mAb specificity was evaluated using the conjugate-coated format, which provided sensitive assays for all MAbs. Cross-reactivity data of several triazole compounds are presented in **Table 4**. As shown, MAbs with diverse cross-reactivity pattern have been obtained even for antibodies derived from the same hapten. This fact became clearly patent by MAbs derived from hapten HH, because HH-14 mAb was hexaconazole-specific, whereas HH-15 mAb recognized four of the six triazoles besides DTP. With regard to DTPH MAbs, all of them recognized tetraconazole and penconazole and one or two of the other triazoles, although again with diverse recognition pattern. Thus, DTPH-11 mAb showed cross-reactivity toward myclobutanil; DTPH-22 mAb showed cross-reactivity toward propiconazole; and mAb DTPH-41 showed cross-reactivity toward cyproconazole and myclobutanil. Furthermore, all of them recognized, to a different extent, DTP, and among them, DTPH-11 mAb afforded the most sensitive assay for this degradation product (I_{50} = 4 nM for DTP). To go more deeply into mAb specificity, other triazole fungicides were assayed. Particularly, diclobutrazol, tebuconazole, and triadimefon also contain a chloroaromatic and triazole ring but in an arrangement other than that depicted in **Figure 1**. As shown in **Table 4**, both types of MAbs exhibited poor recognition of these triazole fungicides. This finding pointed out the high specificity of these MAbs toward fungicides containing the triazole structure under study. Furthermore, cross-reactivities toward triazole fungicides containing other heterocyclic rings (imidazole and pyrimidine) were negligible (data not shown). Interestingly, imazalil, with an imidazole ring instead of the triazole ring as the unique structural change, was almost unrecognized.

On the basis of the above data, it seems reasonable to conclude that mAb specificity is directed to triazole fungicides that fit the common structure shown in **Figure 1**, characterized by a central chiral carbon with a triazole, chlorinated phenyl, and R_1 and R_2 groups attached to it. A similar behavior in terms of triazole fungicide specificity has been found for rabbit polyclonal antibodies derived from DTPO (*16*) and HH (*19*) immunogens. As exemplified in this work, it is worth noting that MAbs, even those derived from the same hapten, may have different recognition patterns of a family of structurally related compounds, and they could be identified providing that appropriate screening protocols are followed.

Development and Characterization of Triazole Immunoassays. mAb characterization proved that both analyte- and class-specific immunoassays could be developed for triazole fungicides. Thus, HH-14 mAb provides a hexaconazole-specific immunoassay, while HH-15 and DTPH-11, -22, and -41 MAbs provide triazole-specific immunoassays because at least three of the triazole studied were recognized. Among them, mAb DTPH-41, recognizing four of the triazole fungicides (tetraconazole, penconazole, cyproconazole, and myclobutanil) with suitable affinity, was selected to develop a class-specific triazole immunoassay (**Figure 4**). For both types of immunoassays, heterologous assays in the conjugate-coated format gave the most sensitive ELISAs (**Table 3**). Then, the selected immu-

Table 5. Influence of Matrix Dilution on the Performance of the Fungicide Immunoassays

			hexaconazole[a]			tetraconazole[b]		
	spiked levels (μg/L)[c]	dilution factor[d]	found (μg/L)[e]	recovery (%)	CV (%)	found (μg/L)[e]	recovery (%)	CV (%)
apple juice	10	10	8	80	34	5	50	34
	20	20	17	85	12	14	70	32
	50	50	54	108	22	65	130	10
	100	100	133	133	26	103	103	10
orange juice	10	10	45	450	26	13	128	7
	20	20	27	135	23	26	130	7
	50	50	51	102	27	59	118	14
	100	100	99	99	19	121	121	4
tomato juice	10	10	17	170	45	22	220	20
	20	20	26	130	5	24	120	8
	50	50	67	134	12	68	136	11
	100	100	102	102	9	121	121	5

[a] Samples analyzed by the hexaconazole-specific immunoassay (HH-14 mAb). [b] Samples analyzed by the triazole immunoassay (DTPH-41 mAb). [c] Samples were spiked separately with hexaconazole or tetraconazole and analyzed by its respective immunoassay. [d] Sample dilution factor before adding to ELISA wells. [e] Data obtained from five independent determinations.

noreagents were HH-14 as mAb and OVA−DTPH as coating conjugate for the hexaconazole immunoassay and DTPH-41 as mAb and OVA−HH as coating conjugate for the triazole immunoassay.

For assay optimization, immunoreagent concentrations were first adjusted to obtain optimum assay parameters. Next, the composition of the assay buffer was studied. With regard to ionic strength and pH, the buffer used throughout this work (1 × PBS at pH 7.5) afforded optimum parameters. The influence of two common additives, the non-ionic surfactant Tween 20 and BSA, was also investigated. In this case, the reduction of Tween 20 concentration to 0.0025% afforded the highest sensitivity assays while maintaining acceptable reproducibility (data not shown).

Typical standard curves obtained with the optimized hexaconazole and triazole immunoassays are presented in **Figure 4**. Hexaconazole can be determined from 0.5 to 3.8 μg/L (20−80% inhibition) with the hexaconazole-specific immunoassay in the assay buffer, with an I_{50} value of 1.3 μg/L (4 nM) and a limit of detection (10% inhibition) of 0.3 μg/L. On the other hand, the triazole immunoassay allows for the determination of tetraconazole, penconazole, cyproconazole, and myclobutanil with I_{50} values ranging from 0.5 to 2.5 μg/L (1.3−8.6 nM) and limits of detection ranging from 0.1 to 0.7 μg/L.

Analytical Performance of Triazole Immunoassays. The optimized immunoassays were applied to the determination of triazole fungicides in fruit juices. To carry out this study, tetraconazole, the triazole fungicide detected with the lowest LOD, was selected for the class-specific immunoassay and, obviously, hexaconazole was selected for the hexaconazole-specific immunoassay.

Evaluation of Matrix Effects. First, the influence of fruit juice matrixes on the triazole immunoassays was studied. Dealing with liquid matrixes, the simplest way to perform this study is to find out the minimum sample dilution to minimize the matrix effects. Accordingly, apple, orange, and tomato juice samples were spiked with triazole fungicides at concentrations in the 10−100 μg/L range and properly diluted with assay buffer to a common concentration (1 μg/L) entering the assay working range. After diluting, all of the samples had the same fungicide concentration but different proportions of each juice. The results of the immunoanalysis of these spiked samples are shown in **Table 5**. As can be seen in this table, when juice samples were diluted $^1/_{10}$ ($^1/_{20}$ in the assay), the recoveries and the coefficients of variation (CV) were relatively poor. However, matrix effects were minimized when apple, orange, and tomato juice samples were diluted at least $^1/_{20}$ ($^1/_{40}$ in the assay), as proven by the acceptable recovery and precision data obtained for both hexaconazole and tetraconazole immunoassays.

Table 6. Reproducibility and Accuracy of Triazole Immunoassays in Spiked Juice Samples

		hexaconazole[a]			tetraconazole[b]		
	spiked levels (μg/L)[c]	found (μg/L)[d]	recovery (%)	CV (%)	found (μg/L)[d]	recovery (%)	CV (%)
apple juice	10	21	210	16	6	60	61
	20	22	110	28	18	90	22
	50	52	104	17	31	62	15
	100	91	91	12	83	83	14
orange juice	10	16	160	18	18	180	36
	20	27	135	8	27	135	21
	50	66	132	16	64	128	9
	100	117	117	24	117	117	13
tomato juice	10	18	180	26	14	140	60
	20	22	110	7	22	110	30
	50	58	116	7	46	92	21
	100	106	106	18	98	98	16

[a] Hexaconazole samples were diluted $^1/_{25}$ in assay buffer before adding to ELISA wells and analyzed by the hexaconazole-specific immunoassay (HH-14 mAb). [b] Tetraconazole samples were diluted $^1/_{50}$ in assay buffer before adding to ELISA wells and analyzed by the triazole immunoassay (DTPH-41 mAb). [c] Samples were spiked separately with hexaconazole or tetraconazole and analyzed by its respective immunoassay. [d] Data obtained from five independent determinations.

Analysis of Spiked Juice Samples. Apple, orange, and tomato juices were spiked with hexaconazole or tetraconazole at 10, 20, 50, and 100 μg/L and directly analyzed by immunoassay, just diluting $^1/_{25}$ (hexaconazole) or $^1/_{50}$ (tetraconazole) in assay buffer. After diluting, these fortification levels entered into their respective assay working ranges, with the exception of the 10 μg/L concentration, which is close to the LOD. **Table 6** summarizes the results of the immunoanalysis of spiked juice samples. For both immunoassays, the analytical parameters of the fortification levels entering the working range were fairly good for the three juices, with CV below 30% and recoveries ranging from 62 to 135%. As expected, the analytical data of samples spiked at 10 μg/L were on the borderline of what can be considered as acceptable, pointing out that the LOD for these immunoassays has been reached. However and despite the fact that samples were properly diluted to minimize matrix effects, there was a tendency to overestimate both triazoles in orange juice samples and underestimate tetraconazole in apple juice samples.

In addition and taking advantage of the class specificity of the triazole immunoassay, juice samples were also spiked with

penconazole and analyzed by this immunoassay. The results of the immunoanalysis of penconazole spiked juice samples were very similar, in terms of reproducibility and accuracy, to those of the tetraconazole analysis (data not shown).

The study of the analytical performance in spiked samples proved that these immunoassays are able to determine triazole fungicides in apple, orange, and tomato juices at concentrations down to 20 μg/L, which is the lowest reported MRL, with acceptable precision and accuracy. Consequently, the described immunoassays can be valuable analytical tools for the rapid and simple determination of triazole fungicide residues in processed fruit and vegetables.

ABBREVIATIONS USED

BSA, bovine serum albumin; CR, cross-reactivity; CV, coefficients of variation; DMF, *N*,*N*-dimethylformamide; DTP, 2-(2,4-dichlorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propanol; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LOD, limit of detection; mAb, monoclonal antibody; I_{50}, concentration giving 50% inhibition of maximum signal; MRLs, maximum residue levels; NMR, nuclear magnetic resonance; OVA, ovalbumin; PBS, phosphate buffer saline; PBST, PBS containing 0.05% Tween 20.

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