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Monoclonal Antibody Generation and Direct Competitive Enzyme-Linked Immunosorbent Assay Evaluation for the Analysis of the Fungicide Fenhexamid in Must and Wine

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Fenhexamid is a common fungicide used to fight botrytis infections in a great variety of plant crops, particularly in vineyards. In this study, high-affinity and specific monoclonal antibodies against fenhexamid were produced for the first time, and an enzyme-linked immunosorbent assay (ELISA) in the antibody-coated direct competitive format was developed. The derivative 4-[2,3-dichloro-4-(1-methylcyclohexanecarboxamido)phenoxy]butanoic acid, with a carboxylic spacer arm attached to the phenolic hydroxyl group of fenhexamid, was synthesized for antibody production and ELISA development. To optimize this assay, the dependence of assay parameters on ionic strength and pH was assessed, and the influence of several organic solvents was determined. The IC_{50} value of the optimized assay for fenhexamid and the calculated limit of detection in phosphate buffer were 0.52 ± 0.06 and 0.13 ± 0.03 μg/L, respectively. Preliminary studies using fortified must and wine samples indicated that the presence of fenhexamid below the maximum residue limits in grapes could be easily determined with the developed immunoassay by a simple dilution of the sample in water.

KEYWORDS: Immunoassay; immunochemical methods; pesticide; botrycide; botrytis; hapten synthesis; food safety; coupling site; alkyl spacer arm

INTRODUCTION

Botrytis cinerea is the causative pathogen of gray mold, a plant infection also commonly named botrytis. It is a fungal disease found and dreaded the world over, especially in high-value crops such as wine grapes, soft fruits, pome and stone fruits, many vegetables, and some ornamental plants. To avoid or diminish culture damage, a widely used botrycide is fenhexamid (FH), the only member of the new hydroxyanilide group of fungicides (*1*). It is used as either a preharvest or a postharvest fungicide treatment, being highly effective not only against *B. cinerea* but also against related pathogens such as *Monilinia* spp. and *Sclerotinia sclerotiorum*, with excellent plant compatibility. FH has experienced great success during the present decade due in part to its new biochemical mode of action, consisting of the inhibition of the enzyme C_3-ketoreductase, which is involved in the biosynthesis of sterols (*2*). FH inhibits the growth of the germ tube and mycelium of the fungi at very low concentrations (500−750 g of active ingredient per ha.), and it is widely used particularly in strawberry fields and vineyards, where it exhibits an outstanding lasting activity (*3*−*6*). As a consequence, residues of this pesticide have been frequently found in strawberries and grapes in comprehensive pesticide residue monitoring programs (*7*, *8*) and even in commercial bottled wines (*9*, *10*). The new rules on pesticide residues applicable in the European Union from September 1, 2008, have set maximum residue limits (MRLs) of FH from 1 to 10 mg/L, depending on the crop (*11*).

Immunoassays (IAs) are a very much adaptable technology, ranging from fully automated sensors to portable rapid tests such as lateral flow assays and enzyme-linked immunosorbent assays (ELISA). Nowadays, IAs are a mature technology with broad application to pesticide analysis. Since the first immunochemical methods were developed for the analysis of agrochemicals (*12*−*14*), different IAs have been described for many pesticides belonging to a variety of families (*15*, *16*). However, to continue with the enhancement of this technology, the development of novel antibodies for individual new generation pesticide compounds is demanded. This includes the design and synthesis of new haptens, the understanding and control of the combination of hapten molecules and macromolecular carriers, and the improvement of the efficiency of existing laboratory procedures to increase the yield of antibodies having the desired characteristics (*17*). To our knowledge, no articles have been published so far describing the production of antibodies against FH and the development of IAs for this pesticide. The aim of the present study was the synthesis of FH derivatives for protein conjugation and the generation of specific and high-affinity monoclonal antibodies against this fungicide. These reagents were used for the development of an IA using the antibody-coated direct competitive ELISA format (dc-ELISA), and finally, the performance of the optimized assay was evaluated in fortified must and wine.

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MATERIALS AND METHODS

Chemicals and Instrumentation. FH [*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methyl cyclohexanecarboxamide] (CAS Registry no. 126833-17-8; MW 302.2) and other pesticide standards, Pestanal grade, were purchased from Fluka/Riedel-de-Haen (Seelze, Germany) or were acquired directly from the manufacturer. Certified tebuconazole, mepanipyrim, (*E*)-metominostrobin, and orysastrobin were from Dr. Ehrenstorfer (Augsburg, Germany). Horseradish peroxidase (HRP), ovalbumin (OVA), and *o*-phenylenediamine (OPD) were purchased from Sigma-Aldrich (Madrid, Spain). Sephadex G-25 HiTrap Desalting columns and HiTrap Protein G HP columns from GE Healthcare (Uppsala, Sweden) were used for conjugate and antibody purification, respectively. Polyclonal rabbit antimouse immunoglobulin peroxidase conjugate (RAM-HRP) was from Dako (Glostrup, Denmark). Bovine serum albumin (BSA) fraction V and Hybridoma Fusion and Cloning Supplement (HFCS) were purchased from Roche Applied Science (Mannheim, Germany). P3-X63-Ag 8.653 mouse plasmacytoma cell line was from the European Collection of Cell Cultures (Wiltshire, United Kingdom). HT and HAT supplements and gentamicine solution were obtained from Gibco BRL (Paisley, Scotland). Cell culture media (high-glucose Dulbecco's modified Eagle's medium), poly(ethylene glycol) (PEG1500), fetal bovine serum (FBS), 200 mM alanyl-glutamine solution, Red Blood Cell Lysing Buffer Hybri-Max, MEM nonessential amino acid solution, and Freund's adjuvants were from Sigma-Aldrich. Culture plastic ware and Costar flat-bottom high-binding polystyrene ELISA plates were from Corning (Corning, NY). Ultraviolet−visible (UV−vis) spectra and ELISA absorbances were read (in dual wavelength mode, 492−650 nm) with a PowerWave HT microplate reader from BioTek Instruments (Winooski, VT). Microplates were washed with an ELx405 washer also from BioTek Instruments.

Ethyl 4-bromobutyrate was supplied by AlfaAesar (Karlsruhe, Germany). All of the other chemical reagents and solvents used for hapten synthesis and conjugate preparation were obtained from commercial sources and employed without further purification. The reactions were monitored with the aid of thin-layer chromatography using 0.25 mm precoated silica gel plates. Chromatography refers to flash column chromatography, and it was carried out with the indicated solvents on silica gel 60 (particle size, 0.040−0.063 mm). All melting points were determined using a Kofler hot-stage apparatus and are uncorrected. NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at room temperature on a Bruker AC-300 spectrometer (300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). The spectra were referenced to residual solvent protons in the ^{1}H MR spectra (7.26 and 2.50 ppm) and to solvent carbons in the ^{13}C NMR spectra (77.0 and 39.43 ppm). Carbon substitution degrees were established by distortionless enhancement by polarization transfer pulse sequences. A combination of correlation spectroscopy and heteronuclear single quantum coherence experiments was utilized for the assignment of ^{1}H and ^{13}C chemical shifts. IR spectra were measured as NaCl pellets using a Nicolet Avatar 320 spectrometer. High-resolution mass spectra were recorded with a VG AutoSpec spectrometer.

Buffers, Media, and Solutions. (1) PB, 100 mM sodium phosphate buffer, pH 7.4. (2) PBS, 10 mM phosphate buffer, pH 7.4, with 137 mM NaCl and 2.7 mM KCl. (3) PBST, PBS containing 0.05% (v/v) Tween 20. (4) CB, 50 mM carbonate−bicarbonate buffer, pH 9.6. (5) Washing solution, 0.15 M NaCl containing 0.05% (v/v) Tween 20. (6) Developing buffer, 2 mg/mL of OPD and 0.012% (v/v) H_2O_2 in 25 mM citrate and 62 mM sodium phosphate buffer, pH 5.4. (7) Stop solution, 2.5 M H_2SO_4. (8) Stock standard solutions, FH, and all other analytes were prepared as concentrated solutions in *N*,*N*-dimethylformamide (DMF) and kept at −20 °C in amber glass vials.

Hapten Synthesis. The derivative 4-[2,3-dichloro-4-(1-methylcyclohexanecarboxamido)phenoxy] butanoic acid (hapten FHo4) was prepared by reaction with ethyl 4-bromobutyrate and subsequent hydrolysis of the ester (**Figure 1**). A solution of 4.0 g of FH (13.2 mmol) in 60 mL of dry acetone was treated with 3.6 g of K_2CO_3 (26.5 mmol) under reflux during 15 min. Then, 2.9 mL of ethyl bromobutyrate (20.0 mmol) was added, and the reaction was allowed to continue overnight under reflux. The next day, the solvent was evaporated, the residue was partitioned between ethyl acetate and water, the organic phase was washed with a 1 N NaOH solution and dried, and the solvent was evaporated. The resulting liquid product was purified by chromatography in silica gel using 95:5 (v/v) dichloromethane:ethyl acetate as the eluent. Pure ethyl ester was hydrolyzed by reflux for 2.5 h in ethanol containing NaOH. Then, the ethanol was evaporated, and ethyl acetate was added and extracted with water. Next, the hapten was precipitated from the aqueous phase with concentrated HCl and dissolved with ethyl acetate. Finally, the dried solvent was evaporated, and from the resulting viscous oil, the pure product (2.8 g, 54% yield) was obtained as a white solid by addition of hexane: mp 102−103 °C (from benzene−hexane). ^{1}H NMR ($CDCl_3$): δ 12.17 (1H, s, CO_2H), 9.12 (1H, s, NHCO), 7.27 (1H, d, J = 9.0 Hz, H-5 Ph), 7.14 (1H, d, J = 9.0 Hz, H-6 Ph), 4.12 (2H, t, J = 6.8 Hz, H-4), 2.42 (2H, t, J = 7.2 Hz, H-2), 2.10−1.94 (4H, m), 1.55−1.20 (8H, m), 1.19 (3H, s, Me). ^{13}C NMR (DMSO-d_6): δ 175.80 (CO_2H), 173.93 (CON), 152.97 (C-1 Ph), 130.12 and 129.45 (C-3 and C-4 Ph), 127.61 (C-5 Ph), 120.33 (C-2 Ph), 111.52 (C-6 Ph), 68.25 (C-4), 42.61 (C-1 Cy), 35.07 (C-2/C-6 Cy), 29.88 (C-2), 26.50 (Me), 25.33 (C-4 Cy), 24.03 (C-3), 22.68 (C-3/C-5 Cy). IR (NaCl): 2437, 3191, 2924, 2853, 1758, 1713, 1669, 1645, 1506, 1468, 1274, 1249, 1046, 978 cm^{-1}. MS (EI): m/z (%) 387 (M^+, 8), 303 (8), 301 (13), 179 (26), 177 (40), 125 (4), 98 (11), 97 (100). HRMS (EI): calcd for $C_{18}H_{23}{}^{35}Cl_2NO_4$, 387.10041; found, 387.09922. UV (PB): ε (280 nm) = 0.91 mM^{-1} cm^{-1}.

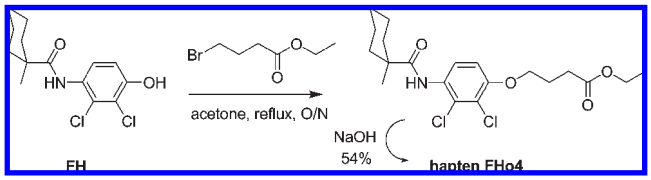

Figure 1. Synthesis of hapten FHo4.

Preparation of Protein−Hapten Conjugates. All conjugates used in this study were prepared by activation of the free carboxylic group of the synthesized hapten and reaction with the free amine groups of the carrier protein. Three carrier proteins were used as follows: BSA for the immunizing conjugate, OVA for the screening conjugate, and HRP for the assay conjugate.

Immunizing Conjugate. Fifty micromoles of FHo4 in DMF was mixed with 50 μmol of *N*-hydroxysuccinimide and 50 μmol of *N*,*N*-dicyclohexylcarbodiimide also in DMF. Additional DMF was added to the mixture to bring the final concentration of all reagents to 50 mM. The hapten was activated overnight at room temperature in amber vials. The day after, the reaction was centrifuged, and the supernatant was collected. Next, 400 μL of activated hapten solution was added dropwise to 2 mL of a 15 mg/mL BSA solution in CB. The coupling reaction proceeded for 4 h at room temperature with moderate stirring. The initial hapten-to-protein molar ratio (MR) in the mixture was approximately 45:1. Finally, the conjugate was separated from uncoupled hapten by gel filtration on Sephadex G-25, using PB as the eluent. The degree of hapten-to-protein conjugation was measured spectrophotometrically. If conjugation occurred, the UV−vis spectrum of the conjugate was slightly different from that of the free protein. Therefore, the hapten-to-protein MR was calculated from the absorbance value at 280 nm by assuming that the molar absorption coefficients of the hapten and the protein did not change upon conjugation. The purified conjugate was diluted to 1 mg/mL with PB and stored at −20 °C.

Screening Conjugate. The mixed anhydride method was used to prepare a conjugate of hapten FHo4 to OVA. Briefly, 18 μmol of hapten was dissolved in 180 μL of DMF and mixed with 18 μmol of tributylamine and 18 μmol of isobutyl chloroformate also in DMF. Then, the concentration of all reagents in the mixture was brought to 90 mM with DMF, and the activation reaction was allowed to occur for 1 h at room temperature. Next, 100 μL of the solution containing the activated hapten was added dropwise to 2 mL of a 15 mg/mL OVA (0.68 μmol) solution in CB. The coupling reaction was performed for 2.5 h at room temperature with moderate stirring. The initial hapten-to-protein MR in the mixture was approximately 13:1. Finally, the conjugate was separated from uncoupled hapten by gel chromatography as described for the immunizing conjugate, and the final hapten-to-protein MR was estimated as before. The conjugate was stored at −20 °C at 1 mg/mL in PB.

Assay Conjugate. The mixed anhydride method was also used to prepare an enzyme conjugate of hapten FHo4. Briefly, 27 μmol of FHo4 was dissolved in DMF and mixed with 27 μmol of tributylamine and

27 μmol of isobutyl chloroformate also in DMF. The same solvent was added to bring the final concentration of all reagents in the mixture to 90 mM. The hapten was activated for 1 h at room temperature. Next, 100 μL of a 1:10 (v/v) dilution in DMF of activated hapten solution was added dropwise to a 1 mL solution of HRP at 2.2 mg/mL (0.05 μmol) in CB. The coupling reaction was allowed to take place for 4 h at room temperature with moderate stirring. The initial hapten-to-protein MR in the mixture was approximately 18:1. The conjugate was separated from uncoupled hapten by gel chromatography as described before, and the final hapten-to-protein MR was calculated using, in this case, the absorbance values of the conjugate at 400 and 280 nm. The purified conjugate was brought to 1 mg/mL with elution buffer, and then, it was diluted 1:1 (v/v) with PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal. The tracer conjugate was stored at −20 °C in amber glass vials, and a working aliquot was kept at 4 °C.

Production of Monoclonal Antibodies. Animal manipulation has been performed in compliance with the laws and guidelines of the Spanish Ministry of Agriculture, Fisheries, and Food.

Immunization. BALB/c female mice (8−10 weeks old) were immunized with the BSA−FHo4 conjugate by intraperitoneal injections. Doses consisted of an emulsion of 100 μL of PBS containing 100 μg of protein conjugate, estimated as protein concentration, and 100 μL of Freund's adjuvant. The first dose contained complete Freund's adjuvant, and subsequent doses were given at weeks 3 and 6 using incomplete Freund's adjuvant. The antiserum from each mouse was obtained by submandibular bleeding 10 days after the third injection. Sera were diluted one-fifth with PBS containing 0.01% thimerosal and stored at 4 °C in amber glass vials. After a resting period of at least 3 weeks from the last injection with adjuvant and 4 days before cell fusion, mice received a booster intraperitoneal injection of 100 μg of protein conjugate in 200 μL of PBS.

Cell Fusion and Culture. P3-X63/Ag 8.653 murine myeloma cells were cultured in high-glucose DMEM supplemented with 2 mM alanylglutamine, 1 mM MEM nonessential amino acids, and 25 μg/mL 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 PEG1500 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 of s-DMEM with 15% FBS. Twenty-four hours after plating, 100 μL of HAT selection medium (s-DMEM supplemented with 100 μM hypoxanthine, 0.4 μM aminopterine, and 16 μM thymidine) with 20% FBS and 1% (v/v) HFCS was added to each well.

Hybridoma Selection and Cloning. Twelve days after cell fusion, hybridoma culture supernatants were screened following a double-assay strategy as first described by our group (*18*). This strategy consisted of two competitive assays that were run one after the other with different assay conditions to increase the characterization of the hybridomas being explored. First, culture wells were examined by simultaneous indirect noncompetitive and competitive ELISA with OVA−FHo4 conjugate under low selective pressure as described by Abad et al. (*19*). The signal in noncompetitive conditions was compared with the competitive one when FH was used as the competitor. The ratio of both absorbances was used as the criterion for selecting good antibody-secreting clones. Next, competitive ELISAs were performed using serial dilutions of the culture supernatant from those wells that afforded saturated signals under noncompetitive conditions in the first screening experiment. Also, the selective pressure was increased in the second assay using lower OVA−FHo4 conjugate and FH concentrations. The selected hybridomas were cloned by limiting dilution in HT cloning medium (s-DMEM containing 20% FBS and supplemented with 100 μM hypoxanthine, 16 μM thymidine, and 1% HFCS). Stable antibody-producing clones were expanded and cryopreserved in liquid nitrogen.

Purification of Monoclonal Antibodies. Immunoglobulins were purified from late stationary-phase culture supernatants by ammonium sulfate precipitation and protein G affinity chromatography following the column manufacturer's instructions. A fraction of the purified mAb was diluted with 1 volume of PBS containing 1% (w/v) BSA and 0.01% (w/v) thimerosal and stored at 4 °C in amber glass vials for daily usage. The remaining mAb solution was stored at 4 °C as ammonium sulfate precipitate. The immunoglobulin isotype was determined using the Mouse MonoAb-ID kit (HRP) from Invitrogen (Carlsbad, CA).

Direct Competitive ELISAs. The assays were performed in the antibody-coated dc-ELISA format. For coating, 100 μL per well of a 1 μg/mL mAb solution in CB was added, and plates were incubated overnight at room temperature. Coated plates were washed four times with washing solution and received, afterward, 50 μL per well of analyte in PBS plus 50 μL per well of HRP tracer solution in PBST. The optimum assay conjugate concentration was established so to reach a maximum absorbance around 1.0. The immunological reaction took place for 1 h at room temperature, and plates were washed again as described. Finally, retained peroxidase activity was determined by addition of 100 μL per well of freshly prepared 2 mg/mL OPD and 0.012% (v/v) H_2O_2 in developing buffer. The enzymatic reaction was stopped after 10 min at room temperature by the addition of 100 μL per well of stop solution. The absorbance was immediately read at 492 nm with a reference wavelength at 650 nm.

Sigmoidal curves were mathematically fitted to a four-parameter logistic equation using the SigmaPlot software package from SPSS Inc. (Chicago, IL). Assay sensitivity was estimated as the concentration of analyte that reduced 50% (IC_{50}) the value of the upper asymptote of the sigmoidal curve (A_{max}). The limit of detection (LOD) and the working range of the standard curve in buffer were estimated as the concentrations of FH that provided a 10 and 20−80% reduction of A_{max}, respectively. The lower limit of the working range was considered as the limit of quantitation (LOQ).

Assay Optimization and Characterization. *Tolerance to Solvents.* Assay tolerance to methanol, ethanol, acetone, acetonitrile, DMF, and dioxane was assessed between 0 and 10% solvent concentration (v/v). Standard curves of FH were prepared in PBS containing different solvent concentrations, and 50 μL of each FH solution was mixed with 50 μL of assay conjugate in PBST.

Buffer Conditions. Antibody-coated competitive assays were performed as described above. Now, the influence of buffers with different pH or ionic strength was evaluated. Standard curves were prepared in water, and the enzyme tracer solution was prepared in different buffers. To study the behavior of the assay under different ionic strength conditions, a 40 mM phosphate buffer, pH 7.4, containing 2 M NaCl solution was serially diluted with 1 volume of the same buffer without NaCl. Afterward, each dilution received 1 volume of a 0.1% Tween 20 solution and a constant concentration of assay conjugate. To evaluate the influence of the pH, a set of buffers was prepared with constant ionic strength but with different pH values. First, a stock solution of 40 mM citrate, 40 mM hydrogen phosphate, and 40 mM Tris was prepared (the final pH was 9.95). Different volumes of 5 N HCl were added to aliquots of the stock solution to reach the desired pH. The ionic strength of each solution was determined at 25 °C according to the formula:

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

where I is the ionic strength value, c is the concentration of each ion at equilibrium, and z is its charge. Afterward, the appropriate volume of a 2 M NaCl solution was added to bring each buffer to the same ionic strength value (I = 360 mM), and volumes were made equal with water.

Must and Wine Analysis. Commercial musts (red and white) and wines (red and white) were acquired from a local supermarket. Competitive assays were performed using the dc-ELISA under the above stated conditions and the optimized buffer and assay conjugate concentration. Each antibody-coated microplate well received 50 μL of FH standards in Milli-Q water or 50 μL of samples fortified with FH and diluted in Milli-Q water plus 50 μL of a 12 ng/mL HRP−FHo4 conjugate solution in 200 mM phosphate buffer, pH 7.4, containing 0.05% Tween 20.

Matrix Effects. FH standard curves were prepared in water containing different proportions of must or wine. Curves were run in triplicate wells, and the mean absorbances were adjusted to the four-parameter logistic equation. The evaluation of matrix effects was done by comparison of the inhibition curves obtained in the diluted sample and those obtained in buffer.

Recovery Analysis. Stocks of FH were prepared at 30, 25, 10, and 5 mg/L in DMF. For ELISA determination, sets of samples were spiked with FH to obtain, after the desired dilution with water, a concentration of FH of 0.3, 1.0, 1.5, and 2.0 μg/L. These dilutions were analyzed with the optimized dc-ELISA without any further sample pretreatment. A stan-

dard curve prepared in water was also run in each plate. Each sample dilution was analyzed in triplicate wells, and the mean absorbance values were interpolated to the standard curve run in the same plate also in triplicate wells. A blank for each sample was also included as control.

RESULTS AND DISCUSSION

Hapten Synthesis and Conjugate Preparation. Proper hapten design is essential for antibody production and ELISA development. However, the chemical synthesis of functionalized molecules is often a laborious and time-consuming task. In the three-dimensional structure of FH, three parts can easily be distinguished: the aromatic ring and its substituents (arranged in the same plane and constituting the core of the anilide pesticides), the amide bridge, and the methylcyclohexane (disposed in a different plane with respect to the aromatic ring). The resulting molecule has a free phenolic hydroxyl group that confers a weak acidic nature to the active ingredient. This free phenolic OH group can be used to easily introduce a spacer arm by O-alkylation of this position using the classical Williamson ether reaction. This approach could however be controversial, because hydroxyl groups located at aromatic rings have the ability to participate in hydrogen-bonding systems, and they are often considered as strong antigenic determinants, particularly in the case of simple phenolic compounds (*20*−*23*). Nevertheless, this simple strategy has been used successfully to obtain valuable antibodies to more complex analytes (*24*−*27*). Consequently, we finally decided to functionalize the analyte through the most straightforward strategy, consisting of a two-step procedure aimed at introducing a four-carbon linear spacer with a terminal carboxylic acid at the planar aromatic part of the molecule (**Figure 1**). In this way, although the phenolic group was blocked, other important immunodeterminant moieties were preserved, including the cyclohexane ring, which is probably a very relevant moiety due to its size. In addition, selectivity issues are not of great concern in the case of FH because other agrochemical compounds with a similar structure are not commercial.

Conjugates of hapten FHo4 with different carrier proteins were prepared. The immunizing conjugate BSA−FHo4 was prepared by the active ester method using a high initial hapten-to-protein ratio to achieve a substantial modification of the protein. As determined by differential absorbance measurements, a final MR of 20 was obtained. On the other hand, the screening conjugate (OVA−FHo4) and the assay conjugate (HRP−FHo4) were prepared by a different coupling method—the mixed anhydride method. In these cases, the measured final MRs were 6 and 1.5, respectively.

Monoclonal Antibody Production and Selection. Three mice were immunized with a conjugate of FHo4 according to the described procedure. The immunization process was monitored by indirect competitive ELISA as previously described (*28*) using the collected antisera and OVA−FHo4 as conjugate. Although the titers of the antisera were higher when the OVA conjugate was used at 1.0 μg/mL, lower IC_{50} values were obtained with a lower coating conjugate concentration. **Table 1** lists the results for the two serum dilutions that afforded A_{max} values immediately above and below 1.0 in competitive assays using 0.1 μg/mL of coating conjugate. This study revealed a good response after the third injection in two of the three mice, with low IC_{50} values for FH. Subsequently, a final booster injection was given, and 4 days later, hybridoma cells were prepared from lymphocyte B cells of each immunized animal. For the screening of cell culture supernatants, a double-assay strategy was followed with increasing selective pressure as described in the Materials and Methods. The initial screening of the cell culture supernatants of the first cell fusion (mouse #1) was performed using a low selective pressure (1 μM FH and 1.0 μg/mL coating conjugate). This assay revealed

Table 1. Curve Parameters Obtained with Mouse Antisera Collected after the Third Injection

mouse no.	antiserum dilution[a]	A_{max}	A_{min}	IC_{50} (nM)	slope
1	30	0.86	0.07	29.6	−0.48
	10	1.81	0.11	39.6	−0.47
2	30	0.98	−0.03	102.0	−0.46
	10	2.08	−0.11	122.6	−0.44
3	30	0.80	0.06	18.7	−0.52
	10	1.64	0.15	26.4	−0.63

[a] Serum dilutions × 10^3.

138 positive wells (absorbance signals higher than 0.5 under noncompetitive conditions), 50 of which presented saturated signals in the noncompetitive assay and little inhibition under competitive conditions. Therefore, a second screening was performed with serial dilutions of the culture supernatants using higher selective pressure. Two FH concentrations were assayed (5 and 50 nM) with 1.0 and 0.1 μg/mL of protein conjugate. Finally, six hybridomas were identified as producers of high-affinity mAbs, thus evidencing the usefulness of the double-assay strategy to identify the best hybridomas. These hybridomas were cloned and stabilized, and the mAbs were purified. Two of these mAbs were of the IgG_{2a} isotype and four of the IgG_1 isotype, all of them containing a κ light chain. Subsequent cell fusions (mice #2 and #3) were screened under higher selective pressure already in the first screening (either 0.5 or 0.1 μM FH and 0.1 μg/mL coating conjugate). Now, the rescreening of positive wells was done with serial dilutions of the culture supernatants and several concentrations of FH (50 nM as the highest concentration). This approach provided two additional clones that produced mAbs against FH with affinities in the low nanomolar range. These mAbs were of the IgG_{2a} isotype with a κ light chain.

To choose the best mAb, assays were performed in the dc-ELISA format with all of the purified antibodies coated on polystyrene plates and serial dilutions of HRP−FHo4 assay conjugate in PBST. All of the mAbs, except one, showed IC_{50} values for FH below 5.0 nM, with three of them (FHo4#22, FHo4#26, and FHo4#27) featuring equivalent IC_{50} values below 2.0 nM. According to overall results, mAb FHo4#27 was finally selected for further characterization and ELISA development.

Antibody Characterization. *Cross-Reactivity*. An exhaustive analysis was performed to find other molecules that could be recognized by mAb FHo4#27, but no cross-reacting pesticides were found. For example, none of the fungicides of the strobilurin family (kresoxim-methyl, trifloxystrobin, pyraclostrobin, azoxystrobin, picoxystrobin, dimoxystrobin, metominostrobin, orysastrobin, and fluoxastrobin) was recognized. In addition, other fungicides that are usually formulated in combination with FH, or other pesticides potentially present in food samples for which FH is also widely used, were tested up to 1 μM, and no binding was observed. The assayed fungicides were procymidone, tolylfluanid, boscalid, cyprodinil, tebuconazole, mepanipyrim, fludioxonil, vinclozolin, fenamidone, famoxadone, captan, and pyrimethanil. Accordingly, no interferences from other active substances are expected in the application of this IA to food sample analysis, which confirms the suitability of the hapten structure for this particular chemical.

Tolerance to Solvents. The influence of organic solvents over the IA was also investigated. Most common extraction procedures for FH use methanol, acetonitrile, or acetone. As seen in **Figure 2**, methanol, acetonitrile, and acetone (in the assayed concentrations) had a very low effect over the A_{max} value of the assay, whereas the IC_{50} value increased with the presence of any

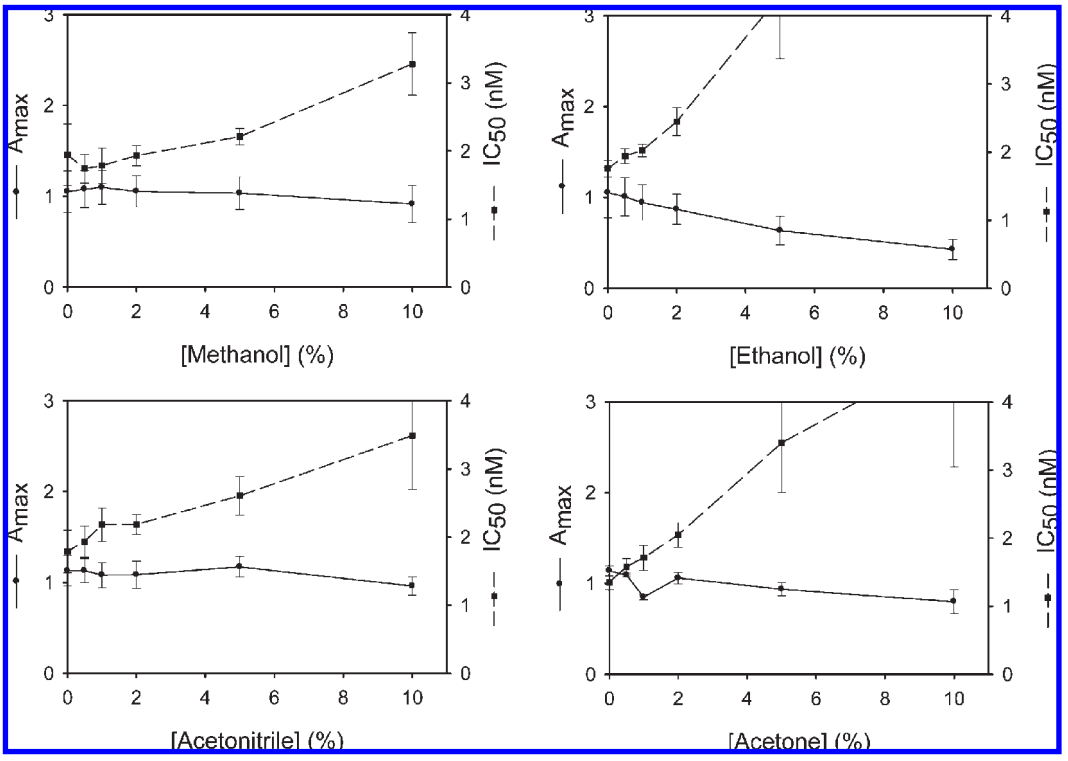

Figure 2. Tolerance to organic solvents. Values are the means of three independent experiments.

of the assayed organic solvents. The best tolerated solvent was methanol, which produced a slower increase of the IC_{50} value. On the contrary, the IC_{50} value of the assay increased rapidly with the presence of acetone or ethanol. This result should be taken into account if the developed IA is to be applied to the analysis of FH in fermented must. Additionally, DMF and dioxane were also assayed. The exerted influence of these solvents was similar to that of ethanol, but even a stronger effect over the A_{max} value was observed (results not shown).

Influence of Buffer Conditions. Competitive assays were performed with mAb FHo4#27 and HRP−FHo4 conjugate using different assay buffers. **Figure 3** shows the influence of varying salt concentrations and pH over the IC_{50} and A_{max} values. Buffers whose ionic strength was lower than that of PBS (I < 164 mM at 25 °C) generated assays with lower IC_{50} values, but using a phosphate buffer without NaCl (I = 24 mM at 25 °C) afforded assays with too low A_{max} values. On the contrary, ionic strength values higher than 300 mM did not modify the A_{max}, although a slight increase of the IC_{50} value was observed. Regarding pH, values between 5.5 and 7.5 were very well tolerated. It should be remarked that pH values higher than that of PBS (pH > 7.4) drastically lowered the sensitivity of the assay. This result is compatible with the weak acidic properties of FH in aqueous systems (pK_a = 7.3). The introduction of the spacer arm at the phenolic OH group to prepare the immunizing conjugate has probably favored the production of antibodies that recognized the protonated species and not the phenolate anion. It could be anticipated that concentrated buffer systems will be required for the analysis of food samples to avoid possible interferences from strong acidic matrices or with pH values higher than 7.5.

Food Sample Analysis. *Optimized IA*. Food analysis experiments were performed with the optimized dc-ELISA. Plates were coated with mAb FHo4#27 at 1.0 μg/mL, the FH standards were prepared in water, and a 12 ng/mL HRP−FHo4 tracer solution in 200 mM phosphate buffer, pH 7.4 (I = 262 mM at 25 °C), containing 0.05% Tween 20 was applied. No relevant improvement of the analytical parameters was accomplished if higher or lower coating antibody concentrations were employed. The inhibition curve for FH of the optimized IA obtained from independent experiments is shown in **Figure 4**. The IC_{50} value of the fitted curve was 0.52 ± 0.06 μg/L, and the estimated LOD was 0.13 ± 0.03 μg/L.

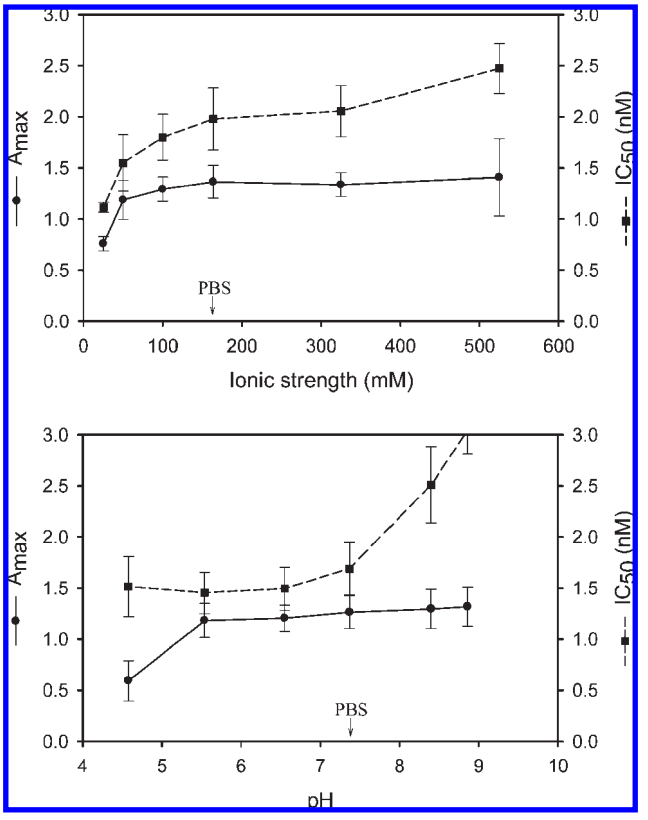

Figure 3. Influence of the ionic strength and pH over the A_{max} values (solid lines) and the IC_{50} values (dashed lines). Values are the means of four independent experiments.

Must and Wine Samples. Botrytis is a very important disease in vineyards all over the world, and to fight this infection, FH is one of the most important antifungal agrochemicals being used. For this reason, it seems feasible that residues of this chemical may remain in must and wine. Therefore, our ultimate objective was to evaluate the developed dc-ELISA in these important food samples. As a previous step, matrix interferences were evaluated by comparison of the inhibition curve obtained in buffer with the curves prepared in must or wine diluted with water. As shown in **Figure 5**, undesired matrix effects were found, which could easily be neutralized by diluting the samples with water. In the case of white must, a 100-fold dilution seemed to be enough, whereas for red must and wine samples, a 250-fold dilution was necessary to completely remove the interferences. Thereafter, recovery values were evaluated by calculation of the FH contents from spiked samples analyzed with the developed dc-ELISA, using standard curves performed in buffer. Because of the high slope of the assay (−1.6), the calculated working range was rather narrow (0.21−1.28 μg/L). Must and wine samples were fortified to reach final concentrations around this working range in the assay after a given dilution factor. As listed in **Table 2**, excellent recoveries (between 95 and 118%) were found with red and white must and with red and white wine samples when appropriately diluted with water. Also, coefficients of variation were generally below 10%. Blank samples always resulted in values below the theoretical LOD. The high dilution factors that had to be applied for the analysis of FH in must and wine were counteracted by the good performance of mAb FHo4#27 in combination with assay conjugate HRP−FHo4. In fact, under these conditions, this assay

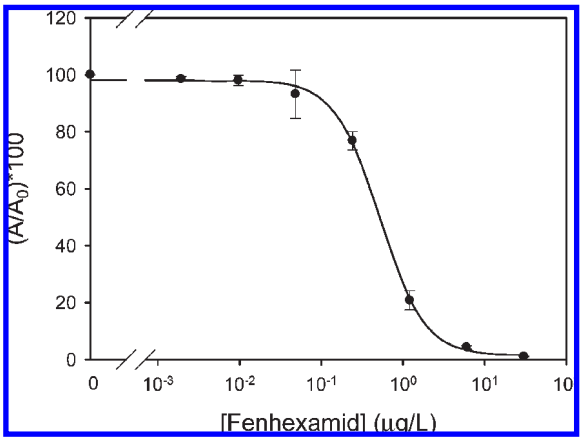

Figure 4. Sigmoidal curve obtained from the mean of four independent experiments, where A_0 is the absorbance obtained at zero dose of analyte. The A_{max} value of each curve was around 1.5 absorbance units.

Table 2. Recovery Studies Performed in Red and White Must and Red and White Wine[a]

	spiked concn (μg/L)	measured concn (μg/L)	recovery (%)	CV (%)
red must[b]	75	71 ± 6	95	8
	250	273 ± 20	109	7
	375	443 ± 39	118	9
	500	546 ± 39	109	7
white must[c]	30	33 ± 3	110	9
	100	118 ± 14	118	12
	150	171 ± 11	114	6
	200	212 ± 13	106	6
red wine[b]	75	80 ± 8	107	10
	250	265 ± 11	106	4
	375	427 ± 31	114	7
	500	541 ± 45	108	8
white wine[b]	75	73 ± 2	97	2
	250	272 ± 2	109	1
	375	401 ± 45	107	11
	500	496 ± 27	99	5

[a] Each value corresponds to the mean of three independent experiments. [b] Diluted 1/250 with water before the assay. [c] Diluted 1/100 with water before the assay.

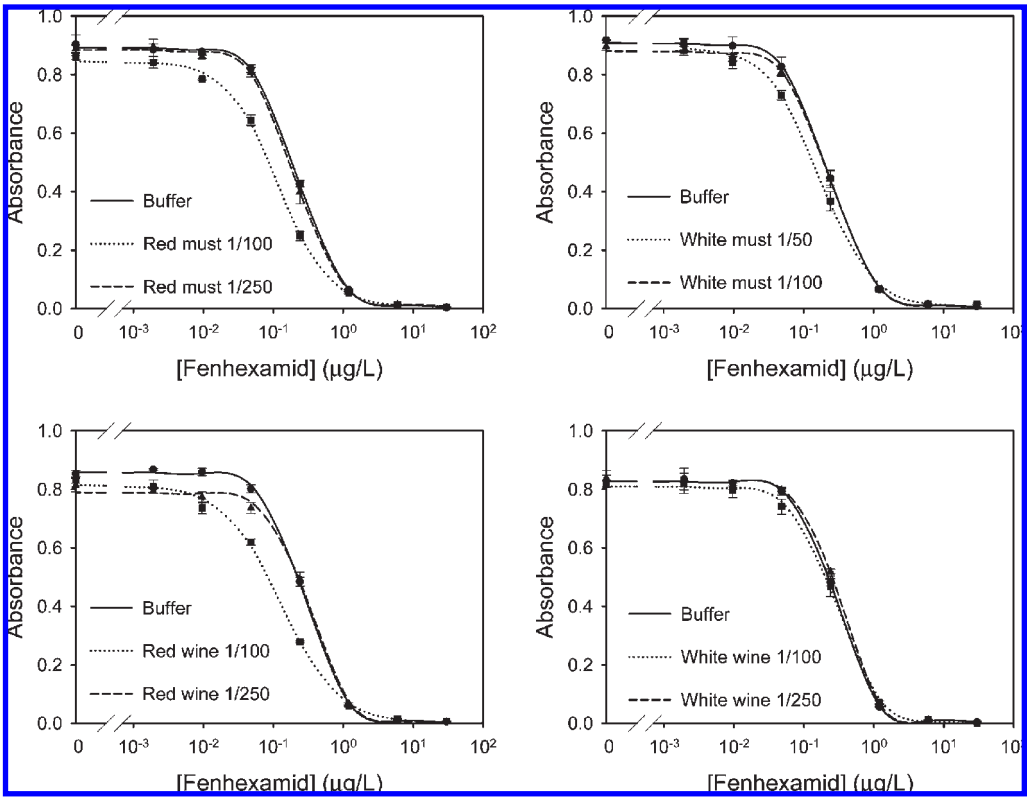

Figure 5. Interferences produced by two types of must and two types of wine over the developed dc-ELISA.

enabled the determination of FH at concentrations as low as 75 μg/L in red must, red wine, and white wine and 30 μg/L in white must. These results indicate the viability of the developed dc-ELISA to determine FH in grape-derived foods without any extraction, purification, or concentration steps at levels below the MRLs established in the EU for grapes (5000 μg/L).

In conclusion, the immunoreagents reported here constitute promising tools for the development of simple and rapid analytical devices valid for the monitoring of FH in food products. In this study, the derivatization of the phenolic OH of FH was demonstrated as an appropriate synthetic strategy for the production of mAbs specific to this fungicide. Nevertheless, other synthetic approaches will be explored in the near future to check whether leaving the OH free would lead to the generation of antibodies with superior characteristics and the influence of that moiety over the selectivity of the immunoreagents. Following an optimization process, an IA was proposed with an IC_{50} value for FH in buffer of 0.52 μg/L. The observed increase of the IC_{50} value of the assay at high pH values could be an indicator of the influence exerted by the derivatization site of the immunizing hapten. Finally, the performance of the dc-ELISA for the analysis of FH in food samples was assessed in a preliminary study using fortified must and wine. Most probably, the chemical properties of this pesticide and the complexity of the samples account for the interferences that were encountered. Despite these matrix effects, FH could be accurately determined in these important food products at relevant analytical levels. Further studies will be performed for additional validation of this dc-ELISA.

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LITERATURE CITED

(1) Kruger, B. W.; Etzel, W.; Goehrt, A. Fenhexamid (KBR 2738)—A botrycide from a new chemical class. *Pflanzenschutz-Nachr. Bayer* **1999**, *52*, 119–126.

(2) Debieu, D.; Bach, J.; Hugon, M; Malosse, C.; Leroux, P. The hydroxyanilide fenhexamid, a new sterol biosynthesis inhibitor fungicide efficient against the plant pathogenic fungus *Botrytis cinerea*. *Pest Manage. Sci.* **2001**, *57*, 1060–1067.

(3) Mosleh, Y. Y.; Paris-Palacios, S.; Couderchet, M.; Biagianti-Risbourg, S.; Vernet, G. Metallotionein induction, antioxidative responses, glycogen and growth changes in *Tubifex tubifex* (Oligochaete) exposed to the fungicide fenhexamid. *Environ. Pollut.* **2005**, *135*, 73–82.

(4) Suty, A.; Pontzen, R.; Stenzel, K. Fenhexamid—Sensitivity of *Botrytis cinerea*: Determination of baseline sensitivity and assessment of the risk of resistance. *Pflanzenschutz-Nachr. Bayer* **1999**, *52*, 145–157.

(5) Roßlenbroich, H. J. Efficacy of fenhexamid (KBR 2738) against *Botrytis cinerea* and related fungal pathogens. *Pflanzenschutz-Nachr. Bayer* **1999**, *52*, 127–144.

(6) Nußlein, F. Method for liquid-chromatographic determination of the fungicidal active ingredient fenhexamid (KBR 2738) in plant materials. *Pflanzenschutz-Nachr. Bayer* **1999**, *52*, 187–226.

(7) USDA Pesticide Data Program. www.ams.usda.gov/pdp.

(8) Annual EU-wide Pesticide Residues Monitoring. http://ec.europa.eu/food/fvo/specialreports/pesticides_index_en.htm.

(9) Pesticide Action Network Europe. www.pan-europe.info/Media/PR/080326.html.

(10) Edder, P.; Ortelli, D.; Zali, O. Survey of pesticide residues in Swiss and foreign wines. 2nd International Symposium on Recent Advances in Food Analysis, November 2−4, 2005, Prague, Czech Republic; http://etat.geneve.ch/des/site/sante/protection-consommation.html.

(11) Pesticide EU-MRLs Database. Regulation (EC) No. 396/2005. http://ec.europa.eu/sanco_pesticides/public/index.cfm.

(12) Langone, J. J.; Van Vunakis, H. Radioimmunoassay for dieldrin and aldrin. *Res. Commun. Chem. Pathol. Pharmacol.* **1975**, *10*, 163–171.

(13) Wing, K. D.; Hammock, B. D.; Wustner, D. A. Development of an S-bioallethrin specific antibody. *J. Agric. Food. Chem.* **1978**, *26*, 1328–1333.

(14) Ercegovich, C. D.; Vallejo, R. P.; Gettig, R. R.; Woods, L.; Bogus, E. R.; Mumma, R. O. Development of a radioimmunoassay for parathion. *J. Agric. Food Chem.* **1981**, *29*, 559–563.

(15) Gabaldón, J. A.; Maquieira, A.; Puchades, R. Current trends in immunoassay-based kits for pesticide analysis. *Crit. Rev. Food Sci. Nutr.* **1999**, *39*, 519–538.

(16) Morozova, V. S.; Levashova, A. I.; Eremin, S. A. Determination of pesticides by enzyme immunoassay. *J. Anal. Chem.* **2005**, *60*, 202–217.

(17) Van Emon, J. M.; Chuang, J. C.; Dill, K.; Xiong, G. Immunoassays and biosensors. In *Analysis of Pesticides in Food and Environmental Samples*; Tadeo, J. L., Ed.; CRC Press, Taylor and Francis Group: Boca Raton, FL, 2008; pp 95−123.

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

(19) Abad, A.; Manclús, J. J.; Mojarrad, F.; Mercader, J. V.; Miranda, M. A.; Primo, J.; Guardiola, V.; Montoya, A. Hapten synthesis and production of monoclonal antibodies to DDT and related compounds. *Anal. Chim. Acta* **1997**, *45*, 3694–3702.

(20) Estévez, M. C.; Kreuzer, M.; Sánchez-Baeza, F.; Marco, M. P. Analysis of nonylphenol: Advances and improvements in the immunochemical determination using antibodies raised against the technical mixture and hydrophilic immunoreagents. *Environ. Sci. Technol.* **2006**, *40*, 559–568.

(21) Manclús, J. J.; Montoya, A. Development of an enzyme-linked immunosorbent assay for 3,5,6-trichloro-2-pyridinol. 1. Production and characterization of monoclonal antibodies. *J. Agric. Food Chem.* **1996**, *44*, 3703–3709.

(22) Watanabe, E.; Eun, H.; Baba, K.; Arao, T.; Endo, S.; Ueji, M.; Ishii, Y. Synthesis of haptens for development of antibodies to alkylphenols and evaluation and optimization of a selected antibody for ELISA development. *J. Agric. Food Chem.* **2005**, *53*, 7395–7403.

(23) Nichkova, M.; Galve, R.; Marco, M. P. Biological monitoring of 2,4,5-trichlorophenol (I): Preparation of antibodies and development of an immunoassay using theoretical models. *Chem. Res. Toxicol.* **2002**, *15*, 1360–1370.

(24) Ohkuma, H.; Abe, K.; Ito, M.; Kokado, A.; Kambegawa, A.; Maeda, M. Development of a highly sensitive enzyme-linked immunosorbent assay for bisphenol A in serum. *Analyst* **2002**, *127*, 93–97.

(25) Bennetau-Pelissero, C.; Le Hourou, C.; Lamothe, V.; Le Menn, F.; Babin, P.; Bennetau, B. Synthesis of haptens and conjugates for ELISAs of phytoestrogens. Development of the immunological tests. *J. Agric. Food Chem.* **2000**, *48*, 305–311.

(26) McConnell, R. I.; Fitzgerald, S. P.; Benchikh, E. O.; Lowry, A. P. Phenethanolamine-derived haptens, immunogens, antibodies and conjugates. U.S. Patent 7192722, 2007.

(27) Skerritt, J. H.; Guihot, S. L.; McDonald, S. E.; Culvenor, R. A. Development of immunoassays for tyramine and tryptamine toxins of *Phalaris aquatica* L. *J. Agric. Food Chem.* **2000**, *48*, 27–32.

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

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