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Preparation of Antibodies and Development of an Enzyme-Linked Immunosorbent Assay for Determination of Dealkylated Hydroxytriazines

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The development of an indirect competitive enzyme-linked immunosorbent assay (ELISA) for dealkylated hydroxytriazines is reported here for the first time. The assay uses polyclonal antibodies raised against *N*-(4-amine-6-hydroxy-[1,3,5]triazin-2-yl)-4-aminobutanoic acid (hapten **2g**) conjugated to keyhole limpet hemocyanin by the active ester method. The immunizing hapten was synthesized by first introducing the amino group to the triazine ring in a protected form in order to increase its solubility in organic media. Subsequent steps consisted of reacting this compound with an appropriate spacer arm, followed by removal of the protecting group in acidic media. The resulting assay uses a homologous competitor hapten coupled to conalbumin by the mixed anhydride method. Coating antigens prepared using a homologous covalent coupling procedure failed to produce competitive immunoassays. The assay tolerates media with high ionic strength (up to 70 mS cm^{-1}) and basic pH values (7.5−9.5 units). Under the optimized conditions, this ELISA is specific for dealkylated hydroxytriazines, reaching suitable limits of detection.

KEYWORDS: ***s*-Triazines; hydroxylated atrazine degradation products; DEHA; DIHA; immunoassay; hapten design; hapten heterology**

INTRODUCTION

Atrazine and other related *s*-triazines have been widely used as selective herbicides for the control of annual grasses and broad-leaf weeds. Triazine residues have been found in many compartments of the environment such as sediments and surface, well, and drinking water due to their extensive use and their persistence in the environment (*1*−*5*). The use of triazines has been limited in several countries, and their levels in natural waters are frequently controlled (*6*−*12*). However, all the restrictions adopted in the use of these herbicides have not solved the problem of the degradation products found in groundwater and surface water.

Triazine herbicides might be degraded by photochemical, chemical, and/or biochemical processes (*13*). Hence, several microorganisms can dealkylate atrazine to produce deethylatrazine (DEA), deisopropylatrazine (DIA), and deethyldeisopropylatrazine (DEDIA) (*14*), although it has been postulated that these metabolites can also be formed by photochemical catalysis (*1*, *15*). Moreover, nonbiological detoxification processes, such as hydrolytic reactions, lead to the formation of the so-called hydroxylated atrazine degradation products (HADPs): hydroxyatrazine (HA), deethylhydroxyatrazine (DEHA), deisopropylhydroxyatrazine (DIHA), and deethyldeisopropylhydroxyatrazine (DEDIHA). Other important triazine herbicides, such as propazine, simazine, and *tert*-butylazine, may lead to the same kind of metabolites (see **Figure 1**). It is important to notice that the half-life of HADP is higher than those of other *s*-triazines (in soil: atrazine 14−50 days, HADP 32−162 days) (*16*), creating a risk for a continuous increase of the concentration of these metabolites in the environment, the impact of which in the ecosystem is still unknown (*17*).

In the past decade, most of the studies performed to establish the impact of these metabolites in the environment have been focused on the dealkylated degradation products such as DIA, DEA, and DEDIA. The presence of these compounds in lake watersheds (*18*), groundwater (*19*, *20*), and rainfall (*1*, *21*) at concentrations around 1−5 $\mu g\ L^{-1}$ has been reported. However, only few studies have been reported regarding HADPs. While these compounds have been rarely detected in groundwater, concentrations from 2 to 6 $\mu g\ L^{-1}$ have been reported in surface water (*22*, *23*). For example, studies performed in watersheds of northeastern Missouri showed that levels of HA were generally higher than or equal to those of atrazine or DEA (*24*).

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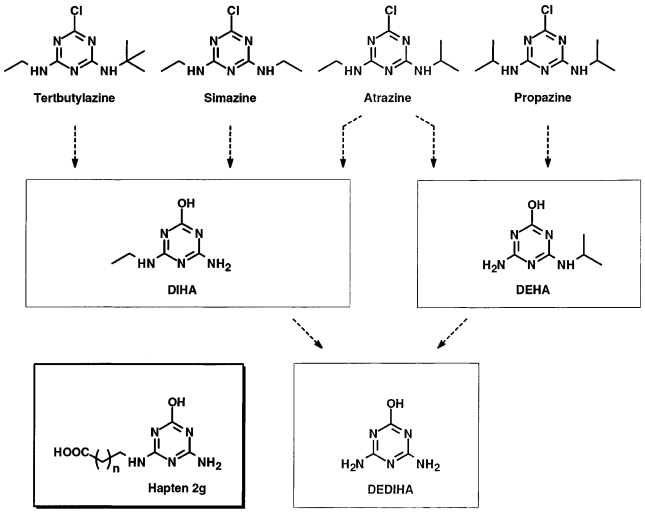


Figure 1. Chemical structures of the most important *s*-triazine herbicides (atrazine, *tert*-butylazine, propazine, and simazine) and their dealkylated hydroxy metabolites. The chemical structure of hapten **2g** is also shown.

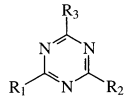
Moreover, it has been shown that HADPs constitute the most important fraction of the atrazine residues found in soil (*16*, *25*) The higher pK_a values of these metabolites (HADPs pK_a = 4−5; atrazine pK_a = 2) may allow them to establish mixed-mode binding mechanisms based on hydrophobic and cationic-exchange interactions with soil components, while atrazine and its chlorinated metabolites are limited to hydrophobic interactions (*26*).

Environmental monitoring of these metabolites is based on chromatographic techniques such as high-performance liquid chromatography with ultraviolet detection (HPLC/UV) or gas chromatography with mass spectrometry identification (GC/MS) after organic solvent extraction followed by purification/concentration steps (*7*, *27*−*29*). Due to their high polarity and water solubility, an important drawback of these procedures is the low efficiency of the procedures for extraction from aqueous samples (water solubility data: atrazine, 0.15 mM; HA, 0.24 mM; DEA, 2 mM; DIA, 1.20 mM; DEDIA, DEHA, and DIHA, >2 mM) (*30*−*32*). In this context, one of the advantages of the immunochemical techniques is that they often provide the necessary detection limits and specificity to directly analyze very low concentrations of the target analyte in water samples (*33*−*37*). Moreover, their reliability, low cost, speed of analysis, and easy-to-use features are well known (*33*, *34*, *38*−*40*). The preparation of antibodies and the development of ELISAs for DEA, DIA, and DEDIA (*41*, *42*), and also for HA (*43*−*45*), have been described. However, despite the above-mentioned analytical difficulties, to our knowledge no immunoassay is available or has been reported to date for the analysis of DEHA, DIHA, and DEDIHA. In this paper we describe the preparation of an immunizing hapten and the production of polyclonal antibodies against dealkylated hydroxytriazines. These antibodies have been used to develop an immunoassay to determine dealkylated hydroxytriazines.

EXPERIMENTAL SECTION

General Methods and Instruments. Thin-layer chromatography (TLC) was performed on 0.25-mm precoated silica gel 60 F254 aluminum sheets (Merck, Darmstadt, Germany), and unless otherwise indicated, the mobile phase employed was THF/AcOEt/hexane 2:13:35. Anhydrous solvents used were distilled immediately before the reaction in the presence of Na and under Ar atmosphere (benzophenone was added as indicator). ^{1}H and ^{13}C NMR spectra were obtained with a Varian Unity-300 (Varian Inc., Palo Alto, CA) spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C) or a Gemini 200 apparatus (199.975 MHz for ^{1}H and 50.289 for ^{13}C). Infrared (IR) spectra were measured on a Bomen MB 120 FTIR spectrophotometer (Hartmann & Braun, Québec, Canada). Elemental analyses were performed by Microanalysis Service of the IIQAB-CSIC in Barcelona. Gas chromatography−mass spectrometry (GC-MS) was performed on an MD-800 capillary gas chromatograph with an MS quadrupole detector (Fison Instruments, VG, Manchester, UK), and the data are reported as *m*/*z* (relative intensity). The ion-source temperature was set at 200 °C; a 15-m × 0.25-mm-i.d. × 0.15-μm-film thickness DB-225 fused capillary column (J&W, Folsom, CA) was used; He was the carrier gas employed at 1 mL/min. GC conditions were as follows: temperature program, 70−80 °C (10 °C/min), 80 °C (5 min), 80−300 °C (10 °C/min); injector temperature, 250°C.

Table 1. Chemical Structure of the Atrazine Haptens Used in This Study[a]



triazine	R_1	R_2	R_3
2a	$NHCH_2CH_3$	$NH(CH_2)_3COOH$	Cl
2b	$NHCH(CH_3)_2$	$NH(CH_2)_3COOH$	Cl
2c	$NHCH_2CH_3$	$NH(CH_2)_5COOH$	Cl
2d	$NHCH(CH_3)_2$	$NH(CH_2)_5COOH$	Cl
2e	$NHC(CH_3)_3$	$NH(CH_2)_3COOH$	Cl
2f	$NHCH(CH_2–CH_2)$	$NH(CH_2)_3COOH$	Cl
2g	NH_2	$NH(CH_2)_3COOH$	OH
4a	$NHCH_2CH_3$	$NHCH_2CH_3$	$S(CH_2)_2COOH$
4b	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	$S(CH_2)_2COOH$
4c	$NHC(CH_3)_3$	$NHCH(CH_2–CH_2)$	$S(CH_2)_2COOH$
4d	$NHC(CH_3)_3$	$NH(CH_2)_3COOH$	SCH_3
4e	$NHCH(CH_2–CH_2)$	$NH(CH_2)_3COOH$	SCH_3

[a] The preparation of the haptens **2a**–**f** and **4a**–**e** has already been reported (*46*–*48*). The synthesis of **2g**, used as an immunizing and coating antigen, is reported here.

Synthesis of the Haptens. Most of the chemical reagents needed for the synthesis of the haptens were obtained from Aldrich Chemical Co. (Milwaukee, WI). The preparation of the haptens **2a**−**f** and **4a**−**f** has already been described (*46*−*48*) (see **Table 1** for chemical structures). The synthesis of the hapten **2g** (**6**) is described below.

*(4,6-Dichloro-[1,3,5]triazin-2-yl)tritylamine (**3**).* A solution of cyanuric chloride **1** (1.00 g, 5.4 mmol) in anhydrous Et_2O (50 mL) in a round-bottom flask provided with a $CaCl_2$ tube was cooled to −20 °C. Subsequently, a solution of tritylamine **2** (1.46 g, 5.6 mmol) and *N*,*N*-diisopropylethylamine (DIEA, 0.98 mL, 5.6 mmol) in anhydrous Et_2O (30 mL) was added dropwise over 1 h. The temperature of the mixture was then allowed to reach room temperature, and the precipitation of a white solid corresponding to the DIEA chlorohydrate was observed. The mixture was stirred for 24 h at room temperature until the reaction was completed according to the TLC analysis. The mixture was filtered and washed with aqueous 1 N HCl (50 mL), 10% $NaHCO_3$ (50 mL), and saturated NaCl (2 × 50 mL). The solution was dried with anhydrous $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The white solid obtained was then dissolved in Et_2O and purified by "flash" chromatography using silica gel as stationary phase and hexane/AcOEt 4:1 as mobile phase to isolate the triazine **3** as a white powder (1.76 g, 80% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.08 (bs, 1H, NH), 7.27 (m, 15H_{ar}). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 71.78 (CHN), 127.42 (C-4), 128.10 (C-2, C-6), 128.64 (C-3, C-5), 143.09 (C-1), 165.51 (CN_3), 169.43 (CN_2Cl), 169.88 (CN_2Cl). EM: *m*/*z* (relative intensity) 406 (M^+, 25), 329 ($M^+ - C_6H_5$, 31), 243 ($C_{19}H_{15}^+$, 39), 165 ($C_{13}H_9^+$, 100), 104 ($C_7H_6N^+$, 63), 77 ($C_6H_5^+$, 47). IR (KBr, cm^{-1}): ν 3404 (NH st), 1546 (C=N st), 1512 (C_{Ar}N st), 700 (ArC−H δ opp). Melting point: 194−196 °C. Anal. Calcd for $C_{22}H_{16}N_4Cl$: C, 64.88; H, 3.96; N, 13.75. Found: C, 64.93; H, 4.10; N, 13.53.

*N-(6-Chloro-4-tritylamine-[1,3,5]triazin-2-yl)-4-aminobutanoic acid (**5**).* In a round-bottom flask provided with a Dimroth refrigerant and

a $CaCl_2$ tube, a mixture of **3** (1.95 g, 4.8 mmol), 4-aminobutanoic acid **4** (0.54 g, 5.3 mmol), and DIEA (1.75 mL 10.1 mmol) were dissolved in absolute ethanol (100 mL). After 4 h at 78 °C, the precipitation of a white solid corresponding to the DIEA chlorohydrate was observed, together with the complete disappearance of the starting material, as evidenced by TLC. The mixture was filtered under vacuum and the solvent evaporated under reduced pressure. The resulting white solid was then dissolved in a 10% $NaHCO_3$ aqueous solution (200 mL) and washed with CH_2Cl_2 (2 × 40 mL). Subsequently, the aqueous layer was acidified to pH 1 with 0.1 N HCl to precipitate the acid **5**. The white solid, separated by vacuum filtration, was washed with H_2O to remove the resting salts (5 × 20 mL) and freeze-dried to rend the triazine **5** as a white powder (2.19 g, 98% yield). ^{1}H NMR (300 MHz, CD_3OD): δ (ppm) 1.23 (m, 2H, J = 5.1 Hz, CH_2), 1.81 (t, 2H, J = 7.2 Hz, CH_2COO), 2.53 (t, 2H, J = 7.2 Hz, CH_2N), 7.17 (m, $15H_{ar}$). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 24.26 (CH_2), 31.85 (CH_2-COO), 47.76 (CH_2N), 69.73 (CHN), 126.21 (C-4), 127.30 (C-2, C-6), 128.42 (C-3, C-5), 144.51 (C-1), 164.13 (CN_3), 164.97 (CN_3), 167.13 (CN_2Cl), 175.21 (CO). IR (KBr, cm^{-1}): ν 3427 (NH st), 3269 (NH st), 1712 (C═O), 1577 (C═N st), 1537 ($C_{Ar}N$ st), 700 (ArC−H δ opp).

*N-(4-Amine-6-hydroxy-[1,3,5]triazin-2-yl)-4-aminobutanoic Acid (**6**) (Hapten **2g**).* A mixture of trifluoroacetic acid (TFA), H_2O, and CH_2Cl_2 (45:45:10, 20 mL) was added to the triazine **5** (1.17 g, 2.5 mmol) in a round-bottom flask. The resulting mixture was heated to reflux for 2 h until the complete disappearance of the starting material was observed by ^{1}H NMR. The solvent was evaporated under reduced pressure to dryness, and the resulting beige solid was washed with *n*-pentane (6 × 10 mL) and dried in the presence of P_2O_5 to obtain hapten **6** (0.37 g, 70% yield) as a pale beige solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 1.72 (m, 2H, J = 6.9 Hz, CH_2), 2.24 (t, 2H, J = 7.5, CH_2COO), 3.25 (bs, 2H, CH_2N). The elemental analysis showed the absence of chlorine atoms in the molecule.

Immunochemistry. *Chemicals.* Atrazine and simazine, used as standards for cross-reactivity studies, were purchased though Riedel de Häen AG (Seelze-Hannover, Germany); desmentryne, 2-hydroxysimazine, 2-hydroxypropazine, DEA, DIA, DEHA, DIHA, DEDIHA, and 2-hydroxyatrazine were obtained from Dr. Ehrenstorfer (Ausburg, Germany); prometryne was purchased from PolyScience Corp. (Niles, IL); finally, irgarol 1051 was prepared in our group by M. Carmen Estévez.

Buffers and Solutions. PBS is 0.01 M phosphate buffer, 0.8% saline solution, and unless otherwise indicated the pH is 7.5. PBST is PBS with 0.05% Tween 20. PBST-I is 0.05 M PBS, pH 7.5, with 0.1% Tween 20. Borate buffer is 0.2 M boric acid−sodium borate, pH 8.7. Coating buffer is 0.05 M carbonate−bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer. Enzymatic reactions were stopped by adding 4 N H_2SO_4.

Immunochemicals. Immunoreagents such as goat anti-rabbit IgG coupled to horseradish peroxidase (antiIgG-HRP) and proteins such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), conalbumin (CONA), and ovalbumin (OVA) were obtained from Sigma (St. Louis, MO). The conjugation of the haptens **2a**−**f** and **4a**−**e** to BSA, CONA, and OVA by the MA method has already been reported (*46*−*48*). The rest of the hapten−protein conjugates were prepared as described below. All the protein conjugates were stored freeze-dried at −40 °C, and unless otherwise indicated, working aliquots were stored at 4 °C in 0.01 M PBS at 1 mg mL^{-1}.

*Preparation of **2g**-KLH (Immunogen) and **2g**-BSA (Homologous Antigen): Active Ester (AE) Method.* Following previously described procedures (*46*), hapten **2g** (4.63 mg, 10 μmol) was activated using freshly prepared solutions of *N*-hydroxysuccinimide (NHS, 11.50 mg, 50 μmol) and dicyclohexylcarbodiimide (DCC, 41.26 mg, 100 μmol) in anhydrous dimethylformamide (DMF, 120 μL) and reacted with the proteins (BSA or KLH, 10 mg) in 0.2 M borate buffer (1.8 mL).

*Coating Antigens **2g**-BSA, **2g**-CONA, and **2g**-OVA: Mixed Anhydride (MA) Method.* According to previously described procedures (*47*, *49*), freshly prepared solutions of tributylamine (14.1 μL, 19.8 μmol) and isobutyl chloroformate (9.4 μL, 22.8 μmol) in anhydrous DMF (40 μL) were used to activate hapten **2g** (12.51 mg, 18 μmol) in the same solvent (190 μL). After the solution was stirred for 45 min at room temperature, the activated hapten was added dropwise to a solution of the protein (BSA, CONA, or OVA, 10 mg) in 0.2 M borate buffer (1.8 mL).

Polyclonal Antisera. Rabbits 71, 72, and 73 (female New Zealand white rabbits), weighing 1−2 kg, were immunized with **2g**-KLH (EA) according to the immunization protocol previously reported (*46*). Evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the different antisera to microtiter plates coated with **2g**-BSA (EA). After an acceptable antibody titer was observed, the animals were exsanguinated, and the blood was collected on vacutainer tubes provided with a serum separation gel. Antiserum (As) was obtained by centrifugation and stored at −40 °C in the presence of 0.02% NaN_3. Working aliquots were stored at 4 °C.

Instrumentation. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) used for analyzing the protein conjugates was a Perspective BioSpectrometry Workstation provided with the software Voyager-DE-RP (version 4.03), developed by Perspective Biosystems Inc. (Framingham, MA), and Grams/386 (for Microsoft Windows, version 3.04, level III), developed by Galactic Industries Corp. (Salem, NH). The pH and the conductivity of all buffers and solutions were measured with a pH meter (pH 540 GLP) and a conductimeter (LF 340), respectively (both from WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). The vacutainer blood collection set was acquired from Becton Dickinson (Meylan Cédex, France). Washing steps were carried out using an SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA) at a single wavelength of 450 nm. The competitive curves were analyzed with a four-parameter logistic equation using the software GraphPad Prism (GraphPad Software Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates. The pK_a and log P theoretical calculations were carried out with the program ACD/log P and ACD/pK_a (Advanced Chemistry Development, Inc., Toronto, Canada) at the University of Lund (Sweden).

Hapten Density Analysis. Hapten densities were determined by MALDI-TOF-MS by comparing the molecular weight of the standard BSA and those of the conjugates. MALDI spectra were obtained by mixing 1 μL of the matrix [(*E*)-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1}, in CH_3CN/H_2O 70:30, 0.1% TFA) and 1 μL of a solution of the conjugates or proteins (5 mg mL^{-1} in CH_3CN/H_2O 70:30, 0.1% TFA).

ELISA Development and Evaluation. *General Protocol.* The plates were coated with the antigens (100 μL/well in coating buffer) overnight at 4 °C, covered with adhesive plate sealers. The next day, the plates were washed four times with PBST (300 μL/well), and the solutions of the analyte (50 μL/well in PBST; zero analyte is only PBST) and/or the antisera (50 μL/well in PBST, 100 μL/well for the noncompetitive assays) were added and incubated for 30 min at room temperature. The plates were washed again as before, and a solution of antiIgG-HRP (1/6000 in PBST) was added to the wells (100 μL/well) and incubated for 30 min more at room temperature. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at room temperature with 4 N H_2SO_4 (50 μL/well), and the absorbances were read at 450 nm.

Combined Competitive Immunoassay Studies. The ability of DEHA to shift the equilibrium of the binding of the antiserum to the coating antigen was analyzed by coating each of two consecutive columns of the microtiter plates with the same coating antigen (1 μg mL^{-1}). Solutions of PBST ("zero DEHA") and DEHA (5 μM) were added (50 μL/well) to two consecutive columns. Serial dilutions of the corresponding antiserum were then added to both columns (1/500 to 1/32 000 in PBST, 50 μL/well). The mixture was incubated for 30 min at room temperature, and the plates were processed as described before. Coating antigen/antiserum combinations showing an inhibition of the absorbance in the presence of DEHA higher than 10% for the same

antiserum dilution were chosen for further studies on competitive assays (see below).

Noncompetitive Indirect ELISA. For each coating antigen/antiserum combination selected, the avidity of the different antiserum versus the coating antigens was determined by measuring the binding of serial dilutions (1/500 to 1/32 000, 100 μL/well) of each antiserum to the antigen-coated microtiter plates (10 μg mL^{-1} to 9 ng mL^{-1}, 100 μL/well). The plates were processed as described above. From these experiments, concentrations for the coating antigens and the antisera were chosen to produce around 0.7−1 unit of absorbance in 30 min.

Screening for Competitive Indirect ELISAs. For each coating antigen/antiserum combination selected, 12 serial dilutions of the analyte (10 000 nM to 1 pM in PBST) were added (50 μL/well), followed by the corresponding appropriately diluted antiserum (50 μL/well in PBST), to the plates coated with the selected antigen concentration. The mixture was incubated for 30 min, and the plates were then processed as described above. The standard curve was fitted to a four-parameter logistic equation.

To improve the immunoassay features, using the best coating antigen/antiserum combination, a set of experimental parameters (detergent concentration, ionic strength, length of the competition time, preincubation effect, length of the coating step, and pH) were studied sequentially as previously described (*50*).

Optimized Competitive Indirect ELISA. Microtiter plates were coated with **2g**-CONA (1.25 μg mL^{-1} in coating buffer, 100 μL/well) and left to stand for 24 h at 4 °C, covered with adhesive plate sealers. The following day, the coated plates were washed with PBST (four times, 300 μL/well), and As72-DEHA solutions (As72 was diluted 1/500 and DEHA at different concentrations from 5000 to 0.32 nM, both in PBST-I) that had been preincubated for 1 h at room temperature were added to the wells (100 μL/well) and incubated for 1 h at room temperature. The plates were processed as previously described.

Cross-Reactivity Determinations. Stock solutions of the *s*-triazines (desmetryne, prometrine, simazine, atrazine, DEA, DIA, and irgarol 1051) were prepared in DMSO (50 mM) and stored at 4 °C. Standard curves for each of these compounds were constructed (5000 to 0.32 nM) in PBST-I. The preparation of the standard curves of the dealkylated hydroxytriazines, 2-hydroxysimazine and 2-hydroxypropazine, was done using the commercial stocks in acetonitrile (10 ng μL^{-1}). Each IC_{50} was determined in the competitive experiments following the optimized protocol described above. The cross-reactivity (CR) values were calculated according to the equation [IC_{50}(DEHA)/IC_{50}(triazine)] × 100.

Accuracy Studies Using Blind Spiked Samples. This parameter was assessed by preparing different blind spiked samples of DEHA in MilliQ water. The samples were mixed with the antiserum, diluted in 0.1 M PBS to obtain the appropriate ionic strength for the assay (0.05 M PBS, 70 mS cm^{-1}). After the mixture was preincubated for 1 h at room temperature, the samples were measured in the ELISA. Measurements were performed in triplicate. The correlation was evaluated by establishing a linear regression between the spiked and the measured values.

RESULTS AND DISCUSSION

Hapten Synthesis. The design of the most suitable immunizing hapten has been considered the most crucial step in the development of an immunochemical technique for a low-molecular-weight analyte. The synthetic preparation of the appropriate hapten can be sometimes tricky and time-consuming, but the effort is worthwhile, considering that important features, such as the specificity and selectivity of the resulting antibodies, are mainly determined by the chemical structure of the immunizing hapten used to raise antibodies (*36*, *51*−*53*). With the idea of producing antibodies to recognize a broad range of HADPs for screening purposes, we thought of hapten **2g** (compound **6**) as a suitable chemical structure to produce antibodies against DEHA, DIHA, and DEDIHA. The linker substituting one of the amino groups would mimic the isopropyl and ethyl groups present in DEHA and DIHA, respectively,

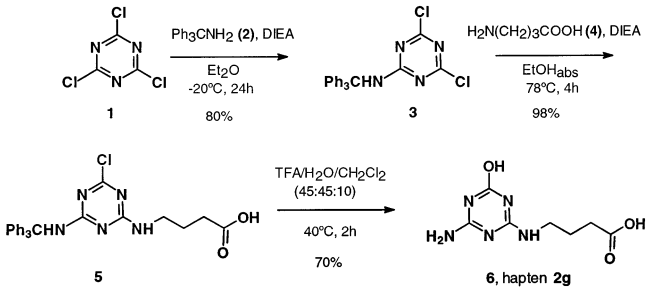


Figure 2. Synthetic pathway used to prepare hapten **2g**.

while preserving the hydroxyl group. Moreover, previous studies performed by our group showed that symmetric molecules were highly recognized by antibodies raised against a hapten when only one of the symmetric sides of the molecule was exposed to the immunesystem (*54*). Therefore, when hapten **2g** was used as the immunizing hapten, there was also a chance for DEDIHA to be recognized by the antibodies raised.

The high polarity of these kinds of substances was the main problem arising during the investigation of several synthetic approaches to prepare hapten **2g**. As mentioned in the Introduction, the high solubility of these aminotriazines in aqueous media and their low extraction efficiency with organic solvents has been reported (*55*). Hence, in the first series of experiments, a lack of reactivity was observed in most of the reactions assayed due to the low solubility of these metabolites in the different organic media. For the same reasons, when the reaction took place, the desired product was obtained in low yield and its isolation and purification from the reaction mixture was extremely difficult. All these questions led us to the idea of working with the amino group protected until the last step. This strategy would surely reduce the polarity of the compound and therefore would allow working in organic media using the classical organic synthesis procedures. Therefore, we proposed using cyanuric chloride (**1**) as starting material and sequentially introducing the different groups by nucleophilic substitutions of the chlorine atoms. The amino group would be introduced in a protected form from the beginning, followed by the introduction of the spacer arm. In the last steps, the third chlorine atom would be substituted by a hydroxyl group, and finally the protecting group would be removed (see **Figure 2**).

The trityl group, commonly used in peptide synthesis, was selected as the protecting group since its hydrolysis takes place only under acidic conditions (*56*, *57*). Consequently, in the first step, tritylamine (triphenylethylamine, **2**) was introduced by nucleophilic substitution of one chlorine atom of the cyanuric chloride (**1**) to obtain compound **3**. The reaction was performed at −20 °C to avoid the substitution of more than one chlorine atom. Subsequently, compound **5** was obtained after another nucleophilic substitution reaction by 4-aminobutanoic acid **4** on a second chlorine atom of **1**. Although such substitutions usually take place at room temperature (*46*−*48*, *58*), we needed to heat the reaction mixture at 78 °C to add energy to the chemical reaction, probably due to the steric hindrance resulting from the great volume of the tritylamine group (*58*, *59*). The hydrolysis of the remaining chlorine atom has been reported to occur upon heating the triazine herbicides in aqueous acidic media (*45*). Consequently, the reaction conditions needed to remove the protecting trityl group could also favor the replacement of the chlorine atom by the hydroxyl group. As expected, hapten **2g** was obtained in 70% yield using a mixture TFA/H_2O/CH_2Cl_2 45:45:10. The overall yield of this synthetic procedure from the cyanuric chloride **1** was 55% (see **Figure 2**).

Table 2. Results from the Combined Competitive Experiments Used for Screening the Different Antiserum/Coating Antigen Combinations[a]

antiserum	coating antigen	absorbance: no DEHA	absorbance: 5 μM DEHA	inhibition[b] (%)
As71	**2f**-BSA	0.419	0.418	0
	2f-CONA	0.733	0.702	4
As72	**2d**-CONA	0.631	0.618	2
	2e-CONA	0.481	0.408	15
	2f-BSA	0.731	0.548	25
	2f-CONA	1.067	0.701	34
	4a-BSA	1.693	1.643	3
	2g-BSA (EA)	0.555	0.541	3
	2g-BSA (MA)	0.386	0.119	69
	2g-CONA (MA)	0.425	0.122	71
As73	**2e**-BSA	0.503	0.413	18
	2e-CONA	0.719	0.682	5
	2f-BSA	1.244	1.009	19
	2f-CONA	1.592	1.446	9
	2d-OVA	0.618	0.561	9
	2g-BSA (EA)	1.952	1.703	13
	2g-BSA (MA)	0.341	0.138	60
	2g-CONA (MA)	1.09	0.378	65

[a] The experiments were performed by coating with each antigen two columns of the plates at 1 μg mL^{-1} and adding serial dilutions (1/500 to 1/32000) of the antiserum in the presence or in the absence of DEHA. [b] Inhibition was calculated according to the following formula: [absorbance(no DEHA)/absorbance(5 μM DEHA)] $\times$ 100.

Development of an Indirect ELISA. Hapten **2g** was conjugated to KLH and to BSA following the active ester method. The coupling reaction was verified by analyzing the BSA derivative by MALDI-TOF-MS. We estimated a hapten density of around 15 haptens covalently attached to each molecule of BSA. Three rabbits were inoculated with 100 μg of **2g**-KLH and boosted each month for 6 months until no significant increase in the antibody titer was observed. The antiserum obtained from each rabbit was named As71, As72, and As73, respectively.

To prepare coating antigens, this same hapten was conjugated to BSA, CONA, and OVA using the mixed anhydride method, to avoid potential interferences due to the potential side reactions that may have occurred during the preparation of the immunogen (*60*, *61*). The competitors **2a**−**f** and **4a**−**e** (see **Table 1** for chemical structures) coupled to BSA, CONA, and OVA had been previously prepared by this same method (*46*−*48*).

The avidity of the As71−73 versus the 36 competitors was tested by using noncompetitive ELISAs (data not shown). Antibody titers were especially high for the homologous **2g**−protein conjugates. The conjugates of the haptens **2a**−**f** and **4a**,**b** were recognized on a lesser extent, and the conjugates of the haptens **4c**−**e** were not recognized at all, probably due to important structural differences between the immunizing haptens, such as the presence of bulky groups (methylthio and *tert*-butylamino groups). The antiserum/coating antigens combinations producing absorbance values higher than 0.5 were tested on a combined competitive screening assay (see Experimental Section) to determine the antiserum's ability to recognize the analyte at a constant concentration of 5 μM. All the experiments were performed with DEHA as target analyte, since it has been reported to be more frequently found in the environment than DIHA (*24*). Only the binding of the As72 and As73 to certain coating antigens, such as those from haptens **2f** and **2g**, was significantly inhibited (see **Table 2**). It is worth noting that the homologous competitor showed the highest inhibition, but only when the coating antigen had been prepared by a different coupling procedure, supporting the possibility that formation of nondesired side conjugation reactions had occurred when using the active ester method, as reported (*61*). Those combinations showing an inhibition of the assay titer higher than 10% in the presence of the analyte were selected to perform two-dimensional titration experiments to establish the appropriate concentrations of the immunoreagents for the competitive immunoassays. **Table 3** shows the features of the indirect ELISAs obtained. Immunoassay As72/**2g**-CONA was selected for further optimization and evaluation because of its acceptable detectability (limit of detection), slope, and the low background noise.

Table 3. Immunoassay Features of the Competitive ELISAs Using DEHA as Analyte

antiserum	coating antigen	A_{max}	A_{min}	slope	IC_{50}[a]	r^2
72	**2f**-BSA	0.89	0.31	–0.71	29.01	0.97
	2f-CONA	1.14	0.54	–0.77	5.52	0.97
	2g-BSA	0.40	0.21	–0.10	4.31	0.97
	2g-CONA	1.37	0.20	–0.98	17.03	0.90
73	**2g**-BSA	0.47	0.11	–0.19	5.03	0.96
	2g-CONA	1.34	0.02	–0.63	20.10	0.91

[a] IC_{50} values are expressed in micrograms per liter. The parameters were extracted from the four-parameter equation used to fit the standard curves.

ELISA Evaluation. Figure 3 shows the results of the studies made to evaluate immunoassay performance. Concerning the effect of the *concentration of Tween 20*, it was observed that, while the maximal absorbance of the assay was not affected, the detectability improved slightly with the concentration of the detergent (see **Figure 3A**). Thus, a 0.1% concentration of Tween 20 was selected for further experiments. Regarding the *ionic strength* effect, the detectability improved significantly from 0 to 30 mS cm^{-1} (20 mM in terms of PBS), remaining constant between 30 and 70 mS cm^{-1} (50 mM in terms of PBS). The maximal absorbance diminished from 0 to 15 mS cm^{-1} (10 mM in terms of PBS), although it did not vary as much between 15 and 70 mS cm^{-1} (see **Figure 3B**). Therefore, 70 mS cm^{-1} was the conductivity chosen to perform this assay. The *length of the competitive step* was set at 60 min as a compromise, since after this time the IC_{50} value increased, diminishing the immunoassay detectability (see **Figure 3C**). Similarly, a *preincubation time* of 60 min for the As72 and the analyte, before their addition to the coated plate, was found to improve slightly the immunoassay detectability (from 7.6 to 5.4 μg L^{-1}). It was also observed that a decrease of the *length of the coating step* below 24 h led to a diminution of the immunoassay detectability. Finally, studies on the *effect of the pH* showed a better tolerance of the assay to basic than to acidic media. At pH values lower than 7.5, the IC_{50} increased drastically (see **Figure 3D**).

A working protocol was established by taking into consideration the results of these studies. The microplates were always coated for 24 h at 4 °C. A preincubation step of the As72 with DEHA for 60 min at room temperature was introduced before the competitive step. The length of the competition was set at 60 min. The concentration of the PBS was increased from 10 to 50 mM in order to produce a conductivity value near 70 mS cm^{-1}. The pH was kept at 7.5, and the concentration of Tween 20 was increased to 0.1%. **Figure 4** shows the standard curve obtained under these conditions, and **Table 4** summarizes the parameters defining the calibration graph of the immunoassay AS72/**2g**-CONA. The assay shows an IC_{50} value of 3.75 μg L^{-1} and a limit of detection of 0.32 μg L^{-1} for DEHA.

Immunoassay specificity was evaluated by preparing standard curves of different related triazines and measuring them in the assay. As expected, only DIHA was better recognized in this

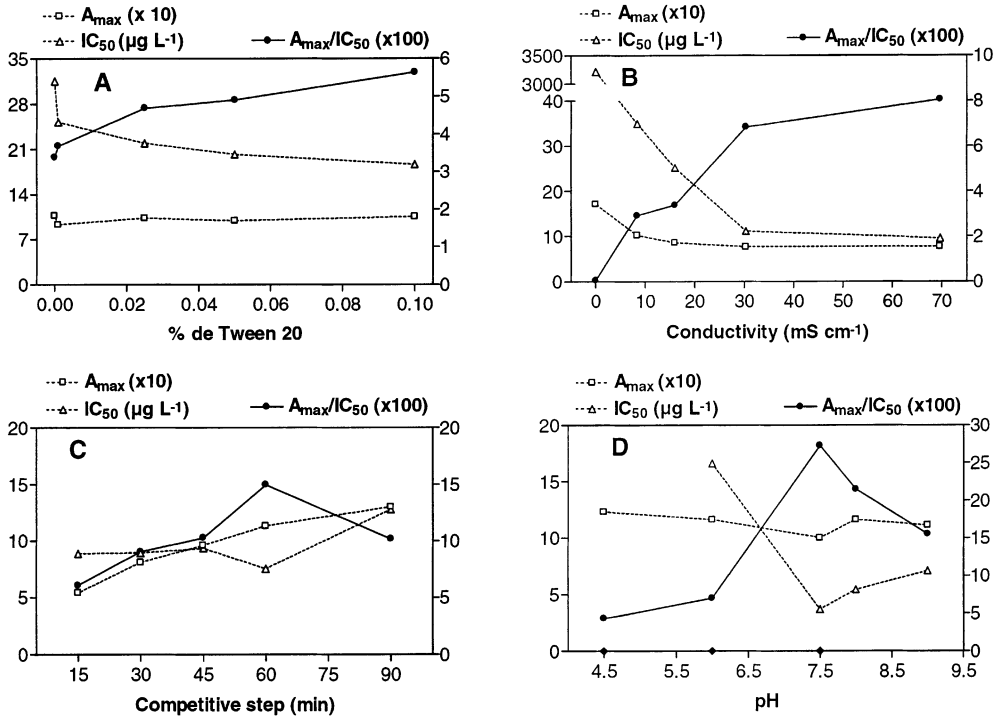


Figure 3. Graphs showing the influence of several parameters on the performance of immunoassay As72/**2g**-CONA. Left axes indicate IC_{50} and A_{max} (×10) of the assay. Right axes indicate A_{max}/IC_{50} (×100). (A) Effect of the detergent concentration. (B) Effect of the ionic strength. (C) Effect of the length of the competitive step. (D) Effect of the pH. The data presented are extracted from the four-parameter equation used to fit the standard curve. Standard curves were prepared using two well replicates.

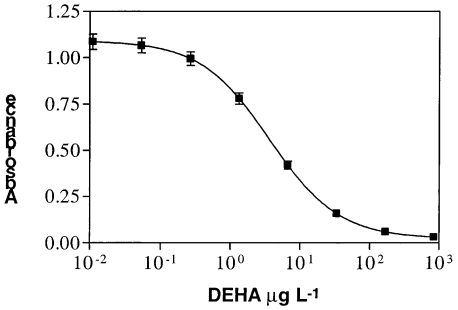


Figure 4. Calibration curve of the immunoassay As72/**2g**-CONA for DEHA obtained after optimizing the protocol. The data presented correspond to the average and the standard deviation of six assays run on six different days. The curves were run using well duplicates. See **Table 4** for the features of the optimized immunoassay.

Table 4. Features of the Optimized Immunoassay As72/**2g**-CONA[a] To Analyze DEHA

A_{min}	0.02 ± 0.01
A_{max}	1.09 ± 0.10
slope	–0.87 ± 0.05
IC_{50}, μg L^{-1}	3.72 ± 0.22
LOD, μg L^{-1}	0.32 ± 0.08
dynamic range	37.79 ± 3.67 to 0.79 ± 0.27
r^2	0.998 ± 0.002

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. The data presented correspond to the average of six calibration curves run on six different days. Each curve was built using two-well replicates.

assay than DEHA, since the ethyl group remaining in the molecule is better mimicked by the spacer arm of the immunizing hapten. The results of the cross-reactivities obtained are shown in **Table 5**. The assay is quite specific for the dealkylated hydroxytriazines, since none of the other triazines assayed were

Table 5. Cross-Reactivity Data Obtained To Characterize the Specificity of the Immunoassay As72/**2g**-CONA for DEHA[a]

s-triazine	log *P*	pK_a	IC_{50} (nM)	CR (%)
DEHA	–0.97	6.56	22.16	100
DIHA	–1.32	6.70	7.27	305
DEDIHA	–2.18	6.75	1250	4%
desmetryne	1.06	3.82	>5000	<0.4
prometryne	1.41	3.91	>5000	<0.4
irgarol 1051	1.24	4.53	>5000	<0.4
atrazine	1.03	2.35	>5000	<0.4
simazine	0.69	3.10	>5000	<0.4
2-hydroxypropazine	0.24	7.68	>5000	<0.4
2-hydroxyatrazine	–0.10	7.59	>5000	<0.4
2-hydroxysimazine	–0.45	7.50	>5000	<0.4
DEA	0.17	2.44	>5000	<0.4
DIA	–0.18	2.55	>5000	<0.4

[a] Cross-reactivity (CR) is expressed as a percentage of the IC_{50} of the DEHA divided by the IC_{50} of the triazine. Log *P* and pK_a values have been introduced to explain the lack of recognition observed for the different triazines evaluated.

recognized in the assay. The recognition pattern of the competitors (see above) had already shown the great importance of the simultaneous presence of a free primary amino group and the hydroxyl group in the chemical structure of the analyte. Thus, the absence of recognition of several hydroxytriazines, such as hydroxyatrazine, hydroxypropazine, and hydroxysimazine, demonstrated that the presence of a hydroxyl group alone was not sufficient for antibody recognition. Looking at the pK_a values of these triazines, it can be observed that the pK_a of the hydroxylated triazines is around 7.5, and therefore both the protonated and the anionic forms will coexist at equilibrium during the competition step. However, the pK_a value of DEHA, DIHA, and DEDIHA are lower, which means that these molecules will be in their anionic form at the assay pH. Similarly, the presence of the free primary amino group alone

Table 6. Results from the Preliminary Accuracy Studies Performed with the Immunoassay As72/**2g**-CONA[a]

spiked concn (nM)	measd concn (nM)	recovery (%)
380	370.37 ± 1.96	97
200	219.48 ± 3.55	110
150	142.58 ± 15.22	95
25	27.04 ± 3.42	108

[a] The accuracy was evaluated by preparing blind spiked samples in water and analyzing them by the optimized immunoassay protocol. Each value corresponds to the mean of three replicates.

is also not enough, as demonstrated by the lack of cross-reactivity shown by DEA and DIA. Triazines such as desmetryne, prometrine, and irgarol, with a methylthio group, were not recognized at all. The same was observed for atrazine and simazine. The low log *P* values of DEHA, DIHA, and DEDIHA tested show their greater polarity compared to the other triazines. Consequently, these analytes can probably establish with the antibodies other kinds of interactions that may predominate over those of more hydrophobic nature. Although, as mentioned in the Introduction, it was expected that the symmetric molecule DIDEHA (two times the epitopes of the immunizing hapten) would be highly recognized, this analyte cross-reacted only 4% in this assay.

Finally, to evaluate *immunoassay accuracy* of the ELISA, blind spiked samples were measured. The results shown in **Table 6** indicate a good accuracy of the method to analyze water samples, since all recovery values are close to 100%, proof that the measured values match very well the spiked concentration values. The linear regression analysis shows a slope close to 1 ($y = 0.97x + 5.96$) and good correlation coefficient ($r^2 = 0.992$).

Conclusions. The analysis of highly polar molecules in water samples at the trace level is often limited by the efficiency of the extraction procedures. Due to their high solubility in water, these molecules are frequently not sufficiently retained in the stationary phases of the chromatographic or solid-phase extraction supports. Immunochemical techniques present the advantage to allow direct measurements in aqueous media. Many HAPDs are common degradation products of several triazine herbicides that are widely used for crop protection. Because they are more stable to the degradation process than their parent compounds, an increased risk of contamination of natural waters by these contaminants exists. Therefore, it is necessary to develop screening methods to efficiently monitoring contamination by these triazine degradation products. In the present paper, we have reported the preparation of antibodies against dealkylated hydroxytriazines. Due to the high polarity of these substances, the preparation of the immunizing hapten **2g** has required keeping the amino group protected until the end of the synthetic pathway. The antibodies raised have been shown to be highly specific for DIHA and DEHA. The simultaneous presence of both the free amino and the hydroxyl group is necessary to observe a recognition. The assay has an IC_{50} of 3.75 $\mu g\ L^{-1}$ and shows a limit of detection of 0.32 $\mu g\ L^{-1}$ for DEHA in water. Further studies will evaluate its performance in natural water samples.

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