Development of an ELISA for the Analysis of Atrazine Metabolites Deethylatrazine and Deisopropylatrazine†

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A highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) is described for the detection of the atrazine metabolites deethylatrazine and deisopropylatrazine. Polyclonal antibodies were raised in rabbits by immunization with a hapten–bovine serum albumin (BSA) conjugate containing 32 hapten residues/molecule of BSA. An ELISA with a peroxidase (POD) hapten tracer was optimized in microtiter plates. A concentration of 50% B/B_0 was found at 0.20 μg/L for deethylatrazine and at 0.28 μg/L for deisopropylatrazine. Limits of determination for deethylatrazine and for deisopropylatrazine were reached at approximately 0.01 μg/L, i.e., well below the maximum concentration permitted by EC guidelines for drinking water. The assay did not require concentration or cleanup steps for drinking water or groundwater samples. Validation experiments confirmed a good accuracy and precision. Since deethylatrazine is the main atrazine metabolite found in water samples from these regions, this test yields fairly accurate results for deethylatrazine concentrations in environmental water samples. If deisopropylatrazine is present in the water samples, estimates for the sum parameter of the two atrazine metabolites are obtained.

INTRODUCTION

The EC guidelines and the drinking water ordinance of the Federal Republic of Germany in combination with the continuous application of pesticides necessitate the availability of fast screening methods. Regular surveys of natural water and drinking water by various research laboratories including the Central Laboratory of Gelsenwasser AG revealed that surface water and enriched ground water are especially endangered by pesticide pollution. Because of its persistence and wide application, atrazine and its main metabolites (hydroxyatrazine, deethylatrazine, and deisopropylatrazine; cf. Figure 1) belong to the most critical compounds and should be monitored continuously. Deethylatrazine and atrazine in particular were repeatedly detected in drinking waters in concentrations above the upper limit of the drinking water ordinance of 0.1 μg/L. Established methods in trace analysis (GC/MS and HPLC) are unsuitable if a great number of samples must be analyzed in a short time. For this purpose, serological methods such as the ELISA are advantageous if sensitive and specific antibodies are available. Worldwide, several groups are working on the development of sensitive enzyme immunoassays (EIAs) for the analysis of *s*-triazines with polyclonal and monoclonal antibodies (Bushway et al., 1988; Goodrow et al., 1989; Schlaeppi et al., 1989; Giersch and Hock, 1990). An ELISA has been described which detects atrazine and propazine to a major extent (Wittmann and Hock, 1989). Even some *s*-triazine EIAs are now commercially available.

The aim of the present paper was the development of a sensitive and specific ELISA for the analysis of deethylatrazine and deisopropylatrazine. It is shown that with polyclonal antibodies and the conventional ELISA technique detection limits for deethylatrazine and deisopropylatrazine of about 10 ng/L can be reached.

MATERIALS AND METHODS

Reagents. *1. Chemicals.* The hapten 2-aminohexanecarboxylic acid-4-amino-6-chloro-1,3,5-triazine was synthesized by Dr. U. Doht, Riedel de Haen, according to the procedure of Pearlman and Banks (1948), Thurston et al. (1951), and Goodrow et al. (1990). The hapten was used for immunoconjugate and enzyme tracer synthesis. The triazine standards were provided by Riedel de Haen and Ciba Geigy. In addition, the following reagents were used: alkaline phosphatase (aP) from calf intestine (2500 units/mg; Boehringer, Mannheim); bovine serum albumin, lyophilized, pure (Serva); 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-4-toluenesulfonate (CMC; Sigma); dicyclohexylcarbodiimide (DCC; Sigma); ethanol absolute, p.a. (Merck); Freund's complete adjuvant (Sigma); horseradish peroxidase (1490 units/mg; Serva); hydrogen peroxide, 30% (Merck); *N*-hydroxysuccinimide (NHS; Sigma); *p*-nitrophenyl phosphate (Serva); tetramethylbenzidine (TMB; Riedel de Haen). All other chemicals were of analytical grade.

2. Buffers and Solutions. The following were used: carbonate buffer, 50 mmol/L, pH 9.6, for coating; phosphate-buffered saline (PBS), 40 mmol/L, pH 7.2 (containing 8.5 g of NaCl/L) for the preparation of standards and the peroxidase tracer in the POD test; PBS washing buffer, 4 mmol/L, pH 7.2 (containing 0.85 g/L NaCl and 0.5 mL of Tween 20/L), for washing the microtiter plates in the POD test; Tris-buffered saline (TBS), 50 mmol/L, pH 7.2 (containing 0.2 g/L $MgCl_2$ and 0.6 g/L NaCl), for the preparation of standards and the phosphatase tracer in the aP test; TBS washing buffer, 5 mmol/L, pH 7.2 (containing 0.02 g/L $MgCl_2$, 0.06 g/L NaCl, and 0.5 mL of Tween 20/L), for washing the microtiter plates in the aP test; substrate buffer for POD, 0.1 mol/L sodium acetate (NaAc) (the pH was adjusted to 5.5 by adding 1 mol/L citric acid); substrate for POD, 400 μL of tetramethylbenzidine (TMB; 6 mg of TMB was dissolved in 1 mL of dimethyl sulfoxide) + 100 μL of 1% H_2O_2 filled to 25 mL with substrate buffer; stopping reagent for POD, 2 mol/L H_2SO_4; substrate buffer for aP, 70 mmol/L diethanolamine, adjusted to pH 9.8 with 37% HCl (v/v) (containing 0.2 g/L NaN_3); substrate for aP, 0.5 mg of *p*-nitrophenyl phosphate/mL of substrate buffer.

3. Preparation of Triazine Standards. Five milligrams of *s*-triazine was dissolved in 50 mL of ethanol absolute with the aid of an ultrasonic bath (20 min). Starting with this solution a stock solution was prepared containing 10 mg of *s*-triazine/L (=excess). A standard series was prepared by making several dilutions of the stock solutions to yield the following *s*-triazine concentrations: 0.01, 0.033, 0.1, 0.33, 1, and 10 μg/L. The stock solution and the standard series were made up either in 40 mmol/L PBS buffer, pH 7.2, for the POD test, in 50 mmol/L TBS buffer, pH 7.2, for the aP test, or in distilled water.

4. Equipment. The following were used: EIA photometer,

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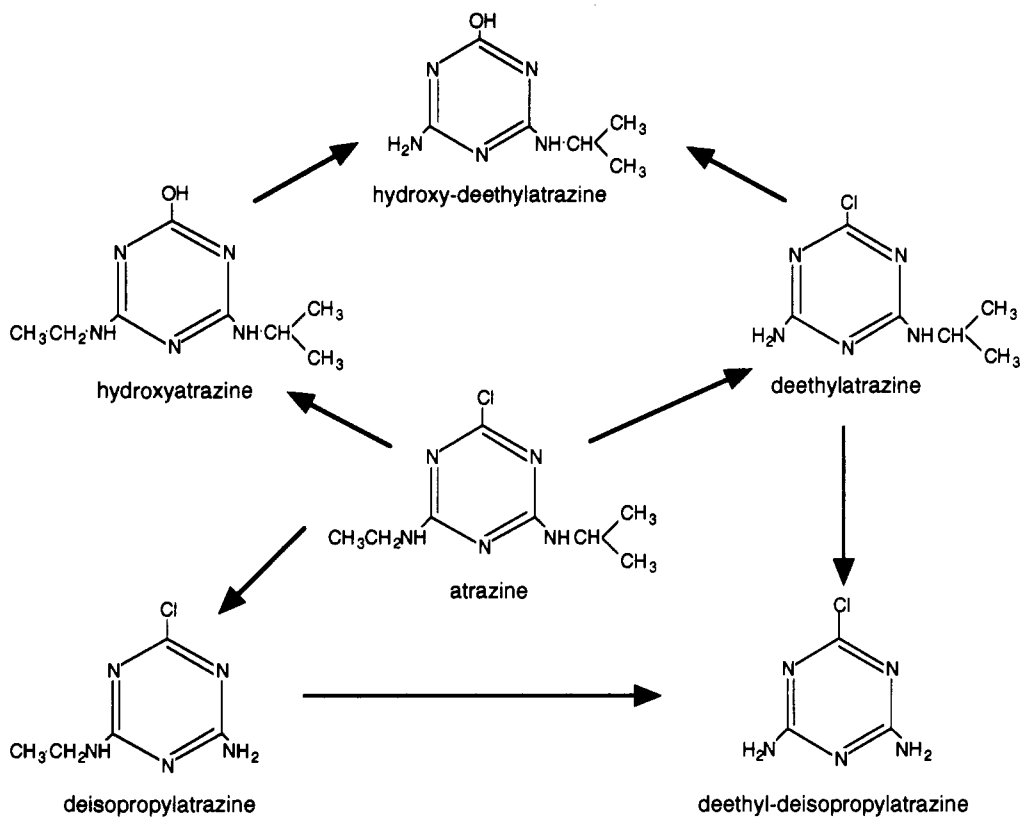

Figure 1. Atrazine and its metabolites relevant for water.

SLT Easy Reader EAR 400 (SLT, Gröding/Salzburg, Austria); microtiter plate washer, SLT Easy Washer EAW 8112 (SLT, Austria); ultrasonic bath, Sonorex (Bandelin).

5. Further Materials. Also used were 96-well microtiter plates, type F-form, high binding capacity, No. 655061 (Greiner Labortechnik, D-7443 Frickenhausen, FRG).

Procedure. *1. Preparation of Antigens.* 2-Aminohexanecarboxylic acid–4-amino-6-chloro-1,3,5-triazine was used as the hapten for the synthesis of the immunogenic conjugate. The hapten was synthesized by Dr. U. Doht, Riedel de Haen. It was coupled to the carrier protein bovine serum albumin via an active ester in a carbodiimide reaction. Figure 2 shows the synthesis of the hapten and the subsequent immunogen. To achieve a coupling rate between 10 and 35 molecules of hapten/molecule of BSA, the CMC procedure was performed with two different carbodiimide/hapten ratios.

CMC Procedure. Two immunogenic conjugates (a and b) were prepared. BSA [200 mg (3×10^{-6} mol)] was dissolved in 10 mL of distilled water. After the pH was adjusted to 5.5 by 0.1 mol/L HCl, (a) 93 mg (2×10^{-4} mol) or (b) 186 mg (4×10^{-4} mol) of CMC was added gradually while the pH was kept constant. Eighty milligrams (3×10^{-4} mol) of the hapten (with a free carboxyl group at the terminal end of the spacer) was dissolved in 2 mL of dimethylformamide and added dropwise to solutions a and b with BSA and carbodiimide under constant stirring, while the pH was kept at 5.5 with 0.1 mol/L NaOH. Milky solutions were obtained. After 15 min, (a) 42 mg (1×10^{-4} mol) or (b) 84 mg (2×10^{-4} mol) of solid CMC was added, and the mixture was stirred overnight and dialyzed for 48 h against 1.5 L of distilled H_2O with four changes of water. The two conjugates a and b were then freeze-dried.

The molar ratios of the two conjugates achieved were determined from the UV spectra by assuming that the absorbances of BSA and the hapten are additive.

2. Preparation and Characterization of Rabbit Antibodies. Three White New Zealand rabbits (C10, C20, C30) were subcutaneously (neck area) and intradermally (caudal area) injected three times at weekly intervals with 1 mg of hapten–BSA conjugate b, dissolved in 0.5 mL of saline (9 g of NaCl/L) and emulsified with 0.5 mL of Freund's complete adjuvant. An intravenous booster injection (0.5 mg of conjugate b in 0.5 mL of saline) into the ear vein followed 2 weeks later. Blood was collected from the ear vein 1 week after the last injection. The animals were boosted at 4-week intervals by subcutaneous and intradermal injections of conjugates a and b alternately, if a decline of the antibody titer has been observed, and bled 2 weeks later. The crude antisera were prepared as described earlier (Huber and Hock, 1986), sterile-filtered (Sartorius filters, 0.2 μm), and lyophilized in portions of 1 mL. The protein concentrations of the sera were determined according to the method of Warburg and Christian (1941). The immunoglobulin G (IgG) fraction of the crude sera was purified by protein A affinity chromatography according to the procedure of Compton et al. (1989). The affinity column was first washed with 0.1 molar phosphate buffer, pH 7.0, and loaded with the crude serum. After an incubation of 15 min, the column was washed with starting buffer until the eluate was free of protein which was continuously measured by the absorption at 280 nm. IgG was eluted with 0.1 M glycine hydrochloride buffer, pH 2.0, in 0.5-mL fractions which were immediately adjusted to pH 7.0 with 0.1 M NaOH. The IgG fractions were combined and lyophilized in portions of 1 mL after protein determination.

3. Enzyme Tracers. In most cases POD was used for tracer synthesis, as the chromogen tetramethylbenzidine and the substrate H_2O_2 permit a very sensitive enzyme reaction [cf. Wittmann and Hock (1989)]. The hapten was coupled to POD in a manner analogous to that used before (Wittmann and Hock, 1989). For this purpose, the carbodiimide/*N*-hydroxysuccinimide procedure [modified from Märtlbauer and Terplan (1988)] was used; 1 mg of the hapten together with 1.7 mg (=15 μmol) of *N*-hydroxysuccinimide and 6.2 mg (=30 μmol) of dicyclohexylcarbodiimide was dissolved in 130 μL of dry dioxane and incubated for 18 h at room temperature. Then the mixture was slowly added to a solution of 2 mg (=0.05 μmol) of POD (1490 units/mg = 24 839 nkat) in 3 mL of sodium hydrogen carbonate (0.13 mol/L), incubated for another 3 h and either dialyzed for 3 days against 40 mmol/L PBS buffer, pH 7.2, or desalted with a Sephadex G-25 PD 10 column (Pharmacia). The POD tracer was stored at 4 °C after sterile filtration.

The epitope densities of the POD conjugates could be determined by measuring the absorption at 403 nm, where only POD absorbs, and at 280 nm, where both POD and the tracer absorb.

For comparison, an aP tracer was synthesized by using a similar

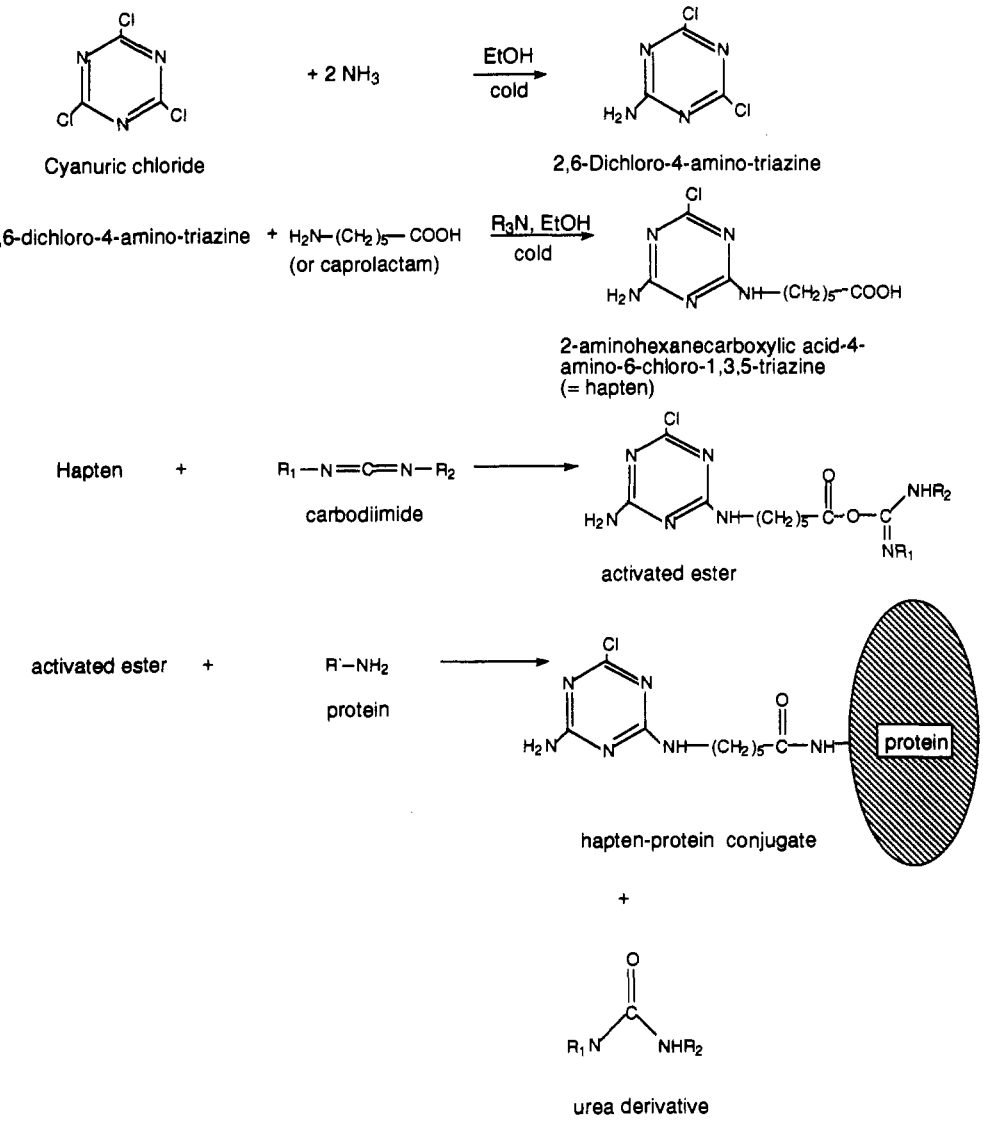

Figure 2. Hapten and subsequent immunogen synthesis with BSA as carrier protein by means of a carbodiimide activation.

method with slightly modified solutions. Alkaline phosphatase from calf intestine (Boehringer, Mannheim) was coupled to the hapten by the carbodiimide/*N*-hydroxysuccinimide procedure; 1 mg of the hapten together with 1.7 mg of *N*-hydroxysuccinimide and 6.2 mg of dicyclohexylcarbodiimide was dissolved in 130 μL of dry dioxane and incubated for 18 h at room temperature. Then the mixture was slowly added to a solution of 0.1 mL of aP (=1 mg; 2500 units/mg = 41 675 nkat) in 3 mL of aP buffer (3 mol/L NaCl, 1 mmol/L $MgCl_2$, 0.1 mmol/L $ZnCl_2$, and 30 mmol/L triethanolamine, adjusted to pH 7.0). The pH was kept constant at pH 7.0 with aP buffer. The whole mixture was incubated for 3 h and then dialyzed for 3 days against 50 mmol/L TBS buffer or desalted with a Sephadex G-25 PD 10 column (Pharmacia). Again, the aP tracer was stored after sterile filtration at 4 °C.

The epitope density of the aP conjugate could not be determined from the UV spectra because of the low concentrations, resulting in weak UV absorptions.

The percentages of aP and POD of the hapten–enzyme conjugates were compared to those of the unconjugated enzymes on the basis of enzyme activities.

4. Enzyme-Linked Immunosorbent Assay (ELISA). *s*-Triazine standards were used for the test with the following concentrations: 0, 0.01, 0.033, 0.1, 0.33, 1, 10, and 10 000 (=excess) μg/L. The pH of unknown samples was adjusted, if necessary, to between 7.0 and 7.5 (1 volume of buffer + 9 volumes of sample). TBS buffer was used for the aP test and PBS buffer for the POD test. ELISAs were performed in microtiter plates (96-well plates, type F-form, high binding capacity) at room temperature.

The protocol for the optimized assay includes the following steps:

Coating. Three hundred microliters of an appropriate antiserum or IgG dilution, respectively, is incubated in carbonate buffer, pH 9.6, at 4 °C overnight or at 37 °C for 4 h.

Washing. Three washes are performed with 300 μL of washing buffer (TBS washing buffer for aP test; PBS washing buffer for POD test) by using the microtiter plate washer. The buffer is removed after the last step.

Immunoreaction. Two hundred microliters of standard or sample is added, followed by 50 μL of enzyme tracer dilution in TBS buffer for the aP test and in PBS buffer for the POD test. After the plate is agitated on a horizontal shaker for 1 min, it is incubated for 2 h at room temperature.

Washing was done three times as before.

Enzyme Reaction. Two hundred microliters of substrate is added by using *p*-nitrophenyl phosphate as the aP substrate and the POD substrate for POD. The absorption of the aP reaction is read after 70 min at 405 nm with the EIA photometer. The POD reaction is stopped after 20–30 min with 100 μL of 2 mol/L H_2SO_4. Then the absorption is read at 450 nm.

Each determination is performed in quadruplicate. The absorptions (A) are converted to % B/B_0 values according to the formula

$$\% B/B_0 = \frac{A - A_{excess}}{A_{control} - A_{excess}} \times 100$$

and finally transformed to logit values for the linearization of

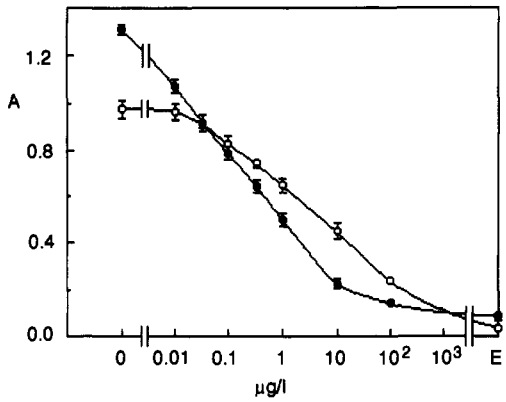

Figure 3. Influence of different antisera on the deethylatrazine calibration curves (five independent analyses). (●) The antiserum C10 (blood collection, February 27, 1990) was diluted 1:50 000 and the POD tracer 1:50 000. The absorption was measured 25 min after substrate addition at room temperature. (○) The antiserum C30 (blood collection, February 28, 1990) was diluted 1:50 000 and the POD tracer 1:50 000. The absorption was measured 20 min after substrate addition at room temperature. E, excess of deethylatrazine.

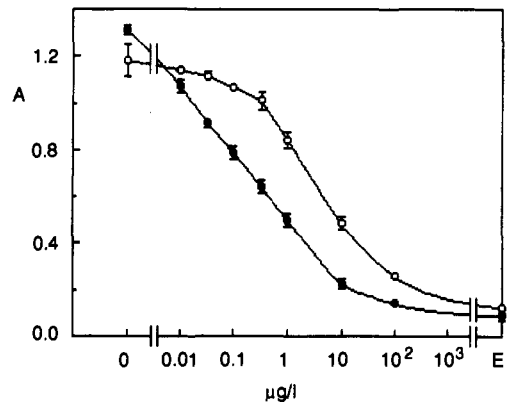

Figure 4. Influence of different enzyme tracers (aP and POD tracer) on the deethylatrazine calibration curves (five independent analyses). (●) POD tracer. The antiserum C10 (blood collection, February 27, 1990) was diluted 1:50 000 and the POD tracer 1:50 000. The absorption was measured 25 min after substrate addition at room temperature. (○) aP tracer. The antiserum C10 (blood collection, February 27, 1990) was diluted 1:50 000 and the aP trace 1:50 000. The absorption was measured 70 min after substrate addition at room temperature.

the calibration curve according to the formula

$$\text{logit}\ (\%\ B/B_0) = \ln \frac{\%\ B/B_0}{100 - \%\ B/B_0}$$

5. Cross Reactivities. The cross reactivities of the antisera were based upon deethylatrazine (=100%). Standard calibration curves were obtained from 15 different *s*-triazines and 10 other herbicides. No difference between the absorptions of the zero control and the corresponding excess sample indicated the lack of cross reactivity of the antiserum with the assayed substance. Cross reactivities were calculated from the calibration according to the formula

% cross reactivity = (hapten concentration at 50% B/B_0/ concentration of the cross-reacting hapten at 50% B/B_0) × 100

6. Sum Parameters. Standard solutions for deethylatrazine and deisopropylatrazine of 0.05, 0.15, and 0.50 µg/L were prepared in distilled water. The deethylatrazine and deisopropylatrazine contents of the standard solutions were determined by two separate ELISAs with the appropriate standard series consisting of the following concentrations: 0, 0.010, 0.033, 0.10, 0.33, 1, 10, and 10 000 (=excess) µg/L. The deethylatrazine and deisopropylatrazine standard solutions were mixed 1:1 (v/v), yielding a final sum for the two metabolites of 0.10, 0.30, and 1.0 µg/L. The sum contents of the two metabolites were determined by two different ELISAs using a deethylatrazine standard series for the first one and a deisopropylatrazine standard series for the second one. The results were related either to deethylatrazine or to deisopropylatrazine.

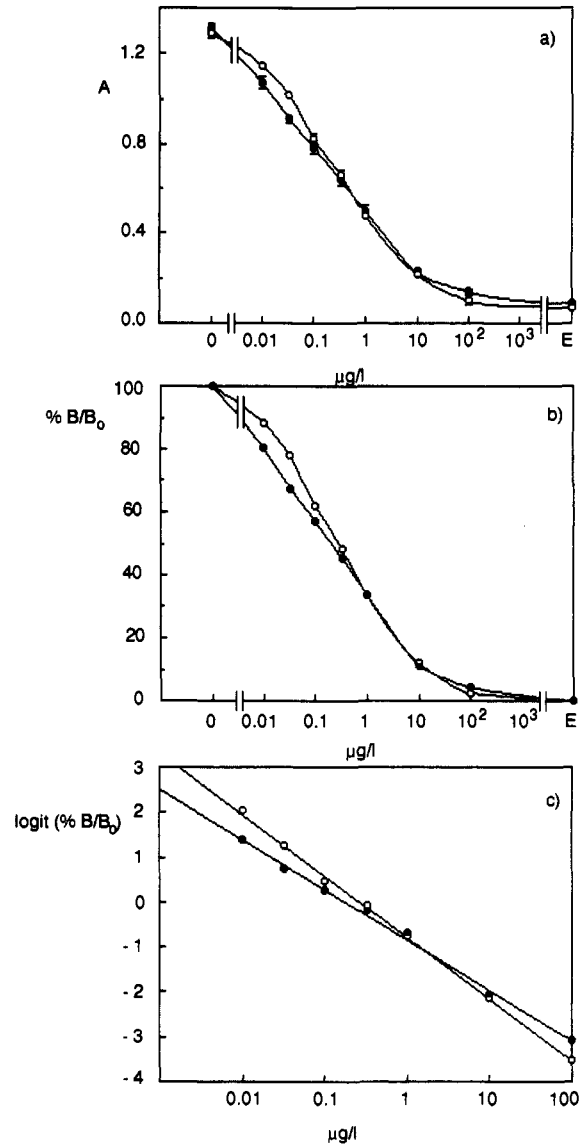

Figure 5. Optimized assay for the analysis of deethylatrazine and deisopropylatrazine with antibody C10 (blood collection, February 27, 1990) and POD tracer, both in a 1:50 000 dilution. The absorption was measured 25 min after addition of the substrate at room temperature. (a) Absorption (mean ± standard deviation of 20 determinatons); (b) % B/B_0; (c) logit (% B/B_0). (●) Deethylatrazine; (○) deisopropylatrazine.

RESULTS

A deethylatrazine derivative was coupled to the carrier protein BSA to produce an immunogenic conjugate. To achieve a coupling rate between 10 and 35 molecules of hapten/molecule of BSA, the coupling method (carbodiimide procedure) was varied with respect to the carbodiimide/hapten ratio. The coupling was evaluated by measuring the change in the UV absorption of BSA. The spectrum of the hapten used for coupling showed a shoulder at A_{max} = 256 nm and BSA an A_{max} at 277 nm. The absorption increase at 256–272 nm depending upon the hapten density after coupling is evident. In the case of conjugate b, the absorption maximum at 265 nm was used to calculate the coupling rate. Conjugate a exhibited

Table I. Cross Reactivities of (a) Antiserum C10[a] (Blood Collection, February 27, 1990) and (b) Antiserum C30[b] (Blood Collection, February 28, 1990)[c]

compd	lower detection limit, μg/L	50% B/B_0, μg/L	cross reactivity, %, based on deethylatrazine
(a) deethylatrazine	0.010	0.200	100
(a) deisopropylatrazine	0.010	0.280	70
(b) deethylatrazine	0.050	5.08	100
(b) deisopropylatrazine	0.030	2.03	250

[a] Dilution 1:50 000, determined with the POD tracer (dilution 1:50 000), which was prepared by the carbodiimide/*N*-hydroxysuccinimide procedure (substrate incubation for 25 min at room temperature). [b] Dilution 1:50 000, determined with the POD tracer (dilution 1:50 000), which was prepared by the carbodiimide/*N*-hydroxysuccinimide procedure (substrate incubation for 20 min at room temperature). [c] The following *s*-triazines could not be detected at concentrations of 100 μg/L: atrazine, deethyldeisopropylatrazine, hydroxyatrazine, simazine, propazine, terbuthylazine, ametryn, aziprotryn, simetryn, prometryn, terbutryn, 2-(ethylamino)-4-(thiomethyl)-6-aminotriazine, 2-amino-4-(thiomethyl)-6-(isopropylamino)triazine and 2-amino-4-methoxy-6-(isopropylamino)triazine. The following other herbicides were not detectable at concentrations of 10 mg/L: acetochlor, alachlor, butachlor, diethyl-ethyl, dimetachlor, metazachlor, metolachlor, propachlor, pretilachlor, methabenzthiazuron.

Table II. Test for Sum Parameters of Deethylatrazine and Deisopropylatrazine with Antiserum C10 (Blood Collection, February 27, 1990) and POD Tracer (Date of Synthesis, March 15, 1990)[a]

sample no.	deethylatrazine single content, μg/L	deisopropylatrazine single content, μg/L	sum of the contents of the two metabolites in one sample: related to deethylatrazine, μg/L	sum of the contents of the two metabolites in one sample: related to deisopropylatrazine, μg/L
1 (nominal)	0.050	0.050	0.100	0.100
1 (measd)	0.058	0.053	0.075	0.125
2 (nominal)	0.150	0.150	0.300	0.300
2 (measd)	0.140	0.135	0.290	0.316
3 (nominal)	0.500	0.500	1.000	1.000
3 (measd)	0.520	0.490	1.020	1.080

[a] The deethylatrazine and the deisopropylatrazine contents of the standard solutions were each determined by two separate ELISAs. The deethylatrazine and deisopropylatrazine standard solutions were mixed 1:1 (v/v), yielding a sum for the two metabolites. The sum contents of the two metabolites were determined by two different ELISAs. The results were related either to deethylatrazine or to deisopropylatrazine.

a coupling rate of 12 molecules of hapten/molecule of BSA. The highest yield, 32 hapten residues/BSA molecule, was obtained by conjugate b using a hapten/BSA ratio of 100 and a carbodiimide/hapten ratio of 2. The protein yield after desalting was 70% with all hapten–BSA conjugates.

First, conjugate b with the higher coupling rate was applied for an immunization period of 2 months. The affinity of the antisera could be improved by continuing the immunization alternately with conjugates a and b. After an immunization period of about 6 months, acceptable antisera with respect to titers and sensitivities were obtained. Coating of the microtiter plates was carried out with 300 ng of lyophilized crude serum or 30 ng of IgG, respectively, per cavity. The yield of the protein A chromatography in terms of antibody activity was almost 100%. However, the IgG-coated and washed plates could only be stored for ca. 2 weeks at 4 °C without activity losses. After this time, a rapid decrease of the absorptions below 0.6 for the zero controls was observed. The plates, which were coated with crude antiserum, could be stored at least for 2 months in the refrigerator before the ELISA was carried out. The results with these plates were highly reproducible. Therefore, a purification of this antiserum does not provide any advantages. The titers (i.e., the antiserum dilution, which is used in the ELISA) turned out to be 1:50 000 for antiserum C10, 1:8000 for antiserum C20, and 1:50 000 for antiserum C30. A screening of the different antisera using preliminary ELISAs proved the superiority of antiserum C10 with respect to the limits of determination for deethylatrazine and deisopropylatrazine, followed by C30. Therefore, the final test was optimized with these antisera.

Table III. Reproducibility of the Deethylatrazine ELISA[a]

analysis no.	concn of unknown samples: 0.100 μg/L	0.300 μg/L	1.000 μg/L
1	0.105 ± 0.006	0.315 ± 0.06	1.020 ± 0.100
2	0.099 ± 0.008	0.295 ± 0.02	1.030 ± 0.080
3	0.110 ± 0.005	0.286 ± 0.04	1.080 ± 0.099
4	0.095 ± 0.010	0.320 ± 0.08	0.986 ± 0.110
5	0.090 ± 0.003	0.295 ± 0.02	0.990 ± 0.060
6	0.102 ± 0.002	0.299 ± 0.01	0.995 ± 0.042
7	0.115 ± 0.001	0.318 ± 0.02	1.060 ± 0.029
8	0.089 ± 0.008	0.330 ± 0.06	1.040 ± 0.034
9	0.120 ± 0.001	0.285 ± 0.08	1.090 ± 0.097
10	0.108 ± 0.006	0.290 ± 0.09	0.985 ± 0.120
means of 10 analyses	0.103	0.303	1.028
CV, %	4.5	3.8	4.8

[a] The ELISA was run on 10 different days. In addition to the zero control and the deethylatrazine excess, the standards comprised seven different deethylatrazine concentrations ranging from 0.010 to 100 μg/L. Three unknown samples (prepared in a different laboratory) and the standards were run in duplicates. Means, standard deviations (in μg/L), and variation coefficients (CV) are incidated.

Figure 3 shows the calibration curves of the two antisera C10 and C30 for deethylatrazine. Antiserum C10 turned out to be superior in terms of the determination limit. Two different enzyme tracers were assayed: aP and POD conjugates of the hapten, both of which were produced by the same carbodiimide/*N*-hydroxysuccinimide method. The enzyme activity of both tracers remained almost unchanged after coupling. With POD, the coupling rate could be determined on the basis of the absorption spectra with a distinct peak at 403 nm. The POD concentration of the tracer could be determined from the absorption at 403 nm. The absorption increase at 280 nm after coupling was related to the epitope density. Under optimal conditions, the carbodiimide/*N*-hydroxysuccinimide procedure yielded approximately 5 hapten residues/POD. The POD yield was found to be close to 100% on the basis of UV spectra. The same strategy could not be applied to aP because of the weak UV absorption at the applied low enzyme concentrations. Therefore, the aP tracer was tested directly by the ELISA. Figure 4 compares the calibration curves for deethylatrazine obtained with antiserum C10 in a dilution of 1:50 000 and for the POD and aP tracer, both in a dilution of 1:50 000. The superiority of the POD tracer concerning the determination limit and the time needed for substrate incubation is evident.

On the basis of these results, an optimized ELISA with the POD tracer was worked out as shown in detail by Figure 5 for antiserum C10. The 50% B/B_0 values, i.e., the regions with the highest assay sensitivities, are reached at 0.20 μg/L for deethylatrazine and at 0.28 μg/L for deisopropylatrazine. The antibody allows a limit of determination of about 0.01 μg/L for deethylatrazine and deisopropylatrazine. The limit of determination is defined as the smallest amount of a substance that can be quantitatively assayed with the required statistical certainty at a single analysis with a statistical error of <5% (Funk et

Table IV. Analysis of Tap Water (D-8050 Freising) from March 15, 1990, before and after It Was Spiked with Defined Deethylatrazine Amounts

sample	deethylatrazine concn of spiked sample, μg/L	total deethylatrazine concn determined with ELISA, μg/L	estimated concn of deethylatrazine amount, μg/L (value of col 3 minus 0.066)	deviation, %
tap water		0.066		
tap water	0.010	0.080	0.014	40
tap water	0.100	0.182	0.116	16
tap water	1.000	1.200	1.134	13

al., 1985). It was graphically determined for deethylatrazine and deisopropylatrazine from more than 20 calibration curves. The cross reactivities are listed in Table I for the two antibodies C10 and C30 with the best properties for deethylatrazine and deisopropylatrazine determination.

Antibody C10 was characterized with the POD tracer and the aP tracer, which yielded similar results for the cross reactivities but different ones with respect to the determination limits for deethylatrazine and deisopropylatrazine. When the aP tracer was used, the 50% B/B_0 values were found at 4.92 μg/L for deethylatrazine and at 5.08 μg/L for deisopropylatrazine. Antibody C30 was characterized with the POD tracer and the aP tracer. In contrast to antibody C10, antibody C30 showed the highest affinity to deisopropylatrazine. The use of different enzyme tracers with the same antibody had no significant effect on the cross reactivities. However, the POD tracer proved to be superior because it allowed lower determination limits and shorter substrate incubation periods. The two antibodies differed remarkably with respect to the cross reactivities. Antibody C10 exhibited the highest affinity to deethylatrazine, followed by deisopropylatrazine (70%).

Table II shows that, within certain limits, the sum parameters of the two metabolites can be determined. For this purpose, synthetic samples were prepared which contained deethylatrazine, deisopropylatrazine, or deethylatrazine and deisopropylatrazine together. Then the samples were measured with the antibody C10 ELISA, using alternately deethylatrazine or deisopropylatrazine standards. Considering the percent calibration curves (cf. Figure 5b), it is obvious that the largest deviations from the sum parameters are found at lower triazine concentrations.

Antibody C30 exhibited the highest affinity to deisopropylatrazine. Therefore, the optimized ELISA was performed with antibody C10 and the POD tracer. The reproducibility of the ELISA was tested by analyzing deethylatrazine concentrations of 0.1, 0.3, and 1.0 μg/L as "unknown samples" 10 times (Table III). The standard deviations proved to be very low, and the coefficient of variation (CV) was determined to be 4.5%.

To detect potential matrix effects in drinking water, a tap water sample was spiked with defined amounts of deethylatrazine as an example. Satisfactory estimates (Table IV) of the fortified samples (column 4) were obtained by considering the concentrations used. Various natural water samples including tap water and seawater were assayed with the ELISA at least at four different times in the natural state and after the samples were spiked with known amounts of deethylatrazine. A representative example with tap water is shown in Table IV. The unspiked sample was already contaminated with deethylatrazine, deisopropylatrazine, or both metabolites. A GC analysis, which had been carried out by Thomas Ruppert in the laboratory of Dr. L. Weil, Institute of Water Chemistry and Chemical Balneology, TU München, confirmed the deethylatrazine concentration of 66 ng/L in the tap water. This result had been extrapolated by considering a recovery of 60%. The experimental error is ca. 20%. GC analyses require sample volumes of at least 1 L to obtain a detection limit of 25 ng/L.

DISCUSSION

The production of anti-hapten antibodies depends upon the availability of suitable conjugates with an optimal coupling rate. We started the immunization with conjugate b, which exhibited a very high coupling rate of 32 hapten residues/BSA. After about 2 months, high antibody titers were obtained. However, the affinities of the antibodies could be improved by continuing the immunization alternating between conjugate a (12 hapten residues/BSA) and conjugate b. Suitable antibodies with respect to the limits of determination and titers were obtained after about 6 months.

There is no general rule for the number of haptens that should be bound to the immunogenic carrier protein. Erlanger (1980) mentions that good antibody titers can usually be obtained with coupling rates anywhere between 8 and 25. Too many hapten molecules often lead to insoluble reaction products. On the other hand, very low epitope densities may require longer immunization periods. However, they are reported to yield antibodies with higher affinities (Pinckard, 1978). In our case, with the high coupling rate of conjugate b, high antibody titers were obtained in a relatively short time. To combine high antibody titers with high affinities of the antibodies, we used an alternate immunization with conjugates a and b. It can be assumed that a high coupling rate of the immunogenic conjugate leads to a high antibody titer, whereas a lower coupling rate seems to be responsible for a high antibody affinity.

A comparison between the crude antisera and their corresponding IgG fractions yielded the surprising result that the plates coated with crude sera could be stored in the refrigerator for a considerably longer time than IgG-coated plates. It was expected that the affinity chromatography removed serum proteases together with other proteins that could cause unspecific binding. The superior results with the crude antiserum indicate a protecting function of the other proteins leading to the observed stabilization.

A prerequisite for a potent ELISA is the availability of not only antibodies with a high affinity toward the analyte but also an enzyme tracer which exhibits a high activity and can be easily replaced by the analyte. As it was demonstrated, the use of the POD tracer, which had been synthesized by the carbodiimide/*N*-hydroxysuccinimide procedure, provided distinct advantages over the aP tracer. The very high enzyme substrate turnover and the sensitive color reaction with the chromogen tetramethylbenzidine guarantee low detection limits in the nanograms per liter range for deethylatrazine and deisopropylatrazine. Moreover, the substrate incubation could be shortened to 25 min with the POD tracer as compared to 70 min with the aP tracer.

One interesting apsect is the different cross reactivities of the two antisera C10 and C30. Antibody C10 exhibited

the highest affinity to deethylatrazine; deisopropylatrazine was detected with a cross reactivity of 70%. As the calibration curves do not run in parallel, but converge at higher concentrations, the sum parameters of the two metabolites could be determined in concentration ranges above 0.1 μg/L. Antibody C30 showed the highest affinity to deisopropylatrazine, whereas the cross reactivity with deethylatrazine amounted to only 40%. Therefore, antibody C30 should be used for a deisopropylatrazine ELISA.

The novel ELISA described in this paper is sensitive and, when used with the antibody C10, very specific for deethylatrazine and deisopropylatrazine, which could be detected in similar ranges. Therefore, an approximate sum parameter for the determination of the two metabolites is provided. Deethylatrazine, the main atrazine metabolite, could be detected repeatedly in drinking water and ground water samples above the upper limit of the EC guidelines of 0.1 μg/L (personal communication from Dr. C. Schlett). The validation assays with drinking water and ground water samples showed no matrix effects yet. Therefore, no cleanup procedure for the water samples is necessary prior to measurement. A comparison of the ELISA presented here with available commercial ELISAs for *s*-triazines yielded significantly lower limits of determination combined with many fewer cross reactivities. To our knowledge, presently available commercial enzyme immunoassays for *s*-triazine analysis detect deethylatrazine to a minor extent; i.e., the lower detection limit for deethylatrazine lies above 0.1 μg/L. Therefore, there is no possibility of monitoring water samples according to the upper limit of the EC guidelines concerning deethylatrazine with these tests. Furthermore, the ELISA described in this paper offers considerable advantages over the classical methods of trace analysis (GC/MS and HPLC). The easy handling, the small sample volumes needed (200 μL), the omission of cleanup and concentration steps, the relatively fast measurement (ca. 2.5 h with coated and washed plates), the high sample turnover (up to 16 samples per plate run in 4 parallels), the low determination limits, and the acceptable costs are important criteria. On the other hand, the disadvantages of the ELISA should not be overlooked. They are mainly seen in the cross reactivity of antiserum C10, leading to a compound response to deethylatrazine and deisopropylatrazine. Only GC or LC methods will allow a quantification of both metabolites. However, the ELISA could be used as a screening method for the analysis of the atrazine metabolites deethylatrazine and deisopropylatrazine in water samples. Work is in progress to demonstrate the application of this ELISA for environmental water samples.

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LITERATURE CITED

Bushway, R. J.; Perkins, B.; Savage, S. A.; Lekousi, S. L.; Ferguson, B. S. Determination of atrazine residues in water and soil by enzyme immunoassay. *Bull. Environ. Contam. Toxicol.* **1980**, *40*, 647–654.

Compton, B. J.; Lewis, M. A.; Wigham, F.; Gerald, J. S.; Countryman, G. E. Analytical potential of protein A for affinity chromatography of polyclonal and monoclonal antibodies. *Anal. Chem.* **1989**, *61*, 1314–1317.

Erlanger, B. F. The preparation of antigenic hapten–carrier conjugates: A survey. *Methods Enzymol.* **1980**, *70*, 85–104.

Funk, W.; Dammann, V.; Vonderheid, C.; Oehlmann, G. (Herausgeber) *Statistische Methoden in der Wasseranalytik- Begriffe, Strategien, Anwendungen*: VCH-Verlagsgesellschaft: Weinheim, 1985.

Giersch, T.; Hock, B. Monoclonal antibodies for the determination of s-triazines with enzyme immunoassay. *Food Agric. Immunol.* **1990**, *2*, 85–97.

Goodrow, M. H.; Harrison, R. O.; Hammock, B. D. Hapten synthesis, antibody development and competitive inhibition enzyme immunoassay for s-triazine herbicides. *J. Agric. Food Chem.* **1990**, *38*, 990–996.

Huber, S. J.; Hock, B. Atrazine in water, p. 438–451. In *Methods of enzymatic analysis. Vol. XI, Drugs and pesticides*; Bergmeyer, H. V., Ed.; VCH Verlagsgesellschaft: Weinheim, 1986.

Märtlbauer, E.; Terplan, G. *Arch. Lebensmittelhyg.* **1988**, *39*, 133–156.

Pearlman, W. M.; Banks, C. K. Substituted chlorodiamino-s-triazines. *J. Am. Chem. Soc.* **1948**, *70*, 3726–3728.

Pinckard, R. N. Equilibrium dialysis and preparation of hapten conjugates. *Handbook of Experimental Immunology. Vol. 1, Immunochemistry*; Weir, D. M., Ed.; Blackwell: Oxford, U.K., 1978; pp 17.1–17.23.

Schlaeppi, J.-M.; Föry, W.; Ramsteiner, K. Hydroxyatrazine and atrazine determination in soil and water by enzyme-linked immunosorbent assay using specific monoclonal antibodies. *J. Agric. Food Chem.* **1989**, *37*, 1532–1538.

Thurston, J. T.; Dudley, J. R.; Kaiser, D. W.; Hechenbleikner, I.; Schaefer, F. C.; Holm-Hansen, D. Cyanuric chloride derivatives. I. Aminochloro-s-triazines. *J. Am. Chem. Soc.* **1951**, *73*, 2981–2983.

Warburg, O.; Christian, W. *Biochem. Z.* **1941**, *310*, 384–421.

Wittmann, C.; Hock, B. Improved enzyme immunoassay for the analysis of s-triazines in water samples. *Food Agric. Immunol.* **1989**, *1*, 211–224.

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