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Hydroxyatrazine and Atrazine Determination in Soil and Water by Enzyme-Linked Immunosorbent Assay Using Specific Monoclonal Antibodies

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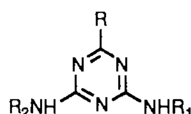
Monoclonal antibodies (MAbs) were obtained against the herbicide atrazine and its metabolite hydroxyatrazine by immunizing mice with protein conjugates of both compounds. By competitive ELISA, we observed that the anti-hydroxyatrazine MAbs cross-reacted predominantly with hydroxypropazine. The anti-atrazine MAbs cross-reacted with propazine and, to a much lower extent, with a few other *s*-triazines and hydroxy-*s*-triazines. Atrazine could be detected in water samples with a sensitivity of 0.05 ng/mL. Average recoveries from soil samples fortified with atrazine or hydroxyatrazine, measured by ELISA, were comparable to those measured by GLC or HPLC. Soil samples of unknown atrazine and hydroxyatrazine content were analyzed by GLC, HPLC, and ELISA. Interference during UV monitoring of hydroxyatrazine by HPLC analysis was observed. Despite limited specificity due to cross-reacting substances, the results demonstrate that the ELISA immunoassay represents a valuable method for detecting trace amounts of atrazine and hydroxyatrazine in the soil.

Derivatives of *s*-triazines (atrazine, propazine, simazine) have been extensively used as herbicides during the past 25 years. Atrazine undergoes degradation in the soil, an important pathway being its conversion to the nonphytotoxic hydroxyatrazine (Jordan et al., 1970). The grow-



atrazine: $R = Cl$, $R_1 = CH_2CH_3$, $R_2 = CH(CH_3)_2$
hydroxyatrazine: $R = OH$, $R_1 = CH_2CH_3$, $R_2 = CH(CH_3)_2$

ing number of soil samples to be analyzed has encouraged the development of simple and inexpensive assays, able to monitor not only the concentration of the active substances but also major metabolites remaining in the soil. The determination of hydroxyatrazine was done previously by TLC (Koudela, 1970; Cee and Gasparic, 1971) or UV spectrophotometry (Hurter, 1966; Sirons et al., 1973) and is currently done by HPLC (Ramsteiner and Hörmann, 1979). HPLC determination of hydroxyatrazine in the soil requires a cumbersome cleanup procedure. The latter could be avoided by using an immunoassay as an alternative approach to residue analysis. Such immunochemical determination based on competitive binding of residues to an antibody has been recently developed for the determination of several herbicides such as 2,4-dichlorophenoxyacetic acid (Fleeker, 1987) and chlorosulfuron (Kelley et al., 1985) or pesticides such as diflubenzuron (Wie and Hammock, 1982), metalaxyl (Newsome, 1985), and parathion (Ercegovich et al., 1981). [For a review, see Hammock and Mumma (1980).] For the determination of *s*-triazines in water, a kit is available commercially (ImmunoSystems Inc., Biddeford, ME) and several immunoassays for atrazine have been described (Dunbar et al., 1985; Huber, 1985; Bushway et al., 1988).

To develop our present immunoassay for atrazine and hydroxyatrazine, we took advantage of hybridoma technology to obtain MAbs, which allowed better definition of the specificity of the assay, as well as an unlimited supply of reagents.

EXPERIMENTAL SECTION

Materials. Atrazine and its analogues were synthesized in our laboratories. Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland) and keyhole limpet hemocyanin (KLH) from Calbiochem (Lucerne, Switzerland). Freund's adjuvant was obtained from Difco (Detroit, MI), 2,6,10,14-tetramethylpentadecane (pristan oil) from Aldrich Chemical Co. (Steinheim, FRG), poly(ethylene glycol) 4000 (PEG) from Merck (Zurich, Switzerland), and Tween 20 from Serva (Heidelberg, FRG). All other reagents were of the highest purity grade. RPMI 1640 medium from Seromed (Biochrom, Berlin, FRG) was supplemented with 15% fetal calf serum (Gibco), 0.01% gentamy-

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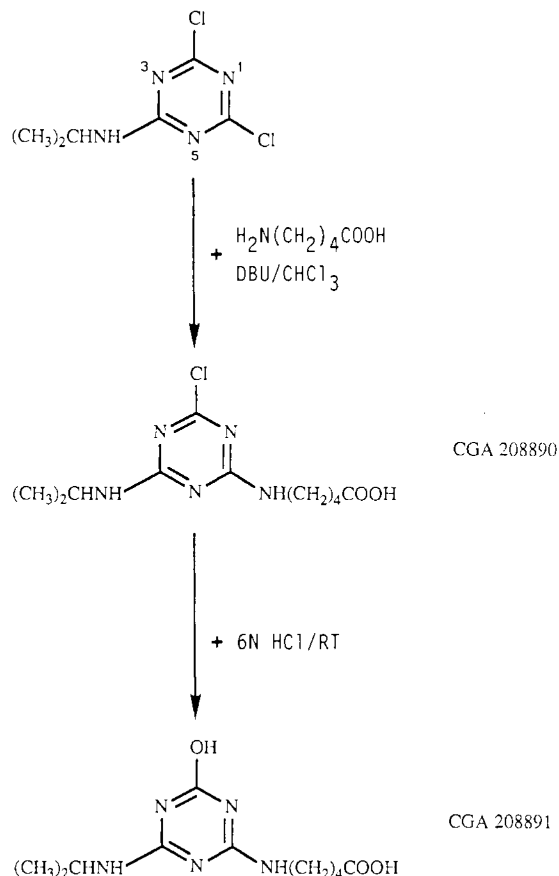

Figure 1. Synthesis of the atrazine and hydroxyatrazine derivatives used for coupling the haptens to the carrier proteins.

cin, 4 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 5 μg/mL insulin/transferin/selenium (Collaborative Research, Bedford, MA). For HAT medium, aminopterine, thymidine, and hypoxanthine (Boehringer, Mannheim, FRG) were added accordingly (Campbell, 1984). Phosphatase-labeled affinity-purified goat antibody specific to mouse IgG was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD), and the isotype and subclass determination ELISA kit was from Bio-Rad (Glattbrugg, Switzerland). We used the Titertek Multiskan ELISA reader (Flow, Herts, U.K.) linked to an IBM XT PC.

Synthesis of Atrazine and Hydroxyatrazine Conjugate. In order to couple the antigen to a carrier protein, two derivatives of atrazine and hydroxyatrazine were synthesized according to the following reactions (Figure 1): The initial substance, 2,6-dichloro-4-(isopropylamino)-*s*-triazine (10.35 g), was mixed with 5-aminovaleric acid (6.15 g) in 150 mL of chloroform, and the resultant mixture was reacted with 15.66 mL of diazobicyclo[5.4.0]undec-5-ene. After 1-h stirring under reflux, the mixture was evaporated at 22 °C. The residual oily product was redissolved at 0 °C in 60 mL of 2 N HCl. After 2 h, the precipitate was filtered, washed with water and diethyl ether, and dried. We obtained 4.3 g of the resulting 2-chloro-4-(isopropylamino)-6-[(1-carboxybut-4-yl)amino]-*s*-triazine (CGA 208890) (mp 191–192 °C). In an additional step, 1 g of 2-chloro-4-(isopropylamino)-6-[(1-carboxybut-4-yl)amino]-*s*-triazine was converted to the hydroxy analogue by adding 7 mL of 6 N HCl at 22 °C. After 4 h, the mixture was dried at 45 °C under vacuum. We obtained 0.98 g of the hydroxy analogue (CGA 208891) (mp 149–151 °C).

Both derivatives were analyzed by mass spectrometry on a Finnigan 4500 instrument with a direct-exposure probe (DEP). The mass spectrum showed intense fragments for the following mass, confirming the structures shown in Figure 1: CGA 208890, *m/e* M^+ 287 (33), 272 (31), 228 (72), 214 (61), 202 (39), 201 (100), 200 (95), 186 (58), 172 (55), 159 (63), 158 (81); CGA 208891, *m/e* M^+ 251 (28), 236 (12), 210 (11), 209 (19), 183 (11), 181 (14), 153 (54), 140 (11), 125 (22), 112 (27), 111 (28), 99 (100).

Atrazine and hydroxyatrazine derivatives were conjugated to either BSA or to KLH by the active ester method (Kulkarni et al., 1981). Briefly, the carboxyl group of the derivative solubilized in *N,N*-dimethylformamide (6 mg/200 μL) was reacted with a 4 M excess of *N*-hydroxysuccinimide (9.1 mg/200 μL) and *N,N'*-dicyclohexylcarbodiimide (16 mg/200 μL). The reaction mixture was stirred for 1 h at 22 °C and then 18 h at 4 °C. The precipitate was removed by centrifugation, and the clear supernatant containing the active ester was added to BSA or KLH (24 mg) solubilized in 5.4 mL of phosphate-buffered saline (PBS) (0.01 M sodium phosphate and 0.145 M NaCl, pH 7.0). After a 4-h incubation at 4 °C, the white precipitate was removed by centrifugation and the supernatant containing the protein conjugate was extensively dialyzed in PBS before it was used for immunization. The protein concentration was determined by the Lowry assay (Lowry et al., 1951).

The extent of coupling was assessed by UV absorbance spectrophotometry at 250 and 280 nm (for protein determination). By assuming that the absorbances at 250 nm of the free and conjugated forms of the derivative were comparable, we calculated that the molar ratio of atrazine and hydroxyatrazine to BSA varied between 9/1 and 11/1. In addition, the coupling of the haptens to BSA was evidenced by a rather diffuse band migrating slower than BSA on sodium dodecyl sulfate gel electrophoresis (data not shown).

Immunization and Fusion Protocol. Groups of five BALB/c female mice (4–6 weeks old) were given a series of three injections with KLH-conjugated atrazine or hydroxyatrazine (50 μg/injection). The first injection consisted of 0.1 mL of conjugate in PBS mixed in a 1/1 ratio with 0.1 mL of complete Freund's adjuvant; 50 μL was injected intraperitoneally and 150 μL subcutaneously. In the second (day 14) and third series of injections (day 30), incomplete Freund's adjuvant replaced the complete one. One week after the last injection, sera were collected and titers were determined by ELISA using the BSA-conjugated hapten to coat the microtiter plate. After a rest period of 2 months, the mice were boosted intraperitoneally with 500 μg of KLH conjugate in PBS (200 μL).

Three to four days later, the mice were sacrificed and the spleen cells were fused with the murine myeloma cell line Sp 2/0.Ag14 (Shulman et al., 1978), using PEG 4000, by a modification of the original Köhler and Milstein method (Galfre et al., 1977). After 2–4 weeks, wells containing growing hybridomas in HAT medium were tested for specific MAbs by ELISA; only the IgG isotype was taken into consideration, by using a phosphatase-labeled goat antibody specific to mouse IgG (γ). Positive hybridomas were cloned by limiting dilution (Goding, 1980), and clones were further selected by ELISA. Positive clones were expanded in mice pretreated with 200 μL of pristan oil. Following standard procedures (Campbell, 1984), the ascites fluid was purified by centrifugation, ammonium sulfate precipitation, and DEAE-cellulose anion-exchange chromatography in order to obtain pure MAbs (IgG) specific for atrazine or hydroxyatrazine. Finally the isotype subclass of each MAbs was determined by ELISA.

Competitive ELISA for Atrazine or Hydroxyatrazine Determination. For the two-step competitive ELISA, 96-well microtiter plates (Dynatech, Type M 129A) were coated with BSA-Conjugated hapten (200 ng/100 μL of 50 mM sodium carbonate buffer, pH 9.6) and incubated overnight at 4 °C. Unbound antigen was removed by washing five times with PBS supplemented with 0.1% (v/v) Tween 20. The remaining free sites on the solid support were blocked with PBS supplemented with 1% BSA (w/v) and incubated for 2 h at 22 °C. The plates were then washed with 0.1% PBS–Tween. In separate test tubes, 50 μL of purified MAbs (2 μg/mL) or of clone supernatants (diluted 1/5 to 1/22.5) was incubated with either 950 μL of standard solutions containing increasing amounts of *s*-triazine analogues or water samples or diluted soil extracts (all dilutions were performed in 0.1% PBS–Tween). After 1-h incubation at 22 °C, 200 μL of the antibody–antigen mixture was transferred to each well and incubated for an additional 1 h. The wells were then washed five times with 0.1% PBS–Tween, and 100 μL of goat anti-mouse antibody conjugated to alkaline phosphatase (diluted 1/1500) was added to the wells and incubated for 1.5 h. After washing, 150 μL of the substrate *p*-nitrophenyl

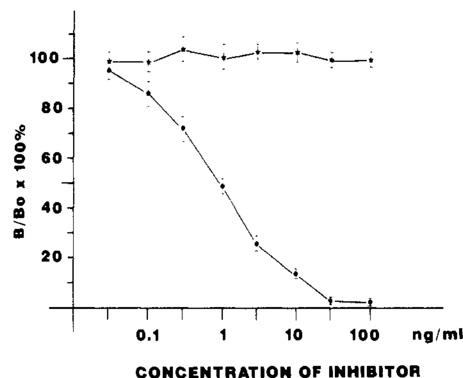

Figure 2. Inhibition of binding of the anti-hydroxyatrazine MAb 4009-85-3 by atrazine (★) and by hydroxyatrazine (●) in the competitive ELISA with BSA–hydroxyatrazine conjugate as the coating antigen. $B/B_0 \times 100\%$ represents the percentage of MAb bound to the plate. Vertical bars represent the standard deviations between assays.

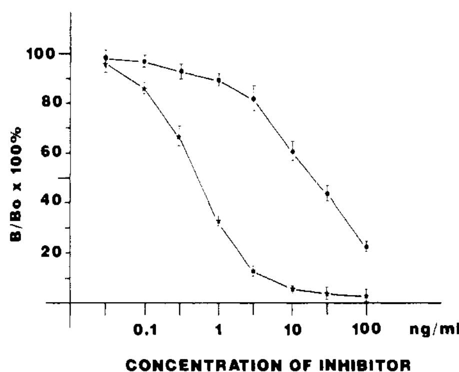

Figure 3. Inhibition of binding of the anti-atrazine MAb 4063-21-1 by atrazine (★) and by hydroxyatrazine (●) in the competitive ELISA with BSA–atrazine conjugate as the coating antigen. $B/B_0 \times 100\%$ represents the percentage of MAb bound to the plate. Vertical bars represent the standard deviations between assays.

phosphate (1 mg/mL) in diethanolamine buffer (1 mM, pH 9.8, supplemented with 0.5 mM $MgCl_2{\cdot}6H_2O$) was added to each well. After 2-h incubation at 22 °C, the change of color, which was proportional to the amount of antibody reacting with the antigen bound to the solid phase, was monitored at 405 nm. The dilutions of the MAbs were chosen to give absorbance values, without inhibitor (B_0), between 0.3 and 0.5. Controls, including zero antibody and infinite dose blanks, gave absorbance values below 0.005. All samples were done in triplicate.

Typical inhibition curves (as shown in Figures 2 and 3) were obtained by plotting $B/B_0 \times 100$ (percent bound) vs the concentration of inhibitor present (B_0 represented the absorbance measured without *s*-triazines inhibitor added to the antibody, and B, the absorbance measured with various concentrations of *s*-triazines inhibitor). I_{50} represented the concentration of atrazine or analogues reducing the ELISA signal to 50% of the control. I_{50} was calculated with use of an adaptation of the curve-fitting program ENZFITTER (R. J. Leatherbarrow, Elsevier-Biosoft) based on a four-parameter logistic curve (Raab, 1983).

Quantitative measurements of atrazine or hydroxyatrazine in soil extracts or water samples, by ELISA, were also calculated with use of the ENZFITTER program, the curve fitting being based on standards run on each microtiter plate.

Soil Samples Analysis. Standard soil samples from various locations were used for this study (only one sample per location). Aliquots (2 g) were extracted in a Soxhlet extractor for 4 h with 20 mL of methanol/water (80/20, v/v). For the competitive ELISA, soil extracts were routinely diluted 1/40 (occasionally 1/20) in 0.1% PBS–Tween, in order to avoid a possible denaturation of the MAbs due to the methanol. We observed some interferences due to the methanol when dilutions lower than 1/20 were used (data not shown). The diluted soil sample (950 μL) was then incubated with 50 μL of specific MAb (see above). All soil determinations were repeated at least three times. For HPLC determination of hydroxyatrazine, the methanol extract was cleaned up further by cation-exchange chromatography on a Dowex 50W-X4 column, followed by the adsorption on a Amberlite XAD-2 poly(styrene–divinylbenzene) resin and by gel filtration on a Bio-Gel P-2 column (Ramsteiner and Hörmann, 1979). The samples were injected in the HPLC (column Lichrospher, SI 60), and hydroxy-*s*-triazines were detected at 240 nm.

The determination of atrazine was performed by GLC, after further cleanup of the methanol extract, using a thermoionic (P–N) detector (Ramsteiner, 1985). GC–MS for atrazine determination was completed as described previously (Karlhuber et al., 1975).

Water Samples Analysis. For the competitive ELISA, 100 μL of PBS–Tween (10× concentrated) was added to 850 μL of the water samples and the resultant mixture then incubated with 50 μL of anti-atrazine MAb 4063-21-1 (see above). For HPLC analysis of atrazine, 20 mL of water samples was concentrated by an on-line HPLC system, consisting of a RP-C_{18} precolumn and an analytical RP-C_{18} column for the further separation of the *s*-triazines. The samples were detected at 230 nm (Ramsteiner, 1989).

RESULTS AND DISCUSSION

MAbs Characterization. One week after myeloma cells were fused with the spleen cells of a mouse immunized with the hydroxyatrazine conjugate, growing colonies were observed in 93 out of 96 wells (97% fusion efficiency), of which 10 secreted MAbs reacted strongly by ELISA. They were cloned by limiting dilution. Nine MAbs were of the IgG_1 subclass, and one was of the IgG_{2b}. These MAbs could be distributed into two groups, based on their cross-reactivity pattern obtained by competitive ELISA. In the first group (represented only by the MAb 4009-85-3), the cross-reactivity was restricted mainly to hydroxypropazine (Table I), whereas in the second group (represented by the MAb 4009-77-20), the cross-reactivity encompassed other hydroxy-*s*-triazines (Table I). In both groups, no cross-reactivity with active *s*-triazines (such as atrazine) was observed; i.e., the binding of the MAbs was restricted to the presence of a hydroxyl group in position R of the triazine ring. The I_{50} values, representing the concentration of hydroxyatrazine reducing the ELISA signal to 50% of the control were 0.95 and 0.5 ng/mL for MAb 4009-85-3 and MAb 4009-77-20 respectively (Table I; Figure 2). The minimum detectable amounts of hydroxyatrazine, defined as being the concentration required to produce a decrease in the percent of MAb bound equal to 2 times the standard deviation of the blank response, were 0.1 and 0.05 ng/mL, respectively. In the case of mice immunized with the atrazine conjugate, all five MAbs obtained from one fusion experiment showed a comparable pattern of cross-reactivity as represented by clone 4063-21-1 (Table I). In this case, the MAbs showed 90% cross-reactivity with propazine and cross-reacted to a lower extent with a few other *s*-triazines and hydroxylated metabolites (Table I; Figure 3). For MAb 4063-21-1, the I_{50} was 0.45 ng/mL and the limit of detection for atrazine in buffer was 0.05 ng/mL. From this cross-reactivity study two interesting findings can be underlined: (i) For the MAb 4009-85-3, the specificity was restricted not only to the substituent R of the triazine ring but also to R_2, suggesting that the antigenic determinant encompassed both substituents. Hydroxypropazine differs from hydroxyatrazine only in R_1, which is the substituent involved in the cross-linking to the carrier protein and is less important in epitope specificity (Eisen and Siskind, 1964). (ii) Replacement in position R of the hydroxyl group by the larger

Table I. Cross-Reactivity of Various s-Triazine Analogues with One Anti-Atrazine MAb (MAb 4063-21-1) and Two Anti-Hydroxyatrazine MAbs (MAb 4009-85-3 and MAb 4009-77-20)

				MAb 4063-21-1		MAb 4009-85-3		MAb 4009-77-20	
compound	R	R_1	R_2	I_{50},[a] ng/mL	% cross-react[b]	I_{50},[a] ng/mL	% cross-react[c]	I_{50},[a] ng/mL	% cross-react[c]
atrazine	Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$	0.45	100	>1000	<0.1	>1000	<0.05
simazine	Cl	$NHCH_2CH_3$	$NHCH_2CH_3$	18	2.5				
propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	0.5	90	>1000	<0.1	>1000	<0.05
terbutylazine	Cl	$NHCH_2CH_3$	$NHC(CH_3)_3$	9	5.0				
trietazine	Cl	$NHCH_2CH_3$	$N(CH_2CH_3)_2$	4	11				
desmetryne	SCH_3	$NHCH_3$	$NHCH(CH_3)_2$	90	0.5				
ametryne	SCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$	9	5.0	>1000	<0.1	>1000	<0.05
atratone	OCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$	7	6.4	>1000	<0.1	>1000	<0.05
hydroxyatrazine	OH	$NHCH_2CH_3$	$NHCH(CH_3)_2$	21	2.1	0.95	100	0.5	100
hydroxysimazine	OH	$NHCH_2CH_3$	$NHCH_2CH_3$	180	0.3	65	1.5	1.2	42
hydroxypropazine	OH	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	5.5	8.2	0.95	100	0.6	83
hydroxyterbutylazine	OH	$NHCH_2CH_3$	$NHC(CH_3)_3$	8	5.6	19	5.0	3.3	15
hydroxydesmetryne	OH	$NHCH_3$	$NHCH(CH_3)_2$	360	0.1	8	12	0.7	71
G 28279	Cl	$NHCH_2CH_3$	NH_2	>1000	<0.05	>1000	<0.1	220	0.2
G 30033	Cl	$NHCH(CH_3)_2$	NH_2	52	0.9	>1000	<0.1	250	0.2
G 28273	Cl	NH_2	NH_2	>1000	<0.05	>1000	<0.1	>1000	<0.05
G 11355	SCH_3	$NHCH_2CH_3$	NH_2	>1000	<0.05			>1000	<0.05
G 11354	SCH_3	$NHCH(CH_3)_2$	NH_2	250	0.2			>1000	<0.05
G 26831	SCH_3	NH_2	NH_2	>1000	<0.05			>1000	<0.05
G 31709	OCH_3	$NHCH_2CH_3$	NH_2	>1000	<0.05	>1000	<0.1	>1000	<0.05
G 14626	OCH_3	$NHCH(CH_3)_2$	NH_2	1000	0.05	>1000	<0.1	>1000	<0.05
G 12853	OCH_3	NH_2	NH_2	>1000	<0.05	>1000	<0.1	>1000	<0.05
GS 17792	OH	$NHCH_2CH_3$	NH_2	>1000	<0.05	>1000	<0.1	15	3.3
GS 17794	OH	$NHCH(CH_3)_2$	NH_2	250	0.2	29	3.3	4	13
ammeline	OH	NH_2	NH_2	>1000	<0.05	>1000	<0.1	30	1.7
cyanuric acid	OH	OH	OH	>1000	<0.05	>1000	<0.1	>1000	<0.05

[a] Inhibitor concentration for 50% inhibition in the competitive ELISA. [b] Cross-reactivity defined as (atrazine concentration for 50% inhibition)/(s-triazine analogue concentration for 50% inhibition) × 100. [c] Cross-reactivity defined as (hydroxyatrazine concentration for 50% inhibition)/(s-triazine analogue concentration for 50% inhibition) × 100.

Table II. Soils Composition

soil	% humus	% sand	% silt	% clay	pH
Aim Corma, Morocco			nd[a]		
Balanggandu, West-Java			nd		
Bandung, Indonesia			nd		
Collombey, Switzerland	1.4	83.9	13.6	2.5	7.4
Evenrond, South-Africa	6.4	41.9	33.6	24.4	5.8
Fukuchijama, Japan			nd		
Gisira, Soudan			nd		
heavy soil, Brazil			nd		
humus, England			nd		
Ibaragi, Japan	7.5	54.5	42.9	2.6	6.1
Kagawa, Japan	11.7	47.5	37.8	14.7	5.5
Kikukawa, Japan	2.1	21.7	51.6	26.7	5.7
Les Evouettes, Switzerland	2.6	25.7	64.0	10.3	6.2
light soil, Brazil			nd		
Lowveld, South Africa			nd		
Negba, Israel	0.8	76.7	14.6	8.0	8.3
Risilon Le Zion, Israel	1.8	80.8	3.8	13.6	7.8
Russian soil			nd		
Speyer, Germany	1.0	93.0	3.1	2.9	7.4
Stein, Switzerland	5.0	43.0	17.4	34.6	7.1
Tel Mond, Israel	1.0	89.7	1.5	1.5	6.0
Vaalhartz, South Africa			nd		
Valley, Israel	8.2	3.6	16.0	35.0	8.2
Vetroz, Switzerland	9.3	18.1	60.4	21.5	7.3

[a] Not determined.

chloride group, in the case of immunization with atrazine, significantly altered the specificity pattern of anti-atrazine MAbs, since the latter showed lower specificity for the substituent R, as evidenced by some cross-reactivity with few hydroxy-s-triazines, atratone and ametryne. An immunoassay for quantitative determination of atrazine, based on the use of MAb 4063-21-1, would not be fully specific, the ELISA values representing a compound response to all cross-reacting substances. One way to improve the specificity would be to combine the use of MAb 4063-21-1 with that of the anti-hydroxyatrazine MAb 4009-85-3, allowing a clear distinction between the response due to s-triazines and that of cross-reacting hydroxylated metabolites. However, the immunoassay would still not discriminate between atrazine and propazine, which in some circumstances could appear together, such as in groundwater.

Table III. Percentage of Added Hydroxyatrazine Detected in Fortified Soil Extracts[a]

soil	hydroxyatrazine added, ppb	hydroxyatrazine recd,[b] % (MAb 4009-85-3)
Risilon, Israel	20	91
	60	92
	200	90
	600	93
Vetroz, Switzerland	100	96
Stein, Switzerland	100	110
Collombey, Switzerland	100	107
Les Evouettes, Switzerland	100	94

[a] Fortified soil extracts (methanol extracts) were diluted 1/40 in 0.1% PBS–Tween for the ELISA determination, (average of four determinations). [b] The percentage of hydroxyatrazine recovered defined as [(ppb measured after addition − ppb measured before addition)/ppb added] × 100.

Soil Extract and Soil Fortification Studies. In the soil extract fortification experiment, we tested for possible interfering soil coextracted materials. A given amount of hydroxyatrazine was added to the methanol/water extract of five soils of known composition (see Tables II and III). Hydroxyatrazine was measured by ELISA using MAb 4009-85-3 before and after the addition of hydroxyatrazine to the soil extract. As shown in Table III, all soil types tested gave acceptable recoveries (97% mean recovery), suggesting that, in these samples, no interfering substances were coextracted.

In the second type of experiment, five soil samples, which showed no detectable residues of hydroxyatrazine

Table IV. Recovery of Hydroxy-s-triazines from Various Fortified Soils[a]

soil	OH-At added, ppm	OH-Prop added, ppm	sum of OH-At and OH-Prop recovered: HPLC: ppm (%)	ELISA (MAb 4009-85-3): ppm[b] (%)	ELISA (MAb 4009-85-3): SD (CV)[c]
Gisira, Soudan	0.5	0.5	0.46 (46)	0.31 (31)	0.04 (12.9)
Fukuchijama, Japan	0.5	0.5	0.67 (67)	0.60 (60)	0.10 (16.7)
Vaalhartz, South Africa	0.5	0.5	0.48 (48)	0.32 (32)	0.03 (9.4)
Lowveld, South Africa	0.5	0.5	0.46 (46)	0.37 (37)	0.08 (21.6)
Collombey, Switzerland	0.1		0.075 (75)	0.092 (92)	0.012 (13.0)
	0.5		0.50 (100)	nd	
	1.0		nd	1.05 (105)	0.13 (12.4)

[a] Methanol extracts from fortified soils were either diluted 1/40 in 0.1% PBS–Tween for ELISA determination or cleaned up for HPLC analysis. [b] Calculated according to standards made of hydroxyatrazine in PBS–Tween and by assuming a 100% cross-reactivity with hydroxypropazine (see Table I). [c] Key: SD, standard deviation; CV, coefficient of variation (average of four to seven determinations).

Table V. Determination of Hydroxyatrazine in Soil Extracts

group	soil	HPLC determin, ppb: OH-At	HPLC determin, ppb: OH-Prop	MAb 4009-85-3 extractn,[a] ppb: MeOH	MAb 4009-85-3 extractn,[a] ppb: HPLC fractn	MAb 4009-77-20 extractn, ppb: MeOH	MAb 4009-77-20 extractn, ppb: HPLC fractn
A	light soil, Brazil	4100	700	5355	nd[c]	6814	nd
B	Ibaragi, Japan	<50	<50	<50	nd	<50	nd
	Kagawa, Japan	<50	<50	<50	nd	<50	nd
	Russian soil	<50	<50	<50	nd	<50	nd
	Collombey, Switzerland	<50	<50	54	nd	<50	nd
	Tel Mond, Israel	<50	<50	60	nd	149	nd
	Aim Corma, Morocco	<50	50	<50	nd	<50	nd
C	humus, England	250	<50	<50	nd	<50	nd
	Kikukawa, Japan	<50	100	<50	nd	<50	nd
	Evenrond, South Africa	<50	110	<50	nd	<50	nd
	Bandung, Indonesia	120	<50	<50	nd	<50	nd
	Balanggandu, West Java	80	80	<50	nd	<50	nd
	Risilon Le Zion, Israel	<50	90	<50	nd	<50	nd
	heavy soil, Brazil	130	50	53	nd	111	nd
	Stein, Switzerland	<50	340	51	nd	69	nd
	Valley, Israel	215	<50	<50	nd	111	nd
	Vetroz, Switzerland	1070	<50	110	66	135	110
	Les Evouettes, Switzerland	2460	<50	<50	<50	<50	<50
	Speyer, Germany	1060	<50	<50	<50	<50	<50

[a] The methanol extract and in few cases the HPLC fraction were used for ELISA determination (see Materials and Methods). [b] Calculated according to standards made of hydroxyatrazine in PBS–Tween (average of three to seven determinations). [c] Not determined.

or hydroxypropazine by the HPLC analytical procedure (limit of detection of 50 ppb), were fortified with both metabolites in equal concentrations. After the methanol extraction, they were analyzed by ELISA and HPLC. As shown in Table IV, recoveries determined by ELISA (60% mean recovery) were comparable to those determined by HPLC (64% mean recovery); the higher recovery measured by HPLC was not significant ($p < 0.01$). The intra- and interassay coefficients of variation obtained by ELISA were 6.3% (23 assays) and 14% (29 assays), respectively (all assays were done in triplicate). The overall recovery showed variations ranging from 46 to 100%. These variations could be attributed to soil adsorption of the hydroxytriazines. For the soil of Collombey, a sandy type of soil, the recovery of hydroxyatrazine measured by ELISA was independent of the fortification procedure (before or after extraction with methanol) (see Tables III and IV), whereas a possible interference due to the high organic material content in the other soil could explain the greater variation of the recoveries observed both by HPLC and ELISA.

The fortification experiment was repeated with atrazine. One soil sample (Stein) was fortified with 100 ppb atrazine and then extracted and analyzed by GLC, GC–MS, and ELISA using MAb 4063-21-1. The recovery measured by all three methods was almost complete (>90%) (see below).

Determination of Hydroxyatrazine in Soil. Nineteen soil samples of our collection were analyzed for their hydroxyatrazine content by either HPLC or ELISA. As

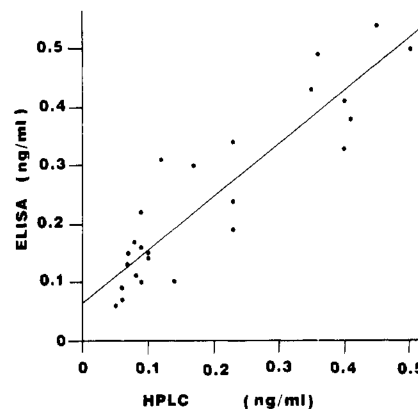

Figure 4. Comparison of atrazine level in water samples measured by HPLC and by ELISA with MAb 4063-21-1: $y = 0.06 + 0.91x$; $r = 0.91$, $n = 25$.

shown in Table V, the soil samples could be distributed into three groups. In groups A and B, a good correlation between HPLC and ELISA was obtained. The unique soil sample of group A contained a high amount of hydroxyatrazine and hydroxypropazine as determined by both methods, whereas the six samples of group B were devoid of these metabolites. However, for the 12 HPLC-positive soil samples of group C, the ELISA values were close to the background level. It should be pointed out that, due to the high dilution of the soil extract necessary for the ELISA analysis (see Materials and Meth-

Table VI. Determination of Atrazine in Soil Extracts

group	soil	GLC, ppb			GC–MS, ppb: At	ELISA (MAb 4063-21-1)[a] ppb (SD)[c]
		At	Prop	Simaz		
A	Negoba, Israel	<50	<50	<50	nd[d]	<40
	Kikukawa, Japan	<50	<50	<50	nd	<40
	Russian soil	<50	<50	<50	nd	<40
	Risilon Le Zion, Israel	<50	<50	<50	nd	<40
	Aim Corma, Morocco	<50	<50	<50	nd	<40
	Evouettes, Switzerland	<50	<50	<50	nd	50 (13)
	Speyer, Germany	<50	<50	<50	nd	56 (8)
	Stein Switzerland	<50	<50	<50	nd	<40
B	Evenrond, South Africa	100	<50	<50	<30	<40
	Bandung, Indonesia	150	<50	<50	<30	<40
	Collombey, Switzerland	170	<50	<50	<30	<40
	Vetroz, Switzerland	70	<50	<50	<30	<40
	Balanggandu, West Java	<50	150	<50	nd	<40
C	heavy soil, Brazil	<50	<50	<50	nd	171 (35)
	Ibaragi, Japan	<50	<50	<50	nd	72 (19)
	Valley, Israel	50	<50	<50	nd	64 (5)
D	light soil, Brazil	1200	<50	150	<30	2482 (296)
	Tel Mond, Israel	150	<50	800	nd	189 (40)
	Kagawa, Japan	<50	180	<50	nd	64 (3)
	recovery 100 ppb[b]	93			97	94 (11)

[a] Calculated according to standards made of atrazine in PBS–Tween. [b] The soil from Stein was fortified with 100 ppb atrazine. [c] Standard deviation (average of six determinations). [d] Not determined.

ods), values around 50 ppb were close to the detection limit of the assay. No significant differences were observed by using the MAb 4009-77-20. To test whether some interferences may have biased the monitoring at 240 nm during HPLC, three soils (Vetroz, les Evouettes, Speyer) were extracted with methanol, cleaned up, and run by HPLC as described in Materials and Methods. The putative hydroxyatrazine peak monitored at 240 nm was collected, concentrated, and neutralized before being analyzed by ELISA for its hydroxyatrazine content. As shown in Table V, the HPLC fraction obtained from the Vetroz soil contained only a small amount of hydroxyatrazine, representing less than 10% of the values calculated on the basis of the absorbance at 240 nm, whereas, for the two other soils, no hydroxyatrazine was measured by ELISA in these HPLC fractions. Moreover, the hydroxyatrazine content measured by ELISA in the HPLC fraction was consistent with the one measured in an aliquot of the methanol extract taken before cleanup. The discrepancies between HPLC and ELISA were very likely due to some interferences biasing the monitoring at 240 nm.

Determination of Atrazine in Water. A total of 28 water samples were analyzed by HPLC (after a concentration step) (Ramsteiner, 1989) and by competitive ELISA using MAb 4063-21-1 (without concentration of the water samples). Atrazine could be detected by both methods down to 0.05 ppb. For the 25 samples containing atrazine ($\geq$0.05 ppb), the correlation between ELISA and HPLC measurements was calculated by regression analysis (Figure 4). A good correlation between methods was obtained ($r = 0.91$, $p < 0.0005$); the slight excess of atrazine measured by ELISA was not significant ($p < 0.005$).

Water samples were also analyzed for hydroxyatrazine by HPLC and by ELISA using the MAb 4009-85-3. No traces of this metabolite were detected by either method (data not shown).

Determination of Atrazine in Soil. The soil samples analyzed for hydroxyatrazine (see above) were also analyzed by GLC and by ELISA for their *s*-triazines content, one soil devoid of atrazine (Stein) being also used for atrazine fortification experiments (Table VI). Of 19 soil samples, eight were essentially devoid of *s*-triazines based on either ELISA or GLC determination (group A), whereas five other samples (group B) contained *s*-triazines based on the GLC determination but scored negative by ELISA. Four of these soils were further analyzed by GC–MS for confirmatory results. It appeared that no atrazine was detected by this method, suggesting that some interferences biased the detection of atrazine by GLC. On the other hand, three soil samples (Group C) were negative by GLC but slightly positive by ELISA. The presence of cross-reacting *s*-triazines analogues may very likely explain these discrepancies. Finally, three samples (Group D) contained some atrazine as determined by either ELISA or GLC. However, in the case of the light soil of Brazil, which contained a high amount of hydroxyatrazine (Table V), the presence of atrazine was not confirmed by GC–MS, suggesting the presence of interfering coextracted materials not yet identified.

CONCLUSION

We used the hybridoma technology to obtain stabilized cell lines secreting MAbs specific for atrazine, a widely used herbicide, and for its metabolite, hydroxyatrazine. A good correlation was observed between the current detection method (HPLC or GLC) and a MAbs based immunoassay (ELISA) when either fortified soils or water samples were analyzed. The limit of detection for atrazine and hydroxyatrazine in soils, was comparable by both methods (around 50 ppb); however, replacing organic by aqueous solvents during the soil extraction would avoid the necessity to highly dilute the sample and would therefore reduce the limit of detection by ELISA down to the ppb level. An additional advantage of the ELISA was that no cleanup steps were necessary for measurement of both compounds. The analysis of undefined soil samples with respect to their herbicide content showed that, for atrazine and hydroxyatrazine, some discrepancies were observed between the two methods due to biased detection during HPLC and GLC measurements. For atrazine this was confirmed by additional GC–MS analysis. No specific confirmatory test for hydroxytriazines in trace analysis currently exists. GC–MS cannot be performed due to the low volatility of the compound, and LC–MS is not yet available on a routine basis. Therefore, the

ELISA immunoassay represents a valuable detection method for trace amounts of atrazine and hydroxyatrazine in soil, despite its limited specificity due to cross-reacting substances.

ABBREVIATIONS USED

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GLC, gas–liquid chromatography; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; KLH, keyhole limpet hemocyanin; MAb, monoclonal antibody; PBS, phosphate-buffered saline.

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Registry No. Atrazine, 1912-24-9; hydroxyatrazine, 2163-68-0; 2,6-dichloro-4-(isopropylamino)-*s*-triazine, 3703-10-4; diazobicyclo[5.4.0]undec-5-ene, 6674-22-2; water, 7732-18-5; 2-chloro-4-(isopropylamino)-6-[(1-carboxy-4-butyl)amino]-*s*-triaazine, 123100-37-8; 2-hydroxy-4-(isopropylamino)-6-[(1-carboxy-4-butyl)amino]-*s*-triazine, 123100-38-9.

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