Application and Performance Characteristics of a Novel ELISA for the Quantitative Analysis of the Atrazine Metabolite Deethylatrazine

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A highly sensitive enzyme-linked immunosorbent assay (ELISA) was applied to the analysis of the atrazine metabolite deethylatrazine in natural water samples. The ELISA, with a detection range between approximately 10 ng/L and 10 μg/L, was performed and validated in microtiter plates; 120 environmental water samples were analyzed with the ELISA. A close correspondence was found between the results of the ELISA and those from GC or HPLC measurements. The correlation coefficient between ELISA and HPLC, based upon 70 water samples, was 0.98 and between ELISA and GC, based upon 50 water samples, 0.99.

INTRODUCTION

The increasing interest in the application of enzyme-linked immunosorbent assays (ELISA) for the detection of pesticides in water is due to their sensitivity, ease of use, and inexpensiveness. Such qualities make them an ideal screening procedure. Of special interest are the *s*-triazines because of their persistence and their widespread occurrence as being revealed by regular surveys of natural water and drinking water by various research laboratories in many countries (Funari et al., 1989; Grandet et al., 1989; Buser, 1990; Frank et al., 1990a–c; Ritter, 1990; Pick et al., 1992).

Little attention has been paid, as yet, to the use of serological techniques to detect the triazine metabolites. To our knowledge, ELISAs are presently available only for the atrazine derivatives hydroxyatrazine (Schlaeppi et al., 1989) and deethylatrazine together with deisopropylatrazine (Wittmann and Hock, 1991). The latter assay has a measuring range between about 10 ng/L and 10 μg/L for the sum of the two metabolites. As deisopropylatrazine residues have not yet been detected in natural water samples, this ELISA could be used as a rapid screening method for deethylatrazine.

The aim of the present paper is the validation of this ELISA as a sensitive test for monitoring deethylatrazine residues in natural water samples. For this purpose we analyzed 120 environmental water samples of different origins and compositions obtained from the northwestern part of Germany and the area around Munich with the ELISA and compared the results with GC or HPLC data. The study was directed toward the verification of the ELISA with the conventional methods in residue analysis (GC and HPLC) rather than toward a monitoring program for atrazine and deethylatrazine in Germany. It is shown that there is no need for any cleanup and that the ELISA indeed provides fairly accurate estimations of deethylatrazine concentrations.

MATERIALS AND METHODS

Apparatus. The laboratory equipment consisted of an ELISA photometer [SLT Easy Reader EAR 400 (SLT, Gröding/Salzburg, Austria)], a microtiter plate washer [SLT Easy Washer EAW 8112 (SLT, Austria)], and an ultrasonic bath (Sonorex, Bandeling). Microtiter plates were provided by Greiner Labortechnik (72636 Frickenhausen, Germany).

Reagents. *1. Chemicals.* 2-Aminohexanoic acid 4-amino-6-chloro-1,3,5-triazine was provided by Riedel de Haen (Seelze, Germany) as the hapten for the synthesis of the immunoconjugate and the enzyme tracer. The triazine standards were obtained from Riedel de Haen and Ciba Geigy (Basel). In addition, the following reagents were used: bovine serum albumin (BSA), lyophilized, pure (Serva, Heidelberg); horseradish peroxidase [POD, 1350 units/mg (Serva)]; tetramethylbenzidine (Riedel de Haen); hydrogen peroxide, 30% (Merck, Darmstadt); and ethanol absolute, p.a. (Merck). All other chemicals used were of analytical grade.

2. Buffers and Solutions: (1) carbonate buffer, 50 mmol/L, pH 9.6, for coating; (2) phosphate-buffered saline (PBS), 40 mmol/L, pH 7.2 (containing 8.5 g of NaCl/L) for the preparation of standards and dilution of the peroxidase tracer; (3) PBS washing buffer, 4 mmol/L, pH 7.2 (containing 0.85 g of NaCl/L and 0.5 mL of Tween 20/L) for washing the microtiter plates; (4) substrate buffer for peroxidase (0.1 mol of sodium acetate buffer/L; the pH was adjusted to 5.5 by adding 1 mol of citric acid/L); (5) substrate for peroxidase [400 μL of tetramethylbenzidine (TMB); 6 mg of TMB dissolved in 1 mL of dimethylsulfoxide] plus 100 μL of 1% (v/v) H_2O_2 made up to 25 mL with substrate buffer; (6) stopping reagent H_2SO_4, 2 mol/L.

s-Triazine Standards. Five milligrams of *s*-triazine, e.g., 5 mg of deethylatrazine, was dissolved in 50 mL of absolute ethanol with the aid of an ultrasonic bath (20 min). The deethylatrazine content of this ethanolic solution was checked by a GC analysis yielding a deethylatrazine concentration of 4.998 mg/50 mL of ethanol. Starting with this solution, a stock solution was prepared consisting of 1 mg/L *s*-triazine (= excess). A standard series was prepared by repeated dilutions of the stock solution yielding the following *s*-triazine concentrations: 0.01, 0.03, 0.1, 0.3, 1.0, and 10 μg/L. The stock solution and the standard series were made up either in 40 mmol/L PBS buffer, pH 7.2, or in distilled water.

3. Water Samples. (*1*) Fifty natural water samples were provided by the group of Dr. L. Weil (Institute of Water Chemistry and Chemical Balneology, TU München), who also carried out GC analyses for atrazine and deethylatrazine. For a better survey of the results, only 7 samples, reflecting the worst case situation in the Munich area with respect to atrazine and deethylatrazine contaminations, are shown in Table I.

For GC analysis, water samples were enriched by solid-phase extraction (on Bakerbond Octadecyl C_{18}, 40 μm, 600 Å, 7025-00 Baker), eluted with acetone and dichloromethane, and determined via GC (Grandet et al., 1988). The GC conditions were as follows: column, 30-m quartz capillary column covered with DB-5; detector, nitrogen–phosphorus selective detector (Hewlett-Packard); internal standard, desmetryn.

(*2*) In addition, 70 environmental water samples of different origins and compositions were obtained by Dr. C. Schlett (Central Laboratory of Gelsenwasser AG, Gelsenkirchen), who also

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0021-8561/93/1441-1795$04.00/0

Table I. Analysis of Natural Water Samples by ELISA and GC[a]

sample	origin	deethylatrazine concn: determined with the ELISA, μg/L ± SD[b]	deethylatrazine concn: determined with GC,[c] μg/L	atrazine concn: determined with the ELISA, μg/L ± SD	atrazine concn: determined with GC, μg/L
1	river (surface area)	0.03 ± 0.001	0.02	0.03 ± 0.002	0.02
2	ground water	0.02 ± 0.002	0.03	0.08 ± 0.003	0.06
3	ground water	0.09 ± 0.003	0.10	0.07 ± 0.004	0.05
4	ground water	0.05 ± 0.004	0.07	0.03 ± 0.001	0.04
5	well (ground water)	0.09 ± 0.002	0.10	0.12 ± 0.005	0.12
6	well (ground water)	0.24 ± 0.009	0.28	0.27 ± 0.009	0.24
7	well (ground water)	0.04 ± 0.001	0.06	0.05 ± 0.002	0.04

[a] The environmental water samples were obtained by Dr. L. Weil (Institute of Water Chemistry and Chemical Balneology, TU München). The atrazine and deethylatrazine concentrations were measured with the two ELISAs in quadruplicate and independently by Dr. Weil with GC. [b] SD, standard deviation. [c] The detection limit for GC analysis of atrazine was 0.01 μg/L and ca. 0.02 μg/L for deethylatrazine. The recovery rate for atrazine amounted to 99% and for deethylatrazine to 67% with coefficients of variation of 10% for atrazine and 20.7% for deethylatrazine. The GC data were projected to 100% (personal communication by Dr. Thomas Ruppert).

Table II. Analysis of Natural Water Samples by ELISA and HPLC[a]

sample	deethylatrazine concn: determined with the ELISA, ng/L ± SD	deethylatrazine concn: determined with HPLC,[b] ng/L	atrazine concn: determined with the ELISA, ng/L ± SD	atrazine concn: determined with HPLC, ng/L
1	272 ± 10	210	84 ± 2	120
2	31 ± 2	<25	17 ± 1	<25
3	37 ± 1	<25	19 ± 1	42
4	<10	<25	16 ± 1	27
5	284 ± 11	240	88 ± 2	130
6	124 ± 9	100	50 ± 1	57
7	99 ± 5	110	45 ± 1	52
8	126 ± 7	180	48 ± 1	75
9	<10	<25	17 ± 1	<25
10	266 ± 8	240	725 ± 30	730
11	100 ± 4	80	190 ± 8	nd[d]
12	130 ± 3	110	160 ± 5	nd
13	150 ± 4	130	630 ± 20	nd
14	70 ± 2	40	400 ± 15	nd
15	90 ± 2	60	230 ± 11	nd
16	20 ± 1	<25	40 ± 2	nd
17	50 ± 1	40	260 ± 5	nd
18	20 ± 1	100	80 ± 1	nd
19	110 ± 4	90	510 ± 20	nd
20	50 ± 3	50	150 ± 4	nd
21	40 ± 2	40	150 ± 3	nd
22	100 ± 5	<90[c]	160 ± 5	nd

[a] The samples were provided by Dr. C. Schlett, Central Laboratory of Gelsenwasser AG, Gelsenkirchen. Samples 1–10 and 16 contained drinking water and samples 11–15 and 17–22 surface water. The atrazine and deethylatrazine concentrations were measured with the two ELISAs and independently by Dr. Schlett with HPLC. [b] The detection limits for atrazine and deethylatrazine when analyzed by HPLC were about 25 ng/L. [c] The estimate <90 ng/L is due to a double peak at the position of deethylatrazine. However, a deethylatrazine concentration of 95 ng/L was independently determined by GC. [d] nd, not determined.

provided HPLC data for the atrazine and deethylatrazine concentrations. Twenty-two of these samples are presented in Table II, reflecting the worst case situation in the area of Gelsenkirchen, situated in the northwest of Germany.

For HPLC analysis, the water samples were extracted and enriched by solid-phase extraction on C_{18} modified silica gel. The extracts were then analyzed by HPLC (Schlett, 1991). The HPLC conditions were as follows: column, Hypersil ODS, 250 × 4.6 mm, 3 μm; column temperature, 40 °C; flow rate, 0.80 mL/min; injection volume, 50 μL; mobile phase, (A) 0.002 mol/L sodium acetate, (B) acetonitrile; gradient

time, min	% A	% B
start	95	5
2	95	5
70	55	45
73	55	45
82	10	90

detector, diode array detector.

(3) Dr. U. Raeder (Limnological Station of the TU München at Iffeldorf) collected a total of 25 samples from the Osterseen, 22 mi south of München: 16 samples were taken from lakes 1–16 on March 4, 1990, and another 7 samples from lakes 2, 9–11, 13, and 14 on July 4, 1990, from a depth of 3 m. To verify the ELISA results, four representative samples from lakes 10 and 13–15 were measured with GC by the group of Dr. L. Weil (Institute of Water Chemistry and Chemical Balneology, TU München). All ELISAs were carried out before GC and HPLC analyses.

Procedure. Details of antibody production and enzyme tracer synthesis for the development of a deethylatrazine enzyme immunoassay have been described previously (Wittmann and Hock, 1991). Briefly, the hapten was coupled to BSA via a carbodiimide reaction yielding a hapten density of 32 per molecule of BSA as determined by UV spectra. It was used for the immunization of rabbits over several months by multiple subcutaneous and intradermal injections of 1 mg of conjugate dissolved in saline and mixed with Freund's complete adjuvant 1:1 (v/v). Blood was collected every 4 weeks. The serum was prepared, sterile-filtered, and freeze-dried as described elsewhere (Huber and Hock, 1986).

The enzyme tracer was synthesized by covalently linking the hapten to horseradish peroxidase with a carbodiimide/*N*-hydroxysuccinimide procedure. A hapten density of 5 molecules of hapten per molecule of peroxidase was determined from the UV spectra. The enzyme tracer was stored after sterile filtration in solution at 4 °C and could be kept for at least 12 months.

If the pH of the water sample was lower than pH 4.0 or exceeded pH 9.0, the environmental water samples were adjusted to a pH between 7.0 and 7.5 (usually with 1 volume of PBS buffer, pH 7.2, plus 9 volumes of sample) before analysis with the ELISA. If the deethylatrazine concentration of a sample exceeded 10 μg/L, dilutions of the sample were carried out until the deethylatrazine concentration was in the range between 0.01 and 10 μg/L.

The ELISA and the evaluation of the cross-reactivities were performed on microtiter plates as described previously (Wittmann and Hock, 1991). The cross-reactivities of the deethylatrazine EIA were 100% for deethylatrazine and 70% for deisopropylatrazine. All other *s*-triazines exhibited cross-reactivites of less than 0.2%.

The cross-reactivities of the atrazine ELISA were 195% for propazine (not being applied in Germany), 100% for atrazine, 20% for simazine, 4% for deisopropylatrazine, 3% for terbutylazine, 1.6% for deethylatrazine, 1.2% for hydroxyatrazine, 0.6% for ametryn, 0.4% for prometryn, and less than 0.1% for all other *s*-triazines.

The assays were performed in quadruplicate. A deethylatrazine standard series was run on each microtiter plate. In addition, atrazine was assayed by a specific ELISA as described by Wittmann and Hock (1989, 1990).

RESULTS

We recently described a new ELISA that detects the sum parameter of the two atrazine metabolites deethylatrazine and deisopropylatrazine (Wittmann and Hock, 1991). Its qualification for routine screening depends upon validation by classic GC and HPLC analyses. For this purpose, environmental water samples were independently assayed by ELISA and GC or HPLC. In addition, we addressed the question whether a relationship could be found between the concentrations of atrazine and its main metabolites. For this purpose, our atrazine ELISA (Wittmann and Hock, 1989, 1990) was also included.

Table I compares the results of ELISA and GC measurements obtained from natural water samples of different origins and compositions within southern Germany. The ELISA data closely match the GC results. The atrazine and deethylatrazine concentrations of ground water sample 6 clearly exceed the upper limit of 0.1 μg/L set by the drinking water ordinance of the Federal Republic of Germany. Sample 5 contained both atrazine and deethylatrazine in concentrations in the range of the limiting value. According to the GC analyses, deethylatrazine was the only atrazine metabolite detected in the analyzed environmental water samples. Although the atrazine and deethylatrazine concentrations were found in comparable ranges, no consistency of the ratios could be detected.

Table II compares further natural water samples from the whole of 70 samples which were collected in northwestern Germany and contained drinking water and surface water. Again, the deethylatrazine and atrazine concentrations were analyzed by two separate ELISAs. HPLC data for atrazine were only available for samples 1–10. With the exception of sample 18, a close agreement between the results of HPLC and ELISA measurements was found.

To detect possible matrix effects, sample 18 was spiked in several tests with defined amounts of deethylatrazine and subsequently analyzed with the ELISA. Furthermore, several ELISA analyses were performed with defined dilutions of this sample. A recovery of 100% was determined, and just as with other water samples treated the same way, no matrix effects were observed. A GC analysis of sample 18 yielded a deethylatrazine concentration of 35 ng/L and confirmed the ELISA result.

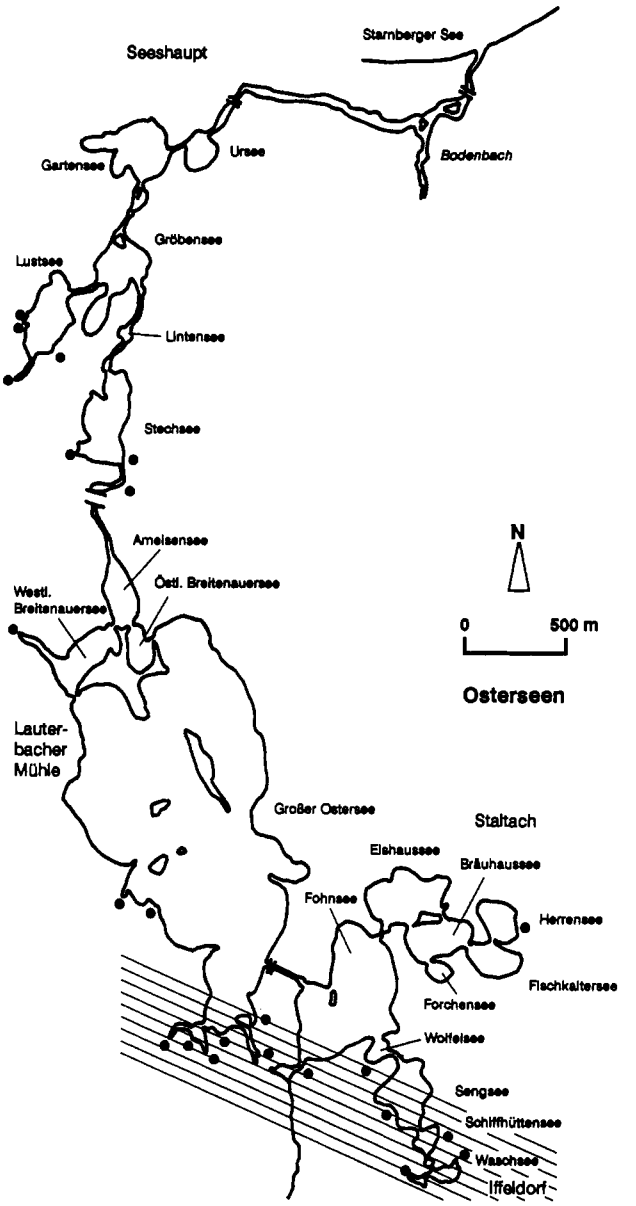

Figure 1. General map from the area of the Osterseen, comprising 16 lakes situated south to München [taken from Raeder (1990)]. The water flow direction is from the south to the north. The shaded (\\\ ) area is the subglacial solid outcrop, composed of rock of the arched limb of the unfolded molasse, and the solid dots (●) represent funnel-shaped sources of ground water.

It can be assumed that there was an overestimation with HPLC measurement being due to matrix effects caused by interferences with a sample compound. Again, no other atrazine metabolites except deethylatrazine were detected with HPLC in the water samples.

In another set of experiments, water samples from the Osterseen in southern Bavaria were assayed for atrazine and deethylatrazine contaminations on two different dates. Figure 1 gives a geographic survey of the Osterseen area [taken from Raeder (1990)].

Table III shows the atrazine and deethylatrazine concentrations. Most samples were obtained at the spring date and some 3 months later. For verification, four samples were measured with GC (Dr. L. Weil), again showing a close correlation between the ELISA and GC results. In general, an increase of the atrazine concentration is observed from spring to summer, which can be explained by the application of atrazine during the early

Table III. Atrazine and Deethylatrazine Concentrations Found in the Osterseen[a]

lake no.	lake name	atrazine concn, ng/L ± SD[b]		deethylatrazine concn, ng/L ± SD	
		April 3, 1990	July 4, 1990	April 3, 1990	July 4, 1990
1	Gartensee	17 ± 0.7	nd[c]	35 ± 12	nd
2	Lustsee	<dl[d]	<dl	<dl	<dl
3	Gröbensee	18 ± 0.5	nd	10 ± 0.1	nd
4	Stechsee	23 ± 0.3	nd	<dl	nd
5	Östlicher Breitenauersee	22 ± 0.3	nd	<dl	nd
6	Ameisensee	22 ± 0.6	nd	42 ± 0.7	nd
7	Westlicher Breitenauersee	18 ± 0.5	nd	16 ± 0.8	nd
8	Ostersee	24 ± 0.3	nd	21 ± 0.9	nd
9	Waschsee	<dl	20 ± 0.5	34 ± 1.1	<dl
10	Schiffhüttensee	12 ± 0.1	40 ± 1.0	59 ± 1.5	10 ± 0.6
		10[e]		*70*	
11	Sengsee	22 ± 1.0	60 ± 1.1	29 ± 1.2	50 ± 0.9
12	Fohnsee	26 ± 0.8	nd	92 ± 3.6	nd
13	Eishaussee	46 ± 1.6	90 ± 2.8	143 ± 4.0	40 ± 1.0
		30		*120*	
14	Bräuhaussee	54 ± 2.0	90 ± 2.6	72 ± 2.8	170 ± 2.0
		40		*80*	
15	Herrensee	92 ± 4.2	140 ± 1.9	220 ± 8.1	210 ± 1.9
		80		*240*	
15a	creek flowing into the Herrensee	nd	230 ± 3.2	nd	380 ± 3.6
16	Fischkaltersee	178 ± 9.1	nd	86 ± 3.0	nd
16a	creek flowing into the Fischkaltersee	nd	470 ± 4.0	nd	150 ± 1.8

[a] The water samples were taken by Dr. U. Raeder (Limnological Station of the TU München at Iffeldorf) from a depth of 3 m. Each sample was determined in quadruplicate with the atrazine and the deethylatrazine ELISA. [b] SD, standard deviation. [c] nd, not determined. [d] <dl, below detection limit (=1 ng/L for the atrazine ELISA and 10 ng/L for the deethylatrazine ELISA). [e] Figures in italic type represent GC analyses carried out by Dr. L. Weil (Institute of Water Chemistry and Chemical Balneology, TU München).

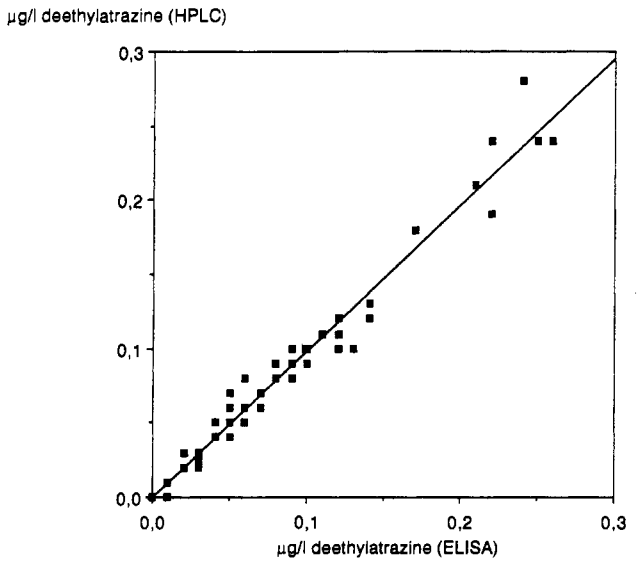

Figure 2. Data used to determine the correlation coefficient for the deethylatrazine assay. Seventy natural water samples were analyzed in independent laboratories with the ELISA and with HPLC.

summer. The highest concentrations of atrazine and deethylatrazine were found in the lakes located at the southeast, with a peak at the Herrensee. Since relatively high loads of atrazine are carried into the Herrensee and the Fischkaltersee by creeks, it seems likely that the pollution of the lakes is due to those creeks. As seen before, no regularities of the atrazine/deethylatrazine ratios were observed.

The availability of a relatively large number of comparisons between ELISAs and HPLC (a total 70 samples were analyzed) with respect to deethylatrazine determinations allowed the estimation of the correlation coefficient. Figure 2 shows the data. The deethylatrazine contamination of the samples was usually found to be in the range 0.01–0.15 µg/L. The coefficient of correlation was 0.98. For the calculation the values below the detection limit (HPLC < 25 ng/L) were taken as 0 ng/L. Furthermore, a correlation coefficient between ELISA and GC of 0.99 was calculated on the basis of ELISA and GC analyses of 50 environmental water samples (data not shown).

DISCUSSION

Herbicides such as the *s*-triazines have long been recognized as an ecologically relevant class of substances because of their biocidal activity, their persistence, and their intensive use in modern agriculture. A considerable percentage of the active substance does not reach its destination in the plant but is distributed in the ecosphere, where it is either metabolized or more or less reversibly bound to soil material, from where it can be made available again (Kreuz et al., 1990). Health protection in combination with the requirements of the drinking water ordinance of the Federal Republic of Germany makes an effective screening procedure necessary.

The present paper deals with the validation of a sensitive ELISA which had recently been introduced for the determination of deethylatrazine (Wittmann and Hock, 1991). Our main goal was to show that environmental water samples of various compositions (as, for example, ground and surface water) and from different origins (as, for example, well water or river water) can be precisely analyzed with the ELISA to gain knowledge about the deethylatrazine contamination of the sample. However, the collected data do not allow for a survey on atrazine and deethylatrazine contaminations in Germany or for a mapping of the German drinking water quality with respect to atrazine and deethylatrazine pollution.

A measuring range between approximately 10 ng/L and 10 µg/L was covered. As shown by the results of the analyzed environmental water samples, this range proved to be very advantageous because the samples often contained rather low amounts of atrazine and deethylatrazine which could not be detected by either GC or HPLC. However, samples were occasionally found that exceeded the upper limit of 0.1 µg/L single pesticide substance, as established in the European Community (EC) guidelines and the drinking water ordinance of the Federal Republic of Germany. A further interesting aspect is that no cleanup

procedure was required for water samples because no matrix effects were observed. The assay turned out to be very robust and free from interferences.

Acceptable variation coefficients were found for the ELISA. It amounted to only 2% in the range between 0.1 and 10 μg/L and to 10% below 0.1 μg/L. With GC a coefficient of variation of 10% for a concentration of 0.1 μg of deethylatrazine/L was determined (Thomas Ruppert, personal communication). The test also proved to be very accurate. It compared favorably to GC or HPLC measurements. The coefficient of correlation between ELISA and HPLC measurements was 0.98 and between ELISA and GC measurements 0.99. Therefore, the ELISA described in this paper allows a very precise and sensitive determination of deethylatrazine. It is suitable for routine analysis of environmental water samples and can serve especially as a screening tool in the range of the limit of the drinking water ordinance of the Federal Republic of Germany and the EC guidelines.

The observed data do not reveal a consistent relationship between atrazine and deethylatrazine concentrations. No constant ratio was observed, even with places as close to each other as some of the lakes shown in Table III.

The possibility has to be considered that, in addition to atrazine pollution and subsequent metabolization, independent contamination with deethylatrazine may occur depending upon changes of water movements, microbial activities, or weather conditions. Many factors affect how long it takes for the atrazine applied by farmers to their fields to reach the ground water via the soil or other ways.

In any case, the availability of fast screening methods such as the ELISA offers us the possibility of establishing local gradients of pesticide concentrations which may reveal the sources of contamination, as is indicated in Table III for the creeks flowing into lakes 15 and 16. Here the water flows from south to north through the Osterseen. Comparison of the atrazine pollution of the lakes situated in the south (e.g., Waschsee, Schiffhüttensee, and Sengsee) with that of the lakes in the north showed no dilution or degradation of atrazine. The main atrazine pollution may be derived from the lakes in the southeastern part, especially from the Herrensee and the Fischkaltersee, where the two creeks flowing into the lakes appear to be the main source of atrazine contamination. However, a final conclusion on the source of atrazine pollution would require a long-term study of at least 1 or 2 years.

In general, the consistent and critical use of ELISAs may contribute to the goal of keep drinking water, one of the most important of our basic foodstuffs, free of pesticides and other toxic substances.

ACKNOWLEDGMENT

The work was supported by the Deutsche Forschungsgemeinschaft (Grant Ho 383/26-1). We thank Dr. U. Doht (Riedel de Haen) for the hapten synthesis and Ciba Geigy for donating several *s*-triazine samples. We are grateful to Dr. U. Raeder (Limnological Station of the TU München at Iffeldorf) for supplying water samples from the Osterseen. We also thank Dr. C. Schlett (Central Laboratory of Gelsenwasser AG) for several drinking water samples and the corresponding HPLC analyses. The group of Dr. L. Weil (Institute of Water Chemistry and Chemical Balneology, TU München) kindly provided us with environmental water samples and the corresponding GC data.

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Received for review December 7, 1992. Revised manuscript received April 20, 1993. Accepted June 21, 1993.*

* Abstract published in *Advance ACS Abstracts*, August 15, 1993.