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EVALUATION OF AN ENZYME LINKED IMMUNOASSAY TECHNIQUE FOR THE ANALYSIS OF ATRAZINE AND DEETHYLATRAZINE (DEA) IN WATER WITH APPLICATION TO UNSATURATED ZONE MONITORING AT L'EMPORDA, SPAIN

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A sensitive Enzyme Linked ImmunoSorbent Assay (ELISA) based on polyclonal antibodies was tested in a water sampling exercise at field scale. In the absence of deethylatrazine (DEA), results indicate that the method is useful for the determination of atrazine concentrations between 0.1 and 10 micrograms/liter. When compared with gas chromatographic analysis, ELISA overestimates atrazine concentration. If these tests are used as a semiquantitative screening tool, this tendency for overprediction does not diminish the test's usefulness. The test appears to be a valuable method for monitoring triazine herbicides in water samples from wells and soil water suction cups.

INTRODUCTION

Pesticides have been used for nearly half a century to increase the yields of agricultural crops through general pest control. In principle, the quality of water resources is threatened where pesticides are manufactured, stored, transported, processed and applied. Pathways of pesticides in the environment still cannot be adequately quantified because of their complex behavior and insufficient validation of sampling technology. In addressing this problem, development of suitable methods for detecting pesticide residues and their transformation products at low concentrations (0.1 μg/L) in water and aquifer material, is fundamental.

To investigate leaching mechanisms, pesticide presence must be measured at the field scale by representative sampling of soil and water at high spatial and temporal resolution (Honeycut and Schabacker, 1994; Boulding, 1995; Walker et al., 1995). The methods generally used to measure pesticides are HPLC and GC/MS involving extraction of large volumes of water, extensive purification, and often derivatization, and expensive equipment. As a consequence, attention has been directed to newer methods, and Enzyme Linked ImmunoSorbent Assay (ELISA) appears to be a good alternative, at least for screening purposes. Thepossibilityto apply the immunoassay methods to environmental studies was recognized more than a decade ago. Since that time, numerous assays have been developed, many of which are for pesticides (Bushway et al. 1988; Wittman and Hock, 1989; Wittmann and Hock, 1990; Hock, 1991; Wüst and Hock, 1992; Ferguson et al., 1993; Stearman and Wells, 1993). Good selectivity, sensitivity, precision, and ease of measuring many samples in one run, makes immunoassay a cost-effective method for routine analysis. Immunoassay can be a suitable alternative for analytes that are not easily analyzed by conventional methods, such as polar pesticides in aqueous samples (Meulenberg et al., 1995).

Antibodies are the key components of immunoassay methods. They are used for the detection of compounds that have a structural affinity to a specific region of the antibody molecule. The degree to which a particular antibody selectively binds the analyte of choice determines its applicability. After addition of an enzyme substrate containing a chromogen, the bound enzyme causes a color change which is inversely related to the amount of analyte present.

ELISA techniques are limited by cross-reacting compounds and matrix effects, and a low degree of cross-reactivity makes it suitable for single compound assays. Interfering matrix effects derive from two sources; the interference can occur naturally in the sample itself or be caused by substances present in water and soil (Stearman and Wells, 1993; Ruppert et al., 1992; Gascon et al., 1995).

According to the literature, ELISA has proved to be a relatively simple, fast analytical method and is especially effective when a small volume of water samples has to be analyzed for residues. However, increasing evidence shows on the semi-quantitative character of this type of analysis, its low accuracy compared to chromatographic techniques, and associated problems to pesticide cross-reactivity (Brady et al., 1990; Thurman et al., 1990; Candela et al., 1996). From this perspective, an attempt to compare ELISA methodology with instrumental methods (GC/HPLC) for monitoring atrazine and DEA in water has been made.

Sampling and analytical protocols for immunoassay techniques for atrazine and DEA in water from the unsaturated zone and from groundwater was validated in an experimental area located in L'Emporda, north of Barcelona, Spain.

EXPERIMENTAL FIELD AND PESTICIDE SAMPLING

The area of study extends for 1.4 ha and is characterized by shallow groundwater, loamy soil, long term irrigated monoculture of maize and atrazine application for more than 20 years. Water table variations ranged between 0.5 and 0 m above sea level during the period.

The experimental plot was supplied with tensiometers to monitor water infiltration and suction cups for soil-pore water sampling (Wilson et al., 1995). Leaching losses were collected in nine suction cup samplers at 1, 1.5 and 2 m depth operated under a falling head vacuum of 50 centibars during a six months period. Groundwater was sampled from the underlying (unconfined) aquifer in the existing well.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a triazine herbicide used for weed control. Major transformation products are deethylatrazine (DEA), deisopropylatrazine (DIA), dealkylatrazine (DAA) and hydroxyatrazine (HYA). DEA was the only metabolite found in the natural water samples. Gas chromatography was applied for the analysis of atrazine and DEA using HP 5890 series II gas chromatography (Hernández et al., 1993).

MATERIALS AND METHODS FOR ENZYME IMMUNOASSAY

Water samples were assayed with the Envirogard High Sensitivity Triazine Plate kit (ENVR P0048, Millipore®). Standards of atrazine, DEA and atrazine-DEA solutions were prepared following regular procedures (Brady et al., 1995; Thurman et al., 1992, Millipore®, 1991). Final concentrations of pesticide were determined through absorbance measurement by using a spectrophotometer at 450 nm of wavelength; the reference wavelength was 620 nm.

The absorbance of samples divided by the absorbance of a negative control (0 μg/L of atrazine) in a percentage form, %Bo, was used to standardize the differences in optical density (OD) caused by the external variables of time and temperature among the sample runs. The linear regression line between %Bo and the log concentration for a series of standard solutions was used to predict atrazine concentration (named atrazine equivalents) in water samples. Dose-response curves showed linear behavior between 0.01 and 0.5 μg/L.

Standard solutions for ELISA

The precision and accuracy of the kit for analyzing atrazine in water was tested by running quadruplicate assays with standard calibrators provided by the kit (0, 0.01, 0.05, 0.1 and 0.5 μg/L of atrazine) plus five intermediate concentrations (0.005, 0.075, 0.5, 0.86 and 4.3 μg/L of atrazine).

A 500 μg/L of DEA in acetone was diluted with water free of DEA residues to obtain final concentrations of 0.05, 0.1, 0.5, 2.5, 10 and 100 μg/L of DEA.

Water solutions with different atrazine-DEA concentrations were prepared from previous standards to test cross-reactivity. Final concentrations for the standard curves were the sum of all possible combination of atrazine concentrations (0.025, 0.075, 0.125, 0.225 μg/L) and DEA (0.5, 1, 1.5, 2 μg/L).

RESULTS AND DISCUSSION

Reproducibility and consistency of the dose-response curve of atrazine.

A standard curve was fit to concentrations ranging from 0 to 4.3 μg/L of atrazine, showing a correlation coefficient always greater than 0.99 with a good linear adjustment between 0.01 and 0.5

µg/L of atrazine.

Four different dose-response curves were calculated at different periods of time (C1-May, C2-June and C3,C4-July). Table 1 shows the statistical analysis of the results which was carried out to assess curve reproducibility and consistency at different test conditions for comparison purposes.

The objective of the statistical analysis was to assess the presence of significant differences in the linear part of the curve through the test of equality of adjusted means. The SPSS-X package (release 6.01 for Windows) was used to perform the analysis. Results allow the rejection of the equality of means at any level of confidence. According to the significance level in a two sided test, two groups of similar data can be detected: curves 1-4 (a= 0.8097) and curves 2-3 (a = 0.8736). The remaining significance levels are below 3%.

Values of atrazine concentration at 50% of %Bo for the experimental curves are: C1= 0.0765597, C2=0.0968278, C3= 0.1037528 and C4= 0.0751623 (Figure 1). This variation may explain why atrazine concentration calculated from the highest (C3) and the lowest regression curve (C1) differed by about 0.03µg/L.

To assess and quantify the test applicability, statistics of the four dose-response curves were calculated. Results are shown in Table 2. The most important results are: (1) the variation coefficient of calibrators was always lower than 10% (following test applicability, Bo values should not exceed 15%); and (2) the mean shows significant differences at the 0.01 µg/L level for the four replicates. This fact clearly shows in Figure 1, and is confirmed by the statistical analysis.

The statistical study confirms that inconsistencies of the method can be present when applying ELISA. Because dispersion is higher at low concentrations, a lack of accuracy may appear in the final results. In order to obtain accurate and consistent readings, standard curves need to be calculated simultaneously with the water sample analysis.

Table 1. Dose-response Curve Statistics Performed on 12 May (*Bo1*), 9 June (*Bo2*) and 4 July (*Bo3* and *Bo 4*) of 1995

Std	**Mean**				**Standard deviation**				**Variation coefficient**			
µ/L	*Bo* 1	*Bo* 2	*Bo* 3	*Bo* 4	*Bo* 1	*Bo* 2	*Bo* 3	*Bo* 4	*Bo* 1	*Bo* 2	*Bo* 3	*Bo* 4
0	100	100	100	100	4.56	1.32	1.94	5.74	4.56	1.32	1.94	5.74
0.005			90.4				2.18				2.42	
0.01	79.80	85.05	89.04	78.13	2.99	1.41	2.97	4.49	3.75	1.66	3.33	5.74
0.05	56.82	60.63	60.63	59.70	3.59	1.71	1.31	2.47	6.32	2.83	2.16	4.13
0.075			52.79				1.97				3.74	
0.1	44.33	48.99	50.06	47.70	1.41	3.55	1.39	0.94	3.19	7.24	2.78	1.98
0.5	23.70	24.88	25.05	22.85	1.29	2.42	1.56	1.34	5.46	9.71	6.21	5.88
0.86			13.02				1.35				10.34	
4.3			5.04				0.63				12.52	
10		0.56				0.40				70.99		

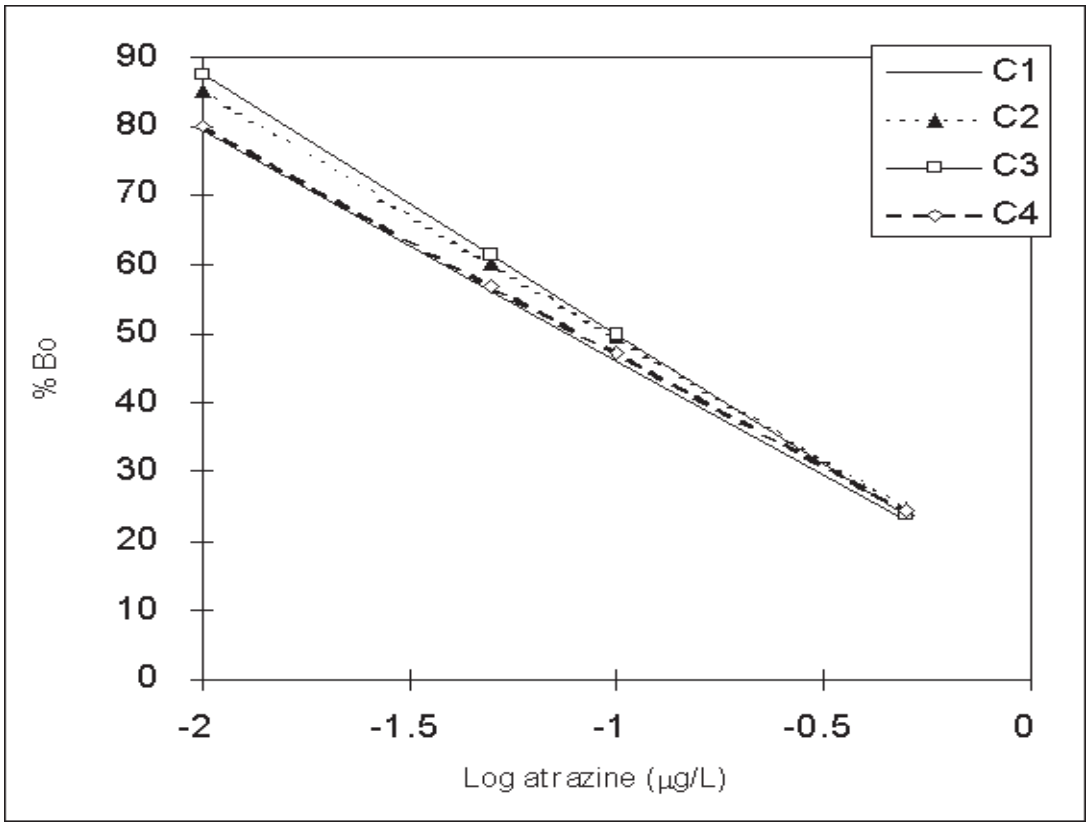

Figure 1. Graph of standard curves obtained at different periods of time (C1: May 12th; C2: June 6th; C3: July 4th; C4: July 4th of 1995) showing differences of atrazine concentration for high %Bo values.

DEA dose response curve

Antibodies used in the kit have high affinity of binding to DEA, which is structurally very similar to the atrazine molecule (Millipore®, 1991). One of the objectives of this exercise was to evaluate and quantify DEA presence in water samples using immunoassay techniques. The dose-response curve was calculated from four replicates of DEA solutions obtained as in the previous procedure for atrazine. Linearity is attained between 0.1 and 10 µg/L with a correlation coefficient of 0.99.

Atrazine-DEA cross-reaction

The polyclonal antibodies do not differentiate between various triazine analytes showing different degrees of cross-reactivity (Millipore®, 1991). Because only atrazine and DEA were detected in natural water samples, cross-reactivity analysis will focus on these two compounds.

To evaluate the ELISA performance for simultaneous presence of atrazine and DEA, all possible combinations of atrazine+DEA standard solutions were used:

Atrazine: 0.025, 0.075, 0.125, 0.225 µg/L

DEA: 0.5, 1, 1.5, 2 µg/L

Figure 2 shows concentrations of DEA (0.05, 1, 1.5 and 2 µg/L) and atrazine (between 0.025 and 0.225) against atrazine equivalents. The 0.00 µg/L of DEA line was also included for comparison purposes. As seen in the graph, for a given value of atrazine equivalent, several atrazine+DEA combinations exist, showing the difficulty to discriminate between the two selected triazines through

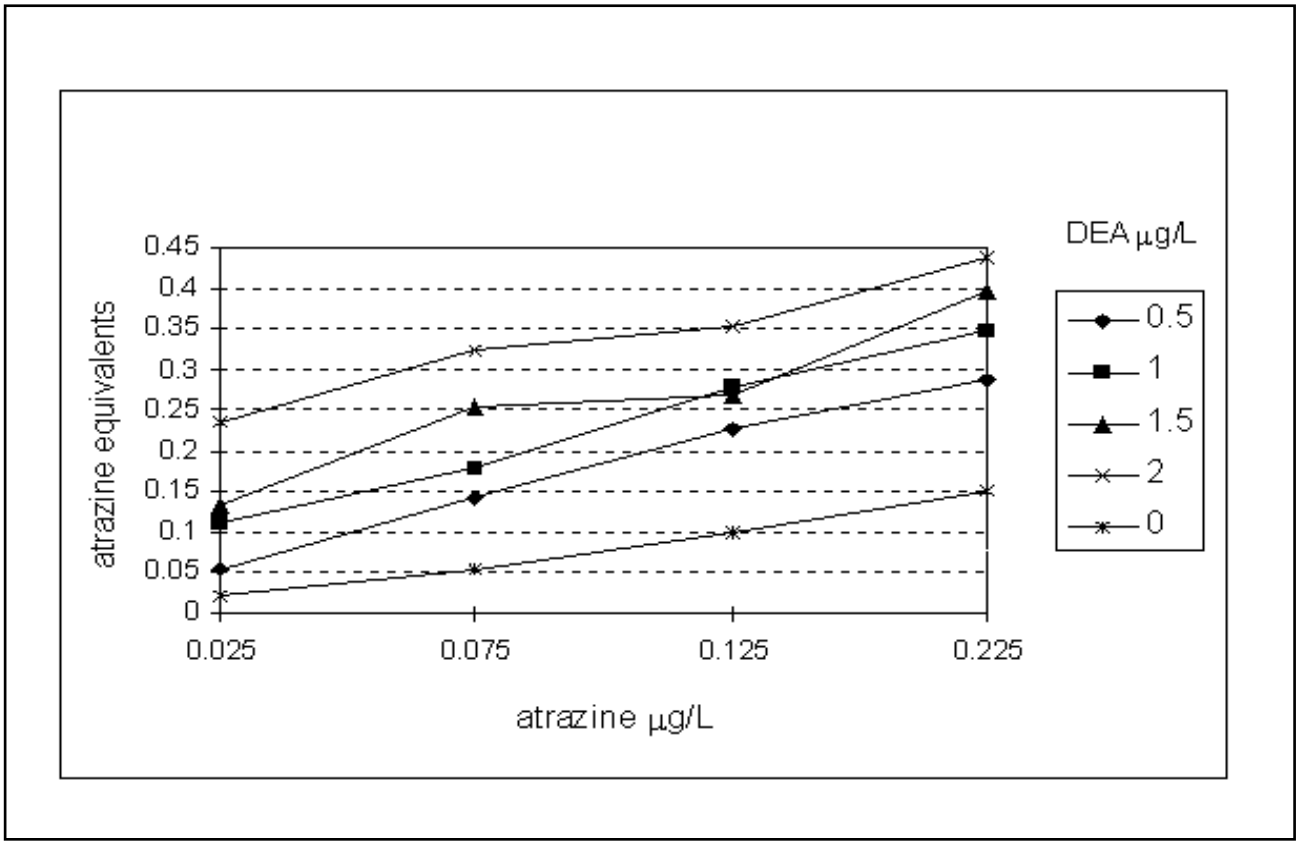

Figure 2. Graph of atrazine equivalent versus atrazine concentration (µg/L) and their correspondence with several DEA concentrations (µg/L).

direct readings. Atrazine equivalents are the result of a linear combination of atrazine and DEA as assessed by trend analysis (Candela et al., 1996).

Immunoassay validation with natural samples

Immunoassay validation was accomplished by using water samples from suction cups at 100, 150 and 200 cm depth and from the well located in the experimental field. Twenty seven water analyses were run in parallel with ELISA and GC to validate immunoassay application for the atrazine and DEA presence (Table 2).

The ELISA test always predicted the presence of triazines in all samples and no false negative was detected. The presence of six false positives may be due to undetected cross-reacting substances or competitive inhibitors showing the same antibody affinity as the analyte atrazine. In the absence of DEA, ELISA generally overestimated GC atrazine values, which agrees with other field studies developed by different authors (Schulze et al., 1992; Thurman, 1992; Brady et al., 1995). The small number of available samples at one depth, and presence of correlation or colinearity between variables does not allow immunoassay validation by conventional GC-ELISA regression to provide acceptability of the method.

When both atrazine and DEA simultaneously appear in water, ELISA readings (or atrazine equivalents) are the result of a linear combination of both analytes. In order to test this assumption, atrazine equivalents estimated by atrazine-DEA regression were compared with the ELISA atrazine equivalents obtained by direct reading. Differences of values between the two calculations range between 0.08 and 0.01 µg/L (Candela et al., 1996).

Table 2. Atrazine and DEA Results from Gas Chromatography (GC) and ELISA. (Suction Cups at 100, 150, and 200 cm depth; *Missing Values, C' Duplicate Sample

WATER SAMPLES ANALYSIS			
22/2/95	GC : Atrazine (µg/L)	GC : DEA (µg/L)	ELISA (µg/L)
C100	0.08	0.26	0.10
C150	0.10	0.00	0.08
C'150	0.09	0.10	0.06
C200	0.08	0.00	0.17
C'200	0.00	0.00	0.00
well'	0.06	0.10	0.09
ATRAZINE APPLICATION			
22/5/95			
C100	12.57	0.00	.10
C150	0.45	0.12	0.63
C'150	16.57	0.27	.10
C200	0.17	0.00	0.53
C'200	0.00	0.00	*
29/5/95			
C100	0.00	0.00	*
C150	0.34	0.20	0.42
C'150	11.12	0.28	*
C22	1.23	0.00	1.98
C'200	0.00	0.00	*
7/6/95			
C100	0.18	0.41	0.30
C150	0.13	0.10	0.50
C'150	8.16	0.25	*
C200	0.00	0.00	0.20
C'200	0.00	0.00	0.09
20/6/95			
C100	0.86	0.35	2.01
C150	0.14	0.00	0.28
C'150	3.06	0.00	4.13
C200	0.00	0.00	0.09
C'200	0.00	0.00	0.20
well'	0.72	0.00	*
26/6/95			
C100	0.75	0.72	0.95
C150	0.24	0.00	0.35
C'150	3.15	0.00	3.48
C200	0.00	0.00	0.15
C'200	0.00	0.00	0.00
well	0.00	0.00	0.23

CONCLUSIONS

From the evaluation of the ELISA methodology in a field study, the following conclusions can be obtained.

Validation of GC-ELISA techniques for detection of pesticide residues in small volumes of water has shown that immunoassay techniques are a cheap, fast and simple semi-quantitative technique for detection of atrazine-DEA concentrations above 0.1 µg/L.

Application of ELISA technique must still considered as semiquantitative tool according to the results of the evaluation exercise. Results may show some inconsistencies when the method is applied. In order to avoid a lack of accuracy, the standard curve should be calculated simultaneously with the sample analysis.

Water analysis of atrazine by ELISA normally overestimates results obtained by gas chromatography; however, no false negative was detected, showing the interest of this technique for groundwater screening purposes.

The simultaneous presence of atrazine-DEA and their possibility to be detected by ELISA techniques in natural water samples was evidenced by the existence of a linear correlation between atrazine concentration calculated by optical density and atrazine-DEA GC values. Expected atrazine equivalents obtained by statistical regression from GC analysis are similar to those obtained by ELISA readings. If the presence of DEA is known, the use of multiple regression may constitute a useful tool to evaluate atrazine concentration in water.

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REFERENCES

Boulding, R.J.; 1995. Practical handbook of soil, vadose zone and groundwater contamination. Assessment, prevention and remediation. Lewis Publishers, Boca Raton, 948 pp.

Brady, J.F., G.S. Lemasters, R.K. Williams, J.H. Pittman, J.P. Daubert, M.W. Cheung, D.H. Skinner, J.L. Turner, M.A. Rowland, J. Lange, and S.M. Sobek; 1995. Immunoassay Analysis and Gas Chromatography Confirmation of Atrazine Residues in Water Samples from a Field Study Conducted in the State of Wisconsin. J. Agric. Food Chem., 43: 268-274.

Bushway, R.J., B. Perkins, S.A. Sabage, S.J. Lekousi, and B.S. Ferguson; 1988. Determination of Atrazine Residues in Water and Soil by Enzyme Immunoassay. Bull. Environmental Contamination and Technology. 40: 647-654.

Candela, L., J. Caballero, T. Melo, and E. Torres; 1996. Development of analytical and sampling methods for priority pesticides and relevant transformation products in aquifers. Final Report. EC contract EV5CT93-032293. Unpublished.

Ferguson, B.S., D.E. Kelsey, T.S. Fan, and R.J. Bushway; 1993. Pesticide Testing by Enzyme Immunoassay at Trace Levels in Environmental and Agricultural samples. The Science of the Total Environment. 132: 415-428.

Gascon, J., E. Martinez, and D. Barceló; 1995. Determination of atrazine and alachlor in natural waters by rapid-magnetic particle-based ELISA. Influence of common cross-reactants: deethylatrazine, deisopropylatrazine, simazine and metolachlor. Anal. Chim. Acta., 311: 357-364.

Hernández, F., J. Beltran, and J.V. Sancho; 1993. Study of multiresidue methods for the determination of selected pesticides in groundwater. The Science of the Total Environment 132: 297-312.

Hock, B.; 1991. The immunoassay study group: enzyme immunoassays for the determination of s-triazines in water samples. Two interlaboratory tests. Anal. Letters. 24(4).

Honeycut, R.C. and D.J. Schabacker; 1994. Mechanisms of pesticide movement into groundwater. Lewis Publishers, Boca Raton, 189 pp.

Meulenberg, E.P., W.H. Mulder, and P.G. Stoks; 1995. Immunoassays for Pesticides. Environmental Science &

Technology, 29 (3) 553-361.

Millipore; 1991. EnviroGard Triazine Plate Kit. Extraction and quantitation for triazine residues in soil.

Ruppert, T., L. Weil, and R. Niesser; 1992. Influence of Water contents on an Enzyme Immunoassay for Triazine Herbicides. Vom Wasser, 78: 387-401.

Stearman, G.K. and M.J.M. Wells; 1993. Enzyme Immunoassay Microtiter Plate Response to Atrazine and Metolachlor in Potentially Interfering Matrices. Bull. of Environmental Contamination and Toxicology., 51: 588-595.

Thurman, E.M., M. Meyer, M. Pimes, C.A. Perry, and A.P. Schwab; 1990. Enzyme-Linked Immunosorbent Assay Compared with Gas Chromatography /Mass Spectrometry for the Determination of Triazine Herbicides in Water. Anal. Chem., 62: 2043-2048.

Thurman, E.M., D.A. Goolsby, M.T. Meyer, M. Mills, M.L. Pomes, and D. Kolpin; 1992. A reconnaissance study of herbicides and their metabolites in surface water of the midwestern United States using immunoassay and gas chromatography/mass spectrometry. Environ. Sci. Technol, 26(12): 2440-2447.

Walker, A., Allen, R. and S. Bayley, 1995. Pesticide movement to water. Monograph no 62, BCPC, Surrey, UK

Wilson, L.G., L. G. Everett, and S.J. Cullen; 1995. Handbook of vadose zone characterization and monitoring. Lewis Publishers, Boca Raton, 730 pp.

Wittmann, C. and B. Hock; 1989. Improved Enzyme Immunoassay for the Analysis of S-Triazines in Water Samples. Food & Agricultural Immunology. 1: 211-224.

Wittmann, C. and B. Hock; 1990. Evaluation and Performance Characteristics of a Novel ELISA for the Quantitative Analysis of Atrazine in Water, Plants and Soil. Food & Agricultural Immunology., 29: 65-74.

Wüst, S. and B. Hock; 1992. A sensitive enzyme immunoassay for the detection of atrazine based upon sheep antibodies. Analytical Letters, 25: 1025-1037.

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