

**JOURNAL OF  
ENVIRONMENTAL  
SCIENCE  
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**PART A: ENVIRONMENTAL SCIENCE AND  
ENGINEERING & TOXIC AND  
HAZARDOUS SUBSTANCE CONTROL**

**PART B: PESTICIDES, FOOD CONTAMINANTS,  
AND AGRICULTURAL WASTES**

**PART C: ENVIRONMENTAL CARCINOGENESIS  
& ECOTOXICOLOGY REVIEWS**

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## EVALUATION OF ATRAZINE POSITIVE AND FALSE POSITIVE IMMUNOASSAY DETECTIONS IN GROUND WATER

Key words: immunoassay, false positive, atrazine, prometon

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### ABSTRACT

False positive responses on an atrazine (6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine) immunoassay kit were investigated to explain possible causes for these occurrences. Ground water samples were evaluated with the immunoassay kit and positive responses ( $>0.20 \mu\text{g L}^{-1}$ ) were confirmed using gas chromatography/mass spectrometry (GC/MS). Non-confirming samples (false positives) were analyzed for seven additional compounds on GC. Resulting GC/MS and GC analyses showed that 70% of the false positives could be attributed to two compounds. Prometon (6-methoxy-N,N'-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine) was responsible for the majority (64%) of the false positive responses. The atrazine metabolite, deethylatrazine (2-chloro-4-amino-6-isopropylamino-1,3,5-

triazine), was responsible for the other 6% of the false positives measured. Unattributed false positives (30%) were probably due to an overestimation of pesticide concentrations in the kit's lower detection range.

## INTRODUCTION

In recent years there has been an increase in the number of wide scale monitoring programs evaluating the presence of pesticides and metabolites in ground and surface waters (Brueggman et al., 1995; Gruessner et al., 1995; Kolpin et al., 1996; Novak et al., 1996; Thurman et al., 1996). These programs have included the collection and analyses of large number of samples. Traditional extraction (liquid-liquid and solid phase extraction, SPE) and quantification (GC, GC/MS, and high pressure liquid chromatography, HPLC) techniques can be time and capital intensive.

To overcome time and cost constraints, enzyme-linked immunosorbent assay (ELISA) technology, as described by Hammock and Mumma (1980), has become widely used as a quick and relatively inexpensive analytical method for screening and quantifying pesticides in environmental samples (Aga et al., 1994, Gruessner et al., 1995, Novak et al., 1996). The key components of an ELISA kit are the antibodies to which the investigated compounds selectively bind. Meulenberg et al. (1995) noted that inherent to the use of antibodies is a certain degree of binding of structurally similar compounds (cross-reactants). This cross-reactivity will result in a positive response when the compound of interest is not present (false-positive) but a chemically similar compound is present. Cross-reactivity, in environmental

monitoring, can confound results since the source of the positive response is unknown. Due to this fact, it is necessary to use a traditional method of extraction and quantification to positively identify the source of the response.

However, the cross-reactivity of an immunoassay kit could prove useful as a preliminary screening method if one was interested in the presence of a compound, such as atrazine, and its degradation products (Gascon et al., 1995). Additionally, if a particular cross-reactant can be isolated, then the ELISA method could be useful in screening for that particular compound. Aga et al. (1994) developed a SPE-ELISA method that allowed for separation of alachlor (2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide) and its metabolite 2-[(2,6-diethylphenyl)(methoxymethyl)amino]-2-oxoethanesulfonic acid (ESA) from a water matrix. The extract containing ESA, was then quantitatively analyzed using an alachlor immunoassay kit which had been recalibrated with ESA standards.

An atrazine immunoassay kit was utilized to screen ground water samples collected as part of a water quality demonstration project conducted on the southeastern U.S. Coastal Plain (Novak et al., 1996). In this study, it was noted that several strong positive atrazine immunoassay results ( $>1.0 \mu\text{g L}^{-1}$ ) failed to confirm under GC/MS analysis. A SPE and GC method (Novak and Watts, 1996) was developed that would detect many of the compounds that had been found to cross react with this atrazine ELISA kit. The objective of this study was to investigate the positive atrazine responses measured and identify potential cross-reactive compounds which would explain the false-positive results.

## MATERIALS AND METHODS

### Sample Collection

Ground water samples used in this study were collected as part of an ongoing USDA Water Quality Demonstration Project in the North Carolina Coastal Plain (Novak et al., 1996). On a monthly basis, samples were collected from 85 to 92 ground water wells, packed in ice and transported to the laboratory. All samples were assayed for atrazine within 72 hours after collection using a magnetic based ELISA kit.

### Immunoassay Analysis

Atrazine RaPID Assay kits (Ohmicron, Inc., Newtown, Pa.), stated minimum detection limit (MDL) of  $0.05 \mu\text{g L}^{-1}$ , were used for ELISA analyses. Analyses were conducted according to instructions provided with the kits. Development, use and expected results of this immunoassay kit are detailed by Rubio et al. (1991). Samples showing a positive response of  $0.20 \mu\text{g L}^{-1}$  (MDL of the GC/MS) were subsampled and shipped on ice to the USDA-ARS National Soil Tilth Laboratory (Ames, IA) for SPE and GC/MS analysis. The remaining portion of the samples were kept frozen ( $-5^{\circ}$ ) until further SPE and GC analyses were conducted.

### GC/MS Analysis.

Prior to GC/MS analysis, 100 mL of sample was extracted through a preconditioned, C-18 SPE cartridge and concentrated 50:1. The extraction procedure was accomplished with an automated Zymate II robotic extraction system (Zymark, Hopkinton, MA.) as outlined by Pfeiffer (1992). The extraction procedure was

determined to be 92% efficient at recovering atrazine in water samples spiked at the 2.0  $\mu\text{g L}^{-1}$  level. Extracts were subsequently analyzed on a Hewlett-Packard (HP) 5890 Series II GC (Palo Alto, CA) equipped with an HP 5970B mass selective detector reported in selective ion mode. The GC/MS detection limit of atrazine was 0.2  $\mu\text{g L}^{-1}$  on a water basis. When the extraction efficiency rate was taken into account the corrected MDL for the GC/MS, on a water basis, was set at 0.22  $\mu\text{g L}^{-1}$ . A detailed description of GC/MS operation conditions is presented by Watts et al. (1996).

#### GC Analysis

Samples were extracted with a Waters tC-18 Plus cartridge (Milford, MA) and concentrated 50:1. Analyte determination and quantification was accomplished with a Varian 3600 GC (Sugarland, TX) equipped with a nitrogen-phosphorus detector. A detailed description of GC operational conditions is described in Novak and Watts (1996). Extraction efficiency and MDL for the compounds investigated are presented in Table 1.

## RESULTS

After 25 samples had been GC/MS analyzed, it was noted that in several samples a strong positive response ( $>1.0 \mu\text{g L}^{-1}$ ) was found with the immunoassay kits, but no atrazine was confirmed by GC/MS analysis. It was speculated that the false positives were due to the presence of structurally similar compounds that would cross-react with the ELISA kit.

**TABLE 1**  
**Chemical Name, SPE Efficiency and MDL<sup>†</sup> of Compounds Investigated in this Study**

Compound	Chemical Name	SPE Recovery (%)	MDL ( $\mu\text{g L}^{-1}$ )
ametryn	N-ethyl-N'-(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine	87	0.15
atrazine	6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine	92	0.20 <sup>‡</sup>
cyanazine	2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropanenitrile	98	0.10
deethylatrazine (DEA)	2-chloro-4-amino-6-isopropylamino-1,3,5-triazine	60	0.15
deisopropyl-atrazine (DIA)	2-chloro-4-ethylamino-6-amino-1,3,5-triazine	19	0.15
metribuzin	4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one	92	0.20
prometon	6-methoxy-N,N'-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine	88	0.20
prometryn	N,N'-bis(1-methylethyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine	85	0.15

<sup>†</sup>Novak and Watts, 1996.

<sup>‡</sup>Atrazine SPE recovery and MDL are for the GC/MS analysis.

To substantiate any future ELISA data, all samples which were GC/MS analyzed were subsampled for subsequent GC analysis. This strategy of screening all samples with immunoassay and using both GC/MS and GC to substantiate positive ELISA responses was used on over 1800 samples. Out of 1800 samples analyzed, only 58



showed a sufficient positive response ( $>0.22 \mu\text{g L}^{-1}$ ) to the atrazine immunoassay kit to warrant confirmation by GC/MS analysis.

### Atrazine Confirmation

Only 25 of the 58 samples that were GC/MS analyzed for atrazine were positively confirmed for atrazine (Table 2). Unlike the high correlation between GC/MS and immunoassay methods reported for both fortified (Rubio et al., 1991) and unfortified (Gruessner et al., 1995) samples, an initial comparison of the 25 samples in this study showed a poor correlation ( $r = 0.55$ ) between the immunoassay and GC/MS results. However, the poor correlation was explainable when further GC analysis showed that 13 of the 25 samples contained either DEA or prometon. A fairly high percent (65.5%) cross-reactivity for DEA in ground water has been found with this immunoassay kit (Gascon et al., 1995), and appears to have been present at sufficient concentrations in this study to elevate the concentrations measured by the immunoassay.

### Prometon and DEA Detections

Of the 33 samples that did not contain a confirmable level of atrazine, subsequent GC analysis identified prometon in 21 of the samples (Table 2). No other compounds were detected in those 21 samples. The presence of the compound is not unexpected since its use in the area has been documented (Novak et al., 1996). After the prometon GC values were corrected for SPE recovery efficiencies, a comparison of the "atrazine" immunoassay values and GC prometon values showed that the immunoassay underestimated the amount of prometon in the sample by an average

**TABLE 2**  
**Number of Positive ELISA Detections that were Confirmable  
 or Attributable**

Positive ELISA detections	58	100%
GC/MS confirmed atrazine	25	43.1%
Positive detection attributed to prometon	21	36.2%
Positive detection attributed to DEA	2	3.5%
Unexplained false positives	10	17.2%

of 47% ( $\pm 11\%$ ) yielding a cross-reactivity of 53%. This response is slightly lower than that reported by Rubio et al. (1991) who found a cross-reactivity of 73% for water samples spiked with prometon. Linear regression analyses between prometon values obtained by the two different methods indicated a significant correlation coefficient ( $r = 0.89$ ) (Fig.1). These data would indicate that when working in a controlled environment, where prometon is the only triazine input, this kit would be fairly accurate at monitoring the presence of prometon as long as the kit is calibrated with prometon standards. The recalibration would correct for the reduced response of the atrazine antibodies to prometon and would also establish the kits linear response to prometon.

Two additional samples that had a positive immunoassay response but did not contain atrazine were found under GC analysis to contain the atrazine metabolite, DEA (Table 2). The presence of this compound, individually, in the samples is not

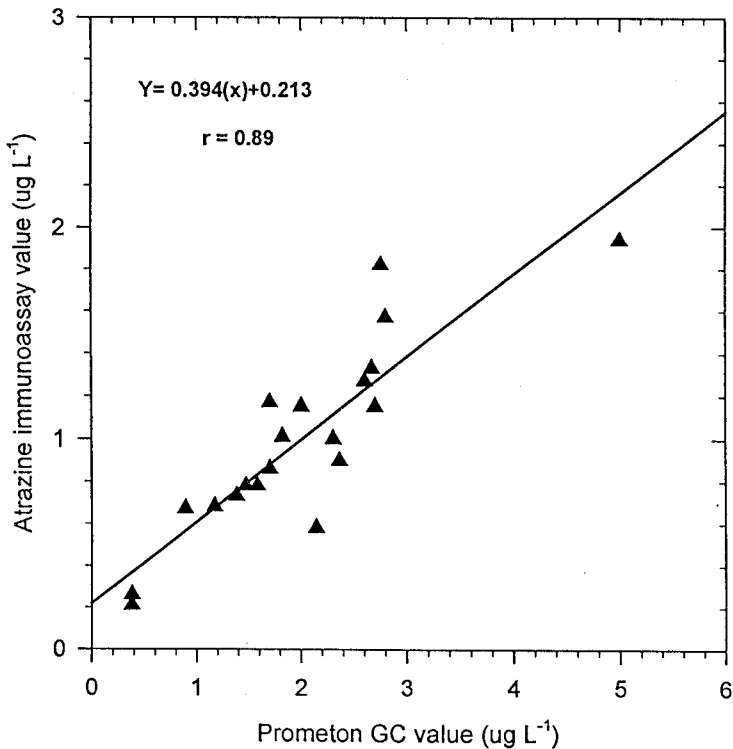


FIGURE 1

Regression comparison of ground water prometon concentrations as determined by an atrazine immunoassay and GC/MS analysis.

unexpected as it was also identified in conjunction with atrazine in 12 additional samples.

Unexplained False Positives

After GC/MS analysis for atrazine and separate GC analysis for seven additional compounds (listed in Table 1.) known to cross-react with the immunoassay kit, 10 false-positive immunoassay detections were still unexplainable. Immunoassay values

of these ten samples ranged from 0.29 to 1.03  $\mu\text{g L}^{-1}$ . These detections could not be explained geographically as the 10 values were recorded from nine separate wells. Additionally, the improper storage of chemicals or containers seems unlikely since no storage facilities are located in close proximity of the nine wells that produced these values.

The false positive responses may be attributed to the fact that seven of the ten samples had immunoassay values that exceeded the GC/MS detection limits by only 0.2  $\mu\text{g L}^{-1}$ . In a comparison of atrazine values measured in root zone leachate, Amistadi (personal communication, 1997) found a weak correlation ( $r=0.55$ ,  $n=42$ ) between the Ohmicron ELISA and GC atrazine values in the range of 0.5 to 1.0  $\mu\text{g L}^{-1}$ . The weak agreement noted both in this study and by Amistadi could be attributed to either the variability within the kit or the combined presence of two reactive compounds which were present, individually, at concentration that were below the detection limits of either the GC or GC/MS.

Another explanation is the possible presence of a chemically similar compound that was not measured by either GC/MS or GC analysis. Product literature provided with the immunoassay kits lists five additional compounds, besides those listed in Table 1, that have shown varying degrees of cross-reactivity with this kit. Those compounds are propazine (6-chloro-N,N'-bis(1-methylethyl)-1,3,5-triazine-2,4-diamine), simazine (6-chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine), terbutylazine (2-*tert*-butylamino)-4-chloro-6-(ethylamino)-*s*-triazine), terbutryn (N-(1,1-dimethylethyl)-N'-ethyl-6-(methylthio)-1,3,5-triazine-2,4-diamine), and hydroxyatrazine (6-hydroxy-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine).

Pesticide application records collected from farmers in the watershed indicated that neither simazine nor propazine was applied during the six growing season (1990-1995) (Novak et al., 1996) which included the time that these unexplainable samples were collected (Dec., 1993 - March 1995). Additionally, it is not likely that any of the unexplained positive response could be attributed to terbutryn or terbuthylazine owing to the fact that neither compound is marketed in the United States. The final compound, hydroxyatrazine, is a degraditive product of atrazine which has been rarely detected in ground water. Reported detections of this compound are at levels (10-30 ng L<sup>-1</sup>) (Cai et al., 1994) well below the kits least detectable dose for hydroxyatrazine (1.1 µg L<sup>-1</sup>).

### CONCLUSION

After both GC/MS and GC analysis had been completed, 85% of the 58 samples with positive immunoassay response could be attributed to either atrazine, prometon, or DEA. The atrazine immunoassay kit was found to be subject to cross reactivity with structurally similar compounds, especially prometon. Although, this ELISA kit underestimated the concentration of prometon by 47%, a linear regression analysis between GC and ELISA results showed a fairly good correlation coefficient ( $r= 0.89$ ) indicating that this kit, when properly calibrated, could be an effective tool for measuring prometon concentration in water samples. It is speculated that the 15% unexplainable false positives are due to the variability of this ELISA kit in the lower portion of its detectable range.

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