TWO QUALITY ASSURANCE MEASURES FOR PESTICIDE ANALYSIS OF WELLWATER: DEGRADATION IN STORAGE AND GC/ELISA COMPARISON

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ABSTRACT. *At the request of Environmental Protection Agency (EPA) project coordinators, two special quality assurance components were included in a study of herbicides in rural wells in Iowa. Since the study involved daily sampling of 88 rural wells for a period of four to five weeks, it was anticipated that samples would be in refrigerated storage for up to eight weeks during which microbial and chemical activity could lead to analyte loss. The sample degradation study reported here was conducted to insure that water samples containing three herbicides (atrazine, alachlor, and metolachlor) did not undergo excessive losses during storage. Results indicate no reduction in pesticide concentrations in six refrigerated water samples analyzed weekly during an eight-week storage period.*

Due to budget and time limitations, the rural well-water study employed enzyme-linked immunosorbent assay (ELISA) techniques to determine atrazine, alachlor, and metolachlor concentrations in well-water samples. Since ELISA techniques generally are considered to be less accurate than the more costly gas chromatography (GC) technique, a GC/ELISA comparison study was designed to evaluate the accuracy of the water quality data from this study. Results of the GC/ELISA comparison show that the ELISA method may over-estimate pesticide concentrations or register a positive detection for a chemical that is not present. Based on results of this study and information from the ELISA test kit manufacturers, it is believed that ELISA results for this study were affected by cross-reactive parent compounds and/or metabolites. Deethylatrazine and prometon were identified as compounds that may have cross-reacted with the antibody of the atrazine test kit. **Keywords.** *Degradation, Water quality, Pesticides, Quality control, Quality assurance, Immunoassay.*

Qualification for funding from the U.S. Environmental Protection Agency (EPA) requires researchers to adhere to strict guidelines for ensuring that accurate data are obtained. Two studies described here; one evaluating sample degradation during refrigerated storage, and the other comparing enzyme-linked immunosorbent assay results with those obtained by gas chromatography, comprised part of the quality assurance/quality control plan for a rural well-water quality study funded by the EPA through the Iowa Department of Agriculture and Land Stewardship (IDALS).

The EPA-funded rural well-water quality study conducted in 1993 and 1994 by the Department of Agricultural and Biosystems Engineering at Iowa State University (ISU) involved sampling of 88 rural wells on a daily basis for five weeks (Newman, 1994). This extensive sampling over time and space made immediate pesticide analyses impractical. To make the project logistics feasible, samples were collected by well owners and stored in a refrigerator until they could be transported to the laboratory at the end of the five-week sampling period. Although refrigeration of water samples before analysis is a common preservation practice in water-quality research, project investigators performed the pesticide degradation study described here to verify the hypothesis that no significant degradation of pesticides occurred in refrigerated well-water samples during an eight-week storage period.

ELISA methods of pesticide analysis have gained popularity in recent years because of their cost effectiveness and speed in delivering results. Cross-reactivity of the ELISA antibodies with compounds other than the target analyte, however, may result in reported concentrations exceeding those of the target analyte. The possibility of cross-reactivity is a concern in ELISA analysis of groundwater where pesticides often co-exist with one or more of their metabolites and/or with other pesticides of similar structure (Baker et al., 1993). The second study described in this article compares the results of the ELISA method with GC results for pesticide analysis of water samples from six wells. The objective of the GC/ELISA comparison study was to test the accuracy of the ELISA method of pesticide analysis and to identify cross-reactive compounds that may affect the ELISA results in groundwater studies.

MATERIALS AND METHODS

PESTICIDE DEGRADATION STUDY

The pesticide degradation study was designed to test the hypothesis that no significant degradation of the herbicides

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atrazine, alachlor, or metolachlor occurs in well-water samples maintained at 4.4°C (40°F) for up to eight weeks. Six samples were prepared for weekly subsampling: Sterile A and Sterile B, prepared with distilled water that was sterilized by boiling for 10 min; two water samples taken from study wells J6 and S7; and two duplicates prepared from a sample of study well B3. The original herbicide concentrations of the three study-well samples were determined from previous ELISA analyses. Additions of atrazine, alachlor, and metolachlor at 1.51 µg/L, 1.54 µg/L, and 1.35 µg/L, respectively, were made to each of the six samples. Nominal additions of 1.5 µg/L were selected to provide a significant increase in concentration while maintaining total concentrations within the analytical range of 0.05 to 5.0 µg/L.

Samples were analyzed on the day of preparation, and at weekly intervals using ELISA test kits manufactured by Ohmicron. The Ohmicron protocol utilizes a special magnetic rack that permits processing of 60 samples at a time. To help insure the accuracy of the colorimetric ELISA procedure, each rack of samples included: (1) a deionized/distilled water blank, so that the absorbance of the sample reagents can be measured in the absence of the analyte of interest; (2) three samples of known concentration, tested in duplicate to produce a calibration curve; and (3) a control sample prepared by different laboratory personnel using different reagent batches than were used to prepare the calibration samples. If the measured value for the control sample is sufficiently close to its known concentration, the work is considered to be within tolerance, and the data are accepted. Since Ohmicron recommends duplication of all samples, the remaining 50 sites in each rack are occupied by 25 field samples. According to information obtained from the manufacturer at the time this study was conducted, the estimated minimum detectable concentration for the Ohmicron test kits for atrazine, alachlor, and metolachlor is 0.05 µg/L, and ELISA results were claimed to vary by as much as 20% of the true analyte concentration. The precision of the results depends largely on the pipetting skills of the laboratory technician, but ambient temperatures in the laboratory also can affect test results.

Inclusion of the spiked sterile-samples in this study is important for two reasons. Comparison of ELISA test results with the calculated concentration provides a check on the accuracy of the spiking process. Furthermore, comparison of the time series of herbicide concentrations from the sterile samples with those from the well-water samples provides an indication of the extent to which microbial degradation may have occurred.

GC/ELISA Comparison Study

The second study described in this article, the GC/ELISA comparison study, was designed to check the accuracy of the ELISA method. To accomplish this, aliquot portions from six samples were submitted to the University of Iowa Hygienic Laboratory (UHL) for GC analysis which has a lower detection limit of 0.1 µg/L. GC results were then compared with ELISA results obtained in the water quality laboratory at the Department of Agricultural and Biosystems Engineering at ISU.

Samples were selected from six project-wells found to contain atrazine, alachlor, and/or metolachlor by the ELISA method during the spring or summer of 1993. UHL originally was contracted to test for atrazine, alachlor, metolachlor, cyanazine, metribuzin, trifluralin, and butylate using EPA GC-analysis method 507/8141. EPA method 507/3510 was used to extract the samples. During these tests, two additional unknowns were detected, and further testing identified them as deethylatrazine (a metabolite of atrazine), and prometon, both of which cross-react with the ELISA atrazine antibody. ISU tested for atrazine, alachlor, and metolachlor using the Ohmicron ELISA kits.

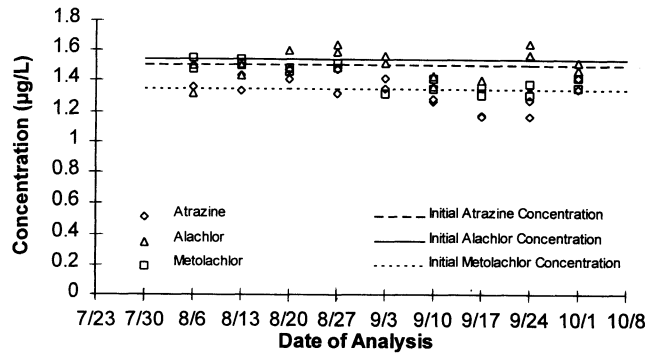

Figure 1–Weekly duplicate analyses results and initial concentrations, based on known additions, for spiked sample Sterile A.

Results and Discussion

Pesticide Degradation Study

The results of this study show little evidence of chemical or biological pesticide degradation during the eight-week period of refrigerated storage. Plots of weekly herbicide concentrations versus storage time for samples Sterile A and J6 6/8/93 (figs. 1 and 2) illustrate typical analytical results. The sterile samples and duplication of sample B3 5/22/93 provided control data for the degradation study. The mean values for atrazine, alachlor, and metolachlor in the sterile spiked samples as determined by the ELISA test correspond well to the measured additions. That is, the values are within the ELISA method range of accuracy of ± 20%. Mean values of the herbicide concentrations for duplicate samples B3 A 5/22/93 and B3 B 5/22/93, presented in table 1, also compare very well with each other.

Important statistical parameters for each sample are given in table 1. The means of nine weekly concentration

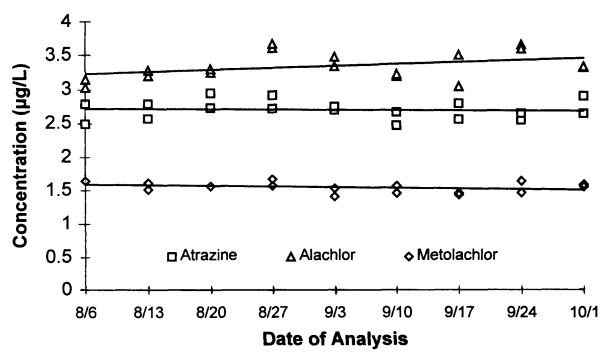

Figure 2–Weekly duplicate analysis results and linear regression lines for spiked sample J6 6/8/93.

Table 1. Statistical analysis of eight-week pesticide degradation study

(1) Sample	(2) Pesticide	(3) Mean (µg/L)	(4) Slope (µg/L/day)	(5) Intercept (µg/L)	(6) Prob > T Ho: Slope = 0
Sterile A	Atrazine	1.35	–0.00305	1.43	0.070
	Alachlor	1.50	0.00067	1.49	0.717
	Metolachlor	1.42	–0.00345	1.52	0.012
Sterile B	Atrazine	1.33	–0.00110	1.36	0.366
	Alachlor	1.56	0.00162	1.51	0.344
	Metolachlor	1.46	–0.00236	1.53	0.104
J6 6/8/93	Atrazine	2.70	–0.00056	2.72	0.774
	Alachlor	3.35	0.00472	3.22	0.184
	Metolachlor	1.55	–0.00132	1.58	0.285
S7 5/21/93	Atrazine	1.93	–0.00010	1.93	0.955
	Alachlor	1.55	0.00126	1.51	0.588
	Metolachlor	1.67	–0.00194	1.72	0.195
B3 A 5/22/93	Atrazine	1.36	0.00005	1.36	0.965
	Alachlor	1.98	0.00350	1.88	0.110
	Metolachlor	1.47	–0.00188	1.52	0.145
B3 B 5/22/93	Atrazine	1.39	–0.00229	1.45	0.063
	Alachlor	2.00	0.00131	1.96	0.636
	Metolachlor	1.47	–0.00279	1.55	0.069

measurements for each pesticide and sample are listed in column 3. The slope (column 4) is that of the linear regression line through the time-versus-concentration data for each sample and analyte. The results show both positive and negative slopes; a negative slope indicates the possibility of some pesticide degradation with time. The intercept (column 5) can be considered to be an estimate of the true initial concentration, but only if one assumes that the corresponding slope estimates the true rate of degradation.

Because of the variability associated with the ELISA analysis technique, it is unlikely, if not practically impossible, for the best-fit line to have a slope of exactly zero, even when there is no degradation. Therefore, the slope of the best-fit line alone does not provide enough information to make a determination of degradation in the samples. The T-statistic (column 6) provides additional information to further interpret the significance of the corresponding slopes. The Prob > T value is the probability of obtaining the corresponding slope value simply by chance, if the true slope of the line is zero. For example, if there was really no degradation of atrazine in sample Sterile A, table 1 indicates a 7.0% chance that the slope of the regression line would equal –0.00305 µg/L/day simply because of random variation of the data points.

Probabilities of T less than 0.05, (5%) may be considered sufficient cause to reject the null hypothesis, Ho, that the slope is truly zero. As shown in table 1, however, the data give little reason to believe that significant degradation of atrazine, alachlor, or metolachlor occurred in the well-water samples maintained at 4.4°C (40°F) over an eight-week period. Only the data for metolachlor in sample Sterile A resulted in a slope having a Prob > T of less than 5%. Furthermore, while the Prob > T value of 0.012 (1.2%) appears convincingly low, it is only one of eighteen such analyses. As such, it is not entirely unlikely that one of eighteen values could be low, simply by chance, even if no degradation occurred in any of the

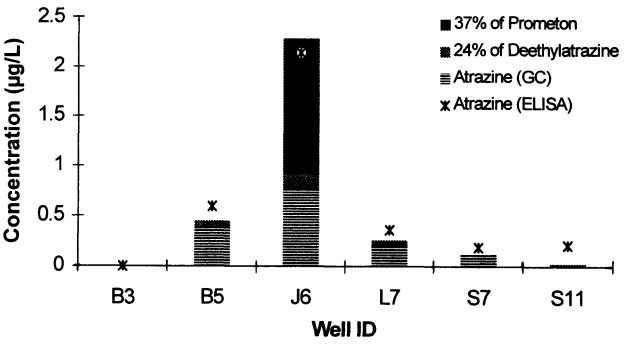

Figure 3–Comparison of ELISA results with GC data for atrazine combined with calculated cross-reactant contributions for prometon and deethylatrazine using percent cross-reactivities of 37% and 24%, respectively.

samples. Furthermore, if the slope is truly negative, its magnitude is so small (–0.00345) that it poses little concern. Even if degradation does occur at this rate, it would result in a reduction in concentration of only about 0.20 µg/L over the eight-week storage period. Assuming a true initial concentration of 1.52 µg/L (the intercept of the regression line), such a reduction is only about 13% of the initial value, which is less than the ELISA range of accuracy of ± 20%.

As a point of reference, weekly herbicide concentrations measured in the refrigerated samples can be compared with values predicted using published soil half-life values. Using half-lives of 60 days (atrazine), 15 days (alachlor), and 90 days (metolachlor) (Wauchope et al., 1992), losses of 47.6%, 92.5%, and 35.0%, respectively, are predicted for the 56-day duration of the degradation study. As would be expected, the predicted losses in soil are well above any suggested by the regression line data in table 1.

GC/ELISA Comparison Study

Results of the GC/ELISA comparison study suggest that the presence of cross-reactive compounds affected the ELISA test results for atrazine, alachlor, and metolachlor. ELISA and GC results for atrazine and selected cross-reactants are compared in figure 3. Similar data for metolachlor are displayed in figure 4. Although all samples were tested for cyanazine, metribuzin, trifluralin, and butylate (using GC methods), none of these herbicides was detected.

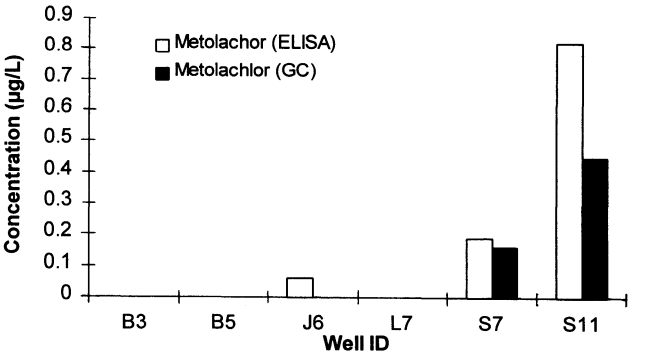

Figure 4–Comparison of GC and ELISA results for metolachlor in six water well samples.

The ELISA method consistently gave concentrations greater than those obtained via GC. Similar discrepancies have been observed in other well-water studies (Baker et al., 1993; Libra et al., 1993) and are thought to be due to the presence of cross-reactive compounds having chemical structures similar to those of the target analyte of the ELISA test. Negative results of the ELISA method are, however, widely accepted as accurate indications of concentrations below the detection limit of the analyte.

Recent information provided by Ohmicron lists 11 triazine analogues having some degree of cross-reactivity with their ELISA test for atrazine. Similarly, five potentially cross-reactive chemicals are listed for their metolachlor test, and four such compounds for their alachlor test.

Since the degree of cross-reactivity for a particular chemical varies with its concentration, several measures of cross-reactivity are possible. One of these, referred to as the "least-detectable dose" (LDD), is defined by Ohmicron as "the lowest level . . . which can be reliably distinguished from zero" (Ohmicron, 1991). The Ohmicron ELISA method involves a colorimetric measurement in which color intensity is inversely proportional to the concentration of the target analyte, Ohmicron operationally estimates the LDD at the point where measured light absorbance (B) is 90% of the absorbance (B_0) produced when no analyte is present (i.e., $B/B_0 = 90\%$), and the lower the LDD, the more cross-reactive the compound. To achieve maximum specificity, the target analyte must have a lower LDD than potential cross-reactants.

A second measure of cross-reactivity that is commonly used is referred to as percent cross-reactivity at ED_{50}. This is obtained for a particular cross-reactant by dividing the ED_{50} ("estimated dose" of target analyte resulting in $B/B_0 = 50\%$) for the target analyte, by ED_{50} for the cross-reactant, and multiplying the resulting quotient by 100. Using this measure, highly cross-reactive compounds have percent cross-reactivities at ED_{50} which are greater than 100%, while chemicals with low or moderate cross-reactivity generally exhibit percent cross-reactivities well below 50%.

Atrazine. Deethylatrazine and prometon are compounds reported by Ohmicron to have atrazine ELISA LDDs of 0.062 µg/L and 0.056 µg/L, respectively. Deethylatrazine is a metabolite of atrazine, and prometon is an s-triazine herbicide similar in structure to atrazine. As indicated by the GC results, four of six samples contained deethylatrazine, and one sample contained prometon. The presence of these two compounds may explain the higher concentrations of atrazine indicated by the ELISA method.

According to information provided by Ohmicron at the time of this study, deethylatrazine and prometon had percent cross-reactivities of 24% and 37%, respectively (David P. Herzog, Ohmicron, Personal Communication). Product information supplied in 1996 indicates slightly lower ED_{50} cross-reactivities of 22% for deethylatrazine and 32% for prometon. Multiplying the percent cross-reactivity values by cross-reactant concentrations obtained via GC provides a first approximation of cross-reactant contributions to the ELISA test results. The stacked bars in figure 3 illustrate the GC results for atrazine combined with 24% of the deethylatrazine and 37% of the prometon.

Deisopropylatrazine, another atrazine metabolite, was not included in the GC analyses but may have been present in the samples. Information recently provided by Ohmicron indicates the percent cross-reactivity at ED_{50} for this compound is about 0.3% so, unless present in high concentrations, it was not likely to have significantly affected the ELISA test results.

Recent information from Ohmicron lists several additional products (propazine, ametryn, prometryn, terbutryn, terbutylazine, and simazine) as potential cross-reactants with the atrazine ELISA test. Since these are not widely used in Iowa, specific GC analyses were not run for them. Had unidentified spikes shown up in the GC analyses, however, further tests would have been undertaken to identify the unknowns. This was, in fact, how prometon, another product not widely used in Iowa, was discovered in several project samples.

It must be stressed that use of percent cross-reactivities and GC cross-reactant concentrations to quantify the impacts of cross-reactive compounds on ELISA test results is not precise since the concentrations at which the cross-reactivity percentages were determined may differ from concentrations present in samples during this study. Despite these uncertainties, this approach demonstrates a reasonable explanation for the discrepancies in GC and ELISA results in this study. As shown in figure 3, ELISA results for most samples appear to provide a reasonable estimate of the sum of GC atrazine results and the estimated cross-reactant contributions derived using percent cross-reactivity values and GC results for the cross-reactants.

Alachlor. GC analysis indicated that none of the six samples contained detectable levels of alachlor, but five samples contained detectable levels of alachlor according to the ELISA method. Previous research indicates that this discrepancy is common, particularly in groundwater samples (Baker et al., 1993; Libra et al., 1993). Feng et al. (1990) reported that 2-[(2,6-diethylphenyl)(methoxymethyl)amino]-2-oxoethanesulfanate (ESA), a metabolite of alachlor, may be responsible for false positive detections of alachlor in groundwater by ELISA methods. Macomber et al. (1992) later supported this finding. Metabolism of alachlor is rapid in the soil, but ESA may be more persistent (Baker et al., 1993). Recent data provided by Ohmicron indicates an ESA percent cross-reactivity at ED_{50} of 33% can be expected in their alachlor ELISA test. As a result, the presence of ESA may have been responsible for the positive ELISA alachlor detections in the five samples of this comparison study. Unfortunately, ESA analytical data are not available to confirm this.

Metolachlor also can influence ELISA results for alachlor. However, the LDD for metolachlor (6.0 µg/L) is well above the concentration of metolachlor found in any of the six samples, and the cross-reactivity at ED_{50} is only 1.3%. Therefore, metolachlor probably did not influence the alachlor ELISA results.

Metolachlor. Like the analyses for atrazine and alachlor, ELISA results for the three metolachlor detections are higher than corresponding GC results. As shown in figure 4, however, one sample (S7) is within the range of ELISA accuracy of ± 20%, and another (J6) was detected by ELISA at 0.06 µg/L which is below the GC detection limit of 0.10 µg/L. Furthermore, three negative metolachlor results obtained by the ELISA method agree with negative GC results. The ELISA results for metolachlor in five of the six samples, therefore, can be considered essentially the same as the corresponding GC

results. Contrasting with this, the ELISA method yielded nearly twice the level of metolachlor as the GC method for sample S11. The reason for this large difference is not clear, but the possibility exists that cross-reactants of the metolachlor antibody, such as butachlor, propachlor, acetochlor, or metalaxyl, were present in the sample. Since the GC analysis did not include these compounds this cannot be confirmed. With respective LDDs of 0.26, 1.0, 0.06, and 0.06 µg/L, and ED_{50} cross-reactivities of 1.6, 0.03, 13.0, and 15.2%, however, even the most reactive of these chemicals would have had to be present in concentrations of 2.5 to 3 µg/L to account for the discrepancy between the GC and ELISA data for sample S11.

Alachlor affects the results of metolachlor ELISA tests (LDD = 1.3 µg/L, ED_{50} cross-reactivity = 1.0%) just as metolachlor can influence the results of alachlor tests. Alachlor cross-reactivity, however, is not likely to have affected ELISA metolachlor results in this instance because GC analysis indicated that no alachlor was present. The metabolite of alachlor, ESA, is also unlikely to have affected the metolachlor results because cross-reactivity of ESA in the metolachlor ELISA kit is less than 0.01% (David P. Herzog, Ohmicron, Personal Communication).

Conclusions

Results of the pesticide degradation study and the GC/ELISA comparison study provide valuable information for conducting pesticide-contamination studies of groundwater. The pesticide-degradation data show that refrigeration of water samples for as long as eight weeks prior to analysis for atrazine, alachlor, and metolachlor is an acceptable practice. Other pesticide solutions may show similar stability under the same conditions.

The GC/ELISA comparison study shows that ELISA detections of atrazine and alachlor in well-water are likely to include some cross-reactant compounds. Deethylatrazine and prometon were identified as possible cross-reactants in the atrazine tests, and it is believed that the alachlor metabolite ESA may be responsible for many if not all of the ELISA alachlor-detections in this study. Cross-reactivity is also a possibility for ELISA metolachlor-detections, although no specific compounds were identified as responsible for elevated ELISA results in this project.

Although the ELISA method may have detected metabolites or structurally similar compounds during the degradation study, the general conclusion that no significant degradation occurred during the eight-week storage period is still considered valid. Although degradation of atrazine can result in formation of cross-reactive metabolites, these compounds are much less reactive to the ELISA antibody than atrazine itself. The atrazine metabolite deethylatrazine, for example, is only about 24% reactive in the ELISA tests (David Herzog, Ohmicron, Personal Communication). As a result, had degradation of atrazine to deethylatrazine occurred, it would have resulted in lower detections by the ELISA method. In this degradation study, detection levels remained constant throughout the eight-week study period, indicating no herbicide metabolism.

The results of the GC/ELISA comparison study provide insight into ELISA-determined herbicide concentrations in Iowa well-water samples. Not all of the compound detected by ELISA actually may be the target analyte. In atrazine tests, some of the detected contaminant may be the metabolite deethylatrazine; or the compound prometon, another s-triazine herbicide.

Identification of deethylatrazine and prometon as cross-reactive components in well-water samples does not lessen the concern for contamination of Iowa groundwater. In fact, deethylatrazine is nearly as phytotoxic as its parent compound (Winklemann and Klaine, 1991), although health advisory limits for humans have yet to be established for that metabolite. Prometon is believed to be less toxic to humans than atrazine, and has a lifetime health advisory level of 100 µg/L compared with 3 µg/L for atrazine.

Based on the results of this study and previous research, detection of alachlor in groundwater by ELISA methods may be misleading. Positive detections may not necessarily indicate the presence of any alachlor at all, but rather may be caused by the alachlor metabolite ESA.

In only one sample out of six were ELISA metolachlor results substantially higher than the GC results. Since no attempt was made to quantify possible cross-reactive compounds by GC in this study, more concrete conclusions about the metolachlor results cannot be postulated.

Possibly most important, all cases of non detections by the ELISA method corresponded to non detections by the GC method. These data indicate that negative results of the ELISA method are an accurate indication of concentrations below the detection limit of the target analyte.

Practical limitations such as cost and project logistics often dictate the methods used for water quality studies. The data presented in this article indicate that refrigerated storage of water samples for eight weeks prior to pesticide analysis is an acceptable practice. The relatively low cost of the ELISA method of pesticide analysis compared with the GC method has encouraged ELISA use. The data presented in this article suggest that ELISA methods provide a good estimate of the total concentration of the target analyte and reactive fraction of cross-reacting compounds present in the sample. While the ratio of cross-reactive compounds to the target analyte cannot be ascertained by the ELISA method, ELISA methods can be useful in identifying the presence or absence of anthropogenic contaminants in groundwater, and they also can be a useful analytical tool for quantifying increasing or decreasing trends in the total concentration of target analytes and cross-reactive compounds.

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