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Determination of Triazine Residues in Water: Comparison Between a Gas Chromatographic Method and an Enzyme-Linked Immunosorbent Assay (ELISA)

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The triazine group includes a number of widely used herbicides. From these, the most important in Greece are atrazine, simazine and prometryn. Triazines have a high solubility in water and are quite stable in soils. During recent years triazines have been detected in groundwater samples (Goolsby et al. 1991, Fleeker and Cook 1991, Bushway et al. 1992, Muldoon et al. 1993). Because of their importance for agriculture and environmental concerns, their presence in water should be monitored.

Several studies have shown that immunoenzymatic techniques, such as enzyme-linked immunosorbent assays (ELISAs) are useful analytical techniques for the determination of residues of pesticides as an alternative to instrumental methods (Hammock and Mumma 1980, Jung et al 1989, Bushway et al. 1988, Kaufman and Clower 1991). Ready-made kits are available which enable fast and cost-effective handling of large number of samples.

The objectives of this study were a) to compare ELISA to gas-liquid chromatography (GLC) for qualitative and quantitative determination of atrazine in water and b) to monitor atrazine, simazine and prometryn residues in water samples from Greece using both techniques.

MATERIALS AND METHODS

Forty samples of natural water were randomly collected by local government officials from lakes, rivers, streams and wells throughout Greece from November to December 1993. Some of these were destined for human consumption, while others were not. The samples were collected in amber glass bottles of 2.5-L capacity and were sent to the laboratory for analysis. Most samples were analysed on arrival at the laboratory, but where this was not possible, they were stored at 4° C for an average of 2 days. In addition, 36 samples of tap water from the city of Athens were collected in April and May 1994 and analyzed.

For the comparison between GLC method and ELISA, spiked samples were prepared using water of high purity (HPLC grade, LAB-SCAN Analytical Sciences) fortified with atrazine at the 0.05, 0.10, 0.20, 0.50 and 1.00 ppb level. The fortification was made in such a way that the final concentration of the solvent (acetone) in the fortified sample was minimal (<1o/oo), in order to avoid any adverse effect on the immunoassay. Spiked samples fortified with simazine and prometryn at 0.1 ppb were also prepared in order to check the efficiency of the gas-chromatographic method for the analysis of the real samples.

For ELISA analysis four kits produced by TRANSIA-DIFFCHAMP, Lyon, France were used. Each kit consisted of one antibody-coated microplate with breakable strips, triazine enzyme conjugate, substrate, chromogen, stop solution (H_2SO_4) and atrazine calibrators (standard solutions) at 0 ppb (negative control), 0.1, 0.25 and 1 ppb. This kit does not differentiate between the various triazines, but detects them to differing degrees. The % cross-reactivities reported by the manufacturer were 0.2 for atrazine, 0.37 for prometryn and 2.5 for simazine. The procedure followed was that recommended by the manufacturer, Briefly, 100 µL of sample was distributed to each antibody-coated well, to which 100 µL of conjugate was added. After incubation for 30 min at room temperature, the wells were rinsed 5 times with distilled water to remove excess unreacted sample and enzyme conjugate, and 100 µL of a mixture of substrate/chromogen (1/1) was added. After incubation for 30 min with agitation, the reaction was stopped and the colour (blue) was fixed (turned to yellow) with 50 µL of "stop" solution, A DYNATECH MR 5000 ELISA spectrometer was used to read the optical densities at 450 nm (blank on air). All samples and calibrators were run in triplicate, i.e., each sample was placed in three separate wells. The concentration of each sample was determined by finding its percent inhibition which is the difference in optical density at 450 nm between the negative control and the sample, divided by the optical density of the negative control, times 100. This normalizes the absorbance readings of each set of samples to the variability of the negative control, which is a sample run with each set that contains no herbicide and has the maximum absorbance reading possible (Thurman et al. 1990). The percent inhibition is referred to as the % activity in the instructions accompanying the kit, and this is the term used in the analysis below. The % activity values were plugged into the equation of the line calculated by linear regression of the % activity of each calibrator against the logarithm of its atrazine concentration.

For GLC analysis the method of Ambrus et al. (1981) was used for the extraction of triazine residues from water. According to this method 1-L analytical sample is extracted 3 times with dichloromethane in the presence of 4% Na_2SO_4 solution. The extract is evaporated to dryness and made-up in a solvent suitable for direct GLC injection. A VARIAN 3700 gas-chromatograph was used equipped with Nitrogen-Phosphorus Detector (NPD) and borosilicate glass columns. Two columns of different polarity were used for this study. The first was 1m long x 2mm o.d.x 1/4 inch i.d. packed with 5% OV-17 on 80/100 mesh Gas - Chrom Q, operating at the following conditions (programmed temperature): inlet and detector temperature 250°C, oven

initial 180°C, time 5 min, rate 10 °/min, final 230°C time 2 min, carrier (helium) flow 40 mL/min, bead toggle adjusted to 700. The second column was 2m long x 2mm o.d. x 1/4 inch i.d. packed with 2% DEGS on 80/100 mesh Chrom WAW operating at the following conditions (isothermal analysis): inlet and detector temperature 250°C, oven 180°C, carrier (helium) flow 30 mL/min, bead toggle adjusted to 700.
On the first column atrazine and simazine had the same retention time (2.5 min) while prometryn was eluted in 4.5 min. On the second column atrazine and prometryn had the same retention time (3.8 min), while that of simazine was 4.9 min. The limit of detection was 0.05 ppb for both columns. In order to achieve a better separation of the three compounds, two additional chromatographs were tested: The first was a Fisons HRGC Mega 2 Series chromatograph, equipped with a Nitrogen-Phosphorus Detector and an SE 54 column 30m long x 0.32mm i.d., film thickness 0.25 µm operating at the following conditions: standby isothermal 70° C, rate 1 7°/min, isothermal 2 150°C time 1 zero, time 2 zero, rate 2 10°/min, isothermal 3 270°, time 10 min, injector (splitless injection) temperature 220°C detector temperature 280°C, makeup gas (nitrogen) pressure 60 kPa and flow 30 ml/mm , carrier (helium) flow 2 ml/mm, hydrogen pressure 60 kpa and air pressure 110 kPa. Under these conditions the retention times were 17.1 for simazine, 17.2 for atrazine and 19.3 min for prometryn.The second was a Hewlett-Packard 5890 Series II plus, equipped with an HP-1 crosslinked methylsilicone gum 30m length x 0.53mm i.d., film thickness 0.88µm operating at the following conditions: initial temperature 80° C, initial time 1 min, level 1 rate 15°/min, temperature 185°C , time 1 min, level 2 rate 8°/min, temperature 230°, time 20 min, injector temperature 80°C (on column injection), detector temperature 280°C, carrier (helium) flow 6.09 mL/min, hydrogen flow 3.5 mL/mm and air flow 110 ml/mm. Under these conditions the retention times were 10.6 for atrazine, 10.5 for simazine and 12.6 min for prometryn.

To ascertain the precision of both methods the coefficient of variation (CV) was calculated for the spiked samples, For quantitative determination of residues by ELISA and to compare ELISA and GLC results linear regression analysis was carried out using the STATGRAPHICS program.

RESULTS AND DISCUSSION

The ELISA kits were validated by calculating the % activity of the calibrators and their CV in order to ensure that all reagents were functional To be accepted, the % activity of each calibrator should fall within given ranges and the CV for the calibrators' optical density (O.D.) values should not exceed 15%. From the 4 plates used, one (plate 1) did not meet the guideline concerning the % activity (Table 1). Thus, the results of this plate were not taken into account. However, the CV fell well below the recommended level (2.2-9.6 %). The between-wells CV for the calibrators varied from 0.9 to 6.5 (Table 1) and those of the fortified samples between 1.0 to 12% (Table 2). The control samples (unfortified HPLC water) gave optical densities close to those given by the 0 ppb calibrators of the kit on plates #3 and #4 (0.60 and 0.63 compared to 0.57 and

Table 1. Validation of the ELISA kits

Plate #	Calibrator, ppb	Mean Optical Density (OD)	% Activity Measured	Recommended Range of % Activity	CVs of ODs
1	0	0.55			2.2
	0.1	0.22	40	50-70	4.0
	0.25	0.14	24	30-50	9.6
	1	0.07	12	15-30	4.3
2	0	0.60			2.6
	0.1	0.32	53.47	50-70	1.2
	0.25	0.20	33	30-50	5.0
	1	0.10	17.38	15-30	3.8
3	0	0.57			0.9
	0.1	0.33	58.10	50-70	1.8
	0.25	0.23	40.98	30-50	1.3
	1	0.14	24.69	15-30	3.5
4	0	0.69			6.1
	0.1	0.39	56.58	50-70	3.8
	0.25	0.28	39.79	30-50	4.7
	1	0.15	22.28	15-30	6.5

0.69) while on plate #2 the optical density of the control sample was slightly lower than that of the 0 ppb calibrator (0.50 compared to 0.60). However, this optical density was much higher than that of the calibrator of 0.1 ppb and that of the sample spiked at the 0.05 ppb level. The real samples, which were shown by GLC analysis to be triazine-free or to contain triazine residues, gave the same result when they were analyzed by ELISA. It is thus reassuring that the test did not give any false-positive or false-negative results.

As stated before, the kit does not differentiate between the various triazines.This can be considered as a drawback. However, similar problem can be encountered with GLC analysis, as shown from the retention times of the three compounds on several columns.

The equations calculated by linear regression for the fortified samples were y=15.8-35.3x for plate #2, 23.7-32.9x for plate #3 and 21.5-33.9x for plate #4 (Table 2). The R^2 were 96.3, 98.3 and 98.9 % respectively.

The between-plates variation for the fortified samples ranged between 43% (for samples spiked at the 0.05 ppb level) to 1.2% (for samples spiked at the 1.00 ppb level)(Table 3). The high variation for the samples spiked at the 0.05 ppb level was due

Table 2. The ELISA response for water samples spiked with atrazine.

Plate #	Fortification Level ppb	Mean Optical Density	CV	% Activity	Conc/tion Det/ed by Linear Regression ppb
2	0	0.50	2.4	81.95	
	0.05	0.31	3.6	50.82	0.101
	0.1	0.25	3.2	41.72	0.184
	0.2	0.19	1.0	31.29	0.360
	0.5	0.12	7.3	20.36	0.742
	1.0	0.10	3.1	16.05	0.983
3	0	0.60	11.6	105.25	
	0.05	0.38	4.8	66.02	0.051
	0.1	0.31	1.6	53.76	0.122
	0.2	0.24	7.5	42.03	0.277
	0.5	0.16	6.2	28.37	0.720
	1.0	0.13	3.1	22.59	1.079
4	0	0.63	6.4	90.73	
	0.05	0.46	8.3	65.99	0.048
	0.1	0.46	11.6	67.14	-
	0.2	0.26	12.0	37.48	0.336
	0.5	0.18	7.1	26.33	0.718
	1.0	0.13	7.9	18.23	1.245

$y_2 = 15.8-35.3x$ $y_3 = 23.7-32.9x$
$y_4 = 21.5-33.9x$

to the low intercept estimated for plate #2 (15.8 compared to 23.7 and 21.5 for plates #3 and #4, respectively). However, the CVs for the samples fortified at the 0.50 and 1.00 ppb level were lower than those obtained by GLC, indicating a lower degree of variation.

Table 3 gives the results for atrazine-spiked samples determined by GLC and ELISA. In almost all the cases the concentrations detected by ELISA where a certain degree higher than the actual while those determined by GLC were lower (recoveries ranging from 90 to 99.5 %) except for the samples fortified at 0.05 ppb, where the recovery was 114%.

In order to study the possibility of using the values determined by ELISA for the fortified samples as reference values for positive real samples and in this way to normalize for the observed matrix effect, a best fitting test was carried out on the data of the three plates averaged, using the x^2 test with the fortification levels on the x axis

Table 3. Atrazine determined in fortified water samples

Fortification Level ppb	Concentration Determined by GLC	Concentration Determined by ELISA
0	< 0.05	< 0.05
0.05	0.06, 0.08, 0.04, 0.048 m=0.06±0.017 CV=30.5%	0.010, 0.051, 0.048 m=0.06±0.029 CV=43%
0.10	0.076, 0.120, 0.087, 0.104 m=0.09±0.019 CV=21.1%	0.184, 0.122 m=0.15±0.043 CV=28%
0.20	0.190, 0.175, 0.165, 0.200 m=0.18±0.015 CV=8.5%	0.360, 0.277, 0.336 m=0.32±0.042 CV=13%
0.50	0.50, 0.49, 0.55, 0.45 m=0.5±0.041 CV=8.3%	0.742, 0.72, 0.718 m=0.73±0.013 CV=1.8%
1.00	1.0, 0.95, 0.88, 0.90 m=0.93±0.053 CV=5.7%	0.983, 1.10, 1.25 m=1.11±0.131 CV=1.2%

and the averaged % activity on the y axis. The best fitting was showed to be the hyperbolic function (x^2=0.051 compared to 0.637 for semilog and 4.27 for exponential functions). However, this approach was not followed up due to the fact that most of the real samples, were found to be triazine free.

The recoveries from spiked samples fortified with simazine or prometryn were 87% ± 0.015 and 85% ± 0.018, respectively (mean of 5 samples).

The analysis of real samples using both methods showed no detectable residues in all samples except two. These two positive samples were untreated water from the rivers Evros and Loudias and were collected on November 10 and December 16, 1993, respectively. They were found using ELISA to contain triazine at approximately the limit of detection. Using the dual column system for GLC analysis, the compound detected was identified to be atrazine and the concentration determined was again close to the limit of detection.

Figure 1 shows the correlation of GLC and ELISA values to spiked sample input values. The GLC slope (R^2) was 0.94 and that of ELISA 1.09 indicating that the immunonoassay results are slightly higher than GLC. However, the results with real samples showed that ELISA is an excellent tool for the analysis of large numbers of samples at the designated level of interest permitting the elimination of negative test samples. Positive samples can be confirmed using alternative methods, as is always required by quality assurance guidelines.

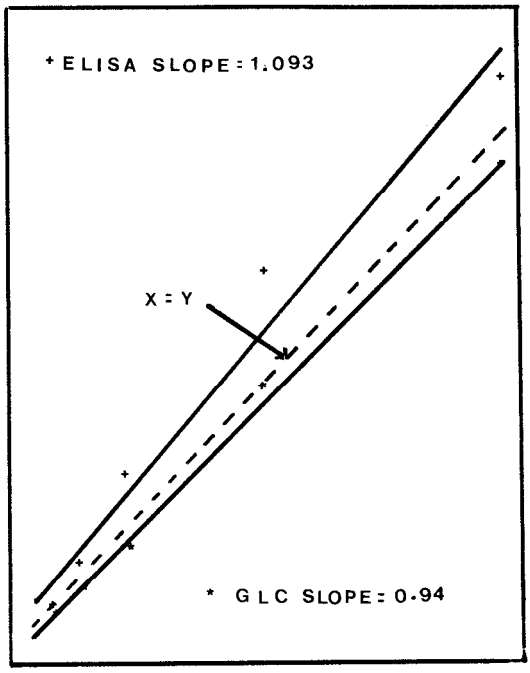

Figure 1. Correlation between GLC and ELISA results for spiked samples

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REFERENCES

Ambrus A, Lantos J, Visi E, Csatlos I, Sarvari L (1981) General method for determination of pesticide residues in samples of plant origin, soil, and water. I. Extraction and cleanup. J Assoc Offic Anal Chem 64: 733-742

Bushway RJ, Perkins B, Savage SA, Lekousi SJ, Ferguson BS (1988) Determination of atrazine residues in water and soil by enzyme immunoassay. Bull Environ Contam Toxicol 40: 647-654

Bushway RJ, Hurst, HL, Perkins LB, Tian L, Guiberteau Cabanillas C, Young BES, Ferguson BS, Jennings HS (1992) Atrazine, alachlor and carbofuran contamination of well water in Central Maine. Bull Environ Contam Toxicol 49: 1-9

Fleeker JR, Cook LW (1991) Reliability of commercial enzyme immunoassay in detection of atrazine in water. In: Immunoassays for trace chemical analysis. M

Vanderlaan, LH Stanker, BE Watkins, DW Roberts eds. ACS Symposium Series No 451. pp 78-85

Goolsby DA, Thurman EM, Clark ML, Pomes ML (1991) Immunoassay as a screening tool for triazine herbicides in streams. Comparison with gas chromatographic - mass spectrographic methods. In: Immunoassays for trace chemical analysis. M Vanderlaan, LH Stanker, BE Watkins, DW Roberts eds. ACS Symposium Series No 451. pp 86-99

Hammock BD, Mumma RO (1980) Recent advances in pesticide analytical methodology. J Harvey Jr & G. Zweig (Eds) American Chemical Society, Washington, DC, pp 321-352

Jung F, Gee SJ, Harrison RO, Goodrow MH, Kant AE, Brown AL, Li QX, Hammock BD (1989) Use of immunochemical techniques for the analysis of pesticides. Pestic Sci 26: 303-317

Kaufman BM, Clower Jr, M (1991) Immunoassay of pesticides. J Assoc Offic Anal Chem 74: 239-247

Muldoon MT, Fries GF, Nelson JO (1993) Evaluation of ELISA for the multianalyte analysis of s-triazines in pesticide waste and rinsate. J Agric Food Chem 41: 322-328

Thurman EM, Meyer M, Pomes M, Perry C A, Schwab AP (1990) Enzyme-linked immunosworbent assay compared with gas chromatography/mass spectrometry for the determination of triazine herbicides in water. Anal Chem 62: 2043-2048