Comparison of Capillary Electrophoresis, HPLC, and Enzyme Immunoassay for Terbuthylazine Detection in Water

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Tap water and groundwater samples spiked with terbuthylazine were analyzed by capillary electrophoresis (CE), HPLC, and a commercial enzyme immunoassay kit. Over the range of concentrations tested (0.2–2.4 $\mu g\ L^{-1}$), the results obtained by the different methods were highly correlated. CE proved to be viable for the detection of terbuthylazine, with good resolution and reproducibility. The detection limit of CE is higher than that of HPLC to detect the lowest concentration of terbuthylazine (0.2 $\mu g\ L^{-1}$), but retention times were shorter. Compared to enzyme immunoassay, CE and HPLC needed sample extraction and concentration before analysis but showed higher accuracy and lower variation.

Keywords: *Terbuthylazine; HPLC; CE; enzyme immunoassay*

INTRODUCTION

Drinking water, surface water, and groundwater pollution by herbicides has stimulated the development of rapid and sensitive methods for the detection of herbicides in water. Terbuthylazine [6-chloro-*N*-(1,1-dimethylethyl)-*N'*-ethyl-1,3,5-triazine-2,4-diamine] is an *s*-triazine herbicide closely related to atrazine. It is not licensed for agricultural use in the United States (Sheperd *et al.*, 1992; Steinheimer, 1993) but is widely used in other countries and has been detected in surface (Hall, 1974; Rossi *et al.*, 1994), lake (Buser, 1990), drinking, and ground waters (Hurle *et al.*, 1987; Meinert and Hafner, 1987; Bowman, 1989).

Several methods for the determination of terbuthylazine in water have been reported. Usually, high-performance liquid chromatography (HPLC) (Coquart and Hennion, 1991; Schlett, 1991; Di Corcia and Marchetti, 1992; Steinheimer, 1993) and gas chromatography (GC) (Meyer *et al.*, 1981; Davì *et al.*, 1992) are used to detect terbuthylazine in water. Recently, capillary electrophoresis (CE) has been reported as a novel approach for the determination of sulfonylurea herbicides (Dinelli *et al.*, 1993a,b) and of *s*-triazine herbicides in tap and surface waters (Dinelli *et al.*, 1992). Immunoassays have been used successfully to determine terbuthylazine (Ulrich *et al.*, 1992) and other *s*-triazines in water (Bushway *et al.*, 1988; Lucas *et al.*, 1991; Ferguson *et al.*, 1993; Lawruk *et al.*, 1993), but they are nonspecific and other *s*-triazine compounds may react (Thurman et al., 1990; Goh *et al.*, 1991; 1992; Schneider and Hammock, 1992). Recently, enzyme immunoassay kits have become commercially available in Italy to detect residues of terbuthylazine in water, because atrazine is forbidden since 1989 and was replaced for agriculture purposes by terbuthylazine. Available kits are specific for *s*-triazines and present high cross-reactivity with terbuthylazine.

In this work HPLC was chosen as a reference method to test the reproducibility and accuracy of a commercial immunoassay kit and CE for the detection of terbuthylazine in tap and ground waters at the micrograms per liter level.

EXPERIMENTAL PROCEDURES

Materials. A wettable powder formulation [50% active ingredient (ai)] of terbuthylazine, supplied by Ciba-Geigy Ltd., Basel, Switzerland, was used. Water samples for method comparison were prepared by adding 0, 0.2, 0.8, 1.6 and 2.4 $\mu g\ L^{-1}$ of terbuthylazine to duplicate samples of drinking and ground waters. Drinking water was tap water from the municipal water system of Bologna (pH 7.2; hardness, 2.5 g L^{-1}; residue, 0.8 g L^{-1}), whereas groundwater was collected at 1 m depth from lysimeters located at Bologna, near the Department of Agronomy (Rossi Pisa *et al.*, 1992). For enzyme immunoassay, additional tap water and groundwater samples were prepared at 0.1, 0.4 and 3.2 $\mu g\ L^{-1}$ of terbuthylazine. Preliminary results showed that water samples were *s*-triazine free (data not shown). Furthermore, cross reactivity of immunoassay with metolachlor, a herbicide often used with terbuthylazine, was evaluated using duplicate samples of tap and ground waters at 0.2 and 1.6 $\mu g\ L^{-1}$ terbuthylazine soaked with 0, 100, 500, or 1000 $\mu g\ L^{-1}$ metolachlor. Reagents for the electrolyte buffer for capillary washing and for the HPLC separation were supplied by Sigma Chemical Co. St. Louis, MO. All solvents, supplied by Bakerbond, were pesticide free.

Immunoassay Procedure. The immunoassay analyses of water samples were carried out in 96-well microplates (EnviroGard Triazine Plate Kit, Millipore Corp., Bedford, MA). The commercial kit is based on the use of polyclonal antibodies that bind both triazines and an atrazine–enzyme conjugate which presents high cross-reactivity to terbuthylazine (Ferguson *et al.*, 1993). The detection limit reported in the kit for terbuthylazine in water is 0.06 $\mu g\ L^{-1}$. The procedure was similar to that described in the kit. Briefly, 160 μL of each water sample was added to the respective well with 160 μL of atrazine–enzyme conjugate. The wells were mixed with rapid circular motions for 1 min and then incubated at room temperature for 1 h. After incubation, wells were rinsed five times with tap water, and 160 μL of substrate was added followed by 80 μL of chromogen. The wells were mixed for 1 min and incubated for 30 min. After incubation, color was fixed with 40 μL of stop solution, mixing the wells until all of the blue color changed to yellow.

Samples and standards were analyzed by measuring the relative absorbance (A/A_0), which is the absorbance at 450 nm observed for a sample (A) divided by the absorbance of the negative control (A_0). Measurements at 450 nm were made using a microtiter plate reader (Microwell EL301).

Calibration Curves by CE and HPLC. A stock solution of the concentration of 1000 mg L^{-1} was prepared by dissolving 100 mg of terbuthylazine in 100 mL of methanol. Appropriate dilutions of this stock solution were made in tap water to obtain final concentrations of 1, 2, 4, 6, and 8 mg L^{-1} for CE calibration curves and in methanol/tap water solution (50/50 v/v) to obtain final concentrations of 0.5, 1, 2, 4, 6, and 8 mg

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Table 1. Absorbance of Immunoassay as a Function of Terbuthylazine Concentration in Tap Water and Groundwater Samples

water sample	terbuthylazine (μg L^{-1})	absorbance
tap water	0	1.206
	0.1	1.144
	0.2	1.116
	0.4	0.940
	0.8	0.873
	1.6	0.761
	2.4	0.613
	3.2	0.562
mean ± SE[a]		0.902 ± 0. 040
CV (%)		4.4
groundwater	0	1.084
	0.1	0.985
	0.2	0.955
	0.4	0.910
	0.8	0.874
	1.6	0.714
	2.4	0.634
	3.2	0.530
mean ± SE[a]		0.836 ± 0.114
CV (%)		13.6

[a] Standard error (n = 16).

L^{-1} for HPLC calibration curves. For each concentration, triplicate injections were made in HPLC or CE.

Extraction for CE and HPLC Analysis. Extraction of the ai from the aqueous solutions was performed by a C_{18} solid-phase extraction (SPE) column (Bakerbond), consisting of 500 mg of C_{18} (octadecylsilane) resin linked to silica gel with an average 40 μm particle size. Prior to analysis, the 1 L samples (tap and ground waters) were filtered through Whatman No. 3 paper. However, preliminary experiments showed that 1 L of sample was not sufficient to concentrate detectable amounts of terbuthylazine at 0.2 μg L^{-1} by CE. Consequently, for this concentration and for CE only the extraction was effected from a 2 L sample. The SPE columns were conditioned with 3 mL of ethyl acetate, followed by 2 mL of methanol and 2 mL of HPLC-grade water; the solvents were run through by gravity drop. Then, 2 mL of HPLC-grade water was added and the sample aspirated by vacuum pump at a flow rate of 25 mL min^{-1}. The column was subsequently vacuum-dried for 10 min and, with the vacuum pump off, was eluted by gravity with an appropriate amount of ethyl acetate. The 0.2 and 0.8 μg L^{-1} samples were concentrated 2000 times, while the 1.6 and 3.2 μg L^{-1} samples were concentrated 1000 times. All samples were stored at −12 °C until analysis.

CE Analysis. Separations were performed using micellar electrokinetic capillary chromatography (MECC), by means of the capillary electrophoresis apparatus P/ACE System 2000 (Beckman). Separations were made with a silica-fused capillary 50 cm long (from injection point to detector), 75 μm internal diameter (i.d.), at a costant temperature of 30 ± 0.2 °C. Applied voltage was 25 kV. Detection wavelength was at 214 nm. Samples were injected at a constant pressure of 3.44 × 10^3 Pa at the capillary's anode end for 10 s. The electrolyte buffer was 50 mM sodium borate, 22.5 mM sodium dodecyl sulfate, and 10% acetonitrile (v/v), pH 8.0.

HPLC Analysis. The HPLC system was a Beckman System Gold 126 with two pumps and a Rheodine valve Model 7725-i (20 μL loop). The detector was a Beckman diode array Module 168. The column was a reversed-phase Ultrasphere (Beckman, C_{18}, 25 cm × 4.6 mm i.d., 5 μm particle size). The analyses were performed in isocratic and gradient conditions, because both are reported in the literature for terbuthylazine detection (Galassi *et al.*, 1990; Coquart and Hennion, 1991; Steinheimer, 1993). For the isocratic separations, the mobile phase was methanol/water (60/40 v/v) with a 1 mL min^{-1} flow rate, whereas for the gradient separations, the gradient elution was performed by increasing linearly the methanol percentage from 50% to 70% in 20 min. An injection volume of 20 μL and UV detection at 220 nm were used.

Table 2. Recovery of Terbuthylazine in Tap Water (1) and Groundwater (2) Samples by Immunoassay, HPLC (Gradient Separations, G; Isocratic Separations, I), and CE

sample concn (μg L^{-1})	% recovery			
	immuno	HPLC-G	HPLC-I	CE
	(1) Tap Water			
0.2	78.6	101.2	93.0	96.8
0.8	95.0	99.5	93.0	104.9
1.6	89.8	96.5	96.5	98.1
2.4	120.1	97.8	93.1	97.4
mean ± SE[a]	95.8 ± 7.6	98.7 ± 4.4	93.8 ± 1.1	99.3 ± 1.8
	(2) Groundwater			
0.2	101.1	99.6	105.6	87.5
0.8	78.5	85.0	97.7	97.7
1.6	77.3	93.1	99.1	98.6
2.4	108.3	82.1	89.3	93.3
mean ± SE[a]	91.3 ± 7.6	89.9 ± 3.2	97.9 ± 2.6	94.3 ± 2.5

[a] Standard error (n = 8).

RESULTS AND DISCUSSION

Standard Curves by Immunoassay. For tap water samples, a linear relationship between the natural logarithm of terbuthylazine concentration, in the range of 0.1−3.2 μg L^{-1}, and the relative absorbance (A/A_0) was found. The regression equation was $y = 0.67 - 0.13 \ln x$ ($r^2 = 0.975$), where x is the terbuthylazine concentration and y is A/A_0. By contrast, the equation that best related terbuthylazine concentration in groundwater to A/A_0 was a second-order polynomial ($y = 0.74 - 0.16 \ln x - 4.13 \ln x^2$, $r^2 = 0.988$). The two equations were employed to assign a concentration value to spiked tap water and groundwater samples, respectively. Table 1 shows that the coefficient of variation (CV) was significantly higher in groundwater than in tap water, suggesting that some matrix effect was present in the groundwater, and this may cause a less accurate detection of terbuthylazine, especially at low concentrations. The presence of chemical interferences is suggested by lower absorbances in blanks (−10.1%, Table 1) compared to tap water blanks.

Standard Curves by CE and HPLC. The calibration curves for CE quantitative determinations showed a linear instrumental response of the capillary-injected terbuthylazine in the 30−480 pg range, which matched the 10 s (60 nL) injection of standards with concentration in the range of 0.5−8 mg L^{-1}. The regression equation was $y = 0.065x$ ($r^2 = 0.995$), where y is the peak area and x is the concentration of the active ingredient (ai) in milligrams per milliliter.

The calibration curves for quantitative determination by isocratic and gradient HPLC separations showed linear responses in the 10−160 ng range of the injected terbuthylazine. These concentrations match the 20 μL injection of standard with concentrations from 0.5 to 8 mg L^{-1} ai. Regression equations obtained by isocratic and gradient separations were $y = 3.52x$ ($r^2 = 0.997$) and $y = 3.51x$ ($r^2 = 0.998$), respectively.

Determination of Terbuthylazine. Good recoveries were obtained for all methods tested (Table 2). The average recoveries by HPLC and CE were not different from those obtained by immunoassay. However, the mean coefficients of variation for immunoassay in tap water and in groundwater were 22.7% and 33.5%,

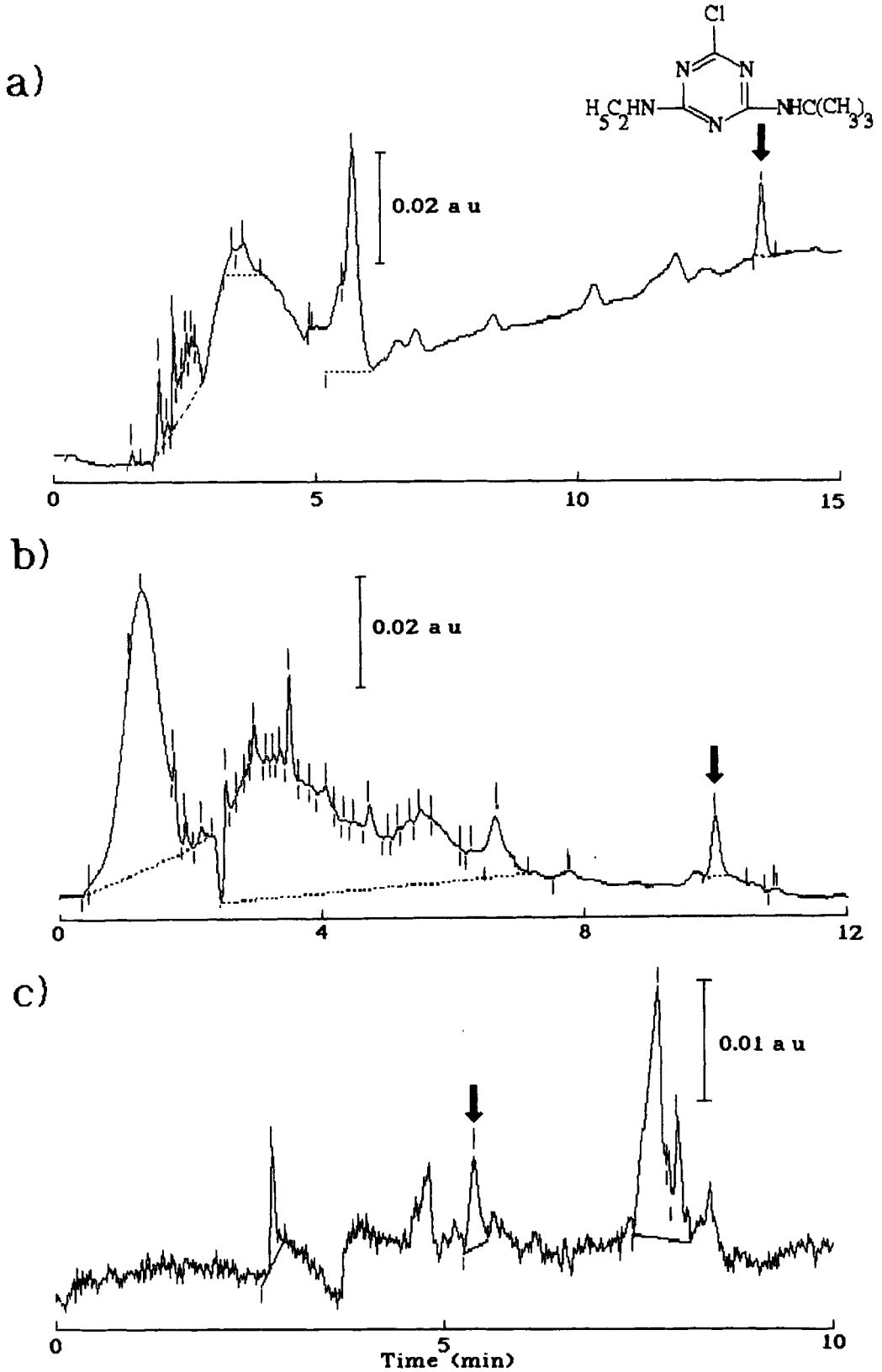

Figure 1. Terbuthylazine detection in groundwater samples spiked at 0.2 μg L^{-1} by (a) HPLC in gradient conditions, (b) HPLC in isocratic conditions, and (c) CE. The arrows indicate the terbuthylazine peak (au = absorbance unit). The chemical structure of terbuthylazine is also reported.

respectively, while the mean coefficients of variation for the instrumental determinations were in all cases less than 9% (data not shown). This indicates that the accuracy and precision of the immunoassay determination over the range of concentration tested were lower than those obtained for HPLC and CE, especially in groundwater samples.

The different positions of the matrix interferences in HPLC and in CE analysis, in advance and next to the ai peak, respectively, are shown in Figure 1. CE showed an on-column purification effect, as demonstrated by the higher percent area of the terbuthylazine peak in CE (20%) than that observed in HPLC (5%) with respect to the total compounds detected. This suggests an on-

Table 3. Effect of Increasing Rates of Metolachlor in Water on Terbuthylazine Detection by Immunoassay

spiked terbuthylazine + metolachlor (μg L^{-1})	terbuthylazine detected (μg L^{-1})	
	tap water	groundwater
0.20 + 0	0.16	0.29
0.20 + 100	0.19	0.67
0.20 + 500	0.20	1.00
0.20 + 1000	0.28	0.87
mean ± SE[a]	0.21 ± 0.03	0.71 ± 0.13
1.60 + 0	1.36	1.60
1.60 + 100	1.61	1.63
1.60 + 500	1.29	1.56
1.60 + 1000	1.07	2.02
mean ± SE[a]	1.33 ± 0.14	1.70 ± 0.13

[a] Standard error (n = 8).

Table 4. Correlation Parameters (Coefficient of Correlation, Slope, and Intercept, n = 8) among Methods (HPLC-G, Gradient Separations; HPLC-I, Isocratic Separations)

methods	R[a]	slope ± SE[b]	intercept
(1) Tap Water			
HPLC-G vs HPLC-I	0.997***	0.963 ± 0.027	− 0.006
HPLC-G vs CE	0.994***	0.990 ± 0.043	0.024
HPLC-I vs CE	0.996***	1.028 ± 0.036	0.025
HPLC-G vs immuno	0.975***	1.241 ± 0.113	− 0.209
HPLC-I vs immuno	0.967***	1.275 ± 0.137	− 0.191
CE vs immuno	0.963***	1.231 ± 0.139	−0.211
(2) Groundwater			
HPLC-G vs HPLC-I	0.992***	1.073 ± 0.052	0.015
HPLC-G vs CE	0.995***	1.133 ± 0.044	− 0.035
HPLC-I vs CE	0.989***	1.042 ± 0.062	− 0.037
HPLC-G vs immuno	0.915**	1.261 ± 0.226	− 0.202
HPLC-I vs immuno	0.905**	1.154 ± 0.221	− 0.195
CE vs immuno	0.916**	1.108 ± 0.198	− 0.156

[a] ***, significant at $P \leq 0.001$; **, significant at $P \leq 0.01$. [b] Mean ± standard error.

column exclusion of some interference compounds during the CE separation. Furthermore, CE separation (Figure 1c) is more rapid than HPLC. In isocratic and gradient conditions, HPLC retention times of terbuthylazine were 10.25 ± 0.15 and 13.01 ± 0.08 min, respectively, while in CE it was 5.37 ± 0.07 min.

In spite of apparent values up to 3.4-fold higher than the spiked level of terbuthylazine, analysis of variance revealed that increasing metolachlor concentration from 0 to 1000 μg L^{-1} had no significant effect on the determination of terbuthylazine with immunoassay at 0.2 and 1.6 μg L^{-1} in tap water and groundwater (Table 3). However, terbuthylazine determination was significantly overestimated in groundwater at 0.2 μg L^{-1}, probably as an effect of the lower accuracy of immunoassay for groundwater samples at lower concentrations.

Correlation Among Methods. Regression analysis yielded coefficients of correlation (R) between the immunoassay and the instrumental determinations (HPLC and CE) in tap water and groundwater significant at $P < 0.001$ and $P < 0.01$, respectively, while the coefficients of correlation between CE and HPLC were all highly significant at $P < 0.001$ (Table 4). These results confirm the lower accuracy of immunoassay for terbuthylazine determination in groundwater samples. The slope of the regression lines correlating HPLC and CE, for tap and ground waters, was not greater than 1, and the intercepts were not different from 0, demonstrating that HPLC and CE were correlated near the theoretical optimum value. In contrast, the slope of the regression lines for the correlation between instrumental methods and immunoassay was greater than 1 and the intercepts were not different from 0. This indicates that the immunoassay kit overestimates the dose of terbuthylazine.

Conclusion. The present data confirm the viability of CE in the detection and quantitation of terbuthylazine in water at micrograms per liter levels. CE shows potential advantages over HPLC, such as shorter retention times and a related interesting cost-benefit analysis, due to low consumption of solvents in CE too. However, CE has a higher detection limit than HPLC, because CE requires a volume of injection in the nanoliter order. In general, CE application on a routine scale for the analysis of herbicides is restricted by the relatively fewer developed methods and literature sources on CE, mainly due to the much shorter history of CE with respect to the other chromatographic techniques.

The immunoassay kit for the detection of terbuthylazine offers many advantages over chromatographic procedures (HPLC and CE) used to detect and quantitate the ai in water at micrograms per liter level. These advantages include the speed of analysis, the high number of samples that can be processed in a day, and time reductions in sample preparation and cleanup procedures. In effect, our data show that mean recoveries from immunoassay are comparable to those obtained by HPLC and CE, that immunoassay and instrumental determinations are well correlated, and that metolachlor, a herbicide often used with terbuthylazine, has a negligible cross-reactivity with the immunoassay. However, immunoassay is less precise and accurate than HPLC and CE, especially in groundwater, but has a great potential as a rapid screening qualitative test prior to accurate HPLC or CE measurements. Great attention has to be paid when this kit is applied to basin- and territorial-scale researches, where other s-triazines or terbuthylazine metabolites may cross-react with immunoassay.

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