Biomarkers, July–August 2006; 11(4): 291–305

informa
healthcare

Analysis of herbicides: demonstration of the utility of enzyme immunoassay verification by HPLC

R. G. PRICE[1], I. BARANOWSKA[2], H. M. T. GRIFFITH[1], R. A. ABUKNESHA[1], & H. BARCHANSKA[2]

[1]*School of Biomedical and Health Sciences, Analytical Research Group, Pharmaceutical Science Division, King's College London, University of London, London, UK and* [2]*Department of Analytical and General Chemistry, The Silesian University of Technology, Gliwice, Poland*

Abstract

Evidence has accumulated that herbicides in the environment present a significant health hazard to the population. Therefore, the levels of heavily used substances such as atrazine and simazine and their metabolites need to be regularly assessed. The objective was to develop a rapid and simple tube ELISA procedure suitable for use in field studies and non-specialized laboratories. The antisera used were polyclonal antibodies raised in sheep against atrazine or simazine amido caproic acid conjugated to bovine serum albumin. The antibodies were first used to construct a two-step competitive ELISA procedure in 96-well microtitre plates. The 96-well format was then adapted to a coated-tube enzyme immunoassay, by immobilization of hapten-gelatine conjugates on polystyrene tubes. This enabled the colour to be read using a basic spectrophotometer. Soil samples were collected from agricultural and non-agricultural sites in Poland. Atrazine and simazine were extracted by liquid extraction from soil and assayed by tube ELISA. In addition, the samples were extracted by solid-phase extraction before analysis by HPLC. The immunoassays and chemical analysis were carried out by different individuals who were unaware of each other's results, which were then compared at the end of the study. Correlation of the two methods was excellent, with $R = 98.7$ and 81.3 for atrazine and simazine, respectively. The immunoassay yielded the same order of results without having to perform solid-phase extraction before analysis. The study has demonstrated that the simple antigen-coated tube assay provides a cost-effective and valuable screening test. Comparison with the more elaborate, heavily labour-intensive HPLC analysis demonstrated that the results obtained by the simpler enzyme-immunoassay tests were within the same order.

Keywords: *Atrazine, simazine, HPLC, tube ELISA, soil samples*

(Received September 2005; accepted February 2006)

Introduction

Atrazine and simazine are systemic herbicides that are absorbed via the roots (simazine) or both roots and leaves (atrazine) of plants. They affect photosynthesis by disruption of the electron transport chain in photo complex II (Gysin & Knuesli 1960, Kobbia et al. 2001). Atrazine is most widely used on corn and sorghum; whereas simazine is used on higher value crops, e.g. citrus (US Environmental

Correspondence: R. G. Price, School of Health and Life Sciences, Analytical Research Group, Pharmaceutical Science Division, King's College London, University of London, 150 Stamford Street, London SE1 9NN, UK. Tel: 44-(0)7848-4451. Fax: 44-(0)7848-4500. E-mail: robert.price@kcl.ac.uk

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500600625729

Protection Agency 2005a, 2005b). They are low cost and atrazine is used more heavily (between 70 000 and 90 000 tons worldwide per year (Hayes et al. 2002). They are relatively mobile in the environment and migrate readily through soil into the aquatic environment (Lee et al. 1999). Their half-life in freshwater is 8–350 days (Diana et al. 2000). Both atrazine and simazine have been detected in rainfall, river and lake water, with levels varying between 0.1 and 40 ng l^{-1} (Hayes 2004). However, much higher levels of 445 ng l^{-1} have been reported in surface waters (Hall et al. 1993). The triazine herbicides are endocrine-disrupting compounds (Bisson & Hontela 2002, Hayes 2004). Atrazine affects the sexual maturation in males of a number of species including frogs (Hayes 2004), rats (Stoker et al. 2002) and Atlantic salmon (Moore & Waring 1998). In humans there appears to be a causal link between long-term exposure of triazines (especially atrazine) with breast, ovarian (Kettles et al. 1997) and prostate cancer (Hayes et al. 2002).

Simazine and atrazine are also used to control weeds on roadside verges, railway lines and footpaths. They are easily leached from the sites of application to surface water, drains or water courses (Meakins et al. 1995). In addition, they are used seasonally (Albanis et al. 1998, Garmouma et al. 1998, Steen et al. 2001). Recently, the European Union Commission published policy documents regarding the use of these herbicides (EU 2004a,b) and under this legislation a number of countries are still permitted to use atrazine and simazine, but the total contamination level of these pesticides and their breakdown products should not exceed 0.5 μg l^{-1} (EU 1980). In the USA, higher levels are acceptable, e.g. contamination in drinking water is of 4 and 3 μg l^{-1} for atrazine and simazine, respectively (US Environmental Protection Agency 1988). Evidence has accumulated that this heavily used class of herbicides may present a significant health hazard to consumers with emphasis on the possible long-term effects on developing endocrine systems. Therefore, the levels of the various parent compounds (mainly atrazine and simazine) and metabolites need to be assessed regularly to meet the legislative minimum levels.

If a management programme is to be effective, frequent analysis of samples from the same areas is required throughout the year. This will necessarily place considerable costs on laboratories, which will require complex equipment and highly skilled laboratory staff trained in different areas of analysis. Soil samples need special treatment before analysis since pesticides have to be extracted from soil matrices. These compounds are usually measured following a variety of extraction procedures (Pacakova et al. 1996, Tekel & Hatrik 1996, Tadeo et al. 2000). Liquid chromatography using thermospray mass spectrometric (LC-TSP-MS) is one of the best methods available for pesticide analysis. Liquid chromatography with ultraviolet detection (LC-UV) (Tekel & Hatrik 1996, Matsui et al. 2000, Pinto & Jardim 2000) and diode array detection (HPLC-DAD) are used for the determination of triazines. Diode-array detection can be used to scan the entire range of the UV-VIS spectrum, allowing interfering or overlapping peaks to be identified (Dean et al. 1996, Scriber et al. 2000, Prosen & Zupancic-Kralj 2005).

The advantage of immunological methods is that they require a less complex sample treatment, are cost-effective, are rapid to perform and allow a higher throughput (Durand & Barcelo 1991, Albanis et al. 1998, Kramer et al. 2001) than HPLC. Procedures have been developed for the various classes of herbicides, pesticides and pollutants from wastewater and industrial pollutants (Goodrow et al. 1990, Harrison et al. 1991, Sherry 1997, Lee & Kennedy 2001). Enzyme-linked immunosorbent assay

(ELISA) is normally carried out in microtitre plates that require specialized instruments and experienced personnel. This limits the use of ELISA to well-equipped laboratories. Initially solid-phase immunoassays based on antibody-coated polystyrene test tubes were used (Catt & Tregear 1967, Engvall & Perlmann 1972), which only required a basic spectrophotometer or fluorimeter. This method is easier to carry out since the larger volumes are easier to manipulate for less experienced workers and avoids the use of the 96-well format and associated equipment. Some tube-based procedures are available commercially Atrazine Tube Kit (Beacon Analytical Systems, Inc., Portland, ME 04103, USA) as well as an assay based on microparticle agglutination inhibition for atrazine (Li et al. 2003; Sherry & Borgmann 1993).

In the present study, the utility and advantages of an alternative antigen-coated tube ELISA using antisera developed in-house are described. Soil samples from several areas in Poland were analysed for atrazine and simazine using both the coated tube enzyme immunoassay and by an established HPLC procedure. Analysis of extracted soil samples was carried out by blind procedures and the results for ELISA and HPLC were compared at the end of the study.

Materials and methods

Materials

Atrazine and simazine were obtained from Reidel-de-Haën (Seelze, Germany). Polystyrene ELISA star-bottomed high-bind tubes and 96-well microtitre high-bind ELISA plates were obtained from Greiner Bio-One Ltd., Brunel Way, Stroudwater Business Park, Stonehouse, Gloucestershire, UK. Phosphate-citrate tablets, pH 5, PBS sachets (0.138 M NaCl, 0.0027 M KCl, pH 7.4), fish gelatine, Tween 20, goat anti-sheep-horse radish peroxidase conjugate, 2,2′-azino-*bis*(3-ethylbenzothiazolone-6-sulphonic acid) di-ammonium salt (ABTS) tablets were from Sigma Chemical Co. (Poole, UK). Acetonitrile, methanol and water for HPLC analysis (HPLC grade) were obtained from Merck (Darmstadt, Germany). Bakerbond solid-phase extraction (SPE) columns packed with 500 mg aromatic sulfonic acid columns were obtained from J. T. Baker (Deventer, the Netherlands). The following reagents were obtained from POCh S.A. Gliwice Poland: acetonitrile, acetic acid, citric acid, hydrogen peroxide, methanol, potassium phosphate dibasic, sodium acetate and sodium fluoride, all analytical grades.

Apparatus

HPLC analysis was carried out using an L-6200 A liquid chromatograph with an L-4500 diode array detector (Merck Hitachi). The columns used were C_{18} columns (25 cm $\times$ 4.6 mm i.d., 5 μm); SPE-12G and $PhSO_3H$ SPE columns were purchased from J. T. Baker; spectrophotometric measurements were made with a SPEKOL 10 (Carl Zeiss, Jena, Germany).

Methods

Extraction of soil samples. The soil samples were collected from a range of different Polish sites, according to the Polish Norm (PN-11466) protocol (Table III). The soil samples were dried by heating them in an oven and sieved through a 0.102 mm mesh. Chloroform (100 ml) and soil (100 g) were placed in a 300-ml flask. The mixtures were

allowed to stand for 24 h after which they were shaken vigorously using a shaker for 2 h (Pieszko & Baranowska 2000). After filtration through Whatman filter paper, the chloroform was allowed to evaporate at ambient temperature until a residue remained.

Sample preparation for HPLC analysis. The residue from the chloroform extraction was dissolved in 5 ml acetonitrile. Columns packed with 500 mg aromatic sulfonic acid washed with 25 ml acetic acid/HPLC grade water (1:99) and 5 ml soil extract were aspirated through the column. The column was then washed with 2 ml acetic acid/HPLC-grade water (1:99), followed by 1 ml acetonitrile, 3 ml HPLC-grade water and finally 1 ml 0.1 M potassium phosphate dibasic. The column was allowed to air dry under vacuum after each addition. Triazine herbicides were eluted with 2 ml acetonitrile/0.1 M potassium phosphate dibasic (1:1). Three samples were prepared simultaneously from each soil sample and three determinations were carried out on each sample.

Chromatographic conditions. Samples were first concentrated on $PhSO_3H$ solid-phase extraction columns and then analysed by HPLC using a C_{18} column. Maximum sensitivity for atrazine and simazine was achieved at 224 nm. Analyses were performed using isocratic elution at flow rate of 1.0 ml min^{-1}. The mobile phase contained methanol:water (1:1) (Baranowska et al. 2005).

Sample preparation for ELISA analysis. The residues from 100 g extracted soil were taken up into methanol (1 ml). Phosphate-citrate buffer (9 ml) was added to the methanol solution; aliquots (0.1 ml) of the buffered solution were then further diluted to 5 ml in the same buffer (1/50 dilution). Duplicate samples (1.5 ml) of the diluted soil extracts were transferred into glass test tubes. Finally, 0.75 ml of the appropriate diluted antisera were added and the assay performed as described below.

Antisera. Polyclonal antisera to atrazine and simazine were generated in sheep using hapten-caproic acid derivatives according to Goodrow et al. (1990). The plate-coating antigens were prepared using triazine-*O*-phenoxy benzoic acid derivatives and gelatine according to a published procedure. The characterization of antisera and optimization of the solid-phase competitive enzyme immunoassays were performed according to a published procedure (Abuknesha & Griffith 2005).

Tube and plate enzyme-immunoassays. ELISA tubes (Greiner BioOne) were coated with 1 ml gelatine conjugates (tube-coating antigen complexes) of either atrazine-*O*-phenoxybenzoic acid or simazine-*O*-phenoxybenzoic acid at 0.2 μg ml^{-1} diluted in buffer made from PBS sachets (Sigma). The coating of the ELISA tubes was carried out for 3 h at room temperature before storage at $-20^{\circ}C$ for 16 h. After washing four times with 2.5 ml PBS containing 0.05% Tween 20, ELISA tubes were blocked for 50 min with 2.5 ml blocking buffer (PBS with 0.5% fish gelatine; Sigma Aldrich Ltd). The coated and blocked ELISA tubes were washed four times before assay. Competitive immunoassays for both compounds were performed in the same format. The appropriate antiserum (0.75 ml) was incubated in duplicate in glass test tubes. The optimal dilutions of antisera made in PBS with 0.5% fish gelatine were 1/20 000 and 1/40 000 for atrazine and simazine, respectively. Analyte solutions (atrazine and simazine, 1.5 ml) were prepared in phosphate-citrate buffer pH 5 starting at

10 ng ml^{-1} and diluted by one-third to provide a range of 10, 3.34, 1.12, 0.37, 0.12, 0.04 and 0.01 ng ml^{-1}; zero reference was buffer only. The incubation of antibody and analyte was carried out for 1 h at 37°C with washing as above. At the end of the incubation period, 1 ml of the antibody and of the analyte mixture were transferred to the antigen-coated blocked ELISA tubes and left for 20 min with shaking at room temperature before the ELISA tubes were washed as above.

Goat-anti-sheep-HRP conjugate (1/2000, 1 ml) was added after washing as above to the ELISA tubes and incubated for 1 h at 37°C. The colour was developed, after washing as above, by adding 1 ml of a solution of ABTS (50 mg) and hydrogen peroxide (40 μl) in 100 ml sodium acetate-citrate buffer, pH 4.1. After standing for 10 min, the reaction was stopped with 50 μl 2% sodium fluoride. The absorbance was measured at 405 nm using a spectrophotometer (SPEKOL 10).

Standard ELISA assays using 96-well microtitre plates were performed using the same reagents and procedure except that the assay volumes were 0.15 ml, plates were washed using a plate washer (Ultrawash plus, Dynex, West Sussex, UK) and the colour was read using a standard microtitre plate reader (Anthos 2001, Salzburg, Austria).

The cross-reactivities of the antisera and assays were assessed by ELISA according to Abraham (1975) and Butler (1975), and the cross-reaction levels of related triazines were calculated using the 50% inhibition procedure, taking atrazine or simazine as 100% for the respective assays.

The recovery of spiked atrazine and simazine aqueous samples was assessed by using three levels of the analytes: 0.1, 2 and 5 ng ml^{-1}. Each spiked level was assessed in triplicate with the respective tube assays on each of 2 days, alongside the calibrant solutions.

The effect of added methanol (5, 10 and 20%, v/v) on the analyte dose–response curves was assessed according to the method of Abuknesha & Griffith (2005).

Statistics

Fitted line regression analysis and Spearman rank correlation were carried out using Minitabs Statistical Software (v.13 for Windows; Minitab Ltd, Coventry, UK).

Results

Validation of immunoassay procedures

Dose–response curves for atrazine and simazine were virtually identical when the antigen-coated procedure was carried out using tubes or microtitre plates (Figures 1 and 2). Interestingly, the coating antigen complexes and antisera were used at the same dilution for both the tube and the microtitre plate assays. This indicates that the assay signal was proportional to the concentration rather than to the total amount of reagents or assay volume. It was therefore possible to compare directly the standard ELISA procedure with the coated tube assay.

The response (absorbance) of the calibration curves shown by the two assays, the standard plate format and in the coated-tube format, was highly comparable. Figure 1A and 2A show the similarity of these two formats for atrazine and simazine, respectively. To compare directly the dose–response curves, the obtained response (absorbances) using the two formats were normalized (Figure 1B and 2B) for atrazine

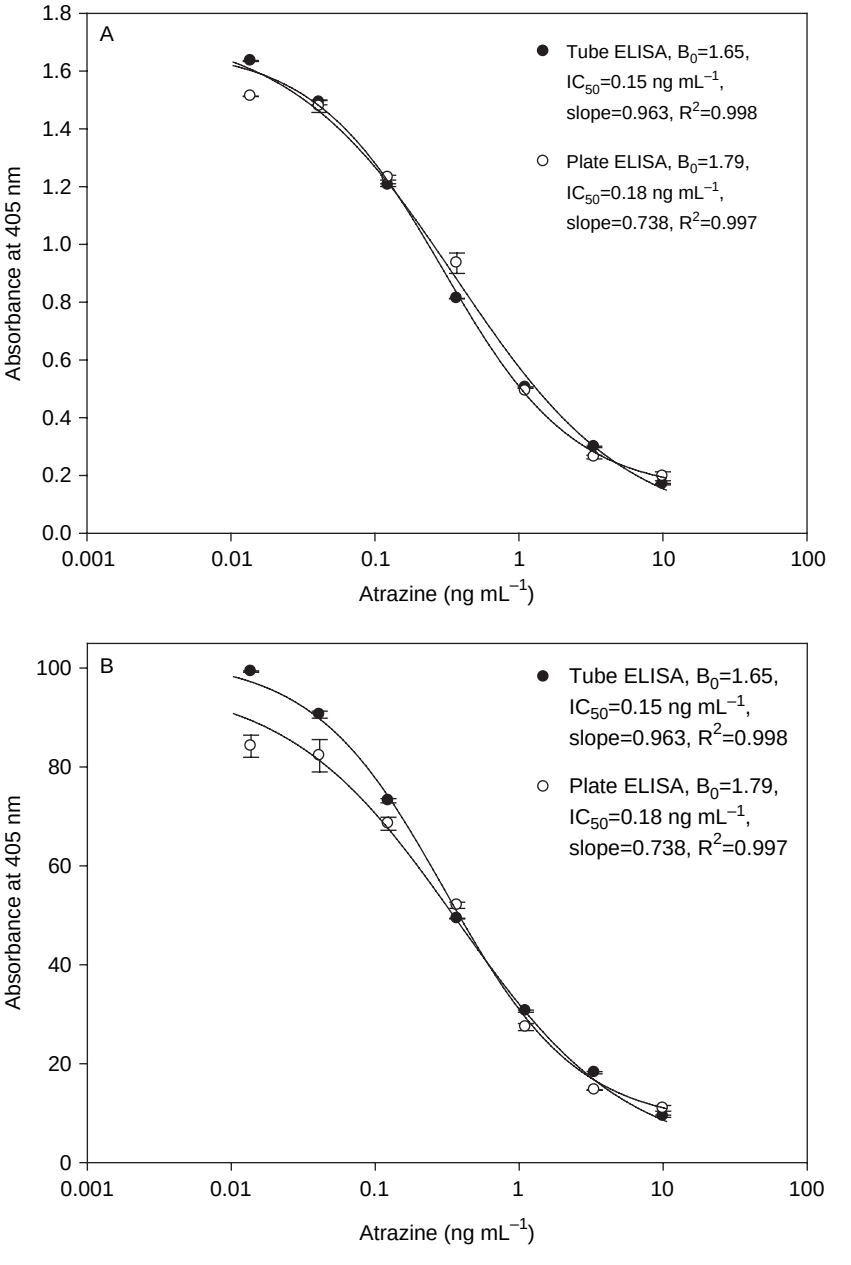

Figure 1. Atrazine dose–response curves using the tube and plate format. Both ELISA formats were constructed using atrazine-*O*-phenoxy acetic acid-gelatine conjugate as the coating antigen. The assay parameters (B_0, slope, IC_{50}, R^2) are essentially the same for both assays. The dose–response graphs were taken from concentration against absorbance (A) and the normalized response (B).

and simazine, respectively. The concentration of standard analyte that inhibited antibody binding by 50% (IC_{50}), the binding at zero analyte concentration (B_0) and the slope of the curves were virtually identical when the enzyme immunoassay was carried out in either a plate or coated-tube format. The agreement between

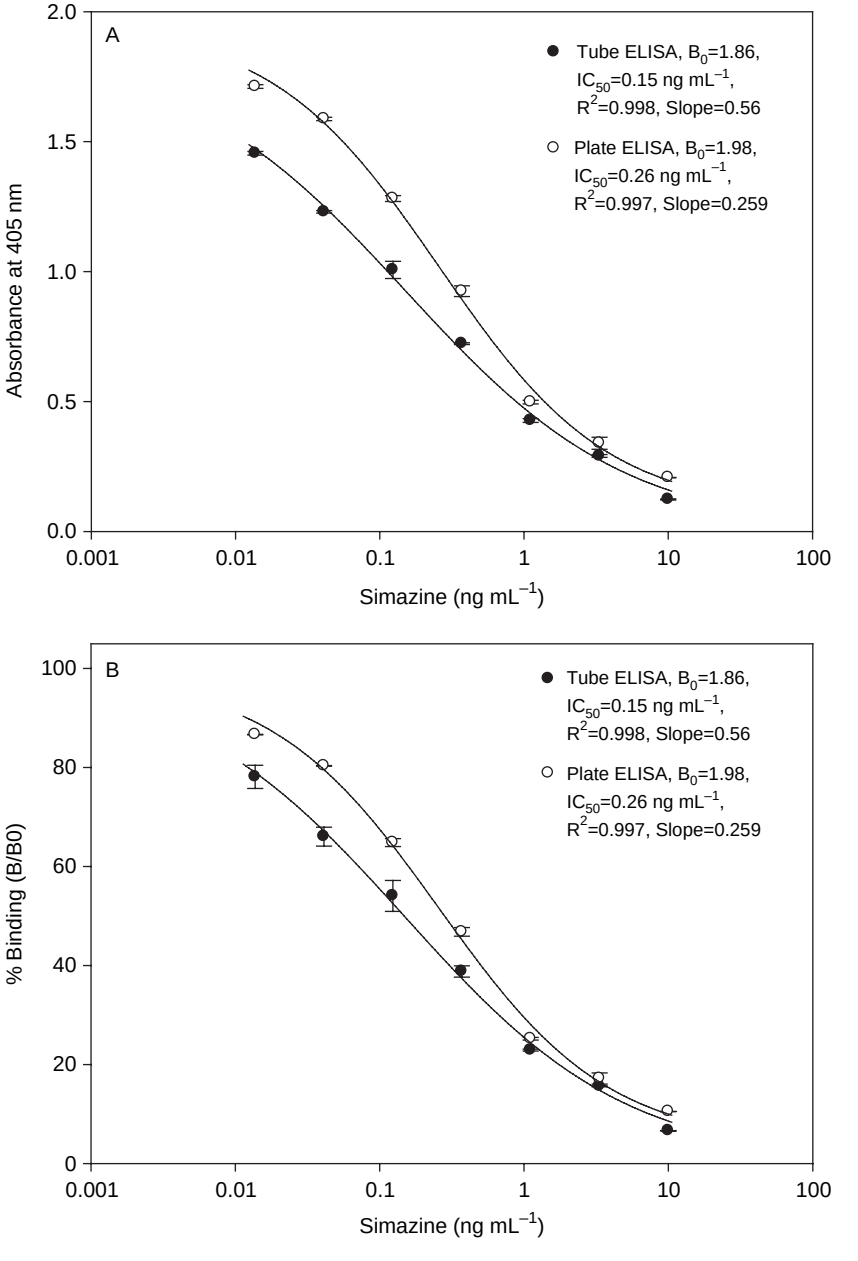

Figure 2. Simazine dose–response curves using the coated tube and microtitre plate formats. See Figure 1 for further details.

concentration and response was good as defined by the R^2 values. The minimum detection limits (the concentration that gave 90% inhibition) were estimated to be about 0.012 ± 0.002 ng ml^{-1} (0.012 µg l^{-1}) for both assays. The cross-reaction values shown by the two antisera are given in Table I. Both antisera showed low cross-reactivities to simetryn and, importantly, to desethylatrazine, which is a major metabolite of atrazine. However, as is well documented (Wortberg et al. 1996,

Table I. Cross-reactivity of the atrazine and simazine antisera with a selection of *s*-triazine compounds.

Compound	Percentage cross-reaction	
	Anti-atrazine	Anti-simazine
Atrazine	100	101.1
Prometryn	177.4	0.4
Ametryn	126.5	3.6
Propazine	94.9	18.0
Desethylatrazine	6.9	2.9
Simazine	59.7	100
Simetryn	3.9	1.6
Irgarol 1051	7.4	0.5
Terbuthylazine	1.2	127.7

Values are percentage cross-reactivity.

Thurman & Aga 2001) with other immunoassays for this class of compounds both antisera showed high cross-reactivities to a number of the *s*-triazine compounds.

Recoveries of the three spiked levels of aqueous samples were between 83 and 111%, which is within the acceptable values for immunoassay screening methods.

The addition of increasing percentages of methanol (5, 10 and 20%) to the analyte buffer over the range 0.002–10 ng ml^{-1} atrazine or simazine for the respective assays (Figure 3) did not adversely affect the performance of either assays. The dose–response curves were only marginally affected by 5 and 10% methanol. However, 20% methanol caused the dose–response curves to show slightly lower absorbances and become flatter.

Analysis of the herbicide content in soil samples by ELISA and HPLC

The amount of each herbicide recovered in the soil samples determined by HPLC and ELISA are shown in Tables III and IV. Regression analysis of the two sets of data found that atrazine values had $r = 98.6\%$ and simazine $r = 81.4\%$ (Figures 4 and 5).

The Spearman rank correlation coefficients between HPLC and ELISA were 0.993 for atrazine with $p < 0.0005$ and 0.936 for simazine with a $p < 0.0005$, using HPLC as the predictor and ELISA as the response. These data indicate that the procedure of extraction atrazine and simazine from soil samples was suitable for both methods. In addition, the determination of these compounds could be carried out effectively by

Table II. Coefficient of variation (%) and percentage recovery of atrazine or simazine recovered in the tube assays. The assays were performed with spiked samples in the Polish laboratory as described in the Materials and methods.

Spiked level (ng ml^{-1})	Atrazine assay				Simazine assay			
	Day 1		Day 2		Day 1		Day 2	
	Percentage recovery	% CV	Percentage recovery	% CV	Percentage recovery	% CV	Percentage recovery	% CV
5	111.6	0.4	97.8	3.6	86.9	0.7	85.6	1.5
2	106.4	3.3	117.2	1.0	83.6	5.2	98.5	1.9
0.1	101.9	4.1	102.3	3.7	87.8	8.4	86.2	1.9

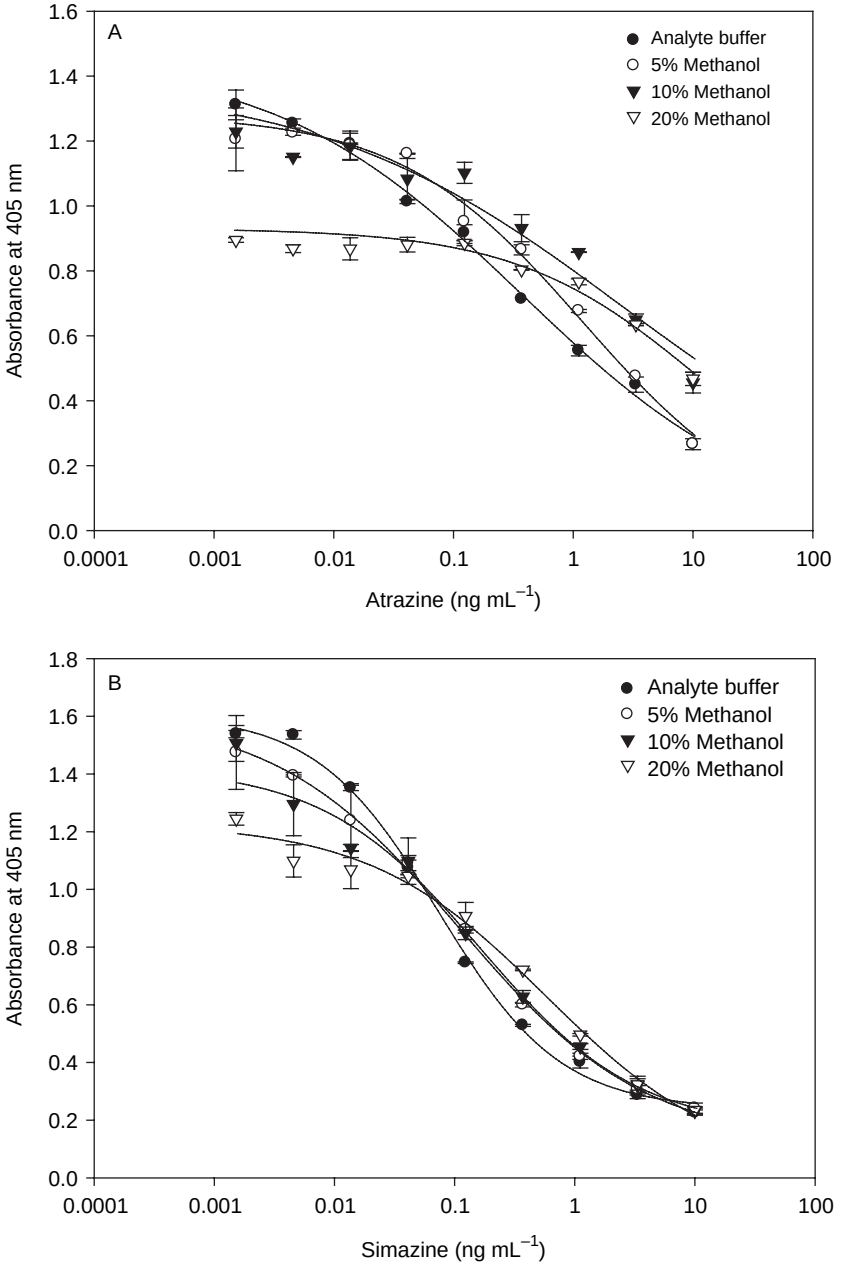

Figure 3. Effect of methanol on the dose–response curves for atrazine (A) and simazine (B) over the calibration range of 0.002–10 ng ml^{-1}. Both assays appear to tolerate up to 10% methanol.

either procedure. However, the ELISA procedure proved to be more sensitive since in some of the soil samples the levels of atrazine and simazine were too low to be detected by HPLC. The results obtained with samples obtained from various localities and type therefore provided an opportunity to assess the value of the simpler coated-tube enzyme-immunoassay method carried out in a non-immunoassay laboratory, analytical chemistry environment.

Table III. Estimation of atrazine levels in soil samples by ELISA and HPLC (ng atrazine g^{-1} of soil) as determined separately by either ELISA or HPLC analysis. Analysis was carried out by different workers who were unaware of the results until the completion of the study. The type of cultivation of the soil is indicated.

		ELISA				HPLC			
Sample	Cultivation type	Day 1	Day 2	Day 3	Mean±SD	Test 1	Test 2	Test 3	Mean±SD
1	Meadow	2.0	1.6	2.0	1.9±0.3	2.6	3.0	2.8	2.9±0.2
2	Meadow	6.1	6.1	6.8	6.3±0.4	6.1	6.6	6.2	6.0±0.2
3	Meadow	4.3	3.2	3.9	3.8±0.6	3.0	3.8	3.4	3.4±0.4
4	Meadow	0.6	0.7	0.6	0.7±0.1	0.7	0.8	0.8	0.8±0.1
5	Meadow	2.1	1.5	1.0	1.5±0.6	1.0	1.2	1.0	1.0±0.1
6	Meadow	4.5	4.0	4.6	4.4±0.3	5.3	5.1	5.8	5.4±0.4
7	Orchard	4.9	2.6	2.1	3.2±1.5	2.8	3.0	4.2	3.3±0.7
8	Wheat field	1.0	0.8	0.9	0.9±0.1	1.0	1.5	1.1	1.2±0.2
9	Wheat field	11.3	9.4	10.9	10.6±1.0	12.6	13.8	12.3	12.4±0.2
10	Strawberry field	2.4	2.3	2.0	2.2±0.2	2.7	3.0	2.2	2.6±0.4
11	Strawberry field	6.5	2.7	4.5	4.6±1.9	6.1	5.5	5.2	5.6±0.4
12	Beet fields	11.5	4.8	7.8	8.0±3.4	7.2	6.6	6.0	6.6±0.6
13	Orchard	1.1	0.6	0.6	0.8±0.3	n.d.	n.d.	n.d.	–
14	Wheat field	25.0	17.9	16.5	19.8±4.6	18.0	20.1	18.8	19.0±1.1
15	Wheat field	21.1	19.8	21.7	21.0±1.1	18.6	25.4	17.2	21.4±4.4
16	Wheat field	46.9	38.6	52.5	45.9±7.2	39.2	37.2	40.8	39.1±1.8
17	Meadow	9.3	9.4	8.8	9.2±0.3	8.9	7.8	8.5	8.4±0.5

Discussion

The principal objective of this study was to assess the utility and value of antigen-coated tube enzyme-immunoassays in the measurement of two commonly used herbicides. It was particularly important to establish whether this procedure was

Table IV. Estimated concentrations of simazine levels in soil samples by ELISA and HPLC (ng simazine g^{-1} soil) in the extracted soil samples analysed by either ELISA or HPLC. For further details, see Table III.

	ELISA				HPLC			
Sample	Day 1	Day 2	Day 3	Mean±SD	Test 1	Test 2	Test 3	Mean±SD
1	1.3	1.7	2.4	1.8±0.6	1.5	1.7	1.7	1.6±0.2
2	3.0	3.9	3.0	3.3±0.5	2.2	4.4	2.6	2.4±0.3
3	1.1	2.5	1.6	1.8±0.7	1.7	2.9	1.1	1.9±0.9
4	–	0.2	0.0	0.1±0.1	–	–	–	–
5	2.6	5.0	6.2	4.6±1.8	4.4	4.3	4.8	4.5±0.3
6	0.9	2.0	1.6	1.5±0.6	1.6	1.7	1.4	1.5±0.2
7	0.3	1.2	1.3	1.0±0.5	–	–	–	–
8	2.1	0.4	0.1	0.9±1.1	–	–	–	–
9	1.5	2.9	2.5	2.3±0.7	3.9	3.1	3.5	3.8±0.4
10	3.2	6.3	7.8	5.8±2.3	6.7	6.6	6.9	6.7±0.2
11	8.7	7.2	12.8	9.6±2.9	8.7	4.8	7.8	8.2±0.6
12	3.7	4.4	4.4	4.2±0.4	3.2	5.0	5.1	4.4±1.0
13	–	0.4	0.7	0.4±0.3	1.5	0.9	1	1.1±0.1
14	5.8	8.4	6.5	6.9±1.3	6.5	5.1	5.5	5.7±1.0
15	3.6	5.4	10.6	6.5±3.6	2.0	2.8	3.1	2.6±0.5
16	5.1	9.1	19.2	11.1±7.3	10.9	15.4	12.5	12.9±2.3
17	0.2	0.2	0.2	0.2±0.0	1.1	1.0	1.2	1.1±0.1

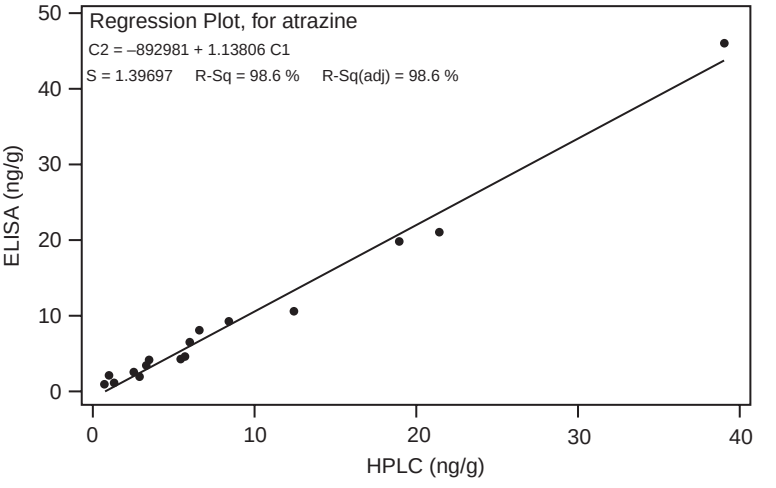

Figure 4. Regression plot of atrazine ELISA data compared with HPLC results with the same samples; the estimation of levels is in ng g^{-1} soil.

suitable for use in a laboratory that lacked immunoassay equipment, therefore making the procedure more widely available. The advantages of the coated-tube enzyme-immunoassay procedure are its high sensitivity, basic specificity, simplicity and ease of performance. The difficulties encountered in handling small volumes are avoided and the procedure is much more flexible than the 96-well plate format, making it more useful for environmental and field screening studies. The coated-tube enzyme-immunoassays can be performed by less skilled workers in a field situation, allowing the test to be performed nearer the sites of potential contamination. In the present study, the developed test proved robust and could be readily incorporated into screening programmes and would not require the involvement of a high-tech laboratory.

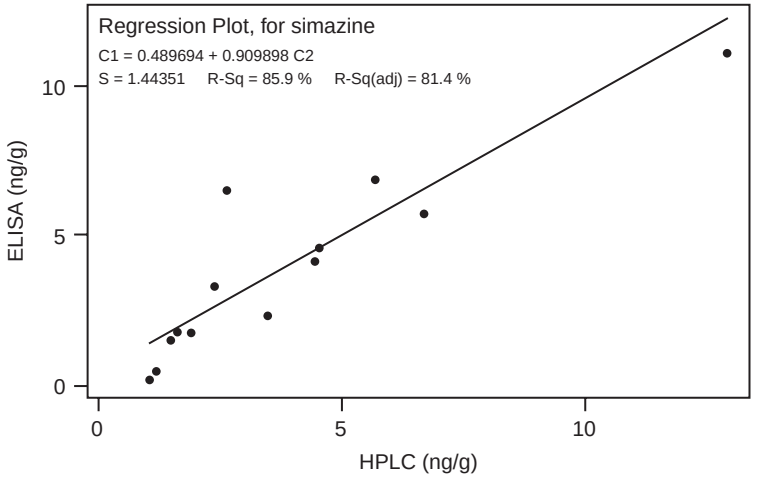

Figure 5. Regression plot of simazine ELISA data compared with HPLC results with the same samples; the estimation of levels is in ng g^{-1} soil.

The results obtained demonstrated that the tube assay for atrazine and simazine showed a high degree of correlation with those obtained using the standard 96-well plate ELISA, indicating that the tube assay could be used for direct assay measurements if required. The minimum detection limit ($0.012\ \mu g\ l^{-1}$) was within the requirements of most regulatory bodies (EU 1980, US Environmental Protection Agency 1988). Both assays had a high level of sensitivity, and the incorporated reagents had a long shelf-life (2.5 years). The present study has demonstrated their utility for the assay of herbicides in soil, but they could easily also be applied to river water or wastewater samples.

Tube assay kits for atrazine and simazine and many other herbicides have been described (Fleeker & Lovett 1985, Sherry & Borgmann 1993), however they are based on the adsorption of antibodies and ligand–enzyme tracers not on the coated-antigen format, adsorbed to tubes. In our hands, the antigen-coated tube approach is more convenient because of the stability of the coated antigen and the faster kinetics of binding of antibodies in solution. In addition, the antisera could be stored for several months or years under ammonium sulphate and the second antibody-labelled tracer is a generic reagent and can be obtained worldwide. The coated tube assay as described avoids contact between the tracer enzyme and samples and, therefore, the possible effect by sample components on the enzyme tracer. Extracts from soil samples may vary widely with unpredictable effects on the tracer enzyme, which could lead to misleading results.

There was cross-reactivity of these antisera with related triazines. However, the levels of both atrazine and simazine estimated by the individual ELISAs were in line with the HPLC results. The cross-reactivity of the two antisera to the related triazine compounds as shown by the ELISA (Table I) did not appear to affect the levels of these two compounds detected by the immunoassays in soil samples when compared with the results obtained by HPLC-DAD. This is probably because the actual interference within the assay is rather less that suggested by the method used to estimate the cross-reactivity. The proportions of the target analyte and the analogues in the actual sample may also be a factor. However, the critical issue here is not absolute specificity, but rather the convenience and practicality of the coated-tube assay coupled with a good specificity range that provides a useful answer.

Analysis of the soil samples by the coated ELISA method and HPLC were carried out by different workers as part of a blind study. The excellent correlation found between the results for both herbicides (Figures 4 and 5) indicated that the methods were interchangeable and that the convenience and greater sensitivity of the tube method would allow its widespread use in environmental studies and monitoring. Further, in some samples there were some differences between results obtained by HPLC and ELISA methods. One difficulty encountered when using HPLC for the analysis of complex soil sample matrix was the incomplete fractionation of compounds in the concentrated extracts, which may cause interference with the analysis. In contrast, this possible source of interference was much reduced in the immunoassay by virtue of diluting the samples before analysis. HPLC had limits in its sensitivity probably due to the detection system used in this instance. Whereas ELISA was a much more sensitive method which avoided interference by other compounds or derivatives present.

The principal objective was to demonstrate the utility of atrazine and simazine enzyme immunoassays for the screening of soil samples and particularly to

demonstrate the performance of the coated-tube enzyme immunoassay format in non-immunoassay laboratory environment. This assay format offers a number of technical and logistical advantages including the obviation of handling small volumes and the use of multi-pipettes, plate washer and readers. In addition, the larger assay volumes allows diluting samples, a much reduced dependence on workers' experience in liquid handling, and possibility of using standard spectrophotometers for measuring absorbances. The method is ideal for exploiting the many advantages of antibody-based analysis by non-immunoassay laboratories.

Acknowledgements

The study was supported by The British–Polish Research Partnership Programme.

References

Abraham GE. 1975. Radioimmunoassay of steroids in biological fluids. Journal of Steroid Biochemistry 6:261–270.

Abuknesha RA, Griffith HMT. 2005. Generation of antiserum to Irgarol 1051 and development of a sensitive enzyme immunoassay using a new heterologous hapten derivative. Annals of Bioanalysis and Chemistry 381:233–243.

Albanis TA, Hela DG, Sakellarides TM, Konstantinou IK. 1998. Monitoring of pesticide residues and their metabolites in surface and underground waters of Imathia (N. Greece) by means of solid-phase extraction disks and gas chromatography. Journal of Chromatography A 823:59–71.

Baranowska I, Barchanska H, Pyrsz A. 2005. Distribution of pesticides and heavy metals in trophic chains. Chemosphere 60:1590–1599.

Bisson M, Hontela A. 2002. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicology and Applied Pharmacology 180:110–117.

Butler VP, Jr. 1975. Drug immunoassays. Journal of Immunology Methods 7:1–24.

Catt K, Tregear GW. 1967. Solid-phase radioimmunoassay in antibody-coated tubes. Science 158:1570–1572.

Dean JR, Wade G, Barnabas IJ. 1996. Review: Determination of triazine herbicides in environmental samples. Journal of Chromatography A 733:295–335.

Diana SG, Resetarits WJ, Jr, Schaeffer DJ, Beckmen KB, Beasley VR. 2000. Effects of atrazine in amphibian growth and survival in artificial aquatic communities. Environmental and Toxicology Chemistry 19:2961–2967.

Durand G, Barcelo D. 1991. Confirmation of chlorotriazine pesticides, their degradation products and organophosphorus pesticides in soil samples using gas-chromatography mass-spectrometry with electron-impact and positive-ion and negative-ion chemical ionization. Analytica et Chimica Acta 243:259–271.

EU. 2004a. 2004/247/EC: Commission decision concerning the non-inclusion of simazine into Annex 1 to council directive 91/414/EEC.

EU. 2004b. 2004/248/EC: Commission decision concerning the non-inclusion of atrazine into Annex 1 to council directive 91/414/EEC

EU. 1980. EU Directive 80/778/EEC 1980. Brussels: Commission of the European Union.

Engvall E, Perlmann P. 1972. Enzyme-linked immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. Journal of Immunology 109:129–135.

Fleeker JR, Lovett LJ. 1985. Enzyme immunoassay for screening sulfamethazine residues in swine blood. Journal of the Association of Analytical Chemistry 68:172–174.

Garmouma M, Teil MJ, Blanchard M, Chevreuil M. 1998. Spatial and temporal variations of herbicide (triazines and phenylureas) concentrations in the catchment basin of the Marne river (France). Science of the Total Environment 224:93–107.

Goodrow MH, Harrison RO, Hammock BD. 1990. Hapten synthesis, antibody development, and competitive inhibition enzyme immunoassay for s-triazine herbicides. Journal of Agriculture and Food Chemistry 38:990–996.

Gysin H, Knuesli E. 1960. Chemistry and herbicidal properties of triazine derivatives. In: Metcalf R, editor. Advances in pest control research Vol. III". New York, NY: Wiley Interscience. p. 289–358.

Hall JC, Van Deynze TD, Struger J, Chan CH. 1993. Enzyme immunoassay based survey of precipitation and surface water for the presence of atrazine, metachlor and 2,4-D. Environmental Sciences Health B 28:577–598.

Harrison RO, Goodrow MH, Hammock BD. 1991. Competitive inhibition ELISA for the s-triazine herbicides: assay optimization and antibody characterization. Journal of Agriculture and Food Chemistry 39:122–128.

Hayes TB. 2004. There is no denying this: defusing the confusion about atrazine. Bioscience 54:1138–1149.

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A. 2002. Hermaphrodite, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences. USA 99:5476–5480.

Kettles MK, Browning SR, Prince TS, Horstman SW. 1997. Triazine herbicide exposure and breast cancer incidence: an ecologic study of Kentucky counties. Environment Health Perspectives 105:1222–1227.

Kobbia IA, Battah MG, Shabana EF, Eladel HM. 2001. Chlorophyll a fluorescence and photosynthetic activity as tools for the evaluation of simazine toxicity to Protosiphon botryoides and Anabaena variabilis. Ecotoxicology and Environmental Safety 49:101–105.

Kramer K, Lepschy J, Hock B. 2001. Long-term monitoring of atrazine contamination in soil by ELISA. Journal of AOAC International 84:150–155.

Lee KE, Blazer VS, Denslow ND, Goldstien RM, Tallmadge PJ. 1999. Use of biological characteristics of common carp (Cyprinus carpio) to indicate exposure to hormonally active agents in selected Minnesota streams, 21999. Water Resources Investigation Report 00-4202.

Lee NA, Kennedy IR. 2001. Environmental monitoring of pesticides by immunoanalytical techniques: validation, current status, and future perspectives. Journal of AOAC International 84:1393–1406.

Li M, Wu RS, Tsai S-F, Rosen SM, DiCesare J, Tsaia JSC, Salamone SJ. 2003. Development of a microparticle-based on-site immunoassay for the detection of atrazine in soil and water samples. Analyst 128:65–69.

Matsui J, Fujiwara K, Ugata S, Takeuchi T. 2000. Solid-phase extraction with a dibutylmelamine — imprinted polymer as triazine herbicide — selective sorbent. Journal of Chromatography A 889:25–31.

Meakins NC, Bubb JM, Lester JN. 1995. The mobility, portioning and degradation of atrazine and simazine in the salt marsh environment. Marine Pollution Bulletin 30:812–819.

Moore A, Waring CP. 1998. Mechanistic effects of triazine pesticide on reproductive endocrine function in mature male Atlantic salmon (Salmo salare L.) Parr. Pesticide Biochemistry and Physiology 62:41–50.

Pacakova V, Stulik K, Jiskra J. 1996. Review: High-performance separation in the determination of triazine herbicides and their residues. Journal of Chromatography A 754:17–31.

Pieszko C, Baranowska I. 2000. Determination of selected herbicides and phenols in water and soils by solid-phase extraction and high performance liquid chromatography. Journal of Chromatography Science 38:211–218.

Pinto GM, Jardim IC. 2000. Use of solid-phase extraction and high-performance liquid chromatography for the determination of triazine residues in water: validation of method. Journal of Chromatography A 869:463–469.

Prosen H, Zupancic-Kralj L. 2005. Evaluation of photolysis and hydrolysis of atrazine and its first degradation products in the presence of humic acids. Environmental Pollution 133:517–529.

Scriber EA, Battaglin WA, Goolsby DA, Thurman EM. 2000. Changes in herbicide concentrations in midwestern streams in relation to changes in use, 1989–1998. Science of the Total Environment 248:255–263.

Sherry J. 1997. Environmental immunoassays and other bioanalytical methods: overview and update. Chemosphere 34:1011–1025.

Sherry JP, Borgmann A. 1993. Enzyme-immunoassay techniques for the detection of atrazine in water samples: Evaluation of a commercial tube based assay. Chemosphere 26:2173–2184.

Steen RJ, Van der Vaart J, Hiep M, Van Hattum B, Cofino WP, Brinkman U. 2001. Gross fluxes and estuarine behavior of pesticides in the Scheldt estuary (1995–1997). Environmental Pollution 115:65–79.

Stoker TE, Guidici DL, Laws SC, Cooper RL. 2002. The effects of atrazine metabolites on puberty sand thyroid function in the male Wistar rat. Toxicology Sciences 67:198–206.

Tadeo JL, Sanchez-Brunete C, Perez RA, Fernandez MD. 2000. Review: Analysis of herbicide residues in cereals, fruits and vegetables. Journal of Chromatography A 882:175–191.

Tekel J, Hatrik S. 1996. Pesticide residue analyses in plant material by chromatographic methods: clean-up procedures and selectivity detectors. Journal of Chromatography A 754:397–410.

Thurman EM, Aga DS. 2001. Detection of pesticides and pesticide metabolites using the cross reactivity of enzyme immunoassays. Journal of AOAC International 84:162–167.

US Environmental Protection Agency. 1988. Atrazine: drinking water exposure assessment for atrazine and various chlorotriazine and hydroxytriazine degradation, sub appendix EPA. Washington, DC: US EPA.

US Environmental Protection Agency. 2005a. Technical fact sheet on: ATRAZINE. Washington, DC: US EPA. (available at: http://www.epa.gov/safewater/dwh/t-soc/atrazine.html).

US Environmental Protection Agency. 2005b. Technical fact sheet on: SIMAZINE. Washington, DC: US EPA. (available at: http://www.epa.gov/safewater/dwh/t-soc/simazine.html).

Wortberg M, Goodrow MH, Gee SJ, Hammock BD. 1996. Immunoassay for simazine and atrazine with low cross reactivity for propazine. Journal of Agriculture and Food Chemistry 44:2210–2219.