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Analysis for Atrazine in Fortified Cornmeal and Corns Using a Commercially Available Enzyme Immunoassay Microtiter Plate

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Atrazine is a selective pre-emergence herbicide used in crops such as asparagus, maize, pineapple, and sorghum, and for forestry and non-crop areas (Worthing 1987). In Canada during 1988, the sales of atrazine-containing products were approximately 2,000,000 kg of the active ingredient (*E.D. Brien, Commercial Chemicals Branch, Environ Can, Private Communication*). It is believed that the vast majority of atrazine was applied to corn, but the use patterns allowed on the labels of various products registered in 1986 also included bare-ground maintenance on non-croplands as well as weed control in lowbush blueberries and triazine-resistant rapeseed. Since atrazine is one of the more persistent herbicides, Agriculture Canada has a program to monitor atrazine residues in cornmeal to ensure that atrazine is used properly and that there are no residues in corns for food and livestock feed. The conventional residue analysis, using gas chromatography (GC) or high performance liquid chromatography (HPLC), is time consuming and expensive due to the long cleanup procedure and the sophisticated analytical instruments required. In contrast, enzyme immunoassays (EIA) are quick and simple to perform, and allow a large number of samples to be measured simultaneously. With the exceptions of a microplate reader, plate washer (if all 96 wells are used simultaneously), and a bench top orbital motion shaker, there is no need of any other expensive equipment.

Several groups have developed sensitive enzyme immunoassays for s-triazine analysis using polyclonal (Bushway et al. 1988, 1989; Wittmann & Hock 1989) or monoclonal (Karu et al. 1990, Goodrow et al. 1990, Giersch et al. 1990, Schlaeppi et al. 1989) antibodies. Some triazine enzyme immunoassay kits are now commercially available both in tube and in microtiter-plate formats. Thus far, using the commercial kit, the tube format (Bushway et al. 1988, 1989, Goh et al. 1990, Thurman et al. 1990) is most popular in the analysis of atrazine residues in soil and water; although the plate format has also been used (Stearman et al. 1992). The plate format is designed to perform 48 analyses in duplicate simultaneously whereas the tube format can only perform up to 10 analyses at a time due to the time (2-4 min) restriction for the color development. Furthermore, the 96-well plate format would allow a standard curve to be run with each set of

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test samples to facilitate quantitation, while the tube format is a quick "yes/no" screening method.

The purposes of this study are to report on (1) the application of a commercially available microtiter plate-based EIA method to quantitate atrazine residues in cornmeal and corns, (2) the limit of detection (LOD) of the method, and (3) the cross reactivities of the kit with the chemically-related substances, simazine and cyanazine, that are also registered to be used on corn grown in Canada.

MATERIALS AND METHODS

The EIA plate kits (EnviroGard™ Triazine Plate kit) were obtained from Millipore (Mississauga, Ontario). Each kit consisted of a plate with 96 antibody-coated wells, atrazine enzyme conjugate, substrate, chromogen and stop solution. Other equipment included a microplate reader (Model EL340, Bio-Tek®, Winooski, VT), bench-top orbital shaker (Flow Laboratories, Mississauga, Ontario), two digital multichannel micropipettes (20-100 uL, Johns Scientific, Toronto, Ontario) and two digital repeater pipette systems (P100 and P1000, Mandel Scientific, Guelph, Ontario).

Glass-distilled methanol (BDH, Toronto) and Milli-Q purified water were used throughout this work. Standard of atrazine (99.0%) was obtained from Caledon (Georgetown, Ontario), simazine (99.8%) from Agan Chemical (Ashdod, Israel) and cyanazine (99.4%) from Du Pont (Wilmington, DE). All standards were used without further purification.

Stock standard solutions of atrazine (1.08 mg/mL), simazine (106 ug/mL) and cyanazine (960 ug/mL) were prepared by dissolving the respective pesticides in methanol. Working standard solutions of atrazine (21.6, 5.4 and 0.216 ug/mL), simazine (10.6, 1.06 and 0.106 ug/mL) and cyanazine (100, 10, and 1 ug/mL) were prepared by serial dilution of the corresponding stock solutions.

A sample of cornmeal was obtained from the field and was confirmed to not contain atrazine above 0.05 ppm using an in-house multiple residue method (Agr. Can., 1990). This sample (1 g) was extracted with methanol (10 mL). After allowing the mixture to settle, an aliquot (1 mL) was diluted to 10 mL with water. The calibration standard solutions were prepared by adding the appropriate amount of triazines to aliquots of this diluted extract to give atrazine concentrations of 0.1, 0.25, 0.75, 1.5; 3.0 ng/mL, simazine levels of 1, 3, 5, 10 and 30 ng/mL and cyanazine levels of 10, 25, 50, 100 and 200 ng/mL.

A triazine-free corn sample was obtained from a local organic farmer and was not analytically tested for triazine residues. The blank extract was prepared from this sample by chopping it with a Hobart food chopper (Robot Coupe, Jackson, Mississippi). For atrazine assays, a chopped sample (10 g) was homogenized with water:methanol (9:1, 100 mL) using a mechanical homogenizer (Polyron®,

Luzern, Switzerland) followed by diluting to 1 L with water. The calibration standard solutions were prepared by adding appropriate amounts of atrazine standard solutions to a 25-mL volumetric flask and diluting to volume with this extract to give atrazine concentrations of 0.03, 0.09, 0.2, 0.6 and 1.0 ng/mL.

The extracts of fortified cornmeal or corn samples were prepared by adding to the samples 100 times the amount of triazines used in the corresponding calibration standard solutions. After standing at room temperature for 5-18 h, the samples were processed in the same manner as for the blank sample.

For simazine assays, the blank corn extract was obtained by filtering the homogenized chopped sample (5 g) in water: methanol (9:1, 50 mL) through Celite 545 (10 g, Fisher Scientific, Nepean, Ontario) on a Whatman No. 4 filter paper. The calibration standard solutions were prepared by adding the appropriate amount of simazine standard solutions to aliquots of this extract to give simazine concentrations of 1.0, 2.0, 5.0, 10.0 and 30.0 ng/mL. The extracts of fortified corn samples were prepared by adding to the samples 10 times the amount of simazine used in the corresponding calibration standard solutions.

The EIA procedure was performed according to the manufacturer's instruction provided with the kits (Millipore 1990). The % control (total binding, % B/B_0) was calculated as $(B/B_0) \times 100$ where B was the average absorbance (A450) of the calibration standard solutions or sample extract at 450 nm and B_0 was that of the blank extract.

RESULTS AND DISCUSSION

The EIA test principles are based on competitive binding of atrazine and atrazine enzyme conjugate to a limited number of antibody binding sites. In these assays, EIA tests of a blank extract and atrazine-fortified blank extract at 5 different levels were performed 4 times on 4 separate days. Since the expected accuracy of quantitation is the highest in the region of 20 - 80 % B/B_0 (Harrison et al. 1989), values that fell outside of this working range were not used.

A 1:10 dilution step for the calibration standard solutions and samples was used to reduce the matrix effects. The calibration curves were established by plotting the % B/B_0 against the logarithm of the atrazine concentrations. Figure 1A shows that the response curve for atrazine in cornmeal was linear over the range of 0.1 - 3.0 ng/mL with a correlation coefficient of -0.997. In corn, it was linear over the range of 0.09 - 1 ng/mL with a correlation coefficient of -0.999. The initial atrazine concentration in sample, expressed as ng/g, was calculated from values found from the calibration curve and multiplied by 100 which was the solution to sample ratio (10:1) and the dilution factor (10:1). Recoveries were calculated by dividing the atrazine found by the atrazine added to the samples multiplied by 100 (see Tables 1 and 2).

Table 1. Percentage recovery from atrazine-fortified cornmeal samples and limit of detection using EIA.

	Atrazine added (ppb, ng/g cornmeal)				
Atrazine found[a] (ppb):	10	25	75	150	300
Mean ($\bar{x}$, ppb)	10.1	26.0	77.0	176.0	303.0
SD (ppb)	0.6	8.9	18.2	26.0	45.8
CV (%)	6.0	34.0	23.5	14.7	15.1
LOD	10.0				
Mean recovery (%)	101	104	103	117	101

[a]Average of a total of 4 determinations performed on 4 separate days, SD = standard deviation, CV = coefficient of variation, LOD = limit of detection which was 80% B/B_0.

Table 2. Percentage recovery from atrazine-fortified corn samples and limit of detection using EIA.

Atrazine found (ppb):	Atrazine added (ppb, ng/g cornmeal)			
	9.0	20	60	100
Mean ($\bar{x}$, ppb)	9.3	18.6	56.8	96.7
SD (ppb)	1.8	2.6	9.8	9.5
CV (%)	19.0	14.4	17.2	9.8
n[a]	8	8	4	6
LOD (ppb)	7.0			
Mean recovery (%)	103	93	95	97

[a]n = Total number of determinations performed on 4 days.

The coefficient of variation (CV) for the sample extracts ranged from 6 - 34%. The limit of detection (LOD) defined as 80% B/B_0, was 10.0 and 7.0 ppb respectively for cornmeal and corn samples. The mean recoveries (93 - 117%) at these levels suggested quantitative extraction of atrazine from the samples.

For the simazine study, because the response of the kit to this chemical in blank extract was low, a 5-g corn sample was extracted with 10% methanol (50 mL). The mixture was filtered through Celite 545 and was not diluted subsequently. The concentration of corn was 0.1 g/mL. The response curve was linear for the range of 1.0 - 30 ng/mL (equivalent to 10 - 300 ng/g corn) with a correlation coefficient of -0.999. However, the recoveries of fortified corn sample varied widely, ranging from 36-147%. The high CV values (Table 3) resulted probably from the fact that simazine was added directly to the sample before the workup

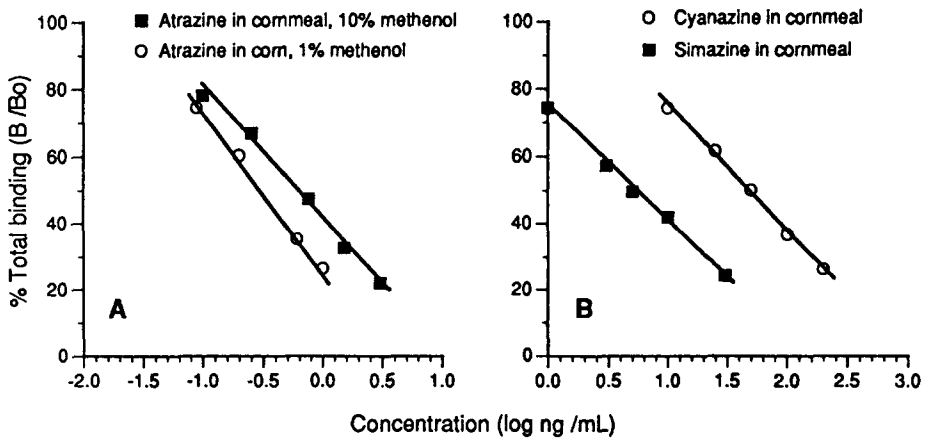

Figure 1 % total binding of: A, atrazine in cornmeal (■), n = 4 and atrazine in corn (○), n = 4, and B, simizine in cornmeal (■), n = 4 and cyanazine in cornmeal (○), n = 5.

Table 3. Average recoveries[a] (%) of simazine from fortified corn.

	Fortified simazine (ppb, ng/g corn)				
	10	20	50	100	300
Average recovery (%)	83.2	74.2	97.2	103.0	99.9
SD	28	24	22.9	33.4	22.8
CV	33.7	32.3	23.5	32.2	22.8

[a]n = A total of 4 determinations performed on 4 separate days.

procedure for the extracts. A sample of cornmeal was used for recovery study and the final extracts were diluted 10 times with water to give sample concentration of 0.01 g/mL. The response curve was linear over 1.0 - 30 ng/mL (i.e. 100 - 3000 ng/g cornmeal) (Figure 1B) and the average recoveries from a total of 4 determinations performed on 4 separate days are shown in Table 4.

Table 4. Average recoveries[a] (%) of simazine from fortified cornmeal.

	Fortified simazine (ppb, ng/g cornmeal)				
	100	300	500	1,000	3,000
Average recovery (%)	88	112	103	116	90
SD	19.2	38.2	25.4	38.4	14.2
CV	21.9	33.9	24.7	33.2	15.7

[a]n = A total of 4 determinations performed on 4 separate days.

The cyanazine study was conducted in the same manner as the simazine in cornmeal. The response curve was linear for the range of 10 - 200 ng/mL which was equivalent to 1.0 - 20 ug/g cornmeal (Figure 1B) with a correlation coefficient of -0.998. The recoveries of this triazine from corn and cornmeal were not performed because the kit is not sufficiently sensitive to detect this analyte to below 0.1 ppm level which is the maximum residue limit (MRL) of pesticides in foods unless otherwise speicified.

Table 5 and Figure 1A show that a slight sample matrix effect of the responses of atrazine in cornmeal and corn samples was observed. Thus, to use EIA as a quantitative method, it is important to use blank extracts, from the same source, containing known amount of analyte to develop the calibration curve. Otherwise, it may be used as a screening method to reduce to only positively responding samples for futher instrumental analysis.

Table 5. Sensitivity comparison of blank extracts[a] fortifed with triazines using EIA plate kit.

Matrix	M.C.[b] (g/mL)	Triazine	L.R.[c] (ng/mL)	50% B_0 (ng/mL)	Slope	Methanol (%)
Cornmeal	0.01	ATR[d]	0.1-3.0	0.1-3.0	-39.1	10
Cornmeal	0.01	ATR	0.1-3.0	0.1-3.0	-33.6	1
Corn	0.01	ATR	0.09-1.0	0.3	47.0	1
Cornmeal	0.01	SIM[e]	1-30	5.2	34.1	10
Corn	0.1	SIM	1-30	5.2	34.9	10
Cornmeal	0.01	CYN[f]	10-200	47.0	37.7	10

[a]n = 4 except for CYN (n = 5) and ATR in cornmeal using 1% methanol (n = 1), [b]M.C. = matrix concentration, [c]L.R. = linearity range, [d]ATR = atrazine, [e]SIM = simazine and [f]CYN = cyanazine.

In conclusion, the EIA plate kit used in this study shows promise in rapid and simple analysis of atrazine residues in cornmeal and corns. Since in Canada, the MRL of any pesticides if not specified in the Federal Food and Drug Act is assumed to be 0.1 ppm, the limits of detection of atrazine (7-10 ng/g sample) are thus sufficiently sensitive for residue analysis. It also provides additional advantages that no cleanup is required and a large number of analyses (96) can be completed in 8 h/day which is significanly quicker than using the traditional methods.

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