Immunoassay Detection of Herbicide Residues in Corn[1]

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ABSTRACT

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Immunoassay detection kits were used to detect herbicide residues in spiked corn samples. Five herbicide compounds (atrazine, alachlor [Lasso], metolachlor [Dual], cyanazine [Bladex], and 2,4-dichlorophenoxy acetic acid [2,4-D]) commonly used by corn producers were selected as analytes. Selection was based on a survey of 200 Iowa Corn Grower Association members. Commercial test kits for water analysis for these compounds were available from Ohmicron Corporation, Newtown, PA. Two extraction methods, blender and heated shaker, were compared for efficiency of herbicide recovery at three spike levels. The blender method yielded recoveries of 80–100% of herbicide spike in the immunoassay for all compounds. Recoveries from the heated shaker method were comparable for four of the five compounds. Low detection limits for the

immunoassay in corn were 20 ppb, with greater variability near the low end of the calibration. Variability decreased with increasing levels of herbicide spike. Recoveries on selected spiked samples were confirmed by traditional detection methods. Blank adjustments were made to compensate for the interferences caused by the corn. Iowa farmers provided 200 corn samples and accompanying two-year herbicide usage history. These samples were assayed for the five compounds, using the immunoassay test kits. Herbicide usage patterns were close to those reported in Iowa and Illinois industry data. No samples tested positive for atrazine, alachlor, metolachlor, or cyanazine. There were two positive, above-tolerance results, not confirmed by chromatography, for 2,4-D. The immunoassay required 0.5 hr of labor per sample in addition to the cost of the kit.

Pesticide residue monitoring has become a necessity for food processors. Whether or not pesticide residues exist in the food supply is a broad question frequently asked by consumers, food processors, foreign buyers, and monitoring agencies throughout the world. Despite assurances to the contrary, food safety in general, and chemical contamination in particular, remain major concerns of food safety professionals and consumers (Wixom 1993).

In the United States, the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) are charged with setting and enforcing tolerance levels and with monitoring the food supply for violative levels of pesticides. Organic farmers in some parts of the country have elected to have their crops certified as *residue-free* by laboratories within their own states. The 1990 Farm Bill gave responsibility for monitoring contaminants in grain to the Federal Grain Inspection Service (FGIS). FGIS is to support FDA in its enforcement program (US Congress 1990).

The goal of the FDA Pesticide Monitoring Program is to ensure that the food supply is safe and free of excess residue, including selective surveys of chemicals not ordinarily regulated in the program (Lombardo 1989). These surveys cover 298 pesticide compounds, mostly insecticides and preservatives applied directly to products. FDA surveillance data in 1991 included 84 samples of corn and popcorn; 71% of those samples contained no residues (FDA 1992). Sources of the FDA samples were not given.

The demand for faster and easier screening methods to determine which samples require more extensive analysis means that rapid methods, such as immunoassay, will become important to both the FDA and private food processors.

Immunoassay offers a number of potential advantages over traditional detection methods. It is potentially faster and less expensive because immunoassay samples can be processed in batches of 20–25 per hour, compared with a single sample through a chromatograph (Herzog 1991). Less operator training and experience are required, and the statistical evaluation of quality control samples can give clear feedback on operator error. The extraction and assay use limited amounts of toxic solvents.

According to data collected by the respective Agricultural Extension Services (Pike et al 1991), the six most commonly used corn herbicide compounds in Iowa and Illinois were

1. Atrazine (Atrazine): 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine. Atrazine alone was used on about 60% of corn acres in 1991 and 1992.
2. Alachlor (Lasso) : 2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide
3. Metolachlor (Dual): 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide
4. Cyanazine (Bladex): 2-[[4-chloro-6(ethylamino-1,3,5-triazin-2-yl]amino]-2 methylpropionitrile
5. 2,4-D (2,4-D): (2-4-dichlorophenoxy) acetic acid
6. Dicamba (Banvel): 3-6-dichloro-2-methoxy benzoic acid

The objectives of this study were to adapt commercially available immunoassay test kits for herbicide residue detection in corn, and to screen a diverse representation of corn samples for five corn production herbicides. There was no kit available for dicamba.

MATERIALS AND METHODS

Corn Samples

There were two sample sets in the study. First, two corn samples were spiked with known concentrations of analytical-grade herbicide. Second, 200 samples of unknown concentration were received from Iowa farmers, along with herbicide-use history.

Spiked Samples

Two dent corn samples were spiked individually with each of five herbicide compounds (Snyder and Kirkland 1979). Each sample contained only one hybrid, although the exact names were not known. The two samples were chosen, based on visual inspection and density data, to be one hard and one soft endosperm type. These samples were divided with a Gamet rotary divider into 400-g portions, ground so that the particles would pass through a 20-mesh sieve, and then weighed in 50-g portions before spiking.

Analytical-grade herbicides were obtained from Chem Service, West Chester, PA. All samples were screened initially for the five compounds by Stephen Ross, Illinois Department of Agriculture, Animal Disease Lab, Centralia, IL. This lab is accredited for pesticide analysis by USDA Food Safety and Inspection Service and by the American Association of Veterinary Laboratory Diagnosticians. The background screening methods were derived from those approved by EPA and AOAC (AOAC 1984, EPA 1976). No initial concentrations of herbicide were present.

To prepare the spiking solution for each compound, 100 mg of the analytical grade compound was diluted in methanol to a 1 mg/ml concentration. After the herbicide was thoroughly dissolved, the solution was further diluted with methanol to a 1g/ml concentration. Two 50-g samples were spiked at each of three levels for each compound. With a few exceptions created by the mechanics of the kits, the spike levels were set at 50, 100, and 200% of EPA tolerance. Methanol was added to equal 50 ml to increase the liquid available for mixing with the corn sample.

Appropriate aliquots of spiking solution were measured into a beaker with enough methanol to equal the 50 ml necessary to make a slurry of a 50-g corn sample. The entire slurry was poured into a blender jar, blended for 1.0 min, and then hand-shaken for 15 sec before being poured into weighing boats to dry overnight. Dried samples were then reblended for 1.0 min to pulverize hardened corn into powder before samples were weighed for extraction.

Farm Samples

Corn samples (approximately 600 g) were mailed to Iowa State University by producers. From a mailing list provided by the Iowa Corn Growers Association, eight producers per county were sent sample bags for corn samples and a survey requesting information about their 1989 and 1990 herbicide usage. Of the 800 requests distributed across the state, 200 samples and surveys were returned. Samples were refrigerated until the assays were done.

The survey information about compounds used and rates of application was compiled. Samples were assayed for herbicide residues based on the usage information. For each compound, samples were assayed if the soil in which they were grown was exposed to any of the five herbicides in 1990 (the growing season), 1989 (the previous year), or in both years.

Test Kits

Ohmicron Rapid Pesticide Immuno Detection Assays (Ohmicron, Inc., Newtown, PA), enzyme-linked immunosorbent assays (ELISA), were modified for grain analysis. Individual kits do single residue analysis with standard solutions of pesticide, pesticide analog, and antibodies attached to magnetic particles, which separate the analyte from the sample matrix. A pesticide analog labeled with horseradish peroxidase competes with the analyte for binding sites on the antibodies. Tetramethylbenzidine (color reagent oxidized with 0.02% hydrogen peroxide) reacts with analog enzyme label. After 20 min of incubation, color development is stopped with $2M$ sulfuric acid, and color absorbance is read at 450 nm. Sample pesticide concentration is inversely proportional to color absorbance.

Laboratory Procedures

Spiked corn. Two extraction methods, developed jointly by Ohmicron and ISU, were compared for efficiency of herbicide recovery. Each method required that 20 g (weighed to 0.001 g) of ground corn be mixed with 80 ml of 80:20 methanol-water, as recommended by Ohmicron. Corn was ground in a Magic Mill III+ home flour mill (Magic Mill, Inc., Salt Lake City, UT). This 4:1 concentration of methanol was considered sufficient to extract hydrophobic herbicide compounds without interfering with the antibody performance in the assay.

The blender method required blending with a kitchen blender for 2.0 min at room temperature. The heated shaker method required combining the ground corn with methanol and water in a heat-resistant bottle, then shaking the sample at 70°C, 35 rpm, in a water bath for 2.0 hr. For both methods, the samples were allowed to settle 30 min at room temperature before cooling at −10°C for 12 hr. This cooling step was necessary to reduce matrix interference in the assay. The samples were allowed to reach room temperature before analysis.

The 0.1-ml sample extract was diluted with 4.9 ml of distilled water for atrazine, cyanazine, alachlor, and metolachlor. This 1:50 dilution not only adjusted the concentrations to fit the cali-

bration curve, but it also reduced the methanol content to less than 2.5%, which the assay could tolerate. A 1:100 dilution was required for the 2,4-D extract to minimize interferences.

For 2,4-D, a 0.1-ml sample was diluted in 1.0 ml of distilled water. Then 0.1 ml of the new concentration was diluted in 1.0 ml of 2,4-D Zero Standard Diluent provided in the test kit.

For all compounds, diluted extract was added as *sample* according to the kit instructions. The sample size range was 100–250 μl; all samples were run in duplicate in the assay. Dilution ratios were chosen to place the spike levels within the working range of the calibration curves.

$$\text{Dilution factor} = 80 \text{ ml methanol-water}/20 \text{ g corn}$$
$$\times \, x \text{ ml water}/1 \text{ ml extract}$$

Enzyme conjugate and magnetic particles were added in a 1:2 ratio to the sample in Ohmicron test tubes. Tubes were allowed an incubation period of 15–30 min, depending on the kit used.

After this incubation period, the entire rack of tubes was placed in a magnetic rack to affix the magnetic particles and bound analyte to the sides of the test tubes. After each of two successive washings with sterile water, the tubes were inverted and blotted to remove residual liquid. Color reagent was added to each tube and developed for 20 min before $2M$ sulfuric acid was added to stop development. Tubes were read within 15 min in a RPA-I RaPID Photometric Analyzer to determine analyte concentration.

The RPA-I Photometric Analyzer is a bichromatic photometer with an overall wavelength range of 340–650 nm. It uses a filter block to set the primary and reference wavelengths to 450 nm and 600 nm, respectively. Standard solutions of three known concentrations of analyte and one blank are provided with the kit. These are to be run in duplicate with each assay, as calibration standards.

Absorbances of these solutions are read at 450 nm, then transformed by the Logit function as follows:

$$\ln (B/B_0)/(1 - B/B_0) = m(\ln C) + b \qquad (1)$$

where $B =$ absorbance of the known concentration standard, $B_0 =$ absorbance of zero concentration standard, $C =$ concentration of the analyte. The m and b values for the Logit function were found by linear regression.

This calibration was applied to procedures for atrazine, cyanazine, alachlor, and metolachlor. The data transformation for 2,4-D was semilog, using absorbance (B) values rather than the Logit function in equation 1. All compounds had extremely high linearity of transformed data ($R^2 > 95\%$), although only three points were used to determine the calibration curve.

All unknown sample concentrations were determined by this calibration and the dilution factor based on extraction of the 20-g sample. A new calibration curve was established for each group (kit) of samples assayed. According to the manufacturer, the kits required individual calibrations to compensate for variation in the standard solutions and for environmental conditions.

Predicted concentrations for each spiked sample were adjusted by subtracting the response of unspiked (control) corn samples in the same immunoassay run. The blank adjustment was determined as a mean predicted concentration of unspiked samples of the same corn type (hard or soft) extracted by the same method (heated shaker or blender). The blank adjustment value was subtracted from the predicted concentration of each spiked sample.

Farm samples. For each compound to be assayed, a 20-g portion was divided with a laboratory divider. Portions were ground and extracted in 80:20 methanol-water solution, as before.

The best (as judged by accuracy and variability on the spiked corn) extraction method for each compound was used for these samples. For atrazine and alachlor, the heated shaker extraction was used. The blender extraction was used for cyanazine, metolachlor, and 2,4-D analysis. The same immunoassay operating procedure was used for these samples as was used for the spiked corn, including the blank adjustment. Any samples with positive response after blank adjustment were sent to Stephen Ross, Illinois Department of Agriculture, Animal Disease Lab, for confirmation.

TABLE I

TABLE I
Recoveries of Five Herbicides from Spiked Corn by Immunoassay

Herbicide (EPA Tolerance)	Average Recovery Percentage by Spike Level and Extraction[a]					
	50% of Tolerance		100% of Tolerance		200% of Tolerance	
	Blender	Heated Shaker	Blender	Heated Shaker	Blender	Heated Shaker
Atrazine (250 ppb)	99[b] (33)	78[b] (18)	91 (21)	75 (21)	88 (13)	78 (5)
Cyanazine (50 ppb)	63 (50)	51 (14)	97 (15)	82 (18)	102[c] (9)	72[c] (19)
Metolachlor (100 ppb)	132 (31)	104 (38)	147 (19)	142 (17)	145 (12)	143 (10)
Alachlor (200 ppb)	67[d] (26)	55[d] (14)	91 (17)	77 (14)	88 (7)	91 (5)
2,4-D (500 ppb)	60 (43)	132 (58)	61 (19)	120 (87)	94 (27)	164 (169)

[a] Two corn samples, duplicate extractions, two assay tubes per extraction = 8 data points. Standard deviation in parenthesis. All recoveries adjusted for response of blank corn samples. Atrazine $LSD_{0.05} = 10$, Cyanazine $LSD_{0.05} = 16$, Metolachlor $LSD_{0.05} = 20$, Alachlor $LSD_{0.05} = 16$, 2,4-D $LSD_{0.05} = 20$.
[b] 40% of tolerance (100 ppb).
[c] 500% of tolerance (250 ppb).
[d] 25% of tolerance (50 ppb).

TABLE II
Recovery of Four Herbicides from Selected Spiked Corn Samples, by Immunoassay and Chromatography[a]

Herbicide (EPA Tolerance)	Spike Level (ppb)	Corn	Assay Method		
			Chromatography[b]	Immunoassay	
			Shaker	Blender	Heated
Atrazine (250 ppb)	100	Hard	122 a	70 b	62 b
	250	Soft	112 a	110 a	89 b
Metolachlor (100 ppb)	50	Soft	104 a	153 b	84 a
	100	Hard	99 a	135 b	148 b
Alachlor (200 ppb)	200	Hard	79 a	100 a	78 a
	400	Soft	91 a	85 a	88 a
2,4-D (500 ppb)	250	Soft	95 a	42 b	126 a
	500	Hard	96 a	62 a	108 a

[a] Across a row, means with same letter not significantly different ($P = 0.05$).
[b] Chromatography detection performed by Stephen Ross, Illinois Department of Agriculture, Centralia, IL.

TABLE III
Blank Adjustments (ppb) by Compound

	Average, All Types & Extractions	By Extraction, Across Type		By Extraction, Corn Type[a]			
				Bl[b]		Sh[c]	
		Bl	Sh	S[d]	H[e]	S	H
Atrazine	−17	−23	−12	−16	−30	−4	−19
Cyanazine	−4	−6	−2	0	−12	0	−4
Metolachlor	−11	−8	−15	−6	−10	−11	−18
Alachlor	−7	−6	−7	−4	−9	0	−13
2,4-D	−307	−325	−288	−368	−282	−364	212

[a] Blank adjustments used in Table IV.
[b] Blender extraction.
[c] Heated shaker extraction.
[d] Soft corn.
[e] Hard corn.

Experimental Design and Statistics

Spiked samples. For each compound, the two corn types were extracted in duplicate for both methods at each spike concentration. Duplicate assays were run on the extractions. This yielded a split-plot design (two samples as blocks, two extractions as main treatments, two concentrations as subtreatments). There were 48 observations per compound, given the duplicate extraction and duplicate assays per extraction. The analysis variable was the percentage of recovery computed by comparison of RPA-predicted concentration multiplied by the dilution factor with the known spike level.

Farm samples. The farmer samples were selectively tested, based on herbicide-use information provided by the farmer. The number of positives, above-tolerance positives, and confirmed (by chromatography) positives were tabulated.

RESULTS

Immunoassay Recovery

The immunoassay generally recovered 80–100% of the spike concentrations (Table I). Although there was variability, most recoveries were within 20% of the spike level, which is comparable to results by chromatography detection of pesticides in oil (Di Muccio et al 1991). The standard deviations were based on eight observations, because the duplicate extractions and assays per extraction are both contributors to precision of the procedure. The least significant difference values were formed using the listed standard deviations, therefore using the precision to assess accuracy.

Comparisons of recoveries between immunoassay and chromatography detection are shown in Table II. Herbicide detection using chromatography showed that recovery from the spiked samples could vary as much as 22% from the intended spike level, but was generally within 10% of the immunoassay recovery. Table II reflects only one of the two original spiked samples, whereas Table I reflects data from both samples. Therefore, the statistical evaluation in Table II was based on four data points, rather than the eight in Table I. Cyanazine was not measured by chromatography because of an unknown interference found in the samples.

The blank (uncontaminated) corn never read zero concentration. The response (in parts per billion) of the blank was subtracted from all samples. Possible blank subtractions are given in Table III. Most blank subtractions were relatively small, and the differences between types of corn were not large. For samples of unknown corn type, we propose that the overall extraction-specific (across-type) blank correction be used, unless several known clean samples are included in each assay run.

Triazines

Atrazine and cyanazine responded similarly according to corn type, extraction method, and level of analyte concentration. Recovery of atrazine was dependent on the corn type and extraction method. Greater matrix interference was caused by the blender extraction method and the hard corn type. Heated shaker extraction involved temperature changes that could have denatured corn proteins, thus decreasing their interference in the assay and reducing variability in the results. Concentration levels did not show significant differences in recovery, except between corn types.

TABLE IV
Herbicide Distribution and Usage Rate in Iowa Farm Samples[a]

Herbicide	Number of Samples Where Compound was Used[b]			Average Rate (lb/acre)[d]	Percentage Use	
	1989[c]	1990	Both Years		Iowa 1990	Illinois 1990
Atrazine	11	68	54	1.1 (0.6)	61	70
Cyanazine	8	43	9	2.1 (0.9)	26	18
Metolachlor	8	36	32	2.4 (1.0)	34	36
Alachlor	13	19	32	2.5 (0.9)	26	24
2,4-D	2	17	7	0.4 (0.3)	12	12

[a] 200 samples collected, 1990 crop corn.

[b] According to producer responses to survey questions.

[c] 1989 usage would be indicative of corn-on-corn cropping pattern.

[d] Reported pounds of active ingredient per acre, standard deviation in parentheses.

TABLE V
Screening of Farmer-Collected Corn Samples[a] for Herbicide Residues[b]

Herbicide (Extraction Method)[c]	Number Screened	EPA Tolerance (ppb)	Number of Positives	
			Any Level	Above Tolerance
Atrazine (Heated shaker)	130	250	1[d]	0
Cyanazine (Blender)	62	50	0	0
Metolachlor (Blender)	76	100	1[d]	0
Alachlor (Heated shaker)	64	200	0	0
2,4-D (Blender)	26	500	20	2[e]

[a] 200 samples collected, 1990 corn crop.

[b] Tested according to farmer response to usage questions.

[c] Chosen as best from spiked samples.

[d] Positive atrazine and metolachlor same sample. None detected in chromatography.

[e] 10% of tolerance (59 ppb and 62 ppb) confirmed with chromatography.

Corn type, extraction method, and concentration all affected the recovery of cyanazine. However, recovery was consistent across all levels of concentration within each extraction method. Recoveries of cyanazine decreased with the heated shaker method, probably because cyanazine is heat-sensitive. Therefore, the blender method gave more accuracy.

Chloroacetamides

Alachlor and metolachlor were not similar to each other in their response to corn type, extraction, and level of analyte concentration. Metolachlor showed significant recovery differences that depended on extraction method, analyte concentration, and corn type within the extraction method. Recovery of alachlor was generally less than that of the spike levels, and the heated shaker extraction resulted in less variability at all concentrations.

Of the two methods, the blender extraction produced less matrix interference, so its blank adjustment was lower than that for the heated shaker extraction. Hard corn gave slightly more interference than did soft corn, probably because of higher protein content. Even after blank adjustments were made, recoveries were above 100%, which suggests that there is still interference beyond that caused by the corn.

Recovery of alachlor was generally less than that of the other compounds, which suggests that alachlor could be slightly altered by the solvent and its binding inhibited. Above EPA tolerance level (200 ppb), recovery of alachlor improved. RaPID Assays for alachlor have low cross-reactivity with compounds similar to alachlor, so it is unlikely that the test would detect derivatives of alachlor.

2,4-D

Extraction method was the only test variable that made a difference in recovery for 2,4-D. Although blank adjustments were high, relative to those for the other analytes, the blender extraction resulted in more uniform recoveries than did the heated shaker extraction. Though the comparison between immunoassay and chromatography recovery would favor the heated shaker method, this extraction produced extremely high variability. Sample interference (and the blank adjustment) might be reduced by filtering or centrifuging the extract. Recoveries ranged from 60 to 90%, with increasing spike concentrations.

Farm Samples

Table IV shows the distribution and usage rates of the five compounds as reported by the farmers. For comparison, data from annual surveys in Iowa and Illinois are included. The use patterns in our samples were similar to those in the two states.

One sample showed atrazine and metolachlor residues that were not confirmed at any level. Several showed 2,4-D residues. (Table V). Chromatography data on the two samples with high 2,4-D responses did not confirm the immunoassay response, but detected a 2,4-D residue at 56 and 63 ppb (10% of EPA tolerance). The herbicide 2,4-D is occasionally applied, within label limits, over mature corn as a rescue treatment for broadleaf weeds. Thus, its presence in the kernels is possible.

DISCUSSION

All compounds showed the most variability in the lowest concentration levels. Standard deviations of recovery percentages were generally less than 20% at the EPA tolerance level and at the level representing 2–5 times the EPA tolerance level. For all compounds, the concentration levels were in the lower half of the calibration curve. The low concentration level approached or exceeded the lower calibration limit for each of the herbicide assay kits.

A weakness in the procedure is that the calibration equation was not capable of reading 0 ppb. Both sides of equation 1 are undefined for $C = 0$ (and presumably $B = B_0$). Therefore, measuring concentrations less than the lower calibrator is automatically going to be less certain. Equation 1) also incorporates B_0 in every point.

Immunoassay can be used as a relatively rapid screening method for herbicide residue detection in corn. There were no unreasonable test results (e.g., negative concentrations, extreme high concentrations, etc.). Although the elapsed time of a test was 12–16 hr because of the overnight chilling, the estimated labor required per sample was 0.5 hr. Further work to eliminate or shorten the chilling step is needed. As an alternative, Ohmicron suggests that trichloroacetic acid and Celite 545 (purified volcanic ash) be added to the sample extract and centrifuged to aid precipitating interferences. This has not been proven.

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