Determination of Paraquat in Fruits and Vegetables by a Magnetic Particle Based Enzyme-Linked Immunosorbent Assay

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A competitive enzyme-linked immunosorbent assay (ELISA) for the quantitation of the free paraquat cation was developed using polyclonal rabbit anti-paraquat antibodies bound to a magnetic particle solid phase. A method for the extraction of the paraquat cation from various fruits and vegetables in less than 30 min was developed for use in conjunction with the immunoassay. The procedure has an estimated detection limit of 10 ng/g (ppb) paraquat cation based on the 20 pg/mL estimate in buffer. Results can be easily converted to the dichloride result by dividing the cation concentration by 0.724. Paraquat cation recoveries averaged 99% using this extraction method and immunoassay for cabbage, apples, and potatoes. A collaborative study in which paraquat-fortified zucchini samples were extracted using the traditional 5 h reflux prior to spectrophotometric and immunoassay measurement yielded a correlation of 0.994 with a slope of 1.05.

Keywords: *Paraquat; ELISA; immunoassay; acid extraction*

INTRODUCTION

Despite its high toxicity to humans, paraquat poisoning is usually associated with accidental ingestion or attempted suicides (Hayes et al., 1991; Sellero et al., 1993). However, because of its widespread use on some crops, there is the potential for human exposure to paraquat through residues on crops (Chichila et al., 1991). Paraquat (1,1′-dimethyl-4,4′-bipyridylium ion) is a highly effective, nonselective contact herbicide that is absorbed by foliage with some translocation through the xylem (Hamish and James, 1991). It is used for the broad-spectrum control of broad-leaved weeds and grasses in fruit orchards, plantation crops, ornamentals, and shrubs (Hamish and James, 1991). Paraquat is also used for general weed control on noncrop land and has been recommended for the control of aquatic weeds. It has been applied as a defoliant on crops such as cotton and as a desiccant for pineapples, potatoes, sugarcane, and sunflower.

Paraquat is strongly hygroscopic and available as a dichloride salt hydrate (mono-, di-, trihydrate). Once in solution, paraquat dichloride dissociates into the positive cation (2+), which presents unique analytical challenges. Paraquat adsorbs strongly to organic and inorganic adsorbents, e.g., clays and soils, plant tissues, chromatography and cleanup materials, and glass (Worobey, 1987). Therefore, strong acid refluxing is required to solubilize both the adsorbate and adsorbent in order to free bound residues (Sellero et al., 1993).

Traditional methods for the determination of paraquat in fruit and vegetable crops include isolation with silica columns followed by HPLC and UV detection (Worobey, 1987; Lawrence, 1982), HPLC with diode ray detection (Chichila et al., 1991), and spectrophotometric methods (Chevron Chemical Co., 1967; Zeneca Agrochemicals, 1993), all of which require lengthy cleanup procedures and refluxing from 1 to 5 h in concentrated acid. The EPA requires the use of the ICI spectrophotometric method (Zeneca Agrochemicals, 1993) for use on crops and plant juices. For this method, the limit of quantitation is 0.01–0.05 mg/kg depending on the crop analyzed.

This paper describes a quick and simple acid extraction that is followed by the use of an enzyme-labeled immunosorbent assay (ELISA) that uses magnetic particles as a means of solid support and separation. Magnetic particle based enzyme-linked immunosorbent assays for the determination of pesticides in fruits and vegetables have been previously described (Itak et al., 1993, 1994).

MATERIALS AND METHODS

Immunochemicals. Rabbit anti-paraquat was produced using immunization protocols similar to those described by Tijssen (1985). The immunogen, paraquat conalbumin, was prepared according to the method of Van Emon et al. (1986). Conalbumin was purchased from Sigma Chemical Co. (St. Louis, MO). The procedure for coupling anti-paraquat antiserum to magnetic particles was previously described (Rubio et al., 1991) using paramagnetic particles purchased from Advanced Magnetics (Cambridge, MA). The prepared particle stocks were diluted 1:1000 for use in the assay.

The enzyme conjugate ligand, *N*-(4-carboxybut-2-yl)-*N*′-methylbipyridinium dichloride, was prepared according to the method of Van Emon et al. (1986), using monoquat and ethyl 5-bromovalerate. Paraquat hapten–horseradish peroxidase (HRP) enzyme conjugate was synthesized by a mixed anhydride coupling of the enzyme (Fickling et al., 1990). Horseradish peroxidase was purchased from Sigma. Tetramethylbenzidine and hydrogen peroxide were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD).

Additional Chemicals. Sulfuric acid (36 N) and pesticide grade methanol were purchased from J. T. Baker (Phillipsburg, NJ). Cation exchange resin (4R, strong acid, sodium form, chromatographic grade, 0.150–0.300 mm) was purchased from Gallard-Schlesinger Industries (Carle Place, NY).

Paraquat dichloride was purchased from ChemService (West Chester, PA). A stock solution was prepared from paraquat dichloride powder dried at 100 °C for 5 h. This is consistent with the analytical procedures of Zeneca Agrochemicals (1993)

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and Worobey (1987). Dried paraquat dichloride (173 mg) was dissolved in 50 mL of methanol (2.5 mg/mL paraquat cation). Paraquat cross-reactants purchased from ChemService were diquat, difenzoquat, chlormequat, allyltrimethylammonium bromide, and (2-bromoethyl)trimethylammonium bromide. Morfamquat, methylbipyridyl methylsulfonium salt, 4-picolinic acid, 4,4-bipyridyl, and diethylparaquat were gifts from Zeneca Agrochemicals, Jealott's Hill Research Station, Berkshire, U.K. 1-Methyl-4-carboxypyridinium and methylamine hydrochloride were purchased from Sigma.

Buffers. Tris-buffered saline (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% BSA was used for diluting the rabbit anti-paraquat magnetic particles. A Tris-based saline (pH 6.0) was used for diluting the paraquat–HRP enzyme conjugate. Sodium acetate buffer (pH 4.0) with 1 mM EDTA and 0.1% gelatin was used to dilute the paraquat cation standards and the extracted samples.

Equipment. The magnetic separation rack and RPA-I Analyzer were supplied by Ohmicron Corp. (Newtown, PA). A Multipractic food processor was obtained from Braun Inc. (Germany), and 15 mL graduated polypropylene tubes with screw caps for sample processing were obtained from VWR Scientific (South Plainfield, NJ). The sonicator (200 W/40 kHz) was from Sonic Systems, Inc. (Newtown, PA), and the Microfuge was from Beckman, Inc. (Palo Alto, CA). A Beckman DU640 spectrophotometer from Beckman, Inc. (Fullerton, CA), was used for paraquat cation determination.

Paraquat Cation Extraction from Fruits and Vegetables. An extraction procedure modified from that of Van Emon and Hammock (1987) was used. Cabbage, potatoes, and apples were obtained from local markets. These items were individually washed and pureed in a food processor. Subsamples (2.5 g) were weighed into 15 mL calibrated polypropylene tubes, and 2.5 mL of 6 N sulfuric acid was added to each. These were vortexed and brought up to 5 mL total volume with 6 N sulfuric acid if necessary. The samples were sonicated for 20 min followed by a 5 min centrifugation to remove any remaining particulate matter. Using a plastic test tube, the top layer was diluted 1:250 (20 μL + 4.98 mL of sodium acetate buffer) and assayed according to the ELISA protocol listed below.

Competitive Immunoassay Procedure. Two hundred microliters of the standard, control, or diluted sample to be tested was added to a disposable test tube along with 250 μL of enzyme-labeled paraquat analog and 500 μL of magnetic particles with paraquat specific antibodies attached. After mixing, the samples were incubated for 15 min at room temperature. The reaction mixture was separated using the magnetic separation rack and washed twice; 500 μL of color solution (1:1 mixture of tetramethylbenzidine and hydrogen peroxide) was then added and allowed to develop for 20 min at room temperature. The color reaction was stopped with 500 μL of stopping solution (2 M sulfuric acid solution). Photometric analysis of the final colored product was made using the RPA-I Analyzer set at 450 nM, which records the absorbance and compares the observed sample results to a linear regression line using a log/logit standard curve prepared from calibrators containing 0, 50, 250, and 500 pg/mL of the paraquat cation. The functions of the RPA-I photometric analyzer were previously described (Rubio et al., 1991). The actual paraquat cation concentration in nanograms per gram was calculated by using the following equation:

$$\text{(assay result)} \times \text{(diln)} \times \frac{\text{vol of extract (mL)}}{\text{wt of sample (g)}} \times \frac{\text{ng}}{1000\ \text{pg}} =$$
$$\text{(pg/mL)} \times (250) \times (5\ \text{mL}/2.5\ \text{g}) \times (1/1000) =$$
$$\text{assay result} \times 0.500 = \text{concn of paraquat cation (ng/g)}$$

Since the paraquat cation is 72.4% of paraquat dichloride (w/w), dichloride results can be calculated by dividing the cation result by 0.724.

Determination of Cross-Reactivity. The relative sensitivity of the assay was determined by assaying a dilution series of each compound in the sodium acetate-based buffer and comparing the IC_{50} values of each analyte (concentration of analyte that produces a 50% decrease in the maximum normalized response) to the IC_{50} value of the paraquat cation.

Table 1. Immunoassay Cross-Reactivity of the Paraquat Cation and Related Compounds in Buffer

compound	90% B/B_0[a] LDD[b] (ng/mL)	IC_{50}[c] (ng/mL)	% cross-reactivity[d]
paraquat cation	0.020	0.30	100
methylbipyridyl methylsulfonium salt	0.002	0.96	33.0
diethylparaquat	0.005	13	2.5
monoquat	0.94	43	0.70
morphamquat	13	746	0.04
diquat	112	7502	<0.01
chlormequat	3300	NR[e]	<0.01
difenzoquat	NR	NR	<0.01
allyltrimethyl-ammonium bromide	6600	NR	<0.01
4,4-bipyridyl	1860	NR	<0.01
1-methyl-4-carboxypyridinium[f]	7100	NR	<0.01
methylamine hydrochloride[f]	NR	NR	<0.01
picolinic acid	NR	NR	NR
(2-bromoethyl)tri-methylammonium salt	NR	NR	NR

[a] B/B_0 was defined as the absorbance observed for a sample or standard divided by the absobance at a zero analyte concentration. [b] The least detectable dose (LDD) was estimated at 90% B/B_0. [c] The IC_{50} was expressed as the amount of compound required to displace 50% of the paraquat enzyme conjugate. [d] The cross-reactivity was expressed as a percentage relative to the paraquat cation at 50% B/B_0. [e] NR was defined as no reaction seen up to 10 μg/mL. [f] Plant breakdown products.

Acid Refluxing and Spectrophotometric Analysis for Correlation Study. The spectrophotometric method was performed with Dr. James Fleeker at the IR-4 Pesticide Analysis Laboratory at North Dakota State University. Zucchini samples were bought from a local market (Hornbachers, Fargo, ND) and prepared and analyzed according to the recommended procedure by Zeneca Agrochemicals (1993), ICI Method 1B. The samples were first macerated in a blender/homogenizer and then boiled under reflux in 0.5 M sulfuric acid for 5 h. Boiled samples were allowed to cool overnight. The digest from these samples was filtered and percolated through a cation exchange column (sodium form, 52–100 mesh). The paraquat was eluted with a saturated ammonium chloride solution at approximately 1 mL/min until 50 mL total was collected.

A 10 mL portion of this eluent was treated with 0.2% sodium dithionite in 0.3 M sodium hydroxide. The resulting paraquat free radical was measured using the height of the second derivative of the spectrum at 396 nm on the Beckman DU640 spectrophotometer. Expected recoveries for fruits and vegetables utilizing this extraction method and a 250 g sample range from 70 to 85% (Zeneca Agrochemicals, 1993). A 1 mL portion of the saturated ammonium chloride eluent was diluted at 1:1000 (10 μL + 9.99 mL) in the sodium acetate buffer and analyzed in the immunoassay described above.

RESULTS AND DISCUSSION

Sensitivity and Cross-Reactivity. The described immunoassay utilizes a competitive format. Since the enzyme-labeled paraquat competes with the unlabeled (sample) paraquat cation for the antibody sites, the color developed is inversely proportional to the concentration of the paraquat cation in the sample.

Three sets of 10 replicates of the zero standard were assayed, and the mean absorbance value and standard deviation were calculated for each run. The mean absorbance value minus four standard deviations was equivalent to a 90% B/B_0, which corresponded to an estimated detection limit of 20 pg/mL paraquat cation. Table 1 summarizes the immunoassay cross-reactivity

Table 2. Recovery of Paraquat Cation from Fruits and Vegetables

amt of paraquat cation added (ng/g)	paraquat cation recovered (ng/g)		
	apples	cabbage	potato
37.5	30.0	44.0	30.0
75.0	74.0	78.0	71.0
125.0	148.0	158.0	115.0
mean % recovered	99.0	109.0	89.0

Table 3. Paraquat Assay Precision in Buffer

	sample			
	1	2	3	4
N	25	25	25	25
mean (pg/mL)	45.46	106.1	225.5	427.7
%CV intra	17.2	14.4	7.5	7.0
%CV inter	<0.1	11.8	<0.1	<0.1
%CV total	17.2	17.9	7.4	6.9

Table 4. Precision of Paraquat Cation Extraction and Measurement in Potatoes by ELISA

	sample	
	1	2
replicates (*N*)	10	10
mean (ng/g)	59.2	125.3
%CV	13.1	8.4

and sensitivity to the paraquat cation and other related quaternary nitrogen compounds in buffer.

Recovery. The accuracy of the shortened extraction procedure and the paraquat immunoassay was assessed by evaluating single lot samples of three fruits and vegetables at three levels of paraquat cation. Blank samples were also assessed according to the recommended protocol and run in the immunoassay. Data showing the accuracy of the spiked recovery of the paraquat cation from apples, cabbage, and potatoes are shown in Table 2. Added amounts of the paraquat cation were recovered accurately in all cases with a mean assay recovery of 99%. The estimated sensitivity of the combined extraction/immunoassay procedure is 10 ng of paraquat cation/g of fruit or vegetable. The current residue tolerance for the paraquat cation in potatoes is 0.5 μg/g and is 0.05 μg/g for cabbage, squash, and apples in the United States (Codex Alimentarius Commision, 1991; U.S. EPA, 1990) and 0.05 μg/g for fruits and vegetables in Europe (Hamish and James, 1991). The immunoassay method described detects levels of the paraquat cation on fruits and vegetables at less than or equal to 20% of the these tolerances.

Precision. The precision of the ELISA was determined by running individual samples in buffer spiked with four different levels of the paraquat cation over 5 days, five assays per day. The within- and between-day and total variations were determined according to the method of Bookbinder and Panosian (1986). The precision of the combined extraction and immunoassay analysis of the paraquat cation from potatoes was also determined using two levels of paraquat-spiked potatoes which were extracted and assayed 10 times over 10 days. Results are shown in Tables 3 and 4.

Correlation to Spectrophotometric Method. To compare the results from the immunoassay and the traditional spectrophotometric method, zucchini was spiked with four levels of paraquat in triplicate at the following levels: 50, 65, 85, and 110 ng/g. Samples were prepared according to ICI method 1B (Zeneca Agrochemicals, 1993). For immunoassay analysis, aliquots

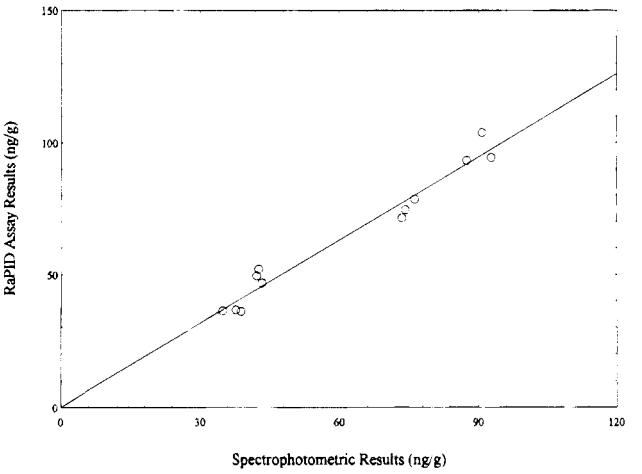

Figure 1. Paraquat cation recoveries: spectrophotometric method vs immunoassay. $y = +1.051 + 0.0324$, $R = 4.15$, max dev = 8.40, $r = 0.9937$, $n = 16$.

Table 5. Recovery of the Paraquat Cation from Zucchini

level (ng/g)	spectrophotometric			immunoassay		
	recovered (ng/g)	mean ± SD (ng/g)	mean (%R)	recovered (ng/g)	mean ± SD (ng/g)	mean (%R)
50	37.8	37.2 ± 1.7	75	36.5	36.2 ± 0.2	73
	38.9			36.0		
	34.9			36.2		
65	43.4	42.8 ± 0.4	67	46.8	49.4 ± 2.1	77
	42.7			51.9		
	42.3			49.4		
85	76.2	74.6 ± 1.1	88	78.6	74.8 ± 2.9	88
	74.2			74.5		
	73.5			71.4		
110	92.8	90.4 ± 2.2	80	94.2	97.1 ± 4.8	86
	90.8			103.8		
	87.5			93.2		

of the saturated ammonium chloride extracts were diluted 1:1000 in sodium acetate buffer and analyzed in the assay. The results comparing the ELISA method (y) and the spectrophotometric method (x) are shown in Table 5 and Figure 1. The correlation between the methods was good, yielding a correlation of $r = 0.994$ and a slope of 1.05. Immunoassay recoveries averaged 81%, while the spectrophotometric recoveries averaged 78%, showing that with this extraction process the ELISA method is equivalent to spectrophotometric measurement of the paraquat cation.

Conclusions. This study demonstrates the feasibility of applying magnetic particle based immunoassays to the detection of pesticide residues on fruits and vegetables. The speed and sensitivity of the described protocol make it suitable as a screening method for large numbers of samples in short periods of time. This assay compares favorably with the EPA-recommended spectrophotometric method for the determination of the paraquat cation at levels below the established tolerances.

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