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Application of an Enzyme-Linked Immunosorbent Assay for the Analysis of Paraquat in Human-Exposure Samples

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Abstract. Paraquat is a toxic quaternary ammonium compound used as an herbicide around the world. Easy, fast, and inexpensive but sensitive methods are needed to study the effects of long-term, low-level exposure of paraquat on human health. An enzyme-linked immunosorbent assay (ELISA) was used for quantification of paraquat in urine and air-filter samples collected in a human-exposure study among farm workers in Costa Rica. A sample pretreatment consisted of removal of interfering substances using solid-phase extraction resin columns. The precision and accuracy of the method were tested using duplicate spiked urine samples. The correlation between results for blind samples obtained using ELISA and liquid chromatography–mass spectrometry was significant (R^2 = 0.945 and 0.906 for spiked and field samples, respectively). With an LOQ of 2 ng mL^{-1}, this ELISA method was able to distinguish the exposed from the nonexposed farm workers. For the air-filter analysis, paraquat was extracted by 9 M H_2SO_4 at 60°C for 12 hours, and the results obtained by ELISA showed good correlation (R^2 = 0.918) with the spectrophotometric (256 nm) measurements. Paraquat in acid-stabilized urine samples was very stable, and no significant losses were detected during a 3-month storage at room temperature, at 4°C, or at −20°C.

Paraquat (1,1′-dimethyl, 4,4′-bipyridinium) is a fast-acting, quaternary ammonium, nonselective, contact herbicide that inhibits photosynthesis when applied to plant foliage. It is used extensively for both weed control and as a preharvest desiccant and defoliant. Although paraquat is highly water soluble, it is not easily leached from soil or taken up into plant root systems because it is quickly and strongly adsorbed to clay and soil organic matter (Tucker *et al*. 1967). The extensive research on the fate of paraquat in agroecosystems has been reviewed by Summers (1980) and recently by Roberts *et al*. (2002).

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Despite its high acute oral toxicity (Pasi 1978; Summers 1980), paraquat is still used in >120 countries, mostly in Asia and North and South America. Respiratory failure is a prominent outcome in fatal paraquat ingestions (Smith and Heath 1975), which demonstrates the ability of paraquat to accumulate in lung tissue (Smith *et al*. 1974; Rose *et al*. 1976) and cause pulmonary fibrosis (Hudson and Patel 1991). However, the hazard to humans through inhalation or other routes of low-level, long-term exposure has not been fully investigated (Castro-Gutierrez 1997; Dalvie 1999).

Determination of the paraquat concentration in urine is a valuable tool for diagnosis in accidental, suicidal, and occupational intoxications, and it can be used for biological exposure assessment as well. After an oral dose, more paraquat is excreted in feces than in the urine (Van Dijk *et al*. 1975). However, large amounts of paraquat have been measured in the urine within 12 hours of ingestion (Matthew *et al*. 1968), and after a subcutaneous administration paraquat is mainly excreted in urine. In all cases, blood levels of paraquat are low compared with those in the urine (Tompsett 1970).

Analytical methods for detection of paraquat in water, food, and biological samples are based either on spectrophotometry (Calderbank and Yuen 1965; Faust and Hunter 1965; Yuen *et al*. 1967; Ganesan *et al*. 1979; Rai *et al*. 1997); on polarography (Engelhardt and McKinely 1966); on thin-layer chromatography (Ikebuchi *et al*. 1988); on gas chromatography (Martens and Heyndrickx 1974; Hajslova *et al*. 1989); on liquid chromatography (LC) with either UV detection (Worobey 1987; Paixão *et al*. 2002; Blake *et al*. 2002) or mass spectrometry (MS) (Taguchi *et al*. 1998; Castro *et al*. 2001; Blake *et al*. 2002; Grey *et al*. 2002); on ion-pair chromatography (Kuo 1987); on photokinetic methods (Martinez *et al*. 1988); on flow-injection analysis (Jain *et al*. 1993); on immunochemical assay (Van Emon *et al*. 1986, 1987); and more recently, on electrophoretic methods with either UV detection (Nuňez *et al*. 2002a, 2002b) or mass spectrometry (Nuňez *et al*. 2002c). The drawback of most chromatographic and spectrophotometric methods is the high limit of detection (LOD; 30 to 50 ppb), which makes them unsuitable for biological samples with low levels of paraquat. The new LC-MS methods have a lower limit of detection, but for them, an extensive sample cleanup is generally required. Van Emon

et al. (1986) developed an enzyme-linked immunosorbent assay (ELISA) to measure paraquat in human-exposure samples (air filters, personal air monitors, clothing patches, and hand washes). Compared with a GC method, the ELISA gave better recoveries, was less labor intensive, and was more sensitive (LOD 0.1 to 1.0 ppb).

In most cases, the analysis of paraquat in urine, plasma, and serum involves a sample pretreatment, which removes interfering substances. The cleanup is usually achieved by using ion-pair extraction either with bromothymol blue (Åkerblom 1990) or on disposable solid-phase extraction (SPE) cartridges (Smith and Henderson 1986; Gill *et al*. 1983; Croes *et al*. 1993; Picó *et al*. 2000; Blake *et al*. 2002). Recently, Paixão *et al*. (2002) reported a simple plasma pretreatment method involving protein precipitation with 6% perchloric acid in methanol.

In human-exposure studies, air monitoring for dust exposure is often used concurrently with biological monitoring. Before analysis, paraquat collected in air filters has to be extracted. The official National Institute for Occupational Safety and Health (NIOSH) method 5003 (NIOSH 1994) for extraction of paraquat from polytetrafluoroethylene (PTFE) filters involves a rapid rinse of the filter with 5 mL water. Van Emon *et al*. (1986) extracted paraquat from glass fiber filters with either 6 M HCl or a paraquat-specific antibody. The average efficiency for the antibody extraction was 60% and 45% for a 2-day and 1-day incubation, respectively. Because paraquat is strongly retained by soil particles, more powerful extraction methods are recommended for filters with dust. According to Roberts *et al*. (2002), the extraction method for soil developed by Syngenta (Cheshire, UK) involves refluxing the sample with 6 M sulfuric acid followed by filtration through a cation exchange resin. However, in the original extraction method by Calderbank and Yuen (1965) and Tucker *et al.* (1967), the sample is refluxed in 9 M H_2SO_4 for 5 hours for complete extraction of paraquat.

The current methods for quantification of paraquat in biological and human-exposure samples require special equipment or time-consuming steps, and in most cases they are not sensitive or fast enough for efficient monitoring of biological samples. This article presents a simple yet sensitive ELISA method for paraquat. We have used it for both urine and air-filter samples collected in a human-exposure study among Costa Rican farm workers. For the paraquat ELISA, we developed a urine cleanup procedure using resin SPE cartridges, and the same procedure was successfully used for the air-filter extracts. The ELISA method was validated by using external standards as quality control samples, duplicate measurements, and comparisons with UV-absorption and current LC-MS methods for paraquat.

Material and Methods

Chemicals and Reagents

Paraquat dichloride tetrahydrate was obtained from Chem Service (West Chester, PA). Bovine serum albumin (fraction V), goat anti-rabbit immunoglobulin (IgG) horseradish peroxidase conjugate, and 3,3′,5,5′-tetramethylbenzidine sodium salt and Tween 20 were from Sigma Chemical (St. Louis, MO). All other chemicals were of reagent grade or better from Fisher Scientific (Pittsburgh, PA). The antibody (Ab21) was prepared in this laboratory previously (Van Emon *et al*. 1986). Hapten and coating antigens were freshly synthesized using previously reported methods (Fatori and Hunter 1980; Van Emon *et al.* 1986).

Urine Sample Preparation

The 24-hour urine collections were cooled, stabilized by boric acid (12 g H_3BO_3/4-L plastic container), and aliquoted into 8 separate 15-mL polypropylene tubes. After freezing, the samples were shipped to the laboratory where they were kept frozen until the analysis. Frozen urine samples were thawed for 16 hours at room temperature after which they were vortexed and centrifuged at 15°C for 10 min at 2500g and the supernatant transferred to another 15-mL polypropylene tube. For the urine cleanup, 3-mL Oasis MCX resin cartridges (Waters, MA) were equilibrated with 2 mL 20 mM sodium phosphate buffer (SPB; pH 6.5). A 1-mL urine sample was extracted by vacuum aspiration through the SPE cartridge at a flow rate of approximately 2 mL min^{-1} and washed down with another 2-mL batch of SPB. For removal of interfering substances, the cartridge was then rinsed sequentially with 4 mL 50% (v/v) methanol in water and 10 mL 5% (v/v) NH_4OH in acetonitrile. Fresh 5% NH_4OH in acetonitrile was prepared daily. Paraquat was eluted from the SPE cartridge using 4 mL 1 M NH_4Cl in 50% methanol. Samples were stored in polypropylene tubes in a refrigerator (at 4°C) until analysis. Spiked urine samples with known concentrations of paraquat were purified with each sample set for recovery and quality assessment.

Air-Filter Sample Preparation

The extraction procedure is a modification of the method described by Tucker *et al*. (1967) for determining paraquat in soils. It was validated for paraquat in PTFE filters by spiking dust-treated filters with methyl-^{14}C paraquat (Sigma, St. Louis, MO) and measuring the recovery using different extractants (H_2O, saturated NH_4Cl, HCl, and H_2SO_4). The effects of extractant, acid concentration (6 M and 9 M), extraction time (5 and 12 hours), and temperature (30°C, 60°C, and 90°C) on the recovery of ^{14}C-paraquat were tested, and the extraction method with best recovery was chosen. For extraction, each PTFE filter was placed in a plastic 20-mL vial with a 5-mL aliquot of 9 M H_2SO_4. Capped vials were shaken horizontally in a water bath at 60°C for 12 hours after which each filter was rinsed with 5 mL deionized H_2O. The rinse was mixed with the extract, and the solution was neutralized with 9 M NaOH. A 4-mL sample of each solution was extracted by vacuum aspiration through a 3-mL MCX cartridge at a flow rate of approximately 2 mL min^{-1}. After washing the cartridge with 4 mL of 50% (v/v) methanol in water, paraquat was eluted from the cartridge using 4 mL 1 M NH_4Cl in 50% methanol.

ELISA

A solid-phase indirect competitive ELISA was used for measurement of paraquat in prepared samples. Regular 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with the coating antigen PQ-C2-BSA (1/4600 dilution of a stock solution with 0.3 mg protein mL^{-1}) in a 0.05 M sodium carbonate–bicarbonate buffer (pH 9.6). The following day, the coating antigen was washed off the plate with a phosphate-buffered saline solution (PBS; pH 7.5) containing 0.05% Tween 20, and 50 μL of each sample or standard in triplicate

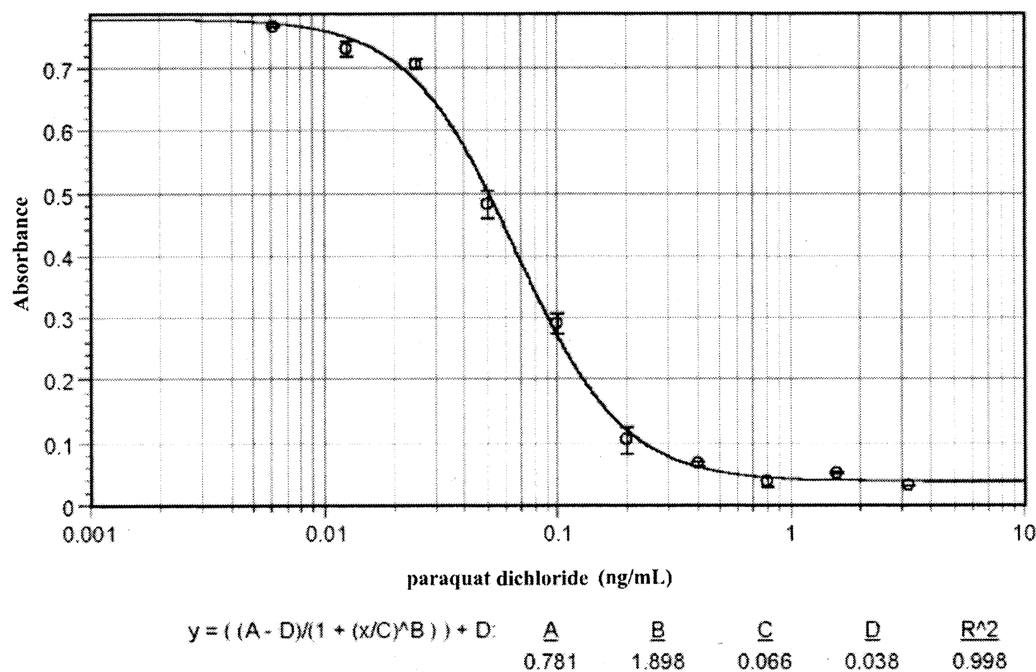

Fig. 1. Standard curve for paraquat dichloride (0 to 3.2 ng mL^{-1}) for the average of three replicates.

were pipetted into each well. The standard curve for paraquat was prepared from the frozen stock standard (1 μg mL^{-1} paraquat dichloride in water) and contained 0 to 3.2 ng paraquat dichloride (corresponding to 0 to 2.32 ng paraquat ion) in 1 mL PBS containing 0.05% Tween 20 and 0.05% BSA (PBSTB). Before loading onto the plate, samples were diluted with PBSTB so that the concentration of paraquat would fit into the linear range of the standard curve. Because the shape of the standard curve was affected by the amount of methanol in the sample, the standard curves were prepared to match the methanol content in each sample batch. To each sample well, 50 μL of the paraquat polyclonal antibody was added in 1/10,000 dilution, and the mixture was incubated at room temperature for 20 minutes. The sample matrix was washed away leaving only the antibodies bound to the coating antigen. Then, 100 μL of the anti-rabbit IgG-horseradish peroxidase conjugate (IgG-HRP) was added into each well, and the IgG-HRP was allowed to bind to IgG sites on the bound antibodies for 40 minutes. Unbound IgG-HRP was washed away to leave an amount of HRP enzyme that (IfG-HRP) was inversely proportional to the paraquat concentration in the samples or standards. Finally, a colorless substrate—1% H_2O_2—and a chromogen—3,3′4.4′-tetramethylbenzidine—were incubated with the bound enzyme to produce a blue color. A 50-μL aliquot of 2 M H_2SO_4 was added as a stop solution to change the color to a stable yellow. ELISA absorbances at 450 nm were measured with a microplate reader (Molecular Devices, Menlo Park, CA). The content of paraquat in the unknown samples was calculated based on the standard curves in each plate. The software package Softmax (Molecular Devices) was used for fitting the 11-point sigmoidal standard curve based on the 4-parameter logistic method of Rodbard (1981).

Quality Assurance

Because paraquat at low concentrations is known to adsorb tightly on glass (Ross and Krieger 1980), all samples and standards were prepared and stored in plastic (polypropylene or polystyrene) containers and tubes. Degradation and adsorption of paraquat during sample storage and handling was assessed in separate stability studies in which multiple measurements were taken in ^{14}C-fortified urine samples stored at room temperature for extended periods of time. Similarly, stability of paraquat in urine samples stored in daylight at room temperature was tested during a 3-month period. Stability of paraquat in NH_4Cl extracts stored in the refrigerator was tested using duplicate measurements over time. The effect of multiple freeze–thaw cycles on the recovery of paraquat in urine samples was also tested.

For validation of the ELISA method and quality control, a set of both spiked and farm-collected urine samples were analyzed by Syngenta (Cheshire, UK) using an LC-MS method (Blake *et al.* 2002), and the results were compared with those obtained by ELISA in a double-blind study. The accuracy of the paraquat standards used in ELISA was verified using a certified standard (100 μg mL^{-1} paraquat dichloride tetrahydrate Chem Service, West Chester, PA) with a UV spectrophotometric method (Summers 1980) at 256 nm (log ϵ = 4.30).

Results and Discussion

An ELISA method for paraquat developed by Van Emon *et al.* (1986)—previously applied to determination of paraquat residues in human exposure samples as well as in milk, beef, and potatoes (Van Emon *et al.* 1986, 1987)—was used to analyze approximately 200 urine samples and 13 air-filter samples for paraquat. The standard curve created using paraquat dichloride in PBSTB was linear between 0.03 and 0.15 ng mL^{-1} giving an IC_{50} value of 0.066 ng mL^{-1} corresponding to 0.04 ng paraquat ion mL^{-1} (Figure 1). Paraquat dichloride standards were validated against a certified standard using absorption at 256 nm, and their accuracy was found to be 96.4% ± 3.6%. Addition of methanol in the standards resulted in a significant decrease in the slope and increase in the IC_{50} of the standard curve in ELISA.

The urine cleanup procedure was optimized using blank urine samples spiked with methyl–^{14}C paraquat to give final concentrations of 5 ng mL^{-1} and 20 ng mL^{-1}. The recovery of paraquat in five replicates of 1-mL spiked samples was 91.5 ± 1.1% for both concentrations. According to a separate loading study, approximately 100 ng paraquat in urine could be retained by one 3-mL Oasis MCX resin cartridge, and the amount of urine was adjusted accordingly (0.1 to 3 mL).

The intra-assay (intraplate) precision of ELISA, expressed by a coefficient of variation (%CV) between three replicates, was always <15% and in most cases <5%. The interday precision (%CV) measured for the same NH_4Cl–methanol extracts during 4 different days did not exceed 13%. The precision and accuracy of the whole system, including the cleanup and ELISA for spiked urine samples, are listed in Table 1. For quality assessment, urine samples of seven different concentrations (between 0.4 and 90 ng paraquat mL^{-1}) were studied during multiple days (3, 4, 5, or 17 days depending on the concentration). When urine samples spiked with known concentrations of paraquat were cleaned up and analyzed by ELISA, the average recovery efficiency for all tested concentrations was 95.2%. The minimum concentration at which acceptable recovery and precision were obtained was 2 ng mL^{-1}. This was also accepted as a limit of quantification (LOQ) for the whole system including urine cleanup and ELISA. For the tested concentrations, added paraquat was less completely recovered at <2 ng mL^{-1} and at >60 ng mL^{-1}.

Comparison of the results obtained by using both ELISA and LC-MS methods for the same set of samples run in a double-blind study is presented in Figure 2. ELISA and LC-MS methods provided consistent measurements for analysis of paraquat in urine. There seemed to be a slight tendency for lower readings acquired by ELISA compared with the LC/MS method, but overall the agreement between ELISA and LC-MS results for the spiked samples was good (R^2 =0.945) with slope = 0.94 and intercept = 1.23. The association between ELISA and LC-MS results was lower for the field samples with R^2 = 0.91, slope = 0.73, and intercept = 2.59. With the field samples, the smaller correlation coefficient (slope) could simply be an indication of a proportionally larger number of samples with low paraquat concentrations within the farm sample population compared with the spiked samples used for the assessment. As listed in Table 1, for urine samples with low levels of paraquat, ELISA seemed to slightly overestimate the paraquat concentrations. In contrast, the recovery of paraquat measured by ELISA decreased with increasing urinary paraquat concentrations. The reproducibility of ELISA, including the urine cleanup procedure, was verified using duplicate blind urine samples. A small set of farm urine samples (n = 52) was divided in half, and each half was analyzed as a separate sample with ELISA during different days. Figure 3 presents the results from samples measured by ELISA as blind duplicate samples indicating a satisfactory correlation (R^2 = 0.945) between the two measurements.

In the assessment of the air-filter extraction method, the recovery of radiolabeled paraquat added to the filter and extracted with 9 M H_2SO_4 for 12 hours at 60°C was found to be the highest: 96.0% ± 0.5% compared with 72.5% ± 1.0%, 71.0% ± 1.7%, and 67.1% ± 2.5% extracted with 9 M HCl, saturated NH_4Cl, and water, respectively. Approximately 20% more paraquat was extracted at 60°C than at 30°C, but recovery was not further improved by increasing the temperature to 90°C. At all temperatures, an increase in extraction time from 5 to 12 hours, or an increase in H_2SO_4 concentration from 6 to 9M, increased paraquat recovery. For verification, both UV and ELISA methods were used for determination of paraquat concentration in a small, randomly chosen set of air-filter samples (n = 13 or 35% of all filters). The correlation between both results is presented in Figure 4 (R^2 = 0.918).

Table 1. Overall precision (reported as %CV including both inter-cleanup and inter-ELISA variation), recovery and accuracy of the determination of paraquat in urine using SPE cleanup and ELISA

Paraquat added (ng mL^{-1})	No. of samples	Paraquat found (ng mL^{-1})	Recovery (%)	CV (%)
0.4	5	0.29 ± 0.05	73.0	17.5
1.0	5	0.90 ± 0.46	90.0	43.0
2.0	17	2.3 ± 0.48	115.6	20.9
15.0	4	15.9 ± 1.55	106.2	9.7
30.0	17	30.5 ± 3.58	101.7	11.7
60.0	4	55.9 ± 7.23	93.2	12.9
90.0	3	77.9 ± 13.15	86.6	16.9

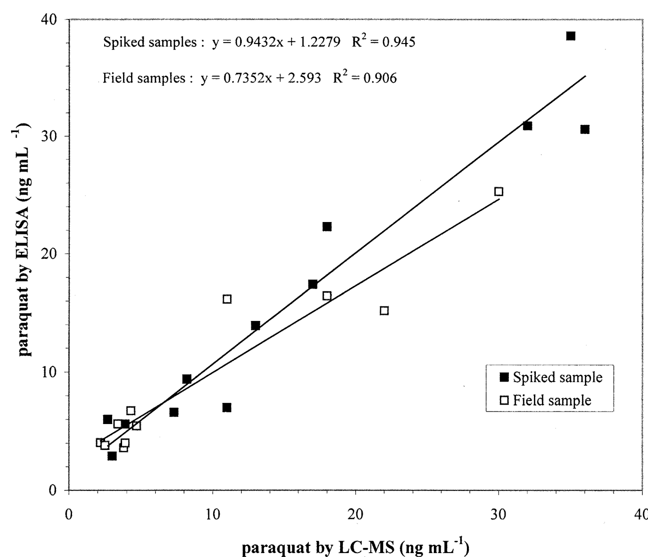

Fig. 2. Comparison between urine paraquat concentrations (ng mL^{-1}) analyzed by LC-MS and ELISA methods.

The LOQ for paraquat in urine samples was determined experimentally as described previously by choosing the lowest concentration that over multiple determinations gave acceptable precision, recovery and accuracy (Table 1). An ELISA, in combination with the urine pretreatment, detected paraquat levels as low as 2 ng mL^{-1} in spiked urine samples. However, the LOD for paraquat in the NH_4Cl–methanol extracts was much lower, and ELISA could detect concentrations as low as 0.02 ng mL^{-1}. For the UV spectrophotometric method for paraquat, LOD was determined by the formula LOD = (3.3SD)/S, where S is the slope of the standard curve, and SD is the deviation of the y-intercept (Paixão

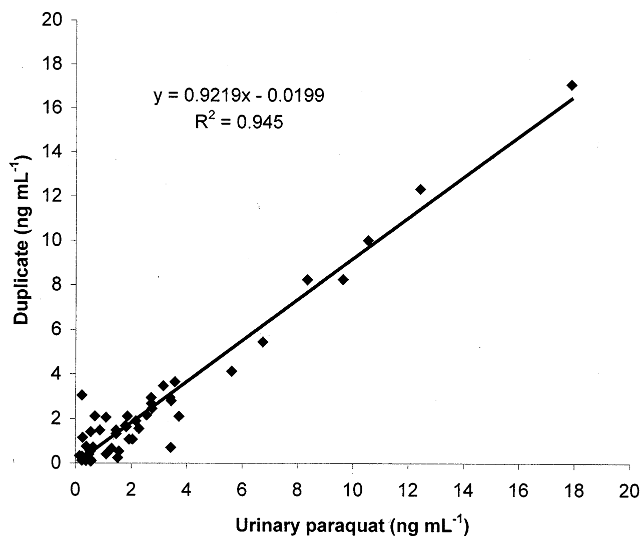

Fig. 3. Correlation between duplicate urine samples analyzed by ELISA. A small set of farm urine samples ($n = 52$) was divided in half, and each half was analyzed as a separate sample with ELISA during different days. The data have not been adjusted by the LOQ.

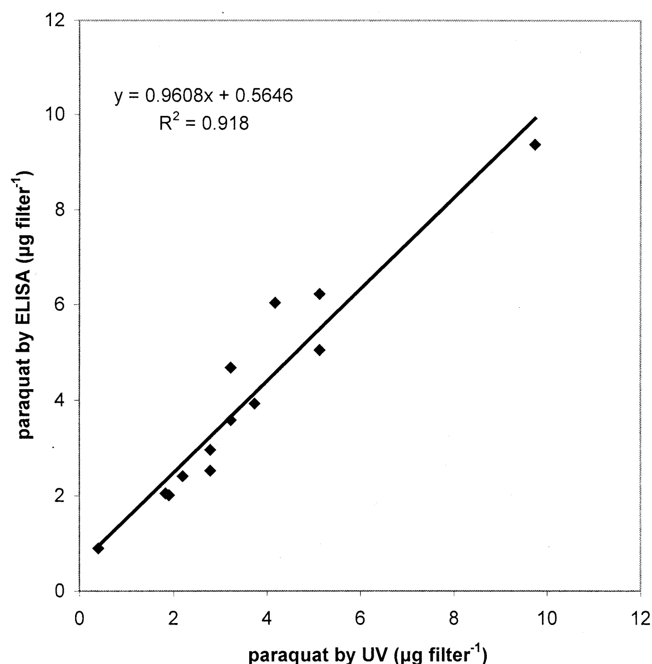

Fig. 4. Comparison of results obtained by the UV and ELISA methods used for analysis of paraquat in air filter samples.

et al. 2002). Similarly, the LOQ was determined by the formula LOQ = (10SD)/*S*. The LOD was approximately 0.07 µg mL^{-1}, and the LOQ for paraquat in the air-filter extracts was approximately 0.2 µg mL^{-1} corresponding to 1.3 µg paraquat/ filter.

ELISA was able to detect a difference in urinary paraquat concentration between the nonhandler and handler groups. Table 2 lists the amount of paraquat found in 24-hour urine samples from nonhandlers and handlers in Costa Rican farms. Urine samples from farm workers with no paraquat-handling activity were low. All but four nonhandlers had nondetectable urinary paraquat. The four positive samples with paraquat levels of 2.0, 2.2, 4.7, and 6.8 µg 24 h^{-1} were collected on farms where paraquat was used. This indicates that a few nonhandlers may have been exposed, but the level and frequency were low. Urine samples of workers who handled paraquat had a mean level of 5.6 ± 10.8 µg urinary paraquat 24 h^{-1} ranging from nondetactable to 75.4 µg 24 h^{-1}. More than 50% of the urine samples of handlers had detectable urinary paraquat, and the highest paraquat concentrations measured in the urine samples were 2 orders of magnitude higher than the LOQ. A detailed presentation of the epidemiological part of the study with farm worker urinary paraquat levels before, during, and after pesticide application among different job and crop types will be published elsewhere (Lee K *et al.*, manuscript in preparation).

Because of the relatively high concentrations of paraquat in the air filters, UV spectroscopy was the main method used for their analysis. The range of paraquat found in a filter was 0.4 to 16.7 µg with an average of 5.1 µg, a median of 3.7 µg, and a mode of 2.9 µg/filter.

No losses of paraquat were observed during storage at room temperature, in the refrigerator, or in the freezer. Paraquat in both urine and NH_4Cl extracts was stable during the 3-month test period, and both photodegradation and adsorption on the surfaces of plastic containers were nonsignificant for paraquat in urine samples.

Table 2. Urinary paraquat levels measured by ELISA among nonhandlers and handlers of paraquat (µg 24 h^{-1}).

Group	No. of samples	Paraquat in urine (µg 24 h^{-1}) Mean	SD	Median	Range	Samples higher than LOQ[a] (%)
Nonhandler	83	0.20	0.98	0	0–6.81	4.8
Handler	119	5.64	10.77	1.45	0–75.37	52.9

[a] LOQ = 2 ng mL^{-1}.

Overall, the ELISA method for measuring paraquat in urine and air-filter samples combined with a simple SPE cleanup proved to be specific, sensitive, and fast enough to accommodate a large number of samples with low analyte concentrations. With an LOQ for urinary paraquat significantly lower than the levels reported in previous exposure studies (Chester and Woollen 1982; Dalvie *et al.* 1999), the ELISA is a feasible and cost-effective method of choice for the analysis of paraquat in large sample sets collected in epidemiological studies.

Conclusion

An easy, fast, inexpensive, and sensitive ELISA method is presented for the quantification of paraquat in urine and air-filter samples. The sample pretreatment before analysis consists of removal of interfering substances using SPE resin columns. The method is easily adapted even in field conditions, and provides excellent precision and accuracy for human exposure samples with low levels of paraquat. The method is best suited for extracts with paraquat dichloride concentrations within the linear range of the ELISA standard curve (0.03 to

0.15 ng mL^{-1}), and according to our experience, the precision is lower at high concentrations because of the dilution errors. To make the method more versatile, the paraquat concentration in the extract can be easily modified by changing the amount of original sample used for clean up or the amount of extractant (1 M NH_4Cl in 5% methanol) used for elution of the analyte from the SPE column. With this ELISA method, the LOQ for paraquat in urine is 2 ng mL^{-1}, which is one order of magnitude lower than reportedly obtained by spectrophotometric and chromatographic methods.

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