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Foliar Dislodgeable Residue Analysis: A New Scientific Approach to a Regulatory Concern

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Summary

The U. S. Environmental Protection Agency and other federal and state regulators are interested in the presence and bioavailability of pesticide residues that remain on vegetation after application. These compounds can become an issue in human exposure if they become airborne and are inhaled, or if they are dermally absorbed by agricultural workers. In an effort to measure the level and extent of these foliar dislodgeable residues, the EPA's Office of Prevention, Pesticides, and Toxic Substances has developed guidelines for sampling and analysis of leaf sections. The testing methods used meet the requirements of the Federal Insecticide, Fungicide, and Rodenticide Act. The advent of immunochemical methods for the analysis of many pesticides offers new, rapid, and sensitive analytical procedures for residue studies of these leaf extracts. This article describes the use of indirect competitive enzyme-linked immunosorbent assay on representative samples for the analysis of the pesticide chlorpyrifos. Results will be compared with those from the traditional high-performance liquid chromatography method.

Keywords: chlorpyrifos, exposure monitoring, foliar dislodgeable residues, immunoassay.

Introduction

The analysis of pesticides in various matrices is conducted to optimize for human safety while allowing reasonable leeway for beneficial pesticide usage and ensuring crop-to-market timeliness. Human exposure studies can focus on several aspects, including long-term exposure to ingested pesticide residues in food, trace pesticide levels in drinking water, or foliar dislodgeable residues (FDRs) on edible and non-edible plants. For analytical purposes, FDR is the amount of pesticide residue that can be washed off leaf disks with a dilute surfactant. Leaf disks are typically 2.5 or 5 cm^2 and are representative of the crop site.

When farmers or other registered applicators spray pesticides on crops, a certain amount remains on the leaves of the plants. Later, agricultural workers may be exposed to these residual pesticides by touching the leaves (a pathway for dermal absorption), by disturbing the plants and inhaling the airborne residue, or by contacting the residue with their clothes and inhaling or ingesting it later. The U. S. Environmental Protection Agency (EPA), through its Office of Prevention, Pesticides, and Toxic Substances (OPPTS), has established guidelines to support the investigation of new methods for collection of representative samples, washing of leaf samples for maximum recovery, and rapid, field-portable analytical methods capable of reliably measuring the relatively small amounts typically found on vegetation *(1)*. One avenue of development, evaluation, and validation is through the EPA Office of Research and Development, National Exposure Research Laboratory.

Recognition of the toxic potential of organophosphate pesticides developed slowly in the first few decades following the introduction of parathion into U. S. agriculture in 1949 *(2–5)*. Some scattered incidents of agricultural worker poisonings were reported but, as they were contradicted by widespread pesticide use without incident, were attributed to other causes. Additionally, the analytical instrumentation and methods of the 1950s

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were not sufficient to the task of subtle separations and quantitation. With the advent of gas-liquid chromatography (GLC) in the early 1960s, it became possible to rapidly separate and quantitate parathion from its oxygen analog, paraoxon *(6)*.

An understanding of the various pathways of exposure and elucidation of organophosphate pesticides as acetylcholinesterase inhibitors provided researchers with industrial hygiene models upon which to base worker protection programs. The difficulty was the erratic and abrupt nature of poisoning incidents and the impracticality of imposing lenghty post-application reentry intervals. The quantitative residue decay dose-response portion of a unified field model *(ibid.)* was used to predict acetylcholinesterase (AChE) inhibiton both from single exposures and series of exposures. But, because exposure to foliar residue is neither daily nor uniform, studies were conducted to measure the variability of exposure levels over a 5-day work week throughout a harvest season and with varying residue concentrations. Variability in response is reduced primarily as the daily AChE inhibitions increase, and secondarily as the variability in the residues increases *(7)*.

Our laboratory has developed and evaluated an immunoassay for chlorpyrifos, a widely used organophosphate pesticide that is sprayed on crops and in the home for insect control. Chlorpyrifos, introduced in 1965 and commercially available as formulated Dursban® for indoor use and Lorsban® for crop protection, has the chemical formula O,O-diethyl-O-[(3,5,6-trichloro-2-pyridyl]phosphorothioate. Chlorpyrifos is suspected of being an AChE inhibitor and, as such, may block normal functioning AChE at the nerve endings and cause neurological damage to exposed individuals. Neurotoxic potency for all organophosphate and carbamate insecticides are calculated on the basis of chlorpyrifos equivalents (Table 1).

An occupational study on 22 pest control operators exposed to an 8-hour level of 27.6 $\mu g/m^3$ of Dursban® showed inhibition of cholinesterase when compared to a control group of the same age and gender *(10)*. Low blood cholinesterase levels can persist from two to six weeks with long-term exposure to chlorpyrifos *(11)*.

The Code of Federal Regulations has established tolerance levels for residues of chlorpyrifos in or on several raw agricultural commodities *(12)*, including cucumbers, nectarines, peaches, and turnip greens. Safe worker reentry times are based on the tolerance levels. The reentry times are presently universal (for chlorpyrifos, reentry time is 24 hours after tolerance level is exceeded) and do not vary with meteorological conditions. Exposure can be monitored using either passive dosimetry which estimates the amount of a chemical impinging on the surface of the skin or the amount available for inhalation, or by biological monitoring methods that measure internal dose of the pesticide or its metabolites in eliminated fluids (typically urine). In many instances passive dosimetry is preferable because it allows early detection of exposure. Field-portable analytical methods provide a secondary means of real-time exposure monitoring, combining the convenience of dosimetry with the reliability of laboratory methods. Our laboratory proposes a rapid immunoassay method for analyzing FDR of chlorpyrifos that may be used on-site by trained personnel. The practical advantage of this method is that real-time chlorpyrifos concentrations can be measured and reported, and workers may be able to enter agricultural fields sooner in certain conditions (*e.g.*, rain may wash off residue, sun may photodegrade residue, etc.).

We obtained samples of various garden vegetables from local markets and gardens and sprayed them with a dilute solution of commercial grade Dursban® (obtained at a local nursery). The greens were moist but not dripping. Then the greens were washed with a dilute surfactant (sodium dioctylsulfosuccinate). Leaf punches were made 24 hours later, based on the safe reentry time for chlorpyrifos. Leaf punch washes were directly analyzed by immunoassay, but had to be extracted into ethyl acetate for high-performance liquid chromatography (HPLC).

The sampling procedure for obtaining representative and uniform leaf punches has been described in detail *(13)*. The recommended method is to use a reliable leaf punch to obtain a circular leaf disk, 1 inch (or 2.54 cm) in diameter, from or near the center of the leaf. The leaf disk may need to be smaller for plants with narrow leaves *(14)*. The leaf disks are collected in a jar, usually 8 ounce (240 mL) capacity. Generally, it is recommended that 40–50 leaf disks be collected per sample. These disks should be free of excess moisture from spray application, rain, sprinkler systems, or dew, but not necessarily dried.

Table 1. Relative neurotoxic potencies of several pesticides (adapted from ref. *8*).
Total average neurotoxic potency = Sum of mean residue × chlorpyrifos equivalency factor for each cholinesterase inhibiting pesticide (with a sample size greater than 20)

Pesticide	Average residue concentration ng/g	Chlorpyrifos equivalency factor*	Potency of average residue in chlorpyrifos equivalency factor × 10^{10}
Acephate	0.60	0.83	335
Carbaryl	2.50	0.030	54
Chlorpyrifos	0.40	1.0	279
Diazinon	0.40	4.0	1060
Dimethoate	0.05	0.40	13
Methamidaphos	8.20	2.0	11800
Parathion	0.02	10.0	166

* Chlorpyrifos equivalency factors were calculated using the methodology described by NAS *(9)*, with chlorpyrifos assigned a value of 1.

Some leaves have physical characteristics which make them difficult to analyze. For example, the hairy leaves of squashes, cucumbers, and raspberries can retain pesticide residue, affecting recovery and giving a low-baised analytical result. The washing procedure may need to be modified to optimize efficiency for a particular vegetation.

Experimental

Reagents

Monoclonal anti-chlorpyrifos antibodies *(15)*, radiolabeled carbon-14 chlorpyrifos, thyroglobulin- chlorpyrifos antigen (generous gifts from Dow Elanco, Midland, MI), goat anti-mouse IgG alkaline phosphatase conjugate, *p*-nitrophenyl phosphate substrate tablets (Sigma, St. Louis, MO), and purified chlorpyrifos powder (Chem Service, Westchester, PA).

Phosphate-buffered saline with Tween 20® (PBST) at pH=7.4 was prepared according to standard procedures *(16)*. Purified chlorpyrifos powder was used to prepare standards. Solvents for HPLC confirmation were pesticide-grade methanol and acetonitrile (Burdick and Jackson, Muskegon, MI). Ecoscint A solution (National Diagnostic, Manville, NJ) was used for liquid scintillation counting procedures. Polystyrene 96-well microtiter plates (Maxisorb I, A/S NUNC, Roskilde, Denmark), multichannel pipettors, and positive displacement micropipettors were used. The confirmatory instrument was an isocratic high performance liquid chromatograph, configured with a C18 column, 15 × 2.1 mm, UV detector at 290 nm. A standard and uniform leaf spray was prepared using commercial concentrate Dursban® (6.6% active ingredient) obtainable from any plant nursery. Surfactant used was sodium dioctylsulfosuccinate (as the sodium salt) (Aldrich Chemical Co., Milwaukee, WI).

ELISA procedure

The development, characterization and cross reactivity of the monoclonal antibody in a magnetic particle-based immunoassay has been previously reported *(15)*. The assay in the current study is an indirect competitive enzyme-linked immunosorbent assay (ELISA) format *(17)*. Standards, controls, blanks, and unknowns are allowed to equilibrate overnight at 4 °C (≈12 hrs.) with an equal volume of antibody at a final dilution of 1 to 8000 (250 ng/mL ascites fluid) in a glass 12 × 75 mm test tube. A volume of 200 μL each of antibody and test material was used. Standards and samples were then placed on a microtiter plate in 100 μL volumes. Microtiter plates had been previously coated with chlorpyrifos antigen at 50 ng per well. Antibody and antigen dilutions were optimized for ELISA by checkerboard titration. Standards were prepared fresh for each assay in a matrix similar to the leaf washes that were analyzed. Standards of 200, 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 ng/mL were prepared from a stock solution of chlorpyrifos (20 μg/mL) in methanol.

Immunoassays were performed the same day as HPLC confirmation and always on leaf washes or extracts that were prepared the previous day to reduce the amount of analyte loss. 100 μL of the overnight equilibrated test material was added to the appropriate well of the antigen-coated microtiter plate. Plates were sealed with an acetate film and shaken on an orbital shaker for two hours at room temperature. After incubation, plates were washed twice with PBST and blotted dry on absorbent paper. 100 μL of goat anti-mouse IgG alkaline phosphate enzyme-conjugate was then added to each well with a multichannel pipettor, the plate was sealed with acetate, and incubated for two hours on the orbital shaker. After incubation, plates were again washed twice with PBST buffer and blotted dry. 100 μL of a freshly prepared 1 mg/mL *p*-nitrophenyl phosphate solution in diethanolamine buffer (pH=9.8) was added for color development. Plates were read for endpoint readings after 30 min., using a Vmax plate reader (Molecular Devices, Menlo Park, CA) at 405 nm. Data were analyzed using SOFTmax software, with a four-parameter logistic curve (Molecular Devices, Menlo Park, CA).

Leaf wash and leaf extracts

Leaf wash

Leaf punches based on the design of Smith and Little *(18)* were used to sample the various crops in this study. Leaf punch size varied with the size of the leaf. A total one-side surface area of 125 cm^2 was used regardless of leaf punch size (*e.g.*, 50 × 2.5 cm^2 punches were analyzed for nectarine leaves, whereas 25 × 5 cm^2 were used for apple and cucumber, and 111 × 1.13 cm^2 punches were used for tomatoes). All punches were collected in separate glass jars that are attached to the punch by screw caps with Teflon® gaskets. Jars had been previously rinsed with methanol and dried to avoid cross contamination of analyte. After collection of the appropriate number of punches, leaves were washed with 150 mL of a dilute surfactant solution, 0.0026% sodium dioctylsulfosuccinate, prepared by mixing 0.65 mL of a 2% stock in 500 mL of nanopure water. Jars containing the leafs punches and wash solution were placed on a mechanical wrist-action shaker for ten minutes and shaken vigorously. This step was repeated and a total of 300 mL of wash was collected, split, and placed in stoppered polypropylene storage containers. Unsprayed leaf washes from the same batch of crops were used to prepare a standard curve to overcome matrix effects in both the ELISA and the HPLC methods.

Leaf extract

On the same day the leaf washes were analyzed by immunoassay, a control standard for HPLC was prepared in unsprayed leaf wash by diluting 80 μL of a 0.1 mg/mL chlorpyrifos standard in methanol with 20 mL control leaf wash to obtain a 400 ng/mL stock standard. Serial dilutions of 10 mL of each of the 400 ng/mL standard and subsequent dilutions were made with 10 mL of leaf wash resulting in five concentrations (200, 100, 50, 25, and 12.5 ng/mL) for the standard curve. From each of these standards, 8 mL was taken, mixed with 4 mL ethyl acetate and NaCl, and then centrifuged to break the emulsion. Then, 3 mL of the ethyl acetate layer was removed and evaporated under a nitrogen stream in a 15 mL conical polypropylene test tube. The remaining residue was reconstituted with 0.3 mL of the

mobile phase (37% methanol, 33% acetonitrile, 30% water). Then, 20 μL of this extract was injected onto the HPLC. A standard curve was prepared by plotting peak area versus concentration in order to quantitate unknowns. Only curves with a correlation coefficient (r^2) of ≥0.999 were used.

Sample preparation

Tomato, cucumber, apple, and nectarine leaves from local area gardens known not to have been sprayed with any insecticides or herbicides were obtained freshly cut and were separated into control group and test group. Each group was sampled and washed in the same manner and kept fresh by placing the stems in water. The test group crops were individually placed in a spray box within a fume hood and sprayed with a solution of Dursban® at a rate that is similar to applications done in agricultural fields in the United States (i.e., 30 – 60 mL of 6.6% Dursban® per 3.79 L of water for every 30.5 meters × 10.2 cm width). Leaves were sprayed with a plastic spray bottle that delivered a measurable amount of solution (1.6 mL of concentrated Dursban® in 200 mL nanopure water), replicating manufacturer-recommended field application. Leaves were wetted with five squirts, allowing no dripping. The leaves were allowed to dry 24 hours before leaf punches were collected. Leaf washes were done as previously described, samples were split, and immunoassay and HPLC analyses were done on the wash or extract prepared that day.

HPLC confirmation

All leaf washes from control and treated test groups were analyzed by HPLC (using extracts prepared from the split wash sample). A carbon-18 column, 15 × 2.1 mm was used for separation, with a mobile phase of 33% acetonitrile, 37% methanol, and 30% water at a flow rate of 0.4 mL per minute. The UV was set at 290 nm, AUF was 0.005. Chlorpyrifos eluted at a retention time of 5.5 minutes (20 μL injection). Ethyl acetate extracts of leaf washes with known values of chlorpyrifos were used to construct standard curves. Unknowns were evaluated from individual standard curves for each crop.

Extraction efficiency

Radiolabeled carbon-14 chlorpyrifos was used to determine the efficiency of the leaf wash in removing the dislodgeable residue from the leaf. Stock solutions of carbon-14 chlorpyrifos were made using 1 mL of a 10 μg/mL »carrier« chlorpyrifos and 114 μL of 1.16×10^7 dpm/mL of radiolabeled material. If 50 μL of this solution were mixed with 5 mL leaf wash, then 100 ng/mL »carrier« chlorpyrifos could be measured in the HPLC peak (a typical expected dislodgeable residue peak). This same solution (50 μL with 5 mL leaf wash) would provide 1.16×10^4 dpm/mL (or 1.16×10^3 dpm/μL in 4.9 mL of Ecoscint A scintillation counting solution).

Recovery rates show that the surfactant wash resulted in >90% recovery of spiked chlorpyrifos.

Extraction efficiency of the leaf wash when ethyl acetate was used was 92.5% by ELISA with 89.6% recovery in the counting procedure.

Immunoassay results

Immunoassay and HPLC results were in close agreement (Table 2; Fig. 1). The ELISA described here is applicable to several vegetation types. ELISA analysis on crude leaf wash samples without extraction by organic solvents or cleanup was found to be sufficient. Crude leaf wash results can easily be converted to provide surface residue concentrations. In addition, the ELISA procedure is easier than the analogous HPLC procedure, provides rapid results, and is field-portable for on-site applications. High sample throughput allowed by immunoassay methods is an important feature in human exposure studies and large-scale surveys of agricultural processes and settings.

Table 2. FDR values obtained by ELISA and HPLC for various composite leaf punch disks

Matrix		ELISA in ng/mL*	ELISA in μg/cm^2 (125 cm^2 total area)	HPLC in ng/mL*
Apple	#1	22.6 ± 1.5	0.054	29 ± 1.8
	#2	24.4 ± 0.9	0.058	28 ± 1.5
Tomato	#1	124.4 ± 1.7	0.299	139 ± 1.0
	#2	124.1 ± 1.5	0.298	136 ± 8.7
Nectarine	#1	46.7 ± 0.9	0.11	37 ± 1.5
	#2	43.4 ± 2.6	0.10	39 ± 1.0
Cucumber	#1	122.1 ± 12.1	0.293	179 ± 2.6
	#2	124.1 ± 8.9	0.298	181 ± 2.5

*Triplicate values for each ELISA, triplicate injections for HPLC

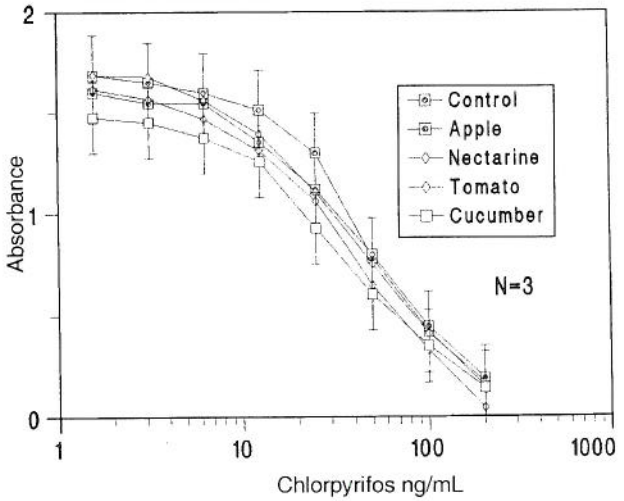

Fig. 1. ELISA standard curves for FDR wash samples of various matrices

Discussion

There are several variabilities associated with FRD sampling and analysis. Leaf size and shape dictate the type of punch required. Intrinsic leaf characteristics, such as waxiness, hairiness, thickness, and smoothness can affect the physical action of adherence to leaf surface, the operational surface area itself, and the readiness of that surface to release the material adsorbed to it. For

example, ELISA results were lower than HPLC results for cucumber leaves. This could be due to physical characteristics of the rough cucumber leaves and their tendency to retain confounding heterogeneous substances. It is possible that the ethyl acetate extraction of the wash prior to HPLC analysis serves as a cleanup step. A simple cleanup step could be performed prior to ELISA analysis in the field, too.

An interesting point made in the literature *(19)* is that practitioners are philosophically split about the calculation of residue per unit area, with about half reporting this concentration based on one side of the leaf, the other half routinely using both sides for calculation. Our calculations are based on one-side as expressed as the size of the punched area. Because so many factors (*e.g.*, porosity and texture) are involved, we believe that the most expedient calculation is reported as concentration per leaf punch area but caution readers that some researchers may report results based on double area.

The analytical results presented here demonstrate the comparability of immunoassay results with results from HPLC. Immunoassay methods are easier to perform on site than chromatographic procedures, require little or no organic solvents, and can be easily learned by field personnel. This ease of use means that agricultural worker reentry times can be adjusted for meteorological conditions, which may improve present universal standards. Customization of field methods to provide a better estimate of the true exposure potential in agricultural settings exploits current, sensitive analytical procedures to optimize crop and worker protection.

Conclusions

The benefits of rapid analysis for trace residue levels of pesticides is not limited to the analysis of vegetation. The expansion of immunoassay applications to worker clothing, carpet coupon samples *(20)*, and other indications of human exposure is a logical extension of this type of application. As research laboratories develop rugged methods for agricultural applications, the user community will benefit from the real-time data that on-site methods can provide. Our laboratory is interested in participating in the expansion of this and other applications of environmental immunochemical methods. For example, we believe that the FDR method described herein is ready for a field pilot study to define its niche in both occupational and nonoccupational human exposure studies.

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Analiza ostataka pesticida na listovima bilja: novi znanstveni pristup zakonskom prijedlogu

Sažetak

Američku agenciju za zaštitu okoliša (U. S. Environmental Protection Agency, EPA), a i druge federalne i državne ustanove, interesira prisutnost i biološka štetnost pesticida koji zaostaju na bilju nakon njihove primjene. Ti spojevi mogu biti opasni ako su hlapljivi, tj. kada ih ljudi inhaliraju ili kad ih poljoprivrednici dodirom apsorbiraju preko kože. U nastojanju da se izmjeri stupanj i doseg pesticidnih ostataka na listovima biljaka, ured EPA za prevenciju pesticida i toksične spojeve razradio je upute za uzimanje uzoraka i analizu dijelova listova. Postupci testiranja u skladu su s federalnim zakonom o insekticidima, fungicidima i rodenticidima. Pojava imunokemijskih postupaka za analizu mnogih pesticida omogućila je nove, brze i precizne analitičke postupke za studij ostataka pesticida na razdrobljenim listovima. U radu je opisan neizravni ELISA-postupak za analizu pesticida Klorpirifosa. Dobiveni rezultati uspoređeni su s uobičajenim HPLC-postupkom.