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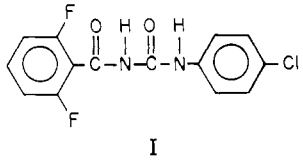
Analysis of Diflubenzuron Residues in Environmental Samples by High-Pressure Liquid Chromatography

Susan J. DiPrima,* Richard D. Cannizzaro, Jean-Claude Roger, and C. Duane Ferrell

Residue methods for the routine analysis of diflubenzuron in a wide range of samples from both agricultural and nonagricultural ecosystems were developed. The procedures involve Celite liquid–liquid partition, and Florisil–alumina–silica gel colum chromatography, followed by detection using high-pressure liquid chromatography (HPLC), with a μ-Bondapak C-18 (reverse phase) or μ-Porasil (normal phase) column. The methods are reproducible and sensitive to 0.01 ppm in water and 0.05 ppm in soil, sediment, aquatic and forest foliage, fish and shellfish, agricultural crops, milk, eggs, and animal tissues.

Diflubenzuron, (I) TH-6040, *N*-[[4-chlorophenyl)-amino](carbonyl)]-2,6-difluorobenzamide, is an insect growth regulator developed under the trade name of Dimilin. Due to its inhibition of chitin biosynthesis,



I

excellent control of a variety of insects has been observed. Some of the more important insects include various forest insects, soybean and cotton insects, mosquitoes, and citrus pests.

Several residue procedures have been developed previously for the analysis of diflubenzuron. Corley et al. (1974) reported the determination of residues of diflubenzuron in milk by extraction with ethyl acetate, a partition between *n*-hexane and acetonitrile, followed by detection with high-pressure liquid chromatography. DeWilde et al. (1975) described a method in crops, soil, mud, and water by extraction with dichloromethane, cleanup of the extract on a Florisil column, and detection by HPLC. Schaefer and Dupras (1976) reported the stability and persistence of diflubenzuron in water utilizing HPLC methodology. The determination of diflubenzuron by HPLC in manure has been described by Oehler and Holman (1975).

Based on ^{14}C-radiotracer studies, residue methods for water, soil, sediment, fish, shellfish, agricultural crops, aquatic vegetation, forest litter and foliage, cow and poultry tissues, milk, and eggs were developed in order to assess the fate of diflubenzuron in both agricultural and nonagricultural ecosystems. The procedures reported here describe extraction, cleanup, and detection techniques routinely utilized for the determination of diflubenzuron residues at 0.01 ppm in water and 0.05 ppm in all other samples.

EXPERIMENTAL SECTION

Reagents. All reagents used were doubly distilled in glass from Burdick and Jackson Laboratories, Inc., Muskegon, Mich. All aqueous solutions were prepared with water which had been deionized and distilled in glass. Mobile phases used for normal phase HPLC chromatography were isooctane–isopropyl alcohol (93:7, v/v) and dichloromethane–methanol (500:1, v/v). Mobile phases used for reverse phase were acetonitrile–water (60:40, v/v) and methanol–water (75:25, v/v). HPLC mobile phases were degassed by evacuation coupled with ultrasonic vibration.

The Florisil was Floridin's 60–100 mesh, pesticide grade. The silica gel and neutral alumina were Woelm's Activity

Thompson-Hayward Chemical Company, Kansas City, Kansas 66110 (S.J.D., R.D.C., C.D.F.) and Cannon Laboratories, Inc., Reading, Pennsylvania 19603 (J.C.R.).

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I. Celite Hyflo Super-Cel was from Johns-Manville.

Standards. A standard solution of diflubenzuron was prepared by dissolving 100 mg of the analytical standard in 100 mL of methanol. One-tenth of a milliliter of this solution was diluted with 100 mL of methanol to give a solution containing 1 ng/μL. This standard was used to fortify control samples for recovery experiments. Solutions used for injection standards were diluted with HPLC mobile phase to a concentration of 1 ng/μL.

High-Pressure Liquid Chromatography Equipment and Operation Conditions. A Model ALC/GPC 204 high-pressure liquid chromatograph (HPLC) with a Model M-6000A pump, U6K injector, and Model 440 UV detector at 254 nm (Waters Associates, Milford, Mass.) was used. Mobile phase flow rates were 1.0–1.5 mL/min at ambient temperature.

Column. A Waters Associates 30 cm × 4 mm i.d. μ-Bondapak C-18 column (octadecyltrichlorosilane chemically bonded to 10 μ-Porasil packing) was used for reverse phase chromatography. A Waters Associates 30 cm × 4 mm i.d. μ-Porasil column was used for normal phase chromatography.

Apparatus. A Buchi rotary flash evaporator with a water bath was used for removal of solvents from sample extracts. Chromatographic columns were Pyrex glass equipped with a Teflon stopcock and 250 mL reservoir.

Standardization of Celite Liquid–Liquid Partition Chromatography. This column chromatography was standardized as follows: Solvent systems were prepared by adding 760 mL of hexane, 200 mL of acetonitrile, and 40 mL of dichloromethane to a 1-L separatory funnel and shaking vigorously for 1 min. The upper layer was designated solvent A and the bottom layer solvent B. After separation, 7 mL of solvent B was added to 20 g of Celite and stirred with a spatula for 1.5 min to insure a uniform mixture. A glass wool plug was placed in the bottom of a 19 mm i.d. × 250 mm chromatographic column equipped with a Teflon stopcock and 250 mL reservoir. The Celite mixture was added to the column in 4-g portions, each portion packed down tightly by applying pressure with a 18 mm o.d. glass rod. A 1-μg standard of diflubenzuron was transferred to a round-bottom flask, mixed with 3 g of Celite, evaporated to dryness on a rotary flash evaporator, and transferred to the column. The column was packed again. The flask was rinsed with two successive 10-mL portions of solvent A, allowing each to penetrate into the column before the next additions. Pressure was applied to the top of the column with nitrogen to increase the flow rate to 3–4 mL/min. An additional 500 mL of solvent A was added to the column and allowed to drain as above, and 50-mL fractions were analyzed for compound recovery. Normally the first 250 mL of solvent was discarded and the second 250 mL collected for routine analysis of residue samples.

Standardization of Florisil–Alumina–Silica Gel. The column packings used for this column chromatography step were standardized as follows: All the column packing materials were activated in an oven at 130 °C for 24 h and cooled in a desiccator prior to use. They were deactivated to the appropriate percentage of moisture by adding distilled water, capping the vessel tightly, and shaking for 30 min. Florisil is deactivated by the addition of 7% moisture, neutral alumina and silica gel by the addition of 12% moisture. Water content of the adsorbents can be monitored by electronic Karl-Fischer titration using an Aquatest II (Photovolt Corp.) following the manufacture's instructions. A glass wool plug was placed in the bottom of three 15 mm i.d. × 250 mm chromatographic columns equipped with a Teflon stopcock and 250-mL reservoir. Three milliliters of Florisil was added to one column, 12 mL of neutral alumina to the second, and 12 mL of silica gel to the third column. A 1-μg standard of diflubenzuron was added to a flask containing 5 mL of dichloromethane and transferred to the Florisil column, and the column was allowed to drain. The flask was rinsed with two successive 5-mL portions of dichloromethane, allowing each to penetrate into the column before the next addition. An additional 80 mL of dichloromethane was added and allowed to drain, and the entire effluent was analyzed for diflubenzuron. If the compound was not quantitatively eluted from the column within the 100 mL of solvent, the addition of moisture was indicated. This procedure was repeated with the neutral alumina and silica gel packings.

Analytical Procedure. *Extraction.* A 20-g sample of the homogenized crop was weighed into a 1-qt blender jar, 150 mL of acetonitrile was added, and the mixture was blended for 10 min. The extraction mixture was vacuum filtered through Whatman No. 4 filter paper and the filtrate was quantitatively transferred to a 500-mL round-bottom flask and evaporated to dryness on a rotary flash evaporator at 40 °C.

Fish, animal tissue, or egg samples were weighed into a 1-qt blender jar, and 150 mL of acetonitrile was added, and the mixture was blended for 10 min. Fish required the addition of 40 g of anhydrous sodium sulfate. The extraction mixture was vacuum filtered through Whatman No. 4 filter paper and the filter cake reblended with 75 mL of acetonitrile. The extract was filtered as above, and the extracts were quantitatively transferred to a 500-mL round-bottom flask and evaporated on a rotary flash evaporator at 40 °C.

Soil samples were homogenized and sieved to remove rocks, sticks, etc. Excess water was decanted off sediment samples. A 20-g sample was weighed into a 500-mL round-bottom flask, 15 mL of water and 150 mL of acetonitrile were added, and the mixture was refluxed for 30 min. The flask was cooled and the condenser rinsed with acetonitrile. The extraction mixture was vacuum filtered and evaporated to dryness as previously described.

Milk samples were extracted by blending a 30-g sample with 120 g of sodium sulfate and 150 mL of ethyl acetate for 5 min. The mixture was vacuum filtered through Whatman No. 4 filter paper and the filter cake reblended with 75 mL of ethyl acetate. The extract was filtered as above, and the extracts were quantitatively transferred to a 500-mL round-bottom flask and evaporated to dryness on a rotary flash evaporator at 40 °C.

It should be noted that in some instances we have experienced difficulties in quantitatively transferring the Celite-coated residue when evaporating in a round-bottom flask. The use of an Erlenmeyer flask has eliminated this problem.

Water samples were filtered through Whatman No. 2V filter paper, and a 100-mL aliquot was transferred to a 250-mL separatory funnel. A 25-mL aliquot of 5% NaCl was added and then extracted successively with three 50-mL portions of hexane. The extracts were pooled, filtered through a 20-g pad of sodium sulfate into a 250-mL round-bottom flask, and evaporated to dryness on a rotary flash evaporator at 40 °C. These samples require no further cleanup. Analysis was performed as outlined in the HPLC Analysis section.

Celite Liquid–Liquid Column Chromatography. The sample residues were dissolved in 50 mL of solvent A, 3 g of Celite was added to the flask, and the solvent was

Table I. Diflubenzuron Recovery Data

Sample	Ppm fortification range	No. of detns.	Recovery % Av	Recovery % Range
Water	0.01-0.10	18	86	77-92
Soil	0.05-0.40	15	91	77-103
Sediment	0.05-0.40	19	96	78-120
Litter, yellow birch	0.05-0.50	7	88	78-128
Litter, pitch pine	0.05-0.50	7	84	70-100
Foliage, pitch pine	0.05-4.0	8	84	77-90
Foliage, hemlock	0.05-2.0	10	81	70-94
Foliage, red oak	0.05-5.0	10	82	73-100
Foliage, red maple	0.05-5.0	10	80	72-100
Foliage, chestnut oak	0.05-2.0	9	92	86-116
Foliage, white oak	0.05-2.0	8	82	70-95
Foliage, yellow birch	0.05-4.0	10	80	73-96
Pasture grass	0.05-100.0	18	94	75-125
Cow liver	0.05-0.50	6	95	80-110
Cow kidney	0.05-0.50	5	89	70-98
Cow fat	0.05-0.50	6	91	80-100
Cow muscle	0.05-0.50	6	90	75-105
Chicken liver	0.05-0.50	3	78	70-83
Chicken muscle	0.05-0.50	3	73	70-76
Chicken fat	0.05-0.50	3	92	72-104
Chicken kidney	0.05-0.50	3	93	75-108
Eggs	0.05-0.50	3	80	70-92
Milk	0.05-0.50	6	86	80-100
Fish	0.05-0.10	16	93	70-125
Crustaceans	0.05-0.10	10	86	70-96
Shellfish	0.05-0.10	10	85	75-97

removed on a rotary flash evaporator at 40 °C. The sample was subjected to Celite column chromatography, as previously outlined. The eluent was evaporated to dryness on a rotary flash evaporator at 40 °C.

Mixed Column Chromatography. Following Celite chromatography, the samples were chromatographed on a Florisil–alumina–silica gel column using adsorbents which have been previously standardized. A glass wool plug is placed in the bottom of a 15 mm i.d. × 250 mm chromatographic column equipped with a Teflon stopcock and a 250-mL reservoir. A mixture of 12 mL of alumina and 12 mL of silica gel was added to the column, followed by 3 mL of Florisil. A glass wool plug was placed on top of the packing. The sample residue was dissolved in 5 mL of dichloromethane–hexane (1:1, v/v), transferred to the column, and the column allowed to drain. The sample flask was rinsed with two successive 10-mL portions of dichloromethane–hexane (1:1, v/v), and the rinses were transferred to the column. Pressure was applied to the top of the column with nitrogen to obtain a flow rate of 3–5 mL/min, and the eluent was discarded. One-hundred milliliters of dichloromethane was added to the column and drained as above and the eluent was collected in a 250-mL round-bottom flask. The eluent was evaporated to dryness on a rotary flash evaporator at 40 °C.

HPLC Calibration. Analysis of diflubenzuron residues were performed by either reverse phase or normal phase chromatography. The conditions were chosen on the basis of the best resolution using flow rates at 1.0–1.5 mL/min and mobile phases specified under the Apparatus Section. A solution containing 1 ng/μL of diflubenzuron was used as a standard to determine retention time and establish operating conditions. The injection volume was usually 2–50 μL. A series of standards ranging from 2 to 30 ng was injected to determine the response curve. Peak heights were used to plot detector response vs. nanograms of diflubenzuron.

HPLC Analysis. The sample residue remaining in the flask following evaporation of the column eluent was dissolved in an appropriate volume of HPLC mobile phase for detection and measurement by high-pressure liquid

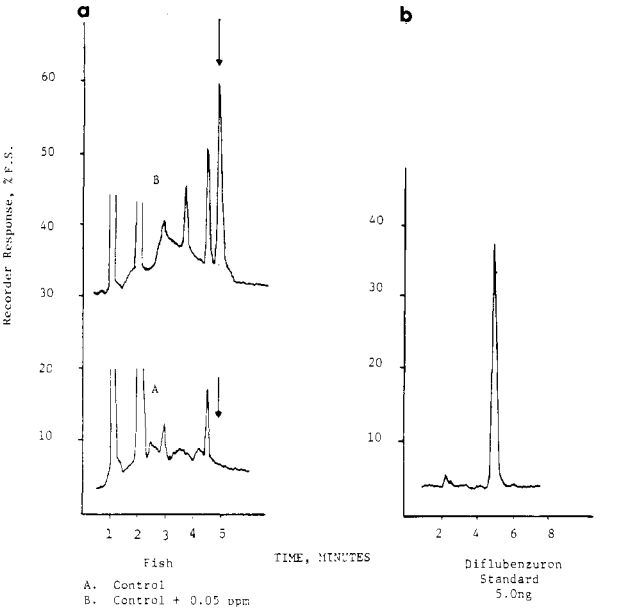

Figure 1. (a) Fish: (A) control, (B) control + 0.05 ppm; (b) diflubenzuron: standard 5.0 ng.

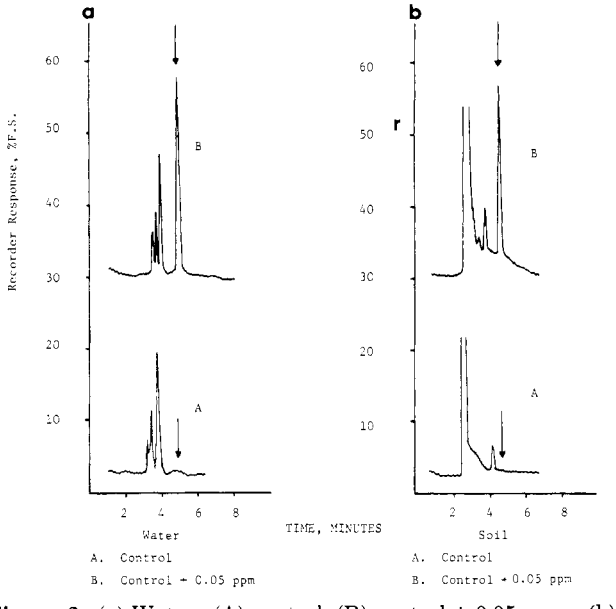

Figure 2. (a) Water: (A) control, (B) control + 0.05 ppm; (b) soil: (A) control, (B) control + 0.05 ppm.

chromatography. Either normal or reverse phase is applicable to any of the matrix analyses. All extracts were normally dissolved in 1 mL, then diluted further depending on the anticipated level of diflubenzuron residues. When the concentration of diflubenzuron in a sample was greater than the highest point on the standard curve, the sample was diluted with mobile phase so that it would fall within the curve. The peak height in the sample injected was measured and the nanograms of diflubenzuron determined using the calibration curve. The concentration of diflubenzuron in the sample in terms of parts per million is calculated by dividing the nanograms of chemical found by the milligrams of sample injected.

RESULTS AND DISCUSSION

The efficiency of these methods for determination of diflubenzuron residues were determined by fortifying control samples over a concentration range of 0.01 to 0.15 ppm for water and 0.05 to 25.0 ppm for crop, plant, fish,

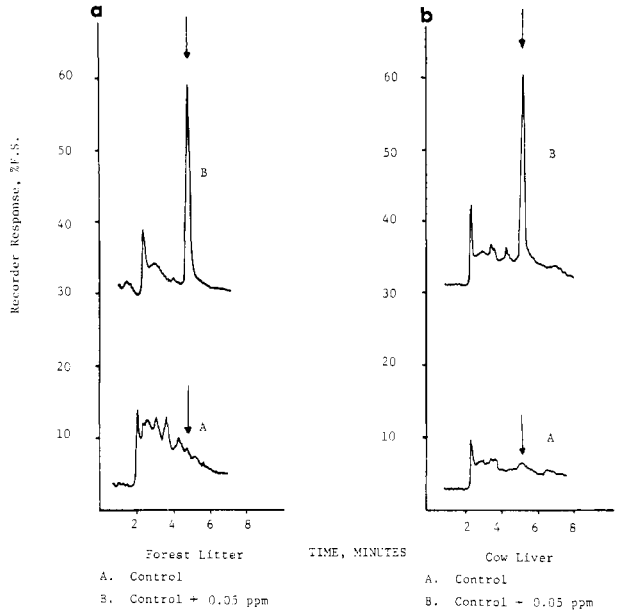

Figure 3. (a) Forest litter: (A) control, (B) control + 0.05 ppm; (b) cow liver: (A) control, (B) control + 0.05 ppm.

and soil samples utilizing the analytical procedures described here. The results are shown in Table I. Typical chromatograms representative of those normally encountered during the course of routine analyses for controls and fortified samples of water, soil, fish, forest litter, and cow liver, as well as a diflubenzuron standard, are presented in Figures 1–3.

Pesticides having a tolerance established in water, animal tissues, and cotton seed, as well as pesticides registered for use on forest insects, have been studied for interference when diflubenzuron is analyzed by HPLC. None of the compounds were found to have a retention time within 10% of diflubenzuron.

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Separation of Amino Acid Conjugates of 2,4-Dichlorophenoxyacetic Acid by High-Pressure Liquid Chromatography Employing Ion-Pair Techniques

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A high-pressure liquid chromatographic procedure was developed for the analysis of 15 amino acid conjugates of 2,4-dichlorophenoxyacetic acid. The procedure employs a μBondapak C_{18} column with a methanol–water solvent and the use of ion-paired chromatography. The method is sensitive (<50 ng) and quantitative over a wide range (50 ng to 100 μg), and conjugates are easily recoverable.

Amino acid conjugates of 2,4-dichlorophenoxyacetic acid (2,4-D) have been shown to be important metabolites of 2,4-D in plants and plant tissue cultures (Andrea and Good, 1957; Klämbt, 1961; Feung et al., 1971, 1972, 1973b, 1975). Other investigations have demonstrated the herbicidal properties of these conjugates (Krewson et al., 1956; Wood and Fontaine, 1952; and Feung et al., 1974). Analytical methods for the analysis of the amino acid conjugates are needed so that we can better understand their significance in our environment. Recently, Arjmand and Mumma (1976a,b) reported the gas chromatographic separation of the methyl esters and of the trimethylsilyl ethers of the amino acid conjugates of 2,4-D. These techniques gave good resolution, but sensitivity was relatively low and recovery of parent conjugates could be difficult. We now report the development of a high-pressure liquid chromatographic (LC) technique for the analysis of 15 amino acid conjugates of 2,4-D employing ion-pair methods (Kissinger, 1977; Walters Associates Inc., 1975). The procedure is more sensitive than existing methods, yields quantitative results over a wide range, and allows for ease of recovery.

Pesticide Research Laboratory and Graduate Study Center and the Department of Entomology (M.A., R.O.M.) and the Department of Biology, (R.H.H.) The Pennsylvania State University, University Park, Pennsylvania 16802.

EXPERIMENTAL PROCEDURE

Reagents and Materials. All solvents were distilled in glass (Burdick and Jackson) and filtered through a 0.45-μm Millipore filter before use. Water was doubly distilled and also filtered just prior to use. The amino acid conjugates of 2,4-D were synthesized as previously reported (Feung et al., 1973a). The conjugates used were 2,4-D-Ala, 2,4-D-Arg, 2,4-D-Asp, 2,4-D-Cys, 2,4-D-Glu, 2,4-D-Gly, 2,4-D-Ile, 2,4-D-Leu, 2,4-D-Met, 2,4-D-Pro, 2,4-D-Phe, 2,4-D-Ser, 2,4-D-Thr, 2,4-D-Trp, and 2,4-D-Val. The ion-pair chromatographic agent was PIC Reagent A (tetrabutylammonium phosphate, Waters Associates, Inc., Milford, Mass.).

Stock solutions of each conjugate and of 2,4-D (4 mg/mL) were prepared in methanol. A small amount of ammonia was added to the vials containing 2,4-D-Gly and

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