Characterization and Microdetermination of a Water-Soluble Metabolite from Bladex Herbicide by Conversion to 5,5-Dimethylhydantoin

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A gas chromatographic method has been designed for the characterization and measurement of Bladex herbicide (propionitrile, 2-(4-chloro-6-ethylamino-*s*-triazin-2-ylamino)-2-methyl-) and its various metabolites. It involves the simultaneous cleavage of the Bladex triazine ring and the cyclization of the characteristic Bladex fragment to 5,5-dimethylhydantoin. Measurement of the 5,5-dimethylhydantoin provides a quantitative assessment of the amount of Bladex and/or its metabolites present. This is readily accomplished by gas–liquid chromatography or by thin-layer chromatography. As an example, a procedure for the electron-capture gas chromatographic determination of a water-soluble metabolite derived from Bladex herbicide by conversion to 5,5-dimethylhydantoin is described. Following the procedure, 1 ng of the metabolite gives 25% full-scale response on the gc recorder chart. Recovery data from experiments run on crops and soil were generally in the 75 to 110% range when equal amounts of sample and reference solution in the same concentration range were analyzed. A sensitivity of 0.1 ppm is achieved in crops and 0.02 ppm is achieved in soil and water.

The *s*-triazine herbicides have established themselves in agriculture as valuable aids for increased crop yields by controlling undesirable weed growth. After application to soil or crops, these herbicides give rise to 2-hydroxy analogs as major metabolites (Castelfranco *et al.*, 1961; Harris, 1967; Shimabukuro, 1967, 1968). These metabolites are nonvolatile and not amenable to gas chromatography. They therefore require suitable derivatization in order to be successfully analyzed by gc.

Bladex herbicide is a selective *s*-triazine herbicide developed to control annual grasses and broad-leaved weeds in corn fields. Like other *s*-triazine herbicides, Bladex also undergoes 2-hydroxylation. Figure 1 shows the chemical structure of Bladex and its breakdown in soils.

As shown in Figure 1, IV represents the major metabolite of Bladex. Beynon *et al.* (1972) studied the breakdown of Bladex herbicide in wheat and potatoes grown in soils treated with [^{14}C]Bladex. They found the breakdown products were mainly those formed by hydrolysis of the cyano group to give an amide (II) and an acid (III), followed by hydrolysis of the chlorine to hydroxyl (IV). The present report describes details of the analytical procedure, involving derivatization to 5,5-dimethylhydantoin, used for the analysis of residues of IV in crops (corn silage, stover, ears; green alfalfa, etc.) and in soil.

EXPERIMENTAL SECTION

The method consists of the following steps: removal of fat-soluble interferences by washing the subdivided tissues with organic solvents; discarding the washings; extracting IV residues from the crop matrix with a polar solvent; cleanup by selectively sorbing IV onto Darco G carbon, followed by chromatography through cation exchange resin; conversion to a derivative with high electron-capture response by cleavage and cyclization to 5,5-dimethylhydantoin (DMH), which is then coupled with trichloromethanesulfenyl chloride (TCMS) to form 3-(trichloromethylthio)-5,5-dimethylhydantoin (TCMS-DMH); and measurement of the TCMS-DMH adduct by ec-glc. Figure 2 shows a flow diagram of the method described above and Figure 3 gives the chemical reactions involved in the final determinative step.

Apparatus and Reagents. A Packard Model 7300 gas chromatograph with tritium electron-capture detector, equipped with a 4 ft × 4 mm glass column packed with 10% OV 101 on 60–80 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.) was used. Conditions were: injection port, 190°; column, 190°; detector, 190°, nitrogen carrier gas flow, 70 ml/min.

"Lab Crest" combustion tubes and accessories, 3 in. × ½ in., obtained from Fisher and Porter Co., Warminster, Pa., and a chromatographic column (see Figure 4) were used in the cleavage and clean-up procedures, respectively.

Crop Preparation. Chop representative crop material into small pieces. Prewash 50 g by blending successively in organic solvents (methylene chlorine, benzene, hexane, ethyl acetate, and ether) using enough solvent to cover the crop material in the blender cup, blending 3 min, allowing the solids to settle, and draining and discarding the washings. By such a prewash the amount of crop coextractives found in the subsequent extract was considerably decreased, while less than 2% of added [^{14}C]IV was lost in the discarded organic washings.

Extraction. Residues of IV were quantitatively extracted from the washed crop by blending three times, 200 + 100 + 100 ml, respectively, with a solvent mixture consisting of methanol, water, and acetic acid (80 + 30 + 3 v/v). The blended mixtures are centrifuged to separate the solid and liquid fractions.

Neutralization and Precipitation of Solids. Heat the pooled extracts above on a steam bath in an open beaker to evaporate off all the methanol. Cool and neutralize with NaOH. Add Tween-20 to a final concentration of 0.5%. This addition assures high recoveries by avoiding nonspecific adsorption of IV to the subsequent precipitate. Add 0.3 g of phosphotungstic acid/50 g of crop (a precipitate immediately forms). Shake thoroughly and centrifuge 5 min to effect separation of a clear top liquid layer (light straw color) and a compacted bottom layer of precipitated solids. Neutralize if necessary and adjust volume to 1 g/ml. Measure 20 ml of the liquid layer (≃20 g crop) into a 125-ml Erlenmeyer flask. Add 0.5 g of Darco G carbon and shake 10 min on a mechanical shaker to effect selective sorption of IV residues onto the Darco G carbon. Transfer contents to a centrifuge tube, centrifuge 5 min, and decant the clear water-white supernatant back into the original 125-ml Erlenmeyer flask, keeping the carbon bottom layer in the centrifuge tube. Treat supernatant with 0.5 g of Darco G a second time and discard liquid phase. Extract the sorbed IV residue from the Darco G carbon with a methanol–water–HCl mixture (95 + 4½ + ½ v/v), shaking four times with 30 + 20 + 10 + 10 ml, re-

Shell Development Company, Modesto, California 95352.

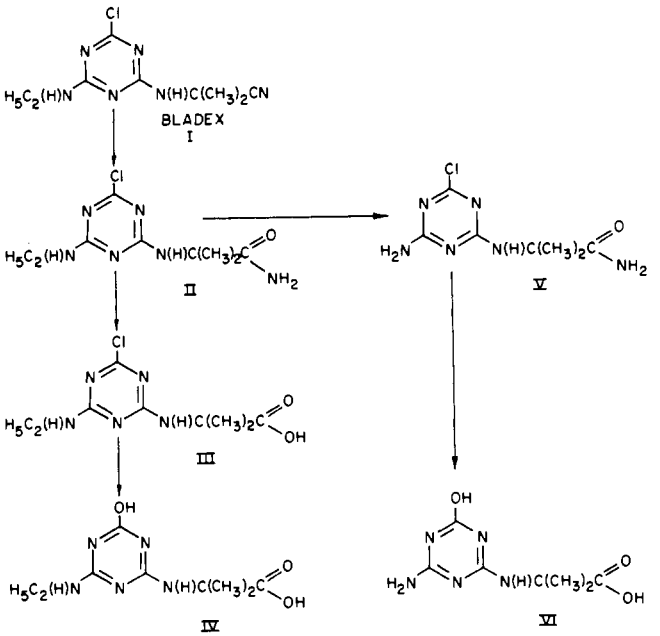

Figure 1. Breakdown products of Bladex in soil.

spectively, and centrifuging after each shaking. Combine the extracts, neutralize with 2 *N* NaOH, and concentrate on a steam bath under a stream of air to approximately 5 ml.

Cation-Exchange Chromatography. Chromatography on a strongly acidic cation-exchange resin was used to further isolate IV from interferences. Wash 20 g of Amberlite CG-120 (hydrogen form, 100–200 mesh, Mallinckrodt) successively with 3 *N* NH_4OH, water to near neutral, 3 *N* HCl, water to near neutral, and load as a slurry into a glass chromatographic column. Transfer the prepared extract onto the column with about 10 ml of wash water, allow extract and washing to percolate into the column, then wash column successively with 200 ml of distilled water and then with 20 ml of 2 + 1 methanol-ammonia mixture, discarding the washings. Elute IV residues from the resin with 30 ml of 2 + 1 methanol-ammonia followed by 100 ml of 1 + 1 methanol-ammonia. Collect the light-colored effluent and concentrate on the steam bath under a stream of air to about 2 ml. Transfer quantitatively to a "Lab Crest" combustion tube with a minimum amount of water and concentrate to a final volume of 1 ml.

Derivatization. Add 1 ml of glacial acetic acid to the sample (in the "Lab Crest" tube), two drops of 10% aqueous potassium ferricyanide, and 0.4 g of anhydrous sodium acetate. Mix to dissolve all of the sodium acetate, seal

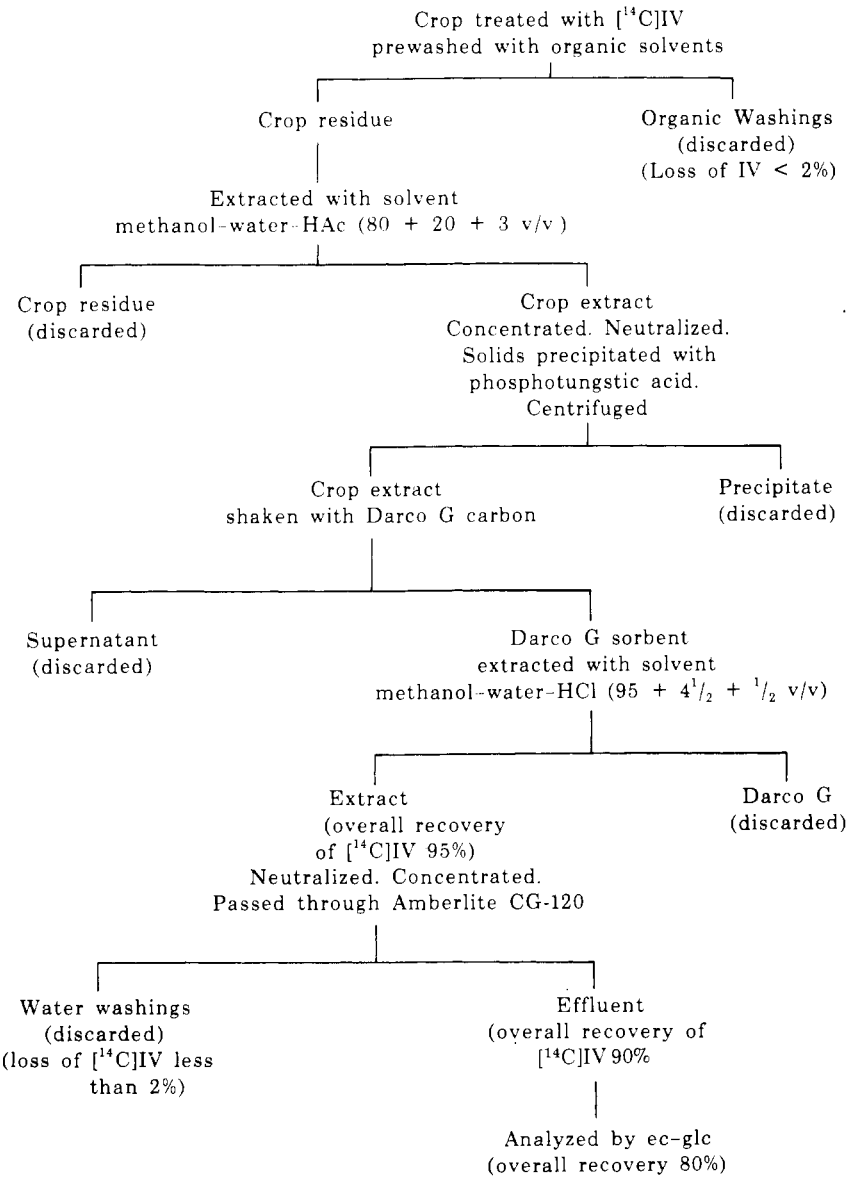

Figure 2. Flow diagram for the clean-up procedure.

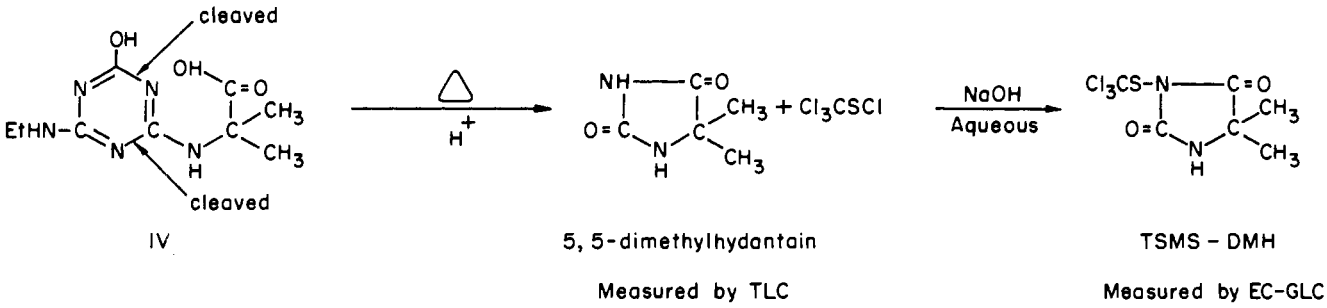

Figure 3. Chemical reactions used in the determination of Bladex metabolite IV.

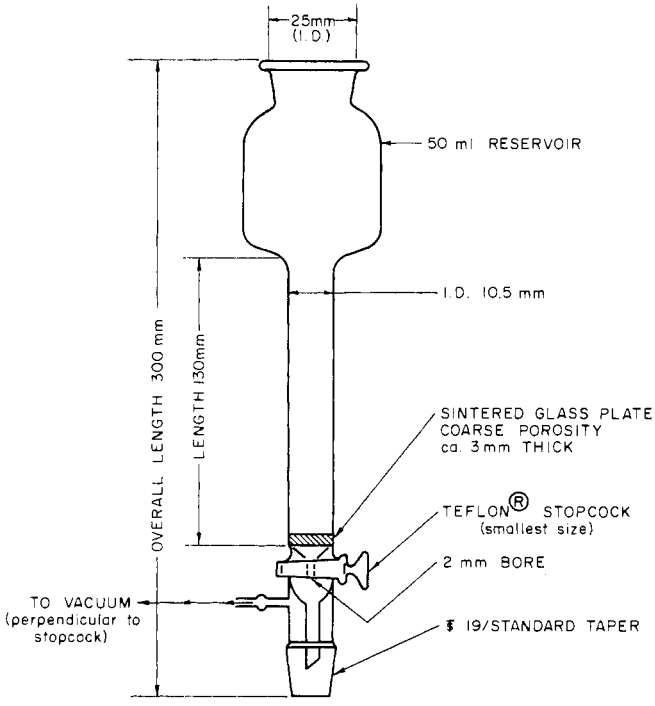

Figure 4. Miniature chromatograph column.

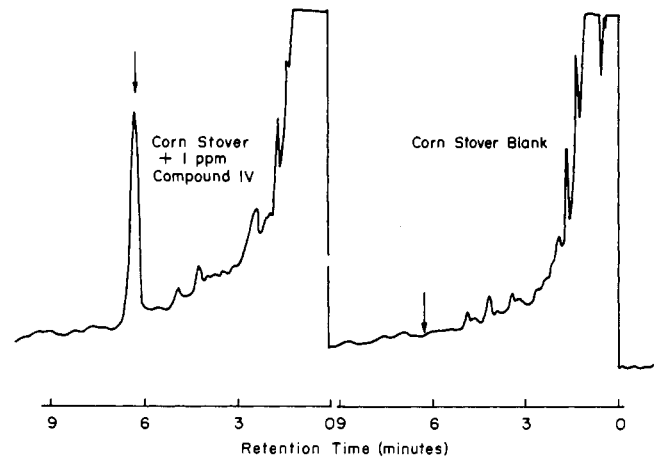

Figure 5. Gas–liquid chromatograms of derivatized corn stover extracts in the absence and presence of added compound IV. Each injection represents the equivalent of 5 mg of sample.

tightly, and heat for 3 hr at 165 to 170°, at which time all IV residues will have been derivatized to the cyclic ketone 5,5-dimethylhydantoin.

Cool and transfer the samples quantitatively into 50-ml centrifuge tubes with about 2 ml of pH 10 buffer. Add enough 10 *N* NaOH (about 2.5 to 3.0 ml) to make the pH between 10 to 11. Add 200 μl of trichloromethanesulfenyl chloride (TCMS). Shake vigorously for 2 min. Allow the reaction to proceed at room temperature for 15 min, shaking the tubes every 5 min. Check the pH of the sample. If it is still basic, add 100 μl of TCMS. The reaction has gone to completion as soon as the sample becomes neutral or slightly acid. Add 10 ml of 1:1 ethyl acetate–hexane to the sample. Shake vigorously for 2 min to extract the trichloromethanesulfenyl derivative of 5,5-dimethylhydantoin (TCMS-DMH). Allow the layers to separate. Centrifuge the sample if necessary.

Florisil Column Chromatographic Cleanup. To remove excess reagent prior to ec-glc, the TCMS-DMH work-up mixture is processed as follows.

Pipet a 5-ml aliquot (25 g) of the upper organic phase into a 15-ml centrifuge tube. Evaporate the sample to dryness in an air jet. Take up the residue with about 1 ml of benzene. Apply the sample into a 3-g Florisil column topped with about an inch of granular anhydrous sodium sulfate. The Florisil column is prepared as follows.

Activate the Florisil at 110° overnight (suggested amount 300 g). Add water to make 10% v/w (30 ml) deactivation. Mix thoroughly and allow to equilibrate overnight. Add about 20 ml of benzene to a miniature column (Figure 4). With stopcock opened, slowly add 3 g of Florisil slurried in benzene. Tap the column gently to attain an absorbent bed without trapped air bubbles. Without using vacuum, adjust the flow rate to about one or two drops/sec. Add about an inch of anhydrous granular Na_2SO_4 to the top of the Florisil.

Apply the sample to the column. First elute with 50 ml of benzene and discard the eluate, then elute with 15–20 ml of 1:1 ethyl acetate–hexane. The elution profile of the Florisil should be predetermined prior to analyzing a given set of samples. This is accomplished by applying 10 μg of TCMS-DMH (3-trichloromethylthio)-5,5-dimethylhydantoin standard in benzene, eluting first with benzene, followed by 1:1 ethyl acetate–hexane (three 10-ml fractions). Every new batch of deactivated Florisil should be profiled prior to use. The collected ethyl acetate–hexane fraction is evaporated to dryness in an air jet and the residue is taken up with 10 ml of hexane. An aliquot of the cleaned-up extracts is injected into the gas chromatograph equipped with an electron-capture detector.

Gas Chromatographic Analysis. The following gc parameters were employed for the analysis of IV residues in soil, water, and crops. Two gc columns were found ideal using either instrument. Instrument: Packard 7300 EC, tritium or Varian 1200, tritium. Column: 4 ft × 4 mm glass containing 10% OV 101 on Gas Chrom Q 60–80 mesh or 8 ft × ⅛ in. containing 1:1 5% DC 200 + 10% QF-1 on Chrom W, hp 80–100 mesh. Temperature: column, 190°, inlet, 190°, detector, 190°, or column, 200° (210°), inlet, 200° (210°), detector, 200° (210°). Carrier gas: 60–80 ml/min or 50–60 ml/min (50 lb psi). Voltage: 25. Range and suppression: 1×10^{-9}, 1×10^{-7} or range and attenuation: 1 × 32 or 1 × 16. Retention time of TCMS-DMH: 6 to 8 min or approximately 8.0 min. Sensitivity: 1 ng of TCMS-DMH = 25% F.S. or 1.5 ng = 25% F.S.

Preparation of Standard Curve. Prepare a series of IV standards in 2% aqueous acetic acid containing 5, 10, and

20 μg/ml, respectively. Cyclize, derivatize, and clean up as described in the above steps. When this procedure is followed, concentrations of 0.25, 0.5, and 1.0 γ/ml are obtained, respectively. Inject 4 μl of cyclized and derivatized IV solutions into the gc, giving 1.0, 2.0, and 4.0 ng, respectively. In order to verify the identity of the peak desired and to check its elution time, inject TCMS-DMH standard (prepared as described by Cremlyn, 1964). Thus, the efficiency of the cyclization and derivatization reaction can be checked with this procedure. Variation in the cyclization–derivatization procedure could be corrected by running IV standards (three levels) for every set of samples analyzed. Plot peak height (or area) *vs.* nanograms of chemical injected.

Sample Analysis. Inject a 4-μl aliquot of the cleaned-up extract. Measure the peak height (or area) and compare with the standard curve previously established. During routine analyses, it is recommended to inject a standard after every third sample to ensure the integrity of the sample analysis, as well as to correct for variations in detector response.

In addition to nontreated check samples and recovery samples (fortified nontreated check samples), each series of samples should include a "reagent blank" which is also carried through from the extraction to the final analysis. The background noise levels from check sample and "reagent blank" are related to the degree of interference. Calculate the IV residues by means of the equation $C = W/S$, where C = ppm of compound, W = weight of compound, in μg, found in the aliquot of the sample injected, and S = weight of the sample, in g, represented by the aliquot injected.

RESULTS AND DISCUSSION

The conversion of amino acids to hydantoins is well known. Thus the classical Edman degradation (Edman, 1956) cleaves the terminal amino acid group in a peptide chain to the corresponding phenylthiohydantoin derivative. More recently, Suzuki *et al.* (1969) converted amino acids to methylthiohydantoin for measurement by gc. The amino acid side chain of IV lends itself to the formation of hydantoin. This property is exploited in the method described. Bladex and its other metabolites also respond to this method of analysis, whereas other *s*-triazine herbicides such as atrazine (2-chloro-4,6-bis(isopropylamino)-*s*-triazine) and simazine (2-chloro-4,6-bis(ethylamino)-*s*-triazine) do not. The preliminary prewashing of the crop material with various organic solvents described under "Crop Preparation" effectively separates the parent Bladex herbicide and its other fat-soluble metabolites from IV.

Identification of 5,5-Dimethylhydantoin. Identification of 5,5-dimethylhydantoin as the active product resulting from the hydrolysis of IV was made by comparing that product with authentic 5,5-dimethylhydantoin purchased from Aldrich Chemical Co. (item number D16,140-3, mp 176–178°). Five parameters were used for comparison: ir, nmr, glc, tlc, and melting point. All five comparisons agreed.

Factors Affecting the Hydrolysis of IV to 5,5-Dimethylhydantoin. Hydrolysis efficiency was found to depend on a number of variables, the most influential being concentration of acetic acid, temperature and time of reaction, presence of sodium acetate, and presence of a catalyst. Although the hydrolysis will take place under less stringent conditions, the parameters required for a reproducible reaction are 1 ml of glacial acetic acid; heating at 165° for 3 hr in a closed tube in the presence of 0.4 g of sodium acetate and trace amounts of $K_3Fe(CN)_6$. Under these conditions reproducible results were obtained. The sodium acetate served as a buffer to prevent the solution from becoming too acidic to cause hydrolysis to α-aminoisobutyric acid. Initially sodium dichromate was used as the catalyst, but $K_3Fe(CN)_6$ was substituted when the latter gave better reproducibility. Its use was found to be a key to the success of the method. Figure 5 shows typical gas–liquid chromatograms of derivatized corn stover extracts in the absence and presence of added IV. Each injection represents the equivalent of 5 mg of sample.

Ec–Glc. Although 5,5-dimethylhydantoin is well suited for gas chromatography, it lacks sensitive groups capable of being measured in the subnanogram range. For residue analysis requiring high sensitivity it is common practice to enhance the response of such compounds by introducing several halogen atoms into the molecule. This is often accomplished by reaction with one of the halogenated anhydrides. Initially, heptafluorobutyric anhydride was reacted with 5,5-dimethylhydantoin, with a corresponding enhancement in analytical sensitivity. However, the use of this reagent has the disadvantage of requiring anhydrous reaction conditions. Since trichloromethanesulfonyl chloride may be reacted with 5,5-dimethylhydantoin in an aqueous environment, and since the resulting derivative TCMS-DMH responds well to ec–glc measurement and is very soluble and stable in organic solvents, this reagent was chosen for use in the ec–glc method adopted.

In practice, a standard curve is carried through the complete procedure in exactly the same manner as the sample. In this way, both standards and unknowns are compared on the same basis and any disturbances such as incomplete reactions, adsorption losses by the derivatization procedure or in the chromatographic system, and interferences from other compounds in the solvent or in the reagents are equalized.

Using this method, recovery experiments were conducted on crops, soils, and water by adding known amounts of IV at the blending step and processing the sample as described above. Recoveries were conducted at the 0.2-, 0.5-, and 1.0-ppm levels. Recoveries in soil and water averaged about 90%. Crop recoveries averaged about 80%, ranging between 75 and 110% (see chromatograms, Figure 5).

The procedure is capable of measuring IV residues of 0.02 ppm in soil and water and about 0.1 ppm in crops.

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