

US EPA ARCHIVE DOCUMENT

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NOT FOR PUBLICATION

DOCUMENT NO. AMR-04-81

DETERMINATION OF DPX-4189 RESIDUES IN GRAIN, STRAW, AND GREEN PLANTS
OF CEREALS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

By

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January 1981

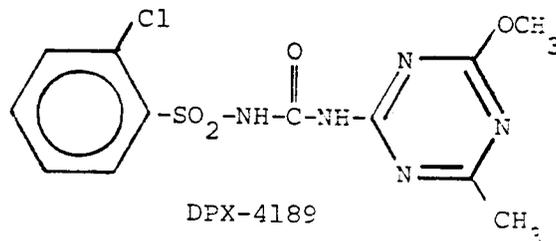
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DETERMINATION OF DPX-4189 RESIDUES IN GRAIN, STRAW, AND GREEN
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INTRODUCTION

This report documents the current analytical method for determination of residues of Du Pont's experimental herbicide DPX-4189 in the grain, straw, and green plants of barley, wheat, oats, and rye. DPX-4189 has the following structure:



This compound is being evaluated as a herbicide for control of weeds in cereals.

DPX-4189 is isolated from the samples by ethyl acetate extraction and is separated from major interfering components by an aqueous filtration, a size-exclusion chromatographic separation for green or particularly oily samples, and by solvent extraction. DPX-4189 is then determined in the sample extracts by high performance liquid chromatography (HPLC). The detection limit for the method is 5 ng which is equivalent to 10 ppb in grain (20 ppb in oats grain) and 50 ppb in straw and green plants. The average recovery efficiency for determination of DPX-4189 in these cereals is approximately 70%.

APPARATUS AND REAGENTS

A Hobart Model 84142 Commercial Food Cutter (Hobart Manufacturing Company, Troy, OH) is used to cut straw and green plant samples into lengths of less than 1 cm. Although rather tedious, scissors are an acceptable substitute.

Waring Commercial Blenders^a (Waring Products Corp., New York, NY) with 40-ounce Blendor^a jars are used to homogenize samples during solvent extraction. These are operated through variable power transformers to provide speed control.

a. Blendor is a trade name of the Waring Products Corporation, New York, NY.

Millipore^b 47-mm Teflon^c-faced glass filters (Millipore Corp., Bedford, MA, Cat. No. XX 1004720) with 47-mm Millipore^b Microfiber glass disk prefilters (Millipore Corp., Cat. No. AP4004705) are used with Fisher Filtrators^d (Fisher Scientific Co., Pittsburgh, PA, Cat. No. 9-788) for all filtrations.

Rotary evaporators with temperature-controlled water baths equivalent to Rotavapor R^c (Fisher Scientific Co., Pittsburgh, PA, Cat. No. 9-548-151) are used to evaporate and concentrate solutions. 1000-mL and 200-mL round-bottom evaporating flasks are used with the rotary evaporators.

Solvent extractions are performed with 125-mL separatory funnels with Teflon^c plugs (Fisher Scientific Co., Pittsburgh, PA., Cat. No. 10-437-10B).

Size-exclusion chromatographic separations are performed on an Autoprep^e 1001 instrument (Analytical Biochemistry Laboratories, Inc., Columbia, MO) using a column of Bio-Beads^f S-X3 styrene-divinylbenzene copolymer (Bio-Rad Laboratories, Richmond, CA).

A Du Pont Model 850 Liquid Chromatograph with a 4.6 mm x 25 cm Zorbax^g Sil column (Du Pont Instruments Division, Wilmington, DE) and a Tracor Model 965 photo-conductivity detector (Tracor Instruments, Inc., Austin, TX) are used for HPLC determinations.

Ethyl acetate, cyclohexane, methyl alcohol, isopropyl alcohol, chloroform*, methylene chloride, and toluene are "Distilled in Glass" quality manufactured by Burdick and Jackson Laboratories, Muskegon, MI.

Acetic Acid is "Reagent ACS" grade (Cat. No. A-38) manufactured by Fisher Scientific Co., Pittsburgh, PA.

Sodium hydroxide solution (Cat. No. SO-S-272), hydrochloric acid solution (Cat. No. SO-A-48), granular Na₂HPO₄ (Cat. No. S-374), and sodium chloride (Cat. No. S-271) are "Certified" grade manufactured by Fisher Scientific Co., Pittsburgh, PA.

The reference standard of DPX-4189 is synthesized and assayed in the Biochemicals Department, Agrichemicals Research Division Laboratories.

- b. Millipore is a trade name of the Millipore Corporation, Bedford, MA.
- c. Teflon is a trade name of the E. I. du Pont de Nemours & Co., Inc., Wilmington, DE.
- d. Filtrator and Rotavapor R are trade names of the Fisher Scientific Co., Pittsburgh, PA.
- e. Autoprep is a trade name of Analytical Biochemistry Laboratories, Inc. Columbia, MO.
- f. Bio-Beads is a trade name of Bio-Rad Laboratories, Richmond, CA.
- g. Zorbax is a trade name of E. I. du Pont de Nemours & Co., Inc., Wilmington, DE.

*Chloroform should be used only in a well-ventilated hood. Skin contact should be avoided. Use of neoprene gloves is suggested.

EXPERIMENTAL PROCEDURE

Sampling

For grain analysis, separate grain from chaff and straw, weigh out a 50 g sample, and transfer it to a blender jar. For straw and green or whole plants, cut the entire sample to a homogeneous relatively-small (< 1 cm) length using a Hobart Commercial Food Cutter. Mix the cut sample thoroughly. Weigh out 10 g of straw or 20 g of green plants and transfer the sample to a blender jar. If the sample is to be a spiked control to determine recovery efficiency, pipet the required volume of 1- μ g DPX-4189/mL CH_2Cl_2 solution onto the sample in the blender and evaporate the solvent.

Weight Loss

Determine weight loss on drying to constant weight in air at room temperature on 20 g of all samples that contain a significant quantity of water. Generally only green plant samples contain sufficient water to warrant weight-loss determination. The weight-loss data permit calculation of DPX-4189 residues on a dry weight basis for direct comparison with other data.

Sample Extraction

Extract the sample three times with 150 mL of ethyl acetate per extraction as follows: Add 150 mL of ethyl acetate to the blender and blend at a high speed for three minutes. Transfer most of the ethyl acetate extract from the blender to the filter by decanting or by using a syringe while retaining the solids in the blender for subsequent extractions. Add the second 150 mL of ethyl acetate to the blender and blend at a high speed for three minutes. Transfer most of the extract to the filter while again retaining solids in the blender. Add the third 150 mL of ethyl acetate to the blender, blend three minutes at a high speed, transfer all extract and solids to the filter, and filter under vacuum. Rinse the blender, filter, and sample solids with 25 mL of ethyl acetate, filter the rinse solution, and combine it with the three ethyl acetate extracts. Discard the sample solids.

Transfer the combined ethyl acetate extracts to a 1000-mL flask and evaporate the solvent on a rotary evaporator at $\leq 35^\circ\text{C}$ until all of the ethyl acetate has been evaporated or until further evaporation of ethyl acetate from the oily residue is impractical. Be careful not to heat the sample excessively at or near dryness. Proceed to the Aqueous Filtration procedure for all samples.

Aqueous Filtration

Prepare a pH-10 buffer solution by dissolving 1.42g Na_2HPO_4 and 40.0g NaCl in 400 mL water. Use a pH meter and adjust the pH of this solution to 10.0 by adding 1M NaOH slowly in small increments with stirring.

Add 10 mL of CH_2Cl_2 to the sample flask to dissolve the sample residue after evaporation of ethyl acetate. Add 25 mL of pH-10 buffer solution to the flask containing the sample residue in CH_2Cl_2 . Evaporate the CH_2Cl_2 on a rotary evaporator at $\leq 35^\circ\text{C}$. The CH_2Cl_2 must be completely evaporated or problems will develop during the subsequent filtration. After complete evaporation of the

CH_2Cl_2 , check the pH of the aqueous solution with a limited-range pH paper. If necessary, add 1M NaOH solution dropwise to stabilize the pH at 10.

When the sample solution has been stabilized at pH 10, cool the solution in an ice-water bath for at least 10 minutes to promote solidification of oily components. Decant the aqueous phase cold onto a Millipore^b glass filter with glass disk prefilter and filter cold under vacuum. Rinse the flask which originally contained the aqueous sample solution with 10 mL of cold pH-10 buffer solution. Filter this rinse solution cold and combine it with the previously filtered sample solution. Discard the filter residue.

If the sample is to be cleaned up on the Autoprep^e size-exclusion column, proceed to the Size-Exclusion Cleanup procedure. Size-exclusion cleanup is usually required for green plant samples and for oat grain samples. Most other samples do not require size-exclusion cleanup. If the sample is not to be cleaned up by size-exclusion chromatography, proceed to the Solvent Extraction Cleanup procedure.

Size-Exclusion Cleanup

Quantitatively transfer the 35-mL aqueous filtrate to a 125-mL separatory funnel. Acidify the filtrate in the separatory funnel by adding 1M HCl slowly with mixing until the pH is 2 as measured by a limited-range pH paper. At pH 2, DPX-4189 can be efficiently extracted into CHCl_3 . Add 25 mL of CHCl_3 to the flask which previously contained the aqueous phase. Rinse the flask well and add the CHCl_3 rinse to the acidified aqueous phase in the separatory funnel. Shake the separatory funnel vigorously for two minutes, separate phases (centrifuge if necessary to break emulsions), and transfer the CHCl_3 phase to a 200-mL round-bottom flask. Extract the aqueous phase again with 25 mL of CHCl_3 . Shake vigorously for two minutes as before, separate phases, and combine the two CHCl_3 extracts in the 200-mL flask. Discard the aqueous phase.

Evaporate the combined CHCl_3 extracts to dryness on a rotary evaporator at $\leq 35^\circ\text{C}$. Use several small quantities of 25% toluene in ethyl acetate to dissolve and quantitatively transfer the sample residue to a 10-mL volumetric flask. Make the flask to volume with additional 25% toluene in ethyl acetate and mix well.

Use the 10-mL sample solution to load the 5-mL loop of the Autoprep^e. Process the sample on the Autoprep^e size-exclusion chromatographic column using 25% toluene in ethyl acetate as the mobile phase at 5 mL/min. Set the dump, collect, and wash times of the Autoprep^e to appropriate settings as determined by a recent calibration for DPX-4189. A calibration procedure for the Autoprep^e size-exclusion chromatographic column is given in the Appendix.

Upon completion of the size-exclusion cleanup, evaporate the collected fraction to dryness on a rotary evaporator at $\leq 35^\circ\text{C}$. Add 25 mL of pH-10 buffer solution to the sample flask. Swirl the flask several times to dissolve any residue. Proceed to the Solvent Extraction Cleanup procedure.

b. Millipore is a trade name of the Millipore Corporation, Bedford, MA.

e. Autoprep is a trade name of Analytical Biochemistry Laboratories, Inc., Columbia, MO.

Solvent Extraction Cleanup

Transfer the aqueous solution from the Aqueous Filtration procedure or from the Size-Exclusion Cleanup procedure to a 125-mL separatory funnel. Rinse the container which previously held the aqueous phase with 25 mL CHCl_3 and transfer this to the separatory funnel. Shake the separatory funnel vigorously for 2 minutes, separate phases (centrifuge if necessary to break emulsions), and discard the CHCl_3 phase.

Add 25 mL more CHCl_3 to the separatory funnel, shake 2 minutes, separate phases, and discard the CHCl_3 phase.

Add a third 25-mL quantity of CHCl_3 to the separatory funnel, shake vigorously for 2 minutes, separate phases, and discard the CHCl_3 phase.

Check the pH of the aqueous phase with a limited-range pH paper to make certain it is still pH 10. Add 1M NaOH dropwise with stirring and adjust the pH to 10 as determined by pH paper if it is not already pH-10.

Extract the aqueous phase two times with 25-mL quantities of cyclohexane. Discard the cyclohexane extracts.

Acidify the aqueous phase in the separatory funnel to pH 2 with 1M HCl as determined by a limited-range pH paper. Proceed promptly with sample processing to minimize hydrolysis of DPX-4189. Add 25 mL CHCl_3 , shake for two minutes, separate phases, and transfer the CHCl_3 phase to a 200-mL round-bottom flask.

Add 25 mL more CHCl_3 to the aqueous phase in the separatory funnel, shake two minutes, separate phases, and add the second CHCl_3 extract to the 200-mL round-bottom flask. Discard the aqueous phase in the separatory funnel.

Concentrate the combined CHCl_3 extracts to 2 to 3 mL and transfer the concentrate quantitatively to a 5-mL volumetric flask. Rinse the extract container with more CHCl_3 as needed to complete the quantitative transfer. Evaporate the CHCl_3 completely from the 5-mL volumetric flask using a stream of dry air or nitrogen. Proceed to the HPLC Calibration and Analysis procedure.

HPLC Calibration and Analysis

DPX-4189 is determined in the sample solution by comparing the chromatographic peak height for DPX-4189 in the sample solution with the corresponding peak heights for standard solutions containing known quantities of DPX-4189.

Prepare HPLC mobile phase by combining and thoroughly mixing 750 mL cyclohexane, 125 mL isopropyl alcohol, 125 mL methyl alcohol, and 1 mL of glacial acetic acid:water solution, 10:1 parts respectively by volume. Make each sample from the Solvent Extraction Cleanup procedure to 5 mL volume with HPLC mobile phase.

Prepare a 100- $\mu\text{g}/\text{mL}$ stock standard by dissolving 10.0 mg of DPX-4189 in 100 mL of CH_2Cl_2 in a volumetric flask. Prepare a 1- $\mu\text{g}/\text{mL}$ standard of DPX-4189 in CH_2Cl_2 by diluting 1 mL of the 100 $\mu\text{g}/\text{mL}$ stock standard to 100 mL with CH_2Cl_2 .

Prepare HPLC standards containing 0.20, 0.40, 0.60, and 0.80 $\mu\text{g}/\text{mL}$ by pipetting 5, 10, 15, and 20 mL, respectively, of 1 $\mu\text{g}/\text{mL}$ DPX-4189 in CH_2Cl_2 into 25-mL volumetric flasks, evaporating the CH_2Cl_2 with a stream of dry air or nitrogen, and making each flask to 25 mL volume with HPLC mobile phase. The stock standard can be stored for several weeks and the lower concentration standards can be stored for several days with negligible decomposition if refrigerated when not in use.

Analyze samples and HPLC standards by high performance liquid chromatography using the following conditions:

Column: Zorbax^g Sil, 4.6 mm x 25 cm
Column Oven Temperature: 35°C
Mobile Phase: Composition given above
Flow Rate: 0.50 mL/min
Injection Volume: 50 μL
Detector: Tracor Model 965 photo-conductivity
Retention Time: \approx 14 min

Unspiked control samples and control samples spiked with 0.5, 1.0, or 2.0 μg of DPX-4189 (spiked with 1.0, 2.0, or 4.0 μg of DPX-4189 if size-exclusion cleanup is used) should be analyzed at random intervals or at predetermined uniform intervals during a series of sample analyses to demonstrate the absence of interferences, to provide a check on recovery efficiency, and to confirm that the retention time in the sample matrix is consistent with that of the standards. HPLC standards should be analyzed at random or predetermined intervals during a series of sample analyses to confirm the stability of instrument sensitivity and retention time. Analysis of one unspiked control, one spiked control, and two working standards for every two or three samples provides adequate assurance of reliable determinations.

Calculations

Prepare a standard calibration curve as in Figure 1 by plotting peak height for DPX-4189 versus μg DPX-4189 injected for the HPLC standards. Draw the best straight line through the origin and experimental data points and calculate the slope of the line, M_0 , in mm/ μg . Calculate ppm DPX-4189 in samples which have not been cleaned up on the size-exclusion column by using Equation (1). Calculate ppm DPX-4189 in samples which have been cleaned up on the size-exclusion column by using Equation (2).

Calculate percent recovery of DPX-4189 in spiked control samples by using Equation (3) for samples which were not cleaned up on the size-exclusion column and by Equation (4) for samples which were cleaned up on the size-exclusion column. The variables in these equations are defined as follows:

^g. Zorbax is a trade name of E. I. du Pont de Nemours & Co., Inc., Wilmington, DE.

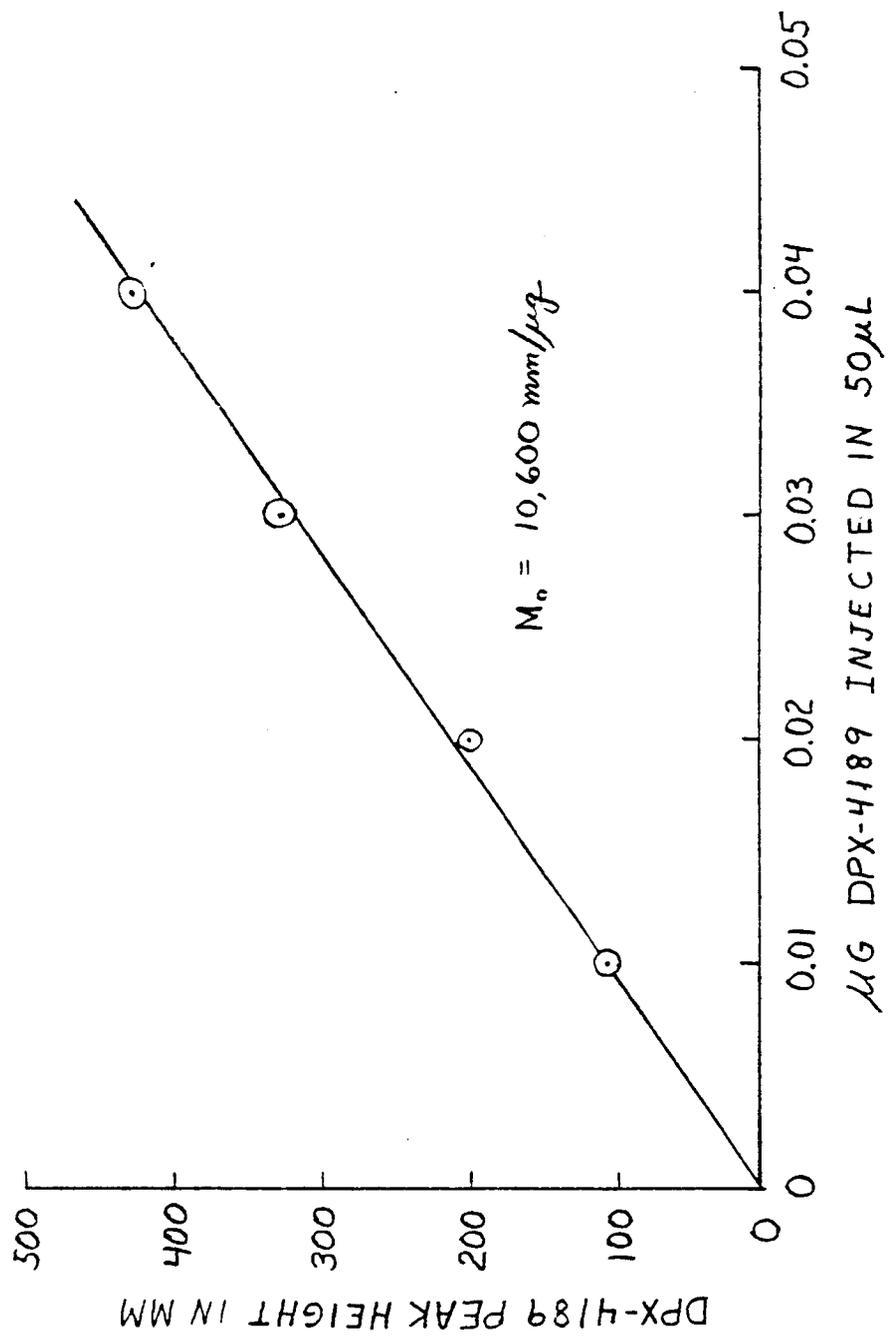


FIGURE 1 STANDARD CURVE FOR DPX-4189

PK = Peak Height for DPX-4189 in mm

M₀ = Slope of Standard Calibration Curve in mm/μg

VS = Volume of Sample Solution in mL

VI = Volume of Sample Solution Injected in mL

SW = Weight of Sample in g

SP = Weight of DPX-4189 Spiked onto Sample in μg

$$\text{ppm} = \frac{\text{PK} \times \text{VS}}{\text{M}_0 \times \text{VI} \times \text{SW}} \quad (1) \quad \text{No Size-Exclusion Cleanup}$$

$$\text{ppm} = \frac{\text{PK} \times \text{VS} \times 10}{\text{M}_0 \times \text{VI} \times \text{SW} \times 5} \quad (2) \quad \text{Size-Exclusion Cleanup}$$

$$\text{Percent Recovery} = \frac{100 \times \text{PK} \times \text{VS}}{\text{M}_0 \times \text{VI} \times \text{SP}} \quad (3) \quad \text{No Size-Exclusion Cleanup}$$

$$\text{Percent Recovery} = \frac{100 \times \text{PK} \times \text{VS} \times 10}{\text{M}_0 \times \text{VI} \times \text{SP} \times 5} \quad (4) \quad \text{Size-Exclusion Cleanup}$$

The factor 10/5 arises in the equations for samples cleaned up by size-exclusion chromatography because the Autoprep^e sample loops hold only 5 mL of the 10 mL sample solution.

DISCUSSION

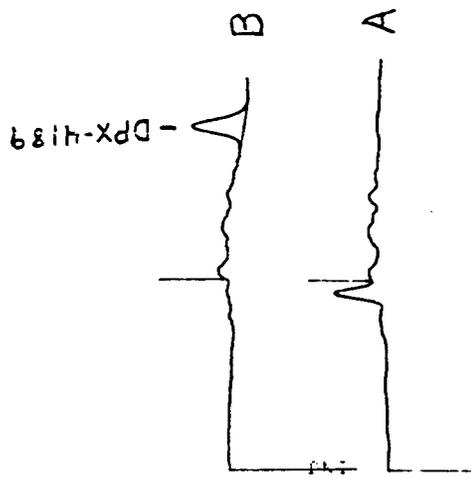
Typical chromatograms for determination of DPX-4189 in cereals are illustrated in Figure 2 by wheat grain and barley straw control samples which were spiked respectively at the 10 ppb and 200 ppb levels before sample preparation. Chromatograms for oats samples and for green plant samples generally show somewhat more interferences than the chromatograms of Figure 2; however, background interferences are usually small because the photo-conductivity detector is quite selective and highly sensitive to DPX-4189.

Results of a recovery study are presented in Table 1. These data were obtained during routine analysis of cereal samples by spiking known quantities of DPX-4189 onto control samples and then determining DPX-4189 in the spiked samples by the analytical method described here. The average recovery efficiency of this method is 70%.

The detection limit for this method was calculated for 95% confidence using the procedure of Hubaux and Vos¹. The detection limit, which is determined primarily by the chromatographic analysis, is 5 ng. This is equivalent to 10 ppb in grain and 50 ppb in straw and green plants.

Routine analyses have demonstrated the applicability of this method for determination of DPX-4189 in grain, straw, and green plant samples of common cereals. The detection limits and recovery efficiency are adequate for initial evaluation of DPX-4189 in cereals. Work is currently in progress to simplify the sample cleanup and to improve recovery efficiency.

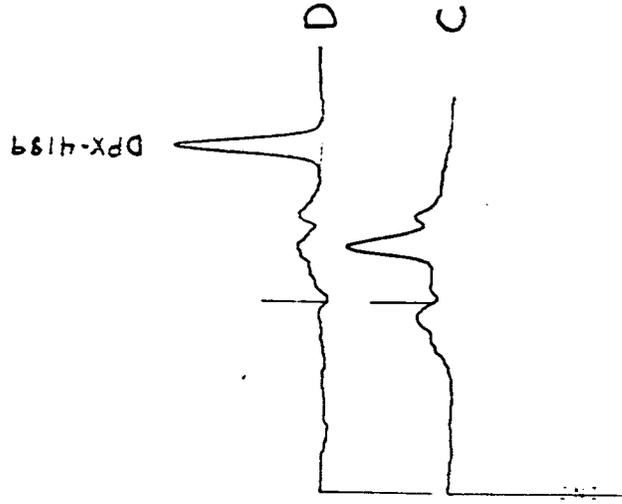
e. Autoprep is a trade name of Analytical Biochemistry Laboratories, Inc., Columbia, MO.



A. WHEAT GRAIN UNSPIKED

B. WHEAT GRAIN SPIKED 10PPB

RECOVERY = 78%



C. BARLEY STRAW UNSPIKED

D. BARLEY STRAW SPIKED 200PPB

RECOVERY = 76%

FIGURE 2 TYPICAL CHROMATOGRAM FOR DETERMINATION OF DPX-4189 IN CEREALS

TABLE I

RECOVERY STUDY FOR DPX 4189

Sample Type	Sample Wt. (g)	"Spike" Level (ppm)	$\mu\text{g DPX-4189}$		% Recovery
			Added	Found	
Barley Seed	50	0.01	0.50	0.45	90
Barley Seed	50	0.01	0.50	0.30	60
Barley Seed	50	0.02	1.0	0.71	71
Barley Seed	50	0.04	2.0	1.2	60
Wheat Seed	50	0.02	1.0	0.85	85
Wheat Seed	50	0.04	2.0	1.5	75
Barley Straw	10	0.05	0.50	0.27	54
Barley Straw	10	0.10	1.0	0.68	68
Barley Straw	10	0.20	2.0	1.5	75
Wheat Straw	10	0.05	0.50	0.45	90
Wheat Straw	10	0.10	1.0	0.60	60
Oats Whole Plant	25	0.08	2.0	1.0	50
Rye Whole Plant	10	0.05	0.50	0.41	82
Wheat Whole Plant	10	0.10	1.0	0.59	59
AVG. 70%					

REFERENCES

¹André Hubaux and Gilbert Vos, "Decision and Detection Limits for Linear Calibration Curves," *Analytical Chemistry*, 42, 849-855 (1970).

APPENDIX

Calibration of the Autoprep^e Size-Exclusion Column

Prepare a 4- $\mu\text{g}/\text{mL}$ DPX-4189 calibration standard by pipetting 1 mL of 100- $\mu\text{g}/\text{mL}$ DPX-4189 in CH_2Cl_2 into a 25-mL volumetric flask, evaporating the CH_2Cl_2 with a stream of dry air or nitrogen, and making to volume with 25% toluene in ethyl acetate. Adjust the Autoprep^e mobile phase flow rate to 5.0 mL/min. Fill the 5-mL loop 1 of the Autoprep^e with the calibration standard and fill all other loops with 25% toluene in ethyl acetate.

With the system in Run Mode and the Dump, Collect, and Wash dials set respectively to 0 min, 4 min, and 0 min, collect 23 fractions. Transfer 2 mL of each fraction respectively, to 23 10-mL graduated centrifuge tubes and evaporate the mobile phase from each tube with a stream of dry air or nitrogen. Make each tube to 2-mL volume with HPLC mobile phase and mix well to dissolve the DPX-4189.

Analyze the solutions in the 23 centrifuge tubes by HPLC using the analytical conditions of the HPLC Calibration and Analysis procedure. Calculate the percent of the DPX-4189 eluted in each fraction as follows:

$$\% \text{ DPX-4189 in Fraction} = \frac{100\% \times \text{PK} \times 4 \text{ min} \times 5 \text{ mL/min}}{M_0 \times .05 \text{ mL} \times 20 \mu\text{g}}$$

In the above equation, PK denotes the DPX-4189 peak height in mm from the analysis of the fraction, and M_0 denotes the slope of the standard calibration curve in mm/ μg . Calculate the DPX-4189 recovery efficiency of the size-exclusion column by summing the percentages for the 23 fractions.

Prepare a calibration plot for the size-exclusion column by graphing the percent DPX-4189 recovered in each fraction against the elution volume for the fraction. A typical calibration plot for elution of DPX-4189 from a 50-g (2.5-cm x 33-cm) column of Bio-Beads^f S-X3 by 25% toluene in ethyl acetate at 5 mL/min is shown in Figure 3. Dump, Collect, and Wash setting for the Autoprep^e of 16, 32, and 12 minutes are illustrated in Figure 3.

e. Autoprep is a trade name of Analytical Biochemistry Laboratories, Inc., Columbia, MO.

f. Bio-Beads is a trade name of Bio-Rad Laboratories, Richmond, CA.

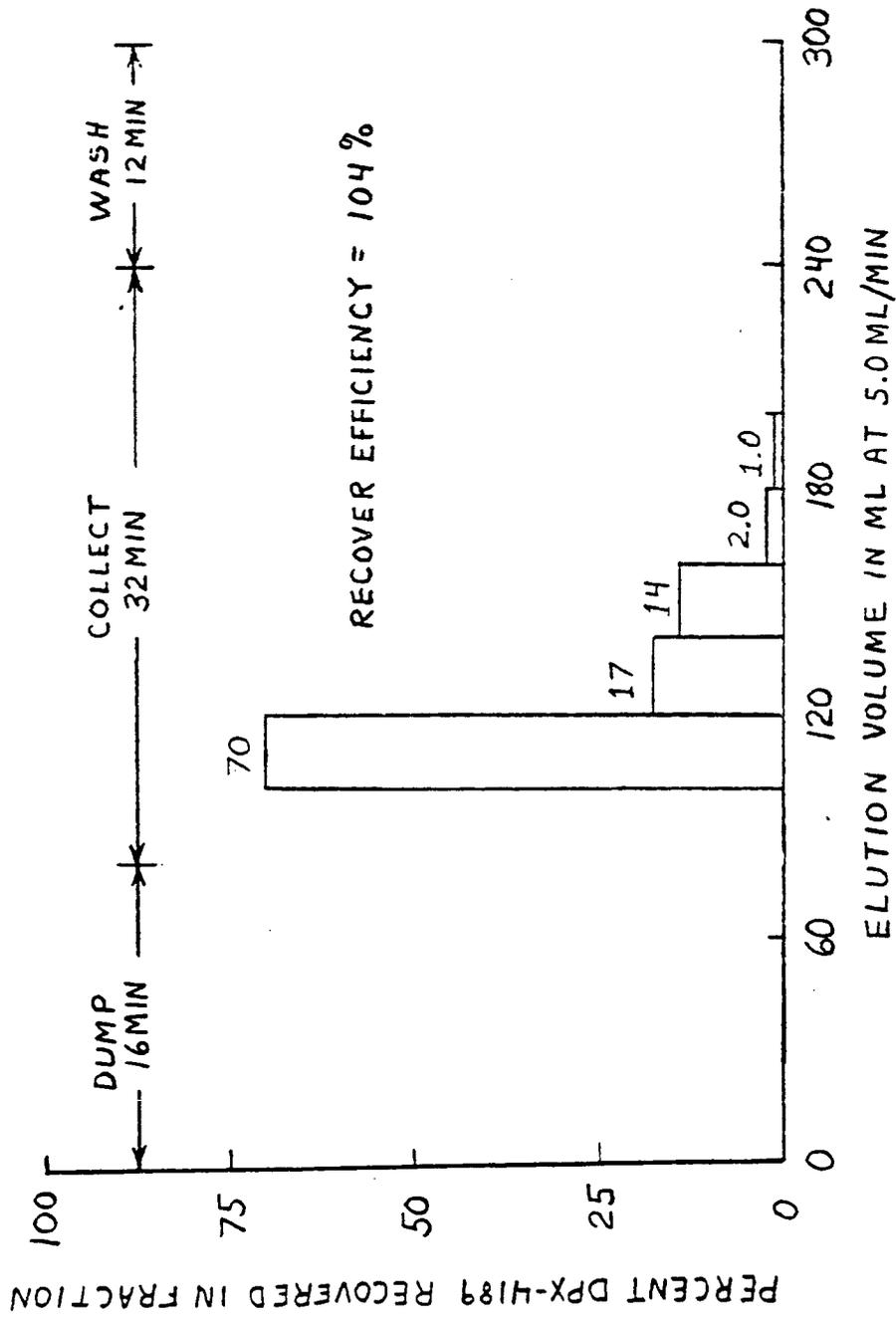


FIGURE 3 SIZE-EXCLUSION COLUMN CALIBRATION PLOT

NOT FOR PUBLICATION

ANALYSIS OF CHLORSULFURON AND METABOLITE A

By

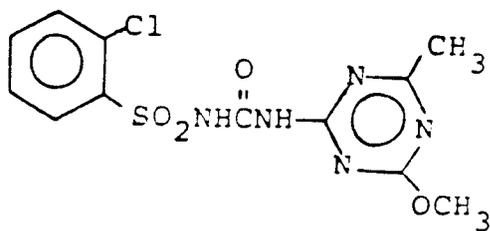
Bob SLATES

E. W. Zahnow

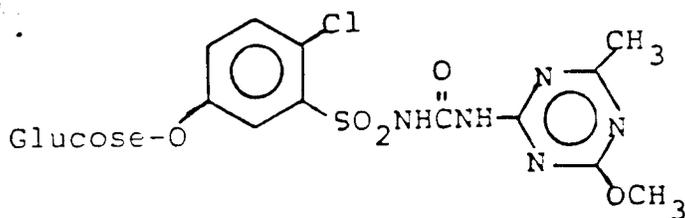
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INTRODUCTION

Living plants of various types are known to be capable of metabolizing chlorsulfuron, 2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl] benzenesulfonamide, to a compound in which the chlorsulfuron molecule is linked to a glucose molecule (Metabolite A). The structures of these compounds are shown below.

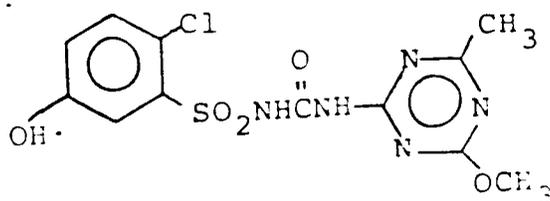


Chlorsulfuron



Metabolite A

A method has been developed to determine the amount of Metabolite A which is present in various plant tissues. The approach used is based on the ability of the enzyme, β -glucosidase, to promote the hydrolysis of Metabolite A to glucose and 5-hydroxychlorsulfuron (see structure below).



5-hydroxychlorosulfuron

5-hydroxychlorosulfuron can be isolated and measured in the plant extracts after suitable purification of the extracts. The measurements are made by liquid chromatography using a photoconductivity detector.

The method has been applied to green wheat, wheat straw, wheat grain, green oats, and barley straw. The detection limit is 50 ng/g (50 ppb), based on detectability of one nanogram of 5-hydroxychlorosulfuron through the liquid chromatography column, and recoveries of 70-80% can be achieved.

Chlorosulfuron can also be determined in the same sample of green wheat or wheat grain. For this compound the detection limit is also 50 ng/g (50 ppb), and recoveries are in the 80-90% range.

EXPERIMENTAL

Preliminary Treatment

Green wheat, wheat straw, barley straw, or green oats are homogenized in a Hobart blender with copious amounts of dry ice (at least equal parts by weight). The entire sample

should be treated at the same time to insure uniformity. The resulting material is a mixture of finely-divided plant material and small particles of dry ice which will have a powdery appearance. This powder is packaged and returned to a freezer where the dry ice sublimates slowly. All samples are stored frozen until they are to be analyzed.

Wheat grain can be analyzed directly without being processed in the Hobart blender.

Extraction and Cleanup Procedure

A 10 g homogenized sample is weighed out and transferred into a Waring blender jar where it is blended at high-speed with 80 mL of acetone and 20 mL of Buffer B (0.82 g anhydrous sodium acetate and 0.5 mL of a solution of 1 part glacial acetic and 10 parts water in 1 liter of solution; pH=5.7) for 1 minute. The resulting slurry is centrifuged at 1500 rpm for 15 minutes, and the supernatant liquid is then decanted through a small plug of glass wool held in a funnel into a 500-mL pear-shaped flask. When centrifuging, only glass bottles should be used. The residual material in the blender jar is treated again with 80 mL of acetone and 20 mL of Buffer B, using the same blending and separation processes. The liquid is combined with that from the first extraction.

Acetone is removed under vacuum from the combined liquids using a rotary evaporator and a water bath set at 40°C.

The aqueous solution is transferred into a 125-mL separatory funnel where it is extracted three times with 25-mL portions of methylene chloride, shaking for one minute. If chlorsulfuron is to be determined, the methylene chloride should be saved for further processing. Refer to Appendix A. Metabolite A remains in the aqueous phase, whereas chlorsulfuron extracts into methylene chloride.

The aqueous solution is drained into a small beaker, and the pH is adjusted to 4.5 with ^{0.25 M HCl} ~~10% hydrochloric~~ acid using a pH meter. This pH adjustment is important for the enzyme, β -glucosidase, to function efficiently. The solution is then transferred to a 500 mL, glass-stoppered, Erlenmeyer flask.

A 60-mg sample of β -glucosidase (Sigma Chemical Co.) is weighed out and dissolved in the aqueous solution. Once the enzyme is completely dissolved, 300 mL of chloroform is quickly added to the flask, the flask is stoppered, and the flask is then placed in a shaker bath set at 35°C. The sample is agitated vigorously for six hours. Note: Chloroform is known to be a weak animal carcinogen. Polyvinyl alcohol gloves should be worn when handling this liquid, and adequate ventilation should be provided.

At the end of this period the entire sample is transferred into a 500-mL separatory funnel, and the chloroform layer is drained off into a 500-mL, pear-shaped flask. The chloroform solution is taken to dryness on a rotary evaporator at 45°C under vacuum.

The residue is then dissolved in 50 mL of acetonitrile with gentle warming. This solution is transferred to a 250-mL separatory funnel and extracted three times with 50-mL

portions of hexane, shaking for one minute. The hexane (upper layer) is discarded. The acetonitrile solution is transferred into a 100-mL, pear-shaped flask and taken to dryness on a rotary evaporator at 45°C under vacuum.

The residue is dissolved in Solution R (750 mL cyclohexane, 125 mL isopropanol, 125 mL methanol, 1 mL of 9 parts glacial acetic acid - 1 part water) by washing the flask with three small (1 mL) portions.

These washings are put through a silica Sep-Pak[®] (Waters Associates) which has been previously washed with 10 mL of Solution R, and the effluent, which contains 5-hydroxychlor-sulfuron, is collected in a 15-mL centrifuge tube. The flask is rinsed with two 1-mL portions of Solution R, and these are put through the Sep-Pak[®] also (collect effluent). Then the Sep-Pak[®] is eluted with 10 mL of Solution R which is also collected in the 15-mL centrifuge tube. (All liquids are put through the silica Sep-Pak[®] by means of a 10-mL hypodermic syringe at a flow rate of ca. 2 mL/min.) A gentle stream of nitrogen is used to evaporate this combined solution to dryness at room temperature. The residue is taken up in Solution R to a final volume of 5 mL.

This sample is analyzed by liquid chromatography. If, for some reason, the sample cannot be analyzed during the same working day, it can be stored dry (refrigerated) after the evaporation step and then dissolved and analyzed at a later time.

Liquid Chromatography

A Du Pont Model 850 liquid chromatograph was used in the development of this method. Since the photoconductivity detector must be used near its maximum sensitivity to achieve the desired lower detection level, it is essential that the chromatographic system provide good temperature control of the column and reasonably pulse-free delivery of mobile phase to minimize baseline fluctuations.

The photoconductivity detector (Tracor Model 965) must be used for this analysis to obtain adequate sensitivity and selectivity. The mercury lamp is used in the detector since it provides much greater sensitivity than the zinc lamp. The detector, including the lamp, is left on at all times to insure greater stability. The flow of the mobile phase through the reference and analytical loops is balanced to within $\pm 2\%$. This is accomplished by installing a metering valve (Nupro Model SS-2SA-TFE) in the solvent line which exits from the reference compartment of the conductivity cell. The "T" which brings the two solvent lines from the conductivity cell back together is eliminated from the instrument. Also, the ion exchange resin tube is not needed to purify the mobile phase and might actually introduce unwanted materials into the system if it is not removed.

The mobile phase is prepared by mixing 750 mL of cyclohexane, 125 mL of isopropanol, 125 mL of methanol, 1 mL of a solution consisting of 9 parts of glacial acetic acid with 1 of water, and 2 mL of glacial acetic acid.

The column is a Du Pont Zorbax[®] SIL (25 cm x 4.6 mm) controlled at 35°C. A new column has to be conditioned by pumping a solution which consists of 40 ^{ml} parts ~~by volume~~ isopropanol, 10 ^{ml} parts glacial acetic acid, and 1 ^{ml} part water, through it for several hours at 1 mL/min. This treatment can also be used to clean columns which have started to lose their efficiency because of contamination from samples. A contaminated column is characterized by broad peaks which tail very badly, poor sensitivity, and by shifting retention times. This conditioning solvent must be thoroughly flushed from the column with the mobile phase. An hour of flushing at 0.5 mL/min is usually sufficient. The conditioning solvent, Solution R, and the mobile phase are all filtered before use through a 0.5 µm Millipore[®] filter (FHUP 04700) held in a Millipore[®] filter apparatus (XX15 04700).

The sample valve is a Valco Model CV-6-UHPa-N60 for manual injection of standards and samples. The loop volume is 10 µL to minimize contamination of the HPLC column and broadening of the chromatographic peak.

During normal operation, mobile phase is pumped through the column at 0.5 mL/min which is judged to be the minimum practical rate. At this flow rate 5-hydroxychlorsulfuron elutes from the column in 14-15 minutes, depending on the extent of column deactivation. This rate is selected because the detector response increases with decreasing flow rate due to the longer residence time of the sample in the quartz reactor coil. At 1 mL/min the peak height for 5-hydroxychlorsulfuron is substantially less than when the mobile phase is pumped at 0.5 mL/min.

Before injection into the chromatograph all samples and standards are filtered through a 5 μm Millipore[®] filter (LSWP 01300) mounted in a Millipore[®] Swinny filter holder (XX3001200) which is, in turn, attached to a 1-mL hypodermic syringe.

Standardization

A standard stock solution of 5-hydroxychlorsulfuron is prepared by weighing out 10.0 mg, dissolving it in methylene chloride, and diluting to 100 mL in a volumetric flask. This solution is quite stable and can be used for many months. It should be stored in a refrigerator.

The working standards used for liquid chromatography are prepared by pipetting 1.0 mL of the stock solution into a clean, dry, 100-mL volumetric flask, evaporating the methylene chloride with a gentle nitrogen stream, dissolving the residue in Solution R, and diluting to volume with Solution R. Standards with concentrations of 0.50, 0.20, and 0.10 $\mu\text{g/mL}$ are prepared from the 1.0 $\mu\text{g/mL}$ standard by appropriate dilution with Solution R. The set of standards prepared in Solution R is replaced with a fresh set every month. Over this time period no change in detector response is observed. All standards should be stored in a refrigerator when not in use.

The detector output is linear over this particular concentration range of 5-hydroxychlorsulfuron. To achieve this sensitivity, it is necessary to use a pump that produces only

small pressure pulses as well as a column of high efficiency. Also, the detector lamp usually needs to be replaced after 500-1000 h of operation, and periodic ultrasonic cleaning of the conductivity cell and electrodes with 0.1% nitric acid is required.

The stock solution (10 $\mu\text{g}/\text{mL}$) used for recovery studies is prepared by dissolving 0.5 mg of Metabolite A in distilled water in a 50-mL volumetric flask and diluting to volume with water. Lower concentrations of Metabolite A are prepared by appropriate dilution.

Calculations

Due to the fact that the background of sample extracts produces a baseline which is irregular, quantitation has been based on peak height measurements.

A chromatogram of the 0.10 $\mu\text{g}/\text{mL}$ 5-hydroxychlor-sulfuron standard is shown in Figure 1.

Response factors for a series of standards are shown in Table 1.

The amount of Metabolite A in a given sample is calculated from the following equation:

$$\frac{\mu\text{g}}{\text{g}} \text{ of Metabolite A (ppm)} = H \times \frac{1}{\text{RF}} \times \frac{1}{1000} \times V_E \times \frac{1}{V_I} \times \frac{536}{374} \times \frac{1}{W}$$

(a) (b) (c) (d) (e) (f) (g)

- (a) H is the peak height in millimeters
- (b) RF is the response factor in millimeters per nanogram
- (c) $\frac{1}{1000}$ converts nanograms to micrograms
- (d) V_E is the total volume of sample extract
- (e) V_I is the volume of sample injected
- (f) $\frac{536}{374}$ converts 5-hydroxychlorosulfuron to Metabolite A
- (g) W is the weight of sample processed

If the quantities described in this procedure are substituted in the equation, the result is:

$$\frac{\mu\text{g}}{\text{g}} \text{ of Metabolite A (ppm)} = \frac{0.0717H}{\text{RF}}$$

Recoveries

Recovery values of Metabolite A from green wheat are shown in Table 2. Recoveries from wheat grain are comparable, whereas those from wheat straw, barley straw, and green oats tend to be about 10% lower.

Representative chromatograms of control and recovery extracts of green wheat are shown in Figures 2 and 3. The spike level was 0.05 ppm Metabolite A, and the measured recovery was 115%.

APPENDIX A

The methylene chloride extract, obtained during the procedure isolating Metabolite A, can be processed to determine chlorsulfuron.

The methylene chloride solution is placed in a 300-mL, pear-shaped flask, and 1 mL of glacial acetic acid is added and mixed in. The solution is then taken to dryness on a rotary evaporator at 35°C.

The residue is dissolved in 1-2 mL of Solution R. This is transferred to a 250-mL separatory funnel containing 100 mL of aqueous 0.1 M Na_2CO_3 - 0.1 M NaHCO_3 . The flask is rinsed three times with small portions (≈ 1 mL) of Solution R, and these are transferred to the separatory funnel also. The excess organic solvent is evaporated by directing a gentle nitrogen stream across the surface of the solution. The remaining solution is mixed thoroughly when the organic layer has evaporated.

The aqueous solution is extracted three times with 50-mL portions of methylene chloride, shaking for 1 minute.

Since the pK_A of chlorsulfuron is about 3.8, the compound remains in the aqueous solution in its anionic form. The methylene chloride layers are discarded. A rotating tumbler unit is useful for this step and should be operated at low speed. Care has to be taken with this operation to avoid the formation of an emulsion which is difficult to break. When a persistent emulsion does form, it can usually be broken by centrifuging. When centrifuging is necessary, only glass centrifuge bottles should be used.

The aqueous solution is drained from the separatory funnel into a 400-mL beaker, and the pH is adjusted to 3-4 by adding 10% hydrochloric acid dropwise while measuring with a calibrated pH meter. In this pH range chlorsulfuron exists in the nonionic form and can be extracted into various organic solvents. The pH adjustment has to be performed carefully since a considerable amount of foaming can occur. Also, due to the chemical equilibria involved, the pH changes slowly. If the final pH is too low (<2), there is a danger of chemical decomposition of chlorsulfuron, whereas if it is too high (>5), extraction may be incomplete.

The solution is then transferred back into a 500-mL separatory funnel with 5 mL of distilled water being used to rinse the beaker. It is extracted three times with 50-mL portions of toluene by shaking vigorously for one minute. When a rotating tumbler is used, it is operated at high speed. The toluene layers are separated from the aqueous phase and are then combined in a 250-mL round-bottom flask. Again, when centrifuging is required to break an emulsion, only glass centrifuge bottles should be used. The combined extracts should be examined to insure that they are free of water droplets.

To the toluene extract is added 1 mL of glacial acetic acid, and the solution is taken to dryness with a rotary evaporator at about 45°C using a water aspirator as the vacuum source.

The residue is dissolved in Solution R using several small washings which are transferred with a Pasteur capillary

pipet to a graduated, 10-mL centrifuge tube. The total volume should not be allowed to exceed 2 mL.

This is put through a silica Sep-Pak[®] (Waters Associates) which has been previously washed with 5 mL of Solution R, and the effluent is collected in a 10-mL centrifuge tube. Then the Sep-Pak[®] is eluted with 5 mL of Solution R which is also collected in the 10-mL centrifuge tube. (All liquids are put through the silica Sep-Pak[®] by means of a 10-mL hypodermic syringe at a flow rate of ca. 2 mL/min.) A gentle stream of nitrogen is used to evaporate this combined solution to dryness at room temperature. The residue is taken up in Solution R to a final volume of 1 mL.

This final extract is analyzed by liquid chromatography using the same experimental conditions described for the analysis of Metabolite A. Under these conditions chlorsulfuron elutes in 15-16 minutes, depending on the condition of the column.

A standard stock solution of chlorsulfuron is prepared by weighing out 10.0 mg, dissolving it in methylene chloride, and diluting to 100 mL in a volumetric flask. This solution is quite stable and can be used for many months. It should be stored in a refrigerator.

The working standards used for liquid chromatography as well as for the spiking of recovery samples are prepared by pipetting 1.0 mL of the stock solution into a clean, dry, 100-mL volumetric flask, evaporating the methylene chloride with a gentle nitrogen stream, dissolving the residue in Solution R, and diluting to volume with Solution R. Standards with concentrations of 0.50, 0.20 and 0.10 $\mu\text{g/mL}$ are prepared from the 1.0 $\mu\text{g/mL}$

standard by appropriate dilution with Solution R. The set of standards prepared in Solution R should be replaced with a fresh set every month. Over this time period no change in detector response is observed. All standards must be stored in a refrigerator when not in use.

The detector output is linear over this particular concentration range of chlorsulfuron. Response factors for a series of standards are shown in Table 3. Chlorsulfuron concentrations are calculated using the same equation as for Metabolite A with the factor $\frac{536}{374}$ omitted.

A chromatogram of the 0.10 $\mu\text{g/mL}$ chlorsulfuron standard is shown in Figure 4.

Recovery values of chlorsulfuron from green wheat are summarized in Table 4. Recoveries from wheat grain are comparable. Wheat straw, barley straw, and green oats have not been tested using this procedure.

Figures 5 and 6 show representative chromatograms of green wheat extracts, including control and recovery samples. The spike level was 0.05 ppm chlorsulfuron, and the measured recovery was 88%.

TABLE 1

Response Factors for 5-Hydroxychlorsulfuron

<u>5-Hydroxychlorsulfuron ($\mu\text{g/mL}$)</u>	<u>Peak Height (mm)</u>	<u>Detector Attenuation</u>	<u>Response Factor (mm/ng)</u>
0.10	85	1 x 1	85
0.20	166	1 x 1	83
0.50	214	1 x 2	86
1.00	173	1 x 5	<u>87</u>
		Average	85

TABLE 2

Metabolite A Recoveries from Green Wheat

<u>Spike</u> <u>(ppm)</u>	<u>Found</u> <u>(ppm)</u>	<u>Recovery</u> <u>%</u>
0.05	0.041	82
"	0.058	115
"	0.049	97
0.10	0.084	84
0.20	0.20	100
0.50	0.34	69
"	0.39	79
1.0	0.68	68
"	0.80	80
2.0	1.40	<u>70</u>
		Average 84

TABLE 3

Response Factors for Chlorsulfuron

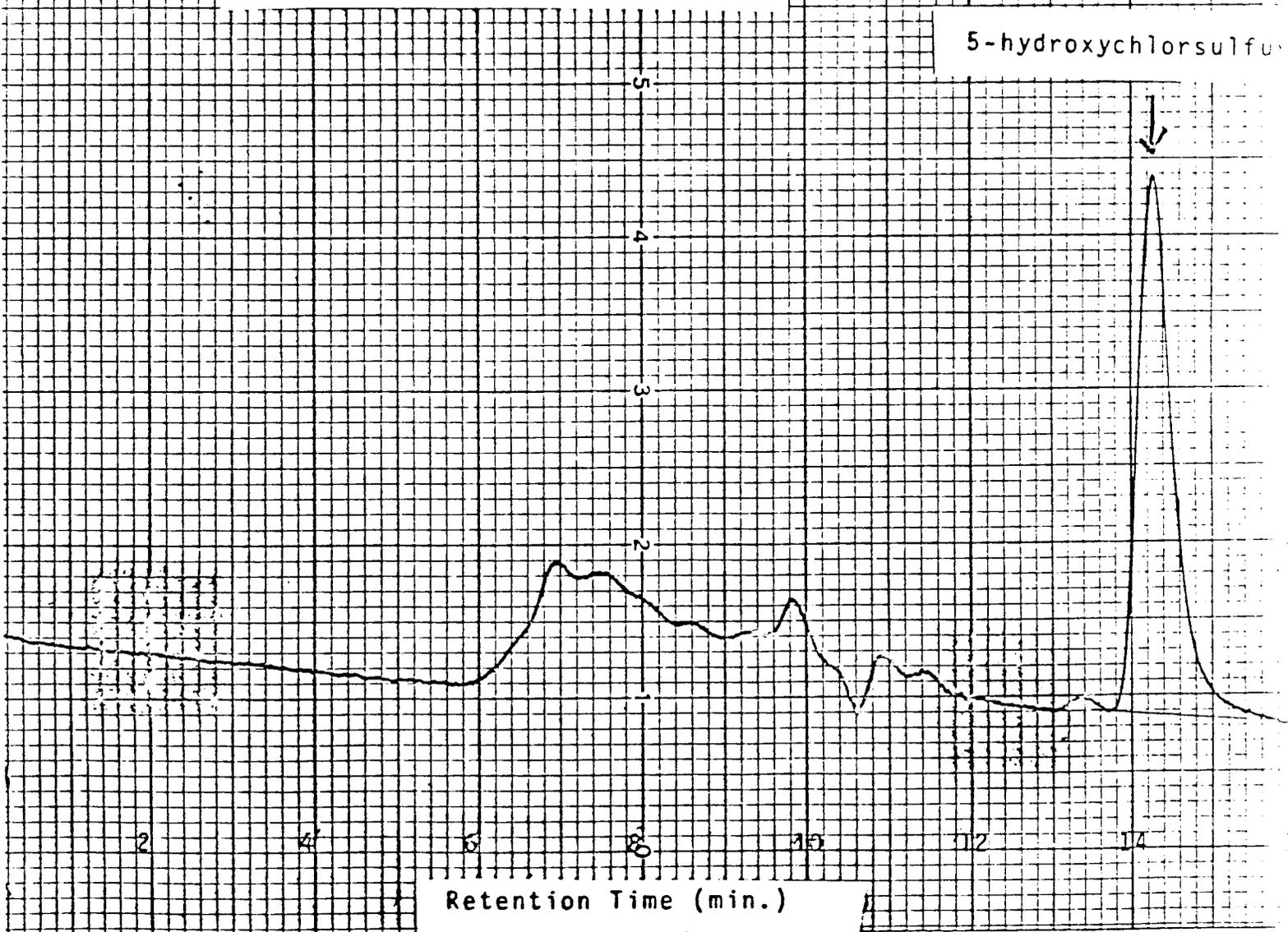
<u>Chlorsulfuron</u> <u>($\mu\text{g/mL}$)</u>	<u>Peak Height</u> <u>(mm)</u>	<u>Detector</u> <u>Attenuation</u>	<u>Response Factor</u> <u>(mm/ng)</u>
0.10	155	1 x 1	155
0.20	151	1 x 2	151
0.50	159	1 x 5	159
1.00	160	1 x 10	<u>160</u>
		Average	156

TABLE 4

Chlorsulfuron Recoveries from Green Wheat

<u>Spike</u> <u>(ppm)</u>	<u>Found</u> <u>(ppm)</u>	<u>Recovery</u> <u>%</u>
0.05	0.047	94
"	0.044	88
0.10	0.075	75
0.20	0.16	82
"	0.17	85
0.50	0.43	87
1.0	1.19	119
2.0	1.67	84
5.0	3.82	76
10	8.65	<u>87</u>
		Average 88

FIGURE 1
5-HYDROXYCHLORSULFURON STANDARD
(Detector Sensitivity, 1 x 1)



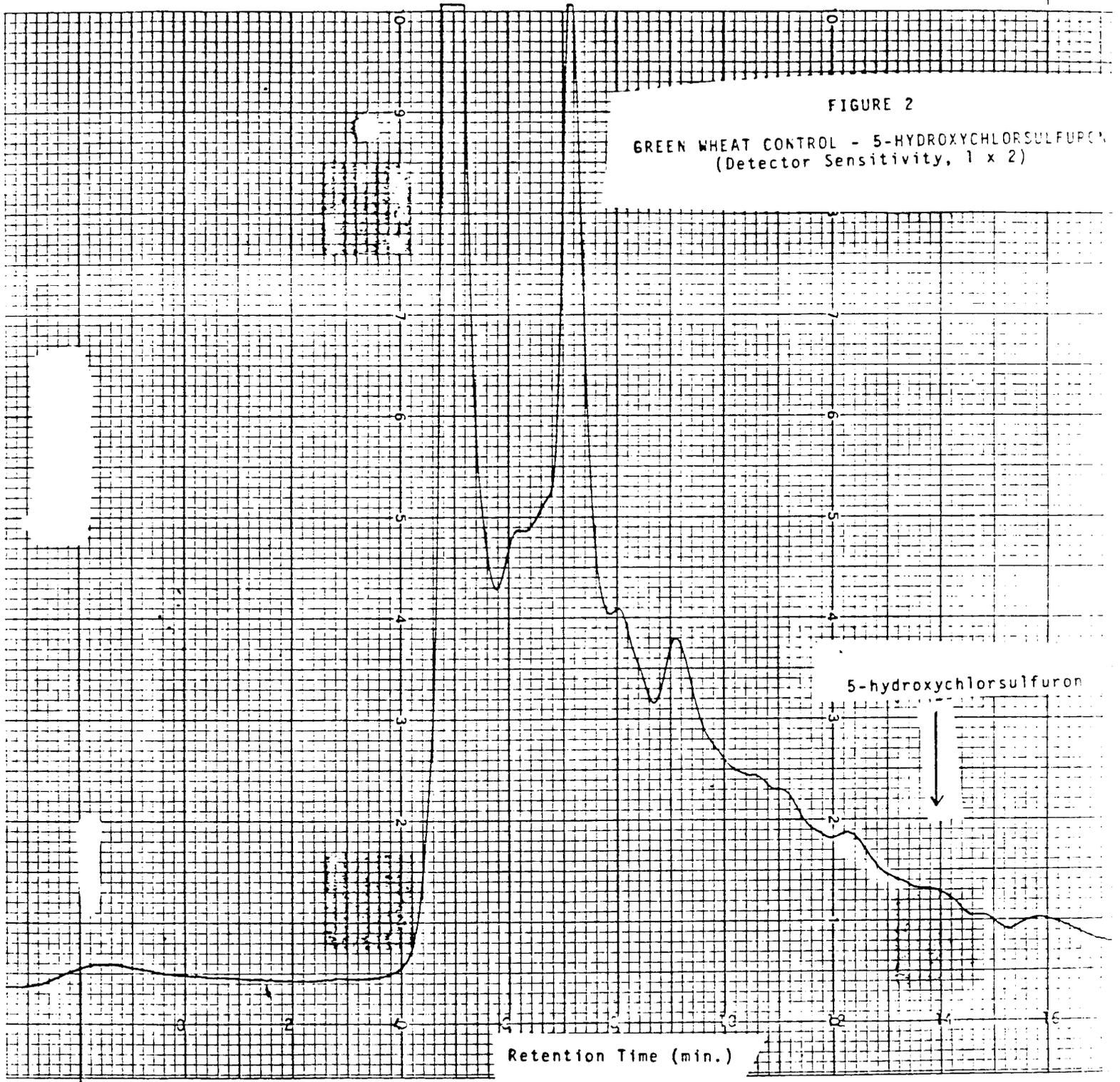


FIGURE 2
GREEN WHEAT CONTROL - 5-HYDROXYCHLORSULFURON
(Detector Sensitivity, 1 x 2)

FIGURE 3
GREEN WHEAT RECOVERY - 5-HYDROXYCHLORSULFURON
(Detector Sensitivity, 1 x 2)

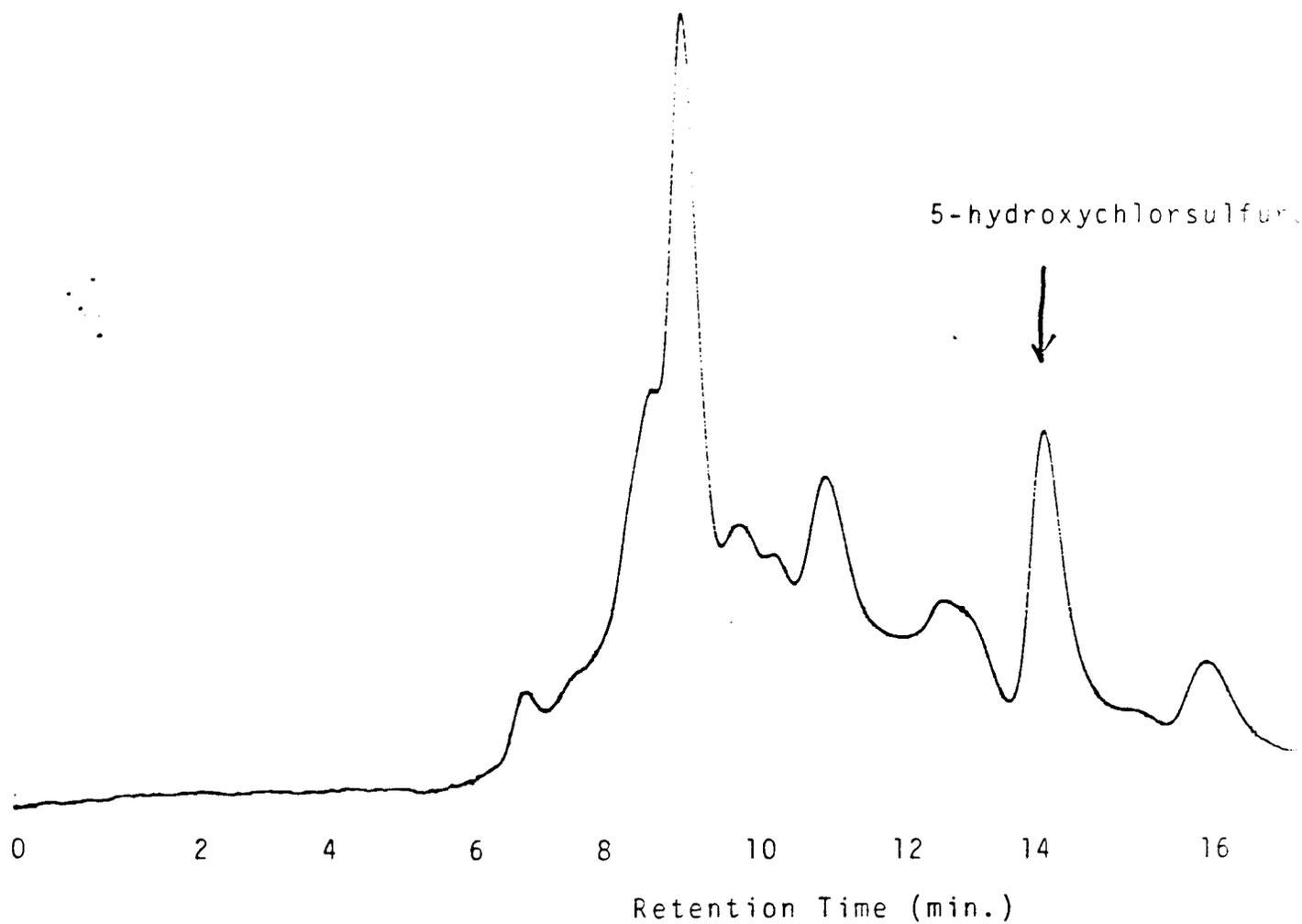
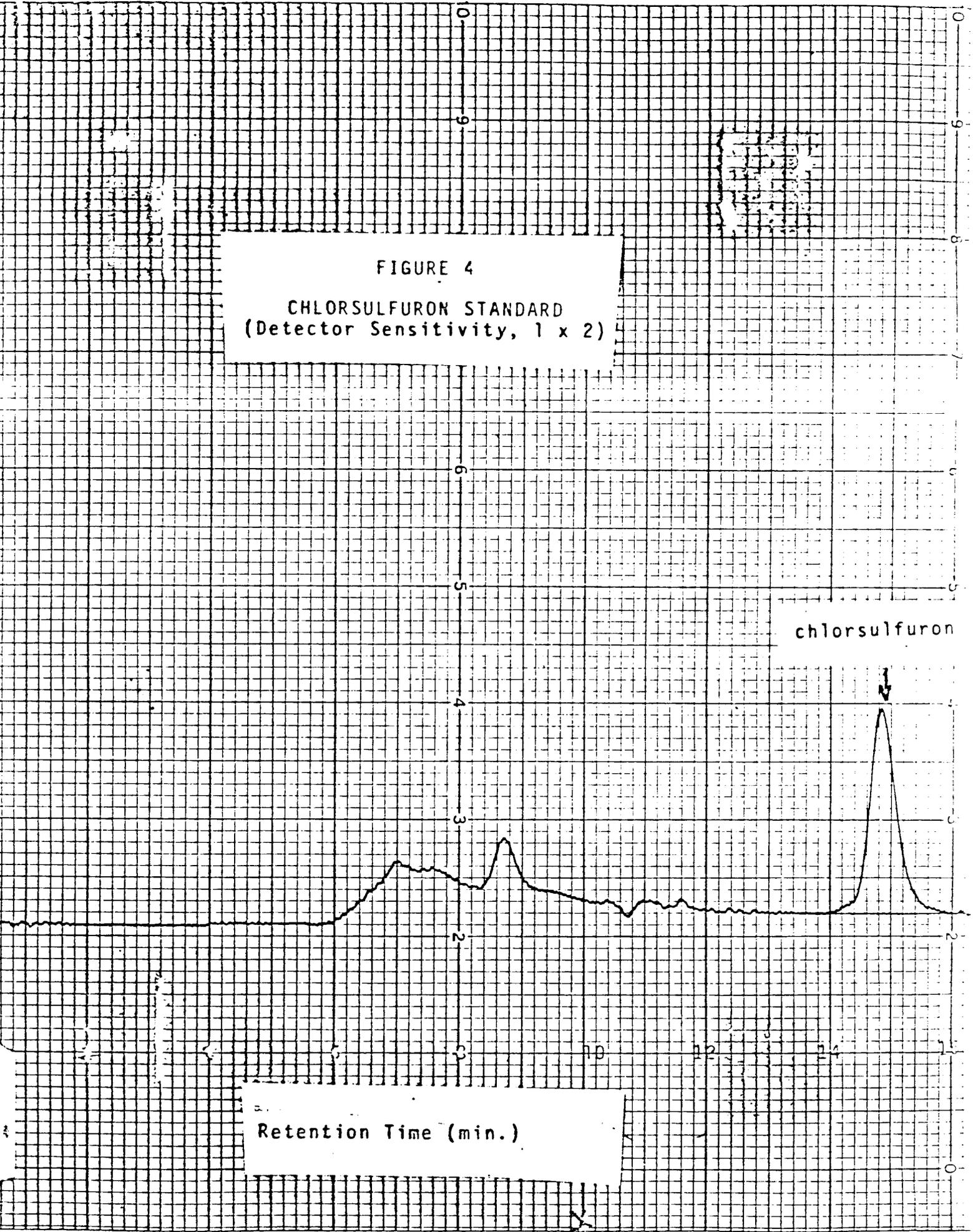


FIGURE 4
CHLORSULFURON STANDARD
(Detector Sensitivity, 1 x 2)

chlorsulfuron

Retention Time (min.)



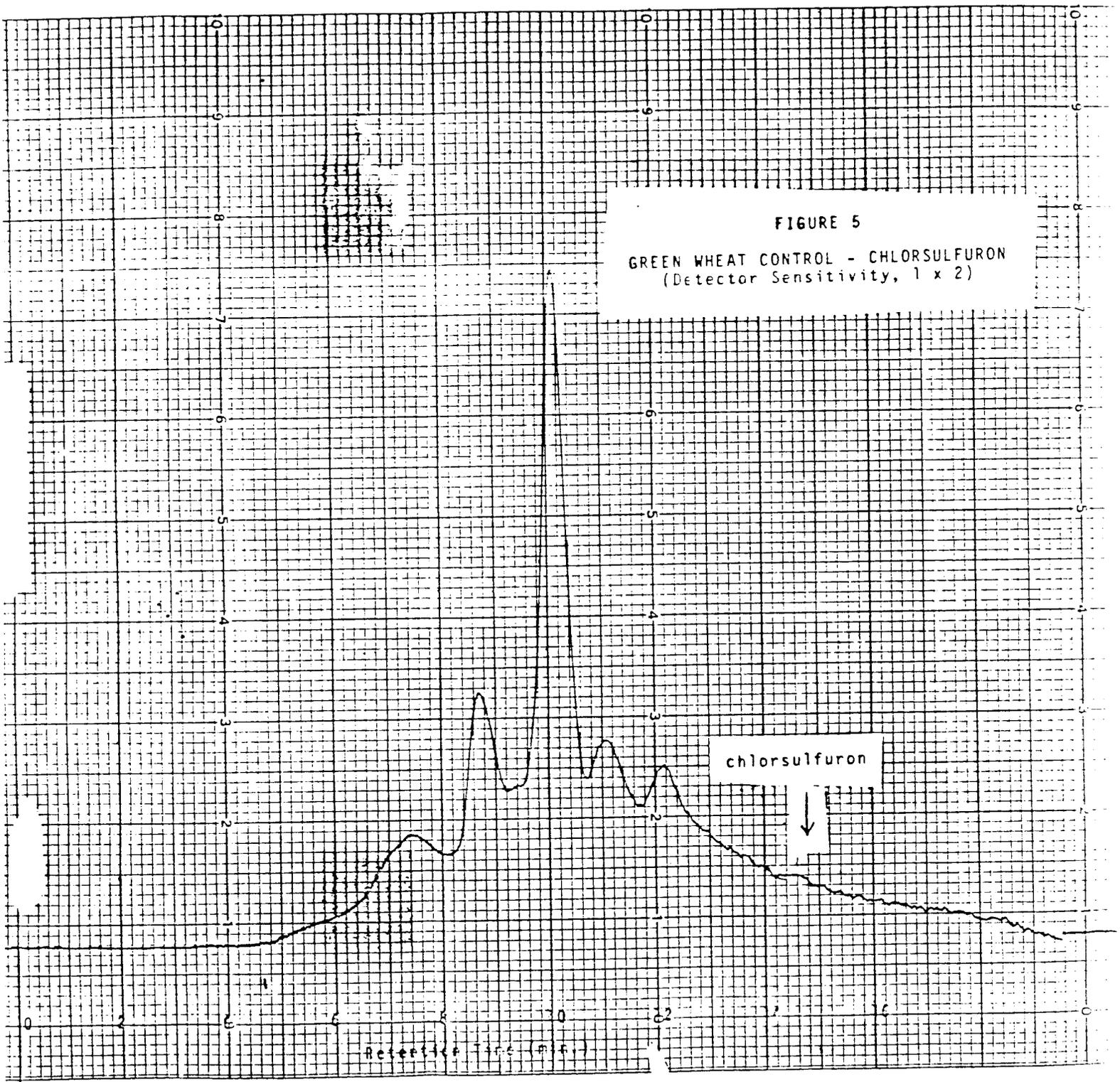
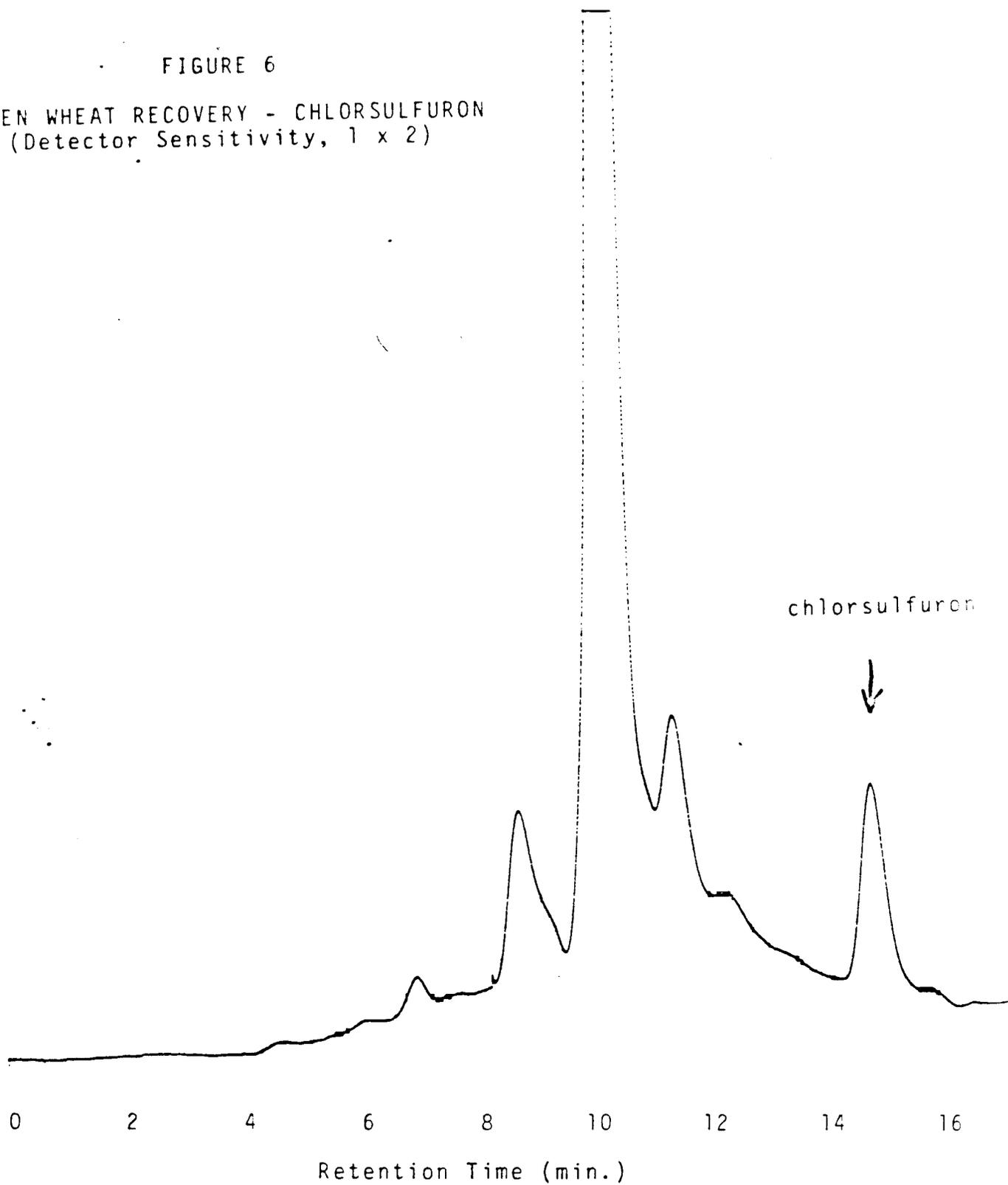


FIGURE 5
GREEN WHEAT CONTROL - CHLORSULFURON
(Detector Sensitivity, 1 x 2)

Retention Time (min)

FIGURE 6

GREEN WHEAT RECOVERY - CHLORSULFURON
(Detector Sensitivity, 1×2)



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DETERMINATION OF CHLORSULFURON IN MILK AND IN LIVER,
LEAN MUSCLE, AND KIDNEY TISSUE OF CATTLE

By

Robert V. Slates

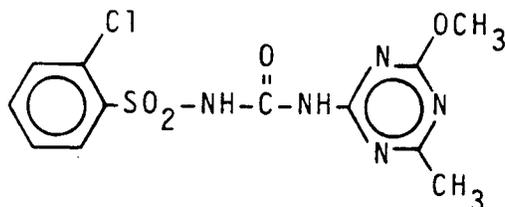
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DETERMINATION OF CHLORSULFURON IN MILK AND IN LIVER,
LEAN MUSCLE, AND KIDNEY TISSUE OF CATTLE

Robert V. Slates

INTRODUCTION

This report documents the analytical method for determination of chlorsulfuron in milk and in liver, lean muscle, and kidney tissue of cattle. Chlorsulfuron, formerly designated DPX-W4189, is the active ingredient in Du Pont GleanTM weedkiller which is used primarily for agricultural control of weeds in cereals. Chlorsulfuron has the following structure:



Chlorsulfuron

Chlorsulfuron is isolated from acidified milk samples by toluene extraction or from liver, lean muscle, and kidney samples by ethyl acetate extraction. Each kidney sample extract requires a preliminary cleanup by refrigeration to precipitate slightly soluble sample components followed by size-exclusion chromatographic separation to separate chlorsulfuron from sample components on a molecular size basis.

The extract for each type of sample is then evaporated to dryness. The resulting residue is redissolved in a two-phase system of CHCl_3 or n-hexane and an aqueous sodium chloride solution buffered to pH 10. Chlorsulfuron partitions into the aqueous phase which is washed several times with CHCl_3 , n-hexane, or cyclohexane to remove interfering sample components while retaining chlorsulfuron in the aqueous phase at pH 10. After the wash steps, the aqueous solution is acidified to pH 2, and chlorsulfuron is extracted into CHCl_3 . The CHCl_3 extract is evaporated to dryness, and the residue is redissolved in a mobile phase for determination of chlorsulfuron by normal-phase high performance liquid chromatography (HPLC) using a photoconductivity detector.

The detection limit of the method is 0.01 ppm. Recovery efficiencies for control samples spiked before sample preparation with known quantities of chlorsulfuron are in the range of 80% to 100% depending upon the type of sample analyzed.

APPARATUS AND REAGENTS

A Hobart commercial meat grinder (Hobart Manufacturing Company, Troy, Ohio) is used to grind tissue samples.

Waring Commercial Blenders[®] (Waring Products Corporation, New York, N.Y.) with 40 ounce Blendor[®] jars are used to homogenize samples during solvent extraction. These are operated through variable power transformers to provide speed control.

Millipore® 47-mm Teflon®-faced glass filters (Millipore Corporation, Bedford, Mass., Cat. No. XX 1004720) with 47-mm Millipore® Microfiber glass disk prefilters (Millipore Corporation, Cat. No. AP 4004705) are used with Fisher Filtrators® (Fisher Scientific Company, Pittsburgh, Pa., Cat. No. 9-788) for all filtrations.

Rotary evaporators with temperature-controlled water baths equivalent to Rotavapor R® (Fisher Scientific Company, Pittsburgh, Pa., Cat. No. 9-548-151) are used to evaporate and concentrate solutions. 1000-mL and 200-mL round-bottom evaporating flasks are used with the rotary evaporators.

Solvent extractions are performed with 125-mL separatory funnels with Teflon® plugs (Fisher Scientific Company, Pittsburgh, Pa., Cat. No. 10-437-10B).

Size-exclusion chromatographic separations are performed on an Autoprep® 1001 Instrument (Analytical Biochemistry Laboratories, Incorporated, Columbia, Mo.) using a 2.5 cm x 29 cm column of Bio-Beads® S-X3 styrene-divinylbenzene copolymer (Bio-Rad Laboratories, Richmond, Cal.).

An International centrifuge Model BE-50 manufactured by International Equipment Company, Boston, Mass. with 200-mL capacity thick-wall glass centrifuge bottles is used for centrifuging samples.

Chlorsulfuron analyses are performed on a Du Pont Model 850 HPLC instrument fitted with a 4.6 mm x 25 cm Du Pont Zorbax® Sil Column, a Tracor Model 965 photoconductivity detector with a mercury lamp, and a Hewlett Packard Model 3380A integrating

recorder. To permit accurate balancing of the mobile phase flows through the reference and analytical cells of the detector, a metering valve was installed on the reference cell discharge line. The ion exchange resin tube and the micropump of the detector were not used because deionization of the mobile phase is not necessary and because the resin could actually introduce interfering contaminants into the mobile phase.

MN-Universal Indikatorpapier (pH 1-11, Art. 90201) manufactured by Macherey-Nagel and distributed in the U.S.A. by Gallard-Schlesinger Chemical Manufacturing Corporation, Carle Place, N.Y. is used for solution pH determination.

Ethyl acetate, cyclohexane, methyl alcohol, isopropyl alcohol, chloroform*, methylene chloride, and toluene are "Distilled in Glass" quality manufactured by Burdick and Jackson Laboratories, Muskegon, Mich.

Acetic acid is "Reagent ACS" grade (Cat. No. A-38) manufactured by Fisher Scientific Company, Pittsburgh, Pa.

Sodium hydroxide solution (Cat. No. SO-S-272), hydrochloric acid solution (Cat. No. SO-A-48), granular Na_2HPO_4 (Cat. No. S-374), anhydrous Na_2SO_4 (Cat. No. S-421), and NaCl (Cat. No. S-271) are "certified" grade manufactured by Fisher Scientific Company, Pittsburgh, Pa.

* Note: Chloroform is classified as an A2 carcinogen by the American Conference of Governmental Industrial Hygienists. When working with chloroform, adequate ventilation should be provided and skin contact should be avoided by use of polyvinyl alcohol gloves.

The chlorsulfuron reference standard is synthesized and assayed in the Du Pont Biochemicals Department, Agrichemicals Research Division Laboratories.

The working standard of 1 $\mu\text{g}/\text{mL}$ chlorsulfuron in methylene chloride is prepared for spiking purposes by dilution of a 100- $\mu\text{g}/\text{mL}$ stock solution. Standards for HPLC analysis (0.20, 0.40, 0.60, and 0.80 $\mu\text{g}/\text{mL}$) are prepared by diluting a 1.0 $\mu\text{g}/\text{mL}$ standard of chlorsulfuron in HPLC mobile phase. The 1.0 $\mu\text{g}/\text{mL}$ standard of chlorsulfuron in HPLC mobile phase is prepared by pipeting 1 mL of the 100 $\mu\text{g}/\text{mL}$ stock standard into a 100-mL volumetric flask, evaporating the methylene chloride with a gentle stream of dry nitrogen or air, and making to volume with HPLC mobile phase.

The HPLC mobile phase consists of

750 mL	cyclohexane
125 mL	isopropyl alcohol
125 mL	methyl alcohol
1 mL	{ 10 mL glacial acetic acid
	{ 1 mL water

The HPLC column cleaning solution consists of 400 mL isopropyl alcohol, 100 mL glacial acetic acid, and 10 mL water.

The pH-10 buffer solution is prepared by dissolving 1.42 g anhydrous Na_2HPO_4 and 40.0 g NaCl in 400 mL water. Using a pH meter, the pH of the solution is adjusted to 10.0 by adding 1 M NaOH in small increments with stirring.

EXPERIMENTAL PROCEDURE

Sampling

To determine chlorsulfuron in milk, thoroughly mix the bulk milk sample to distribute the cream homogeneously. Weigh out a 50-g sample of the milk and transfer it to a centrifuge bottle. If the milk sample is to be a spiked control to determine recovery efficiency, pipet the required volume of a 1.0- $\mu\text{g}/\text{mL}$ standard solution of chlorsulfuron in CH_2Cl_2 into an empty centrifuge bottle, evaporate the CH_2Cl_2 with a gentle stream of dry air or nitrogen, then add the 50 g of milk, and mix thoroughly to dissolve chlorsulfuron.

For liver, lean muscle, or kidney, grind a representative bulk sample to the consistency of hamburger and mix the ground sample to ensure homogeneity. Weigh out a 50-g sample and transfer it to a blender. If the liver, lean muscle, or kidney sample is to be a spiked control, pipet the required volume of chlorsulfuron standard in CH_2Cl_2 onto the tissue in the blender and gently evaporate the CH_2Cl_2 with a gentle stream of dry air or nitrogen.

Sample Extraction

To extract chlorsulfuron from milk, acidify the milk to approximately pH 2 as determined by pH paper by adding 1.0 M HCl with stirring. Add 100 mL of toluene to the acidified milk, stopper the centrifuge bottle (a green rubber stopper is acceptable), shake moderately for three minutes, and centrifuge at

12000-15000 rpm to separate the phases. Draw off as much of the toluene phase as possible with a syringe and needle and transfer it through a glass wool plug to a 1000-mL round-bottom flask. Extract the acidified milk two more times with 100-mL quantities of toluene in a similar manner. Combine the toluene extracts and evaporate the toluene on a rotary evaporator at 40°C. A light-colored oily residue containing chlorsulfuron from the sample will remain.

During extraction of chlorsulfuron from the milk, the pH of the aqueous phase must be maintained at approximately 2. If the pH is significantly higher than 2, chlorsulfuron will not extract efficiently; if the pH is significantly lower than 2, chlorsulfuron may undergo acid hydrolysis.

Extract chlorsulfuron from liver, lean muscle, or kidney as follows: Add 50 g anhydrous Na_2SO_4 to each kidney sample to absorb water and to improve extraction and filtration. Na_2SO_4 need not be added to lean muscle or liver samples. Add 150 mL of ethyl acetate to each sample and blend at a moderate to high speed for three minutes. Let the sample solids settle to the bottom of the blender and then transfer most of the ethyl acetate extract from the blender to the filter by decanting. Retain the sample solids in the blender for subsequent extractions. Extract the tissue sample two more times with 150-mL quantities of ethyl acetate in a similar manner. On the third extraction, transfer all of the sample solids with the ethyl acetate to the filter and filter under vacuum into a 1000-mL round-bottom flask. Rinse the blender, filter, and sample solids with 25 mL of ethyl acetate.

Filter the rinse solution and combine it with the three ethyl acetate extracts. Discard the sample solids. For kidney samples, proceed to the "Kidney Preliminary Cleanup" section. For all other samples, evaporate the solvent from the sample extract on a rotary evaporator at 35°C until all of the ethyl acetate has been evaporated or until further evaporation of ethyl acetate from the oily residue is impractical. Be careful to not heat the samples excessively at or near dryness. Proceed to the "Solvent Partitioning Cleanup" section.

Kidney Preliminary Cleanup

Evaporate the ethyl acetate extract for each kidney sample to 50-60 mL on a rotary evaporator at 35°C and transfer the solution quantitatively to a 200-mL glass centrifuge bottle using additional ethyl acetate to complete transfer. Make final volume to 75 mL. Refrigerate at approximately 10°C for several hours or preferably overnight. During refrigeration, a white precipitate will form and will settle to the bottom of the centrifuge tube. Without disturbing the precipitate, remove as much of the solution as possible with a syringe and needle and transfer the solution to a round-bottom flask. The volume of the solution transferred to the round-bottom flask must be determined. This is conveniently done by weighing the bottle before and after removing the ethyl acetate. The difference in weight times the specific gravity of ethyl acetate (0.90) is the volume taken for analysis. Evaporate the sample solution in the round-bottom flask to 25 mL for size-exclusion chromatographic separation.

Use the 25 mL sample solution to load two adjacent 5-mL loops of the Autoprep® 1001 size-exclusion chromatography instrument. Process the contents of the two loops through the column using ethyl acetate mobile phase at 5.0 mL/min, and combine the collected fractions for the 2 loops. Set the dump, collect, and wash times for the Autoprep® to appropriate settings as determined by a recent calibration for chlorsulfuron. (For comparison, the dump, collect, and wash settings for the column currently in use in this laboratory are 15, 17, and 28 minutes respectively.) Procedures for column preparation, column calibration, and instrument operation are given in the manufacturer's literature. Upon completion of the size exclusion cleanup, evaporate the collected fractions to dryness and proceed to the "Solvent Partitioning Cleanup" section.

Solvent Partitioning Cleanup

For determination of chlorsulfuron in milk, add 25 mL of CHCl_3 to the sample residue after evaporation of all toluene and swirl to dissolve the oil. Then add 25 mL of the pH-10 buffer solution described in the "Apparatus and Reagents" section. Swirl to rinse the flask walls and transfer the mixture to a separatory funnel. Shake the separatory funnel moderately for 3 minutes to partition chlorsulfuron into the aqueous phase and interfering sample components into the CHCl_3 phase. Let the phases separate (centrifuge if necessary to break emulsions) and discard the CHCl_3 phase.

Add 5 mL of the pH-10 buffer solution and 25 mL of CHCl_3 to the flask which originally contained the sample. Rinse the flask thoroughly to recover residual chlorsulfuron and transfer the mixture to the separatory funnel. Shake the separatory funnel moderately for 3 minutes, let phases separate and discard the CHCl_3 phase. Wash the aqueous phase one more time with 25 mL of CHCl_3 for a total of three CHCl_3 washes.

After completing the three CHCl_3 washes, wash the aqueous phase two times with 25-mL quantities of cyclohexane in a manner similar to that used for the CHCl_3 washes. Discard the cyclohexane washes. Chlorsulfuron from the sample is retained in the aqueous phase.

For determination of chlorsulfuron in liver, lean muscle, and kidney samples, solvent partitioning cleanup is performed in centrifuge bottles because frequent centrifuging is required. Add 25 mL of n-hexane to the sample residue after evaporation of the ethyl acetate. Swirl until residue is dissolved or at least thoroughly broken into small particles. Add 25 mL of the pH-10 buffer solution described in the "Apparatus and Reagents" section. Swirl to rinse flask walls and transfer the mixture to a glass centrifuge bottle. Stopper the centrifuge bottle (a green rubber stopper is acceptable) and shake moderately for 3 minutes to partition chlorsulfuron into the aqueous phase and fats and oils into the n-hexane phase. Centrifuge at 12000-15000 rpm to separate the phases, then using a syringe and needle, remove and discard as much of the n-hexane phase as possible without disturbing any emulsion.

Add 5 mL of the pH-10 buffer solution and 25 mL of n-hexane to the original sample flask, swirl to recover residual chlorsulfuron, and add this mixture to the sample mixture in the centrifuge bottle. Shake 3 minutes, centrifuge, and again discard as much as possible of the n-hexane phase. Wash the aqueous phase two more times with 25 mL quantities of n-hexane. After completing four n-hexane washes, wash the aqueous phase two times with 25-mL quantities of CHCl_3 in a manner similar to that used for the n-hexane washes. Discard both CHCl_3 washes. Chlorsulfuron from the sample is retained in the aqueous phase.

NOTE: During the solvent partitioning cleanup for all samples, the aqueous phase must be maintained at approximately pH 10 as determined by frequent monitoring with pH paper. 1-M NaOH or 1-M HCl should be added as necessary with stirring to maintain the required pH. If the pH is significantly lower than 10, chlorsulfuron will be lost during the wash steps; if the pH is significantly higher than 10, basic hydrolysis of chlorsulfuron may occur.

After the solvent partitioning cleanup wash steps, the milk, liver, lean muscle, and kidney samples are all processed by the same procedure as follows: Acidify the aqueous phase to pH 2 as determined by pH paper by adding 1-M HCl with stirring. Add 25 mL of CHCl_3 , shake moderately for 3 minutes, and let phases separate (centrifuge if emulsions persist or if phases do not separate cleanly). Transfer the CHCl_3 extract to a round-bottom flask without taking any emulsion. Extract the acidified aqueous phase two more times with 25-mL quantities of CHCl_3 in a similar manner and combine the three CHCl_3 extracts.

Add approximately 5 drops of concentrated acetic acid to the combined CHCl_3 extracts and evaporate to dryness on a rotary evaporator at 35°C . Redissolve kidney sample residues in 2 mL of HPLC mobile phase for chlorsulfuron analysis. Redissolve milk, liver, and lean muscle sample residues in 5 mL of HPLC mobile phase for analysis.

HPLC Analysis

Chlorsulfuron is determined by high performance liquid chromatography using a photoconductivity detector by comparing the chromatographic peak heights for chlorsulfuron in the sample solution with the corresponding peak height for standard solutions containing known quantities of chlorsulfuron. The preparation of HPLC mobile phase, HPLC column cleaning solution, and chlorsulfuron standards is described in the "Apparatus and Reagents" section.

Care should be taken to properly condition and equilibrate the HPLC column before analysis. If the column is not properly conditioned, low sensitivity or rapidly drifting sensitivity may be experienced. Condition the column by pumping the HPLC cleaning solution through the column and detector at 0.5 mL/min for at least four hours but preferably overnight. Then pump the HPLC mobile phase through the column and detector for about two hours to establish equilibrium between the column and the mobile phase.

Analyze samples and standards alternately by HPLC using the following conditions:

Column:	Du Pont Zorbax® Sil, 4.6 mm x 25 cm
Column Oven Temperature:	35°C
Mobile Phase:	Composition given in "Apparatus and Reagent" section
Mobile Phase Flow-Rate:	1.0 mL/min
Injection Volume:	10 µl
Detector:	Tracor Model 965 photoconductivity
Retention Time:	7 min

At least one unspiked control sample and one control sample spiked with 0.50, 1.0, or 2.0 µg chlorsulfuron should be prepared with each batch of samples and analyzed at random intervals or at predetermined uniform intervals during a series of sample analyses. This will demonstrate the absence of interferences, provide a check on recovery efficiency, and confirm that the retention time in the sample matrix is consistent with that of the standards.

Calculations

Standard curves of chlorsulfuron peak height versus ng chlorsulfuron injected are linear with zero intercept for injections of at least 10 ng chlorsulfuron when analyzed under carefully controlled conditions. However, under typical sample analysis conditions, some drifting of the detector sensitivity is generally observed. This is conveniently accounted for by analyzing samples and standards alternately. Any drift in

sensitivity can be readily monitored, and an average sensitivity, M_0 (see page 15), can be used for each sample calculation.

Calculate ppm chlorsulfuron in milk, liver, and lean muscle samples by using Equation 1. Calculate ppm chlorsulfuron in kidney samples by using Equation 2. Calculate percent recovery for spiked control samples of milk, liver, and lean muscle by using Equation 3. Calculate percent recovery for spiked control samples of kidney by using Equation 4.

$$\begin{array}{l} \text{PPM chlorsulfuron} \\ \text{in milk, liver, or} \\ \text{lean muscle} \end{array} = \frac{\text{PK} \times \text{ATTEN} \times \text{VS}}{M_0 \times \text{VI} \times \text{SW}} \quad (1)$$

$$\begin{array}{l} \text{PPM chlorsulfuron} \\ \text{in kidney} \end{array} = \frac{\text{PK} \times \text{ATTEN} \times \text{VS} \times 25 \times 75}{M_0 \times \text{VI} \times 10 \times \text{VSAF} \times \text{SW}} \quad (2)$$

$$\begin{array}{l} \text{Percent recovery} \\ \text{from milk, liver,} \\ \text{or lean muscle} \end{array} = \frac{100 \times \text{PK} \times \text{ATTEN} \times \text{VS}}{M_0 \times \text{VI} \times \text{SP}} \quad (3)$$

$$\begin{array}{l} \text{Percent recovery} \\ \text{from kidney} \end{array} = \frac{100 \times \text{PK} \times \text{ATTEN} \times \text{VS} \times 25 \times 75}{M_0 \times \text{VI} \times 10 \times \text{VSAF} \times \text{SP}} \quad (4)$$

Variables in the above equation are defined as follows:

ATTEN = Detector attenuation setting

C = Concentration of chlorsulfuron standard in $\mu\text{g/mL}$

$M_o = \left[\frac{PK \times ATTEN}{2 \times C \times VI} \right]_{\text{Std before Sample}} + \left[\frac{PK \times ATTEN}{2 \times C \times VI} \right]_{\text{Std after Sample}} = \text{Average detector sensitivity in mm}/\mu\text{g}$

PK = Chlorsulfuron peak height in mm

SP = Weight of chlorsulfuron spiked onto sample in μg .

SW = Weight of sample in g

VI = Volume of sample solution injected in mL

VS = Volume of sample solution in mL

VSAF = Volume of kidney sample in mL that was removed from centrifuge bottle by syringe after refrigerating.