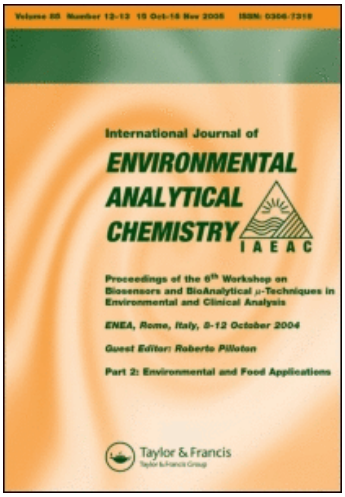

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A Method for Determination of Low Levels of Exposure to 2,4-D and 2,4,5-T

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A Method for Determination of Low Levels of Exposure to 2,4-D and 2,4,5-T

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A method has been developed for the determination of trace quantities of 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenol (2,4-DCP), and 2,4,5-trichlorophenol (2,4,5-TCP) in human and rat urine. The method involves acid hydrolysis of the phenolic conjugates, extraction of the free phenols and acids, ethylation with diazoethane, silica-gel column chromatography clean-up of the derivatized urine extract, and gas chromatographic determination using the electron-capture detector. The average recoveries of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP from rat urine spiked with known amounts of the herbicides and their phenols were 94%, 98%, 92%, and 90%, respectively. The limits of detection for 2,4-D, 2,4,5-T, DCP, and TCP in rat urine were: 0.05, 0.01, 0.10, and 0.01 ppm, respectively. The method was used to analyze urine of rats given various levels of 2,4-D and 2,4,5-T by gavage. Results showed that levels of exposure of 3.75 mcg/kg for 2,4-D and 5.0 mcg/kg for 2,4,5-T in rats can be detected in urine within 24 hr from exposure. Urine samples from occupationally exposed people were analyzed and found to contain 0.2 to 1.0 ppm 2,4-D and 0.05 to 3.6 ppm 2,4,5-T.

INTRODUCTION

A number of derivatives of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are applied extensively as selective herbicides in the control of terrestrial and aquatic broadleaf plants. Because

of the widespread use of these compounds and their relatively lengthy persistence, particularly in treated lakes and streams,[1] potential human exposure may occur via several routes. These include consumption of contaminated plants, livestock, and water, as well as the direct exposure of agricultural spraymen and herbicide formulators. For toxicological and environmental monitoring of these herbicidal compounds, a rapid, sensitive procedure is needed for the detection in human and animal urine of the free acids and the chlorinated phenol degradation products which result from exposure.

Several methods are described in the literature for the gas chromatographic determination of the methyl ester derivatives of 2,4-D and 2,4,5-T isolated as residues from a variety of natural materials.[2–6] Body tissues of sheep have been analyzed for 2,4-D by electron-capture gas chromatography of the free 2,4-dichlorophenol (2,4-DCP) formed by enzymatic hydrolysis of the herbicide residue.[7] In addition, 2,4,5-T residues derivatized as the methyl ester have been determined in sheep urine[6] by microcoulometric gas chromatographic analysis preceded by a Florisil column clean-up procedure. However, the data reported in both studies were based entirely on the analysis of fortified samples.

The purpose of this investigation was to develop a procedure for the simultaneous determination of trace quantities of 2,4-DCP, 2,4,5-trichlorophenol (2,4,5-TCP), 2,4-D, and 2,4,5-T in animal urine. The level of the free acids of 2,4-D and 2,4,5-T excreted in the urine of exposed animals can be used as an index of exposure to these herbicides.[8,9]

EXPERIMENTAL

Apparatus and equipment Micro-Tek gas chromatograph, model MT 220, with a tritium electron-capture detector operated under the following conditions: a glass column, 180 cm × 0.4 cm i.d., packed with 20% OV-101 on Gas-Chrom Q, 60/80 mesh; nitrogen carrier gas with a flow rate of 60 ml/min; column temperature 175°C; injection port 210°C; detector 210°C; transfer line 250°C. Another glass column, 180 cm × 0.4 cm i.d. packed with 4% SE-30 and 6% QF-1 or 4% SE-30 and 6% OV-210 on Chromosorb W, high performance, 80/100 mesh can be used at the same operating temperatures and flow rates mentioned above, where 2,4-D and 2,4,5-T will have shorter retention times, 7 and 12 minutes, respectively. The 20% OV-101 column can also be operated at 195°C, with a flow rate of 60 ml/min., to produce retention times of approximately 5 minutes for 2,4-D and 9 minutes for 2,4,5-T.

Chromatographic Columns Size 22, i.d. 7 mm, length 200 mm, Kontes No 420100.

Standards Analytical standards of 2,4-D, 2,4,5-T, 2,4-DCP, and 2,4,5-TCP were obtained from the Dow Chemical Co., Midland, Mich. The standards were used as received after the ethyl esters were checked for purity by gas chromatography.

Reagents Benzene and hexane, Mallinckrodt Nanograde solvent or equivalent. *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine, Aldrich Chemical Co., Milwaukee, Wis.

Silica gel, Woelm, activity grade I, Waters Associates, Inc., Framingham, Mass. Dry adsorbent for 48 hr at 170°C and store in a desiccator. On the day of use, deactivate 1 g of the silica gel by adding 1.5% by weight of water in the following manner. Add 15 mcl water and 1 g silica gel to a 125-ml Erlenmeyer flask. Stopper and rotate the flask until the water is evenly distributed throughout the adsorbent. Allow to equilibrate for 2 to 3 hr with periodic shaking. Prepare the chromatographic columns just before use.

Deionized or distilled water, extracted with benzene, is used throughout the procedure.

Preparation of ethylating reagent

Prepare diazoethane reagent [4] as follows: Dissolve 2.3 g potassium hydroxide in 2.3 ml water in a 125-ml Erlenmeyer flask and allow the solution to cool to room temperature. Add 25 ml hexane and cool the flask in a freezer for 15 min. In a well-ventilated hood, add 1.6 g *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine in small portions and mix the contents of the flask after each addition. Decant the hexane layer into a bottle with a Teflon-lined screw cap. The diazoalkane solution may be stored at −20°C for as long as one week. Because of their demonstrated carcinogenicity, do not allow the nitrosoguanidine or the diazoethane solution to come in contact with the skin. Avoid the use of ground glass-stoppered containers or etched glassware for storing or preparing the reagent.

Preparation of standard solutions

Prepare stock standard solutions of 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T in benzene by weighing 100 mg of each into separate 100-ml volumetric flasks. Dilute to volume with benzene. From these stock standards prepare an ethylated stock standard mixture by pipetting 2 ml 2,4-DCP, 0.1 ml 2,4,5-TCP, 0.5 ml 2,4-D, and 0.2 ml 2,4,5-T into a 50-ml volumetric flask. Add 15 ml benzene and then add diazoethane reagent, dropwise with a disposable pipet, until a definite yellow color persists. (Diazoethane reagent must be used in a well-ventilated hood.) Let stand 15 min; then bubble nitrogen through the solution until the yellow color disappears (5-10 min). Dilute to

volume with benzene. The ethylated stock standard mixture will contain, per milliliter, 40 mcg 2,4-DCP, 2 mcg 2,4,5-TCP, 10 mcg 2,4-D, and 4 mcg 2,4,5-T.

Preparation of standard curves

Pipet aliquots ranging from 0.05 to 1 ml from the ethylated stock standard mixture into separate 5-ml volumetric flasks. Dilute to volume with benzene. Five-microliter injections from these dilutions will contain 2.0–40.0 ng 2,4-DCP, 0.1–2.0 ng 2,4,5-TCP, 0.5–10.0 ng 2,4-D, and 0.2–4.0 ng 2,4,5-T. Separate calibration curves must be prepared for each compound. With an electrometer setting of 10×32 the following amounts will give approximately a 50% full-scale deflection on the recorder: 14 ng 2,4-DCP, 0.8 ng 2,4,5-TCP, 5 ng 2,4-D, and 1.7 ng 2,4,5-T. The sensitivity of the detector to the ethylated derivatives is 0.20 ng 2,4-DCP, 0.02 ng 2,4,5-TCP, 0.10 ng 2,4-D, and 0.04 ng 2,4,5-T.

Elution of ethylated 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T from silica gel

Determine the elution pattern of the four ethylated compounds before using the silica-gel column chromatography for clean-up of ethylated urine extracts. To a glass chromatographic column lightly plugged with glass wool and containing 1 g of the partially deactivated silica gel (1.5% water), add 10 ml hexane to prewash the column. When the last of the hexane has reached a point approximately 2 cm above the silica gel, add 0.3 ml of the ethylated standard stock mixture and begin collecting the eluate. Elute successively with 10 ml of each of the solvents listed in Table I, collecting each fraction separately. Inject 5 or 10 mcl from each fraction into the gas chromatograph and calculate the per cent of each compound present in the fraction. A typical elution pattern is shown in Table I.

TABLE I

Elution pattern of ethylated compounds from silica-gel column

Eluting solvents	2,4-DCP 20 mcg	2,4,5-TCP 1 mcg	2,4-D 5 mcg	2,4,5-T 2 mcg
20% Benzene–hexane	100%	100%	0	0
40% Benzene–hexane	0	0	0	0
60% Benzene–hexane	0	0	0–2%	20–25%
80% Benzene–hexane	0	0	98–100%	75–80%
100% Benzene	0	0	0	0

Analysis of urine

Pipet 1 to 5 ml (the actual volume may be determined by the anticipated residue level) of urine into a 25-ml concentrator rube. Add dropwise a volume of concentrated HCl equal to one-fifth the amount of urine, and mix well. Fit a stoppered reflux condenser to the tube and heat in a steam bath for 1 hr while cooling the condenser with circulating ice-water. Remove from the bath, cool, and rinse the sides and tip of the condenser with a total of 3 ml benzene. Mix contents of tube vigorously on a Vortex mixer for 1 min, then centrifuge, and transfer the benzene layer to a 15-ml centrifuge tube with a disposable pipet. Repeat the extraction with an additional 3-ml volume of benzene, adding the second benzene extract to the centrifuge tube. Add diazoethane dropwise with a disposable pipet until the yellow color persists. Let the solution stand 15 min; then bubble clean, dry nitrogen through the solution to remove the excess reagent. Concentrate the ethylated urine extract to 0.3 ml ($\pm$0.1 ml) under a gentle stream of nitrogen at 40°C in a water bath.

Prepare a silica-gel chromatographic column as previously described. Prewash the column with 10 ml hexane and discard the washing. When the hexane level reaches a point approximately 2 cm above the silica gel, transfer

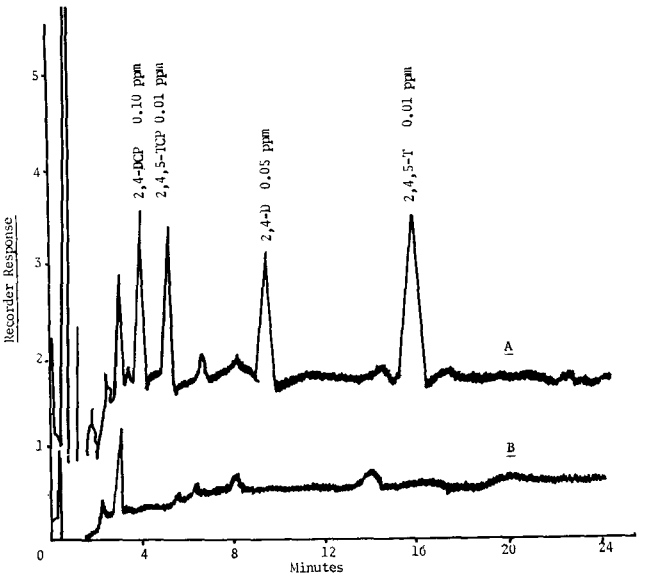

FIGURE 1 Representative chromatograms at a column temperature of 175°C. *A*, fortified control rat urine; *B*, control rat urine.

the concentrated urine extract to the column and begin elution with 10 ml of 20% benzene in hexane. This fraction contains ethylated 2,4-DCP and 2,4,5-TCP. Follow with 10 ml of 40% benzene-hexane and discard this fraction. Finally, add 10 ml of 60% benzene-hexane followed by 10 ml of 80% benzene-hexane and collect these fractions in a single tube. The combined 60% and 80% fraction contain ethylated 2,4-D and 2,4,5-T. Adjust the volume of each fraction to 10 ml with benzene. If the anticipated residue level is low, the fractions may be concentrated to 5 ml under a gentle stream of nitrogen at 40°C in a water bath. Inject into the gas chromatograph 5–10 mcl of the 20% fraction for the determination of the phenols and 5–10 mcl of the combined 60–80% fraction for the determination of the chlorophenoxyacetic acids. The elution pattern of the four compounds extracted from a fortified urine sample must be established and should be the same as previously indicated. The limits of detection for 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T are 0.10, 0.01, 0.05, and 0.01 ppm, respectively. A typical chromatogram is shown in Figure 1.

RESULTS AND DISCUSSION

The ethylated derivatives of 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T were selected because of their stability and minimal hydrolysis during sample manipulation and silica-gel column chromatography. The gas chromatographic resolution of the phenols having short retention times from those peaks which interfere with them was improved by ethylation as compared to the more commonly used methyl derivatization.

Hydrolysis conditions

Urine samples from rats which had been fed 2,4-D and 2,4,5-T (10^{-1} the LD_{50}) were analyzed to determine the optimum hydrolysis conditions for complete recovery of herbicide residues. The samples were acidified and extracted without heat hydrolysis, or were hydrolyzed in a steam bath for 10, 60, 90-min intervals and then extracted. The derivatives were prepared and cleaned up, and the herbicide residues were quantitatively determined by gas chromatography. Results are shown in Table II. The recoveries increased significantly when the samples were heated for 10 min, and then remained relatively constant up to the 90-min hydrolysis period. One hour was selected as the optimum time of heat hydrolysis because the extraction efficiency was maximum at this time. In addition, fewer emulsification problems during the extraction step were observed for samples hydrolyzed for 1 hr.

TABLE II

The effect of acid hydrolysis time on recovery of herbicide residues from rat urine[a]

Compound	Room temperature	100°C			
		10 min	30 min	60 min	90 min
2,4-D	10300	12500	12400	12700	12600
2,4,5-TCP	356	448	434	460	402
2,4,5-T	6350	7745	7550	8910	8010

[a]Rats were fed 10^{-1} the LD_{50}. Results are expressed in micrograms.

Recovery studies

Recovery studies were performed using fortified control rat urine. Five-milliliter samples or rat urine were spiked with different levels of 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T in aqueous NaOH. The samples were hydrolyzed with acid for 1 hr at 100°C, extracted, derivatized, cleaned up by silica-gel chromatography, and analyzed gas-chromatographically by the described procedure. The levels of fortification and recovery data for each compound are shown in Table III.

TABLE III

Recovery of 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T from 5 ml of fortified rat urine[a]

2,4-DCP		2,4,5-TCP		2,4-D		2,4,5-T	
Added mcg	Recovered %	Added mcg	Recovered %	Added mcg	Recovered %	Added mcg	Recovered %
20.0	89–91	1.0	91–95	5.0	84–88	2.0	104–107
10.0	94–96	0.5	85–90	2.5	96–98	1.0	97–99
4.0	87–89	0.2	85–86	1.0	92–96	0.4	93–97
2.0	94–96	0.1	91–94	0.5	97–99	0.2	94–96

[a]Average of three determinations.

Application of the method for the determination of low-level exposure to 2,4-D *and* 2,4,5-T

Male Sprague–Dawley rats weighing 420 to 470 g were dosed by gavage with 10^{-1}, 10^{-2}, 10^{-4}, and 10^{-5} of the LD_{50} of 2,4-D and 2,4,5-T. The LD_{50} of 2,4-D was considered to be 375 mg/kg and of 2,4,5-T, 500 mg/kg.[10] The doses were administered daily for 3 days to two rats at each dose level. The

animals were maintained in stainless steel metabolism cages (Acme Metal Products, Inc., Chicago, Ill.); the two rats given the same dose level were maintained in the same cage. The urine samples were collected in a manner which minimized fecal contamination before the exposure period and at timed intervals during and after exposure. The described procedure was used to determine 2,4-DCP, 2,4,5-TCP, 2,4-D, and 2,4,5-T in the urine, and the results were calculated in terms of micrograms excreted in 24 hr. The excretion rates of 2,4-D and 2,4,5-T are shown graphically in Figures 2 and 3. The total dosages and urinary excretion of the different levels of exposure for both 2,4-D and 2,4,5-T are shown in Table IV.

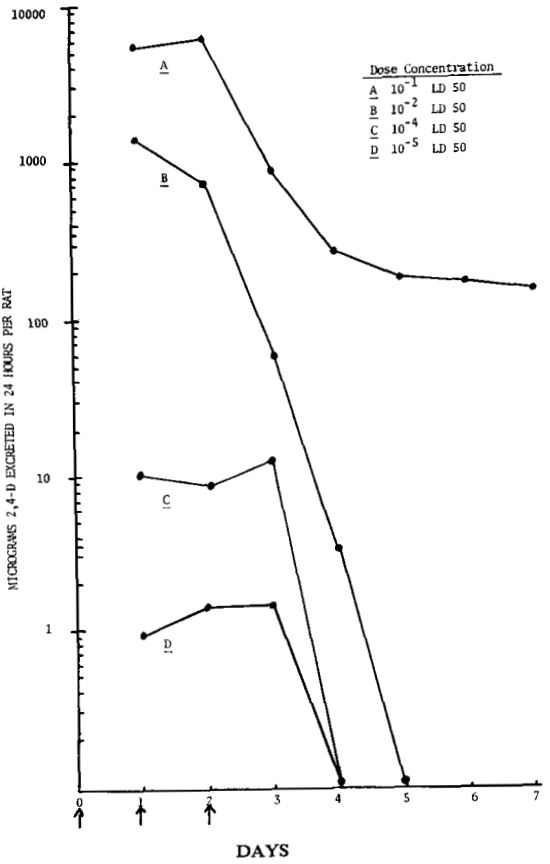

FIGURE 2 The rate of urinary excretion of 2,4-D from rats fed three consecutive doses. Arrows indicate time of dosage.

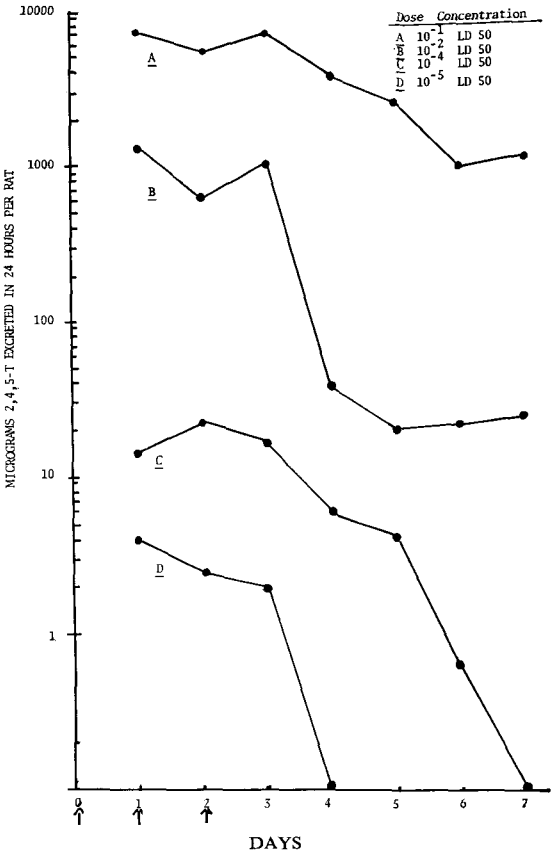

FIGURE 3 The rate of urinary excretion of 2,4,5-T from rats fed three consecutive doses. Arrows indicate time of dosage.

TABLE IV

The relation between total dosage and urinary excretion of 2,4-D and 2,4,5-T

Total dosage mg/rat		Total mg in urine/rat		No. of days for total excretion		Percent of total dose excreted in urine	
2,4-D	2,4,5-T	2,4-D	2,4,5-T	2,4-D	2,4,5-T	2,4-D	2,4,5-T
48.6	64.5	13.82	31.494	14	35	28	49
4.86	6.45	2.24	3.2135	4	12	46	50
0.0507	0.0645	0.0319	0.0570	3	6	63	88
0.00507	0.00645	0.0038	0.0065	3	3	75	100

The data in Figures 2 and 3 and Table IV show that the excretion rates of 2,4-D and 2,4,5-T were dependent on the dose administered. The per cent excretion decreased in rats fed the higher doses; this finding is in agreement with those of Clark *et al.*[8] and Khanna and Fang,[9] who reported similar results in their studies of the metabolism of 2,4-D. The excretion of 2,4-D and 2,4,5-T in urine of rats fed the lowest level (3.75 mcg/kg/day for 2,4-D; 5.00 mcg/kg/day for 2,4,5-T) was complete 2 days after the final dosage.

TABLE V

Results of analysis of urine from people occupationally exposed to 2,4-D and 2,4,5-T[a]

Sample	Exposure compound	Results (ppm)	
		2,4-D	2,4,5-T
Spray operator			
1	2,4,5-T	N.D.[b]	1.1
2	2,4,5-T	N.D.	2.8
3	2,4,5-T	N.D.	2.6
4	2,4,5-T	N.D.	3.6
5	2,4,5-T	N.D.	3.0
6	2,4,5-T	N.D.	1.3
Farmer			
7	2,4-D and 2,4,5-T	0.20	N.D.
8	2,4-D and 2,4,5-T	0.19	N.D.
Foreman, spray crew			
9	2,4,5-T	N.D.	1.2
10	2,4,5-T	N.D.	0.5
Herdsman			
11	2,4-D	N.D.	N.D.
12	2,4-D	N.D.	N.D.
Farm laborer			
13	2,4-D	N.D.	N.D.
14	2,4-D	N.D.	N.D.
15	2,4-D	N.D.	N.D.
16	2,4-D	N.D.	N.D.
Pesticide project officer			
17	2,4-D	N.D.	N.D.
18	2,4-D	N.D.	N.D.
Spray operator			
19	2,4-D	1.0	N.D.
20	2,4-D	0.2	N.D.
Aircraft spray operator			
21	2,4-D and 2,4,5-T	0.4	0.05
22	2,4-D and 2,4,5-T	1.0	0.05

[a]The derivative is unknown.

[b]N.D., not detected.

In all rats given 2,4-D, the major portion of the administered herbicide was excreted as the unchanged compound in the urine. Under the conditions of the described procedure it was not possible to detect the presence of metabolized residues. On the other hand, rats given 50 mg/kg of 2,4,5-T excreted at least three metabolites in addition to 2,4,5-T. 2,4,5-TCP was detected at high levels of exposure and represented about 0.5 to 9.0% of the 2,4,5-T excreted at a given time following initial dosage. The level of 2,4,5-TCP in the urine decreased more rapidly with time as compared with 2,4,5-T, and no detectable amounts of 2,4,5-TCP were found for the low levels of exposure (10^{-4} and 10^{-5} the LD_{50}), indicating that the determination of 2,4,5-TCP cannot be used as an index of exposure to low levels of 2,4,5-T. Mass spectrometric analysis of the two additional metabolites indicated that one is a hydroxylated trichlorophenoxyacetic acid and the other is a hydroxylated trichlorophenol. Further confirmation by comparison with authentic materials is required. The presence of 2,4-D, 2,4,5-T, and 2,4,5-TCP in rat urine was confirmed by gas chromatography, silica-gel column chromatography employing selective differential elution, and combined gas chromatography-mass spectrometry of the derivatized residues isolated from rat urine and compared with authentic compounds.

To demonstrate the applicability of the method for monitoring occupational exposure, urine from people directly and indirectly involved in the application of 2,4-D and 2,4,5-T derivatives was analyzed. The results, as shown in Table V, indicate a higher degree of exposure for spray operators than those whose occupations afforded less direct contact.

It can be generally concluded that the method suggested for the determination of 2,4-D and 2,4,5-T in urine may be used to determine low levels of exposure to these herbicides. Levels of exposure of 3.75 mcg/kg for 2,4-D and 5.00 mcg/kg for 2,4,5-T in rats can be determined in urine within 24 hr from exposure.

References

1. O. M. Aly and S. D. Faust, *J. Agr. Food Chem.* **12**, 541 (1964).
2. G. Yip, *J. Ass. Offic. Anal. Chem.* **47**, 343 (1964).
3. P. L. Pursley and E. D. Schall, *J. Ass. Offic. Anal. Chem.* **48**, 327 (1965).
4. C. W. Stanley, *J. Agr. Food Chem.* **14**, 321 (1966).
5. W. L. Zielinski, Jr. and L. Fishbein, *J. Agr. Food Chem.* **15**, 841 (1967).
6. D. E. Clark, *J. Agr. Food Chem.* **17**, 1168 (1969).
7. D. E. Clark, F. C. Wright, and L. M. Hunt, *J. Agr. Food Chem.* **15**, 171 (1967).
8. D. E. Clark, J. E. Young, R. L. Younger, L. M. Hunt, and J. K. McLaran, *J. Agr. Food Chem.* **12**, 43 (1964).
9. S. Khanna and S. C. Fang, *J. Agr. Food Chem.* **14**, 500 (1966).
10. W. J. Hayes, *Clinical Handbook on Economic Poisons*, Public Health Publication No. 476 (1964) p. 107.