Acta vet. scand. 1966, 7, 77—96.

From the National Veterinary Institute, Stockholm, Sweden.

DETERMINATION OF PHENOXYACETIC HERBICIDE RESIDUES IN BIOLOGICAL MATERIALS*)

By
Kurt Erne

The wide-spread and increasing use of chlorinated phenoxy-aliphatic acids as herbicides and growth regulators in agriculture, horticulture and forestry has created a need for adequate methods of detecting and determining residues of such compounds in a variety of biological materials — a need intensified by the growing interest in environmental factors as possible health hazards to man and animals.

The most widely used of the phenoxy herbicides are 2,4-dichloro-, 4-chloro-2-methyl- and 2,4,5-trichlorophenoxyacetic acids (in the following abbreviated 2,4-D, MCPA and 2,4,5-T, respectively), but recently also the analogous derivatives of 2-phenoxypropionic acid (2,4-DP (dichlorprop), MCPP (mecoprop) and 2,4,5-TP) and of 4-phenoxybutyric acid (2,4-DB, MCPB and 2,4,5-TB) have gained some importance. In formulations these compounds occur as the free acids or as salts with alkali metals or amines or as esters.

Published methods for analysis of phenoxy herbicides in biological materials are based on either photometric determination of a suitable coloured derivative, or spectrophotometry in the ultraviolet, or gas chromatography.

The colour reaction with chromotropic acid, originated by *Freed* (1948), was applied by *Marquardt & Luce* (1951, 1955) for determining 2,4-D in milk and in grain. Extractives inter-

*) This investigation was supported by grants from "Albert Hjärrefonden" and from "Knut och Alice Wallenbergs Stiftelse".

fering with the colour development were removed by deproteination with phosphotungstic acid, liquid-liquid extraction and — in the method for grain — column chromatography. Other methods, based on the chromotropic acid reaction in conjunction with a column chromatographic "clean up", were described by *Daoud & Luh* (1962) and *Coakley et al.* (1964) for residue determination of 2,4,5-T in apricots and of 2,4-D in shellfish, respectively. Another photometric residue method for agricultural products, developed by *Marquardt & Luce* (1961), involves cleavage of the phenol ether linkage with pyridine chloride and subsequent photometric determination of the phenol using 4-amino-antipyrine. *Kuznetsov & Gagarina* (1962) utilized the formation of an extractable complex with rhodamine B for a photometric determination of phenoxy acids, separated from plant material by paper chromatography.

Ultraviolet spectrophotometry was employed for residue determination of 2,4-D in soil extracts, purified by counter-current distribution, by *Warshowsky & Schantz* (1950) and in forensic material by *Nielsen et al.* (1965).

Gas chromatographic residue methods for phenoxy herbicides in plant material have been described by, among others, *Erickson & Hield* (1962), *Yip* (1962) *Bevenue et al.* (1962, 1963), *Gutenmann & Lisk* (1963).

In a previous study of phenoxy herbicide residues in natural waters, a reproducible and sensitive method, based on the chromotropic acid reaction, was developed (*Erne* 1963). This procedure, supplemented with "clean up" steps including solvent partitioning and thin-layer chromatography, forms the basis of the residue method for phenoxyacetic herbicides in biological materials described below. The method is applicable to widely differing types of materials, such as animal tissues, body fluids, foods, feeds and soils.

MATERIAL AND METHODS

Principle

The sample is extracted at low pH with an organic solvent, and phenoxy acids are separated from neutral and weakly acidic extractives by partitioning between solvent and aqueous buffer of pH 6.2. Remaining extractives are separated and the phenoxy acids identified by thin-layer chromatography. Quantitative data

are obtained by elution of the phenoxy acids from the adsorbent and photometric determination applying the modified chromotropic acid technique, as previously described (*Erne* 1963).

Reagents and apparatus

All reagents should be of analytical grade, unless otherwise stated.

Celite, Hyflo Super-cel.

2-Propanol, isopropyl alcohol.

Acetone.

Ethanol.

Ethyl ether.

Benzene.

Phosphate buffer, pH 6.2. Mix one volume of 0.25 M disodium hydrogen phosphate with three volumes of 0.25 M potassium dihydrogen phosphate. Saturate with chloroform.

Silica gel G, obtainable from E. Merck, Darmstadt.

Kieselguhr G, „ „ „ „ „

Ethyl acetate.

n-Hexane, min. 95 %, boiling range 68—69°C.

Formic acid, 98—100 %.

Standard solutions of phenoxyacetic acid derivatives. Freshly prepared solutions of 2,4-D, MCPA and 2,4,5-T in ethyl acetate, containing 1 μg per μl.

Silver-phenoxyethanol reagent. Dissolve 0.4 g of silver nitrate in 5 ml of water, add 20 ml of 2-phenoxyethanol (commercial grade will suffice) and dilute with acetone to 200 ml. Add one drop of 30 % hydrogen peroxide and keep in a glass-stoppered vessel protected from light.

Methylumbelliferone reagent. 0.02 % (w/v) solution of 4-methylumbelliferone in 30 % ethanol, adjusted to pH about 8 by means of a few drops of ammonia.

Chromotropic acid reagent. Dissolve 0.40 g of chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulphonic acid, disodium salt, Merck) in 1 ml of distilled water by warming slightly and then cautiously add, while cooling, 100 ml of concentrated sulphuric acid (95—97 % w/v). Keep in a glass-stoppered vessel protected from light. Discard when distinctly discoloured.

High-speed blender. Preferably of the top-drive type, e.g. M.S.E. Homogeniser (Measuring & Scientific Equipment, London).

Concentrating tubes. Test tubes of approximately 25 ml volume, with the lower end narrowing off to form an ampoule of 0.5 ml capacity graduated to 5/100 of a ml (*Erne* 1958) or, alternatively, conical graduated centrifuge tubes.

Thin-layer spreading apparatus. Any type, suitable for preparing chromatoplates with a layer thickness of about 0.3 mm. The Camag (Muttenz, Switzerland) apparatus has been found suitable.

Glass plates, 10×20 cm or 20×20 cm.

Developing tank.

Test tubes, borosilicate glass, 16×160 mm.

Glass filter tubes, medium porosity, e. g. 12 g 3, Schott & Gen., Mainz.

Ultraviolet light source. Any source giving intense mercury radiation. A Hanau analysis mercury lamp with a Q 81 quartz tube and a detachable filter transmissible to the long-wave mercury radiation (366 mμ) has proved useful.

Procedure

1. *Extraction*

Animal tissues and ingesta. Tissues should be minced before analysis. Mix 25 g of sample with 2 ml of 5 M sulphuric acid, add c. 75 ml of 2-propanol and homogenize for 5 min. in a high speed blender. Stir in a few g of Celite and filter the mixture through paper under reduced pressure, rinse the blender cup and the filter with a little 2-propanol, return the filter cake to the blender cup and homogenize with further c. 50 ml of 2-propanol for 1 min. at high speed, filter again and rinse the cup and filter with two further portions of the alcohol. Combine the filtrates, add 5 ml of 5 M sodium hydroxide and evaporate cautiously on a water bath to 20—30 ml.

Blood. To 25 ml of serum, plasma or whole blood in a glass-stoppered flask add, while swirling the sample, 75 ml of acetone and then 1 ml of concentrated phosphoric acid. Stopper the flask, shake vigorously for a few min., let stand for 5—10 min. and filter off the precipitated proteins through a wet filter. Rinse the flask and the residue on the filter with three 10-ml portions of 75 % aqueous acetone. Evaporate the combined filtrates cautiously on a water bath to 20—30 ml, add 10 ml of 5 M sodium hydroxide and continue the heating for 15 min..

Milk. Can be treated as described for blood.

Urine. To 25 ml of sample add 3 ml of 5 M sulphuric acid and boil under reflux for 30 min. Without filtering transfer the mixture with 25 ml of water to a separatory funnel and extract three times with 20-ml portions of chloroform. Filter the extracts through a dry filter and then proceed as described in the next section (2. *Liquid-liquid extraction*), beginning with "Extract the combined ...".

Eggs. Fresh eggs should be boiled for 10 min. before analysis and the contents forced through a coarse sieve and mixed. Mix 25 g of sample with a few g of Celite, add 2 ml of 5 M sulphuric acid and c. 100 ml of 75 % aqueous acetone, transfer to a glass-stoppered flask and shake mechanically for 30 min.. Decant through a filter and repeat the extraction by shaking with 50 ml of 75 % aqueous acetone for 15 min., filter through the same filter and rinse the flask and the filter with two further portions (10 ml each) of solvent. Add 5 ml of 5 M sodium hydroxide to the combined filtrates and evaporate cautiously on a water bath to about 20—30 ml.

Vegetable materials. a) *Materials of high moisture content* (silage, green crops, etc.). In a glass-stoppered flask mix 25 g of chopped sample with 2 ml of 5 M sulphuric acid and c. 100 ml of 2-propanol and shake mechanically for 30 min.. Decant through a filter and repeat the extraction by shaking with c. 50 ml of 2-propanol for 15 min., filter through the same filter and rinse the flask and filter with two further portions (10 ml each) of solvent. Add 5 ml of 5 M sodium hydroxide to the combined filtrates and evaporate carefully to about 20—30 ml.

b) *Materials of low moisture content* (grain, seed, hay, etc.). To 10 g of finely ground sample in a glass-stoppered flask add 15—20 ml of water and mix until the water has been evenly distributed throughout the sample, add 2 ml of 5 M sulphuric acid and c. 100 ml of 2-propanol and shake mechanically for 30 min., then continue as directed under a), beginning with "Decant through a filter ...".

Soil. In a glass-stoppered flask mix 25 g of sample with 25 ml of water, add 2 ml of 5 M sulphuric acid and c. 75 ml of 2-propanol and shake mechanically for 30 min.. Centrifuge at about 2000 r.p.m. for 5—10 min.. Decant the supernatant through a filter. Suspend the sediment in 30 ml of 2-propanol, stir intermittently for several min., centrifuge again and decant through the same filter. Repeat the extraction, centrifugation and decantation with further 30 ml of solvent. Add 5 ml of 5 M sodium hydroxide to the combined filtrates and evaporate cautiously on a water bath to 20—30 ml.

2. *Liquid-liquid extraction*

Transfer the concentrated alkaline extracts, obtained according to the preceding section, with 150 ml of water to a separating funnel, acidify with 5 M sulphuric acid to pH below 2, add 5 ml of 25 % sodium chloride and extract the mixture with benzene, first 25 ml and then two 20-ml portions. (With blood extracts chloroform can be substituted for benzene in this extraction.) Filter the combined benzene, or chloroform, extracts through anhydrous sodium sulphate.

Then extract the combined extracts with three successive 20-ml portions of phosphate buffer pH 6.2, shaking cautiously for 1 min. each time. Any emulsion forming in the buffer phase usually breaks up spontaneously in a short time or after the addition of a little 25 % sodium chloride. Filter the combined buffer extracts through a wet filter, wash them by shaking in a separatory funnel with two 5-ml portions of chloroform, then adjust the pH to 2 with 5 M sulphuric acid and extract with three successive 15-ml portions of chloroform. Filter the chloroform extracts through a little anhydrous sodium sulphate, rinse the filter with several small portions of chloroform and evaporate the filtrate cautiously to about 5 ml. Quantitatively transfer the extract to a graduated concentrating tube and continue the evaporation at 30—40°C in a weak current of air to about 0.1 ml. Rinse down the sides of the tube with a little chloroform and adjust to an appropriate small volume. Use aliquots of this solution for thin-layer chromatography.

3. *Thin-layer chromatography*

Using standard equipment prepare chromatoplates with a coating of Silica gel G and Kieselguhr G in the proportion 3:2 with a wet layer thickness of 0.30 mm, and allow the plates to dry at room temperature for 30 min.. Plates which are to be treated with the silver reagent should be developed with distilled water and again dried at room temperature for 15 min.. Activate the plates at 110°C for 1 hr. and store in a desiccator.

With the aid of a micropipette apply the sample solutions 2.5 cm from the bottom edge of a washed plate, as spots at least 1.5 cm apart and 2 cm from the lateral edges. With extracts of tissues and other solid samples it has proved practical to apply aliquots equivalent to 1 g and 3 g of each sample. With samples low in extractives the aliquots often may be increased considerably. In addition, pure standards and extracts with added standards should be spotted on each plate.

Add the developing solvent, a mixture of ethyl acetate, n-hexane, formic acid, in the volume ratio 20 : 80 : 0.3, to the tank to a depth of 1 cm, line the tank with thick filter paper and allow to equilibrate for at least 30 min.. Insert the plate in the tank and develop until the solvent front has reached a line marked 10 cm above the origin (about 15 min.), remove the plate from the tank and, after 1—2 min. in open air, return it to the same tank and develop for the second time. After drying in a hood for 1 hour place the plate for several seconds in the steam from a water bath, dry in air for a few minutes, then spray with the silver-phenoxyethanol reagent and immediately expose to strong, unfiltered, ultraviolet radiation from the mercury lamp mounted about 10 cm above the plate, until the spots are fully developed (5—10 min.). Dark spots in the R_F-region 0.3—0.4, usually appearing after about 2—3 min. of exposure, indicate that chlorophenoxyacetic acids may be present. (Phenoxypropionic and -butyric acids appear in the region 0.4—0.5.) The spot area permits a rough estimate of the amount present. A negative silver test conclusively proves the absence of chlorinated acids. The test is sensitive to about 0.1 μg of the common chlorophenoxy acids.

(*Note.* During the chromatographic work the plate must be protected from vapours of halogen compounds, hydrogen sulphide and other silver-reactive agents.)

If spots appear, prepare a duplicate chromatoplate for the quantitative determination. (In this case pre-washing is not needed.) Of each sample solution apply two aliquots estimated with the aid of the first chromatogram to contain 10—50 μg of phenoxy acid, and also one or more spots of the appropriate standard. Develop the chromatogram twice in the manner described above, dry in a hood for 1 hour, steam for a few seconds, spray with the methylumbelliferone reagent and locate the phenoxy acids to be eluted by observing the chromatogram in filtered long-wave ultraviolet light (366 mμ). The phenoxy acids, and certain other acidic compounds, appear as bluish-white fluorescent spots against a dark background.

4. *Elution and photometric determination*

Scrape off the adsorbent from the relevant areas of the plate and also from a similarly located area containing no phenoxy acid, for the reagent blank, transfer quantitatively to small glass-filter tubes of medium porosity and elute the phenoxy acids by percolating with methanol containing 1 % of concentrated ammonia, first 1 ml and then three 0.5-ml volumes. Allow the first solvent portion to penetrate the adsorbent for 2 min. before applying suction. Collect the eluates in test tubes (16×160 mm) and remove the solvent completely at 30—40°C in a slow current of air.

Pipette 5.00 ml of chromotropic acid reagent into each of the tubes containing eluted samples, standard and reagent blank, respectively, rotate the tubes to dissolve the residue, place them in a rack and heat in a thermostatically controlled oven at 150 ± 2°C for 25 ± 1 min. and then cool to room temperature in water. At this stage the colour is stable for several hours. Measure the absorbance of the solutions by means of a recording spectrophotometer, in 1-cm cells, over the range 500—660 mμ (or — if a recording spectrophotometer is not available — at the wevelengths 505, 580 and 655 mμ) against the reagent blank, and calculate for each solution a base-line absorbance, A_{corr}, by means of the formula:

$$A_{corr} = A_{580} - 0.5\ (A_{505} + A_{655})$$

By means of a calibration graph, relating A_{corr} to concentration of phenoxy derivative and prepared by carrying the appropriate standard through the described procedure, starting with the thin-layer chromatographic step, calculate the phenoxyacetic acid content of the sample. Use the result obtained with the simultaneously run standard to check the calibration graph.

RESULTS AND DISCUSSION

Photometric determination

The details of the photometric procedure are based on a previous study of the chromotropic acid colour reaction (*Erne* 1963). The base-line technique then employed proved applicable also to extracts of biological materials, purified according to the present method, the background absorption over the range 500—660 mμ consistently being only faint and essentially linear (*cf.* Sample blank, below).

Chromatographic separation

The paper-chromatographic technique developed for the analysis of water (*Erne* 1963) can be applied to extracts of biological materials, but like other paper-chromatographic methods it is sensitive to interference from extractives. In a search for an

alternative technique thin-layer chromatography was tried. (Since the completion of the present work the application of this technique to the separation and determination of phenoxy herbicides has been described by *Abbott et al.* 1964). Preliminary experiments indicated a satisfactory resolution to be obtainable with silica gel and mixed solvents containing a light hydrocarbon and a polar component, and acid as an ionization depressant. A mixture of silica gel with kieselguhr showed an enhanced resolving power, and the chromatographic system finally adopted had the composition, Silica gel G, Kieselguhr G (3 : 2)/ ethyl acetate, n-hexane, formic acid (20 : 80 : 0.3, by volume). In this system the phenoxyacetic acids travelled as dense spots, as a rule distinctly separated from any extractives. In rare instances, with heavily contaminated extracts, the resolution was unsatisfactory. This proved to be easily remedied, however, by employing a multiple development technique, and since the time expense for

Table 1. Chromatographic mobility of phenoxy acids (and some other halo acids and phenols) in the system, Silica gel G—Kieselguhr G (3:2)/ethyl acetate—n-hexane—formic acid (20:80:0.3, by volume), with a double development. Values given are means of four runs.

Compound	$R_F \times 100$	R_D *)
Trichloroacetic acid	13	0.46 ± 0.02
2,4-D	28	1.00
MCPA	35	1.25 ± 0.02
2,4,5-T	37	1.31 ± 0.01
Monochloroacetic acid	38	1.35 ± 0.02
2,2-Dichloropropionic acid	40	1.44 ± 0.03
MCPB	41	1.47 ± 0.02
Dichlorprop (2,4-DP)	43	1.54 ± 0.03
2,2-Dichlorobutyric acid	45	1.60 ± 0.03
Mecoprop (MCPP)	49	1.77 ± 0.03
2,3,6-Trichlorobenzoic acid	50	1.80 ± 0.05
	59	2.10 ± 0.06
2,4-Dichlorophenol	65	2.35 ± 0.04
2,4,5-Trichlorophenol	70	2.50 ± 0.05
2-Methyl-4,6-dinitrophenol (DNOC)	73	2.60 ± 0.03
Pentachlorophenol	81	2.90 ± 0.03
2-(1-Methylpropyl)-4,6-dinitrophenol (dinoseb)	86	3.05 ± 0.04
2,4-D butylester	99	3.5 ± 0.02

*) Mobility relative to 2,4-D

each additional run was only about 10 min., a double development was included in the standard procedure. The mobilities of the phenoxyacetic acids and some other herbicides and related compounds in the system described are recorded in Table 1.

As is seen, the phenoxyacetic acids travelled as a group (R_F values about 0.3—0.4) relatively well separated from the other compounds, although the resolution within the group usually was sufficient for permitting a differentiation of the acids. Phenoxypropionic and -butyric acids moved somewhat more rapidly, the phenolic compounds even more and the 2,4-D ester, in accordance with the weakly polar character of carboxylic esters, with the solvent front. Extractives rarely moved beyond R_F 0.2.

The relative chromatographic mobilities (R_D values) showed a good reproducibility.

The adsorbent had a high tolerance for extractives; with tissue extracts, purified according to the procedure given, aliquots equivalent to as much as 5 g of sample could usually be applied without overloading the chromatogram.

For detecting the chlorophenoxy acids on the plates the silver-phenoxyethanol reagent of *Mitchell* (1958) proved excellent. Reducing the silver concentration of the reagent, to about 0.01—0.02 M, was found helpful, however, in enhancing the contrast between spots and background, as naturally did preliminary washing of the plate. Furthermore, the removal of the acid of the developing solvent proved essential for attaining the full sensitivity of the test. Performed as directed in "Procedure", the silver reaction thus could detect quantities as small as 0.1 μg of the phenoxyacetic acids and of the propionic and butyric acid analogues.

For locating the spots to be eluted in the quantitative determination, the methylumbelliferone reagent, being non-destructive and non-interfering with the photometric determination could be applied successfully. The phenoxy acids appeared in long-wave ultraviolet light as white-bluish fluorescent spots against a dark background, and, provided the excess of developing acid was removed, 1—2 μg of phenoxy acids were detectable.

Elution

An effective elution of the phenoxy acids from the adsorbent after removal from the plate was obtainable by repeated leaching with solvent and centrifugation, as well as by percolation with

solvent in a glass filter tube. The last mentioned technique, being considered the most convenient, was studied quantitatively. Thirty-μg amounts of 2,4-D were chromatographed, eluted in glass filter tubes with ammoniacal methanol and then determined photometrically by the chromotropic acid reaction as directed in "Procedure". The recovery (calculated on unchromatographed standard) in 20 replicate determinations was 93 %, with a standard deviation of 2.9. The reagent blank, obtained under identical conditions, corresponded to about 0.10 μg of apparent 2,4-D.

Liquid-liquid extraction

Since chlorophenoxyacetic acids are relatively strong acids, the pk_a values for 2,4-D, MCPA and 2,4,5-T being of the order of 3, separation from more weakly acidic materials conceivably could be achieved by extraction from organic into aqueous phase at relatively low pH values. Accordingly, the distribution of the phenoxy acids between organic solvents and aqueous buffers was examined. The organic phase, being approximately 0.3 mM with respect to phenoxy acid and saturated with the aqueous phase, was shaken for 10 min. at 20°C in a separatory funnel with an equal volume of 0.1 M buffer (saturated with the organic phase). The buffers were either phosphate, phosphate-citrate or borate buffers. The phenoxy acid content or the organic phase was determined, by ultraviolet spectrophotometry at the absorption maximum in the 280—290 mμ region, before and after the partitioning, and the partition ratio, D (total concentration in organic phase / total concentration in aqueous phase), was calculated (Table 2). The last column of the table also gives the partition coefficients, K_d, of the undissociated acids, calculated by means of the formula:

$$\log K_d = \log D + pH + \log [(h) + K_a],$$

where (h) denotes the hydrogen ion activity and K_a the apparent dissociation constant of the acid. The calculations have been based on D-values in the range 0.3—3, being the most accurate ones. The degree of dimerization of the acids in the organic phase has been considered negligible.

On the basis of the tabulated data, conditions for a quantitative extraction of the acids into the phases studied were calculated, and schemes of consecutive liquid-liquid extractions devised. Then, the procedures were applied to extracts of biological

Table 2. Partition ratios, D, and partition coefficients, K_d, of phenoxy acids in various systems.

Phenoxy acid	pK_a	Solvent	pH of aqueous phase	D	log K_d
2,4-D	2.90[1])	Chloroform	5.40	0.12	
			4.82	0.31	
			4.20	0.88	1.3 ± 0.1
			3.61	3.25	
			2.85	7.9	
		Chloroform--ether (3+1)	5.40	0.53	
			4.20	5.0	2.1 ± 0.1
		Ether	6.05	0.31	
			5.40	1.10	2.5 ± 0.1
			4.20	10.2	
		Dichloro-methane	4.82	0.44	
			4.20	1.53	1.4 ± 0.1
			3.61	4.6	
		Benzene	4.20	0.38	
			3.61	1.24	0.9 ± 0.05
			2.85	4.7	
		Benzene Aq-prop.	(1+1)[3])	7.1	
		„	(1+2)[3])	17	
		„	(1+4)[3])	21	
MCPA	3.11[1])	Chloroform	5.40	0.20	
			4.82	0.75	
			4.20	2.23	1.5 ± 0.1
			3.61	7.5	
			2.85	25	
2,4,5-T	2.83[1])	Chloroform	6.05	0.10	
			5.40	0.32	
			4.82	1.10	2.0 ± 0.1
			4.20	3.60	
			3.61	9.7	
MCPP	3.0[2])	Chloroform	6.31	0.10	
			5.40	0.59	
			4.82	2.40	c. 2,2
			4.20	7.1	
			3.61	21	
MCPB	4.5[2])	Chloroform	10.1	0.37	
			9.05	2.34	c. 5
			8.20	7.6	

[1]) *Matell & Lindenfors* (1957)

[2]) Estimates based on the measurements of *Phillips* (1958) and *Behrens & Morton* (1963)

[3]) Aqueous solutions of 2-propanol 0.05 M in sulphuric acid

materials and compared as to selectivity (by thin-layer chromatography and u.v.-measurement) and practicability (ease of phase separation, etc.). The finally adopted scheme involves transfer of the phenoxy acids from aqueous phase of pH 2 into benzene, then into aqueous phase of pH 6.2 and (after adjusting pH to 2) into chloroform. (With sample extracts low in extractives benzene may be replaced by chloroform.) In model experiments with phenoxy acids carried through the steps of this scheme, the details following "Procedure", and then determined by ultraviolet spectrophotometry of the chloroform extracts, overall recoveries of 95 % or higher were obtained with the three phenoxyacetic acids, as well as with the propionic acid analogue. The butyric acid analogue required a buffer pH of about 10 for a similar recovery to be obtained.

Extraction

In preliminary extraction experiments with liver tissue, with added 2,4-D, both repeated stripping with solvent, Soxhlet extraction and solvent homogenizing techniques showed some promise. (Homogenizing of the tissue with aqueous buffer and subsequent solvent extraction gave poor yields.) Solvent homogenization, being the most rapid technique, as well as technically simple, was selected for further study. Thus, liver samples with added 2,4-D were homogenized with solvents of different polarity, the purity of the extracts was examined by thin-layer chromatography and the recovery of 2,4-D was determined photometrically according to "Procedure". Yields from 70 % and upwards were obtained with polar solvents, such as the lower alcohols, ketones and acetonitrile. With less polar solvents there was an objectionable co-extraction of lipid matter, and the recovery of the phenoxy derivatives often was unsatisfactory. 2-Propanol was considered most promising of the solvents tried because of a certain selectivity and a minimum tendency of the extract to form emulsions during the subsequent steps of the procedure.

The course of the extraction of animal tissues was studied by repeatedly extracting a liver sample, with 2,4-D added, with fresh portions of 2-propanol and analyzing the ensuing extracts according to "Procedure". An analogous experiment was performed under physiological conditions with liver from a pig given 2,4-D orally (Fig. 1).

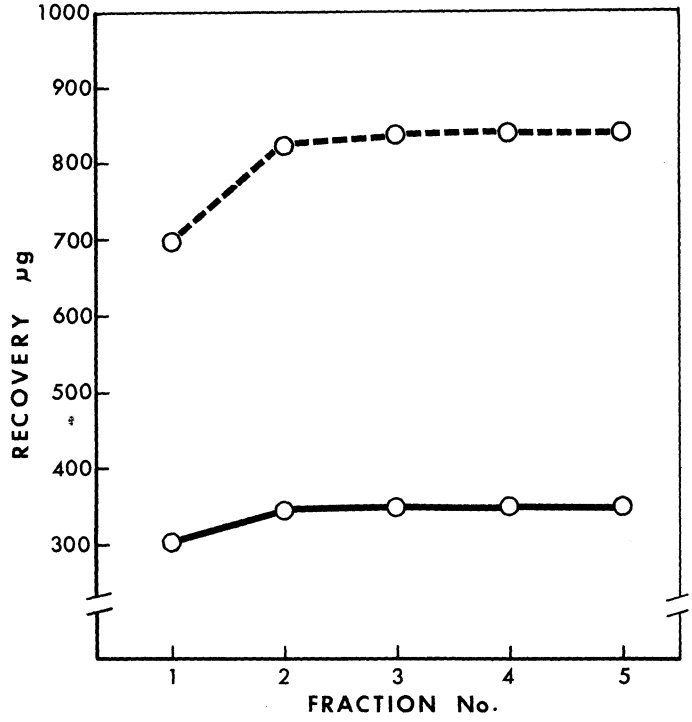

Figure 1. Cumulative recovery of 2,4-D from liver tissue on repeated extraction with 2-propanol.

o———o Liver (10 g) with 380 µg of 2,4-D added.

o- - - -o Liver (10 g) from 2,4-D-treated pig (500 p.p.m. of 2,4-D in feed for 5 months; killed 2 hours after last dose).

It is seen that the extraction curves level off after two extractions, the yield of the double extraction in both instances being about 98 % of the (asymptotic) value obtainable by five extractions.

The principle illustrated above with animal tissues was followed also in establishing optimum extraction conditions for the other types of sample materials.

During the evaporation of the sample extracts, an alkaline hydrolysis step was inserted in order to split possible phenoxy esters, which otherwise might be lost during the separations. This treatment was shown to bring about a quantitative hydrolysis of the esters common in formulations, and it can be expected to hydrolyze also certain conjugates of 2,4-D, such as amides and

Table 3. Sample blanks and overall recoveries of phenoxyacetic acids from animal and vegetable materials fortified before extraction. Recoveries were calculated relative to standards directly subjected to colour development; otherwise experimental details follow "Procedure". Results are means of duplicate determinations starting from the sample.

Sample	Added			Found p.p.m.*)	Recovery %
	Compound	µg	p.p.m.		
Liver (pig) 10 g	2,4-D	0	0	0.11	—
		50	5	3.9	78
		250	25	20.1	80
		1000	100	87.2	87
	MCPA	250	25	20.7	83
	2,4,5-T	250	25	19.1	76
Muscle (pig) 10 g	2,4-D	0	0	0.06	—
		250	25	19.8	79
Blood serum (cattle) 10 ml	2,4-D	0	0	0.05	—
		100	10	8.2	82
Urine (cattle) 25 ml	2,4-D	0	0	0.03	—
		100	4	3.4	85
Milk 25 ml	2,4-D	0	0	0.06	—
		100	4	2.8	70
Egg 25 g	2,4-D	0	0	0.04	—
		250	10	7.8	78
Hay (timothy) 10 g	2,4-D	0	0	0.08	—
		250	10	20.8	83
Oat grain 10 g	2,4-D	0	0	0.10	—
		250	25	19.7	79
Silage 25 g	2,4-D	0	0	0.05	—
		250	10	8.8	88
Soil (loam) 25 g	2,4-D	0	0	0.09	—
		250	10	9.1	91

*) Sample blank values are calculated on a 3-g sample aliquot

glucose esters, which might be present in growing vegetable tissues (*Andreae & Good* 1957; *Klämbt* 1961, and others).

In the case of urine there should be no need for hydrolysis, since conjugation of phenoxy acids in the animal organism seems not to have been reported, and some attempts by the present author to detect alkali- or acid-hydrolyzable derivatives of phenoxy acids in experimental animals also were negative (*Erne* to be

Table 4. Precision of the quantitative determination of 2,4-D added to biological materials. (Recoveries calculated relative to standards directly subjected to colour formation.)

Sample	2,4-D added p. p. m.	Number of dertermi-nations	2,4-D found p. p, m. (range)	Recovery % (mean ± s)
Liver	5.0	10	3.5— 4.1	76 ± 5.1
	25.0	10	19.0—21.3	81 ± 3.7
Serum	10.0	12	7.3— 8.5	79 ± 4.2
Hay	25.0	8	20.5—23.0	87 ± 3.8

published). However, an acid hydrolysis step was included in this case mainly for analytical-technical reasons, the subsequent processing thereby being facilitated.

Accuracy and precision

Various sample materials of animal and vegetable origin were quantitatively analyzed according to the described procedure before and after fortification with phenoxyacetic acid standards (Tables 3 and 4). The standards were added before the extraction step, at several concentration levels.

For most sample materials the procedure worked satisfactorily, and the overall uncorrected recoveries ranged from 70 to above 90 % of the added amount, with a standard deviation of 3—5 %. The coefficient of variation accordingly was about 4—7 %.

The recovery studies, reported above, with the individual steps of the procedure permit an estimate of their contribution to the overall losses. Thus, on the average 3 and 7 % of added standard may be lost in the liquid-liquid extraction and chromatographic steps, respectively.

Sample blank

The level of apparent phenoxy derivatives found when control samples (containing no phenoxy derivatives) were carried through the complete procedure consistently were low (Table 3), the average being 0.07 p.p.m., or about 0.2 μg of apparent 2,4-D. Because of the low level and the slight variability of the sample blank encountered with the present method, the blank will not seriously affect the result of a sample determination unless at low residue levels (below 1 p.p.m.). For a precise determination

at the sub-p.p.m.-level, however, a sample blank value — obtained by carrying a control sample, as nearly identical to the test sample as possible, through the full procedure — should be subtracted from the analytical result.

Sensitivity and limit of determination

The sensitivity of the silver reaction is about 0.1—0.2 μg of phenoxy derivative, and concentrations as low as 0.1 p.p.m. (in a 3-g aliquot), therefore, should be detectable on the chromatograms. In many instances it has proved possible to reduce the detectability level by adjusting sample size or aliquot taken to analysis, or both.

The lower limit of determination of the method, the smallest amount of residue that can be satisfactorily determined, can be set at approximately 1 μg of phenoxy derivative, or 0.3 p.p.m., in a 3-g sample aliquot.

Selectivity

Owing to the added discriminating effects of the buffer extraction, the chromatographic separation and the photometric determination, the method has a reasonably high degree of selectivity. Only distinctly acidic, halogen-containing compounds of the appropriate chromatographic mobility and reactivity towards chromotropic acid will be determined. Thus, other halogen-containing, acidic herbicides, such as aliphatic and aromatic acids and homologous phenoxyaliphatic acids, although being detected on the chromatograms, will not be included in the determination.

Homologues and dinitrophenols

During the fractionation steps of the procedure the 2-phenoxypropionic acids will closely follow the phenoxyacetic acids owing to a similarity in physico-chemical properties (*Phillips* 1958; *Behrens & Morton* 1963). A quantitative determination can be based on spectrophotometry at the absorption band near 280 mμ. Thus, when carried through the solvent partitioning, thin-layer chromatography and elution steps of the procedure, these acids were recovered to about 90 %, with a limit of determination of about 5—10 μg, in 5 ml of final solution. The distinctly less acidic 4-phenoxybutyric acids were recoverable to a similar extent, if

the buffer extraction was carried out at pH 10, conditions otherwise being equal.

Extraction at an elevated pH enables also the dinitrophenol herbicides to be isolated with the procedure. Thus, DNOC and dinoseb proved to be quantitatively extractable at pH values above 9. In the chromatographic step the intense yellow colour of their anions permitted the detection of amounts well below 0.05 µg. A quantitative determination after elution can be achieved by spectrophotometry in the 360 to 380 mµ region, amounts down to 1—2 µg in 5 ml of final solution being measurable.

Application

The extraction and "clean up" steps of the method are sufficiently broad in scope for the method to be applicable to a wide range of biological materials with only minor modifications. Thus, during several years of use in toxicological and residue-analytical work it has been successfully applied to animal tissues (of mammalian, fish and insect origin), body fluids, vegetable tissues (green and dry crops, fruit and grain), various feedstuffs (including silage and molasses), soils, etc.. Usually the degree of "clean up" attained has been adequate. Only in rare instances, as with certain highly decomposed post-mortem materials, the purified extract contained objectionable amounts of extraneous matter which could be reduced to an acceptable level, however, by an additional partitioning between chloroform and buffer followed by reextraction into chloroform.

Table 5. Tissue levels of 2,4-D found in experimental animals given 2,4-D orally at the rates indicated.

Animal	Dose	2,4-D found, mg/kg			
		Liver	Kidney	Muscle	Plasma
Chicken no. 6 B	200 mg/kg as single dose. Killed 2 hrs. after dose	125	165	3	120
Pig no. 12 A	100 mg/kg/day for 3 days. Severely affected. Killed 6 hrs. after last dose	225	185	120	530
Pig no. 16 B	500 p.p.m. in diet for 2 months. Killed 4 hrs. after last meal	53	142	19	160

The application of the method to the analysis of animal tissues is illustrated in Table 5. The results form part of a study of the distribution and elimination of phenoxy derivatives in animals (*Erne* to be published).

REFERENCES

Abbott, D. C., H. Egan, E. W. Hammond & J. Thomson: The detection and determination of organo-chlorine herbicides in soil and water. Analyst 1964, *89*, 480—488.

Andreae, W. A. & N. E. Good: Studies on 3-indoleacetic acid metabolism. IV. Conjugation with aspartic acid and ammonia as processes in the metabolism of carboxylic acids. Plant Physiol. 1957, *32*, 566—572.

Behrens, R. & H. L. Morton: Some factors influencing activity of 12 phenoxy acids on Mesquite root inhibition. Plant Physiol. 1963, *38*, 165—170.

Bevenue, A., G. Zweig & Nancy L. Nash: Residue determination of 2,4-dichlorophenoxyacetic acid in dry crops and walnuts. J. Ass. Offic. Agric. Chemists 1962, *45*, 990—993.

Bevenue, A., G. Zweig & Nancy L. Nash: Cleanup for residue analysis of some 2,4-D esters in potatoes. J. Ass. Offic. Agric. Chemists 1963, *46*, 881—883.

Coakley, J. E., J. E. Campbell & E. F. McFarren: Determination of butoxyethanol ester of 2,4-dichlorophenoxyacetic acid in shellfish and fish. J. Agric. Food Chem. 1964, *12*, 262—265.

Daoud, H. N. & B. S. Luh: Spektralphotometrische Bestimmung von 2,4,5-Trichlorphenoxyessigsäure (2,4,5-T) in dosenkonservierten Aprikosen. Fruchtsaft-Ind. 1962, *7*, 33—40.

Erickson, L. C. & H. Z. Hield: Determination of 2,4-dichlorophenoxyacetic acid in Citrus fruit. J. Agric. Food Chem. 1962, *10*, 204—207.

Erne, K.: The toxicological detection and determination of pentachlorophenol. Acta pharmacol. (Kbh.) 1958, *14*, 158—172.

Erne, K.: Detection and determination of chlorophenoxyacetic acid derivatives in water. Acta chem. scand. 1963, *17*, 1663—1676.

Freed, V. H.: Qualitative reaction for 2,4-dichlorophenoxyacetic acid. Science 1948, *107*, 98—99.

Gutenmann, W. H. & D. J. Lisk: Rapid determination of 4-(2,4-DB) and a metabolite, 2,4-D, in treated forage by electron affinity spectroscopy. J. Agric. Food Chem. 1963, *11*, 304—306.

Klämbt, H. D.: Wachstumsinduktion und Wuchsstoffmetabolismus im Weizenkoleoptilzylinder, III. Stoffwechselprodukte der Naphthyl-l-essigsäure und 2,4-Dichlorphenoxyessigsäure und der Vergleich mit jenen der Indol-3-essigsäure und Benzoesäure. Planta 1961, *57*, 339—353.

Kuznetsov, V. I. & I. Gagarina: Fotometricheskoe opredelenie 2,4-dikhlorfenoksiuksusnoi kisloty s butil-rodaminom. Zh. Analit. Khim. 1962, *17*, 235—238.

Marquardt, R. P. & E. N. Luce: Determination of small amounts of 2,4-dichlorophenoxyacetic acid in milk. Anal. Chem. 1951, *23*, 1484—1486.

Marquardt, R. P. & E. N. Luce: Determination of 2,4-D in grain and seed. J. Agric. Food Chem. 1955, *3*, 51—53.

Marquardt, R. P. & E. N. Luce: A new basic procedure for determining phenoxy acid herbicides in agricultural products. J. Agric. Food Chem. 1961, *9*, 266—270.

Matell, M. & S. Lindenfors: Thermodynamic dissociation constants of 2,4-dichlorophenoxyacetic acid (2,4-D) and some related plant growth regulators. Acta chem. scand. 1957, *11*, 324—328.

Mitchell, L. C.: Separation and identification of chlorinated organic pesticides by paper chromatography. XI. A study of 114 pesticide chemicals: technical grades produced in 1957 and reference standards. J. Ass. Offic. Agric. Chemists 1958, *41*, 781—806.

Nielsen, K., B. Kaempe & J. Jensen-Holm: Fatal poisoning in man by 2,4-dichlorophenoxyacetic acid (2,4-D): Determination of the agent in forensic materials. Acta pharmacol. (Kbh.) 1965, *22*, 224—234.

Phillips, J. N.: Strength of chloro-substituted phenoxyacetic and related phosphorus-containing acids. J. chem. Soc. 1958, 4271—4276.

Warshowsky, B. & E. J. Schantz: Determination of 2,4-dichlorophenoxyacetic acid in soil. Anal Chem. 1950, *22*, 460—463.

Yip, G.: Determination of 2,4-D and other chlorinated phenoxy alkyl acids. J. Ass. Offic. Agric. Chemists 1962, *45*, 367—376.

SUMMARY

The development of a method for separating and determining phenoxyacetic herbicide residues in biological materials is described.

The sample is extracted with solvent at low pH and the phenoxy acids are separated from coextracted matter by partitioning between solvent and aqueous buffer at pH 6.2 and subsequent thin-layer chromatography. Quantitative results are obtained by eluting the phenoxy acids from the adsorbent and photometric determination by means of the chromotropic acid reaction.

Phenoxyacetic derivatives added to a variety of biological materials at the levels 4—100 p.p.m. were recovered to about 70—90 %, with a standard deviation of 3—5 %.

The detection limit of the thin-layer chromatographic step is about 0.1—0.2 μg of phenoxy derivative, or less than 0.1 p.p.m., with a 3-g aliquot of sample, and the photometric step allows the determination of about 0.3 p.p.m. of phenoxy derivative (in a 3-g aliquot).

The method can be modified to include also homologous phenoxy-aliphatic acids and dinitrophenols.

The method has been used during several years for toxicological and residue analysis in a variety of biological materials.

ZUSAMMENFASSUNG

Bestimmung von Phenoxyessigsäureherbiziden in bioligischem Material.

Eine Methode zur Isolierung und Bestimmung von Phenoxyessigsäurerückständen in biologischem Material wird beschrieben.

Die Probe wird mit Lösungsmittel bei niedrigem pH extrahiert, die Phenoxyessigsäuren werden von Extraktivstoffen durch Verteilung zwischen Lösungsmittel und Pufferlösung vom pH 6,2 und Dünnschichtchromatographie getrennt. Quantitative Resultate werden durch Eluierung der Phenoxyessigsäuren vom Adsorbens und photometrische Bestimmung nach früher beschriebenem Chromotropsäureverfahren erhalten.

In Zusatzversuchen mit biologischem Material verschiedener Typen wurden die zugesetzten Phenoxyessigsäurederivaten zu rund 70—90 % wiedergewonnen, mit einer Standardabweichung von 3—5 %.

Die Nachweisgrenze der dünnschichtchromatographische Methode liegt bei zirka 0,1—0,2 µg Phenoxyderivat, was weniger als 0,1 p.p.m. entspricht, auf 3 g Probe berechnet. Die photometrische Bestimmung erlaubt Bestimmung von zirka 0,3 p.p.m. (auf 3 g Probe berechnet).

Diese Methode lässt sich auch für die Bestimmung homologer phenoxyaliphatischer Säuren und Dinitrophenole modifizieren.

Das beschriebene Verfahren hat sich für Analyse von biologischem Material verschiedener Art bewährt und ist seit mehreren Jahren für die toxikologische Analyse und Rückstandsanalyse angewandt worden.

SAMMANFATTNING

Restanalys av fenoxiättiksyreherbicider i biologiskt material.

En metod för isolering och bestämning av fenoxiättiksyrederivat i biologiskt material beskrives.

Provet extraheras med lösningsmedel vid lågt pH och fenoxiättiksyrorna separeras från extraktivämnen genom fördelning mellan lösningsmedel och vattenfas av pH 6,2 samt tunnskiktskromatografi. Efter eluering från adsorbens bestämmas fenoxiättiksyrorna kvantitativt enligt en tidigare beskriven kromotropsyremetod.

Vid analys av olika typer av biologiskt material, försatta med fenoxiättiksyrederivat i koncentrationsområdet 4—100 p.p.m., erhölls utbyten från 70 till c:a 90 %, med en standardavvikelse av 3—5 %.

Påvisbarhetsgränsen i det tunnskiktskromatografiska steget är c:a 0,1—0,2 µg fenoxiderivat, motsvarande mindre än 0,1 p.p.m. beräknat på 3 g prov. Den fotometriska bestämningen tillåter bestämning av c:a 0,3 p.p.m. (beräknat på 3 g prov).

Metoden kan modifieras för bestämning även av homologa fenoxialifatiska syror och dinitrofenoler.

Den beskrivna metoden har visat sig tillämplig på biologiska material av vitt skiftande typer och har använts under flera år för toxikologisk analys och restanalys.

(Received December 4, 1965).