Detection and Determination of Chlorophenoxyacetic Acid Derivatives in Water

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The development of a method for detecting and determining trace amounts of chlorophenoxyacetic acid derivatives and its application to analysis of natural waters is described. Basically the method involves a paper chromatographic separation of the phenoxyacetic acids isolated by a series of extractions including extraction from organic solvent into aqueous buffer. Quantitative results are obtained by photometric determination of the compounds eluted from the chromatogram using a colour reaction with chromotropic acid. An enhanced precision is achieved by applying a geometric correction procedure.

The paper-chromatographic step is sensitive to about 0.5 μg of phenoxy derivative in 100 ml of water, or 0.005 p.p.m., and the lower limit of determination by the photometric technique is about 0.5 μg per ml of final solution.

In recovery experiments with phenoxyacetic derivatives added (in concentrations of 0.15–1 p.p.m.) to water samples, recoveries of 84 to 102 %, with coefficients of variation of approximately 4 to 6 %, were obtained.

According to experimental evidence the method affords a high degree of selectivity.

The method has been used during several years for the routine analysis of herbicide residues in natural waters. A study has also been made of the stability of phenoxyacetic derivatives in well waters.

The recent wide-spread and still increasing use of chlorinated aryloxyalkyl carboxylic acid derivatives as selective herbicides in agriculture, horticulture, and forestry has raised a need for means of detecting and quantitatively determining such compounds in a variety of materials ranging from natural waters and vegetable materials to animal tissues. Reports of accidental contamination of water supplies and various feed stuffs and of actual or suspected poisoning of domestic animals and wildlife by phenoxy herbicides have been frequent during recent years in this country. The present study aims at contributing to the solution of one of the problems arising from this situation, the analysis of phenoxyacetic herbicides in natural waters.

By far the most important of these compounds are 2,4-dichloro-, 2-methyl-4-chloro- and 2,4,5-trichlorophenoxyacetic acids (in the following abbreviated 2,4-D, MCPA and 2,4,5-T, respectively) and their esters and salts.

Of the methods available for detecting or determining phenoxyacetic derivatives some are non-specific, such as biological methods utilizing the growth stimulating effect on various vegetable tissues and chemical methods based on a determination of the acidic group of the molecule or of the chlorine content. More specific are the spectroscopic methods involving measurement in the ultra-violet ¹⁻⁵ or the infra-red ⁶⁻⁸ or, after conversion of the compounds into coloured derivatives, in the visible region. A sensitive colour reaction based on the formation of a red-violet reaction product upon heating phenoxyacetic acid derivatives with chromotropic acid in sulphuric acid was first described by Freed ⁸ and later modified by others ⁹⁻¹². Other principles utilized for determining phenoxyacetic derivatives include polarography,¹³ isotope dilution ^{14,15} and, recently, gas chromatography ¹⁶⁻¹⁹. Column chromatography in conjunction with a volumetric ²⁰⁻²⁴ or a spectrophotometric ^{25,26} determination has been employed for separating and determining the components of technical products. However, most of the analytical methods for phenoxy herbicides have apparently been intended for quality control of technical preparations or formulations and are not well suited for trace analysis because of limited sensitivity or specificity, or both.

Only very few published methods may be considered sufficiently sensitive and selective for residue analysis. Roth and Schuster ²⁷ attempted the determination of phenoxyacetic acids in certain biological materials by performing as well a total chlorine determination as an acidimetric titration on a solvent extract of the sample. Counter current distribution followed by spectrophotometric determination was used by Warshowsky and Schantz ²⁸ for determining 2,4-D in soil. Marquardt and Luce described methods for the determination of 2,4-D residues in milk ¹¹ and grain,¹² involving deproteination with phosphotungstic acid, extraction alternately into organic solvent and into aqueous alkali and — in the case of grain — partition chromatography, and subsequent measurement at 565 m μ of the reaction product with chromotropic acid. Recently the same authors described a method for determining phenoxy herbicides in agricultural products which was based upon cleavage of the ether linkage with pyridinium chloride and photometric determination of the liberated phenol by means of 4-aminoantipyrine ²⁹. Apparently no method for the analysis of phenoxy herbicides in waters has been reported.

Below will be described the development and application of a rapid method for detecting and determining phenoxyacetic herbicide residues in natural waters involving extractive isolation, paper chromatographic separation and photometric determination of the phenoxy derivatives.

EXPERIMENTAL

D e t e r m i n a t i o n

A spectrophotometric determination of chlorophenoxyacetic acids may be accomplished by measuring the ultra-violet absorption at the maximum in the 280-m μ region. The molar absorptivities (ϵ) of the commonly used phenoxyacetic acids are of the order

of 2000, which means that concentrations of about 2 μg of phenoxy derivatives per ml solution may be estimated (using 1 cm cells). The limited selectivity of the method, however, detracts from its applicability. A determination utilizing the infra-red absorption affords a high degree of specificity, but the sensitivity is less than in the ultra-violet determination. For the present purpose a determination based on the colour reaction with chromotropic acid was preferred because of its superior sensitivity and fairly good selectivity. Moreover such a method does not require any expensive equipment.

The *absorption curves* obtained from phenoxyacetic derivatives after reaction with chromotropic acid in sulphuric acid have the form shown in Fig. 1. 2,4-D, MCPA and 2,4,5-T were found to give curves of identical shape. When read against the reagent blank in a Beckman Model DK-2 recording spectrophotometer, the curves exhibited maxima at 480 and 580 $m\mu$ (ϵ being approximately 10 000 and 19 000, respectively) and a minimum at 504 $m\mu$ (ϵ about 8 000). The subsequent photometric measurements were performed at the 580- $m\mu$ maximum, employing the following tentative procedure.

Chloroform solutions containing 25 μg of phenoxyacetic derivative were evaporated in test tubes in a current of air, 5.00 ml of reagent (0.25 % w/v of chromotropic acid in concentrated sulphuric acid) were added to each tube, and the tubes together with a reagent blank were heated in a drying oven at the desired temperature for a given length of time and then cooled in water. The absorbance of the solutions was then read at 580 $m\mu$ against the reagent blank, using a Beckman Model B photometer.

Since the absorbance values so obtained showed a poor reproducibility, a systematic study of the factors affecting colour formation was undertaken.

At an early stage of the investigation it appeared that the thermal degradation of the reagent during the heating step, giving rise to a variable background absorption, might constitute an important source of error, and therefore a *geometric correction* for irrelevant absorption was applied. This measure proved highly successful. The correction principle is illustrated in Fig. 1.

A base-line is drawn tangentially to the curve at two points on either side of the maximum, and then the peak height above the base-line (A_{corr}) can be calculated by simple geometry (*cf.* Ref.³⁰). Provided the background absorption curve is linear within the base-line region, A_{corr} will be independent of the background. For the chlorophenoxy-acetic acids suitable measuring points are at 505, 580, and 655 $m\mu$, and consequently A_{corr} will be given by the expression

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

In order to check the validity of this correction procedure, samples representing various types of natural waters and containing no phenoxy derivatives were passed through the full analytical procedure (see "Method"), and the absorption curve was measured in the region 500 to 660 $m\mu$. The background absorption detected in this way, if any, was only faint, and the absorption curves invariably approximated linearity. The A_{corr} values found were in the range 0–0.006, for 100 ml of sample.

Applying the correction procedure, the *effects of temperature and of time of heating* on the colour formation were studied (Figs. 2 and 3).

As is seen in Fig. 2, a maximum colour intensity was attained with 2,4-D in approximately 20 min at 150°C, the necessary heating times at 140 and 130°C being about 30 and 40 min, respectively. Although MCPA and 2,4,5-T reacted slightly more slowly (Fig. 3), a heating period of about 25 min appears to ensure a complete colour development with any of the three phenoxy compounds, under the experimental conditions given. The use of a thermostatically-controlled oven as a source of heat, as originally proposed by Marquardt and Luce,¹¹ proved most convenient; with a little experience the temperature control could easily be regulated so as to compensate for the initial temperature drop accompanying the insertion of the rack with the tubes.

Employing a standard thermal treatment of 30 min at 150°C, the *effect of the chromotropic acid concentration* on the colour development was studied with 2,4-D, using analytical reagent quality of chromotropic acid purified further according to Bricker and Johnson³¹ (Fig. 4).

As is seen, the chromotropic acid concentration of the reagent ought to be above 0.10 % w/v, in order to attain a maximum colour intensity when using 5 ml of reagent to 25 μg of 2,4-D. It follows that a complete reaction requires a minimum ratio of chromotropic acid to phenoxy derivative of about 200 (or on a molar basis about 100). In

Fig. 1. Absorption spectrum of coloured product formed from phenoxyacetic derivatives in chromotropic acid reaction (50 μ g of 2,4-D in 5 ml of reagent). The figure also shows the base-line construction employed in correcting absorbance values.

order to extend the useful range of the reagent, the concentration of chromotropic acid was adjusted to 0.4 % w/v for subsequent experiments.

Keeping the chromotropic acid concentration at 0.4 % w/v, the effect of the water content of the reagent on the colour development was examined (Fig. 5).

It can be seen that the rate of colour development varied inversely with the water content of the system. For example an increment of the water content by 1 % (from 5 % w/w, as in concentrated sulphuric acid, to 6 % w/w) necessitated an extension of the heating period by some 10 min to avoid low absorbance values. On the other hand the maximum colour intensity attainable in each case apparently was independent of the water content. It may be concluded that any dilution of the acid of the reagent, be it by using an aqueous stock solution of chromotropic acid for preparing the reagent or by using aqueous sample solutions, should be avoided.

The stability of the chromogen formed in the chromotropic acid reaction was checked by keeping the coloured solutions in covered cells in the dark. The A_{corr} values did not change materially during 15 h.

The precision of the photometric determination supplemented with the correction procedure proved excellent. Ten replicate determinations of 25- μ g amounts of 2,4-D standard performed over a week gave a coefficient of variation of 2.0 %.

Calibration data, relating A_{corr} to concentration of phenoxy derivative, were obtained using recrystallized samples and reagent (Table 1). The Kofler melting points of the samples of 2,4-D, MCPA and 2,4,5-T were 138–142°, 121–122°, and 155–157°C, respectively.

The table shows an adherence to Beer's law up to a concentration of at least 80 μ g of each phenoxy derivative, when using 5 ml of reagent containing 0.4 % w/v of chromotropic acid. The slopes of the three calibration graphs agree fairly well if expressed on a molar basis, the molar base-line absorptivities (ϵ_{corr}) for 2,4-D, MCPA and 2,4,5-T being 13 300, 12 700 and 13 100, respectively.

Paper chromatography

For paper chromatography of phenoxyacetic acids solvent systems containing an alcohol and ammonia have been proposed³²⁻³⁴. A combination of alkaline and acidic systems has also been described³⁵.

After a trial of various solvent systems, both alkaline and acidic (including non-aqueous systems), one system containing *sec*-butanol and M ammonia was selected for the present work because of the sharpness of the ensuing spots and the efficient separation from extractives achieved. With this system and using circular development the chlorophenoxyacetic acids had R_F values in the range 0.6–0.7, average values of ten

Figs. 2–5. Factors affecting colour development in chromatotropic acid reaction. *Fig. 2.* Effect of temperature on colour development with 2,4-D (25 μ g, in 5 ml of reagent containing 0.25 % [w/v] of chromatotropic acid). *Fig. 3.* Rate of colour development at 150°C with 2,4-D, MCPA and 2,4,5-T (25 μ g of each). *Fig. 4.* Effect of chromatotropic acid content on colour development with 2,4-D (25 μ g, heating at 150°C for 30 min). *Fig. 5.* Effect of water content of reagent on colour development with 2,4-D (25 μ g).

chromatograms for 2,4-D, MCPA and 2,4,5-T being 0.63, 0.61, and 0.69, respectively. On Whatman paper No. 1 the solvent front advanced 6–7 cm in an hour at 20°C.

Since the completion of the present work Mitchell³⁶ described a solvent system containing *tert*-butanol, methyl ethyl ketone, and ammonia. Although offering some advantage in being homogeneous and moving rapidly, such a system, however, was found inferior in resolving power to the *sec*-butanol-ammonia system.

Several ways of detecting the phenoxy derivatives on the chromatogram were tested. The sensitivities attained were, by using acid-base indicators about 5 μ g, by using ultra-violet light and a fluorescent screen 10–20 μ g, and by using a fluorescence indicator (4-methylumbelliferone³²) and ultra-violet light 2 μ g of phenoxy acid. The latter test, involving an inhibition by the test compound of the fluorescence of the indicator, is a general test for acidic substances. It was found to be positive with acids having pK_a values roughly below 7–8.

In a search for a more specific means of detecting, the silver-phenoxyethanol reagent proposed by Mitchell³⁷ for the detection of chlorinated pesticides proved particularly useful. On spraying a chromatogram with this reagent and exposing it to strong ultra-violet light, halogen compounds appear as dark spots. The limit of detection using this

Table 1. Relation between concentration of phenoxyacetic acids and base-line absorbance (A_{corr}) of coloured product formed in chromotropic acid reaction.

Concentration μg per 5 ml	A_{corr}		
	2,4-D	MCPA	2,4,5-T
10	0.122	0.125	0.105
20	0.242	0.248	0.209
30	0.361	0.369	0.309
40	0.484	0.501	0.411
50	0.607	0.630	0.514
60	0.724	0.758	0.613
70	0.851	0.882	0.721
80	0.973	1.005	0.821

test proved to be less than 0.5 μg of chlorophenoxy acid. As might be expected, the rate of the photochemical reaction proved to be a function not only of the concentration of the halogen compound but of the nature of the compound as well. Thus, with the experimental conditions of "Method" and using 10 μg -amounts of test compounds, inorganic or easily ionized organic halogen reacted immediately to give dark spots, while aliphatic halogen became visible after some 10 sec and aromatic halogen after 1–2 min of exposure to the ultra-violet light. Observing the time course of the reaction, therefore, might enhance the selectivity of the test.

Since most grades of chromatographic paper contain impurities interfering with the silver reaction, a preliminary washing of the paper is necessary. A washing with aqueous ammonia and distilled water proved satisfactory. A final washing, with distilled water, after development and detection also is advisable in order to remove excess silver ions which otherwise might give rise to background darkening during storage of the chromatogram.

Whenever spots appear in the phenoxy area of the paper chromatogram, a photometric determination after elution from the chromatogram should be carried out in order to obtain further criteria of identity in addition to quantitative results. For locating the zones to be eluted from the paper the fluorescence test using methylumbelliferone was found very useful. It was sensitive and did not interfere with the subsequent photometric determination. Experiments proved that a rapid elution could be achieved by either a Soxhlet type extraction in the condensing vapours of a suitable solvent or by direct leaching with the solvent. Using methanol containing a few per cent of ammonia as the solvent the elution of the phenoxy acids proved quantitative in 10 min by the Soxhlet technique, in approximately 1 h by leaching at room temperature and in 5 min by digesting at 50°C.

The efficiency of the quantitative determination was checked in recovery experiments. In ten replicate analyses of 25- μg portions of 2,4-D by combined paper chromatography, elution and photometric determination (for experimental conditions see "Method") a mean recovery of 95% with a coefficient of variation of 3.9% was obtained. The paper blank value consistently was low and rarely exceeded 0.015 A_{corr} units.

Isolation

Owing to the fairly high acid strength of the chlorophenoxyacetic acids (the $\text{p}K_{\text{a}}$ values are about 3³⁹) it proved possible to achieve an effective separation from neutral and more weakly acidic compounds by extraction from an organic solvent into a buffered aqueous phase. With a knowledge of the pH-dependence of the partition ratios of the phenoxyacetic acids,³⁹ the conditions for quantitative extraction could be calculated. Thus the extraction of 2,4-D, and also of MCPA and 2,4,5-T, from aqueous phase into

chloroform would be quantitative at pH 2.5, provided the volume ratio, organic to aqueous phase, were 1:3 and three portions of extractant were used. Conversely, a complete extraction of the phenoxyacetic acids from chloroform should be attained at pH 6 using three one third-volume portions of buffer. Experiments proved these predictions to hold.³⁹

METHOD

Reagents and apparatus. Unless otherwise stated, analytical grade chemicals should be used.

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.

sec-Butanol, chromatographic grade.

Silver-phenoxyethanol reagent. Dissolve 1.70 g of silver nitrate in 5 ml of water, add 20 ml of 2-phenoxyethanol (commercial grade) and dilute with acetone to 200 ml. Keep in a closed vessel protected from light.

4-Methylumbelliferone. 0.02 % w/v solution 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 (disodium salt, Merck) in 100 ml of concentrated sulphuric acid (95–97 % w/w) and swirl to ensure complete dissolution. Keep in a closed vessel protected from light. Prepare freshly each week.

Chlorophenoxyacetic acid standard solutions. Freshly prepared solutions of 2,4-D, MCPA, and 2,4,5-T in chloroform, containing 1 μ g per μ l.

Ultra-violet light source. Any source giving short-wave ultra-violet radiation (about 254 $m\mu$) will serve. A Hanau analysis mercury lamp with a Q 81 quartz tube and with a detachable filter has proved very useful.

Concentrating tubes. Wide test tubes with a constricted lower part of 0.5 ml capacity and graduated to 5/100 of a ml³⁰ or, alternatively, conical graduated centrifuge tubes. Used for concentrating extracts to a definite, small volume before chromatography.

Procedure

Isolation. To a 300-ml sample of water add 5 M sodium hydroxide to pH above 12 and evaporate on the steam bath to about 25 ml. Without filtering transfer with a little distilled water to a separating funnel and acidify the aqueous phase to pH below 2 with 5 M sulphuric acid and extract with three 10-ml portions of chloroform, filtering the extracts through a dry filter. Extract the combined chloroform portions with phosphate buffer pH 6.2 (3×10 ml), shaking for one minute each time. Wash the combined buffer extracts with 3 ml of chloroform, adjust pH to below 2 and extract with chloroform (3×10 ml). Filter the chloroform extracts through a little anhydrous sodium sulphate into a flask, rinse with several small portions of chloroform and reduce the volume on a water bath to about 5 ml, transfer quantitatively to a graduated concentration tube and continue the evaporation at 30–40°C in a weak current of air to 0.1–0.2 ml. Rinse down the walls of the tube and adjust the volume to a suitable value with a little methanol (in order to prevent the separation of an aqueous phase) and use aliquots of this solution for paper chromatography.

Paper chromatography. On circular filter papers (Whatman No. 1 or equivalent grade; 16–18 cm in diameter), mark a concentric starting circle 40–45 mm in diameter with a hard pencil, wash with 5 M ammonia and then with distilled water (by chromatographic development or by suction on a Büchner funnel) and dry in air. By means of a micropipette apply sample solution and the relevant standard as spots on the starting circle. It has proved practical to apply three separate spots of each, corresponding to 25, 50 and 100 μ l of sample and 5, 10 and 20 μ g of standard, respectively. More than one sample may be chromatographed at a time, since the paper can accommodate up to 12 spots. In order to keep the spot area small, apply the solutions in small portions with intermittent drying. (Hot air should be avoided because of possible losses by evaporation.)

Equilibrate the paper for 5–10 min in the vapours of the developing solvent, the lower phase of the mixture, *sec*-butanol, 5 M ammonia, water (25:5:20, by vol.), kept in a 15-cm

Petri dish. Then attach a wick of ether-washed and dried cotton to the centre of the paper and develop the chromatogram with the clear upper phase of the solvent mixture kept in a smaller dish inserted into the other one, until the solvent front approaches the rim of the larger dish (about 1 h). Remove the paper, mark the front, dry for about 30 min in a hood and examine in filtered ultraviolet light to detect possible extraneous matter in the R_F region around 0.6.

Spray the dried paper evenly with the silver-phenoxyethanol reagent and immediately expose to *strong* ultra-violet light, first the back side of the paper and then the front side, until the dark spots are fully developed. Using the unfiltered radiation from a Hanau analysis mercury lamp (with a Q 81 quartz tube fitted 10 cm above the chromatogram) exposure times of 30 sec for the back side and 1–2 min for the front side are usually adequate. A repeated examination of the chromatogram in filtered ultra-violet light at this stage will assist in detecting potentially interfering materials. Mark the spots and wash the chromatogram to remove excess silver ions, first once in very dilute ammonia, then repeatedly in distilled water and finally once in methanol, and dry in air.

(Note. During the chromatographic work the paper must be protected from vapours of hydrogen halides, hydrogen sulphide and other silver-reactive agents.)

Dark spots in the R_F -region 0.6–0.7, usually appearing after about 1 min of exposure, indicate the probable presence of chlorophenoxyacetic acids. The spot area permits a rough estimate of the amount present.

If spots appear, prepare a duplicate chromatogram for the quantitative determination, applying two spots of each sample solution calculated from the qualitative chromatogram to contain 10–50 μ g of phenoxy derivative, and two or more spots of the appropriate standard. Equilibrate and develop the chromatogram in the manner described above, dry in air, spray lightly with the methylumbelliferone reagent and locate the phenoxy acids by observing the chromatogram in filtered ultra-violet light. Cut out the relevant areas and also an adequate paper blank of the same size and location and elute the phenoxy acids from the excised sections, preferably cut into smaller pieces, by refluxing for 10 min in a little flask at 50°C with 3 ml of methanol containing 3 drops of concentrated ammonia. Alternatively, the flask may be simply kept at room temperature with occasional rotating during 1 h. Filter the eluate through a small pledget of glass wool into a borosilicate glass test tube (16 × 160 mm), rinse the flask and the filter with two 1-ml portions of methanol and remove the solvent at 30–40°C in a slow current of air.

Photometric determination. Employing a careful pipetting technique, measure 5.00 ml of chromotropic acid reagent into each of the tubes containing eluted samples, standards and paper blank and into a clean tube (reagent blank). Rotate the tubes at intervals during 5 min to dissolve the residues. Heat all the tubes, placed in a rack, in a thermostatically-controlled oven at 150°C for 30 min and then cool in running water.

Measure the absorbance of the solutions, in 1 cm cells, at the wavelengths 505, 580, and 655 m μ , against the reagent blank, and calculate for each solution a base-line absorbance, A_{corr} , by means of the formula:

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

Subtract the paper blank from each value and by means of a calibration graph, relating A_{corr} to concentration of phenoxy derivative, and prepared under identical conditions, calculate the phenoxyacetic acid content of the sample. Use the simultaneously determined standard value to check the calibration graph.

RESULTS AND DISCUSSION

Accuracy and precision. The proposed method was tested in recovery experiments with phenoxy derivatives added to well-water samples in concentrations of 0.15 to 1 p.p.m. (Table 2).

As can be seen, the overall recoveries ranged from 84 to 102 % with a standard deviation of 4.2 to 5.7 %, the coefficient of variation accordingly being 4.5 to 6.2 %. The table also indicates that the apparent content of phenoxy derivatives of normal well waters, as calculated from the photometric

Table 2. Recovery of phenoxyacetic acids from well-water samples using the described procedure. Figures in parentheses denote number of experiments.

Phenoxyacetic acid	Added p.p.m.	Found p.p.m.	Recovery, %	
			Range	Mean, with S.D.
—	0	0—0.005 (4)	—	—
2,4-D	0.15	0.126—0.151 (6)	84—101	93 ± 5.1
2,4-D	0.30	0.257—0.294 (6)	86—98	92 ± 5.7
2,4-D	1.00	0.87—1.02 (10)	87—102	94 ± 4.2
MCPA	1.00	0.85—0.98 (6)	85—98	92 ± 5.0
2,4,5-T	1.00	0.89—0.97 (6)	89—97	93 ± 4.5

results, was negligible. The paper-chromatographic tests in these instances were negative throughout.

Sensitivity. Since the paper-chromatographic step is sensitive to less than 0.5 μg of chlorophenoxy derivative, the detection of concentrations below 0.005 p.p.m. in an 100-ml aliquot of water sample is possible. This sensitivity seems quite sufficient for most practical purposes, but if desirable in special instances, it might be enhanced by increasing the sample size or the aliquot of chloroform extract taken to chromatography.

The lower limit of estimation in the photometric step is of the order of 0.5 μg of phenoxy derivative per ml of final solution (*cf.* Table 1).

Specificity. The method derives its specificity from the added discriminating effects achieved in the consecutive steps of the procedure. Thus, the extraction step effectively eliminates any non-acidic compounds present in the sample. (The initial addition of alkali serves the dual purpose of hydrolyzing any phenoxyacetic esters present and of fixing the phenoxy acids during the evaporation.)

The separating efficiency of the paper chromatographic step was tested with a number of compounds representing varying degrees of acid strength and polarity and expected capable of passing, at least to some extent, the extraction step of the procedure. The test substances included aliphatic and aromatic acids, halogenated as well as non-halogenated, substituted phenols and compounds of biological interest. In Table 3 they are arranged according to increasing R_F values.

As can be seen, a separation of the chlorophenoxyacetic acids from most of the other compounds was effected. The sensitivity of the silver reaction, as might be expected, was found to vary considerably with the type of compound, from the extreme sensitivity displayed with halogenated compounds (except fluorine compounds) to virtually none with saturated aliphatic acids. Aromatic compounds took an intermediate position. Of the few compounds found to have R_F values closely to those of the chlorophenoxyacetic acids, only three, trichloroacetic, 2,2-dichlorobutyric and 2,3,6-trichlorobenzoic acid, reacted with the silver reagent to form typical dark spots. Salicylic acid reacted faintly, whereas the characteristic yellow spots of the nitrophenols appeared already before the treatment.

Table 3. Approximate R_F values (in the system *sec*-butanol-M aqueous ammonia) and behaviour towards the silver reagent of selected acidic compounds. A plus within parenthesis indicates that spots appeared although not of the characteristic, dark-coloured type. Compounds not being detectable by the silver reagent were located by means of the methylumbelliferone reagent (see "Method").

Compound	R_F	Silver reaction	
		Response	Sensitivity, μg
Oxalic acid	0.08	(+)	5
Tartaric acid	0.08	(+)	2
Succinic acid	0.10	(+)	5
Dialuric acid	0.16	(+)	2
Phthalic acid	0.18	(+)	1-2
Uric acid	0.19	—	
Glycolic acid	0.22	(+)	5
Lactic acid	0.26	(+)	2
Acetic acid	0.28	—	
Thioglycolic acid	0.29 (0.14)	(+)	2
Fluoroacetic acid	0.29	(+)	1-2
Propionic acid	0.30	—	
Pantothenic acid	0.33	—	
Chloroacetic acid	0.38	+	0.3
Bromoacetic acid	0.39	+	0.3
Nicotinic acid	0.39	(+)*	2
Pyruvic acid	0.40 (0.54)	(+)*	3
Butyric acid	0.41	—	
Iodoacetic acid	0.44	+	0.2
Mandelic acid	0.44	(+)	1-2
Hippuric acid	0.45	(+)	5
3-Indoleacetic acid	0.46	(+)*	1
Dichloroacetic acid	0.47	+	0.2
Phenylacetic acid	0.48	(+)	3
Benzoic acid	0.50	(+)	3
Phenoxyacetic acid	0.51	(+)	3
2,2-Dichloropropionic acid	0.52	+	0.2
Sorbic acid	0.53	—	
Trichloroacetic acid	0.56	+	0.1
Salicylic acid	0.57	(+)	1-2
2,2-Dichlorobutyric acid	0.58	+	0.3
4-Nitrophenol	0.58	— (yellow)	0.2
MCPA	0.62	+	0.3
2,4-D	0.63	+	0.2
2,3,6-Trichlorobenzoic acid	0.64	+	0.5
2,4,5-T	0.69	+	0.2
Warfarin	0.72	—*	
4-Methylumbelliferone	0.74	—	
4,6-Dinitro-2-methylphenol	0.78	— (yellow)	0.1
2,4,5-Trichlorophenol	0.81	+	0.2
Pentachlorophenol	0.82	+	0.5
Coumachlor	0.87	—*	
4,6-Dinitro-2- <i>sec</i> -butylphenol	0.91	— (yellow)	0.1
2,4-Dichlorophenol	0.97	+	0.3
4-Chlorophenol	0.98	+	0.3
2-Methylphenol (<i>o</i> -cresol)	0.99	(+)	5

* Fluorescent after silver reaction.

Aromatic halogen compounds when subjected to the silver test required exposure times of the order of minutes whereas most inorganic and aliphatic halogen compounds reacted in a few seconds to produce visible spots. Therefore, an enhanced selectivity of the test could be attained by observing the time course of the reaction. Furthermore, an examination of the silver-developed chromatogram in filtered ultra-violet light proved useful in detecting foreign materials, since several compounds, giving no or only faint silver spots, were found to give rise to intensely fluorescent spots under the conditions of the silver test. In this way compounds as widely differing as 3-indoleacetic acid, nicotinic acid, pyruvic acid and coumarin derivatives, such as warfarin and coumachlor, could be readily detected.

The colour reaction with chromotropic acid is not entirely specific for phenoxyacetic derivatives, as was also noted by Freed⁸. Being in principle an extension of the Eegriwe test for formaldehyde,⁴⁰ it could be expected to give a positive result with any compound capable of forming formaldehyde under hot acid conditions. In a study of the specificity of the reaction, Le Tourneau and Krog⁹ observed a number of compounds to react qualitatively as the phenoxyacetic acids, all of which could conceivably split off formaldehyde under the experimental conditions. With formaldehyde, glycolic acid, 3-indoleacetic acid and several salts and esters of substituted phenoxyacetic acids even the molar absorptivities were found to agree. These facts point to a common reaction mechanism involving the intermediate formation of formaldehyde.

In order to check the scope of the chromotropic acid reaction, as performed according to the directions of the present method, a wide selection of compounds were tested (Table 4). One hundred μg or, if necessary to avoid unduly high absorbance values, smaller amounts, of each sample were reacted with 5 ml of reagent, the absorption curves were recorded and the A_{corr} values were calculated.

It can be seen that a number of compounds — glycolic acid, glyoxal, 3-indoleacetic acid, ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid and dialuric acid — gave rise to intense violet-red colours like the phenoxyacetic acids. A faint reaction was obtained with alloxan, hippuric acid, uric acid, tryptophan, pyruvic acid and lactic acid. Tartaric acid gave a brown colouration which, however, did not affect the A_{corr} value. Thioglycolic acid and mandelic acid yielded yellowish-brown and green colours, respectively, and negative A_{corr} values. It is interesting to note that under the experimental conditions given several potentially reactive compounds such as the monohaloacetic acids, oxalic acid, citric acid and glycine were found not to react.

For the compounds in the top of the table the A_{corr} values were recalculated on a molar basis (ϵ_{corr}), and, as is seen, these values agreed fairly well, except for unsubstituted phenoxyacetic acid and EDTA, suggesting one mole of formaldehyde to be liberated for each mole of compound. The molar yield of formaldehyde from EDTA, as might be expected, exceeded 1.

It is evident from Tables 3 and 4 that none of the compounds found to behave in a chromatographic sense similarly to the chlorophenoxyacetic acids respond to the chromotropic acid reaction. Conversely, any of the compounds found to react with chromotropic acid are effectively separated from the

Table 4. Specificity of the chromotropic acid reaction. Recorded A_{corr} values refer to a concentration of 100 μg of sample in 5 ml of solution, if not otherwise stated.

Compound	Colour	A_{corr}	ϵ_{corr}^*
2,4-D	Violet red	0.604 (50 μg)	13 300
MCPA	» »	0.632 (50 μg)	12 700
2,4,5-T	» »	0.514 (50 μg)	13 100
Phenoxyacetic acid	» »	0.755	6 500
Paraformaldehyde	» »	0.800 (10 μg)	12 000
Glycolic acid	» »	0.722 (20 μg)	13 700
Glyoxal	» »	0.860 (20 μg)	12 500
3-Indoleacetic acid	» »	0.695 (50 μg)	12 200
EDTA, disodium salt	» »	0.865	16 100
Nitrilotriacetic acid	» »	0.585 (50 μg)	11 100
Dialuric acid	» »	0.110	
Alloxan	» »	0.050	
Hippuric acid	» »	0.030	
Uric acid	» »	0.007	
Tryptophan	» »	0.005	
Pyruvic acid	Brownish red	0.025	
Lactic acid	» »	0.012	
Chloral hydrate	» »	-0.044	
α -Chloralose	» »	-0.010	
Tartaric acid	Brown	0	
Thioglycolic acid	Yellowish brown	-0.290	
Mandelic acid	Green	-0.092	
Chloroacetic acid	None	0	
Bromoacetic acid	»	0	
Iodoacetic acid	»	0	
Dichloroacetic acid	»	0	
Trichloroacetic acid	»	0	
2,2-Dichloropropionic acid	»	0	
2,2-Dichlorobutyric acid	»	0	
Sorbic acid	»	0	
Oxalic acid	»	0	
Malonic acid	»	0	
Succinic acid	»	0	
Citric acid	»	0	
Serine	»	0	
Glycine	»	0	
Histidine	»	0	
Proline	»	0	
Aspartic acid	»	0	
Pantothenic acid	»	0	
2,3,6-Trichlorobenzoic acid	»	0	
Phthalic acid	»	0	
Nicotinic acid	»	0	
Barbituric acid	»	0	
Thiobarbituric acid	»	0	
Uracil	»	0	
Creatinine	»	0	
Guanine	»	0	
Warfarin	»	0	
4-Methylumbelliferone	»	0	
4,6-Dinitro-2-methylphenol	»	0	
2,4,5-Trichlorophenol	»	0	
DDT	»	0	
Aldrin	»	0	
Tris(hydroxymethyl)amino-methane	»	0	

* $A_{\text{corr}}/\text{mole/l.}$

chlorophenoxyacetic acids during the chromatographic step (if not removed earlier in the procedure). Taking into consideration the wide range of chemical and physical properties covered by the test compounds, it may be concluded, therefore, that the proposed method has an adequate degree of selectivity.

Application. The described method has been applied successfully during some years to the analysis of natural waters of various types, such as well waters and ditch, stream, and river waters. So far chromotropic acid-positive material has never been encountered in the "phenoxy area" of the chromatograms, unless the preceding silver reaction was also positive, which may be taken as a further indication of the good selectivity. In samples suspected of being contaminated with herbicides, trace amounts of phenoxyacetic derivatives were detected occasionally; in a few cases concentrations as high as 100 p.p.m. were observed. In accordance with other reports, several of the contaminated waters were found to display a characteristic taste and odour, probably arising from traces of halophenols being present as impurities in the herbicide formulations used. Such phenomena generally were rather persistent, and in one of the samples the taste was perceptible even at a concentration of phenoxy derivatives as low as 0.005 p.p.m. The content of halophenols, determined spectrophotometrically was below 0.003 p.p.m.

Stability. In order to get an idea of the persistence of phenoxy herbicides in natural waters, some stability tests were made. 2,4-D was added to two water samples rich in organic matter, one ditch water sample with an ample amount of solid particles and one well-water sample with a moderate sediment, and the samples were kept in glass-stoppered flasks at room temperature (15–20°C) in diffuse daylight. At intervals the flasks were shaken and aliquots taken to analysis without filtering (Table 5).

The results indicate only a slight decrease in concentration during storage for six months at room temperature. On the other hand, the distinctly lower values obtained after filtration of the samples (and not after filtering standards) suggest that some adsorption to the solid phase of the samples had taken place.

Table 5. Stability of 2,4-D in ditch-water and well-water samples at 15–20°C in diffuse daylight. Aliquots were taken to analysis without filtering. Recorded values are means of duplicate determinations.

Time of storage days	2,4-D found, p.p.m.	
	Ditch water (8 p.p.m. of 2,4-D added)	Well water (1 p.p.m. of 2,4-D added)
0	7.9	0.95
4	8.0	—
22	7.6	—
41	7.3	0.96
90	7.4	0.94
170	7.2 (5.4) *	0.87 (0.70) *

* Sample aliquot filtered before analysis.

It appears from these preliminary results, as well as from a study of the rate of disappearance of phenoxy herbicides from the water of a contaminated well, that phenoxyacetic derivatives may have a marked stability in natural waters, as contrasting with the relatively rapid break down in soil ⁴¹.

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