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*Chromatographic
Separation and Identification
of Organic Acids*

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ILLINOIS STATE WATER SURVEY
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URBANA
1960



(20483-7-60)

Printed by authority of the State of Illinois

Chromatographic Separation and Identification of Organic Acids

Application to Detection of Organic Acids in River Waters

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Although organic acids are present in river waters in relatively low concentrations, they can to some extent be separated and identified by chromatographic methods. Preliminary data from 25 river samples illustrate the potential of chromatography for the evaluation of water quality. An alkaline evaporation of the sample, followed by an ether extraction of the acidified aqueous residue, is used to prepare the sample for the separation of individual acids or groups of acids by column partition chromatography. Identity of some acids is confirmed by a comparison of the R_f values obtained for pure acids by partition chromatography on paper.

ALTHOUGH organic acids are seldom responsible for tastes and odors (6), their concentrations in surface water supplies may be several times the concentration of neutral and basic organics present. This report concerns the effective application of the combined techniques of column and paper partition chromatography for the separation and identification of water-soluble acids. The acids are separated by the column method and are tentatively identified

by their respective elution patterns. Confirmatory identification of some of the separated acid fractions is made by partition chromatography on paper.

EXPERIMENTAL

Sample Preparation. The water samples are collected in a container to which a few pellets of sodium hydroxide have been added. After filtration, a sample volume of 3 to 4 liters is heat-concentrated to approximately 30 ml. The concentrate is acidified with mineral acid and clarified by filtration when necessary, and the organic acids are extracted with ethyl ether for 15 hours. After extraction, the acids are titrated with dilute sodium hydroxide using bromothymol blue as the indicator. The neutralized solution is then warmed on a steam bath to volatilize the ether layer, and the aqueous phase containing the acid salts is concentrated to a volume of 1 ml. This sample extract is acidified and adsorbed on silicic acid for addition to the column.

Chromatography. **COLUMN PARTITION METHOD.** The acids are separated initially by the method of Bulen, Varner, and Burrell (5) as modified by Mueller, Larson, and Lennarz (12) for the quantitative determination of the lower volatile acids. Silicic acid

is used as the adsorbent, onto which is fixed dilute sulfuric acid as the stationary phase. The mobile phase consists of various concentrations of 1-butanol in chloroform. The procedure differs from that described only in that effluent fractions of 5 ml. are collected and titrated with 0.02N alcoholic sodium hydroxide using 0.1% bromothymol blue as the indicator. Blank values are determined for each solvent system and subtracted from the respective effluent titration values. By this technique 20 or more acids could be partially separated.

Each acid has a characteristic peak effluent volume, which is that fraction having the highest titration value. The positions of the acids separated by this method are shown in the reference chromatograms in Figures 1, 2, and 3. The locations of these acids were determined by repeated appearance within one fraction of the position indicated. Recoveries in excess of 90% were obtained for all acids.

All of the acids are not separated completely by this method, because two or more acids often have the same elution pattern. In some cases these can be separated by further column treatment modified for the particular acids in question. An example is the peak

common to formic, fumaric, and alkyl benzenesulfonic acids (ABS). These acids are completely separated by changing the inside diameter of the column from 10 to 7.0 mm. and using 15% butanol in chloroform as the developing solvent. Supplementary separations of this type are somewhat laborious and are recommended only where the acids are desired for further chemical tests; otherwise, confirmatory identification is made by partition chromatography on paper. The 5-gram column permits the detection of 1.0 μ eq. of acid, but is best suited for separation of 2 to 75 μ eq. of each acid.

PAPER PARTITION METHODS. After quantitative separation and grouping of the acids by column chromatography, the fractions associated with each of the identifying peaks are combined and the sodium salts extracted into water from the eluting solvent. After

concentration to dryness over steam, the acid salts, hydroxamate derivatives, or the free acids are chromatographed according to the methods described.

The lower fatty acids may be chromatographed as their sodium (3), ammonium (8, 10, 13, 14), or diethylamine salts (9), or their hydroxamate derivatives (2, 7, 15). In the initial analyses on the river samples, the sodium salts of the acids were chromatographed by a modification of the methods of Brown (3) and Reid and Lederer (14). However, resolution of the acids was not entirely adequate and the use of the method was limited to a few water samples.

Consequently, the method of Thompson (15) was modified to accommodate at least 20 μ moles of acid, because this concentration was found to be limiting for the successful preparation of the acid hydroxamates.

For the identification of unknown acids recovered by column chromatography, the sodium salts of the acids, after concentration to dryness, are acidified with 0.4*N* alcoholic hydrochloric acid. Acids recovered from the column separation in concentrations less than 20 μ moles are supplemented with an acid of known purity such as butyric or propionic acid, prior to the hydroxamate preparation. The acids are esterified at 0° C. by the addition of ethereal diazomethane to the appearance of a yellow coloration, which persists for a minimum of 10 minutes. The excess diazomethane is destroyed by the addition of several drops of 0.4*N* alcoholic hydrochloric acid, just prior to the formation of the hydroxamate.

For 20 μ moles of acid, 8 ml. of ethyl ether is added to the ester, followed by 0.5 ml. of hydroxylamine reagent. (This reagent is prepared at 5° C. just prior to use from equal volumes of 5% $\text{NH}_2\text{OH}\cdot\text{HCl}$ and 12.5% NaOH in absolute methanol; the precipitated NaCl is removed after 5 minutes by filtration.) After a reaction time of 30 minutes at 25° C., 0.02 ml. of glacial acetic acid is added and the solution is filtered again. After evaporation to dryness over steam, the hydroxamates are dissolved in 0.1 to 0.2 ml. of absolute methanol and an aliquot containing a minimum of 0.05 μ mole of the unknown acid is chromatographed on Whatman No. 1 filter paper by the ascending technique. The chromatogram is developed with the following solvent system—1-butanol-acetic acid-water in proportions of 4:1:5. The purpose of the acetic acid in this system, referred to as a "swamp" acid, is to repress ionization of the hydroxamic acids and thus prevent tailing of the spot. The presence of a swamp acid also increases the solubility of the water-immiscible solvent for water. After development for 15 hours the chromatograms are removed, dried for 1 to 2 hours, and sprayed with 1% alcoholic ferric chloride, containing 0.1% hydrochloric acid. The red-purple spots that appear on a yellow background are specific for the hydroxamates.

Although the sensitivity of the method has been reported to be 0.01 μ mole for most hydroxamates (2), it was desirable to have 0.03 to 0.05 μ mole as the limiting concentration.

Identification of the dicarboxylic acids as their hydroxamate derivatives is not entirely satisfactory and has been limited to a few acids. Two, and oftentimes three, values were observed for most of these acids. These values are given in Table I in the order of their intensity. Fink (7) has reported the R_f values for hydroxamate derivatives of several dicarboxylic and other aliphatic acids using water-saturated isobutyric acid and water-saturated phenol as the solvent systems. Identification of these acids by two-dimensional partition chromatography has also been

Table I. Comparison of R_f Values of Separated Acids with Reference Acids Showing Similar Elution Patterns

Peak	Reference Acid	Sodium Salt ^a		R_f Values Hydroxamate ^b		Acid ^c	
		Reference	Unknown ^d	Reference	Unknown ^d	Reference	Unknown ^d
A	Butyric	0.37	0.14 0.16	0.78
	Crotonic	0.31	0.07	0.78
B	Propionic	0.28	0.15	0.66	0.40 0.47 0.50	...	0.55
	Acetic	0.16	0.16	0.58	0.58
D	Pyruvic		0.18 0.13 0.11 0.07	0.55	0.56	0.06 0.62	0.04
	Formic	0.14	0.14	0.48	0.48 0.26	...	0.72 0.04
E	ABS	0.77		...		0.72 0.04	
	Lactic	0.14	0.14	0.49	0.48 0.39 0.54	0.55	0.55
F	Succinic			0.55 0.52 0.47		0.60	
	Gallic	0.09	0.18	...	0.23	0.48	0.39
G	Malonic	0.02	0.15	...	0.47	0.50	0.58
	Oxalic	...	0.07	0.59 0.55	0.44 0.54		0.68
	Aconitic			0.53 0.66	0.59 0.38	0.67	
H	1-Malic		0.18	0.57 0.52 0.44 0.49	0.53 0.44	0.32	...
	Citric	0.23	0.23	0.52	...	0.23	...
J	Isocitric			0.59 0.45			...
					0.54 0.44		
L	Tartaric			0.52	0.54 0.61	0.15	

Solvent systems.

^a 5% ethanol-45% 1-butanol-50% NH_4OH (concd.)

^b 1-Butanol-acetic acid-water (4:1:5).

^c 1-Pentanol-5*M* formic acid (1:1, v./v.).

^d R_f values obtained on different samples.

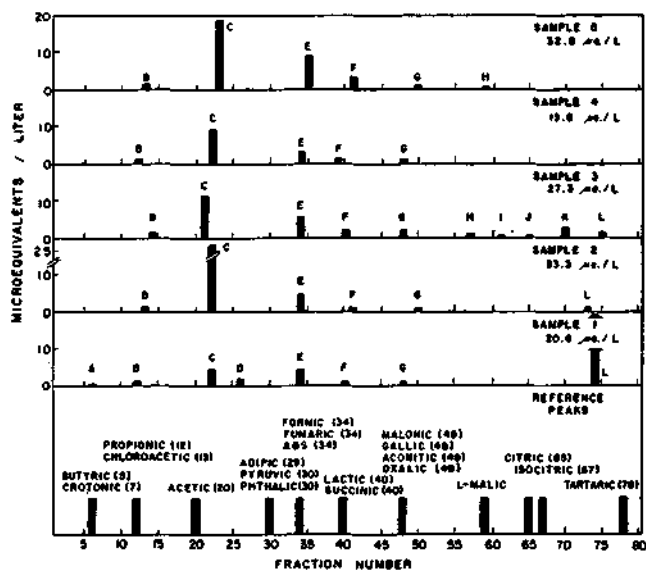


Figure 1. Organic acids in Ohio River water

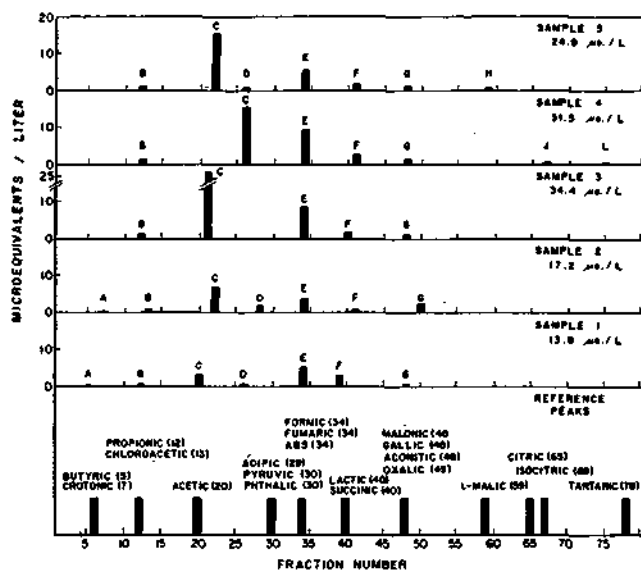


Figure 2. Organic acids in Mississippi River water

recommended by Bergmann (2). These have not been tried.

The dicarboxylic acids may be more conveniently identified by direct chromatography of the acidified fraction in question. A modification of the procedure of Lugg and Overell (11), employing the alcoholic phase of 1-pentanol and 5*M* formic acid (v./v.) as the developing solvent, has given highly reproducible R_f values for several dicarboxylic acids.

The aqueous phase of the solvent system is retained in a beaker placed in the bottom of the chromatography jar. After the acids are applied to the paper, an equilibration time of 1 to 2 hours is allowed prior to lowering the paper into the solvent to obtain reproducible R_f values. The "life" of the solvent was not extended beyond 5 days. After development overnight, the chromatograms are removed and dried for 1 to 2 hours in a flowing air current for the removal of the formic acid, which serves as the swamp acid. The acids are located by spraying the chromatogram with a 0.04% solution of bromophenol blue in 95% alcohol adjusted to pH 6.7. The acids appear as yellow spots against a blue background. The method is sensitive to 0.1 μ mole of acid. A 0.1% mercurochrome solution made neutral in alcohol has been used by Airan *et al.* (1) for similar separations. When this spray is used, the spots due to the acids are distinct and appear violet against the fluorescence of a greenish yellow background.

RESULTS AND DISCUSSION

Separations of the water-soluble acids from samples typical of approximately 20 waters of the Ohio and Mississippi Rivers are graphically illustrated in

Figures 1 and 2. The concentrations of these acids ranged from 13.8 to 34.4 μ eq. per liter. For simplicity, the chromatograms are not shown in their entirety, but rather the microequivalents and peak effluent volumes characteristic of each acid. Several peaks—designated as B, C, E, F, and G—appeared to be common to all of the samples analyzed. However, a quantitative relationship of the acid concentrations was not observed. Other peaks, such as A, H, I, J, K, and L, were present in lesser amounts with considerable variation among the samples, both within and between river waters.

Confirmatory identification of the separated acids by partition chromatography on paper has been satisfactory for some of these acids. However, much is to be desired in the identification of the acids present in low concentrations. A comparison of the R_f values of the separated peaks with those obtained for the reference acids is presented in Table I. Because of limited concentrations of some acids, the values for each procedure were obtained on separate samples. The data can best be interpreted by separate discussion of the acid peaks.

A. This peak, when present, is always in trace amounts and could not be sufficiently concentrated for the satisfactory preparation of the hydroxamate derivative. When chromatographed as the sodium salt, several R_f values were observed, indicating the possibility of more than one acid. These values are of little assistance in the identification of this acid(s). However, the absence of butyric and crotonic acids is indicated.

B. The elution pattern of peak B would tentatively identify this acid as propionic acid; however, in no instance, as the salt or as the hy-

droxamate derivative, has a correlation of R_f values been shown. Therefore, its identity remains unknown.

C. This peak has been predominant in nearly all of the samples analyzed. Chromatographs obtained from the sodium salt or the hydroxamate derivatives of peak C leave little doubt as to its identity as acetic acid. This would be expected, as this acid is a common product of metabolic activity.

D. The appearance of this peak is inconsistent and may represent a false peak of acid C, due to slight pressure variation at the time of solvent change. In sample 4 from the Mississippi River, peak C, confirmed as acetic acid, was eluted entirely in the fractional range of peak D. This elution pattern has also been observed occasionally for pure acetic acid. The R_f data, however, are variable and the possible presence of other trace acids "masked" by the overrun of acetic acid is recognized.

E. This peak is of considerable interest, for several acids are eluted in this fractional range—namely, formic and fumaric acids, and the anionic detergent, ABS. R_f values obtained from chromatograms on the sodium salt and the sodium hydroxamate derivative have confirmed the presence of formic acid. In some samples, the chromatograms showed some indistinct trailing spots. This could be due to the presence of other acids. In addition to formic acid, the presence of ABS has been shown by R_f values obtained from chromatograms of the free acids. In further elucidation of this fraction, steam distillation has shown that 40 to 60% of the eluted acid was steam volatile.

F. Confirmatory data by paper chromatography indicate the presence of lactic or succinic acid. Both or either of these acids could be present as metabolic products. By paper chromatography, these acids have similar

mobilities and are not separated by the chromatographic methods employed; however, slight differences in R_f values have been noted when the free acids are chromatographed. Buch, Montgomery, and Porter (4) have proposed several spray materials for the differentiation of these acids by specific color reactions. Ammoniacal silver nitrate (0.1N), mixed just prior to use, distinguishes between lactic and succinic acids when used as the spray in the color development of the chromatograms of the free acids by the method of Lugg and Overell (11). Succinic and lactic acids appear as white and yellow spots, respectively, on a tan background. Maximum color differences are observed after 4 hours in subdued light. In one sample, peak F was identified as lactic acid by this technique.

G. The R_f values noted for this peak have not been distinct in any one chromatogram when spotted as the salt or as the hydroxamate derivative. The most recent separations observed when chromatographing the free acids have given three distinct spots of equal intensity. A correlation of these spots with the R_f values obtained for the reference acids, however, is limited to aconitic acid.

H and I. These peaks have appeared infrequently and in concentrations too small for identification by this technique.

J. This peak appeared in the fractional area for citric and isocitric acids. Sufficient concentrations were not present to determine its identity.

K. This peak was observed in the Ohio River sample 3 and only in trace amounts. Dissimilar elution to the column reference acids did not permit its tentative identification.

L. Peak L appeared in sufficiently high concentration in one sample and to a lesser extent in two samples from the Ohio River. The hydroxamate preparation was successful and gave a distinct R_f value at 0.54. Unfortunately, the column elution pattern could not be correlated with any of the reference acids. An R_f value of 0.61 was noted for the hydroxamate derivative when present in trace amounts. This acid has not appeared in subsequent samples in concentrations sufficient for identification.

Composite samples taken from the Illinois River and from test well 19 at Peoria, Ill., which receives its supply from the ground water recharge pits located adjacent to the river, were chromatographed by the technique described. The data for these separations are graphically summarized in Figure 3. The acid types that appeared in both samples were essentially the same, with the exception of peak J, which was present only in the sample from test well 19. The appearance of this peak in such a high concentration is somewhat puzzling, but not unusual, for a similar peak has been observed on analyses of sewage plant effluents. The quantitative relationship of many of

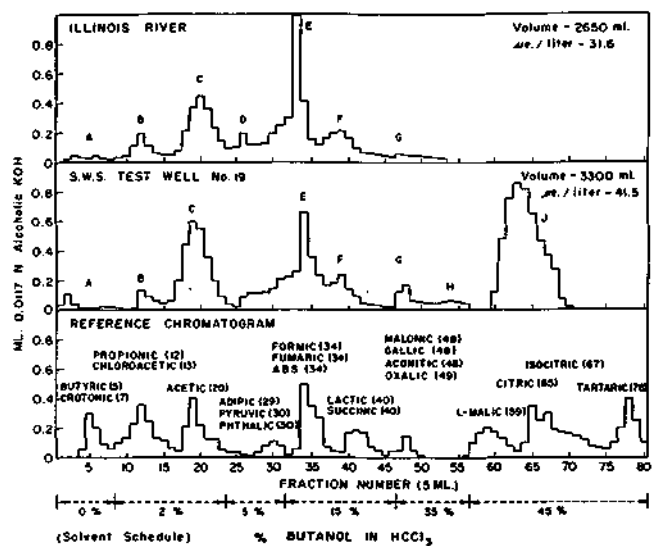


Figure 3. Organic acids in Illinois River and test well 19 waters

the other acids separated, as well as the fact that this acid was absent from the Illinois River water, suggests that it may have resulted from bacterial activity in the ground.

The R_f values observed for the commonly occurring acids conform to those observed for both the Mississippi and Ohio River waters for these acids. On steam distillation of the acids at peak E, it was noted that 60 and 42% of the acidity was steam-volatile for the respective Illinois River and test well samples. These observations substantiate the confirmed presence of formic acid. The R_f value for peak J, when chromatographed as the hydroxamate derivative, was 0.54. Although the elution patterns of peak J and citric acid are similar, the R_f values were not in sufficient agreement for identification.

SUMMARY

The data presented for these river waters illustrate the use of chromatography as a general technique directed for the identification of specific organic materials in water. The organic acids recovered from water samples from the Ohio and Mississippi Rivers during the last 6 months of 1958 ranged from 1000 to 2400 p.p.b., 33 to 62% of which was attributed to the nonvolatile components. Carbon filter samples during the same period showed a variation from 194 to 383 p.p.b. for the total extractables recovered. The water-solubles and the acidic components varied from 16 to 30 and from 5 to 13 p.p.b., respectively. These comparative data indicate the relative magnitude of acid concentrations in

streams, without attempting to evaluate the relative significance as to potability or toxicity. The data presented are not considered complete, for much is to be desired in confirmative identification of these components.

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RECEIVED for review November 18, 1959
Accepted January 28, 1960. Division of Water, Sewage, and Sanitation Chemistry, Symposium on Organic Chemicals in Water, 136th Meeting, ACS, Atlantic City, N. J., September 1959. Investigation supported in part by Research Grant 6406 from the National Institutes of Health, U. S. Public Health Service.