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Identification of Organic Acids on Paper Chromatograms

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In the course of investigations by means of paper chromatography on the acidic components of maple sap and maple sirup and the action of alkali on sugars, a number of acids having similar mobilities were found which could not be identified by *R* values alone. A method was developed which utilizes four spray materials—silver nitrate–ammonium hydroxide, acetic anhydride–pyridine, ammonium vanadate, and ceric ammonium nitrate—to produce color reactions that aid in the differentiation of certain groups of these acids. The procedure increases the usefulness of the paper chromatogram technique as applied to organic acids by aiding in identification of certain individual acids.

USE of paper chromatography for qualitative determination of organic acids has been limited for the following reasons: R_F values are not sufficient evidence in many cases for positive identification of an unknown acid, because several of the acids have R_F values so close together that useful separation cannot be obtained; and the R_F values vary with the distance moved by the solvent front. In the authors' experience a difference of 5 to 10 cm. in solvent front movement may produce variations of 0.05 or more in R_F units. For this reason *R* values expressed as the ratio of the distance moved by an unknown compound to the distance moved by a known pure compound may be preferred to the usual R_F referred to the solvent front movement. However, the former values are also subject to errors due to poor resolution.

In the course of investigations on the acidic components of maple sap and maple sirup (*14, 17*) and the acids produced by the action of alkali on sugars, a study was made of the behavior of various organic acids when subjected to the slightly modified procedure of Lugg and Overell (*11*). The method described in this paper increases the usefulness of the chromatogram technique by utilizing certain color reactions which aid in the differentiation of certain groups of acids that cannot be properly resolved.

MATERIALS AND REAGENTS

Whatman No. 1 paper was used in all experiments.

Solvents were checked for purity and distilled if necessary. The solvent mixture employed was 1-pentanol–5 *M* aqueous formic acid, prepared at least 3 hours before use by mutually saturating equal parts (by volume) of the two components.

Most of the acids were commercial products purified before use.

Many detecting reagents were tried as spray solutions in an attempt to find those specific for the different reactive groups of the organic acids. For example, 1,3-naphthalenediol–phosphoric acid (*13*) and sodium dinitrosalicylate (*16*) indicated only the uronic acids. However, many of the well-known reagents were either too insensitive or did not show the desired specificity. Unreliable results were obtained with *p*-phenylenediamine hydrochloride (*4*) and *p*-aminodimethylaniline sulfate (*4*), and these compounds were rejected. Phenol–ferric chloride (*2*), diphenylamine–ethyl alcohol (*8*), 2% ferric chloride, saturated ferric chloride, Fenton's reagent (*9*), bromine water (*7*), resorcinol–sulfuric acid (*5*), and ferric chloride–ammonium hydroxide (*1*) all proved too insensitive. Diazotized sulfanilic acid (*3*), neutral potassium permanganate, and potassium permanganate–sodium carbonate reacted with all the acids but were not so convenient to use as bromophenol blue.

Spray Reagents. Bromophenol blue. A 0.04% solution in 95% ethyl alcohol was adjusted to a definite blue color (pH 6.7 as determined with a glass electrode) with dilute sodium hydroxide.

Ammoniacal silver nitrate (*12*). Equal parts of 0.1 *N* silver nitrate and 0.1 *N* ammonium hydroxide were mixed just prior to use. The sprayed chromatogram was dried at room temperature away from direct sunlight, and the spots that developed were observed after the background became a light tan (maximum contrast), usually in about 4 hours. The observations were made both in daylight and under an ultraviolet lamp of the "black light" fluorescent-tube type with a purple envelope.

Acetic anhydride–pyridine (*6*). A 10% solution (by volume) of acetic anhydride in pyridine was sprayed, and the paper was then heated at 100° C. for 5 minutes. Observations were made in daylight immediately after heating and again after 20 hours but under ultraviolet illumination.

Ammonium vanadate (*10*). A saturated solution of ammonium vanadate in water was used. The spots produced were observed only in daylight while the paper was still wet and again after 20 hours.

Ceric ammonium nitrate (*15*). A 2% solution of ceric ammonium nitrate was prepared in 1 *N* nitric acid. The observations were made by daylight 10 minutes after spraying or when dry enough to handle. Ultraviolet illumination gives better contrasts, but the spots are not truly fluorescent.

These reagents did not indicate any specific groupings in the acids studied.

APPARATUS

A wooden developing tank lined with stainless steel and having a large glass window in the front and back was employed. The sheets of paper were hung from stainless steel trays suspended on shelves near the top of the tank. Openings placed in the tank cover directly over the trays facilitated addition of the organic phase of the solvent system without disturbing the vapor-liquid equilibrium in the tank. There was no special temperature control; however, the laboratory temperature was 25° ± 4° C.

When elevated temperatures were necessary, the samples were dried in a special oven made in this laboratory, the details of which are to be described in a later publication.

The sprayer was an all-glass type operated with air at low pressure.

METHODS

The acid test solutions (15 mg. per ml.) were spotted on the paper sheets (46 × 57 cm.) by use of a 0.01-ml. Breed and Brew pipet. The spot diameters were approximately 1 cm. In most cases this quantity of each acid (150 micrograms per spot) was optimum for both sensitivity and resolution, for known solutions of single acids as well as known and unknown mixtures. Lower quantities can be used, but the sensitivities are generally decreased. Fumaric acid can be run at somewhat lower concentrations but sometimes, because of its low solubility, several drops must be superimposed on the starting line to give sufficient acid. The spots were allowed to air-dry. Individual sheets were prepared for each spray investigated.

The aqueous phase of the two-phase solvent system was placed in trays at the bottom of the tank. The paper sheets were hung from the trays at the top of the tank and held in place by small stainless steel bars (0.25 × 0.125 × 4 inches). The tank cover was clamped in position, and the sheets were equilibrated for 3 hours with the vapor from the aqueous phase. After equilibration, the organic phase of the solvent system was placed in the trays, through the openings in the cover, and the chromatograms were developed overnight, or until the solvent front had moved 35 to 40 cm.

After development of the chromatograms, the sheets were removed from the tank, the solvent front was marked, and the solvents were removed by evaporation by hanging at room temperature for 2 hours in a stream of air in a laboratory hood. The use of elevated temperatures increased the time necessary for complete removal of formic acid.

The spot locations were determined by spraying one of the

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Table I. *R* Values of Some Organic Acids on Paper Chromatograms and Results of Color-Producing Reactions for Further Identification[a]

Acid[b]	*R* Values (Bromophenol Blue)			Reagents and Conditions of Observation: Silver Nitrate + Ammonium Hydroxide		Acetic Anhydride + Pyridine		Ammonium Vanadate		Ceric Ammonium Nitrate	
	R_F	R_{lactic}	R_{malic}	Daylight	UV	Daylight	UV (20 hours)	Daylight	Daylight (20 hours)	Daylight or UV (10 min.)	UV (20 hours)
Gluconic	0.02	0.04	0.07	Yellow	F	–	–	–	Gray	Decolorized	+
Glucosaccharic	0.02	0.03	0.06	White	F	–	Yellow F	Yellow	Gray	Decolorized	–
Galacturonic	0.02	0.03	0.05	Yellow with red edge	Yellow F	–	F	Yellow	Gray	Decolorized	+
Sulfamic	0.08	0.14	0.26	White	Blue F	–	F	Yellow	–	–	++
Glucuronic	0.10	0.15	0.29	Yellow with red edge	F	Yellow	F	–	Gray	Decolorized	–
Ascorbic	0.12	0.19	0.37	Immed. black	Dark	Yellow	F	Blue gray	Gray	Decolorized	+
Tartaric	0.14	0.22	0.42	White	F	–	F	Red	Red	Decolorized	+
Glyceric	0.22	0.36	0.70	Yellow	F	–	–	–	Gray	Decolorized	–
Citric	0.23	0.36	0.70	Pink	F	–	F	Yellow	W. gray	Decolorized	++
α,γ-Dihydroxybutyric	0.29	0.46	0.91	White	F	–	–	Yellow	–	–	–
Malic	0.32	0.51	1.00	Gray	F	–	–	Yellow	Gray	Decolorized	++
α,β-Dihydroxybutyric	0.40	0.65	1.24	Yellow	Yellow F	–	–	–	Gray	Decolorized	–
Glycolic	0.43	0.67	1.30	Yellow	Yellow F	–	–	Yellow	Gray	–	–
Diglycolic	0.43	0.70	1.34	White	Yellow F	–	–	Yellow	–	–	–
Ketoglutaric	0.48	0.80	1.50	White	Pink F	–	W	Yellow	W. gray	Decolorized	–
Maleic	0.53	0.84	1.65	White	F	–	–	Yellow	–	–	–
Tricarballylic	0.53	0.85	1.65	White	F	–	–	Yellow	–	–	–
Malonic	0.53	0.86	1.67	White	F	–	–	Yellow	–	Decolorized	++
Succinic	0.61	0.98	1.93	White	White F	–	–	Yellow	–	–	–
Lactic	0.62	1.00	1.97	Yellow	Yellow F	–	–	W. yellow	Gray	Decolorized	+
Pyruvic	0.65	1.03	1.95	W. white	W. white F	–	–	–	–	–	–
Aconitic	0.66	1.06	2.09	White	White F	Brown	F	Yellow	–	–	+
Citraconic	0.67	1.07	2.16	Gray	F	–	–	Yellow	–	–	–
Itaconic	0.71	1.14	2.24	Gray	Yellow F	Orange	F	Yellow	–	Decolorized	++
α-Hydroxyisobutyric	0.75	1.20	2.32	Yellow	F	–	–	–	–	Decolorized	–
Fumaric	0.79	1.28	2.49	White	F	–	–	Yellow	–	–	–
Furoic	0.83	1.33	2.59	White	F	–	–	Yellow	–	Decolorized	–
Mesaconic	0.85	1.34	2.65	White	F	–	–	Yellow	–	–	–
Sorbic	0.88	1.35	2.80	White	F	–	–	Yellow	–	–	–

[a] F = fluorescent; W = weak; – = no reaction; + = weakly positive spot; ++ = strongly positive spot.
[b] Concentration: 15 mg./ml. Of this, 0.01 ml., or 150 γ, was used per test.

sheets with bromophenol blue. The remaining sheets were sprayed with the identifying reagents and observed under the conditions given under "Materials and Reagents."

RESULTS

The data in Table I are averages of several chromatograms for each of the individual acids and color-producing sprays. The colors indicated are those generally obtained. However, differences in acid concentration, such as below 50 or above 200 micrograms per spot, may cause a strengthening or weakening of the hue, sometimes with a shift to different dominant wave lengths. For this reason, the data presented are offered as a guide and may have to be checked for different circumstances.

The data in Tables I and II in the columns headed R_{lactic} and R_{malic} are presented for reference purposes. The R_{lactic} values are the ratio of the distance moved by the test acid to that moved by pure lactic acid. The R_{malic} values were calculated in reference to the distance moved by pure malic acid.

The number of spots given by lactic acid solutions depends upon the concentrations in the solution analyzed. It is well known that dehydration of a dilute aqueous solution of lactic acid results in concomitant autoesterification, with the formation of lactyllactic acid, lactyllactyllactic acid, and eventually the higher polymers. Thus with 10% lactic acid solution, it was possible to detect only monomeric lactic acid (R_F = 0.62). Solutions of 18% lactic acid showed the monomer and some lactyllactic acid (R_F = 0.74). Solutions of 80% lactic acid could be separated into monomer, dimer, and trimer (R_F = 0.80).

Several acids in addition to those reported in Table I have been chromatographed under the same conditions to determine the *R* values, but their color reactions have not been investigated. Adipic acid, if present, may interfere with the identification of α-hydroxyisobutyric acid. The results are summarized in Table II.

DISCUSSION

The fact that the R_F value alone is not sufficient evidence, in many cases, to identify an unknown material is not peculiar to the paper chromatography of organic acids. Errors due to such factors as temperature variations, solvent concentrations, and sloping fronts are possible in all applications. In addition, compounds having similar mobilities in a particular solvent pair cannot be resolved sufficiently to give reliable R_F values for positive identification. The use of color-producing reagents in conjunction with known acids run simultaneously with the sample increases the possibilities of success.

The acids chosen for this study were representative of acids found in natural materials, and the results may be considered as illustrative of the possibilities of the general procedure. Use of the five spray materials differentiated all but four of the possible pairs of acids tested; the R_F values of these were too similar for complete resolution. These pairs of acids were: tricarballylic-maleic; succinic-citraconic; mesaconic-fumaric; and mesaconic-sorbic. In practice, however, combinations with other acids may be encountered which could increase the doubtful number. For example, if a spot of R_F 0.53 was found, the possibilities would be tricarballylic, maleic, and malonic acids. If only tricarballylic acid or maleic acid was present, the spray technique would not differentiate between them, and final identification could not be made. If either tricarballylic or malonic acid was present, identification would be possible by the color test. The same holds true for maleic or malonic acids. However, if any two of the acids or all three were present, identification by color might lead to serious errors. If all three acids were present in a

Table II. *R* Values of Organic and Inorganic Acids
(Bromophenol blue indicator)

Acid	R_F	R_{lactic}	R_{malic}	Acid	R_F	R_{lactic}	R_{malic}
Aspartic	0.03	0.06	0.09	Hippuric[a]	0.78	1.26	2.44
Glutamic	0.05	0.08	0.16	Glutaric[a]	0.78	1.26	2.44
Sulfuric	0.10	0.16	0.31	Azelaic[a]	0.86	1.39	2.69
Oxalic[b]	0.15	0.24	0.47	*n*-Caprylic[a]	0.88	1.42	2.75
Phosphoric	0.23	0.37	0.72	Sebacic[a]	0.89	1.44	2.78
Levulinic[a]	0.74	1.19	2.31	Hendecanoic	0.90	1.45	2.81
Adipic[a]	0.75	1.21	2.34	Lauric[a]	0.91	1.47	2.84

[a] These materials are weakly acidic and do not show up clearly with indicator spray.
[b] Spot appeared as streak. *R* values are averages from several runs.

mixture, the color reactions would indicate malonic acid to the exclusion of the other two acids.

In practice the technique should be used as follows:

Develop a chromatogram of the unknown material and spray with bromophenol blue. Calculate the R_F values for each spot. From these R_F values, select acids for standards of comparison which have corresponding R_F values. Prepare new chromatograms in quadruplicate, so that each standard acid is run adjacent to an unknown mixture. After drying the paper sheets, spray each chromatogram with the appropriate color-producing reagent and treat and observe as described under "Materials and Reagents."

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