Chromatography of Organic Acids in Cured Tobacco[1]

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In recent years a number of papers have dealt with the separation of organic acids by partition chromatography. (In this paper the term "organic acids" applies to polybasic acids such as oxalic, malic, and citric, excluding amino acids.) Isherwood (1946) described a method for separating similar organic acids on a silica gel column by eluting the acids with n-butyl alcohol-chloroform. Several other investigators have published similar procedures (Frohman *et al*, 1951; Marshall *et al*, 1952; 1949). Bulen, Varner, and Burrell (1952) published a method for separating 16 biologically important organic acids by elution from a silica gel column. Marvel and Rands (1950) reported a method for separating organic acids on a silica gel column without initial addition of mineral acid to the gel. Others (Bulen *et al*, 1952; Isherwood, 1946) have found this to be unsatisfactory for some acids; incorporation of sulfuric acid in the gel itself effectively suppresses ionization of the organic acids, thereby greatly decreasing the amount of "tailing." On the other hand, a recent paper (Roberts and Martin, 1954) has described a modification of the Marvel and Rands technique which still employed an unacidified column.

Titrations of the nonaqueous eluate fractions from silica gel columns have been extremely difficult. Isherwood (1946) titrated his eluates with barium hydroxide to a pH of 8.4 in a nonmiscible system which was agitated by blowing carbon dioxide-free air through the titration vessel. Even under these conditions a correction of approximately 2% had to be added to the observed titer. Similarly, Bulen and others (1952) titrated a nonmiscible system with dilute aqueous sodium hydroxide; near the end point vigorous agitation was necessary to ensure intimate contact between the two phases. Houston and Hamilton (1952) added excess barium hydroxide to the acid fractions and then back-titrated with dilute hydrochloric acid. Frohman and Orten (1953) recommended preparation of derivatives of the organic acids by reaction with resorcinol and subsequent fluorometric estimation, in place of direct titration with standard base. In the experimental section, a method is described which obviates the titration difficulty.

Reagents

Silica gel, grade 70, Davison Chemical Corp.

Dowex 1, X8, 50 to 100 mesh, Dow Chemical Co.

Amberlite IR-112, Rohm and Haas Co.

8-Quinolinol, recrystallized from ethyl alcohol, Eastman White Label, Distillation Products Industries.

Hydrochloric acid, C. P. or equivalent.

Sodium carbonate, C. P. or equivalent.

Ammonium carbonate, C. P. or equivalent.

Anhydrous ether, Mallinckrodt absolute ether, Mallinckrodt Chemical Works.

Chloroform, C. P. or equivalent.

tert-Butyl alcohol, C. P. or equivalent.

All other organic solvents were distilled prior to use.

Apparatus

Rapid evaporator (Numerof and Reinhardt, 1953).

Chromatographic column, 50 ml. buret.

Anion exchange column, 50 ml. buret.

Cation exchange column, borosilicate glass tube, 20 X 1 cm.

Ultraviolet Mineralight, Model SL 2537, Fisher Scientific Co., Catalog No. 111.

Preparations

Purification of Silica Gel. A 50-gram sample of silica gel (80- to 120-mesh) was suspended in 300 ml. of concentrated hydrochloric acid and allowed to stand overnight. The yellow supernatant solution was decanted, fresh concentrated hydrochloric acid was added, and the mixture was shaken, and again allowed to stand. This process was repeated until the solution was colorless. The mixture was next filtered with suction on a sintered-glass funnel. The residue on the filter was suspended in water and washed by decantation until free of chloride, filtered as before, and the material was then suspended in 95% ethyl alcohol. This suspension was filtered and washed with 200 ml. of 95% ethyl alcohol on the sintered-glass disk. The gel was then washed with 200 ml. of absolute ethyl alcohol, suspended in anhydrous ether, filtered, and washed with 500 ml. of anhydrous ether. The gel was heated for 24 hours at 100°C. and finally dried for 24 hours in a desiccator over phosphorus pentoxide in vacuo.

Davison Chemical Corp. Grade 70 silica gel, purified and dried in this way, will adsorb its weight of water and still remain dry enough to "gel" with chloroform. This property is desirable because it allows solutions of organic acids to be taken up with a relatively small amount of gel. The mixture of gel and acids can then be placed on top of the major portion of the column in a compact zone, which permits sharp eluate fractions.

Treatment with hydrochloric acid

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is necessary to remove inorganic cations which form insoluble salts with the organic acids; incomplete washing with hydrochloric acid leads to low recoveries of the acids. The adsorbent properties which are present in some silica gels also are removed in the purification process (Isherwood, 1946).

Preparation of the Developing Solvents. Mixtures of *tert*-butyl alcohol and chloroform were prepared in definite proportions by volume (8, 13, 20, 25, and 30% *tert*-butyl alcohol). Each mixture was shaken with one tenth its volume of water in a separatory funnel in order to saturate the mixture with water.

Preparation of Anion Exchange Column. A piece of glass wool was placed in a 50 ml. buret and then 2 grams of Dowex 1 (wet basis, chloride form) was washed into the buret. After the column had been backwashed with water, a piece of glass wool was placed on top of the column to prevent disturbance of the resin during the addition of eluents. The resin was then treated with about 500 ml. of 2N sodium carbonate (until the eluate gave a negative test for chloride). Two hundred milliliters of 1.5N ammonium carbonate were passed through the column and the column was washed exhaustively with water to remove excess carbonate.

Preparation of Cation Exchange Column. Three grams of Amberlite IR-112 (wet basis, hydrogen form) were placed in a 20 x 1 cm. column. The column was then washed thoroughly with water to remove any excess acid, the eluate being checked with bromocresol purple indicator.

Preparation of Silica Gel Chromatographic Column. Four grams of the silica gel purified as above was weighed into an Erlenmeyer flask, and 4 ml. of 1N sulfuric acid were added to the flask. The dry material was mixed thoroughly with a glass stirring rod. Approximately 25 ml. of water-saturated chloroform were added to the flask, which was stoppered and shaken vigorously to ensure complete homogeneity. The gelled material was transferred to a 50 ml. buret and allowed to pack slowly, by partially opening the buret stopcock. The column was then washed with water-saturated chloroform until it had completely settled.

Preparation of Solvent Systems for Paper Chromatography. The amyl alcohol system (Lugg and Overell, 1948) was prepared by refluxing a mixture of *n*-amyl alcohol, water, and formic acid (20:12:1) for 1 hour. After the water had been drawn off, 0.05% purified 8-quinolinol was added to the organic phase. The miscible phenol system (Stark *et al*, 1951) contained phenol, water, and formic acid (75:24:1) with 0.5% purified 8-quinolinol added. The mesityl oxide system (Bryant and Overell, 1953) was prepared by shaking mesityl oxide, water, and formic acid (7:10:1) in a separatory funnel and separating the two phases. The benzyl alcohol system (Stark *et al*, 1951) was prepared by mixing benzyl alcohol, *tert*-butyl alcohol, isopropyl alcohol, water, and formic acid (30:10:10:10:3).

Procedure

Weigh out an amount of stemmed tobacco to contain approximately 0.5 gram of total organic acids. Suspend this tobacco in 500 ml. of water in a Waring Blendor and homogenize for 10 minutes. Add enough solid sodium hydroxide to bring the solution to a pH of 8 (approximately 200 mg. of sodium hydroxide), blend the basic solution for another 10 minutes, and filter.

Dilute 25 ml. of the filtrate (approximately 25 mg. of total acids) to 100 ml. with water. Pass this water solution slowly (1 ml. per minute) through the Dowex 1 anion exchange column, according to the Bryant and Overell method for the ion exchange isolation of acids from plant tissue extracts (Bryant and Overell, 1953). This eliminates the interfering substances present in the tobacco extract, such as sugars, pigments, and cations. After the meniscus of the filtrate has reached the top of the resin, wash the column thoroughly with water and elute the acids with 100 ml. of 1.5N ammonium carbonate, collecting the eluate in a 100 ml. volumetric flask.

Total Organic Acids. For determination of total organic acids, pipet 25 ml. of the ammonium salt eluate from the anion exchange column into a 125 ml. Erlenmeyer flask. To decompose the ammonium carbonate, place the flask in a water bath and heat the solution to 70° C. until no odor of ammonia can be detected and then cool to room temperature. Pass this concentrated solution slowly (1 ml. per minute) through the cation column and rinse the flask with several small portions of water, which are added to the cation exchange column. Wash the column thoroughly with water (about 50 ml.) Titrate the eluate containing free organic acids with 0.02N sodium hydroxide, using phenolphthalein as indicator.

Evaporate 25 ml. of 1.5N ammonium carbonate and pass it through the cation exchanger for use as a blank. Calculate the total organic acids (per gram of tobacco) as follows:

$$\text{Total Acidity (Meq.)} = \frac{80 \times (\text{Meq. of NaOH for titration} - \text{Meq. of NaOH for blank})}{\text{Weight of tobacco sample (grams)}}$$

Individual Acids. The procedure described here was developed using a mixture of known acids and has been found to work equally as well for the individual acid analysis of tobacco. The acids from different samples of tobacco are first identified qualitatively by paper chromatography.

For quantitative measurement of the amount of each acid present, pipet 50 ml. of the ammonium salt eluate from the anion exchange column into a 200 ml. Erlenmeyer flask with a standard-taper 24/40 joint. Evaporate the ammonium salts to dryness on the rapid evaporator (Numerof and Reinhardt, 1953), using a water bath kept at 60° C., to decompose the ammonium carbonate. Add 1 ml. of 1N sulfuric acid to the residue of dry ammonium salts and swirl the flask to dissolve the acids. Add 1 gram of purified silica gel to the flask and mix the dry material thoroughly with a stirring rod (Bulen *et al*, 1952). Add 25 ml. of water-saturated chloroform to the flask, stopper, and shake vigorously.

Transfer the gelled material to the silica column and rinse the flask with four 5 ml. washings of water-saturated chloroform. Rotate a piece of dry glass wool around the walls of the flask to ensure quantitative removal of the gel and then place the glass wool just inside the buret. Adjust the flow rate to 1 ml. per minute and collect the eluate in a 200 ml. Erlenmeyer flask. After the added gel has packed down, close the stopcock and press the piece of glass wool lightly on top of the gel.

Pass a total of 50 ml. of water-saturated chloroform through the column. When the meniscus is resting just on top of the gel, pass 75 ml. of 8% *tert*-butyl alcohol-chloroform through the column. Collect this eluate, which contains fumaric acid, in a 200 ml. Erlenmeyer flask. Next, pass 75 ml. of 13% *tert*-butyl alcohol-chloroform through the column and collect the eluted succinic acid in another flask. Follow the same procedure using 115 ml. of 20% *tert*-butyl alcohol-chloroform to elute oxalic acid, 90 ml. of 25% *tert*-butyl alcohol-chloroform for malic acid, and 110 ml. of 30% *tert*-butyl alcohol-chloroform for citric acid.

After collection of each fraction, add 25 ml. of water and a piece of

Table 1. Recovery of acids after silica gel chromatography.*

Acid	Amount Added (meq.)	Amount Found (meq.)	Recovery (%)
Fumaric	0.074	0.073	99
Succinic	0.070	0.069	99
Oxalic	0.064	0.061	96
Malic	0.060	0.058	97
Citric	0.065	0.063	97

*Figures constitute an average of five runs.

porous clay plate to each flask and place the flask on a steam bath. When the chloroform has evaporated, leaving a one-phase system, cool the solution to room temperature and wash down the sides of the flask with water. Add 2 drops of 1% alcoholic phenolphthalein to the flask and titrate the acid with 0.02N sodium hydroxide. A blank column should be run for each batch of purified gel.

Calculate the percentage of each acid in the tobacco sample as follows:

$$\% \text{ of acid} = 4 \times \frac{(\text{Meq. of NaOH for titration} - \text{Meq. of NaOH for blank})}{\text{Weight of tobacco sample (grams)}} \times \text{equiv. wt. of acid}$$

Paper Chromatography. Pour the remainder of the ammonium salt eluate from the anion exchange column (approximately 25 ml.) into a 125 ml. Erlenmeyer flask and carry it through the "Total Acid Procedure." Concentrate the eluate, which contains free acids, on the rapid evaporator to approximately 1 ml. Spot the acids on Whatman No. 1 paper and run overnight in the amyl alcohol system described. A few minutes after the paper has been removed from the chamber, examine it under ultraviolet light. The acids are disclosed as dark spots against a light fluorescent background, this fluorescence being stable for at least 24 hours. After drying, the chromatogram can be sprayed with 0.2% alcoholic bromothymol blue solution, revealing the acids as yellow spots on a blue background.

For further confirmation of the identity of an acid spot, chromatograph the mixture in the phenyl, benzyl alcohol, or mesityl oxide system. For chromatograms in the strongly acidic phenol system, it is not necessary to convert the ammonium salts to the free acids prior to chromatography.

Table 2. Paper chromatography of organic acids.

Acid	$R_f \times 100$ A	B	C	D
Caffeic	—	80	63	62
Chlorogenic	—	—	29	66
Citric	41*	34*	22*	36*
Fumaric	80	83	80	57
Galacturonic	11*	7*	2*	12*
Glutaric	80	73	66	76
α—Ketoglutaric	—	65	25	43
Malic	50*	43*	30*	44*
Oxalic	10*	—	11*	23*
Succinic	71	69	60	65
Tartaric	25	—	10	10
Unknown	—	—	43*	—

A. Benzyl alcohol system.
B. Mesityl oxide system.
C. Amyl alcohol system.
D. Phenol system.
*Found in bright tobacco (grades PO-4 and G.D.).

Table 3. Analysis of tobacco samples.

Acid	Developer*	Grade PO-4 Amount titrated meq.	Grade PO-4 % In tobacco sample	Grade G.D. Amount titrated meq.	Grade G.D. % In tobacco sample
Unknown	8	0.008	——	0.006	——
Unknown	13	0.013	——	0.008	——
Oxalic	20	0.009	0.33	0.002	0.07
Malic	25	0.106	5.68	0.071	3.80
Citric	30	0.112	5.73	0.029	1.48
Total		0.248 meq.		0.116 meq.	
Total per gram of sample, meq.		1.984		0.928	

*% *tert*-butyl alcohol in chloroform.

Results

To determine the accuracy of the method, known mixtures of five acids were separated and analyzed according to the above procedure, after preliminary passage through the anion exchange column. As the results in table 1 show, recoveries of from 96 to 99% were obtained. Similar percentages were obtained with quantities as low as 0.01 meq. of each acid.

The data obtained by paper chromatography of known acids and of acids present in tobacco after anion and cation exchange are given in table 2. Amino acids were absent in the anion exchange eluate of tobacco samples as shown by spraying the paper chromatograms with ninhydrin.

Analysis of two samples of bright tobacco PO-4, a poor grade tobacco, and G. D., a good grade tobacco, is shown in table 3. (The tobacco was graded according to market specifications.) The results in table 3 are similar to those obtained by Phillips and Bacot (1953)—that is, the percentages of oxalic and citric acids

appear to vary inversely with the quality of the tobacco.

The total organic acid content of these tobacco samples determined by ion exchange was found to be 3.761 meq. per gram of grade PO-4 and 2.082 meq. per gram of grade G. D., compared with 1.984 and 0.928 meq., respectively, from table 3. The fact that the total acidity was greater than the sum of the amounts of individual acids obtained from a silica gel column may be partially explained by the high uronic acid content of cured tobacco as shown by Phillips and Bacot (1953). The presence of uronic acids in the total acid analysis of tobacco was shown by identifying galacturonic acid by paper chromatography, using several specific spray reagents for its detection (Buch *et al*, 1952). However, paper chromatography of the individual acid eluates from a tobacco sample chromatographed on silica gel did not reveal the presence of galacturonic acid. In fact, no galacturonic acid could be detected even when the polarity of the developing solvent was further increased by using a 50% *tert*-butyl alcohol-chloroform mixture.

The discrepancy between the sum of the individual acids and the total acidity might also be attributed to the inorganic anions which would probably be titrated as a part of the total organic acid fraction but would not be eluted from the silica gel column.

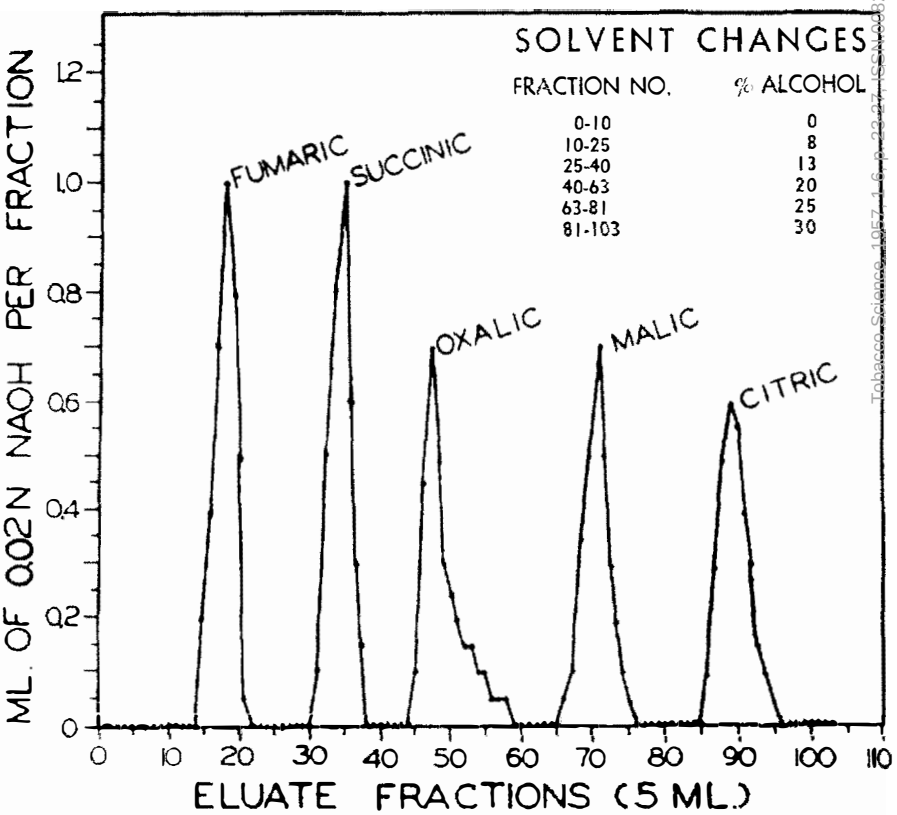

Figure 1. Separation of mixture of known organic acids.

Discussion

It is apparent from table 1 that the acids are eluted from the silica gel column with *tert*-butyl alcohol-chloroform almost quantitatively. By using *tert*-butyl alcohol it is possible to make the silica gel eluate miscible with water, which permits titrations to a sharp end point and eliminates the disadvantages of a two-phase system.

The tertiary alcohol has the further advantage that it prevents the possibility of ester formation, which is likely to occur on the column because of the presence of mineral acid, reactive organic acids, and the large surface afforded. A loss of 77% of oxalic acid was incurred when oxalic acid was allowed to remain on a column containing *n*-butyl alcohol for 2 days. The fact that ester formation could take place in a methanolic solution of oxalic acid was demonstrated in the following manner. A fresh alcoholic solution of oxalic acid was chromatographed on paper and only one spot appeared with an R_f of 0.23 in the phenol system. After this solution had stood for several weeks, it was rechromatographed and two spots appeared. These had R_f values of 0.23 and 0.55 corresponding to oxalic acid and monomethyl oxalate, respectively. On prolonged standing neither spot could be detected from this solution by spraying the chro-

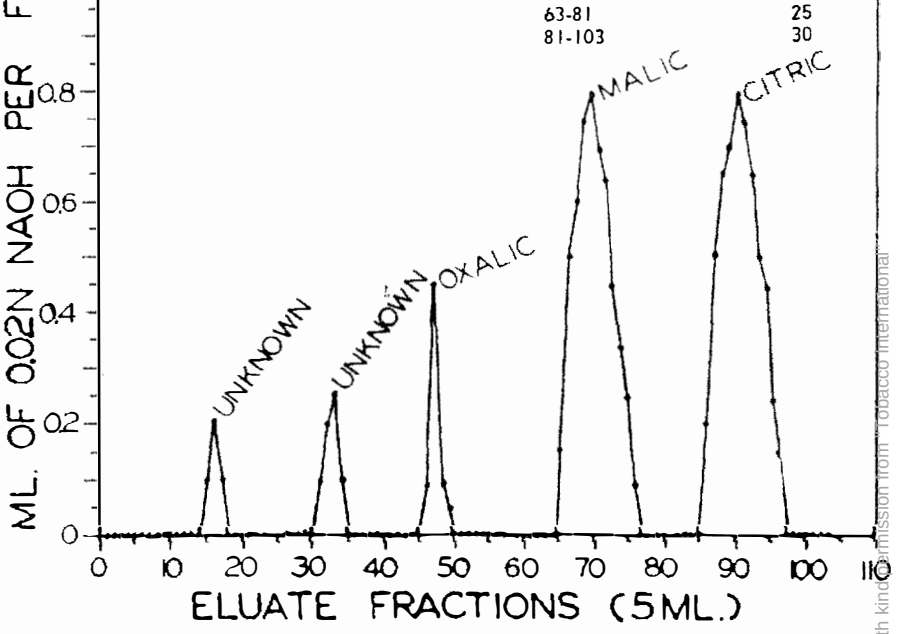

Figure 2. Separation of organic acids from bright tobacco.

matogram with bromothymol blue, indicating complete conversion to the dimethyl ester of oxalic acid.

The solvent schedule for the elution of the acids from the silica gel column was determined by collecting consecutive 5 ml. fractions. The fractions were collected in test tubes containing enough thymol blue indicator solution, prepared with added alkali according to Isherwood's direction (Isherwood, 1946), just to neutralize the sulfuric acid being eluted from the column. Figure 1 shows the separations obtained for a mixture of five acids. The mixture of acids was passed through the anion exchange column prior to separation on the silica gel column.

Similar separations were obtained when a tobacco sample was chromatographed to determine whether the solvent schedule was satisfactory for that particular concentration of tobacco extract. A typical analysis of grade PO-4 bright tobacco is shown in figure 2.

Analysis of several other types of tobacco, not presented here, showed similar separations using this solvent schedule.

When a mixture of five acids (fumaric, succinic, oxalic, malic, and citric) was placed on a silica gel column prepared from unpurified Davison Grade 70 gel, only four acids were recovered, oxalic acid remaining on the column. In another experiment unpurified silica gel was suspended in a saturated oxalic acid solution, following which excess oxalic was removed by passing the appropriate developer through the column. When a known amount of oxalic acid was added to this column, almost 90% was recovered. On the other hand, purification of silica gel with hydrochloric acid, as described above, permits 96% recovery of oxalic acid.

Use of 8-Quinolinol. The addition of 8-quinolinol to solvent systems used in paper chromatography should have wide application because of its fluorescence when exposed to ultraviolet light. In the cases where 8-quinolinol moves with the solvent front, it facilitates the determination of the position of the front in volatile systems or in other systems where the front is not readily distinguishable. Another advantage is that less time is consumed, as papers do not have to be dried and sprayed; instead they can be observed immediately after removal from the chamber. Complex mixtures could be identified in this manner on a single chromatogram—for example, both sugars and acids would show up under ultraviolet light; subsequently the paper could be sprayed with an acid-base indicator to distinguish acid spots from those of sugars.

Summary

A method is described for the analysis of some poly-basic organic acids in tobacco. One sample is sufficient for the determination of total organic acidity, the qualitative identification of these acids by paper chromatography, and the quantitative determination of these individual acids by column partition chromatography. The purification of a commercial silica gel is described. In the quantitative analysis of the organic acids *tert*-butyl alcohol-chloroform mixtures are used for elution of the acids from the silica gel column. These eluates are made water-miscible by evaporation of the chloroform, permitting titrations to sharp end points with dilute aqueous sodium hydroxide. A fluorescence technique for locating organic acids on paper chromatograms has also been developed.

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