Determination of Organic Acids in Silage

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Celite columns with an internal indicator have been developed for the quantitative separation of silage acids ranging from butyric to succinic. The aqueous internal phase employs minimal amounts of sulfuric acid to prevent retention of organic acids; sugar is added to the phase to increase elution resistance. Aqueous samples, 2 ml. or less, are added directly to a dry column cap. Eluting solvents are mixtures of acetone with Skellysolve B. The method is designed to eliminate steam distillation, or ether extraction of acids, and mechanical equipment for collection of fractions. Single zone collections make possible a reduced number of titrations with increased accuracy. The column permits fairly wide separations of lactic and succinic acids, often difficult on silicic acid columns.

In studies of biochemical changes in silages, a method with the advantages of an internal indicator type of chromatograph column was desired to separate butyric, propionic, acetic, formic, lactic, and succinic acids.

Smith (*14*) first demonstrated the separation of volatile organic acids on the partition chromatogram, and the technique has been widely used and extended. Bulen, Varner, and Burell (*3*), Frohman, Orten, and Smith (*6*), Isherwood (*8*), Marshall, Orten, and Smith (*10*), and Marvel and Rands (*11*) have separated a wide range of acids beginning with acetic and stronger acids and extending through the fruit acids. Generally, these authors used adsorbents acidified to a degree that would preclude use of an internal indicator.

Methods using such indicators, but designed to separate acetic and weaker acids, have been published by Elsden (*4*), Fairbairn and Harpur (*5*), Gray (*7*), and Ramsey and Patterson (*13*).

Neish (*12*) and Zbinovsky (*16*) have published methods which separate acids in a range that would include butyric and succinic acids; Zbinovsky used an internal indicator. He recommended that selected groups of acids be run on columns of different size to avoid excessive time.

Details are reported here on a procedure which has been developed to separate silage acids. The method employs Celite columns with alphamine red-R (*9*) as an internal indicator and acetone–Skellysolve B solutions as eluents. A distinctive feature is the use of concentrated sugar solution as the stationary phase to resist leaching action of solvents. In addition, minimal amounts of sulfuric acid are added to the phase to avoid retention of acids by the column. The acids in aqueous solution are transferred directly to the column (*12*).

Apparatus

Chromatographic tube with a fritted-glass filter and delivery stopcock with an inside diameter of 18 mm. and a length (top to stopcock) of 50 cm. was used. A filter, extra porous, was sealed in 2 cm. above the stopcock.

Nitrogen tank with regulator valve to give 0 to 15 pounds per square inch.

Tamping rod, consisting of stainless steel rod, 3.2 cm. in diameter, 60 cm. in length, silver-soldered to the center of the stainless steel disk, 16 mm. in diameter, punched from 16-mesh screening, 24-gage wire.

Cap material stirrer, tamping rod suspended vertically from a chuck of a drill motor attached at an elevated position on a ring stand.

Titration assembly, 25-ml. buret with a two-way stopcock, gravity-fed from a 4-liter polyethylene bottle provided with a carbon dioxide adsorption tube for an air inlet.

Magnetic stirrer.

Materials and Reagents

Celite, Johns Manville's analytical filter aid.

Skellysolve B (Skelly Oil Co.), purification described elsewhere (*15*).

Acetone, distilled through a 50-cm. Vigreux column.

Cresol red indicator. Add 1.3 ml. of 0.1*N* sodium hydroxide to 50 mg. of *o*-cresolsulfonphthalein in 20 ml. of alcohol, make to 50 ml. with water.

Sugar, extra fine granulated (Domino brand).

Organic acids, purified by fractional distillation or recrystallization.

Formic acid dried over anhydrous copper sulfate before distillation. Lithium lactate was used as the lactic acid standard.

Alphamine red-R indicator, 0.4 gram in 100 ml. of water.

Sulfuric acid, 0.1*N*.

Sodium sulfate, anhydrous powder.

Ammonium sulfate, anhydrous powder.

Experimental

Adsorbent. Twelve milliliters of alphamine red-R indicator solution are mixed in a 100-ml. beaker with 30 ml. of sugar solution (2 sugar to 1 water) and 0.5 ml. of 0.1*N* sulfuric acid, resulting in a stationary phase of approximately 50% sugar solution. This mixture is added slowly to a swirling suspension of 75 grams of Celite in 750 ml. of Skellysolve B and acetone solvent, 1 to 1 by volume in a Waring Blendor. Stirring is continued vigorously for 3 minutes. Adsorbent, thus prepared, may be stored in glass-stoppered flasks in a refrigerator for several months.

Developing Solvents. Various percentages by volume of acetone in Skellysolve B are made as follows: 1-, 5-, 10-, 15-, 20-, 30-, and 40%; these are referred to as BA_1, BA_5, etc. To prevent gradual removal of water from the column by dry eluents, concentrations above BA_5 are equilibrated against

the static phase as follows: Four liters are stirred vigorously with 100 ml. of 50% sugar solution to which have been added 2 ml. of saturated barium hydroxide solution and a few drops of cresol red indicator to free the solvent of carbon dioxide and traces of acids; after settling, the solvent is freed of suspended droplets by passing through filter paper.

Standard Acids. Stock solutions of butyric, propionic, acetic, and formic acids are made by pipetting 1.6, 1.3, 1.1, and 0.7 ml., respectively, into 100-ml. volumetric flasks and bringing to volume with carbon dioxide–free water—distilled water boiled for 0.5 hour in a round-bottomed flask, stoppered, and cooled. One and three hundredths grams of succinic acid are made to 100 ml.; 0.840 gram of lithium lactate is made to a 50-ml. volume. A small crystal of thymol is added to each stock solution. A composite test solution is made by pipetting 10 ml. of each stock solution and 10 ml. of 0.7*N* sulfuric acid into a glass-stoppered flask; a crystal of thymol is also added. From this composite test solution, which contains each acid at $^1/_7$ of the concentration of its stock solution, 2-ml. aliquots are used for chromatographic recovery tests. For comparison, titers against 0.005*N* barium hydroxide are obtained on 2-ml. aliquots of seven-fold dilutions of each of the stock solutions with the exception of lithium lactate. Two milliliters of these dilutions are pipetted into a titration flask; approximately 50 ml. of carbon dioxide–free water are added and the solution is stirred (magnetic stirrers) for 3 minutes while a stream of carbon dioxide–free nitrogen is bubbled through the solution to remove traces of carbon dioxide before titration. The solution is then titrated with 0.005*N* barium hydroxide to the cresol red end point.

Column Preparation. Sufficient adsorbent slurry in a separatory funnel is allowed to flow into a well-clamped chromatographic tube nearly to full capacity. A tamping rod is passed lightly through the slurry to dislodge air bubbles. With the stopcock open, a pressure of 10 pounds per square inch is transmitted from a tank of nitrogen to the top of the chromatographic tube; pressure is applied to compress the adsorbent to a fixed volume in the rapidly moving solvent stream and released when the solvent has been expressed to the top level of the adsorbent. Uneven surfaces are leveled by light tamping. BA_1 is added as a fine stream down the side of the tube to a depth of 5 cm., isolating the top surface from further turbulent effects. Eight grams of sodium sulfate, Celite, and ammonium sulfate in the proportions 12 to 8 to 1, hereafter referred to as "cap material," are added as a slurry in about 25 ml. of BA_1. Pressure is applied to compress the cap material. Approximately 75 ml. of BA_1 are forced through the column to remove the BA_{50} solvent initially present; this results in a slightly darker tint of indicator on the adsorbent. Supernatant liquid is expressed to the level of the cap material, the pressure is removed, and the stopcock closed.

Sample Introduction. A cavity, extending about $^2/_3$ down the center of the cap, is made by use of a pointed glass rod 7 mm. in diameter and 50 cm. long. The cavity is enlarged by an off-center motion of the rod sufficiently to permit a 2-ml. sample of acids in water to sink below the top surface. The sample is drained into the cavity from the 2-ml. pipet and approximately 25 ml. of BA_1 are added. The chromatographic tube is detached and placed under the cap material stirrer with the tamper disk poised just above the cavity. The motor is started and the tube moved repeatedly up and down so that the rotating disk homogenizes the charge and cap material by disintegrating all clumps. The disk is permitted to spin itself free of cap material above the solvent, and rinsed with BA_1 after the motor has been stopped.

The chromatograph tube is removed from the stirrer and clamped in position. The cap material is compressed to 5 to 6 cm. in height by a succession of light taps with the tamping rod, which is rinsed and removed. A temporary accumulation of solvent between the stopcock and filter plate is removed by a series of short gentle pulls by vacuum applied to the bottom of the delivery stopcock.

Column Development. Pressure is adjusted to 1 to 2 pounds per square inch to give a drip rate of 2 to 3 drops per second. BA_1 elutes butyric and higher acids, the progress of the acids down the column being followed by an indicator change from orange to blue. The indicator is sufficiently sensitive that valeric acid registers fairly well and may be separated when present at favorable levels. BA_5, BA_{10}, or BA_{15} may be used individually to remove propionic and acetic acids in order. Lower concentrations of acetone give wider separations; higher concentrations give faster elutions in smaller collection volumes. Generally, the choice of eluent for a given column is governed by relative amounts and kinds of acids in adjacent zones. Receiver flasks are changed when the blue zones have been brought down to within 1 cm. of the filter plate. Formic acid is removed by BA_{30} ahead of lactic and succinic acid zones. Although BA_{20} gives wider separations of the latter two zones, BA_{30} is used to speed them down the column until lactic is partially eluted. Then BA_{20} is used to complete the elution of lactic acid while snubbing the progress of the succinic zone. BA_{40} is used to remove succinic acid. Elution volumes on recovery tests are approximately 100 ml. for each of the first three acids and 200 ml. for each of the other three. The time of elution for ten columns run simultaneously is approximately 3 hours; one column by itself requires approximately 1.5 to 2 hours.

Results

Recoveries. Known amounts of acids—2-ml. aliquots of a composite test solution—were chromatographed through 22-cm. columns with BA_1, BA_{15}, BA_{30}, BA_{20}, and BA_{40} in the order as described. Appropriate blank corrections were obtained from titers of equal volumes of eluents passed through blank columns under similar conditions. Blank titers tend to increase with the percentage of acetone and generally range from 0.05 to 0.20 ml. of 0.005*N* barium hydroxide per 100 ml. of eluent. Average recoveries of 97.5 to 101.0% were obtained, as shown in Table I. A recovery test was also made on a composite acid solution in which the propionic, formic, and succinic acids were 4.80, 4.99, and 9.76 microequivalents, respectively, while the butyric, acetic, and lactic acids were 47.8, 54.6, and 50.0 microequivalents. Recoveries for propionic, formic, and succinic acids were

Table I. Recovery of Acids in 2 Ml. of Composite Test Solution

	Butyric	Propionic	Acetic	Formic	Lactic	Succinic
	Added					
Column Number	47.6	47.4	51.7	50.7	49.7	49.7
	Recovered					
1	46.7	47.1	51.6	50.0	48.0	47.9
2	47.0	47.9	51.8	50.7	48.3	49.5
3	47.0	47.5	51.4	50.0	47.9	48.4
4	47.1	48.0	51.7	50.5	49.2	49.7
5	47.3	47.2	51.1	50.0	47.8	48.3
6	47.1	48.5	50.7	50.9	47.7	50.7
7	47.1	47.5	51.5	50.7	49.0	47.5
8	47.6	48.4	51.8	50.6	49.8	49.8
9	46.6	48.1	50.6	50.6	47.4	49.3
10	48.1	48.4	52.1	51.0	49.2	49.1
Av. microequivalents	47.2	47.86	51.55	50.50	48.43	49.02
Av. recovery	99.2	101.0	99.7	99.6	97.5	98.6

5.04, 4.78, and 9.30 microequivalents, respectively, or 105, 96, and 95%; recoveries for butyric, acetic, and lactic acids were 98.3, 99.4, and 98.6%, respectively, where 0.1 ml. of 0.005*N* titrant represented 0.5 microequivalents.

In the latter recovery experiment, blank titers were reduced to practically zero, less than 0.05 ml. per 100 ml. of eluent, by passing the solvents through an alkali-loaded adsorbent column of Hyflo SuperCel; this adsorbent preparation is made as described for adsorbent, 10 ml. of saturated barium hydroxide solution replacing the 0.5 ml. of sulfuric acid.

Table II. Development of Organic Acids in Orchard Grass Silage

Sample No.	Days in Silo	pH	Dry Matter, % Butyric	Propionic	Acetic	Formic	Lactic	Succinic
			Silo 2					
2	0	5.61	0.00	0.04	0.50	0.00	0.17	0.00
60	5	5.64	0.28	0.04	0.99	0.08	2.78	0.54
72	8	4.27	0.00	0.00	1.43	0.12	7.95	1.08
126	15	4.05	0.19	0.04	1.18	0.09	7.41	0.46
150	27	4.42	0.66	0.10	2.30	0.08	9.24	0.94
185	62	4.39	1.18	0.36	3.71	0.05	9.64	0.30
			Silo 6					
1	0	5.84	0.00	0.00	0.25	0.00	0.12	0.00
62	5	5.82	0.08	0.04	0.96	0.05	2.80	0.45
74	8	4.65	0.69	0.04	1.26	0.15	5.46	1.12
128	15	5.00	1.58	0.14	0.91	0.12	2.22	0.74
152	27	5.31	5.42	1.46	5.84	0.05	1.15	0.68
187	62	4.68	5.78	1.28	4.70	0.33	0.13	0.53

Discussion

The use of Celite in preference to silica columns has been suggested by Bueding and Yale (*2*) and by Gray (*7*) because of unpredictable chromatographic properties encountered with various preparations of the latter. Gray reported losses on passing the first sample of acids through his Celite columns; these results were rejected. The authors found that this loss could be prevented by an addition to the stationary phase of either 3 grams of ammonium sulfate or, as described above, 0.5 ml. of 0.1*N* sulfuric acid. The 10 ml. of 0.7*N* sulfuric acid added to the composite test solution of acids (see Standard Acids), serves not only to liberate the lactic acid from its lithium salt, but also to prevent retention of organic acids by the cap material.

Although more sulfuric acid may be desirable in the internal phase for the elution of stronger organic acids, the use of increasing amounts of sulfuric acid renders the column indicator increasingly blue; eventually, a point is reached where weaker acids fail to stand out clearly against the dark background. Approximately 2 to 3 times as much sulfuric acid may be added before this situation prevails.

The prescribed amount of sulfuric acid permits butyric acid to register as a blue zone against a dull orange background when BA_1 is present. BA_5 and higher concentrations of acetone turn the indicator to a bright orange color. Although approximately 50 microequivalents of each acid were used in recovery experiments, the column is sufficiently sensitive to reveal 1 microequivalent of a single acid. Succinic acid, however, gives diminished color with higher concentrations of acetone and 10 microequivalents is the minimal practical amount for visual observation.

Concerning the preparation of the adsorbent, consideration has been given to the tendency of a column to shrink upon addition of eluents richer in acetone. The blending with BA_{50} gives essentially a preshrunk adsorbent.

Acetone also serves to protect alpha-mine red-R from decomposition; this indicator decomposes within a few days if the adsorbent preparation is blended in Skellysolve B alone. Similar protective action of acetone against destruction of carotene by Celite in Skellysolve B has been noted (*15*).

The addition of sugar was designed to stabilize the stationary phase against elution by raising its water-retentive properties. This is reflected by improved resistance of the indicator to elution by stronger solvents such as BA_{50}.

Applications to Silage Extracts and Other Acids. Extracts of silage samples are made as follows: Fifty grams of finely chopped silage are tamped into a 4-ounce wide-mouthed bottle provided with a tight-fitting plastic screw cap. The sample is covered with 50 ml. of 0.6*N* sulfuric acid and a crystal of thymol is added. The bottle is capped and stored in a refrigerator for a week. The contents are mixed and compressed with a stout flat-headed glass rod so that the liquor can be drained into a plastic centrifuge tube. After being centrifuged, the supernatant liquid is drained off in a bottle and stored for analysis. If 2 ml. are taken for analysis, the equivalent dry matter is calculated from, or read from a graph of the equation:

$$\text{Dry matter aliquot} = \frac{2 \times \text{dry matter (\%)}}{200 - \text{dry matter (\%)}}$$

Smaller aliquots may be desirable when individual organic acids exceed 50 microequivalents on the column, in which case correspondingly less cap material is used.

Although the column, as designed, is not practical for the chromatographing of isocitric, citric, and oxalic acids, its usefulness may apply to such acids as pyruvic, glycolic, fumaric, malonic, and α-ketoglutaric which individually have been observed to proceed smoothly down the column.

When used to follow development of organic acids in orchard grass silages, the results in Table II were obtained. Low butyric–high lactic and high butyric–low lactic types of silages are shown in Silos 2 and 6, respectively. Limited data in this laboratory have shown good agreement of acid analysis of extracts obtained by the soaking procedure described above to analyses of blender extracts made according to the American Association of Official Agricultural Chemists' manual (*1*).

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