1963

A Column Chromatographic Analysis of Organic Acids in Silage

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A COLUMN CHROMATOGRAPHIC ANALYSIS OF ORGANIC ACIDS IN SILAGE

BY

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A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Department of
Bacteriology, South Dakota State
College of Agriculture
and Mechanic Arts

August, 1963

A COLUMN CHROMATOGRAPHIC ANALYSIS

OF ORGANIC ACIDS IN SILAGE

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

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ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Doctor Edward C. Berry for his kind guidance, assistance, and encouragement throughout the course of the work reported here and the preparation of this thesis.

The generous help of Doctor John Tanaka and Doctor Robert M. Pengra with matters of procedure, construction, and grammar, of my wife Marlys L. Bawdon with proofreading, and of Sharon Bothwell who typed the thesis, is gratefully acknowledged.

REB

REVIEW OF LITERATURE

Chromatographic analysis of organic compounds is a comparatively new field. In the last twenty years it has been used in an extremely high percentage of the quantitative research done in the biological sciences.

Column Chromatography

The first person to publish observations of chromatographic analysis was a Russian botanist. Tswett (24) in 1906 discovered that if a solution containing a mixture of colored solutes was allowed to run through a vertical glass tube filled with a suitable powdered absorbing material, the material absorbed in the column appeared in a series of colored bands. This indicated that a partial separation of the compounds in the solution had taken place. As more solvent was added, the bands separated more distinctly and eventually passed through the opening at the bottom of the tube. Chromatographic analysis progressed little until in 1931 Lederer and Lederer (16) resolved plant carotene into its various components. Analytic chromatography really started to develop when Claesson (3) recognized the techniques of frontal and displacement analysis.

In 1940 Wilson (27) developed the first theory of chromatography. He took into consideration that equilibrium between solution and absorbant is instantaneous, but he neglected to include the effects of diffusion.

In 1941 Martin and Synge (18) published the first theory which covered the effects of diffusion that Wilson had neglected to include. This theory, which was developed by Martin and Synge, is still held today. Even though the theory was considered accurate, it was criticized by Smith (23), who stated that it is inaccurate because of the adsorption of acids by the column and because of the neutralization of the acids by the alkali indicator. For these reasons, Smith says that the actual yield is nowhere near their theoretical yield.

Isherwood (11) made only slight modifications in the procedure of Martin and Synge (18) and was successful in quantitatively determining acetic, fumaric, glutamic, formic, succinic, and other organic acids.

In 1950 Marvel and Rands (19) separated organic acids on a column of silicic acid. Water and chloroform were used as the eluants. They collected the acids in 10 milliliter fractions and titrated them with 0.0203 normal sodium hydroxide. The acids were eluted from the column by increasing the percent concentration of butanol by 5 percent increments to 30 percent in chloroform. They had a recovery rate of 99 to 102 percent. They separated 39 different low molecular weight organic acids which included those likely to be in biological material. Bulen (1) in 1952 used essentially the same procedure as Marvel and Rands (19). Their recovery rate, however, was only 85 to 100 percent.

Wiseman and Irving (28) in 1957, using modifications in the procedure of Marvel and Rand (19), used 70 grams of Celite to which 30 milliliters of 50 percent sugar water solution and 12 milliliters of

alpha-amine red indicator was mixed in a slurry which was formed in 50 percent acetone in hexane. Several drops of 0.6 normal sulfuric acid solution was added to the column to prevent retention of the organic acids. The eluting solvent was hexane which contained increasing amounts of acetone. They obtained complete separation and elution of the organic acids from the column. Recovery rates were quite high, ranging from 97.5 to 102 percent.

In 1958 Hankinson et al. (9) used a slightly modified procedure of Martin and Synge (18) in which they successfully recovered the fatty acids in milk. Their recovery rate was quite low ranging from 57 to 86 percent.

Frazeur (7) published a paper on column chromatographic methods for determining lactic and citric acid in milk. The methods he used were only slightly modified from those used by Marvel and Rand (19) in 1950. The modification being that the column was three sectioned and was composed of unbuffered sulfuric acid and silicic acid. The eluate was collected in three portions of varied sizes. Lactic acid was the only alpha-hydroxy acid in the first eluate portion, and citric acid was the only acid in the third portion. Citric acid was directly titrated, and lactic acid was converted to the ferric chloride-hydroxy-organic acid and determined colorimetrically. Recoveries were from 94.5 to 100.7 percent.

Gas-liquid Chromatography

Besides the method of column chromatography, there is the new and different method of gas-liquid chromatography. James and Martin (12)

alpha-amine red indicator was mixed in a slurry which was formed in 50 percent acetone in hexane. Several drops of 0.6 normal sulfuric acid solution was added to the column to prevent retention of the organic acids. The eluting solvent was hexane which contained increasing amounts of acetone. They obtained complete separation and elution of the organic acids from the column. Recovery rates were quite high, ranging from 97.5 to 102 percent.

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Gas-liquid Chromatography

Besides the method of column chromatography, there is the new and different method of gas-liquid chromatography. James and Martin (12)

in 1952 used gas partition chromatography to determine the fatty acids from formic acid to dodecanoic acid. They used a column of ethylene glycol monoethyl ether and stearic acid as the stationary phase which was placed on a satisfactory supporting material. The stearic acid is used to prevent tailing of the organic acids being determined. They used a recording burette coupled with a photoelectric cell as a detector. This apparatus made titrations automatic.

In 1956 James and Martin (13) developed a sensitive method of detection in which a thermocouple responded to a change in the density of the gases flowing through it. This is now known as a thermal conductivity cell.

Again in 1956 James and Martin (14), using the above method of detection, determined methyl esters of fatty acids using diazomethane. They included analysis of both low and high molecular weight fatty acids.

In 1960 Hunter et al. (10), using gas liquid chromatography with the use of the thermal conductivity cell and a diethylene glycol adipate polyester column and helium as a carrier gas, successfully separated the volatile fatty acids including iso-butyric and iso-valeric acid. One advantage of this method was the nearly complete resolution of the acids in the presence of water in concentrations up to 50 percent.

In 1961 McWilliam and Dewar (20) developed a new type of detection for the analysis of gases and vapors of organic nature. This detector was called the flame ionization detector and greatly increased

the sensitivity of chromatographic analysis. In some cases complete analysis may be made on only 0.01 microgram of material.

Emery and Koener in 1961 (4), using a column of 20 percent Tween 80 and 2 percent phosphoric acid on a packing of Chromsorb W, successfully determined the organic acids of blood and rumen juice. The acids were extracted from the juice with 1 milliliter of 25 percent meta-phosphoric acid in 5 milliliters of juice. Also, they state that the methyl ester-methyl ester of lactic acid may be formed by the use of diazomethane. The lactic ester may be easily detected by the use of the flame ionization detector. Standard deviation was reported at less than 0.1 percent. Later in 1961 Erwin et al. (5) published results on fatty acid analysis similar to their previous publication (4). The only change in this procedure was that the flame was extinguished while water was passing through the detector cell.

In 1961 Metcalfe (21) used a column of 20 percent diethylene glycol polyester adipate ester and 2 percent phosphoric acid on 60/80 mesh acid washed firebrick. He found it necessary to extinguish the flame of the flame ionization detector. This column was excellent for separating the lower molecular weight fatty acids found in biological material.

In 1962 Gehrke and Lamkin (8) used a thermal conductivity cell to determine the methyl esters of the steam volatile fatty acids and some of the Krebs Cycle acids by formation of barium salts of the organic acids; then, he esterified them with iodomethane. The column used was a silicone 550 (Dow Corning) plus 10 percent stearic acid on Celite 545. The recovery rate was from 93 to 110 percent.

PROCEDURE

Silage Sample Preparation

The silage that was to be analyzed by both chromatographic procedures came from gas tight silos and 55 gallon barrels. It was collected in polyethylene bags and was sealed tightly and stored in the refrigerator at 15 degrees Centigrade until the material was extracted for chromatographic analysis.

In pilot studies, material was ensiled in pint, quart, and one-half gallon jars in the same manner that Cadman (2) used in 1961 when he made bacterial counts on silage fermentations. He steamed the jars and their lids to prevent bacterial contamination. The material which was to be fermented was placed in the jars and the lids which were equipped with "escape valves" were screwed on tightly.[1] The jars were incubated at room temperature and were sampled every seven days. The fermentation lasted for a total of 42 days. When a jar was opened and a sample taken for chromatographic analysis, the remainder of the fermented material was discarded. This was done because it was felt that the introduction of the aerobic conditions would interfere with the natural fermentation. Therefore, for a fermentation which lasted 42 days, a total of six jars would be used in the sampling. In this pilot study, two sets of samples were setup using alfalfa from the

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[1]This was a device placed in the lid to allow the carbon dioxide to be expelled from the jar and, too, at the same time maintain anaerobic conditions.

same field and packing the same amount of material into each jar. Special care was taken to make sure each jar was sealed from the outside air. Also, other conditions were the same for both series of samples.

To prepare a sample for chromatographic analysis, the following procedure was followed. A 20 gram sample of silage was extracted by the addition of 20 milliliters of 20 percent meta-phosphoric acid. Approximately 30 minutes was allowed for the extraction of the organic acids to take place. During this time they were mixed occasionally. Then to extract the liquid from the solid material, the liquid and the silage was mixed, and the liquid was poured into a centrifuge tube. Centrifugation was used only to remove debris that may plug pipettes and syringes during the analysis. After centrifugation the liquid was decanted into tubes that were then stoppered with plastic caps. The sample was then ready for analysis. Every 2 milliliters of liquid sample represented 1 gram of the silage sample.

Sample Analysis by Gas-liquid Chromatography

The instrument used for the analysis was a Wilkens Aerograph Hy-Fi Model A 600-B which employs the flame ionization detector method. To the Model A 600-B is attached a Brown Honeywell integral recorder with the disc integrator attachment which limits instrument error to plus or minus 0.1 percent.

The conditions for the chromatographic analysis were as follows: injector temperature 250 degrees Centigrade, column oven temperature

155 degrees Centigrade, input impedance 10^9 ohms, output impedance 10X. Input impedance according to Wilkens Instrument and Research, Incorporated (25) is defined as follows:

> This two-position switch selects the grid resistance to the electrometer input of either 10^9 or 10^7 ohms. The sensitivity of the instrument is exactly 100 times greater at the 10^9 ohm setting than the 10^7 ohm setting.

The output impedance is defined as follows:

> This two-position switch can be used to increase the signal output of the electrometer 10 fold. The highest sensitivity occurs at the setting of 10X.

The attenuator was set at varied attenuation from 8X to 64X. The attenuator according to Wilkins Instrument and Research, Incorporated (25) is defined as follows:

> This binary 11-position stepping switch cuts the electrometer signal output by one-half every step. Maximum sensitivity of the instrument is at the setting of 1.

Each position of the switch actually has a resistance twice that of the preceding switch setting. The flow of the hydrogen was 30 milliliters per minute and for nitrogen it was 10.6 milliliters per minute.

The sample size was 0.0002 milliliters and 1 percent accuracy between samples may be obtained from the Hamilton microliter syringe with the use of the Chaney adapter.

The column used was 5-feet long with an outside diameter of 1/8 inch. The column contained 20 percent diethylene glycol adipate ester with 2 percent phosphoric acid absorbed on 60/80 mesh firebrick. The phosphoric acid was added to prevent tailing of the organic acids and hence possible error in analysis. The column described above did an

excellent job of separating the organic acids as indicated by the retention time. The retention time for acetic acid was 4.4 minutes, for propionic acid it was 5.7 minutes, and for butyric acid it was 7.9 minutes.

Retention time is defined as the time from injection to the maximum height of the peak made by the unknown organic compound. Retention time is important in the identification of components of the sample. This may be substantiated by a quotation from Keulemans, A.I.M. (15).

> Under a definite set of operational conditions the retention volume or retention time is characteristic of a certain component. In many cases--particularly in routine work--the components present in the mixture to be analyzed will be known with fair certainty, and so long as conditions remain unaltered the constituents can then be identified by a simple comparison of their retention volumes with those found in some previous analysis. The time axis of a constant-speed recorder chart can obviously be used for this purpose instead of a volume axis.

According to Lovelock (17) formic acid is one of the few organic compounds which cannot be detected by the use of gas-liquid chromatography using this type of detector. Therefore, this is one of the disadvantages of the use of the flame ionization detector when working with unesterified fermented material. It must also be included that there were other columns tried such as Tween 80 (polyoxyethylene sorbiton mono oleate ethers of mixed partial oleic ester of sorbitol anhydride) and carbowax 20 M (salbase, solid polyethylene glycols of the general formula $HO{\cdot}CH_2(CH_2)(CH_2)_x\ (CH_2OH)$. It was found that they were unstable and caused base line drift which

produced a significant error in analysis. This unstable base line is caused as a result of the high operation temperature and the bleeding of the column packing into the detector. This could not be remedied because the high temperature is necessary for the removal of the organic acids from the column. However, the 20 percent diethylene glycol adipate ester column used has a much higher decomposition point, hence it has less base line drift and can be used practically indefinitely.

Also it must be noted that lactic acid, one of the most important constituents of silage, cannot be determined using this type of analysis. According to Feiser and Feiser (6) lactic acid when boiled becomes anhydrous, forming the cyclic compound d-l lactide. This may be decomposed by heat into carbon dioxide and water which cannot be determined using gas-liquid chromatography. Since this is a problem which hinders the complete analysis of silage using gas-liquid chromatography, it is probable that a methyl ester of lactic acid may be formed by the use of diazomethane.

There is another method of analysis of lactic acid whereby the silver salt of the acid is formed, and then it is esterified with iodomethane. Since Gehrke and Lamkin (8) did not obtain a recovery rate significantly better than the procedure described here, this method was not used as a method of analysis.

Sample Analysis by Column Chromatography

The analytic columns were prepared in the following manner. Seven hundred milliliters of 50 percent acetone-hexane were added to

80 grams of Celite in a Waring blendor. This mixture was added to a solution containing 30 milliliters of 50 percent sugar and water plus 12 milliliters of alpha-amine red indicator. The solution was added slowly to the Celite solvent slurry and blended for several minutes to insure that the slurry was uniform. One to two drops of 20 percent meta-phosphoric acid was added to the slurry; then blended again for a time. The phosphoric acid was added to the slurry to prevent the columns from retaining minute quantities of the organic acids from the standards or the silage sample. More phosphoric acid may be added to the column, but if an excess is added, the column becomes a very intense blue and will mask the elution of the organic acids. With the slurry prepared in the above manner, there was sufficient quantity to prepare five columns for organic acid analysis.

As the slurry was added to the columns, it was compressed with approximately 10 pounds of nitrogen pressure. It must be noted that when the pressure is applied, the solvent level should not be allowed to become lower than the column packing level. If this occurs, it will be difficult to resaturate the column packing. After the columns, which were 2.2 centimeters in diameter and 40 centimeters in length, had been filled with Celite slurry to a depth of approximately 20 centimeters, a solution of 1 percent acetone in hexane was added. This was added to each column to remove the 50 percent acetone-hexane solution. Approximately 30 milliliters of the solution were added. After this was allowed to drain from the column, an anhydrous cap was

prepared to cover the previously added Celite slurry. The cap consisted of 12 parts of anhydrous sodium sulfate, 8 parts Celite, and 1 part anhydrous ammonium sulfate. A mixture of this ration was made containing 24 grams of sodium sulfate, 16 grams of Celite, and 2 grams of ammonium sulfate. This was sufficient material to cap five columns. The cap material was added to the column in a slurry formed by the addition of 1 percent acetone-hexane. This material was compressed with nitrogen gas pressure.

The sample containing the organic acids was introduced into the cap material with the solvent level slightly below the upper level of the cap. This was to allow the maximum absorption of water by the cap material. Between 20 and 40 milliliters of a 1 percent mixture of acetone-hexane was added to the column. The column was placed under a stirring motor with a stirring rotor to fit into the small column opening. The cap material and sample were homogenized to complete the removal of water and to make the cap and sample a uniform mixture. When the rotor was removed from the column, it was rinsed with 1 percent acetone-hexane to remove the organic acids. After the removal of the rotor, the cap material was gently packed and the columns were ready for elution. Before the solvent was added, a 250 milliliter separatory funnel was placed on each column to act as a solvent reservoir.

The solvents used for the extraction of the organic acids from the sample were varying concentrations of acetone in hexane. Both components were Fisher Analytical Reagents and were free of carbon

dioxide. The concentration of solvents during the elution consisted of 1, 10, 20, 30, and 40 percent acetone in hexane. The solvent was made carbon dioxide free by the addition of a solution of 97 milliliters of 50 percent sugar water solution, 3 milliliters of 0.05 normal sodium hydroxide solution, and several drops of phenol red indicator. This was added to 4 liters of the acetone-hexane solvent solution. The solvent and the carbon dioxide extracting solution were stirred on a magnetic stirrer to allow complete removal of carbon dioxide from the solvent solution. The blank titration on the solvent using phenolphthalein as an indicator and 0.005 normal sodium hydroxide was zero. The solution prepared earlier and added to remove carbon dioxide also served to saturate the solvent with water. This solution also prevented leaching of the water from the column. This was added to all the solvent solutions but the 1 percent acetone-hexane solution because there was not enough acetone present to cause serious leaching of water from the stationary phase of the column.

The organic acids were eluted from the column in the following order using the following solvents: 1 percent acetone-hexane eluted butyric before propionic acids, 10 percent acetone-hexane eluted acetic acid from the column quite rapidly but hindered the elution of lactic acid and succinic acid if they were present, 30 percent acetone-hexane rapidly eluted lactic acid from the column and eluted succinic acid very slowly, 40 percent acetone-hexane rapidly eluted succinic acid from the column when it was present in the sample.

As the organic acids were eluted from the column under 2 pounds of nitrogen gas pressure, they formed blue bands in the presence of the alpha-amine red indicator. As the individual bands moved close to the end of the column, each was collected and tightly stoppered in an individual erlenmeyer flask until titration. See Appendix, page 42, for a photograph of the apparatus used in the analysis.

To titrate the individual samples of organic acids, 50 milliliters of recently boiled carbon dioxide free water was added. Also to each flask was added several drops of phenolphthalein indicator. Before titration the flasks were placed on a magnetic stirrer and nitrogen gas was bubbled through the liquid to expel the carbon dioxide present. The magnetic stirrer was used to make sure that the acetone and organic acid was extracted from the hexane into the water layer.

The organic acid was then titrated with 0.005 normal sodium hydroxide. The 0.0502 normal sodium hydroxide solution was used when a sample larger than 2.0 milliliters was used or if there was an excessive amount of the organic acid. The 0.005 normal sodium hydroxide solution was checked daily with potassium acid phthalate using the procedure described in the Official Methods of Analysis of the Association of Official Agricultural Chemists (22). The 0.0502 normal sodium hydroxide solution was checked at least weekly by the same procedure.

RESULTS AND DISCUSSION

Standard Preparation and Calculations

In order to check the maximum and the minimum limits of the column chromatographic method and to check the rate of recovery of this method, two sets of standards were made which contained varied amounts of the organic acids found in silage and possibly in other fermented materials.

The standards were prepared by weighing out a predetermined amount of the analytical reagent grade organic acid on the Mettler analytical balance. The amount of each organic acid was determined by the following formula:

$$\frac{\text{Weight in grams}}{\text{Equivalent weight}} = \text{the number of gram-equivalents}$$

$$\frac{\text{Number of gram-equivalent}}{\text{Total volume of liquid in milliliters}} = \text{the number of gram-equivalents per milliliter}$$

The contents of standard number I may be found in Table 1.

Table 1. Contents of Standard Number I

Organic acid	Micro-equivalents
Butyric	13.23
Propionic	13.23
Acetic	19.94
Formic	24.50
Lactic	9.50

The contents of standard number II may be found in Table 2.

Table 2. Contents of Standard Number II

Organic acid	Micro-equivalents
Butyric	253.23
Propionic	245.69
Acetic	251.54
Formic	257.74
Lactic	214.17

The amount of the organic acids in the sample was calculated using the following formula:

V x N = the micro-equivalents of organic acid present in one gram of silage

V = the volume of the standard base in microliters
N = the normality of the standard base

Since 2 milliliters of the sample extract is very closely equal to 1 gram of silage, there is no dilution.

For gas-liquid chromatography the total disc units for the individual acids were averaged and setup proportional to the concentration of the organic acids in the following formula. Disc units are an arbitrary value which determines the area under the peaks.

$$\frac{AD}{DU} = \frac{CSO}{CUO} \times D = \text{the amount of organic acid present per gram}$$

AD = the average disc units of the standard organic acid
DU = the disc units of the unknown quantity of the organic acid
CSO = the concentration of the standard organic acid in equivalents per milliliter
CUO = the unknown concentration of the organic acid sample in micro-equivalents per milliliter
D = the conversion factor for comparing the samples analyzed by the two methods

Discussion of Data

Two milliliters of standard I on Table 1 were analyzed by column chromatography, and it was found on Table 3 that this standard containing such small quantities of the organic acid approached the lower limit of column efficiency. Even at this low level butyric, propionic, and acetic acids were still quite distinct and the bands were quite bright. The formic and lactic acids were quite difficult to determine quantitatively because of the difficulty in distinguishing the edges of the colored bands formed by these acids. It must be noted that on Table 3 the recovery rate for propionic acid was 78 percent and for acetic acid was 97 percent. The recovery rates for butyric, formic, and lactic acids were very high; they were 130, 120, and 190 percent respectively.

One milliliter of standard II on Table 2 was analyzed for organic acids, and it was found on Table 4 that the concentrations of propionic and butyric acids in this sample (which contained approximately 20 times the amount of material contained in standard number I) were not sufficiently well separated by the column to allow accurate analysis of these acids, although the remainder of the acids in the sample were sharply separated. The recovery rates for acetic, formic, and lactic acids were 98, 103, and 91 percent respectively. One-half milliliter of standard number I was analyzed for organic acids, and it was found on Table 5 that there was still insufficient separation to quantitatively determine butyric and propionic acids. The recovery rates for the acetic, formic, and lactic acids were

Table 3. Column Chromatographic Analysis of
2.0 Milliliters of Standard Number I

	Butyric		Propionic		Acetic		Formic		Lactic	
	Amount*		Amount*		Amount*		Amount*		Amount*	
Sample Number	Added	Recovered	Added	Recovered	Added	Recovered	Added	Recovered	Added	Recovered
1	26.46	34.00	26.46	32.00	39.88	35.00	49.00	65.00	19.04	24.00
2	26.46	35.00	26.46	30.00	39.88	37.00	49.00	62.00	19.04	39.00
3	26.46	35.00	26.46	33.00	39.88	36.00	49.00	62.00	19.04	46.00
4	26.46	35.00	26.46	30.00	39.88	40.00	49.00	57.00	19.04	31.00
5	26.46	34.00	26.46	30.00	39.88	32.00	49.00	58.00	19.04	30.00
6	26.46	33.00	26.46	28.00	39.88	36.00	49.00	52.00	19.04	65.00
7	26.46	33.00	26.46	33.00	39.88	-	49.00	56.00	19.04	65.00
8	26.46	41.00	26.46	31.00	39.88	52.00	49.00	65.00	19.04	17.00
9	26.46	44.00	26.46	31.00	39.88	44.00	49.00	56.00	19.04	27.00
10	26.46	20.00	26.46	31.00	39.88	43.00	49.00	59.00	19.04	32.00
11	26.46	36.00	26.46	20.00	39.88	26.00	49.00	54.00	19.04	17.00
Average Recovery		34.00		30.00		38.00		59.00		36.00
Percent Recovery		130.00%		78.00%		97.00%		120.00%		190.00%

*Expressed in micro-equivalents.

Table 4. Column Chromatographic Analysis of
1.0 Milliliter of Standard Number II

	Butyric		Propionic		Acetic		Formic		Lactic	
	Amount*		Amount*		Amount*		Amount*		Amount*	
Sample Number	Added	Recovered	Added	Recovered	Added	Recovered	Added	Recovered	Added	Recovered
1	253.23	#	245.69	#	251.54	251.00	257.74	256.00	214.17	210.00
2	253.23	#	245.69	#	251.54	245.00	257.74	266.00	214.17	215.00
3	253.23	#	245.69	#	251.54	245.00	257.74	270.00	214.17	200.00
4	253.23	#	245.69	#	251.54	240.00	257.74	271.00	214.17	210.00
5	253.23	#	245.69	#	251.54	245.00	257.74	266.00	214.17	230.00
6	253.23	#	245.69	#	251.54	245.00	257.74	266.00	214.17	175.00
7	253.23	#	245.69	#	251.54	256.00	257.74	271.00	214.17	160.00
8	253.23	#	245.69	#	251.54	256.00	257.74	271.00	214.17	160.00
Average Recovery						247.00		267.00		195.00
Percent Recovery						98.00%		103.00%		91.00%

*Expressed in micro-equivalents.
#Not sufficient separation for quantitative determinations.

Table 5. Column Chromatographic Analysis of
0.5 Milliliter of Standard Number II

	Butyric		Propionic		Acetic		Formic		Lactic	
	Amount*		Amount*		Amount*		Amount*		Amount*	
Sample Number	Added	Recovered	Added	Recovered	Added	Recovered	Added	Recovered	Added	Recovered
9	126.61	#	127.35	#	125.77	135.00	128.87	150.00	107.34	96.00
10	126.61	#	127.35	#	125.77	114.00	128.87	154.00	107.34	102.00
11	126.61	#	127.35	#	125.77	131.00	128.87	145.00	107.34	99.00
12	126.61	#	127.35	#	125.77	135.00	128.87	124.00	107.34	92.00
13	126.61	#	127.35	#	125.77	132.00	128.87	127.00	107.34	97.00
Average Recovery						129.00		140.00		97.00
Percent Recovery						103.00%		109.00%		91.00%

*Expressed in micro-equivalents.
#Not of sufficient quantity for quantitative determination.

103, 109, and 91 percent respectively. The results are quite comparable to those found using 1 milliliter of standard number I.

Using 0.2 of a milliliter of standard number II, it was found on Table 6 that the column would efficiently separate the butyric and propionic acids quite well. If the columns were evenly packed, there was no tailing of the butyric acid into the propionic acid. The recovery rates for 0.2 of a milliliter of standard number II were 80.00, 110.00, 92.00, 120.00, and 100.00 percent for butyric, propionic, acetic, formic, and lactic acids respectively.

From the data in Tables 3, 4, 5, and 6 it can be concluded that the minimum amount for an accurate determination was approximately 12 micro-equivalents of each acid per milliliter, and the maximum amount was approximately 50 micro-equivalents of each acid. This was not a limiting factor for the determination of acetic, formic, and lactic acids. These acids may be accurately determined in amounts exceeding 250 micro-equivalents. In most cases this is a higher concentration than will be found in most silage or fermented material.

The acids in standards I and II were also determined using gas-liquid chromatography and, according to the data in Tables 7 and 8, there was very little difference in the quantity shown by the two methods. Since these samples contained water, and water according to Wilkens Instrument and Research, Incorporated (26) cannot be quantitatively determined using gas-liquid chromatography; the standards were setup using the proportion described in the procedure on page 17. Presently there is no other method of determining the quantity of the

Table 6. Column Chromatographic Analysis of 0.2 Milliliter of Standard Number II

	Butyric		Propionic		Acetic		Formic		Lactic	
	Amount*		Amount*		Amount*		Amount*		Amount*	
Sample Number	Added	Recovered	Added	Recovered	Added	Recovered	Added	Recovered	Added	Recovered
14	50.65	19.00	49.14	53.00	50.31	43.00	51.55	62.00	42.94	42.00
15	50.65	46.00	49.14	58.00	50.31	46.00	51.55	65.00	42.94	34.00
16	50.65	43.00	49.14	56.00	50.31	49.00	51.55	54.00	42.94	39.00
17	50.65	49.00	49.14	55.00	50.31	49.00	51.55	65.00	42.94	41.00
18	50.65	47.00	49.14	50.00	50.31	39.00	51.55	68.00	42.94	37.00
19	50.65	39.00	49.14	59.00	50.31	37.00	51.55	62.00	42.94	43.00
20	50.65	44.00	49.14	58.00	50.31	47.00	51.55	60.00	42.94	45.00
21	50.65	32.00	49.14	59.00	50.31	43.00	51.55	74.00	42.94	45.00
22	50.65	45.00	49.14	59.00	50.31	56.00	51.55	56.00	42.94	51.00
23	50.65	35.00	49.14	59.00	50.31	53.00	51.55	56.00	42.94	48.00
Average Recovery		40.00		57.00		46.00		62.00		42.00
Percent Recovery		80.00%		110.00%		92.00%		120.00%		100.00%

*Expressed in micro-equivalents.

Table 7. Gas-liquid Chromatographic Analysis of
0.0002 Milliliters of Standard Number I

	Butyric		Propionic		Acetic	
Sample Number	Disc Units	Micro-equivalents	Disc Units	Micro-equivalents	Disc Units	Micro-equivalents
1	7.2	30.00	4.6	27.00	2.9	39.00
2	4.9	21.00	3.4	20.00	3.6	44.00
3	6.5	27.00	4.6	27.00	3.0	37.00
4	6.0	25.00	4.6	27.00	3.2	39.00
5	6.7	28.00	5.4	32.00	3.4	41.00
6	5.6	24.00	4.1	24.00	3.2	40.00
Average Recovery		25.00		26.00		40.00
Average Disc Units	6.1		4.4		3.2	

Table 8. Gas-liquid Chromatographic Analysis of 0.0002 Milliliters of Standard Number II

	Butyric		Propionic		Acetic	
Sample Number	Disc Units	Micro-equivalents	Disc Units	Micro-equivalents	Disc Units	Micro-equivalents
1	30.00	491.00	19.70	489.00	10.30	504.00
2	32.00	521.00	21.00	521.00	10.00	489.00
3	32.40	531.00	19.30	479.00	10.00	489.00
4	32.00	526.00	20.40	566.00	9.90	485.00
5	33.00	540.00	20.40	506.00	9.90	485.00
6	32.90	539.00	21.00	521.00	10.10	499.00
7	30.70	503.00	19.80	487.00	12.30	612.00
8	31.70	519.00	20.20	501.00	10.60	519.00
9	28.20	462.00	19.00	472.00	9.60	470.00
10	30.60	501.00	20.20	501.00	10.20	499.00
11	29.70	486.00	18.70	465.00	10.30	504.00
12	31.90	522.00	20.00	496.00	10.60	519.00
13	28.80	471.00	19.00	472.00	10.10	494.00
14	28.80	471.00	17.30	429.00	9.80	463.00
Average Recovery		506.00		493.00		502.00
Average Disc Units	30.80		19.70		10.20	

unesterified organic acids in material such as silage because of the variable concentration of the water and the many volatile compounds that are usually present.

To compare the two procedures, 36 silage samples were analyzed by the column chromatographic procedure and gas-liquid chromatographic procedure. Tables 9, 10, 11, and 12 show the actual results expressed in micro-equivalents and Figures II and III show the actual comparison of the samples determined by the two procedures.

The results of the two procedures compare quite favorably and, as a general rule, the column chromatographic results are slightly lower than those of the gas-liquid chromatographic results. There are three exceptions where there was no close relationship between the two methods of determination. In trying to determine the cause of the variation, sample number 5 was analyzed again using both methods of determination, but the results came out identical to those of the first determination. Therefore, there is no easy explanation as to why there was so much variation in the determination of this sample.

Method of Analysis

The formation of the methyl ester of the organic acids was not used as a method of analysis because of the following four reasons: (1) lactic acid and other organic acids are in micro-equivalent quantities, and losses resulting from handling during the esterification process may become excessive; (2) under laboratory conditions, it was

Table 9. Column Chromatographic Analysis
For Organic Acids in Silage

Silage Sample Number	Butyric Acid*	Propionic Acid*	Acetic Acid*	Formic Acid*	Lactic Acid*	Succinic Acid*
1	#	#	100.00	#	#	#
2	#	#	120.00	#	330.00	#
3	#	#	390.00	#	390.00	#
4	#	#	330.00	170.00	190.00	#
5	#	#	540.00	150.00	300.00	#
6	#	#	180.00	320.00	320.00	#
7	Trace	#	100.00	50.00	390.00	#
8	#	#	160.00	90.00	300.00	#
9	#	#	250.00	Trace	320.00	#
10	#	#	100.00	Trace	360.00	#
11	#	#	220.00	50.00	60.00	#
12	#	#	70.00	Trace	80.00	#
13	#	#	40.00	140.00	30.00	#
14	#	#	-	100.00	410.00	#
15	#	#	100.00	-	-	#
16	#	#	460.00	Trace	180.00	#
17	#	#	150.00	Trace	220.00	#
18	#	#	240.00	Trace	240.00	#
19	#	#	150.00	Trace	160.00	#
20	#	#	120.00	Trace	510.00	#
21	#	#	100.00	100.00	470.00	#
22	#	#	100.00	Trace	300.00	#
23	#	#	150.00	Trace	420.00	#

*Expressed in micro-equivalents.
#Not of sufficient quantity for quantitative determination.
-Separated acid was lost in analysis.

Table 10. Column Chromatographic Analysis
For Organic Acids in Silage

Silage Sample Number	Butyric Acid*	Propionic Acid*	Acetic Acid*	Formic Acid*	Lactic Acid*	Succinic Acid*
24	#	#	110.00	Trace	20.00	#
25	#	#	100.00	Trace	240.00	#
26	#	#	170.00	50.00	400.00	#
27	#	#	150.00	Trace	320.00	#
28	#	#	250.00	Trace	170.00	#
29	#	#	220.00	80.00	300.00	#
30	#	#	102.00	30.00	350.00	#
31	#	#	90.00	50.00	350.00	#
32	#	#	130.00	70.00	360.00	#
33	#	#	140.00	50.00	330.00	#
34	#	#	40.00	330.00	300.00	#
35	#	#	130.00	80.00	120.00	70.00
36	#	150.00	40.00	100.00	350.00	#
37	#	#	210.00	30.00	170.00	120.00
38	#	#	340.00	50.00	630.00	#
39	#	#	410.00	70.00	70.00	140.00
40	#	250.00	140.00	60.00	-	#
41	#	#	370.00	40.00	450.00	#
42	#	#	430.00	Trace	430.00	#
43	#	#	250.00	Trace	250.00	#

*Expressed in micro-equivalents.
#Not of sufficient quantity for quantitative determination.
-Separated acid was lost in analysis.

Table 11. Gas-liquid Chromatographic Analysis For Organic Acids in Silage

Sample Number	Attenuation*	Acetic		Propionic		Butyric	
		Disc Units	Micro-Equivalents	Disc Units	Micro-Equivalents	Disc Units	Micro-Equivalents
1	32	8.2	100.00	Trace	#	Trace	#
2	32	10.7	132.00	#	#	#	#
3	64	17.7	437.00	#	#	#	#
4	64	14.0	345.00	#	#	#	#
5	32	12.4	153.00	#	#	#	#
6	32	12.2	190.00	#	#	#	#
7	32	9.3	114.00	#	#	#	#
8	32	13.4	165.00	#	#	#	#
9	64	10.6	261.00	#	#	#	#
10	64	3.6	88.00	#	#	#	#
11	32	17.2	212.00	1.1	65.00	.3	10.00
12	64	3.7	91.00	#	#	#	#
13	64	18.1	446.00	#	#	#	#
14	64	3.6	88.00	#	#	#	#
15	32	20.6	254.00	#	#	#	#
16	64	17.0	419.00	.2	1.00	#	#
17	32	12.3	151.00	#	#	#	#
18	32	19.6	241.00	.6	3.00	#	#
19	32	9.2	113.00	#	#	#	#
20	64	3.7	91.00	#	#	#	#
21	32	10.0	123.00	#	#	#	#
22	32	8.9	109.00	#	#	#	#
23	32	13.6	168.00	#	#	#	#

*Change in instrument sensitivity.
#Not of sufficient quantity for quantitative determination.

Table 12. Gas-liquid Chromatographic Analysis
For Organic Acids in Silage

Sample Number	Attenuation*	Acetic		Propionic		Butyric	
		Disc Units	Micro-Equivalents	Disc Units	Micro-Equivalents	Disc Units	Micro-Equivalents
24	32	7.9	97.00	#	#	#	#
25	32	9.4	116.00	#	#	#	#
26	32	17.2	212.00	#	#	#	#
27	32	12.1	149.00	#	#	#	#
28	32	22.5	277.00	Trace	#	#	#
29	32	22.0	271.00	#	#	#	#
30	32	7.8	97.00	#	#	#	#
31	16	12.0	149.00	#	#	#	#
32	32	11.0	135.00	#	#	#	#
33	32	12.6	155.00	Trace	#	#	#
34	64	2.4	59.00	#	#	#	#
35	64	7.2	177.00	#	#	#	#
36	64	17.1	422.00	.3	179.1	#	#
37	32	--	--	#	#	#	#
38	32	2.6	321.00	.5	298.6	#	#
39	32	10.7	132.00	#	#	#	#
40	32	12.3	151.00	.3	179.1	#	#
41	32	17.6	217.00	#	#	#	#
42	32	10.0	120.00	#	#	#	#
43	32	30.3	395.00	Trace	#	#	#

*Change in instrument sensitivity.
#Not of sufficient quantity for quantitative determination.

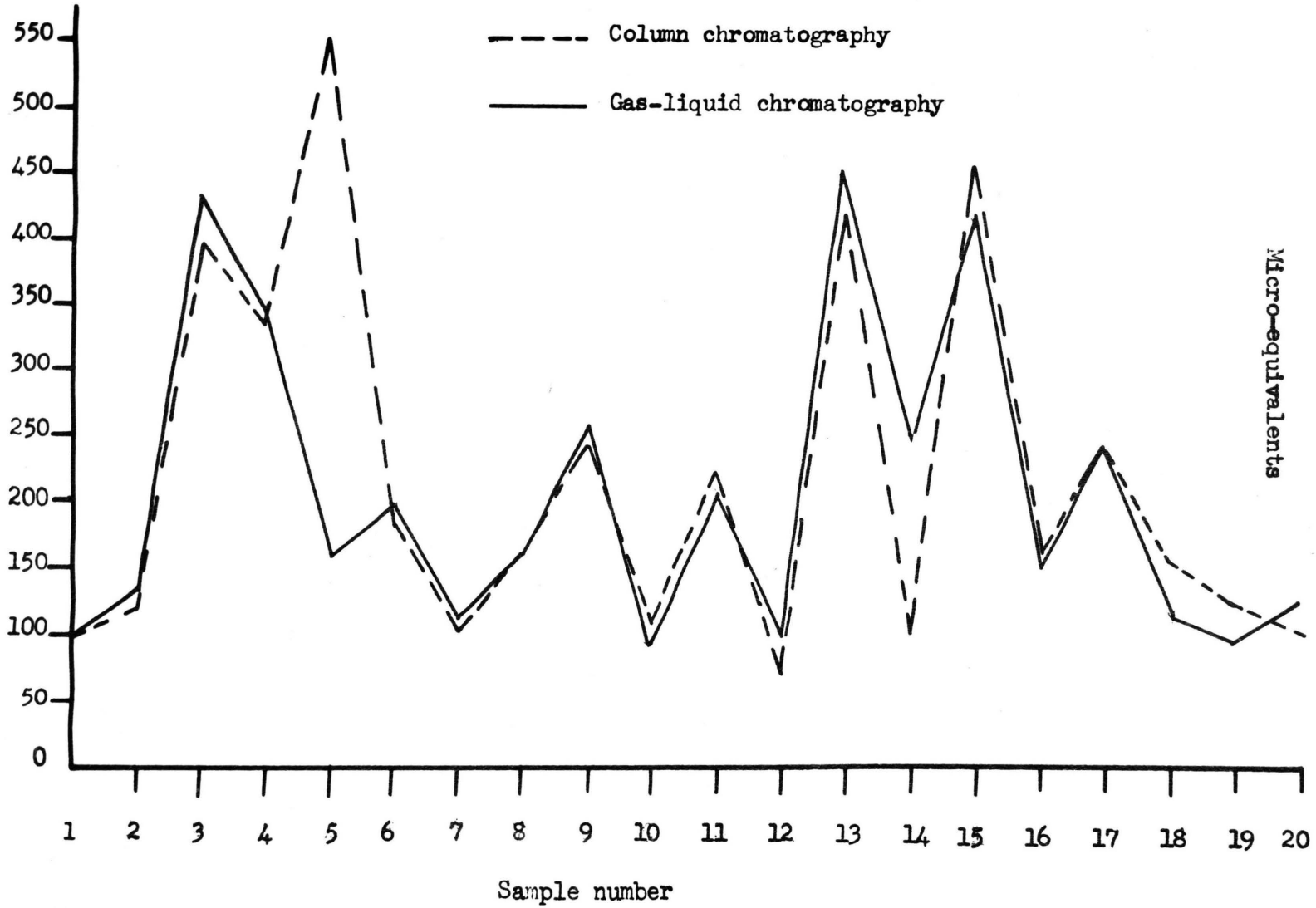

Figure II. The comparison of column chromatographic and gas-liquid chromatographic analysis in the determination of acetic acid in silage.

Figure III. The comparison of column chromatographic and gas-liquid chromatographic analysis in the determination of acetic acid in silage.

found that it was difficult to form the esters of lactic acid; (3) the methyl esters of acetic, propionic, and butyric acids are very volatile and are difficult to keep especially when quantitative analysis is necessary; and (4) the mixture of the organic acids to be methylated must be completely anhydrous to prevent hydrolysis of the methyl esters. At this time there is no such equipment available to use for this part of the procedure.

Evaluation of the Pilot Studies

In determining the validity of the two series of silage samples, it was found on Table 13 that there seemed to be no direct relationship between the two groups. Although all the determinations of lactic acid were not made on two sets of samples, it was quite obvious that there was no direct relationship.

Table 13. The Comparison of Organic Acid Concentration In Controlled Pilot Studies

	Butyric		Propionic		Acetic		Formic		Lactic	
	Control		Control		Control		Control		Control	
Sampled At	1	2	1	2	1	2	1	2	1	2
7 days	#	#	#	#	*150.0	* 62.0	*10.0	*10.0	*226.0	*200.0
14 days	#	#	#	#	*100.0	*153.0	* 5.0	*10.0	—	*290.0
21 days	#	#	#	* 5.0	*150.0	*130.0	* 5.0	*10.0	—	*390.0
28 days	#	#	#	#	*250.0	*155.0	* 7.0	*10.0	*175.0	*160.0
35 days	#	#	#	* 5.0	*220.0	*542.0	*15.0	*20.0	* 62.0	*160.0
42 days	#	#	#	*10.0	*340.0	*396.0	*17.0	*10.0	* 95.0	* 65.0

*Expressed in micro-equivalents.
#Not of sufficient quantity for quantitative determination.

SUMMARY

The purpose of this work was to determine the quantity of organic acids present in silage. To be sure that the methods of analysis were correct, two different procedures were used. The two procedures used were that of column and gas-liquid chromatographic analysis. To determine the actual quantities of the organic acids, 36 samples from various containers were used. The containers used were concrete silos, 55 gallon barrels, and also some samples were analyzed which were ensiled in pint, quart, and one-half gallon jars. It was found that there is no real definite amount of organic acid produced in different silage fermentations.

The accuracy of the gas-liquid chromatographic analysis has been well established as being practically without error. The only exception to this is the error of the operator. It was found that the column chromatographic analysis in some instances gave very excessive recovery rates. However, in the actual field comparison studies, the method seemed to be very accurate. The accuracy of the recovery rate was very low when the minimum limit of 12 micro-equivalents per milliliter was reached. The accuracy increased and the recovery rate was better as the amounts increased to 250 micro-equivalents per milliliter. The results of Gherke and Lamkin (8) gave a recovery rate of 93 to 110 percent in the determinations of organic acid analysis by gas-liquid chromatography. The results in this work showed a recovery rate of 78 to 190 percent using column chromatography. This included

the determination of maximum and minimum limits of standard number I and II.

Both determinations are believed to be practical, although it was felt that the column chromatographic procedure was more versatile in that it can be employed to easily determine formic and lactic acids in their unesterified form. The gas-liquid chromatographic analysis requires the esterification of both formic and lactic acids, so that they may be quantitatively determined.

The time consumed is quite variable, especially with the column chromatographic procedure. If everything is prepared the day before, 15 samples may easily be determined in an eight hour day. Since the methyl ester preparation of the organic acids was quite lengthy, it is doubtful if this many samples could be run using this procedure. However, an undetermined number of samples, exceeding 30, may be easily determined in the unesterified form using gas-liquid chromatography. This determination would include the analysis of butyric, propionic, and acetic acids.

The validity of the small scale pilot studies under controlled conditions was definitely established as not being valid. The most probable explanation for this is that no two fermentations proceed in the same exact manner.

CONCLUSION

It appears that the quantity of organic acids in silage is variable. The field studies and the controlled pilot studies indicate that it is inadvisable to try to establish predictable levels of organic acids present in silage.

The column chromatographic analysis seems to be the easiest and the most timesaving method for determining silage acids. If it is desired to determine only butyric, propionic, and acetic acid, there is no question as to the rapidity and effectiveness of the gas-liquid chromatographic procedure.

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APPENDIX

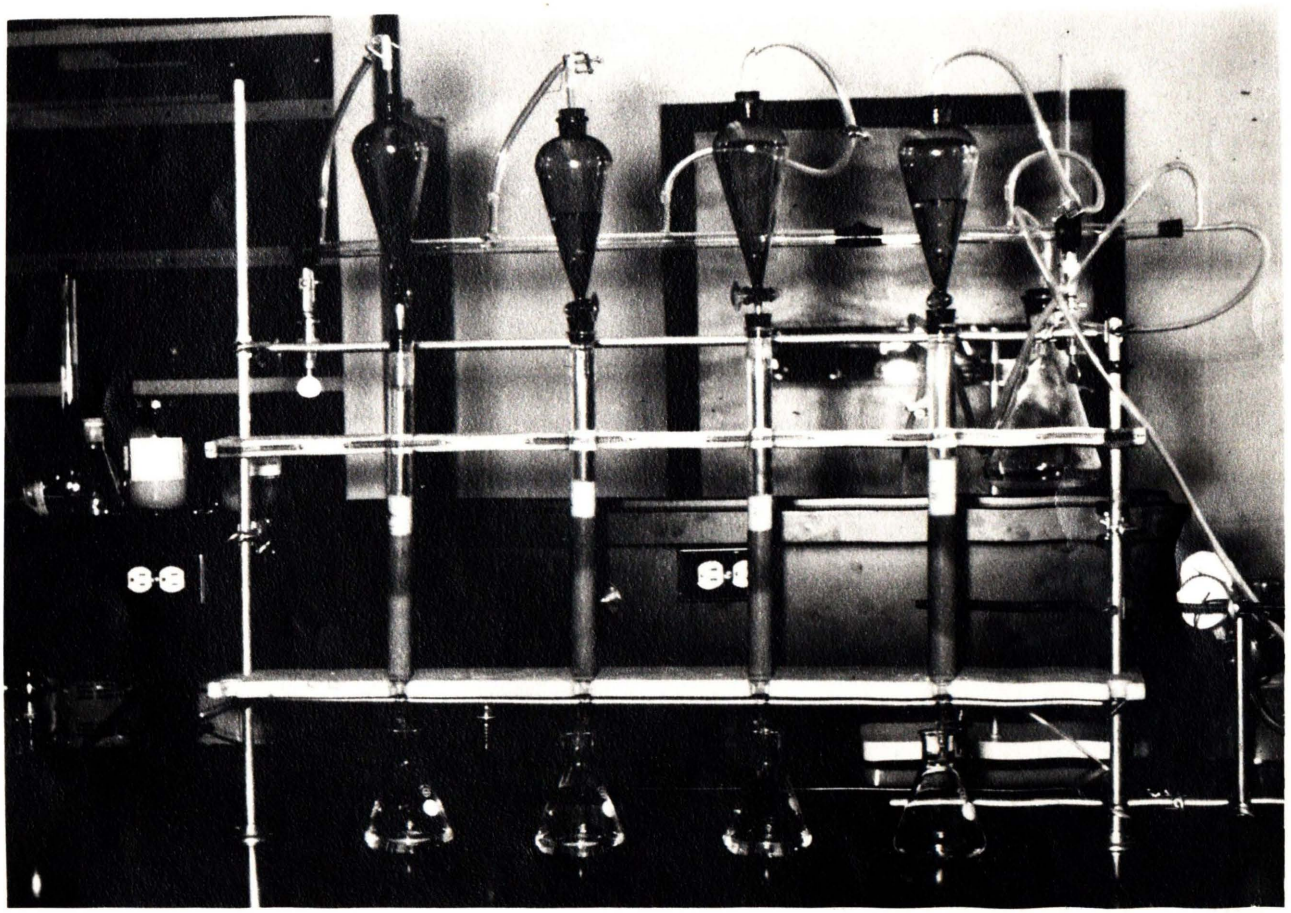

Figure I. Apparatus used in column chromatographic analysis of silage