Quantitative Determination of Steam-Volatile Fatty Acids by Gas-Liquid Chromatography

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A quantitative gas chromatographic procedure has been developed for the determination of steam-volatile fatty acids in biological materials. It is a modification of the original procedure of James and Martin and uses commercially available gas chromatographic apparatus. Techniques for low temperature vacuum concentration of samples and for the column removal of water are included. The removal of water is so complete that problems due to its presence are eliminated. Recovery in each step approaches 100%. Thermal conductivity detection is used, and an independent detector temperature control system is not required.

A GAS CHROMATOGRAPHIC TECHNIQUE for steam-volatile fatty acids offers advantages over older methods because of its sensitivity, speed, and simplicity, and the refined separations possible. James and Martin (*6*) reported the gas chromatographic separation and estimation of both straight and branched-chain fatty acids from formic to dodecanoic. Modifications on the method of James and Martin were reported in 1955 by Van de Kamer, Gerritsma, and Wansink (*13*). Cropper and Heywood (*2*) extended the technique to acids in the range of C_{12} to C_{22} by converting the acids into their methyl esters prior to chromatography. Further improvements and extensions have been reported by James and Martin (*7*), James and Wheatley (*8*), Beerthuis and Keppler (*1*), and Hankinson, Harper, and Mikolajcik (*4*). Hawke (*5*) reported the use of a new stationary phase for separating the free acids. Recent work by Orr and Callen (*10, 11*), Stoffel, Insull, and Ahrens (*12*), and Lipsky (*9*) has been concerned with higher fatty acids of biological interest and has resulted in the gas chromatographic separation of the methyl esters of unsaturated as well as saturated acids.

In attempts to apply the findings of previous investigators to the determination of steam-volatile fatty acids in rumen fluid, rumen digesta, silage, etc., certain obstacles were encountered. The sample introduced onto the chromatographic column must contain a rather high concentration of the acids of interest. Otherwise, the peaks obtained will be too small for a good quantitative analysis. Also, the sample should be anhydrous, especially for the chromatography of free acids, since water gives a very poor chromatographic peak which comes over quickly and tails badly into subsequent peaks, and even traces of water tend to upset the chromatographic separation of the acids, making frequent replacement of columns necessary and casting doubt on all results. The thorough removal of water allows free acids to be chromatographed directly without prior conversion to their methyl esters.

Experimental

Apparatus. A Perkin-Elmer 154-C Vapor Fractometer with a Leeds & Northrup Speedomax Type G recorder (0- to 5-mv.) and a dual-type thermistor thermal conductivity cell having a 0.5-second time constant and 0.25-ml. volume was used in this work. The only modification of the instrument was the detachment of the vent line from the solenoid valve necessitated by the elevated column temperatures employed. If this change were not made, stationary phase would elute from the columns, condense in the solenoid valve, and clog the system. The instrument was equipped with a Perkin-Elmer microdipper sample introduction system which was used in all cases where accurate reproduction of sample volume was required. A Welch Duo-Seal vacuum pump with 140-liter-per-minute capacity was used in the final concentration step described below.

Sample Preparation and Initial Concentration. Water solutions containing known amounts of the sodium salts of acetic, propionic, *n*-butyric, isovaleric, and *n*-valeric acids (totaling about 4 meq. per sample) were prepared from standard solutions of the individual acids. The standard solutions were prepared by weighing pure acids which had been checked chromatographically for purity and redistilled where necessary. The final concentration was determined in each case by titration. Values obtained in this way agreed closely with those computed from weights. In an actual analysis of a sample of biological origin, a solution of the sodium salts of the various acids may be obtained from a steam distillate of the sample to be analyzed or by a solvent extraction procedure. One of the objectives was to check the accuracy and reliability of the proposed procedure; therefore, carefully prepared synthetic samples were used. Details on steam distillation or solvent extraction may be found in works dealing with fatty acid analysis.

Each solution was evaporated to dryness on a steam bath with the final step of the evaporation carried out in a 25-ml. Erlenmeyer flask to facilitate subsequent handling. Evaporation was speeded up by blowing filtered air over the samples. Some loss may occur if the evaporation is carried out on a hot plate; use of a steam bath guards against this possibility.

Column Removal of Water. One milliliter of distilled water was added to the residue in each flask, and the sodium salts were redissolved. The samples were then cooled to 0° C. in an ice bath, and 0.4 ml. of concentrated sulfuric acid (also at 0° C.) was added to each to liberate the free acids. Each solution was then poured onto the top of a 7-mm. column packed with 7 cm. of Celite 545 (Johns-Manville) over 12 cm. of 20- to 40-mesh anhydrous calcium sulfate. Both the Celite and calcium sulfate were previously dried by heating overnight in a muffle furnace at 300° C. The 20- to 40-mesh calcium sulfate was prepared by grinding and screening larger lumps. This mesh size gave a satisfactory flow rate for the purpose at hand.

The acids remaining in each flask were washed onto the respective columns with several small portions of reagent grade ethyl ether totaling about 5 ml. per sample. When the level of liquid in a column had run down to just above the top of the Celite layer, another small portion (1 to 2 ml.) of ether was added, and the process was repeated until 15 ml. of eluate had run through. The eluate was collected in a tube especially designed to facilitate subsequent concentration (Figure 1). Removal of

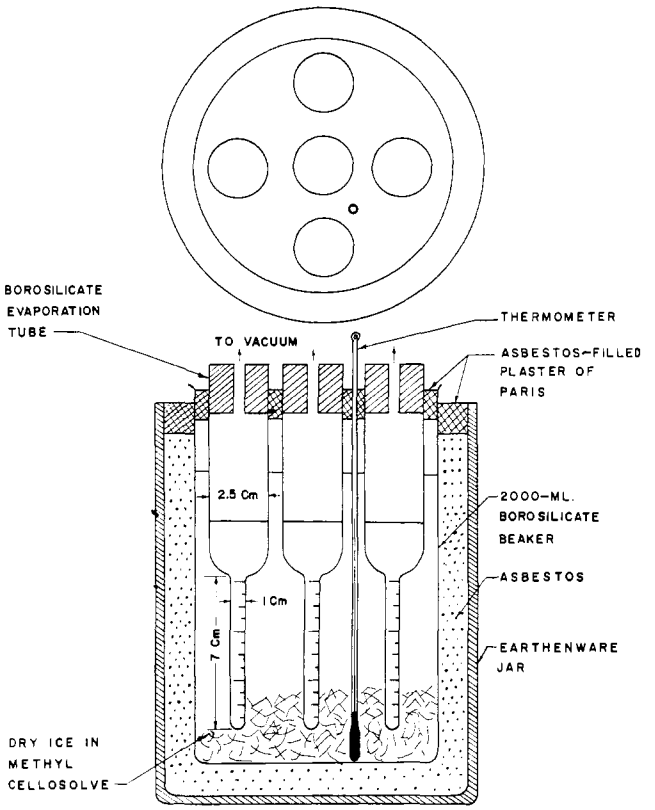

Figure 1. Low temperature concentration bath

Table I. Results of Analysis of Synthetic Mixtures of Volatile Fatty Acids

Sample No.		Mole % Acetic	Propionic	n-Butyric	Isovaleric	n-Valeric	Recovery, %
41	Actual	4.96	74.74	9.65	5.29	5.36	
	Found	5.78	72.54	10.92	5.58	5.18	110.68
	Error	+0.82	−2.20	+1.27	+0.29	−0.18	
42	Actual	11.45	46.03	15.61	24.43	2.47	
	Found	11.55	46.90	15.96	22.65	2.93	107.42
	Error	+0.10	+0.87	+0.35	−1.78	+0.46	
43	Actual	20.77	20.87	40.43	2.21	15.72	
	Found	18.93	20.71	41.88	2.65	15.83	102.85
	Error	−1.83	−0.16	+1.45	+0.44	+0.11	
44	Actual	40.13	10.08	19.53	8.56	21.69	
	Found	41.53	10.40	18.50	8.02	21.55	106.37
	Error	+1.40	+0.32	−1.03	−0.54	−0.14	
45	Actual	68.97	3.96	3.84	14.72	8.52	
	Found	68.85	4.14	3.57	14.78	8.66	110.50
	Error	−0.12	+0.18	−0.27	+0.06	+0.14	

Table II. Precision in Analysis of Volatile Fatty Acids

Sample No.[a]	Mole %, Found Acetic	Propionic	n-Butyric	Isovaleric	n-Valeric	Recovery, %
46-A	40.19	10.15	19.83	8.25	21.59	95.68
-B	38.07	10.11	20.19	8.37	23.26	93.24
-C	39.94	8.75	19.83	8.29	23.20	109.49
-D	41.60	9.23	20.04	7.45	21.68	103.93
-E	41.22	9.95	19.48	7.44	21.90	108.19
Av. found	40.20	9.64	19.87	7.96	22.33	102.11
Actual	39.97	10.01	19.84	7.70	22.48	
Std. dev.	±1.38	±0.62	±0.27	±0.47	±0.83	

[a] All samples contained same mole per cent of various acids.

water by this procedure was quantitative. The Celite removes most of the water, and remaining traces are taken out by calcium sulfate. Sodium sulfate gave unsatisfactory results because of its lower efficiency as a drying agent. Recovery of the volatile acids is quantitative if 15 ml. of eluate are collected. Sulfuric acid is adsorbed and retained quantitatively on the column.

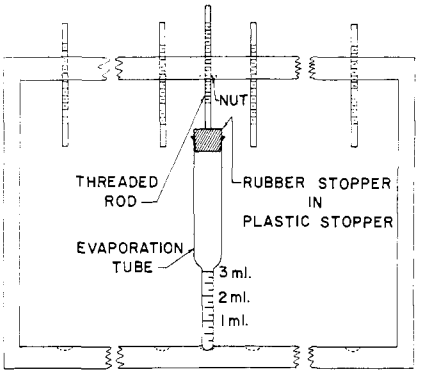

Figure 2. Rack for evaporation tubes

Final Concentration of Acids. The anhydrous solutions of the acids in 15 ml. of ether were too dilute for a good quantitative analysis. Also, attempts to strip the solutions to a smaller volume at room temperature resulted in appreciable losses of the more volatile acids. However, these losses were prevented by the following concentration procedure.

The tubes containing the samples were transferred to the specially constructed low temperature bath shown in Figure 1, which was maintained at a temperature of −50° C. by dry ice in methyl Cellosolve (ethylene glycol monomethyl ether). The samples were then evaporated to just less than 2.00 ml. at a pressure of 6 mm. of mercury. (The conditions of temperature and pressure were arrived at as the result of a rather extensive series of recovery experiments in which various temperatures and pressures were tried.) Samples were run in groups of five; between 2 and 3 hours were required for the evaporation of this number under the temperature and pressure conditions employed. The synthetic samples each contained about 4 meq. of total acidity; for smaller sample sizes, the evaporation may be carried to 0.5 ml. or less, thus increasing the sensitivity.

After removal from the low temperature bath, the tubes containing the samples were placed in the rack shown in Figure 2. The design of this rack made it possible to keep samples for a few days without loss of solvent or acids. After the samples had come to room temperature and just prior to chromatography, each was diluted to 2.00 ml. with ethyl ether dried by passage through a column of anhydrous calcium sulfate.

Chromatographic Separation. A 20-μl. aliquot of each sample was run gas chromatographically, and the areas under the peaks corresponding to the various acids were determined with a planimeter. Peaks were identified by retention times, which were previously determined using known pure acids. Separation was brought about by a column similar to that employed by James and Martin (*6*). Figure 3 shows

a typical chromatogram. Instrumental conditions employed represent a compromise between resolution and retention time. No attempt was made to obtain maximum resolution. Duplicate 2-μl. portions of redistilled acetic acid were run with each group of samples so that a correction factor could be computed for daily variations in instrumental response.

A series of 10 standard mixtures representing five levels of each of the five acids in question was run prior to all samples to establish the relationship between peak area and concentration (total milliequivalents). Each mixture was taken through the entire procedure outlined above, and a single standardization was valid for the life of a column. These standards were so prepared that each contained approximately the same number of milliequivalents of acid as the samples, and no combination of acids was used in a given mixture which would give any overlapping of peaks when chromatographed. Figure 4 shows the relationships obtained between peak areas and total milliequivalents. Because a 20-μl. aliquot from 2.00 ml. was taken in each case, the amount actually chromatographed to produce a given peak area (at $^1/_8$ maximum sensitivity) was 0.01 of the amount shown on the abscissa.

Two 2-μl. portions of pure redistilled acetic acid were run with each group of standard mixtures so that daily variations in instrumental response could be corrected. This correction was made in the following manner. The areas were averaged for the two 2-μl. portions of acetic acid run on a given day. This average value was then divided into an area arbitrarily chosen as standard to give a correction factor by which all peak areas obtained on that day were multiplied. Thus samples and standard mixtures were corrected to the same instrumental response, so that milliequivalents as well as mole per cents could be computed from the chromatographic curves. The correction is usually of minor consequence. However, since dirty thermistors, etc., can cause instrumental response to change radically, it is necessary if per cent recovery is to be computed.

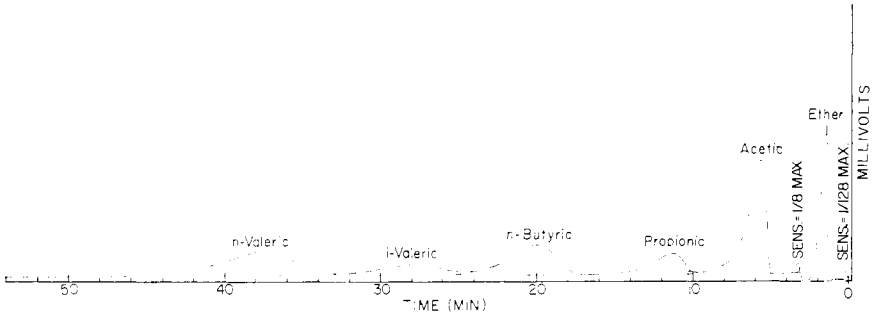

Figure 3. Typical chromatogram showing separation of five steam-volatile fatty acids

Carrier gas, He. Column, 1.5 meters × 0.4 cm. Silicone 550 (Dow Corning) + 10% (w./w.) stearic acid on Celite 545 [Johns-Manville, size-graded and acid-washed as described (6); 0.5 gram of liquid phase per 1.0 gram of Celite]. Column temperature, 137° C. Pressure, 13.0 p.s.i. Flow, 34 ml. per min. at 760 mm. and 70° F. Sample volume, 20 μl.

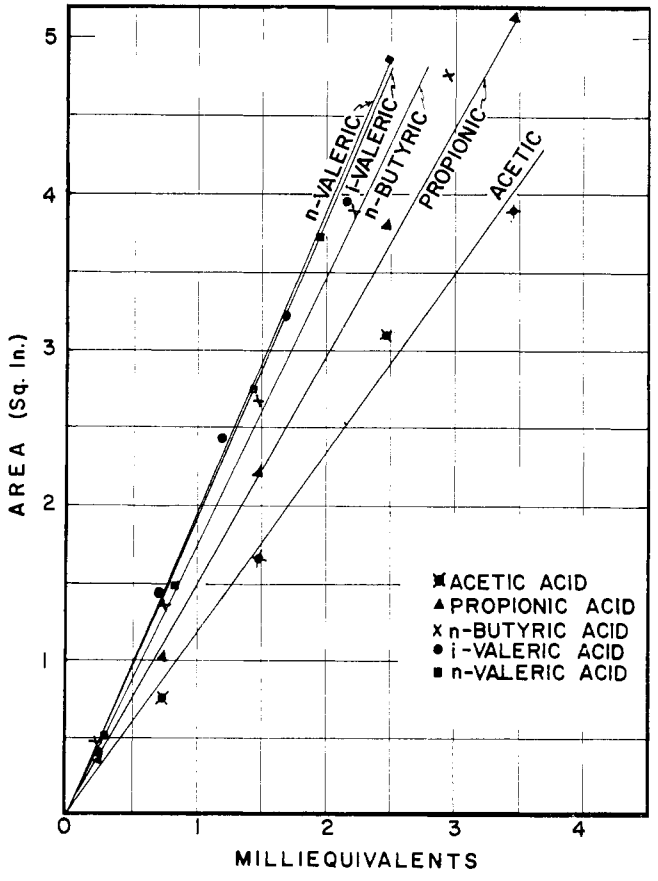

Figure 4. Peak area as a function of concentration

Results and Discussion

Results obtained in the analysis of five synthetic mixtures containing various concentrations of the fatty acids are summarized in Table I. Table II shows the precision obtained in the analysis of five identical mixtures. Standard deviations varied from ±0.27 to ±1.38 mole %; the average coefficient of variation was 4.2%. The procedure has been used for the routine analysis of several hundred samples of rumen digesta. It is valid for the determination of all possible isomers of the saturated fatty acids from acetic through valeric as well as other acids such as caproic. Changes in column length and operating conditions would, of course, be required in some of the more difficult separations. Preliminary experiments indicate that the recovery of formic acid in the evaporation step is in the neighborhood of 90%. Therefore, it is probable that this acid may also be determined.

Samples carried through the procedure are dried so thoroughly that deterioration of the chromatographic column due to the presence of water is eliminated. A gradual loss of stearic acid from the stationary phase over a period of time at the temperature employed (137° C.) does cause the column to deteriorate slowly. However, if special precautions are taken, such as reversing the column each day, column life can be extended to over 2 months of continuous use (10 hours per day) without appreciable loss of resolution.

The mole per cent obtained for a given acid may occasionally deviate from the correct value by 1% or more, chiefly because of the slight overlapping of peaks. The variation of the per cent recovery from 100% by about ±10% is due primarily to sample introduction errors. Because the mole per cent of a given acid is computed by dividing the milliequivalents of that acid found chroma-

tographically by the total number of milliequivalents of acid found chromatographically, such variations in per cent recovery have no effect on the mole per cents obtained for the various acids. If per cent recovery must be known more exactly, use of a column with higher resolution should allow an internal standard to be employed.

If the recorder is operated at maximum sensitivity and the evaporation is carried to 0.5 ml., samples containing in the neighborhood of 0.1 meq. of total acidity may be successfully analyzed by the procedure as outlined. Samples containing total acidity in the range of about 0.1 to 4 meq. thus may be analyzed by chromatography of 20 μl. of the final solution. The introduction of such a large volume provides sensitivity at the expense of resolution. For those samples containing 1 meq. or more of total acidity, resolution may be improved by reducing the volume introduced to 2 μl. If the sample contains less than 0.1 meq. of total acidity, it may be necessary to carry the evaporation to less than 0.5 ml. Instrumental conditions may be varied widely to meet the requirements of a particular analysis or investigator.

Conclusions

The procedure may be readily adapted to methyl esters by reacting the acids with diazomethane (*3*) following the final concentration step. However, the Silicone 550–stearic acid column used for separating the free acids has been studied in detail by James and Martin (*6*) and found capable of resolving a wide spectrum of acids. Thus, for many applications, chromatography of the free acids is not only adequate but desirable. The techniques for sample concentration and water removal can undoubtedly be extended to other types of mixtures analyzed by gas chromatography.

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LITERATURE AVAILABLE

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