QUANTITATIVE GAS–LIQUID CHROMATOGRAPHY OF VOLATILE FATTY ACIDS

A METHOD FOR THE DETERMINATION OF C_1 TO C_6 ACIDS IN BIOLOGICAL MATERIAL

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(Received June 29th, 1964)

INTRODUCTION

The classical work of JAMES AND MARTIN[1] demonstrated that the separation and quantitative estimation of saturated monocarboxylic acids, up to and including dodecanoic acid, could be achieved by means of gas–liquid chromatography (GLC). However, routine determination of volatile acids present in biological materials has remained a formidable task. Such acids are usually separated from materials in which they occur by steam distillation and recovered as an aqueous solution of their sodium salts. Part of the difficulty entailed in the analysis of these acids by GLC lies in the requirement, with most column packings, that they be applied in free form and in an anhydrous state. In the procedure of JAMES AND MARTIN[1] a micro-partition column was employed to convert sodium salts to an anhydrous ethereal solution of the free acids, which was then distilled onto the chromatographic column for analysis. It was claimed that the method gave quantitative results. Often, however, difficulties have been experienced with the method and various alternative procedures have been proposed. See, for example, MCINNES[2], GEHRKE AND LAMKIN[3], and SHELLEY, SALWIN AND HORWITZ[4]. SMITH[5] demonstrated the possibility of analysing aqueous solutions of free fatty acids by GLC, using a stationary phase of Tween 80 on Celite but no means of applying the process to sodium salts was described.

In the present communication, a simplified procedure for the analysis of mixed sodium salts of volatile fatty acids by means of GLC is described. It gives quantitative results, with complete separation of saturated monocarboxylic acids from formic to caproic, inclusive. The method is based upon the observation that a liquid phase consisting of behenic and orthophosphoric acids tolerates the introduction of a small amount of water with the sample of acids. JACKSON[6] showed that this liquid phase gave excellent separation of volatile acids from C_1–C_5, at 135°, with helium as the carrier gas, but found some evidence of partial decomposition of formic acid under the conditions used. We have since established that such decomposition is prevented by using wet nitrogen as the carrier gas.

A gaseous phase containing water vapour precludes the use of a thermal conductivity detector. Flame ionisation detectors, which may be used with a wet gas, unfortunately do not respond to formic acid. The response of an argon ionisation

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detector to organic compounds is damped by the presence of water vapour in the carrier gas and, although this type of detector has not been tried for our purpose, it therefore appears unlikely that it could be used satisfactorily. Thus, in the method of analysis described below, titration was chosen as the means of detecting acids eluted from the column. As an alternative to the photo-electric titration apparatus described by JAMES AND MARTIN[1] and MCINNES[2], the present authors have found that a glass electrode, pH-stat assembly is suitable as a recording detector for the acids.

EXPERIMENTAL

Apparatus

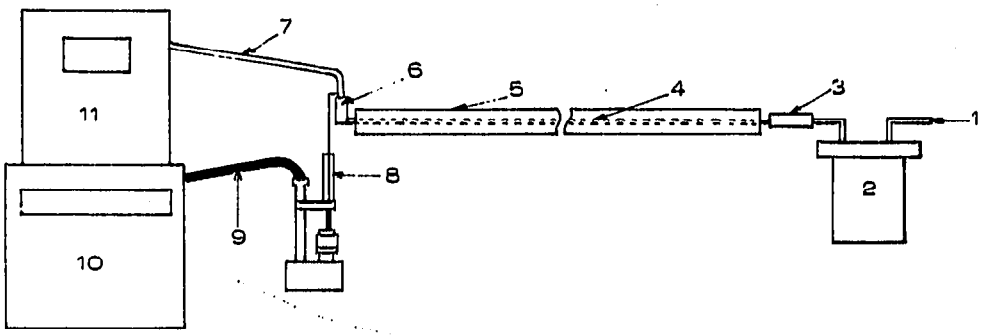
The chromatographic apparatus used for this study was constructed in the laboratory (see Fig. 1). It consisted of a water bubbler through which the carrier gas (nitrogen) could be passed before entering the column, a detachable sample heater, a straight chromatographic column, 0.4 cm I.D. by 125 cm long, within a cylindrical heating oven, a titration vessel and the automatic titrator. With the exception of the oven, borosilicate glass was used for all parts. Demountable joints were secured by clamps or spring-loading so as to withstand an internal pressure of 25 lb./sq. in. The heating element for the sample heater consisted of an externally wound nichrome ribbon (40 Ω total resistance) which was covered by an insulating layer of fibreglass adhesive tape. Operating at an applied potential of 18–20 V gave an internal temperature between 140° and 160°. The oven walls consisted of two aluminium tubes approximately 125 cm long, an inner tube (2.5 cm diameter) being located coaxially with the outer (6.4 cm diameter) by means of cork spacing rings. The inner tube was wound with a 150 W heating tape, 183 cm long by 5 cm wide, and the space between the heating tape and the outer aluminium tube was packed with asbestos. The partitioning column was supported in the oven also by means of cork spacers, the one at the input end being split in order to facilitate assembly and dismantling. Within the oven, the column was enclosed in a loosely fitting glass tube which enabled thermocouples to be held in a suitable position for column temperatures to be determined. Analyses were carried out with (rising) temperature programming. Adequate temperature control was obtained by means of a variable transformer. Although this method of control did not give linear programming, the temperature–time curve was sufficiently reproducible. Any effects of variations in this were insignificant.

Emerging acids were determined by means of the recording pH-stat assembly (Radiometer, Copenhagen: Titrator TTT1c, Titrigraph SBR2c and Syringe Burette SBU1c). When 0.02 *N* alkali was used as titrant, the limit of readability on a titration curve corresponded to $\pm$ 0.01 μmole.

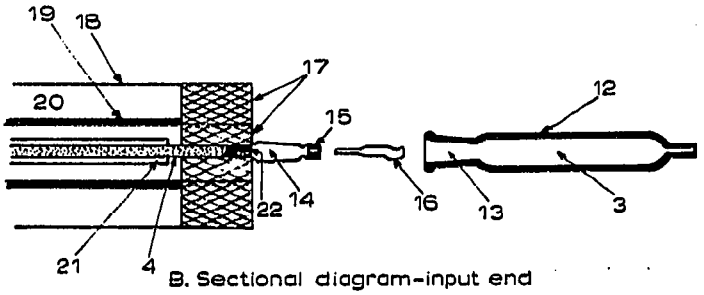
Since the gas stream from the column tends to heat the titration cell contents, an upward slope in the baseline may be evident. The extent of the rise in baseline may be limited by starting an analysis with the titration liquid at 35° and making use of the cooling effect of the nitrogen stream through the auxiliary bubbler which is employed to mix the cell contents. Allowance may readily be made for a rising baseline when reading results from the curve.

Stationary phase

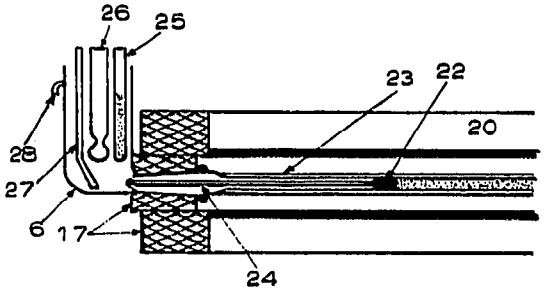
The stationary phase consisted of behenic acid, 20 g, orthophosphoric acid, 4 g,



A. Chromatograph assembly (not to scale)



B. Sectional diagram-input end



C. Sectional diagram-titration end

Fig. 1. Gas–liquid chromatographic apparatus (Figs. 1B and 1C are approx. $^1/_4$ size):

1. Gas inlet.
2. Water bubbler.
3. Sample heater.
4. Chromatographic column.
5. Column oven.
6. Titration vessel.
7. Electrode leads.
8. Micro-burette.
9. Flexible drive.
10. Recorder.
11. pH meter-titrator.
12. Insulated heater (40 Ω).
13. Ground glass socket-B10.
14. Modified glass cone-B10.
15. Silicone rubber plug.
16. Glass sample boat.
17. Cork spacers.
18. Aluminium tube.
19. Aluminium tube with heating tape (150 W).
20. Asbestos packing.
21. Glass tube for thermocouples.
22. Glass yarn plug.
23. Capillary end of column.
24. Glass cone and socket-B7.
25. Calomel electrode.
26. Glass electrode.
27. Bubbling tube.
28. Vacuum connection.

on acid-washed Chromosorb W (F & M Scientific Corporation) 80–100 mesh, 100 g. This mixture gives excellent separation of C_1–C_6 monocarboxylic acids, with wet nitrogen as the carrier gas. It has, moreover, a substantial tolerance of water vapour in the gaseous phase, a feature of basic importance to the technique to be described here. Reagent grade (syrupy) phosphoric acid was used as received but laboratory grade behenic acid was re-crystallized from acetone. The column packing was prepared by dissolving the acids in acetone, adding the Chromosorb, then evaporating the solvent by heating over a steam bath with constant stirring. Columns were packed, with the aid of a vibrator, to a density such that, at 90–100°, a flow rate of 50 ml/min

was obtained with a head pressure of 18–20 lb./sq. in. Before use, the packed column was conditioned by heating overnight at 100° while passing through it a slow stream (10 ml/min) of wet nitrogen. Omission of this step resulted in low recoveries of formic acid, and high retention volumes with incomplete separation of formic and acetic acids during the first few analyses. The reason for this beneficial effect of hydration of the column packing on its performance is obscure.

Sample preparation

Volatile acids were distilled from biological material according to FRIEDEMANN[7] and titrated to the phenolphthalein end point with NaOH (0.1 *M*). One ml excess of the alkali was then added and the sodium salts solution concentrated by boiling. It was then made up to a volume such that the total concentration of acids was approximately *M*. A sample of this solution (10 μl) was transferred to a glass boat (see Fig. 1) by means of a micro-syringe. The syringe needle had a square cut tip and the last few mm of its outer surface were smeared lightly with silicone grease. Gentle suction was applied to the boat while it was warmed carefully with a micro-flame to dry the sample. A further 10 μl volume of the salts solution was then placed in the boat and similarly dried, the process being repeated until a sufficient quantity of salts had accumulated (5–10 μmoles). Best results were obtained when the salts were dried in a compact mass. Drying to the extent of removing water of crystallization from the salts was found undesirable since this usually led to loss by spattering. Boats containing dried salts could be stored, in a desiccator, without loss of fatty acids.

Chromatography

The column temperature was raised to 90° and maintained at this level by appropriate adjustment of the applied voltage. At the same time, the sample heater was switched on (20 V). Reduced pressure equal to 0.5 atm was applied to the column via the titration vessel and a boat containing a sample of dried salts was attached to the input end by means of the perforated silicone rubber plug. Phosphoric acid solution (15 μl of 30 % w/v) was dropped onto the salts in such a manner that the whole sample was wetted at the same time. The sample heater was then attached to the column and suction continued for 5 min. At the end of this period the vacuum was gradually replaced by a flow of wet nitrogen at 8–10 ml/min. Five minutes later, this flow rate was increased to 30 ml/min and the oven heater voltage raised to 180 V. Acids were neutralized as they emerged from the column and the volume of alkali (0.02 *M*) consumed was recorded with the pH-stat assembly. The volume of liquid in the titration vessel was 15 ml and the end-point setting pH 7.5.

Formic acid, if present, emerged from the column about 5 min after increasing the nitrogen flow to 30 ml/min. The order of emergence of the acids was the same as found by JAMES AND MARTIN[1] using stearic acid/silicone columns. When *n*-butyric acid appeared (31–32 min) the nitrogen flow was increased to 40–50 ml/min in order to keep the time required for the analysis to a minimum (60–70 min for caproic acid). The various acids present in the column could be identified by their time of emergence under standard conditions of operation and the amounts read directly in micro-equivalents from the chromatogram by means of the chart graduation lines (see Fig. 2).

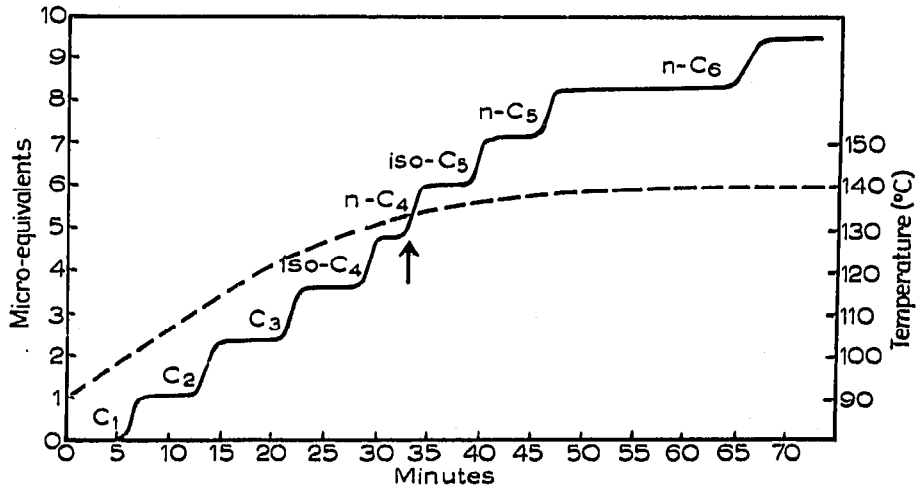


Fig. 2. Gas–liquid chromatogram from a synthetic mixture of sodium salts of volatile fatty acids, using a 125 cm behenic acid–phosphoric acid column with wet nitrogen as carrier gas. Acids: C_1 = formic; C_2 = acetic; C_3 = propionic; iso-C_4 = isobutyric; n-C_4 = n-butyric; iso-C_5 = isovaleric; n-C_5 = n-valeric; n-C_6 = n-caproic. ——— alkali consumed; – – – column temperature. For other details see Fig. 1 and text.

RESULTS

Evaluation of the method

Fig. 2 illustrates the chromatogram obtained from a synthetic mixture of sodium salts of eight acids within the range C_1–C_6. It can be seen that the titration curves display an almost complete lack of tailing. The isovaleric curve does lack symmetry, probably because of contamination of the sample used with α-methylbutyric acid (see JAMES AND MARTIN[1]). These two isomers are not completely separated on a 125 cm behenic acid–phosphoric acid column under the conditions employed.

The accuracy and reproducibility of results obtainable with the method were examined by replicate analyses of 10 μl portions of a synthetic mixture of sodium

TABLE I

ANALYSIS OF A SYNTHETIC MIXTURE OF VOLATILE FATTY ACIDS (6 REPLICATES) BY GAS–LIQUID CHROMATOGRAPHY ON A BEHENIC ACID–ORTHOPHOSPHORIC ACID COLUMN

Acid determined	*Found (mean μmoles)*	*Standard deviation**
Formic	0.827	± 0.008
Acetic	0.915	± 0.011
Propionic	0.861	± 0.012
Isobutyric	0.843	± 0.009
n-Butyric	0.861	± 0.010
Isovaleric	0.803	± 0.005
n-Valeric	0.788	± 0.008
n-Caproic	1.035	± 0.006
Total acids	6.935	± 0.025
Total acids taken (μmoles)	7.020	
Total recovery (%)	98.9	± 0.35

* Differences between the standard deviations for individual acids were not significant.

salts, the total acid content of this solution being determined by steam distillation and titration of a larger sample. Results from this investigation shown in Table I indicate that the method possesses high levels of accuracy and precision. No evidence of specific differences in recoveries of individual acids is apparent.

Application of the method

The method described has been used by the authors as a means of estimating components of volatile acidic fractions from silages and rumen contents. Fig. 3 illustrates the chromatogram obtained with material from a poorly preserved,

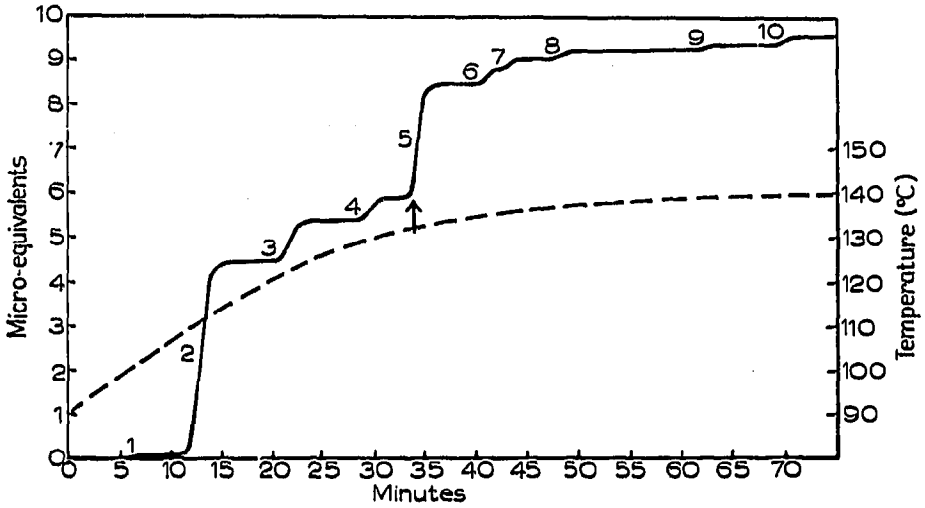


Fig. 3. Gas–liquid chromatogram from sodium salts of volatile acids isolated from a poorly preserved ryegrass silage. Acids: 1 = formic; 2 = acetic; 3 = propionic; 4 = isobutyric; 5 = *n*-butyric; 6 = isovaleric; 7 = α-methylbutyric; 8 = *n*-valeric; 9 = "isocaproic"; 10 = *n*-caproic. ——— alkali consumed; – – – column temperature. Chromatography as in Fig. 2.

ryegrass silage. Ten acids within the range C_1–C_6 have been resolved. The identity of acid No. 9 has not been established, since only the normal isomer of the hexanoic acids has so far been available for examination by this method. Although the difficult pair isovaleric and α-methylbutyric acids have not been separated completely in this analysis, it is apparent that the degree of resolution on the 125 cm column is adequate for most purposes. All other separations are complete. Absence of tailing of formic acid is especially noteworthy.

To date, approximately 150 silage samples, representing several plant species and a wide range of qualities, have been analysed for volatile fatty acids by the method described above. Recoveries have ranged from 96 to 102 % of values obtained by titration of distillates, with a bias towards slightly low values. Incomplete acidification of salts, when they have been allowed to spread while drying in the boat, seems to be the most likely cause of losses. No evidence has yet been found for the presence in silage of volatile acids above C_6 in the series.

Life of stationary phase

The useful life of a behenic acid–phosphoric acid/Chromosorb column has not yet been determined; but more than 100 analyses have been made on the column in current use, without detectable loss in efficiency. Occasional blocking of the capillary

outlet does occur as a result of "bleeding" of the liquid phase. The plugs are, however, readily removed by immersing the capillary in chloroform, without effect on the column's performance.

DISCUSSION

The particular virtues of the method described lie in its simplicity of application to biological materials, and the comparative ease with which accurate quantitative results may be obtained. Although it has only been evaluated for acids from formic to caproic, inclusive, the method would seem to be useful also for higher homologues within the steam volatile group. JAMES AND MARTIN[1] were able to separate saturated monocarboxylic acids up to and including dodecanoic on their silicone/stearic acid columns by prolonged operation at 137°. However, retention volumes with stationary phases containing orthophosphoric acid are about four times greater than with those not containing this acid and, consequently, elution times for higher acids are so long as to render behenic acid–phosphoric acid columns inconvenient for acids containing more than about six carbon atoms. Other stationary phases, *e.g.* polyesters, are preferable when interest lies in the longer chain acids.

The advantage of the pH-stat detector resides in its specificity and ability to yield a direct measurement of acids eluted from a column. No calibration problems arise as in the case of most other detectors. Thus a troublesome feature, which is a common source of errors in quantitative GLC, is eliminated.

ACKNOWLEDGEMENT

This investigation was financed, in part, by funds received from the Australian Dairy Produce Research Committee.

SUMMARY

A stationary phase comprising behenic acid, 20 parts, and phosphoric acid, 4 parts, on Chromosorb W (acid-washed), 100 parts, gives excellent gas–liquid chromatographic separations of C_1–C_6 saturated monocarboxylic acids when wet nitrogen is used as the carrier gas. The tolerance of this column packing for substantial amounts of water vapour in the gas phase has permitted development of a much simplified procedure for the quantitative analysis of volatile fatty acid mixtures isolated as sodium salts. A readily made laboratory chromatograph, using a commercial, glass electrode, auto-titrator as the detector, is described.

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