

Determination of Steam-Volatile Organic Acids in Fermentation Media by Gas-Liquid Chromatography

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ABSTRACT

PACKETT, L. V. (Purdue University, Lafayette, Ind.), AND R. W. McCUNE. Determination of steam-volatile organic acids in fermentation media by gas-liquid chromatography. *Appl. Microbiol.* **13**:22-27. 1965.—Five gas chromatographic liquid phases (25% Carbowax 20 M plus 4% H_3PO_4 , 17.5% dioctyl sebacate plus 7.5% sebacic acid, 17.5% dioctyl sebacate plus 7.5% docosanoic acid, 5% Tween 80, and 20% LAC-296 [poly(diethylene glycol adipate)] plus 2% H_3PO_4) were studied with respect to their utility in the separation and quantitation of steam-volatile organic acids commonly produced in fermentation. Optimal operating conditions and column stability for routine analysis were established. An Aerograph Hy-Fi gas chromatograph was used for all work, except the studies with Tween 80 in which an Aerograph A-90-C was employed. Chromatographic traces are presented of volatile fatty acid analyses with each of the liquid phases. Complete separation of all isomers of the fatty acids from C_2 to C_5 was accomplished by the Carbowax 20 M plus H_3PO_4 , dioctyl sebacate plus sebacic acid, and dioctyl sebacate plus docosanoic acid columns. The latter two liquid phases were extremely unstable and proved to be unsatisfactory for analysis of aqueous samples. A column of Carbowax 20 M + H_3PO_4 separated steam-volatile organic acids completely. The volatile fatty acid isomers were separated by 5% Tween 80 somewhat less completely, and the peak shapes were not as sharp and symmetrical as that desired for good quantitative work. LAC-296 (20%) plus 2% H_3PO_4 proved to be the most satisfactory of the liquid phases for routine analysis of deproteinized *in vitro* rumen fermentation media. The column has been used for routine analysis of rumen fermentation fluid and *in vitro* rumen incubation fluid. All the organic acids from C_2 to C_5 , except isobutyric, could be quantitated with this column. Stability of the column with the aqueous solutions was extremely good. The standard deviation of the analysis of each volatile acid component in a fermentation fluid was less than 0.5 molar per cent. The short-chain organic acids (C_2 to C_5) were shown to be extremely stable in aqueous solution for as long as 6 months after preparation for gas chromatographic analysis by protein precipitation with metaphosphoric acid- H_2SO_4 and refrigeration at 4 C in stoppered tubes.

Investigations of microbial fermentations require rapid, precise methods of quantitative analysis of metabolic end products that are excreted in the media. The steam-volatile fatty acids (VFA) are a group of metabolites commonly found under such conditions of fermentation.

Column chromatography methods similar to that developed by Elsdon (1946) have been used for some time for the separation of the VFA. These methods are time-consuming and require the use of large quantities of organic solvents. Since the development of gas-liquid chromatography (GLC) by James and Martin (1952), investigators have extracted the VFA with subsequent conversion to esters, usually methyl (James and Martin, 1956), prior to GLC analysis.

With the development of the hydrogen flame (HF) ionization detector, attention was turned to the analysis of free aqueous VFA.

The purpose of this investigation was to find the most suitable liquid phase for the direct GLC analysis of VFA in aqueous fermentation fluids. Emphasis was placed on column separation, stability, and the adaptation of a column for rapid routine analysis of normal C_2 to C_5 VFA and their isomers. It was also of interest to determine the precision which might be expected of such an analytical method, and the precautions necessary to store samples for later analysis.

MATERIALS AND METHODS

The gas-chromatographic instruments were obtained from Wilkens Instrument and Research,

Inc., Walnut Creek, Calif. The majority of the investigations were carried out with an Aerograph Hy-Fi 600 B. An Aerograph A-90-C, equipped with a thermal conductivity (TC) detector, and an Aerograph Hy-Fi 600 were used to a limited extent. Both Hy-Fi instruments were equipped with HF detectors. Detector temperature in all instruments was the same as the column temperature, because they were in the column oven.

The hydrogen supply for the flame detector was an Aerograph model 650 hydrogen generator. The air-flow rate was approximately 320 ml/min. A model 143 \times 57 Brown Electronik recorder (Minneapolis-Honeywell, Inc., Philadelphia, Pa.) equipped with a Disc integrator was used to record

and integrate the areas of component chromatograms.

Carrier gases employed were helium (passed through a tube of Drierite and Ascarite) for the A-90-C, and N₂ direct from the bottle was used for the Hy-Fi instruments.

Carbowax 20 M (Wilkins Instrument and Research, Inc., *personal communication*) and LAC-296 [poly(diethylene glycol adipate)], reported by Hunter, Ortegran, and Pence (1960), were obtained from Wilkins Instrument and Research, Inc. Dioctyl sebacate (Raupp, 1959; Fukui, Nagatomi, and Murata, 1962) was obtained from K & K Laboratories, Inc., Jamaica, N.Y. Sebacic acid (Raupp, 1959) was from Eastman Organic Chemi-

TABLE 1. Summary of the performance of GLC liquid phases on Chromosorb W (60 to 80) Regular for the separation of an equimolar mixture of steam-volatile fatty acids

Column packing	Operating conditions*					Separation† achieved	Stability
	Column size		Column temp	Gas flow (ml/min)			
	Length	Width		N ₂	H ₂		
	cm	cm	C				
Carbowax 20 M (25%) + 4% H ₃ PO ₄	243.8	0.3	165	20	20	C ₂ , C ₃ , C ₄ , C _{4-i} , C ₅ , and C _{5-i} all separated (Fig. 1).	Stable under stated operating conditions. Stable to aqueous injections.
Dioctyl sebacate (17.5%) + 7.5% seb- acic acid	182.9	0.3	160	16	20	C ₂ , C ₃ , C ₄ , C _{4-i} , C ₅ , and C _{5-i} all separated (Fig. 2).	Not stable. Bled off after 1 to 2 weeks at stated operating conditions. Extreme deterioration after aqueous injections.
Dioctyl sebacate (17.5%) + 7.5% do- cosanoic acid	182.9	0.3	160	16	20	C ₂ , C ₃ , C ₄ , C _{4-i} , C ₅ , and C _{5-i} all separated (Fig. 3).	Not stable. Bled off after 1 to 2 weeks. Extreme deterioration after aqueous injections.
Tween 80 (5%) with 45.7 \times 0.6 cm tail of 25% Carbowax 20	213.4	0.6	110			Overlap between C ₃ and C _{4-i} . Other wise all VFA from C ₂ to C ₅ were separated. Peaks were not sharp or symmetrical (Fig. 4).	Stable under stated operating conditions. Water stability not tested.
LAC-296 (20%) + 2% H ₃ PO ₄	182.9	0.3	134	16	20	C ₃ and C _{4-i} same peak. Slight overlap of C ₄ and C _{5-i} . All other VFA between C ₂ and C ₅ were separated (Fig. 5 and 6).	Stable under stated operating conditions. Stable to aqueous injections.

* Instrument used was an Aerograph Hy-Fi with an HF detector (injector temperature of 200 C), except that an Aerograph A-90-C instrument was used with the Tween 80 column. This A-90-C instrument was used with a 180 C injector temperature and a helium flow rate of 110 ml/min. It was equipped with a thermal conductivity detector.

† VFA notations are the following: acetate (C₂), propionate (C₃), isobutyrate (C_{4-i}), butyrate (C₄) isovalerate (C_{5-i}), and *n*-valerate (C₅).

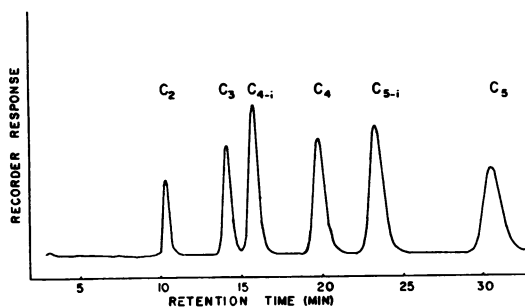


FIG. 1. Separation of VFA in an artificial mixture by Carbowax 20 M + H_3PO_4 . Operating conditions are given in Table 1.

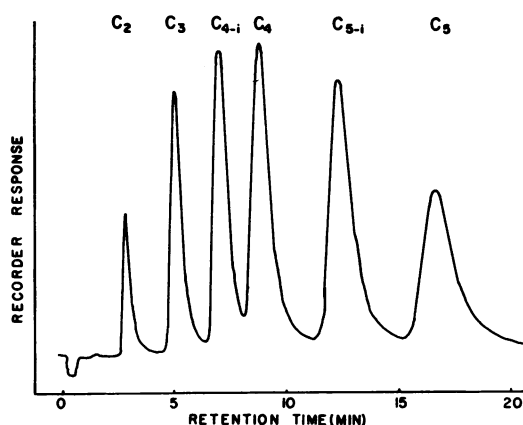


FIG. 2. Separation of VFA in an artificial mixture by dioctyl + sebacate sebacic acid. Operating conditions are shown in Table 1.

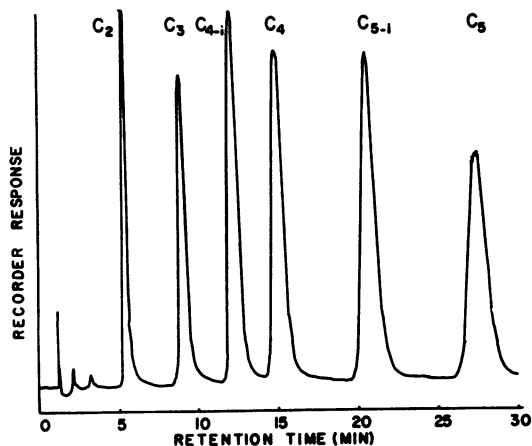


FIG. 3. Separation of VFA in an artificial mixture by dioctyl sebacate + docosanoic acid. Operating conditions are given in Table 1.

cals, Rochester, N.Y., as was the docosanoic acid (Fukui et al., 1962). Polyoxyethylene (20) sorbitan mono-oleate (Tween 80), reported by Smith (1959), was from Atlas Powder Co., Wilmington, Del. Orthophosphoric acid (85%) was obtained from the J. T. Baker Chemical Co., Phillipsburg, N.J. All the liquid phases were coated on Chromosorb W (60 to 80) Regular from Wilkens Instrument and Research, Inc. The coating of liquid phases and packing of columns were done in our laboratory. All columns were copper tubing. The columns were conditioned before use by purging with nitrogen for 24 hr at 200 C.

Several criteria were established to compare the liquid phases. First, the column was evaluated as to its ability to separate the VFA dissolved in an organic solvent (either carbon disulfide or diethyl ether). Secondly, the stability of the liquid phase upon repeated use and continued holding at operating temperature was examined. Thirdly, the ability of the column to withstand repeated aqueous injections was evaluated.

Aqueous solutions were prepared for analysis by adding to 5 ml of the fermentation fluid (or a standard VFA aqueous mixture) 1 ml of 5 N H_2SO_4 containing 25% metaphosphoric acid to precipitate the protein. The solutions were mixed, allowed to stand at least 30 min, and then centrifuged at $1,360 \times g$ for 10 min. An amount of 1 to 2 μ liters of the resulting supernatant fluid was injected into the chromatograph, or refrigerated for later analysis.

For quantitative work, peak areas were normally determined by the use of the Disc integrator. Molar percentages of the VFA were determined by the internal normalization method (Burchfield and Storrs, 1962) with corrected peak areas. Correction factors for the peaks were established by GLC separation of an equimolar mixture of the pure VFA containing acetic, propionic, butyric, isovaleric and *n*-valeric acids. Butyric acid was the VFA upon which the correction factors were based. The following formula

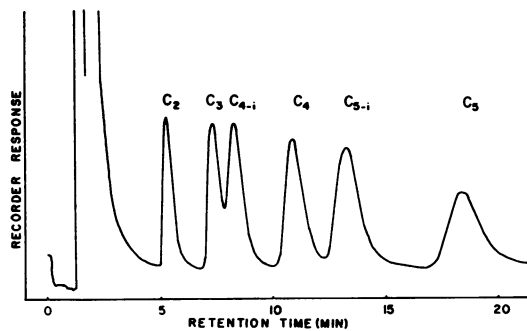


FIG. 4. Separation of VFA in an artificial mixture by Tween 80. Operating conditions are given in Table 1.

was used:

Correction factor for VFA_x

$$= \frac{\text{Area of butyric acid peak in an equimolar sample}}{\text{Area of } VFA_x \text{ peak in the same equimolar sample}}$$

Total VFA was determined by steam distillation (Olmstead, Whitaker, and Duben, 1929).

Extensive routine analytical work was done by use of the 20% LAC-296 plus 2% H_3PO_4 column with the Aerograph Hy-Fi 600 B. In vitro ovine rumen fermentation media were analyzed on this

column after the aqueous samples had been prepared by the method outlined previously.

RESULTS AND DISCUSSION

Table 1 summarizes the evaluations made of the tested liquid phases under the conditions stated. The operating conditions shown were established as optimal for the experimental conditions and column.

Characteristic chromatographic curves for each of the tested liquid phases are illustrated in Fig. 1 to 6.

LAC-296 (20%) plus 2% H_3PO_4 afforded good separation of the major VFA (Fig. 5 and 6). Similar results have been observed by Rumsey (1963). The chromatogram of a typical artificial rumen sample is illustrated in Fig. 7. The column was best prepared by dissolving the liquid phase in dichloromethane and coating the Chromosorb W in a flash evaporator (Rinco Instrument Co., Greenville, Ill.), followed by the coating of aqueous H_3PO_4 in the same manner. This column is used extensively in this laboratory to separate VFA in the rumen liquor or in vitro fermentation media. The columns have been stable up to 3 months of continuous use and 500 determinations.

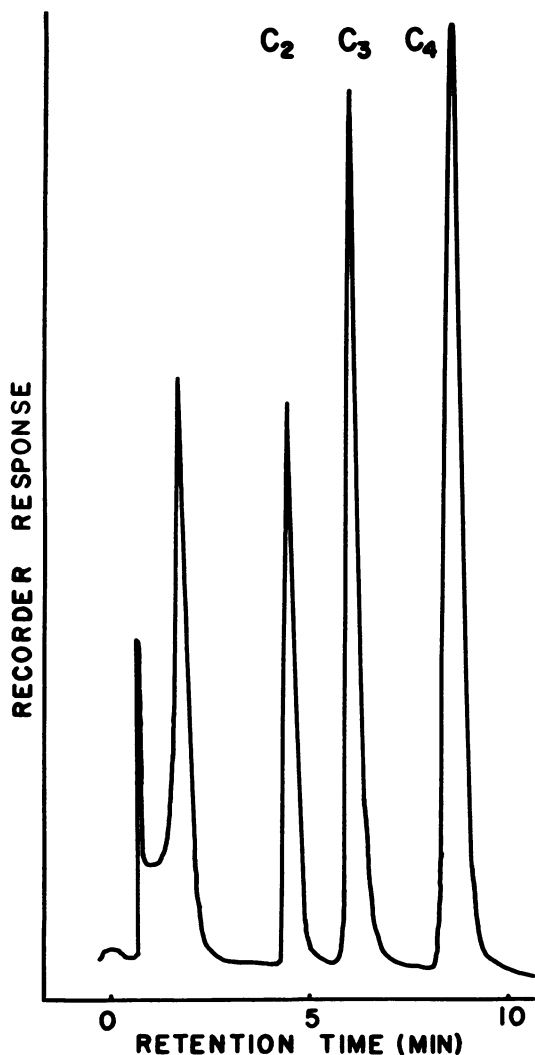


FIG. 5. Separation of VFA (C_2 , C_3 , and C_4) in an artificial mixture by LAC-296 + H_3PO_4 . Operating conditions are given in Table 1.

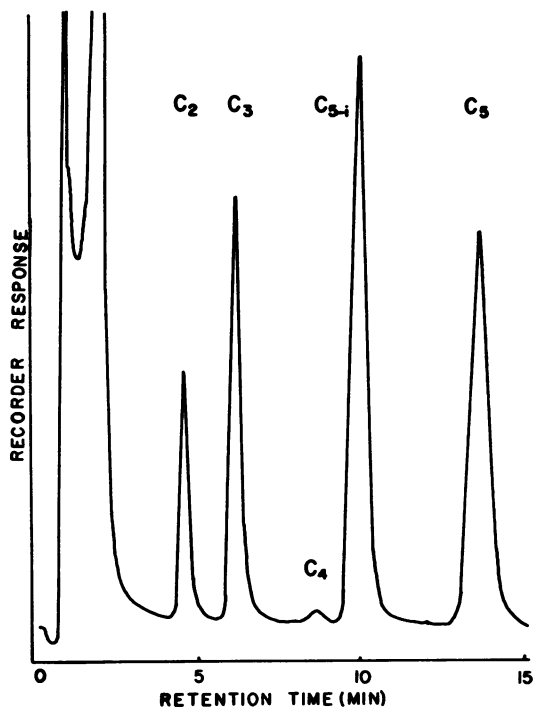


FIG. 6. Separation of VFA (C_2 , C_3 , C_4 , C_{5-1} , C_5) in an artificial mixture by LAC-296 + H_3PO_4 . Operating conditions are given in Table 1.

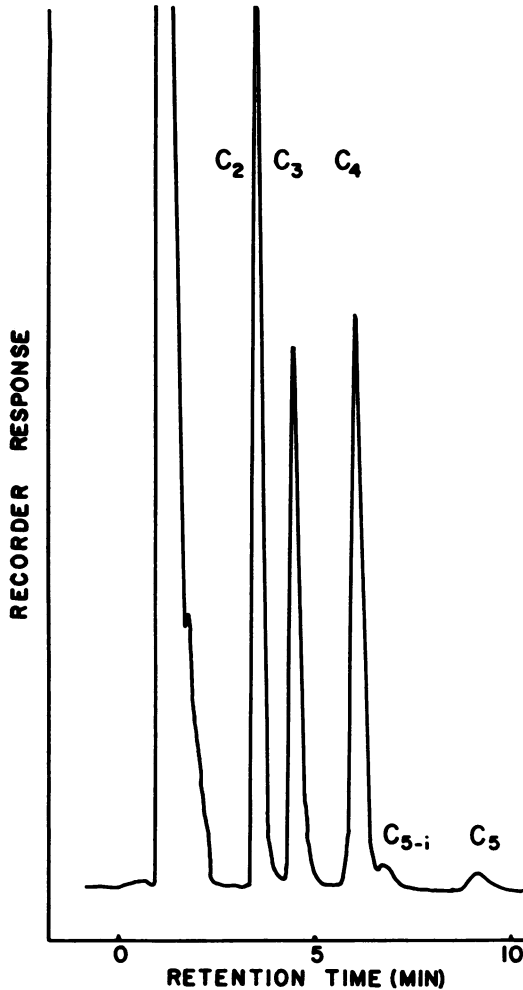


FIG. 7. Gas-liquid chromatographic analysis of the VFA in a typical *in vitro* rumen sample. The liquid phase is 20% LAC-296 + 2% H_3PO_4 , and the operating conditions are as in Table 1.

A series of determinations were made on duplicate samples from an artificial rumen experiment (Table 2) with the LAC-296 plus H_3PO_4 column. These values are indicative of the precision that may be expected with aqueous fermentation fluids containing low levels of VFA (30 μ eq/ml). Rumen and *in vitro* rumen fluid are well above this range. The two samples were also analyzed 6 weeks prior to the above work. It can be noted that little, if any, change occurred in the mean values of the samples (Table 2). These samples had been held in rubber-stoppered tubes at 4 C after preparation for analysis by the H_2SO_4 -metaphosphoric acid reagent as previously outlined. This points out the feasibility of holding samples in this manner for long periods of time without the fear of loss of the VFA. In this form, samples are always ready for immediate analysis. Further evidence of the stability of the VFA in unfrozen aqueous acid solution is the comparison of the peak correction factors as determined by the analysis of a standard VFA mixture at three different times after preparation for analysis (Table 3). The same standard solutions were used over a 6-month period of time, being held at 4 C in rubber-stoppered test tubes. It is quite obvious

TABLE 3. Constancy of the VFA peak correction factors with time

Time elapsed	Peak correction factors*				
	Acetate	Propionate	Butyrate	Isovalerate	n-Valerate
Initial	2.92	1.43	1.00	0.75	0.83
9 days	2.89	1.48	1.00	0.76	0.85
6 months	2.82	1.41	1.00	0.76	0.88

* The standard solutions were diluted so that analysis was done at the same electrometer attenuation as the fermentation samples.

TABLE 2. Precision of analysis of VFA in duplicate *in vitro* rumen fermentation flasks by gas-liquid chromatography*

Fermentation flask	Molar percentage of acids				
	Acetic	Propionic	Butyric	Isovaleric	n-Valeric
Flask A	57.0 (0.4)	31.0 (0.3)	9.4 (0.1)	0.6 (0.1)	2.1 (0.2)
Flask A'	56.9 (0.6)	31.0 (0.5)	9.3 (0.3)	0.5 (0.2)	2.2 (0.3)
Flasks A and A' combined	56.9 (0.5)	31.0 (0.3)	9.3 (0.3)	0.5 (0.1)	2.2 (0.2)
Routine analysis†	56.9	30.7	9.7	0.6	2.4

* The GLC column was 20% LAC-296 + 2% H_3PO_4 . Operating conditions were as in Table 1. Ten determinations were run on flask A and 13 on A'. Values reported are the mean with the standard deviation in parentheses.

† Single determinations were made on each of these two flasks and averaged as part of the routine analysis of a fermentation experiment 6 weeks prior to the time the other values in this table were determined.

that little or no change had occurred in any of the fractions during that period of time.

Examination of Table 1 and Fig. 1 indicates that 25% Carbowax 20 M plus 4% H₃PO₄ would have been the most suitable liquid phase for routine analysis of aqueous VFA solutions. However, these observations were made with a large number of trials on one column. Repeated attempts to make additional working columns of this material were unsuccessful. Due to this difficulty in making the column again, it was not used for routine analysis.

Both the dioctyl sebacate plus sebacic acid and dioctyl sebacate plus docosanoic acid columns (Fig. 2 and 3) yielded excellent separations of the VFA. However, the dioctyl sebacate plus sebacic acid liquid phase was not stable. Increased stability, expected by substituting docosanoic acid for sebacic acid according to Fukui et al. (1962), was not observed in this work. The lack of stability of both types is even more marked upon injection of aqueous solution.

Tween 80, although reported by Smith (1959) to be useful for the analysis of aqueous solutions, did not separate ethereal VFA into desired sharp, symmetrical peaks (Fig. 4). It was not tested with aqueous solutions of the VFA.

A problem encountered in the analytical procedure as outlined was the incomplete precipitation of the protein in the *in vitro* fermentation media. Apparently, the small amount of protein sometimes remaining in solution caused baseline difficulties in a number of the analyses. When this occurred, peak areas were approximated by the "height \times width at half-height method" ($0.84 \text{ area} = \text{height} \times \text{width at half-height}$; Burchfield and Storrs, 1962). This procedure, although accurate, is tedious and time-consuming. A recent report by Hoehne, Cramer, and Daugherty (1963) deals with the problem of protein precipitation in the preparation of fermentation samples for GLC analysis. These workers precipitated the protein with ethanol, and this necessitated subsequent evaporation of the ethanol. A procedure requiring less handling of the samples would be desirable.

The method described for the routine storage and analysis of the VFA is suitable for use with

aqueous biological fluids. Because the separation of the branched VFA (isobutyric and isovaleric) is not complete, a liquid phase such as Carbowax 20 M plus H₃PO₄ may be more suitable. However, disadvantages of this column are the difficulty in reproducing its packing, as discussed in the text, and the longer time of analysis that is required (Fig. 1).

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