Journal of Chromatography, 244 (1982) 337–346
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,945

ANALYSIS OF C_2–C_6 MONOCARBOXYLIC ACIDS IN AQUEOUS SOLUTION USING GAS CHROMATOGRAPHY

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(First received July 23rd, 1981; revised manuscript received April 2nd, 1982)

SUMMARY

The determination of short chain fatty acids is a key requirement in many fermentations and has been studied by gas chromatography.

A simple acidification of the fermenter broth with oxalic acid allows direct injection on untreated porous polymer columns with virtually no ghosting problems.

The method is suitable for the routine quantification of *normal*- and *iso*-monocarboxylic acids with an analysis time of 40 min. For triplicate analyses, the 95% probability confidence limits are ± 0.05 for a mean of 1 g/l.

INTRODUCTION

Free fatty acids are produced in a number of biological processes[1–4] and their accurate determination is of considerable interest to biochemists and physiologists alike.

The analysis is of particular interest in anaerobic digestion processes as most of the macromolecular input is degraded to C_2–C_6 acids prior to methane production. Certain individual acid levels are indicators of digester malfunction whilst overall acid levels must be controlled to prevent the inhibition of methane generation.

Separations of the free fatty acids by gas chromatography (GC) have been limited by (a) Adsorption of the acids in the column and apparatus[5–7], and (b) Postulated molecular association of the acids in the vapour state.

Adsorption, recognised as the worst of the problems, generally gives rise to tailing of the acid peaks, irregularly shaped peaks and "ghosting"[8]. Molecular association may also result in the first two effects. Ghosting occurs when a solution, sufficiently more polar than the acids being analysed, is injected subsequent to a solution of free fatty acids, with the result that acids which were previously adsorbed in the column are eluted. From the work of Van Eenaeme *et al.*[9] ghosting has been shown to be dependent on the column packing, acid nature, injector type, nature of the ghost eluter and injection sequence.

Due to the difficulties mentioned above, free fatty acids have frequently been analysed after conversion to their esters which are more easily analysed. However, Metcalfe and Schmitz[10] have reported problems with the loss of lower fatty acids in the aqueous layer.

0021-9673/82/0000–0000/$02.75

As noted by Nikelly[7], four main methods have been used for overcoming the problems associated with the analysis of free fatty acids by GC. These consist of using a highly polar liquid phase, of modifying the liquid phase, the inert support or the carrier gas.

The use of polar liquid phases has had little success. Emery and Koerner[11] reported the efficient separation of aqueous solutions of the lower free fatty acids but Erwin *et al.*[8] and Arni *et al.*[12] were unable to repeat the work due to excessive tailing and ghosting. Baker[13] used 10% FFAP on Chromosorb W for separation of aqueous C_2–C_6 acids. At low levels the shape of the acetic acid peak was so poor that quantitation was impossible.

Modification of the liquid phase has been widely used. It normally involves the addition of a non-volatile acid to the liquid phase. This is believed to function by suppressing hydrogen bonding and therefore dimerisation[14]. It also overcomes the alkaline effect of random hydroxyl groups present in the column and packing. Recent evidence indicates that acid additives have most effect on the support[15] with phosphoric acid being normally used. Vandenheuvel[16] used a 20% DEGS–2% H_3PO_4 packing in a stainless steel column to determine C_2–C_4 acids. However, the acids had to be converted to their sodium salts and dissolved in carbon disulphide containing formic acid before injection. With meticulous conditioning of the column good accuracy and reproducibility were achieved. Dees and Moss[17] used a packing of 15% SP 1200–1% H_3PO_4 to separate C_2–C_7 free fatty acids. The acid level used was not quoted and the peaks tailed badly, acetic acid eluting on the tail of the solvent peak. Di Corcia and Samperi[3] and Du Preez and Lategan[18] have both used the novel support, graphitised carbon black, modified with FFAP or Carbowax 20M–H_3PO_4, respectively but in both cases formic acid had to be added to the solution to suppress ghosting. Furthermore, such columns are expensive and difficult to prepare.

Porous polymers have been used for the analysis of free fatty acids both with[19] and without[2,20] modification. Henkel[2] utilised Poropak N to quantitatively determine low levels of free fatty acids in aqueous solution but unpublished work by the author failed to reproduce the published results.

As was realised by Ackman and Burgher[21], if the analysis of free fatty acid is carried out in the continually polar atmosphere of formic acid, the strongest acid of the monocarboxylic acid series, the difficulties associated with adsorption should be overcome. Cochrane[14] introduced the acid by passing the carrier gas over the surface of formic acid contained in a small glass vessel. However, corrosion of the system will occur if water is present in any quantity.

Although the difficulties surrounding the analysis of free fatty acids by GC are legion, as illustrated by the foregoing, the advantages of gas chromatography over other techniques in terms of sensitivity of detection, speed of analysis and high degree of resolution make it worth the effort to overcome the problems.

This paper details a method whereby free fatty acids may be determined individually in aqueous solution with none of the laborious intricacies previously mentioned. The method produces virtually no ghosting, even at concentrations of fatty acids as low as 50 ppm.

EXPERIMENTAL

Materials

Acetic acid, propionic acid, isobutyric acid, *n*-butyric acid, pivalic acid, isovaleric acid, *n*-valeric acid, isocaproic acid and *n*-caproic acid were obtained from commercial sources and were ≥97% pure. Oxalic acid, Analar reagent, was purchased from Fisons Scientific, acetone, Analar reagent, from Hopkins & Williams and Porapak Q (80–100 mesh) (Waters), from Phase Separations.

Apparatus

A Pye 104 gas chromatograph fitted with dual heated flame ionisation detectors and a wide range amplifier was utilised. The columns were glass coils (1.5 m × 4 mm I.D.) packed with Porapak Q (80–100 mesh). Prior to packing, the Porapak Q was exhaustively extracted in a Soxhlet apparatus for at least 30 min each with two separate 100-ml portions of acetone.

The GC conditions were as follows: helium carrier flow-rate, 60 ml/min at NTP; hydrogen flow-rate, 30 ml/min at NTP; air flow-rate, 500 ml/min at NTP; oven temperature, 220°C; injection block temperature, 200°C; detector temperature, 200°C; injection volume, 1 μl on-column injection; recorder, Smiths Servoscribe, 10 mV full scale.

The peak areas were measured with a Kemtronix Supergrator 3 computer integrator, which also calculated the results.

Conditioning of the column is essential for reproducible analysis. For this, the Porapak Q must be thoroughly extracted with acetone before use to remove monomeric material. The packed column must be heated to 220°C for several days whilst passing the normal flow of carrier gas. Extended heating of the column at 220°C improves peak shape and resolution, probably as a result of the loss of the last traces of monomeric material. It is advisable to prepare a spare column running in parallel with the column in use, so that the analytical column can be replaced when contamination eventually degrades its performance.

Calibration

To establish the concentration working range for each acid a number of solu-

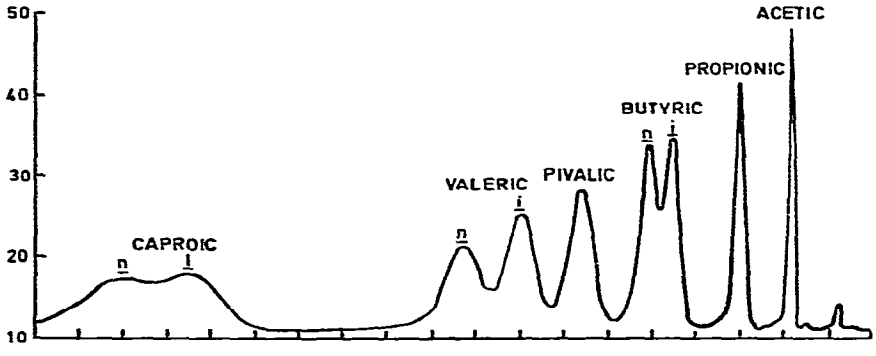

Fig. 1. Chromatogram of standard acid mixture C_2–C_6. Column: glass, 1.5 m × 6 mm, Porapak Q (80–100 mesh) (acetone extracted). Oven temperature: 220°C. Helium flow-rate: 60 ml/min. Chart speed: 1 cm/min. Attenuation: 8×10^2.

tions with concentrations varying between 0.005 g/l and 10 g/l were prepared and injected. From the results a practical linear working range of 0.05–5 g/l was obtained.

In order to use the computing integrator a spot calibration within that range was carried out weekly.

The calibration sample of acids in the range C_2–C_6 was prepared by accurately weighing approximately 0.1 g of each acid into a 100-ml volumetric flask and making-up to the mark with 0.5 *M* aqueous oxalic acid solution. A 1-μl portion of this mixture, when injected into the gas chromatograph using the conditions described previously, produced the chromatogram shown in Fig. 1.

Integration areas were allocated for merged peaks by dropped perpendiculars except in the case of *n*-valeric acid which was tangent skimmed. Using these areas the response factors of area/unit weight were calculated for each acid. Further injections were made and the response factors for each acid averaged.

Preparation of samples

The anaerobic digester broth was spun at a relative centrifugal force of 12,000 for 10 min to produce a clear supernatant. To 1 ml of this supernatant, contained in a 2-ml septum vial fitted with a Teflon faced septum, were added approximately 0.06 g solid oxalic acid. The vial was well shaken and allowed to stand for 5 min before samples were chromatographed. The volume change due to the oxalic acid was negligible for these analysis.

The pH of the sample solution after addition of oxalic acid was found to be <2.5 when tested with wide range pH paper.

RESULTS

This analysis will handle a wide variety of aqueous solutions of C_2–C_6 free fatty acids. For example, Fig. 2 shows the chromatogram given by an oxalic acid treated pre-digestion feedstock of molasses plant effluent. The post-anaerobic digestion supernatant analysis may be seen in Fig. 3.

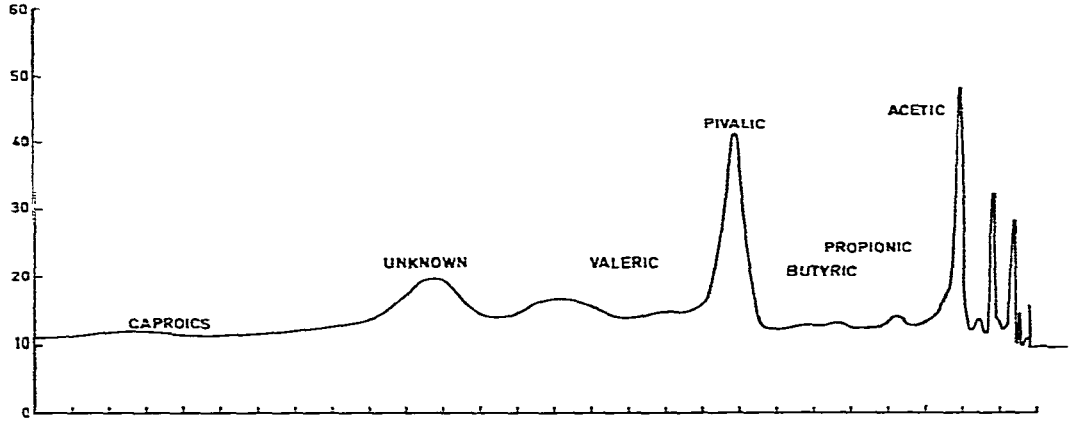

Fig. 2. Chromatogram of molasses effluent before anaerobic digestion. Conditions as in Fig. 1, except attenuation: 2×10^2.

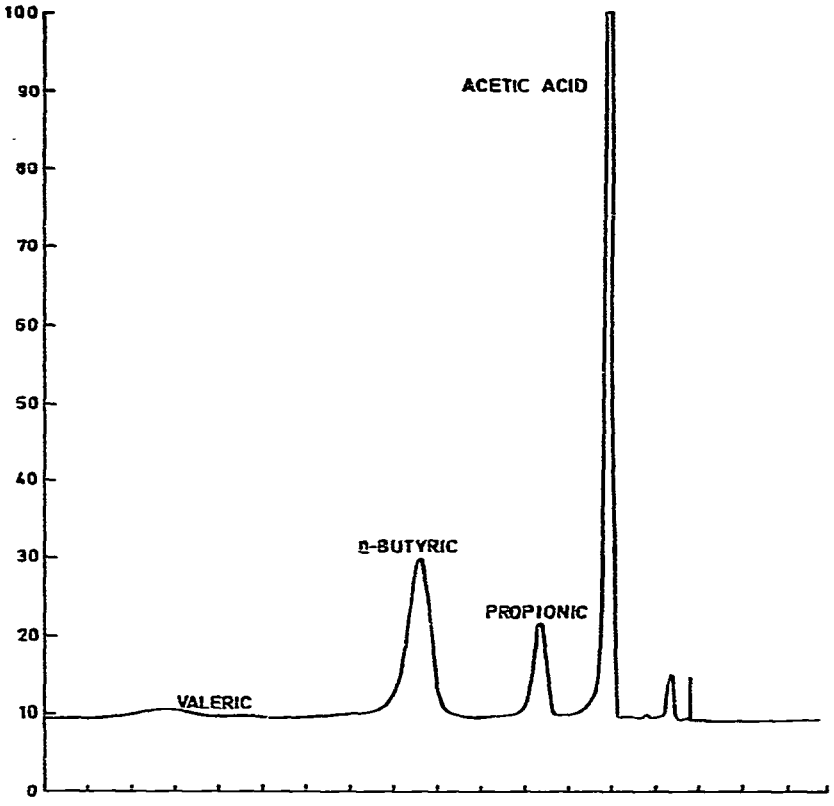

Fig. 3. Chromatogram of molasses effluent after anaerobic digestion. Conditions as in Fig. 1.

Similarly Fig. 4 shows the chromatogram produced by an anaerobically digested sample of pig manure slurry.

Precision

Repeated injections of the C_2–C_6 standard fatty acids sample mixture were made to test the repeatability of the analysis. The results are shown in Table I. The relative standard deviation for triplicate determinations is quite uniform and substantially within $\pm 5\%$ at the level of 1 g/l.

The precision of a series of actual fermenter samples may be seen in Table II where the results reported for triplicate analyses confirm the expectations, at least in the 4–0.3 g/l range.

Ghosting

The ghosting between injections was checked by injecting a 0.5 *M* aqueous solution of oxalic acid immediately after a standard acid mixture containing approximately 1 g/l of each acid.

The results obtained may be seen in Table III. Only acetic acid shows any degree of ghosting but even this is below the minimum value set for the analysis, *i.e.*,

TABLE I

DETERMINATION OF THE REPEATABILITY OF ANALYSIS OF A STANDARD SOLUTION OF FATTY ACIDS AT THE 1 g/l LEVEL

No. of determinations = 7.

	Integrator readings								
	Acetic	*Propionic*	*Isobutyric*	*n-Butyric*	*Pivalic*	*Isovaleric*	*n-Valeric*	*Isocaproic*	*n-Caproic*
Mean value	294,286	443,591	522,361	586,555	610,489	882,155	323,228	1,177,343	1,177,343
Standard deviation	2943	8372	9035	10,144	11,860	32,826	4594	22,827	22,827
Relative standard deviation (%) for a triplicate analysis	1.4	2.6	2.4	2.4	2.6	5.1	1.9	2.6	2.6

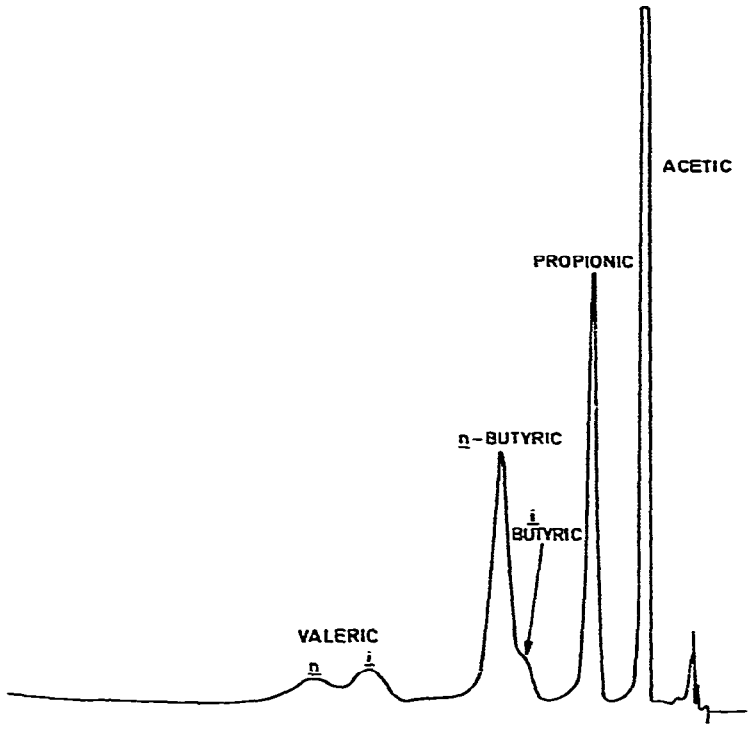

Fig. 4. Chromatogram of an anaerobically digested sample of pig slurry. Conditions as in Fig. 1.

0.05 g/l (equivalent to 5% of the amount injected). Subsequent injections of the aqueous 0.5 *M* oxalic acid solution showed no trace of ghosting.

Real samples were also tested for ghosting in a like manner, with results similar to those given by pure standards, being obtained.

DISCUSSION

The quantitative results obtained by the addition of oxalic acid to the sample before chromatographing are consistent. Experimental work shows good column life in spite of the dirty nature of the sample; lives of 8 months or more are possible even with multiple analysis.

As Table III shows, very little ghosting is evident. The quantity of acetic acid found on subsequent injection of oxalic acid solution was less than 4% of the original quantity injected.

The mechanism of the process whereby oxalic acid prevents the other organic acids from adsorbing is uncertain. Differential thermal analysis of oxalic acid revealed that little breakdown occurred when it was heated to 220°C, the temperature pertaining to the injection port and column oven. Most of the oxalic acid sublimes at or before that temperature.

Rapid heating of oxalic acid yields formic acid[22]. Chromatographing 0.4 *M* aqueous oxalic acid on a Porapak Q column connected to a katharometer detector

TABLE II

THE REPEATABILITY OF A SERIES OF ANAEROBIC DIGESTER SAMPLES

C_2–C_5 Acids determined; all values in g/l.

	Acetic	*Propionic*	*Isobutyric*	*n-Butyric*	*Isovaleric*	*n-Valeric*
Sample No. 1.						
No. of determinations	3	3	–	3	3	3
Mean value	2.77	2.34	–	2.63	0.09	3.58
Relative standard deviation (%)	1.3	1.5	–	1.1	–	1.5
Sample No. 2						
No. of determinations	3	3	–	3	3	3
Mean value	2.10	1.14	–	1.19	0.06	0.81
Relative standard deviation (%)	1.0	1.5	–	0.5	9.6	1.2
Sample No. 3						
No. of determinations	3	3	3	3	3	3
Mean value	3.52	1.61	0.13	0.28	0.11	0.13
Relative standard deviation (%)	0.9	1.6	–	29	15	

showed that 16% of the oxalic acid was released from the column as formic acid. The formic and oxalic acids may be acting independently or in concert to block active sites, thus preventing adsorption and subsequent ghosting. The low degree of ghosting exhibited, enabled a linear working range of 0.5–5 g/l to be attained.

Use of oxalic acid, results in an analysis in which sample handling and pretreatment is minimised, this is suited to routine fermenter analysis. With sufficient oxalic acid being added to maintain the pH of the sample solution at or below 2.5, it may be calculated from the pK_a values of the C_2–C_6 acids listed in Table I that all will be less than 1% dissociated. Thus, there will be no losses due to the salt form being deposited in the injection port.

The acids present in the samples were identified by co-elution with known standards. Pivalic acid (identified this way) appeared in some samples and seemed to be a function of a poor fermentation. Attempts were made to confirm the identity of the acids present by means of combined gas chromatography–mass spectrometry. This proved impossible as the acids were adsorbed in the separator between the chromatograph and the mass spectrometer.

Further work is being undertaken to find column packing materials which will give improved separation of these acids.

CONCLUSIONS

A rapid, reliable and precise chromatographic method for the direct determination of free fatty acids in the range C_2–C_6 in aqueous solution has been developed.

Oxalic acid, added to each sample, has proved to be a convenient and reliable

TABLE III

DETERMINATION OF THE DEGREE OF GHOSTING BETWEEN INJECTIONS

All values in g/l unless stated otherwise.

Injection	*Acetic*	*Propionic*	*Isobutyric*	*n-Butyric*	*Pivalic*	*Isovaleric*	*n-Valeric*	*Isocaproic*	*n-Caproic*	
Leve. of C_2–C_6 acid injected	1.00	1.10	1.00	1.00	1.00	1.04	1.00	1.00	1.00	
Correction reqd. due to ghosting	0.04 (4.0%)					Less than 0.01				→
Level of C_2–C_6 acid injected	1.01	0.99	1.0	0.99	0.99	1.00	1.01	0.98	0.98	
Correction reqd. due ghosting	0.04 (4.0%)					Less than 0.01				→
Level of C_2–C_6 acid injected	0.99	0.99	1.0	1.02	1.0	1.03	0.99	1.01	0.98	
Correction reqd. due to ghosting	0.038 (3.8%)					Less than 0.01				→

method for overcoming the ghosting problems normally associated with this type of chromatography.

Furthermore, its use has extended column life so that the routine determination of acids in anaerobic digester effluents may be accomplished.

ACKNOWLEDGEMENTS

Permission to publish this paper has been given by The British Petroleum Company Ltd.

The assistance of Mrs. I. Paschentis with the statistical analysis is gratefully acknowledged.

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