Packed and Capillary Column Gas Chromatographic Analysis of Fecal Samples for Volatile Fatty Acids

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Procedures for analyzing volatile fatty acids content of aqueous samples using packed and capillary chromatography columns are described and compared. A packed column system tolerant of aqueous samples was used that incorporated a formic acid vapor entrainment device. This permitted a simple sample preparation and concentration scheme. This system was compared to capillary column analysis with split, manual, or autosampler injection. The autosampler/capillary system used a solvent trapping technique not previously applied to aqueous sample chromatography. The capillary column gave greatly increased resolution, resulting in more precise quantitation and lower detection limits but with slightly longer analysis time. These procedures have been successfully applied to the analysis of volatile fatty acids in large numbers of fecal samples.

Volatile fatty acids (VFAs), acetic through valeric, are common products of bacterial fermentation of carbohydrates. The quantitative analysis of the common VFAs involves two major areas: sample preparation and chromatographic separation. Sample preparation has been performed by several different methods including solvent extraction (Spiller et al., 1980) vacuum microdistillation (Vreman et al., 1978), derivatization (Salanitro and Muirhead, 1975; Larsson and Roos, 1983), and combinations of these. Quantitative separation of the VFAs has been reviewed (Cochrane, 1975; Kuksis, 1977). The choice of gas chromatography (GC) column and conditions for its use must take into consideration the method of sample preparation and the kind of information desired from the analysis.

In the normal mammalian digestive tract, carbohydrates and other non- or malabsorbed food components may be fermented to VFAs and other metabolites by the bacterial flora residing in the large intestine (Spiller et al., 1980; Ehle et al., 1982). This work describes and compares simple sample preparation methods in conjunction with packed column analysis and capillary column analysis with autosampler to provide relative data on the VFA content of human fecal samples.

MATERIALS AND METHODS

Reagents. Formic acid was obtained from Aldrich Chemical Co., Milwaukee, WI, and was redistilled and stored over $MgSO_4$ prior to use. Acetic, propionic, isobutyric, butyric, pivalic, isovaleric, 2-methylbutanoic, and valeric acids were obtained from Eastman Organic Chemicals, Rochester, NY, and used without further purification. Sodium hydroxide and hydrochloric acid were analytical grade; water used in sample preparation was purified with a Milli-Ro/Milli-Q deionizer (Millipore Corp., Bedford, MA).

Standard mixtures of acetic through valeric acids were prepared by weighing the individual acids into 3 mL of 4 N NaOH, adding 20 mL of 1 N HCl, and diluting to the required volume with deionized H_2O.

Sample Preparation. Human feces were prepared by diluting 3:1 (v/w) a freshly collected sample with 0.9% NaCl (w/v) and homogenizing at low speed in a blender for ca. 1 min under a blanket of flowing argon. One-milliliter aliquots were withdrawn and added to 1.5 mL of water in incubation tubes, purged with argon, sealed, placed in a boiling water bath for 6 min, and quickly frozen. Dry weights were obtained by air-drying a 1-mL aliquot for 72 h at 70 °C. Fecal samples were prepared for VFA analysis by adding 2 mL of 0.2 N NaOH to 2.5-mL frozen aliquots, thawing, mixing thoroughly, and centrifuging for 20 min at 1300g. A 1-mL aliquot of the supernatant was dried at 50 °C under N_2 and reconstituted with 200 μL of 2.0 N HCl. This was centrifuged for 20 min at 10000g, and 1–3 μL of the supernate was injected onto the packed column. For autosampler capillary column analysis frozen aliquots were thawed, mixed thoroughly, and centrifuged 10 min at 1300g, and the supernatant was filtered through 0.45 μM disposable filters (Millex, Millipore Corp., Bedford, MA). An aliquot of the filtered supernatant, 100 μL, was immediately transferred to an autosampler vial, which was frozen until analysis. Prior to analysis 10 μL of 4.0 N HCl was added to each frozen sample.

Packed Column Chromatography. A 6 ft long × 2 mm i.d. glass column packed with Super Q, 80–100 mesh, (Alltech Associates, Inc., Deerfield, IL) retained with H_3PO_4-treated glass wool was used in a Hewlett-Packard 5830A gas chromatograph. The column was installed in an "on-column injection" configuration, but with 1.5 mm × 100 mm glass capillary tubes (Kimble Products, Owens-Illinois) used between septum and column packing as disposable injection liners.

The VFAs were eluted with helium at 50 mL min^{-1} flow. The column oven temperature was held at 190 °C for 3 min, then programmed from 190 to 210 °C at 4 °C min^{-1}, and held for 5.4 min. The separated VFAs were detected by using a flame ionization detector (FID) operated at 250 °C. The injector temperature was held at 200 °C.

The carrier gas was treated with formic acid vapor to improve the chromatography. The device used to entrain the vapor in the carrier stream is shown in Figure 1. It was placed between the GC flow controller and the injection port. The device was constructed by using a thick walled glass sediment trap with stainless steel top and a stainless steel $^1/_4$-in. union cross. Carrier gas flow was routed through $^1/_8$-in. down to $^1/_{16}$-in. stainless steel tubing that runs through the union cross to end slightly below the lip of a 1-dram (15 × 45 mm) shell vial. The carrier gas impinged on the 1-mL formic acid pool in the shell vial and was routed back to the injection port through $^1/_8$-in. stainless steel lines and fittings. The reservoir was filled by shutting off the carrier gas flow and removing the top fitting of the union cross, which contained a $^1/_4$-in. Teflon rod as a fitting plug. A syringe with a 4 in. long 18-ga Teflon-coated stainless steel needle was used to introduce formic acid into the vial reservoir. To shut off the carrier

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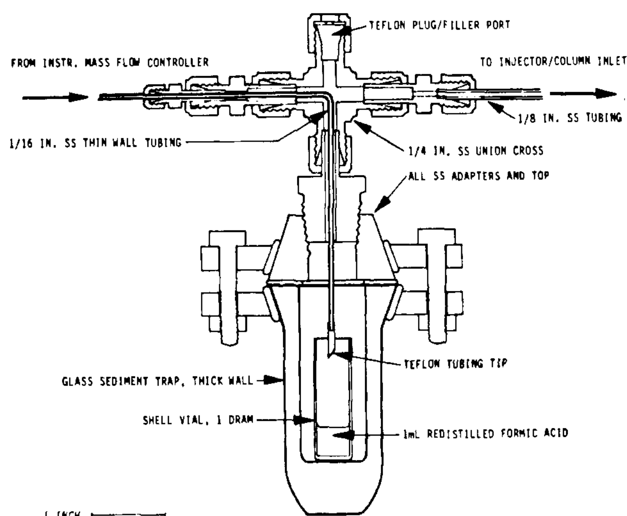

Figure 1. Formic acid vapor entrainment apparatus.

gas flow to the vapor entrainer while maintaining column flow, a four-port ball valve was installed between flow controller, vapor entrainer, and injection port.

Capillary Column Chromatography. A fused silica capillary column, 0.25 mm i.d. × 30 m long, with OV-351 (a Carbowax nitroterephthalic acid polymer) coating was obtained from J & W Scientific, Rancho Cordova, CA. It was used in a Hewlett-Packard 5840A gas chromatograph in the split injection mode. The split ratio in the 200 °C injection port was 1:30 with a column flow of 1.3 mL min^{-1} of helium. The split liner was packed with Chromosorb W (HP), 80–100 mesh, coated with 3% OV-1 retained by H_3PO_4-treated glass wool. The column oven temperature was programmed from 140 to 158 °C at 2 °C min^{-1} and held 5 min. Peak detection was by FID at 250 °C with N_2 makeup gas.

Manual injections of from 1 to 3 μL were made with a 5-μL syringe (Dynatech Precision Sampling, Baton Rouge, LA) with a Teflon-tipped plunger, rinsed between injections with 0.5 N NaOH, 0.5 N HCl, and deionized H_2O.

The same capillary column was also installed in a Hewlett-Packard 5880A gas chromatograph in the same mode, split liner, injection and detector configurations, and temperatures. The column oven temperature was held at 50 °C for 0.1 min and then programmed from 50 to 130 °C at 30 °C min^{-1}; the temperature was then programmed from 130 to 150 °C at 2 °C min^{-1} and held for 1 min. A postrun temperature of 175 °C was used to elute any other compounds. A Hewlett-Packard 7671A automatic sampler was used to inject 1-μL samples by using a 5-μL Dynatech Precision Sampling Teflon-tipped plunger syringe. The syringe was rinsed with distilled water between sample or standard injections.

Data Preparation. Fecal VFA content was calculated by comparing sample peak areas to standard mixture peak areas. All samples were routinely chromatographed in triplicate and results averaged. The method of standard additions at three addition levels of known mixtures was used to ensure peak identification, purity, and recovery.

RESULTS AND DISCUSSION

Sample preparation for VFA analysis by GC has been performed by several methods including methylation, extraction, and microdistillation. These procedures were considered too time consuming for large numbers of samples. In this work, large numbers of samples were to be analyzed and an uncomplicated sample preparation was needed. Initially, direct acidification of the sample with HCl (1 part of 4.5 N HCl to 5 parts of fecal sample) was used for packed column analysis, but it was difficult to quantitate the smaller peaks and larger injection volumes caused poor peak shape. To avoid these problems, forming the salts with NaOH, drying, and reconstituting in a smaller volume of HCl were found to be an efficient method of sample preparation. This method was compared to direct acidification and found to give identical results on identical samples and allow quantitation of, for example, isobutyric or isovaleric acids, which were often in concentrations too low to reliably quantitate.

Table I. Amounts and Retention Times of VFA Chromatograms in Figures 2 and 3

	Ac	Pr	iBu	Bu	Piv	iVal	Val
	μg of Each Component						
standard mixture	2.6	1.5	0.30	1.0	0.20	0.31	0.68
fecal sample	0.87	0.28	0.06	0.42		0.11	0.09
	Retention Times (Minutes)						
packed column[a]	1.55	3.22	5.50	6.17	7.95	9.20	10.37
capillary[a]	4.40	5.26	5.62	6.47	5.75	7.18	8.49

[a] 1-μL injection packed column, direct injection; capillary column, 1:30 split ratio.

The selection of a column packing material was limited to those able to tolerate aqueous samples. Super Q was used in a glass column with glass disposable injection liners inside the column inlet. Initial operation gave good separation, but the system exhibited ghosting and peak tailing. The installation of the formic acid vapor entrainment apparatus into the carrier gas line improved peak shape and eliminated ghosting. Using Teflon-tipped plunger syringes eliminated HCl-accelerated corrosion of the stainless steel plungers. Rinsing the syringe between manual injections sequentially with 0.5 N NaOH, 0.5 N HCl, and deionized water was performed to ensure removal of traces of previous sample. Subsequently, it was found the water alone adequately cleaned the syringe for capillary injection and was the preferred procedure for the autosampler.

Other configurations of formic acid vapor entrainment devices have been reported (Woo and Lindsay, 1980; Duthie et al., 1983). The construction shown in this work had a considerable safety factor in that the only glass part was extremely heavy walled and therefore very resistant to physical damage. This was a consideration in view of the hazards of formic acid, liquid or vapor, under 70 psi gas pressure during GC operation and maintenance. While the vapor entrainer was being refilled, the four-port valve allowed continuous flow to the column, which ensured that the detector flame remained lit to destroy any acid vapor still in the flow stream. Varying the amount of formic acid taken up into the carrier gas (by changing the carrier gas tube to formic acid pool distance) showed that the minimum amount of acid that eliminated tailing and ghosting would be the optimum for reproducibility; too much formic acid produced a base-line upset in the vicinity of the acetic acid elution time. A 1-mL charge of formic acid gave consistent and reproducible results over a 2-day period. Less formic acid was consumed under conditions described here than in previously reported studies (Woo and Lindsay, 1980; Cochrane, 1973).

Typical chromatograms from the packed column are shown in parts A, standard mixture, and B, fecal sample, of Figure 2. Peak retention times and amounts are listed in Table I. The amounts listed for the standard mixture were those contained in 1 μL injected onto either column. The amounts shown for the fecal sample were calculated from a comparison of peak areas to standard mixture peak areas. Detector response was linearly proportional to

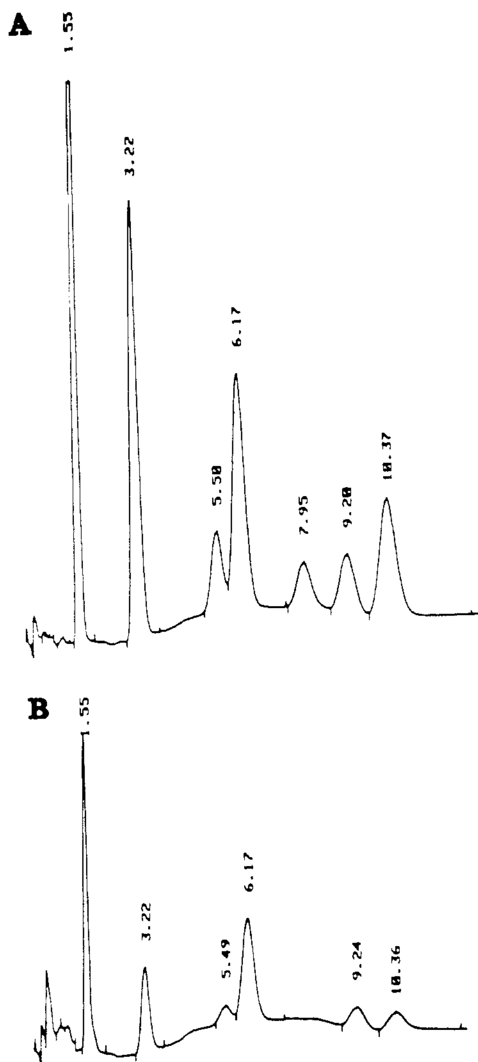

Figure 2. (A) Chromatogram of VFA standard mixture and (B) chromatogram of fecal sample: on Super Q; 6 ft × 2.2 mm; 50 mL min^{-1} helium; 190–210 °C at 4 °C min^{-1}; FID, formic acid entrained carrier gas.

amounts injected on both column systems from the (lower) limit of reliable integration to 8 mg mL^{-1} acetic, 5 mg mL^{-1} propionic, 0.8 and 4 mg mL^{-1} isobutyric and butyric, and 0.8 and 3 mg mL^{-1} isovaleric and valeric acids. An oven temperature that gave a good balance between resolution and speed resulted in a run time of 13.4 min. Increased resolution could be obtained with lower oven temperatures or carrier gas flow rates, but the slight advantage in isobutyric and butyric separation was not considered worth the time required.

The capillary column was selected for VFA analysis for three reasons: the inertness of the fused silica column walls; enhancement of the separation of straight-chain and branched-chain VFAs; more reproducible integration of small VFA peaks. The capillary column was operated without any modification to the GC for VFA analysis. The use of H_3PO_4-treated glass wool instead of silanized in the injector split liner was required to prevent ghosting upon injection of water blanks and eliminated a small amount of tailing. Formic acid in the carrier gas was not required to eliminate ghosting.

The use of the autosampler for VFA sample injection was initially quite disappointing in both reproducibility and peak shape compared to manual injection. It was soon determined that the autosampler injection rate was not rapid enough for the split injection mode of operation to deliver a discrete portion of the sample to the column head. Splitless injection was attempted and found to be much worse in terms of peak shape. This problem was solved by lowering the column oven temperature to 50 °C, causing the vaporized sample to be condensed in the column inlet, and then rapidly (30 °C min^{-1}) raising the oven temperature to the analytical range. While the solvent trapping technique (Grob, 1982) has been used and reported widely with organic solvents, this work shows that the effect may be used with an aqueous sample, with split injection, and for samples that contain analytes with boiling points below that of the solvent (given an appropriate column and retention mechanisms). This solvent trapping effect also changed the apparent split ratio, as was seen by an approximate doubling in peak areas when changing from 140 to 50 °C column oven start temperature.

Table II. VFA Content of Some Human Fecal Samples

subject	date	Ac	Pr	iBu	Bu	iVal	Val	total
		VFA content of fresh fecal samples, μmol g^{-1} dry weight						
1	5-4-82	180	68	4.9	54	5.8	10	324
1	6-9-82	176	56	2.4	47	2.8	5.2	289
2	5-19-82	71	12	3.8	14	6.4	2.9	110
2	12-1-82	245	63	2.0[a]	63	16	13	402
2	12-8-82	73	16	2.0[a]	13	5.7	2.0	111

[a] Estimated from unintegrated peak size.

Table III. Statistical Comparison of Packed and Capillary Columns

	% standard deviations for repeat injections[a]						
	Ac	Pr	iBu	Bu	Piv	iVal	Val
packed column	5.35	5.28	5.49	5.27	6.02	6.80	6.63
capillary column (manual injection)	7.47	7.97	8.07	8.07	7.52	7.90	7.90
capillary column (autosampler)	2.99	3.30	3.82	3.67	4.39	3.95	3.99

[a] n = 24; injections over a 3-day period.

Typical capillary column chromatograms of a standard mixture and a fecal sample are shown in parts A and B of Figure 3. Reliable detection limits with the autosampler/capillary column system using 1-μL sample volumes and an estimated 1:15 split ratio were 10 μg mL^{-1} acetic, 6 μg mL^{-1} propionic, and 4 μg mL^{-1} the remaining acids or approximately 0.3, 0.2, and 0.1 ng per peak, respectively. The enhanced detection using the capillary column meant that direct acidification of the filtered fecal samples was a satisfactory sample preparation procedure. The reliable detection limit on the packed column was affected by the broad late-eluting peaks, isovaleric and valeric acids, and the difficulty in integrating slowly eluting peaks.

The separations of the packed column and the capillary column were slightly different. The pivalic acid peak eluted between butyric and isovaleric on the packed column and eluted between isobutyric and butyric on the capillary column. In addition to pivalic acid, 2-methylbutanoic acid was injected and was found to elute with 3-methylbutanoic (isovaleric) acid on both columns; therefore, values for isovaleric were for the combination. It is not known whether the 2-methyl isomer is found among bacterial fermentation products.

Fresh human fecal samples were analyzed for VFA content, and the results from two sources are shown in Table II. The data indicate a range and distribution of the acids found on several different dates. The precision observed in this work is summarized in Table III. The relative standard deviations listed indicate that errors due

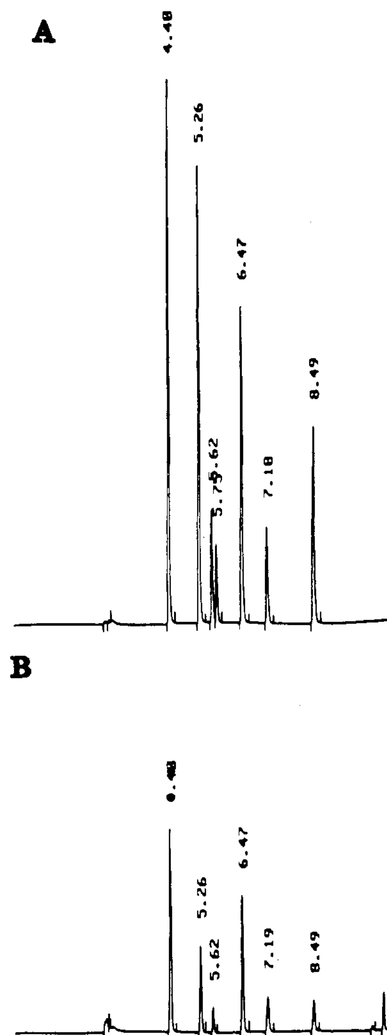

Figure 3. (A) Chromatogram of VFA standard mixture and (B) chromatogram of fecal sample: on OV-351; 0.25 mm × 30 m; 1.3 mL min^{-1} helium; 140–158 °C at 2 °C min^{-1}; FID; nitrogen makeup gas; manual injection; 1:30 split ratio.

to detection and integration have little relationship to retention time, which was an initial concern for the later eluting peaks on the packed column. The theoretical advantages of peak shape and separation on the capillary column with manual injection were not evident here, as is shown by a 35% increase in the relative standard deviation compared to that of the packed column.

The method of standard additions was used to verify peak identity and to check the sample preparation and analysis for recovery. Peak enhancement upon spiking samples with known compounds and analysis on both column systems verified peak identities. Recovery levels on the packed column were 95% for acetic, 97% propionic, 86% isobutyric, 88% butyric, and 85% and 97% for isovaleric and valeric acids. The range was attributed to the difficulty in integrating the smaller peaks preceding larger peaks such as isobutyric and isovaleric acids. This was not considered a problem in this work because acetic, propionic, and butyric acids account for more than 90% of the VFAs present.

On the capillary column (either manual or autosampler injection) the recoveries of standard additions were 99% acetic, 99% propionic, 99% isobutyric, 99% butyric, 98% isovaleric, and 98% valeric acids. The enhanced recovery was attributed to both the inertness of the column and the peak shape and separation improvements over the packed column, and, therefore, more accurate detection and integration.

CONCLUSION

The sample preparation scheme described in this work was simple and reliable. The use of the Super Q packed column tolerant of aqueous solutions contributed to the sample preparation simplicity. Formic acid vapor entrained carrier gas eliminated problems of ghosting and poor peak shape related to the aqueous solutions and the column packing material. The major attribute of the packed column system is low cost; the packing material and glass column are inexpensive. The vapor entrainment device may be constructed in several different ways dependent on available resources and projected use.

The capillary column gave an excellent separation of VFAs. There are significant advantages the capillary system offers. The first is the elimination of the requirement of formic acid vapor entrained carrier gas and the plumbing complexity. The second is simplified maintenance compared to a packed column, i.e., no packing voids, no contaminated glass wool to replace, and a flexible column with little column bleed at higher temperatures.

With the use of the autosampler and low initial oven temperature solvent trapping effect, the disadvantage of difficult quantitation when using split injection was minimized. This, in conjunction with the resolving power of the capillary column and the resulting easier integration of peak areas, improved the analysis by lowering detection limits and increasing the precision of the results.

The problems associated with VFA chromatography have historically presented constraints on sample preparation methods. The two chromatographic systems described here enable the analyst to prepare aqueous samples in uncomplicated fashion and to obtain relative results within a group of samples. Fecal VFA analyses present a particular set of sample preparation difficulties ranging from the method of collection and sampling to the contained biological activity and volatility during preparation. The scope of this work does not permit a review of the multitude of feces collection and sampling methods. The methods of analysis described here do, however, permit one to compare VFA concentrations of materials, collected and prepared identically, with a satisfactory level of confidence.

Registry No. Ac, 64-19-7; Pr, 79-09-4; *i*-Bu, 79-31-2; Bu, 107-92-6; *i*-Val, 503-74-2; Val, 109-52-4; pivalic acid, 75-98-9; 2-methylbutanoic acid, 116-53-0; formic acid, 64-18-6.

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Received for review July 27, 1984. Accepted December 3, 1984. Reference to a company and/or product named by the U.S. Department of Agriculture is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others that may also be suitable. Presented in part at the Pacific Conference on Chemistry and Spectroscopy, San Francisco, CA, Oct 27–29, 1982.