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Direct Quantitative Gas Chromatographic Separation of C_2-C_6 Fatty Acids, Methanol, and Ethyl Alcohol in Aqueous Microbial Fermentation Media

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A method is described for the direct quantitative gas chromatographic separation of C_2-C_6 lower fatty acid homologues, methanol, and ethyl alcohol in aqueous microbial fermentation media. A hydrogen flame detector and a single-phase solid column packing, comprising beads of a polyaromatic resin (polystyrene cross-linked with divinyl benzene), were employed. Direct injections of 1 to 10 μliters of aqueous culture supernatant fluids were made. Quantitative recoveries of C_2-C_6 acids added to culture supernatant fluids were obtained.

The determination of steam volatile lower fatty acids in fermentation medium gives rise to problems in quantitative separation and purification, esterification, preparation for column chromatography, and, very importantly, expenditure of time. With advances in gas-liquid chromatography, it has been recognized (4, 7) that the ability to analyze aqueous fermentation liquids directly would offer distinct advantages. Kaplanová and Janák (6) have discussed some of the difficulties in gas chromatographic analysis of the lower homologues. Among these are their high polarity and tendency to associate, their presence in water solutions and the interference of water in quantitative esterification, the complexity of drying the acids or isolation of acid salts, the various factors influencing dissociation and absorption, and the resultant overlapping and tailing of peaks when biphasic liquid-solid column packings are used (4, 6, 7).

Recently, Hollis (3) investigated the use of uncoated porous polyaromatic beads of styrene, ethylvinylbenzene, or tertiary butyl styrene cross-linked with divinylbenzene as column packings for the separation of water, alcohols, glycols, certain gases, hydrocarbons, and C_1-C_3 volatile acids. The degree of cross-linking with divinylbenzene and the porosity of these packing materials serve to confer many of the desirable properties of the polymer beads. Chief among these is the ability to use the porous polyaromatic polymer beads as a homogenous column packing, thus eliminating such serious problems as the complexities and frequent lack of reproducibility of column preparation, bleeding, etc., associated with biphasic column materials.

The recent commercial availability of these polyaromatic resins encouraged us to investigate their use as uncoated column packings for the quantitative direct separation of lower fatty acids in aqueous fermentation liquids. In addition, as this report will demonstrate, methanol and ethyl alcohol may be quantitatively separated on the same column.

MATERIALS AND METHODS

Equipment. A Microtek dual flame MT-220 gas chromatograph (Microtek Instruments, Inc., Baton Rouge, La.) was used. The output of the hydrogen flame detector circuit was recorded on a Sargent MR recorder with a 1.25-mv full-scale sensitivity and a chart span of 25 cm.

The carrier gas was 99.99% minimum purity helium (Matheson Co., Inc., East Rutherford, N.J.). To the helium tank was attached a two-stage model 9-590 Matheson regulator. The hydrogen and air tanks (Southern Oxygen Co., Washington, D.C.) were fitted with the appropriate series eight two-stage automatic Matheson regulators. Each regulator was fitted with a Cajon ¼-inch (0.6-cm) hex coupling (part no. 4-HCG) to which in turn was connected a stainless-steel swagelock quick-connect fitting-"body assembly" (part no. ¼-inch GC-100-4-316, Crawford Fitting Co., Cleveland, Ohio). To this, by means of another hex coupling, was joined a filter drier cartridge containing molecular sieve 5A (no. 830051, Microtek Instruments). The distal end of the cartridge was fitted with another hex coupling to which was fitted ¼-inch soft copper tubing (helium) or ¼-inch high molecular weight precision instrument polyethylene tubing rated

for a maximal working pressure of 105 psi at 125 F (51.7 C; hydrogen and air; type P, Crescent Insulated Wire and Cable Co., Inc., Trenton, N.J.). The tubing was then connected by hex fittings to the appropriate ports in the chromatograph.

Columns and packing. Dual U-shaped glass columns (4 × ¼ inch, 10.2 × 0.6 cm) were treated with acid dichromate solution, washed with water, and then rinsed thoroughly with acetone, followed by ether. The packing material was Polypack-2 (uncoated, 80/120 mesh, Hewlett Packard F & M Scientific Division, Palo Alto, Calif.). This is a polyaromatic resin consisting of polystyrene cross-linked with divinylbenzene. The hydrophobic resin was washed with distilled water and any fines were decanted. The resin, in a Büchner funnel, was then washed with acetone and four volumes of ether. After air-drying the resin overnight, the columns were packed by use of the normal vacuum-vibration technique. The columns were thoroughly vibrated with an electric vibrator along the whole column lengths, while the resin was very slowly poured to ensure a very close packing. This thorough vibrating could be done without fear of forming excessive fines because the beads are highly resistant to physical disintegration in normal handling.

Gas flows. The helium flow rate was adjusted to 190 ml/min at standard temperature and pressure (STP), the air flow to a rate of 570 ml/min STP, and hydrogen flow rate to 60 ml/min STP for each flame. The sample and reference columns were conditioned by flowing helium through them for 24 hr at an oven temperature of 230 C. This was generally sufficient to establish stable base lines at the working attenuations of 400 to 3,200 ×. At the end of a working day, the columns were kept in condition by a reduced flow of 25 ml/min STP of helium overnight and at an oven temperature of 100 C.

Operation. After numerous trials, the following parameters were chosen: gas flows as described; an inlet temperature of 250 C; an initial oven temperature of 100 C; a temperature program comprising a 2-min hold at the initial temperature followed by a 5 C per min temperature rise until 200 C was reached; and finally followed by a 10-min cooling and equilibration period. The total time for a run was 32 min.

Sample preparation. Fermentation samples were prepared by removing the supernatant fluid from centrifuged cells and adding 0.2 ml of concentrated HCl per 15 ml. Usually, the resultant *p*H was about 2. Occasionally, a slight precipitate formed after the addition of acid. In this case, the cell-free fermentation liquor was recentrifuged and the clarified supernatant fluid was stored in tightly screw-capped tubes at 4 C.

The free acid standards were made up from fresh reagents as follows: 0.1-ml amounts of acetic, propionic, isobutyric, and butyric acids were diluted to 5 ml in water, resulting in the following respective concentrations (μmoles per μliter), 0.349, 0.265, 0.214, and 0.216; 0.1-ml amounts of valeric and caproic acids were diluted to 10 ml in 50% aqueous methanol to concentrations of 0.092 and 0.080 μmoles per μliter, respectively. Mixtures of the C_2-C_6 acids were dissolved in 50% methanol in the same relative concentrations. Only freshly made dilutions in tightly capped tubes were used in critical analyses. Each of these acids was chromatographically homogenous, exhibiting single sharp peaks.

RESULTS AND DISCUSSION

Column stability was excellent. After hundreds of injections of aqueous solutions of acids and highly complex media containing amino acids, salts, glucose, and many other compounds, column usefulness was unimpaired. Injections of 10 μliters of aqueous solutions were very frequent. The flame detector does not respond to water at the sensitivities used here, and therefore water can not give rise to responses obscuring peaks from other substances. The injections of 10 μliters of water sometimes extinguished the flames, but generally this occurred when the flame detector elements required cleaning. It was important to clean these elements periodically by burnishing them to a high polish. The column packing should not be heated appreciably higher than 250 C for extended periods of time. However, we chose an inlet temperature of 250 C to enhance vaporization. This high inlet temperature seemed to have no adverse effect on the column.

The flame detector also does not respond to formic acid, and we shall not report on the analysis of formic acid in this paper. However, it can be detected with a thermal conductivity detector (3) at lower sensitivities.

Isovaleric and isocaproic acids are not included in this presentation because the obtainable reagents were impure. For example, two different isovaleric acid samples gave multiple peaks indicating considerable probable admixture of propionic, butyric, and valeric acids. The major peak, probably corresponding with isovaleric acid, appeared immediately before the valeric acid was eluted. Thus, it is likely that isovaleric and isocaproic acid can be quantitatively separated from their isomers in the same way as the other C_2-C_6 acids.

A typical record (Fig. 1) illustrates the chromatographic separation of a mixture of C_2-C_6 acids solubilized in 50% methanol. A small injection peak appeared within 1 min, followed by a large methanol peak 12 sec later. When the acids were chromatographed singly, the methanol peak appeared only with valeric and caproic acids where methanol was used for solubilization. The retention times were 4.3, 9.5, 13.1, 14.5, 18.0, and 20.7 min for acetic, propionic, isobutyric, butyric, valeric, and caproic acids, respectively. For the same order of the acids, the approximate temperatures at which peaks

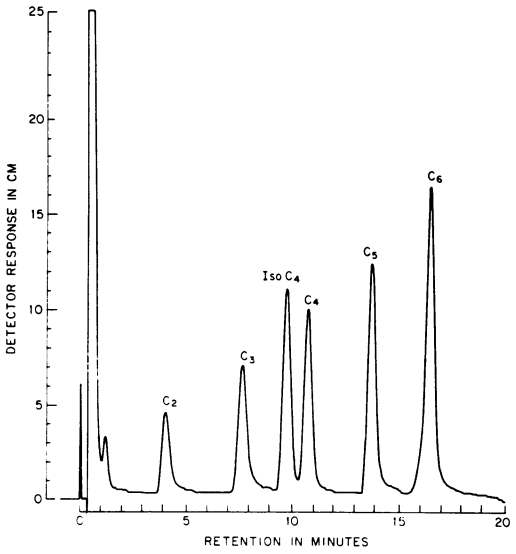

FIG. 1. *Chromatographic separation of a mixture of acetic, propionic, isobutyric, butyric, valeric, and caproic acids.*

TABLE 1. *Reproducibility of peak heights*

Acids	Height (cm)			Deviations from mean	
	Run 1	Run 2	Mean	Per cent	σ
C_2	4.6	4.8	4.70	±2	±0.1
C_3	6.7	6.7	6.70	0	0
iso C_4	10.7	10.7	10.70	0	0
C_4	9.7	9.7	9.70	0	0
C_5	12.0	12.1	12.05	±0.4	±0.1
C_6	15.9	16.3	16.10	±1.2	±0.2

[a] Each run was the mean of triplicate determinations on two successive days. Measurements were made to the nearest 0.1 cm, and therefore 0 deviations from the mean are not to be interpreted absolutely. Standard deviations (σ) were also calculated to the nearest 0.1 cm and were never greater than those shown in any single replicate determination on the same day.

began to appear in the program were 110, 130, 145, 150, 170, and 180 to 185 C.

If the C_2-C_6 acids were neutralized as the sodium salts, the compounds were not eluted from the column, no peaks appeared, and the chart record remained at the zero base line during the entire program. If 10 μliters of 1.6 N HCl was then injected, and the program was rerun, the previously retained salts were eluted from the column as the free acids. The peaks were of the expected order of magnitude, and the acids were eluted in the same order as is shown in Fig. 1. When a mixture of C_2-C_6 acids was injected and a normal program was run as in Fig. 1, a blank program was often rerun immediately afterwards without a second injection. In these cases, the base line remained at the normal zero throughout the program. The absence of peaks in the rerun would indicate that the acids which were initially injected had been completely eluted.

The reproducibility of peak heights was excellent when the same quantity of a mixture of acids was injected in repeated runs. Table 1 presents some typical data. The largest deviation from the mean was ± 2% and was generally smaller. The greatest mean deviations occurred with acetic and caproic acids.

Peak areas were proportional to the attenuations. For example, if 5 μliters of the same mixture of acids was run at attenuations of 400 × and 800 ×, the peak areas of each of the acids at 400 × should be twice as great as the areas obtained at an attenuation of 800 ×. In general, the areas were 97% of expected values. When different quantities of acids were injected at a

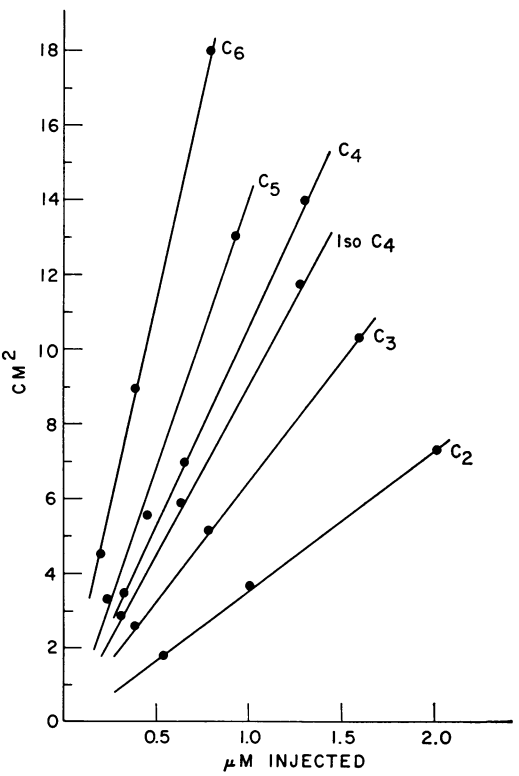

FIG. 2. *Linear correlation of peak area to variable concentrations of C_2-C_6 acids at a constant attenuation of 1,600 ×.*

TABLE 2. *Concentration, retention, and efficiency data for a mixture of C_1-C_6 acids, methanol, and ethyl alcohol*[a]

Compound	Amt injected (μmoles)	Cm^2/μmole at 1 × attenuation	Theoretical plates	HETP	Retention relative to butyric acid
Acetic acid....	1.0480	5,648	564	0.216	0.30
Propionic acid....	0.7948	8,352	1,600	0.076	0.66
Isobutyric acid....	0.6417	14,704	2,836	0.042	0.91
Butyric acid....	0.6485	17,264	3,285	0.037	1.00
Valeric acid....	0.4580	22,944	6,006	0.020	1.24
Caproic acid....	0.3971	36,256	6,951	0.018	1.43
Methanol.	0.9848	3,680	315	0.387	—
Ethyl alcohol...	0.6853	9,792	339	0.360	—

[a] The acids were run at an attenuation of 1,600 ×, the alcohols at 3,200 ×. HETP = height in centimeters equivalent to a theoretical plate. Data are means of triplicate runs. Chromatographic profiles of methanol and ethyl alcohol peaks are shown in Fig. 5.

single attenuation of 1,600 ×, the peak areas correlated linearly with the amounts injected, and extrapolation of the lines for the C_2-C_6 acids (Fig. 2) intersected at the origin.

Table 2 presents representative data on the efficiency in terms of theoretical plates and the height equivalent to a theoretical plate (HETP). Retentions relative to butyric acid compare very favorably with results obtained with liquid coated packings where thermal conductivity detectors were employed. For example, in the present study, the relative retentions of valeric and caproic acids were 1.24 and 1.43, whereas cited (4, 5, 8) values ranged from 1.55 to 2.17 and 2.4 to 3.58, respectively.

The ratios of peak areas to concentrations, expressed as square centimeters per micromole at an attenuation of 1 ×, are listed in Table 2. The calculations were made by (i) determining micrograms per microliter from the known purity and density of the compounds and the dilutions of the standards as previously described; (ii) multiplying by the number of microliters injected; (iii) dividing (ii) by the molecular weight (= micromoles injected); (iv) dividing square centimeters of the peaks (as determined by planimetry) by micromoles injected; and (v) multiplying by the attenuation (usually 400 to 1,600 ×). The data for the C_2-C_6 acids show clearly that detector response increases generally with carbon number, that is, the sensitivity or peak area per micromole of caproic acid is approximately 6.4 times that of acetic acid. The only exception to this may be the case of the C_4 acids where the flame detector appears to be somewhat more sensitive to butyric acid than to isobutyric acid. However, the peak bases are not completely separated. Thus, a small amount of the isobutyric acids may be intermixed in both peaks. Calculations based on the theoretical treatment of Glueckauf (2) predict that a 6-ft (1.8 meter) column would permit at least a 99% separation of the C_4 acids.

When square centimeters per micromole are plotted arithmetically as a function of carbon number of the acids, an ascending exponential curve of the family $b(1 - e^{-ax})$ results. If the logarithms of square centimeters per micromole are graphed against carbon number, the plot is a straight line. The following equation fits the data: $\log_{10} cm^2/\mu mole = 3.3444 + 0.2055\ C_n$, where C_n = number of carbon atoms in the acid molecule. Such a plot is shown in Fig. 3, in

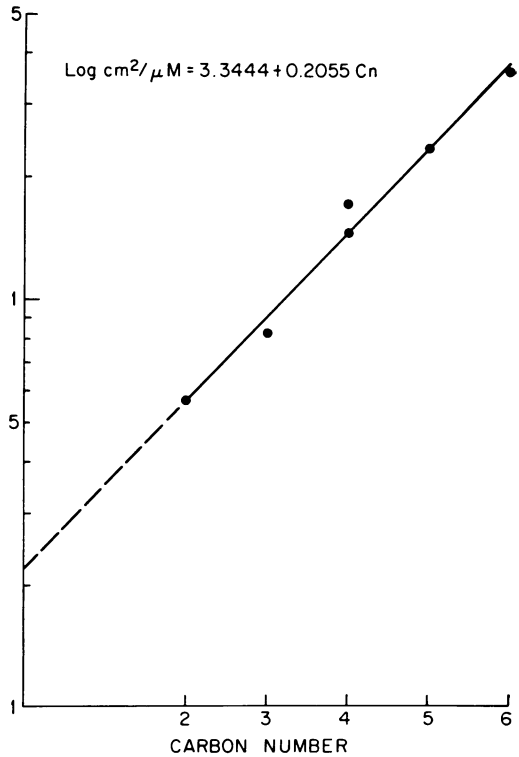

FIG. 3. *Relation between peak area per micromole injected (cm^2/μmole) and number of C atoms in the acid molecules.*

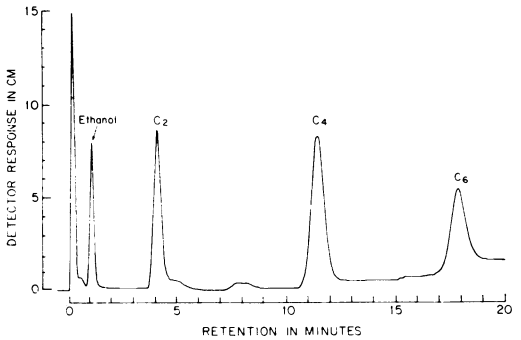

FIG. 4. *Record of the chromatographic separation of the lower fatty acids after the direct injection of aqueous fermentation medium.*

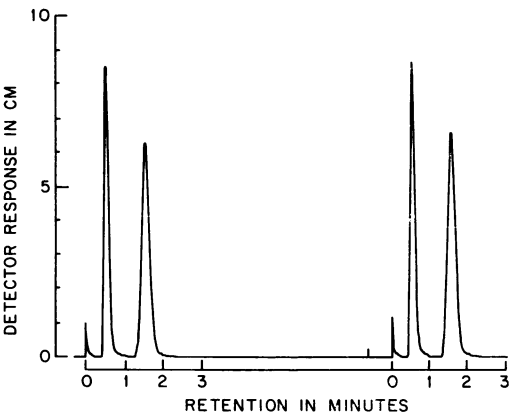

FIG. 5. *Chromatographic separation of aqueous dilutions of methanol and ethyl alcohol.*

which the equation and the line of best fit were derived by the method of least squares. The points are experimental values.

This method was applied to the study of fermentation end products accumulating in growing cultures of a group of gram-negative anaerobic cocci isolated from the pig gut by Fuller (1).

Fourteen representative strains were selected, based on studies of morphological, cultural, biochemical, and nutritional characteristics. These general data will be reported elsewhere. The strains were grown in a medium containing 2% acid-hydrolyzed casein supplemented with 0.01% tryptophan and 0.035% L-cysteine-HCl as a source of amino acids; 0.0375% Na_2HPO_4 and 0.0125% KH_2PO_4; 0.001% guanine, uracil, and hypoxanthine; known vitamins; and trace salts consisting of Mg, Mn, Zn, Ca, Fe, Co, V, Mo, and Cu. The *p*H was adjusted to 7.0 with solid K_2CO_3 and autoclaved for 10 min at 121 C in screw-capped tubes. The fresh medium was inoculated (10%, v/v), and the tubes were filled with about 17 ml of medium so that, at most, a small air bubble persisted beneath the cap. Incubation was at 36 C for 24 hr. The cultures were also grown in this medium supplemented with 0.5% glucose (autoclaved in the medium). Since the products with glucose were the same as without glucose, only the latter results will be presented.

A typical chromatographic record of a culture supernatant fluid is shown in Fig. 4. The following peaks appeared: (i) ethanol; (ii) acetic acid; (iii) butyric acid; and (iv) caproic acid. Of these, the ethyl alcohol and caproic acid peaks were obtained with *uninoculated* media and were of the same dimensions per microliter injected as in the cultures. The ethyl alcohol peak most probably had its origin in the alcohol used to solubilize some of the vitamin solutions, and was calculated to be present in the medium at a concentration of 0.00629 μmole/μliter $\sim$ 0.2898 g/liter $\sim$ 0.3671 ml/liter. This was the approximate amount added in the preparation of the medium. It appears that there is no significant boil-off of this dilution of ethyl alcohol with the present conditions of autoclaving. The integrity of the caproic acid peak in the uninoculated medium was investigated by adding known amounts of reagent (0.1 to 0.3 μmole/10 μliters culture supernatant fluid). The added acid co-chromatographed with the compound already present in the uninoculated medium so that a single peak was exhibited with the expected retention of 1.43 relative to butyric acid. Calculated recoveries of the added caproic acid (using the standard of 36,304 cm^2/μmole at an attenuation of 1 $\times$) were 95 to 99%. Thus, only the acetic and butyric acid peaks were significant in the sense that these compounds were produced by microbial metabolism. Furthermore, when acetic and butyric acid reagents were added to culture supernatant fluids, these acids co-chromatographed with the fermentation acids. The resultant increased peak sizes were commensurate with the quantities of added acids (recoveries = 101 to 106%).

The range of acetic acid produced by different strains was from 0.262 to 0.304 μmole/10 μliters (mean = 0.277) and that of butyric acid was from 0.115 to 0.145 μmole/10 μliters (mean = 0.132). This is equivalent to a mean accumulation of 0.0277 mole/liter (1.6634 g) and 0.0132 mole/liter (1.1629 g) of acetic and butyric acids, respectively. For the 14 different strains, the molar ratio of C_2/C_4 acids ranged from 1.94 to 2.20 with a mean ratio of 2.09.

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