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A CONVENIENT CHROMATOGRAPHIC PROCEDURE FOR THE SEPARATION OF LOWER FATTY ACIDS

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SUMMARY

A simplified preparative procedure for the separation of homologous fatty acids lower than hexanoic has been developed. The method employs partition chromatography of the free fatty acids on silicic acid. Glycine solutions (1 *M*), with indicators incorporated, comprise the aqueous phase; chloroform is the mobile phase. It is particularly applicable to separation of "carrier" quantities of mixed fatty acids (60–500 mg range, total) which accrue from Schmidt, Kuhn–Roth, or permanganate degradations of radioactive metabolites. The methodology requires neither extensive collection of fractions nor titrations and provides high recoveries of well-resolved components which are analytically pure, as judged by gas–liquid chromatographic analysis.

INTRODUCTION

Among the technique available[1] for sequential stepwise degradation of labelled fatty acid fragments from radioactive metabolites, perhaps none enjoys such widespread use as does the Schmidt reaction, as modified by MOSBACH *et al.*[2] for this purpose. When this procedure is carried out, overoxidation by permanganate of the intermediary primary amines usually causes the appearance of homologous lower fatty acids[1], which may in turn tend to obscure meaningful radioactivity determinations on the subsequent decarboxylation steps. Of perhaps equal importance in biological tracer experiments is the Kuhn–Roth degradation. Where applicable, compounds which contain C-methyl or higher *n*-alkyl side residues yield predominantly acetic acid from their terminal methyl ends. Although reaction conditions have been made optimal[3] to the point where 98% titratable "acetic acid" accrues following this oxidation, the fact remains that here too, a mixture of lower fatty acids results[1]. The same remarks can be applied to the products of permanganate oxidation of aliphatic olefins. When employing the above degradations with radioactive substances, it may become necessary to resolve and to recover centigram to decigram quantities of mixtures of lower fatty acids, especially when they follow dilution steps with inactive carriers.

This report is concerned with an improved and simplified method of partition chromatography which gives a facile separation of such mixtures. It not only pro-

vides analytically pure components as judged by gas–liquid chromatographic examination, but also obviates the necessity of titrations, solvent gradients, or the use of a fraction collector. The application of this procedure is illustrated by the separation of fatty acids which accumulate from carbon-by-carbon Schmidt degradations beginning with hexanoic acid; and by resolution of the acids which obtain from Kuhn–Roth oxidation of a model mold metabolite which has an *n*-pentyl side chain.

EXPERIMENTAL

Column preparation

Silica gel (100–200 mesh, Davison) was inactivated by treatment with concentrated HCl for 18 h. It was serially washed with distilled water, first by decantation and then by vacuum filtration, until the washings were neutral. The neutral silica was dried first by prolonged suction on a buchner funnel, then was spread out and air dried for 24 h, and was finally placed in a desiccator over H_2SO_4. Further washing with methanol, as suggested by CORCORAN[4], gave poorer resolution of acids. For the separation of acetic, propionic and butyric acids, the aqueous phase was prepared by mixing 170 mg Bromcresol Green in 100 ml 1.0 *M* glycine at pH 7.5. For resolution of butyric, valeric and caproic acids, the aqueous phase consisted of 12 mg Cresol Red dissolved in 100 ml 1.0 *M* glycine at pH 11.0.

With efficient mixing, 60 ml of the appropriate glycine-indicator solution[5] was added to 400 g of the inactivated silica gel. The interaction of these components lowered the pH to approximately 4.9 (green) for the Bromcresol Green indicator and to 7.6 (magenta) in the case of the mixture with Cresol Red. With different lots of silica gel, it was necessary in some instances to add more alkali to adjust the final pH to these values. It was also found with some batches that addition of more water was required in order to attain a properly moist, though not wet, column packing. The column materials thus prepared were loaded to a height of 27 cm into glass columns of 5.5 cm diameter, using chloroform (Mallinkrodt, A.R.) as the non-aqueous phase.

Chromatographic procedure

The mixture of fatty acids (range, 60–500 mg) was dissolved in a minimal amount of chloroform, and was applied to the top of the column. Chloroform was used for development, and the progression and separation of acids as yellow or pink bands could readily be followed because of the indicator color change[5] against the background colors. With the Bromcresol Green column, the limited solubility of the indicator in chloroform often necessitated addition of an extra 15 g of indicator–silica gel mixture to the top of the column. This step was carried out after sample addition and prior to development, and usually insured that an adequate amount of indicator was present during the run. A flow rate of 600 ml/h was found to give most satisfactory results. As each band of acid was eluted it was treated with excess methanolic NaOH, and the solvents were removed by distillation *in vacuo*. Acidification, steam distillation, and neutralization (pH meter) gave indicator-free sodium salts of the individual fatty acids.

Schmidt degradations

The procedure of MOSBACH *et al.*[2] was followed, with the following minor modi-

fications: for each mmole of fatty acid dissolved in 1.5 ml conc. H_2SO_4, 250 mg of sodium azide was added. The resultant lower homologous amines were oxidized by heating in a boiling water bath for 15 min with 0.5 ml 1.0 *N* NaOH and 10 ml of 5 % $KMnO_4$.

Kuhn–Roth oxidation

Pulvilloric acid (576 mg) was treated for 1 h by boiling with 245 ml oxidation mixture[6] containing 21.6 mg $MnSO_4 \cdot H_2O$[3]. The fatty acids were removed by steam distillation, neutralized with 0.03 *N* NaOH, and were concentrated by evaporation *in vacuo*. The fatty acids were liberated from their sodium salts with HCl, and were extracted into chloroform. A small aliquot was analyzed by GLC, and the bulk was separated by the above described chromatographic procedure.

Gas chromatography

Identification of mixtures and homogeneity of separated fatty acids was checked by gas–liquid chromatography. An F and M Model 402, equipped with a Disc Instruments 228 A chart Integrator was employed. The columns were 6 ft. 6 % poly(diethyleneglycol succinate) on 80–100 mesh Diatoport S, maintained under isothermal conditions. The free fatty acids in $CHCl_3$ solution were injected into the port directly. Injection port, column, and flame detector block temperatures were 135°, 110°, and 150°, respectively. Quantitation of GLC data was carried out by the total peak area method, and where applicable by the use of hexanoic acid as an internal standard.

RESULTS

When hexanoic acid was subjected to the standard Schmidt rearrangement and subsequent permanganate oxidation[2], GLC analysis of the distillate showed that the main product, valeric acid, was contaminated with acetic acid (21 %), propionic acid (4 %) and butyric acid (7 %). No unreacted hexanoic acid remained. Treatment of the foregoing mixture of fatty acids in a second cycle of rearrangement–oxidation steps gave butyric acid, which was shown upon analysis to contain approximately the same amount of C_3 (6 %) and of C_2 (18 %) contaminants. These results led to initiation of exploration of procedures for preparative resolution of such fatty acid mixtures.

The separation of an artificial mixture of C_2, C_3, and C_4 fatty acids using glycine at pH 4.9 with Bromcresol Green is schematically represented in Fig. 1A. The recovery of individual fatty acids was almost quantitative. Gas–liquid chromatographic analyses of the starting mixture, and of each visually selected acidic band from the column, are presented in Fig. 2. These data clearly demonstrate the resolution of acetic, propionic, and butyric acids by this approach.

The chromatographic separation of C_2 through C_6 acids with the more alkaline (pH 7.6) glycine system in conjunction with Cresol Red indicator is depicted in Fig. 1B. This particular weight ratio of fatty acids chosen in this experiment corresponds to the composition of a steam-distillable mixture which can be obtained by the permanganate oxidation of 1.5 g of 1-(3,5-dimethoxyphenyl)-heptene-2. Although acetic and propionic acids were not individually resolved, this latter pair was well separated from each of the C_4, C_5, and C_6 acids. They can further be resolved from one another by re-chromatography under the protocols given in Fig. 1A. Gas–liquid chromato-

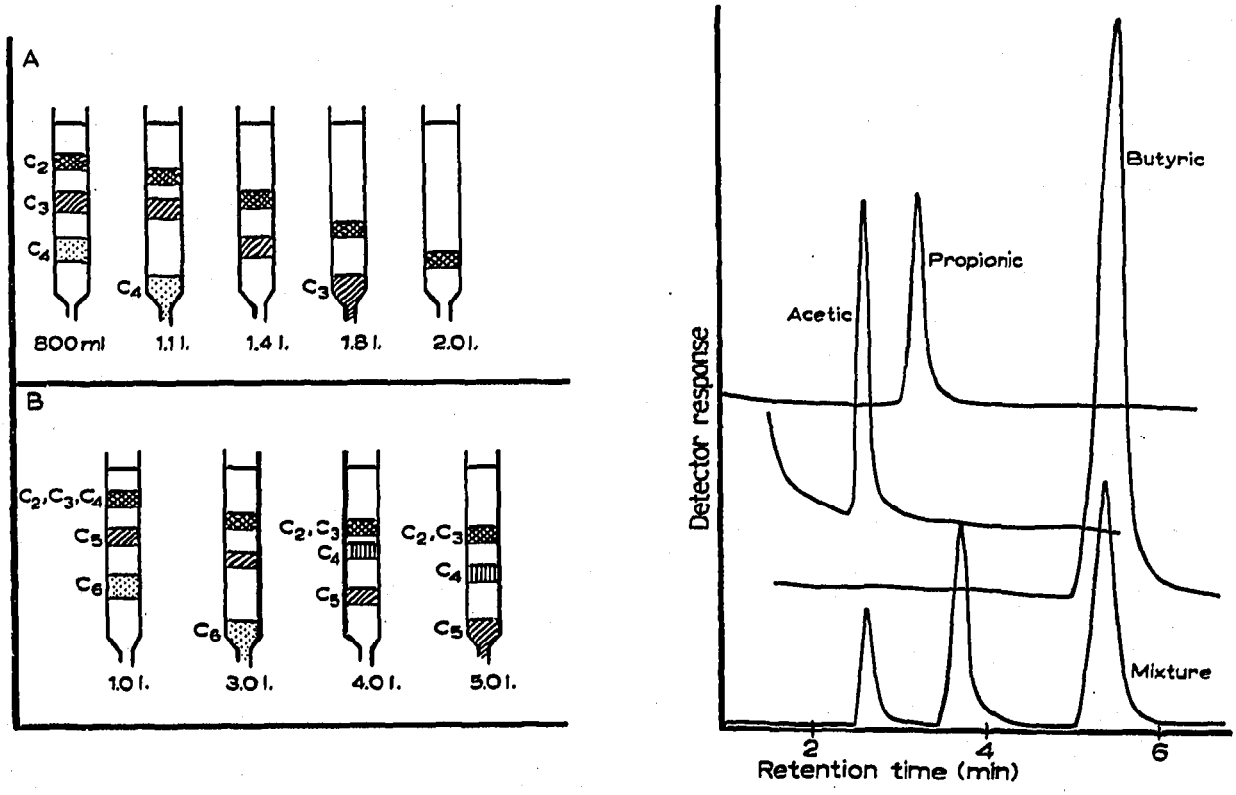


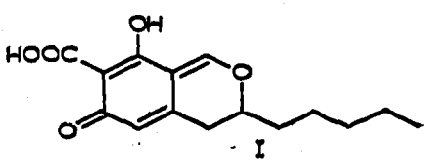
Fig. 1. Chronologic appearance of partition chromatography columns during the resolution of lower fatty acids. (A) Separation of acetic, propionic and butyric acids (60 mg each in mixture) on glycine-buffered silica gel, pH 4.9, with Bromcresol Green indicator incorporated. The flow rate of chloroform was 10 ml/min. (B) Separation of $C_2 + C_3$ acids from resolved butyric, valeric and hexanoic acids. Mixture contained C_6, 209 mg; C_5, 112 mg; C_4, 39 mg; C_3, 12 mg; C_2, 28 mg. The column contained glycine-buffered silica gel at pH 7.6 with Cresol Red indicator. Flow rate approximately the same as above.

Fig. 2. Gas chromatographic examination of the starting mixture and of the individual bands obtained after the chromatogram run in Fig. 1A.

graphic analyses of each of the acidic indicator bands corresponding to C_6, C_5, and C_4 acids were also satisfactory for homogeneity.

APPLICATION

Kuhn–Roth oxidation of pulvilloric acid (I), a fungal metabolite which contains



I

an *n*-pentyl side chain, was shown by GLC to provide acetic acid (73%), propionic acid (19%), butyric acid (5%), valeric acid (2%), and hexanoic acid (1%). When these fatty acids (acetic acid content 54 mg) were purified by the foregoing chromatographic method, 80% of the acetic acid was recovered. The purity of the C_2 acid thus obtained is evident from the analysis given in Fig. 3.

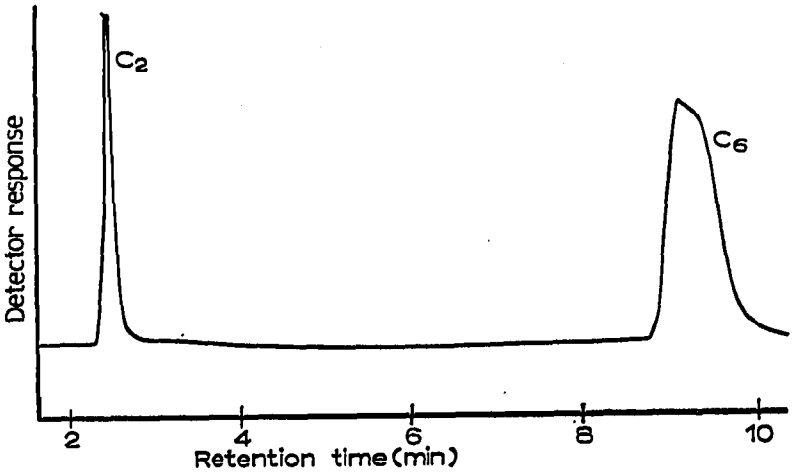


Fig. 3. Homogeneity of acetic acid obtained after Kuhn–Roth oxidation of pulvilloric acid and subsequent partition chromatographic resolution. In this run, hexanoate was added as an internal standard. In the absence of the C_6 marker, only the C_2 peak was present.

DISCUSSION

A voluminous literature exists which describes a variety of anion-exchange, paper, thin-layer, and partition chromatographic separations for organic acids (*cf.* E. LEDERER AND M. LEDERER[7] or HEFTMANN[8] for reviews). Many of these methods are often time consuming, difficult to reproduce, accommodate only small quantities of material, involve extensive manipulations by the experimenter, or require elaborate ancillary collection apparatus. Even limiting consideration to those procedures which have outlined separation of homologous lower fatty acids by partition chromatography on silicic acid would result in an extensive bibliography. However, the authors considered the work of ELSDEN[5], MARVEL AND RANDS[9], BULEN *et al.*[10] and of CORCORAN[4] to be either germinal or applicable to the problem at hand, *i.e.*, the separation of C_2–C_6 fatty acids on the decigram scale.

The method herein described is based on one which was developed by CORCORAN[4], and also embodies direct incorporation of indicators into the partition chromatographic column[5]. Several distinct and important changes in the original protocols were found, in our hands, to be requisite both for optimum separation of the lower fatty acids, as well as to enable the internal indicator technique to be employed. These included: avoidance of the methanol wash of the 10 *N* hydrochloric acid inactivated silica gel; use of 1 *M* rather than 2 *M* glycine in the stationary phase; adjustment of the pH of the silicic acid–aqueous phase mixture rather than that of the aqueous phase prior to its mixing with the gel; use of a single rather than a mixed solvent mobile phase during column development; use of a much lower ratio of aqueous phase to silica gel (*ca.* 60 ml per 400 g) for column preparation; use of a small pressure head (gravity-flow) rather than nitrogen under pressure; and, circumvention of the collection of fractions with attendant titrations.

Adherence to these protocols has provided analytically pure C_2–C_6 lower fatty acids from mixtures on a centigram to decigram scale. The method has been applied successfully to the separation of homologous fatty acids which result from Schmidt degradations and from permanganate oxidations of olefins. It is especially applicable

to radioactive tracer experiments which employ the Kuhn–Roth reaction for obtaining acetic acid from complex metabolites. Thus, it is customary in such biosynthetic studies to purify the resultant acetic acid via derivatization, followed by preparative thin-layer chromatography or by repeated crystallizations. Such derivatives are often scintillation-quenching. Application of this single-stage chromatographic method to the products of Kuhn–Roth oxidation of pulvilloric acid gave better than 50% yield of analytically pure acetic acid. The technique is once begun, sufficiently non-critical to allow other laboratory operations to be carried out simultaneously. It has the further potential of being readily extended for separating other acidic substances, using columns maintained at different pH values, provided that the indicators to be used exhibit low solubility in the solvents chosen for elution.

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