Gas Liquid and Thin Layer Chromatographic Analysis of 12-Aminostearic Acid[1]

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Abstract

Products from the reductive amination of 12-ketostearic acid to 12-aminostearic acid have been monitored by gas liquid chromatography (GLC) and thin layer chromatography (TLC). The nonvolatile amino acid was analyzed by TLC, but its trifluoroacetylated methyl ester was analyzed by TLC and GLC. Conditions for obtaining high conversions of the amino acid to this derivative are described. The derivative was quantitated by GLC using methyl 12-ketostearate as an internal standard. Various developing solvents, types of TLC plates, and visualization spray reagents were examined and compared for efficiency and convenience regarding the TLC separations. Satisfactory TLC systems were developed for crude reaction mixtures containing 12-aminostearic acid and these same mixtures, which were trifluoroacetylated. The influence of water in the developing solvent on the ionic forms of the amino acid is discussed.

Introduction

To monitor the reductive amination of 12-ketostearic acid to 12-aminostearic acid (1), an analytical method was needed to determine the extent of conversion and to assess product purity. Because the amino acid is not volatile enough to be analyzed by gas liquid chromatography (GLC), efforts were first directed toward developing a thin layer chromatography (TLC) system which could show the presence of both the amino acid and reaction by-products with higher and lower R_f values than the amino acid. This method was required to separate hydroxy, keto and secondary amino dicarboxylic fatty acids from the desired 12-aminostearic acid rather than separate positional isomers of amino acids from one another.

To obtain more quantitative data from crude mixtures containing little nonvolatile material a GLC procedure was also desired. In order to convert the amino acid into a volatile derivative, the trifluoroacetylated (TFA) methyl ester seemed the best choice. To determine that conversion to the TFA derivative was quantitative, and to further examine components of the reductive amination mixture, a TLC system was also developed for the TFA derivative.

Many workers have used TFA methyl esters (2–5) and butyl esters (6) for gas chromatographic analysis of α-amino acids. TLC procedures for α-amino acids have also been investigated extensively (7). The extension of these analytical techniques from the α-amino acids to amine derivatives of fatty acids has not been reported. The present research was undertaken, therefore, with the additional object of developing chromatographic techniques which would be useful in analysis of crude fatty amino acid mixtures.

Experimental Procedures

Thin Layer Chromatography

Plates and Strips. Applied Science Laboratories, Inc. Adsorbosil-5 precoated plates, 250 μ thick and Brinkmann Instruments Inc. precoated plates with Silica Gel (5763) hereafter called commercial Silica Gel, 250 μ thick were used. For screening developing solvents laboratory-made Silica Gel G chromatostrips and plates 350 μ thick were employed (8). The plates were spotted 20 mm from their ends and developed along a path of 15 cm from origin to solvent front. The chromatostrips were spotted 15 mm from the end and developed along a 10 cm path.

Sample Application. Solutions were prepared by dissolving 10 mg of 12-aminostearic acid in 1 ml boiling methanol and applying 5–10 μl of the cooled solution with Drummond disposable micro-pipettes. The TFA derivative was used at the same concentration, but was dissolved in acetone rather than methanol.

Developing Solvents. Of several dozen developing solvents evaluated for the amino acid, mixtures of methylene chloride-methanol-water performed most satisfactorily. Two of these mixtures, 70:30:5 (v/v/v) and 80:20:3, are discussed later. The best developing solvent for the TFA derivative is Skellysolve F-ether (80:20).

Visualization. Both the amino acid and the TFA derivative when used with Adsorbosil plates and chromatostrips were best visualized by spraying with chromic-sulfuric acid solution followed by charring on a hot plate. Because the commercial Silica Gel plates contain an organic binder, they can be heated only to 120 C which is best accomplished in an oven. In this case aqueous 50% sulfuric acid (v/v) is more effective than chromic-sulfuric acid solution although with both sprays 16 hr heating is required to visualize the spots. The Adsorbosil plates after acid spray gave quite clear spots when heated only 5–15 min on the hot plate. Because of their much more rapid visualization time, they were preferred over the commercial Silica Gel plates with organic binder. They were also superior to lab-made strips or plates because of greater uniformity of surface and durability. However, strips were more convenient than plates for screening solvent systems.

Ninhydrin solution could be used to give a pink spot for the amino acid only, but chromic-sulfuric acid was generally used because it visualized all spots.

Gas Liquid Chromatography

A Varian Aerograph gas chromatograph model 1525B equipped with dual columns and dual flame ionization detectors was used. Packed columns of 5 ft, 1/8 in. stainless steel, 10% FFAP on 70/80 AW-DMCS Chromosorb W or 7 ft, 1/8 in. stainless steel, 10% Carbowax 20 M TPA on 70/80 AW-DMCS Chromosorb W were heated isothermally in the range of 215–230 C at a helium flow of 30 ml/min. Before use columns were conditioned for 48 hr at 235–240 C.

12-Aminostearic Acid

This compound was prepared by reductive amination of 12-ketostearic acid (1) by modifying the procedure of Hanford (9). Its purity and structure were confirmed by melting point, elemental analysis, and mass, NMR and IR spectra. The purified amino acid was used as a standard for both GLC and TLC analyses.

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Chemicals

Methanol-HCl solution was made by saturating cooled methanol with gaseous HCl. This solution when stored in the refrigerator was useful for several months. Thionyl chloride was reagent grade purchased from Eastman Organic Chemicals. Trifluoroacetic anhydride (TFAA) was reagent grade from Matheson Coleman and Bell.

Derivative Preparation

Analytical Scale. To 10 mg of 12-aminostearic acid in a 10 ml flask was added 2 ml of methanol-HCl solution and 0.09 ml of $SOCl_2$. A condenser with drying tube was attached to the flask and the contents refluxed for 30 min. The mixture was then stripped in a rotary evaporator at water aspirator pressure, 2 ml of methanol was added to the residue, and the contents stripped again, first at water aspirator pressure and then at 0.1 mm. The residue was then refluxed with 0.4 ml trifluoroacetic anhydride for 30 min. Excess TFAA was removed by blowing with nitrogen. After the derivative was dissolved in acetone, it was ready for analysis.

Preparative Scale. To a 125 ml Erlenmeyer flask with 24/40 neck and containing a magnetic stirrer was added 1.0 g 12-aminostearic acid and 75 ml of a refrigerated solution of methanol-HCl. The flask was surrounded by an ice bath and the mixture was stirred for 10–15 min until solution was obtained. With the temperature at 4 C, 2.0 ml of $SOCl_2$ was added to the flask dropwise over a few minutes. The solution was refluxed for 30 min, cooled, and allowed to sit overnight at room temperature. The mixture was filtered to remove a small amount of solid residue, the filtrate was evaporated at water aspirator pressure, treated with 50 ml methanol, and evaporated again. The oily residue was heated (steam bath) at 0.1 mm.

To this residue was added 10 ml of TFAA. The resulting clear solution was refluxed for 30 min. Excess TFAA was removed first at water aspirator pressure with a rotary evaporator and then at 71 C/0.7 mm. On cooling to room temperature the oil solidified. The solid was purified by slurrying a 203 mg solution in 10 ml ether with 21 g of neutral alumina (Bio-Rad A6-7) previously saturated with ether. This slurry was filtered, the alumina washed with 50 ml ether, and the filtrate and wash evaporated giving the pure TFA derivative, mp 72.0–72.1 C.

Calculated for $C_{21}H_{38}NO_3F_3$: C, 61.59; H, 9.35; N, 3.42; F, 13.92. Found: C, 61.70; H, 9.42; N, 3.48; F, 13.87. The infrared spectrum of the derivative was consistent with the expected structure.

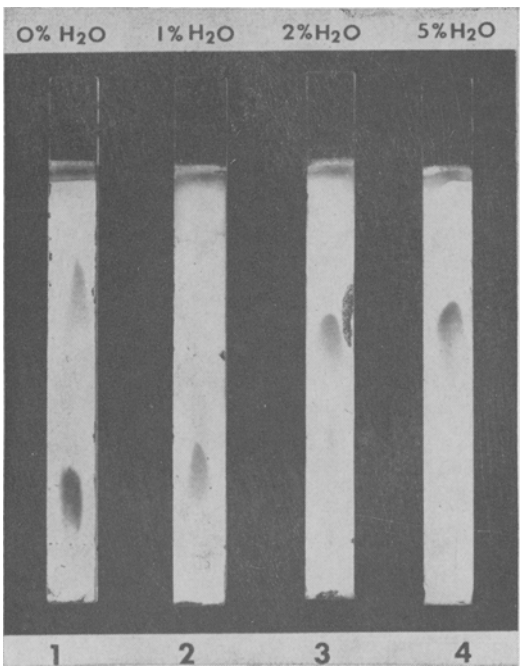

FIG. 1. TLC of 12-aminostearic acid. Influence of water. Developing solvent: 1, CH_2Cl_2-CH_3OH (70:30); 2. CH_2Cl_2-CH_3OH-H_2O (70:30:1); 3. CH_2Cl_2-CH_3OH-H_2O (70:30:2); 4. CH_2Cl_2-CH_3OH-H_2O (70:30:5).

Results and Discussion

TLC of 12-Aminostearic Acid

Developing Solvents. A large number of solvents were evaluated in an effort to find one in which the amino acid appeared as a reasonably compact spot and was separated from other impurities in the mixture. Compounds which were known or suspected to be present in the crude reaction mixture in addition to 12-aminostearic acid were ketostearic acid, hydroxystearic acid, and stearic acid all three of which have R_f values greater than the amino acid as determined by comparison with known compounds in the solvent systems described, and secondary amino dicarboxylic acids which presumably have lower R_f values than the desired amino acid. Solvents which are often used with common α-amino acids gave too high an R_f for 12-aminostearic acid to be useful. The only solvent mixtures that gave relatively compact, nonstreaking spots in addition to adequate separations were those of methylene chloride-methanol with or without water. Several such solvent mixtures gave two spots for the pure amino acid.

Ionic Forms of 12-Aminostearic Acid. One example of a solvent which occasionally gave two spots with the amino acid was methylene chloride-CH_3OH (70:30). Because this occurred with purified amino acid it did not seem likely that the other spot was an impurity. Furthermore, the distribution of the densities and areas of the spots and their R_f values varied unexplainably. Careful study showed that the degree of activation of the chromatostrips strongly influenced the distribution of the two spots and their R_f values. With strips that were used within a few minutes after removal from the oven there was little or no tendency for two spots to form and lower R_f values were observed. With strips that were not activated but only air dried the tendency to form two spots was most pronounced. Strips which had been activated but were stored in a cabinet with desiccant for several days before use showed an intermediate tendency regarding multiple spot formation for the pure amino acid and intermediate R_f values. As solvents were screened using chromatostrips of intermediate activity, and since the amount of water present on these strips varied, so did the R_f values of the resulting spots. Thus the moisture present on the strip appeared to influence both R_f and the tendency for multiple spot formation.

To study this phenomenon further varying amounts of water were added to the developing solvent with the results shown in Figure 1. To enhance multiple spot formation the strips used for Figure 1 had been oven activated and stored in a cabinet, but were exposed to air for 15–30 min before use. An example of a strip having two spots is shown by Strip 1 where no water was used. In contrast, Strip A in Figure 3 which was freshly oven activated, showed essentially

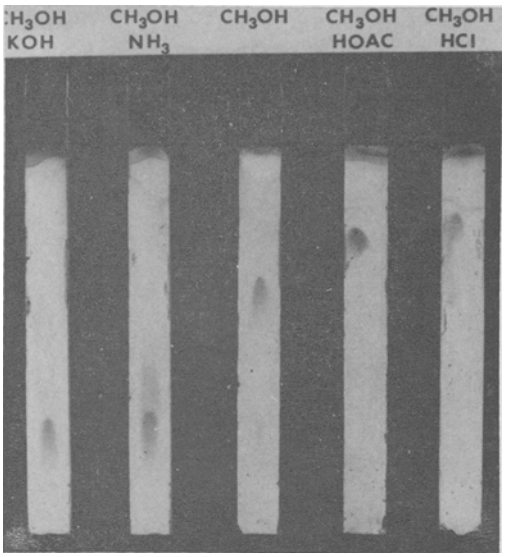

FIG. 2. Ionic forms of 12-aminostearic acid. Developing solvent: CH_2Cl_2-CH_3OH(70:30).

one spot with the same developing solvent. In Strip 2 of Figure 1 where 1% water was present the major spot was the one with lower R_f. With 2% water the major spot was the one with higher R_f. This behavior suggests that the major spot in the 1% H_2O strip has been largely converted to an isomeric form in the 2% H_2O strip. When 5% water was added this conversion was complete and only one spot was observed (Strip 4).

An explanation for this observation is that the amino acid is changing from one ionic form to another. This can be most easily understood by the following equation:

$$\begin{array}{ccccc} & & \mathrm{NH_2 \cdot R \cdot CO_2H} & & \\ & & \downarrow\uparrow & & \\ & \overset{-H^+}{\swarrow} & & \overset{H^+}{\searrow} & \\ \mathrm{NH_2 \cdot R \cdot CO_2^-} & \rightleftharpoons & \mathrm{NH_3^+ \cdot R \cdot CO_2^-} & \rightleftharpoons & \mathrm{NH_3^+ \cdot R \cdot CO_2H} \end{array}$$

It is well known that amino acids exist mostly in zwitter ion form rather than in the non-ionic form (10). Acidification of the zwitter ion produces the cationic form while loss of a proton from the zwitter ion by addition of base gives the anion. Thus amino acids can exist in different ionic forms which are in equilibrium depending on pH.

That the two spots observed were two ionic forms of the amino acid could be corroborated by adding base or acid to the zwitter ion to force it into anionic form or cationic form, respectively. The R_f values of the resulting spots could then be compared to the R_f values obtained in a neutral solvent. This was done with the results shown in Figure 2. In each case the amino acid was dissolved in methanol containing the reagents shown. Each methanolic solution was then spotted on the TLC strip and developed with methylene chloride-methanol.

These strips clearly show three distinct regions: a lower R_f region corresponding to the anionic form, a middle R_f region indicating zwitter on nonionic form, and a higher R_f region corresponding to the cationic form. In the center strip, the amino acid was dissolved only in methanol. Judging from the location of the two spots in this strip relative to the other spots, the major spot would thus appear to be the zwitter ion or nonionic form and the minor spot the anionic form. The relative amounts of these two forms seem to be influenced by the amount of water present in the developing solvent as well as on the TLC strip itself.

To have a TLC procedure in which only one spot was present for the pure amino acid, freshly oven-activated plates were used to give the results shown in Figure 3. Although the use of a freshly oven-activated strip (A) with methylene chloride-methanol (70:30) did produce essentially one spot, the R_f of this spot is too low to be useful. By adding 5% water to the developing solvent as with plate B, the R_f of the amino acid was raised to a more useful range while still giving only one spot for the pure amino acid (B-1). B-2 and B-3 show a comparison between a synthetic mixture consisting of 12-amino, 12-hydroxy and 12-ketostearic acids, respectively, and a crude reductive amination reaction mixture. In each case the impurities are separated from the amino acid. The developing solvent methylene chloride-methanol-water (70:30:5) is particularly good for examining impurities with lower R_f values than the amino acid. A better developing solvent for examining impurities with R_f values higher than the amino acid is methylene chloride-methanol-water (80:20:3), used with plate C. As most of the crude reaction mixtures contained predominantly higher R_f impurities, this became the solvent system of choice. With 80:20:3 the higher R_f impurities are better resolved and the amino acid spots are less elongated than with 70:30:5. By the use of 80:20:3, freshly activated plates, and detection by chromic-sulfuric acid, the conversion to and purity of 12-aminostearic acid could be quickly

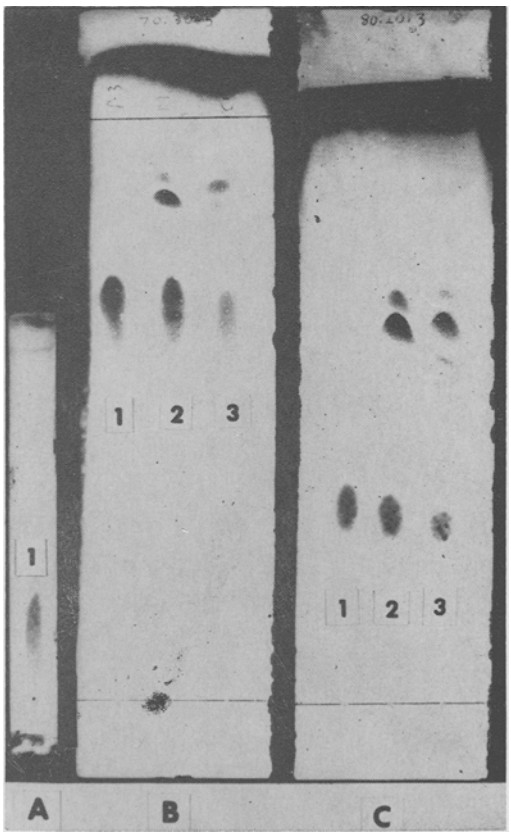

FIG. 3. TLC of pure and crude 12-aminostearic acid on freshly activated plates. A, 10 cm path chromatostrip with methylene chloride-methanol (70:30). B, 15 cm path Adsorbosil Plate (5 × 20 cm) with methylene chloride-methanol-H_2O (70:30:5). C, As in B but with methylene chloride-methanol-H_2O (80:20:3). 1, Pure 12-aminostearic acid. 2, Synthetic mixture of 12-aminostearic acid, 12-hydroxystearic acid and 12-ketostearic acid, respectively. 3, Crude reductive amination reaction mixture.

TABLE I

Effects of Reaction Variables in Conversion of 12-Aminostearic Acid to its TFA Methyl Ester

Run	Undistilled $SOCl_2$		Distilled $SOCl_2$		Undistilled TFAA		Distilled TFAA		% Conversion to derivative	
	Moles	Time, min	Moles	Time, min	Moles	Time, min	Moles	Time, min	TLC[a]	GLC[b]
1			0.03	15	0.2[c]	10			High	99
2	0.03	30			0.2[d]	30			High	....
3	0.03	60			0.2[d]	60			High	95
4	0.03	15			0.2[d]	10			High	....
5	0.03	15			0.2[e]	10			Low	....
6	0.03	15			0.2[e]	10			Low	....
7	0.09	30			0.4[e]	30			Med	....
8			0.03	15			0.2	10	High	95
9			0.09	30			0.4	30	High	97
10	0.09	15					0.4	10	High	....
11			0.09	15	0.4	10			Med	....
12			0.03	30			0.2	30	High[f]	96
13			0.09	30			0.4	30	High[f]	96
14			0.09	30			0.4	30	High[f]	99

[a] High, > 90%; medium, 60–90%; low, < 60% by estimation of TLC spots.
[b] The percentages shown by GLC were determined by peak height times width at half-height.
[c] TFAA was from a freshly opened bottle.
[d] TFAA was 8 months old.
[e] TFAA was 1 year old.
[f] In these runs conversion was essentially quantitative.

assessed. This system with modification where needed should be suitable for use with other fatty amino acids as well.

Preparation of TFA Derivative

Analytical Scale. Because it was important to convert the amino acid into its TFA derivative as completely as possible, reaction conditions for this conversion were examined carefully. TLC was used to monitor the conversion primarily, but GLC was used as an additional check when suitable reaction conditions were developed. The major problem encountered was the difficulty of completely converting the amino acid to the derivative rather than the problem of by-product formation. Under the TLC conditions used, unconverted amino acid remained at the origin while the derivative had an R_f of about 0.4. The amino acid could also be distinguished from its derivative by TLC because it gave a pink color when sprayed with ninhydrin solution whereas the derivative did not react. The conversion occurs in two steps, formation of the methyl ester of the amine hydrochloride, then reaction with TFAA to produce trifluoroacetylated amino methyl ester. $SOCl_2$ reacts with methanol to give dimethyl sulfite which acts as a supplementary methylating agent (2,3).

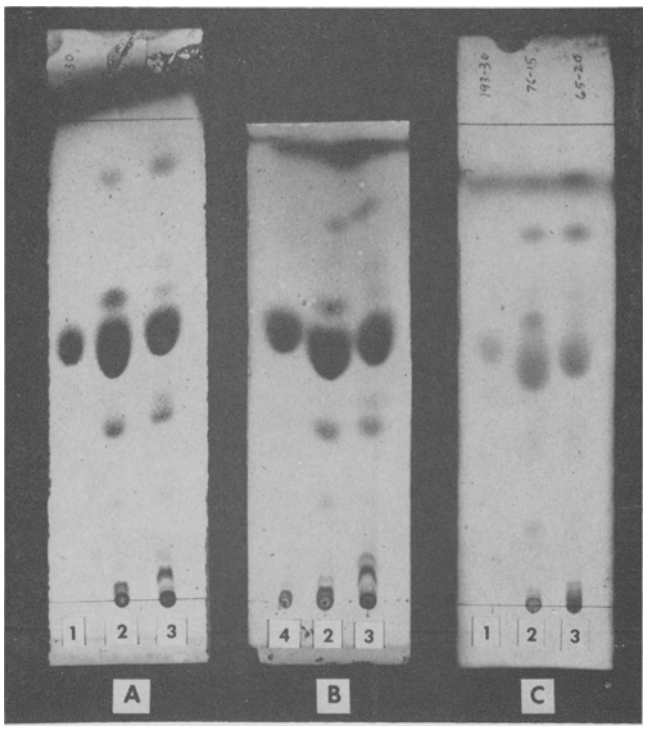

FIG. 4. Comparison of TFA derivative on three different TLC plates. A, Adsorbosil. B, Laboratory-made Silica Gel G. C, Commercial Silica Gel. 1, Pure TFA methyl ester of 12-aminostearic acid. 2 and 3, Different crude 12-aminostearic acid reaction mixtures as their TFA methyl esters. 4, TFA methyl ester of 12-aminostearic acid before alumina treatment.

The most important variables affecting the conversion of the amino acid to its derivative appeared to be the purity and quantities of reagents and reaction times. The results of studying combinations of these variables are shown in Table I. The conditions shown in Run 1, in which the TFAA used was from a freshly opened bottle, gave high conversions. These conditions gave satisfactory conversions for several months. The use of eight-month-old TFAA and undistilled $SOCl_2$ (Runs 2, 3 and 4) under a variety of reaction times did not give as high a conversion as in Run 1.

In Run 5, in which the TFAA was one-year-old, surprisingly most of the amino acid remained unconverted. Use of a freshly made methanol-HCl solution (Run 6) did not give sufficient improvement, nor did use of greater quantities of reagents and longer reaction times (Run 7). The use of distilled TFAA and $SOCl_2$ (Run 8) once again gave satisfactory results. Larger quantities of reagents and longer reaction times (Run 9) increased conversion further.

To determine whether the undistilled $SOCl_2$ or TFAA was causing the low conversions in Runs 5–7, Runs 10 and 11 were performed. The higher conversion of Run 10 compared to that of Run 11 indicated undistilled TFAA was at fault. The use of distilled TFAA and $SOCl_2$ in Runs 12–14, however, gave consistently good results. Increased reaction time in Run 12 compared to Run 8 gave a slightly higher conversion. Although a further increase in the quantities of reagents (Run 13) did not affect conversion, the conditions of Run 13 were adopted as standard. Run 14 was made with methanol-HCl, distilled $SOCl_2$ and distilled TFAA all of which were two months old. The excellent conversion obtained showed the reagents were still effective for at least two months. When crude reductive amination reaction mixtures were derivatized, a control using the pure amino acid was always run at the same time thus insuring that conditions for derivatization were satisfactory.

Preparative Scale. Derivative formation was next scaled up to obtain enough material to characterize the derivative by elemental analysis, melting point and infrared analysis, and to develop a quantitative GLC procedure. The purity of the derivative from the 1 g scale run was estimated by TLC to be about 90%, the remainder being unconverted acid. As described in the experimental section, unconverted acid was removed completely by filtration through alumina giving an analytically pure derivative which was used as a standard for quantitative GLC analysis.

Thin Layer Chromatography of TFA Derivative

Figure 4 shows a comparison between the three types of plates used for TLC analysis of the derivative. Of these three, A (Adsorbosil) gave the greatest clarity and is therefore the most satisfactory. The impurities in A-2 and 3 are well resolved from the TFA derivative. C (commercial Silica Gel) is unsatisfactory mainly because the spots are too light. Also 16 hr are required for visualization in C compared to 15 min for A and B. When C was heated longer the entire plate turned brown. B (lab-made Silica Gel) is less satisfactory than A because clarity is not as good, there is less uniformity in layer thickness, and the layers are much more fragile. B-4 is the TFA methyl eester of 12-aminostearic acid before purification with only a small amount of unconverted acid as an impurity. A-1 shows this impurity removed.

The developing solvent used for the plates in Figure 4 was Skellysolve F-ether (80:20). This solvent system was chosen because it centers the derivative on the plate, and gives an adequate separation of the impurities present. Other volume ratios of these two solvents, 95:5, 90:10, 70:30 and 50:50 were also tried, but were less satisfactory in that the R_f of the main spot was too high or too low.

Visualization of the Adsorbosil plates was so satisfactory with chromic-sulfuric acid solution followed by charring that no other sprays were investigated. This visualization system also worked better than 50% aqueous sulfuric acid solution (v/v) for the laboratory-made plates. Spray reagents investigated for use with the commercial Silica Gel plates were 50% aqueous sulfuric acid, chromic-sulfuric acid, anisaldehyde reagent, phosphomolybdic acid in ethanol and dichlorofluoroscein. Of these 50% aqueous sulfuric acid was the best, but the commercial Silica Gel plates with organic binder were not nearly as satisfactory as the Adsorbosil plates.

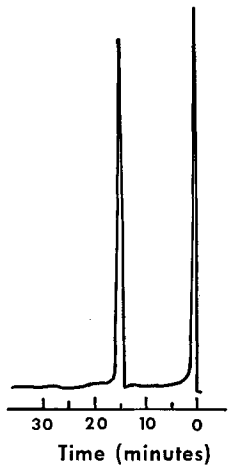

FIG. 5. GLC of pure TFA derivative of methyl 12-aminostearate on 5 ft × ⅛ in. S.S. 10% FFAP 70/80 DMCS Chrom. W; 215 C; helium 30 ml/min.

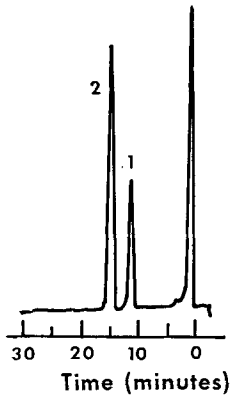

FIG. 6. GLC of methyl 12-ketostearate (Peak 1) and TFA derivative of methyl 12-aminostearate (Peak 2).

Gas Liquid Chromatography

As suitable means for derivative preparation and TLC analysis were now developed, GLC analysis was next investigated. Previous experience had shown that packed columns containing FFAP or Carbowax 20 M were suitable for the GLC analysis of fatty esters containing various functional groups. In Figure 5, where an FFAP column was used, a single, sharp peak was observed for the pure TFA derivative indicating that the FFAP column was suitable. This column also showed better resolution of crude mixtures than one of Carbowax 20 M.

Quantitation of the pure TFA derivative was accomplished using methyl 12-ketostearate as an internal standard. Methyl 12-ketostearate was chosen because it is stable under the conditions used, and because its retention time is close to that of the TFA derivative. Known mixtures of the pure TFA derivative and methyl 12-ketostearate were made up. The GLC of one of these mixtures is shown in Figure 6.

By determining the area under the curve of these

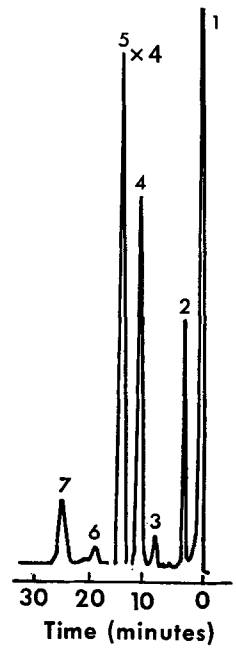

FIG. 7. GLC of crude TFA derivative of methyl 12-aminostearate. Peak 1, Solvent; Peak 2, TFA of methyl 12-hydroxystearate; Peak 4, Methyl 12-ketostearate; Peak 5, TFA of methyl 12-aminostearate; Peaks 3, 6 and 7, unknowns.

two peaks (height × width at half-height) to obtain observed weight percent and plotting this against the actual weight percent, a straightline relationship was obtained. This plot was then used to determine the actual weight percent of the TFA derivative in unknown, crude reaction mixtures.

Figure 7 shows such a reaction mixture in which the compounds present are well resolved. The three major peaks in order of increasing retention times are the TFA derivative of methyl 12-hydroxystearate (3%, Peak 2), methyl 12-ketostearate (10%, Peak 4), and the TFA derivative of methyl 12-aminostearate (82%, Peak 5). Five minor components totaling 5% have not been identified. By the combined use of TLC for qualitative analyses and GLC for quantitative analysis, monitoring the synthesis of 12-aminostearic acid has been greatly facilitated. This information should be of help in analytical studies involving other fatty amino acids as well.

REFERENCES

1. Freedman, B., and G. Fuller, in press.
2. Gee, M., Anal. Chem. *39*, 1677–1679 (1967).
3. Cruickshank, P. A., and J. C. Sheehan, Ibid. *36*, 1191–1197 (1964).
4. Darbre, A., and A. Islam, Biochem. J. *106*, 923–925 (1968).
5. Hagen, P. B., and W. Black, Can. J. Biochem. *43*, 309–315 (1965).
6. Gehrke, C. W., and D. L. Stalling, Separation Science *2*, 101–138 (1967) for leading references.
7. Jones, K., and J. G. Heathcote, J. Chromatog. *24*, 106–112 (1966) for leading references.
8. Applewhite, T. H., M. J. Diamond and L. A. Goldblatt, JAOCS *38*, 609–614 (1961).
9. Hanford, W. E. (Dupont Co.), U.S. Patent 2,312,967 (1943).
10. Noller, C. R., "Chemistry of Organic Compounds," 3rd Edition, W. B. Saunders Co., Philadelphia, 1965, p. 441.

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