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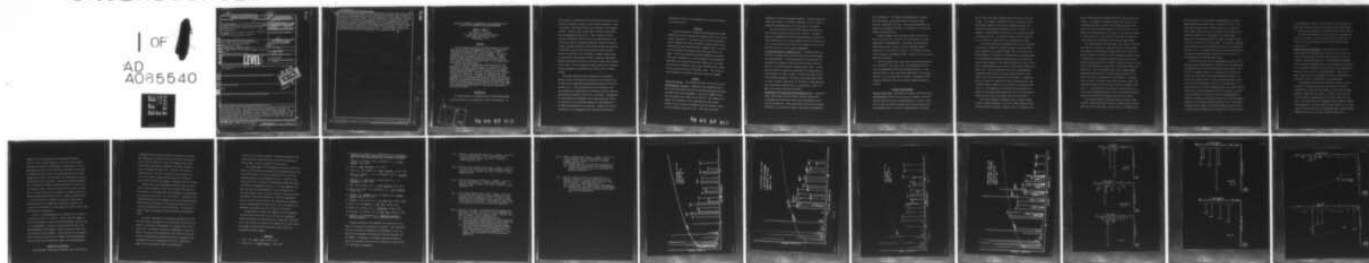
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ANALYSIS OF HYDROXYL, UNSATURATED, AND CYCLOPROPANE FATTY ACIDS--ETC (U)  
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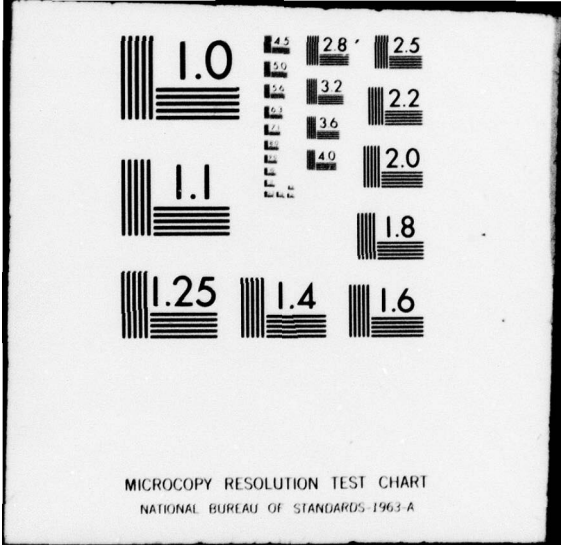
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)  
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A method for the hydrogenation of unsaturated fatty acid mixtures is described.

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and the effects of variations in time, temperature, pressure, and the amount of catalyst are examined on the percent hydrogenation of unsaturated to the saturated forms of the fatty acids. Further, the effects of the different variables are related to the percent of hydrogenation of the cyclopropane fatty acids. Evidence is provided on the hydrogenation of the cyclopropane which does not agree with previously described ring opening mechanisms which have been proposed from GLC data which may be due to the difference in temperatures under which the chromatographic processes are carried out. These findings indicated the possibility of isolating an intermediate in the process by HPLC.

ANALYSIS OF HYDROXYL, UNSATURATED, AND CYCLOPROPANE FATTY ACIDS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

The investigation describes the HPLC separation of hydroxyl fatty acids as their methoxyphenacyl esters. All separations were performed using two types of reverse phase columns,  $\mu$ -Bondapak C 18 and Fatty Acid Analysis columns. The solvent system consisted of acetonitrile:water gradient. A simple method for the trifluoroacetylation of the hydroxyl group is described which permits the identification of these specific fatty acids and decreases the lower limit in chain length determination for these fatty acids.

A method for the hydrogenation of unsaturated fatty acid mixtures is described, and the effects of variations in time, temperature, pressure, and the amount of catalyst are examined on the percent hydrogenation of unsaturated to the saturated forms of the fatty acids. Further, the effects of the different variables are related to the percent of hydrogenation of the cyclopropane fatty acids. Evidence is provided on the hydrogenation of the cyclopropane which does not agree with previously described ring opening mechanisms which have been proposed from GLC data which may be due to the difference in temperatures under which the chromatographic processes are carried out. These findings indicated the possibility of isolating an intermediate in the process by HPLC.

INTRODUCTION

In recent studies (1-4) High Pressure Liquid Chromatography has been utilized in the separation of fatty acid mixtures. In

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those studies, identification of the fatty acids was made by comparison of retention volumes to those of known standards. There are, however, five types of fatty acids important to biological systems: straight chain, branch chain, hydroxyl, unsaturated, and cyclopropane fatty acids (5). Since standards for the variety of different fatty acids are not always available, and retention volumes of standards provide only tentative identification, alternate methods for their identification are necessary. In addition, certain fatty acids may have the same retention volume as other fatty acids; therefore, chemical modifications of the molecule would provide a means to separate these peaks. Chemical modification of fatty acid molecules have been utilized in GLC (6-8). This investigation examines some of the alternate chemical methods for identification of fatty acids as applied in HPLC.

This paper reports the HPLC separation of the methoxyphenacyl esters of hydroxyl fatty acids and the difficulties in the detection of important microbiological hydroxyl fatty acids by HPLC (9-11). Trifluoroacetylation of the hydroxyl acids which permits both identification and detection of the important microbiological hydroxyl fatty acids is described. Hydrogenation of unsaturated and cyclopropane fatty acids along with effects of variations in the operating parameters of hydrogenation on the HPLC chromatograms will be reported. Evidence will be provided which is in disagreement with the previously described

mechanism (6,12,13) for the hydrogenation of the cyclopropane ring in fatty acids.

#### MATERIALS

UV-grade acetonitrile ( $\text{CH}_3\text{CN}$ ) and dimethylformamide (DMF) (Burdick and Jackson Laboratories, Muskegon, Mich.) was used for all HPLC separations. Ultra pure water ( $17.8\text{M}\Omega$ ) was generated using a combination of Milli-RO, Milli-Q reverse osmosis water purification system (Millipore Corp., Bedford, Mass.), and then filtered through  $0.45\mu$  membrane filter (Millipore Corp.) to degas. The UV tag ( $\alpha$ -bromo-m-methoxyacetophone) was obtained from Pfaltz and Bauer, Inc. and the catalyst (N,N-diisopropylethylamine) was obtained from Aldrich Chemical Co. The fatty acids used for standards in this study were quantitative grade and purchased from Supelco, Bellefonte, Penna. The trifluoroacetic anhydride was obtained from Fisher Scientific Company.

#### METHODS

Derivative Process: The procedure of Miller *et al.*(4) was used.

Chromatographic Procedure: Separations were performed using a Waters Associates Model ALC/GPC-244 Liquid Chromatograph equipped with Model 660 Solvent Programmer, an auxiliary M6000A Solvent Delivery System, and a Model 440 UV detector. The standards and samples were separated on two  $\mu$ -Bondapak C18 reverse phase columns in series and two  $\mu$ -Bondapak Fatty Acid Analysis columns in series (Waters Associates, Inc.). The fatty acids were

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monitored at 254 nm for maximum absorption. A water jacket maintained the columns at a constant temperature of 38° in order to improve the reproducibility of the  $t_R$  values in the method.

The fatty acid esters were eluted using an acetonitrile/water solvent system at a flow rate of 1 ml/min. The mobile phase was programmed from 40/60 acetonitrile/water initially to 100% acetonitrile over a 3-hour period for the C18 columns, and over a 6-hour period for the fatty acid columns, using a convex gradient curve No. 5 on the solvent programmer.

Trifluoroacetylation of Hydroxyl Acids: A standard mixture of derivative straight chain and hydroxyl fatty acids was prepared and chromatographed. Trifluoroacetylation of the mixture was performed by the method of Moss and Dees (15) which they had applied to the methyl esters of hydroxyl fatty acids. Two hundred microliters of mixture was evaporated to dryness under  $N_2$  and redissolved in 200  $\mu$ l of  $CHCl_3$ , to which was added 25  $\mu$ l of trifluoroacetic anhydride. The mixture was capped and allowed to stand at ambient temperature for 30 minutes. The mixture was then evaporated to dryness under  $N_2$ , redissolved in 200  $\mu$ l of DMF and an aliquot injected on the HPLC.

Hydrogenation of Unsaturated and Cyclopropane Acids: A bacterial sample (*Escherichia coli* ATCC 25922), known to contain both cyclopropane and unsaturated fatty acids, was prepared as previously described (4). After extraction of the fatty acids into chloroform, the solution was taken to dryness and resuspended in



10 ml of methanol. The sample was hydrogenated in a Supelco Micro Hydrogenator varying the amount of catalyst (PtO<sub>2</sub>), pressure, temperature, and time. Following hydrogenation, the mixture was evaporated to dryness, resuspended in 1 ml DMF and tagged as described above.

A known standard fatty acid mixture which contained palmitic 16:0, oleic 18:1, linoleic 18:2, and linolenic 18:3 was tagged prior to hydrogenation. The mixture was hydrogenated using 100 mg catalyst (PtO<sub>2</sub>), 10 psi of hydrogen, ambient temperature, and 30-minute time period. The sample was evaporated to dryness, resuspended in DMF, filtered twice and injected directly on the HPLC.

A second standard fatty acid mixture containing myristoleic 14:1, palmitoleic 16:1, oleic 18:1, and dihydrosterculic Δ<sup>19</sup> acid was hydrogenated under the following conditions: 10 mg catalyst (PtO<sub>2</sub>), 10 psi, and ambient temperature for 15 minutes duration. This was evaporated to dryness, resuspended in DMF, tagged as described above and an aliquot injected directly on the HPLC.

#### RESULTS AND DISCUSSION

Hydroxyl Fatty Acids: Two families of hydroxyl fatty acids were chromatographed on both C18 and fatty acid columns. The alpha hydroxyl fatty acids elute at approximately 2.7 relative carbon units in from the parent straight chain fatty acid, while the beta hydroxyl fatty acid elutes at approximately 3.32 carbon

units in from the parent straight chain fatty acid on the C-18 columns. For example,  $\alpha$ -hydroxyl palmitic acid elutes 3/10 of the distance between C-13 and C-14,  $\beta$ -hydroxyl palmitic acid elutes 6/10 of the distance between C-12 and C-13 (Fig. 1). In addition, 12-hydroxystearic acid, which is not shown, was found to coelute with C-12. The relative carbon units for both the  $\alpha$  and  $\beta$  acids were consistent with increasing chain length.

The elution profile of the hydroxyl fatty acids on the Fatty Acid Analysis columns had a larger decrease in elution volume when compared to their parent straight chain fatty acids, and greater degree of variability in this decrease in elution volume is demonstrated by comparing  $\alpha$ -hydroxyl myristic which had an elution volume change of 4.33 relative carbon units prior to C-14 while  $\alpha$ -hydroxydocosanoic acid elutes 3.42 relative carbon units before C-22. The two  $\beta$ -hydroxyl fatty acids chromatographed demonstrate both decrease in elution volume relative to the parent chain and the variability in the decrease.  $\beta$ -hydroxyl myristic elutes 4.85 carbon units before the parent chain, and  $\beta$ -hydroxyl palmitic elutes 4.0 units prior to its parent chain (Fig. 2). The variability in decrease in elution volume as a result of the hydroxyl group may be due to the relative greater polarity of the fatty acid columns over the C-18 columns.

The elution profiles of hydroxyl acids on either columns indicate a major difficulty in the use of HPLC analysis of this particular fatty acid group. Bussell *et al.* (3) reported that

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the lower limit of this procedure is approximately C-10 due to tagging reaction by-products which elute prior to C-10. This would limit hydroxyl acids to approximately C-14 or greater chain length, however, at least in bacteria fatty acids, (9-11) the most prominent are C-10 through C-14 in chain length. A solution to this problem would be to modify the hydroxyl group in order to increase its elution volume. An important consideration in modification of the hydroxyl group is steric hindrance from the UV tag. The method utilized was trifluoroacetylation of the hydroxyl group previously described by Moss and Dees (14) for methyl esters of hydroxyl fatty acids.

The TFAA derivatives of the methoxyphenylacyl esters of the hydroxyl fatty acids produced an increase in elution volume where the hydroxyl fatty acids were eluting after their parent chain on the C 18 columns, however, the increase was more marked in the shorter chain acids with  $\alpha$ -hydroxymyristic eluting at 0.8 carbon units greater than C-14 and  $\alpha$ -hydroxylicosanoic eluting at 0.16 carbon units greater than the C-20 parent chain. The TFAA derivatives of the two  $\beta$ -hydroxyl acids elute prior to their parent chain, but both demonstrate that the increase in elution volume was greater in the short chain with  $\beta$ -hydroxymyristic eluting 0.16 relative carbon units prior to C-14 and  $\beta$ OH palmitic eluting at 0.29 relative carbon units before C-16. The explanation may be that change in the polarity region of molecule due to derivation is equivalent for each fatty acid in either series

or the  $\beta$ -hydroxyl series. The shorter chain acids have less of hydrocarbon nature to balance the polarity change which results in a larger net change for the shorter chain fatty acids (Fig. 3).

The TFAA derived hydroxyl fatty acids showed the same relative elution profile on the Fatty Acid Analysis columns as on the C 18, but were shifted out to an overall greater elution volume (Fig. 4).

Unsaturated and Cyclopropane: In our laboratory we had previously applied GLC hydrogenation techniques to our free fatty mixtures before tagging (3), but we were unable to obtain consistently complete hydrogenation. We undertook the investigation to determine optimum condition for hydrogenation and effects of the different components such as pressure, time, temperature, and amount of catalyst, on the hydrogenation. There are two basic types of fatty acids which are affected by hydrogenation unsaturates and to a lesser extent cyclopropane acids.

Cyclopropane fatty acids are difficult to obtain as pure standards, therefore, we used bacterial fatty acid mixture obtained from *E. coli* which was known to contain both cyclopropane and unsaturated fatty acids. In Table 1, the values for relative percent hydrogenation of  $\Delta 19$  and 18:1 fatty acid are given. The table shows that temperature had the greatest effects on hydrogenation, and that 4° gave the best results in that only minimal amounts of the  $\Delta 19$  was hydrogenated while allowing almost complete conversion of the 18:1 to the 18:0 fatty acid.

TABLE 1

Hydrogenation of *Escherichia coli* Fatty Acid Mixture

PtO <sub>2</sub> (mg)	Press. psi	Temp.	Time Min.	Hydrogenated	
				% $\Delta$ 19	%18:1
4.97	10	ambient	10	45	92
9.60	10	ambient	10	35	100
19.90	10	ambient	10	36	9
30.09	10	ambient	10	15	96
5.88	10	ambient	45	2	92
4.81	10	ambient	120	16	83
6.13	10	36	10	5	58
4.92	10	36	30	15	92
12.74	10	36	30	15	96
9.77	25	36	30	8	92
11.91	25	47	30	2	92
11.44	20	4	30	0	66
19.93	20	4	30	0.5	100

The interesting finding in chromatograms (Fig. 5) showed that neither  $\Delta$ 17 nor the  $\Delta$ 19 produced new peaks in the area expected from the breakdown scheme described elsewhere (12, 13). There was, however, a new peak eluting prior to C-15 and had a large UV absorption greater than the total decrease in  $\Delta$ 17 and  $\Delta$ 19 peaks. This observation was confirmed using a standard mixture of 14:1, 16:1, 18:1, and  $\Delta$ 19. The peak in question

appeared in the chromatogram of the hydrogenated mixture (Fig. 6), while no peaks appeared in the area of the chromatogram where the products (nonadecanoic, 9-methylstearic, and 10-methylstearic acids) of the previously described reaction would be expected to occur (6,12). The inward migration of the peak suggests that there is the attachment of a highly polar group to the molecule as the ring opens. The high UV absorption suggests that the molecule can bind an additional molecule of the UV tag to that polar group. Since this reaction was not seen in GC-MS data, it would indicate that the complex is probably unstable at elevated temperatures utilized in GLC. Another important point is that the  $\Delta 19$  and  $\Delta 17$  produced the same peak, which would indicate that the two carbon unit difference between the two acids was not sufficient to allow separation because of the large polar areas in the molecules.

Finally, in GLC hydrogenation is performed after formation of the esters (Fig. 7). There is a report (16) that suggests that mild hydrogenation may not destroy the phenyl ring structure as a chromatophore. A mixture of unsaturated C-18 fatty acid was hydrogenated after tagging. Greater than 90% of the UV tag was destroyed by the technique. This indicates that the sample must be split with one-half to be tagged, then chromatographed, and the second half hydrogenated, tagged, then chromatographed.

#### SUMMARY AND CONCLUSIONS

Chromatographic separations of hydroxyl fatty acids on both

$\mu$ -Bondapak C18 and Fatty Acid Analysis columns was described. The  $\mu$ -Bondapak C18 columns provided a consistent elution profile of the two families,  $\alpha$  and  $\beta$ , of hydroxyl fatty acids tested which permitted the prediction of elution volume for the increasing chain fatty acids. The Fatty Acid Analysis column produced a greater variation in elution volumes relative to the parent straight chain acid due to the greater polarity of the fatty acid column as compared to the C18 column.

Examination of the chromatograms indicated that the lower limit in chain length for detection of hydroxyl fatty acids is C14; however, the hydroxyl fatty acids important in microbiological systems range between C-10 to C-14 (9-11). A method for the trifluoroacetylation of the hydroxyl fatty acids permitting both identification by migration of the peaks and detection of C-10 or greater hydroxyl fatty acids was described. The method was simple, rapid, and produced a 100% conversion of the hydroxyl acids.

In earlier experiments, we noted that hydrogenation of the bacterial fatty acid samples had a considerable variation in the percent of conversion of unsaturated to saturated form of the fatty acids. Variations in pressure, temperature, time and the amount of catalyst were evaluated for their effects on the conversion of unsaturated to saturated form and the opening of the cyclopropane ring in cyclopropane fatty acids. Temperature seems to have the greatest effect on hydrogenation.



The use of an ice bath to obtain 4° temperature permitted 100% conversion of the unsaturated fatty acid while having only a minimal effect on the cyclopropane ring structure.

The chromatograms of the hydrogenated bacterial fatty acid mixture containing both unsaturated and cyclopropane fatty acids did not have peaks in the areas expected from the previously described mechanism for hydrogenation (6,12). There was one large peak with an increased UV absorption exceeding the amount of converted cyclopropane fatty acid. This peak appeared only when there was some hydrogenation of the cyclopropane acid. This peak indicates that we could be seeing a relatively stable intermediate, which would decompose at elevated temperatures that are normally used in GC-MS. The observation was repeated using a standard mixture of fatty acids. More investigation is necessary to determine the exact structure of this peak.

Although some literature (16) suggest that mild hydrogenation would have little effect on our UV tag, our experiments showed that greater than 95% of the tag is destroyed, which indicates that in fatty acid analysis the sample must be split using one aliquot for the initial run and the second aliquot for hydrogenation before tagging.

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Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the authors and are not to be construed as those of the U. S. Army Medical Department.

- Fig. 1 HPLC of  $\alpha$  and  $\beta$  hydroxyl fatty acid. Columns: Two 30 cm X 3.9 mm  $\mu$ -Bondapak C18, eluent acetonitrile:water, gradient curve 5, 180 min., flow rate 1.0 ml/min., column temperature 38°.
- Fig. 2 HPLC of  $\alpha$  and  $\beta$  hydroxyl fatty acids. Columns: Two 30 cm X 3.9 mm fatty acid analysis, eluent acetonitrile:water, gradient curve 5, 6 hours, flow rate 1.0 ml/min., column temperature 38°.
- Fig. 3 HPLC of TFAA hydroxyl fatty acids. Columns: Two 30 cm X 3.9 mm  $\mu$ -Bondapak C18, eluent acetonitrile:water, gradient curve 5, 180 min., flow rate 1.0 ml/min., column temperature 38°.
- Fig. 4 HPLC of TFAA hydroxyl fatty acids. Columns: Two 30 cm X 3.9 mm fatty acid analysis, eluent acetonitrile:water, gradient curve 5, 6 hours, flow rate 1.0 ml/min., column temperature 38°; TFAA hydroxyl fatty acids are indicated by asterisk and an underline. The original elution volumes of the hydroxyl fatty acids are shown by shaded peaks.
- Fig. 5 HPLC of *E. coli* fatty acid mixture used for hydrogenation experiment. Columns: Two 30 cm X 3.9 mm  $\mu$ -Bondapak C18, eluent acetonitrile:water, gradient curve 5, 180 min., flow rate 1.0 ml/min., column temperature 38°.
- Fatty acid mixture, before hydrogenation.
  - Fatty acid mixture hydrogenated at 4° with conversion of the unsaturated fatty acids to saturated forms and no hydrogenation of the cyclopropane fatty acids.
  - Fatty acid mixture hydrogenated at ambient temperature with conversion of the unsaturated fatty acids and partial conversion of the cyclopropane fatty acids. Showing one large peak indicated by ?. This suggests that its origin is from the cyclopropane fatty acids.

Fig. 6 HPLC of standard fatty mixture. Columns: Two 30 cm X 3.9 mm  $\mu$ -Bondapak C18, eluent acetonitrile:water, gradient curve 5, 180 min., flow rate 1.0 ml/min., column temperature 38°.

- (a) Standard fatty acid mixture before hydrogenation.
- (b) Standard fatty acid mixture after hydrogenation showing the same peak seen in *E. coli* fatty acid mixture indicated by ?.

Fig. 7 HPLC of unsaturated fatty acid standard mixture. Columns: Two 30 cm X 3.9 mm  $\mu$ -Bondapak C18, eluent acetonitrile:water, gradient curve 5, 180 min., flow rate 1.0 ml/min., column temperature 38°.

- (a) Standard fatty acid mixture before hydrogenation.
- (b) Standard fatty acid mixture tagged, then hydrogenation showing destruction of the UV tag under mild conditions of hydrogenation.

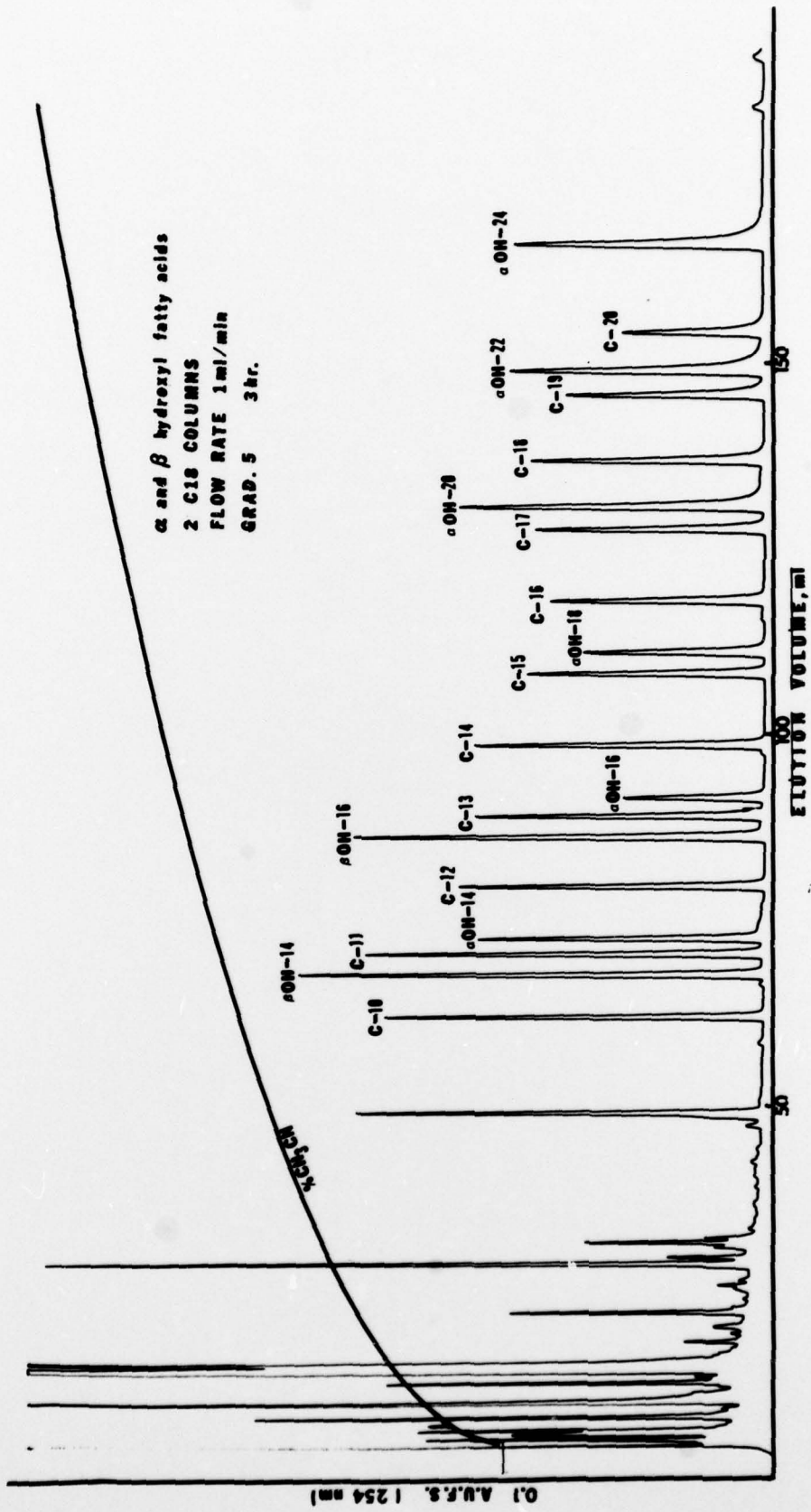


Fig. 1

$\alpha$  and  $\beta$  hydroxyl fatty acids

2 FATTY ACID COLUMNS

FLOW RATE 1ml/min

GRAD. 5 6 hr.

0.1 A.U.F.S. (254nm)

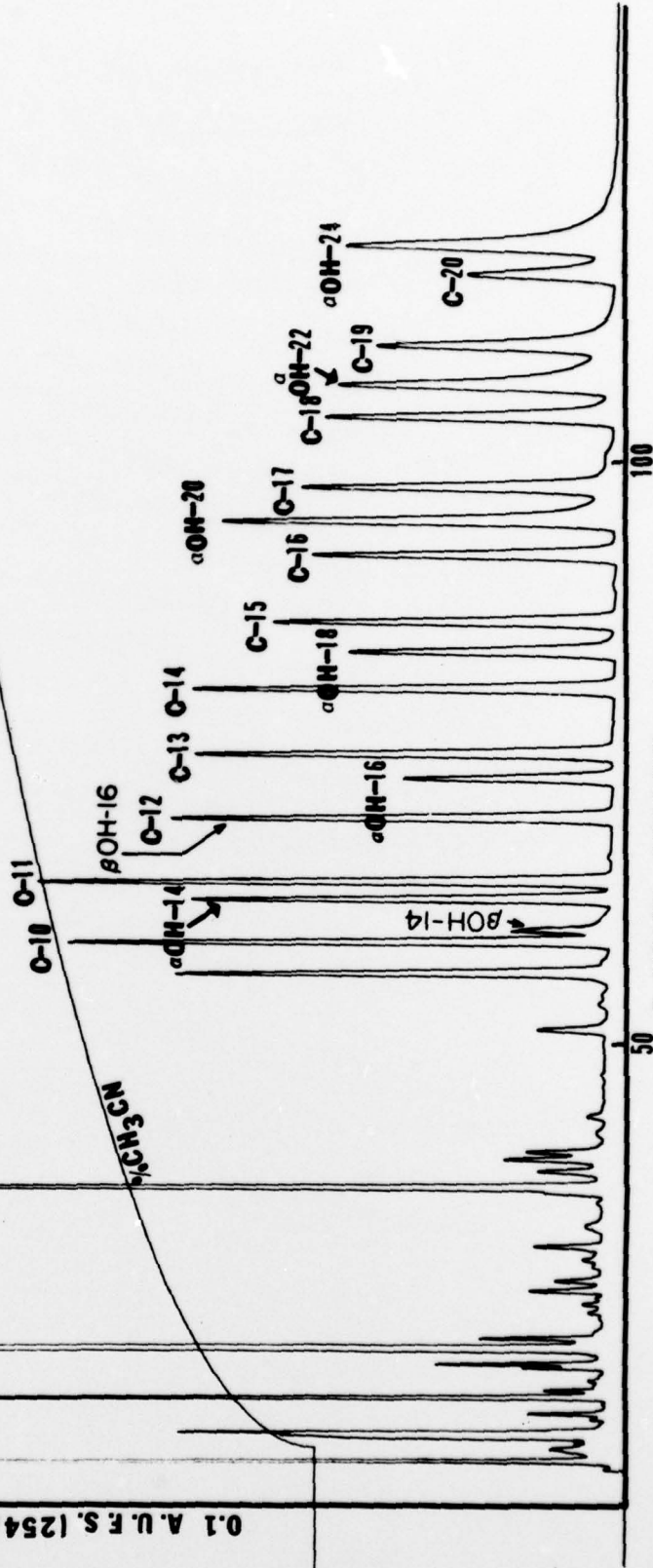


Fig. 2

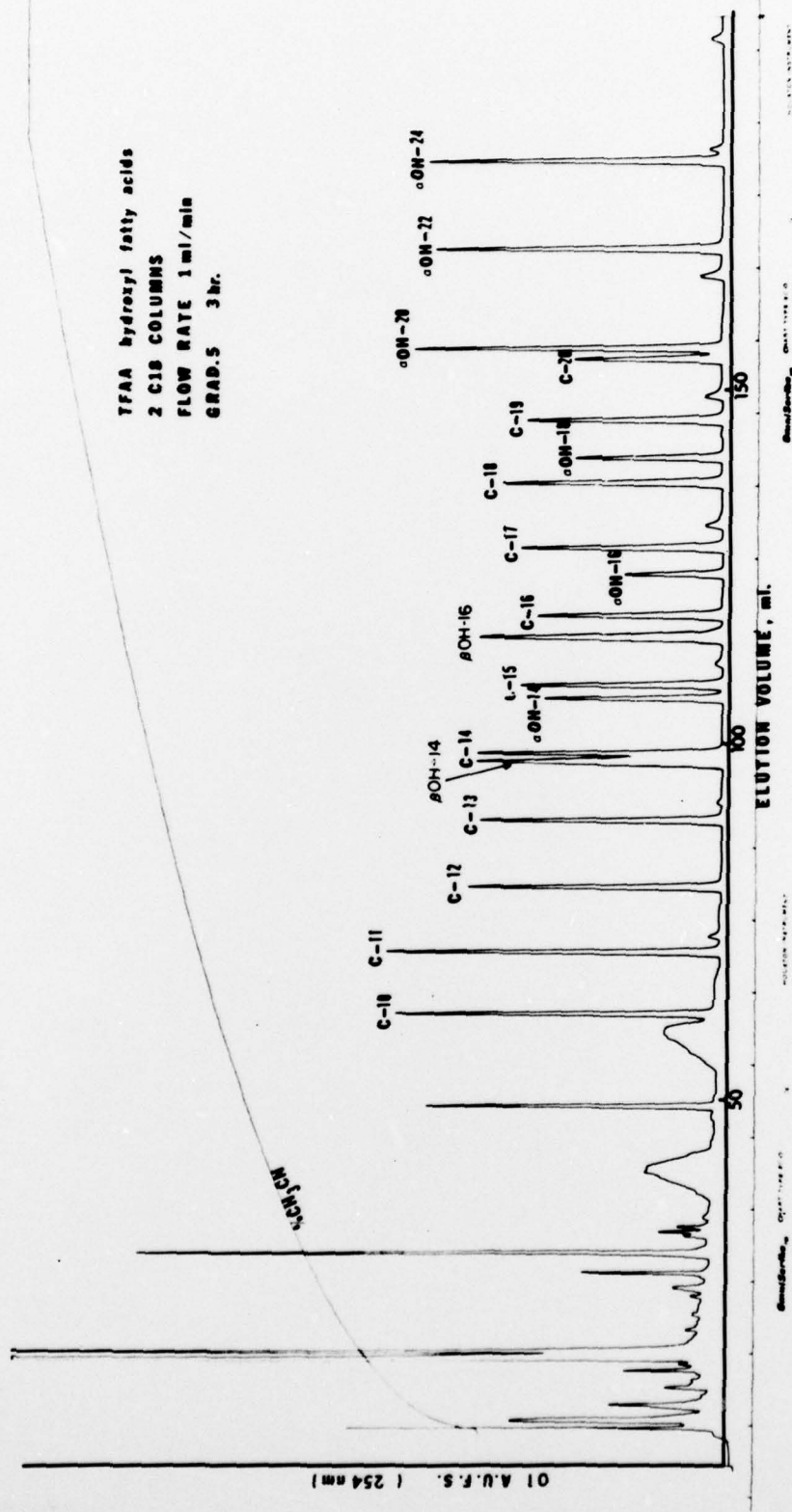


Fig. 3

TFAA hydroxyl fatty acids  
2 FATTY ACID COLUMNS  
FLOW RATE 1 ml/min  
GRAD. 5 Ghr.

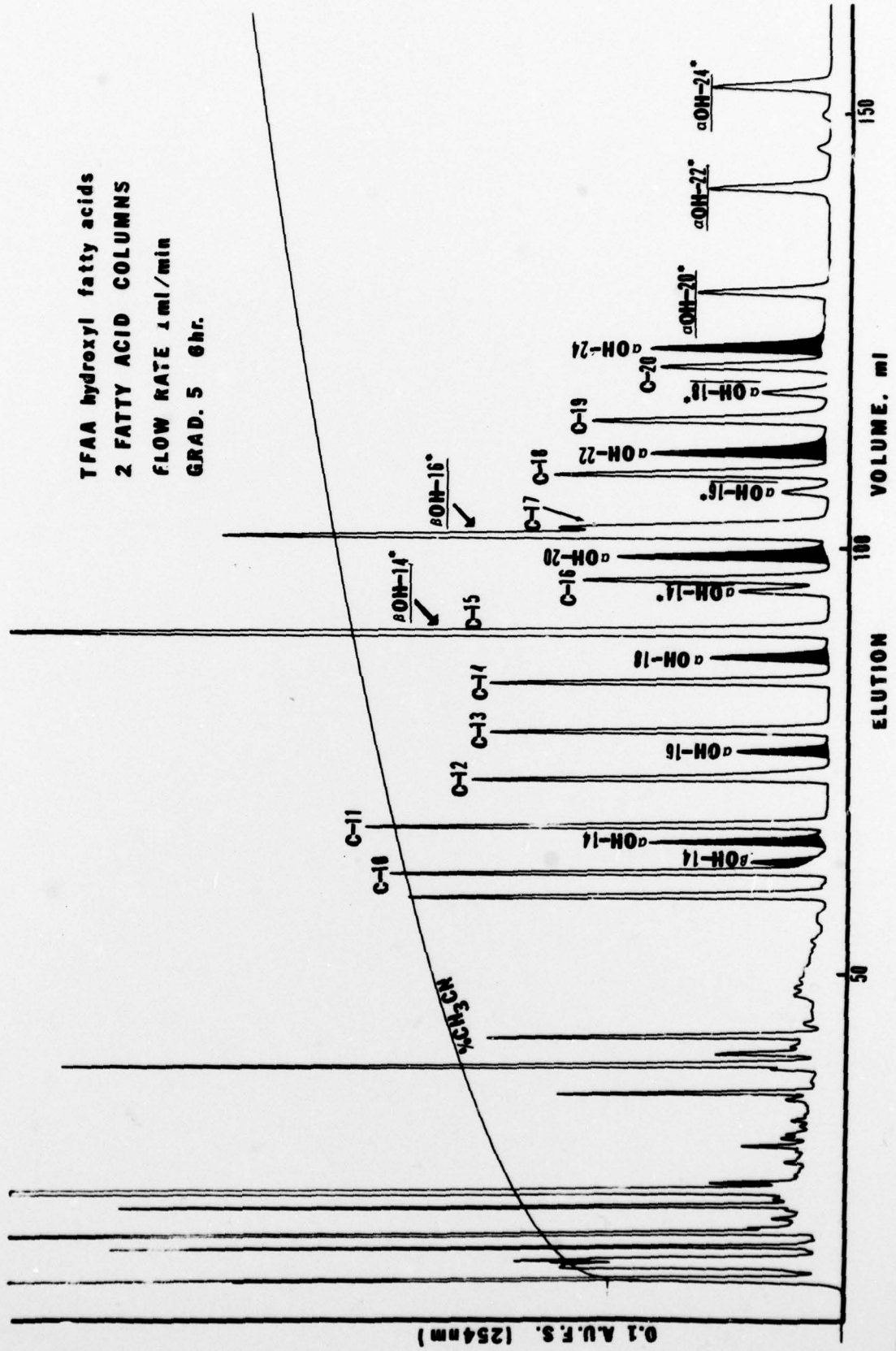
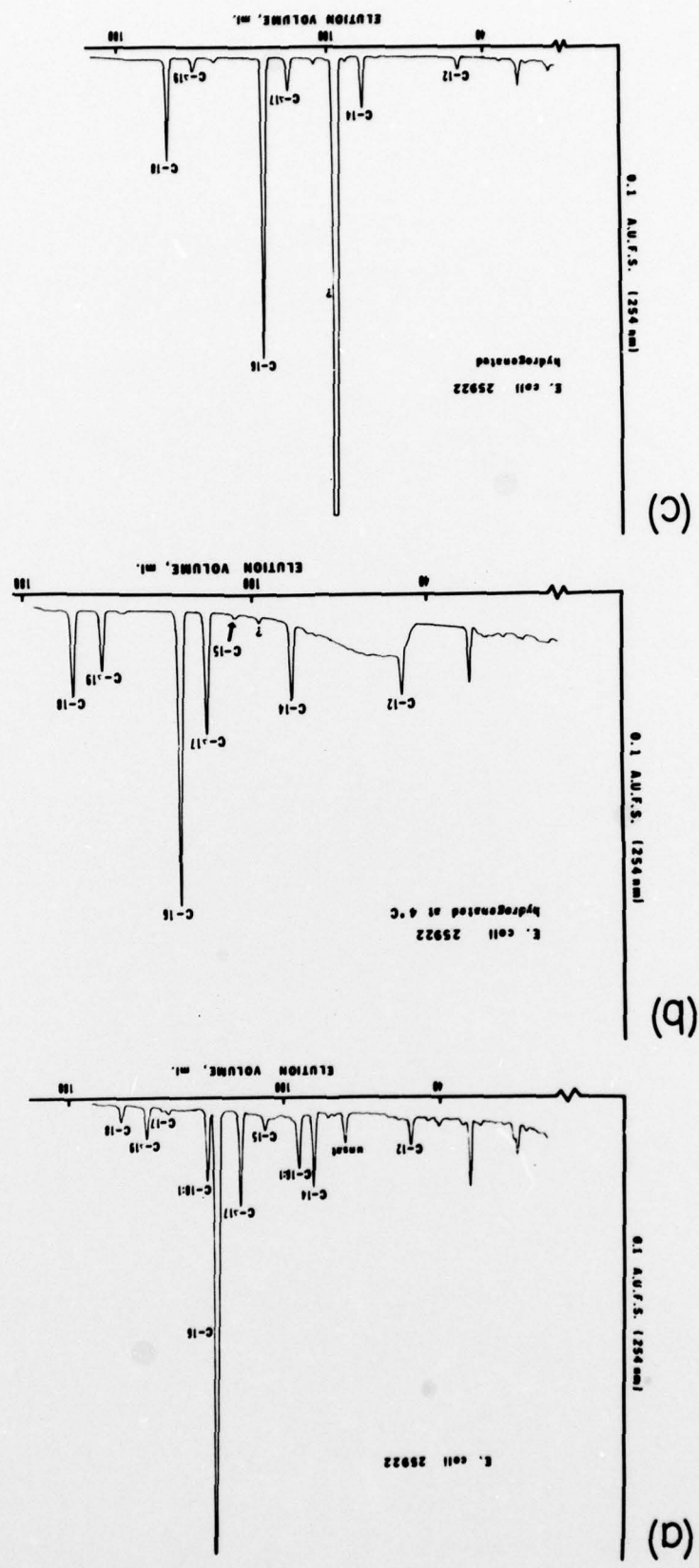
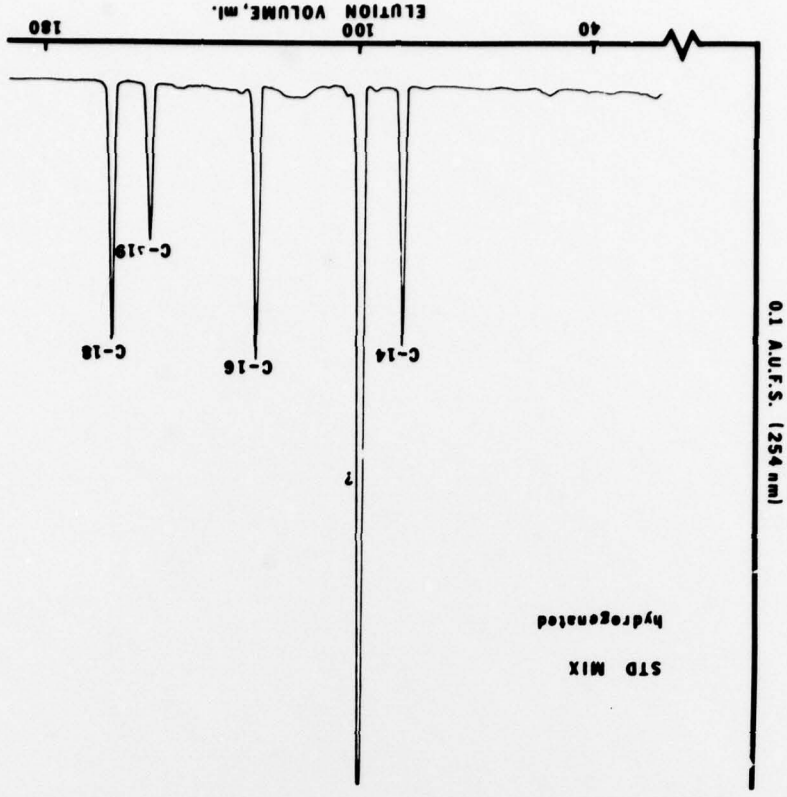


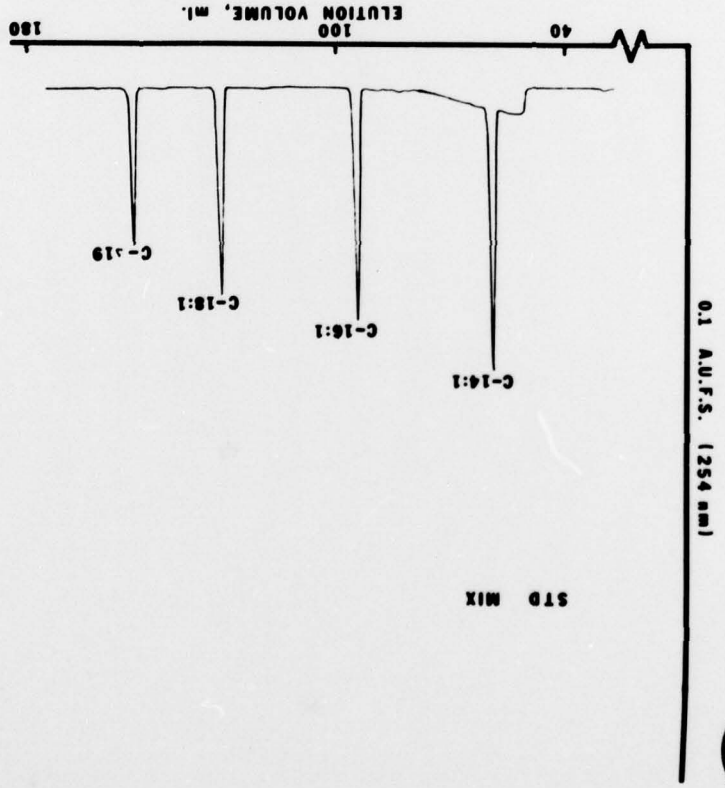
Fig. 4







(b)



(d)

Fig. 7

