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HIGH PRESSURE LIQUID CHROMATOGRAPHY IN THE ANALYSIS OF FATTY AC--ETC(U)
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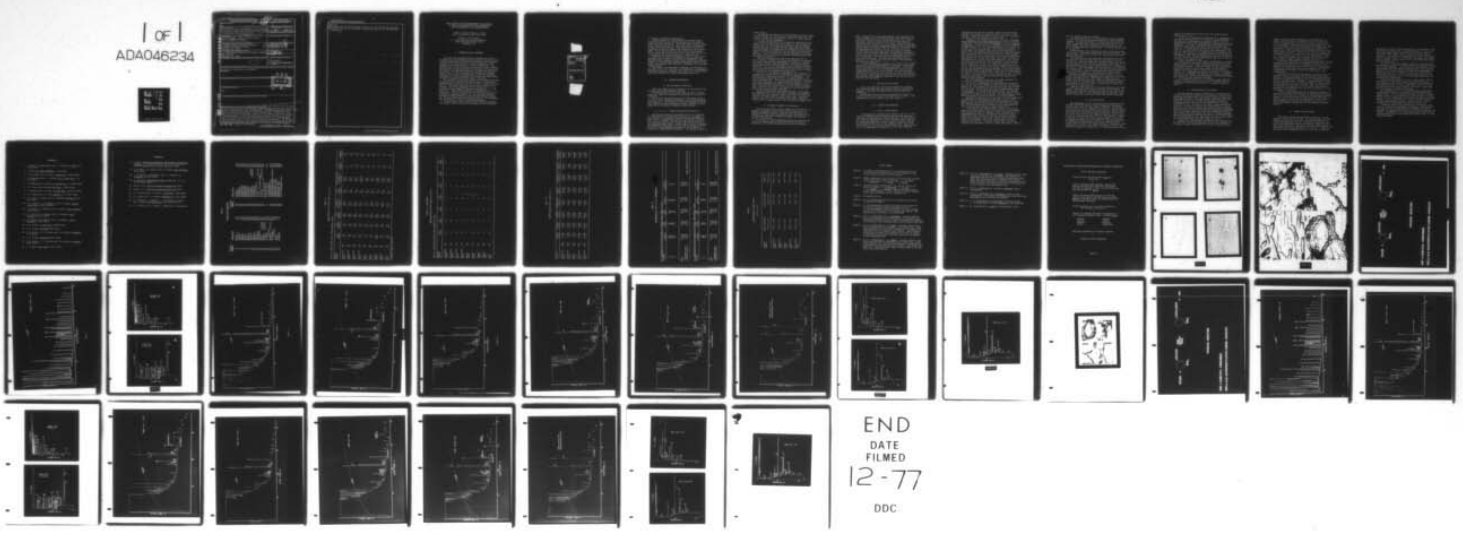

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high pressure liquid chromatography (HPLC) was used to find

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In the bacterial strains used, ~~we found evidence by HPLC of~~ a C20, C22, and C24 present in approximately equal concentrations. ~~We also found~~ evidence of C21, C23, and C25 acids at about 10% of the C20 concentration. These fatty acids had not previously been reported. There was also evidence of two C16:1 fatty acids being present. The GC and HPLC data were similar in respect to the major fatty acid peaks, but the ratios of individual peaks in the GC chromatograms did not correlate well with the ratios in the HPLC chromatograms. The HPLC provided improved separation over standard GC techniques, and sensitivity of the

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HPLC methodology was 100 times that of FID in GC. The authors feel that HPLC methodology will aid in the chemical taxonomic studies for the identification of bacteria.

HIGH PRESSURE LIQUID CHROMATOGRAPHY IN THE ANALYSIS OF FATTY ACID COMPOSITION OF ORAL STREPTOCOCCI AND ITS COMPARISON TO GAS CHROMATOGRAPHY

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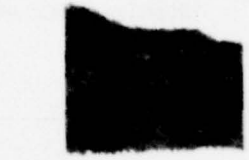
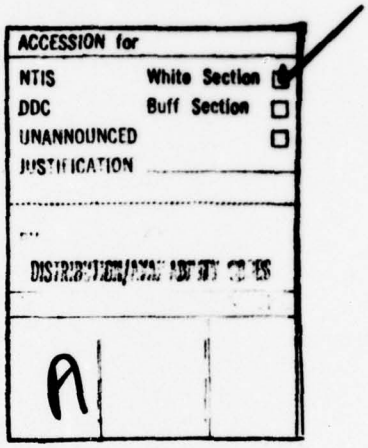
I. INTRODUCTION AND BACKGROUND

The detection and identification of bacteria in clinical specimens is a time consuming process involving isolation in pure culture, determination of numerous physiological and biochemical characteristics, and analysis of the data accumulated. This procedure is illustrated in Fig. 1 which describes steps for identification of oral streptococci used in this study. Definite identification of bacterial species is generally not possible on the basis of colonial morphology (Fig. 2) and/or electron micrographs (Fig. 3). Because standard microbiological techniques require 24-72 hours for identification of infecting organisms, development of a more rapid method would aid in initiating proper therapy at the earliest possible state of infection.

The possibility exists that analysis of bacterial fatty acids may provide such a rapid identification system. In 1963 Abel, DeSchmertzing, and Peterson [1] were the first to show the feasibility of correlating fatty acid composition of bacteria with taxonomy. Since then, knowledge of the chemistry and [illegible]my bacteria has been expanded by laboratories using gas [illegible]og-raphy (GC) to study both cellular fatty acids and metab[illegible] by-products. Shaw [2] has described the three basic criteria of an ideal chemotaxonomic method: (1) the technique should be applicable to large numbers of bacterial genera and/or species, (2) the data should be readily obtained, and (3) the parameters used should differ as much as possible among genera.

In recent years, clear, well defined patterns have emerged from studies using GC and other methodology on lipid composition

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as a guide to bacterial classification.

Lambert and Moss [3] have successfully divided closely related streptococci into two distinct groups on the basis of their fatty acid composition. Prefontaine and Jackson [4] separated "corroding" bacilli into four major groups by comparison of their fatty acid profiles. These groups were readily distinguished by presence, absence, and relative size of the major peaks. It has been shown by the use of GC that cyclopropane [5-7], hydroxyl [8-11], and branch chain fatty acids [11-14], are characteristic and distinctive acids in certain bacteria.

High Pressure Liquid Chromatography (H.P.L.C.) has several advantages over GC such as the ability to chromatograph high molecular weight compounds, greater sample loadability, increased capability of separating and assaying temperature sensitive compounds, and extremely high sensitivity (picograms) when utilizing tagging reagents.

This project was undertaken to determine the feasibility of employing H.P.L.C. for detection and identification of pathogenic bacteria by analysis of cellular fatty acids and to compare sensitivity and advantages of liquid chromatography with GC.

II. METHODS AND MATERIALS

A. Bacteriological Preparation

Fatty acid composition of streptococci is influenced by culture media and growth conditions [15-18].

The cultures used in this study (Table II) were, therefore, grown under identical conditions for an optimal GC to H.P.L.C. comparison.

Each culture was grown aerobically in Todd-Hewitt broth at 37°C for 24 hours, washed three times in saline and harvested by centrifugation. A wet weight of 0.60 gm was suspended in 200 ml of 5% NaOH in 50% aqueous methanol, heated to boiling in a 90°C water bath for 15 min, and acidified to pH2 with HCl.

B. H. P. L. C. Sample Preparation and Analysis

The fatty acid mixture was prepared for H.P.L.C. analysis by a modification of Borch's [19] procedure for UV tagging. A 2 ml aliquot of the saponified bacterial mixture was combined with 2 ml of a saturated NaCl solution, and extracted with five 10 ml portions of $CHCl_3$, and the chloroform extract was dried over anhydrous $MgSO_4$, taken to dryness under a stream of nitrogen and immediately resuspended in 1 ml of DMF containing 30 μmoles of UV tag (α-bromo-m-methoxy-acetophenone) and 60 μmoles of catalyst (N, N-disoprophenylethylamine). The tagging reaction is

shown in Fig. 4.

The mixture was heated in a 60°C waterbath for 60 min, cooled, filtered through a 0.5 µm Fluoropore filter (Millipore) and a 50 to 150 µl aliquot was injected into the H.P.L.C. (Waters Associates 244 with solvent programmer).

It should be emphasized that although the preferred solvent system for extraction of free fatty acids is a $CHCl_3$-CH_3OH (2:1) mixture [20], our observations have indicated that the tagging reaction did not go to completion when using samples extracted with $CHCl_3$-CH_3OH. The completion of the tagging reaction however, could be achieved by addition of more catalyst and reheating the mixture. It is our opinion that even on evaporation to dryness, there were still trace amounts of methanol remaining which would inactivate the catalyst.

Sample hydrogenation was used to aid identification of H.P.L.C. peaks. A second 2 ml aliquot was placed in a hydrogenation vessel (Supelco Micro-Hydrogenator), and 10 ml of methanol and 20 mg of platinum oxide were added to the reaction vessel pressurized to 10 psi with hydrogen, and the contents were mixed for 45 min. The solution was removed from the vessel, filtered, and the filtrate was extracted with five 10 ml portions of $CHCl_3$, and processed for UV tagging as described above.

The H.P.L.C. separation was performed on two µBondapak C18 reverse phase columns (30 cm X 4 mm) (Waters Associates) in combination with an ODS guard column (7 cm X 2 mm)(Whatman). The C18 columns were maintained at 37°C by a heating jacket to increase reproducibility. The solvent system consisted of deionized water and acetonitrile (UV grade Burdick and Jackson). The solvents were programmed from 40/60% to 100/0% acetonitrile:water over a three hour period using curve 5 on the solvent programmer, and a flow rate of 1 ml/min. The separation was continued for 60 min after reaching final conditions.

The derivatized fatty acids were detected at 254 nm using 0.1 Absorption Units Full Scale (AUFS) initially, and changed to 0.02 AUFS after the elution of stearic acid #10 to increase the detection of higher molecular weight fatty acids. The amount injected into the H.P.L.C. varied from 50 to 200 µl sample in order to give a response greater than 0.05 AUFS for palmitic acid.

C. GC Sample Preparation and Analysis

The fatty acid mixture (100 ml aliquot) was prepared for GC analysis by formation of the methyl esters using a boron trihalide in methanol technique. Although other techniques are available for methyl ester formation, several investigators [21, 22] have shown the boron trihalide technique to be the best method of GC analysis.

An aliquot of a 100 ml saponified mixture was combined with 100 ml of saturated NaCl and extracted twice with 100 ml of chloroform. The combined extracts were dried over anhydrous

$MgSO_4$, evaporated to dryness under nitrogen, and resuspended in 3 ml of reagent, BCl_3, 14% (W/V) methanol. After the mixture was boiled for 10 min, 30 ml of petroleum ether and 20 ml of water were added, allowed to separate, and the petroleum ether was evaporated under nitrogen to a 25 µl volume. One microliter was then injected in the gas chromatograph (Varian 2860) equipped with a Flame Ionization Detector (FID) and integrator (Varian CDS 101). The separation was carried out on a 12 ft X 1/4 in X 0.2 mm (inside diameter) coiled glass column packed with 10% DEGS-PS coated on 80 to 100 mesh Supelcoport, was performed isothermally at 195°C with the injector and detector temperatures at 205°C and 210°C respectively. The carrier gas was purified helium at a flow rate of 20 ml/min. The flow rates for hydrogen and compressed air for the FID were 40 and 20 ml/min. The attenuation was 4X and the range 10^{-11}.

A second separation was performed on a 10 ft X 0.2 mm (inside diameter) X 1/4 in (outside diameter) coiled glass column packed with a 3% SP-2100 DOH on 100/120 mesh Supelcoport. The initial column temperature of 150°C was maintained for 5 min. and then programmed to 225°C at a rate of 4°C/min. The column was maintained at 225°C for 20 min, then cooled for 4 min. The injector and detector temperatures were 250°C and the carrier gas flow rate was 20 ml/min. The operating conditions of the FID were same as those for the DEGS column.

D. Identification of Peaks

Fatty acid peaks were tentatively identified by comparing their retention times with the retention times for the purified standards. Previously published results provided additional information.

The standard curve for fatty acid analysis in H.P.L.C. is shown in Fig. 5 with the fatty acids used listed in Table I. The standard curves for GC analysis are shown in Fig. 6.

III. RESULTS AND DISCUSSION

A. H.P.L.C. Chromatograms

The separation of bacterial fatty acids on the H.P.L.C. produced a chromatogram which had major peaks with the same retention times as the major fatty acids reported in the literature to be present in these bacteria [3, 18]. The major fatty acids seen in the chromatograms were: lauric (C12:0) #7, myristic (C14:0) #8, palmitoleic (C16:1 cis Δ^9) #14, palmitic (C16:0 cis Δ^9) #9, oleic (C18:1 cis Δ^9) #15, and stearic (C18:0) #10. Representative H.P.L.C. chromatograms are shown in Fig. 7-11, with tentative

identification of some of the peaks. Some of the minor peaks reported to be present [2] were also seen in the H.P.L.C. chromatogram. These were myristoleic (C14:1 cis Δ^9) #34, pentadecanoic (C15:0) #29, and heptadeconic (C19:0) #30.

Eicosenoic acid had been reported to be present in Streptococcus salivarius and Streptococcus mutans but not in other oral streptococci. We noted in the chromatograms of both S. mutans and S. salivarius cultures there was a shoulder off peak #10 (C18:0) as shown in Fig. 7, 8, 11. Upon hydrogenation of the sample, the shoulder peak was lost indicating that it was an unsaturate. It can be seen in the standard curve that unsaturation moves the peaks in from the parent saturated fatty acid. Since the shoulder peak eluted after the C18:0 #10 fatty acid; this would indicate that the fatty acid is at least C19:0, and from the hydrogenation chromatogram (Fig. 12) that particular fatty acid is probably a C20 unsaturate or in other words C20:1 eicosenoic acid. A question then can be raised, why did the shoulder not correspond to either standard #37 or #49? It is important to point out that H.P.L.C. separates not only on the basis of unsaturation as in GC, but also on the basis of the cis-trans isomers, and the position of the double bond within the molecule. It has been reported that bacteria produce only monoenolic acids and the unsaturation is usually in cis Δ^9 position [15]. Since #37 is a cis Δ^5 and #49 is a cis Δ^{11} eicosenoic acid, it would be expected that the cis Δ^9 eicosenoic acid found in the bacteria would not overlap with either of these standards.

The above discussion points out the extreme dependency of the H.P.L.C. methodology on accurate standards.

The H.P.L.C. chromatograms of oral streptococci had peaks past the C20 fatty acid reported by Drucker [15,16] and Moss and Lambert [3]. We saw peaks with the same retention time as C20:0 #11, C22:0 #12, and C24:0 #13, and they were approximately equal in peak height. In the bacterial strains tested, we saw peaks corresponding to C21:0 #32, C23:0 #33, C26:0 #47, and C28:0 #48, which had not previously been reported. The peak heights of these acids were about 10 to 20% of the C20, C22, and C24 heights.

This may have been due to the fact that in GC the columns used are optimized for investigating a particular range of carbon lengths. Most of the columns [24,25] used previously for fatty acid analysis used columns optimized for acids from C10 through C20. The peak heights of the different fatty acids present in each of the bacterial strains are shown in Table III.

In addition to peak heights shown in Table II, percentage values were calculated for each of the major fatty acids present. These percentages could be compared to those derived from the GC chromatograms in Table IV. The major fatty acids that were grouped were lauric (C12:0) #7, myristic (C14:0) #8, palmitic C16:0) #9, palmitoleic (C16:1) #14, stearic (C18:0) #9, oleic (C18:1) #15, arachidic (C20:0) #11, and eicosenoic (C20:0) (shoulder peak of #10). It should be noted that in GC we are looking at all monoenolic acids in a single chain length, while in

H.P.L.C. specific acids are observed.

In evaluating the H.P.L.C. chromatograms, we found evidence of other fatty acids being present which had not been previously reported. In some cases these fatty acids had similar retention times to standards and are labeled in parentheses in Fig. 7-12. As an example, there was a consistent peak in the area of #44, but on hydrogenation this peak was lost. This would indicate that it was not #44.

Another example of an unknown peak with a similar retention time as one of the standards was in the vicinity of #16/#17. This peak is shown in Fig. 7-11 as (16)/(17), and was lost upon hydrogenation (Fig. 12).

This indicated that the peak was an unsaturated fatty acid, and therefore could be either trans Δ^9 elaidic #16, or cis Δ^{11} vaccenic #17. Moss and Lambert [3] reported finding trace amounts of lactobacillic acid, a 19 carbon cyclopropane fatty acid. It has been shown that this acid is formed from cis Δ^{11} vaccenic acid C18:1 [23]. This would suggest that the peak seen is at least in part vaccenic acid. The reason this acid had not been reported in the GC literature is that it would have eluted under the oleic acid peak in GC techniques. The detection of vaccenic acid illustrates the improved separation power of H.P.L.C. over GC. Another important point to emphasize is the detection of vaccenic acid in low concentration. Both Lambert and Moss [3], and Drucker [14] did not report fatty acids in concentration below 1% of the total fatty acids present. Vaccenic acid shown in this study amounts to less than 0.5% of the total, or in terms of palmitic acid #9, from 0.5% to 3%.

There were other peaks which did not correspond to any standard and these were labeled with an asterisk in the figures. For example, #14 possessed a shoulder peak which was not lost during hydrogenation (Fig. 11, 12), suggesting that the shoulder represents a branch chain, hydroxyl or some other unusual fatty acid.

B. GC Chromatograms

The separations of fatty acid methyl ester standards on both the DEGS and SP-2100 columns, showed a marked peak broadening, with C20 being about the upper limit of analysis. In addition, it can be seen in the GC standard curves (Fig. 5) that the elution time between two carbon units increases with chain length, resulting in the C24 on the DEGS eluting at 59 minutes. The increase in elution time in GC on these columns demonstrates that in order to cover the range obtained with H.P.L.C. with respect to carbon chain length a fairly long GC run would be needed. This is not to suggest that there are no GC columns that give good separation of the higher molecular weight fatty acids. However, these columns would not provide the separation ability achieved by those optimized for the C10 to C20 region. Therefore, to obtain the separation achieved in a single H.P.L.C., would require the use of

several GC columns which could be more time consuming than a single H.P.L.C. run.

In Fig. 13, a separation of fatty acids of S. salivarius on both the 10% DEGS-PS and the 3% SP-2100 DOH columns is shown. A representative chromatogram of S. mutans is shown in Fig. 14. Several points about these representative chromatograms should be emphasized. The first is the solvent peak which shows some considerable tailing during isothermal runs on the DEGS column (Fig. 13). The peak tailing causes some difficulty in interpretation of the early peak in that part of the chromatogram. However, the peak tailing can be compensated for by temperature programming, but temperature programming produces other problems at low concentrations. One is column bleed as indicated by the base line rise on the SP-2100 DOH chromatograms (Fig. 13, 14), and an increase in the separation time.

Another difficulty is that without using GC-MS, it is difficult to determine if the small unidentified peaks present in the chromatograms are fatty acids or reaction by-products from the methylation reaction. It has been stated [22, 26] that methylation should produce no structural changes or side products. However, this is difficult to achieve in bacterial fatty acid studies [27]. Comparing this problem to the H.P.L.C. UV tagging reaction, it was observed that most of the reaction by-products elute before C12; however, the possibility of reaction by-products past C12 has not been completely excluded.

For comparison with H.P.L.C., the values of peak areas obtained for the major fatty acids on the GC integrator, and percentages for individual fatty acids in the group are shown in Table IV.

C. Standardization of Technique

Any new technique requires determination of reproducibility. This was evaluated by taking four 2 ml aliquots of the batch of saponified bacterial mixture and carrying through the extraction and tagging procedure. The peak heights of the five major fatty acids, #9, #10, #15, #8, and #14 were determined from their chromatograms and relative error was calculated for each peak (Table IV, Part A). As shown in the table, a mean relative error was 8.19%. In order to ascertain the reproducibility of values obtained from culture to culture grown under identical conditions, three cultures of a bacterial strain were saponified independently. Three 2 ml aliquots from each of the three independently saponified cultures were then prepared for H.P.L.C. A mean peak height value was determined for the five main fatty acids in each culture. The calculated relative error and a mean relative error of 5.51% for the five main peaks are shown in Table IV, Part B.

Sensitivity is an important consideration when examining any new technique, and several approaches can be used to measure it. We choose to express sensitivity on the basis of bacterial mass

needed to produce a good chromatogram. We used 600 mg (wet weight) of bacterial mass to produce 200 ml of solution. For H.P.L.C. determinations, 2 ml of this solution, equivalent to 6 mg (wet weight) of bacterial mass was concentrated to 1 ml and 50 µl (equivalent to 0.3 mg) was injected per chromatogram. The sensitivity of the UV detector would allow this amount to be decreased ten times by reading at 0.005 AUFS or 0.015 mg of bacterial mass (wet weight). For the GC the 100 ml of solution was concentrated to 50 µl of which 1µl (6 mg) was injected into the GC. The attenuation was set at 4X and a range of 10^{-11}, which would allow the bacterial mass wet weight to be decreased to 1.5 mg, which is 100X the amount required in H.P.L.C.

GC has been established as the principal methodology for both quantitative and qualitative determination of fatty acids. On a semiquantitative basis, H.P.L.C. compares well with GC. For example, the largest peak in both GC and H.P.L.C. chromatograms represents the same fatty acid, namely, palmitic acid. This semiquantitative comparison exists for major peaks in both chromatograms.

We explored the quantitative aspects of GC with H.P.L.C. by comparing ratios of the major peaks within a particular chromatogram. The values obtained for the different ratios (Table V) indicate that peak ratios within an H.P.L.C. chromatogram could not be correlated with GC. However, a response factor is necessary for each fatty acid, and this may explain, at least in part, the discrepancy in the ratios [28, 29]. Also, the differences in volatility and solubility of the different acids and the gentleness of the derivation step may all be responsible for the discrepancy in the ratios.

Finally, using hydrogenation as an augmenting technique, it was found that 30 min was sufficient for greater than 95% conversion to the saturated acid. However, in the saponified bacterial mixtures, the percent conversion varied from 50 to 90% when analyzing oleic acid #15 and its conversion to stearic acid #10. Since hydrogenation was used only qualitatively, we did not explore the possible explanations for this variation in hydrogenation.

IV. SUMMARY AND CONCLUSIONS

This report describes the application of H.P.L.C. in the analysis of bacterial fatty acids. The chromatograms which were produced possessed peaks with the same retention times as the fatty acids previously reported. In addition, there were other fatty acid peaks present that were tentatively identified, and others that could not be identified. These new peaks stemmed from the improved separation power of H.P.L.C. over GC. It was also shown that the UV tagging produced 10 to 100 times increase in

sensitivity over FID, allowing the detection of trace peaks. With the increase in sensitivity, a significant number of fatty acids with chain length greater than C20 has been detected, and as a result the usable range of carbon lengths was double that in standard GC. The standard GC columns provide a good working range from C10 through C20 but to extend beyond this range, the column must be changed. However, with H.P.L.C. the working range extends from C10 through C40.

The use of the chemical tagging reaction in bacterial fatty acid analysis provided an alternative method for derivative formation of these compounds. It was observed in the different H.P.L.C. chromatograms that the chemical tagging reaction did not produce spread of the reaction by-products through the chromatograms, as does methylation in GC. However, the UV reaction by-products limited the use of H.P.L.C. to fatty acids above C10:0. It may be concluded therefore, that GC is the preferred methodology for analyzing short chain acids.

The quantitative aspects of H.P.L.C. still require further investigation to achieve the quantitative performance of GC. It was demonstrated that H.P.L.C. does correlate, on a semiquantitative basis with GC, but direct comparison of the H.P.L.C. and GC quantitative data is not possible. This lack of correlation may be attributed to different techniques.

The technique of H.P.L.C. in bacterial fatty acid analysis yielded additional data to information which had already been obtained by GC. H.P.L.C. therefore provides an excellent complementary method to GC in bacterial fatty acid analysis. The combination of both methods opens new frontiers in chemical taxonomic studies. H.P.L.C. satisfies two of the three basic criteria of an ideal chemicotaxonomic method. Finally, H.P.L.C. offers a possible means of more rapid identification of bacteria.

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 thos of the U. S. Army Medical Department.

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TABLE I

Fatty Acid Standards

Standard Number	Fatty Acid		Standard Number	Fatty Acid	
1.	Acetic	2:0	26.	Nonanoic	9:0
2.	Propionic	3:0	27.	Undecanoic	11:0
3.	Butyric	4:0	28.	Tridecanoic	13:0
4.	Caproic	6:0	29.	Pentadecanoic	15:0
5.	Caprylic	8:0	30.	Heptadecanoic	17:0
6.	Capric	10:0	31.	Nonadecanoic	19:0
7.	Lauric	12:0	32.	Heneicosanoic	21:0
8.	Myristic	14:0	33.	Tricosanoic	23:0
9.	Palmitic	16:0	34.	Myristoleic (cis-9)	14:1
10.	Steric	18:0	35.	Palmitelaidic (trans-9)	16:1
11.	Arachidic	20:0	36.	Linoledaidic	18:2
12.	Behenic	22:0	37.	Cis-5-Eicosenoic	20:1
13.	Lignoceric	24:0	38.	Erucic (cis-13)	22:1
14.	Palmitoleic	16:1	39.	11, 14, 17-Eicosatrienoic	20:3
15.	Oleic (cis-9)	18:1	40.	Brassidic (trans-13)	22:1
16.	Elaidic (trans-9)	18:1	41.	4, 7, 10, 13, 16, 19 Docosahexaenoic	22:6
17.	Vaccenic (cis-11)	18:1			
18.	Linoleic	18:2	42.	5, 8, 11, 14, 17 Eicosapentaenoic	20:5
19.	α - Linolenic	18:3			
20.	Nervonic (cis-15)	24:1	43.	Pentacoseinoic Acid	25:0
21.	Arachidonic	20:4	44.	12-Methyltetradecanoic (Antiso)	15:0
22.	γ - Linolenic	18:3	45.	Hydroxystearic Acid	18:0
23.	Lactic	5:0	46.	Dihydrosterulic Acid	19:0
24.	Valeric	5:0	47.	Hexacosanoic Acid	26:0
25.	Heptanoic	7:0	48.	Octacosanoic Acid	28:0
			49.	Cis-11 Eicosenoic Acid	20:1

TABLE II

QUANTITATION OF FATTY ACIDS IN H.P.L.C.

Part A: Major Fatty Acids (Peak Heights in mm)

Species Strain		#7 Lauric (12:0)	#8 Myristic (14:0)	#9 Palmitic (16:0)	#14/#35 Hexadecenoic (16:1)	#10 Stearic (18:0)	#15/(16)/(17) Octodecenoic (18:1)	#11 Arachidic (20:0)	† Eicosenoic (20:1)
Salivarius	PH	18	31	238	18/10	58	115/3	6	38
13419	%	3.4	5.8	44.5	5.2	10.8	22.0	1.1	7.1
Salivarius	PH	24	39	196	17/6	41	61/1	4	20
55262	%	5.9	9.5	48.2	5.6	10.0	15.2	1.0	4.9
Salivarius	PH	45	79	386	30/6	79	115/2	15	33
9222	%	5.7	10.0	48.9	4.6	10.0	14.8	1.9	4.2
Salivarius	PH	62	71	245	27/13	95	223/15	3	10
9758	%	8.1	9.3	32.1	5.2	12.4	31.2	0.4	1.3
Mutans	PH	5	19	74	4/6	16	25/0	1	5
27352	%	3.2	12.2	47.8	6.4	10.3	16.1	0.6	3.2
Mutans	PH	34	37	235	9/12	55	36/1	6	16
25175	%	7.7	8.4	53.3	4.8	12.5	8.4	1.4	3.6
Sanguis	PH	108	90	290	37/32	94	330/9	3	0
10556	%	10.9	9.1	29.3	7.0	9.5	34.2	0.3	0
Sanguis	PH	18	30	145	20/10	55	147/2	1	0
10557	%	4.2	7.0	33.9	7.0	12.8	34.8	0.2	0
Mitis	PH	57	57	218	23/7	72	125/3	3	0
9811	%	10.1	10.1	38.6	5.3	12.7	22.6	0.5	0

† Shoulder Peak off of #10

TABLE II, CONTINUED

QUANTITATION OF FATTY ACIDS IN H.P.L.C.

Part B: Minor Fatty Acids (Peak Heights in mm)

Species Strain		29 (15:0)	12 (22:0)	13 (24:0)	32 (21:0)	33 (23:0	49 (26:0)	48 (28:0)
Salivarius 13419	PH	10	11	6	1	2	4	5
Salivarius 55262	PH	6	10	4	1	1	1	1
Salivarius 9222	PH	16	4	3	<1	<1	1	1
Salivarius 9758	PH	13	3	4	<1	<1	<1	<1
Mutans 27352	PH	1	4	2	<1	<1	<1	<1
Mutans 25171	PH	15	13	7	<1	<1	<1	<1
Sanguis 10556	PH	32	2	6	<1	<1	1	<1
Sanguis 10557	PH	5	1	1	<1	<1	<1	<1
Mitis 9811	PH	13	3	4	<1	<1	<1	<1

TABLE III

MAJOR CELLULAR FATTY ACIDS IN G.C.

Culture #		Lauric (12:0)	Myristic (14:0)	Palmitic (16:0)	Hexadeconoic (16:1)	Stearic (18:0)	Octodecenoic (18:1)	Arachidic (20:0)	Eicosenoic (20:1)
Salivarius	Area	11,066	21,964	224,072	36,544	124,668	202,836	59,256	128,928
13419	%	1.4	2.7	27.7	4.5	15.4	25.1	7.3	15.9
Salivarius	Area	2,824	26,786	150,932	23,256	19,728	76,940	6,356	14,628
55262	%	0.9	8.3	47.0	7.2	6.1	24.8	2.0	4.6
Mutans	Area	1.616	35,396	134,552	40,080	87,984	122,256	35,424	88,080
27352	%	0.3	6.5	24.7	7.3	16.1	22.4	6.5	16.1
Salivarius	Area	54,810	75,060	307,450	30,504	74,262	145,040	30,976	63,440
9222	%	7.0	9.6	39.3	3.9	9.5	18.6	4.0	8.1
Salivarius	Area	65,882	105,272	268,112	64,340	93,344	131,136	32,504	24,408
9758	%	8.3	13.4	34.2	8.2	11.9	16.7	4.1	3.1

TABLE IV

REPRODUCIBILITY OF H.P.L.C. METHODOLOGY

A. Reproducibility From Aliquot to Aliquote (Peak Height in mm)

Aliquote #	9 Palmitic	10 Steric	15 Oleic	8 Myristic	14 Palmitoleic	
1	127	39	34	24	9	
2	118	38	32	23	8	
3	127	48	35	24	9	
4	137	53	38	26	9	
Mean	125.75	44.50	34.75	24.25	8.75	
± σ	8.38	7.23	2.50	1.25	0.50	
± Standard error	4.19	3.61	1.25	0.62	0.25	
% Relative error	6.66	16.24	7.20	5.15	5.71	Mean Relative Error 8.19%

B. Reproducibility From Culture to Culture (Peak Height in mm)

Culture #	9 Palmitic	10 Steric	15 Oleic	8 Myristic	14 Palmitoleic	
1	125	41	34	26	9	
2	120	44	39	23	8	
3	126	45	35	24	9	
Mean	123.66	43.33	36.00	24.33	8.66	
± σ	3.21	2.08	2.64	1.52	0.57	
± Standard error	1.85	1.20	1.52	0.88	0.33	
% Relative error	2.60	4.80	7.33	6.25	6.58	Mean Relative Error 5.51%

TABLE V

A COMPARISON OF PEAK RATIOS

H.P.L.C.:GC

SPECIES STRAIN	RATIO 16:0/18:0 H.P.L.C.:GC	RATIO 18:1/18:0 H.P.L.C.:GC	RATIO 7/8 H.P.L.C.:GC
Salivarius 13419	4.12 : 1.80	2.04 : 1.62	0.59 : 0.52
Salivarius 55262	4.82 : 7.70	1.56 : 4.06	0.62 : 0.11
Mutans 27352	4.64 : 1.53	1.56 : 1.39	0.26 : 0.05
Salivarius 9222	4.89 : 4.14	1.48 : 1.96	0.57 : 0.73
Salivarius 9758	2.58 : 2.87	2.50 : 1.40	0.87 : 0.62

FIGURE LEGENDS

Figure 1. A schematic representation of the bacteriological steps in the isolation and identification of oral streptococci used in this study along with the time required.

Figure 2. Colony morphology of 24 hr cultures of the different bacterial species used in the study on blood agar. a. S. mitis b. S. salivarius c. S. sanguis d. S. mutans

Figure 3. Scanning electron micrographs of the bacteria used in the study at 15,000X. a. S. salivarius b. S. mitis c. S. sanguis d. S. mutans. These pictures are courtesy of COL John M. Brady, U. S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, DC

Figure 4. UV Tagging Reaction

Figure 5. H.P.L.C. chromatogram of fatty acid standards, with the key to standards in Table I.

Figure 6. a. GC chromatogram of a fatty acid methyl ester mixture on 10% DEGS-PS column performed isothermally at 195°C.
b. GC chromatogram of Supelco bacterial fatty acid standard mixture (Cat #4-5436) and a C20:1 added to the mixture. The separation was performed on a 3% SP-2100 DOH.

Figure 7. H.P.L.C. chromatogram of a S. salivarius. Asterisks indicate fatty acid peaks which did not correspond to any standard runs. Parentheses indicate fatty acid peaks corresponding to standards, but insufficient data establish them as that particular fatty acid.

Figure 8. H.P.L.C. chromatogram of a S. mutans. Asterisks indicate fatty acid peaks which did not correspond to any standard run. Parentheses indicate fatty acid peaks corresponding to standards, but insufficient data to establish their identity. Alpha indicates a peak which we suspect as lactobacillic acid which is similar to dihydrosterulic acid #46, differing from any in the position of the cyclopropane ring.

Figure 9. H.P.L.C. chromatogram of a S. sanguis. Asterisks indicate fatty acid peaks which did not correspond to any standard run. Parentheses indicate fatty acid peaks corresponding to standards, but insufficient data to establish their identity. Large broad peaks under #10 is contaminate which appears inconsistently in the bacterial fatty acid mixtures.

Figure 10. H.P.L.C. chromatogram of a S. Mitis. Asterisks indicate fatty acid peaks which did not correspond to any standard run. Parentheses indicate fatty acid peaks corresponding to standards, but insufficient data to establish their identity. Alpha indicates a peak which we suspect as lactobacillic acid which is similar to dihydrosterulic acid #46, differing only in the position of the cyclopropane ring.

Figure 11. H.P.L.C. chromatogram of a second S. salivarius, before hydrogenation

Figure 12. H.P.L.C. chromatogram of S. salivarius in Fig. 11 after hydrogenation. This sample was hydrogenated before extraction. Approximately 50% conversion was achieved.

Figure 13. a. GC chromatogram of S. salivarius on 10% DEGS column.
b. GC chromatogram of same S. salivarius on 3% SP-2100 DOH.

Figure 14. GC chromatogram of S. mutans on 3% SP-2100 DOH column.

ISOLATION AND IDENTIFICATION PROCEDURE FOR "VIRIDANS" STREPTOCOCCI

Collect specimen aseptically

↓

Streak on blood agar and incubate 24 hr in a candle jar

↓

Pick all alpha and gamma hemolytic colonies that grow on the blood arar, gram stain, and streak all gram (+) catalase (-) cocci on Mitis-Salivarius agar and incubate for 24 hrs.

↓

Observe the Mitis-Salivarius agar to check the purity of each isolate and grow each pure isolate 18 hrs in Todd-Hewitt broth for antigen entraction and subsequent serotyping.

↓

Record all group A, B, D, N, and Q serotypes as non "viridans" streptococci.

↓

Subject the remaining serotypes of streptococci to the following biochemical tests: incubate 24 hrs.

mannitol	arginine
lactose	esculin
hippurate	raffinase
inulin	litmus milk

↓

Additional biochemical tests may be required.

↓

Analysis of data accumulated

Figure 1.

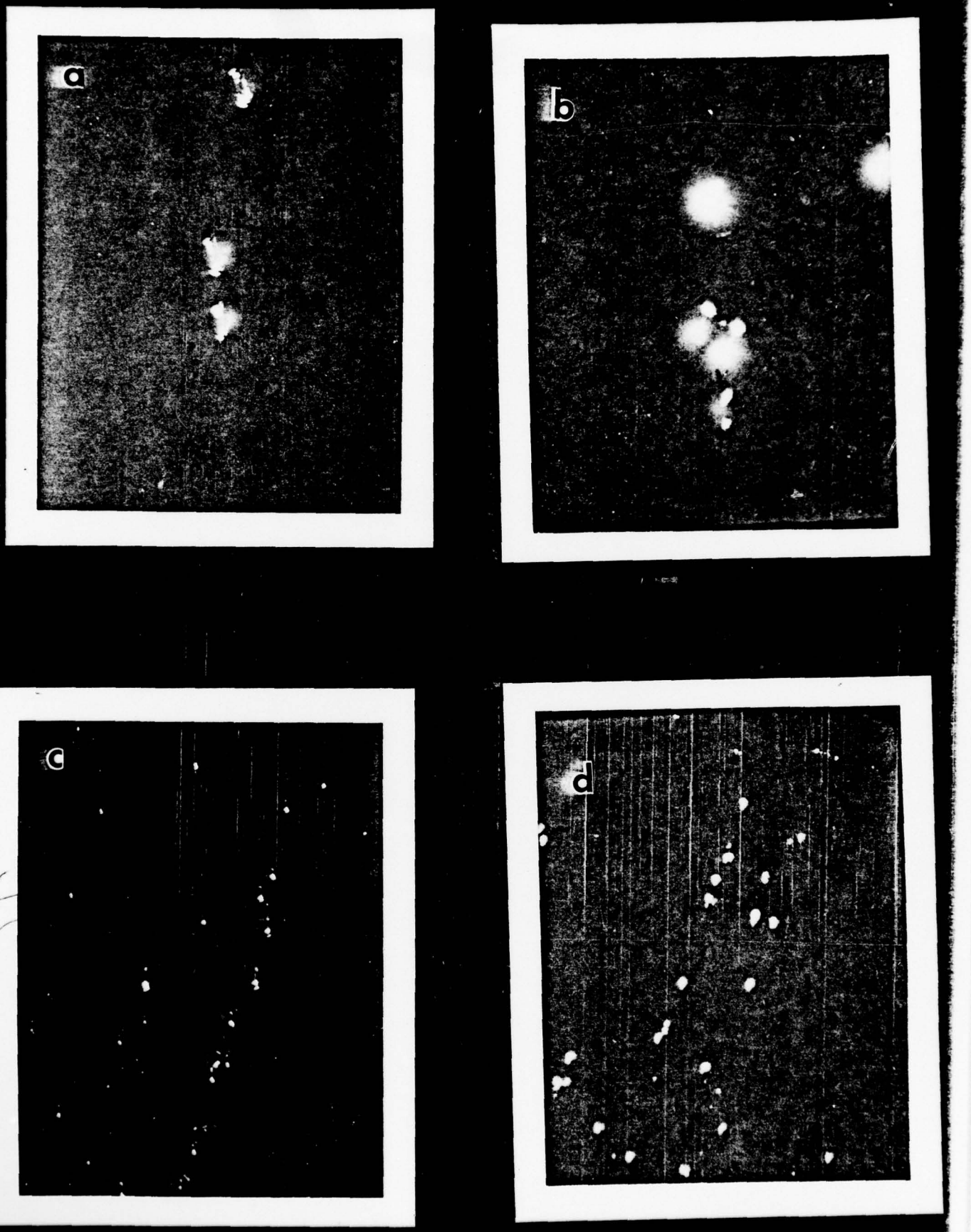


Figure 2.

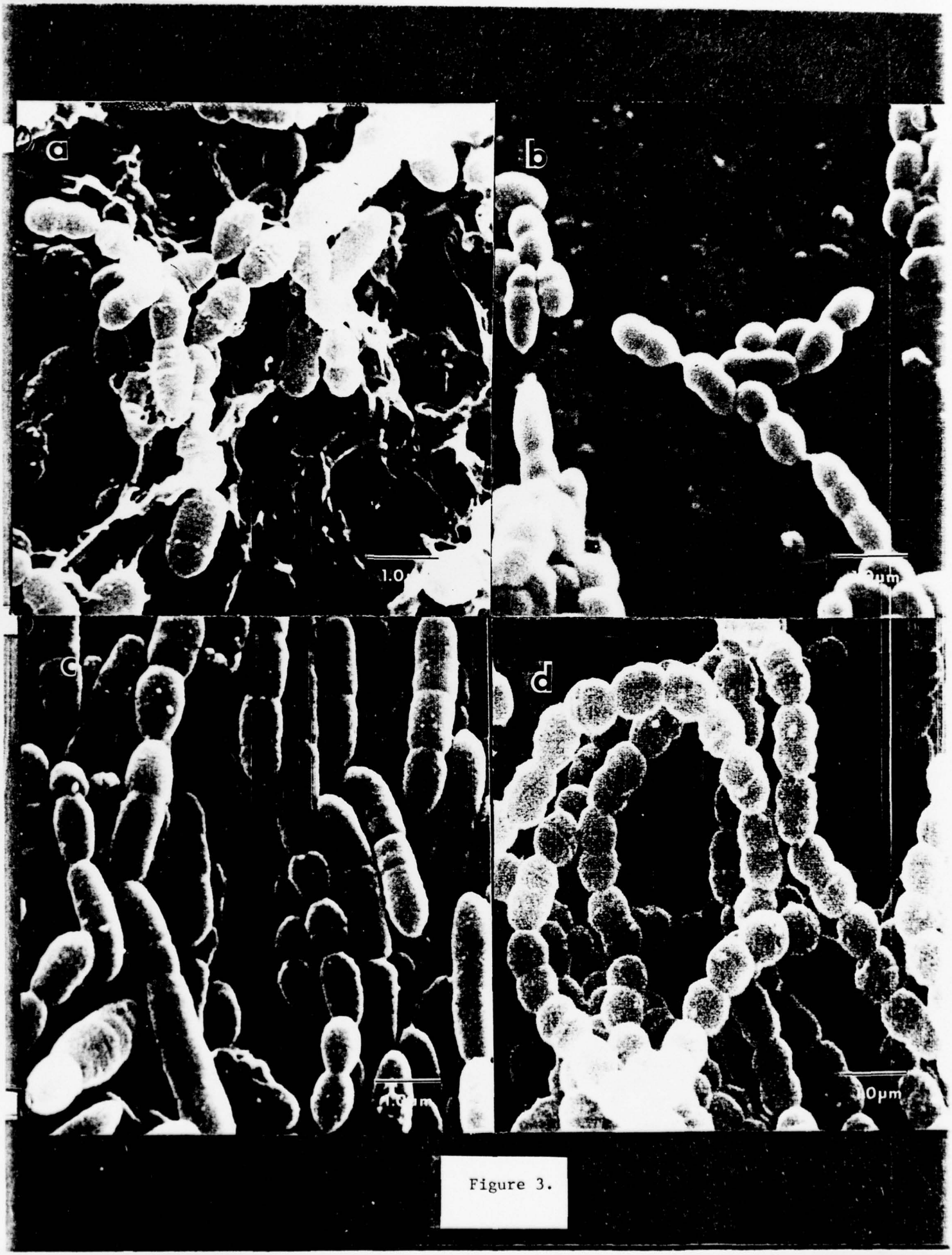


Figure 3.

TAGGING REACTION

DMF = DIMETHYL FORMAMIDE

DIEA = N,N-DIISOPROPYLETHYLAMINE-CATALYST

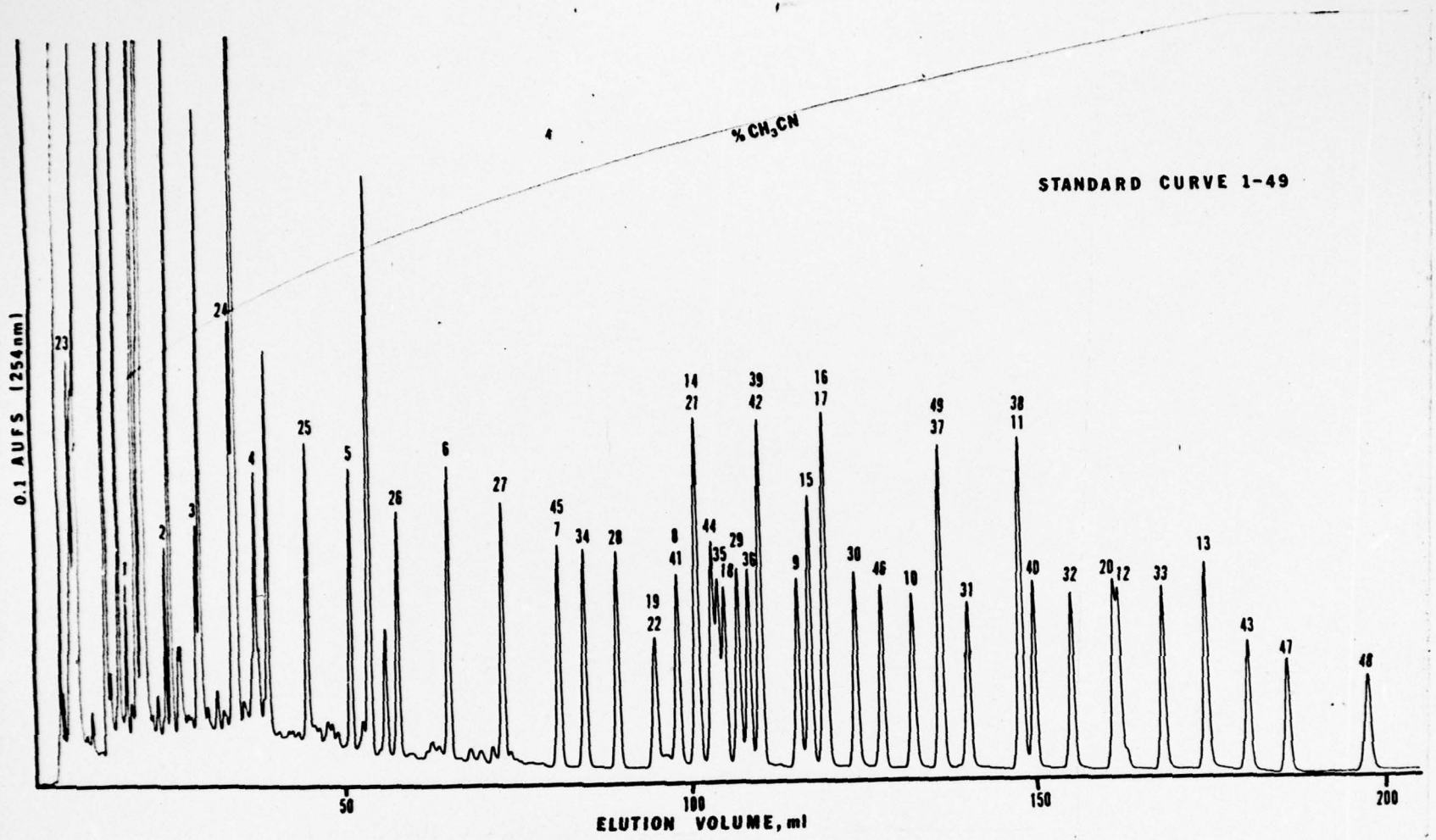
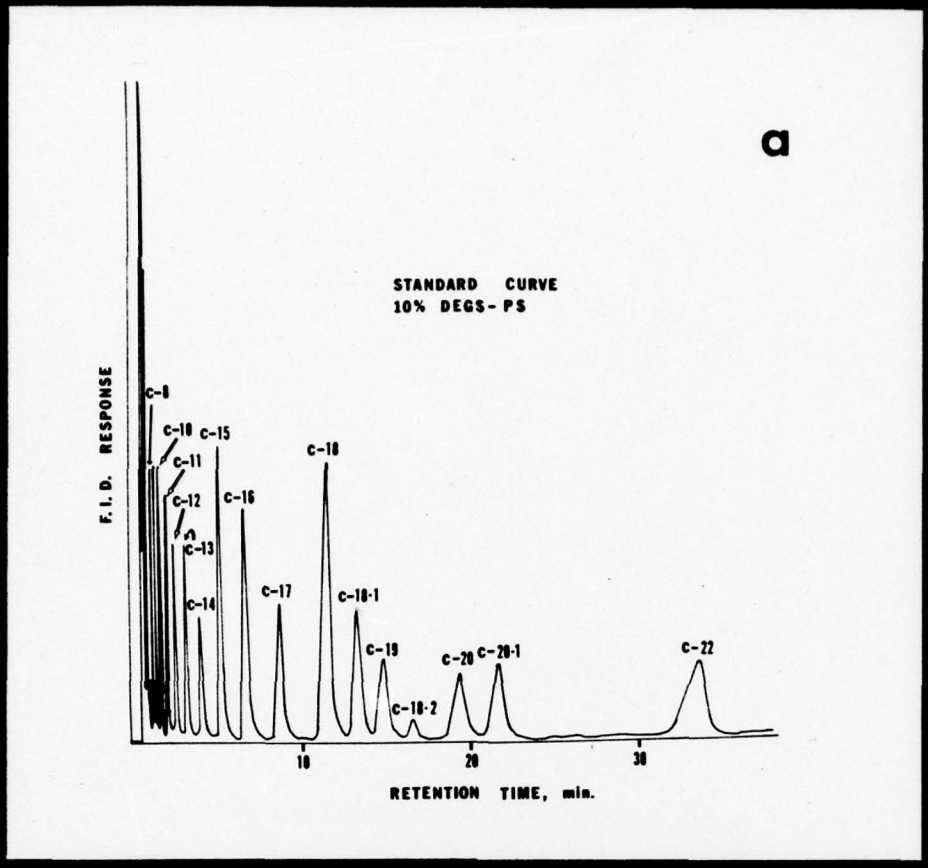

STANDARD CURVE 1-49
% CH_3CN
0.1 AUFS (254nm)
ELUTION VOLUME, ml
50
100
150
200
23
24
1
2
3
4
25
5
26
6
27
45
7
34
28
19
22
8
41
14
21
44
35
18
29
36
39
42
9
15
16
17
30
46
10
49
37
31
38
11
40
32
20
12
33
13
43
47
48

Figure 5.


a
STANDARD CURVE
10% DEGS-PS
F.I.D. RESPONSE
C-8
C-10
C-15
C-11
C-12
C-16
C-13
C-18
C-14
C-17
C-18-1
C-19
C-20
C-20-1
C-18-2
C-22
10
20
30
RETENTION TIME, min.

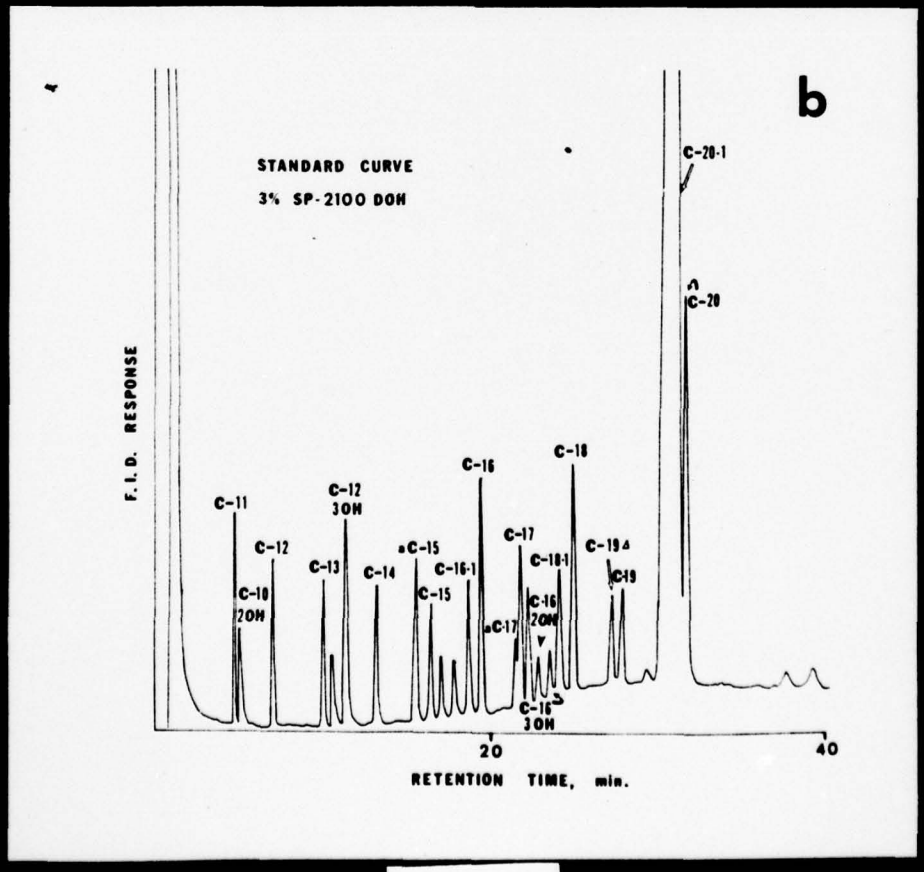

b
STANDARD CURVE
3% SP-2100 DOH
F.I.D. RESPONSE
C-20-1
C-20
C-11
C-10 2OH
C-12
C-12 3OH
C-13
C-14
aC-15
C-15
C-16-1
C-16
aC-17
C-17
C-18
C-18-1
C-16 2OH
C-16 3OH
C-19
C-19
20
40
RETENTION TIME, min.

Figure 6.

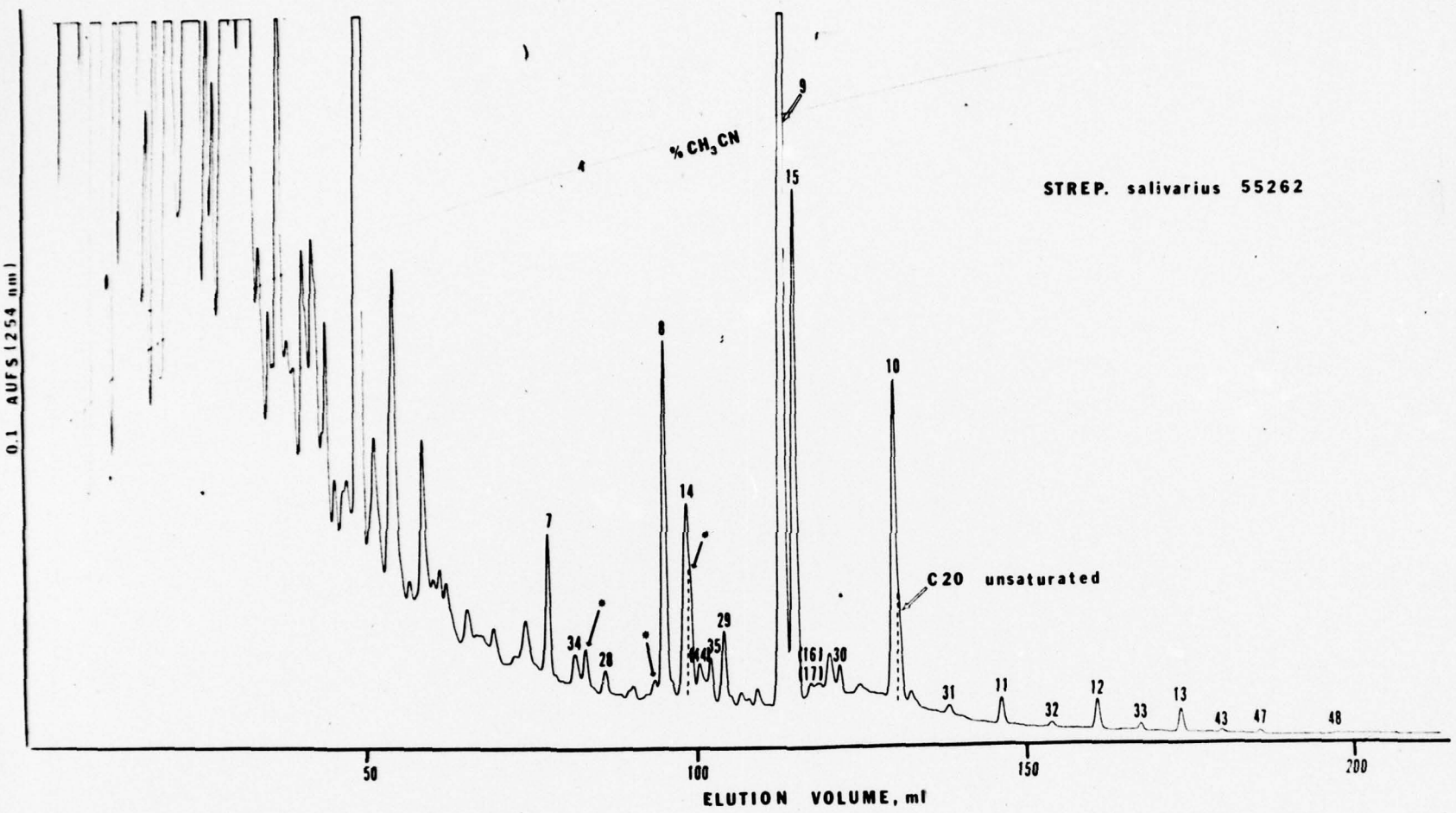

STREP. salivarius 55262
% CH_3CN
0.1 AUFS (254 nm)
C 20 unsaturated
ELUTION VOLUME, ml
50
100
150
200
9
15
8
10
14
7
34
28
29
35
(16)
(17)
30
31
11
32
12
33
13
43
47
48

Figure 7.

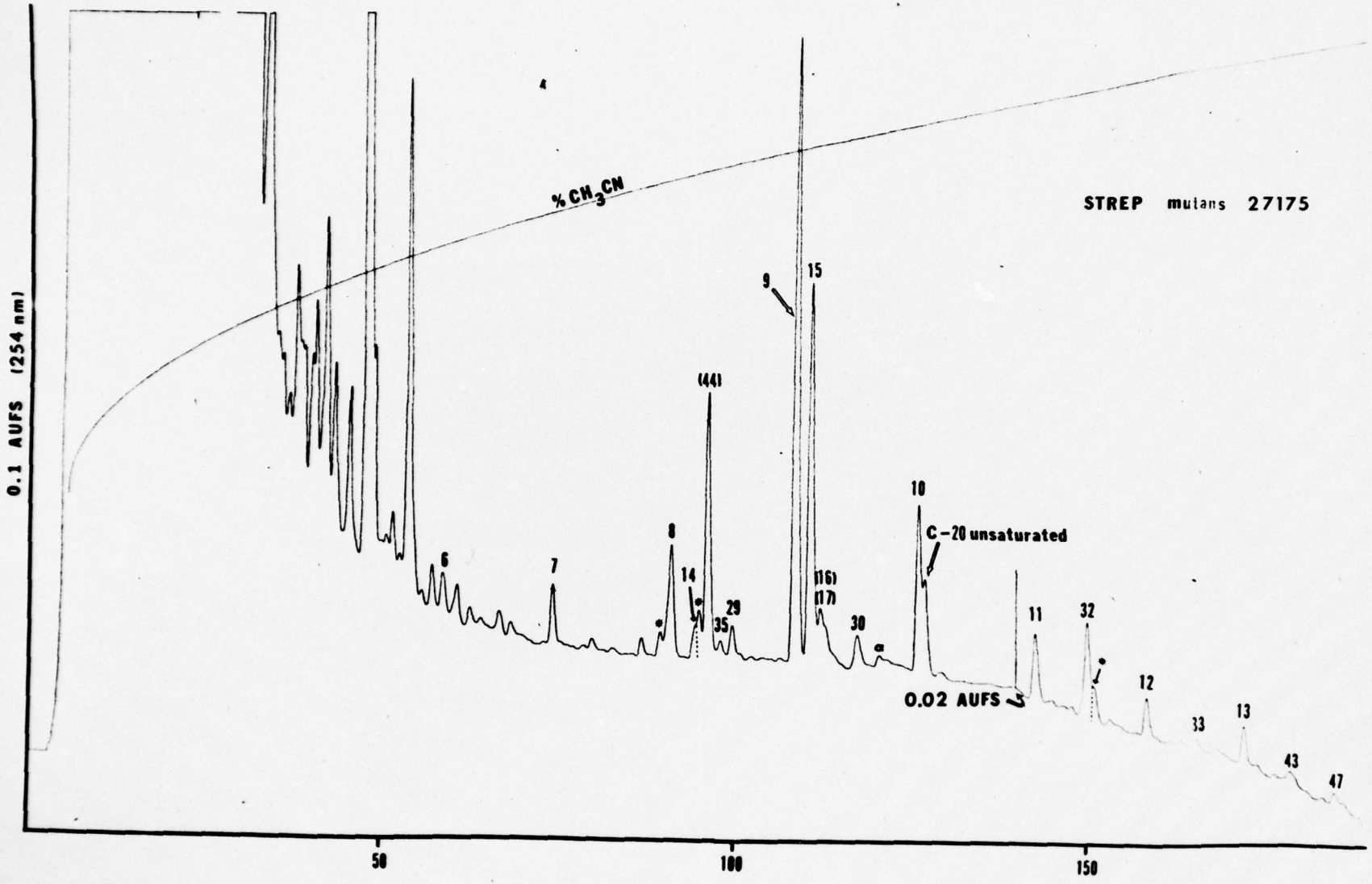

0.1 AUFS (254 nm)
% CH3CN
STREP mutans 27175
9
15
(4)(4)
10
C-20 unsaturated
8
6
7
14
29
35
(16)
(17)
30
11
32
0.02 AUFS
12
33
13
43
47
50
100
150

Figure 8.

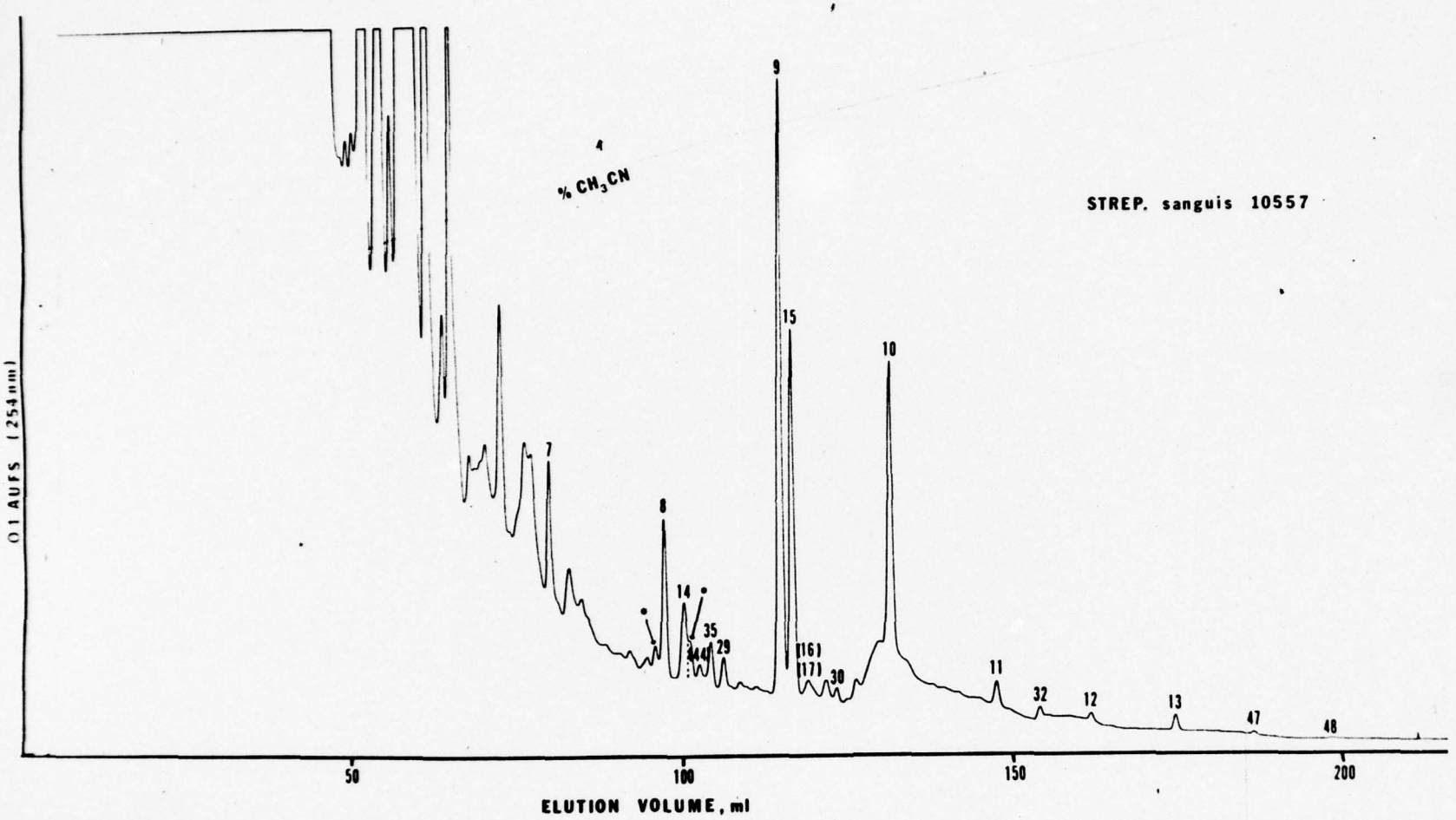

STREP. sanguis 10557
% CH_3CN
0.1 AUFS (254nm)
ELUTION VOLUME, ml
50
100
150
200
9
15
10
8
7
14
35
29
11
32
12
13
47
48
30

Figure 9.

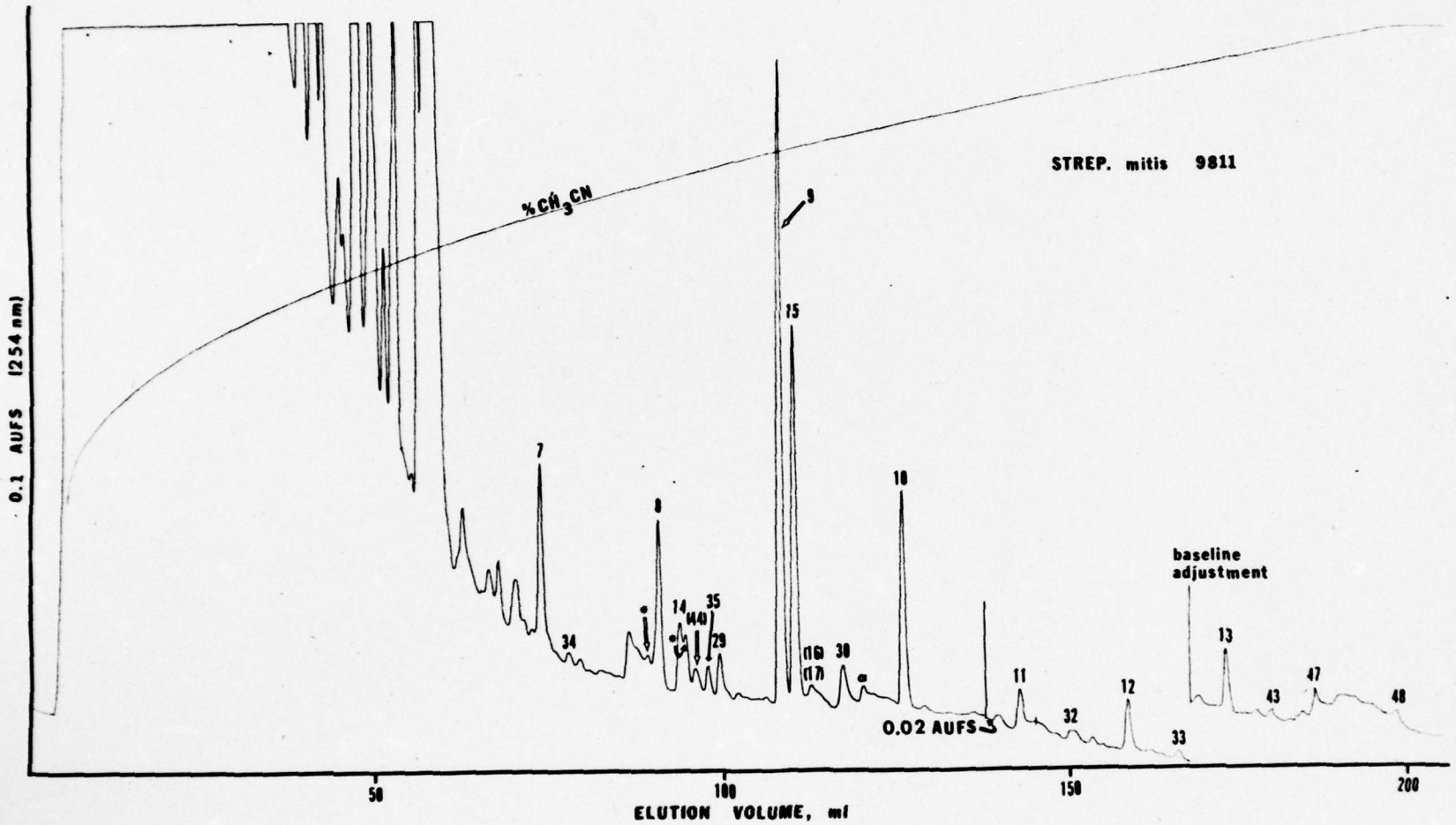

STREP. mitis 9811
% CH3CN
0.1 AUFS (254 nm)
0.02 AUFS
baseline adjustment
ELUTION VOLUME, ml
50
100
150
200
9
15
7
8
18
34
14
35
29
30
11
32
12
33
13
43
47
48

Figure 10

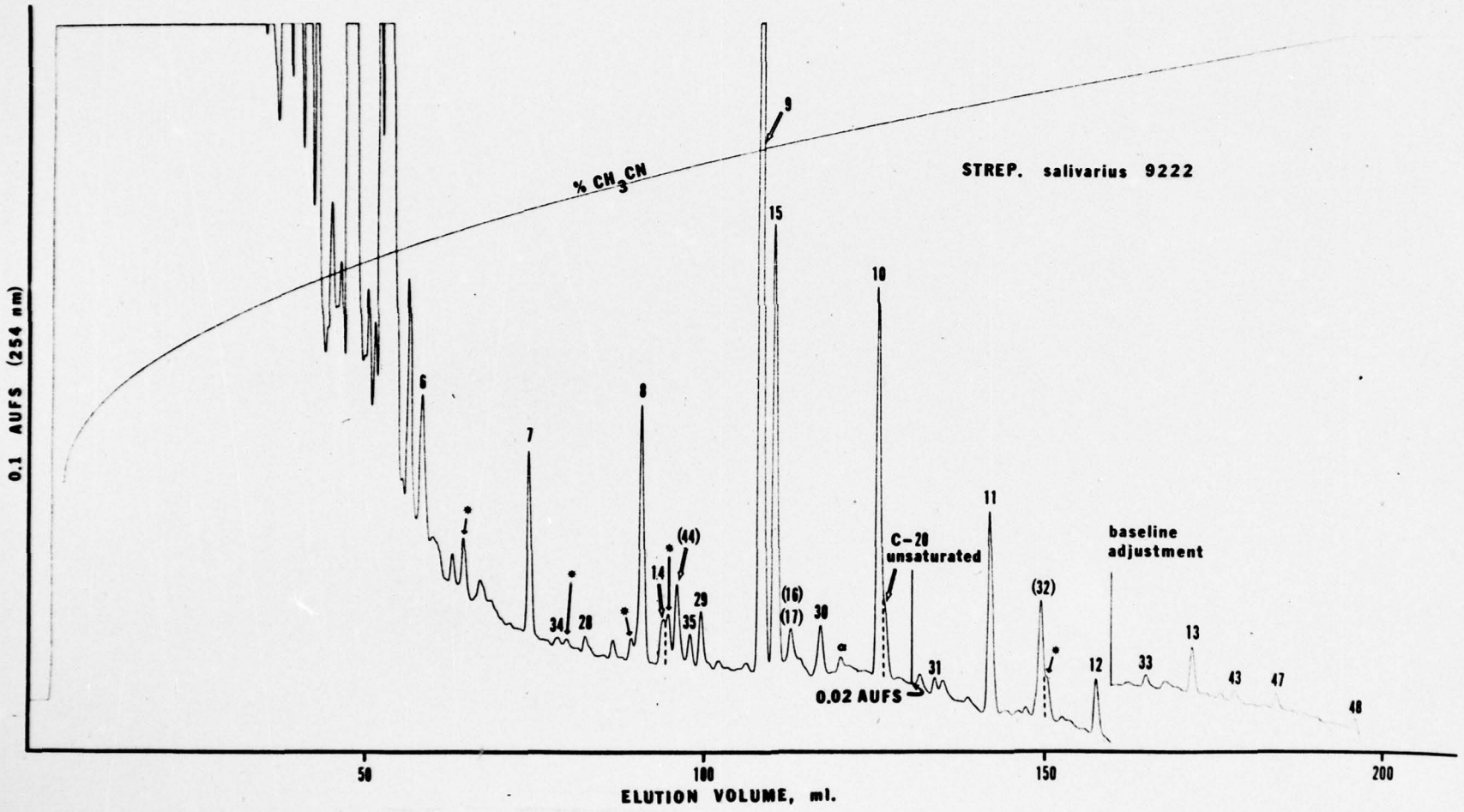

STREP. salivarius 9222
% CH3CN
0.1 AUFS (254 nm)
ELUTION VOLUME, ml.
50
100
150
200
0.02 AUFS
C-20 unsaturated
baseline adjustment
6
7
8
9
10
11
12
13
14
15
16
17
28
29
30
31
32
33
34
35
43
44
47
48

Figure 11.

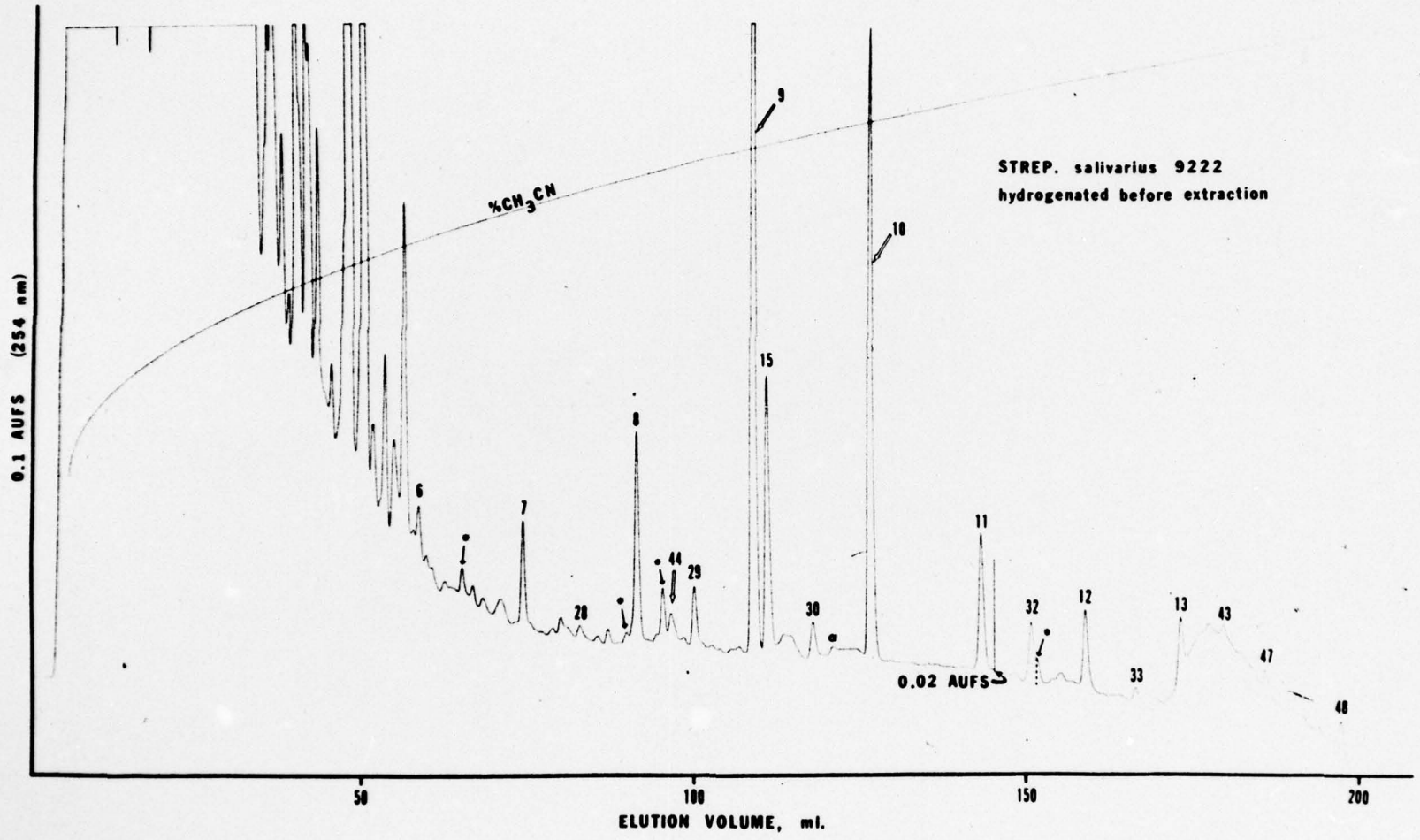
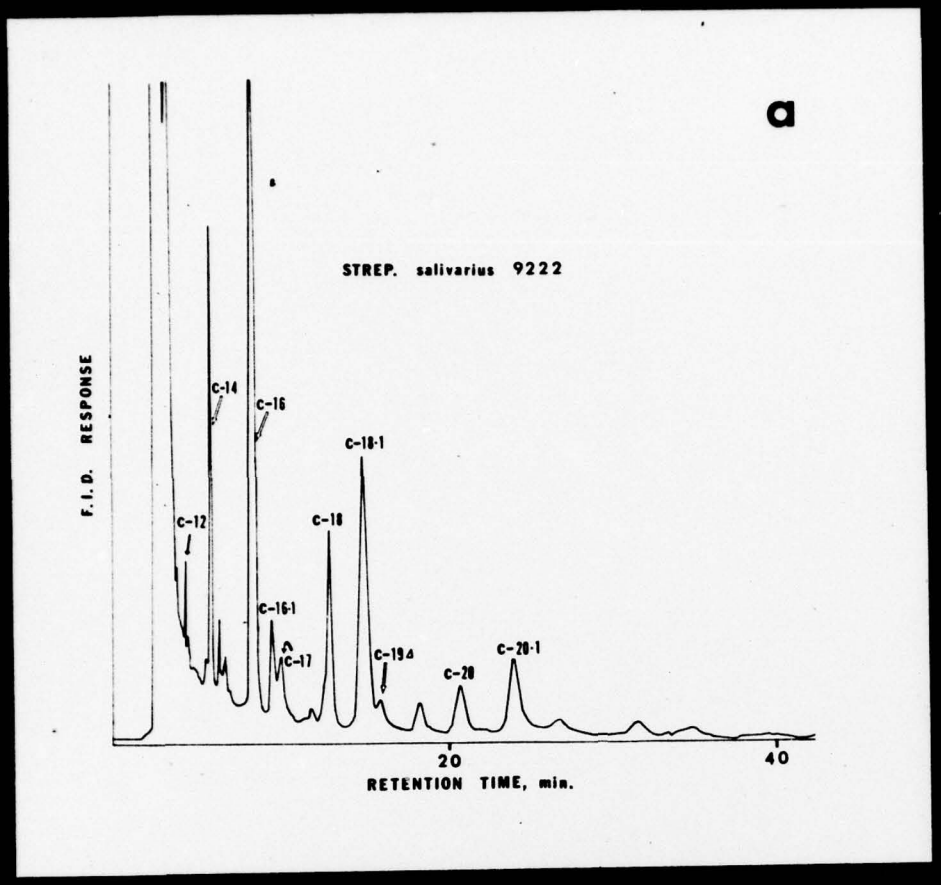

STREP. salivarius 9222
hydrogenated before extraction
%CH3CN
0.1 AUFS (254 nm)
0.02 AUFS
ELUTION VOLUME, ml.
50
100
150
200
6
7
8
9
10
11
12
13
15
28
29
30
32
33
43
44
47
48

Figure 12.


a
STREP. salivarius 9222
F.I.D. RESPONSE
c-14
c-16
c-18:1
c-12
c-18
c-16:1
c-17
c-19:Δ
c-20
c-20:1
20
40
RETENTION TIME, min.

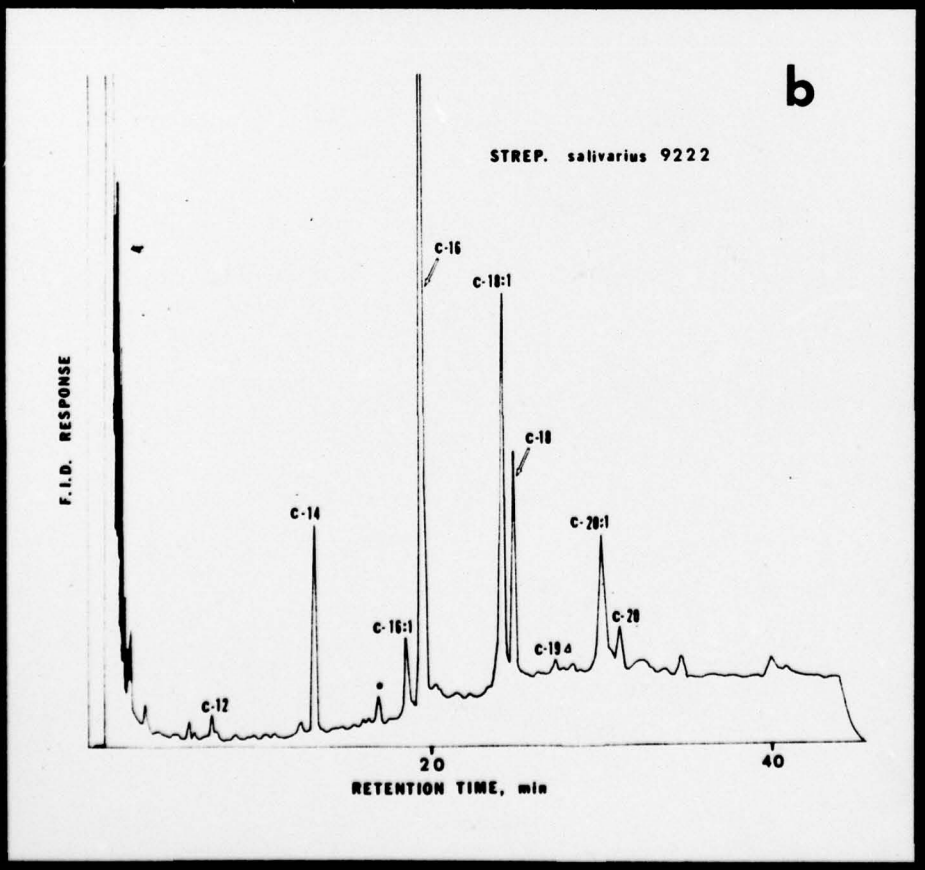
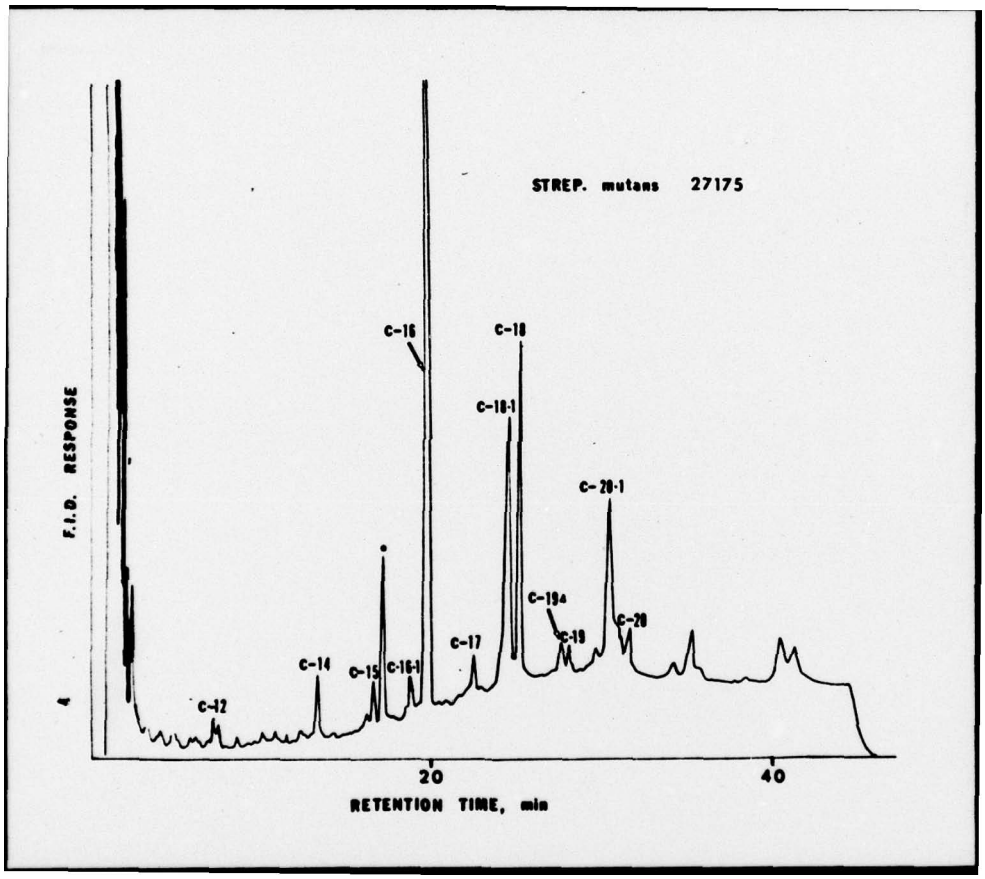
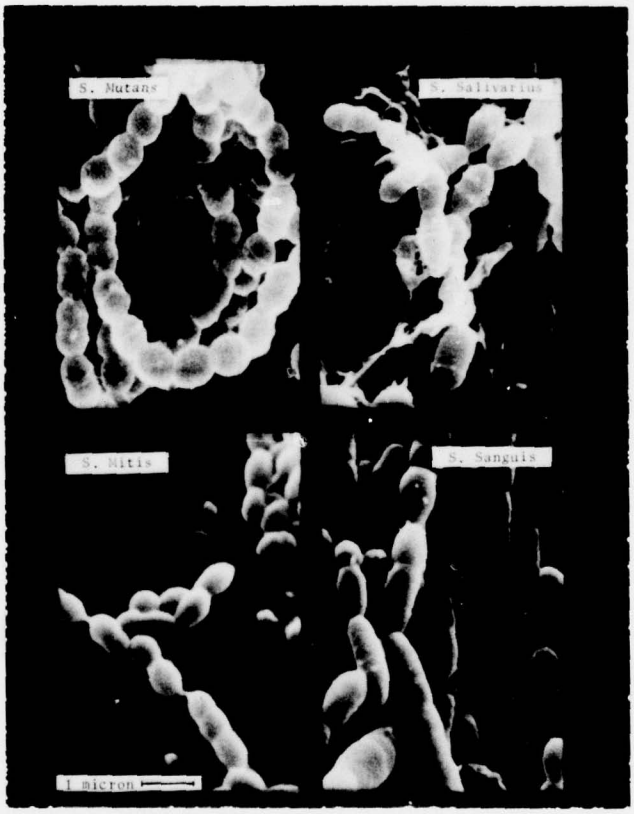

b
STREP. salivarius 9222
F.I.D. RESPONSE
c-16
c-18:1
c-18
c-14
c-20:1
c-16:1
c-20
c-19:Δ
c-12
20
40
RETENTION TIME, min

Figure 13.

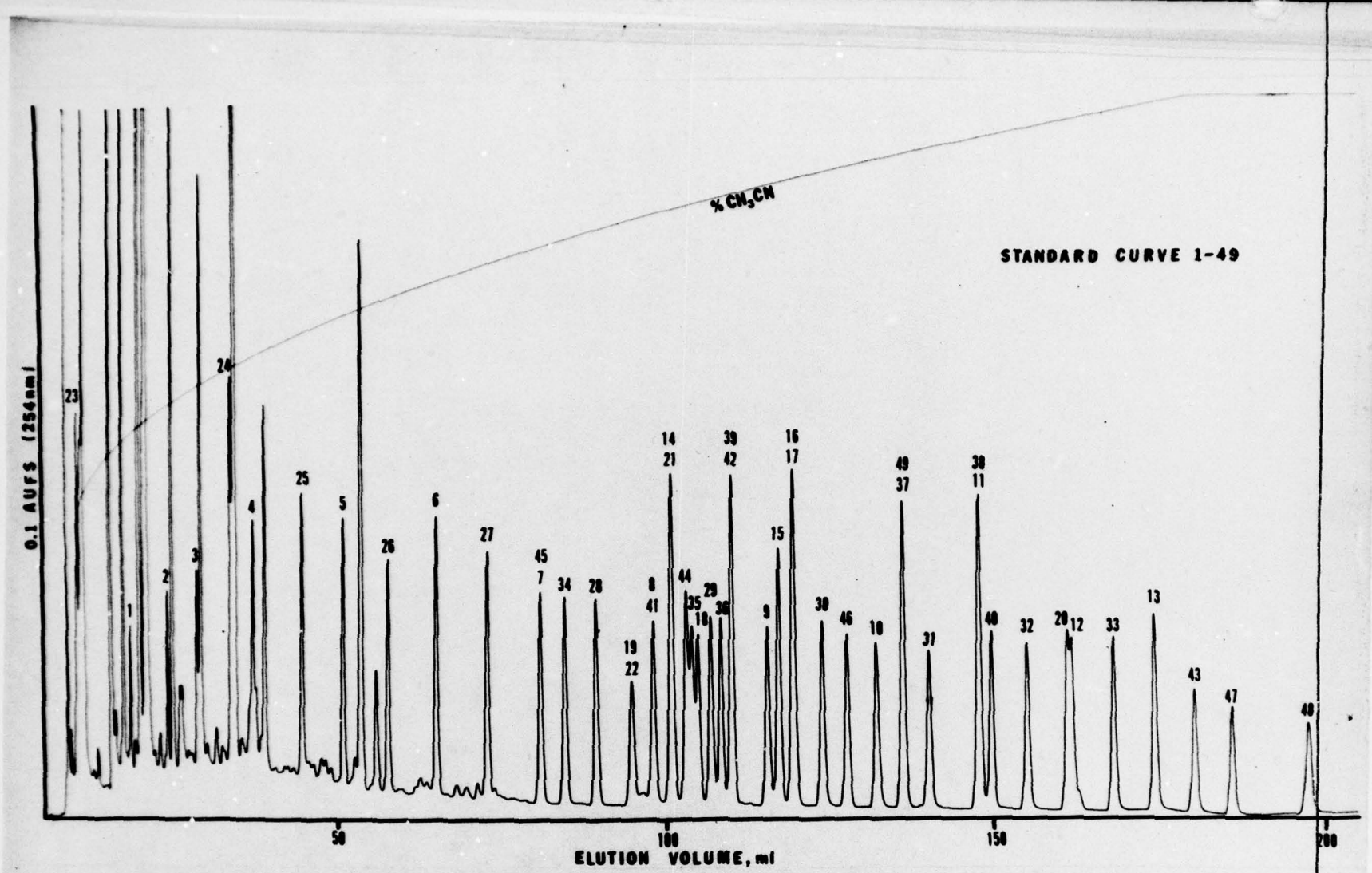

STREP. mutans 27175
C-16
C-18
C-18-1
C-20-1
C-19a
C-19
C-20
C-17
C-14
C-15
C-16-1
C-12
F.I.D. RESPONSE
20
40
RETENTION TIME, min


S. Mutans
S. Salivarius
S. Mitis
S. Sanguis
1 Micron

TAGGING REACTION

DMF = DIMETHYL FORMAMIDE

DIEA = N,N-DIISOPROPYLETHYLAMINE-CATALYST


STANDARD CURVE 1-49
% CH3CN
0.1 AUFS (254nm)
ELUTION VOLUME, ml
50
100
150
200
23
24
1
2
3
4
25
5
26
6
27
45
7
34
28
19
22
8
41
14
21
44
35
18
29
36
39
42
9
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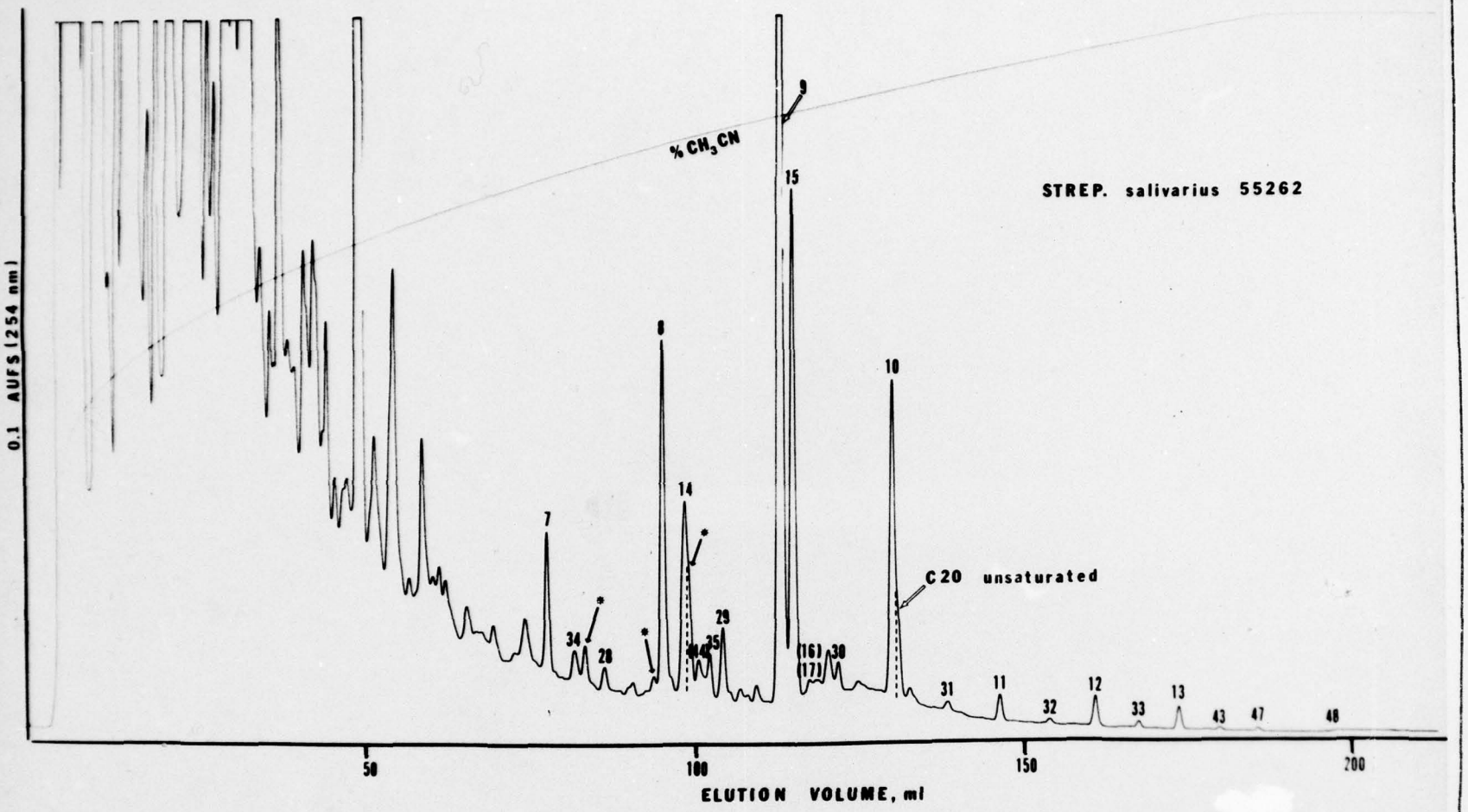
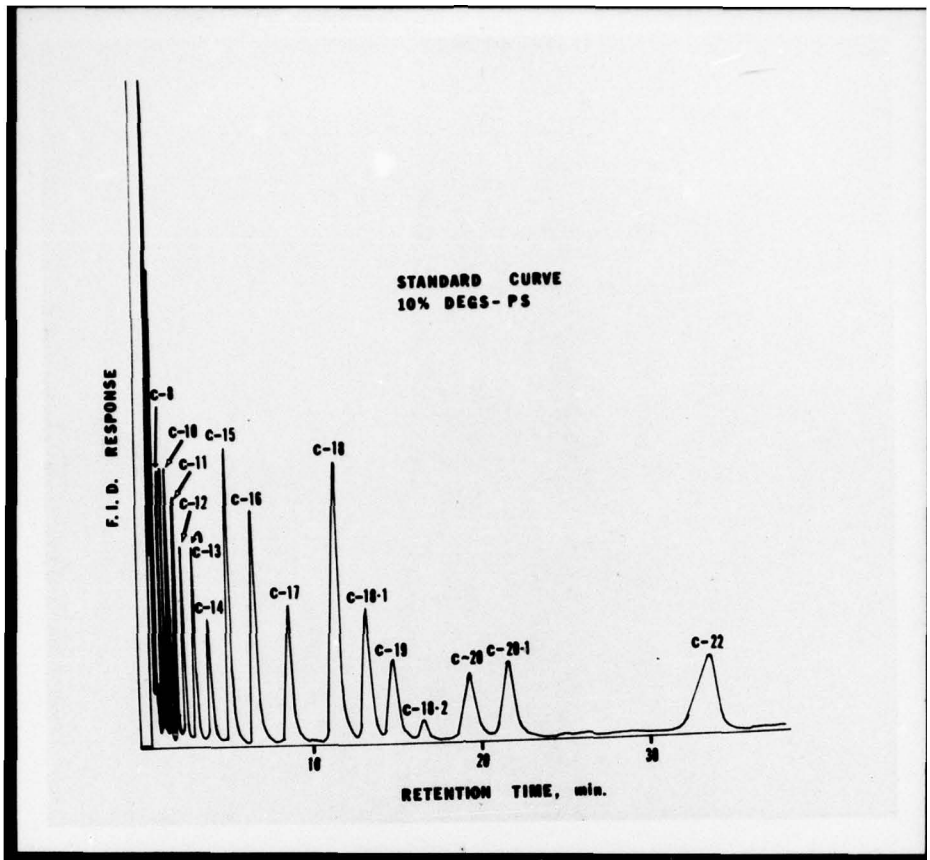
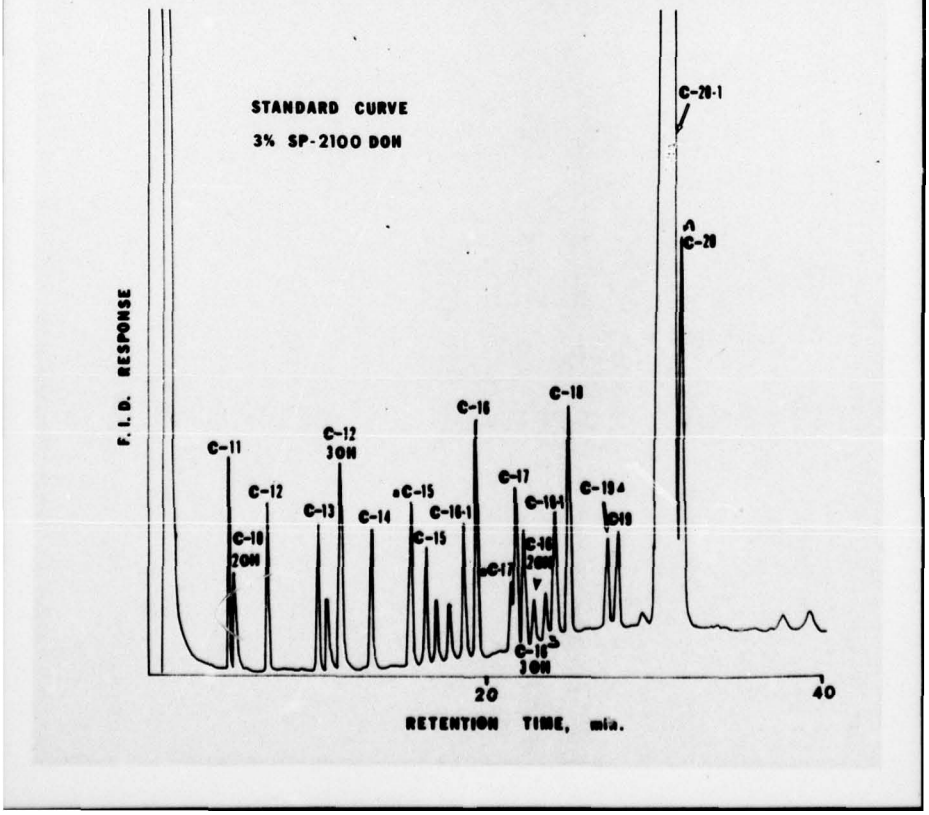
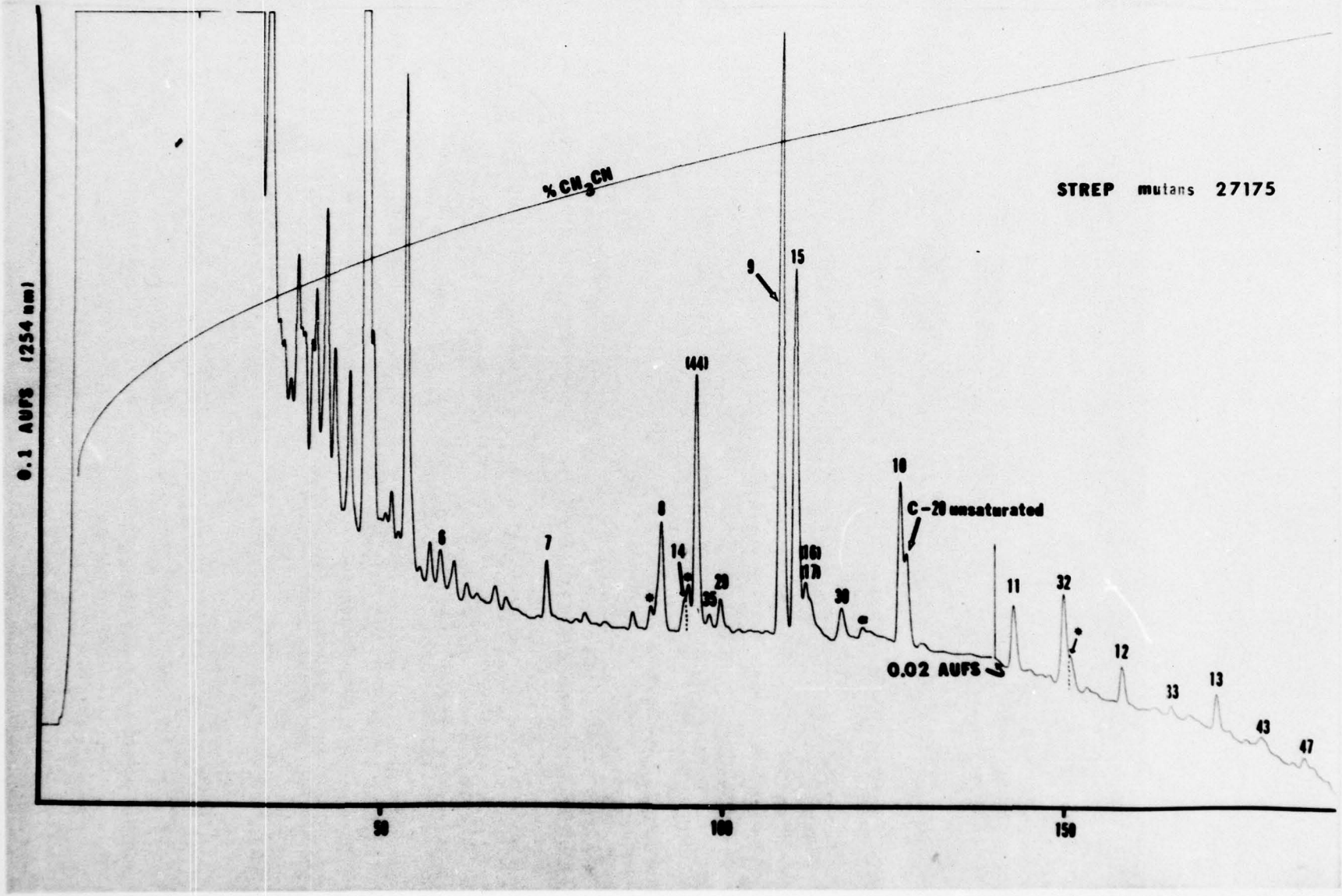
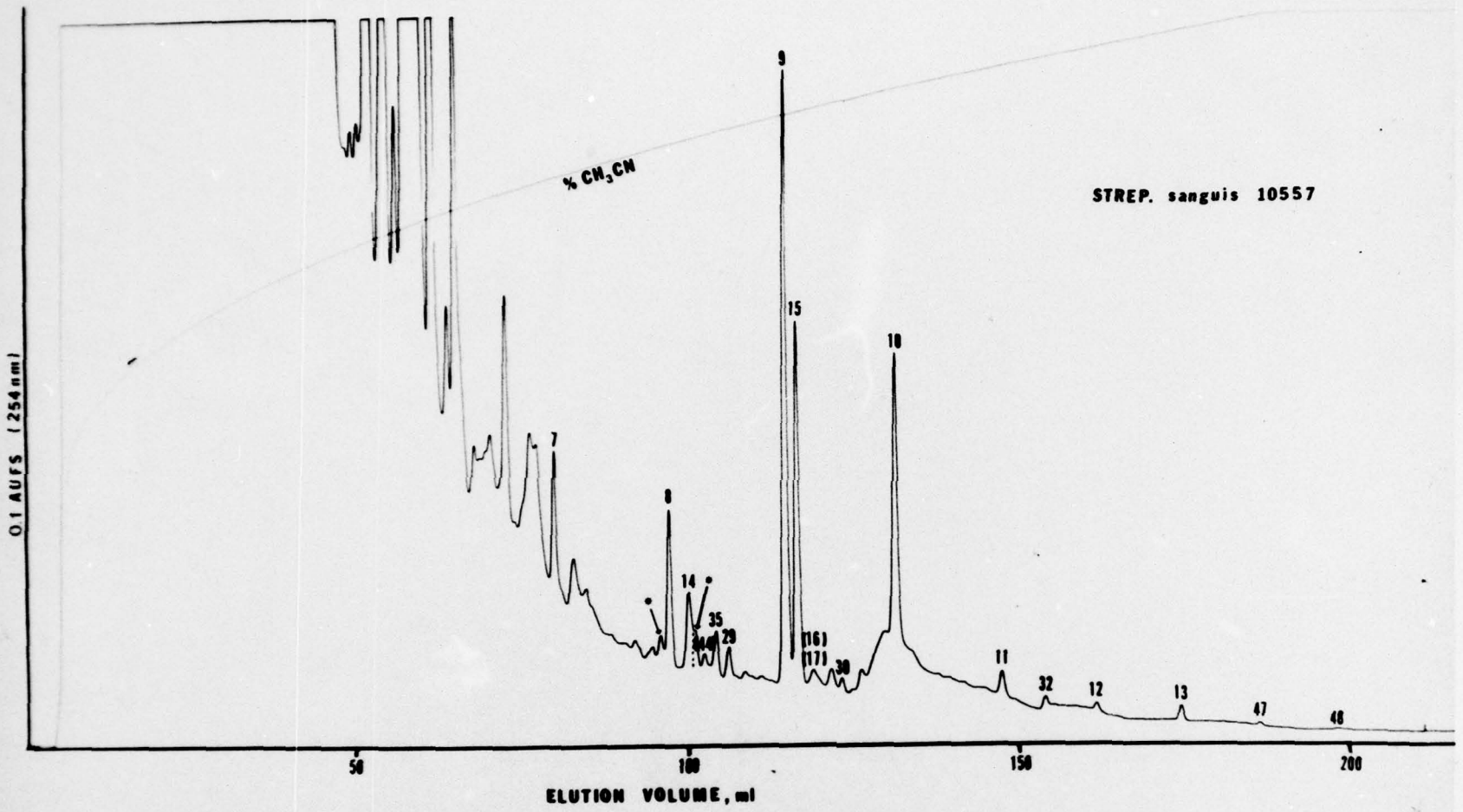
11
40
32
20
12
33
13
43
47
48

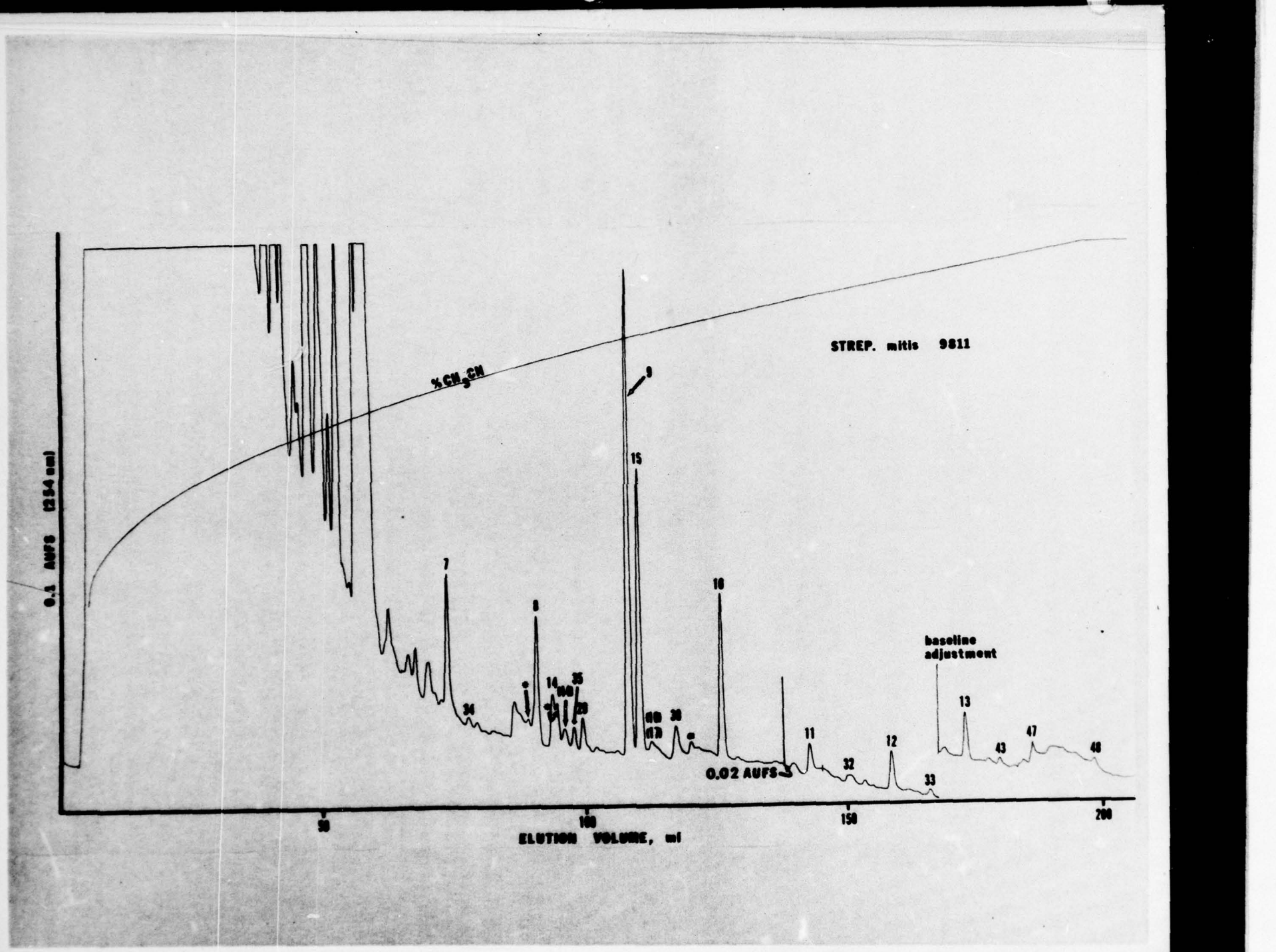

STREP. salivarius 55262
% CH_3CN
0.1 AUFS (254 nm)
9
15
8
10
14
7
C 20 unsaturated
29
34
28
25
(16)
(17)
30
31
11
32
12
33
13
43
47
48
50
100
150
200
ELUTION VOLUME, ml

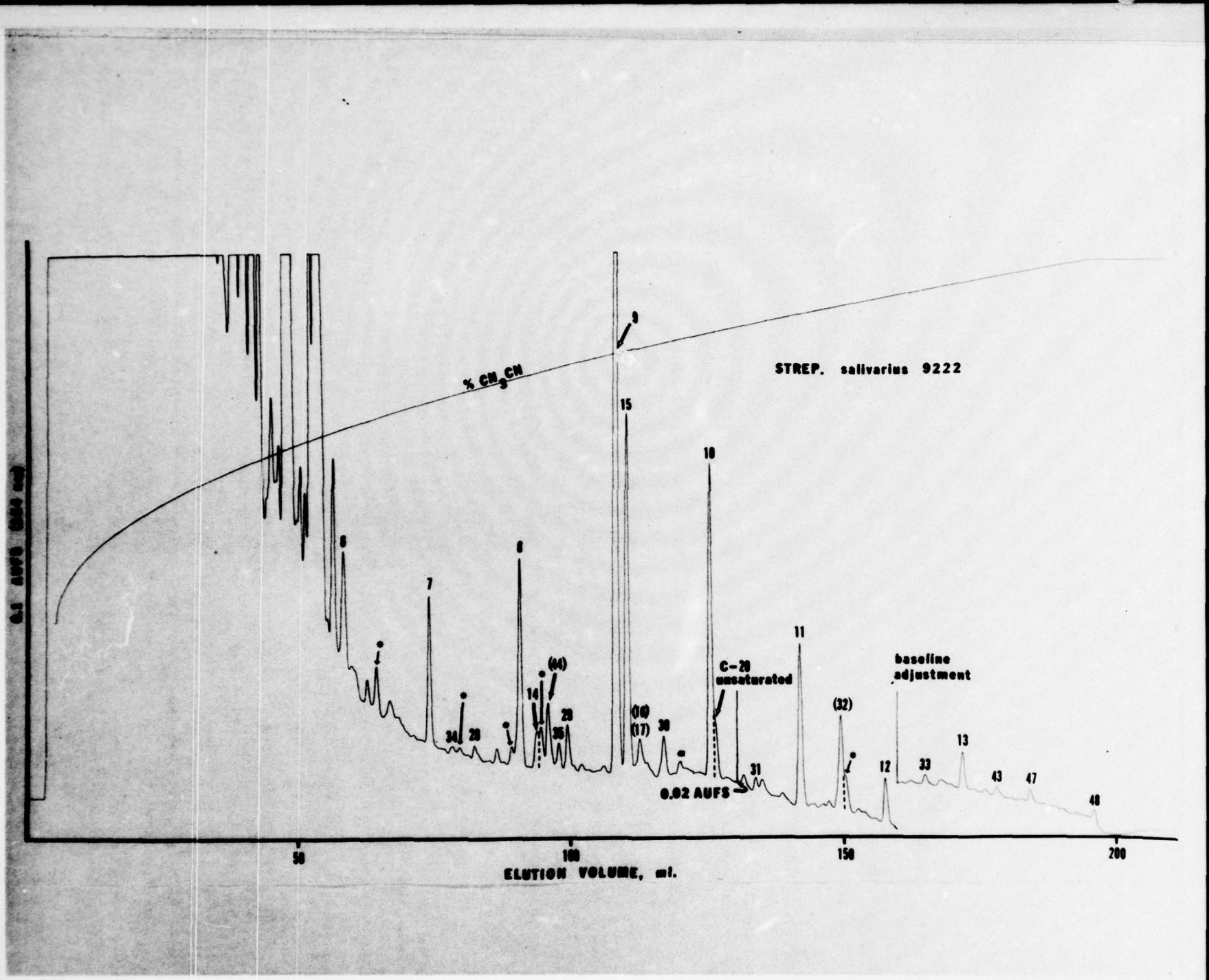

STANDARD CURVE
10% DEGS-PS
F.I.D. RESPONSE
C-8
C-10
C-11
C-12
C-13
C-14
C-15
C-16
C-17
C-18
C-18-1
C-19
C-18-2
C-20
C-20-1
C-22
10
20
30
RETENTION TIME, min.

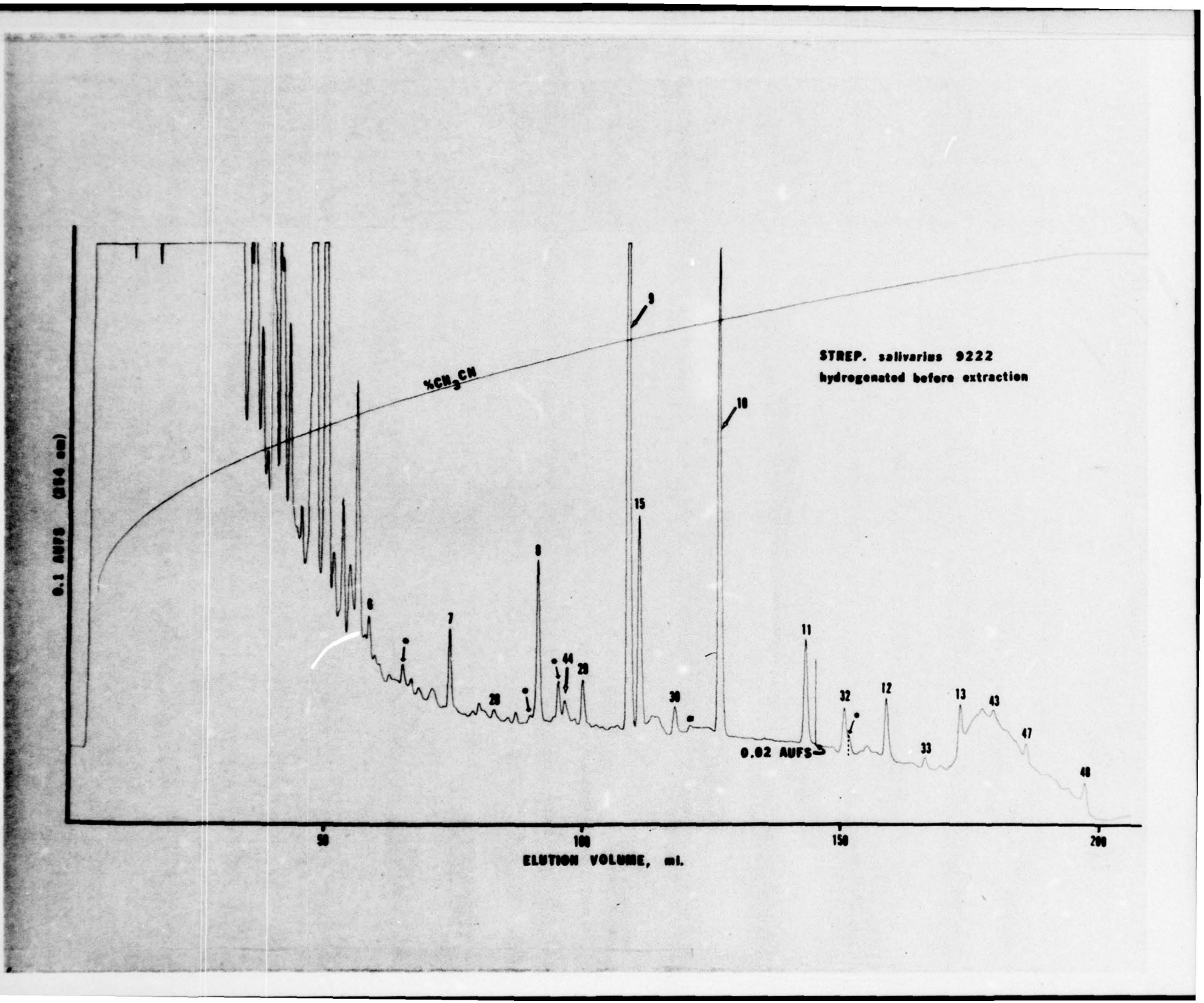

STANDARD CURVE
3% SP-2100 DON
F.I.D. RESPONSE
C-20-1
C-20
C-11
C-12
C-18 2ON
C-12 3ON
C-13
C-14
C-15
C-16
C-15
C-16-1
C-17
C-17
C-18
C-18-1
C-16 2ON
C-16 3ON
C-19
C-19
20
40
RETENTION TIME, min.

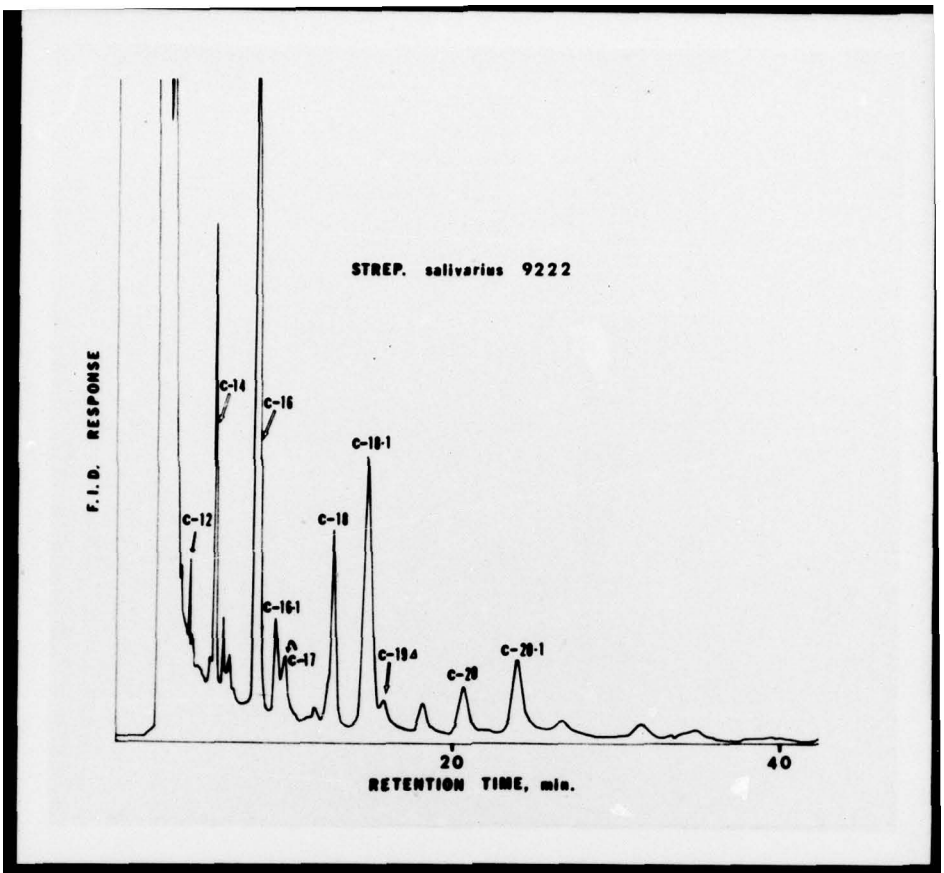

STREP mutans 27175
0.1 AUFS (254 nm)
% CH3CN
9
15
(44)
8
6
7
14
20
35
(16)
(17)
30
10
C-20 unsaturated
11
32
12
33
13
43
47
0.02 AUFS
90
100
150

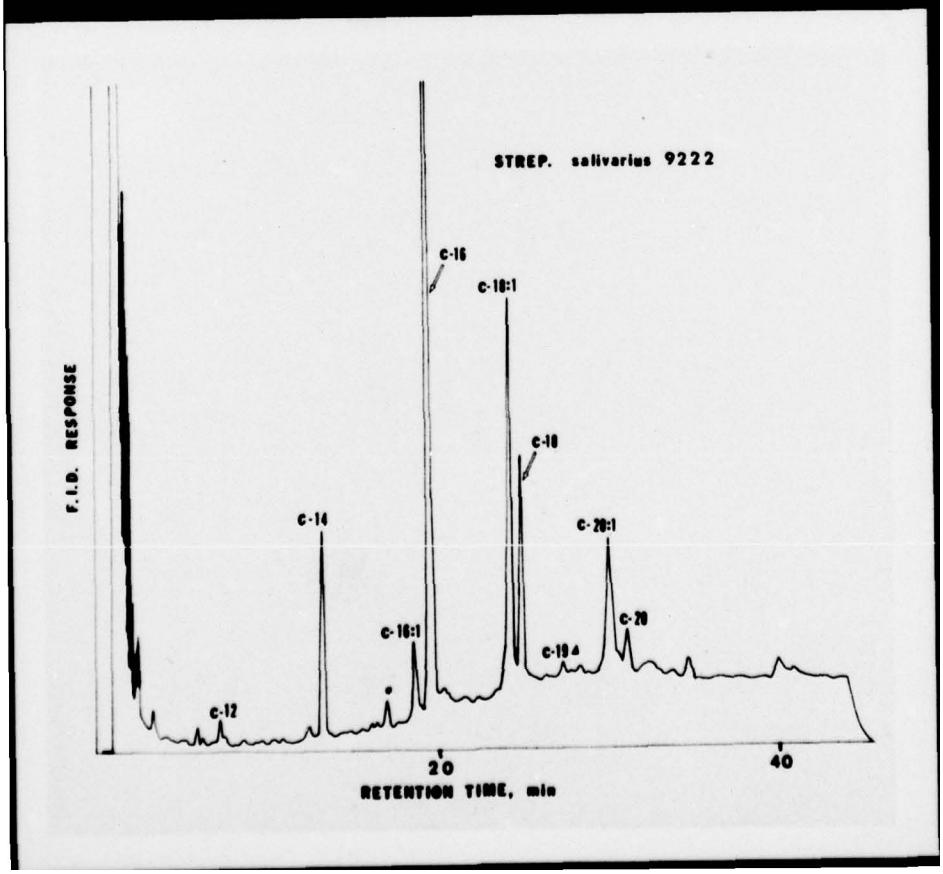

% CH_3CN
STREP. sanguis 10557
0.1 AUFS (254 nm)
9
15
10
7
8
14
35
29
(16)
(17)
30
11
32
12
13
47
48
50
100
150
200
ELUTION VOLUME, ml

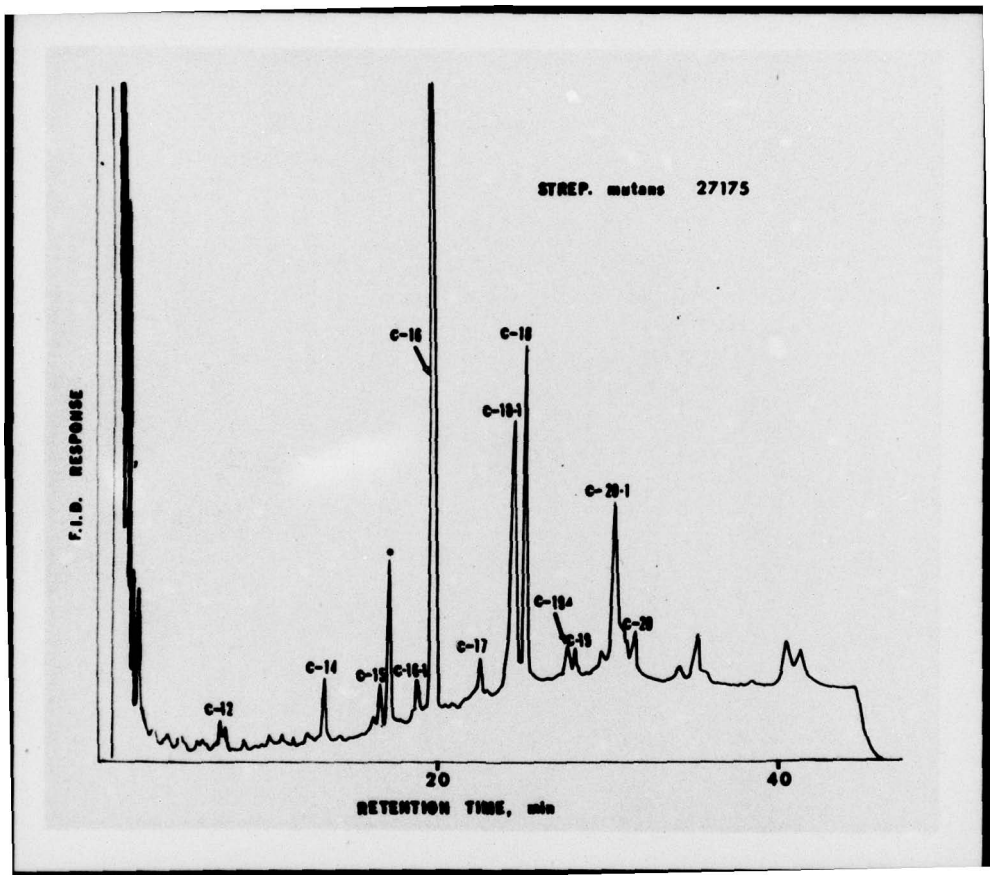

STREP. mitis 9811
%CH3CN
0.1 AUFS (254 nm)
0.02 AUFS
baseline adjustment
ELUTION VOLUME, ml
50
100
150
200
9
15
7
8
16
11
12
13
32
33
43
47
48
34
35
36


STREP. salivarius 9222
% CH3CN
9
15
10
6
7
8
11
(32)
baseline adjustment
C-20 unsaturated
(64)
14
(10)
(17)
30
34
28
35
29
31
12
33
13
43
47
48
0.02 AUFS
50
100
150
200
ELUTION VOLUME, ml.


STREP. salivarius 9222
hydrogenated before extraction
%CH3CN
0.1 AUFS (254 nm)
0.02 AUFS
ELUTION VOLUME, ml.
50
100
150
200
6
7
8
9
10
11
12
13
15
20
28
30
32
33
43
44
47
48


STREP. salivarius 9222
F.I.D. RESPONSE
c-14
c-16
c-18:1
c-12
c-18
c-16:1
c-17
c-19Δ
c-20
c-20:1
20
40
RETENTION TIME, min.


STREP. salivarius 9222
F.I.D. RESPONSE
c-16
c-18:1
c-18
c-14
c-20:1
c-16:1
c-20
c-19Δ
c-12
20
40
RETENTION TIME, min


STREP. mutans 27175
F.I.D. RESPONSE
c-16
c-18
c-18-1
c-20-1
c-18+
c-19
c-20
c-17
c-14
c-15
c-16-1
c-12
20
40
RETENTION TIME, min