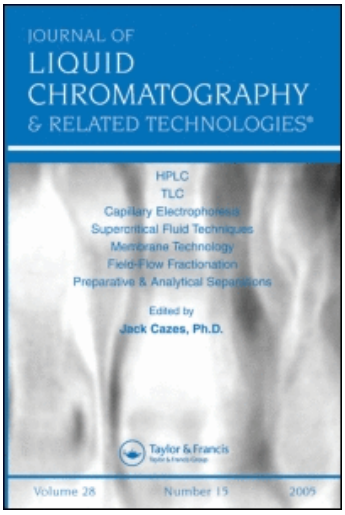

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Norman E. Bussell D.D.S., M.S., Ph.D.[a]; Arthur Gross D.D.S., M.S.[a]; Robert A. Miller B.S.[a]
[a] Division of Oral Biology, U. S. Army Institute of Dental Research Walter Reed Army Medical Center, Washington, D. C.

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ANALYSIS OF FREE FATTY ACIDS ON THE FINGERTIPS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Norman E. Bussell, D.D.S., M.S., Ph.D.
Arthur Gross, D.D.S., M.S.
Robert A. Miller, B.S.
Division of Oral Biology
U. S. Army Institute of Dental Research
Walter Reed Army Medical Center
Washington, D. C. 20012

ABSTRACT

This investigation studied the efficiency of high performance liquid chromatography in the determination of free fatty acids present on the fingertips, and assessed the quantitative relationship between skin fatty acids and the degree of microbial contamination. Automated surgical scrub was utilized to eliminate the microbial contamination.

The high performance liquid chromatography provided excellent separation of skin fatty acids for evaluation with the bacterial counts. The fatty acid peaks identified ranged in chain length from C12 through C32. All the fatty acids evaluated showed positive correlation with the bacterial counts with the exception of one acid which had an inverse relationship but none were statistically significant. Finally, the surgical scrub chromatograms showed that the straight chain acids C19 and C21 were lower in concentration than C23 and C25; also, C26 was lower in concentration than C28 and C30.

It was evident from the data that fatty acids which have been shown to be bacteriostatic *in vitro* do not demonstrate the same property on the fingertips. The finding of lower concentration of C19, C21, and C26 than longer chain acids is inconsistent with simple two carbon addition, and indicates there is possible branching at these points in metabolic pathway of fatty acid synthesis.

INTRODUCTION

For some time it has been apparent that certain individuals have higher bacterial counts on their hands than others. This observation correlates with the findings of an antimicrobial agent found to be present on the skin (1-3). This agent has been characterized as lipid in nature, but its specific chemical structure has not been elucidated. The major lipid class which has been investigated most, in respect to its antimicrobial properties, is the free fatty acids (FFA). As early as 1943, Bergeim *et al.* (4) suggested that fatty acids might play an antimicrobial role on the skin. Later work by Kabara *et al.* (5) showed lauric acid to be active against gram positive microorganisms *in vitro*.

Investigations of the FFA present on the skin are complicated because of the difficulty in identifying their true source. These acids can be derived from sebum triglycerides, stratum corneum cells of the epidermis, and the bacteria present on skin. Furthermore, the variation in position of unsaturation within a single fatty acid and variations in chain branching as discussed by Nicolaides (6) provide technical difficulties in the separation of FFA.

Previous studies (6-10) on fatty acid composition of the skin have utilized either gas-liquid chromatography (GLC) or thin layer chromatography for both qualitative and quantitative analysis. Recently, Borch (11) and Jordi (12) demonstrated

improved chromatographic separation of fatty acids on reverse phase high pressure liquid chromatography (HPLC). Bussell *et al.* (13) reported increased sensitivity of UV detection in GLC, and Miller and Bussell (14) described the close quantitative correlation between GLC and HPLC results.

This investigation was undertaken to study the efficiency of HPLC in the determination of FFA on the fingertips and to assess the quantitative and qualitative relationship between FFA and the degree of microbial contamination on the skin of fingertips.

MATERIALS AND METHODS

Sample Collection

Prior to sample collection, bacteriological cultures of the five fingertips were obtained by touching the surface of blood agar plates. Following incubation at 37C for 48 hours, colony forming units (CFU) of bacteria were counted. In cases of heavy or confluent growth, counts of 50 CFU per fingertip, or a total of 250 as a maximum, for five fingertips were recorded. The bacteria were not identified.

Samples for fatty acid analysis were collected from ten individuals by swabbing each of the five fingertips of one hand with separate cotton pledgets dipped in acetone. The five cotton pledgets were placed in a vial containing 10 ml of acetone.

In order to obtain samples that minimized the contribution of bacteria to the levels of FFA on the fingertips, a second

experiment was carried out in which four individuals scrubbed their hands for 90 seconds utilizing the Hand and Arm Washer developed at the United States Army Institute of Dental Research and described elsewhere (15-17). This procedure has been shown to produce a 97.2% reduction in the microbial counts on the hands.

Immediately after scrub, a fingertip impression culture of the left hand was obtained and the dried right hand was placed in a sterile surgical glove to allow for the endogenous production of lipids to occur without concurrent microbial recolonization on the hand. After three hours the surgical glove was removed and the lipid samples were collected.

Sample Preparation

The cotton pledgets were removed from the vials and the acetone divided into two equal aliquots. Each aliquot was taken to dryness under a gentle stream of nitrogen. Labeling of the FFA by phenylacyl ester groups was done by adding 1 ml of dimethyl formamide (DMF) (Burdick and Jackson Laboratories, L.C. grade) containing 30 μmoles of catalyst (N,N-diisopropylethylamine) (Aldrich Co.) and 15 μmoles of UV Tag (α-bromo-m-methoxyacetophenone) (Pfatz Bauer) to one sample, sealing in a reaction vial and heating for 60 minutes in a 60C water bath. After cooling, the sample was filtered through a 0.5 μm Fluoropore filter (Millipore Corp.). The second sample was hydrogenated by adding 10 ml of methanol and 20 mg of platinum oxide and the mixture placed in a Micro-Hydrogenator (Supelco). The reaction vessel

was pressurized to 10 psi with hydrogen and the contents stirred for 45 minutes. The mixture was removed, filtered, and evaporated to dryness. The sample was then tagged as described above.

Chromatographic Separation

An aliquot of the sample was injected into a High Performance Liquid Chromatograph (Model 244, Waters Associates) equipped with a solvent programmer. The separation was carried out on two μ-Bondapak C18 reverse phase columns in series with a total plate count of greater than 10,000. The columns were placed in a heating jacket at 38C to maintain consistencies within the chromatograms. The fatty acids were eluted using an acetonitrile/water system (Burdick and Jackson) programmed from 40/60 to 100/0 percent during a three hour period using curve five on the solvent programmer. The chromatograms were continued for one hour after final conditions had been reached.

Identification of Fatty Acids

The fatty acids were tentatively identified by comparison times of the unknown peaks with those of standards (Supelco; Alltech; Analabs). The hydrogenation chromatogram was utilized to confirm whether the peaks were unsaturated or saturated.

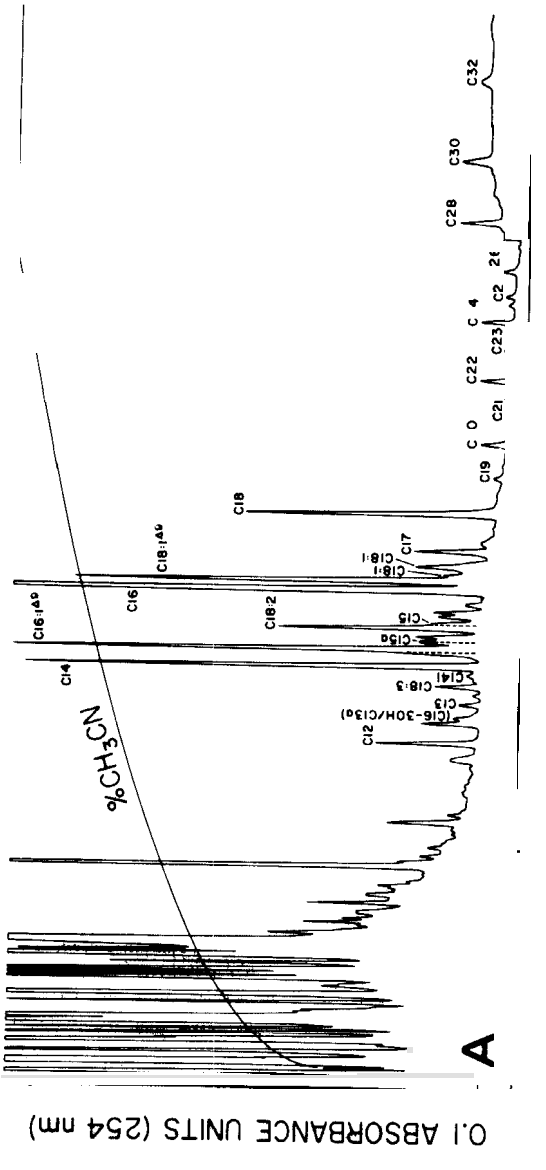
Statistical Methods

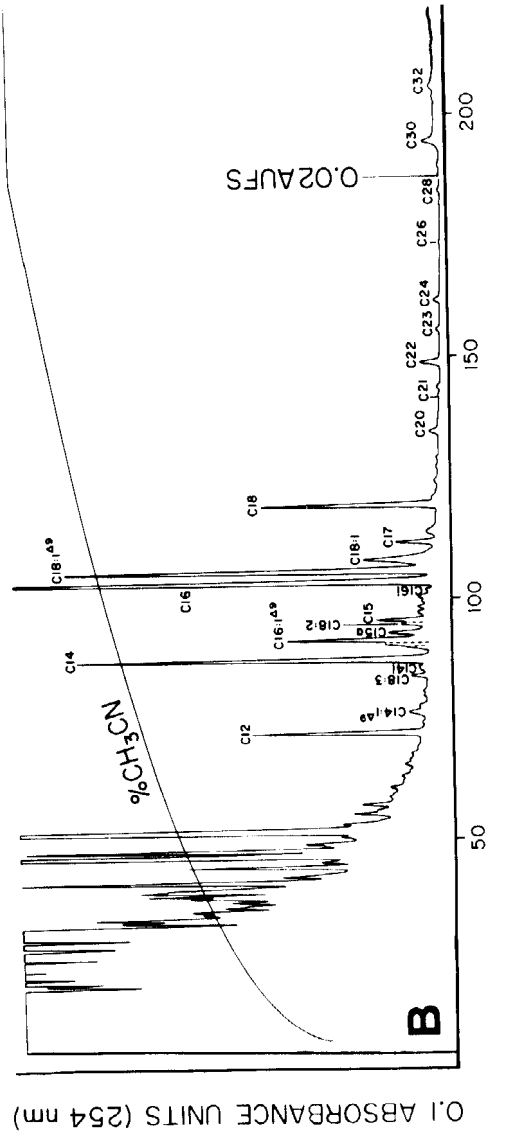
Linear regression correlation coefficients were calculated as described elsewhere (18) with $p < 0.05$ taken as the minimal level of statistical significance.

RESULTS

Fig. 1 shows examples of the HPLC chromatograms obtained. The FFA which were identified ranged from C12 through C32 in chain length and more than 40 individual peaks were seen above C12. The total free fatty acid concentration was calculated by summation of the peak heights of the fatty acids in the chromatograms and are listed in Table 1 along with the bacterial counts observed on the fingertips. Regression analysis was done on the correlation between CFU and total FFA shown in Table 1. There was a positive correlation ($r = +0.47$). Individual 10 was eliminated from the correlation coefficient calculation due to the discrepancy in palmitic acid peak, as a result of being both a *Candida* and *Staphylococcus aureus* carrier. However, this correlation coefficient was not statistically significant at 0.95 confidence level and thus total FFA did not appear to be related to bacterial counts. For simplification of data presentation, the fatty acids were divided into major, minor, and trace. The divisions were arbitrarily based on concentration. The major fatty acids observed were lauric (C12:0), myristic (C14:0), palmitic (C16:0), oleic ($C18:1^{\Delta 9}$), and stearic (C18:0); their peak heights are shown Table 2. As seen in Fig. 1 there were several other C18:1 fatty acids in addition to oleic acid. These were labeled as C18:1 with no indication as to the position of the unsaturation.

The minor fatty acids present were palmitoleic ($C16:1^{\Delta 9}$), linoleic ($C18:2^{\Delta 9,12}$), pentadecanoic (C15:0), and heptadecanoic





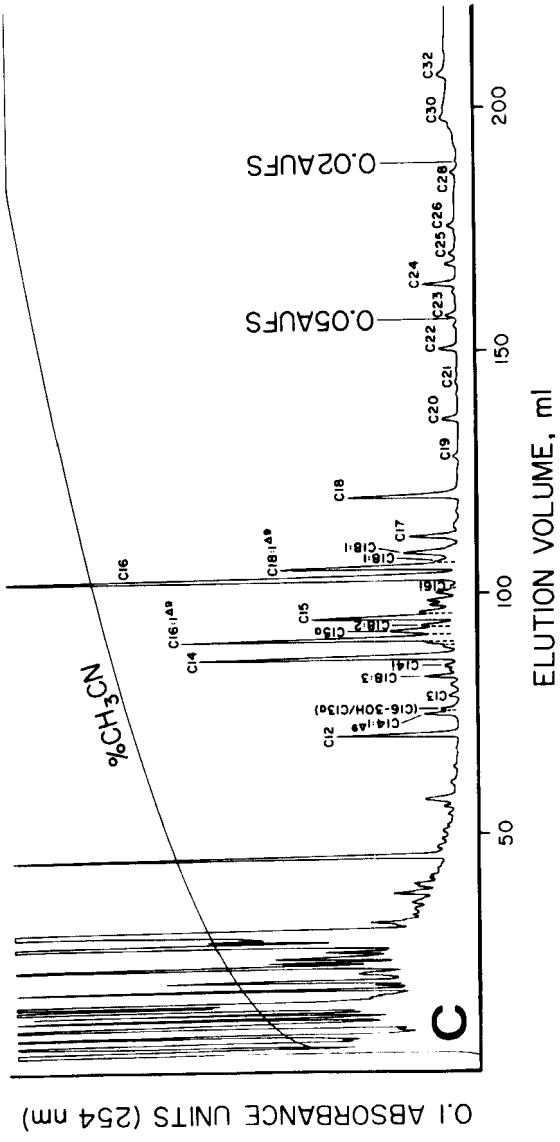


Fig. 1. Examples of HPLC chromatograms of the free fatty acids from the fingertips.

A. Individual #5, 100λ injection CFU 103.
B. Individual #9, 125λ injection CFU 26.
C. Individual #10, 100λ injection CFU 45.

Columns: Two 30 cm x 3.9 mm μBondapak C18, eluent acetonitrile: water, gradient curve 5, 40/60% to 100/0%, 180 min, flow rate 1.0 ml/min.

TABLE 1

BACTERIAL COUNTS (CFU) AND TOTAL FREE FATTY ACID ON THE SKIN OF FINGERTIPS

Individual	CFU[a]	Total Free Fatty Acid Concentration[b]
1	106	1240
2	88	1056
3	250	735
4	250	1020
5	103	1728
6	250	2511
7	250	2909
8	63	736
9	26	978
10	45	2760

[a]Colony Forming Units

[b]The total free fatty acid concentration was determined by summation of the peak heights for each individual fatty acid, (Tables 2, 3, 4,).

TABLE 2

Major Fatty Acids on the Skin of Fingertips, Peak Height (1 mm)

	C12:0	C14:0	C16:0	C18:1	C18:0
Mean ± SE	64±16	183±29	457±72	152±32	103±10
r[c]	+0.36	+0.40	+0.53	+0.30	+0.52

[c]Correlation coefficient for the relationship between bacterial counts and Free Fatty Acids.

(C17:0) (Table 3). In addition, there was a peak which had the same retention volume as β-hydroxypalmitic acid, but β-hydroxypalmitic acid elutes at the same point as 10-methyldodecanoic (C13-0a). Since delineation between the two acids could not be made at this time, the peak was labeled as 30H-C16:0/C13:0a. Also, 12-methyl tetradecanoic (C15:0a) was seen and its peak height recorded (Table 3).

Peak height values for trace fatty acids C19 through C32 are shown in Table 4. The peaks between the straight chain fatty acids which could not be identified which are shown in the figures, were found to be saturated, indicating either branch chain or hydroxy fatty acids.

Tables 2 and 3 show the correlation between the individual FFA identified in each chromatogram and the CFU for each subject as listed in Table 1. All of the fatty acids showed a weak positive correlation to CFU except for the fatty acid 30H-C16:0/C13:0a (Table 3) which showed a negative correlation. None of the correlation coefficients for the individual free fatty acids were statistically significant.

The surgical scrub eliminated most of the bacteria from the fingertips as evidenced by counts below 10 CFU for each of the four individuals tested (Table 5). The individual samples from fingertips of the gloved hands three hours postscrub yielded HPLC chromatograms of the FFA for each subject which were similar to each other, both quantitatively and qualitatively (Fig. 2). The major and minor fatty acids observed were lauric (C12:0),12-

TABLE 3

Minor Fatty Acids on the Skin of Fingertips, Peak Height (1 mm)

	C14:1	C14:0i	C15:0	C15:0a	C16:0i	C17:0	C18:2	30H-C16:0/ C13:0a
Mean ± SE	147±41	6±2	79±19	31±8	5±1	30±4	24±4	21±8
r[c]	+0.44	+0.46	+0.48	+0.51	+0.41	+0.48	+0.58	-0.53

[c]Correlation coefficient for the relationship between bacterial counts and Free Fatty Acids.

TABLE 4

Trace Fatty Acids on the Skin of Fingertips, Peak Height (mm)

	C19:0	C20:0	C21:0	C22:0	C23:0	C24:0	C25:0
Mean ± SE	2±0.6	9±1.8	2±0.8	12±0.9	3±0.4	14±2.8	4±0.7
	C26:0	C27:0	C28:0	C29:0	C30:0	C32:0	
Mean ± SE	6±1.2	1±0.8	9±1.0	2±1.0	7±0.6	3±0.4	

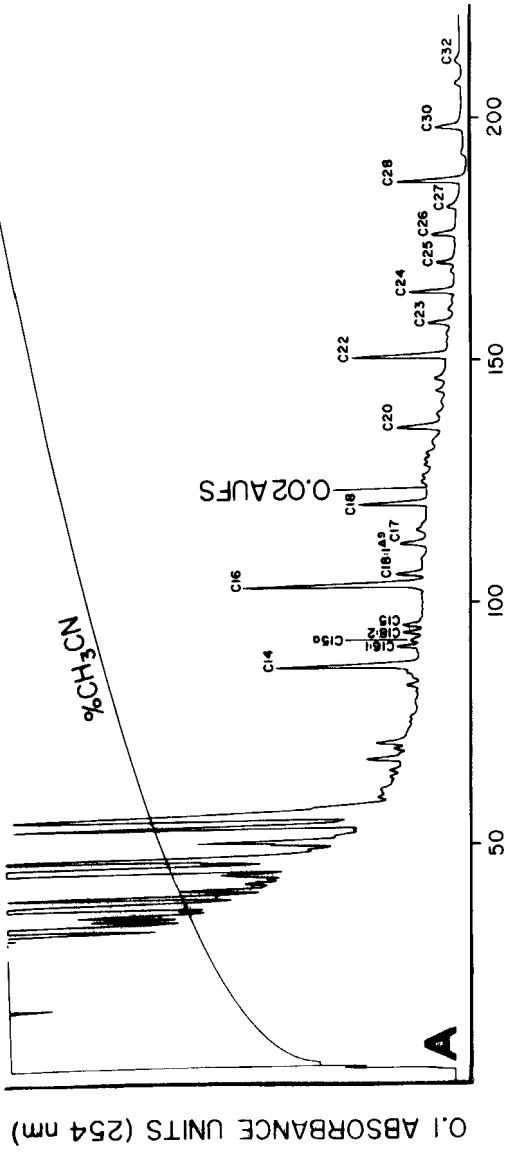
TABLE 5

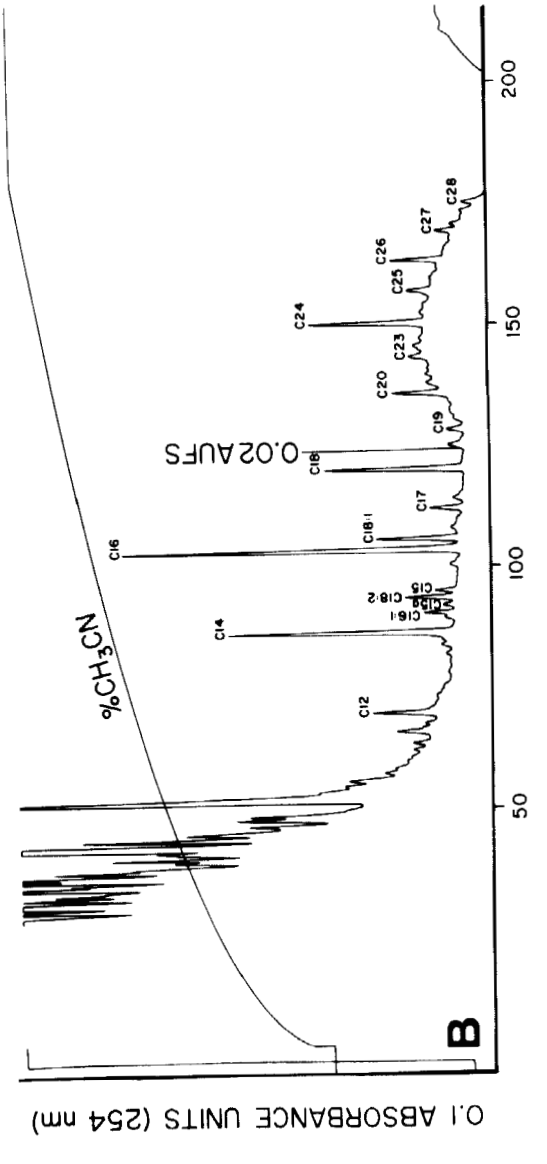
Major and Minor Fatty Acids on the Skin of Fingertips After Surgical Scrub, Peak Height (mm)

	C12:0	C14:0i	C14:0	C15:0a	C15:0
Mean ± SE	24±4	5±3	83±4	12±1	8±1
	C16:0	C17:0	C18:0	C18:1	C18:2
Mean ± SE	104±13	13±1	44±4	25±5	15±4

methyl tridecanoic (C14:0i), myristic (C14), 12-methyl tetradecanoic (C15:0a), pentadecanoic (C15:0), palmitic (C16:0), oleic (C18:1), linoleic (C18:2), heptadecanoic (C17), and stearic (C18:0) (Table 5). Trace fatty acids above C19 were also noted (Fig. 1). However, the intermediate peaks past C19 observed on the prescrub samples were absent in this area. The peak heights for the trace acids are shown in Table 6.

Several points of interest were noted on examination of the postscrub chromatograms of hydrogenated fatty acid samples. The peak for 12-methyl tetradecanoic (C15:0a) increased two to four-fold, or approximately equal to the pentadecanoic acid peak (C15:0) (Fig. 3). In the trace fatty acid group, the C23 and C25 fatty acids were usually higher in concentration than their precursors C19:0 and C21:0. Behenic acid (C22) usually exhibited the highest concentration of any of the trace fatty acids (Tables 3 and 5). Finally, the C26:0 fatty acid was lower in concentration than C28:0 and C30:0.





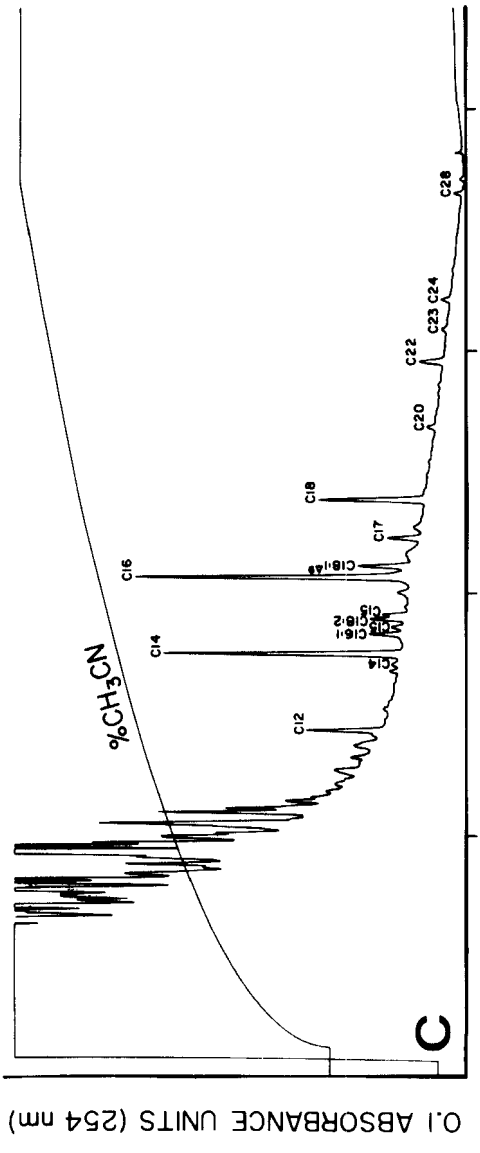


Fig. 2. Examples of HPLC chromatograms of the free fatty acids from the fingertips of individuals after surgical scrub.

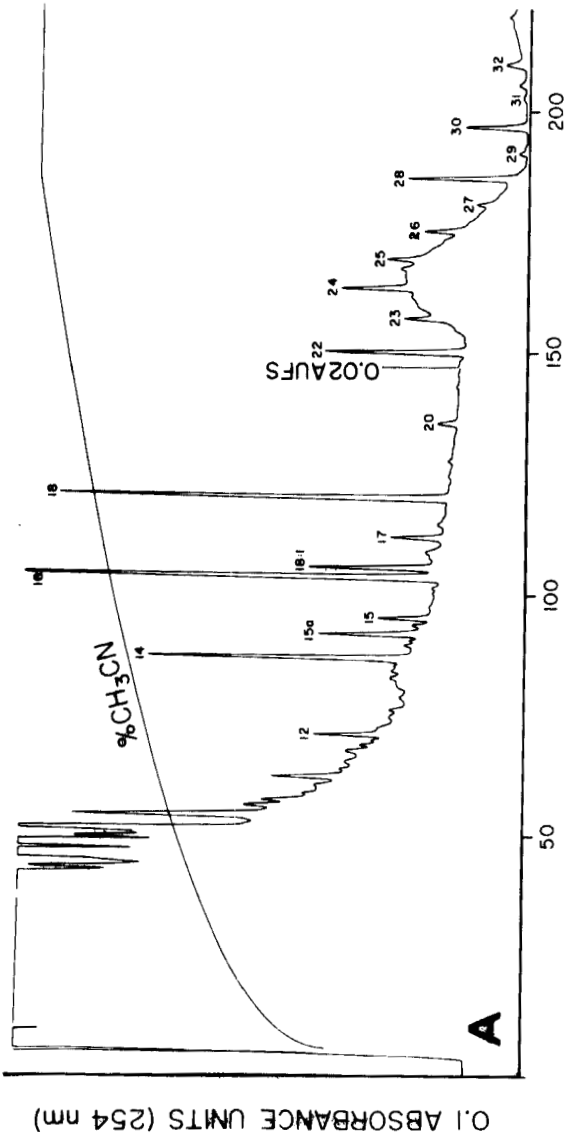
A. Individual #3, 100λ injection CFU 5.
B. Individual #4, 100λ injection CFU 4.
C. Individual #1, 100λ injection CFU 8.

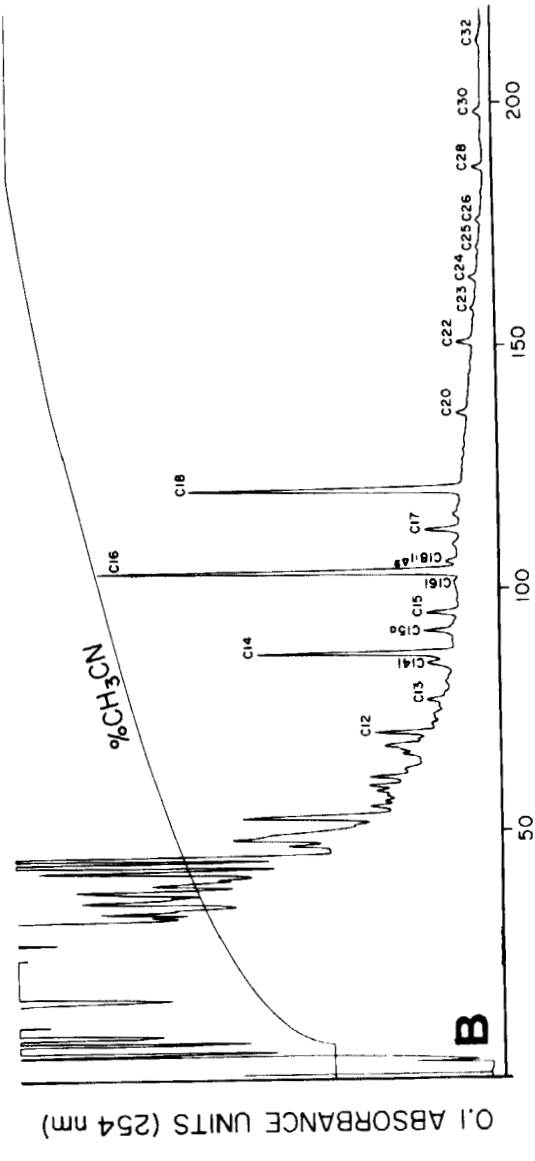
Columns: Two 30 cm x 3.9 mm μBondapak C18, eluent acetonitrile: water, gradient curve 5, 40/60% to 100/0%, 180 min, flow rate 1.0 ml/min.

TABLE 6

Trace Fatty Acids On The Skin of Fingertips After Surgical Scrub, Peak Height (mm)

	C19:0	C20:0	C21:0	C22:0	C23:0	C24:0	C25:0
Mean ± SE	1±1	3.2±.5	--	8.5±.3	1.5±.3	3.3±.3	1.3±.3
	C26:0	C27:0	C28:0	C29:0	C30:0	C32:0	
Mean ± SE	1.5±.3	.8±.2	8±.6	--	6±.6	23±.3	





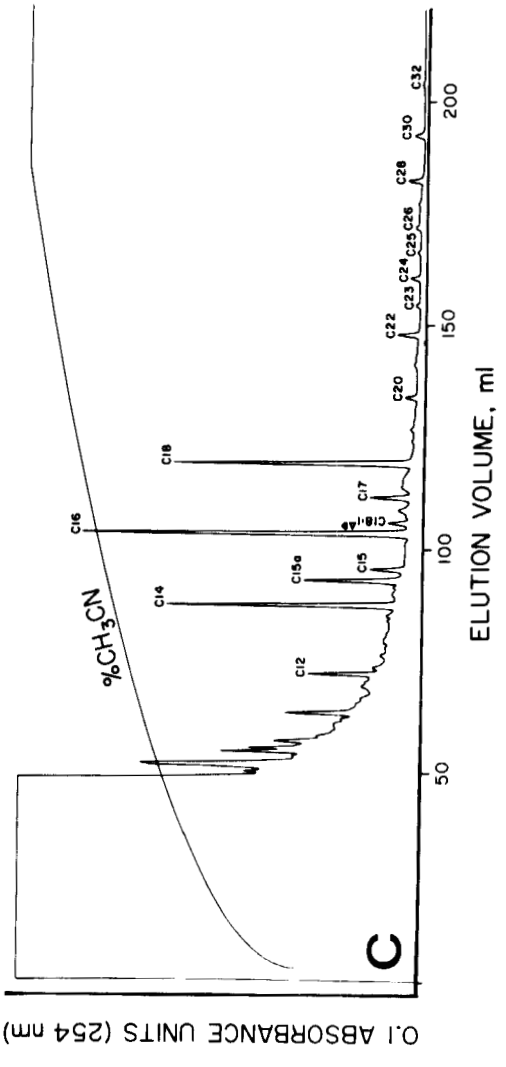


Fig. 3. Examples of HPLC chromatograms of hydrogenated free fatty acids from the fingertips of individuals after surgical scrub.

A. Individual #3, 100λ injection.
B. Individual #4, 100λ injection.
C. Individual #1, 100λ injection.

Columns: Two 30 cm x 3.9 mm μBondapak C18, eluent acetonitrile: water, gradient curve 5, 40/60% to 100/0%, 180 min, flow rate 1.0 ml/min.

DISCUSSION

Although the prescrub total free fatty acid concentration and the microbial counts on the fingertips showed a positive correlation coefficient ($r = +0.47$), this correlation was not statistically significant at the 0.95 confidence level. An explanation for the wide variations in FFA levels seen may be due to the multiplicity of sources of origin of the FFA. Four possible sources are:

1. Breakdown of sebum triglyceride by bacterial lipases (19,20). Lipases, however, are produced only by certain species of bacteria. Therefore, variation in specific colonization bacteria among individuals may influence the contribution of this source to total FFA of the skin. The breakdown of sebum triglycerides is significant in production of FFA, but the contribution of sebum is minimal on the fingertip area as compared to other parts of the body, e.g., the forehead.
2. Production of lipids by the epithelial cells of the stratum corneum, which amounts to a considerable portion of the lipid present in this area (10). This source of lipid has not been examined extensively.
3. In extraction of FFA with acetone, some fatty acids could be removed from the cell walls of bacteria present on the skin.
4. Contamination of the fingertips by touching other areas of the body such as the face and forehead. In

addition, residual FFA could be present from the use of cosmetics and cleaning agents.

By assaying individual fatty acids which have been shown to be produced either by human, e.g., linoleic acid (21) or bacteria exclusively, the origin of fatty acids on the skin could be established. To minimize or eliminate fatty acids of bacterial origin we have used a procedure of removing the bacteria by surgical scrub and then allowing the endogenous lipids to regenerate.

Analysis of individual acids on fingertips before scrub showed positive correlation with CFU counts for all the acids with the exception of 30H-C16:0/C15:0a. Statistically significant positive correlation ($p < 0.05$), however, could be demonstrated for palmitic, stearic, heptadecanoic, and 12-methyl tetradecanoic. While palmitic (C16:0) and heptadecanoic (C17:0) acids have been shown to possess some growth inhibitory properties for certain bacteria *in vitro* (22, 23), these fatty acids may also act as growth factors for other bacteria (24) rather than showing any antimicrobial action. One FFA of particular interest was linoleic acid ($C18:2^{\Delta 9,12}$) because its source is eukaryotic cells (21). The reason this particular acid did not show a significant correlation may have resulted from two factors. The first being the difficulty in handling of unsaturated fatty acids because of the ease in inducing hydrogenation of the double bonds (25). The second reason may be due to the mixed flora present on the hands which may produce varying amounts of

lipases for hydrolysis of the triglycerides, thus affecting the concentrations of linoleic acid on the skin.

No negative correlation could be shown between bacterial counts and lauric acid ($r = +0.36$) or oleic acid ($r = +0.30$), that had been previously shown to be bacteriostatic *in vitro* (26). This finding suggests that these acids may not demonstrate antibacterial activity *in vivo* against skin bacteria or that their concentrations are too low to inhibit bacterial growth.

The only acid that was found to have a negative correlation coefficient ($r = -0.427$) was tentatively identified as 3OH-C16:0/C13a. Postscrub chromatograms did not show a peak in this area which indicated that it was probably of bacterial origin, or that its synthetic rate by the skin is slower than that of other acids. Its antimicrobial properties should be investigated further by *in vitro* and *in vivo* techniques.

Several C18:1 fatty acid peaks were separated by the HPLC methodology. The specific location of their unsaturation could not be determined, although three separate C18:1 isomers were separated with unsaturation at the 6, 9, and 11 positions. Nicolaides (6) reported that the most prominent unsaturations were in the 6 and 8 positions. However, our findings indicated that the prominent position was the 9. The elution volume of the 8 position cannot be predicted from the other three acids since their elution volumes are $C18:1^{\Delta 9} < C18:1^{\Delta 11} < C18:1^{\Delta 6}$ which could indicate that the 8 position coelutes with the 9 position. In addition, several small peaks were noted around palmitoleic

C16:1$^{\Delta 9}$ as seen in Fig. 1; these peaks are probably C16:1 with variation in the position of the unsaturation.

The HPLC chromatograms of the FFA from fingertips from the postscrub experiment showed a similarity both quantitatively and qualitatively as seen in Fig. 2. The only variation noted was in the area of C16:1 peak which showed a slight shift in one individual out of the four. This could indicate a possible variation in position of the double bond, which would correlate with Nicolaides' (6) description of families of fatty acids.

The postscrub chromatograms (Fig. 3) of the hydrogenated fatty acids revealed several interesting points. The peaks which corresponded to 12-methyl tetradecanoic C15a increased to approximately equal to or greater than pentadecanoic peak C15 which indicates that a considerable amount of the 12-methyl tetradecanoic is unsaturated and that this acid is one of the major odd chain fatty acids present on the skin. Furthermore, the hydrogenation chromatograms showed the C19 and C21 were lower in concentration than C23 and C25. This finding is inconsistent with simple chain elongation by a two carbon unit addition, and would suggest either an alteration of their biochemical pathway or their utilization in other lipid classes. The fatty acid C26 was also shown to be lower in concentration than C28 or C30. Further investigations are indicated to evaluate the significance of these findings. The identification of these minor peaks illustrates the improved separation potential of HPLC, and the extended range of the HPLC over previously reported methods. The

use of HPLC for the analysis of FFA on the skin shows great promise.

SUMMARY AND CONCLUSIONS

A quantitative and qualitative analysis of the free fatty acids present on fingertips (before and after surgical scrub) was done on the phenylacyl esters of the fatty acids separated by high pressure liquid chromatography. This procedure allowed for the resolution of more than 40 major, minor, and trace peaks. Regression analysis comparing the bacterial population of the fingertips and the total free fatty acid content eluted from the sample showed a positive but nonsignificant correlation. Regression analysis comparing the bacterial population of the fingertips and the total free fatty acid content eluted from the sample showed a positive but nonsignificant correlation. Regression analysis of the individual FFA versus microbial counts showed positive correlation between all of the acids identified except one which showed a negative correlation. The chromatograms obtained following the surgical scrub indicated that this acid was possibly of bacterial origin.

The elimination of most of the microorganisms by surgical scrub produced less scatter in the elution profiles of the FFA than observed in random samples from fingertips. This suggests that the fatty acid profiles obtained represented mainly the lipid derived from the skin and, in particular, from the cells of the stratum corneum. Finally, it was shown that HPLC provided the improved separation necessary for the study of this complex

group of lipids. The results also indicated that certain long chain fatty acids, above C19, offer new interesting areas of investigation into their metabolic pathways, and that HPLC provides the chromatographic qualities for such investigations.

* * * *

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.

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