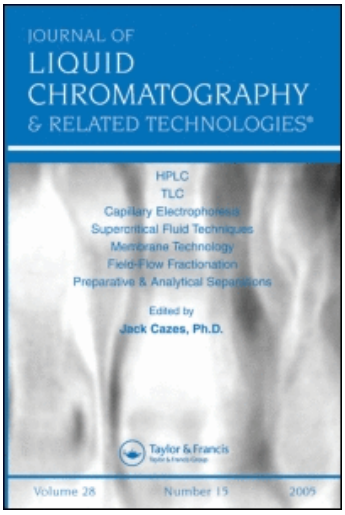

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Quantitation of Long Chain Fatty Acids as the Methoxyphenacyl Esters

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To cite this Article Miller, Robert A. , Bussell, Norman E. and Ricketts, Charles(1978) 'Quantitation of Long Chain Fatty Acids as the Methoxyphenacyl Esters', Journal of Liquid Chromatography & Related Technologies, 1: 3, 291 — 304
To link to this Article: DOI: 10.1080/01483917808060000
URL: http://dx.doi.org/10.1080/01483917808060000

JOURNAL OF LIQUID CHROMATOGRAPHY, 1(3), 291-304 (1978)

QUANTITATION OF LONG CHAIN FATTY ACIDS AS THE METHOXYPHENACYL ESTERS

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ABSTRACT

In recent years High Performance Liquid Chromatography has been used for the separation of long chain fatty acids. This study establishes a procedure for the quantitation of the major fatty acids found in oral bacteria. The acids studied were C-10, C-12, C-14, C-16, C-18, C-18:1, C-20, and C-22. The samples were esterified with α-Bromo-m-methoxyacetophenone, separated by reversed phase chromatography and monitored at both 254nm and 280nm. The fatty acids have approximately the same linear absorbance range at 254nm from 100 picomoles to 50 nanomoles. While the linear absorbance range was similar, the response factors varied by more than 40% when using peak heights, compared to less than a 15% variation when using peak areas. Several A-NHI fatty acid reference mixtures were used to assure the reliability (average relative error = 3.72%) of the method. Subsequent analysis of a bacteria sample was made by both High Performance Liquid Chromatography (HPLC) and Gas Liquid Chromatography (GLC) in order to further substantiate the validity of the technique.

INTRODUCTION

Previously, it has been established that fatty acid analysis (as well as other cellular components and metabolic by-products) can be a useful chemotaxonomic aid for the differentiation of

microorganisms.[1-4] In the past, GLC has been used extensively for bacterial fatty acid analysis, but a recent article by Bussell, *et al.*[5] has demonstrated the advantages of using HPLC over GLC in the fatty acid analysis of microorganisms. HPLC analysis of the phenacyl esters of fatty acids, which was introduced by Borch,[6] and subsequent analysis by Jordi,[7] both pertain to the separation of the long chain acid esters and not their quantitation.

An initial study on qualitative analysis of bacterial fatty acids in our laboratory indicated that there was poor correlation in quantitation by direct comparison of HPLC peak heights to GLC peak areas.[5] In order to be a good quantitative technique, the analysis should meet the following criteria: the standard curve should be linear over a wide concentration range and the method should exhibit good precision as well as be accurate. This paper reports on the quantitation of long chain fatty acids by HPLC.

MATERIALS AND METHODS

The fatty acids used for standards in this study were quantitative grade with a purity greater than 99+%. Myristic, palmitic, and stearic acids were obtained from Polyscience Corp. and arachidic and behenic acids were obtained from Sigma Chemical Co. Quantitative A-NHI reference mixtures and boron trichloride kits were obtained from both Supelco, Inc. and Alltech Associates, Inc. The UV tag (α-bromo-m-methoxyacetophenone) was obtained from Pfaltz and Bauer, Inc. and the catalyst (N,N-diisopropylethyl-

amine) was obtained from Aldrich Chemical Co. UV-Grade acetonitrile and dimethylformamide (DMF) were obtained from Burdick and Jackson Laboratories, Inc.

The fatty acid esters were prepared by a modification of Borch's procedure[6] for UV tagging. A two-fold molar excess of UV tag and a four-fold excess of catalyst were added to the fatty acids in DMF, sealed in a reaction vial, and heated to 60°C in a water bath for one hour. The samples were then filtered on 0.5μm fluoropore (Millipore Corp.) filters prior to injection into the HPLC.

Separations were performed using a Waters High Performance Liquid Chromatograph (HPLC) Model 244, with a solvent programmer. The standards and samples were separated on two μ-Bondapak C_{18} reverse phase columns in series (Waters Associates, Inc.) in conjunction with an ODS guard column (Whatman, Inc.). The fatty acids were monitored at 254nm for maximum absorption and at 280nm for decreased sensitivity. A water jacket maintained the columns at a constant temperature of 38°C in order to improve the reproducibility of the t_R values in the method.

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

Standard solutions of each tagged fatty acid were prepared at various concentrations in order to cover a range of 100 picomoles to 1 micromole per injection (10µl). These standards were chromatographed at various absorbance ranges to establish the linear working range.

Fifty micromoles of each of the five fatty acids standards were weighed into a vial on an analytical balance and their exact weights were recorded. This was repeated until 10 vials with known quantities of fatty acids were obtained. The standards were then esterified as previously described in 10ml DMF giving a final concentration of 25nmoles/5µl, which was injected into the HPLC. Both peak heights and peak areas were determined in order to compare the precision of the method. The response factors were determined using peak heights.

Fatty acid A-NHI mixtures with known compositions were esterified and chromatographed as previously described. Their percent composition was determined by using peak heights in combination with the predetermined response factors. The amounts determined experimentally were then compared to the known concentration.

Finally, a bacterial sample (*S. mutans* ATCC 27175) 0.3gm wet weight was saponified in 100ml 5% KOH in 50% aqueous methanol for 15 min at 90°C in a water bath. The solution was acidified and extracted with 200ml $CHCl_3$. A 4ml aliquot was removed, taken to dryness, and tagged for HPLC. The remaining solution was taken to dryness, and methylated by the boron trichloride

technique. This sample was analyzed using a Varian Gas Chromatograph Model 2860 equipped with a Flame Ionization Detector (FID) and a Varian CDS 101 Integrator. The separations were carried out isothermally on a 10% DEGS-PS Supelcoport 100/120 mesh 12'X ¼" (OD) X 0.2mm (ID) glass column at 185°C. The carrier gas was purified helium at a flow rate of 20ml/min with the injector and detector temperatures at 205°C and 210°C respectively. The results from the GLC analysis were compared to the HPLC results.

RESULTS

The linear absorbance range at 254nm was 100 picomoles to 50 nanomoles. The range of linearity at 280nm is illustrated in Fig. 1. Lauric (C-12), myristic (C-14), and palmitic (C-16) acids have a linear absorbance range from 2.5nM to 500nM and stearic (C-18) acid has a range from 2.5nM to 150nM. Arachidic (C-20) and behenic acids (C-22) have the smallest range from 25nM to 100nM.

The precision of the fatty acid analysis is shown in Tables 1 and 2. The average precision using peak height was 4.80% compared to 7.02% using peak area. The relative response factors were calculated using the mean peak heights and are as follows: C-10 = 1.154, C-12 = 1.000, C-14 = 0.833, C-16 = 0.789, C-16:1 = 0.821, C-18 = 0.672, C-18:1 = 0.863, C-20 = 0.691, and C-22 = 0.712. There is a significant decrease in response factors (40%) from C-10 to C-18. The response factors using peak area for the fatty acids C-10 → C-22 had a variation of less than 15%, indicating that a portion of the decrease in response (peak height) was due to peak broadening.

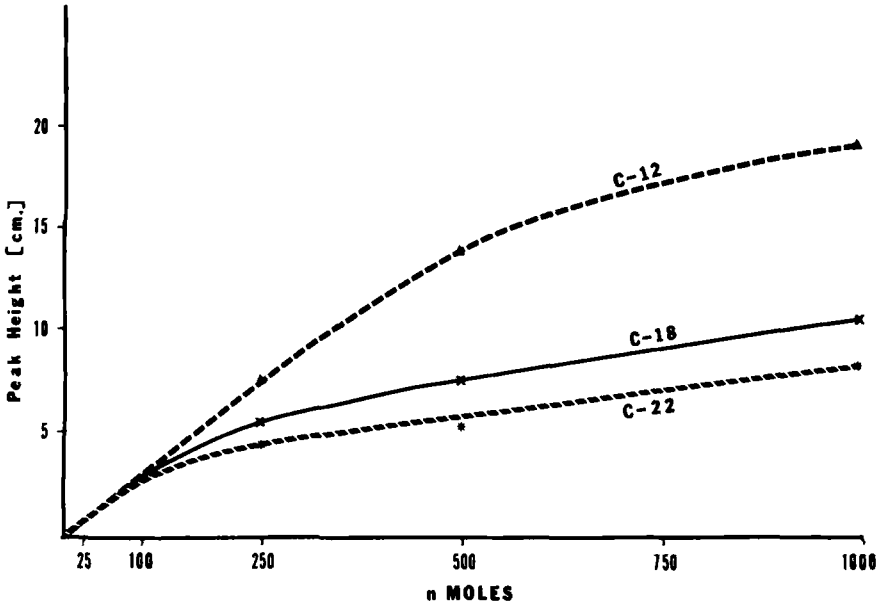

Figure 1.

Standard curve at 280nm for lauric acid C-12, stearic acid C-18, and behenic acid C-22.

Figure 2 is a representative chromatogram of the quantitative A-NHI mixtures which were analyzed to determine the accuracy of the method. Analysis of the A-NHI mixtures as summarized in Table 3 showed an average relative error of 3.72%. The composition of the major fatty acids in a bacteria sample, analyzed by both GLC and HPLC is shown in Table 4. The average relative error between the techniques was 7.2%. The HPLC chromatograph of the bacteria sample is shown in Figure 3.

DISCUSSION

The HPLC method used for this study had a wide linear working range when the UV absorbance of the phenacyl fatty acid

TABLE 1

Precision of Fatty Acids

(25 n M Sample)

Corrected Peak Heights (cm)

RUN NO.	C–12	C–14	C–16	C–18	C–20	C–22
1	14.15	11.95	10.63	9.26	9.07	9.13
2	13.37	11.70	11.58	8.85	9.33	9.14
3	13.45	11.05	10.38	8.84	9.19	9.83
4	13.45	11.20	10.99	9.61	9.58	9.86
5	13.08	10.99	10.35	8.60	9.18	8.41
6	13.34	10.92	10.42	9.01	9.23	9.91
7	13.21	10.73	10.12	9.19	9.35	9.82
8	13.09	10.83	10.28	8.83	8.69	9.48
9	13.07	10.82	10.16	8.48	8.75	8.75
10	12.92	10.71	10.09	8.83	9.68	10.48
Mean	13.31	11.09	10.50	8.95	9.20	9.48
Standard Deviation	± .3448	± .4188	± .4646	± .3311	± .3153	± .6221
Precision	2.59%	3.78%	4.42%	3.69%	3.42%	6.56%

esters at both 254nm and 280nm was monitored. The nonlinearity of the long chain acids (>C-18) at high concentrations may have been due to the decrease in the fatty acids solubility in the eluent or from column overload of these acids. This could possibly be eliminated by using a less polar solvent.

The elevation in precision from 4.08% using the peak height technique to 7.02% using the peak area technique is due to the difference in average relative precision of measurement (1-2% for peak height and 3% for peak area).[8] However, when comparing the

TABLE 2

Precision of Fatty Acids

(25 n M Sample)

Corrected Peak Areas (in.^{2})

RUN NO.	C–12	C–14	C–16	C–18	C–20	C–22
1	.281	.233	.266	.231	.254	.231
2	– –	.222	.271	.221	.243	.234
3	.243	.266	.205	.221	.220	.258
4	.262	.244	.242	.210	.227	.254
5	.252	.225	.231	.208	.234	.229
6	.300	.276	.277	.210	.226	.253
7	.252	.216	.223	.232	.236	.224
8	.251	.217	.226	.211	.223	.236
9	.238	.237	.229	.223	.243	.224
10	.233	.229	.257	.210	.255	.257
Mean	.257	.237	.243	.218	.255	.240
Standard Deviation	±.0214	±.0203	±.0239	±.009	±.012	±.014
Precision	8.33%	8.57%	9.84%	4.18%	5.39%	5.80%

response factors for the peak area technique versus peak height technique, only a 15% variation was observed using peak area compared to a 40% decrease in response from C-10 to C-18 for peak height. For semi-quantitative analysis with good resolution, peak area would be the method of choice with no response correction necessary for fatty acids C-10 → C-22. For quantitative analysis response factors must be used for both methods of measurement, but on peaks with poor resolution, as shown by C-18 and C-20:1 in

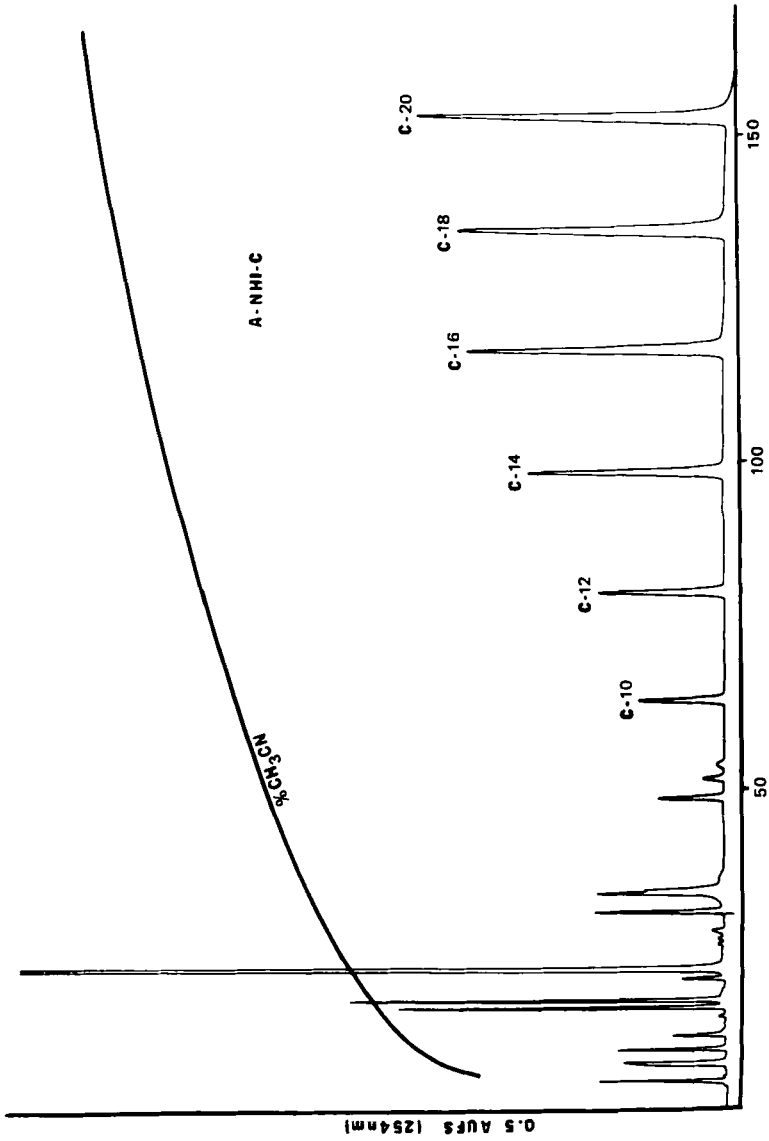

Figure 2. HPLC chromatogram of A-NHI-C fatty acid reference mixture. Columns: Two μBondapak C_{18}; eluent: acetonitrile/water; flow rate: 1ml/min; see text for details.

TABLE 3

Fatty Acid Mixture Composition

(Weight %)

	C–12	C–14	C–16	C–18	C–20	C–22
A–NHI–C						
Certified	6.28	12.62	20.31	26.07	34.76	
Experimental	5.91	12.57	19.88	27.36	34.21	
Relative Error	5.89%	0.40%	2.12%	4.95%	1.58%	
A–NHI–D						
Certified		24.33	48.66	27.01		
Experimental		25.19	48.43	26.38		
Relative Error		3.53%	0.47%	2.33%		
A–NHI–F						
Certified		4.71	7.93	13.77	25.66	47.93
Experimental		5.14	8.06	14.85	26.52	45.42
Relative Error		9.13%	1.63%	7.84%	3.35%	5.23%

Average Relative Error – 3.72% (0.4% – 9.13%)

TABLE 4

Major Fatty Acid Composition of

Streptococcus mutans 27175

(Weight %)

	C–16	C–18	C–18:1
HPLC Analysis	61.0	16.6	22.5
GLC Analysis	64.2	15.8	20.0
Relative Error	5.1%	4.8%	11.8%

Average Relative Error 7.2%

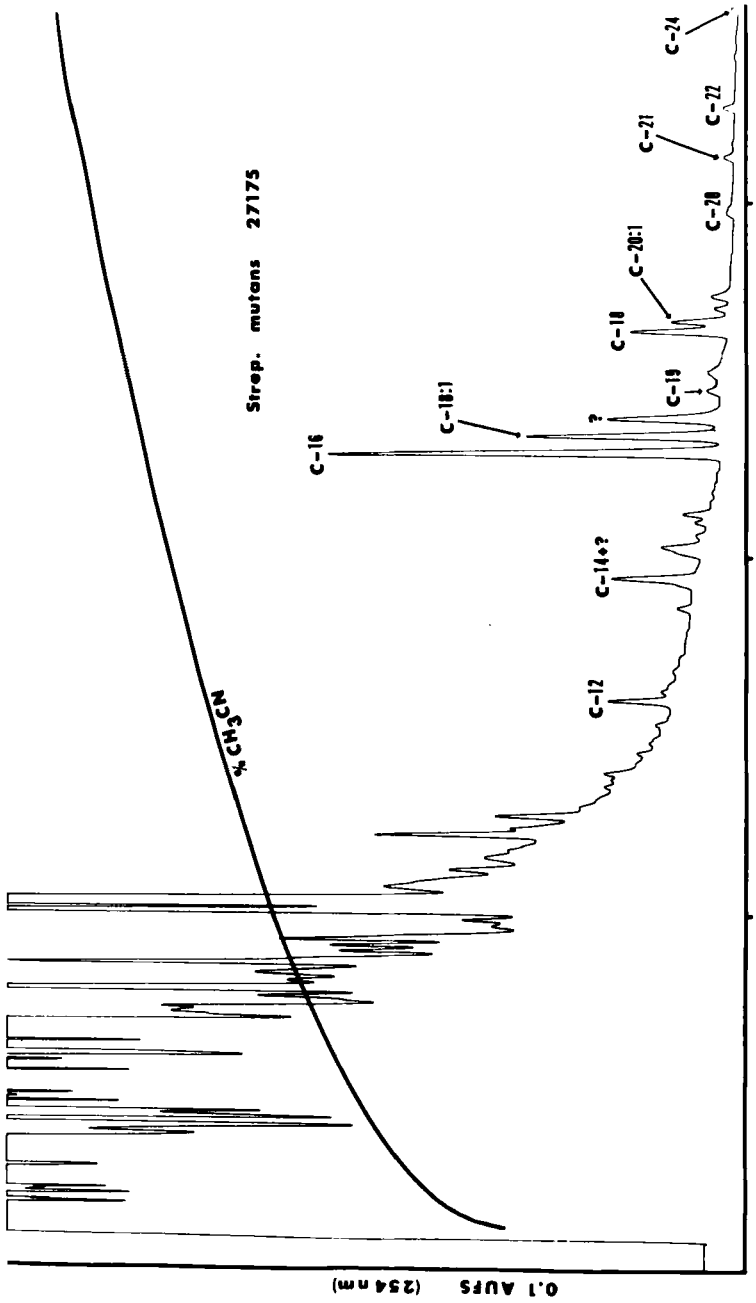

Figure 3. HPLC chromatogram of the fatty acids in *Streptococcus mutans* ATCC 27175. Columns: Two μBondapak C_{18}; eluent: acetonitrile/water; flor rate: 1ml/min; see text for details.

Figure 3, peak height measurement would be the method of choice. Caution is emphasized because, in a previous study, inter-laboratory exchange of response or correction factors had been shown to be a significant source of errors in GLC analysis.[9]

Bussell, *et al.*[5] have indicated that bacterial fatty acid quantitation by HPLC did not show good correlation with data obtained by GLC. In this study we have demonstrated the accuracy of fatty acid quantitation in known mixtures of fatty acids and by comparison of HPLC to GLC analysis of bacteria. The poor correlation previously seen was due to the combination of different response factors for each acid and slightly different extraction techniques in sample preparation for HPLC and GLC. For example, in preparation for GLC only one extraction of 200ml of $CHCl_3$ was made of 100ml saponified bacteria while for HPLC, 2ml of the saponified mixture was extracted 5 times with 10ml of $CHCl_3$. The extraction volume ratio was 1:2 for GLC and 1:25 for HPLC. This difference by itself could account for the different quantities of fatty acids seen. By eliminating the variables in the extraction procedure, HPLC analysis showed good correlation with GLC.

CONCLUSION

A specific method for the quantitation of long chain fatty acids in bacteria has been discussed. The method has a broad linear absorbance range which is required for analysis of bacteria since there are a few major components and many trace components within the microorganisms. Only fatty acids with even carbon numbers were discussed, but this was sufficient enough to demon-

strate the large decrease in UV response of the different fatty acid esters using peak heights. The technique had precision from 2.59% for C-12 to 6.56% for C-22 which includes error from weighing samples, esterification, syringe injection, UV detection and peak height measurement without using an internal standard. The analysis had an average relative error of 3.72% for analysis of known mixtures and a relative error of 7.2% for analysis of a bacteria sample. The simplicity in the preparation of the ester coupled with the chromatographic separation and the quantitation of the fatty acids should make this method a useful aid in the differentiation of microorganisms.

* * * * * *

Commercial materials and equipment are identified in this report to specify the investigative procedure. 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 Army Medical Department.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Howard C. Jordi, of Waters Associates, Inc., Milford, Massachusetts, for his useful discussions concerning the HPLC separations of fatty acid esters.

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