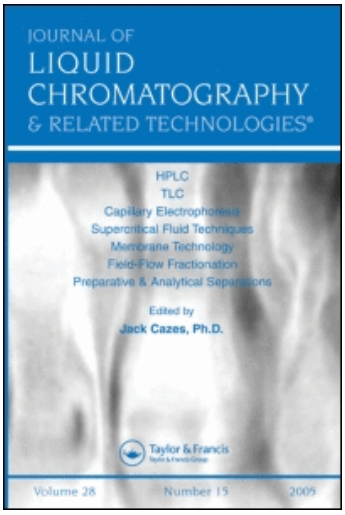

Journal of Liquid Chromatography & Related Technologies

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

Cellular Fatty Acid Composition of Vibrio parahaemolyticus by Reversed-Phase High-Performance Liquid Chromatography

L. D. Mell Jr.[a]; S. W. Joseph[a]; N. E. Bussell[b]
[a] Naval Medical Research Institute, Bethesda, Maryland [b] US Army Institute of Dental Research Walter Reed Army Medical Center, Washington, D.C.

To cite this Article Mell Jr., L. D. , Joseph, S. W. and Bussell, N. E.(1979) 'Cellular Fatty Acid Composition of Vibrio parahaemolyticus by Reversed-Phase High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 2: 3, 407 — 416
To link to this Article: DOI: 10.1080/01483917908060071
URL: http://dx.doi.org/10.1080/01483917908060071

JOURNAL OF LIQUID CHROMATOGRAPHY 2(3), 407-416 (1979)

Cellular Fatty Acid Composition of Vibrio parahaemolyticus by Reversed-Phase High-Performance Liquid Chromatography

L. D. Mell, Jr. and S. W. Joseph
Naval Medical Research Institute
Bethesda, Maryland 20014

N. E. Bussell
US Army Institute of Dental Research
Walter Reed Army Medical Center
Washington, D.C. 20012

ABSTRACT

High-performance liquid chromatography was used to separate and identify cellular fatty acids isolated from Vibrio parahaemolyticus, a gram-negative estuarine microorganism associated with seafood-borne enteritis in man. Fatty acids were isolated from statically grown bacterial cultures, saponified, and derivatized with an ultraviolet tag. Aliquots of derivatized fatty acids were injected onto a reversed-phase column with water:acetonitrile gradient as the mobile phase and ultraviolet detection at 254 nm. The predominant fatty acids found for the V. parahaemolyticus strains studied were C12, C14, C16:1, C16, C18:1, and C18. In addition, previously unreported fatty acids C13, C17, C19, and C21 were identified. Comparison of HPLC with GLC fatty acid separations showed good agreement with the exception that HPLC was able to resolve previously unidentified fatty acid constituents.

INTRODUCTION

Vibrio parahaemolyticus is an important cause of gastroenteritis. It must be differentiated from closely allied

vibrios which are involved in other types of human infection (1). Therefore, emphasis is being placed on criteria that separate microorganisms which have very few distinctly different characteristics (1,2).

Analysis of cellular fatty acids by gas liquid chromatography (GLC) has been used successfully to characterize some bacterial species including V. parahaemolyticus (3-6). When combined with mass spectrometry, rapid identification of fatty acids and other cellular chemical constituents is possible (6-9). Recently, liquid chromatography was used to identify cellular fatty acids from aerobically grown oral streptococci by identifying the fatty acid phenacyl esters using reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet detection (10).

HPLC combines greater sensitivity and better separation capability of high molecular weight fatty acids in comparison with GLC. These factors and the absence of a previous evaluation of this method using gram-negative bacteria led us to analyze the cellular fatty acids of several human V. parahaemolyticus isolates well-characterized by other methods. The HPLC method showed good agreement with results of GLC analysis, while revealing additional, previously undescribed, high molecular weight fatty acids. This suggests that HPLC may prove useful in detecting the presence and relative proportions of gram-negative bacterial cellular fatty acids for taxonomic purposes.

MATERIALS AND METHODS

Cultures and Harvesting

The cultures used were Vibrio parahaemolyticus strains DJ 7131, ATCC 27519, and ATCC 17802. They were grown in brain heart infusion (BHI) broth containing 2% NaCl. One liter of BHI (2% NaCl) in 2 l Erlenmeyer flasks was inoculated with 10 ml of cells grown without shaking in BHI broth overnight at 37°C.

Incubation was continued at 37°C without shaking for 24 h. The bacterial cultures were centrifuged in 250 ml polycarbonate screw-capped bottles at 24,000 x g for 20 min, and the supernatant discarded. The cells were then washed three times with phosphate buffered saline (PBS).

Saponification and Tagging

Washed cells (0.6 g) were added to 200 ml of 5% NaOH in 50% aqueous methanol. A 2 ml volume of this suspension, heated for 15 min in a 90°C water bath and adjusted to pH 2 with concentrated HCl, was added to 2 ml of saturated NaCl, extracted with five 10 ml portions of chloroform, dehydrated with anhydrous magnesium sulfate, and then taken to dryness with nitrogen.

The dried extract was resuspended in 1 ml of dimethylformamide (DMF) containing 30 μmoles of the ultraviolet tagging agent α-ρ-dibromoacetophenone (ρ-bromophenacyl bromide) and 60 μmoles of a catalyst, N,N-diisopropylethylamine. Aliquots of this mixture, after heating in a water bath at 60°C for 60 min and cooling to room temperature, were injected onto the reversed-phase columns.

Hydrogenation of Bacterial Extracts

Identification of unsaturated fatty acids was aided by hydrogenation of bacterial samples. A 2-ml sample of saponified bacterial mixture was placed in a hydrogenation vessel (Supelco Micro-Hydrogenator, Supelco, Inc., Bellefonte, PA 16823) with 10 ml of methanol and 20 mg of platinum oxide, pressurized to 10 psi with hydrogen, and the contents mixed for 45 min. The solution was removed, filtered, and extracted with five 10-ml portions of chloroform. This extract was then processed for ultraviolet tagging as described above.

High-performance Liquid Chromatography

The fatty acid phenacyl esters were analyzed using a Model 244 liquid chromatograph equipped with a Model 660 Solvent Pro-

grammer, Model 440 ultraviolet detector (254 nm), and two 30 cm X 4 mm μBondapak C_{18} reversed-phase columns all purchased from Waters Associates, Inc., Milford, MA 01757, in combination with a 7 cm X 4 cm ODS guard column (Whatman, Inc. Clifton, NJ 07014). The solvent system consisted of deionized water and acetonitrile and was programmed from 40%/60% to 100%/0% acetonitrile:water over a 3-h period using curve 5 (see Figure 1) on the solvent programmer and a flow rate of 1 ml/min. The separation was continued for 60 min after reaching final conditions. Tentative identification of phenacyl esters from V. parahaemolyticus was based on the retention volumes of standard fatty acids and by co-chromatography of known fatty acid standards added to extracted bacterial specimens prior to chromatography. The percent fatty acid composition was determined from the ratio of the peak area for each derivatized fatty acid to the total combined peak area.

Gas-liquid Chromatography

For comparison, cellular fatty acids were identified from V. parahaemolyticus using GLC. Fatty acid methyl esters were formed using boron trichloride and methanol (11, 12). Details of this procedure, including sample extraction and GLC conditions have been described previously (10).

RESULTS

Good separation of the phenacyl esters of all fatty acids of interest was achieved on the μBondapak C_{18} reversed-phase columns (Figure 1 and Figure 2). These chromatograms illustrate the applicability of the analytical procedure for bacterial fatty acid analysis.

Figure 1 shows a representative chromatogram for the HPLC separation of the fatty acid phenacyl esters of a standard mixture from caprylic acid, C8, to lignoceric acid, C24. The presence of fatty acids of chain lengths shorter than C12 was not verified since excess UV tagging reagent and most of the reaction by-

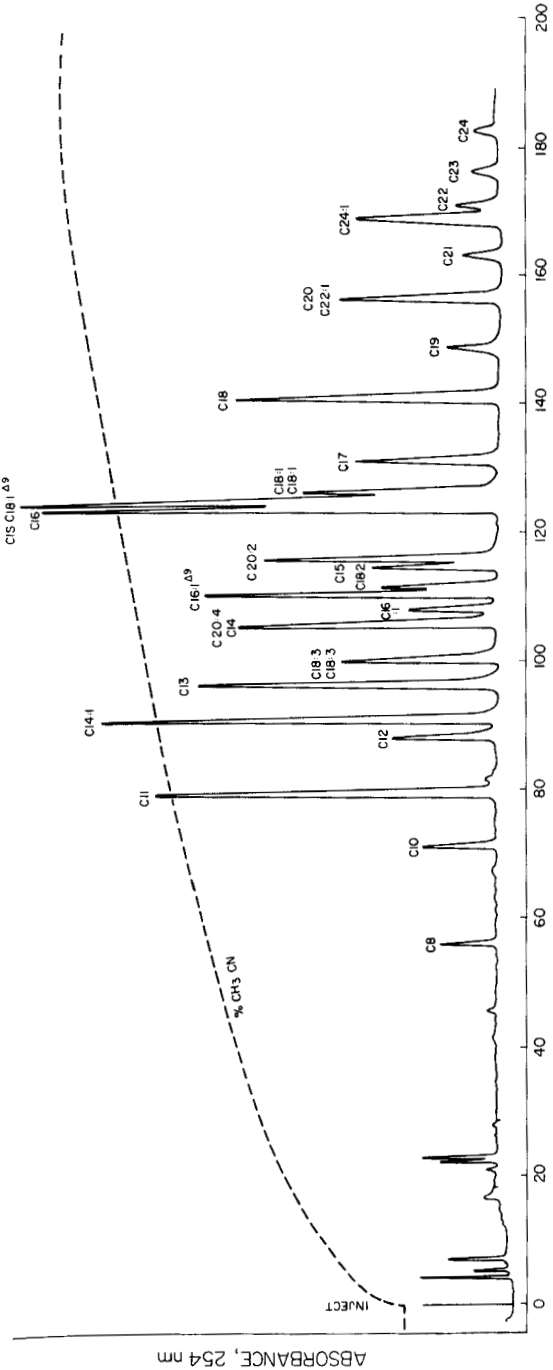

FIGURE 1. High-performance liquid chromatogram of fatty acids as phenacyl esters in a standard mixture. Sample injected, 100 µl of DMF containing 1 µg of each derivatized fatty acid: Detector sensitivity, 0.1 AUFS: Column temperature, 25°C: Chromatographic conditions are given in text. Dashed line represents water: acetonitrile gradient profile. Elaidic (trans 18:1) and vaccenic (18:1) acids co-elute as do α- and γ-linoleic (18:3) acids. The abbreviated formulas, e.g., C18 and C18:1, respectively, indicate the number of carbon atoms with no double bonds and those with double bonds.

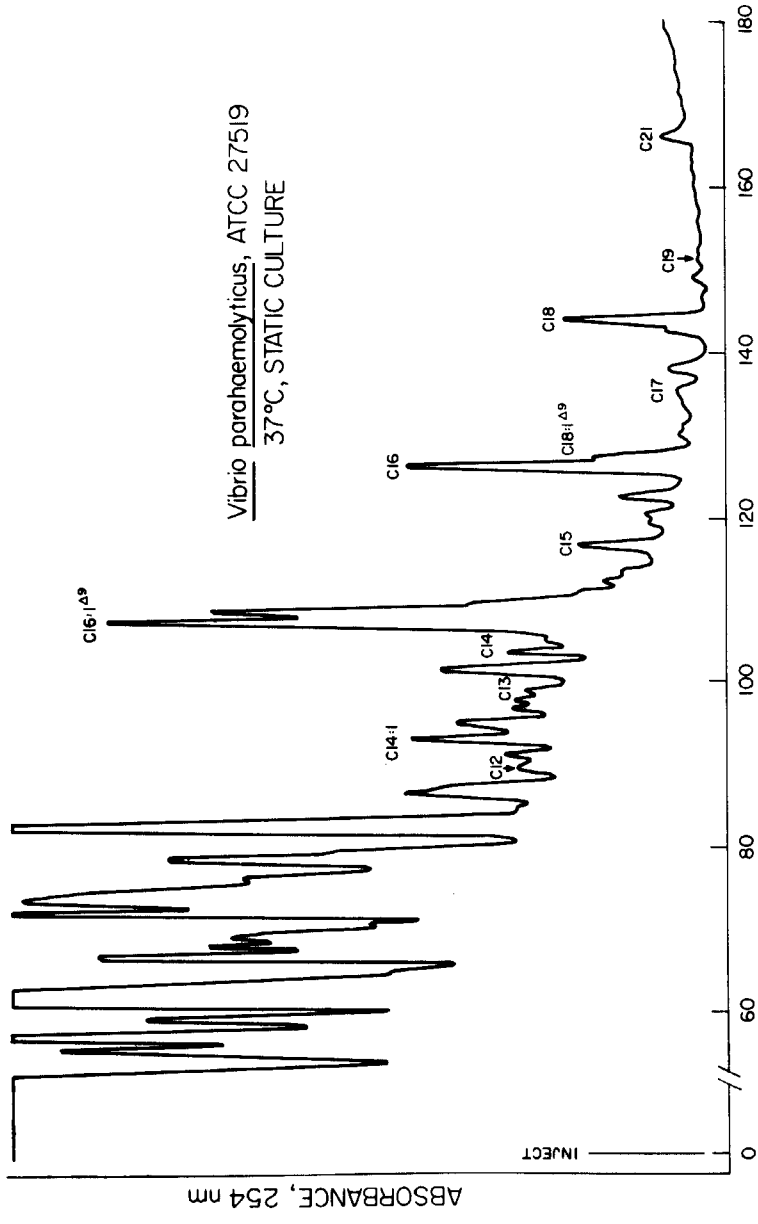

FIGURE 2. High-pressure liquid chromatogram of saponified cellular fatty acids as phenacyl esters extracted from Vibrio parahaemolyticus. Volume injected, 50 µl: Detector sensitivity, 0.1 AUFS: Column temperature, 25°C. Chromatographic conditions are given in text.

products elute before C12. High molecular weight fatty acids of chain length longer than C21, heneicosanoic acid, usually appeared in trace to almost undetectable amounts, and their presence was not listed in Table 1.

The fatty acid compositions obtained by HPLC for three strains of V. parahaemolyticus were qualitatively similar (Table 1). The predominant fatty acids were C12, C14, C16:1, C16, C18:1, and C18. Minor differences in the quantitative distribution of fatty acids for the three V. parahaemolyticus strains were observed. Strain 7131 appeared to lack C14:1 and C21 fatty acids, while strain 27519 showed a relatively higher proportion of C15 fatty acid. Approximately 20% to 30% of the relative fatty acid composition for the three strains in Table 1 could not be identified when compared with retention volumes for the standards in Figure 1. Several of the major unidentified peaks, by comparison to more recently obtained standards, presumptively appear to be iso- and anteiso- C13, C15, and C16 fatty acids (Figure 2).

Table 1 also lists the fatty acid composition obtained by GLC for the same strains and compares these results with previously published GLC data obtained for V. parahaemolyticus. Comparison of HPLC with GLC results shows general agreement between the fatty acid compositions obtained by the two methods for the major fatty acid constituents. The presence of C14:1 and C15 fatty acids in V. parahaemolyticus are confirmed, and previously unreported fatty acids C13, C17, C19 and C21 are identified (3, 4).

DISCUSSION

The use of HPLC for the determination of the fatty acid composition of V. parahaemolyticus, and other closely related gram-negative organisms, has not been reported previously. Comparison of HPLC with GLC fatty acid separations showed good agreement except that the HPLC technique was able to resolve previously unidentified fatty acid constituents. In this case,

TABLE 1

Percent Fatty Acid Composition of Vibrio parahaemolyticus by HPLC and GLC

HPLC	C12	C14:1	C13	C14	C16:1	C15	C16	C18:1	C17	C18	C19	C21	Miscellaneous
ATCC 27519	4.4[a] (0.6)	3.6 (2.1)	1.2 (0.5)	4.2 (0.5)	17.8 (3.2)	14.0 (1.6)	21.3 (3.2)	3.3 (1.3)	1.1 (0.9)	10.1 (.15)	0.8 (0.2)	1.5 (0.1)	19.5 (3.2)
ATCC 17802	4.1 (0.2)	3.6 (0.5)	2.1 (0.4)	3.5 (0.8)	26.9 (0.8)	4.5 (0.4)	13.9 (1.6)	4.9 (0.2)	1.4 (0.3)	6.3 (0.6)	0.4 (0.1)	1.0 (0.1)	28.8 (5.0)
DJVP 7131	4.9 (0.8)		1.2 (0.1)	3.6 (0.6)	28.3 (2.5)	3.5 (0.4)	15.9 (3.6)	3.2 (1.5)	0.5 (0.1)	10.9 (2.9)	1.1 (0.2)		27.0 (5.6)

GLC	C12	C14:1	C13	C14	C16:1	C15	C16	C18:1	C17	C18	Miscellaneous
ATTC 27519	2.8[b] (0.9)			6.0 (1.3)	24.7 (1.4)	0.4 (0.2)	20.7 (0.8)	10.7 (0.4)	0.3 (0.1)	2.2 (0.3)	33.9 (4.1)
ATCC 17802	3.0 (0.7)			3.3 (1.3)	19.8 (5.3)	3.6 (1.0)	15.9 (3.8)	13.4 (3.4)	0.2 (0.1)	2.0 (0.5)	38.7 (13.0)
DJVP 7131	2.9 (0.4)			5.6 (1.1)	22.6 (4.6)	4.8 (0.4)	19.3 (1.6)	12.1 (.17)		2.0 (0.2)	32.0 (9.5)
Reference 4	12.0			29.0		2.0	14.0				23.0
Reference 3[c]	1.7	0.9		5.1	32.2		36.5	18.1		3.1	2.4

[a]Mean, (S.D.), n=3 for separately grown bacterial cultures.

[b]Mean, n=3 for one bacterial culture.

[c]0.5% NaCl in TSB, 37°C.

increased sensitivity over GLC is obtained due to relatively simple ultraviolet tagging procedure, sensitive detection, and improved separation of HPLC over GLC. With increased sensitivity and selectivity a significant additional number of previously unidentified fatty acid components, particularly those having a chain length greater than C20, can be detected. In practice, the working range for HPLC can be extended to C40 (10), giving this technique a distinct advantage over GLC in the number of fatty acids that can be resolved.

The application of HPLC for rapid bacterial identification shows promise (10, 13). Precision and accuracy of the method was confirmed by comparison of analyses for fatty acids of V. parahaemolyticus from our two separate laboratories. In particular, this technique should be a valuable aid in further identification and differentiation of V. parahaemolyticus and other similar organisms which previously have presented problems for taxonomic description.

ACKNOWLEDGEMENTS

We thank Carol Adkins and Richard Gates for their technical assistance. We are grateful to Emilio Weiss for reviewing the manuscript.

This work was supported by the Naval Medical Research and Development Command Research Work Unit Nos. ZF51.524.009.0057 and ZF51.524.023.1007. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Medical Department, the naval service at large, or the Army Medical Department.

REFERENCES

1. Hollis, D. G., Weaver, R. E., and Backer, C. N., Thornsberry, C., J. Clin. Microbiol., 3, 425, 1976.
2. Colwell, R. R., J. Bacteriol., 104, 410, 1970.

3. Beuchat, L. R., and Worthington, R. E., Applied and Environmental Microbiology, 31, 889, 1976.
4. Deneke, C. F., and Colwell, R. R., Can. J. Microbiol., 19, 241, 1972.
5. Kaneda, T., J. Bacteriol., 95, 2210, 1968.
6. Moss, C. W., and Dees, S. B., J. Clin. Microbiol., 4, 492, 1976.
7. Lewis, V. J., Weaver, R. E., and Hollis, D. G., J. Bacteriol., 97, 1, 1968.
8. Moss, C. W., and Dees, S. B., J. Chromatogr., 112, 595, 1975.
9. Moss, C. W., Weaver, R. E., Dees, S. B., and Cherry, W. B., J. Clin. Microbiol., 6, 140, 1977.
10. Bussell, N. E., Miller, R. A., Setterstrom, J. A., and Gross, A., In Biological/Biomedical Applications of Liquid Chromatography, Hawk, G. D., (ed.), Marcel Dekkar, New York, NY, 1978.
11. Edwards, J. C., Williamson, B., and Cunfiffe, W. J., J. Chromatogr., 115, 283, 1975.
12. Moss, C. W., Lambert, M. A., and Merwin, W. H., Appl. Microbiol., 28, 80, 1974.
13. Miller, R. A., Bussell, N. E., and Ricketts, C., J. Liquid Chromatogr., 1, 291, 1978.