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Optimization of bacterial fatty acid methyl esters separation by gas chromatography - mass spectrometry

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ABSTRACT

Analysis of bacterial fatty acids as their methyl esters (FAMEs) has been extensively used for rapid diagnosis of anaerobic infections, bacterial classification and taxonomy. This makes bacterial FAMEs and especially hydroxy FAMEs analyses of primary importance for clinical and taxonomical interests. However, selection of the appropriate column and other chromatographic parameters makes it an intricate issue for the simultaneous separation of various classes of compounds. For the resolution of these complex mixtures, we have compared four bonded capillary columns OV1, HP-INNOwax, DB5, and VF5-ms of the same length, internal diameter and coated film thickness. Experimental conditions such as gas flow rate and oven temperature programming were optimized to achieve the best resolution on these columns. The standard FAMEs used in this study contained 6 hydroxy fatty acids, two geometric isomers, two positional isomers in branch chain, a cyclopropane and short and long chain components. We have found that OV1 capillary column of low polarity could give best separation of all classes of bacterial FAMEs present in a mixture. Identification of the FAMEs was carried out from their mass spectral fragmentation.

Keywords: Bacterial fatty acids; branch chain fatty acids; gas chromatography; hydroxy-FAMEs; mass spectrometry.

INTRODUCTION

Fatty acid (FA) analysis is important in lipid biochemistry, microbiology and clinical manifestations. Branch-chain, iso-, anteiso- and hydroxy fatty acids occur in bacteria as their major acyl constituents, which are important taxonomic indicators (Lu & Harrington, 2010). The iso-, anteiso-, and omega-alicyclic acids support bacteria in normal as well as extreme environmental habitats. These fatty acids and other oxylipins, as major cellular components are

also important markers used in the identification and bacterial classification (Lu & Harrington, 2010; Garcia-Barcelo *et al.*, 1993). Bacterial identification, using phenotypic and genotypic groups is common (Amy *et al.*, 1992) but a comparison of membrane short chain fatty acids and others are also frequently used for this purpose (Kim *et al.*, 2000).

In addition, structural diversity of membrane phospholipids coupled with biological specificity are markers for microbial identification (Muller *et al.*, 1993). It is common knowledge that phospholipid fatty acids (PLFAs) are distinctive components of cell membranes forming about 50 % of eukaryotic and almost 98 % of bacterial membrane lipids (Jiasong *et al.*, 2000). Therefore, analysis of PLFAs mixture is an additional tool in bacterial classification, chemo-taxonomy (Xu *et al.*, 2000), bacterial subgrouping, and infectious diagnosis (Drucker, 1993).

Numerous extraction and chromatographic procedures have been used for analyses of FA (Hauff & Vetter, 2010, Gu, *et al.*, 2010). Head-space gas liquid chromatography and recently introduced two dimensional GC (GC-GC) remain powerful tools in bacterial FAMEs profiling (Hauff & Vetter, 2010, Gu, *et al.*, 2010).

However, to exploit separation techniques, a meticulous column selection is an important prerequisite for an adequate resolution of complex mixture of FAMEs. Imperfect column selection or gas chromatography (GC) experimental conditions may result in an inadequate resolution, especially for hydroxy-FAs. Presence of hydroxy-FAs in a mixture may cause a baseline drift with broad and/or unresolved peaks. Due to their susceptibility to oxidation, hydroxy-FAs may also give rise to their corresponding oxo-derivatives which may interfere in resolving these FA (Akoto *et al.*, 2008). Thus, derivatization of hydroxy-FAs has been proposed for better GC resolution. This includes esterification or silylation of the hydroxyl-group, which may restore peaks for the hydroxy-FAs (Moll *et al.*, 1992). However, this procedure causes a shift in the retention time resulting in co-elution of hydroxy-FAs double derivative of the FAMEs mixture on CP-Sil5 CB WCOT column. For example, C14:0 3-OH trifluoroacetyl derivative (TFA) is known to co elute with i-C15:0 FA (Moss *et al.*, 1973).

Branch-chain, cyclopropane and geometric isomers of FA, present in a mixture add to the difficulty for their GC resolution. Thus, separation of complex FAMEs mixture by GC is a challenge especially when it contains hydroxy-FAs. For this purpose, numerous GC columns have been tried, resulting in partial or near partial resolution. For example, Alltech AT-Silar-90 capillary column has been used to resolve only geometric isomers using GC-MS (Huang *et al.*, 2006). Other polar stationary phases, such as cyanoalkyl polysiloxane, have been used for the separation of complex geometric isomers in

FAMEs mixture (Huang *et al.*, 2006). In the present study, we have examined and compared low, medium and high polarity stationary phases for the simultaneous resolution of different classes of FAMEs of bacterial origin and have achieved an excellent resolution of six hydroxy FAMEs acids in a mixture of 26 FAMEs of bacterial origin.

MATERIALS AND METHODS

A mixture of standard FAMEs CP Mix, # 47080-U containing six hydroxy (2,5,6,11,12,19), two positional (8,9), two geometric isomers (21,22) and one cyclopropane FA (17), commonly found in bacteria, was purchased from Supelco (Bellefonte, PA, USA). An Agilent (Santa Clara, CA, USA) GC model-689 interfaced with an Agilent MSD model-5973 and an Agilent autosampler model-7683 was used in a 1/50 split mode with helium carrier gas at 0.8 mL min^{-1} flow rate. The columns used in this study were (1) OV1 (Marietta, Ohio, USA), (2) HP-INNOwax (Agilent), (3) TR5MS (DB5, Agilent) and (4) VF5-ms (Agilent). All columns were of equal length, id. and film thickness (30m $\times$ 0.25mm $\times$ 0.25μm). The column composition was as follows: OV1-nonpolar, 100% methylpolysiloxane; HPINNOwax-highly polar, bonded and cross linked polyethylene glycol; TR5MS (DB5)-nonpolar low bleed, coated with 5% phenyl 95% dimethylpolysiloxane; VF5-highly inert, coated with 5% phenylmethyl polysiloxane. The HPINNOwax, DB5, and VF5 columns were from Agilent. The inlet temperature was maintained at 250^{o}C and the oven was programmed as follows: initial temperature 50^{o}C, (1 min), ramp 25^{o}C min-1 to 200^{o}C (10 min), ramp 10^{o}C to a final temperature 300^{o}C (10 min). Agilent software was used to analyze data and NIST library was used for identification of the FAME peaks.

The standard 26 component FAMEs mixture was resolved on all four columns and the peaks were identified from their elution order, retention time and mass spectral fragmentation. The peaks after identification were numbered as follows:

1. Undecanoate (11:0); 2. 2-Hydroxydecanoate (2-OH-10:0); 3. Dodecanoate (12:0);

4. Tridecanoate (13:0); 5. 2-Hydroxydodecanoate (2-OH-12:0);

6. 3-Hydroxydodecanoate (3-OH-12:0); 7. Tetradecanoate (14:0);

8. 13-Methyltetradecanoate (i-15:0); 9. 12-Methyltetradecanoate (a-15:0);

10. Pentadecanoate (15:0); 11. 2-Hydroxytetradecanoate (2-OH-14:0);

12. 3-Hydroxytetradecanoate (3-OH-14:0); 13. 14-Methylpentadecanoate (i-16:0);

14. cis-9-Hexadecenoate ($16:1^{9}$); 15. Hexdecanoate (16:0);

16.15-Methylhexadecanoate (i-17:0); 17. Methyl-cis-9,10-mthylenedexadecanoate (17:0);

18. Heptadecanoate (17:0); 19. 2-Hydroxyhexadecanoate (2-OH-16:0);

20. Methyl-9,12-octadecadienoate ($18:2^{9,12}$); 21. 9-Octadecenoate ($18:1^{9c}$);

22. 9-Octadecenoate ($18:1^{9t}$); 23. Octadecanoate (18:0);

24. Cis-9,10-methyleneoctadecanoate (19:0);

25. Nonadecanoate (19:0); 26. Eicosanoate (20:0).

RESULTS AND DISCUSSION

The elution pattern of OV1 and VF5 columns was the same. However, the elution rates were different for two columns. Therefore, the chromatographic time on VF5 column was longer (34 min.) to elute 21 components of the mixture while it took 22 min. to elute all 26 components of the mixture on OV1 column of the same length. This shows that under experimental conditions all components of the FAMEs mixture were not resolved on VF5 column. On VF5 column, the medium chain FAMEs with C11, C12 and C13 did not appear in the chromatogram. In addition, from a total of six hydroxy FAMEs in the mixture, only four hydroxyl FAMEs appeared in the chromatogram. The medium chain hydroxy FAMEs namely 2-OH- and 3-OH-12:0 acids were not detected using VF5 column. The HP-INNOwax column eluted saturated before the corresponding unsaturated FAMEs while their elution was reversed on the OV1 column. The HP-INNOwax column resulted in the separation of only 24 components of the mixture containing 26 FAMEs in 47 min. chromatographic run time. Again 2-OH- and 3-OH-12:0 were not resolved on this column. Therefore, for the resolution of hydroxy FAMEs, HP-INNOwax column behaved similar to VF5. Thus, under the experimental conditions used in this study, HP-INNOwax and VF5 columns may not be appropriate for the separation of a mixture of OH-FAMEs of bacterial origin.

Ineffective resolution of hydroxy-FAs may be a sensitive issue for analysis of FAMEs mixtures especially when these have a clinical significance and are also used as bacterial biomarkers (Alex *et al.*, 2004). A mixture containing antimicrobial 2-hydroxy-FAs of C_{14}-C_{18} chain length has been used as a biomarker for Sacchramycopsis species (Kurtzman *et al.*, 1973). Similarly, dihydroxy and trihydroxy-fatty acids have been used as bacterial biomarkers (Hou & Bagby, 1991). This signifies the importance of hydroxy-fatty acids and their resolution is a prerequisite for FAMEs profiling in bacterial identification.

Elution of monoenoic and dienoic FAMEs, such as ($18:1^{9}$) (21) and ($18:2^{9,12}$) (20) was reversed on HP-INNOwax as compared to OV1, VF5 and DB5 columns

which may be due to differences in stationary phase polarity. On HP-INNOwax column the $18{:}1^{9c}$ (21) eluted first followed by ($18{:}2^{9,12}$) (20) while on OV1, VF5 and DB5 columns, these FAMEs were eluted in the reverse order. The resolution of geometric isomers, $18{:}1^{9c}$ (21) and $18{:}1^{9t}$ (22) was better on OV1, DB5 and VF5 columns as compared to HP-INNOwax column (Figs. 1 and 2). The isomer (22) eluted before the isomer (21) on all three columns. Separation of isomeric components depends on many factors that include chemical nature of the stationary phase, column efficiency, sample capacity and column length and optimizing one of these factors may cause deterioration of the other.

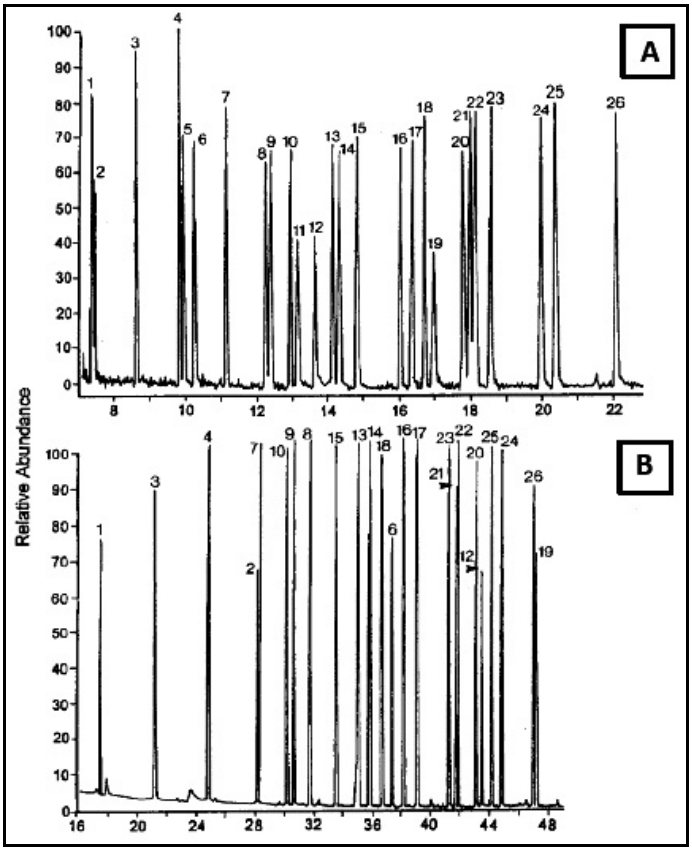

Fig. 1. GC separation of bacterial fatty acid methyl esters on (A) OV1 and (B) DB5 column. Peak number corresponding to compound number and their retention times are given in Table 1.

All 26 FAME components of the mixture were resolved on a DB5 column in 30 min. run time (Fig. 2A). The FAMEs retention on stationary phase is known to occur solely by gas-liquid partitioning, which may explain different run time on different stationary phases of these columns (Collin *et al.*, 2000). Separation

of all 26 FAME components in the mixture was best achieved on a non-polar OV1 column, which showed sharp and symmetrical and well resolved peaks (Fig. 1A).

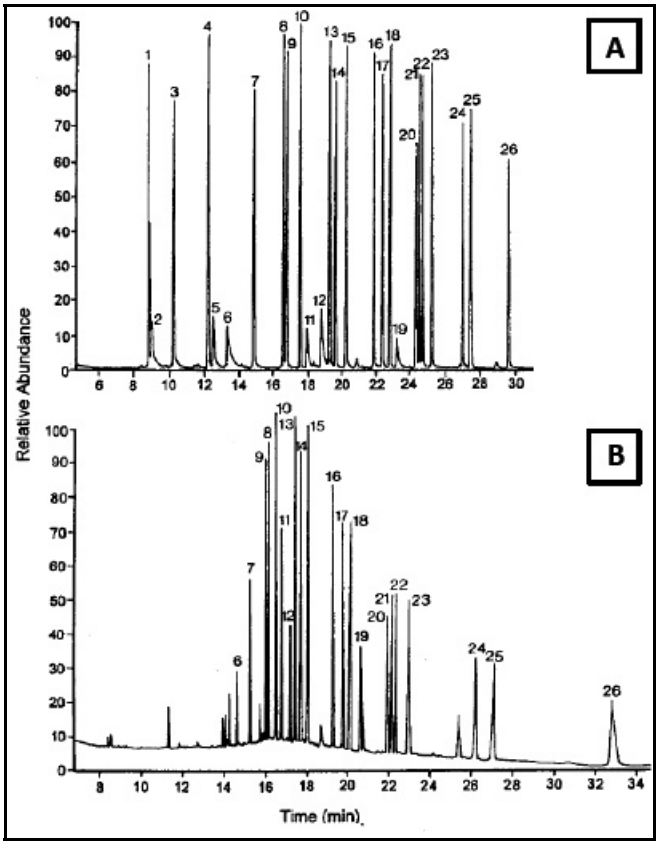

Fig. 2. GC separation of bacterial fatty acid methyl esters on (A) HP-INNOwax and (B) FX5 capillary column. Peak number corresponding to compound number and their retention times are given in Table 1.

All FAME components including six hydroxy-FAs (2,5,6,11,12,19), two branch chain (8,9), cyclopropane (17) and as well as two isomeric congeners (21,22) could be well resolved on this column and were identified from their mass spectral data (Table 1). With its low bleed, the OV1 column had the advantage to give unparallel resolution, especially for hydroxy-FAMEs components and geometric isomers which is a precondition for MS identification. Our results indicate that OV1 capillary column offers better promise over the other columns for separation of bacterial FAMEs including OH-FAMEs.

Table 1. Mass spectral fragmentation and retention time of bacterial FAME mixture on OV1 column

Peak #	FAME	RT	Fragmentation m/z
1	11:0	7.375	M+. 200, 169, 157, 87, 74B, 55
2	2-OH-10:0	7.449	M+. 202, 143, 90, 83, 69B, 55
3	12:0	8.640	M+. 214, 171, 143, 87, 74B, 55
4	13:0	9.840	M+. 228, 185, 143, 87, 74B, 55
5	2-OH-12:0	9.935	M+. 202, 143, 83, 69B, 55
6	3-OH-12:0	10.250	M+. 230, 103B, 71, 61, 55
7	14:0	11.158	M+. 242, 199, 143, 87, 74B, 55
8	i-15:0	12.261	M+. 256, 213, 143, 87, 74B, 55
9	a-15:0	12.399	M+. 256, 199, 87, 74B, 69, 55
10	15:0	12.942	M+. 256, 213, 87, 74B, 55.
11	2-OH-14:0	13.142	M+. 230, 171, 97B, 90, 83, 57
12	3-OH-14:0	13.644	M+. 258, 103B, 71, 61, 55
13	i-16:0	14.147	M+. 270, 227, 143, 129, 87, 74B
14	$16:1^{9}$	14.342	M+. 268, 236, 194, 152, 74, 55B
15	16:0	14.839	M+. 270, 227, 143, 87, 74B, 55
16	i-17:0	16.039	M+. 284, 241, 199, 143, 87, 74B, 57
17	17:0; (9,10-cyclopropane)	16.371	M+. 282, 250, 208, 96, 83, 69, 55B
18	17:0	16.725	M+. 284, 241, 185, 143, 87, 74B, 57
19	2-OH-16:0	16.977	M+. 286, 227, 111, 90, 83, 69, 55B
20	$18:2^{9,12}$	17.822	M+. 294, 263, 109, 81, 67B, 55
21	$18:1^{9c}$	17.988	M+. 296, 264, 222, 180, 111, 55B
22	$18:1^{9t}$	18.125	M+. 296, 264, 222, 180, 111, 97,55B
23	18:0	18.577	M+. 298, 255, 199, 143, 87, 74B, 55
24	19:0; (9,10-methylene)	19.994	M+. 310, 278, 236, 111, 97, 83, 55B
25	19:0	20.366	M+. 312, 269, 143, 87, 74B, 69, 55
26	20:0	22.086	M+. 326, 143, 87, 74B, 69, 55
			B = Base peak

CONCLUSION

OV1 non-polar column coated with 100% dimethylpolysiloxane is suitable for the separation of OH-FAMEs of bacterial taxonomic and physiological importance. However, resolution of PUFA geometric isomers may be difficult to obtain on this column.

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الفصل الأمثل للأحماض الدهنية ذات الاسترات المثيلية في البكتيريا بواسطة مقياس الطيف الكتلي والكروماتوغرافي الغازية

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خلاصة

تستخدم الأحماض الدهنية متمثلة باستراتها المثيلية على نطاق واسع في التشخيص السريع للإصابة بالبكتريا اللاهوائية وفي تصنيف البكتيريا. وهذا يجعل من الأحماض الدهنية وبشكل خاص الهيدروكسيدية منها ذات أهمية أساسية في التطبيقات السريرية والتصنيفية. ومع ذلك فإن اختيار العمود الكروماتوغرافي المناسب والمعايير الكروماتوغرافية الأخرى لفصل مختلف أنواع المركبات بشكل متزامن يشكل مسالة معقدة.

ولفصل هذه المركبات المعقدة قمنا بمقارنة أربعة أنواع من الأعمدة الكروماتوغرافية الشعيرية المتصلة OV1, HP-INNO wax, DB5 and VF5-ms المتماثلة بالطول والقطر الداخلي وسماكة التغليف الداخلي، وأما المعايير المختبرية لهذه الأعمدة مثل معدل تدفق الغاز وبرمجة حرارة الفرن فقد كانت مثالية من أجل الحصول على أفضل فصل للأحماض الدهنية.

وقد احتوت عينة الأحماض الدهنية القياسية التي استخدمت في هذه الدراسة على ستة أحماض دهنية هيدروكسيدية، اثنان من الايزومرات الهندسية، اثنان من الايزومرات الموضوعية المتفرعة السلاسل، سيكلوبروبين واحد، ومكونات ذات السلسلة القصيرة والطويلة.

وقد وجدنا بأن أفضل فصل لكافة أنواع الأحماض الدهنية ذات الاسترات المثيلية في البكتريا قد تم باستخدام عمود الكروماتوغرافي الشعيري OV1 منخفض القطبية. وأما التعرف عليها فقد تم بواسطة تفتيت الكتلة الطيفية لها.