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# Profile of Mycolic Acid Cleavage Products of Isoniazid Resistant *Mycobacterium tuberculosis* Isolate By Gas Chromatography-Mass Spectrometry

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**ABSTRACT**

Mycolic acid cleavage products (MACPs) profile of *Mycobacterium tuberculosis* (MTB) which is resistant to isoniazid (INH) has been studied. This study describes the profile of MAs based on their cleavage products (MACPs) after esterification process, using $BF_3$ in methanol as a catalyst, by gas chromatography-mass spectrometry detector (GC-MSD). The MACPs profile of INH resistant MTB was compared to MACPs of H37Rv strain and MACPs of INH sensitive MTB isolate. Objective of this study was to obtain the characteristic profile of cellular fatty acid and MACPs of INH-resistant MTB isolate using GC-MS. Samples were cultured in Middlebrook 7H10 medium for more than 21 days. MAs and cellular fatty acids were extracted with chloroform prior to esterification for 1 hour at (90-95)° C using methanolic-BF3 as a catalyst. MACPs were extracted with heptane before injected into GC-MSD. This study obtained that the profile of six characteristic peaks of cellular fatty acids and MACPs of samples of INH-resistant MTB isolate was not significantly different to the INH-sensitive MTB isolate. But, the profile of H37Rv strain was significantly different compared to both INH-resistant MTB and INH-sensitive MTB isolate.

**Keywords:** Mycolic acids, INH resistant *M. tuberculosis*, *M. tuberculosis*, GC-MS

## INTRODUCTION

Mycolic acids (MAs) are specific lipid fractions of the outer cell wall of *Mycobacterium* species. MAs have three major structure types including alpha, keto and methoxy mycolic acid. Keto and methoxy mycolic acid have cis and trans-isomers as shown in figure 1. Biosynthesis of MAs involved many enzymes which are the isoniazid (INH) target. Those enzymes are responsible for the initiation, elongation and modification structures of MAs by introduction of methyl, methoxy, keto and cyclopropane ring into meromycolate chain[2,3]. Only pathogenic slow growing mycobacteria (such as *M. tuberculosis* produce significant amounts of cyclopropanated MAs[2]. Different strains of *M. tuberculosis* (MTB) contain a different complex mixture of MAs that can provide a fingerprint[4]. On the other hand, inhA, katG, ahpC and kas A gene mutations, have been found in INH resistant MTB[5]. Those enzymes were responsible in synthesis of MAs meromycolate chain. Therefore, MAs of INH-resistant MTB were estimated to have an anomaly structure and characteristic composition. Yet, no publication has reported the MAs profile of INH-resistant MTB. This research will verify the anomaly of MAs profile of INH-resistant MTB using Gas Chromatography-Mass Spectrometry Detector (GC-MSD). The different of MAs profile between INH-resistant MTB and H37Rv strain indicates that there is a modification of MAs biosynthesis. Identification of MAs composition can be based on both chromatogram profile of MAs molecule[6-10] and MAs cleavage products (MACPs)[11-13]. Identification of MAs molecule profile of mycobacteria species using High Performance Liquid Chromatography (HPLC) with UV detector have confirmed that isolate of drug resistant MTB produced MAs profile that was looked like *M. bovis*[9]. But, that article did not clearly explain the drug resistant MTB isolate criteria and the substance that has made MAs of MTB isolate profile to be different in comparison with *M. bovis*. The MACPs profile of MTB has been used for MTB identification from culture or sputum sample[13,14]. The MACPs profile was obtained by GC-MSD using various esterification procedures. The MACPs profile showed that hexacosanoic acid ($C_{26:0}$) is characteristic as a dominant peak of α-alkyl MAs of MTB and its amount is always greater than that of tetracosanoic acid ($C_{24:0}$)[11,15]. The combination of tuberculostearic acid (TBSA) and hexacosanoic acid found to be the most discriminative biomarker for differentiation of MTB from Non-MTB[14]. TBSA ($C_{20}H_{40}O_2$) is a well known cellular fatty acid that characteristically produced by mycobacterium species, included MTB complex complex[11,16]. However, these markers are not sufficiently specific for MTB because they also occur in several Non-MTB species. Therefore, Dang et.al[13] used 20 biomarker compounds and chemometrics approach for detection the differentiation between MTB and Non-MTB species. The purpose of this study was to obtain the characteristic profile of MACPs of INH resistant

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Table 1: Identification of peaks chromatogram of MTB samples

| Code | tr (minute) | Identity of fatty acids methyl ester base on Wiley 08 | M/e | Similarity | |
|---|---|---|---|---|---|
| 1. | 3.90 | Tetradecanoic acid | 242 | 98% | C14:0 |
| 2. | 5.26 | Pentadecanoic acid | 256 | 98% | C15:0 |
| | | 7-hexadecenoic acid | 268 | 99% | C16:1 |
| 3. | 6.80 | 9-hexadecenoic acid | 268 | 99% | C16:1 |
| 4. | 6.89 | 9-hexadecenoic acid | 268 | 99% | C16:1 |
| 5. | 7.28 | Hexadecanoic acid | 270 | 99% | C16:0 |
| 6. | 9.14 | Cyclopropaneoctanoic acid, 2-hexyl- | 282 | 90% | |
| 7. | 9.76 | Heptadecanoic acid | 284 | 99% | C17:0 |
| | 10.93 | Heptadecanoic acid, 10 methyl | 298 | 93% | C17:0 |
| 8. | 11.73 | 12-octadecadienoic acid | 294 | 94% | C18:2 |
| 9. | 11.96 | 9-octadecenoic acid | 296 | 99% | C18:1 |
| 10. | 12.15 | -octadecenoic acid | 296 | 99% | C18:1 |
| | 12.73 | Heptadecanoic acid, 16-methyl | 298 | 97% | C17:0 |
| 11. | 12.76 | Octadecanoic acid | 298 | 98% | C18:0 |
| 12. | 14.06 | Octadecanoic acid, 10-methyl | 312 | 99% | C18:0 |
| 13. | 19.38 | Eicosanoic acid | 326 | 99% | C20:0 |
| 14. | 24.21 | Docosanoic acid | 264 | 96% | C22:0 |
| 15. | 27.88 | Tetracosanoic acid | 354 | 99% | C24:0 |
| 16. | 29.45 | Pentacosanoic acid | 382 | 99% | C25:0 |
| 17. | 29.88 | Unknown | 422 | | |
| 18. | 30.96 | Hexacosanoic acid | 410 | 95% | C26:0 |

Table 2: Area normalization (%) of cellular fatty acids and MACPs of MTB

| | Analyt area (%) at retention times (minutes) | | | | | |
|---|---|---|---|---|---|---|
| Samples | 7.3 | 11.96 | 12.76 | 14.05 | 27.93 | 30.96 |
| H37Rv strain | 27.68 (±3.69) | 19.24 (±4.45) | 9.73 (±1.21) | 18.15 (±4.26) | 2.46 (±0.69) | 11.13 (±1.30) |
| INH-sensitive isolate | 25.90 (±2.04) | 37.62 (±4.21) | 9.33 (±0.78) | 8.07 (±3.98) | 1.17 (±0.45) | 6.57 (±2.45) |
| INH resistant Isolate | 23.86 (±3.15) | 30.42 (±6.11) | 10.33 (±2.12) | 9.07 (±2.53) | 3.03 (±2.58) | 10.57 (±4.65) |

(Standard deviations are given in the bracket)

MTB based on the dominant and characteristic peaks of GC-MSD chromatogram that can be used for identification of INH-resistant MTB. The characteristic profile of MACPs in INH-resistant MTB will be an important data base for the detection of TB infection. Experimentally, the MACPs profile of INH resistant MTB was compared to both H37Rv strain and INH sensitive isolate.

## MATERIALS AND METHODS

*Materials*

Solid media agar base of Middlebrook 7H10 enriched with Oleic Albumin Dextrose Catalase (OADC) growth supplement (Sigma-Aldrich). Trehalose 6,6' dimycolate (TDM) isolated from *M. tuberculosis* produced by Alexis Biochemical's and tricosanoic methyl ester (TME) (Sigma-Aldrich). The reagents used for sample preparation were: KOH, NaOH, HCl, $Na_2SO_4$ *exciccatus*, NaCl, $BF_3$14% in methanol, methanol, heptanes and chloroform (E.Merck). All of the reagents were pro analyze grade except for heptane which was pro GC.

*Sample*

$H_{37}$Rv strain (n=6), 3 isolate of INH sensitive MTB (n=6) and 6 isolate of INH resistant MTB (n=6). Samples were supplied from Clinical Microbiology Department of Dr. Soetomo hospital, Surabaya, Indonesia. All isolate samples were re-checked for their sensitivity to INH using a MGIT™ sensitivity test prior to being cultured. Samples were cultured at 37°C for 21-30 days. Biomass (25-50 mg) was placed in pyrex tube that contained 2 ml of 25% KOH in a mixture of methanol:water (1:1) and autoclaved for 1 hour at 121°C [1]. After cooling to room temperature the tube was brought out of a BSL-3 facility room for further processed at pharmaceutical analysis laboratory.

*Instrument*

Agilent 6890 Network GC system with autosampler, J&W Scientific, HP-5 5% phenylmethylsiloxane (30 m x 0.32 mm x 0.25 µm) column, Agilent 5973 inert MSD. The optimum operational conditions of GC-MSD were as follows. Inlet and detector temperatures were set at 285°C, carrier gas (helium) flow rate was 1 ml/minute, 5 µl samples was injected by *split-less* technique. Column temperature was programmed as follow. Initial temperature was 180 °C and held for 6 minutes, increased by 2 °C/ minute to 200 °C, maintained at 200°C for 1 minute, increased by 5 °C/minute to 275 °C, maintained at 275 °C for 9 minutes, increased by 5°C/minute to 300 °C and finally maintained at 300°C for 14 minutes. The mass spectra were obtained by electron impact (EI) at ionization energy of 69.9 eV. A mass range of MSD was set to detect analyte ion of 20-700 atomic mass units.

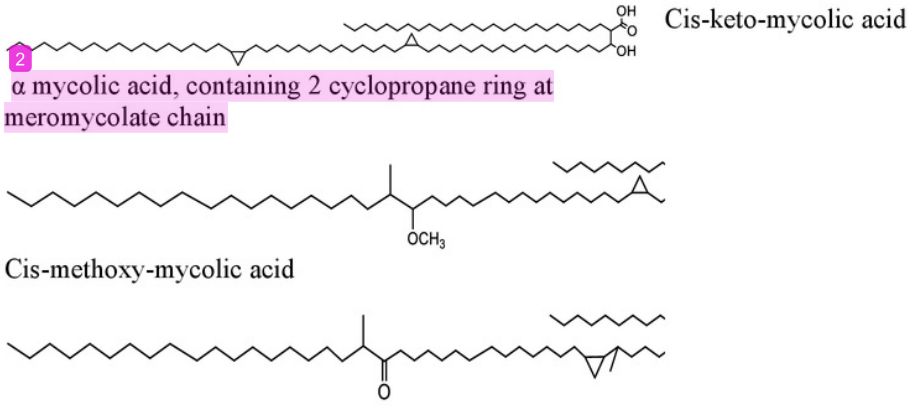


Figure 1: Mycolic acids structure of *M. Tuberculosis*[1]

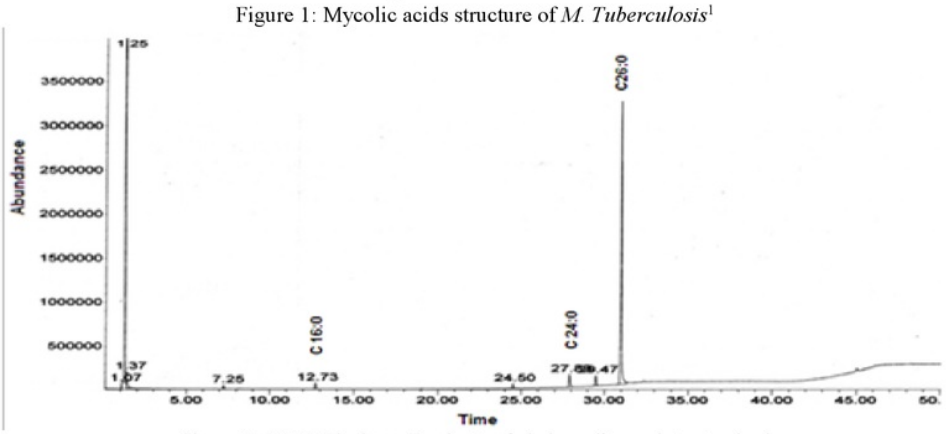


Figure 2: MAS Cleabage Products of ehalose dimycolate standard

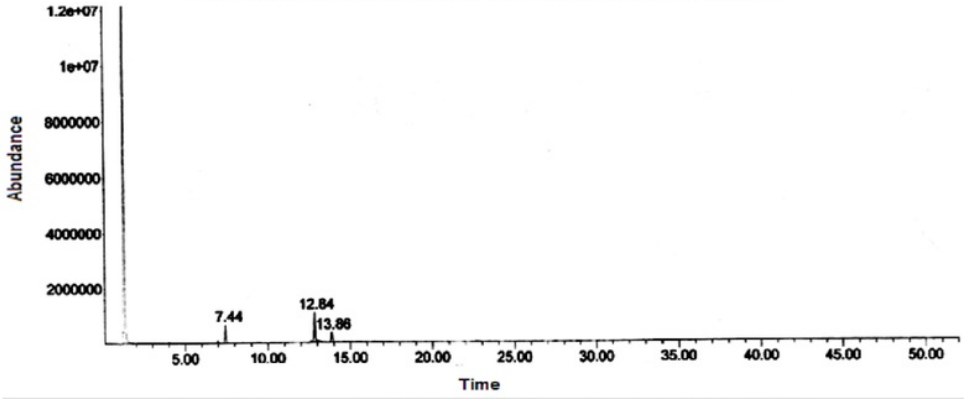


Figure 3: fragments of Middlebrook 7H10 media enriched with OADC

*Isolation and esterification of mycolic acids of Mycobacterium tuberculosis*

The Pyrex tube containing saponified MAs was added with 1.5 ml hydrochloric acid (1:1) prior to extract with 3 x 2 ml of chloroform. The collected chloroform extracts were dried in a gentle stream of $N_2$ gas. 1.0 ml of 0.5N methanolic-NaOH was added to the dried MAs extract in a pyrex cupped tube and heated on the water bath for 5 minutes at 95°C. The sample was cooled to room temperature before the addition of 1.0 ml 14% $BF_3$ in methanol and heating in water-bath at 95°C for 1 hour. The obtained methyl ester MAs cleavage products were extracted using 3x1 ml heptane by agitating on vortex for 2 minutes. Heptane extract was separated by centrifugation at 2000 rpm for 2 minutes and transferred to another clean tube. Heptane extract added with 2 ml saturated NaCl and agitated for 2 minutes before separated to another tube. 1 g of $Na_2SO_4$ *exsiccatus* was added to heptane phase before

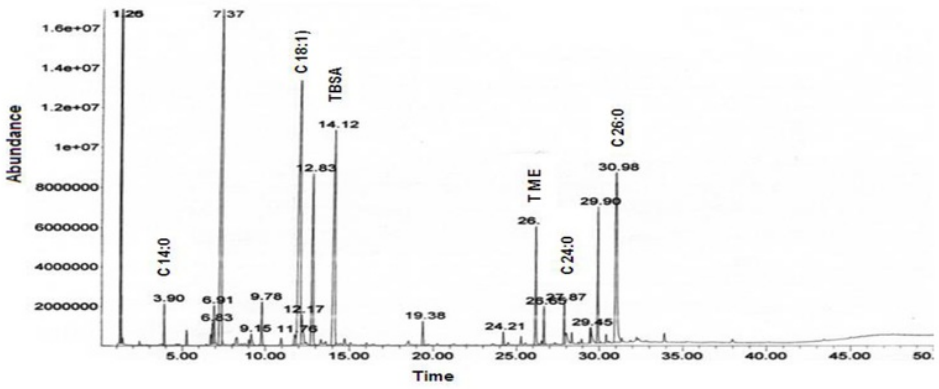


Figure 4: MAS Cleavage products of H 37 RV added with TME

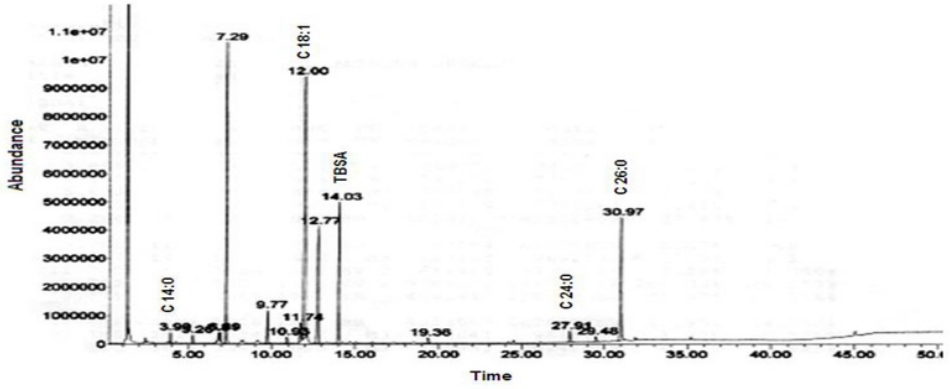


Figure 5: MAs cleavage products of INH sensitive MTB isolate

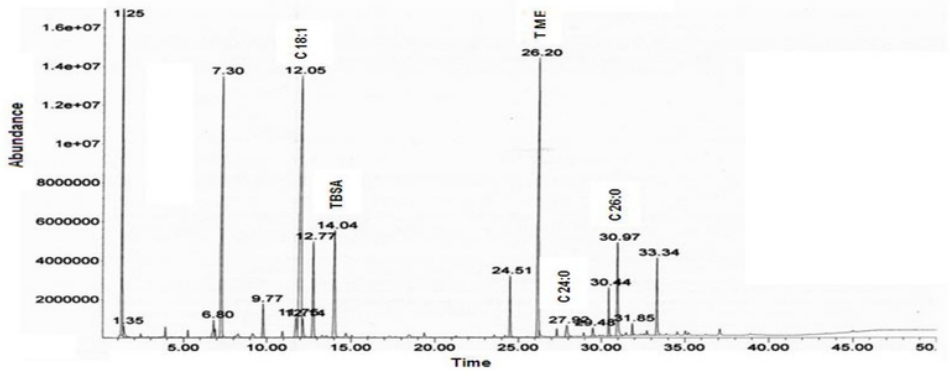


Figure 6: MAs cleavage products of INH resistance MTB isolate added with TME

the extract dried using a stream of $N_2$ gas. The dried extract was re-dissolved in 0.30 ml heptane prior to injection in to GC-FID. TME as internal standard was added to the dried MAs extract in a Pyrex cupped tube, before the esterification process[17].

*Data analysis*

Analyte peaks in chromatogram were identified based on the highest similarity with the data base of Wiley 08. TME ($C_{23:0}$) was used as an internal standard for correcting the analyte retention time ($t_r$) variation[17]. TME was used since this substance was not detected in all of sample chromatograms. Samples of chromatogram profile were analyzed based on characteristic peak areas after the normalization process. The similarity among samples peak profile was determined based on the Pearson correlation test using SPSS program[18]. ANOVAs test at significant level of 0.05 was used to determine the differences among the variation of the six characteristic peak areas in sample

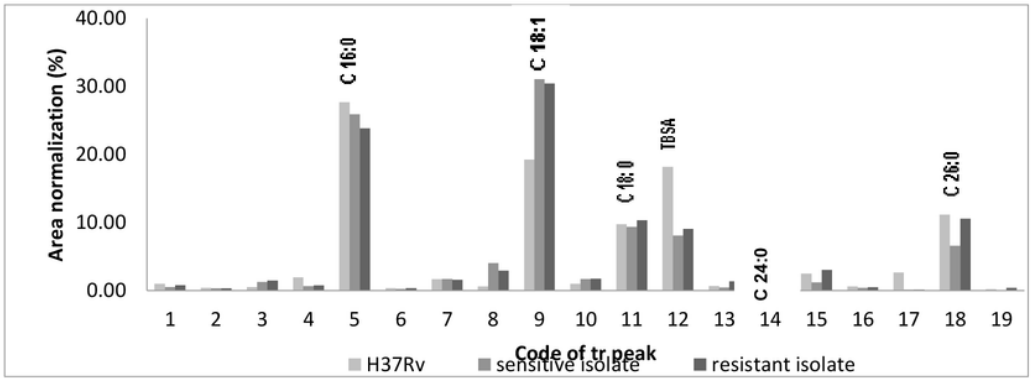


Figure 7: Profile of cellular fatty acids and MACPs based on area normalization (%)

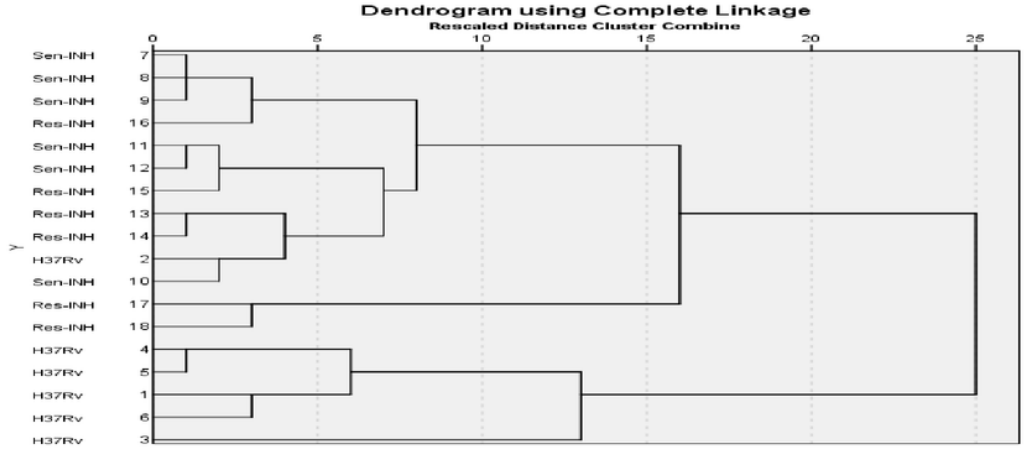


Figure 8: Hierarchical Cluster analysis of MTB samples

chromatogram. Hierarchical cluster analysis of SPSS program was used for grouping samples based on the six characteristic peak areas.

## RESULTS AND DISCUSSION

Saponification and extraction of MAs samples were adopted from the standard procedure of the previous researcher[1]. Esterification procedure using Methanolic-BF3 reagent has been optimalized before execution samples. The optimalization procedure included reagent volume, water-bath temperature and time for complete esterification process were done (data not shown). A modified procedure was used in order to shorten reaction time and avoid the use of benzene[16-19]. MTB-TDM standard contain only alpha mycolate as shown in the certificate of analysis. Trans-esterification of MTB-TDM (100-200) µg using methanolic-$BF_3$ fragmented the MTB-TDM into MACPs with $t_r$ of 7.25, 12.73, 27.89, 29.47 and 30.97 minutes (figure 2). Those peaks were identified as methyl ester of hexadecanoic acid ($C_{16:0}$), heptadecanoic acid-16-methyl ($C_{17:0}$), tetracosanoic acid ($C_{24:0}$), pentacosanoic acid ($C_{25:0}$) and hexacosanoic acid ($C_{26:0}$) respectively. None of those peaks overlapped with the fragment of Middlebrook media. The Profile of Middlebrook media (± 200 mg, 5-8 fold of sample weight) after processed with the same procedure. The $C_{24:0}$, $C_{25:0}$ and $C_{26:0}$ were the cleavage products of the alpha alkyl chain of MAs[16], the largest peak of MACPs was $C_{26:0}$. Whereas $C_{16:0}$ and $C_{18:0}$ were supposed to be obtained from the meromycolate chain fragmentation. Other MACPs of the meromycolate were not detected. As previous researcher report, both high temperature in injection port of GC-MS instrument and $BF_3$–methanolic reagent could cleavage MAs to become $C_{26:0}$ and other MACPs[11,19]. The specific fragments of MTB-TDM were also obtained in chromatogram of H37Rv strain, INH-sensitive MTB isolate and INH-resistant MTB isolate as shown in figure 4, figure 5 and figure 6 respectively. Using Middlebrook 7H10 media enriched with OADC (agar based), MTB was not growing as well as in Lowenstein-Jensen media (egg based). However, the fragments of OADC in Middlebrook media were not interferences of the sample chromatogram (figure 3). The other analyte peaks obtained in sample chromatogram were shown in table 1. Some of analyte peaks in sample chromatogram were identical with the analyte marker that had been used by the previous researcher[13]. But, the other analyte peaks that have been obtained by the previous researcher were not detected. Based on the three sample chromatograms we figured out that abundance of $C_{24:0}$ was always less than $C_{26:0}$, and $C_{14:0}$

always become a minor peak. This composition was identical with the result of the previous study[11]. The profile of those three peaks was characteristic so it can be used to distinguish MTB group from the other slow growing mycobacterium. The majority of $C_{26:0}$ and $C_{24:0}$ amounts supposed to be coming up from alpha mycolate, because 70% of mycobacterium MAs was in the form of alpha mycolic acids, whereas (10-15) % was as methoxy and keto mycolate[3]. Profile of peaks with areas more than 0.1% of the largest analyte area are shown in figure 7. The profile was obtained after normalization process among 19 peak areas. The correlation coefficient among H37Rv strain profile and INH-sensitive MTB isolate or INH-resistant MTB isolate profiles were 0.690 and 0.687 respectively. Whereas correlation coefficient between INH-sensitive MTB isolate and INH-resistant MTB isolate was 0.908 (at significance level of 0.01). Hierarchical cluster analysis (as depicted at figure 8) showed that INH-sensitive MTB and INH-resistant MTB were clustering in one group. Whereas H37Rv strain profile was separated in another cluster. It can be concluded that MACPs profile of MTB isolate was different compared to H37Rv strain profile. Unfortunately, the MACPs profile of INH-sensitive MTB could not be distinguished from INH-resistant MTB. Normalization among six areas which were of MTB characteristic (TBSA, $C_{24:0}$ and $C_{26:0}$) and analytes with relatively high abundance ($C_{16:0}$, $C_{18:1}$, $C_{18:0}$) were shown in table 2. ANOVAs test among three samples obtained that both peaks area of $t_r$ 11.96 minute (9-octadecenoic acid, $C_{18:1}$) and 14.05 minute (TBSA) were significantly different. Both comparison area ratios of $C_{26:0}$/TBSA and $C_{18:1}$/TBSA among H37Rv strain, INH-sensitive MTB and INH-resistant MTB showed that there were significant differences between H37Rv strain and both of INH-sensitive MTB and INH-resistant MTB. Whereas, the ratio of $C_{26:0}$/TBSA and $C_{18:1}$/TBSA among INH-sensitive MTB and INH-resistant MTB was not significantly different (at significance level of 0.05). The similarity of cellular fatty acid/MACPs composition between MTB-isolates implicated that characteristic profile of INH-resistant MTB will need more data of the MAs molecule, not only based on their fragmentation. Although the different composition or structures of MAs of MTB isolate have been identified, the type of MAs which their meromycolate changed is still unknown, because $C_{18:1}$ is also a cellular fatty acid obtained in microbial cell wall. Methanolic BF3 reagent can shorten the esterification time to one hour, whereas methanol-toluene-sulfuric acid (MTS) reagent needs 16 hours. Methanolic-$BF_3$ has been reported to have cleaved the cyclopropane ring, oxidize unsaturated of fatty acid and unfortunately produce methoxy artifacts when used in high concentration, but in low concentration and under controlled conditions some disadvantages of using this reagent can be minimalized. This study showed that methanolic-$BF_3$ made the relative standard deviation of the peak area was relatively higher in comparison with that had been reported by the previous researcher[13]. Although culture age and growth conditions influenced the MAs amount, the type of formed MAs is not affected[12]. However, environmental influences especially missed use of antibiotic or other antimicrobial agents that have action on the cell wall of mycobateria supposed to vary MAs profile of MTB isolate. This is probably the reason that MACPs profile of INH sensitive MTB is not significantly different to INH resistant MTB. So, it is important to increase the number of samples to ensure a significant MAs profile model.

## CONCLUSION

There is a significant difference in MAs profile between H37Rv strain and both isolate of INH sensitive MTB and INH resistant MTB. The increased composition of $C_{18:1}$ and decreased of TBSA showed that the modification MAs structure was happened at meromycolate chain, since the characteristic alkyl chain of $C_{26:0}$ composition is not change. However, the insignificantly different of $C_{18:1}$ and TBSA variation between the two MTB isolate make this method is not sufficient to differentiate INH-resistant MTB isolate from INH-sensitive MTB isolate. Also, this method could not differentiate the $C_{18:1}$ as MAs cleavage and $C_{18:1}$ that produced as cellular fatty acid of mycobacteria.

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