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# Research Journal of Pharmaceutical, Biological and Chemical Sciences

## Method Validation of High Performance Liquid Chromatography for Determination of Mycolic Acids Profile of Mycobacterium Tuberculosis

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### ABSTRACT

This study developed the High Performance Liquid Chromatography (HPLC) for INH resistant *M. tuberculosis* (MTB) isolate identification based on a chromatogram profile of mycolic acids (MAs). The aim of this study was to validate the HPLC for determining the characteristic profile of MAs chromatogram of the INH resistant MTB. The optimum derivatization process obtained was as follows: the minimum biomass weight was 25 mg. The experimental temperature was performed in (80-90) °C using a water bath for minimum 30 minutes in order to complete the MAs derivatization. Reagent volume used in the range of (200-500 µL) were not influenced the MAs chromatogram profile. The optimum condition of HPLC was as follows: mobile phase was methanol:isopropanol (60:40) for 3 minutes, followed by gradient elution (4:96) in 50 minutes. Thereafter, the mobile phase composition change gradually for 40 minutes to a final composition of (60:40). The sample volume was 20 µL and the mobile phase flow rate was 1 mL/minute. The result of this study showed that the MAs chromatogram profile of INH resistant MTB looked like H37Rv MTB strain. The chromatogram profile was a cluster with 6 characteristic peaks at the end of the analysis. The other short chain carbon fatty acids were eluted in the first 15 minutes.

**Keywords** : Validation method, HPLC, MTB, Mycolic Acids, isoniazid.

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## INTRODUCTION

*Mycobacterium tuberculosis* (MTB) is the causative agent of infectious disease of tuberculosis (TB). Tuberculosis diagnosis, especially in the multi drug resistant tuberculosis (MDR-TB), requires a specific, sensitive and rapid method. MTB culture is a gold standard method for TB diagnosis, but requires 4-8 weeks for completion. A long cultivation time is a subject for contamination. Therefore, various MTB identification methods have been improved for MDR-TB detection, such as Polymerase Chain Reaction (PCR), DNA sequencing and chromatography [1] [2].

Isoniazid (INH) is one of the first lines of anti TB drugs that used for TB prevention and therapy. The resistance to INH and Rifampicin, with or without other antiTB drugs called Multi Drug Resistant TB (MDR-TB). Isoniazid active at MTB's cell wall, especially inhibition of mycolic acids (MAs) biosynthesis [3]. *InhA*, *katG*, *ahpC* and *kasA* gene mutations have been found in INH resistant MTB. Those genes were coding the enzymes that involved in initiation, elongation and condensation of MAs biosynthesis [4]. Therefore, INH-resistant MTB was estimated to have a MAs characteristic profile.

Mycolic acids are  $\alpha$ -alkyl  $\beta$ -hydroxy fatty acid which contains (60-90) carbon atoms in each molecule (Figure 1). The main variations of MAs structure are alpha mycolate (>70%), keto mycolate and methoxy mycolate (10-15%) [5][6]. The MAs composition and structure profiles are specific and not influenced by cultivation media [7]. The MAs profile can be used as MTB fingerprint [8].

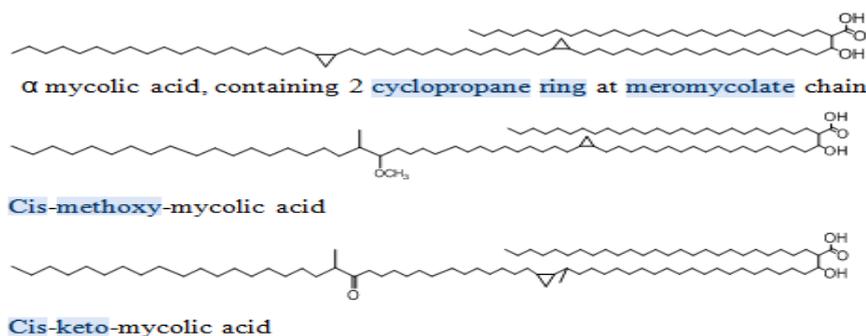


Figure 1. Mycolic acid structures [5]

This study will develop the High Performance Liquid Chromatography (HPLC) for isoniazid (INH) resistant MTB identification based on a chromatogram profile of mycolic acid molecules. The aim of this study was obtaining a valid HPLC method for analysis MAs of MTB isolate using *p*-bromophenacyl bromide reagent. The characteristic profile of MAs chromatogram of INH resistant MTB isolate can be used as database for identification of INH resistant MTB. Method validation of HPLC covered optimization of MAs derivatization and HPLC condition. Parameters of the validity of HPLC method for MTB sample were resolution ( $R_s$ ) between peaks of MAs ( $R_s > 1.2$ ) [9], peaks number of MAs at chromatogram that can be observed and consistency of the MAs characteristic peaks at chromatogram profile.

## MATERIALS AND METHODS

### MATERIALS:

Middlebrook 7H10 (Fluka Analytical, Cat. MO303.500G) with Oleic Acid Albumin Dextrose Catalase (OADC) growth supplement (Sigma MO678), trehalose 6,6' dimycolate (TDM) isolated from H37Rv of MTB strain (Alexis), tricosanoic methyl ester (TME) (Sigma-Aldrich), *p*-bromophenacyl bromide, dicyclohexyl-18-crown-6-ether,  $\text{KHCO}_3$ , KOH, NaOH, HCl,  $\text{Na}_2\text{SO}_4$  exsiccatus, methanol, isopropanol pro HPLC, acetonitril and chloroform. All of the reagents, except other mention, were pro analyzing grade (E.Merck).

Samples of *M. tuberculosis* of H37Rv strain and INH resistant MTB isolate were supplied by the Health Laboratory of Surabaya (Balai Besar Laboratorium Kesehatan Surabaya) and Clinical Microbiology Department of Dr. Soetomo Hospital, Surabaya, Indonesia. All samples were re-checked for their sensitivity to INH using

standard sensitivity test prior to be cultured. The sample was cultured at 37°C for 21-31 days. Biomass was placed in Pyrex glass tube before added 2 mL of 25% KOH in a mixture of methanol:water (1:1) and autoclaved for 1 hour at 121°C [5]. After this process had finished, the sample was brought out of the BSL-3 facility, and ready for HPLC analysis.

## **METHOD:**

### **Instruments**

UFLC Prominence Shimadzu 20A, shim-pack-VP-ODS/C8/Phenyl end-capped column, size 150 mm x 4.6 mm, with diode array detector. Analytical balance (Sartorius), vortex (Genius 3), centrifuges (EBA20 Heich).

### **Extraction of Mas:**

Samples were added with 1.5 mL of HCl (1:1) v/v and agitated for 2 minutes on vortex before extracted (three times) using 2 mL of CHCl<sub>3</sub>. The extract in organic solvent was separated into other screw-thread tube and dried using nitrogen gas. The dried extract was stored in the refrigerator until the next derivatization process [5].

### **Hydrolysis of standard substance:**

Trehalose dimycolate and TME (as internal standard) were hydrolysed by adding 2 ml solution of 25% KOH in methanol and heating for 1 hour at 90°C on water-bath. After cooling to the room temperature, the sample was acidify with 1,5 mL of HCl (1:1) and the free fatty acids were extracted with CHCl<sub>3</sub> ( in accordance with MAs extraction procedure mentioned above ).

### **Derivatization of Mas:**

The sample in a screw-thread tube was added with 40 mg of KHCO<sub>3</sub>, 300 µL of derivatization reagent and 700 µL of CHCl<sub>3</sub>. The sample was heating at 90°C for 30 minutes on the water-bath. After cooling down to the room temperature, the sample was filtered through Whatman filter paper of 0,2 µm pore and ready to inject into HPLC [7] [10]. The hydrolyzed internal standard TME (50 µg) was added in the sample before derivatization process.

Derivatization reagent was a mixture of 0.1 mMol (27,79 mg/mL) p-bromophenacyl bromide and 0,005 mMol (1,86 mg/mL) dicyclohexyl-18-crown-6-ether in acetonitril. This reagent is relatively stable for several months in refrigerator [7].

The sample that has been processed was dried using N<sub>2</sub> and stored in the refrigerator if it was not immediately injected. The dried sample was re-solved again using 1 mL solution of a mixture of acetonitril and CHCl<sub>3</sub> (1:1) before injection.

## **METHOD VALIDATION:**

### **Selectivity:**

various solvents with different composition were used as mobile phase in order to obtain a characteristic MAs profile of the TDM standard and MTB sample. Characteristic of MAs profile parameter was the maximum amount of MAs peaks that obtained consistently similar in TDM standard and MTB sample. The resolution among peaks in the MAs cluster peaks were > 1,2 [9].

### **Optimization of MAs derivatization process:**

various water-bath temperature of (70-90) °C, warming time of (30-60) minutes and the reagent volume were studied in order to obtain the characteristic profile of MAs MTB isolates.

**Minimum sample weight required:**

some samples were processed using the optimum condition in order to obtain the minimum biomass weight by which the characteristic MAs profile achieved..

**Data analysis:**

The sample peaks were identified based on their similar retention time ( $t_R$ ) with H37Rv peaks and the similarity between their UV spectra peaks. The profile of MAs was obtained after area normalization of the six characteristic peaks in the cluster of MAs peak.

$$\text{Normalization of each peak} = (\text{area peak} / \text{total area of 6 characteristic peaks}) \times 100 \%$$

Similarity among MAs chromatogram profile was determined based on correlation test using IBM SPSS statistics 22 programs. The variation among the MAs peaks area of the sample were determined using general linear model statistic test on 0.05% significance level.

**RESULTS AND DISCUSSION**

**Selectivity:**

The optimum mobile phase obtained was a mixture of methanol:isopropanol (60:40) isocratically for 3 minutes, followed by gradient elution (4:96) in 50 minutes. Thereafter, the mobile phase composition change gradually for 40 minutes to a final composition of (60:40). The sample volume was 20  $\mu$ L and the mobile phase flow rate was 1 mL/minute. The MAs signal was detected using a diode array detector (DAD) at 254 nm. Analysis of standard TDM and H37Rv MTB strain using this optimum condition obtained a characteristic profile of MAs as a cluster containing minimal six peaks that can be showed at Figure 2 and Figure 3. This cluster was identical with the result of the previous research [7] [10].

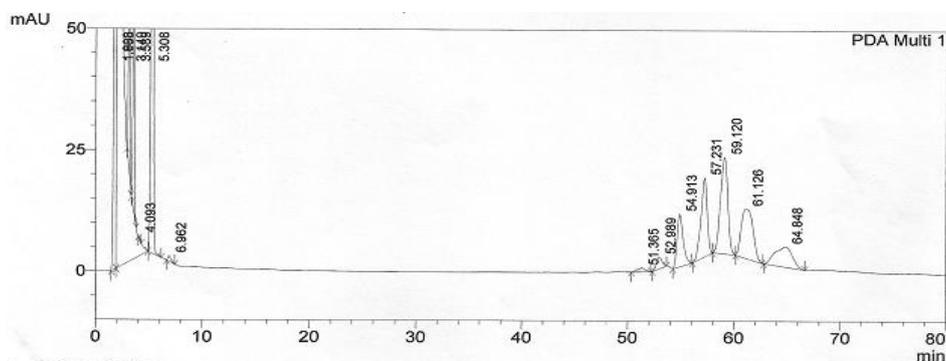


Figure 2. Mycolic acids chromatogram profile of TDM standard

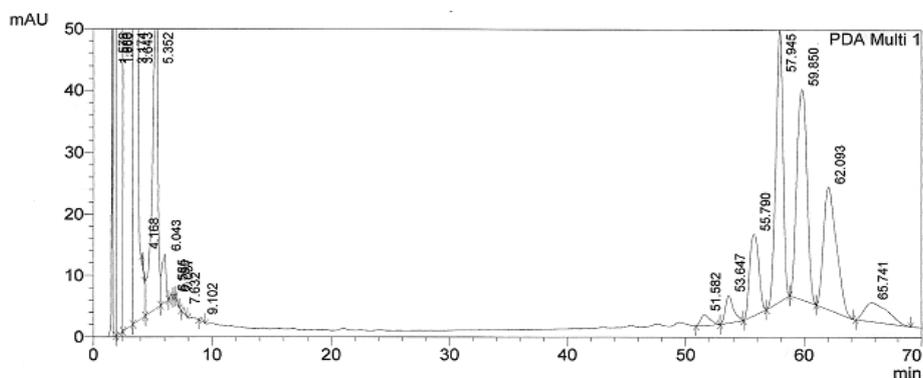


Figure 3. Mycolic acids chromatogram profile of H37Rv strain of MTB

The characteristic peak in a cluster was detected at the minutes of 51.58, 53.64, 55.79, 57.95, 59.89, 62.09 and 65.74, respectively. The peaks that eluted before retention times of 51.58 minutes showed a very small area. The peaks that eluted after retention time of 65.74 minutes usually tailing, or contained two or more peaks that couldn't be separated. So, these peaks were not used in the determination of MAs chromatogram profile.

Resolution ( $R_s$ ) among peaks in the cluster were in the range of 1.12 – 1.63. This resolution was relatively good. At a resolution of 1.0, the two adjacent peaks were overlap their area of 4%. At a resolution of 1.5 the overlap is about 0.3% [11]. In order to obtain peak area accurately, slope, threshold and minimum area of the chromatogram were adjusted appropriately.

The spectrum profile of each 6 characteristic MAs analyte was nearly the same. Their maximum wavelength were very closed range of ( 254-256 nm) (Figure 4). These spectra were as same as a spectrum profile of p-bromophenacyl bromide reagent (not presented in this article).

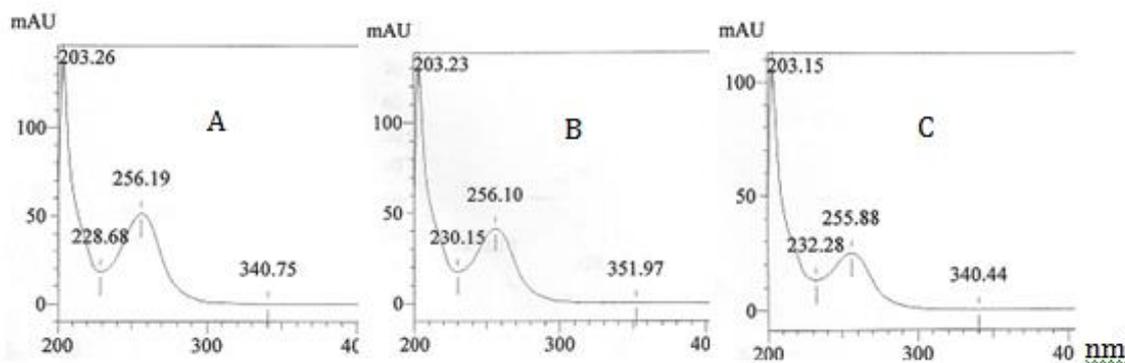
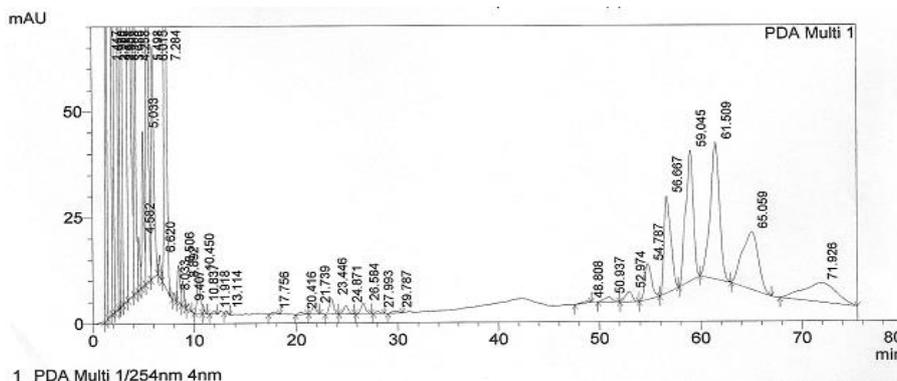


Figure 4. Spectra of MAs of H37Rv strain of MTB at retention times of 57.9 minutes (A), 59.8 minutes (B) and 62.1 minutes (C)

The other fatty acids with a shorter carbon chain that come from OADC of Middlebrook media, LJ-media or TME ( $C_{23}$ ) were eluted at the first 15 minutes. So, those substances do not interfere the MAs analysis (Figure 4). This method is selective for MAs analysis, but require a long time for elution (70 minutes) and caused high variation of MAs retention time. Therefore, identification of MAs peaks also based on the peak in the sample cluster peaks that have a similar spectrum profile with the MAs of H37Rv strain peak that have retention times of 59.65 minutes (Figure 5.). The similarity value of that certain peak would be more than 0.995. This certain peak would be used as internal standard for setting the consecutive number of the other peaks in the same sample. The peak that has retention times of 59.65 minutes was a sharp peak, relatively symmetric and having area with the lowest variation (Figure 3, Table 1). The relative retention time of MAs based on the original internal standard (TME) couldn't improve the precision of MAs peak identification, because the MAs peaks were a cluster peaks in a narrow span time and their position were far from TME peaks.



**Table 1. The profile of MAs of H37Rv strain based on normalization of the peak area.**

Sample codes	Weight (mg)	Normalization of peak area at t <sub>R</sub> (minutes) and order peak no.							
		49.46	51.49	53.57	55.63	57.9	59.87	62.13	65.8
			1	2	3	4	5	6	
H37-1.2	26.4	0.38	2.19	5.75	15.89	25.55	29.02	21.61	7.65
H37-C8	27.9	0.07	1.74	4.07	13.02	28.97	30.61	21.59	0.14
H37 C1	49.3	0.30	1.62	3.75	11.88	27.74	31.01	24.00	6.34
H37 C2	44.3	0.17	1.63	3.54	11.36	29.12	31.79	22.57	6.21
H37-C34	46.2	0.00	1.35	3.88	12.75	26.51	31.44	24.08	9.30
H37 C5 5	42.8	0.00	1.44	3.26	11.06	28.57	31.88	23.79	6.77
H37 C3 5	43.5	0.00	1.65	2.79	10.81	29.78	31.74	23.23	6.36
Mean		0.13	<b>1.66</b>	<b>3.86</b>	<b>12.40</b>	<b>28.03</b>	<b>31.07</b>	<b>22.98</b>	6.11
SD		0.16	0.27	0.93	1.75	1.53	1.02	1.08	2.85
CV (%)		118.95	16.18	24.20	14.10	5.44	3.27	4.68	46.66

The molecular structure of each peak in a sample cluster peaks were not identified. But, the peak cluster of MAs sample was identical with the peak cluster of TDM standard (Figure 2). This method can't identify the structure of each MAs peak, no standard substance to compare with each peak in the cluster peak.

The addition of CHCl<sub>3</sub> in a mixture of mobile phase would shorter the MAs retention times, but the instrument must be equipped with a special piping to avoid the CHCl<sub>3</sub> vapor contact with the leak column detector and made the HPLC system shut down.

**Optimization of derivatization process:**

Reagent volume in the range of (200-500) µL for ± 40 mg sample was not making the MAs chromatogram profile changed. Some references used a different temperature and time for derivatization [7][5]. This study obtained that the temperature range of (70-90)°C were not making a different MAs chromatogram profile. The range of derivatization time of (30-60) minutes were not making a different MAs chromatogram profile also. In this study, MAs were derivatized at 90°C on the water bath for 30 minutes.

**Minimum sample weight required:**

This study obtained that six peaks in a cluster would be obtained in MAs chromatogram when 300 µg/mL of TDM was tested (Table 2.). The minimum weight of biomass that would be produced a characteristic chromatogram profile of MAs was 25 mg.

**Table 2. The minimum weight of the TDM standard for MAs analysis using HPLC**

TDM standard	Weight (µg/mL)	Normalization of peak area at t <sub>R</sub> (minutes) and order of peak no.							
		52.32	53.98	54.9	55.62	58.34	60.1	62.49	66.8
			1	2	3	4	5	6	
TBC -4	100	0.01	2.83	0.17	14.16	23.28	32.91	26.66	10.25
TBC-6	100	0.00	0.00	2.68	15.24	24.89	34.95	22.25	
TBC 21-200	200	0.00	0.00	3.11	14.38	23.74	32.09	26.68	0.00
TBC 22-300	300	0.00	1.32	2.86	14.35	22.41	32.35	26.71	15.99
Mean		0.00	<b>1.04</b>	<b>2.21</b>	<b>14.53</b>	<b>23.58</b>	<b>33.07</b>	<b>25.57</b>	8.74
SD		0.00	1.35	1.37	0.48	1.03	1.30	2.22	8.10
CV (%)		0.00	129.86	61.98	3.32	4.37	3.92	8.67	92.61

**Precision:**

Intraday precision test obtained that areas variation of MAs were in the range of (0.2- 4)% (Table 3.). But, the cluster peaks retention times could be shifted in the range of (53.01 -65,80) minutes.

**Table 3. Intraday precision of MAs analysis using HPLC**

	Normalization of peak area at t <sub>R</sub> (minutes) and order peak no.						
	51.7	53.01	55.08	57.08	59.47	62.13	65.80
Sample codes		1	2	3	4	5	6
REB 14 (1)	0.47	1.72	5.70	15.94	24.23	30.58	21.82
REB 14 (2)	0.90	1.66	5.77	15.76	24.03	30.53	22.25
REB 14 (3)	0.71	1.77	5.87	15.60	23.95	30.46	22.35
REB 14 (4)	0.44	1.75	5.73	15.59	23.88	30.59	22.46
REB 14 (5)	0.44	1.84	5.71	15.58	23.96	30.50	22.41
<b>Mean</b>	0.62	<b>1.75</b>	<b>5.77</b>	<b>15.63</b>	<b>23.96</b>	<b>30.52</b>	<b>22.37</b>
<b>SD</b>	0.23	0.07	0.07	0.08	0.06	0.05	0.09
<b>CV (%)</b>	36.18	4.11	1.26	0.53	0.26	0.18	0.41

**Note:** The peak at t<sub>R</sub> of 62.17 minutes have a similar spectrum profile with the peak of the H37Rv MTB strain at the t<sub>R</sub> of 59.87 minutes with similarity factor of 0.992. REB = INH resistant MTB isolates sample.

**Chromatogram profile of Mas:**

MAs chromatogram profile of INH resistant MTB isolate showed in Figure 5., Table 4. The chromatogram showed that the last analyt was tailing (in other sample it was splitting). Therefore, the area of this characteristic peak could not be taken into account in the normalization area in order to obtain a MAs profile based on area variation peaks. This chromatogram showed that the MAs profile of INH resistant MTB looks alike MAs of H37Rv strain.

**Table 4. The MAs profile of INH resistant MTB isolate based on the normalization of the peak area**

		Normalization of peak area at t <sub>R</sub> (minutes) and order peak no.							
Sample code	Weight	49.98	51.78	53.57	55.66	57.76	59.77	62.17	65.6
	(mg)		1	2	3	4	5	6	
RE 444	30.0	0.00	1.17	4.22	14.20	27.42	32.79	20.20	4.33
RE 55 TME	35.0	0.00	1.71	4.70	15.00	25.09	31.05	22.44	6.92
RE 999	40.0	0.00	2.01	3.68	14.43	27.13	32.98	19.78	4.59
REB 11. 2	42.0	0.00	2.02	5.32	15.66	28.30	28.67	20.03	wide
REB 13-13	45.0	0.00	1.74	4.92	13.73	27.78	31.70	20.12	wide
REB 3.1 +TME	48.6	0.01	2.47	6.77	18.35	27.35	26.94	18.12	wide
REB 14-123	49.2	0.00	1.78	5.56	15.25	23.93	30.85	22.62	17.51
<b>Mean</b>		0.00	<b>1.84</b>	<b>5.02</b>	<b>15.23</b>	<b>26.71</b>	<b>30.71</b>	<b>20.47</b>	8.34
<b>SD</b>		0.00	0.40	1.00	1.52	1.59	2.20	1.58	6.22
<b>CV (%)</b>		168.9	21.5	19.9	10.0	5.9	7.2	7.7	74.7

Comparison of MAs profile between H37Rv MTB strain and INH resistant MTB isolate can be shown in Figure 6. Paired sample T test of the pair peaks of H37Rv strain and INH resistant MTB isolate obtained the correlation ( $r^2$ ) of 0.972. It means that pairs of chromatography profile looking alike. General linear model statistic showed that peak number 3 and peak number 6 of those pair of chromatography profile were significantly different ( at  $\alpha$  0.05).

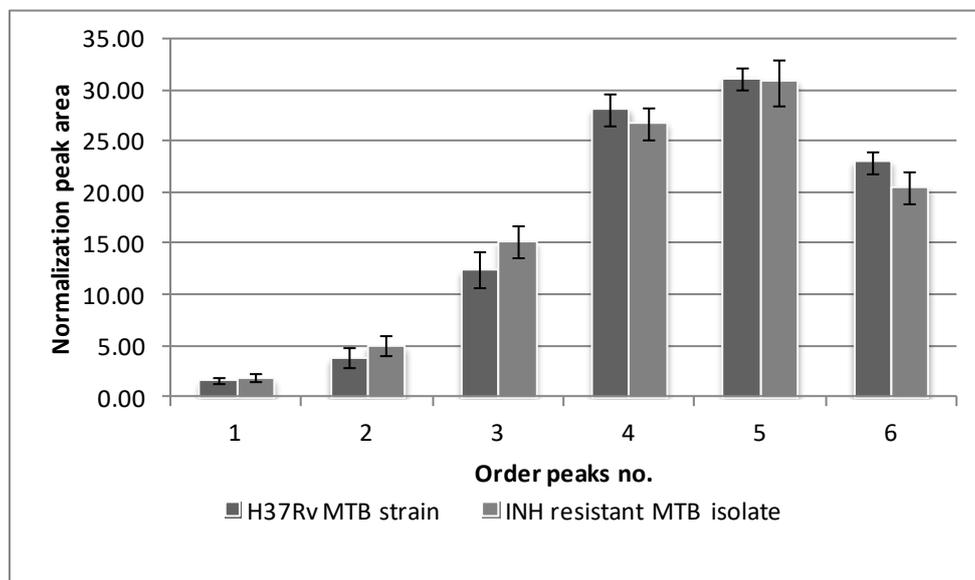


Figure 6. Comparison of MAs chromatogram profile of H37Rv strain and INH resistant MTB isolate.

The similarity MAs chromatogram profile between INH resistant MTB and H37Rv strain caused the composition peak area were difficult to use for identifying INH resistant MTB. But, this study confirmed that MAs profile was characteristic for MTB in general. The similar MAs profile supposed to be caused of the detector used is DAD and the MAs were detected as a coupling substance with the reagent (bromophenacyl bromide and dicyclohexyl-18-crown-6-ether in acetonitril). This reagent increased dominantly the absorptivity of the coupled substance, but a modified in MAs structure does not significantly detected. The more specific detector (i.e. spectrometry massa) supposed to be more efficient for identifying the modified structure of MAs [2]. In the liquid chromatography-tandem mass spectrometry (LC-MS/MS), the MAs molecule can be detected without derivatization [2].

### CONCLUSION

The optimum derivatization process was as follows: minimum biomass sample weight was 25 mg. Reagent volume in the range of (200-500)  $\mu$ L was not influenced the MAs chromatogram profile. The Water-bath temperature was set at (80-90)  $^{\circ}$ C for minimum 30 minutes in order to complete the MAs derivatization. The optimum mobile phase was a mixture of methanol and isopropanol. The elution process was started at mobile phase composition on (60:40) for 3 minutes, followed by the gradient flow rate of (4:96) in 50 minutes. Thereafter, the mobile phase composition change gradually for 40 minutes to a final composition of (60:40). The sample volume was 20  $\mu$ L and the mobile phase flow rate was 1 mL/minute. The MAs chromatogram profile was a cluster with 6 characteristic peaks at the end of the analysis. The other short carbon fatty acids have been eluted in the first 15 minutes. The result of this study showed that the MAs chromatogram profile of INH resistant MTB was looked like H37Rv strain.

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