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Research Article

IDENTIFICATION OF MYCOLIC ACIDS OF MYCOBACTERIUM TUBERCULOSIS BY GAS CHROMATOGRAPHY-FLAME IONIZATION DETECTOR

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ABSTRACT

Objective: The aims of this study were to obtain the optimum condition for mycolic acids (MAs) analysis by Gas Chromatography-Flame Ionization detector (GC-FID) after MAs esterification using methanolic-BF_3 as a catalyst and identify of MAs fragments of *Mycobacterium Tuberculosis* (MTB).

Methods: The MAs of inactivated MTB were extracted with chloroform prior to esterification using methanolic-BF_3 as a catalyst in 0.5N methanolic NaOH. MA fragments were extracted with heptane prior to injection into GC-FID.

Results: Optimum esterification process was obtained by adding 1.0 ml of 0.5 N methanolic NaOH and 1.0 ml 14% BF_3 in methanol before heated at 95°C for 1 hour in waterbath. Operational conditions of GC were as follow, inlet and FID temperatures were 285°C, flow rate of carrier gas was 1 ml/minute, the injection volume was 2 µL with split less mode, the HP-5 capillary column temperature was started at 200°C for 1 minute, increased 5°C/minute to 275°C, maintained at 275°C for 9 minute, increased 5°/minute to 300°C, and finally maintained at 300°C for 30 minutes.

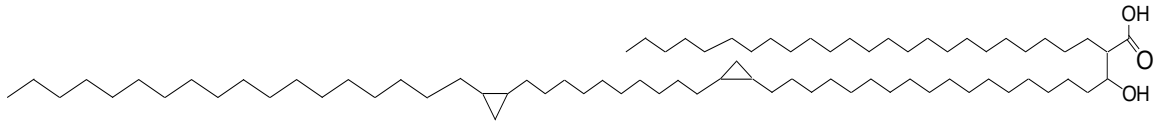
Conclusion: The proposed method can be used for identification of MAs in H37Rv strain and MTB strain isolated from tuberculosis patient. Specific retention time of MAs fragments were 12.43; 13.67 and 15.06 minute respectively.

Keywords: Mycolic acids, *Mycobacterium Tuberculosis*, Gas Chromatography-Flame Ionization Detector.

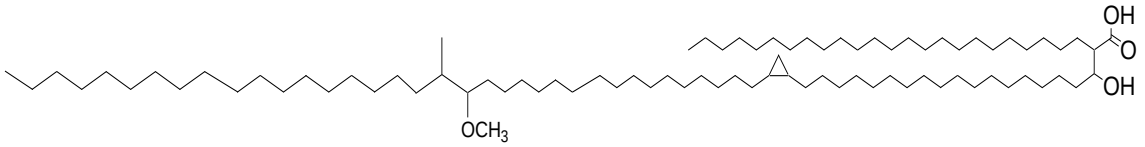
INTRODUCTION

Mycolic acids (MAs) are specific lipid fractions of the outer cell wall of Mycobacterium species. The characteristic structure of MA is *mycolic acid-arabinogalactan-mucopeptide complex* which is not affected by culture conditions. As a result, MA is recognized as a stable *phenotypic property* of Mycobacterium species [1].

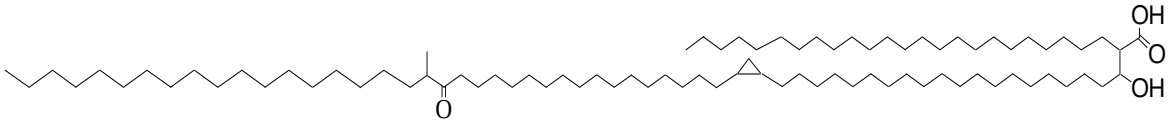
MAs are high-molecular weight of 3-hydroxyl fatty acids with long alkyl chains at position 2. Each molecule contains 60-90 carbon atoms with some cyclopropane ring at meromycolate chain (Fig.1). Mycobacterium Tuberculosis (MTB) produces MAs as alpha MA (at least 70%), methoxy MA and keto MA (10-15) %. Methoxy MA and keto MA have cis and trans-isomer [2, 3]. MTB is considered as the most deadly infectious agent when effecting the HIV infected people [4]. Identification of MA is important for biosynthesis study, identification the formation of unusual MAs in mutant strain, as in MDR-TB, and the effect of antimicrobial drug targeting the cell wall. Various methods that have been used for characterization of MAs from mycobacterium species were Thin Layer Chromatography (TLC) [5], High Performance Liquid Chromatography (HPLC) [1, 6] and Gas Chromatography (GC) [7, 8]. MAs pattern in HPLC/GC chromatograms have been used as a data base for identification of a species or a strain in specific species of mycobacterium [1, 6, 7]. MAs could be identified from their p-bromophenacyl ester derivate [1,5], trimethylsilyl ether of the methyl ester MAs derivate [7] or as MAs fragment as cleavage products after Mas esterification process using Methanol-toluene-concentrated H_2SO_4 (MTS reagent) [8].



α mycolic acid, containing 2 cyclopropane ring at meromycolate chain



cis methoxy-mycolic acid



cis keto-mycolic acid

Fig. 1: Mycolic acids structure containing alkyl branch and meromycolate chain

However, the MAs esterification process using MTS reagent is time consuming [16 hours at 80°C in covered bath]. This study will optimize MAs esterification process using BF_3-methanolat reagent in order to obtain a reproducible MAs fragments and short analysis time.

Boron trifluoride (BF_3) in the form of its coordination complex with methanol is powerful acidic catalyst for trans-esterification of fatty acids. Although, methanolic-BF_3 reagent had been reported could cleave the cyclopropane ring, oxidize unsaturated of fatty acid and produce methoxy artifacts when be used in a high concentration, but it also has same disadvantages as other acidic reagents for fatty acids with labile functional groups in low concentrations [9]. However, methanolic BF_3 have been used as reagent in standard method for determination of omega 3 unsaturated fatty acids as DHA and EPA [10]. Based on that reason, this study aimed to obtain the optimum esterification condition using methanolic-BF_3 as a catalyst to produce the reproducible MA fragments that can be used for MAs identification by GC-FID

MATERIALS AND METHODS

Sample: *M. tuberculosis* i.e. H37Rv strain, MTB isolate that sensitive to Isoniazid-Streptomycin-Rifampicin-Ethambutol (S-I-R-E, the first line of antituberculosis drugs) and MTB isolate that resistance to Isoniazid. All strains were supplied from Clinical Microbiology department of Dr. Soetomo Hospital and 'Balai Besar Laboratorium Kesehatan Surabaya', Surabaya, Indonesia Reagents: Lowenstein-Jensen (L-J) medium**,** Trehalose Dimycolate (TDM) of *M. Bovis* ex Sigma, Tricosanoic acid methyl ester (TME) ex Sigma. KOH, NaOH, HCl, Na_2SO_4 exciccatus, NaCl, BF_3 14% in methanol, methanol, chloroform, (all of the reagents were pro analyze grade) except for heptane (pro GC grade). Instruments: GC Agilent 6890 series, HP5 capillary column (30 m x 0,32 mm x 0,25 µm), with Flame Ionization Detector (FID)**,** GC Agilent 6890N series with Agilent 5973 inert MSD**,** auto sampler, HP5 capillary column (30m x 32mm x 25µm).

Sample preparation: *M. tuberculosis* of H37Rv strain, isolate which was sensitive to isoniazid and isolate which was resistance to S-I-R-E were grown on Lowenstein-Jensen (L-J) slant and harvested after 21-30 days. Biomass (100-200) mg was taken from L-J slant and placed in Pyrex tube containing 2 ml of 25% KOH in a mixture of methanol: water (1:1). The suspension was mixed and autoclaved for 1 hour at 121°C in order to kill the MTB. Before preparation process, the MTB isolates sensitivity were confirmed to the first line of anti-tuberculosis drugs (S-I-R-E) using MGIT (*Mycobacterium Growth Indicator Tube*). This process was conducted in Biosafety level 3 (BSL-3) room of clinical microbiology department of Dr. Soetomo Hospital Surabaya. Isolation of mycolic acids of *M. tuberculosis*: 1.5 ml of hydrochloric acid (1:1) was added to the inactivated MTB sample and agitated carefully. Two ml of chloroform was added to the mixture and agitated with vortex for 2 minute prior to centrifugation at 2000 rpm for 2 minute. Chloroform phase was transferred to another clean tube. (This process was repeated three times). The collected chloroform extracts was dried in a gentle stream of nitrogen gas. Dried chloroform extract was re-dissolved in 2.0 ml heptanes. Aliquots volume of heptanes' solution (equivalent to 50 mg of sample) was dried before added with esterification reagent to be proceeded with the following step. Esterification of mycolic acids: *1.0 ml of 0.5N methanolic-NaOH was added to the dried MAs extract in a pyrex cupped tube, followed by heating on the water bath for 5 minute at 95°C and then cooled to room temperature. A methanolic-BF_3 (1.0 ml, 14% BF_3 in methanol) was added into the MAs extract and heated in waterbath at 95°C for 1 hour. The obtained methyl ester MAs fragment was extracted using 1.0 ml heptane by agitating on vortex for 2 minutes. Heptane extract was separated by centrifugation at 2000 rpm for 2 minute and then transferred to another clean tube. (This extracting process was repeated 3 times). The combined heptanes extract was dried by a stream of nitrogen gas. The dried extract was re-dissolved in 0.50 ml heptanes prior to injection in GC-FID or GC-MS. *In order to quantify MAs fragments, 10.0 µL of 5000 ppm of tricosanoic methyl ester (TME) was added in the sample as internal standard before the esterification process. Optimization of producing MAs methyl ester fragments: Factors that can influence the production of MAs methyl ester fragment were studied i.e. sample saponification period, volume 0.5N methanolic- NaOH, volume methanolic-BF_3 and sample esterification period.

Verification of analysis condition for MAs identification using GC-FID: Methyl ester MAs fragments in heptane extract was injected into the instrument in various conditions i.e. Injection sample program, injection port temperature, column temperature program and carrier gas flow. Various factors were combined to obtain some specific analyte peaks with good resolution (RS), especially from the matrix peaks.

MAs of *M. Tuberculosis* analysis using GC-MS: Methyl ester MAs fragments in heptanes extract was injected into GC-MS using optimum condition obtained in sample analysis using GC-FID. Specific analyte peaks were identified based on their specific retention time and mass data base library.

RESULT AND DISCUSSION

Optimization of producing specific MAs methyl ester fragments: Trehalose dimicolate (TDM) standard produced three specific fragment peaks. Those specific fragments were eluted after the internal standard's peak (TME). The retention time (t_R) of those fragments were TDM (C) of 15.15 minute (dominant), TDM (A) 12.50 minute (minor) and TDM (B) of 13.81 minute (minor). Rs among peaks were good (Rs > 1.15) (Fig. 2). Inter-day precision of that fragment's retention time were 0.04, 0.04 and 0.06 minute respectively. The fragment's area variation among sample injection was significantly different. But, using area normalization manner those three fragments showed a reproducible composition when heated on water bath at 95°C for 1 to 8 hours (table 1). Waterbath temperature and period of sample heating influenced the composition of sample fragments. As previous review [2] high concentration of methanolic-BF_3 solution and the life time of reagent would produce some noises or un-control fragmentation. BF_3 as a Lewis acid, in the form of its coordination complex with methanol, is a powerful acidic catalyst for the esterification of fatty acid [2].Esterification of the heptanes extract of H37Rv strain for 1 hour on the waterbath at 95°C obtained a fragment chromatogram as showed in Fig. 3. The specific MAs fragments composition had been produced when H37Rv had being esterified for 1 hour was not significantly different with the specific MAs fragment composition from 8 hours esterification process (Fig. 4). This esterification period was shorter than previous esterification period using methanol-toluene-H2SO4 (i.e. 16 hours at 80°C) [8].However, chromatogram on Fig.3 showed that L-J medium interfered the mycobacterium lipid chromatogram. The Egg suspension in L-J medium contained fatty acid that could be simultaneously esterified with mycobacterium lipid using BF_3 reagent. The GC chromatogram of fatty acid fragment of L-J medium showed in Fig 5.

Table 1: Area composition of TDM's specific fragments

	TDM	Area (pA.det) at t_R(minute)			Area normalization			Esterification
Sample	**concentration (mg/0.5 mL)**	**12.50 (A)**	**13.81 (B)**	**15.15 (C)**	**A/T x 100**	**B/T x 100**	**C/T x 100**	**periods (hours)**
TDM 1	0.250	209.7	540.9	2557	6.3	16.4	77	0.5
TDM 9	0.250	105.4	81.91	1333	6.9	5.4	88	1
TDM10	0.075*	55.53	58.24	817.8	6.0	6.3	88	2
TDM 6	0.250	82.91	65.9	1142	6.4	5.1	88	3
TDM 4	0.250	235.3	229.8	2371	8.3	8.1	84	8

Note : T = Totally area of A+B+C, * dissolved in 0.2 mL heptanes

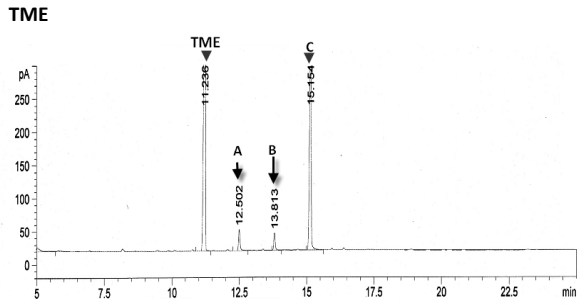


Fig. 2: Chromatogram of TDM fragments (A, B, C) after esterification for 1 hour

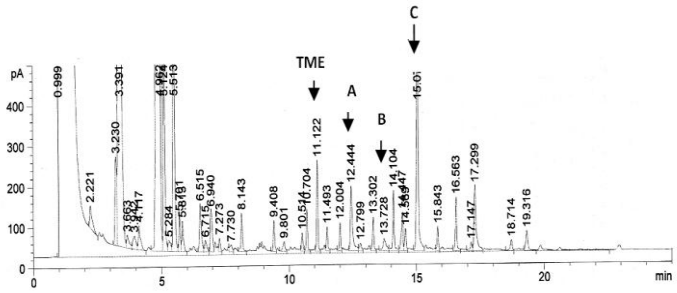


Fig. 3: Chromatogram of fatty acid fragments of H37Rv strain after esterification for 1 hour

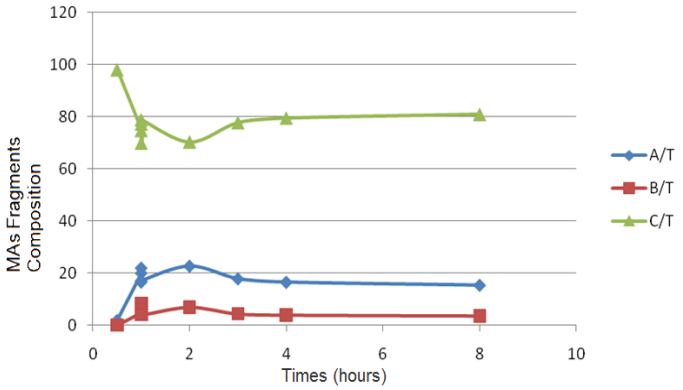


Fig. 4: Composition of MAs fragments of H37Rv strain produced on the different esterification period.

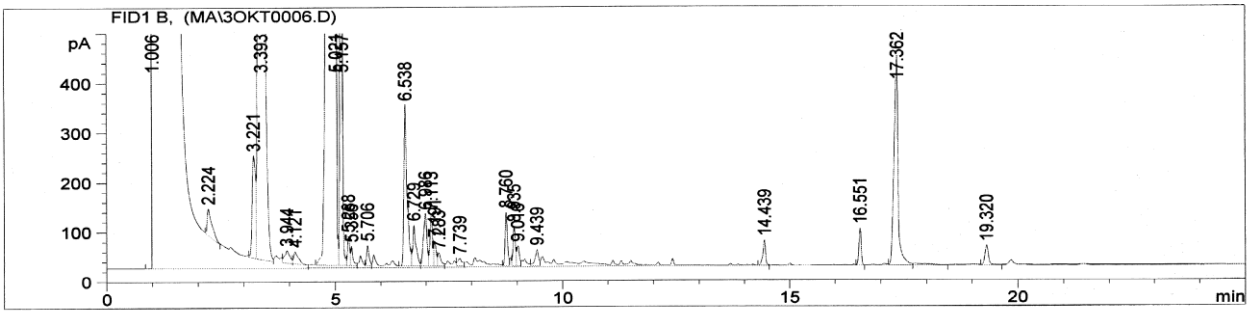


Fig. 5: Chromatogram of fatty acids fragment of L-J medium after esterified for 1 hour

Table 2: The influenced of methanolic-BF3 volume on the esterification products of MTB

	Sample	Area (pA.det) of			Area normalization			Volume
Sample	Concentration	t_R 12.42	t_R 13.67	t_R 15.06	A/T	B/T	C/T	of BF3
	(mg/0.5 mL)	(A)	(B)	(C)	x 100	x 100	x 100	(mL)
LHE1A	78.04	16.3	24.29	80.58	13	20	67	0.5
LHE1C	52.02	173.1	23.65	1072	14	1.9	85	1
LHE1B	78.04	426.9	63.0	2488	14	2.2	84	2

Table 3: Composition of MAs specific fragments among MTB strains

Sample	Sample weight	t_R 12.42 (A)	t_R 13.67 (B)	t_R 15.06 (C)	A/T	B/T	c/T
	mg/0.5 mL	pA.det	pA.det	pA.det	x 100	x100	x100
S1B	53.3	548.6	142	2043	20.1	5.2	74.7
S1C	35.5	565.0	137.4	2456	17.9	4.3	77.8
S3A	65.0	733.9	270.4	2324	22.0	8.1	69.8
S3B	65.0	917.2	278.7	2832	22.8	6.9	70.3
LA1B	79.38	1406.	332.5	6477	17.1	4.0	78.8
LA3B	77.29	622.8	227.8	2886	16.7	6.1	77.2
LA3C	51.52	820.9	193.4	4313	15.4	3.6	81.0
LA4B	77.77	1820	432.5	8728	16.6	3.9	79.5
Mean of Composition of A,B,C fragments of H37Rv strain					18.6 ± 2.3	5.3 ± 1.3	76.1± 3.4
SN1A±TME	40.0	393.7	90.71	1677	18.2	4.2	78
SN1B	40.0	467.9	105.3	2057	17.8	4.0	78
SN2B±TME	57.37	152.3	39.40	589.4	19.5	5.0	75
SN2A	57.37	579.9	138.5	2302	19.2	4.6	76
SN3B±TME	50.10	237.7	18.50	877.3	21.0	1.6	77
SN3A	50.10	458.2	75.15	1525	22.3	3.6	74
Mean of composition of A,B,C fragments of isolate that was sensitive to SIRE					19.7 ± 1.3	3.9 ± 0.8	76.5 ±1.2
RE1B±TME	50.50	492.3	47.16	947.9	33.1	3.2	64
RE1A	50.5	1149	268.5	2134	32.4	7.6	60
RE2A±TME	50.50	206.8	68.6	1223	13.8	4.6	82
RE2B	50.50	474.9	176.8	2755	13.9	5.2	81
RE3B±TME	47.25	208	62.3	972.9	16.7	5.0	78
RE3A	47.25	397.08	112	1935	16.2	4.6	79
Mean of composition of A,B,C fragments of isolate that was resistance to Isoniazid					21.0± 7.8	5.0 ± 0.9	74.0± 8.0

Note :.
S and LA = H37Rv strain
RE = isolate which was resistant to isoniazid
SN = isolate which was sensitive to S-I-R-E

Extended saponification periods of sample with 25% of KOH in a mixture of methanol -water (1:1) for (1-8) hours, (after sample have been autoclaved for 1 hour), did not influence the composition of MAs fragment products. The 0.5N Methanolic NaOH volume in the range of (0.5-1) ml did not increase the number of MAs fragments in chromatogram. However, increasing volume of 14% methanolic BF3 influenced the reaction products (table 2). BF3 reagent has a limited shelf life at room temperature and should be store in refrigerator [9].

Confirmation of MAs analysis using GC-MS

Optimum operational conditions of GC-FID or GC-MS were as follow. The inlet and FID temperature were 285^o C, sample volume injection was 2 µL with split-less mode. The column temperature was programmed initially at 200^{o}C for 1 minute, increased 5^{o}C/minute to 275^o C, maintained at 275^o C for 9 minutes, increased 5^o C/minute to 300^o C and finally maintained at 300^o C for 30 minutes. Using optimum operational conditions above, the three specific fragments of MAs showed a good resolution on GC-MS. The instrument precision was good. The intraday injection of TME obtained its t_R of 11.15 minutes (KV of 0.02%) and area of 1987.62 pA.det (KV 2.78%).

The inter-day precision of TME retention time was 11.20 minute (KV 0.034%) and the recovery of the added TME that have been simultaneously processed with the sample was (107.2 ± 17.9) %. GC-MS showed an identical MAs chromatogram when was compared to MAs chromatogram from GC-FID. Based on library data base of GC-MS, the fragment with t_R of 15.05 minute was hexacosane methyl ester (C26:0) with identical quality of 96%. The fragment with t_R 12.42 minute was tetracosane methyl ester (C24:0) with identical quality of 96% and fragment with t_R 13.71 minute was pentacosane methyl ester (C25:0) with identical quality of 92%. Even the structures of the fragments have not confirmed with NMR, they still can be used as a marker for identification of TDM, because they were reproducibly obtained when TDM was esterified. Those fragment would be produced when the chain of meromycolate and β hydroxyl MA were fragmented.

Analysis of MAs of isolate MTB strains

The previous study [7] concluded that the primer fragment of MAs of MTB was fragment of C26:0 and fragment composition of hexacosane methyl ester and tetracosane methyl ester of MAs from H37Rv was (17:3).

This study obtained the composition of hexacosane methyl ester and tetracosane methyl ester of TDM of M bovis was (88:6) and the composition of hexacosane methyl ester and tetracosane methyl ester of H37Rv strain was (76:19) (table 3). Its means that fragment of C26:0 is not specific fragment for MTB, but its composition with another fragment maybe used as identification marker of MAs structure of MTB.

MAs specific fragments with t_R of 12.42, 13.67 and 15.06 minutes were found in all of MTB strain that have been analyzed i.e. H37Rv strain, isolate that sensitive to S-I-R-E and isolate that resistance to isoniazid, with composition area as showed in table 3.

According to anava test (α 0.05), the composition of the three fragments among strains were not significantly different. It can be concluded that by using only 3 specific peaks cannot identify the different structure of MAs.

This study could not use other peaks of MTB strain fragments because they overlapped with the fragment of L-J medium peaks. But, GC-MS chromatogram also showed the fragments at t_R of 5.87-5.91 minute and 5.488 minute in all MTB strains. According to GC-MS data base, those were tuberculostearate and octadecane methyl ester that were normally found in mycobacterium species. Unfortunately, these fragments were not exactly separated from their adjacent peaks.

In order to omit the inference, it is proposed to use non fatty acid containing medium as broth culture, i.e. Middle brook 7H9 or Middle brook 7H10 [8]. As previous recommendation the existence of different structure of MAs among the MTB strain could be studied using LC-fluorescence detector (as a whole MAs molecule) [1,2] or LC-MS (as Fragmented molecule).

CONCLUSIONS

The optimum condition for MAs analysis using GC-FID was obtained. Three specific fragments of MAs were found to be used as MAs

identification in samples of H37Rv strain and MTB strains which were isolated from TB patients. However, this method could not distinguish different structures among the MAs obtained from MTB samples.

REFERENCES

1. Butler W, Guthertz LS. Mycolic Acid Analysis by High-Performance Liquid Chromatography for Identification of Mycobacterium Species. Clinical Microbiology Reviews 2001; 704-726.
2. Deysel MS. http://upetd.up.ac.za/thesis/available/etd-06062008-155723/unrestricted/ 00front.pdf mycolic acid structure-function relationships. Retrieved August 2012
3. Huang CC, Smith CV, Glickman MS, Jacobs WR, Sacchettini JC. Crystal Structures of Mycolic Acid Cyclopropane Synthases from Mycobacterium tuberculosis. The Journal of Biological Chemistry 2002; 11559-11568.
4. Mukesh M, Manju P, Sabitha M. Structural Model of The Alpha Phosphoglucomutase: A Promising Target for The Treatment of Mycobacterium Tuberculosis. Int. J of Pharmacy and Pharm Sci, 2013; 5, 2: 107-108.
5. Vilcheze CA. Isolation and Analysis of Mycobacterium tuberculosis of Mycolic acid. In Current Protocols in Microbiologi. John Wiley & Sons; 2007. p10 A.3.1
6. Tortoli E, Bartoloni A. High performance liquid chromatography and identification of mycobacteria. Reviews in medical microbiology 1996; 207-219.
7. Kaneda K, Naito S, Imaizumi S, Yano I, Mizuno S, Tomiyasu IB, et al. Determination of Molekular species composition of C80 or longer-chain alpha mycolic Acid in Mycobacterium spp. by gas chromatography-mass spectrometry and Mass Spectograhy. Journal of Clinical Microbiology 1986; 1060-1070.
8. Lambert MA, Moss CW, Silcox VA., Good RC. Analysis of Mycolic Acid Cleave Products and Cellular Fatty Acids of Mycobacterium Species by Capillary Gas Chromatography. Journal of Clinical Microbiology 1986; 731-736.
9. Christie WW. Preparation of Ester Derivatives of Fatty Acids for Chromatographic Analysis. In Christie WW, editor. Advances in Lipid Methodology. Dundee: Oily Press; 1993. p. 69-111
10. 10. Firestone D. Oils and fat. In Official Methods of Analysis of AOAC International, Horwitz W, editor. 17th ed. Washington: AOAC International; 2000. p. 26-28.