

**A new liquid chromatographic method for the identification  
of tuberculosis and other mycobacterium species**

**VOLKER REINHARD SCHILLACK**

## Declaration

I, Volker Reinhard Schillack, declare that the dissertation for the degree MSc in Chemistry at the University of Pretoria, is of my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Volker Reinhard Schillack

.....  
Signed

.....  
Date

**A new liquid chromatographic method for the identification  
of  
tuberculosis and other mycobacterium species**

BY

**VOLKER REINHARD SCHILLACK**

Supervisor

Prof. E.R. Rohwer

Submitted in partial fulfilment of the requirements for the  
degree of

**Master of Science**

**Chemistry**

in the faculty of Natural and Agricultural Sciences

University of Pretoria

May 2006

## SUMMARY

High performance liquid chromatography was used to identify different *mycobacterium* species isolated from clinical specimens. The main focus of the study was to develop a fast, cost effective and reliable analysis method that could be used in a clinical diagnostic environment on a daily basis. This research was a first for South Africa, and is currently successfully applied in private pathology laboratory for the management of mycobacterial diseases.

**‘n Nuwe vloeistofchromatografiese metode in die  
identifisering van tuberkulose en ander  
mikobakterium-spesies**

Deur

**VOLKER REINHARD SCHILLACK**

Promotor

Prof. E.R. Rohwer

Ingehandig in deelse bereik van die behoeftes vir die graad

**Magister Scientiae**

**Chemie**

in die Fakulteit Natuur en Landbouwetenskappe

Universiteit van Pretoria

Mei 2006

## Samevatting

Hoëdruk-vloeistofchromatografie is gebruik om verskillende *mycobacterium*-spesies, geïsoleer uit biologiese monsters te identifiseer. Die doel van die studie was om 'n vinnige, koste-effektiewe en betroubare analisemetode daar te stel wat daagliks in 'n klinies-diagnostiese hoedanigheid gebruik kan word. Die navorsing is 'n eerste vir Suid Afrika en word tans suksesvol kommersiëel toegepas in 'n private patologie-laboratorium vir die bestuur van mikrobakteriële siektes.

## Acknowledgement

### **I acknowledge with gratitude the following persons:**

My wife Nicolene, for her love, support and help in the completion of this thesis. I wish to thank her for her dedication and encouragement during my studies.

To my sons, Reiner and Erwin, for their ongoing companionship during the long hours of writing. Their pre-school drawings were very inspiring.

My promotor Professor Egmond Rohwer for allowing me, with financial support, to do the research work as part of an analytical project. His academic contribution, guidance and support are valued very highly.

Johan Janse van Rensburg for his support and knowledge in the field of microbiology.

I thank all my friends and family for supporting me with their friendliness, ideas and technical support in doing this research.

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## LIST OF ABBREVIATIONS



AFB	: acid fast bacilli
AIDS	: acquired immunodeficiency syndrome
ART	: adjusted retention time
BCG	: Bacillie Calmette Guerin
C <sub>60</sub> – C <sub>90</sub>	: carbon 60 – carbon 90 chain length region
CDC	: Centre of Disease Control, USA
CFU	: colony forming unit
CSF	: cerebral spinal fluids
DOTS	: direct observed treatment program
DNA	: deoxyribonucleic acid
HIV	: human immunodeficiency virus
HMW ISTD	: high molecular weight internal standard
HPLC	: high performance liquid chromatography
HPLC / FL	: high performance liquid chromatography / Fluorescence detector
IV	: intravenous
LAM	: lipoarabinomannan
LMW ISTD	: low molecular weight internal standard
RRI	: relative retention index
MA	: mycolic acid
MAIC	: <i>Mycobacterium avium intracellulare complex</i>
MDR	: multi drug resistance
MIC	: minimum inhibition concentration
MOTT	: mycobacterium other than tuberculosis
MTB	: <i>Mycobacterium tuberculosis</i>
MW	: molecular weight
<i>M. chelonae</i>	: <i>Mycobacterium chelonae</i>

NALC	: n-acetyl-L-cysteine
NCTC	: national culture type collection
nm	: nanometre
NTM	: non tuberculous mycobacteria
PBPA	: p-bromophenacyl esters
PCR	: polymerase chain reaction
pg/L	: picogram per litre
RT-PCR	: reverse transcriptase polymerase chain reaction
SAM	: s-adenosyl methionine
TB	: tuberculosis
WHO	: World Health Organization
µg/L	: micrograms per litre

## Abstract

An important class of fatty acids present in the cell wall of the mycobacterium organisms is the group of high-molecular-weight, long chain C<sub>60</sub> – C<sub>90</sub>,  $\alpha$ -branched,  $\beta$ -hydroxylated, keto, methoxy mycolic acids that were already identified in 1950 by J. Asselineau. Mycolic acids were detected in both reference strains and clinical isolates of mycobacteria by using high performance liquid chromatography together with fluorescence detection (HPLC-FL), to identify the different mycolic acid profiles within the cell wall of different mycobacterium species. Analysis of cellular “mycolic acids” by HPLC indicated a distinct pattern, which is known as the “fingerprint” of that mycobacterium species. This is achieved by profiling the mycolic acid 6,7-dimethoxycoumarin esters. Two internal standards with different molecular masses (C<sub>60</sub> – C<sub>90</sub>) are used to determine the relative retention index (RRI), of the different mycolic acid profiles within the cell wall of the mycobacterium. Peak ratios between two or more prominent peaks are also used to identify different species (eg. *Mycobacterium kansasii* and *Mycobacterium asiaticum*).

The identification of different mycobacteria by conventional tests is often difficult, costly and time consuming. Without the aid of HPLC/FL, it is almost impossible to correctly identify atypical mycobacterium species. Sophisticated techniques, like genetic sequencing and HPLC, by now seem indispensable for differentiating unusual and new mycobacteria from the well established ones. The HPLC technique and the distinct patterns of mycobacteria can be used not just as a research tool but also as a diagnostic tool in the clinical environment. This technique is currently used to identify most of the known mycobacterium species according to their mycolic acid profiles. This technique is able to identify certain multiple mycobacterial infection in patients by comparing the different mycolic acid profiles within the cell wall of the

mycobacterium. Unknown mycobacterium species are not uncommon and are easily recognized with this technique. The HPLC/FL technique together with an optimised culturing process, has been proven to be a powerful tool in the clinical diagnostic field.

## Opsomming

'n Belangrike groep vetsure teenwoordig in die selwand van mikobakterium organismes, is die groep hoë - molekulêre-massa, langketting ( $C_{60} - C_{90}$ ),  $\alpha$ -vertakte,  $\beta$ -gehidroksileerde keto, metoksie, mikoliese sure wat alreeds in 1950 deur J.Asselineau geïdentifiseer is. Mikoliese sure in beide kliniese en verwysings - mikobakterium isolate is ontleed deur gebruik te maak van hoëdrukvlloeistof chromatografie met 'n fluoressensie - detektor (HDVC / FL), om die verskillende mikoliese sure in die selwand van die mikobakterium te identifiseer. Ontleding van selwand - mikoliese sure met behulp van HDVC lewer 'n definitiewe vingerafdruk van die betrokke mikobakterium - spesie. Dit is bereik deur profiele van die 6,7-dimetoksie-kumarien esterderivate van mikoliese sure op te neem. Twee interne standaarde met verskillende molekulêre massas ( $C_{60} - C_{90}$ ) is gebruik om die relatiewe retensie indeks (RRI) van die verskillende mikoliese – suur - profiele binne die selwand van 'n mikobakterium te bepaal. Piekverhoudings tussen twee of meer prominente pieke is ook gebruik vir die identifikasie van verskillende spesies (bv. *Mikobakterium kansasii* en *Mikobakterium asiaticum*).

Die identifikasie van 'n mikobakterium deur konvensionele toetse is dikwels moeilik, duur en tydrowend. Sonder die hulp van HPVC-FL, is dit prakties onmoontlik om atipiese mikobakterium - spesies korrek te identifiseer. Gevorderde tegnieke, soos nukleotied – volgorde - bepaling en HPVC blyk onmisbaar te wees in die onderskeiding van ongewone en nuwe mikobakteria van die goed gevestigde spesies. Die HPVC - tegniek en die bekende patroon mikoliese sure wat mikobakteria lewer, verskaf saam 'n goeie diagnostiese en navorsingsmetode vir die gebruik in die kliniese omgewing. Die tegniek word tans gebruik vir identifikasie van meeste mikobakterium-spesies aan hand van die mikoliese – suur - profiele. Die

tegniek is ook in staat om sekere multi – mikobakterium - infeksies in pasiënte te identifiseer aan hand van die verskillende mikoliese – suur - profiele in die selwand van die mikobakterium. Die HPVC/FL tegniek, tesame met 'n geoptimiseerde kweekproses, het homself bewys as 'n kragtige tegniek in die klinies - diagnostiese veld.

## CHAPTER 1: INTRODUCTION

Tuberculosis is the leading cause of death in the world resulting from a single bacterial infection. Despite the enormous burden on the world health, little is known about the molecular mechanisms of the pathogenesis of *Mycobacterium tuberculosis*.

In the developing countries and with the AIDS pandemic, the way we look at the epidemiological situation of mycobacterial disease has changed. *Mycobacterium tuberculosis* is still effectively treated with the standard regimen of antibiotics. Recently, a number of other species of *Mycobacterium* have become opportunistic pathogens in immuno-compromised individuals, mainly AIDS-sufferers, where they can cause tuberculous or non-tuberculous symptoms of infections [1]. Tuberculosis remains a major public health problem. The World Health Organization (WHO) estimates that eight million new cases and three million deaths are directly attributable to the disease each year. Tuberculosis has been cited as the leading cause of death in many resource poor and developing countries. In the United States, the number of new cases reported to the CDC (Centre of Disease Control) peaked in 1992. Since then a substantial decline in cases has been reported. This is due to the strict monitoring and directly observed treatment program (DOTS) that was implemented in 1989.

Pulmonary mycobacterial infections are usually caused by members of the *M. tuberculosis* complex. Mycobacteria other than tuberculosis (MOTT) are referred to as the non-tuberculous mycobacteria (NTM) or potentially pathogenic environmental mycobacteria (PPEM). The majority of MOTT isolates require incubation at 35°C to 37°C, except where otherwise indicated. These organisms are commonly found in

soil and water and have been implicated as opportunistic pathogens in patients with underlying lung disease, immune suppression or per-cutaneous trauma (skin lesions).

MOTT associated with pulmonary disease are: *M. avium intracellulare complex (MAIC)*, *M. abscessus*, *M. kansasii*, *M. simiae*, *M. asiaticum*, *M. fortuitum complex*, *M. szulgai*, *M. malmoeense*, *M. celatum* and *M. xenopi*. MOTT producing other types of infections (e.g. Scrofula: *Tuberculosis* lymphadenitis of the cervical nodes; Gastrointestinal Tuberculosis;

Urinary tract Tuberculosis; Skeletal Tuberculosis; Cardiac Tuberculosis etc.) are

*M. avium intracellulare complex*, *M. kansasii*, *M. scrofulaceum*, *M. fortuitum complex*, *M. chelonae*, *M. szulgai*, *M. simiae*, *M. genavense*, *M. celatum* and *M. haemophilum*.

In developing countries the rate of initial drug resistance of *Mycobacterium tuberculosis* to first-line drugs has increased to a point where chemotherapy might have a high rate of failure. The rate of MOTT infections in AIDS patients – (and localized pulmonary diseases in non-AIDS patients) - are growing throughout the world. Sub-Saharan Africa in particular, has been hard hit by mycobacterium diseases. Effective treatment is often difficult as the shortest treatment program available lasts at least six months. *Mycobacterium tuberculosis* is very contagious and infected patients should be treated promptly with standardized TB antibiotic regimens. For Africa this is not easy, poor living conditions and almost non existing infrastructure have a negative effect on the fight against mycobacterial disease.



*Mycobacterium tuberculosis* infections are persistent and continuously overwhelm the population in developing countries.

Mortality rates of between 23 – 50% are not uncommon. Slow growing non-tuberculosis (MOTT) infection is an emerging problem. Various diseases predispose humans to become vulnerable to the development of tuberculosis. The most important risk is HIV- infections, which affect the immunity of the infected person. The risk of developing a disease from *M. tuberculosis* is directly related to the state of immunity suppression in the individual. The risk for a non-HIV infected individual to develop tuberculosis in his/her lifetime is about 10%. In individuals infected with HIV or other immune compromising diseases, the risk increases to 10-15% per year [2]. Tuberculosis is the most common infection in HIV infected patients. The current number of co-infections is expected to double in the next decade due to the impact of the HIV epidemic [2].

The *Mycobacterium fortuitum* complex includes *Mycobacterium fortuitum*, *Mycobacterium chelonae* and *Mycobacterium abscessus*. They are relatively rare pathogens and account for only 8% of the MOTT isolated infections in 2003 [3]. However, an increase of the MOTT isolates and infections have already been reported in 1981 [3]. *Mycobacterium smegmatis*, which was commonly considered to be an environmental isolate, has only recently been reported to cause infections in humans [3].

The so-called “environmental mycobacteria” were previously not investigated as human pathogens. These environmental mycobacteria have been further investigated as possible secondary pathogens in humans. It is only recently that the persistent MOTT infections have been further investigated as possible human pathogens.

The deterioration of general hygiene in hospitals and clinics is one of the biggest problems faced in Africa. Other mycobacterial diseases that started to burden our health system with increasing incidence, are infections of *M. avium intracellulare complex (MAIC)*, *M. abscessus*, *M. kansasii*, *M. simiae*, *M. asiaticum*, *M. fortuitum complex*, *M. szulgai*, and *M. xenopi*. These species require a different approach to antibiotic treatment and susceptibility testing than that of the *M. tuberculosis complex*.

Tuberculosis is normally diagnosed by identifying the mycobacteria in sputum samples using the microscope and the “Acid Fast Stain” technique. The microscope has a sensitivity of detecting 5000 -10000 bacilli per millilitre of sputum. Molecular diagnostic, the recently introduced approach, results in more rapid and sensitive detection, but is very expensive and requires trained personnel and sophisticated infrastructure. DNA profiles are specific [3] does not require 10000-100000 organisms for identification. Theoretically one genome or 1 bacterium should suffice and certainly in general PCR practitioners regard ten genomes as adequate. At present, the main focus is the PCR or DNA amplification of parts of the genome of mycobacteria.

Another method developed for diagnosing mycobacterial infection is based on the measurement of mycolic acid profiles - uniquely associated with the cell wall structure of each bacterium – by measuring the unique mycolic acid profiles with high performance liquid chromatography (HPLC).

In general, non-aromatic organic acids like the mycolic acids do not produce strong fluorescence or absorption in the ultraviolet or visible regions. Therefore, derivatization with a suitable labelling reagent has been used to increase their detectability with the HPLC fluorescence detector. Derivatization of the mycolic

acid is a very sensitive method for the evaluation of mycolic acid profiles at picomole amounts [4].

The mycolic acid profiles associated with the cell wall of the bacteria is not only used to identify infections but also provides new insights into the metabolic reaction of the mycobacteria towards external pressure. Short chain fatty acids are intermediates in the metabolism of amino acids such as leucine and isoleucine and are secreted by some bacteria. Understanding the way mycolic acid profiles change under pressure may lead to new drug therapy and vaccination programs. Such program can only be successful through continuous research of the mechanisms of *Mycobacterium tuberculosis* pathogenesis. Early detection of mycobacterium in patients is of vital importance, as it will lead to a reduction of infections.

Continuous research and measurements will assist the scientist in the identification of possible early mutations within the cell wall of *Mycobacterium tuberculosis* and other mycobacteria.

## **1.1 FIELD OF RESEARCH**

The measurement of mycolic acid profiles within the cell wall of the mycobacterium, as a method for identification of mycobacterium species, has only recently been attempted. It was only in the late 1990's that a proper identification method was established [5]. Only a small group of scientists worldwide use this technique in a diagnostic clinical environment. High Performance liquid Chromatography (HPLC) profiling of mycolic acids for the identification of Mycobacteria currently is time consuming and requires visual comparison as well as retention index calculation.

In 2000, we realized that the methods currently used to identify mycobacterium other than tuberculosis (MOTT) only provide us with a limited amount of information.

After an intensive literature search we decided to evaluate the high performance liquid chromatography technique. There are different techniques that could be used to identify mycobacteria, but none as comprehensive and specific as the HPLC technique.

## 1.2 HYPOTHESIS

Reference laboratories use mycolic acid profiles mainly to identify different mycobacteria. Identification of the different mycolic acid profiles originating from the cell wall of the mycobacteria is mostly done in a research environment. Only a few institutions worldwide use this technique as a tool to identify the different mycobacteria on a routine basis.

Mycolic acids are  $\alpha$ -ramified,  $\beta$ -hydroxylated fatty acids in which the number of carbon atoms and degree of saturation of the main and lateral chain, vary according to the genus considered, e.g. *Corynebacterium*, C<sub>22</sub>–C<sub>38</sub>; *Rhodococcus*, C<sub>34</sub> – C<sub>64</sub>; *Nocardia* C<sub>44</sub> –C<sub>60</sub> and the *Mycobacteria* C<sub>60</sub> – C<sub>90</sub>.

Our hypothesis is that by analysing these long chain fatty acids routinely, by means of an HPLC/FL technique, we will be able to increase sample throughput, improve diagnostic ability, and consequently save on the overall cost and time and, eventually, improve the way we diagnose and treat mycobacterium diseases.

## 1.3 OBJECTIVE

In this study, we will examine, develop and introduce an effective high performance liquid chromatographic technique (HPLC) for the identification of mycobacteria by analysis of the mycolic acid profiles associated with the cell wall of the bacilli.

Our primary objective was to obtain unique mycolic acid profiles for the different mycobacterium isolates. This would allow unambiguous identification of unknown mycobacterium species in clinical samples.

A secondary purpose of this study was to optimise the high performance liquid chromatography method first described by Jost, Dunbar, Barth, Headley, Elliott in 1995 [6]. This technique, in combination with a modern culturing system and culture medium, could possibly be improved as far as sensitivity, robustness, time and cost-effectiveness is concerned. For this purpose a liquid growth medium would be evaluated.

The research was undertaken to:

- establish an alternative, more cost effective, rapid method for the identification of mycobacteria
- optimise a High Performance Liquid Chromatographic / Fluorescence (HPLC/FL) detection technique for mycolic acids
- establish an own mycolic acid reference library of the mycobacteria, recorded with new analytical procedure.

## CHAPTER 2: MYCOBACTERIAL LIPIDS: A HISTORICAL PERSPECTIVE

### 2.1 MYCOLIC ACIDS

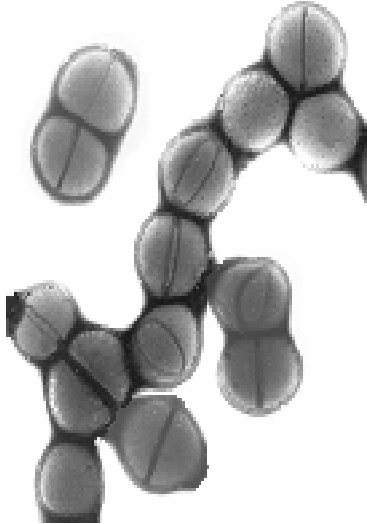
Mycolic acids are complex hydroxylated fatty acids with elevated carbon numbers ( $C_{60} - C_{90}$ ). The mycolic acids may also contain diverse functional groups such as  $\alpha$ -branched,  $\beta$ -hydroxylated, keto, methoxy and epoxy ester groups as well as cyclopropane rings. These fatty acids were isolated and named by Stodala RJ [7] from waxy extracts of the human tubercle bacillus (*Mycobacterium tuberculosis*) with a common structure that was elucidated in 1950 by Asselineau J. [8].

*Mycobacterium tuberculosis* was also studied in 1942 by using electron microscopy [7]. This was done mainly to establish the consistency and the ultra structure of the mycobacteria species. This species and other fast and slow-growing mycobacteria have subsequently been studied by a variety of electron microscopic techniques, thus a considerable amount of the ultra structural data has been documented [9].

Most bacteria do not have the elaborate system of internal compartments found in eukaryotic cells. The information obtained by microscopy is primarily information about the cell wall layers (Diagram 1).

## Diagram 1. Images of bacteria [6].

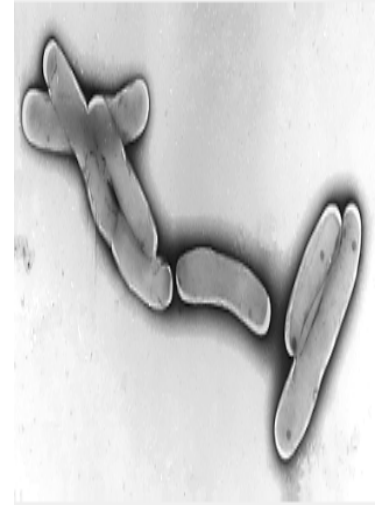
**Gram-positive**  
*S. aureus*



**Gram-negative**  
*E. coli*

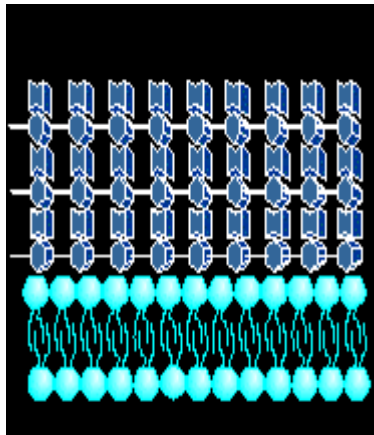


**Acid fast**  
*M. tuberculosis*



Bacterial envelopes (cell walls) provide protection and support for the bacterial cell and also regulate the diffusion of minerals between the cell and the environment. The intrinsic drug resistance of mycobacteria has been attributed, at least partially, to the low permeability of the cell wall [10]. Diffusion or influx of small hydrophilic agents, which are likely to be transported through the porins or channels, is extremely slow [9] due to the small number of porins within the cell wall. However, the mycobacterium cell wall is extremely rich in lipids. Lipophilic and amphiphilic agents may cross the cell wall through the lipid domain. Yet, most mycobacteria exhibit a high level of intrinsic resistance to such lipophilic and amphiphilic agents. A major difference between gram-positive and gram-negative species is the cell structural composition. *Mycobacterium tuberculosis* and other mycobacteria belong to the gram-positive group but have some distinctive features (Diagram 3).

**Diagram 2. Gram-positive organism [10].**



Covered by a porous **peptide glycan layer** which does not exclude most anti microbial agents



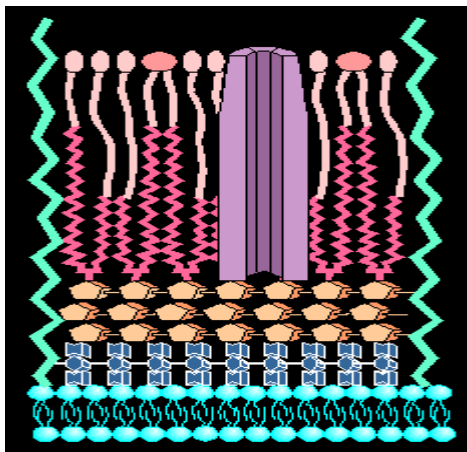
LIPID BILAYER



PEPTIDOGLYCAN

All gram-positive (Diagram 2) bacteria possess peptidoglycan, a cross-linked polymer of amino sugar and amino acid determining the shape of the bacterial cell (Diagram 1).

**Diagram 3. *Mycobacterium tuberculosis* cell structure [10].**



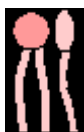
Mycobacteria have a thick mycolate – rich outer covering which functions as an exceptionally efficient barrier.



LAM



ARABINOGALACTAN



ACYL LIPIDS



MYCOLATE



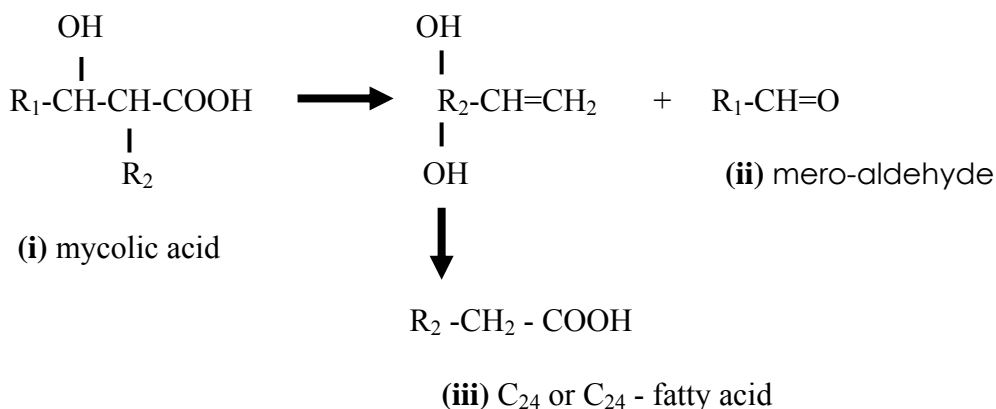
PORINS



In mycobacteria the molecules attached to this polymer are predominantly lipids other than the proteins and polysaccharides found in gram-positive bacteria [12]. Mycobacteria cell walls consist of polysaccharides that resemble that of the gram-positive bacteria in having a disaccharide-phosphoryl link between the peptidoglycan and polysaccharide [13]. Since *Mycobacterium tuberculosis* is one of a small group of species able to survive inside the phagocytic cells of a host, it is thus very likely that the envelope (cell wall) has special properties defending the bacterium against invader host microbiocidal processes. In studying the mycobacterial envelope and its chemical composition, it is necessary to fractionate the cells. Fractionating of mycobacteria presents various problems: they are mechanically tough and the various fractions tend to adhere to one another and to the surfaces. By prolonged saponification of the waxes of tubercle bacillus, an acid called *mycolic acid* was isolated by Anderson in 1939 [14]. The formula  $C_{88}H_{172}O_4$ , was first attributed to this mycolic acid, which shows a deficit of 4 hydrogen atoms, although no unsaturated bonds could be detected (Figure. 1).

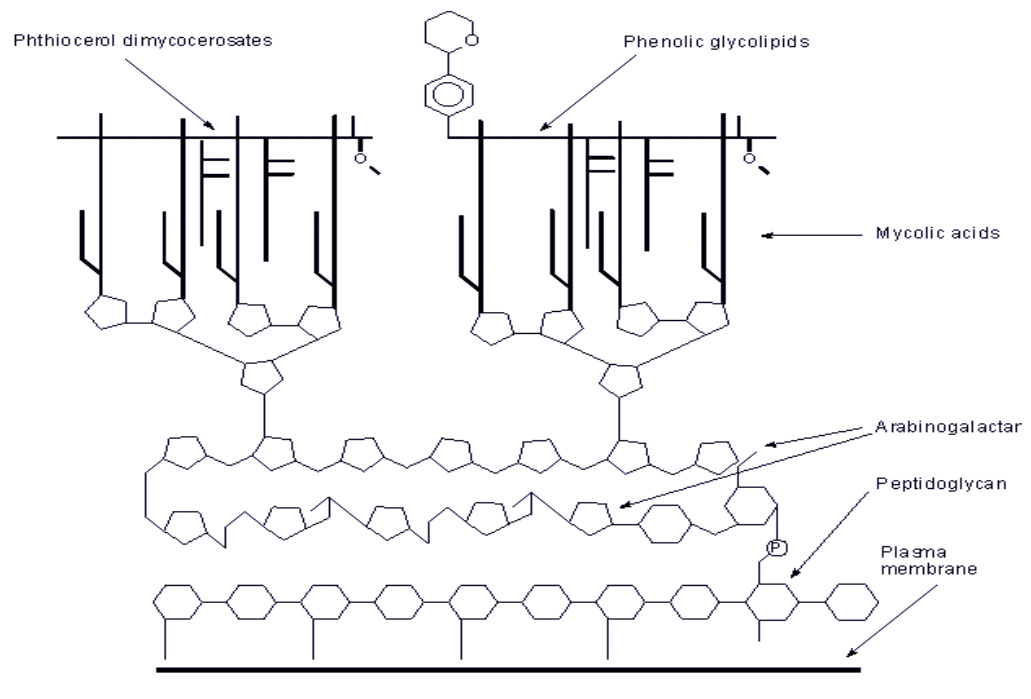
**Figure. 1. Mycolic acid and its pyrolytic cleavage. Asselineau J. [8].**

**Mycolic acid (i) and its pyrolytic cleavage**



To study the mycolic acids within the cell wall it is important to saponify the cell wall of the bacteria in strong alkaline/methanol medium. Membranes of mycobacteria, including *Mycobacterium tuberculosis* appear in ultra thin sections as classic bilayers, outer layer and plasma membrane separated by an electron-transparent layer and an electron-dense layer [15]. Mycobacterial membranes do, however have some distinctive components that differentiate them from the other bacteria e.g. the lipopolysaccharides, lipoarabinomannan (LAM), lipomannan and the phosphatidylinositol mannosides. As already stated, the chemistry of the mycobacterial wall consists of shape forming peptidoglycan, attached to this (by phosphodiester bonds) is a branched chain polysaccharide, the arabinogalactan-peptidoglycan, whose distal ends are esterified with high molecular weight fatty acids (mycolic acids) of sizes and structures unique to mycobacteria (Figure. 2).

**Figure. 2. Diagram of the Mycobacterial cell wall**  
(Jean Asselineau ; Gilbert Laneelle [6])



Mycolic acids are branched 1-alkyl, 2- hydroxy fatty acids, typical with 70-90 carbon atoms with a total saturated alpha branch ( $R_2$ ) of typically between 24-26 carbons and a meromycolate ( $R_1$ ) chain of 40-60 carbons. Mycolic acids are located within the bacterial cell wall, (Fig. 2) covalently esterified to peptides – glycan – linked arabinogalactan polysaccharides, and are also non covalently, hydrophobically associated in the form of 6,6 trehalose dimycolate [16]. Mycolic acids were primarily described as a component of bacterial membranes and their cell envelopes,, which are found only in certain Gram – positive bacteria, including the genera *Mycobacterium*, *Gordona*, *Nocardia* and *Rhodococcus*. Distinct mycolic acid profiles differences in other genera can be seen in table 1.

**Table 1. Genus bacteria of importance**

(Jean Asselineau ; Gillbert Laneelle [6])

Genus	Total no of carbons	Double bonds	$\alpha$ -Branch carbons ( $R_2$ )
Corynebacteria	22 – 36	0 – 2	14 – 18
Gordona	48 – 66	1 – 4	16 – 18
<b>Mycobacteria</b>	<b>60 – 90</b>	<b>2</b>	<b>20 – 26</b>
Nocardia	44 – 60	0 – 3	12 – 18
Rhodococcus	34 – 48	0 – 4	12 – 18
Tsukamurella	64 – 78	1 – 6	20 – 22

In the meromycolate ( $R_1$ ) chain (Fig.1), there are usually only two positions that may be occupied by double bonds, cyclopropane rings, or other functional groups [17].

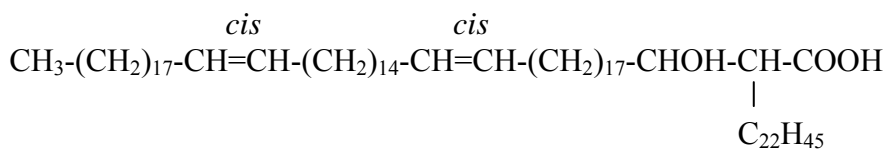
The main branch ( $R_2$  - merolate branch) contains cyclopropyl, methoxyl or keto and methyl groups. The asymmetry between the two alkyl chains is important in the construction of the envelope. For each mycobacterium, the mycolic acids profiles

within the cell envelope differ. This property can be used in the identification of the different mycobacteria.

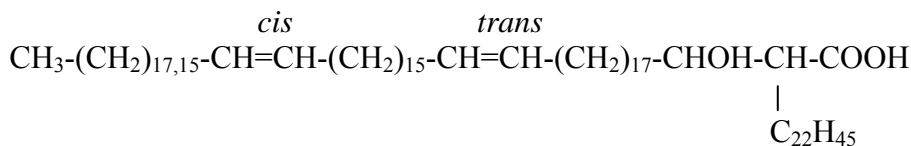
The more distal of the two positions in the R<sub>1</sub> chain may contain oxygen functionalities, which allows us to classify mycolic acids into alpha-mycolates (without oxygen containing functional groups in the meromycolate branch), ketomycolates and methoxymycolates (Figure. 3).

**Figure. 3. Mycolic acids without oxygen function in the mero part (alpha-mycolic acid) (Jean Asselineau ; Gillbert Laneelle [8])**

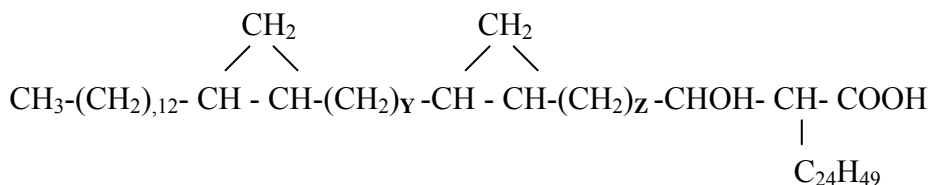
(i) *Mycobacterium phlei*



(ii) *Mycobacterium smegmatis*

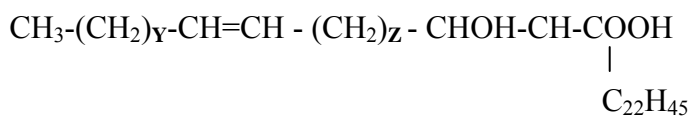


(iii) *Mycobacterium tuberculosis*



Y= Carbon chain length - 10, 14, 16      Z= Carbon chain length - 11, 13, 17, 19

(iv) **Other alpha mycolic acid**



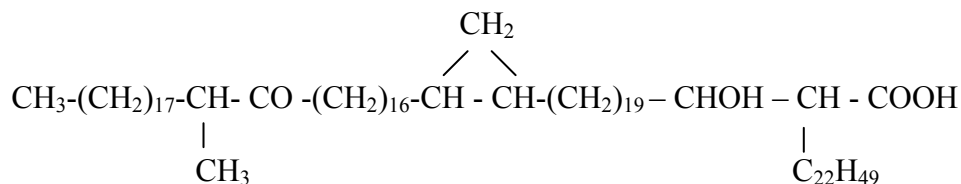
Y= Carbon chain length - 15, 17

Z= Carbon chain length - 17, 19

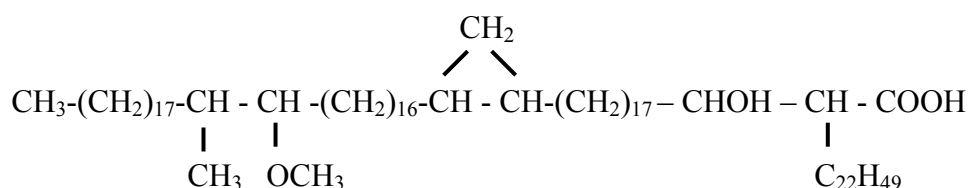
By working on a mycolic acid fraction from *Mycobacterium smegmatis* (C<sub>62</sub>, C<sub>64</sub>) in 1964, the group led by Etemadi [17] was able to provide the first complete structure of a mycolic acid molecule (Fig. 3(ii)). Later on, it was shown that cyclopropane rings could exist instead of the double bonds (leading to an acid devoid of unsaturation), for example, in acids from *Mycobacterium kansasii* and *Mycobacterium tuberculosis*. Shorter chain alpha-mycolic acids are found in some species (Fig. 3(iv)). These alpha-mycolic acids, about C<sub>60</sub>, represent up to about 25% of the total mycolic acids of the mycobacteria. The alpha-mycolic acids are also present in *M. chelonae* (C<sub>64</sub>), *M. fortuitum* (C<sub>68</sub>), *M. parafortuitum* (C<sub>58</sub> - C<sub>60</sub>), *M. vaccae* (C<sub>58</sub> - C<sub>60</sub>). In some species, the oxygen containing groups in the meromycolate branch is a carboxylic ester, producing wax ester mycolates

**Figure. 4. Mycolic acid with an oxygenated function in the mero part**  
**(Jean Asselineau ; Gilbert Laneelle [8])**

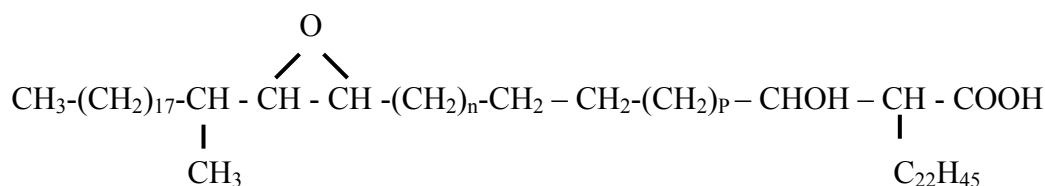
**(v) Keto (or oxy) mycolic acids**



**(vi) Methoxylated mycolic acids**



**(vii) Epoxy mycolic acids**



$n$  = Number of carbons length - 10 to 17     $p$  = Number of carbons length - 13, 15, 17, 19, 21

The more proximal of these positions contains a double bond or cyclopropane, (Fig. 4) and invitro a fraction of these structures is converted from the cis-isomer to a trans-isomer, a reaction that is expected to affect the fluidity of the packed hydrocarbon chain [18].

Keto- (or oxy-) mycolic acids (Fig. 4(v)) and methoxylated mycolic acids have been known to scientist for some time (Fig. 4(vi)). More recently, (omega-1) -methoxy acids were identified in species from the environment [2]. Epoxy-mycolic acids (Fig. 4 (vii)) contains a *trans*-epoxy ring in the mero part of the molecule [19]. These

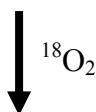
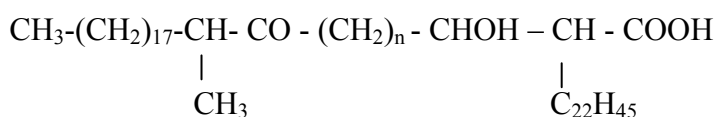
mycolic acids are also present in *Mycobacterium farcionogens*, *Mycobacterium senegalense*, *Mycobacterium chitae* and *Mycobacterium smegmatis*.

Carboxy-mycolic acids and wax-mycolic acids (Fig. 5) are the most abundant of all the mycolic acids. These mycolic acids have a second carboxyl group at a lower carbon molecular weight ( $C_{60} - C_{68}$ ). They are often called "dicarboxylic mycolic acids".

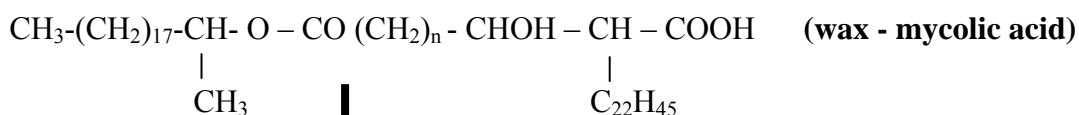
**Figure 5. Carboxymycolic acids and their biogenesis (Jean Asselineau ; Gilbert Laneelle [8])**

Keto or oxy mycolic acid (viii) (first precursor). Carboxy mycolic acid (xi), eicosanol-2 and octadecanol-2 (x) and "Wax" mycolic acid (ix) are oxygenated precursors.

(viii)



(ix)



(x)

(xi)



It was suggested that a keto-mycolic acid could be transformed into esters and carboxy-mycolic acids (wax-esters) and eicosanol-2 or octadecanol-2 by the reaction of Baeyer-Villiger type [20]. This theory was strengthened by isolating the wax-ester mycolic acid from *Mycobacterium paratuberculosis* (Fig.5(xiii)) and by the incorporation of  $^{18}\text{O}$  (from  $^{18}\text{O}_2$ ) into the wax-esters [21]. The stereochemistry of the

group characteristic of mycolic acids (Fig.1(i)) studied in coryno-mycolic acid was established as tetradecyl-2*R* hydroxy-3*R* octadecanoic acid [22]. These configurations of tetradecyl-2*R* hydroxy-3*R* octadecanoic acid were also later identified for mycolic acids of *Mycobacterium tuberculosis*, *Mycobacterium fortuitum*, *Mycobacterium marinum*, and *Mycobacterium ulcerans* [23].

Mycobacterial cell walls also contain several types of “extractable lipids” such as trehalose containing glycolipids, phenolic glycolipids or glycopeptidolipids [18, 24]. Only recently biophysical studies provided some insight into the organization of the cell wall lipids [25].

Purified cell wall of *Mycobacterium chelonae* showed that the bulk of the hydrocarbon chain existed in tightly packed parallel, quasi crystalline array and that they are mainly arranged in the direction perpendicular to the cell wall surface. These findings supported the earlier model as proposed by Minnikin [25] in which the cell is composed of an asymmetric lipid bilayer; the inner leaflet contains mycolic acids covalently linked to the arabinogalactan and the outer leaflet contains other extractable lipids.

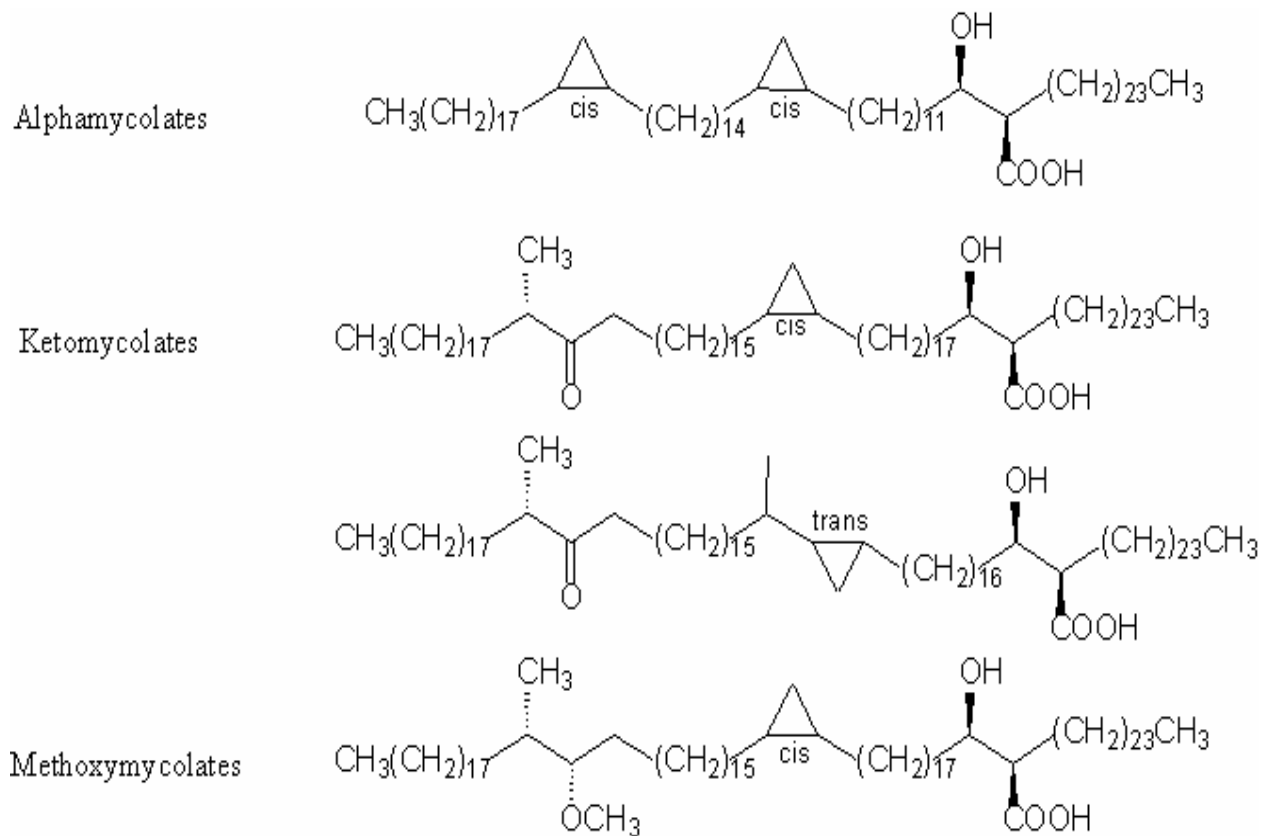
*Mycobacterium* synthesizes three classes of mycolic acid (very long chain,  $\alpha$ -alkyl, beta hydroxyl fatty acid) in its cell envelope: Alpha mycolic acid, methoxy mycolic acid, keto mycolic acid (Fig. 6).

These classes are modified with cyclopropane rings and methylated branches through the combined action of a large family of S-adenosyl methionine (SAM) dependent methyl transferase that modifies double bonds in the mero mycolic chain. The mycolic acid structure of *Mycobacterium tuberculosis* is highly complex. Identifying the structures of each mycolic acid within the cell wall of the mycolic acid



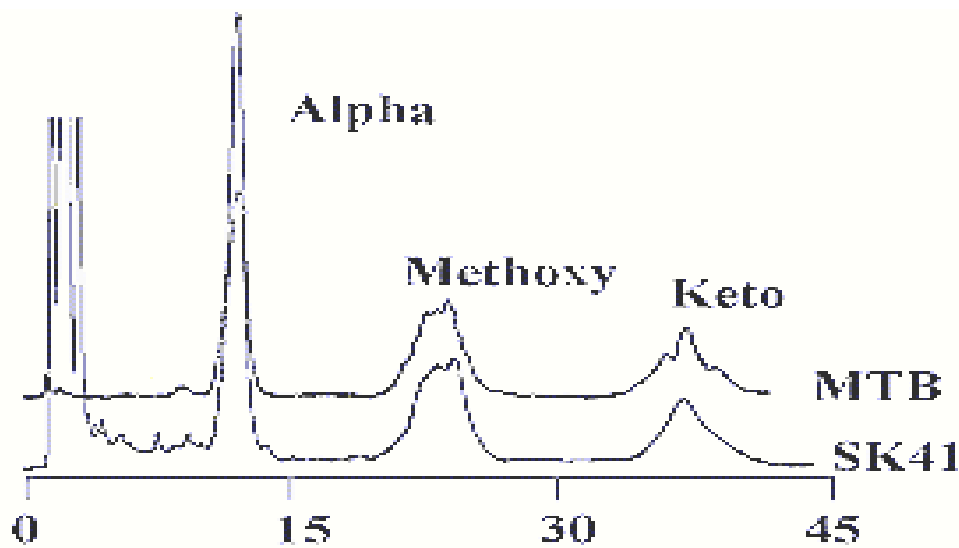
is difficult enough. Finding out the exact position of these mycolic acids within the cell wall is, however, a much more difficult task.

**Figure. 6. The three main mycolate configurations**  
**(D.E. Minnikin, Research in microbiology [16])**



**Figure. 7. Normal phase chromatogram of *Mycobacterium tuberculosis***

Normal phase chromatogram of alpha-, methoxy- and ketomycolic acids in *Mycobacterium tuberculosis* complex species (MTB) and a few thousand year old *Mycobacterium tuberculosis* specimen (SK41) (D.E Minnikin, Research in Microbiology [16]).



It is known that long chain constituents of *Mycobacterium tuberculosis* can survive in archaeological material for over thousand years [23]. Fractions containing different mycolates could be isolated by normal phase liquid chromatography from an ancient skeleton (SK41) from the Newcastle infirmary site (Fig. 7). These mycolic acids have been identified as wax ester, epoxy and methoxy mycolates. These mycolate chains are characterized by the way these groups position themselves, and are thus responsible for some of the inherent properties of the mycobacteria [26].

### 2.1.1 Mycolic acid Class Variation Between Species

The mycolic acids of the mycobacteria are amongst the longest and the most highly functionalised of the mycolates [18]. They occur with a variety of functional groups, both polar and non-polar, which give rise to the characteristic chromatographic profiles (Fig. 7).

The two exceptions to this general statement are the shorter carbon chain  $\alpha$ -mycolates and the  $\omega$ -1 methoxy-mycolates of some rapidly growing mycobacterium species. Polar modifications tend to be restricted to the distal position and include such functional groups as methyl ethers, ketones, esters and epoxides. Non-polar modifications occur at both the distal and proximal positions and includes cis or trans double bonds and cis or trans cyclopropanes (Fig. 6). The composition of these mycolic acids in various mycobacterial species is shown in table 1. The most widespread classes of mycolic acids are the  $\alpha$  mycolates. The second most widespread class is the keto-mycolates. These are found in many different mycobacterium species, regardless of the growth rate or state, and are classified as pathogens or saprophytes. The wax ester mycolates are also widely distributed, but are not present in *Mycobacterium tuberculosis*.

The property of the long chain fatty acid “mycolic acids” contributes to the resistance of the mycobacterium against the elements of nature. The longer chains of mycolic acids are also partially responsible for the resistance of the organism to certain chemo treatments [27]. The way these mycolic acids arrange themselves in the cell wall, determines this resistance.

### 2.1.2 Analysis Technique: Chromatography

Chromatography is an extremely versatile analytical technique in a clinical laboratory. Chromatographic patterns generated by modern instruments are used in a wide variety of quantitative and qualitative analysis.

*Mycobacterium tuberculosis* is the etiologic agent of tuberculosis and can be accurately detected by laboratories using commercial genetic tests. Mycobacteria other than tuberculosis (MOTT) causing other mycobacterioses can be difficult to identify.

Identification processes are confounded by an increasing diversity of the newly characterized MOTT species. The different infection rate in immune-compromised as apposed to the non immune-deficient patients further complicates the problem of early and correct identification.

Early identification of the *Mycobacterium* genus has included observations of staining properties of bacilli, culturing morphology, biochemical tests, and the susceptibility testing on animals with live bacilli for research and observation of animal pathogenicity. These early tests were designed to discriminate among mycobacteria involved in disease and were directed only towards detecting *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium*, or saprophytes [28].

Over the years, unequivocal identification of *Mycobacterium tuberculosis* and species of clinical interest has dominated the taxonomy of mycobacteria [29].

The genetic probes were developed to identify the most commonly found mycobacteria in a clinical laboratory. However, these genetic probes were only developed for a majority of the mycobacteria species, because most of the MOTT's

were not considered a threat for the public health, rarely isolated, and were never transmitted from person to person.

Liquid chromatography principles and instrumentation originated in the field of analytical chemistry during the mid-1960s and widespread applications in organic, inorganic and biochemical areas have since been developed [30].

In the mid to late 1970s, the newly developed method was referred to as high performance liquid chromatography (HPLC). High-pressure pumps forced a liquid referred as buffer or solvent through a packed bed of solid stationary phase, enclosed in a metal column, resulting in the rapid fractionation of the sample components. The separated components were sensed by a detector and recorded as peaks by a recorder. Complex interaction of the compounds within the sample occurring between the mobile liquid phase and solid stationary phase resulted in the separation of the different compounds within the metal column [31]. The sensitivity of mycolic acid detection was increased by derivatization to UV-absorbing p-bromophenacyl esters (PBPA) [32]. The reverse phase chromatography procedure utilized high efficiency C<sub>18</sub>, microparticle bonded stationary phase columns and mobile organic solvents. It was reported that reverse phase HPLC profiles for alpha-mycolate, methoxymycolate, and ketomycolate chemical functional groups demonstrated increasing retention times with increased carbon chain length. However, it was noted that the classes of mycolic acids were not completely chemically resolved by HPLC when subjected only to reverse phase chromatography [33, 34]. Consequently, chromatographic patterns were published of mycolic acids for mycobacteria that demonstrated extensive overlapping of the chemical fractions [30, 33, 35]. The recognition of pattern difference between the

different mycobacteria provided the stimulus for further research with the HPLC instrument as a tool for species identification.

Detection sensitivity can be improved by the use of fluorescence labelling reagents.

Theoretically, fluorescence detection can increase the sensitivity 10 to 1000 times.

Different fluorescence labelling compounds were investigated, as some will influence the separations of the labelled carboxylic acid, an alternative fluorescence-labelling reagent, 4-bromomethyl-7-acetoxycoumarin was suggested [36].

Eventually, 4-bromomethyl 6,7-dimethoxycoumarin was shown to be suitable for gradient elution with reverse-phase chromatography [37]. A 20 to 80-fold increase in sensitivity was observed compared to UV-absorption of the PBPA derivative [38, 39]. An early demonstration of the use of coumarin derivatives with mycobacterial mycolic acid and reverse-phase HPLC was the detection of *Mycobacterium tuberculosis* and *Mycobacterium paratuberculosis*, the causative agent of Johne's disease [38]. Mycolic acids C<sub>48</sub> to C<sub>76</sub> were separated with gradient mobile phase of chloroform and acetonitrile. The fluorescence derivatives were analysed at excitation wavelength of 285 nm and emission wavelength of 340 nm with characteristic co-emerging homologous mycolic acid peaks. The author speculated that mycolic acid patterns for *Mycobacterium para-tuberculosis* would be useful in detecting this bacterium in the feces of infected cattle.

In 1985, scientists at the Centers for Disease Control and Prevention (CDC) proposed the use of HPLC as an aid in mycobacterial classification [30]. In 1989 the method was implemented, and used in a regimen of tests in the CDC Mycobacteriology Reference Laboratory. Only in 1990 mycolic acid profiling was offered as a standard test for identification of *Mycobacterium* species [40].

HPLC techniques are considered sophisticated and costly compared to other laboratory methods, and dedicated, highly trained operators are required. Instrumentation is costly compared to conventional methods. Laboratories that detect acid-fast bacilli only by smear may not have the capacity to develop sophisticated methods by HPLC. However, many high-throughput laboratories that are proficient in all aspects of mycobacteriology have successfully incorporated HPLC into their methodologies [41].

The development of laboratory staff to interpret the chromatographic profiles takes considerable time and training.

The analysis of these mycolic acid by means of HPLC has contributed significantly to the knowledge we currently have of the *Mycobacterium* species. The diagnostic value of the mycolic acids was quickly recognised by early chemical investigators who characterized the different mycolic acid containing genera [26, 42, 43].

Alternative chromatographic (2D-TLC and GC-MS) techniques [44, 45] contributed considerably to the structural evaluation of *Mycobacterium* specie. Analysis of 50 species of mycobacterium by 2D-TLC revealed that mycobacterium were separated into 11 structural groups based on the composition of their mycolic acids, and the analysis technique was accepted in clinical laboratories for the rapid identification of mycobacteria [46].

Presently, mycolic acid containing genera can be distinguished by profiling the length of their mycolic acid chains.

The HPLC ability to separate and analyse complex samples is an integral part of biological and medical research. This technique of mycolic acid analysis has proven to be an indispensable part in the identification of mycobacteria.

## CHAPTER 3: ANALYTICAL PROCEDURE

### 3.1 GENERAL

A suspension of acid-fast bacteria was saponified to break down the mycolic acids bound in the cell wall of the bacteria. The mycolic acids were extracted into acidified chloroform. After conversion to the fluorescence derivative, the different mycolic esters were separated on a reverse-phase C<sub>18</sub> column using an HPLC system. Separation of the mycolic esters was achieved by running a gradient that contains methanol and dichloromethane as mobile phase.

### 3.2 REAGENTS AND MATERIALS

Except where noted, chemicals and solvents must be of a certified analytical grade or HPLC grade. All HPLC solvents and samples must be filtered. All water that is used must be deionised and sterilized.

#### 3.2.1 Chemicals

1. Hydrochloric acid
2. Potassium hydroxide
3. Potassium bicarbonate
4. High and low molecular weight internal standards from Ribi Immno Chem Research , Inc.
5. Derivatization reagent
  - (i) Dimethoxy coumarin (0.1 mmol/ml)
  - (ii) Dicyclohexyl-18-crown-6 ether (0.005 mmol/ml) in dichloromethane.
6. Chloroform
7. Dichloromethane
8. Methanol



### 3.3 REAGENTS FOR SAPONIFICATION, EXTRACTION AND DERIVATIZATION OF MYCOLIC ACIDS

#### 3.3.1 Saponification reagent

Potassium hydroxide (KOH) 200.0 g

Reagent grade water 400.0 ml

Methanol 400.0 ml

While stirring slowly, add KOH to water in glass beaker standing in an ice bath to keep mixture cool. Continue stirring until the KOH has dissolved.

When cool, add the methanol. Store saponification solution in polypropylene container until used.

#### 3.3.2 Acidification reagent

Reagent grade water 200.0 ml

Concentrated hydrochloric acid (HCl) 200.0 ml

While stirring, slowly add the 200.0 ml of conc. HCl to the water in a litre beaker

#### 3.3.3 Potassium bicarbonate reagent

Potassium bicarbonate (KHCO<sub>3</sub>) 4.0 gram

Reagent grade water 98.0 ml

Methanol 98.0 ml

Add KHCO<sub>3</sub> to 98.0 ml H<sub>2</sub>O in glass beaker. Stir until dissolved, add the methanol and stir. If precipitation occurs, heat to 35°C ± 1°C to dissolve.

### **3.3.4 Derivatization reagent**

Prepare and store as described in the supplier instructions.

### **3.3.5 Clarification reagent**

50/50 acidification reagent and methanol

Mix 100.0 ml acidification reagent (step 2.) with 100.0 ml methanol. Store at ambient temperature in glass container.

### **3.3.6 Internal standard**

Add 4.0 mg of LMW ISTD and 2.0 mg of HMW-ISTD to 50 ml volumetric flask with dichloromethane.

Dilute internal standards to achieve a final of 80.0 µg/L LMW and 40.0 µg/L HMW internal standards.

## **3.4 EQUIPMENT**

3.4.1 Certified biological safety cabinet

3.4.2 Certified chemical fume cabinet

3.4.3 HPLC equipment with Fluorescence detector

(HPLC/FL – excitation wavelength 351 nm and emission wavelength 430 nm)

3.4.4 Autoclave

3.4.5 Heating block for 13 mm tubes (85 - 105°C)

3.4.6 Evaporator Nitrogen manifold for 13mm tubes

3.4.7 Vortex-type mixer

3.4.8 Adjustable micropipette (20 - 200.0 µl)

### **3.5 SUPPLIES /MATERIAL/DISPOSABLES**

- 3.5.1 Pasteur pipettes - disposable glass
- 3.5.2 BacT Alert TB culture bottles – Middle-brook 7H9-liquid medium
- 3.5.3 Auto sampler vials
- 3.5.4 pH paper (range 0 – 13)
- 3.5.5 200µl tips for adjustable micropipette
- 3.5.6 Volumetric flasks. 50,100, 250 ml
- 3.5.7. Dark amber glass reagent bottle.

### **3.6 SAMPLE PREPARATION**

#### **3.6.1 Specimens for culturing are divided in two specimen types:**

- (a) Specimens of pulmonary origin, e.g. sputa, laryngeal swabs, gastric lavage fluid, bronchial washing and bronchial brushing, are almost always contaminated with normal host microbiota. Pleural biopsies and trans tracheal aspirations, if done aseptic should render non-contaminated pulmonary specimens.
- (b) Specimens of extra-pulmonary origin, e.g. urine, stool, skin or soft tissue and aspirates of samples i.e. blood, bone marrow, pubic bladder aspirates, cerebral spinal fluids (CSF) and other body fluids, aseptically collected tissue and biopsy samples.

All contaminated samples require a digestion-decontamination and concentration step in order to maximise the mycobacterial yield. When selecting any digestion procedure, the rule of thumb is to select the mildest decontamination procedure that will yield highest mycobacterial growth with the lowest contamination rate [47].

These samples were digested in a mixture of sterile solution of 1% N-acetyl-L-cysteine (NALC) and 1 mol/L sodium hydroxide for a period not longer than 20.0 minutes. The sample was then neutralised with a mixture of 1 mol/L hydrochloric acid and 0.067 mol/L phosphate buffer (pH 6.8) and centrifuged it at 4330 RCF for 15 minutes. Discarding the supernatant, add 1 ml phosphate buffer (pH 6.8) and vortexed the tube to dissolve the sediment completely.

### **3.6.2 The culturing and the identification of mycobacteria.**

Transfer 0.5 ml of the digested supernatant to the BacT/ALERT MP culture bottles. The bottles are then placed into the mycobacteria detection system which uses a colorimetric sensor that monitors the production of carbon dioxide by the mycobacteria. At the time of detection, approximately  $10^6$  -  $10^7$  colony forming units (CFU) will be present in the culture bottle. The time it takes for most of the mycobacteria species to be detected by this technique is anything between 3 - 14 days. All remaining culture bottles are kept incubated for a period of six weeks, and discarded thereafter.

### **3.6.3 Saponification of the mycobacteria and extraction of mycolic acids.**

Transfer the cultured samples (0.5 -1.0 ml) to a digestion tube. Add 2.0 ml of 50% sodium hydroxide in water / methanol (1:1 vol/vol) mixture to the sample, cap, vortex and autoclave for 60.0 minutes at 120°C to saponify the bacterial cells. Cool the saponified cells to ambient temperature. Add 2.0 ml chloroform and 1.5 ml hydrochloric acid (5.0 mol/L) and cap tightly. Vortex tubes for minimum of 20 seconds. Transfer the chloroform mycolic acid containing

layer to new tube, repeat extraction process a second time and evaporate the transferred chloroform to dryness, at 85 -105 °C, under a stream of nitrogen. This method is a modified version of the method described by Jost et al [47].

#### **3.6.4 Derivatisation of the mycolic acid to the 6,7-dimethoxycoumarin mycolic ester.**

Add 100 µL potassium bicarbonate/methanol solution to the dry sample. Dry sample at 85 - 105°C under a stream of nitrogen. Cool sample and add 200µL derivatization solution containing 250.0 µg of 4-bromomethyl 6,7 – dimethoxycoumarin and 30.0 µg of 18-crown-6 ether. Recap the tube and heat the sample to 60°C for minimum of 20 minutes. The function of the crown ether is to catalyse the alkylation of mycolic acids.

#### **3.6.5 Extraction of the derivatized mycolic ester.**

Add 1ml chloroform and 1ml clarification solution (1:1 vol/vol) and recap. Mix tube vigorously for minimum of 20 seconds and allow layers to separate. Remove chloroform layer with glass Pasteur pipette and transfer to new tube. Repeat process, evaporate chloroform at 85 -105 °C under a stream of nitrogen. Store samples at 4 - 6°C in dark container until ready for HPLC analysis. (Diagram 2.)

#### **3.6.6 Chromatographic gradient conditions.**

The HPLC system that was used in the analysis, consisted of a quaternary pump system, auto sampler and a fluorescence detector. Separation of the different mycolic acids was achieved by using a C<sub>18</sub> – reverse phase analytical column and a C<sub>18</sub> pre-column. Solvent flow rate was set at 2.5

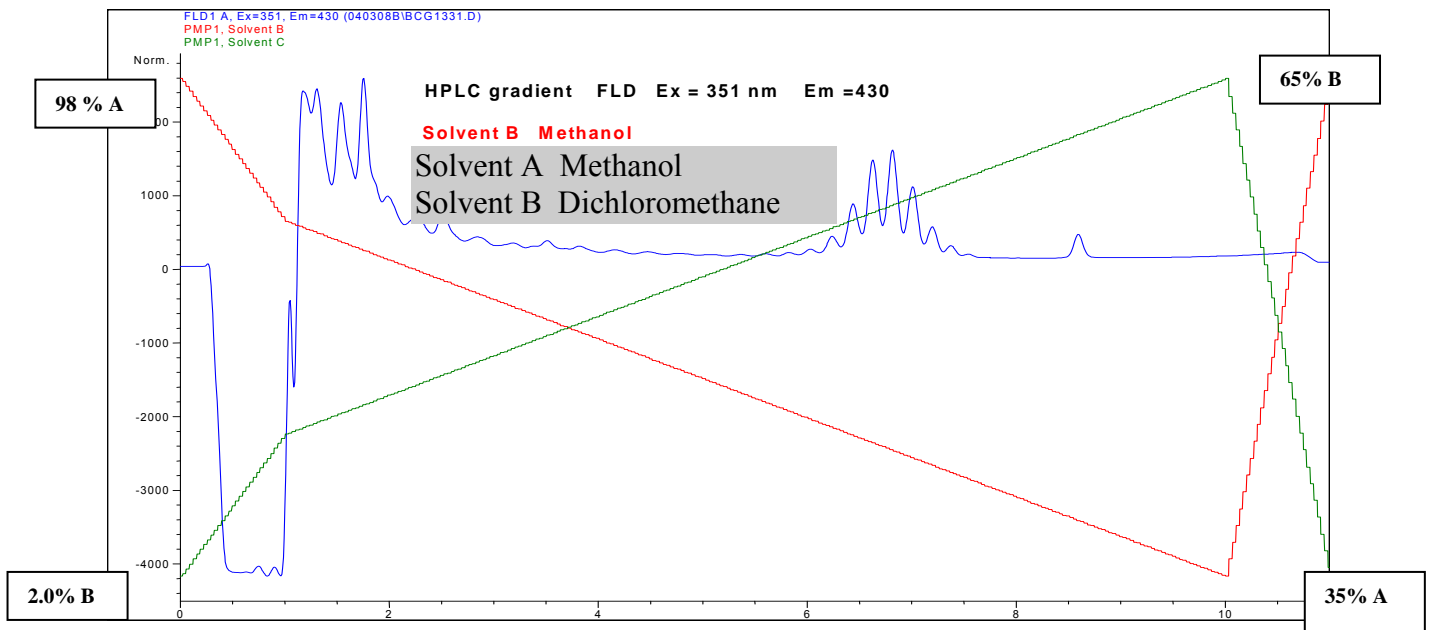
ml/minute. The initial mobile phase setting was 98% methanol (solvent A) and 2% dichloromethane (solvent B) for 5 minutes. This weak solvent setting enabled us to pre-concentrate the injected sample before actual separation of mycolic acids occurred.

One minute after the pre-concentration step the solvent mixture is changed to 80% A solvent to 20% B solvent. During the next 9 minutes the mobile phase was changed from 35% A solvent to 65% B solvent. Finally the mobile phase was set back to its original condition for a duration of 3 minutes before injection of the next sample. Data acquisition only occurs in the 10 minutes after the gradient is activated. The analysis and the re-equilibration time for each chromatogram cycle time is 18 minutes in total. (Diagram 3.)

### **3.6.7 Sample preparation for HPLC analysis.**

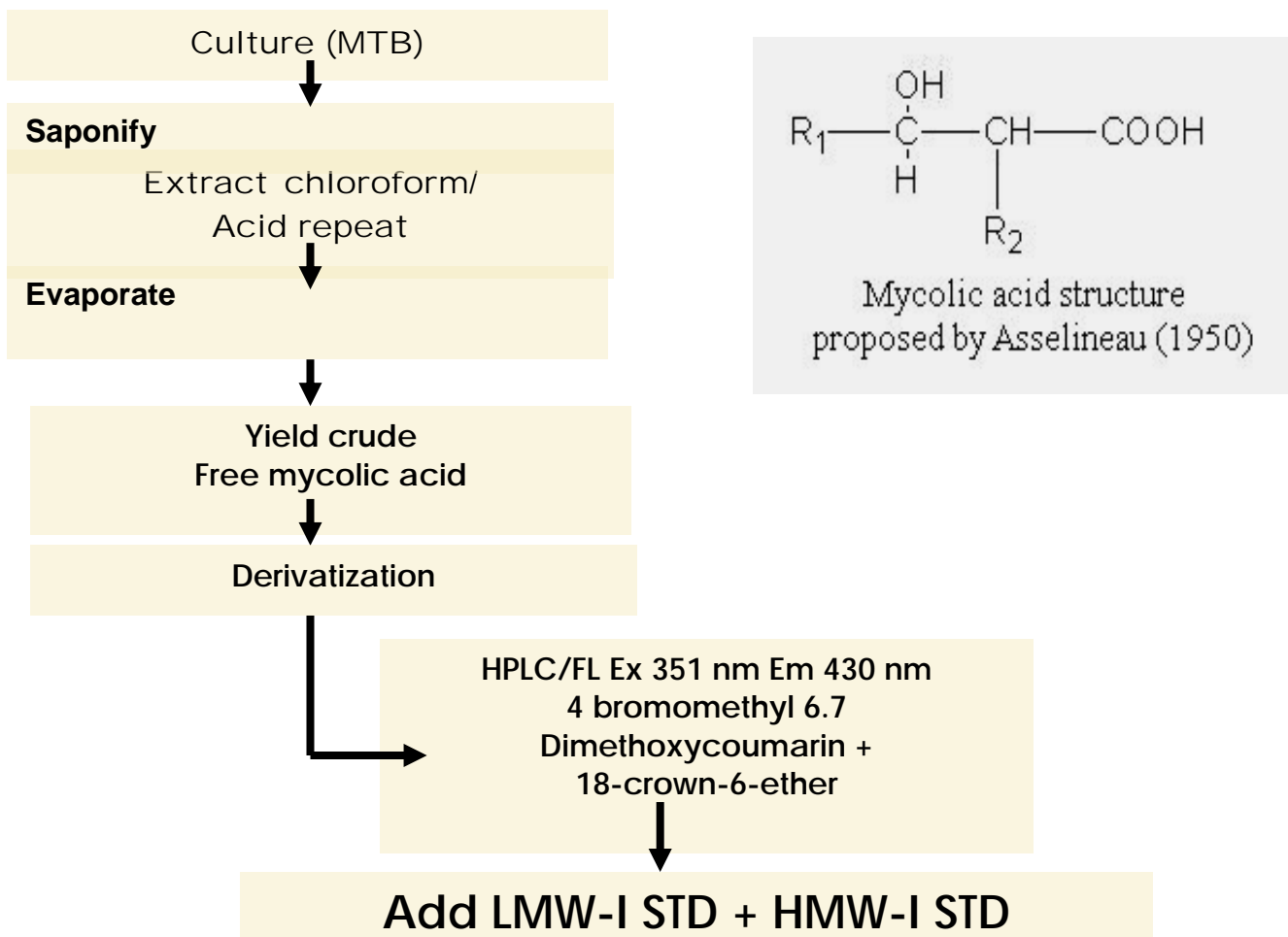
The samples were dissolved in 200.0  $\mu\text{L}$  of dichloromethane containing 80.0  $\mu\text{g/L}$  and 40.0  $\mu\text{g/L}$  LMW and HMW internal standards respectively. With the pre-concentration gradient step, up to 5.0 - 50.0  $\mu\text{L}$  of the sample can be injected without influencing the separation quality. Measurement is done by a fluorescence detector at an excitation wavelength of 351 nm, an emission wavelength of 430 nm, and a scanning time of 0.5 seconds. (Diagram 3.)

**Diagram 3. Schematic pump gradient**



**Diagram 4. Extraction flow diagram**

MTB CULTURE ANALYSIS BY HPLC/FL/UV DETECTION



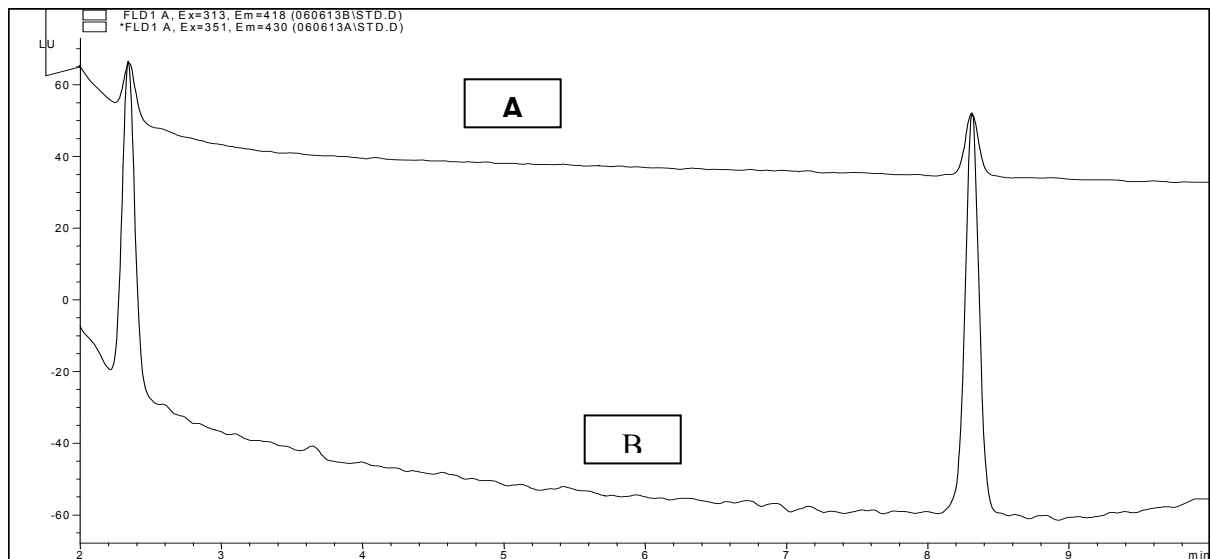
# CHAPTER 4: DEVELOPMENT OF A HPLC METHOD FOR MYCOLIC ACID PROFILING OF MYCOBACTERIA

## 4.1 SENSITIVITY OF THE HPLC/FL TECHNIQUE

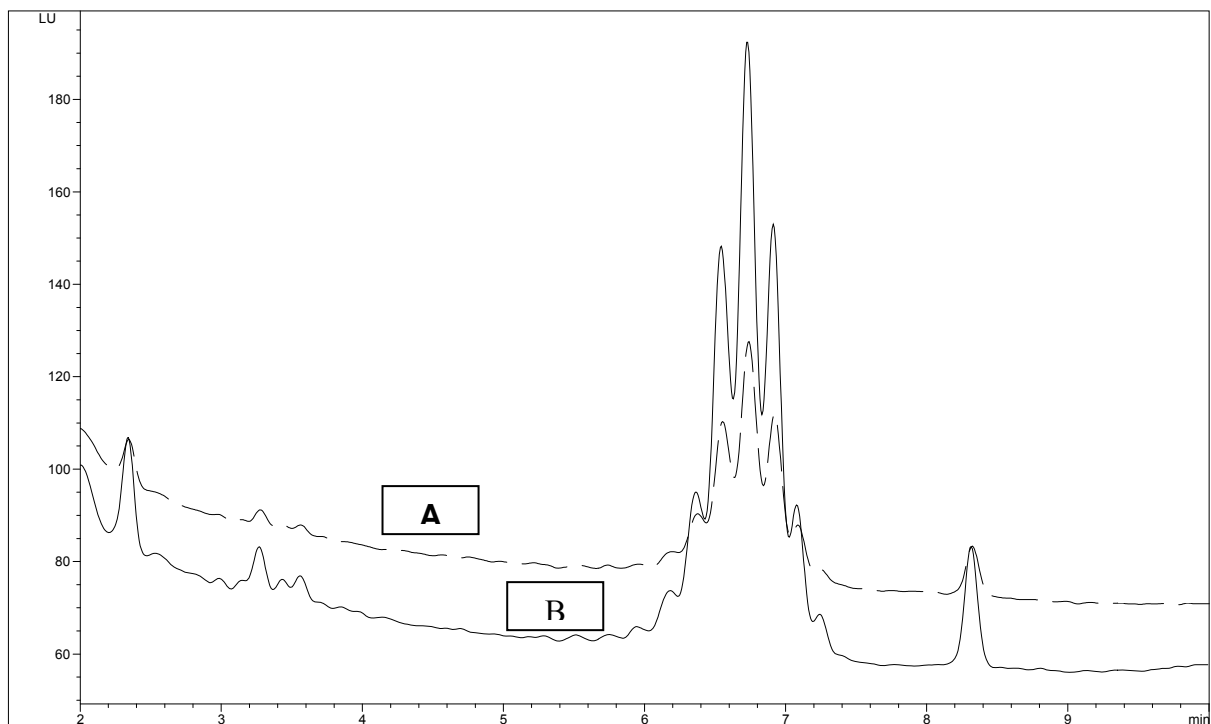
The analysis of mycolic acid profiles of the different mycobacterial isolates, by high performance liquid chromatography, produced very sensitive and reproducible results. High performance liquid chromatography/fluorescence detection technique was at least 200 times more sensitive than UV detection [38]. By optimising the excitation (351nm) and emission (430 nm) wavelengths of the fluorescence detector we were able to increase the sensitivity of mycolic acid detection in the low and high carbon molecular weight range. This has been demonstrated by comparing the LMW and HMW internal standards at different excitation and emission wavelengths (Fig. 8). The original method uses 313 nm and 418 nm as exciting and emission wavelength respectively [47].

Increase in sensitivity can also be observed when comparing mycolic acid profiles of *Mycobacterium tuberculosis* at the different excitation and emission wavelengths (Fig.8a). Optimising the wavelengths contributed to a less noisy baseline, which in turn made the identification of the mycolic acid profiles for mycobacteria less complicated.



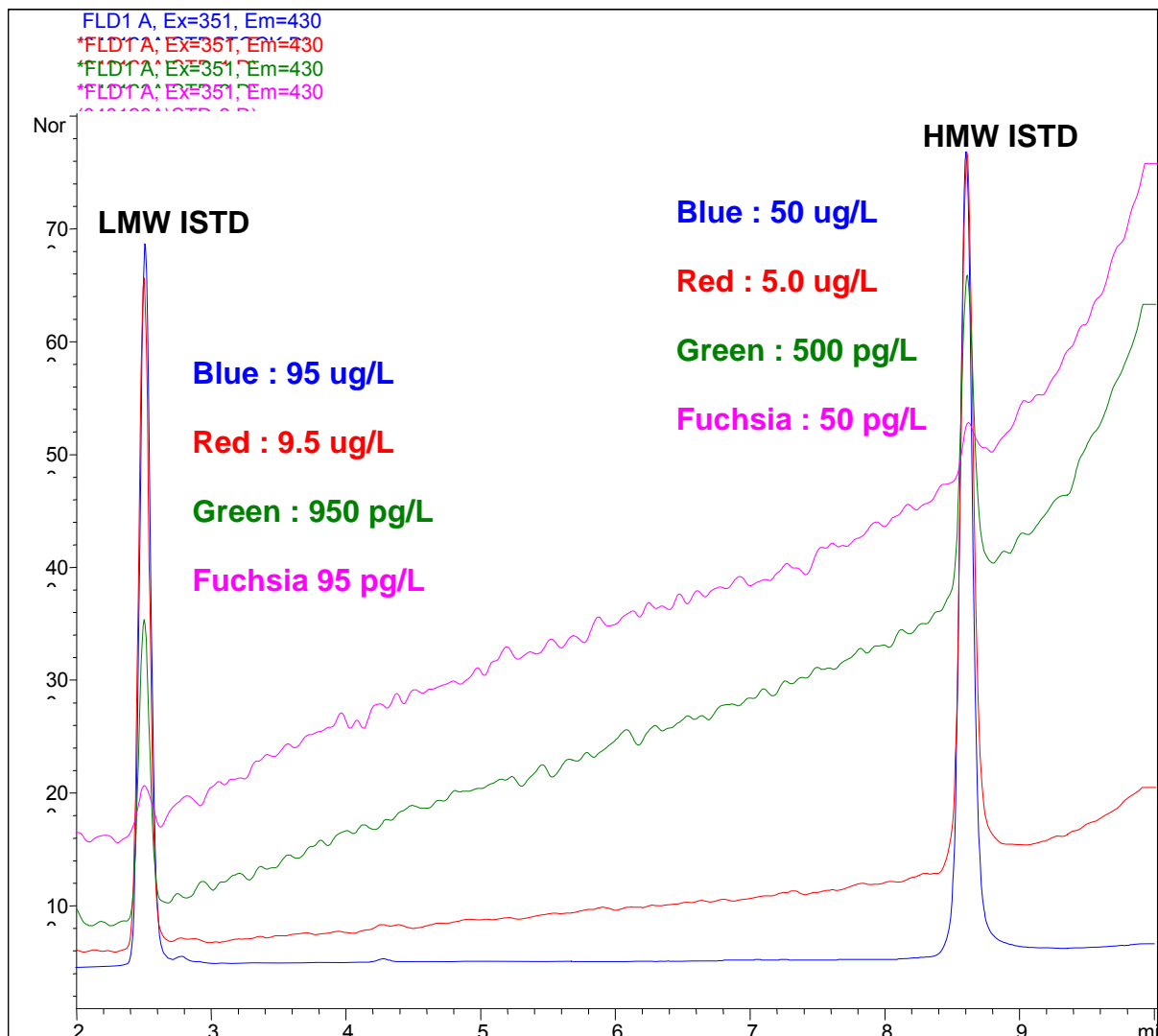


**Figure. 8.** Demonstrates the increase in sensitivity of the internal standards at different excitation and emission wavelengths. (A) Excitation wavelength 313 nm, emission wavelength 418 nm. (B) Excitation wavelength 351 nm, emission wavelength 430 nm.



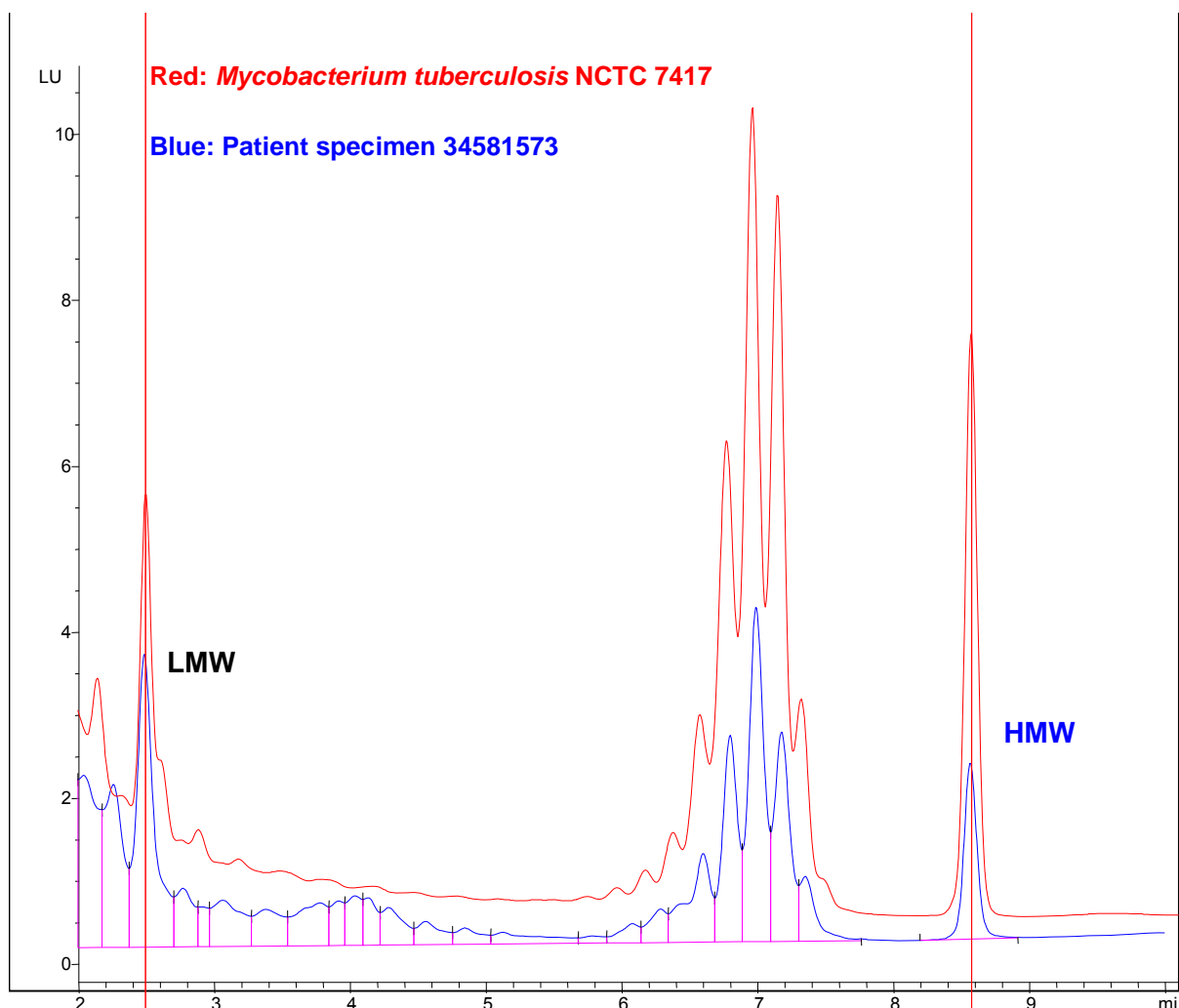
**Figure. 8b.** Demonstrates the increase in sensitivity of mycolic acids of *Mycobacterium tuberculosis* at different excitation and emission wavelengths. (A) Excitation wavelength 313nm, emission wavelength 418 nm. (B) Excitation wavelength 351 nm, emission wavelength 430 nm. Notice the additional peaks between 2 and 3minutes and observe the ratio differences of the mycolic acids.

The sensitivity of the HPLC/FL technique was established by diluting the LMWIS and HMWIS by factor of 10, until a signal to noise ratio of 3:1 was achieved. We were able to measure concentration as low as 50.0 pg/L of mycolic esters for the high molecular weight internal standard (HMWIS) and 95.0 pg/L for low molecular weight internal standard (LMWIS). Detection limits have not been declared in literature for comparison purposes (Fig. 9).



**Figure. 9. Different internal standards concentrations. LMW internal std from 95.0  $\mu\text{g/L}$  – 95.0  $\text{pg/L}$ . HMW internal std from 50.0  $\mu\text{g/L}$  – 50.0  $\text{pg/L}$ .**

This increase in sensitivity enabled us to analyse mycobacteria from smear-positive sputum microscopy slide specimens with a 40x objective in the region of few (1-9 bacilli/10 fields) to moderate (1-9 bacilli/field) bacilli count directly (Fig. 9a). Optimisation of the HPLC/FL parameters enabled us to analyse mycolic acid profiles of mycobacterium isolates within 3 - 7 days, cultured on a Middelbrook 7H11 solid culture medium. Current analysis of mycobacteria is performed on an isolate culture from a liquid BacT/Alert system.



**Figure. 9a. Chromatogram of a direct sputum sample. Blue: Patient - direct sputum sample. Red: Reference culture, *Mycobacterium tuberculosis* NCTC 7417.**

## 4.2 REPRODUCIBILITY OF THE HPLC/FL

A total of 22000 clinical mycobacterial isolates, including 35 reference mycobacterial species (Appendix. A) and complexes were analysed by HPLC/FL the past few years. Mycolic acid profile reproducibility is of utmost importance, as it may influence the reliability of visual and overlaying interpretation of the mycolic acid patterns for mycobacteria species. Identification of unknown mycolic acid profiles is directly related to the quality of the chromatogram. Figure 10 shows the chromatograms of *Mycobacterium tuberculosis* for different patients over a period of five months.

Identification of mycolic acid profiles for mycobacteria was achieved by calculating the adjusted retention time (ART) of each primary peak by using both high and low molecular weight internal standards (Fig. 10a). A second method was by measuring the retention time difference between one of the internal standard and the primary mycolic acid peaks. Overlaying the non-adjusted data, (Fig.10) graphically revealed the standard deviation of the mycolic acids profiles for *Mycobacterium tuberculosis* (Fig. 10a).

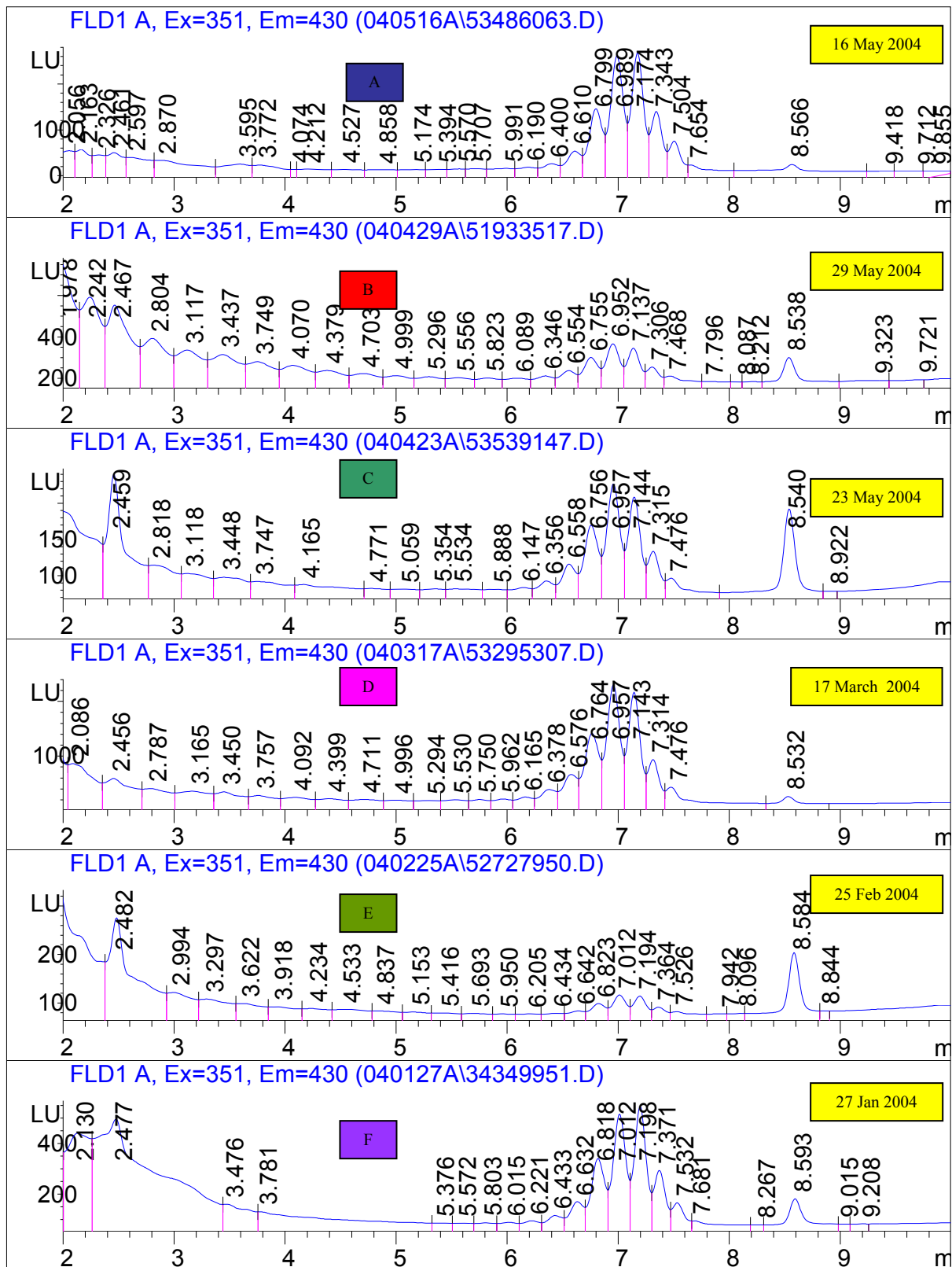
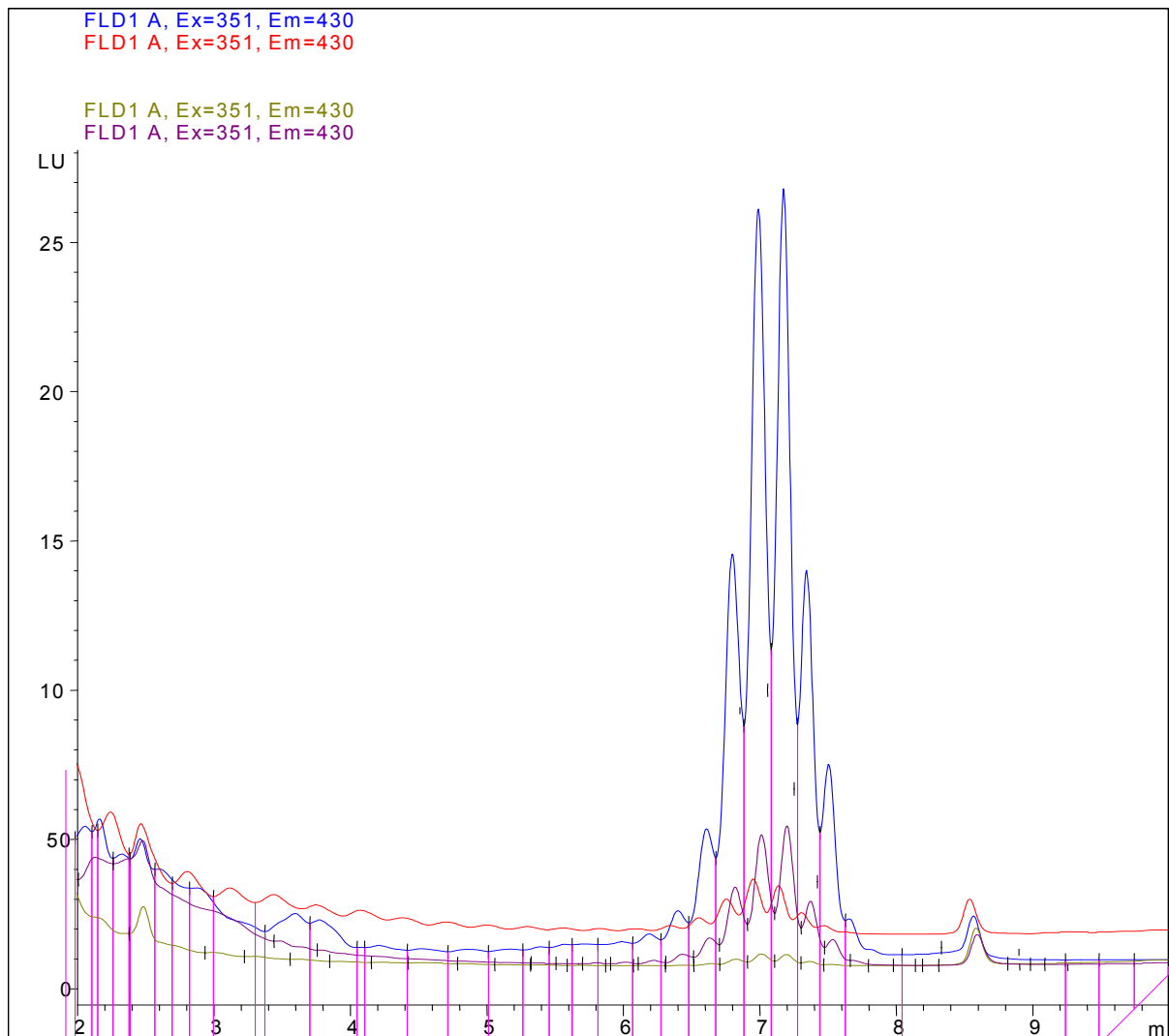


Figure. 10. Mycolic acid profiles of *Mycobacterium tuberculosis* of sputum samples from different patients prior to overlaying. Notice the concentration chromatogram B and D are very close to the detection limit of the detector.

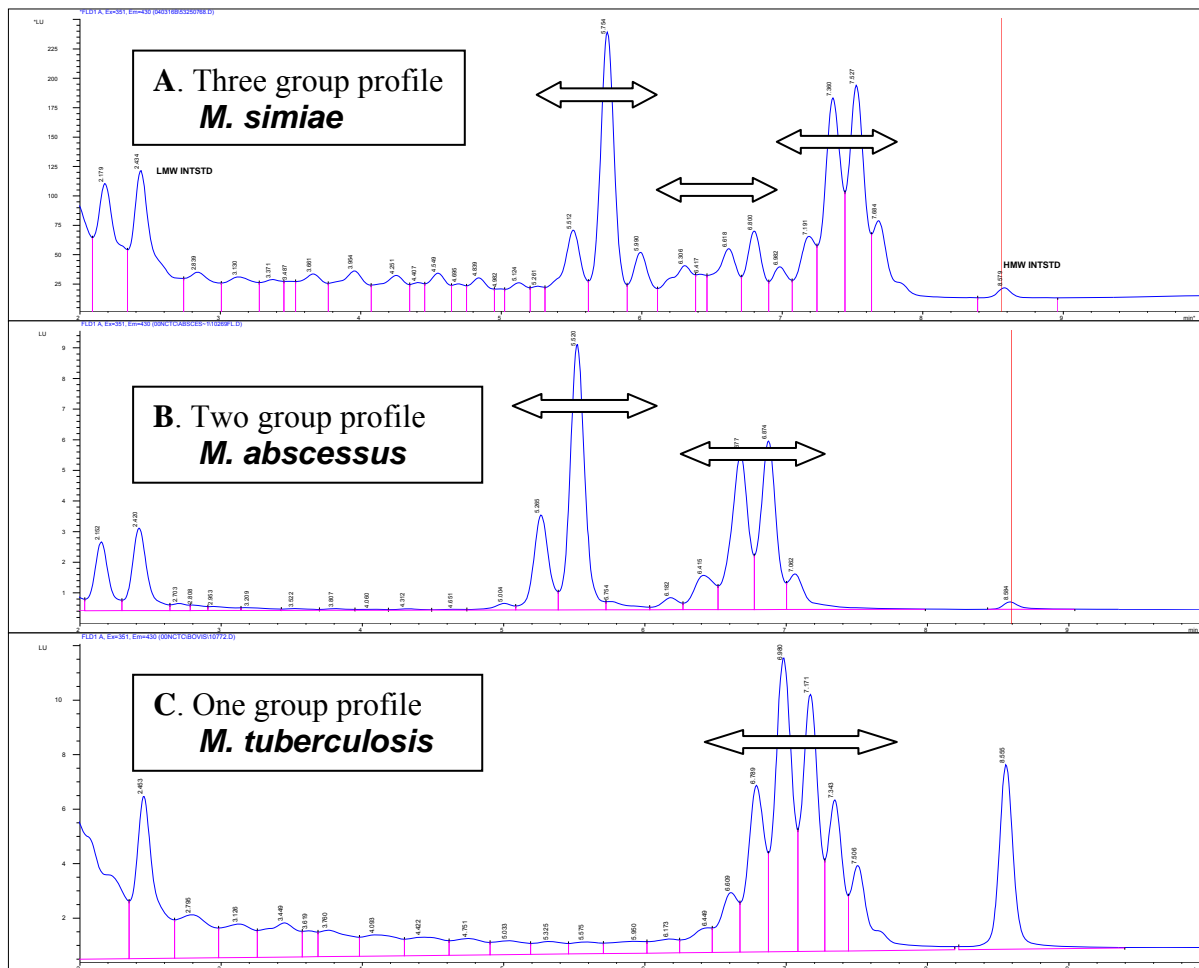


**Figure. 10a. Unadjusted *Mycobacterium tuberculosis* chromatograms. Notice the small variation in retention time before adjustment when overlaying of 6 different patients shown in Fig 10 (A – F).**

In this study, identification of the mycolic acid profiles was done by using a computer program that enables us to overlay the mycolic acid chromatographic profiles of any reference mycobacteria isolate with the unknown mycolic acid chromatographic profile. Visual comparison of the chromatographic mycolic acid profiles for these mycobacteria, with mycolic acid profiles for the different reference mycobacterial species, was the first step in identifying the mycobacterium species.

By visually pre-screening HPLC/FL patterns according to groups of mycolic acid present (e.g. one, two or three group of peaks present), it was easy to tentatively

identify the mycolic acid profiles for each mycobacterium species (Fig. 10b) before computer overlaying confirmation was done.



**Figure. 10b. Different groups of mycolic acid profiles generally encountered.**

Overlaying software assisted in the initialising of both the reference and unknown chromatograms with the internal standards, this technique enabled the identification of each mycobacterium isolate by its specific mycolic acid profile. No arbitrary numbering system was used to identify designated mycolic acid peak or peaks [47]. The small day-to-day variations in retention times have been observed and may affect the interpretation of mycolic acids profiles from mycobacteria. (Fig. 10b.) To adjust for these small day-to-day variations in retention times the reproducibility of the chromatographic profile had to be improved. These corrections can either be

done by calculating the ART (adjusted retention time) for each peak identified, or by using the computerized overlaying technique to automatically convert small variations in retention times and adjusting them according to the internal standards of the known and unknown mycolic acid profile (Fig. 10c).

Reproducibility of the chromatographic profile was improved by the incorporation of both the HMWIS and LMWIS in the calculations of the adjusted retention time. These calculations are done by computer software and can be described as follows:

**4.2.1** Identifying the low (LMW) and high (HMW) molecular weight internal standard peaks in each sample. The peak position of the two internal standards are compared to those from a given reference chromatogram to produce two correction factors. (CF) The correction factor is then used to adjust the corresponding internal standard times in the reference chromatogram.

$$CF = \frac{RT_{INT\ STD} (SAMPLE)}{RT_{INT\ STD} (REFERENCE)}$$

**4.2.2** The retention time fractional (RTF) value is calculated by measuring the position of any peak relative to the two internal standards.

$$RTF_{PEAK} = \frac{RT_{PEAK} - RT_{LMW}}{RT_{HMW} - RT_{LMW}}$$

**4.2.3** The retention time of each peak between the two internal standards was then adjusted by incorporating the local correction factor (LCF) in the equation.

$$LCF_{PEAK} = CF_{LMW} + RTF_{PEAK} (CF_{HMW} - CF_{LMW})$$

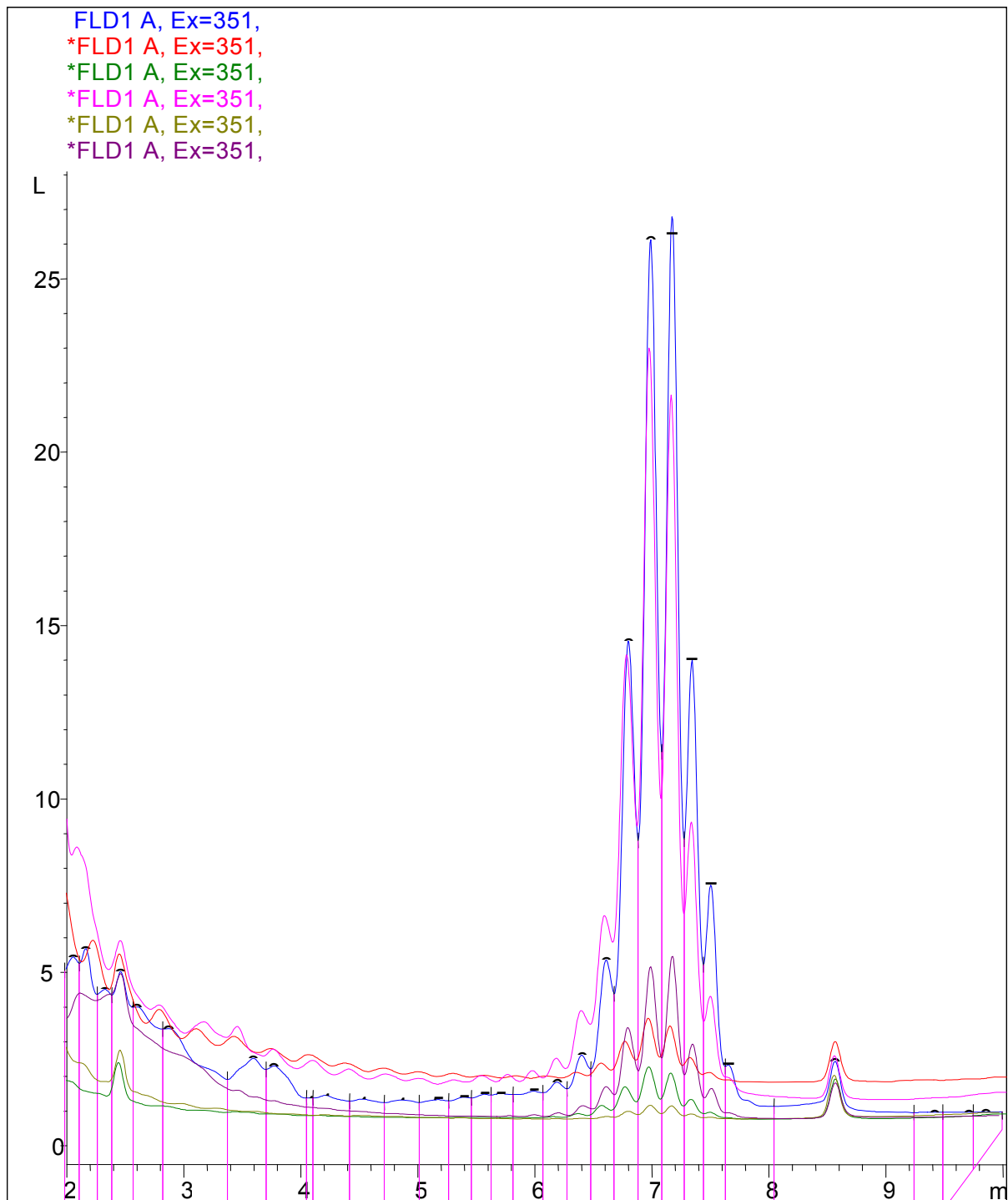


4.2.4 Finally, the local correction factor is used to modify the RT of the peaks within the two internal standards and to produce an adjusted retention time for each peak (ART).

$$\text{ART}_{\text{PEAK}} = \frac{\text{RT}_{\text{PEAK}}}{\text{LCF}_{\text{PEAK}}}$$

$$\text{LCF}_{\text{PEAK}}$$

This method of calculation and verification enables us to correctly match mycolic acid profiles of mycobacteria with reference mycolic acid of known mycobacteria. Mycolic acid profiles for *Mycobacterium tuberculosis* of the different patients with corrected reference mycolic acid profiles of *Mycobacterium tuberculosis* were compared. (Fig.10c.)



**Figure. 10c.** *Mycobacterium tuberculosis* – Mycolic acid profile corrected for retention time variation in chromatographic peaks. Notice the improvement when matching the mycolic acid profiles to the uncorrected data in Fig. 10a.

The reproducibility of the HPLC/FL technique was compared over a period of six months by randomly selecting patients that were infected with *Mycobacterium tuberculosis*. A local correction factor is calculated for any peak position between the

two internal standard peaks. This is an interpolation value of the correction factor at both extremes of retention time (Table 3.). Applying computer software to initialise each mycolic acid chromatogram to the two internal standards and normalising each chromatogram to a full-scale response, we were able to compare the different chromatograms to a reference mycolic acid profile for mycobacteria.

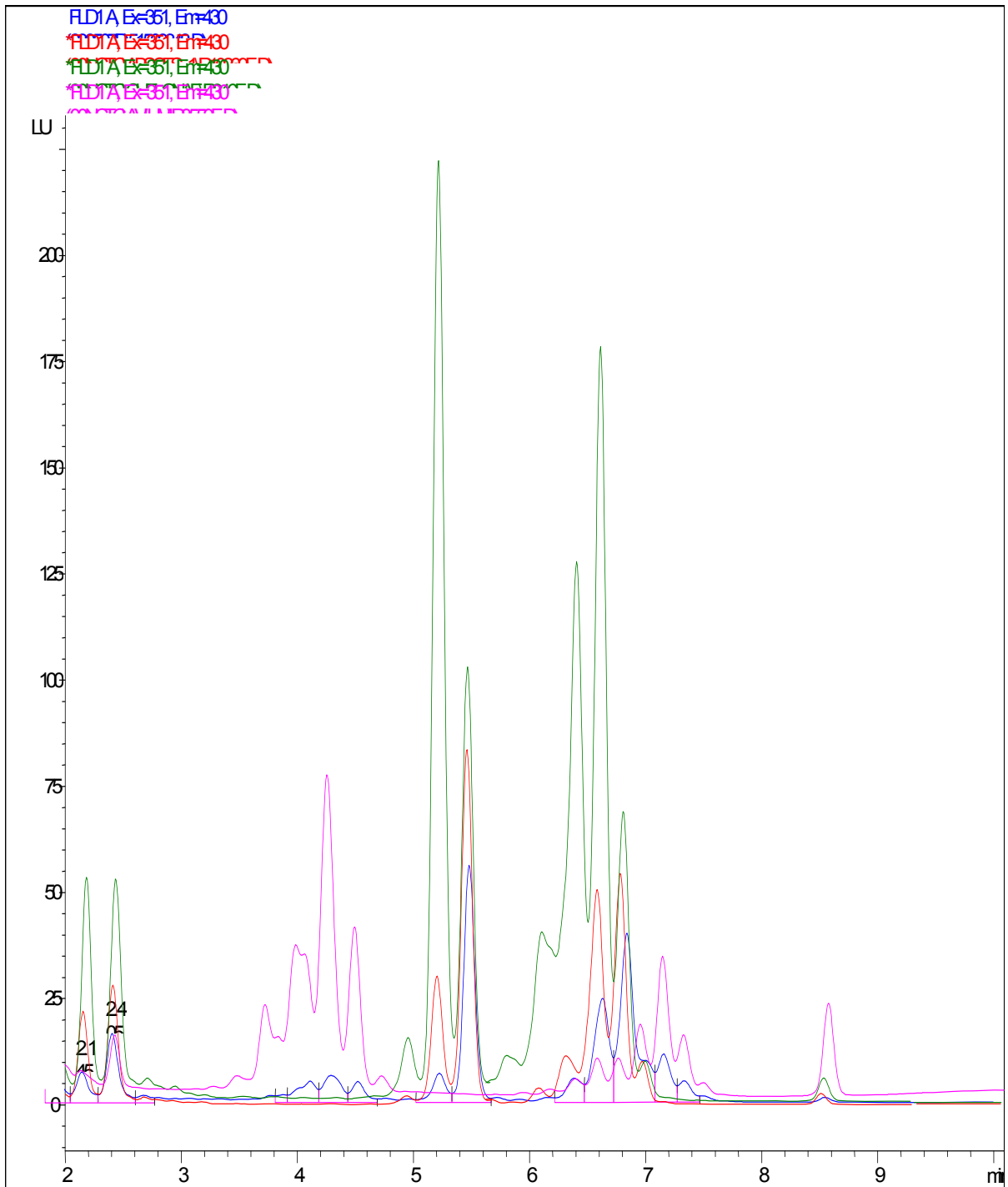
Reproducible chromatographic patterns contained combinations of characteristic mycolic ester peaks that could be used in diagnosing of the mycobacterium infection in the patient.

The chromatographic resolution of a mycolic acid profile determines the quality of the “fingerprint” of a given mycobacterium. Reproducibility of the mycolic acid profile is very important. Identification can only be done when the chromatographic conditions are standardized.

Separation is achieved by using a Luna 5 $\mu$  particle size C<sub>18</sub> reverse phase (75mm x 4,6mm) column. This C<sub>18</sub> column delivered a complete mycolic acid profile for mycobacteria with an increased resolution for some of the mycolic acids peaks within 10 minutes as compared to previous chromatograms [46].

Shorter retention times with increased resolution increased productivity and contributed to an increased confidence in the final result. Reduction of the analysis time has a direct effect on cost of the analysis.

With an increase in resolution, classification of the hard-to-distinguish mycobacterial isolates like *Mycobacterium chelonae* (Appendix. Fig. 8) and *Mycobacterium abscessus* (Appendix. Fig. 1) were made possible (Fig. 10d).



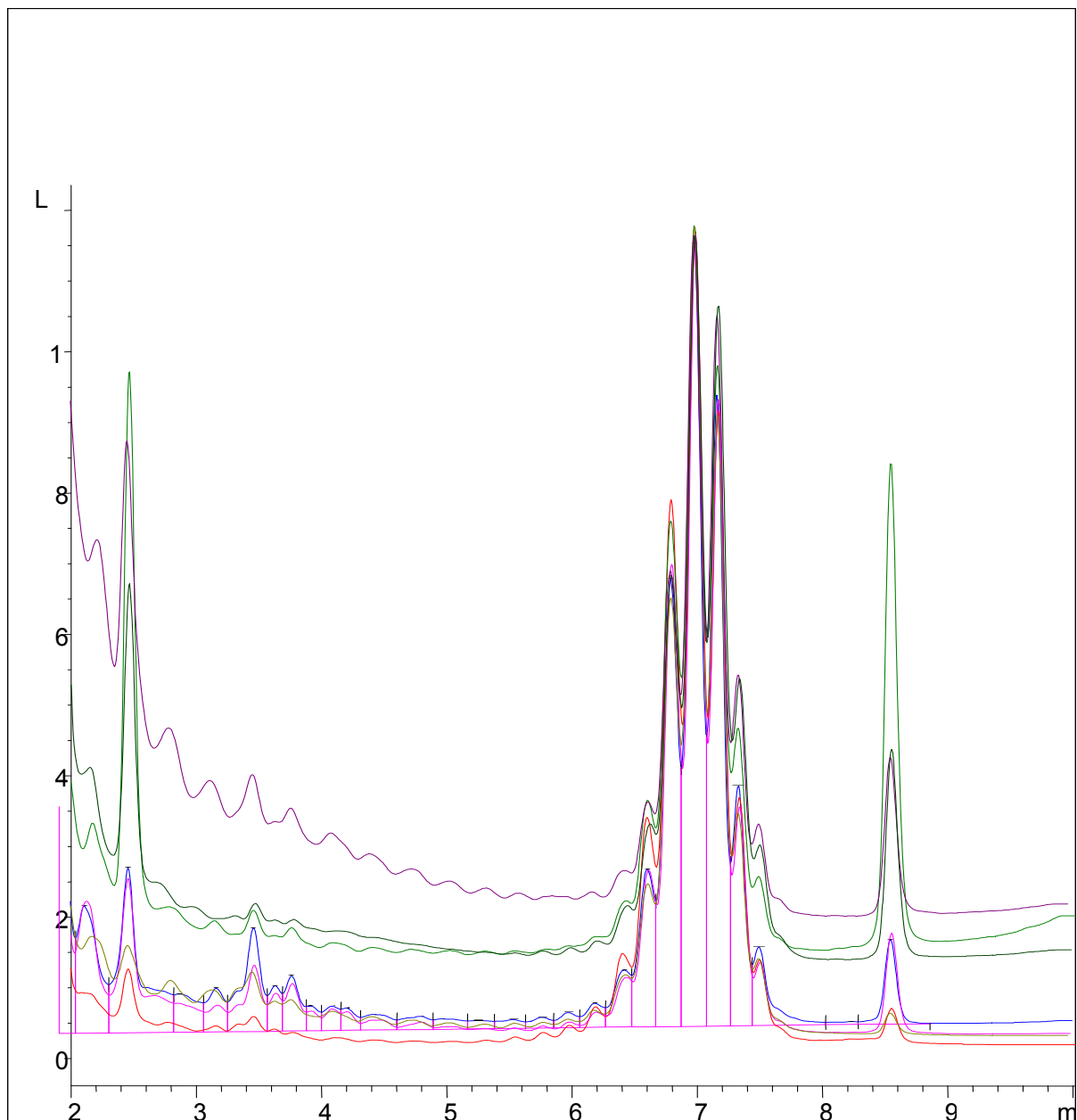
**Figure. 10d. Chromatogram demonstrating the resolution properties of C<sub>18</sub> reverse phase column. Notice how easy it is to identify the patient *Mycobacterium abscessus* infection. Blue: Patient 1503348 – *Mycobacterium abscessus* Green: *Mycobacterium chelonae* NCTC 946. Red: *Mycobacterium abscessus* NCTC 10269. Pink: *Mycobacterium avium* NCTC 8559.**

Comparing the retention times of the major mycolic acid peaks for *Mycobacterium tuberculosis* over a seven month period is summarized, (Table. 2) and shows that the analysis method is reproducible and reliable.

**Table 2. Corrected (A) and uncorrected (B) retention times of six most abundant mycolic acid peaks of *M. tuberculosis* over a period of six months.**

		Different retention times and standard deviations.							
		Month							
		1	2	3	4	5	6	7	
Mycolic acid									SD
1	A	6.483	6.463	6.493	6.523	6.513	6.523	6.423	0.036
	B	6.383	6.400	6.554	6.632	6.487	6.575	6.440	0.094
2	A	6.662	6.642	6.512	6.622	6.622	6.612	6.611	0.046
	B	6.595	6.610	6.755	6.756	6.767	6.787	6.818	0.088
3	A	6.885	6.885	6.785	6.805	6.798	6.855	6.785	0.045
	B	6.780	6.999	6.920	6.972	6.941	7.017	7.012	0.083
4	A	7.053	7.043	7.033	7.050	7.049	7.095	7.053	0.019
	B	7.097	7.134	7.137	7.244	7.143	7.194	7.201	0.050
5	A	7.222	7.211	7.301	7.199	7.255	7.185	7.277	0.042
	B	7.277	7.351	7.196	7.305	7.324	7.397	7.371	0.067
6	A	7.385	7.355	7.315	7.410	7.415	7.412	7.310	0.045
	B	7.501	7.532	7.532	7.532	7.532	7.532	7.683	0.060

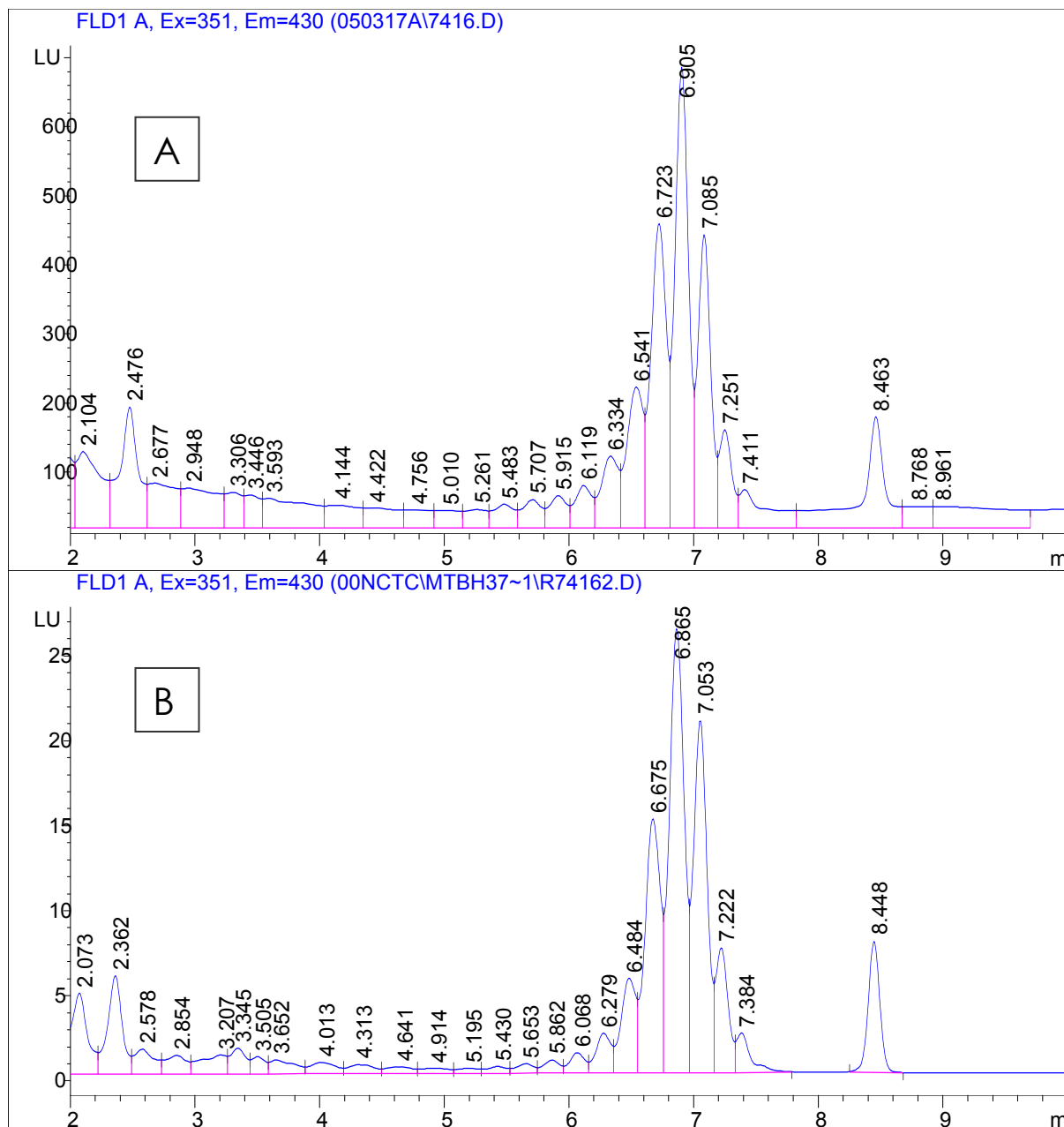
The excellent reproducibility (relative standard deviation <10%) could only be achieved by adjusting the data according to the adjusted retention time (ART). To both the internal standards by computer driven software (Fig. 10e). Improvement in standard deviation can be observed after adjustment (Table 2).



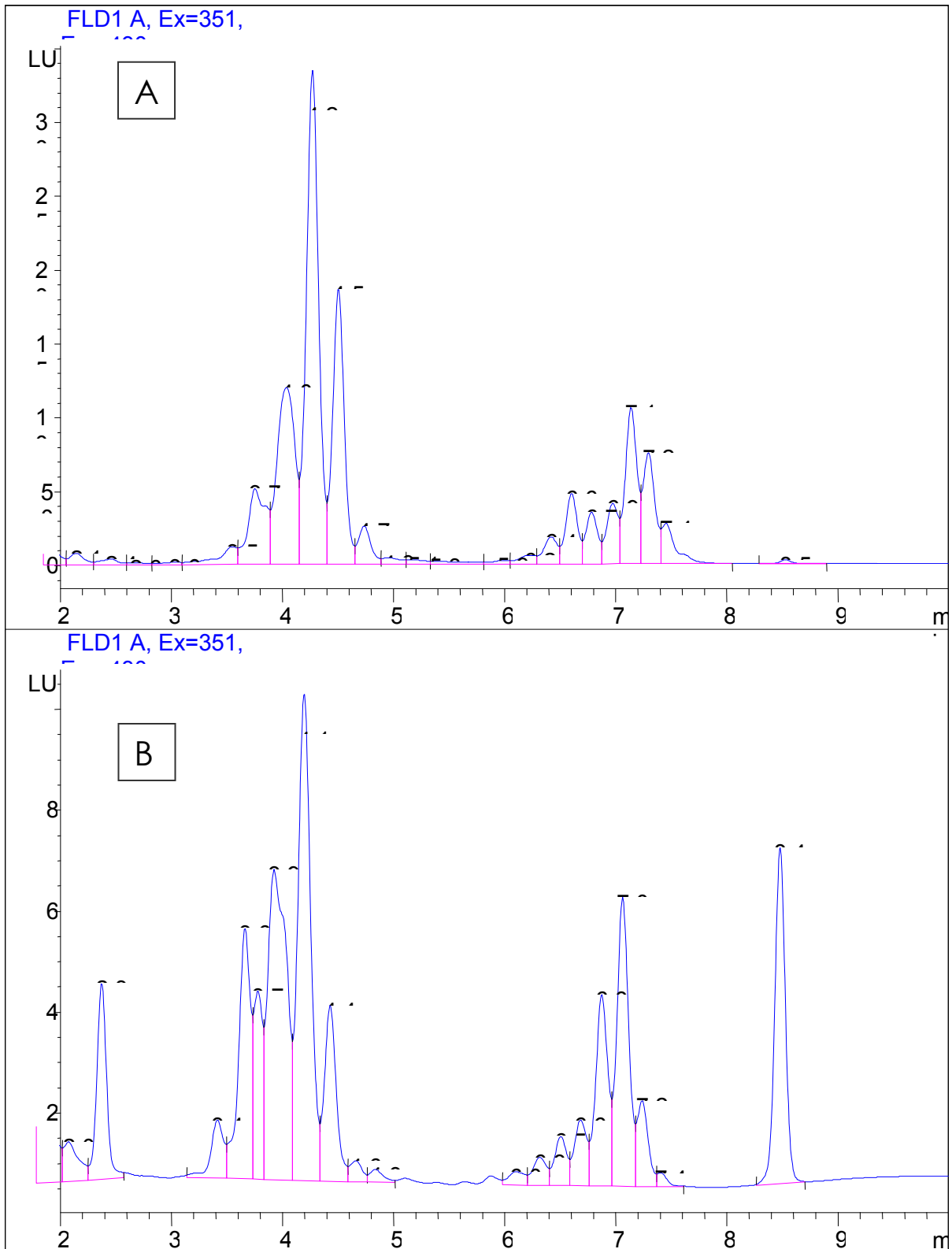
**Figure. 10e. Chromatogram demonstrated the reproducibility of the HPLC/FL technique. Chromatogram shows the repeatability of mycolic acid profiles for *Mycobacterium tuberculosis* of different patients when compared over seven months.**

Figure 10e demonstrates the excellent reproducibility of the chromatograms after 6000 injections without the loss of resolution. Standard practise is to change the HPLC column after 6000 injection.

The robustness of the HPLC technique was demonstrated in comparing the same reference mycolic acid profiles of *Mycobacterium tuberculosis* (Fig. 10 f) and *Mycobacterium avium* (Fig. 10 g) over a period of 2 years.



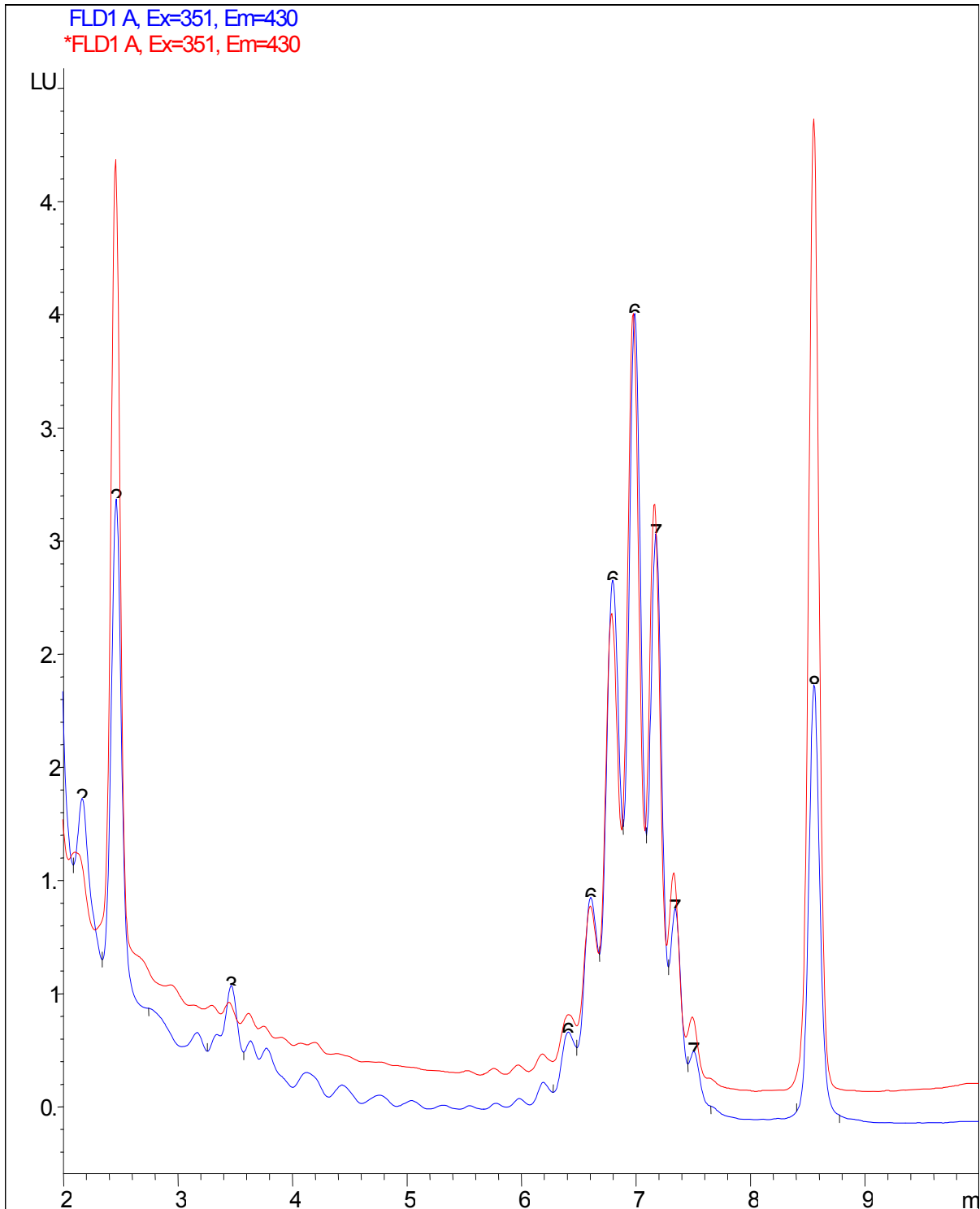
**Figure. 10f. Mycobacterium reference standards analysed in 2003 and 2005. Chromatogram A and B represents mycolic acid profiles of *Mycobacterium tuberculosis* analyzed in March 2003 and March 2005 respectively. Notice the small ratio differences.**



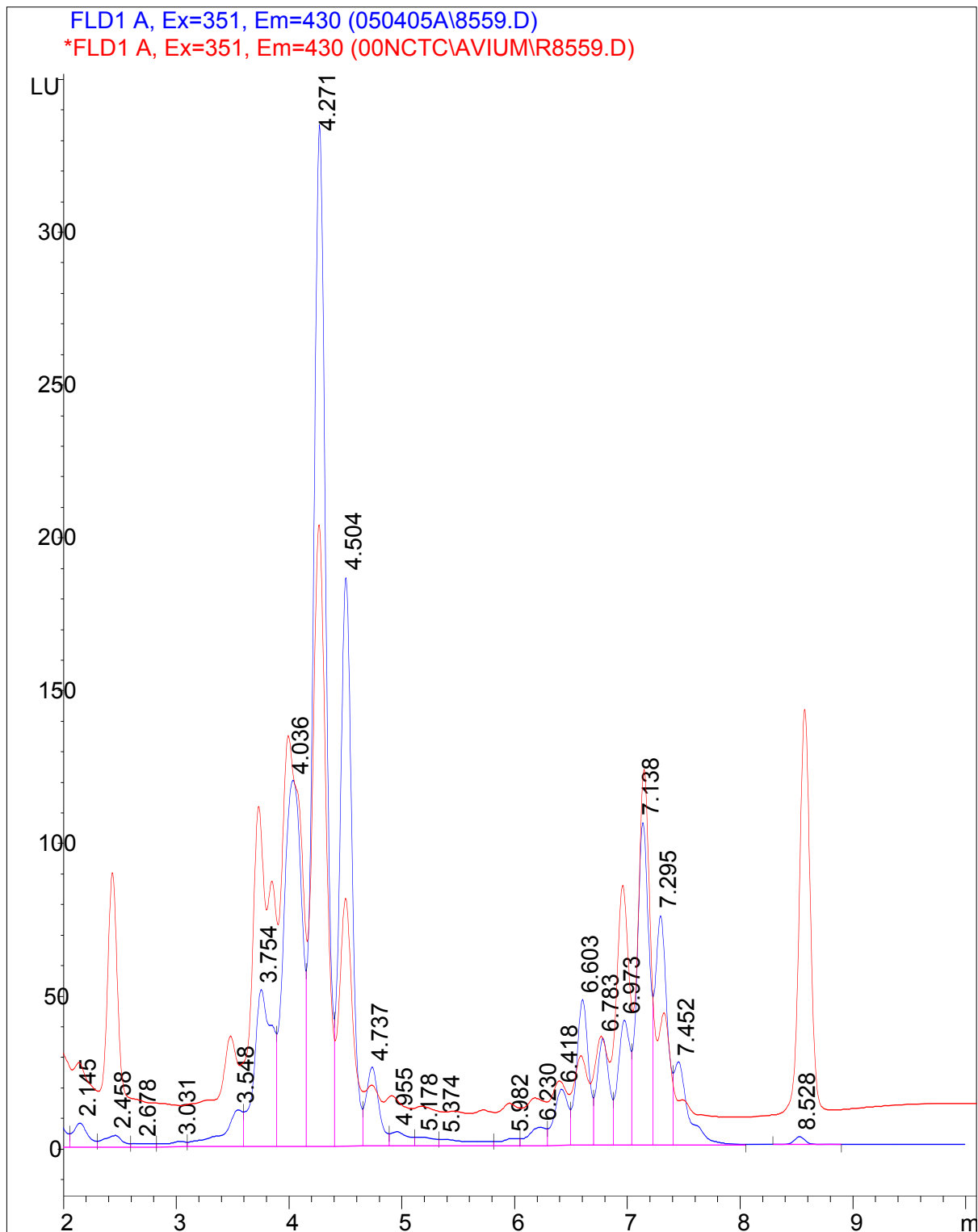
**Figure. 10g. Mycobacterium reference standards analysed in 2003 and 2005. Chromatogram A and B represents mycolic acid profiles of *Mycobacterium avium* analyzed in March 2003 and March 2005 respectively.**



The adjusted chromatograms for both the *Mycobacterium tuberculosis* and *Mycobacterium avium* is shown in Fig 10h and Fig10i respectively.



**Figure. 10h. Chromatogram of *Mycobacterium tuberculosis* corrected for retention time variations in chromatogram peaks as shown in figure 10f. Chromatogram red and blue compares *Mycobacterium tuberculosis* mycolic acid profiles measured in March 2003 and 2005 respectively.**



**Figure. 10i. Chromatogram of *Mycobacterium avium* corrected for retention time variations in chromatogram peaks as shown in figure 10g.** Chromatogram red and blue compares *Mycobacterium avium* mycolic acid profiles measured in March 2003 and 2005 respectively. Notice the small ratio differences in the first group of peaks.

### 4.3 GROWTH MEDIUM STANDARDIZATION

#### 4.3.1 Different growth media generate different mycolic acid profiles –

##### *Mycobacterium avium*

Reliable identification was achieved only when the mycobacterium isolates were cultured under standardised conditions. Mycobacterium species were grown in a BacT /Alert® MP culture medium. Mycobacteria are aerobic, non-spore forming, non motile, acid fast bacilli which have slow to very slow growth rates. Generation times of species varied from 2 to 20 hours depending upon the species. Growth of *Mycobacterium tuberculosis*, using traditional culture media, can take two to eight weeks or longer. The liquid BacT/Alert® MP culture medium [48] provides more rapid growth and, together with the highly sensitive HPLC / FI method, identification of the mycobacterium can mostly be achieved in 3 -14 days (Table 3).

Automated culturing process speeds up the positive identification of mycobacterium and can be of considerable advantage in a routine laboratory.

**Table 3. Performance characteristics of the BacT/Alert® MP culture:**

Micro organism	Inoculum CFU/bottle	Time to detection (days)
TB-complex	$\leq 10^6$	5.8 – 10.8
M.tuberculosis	$\leq 10^2$	13.6 – 21.4
M.tuberculosis (4 strains)	$\leq 10^6$	7.6 – 7.8
M.bovis (2 strains)	$\leq 10^2$	15.0 – 18.7

\*Data supplied by Biomerieux Inc. Durham, North Carolina

Early identification of the mycobacterium by HPLC is essential to prescribe the most appropriate drug treatment program for each infection. The *Mycobacterium avium-intracellulare* complex, for example, does not at all respond to the triple therapy used for *Mycobacterium tuberculosis*, therefore accurate identification of the mycobacterial species is essential.

Standardization on growth medium is important when comparing mycolic acid profiles of unknown mycobacterium isolates to the mycolic acid profiles of NCTC reference data. This was demonstrated by culturing a *Mycobacterium avium* and *Mycobacterium tuberculosis* species on Middelbrook 7H11 medium solid media, and in Middelbrook 7H9 medium liquid media (Fig. 11 & 12).

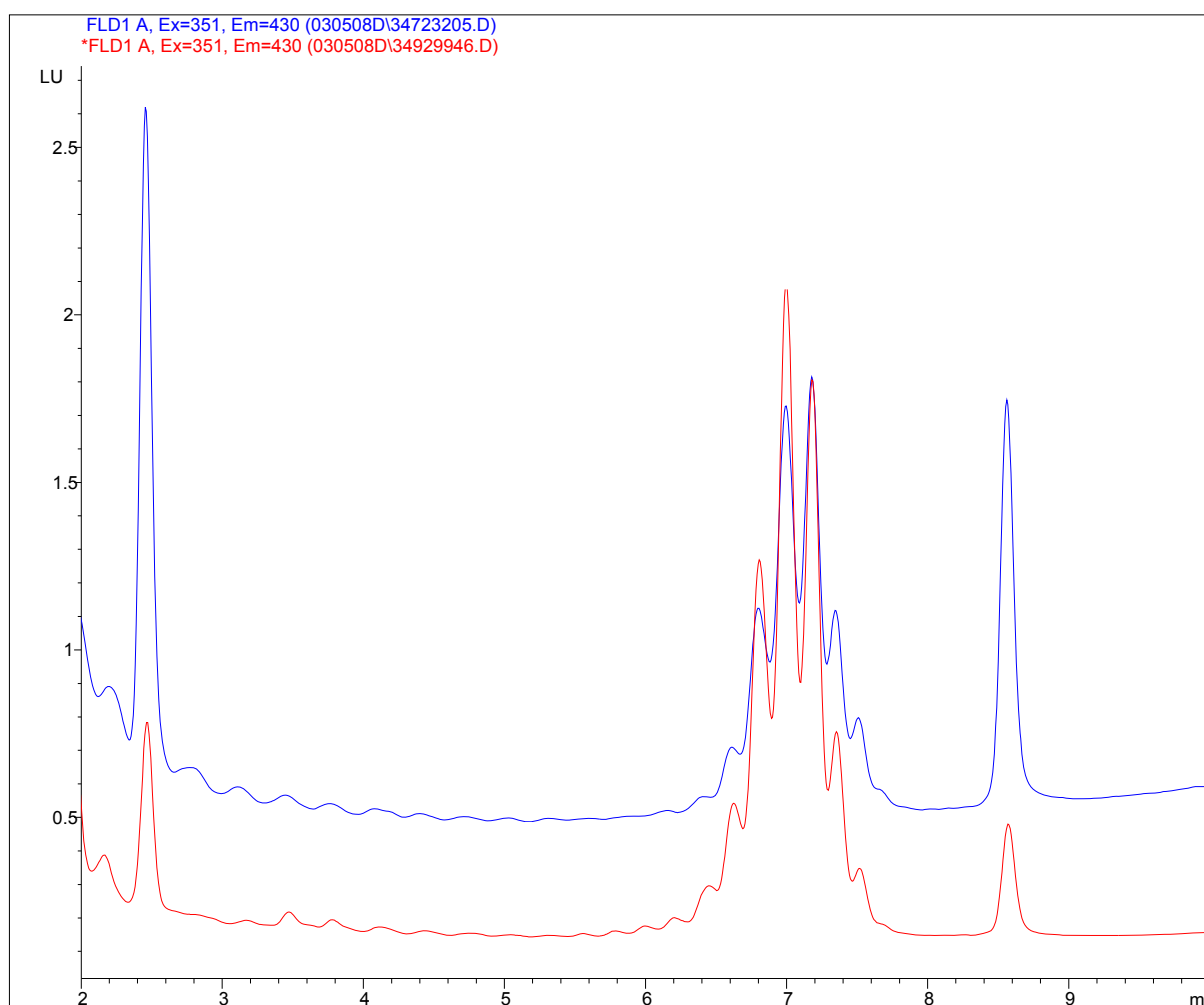
A definite change in the more non-polar, longer-chain mycolic acids was observed. Mycolic acid peaks in the more non-polar long-chain region of the chromatogram experienced an increased concentration in the Middelbrook 7H11 medium solid media. Mycolic acid in the more polar short chain region of the chromatogram experienced no significant profile changes. A number of other differences in concentration of closely eluting acids are clearly observed.



### 4.3.2 Different growth medium generate different mycolic acid profiles – *Mycobacterium tuberculosis*

The mycolic acid profile of *Mycobacterium tuberculosis* species was also compared when cultured on Middelbrook 7h11 and Middelbrook 7h9 media.

The chromatogram of *Mycobacterium tuberculosis* consists of long-chain mycolic acids that are highly non-polar. In comparing the chromatographic mycolic acid profiles, changes in relative peak heights but not in retention times can be observed (Fig. 11).



**Figure. 12. Mycobacterium adjusted retention time chromatographic profiles in different growth mediums. Chromatograms blue and red compare the difference in mycolic acid profiles of *Mycobacterium tuberculosis* cultured in Middelbrook 7H11 solid and Middelbrook 7H9 liquid culture medium.**

Culturing conditions, for instance the growth medium and temperature, do influence the mycolic acid profiles within the cell wall of the mycobacteria.

These variations made it almost impossible to compare the generated chromatographic mycolic acid profiles for mycobacterium with existing profiles in literature. A comprehensive mycolic acid profile library of mycobacteria from the National Collection of Type Cultures (NCTC) had to be established.

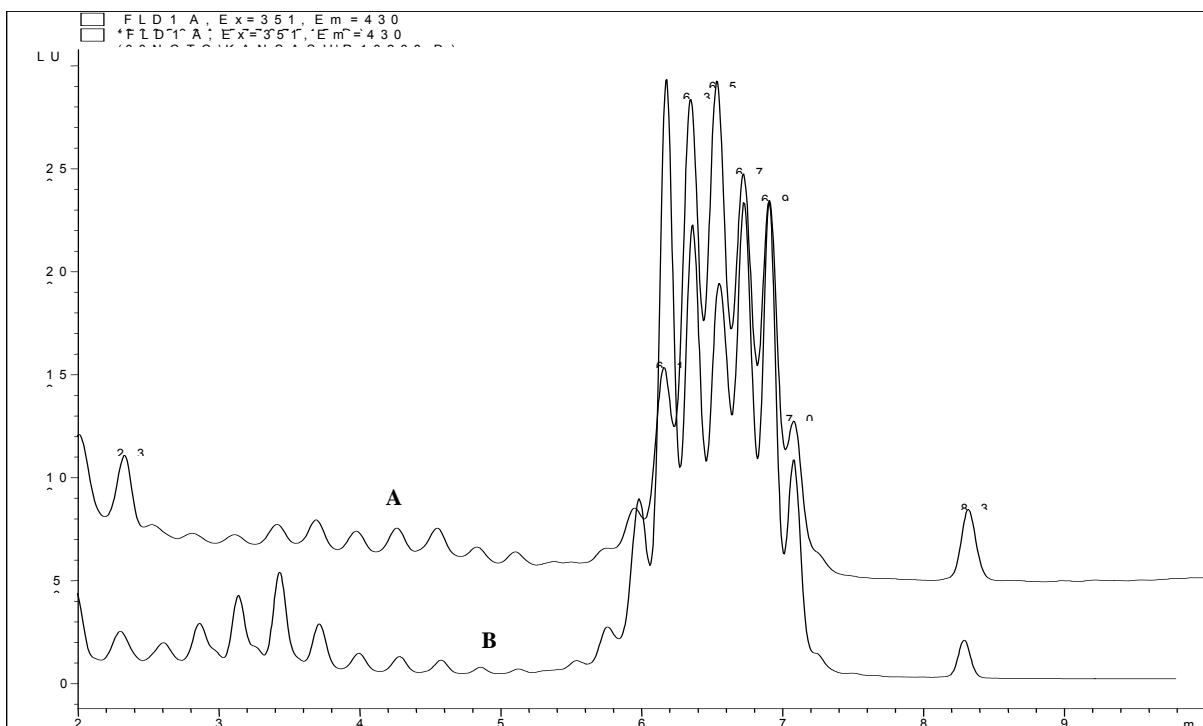
This library contains 35 mycolic acid profiles of different mycobacterium species and was compiled using the BacT/Alert MP culturing bottles containing 7H9 culturing medium (Appendix. Fig.1-35).

#### **4.4 IDENTIFICATION OF SOME OF THE RARE MYCOBACTERIUM INFECTIONS FOUND IN SOUTH AFRICA**

In general, mycobacteria can be separated into two major groups, *slow growers* and *fast growers*. The characteristic slow growth of most mycobacteria is probably due, at least in part, to the hydrophobic character of the cell surface, which renders the cells strongly impermeable to nutrients [4]. Other species such as *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium phlei*, and *Mycobacterium smegmatis* are considered to be fast-growing species that may double every two hours. Species having shorter chain - length lipids grow considerably more rapidly. Some fast and slow growing species like *Mycobacterium tuberculosis*, *Mycobacterium avium* and *Mycobacterium kansasii* produce unique mycolic acid profiles that are generally easily identified with HPLC/FL technique (Appendix A).

#### 4.4.1 *Mycobacterium kansasii*

This pathogen has been frequently isolated from tap water, shower or distribution networks and can be acquired from the environment rather than human-to-human transmission. *Mycobacterium kansasii* commonly causes pulmonary infection similar to pulmonary tuberculosis, with highest incidence in men over 50, who suffer from chronic obstructive pulmonary disease [49]. Currently *Mycobacterium kansasii* is the second species next to *Mycobacterium avium* of nontuberculous mycobacteria (NTM) that causes disseminated diseases in HIV positive patients [50]. As illustrated in chapter 6, *Mycobacterium kansasii* is one of the more frequent NTM infections seen in South Africa.

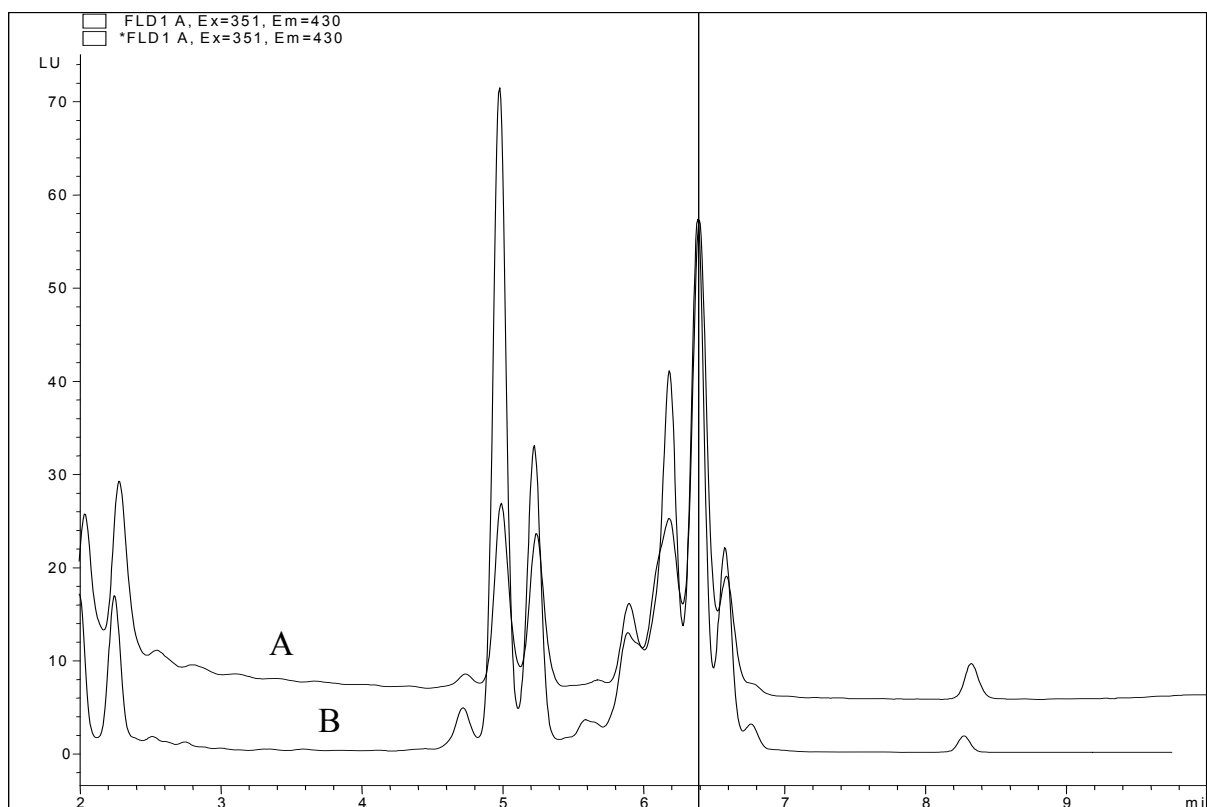


**Figure. 13. Chromatogram of patient infected with *Mycobacterium kansasii*. Chromatogram (A) and (B) illustrates mycolic acid profiles of patient and reference mycobacteria.**



#### 4.4.2 *Mycobacterium chelonae*

Rapid growing mycobacterium such as *Mycobacterium chelonae*, *Mycobacterium abscessus* and *Mycobacterium fortuitum* are wide spread in nature and in hospital environments, generally of low virulence, are capable of causing a wide spectrum of infections. These organisms are notorious for causing infections of soft tissue, tendons, bones and joints. These organisms may cause severe opportunistic infections in humans, including lung disease, tissue infections as well as postoperative wound infections, and prosthetic valve endocarditis. They are highly resistant to antibiotics, antiseptics and disinfectants and hence are important human pathogens [51].



**Figure. 13a. Chromatogram of patient infected with *Mycobacterium chelonae*. Chromatogram (A) and (B) illustrates mycolic acid profiles of patient and reference mycobacteria.**

# CHAPTER 5: APPLICATION OF THE HPLC PROFILING METHOD TO SOUTH AFRICAN CASE STUDIES

## 5.1 APPLICATIONS

In our laboratory an average of 20000 patients for mycobacterium infection are analysed annually. The positive screened mycobacterium specimen mycolic acid profiles are then readily and unambiguously identified by HPLC technique and compared to their mycolic acid profiles of the NCTC library (Appendix: A). This chapter will only deal with some isolates and interesting cases that could not be resolved by simple comparison to reference profiles.

## 5.2 IDENTIFICATION OF MULTIPLE MYCOBACTERIUM INFECTIONS

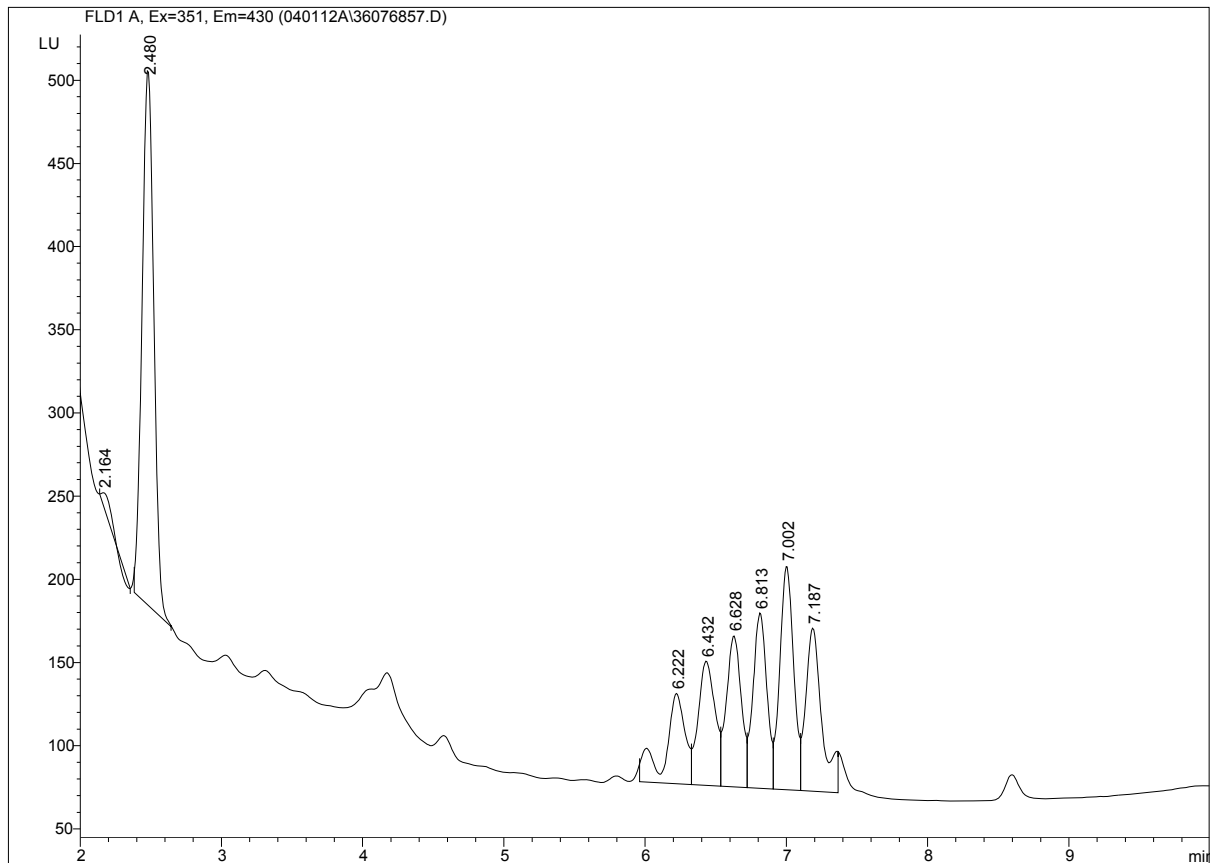
For the first time in South Africa multiple mycobacterium species infections have been identified by this technique. These infections are often misdiagnosed and very difficult to identify with common biological techniques. They have apparently not yet received much attention in scientific literature.

Diagnosing patients infected with multiple mycobacterium species is difficult and these cases are not often reported. Normally, repeated subcultures on solid media have to be performed to identify such multi - infections.

### 5.2.1 Multi - Mycobacterium infection – *Mycobacterium szulgai* and *Mycobacterium avium*

Multiple mycobacterium isolate in patients is not generally diagnosed by conventional methods. Such samples are normally regarded as contaminated and

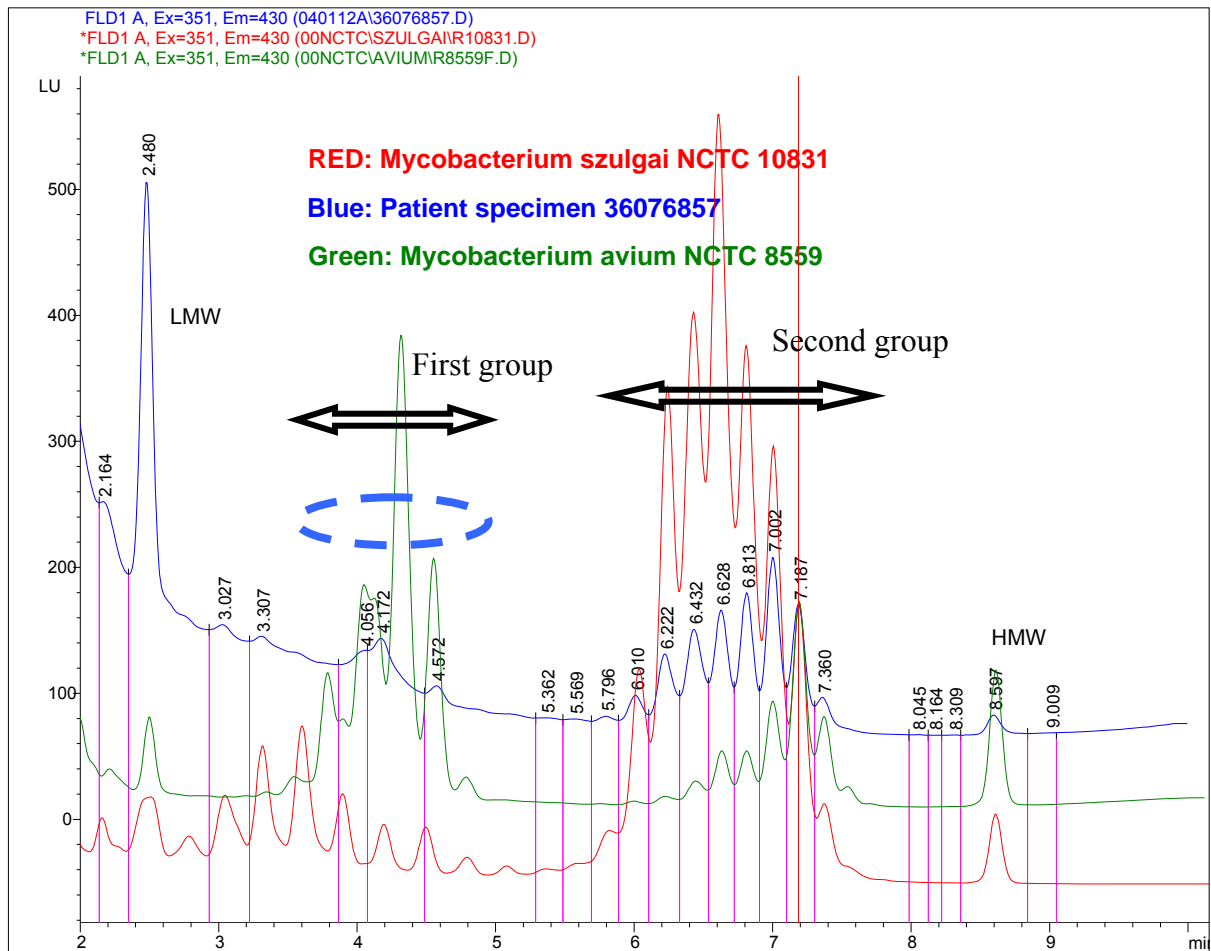
not reported as a possible positive for mycobacteria. *Mycobacterium szulgai* and *Mycobacterium avium* are not generally found together in one isolate. On first glance, the unknown (Fig.14) could easily be mistaken for a *Mycobacterium kansasii* (Appendix: A. Fig.17).



**Figure. 14. Chromatogram demonstrates patient specimen 36076857 measured for the first time on 09 Jan 2004. Chromatogram was identified as a *Mycobacterium szulgai*, a very rare mycobacterium infection. Notice the extra peak at 4.2 minutes, a possible contamination.**

If not discarded as “sample contamination” such multi - infections would previously have been documented as an unknown mycobacterium infection. However, with the improved HPLC/FL technique, including current chromatographic overlaying computer software technology, we were able to differentiate between a real unknown mycobacterium and a multi - mycobacterium infection.

Overlaying the unknown mycobacteria with possible matches from the established reference library for mycolic acids did not produce a definite match.



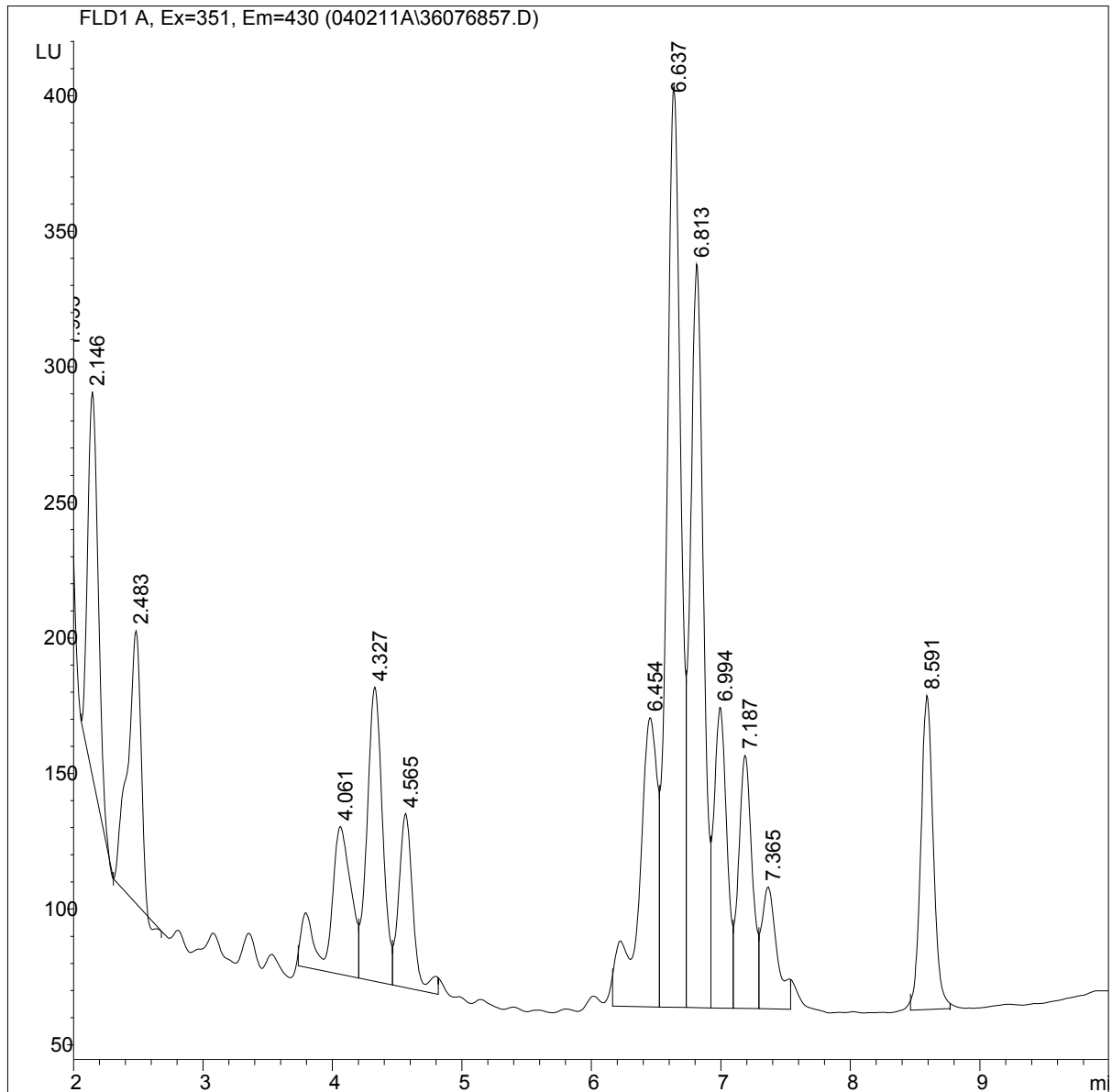
**Figure. 14a. Chromatogram demonstrates the different mycolic acid profiles.** (a) Blue: Patient multiple *Mycobacterium* infection. (b) Red: *Mycobacterium szulgai* NCTC 10831. (c) Green: *Mycobacterium avium* NCTC 8559. Notice the broad peak at 4.0 minutes, a possible *Mycobacterium avium* infection.

The second group of peaks (Fig. 14a.) was well defined; the first group of the patient mycolic acid (Fig.14a. marked with blue circle) could not be identified due to bad resolution. The mycolic acid chromatogram did not match any of the known reference mycobacterium mycolic acid profiles in our library. (See Appendix: A)

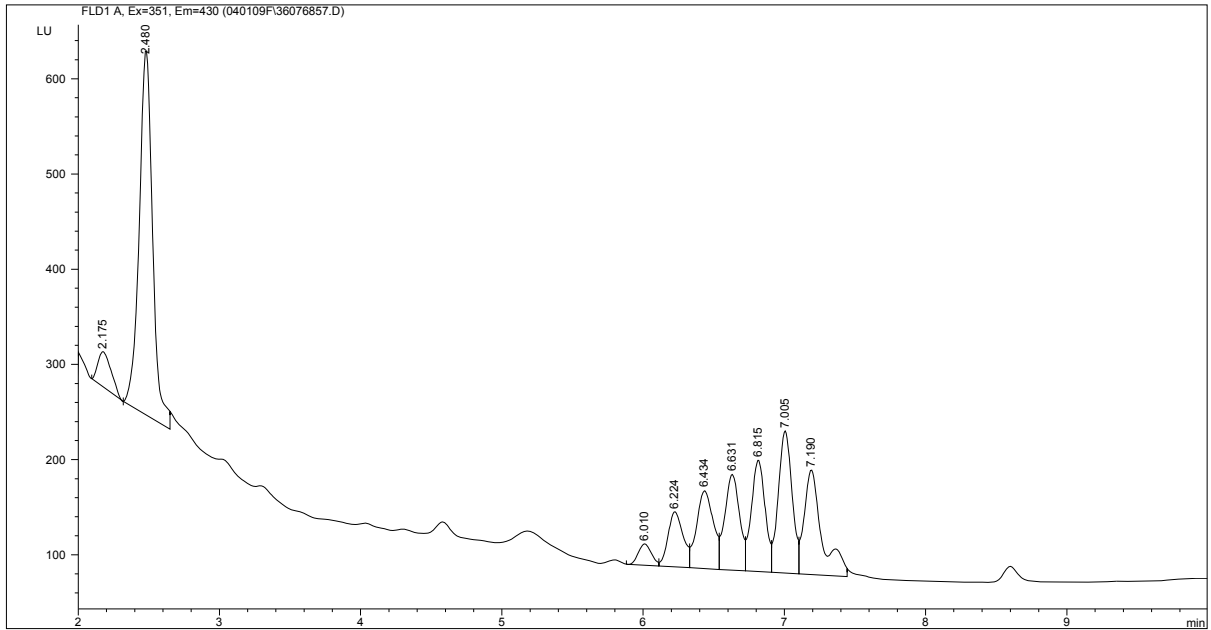
Further investigation confirmed our suspicion of a multi - mycobacterium infection. By sub-culturing the patient mycobacterium species on Middelbrook solid media (BacT 7H11) a different culture medium, we were able to identify these subcultures with the HPLC technique. This confirmed our suspicion; the mycobacteria isolates that infected the patient were *Mycobacterium avium* and *Mycobacterium szulgai*.

Both these species are classified as slow growing mycobacteria and were not easily identified by HPLC technique alone. The multi - mycobacteria infected patient specimen (Nr-36076857) was further analysed by measuring the mycolic acid profile at different culturing time intervals (Fig.14b&c). Only after a month could we positively identify the multi - mycobacterium specie that infected the patient.

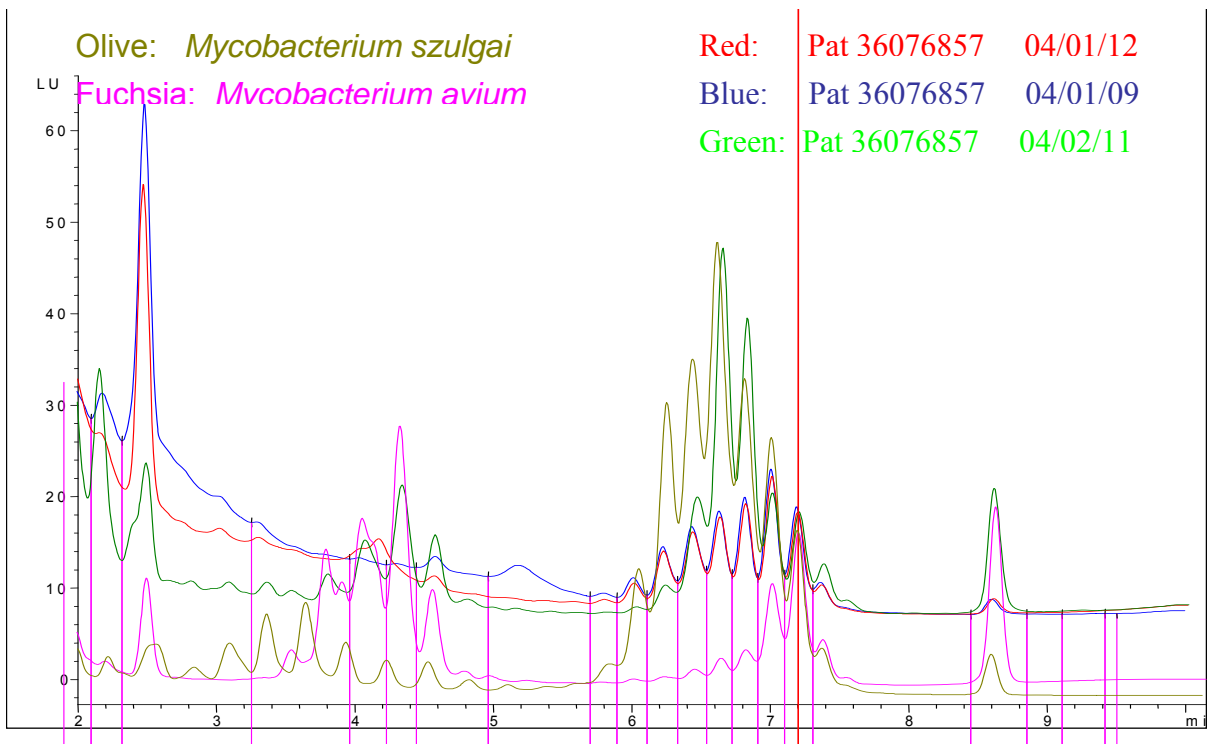
The chromatograms generated at these different time intervals, as indicated in figure 14d, only confirmed that the patient was definitely infected with *Mycobacterium avium* and *Mycobacterium szulgai*.



**Figure. 14b. Chromatogram demonstrates a subculture of patient specimen 36076857 measured on 11 Feb 2004. Subculture was identified as a *Mycobacterium avium*. Notice the extra peak at 4.2 minutes mentioned in figure 14a is well defined.**



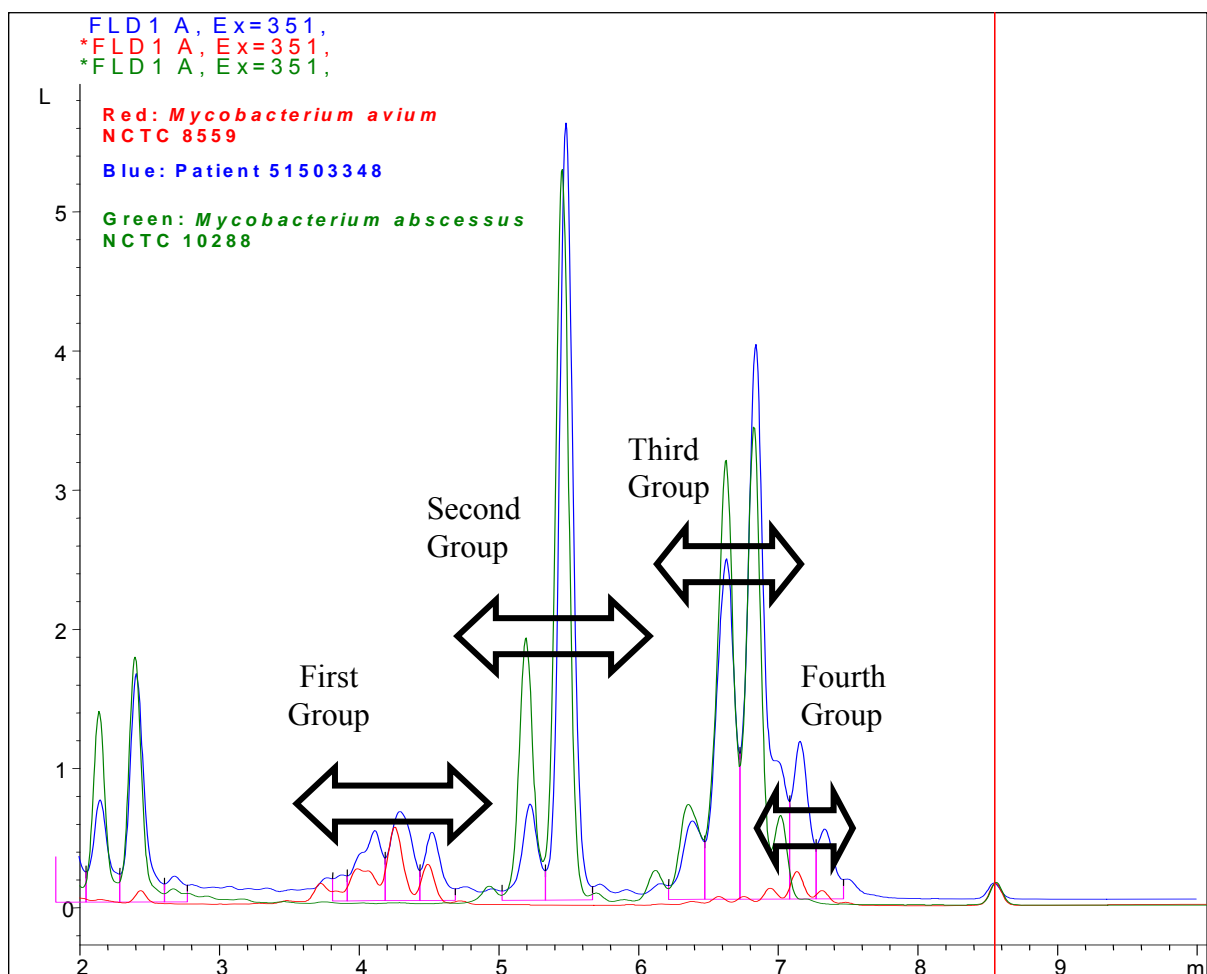
**Figure. 14c. Chromatogram demonstrates a subculture of patient specimen 36076857 measured on 12 Jan 2004. Subculture was identified as a *Mycobacterium szulgai*. Notice the extra peak at 4.2 minutes mentioned in figure 14a is not present.**



**Figure. 14d. Chromatogram demonstrates patient specimen 36076857 measured at different time intervals. Notice the chromatographic difference of each subculture culture measured over time.**

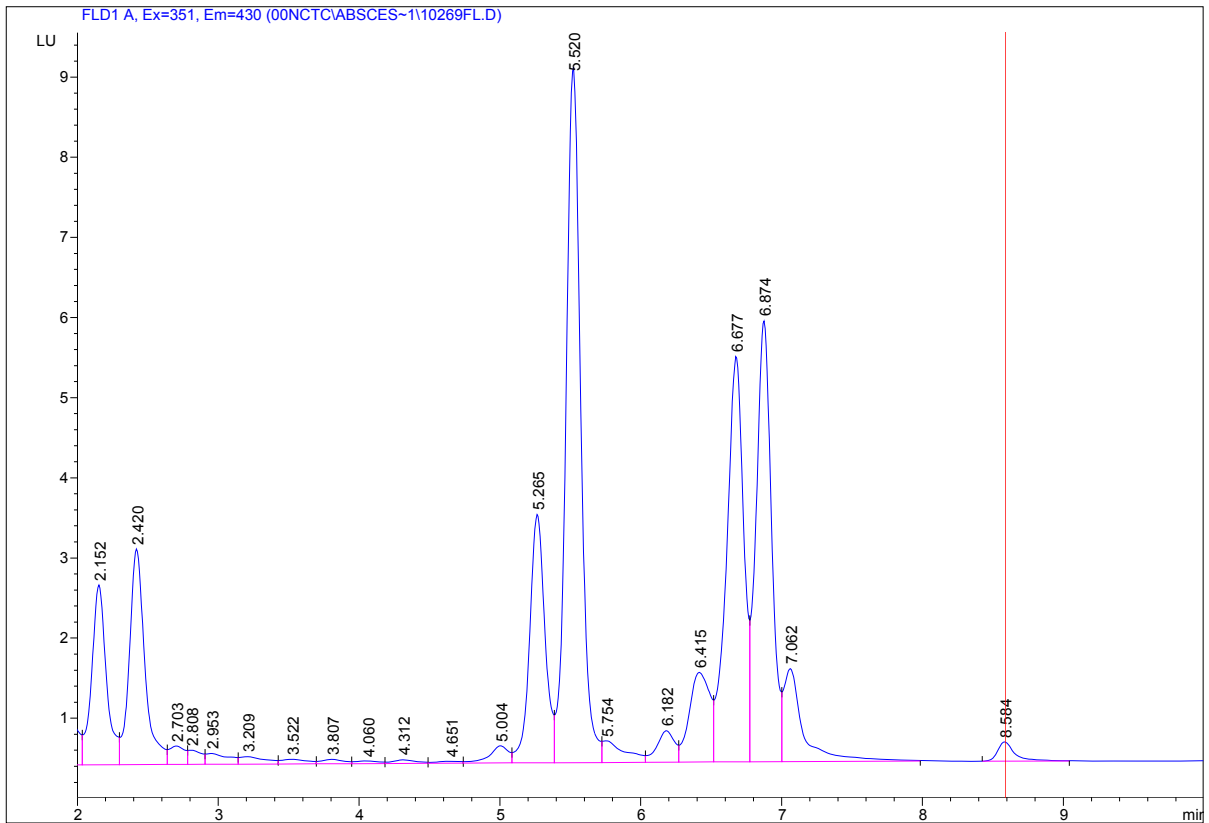
## 5.2.2 Multi - Mycobacterium infection – *Mycobacterium abscessus* and *Mycobacterium avium*

A very rare multiple mycobacterium infection found in two patients was a combination of *Mycobacterium avium* and *Mycobacterium abscessus* (Fig. 15a). The more prevalent mycobacterium isolate, clearly defined by the second and third groups of peaks in this patient, was identified as *Mycobacterium abscessus* (Fig. 15). *Mycobacterium avium* (Fig. 15a) isolate can be identified from the first and fourth groups of mycolic acid peaks.

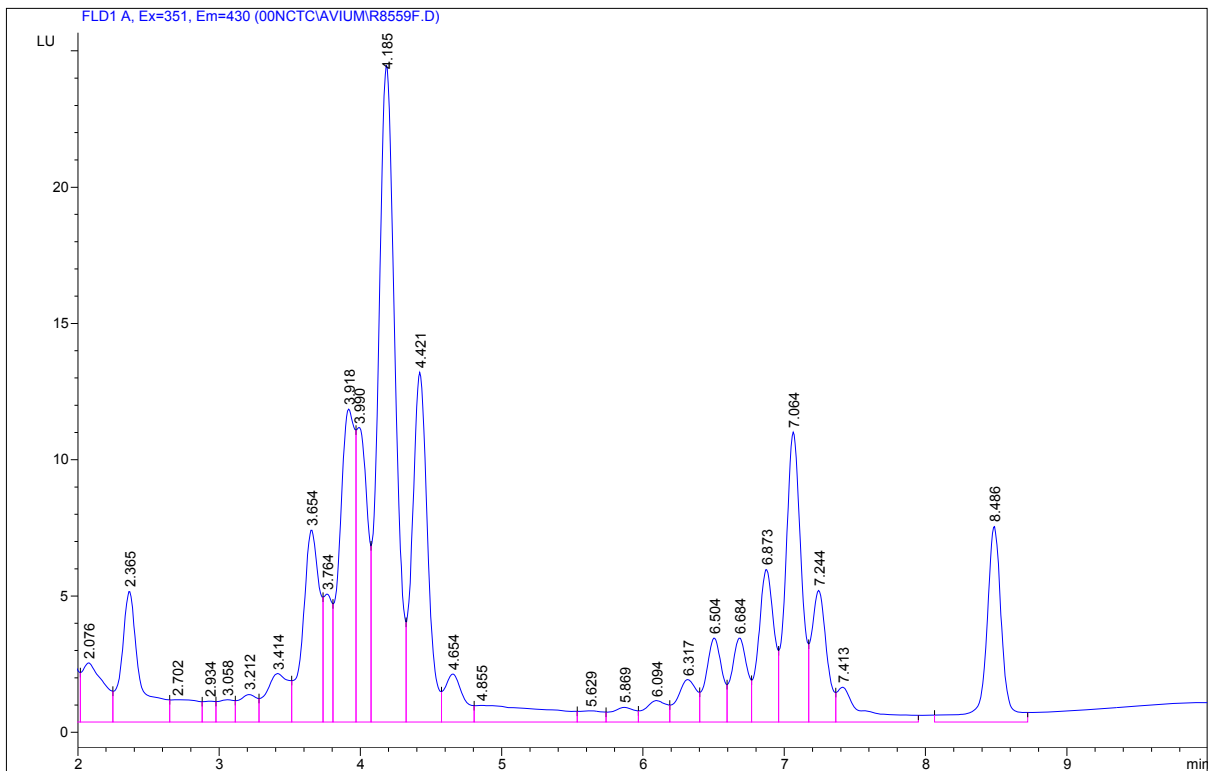


**Figure. 15. Chromatogram demonstrated mycolic acid profiles of a multi Mycobacterium infection. (a) Blue: Patient multiple mycobacterium infection. (b) Red: *Mycobacterium avium* NCTC 8559. (c) Green: *Mycobacterium abscessus* NCTC 10288.**





**Figure. 15a. *Mycobacterium abscessus* NCTC 10269**



**Figure. 15b. *Mycobacterium avium* NCTC 8559**

### 5.2.3. Multi - Mycobacterium infection – *Mycobacterium tuberculosis* and *Mycobacterium avium*

*Mycobacterium tuberculosis* and *Mycobacterium avium* combination are the most prevalent and more frequently identified multi mycobacterium infection (Fig.16).

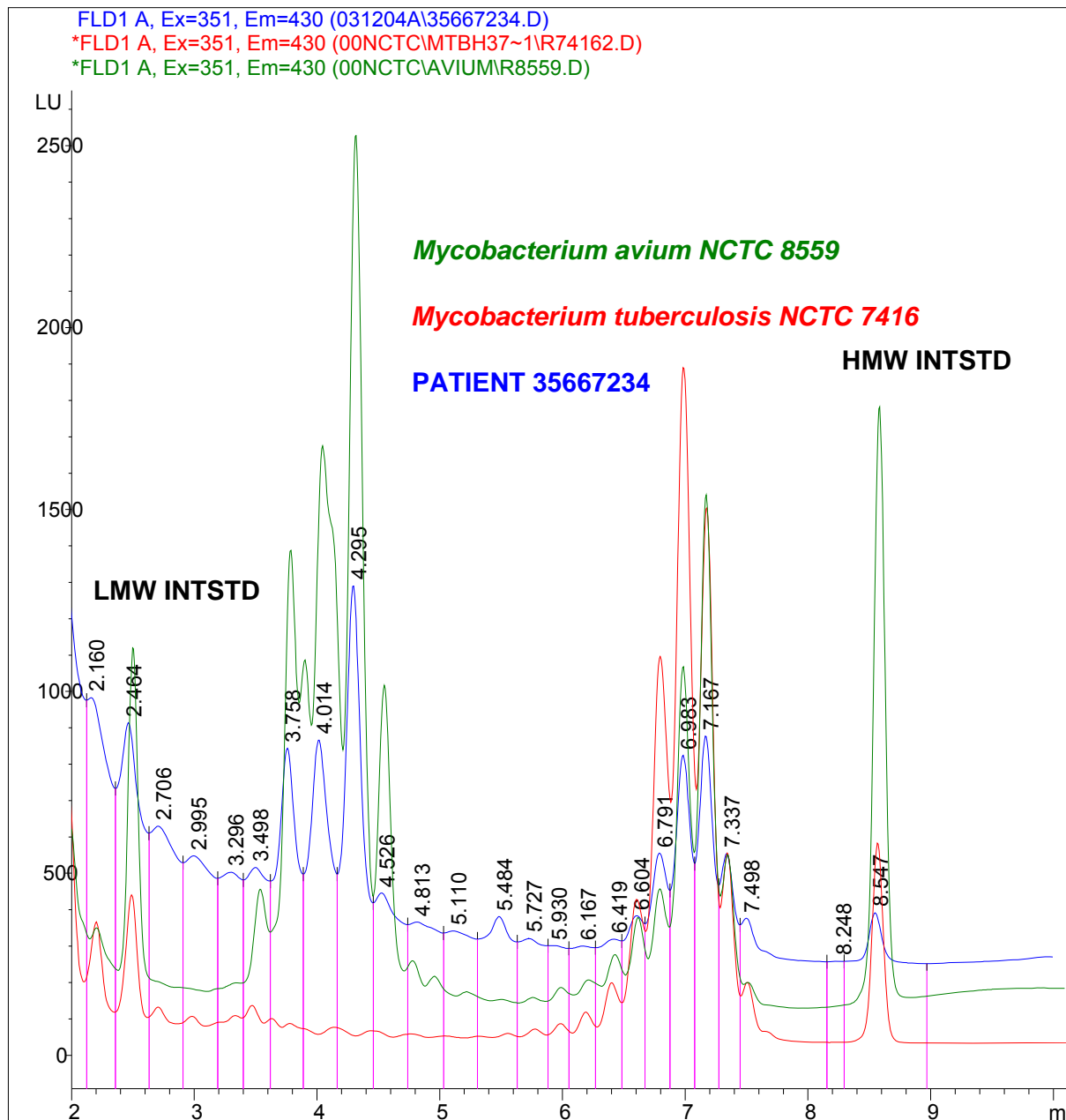


Figure. 16. Chromatogram demonstrated mycolic acid profiles of a multi - Mycobacterium infection. (a) Blue: Patient *Mycobacterium avium* and *Mycobacterium tuberculosis*. (b) Green: *Mycobacterium avium* NCTC 8559. (c) Red: *Mycobacterium tuberculosis* NCTC 7417.

Multi - mycobacterium infections are commonly associated with immune compromised diseases. An increase in *Mycobacterium avium* infections is associated with the high infection rates of HIV in Sub-Saharan Africa. *Mycobacterium avium* infections are the most frequent MOTT infections found in these regions. According to a WHO report in 1999, Africa has been rated the highest regarding TB/HIV co-infection in the world [52], but most probably also the highest in MAIC / HIV co-infections.

### **5.3 MYCOBACTERIUM COMPLEX ISOLATES - *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum***

Mycobacteria that are not easy to distinguish is the group of *Mycobacterium avium intercellulare* complex (MAIC) strains consisting of *M. avium*, *M. intercellulare* and *M. scrofulaceum*. The close phenotype basis existing within the group of these slow growing organisms has been described by the term *Mycobacterium avium intercellulare* complex. In literature positive *Mycobacterium avium intercellulare* complex (MAIC) strains consisting of *M. avium*, *M. intercellulare* and *M. scrofulaceum* are only reported as *Mycobacterium avium intercellulare* complex and not generally differentiated from each other [53].

Separation of the different strains within *Mycobacterium avium intercellulare* complex is only done to demonstrate the resolving power of this technique and to demonstrate that it is possible to separate the different mycolic acids profiles of these mycobacterium complexes.

By computer overlaying the mycolic acid profile chromatograms of the mycobacterium species contained in this complex, we identified small differences in

the mycolic acid profiles of the *Mycobacterium intracellulare* and *Mycobacterium avium* (Fig. 16a).

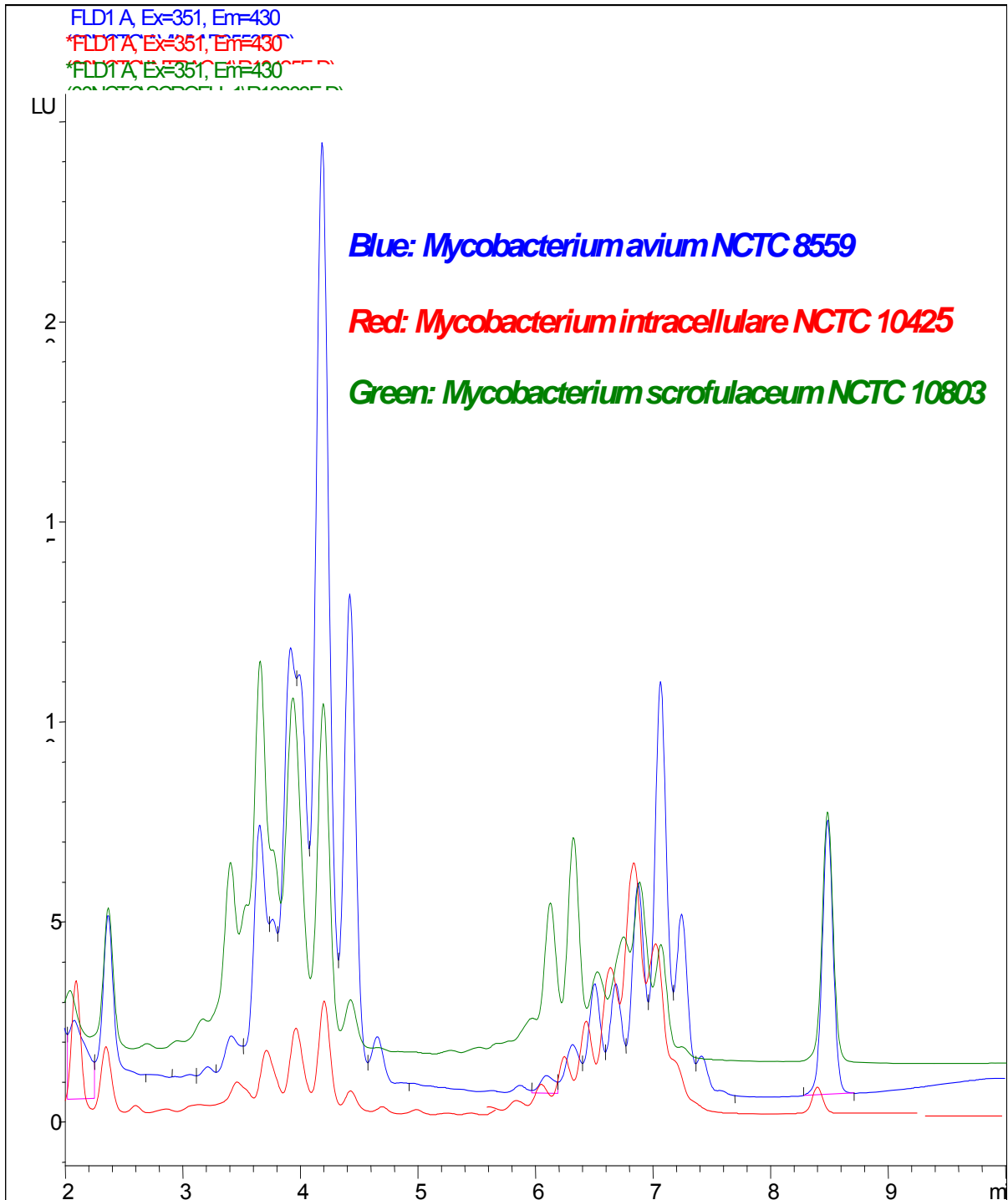
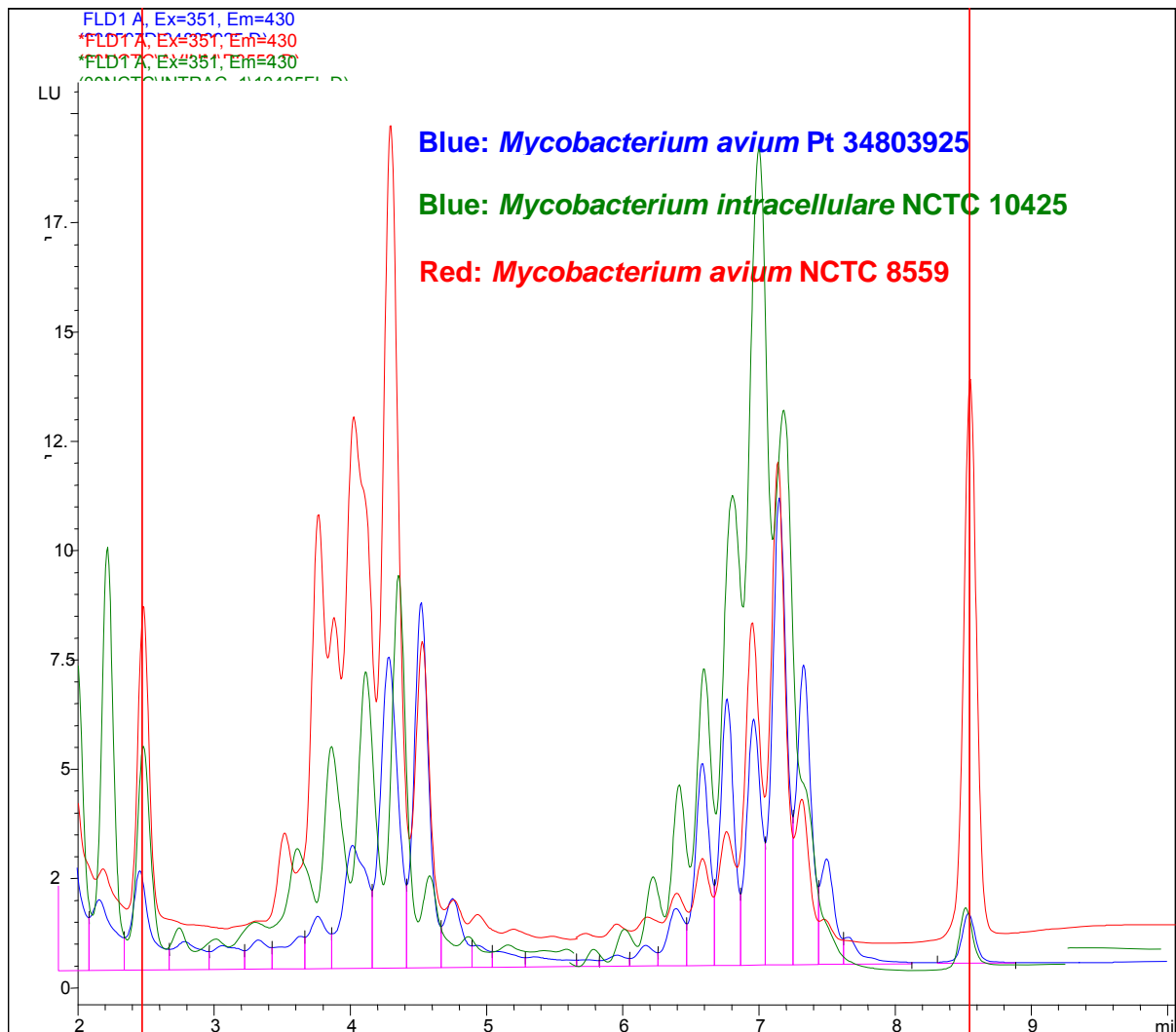


Figure. 16a. Chromatogram demonstrates the small differences in mycolic acid profiles of the *Mycobacterium avium* intercellulare complex specie.

By overlaying the patient profile and the individual reference profiles of *Mycobacterium avium intracellulare* complex it was very easy to identify the patient sample. With these small concentration and ratio differences of the mycolic acid profiles it was still possible to identify patient nr 34803925 as a positive *Mycobacterium avium*. (Fig.17).



**Figure. 17. Chromatogram demonstrates the small variations in mycolic acid peaks when comparing the patient to *Mycobacterium intracellulare* complex infection. (a) Blue: Patient infected with *Mycobacterium avium* (b) Green: *Mycobacterium avium intracellulare* NCTC 10425. (c) Red: *Mycobacterium avium* NCTC 8559.**

The difference in the chromatograms was demonstrated by overlaying the different species of *Mycobacterium avium complex* and by comparing the mycolic acid chromatographic profiles of these mycobacterial isolates separately (Fig.18 - 21).

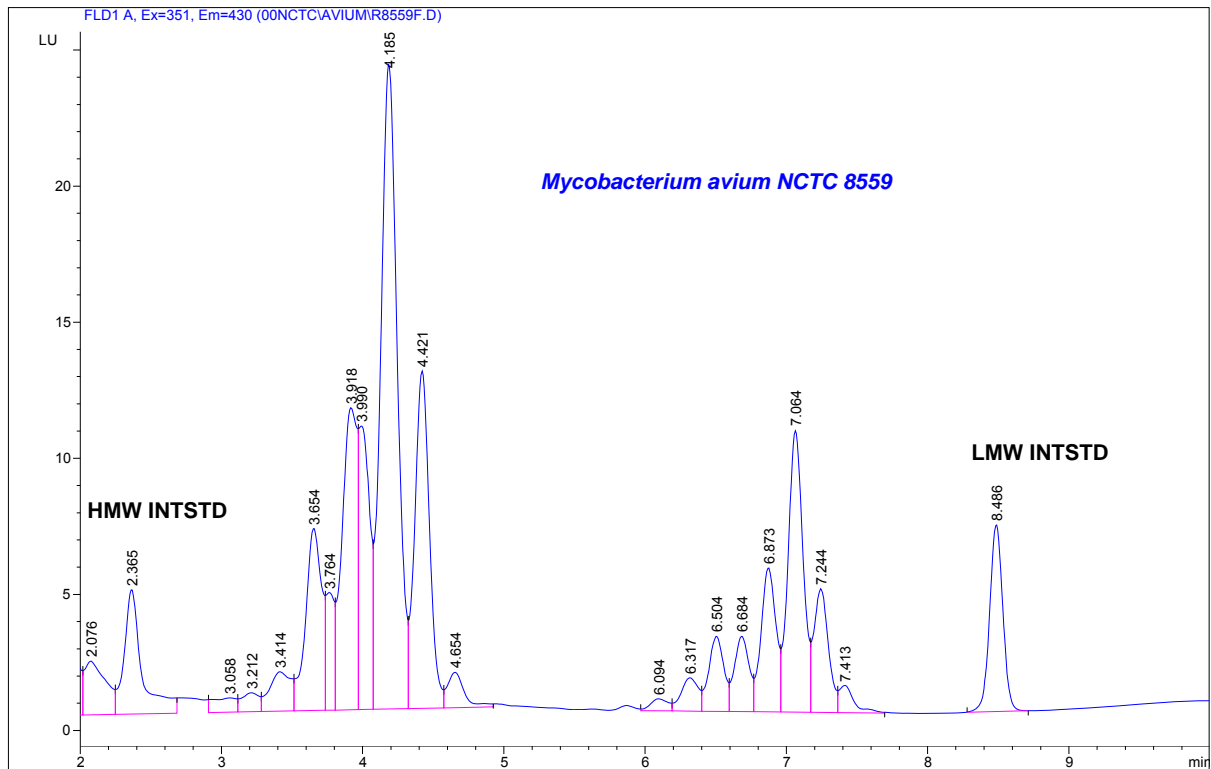


Figure. 18. Chromatogram of *Mycobacterium avium* NCTC 8559

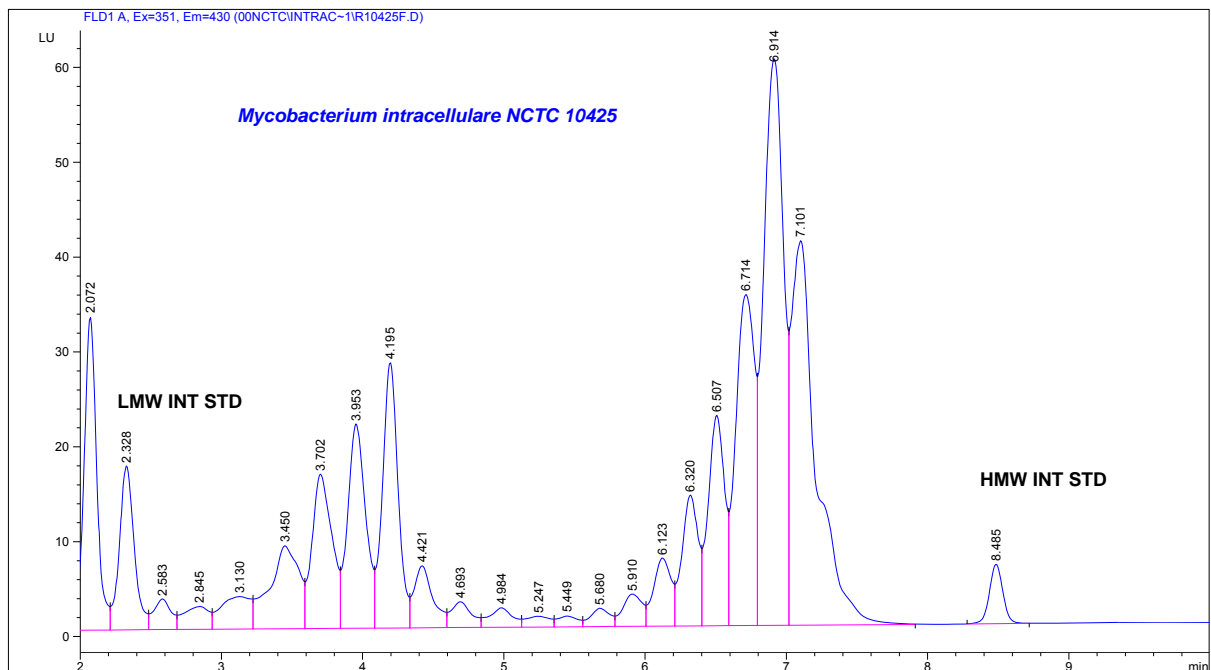


Figure.19. Chromatogram of *Mycobacterium intracellulare* NCTC 10425

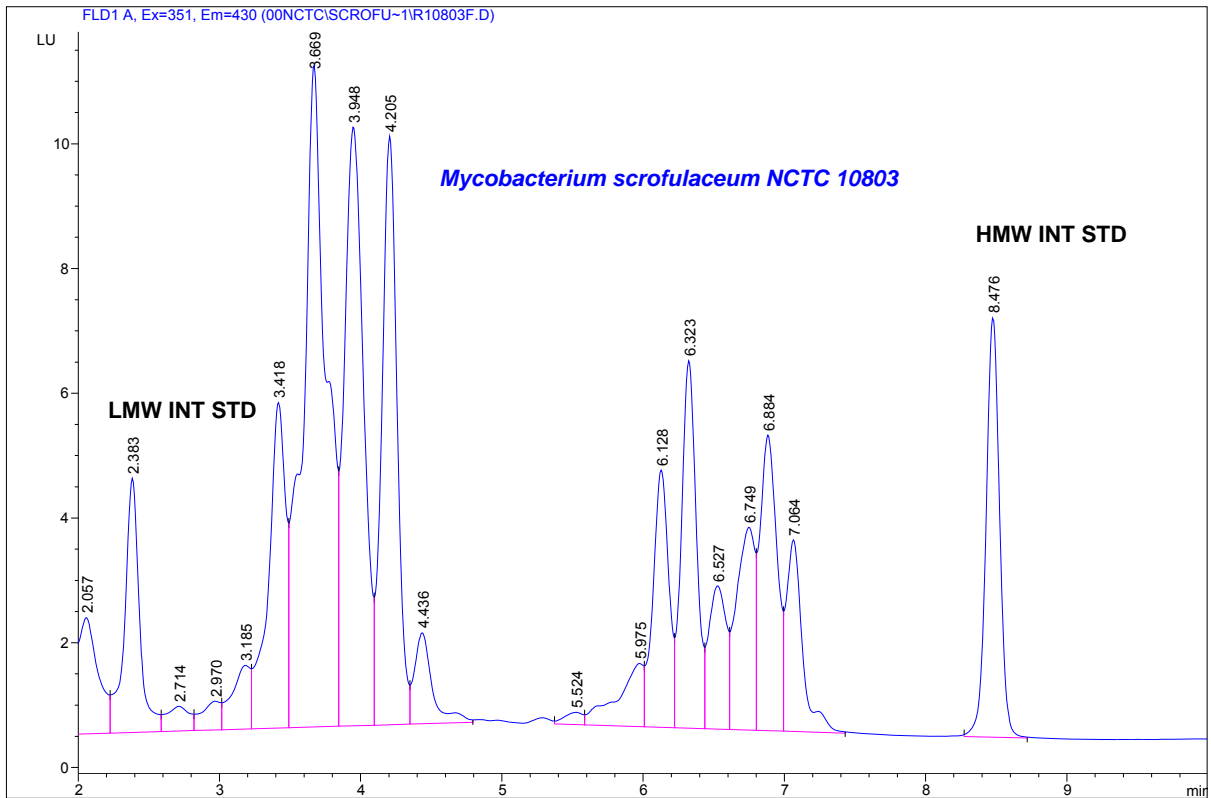


Figure. 20. Chromatogram of *Mycobacterium scrofulaceum* NCTC 10803

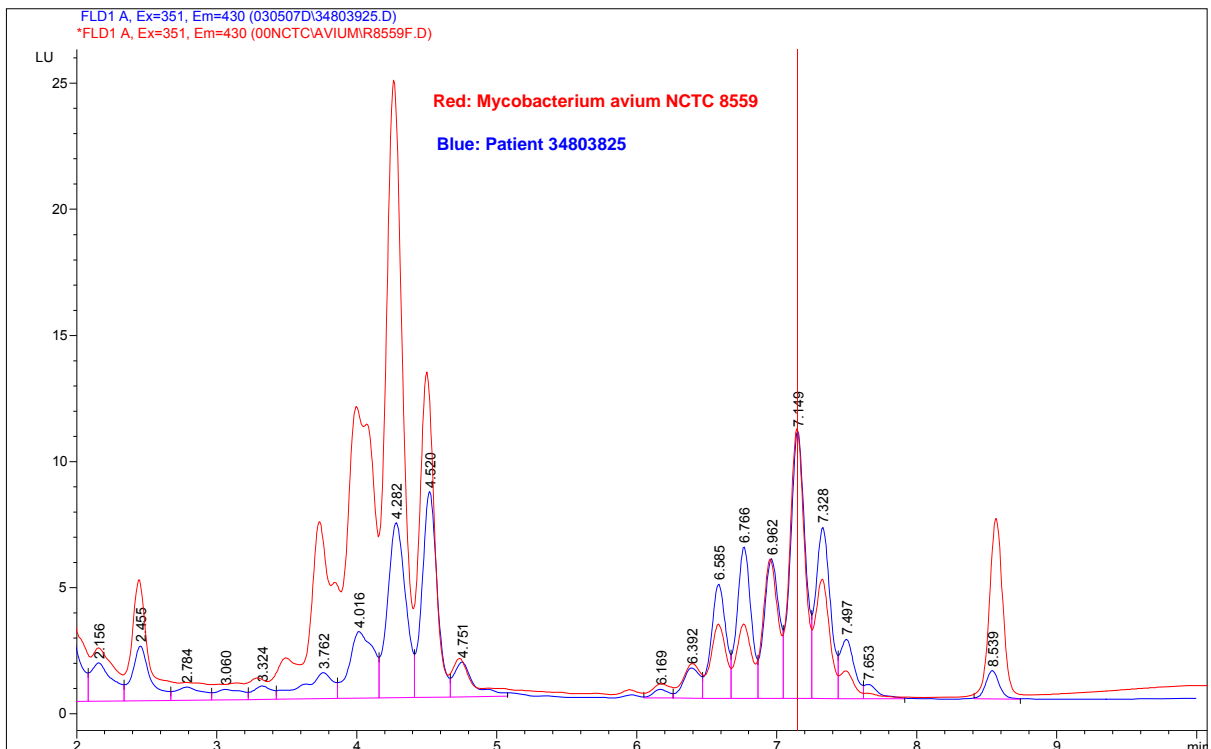
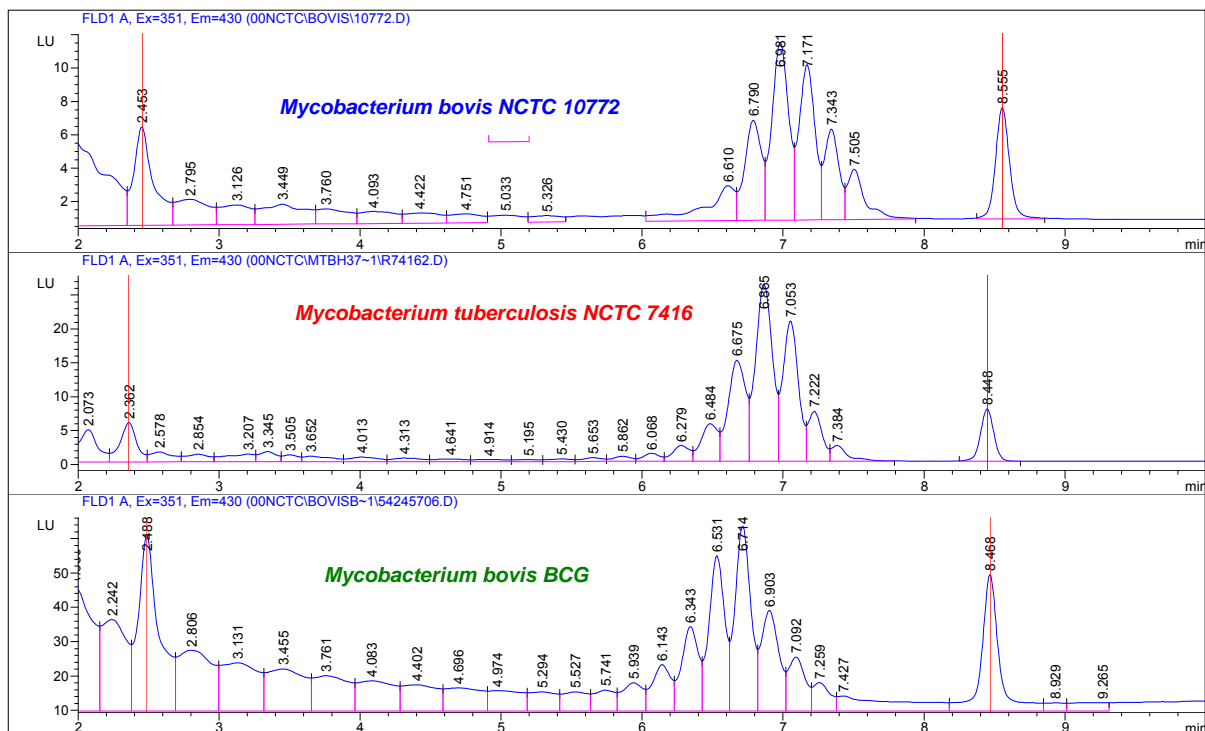


Figure. 21. Chromatogram demonstrates a patient nr 34803825 infected with *Mycobacterium avium*. The red line indicates the peak used in the normalization of the chromatograms.

### 5.3.2 *Mycobacterium tuberculosis* complex infections – *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium bovis* BCG.

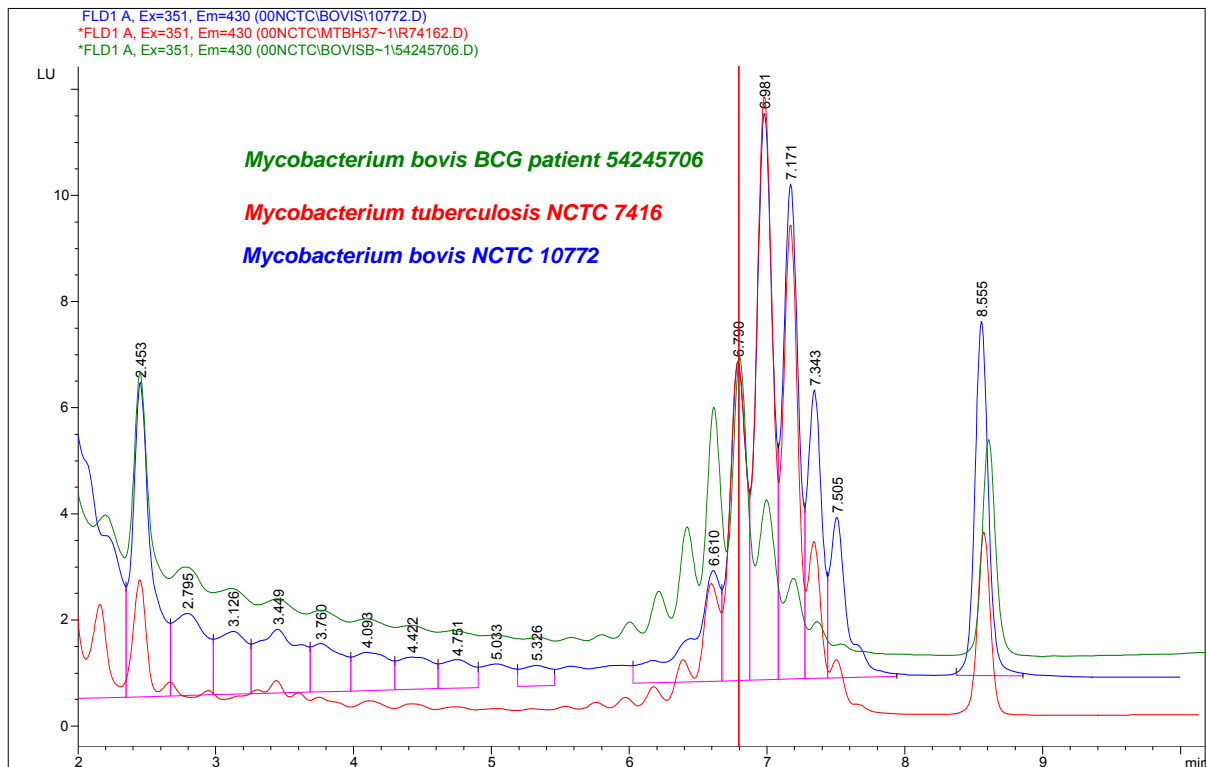
*Mycobacterium tuberculosis* complex consists of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG (Bacillus Calmette-Guérin), *Mycobacterium africanicum* and *Mycobacterium microti*. It is not possible to differentiate between the different species by conventional biochemistry methods. *Mycobacterium bovis* BCG is an attenuated *Mycobacterium bovis* strain and is used in the treatment of bladder cancer in patients. In some countries it is also used to vaccinate people to obtain immunity against *Mycobacterium tuberculosis* complex infection. It is thus very important to distinguish *Mycobacterium bovis* BCG from the other species of the *Mycobacterium tuberculosis* complex (Fig. 22).



**Figure. 22. Chromatogram demonstrates the differences in mycolic acid profiles of the *Mycobacterium tuberculosis* complex. Notice how all three species are clearly distinguished on the basis of accurate retention times (carefully adjusted with the two internal standards). Casual inspection only might mistake them for the same profile.**



This technique is able to differentiate *Mycobacterium bovis* BCG (Isolated from patient infected vaccination area) from *Mycobacterium tuberculosis* and *Mycobacterium bovis*. In overlaying the above *Mycobacterium tuberculosis* complex species it was easy to separate the different mycolic acid profiles from the different *Mycobacterium tuberculosis* (Fig. 23).



**Figure. 23.** Chromatogram demonstrates the difference in the *Mycobacterium tuberculosis* complex and *Mycobacterium bovis* BCG. (a) Green: Patient infected with *Mycobacterium bovis* BCG. (b) Red: *Mycobacterium tuberculosis* NCTC 7416. (c) Blue: *Mycobacterium bovis* NCTC 10772. Notice the different retention times in mycolic acid peaks from *Mycobacterium bovis* BCG and the *Mycobacterium tuberculosis* complex.

## CHAPTER 6: STATISTICAL OVERVIEW OF RESULTS

### FROM SOUTH AFRICA

In a recent survey we compared data from 1997 compiled by the WHO with our current data. Our main aim was to establish the rate of infection caused by *Mycobacterium tuberculosis* (MTB) and infections caused by Mycobacterium other than Tuberculosis (MOTT). This was achieved by analysing each positive sputum sample by means of the established HPLC/FI technique. This technique enables us to distinguish the different species of Mycobacterium as shown in previous chapters. For the last few years we have been using this technique to do proper diagnosis of the MOTT infection in our patients. An increase in *Mycobacterium tuberculosis* complex as well as MOTT infections have been indicated by data collected in our laboratory the last 4 years [52]. Multiple Mycobacterium strain infections are not uncommon in a country where HIV/AIDS is so prevalent. The high incidence of HIV / MTB infection complicates the treatment of Mycobacterium. The incidence of *M. avium intercellular* infection (MAIC), for example, is increased 100 fold for patients with immune compromised disease. Up to now the MOTT infection was not treated differently from that of normal *Mycobacterium tuberculosis* complex. Often patients are treated with standard regimen of antibiotics although this treatment is not suitable for some of the MOTT infections. It is very important to identify the mycobacterium species before submitting the patient to the standard treatment of antibiotics. For instance a MAIC infections cannot be treated effectively with the normal antibiotics. A combination of ethambutol, rifabutin or rifampicin and clarithromycin are to be used for a period of 18 months for the treatment of MAIC infections.

Studies done in our laboratory demonstrated that general regimen of mycobacterium antibiotics are not effective against some of the MOTT infections. It is thus essential that a proper minimum inhibition concentration (MIC) antibiotic study test program has to be implemented if a pathogenic MOTT is identified.

Africa has the highest infection rate of TB/HIV infection in the world. A 1997 survey by the WHO estimate the TB/HIV infection at 7,302 million. In our study we only concentrated on the number of MOTT infections.

In 1997 the WHO compiled a report that revealed the infection rate of *Mycobacterium tuberculosis* worldwide [53]. In our study we used this information (Table 4.) as a baseline to compile a report of the current mycobacterial infection in South Africa.

Comparing the results we obtained for the last 5 years we observed an increase in pathogenic MOTT infections (Graph 1). The infection rate for *Mycobacterium tuberculosis* in 2003 was measured at 18% of a population of 21050 suspected positive patients.

Of the 18% positive mycobacterial cultures, 78% were identified to be *Mycobacterium tuberculosis complex* and 22% were MOTT infections.

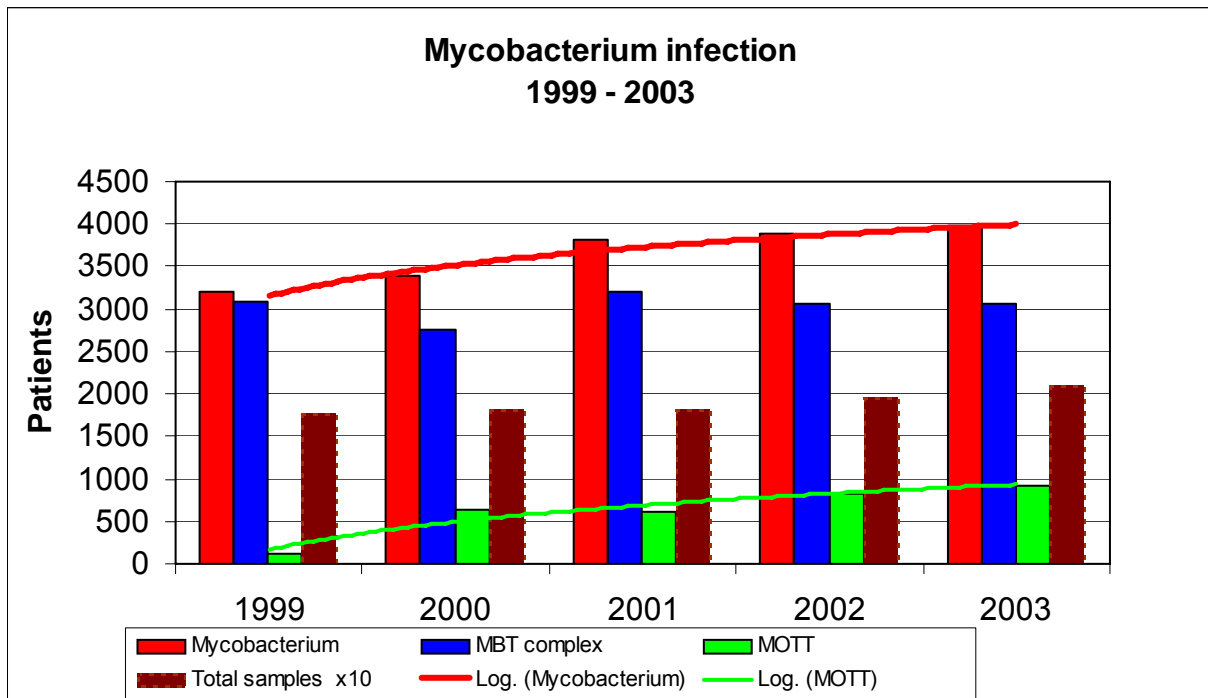
A small portion (1.8%) of the MOTT isolates could not be identified to specie level.

**Table 4. Estimates of *Mycobacterium tuberculosis* burden in the 10 highest incidence countries as compiled in 1997 by the WHO**

Rank	Country	Population Thousands	Population Numbers Thousands									
			Incidence	Notified Cases All	SS+ Incidence	Notified		SS+ Prevalence	Infection Prevalence	TB Death Rate	HIV- Pos. Cases	Pos TB/ HIV
						Cases SS+	Prevalence					
1	India	960 178	1799	1136	805	274	4854	2182	422 569	437	45	4804
2	China	1243 738	1402	419	630	189	271	1132	445 343	258	5	143
3	Indonesia	204 323	583	22	262	19	1606	715	99 920	140	6	25
4	Bangladesh	122 013	300	63	135	33	620	270	56 260	68	1	10
5	Pakistan	143 831	261	0	117	0	583	259	57 110	64	3	25
6	Nigeria	118 369	253	17	110	11	454	197	42 773	69	35	831
7	Philippines	70 724	222	208	100	83	490	219	33 523	48	1	11
8	<b>South Africa</b>	<b>43 336</b>	<b>170</b>	<b>105</b>	<b>69</b>	<b>55</b>	<b>262</b>	<b>114</b>	<b>16 449</b>	<b>72</b>	<b>76</b>	<b>1101</b>
9	Russian fed.	147 708	156	121	70	42	241	108	26 587	26	1	7
10	Ethiopia	60 148	156	59	66	16	221	97	21 471	49	47	928

SS+ Incidence – sputum smear positive, SS+ prevalence – sputum smear positive all form (new and existing) of cases, Infection prevalence – average of population infected with *Mycobacterium tuberculosis*

**Graph 1. Mycobacterium infection 1999 – 2003**



In the last few years we have determined a trend regarding the increase in pathogenic MOTT isolates (Graph 2.). In our laboratory *Mycobacterium avium intracellulare-complex* (MAI-complex) is found to be the most common MOTT infection. We normally relate this to the high incidence of immune compromised patients. After MAI-complex, *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum* and *Mycobacterium gordonae* were the most prevalent MOTT isolates and are considered typical infections due to unhygienic conditions in hospitals and clinics.

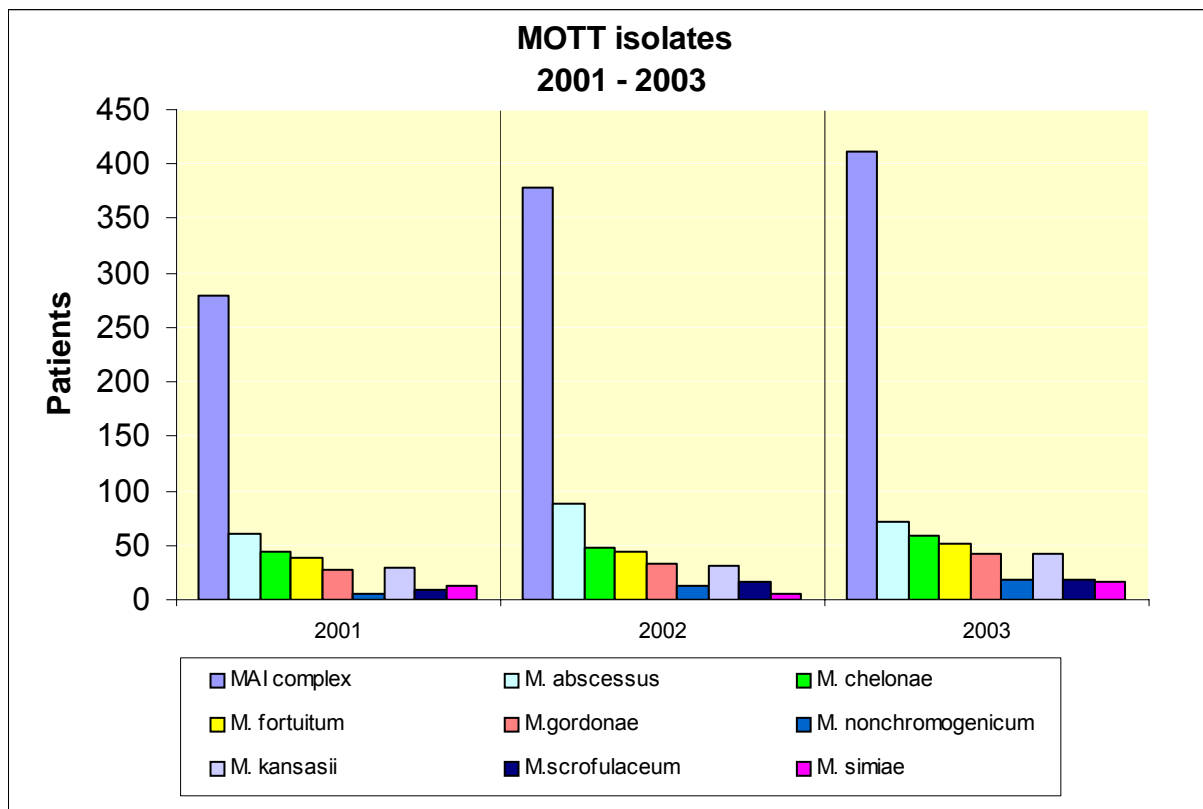
In certain cases the MOTT isolates were found to be environmental contaminants of the sample. In other cases some of these previously non-pathogenic environmental isolates have been proven to be the etiological agent responsible for the infections.

The data does not statistically address the impact of combined TB/HIV infections, as the HIV status of the patients are unknown. In addition, it should be remembered

that the data is from a selected population sample that has a medical fund or can afford these tests.

Mycobacterium management programmes based mainly on case detection by identifying smear positive patients via microscopy alone is dangerous. Treatment of the disease with standardized TB treatment regimen of antibiotics is not justifiable in situations where MOTT infections are as high as 22% of the total mycobacterium infection.

**Graph 2. Mycobacterium other than tuberculosis (MOTT) isolates**



## CHAPTER 7: DISCUSSION and CONCLUSION

### 7.1 SUMMARY

Previous HPLC methods of identification of mycobacterium by profiling different mycolic acids within the cell wall have been verified. It was demonstrated that an improved HPLC/FL method can be used in a routine clinical laboratory as a rapid, easily performed, reproducible and reliable method to identify the mycobacterium species. As mycobacterium infections increase among AIDS patients, there is an increasing need for rapid identification of the different *Mycobacterium* species. This study demonstrated that by using reverse phase liquid chromatography (HPLC) we were able to generate a “fingerprint” chromatogram of the mycolic acids that characterise the cell wall of each *Mycobacterium* species. The facility to identify the different mycolic acid profiles is a first for South Africa and is currently used on a daily basis in the diagnosis of patients infected by mycobacterium. The analysis of reference materials proves that the HPLC/FL technique is able to differentiate clearly between all 35 tested pathogenic and non-pathogenic species. Automation of the improved culturing and HPLC technique provides rapid, cost effective and reliable analysis (1) for routine diagnosis around the clock, (2) for observing multiple infections and (3) for generation of reliable statistics on *Mycobacterium* infection in Southern Africa.

### 7.2 METHOD DEVELOPMENT

Improvements in the method have been achieved by (a) changing the culturing system, (b) modifying chromatographic conditions and (c) improving data handling.

### **7.2.1 LIQUID CULTURING SYSTEM**

By standardizing the growth medium and incubation temperatures and by using the BacT/Alert liquid culture system [48], we were able to culture positive samples much faster and safer than with the solid media culturing plates (Figure. 11&12). The liquid culturing process is done in a closed container, which in turn limits possible cross contamination and produces a more homogeneous sample for analysis. One of the added advantages is the automation and the unattended monitoring of the growth process, which in turn is less labour intensive and more cost efficient. Reference laboratories generally perform identification of mycobacteria to species level. The time it takes to do a clinical diagnosis is generally hampered by the time required to isolate and identify mycobacterial species. This process can take from 2 – 10 weeks, or even longer. The BacT/Alert 3D system employs a colorimetric sensor for continuous detection of carbon dioxide (CO<sub>2</sub>) generated by the organisms in the metabolism of substrates in the culture medium [48]. This technique is performed under controlled conditions and automatically reports a positive mycobacterium culture for HPLC analysis in anything from 3 –14 days.

### **7.2.2 HPLC OPTIMIZATION**

In optimising the HPLC method and the standardisation of the culturing process we were able to provide a species-specific, rapid, reliable and reproducible method that could be used to identify and differentiate primary mycolic acids within the mycobacterium genus. This unique “fingerprint” pattern of the different mycolic acid profile allows the unambiguous identification of mycobacteria. Reference mycobacterium species, accurately identified to the species level and obtained from NCTC, have been used to compile a detailed HPLC library for each of the



mycobacterium species. Small differences in chromatographic peaks could have been better resolved by altering the chromatographic gradient. It must be stressed that each peak did not represent a separate mycolic acid, improved separation can be achieved, but only at the expense of time. The resolution of mycolic acid peaks achieved in this study was sufficient to obtain a proper identification of the mycobacterium species of interest. A comprehensive mycolic acid profile reference library of 35 mycobacterium species generally isolated and identified in South Africa, cultured on a BacT/Alert culturing system, and analysed by HPLC, was compiled. These mycobacterium species were differentiated from each other by the optimisation of this HPLC/FL technique. Increased performance of the fluorescence detector was obtained by the optimisation of the emission and excitation wavelengths that contributed to the improvement in detection and limited interference due to background noise (Figure. 8). The increased response enabled us to measure concentrations of mycolic acid esters as low as 50.0 pg/L. Consequently, the smaller, normally undefined mycolic acid peaks are now reliably measured and more frequently used in the identification of mycolic acid profiles (Figure. 17). This enables us to generate vitally important results much faster and with high confidence. The increased sensitivity also enabled the direct profiling of sputum samples with a moderate bacilli count without culturing each specimen (Figure. 9).

### **7.2.3 CHROMATOGRAPHIC DATA HANDLING**

Identification of mycobacterium is done by comparing the chromatographic mycolic acid profiles to the reference library. Many authors have described a peak-naming system in the identification of mycolic acid profiles [5, 6, 13, 17, 37, 39, 56, 57]. In our

case, computerised standardization of retention times and graphic overlaying of chromatograms achieve matching of the mycolic acid profile of the different mycobacterium species. In this way, comparison of the unknown to the reference chromatograms provides species identification with a high degree of confidence. Close attention was given to mycolic acid profiles of the mycobacterium complexes e.g. *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* (Figure. 16). In using the overlaying program and two reference internal standards it is possible to identify more than one mycolic acid profile in a given sample. This overlaying technique enables us to carefully compare peak area ratios in the identification process. For instance, the mycolic acid profiles of *Mycobacterium abscessus* and *Mycobacterium chelonae* are very similar, requiring reliable measurement of small differences in peak ratios of the mycolic acids for their differentiation (Figure.10d). The careful standardisation of retention times, on the other hand, allows clear differentiation of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG, that show almost identical profiles with only a small difference in retention time.

### **7.3 ROUTINE DIAGNOSTIC SERVICE**

This HPLC analysis technique, combined with the culturing technique, enhances our ability to diagnose the patients with mycobacterium infections more precisely and with a high confidence level. Early identification of mycobacterium species allows the clinician to make the correct diagnosis and then treat the patient accordingly. By identifying the mycolic acid patterns with the HPLC/FL technique, we were able to differentiate between most of the known pathogenic and non-pathogenic MOTT infections.

HPLC/FL did offer advantages over conventional biochemical and genetic probe identification methods. The HPLC produced simple, reproducible, easily identifiable mycolic acid chromatographic profiles from mycobacteria for comparison to all the mycolic acid patterns of the reference mycobacterium species currently analysed in South Africa. An additional advantage of the HPLC method includes the ability to distinguish between strains of *Mycobacterium bovis* BCG and the other members of the *Mycobacterium tuberculosis* complex (Figure. 23). The HPLC/FL technique can also distinguish between very similar mycolic acid profiles of mycobacterium species. For instance, strains of *Mycobacterium avium* and *Mycobacterium intracellulare* (Figure. 17) can be identified without resorting to other more complex expensive methodologies, e.g., DNA probes, biological tests and 16S r-RNA sequencing. Despite the small differences in the mycolic acid profiles of the *Mycobacterium tuberculosis* complex and *Mycobacterium avium intracellulare* complex, it was possible to clearly distinguish between these two species

#### **7.4 MULTIPLE INFECTIONS**

This modified HPLC/FL technique enabled us to generate results that have not yet been reported in literature. For instance, the identification of multi-mycobacterium isolates could be easily be performed with the HPLC technique. This is important as co-infections are difficult to detect but not uncommon in South Africa. *Mycobacterium tuberculosis* and *Mycobacterium avium* are the most frequent multiple infections observed. They are very difficult to identify due to the different growth rates of these bacilli (Figure. 16). It is very important to confirm that a *Mycobacterium avium* infection does not have an underlying *Mycobacterium tuberculosis* infection.

A very rare multiple mycobacterium infection was the combination of *Mycobacterium avium* and *Mycobacterium abscessus*. Chromatographically these isolates are very easily identified with the HPLC/FL technique (Figure. 15a).

Another very rare multiple mycobacterium infection was diagnosed by sub-culturing a patient specimen where the chromatogram presented an unknown peak at 4.2 minutes. Conformation of sub-cultures on solid medium revealed that the patient was infected with *Mycobacterium szulgai* and *Mycobacterium avium* (Figure.14a). From this experiment we realised that it is very important to evaluate each unknown profile before dismissing the result as a possible unidentified mycobacterium. It is entirely likely that mixed infections are far more common than has been indicated, simply because the relative proportion the minor isolate is too low for detection using this technology.

Multiple mycobacterium infections are commonly associated with immune compromised diseases where *Mycobacterium avium* is the most frequent infection described and associated with HIV infections [50]. This, as yet, is an unconfirmed observation made from accumulated data collected over the past four years in South Africa.

## 7.5 STATISTICS

In the statistical overview of the results from South Africa the past five years, a definite increase in *Mycobacterium tuberculosis* complex and MOTT infections could be observed. This increase is probably due to the high rate of opportunistic infections in South Africa. The analysis of 21000 suspected *Mycobacterium* infected patients in 2003 showed an average infection rate of 20% for mycobacterium diseases. The 20% consists of 80% *Mycobacterium tuberculosis* complex and 20%

Mycobacterium other than tuberculosis. Currently the most common MOTT infection found in South Africa is the *Mycobacterium avium intercellulare*. Almost 50% of all the MOTT infections are due to *Mycobacterium avium intercellulare* (Graph. 1). Mycobacterium management programmes based mainly on case detection by identifying smear positive patients via microscopy alone is dangerous. Treatment of the disease with standardized TB treatment regimen of antibiotics is not justifiable in situations where MOTT infections are as high as 22% of the total mycobacterium infection (Graph 2.).

## 7.6 FUTURE OUTLOOK

Many areas remain to be explored for our better understanding of the structure and function of the different mycolic acids present within the mycobacterium cell wall. Our knowledge of the lipids of the outer leaflet of the bi-layer structure of the cell wall is still inadequate. Defining the precise arrangement and function of these lipids remains a major challenge.

In understanding the mechanism and assembly of this extremely complex lipid structure, which appears to have a rather rigid structure and interior, our knowledge of the function of some of these lipids might be able to assist in developing more effective antibiotics. We are only now beginning to understand the possible function and the adaptability of these lipid species when placed under certain stress. Mycobacterium cells synthesize a variety lipids species (mycolic acids) of unusual structure and variations under certain conditions. In an ideal environment, each mycobacterium species performs such an important function in their interaction with the nonliving and living environment. By changing the ideal environment where that specie lives, we are probably also affecting the consistency of the mycolic acid

profiles within the cell wall, e.g. increasing the incubation temperature up to 50 °C, results in an increase in long-chain molecular species of the mycolic acid by elongation of the  $\alpha$ -alkyl unit of the mycolic acid of the rapid growing species *Mycobacterium phlei* [58]. The successful identification of MOTT has become more important over the years. Chromatographic techniques will play a very significant role in solving the complex structure of mycolic acids. Some of these problems may be solved by the application of existing techniques, but others await the development of new methods, particularly the ability to correctly identify individual lipids and carbohydrates. The growing global burden of tuberculosis creates an urgent need to define new classes of therapeutics effective against multiple drug resistant (MDR) strains and with improved activity. The mystery behind the physiology of mycobacteria and the mechanisms associated with drug sensitivity has been the single greatest hurdle in the development of effective therapeutic drugs. It is only recently that the complete genome sequence for *Mycobacterium tuberculosis* H37Rv and other mycobacterial strains have become available. Genomic analysis alone provides only a limited view of the dynamics associated with cellular responses to a particular stress. Proteomic analysis, however, provides the possible tool for measuring changes undergone in peptides and proteins when bacteria are exposed to a certain drug. This and other analytical techniques may assist the research scientist in understanding the pathophysiology of mycobacteria as well as pathways of possible rational drug discovery.

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## Appendix: A. Mycobacterium reference library

Figure. 1. *Mycobacterium abscessus* NCTC 10269

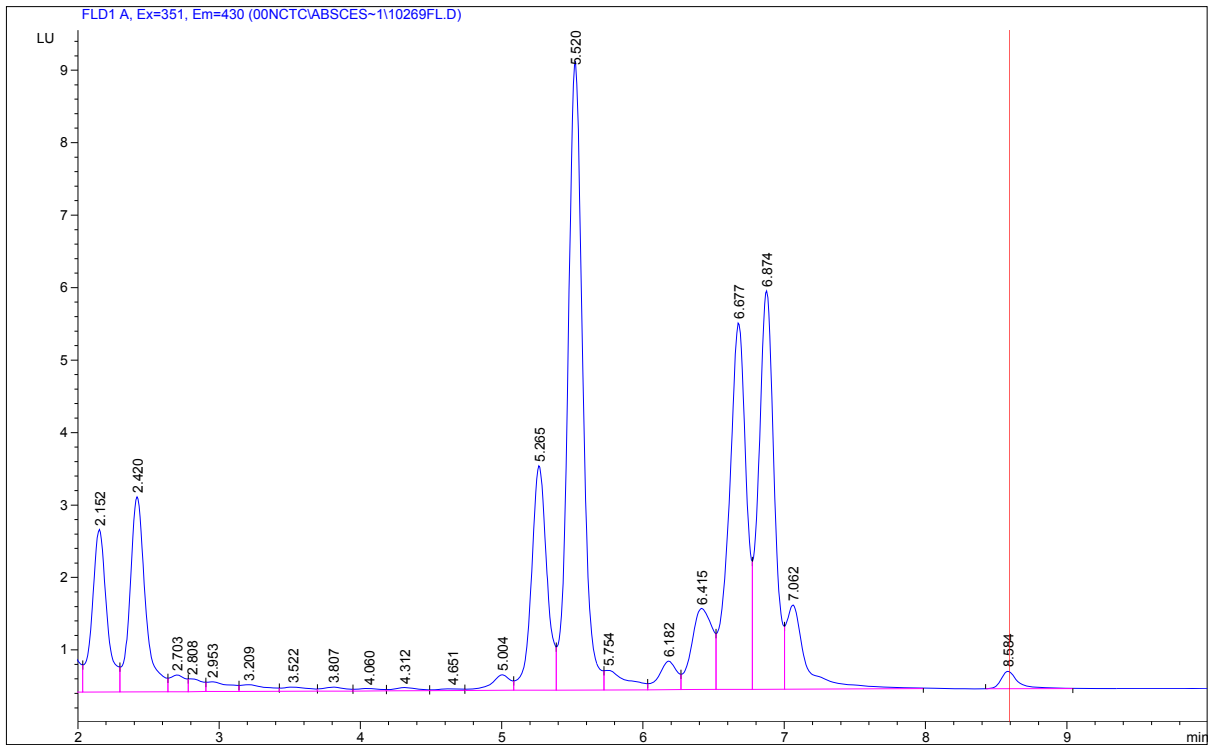
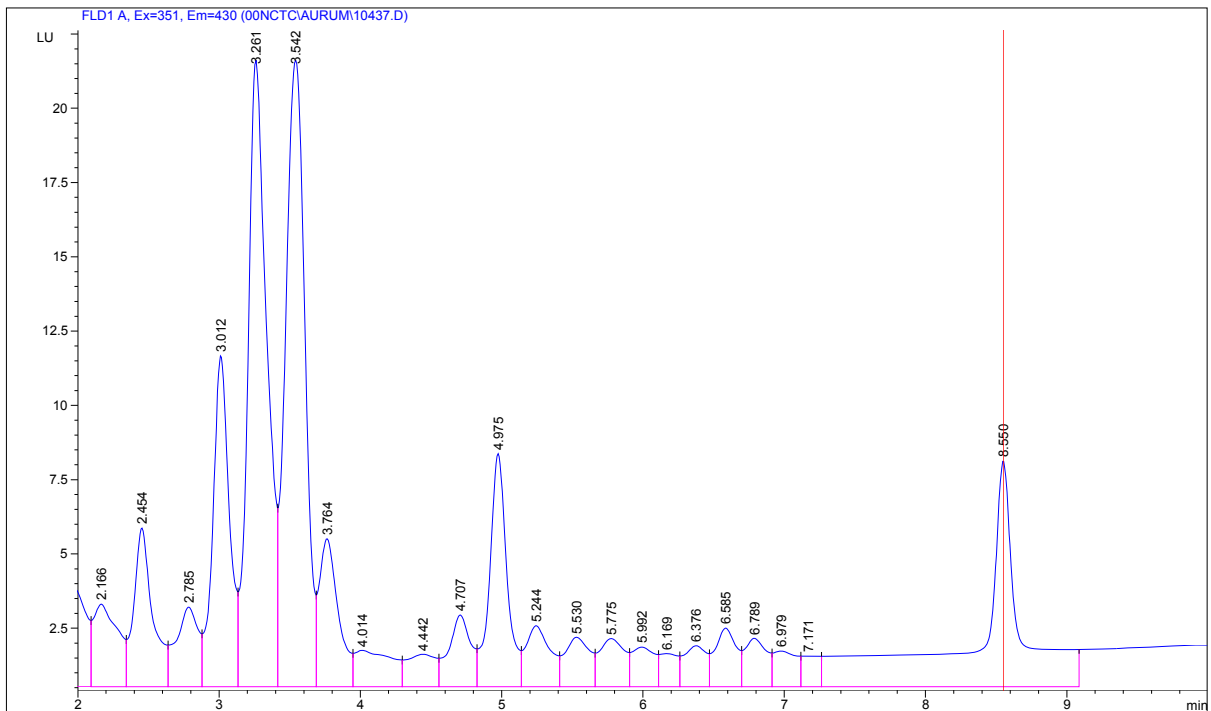
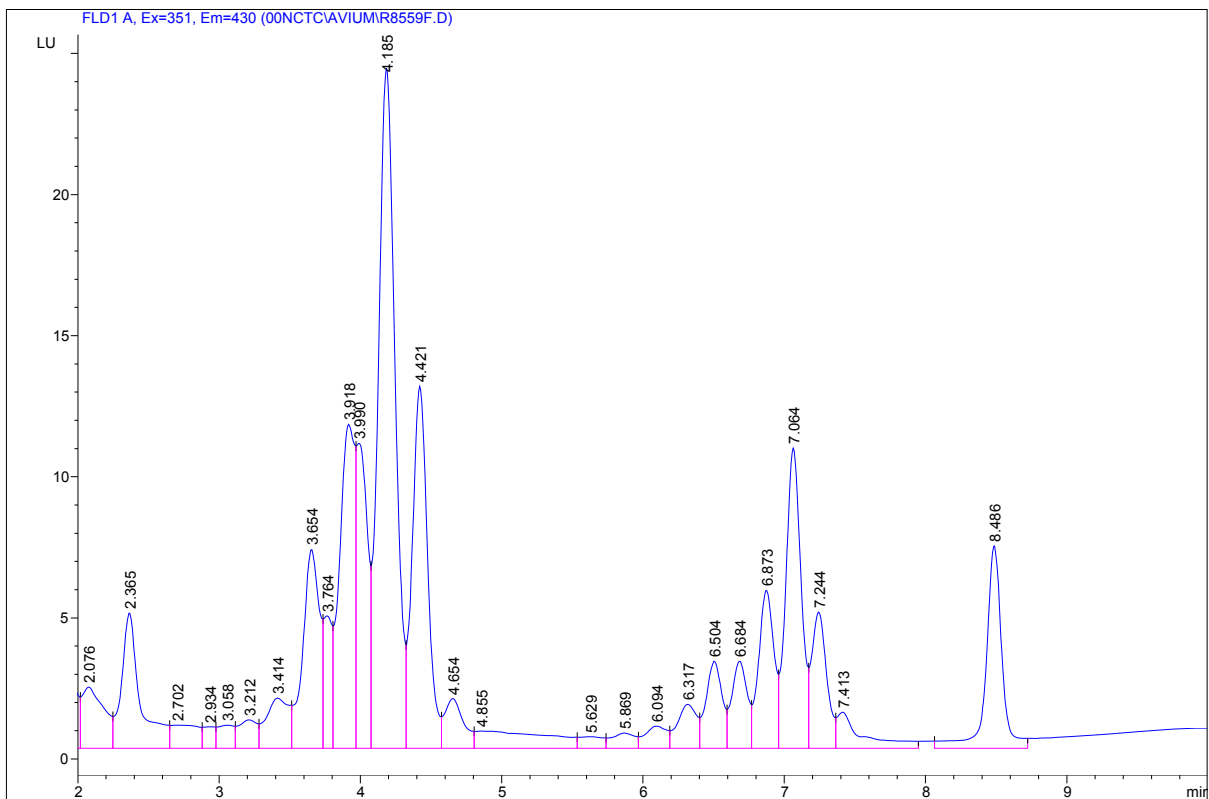


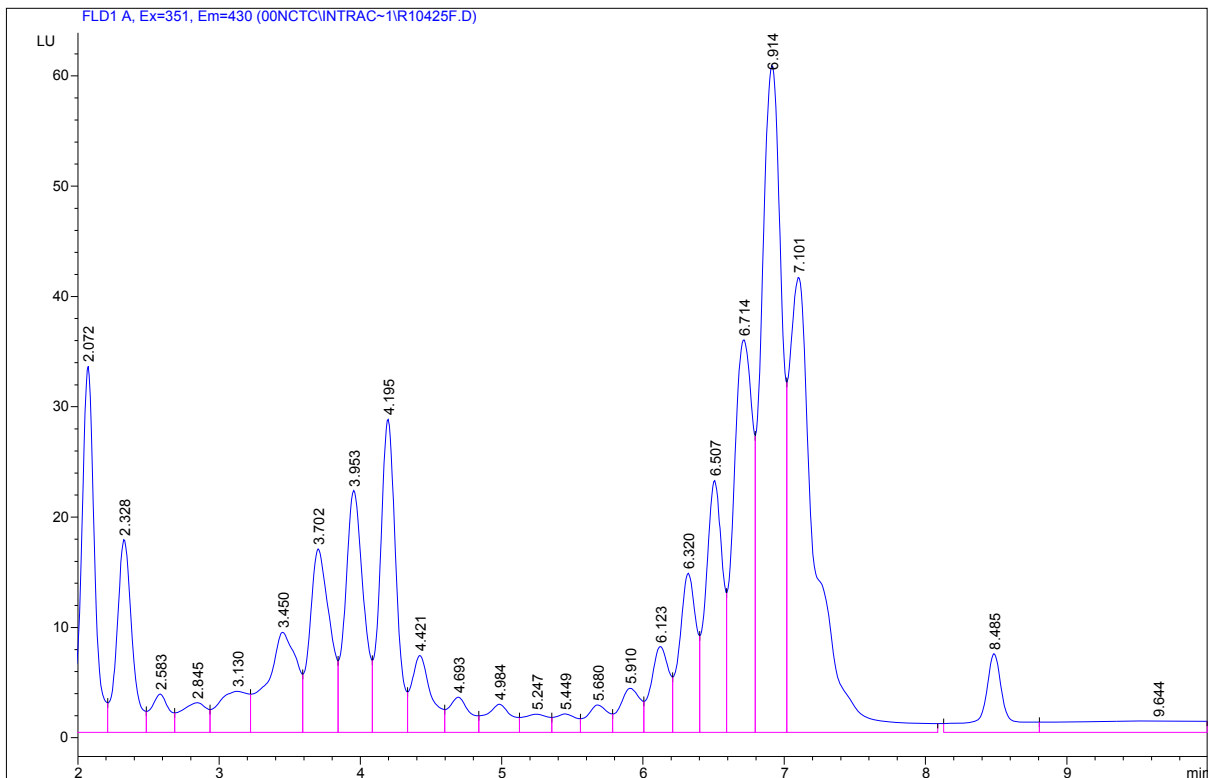
Figure. 2. *Mycobacterium aurum* NCTC 10437



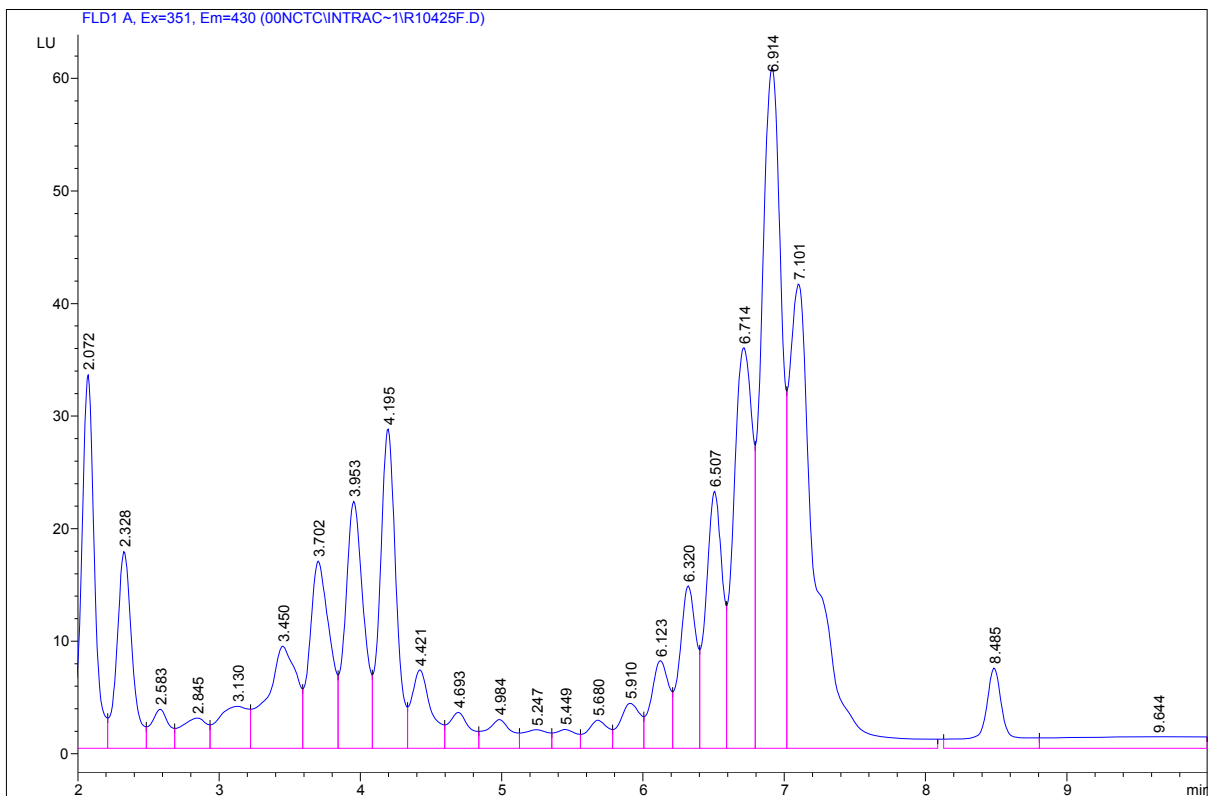
**Figure. 3.** *Mycobacterium avium* NCTC 8559



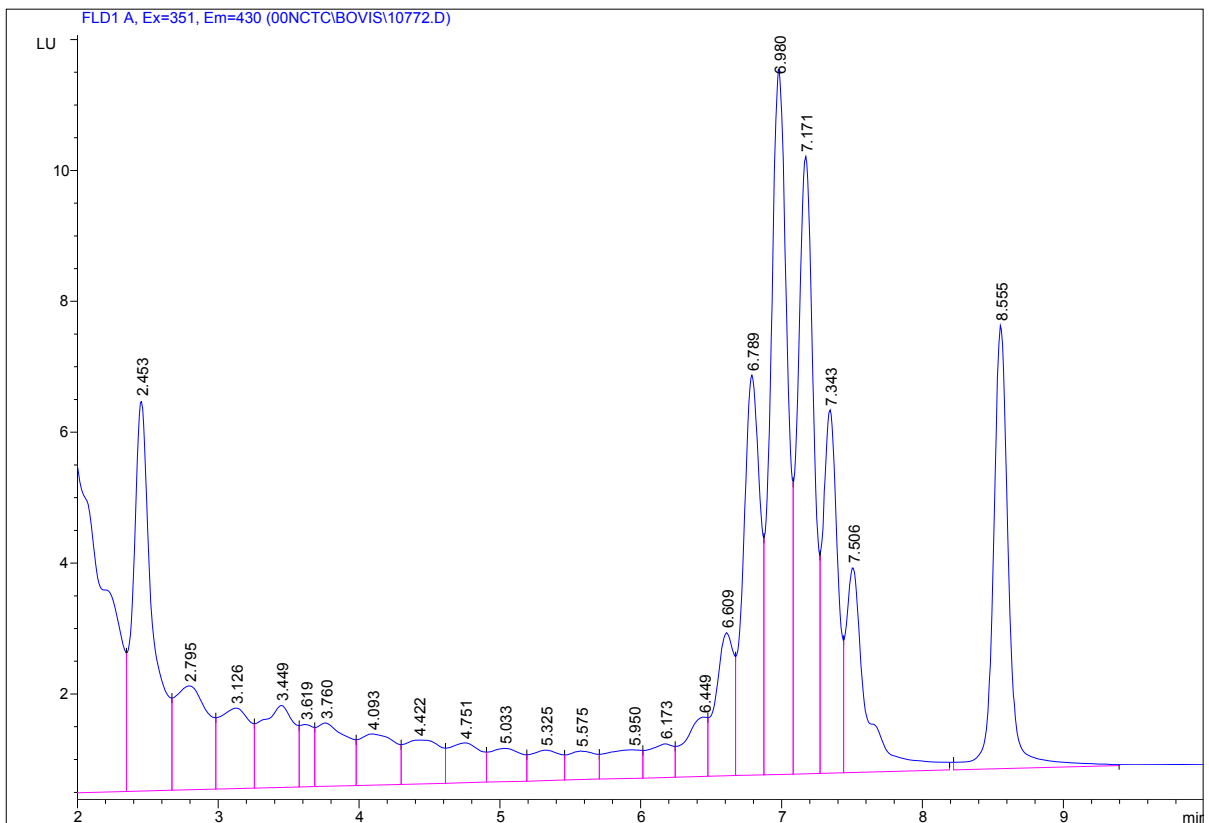
**Figure. 4.** *Mycobacterium avium intracellulare* complex NCTC 10425



**Figure. 5.** *Mycobacterium avium* complex NCTC 104267

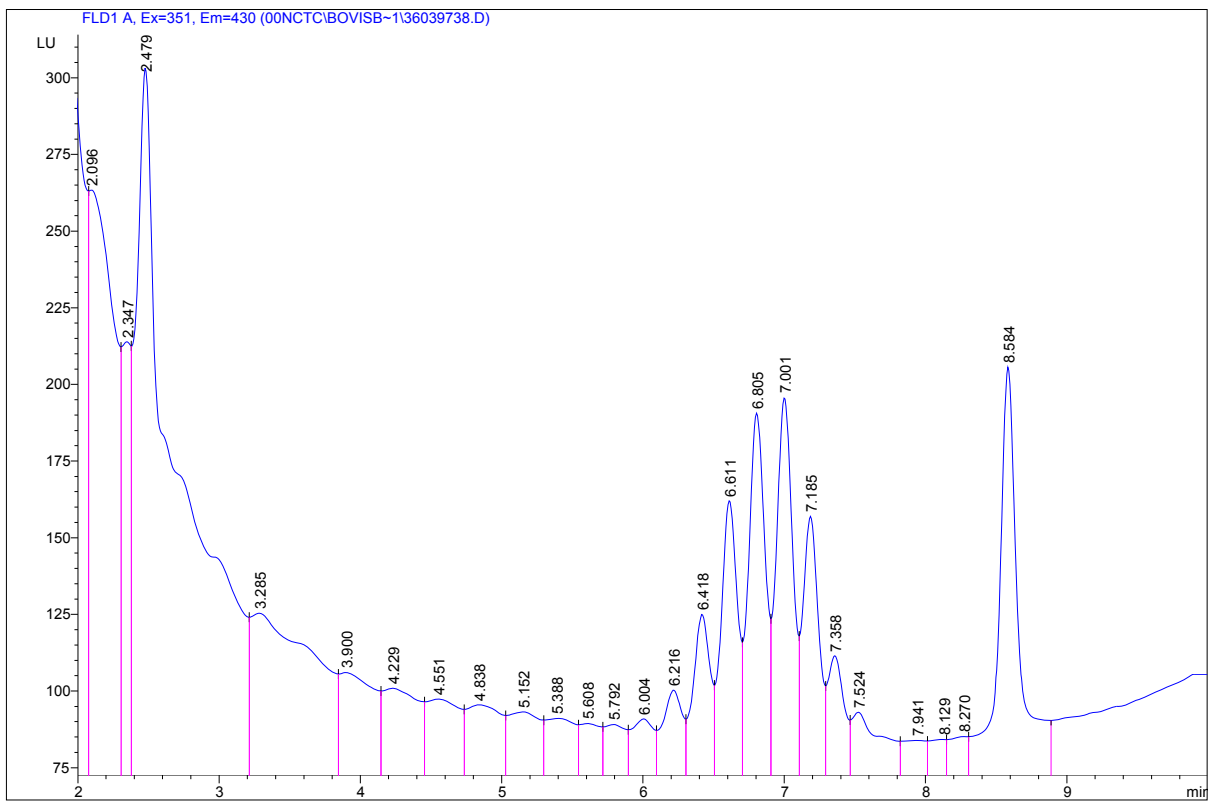


**Figure. 6.** *Mycobacterium bovis* NCTC 10772

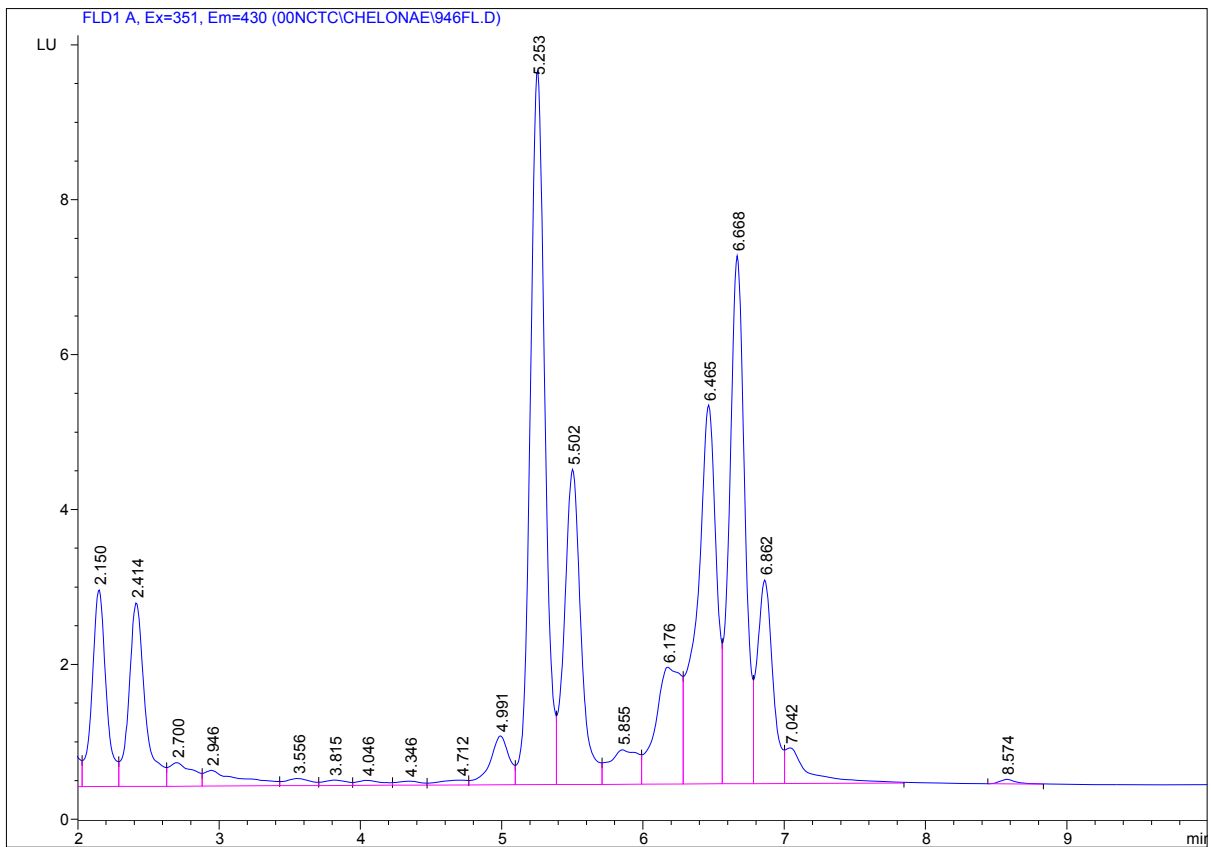




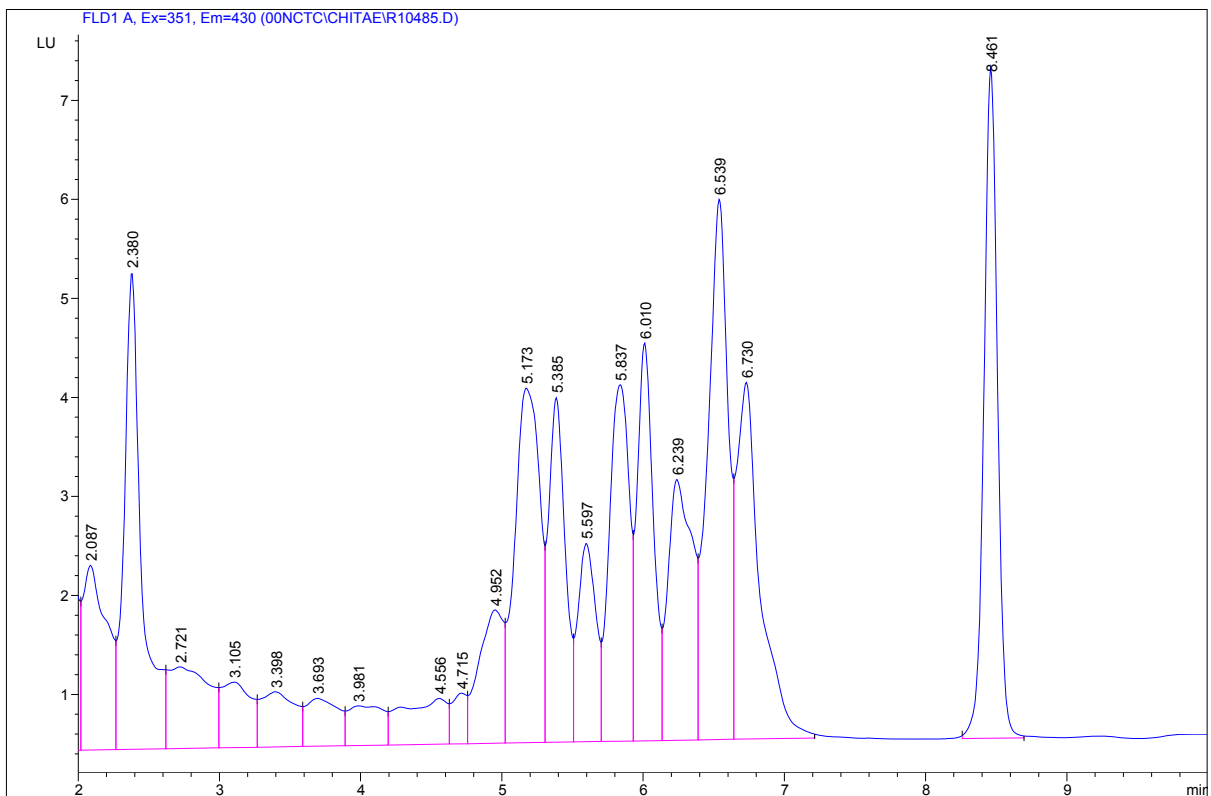
**Figure. 7.** *Mycobacterium bovis* BCG NCTC 5692



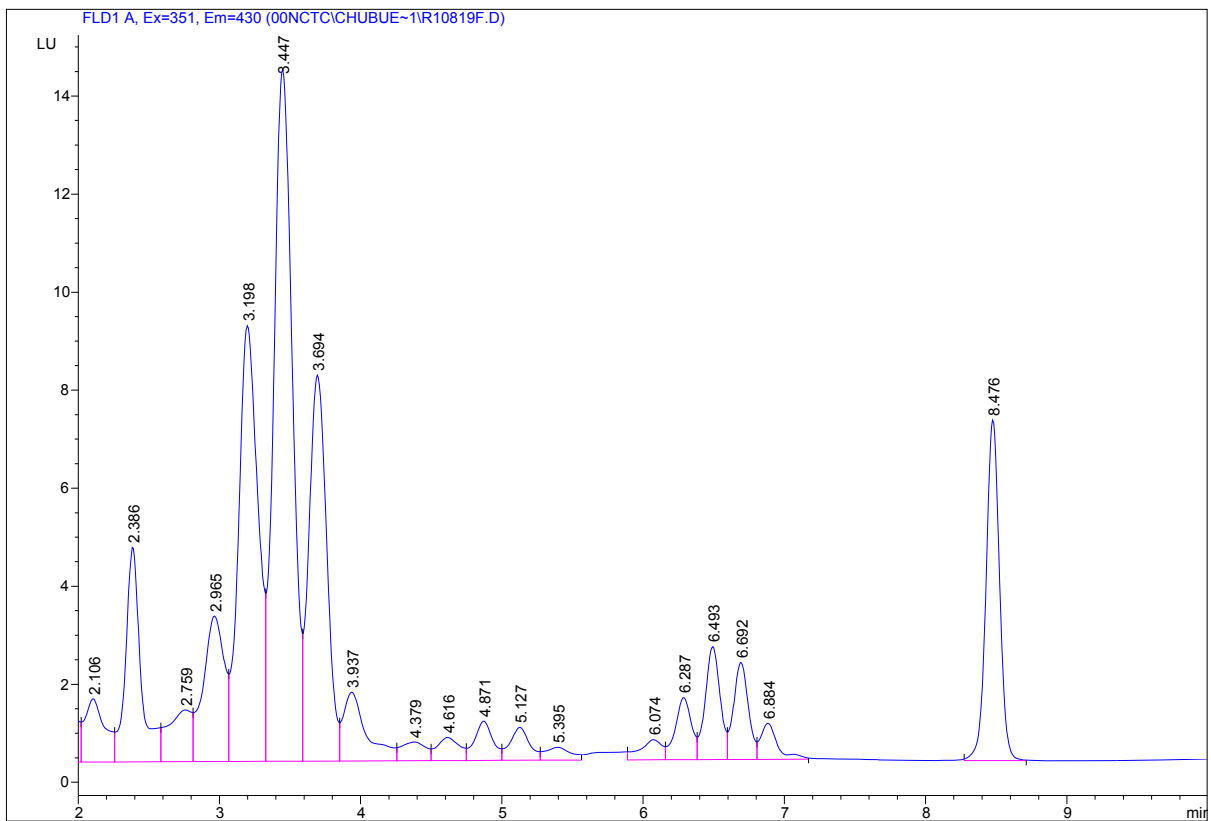
**Figure. 8.** *Mycobacterium chelonae* NCTC 946



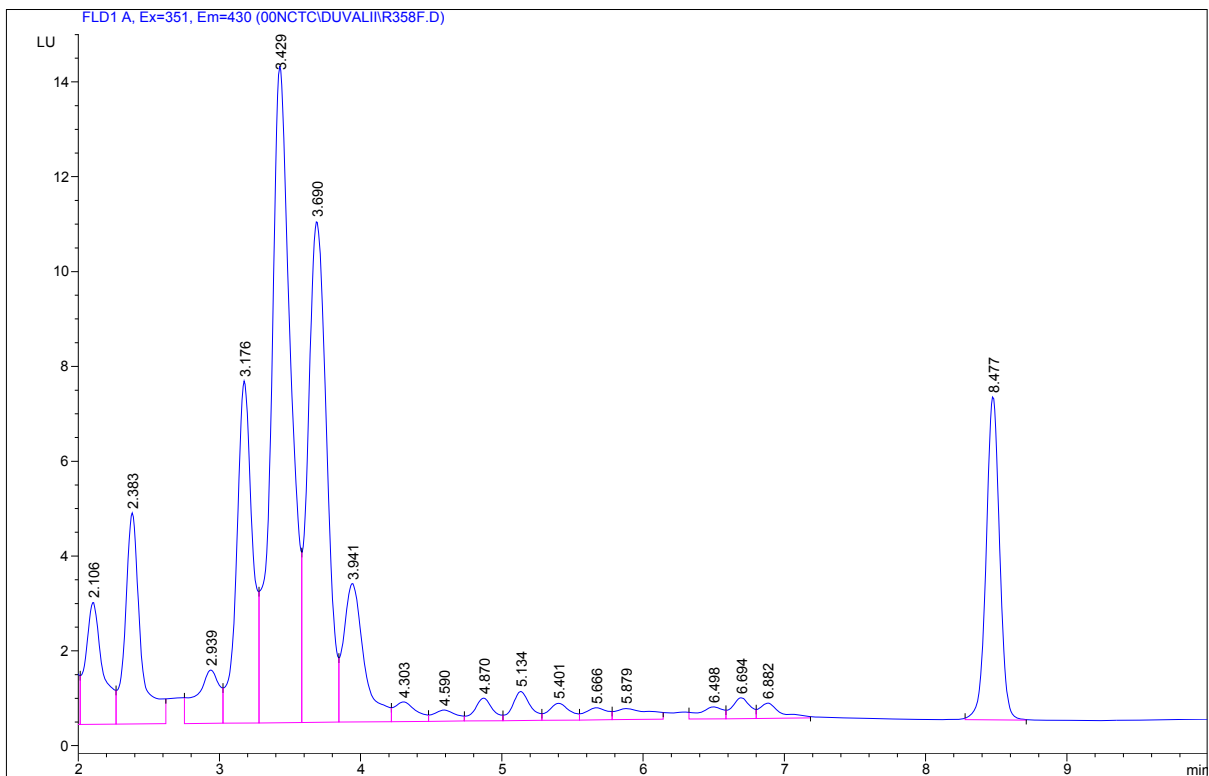
**Figure. 9.** *Mycobacterium chitae* NCTC 10485



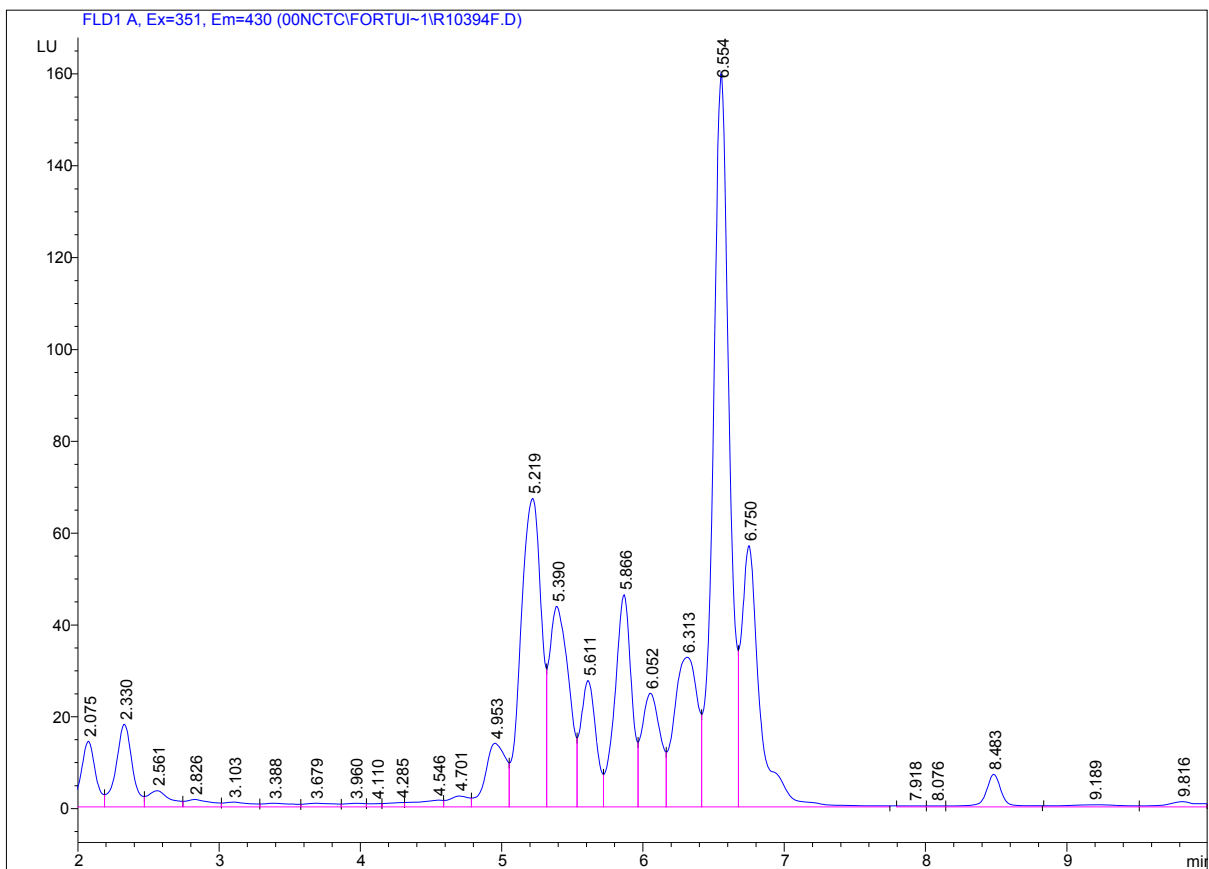
**Figure. 10.** *Mycobacterium chubuense* NCTC 10819



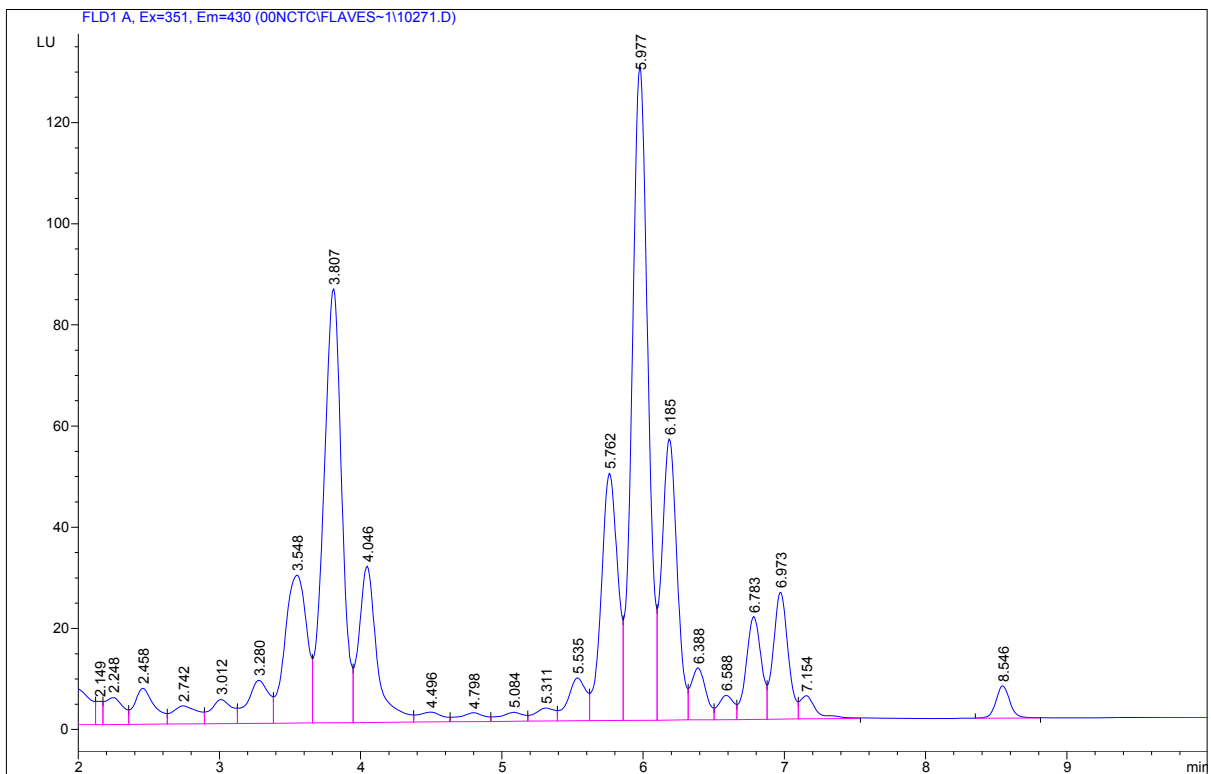
**Figure. 11.** *Mycobacterium duvalii* NCTC 10959



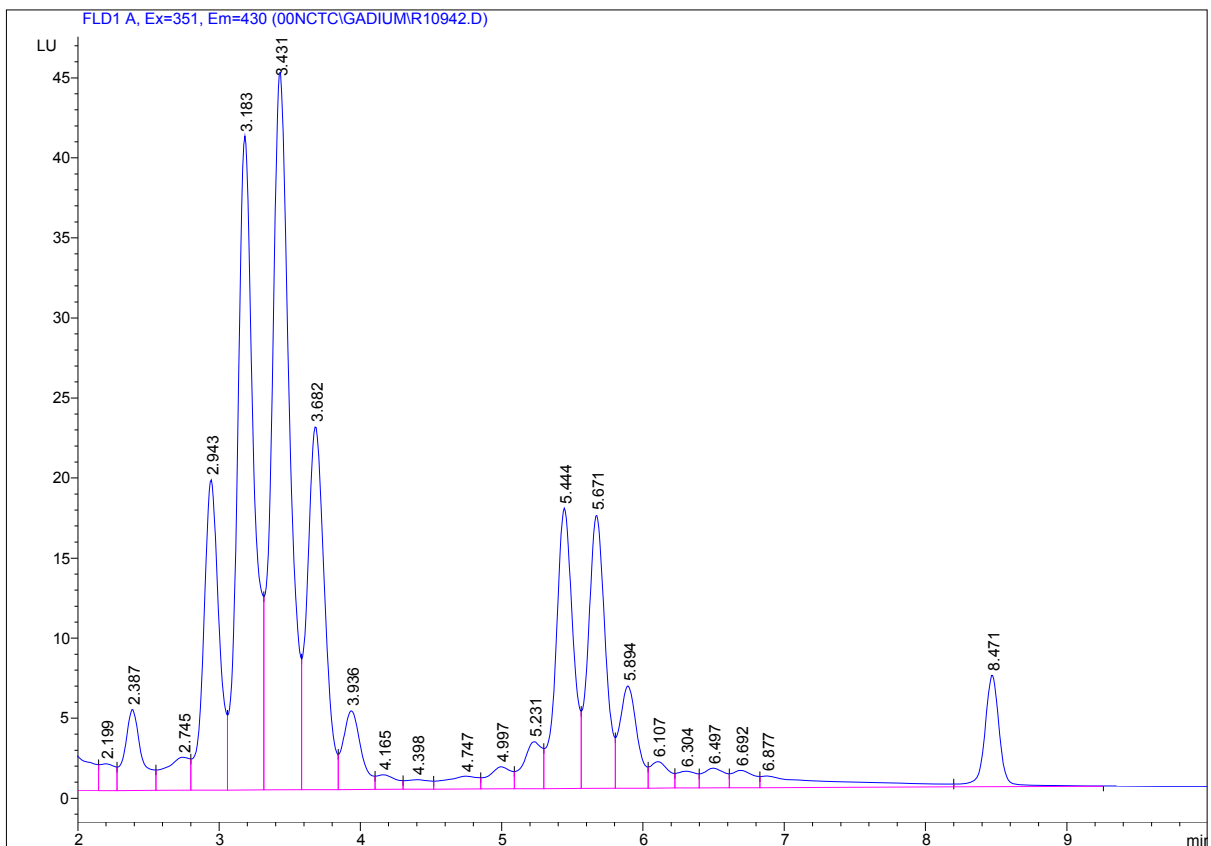
**Figure. 12.** *Mycobacterium fortuitum* NCTC 10394



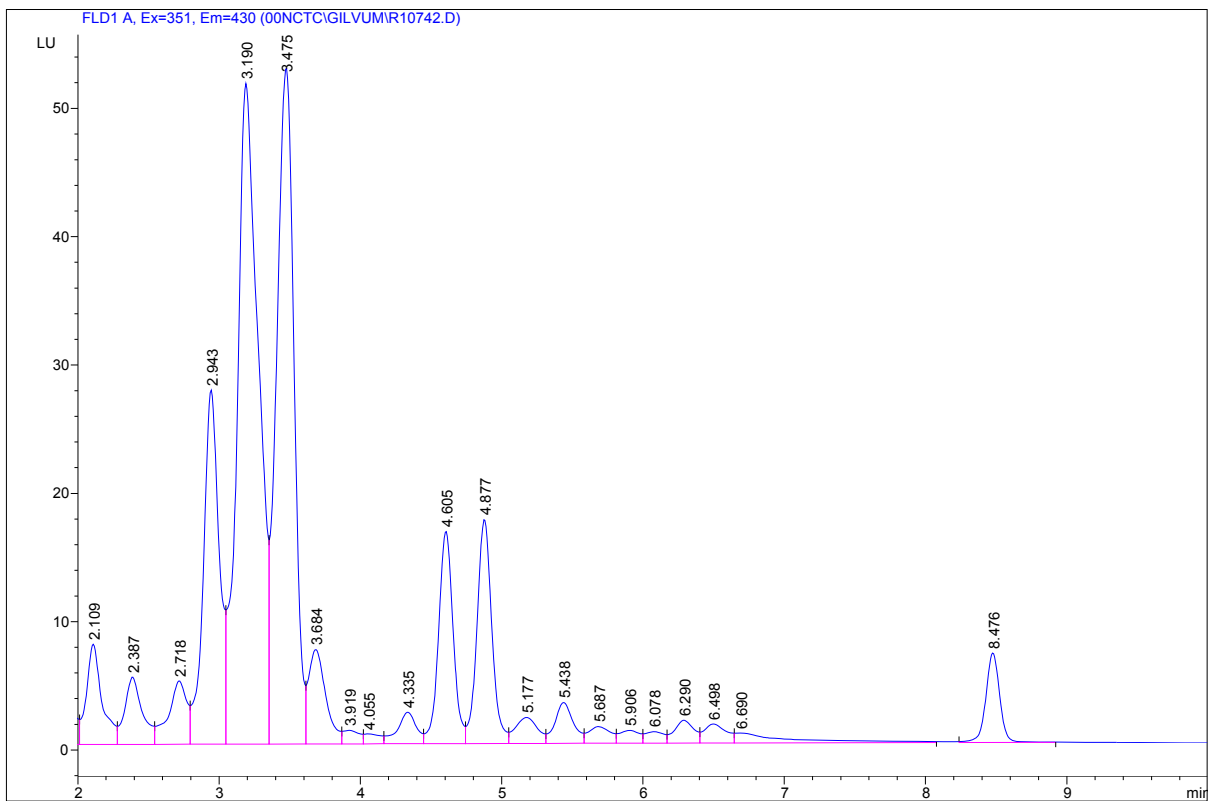
**Figure. 13.** *Mycobacterium flavescens* NCTC 10271



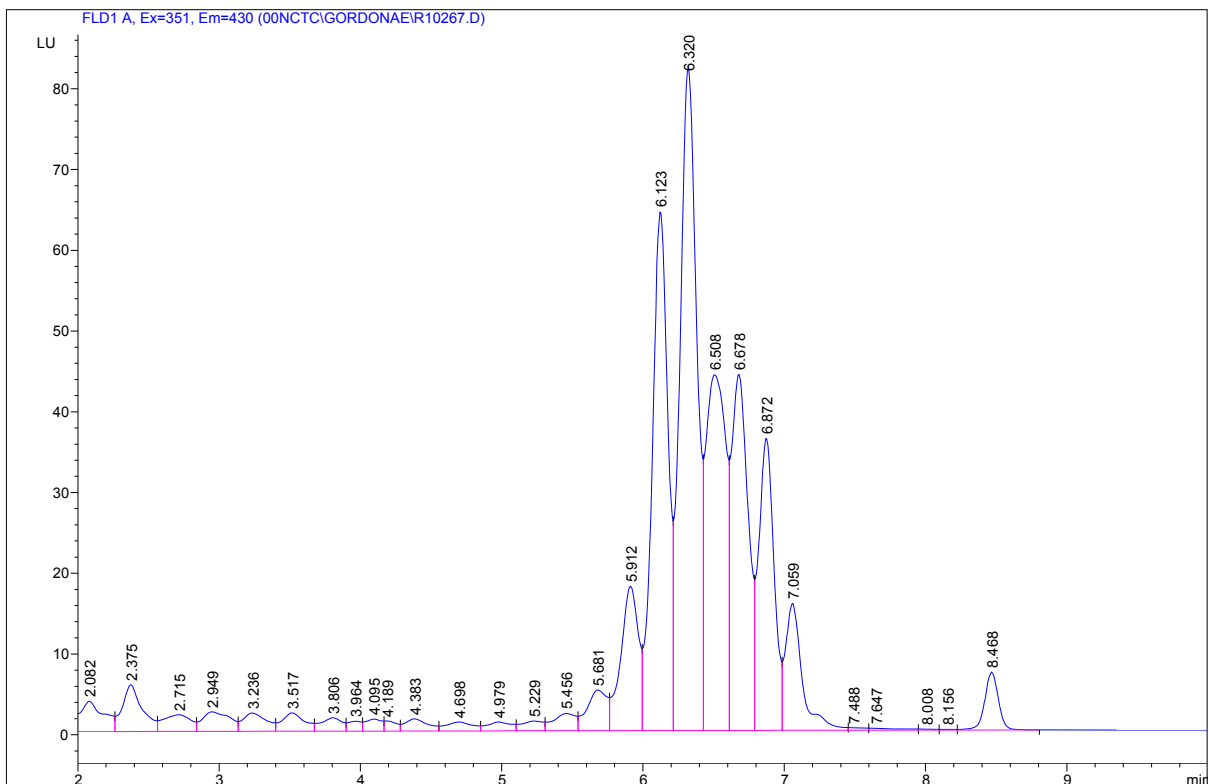
**Figure. 14.** *Mycobacterium gadium* NCTC 10942



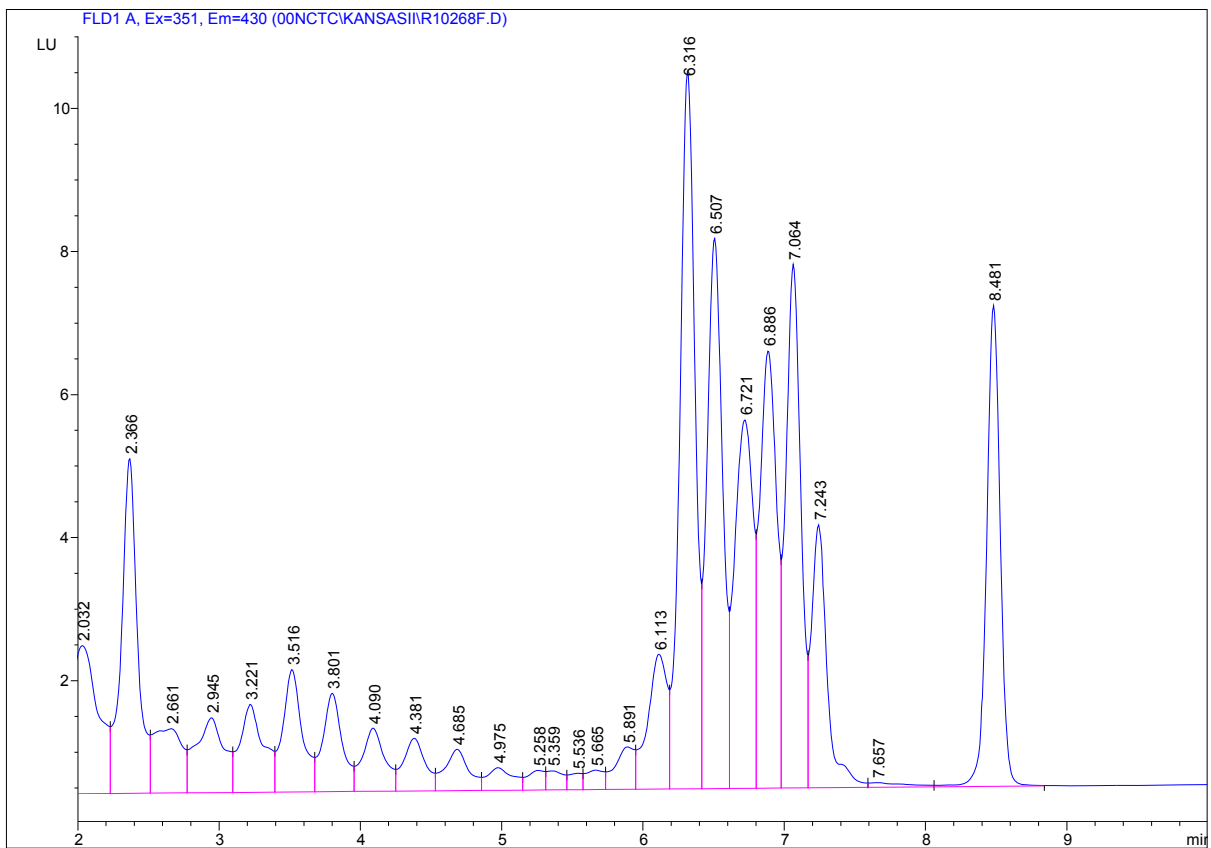
**Figure. 15.** *Mycobacterium gilvum* NCTC 10742



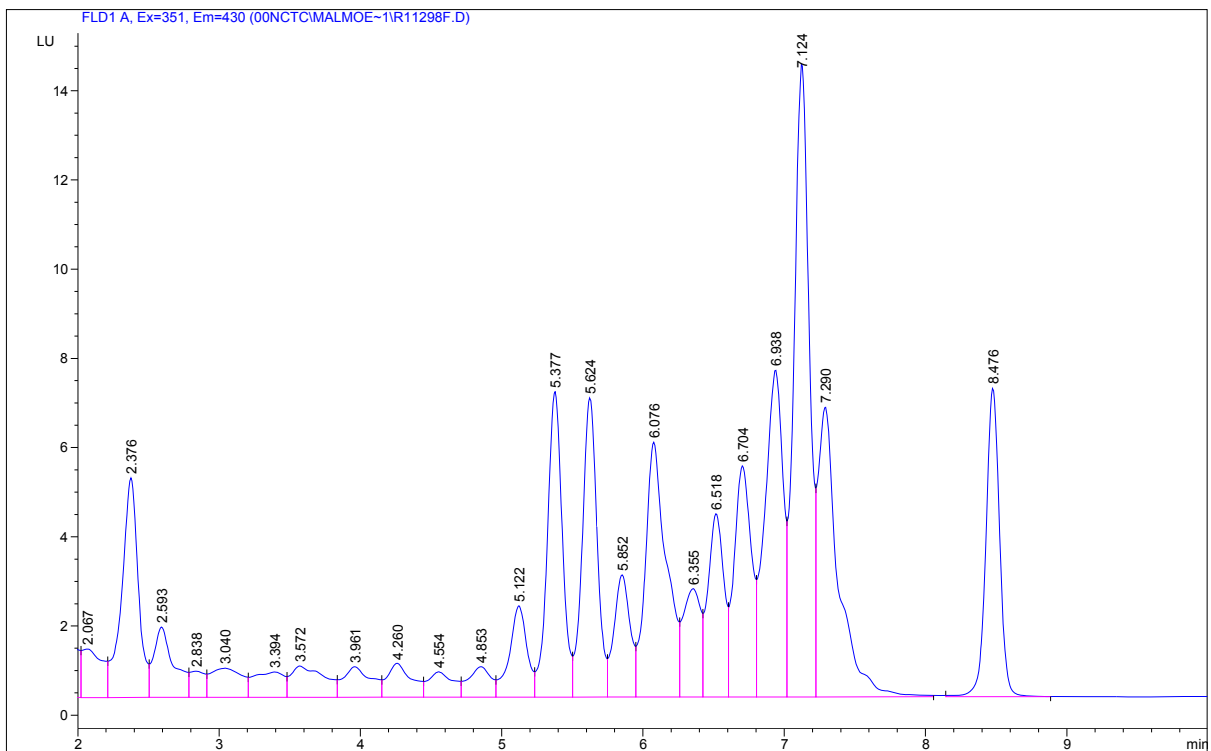
**Figure. 16.** *Mycobacterium gordonae* NCTC 10267



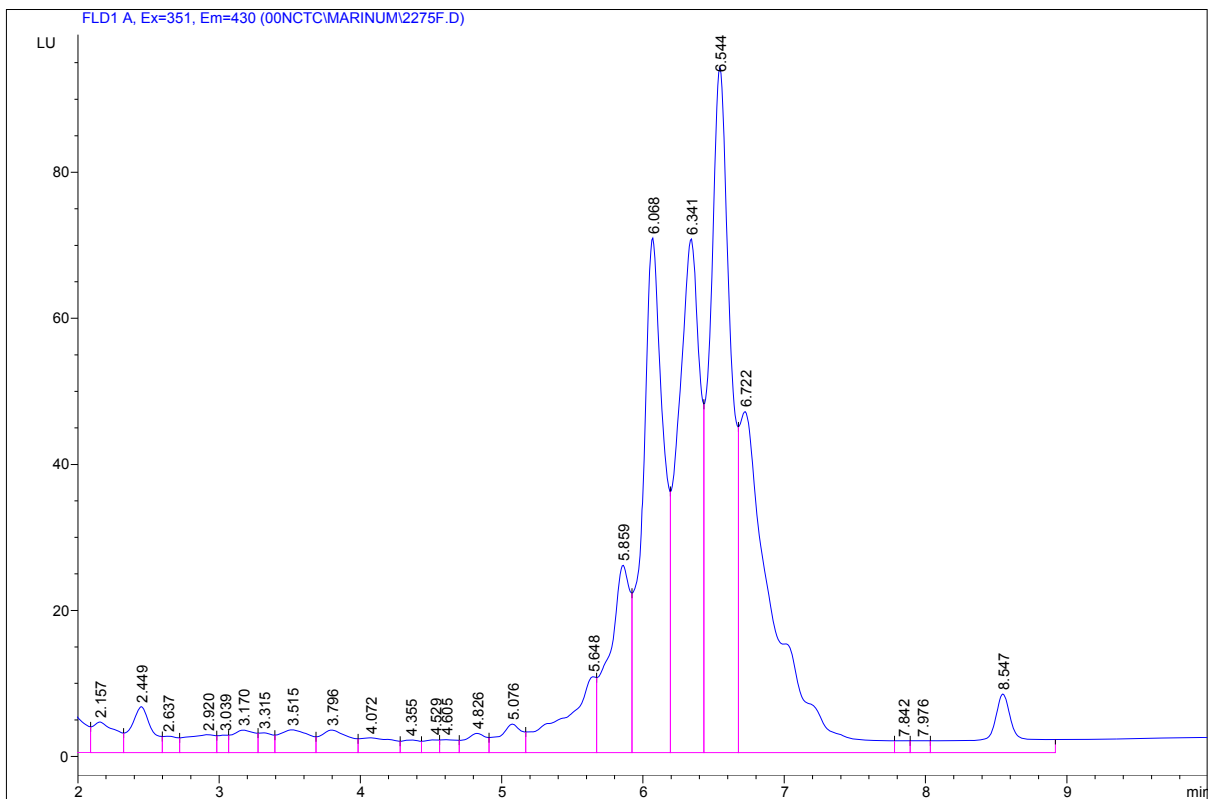
**Figure. 17.** *Mycobacterium kansasii* NCTC 10268



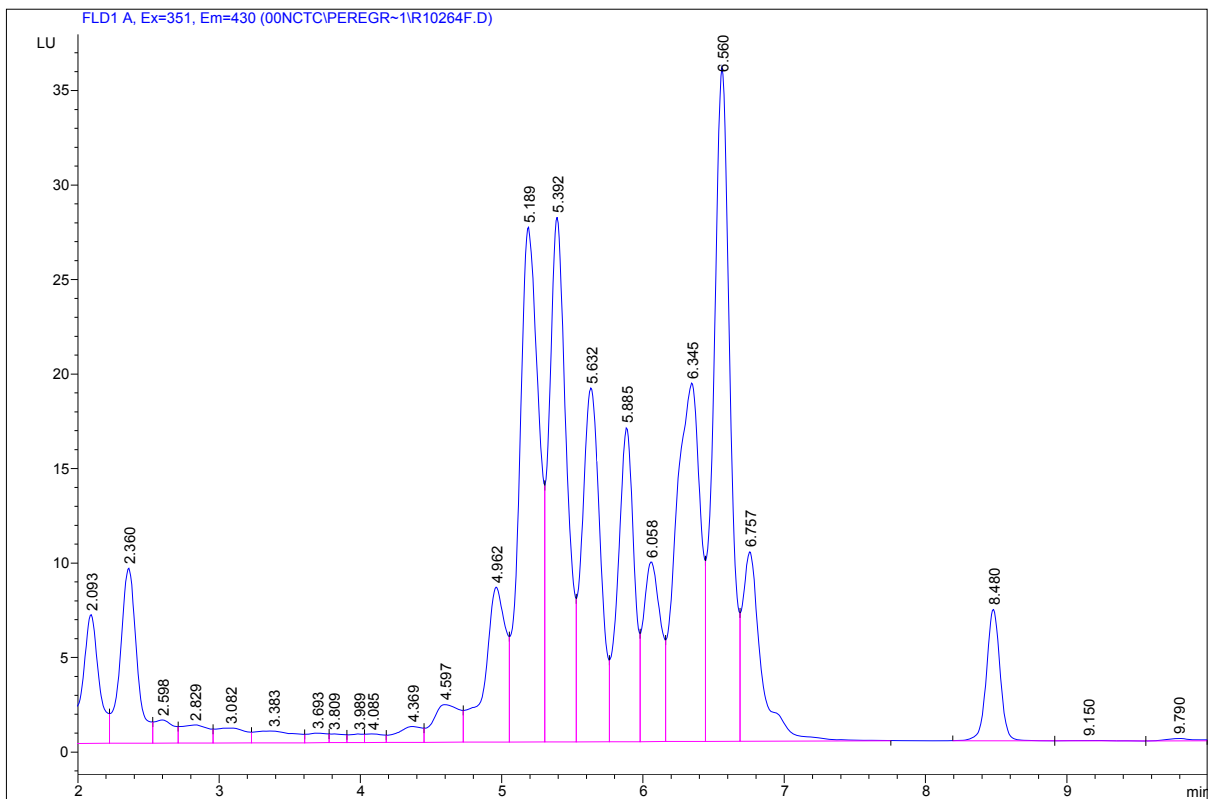
**Figure. 18.** *Mycobacterium malmoeense* NCTC 11298



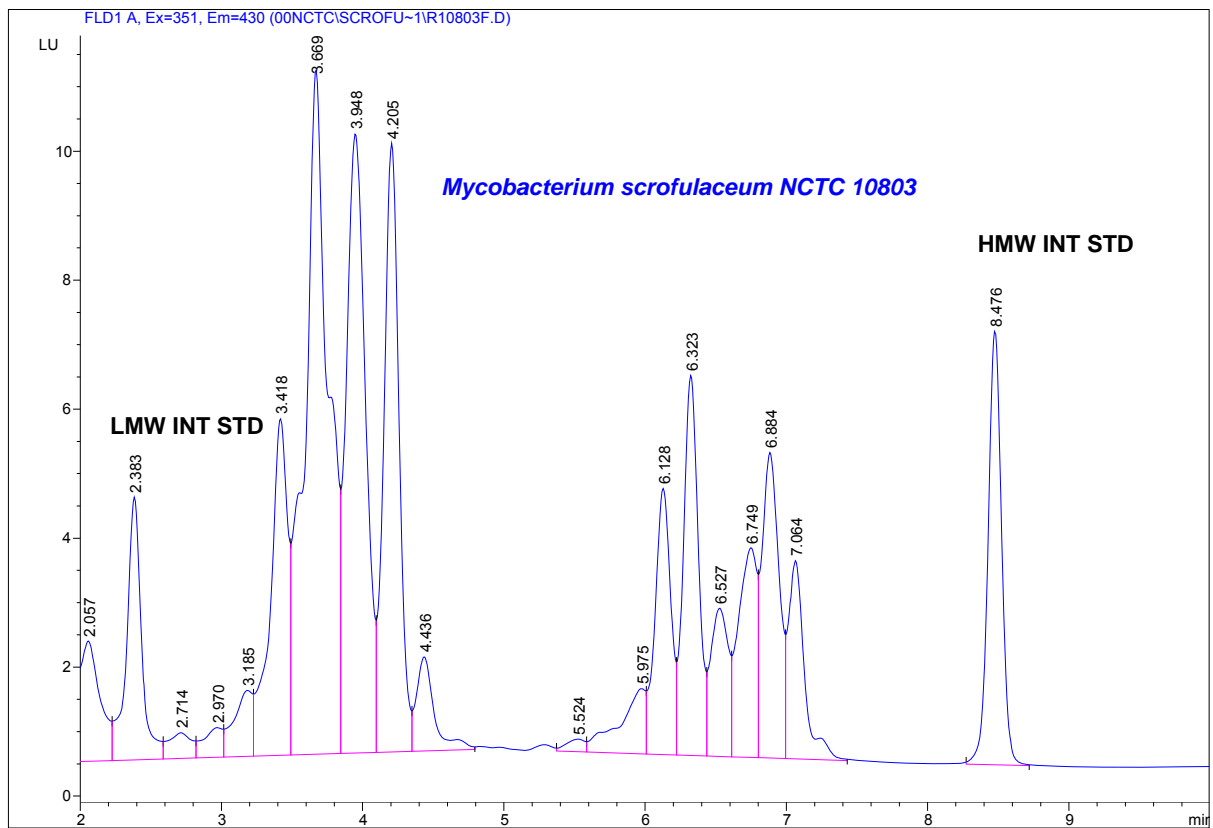
**Figure. 19.** *Mycobacterium marinum* NCTC 2275



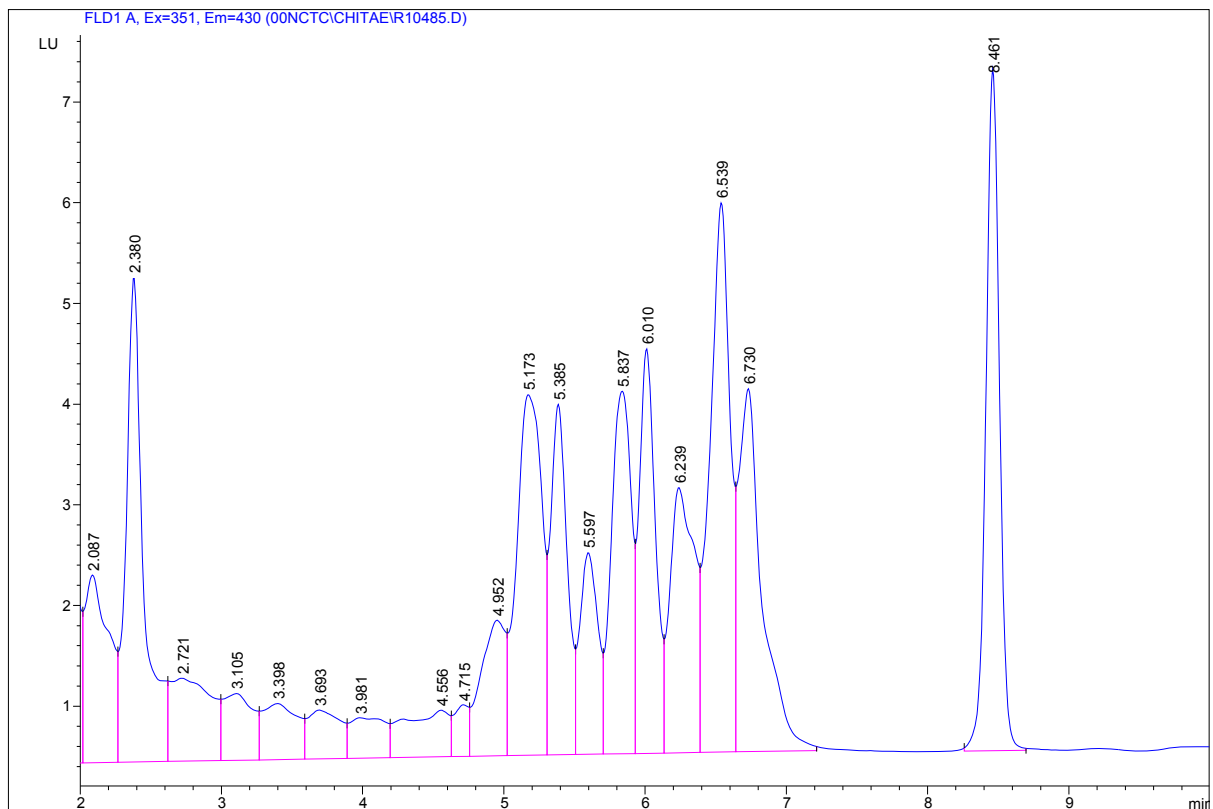
**Figure. 20.** *Mycobacterium perigrinum* NCTC 10264



**Figure. 21.** *Mycobacterium scrofulaceum* NCTC 10803

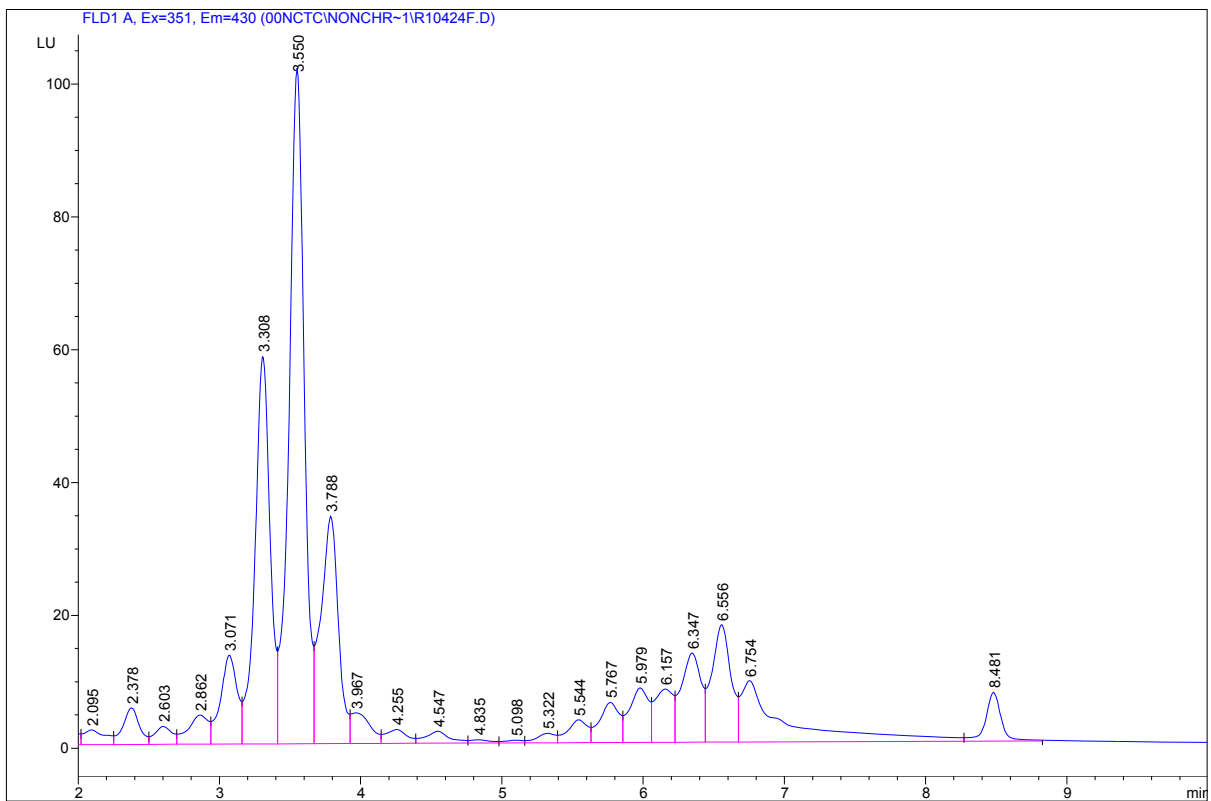


**Figure. 22.** *Mycobacterium neoaurum* NCTC 10818





**Figure. 23.** *Mycobacterium nonchromogenicum* NCTC 10242



**Figure. 24.** *Mycobacterium novum* NCTC 10486

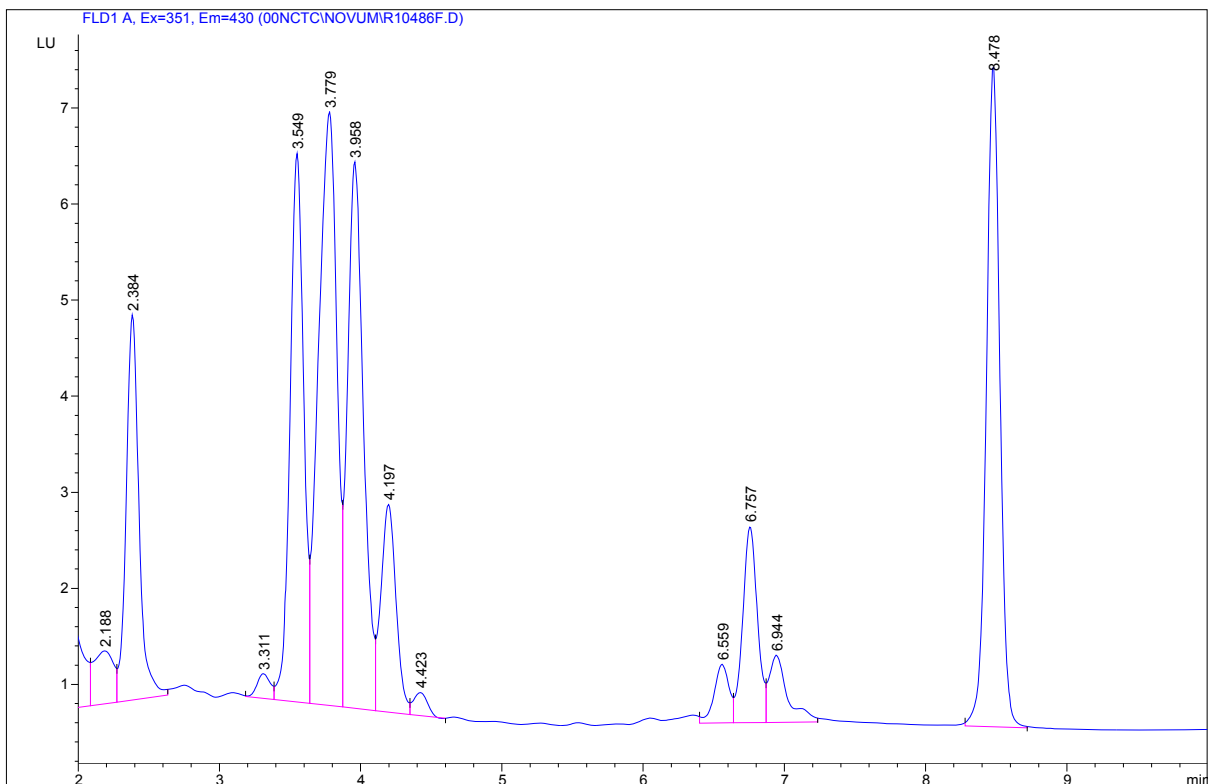


Figure. 25. *Mycobacterium parafortuitum* NCTC 10411

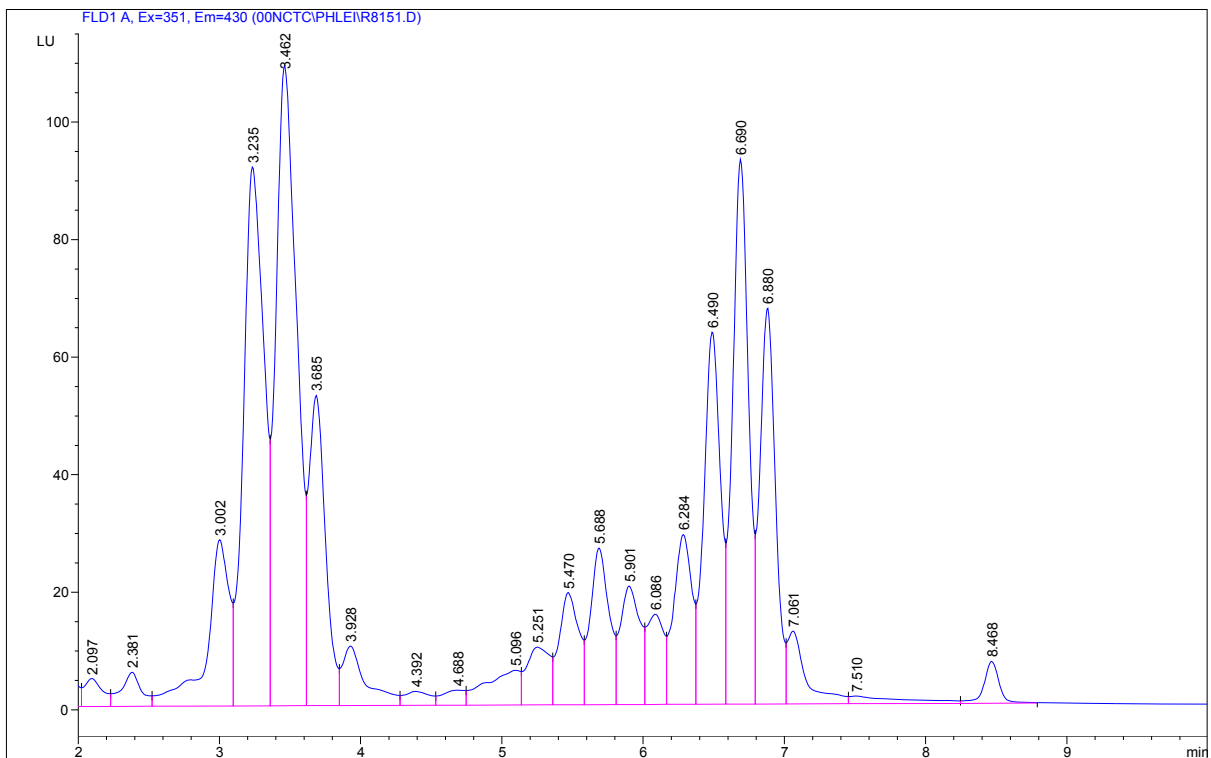
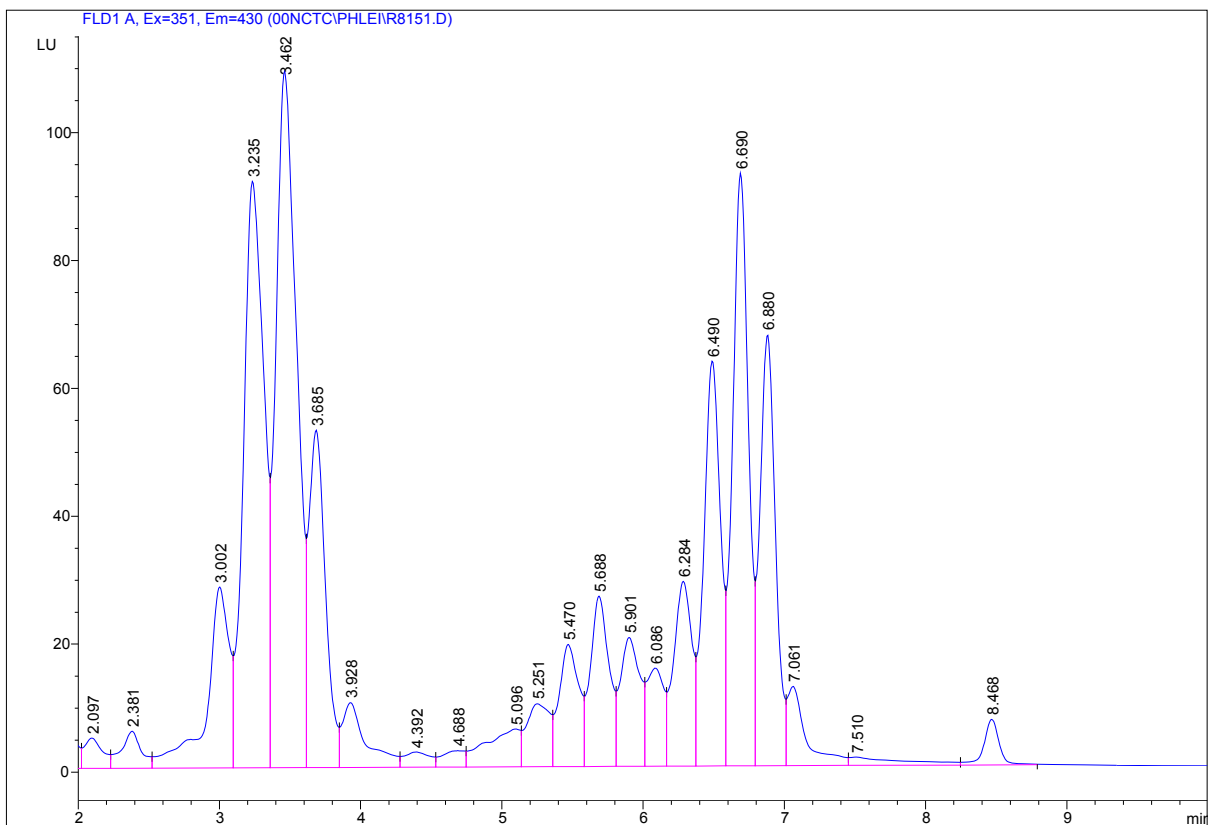
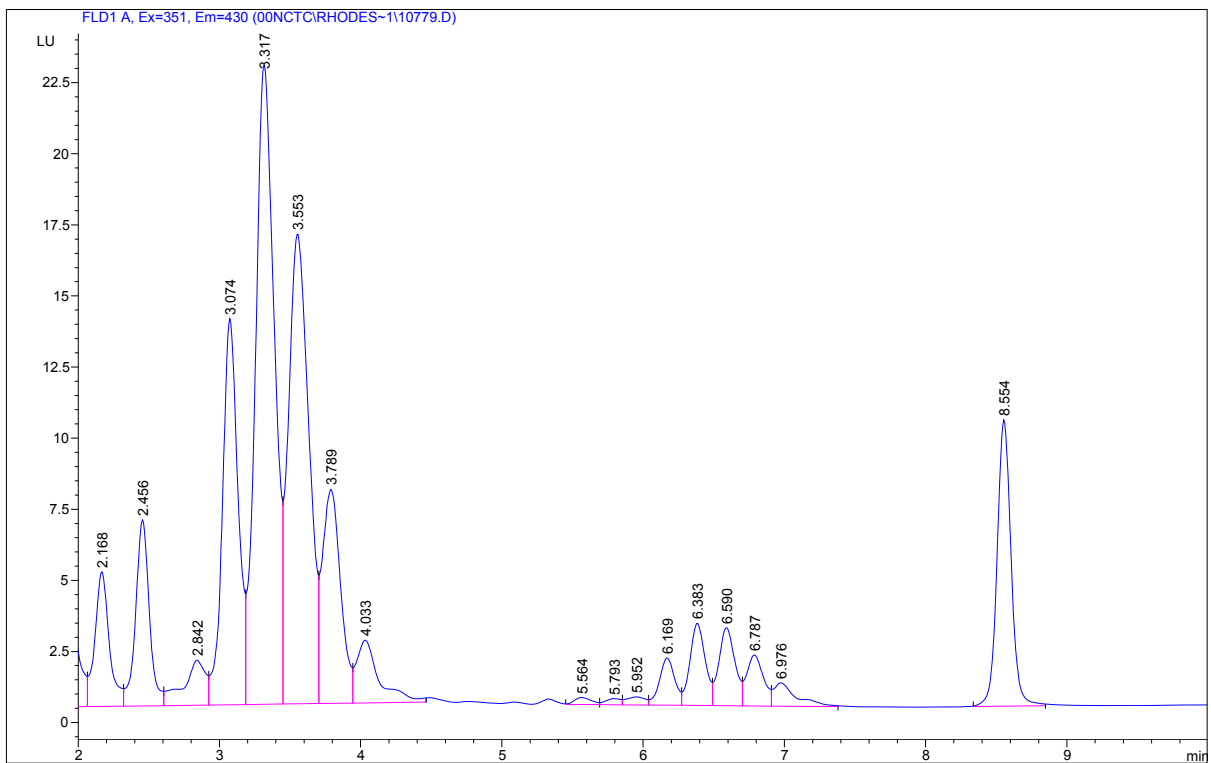


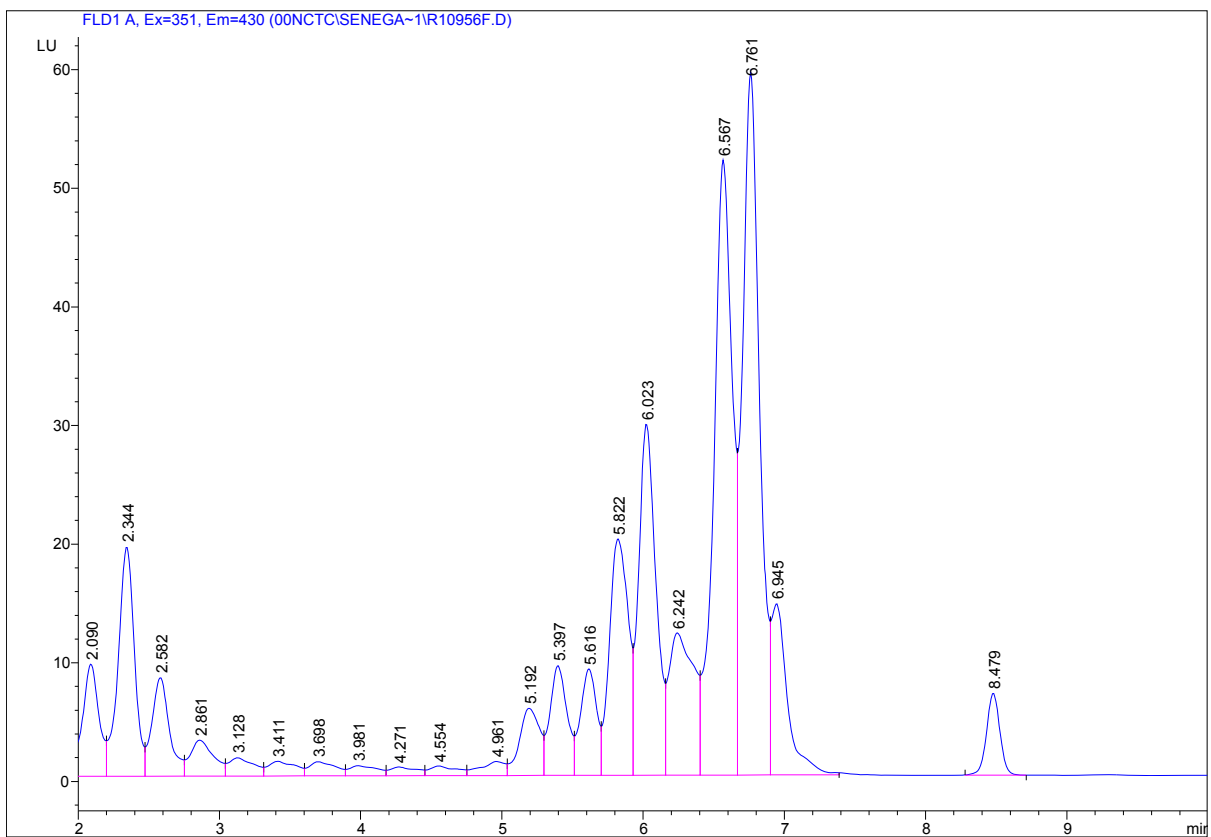
Figure. 26. *Mycobacterium phlei* NCTC 8151



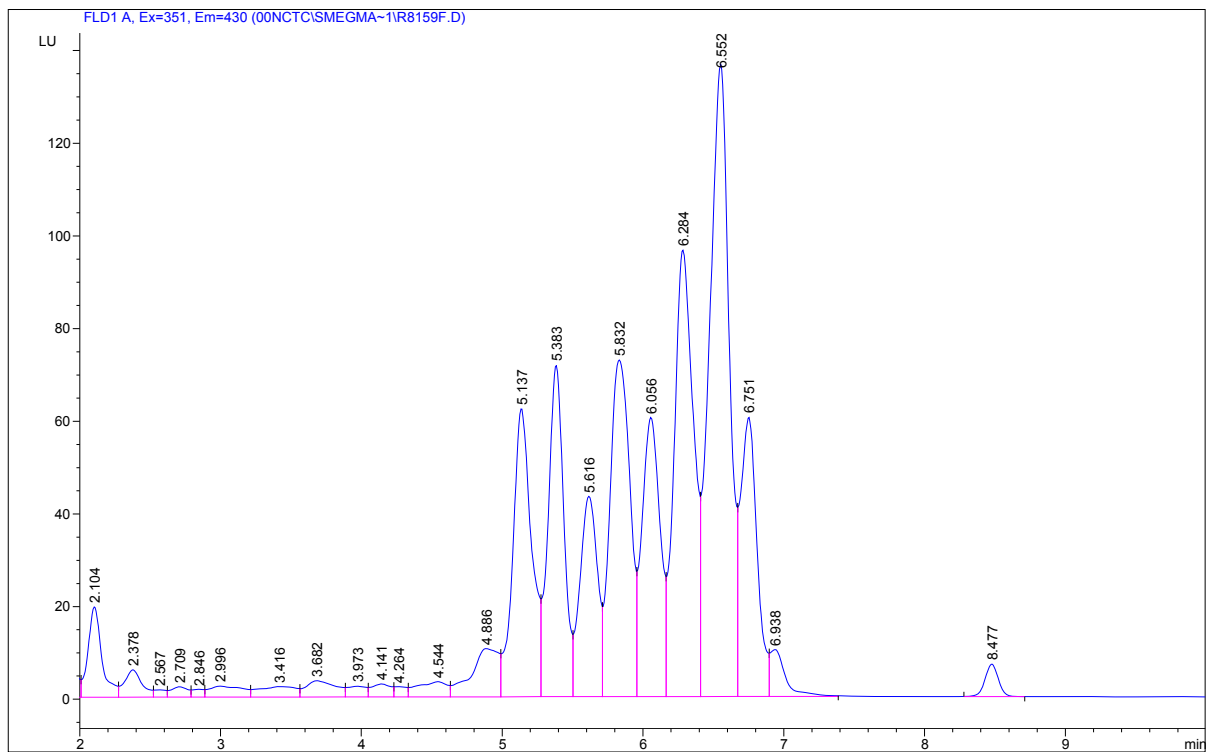
**Figure. 27.** *Mycobacterium rhodesiae* NCTC 10779



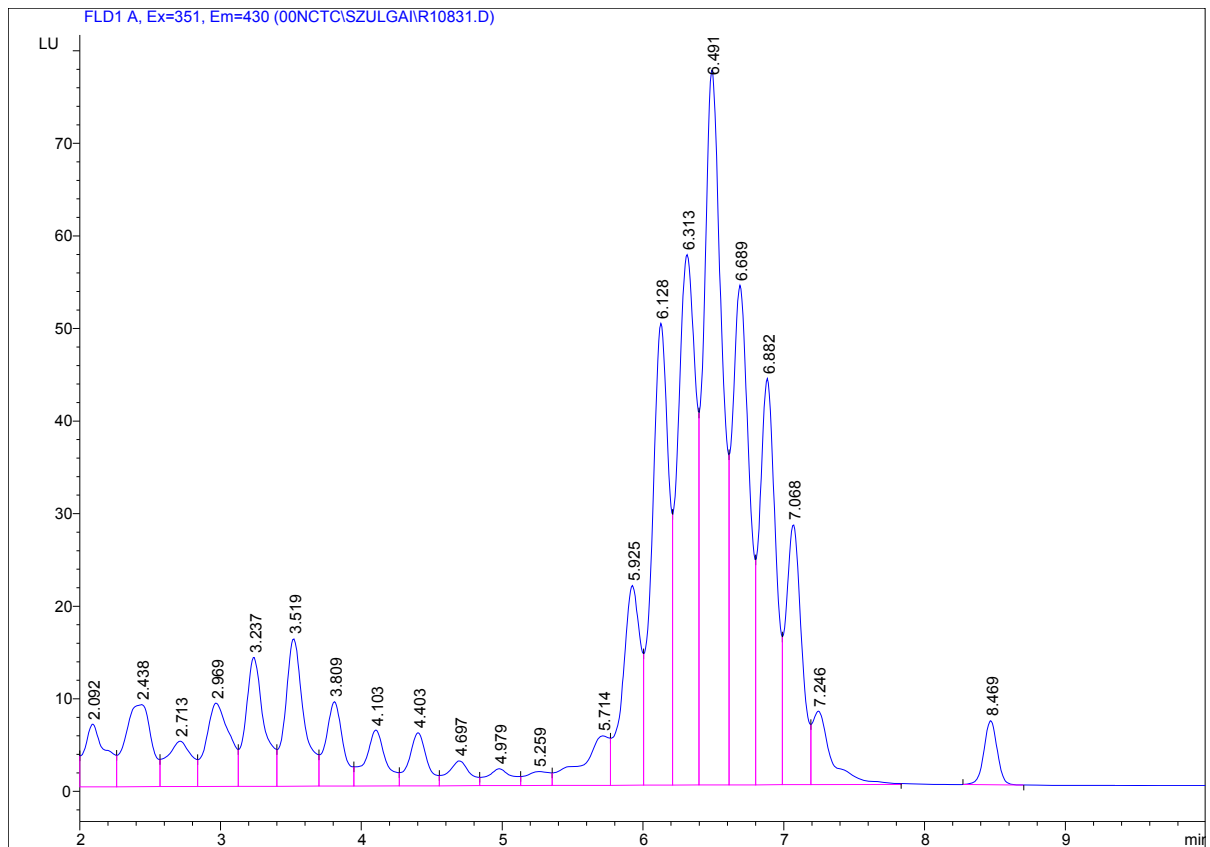
**Figure. 28.** *Mycobacterium senegalense* NCTC10956



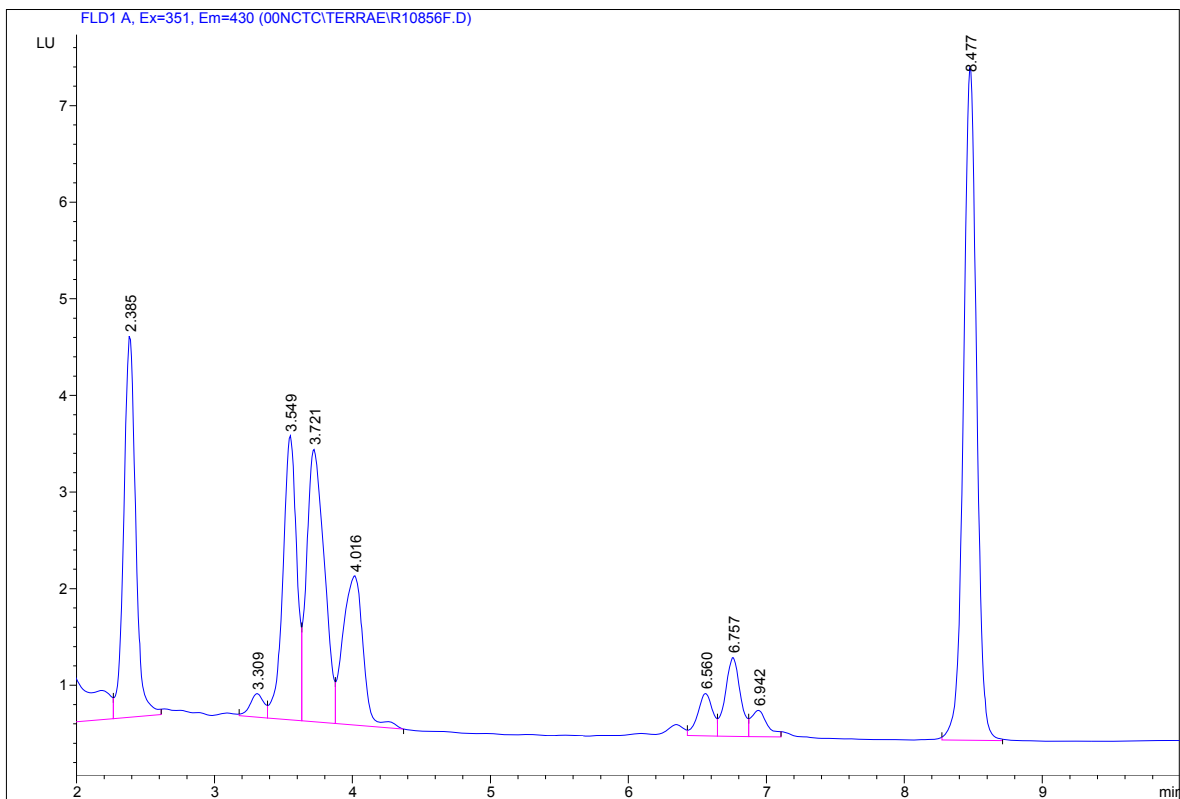
**Figure. 29.** *Mycobacterium smegmatis* NCTC8159



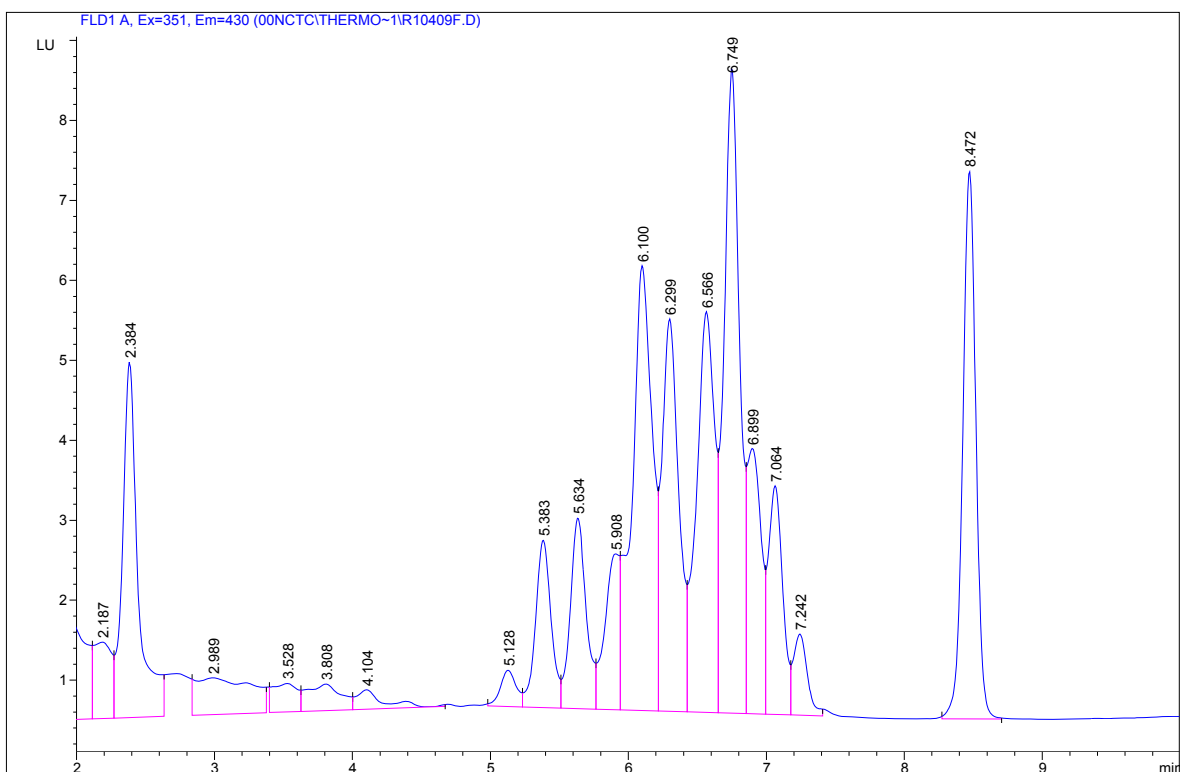
**Figure. 30.** *Mycobacterium szulgai* NCTC10831



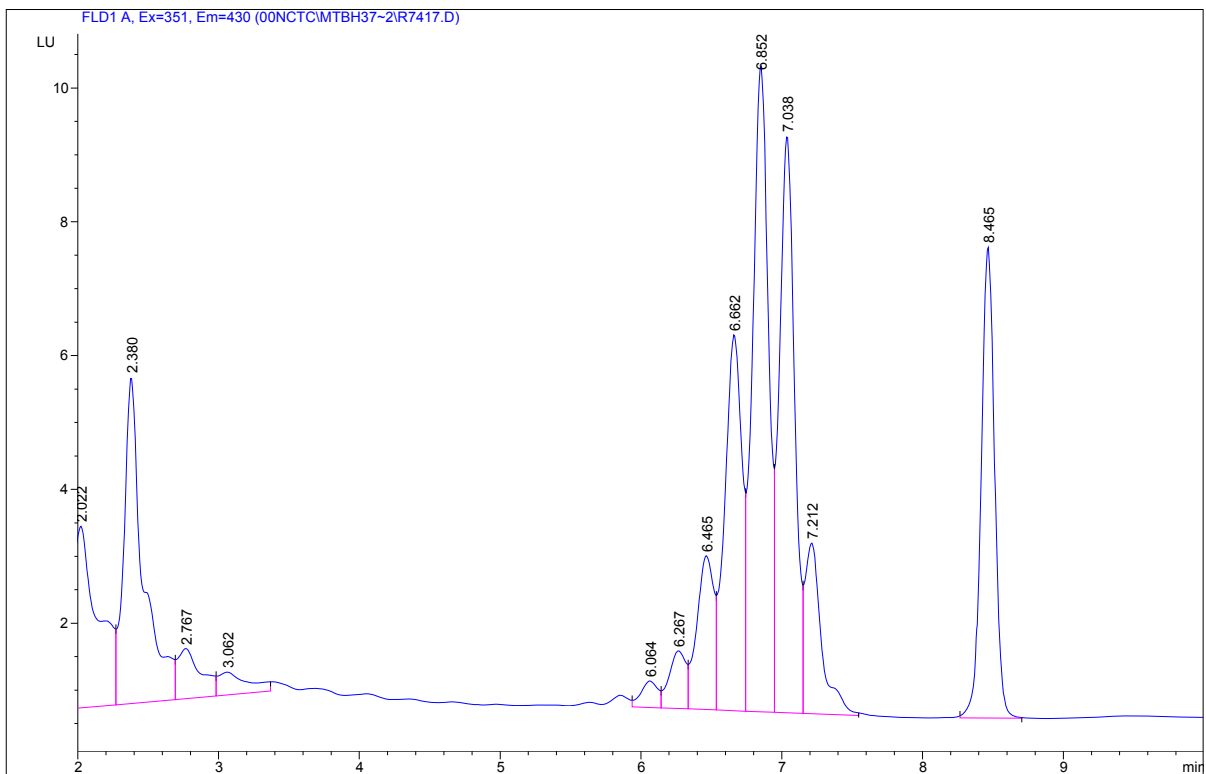
**Figure. 31.** *Mycobacterium terrae* NCTC1085



**Figure. 32.** *Mycobacterium thermoresistibile* NCTC10409



**Figure. 33.** *Mycobacterium tuberculosis* NCTC 7416



**Figure. 34.** *Mycobacterium vaccae* NCTC 10916

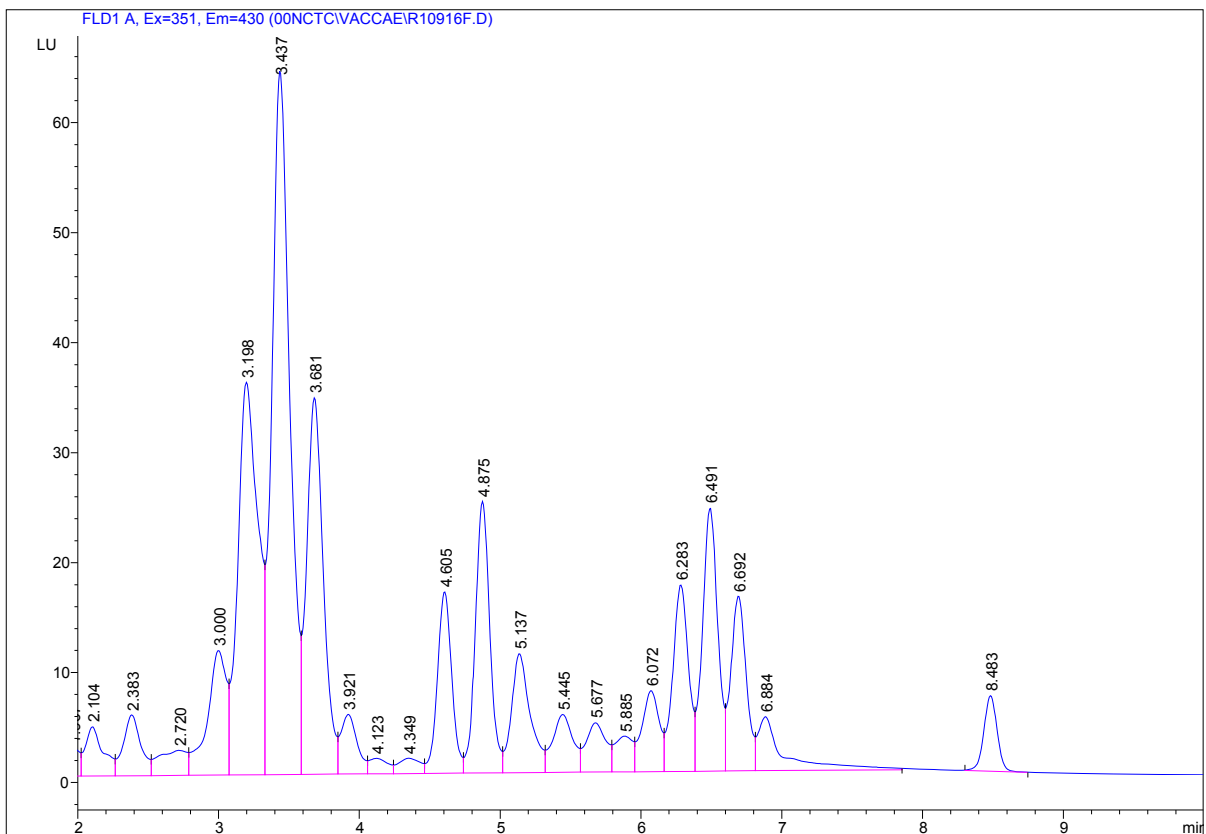


Figure. 35. *Mycobacterium xenopi* NCTC 10042

