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FACULTY OF HEALTH SCIENCES

VIABILITY OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AS A METHOD OF MYCOBACTERIAL IDENTIFICATION IN SOUTH AFRICAN LABORATORIES

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A research report Submitted to the faculty of Health Sciences, University of Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Masters of Science in Medicine (Pharmaceutical Affairs).

Johannesburg, 2001

DECLARATION

I, Shirona Naidoo, declare that this research report is my own work. It is being submitted for the degree of Masters of Science in Medicine (Pharmaceutical Affairs) in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



_____day of _____, 2001

Dedicated to my Mum and Dad - 'my strength through adversity'

ABSTRACT

Pathogenic mycobacterial infection was in recent decades a health concern so well controlled that eradication seemed imminent. However, it is once again reaching epidemic proportions following the increasing prevalence of AIDS. One important means of curbing this resurgence, is a robust method that has the capability of identifying to a species level speciating disease causing mycobacteria in a matter of days. Several new methodologies are now available that enable dramatic reductions in turn-around times.

In this study High Performance Liquid Chromatography was investigated to determine how this system compared with the current mycobacterial system of methodologies adopted in South African laboratories.

Four species of pathogenic mycobacteria, with a high prevalence in South Africa, were tested in a sample size of 80. Samples were subjected to HPLC, Gene Probes and Biochemical testing. HPLC was the most capable of identifying the mycobacteria to species level displaying a sensitivity to the organisms of 96.25 %. Gene probes and Biochemical testing had sensitivity values of 82.5 % and 80 % respectively.

HPLC was also more cost efficient and displayed a wider range of identification. It is therefore suggested that HPLC replace Gene probes and Biochemical testing for purposes of MOTT identification in the comprehensive mycobacterial identification system. The result is a time saving of at least 3 weeks and a cost reduction of approximately 30 %.

The large initial capital investment required for the implementation of the HPLC system is justified by the long term cost saving as well as the additional utility derived from early identification. As a consequence treatment is not empiric but rather tailored to the organism infecting the patient, hence preventing multiple drug resistance developing and ultimately saving a life through rational drug use.

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List of Abbreviations used:

SAIMR	- South African Institute for Medical Research
NTM	- Non Tuberculosis *Mycobacteria*
MOTT	- *Mycobacteria* Other Than Tuberculosis
MDR	- Multiple Drug Resistance
L-J	- Lowenstein Jensen
AFB	- Acid Fast Bacilli
LMW ISTD	- Low Molecular Weight Internal Standard
HMW ISTD	- High Molecular Weight Internal Standard
HPLC	- High Performance Liquid Chromatography

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1. Introduction:

Tuberculosis accounts for more than 80% of all communicable diseases notified in South Africa and is regarded as one of the most serious health problems affecting the country. [1] It is still the largest cause of death from a single pathogen and is still the leading infectious killer of youth and adults and is estimated to kill almost 1000 people every month. [1]

Most significant, however, is it's effect on individuals infected with the Human Immunodefeciency Virus (HIV). Stricken individuals progress to active TB at an occurrence rate that is 40 times faster than HIV negative individuals. HIV has also introduced the opportunistic mycobacteria as further possible life threatening infections. [1]

Epidemiological studies conducted in South Africa have also demonstrated an unusually high incidence of Non-Tuberculosis Mycobacteria (NTM) related diseases; not necessarily linked to the HIV epidemic. [2] It has been shown that the clinically significant mycobacteria other than Tuberculosis (MOTT's) are *M. kansasii* (61.5%), *M. avium* (12.3%) and *M. scrofulaceum* (13.4%). [3]

The need for a rapid, accurate and reliable method for identification of these various mycobacteria is urgent. Diagnostic techniques currently employed in South Africa for the identification of mycobacteria (especially mycobacteria other than Tuberculosis); are time consuming and lengthy. This is due to primarily to the extremely slow growth rate of most members of the genus *Mycobacterium.* In general, mycobacteria require 2 to 8 weeks or longer for detection using traditional mycobacteriology methods. Once the organisms are detected, conventional biochemical testing can commence requiring an additional 3 to 6 weeks. As a result isolation and identification of a mycobacterium from a clinical sample can easily take 8 to 12 weeks. [4] This long turn -around time before a clinically useful report can be generated can cause several problems. Lengthy delays in confirming which species of mycobacterium the clinical isolate is results in uncertainties in patient therapy. Many mycobacteria other than Tuberculosis (e. g. *M. scrofulaceum*) will require different treatment regimines or some (e. g. *M. gordonae*) may require no treatment at all. [4] The end

result of the delayed diagnosis is the emergence of multiple drug resistant strains of pathogenic mycobacteria and the patient being treated with drugs that are ineffective if the patients isolate has developed drug resistance.

The increasing incidence of multiple-dug-resistance (MDR) strains emerging have far reaching consequences for the patients well being. In a study involving more than 200 patients with MDR TB, most patients involved were infected with HIV, and 72% to 89% died within 4 - 16 weeks of diagnosis. [5]

The key to effectively dealing with the reemerging epidemic is to rapidly and accurately identify the mycobacteria to a species level; so as to implement the correct treatment regimin to the patient before the actual onset of the fatal sequalae of the mycobacterial infection.

The current procedure for mycobacteria identification, at the South African Institute of Medical Research, Johannesburg, is as follows: [6]

- All specimens undergo **Radiometric testing** (i.e. BACTEC). BACTEC uses a broth matrix, optimised for the growth of mycobacteria, containing the ^{14}C labelled palmitic acid. Mycobacteria metabolise the labelled palmitic acid and expire CO_2, which is quantified to detect the presence and growth of mycobacteria.

  This process takes approximately 2 - 4 weeks. Radiometric testing assists in establishing the presence of Acid Fast Bacilli. It does not however provide positive identification of the mycobacterium present to a species level.

  A further limitation coming is that procedures and facilities to dispose of the radiometric waste is an important prerequisite.

- Those specimens that are positive for Acid Fast Bacilli undergo **PCR testing** (i.e. A Nucleic Acid Amplification Technique). This is a rapid process requiring

only 24 hours. The SAIMR, however, only uses the DNA sequence sensitive to *M. tuberculosis*. Hence only a positive identification of Tuberculosis is made. Identification of mycobacteria other than Tuberculosis is presumptive.

There does exist a possibility of increasing the number of nucleic acid sequences used, in order to aid the positive identification of Non Tuberculosis Mycobacteria. PCR testing does however have two major setbacks. [7] False - negative reactions caused by inhibitors of *Taq* polymerase that interferes with PCR; and secondly false - positive reactions due to contamination with DNA fragments are characteristic of PCR testing . [1]

- Those specimens that are PCR negative undergo **Microscopy**. Distinguishing colony morphology provides an indication of species. However this technique is also presumptive and its ability to identify mycobacteria other than tuberculosis conclusively is very limited.

- Slides that resemble Tuberculosis or Mycobacterium Avium Complex undergo a **nonradiometric gene probe** (i.e. Accuprobe). In this technique mycobacteria are lysed and their ribosomal RNA is released. This RNA can be hybridised to single stranded DNA probes. The DNA:RNA hybrids are stable and quantitated.[4] Identification once more is rapid, requiring only up to 24 hours for positive identification. [1] Although the nucleic acid amplification technique has demonstrated a sensitivity and speed favourable to the identification of mycobacteria; the range of speciation is narrow. This is due to the limited number of *Mycobacterium* species that can be identified by the use of commercially available DNA probes. The number of species a lab is capable of identifying, is limited by the number of DNA sequences available to the laboratory. Affordability of these expensive DNA sequences limits the range of species that may be identified by a laboratory. Currently the SAIMR only uses *M. tuberculosis* and *M. avium* complex, although *M. kansasii* is also available. Comparative studies conducted on HPLC versus PCR and gene probes have demonstrated that HPLC has the advantage of

providing species - species identification for a much larger percentage of mycobacterial genus. [8] The restrictions with regard to this procedure is that, more than one procedure will be required to make a species level identification of a variety of specimens and the cost per sample is excessive. This will be expanded on in the Chapter 4 (Cost Analysis).

- MOTTS, however, must now undergo a battery of biochemical tests and observations to identify the mycobacteria to a species level. These include:
  - Growth rate in days
  - Growth temperature range (24 ^{0}C, 37 ^{0}C, 42 ^{0}C, 45 ^{0}C)
  - Pigmentation of culture
  - Colony morphology
  - Nitrate reduction
  - Catalase production
  - Tween hydrolysis
  - Urease

  This method of identification is dependant on a suitable amount of growth. The time for completion may range from 3 - 8 weeks. [1][6] They are often subjective, because this technique is based on the experience and skill of the technician. Identification is not conclusive.

There exists an opportunity in South African laboratories for the application of High Performance Liquid Chromatography as a method of differential mycobacterial identification.

The chemical uniqueness of cell wall bound mycolic acids has already been demonstrated by methods of analysis that combine absorption chromatography with High Performance Liquid Chromatography. [8][9] HPLC has also been used to demonstrate the distinctly different mycolic acid "fingerprint" patterns produced by different species of

different species of mycobacteria. [10] The range of speciation and the sensitivity of High Performance Liquid Chromatography Tests were shown to be greater than either of the current methods mentioned. [7]

This, however, does not provide sufficient evidence of the transcendency of High Performance Liquid Chromatography over methods currently in use in South Africa. An evaluation of the process based on factors such as time and cost is necessary in order to prove the value of HPLC.

In this study I made use of simple single step HPLC analysis of *p* - bromophenacyl esters of mycolic acids extracted from saponified whole cells of four clinically significant mycobacteria; relevant within a South African context. Prior to application of the HPLC method; these mycobacteria were identified by conventional laboratory methods and where applicable, Gene Probes.

To assist in the selection of suitable HPLC detection methods, a literature survey was carried out for methods that were previously developed. From the studies conducted; a reliable and proven process to saponify, extract and derivatise the mycobacteria was identified. It was also established that a specially prepared high molecular weight internal standard compound could be used to standardise the real retention times of peaks to the relative retention times. HPLC conditions were stipulated according to the individual HPLC set up in each laboratory. [11][12][13] Hence conditions were standardised to the set up available within the Department of Pharmacy, Witwatersrand University. Methods were established to apply HPLC directly to BACTEC 7H12B medium. [14] However these methods generated chromatograms that could not be interpreted because they lacked adequate or key peaks. Reliable interpretation was thus prevented. Further; working with and disposing of radiometric waste becomes a problem that can be a disadvantage when comparing HPLC to other identification techniques. Therefor HPLC chromatograms were generated from mycobacteria grown on Lowenstein-Jensen Slopes.

Chromatograms were generated for both reference culture strains and clinical isolates; for each of the mycobacteria. The clinical isolate chromatogram was identified by comparing visually against the reference standard chromatogram and identifying relative retention peaks specific to that particular species. An analysis follows in chapter 3 discussing comparison of methods used to identify the specimens.

Time taken to achieve positive, definitive results were also compared for the HPLC method and the methods currently in use.

A cost analysis was also conducted to establish if HPLC is a feasible method. The analysis was based on fixed and variable costs. Fixed costs comprised the cost of the specialised equipment required for each process. Service costs were factored into total cost of the equipment. Variable costs comprised Man and Material operating costs. These costs were calculated as a unit cost per one sample to standardise the analysis. The cost per sample for the currently employed method and for the HPLC method were then compared to establish feasibility. Viability was then also established by demonstrating improved sensitivity and time to positive identification to a species level. Additional utility conferred by use of the HPLC system will be discussed to reinforce viability of the system.

2. METHODOLOGY:

2.1. Introduction:

The objective of the study was to identify mycobacteria by analysis of mycolic acids using HPLC. Identification was achieved through the production of reproducible chromatographic patterns containing combinations of different diagnostic peaks. A suspension of acid–fast bacteria was saponified to cleave the mycolic acids bound to the cell wall. Mycolic acids were then separated by acidification and extraction into chloroform. After derivitisation into ultraviolet (UV) – absorbing *p* – bromophenacyl esters the mycolic acids were analyzed on a reverse – phase C18 column using HPLC. A gradient of methanol and dichloromethane generated by microprocessor - controlled pumps was used to separate the mycolic acids esters. Detection of mycolic acids was achieved by using a UV/Visible detector. Reproducible chromatographic patterns containing combinations of different diagnostic peaks were generated. Each mycobacterial species analyzed produced a different mycolic acid "fingerprint" that was interpreted by visual inspection.

2.2. Specimens:

Reliable identification was achieved when mycobacteria were grown under standardized conditions. Accordingly, the reference culture strains to be identified were grown on LJ slopes, and incubated at 35 – 37 ^{0}C until sufficient growth for preparation of extracts was detected. Clinical isolates used were planted and identified by the technical staff at the SAIMR. Inclusion criteria for this study were that the specimens be previously identified by conventional techniques to their species level and were cultured in the same conditions under which the reference culture strains were grown.

The Mycobacterial species that were analyzed :

- *M. tuberculosis*
- *M. avium*
- *M. scrofulaceum*
- *M. kansasii*

Chromatograms were generated for 20 of each species consisting of clinical isolates and reference culture strains.

The Mycobacterium isolates were attenuated at the SAIMR by autoclaving for one hour at 121 °C, 15 psi.

Before commencing work on the study; *M. smegmatis, M. tuberculosis, M. avium, M. scrofulaceum and M. kansasii* clinical samples were exposed to the above attenuation conditions and then incubated at 25 °C; 37°C; 42°C and 45°C. They were allowed to incubate for three months and were checked at intervals of 1 week; 3 weeks; 6 weeks; 8 weeks and 3 months. No growth was observed in this time period.

It could then be concluded that autoclaving under the aforementioned conditions attenuated the samples adequately.

2.3. Materials:

A. Chemicals:

Solvents must be HPLC grade and filtration is recommended in order to increase resolution of the chromatograms.

The following chemicals are required:

CHEMICALS	SUPPLIER
Hydrochloric Acid	Merck Laboratory Supplies Box 1998, Halfway House, 1685 Tel: (011) 655 6000
Potassium Hydroxide	
Potassium Bicarbonate	
Printol D (or any other phenolic tuberculoidal disinfectant)	
Synthetic Compounds (Internal Standards) ▸ High Molecular Weight ▸ Low Molecular Weight	Corixa 553 Old Covalis Road, Hamilton Montana, 59840, USA Fax: 406 363 6214
Derivitisation Reagent ▸ *p* - bromophenacyl bromide (0.1 mmol/ml) ▸ dicyclohexyl - 18 -crown - 6 ether (0.005 mmol/ml) in acetonitrile	Sigma - Aldrich Box 4853, Atlasville, 1465 South Africa Tel: (011) 397 8886
Chloroform	Labchem Box 2371, Edenvale, 1610 South Africa Tel: (011) 452 1122
Dichloromethane	
Methanol	

B. Reagents for Saponification, Extraction and Derivitisation of Mycolic Acids:

Preparation of:

1. Saponification Reagent

Potassium hydroxide	200 g
Reagent grade water	400ml
Methanol	400 ml

While stirring, slowly added the KOH to the water in a glass beaker, using an ice bath to cool the mixture. Continued stirring until the KOH has dissolved. When cooled, added the methanol.

Stored in an air-tight convenient container at ambient temperature until used.

Expiration: 1 year.

2. Acidification Reagent

Reagent grade water	400 ml
Conc. Hydrochloric acid	400 ml

While stirring, slowly added 400 ml of conc. HCl to the water in a 1L beaker.

Stored in an air tight convenient container at an ambient temperature until used.

Expiration: 1 year

3. Potassium Bicarbonate Reagent

Potassium bicarbonate	4 g
Reagent grade water	98 ml
Methanol	98 ml

Added the $KHCO_3$ to the 98 ml water in a suitable container. Stirred until dissolved, then added the methanol and stirred.

Stored at ambient temperature in an air tight convenient container. Any precipitation is removed by heating to 35 °C +/- 1 °C to re-solubilise, before reagent was used.

Expiration: 1 year

4. Derivitisation Reagent

Prepared and stored as described in the manufactures instructions.

Expiration: Two years from the date of manufacture of the oldest component.

5. Clarification Reagent

Mixed 100 ml acidification reagent with 100 ml methanol.

Stored at ambient temperature in a convenient container.

Expiration: 1 year

C. Sample Diluent with Internal Standard:

Added 0.4 mg of Low Molecular Weight Internal Standard (LMW – ISTD) and 2 mg of High Molecular Weight Internal Standard (HMW – ISTD) to 50 ml dichloromethane in a 50 ml volumetric flask. (8 and 4 ug/ul respectively).

Stored at 4 °C in a tightly capped dark amber bottle.

Expiration: 3 months

D. Equipment:

1. Certified biological safety cabinet

2. Certified chemical fume cabinet.

3. HPLC equipped with a Phenomenex Lunar C18 endcapped column with integral guard column, packed with a 3 ,um silica (4.6 mm x 7.5 cm); and a column oven.

Note that the HPLC conditions during the course of the study were as follows:

- HPLC of the *p* – bromophenacyl esters of the mycolic acids was done with an HPLC 450 data system controller. (Beckman instruments)

- The UV light absorbing *p* –bromophenacyl esters were detected with a variable-wavelength detector (Beckman, Model 168) set at 254 nm.

- A Beckman model 126 solvent delivery pump controlled the solvent gradient system of methanol and dichloromethane.

4. Autoclave (Butterworth High Speed H.D. 158)

5. Heat block for 13 mm tubes (85 – 105 °C) or vacuum evaporator unit.

6. Evaporator air manifold for 13 mm tubes with blunt ended needles or vacuum evaporator unit.

7. Vortex type mixer (Vortex Genie 2 G560 E)

8. Adjustable micropipette (Finnipippette model F28673 and F53158)

9. Safety pipetting device

E. Supplies:

1. Pasteur pipettes with 5 ¾ inch disposable glass

2. Borosilicate glass culture tubes (13 x 100 mm, new defect free, unwashed) with new, Teflon lined screw caps

3. Polyester fiber tipped applicators

4. Inoculation loops (ca. 10 µl)

5. Dark amber glass reagent dispensing bottles; or 1.0 ml and 5.0 ml glass pipettes

6. Volumetric flasks, 50, 100 and 250 ml

7. Dark amber glass bottles with caps, 1 L, 50 and 100 ml

8. 200 ul tips for adjustable micropipette

9. Filtered dry air or nitrogen

10. pH paper (range pH 0 to pH 13)

11. Autosampler vials and caps

2.4. Sample preparation procedure:

A. Cell harvesting procedure:

1. Added 2.0 ml saponification reagent to a new 13 x 100 mm tube.

2. Removed a sample of bacteria from the L-J medium using a sterile polyester swab or transfer loop. An amount equivalent to 1 – 2 full loops is sufficient.

3. Added the bacteria to the tube and capped tightly with a new cap.

4. Mixed vigorously for at least 20 seconds.

B. Saponification and extraction procedures:

1. Autoclaved the tubes for a minimum of 1 hr at 121 °C, 15 psi. The tops of the tubes should be covered with aluminum foil to ensure adequate heat transfer for decontamination. Cooled to an ambient temperature (or below).

2. Added 2.0 ml of chloroform.

3. Added 1.5 ml of acidification reagent. Capped tightly.

4. Vigorously mixed the tubes for a minimum of 20 seconds. Allowed the layers to separate for 20 – 30 seconds. If the bottom layer remained turbid, mixed again for 30 – 60 seconds. If still turbid, proceed.

5. Using a glass Pasteur pipette, removed the bottom (chloroform) layer (containing mycolic acids)

6. Used a heat block to evaporate the chloroform at 85 – 105 °C under a stream of filtered dry air until the sample was thoroughly dry. Avoided excessive air pressure that could have cause the sample to splatter. Alternatively, used a vaccum evaporator to the dry sample.

C. Derivitisation to *p*-bromophenacyl esters:

1. Added 0.1 ml of potassium bicarbonate reagent to the dry sample

2. Using a heat block, evaporated at 85 – 105 °C under a stream of filtered dry air until sample was thoroughly dry. Alternatively used a vacuum evaporator

3. Cooled the sample to ambient temperature or below and added 1.0 ml of chloroform, followed by 50 µl of derivatising reagent.

4. Capped the tube tightly and mixed vigorously for 30 sec.

5. Using a heat block, heated the sample at 85 – 105 °C for a minimum of 20 minutes. After the first 30 – 60 seconds of heating, checked the volumes. If the volume in any

After the first 30 – 60 seconds of heating, checked the volumes. If the volume in any tube appears to be less than 1 ml, adjusted the chloroform to 1.0 ml. Recapped with a new cap and reheat the sample.

D. Clarification by liquid – liquid extraction:

1. Cooled the samples to ambient temperature or below and added 1 ml of clarification reagent. Recapped.

2. Mixed each tube vigorously for a minimum of 20 sec and allowed the layers to separate (5 – 10 seconds minimum).

3. Removed the bottom (chloroform) layer with a glass Pasteur pipette and transferred to a new tube.

E. Completion and storage:

1. Evaporated to dryness at 85 – 105 °C under a stream of filtered dry air. Capped tightly.

2. Stored the sample at 4 – 6 °C in the dark until ready for analysis.

2.5. Chromatographic set up:

A. Quality control:

Before starting the HPLC analysis; confirm the quality control specifications have been met (Refer ***Appendix 1: Quality Control***).

B. Gradient conditions:

Gradient conditions were defined with a Phenomenex Lunar C 18 analytical cartridge column, 4.6 mm X 7.5 cm, packed with a 3 um silica. The instrument method was set up to reproduce the following solvent flow conditions:

(The suggested instrument settings are summarized in the Appendices.

Appendix 2 : HPLC method file set up; Table 1: Instrument Control Settings

The manufactures instruction manual should also be referenced for specific instrument settings)

- The solvent flow rate must be maintained at a constant flow rate of 2.5 ml/min for all samples analyzed.
- The initial solvent mixture is 98 % methanol (solvent A) and 2 % dichloromethane (solvent B).
- During the first minute following injection the solvent mixture is changed to 80 % solvent A and 20 % solvent B using a linear gradient (elapsed time = 1min).
- During the next nine minutes, using a linear gradient, the solvent mixture is changed to 35 % A and 65 % B (elapsed time = 10 min).
- During the next 0.5 min the gradient is returned to 98 % A and 2 % B (elapsed time = 10.5 min).
- Finally, the solvent mixture is held at 98 % A and 2 % B to equilibrate the column for a duration of 4.5 min (total elapsed time = 15 min).

C. Start up instructions:

1. Turned on the chromatograph and the detector lamp 30 min before use or as recommended by the manufacturer.

2. Ensured that the amount of solvents are adequate and that the waste collection container was in place.

3. Verified that the column temperature was at 35 °C +/- 1 °C.

4. Primed the pumps.

5. Ran the analysis method with or without the injection of a dichloromethane blank. Checked for leaks, bubbles and confirmed that the pump pressure to be within the limits.

2.6. HPLC analysis:

A: Sample preparation:

1. The sample was dissolved in approximately 50 – 500 ul of sample diluent. The optimum amount of sample diluent is sample and system dependant. Sample concentration must be adjusted to produce a chromatogram with clearly detectable internal standards and mycolic acid peaks (which elute between the internal standards). For a chromatogram to be acceptable, a mycolic acid peak 2 – 5 % the height of the tallest mycolic peak must be detectable and all mycolic acid peaks must be on scale. Rapidly growing mycobacteria may require more sample diluent. Poorly growing mycobacteria may require less sample diluent.

2. The sample was then transferred to an autosampler vial and labelled appropriately.

B: Analysis:

1. The instrument verification was performed according to the criteria in the section Quality Control. (Refer ***Appendix 1: Quality Control***).
2. The sample table was prepared and the samples were logged in. The samples were loaded into an autosampler tray. Samples may be individually analyzed using manual injection. The order of controls and samples was:

   a. Commenced with a control set which contained the negative and positive controls.

b. Loaded the sample set. A positive control should be added every 12 – 15 samples, and at the end of the sample set.

3. Inject and analyze the control set.

a. Verify that the negative control pattern did not have any peaks in the region between the LMW – ISTD and the HMW – ISTD.
***Appendix 3: Chromatogram 1: Candida albicans* (ATCC 10231)**

b. Verify that the positive control pattern matched the pattern for *M. intracellulare*.
Appendix 4: Chromatogram 2: M. intracellulare

c. For the positive control, determine that the mycolic acid peak was 2 – 5 % the height of the tallest peak is detected.

d. If analysis of the control set demonstrated discrepancies, corrections were made and the controls repeated.

4. When the controls were correct, the samples were injected. Samples that yielded unacceptable results were rerun or reanalyzed after corrective measures were taken. These corrective measures are described in the section on Quality control. (***Appendix 1: Quality Control***)

5. The beginning pressure of each run was recorded and compared. If the pressure varied by more than approximately 10 % of the previous days starting pressure then, appropriate corrective actions are taken. (*Refer* ***Appendix 1: Quality Control, Section Instrument Operating Conditions***).

6. Recorded separately the daily number of runs for the guard column, in line filter and column. Recorded any adjustments or repairs on the sample log and on the equipment maintenance log.

Table 2.8. List of Clinical Isolates Used in Study:

The following clinical isolates identified first at the SAIMR, Johannesburg (by PCR /Gene Probe/ biochemical techniques) correspond to the chromatograms numbered 1 - 20 that appear in Tables 3.3 - 3.6. in Chapter 3: Results Analysis:

Table 2.1. List of clinical isolates for each species							
M. tuberculosis		***M. avium***		***M. scrofulaceum***		***M. kansasii***	
CR no	lab id no	CR no	lab id no	CR no	lab id no	CR no	lab id no
1	70430	1	75894	1	79359	1	69261
2	75812	2	76277	2	83444	2	83438
3	78897	3	1150094	3	83433	3	73462
4	77378	4	1108346	4	1108875	4	79400
5	1104186	5	78007	5	87510	5	82532
6	79281	6	80702	6	83971	6	83669
7	81362	7	1149497	7	87483	7	83676
8	86729	8	1109306	8	88018	8	80477
9	1101296	9	83439	9	92478	9	79158
10	89300	10	83440	10	89447	10	77992
11	80062	11	83443	11	91140	11	77994
12	81049	12	83457	12	87503	12	81168
13	81198	13	84068	13	89640	13	81394
14	77910	14	84069	14	83448	14	81420
15	79141	15	76587	15	84457	15	81421
16	1106188	16	1107037	16	92157	16	83670
17	86735	17	84067	17	93146	17	83676
18	86736	18	1150723	18	95216	18	79059
19	81467	19	82533	19	005514	19	80478
20	86317	20	84452	20	92157	20	86720

3. RESULTS AND DISCUSSION:

3.1. Introduction:

Before attempting to identify the clinical isolates of mycobacteria; a chromatographic profile was generated for the American Type Culture Collection ATCC mycobacterial standards of each of the four species being tested. (Refer figures 3.1; 3.3; 3.5; 3.7). These profiles were used as reference chromatograms against which chromatograms of the clinical isolates were identified and verified (Refer figures 3.2; 3.4; 3.6; 3.8). ATCC mycobacterial standards were also employed as sample controls during each run of a specific species.

The clinical isolates were first identified using conventional MOTT (Mycobacteria other than Tuberculosis) identification techniques, comprising a standard battery of biochemical tests, and where possible genetic probes. (Genetic probes, in the reference laboratory used, were only available for *M. tuberculosis* complex and *M. avium* complex).

Thereafter, results were interpreted visually by manually comparing the sample chromatogram with the reference pattern to determine a match. An approach to facilitate visual interpretation is discussed in detail further in Chapter 3.2.

A comparison was also made of the correct positive identifications made by each of the three identification processes employed. The results of which will be discussed in detail later.

Below is a list of reference standard and typical clinical isolate chromatograms that were generated in the study :

Figure	Chromatogram
3.1	ATCC 18280 - Reference Standard for *M. tuberculosis*
3.2	Clinical Isolate for *M. tuberculosis*
3.3	ATCC 15769 - Reference Standard for *M. avium*
3.4	Clinical Isolate for *M. avium*
3.5	ATCC 7091 - Reference Standard for *M. scrofulacuem*
3.6	Clinical Isolate for *M. scrofulaceum*
3.7	ATCC 25221 - Reference Standard for *M. kansasii*
3.8	Clinical Isolate for *M. kansasii*

The chromatograms in Figures 3.1 - 3.8 were exported from the System Gold software and plotted as X and Y values using LOTUS FREELANCE GRAPHICS. The original chromatograms could only be printed directly from System Gold using a dot matrix printer and appear in the appendix. (Refer ***Appendix 6 - 13***)

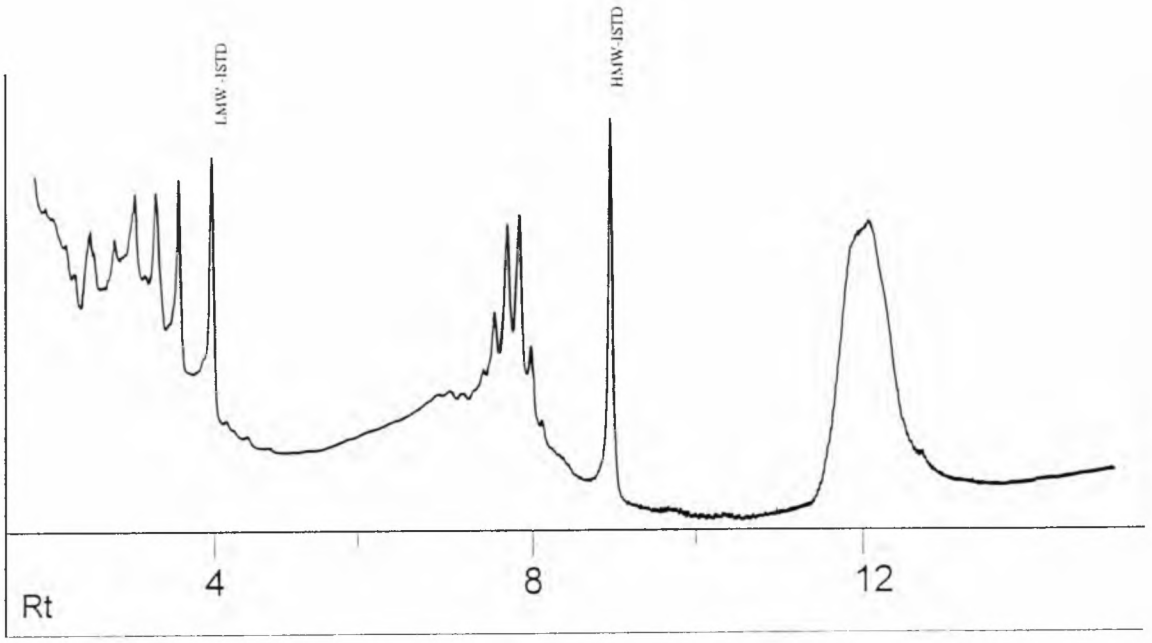


Figure 3.1. *M. tuberculosis* (H 37) reference standard

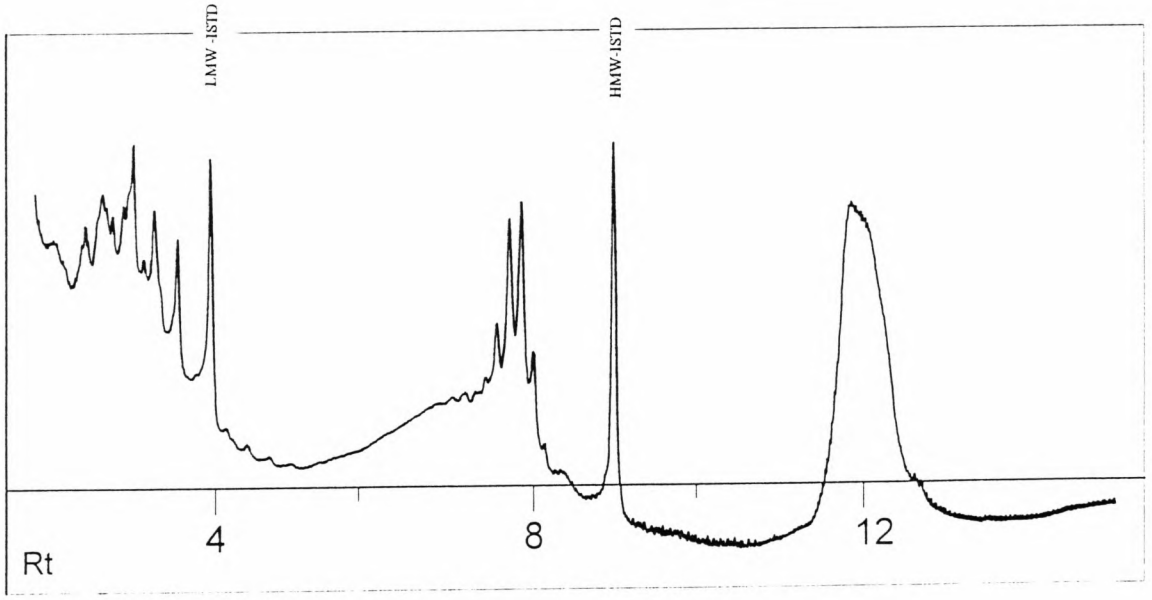


Figure 3.2. *M. tuberculosis* Clinical isolate

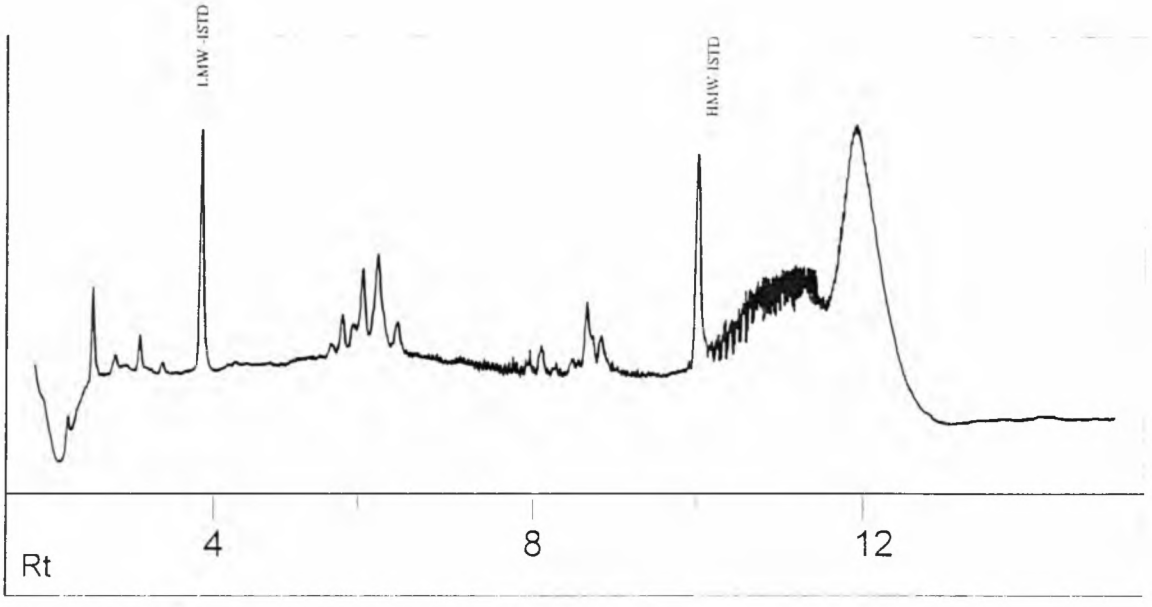


Figure 3.3. *M. avium* reference standard

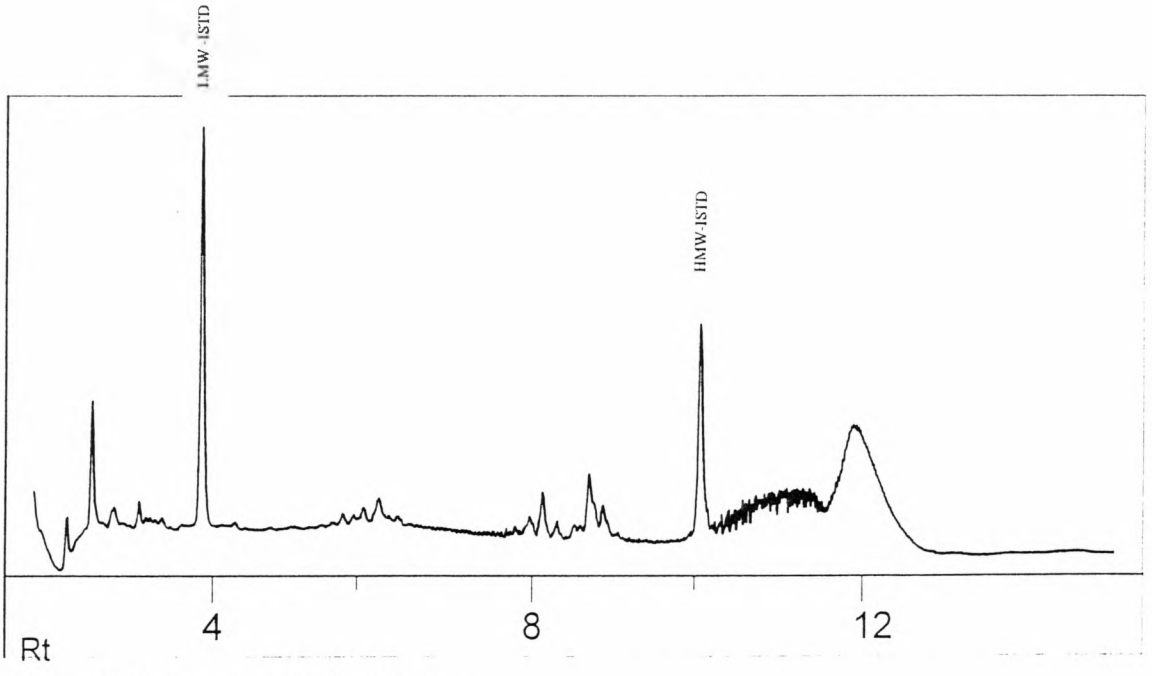


Figure 3.4. *M. avium* clinical isolate

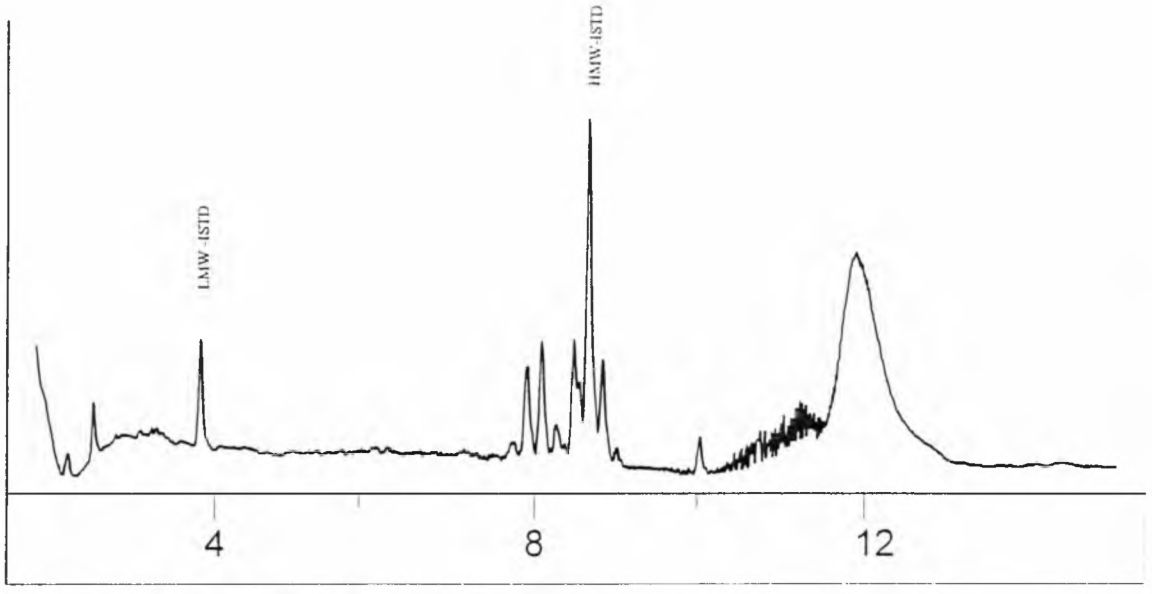


Figure 3.5. *M. scrofulaceum* reference standard

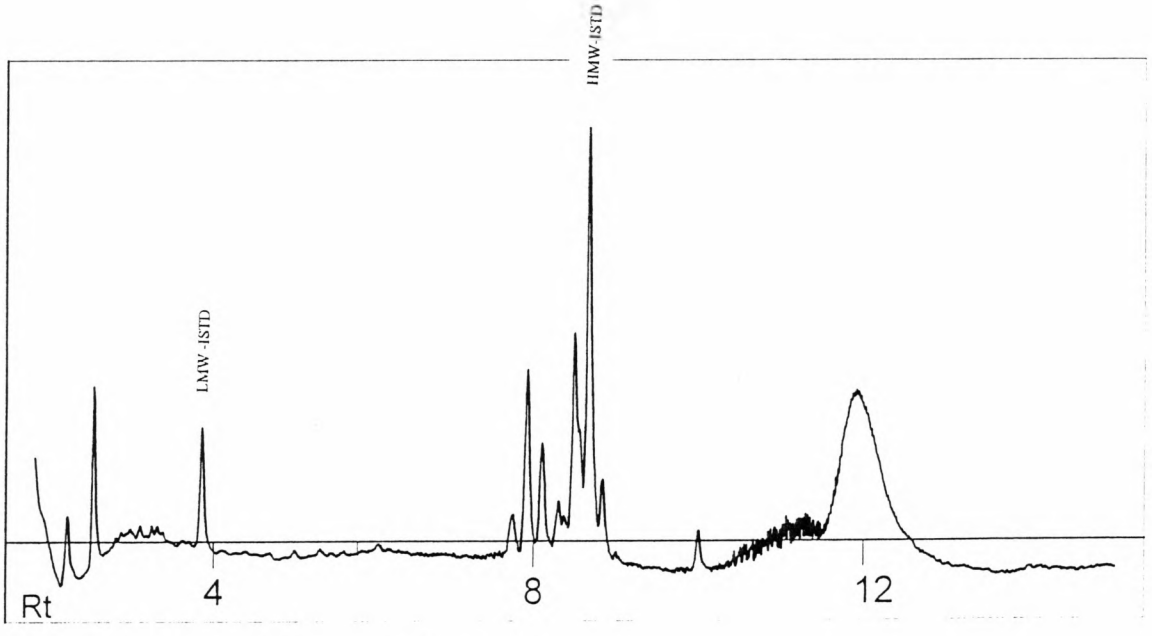


Figure 3.6. *M. scrofulaceum* clinical isolate

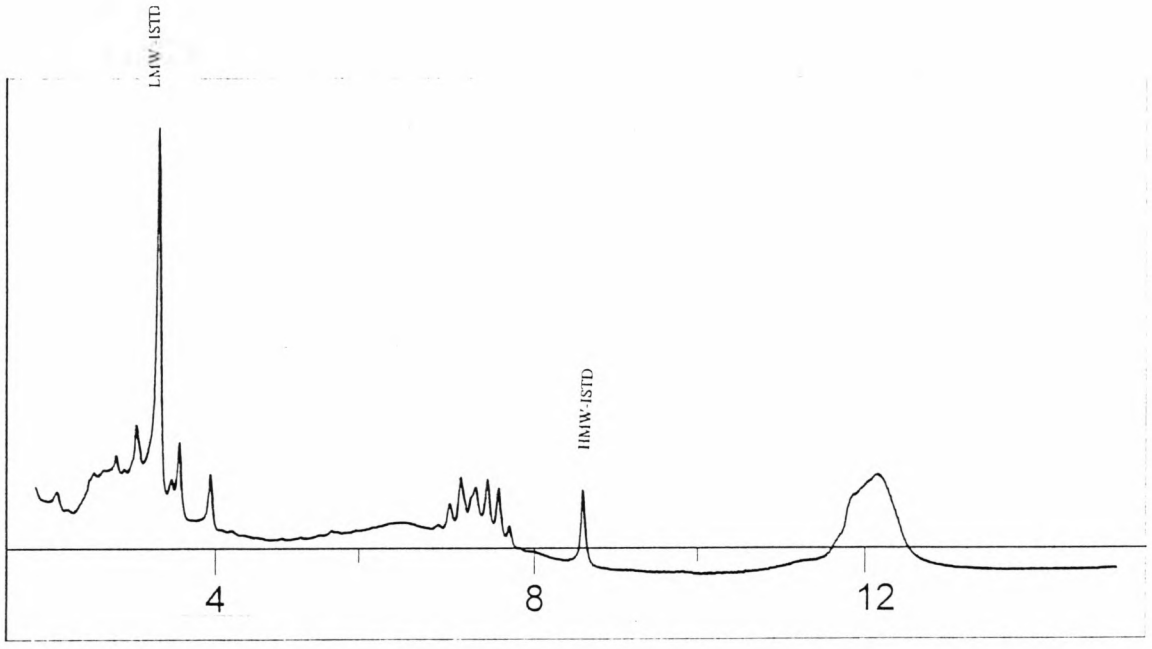


Figure 3.7. *M. kansasii* reference standard

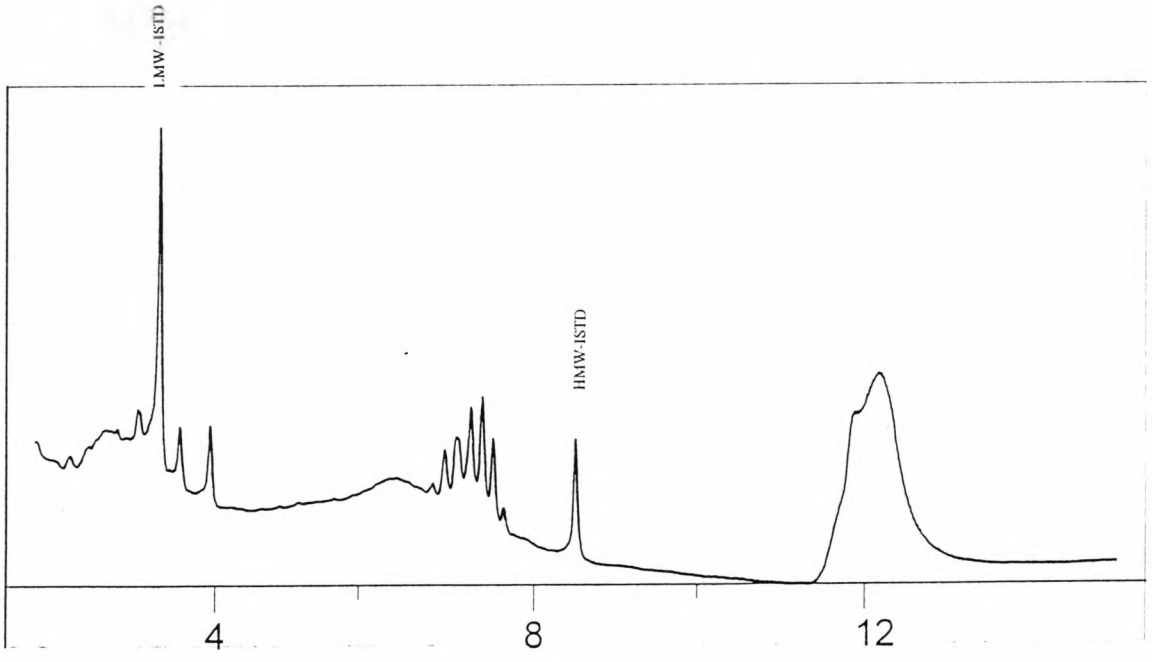


Figure 3.8. *M. kansasii* clinical isolate

3.2. Visual Interpretation Method:

3.2.1. Determine Relative Retention Times

The relative retention times were determined for all significant peaks in the chromatograms generated for the ATCC mycobacterial standard for each species. [*Refer* Table3.1 *: Relative Retention Times for the Reference Standards of each species*]. The chromatograph was set up to calculate the Relative Retention Times automatically.

First the clinical isolate chromatograms generated were matched with the reference standard chromatogram based on similar visual appearance and was verified against the relative retention time values specific to that species as shown in Table 3.1. The relative retention times obtained for the reference standard was verified by generating a summary table of the high frequency relative retention time peaks occurring for all the clinical isolate chromatograms for each species.[*Refer* Table 3.2: Summary of Relative Retention Times for the Clinical Isolates of each species]. Note that this table was generated from the High Frequency Relative Retention Time Peak Tables(3.3 - 3.6) shown in Chapter 3.3.

Species that are not Mycobacteria generally produce no peaks between the LMW-ISTD and the HMW - ISTD or may produce peaks before 3 minutes and ending about 6.5 minutes without any other peaks except the LMW-ISTD and the HMW - ISTD. Hence any sputum specimen that exhibited this chromatogram characteristic may be labelled as "*Not Mycobacterium*".[22]

Appendix 4: Chromatogram 3: Non mycobacterium Specimen

Table 3.1: Relative Retention Times for the Reference Standards of each species:

M. tuberculosis	*M.avium*	*M. scrofulaceum*	*M. kansasii*
	2.00	1.98	1.94
2.27	2.31	2.29	2.39
2.57	2.58		
2.82	2.89	2.87	2.91
			3.19
3.37			3.44
	Low Molecular Internal Standard (3.6 - 3.8)		
			4.95
	5.42		5.36
	5.67		
	5.86		
			6.66
			6.80
			6.94
7.18			
7.32			7.27
7.48			7.41
7.63			7.54
7.77		7.70	
7.87	7.89	7.88	
		8.06	
		8.28	
	8.46	8.46	
	8.64	8.64	
	High Molecular Weight Internal Standard (8.4 - 9.9)		

Table 3.2: Summary of Relative Retention Times for the Clinical Isolates of each species:

M. tuberculosis	*M. avium*	*M. scrofulaceum*	*M. kansasii*
	1.99 - 2.05	1.98 - 2.01	1.90 - 1.95
2.21 - 2.27	2.30 - 2.32	2.29 - 2.32	2.33 - 2.39
2.51 - 2.57	2.57 - 2.60		
2.79 - 2.83	2.88 - 2.91	2.87 - 2.90	2.91 - 2.95
			3.15 - 3.20
3.34 - 3.37			3.42 - 3.45
	Low Molecular Internal Standard (3.6 - 3.8)		
			4.90 - 4.97
	5.42 - 5.50		5.34 - 5.40
	5.63 - 5.70		
	5.86 - 5.93		
			6.59 - 6.66
			6.74 - 6.80
			6.89 - 6.94
7.14 - 7.20			
7.32 - 7.34			7.21 - 7.27
7.48 - 7.50			7.40 - 7.47
7.61 - 7.64			7.54 - 7.60
7.77 - 7.80		7.65 - 7.71	
7.88 - 7.92	7.88 - 7.91	7.88 - 7.92	
		8.03 - 8.06	
		8.28 - 8.32	
	8.46 - 8.55	8.42 - 8.49	
	8.65 - 8.72	8.59 - 8.65	
	High Molecular Weight Internal Standard(8.4 - 9.9)		

3.3. Interpretation of High frequency Relative Retention Time Peak Tables:

The chromatograms generated for each species were analysed. A summary table of the frequently occurring relative retention time peaks for each species was as a result created. These peak tables follow below in Tables 3.3 - 3.6

The Relative Retention time Tables (3.1 - 3.8) and the chromatograms in Figures 3.1 - 3.8; show that each species generates peaks at intervals and retention times in clusters very unique to that species. It can be concluded that HPLC is successful in generating "fingerprint " identification patterns to distinguish between species of *Mycobacterium*.

Table 3.3: High frequency RRT Peaks for *M. tuberculosis*:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2.21	2.23	2.26	2.23	2.26	2.23	2.26	2.21	2.24	2.21	2.26	2.23	2.18	2.26	2.27	2.27	2.23		
2.51	2.56	2.56	2.55	2.56	2.56	2.56	2.57	2.55	2.51	2.56	2.55	2.56	2.56	2.57	2.56	2.55		
2.80	2.83	2.79	2.83	2.81	2.8	2.8	2.8	2.79	2.8	2.81	2.83	2.83	2.79	2.82	2.82	2.83		
3.34	3.36	3.36	3.36	3.35	3.34	3.35	3.35	3.34	3.34	3.35	3.35	3.36	3.36	3.37	3.37	3.35		
3.75	3.76	3.77	3.75	3.76	3.75	3.77	3.75	3.77	3.75	3.76	3.75	3.76	3.77	3.77	3.76	3.75		
7.14	7.17	7.17	7.17	7.19	7.2	7.21	7.2	7.15	7.14	7.13	7.17	7.17	7.17	7.18	7.17	7.17		
7.34	7.34	7.34	7.33	7.34	7.34	7.34	7.34	7.37	7.34	7.34	7.33	7.33	7.34	7.32	7.33	7.33		
7.50	7.48	7.49	7.49	7.5	7.5	7.5	7.5	7.53	7.5	7.5	7.49	7.48	7.49	7.48	7.5	7.49		
7.64	7.61	7.63	7.63	7.64	7.65	7.63	7.64	7.66	7.64	7.64	7.63	7.61	7.63	7.63	7.66	7.63		
7.79	7.77	7.77	7.78	7.78	7.79	7.77	7.79	7.81	7.79	7.78	7.78	7.79	7.77	7.77	7.77	7.78		
7.90	7.91	7.88	7.92	7.89	7.88	7.9	7.9	7.95	7.9	7.89	7.92	7.91	7.88	7.87	7.87	7.92		
8.78	8.74	8.75	8.75	8.76	8.76	8.77	8.78	8.8	8.78	8.76	8.75	8.74	8.75	8.74	8.74	8.75		

Table 3.4: High frequency RRT peaks for *M. avium*:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2.32	2.31	2.3	2.3	2.31	2.31	2.3	2.3	2.31	2.31	2.3	2.31	2.31	2.3	2.31	2.32	2.32	2.33	
2.6	2.59	2.58	2.57	2.58	2.59	2.57	2.57	2.58	2.59	2.57	2.59	2.58	2.58	2.59	2.6	2.59	2.59	
2.91	2.9	2.89	2.88	2.89	2.89	2.88	2.88	2.88	2.89	2.89	2.89	2.9	2.89	2.9	2.91	2.86	2.91	
3.7	3.67	3.67	3.65	3.66	3.67	3.65	3.65	3.66	3.67	3.67	3.67	3.67	3.67	3.67	3.7	3.66	3.65	
5.5	5.43	5.44	5.42	5.42	5.42	5.42	5.42	5.42	5.42	5.42	5.42	5.44	5.44	5.43	5.5	5.48	5.5	
5.63	5.69	5.7	5.67	5.67	5.68	5.67	5.67	5.67	5.67	5.67	5.68	5.7	5.7	5.69	5.63	5.64	5.61	
5.93	5.88	5.88	5.86	5.86	5.87	5.86	5.86	5.86	5.86	5.87	5.87	5.88	5.88	5.88	5.88	5.93	5.93	
7.91	7.93	7.92	7.88	7.89	7.91	7.88	7.88	7.89	7.91	7.91	7.91	7.91	7.92	7.93	7.94	7.93	7.86	
8.55	8.5	8.5	8.46	8.46	8.48	8.46	8.5	8.46	8.48	8.48	8.48	8.48	8.5	8.5	8.55	8.46	8.46	
8.72	8.67	8.67	8.63	8.64	8.65	8.63	8.63	8.64	8.65	8.65	8.65	8.66	8.67	8.67	8.67	8.72	8.67	
9.9	9.85	9.86	9.81	9.82	9.83	9.83	9.83	9.82	9.83	9.83	9.83	9.85	9.86	9.85	9.9	9.9	9.9	

Table 3.5: High Frequency Peaks RRT for *M. scrofulaceum*:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1.99	1.98	1.98	1.97	1.99	1.98	1.99	2.02	1.97	1.97	2.02	1.99	1.98	1.99	1.99	1.98	2.01	1.98	
2.3	2.3	2.3	2.3	2.3	2.29	2.31	2.33	2.29	2.29	2.33	2.31	2.29	2.3	2.3	2.3	2.31	2.32	
2.89	2.87	2.86	2.86	2.58	2.86	2.89	2.9	2.86	2.87	2.9	2.9	2.87	2.86	2.86	2.86	2.87	2.9	
3.63	3.62	3.61	3.62	3.63	3.64	3.67	3.68	3.62	3.62	3.68	3.67	3.64	3.63	3.62	3.61	3.64	3.64	
7.66	7.7	7.7	7.7	7.65	7.7	7.71	7.73	7.67	7.65	7.73	7.71	7.7	7.65	7.66	7.71	7.7	7.71	
7.88	7.9	7.87	7.88	7.88	7.88	7.9	7.92	7.88	7.88	7.92	7.9	7.88	7.88	7.88	7.88	7.92	7.88	
8.04	8.05	8.06	8.06	8.06	8.06	8.03	8.03	8.06	8.04	8.04	8.03	8.06	8.03	8.06	8.06	8.06	8.03	
8.28	8.3	8.28	8.32	8.28	8.29	8.28	8.28	8.28	8.28	8.32	8.3	8.28	8.28	8.28	8.28	8.29	8.29	
8.42	8.42	8.42	8.43	8.43	8.42	8.46	8.48	8.49	8.49	8.49	8.48	8.46	8.42	8.46	8.47	8.43	8.49	
8.59	8.64	8.65	8.59	8.68	8.64	8.65	8.67	8.59	8.65	8.64	8.63	8.65	8.64	8.65	8.65	8.64	8.64	

Table 3.6: High Frequency Peaks RRT for *M. kansasii*:

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1.94	1.92	1.9	1.9	1.95	1.94	1.93	1.93	1.92	1.9	1.9	1.95	1.95	1.95	1.92	1.92	1.9	1.95	1.95
2.39	2.36	2.37	2.39	2.36	2.35	2.39	2.33	2.37	2.36	2.35	2.36	2.33	2.34	2.35	2.33	2.37	2.37	2.35
2.91	2.94	2.95	2.94	2.93	2.95	2.91	2.92	2.92	2.92	2.93	2.94	2.95	2.93	2.95	2.95	2.95	2.95	2.95
3.19	3.2	3.17	3.15	3.15	3.16	3.16	3.16	3.17	3.2	3.2	3.15	3.16	3.17	3.17	3.17	3.2	3.2	3.2
3.44	3.45	3.4	3.42	3.4	3.43	3.4	3.42	3.42	3.41	3.43	3.45	3.45	3.42	3.45	3.42	3.42	3.42	3.45
3.83	3.83	3.8	3.77	3.78	3.8	3.77	3.8	3.8	3.8	3.82	3.83	3.83	3.83	3.83	3.82	3.83	3.83	3.84
4.95	4.93	4.94	4.9	4.94	4.92	4.9	4.92	4.92	4.93	4.92	4.92	4.95	4.95	4.97	4.97	4.94	4.94	4.97
5.35	5.38	5.35	5.34	5.36	5.34	5.34	5.36	5.36	5.38	5.38	5.38	5.38	5.37	5.4	5.4	5.38	5.38	5.4
6.66	6.59	6.59	6.59	6.6	6.61	6.6	6.63	6.65	6.65	6.65	6.65	6.6	6.6	6.66	6.66	6.66	6.64	6.63
6.8	6.74	6.75	6.76	6.76	6.75	6.74	6.8	6.8	6.76	6.75	6.74	6.74	6.74	6.76	6.76	6.76	6.74	6.8
6.94	6.89	6.89	6.89	6.9	6.89	6.9	6.94	6.89	6.94	6.96	6.94	6.89	6.9	6.89	6.89	6.94	6.94	6.94
7.27	7.21	7.24	7.24	7.24	7.25	7.26	7.26	7.27	7.21	7.21	7.21	7.24	7.25	7.26	7.26	7.27	7.27	7.24
7.41	7.47	7.4	7.4	7.45	7.46	7.46	7.46	7.43	7.42	7.42	7.48	7.47	7.5	7.47	7.4	7.47	7.47	7.47
7.54	7.6	7.54	7.54	7.56	7.57	7.57	7.58	7.59	7.59	7.58	7.58	7.54	7.6	7.6	7.6	7.62	7.54	7.6
8.44	8.35	8.38	8.36	8.43	8.44	8.36	8.37	8.37	8.44	8.36	8.43	8.43	8.44	8.40	8.39	8.42	8.35	8.35

The full complement of chromatograms were not generated for every species. The reasons for this are as follows:

- *M. tuberculosis* :

Two of the three clinical isolates, not included in the peak tables above, were processed and reconstituted; but could not be analysed on the HPLC, due to technical malfunctions experienced with the HPLC system. [note: The system used in the department of pharmacy is used primarily for teaching and contract work and hence has multiple users . Technical faults occur with higher frequency in a system not dedicated to mycobacterial analysis only]. The reconstituted specimens do degrade after a period of more than one week. Since the integrity of the processed specimens were compromised; chromatograms generated were not of the quality produced in the other 17 clinical isolates. If peaks were produced, they were of a poor quality; hence these chromatograms were not included in the study.

The third clinical isolate could not be conclusively identified by biochemical tests because of ambivalent test results. This isolate had a positive PCR test but a negative probe result. The chromatogram generated did not match either the reference standard for *M. tuberculosis* or the other clinical isolates generated for *M. tuberculosis*. It may be that the PCR result was a false positive reaction due to contamination with DNA fragments.

- *M. avium* :

Two specimens were identified as *M. avium* although the gene probe yielded a negative result and the biochemical testing was inconclusive. The growth on the L-J slants pigmented a sparse, pale yellow shade and an ambiguous positive urease test result was obtained. The retention times of the chromatograms generated, however, matched the retention times for the *M. scrofulaceum* species.

- *M. scrofulaceum* :

One specimen identified as *M .scrofulaceum* using biochemical techniques, had produced retention times that matched *M. avium*. On further inspection it was confirmed that the biochemical tests were ambiguous because of the atypical pigmentation and an ambiguous negative urease test. The second specimen not included in the study was identified as

M. scrofulaceum in the biochemical tests, but produced a chromatogram that did not match any of the reference standard chromatograms. It was excluded because of the limited number of reference standard chromatograms that were generated in this study against which a visual interpretation identification could be made.

- *M. kansasii:*

One specimen identified as *M. kansasii* in the biochemical tests used produced a chromatogram that did not match any of the reference standards generated for the study and hence could not be identified and was excluded from the study.

The above explanations warrants a closer look into how each identification procedure; namely HPLC, Gene Probe and biochemical applications; compare with regard to number of negative results yielded. A negative result constituted:

- HPLC - Specimen could not be identified
  (Technical errors in processing of sample can be rectified . When this was the reason for not producing a chromatogram, it was not included in this statistic)

- GENE PROBE - produced a false negative test

- BIOCHEMICAL TESTING - inconclusive identification because of ambiguous result.

3.4. Comparison of various Mycobacterial identification procedures:

Table 3.7. Comparison of negative results achieved in identification process by Probes/ biochemical tests and HPLC:

Species	No. of Strains	HPLC (- ve result)	Probe (- ve result)	Biochem. (- ve result)
M. tuberculosis	20	1 (86317)	4 (77378) (1101296) (89300) (77910)	3 (1104186) (77910) (1106188)
M. avium	20	-	3 (82533) (1149497) (84452)	5 (1150094) (78007) (1109306) (82533) (84452)
M. scrofulaceum	20	1 (92157)	n/a*	4 (83448) (84457) (92157) (005514)
M. kansasii	20	1 (86720)	n/a*	2 (83438) (79158)

* No Gene Probe result was generated for *M. scrofulaceum* and *M. kansasii* because no DNA sequence was available at the SAIMR to perform a gene probe identification test on these two species.

The values that appear in Table 3.4 above translate into the following statistics:

- 3 out of 80 (3.75 %) specimens could not be identified using HPLC
  Therefore HPLC is capable of identifying specimens to a species level with a 96.25 % success.
- 7 out of 40 (17.5 %) specimens subjected to Gene Probes had false positive results. This translates to a 82.5 % competency to correctly identify a specimen to a species level.
- 16 out of 80 (20 %) specimens were inconclusively identified using biochemical tests. Therefore biochemical testing is only capable of identifying 80 % of specimens to a species level.

From the figures in Table 3.7. and the percentage values that follow, HPLC seems to be more sensitive to probes and biochemical testing for all four species tested; as HPLC correctly and conclusively identified (to a species level) more specimens per group of species than the other two procedures used.

4. Cost Analysis:

4.1. Introduction:

To establish the viability of the HPLC method to identify Mycobacteria; a comparison must be made to methods already established to identify Mycobacteria. For the purpose of this study, established methods will be those currently employed at the SAIMR. The methods currently employed in the SAIMR were discussed in Chapter 1 and are summarised in the flow chart in Figure 4.2. overleaf.

Viability has been established by comparing characteristics such as cost, time and additional utility and demonstrating the benefit of using HPLC. A suggestion will be made as to how the current *Mycobacterium* identification system could be modified to incorporate HPLC.

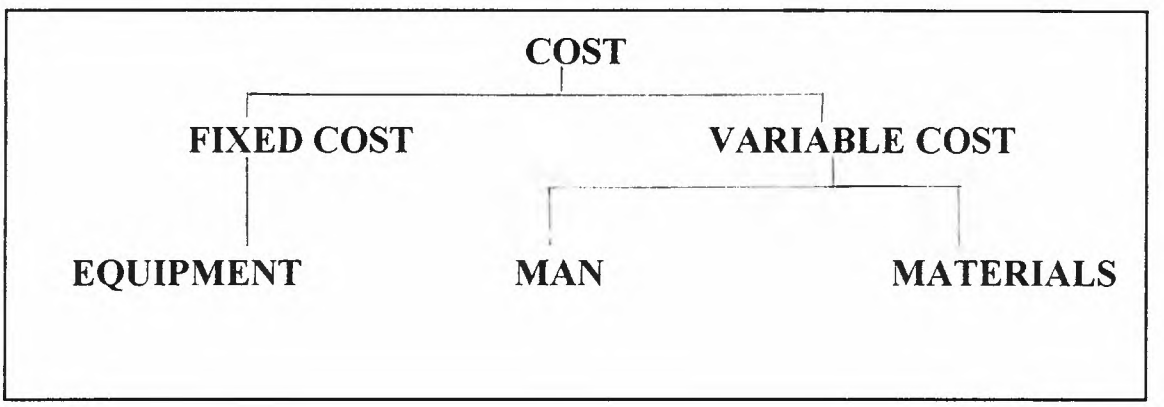
4.2. Cost:

To establish the cost incurred to identify one sputum sample up to a species level; each of the two systems were disassembled into their various procedures and steps and cost for each step was individually calculated. Figure 4.2 overleaf is a flow chart showing the procedure for evaluating one sample of *Mycobacterium* using the procedures employed at the SAIMR Figure 4.3. demonstrates the procedure for evaluating one sample of mycobacterium, to a species level as well, using HPLC.

A brief description of the steps involved in each procedure and time required for execution of each procedure are indicated in the flow charts.

The total cost was delineated into fixed and variable costs per procedure as indicated in Figure 4.1. below.

Figure 4.1. Delineation of costs:



ure 4.2. PROCEDURE FOR EVALUATING ONE SAMPLE AT THE S.A.I.M.R.

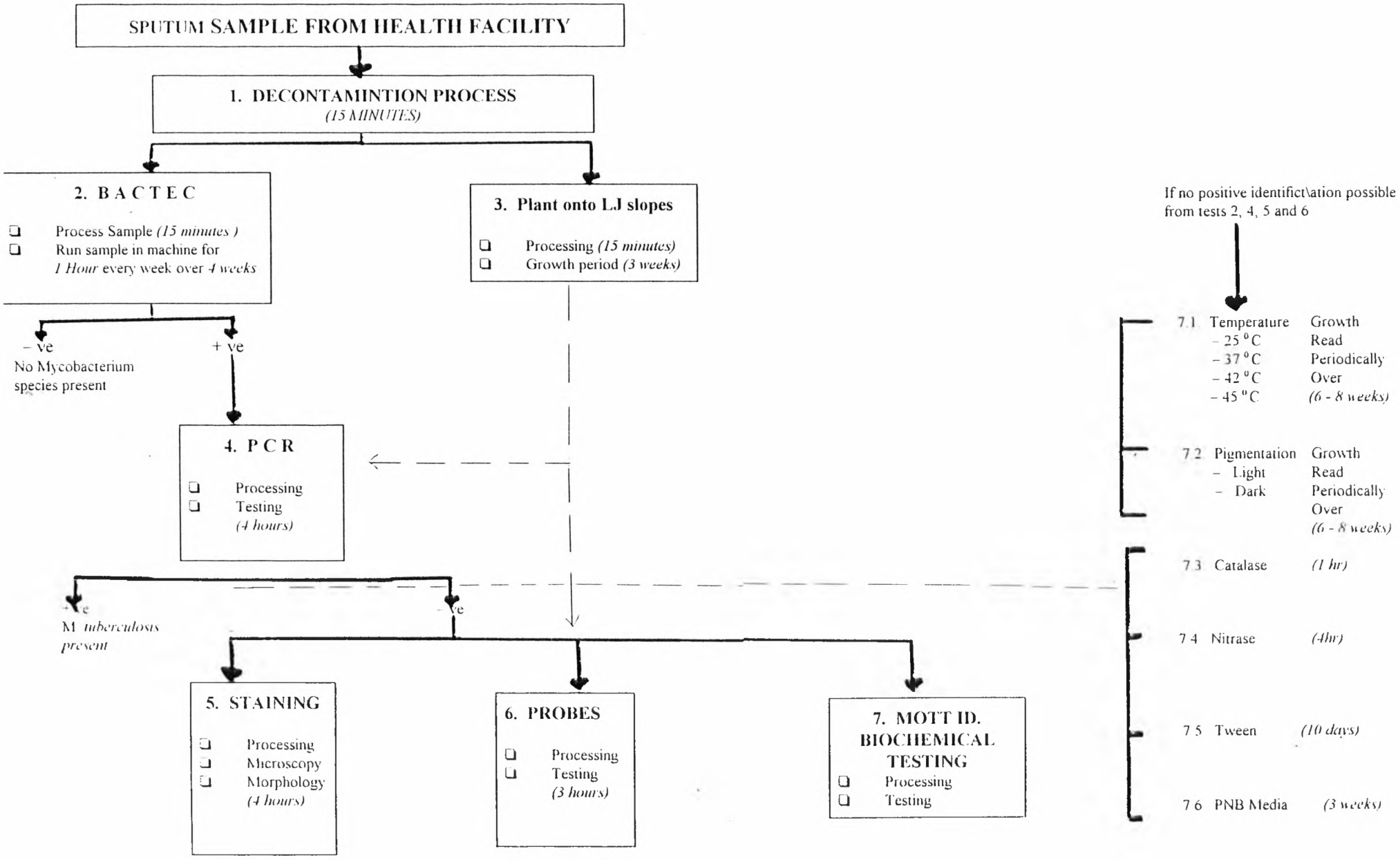
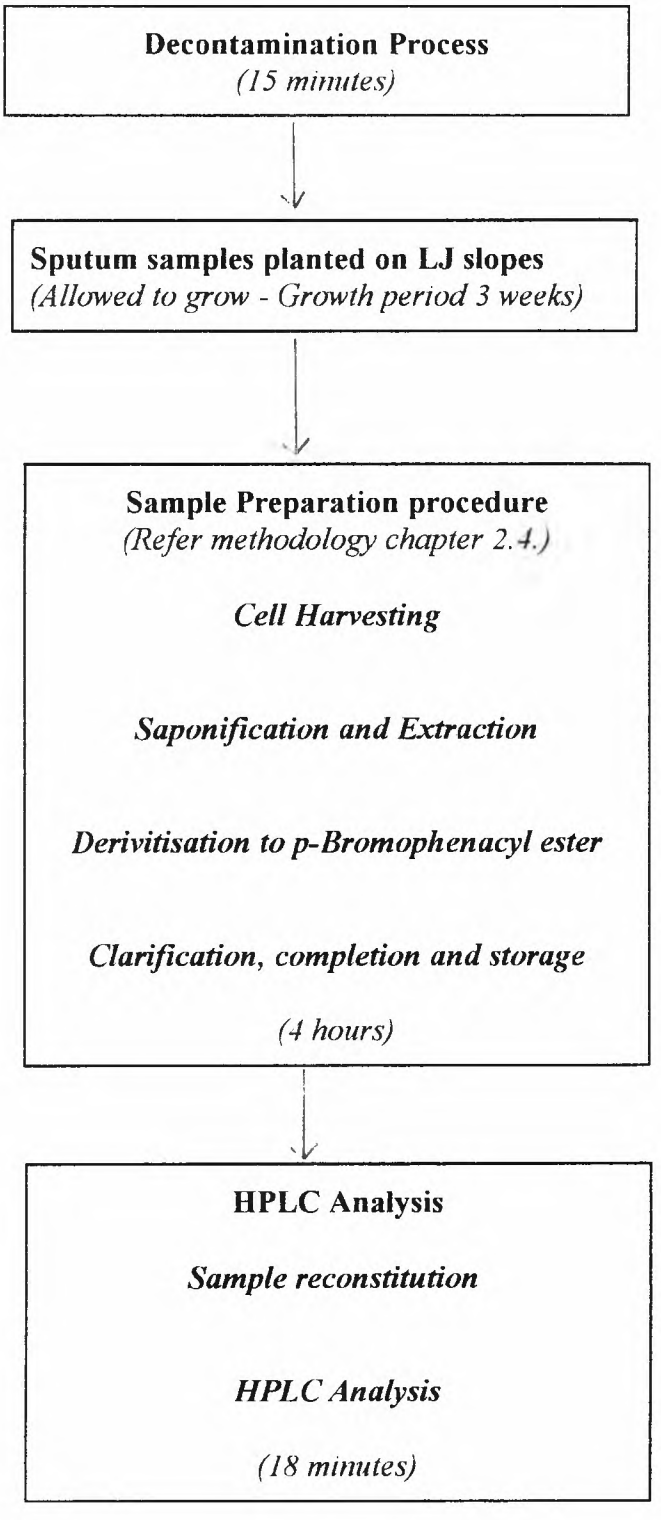


Figure 4.3. Process to evaluate one sample of mycobacteria using High Performance Liquid Chromatography:



4.2.1. Fixed costs:

Equipment:

The cost of the equipment per a single sample was established as follows:

Total Cost of equipment = Instrument cost + Service cost over life span = X

Time usage = 6/7* x 364 days x 10 years = 6/7 x 364 x 10 = 3120 days in lifespan

(**Assume all equipment is used 6 out of 7 days in the week*)

No of samples tested per day = Y

$$\textbf{Cost per sample} = \frac{\textbf{Total cost of equipment}}{\textbf{No of samples tested per day x 3120}} = \frac{\textbf{X}}{\textbf{Y x 3120}}$$

Equipment was costed according to current prices (2001) from the relevant companies.

HPLC Equipment : Beckman Coulter (011) 805 2014

BACTEC Equipment : Bactlab [Becton Dickson] (011) 792 7383

PCR Equipment : Perkin Elmer Biosystems (011) 478 0411

Probe Kits were costed using the figures provided by Mr Sam Padayachee at the SAIMR Purchasing Department (011) 489 9135.

The unit costs for equipment reflected in the cost analysis tables (Table 4.1. and Table 4.2.) were calculated as follows for each procedure.

A: BACTEC MACHINES: (BACTEC 460 TB Mycobacterial Testing System)

COST: Instrument Capital Cost = R123 000

Service Cost = R5000 x 10 years (life Span)

= R50 000

Total Cost = R173 000

TIME: Estimated life Span = 10 years

Used 6 out of 7 days per week = 6/7 x 364 = 312 days per year

therefore: Usage in life span = 312 x 10 years = 3120 days

Number of samples tested per day = 90

therefore: no of samples tested per lifespan = 90 x 3120 = 280 800

$$\text{COST PER SAMPLE} = \frac{\text{R173 000}}{\text{280 800}} = \text{R0.62 per sample}$$

B: PERKIN ELMER MACHINES: (Gene Amp PCR system 9600)

COST: Instrument Capital Cost = R107 000

Service Cost = R3 000 x 10 years (life Span)

= R30 000

Total Cost = R137 000

TIME: Estimated life Span = 10 years

Used 6 out of 7 days per week = 6/7 x 364 = 312 days per year

therefore: Usage in life span = 312 x 10 years = 3120 days

Number of samples tested per day = 48

therefore: no of samples tested per lifespan = 48 x 3120 = 149 760

$$\text{COST PER SAMPLE} = \frac{\text{R137 000}}{149\ 760} = \text{R0.92 per sample}$$

C: GENE PROBES (Kits)

To obtain the unit price per sample the cost price per kit is divided by the number of samples that can be tested by each kit:

MTB complex culture ID test kit = R1254.24 ÷ 20

= R62.71

Accuprobe MAC complex kit = R1254.24 ÷ 20

= R62.71

D: BECKMAN EQUIPMENT: (HPLC)

COST:

Instrument Cost =

Model 126 Binary Gradient pump = R 136 590.44

Model 166 single array UV Detector = R 72 320.00

Model 507 Autosampler (96 position carousel) = R 143 002.94

Phenomenex Lunar C18 column (6mmx75mm) =R 44 880.00*

Software (for Windows) [32 karat] = R 60 021.00

R 456 814.38

* Number of injections per column = 3 000
Samples tested per day 96

Therefore one column lasts = 31.25 days ~ 1 month
Therefore 6 columns would be required for a period of one year

One column costs = R 3 740 x 12
= R 44 880.00

Service Costs = R5 000 x 10 years (life span)

= R 50 000

Total Capital investment = R 456 814.38 + R50 000

= R 506 814.38

TIME: Estimated life Span = 10 years

Used 6 out of 7 days per week = 6/7 x 364 = 312 days per year

therefore: Usage in life span = 312 x 10 years = 3120 days

Number of samples tested per day = 96

therefore: no of samples tested per lifespan = 96 x 3120 = 299 520

COST PER SAMPLE: = R506 814.38 = R1.70
299 520

4.2.2. Variable costs:

Variable costs were calculated using two components:

A: MAN

This component takes into account the labour requirements for each method. Two salary scales were required. One for a laboratory technician and one for the technologist.
The salary scales were provided by the SAIMR salaries department.[21]

To calculate the labour costs for a procedure, the relevant hourly rate was multiplied by the time taken to complete a particular procedure for one sputum sample of mycobacteria.

The monthly rates were estimated as follows:

The monthly salary was divided by the number of hours worked per month.

Number of hours worked per month = 8 hrs/ day x 5 days/ week x 4 weeks/ month

= 8 x 5 x 4

= 160 hours

therefore: Technologist	Technician
= R4839.13	= R 2397.00
160	160
= R30.24/hr	= R14.98/hr

B. MATERIAL COSTS

Materials were costed from the supplying companies according to the quantities supplied by the company. Materials were also costed using data provided by the Purchasing and Media Departments at the SAIMR. [21]

The price of that material per one sample was obtained by dividing suppliers price by suppliers volume to obtain a unit price and multiplying by actual amount used for one sample in the process.

4.2.3. Cost Analysis Tables:

The cost per procedure is presented overleaf in Tables 4.1 Cost per sample per procedure at SAIMR; and Table 4.2. Cost per sample per procedure using HPLC. An analysis and interpretation of these figures will follow in Chapter 4.2.4.

Table 4.1: Cost per sample per procedure - SAIMR

Process	Fixed		Variable				Total
	Equipment	Cost	Material	Cost	Human resources **	Cost	
Decontamination			15 ml Sodium Hydroxide	R 10.18	Technician		
			15 ml M15 Phosphate buffer	R 0.17	1 x R14.98 x 0.25	R3.75	
			2 x plastic disposable pipette	R 0.50			
			1 x specimen bijoux bottle	R 1.00			
		R0.00		R 11.85		R 3.75	R15.60
2. BACTEC	BACTEC 460TB Mycobacterial Testing System *Cost per one sample* *	R0.62	5ml vial BACTEC 12HB Broth	R25.73	Technologist		
			1 x Syringe and needle	R 0.89	1 x R30.24 x 1 (4)	R120.96	
		R0.62		R 26.62		R120.96	R148.20
3.Plant sputum samples on LJ Slopes			1 x 6 - Glycerol LJ slope	R22.59	Technician		
			1 x Innoculation tube	R 0.25	1 x R14.98 x (0.3)	R 4.50	
		R0.00		R 22.84		R 4.50	R 27.34
4. PCR	PERKIN ELMER Machines Gene Amp PCR System 9600 Cost per one sample *	R0.92	2 xThermowell tubes	R 1.66	Technologist		
			2 x Eppendorf tubes	R 0.61	1 x R30.24 x 1	R 30.24	
			250 ul Buffer	R 2.52			
			11 ul P1/P2 Primer Mix	R 3.48	Technician		
			32 ul Taq	R 1.51	1 x R14.98 x 3	R 44.94	
			25 ul dNTP	R 2.85			
			4 x pipette tips	R 6.87			
			1 x Gibson pippete tip	R 6.21			
			PCR Tray	R 1.63			
			Film for instant camera	R 1.33			
		R0.92		R 28.67		R 75.18	R104.77
5. Staining			2 ml fixative	R 1.12	Technologist		

			2 x sterile discs	R 0.05	1 x R30.24 x 0.5	R 15.12	
			1 x needle and syringe	R 0.89			
			5 ml Carbol Fuchin	R 1.45			
			5ml Methylene Blue	R 1.10			
			10 ml 5 % Acid Alcohol	R 2.96			
			5 ml 20 % Sulphuric Acid	R 1.98			
		R 0.00		R15.32		R 67.55	R 82.87
6. Probes	KITS						
	MTB complex culture ID test	R 62.71	Ritipps	R 0.58	Technologist		
	Accuprobe MAC Complex Kit	R 62.71	1x inocculation loop	R 0.25	1 x R30.24 x 3	R 90.72	
	cost per one sample *		2 x sterile plastic pippete	R 0.50			
		R 62.71		R 1.33		R 90.72	R154.75
7. MOTT ID							
7.1 Catalase			0.5 ml M15 phosphate buffer	R 0.01	Technician	R 14.98	
			1.5 ml 10 % tween 80	R0.01	1 x R14.98 x 1		
			0.5 ml Hydrogen peroxide	R 1.95			
			2 x disposable plastic pippete	R 0.50			
			1 x screw cap bottle	R 1.00			
			1 x innoculation loop	R 0.25			
7.2. Nitrase			2 ml M30 phosphate buffer	R 0.02	Technician	R 29.96	
			0.17 mg Sodium Nitrate	R 0.01	1 x R14.98 X 2		
			0.25 ml conc HCl Acid	R 0.03			
			0.5 ml 0.2 % Sulphanamide	R 0.20			
			0.5 ml 0.1 5 N(napthyl)ethylene diamine dihydrochloride	R 0.52			
			1 x innoculation loop	R 0.25			
			0.5 ug powdered zinc	R 0.01			
			1 x sterile bijoux bottle	R 1.00			
			4 x disposable plastic pippete	R 1.00			

7.3. Tween			1.25 ml Tween 80	R 0.03	Technician	R 59.92	
			0.1g Disodium Hydrogen Phosphate	R 0.08	1x R14. 98 x 1 (2)		
			0.06g Potassium dihydrogen Orthophospahte	R 0.04	*refer Calc D*		
			0.6 ml Neutral red indicator	R 0.01			
			4 x plastic disposable pipettes	R 1.00			
			1 x Sterile bijoux bottle	R 1.00			
7.4. Temperature pigmentation & PNB Media			6 x 6-Glycerol LJ slope	R 15.06	Technician	R 18.73	
			1x PNB Media	R 2.51	1 x R14.98 x 0.25(5)		
			1x innoculation loop	R 0.25	*refer Calc E*		
		R 0.00		R 26.74		R123.59	R150.33
		R164.84		R133.37		R486.25	R683.86
		9.5 %		19.5 %		71 %	

Table 4.2. Cost per sample per procedure - HPLC:

Process	Fixed		Variable				Total
	Equipment	Cost	Material	Cost	Human resources	Cost	
Decontamination			15 ml Sodium Hydroxide	R 10.18	Technician		
			15 ml M15 Phosphate buffer	R 0.17	1 x R14.98 x 0.25	R 3.75	
			2 x plastic disposable pippete	R 0.50			
			1 x Specimen Bijoux bottle	R 1.00			
		R 0.00		R 11.85		R 3.75	R 15.60
2. Plant sputum specimen on LJ slopes			2 x 6-glycerol LJ slopes	R 5.02	Technician		
			1 x inocculation loop	R 0.25	1 x R14.98 x 0.1	R 1.50	
		R 0.00		R 5.27		R 1.50	R 6.77
3. Sample Preparation							
3.1. Cell Harvesting			2.0 ml Saponification Reagent	R 2.30	Technician		
			1 x 13x100 mm test tube	R 6.50	1 X R14.98 x 4	R 59.92	
			1 x plastic disposable pippete	R 0.25			
			1 x inocculation loop	R 0.25			
3.2Saponification and Extraction			Alluminum foil	R 0.10			
			2.0 ml Choroform	R 0.10			
			1 x plastic disposable pippete	R 0.25			
			1.5 ml Acidification Reagent	R 3.03			
			1 x plastic disposble pippete	R 0.25			
			1 x syringe and needle	R 0.89			
			1 x 13x100 mm test tube	R 6.50			
3.3 Derivitisation			0.1 ml Potassium Bicarb Reagent	R 0.40			
			1 x disposable plastic pipette	R 0.25			
			1.0 ml chloroform	R 0.05			
			50 ul derivitisation reagent	R 3.85			

			2 x disposable plastic pipette	R 0.25			
3.4. Clarification			1 ml Clarification reagent	R 3.16			
			1 x disposable plastic pipette	R 0.25			
			1 x syringe and needle	R 0.89			
			1x Autosampler vial	R 2.73			
		R 0.00		R 32.50		R 59.92	R 92.42
HPLC Analysis	BECKMAN SYSTEM	R 1.70	100 ul sample diluent	R 1.20	Technologist		
	model 126 pump		40 ml methanol	R 1.30	1 x R30.24 x 0.25	R 7.56	
	model 166 detector		37.5 ml Dichloromethane	R 1.43			
	model 507 Autosampler		1 x microvial	R 1.10			
	32 karat software		1 x plastic disposable pipette	R 0.25			
	Phenomenex Lunar C18 column						
	cost per one sample* calc F *						
		R 1.70		R 5.28		R 7.56	R 14.54
		R 1.70		R 49.62		R72.73	R129.33
		6 %		38%		56 %	

Notes to Table 4.1 and Table 4.2

* Calculation expansions for Equipment for each procedure is reflected in 4.2.1. Fixed Costs - Equipment

** Human resource Calculation Expansions are reflected under 4.2.2. Variable costs - Man

D. Human Resource time for Tween MOTT identification test is multiplied by 2 because the test is conducted twice (days 5 and 10).

E. Human Resource time for temperature, pigmentation and PNB media is multiplied by five because the test is repeated on days 3; 7; 14; 21 and 28.

4.2.4. Interpretation and Discussion of Cost Results:

4.2.4.1. Summary of costs per procedure:

Tables 4.3 below provides a brief summary of the total for each procedure as well as the percentage contribution of each component of the mycobacterial identification system. Following the summary table is a brief interpretation and analysis of these figures with an aim to establish viability of the HPLC system.

A suggestion will be offered following this analysis indicating how the current system may be modified to include HPLC and to increase the efficiency of *Mycobacterium* identification to a species level.

Table 4.3. Summary of costs for all procedures:

PROCEDURE	EQUIPMENT	MATERIAL	HUMAN RESOURCE	TOTAL
SAIMR				
Decontamin.	R 0	R 11.85	R 3.75	R 15.60
BACTEC	R0.62	R 26.62	R 120.96	R 148.20
Plant samples	R 0	R 22.84	R 4.50	R 27.34
PCR	R0.92	R 28.67	R 75.18	R 104.77
Staining	R 0	R 15.32	R 67.55	R 82.87
Probes	R62.70	R 1.33	R 90.72	R 127.61
MOTT ID	R 0	R 26.74	R 123.59	R150.33
Total	**R164.84**	**R133.37**	**R 486.25**	**R683.86**
	9.5 %	19.5 %	71 %	
HPLC				
Decontamin.	R 0	R 11.85	R 3.75	R 15.60
Plant sample	R 0	R 5.27	R1.50	R 6.77
Sample prep	R 0	R 32.50	R 59.92	R 92.42
Analysis	R 1.70	R 5.28	R 7.56	R 14.54
Total	**R 1.70**	**R 49.60**	**R72.73**	**R123.33**
	6 %	38 %	56 %	

Calculation 1:

$$\frac{\text{Cost of HPLC procedure}}{\text{Cost of SAIMR procedure}} = \frac{R123.33}{R683.86} \times 100$$

$$= 0.18 \times 100$$

$$= 18\ \%$$

$$\approx 20\ \%$$

If the aim of this study was to investigate the possibility of replacing the current *Mycobacterial* system with the HPLC system, the above simple calculation would be sufficient to prove the transcendency of the HPLC system with regard to cost, as the HPLC identification procedure is approximately of the cost of the system currently employed at the SAIMR

However the aim of this study is not to simply replace the current system, but to eliminate the costly and/or inefficient procedures with the ultimate aim of having a complete, efficient yet cost effective system to identify *Mycobacteria* to a species level. In order to determine the value of each factor, we must take cognisance of how each procedure contributes to this aim.

BACTEC is used in the initial phases to determine the presence of *Mycobacteria*. This is of value as the battery of tests required to make a species identification need not be conducted if it can be ascertained that no *Mycobacterium* species is present.

Staining may also be included at this early stage to test for the presence of Acid Fast Bacilli (AFB) and to provide an indication, through morphological observations, which species may be present in the specimen.

PCR may also be implemented in these early stages to differentiate between *M. tuberculosis* and mycobacteria other than Tuberculosis. *M. tuberculosis* is the most likely organism to be isolated in the South African environment, and a test that quickly identifies this specific organism is essential. Once a diagnosis of *M. tuberculosis* is made, therapy specific to this organism can be implemented with the assurance that the correct species is being treated with the correct drug. Specificity of treatment is essential if Multiple Drug Resistant (MDR) strains are to be reduced.

The next step in the identification procedure is to identify the mycobacteria other than Tuberculosis. The three procedures investigated compare as follows:

Table 4.4: Comparison of MOTT identification procedures:

Procedure	Cost	Sensitivity
HPLC	R123.33	96.25 %
Probes	R154.75	82.5 %
Biochemical tests	R150.33	80 %

HPLC transcends both the Gene Probe and Biochemical Tests with regard to cost and efficiency in identifying the specimen to a species level. Further, probes can only identify a limited range of species and more than one sample preparation procedure is required for both probes and biochemical testing to complete a species level identification. HPLC is hence the obvious choice for MOTT identification.

4.2.4.2. Suggested Mycobacterial Identification System :

Table 4.5. Summary of costs for all procedures of suggested system:

Decontam.	R 0	R 11.85	R 3.75	R 15.60
BACTEC	R 0.62	R 26.62	R 120.96	R 148.20
Plant samples	R 0	R 22.84	R 4.50	R27.34
PCR	R 0.92	R 28.67	R 75.18	R 104.77
Staining	R 0	R 15.32	R 67.55	R 82.87
HPLC	R 1.70	R 49.60	R 72.73	R 123.33
	R 3.24	**R 154.90**	**R 344.67**	**R 502.81**

Calculation 2:

$$\frac{\text{Cost of Suggested Procedure}}{\text{Cost of SAIMR procedure}} = \frac{R502.8133}{R683.86} \times 100$$

$$= 0.73 \times 100$$
$$= 73\ \%$$

R683.86 - R502.81 = R181.05

$$\frac{\text{Cost difference}}{\text{Cost of SAIMR procedure}} = \frac{R181.05}{R683.86} \times 100$$

$$= 0.27 \times 100$$
$$= 27\ \%$$

The proposed system is approximately one third the cost of the current system for mycobacterial identification.

4.3. Time:

In order to determine the time savings; we must account for the time difference of the two procedures that were eliminated from the identification system proposed in chapter 4.2.4.2. above. The time taken to complete identification of a system using HPLC must be factored in. However; HPLC and Gene probe require approximately the same amount of time to complete. Therefore the time difference is based on the removal of the biochemical testing

system only. This, as indicated in the flow chart in Figure 4.2. , would result in a time saving of approximately 3 - 8 weeks. The proposed system is therefore time efficient.

5. Conclusion:

The resurgence of tuberculosis in South Africa in proportions that have overwhelmed the health system has shifted the emphasis of mycobacterial identification toward early detection, identification and antimicrobial testing of *M. tuberculosis*. [15][16]

Although the importance of *M. tuberculosis* is stressed, the other clinically important species of mycobacteria cannot be overlooked. The initiation of appropriate antimicrobial therapy often depends on the specific organism causing the infection.[17][18]

Identification of mycobacteria in South African laboratories still remains a fastidious, difficult and time consuming procedure. The morphological, cultural and biochemical tests used for identification of *Mycobacteria* require specialised knowledge as well as trained technicians. [19][20]

The novel techniques (Gene Probes) that has been adopted; decreased this identification time from at least three weeks to a few hours. However they have a limited range of speciation, require more than one sample processing method and are extremely expensive.[4][8]

HPLC provides a system that uses one sample preparation procedure to identify a wide range of mycobacterium species with a sensitivity to the mycobacterium species of 96.25 % . By replacing the two methods mentioned above with HPLC, the time required for identification was reduced to approximately 4 hours and decreasing costs by 27 %. Although it was suggested that HPLC be integrated as part of a comprehensive system, HPLC can achieve identification as an independent system thereby decreasing cost requirement by 80 %.

The value of the HPLC system is, however, not simply derived from an increased sensitivity to the species or a reduction in cost and time. The value of implementing the system is derived form the additional benefit and utility resulting from its use.

Quick turn-around- times for identification to a species level allows correct treatment to be implemented. This could mean the difference between life or death for a patient who is immuno-compromised and requires rapid initiation of treatment.

The likelihood of this patient requiring other anti-tuberculosis therapy in the future is high. Avoiding the use of empiric therapy prevents limitations on the choices of drugs that can be used in this patient in the future, as no unnecessary drug resistance has been acquired through irrational drug use. The cost of a saving a life through rational drug use cannot be measured.

The burden on the health system would be decreased if there were a decrease in mycobacterial identification costs. Money spent routinely on expensive novel techniques could be used elsewhere. Furthermore the decreased cost per specimen makes identification more affordable to a greater number of health institutions hence making tailored therapy more available to a larger number of South Africans. The large initial capital investment and training required to use the system can be justified by the long term savings as well as the additional utilities and benefits derived from conducting individual tests.

This study demonstrates the viability of HPLC and necessitates that HPLC be implemented in South African laboratories for routine application in mycobacterial identification.

6. Future Objectives:

For effective implementation of the HPLC system a library containing mycobacterial chromatograms idiosyncratic to South Africa needs to be developed. This library will allow development of a mycobacterial identification flow chart that will facilitate the visual interpretation of the mycobacterial chromatograms. Also pattern recognition software developed to mechanically identify the chromatograms generated could result in further savings with regard to human resource costs and time.

APPENDIX:

Appendix 1: Quality control:

A: Instrument operating conditions:

1. Maintain records of all service, maintenance intervals and calibration done.

a. Service the chromatograph and verify it meets the manufacturer's specifications at least every 18 months.

b. Verify injector carry over is within manufacturer's tolerance. This may be done as part of a periodic maintenance procedure, or as part of the control procedure when performing analyses.

2. Verify the instruments function. Turn on the instrument 30 minutes before use. Check the solvent bottles, waste bottles and the compressed air. Do not operate the chromatograph until all needed repairs or adjustments are performed.

a. Verify that the room temperature is in the range of 15 – 26 °C. (This is important to maintain sample integrity)

b. Verify that the sparger or vacuum degasser (if used) is operating.

c. Prime the pump(s) according to the manufacturer's procedure. Set conditions so that solvent flow bypasses the column. Visually verify that the pump(s) show no evidence of "pulsing "at high flow rate.

d. After stopping the pump redirect the solvent flow through the column according to the procedure for each individual machine, and then increase the flow rate gradually to 2.5 ml/min.

e. Load the HPLC method file containing the specified gradient.

Appendix 2: HPLC Method File Set-up; Table 2 : Gradient Conditions

f. Periodically verify that the pump(s) produce a constant flow, i.e. +/- 2.5 ml/min, while operating the gradient.

g. Verify the fluid path is free of any leaks.

h. Using the solvent conditions for the assay method the suggested tolerance for the systems pressure is 10 % of the previous days starting pressure.

i. Replace the inline filter and/or guard column if the day's starting pressure is the total pressure exceeds the pressure limit recommended by the column manufacture. The guard column should last approximately 250 injections.

j. If replacement of the inline filter and /or the guard column fails to bring the pressure into tolerance; replace the analytical column. The analytical column should last approximately 1000 injections

k. Verify the column oven temperature is 35 °C +/- 1 °C.

l. Verify that the detector baseline is stable while the chromatograph is operating under the initial solvent conditions for the assay.

B: Procedure controls:

1. The internal reference standards (LMW – ISTD and HMW - ISTD) were first added to all samples and controls at 8 ug/100 ul and 4 ug / 100 ul, respectively, assuming a 100ul injection. These standards are included in the sample diluent

The mean retention time of the HMW –ISTD should be in the range of 7.9 to 10.0 minutes (machine and column dependant)

2. A negative derivatisation control is prepared from a non mycolic acid – containing organism such as *Candida albicans* grown on LJ slopes, and containing both the LMW – ISTD and the HMW – ISTD The chromatogram produced by the negative derivitisation control can be used to measure the performance of the HPLC system. Mycolic acid peaks should not be detected in the region between the LMW-ISTD and the HMW-ISTD. ***Appendix 3: Chromatogram 1: Candida albicans***

3. A culture of *M. smegmatis* grown under standardized conditions can be processed with the specimens to serve as a positive control for the sample preparation procedure. The positive control should be run at the beginning of run and after every 15 samples,

and at the end of each sample set.

C: Solvent purity verification:

Chloroform and dichloromethane decompose in the presence of heat, light and air to form; hydrochloric acid, chloride ions, phosgene and free chlorine. [11] The presence of these contaminants interferes with the mycolic acid assay, and can damage the chromatography column. Accordingly, these solvents can be tested to confirm the absence of breakdown products before use and at intervals, thereafter. The solvents should not be used if any of the following tests are positive.

1. Reagents:

a. Silver nitrate reagent (for chloride determination)

Silver nitrate	4.25 g
Reagent-grade water	up to 250 ml

Place the $AgNO_3$ in a 250 ml volumetric flask. Bring to volume with water. Store in a dark amber bottle at ambient temperature.

Expiration: 1 year

b. Potassium Iodide (for free chlorine determination)

Potassium Iodide (KI)	10 g
Reagent-grade water	up to 100ml

Place the KI in a 100 ml volumetric flask. Bring to 100 ml with water. Store in a dark amber bottle at ambient temperature.

Expiration: 1 year

c. Sodium hypochlorite (for positive control)

Dispense 5.25 % sodium hypochlorite (household bleach) into a dark amber bottle and store at an ambient temperature.

Expiration: 1 year

2. Procedure

a. To test for acid and chloride, shake approximately 17 ml of chloroform or 20 ml dichloromethane with 25 ml water for a minimum of 1 min. Allow to separate, and then draw off the upper (aqueous) phase.

- To test for acid, add a small piece of blue litmus to 10 l of the aqueous phase. For a negative test, the litmus should remain blue. If the test is negative, verify the proper function of the litmus by adding a few drops of acidification reagent to cause a positive reaction.
- To test for chloride ion, add 0.25 ml of silver nitrate reagent to another 10 ml of aqueous phase. A negative reaction is indicated by the absence of turbidity (indicates no chloride ions present). If the test is negative, verify the reagent function by adding a few drops of 5.25 % sodium hypochlorite and mixing to cause formation of a precipitate, representative of a positive reaction.

b. To test for free chlorine, combine 10 ml of chloroform or dichoromethane with 10 ml water containing 0.10 ml potassium iodide reagent. Shake for 1 min and allow to separate.

- A negative test is indicated by the lack of a purple tint in the bottom (aqueous) layer and indicates the absence of free chlorine.
- If the test is negative, verify the reagent function by adding a few drops of bleach to the mixture and shaking again to cause formation of a purple tin, representative of a positive reaction.

APPENDIX 2: HPLC METHOD FILE SETUP

The following conditions have been established for stable separation of mycolic acids. Recommended instrument settings are summarised in Tables I through V.

Table I: Instrument control Settings

Table I: Instrument control Settings			
Column Oven	Set point = 35 °C	Low = 34 °C	High = 36 °C
Pressure Limits	Set point = None	Low = 600 psi	High = 3000 psi

Pump compressibility Setting: Adjust to achieve 25.0 +/- 0.5 ml in 10.0 +/- 0.1 minutes when operating the gradient specified in Table I.

U.V. Detector Wavelength: Set Point = 254 or 260 nm

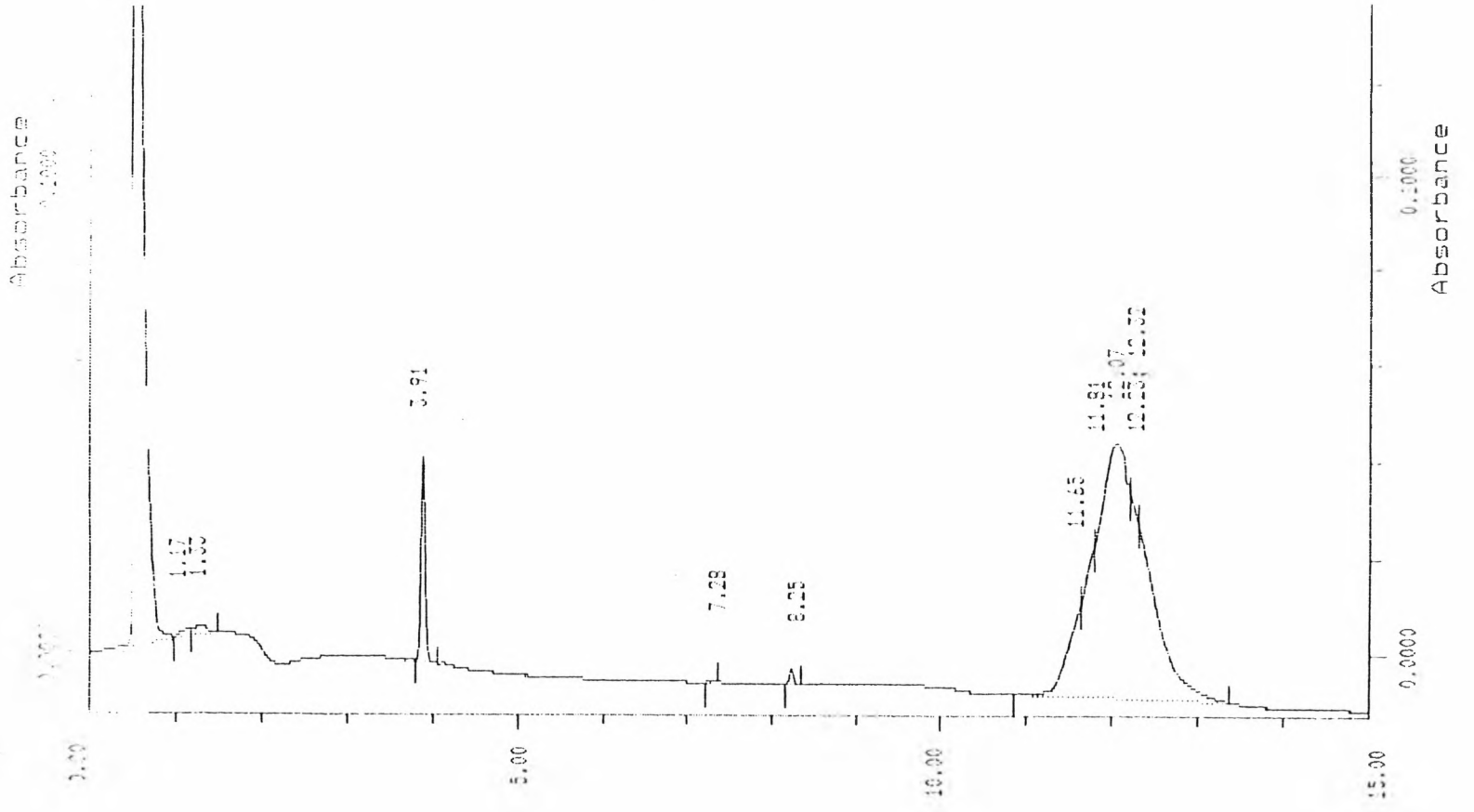
Other Detector settings: Use factory defaults.

Table II: Gradient conditions

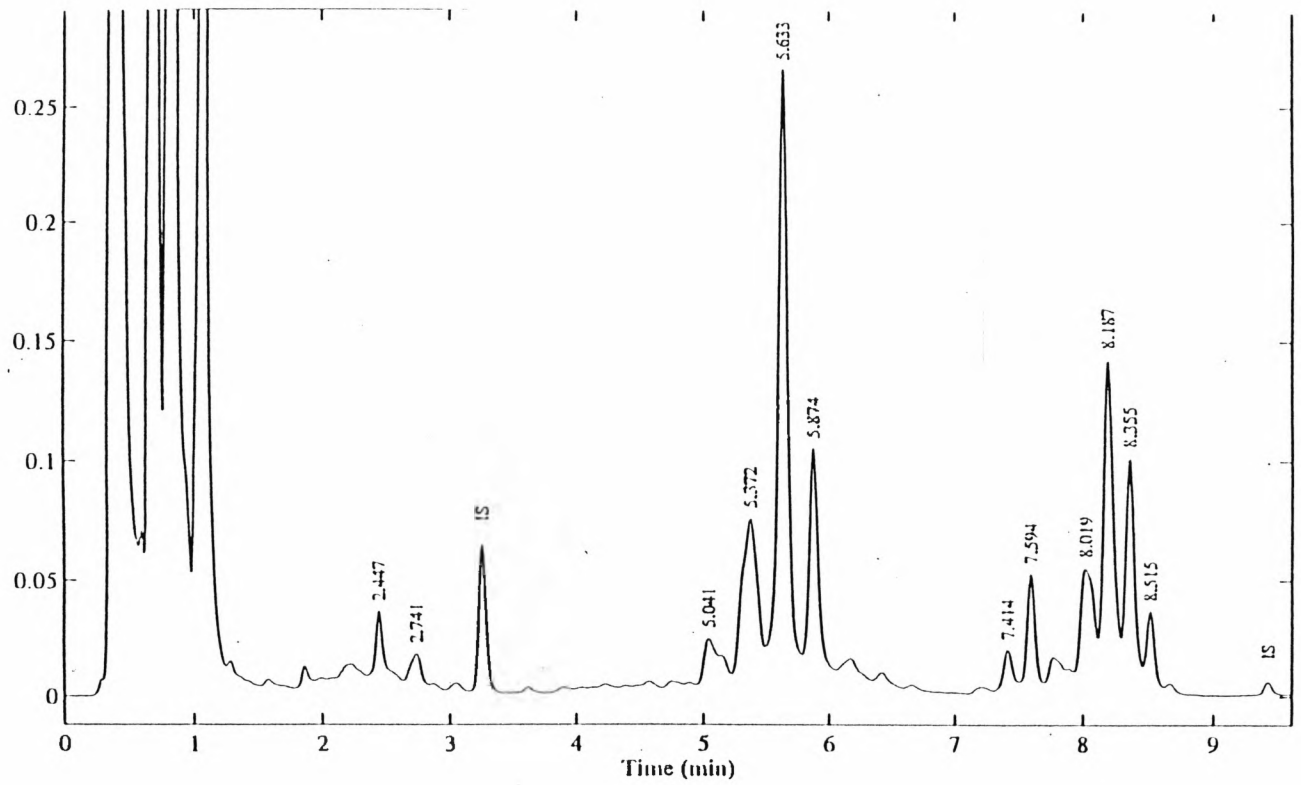
Table II: Gradient conditions[a]			
Time (min)	**Flow Rate (ml/min)**	**Percent Methanol (Solvent A)**	**Percent Dichloromethane (Solvent B)**
Initial	2.5	98	2
1.0	2.5	80	20
10.0	2.5	35	65
10.5	2.5	98	2
15.0[b]	2.5	98	2

a. The solvent concentrations are adjusted using a linear gradient
b. The interval from 10.5 to 15 min is included to standardise the column wash time in order to re-equilibrate the column with approximately 20 void volumes of the initial solvent mixture.

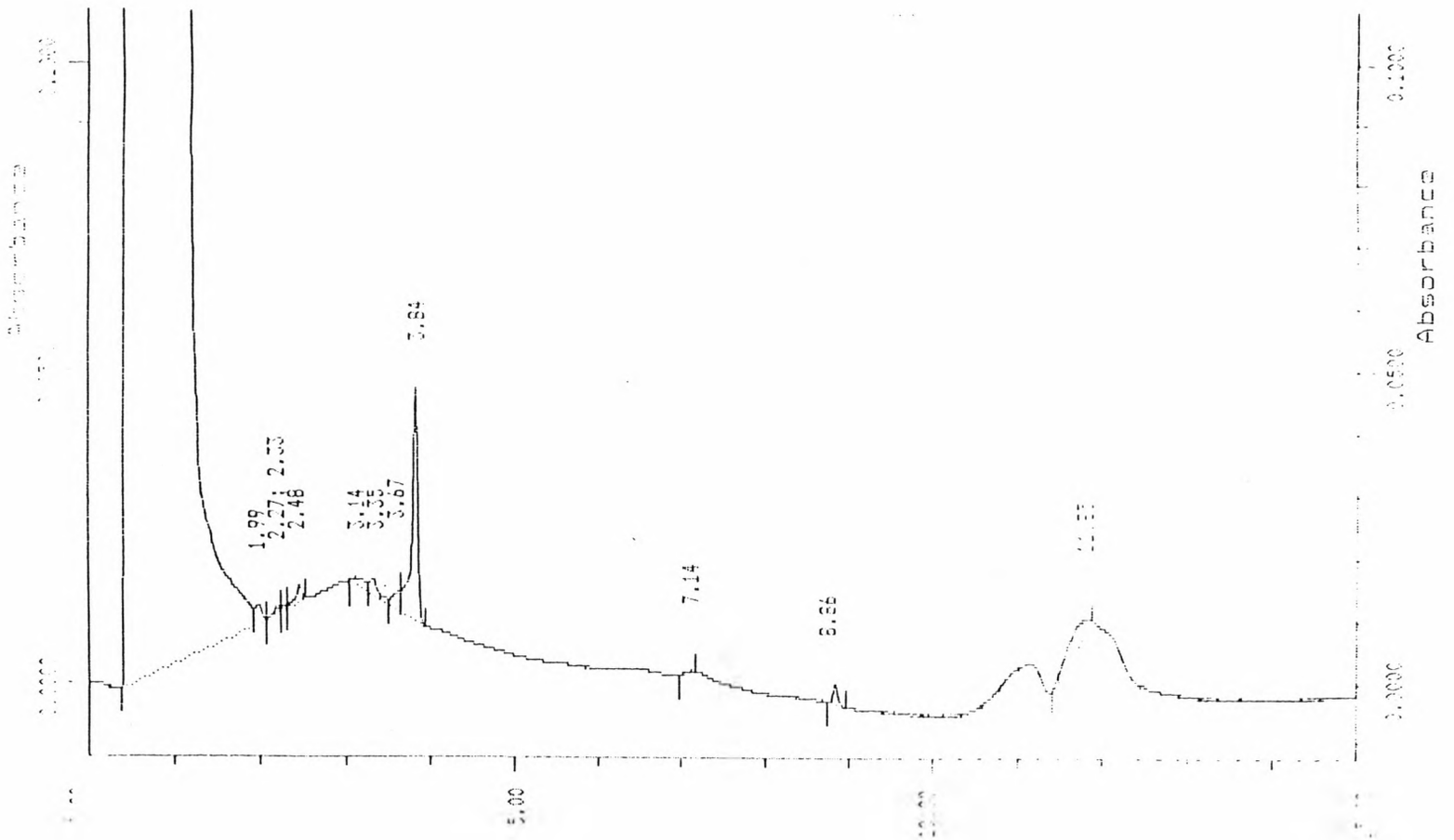
Appendix 3: Chromatogram 1: *Candida albicans* (ATCC 10231)



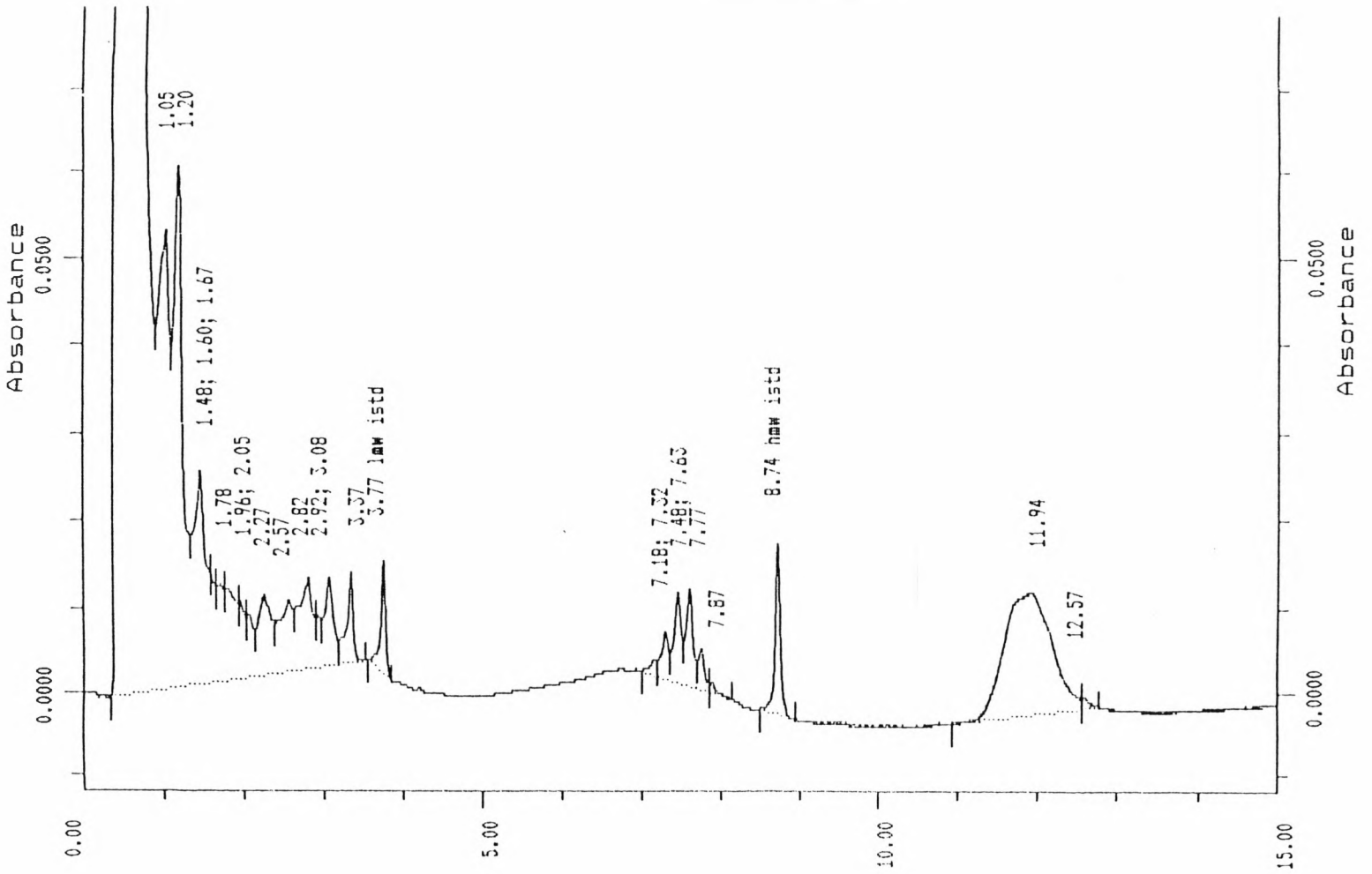
Appendix 4: Chromatogram 2: *M. intracellulare*



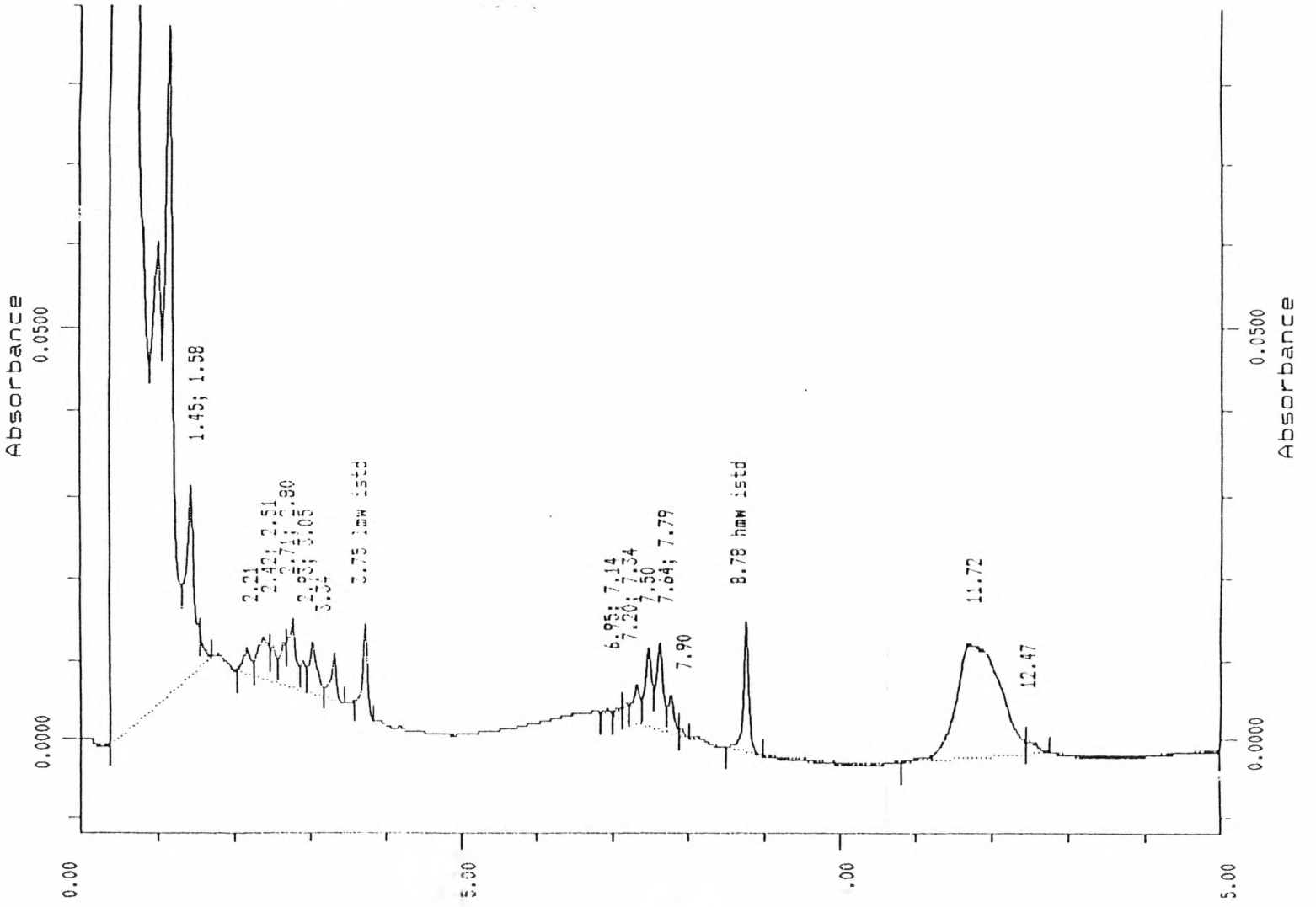
Appendix 5: Chromatogram 3: Non *mycobacterium* Specimen



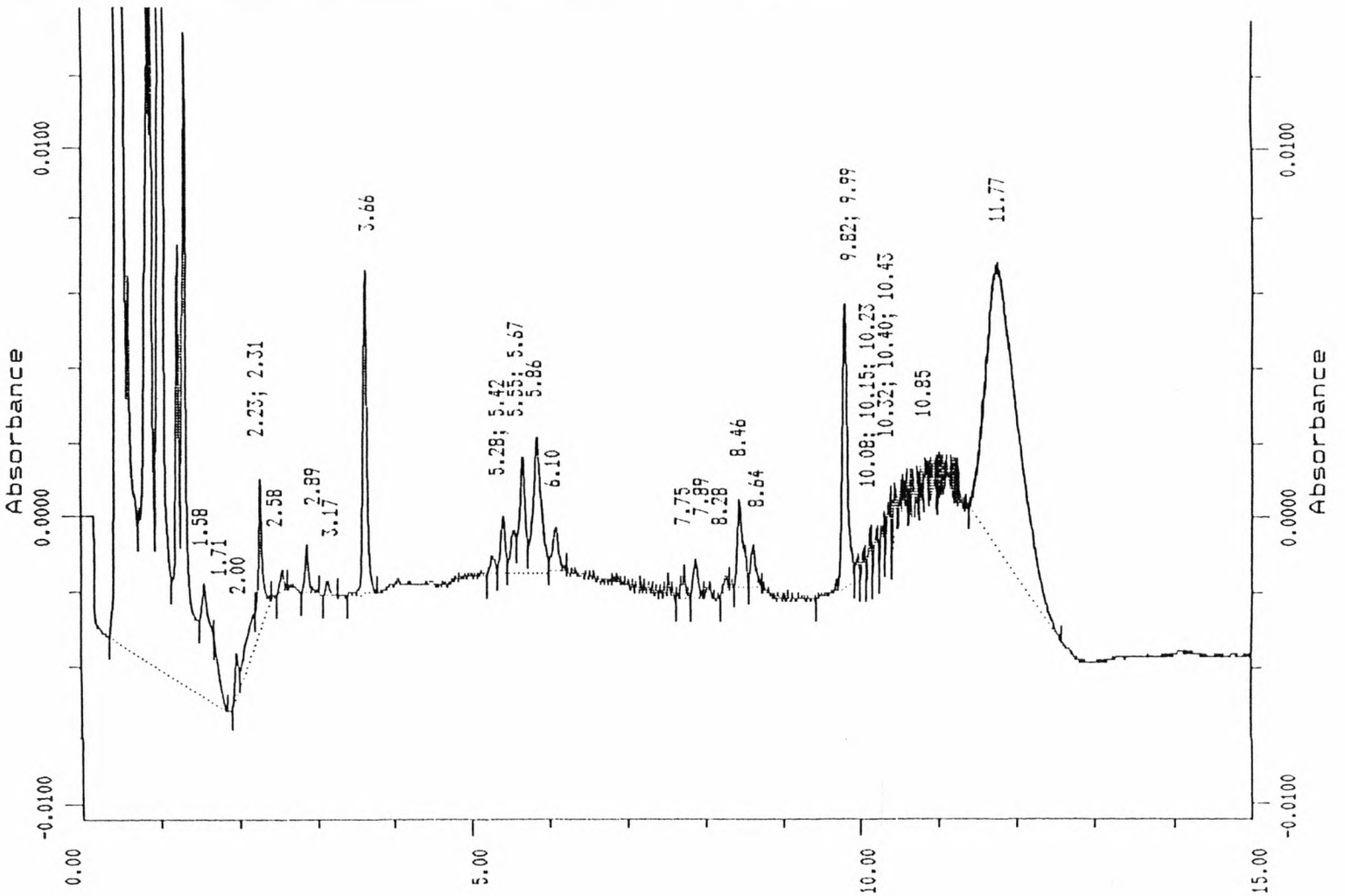
Appendix 6 : Chromatogram : ATCC 18280 - Reference Standard for *M.tuberculosis*



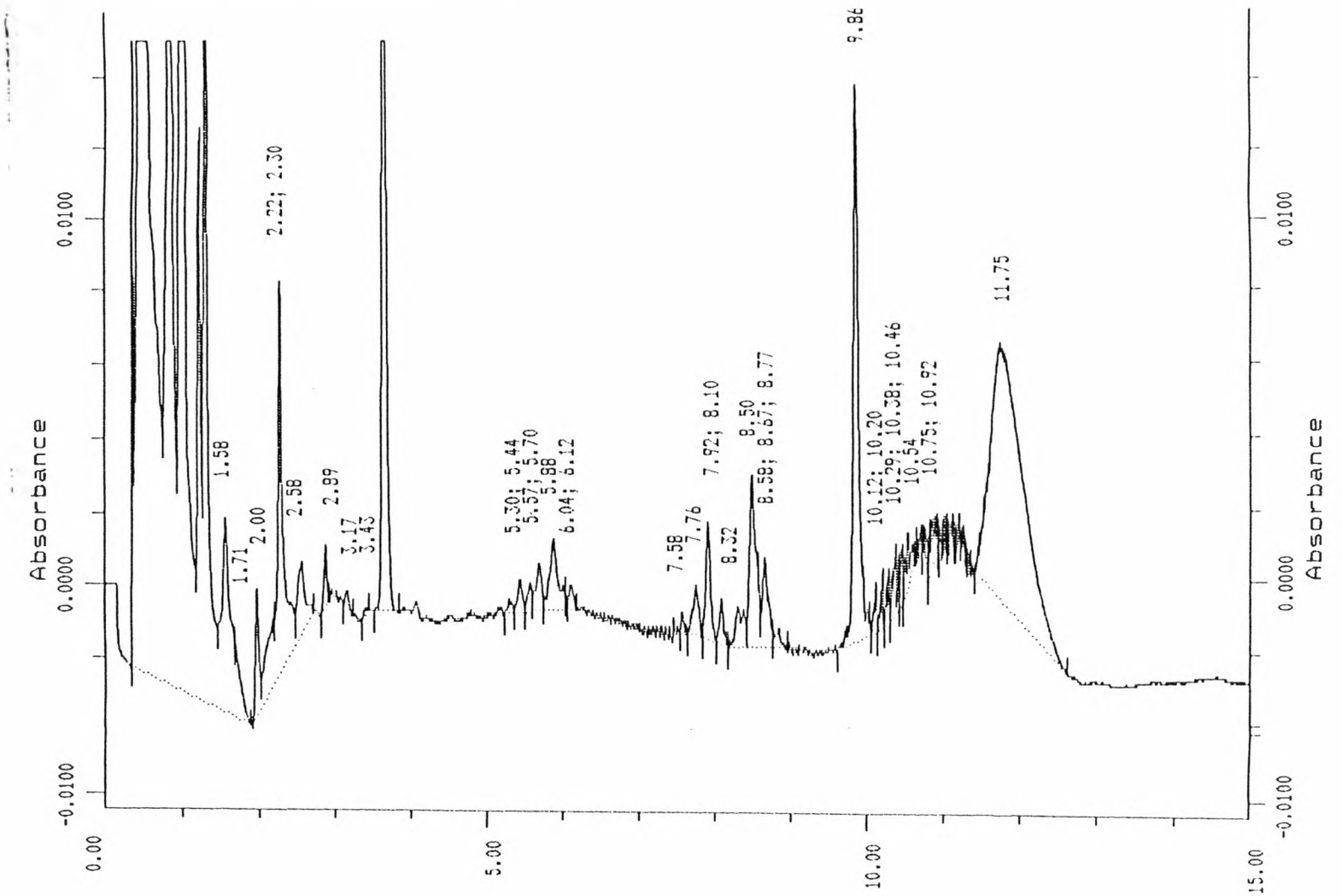
Appendix 7 : Chromatogram : Clinical isolate for *M.tuberculosis*



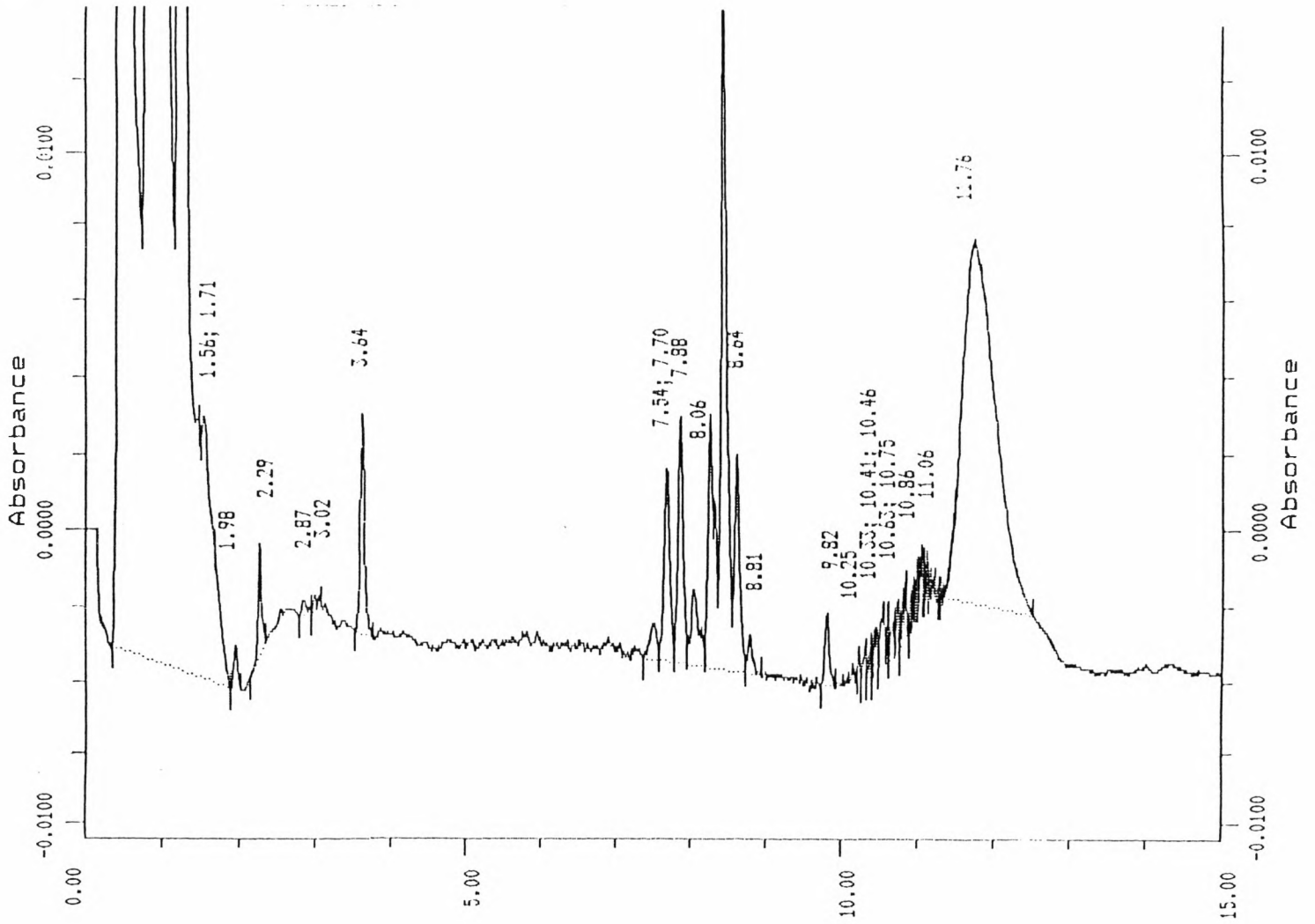
Appendix 8 : Chromatogram : ATCC 15769 -Reference standard for *M. avium*



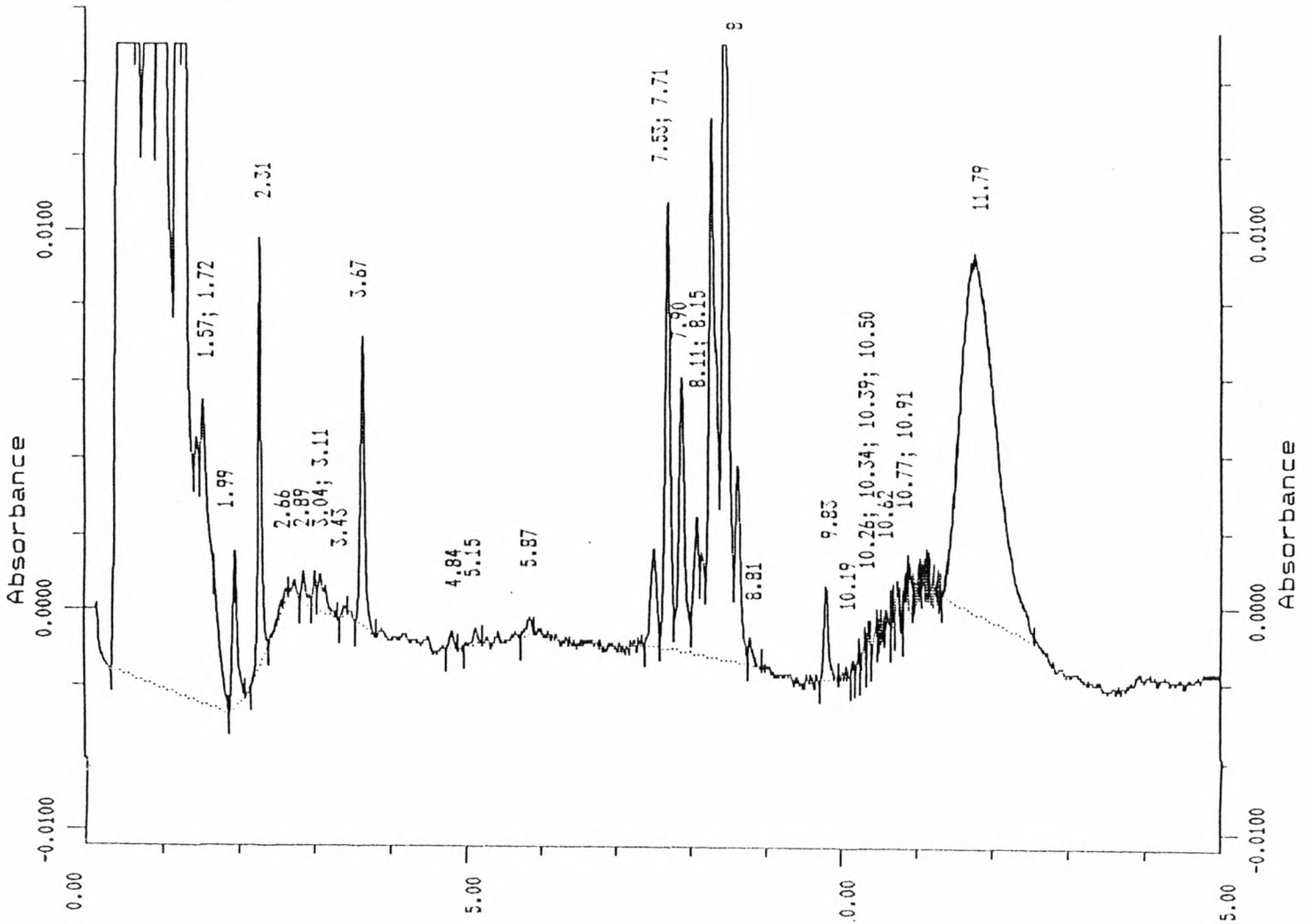
Appendix 9 : Chromatogram : Clinical isolate for *M. avium*



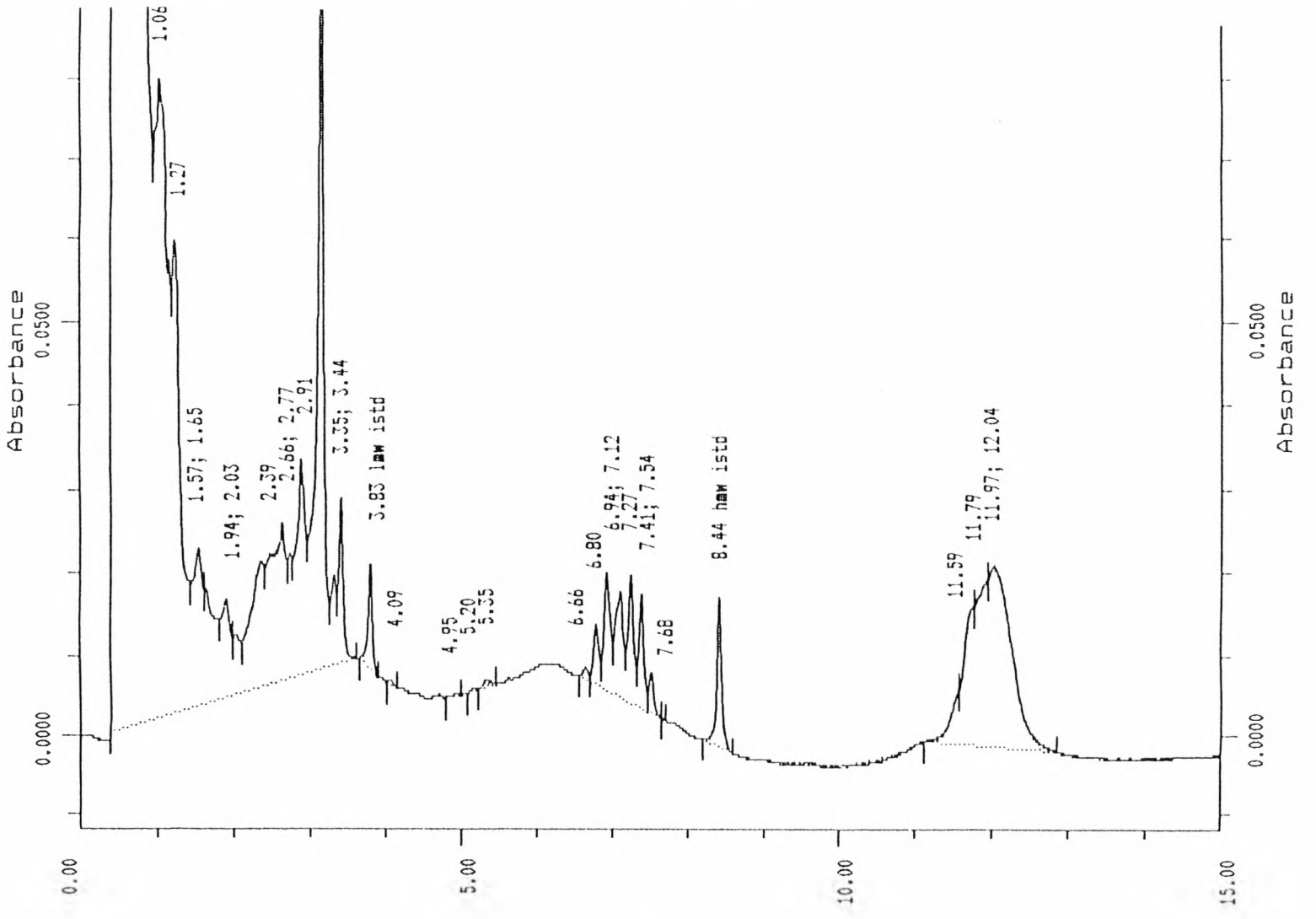
Appendix 10 : Chromatogram : ATCC 7091 -Reference standard for *M. scrofulaceum*



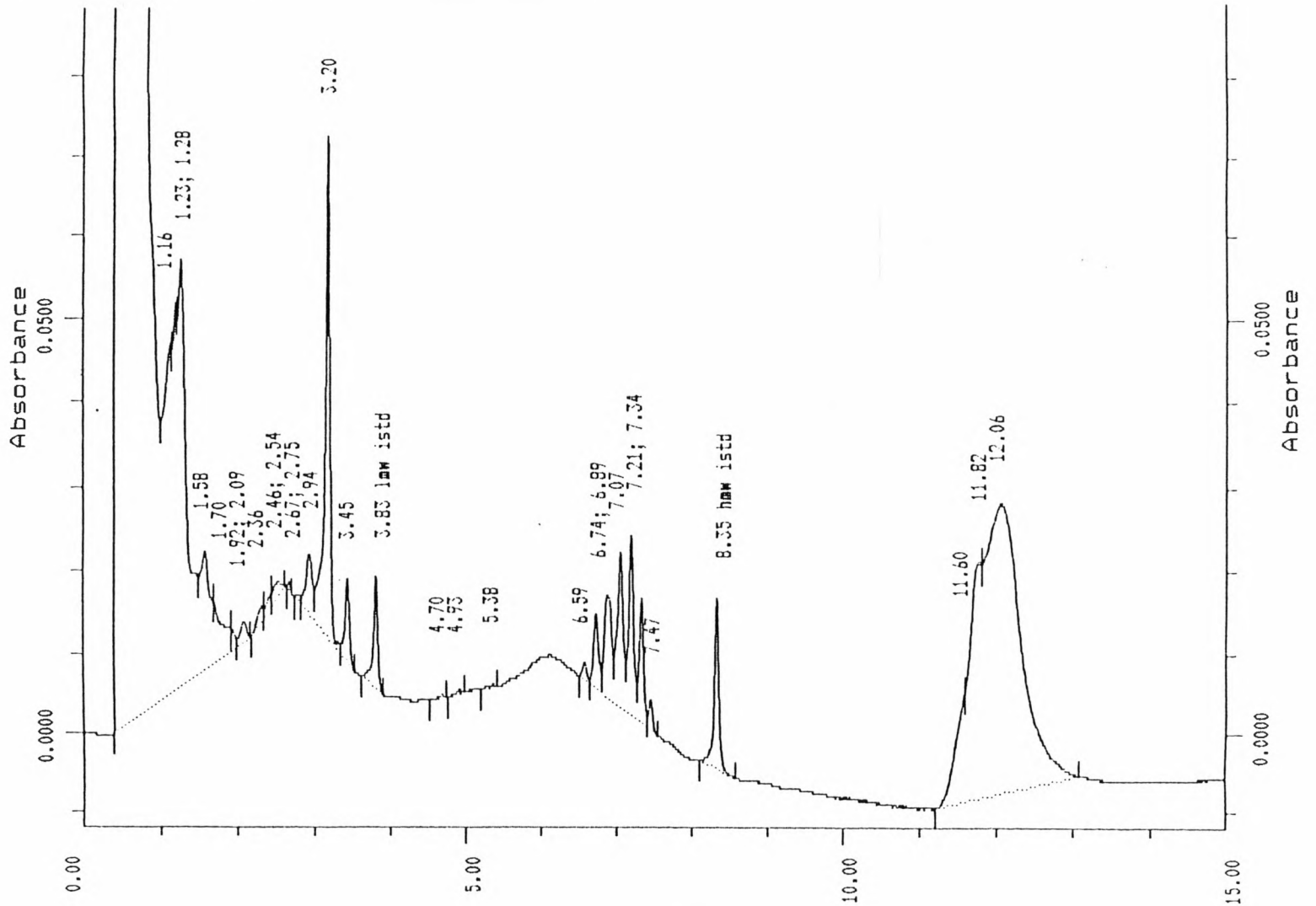
Appendix 11 : Chromatogram : Clinical isolate for *M. scrofulaceum*



Appendix 12 : Chromatogram : ATCC 25221 -Reference standard *M. kansasii*



Appendix 13: Chromatogram : Clinical isolate for *M. kansasii*



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