#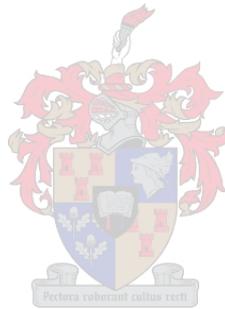 GENERATION OF A DATABASE OF MASS SPECTRA PATTERNS OF SELECTED *MYCOBACTERIUM* SPECIES USING MALDI-ToF MASS SPECTROMETRY

By

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 8 December 2008

SUMMARY

The genus Mycobacterium is a group of acid–fast, aerobic, slow- growing organisms which include more than 90 different species. A member of this genus, *Mycobacterium tuberculosis*, belonging to the Mycobacterium tuberculosis complex (MTB), is the causative agent of tuberculosis (TB). This disease is currently considered a global emergency, with more than 2 million deaths and over 8 million new cases annually. TB is the world's second most common cause of death after HIV/AIDS. About one-third of the world's population is estimated to be infected with TB. This catastrophic situation is further compounded by the emergence of Multi Drug Resistant tuberculosis (MDR-TB) and in more recent times, Extensive Drug Resistant tuberculosis (XDR-TB).   Early diagnosis is critical to the successful management of patients as it allows informed use of chemotherapy. Also, early diagnosis is also of great importance if the menace of MDR-TB and XDR-TB is to be curbed and controlled.

As MTB is highly infectious for humans, it is of paramount importance that TB be diagnosed as early as possible to stop the spread of the disease. Traditional conventional laboratory procedures involving microscopy, culture and sensitivity tests may require turnaround times of 3-4 weeks or longer. Tremendous technological advancement over the years such as the advent of automated liquid culture systems like the BACTEC® 960 and the MGIT^{TM} Tube system, and the development of a myriad of molecular techniques most of which involves nucleic acid amplification (NAA) for the rapid identification of mycobacterial isolates from cultures or even directly from clinical specimens have contributed immensely to the early diagnosis of tuberculosis. Most of these NAA tests are nevertheless fraught with various limitations, thus the search for a rapid, sensitive and specific way of diagnosing tuberculosis is still an active area of research. The search has expanded

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to areas that would otherwise not have been considered 'conventional' in diagnostic mycobacteriology. One of such areas is mass spectrometry.

This study joins the relatively few studies of its kind encountered in available literature to establish the ground work for the application of mass spectrometry, specifically Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS) in the field of diagnostic mycobacteriology. This is an area which is in need of the speed, sensitivity and specificity that MALDI-ToF technique promises to offer. Since this technology is still in its infancy, the use of utmost care in the preparation of reagents, and the handling and storage of the organisms used to generate reference mass spectra for the database cannot be overemphasized. Similarly, the optimization of certain crucial experimental factors such as inactivating method and choice of matrix is of paramount importance.

The main aim of this thesis was to generate a database of reference mass spectra fingerprints of selected (repository) Mycobacterium species. This necessitated the standardization of an experimental protocol which ensured that experimental factors and the various instrument parameters were optimized for maximum spectra generation and reproducibility. A standard operating procedure (SOP) for generating the database of reference mass spectra finger print of selected Mycobacterium species was developed and used to investigate the ability of the database to differentiate between species belonging to the same clinical disease complex as well as the non-tuberculosis complex.

The findings of this study imply that if the defined protocol is followed, the database generated has the potential to routinely identify and differentiate (under experimental conditions) more species of

Mycobacterium than is currently practical using PCR and its related techniques. It is therefore a realistic expectation that when the database is clinically validated and tested in the next phase of the study, it will contribute immensely to the diagnosis of tuberculosis and other mycobacterioses. It will also aid in the identification of emerging pathogens particularly amongst the non-tuberculous mycobacteria.

OPSOMMING

Die genus Mycobakterium is 'n groep suur vaste, aerobiese, stadig groeiende organisms wat meer as 90 spesies insluit. *Mycobacterium tuberculosis*, 'n lid van die genus, is die oorsaak van tuberkulose (TB) en maak deel uit van die *Mycobacterium tuberculosis* kompleks (MTB). Tuberkulose word tans gesien as 'n wêreldwye problem met meer as twee miljoen sterftes en oor die 8 miljoen nuwe gevalle per jaar. TB is die tweede algemeenste oorsaak van sterftes naas MIV/AIDS. Daar word beraam dat een derde van die wêreld se populasie geinfekteer is met TB. Hierdie situasie word verder vererger deur die onstaan van veelvuldig antibiotika weerstandige tuberkulose (MDR-TB), en meer onlangs die onstaan van ekstensiewe antibiotika weerstand TB (XDR-TB). Vroegtydige diagnose is van uiterse belang vir die suksesvolle behandeling van pasiënte en gebruik van geskikte chemoterapie. Verder speel vroegtydige diagnose ook 'n baie groot rol vir die effektiewe voorkoming van verspreiding van MDR-TB en XDR-TB.

Siende dat MTB hoogs aansteeklik is vir mense is dit uiters belangrik dat TB vroegtydig gediagnoseer word om die verspreiding van die siekte te voorkom. Tradisionele laboratorium prosedures wat mikroskopie, kulture en sensitiwiteits toetse insluit neem gewoonlik 3-4 weke of selfs langer. Vorderings in tegnologie oor die jare sluit in ge-outomatiseerde vloeistof kultuur sisteme in die vorm van die BACTEC 960 en die MGIT™ buis sisteem asook die ontwikkeling van verskeie molekulêre tegnieke wat gebaseer is op nukleinsuur amplifikasie (NAA). Hierdie versnelde identifikasie van Mycobakterium isolate vanuit kulture of selfs direk van kliniese monsters het grootliks bygedra tot die vervroegde diagnose van tuberkulose. Meeste van hierdie NAA toetse is egter vol beperkings en dus is die soektog vir 'n vinnige en sensitiewe diagnose vir TB nog steeds 'n aktiewe area van navorsing. Die soektog het uitgebrei en sluit areas in wat nie gewoonlik as konvensioneel beskou sal word in TB diagnose nie. Een van die areas is massa spektrometrie.

Hierdie studie vorm deel van relatief min studies van sy soort in die literatuur wat kyk na die moontlike toepassing van massa spektrometrie in die veld van TB diagnose, spesifiek Matriks-Ondersteunde Laser Desorpsie/Ioniserings Vlugtyd massa spektrometrie (MALDI-ToF MS). Dit is 'n area van diagnose wat die spoed, sensitiwiteit en spesifisiteit wat met MALDI-ToF tegnieke geassosieer word, benodig. Omdat die tegnologie nog nuut is kan dit nie oorbeklemtoon word hoe belangrik dit is nie dat groot sorg geneem moet word met die voorbereiding van reagense en die hantering en berging van organismes wat gebruik word vir die generering van verwysings massaspektra vir die databasis. Net so is die fynstelling van sekere belangrike eksperimentele faktore soos die inaktiverings metode en keuse van matriks van kardinale belang.

Die hoofdoel van die tesis was om 'n databasis te skep met massaspektra vingerafdrukke van spesifieke en uitgesoekte *Mycobacterium* spesies. Dit noodsaak die standardisering van eksperimentele metodes wat verseker dat eksperimentele faktore en verskeie instrument parameters geoptimiseer word vir maksimum spektra generering en herhaalbaarheid. 'n Standaard werksprosedure om 'n verwysings massaspektra databasis op te stel van *Mycobacterium* spesies is ontwikkel. Dit is ook gebruik om ondersoek in te stel of die databasis kan onderskei tussen verskillende spesies wat aan dieselfde kliniese siekte kompleks behoort asook nie-tuberkulêre komplekse.

Die bevindings van die studie wys daarop dat die databasis die potensiaal het om meer spesies te identifiseer en tussen meer te onderskei (onder eksperimentele toestande) as huideglik moontlik met PKR en verwante tegnieke, indien die ontwikkelde protokol gevolg word. Dit is dus 'n realistiese verwagting dat die databasis en tegniek 'n groot bydrae sal lewer tot die diagnose van tuberkulosis

en ander mykobakterioses, sodra dit klinies bewys word in die opvolgende fase van die studie. Verder kan dit bydrae tot die identifisering van nuwe patogene wat deel vorm van die nie-tuberkulose mikobakteriums.

ABBREVIATIONS

ACN: Acetonitrile

ACTH: Adrenocorticothropic hormone

AFB: Acid fast bacilli

AIDS: Acquired immune deficiency syndrome

AK: Amikacin

APCI: Atmospheric pressure chemical ionization

ATCC: American Type Culture Collection

BCG: Bacille Calmette-Guerin

bDNA: Branched deoxyribonucleic acid signal amplification

BMBL: Biosafety in Microbiological and Biomedical Laboratories

BSL 3: Bio-safety level 3

CBA: Columbia blood agar

CHCA/ α-cyano: Alpha-cyano-4-hydroxylcinnamic acid

CI: Chemical ionization

CLED: Cysteine-lactose-electrolyte-deficient (media)

CMBT: 5-chloro-2-mercaptobenzothiazole

CMI: Cell mediated immune response

CO_2: Carbon dioxide

CSF: Cerebrospinal fluid

Da: Daltons

DDI: Distilled de-ionized (water)

DIOS: Desorption/ionization on silicon

DNA: Deoxyribonucleic acid

DOT: Directly observed treatment

EI: Electron impact

ELISA: Enzyme linked immunosorbent assay

ESI: Electrospray ionization

ETB/E: Ethambutol

FAB: Fast atom bombardment

FD: Field desorption

FDA: Food and Drug Administration

FR-ICR: Fourier transform ion cyclotron resonance

GLC: Gas-liquid chromatography

HIV: Human immunodeficiency virus

HPLC: High Performance Liquid Chromatography

IFN-γ: Gamma interferon

INH/H: Isoniazid

ISP: International *Streptomyces* project (medium)

LAMP: Loop mediated isothermal amplification

LCD: Liquid crystal display

LCR: Ligase chain reaction

LJ: Lowenstein-Jensen media

LTBI: Latent *Mycobacterium tuberculosis* infection

m/z: Mass-to-charge

MAC: *Mycobacterium avium-intracellulare* complex

MALDI-ToF MS: Matrix assisted laser desorption/ionization time-of-flight mass spectrometry

MCP: Micro channel plates

MDR: Multi drug resistant tuberculosis

MGIT: Mycobacterium growth indicator tube

MMU: Manchester Metropolitan University

MOTT: Mycobacteria other than tubercle bacilli

MS: Mass spectrometry

MSS: Matrix solvent solution

MTB: *Mycobacterium tuberculosis*

NAA: Nucleic acid amplification

NALC: N-acetyl-L-cysteine

NAP: Beta-nitro-alpha-acetylamine-beta-hydroxyl-propiophenone

NASBA: Nucleic acid sequence based – amplification

NCTC: National Collection of Type Cultures

NHLS: National Health Laboratory Services

NTM: Non-tuberculous Mycobacteria

OADC: Oleic acid, albumin, dextrose and catalase

PANTA: Polymyxin B, Amphothericin B, Nalidixic acid, Trimethoprim and Azlocillin

PAS: Para-aminosalycilic acid

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PPD: Purified protein derivative

PZA/Z: Pyrazinamide

QA: Quality assurance

rDNA: Ribosomal deoxyribonucleic acid

RIF/R: Rifampicin

RMS: Root mean square

RO: Reverse osmosis

rRNA: Ribosomal ribonucleic acid

RTDS: Real time data selection

SDA: Strand displacement amplification

SIMS: Secondary ion mass spectrometry

SM/S: Streptomycin

SNP: Synthetic and natural polymers

SOP: Standard operating procedure

TB: Tuberculosis

TFA: Trifluoroacetic acid

TLC: Thin layer chromatography

TLF: Time lag focusing

TMA: Transcription mediated amplification

ToF: Time-of-flight

TST: Tuberculin skin test

TU: Tuberculin units

WHO: World Health Organization

XDR: Extensive drug resistant tuberculosis

ZN: Ziehl-Neelsen stain

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ACKNOWLEDGMENTS

DEDICATION

To the King eternal, immortal, invisible, the only wise God, who is, was, and will soon come is this work dedicated.

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CHAPTER SIX   DISCUSSION

CHAPTER ONE

LITERATURE REVIEW

1.1.   *Mycobacterium*: The Pathogen.

The genus *Mycobacterium* is currently the only genus in the family *Mycobacteriacae* of the sub order Corynebacterineae within the order *Actinomycetales.* This order is classified under the Phylum *Actinobacteria* in the kingdom Bacteria. The genus is an ever-expanding one that has grown to 95 members by the year 2003 (1). Mycobacteria are non-motile, non-sporing, aerobic, catalase positive, straight or slightly curved rods. The rods measure between 2μm - 4μm in length and 0.2μm – 0.4μm in width (2). Some display coccobacillary, filamentous or branched forms, and some produce yellow to orange pigment in the dark or after exposure to light (3, 4).

The cell wall of *Mycobacterium* is one of the most important features which differentiate them from other bacteria: it has very high lipid content (up to 60% of its dry weight) and it is composed of three covalently linked substructures: Peptidoglycan, which contains N-glycolylmuramic acid instead of the usual N-acetylmuramic acid, this is linked to arabinogalactan via a phosphodiester bridge, and mycolic acids (5, 6).Figure 1.1 shows the schematic representation of the Mycobacterial cell wall. The mycolic acids are long chain, branched fatty acids that have been exploited in differentiating the various species of Mycobacteria (3). In addition to these, there are other lipids loosely associated with the cell wall by hydrophobic forces. These are extractable by organic solvents and include various complex lipids such as: glycopeptidolipids, trehalose-containing glycolipids, and triacylglycerols, amongst others (5, 6).

Figure 1.1: A cross sectional view of the Mycobacteria cell wall
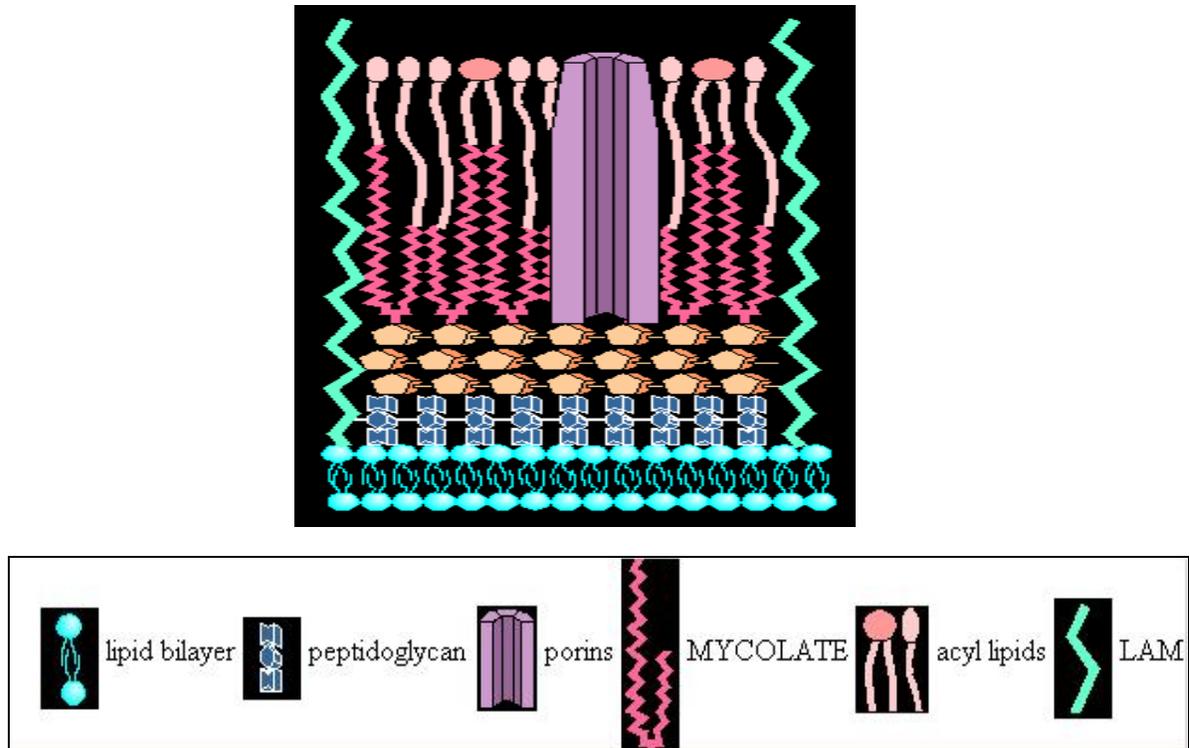
Image adapted from: http://web.uct.ac.za/depts/mmi/lsteyn/cellwall.html (Ref.7)

The waxy, hydrophobic cell wall is responsible for the unique staining pattern seen with the Ziehl-Neelsen (ZN) technique. Poor absorption of the staining dyes followed by their high retention, when eventually absorbed in a process facilitated by the application of heat are exhibited by the members of this genus and some other related bacteria, notably members of the genus *Norcardia, Rhodococcus* and *Corynebacterium* (3). The cell wall of Mycobacteria is also known to play a major role in the virulence of pathogenic strains and their resistance to desiccation. Because of their cell wall, members of this genus are relatively resistant to acids, alkalis, detergents, oxidative bursts, lysis by complement and antibiotics (2).

As mentioned earlier, the cell wall of Mycobacteria is responsible for their rather unique staining pattern. They are routinely stained with a staining technique that uses fairly concentrated dyes (usually carbolfuchin) in a process combined with heat. Once stained, they resist de-colorization by acidified alcohol, and retain the colour of the carbolfuchin even after being counter stained with another dye (usually Methylene-blue). This is why these organisms are referred to as acid-fast (3). Other members of the sub-order Corynebacterineae such as the genus *Norcardia, Rhodococcus* and *Corynebacterium* which also have mycolic-acids in their cell wall, though to a lesser extent, also share this unique acid-fast staining characteristic. Because most are rods, they are generally referred to as acid-fast bacilli, or, AFB (3). The most common staining technique used to identify acid-fast bacteria in routine microbiology laboratory is the Ziehl-Neelsen (ZN) stain. It is a process similar to the one described above: when viewed using a light microscope, the AFB appears red against a blue background as shown in Figure1.2. However, if stained with the standard Gram stain, Mycobacteria may stain weakly Gram positive or not at all.

Figure 1.2: Ziehl-Neelsen stained slide showing Acid Fast Bacilli (AFB)
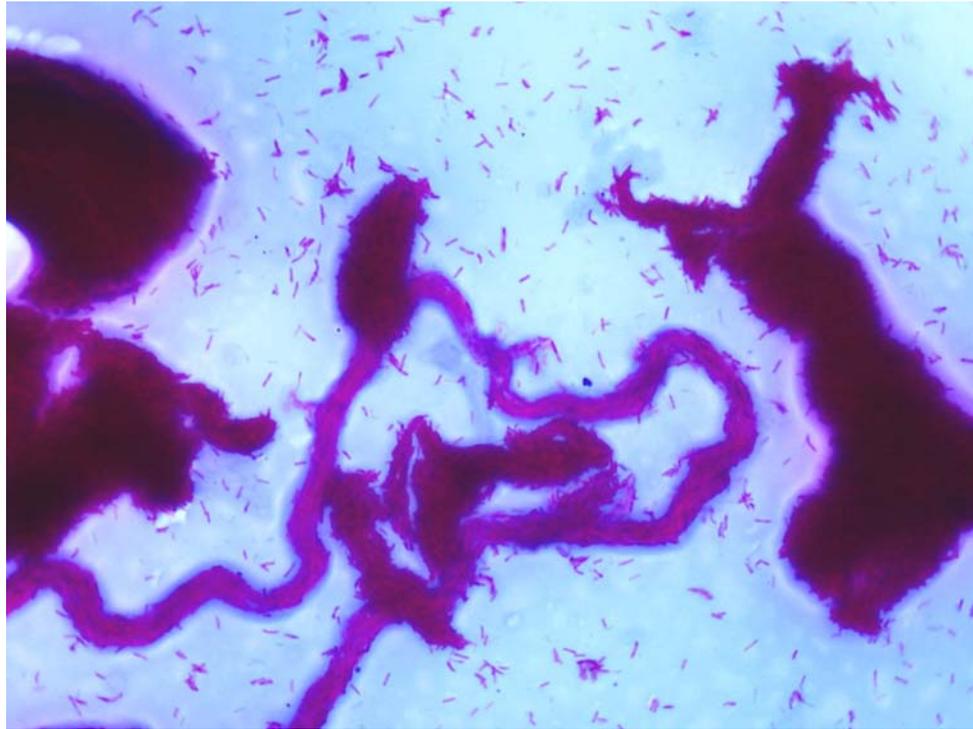
Ziehl-Neelsen stained slide of the laboratory strain of *Mycobacterium smegmatis* used as one of the test organisms in this research project, viewed using an x100 oil immersion lens. Of note is the aggregation of the cell to form the "Serpentine cord" a feature believed to be associated with virulence.

Acid-fast bacteria can also be visualized by fluorescent microscopy using specific fluorescent dyes (auramine-rhodamine stain is a commonly used example) (8).

In the past, the genus Mycobacterium has been classified into several major groups using various criteria such as growth rate, pigmentation, biochemical reactions and pathogenicity. However, for the purpose of diagnosis and treatment, Mycobacteria can be broadly categorized as the *Mycobacterium tuberculosis* complex (**MTB complex**), *M. leprae*, and the non-tuberculous

mycobacteria (**NTM**) (3). A complex is defined as two or more species whose distinction is of little or no medical importance (3). For example, pulmonary tuberculosis caused by *M. africanum* is treated in the same manner as pulmonary tuberculosis caused by *M. tuberculosis*, so it is sufficient to identify the causative agent of pulmonary tuberculosis as a member of the *Mycobacterium tuberculosis* complex. MTB complex comprises of *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canettii* (9). The non-tuberculous mycobacteria (NTM) are other species of this genus that are not members of the MTB complex or *M. leprae*, the causative agent of leprosy, a disease also known as Hansen's disease (10, 11). The NTM organisms have in the past been referred to by various names such as atypical, anonymous, environmental, and opportunistic and mycobacteria other than tubercle bacilli (MOTT) (10). Most NTM are free-living saprophytes present in the environment but can be opportunistic and at times, deadly pathogens. Of the over 90 known species of NTM, about one third has been associated with diseases in humans (1). The most common non-tuberculous Mycobacterium found in clinical specimens is *M. avium* (10), a member of the *M. avium* complex (MAC), which includes both *M. avium* and *M. intracellulare* (12). MAC has been found to be a major cause of pulmonary and non-pulmonary infections in humans (1). Other non-tuberculous mycobacteria also cause disease with varied clinical significance and manifestations: these diseases are usually not transmitted from man to man, and have been broadly grouped as Mycobacterioses (1).

Mycobacterium tuberculosis is the 'type species' of the genus *Mycobacterium*. It is one of the earliest recognized etiologic agents of a human disease, tuberculosis (3). *Mycobacterium tuberculosis* (MTB) is an obligate aerobe and a facultative intracellular parasite (usually of macrophages) with an average generation time of 15-20 hours. (2). When cultured *in vitro*, the cells

aggregate in chains to form distinctive 'serpentine cords', a feature first observed by Robert Koch, who associated cord factor with virulence (2).

1.2 Tuberculosis: The disease.

The term tuberculosis describes a broad range of clinical illnesses caused by *Mycobacterium tuberculosis* or any member of the *Mycobacterium tuberculosis* complex (13, 14). Pulmonary TB is characterized by prolonged cough, hemoptysis, chest pain and dyspnea, while fever, malaise, anorexia, weight loss, weakness and night sweats are manifestations of the systemic or disseminated disease (15).

Tuberculosis has been described since, at least, the time of Hippocrates, who referred to it as "Phthisis", a term reflective of the wasting character of the disease (16). Other names used for TB in the past include: King's Evil, Consumption, lupus vulgaris and the white plague (17, 14). Although TB is a disease of great antiquity, it only became a major public health problem during the industrial revolution when the social conditions prevalent at that time such as overcrowded cities and over stretched and often inadequate public health facilities presented the ideal circumstances for the spread of tuberculosis (18). Tuberculosis is spread almost exclusively by aerosolization of infectious droplet nuclei by people with cavitary pulmonary or laryngeal tuberculosis, also called 'open' tuberculosis (16, 17). Infection is acquired by inhalation of these infective droplets, which are usually less than 5μm in diameter and are capable of remaining airborne up to several hours after expectoration (16, 19).

Studies conducted through the years have revealed how efficiently tuberculosis is spread. In general, about 30% of persons who have sustained contact with an 'index' case patient (smear positive patient) will develop tuberculosis infection, reflected by a positive Tuberculin Skin Test (TST) while persons in contact with smear negative patients have less than a 10% rate of new infection (16). In primary infection, (i.e. infections in individuals encountering the pathogen for the

first time), the organisms are engulfed by the alveolar macrophages in which they can both survive and multiply. Non-resident macrophages are attracted to the site; these ingest and carry the organisms via the lymphatics to the local hilar lymph nodes. Here in the lymph nodes, immune response, predominantly the cell-mediated immune response (CMI) is stimulated. This CMI response is detectable 2-8 weeks after infection. This can be visualized by introducing Purified Protein Derivative (PPD) into the skin intra-dermally in a procedure referred to as Tuberculin Skin Test (TST) mentioned earlier. A positive result is indicated by the size of local indurations and erythema as measured 48-72 hours later (20, 19). It should be noted that these sizes vary for immunocopetent and immunocompromised people, and the results should be interpreted bearing this in mind.

Primary tuberculosis is usually mild and asymptomatic and in 90% of the cases does not proceed further: however, clinical disease develops in the remaining 10%. *M. tuberculosis* that somehow manages to escape phagocytosis by macrophages will set up foci of infection primarily in the lungs. This causes sensitized T cells to release lymphokines that activate macrophages and increase their ability to destroy the Mycobacteria. The body attempts to contain the organisms within 'tubercles' which are small granulomas consisting of epitheloid cells and giant cells. The lung lesion plus the enlarged lymph nodes is referred to as 'Ghon' or primary complex. After some time, the materials within the granulomas become necrotic and caseous or cheesy in appearance (20, 19). The tubercles may heal spontaneously, become fibrotic or calcified and persist as such for a lifetime in people who are otherwise healthy. They are seen as radio-opaque nodules in chest radiographs (20). As mentioned earlier, in a small percentage (about 10%) of people with primary infection and particularly the immunocompromised, the Mycobacteria that are not contained in the tubercles will invade the blood stream to cause disseminated (systemic) disease also referred to as 'miliary'

tuberculosis. The risk of primary infection developing into clinically overt tuberculosis is highest in the first two years after infection (20, 16).

Secondary tuberculosis is due to reactivation of dormant Mycobacteria and is usually a consequence of impaired immune function resulting from some other causes such as malnutrition, underlying malignant disease, chemotherapy, poorly controlled diabetes mellitus, renal failure, extensive corticosteroid therapy, or other infections especially the immune suppression caused by Human Immunodeficiency Virus (HIV) (17, 19). It can also be caused by re-infection with MTB. HIV is the greatest single risk factor for the progression of tuberculosis infection to the active disease in adults (19). HIV exerts an immense influence on the natural course of TB disease. Individuals with latent infection who contact HIV are at risk of developing active TB at the rate of 7-10% per year compared to approximately 8% per lifetime for HIV negative individuals (21). HIV infected persons recently infected with *M. tuberculosis* may progress to active disease at a rate over 35% within the first six months compared to 2 – 5% in the first two years in HIV negative individuals (17). Several authors have documented that HIV infection tends to accelerate the progression of TB: while in turn, the host's immune response to *M. tuberculosis* can enhance HIV replication and may accelerate the natural course of HIV/AIDS (22).

Important and worthy of emphasis is the fact that the host's immune response (basically the CMI response) usually controls and contains MTB infection, but when it is inadequate, infection disseminates or reactivates (20). Consequently, nearly all the pathology and the disease is a consequence of this CMI response as MTB causes little or no direct or toxin-mediated damage.

1.3 Epidemiology

The start of the 20th century witnessed a progressive decrease in the incidence of tuberculosis in developed countries due to improvements in sanitation and housing (23) as well as improved nutritional standards, particularly the pasteurization of milk which eliminates *Mycobacterium bovis*, (16). These trends were accelerated by the introduction of BCG vaccination and the discovery of antimicrobials such as streptomycin, which were used in effective combinations established in a series of landmark trials by the British Medical Research Council, the USA Public Health Service, and their partners (24). As the incidence curve approached the zero baselines in many parts of the world, many microbiologists were confident that tuberculosis was about to be conquered, but in fact the opposite has happened (8). Tuberculosis is now the leading cause of death world wide due to any single infectious agent (13). So serious is the scourge that in 1993, the World Health Organization (WHO) took the unprecedented step of declaring tuberculosis a global public health emergency (13). The WHO also estimates that approximately one third of the global community is infected with Mycobacterium tuberculosis. There were an estimated 8-9 million new cases in 2000, fewer than half of which were reported: while 3-4 million were sputum-smear positive, the most infectious form of the disease (25, 19). There was also an estimated 3 million deaths world wide due to TB in the year 2000 (16).

Tuberculosis has been associated with poverty and poor living conditions. The global distribution of TB cases reflects this fact with most cases being found in the low income and emerging economies. These resource-poor countries bear over 90% of the entire global tuberculosis disease burden, 98% of all TB related deaths occur in these developing countries (17, 26). However, more recent statistics seems to suggest a general improvement in the grim picture painted above. The WHO estimates in 2005 showed that the per-capita incidence of TB was stable or falling in six WHO

regions, after having reached a peak world-wide. However, the total number of TB cases still continues to rise slowly, because of the continued increase of the disease case-load in the African, Eastern Mediterranean and South-East Asia regions (27, 28). Figure 1.3 on the next page illustrates this graphically.

Figure 1.3:
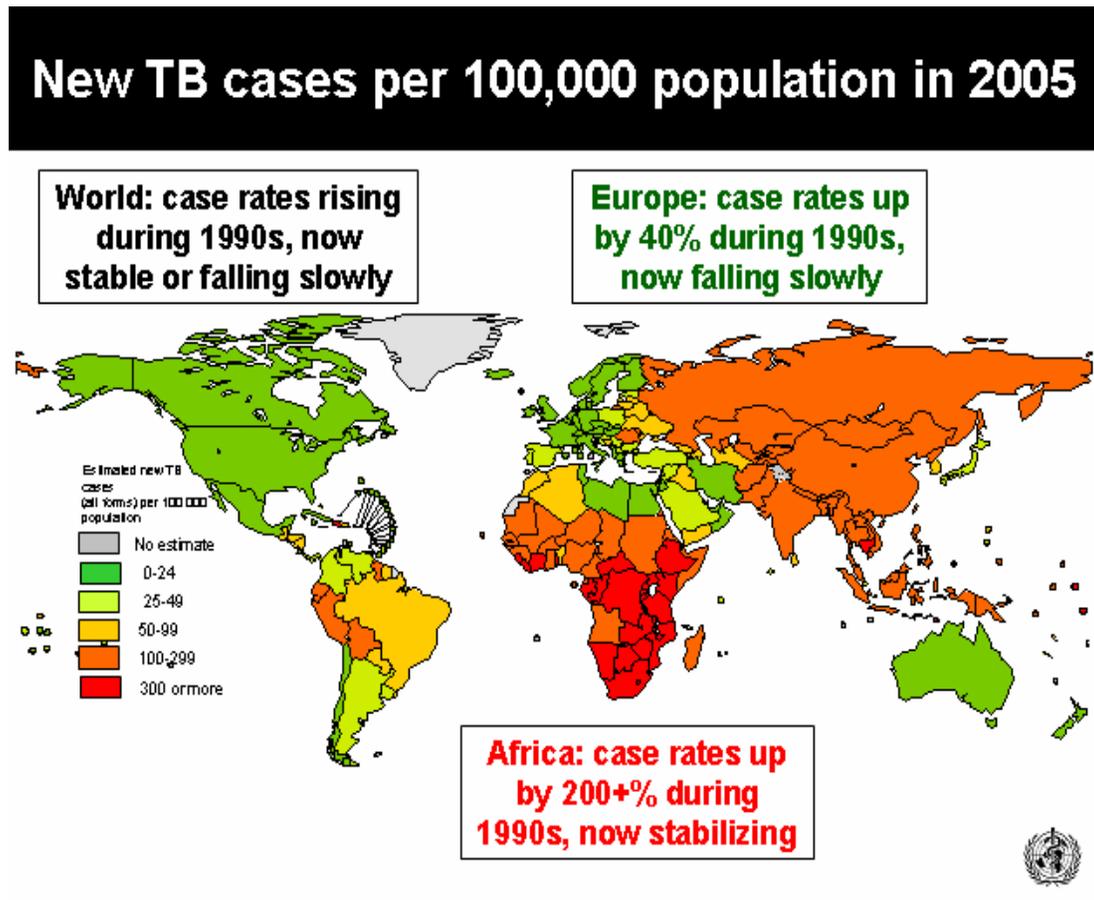
Map obtained from: Estimated TB incident rate, 2005. Available from:

http://www.ecdc.europa.eu/documents/C_Dye.ppt (Ref.28)

This progressive decrease in the incidence of tuberculosis in the developed countries at the turn of last century was a trend that continued till the mid 1980's when a marked rise in the incidence curve of the disease was observed. In the United States of America (USA) several reasons have been given for this resurgence, these include: a rise in the number of homeless persons and persons living in congregate settings, increased influx of immigrants from countries where TB is endemic, the

deterioration of the public health facilities and by far the most important, the HIV/AIDS pandemic (16).

The immense influence of HIV on the incidence of tuberculosis cannot be overemphasized. In the USA, the highest recent increases in the number of TB cases occurred among the Asian, Black and Hispanic persons reflecting the high rates of HIV infection in these groups. In comparison, the rates of infection for non-Hispanic whites, American Indians, and Alaskan Natives have been observed to continue to decrease (16, 37). In the European region, TB notification rates per hundred thousand (100,000) populations are the highest in Russia and the other successor states to the Soviet Union (26). This is attributed to economic decline and the deterioration of the health services since 1991 (19). In other parts of Europe, particularly in Western Europe, the incidence of tuberculosis is slowly decreasing, though at a much slower rate than would otherwise have been expected due to the relatively high degree of infection in migrants and refugees of those countries. For example, in Germany, it is estimated that 30% of all new cases occur in foreign-born population (16, 26). Other parts of the world where TB cases have been observed to decline more or less steadily includes Central Europe, North America and the Middle East (19).

As mentioned earlier, the poorer countries of the world bear the greatest tuberculosis disease burden. Sub-Saharan Africa has the highest incidence rate (290 per 100 000 populations), while the countries of Asia with the highest population have the highest prevalence rate: India, China, Indonesia, Bangladesh, and Pakistan together account for more than half the global burden (19, 17). HIV infection accounts for the recent increase in the global tuberculosis burden (25). Worldwide, an estimated 11% of new adult TB cases in 2000 were infected with HIV, with wide variations amongst regions: 38% in sub-Saharan Africa, 14% in more developed countries, and 1% in the

Western Pacific Region (19). The increase in tuberculosis incidence in Africa is strongly associated with the prevalence of HIV infection (19). Rates of HIV infection among tuberculosis patients are correspondingly high, exceeding 60% in Botswana, South-Africa, Zambia and Zimbabwe. Of the estimated 2 million deaths in 2000 due to tuberculosis, 13% were also infected with HIV (25). Tuberculosis, like its infamous counterpart HIV/AIDS affects predominantly the economically most productive age group (14-49 years) (19). TB is the leading infectious cause of mortality among adults in developing countries, it kills more than 2 million people each year and this constitutes about 26% of avoidable adult deaths in the developing world (30).

The distribution of Non-tuberculous Mycobacteria (NTM) and the incidence of the disease caused by these organisms is perhaps not fully understood in most parts of the world (1). NTM are widely distributed in nature and have been isolated from natural water, tap water, soil, water used in showers, surgical solutions, food, house dust, domestic and wild animals (1, 12). As noted earlier, *Mycobacterium avium* complex (MAC) is the most common NTM found in clinical specimens, it has been observed to be an important cause of morbidity and mortality in the immunocompromised host in Western countries (1). In the United States, most of the NTM isolates from pulmonary sources were MAC, *M. kansasii* and *M. fortuitum* (10). In Canada and some parts of the United Kingdom and Europe, *M. xenopi* ranks second to MAC, whereas in Scandinavia and Northern Europe, *M. malmoense* is next to MAC (31).

1.4   Treatment of Tuberculosis

Tuberculosis, as earlier mentioned, is a disease of great antiquity. In the second half of the 19th century, before the advent of antimicrobials, tuberculosis was treated in specialized sanatoria. Here, treatment was a combination of diet, gentle exercise and rest in the open air and sunlight (32, 33). It

soon became apparent that sanatoria regimes probably benefited the cases diagnosed before cavitations but had little impact on cavitary disease. When it was established that cavitations was a principal event in progressive pulmonary tuberculosis, cavity closures became the focus of most special therapies. These therapies were basically surgical procedures attempting to obliterate the cavities by collapsing part of the lung itself (33, 32).

The treatment of tuberculosis took a turn for the better in the 1940's with the discovery and subsequent clinical use of antimicrobials such as streptomycin (SM/S), and para-aminosalycilic acid (PAS). In 1952, isoniazid (INH/H) that is still the most effective drug at killing actively dividing tubercle bacilli also came into use (33). As the years progressed, other drugs such as pyrazinamide (PZA/Z), ethambutol (ETB/E), ethionamide, and cycloserine were discovered, added to treatment regimes, used in combination with other drugs or dropped depending on their efficacy and side effects. Rifampicin (RIF/R), arguably the most important drug in the treatment of tuberculosis because of its efficacy in killing slowly dividing bacteria (so called "persisters") came into clinical use in the 1970's (33, 32).

The availability of drugs confirmed the efficacy of chemotherapy in rendering treated patients non infectious. This led to new treatment principles and the need for specialized sanatoria ultimately disappeared. The duration of chemotherapy also changed significantly with the discovery and use of new drugs. When SM and PAS were the only drugs in use, standard treatment was for two years, with the addition of INH, the length of treatment was reduced to 18 months (33). This decreased to 9 months if INH and RMP were given together and to just 6 months if a multi-drug therapy comprising of INH, RMP and PZA was used (33, 32). This appears to be the preferred regimen and it forms the core of the current standard treatment for tuberculosis, it is conveniently abbreviated

and referred to by clinicians as 2HZR/4HR, indicating 2 months of isoniazid (H), pyrazinamide (Z), and rifampicin (R) followed by 4 months of isoniazid and rifampicin only (33). If culture is still positive after 2 months of triple drug therapy administration, it is recommended that it be continued until the culture becomes negative. Thereafter, it should be continued for about 4 months more. (9). It is also recommended to use ETB or SM as a fourth drug until the susceptibility of the MTB is known, if it is susceptible to the three drugs in the preferred regimen, then ETB/SM should be discontinued. There are four regimens in the treatment of TB, of which the above seems to be the preferred regimen. If Z cannot be used in the first 2 months, the reasonable alternative will be to administer H and R for 9 months (9). Here, in the republic of South Africa, the preferred regimen is usually made up of these four drugs: INH, RIF, PZA and EMB (34).

Currently there are 10 drugs approved by the United States Food and Drug Administration (FDA) for the treatment of tuberculosis. In addition to these, other drugs such as fluoroquinolones, though not approved by the FDA for the treatment of tuberculosis are commonly used to treat TB caused by drug resistant organisms or for patients who are intolerant of some of the 'first line' drugs (35). Table 1.1 shows some of the first line drugs anti-tuberculosis drug and their side effects.

Table 1.1: First Line Drugs Used In Chemotherapy of Tuberculosis: Dosages and Main Side Effects

Drugs	Daily Regimen	Twice or Three Times Weekly	Side Effects
INH	5 mg/kg (max 300mg)	15 mg/kg (max 900mg)	Hepatitis, peripheral neuropathy, lupus-like syndrome, drug interactions
RIF	10 mg/kg (max 600mg)	10 mg/kg (max 600mg)	drug interactions, orange discoloration of body fluids, GI upsets, hepatitis, fever, hypersensitivity, acute renal failure, hemolytic anemia
PZA	15-30 mg/kg (max 2 g)	50-70 mg/kg (max 4 g)	Hyperuricemia, gouty arthritis rarely hepatitis
ETB	15-25 mg/kg	25-30 mg/kg	optic neuritis, exfoliative rash
SM	15 mg/kg	25-30 mg/kg	cochleo-and vestibulo-toxicity, nephrotoxicity
AK (Amikacin) (same as SM)	7.5-10 mg/kg		

Adapted from: Octavian L, Tomford WJ. Tuberculosis. Medicine Index. Available from:

http://www.clevelandclinicmeded.com/medicalpubs/diseasemanagement/pulmonary/tb/tb.htm

(Ref.9)

Another drug, rifabutin, which is approved for use in preventing *Mycobacterium avium* complex

disease in patients with HIV infection but not approved for the treatment of TB, has been found to

be very useful in treating TB in patients that are currently taking drugs that have unacceptable

interactions with other rifamycins. In a similar vein, two almost identical aminoglycoside drugs,

amikacin and kanamycin; though not approved by the FDA for the treatment of TB are also used in

the treatment of tuberculosis caused by drug resistant organisms (35). Rifabutin and rifapentine are

other drugs that can be considered and used as first line agents in some specific situations such as:

patients receiving medications that have unacceptable interactions with rifampicin, or rifampicin

intolerance. In such cases rifabutin is substituted for rifampicin as earlier mentioned, rifapentine on the other hand is used in a once a week combination with INH in the continuation phase of treatment for HIV- seronegative patients with non-cavitary, drug susceptible pulmonary tuberculosis who have negative sputum smears at the completion of the initial phase of treatment (35).

The importance of chemotherapy in the effective management and control of tuberculosis cannot be overemphasized. Before effective drugs were available, about 50% of patients with active pulmonary tuberculosis died within 2 years and only about 25% were cured (32). But the introduction of anti-tuberculosis drugs and the subsequent development of the various drug regimens brought the chances of cure to 98% by the mid 1980's. The goals of treatment are to ensure cure without relapse, to prevent death, to stop transmission and to prevent the emergence of drug resistance. Successful treatment of TB especially in adult patient is a reality but in practice, failure occurs. This is most commonly due to non-adherence of the patient to the prescribed regimen, other causes such as drug resistance and inappropriate treatment regimen may also be responsible. For this reason, the responsibility of adequate treatment was shifted from the patient to the prescribing physician and health authorities. The treatment of TB is most successful within a comprehensive framework that addresses both clinical and social issues relevant to the patient, it is strongly recommended that patient-centered care be the initial management strategy, a strategy that should always include an adherence plan that emphasizes directly observed therapy (DOT), in which patients are observed to ingest each dose of anti-tuberculosis medications, to maximize the likelihood of completion of therapy, minimize the development of acquired drug resistance and prevent relapse (35, 19).

1.5 Drug Resistance

The history of drug resistance is almost as old as that of the advent of chemotherapy in the treatment of tuberculosis. Streptomycin, the first specific anti-tuberculosis drug discovered in the USA in the mid 1940's and brought into clinical use soon after, provided a seemingly miraculous cure, especially for children dying from tuberculous meningitis (33). Unfortunately, many children relapsed after a few months of treatment because the bacteria had developed resistance to streptomycin. This was the curtain - raiser to the terrible, often disastrous world of anti-tuberculous drug resistance.

Para-aminosalycilic acid (PAS) fortunately was discovered soon after by European scientists and brought into use by the late 1940's. It was discovered that by administering these two drugs together the emergence of resistant strains was largely prevented (33, 16). Resistance to anti tuberculous medications may be either primary or secondary. Primary resistance occurs in patients with active TB who have never received anti tuberculous drugs (9), while secondary resistance occurs when resistant mutations of an initially drug susceptible infection emerge in the setting of incomplete compliance with therapy or incorrect selection of treatment regimen (16).

In theory, it is assumed that there are three separate sub populations of *M. tuberculosis* within the host; these populations are defined by their growth characteristics and the milieu in which they are located (35). Rapidly growing extra cellular bacilli that reside mainly in cavities makes up the largest sub population. This sub population, because of its size, is most likely to harbor organisms with random mutation conferring drug resistance. The frequency of these mutations that confer resistance is about 10^{-6} for INH and SM, 10^{-8} for RIF, and 10^{-5} for EMB: thus the frequency of concurrent mutations to both INH and RIF, for example would be 10^{-14} (10, 35). This makes

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simultaneous resistance to both drugs in an untreated patient a highly unlikely event. This emphasizes the fact that the chances of multi drug resistant MTB occurring in nature is very slim indeed. The unfortunate episode with the initial administration of streptomycin monotherapy underscores an important principle in the treatment of tuberculosis: ***active tuberculosis should never be treated with a single drug***. Neither should a single drug be added to a failing regimen; if this is done, the organism quickly develops resistance to the new drug and so it will continue with subsequent addition of other drugs (19, 33).

Treatment non-compliance is the major cause of secondary resistance, also referred to as acquired resistance (16). Secondary or acquired resistance also occurs when patients are treated inappropriately or are exposed, even transiently, to sub-therapeutic drug levels (17). The need to maintain high drug levels over many months of treatment, combined with the inherent toxicity of the agents, results in reduced patient compliance and subsequently higher likely hood of drug resistance acquisition (36). Therefore, concerted efforts are needed to ensure patient compliance, and the need for intervention with programmes such as DOT can not be over emphasized.

1.5.1 Multi Drug Resistant Tuberculosis (MDR-TB)

Multi drug resistant tuberculosis (MDR-TB), defined as the tuberculosis caused by strains that are resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs (37, 17), was first identified in the Western Cape, South Africa in 1985 (37, 38). Since then, patient non compliance, acute infection with already resistant strains and over 4 decades of ineffective administration of effective medicines have conspired to create a growing number of persons with resistant tuberculosis globally (16). Currently, tuberculosis is treated using one of the four recommended regimens, all of which includes the two major anti tuberculous drugs: isoniazid (INH) and

rifampicin (RIF), and at least one other first line anti tuberculous drug. Here in South Africa, two other first line drugs, ethambutol and pyrazinamide, are administered along with these two. These two drugs are at the core of effective TB treatment and cure, with INH being highly effective at killing actively dividing tubercle bacilli, and RIF being effective at killing the slowly dividing bacteria (persisters), leading to the so called "sterilization" of infected sites (33). Susceptibility to both drugs allows 6-9 month regimens, susceptibility to RIF but not to INH allows 9-12 month regimens, and susceptibility to INH and not RIF allows 12-18 months effective regimens (16). Maintenance of susceptibility to at least one of these two agents is therefore crucial to the control and cure of tuberculosis.

Treatment of MDR-TB is generally more difficult, slower, more toxic, and more expensive than treatment of susceptible disease. It has also been associated with very high mortality and morbidity, prolonged treatment to cure and an increased risk of spreading drug resistant isolates in the community (16, 17). Management of resistant TB varies according to the pattern of drug resistance: if drug resistance is suspected but not yet confirmed, a detailed history of the anti- tuberculous drugs that the patient has had must be obtained. The patient should then be put on an appropriate regimen consisting of at least 3 or preferably four drugs to which they have not had previous exposure, INH and RIF should also be administered as if the organism is susceptible to them. Streptomycin is usually not included in the treatment of MDR-TB, even if the patient has not previously exposed to it, because resistance to it is so common (33). If susceptibility test results are available, a regimen can be chosen based on this. Most authorities recommend 3 or 4 oral drugs plus one injectable drug (such as Capreomycin, Amikacin, or Kanamycin) to which the isolate is susceptible for 3-6 months, and then at least 3 effective oral drugs for 15-18 months for a total of 12-18 months after culture conversion to negative (19, 39). Longer use of injectable drugs has been

associated with improved outcomes (40), but long term administration is commonly complicated by ototoxicity, nephrotoxicity, and local adverse reactions such as pain, indurations and abscess formation (19).

The success rate of drug therapy for resistant tuberculosis is much lower than that of drug sensitive disease; being between 60%-70% cure compared with over 95% cure rate for the latter. Surgery may sometimes be necessary. If the disease is confined to one or at the most two lobes of the lungs, then lobectomy offers a better chance of cure than continued drug therapy (33). Once more, the influence of HIV/AIDS on TB comes to the fore in the management of MDR-TB. MDR- TB is a rapidly fatal disease in patients with AIDS, with most patients dying within 1-3 months. Hence, prompt selection of an effective regimen is a key determinant in the survival of patients with MDR-TB and AIDS (16). Although HIV sero-positivity does not in itself increase the chances of drug resistance, it does raise drug interaction concerns (33). The bactericidal activity of anti-TB drugs on the tubercle bacilli is similar in HIV positive and negative patients; hence the same drugs are employed in the two populations (29). While the 5-6 drug empirical regimens do appear superior when given in the correct setting, administering this regimen, which often contains several toxic second-line agents, to patients with AIDS who often are already receiving several other medications, can further complicate an already complicated clinical picture. Management of common toxicities such as rash, fever or hepatitis is particularly difficult, since it is seldom obvious which of the drugs is the causative agent (16).

As earlier mentioned, drug interaction is of great concern in the management of TB and HIV/AIDS co-infection, whether the TB is resistant or susceptible. It is recommended that some anti-retroviral drugs (such as most protease inhibitors and non-nucleoside reverse transcriptase inhibitors) should

not be used with rifampicin. Rifabutin, a drug with similar activity against MTB but with less effect on the pharmacokinetics of some antiretroviral drugs, should be substituted for it (19).

1.5.2 Extensive Drug Resistant Tuberculosis (XDR-TB)

Extensive drug resistant tuberculosis (XDR-TB), like its infamous progenitor MDR-TB, was first reported in early 2006 in the KwaZulu Natal province of South Africa (37). Later that year, XDR-TB was re-defined by a group of international experts led by the WHO as *Mycobacterium tuberculosis* isolates that are multidrug resistant, with additional resistance to a fluoroquinolone and one or more of the following injectable drugs: Kanamycin, Amikacin and Capreomycin (37, 41). These drugs are second-line drugs used against TB when first-line drugs are no longer effective. Table 1.2 shows some second-line drugs and their possible side effects.

Table 1.2: Second-Line Drugs Used In the Chemotherapy of Tuberculosis: Dosages and Main Side

Effects

Drug	Daily Dose (Maximum Dose)	Adverse Reactions	Monitoring	Comments
Capreomycin	15-30 mg/kg (1g)	Toxicity - auditory - vestibular - renal	Asess - vestibular function - hearing function Measure -blood urea nitrogen -creatinine	After bacteriologic conversion, dosage may be reduced to 2-3 times per week
Kanamycin	15-30 mg/kg (1g)	Toxicity - auditory - vestibular -renal	Asess - vestibular function - hearing function Measure -blood urea nitrogen -creatinine	After bacteriologic conversion, dosage may be reduced to 2-3 times per week
Ethionamide	15-30 mg/kg (1g)	GI upset Hepatotoxicity Hypersensitivity Metallic taste Bloating	Measure hepatic enzymes	Start with low dosage and increase as tolerated May cause hypothyroid condition, especially if used with PAS
Para-aminosalicylic acid (PAS)	150 mg/kg (12g)	GI upset Hypersensitivity Hepatotoxicity Sodium load	Measure hepatic enzymes Assess volume status	Start with low dosage and increase as tolerated Monitor cardiac patients for sodium load
Cycloserine	15-30 mg/kg (1g)	Psychosis Convulsions Depressions Headaches Rash Drug interactions	Assess mental status Measure serum drug levels	Start with low dosage and increase as tolerated Pyridoxine may decrease CNS effects
Ciprofloxacin	500-1000 mg/day	GI upset Dizziness Hypersensitivity Drug interactions Headaches Restlessness	Drug Interactions	Not approved by FDA for TB treatment Should not be used in Children Avoid -Antacids -iron -zinc sucralfate

Table adapted from: Treatment of Tuberculosis. American Thoracic Society, CDC, and Infectious Diseases Society of America. June 20, 2003/52(RR11); 1-77 Available from: http://www.cdc.gov/mmwR/preview/mmwrhtml/rr5211a1.htm (Ref.35)

Several well documented factors including high treatment interruption rates of drug-sensitive TB and consequently low cure rates, together with the HIV epidemic, alongside inadequate health care system response, poverty and global inequity have contributed to the emergence of MDR-TB and XDR-TB in South Africa (42). It has been reported that about 15% of patients nationally default on the first-line six-month treatment, while almost a third of patients default on second-line treatment (42). Since the first WHO report of XDR-TB in the Tugela Ferry area of the KwaZulu Natal province of South Africa, XDR-TB cases have been reported world wide (37). The deadly nature of the disease was also very obvious from the onset. In a study carried out in the afore mentioned area in the year 2005, of the 544 patients studied, 221 had MDR-TB. Of these, 53 cases were identified as XDR-TB cases. This reportedly represented almost one-sixth of all known XDR-TB cases reported world wide. Forty four of the 53 patients tested positive for HIV, and the median survival from the time of diagnosis was 16 days for 52 out of the 53 infected individuals, including 6 health workers and those reportedly taking anti-retrovirals (43, 42). The very high fatality rate within such a short time had not been reported anywhere else in the world. The fatal nature of XDR-TB especially in patients co-infected with HIV, coupled with the fact that is virtually untreatable as it is resistant to most first-line and second-line anti-TB drugs, demands that urgent and concerted efforts be made to prevent and effectively control the emergence and spread of XDR-TB.

In light of the grave danger posed to public health by XDR-TB, WHO set up a global task force in Geneva on the 17th of October 2006 (35, 44). This task force outlined a series of measures that countries must put in place to effectively combat XDR-TB. Such measures include (44):

- ❖ Strengthen basic TB care to prevent the emergence of drug resistance

- ❖ Ensure prompt diagnosis and treatment of drug resistant cases to cure existing cases and prevent further transmission

- ❖ Increase collaboration between HIV and TB control programmes to prevent necessary prevention and care to co-infected patients

- ❖ Increase investments in laboratory infrastructures to enable better detection and management of resistant cases (44).

The task force also made specific recommendations on issues such as drug resistant TB surveillance, laboratory capacity strengthening measures, the implementation of infection control measures to protect patients, health workers and visitors (particularly those who are HIV infected), and access to second-line anti-TB drugs, amongst other things. A full version of the report is available at **http://www.who.int/tb/xdr/news_mar07.pdf**.

It is essential that TB patients are diagnosed early and be treated according to international standards of care so that the emergence of MDR-TB and XDR-TB is prevented by ensuring that TB patients are cured the first time around.

1.6   Synopsis of some selected Non-tuberculous Mycobacteria.

A review of the genus *Mycobacterium* will not be complete without a word about the non-tuberculous mycobacteria, especially since one of the main aims of this project is to generate a database of mass spectra of mycobacteria, and these organisms make up the majority of the species included in the database. Non-tuberculous Mycobacteria (NTM) are *Mycobacteria* species that do not belong to the *Mycobacterium tuberculosis* complex and are not *Mycobacterium leprae*. This group of organisms makes up the majority of the species in the genus Mycobacteria, and is an evolving class of pathogens to be reckoned with in their own right, especially in this era of HIV/AIDS.

As mentioned earlier, most NTM are free-living saprophytes present in the environment but can on occasions be opportunistic or even deadly pathogens. Of the over 90 known species of NTM, about one third have been found to be associated with diseases in humans (10). NTM can be categorized into different groups based on their colony morphology, growth rate, and pigmentation. Although this system is less used today because of the availability of more rapid and reliable diagnostic systems, growth rate still remains a practical way of grouping NTM in many laboratories today. Based on this, NTM are broadly categorized into 3 major groups:

(a) Rapidly growing *Mycobacteria*: These are organisms that produce mature growth on agar plates within 7 days (10). They may or may not be pigmented: among the non-pigmented rapid growers are *M. fortuitum*, *M. peregrinum*, *M. chelonae* and *M. abscessus* (1, 10). Some strains of *M. smegmatis* that are also rapid growers are pigmented while others are not (10). Rapidly growing pigmented NTM occasionally identified in clinical disease includes *M. vaccae*, *M. phlei*, *M. flavescens* and *M. thermoresistible*, amongst others (10).

(b) Slow growing *Mycobacteria*: These are organisms that require more than 7 days of incubation to produce mature growth on agar plates. Some may require nutritional supplementation of routine mycobacteria media (10). The most common, clinically important organism in this group is the *Mycobacterium avium* complex (MAC) comprising of *M. avium* and *M. intracellulare*. Other species of clinical importance found in this group includes *M. kansasii*, *M. xenopi*, *M. simiae*, *M. scrofulaceum*, *M. haemophilum* and *M. malmoense*, amongst others. There are also newer, more rarely isolated species of NTM categorized in this group: *M. confluentis*, *M. interjectum*, *M. triplex*, *M. celatum*, *M. conspicuum* and *M. asiaticum*.

(c) Intermediately growing *Mycobacteria*: these are usually pigmented organisms that require 7-10 days of incubation to produce mature growth on agar plates. Examples are *M. marinum* and *M. gordonae*. *M. marinum* has an optimal growth temperature of 30°C, while *M. gordonae* has a documented preferred growth temperature of 35°C (10), although it grows well at 37°C, as observed and used in this project.

In the pre- AIDS era, most diseases caused by NTM were pulmonary, confined to the cervical lymph nodes, limited to the skin or in rare cases, disseminated (45). Pulmonary diseases were found mostly in middle aged-males, and most had predisposing lung conditions such as pneumoconiosis or 'black lungs', or worked under conditions where they were exposed to dust, such as farming or mining. Organisms of the *Mycobacterium avium* complex (MAC), *M. avium* and *M. intracellulare* and *Mycobacterium kansasii,* were the major NTM pathogens found in such clinical settings (46). Furthermore, in the pre-AIDS era, *Mycobacteria scrofulaceum* was found to be the causative agent of cervical lymphadenitis in children, and *M. marinum* infections that were found principally in the

skin, were associated with cuts, abrasions and exposure to aquaria or swimming pools or an occupation in the fishing industry (45, 46).

As in the case of tuberculosis, the HIV/AIDS epidemic has had a tremendous influence on human diseases caused by nontuberculous mycobacteria, and there has been an accelerated increase in the rate of non-tuberculous mycobacterial disease in AIDS patients since such was first reported in 1982 (46). Before the AIDS epidemic, and even so today in the HIV seronegative patient, NTM diseases are primarily pulmonary. In contrast, it is usually disseminated in immunocompromised patients. Furthermore, NTM infections of the skin or joints in the immunocompetent individual are usually associated with trauma due to injury or surgery, or corticosteroid/immunosuppressant usage. In contrast, skin and joints infections in AIDS patients are not associated with trauma or corticosteroid use (46).

Table 1.3: Major syndromes associated with NTM infections and their etiological agents.

Syndrome	Relative Common Causes	Less Frequent Causes
Chronic bronchopulmonary disease (usually adults)	*M. avium complex, *M. kansasii, *M. abscessus*	*M. xenopi, M. malmoense, M. szugai, *M. smegmatis, *M. scrofulaceum, M. celatum, M. simiae*
Cervical or other local lymphadenitis (especially children)	*M. Avium complex*	*M. scrofulaceum, M. malmoense* (northern Europe) *M. abscessus, M. fortuitum*
Skin and soft tissue disease	*M. fortuitum, *M. chelonae, M. abscessus, *M. marinum*	*M. kansasii, M. haemophilum, M. smegmatis, *M. ulcerans* (Australia, tropical countries only)
Skeletal (bone, joint, tendon) infection	*M. marinum, M. avium complex, M. kansasii, M. fortuitum group, M. abscessus M. chelonae*	*M. haemophilum, M. scrofulaceum, M. smegmatis, M. terrae/nonchromogenicum* group
Disseminated infection HIV-seropositive host	*M. avium, M. kansasii*	*M. haemophilum, M. scrofulaceum, M. genavense, M. xenopi, M. marinum, simiae, * M. intracellulare, M. fortuitum*
HIV-seronegative host	*M. abscessus, M. chelonae*	*M. marinum, M. kansasii, M. haemophilum*
Catheter related infections	*M. fortuitum, M. abscessus, M. chelonae*	*M. marinum, M. kansasii, M. haemophilum, M. mucogenicum*

*Organisms included in this study

Table adapted from: Brown BA, Wallace RJ. Infections due to nontuberculous mycobacteria. In: Mandell GL, Bennett JE, Dolin R, Eds. Mandell, Douglas and Bennett's Principle and Practice of Infectious Diseases. Churchill Livingstone 5th Ed. Philadelphia, Pennsylvania, 2000; vol. 2: pp. 2630-2635. (Ref.10)

CHAPTER TWO

CURRENT DIAGNOSTIC TECHNIQUES AND DILEMMAS

Mycobacteriology is an area in which technological advances are highly warranted, not only because of the clinical importance of members of the genus Mycobacterium but also because of their diversity, fastidiousness and generally slow growth characteristics (47). The highly infectious nature of tuberculosis makes its early detection and diagnosis of paramount importance. This is a challenging problem especially in cases of paucibacillary and extra-pulmonary forms of the disease. Various conventional methods are available for the diagnosis of tuberculosis, these include: radiological examinations (chest X-rays), histopathology of diseased tissues, microscopy and culture of clinical specimens, and various immunological tests such as tuberculin skin test (TST), antibody detection by enzyme linked immunosorbent assay (ELISA) and more recently gamma interferon (IFN-γ) release assay such as the QuantiFERON® assay.

Until recently, diagnostic tests were limited to those that detect the whole organism either by microscopy of stained specimen or by bacterial culture. Now the range of tests has been expanded to include those that can detect various cellular components of the organism. These components include genomic DNA, cell wall components and protein antigens (48). Below is a summary of currently available techniques and their limitations.

2.1   Microscopy:

Since 1882 when Robert Koch first demonstrated his microscopic staining technique for *M. tuberculosis*, microscopy has remained a major cornerstone in the diagnosis of tuberculosis (23).

The standard stain for *M. tuberculosis* is the acid-fast (AFB) stain, which depends on the ability of the organism to resist decolourization with acid-alcohol following staining with arylmethane dyes. Smears for AFB stains can be prepared directly from clinical materials or from concentrated specimens which increases the sensitivity of the method. Most laboratories routinely concentrate their specimens, especially sputum, before processing. A digestion and decontamination step precedes the concentration of sputum specimens. The standard method is to treat the specimen with a solution containing amongst other things, a mucolytic agent; N-acetyl-L-cysteine (NALC) and sodium hydroxide (NaOH). The NALC 'digests' the sputum to free the bacilli from the surrounding proteinaceous material, while the NaOH 'decontaminates' the specimen by getting rid of other bacteria that may be present in the specimen. The digestion and decontamination step is strictly timed for twenty minutes. Timing is critical, as the NaOH may destroy the bacilli if allowed to act for too long. The NALC – NaOH solution is prepared in such a way that when equal quantity of the solution and the specimen is mixed together, the final concentration of NaOH in the specimen is 1%. The action of the NaOH is stopped by the addition of phosphate buffer to the specimen. The specimen is then concentrated by centrifuging at 3,000g for 20 minutes. Part of the deposit is used to prepare smears for staining, and 0.5ml is used for culture.

Generally, smears can be stained by either of the two commonly used acid fast stains: (1) the carbolfuchsin stains; Ziehl-Neelsen (ZN) (hot stain) or Kinyoun stain (cold stain), and (2) the fluorochrome stains; Auramine O with or without a second fluorochrome, rhodamine (8).

The most widely used stain in South Africa is the Ziehl-Neelsen (ZN) stain in which carbolfuchsin is used to stain the cells as described in the previous chapter. The ZN stain requires no more than a standard light microscope and a good deal of patience on the side of the technologist reading the slide. A specimen cannot be called negative unless 300 high power fields have been examined (48).

The sensitivity of this traditional method ranges from 22%-78% compared with culture results, and the limits of detection is approximately $5x10^3$ to 1×10^4 bacilli /mL, depending on several variables. These includes: the type of specimen, the Mycobacterium species present, the efficiency of decontamination, liquefaction and concentration of the specimen where required, and the technical preparation of the slide and expertise of the laboratory personnel performing the microscopic examination (47, 49). All these make the process tedious and error prone. The alternative stain, fluorescence Auramine-O, requires the use of a special fluorescence microscope. Smears prepared by this staining method are examined using the lower power magnification 40X objective lens. The bacilli are seen as bright yellow or orange-red against a dark background. The sharp contrast between the brightly colored Mycobacteria and the dark background offers a distinct advantage in scanning the slide. Because a significantly larger area of smear can be scanned per unit of time with this method than with the ZN method, it offers the advantage of a greater sensitivity (8). However, there is need for a well trained technologist who can distinguish true signals from false ones which may be due to fluorescence of non-specific tissue or cellular debris. This method is also prone to human error, and like the ZN method, has low sensitivity when compared to culture. Dead Mycobacteria will also fluoresce when stained with this method, just as dead cells will stain with the ZN method. This leads to a smear-positive, culture-negative situation about 10% of the time. This fact is an important one to remember when using microscopy smears to assess treatment efficiency (8). This is another disadvantage of microscopy when compared to culture.

2.2   Culture:

The current "gold standard" for TB diagnosis is by culture (50). Culture can be performed on a number of specimens including sputum and bronchial lavage, and also non-pulmonary samples like blood, CSF and urine (51). As few as 10 viable bacilli can be detected by culture, giving it the

advantage of being about 500 times more sensitive than microscopy. In addition, culture provides

viable organisms for further investigations such as drug sensitivity and genotyping. However,

culture using the traditional solid media technique is slow, usually taking between 6-9 weeks for

culture to be confirmed as positive or negative (50). Solid media systems can either be egg-based or

agar based. The most commonly used egg-based media is the Lowenstein-Jensen (LJ) media, while

the best known agar based media are the two variants of the Middlebrook media (Middlebrook

7H10 and Middlebrook 7H11). These are increasingly being superseded by a range of commercially

available liquid culture media systems (50). These systems, unlike the traditional solid media

culture systems which depend on the appearance of visible growth, depend on the measurement of

metabolic products secreted by multiplying bacteria. Thus they have significantly reduced time to

detection (TTD) of growth. Of the available liquid culture systems, the BACTEC® 460 system and

the BACTEC® 960 System MGIT™ System are the most notable.

2.2.1   BACTEC® 460 system:

This assay system developed by Becton Dickinson is based on generation of radioactive carbon

dioxide from substrate palmitic acid (52).   The principle of this system is based on the

measurements of $^{14}CO_2$ released from ^{14}C-palmitic acid during cellular metabolism. Cultures are

sampled periodically using a needle that pierces rubber septum on the broth culture vials, and

growth indices commensurate with bacterial growth on a scale of 0 to 999 are determined (47). This

semi-automated system has been extensively used all over the world and growth can be detected as

early as 5-10 days in this system, although the usual time of incubation before discarding the

specimen as negative remains a minimum of six weeks for all automated systems. Inclusion of NAP

(beta nitro alpha acetylamine beta hydroxyl propiophenone) helps in distinguishing *M. tuberculosis*

(inhibited) from other Mycobacteria. This system has been widely used for drug susceptibility

testing and is currently used as a comparative standard (52, 53). Nevertheless, this system has some well established limitations which include problems with the use of radioactive material, cumbersome manual loading and unloading, potential hazard of needle stick injury, risk of cross contamination and lack of computerized data management (52).

2.2.2 BACTEC® 960 and the MGIT^TM Tube System:

The BACTEC® MGIT^TM 960 instrument is an *in vitro* diagnostic instrument designed for the rapid detection of Mycobacteria in clinical samples other than blood. This system is a non-radiometric, fully automated, continuous monitoring system that was introduced by the same manufacturer as a replacement for the BACTEC®460 system (47, 53). The principle of operation of this instrument is the same as for the BBL® MGIT^TM (Mycobacterial Growth Indicator Tube). The MGIT^TM system uses oxygen – sensitive compound imbedded in silicone at the bottom of tubes containing a liquid culture medium. This medium is modified Middlebrook 7H9 broth base, which can be and is usually supplemented with a mycobacterial growth enhancer; OADC (Oleic acid, Albumin (bovine), Dextrose and Catalase), and a cocktail of antimicrobials: Polymyxin B, Amphothericin B, Nalidixic acid, Trimethoprim and Azlocillin (PANTA) (54) to inhibit contaminants.

A change in the concentration of oxygen in the culture media allows the oxygen-sensitive compound embedded in the silicone at the bottom of the tube to fluoresce, this fluorescence may be manually observed in the MGIT tubes by using a 365 nm UV lamp (the MGIT tube system) or it may be quantitated in the BACTEC® 960 equipment (BACTEC 960 system) (47). In the BACTEC® 960 system, a row of Light Emitting Diodes (LEDs) below the tubes illuminates the tubes, activating their fluorescent sensors. Then the instrument's photo detectors measure the level of fluorescence, which corresponds to the amount of oxygen consumed by organisms in the tubes.

Raw data from the detector is sent to an inbuilt computer where positivity analysis is performed. Positive cultures are immediately flagged by an indicator light on the front of the drawer and an optional alarm sound, and are displayed on the LCD screen of the instrument. When positive tubes are identified, they are removed from the instrument by the technologist. Tubes are incubated for a minimum of 42 days before being flagged negative by the instrument if no sign of viable organism is detected. This system helps in the early detection of Mycobacterial growth (7-12) days (53), and seems to be the preferred system in most laboratories where culture for *Mycobacterium* is routinely done.

A single BACTEC® MGIT™ 960 instrument is capable of monitoring a total of 960 BBL® MGIT™ tubes. The tubes are arranged in three drawers, each of which holds up to 320 tubes. The tubes are continuously incubated at the desired temperature (usually 37°C). A test cycle of all drawers are completed every 60 minutes. The BACTEC® MGIT™ 960 instrument was used to culture all the ATCC® repository strains and other laboratory strains of *Mycobacterium* used in this phase of this project.

A major drawback for this system is that it is unsuitable for the examination of blood and bone marrow specimens since blood interferes with the detection of fluorescence (53). It may also give questionable result with specimen containing blood such as tissue biopsies and bloody sputum.
Several comparative studies involving the BACTEC® MGIT™ 960 system have been carried out over the years in the different parts of the world (55-61). As expected, there were differences in these reports. In 2003, Cruciani and his co-workers (53) carried out a remarkable meta-analysis of ten most suitable of these studies. They demonstrated that though the sensitivity of the BACTEC® 460 in detecting all mycobacteria including the MTB complex but excluding the *M. avium* complex

was higher than that of the BACTEC® MGIT™ 960 system, these differences were not statistically significant. They also found the converse to be true: they found that BACTEC® MGIT™ 960 system showed a higher, though not statistically significant, sensitivity in detecting *M. avium* complex than the BACTEC® 460 system. The difference in the rates of breakthrough contamination for both systems was however found to be statistically significant, with the BACTEC® MGIT™ 960 system having a greater contamination rate than the BACTEC® 460 systems. These rates were however much lower when compared to that of the solid media culture system. This study also demonstrates that the BACTEC® MGIT™ 960 system has a significantly shorter time to detection (TTD) than the BACTEC® 460 system and the solid media culture system for the overall mycobacteria isolates as well as for each of the species evaluated as shown in Table 2.1.

Table 2.1: Time to detection of mycobacterial species

Mycobacteria species	Time To Detection (Days)		
	BACTEC MIGT 960	BACTEC 460	LJ MEDIUM
All Mycobacteria	12.9	15.0	27.0
M. tuberculosis	13.2	15.2	25.8
Smear positive specimen	11.7	11.6	22.3
Smear negative specimen	16.5	18.0	33.7
All nontuberculous mycobacteria	16.3	20.2	24.3
M. avium complex	7.6	10.7	23.3

Table adapted from: Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G, Mengoli C. Meta-Analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without Solid Media for Detection of Mycobacteria. JCM 2004; 42:2321-2325. (Ref. 53)

From this study, Cruciani and his colleagues concluded that the BACTEC® MGIT™ 960 system is a very valuable instrument in the recovery of Mycobacteria from clinical specimen. Nevertheless, they maintained that the radiometric BACTEC® 460 in combination with solid media remains the 'gold standard' for the diagnosis of acid- fast bacilli. Table 2.2 summarizes some of their findings.

Table 2.2: Sensitivity of culture systems according to mycobacterial species

		Sensitivity (95% confidence Interval)		
Mycobacteria species	No of Isolates	BACTEC MGIT 960	BACTEC 460	Solid Media
All species	1,381	0.81 (0.76-0.86)	0.85 (0.81-0.89)	0.67 (0.63-0.72)
M. tuberculosis	810	0.88 (0.84-0.91)	0.90 (0.86-0.93)	0.76 (0.73-0.79)
All nontuberculous mycobacteria	571	0.66 (0.55-0.76)	0.75 (0.68-0.81)	0.51 (0.40-0.60)
M. avium complex	265	0.80 (0.75-0.85)	0.73 (0.67-0.98)	0.57 (0.42-0.70)
Other nontuberculous mycobacteria	306	0.53 (0.42-0.64)	0.64 (0.57-0.75)	0.47 (0.34-0.61)

Table adapted from: Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G, Mengoli C. Meta-Analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without Solid Media for Detection of Mycobacteria. JCM 2004; 42:2321-2325. (Ref. 53)

In general, most investigators agree that the BACTEC® MGIT™ 960 is a practical alternative to the radiometric BACTEC® 460 and a desirable replacement for the traditional Lowenstein-Jensen solid media culture system.

2.2.3 Other Liquid Culture Systems

There are other liquid culture systems that are commercially available for the rapid detection mycobacteria from clinical samples. These include:

(a) The MB/BacT system: Manufactured by Organon Technika Inc. North Carolina, this uses automated colorimetric monitoring of carbon dioxide in a closed system. A solid state sensor is located at the base of each culture vial contains a colorimetric indicator that changes from green to yellow when carbon dioxide is produced within the vial (47). In a multicenter study carried out by Piersimoni and his co-workers (60) the recovery rates of this system was shown to be lower than that of the radiometric method. The rate of breakthrough contamination was also found to be higher in this system than in the BACTEC$^®$ 460 by this same team of investigators.

(b) ESP Culture System II: This is produced by Trek Diagnostic Systems, Ohio. It is a non-radiometric, fully automated, continuous monitoring system. This instrument uses a unique principle of automated monitoring of pressure changes inside inoculated tubes due to production or consumption of gas during growth of organisms (47).The rates of recovery mycobacteria from clinical samples using this system has been found to be comparable to that of BACTEC$^®$ MGIT^{TM} 960 (61). The rate of breakthrough contamination was also found to be similar. However, the ESP system was found to have 3.6 times as many false positive signals than the BACTEC$^®$ MGIT^{TM} 960, it was also seen to have recovered significantly more *M. gordonae* than the BACTEC$^®$ MGIT^{TM} 960 system. The implication of this is that; the recovery and subsequent identification of *M. gordonae* (which rarely is a true pathogen) creates extra laboratory work that does not benefit patient care and could

have a negative impact if patients were treated unnecessarily with anti-tuberculous agents (61)

(c) Septi-Chek AFB® system: This is a biphasic medium system introduced in the early 1990's by Becton Dickinson (47). The essential component of this system consists of culture vials containing Middlebrook 7H9 broth containing antimicrobial agents to suppress the growth of non mycobacterial organisms, an atmosphere containing CO_2, and a paddle containing three different agars. The recovery time using this system has been shown to be longer than that of the BACTEC systems. Nevertheless, its rate of recovery of mycobacterium from clinical samples has been found to be comparable to that of the radiometric BACTEC® 460 (62).

It has been consistently demonstrated that the combination of the solid media culture system (particularly the LJ media) and the liquid media gives the highest rate of recovery of mycobacterium from clinical samples (55, 59, 63, & 64). Therefore, many investigators recommend that both systems should be used together.

Apart from liquid culture systems, solid culture based techniques for the rapid identification of Mycobacterium from clinical samples is also available. The best known amongst them is the FASTPlaqueTB™ test (Biotec laboratories, Ipswich, U.K). This uses phage amplification technique for the production of a plaque on an indicator strain of Mycobacteria (65). This method has been found to be relatively fast. The turnaround time for phage-based tests is 2 days compared to about 2 hours required for microscopy or up to 2 months required for culture. Phage-based assays have been found to have high specificity but lower and variable sensitivity (66). Their performance characteristics have been found to be similar to that of sputum microscopy. Phage-based technology

has also been applied to develop a rapid test to indicate multi-drug resistant TB. This is commercially available as FASTPlaqueTB-MDRi^{TM} (65). The disadvantages of Phage-based techniques include low and variable sensitivity mentioned earlier, chances of missed diagnosis if the phage specific to the particular Mycobacterium is not used, and the general risks associated with handling of bacteriophages.

2.3   Molecular Techniques:

A very important step towards early diagnosis of tuberculosis is the rapid identification of mycobacterial isolates either from traditional solid media cultures or the rapid liquid media cultures such as the BACTEC$^®$ MGIT^{TM} 960 system. In the past, biochemical tests and growth characteristics were used to identify mycobacterial isolates. Needless to say, these were time consuming and at times, ambiguous results are obtained (52). A number of methods and techniques for the rapid identification of mycobacterial isolates from primary cultures and even directly from clinical specimen have emerged in recent years. While some are commercially available, others are "in house" protocols developed and followed by individual institutions.

Nucleic acid amplification tests (NAA's) constitute the bulk of these rapid identification techniques, although other methods such as analysis of lipid profiles by gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC) are also available and has been successfully used (8, 67).One of the GLC procedures involves saponifying the organisms in methanolic NaOH solution, the identification of the organism is the carried out based on the chromatogram tracing and on the characteristics of its colonies. Another method involving the use of acid methanolysis for the isolation of mycolic acids and methyl esters from bacterial cell has also been developed and applied

in the identification of Mycobacterial species. This method has been found to be more sensitive and less time consuming than saponification techniques (8).

The coupling of GLC and HPLC systems with mass spectrometers has also been explored in the identification of Mycobacterial isolates from cultures. These commercially available GLC-MS or HPLC-MS instruments uses computer based pattern recognition files to identify *Mycobacteria* isolates based on previously archived reference mycolic acid and lipid profiles (67). An example of such an instrument is the Microbial Identification System, a product of Microbial ID, Inc, Newark DE. This consists of a gas chromatograph and a computer system that includes a library of cell wall lipids of 26 medically important *Mycobacterium* species derived from ATCC repository organisms and some clinical strains (8). These techniques and equipments are however largely restricted to research laboratories for obvious reasons of costs and technical expertise.

Nucleic acid amplification tests (NAA's) can achieve the goal of reducing the generation time of microorganisms to minutes, and of replacing biological growth on artificial media with enzymatic reproduction of nucleic acid *in vitro* (68).The majority of techniques available for the molecular detection of *Mycobacterium* species are based on the Polymerase Chain Reaction (PCR). Since its first application to the diagnosis of tuberculosis in 1989 by Brisson-Noel et al, PCR has become the most widely used technique for amplifying mycobacterial nucleic acids (68). PCR based sequencing has become the gold standard of NAA tests for the identification of mycobacterial species (69). PCR based assays are comparatively simple, with good availability of reagents.

Other alternative amplification methods are also available. This includes:

    (a)   Strand displacement amplification (SDA) method

    (b)   Transcription mediated amplification (TMA) method

(c)   Nucleic acid sequence based – amplification (NASBA) method

(d)   Q-Beta replicase amplification method

(e)   Ligase chain reaction (LCR)

(f)   Branched deoxyribonucleic acid signal amplification (bDNA)

(g)   Loop mediated isothermal amplification (LAMP) and

(h)   Luciferase reporter mycobacteriophage (firefly luciferace assay)

These all have been used in various forms with varying degree of success (47, 52, 70-78), some are commercially available in kit-based, user friendly formats. For example, the strand displacement amplification (SDA) is an isothermic NAA technique: Becton-Dickenson provides a TB diagnostic assay using SDA with the trade name BD ProbeTec ET direct TB system. Ligase chain reaction (LCR) has also been used to produce a rapid TB diagnostic tool, which is commercially available as LCx MTB assay and ABBOT LCx Probe system developed by Abbott Laboratories (70, 50). GenoType Mycobacteria Direct Assay (GTMD) is another commercially available, user friendly kit. Based on Nucleic acid sequence-based amplification (NASBA), it is manufactured by Hain Lifescience GMBH, Nehren, Germany for the detection of MTB complex, *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. malmoense* directly from clinical specimen. Though a relatively new product, it was evaluated and compared with COBAS AMPLICOR MTB produced by Roche Diagnostics by Franco Alvarez de Luna and his colleagues in 2006 (77). They concluded that GTMD is a relatively fast easy to interpret assay, and results can be obtained within one working day. They nevertheless admitted that the technique involves a laborious extraction process and requires more manipulation than the COBAS AMPLICOR MTB system which is more automated.

In the United States, two NAA based products have been approved for use commercially by the Food and Drug Administration (FDA). These are: The Enhanced Amplified *Mycobacterium Tuberculosis* Test (E-MTD; Gen-Probe, Inc., California) which is approved for use on respiratory specimens regardless of AFB result and The Amplicor *Mycobacterium tuberculosis* Test Assay (Roche Diagnostic Systems, New Jersey) which is approved for use only with AFB positive specimen (69, 70, 72, 76 & 78). The Amplicor test is based on PCR, while the E-MTD test is based on transcription-mediated amplification (TMA) system. Though the performance of both NAA tests has been reported to be excellent (sensitivity 95-96%, specificity 100%) when testing respiratory specimen that are AFB smear-positive, it is however much lower when testing smear-negative specimens (sensitivity 48-53%, specificity 96-99%, depending on the NAA test) (78). As a consequence, in a population where the prevalence of *Mycobacterium tuberculosis* complex in AFB smear-negative specimens is low, NAA would yield many false-positive result relative to additional cases of TB detected (78). The impact of false positive diagnosis of TB is substantial as treatment is lengthy and the drugs used are generally toxic to the patients.

Furthermore, because molecular amplification techniques are capable of detecting low numbers of DNA target, very low level of contamination can lead to false positive results, unless a four room strategy with unidirectional work flow is established and strictly adhered to (50). Another major problem plaguing NAA tests is the difficulty in controlling hybridization conditions for medium or high-density arrays, which may result in some probes binding to mismatched sequences, thus leading to erroneous hybridization result, and this limits the accuracy of the assay. Fragmentation caused predominantly by depurination as a consequence of the protonation of the nucleobases A and G is another inherent problem of NAA methods (79). NAA techniques keeps evolving almost on daily basis, consequently, a vast amount of materials covering all aspects of NAA techniques is

available in literature. Unfortunately, a detailed and technical review of all is beyond the scope of this text.

Despite its elegance, high specificity and sensitivity, nucleic acid amplification diagnostic techniques are still fraught with various limitations. Some of these have been discussed above, others include financial costs and time constrains. All these play an important role in making NAA methods less desirable, thus further lending credence to the fact that the search for an alternative method of diagnosis that is simple, efficient, with high sensitivity and specificity, and with a significantly short turn around time is of utmost importance.

2.4   Immunological Methods

As earlier mentioned, various other conventional methods are available for the diagnosis of tuberculosis. Such methods includes radiological examinations (chest X-rays), histopathology of diseased tissues, and immunodiagnostic methods involving tests such as the tuberculin skin test (TST), others that are based on enzyme linked immunosorbent Assay (ELISA) such as the QuantiFERON® assay. Of these, the tuberculin skin test (TST) is the oldest, it was developed by Robert Koch in the 1880's (old tuberculin) (19). The old tuberculin was an extract of boiled culture of the tubercle bacilli. In the 1990's a simple protein precipitate of the old tuberculin was made known as purified protein derivative (PPD), from this came the more consistent form of tuberculin; standardized purified protein derivative (PPD-S) which has been used to assess latent *M. tuberculosis* infection (LTBI) since 1939 (80, 19, 13).

The TST involves the administration of a 5 tuberculin units (TU) dose (which is equivalent to 0.0001mg of PPD-S protein in 0.1ml of solution) (13, 2). The sensitivity and specificity of this dose were derived from the populations in which the incidence of tuberculosis was accurately known

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(13). There are two ways of administering TST: the multiple puncture technique (Heaf and Tine test) or the intracutaneous injection of the 5TU dose into the fore arm (the Mantoux test). The latter is the more common of the two. The result of the TST is usually read within 48-72 hours. The test is considered positive if the diameter of the resulting lesion is 10mm or greater (13, 2). The interpretation of TST is subjective, and the it's use is fraught with several limitations, the major one being cross reaction with bacille Calmette-Guerin (BCG) vaccine in BCG vaccinated populations. The effect of vaccination may last for over 15 years, and this may influence the results or its interpretation (81, 19). Other limitations of TST includes: inter operator variability, differences in mode of administration (i.e. between the multiple puncture technique and the intracutaneous injection), low sensitivity in immunocompromised patients, false negativity due to clinical conditions such as malnutrition and sarcoidosis, and cross reaction with environmental Mycobacteria, this decreases the sensitivity of the test (81, 19, 2).

More recently, other immunological tests that are more sensitive and specific than the TST have been developed. These measure the gamma interferon (IFN-γ) released by the memory and effector T-cells in the blood of *M*. *tuberculosis* infected individuals (81). The QuantiFERON® (QTF-TB) earlier mentioned is one of such tests. Manufactured by Cellestis limited (Carnegie, Victoria, Australia), it was approved for the detection of latent *Mycobacterium tuberculosis* infection (LTBI) by the FDA in 2001(79). The major advantage of QFT is that is does not cross-react with BCG, and therefore can be used reliably in BCG vaccinated populations (81, 82).

The EliSpot test is another test based on the measurement of gamma interferon (IFN-γ). Although more laborious than the QFT, it has been shown to be less susceptible to false negative results in the lymphopenic patients (81). IFN-γ based tests, in addition to being more sensitive than TST, also

perform better in identifying LTBI in immunocompromised patients (81). Nevertheless, these newer tests, like others before them, do have their limitations. These includes their high costs, requirement of highly trained personnel, inherent dangers of phlebotomy and handling of fresh blood especially in populations with a high prevalence of HIV (81, 79).

Although the past 50 years of active tuberculosis research has brought about the development of rapid diagnostics to identify all new cases and powerful chemotherapy to cure tuberculosis, improvement on these existing methods of diagnosis and the development of new ones still continues to be an attractive area of research. Over the years, this research has spread to the field of mass spectrometry. The analysis of lipid profiles by GL-MS and HPLC-MS earlier mentioned are some of such mass spectrometric methods. The Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-ToF MS) being explored in this study is another mass spectrometry method.

Subsequent chapters of this dissertation catalog the work done in this phase of this study to explore the applicability and the promises MALDI-ToF mass spectrometry holds in the search for a rapid, highly sensitive and specific way of diagnosing the often debilitating, highly contagious and often fatal disease: tuberculosis.

CHAPTER THREE

Introduction to Mass Spectrometry

The search for a more rapid, highly sensitive and specific method of diagnosing tuberculosis is an ever widening circle that has expanded to areas that would otherwise not have been considered 'conventional' in diagnostic mycobacteriology. One such area is mass spectrometry. Even though, as mentioned in the preceding chapter, some form of mass spectrometry (mainly GC-MS and LC-MS) has been used in the analysis of lipid profiles of *Mycobacterium* for their identification (8, 67). This use has mainly been restricted to taxonomic and research purposes due to reasons of cost and the highly infective nature of some of the medically important members of this genus.

This study joins the relatively few studies of its kind encountered in available literature to establish the ground work for the application of mass spectrometry, specifically Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS) in the field of diagnostic mycobacteriology. This is an area which is in need of the speed, sensitivity and specificity that MALDI-ToF technique promises to offer.

3.1   Basic Principles of Mass Spectrometry

In simple terms, a mass spectrometer is an instrument that measures the masses of individual molecules that have been converted to ions, i.e. molecules that have been electrically charged (83). In actual fact, a mass spectrometer does not measure the molecular mass of a molecule directly, but rather, its mass-to-charge (*m/z*) ratio. This is because the minute size of a molecule makes it impracticable for its mass to be measured by units convenient to our everyday macroscopic world such as grams and kilograms. A suitable mass unit known as Daltons (Da) which is defined as 1 Da

= 1/12 of the mass of a single isotope of Carbon 12 (^{12}C) was developed for it. In a similar vein, the charge on an individual ion is measured in a unit convenient for it, which is the fundamental unit of charge; the magnitude of charge on an electron which is denoted by the integer z. The mass to charge ratio (m/z) therefore represents Daltons per fundamental unit of charge. Since the ions most often encountered in mass spectrometry are usually singly charged, ($z = 1$), therefore the m/z value is numerically equal to their molecular (ionic) mass in Daltons (83).

There are different kinds of mass spectrometers used for different kinds of applications/analysis, each with its own peculiar strength and weakness. Nevertheless, there are things that are common to all mass spectrometers. Firstly, all mass spectrometer consists of three basic functional parts:

(a) An **ion source** where gas phase ions are produced;

(b) An **analyzer** which sorts/ separates the ions according to their mass-to-charge (m/z) ratio;

(c) A **detector** in which the ions flux is converted to proportional electrical current. This is then fed into a data handling system which records the magnitude of these electrical signals as a function of their m/z and presents the information as a mass spectrum (83, 84, 88).

Secondly, all mass spectrometers operate on the principle that the paths of gas phase ions in an electric and/or magnetic field are dependent on their mass-to-charge ratio. Therefore, samples (analytes) must be converted to gas phase ions for them to be analysed by mass spectrometry. The generation of gas phase ions takes place in the ion source; the ions produced are directed either by a magnetic field or an electric field (or both) into the mass analyzer. In the ion source samples are evaporated, ionized and accelerated. Traditionally, evaporation was accomplished by simple heating and ionization was accomplished by using 70eV electrons (84). Both processes can result in the decomposition of the analyte. The process of ionization by electrons accelerated through a potential

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of 70 volts is a highly energetic or "hard" process which may lead to excessive fragmentation that may leave very little or no trace of the parent ion (83). This makes the analysis of large biological molecules by conventional mass spectrometry difficult, since sample analysis by mass spectrometry may have to be carried out on molecular (parent) ions rather than daughter ions species.

In the past, analysis of compounds from biological origin was severely limited due to their thermo-labiality and limited volatility (88). These properties made them incompatible with the methods of ionization available at the time, most of which were 'hard' ionization methods. The development of 'softer' methods for sample evaporation and ionization in modern mass spectrometry has tremendously opened up the field of bioanalysis (i.e. analysis of compounds of biological origins) (88). Nonequilibrium energy transfer can eject (desorb) samples into the gas phase without heating, and low energy proton transfer can be used for 'soft' ionization (84). The desorption/ionization steps can even be combined into a single step as used in the Matrix Assisted Laser Desorption/ Ionization Time-of-Flight mass spectrometry (MALDI-ToF MS).

There are different kinds of ionization methods and some of these are shown in Table 3.1 below. Also shown is the mass range over which they can be used and applied:

Table 3.1: Commonly used ionization methods

Ionization Method	Typical Analytes	Sample Introduction	Mass Range	Method Highlights
Electron Impact (EI)	Relatively small, volatile molecules	GC or liquid/solid probe	Up to 1,000 Daltons	Hard method
Chemical Ionization (CI)	Relatively small, volatile molecules	GC or liquid/solid probe	Up to 1,000 Daltons	Soft Method
Electrospray (ESI)	Peptides Proteins	Liquid chromatography or syringe	Up to 200,000 Daltons	Soft method
Fast Atom Bombardment (FAB)	Carbohydrates Organometallics Peptides	Sample mixed in viscous matrix	Up to 6,000 Daltons	Soft method but harder than ESI or MALDI
Matrix Assisted Laser Desorption/Ionization (MALDI)	Peptides, Proteins Nucleotides	Sample mixed in matrix solution	Up to 500 kDa	Soft method Very high mass

Table adapted from The University of Arizona, Department of Chemistry. Introduction to Mass

Spectrometry. Available from: http://www.chem.arizona.edu/massspec/intro_html/intro.html

(Ref.89)

In addition to these, there are other ionization methods such as inductively coupled plasma sources, glow discharge, field desorption (FD), thermospray, desorption/ionization on silicon (DIOS), direct analysis in real time (DART) atmospheric pressure chemical ionization (APCI), secondary ion mass spectrometry (SIMS) spark ionization, thermal ionization, ion attachment ionization, easy ambient sonic-spray ionization (EASI), and desorption atmospheric pressure photoionization (DAPPI) (90). Out of all these, only two are most suitable for the analysis of large biological molecules. They are: Electrospray ionization (ESI) developed by John Bennett Fenn, and Matrix Assisted Laser Desorption/Ionization (MALDI) (88). The later is used in this project and will be discussed in more details subsequently.

As there are different kinds of ionization methods, similarly, there are different kinds of mass analyzers. The mass analyzer is the second basic functional unit of any mass spectrometer; it uses dispersion or filtering to sort ions according to their mass-to-charge (m/z) ratio or a related property (83). All mass spectrometers operate on the dynamics of charged particles in an electric and or magnetic field as earlier mentioned. To prevent collision/interference with air molecules which are ubiquitous, this dynamics must take place in an environment devoid of air molecules that is; in a vacuum. It therefore follows that good vacuum (in the magnitude of 10^{-4} torr or below) is required in the mass analyzer for successful mass spectrometry analysis (91). There are different types of mass analyzers using either static or dynamic fields, or magnetic or electric fields. Each type of analyzer has its own peculiar strength and weaknesses, many mass spectrometers combine two or more mass analyzers for **Tandem** mass spectrometry (MS/MS) (83, 91). Some of the more common analyzers are briefly described below. In addition to these, there are other less common ones designed for special applications.

a. **Magnetic Sector Analyzers:** These function by bending the trajectories of ions into paths of radii that depends on the momentum-to-charge ratio of the ions. Ions of larger m/z are less deflected than ions of smaller m/z values, resulting in the dispersion of these ions in space according to their m/z values. Varying the magnetic field strength changes the ions trajectories, and ions of different nominal mass-to-charge ratios can then be focused on a detector (83).

b. **Quadrupole Mass Analyzers (also known as quadrupole mass filters):** These, as the names implies, consists of four precisely parallel rods equally spaced around a central axis. In this devise, mass sorting depends on ion motions resulting from simultaneously applied constant direct current (dc) and radio frequency (rf) electric fields. By systematically changing the field strengths, ions of different m/z can be scanned through the mass analyzer and transmitted to the detector (91).

c. **Quadrupole Ion Trap Mass Analyzer:** These works on the same physical principles as that of the quadrupole mass analyzers, however, they do not operate as filters. Rather, ions are trapped and sequentially ejected using fields generated by rf (and sometimes dc) voltages applied to the poles (electrodes) (83, 91).

d. **Linear Quadrupole Ion Trap:** this devise is similar to a quadrupole ion trap, it traps ions in a 2D quadrupole field instead of a 3D quadrupole field like in the quadrupole ion trap (91).

e. **Time-of-flight Mass Analyzers:** These are about the simplest mass analyzers to design and understand. Here, ions emitted and accelerated in a brief burst (pulsed) travel through a Time-of-flight tube where their traveling time to the detector is dependent on their different m/z ratios. The ions with the smaller m/z values will arrive first at the detector (83). There are two common variants of the ToF analyzers; they are the **Linear** and the **Reflectron**. The

linear mode is the one used in this project, like the ionization source (MALDI); linear ToF will be discussed in detail subsequently.

f. **Fourier Transform Ion Cyclotron Resonance (FR-ICR) Spectrometer:** This measures mass by detecting the image current produced by ions in a magnetic field (91).

The third basic functional unit of a mass spectrometer is the detector. The detector records the change induced or current produced when an ion passes by or hits a surface. Like the two preceding basic functional units, there are different kinds of detectors employed in different kinds of mass spectrometers. Some of the numerous detectors in use includes: electron multipliers, Faraday cups, ions-to-photon detectors and micro channel plate detectors (MCP). The MCP detector is the type of detector in the instrument used for this project.

All mass spectrometers are connected to a data handling system which usually is a computer. Due to the large amount of information generated by the mass spectrometer, and a relatively large amount of parameters used or changed within a short period of time, computers are necessary for mass spectrometer control and for data acquisition, storage and presentation. Computer based data handling systems also typically include software for quantitation, spectral interpretation and compound identification through use of on or off line spectra libraries (83).

3.2   Introduction to MALDI-ToF technology

In 2002, Koichi Tanaka was jointly awarded the Nobel Prize in chemistry with John Bennett Fenn for the development of 'Soft Laser Desorption' (SLD) in 1987. Later on in that same year, Franz Hillenkamp and Michael Karas improved on the SLD method by developing the Matrix Assisted Laser Desorption/Ionization method (92, 93). Hillenkamp, Karas and their colleagues discovered that the amino acid alanine could be ionized more easily if it was mixed with the amino acid tryptophan and irradiated with a pulsed 266 nm laser. They discovered that the tryptophan was absorbing the laser energy and helping to ionize the non-absorbing alanine. It was also discovered that peptides up to 2.843 kDa such as melittin could be ionized when mixed with this kind of 'Matrix' (92, 93).

Since that time, the use of a 'matrix' to 'assist' in the desorption /ionization has undergone tremendous development. The MALDI matrix is usually an organic aromatic weak acid, which should absorb energy at the wavelength of the laser in use and should not modify or react with the analyte before laser irradiation (94.). The MALDI matrix plays several important roles such as absorbing the laser energy thereby protecting the analyte from excessive energy that may cause decomposition; it also enhances ion formation of the analyte by photoexcitation or photoionization of the matrix molecule (94). Over time, numerous matrices has been developed for use in MALDI-ToF mass spectrometry, these include: nicotinic acid, glycerol, Sinapinic acid (94), alpha-cyano-4-hydroxylcinnamic acid (CHCA) (94-99) 5-chloro-2-mercaptobenzothiazole (CMBT) (99), 2, 5-dihydroxylbenzoic acid, and indole-3-pyruvic acid (94) amongst many others.

As earlier mentioned MALDI is a 'soft' ionization method that allows fragile biomolecules to be introduced into the mass spectrometer as ions without significant decomposition caused by either

the ionization or desorption step (84-87). This major advantage of the MALDI method makes it suitable for use in the analysis of polar biomolecules such as proteins and peptides, and shortly after its development it was routinely used for their analysis. Eventually, proteins from bacterial extracts were also analysed by MALDI, including proteins that can be used for their taxonomic characterization. A major breakthrough came with the observation that taxonomically characteristic proteins can be detected by MALDI mass spectrometry applied directly to whole bacteria cells (84). This opened up a whole new world of possibilities in the area of bacteria identification and characterization. The coupling of MALDI ionization method with the Time-of-flight mass analyzer produces a mass spectrometer; the MALDI-ToF mass spectrometer.

The ToF mass analyzer as earlier mentioned is simple in design. It essentially consists of a "field free" flight tube and a detector. Ions emitted in a brief burst (pulse) from the ion source are accelerated at ground potential; the accelerated ions obtain a mass dependent velocity and move from the ion source into the 'drift' or 'field free' tube where their different velocities (based on mass and charge) differentiates them as they "race" towards the detector (84, 102). In the detector region, the now mass-separated ions are counted giving rise to a signal for each m/z value that is proportional to the number of ions present (84). This describes a linear ToF analyzer. The "reflectron" ToF, which is a more complex variant of the linear ToF, reflects the ions back towards the source, essentially doubling the length of the flight field. This has been shown to increase the maximum achievable resolving power by a factor of about 2 (84). The ToF mass analyzer has three main advantages that make it the analyzer of choice in the identification and characterization of bacteria, they are:

(a) **Sensitivity**. ToF analyzers detect **all** ions of 'like charge'. In the ToF analyzers, ions are separated in time (a very short time), but all are eventually detected. Unlike other analyzers

such as sector, quadrupole and ion trap analyzers that differentiate one *m/z* value from others by simply discarding ions with the non-selected *m/z* values and thereby discard more ions than they detect. This makes them less sensitive and not suitable for bacterial mass analysis (84).

(b) **High mass range.** ToF analyzers are able to detect ions up to and well over 300,000 Da (102, 91).
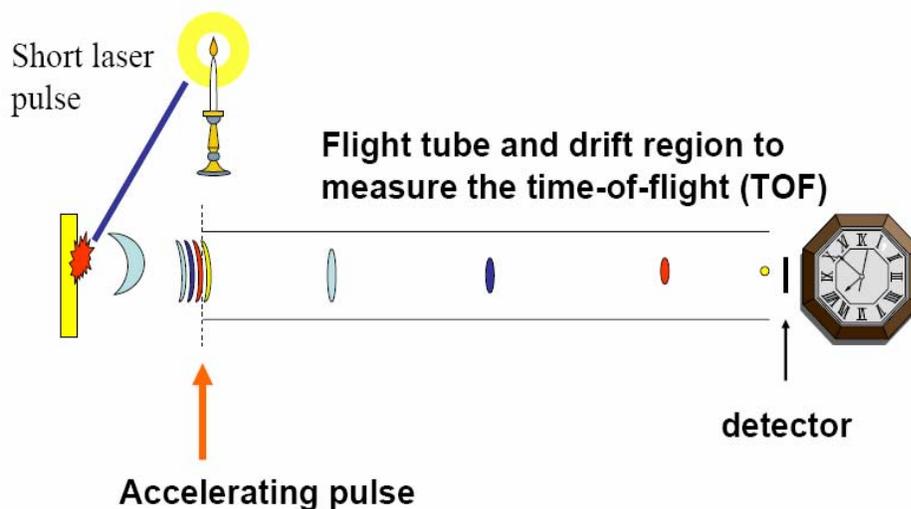
(c) **Speed**. The ToF instrument can produce individual spectra in less than a second; this makes the acquisition of many spectra possible within a short period of time (84).

The MALDI method of ionization and the ToF mass analyzer are both suited for each other as they both work well in the 'pulsed' mode. In addition to the three main advantages of ToF analyzers enumerated above, MALDI-ToF mass spectrometers also has other advantages which includes spectra simplicity due to singly charged ions, low noise levels, little sample consumption, average salt tolerance and minimal fragmentation (94).

Figure 3.1 below shows the schematic set-up and general principle of operation of a linear MALDI-ToF mass spectrometer.

Figure 3.1: General operating principle of MALDI-ToF MS

Matrix-Assisted Laser Desorption Ionization (MALDI)

Short laser pulse

Flight tube and drift region to measure the time-of-flight (TOF)

detector

Accelerating pulse

Obtained from: Barnes S. Mass spectrometry methods in botanicals research Purdue-UAB

Botanical Center Workshop 2002. Available from:

http://www.uab.edu/botanicals/pdfs/09_MALDI.pdf (Ref.101)

As previously explained, molecules of the analyte embedded in the matrix are vaporized and ionized in a vacuum by a short laser pulse, and accelerated in an electric field. According to their masses, they travel faster or slower, resulting in different arrival times (Time-of-flight) at the detector where they are detected as electrical signals (84, 102). The Maldi accomplishes both vaporization and ionization in a single step, hence it is referred to as desorption/ionization technique (102). This ability of the Maldi to achieve both vaporization and ionization in a single non-destructive step is its major advantage, and this makes it the excellent choice for the mass spectrometry of molecules of biological origins.

3.3   MALDI-ToF MS and Bacteriology

MALDI-ToF MS technique has been used in the past for the analysis of proteins, peptides, nucleic acids and synthetic polymers, and sugars (95, 88). Such applications have however been limited mainly to areas such as analytical chemistry, forensics and biotechnology in the industrial and pharmaceutical fields.   Since the first reports demonstrating successful MALDI-ToF MS biochemical analysis were published in the late 1980's from the labs of Karas *et al* and Tanaka *et al*, this technology has become a popular and versatile method to analyze a range of macromolecules from biological origin (95). The distinct advantage of this technique to provide good resolution and mass accuracy from complex biological mixtures in the presence of low levels of salts and other agents that often interferes with other mass spectrometry techniques (103) mentioned earlier makes it an attractive tool to explore in the search for a rapid and accurate method of microbial identification.

As previously explained, the 'soft' ionization method of MALDI-ToF mass spectrometry allows the desorption of peptides and proteins from whole bacteria, vegetative cells or spores and other microorganisms such as viruses and fungi without extensive separation (104). 'Whole cell' (i.e. the desorption of ions from 'intact cells') MALDI-ToF is an emerging sensitive technique and it offers great promise for the rapid identification of bacteria down to species and strain level. The term "Whole cell" as used here does not refer to literally intact or whole bacterial cells. Rather, this term refers to cells that have not been treated or processed in any way specifically for the removal or isolation of any cellular components from the others (102). For safety reasons, it is sometimes necessary to inactivate or 'kill' microorganisms before subjecting them to mass spectrometry analysis. Such inactivation steps may result in cell disruption. Because no additional steps are taken to isolate protein or any other analyte, the process is still referred to as "Whole cell" or "intact cell"

analysis, to differentiate it from procedures where additional steps are included to deliberately disrupt cellular membranes or separate/recover analytes from the cells (102). This is of particular importance in this project, where the database generated consist of spectra of both "Cell Extract" and "Cell Deposit". All the Mycobacterial organisms used in this project were all treated as Bio-safety level 3 (BSL 3) organisms and were rendered non-viable using an acidified solution of Acetonitrile/Water before the removal of the organisms from the BSL 3 facility to the mass spectrometry laboratory. This may have resulted in some form of cell disruption or lysis. More details about this "Sterilization" procedure is given in the following chapter of Materials and Methods.

Whole cell MALDI-ToF MS was originally developed and intended for rapid taxonomic identification of bacteria (102), but its proven efficiency in this area makes it an attractive tool to explore in diagnostic Mycobacteriology. Identifying bacteria by their mass spectra fingerprints is of practical significance only if the spectra are reproducible, and research is still ongoing on ways to generate reproducible results within and between laboratories. Numerous experimental factors have been shown to influence the quality and reproducibility of mass spectra obtained when bacteria are analyzed using MALDI-ToF MS (97). The pre analysis sample preparation step incorporates the most important elements influencing the quality and reproducibility of the spectra. Of these, the choice of matrix is of utmost importance as difficulties with MALDI-ToF MS frequently arise from non-incorporation of the analyte into the solid matrix and non-formation of suitable crystallites (95). Therefore, the matrix of choice should be such that it will be able to absorb energy at the wave length of the laser used, and at the same time protect the analyte from excessive energy (i.e. prevent analyte decomposition). It should also enhance ion formation of the analyte and should not modify or react with the analyte before laser irradiation (94).

In an extensive study carried out by William and her co-workers in 2003, α-cyano-4-hydroxycinnamic acid (CHCA) matrix in 50:50 acetonitrile: water acidified with 2.5% trifluoroacetic acid (TFA) was found to be the optimum of the matrices tested (97). The use of additives or co-matrixes has also been reported to give improved results in some cases (94, 97). Hettick et al (98) used fructose as an additive to α-cyano-4-hydroxycinnamic acid (CHCA) matrix and found that it resulted in good quality, highly reproducible MALDI-ToF spectra. Other experimental factors that can influence the quality of whole cell MALDI-ToF MS include: sample deposition method, culture medium, incubation time, matrix solvent, concentration of cells in the matrix, concentration of acid in the matrix, and several instrumental parameters (102, 97). The effect of all these have been investigated by various authors, a comprehensive review of which is beyond the scope of this text. Nevertheless, it is a well established fact that the optimization and control of all experimental variables will lead to a stable protocol for the MALDI-ToF analysis of bacteria, it has also been established that by following a clearly established protocol, reproducible Maldi spectra can be obtained (96-99, 104).

3.4   MALDI-ToF MS and Diagnostic Mycobacteriology

MALDI-ToF MS is a relatively new and evolving technology in the field of diagnostic Mycobacteriology. Although this technique has been successfully applied in other areas of bacteriology (95, 96, 102-104), its application in diagnostic bacteriology is still in its infancy. Of the comparatively few documents available in literature on this area, the work of Hettick and his colleagues (98) is outstanding. Using six species of *Mycobacterium*, they sought to establish the ground work differentiating *Mycobacterium tuberculosis* from other Mycobacterial strains using MALDI-ToF MS technique. They also compared the use of cell extract versus cell deposit to

generate mass spectrum 'finger print' for each of the species of *Mycobacteria* investigated. This work provided valuable information that was utilized in this current work.

A study of interest was published in 2004 by Lefmann et al (107), who identified 12 type strains of Mycobacterium species after base specific cleavage of PCR amplified and in vitro-transcribed 16S rRNA gene (rDNA) using the MALDI-ToF MS technology. They concluded that the technology delivers an open platform for high-throughput microbial identification on the basis of any specific genotypic marker region. Another work of relevance is that of Michelle Pignone and her co-workers (108), which generated a mass spectra database using 37 strains representing 13 different species of Mycobacteria. They concluded that the identification of diverse *Mycobacterium* species is a tractable task by using the MALDI. They also demonstrated that it is possible to resolve clearly related strains Mycobacteria, and that whole cell MALDI-ToF MS can serve as an effective identification system for *Mycobacterium* species. They however admitted that their work needs to be confirmed by the testing of more virulent strains of *Mycobacterium tuberculosis* and building a robust database.

This current study seeks to fill the knowledge gap highlighted by the last authors. While unique and different from the above mentioned studies and any other so far encountered in available literature in many ways, as evident in subsequent chapters, it is believed that this study in a 'stand alone' mode and as a basis for further studies will contribute immensely to making MALDI-ToF MS technique relevant in the field of diagnostic Mycobacteriology.

CHAPTER FOUR

MATERIALS AND METHODS

Background

The technology of MALDI- TOF MS is a relatively new and evolving technology and its application in the clinical field is still in its infancy. Its use for diagnostic purposes is even more so, therefore the use of utmost care in the preparation of reagents, and the handling and storage of the organisms used to generate reference mass spectra for the database cannot be overemphasized. Similarly, the optimization of certain crucial experimental factors such as inactivating method and choice of matrix is of paramount importance. Several basic criteria have been investigated previously (97-99), some of which after due consideration were used as reported. Other criteria had to be developed, investigated and optimized before being utilized to generate the spectra included in this database.

The main aim of this thesis was to generate a database of reference mass spectra fingerprints of selected *Mycobacterium* species. This necessitated the standardization of an experimental protocol which ensured that the experimental factors previously mentioned and the numerous instrument parameters such as pulse voltage and real time data selection function (RTDS) used were optimized for maximum spectra generation and reproducibility. A standard operating procedure for generating the database of reference mass spectra finger print of selected Mycobacterium species as done in this study was developed. It is included at the end of this thesis as Appendix A.

4.1 Chemicals, Reagents and Culture Media

4.1.1 Solvents

Table 4.1 shows the list of critical solvents, the respective suppliers and corresponding product numbers of the various solvents used in this study. They were all used and stored according to standard laboratory safety practice.

Table 4.1: List of Solvents used and the respective suppliers

Product	Supplier	Product Code
Acetonitrile (HPLC grade)	Romil	H 048 L
Methanol (HPLC grade)	Romil	H 409 L
Trifluoroacetic acid	Merck	8.08260.0100
Nitric Acid	Merck	SAAR 4465080 LC
Glycerol	Merck	SAAR 2676520 LC
Formic acid	Merck	SAAR 2437080 LC

4.1.2 Reagents

All reagents used were commercially obtained from the manufacturers or their accredited representatives. They were prepared, used and stored as per manufacturer's instructions, accompanying product information sheets or as instructed by our collaborators during training in Manchester. Standard laboratory safety practice and regulations were strictly observed. Below is a summary of such reagents, their preparation and storage conditions:

(a) Alpha-Cyano-4-hydroxycinnamic acid (α-Cyano), 99% purity by TLC, powdered form; Product number C2020 (Sigma Aldrich). A working solution was prepared by dissolve 14mg of the

powder in 1ml of Matrix Solvent Solution (MSS). This solution is unstable; it is therefore not stored, but discarded appropriately after use. The dry powder was stored at -20°C.

(b) 5-Chloro-2-mercaptobenzthiazole (CMBT), 90% (technical grade) powder; Product number 125571 (Sigma Aldrich). A working solution was prepared by dissolving 3mg of the powder in 1ml of Matrix Solvent Solution (MSS). This solution is also unstable and therefore not stored, but discarded appropriately after use. The dry powder was stored at -20°C.

(c) Phosphate Buffered Saline (PBS), product number D5652 (Sigma Aldrich). A working solution is made up by dissolve 9.6g of the powder in 1 liter of sterile water. This solution is stable, it is stored at 4°C, and the dry powder is also stored at 4°C

(d) Matrix Solvent Solution (MSS), this solution was made up as follows:

- ➤ Acetonitrile, RO distilled water and Methanol in a 1:1:1 ratio
- ➤ 0.01M 18-crown-6-ether 99% (1, 4, 7, 10, 13, 16 Hexaoxacyclootadecane), product number 186651 (Sigma Aldrich)
- ➤ 0.1% formic acid V/V

The solution is stable with a shelf life of 6 months at room temperature. After this time it begins to deteriorate, and it then discarded and a fresh solution be prepared.

(e) Inactivating solution: this solution was made up as follows:

- ➤ Acetonitrile and RO distilled Water in a 1: 1 ratio
- ➤ Trifluoroacetic acid is added to the mixture to a final concentration of 2.5%

The solution is stable and was stored at room temperature in a dark cupboard.

(f) Pepmix. This is a mixture of seven standard peptides used for the automated calibration of the MALDI-ToF instrument, and to "lock mass" correct for the first order mass drifts that may occur during the course of the experiment. All 7 peptides were purchased from Sigma Aldrich and they were dissolved in RO distilled water (DDI) to make an initial solution at a concentration of 1mg/ml. Table 4.2 shows the list of peptides, product numbers and individual volumes (of the initial 1 mg/ml solution prepared) used to prepare a 1ml volume of the prepared Pepmix.

Table 4.2: List of peptides and corresponding volumes (of 1 mg/ml solution) in 1ml of Pepmix

Peptide	Product Number	Dilution Required in 0.1% (v/v) TFA
Bradykinin	B3259	2.00 : 1000μl
Angiotensin	A9650	2.60 : 1000μl
Glu1-fibrinopepetide B	F3261	3.14 : 1000μl
Renin substrate tetra decapeptide	R8129	3.52 : 1000μl
ACTH (18-39)	A0673	4.94 : 1000μl
Insulin (bovine)	I5500	22.93 : 1000μl
Ubiquitin (bovine)	U6253	171.20 : 1000μl

The individual 1mg/ml solutions of the peptides were stored at -20^OC since they are more stable and can be stored up to 24 months at that constant temperature. Once mixed, the Pepmix cocktail was also stored at -20^OC. However, a fresh mixture was prepared every 6 months as it deteriorates over time.

4.1.3 Media

All the media used for this project, with the exception of Middlebrook 7H9 broth, Middlebrook 7H10 agars, Lowenstein Jensen (LJ) media and Mycobacterium Growth Indicator Tube (BBL^TM MGIT^TM) were commercially obtained from Select-A-Media (Johannesburg) and each batch ordered was delivered with its own Quality Assurance certificate. The other media were obtained as follows:

> ➤ The Lowenstein Jensen (LJ) media used were purchased from the NHLS Central Media Laboratory, Greenpoint, Cape Town through the Tygerberg hospital NHLS TB laboratory.
>
> ➤ The Difco^TM Middlebrook 7H9 broth powder was purchased from the local representatives of Becton Dickinson (Johannesburg). The ingredients and their approximate concentration per 900ml were provided by the manufacturer on the product container. It was prepared according to manufacturers instructions as follows:
>
> > ▪ The recommended 4.7g of the Difco^TM Middlebrook 7H9 broth powder (product number 271310) was dissolved in 900ml of RO distilled water containing 2ml of glycerol.
> >
> > ▪ This was autoclaved at 120°C for 10 minutes.
> >
> > ▪ One hundred (100) mL of Albumin-Dextrose-Catalase (ADC) (product number 211887, Becton Dickinson) mixture was aseptically added to it when cool to touch.
> >
> > ▪ It was then filtered through a Corning^® 0.22μm cellulose acetate 500ml filter system (Corning Incorporated, NY 14831).

- It was stored at 4°C when not in use. The sterility was checked as subsequently described before each use.

➢ The Middlebrook 7H10 agar powder (product number 262710, Becton Dickinson, Johannesburg) used to prepare the agar plates. The ingredients and their approximate concentration per 900ml were provided by the manufacturer on the product container. It was prepared according to manufacturers instructions as follows:

- As stipulated, 19g of the agar powder was dissolved in 900mL of RO distilled water containing 5ml of glycerol.

- It was thoroughly mixed and gently heated with constant agitation until all the powder was completely dissolved.

- It was then autoclaved at 120°C for 10 minutes.

- One hundred (100) ml of Oleic acid-Albumin-Dextrose-Catalase (OADC) (product number 212240, Becton Dickinson) mixture was aseptically added to it when cool to $50-55^{O}$C.

- It was then aseptically poured into commercially available, sterile plastic agar plates to a depth of about 2.5mm. Standard microbiology aseptic measures and procedures were strictly observed.

- Sterility was checked and assured as subsequently described. Like the broth powder, the agar powder was stored at room temperature and the prepared plates were stored at 4°C when not in use.

➢ The Mycobacterium Growth Indicator Tube (BBL^{TM} MGIT^{TM}; product number 245122, Becton Dickinson, Johannesburg) was delivered accompanied with a box of

supplement (6 vials containing Oleic acid–Albumin-Dextrose-Catalase (**OADC**) growth supplement and 6 vials containing the **PANTA** antimicrobial mixture (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin, product number 245724). The approximate formula per liter of RO distilled water of each of these supplements was detailed in the product information sheet. These products were used and stored as recommended.

4.2   Test Organisms

4.2.1   Acquisition of test organisms

Repository strains of Mycobacterium were purchased from the American Type Culture Collection (ATCC$^{®}$) Virginia, USA. The criteria for selection were largely based on the medical relevance of the species and the likelihood of it being isolated from clinical specimen particularly in the Southern African Region. Consideration was also given to species of environmental importance. Table 4.3 shows the comprehensive list of all the repository strains of *Mycobacterium* species used in this phase of the study:

Table 4.3: Comprehensive list of all the repository strains *Mycobacterium* used in the study

Organism	ATCC number	Source	Site of Isolation
Mycobacterium abscessus	23018	Human	Sputum
Mycobacterium africanum	25420	Human	Expectorate from Senegalese with pulmonary TB
Mycobacterium avium sub-avium	35716	Human	Opossum tissues
Mycobacterium bovis	35724	Human	Lung
Mycobacterium chelonae chemovar	19538	Human	Gastric lavage
Mycobacterium chelonae	35752	Human	Gastric lavage
Mycobacterium confluentis	49920	Human	Sputum healthy human male
Mycobacterium conspicuum	700090	Human	27 yr old male with cellular immunodeficiency and pneumonia
Mycobacterium flavescens	23008	Human	Human, Colorado
Mycobacterium gordonae	14470	Human	Gastric lavage
Mycobacterium gordonae	23409	Human	Tonsil
Mycobacterium intracellulare	23436	Human	Sputum
Mycobacterium kansasii	21982	Human	Sputum, lung and G.L.
Mycobacterium marinum	11566	Human	Elbow lesion
Mycobacterium peregrinum	23049	Human	Sputum
Mycobacterium scrofulaceum	23429	Human	Sputum Maryland
Mycobacterium scrofulaceum	23420	Human	Cervical node
Mycobacterium senegalense	BAA-849	Human	Clinical specimen- human right heel bone
Mycobacterium smegmatis	11759	Human	Clinical material
Mycobacterium tuberculosis	35804	Human	TB patient
Mycobacterium tuberculosis	35814	Human	Lung
Mycobacterium ulcerans	19423	Human	Pus from leg ulcer
Mycobacterium vaccae	23005	Human	Human
Mycobacterium xenopi	19971	Human	Human surgical specimen, lobectomy

Three of the organisms, namely *M. africanum* (ATCC[®] 25420), *M. bovis* (ATCC[®] 35724) and *M. ulcerans* (ATCC[®] 19423) failed to grow from the lyophilized state after the first purchase. They had to be re-ordered from the ATCC[®]. *M. xenopi* (ATCC[®] 19423) also had to be re-ordered because of difficulty in propagation after initial recovery from the lyophilized state. Three other

organisms: *Norcardia brevecatena* (ATCC® 15333), *Legionella pneumophila* (ATCC® 33215) and *Rhodococcus equi* (ATCC® 10146) which also possess the acid-fast characteristics, though not as strongly as the members of the Mycobacterium genera, were also selected and purchased from the ATCC® for inclusion in the database. This was to enable their mass spectra to be compared with those of the selected strains of Mycobacterium and to demonstrate the ability of the MALDI-ToF technology to distinguish between phenotypically similar genera.

4.2.2   Reconstitution and recovery

The ATCC® repository strains were received in two batches as lyophilized, powdery cell pellets either in single or double vial preparations, except *M. conspicum* (ATCC® 700090). This was shipped frozen and received thawed. Nevertheless, viable organisms were still recovered from the vial. The vials were carefully and aseptically opened according to the product insert in the biosafety level 3 containment laboratory of the division of Molecular Biology and Human Genetics in the department of Biomedical Sciences of the faculty of Health Sciences of Stellenbosch University, Tygerberg campus and in the TB lab of the National Health Laboratory Services (NHLS), 9th floor 'C' block Tygerberg Academic Hospital.

As stipulated in the accompanying product information sheet, 500μl of the recommended sterile Middlebrook 7H9 broth was used to resuspend the cell pellets. Aliquots of 100μl of the suspension were used to inoculate the following media: 10ml of fresh, sterile Middlebrook 7H9 broth in 50ml tissue culture flasks, Middlebrook 7H10 agar plates, and Lowenstein-Jensen (LJ) slants all clearly and appropriately labeled. The remaining 400μl of the cell suspension was used to inoculate fresh Mycobacterium Growth Indicator Tubes (MGITs) containing 0.8ml of Oleic acid–Albumin-Dextrose-Catalase (OADC) supplement only, without the PANTA antimicrobial mixture which is

usually included in MGIT tubes inoculated with clinical specimen to suppress the growth of other bacteria that do not belong to the genus Mycobacterium that may be present in the specimen (54). PANTA was omitted in the recovery and subsequent pure culture propagation of the standard ATCC® strains so as not to jeopardize the recovery of viable organisms from the lyophilized state. Also, since the primary aim of this phase of the project was to generate a database of reference mass spectra fingerprint pattern of standard strains of selected species of *Mycobacterium*, PANTA was omitted to avoid putting the organism under unknown or undefined conditions which may force/induce the expression of some proteins they may not normally express (which may influence their mass spectral patterns upon examination by MALDI-ToF MS).

However, in the course of the preparation of the database, the spectra of some of the organisms cultured in MGITs with and without the PANTA mixture were considered and compared as a matter of interest and to serve as groundwork for the second phase of the project, namely the clinical validation of the database. An example of such comparative spectra generated for the representative test organism *M. tuberculosis* (ATCC® 35804) is documented and presented in the next chapter: Results.

The Middlebrook 7H9 broth used for the reconstitution and culture of the repository strains of ATCC® organisms was prepared as described earlier. Sterility of the broth was checked and assured by aseptically transferring 10ml of the prepared broth into a sterile capped tube: this was incubated at 37°C overnight and checked visually for any sign of contamination (turbidity). It was also checked after 48 hours. A drop was also 'spotted' on sterile Blood agar plate and incubated at 37°C overnight, and subsequently examined for any contaminant (growth). The media was only used when proved sterile.

4.2.3 Culture of the repository strains

LJ slants were used as 'back-up' for the initial recovery and culture of some of the test organisms. The commercial slants were carefully examined visually for any sign of contamination before use and any suspicious looking slant was discarded. The slants were used well before the recommended expiry dates which differed with each batch. Slants were stored at 4^oC prior to use. Once inoculated, all LJ slants were incubated 37^oC in an ambient incubator. They were examined, 'aired' and drained weekly (except for fast growers like *Mycobacterium smegmatis* which was examined as from 48 hours of incubation). *Mycobacterium marinum* (ATCC® 11566) and the re-ordered *M. ulcerans* (ATCC® 19423) were incubated at 30^oC as stipulated in the accompanying product information sheets supplied by the ATCC®.

The Middlebrook 7H10 agar plates with OADC supplement used in this study were prepared as described above. Standard microbiological aseptic measures were strictly observed: the sterility of the media was checked by examining an uninoculated plate after overnight and 48 hours incubation at 37^oC. As with the slants, uninoculated plates were stored at 4^oC prior to use. Inoculated plates were sealed with Parafilm® to prevent drying out during incubation at 37^oC in an ambient incubator: the plates were examined for growth as from 48 hours after inoculation.

As stated earlier , MGITs inoculated for the recovery and culture of *Mycobacterium* were without the PANTA antimicrobials, however the ones "back inoculated" to confirm that the organisms were actually killed and rendered non viable before being taken out of the BSL 3 facility for mass spectrometry contained PANTA as used in the routine culture of TB specimens. The contents and approximate formulae per liter of purified water were provided by the manufacturer (54). Each

MGIT tube contains 110μl of fluorescent indicator embedded in silicone rubber base at the bottom of the tube and 7ml of modified Middlebrook 7H9 broth. Elaborate details of its principles and mode of operation are available in the product insert (54). A condensed version is provided in Chapter 2 of this thesis. For use, 0.8ml of OADC only or OADC and PANTA mixture (as appropriate) was added to the MGITs just before inoculation. Inoculated tubes were incubated in the BACTEC^{TM} MGIT^{TM} 960 system, where they were continuously monitored every hour for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument positive, that is, whether the inoculum contains viable organisms, or instrument negative. Culture tubes that were flagged negative after a minimum of 42 days incubation are sterilized prior to discarding. Positive tubes with sufficient growth were scanned out of the instrument and are subjected to the appropriate inactivation procedure in the NHLS TB lab before they were taken to the mass spectrometry laboratory. The OADC supplement is stable at temperatures between 2°C-25°C up to the expiry date, but as a precaution, once opened it was stored at a constant temperature of 4°C and used within 14 days. On the other hand, the PANTA mixture once reconstituted, was stored at 4°C and used within five days as stipulated in the product insert.

Ten milliliters (10ml) 'stock' inoculum culture was maintained for all organisms using Middlebrook 7H9 broth in 50ml sterile tissue culture flasks. These were incubated flat on their sides to provide ample surface area for growth: this method has been proved to minimize clumping and chord formation and to give a relatively even and well dispersed cell growth for *Mycobacterium* species. These cultures (referred to as 'primary cultures' when obtained directly from the re-suspended, lyophilized cell pellets) were incubated in the ambient incubator at 37°C for 7days and were used to inoculate another fresh 10ml of Middlebrook 7H9 broth. These were referred to as the "first generation" cultures: this indicates that they were sub cultured at least once after recovery

from the lyophilized state before being used as inoculum. They were removed from the incubator after use, tightly capped, wrapped, sealed securely and stored in well labeled polythene bags in a crate at 4°C for a while before being discarded in the appropriate manner if not needed for further use.

4.2.4   Storage of repository strains:

Two milliliters (2ml) aliquots of 7-14 days broth cultures cultured in the method described above were aseptically transferred into clean cuvettes with tight fitting lids reinforced with Parafilm®. They were moved with utmost care to the spectrophotometer in the clinical chemistry lab of the NHLS where their Optical Density (OD) reading was determined at a wavelength of 600nm. The instrument was 'zeroed' using 2ml of uninoculated Middlebrook 7H9 broth as blank. Cultures having an OD of 0.5 or more at 600nm were ready for long-term storage. This procedure was carried out for some of the fast growing, non-pathogenic *Mycobacterium* such as *M. smegmatis and M. gordonae*, and also for one representative species of the pathogenic slow growers (*M. tuberculosis*). It was discovered that both fast growers and slow growers achieved an optical density above 0.5 at 600nm before 14 days incubation. Subsequently, visual turbidity was used to assess if sufficient growth has been achieved for culture storage purposes to avoid the risk associated with moving live cultures around in the laboratory.

For the long term storage, seven hundred micro liters (700µl) of the ready cultures were aseptically transferred into previously sterilized, well labeled 2ml screw capped plastic cryo-tubes containing 300µl of sterile glycerol. The culture and the glycerol were lightly vortexed to ensure thorough mixing before storage at –80°C. Glycerol stocks were prepared in triplicates for each organism. When required, one cryo-tube was removed at a time, allowed to thaw at room temperature and the

content used to inoculate 10ml of freshly prepared Middlebrook 7H9 broth and MGITs. From this (the Middlebrook 7H9 broth), other subcultures were made as necessary. To ensure the integrity of the cultures used to prepare the glycerol stock, a drop of the culture was spotted on Columbia Blood Agar (CBA), and another drop onto a new microscope slide for ZN staining. The CBA plate was examined after overnight incubation, and the ZN slide thoroughly examined field by field for any contaminant. If the result of the CBA plate and the ZN stain were both satisfactory, the culture was used for glycerol stock preparation and/or as inoculum. If there is any shadow of doubt, the CBA was incubated for another 24 hours after a duplicate smear of the suspicious 'growth' has been made. One smear was Gram stained and the other ZN stained. The result of both slides was used in conjunction with that of a fresh overnight culture on CBA of the broth to determine if the culture was suitable for use. If a culture was suspected to be contaminated, it was discarded and a fresh broth culture prepared by inoculating 5ml of sterile broth with inoculum from a previously stored broth culture or a 'clean' Middlebrook 7H10 plates or 'clean' LJ slant. When sufficient growth was achieved, 0.5ml of the culture was used to inoculate 10ml of sterile broth. When sufficient growth was achieved, another round of "culture integrity check" is carried out before the culture was used or stored.

Information/advice received from colleagues in the NHLS TB lab was that *Mycobacterium* species are preserved for much longer if stored on sterile beads like other conventional bacteria. Therefore, in addition to the 'glycerol stock' storage procedures, the organisms were stored on sterile beads in replicates of 5 vials per organism. This was done by taking one glycerol stock and culturing it in the method described above. The 'first generation' culture was used to inoculate fresh MGITs without PANTA which was incubated for 14 days in the BACTEC^{TM} MGIT^{TM} 960 instrument. The contents of the MGITs were checked for contamination in a manner similar to the one detailed above. If fit

for use, the MGITs were vortexed lightly to ensure even distribution of cells in the culture. Approximately 1ml was added to one cryo-vial of sterile 'dry' beads. Cryo-vials of sterile, 'dry' beads (i.e. vials not containing any cryo-preservative fluid) were obtained commercially (MicroBank[TM] –Dry, PRO-LAB, Davies Diagnostics, Johannesburg). These were clearly labeled before the addition of the culture, excess fluid was aseptically aspirated leaving the beads as free of fluid as possible. The inoculated cryo-vials were stored at $-80^{\circ}C$.

4.3   Quality Assurance (QA) Organisms

4.3.1   Acquisition of QA organism

Representative species of eight different genera (7 aerobes and 1 anaerobe) comprising of both Gram positive and Gram negative organisms were purchased from the National Collection of Type Cultures (NCTC) London, United Kingdom. These were used as quality assurance (QA) organisms as recommended by our collaborators at Manchester Metropolitan University (MMU). These organisms have been extensively tested and reported to yield reproducible spectra and good matches when searched as 'unknown' against existing databases. The successful identification of these QA organisms was based on several factors including their Root Mean Squares (RMS) values were generally less than the maximum recommended value of 3.0, the relative probability values very high (often over 90%) and absolute probability values within acceptable range. Names of the organisms, media of culture, temperature, time and conditions of incubation are documented in Appendix B.

In this study, two QA organisms were included on each MALDI-ToF target plate containing one test (Mycobacterium) organism. The test organisms were put in 24 or 12 replicate wells on the target plate (depending on the quantity of cell pellets available), while the QA organisms were put

in 12 replicate wells as advised by our collaborators. The two organisms used for QA were: *Vibrio parahaemolyticus* (NCTC 10903) and *Micrococcus lylae* (NCTC 11037). The former is an aerobic Gram-negative rod that uses α-Cyano as matrix, the same matrix used for the *Mycobacterium* test organisms, while the latter is a Gram-positive aerobic coccus that uses CMBT as matrix.

4.3.2   Reconstitution and recovery

All NCTC organisms, like their ATCC® counterpart, were received as lyophilized powdery cell pellets in vacuum-sealed vials. These were opened with utmost care and with strict adherence to standard laboratory safety practices, and according to the enclosed instructions. Five hundred micro liters (500μl) of sterile nutrient broth obtained commercially from Selecta-MEDIA (Johannesburg) was used to rehydrate the cell pellets. These were then inoculated onto appropriate agar plates and incubated at 37^oC under the appropriate incubation conditions for 24 hours. The initial plates were made in duplicates. Two further subcultures were made from the 24 hours culture to enable the organisms to fully recover from the lyophilized state and to express all the necessary proteins that give them their characteristic unique mass spectra.

4.3.3   Culture of QA organisms:

All the organisms except *Escherichia coli* were cultured on commercially obtained, quality assured, sterile CBA plates from Selecta-MEDIA. *E. coli* was cultured on Cystein-Lactose-Electrolyte-Deficient (CLED) agar from the same commercial supplier, because it has been found out that *E. coli* cultured on CLED gives more species specific spectra than *E.coli* cultured on CBA. The latter gives a spectrum that is so similar to that of some of the other members of the *Enterobacteriaceae*, that it makes differentiation of the various species difficult. All of the organisms with the exception of *Bacteroides fragilis* were incubated in ambient incubator maintained at 37^oC. *Bacteroides*

fragilis was incubated in the anaerobic incubator also maintained at 37°C, which was flushed, when necessary, with nitrogen gas. Our collaborators in MMU had observed that the 3rd subculture from storage was the best to use for mass spectrometry. This procedure was strictly adhered to: all organisms sub cultured from beads were sub cultured thrice (3 consecutive 24 hours incubation) in the appropriate incubator before use, the only exception being *E. coli* on CLED. The 3rd subculture of *E. coli* on CLED was only incubated for 16 hours prior to mass spectrometry, as was instructed during training in Manchester.

4.3.4   Storage of QA organisms:

Young colonies of 18-24hrs cultured under appropriate culture conditions were emulsified in sterile nutrient broth and used to prepare cell suspensions of approximately 3-4 McFarland standards. One milliliter (1ml) aliquot of this was used to prepare stock cultures on dry beads in a manner similar to that of the test Mycobacterial strains. The clearly labeled tubes were tightly capped before storage at –80°C. Stock cultures were prepared in quadruplicates for each organism. For use, 1 bead from the appropriate cryo-vial was aseptically removed and used to inoculate sterile CBA plate, which was subsequently incubated under appropriate culture conditions. It must be stressed that utmost care was exercised in the preparation and use of the cryovials and the manufacturer's instructions was strictly adhered to. It was also ensured that the vial was not allowed to thaw completely. As soon as a bead could be detached from the whole frozen content, the vial was returned back to the -80 freezer to minimize the risk of contamination and damage to the organisms due to repeated freeze-thaw cycles.

4.4 Other Bacterial Strains

As earlier mentioned, three other organisms that do not belong to the *Mycobacterium* genera but share the acid-fast characteristic were included in this phase of the study. These were also purchased from the ATCC®. They were opened, reconstituted, cultured and stored in a fashion similar to that of the quality assurance organisms, except for *Norcardia brevecatena* (ATCC® 15333). Although this organism grew on the recommended glycerol agar and even on CBA, all attempt to get the colonies off the culture plates and onto the MALDI-ToF target plate were futile. This was because the colonies "adhered" strongly to the culture media and all attempts to dislodge or 'scrape' them off the surface of the culture media resulted in removal of the culture media. This, for obvious reasons, was not an appropriate sample for mass spectrometry. It was advised by a senior technologist that culturing the organism in plain MGITs could be a solution to the predicament. From the primary broth culture in ISP medium as recommended in the product information sheet, the organism was sub-cultured three times. Five hundred micro liters (500μl) of the third subculture was used to inoculate 'Plain' MGITs (i.e. MGITs without OADC or PANTA): these were incubated in the BACTEC^{TM} MGIT^{TM} 960 instrument. By the 7th day, sufficient growth was observed in the tubes to allow for MALDI-ToF assay. The content of the tubes were centrifuged the cell pellet used for mass spectrometry after the integrity of the cultures were checked and assured in the manner previously described.

Cultures grown in ISP medium were used to prepare stock cultures stored on beads at –80°C in a manner similar to that used for the storage of the QA organisms.

4.5   Inactivation of Mycobacterium species

Although some of the species of *Mycobacterium* used in this study such as *M. kansasii* and *M. marinum* are classified as Risk Group 2 (RG 2) agents (109), the inclusion of *Mycobacterium tuberculosis, M. avium* and *M. intracellulare* which are all RG 3 organisms and others such as *M. abscessus*, *M. gordonae* and *M. smegmatis* which were not categorized in a definite group made it necessary to handle and treat all the species of Mycobacterium used in this study as RG 3 organisms. This meant that all culture and other relevant procedures were carried out in a Biosafety Level 3 (BSL 3) containment facility/ laboratory. BSL 3 refers to a containment level consisting of a combination of primary and secondary barriers and standard and special practices as defined in the CDC's Biosafety in Microbiological and Biomedical Laboratories (BMBL) document (109). This containment level is typically used to work with RG 3 agents that are known or suspected to be present in high concentrations or large volumes, which in this context includes pure cultures typical of research preparations.

All Mycobacterium organisms were 'killed' (i.e. rendered non-viable) before being taken out of the BSL 3 facility to the analytical laboratory for mass spectrometry. Of the different methods of inactivation possible for *Mycobacteria*, chemical inactivation was considered and chosen for use in this research project. Other methods were not suitable for use in this study for the following reasons:

(a) Application of heat was not considered because of the length of time required to assure proper 'sterility' of the cultures, (average inactivation time for heavy inocula typical of research preparations like the ones used in this study is about one hour). This would have defeated one of the overall aims of this project which is to diagnose TB in the shortest possible time. Also, heat

application has the potential of producing aerosols which could be an additional safety hazard to personnel and the environment.

(b) Nitrogen laser (337nm) irradiation is another possible method of inactivation. The effect of this has however been shown to be transient, with cells showing significant recovery of viability after incubation in a suitable broth media for 3 days (110).

In recent literature, inactivation by chemical methods was the method used and documented for mass spectrometry of Mycobacterial organisms (98 & 108). The efficacy of this method has been proven and is alluded to in this current work, also the comparatively shorter time required for inactivation (from 30 seconds to two minutes) made this the preferred method of inactivation for this work. Two chemical methods of inactivation were explored and compared in this study:

➢ The first method was a modification of that used by Hettick and his co-workers in 2004 (98). This involves the use of a solution (Solution A) made up of a mixture of 50:50 Acetonitrile (ACN)/Water (H_2O) containing 4% Trifluoroacetic acid (TFA).

➢ The second method involved the use of a solution (Solution B) made up of 90% ACN and 5% TFA as documented in Application Notes #MT-80 by Bruker Daltonics® (111).

Heavy growth of the test organism (a laboratory strain of *Mycobacterium smegmatis*) was emulsified in 1ml of the above solutions contained in clearly labeled, safety-lock Eppendorf® tubes. They were well vortexed to ensure thorough contact between cells and the solutions. The tubes were subsequently centrifuged at 13,400rpm in an Eppendorf® MiniSpin centrifuge for 15 minutes.

The supernatant was carefully aspirated and a good portion of the cell deposit used to inoculate freshly prepared MGITs containing 0.8ml of OADC and PANTA mixture as used in the routine culture of TB specimen. These were incubated in the BACTEC™ 960 where they were monitored

hourly. They were flagged negative after 42 days incubation. The tubes were scanned out and rescanned in as fresh tubes. They completed another 42 days of hourly monitoring and were flagged negative for the second time. This made a total of 84 days of incubation with no evidence of growth or viability. We therefore concluded that this treatment rendered the organisms no-viable and safe to handle in the analytical unit.

This experiment was repeated using *Mycobacterium tuberculosis* H37Rv (ATCC® 27249) cultured on Middlebrook 7H10 agar for 3 weeks. The results were the same: the tubes were instrument negative meaning no viable organism was present. By inference, both solution A and B were considered effective killing agents for the two species of Mycobacterium tested. This inactivation experiment was the first in a series of experiments dubbed 'pilot projects' carried out to investigate and optimize some crucial experimental factors. The second part of this inactivation pilot project entailed investigating which of the two solutions gave better and more reproducible mass spectra. This was done by subjecting both the supernatant (cell extract) and the cell deposit of the above named organisms cultured in a similar fashion to MALDI-ToF mass spectrometry. Details of the outcome of this experiment are presented in the next chapter: Results.

4.6   Choice of Matrix for use:

Having determined the best inactivating solution to use, the choice of matrix was the next important experimental factor to consider. Two matrices were considered and compared for the *Mycobacterium* test organisms. The first matrix consists of 14mg of α-Cyano dissolved in 1ml of Matrix Solvent Solution (MSS) made up as described above. This matrix has been used extensively by our collaborators in MMU and has been documented in literature for use with Gram-negative bacteria (96, 97).

The other matrix considered was 3mg of CMBT dissolved in 1ml of matrix solvent solution. This matrix has also been used extensively for Gram-positive bacteria (99). The experimental approach was similar to the one used for the inactivation experiments: the difference was that only solution A was used for the inactivation of the test organism, which in this case was only the laboratory strain of *Mycobacterium smegmatis*. A single MALDI-ToF target plate was prepared by depositing 1μl of the cell extract of the repository strain of *M. smegmatis* in 48 replicate wells whereupon α-Cyano was used as matrix on 24 wells and CMBT was used as matrix on the other 24 wells. Similarly, another 48 replicate wells the cell deposit of the organism was prepared and analysed using either α-Cyano (24 wells) or CMBT (24 wells). The wells were given an hour to air dry before the application of 1μl of the above matrices. A further quarter of an hour was allowed for the matrix to air dry on the samples before the target plates were put through the by MALDI-ToF mass spectrometer. The details of this experimental step is provided and discussed in the next chapter: Results.

4.7   Choice of Cellular Part

As explained in the previous chapter, it is again emphasized that the inactivation step is not a deliberate effort to disrupt the *Mycobacterium* cells or extract cellular components for MALDI assay. The pathogenic nature of the species included in this study made the inactivation step necessary. The term 'cell extract' as used in this study, refers to the supernatant aspirated from the cells after they had been exposed to the killing action of the inactivating solution. The presence of potential species-differentiating peptides in this solution has previously been reported (98): no consensus exists on which sample preparation methodology is preferable. Hettick and his co-workers (98) documented that cellular extracts of *Mycobacterium fortuitum* extracted with 50:50

ACN: H_2O/4% TFA for 30 seconds gave very similar spectra in terms of observed peaks and their relative abundance to that of whole cell deposit spectra. They chose to use the cell extract because the species-specific peaks observed for *M. fortuitum* was more intense than the ones observed with the whole cell deposit and also because it is believed that the use of cellular extract minimizes personnel contact with the infectious agent. In this study however, both the cell extract and the cell deposit spectra were included in the database for reasons enumerated below.

> According to the protocol developed and followed and the instrument used in this study, the whole cell deposit spectra had more peaks at the higher mass range than the cell extract. This condition (as earlier explained) is best suited for species differentiation.

> Also, being confident of the effective inactivation procedure, the possible risk that contact with the inactivated cells may pose to the well trained and careful personnel is far out-weighed by the enormous advantages to be derived by the inclusion of the whole cell spectra in the database.

> On the other hand, the cell extract spectra is included in view of the fact that one of the ultimate aims of this entire research project is to explore the possibility of bypassing the culture stage to the direct identification of the selected *Mycobacterium* species present in clinical samples. The cell extract is the logical analyte to use if this lofty aim is to be achieved.

The protocol of this experiment was similar to the one described for the 'Choice of Matrix' experiment: the findings of this experiment are documented and illustrated in the Results chapter of this dissertation. In view of the above, efforts were made to increase the quality and quantity of ions in the cell extract, especially in the higher mass range region, a quest which lead to two additional pilot projects summarized below.

4.8   Variation of Water in Inactivation solution:

It is common knowledge that in reversed phase Liquid Chromatography Mass Spectrometry (LCMS), polar compounds are more readily eluted than their not so polar counterparts by a polar mobile phase (i.e. a mobile phase with more hydroxyl ions [OH^-]). In other words, mobile phase with higher water content readily elutes polar compounds. Conversely, a mobile phase with less water content (i.e. less polar mobile phase) does the same for less polar compounds. Since the polarity of the spectral ions are not known, two solutions C and D, one more polar and the other less polar than the original solution A were used for the extraction and compared with the original solution A made up of 50:50 ACN: H_2O/4% TFA.

Using a protocol similar to the one previously described, the effects of these two solutions of opposing polarity on spectra production were investigated:

➢ Solution C was the more polar solution of the two, being made up as a 25% ACN: 75% H_2O ratio

➢ Solution D was the less polar solution, namely a solution of 75% ACN: 25%H_2O.

The effect of the two solutions had no significant impact on the mass spectra of the test organism when compared to the original Solution A. Therefore, the details and results of this experiment are not documented in this thesis.

4.9   Heat "Extraction" Experiment:

The possibility that by increasing the temperature of inactivation more ions could be released into the cell extract, resulting in more peaks at the higher mass range, was also considered. With the use

of an adaptation of the previously described protocol, an experiment was designed and carried out that investigated the effect of increased temperature on spectra production. Because of the volatile nature of Acetonitrile, the highest temperature considered safe for this experiment was $37^{o}C$. The result compared spectra from extracts obtained at room temperature to that obtained from inactivation carried out at $37^{o}C$ for 30 minutes. The outcome of this experiment showed that there was no significant impact of increased temperature spectra production at the temperature investigated. Moreover, the spectra of the room temperature extract appeared to be better than the spectra of the $37^{o}C$ extract. Therefore, further details of this experiment are not documented in this thesis and we decided that subsequent inactivation would be carried out at room temperature.

Hettick *et al* (98) documented that extraction (inactivation) time had no significant impact on the spectra obtained. Our findings from the results of some side experiments (not recorded in this work) corroborated these findings. Their extraction time was 30 seconds, but in this study, the average extraction time (which also doubles as the inactivation time) was 2 minutes. This was to ensure thorough killing of even heavy inocula of *Mycobacterium* species.

4.10   Target Plate Preparation:

The standard MALDI-ToF target plate of 96 sample wells with 24 "near point lock mass correction" wells were purchased from Micromass[TM] in the United Kingdom through their local Waters[TM] representatives (MicroSep, Cape Town). A diagram of the target plate is shown in figure 4.1:

Figure 4.1: Picture of a MALDI-ToF target plate

Each group of four sample wells is lock mass corrected from the central lock mass well. For example A1, A2, B1 and B2 have the same lock mass well.
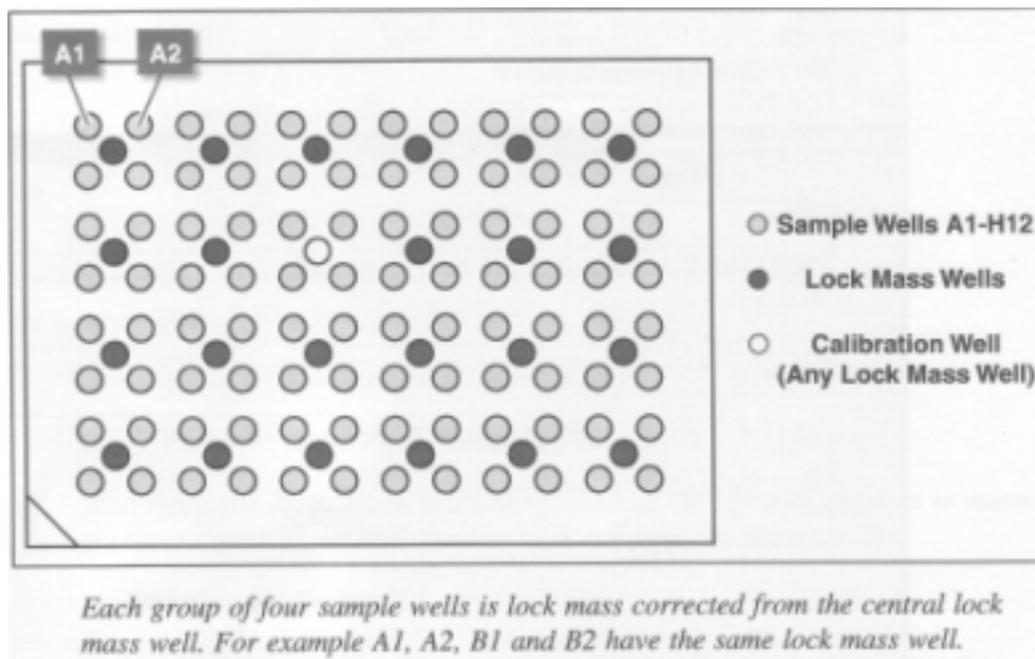
Obtained from: M@LDI user's guide. Micromass UK Limited. Floats Road, Wythenshawe, M23 9LZ. (Ref.112)

These plates have a well-defined geometry of 12 wells per row and 8 wells per column, each well having a constant diameter of 2mm and a pitch of 4.5mm. Between these rows are the "near point lock mass correction" wells. There are 6 of these wells per row and 4 per column; they also have a diameter of 2mm each but a much deeper pitch of 9mm. Each well is referred to using the alpha-numeric system for example A1, B6, and the lock mass wells as A1LM, B6LM etc.

The bottom of the sample wells were evenly coated with a small inoculum of the bacterial growth (in the case of quality assurance organisms and other non-mycobacterium bacteria) or inactivated 'whole cell' deposit of *Mycobacterium* using a 1μl loop. For analysis of the cellular extract, 1μl of the supernatant from the inactivation of the *Mycobacterium* cells was transferred to the sample wells using a 10μl Gilson® positive displacement pipette. The samples were allowed to air dry for a

minimum of one hour before being overlaid with 1μl of appropriate matrix and then allowed to dry for a minimum of 15 minutes.

A mixture of seven standard peptides (Pepmix) was prepared as previously described. A 1μl volume of a 1:1mixture of α-Cyano matrix solution and the Pepmix was spotted onto the lock mass wells. The Pepmix was used for the automated calibration of the instrument, which was done before every target plate was processed. It was also used during automation to improve and validate external mass measurement accuracy. It involved a slight adjustment to the calibration function to correct for first order mass drifts that may occur during the course of the experiment. This mixture was also given a minimum of 10 minutes to air dry before the plate was loaded into the instrument for data acquisition. The quality of a new batch was assured by comparing the spectra, calibration profile, RMS and "peak width at half height" values with that of the preceding batch.

For the database generated in this study, a prepared Maldi target plate contained two separate rows of 12 replicate wells of QA organisms. The two QA organisms used in this project were: *Vibrio parahaemolyticus* NCTC® 10903 and *Micrococcus lylae* NCTC® 11037 as previously mentioned. The plate also contained 24 replicate wells of cell extracts and at least 12 replicate wells for the cell deposit of the inactivated test organisms. Appropriate numbers of lock mass wells containing the standard peptides (Pepmix) were included on all plates. The plate was allowed to air dry thoroughly before being loaded in the MALDI-ToF for processing.

4.11 Brief Description of the Instrument:

The Matrix Assisted Laser Desorption/ Ionization– Time of Flight Mass Spectrometer (MALDI-ToF MS) used for this study was obtained from the local WATERS™ representative (MicroSep, Cape Town). It was installed, validated, serviced and maintained by a team of efficient, qualified staff. The instrument is a Micromass™ M@LDI™ bench top linear system and is optimized for applications including bacterial finger printing and analysis of oligonucleotides for synthetic and natural polymers (SNP) detection. The Maldi linear instrument is fully MicrobeLynx™ software compatible (112). The instrument was fitted with a nitrogen laser (wavelength 337nm; time lag focusing (TLF) of 500ns). The TLF is defined as the delay between the laser firing and the ions leaving the source.

With the exception of the Mains Power On light situated in the middle of the instrument cover, all instrument control and monitoring is provided in the MassLynx™ user interface. The student was trained by our collaborators Dr. Diane Dare and Ms Helen Sutton of MMU on the basic settings and operation of the instrument and its operating software MassLynx™, for optimum data generation, acquisition, processing, interpretation and storage.

The M@LDI™ linear system is divided into three units that require mains power:

➤ The instrument itself.

➤ The embedded personal computer (PC). This is connected to the instrument via the optical loop cable, it runs under the VXWORKS operating system: its main functions are data acquisition and direct instrument control. It performs 'real time' remote processing of the raw data.

➢ The MassLynx^{TM} PC and monitor. The MassLynx PC is the one connected to the mouse, monitor and keyboard: it runs under *Windows XP* and displays the software user interface. It is connected to the embedded PC by a local Ethernet network cable (112).

4.11.1 Basic Operation and Typical Settings of the Instrument

As mentioned earlier, the instrument is fully MicrobeLynx^{TM} system compatible, all its parameters are controlled from the Tune page user interface. Once these parameter files have been set and saved, then automated control of the system (multiple sample acquisition) is activated from the sample list user interface of the main MassLynx window. A screen capture of the tune page is shown in figure 4.2

Figure 4.2: A screen capture of the tune page
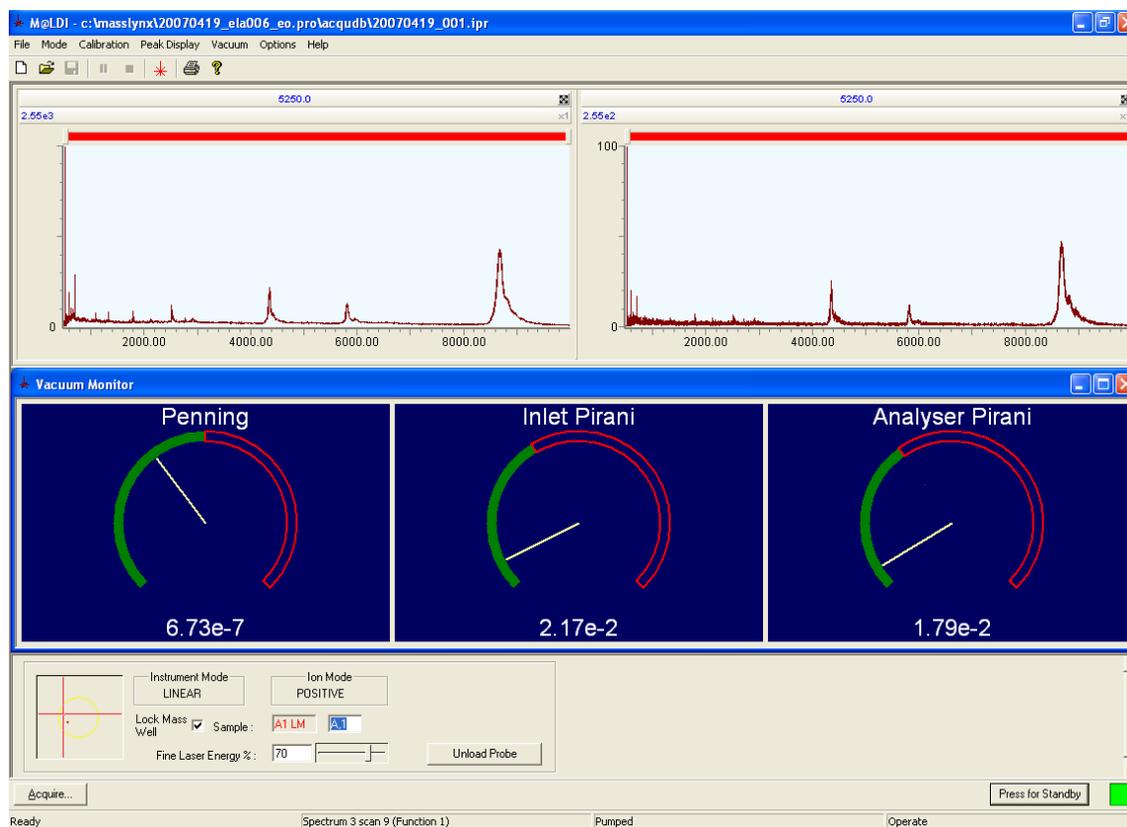
Vacuum is one of the most important factors to the success of any mass spectrometer and the M@LDI^{TM} linear system is no exception. Its vacuum system consist of a single 240Liters /second turbomolecular pump backed by a $1.5m^3$ rotary vacuum pump. The analyzer fine vacuum is monitored by Penning gauge and the analyzer Pirani gauge monitors the analyzer "rough" or backing pressure. The inlet line (vacuum lock) is monitored the Inlet Pirani gauge. It is essential to check the vacuum gauges daily and to ensure that there is good pressure: they are also routinely checked to see that vacuum is restored after loading a plate or venting the instrument. The system is not operated if the pressure (i.e. the value shown by the Penning gauge) is higher than $3e^{-6}$ mbar.

The instrument's operating voltage is another important parameter to be set and saved in the instrument parameter file which has an .ipr extension. This is saved in the ACQUDB folder which is a sub folder of a main project folder that is automatically generated each time a new project is opened. The Source voltage is essentially the accelerating voltage of the ions: it is usually set and left for all experiments at its highest value, namely 15,000V. The Pulse voltage on the other hand is the time lag focusing voltage. This affects resolution and its optimum is mass dependent. For each plate loaded, it is manually adjusted to provide the best resolution for the rennin peptide peak at 1,760Da. This resolution (Peak width at half height) value must not exceed 3.5, while values less than 3 are excellent. Most of the peak width at half height values obtained for target plates processed in this study was below 3, and none was above 3.5 as stipulated by our collaborators.

The Micro Channel Plate (MCP) detector voltage was set at 1800V and the matrix suppression delay was set at 300 mass units. The instrument was operated in the positive ion mode with a sampling rate of 2ns.

On loading each target plate, automatic accurate indexing of the sample/reference (i.e. lock mass) wells was performed using the Spatial Calibration function. When all the tune page parameters were set, checked and saved, the instrument was calibrated. This process includes automated data acquisition whereby the real time data selection (RTDS) algorithm was invoked. RTDS is a process whereby laser energy was automatically tuned to provide data that is on scale. The key aspect of this algorithm is that data that is either too intense or too weak is not saved to disk (99).

During the calibration process the system automatically acquires on-scale data from the Pepmix and matches the known peaks against those in the reference file. A Calibration file was created and saved with a .cal file extension in the AQCUDB folder under the main project folder. Sometimes, known peaks were manually matched to those of the reference file by using the right mouse button. All 7 peaks had to be correctly matched and the mean residual value had to be 0.1or less for the calibration to be considered successful and saved for use. Once the calibration file had been created and loaded using this procedure, all subsequent data acquisition (both manual and automated) were mass measured using this calibration file.

After calibration, a unique sample list for each target plate, like the tune page parameter file and calibration file was created. This is basically in an Excel worksheet format consisting of various columns in which mandatory information and parameters governing the automated process were stored. Such information and parameters included:

➢ The raw data file names

➢ Sample locations

➢ Family

➢ Genus

➢ Species

➤ Strain (if applicable).

➤ 'Identifier' column: this is used by the MicrobeLynx™ application to determine replicates to be grouped together using unique 'identifiers' such as ATCC®/NCTC numbers or any other specified identifier.

➤ Culture column that contains the same information as the identifier column

➤ Parameter column with the parameter files specifying the databases to be searched

➤ Process column where an executable file is loaded, for this study, the MicrobeLynx™ file was the only one loaded on the sample list

➤ Inlet column which is just set as default

➤ MS method column where the experiment control file containing the instrument automation parameters (MS methods) when created is loaded. A screen capture of a typical MassLynx™ sample list is shown in Figure 4.3 below:

Figure 4.3: A screen capture of a typical MassLynx^{TM} sample list

When a sample list was correctly set up with all the necessary information filled in and the parameter files loaded, it was saved. It was saved in a Sample DB folder with a .spl file extension (the Sample DB folder like the ACQUDB folder, is a sub folder of the main project folder). When a new project was created it was uniquely named using the date (starting with the year), the instrument number (which in this case is ELA 006), and the initials of the operator, for example; 20071228_ELA006_EO. In a similar vein all files generated in a project were given the same date and the target plate's serial number e.g. 20071228_001.cal. (for the calibration files of target plate number 001of the project). This main project folder has a .PRO extension and is stored in the MassLynx main folder in the 'C' drive of the MassLynx computer. All the information relating to a

particular   project   could   be   accessed   or   recalled   by   interrogating
C:\MassLynx\20071228_ELA006_EO.PRO.

As mentioned earlier, the main project folder contained subfolders: there are six of them in all as
explained below:

> ACQUBD folder: this contained the tune page parameter files with .ipr extension;
   calibration files (.cal extension) and the MS method file (.exp extension).

> Data DB: here all the raw data files (.raw extensions) for the experiment were saved.

> Sample DB: all the sample lists (.spl extension) for the experiment are saved.

> The three other folders, namely, the Curve DB, the Method DB and the Peak DB were
   currently not in use for this version of MassLynx^{TM} (MassLynx^{TM} 4.0). This was the latest
   version of the MassLynx^{TM} software at the commencement of this project.

The sample list was loaded with an experimental file (MS method file) which was an instrument
control file for automated acquisition. Two experiment functions were set up: the first for the
acquisition of the near well lock mass data for each sample and the second for the acquisition of the
sample data. These files contained mass range specifications. For this study, the mass range for
both the sample data acquisition (Maldi scan) and the lock mass wells (Maldi lock mass scan) were
set at 500 – 10,000*m/z*. The instrument was set to acquire 10 shots per spectrum at a laser firing
rate of 20Hz. It was also set to collect a total number of 15 spectra per well for the Maldi scan and
10 per well for the lock mass scan. The laser pattern for both was set to 'Random', the real time
data selection for optimal automated data acquisition was selected and the laser energy set just
above the threshold of ion production. Values for low intensity and high intensities were set at 5%
and 98% respectively. Low mass for the Maldi scans was set at 800Da while that of the lock mass

scans was 1000Da. but high mass for both was set at 3000Da. Other parameters such as 'Maximum fail per aim' (2 for both), 'Maximum pass per aim' (5 for both), 'Maximum pass per well' (15 for Maldi scan) and 10 for lock mass scans were all set here in the experimental file using the MS method editor. When all was set for optimum data acquisition as indicated above, the automated data acquisition was triggered by selecting the target plate sample list 'run' function.

The data acquired was processed with the aid of MicrobeLynx^{TM} software, a MassLynx^{TM} software add-on. Individual spectral profiles were lock mass corrected. The 15 spectral profiles collected for each sample well were then combined. This process facilitated the enhanced mass accuracy and produced a reproducible Mycobacterial spectrum for each replicate sample. The spectrum for each sample well was 'background subtracted' using a polynomial order of 99; 5% below the curve and smoothed with the minimum peak width at half height set to seven channels, and two smoothes were performed using the Savitzky Golay algorithm (99).

For database inclusion, the spectral reproducibility between the 12/24 replicates per sample was tested using a root mean square (RMS) calculation to identify and reject outliers at a value greater than 3.0 as the screen capture (Figure 4.4) illustrates.

Figure 4.4   Screen capture of a typical pre & post RMS calculation screen
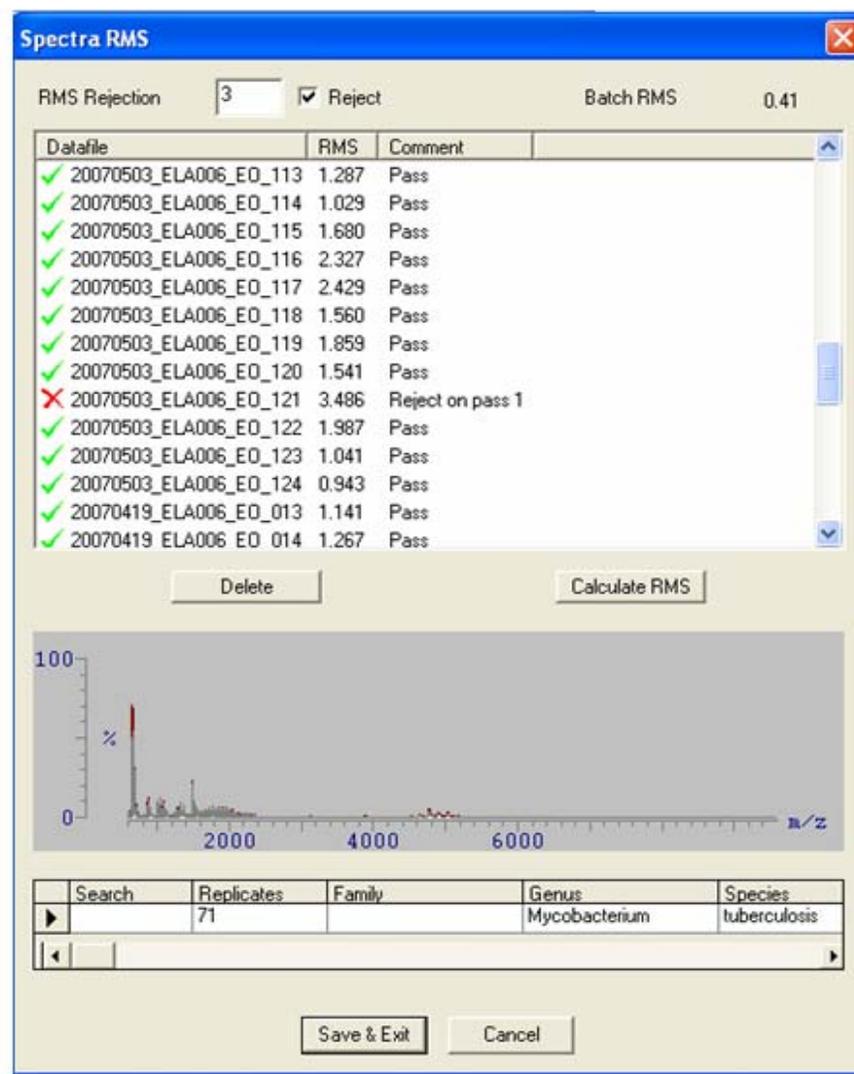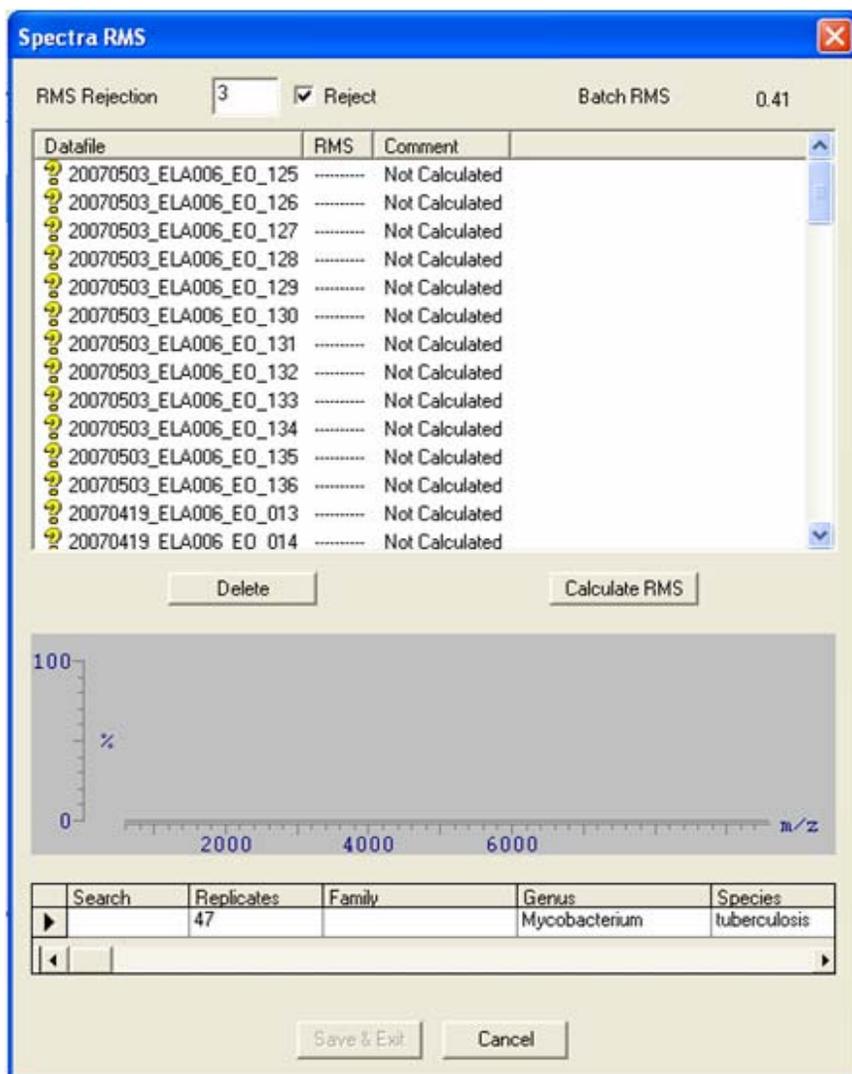
To qualify for database entry, a minimum of 8 of 12 replicates had to meet the RMS <3.0 criterion. The RMS is the normalized deviation of the median test spectra from the spectral average: therefore, it was used to compare each replicate spectrum in turn to the composite spectra of the remaining replicates. All verified spectra were combined to produce a composite spectral entry for each test organism included in the database.

4.12   Example of How an Entry Is Made Into the Database

For the data acquired for a test organism on a target plate to be considered for entry into the database, at least one of the two quality assurance organisms included with it on the target plate had to be recognized down to strain level when searched against the MMU's 2005 database containing 4,123 spectral entries covering over 500 different species of bacteria. The spectra of the test organisms that met this criterion were subjected to some automated processes which included background (noise) subtraction and root mean square (RMS) calculations: they were also verified using the Savitzky Golay algorithm of the MicrobeLynx^{TM} application (a MassLynx^{TM} software 'add on' as explained earlier). Figure 4.5 is an example of a well processed target plate which was processed using the Search_ None parameter file. This is the parameter file used to make an entry into a database. Similarly, Figure 4.6 represents a screen capture of part of the database generated in this study, with the entry of the representative test organism highlighted. Below the database is the organism's composite spectrum.

Figures 4.7 and 4.8 show the result of a search of the database generated in this study. The representative test organism was used as an example: in these diagrams, Figure 4.7 shows the result for the cell extract while figure 4.8 shows that of the cell deposit of the same organism.

Figure 4.5   Typical example of a "Search_None" database result
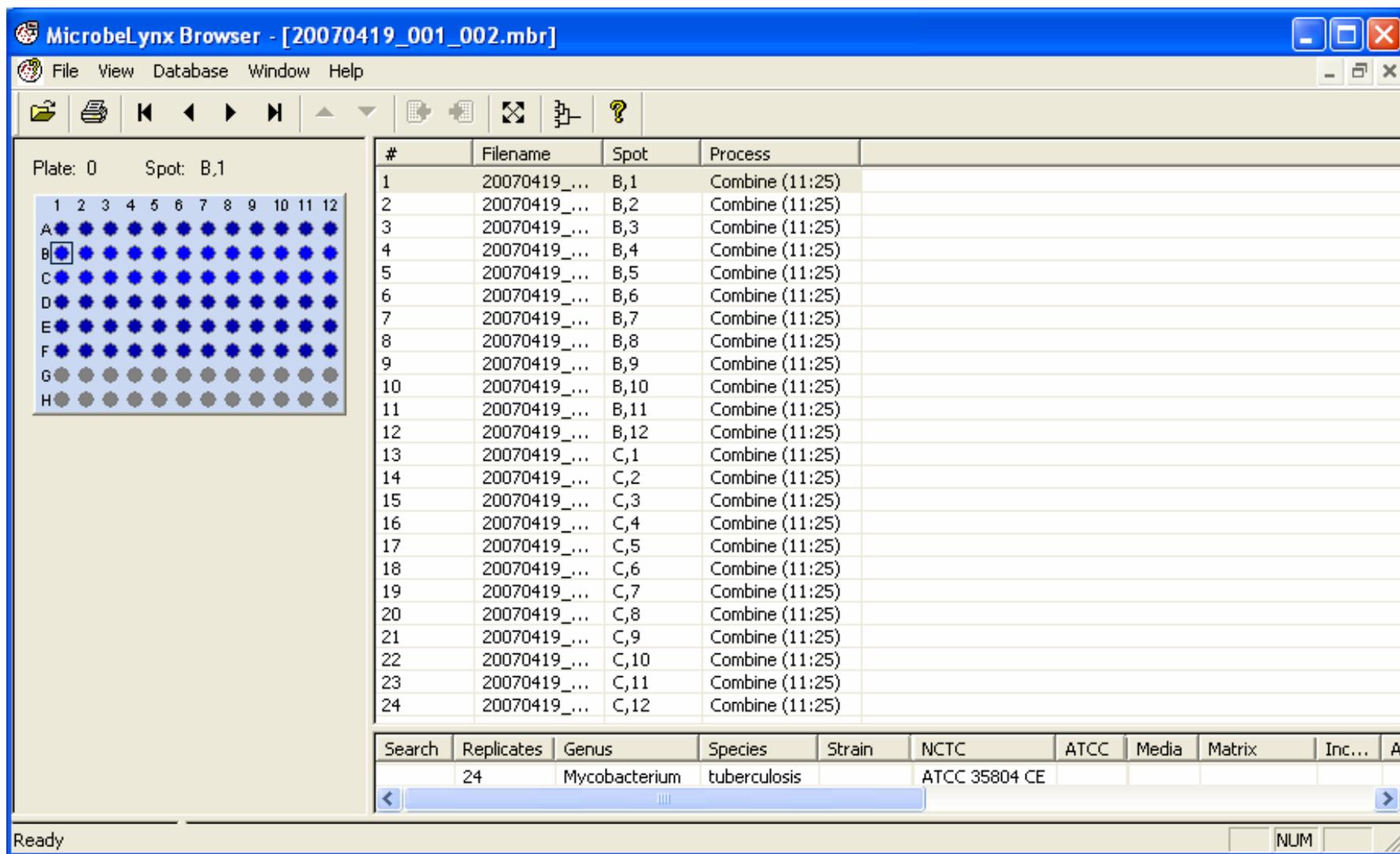
Figure 4.6   Graphic representation of the generated database
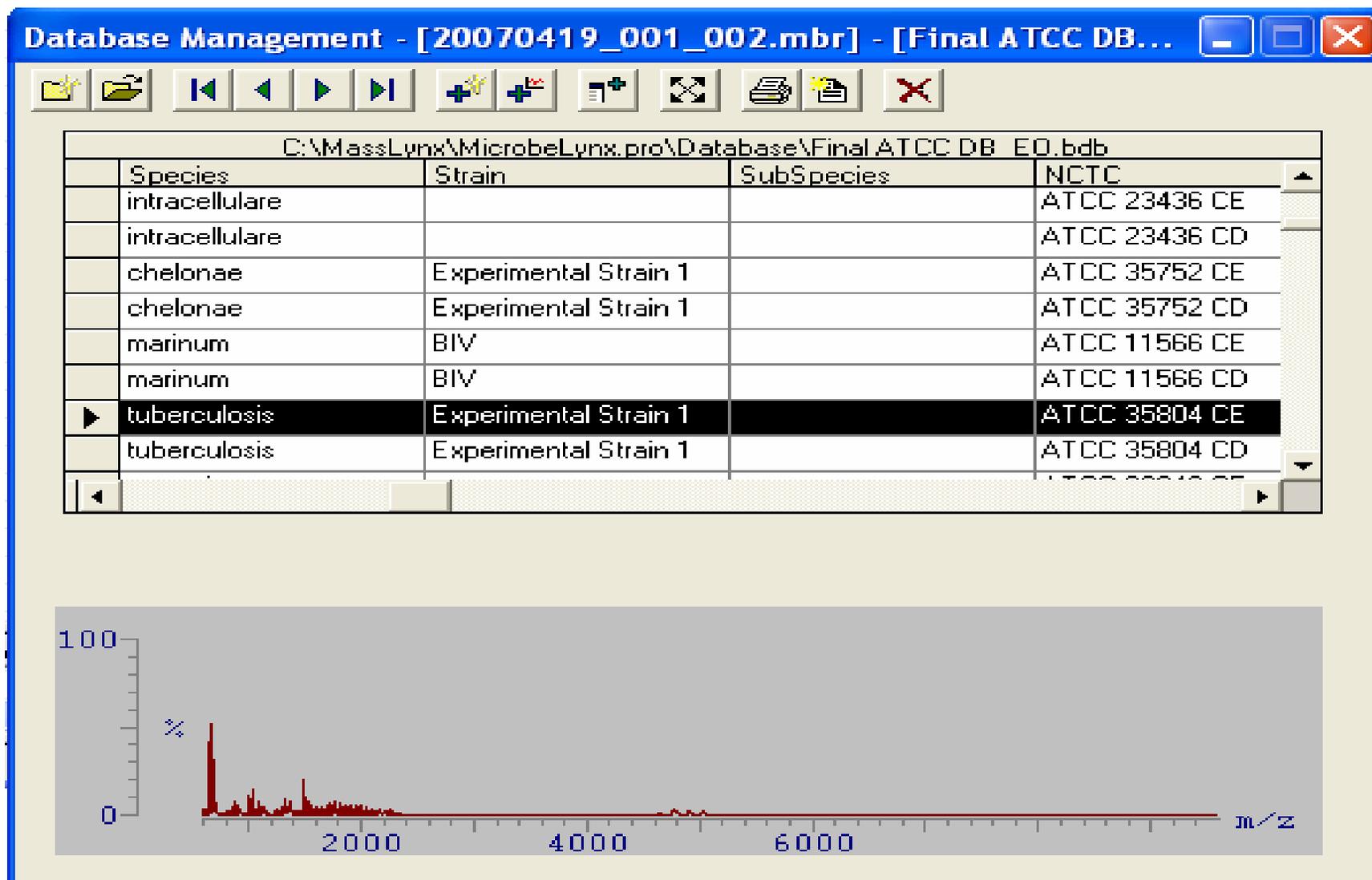
Fig 4.7: Result of a typical database generated search of the representative test organism; cell extract (*Mycobacterium tuberculosis* ATCC® 35804)
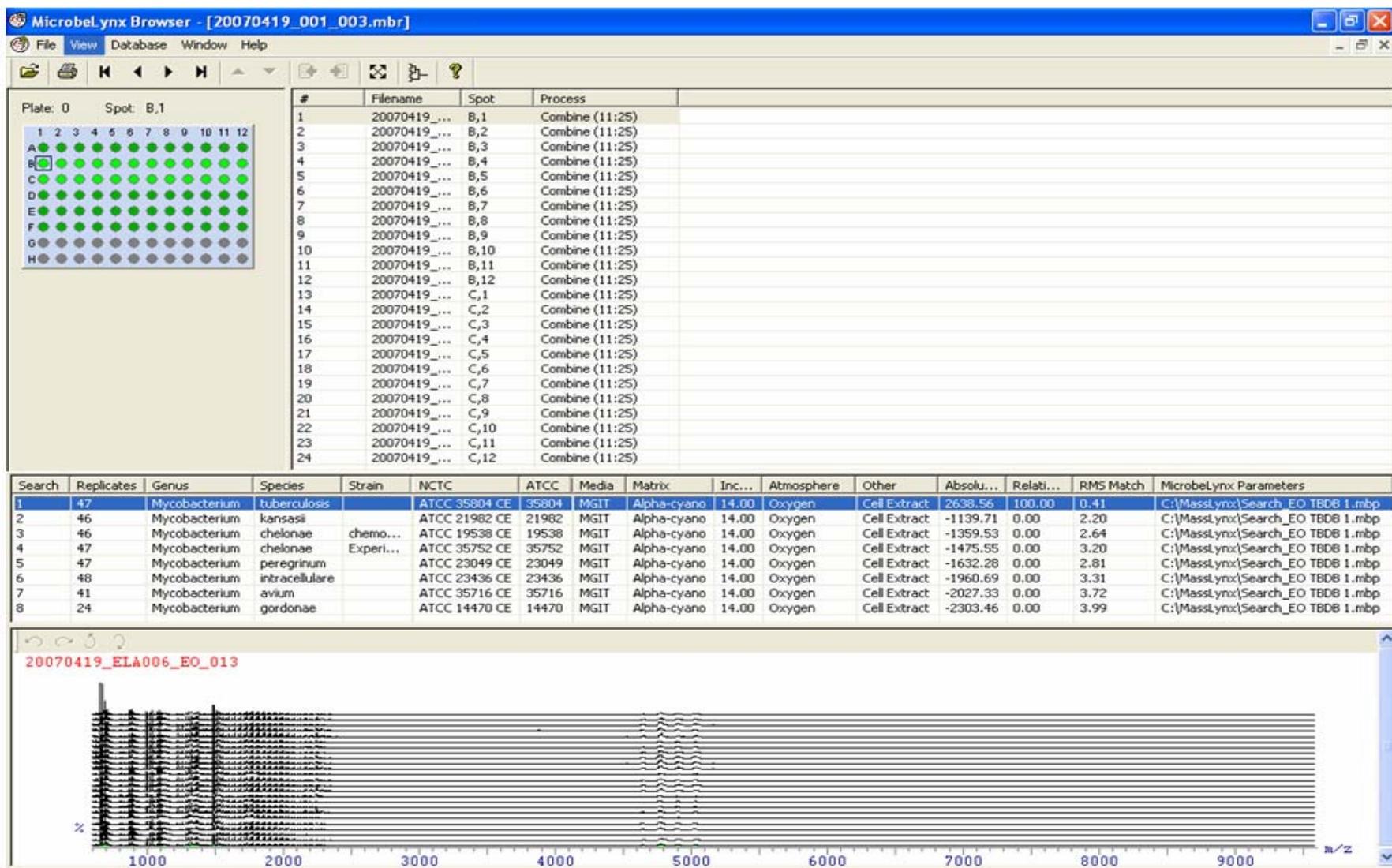
Fig 4.8: Result of a typical database generated search of the representative test organism; cell deposit (*Mycobacterium tuberculosis* ATCC[®]35804)
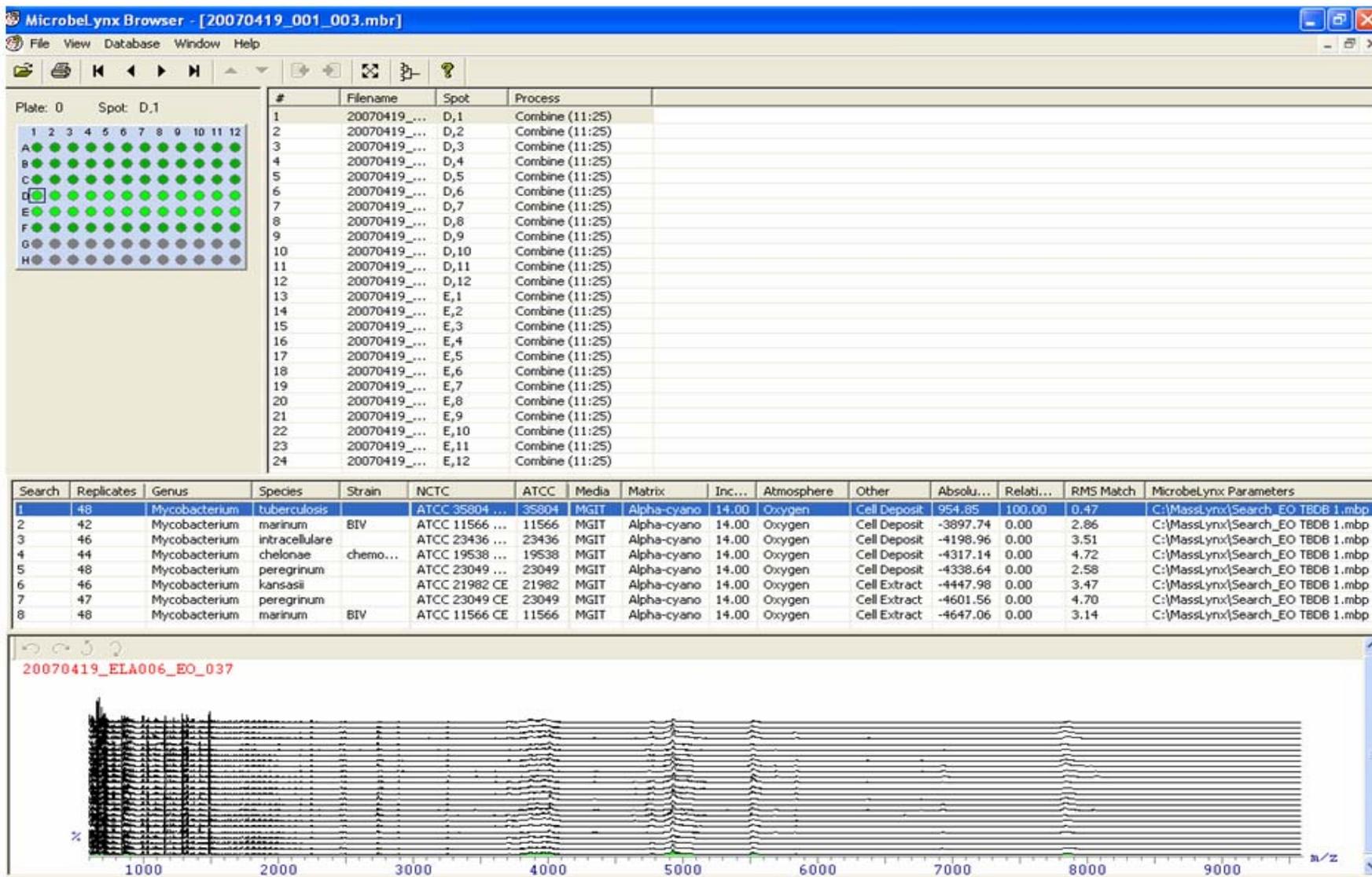
CHAPTER FIVE

RESULTS

Introduction

The application of MALDI-ToF MS is the field of diagnostic Mycobacteriology is still at a tentative stage and therefore the need for optimization and standardization of crucial experimental factors are of great importance. This is to ensure that the database of reference mass spectra of the selected repository strains of Mycobacterial species generated is reproducible and can be applied to clinical samples. The results of these optimization experiments and some examples of spectra representative of some of the entries in to the database are presented in this chapter. The current study is the first phase of a larger, more comprehensive study. This phase requires no statistical inference as stated in the study proposal.

5.1 Choice of Inactivating Solution for routine use:

When we considered the infectious nature of the samples with which we were working, we had to find an acceptable and reproducible manner to render the samples non-infectious without inducing changes to the samples prior to them being analysed by the technology. In order to achieve this, we had to consider alternative methods whereby all the *Mycobacterium* repository strains were inactivated (that is, rendered non-viable) before being subjected to mass spectrometry processes. After due consideration of other probable inactivation methods (irradiation included), two chemical methods involving the use of solutions containing ACN and TFA were considered and compared. These were constituted as follows:

- o Solution A   50:50 Acetonitrile (ACN)/Water (H_2O) mixture containing 4%TFA

- o Solution B   90% ACN: 10 % H_2O containing 5% TFA.

Both solutions proved to be effective killing agents for the two laboratory strains of *Mycobacteria* (*M. tuberculosis* and *M. smegmatis*) tested. This was also found to be true for all other species of Mycobacterium tested and included in the database generated. Details of the protocol, procedure and efficiency check of the inactivation steps are documented in the preceding chapter, namely Materials and Methods. The efficacy of the procedure was based on the lack of growth (hence no viable organisms following the incubation with the solution being tested) after 2 cycles of 42 days of culture.

Representative spectra of the organisms treated with these solutions are shown in Figure 5.1 below: this figure shows the full spectra of the cell deposit obtained for both solutions A & B using the laboratory strain of *Mycobacterium smegmatis.* The spectrum obtained using Solution A is shown in the insert above while that for Solution B is below. In both cases, the organisms were non-viable upon culture. For clarity purposes, the whole spectrum has been expanded in three mass ranges: Figure 5.1(a) shows the spectrum in the lower mass range of 600-2000 *m/z,* figure 5.1(b) the same spectrum in the intermediate region of 2000-6000 *m/z* and figure 5.1(c) the spectrum in the higher mass region of 6000-95000 *m/z.* We have also represented the spectra obtained for each well of the target plate in order to demonstrate the reproducibility of the data rather than show a composite spectrum which is the average spectrum of the replicates.

For further clarity and easy understanding, we have also included a comparative spectra obtained by using the MicrobeLynx[TM] software to superimpose the two spectra patterns of interest against each other (Figure 5.1d).

This was achieved by using these spectra patterns of interest to generate a mini database containing just the two spectral patterns and 'searching' it against the appropriate part of the of the sample list of the experimental factor or organism under consideration. This comparative figure shows the spectra from the 2000 *m/z* to 9500 *m/z*. The MicrobeLynx^{TM} software generated comparative spectra is included for all the comparative figures presented in this chapter.

Our results indicated that, although both solutions were effective in killing the *Mycobacterium* cells and rendering them non-viable, solution A was more suitable for mass spectrometry as it gave more peaks for both cell extract (spectra not shown) and cell deposit, especially in the intermediate and higher mass range when compared to those obtained when using solution B. This is evident in the representative figure 5.1. Furthermore, this solution appears to yield better resolution of the peaks (higher intensity) in most *m/z* regions as shown in figure 5.1(d), this we considered as important when making our choice for inactivation solution to be used routinely.

Figure 5.1 **Full Spectrum of laboratory strain of *Mycobacterium smegmatis* (Cell Deposit) using Solution A as inactivation solution:**
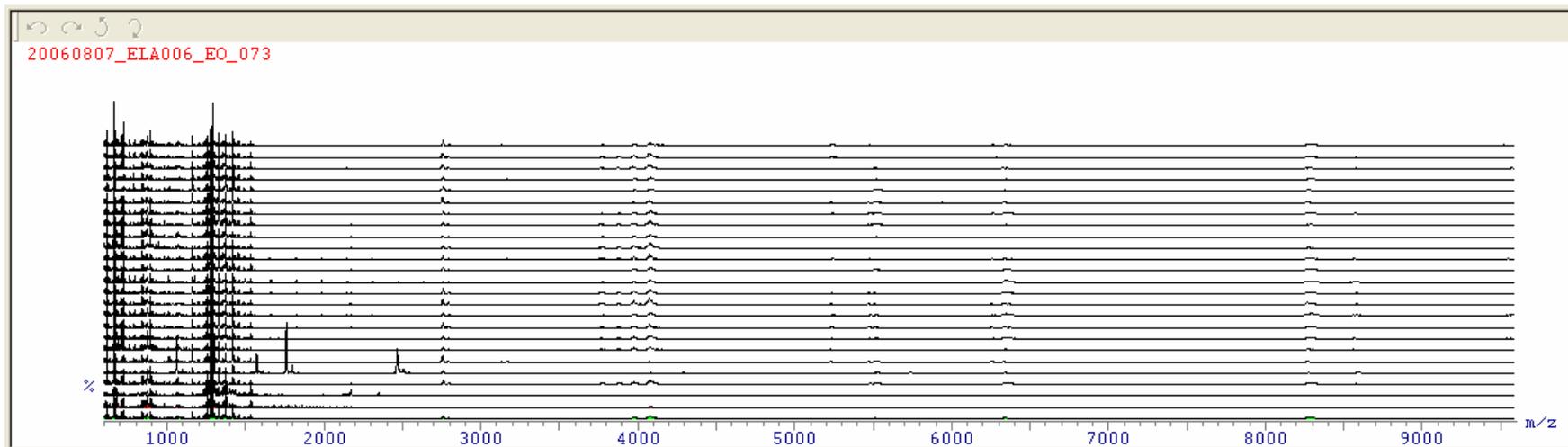
Full Spectrum of laboratory strain of *Mycobacterium smegmatis* (Cell Deposit) using Solution B as inactivation solution:
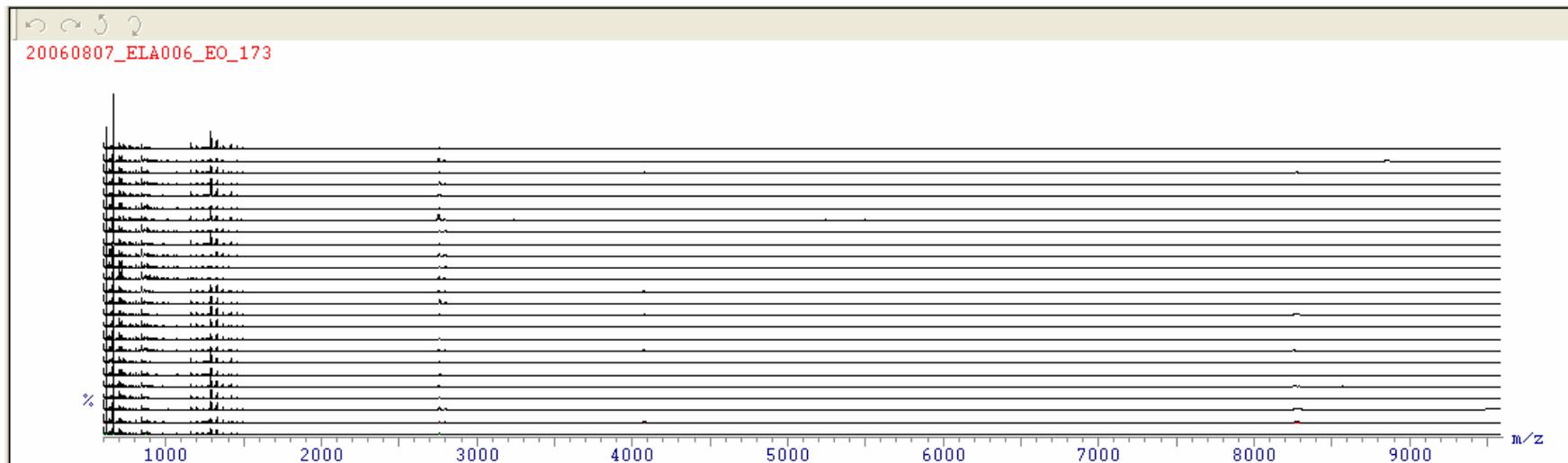
Figure 5.1 (a) Spectrum of laboratory strain of *Mycobacterium smegmatis* (Cell Deposit): solution A in lower mass range of 600- 2000 *m/z*.
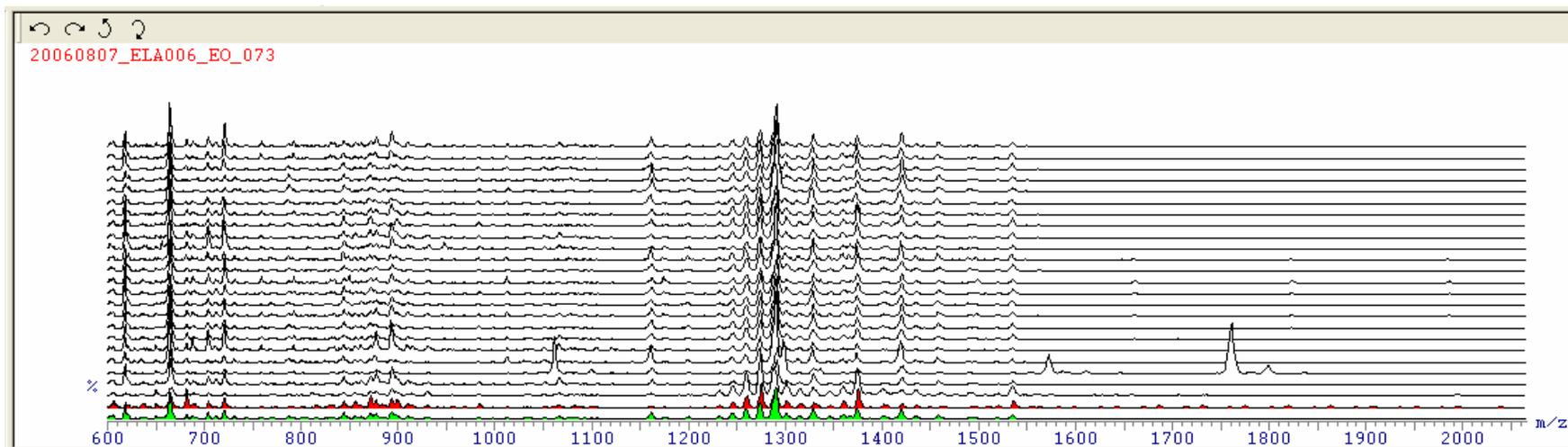

Spectrum of laboratory strain of *Mycobacterium smegmatis* (Cell Deposit): solution B in lower mass range of 600- 2000 *m/z*.
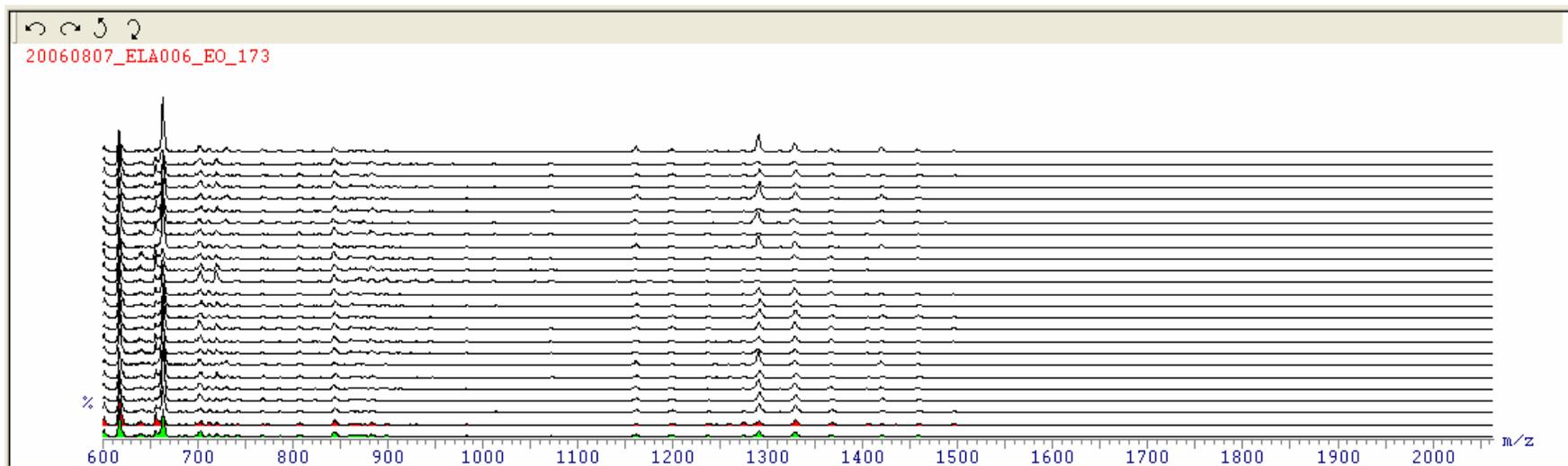
Figure 5.1 (b) Spectrum of laboratory strain of *Mycobacterium smegmatis* (Cell Deposit): solution A in intermediate mass range of 2000 -6000 *m/z*
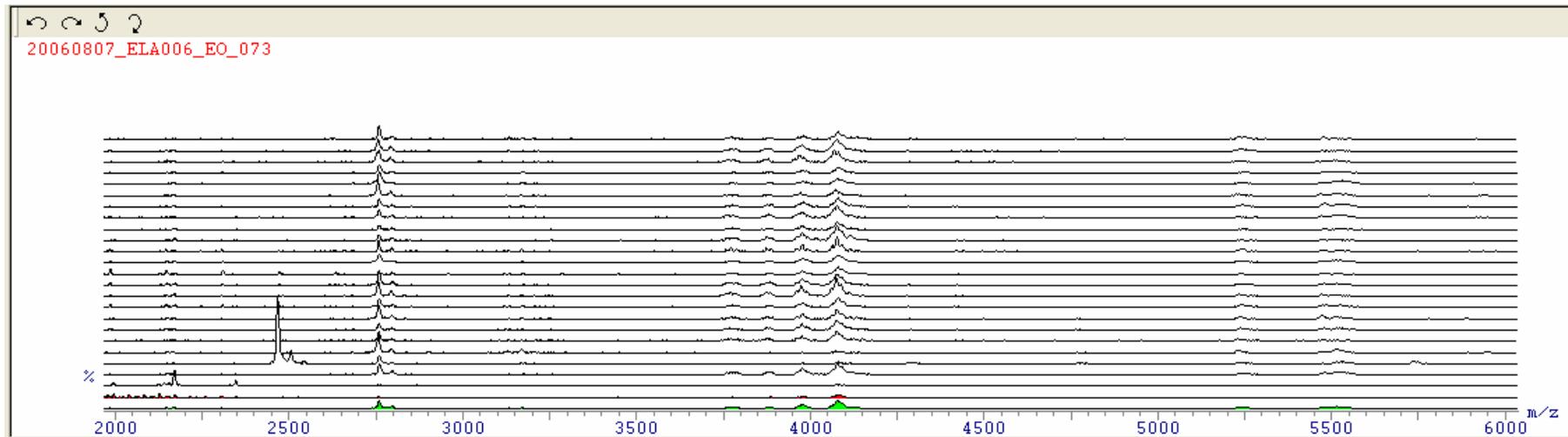
Spectrum of laboratory strain of *Mycobacterium smegmatis* (Cell Deposit): solution B in intermediate mass range of 2000 -6000 *m/z*
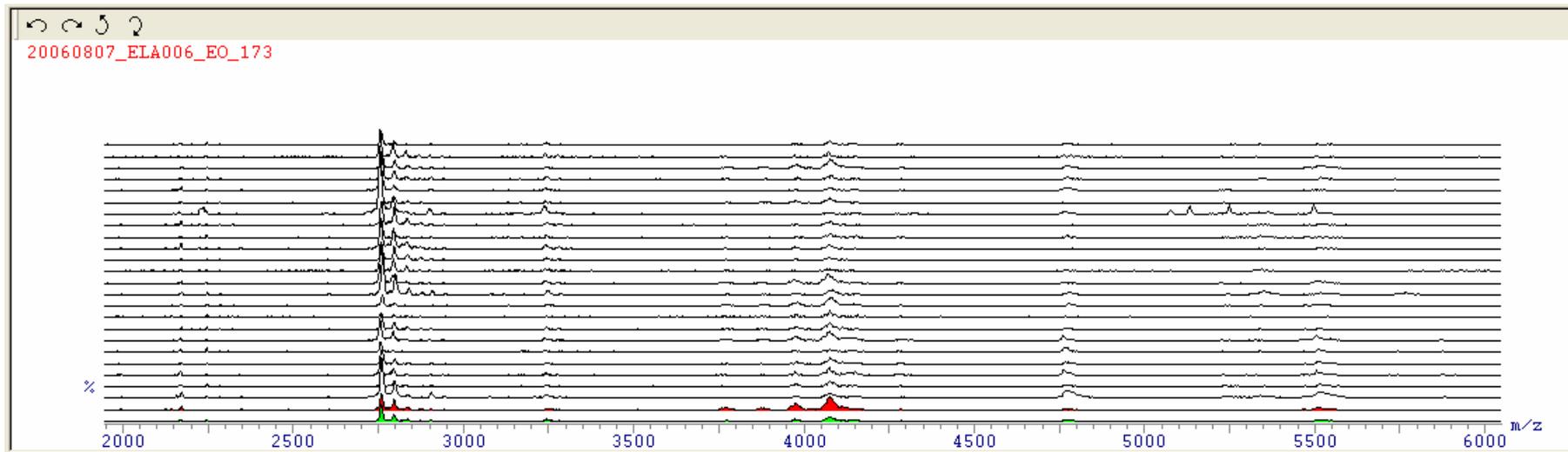
Figure 5.1 (c) Spectrum of laboratory strain of *Mycobacterium smegmatis* (Cell Deposit): solution A in higher mass range of 6000 -9500 *m/z*
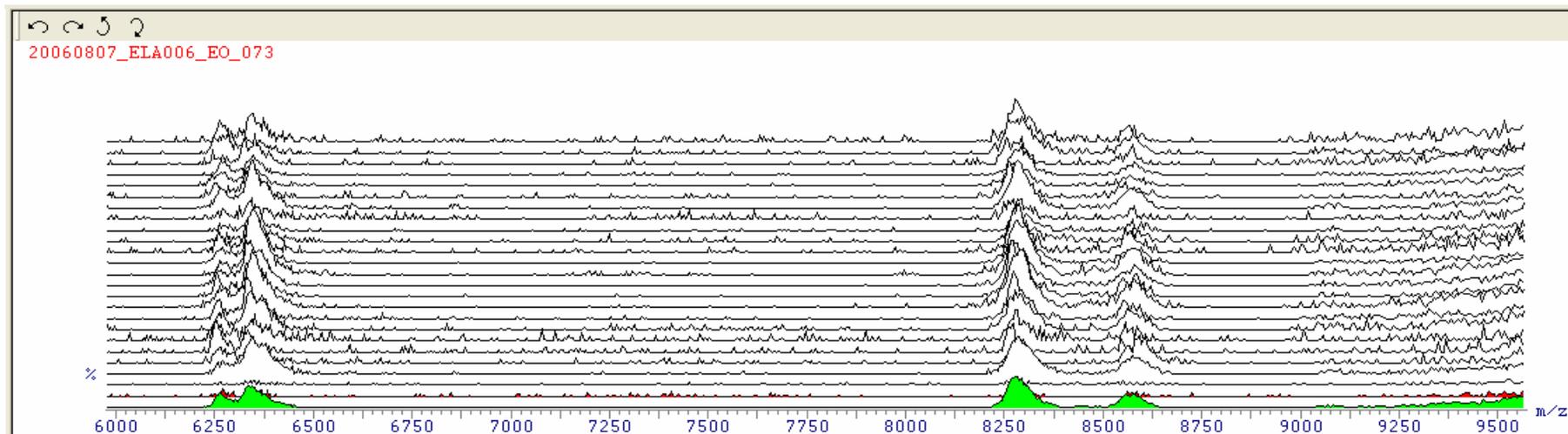
Spectrum of laboratory strain of *Mycobacterium smegmatis* (Cell Deposit): solution B in higher mass range of 6000 -9500 *m/z*
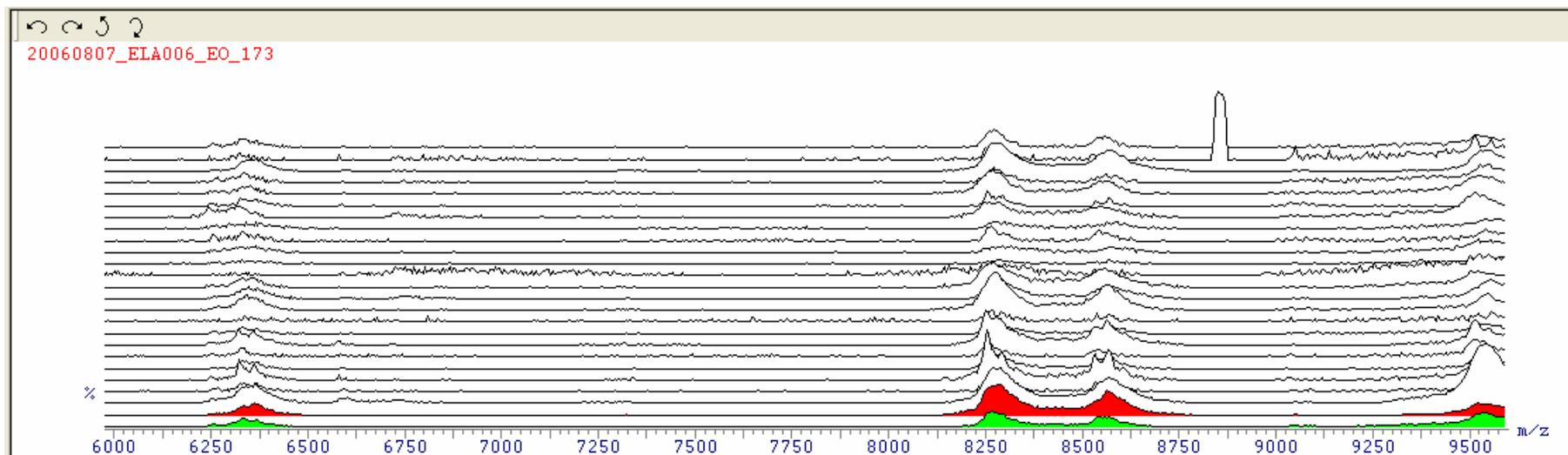
Figure 5.1(d) Spectra of laboratory strain of *M. smegmatis* (cell deposit) comparing both inactivating solutions using MicrobeLynx^{TM} software
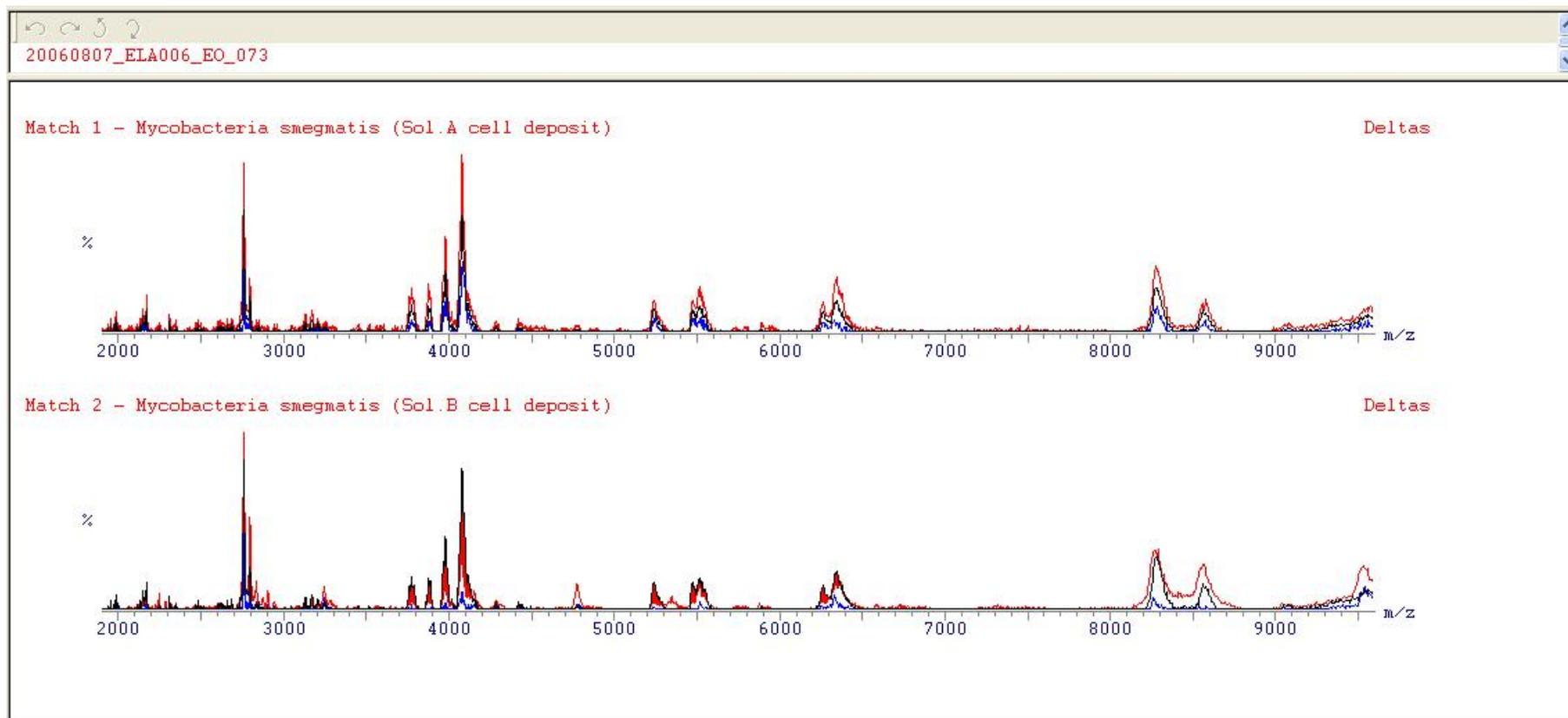
5.2   Choice of Matrix for routine use:

Having determined the best inactivating solution for routine use (based on both the ability of the solution to render the organism non-viable and its ability to yield reproducible spectra), the choice of matrix was the next important experimental factor that we had to consider. Two matrices were considered and compared for use: these were Alpha-Cyano-4-hydroxy-cinnamic acid (α-Cyano) and 5-chloro-2-mercaptobenzothiazole (CMBT). The matrices were made up in 1ml of the matrix solvent solution (MSS) as described in the preceding chapter.

In Figure 5.2 hereunder, we show representative data generated using α-Cyano (above) and CMBT (below) for cell deposit of the laboratory strain *M. smegmatis* cultured as previously described. Figure 5.2 (a-c) shows the spectra expanded in the lower, intermediate and higher mass ranges respectively. Once again, the data is presented as the individual spectra per well of the target plate. Also, the comparative spectra obtained using the MicrobeLynx™ software has been included (Figure 5.2 d).

As stated in the preceding chapter, CMBT did give good spectra at the lower mass range when the cell extract was used as source material (spectra not shown). Nevertheless, based on several experiments, α-Cyano proved to be the matrix of choice because it gave more peaks with higher intensities where it mattered most: at the intermediate and higher mass ranges (this is where most 'species-specific' ions are likely to be found) α-Cyano produced a wide variety of ions with good intensities and this can be clearly seen in figures 5.2 (b, c, & d). Furthermore, the α-Cyano matrix also gave better spectra at the intermediate mass range with the cell extract when compared to the CMBT matrix (spectra not shown) and since we considered the cell extract as a possible alternative source material when it comes to the analysis of clinical samples, we decided to opt for the α-Cyano as the matrix of choice for routine use. All subsequent experiments were therefore conducted using Solution A as inactivation solution and α-Cyano as matrix.

Figure. 5.2 Full Spectrum of laboratory strain *Mycobacterium smegmatis* (Cell Deposit) using α-Cyano as matrix
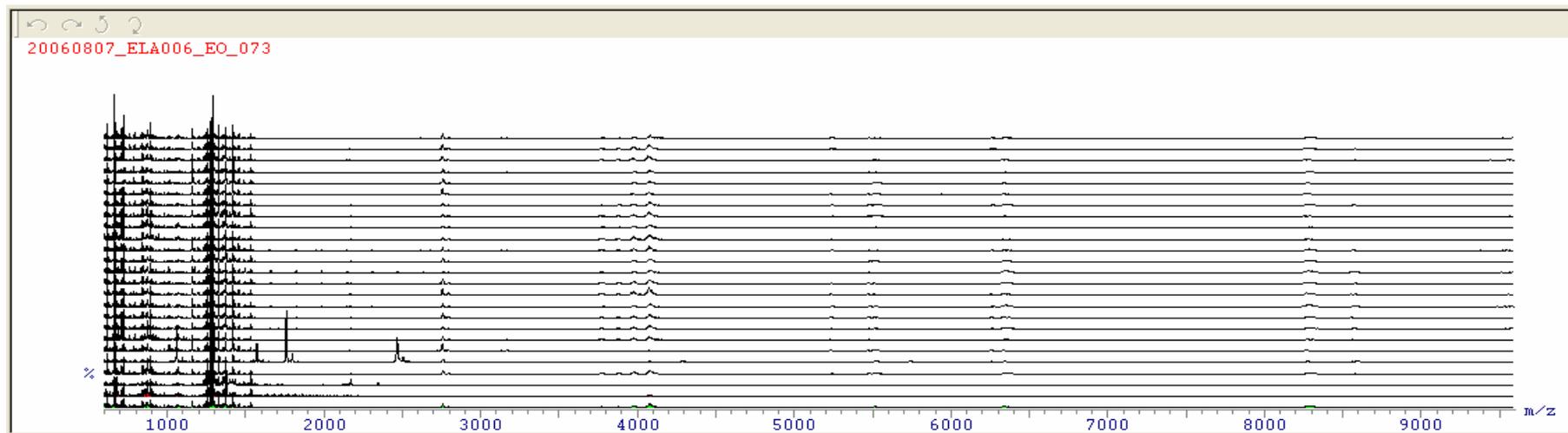
Full Spectrum of laboratory strain *Mycobacterium smegmatis* (Cell Deposit using CMBT as matrix
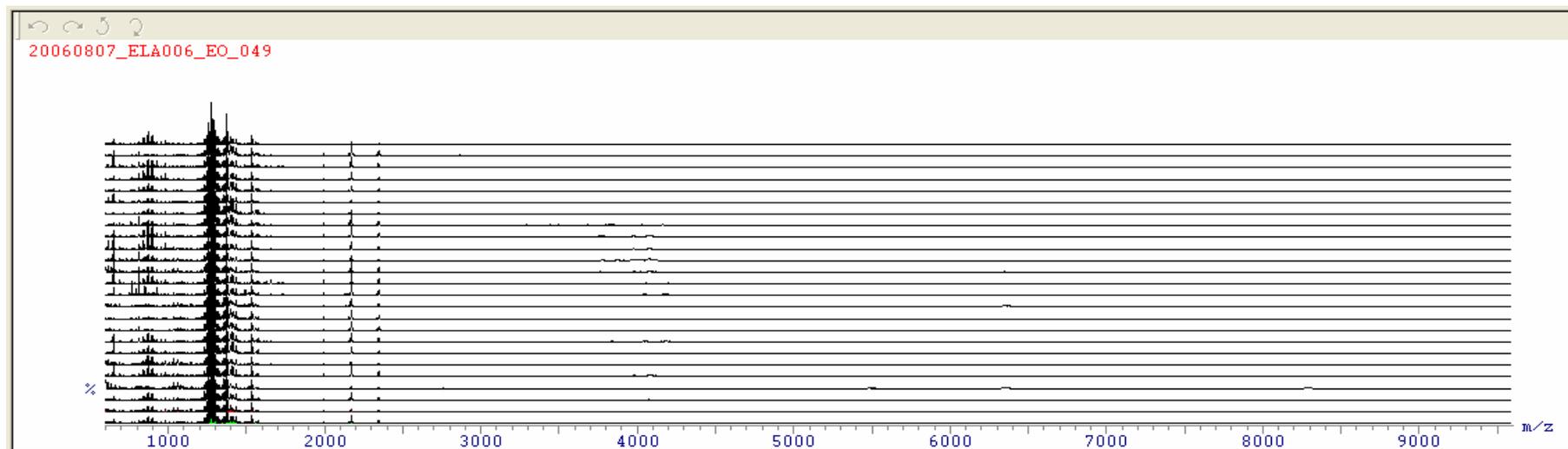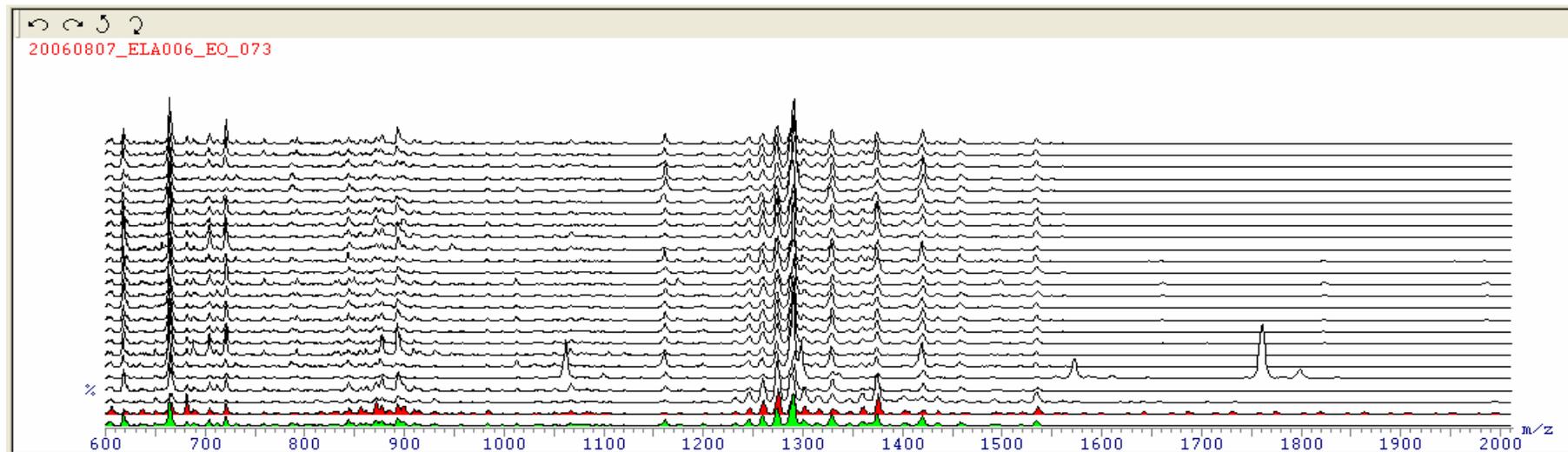
Figure. 5.2 (a) Spectrum at lower mass range 600- 2000 *m/z* **with α-Cyano as matrix (organism =** *M smegmatis***)**

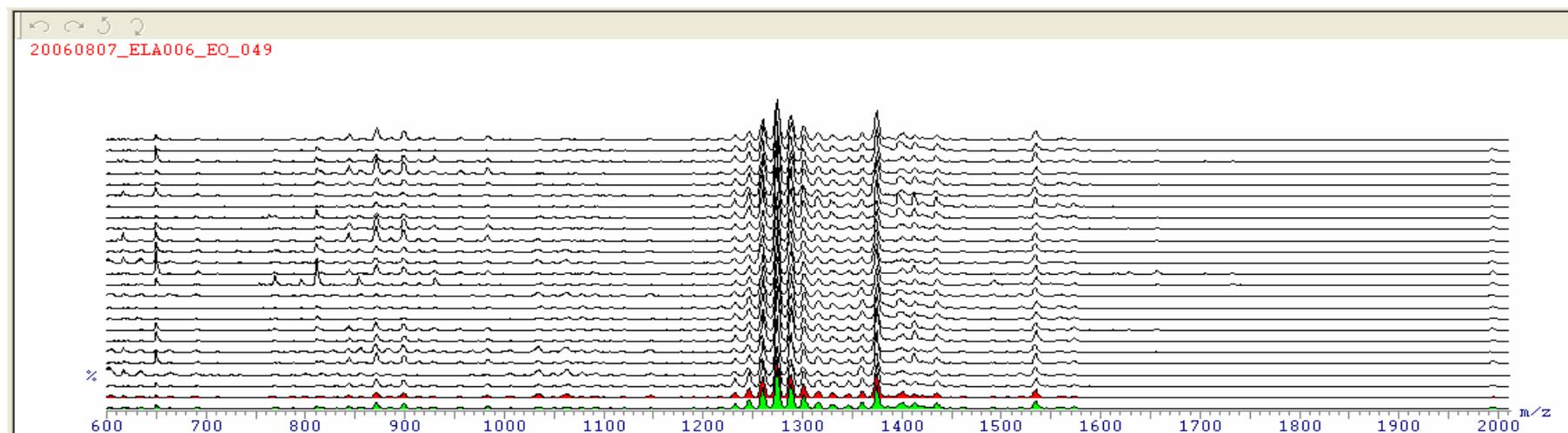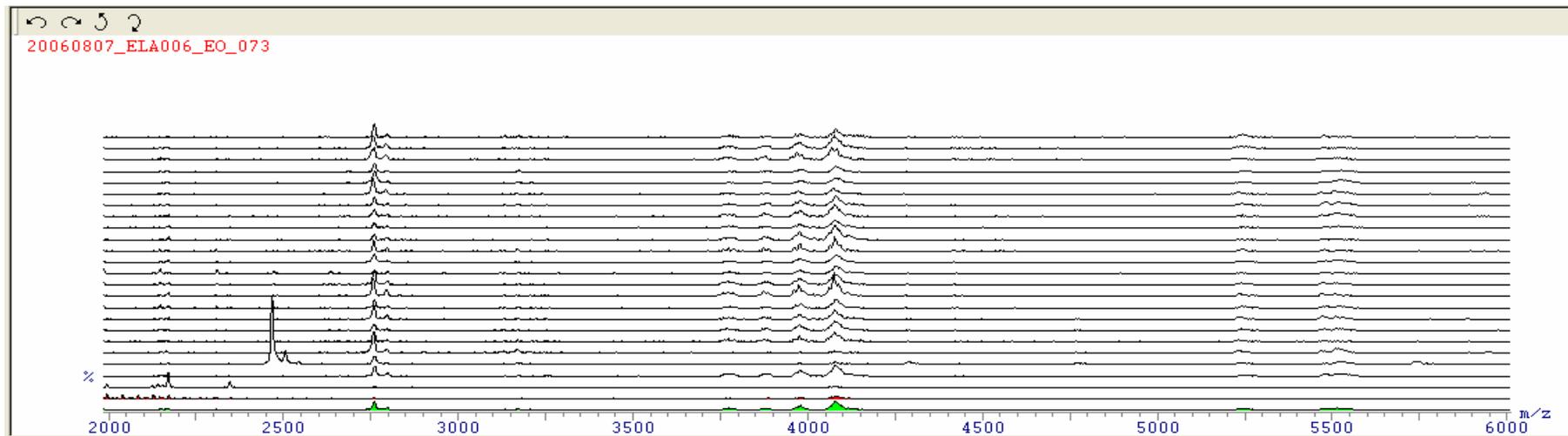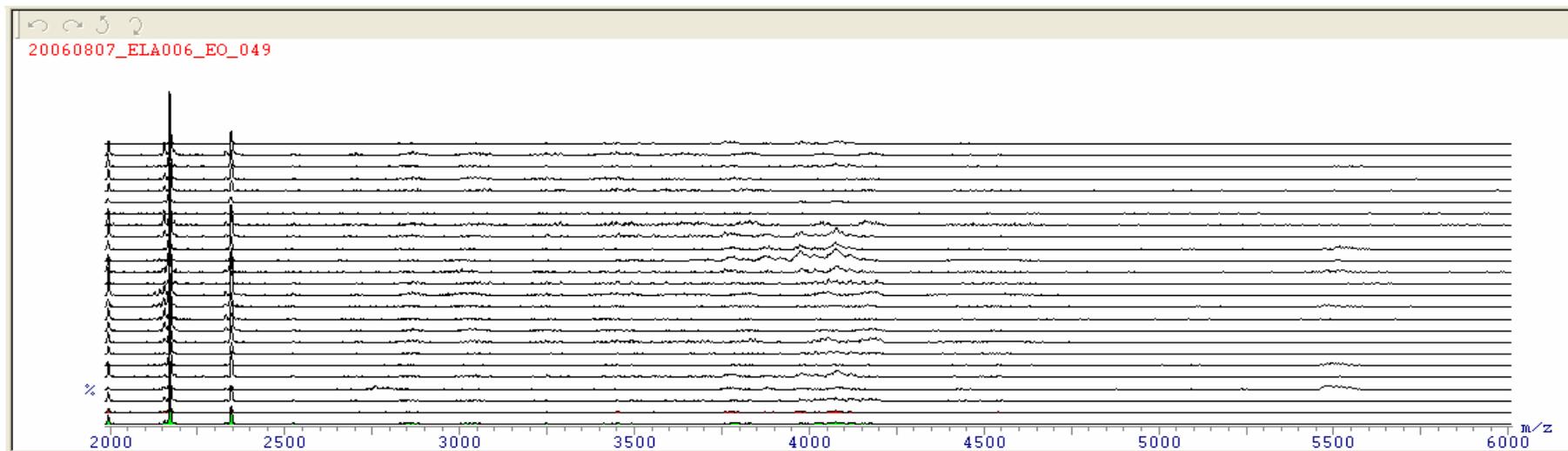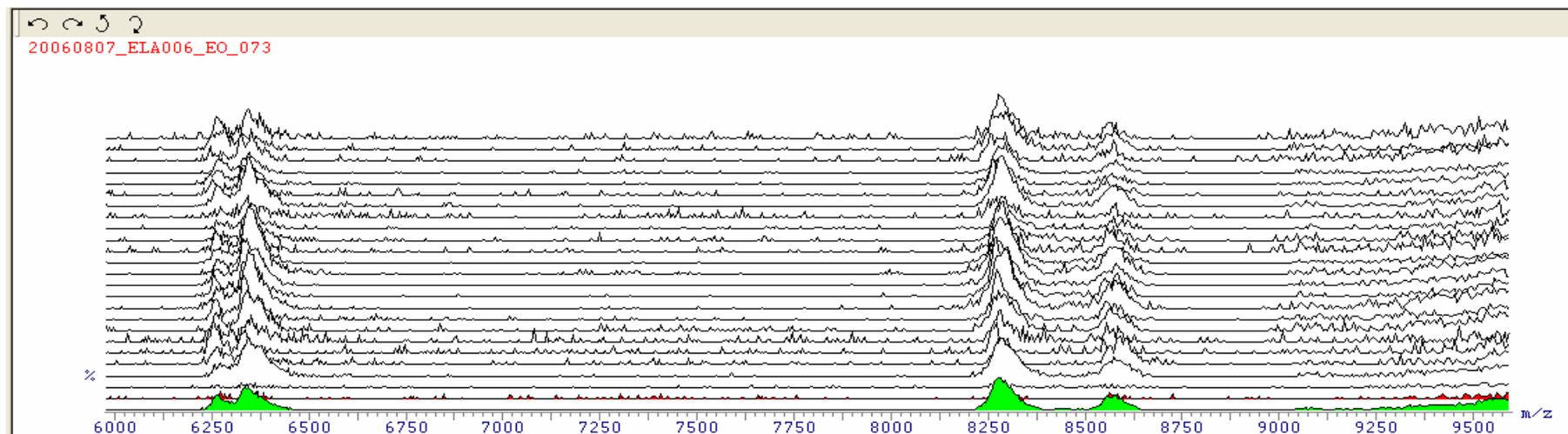**Spectrum at lower mass range 600- 2000** *m/z* **with CMBT as matrix (organism =** *M smegmatis***)**

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Figure 5.2 (b)   Spectrum at intermediate mass range 2000 - 6000 *m/z* with α-Cyano as matrix (organism = *M smegmatis*)

Spectrum at intermediate mass range 2000 - 6000 *m/z* with CMBT as matrix (organism = *M smegmatis*)

Figure 5.2 (c)   Spectrum at higher mass range 6000 - 9500 *m/z* with α-Cyano as matrix (organism = *M smegmatis*)

Spectrum at higher mass range: 6000 - 9500 *m/z* with CMBT as matrix (organism = *M smegmatis*)
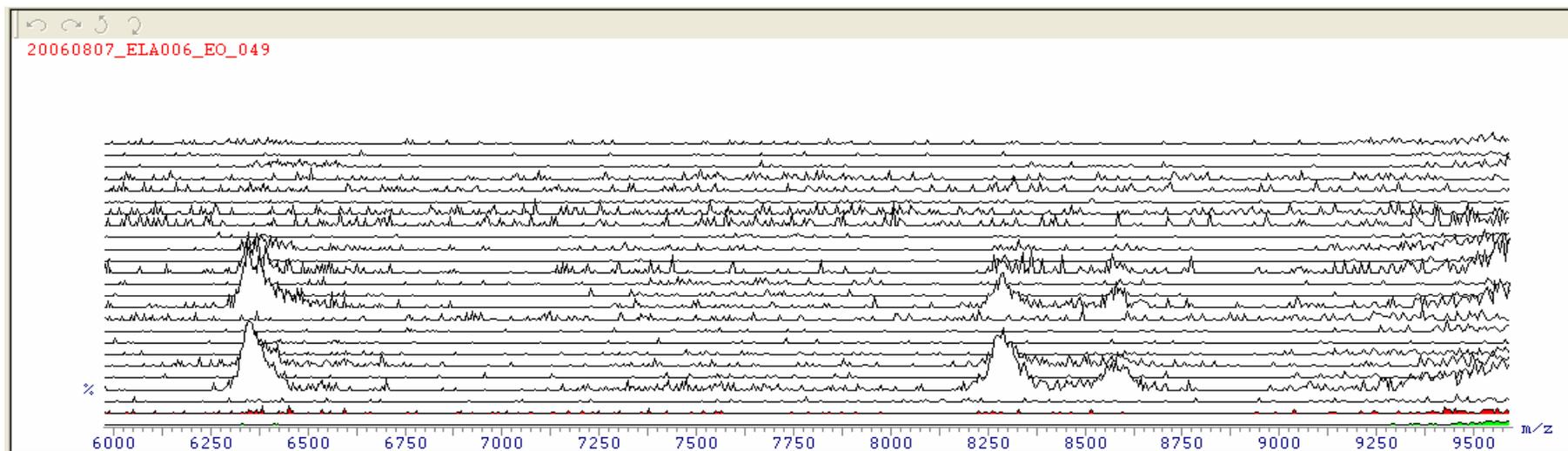
Figure 5.2(d) Spectra of laboratory strain of *M. smegmatis* (cell deposit) comparing both matrices using MicrobeLynx™ software
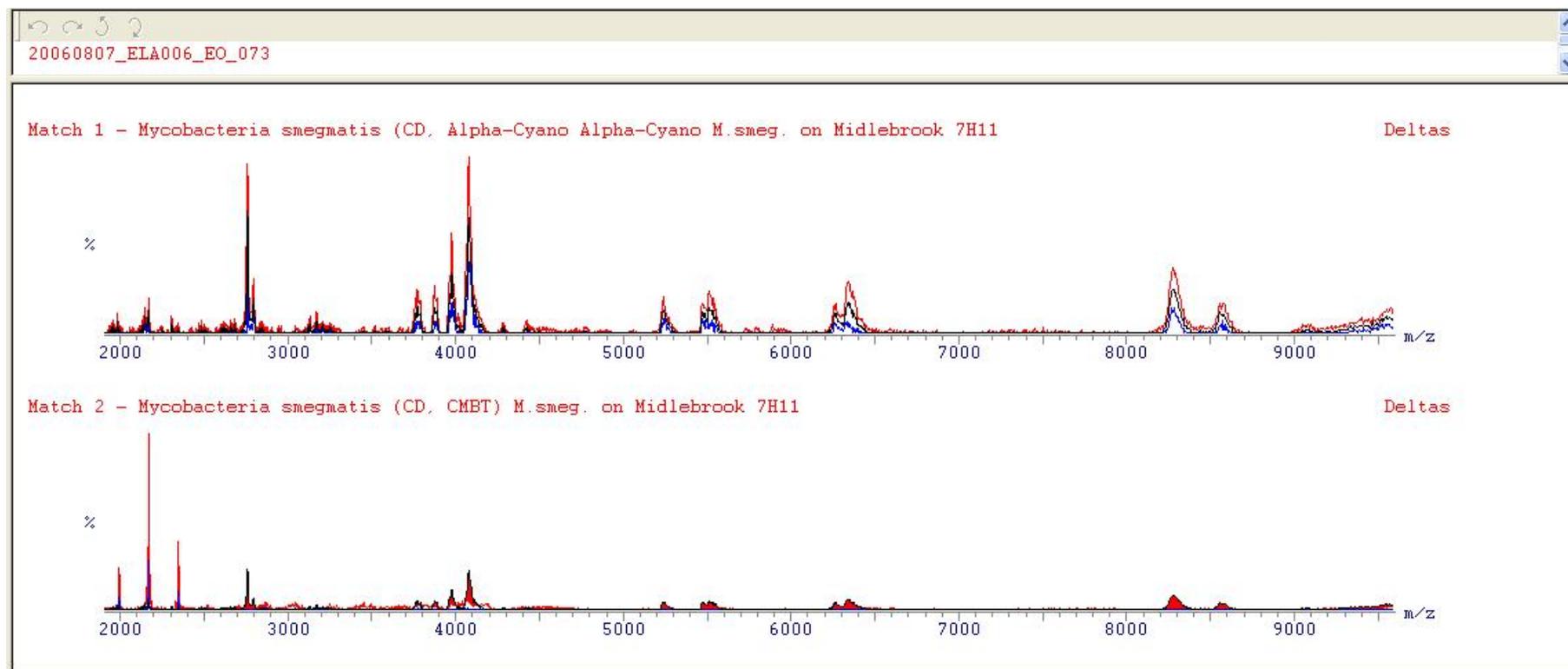
5.3   Choice of Cellular Part as source material:

It is again emphasized that the use of the inactivation solution was not a deliberate effort to disrupt the *Mycobacterium* cells or extract cellular components for MALDI assay. The pathogenic nature of most of the species included in this study made the inactivation step necessary in order to work in a non BSL3 environment for the post-culture analysis. However, since treatment with the inactivating solution lead to the generation of an acellular part or cell supernatant (referred to as cell extract), we decided to investigate the use of this preparation for generating mass spectra of the Mycobacteria test organisms. A laboratory strain of Mycobacterium *smegmatis* was used as a model organism for this purpose: it was treated with solution A (as decided upon in earlier experiments) and the mass spectra generated were compared, that is the spectrum generated using the cell deposit was compared to its corresponding cell extract (acellular part).

In figure 5.3, we show the comparison of the whole spectra of cell extract (above) with that of cell deposit (below). These are once again expanded in figures 5.3(a)-5.3(c), with the comparative spectra obtained using the MicrobeLynx^{TM} software presented in figure 5.3 (d) in order to make the differences clearer. The cell deposit spectrum had more peaks where it mattered most: at the higher mass range as figure 5.3(c & d) clearly shows. This implied that the cell deposit had potentially more 'species-specific' (that is species differentiating) ions than the corresponding cell extract. A similar pattern was observed for all species of Mycobacterium included in this study: all the strains had more ions in the higher mass ranges when the cell deposits were analysed. Nevertheless, both spectra of cell extract and cell deposits of all the *Mycobacterium* test organisms were considered and included in the database as separate entries for the above mentioned reason and others whose details are provided in the preceding chapter.

An in-house validation of the database was conducted as the database was populated. For this, a search of the database using organisms that had complete spectral entries in the database as "unknowns" was conducted. These organisms were cultured under identical conditions and prepared for MALDI-ToF analysis. When the database was interrogated, excellent results were obtained whether we used the cell deposit or the cell extract as source material: the database came up as first "Hits" with very high relative probabilities (usually 100%) and corresponding very low R.M.S values (usually less than 3). In exceptional cases of closely related strains, the second possibility is sometimes recognized as the first hit, but the test strain would have been proposed as the 2nd or 3rd hit with an R.M.S usually below 3. This was occasionally observed for cell extract of the organism used but never when the cell deposit was used. As previously stated, four species of the repository strains purchased for this study had duplicate strains (having different accession numbers) and these were *M. tuberculosis* (ATCC 35814 and 35804), *M. scrofulaceum* (ATCC 23420 and 23429), *M. gordonae* (ATCC 23409 and 14470), and *M. chelonae* (ATCC 35752 and 19538). These strains were handled as separate entries and when the "in-house validation" was conducted as described above; the results confirmed the ability of the database to differentiate between the two strains of the same organism, especially when the cell deposit was used as source material. Some of the results of the in-house validation tests are presented in table 5.1.

Table 5.1 showing some of the in-house validation test results

Organism Used As Unknown	ATCC Num.	1st Hit Identification	RMS	Relative Probability	Absolute Probability
Mycobacterium marinum	11566	*Mycobacterium marinum*	1.42 1.59	99.98 99.98	-2063.47 -2156.57
Mycobacterium peregrinum	23049	*Mycobacterium senegalense* (cell extract) *Mycobacterium peregrinum*(cell deposit)	5.28 2.62	63.18 78.41	-811.26 3057.65
Mycobacterium smegmatis	11759	*Mycobacterium smegmatis*	1.84 1.10	100.00 100.00	-3267.24 -2519.26
Mycobacterium intracellulare	23436	*Mycobacterium intracellulare*	2.24 1.68	52.27 99.68	-1828.45 -846.90
Mycobacterium gordonae (Exp. Strain 1)	14470	*Mycobacterium gordonae* (Exp. Strain 2 , cell extract) *Mycobacterium gordonae* (Exp. Strain 1 , cell deposit)	1.75 0.95	82.00 100.00	-2023.28 -545.86
Mycobacterium bovis	35724	*Mycobacterium ulcerans* (cell extract) *Mycobacterium bovis* (cell deposit)	1.26 2.49	75.81 99.99	534.74 -3340.76
Mycobacterium africanum	25420	*Mycobacterium africanum*	1.00 1.45	94.11 100.00	1175.69 -151.14
Mycobacterium ulcerans	19423	*Mycobacterium ulcerans*	1.34 1.60	97.61 100.00	782.74 -1152.40
Mycobacterium tuberculosis(Exp. Strain 1)	35804	*Mycobacterium tuberculosis*(Exp. Strain 1)	1.21 1.47	98.42 100.00	324.55 -1833.22
Mycobacterium tuberculosis(Exp. Strain 2)	35814	*Mycobacterium tuberculosis*(Exp. Strain 2)	0.68 0.87	100.00 100.00	2188.34 1334.24
Mycobacterium confluentis	49920	*Mycobacterium confluentis*	1.09 0.93	99.42 100.00	112.40 -1310.10
Mycobacterium flavescens	23008	*Mycobacterium flavescens*	0.59 1.34	100.00 89.16	138.52 -3452.57
Mycobacterium conspicuum	700090	*Mycobacterium conspicuum*	0.86 1.94	100.00 100.00	-712.64 -953.40
Mycobacterium gordonae (Exp. Strain 2)	23420	*Mycobacterium gordonae* (Exp. Strain 1)	1.61 0.75	99.66 100.00	-441.94 1629.17
Mycobacterium xenopi	19971	*Mycobacterium xenopi*	0.69 0.84	100.00 100.00	2898.71 50.59
Mycobacterium abscessus	23018	*Mycobacterium abscessus*	2.32 5.27	91.47 99.99	257.70 -808.78
Norcardia brevicatena	15333	*Norcardia brevicatena*	0.98	100.00	2633.34
Legionella pneumophila	33215	*Legionella pneumophila*	0.89	100.00	3851.86

Figure 5.3   Full Spectrum of the cell extract of the laboratory strain *M. smegmatis*
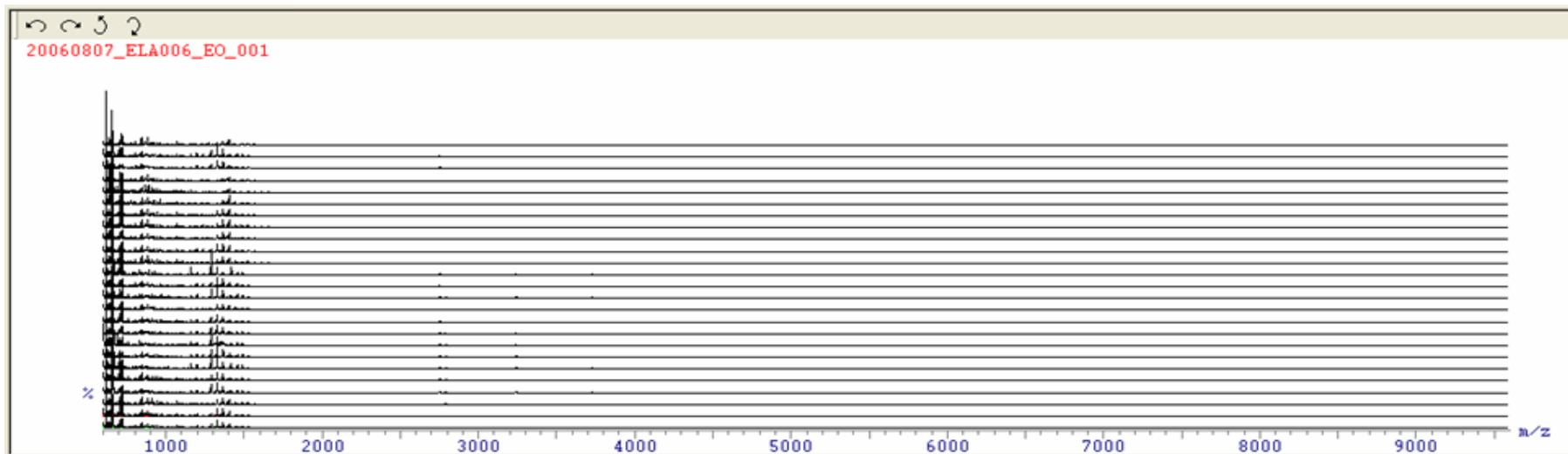
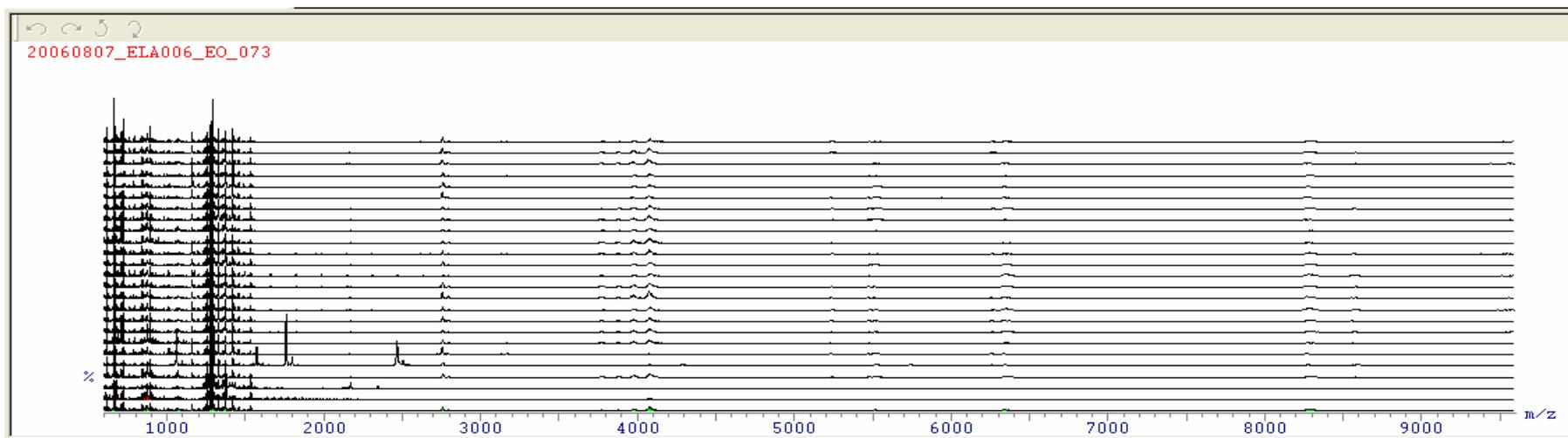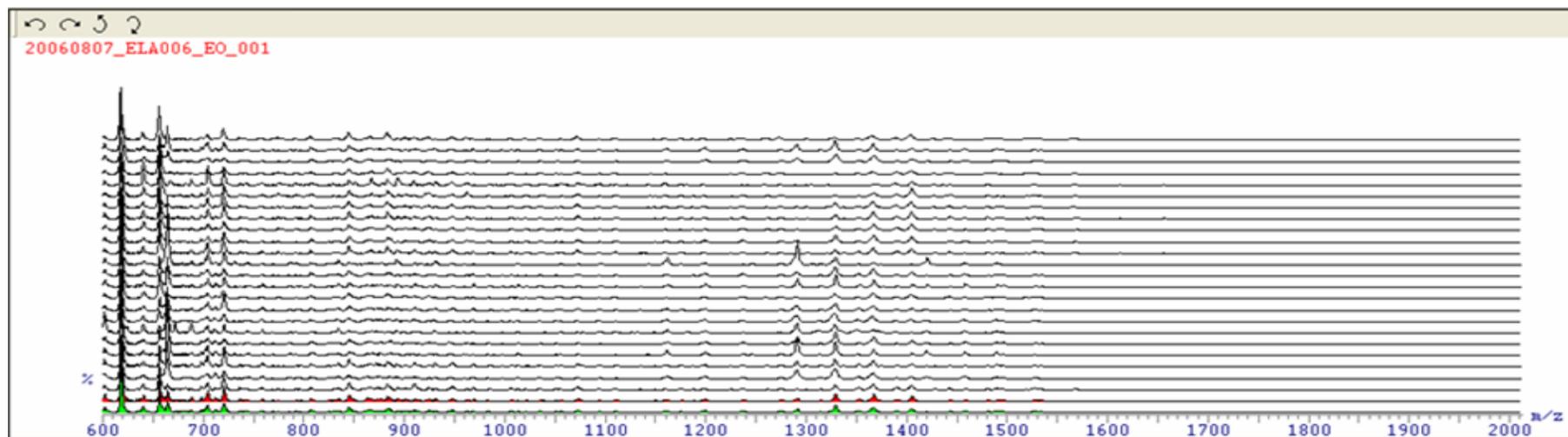**Full Spectrum of the cell deposit of the laboratory strain *M. smegmatis***

Figure 5.3 (a)   Lower mass range: 600- 2000 *m/z* spectrum of the cell extract of the laboratory strain *M. smegmatis*

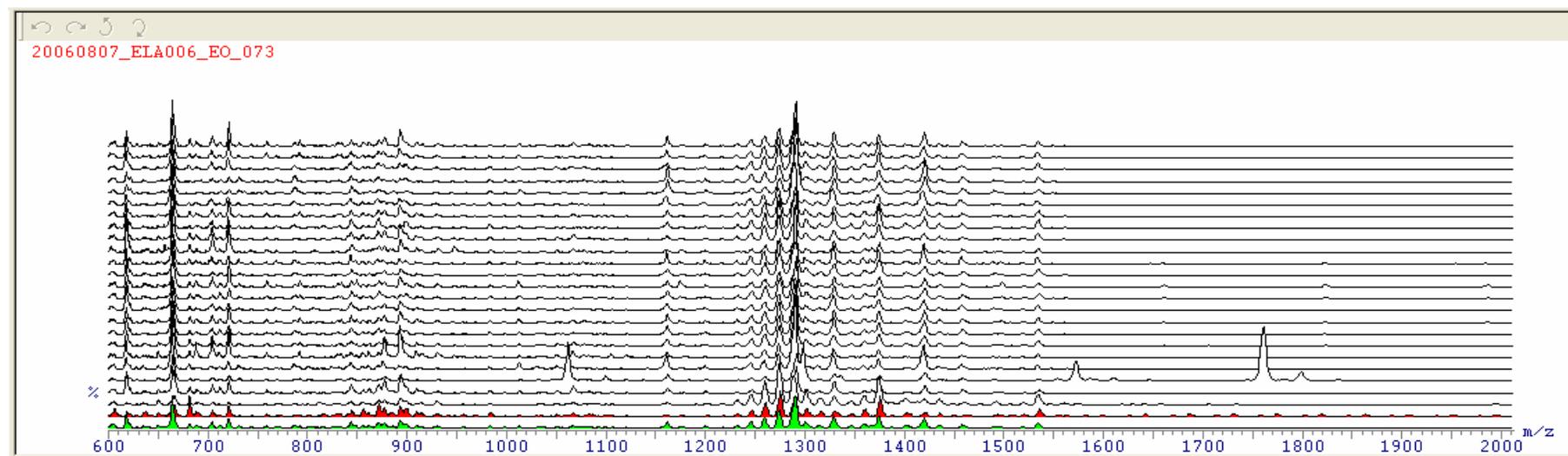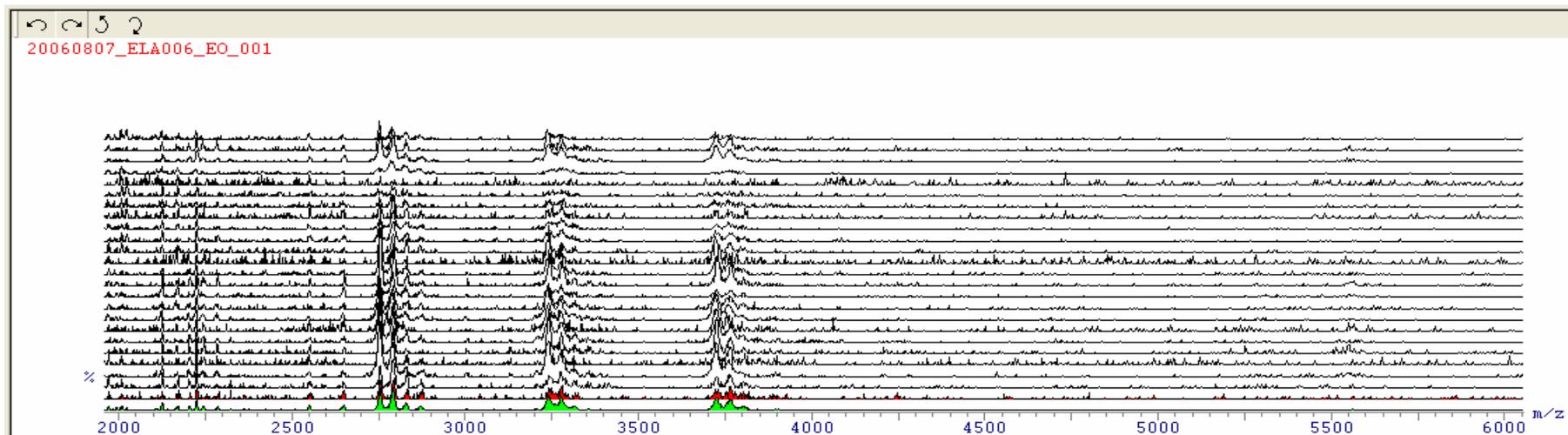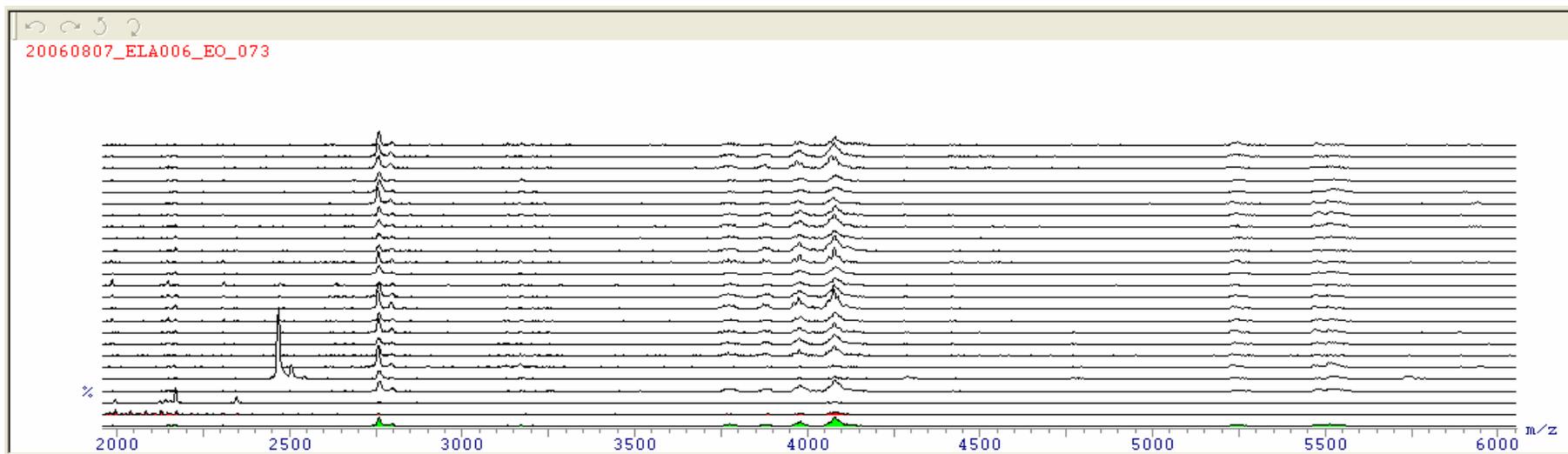**Lower mass range: 600- 2000 *m/z* spectrum of the cell deposit of the laboratory strain *M. smegmatis***

Figure 5.3(b) **Intermediate mass range: 2000 -6000 *m/z* spectrum of the cell extract of the laboratory strain *M. smegmatis***

Intermediate mass range: 2000 -6000 *m/z* spectrum of the cell deposit of the laboratory strain *M. smegmatis*
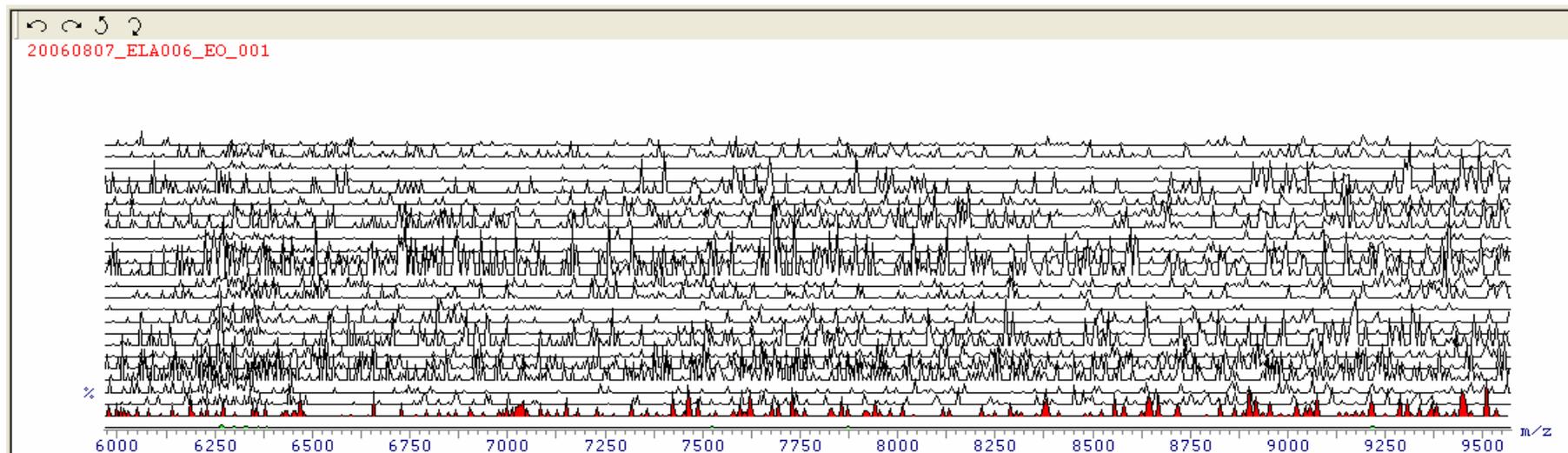
Figure 5.3(c). Higher mass range: 6000 - 9500 *m/z* spectrum of the cell extract of the laboratory strain *M. smegmatis*

Higher mass range: 6000 - 9500 *m/z* spectrum of the cell deposit of the laboratory strain *M. smegmatis*
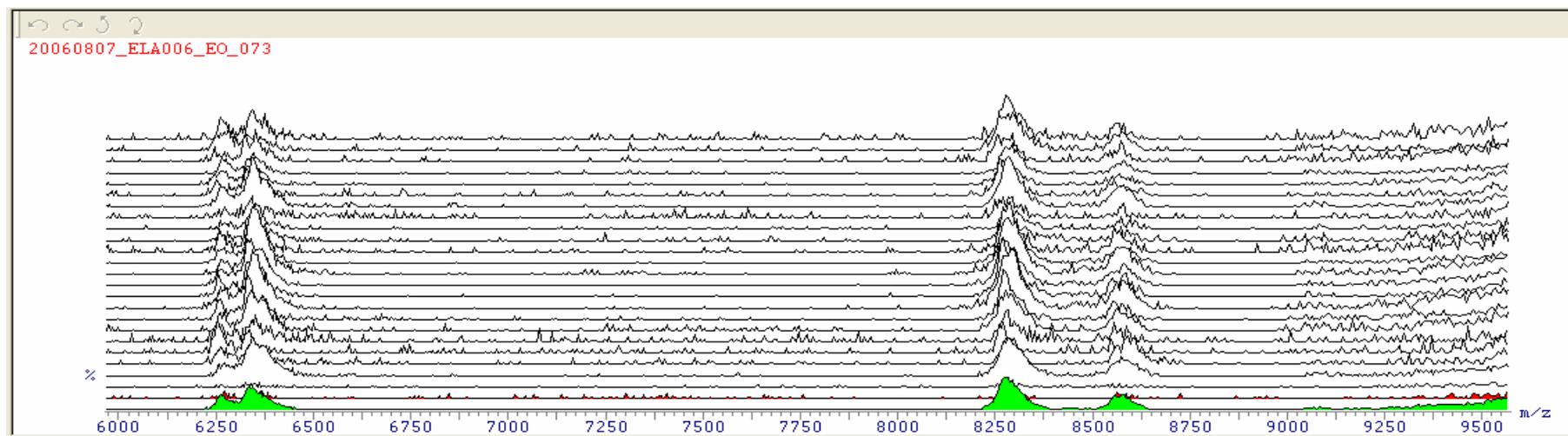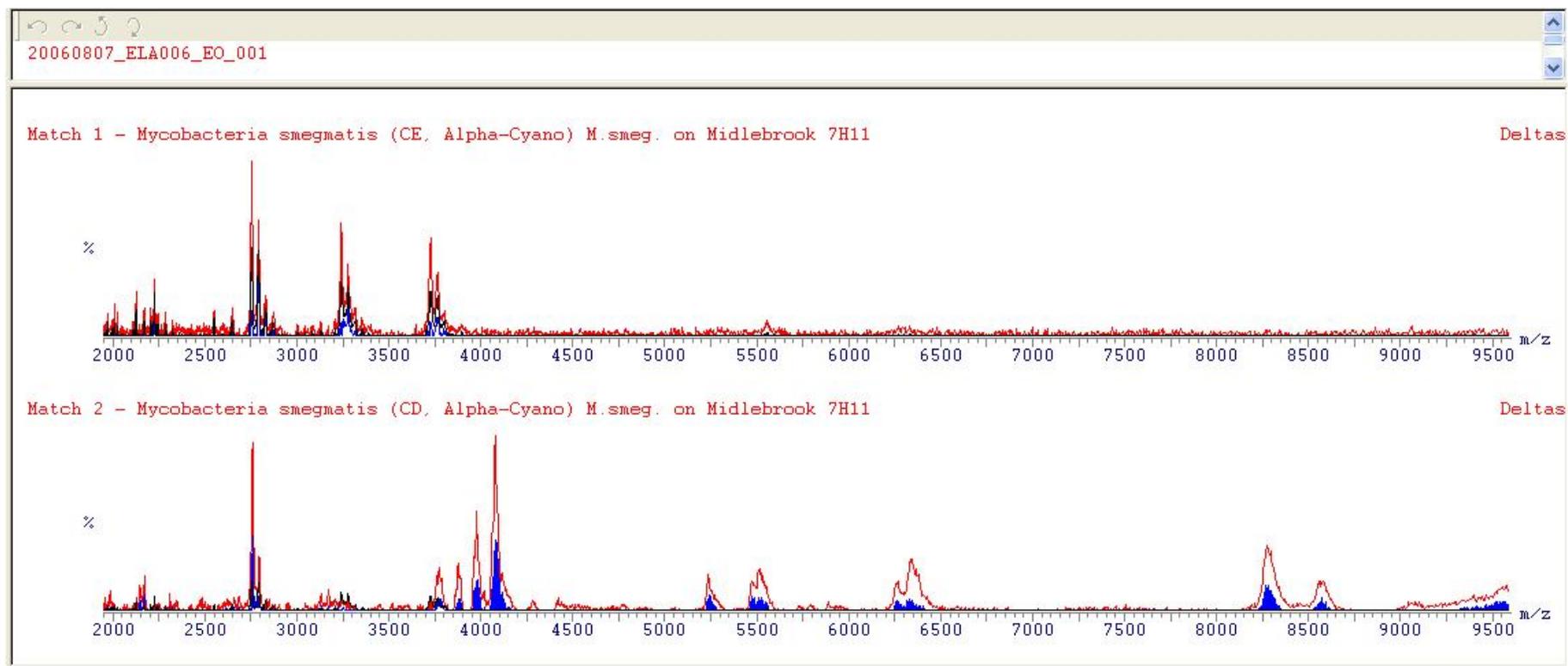
Figure 5.3(d) Spectra of laboratory strain of *M. smegmatis* comparing both cell extract and cell deposit using MicrobeLynx^{TM} software

5.4   Representative spectra of organisms belonging to the MTB complex

Mycobacterium tuberculosis is the archetypical species of the genera Mycobacterium and is one of the species which belong to the so-called MTB complex. In this section, one of its species, namely, *Mycobacterium tuberculosis* (ATCC$^®$ strain 35804) was used as the representative test organism. In comparison, we present the data of another species belonging to the same MTB complex group of organisms in order to demonstrate the differences detected by the MALDI-ToF technology: this technology is sensitive enough to differentiate between closely-related species and is therefore a powerful tool in the clinical Mycobacteriology discipline. The composite spectra are shown in Figure 5.4 (cell deposit) and figure 5.5 (cell extract). For further clarity, the MicrobeLynx^{TM} software generated comparative figures are presented in figures 5.6 & 5.7.

The figure 5.6 in particular makes it more evident that the technology can differentiate between these related species.

Figure 5.4: Composite spectra of *Mycobacterium tuberculosis* (A) and *M. bovis* (B) cell deposits
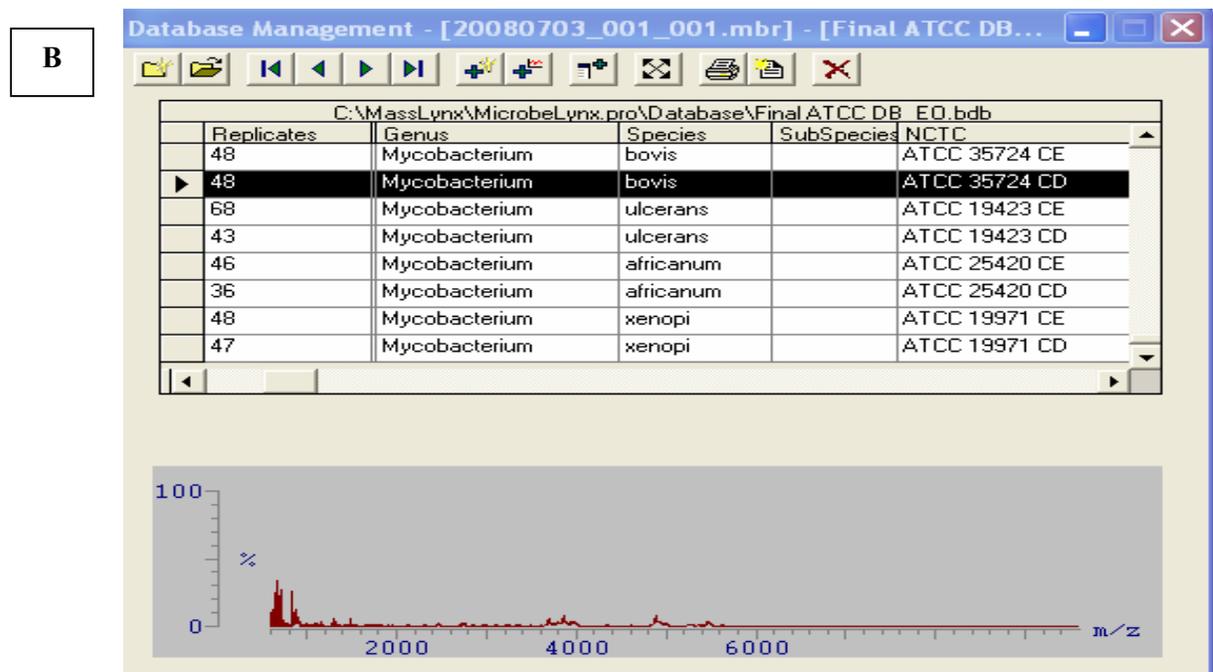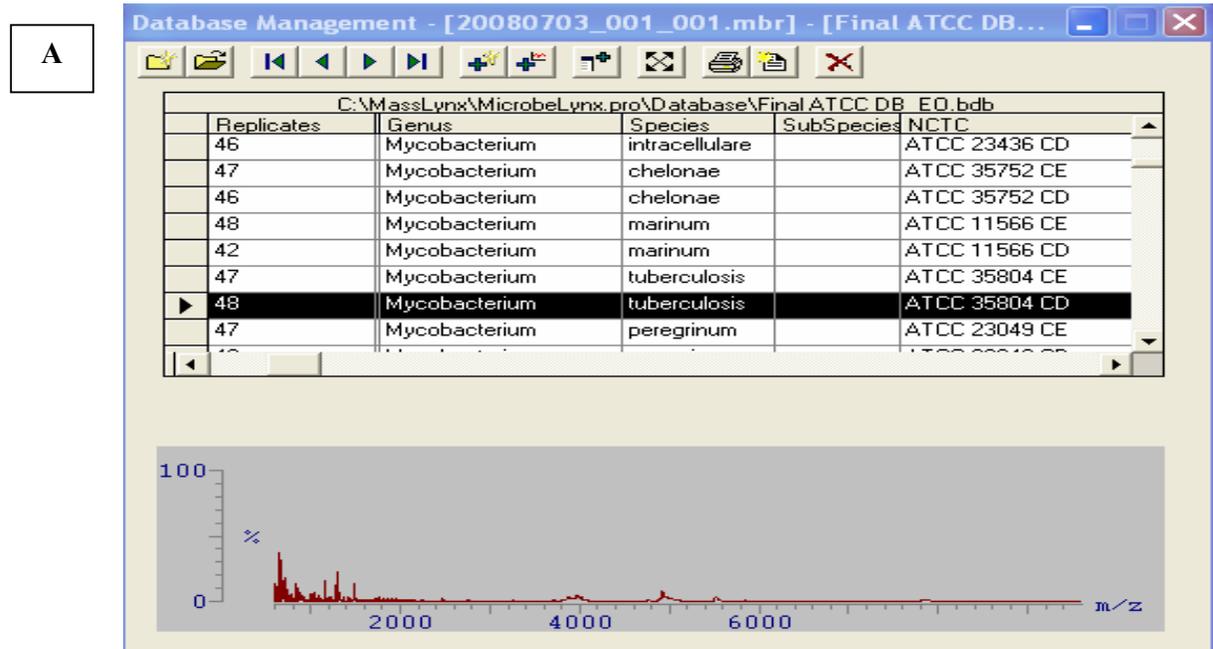
Figure 5.5: Composite spectra of the cell extracts of *M. tuberculosis* (A) and *M. bovis* (B)
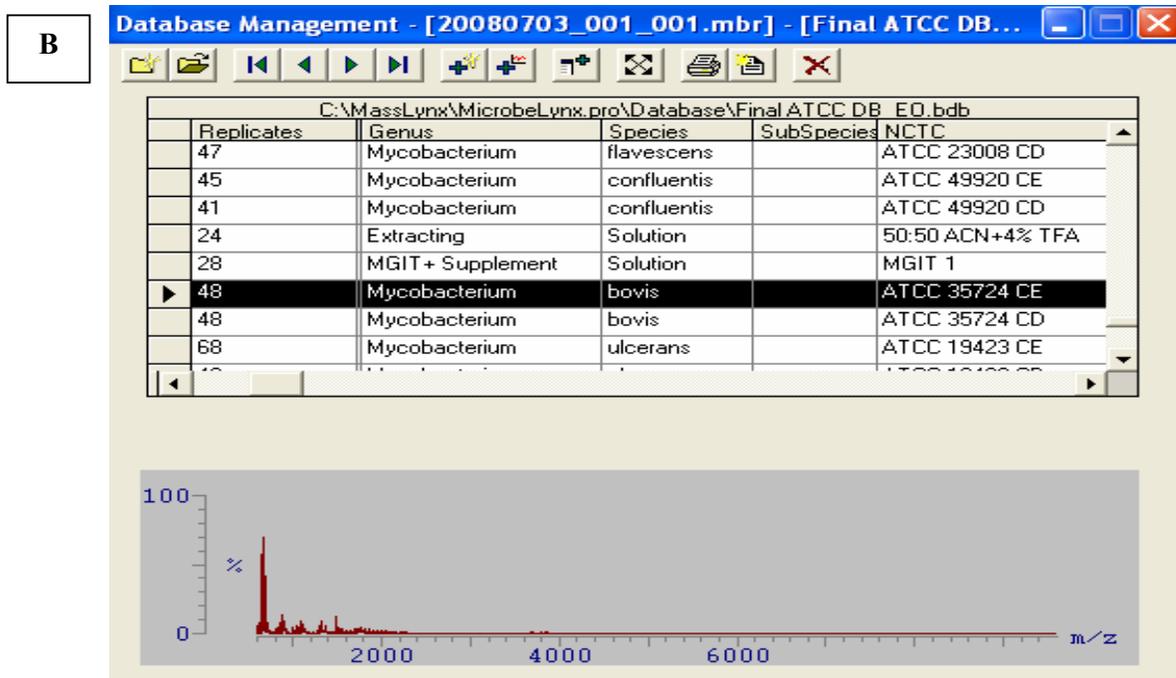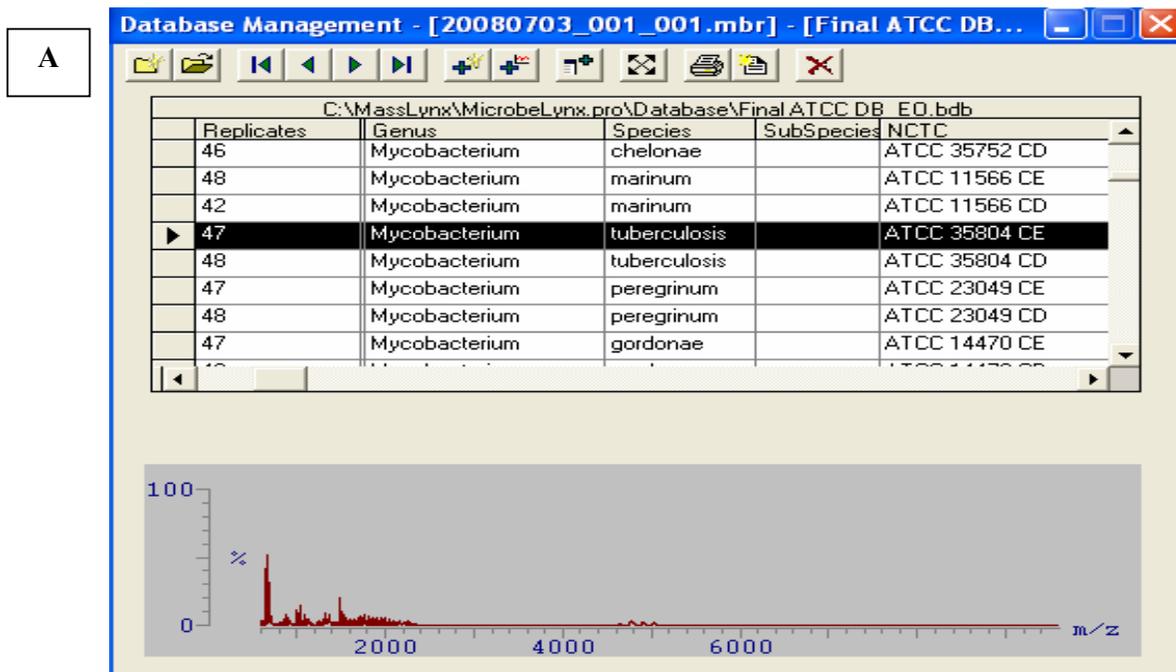
Figure 5.6 Comparative spectra of *Mycobacterium tuberculosis* and *M. bovis* (cell deposit) using MicrobeLynx™ software
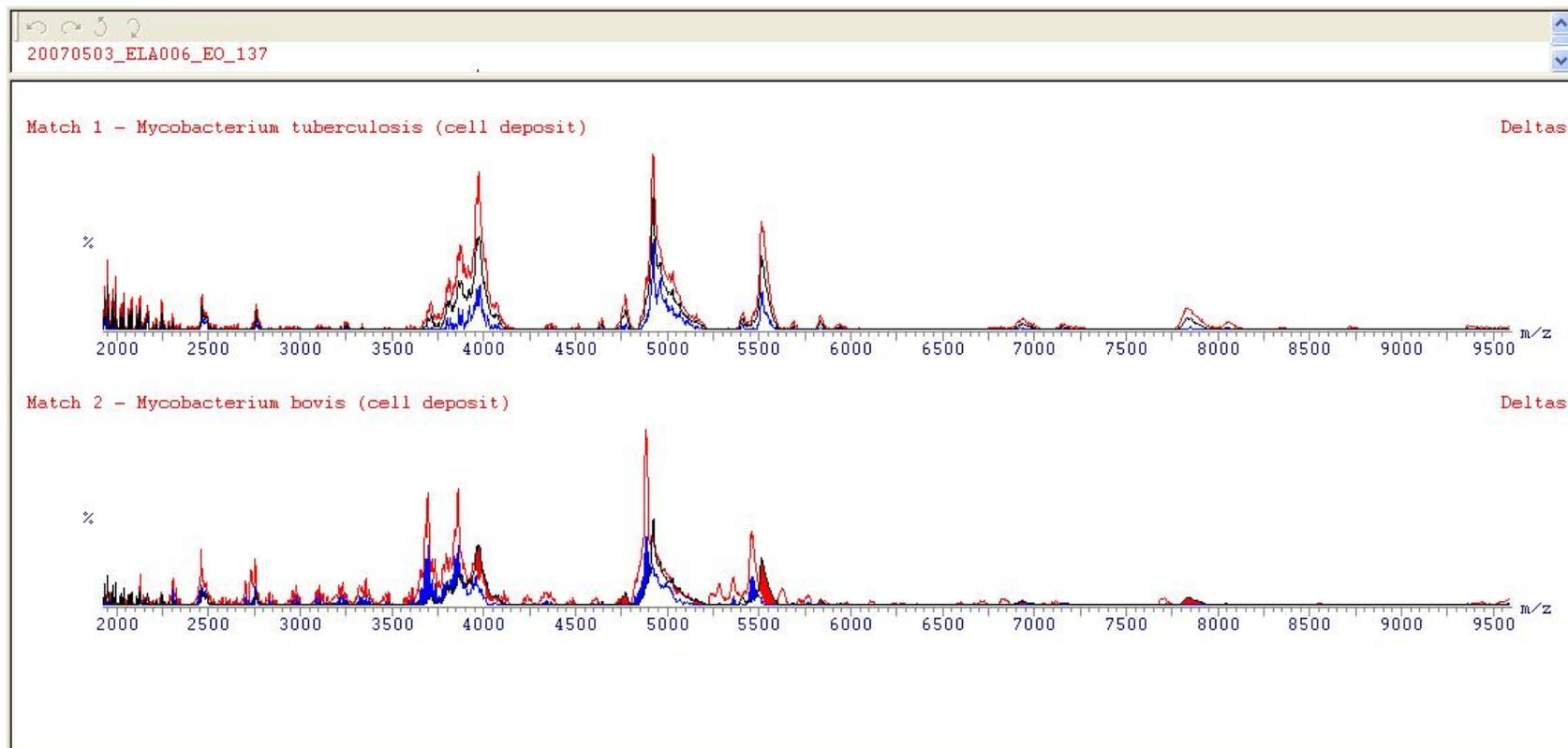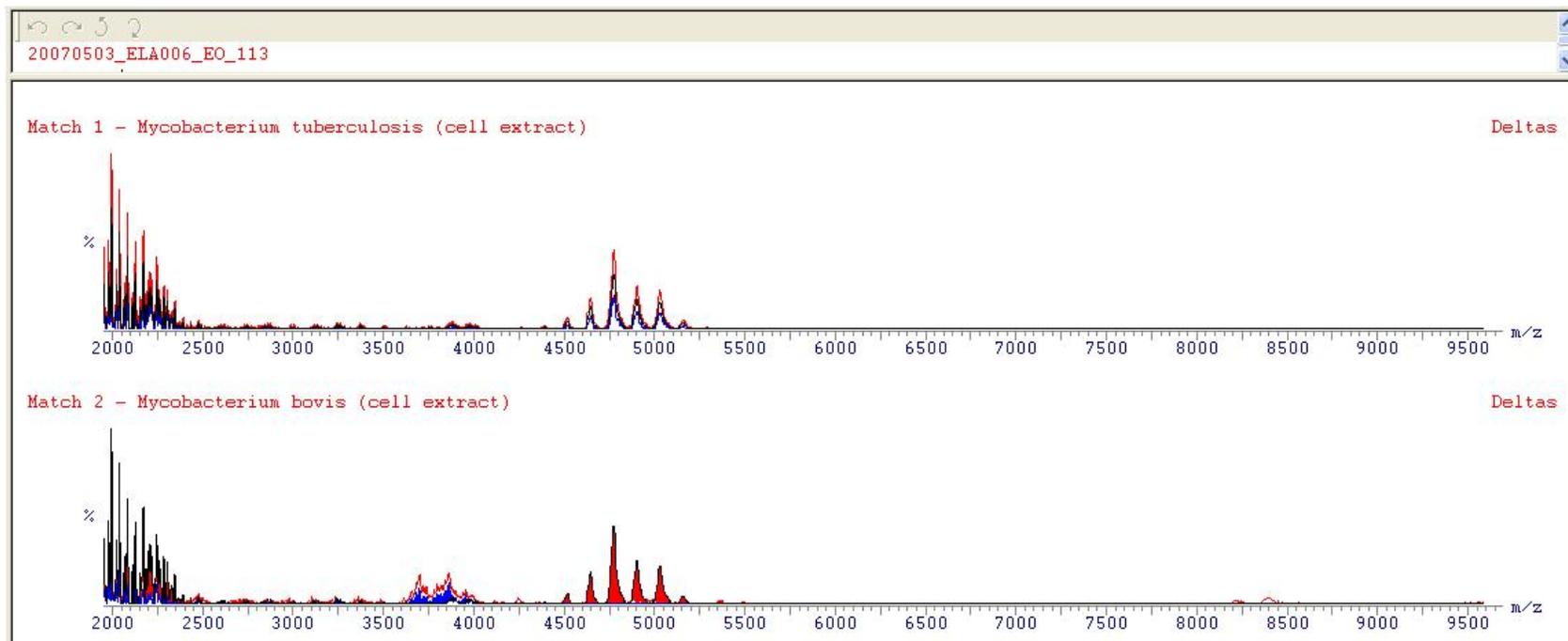
Figure 5.7 Comparative spectra of *Mycobacterium tuberculosis* and *M. bovis* (cell extract) using MicrobeLynx™ software

5.5   **Representative spectra of Non- tuberculous *Mycobacteria* (NTM) species**

Twenty-six of the twenty-nine *Mycobacterium* species purchased from the repository and included in the database are non- tuberculous *Mycobacterium*. Some such as *M. gordonae* are free living saprophytes while others such as *M. avium* and *M. intracellulare* could be fatal pathogens especially in the immune compromised patient. Once again, in order to demonstrate the ability of the technology to differentiate between species belonging to the same group of organisms, *M. avium* (ATCC$^{®}$ 35716) and *M. intracellulare* (ATCC$^{®}$ 23436) were used as the representative organisms and the composite spectra of their cell deposits are shown hereunder in Figure 5.8 and that obtained using the cell extracts as source material is shown in Figure 5.9.

In addition to these and as previously done, the MicrobeLynx^{TM} software generated comparative figures are presented in figures 5.10 & 5.11.

Figure 5.8: Composite spectra of cell deposits of *M. avium* (A) compared to that of *M. intracellulare* (B):
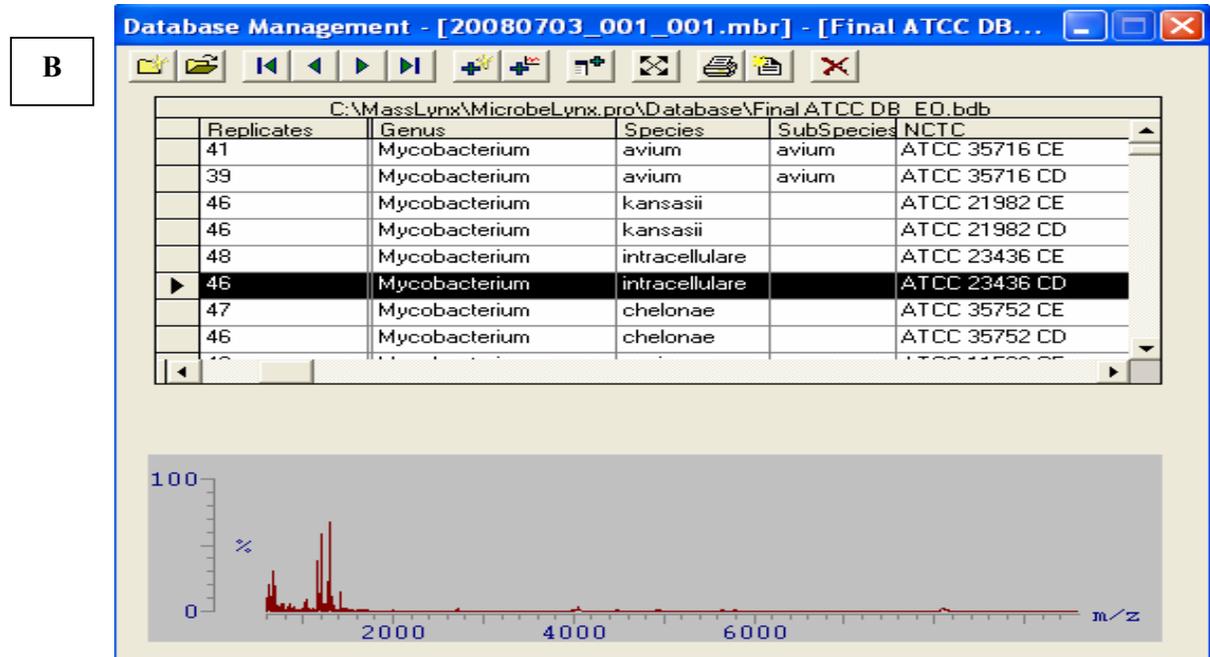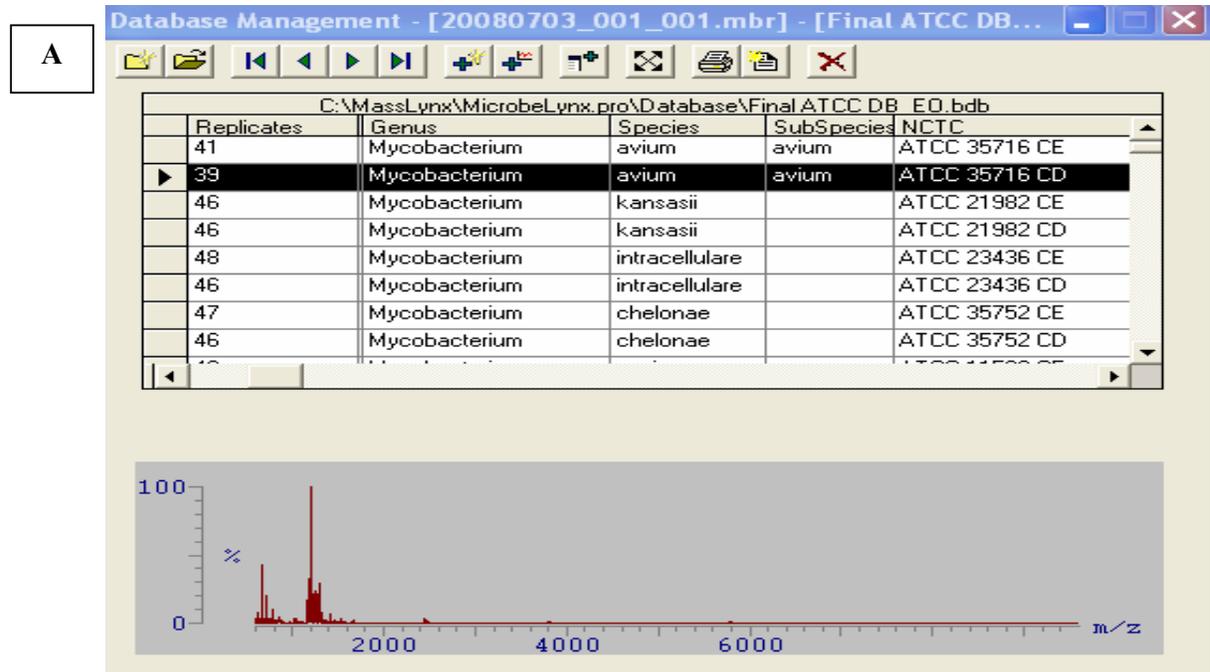
Figure 5.9: Composite spectra of the cell extracts of *M. avium* (A) compared to that of *M. intracellulare* (B)
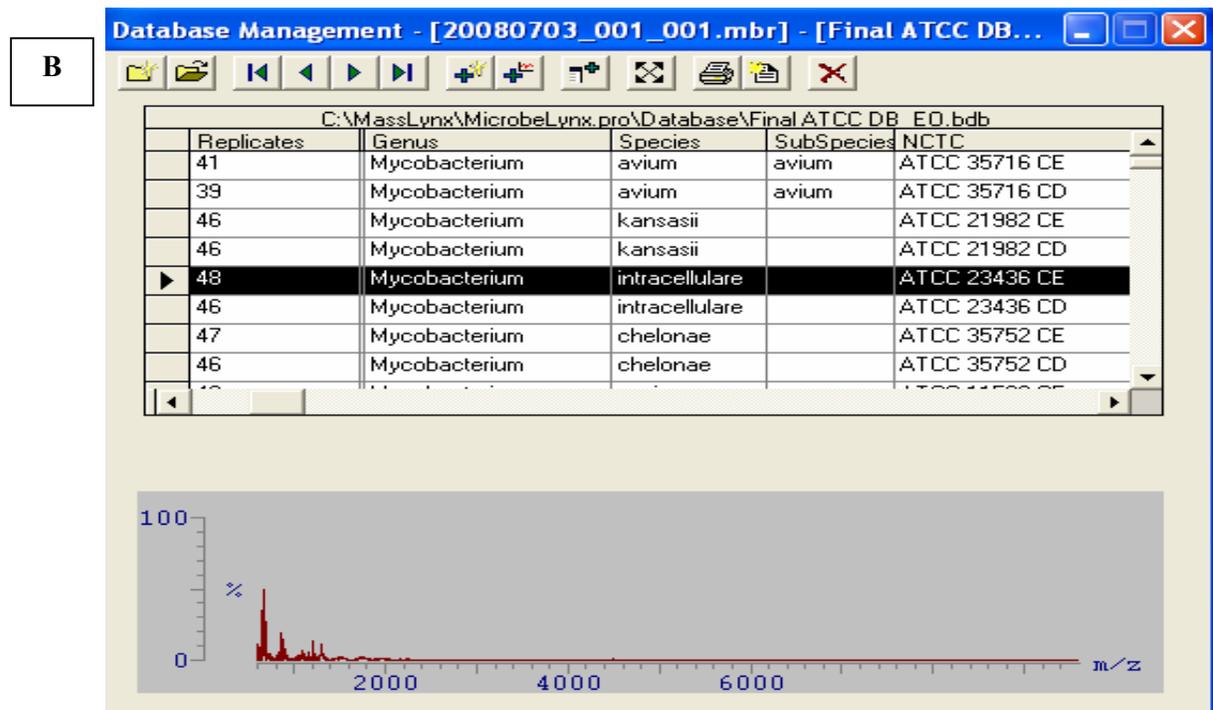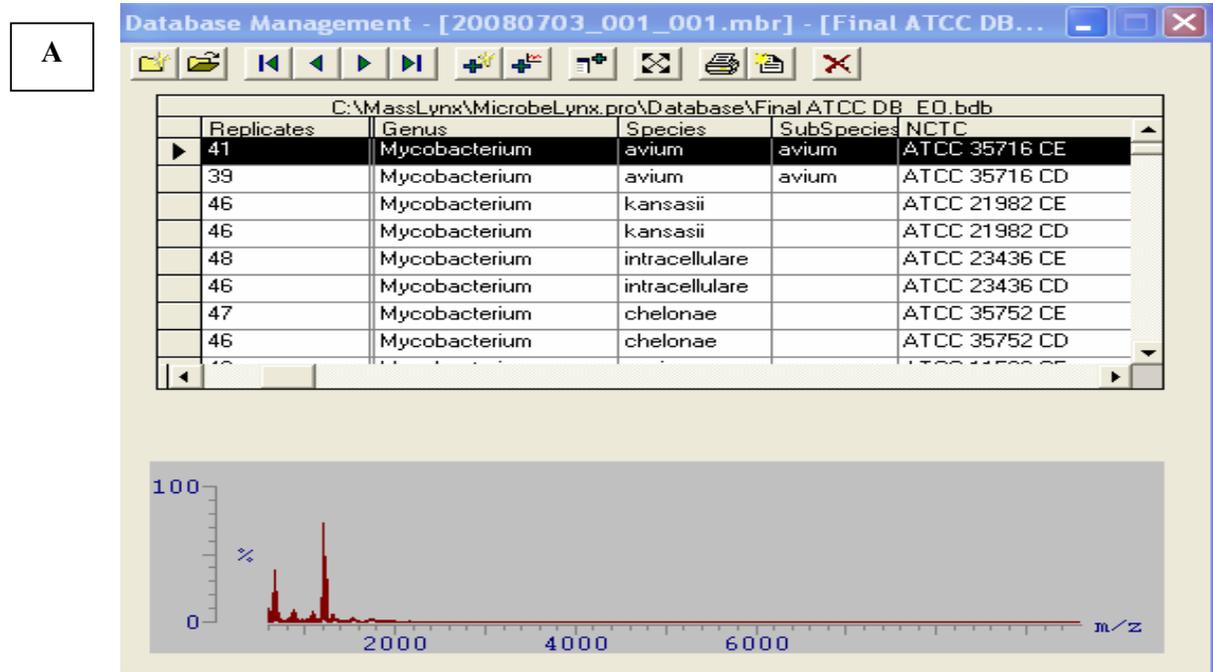
Figure 5.10 Comparative spectra of *M. intracellulare and M. avium* (cell deposit) using MicrobeLynx^{TM} software
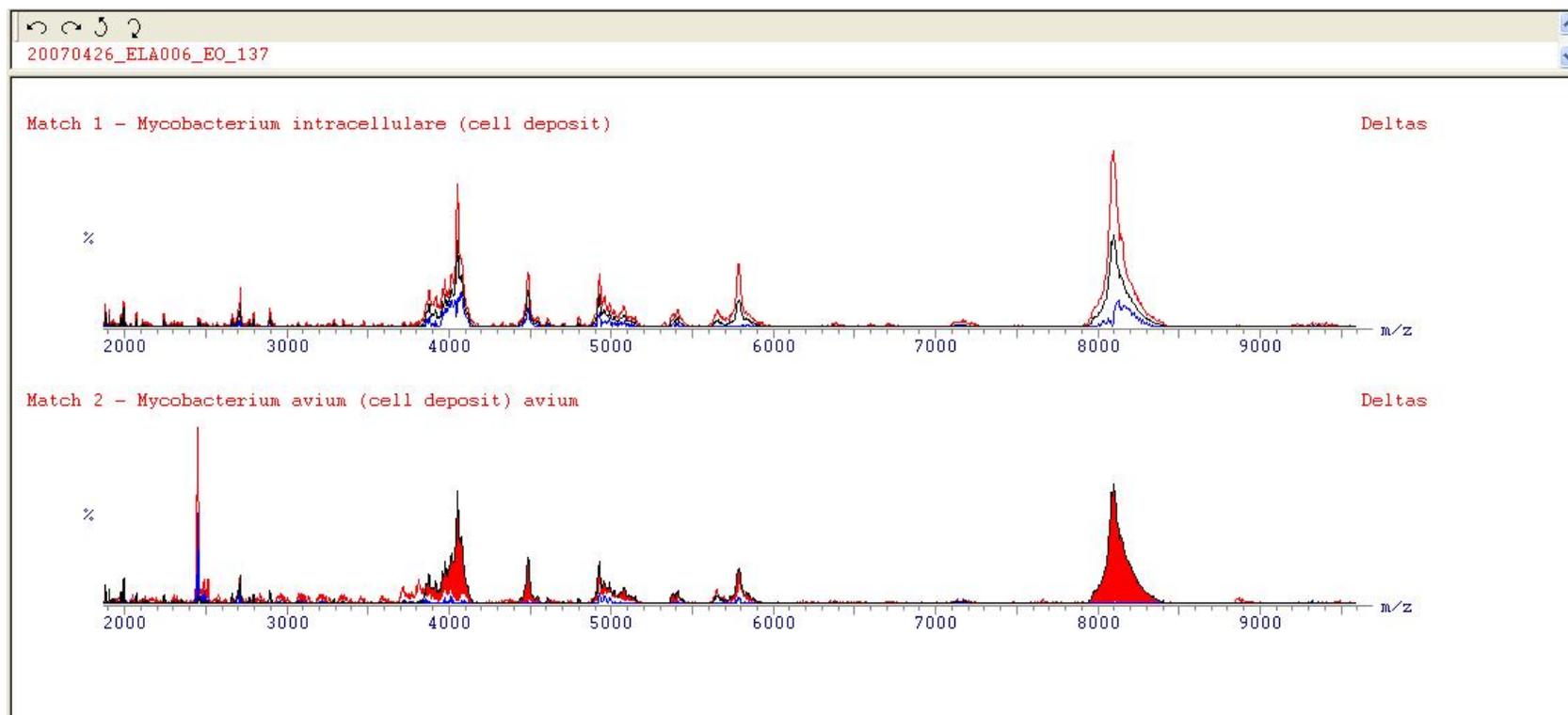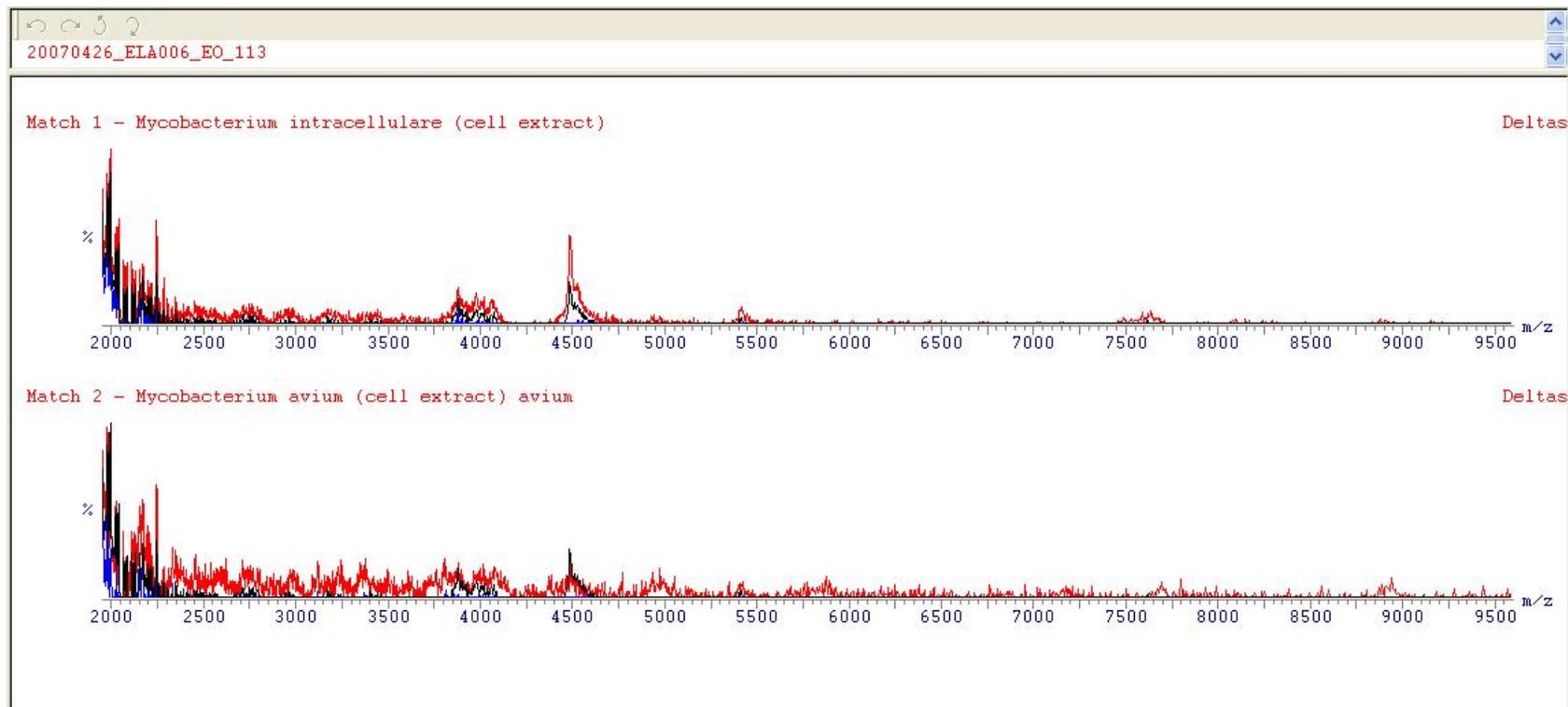
Figure 5.11 Comparative spectra of *M. intracellulare and M. avium* (cell extract) using MicrobeLynx™ software

5.6   Comparison of Spectra of Representative Non-Mycobacteria Test Organisms:

As mentioned in the preceding chapter, 3 other organisms that are not members of the genus *Mycobacterium* but share their acid fast characteristics were included in the database generated. The aim of including such species in this study was to demonstrate the ability of MALDI-ToF mass spectrometry (as applied in this study) to distinguish between phenotypically similar genera. Representative results are shown in Figures 5.12: we compared the whole spectrum of a representative Mycobacterium test organism, namely *Mycobacterium tuberculosis* (ATCC$^®$ 35804) with that of a representative non-Mycobacteria acid fast organism *Rhodococcus equi* (ATCC$^®$ 10146).

As expected, the two spectra were similar at the lower mass range (figure 5.12a). This is due to their common cell wall components which confer on them the acid fast characteristics. However, the difference between the spectra of these organisms is very obvious in the higher mass range (figure 5.12 b & c). This difference is made clearer by the inclusion of the MicrobeLynx^{TM} generated comparative spectra presented in figure 5.12 (d). It should be noted that the spectra of the test organism shown here is that generated using the cell deposit as source material. For reasons explained in the previous chapter, *Rhodococcus equi* does not require a separate inactivation step. Neither did we consider conducting an "inactivation" step using this organism in order to compare the spectra generated using the non-inactivated versus the inactivated cell deposit or cell extract data.

Figure 5.12   **Full Spectrum of the cell deposit of *Mycobacterium tuberculosis* ATCC® 35804**

20070419_ELA006_EO_037
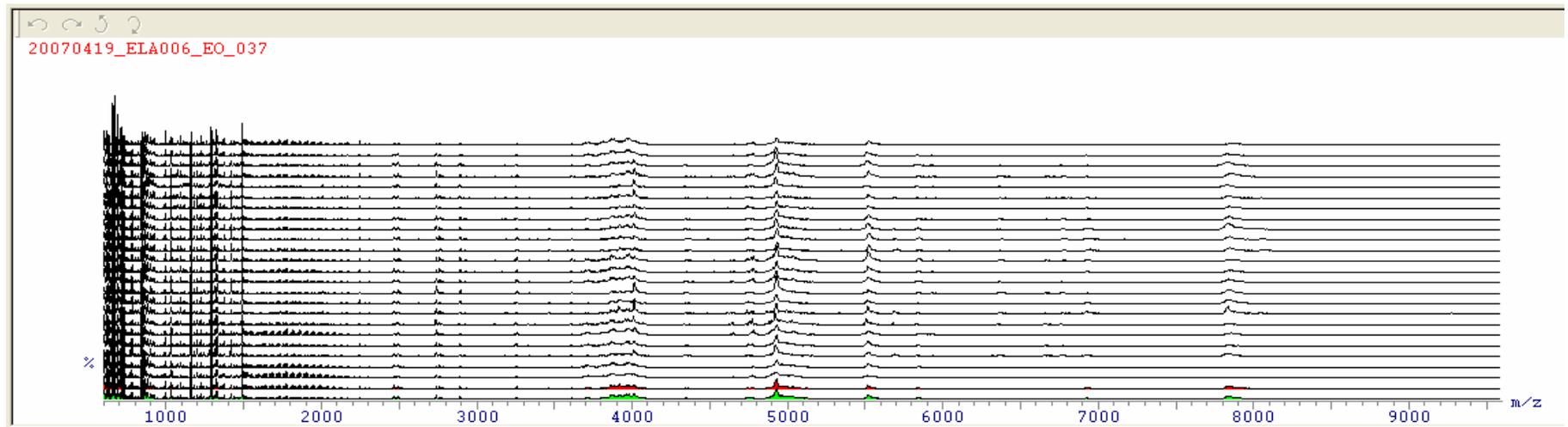
Full Spectrum of the cell deposit of a representative AFB non-Mycobacteria organism, *Rhodococcus equi* ATCC® 10146

20070706_ELA006_EO_037

Figure 5.12(a) Lower mass range: 600- 2000 *m/z* spectrum of the cell deposit of *Mycobacterium tuberculosis* ATCC® 35804

Lower mass range: 600- 2000 *m/z* spectrum of the cell deposit of *Rhodococcus equi* ATCC® 10146

Figure 5.12(b) Intermediate mass range: 2000 - 6000 *m/z* spectrum of the cell deposit of *Mycobacterium tuberculosis* ATCC® 35804

Intermediate mass range: 2000 - 6000 *m/z* spectrum of the cell deposit of *Rhodococcus equi* ATCC® 10146
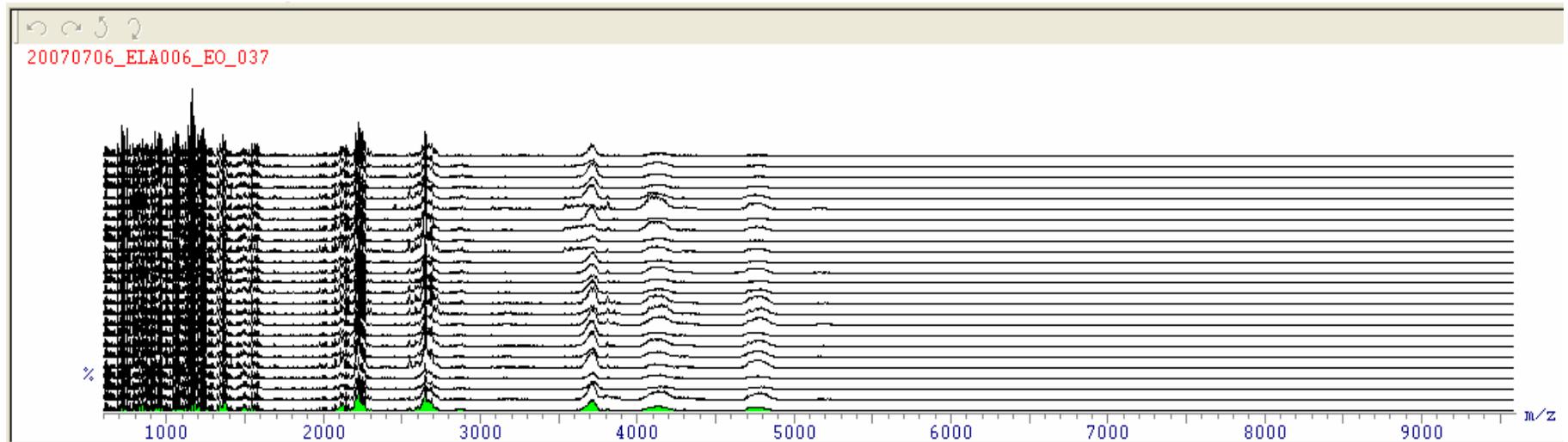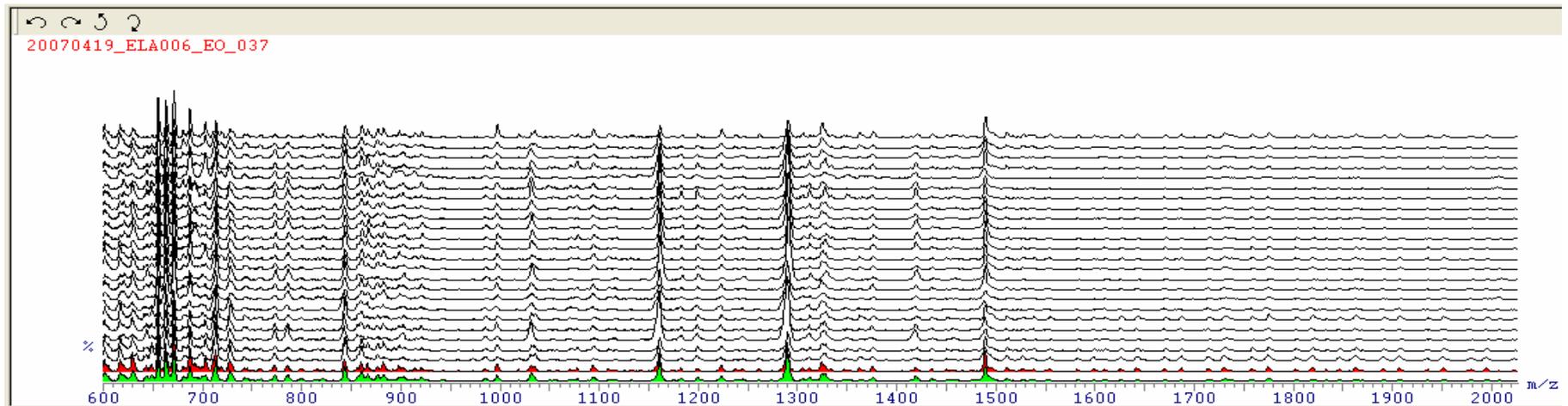

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Figure 5.12(c) Higher mass range: 6000 - 9500 *m/z* spectrum of the cell deposit of *Mycobacterium tuberculosis* ATCC[®] 35804

Higher mass range: 6000 - 9500 *m/z* spectrum of the cell deposit of *Rhodococcus equi* ATCC[®] 10146
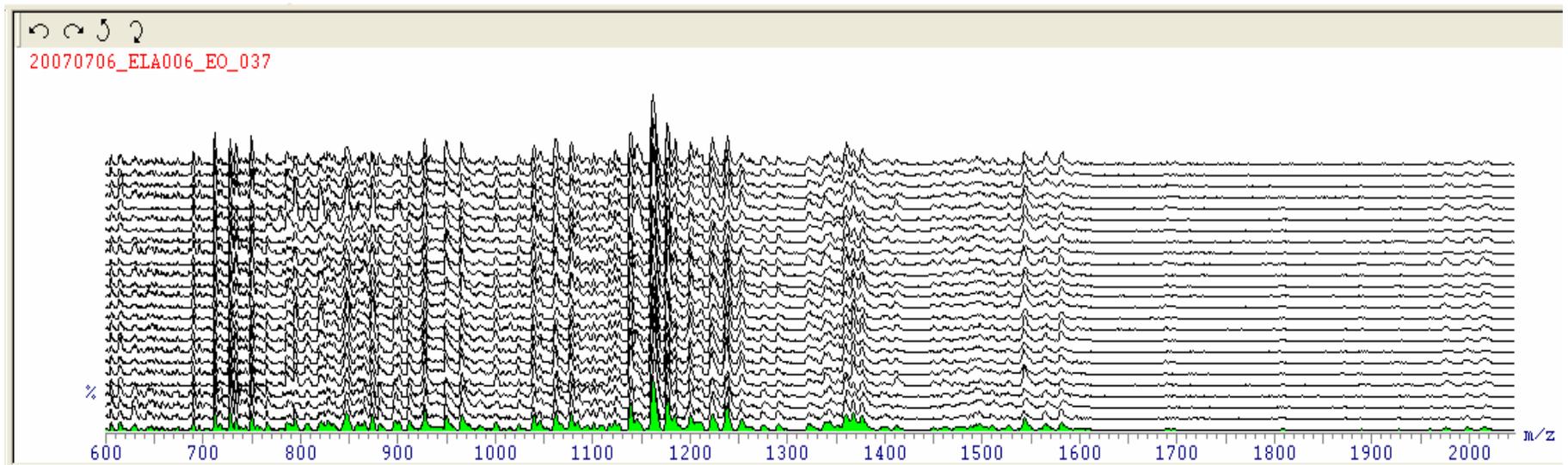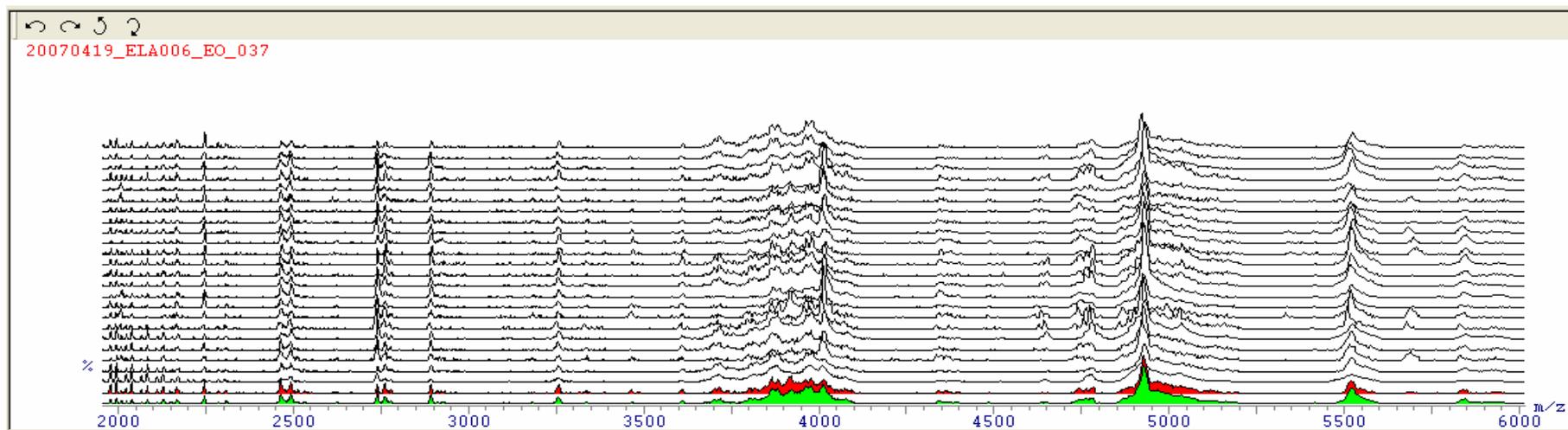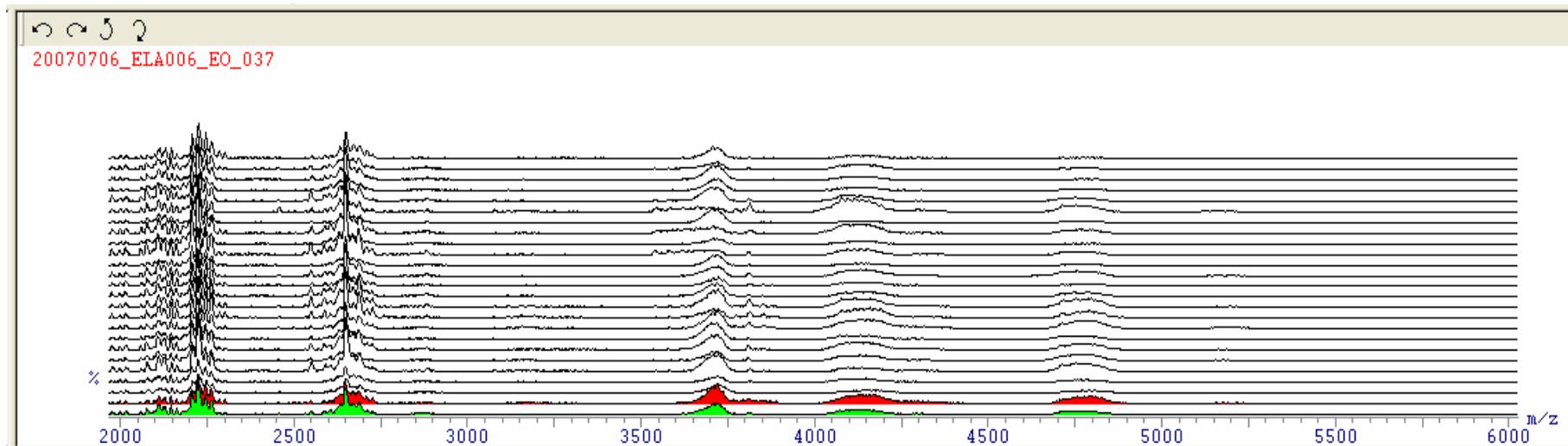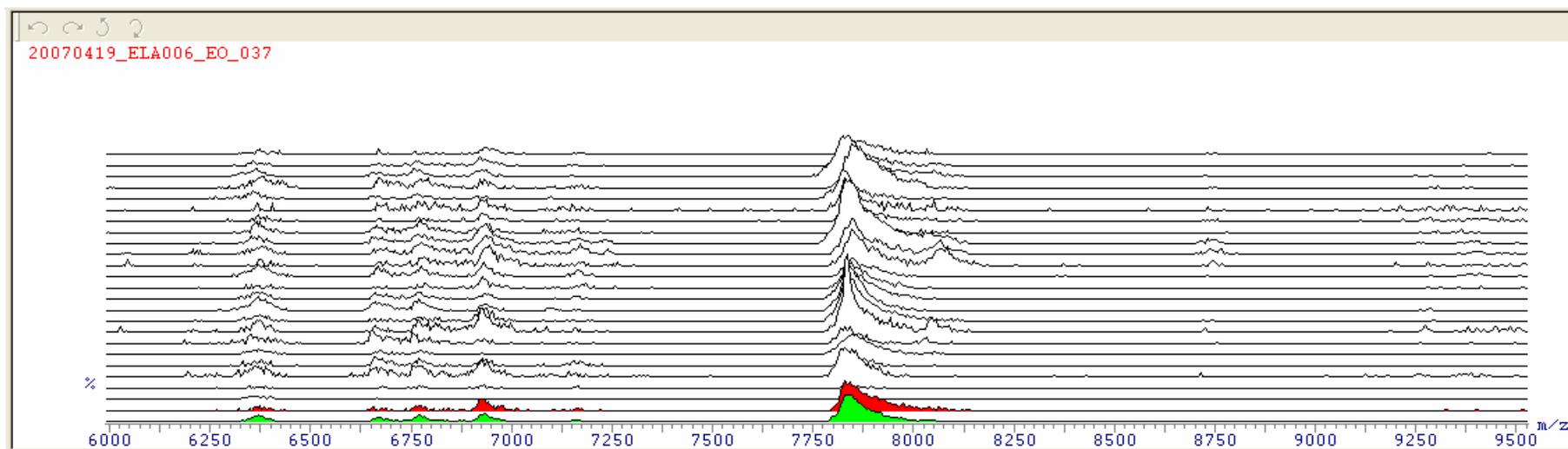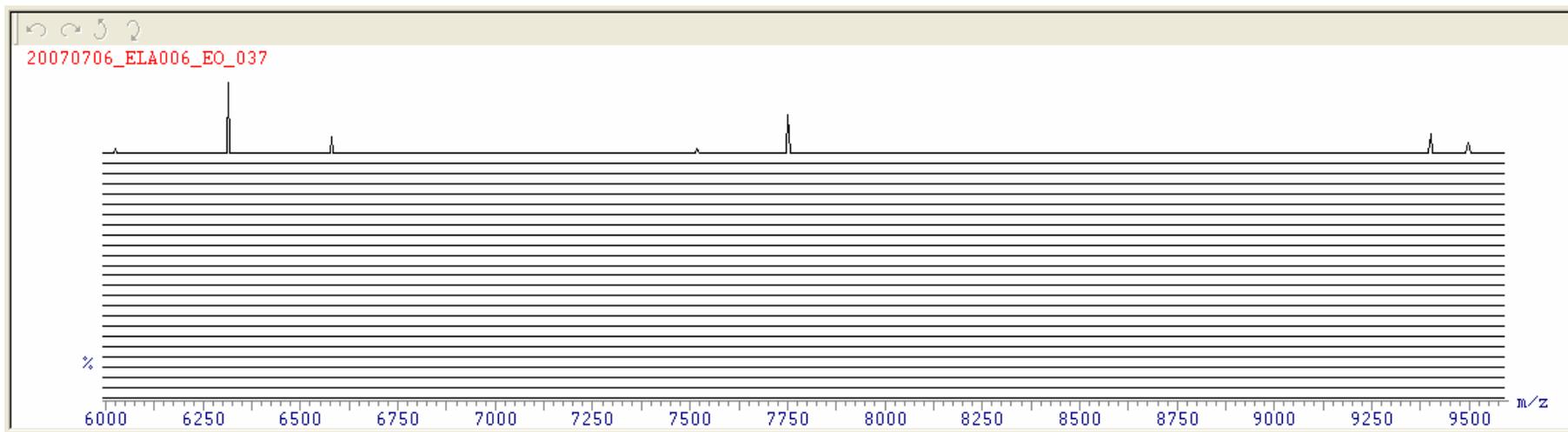
Figure 5.12(d) Comparative spectra of *Rhodococcus equi* and *Mycobacterium tuberculosis* using MicrobeLynx^{TM} software
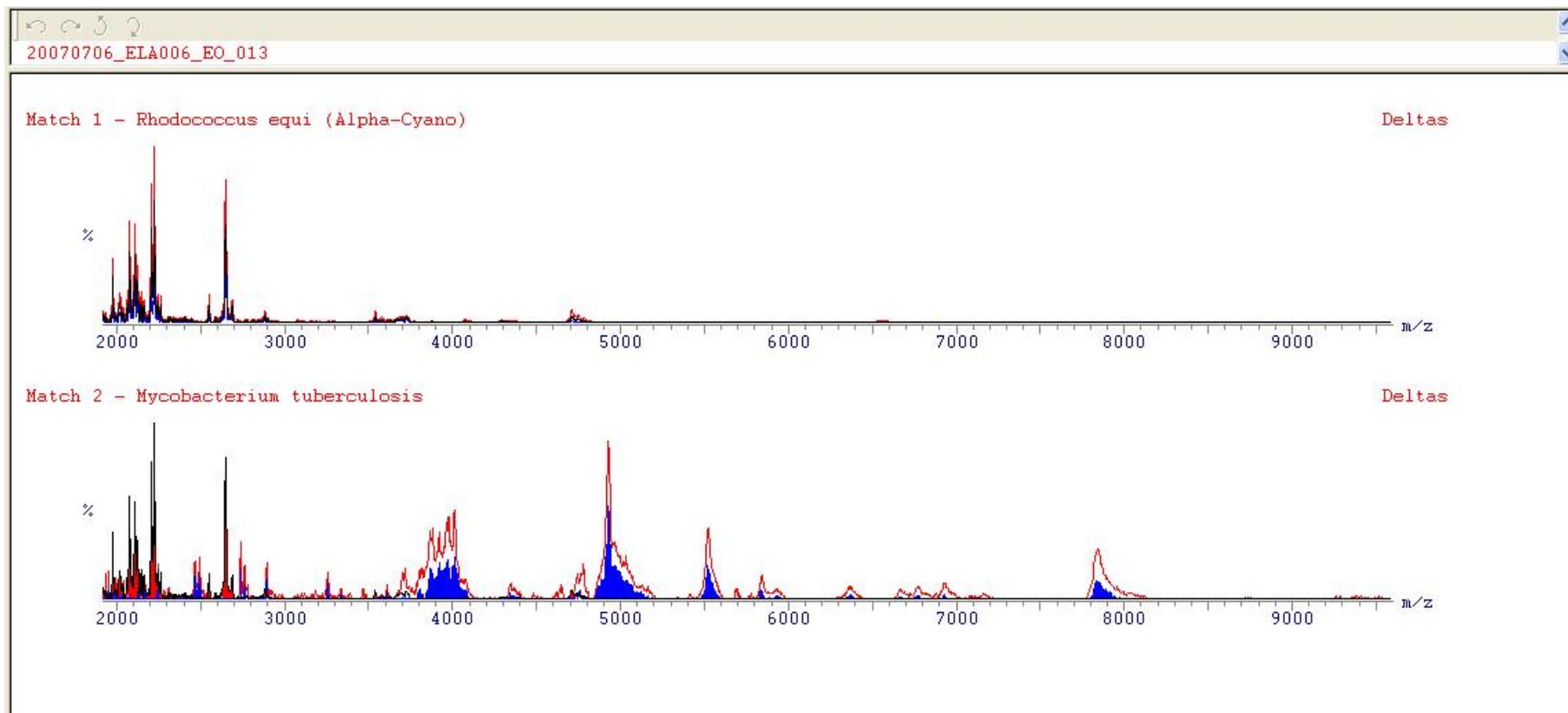
5.7   Comparison of Spectra of Representative Test Organism Cultured with and without PANTA.

In the routine culture of clinical specimen for *Mycobacterium* using MGIT culture medium, 0.8ml of a combination of two additives is usually included in each inoculated MGIT tube as stated in the preceding chapter. These are: OADC and PANTA: OADC is a mixture of Oleic acid, Albumin, Dextrose and Catalase, this is a growth enhancer for *Mycobacterium* species. PANTA on the other hand, is an antimicrobial cocktail included to eliminate or suppress the growth of other bacteria that may be present in the specimen. It is made up of Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin.

As previously explained, PANTA was omitted in the recovery and culture propagation of the ATCC[®] repository strains. This was to avoid jeopardizing the recovery of viable organisms from the lyophilized state and to avoid putting the organism under undefined stress, thereby possibly forcing them to express proteins that they may not normally express. This may influence their mass spectral patterns and so it was decided to culture all organisms included in the database without the mixture of antimicrobial compounds. However, in the course of the compilation of the database, the spectra of some of the organisms cultured in MGIT tubes in the presence and absence of the PANTA mixture were considered and compared as a matter of interest. Since the following phase of the study is to clinically validate the database and normally, clinical samples are cultured in the presence of PANTA, we felt that these experiments would serve as groundwork for the next stage of research and that the results would be an indication of what to expect. An example of such spectra generated for the representative test organism *M. tuberculosis* (ATCC[®] 35804) is presented in figure 5.13.

When one considers the full spectra of the organism grown with and without PANTA, our original thoughts that PATNA could indeed induce differences was suggested when one compared the full spectra: in figure 5.13 below, it seems that the organism grown in the absence of PANTA had a more diverse source of ions when compared to the same organism grown with PANTA. However, as shown in Figure 5.14, this is not confirmed in the comparative spectra. Hence, our original thoughts that PANTA may lead to the suppression of several surface proteins could not be confirmed since the current data is limited to a single organism. This requires further experimentation and will need to be addressed before the clinical validation of the current database. This has important implications since the clinical samples are all cultured in the presence of this antimicrobial mixture.

Figure 5.13 Full Spectrum of the cell deposit of *Mycobacterium tuberculosis* ATCC 35804 cultured without PANTA
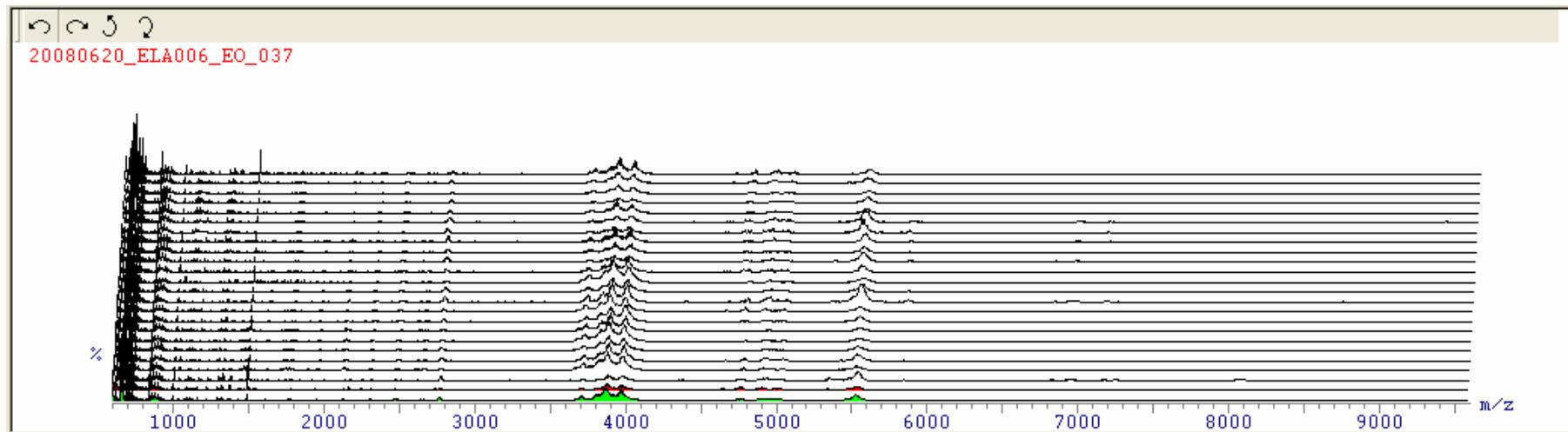
Full Spectrum of the cell deposit of *Mycobacterium tuberculosis* ATCC 35804 cultured with PANTA:

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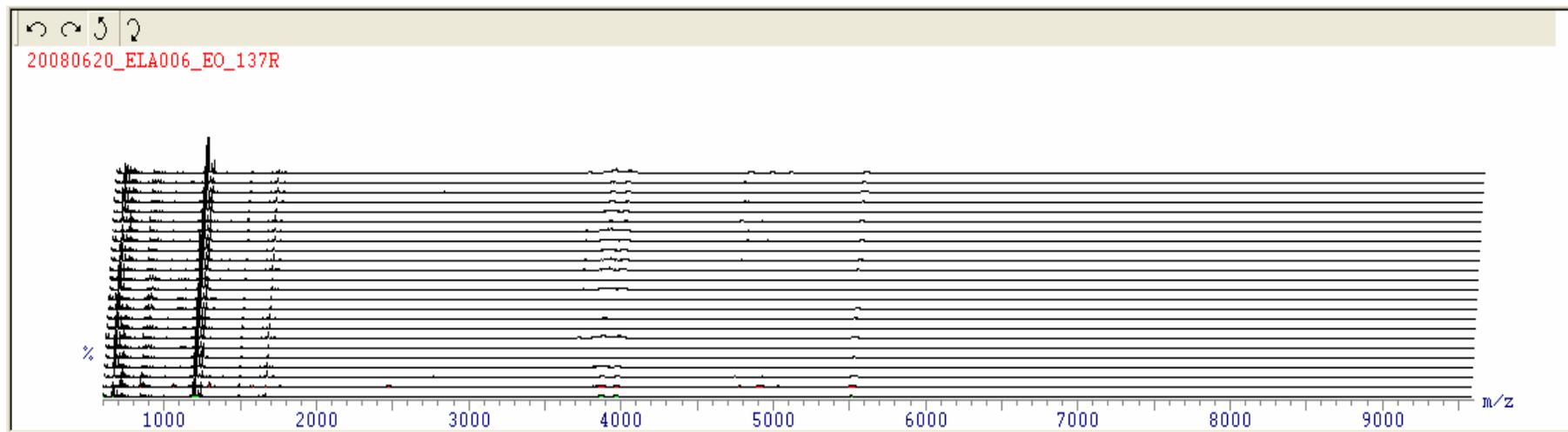
Figure 5.14 Comparative spectra of *Mycobacterium tuberculosis* cultured with and without PANTA using MicrobeLynx[TM] software
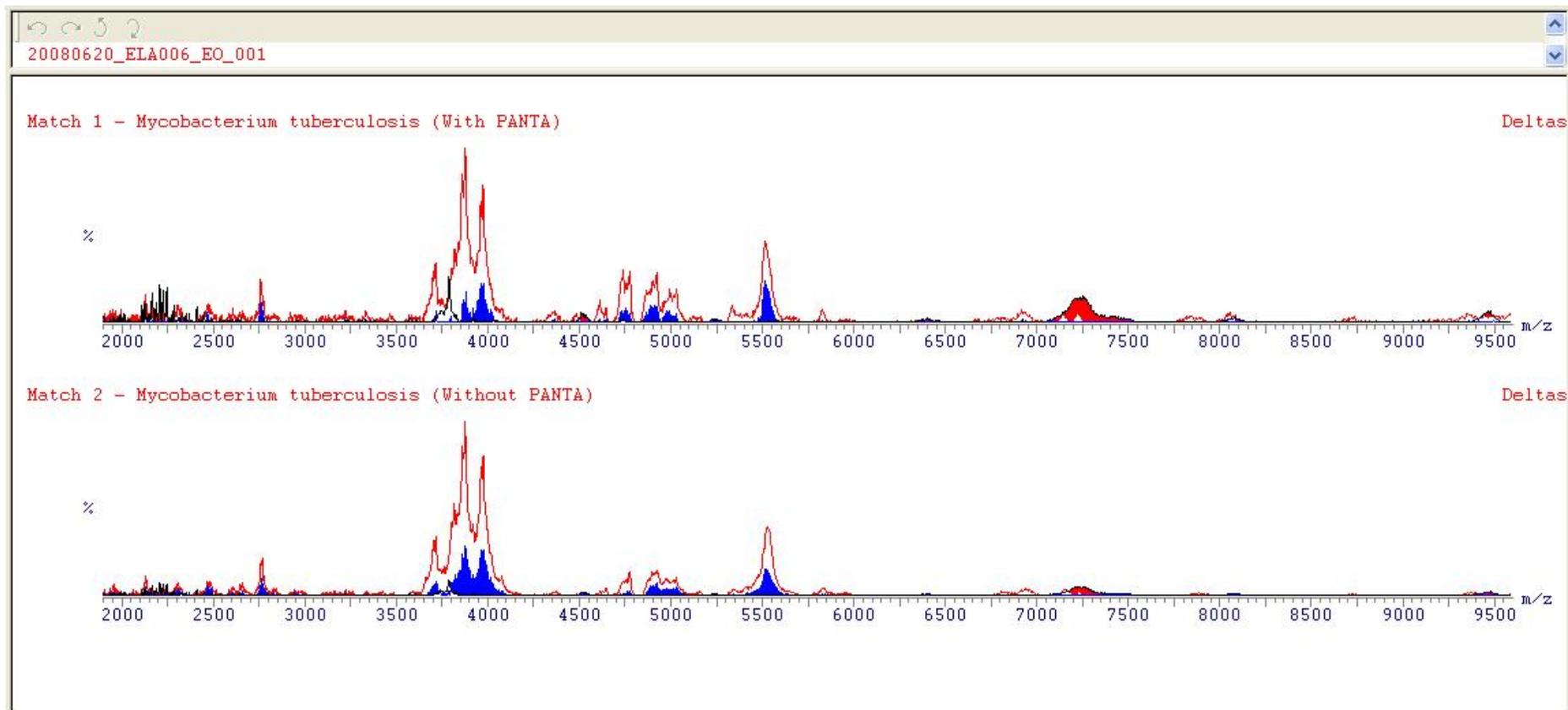
5.8 **Generation of the Mycobacterium Database:**

In order for the data acquired for a test organism on a target plate to be considered for entry into the database, at least one of the two quality assurance organisms included with it on the target plate had to be recognized down to strain level when searched against the MMU's 2005 database containing 4,123 spectral entries covering over 500 different species of bacteria. The spectra of the test organisms that met this criterion were subjected to some automated processes which included background (noise) subtraction and root mean square (RMS) calculations. They were also verified using the Savitzky Golay algorithm of the MicrobeLynx^{TM} application, a MassLynx^{TM} software 'add on' as explained earlier.

As examples, Figure 5.15 shows a well processed target plate having been interrogated using the "Search_ None" parameter file. This is the parameter file used to make an entry into a database. Figure 5.16 is a screen capture of part of the database generated in this study, with the entry of the representative test organism highlighted. Below the database is the organism's composite spectrum. Figures 5.17 and 5.18 show the result of a search of the database generated in this study. The representative test organism is used as an example. Figure 5.17 shows the result for the cell extract while figure 5.18 shows that of the cell deposit.

Figure 5.15   Typical example of a "Search_None" database result

Figure 5.16   Screen capture of part of the generated database
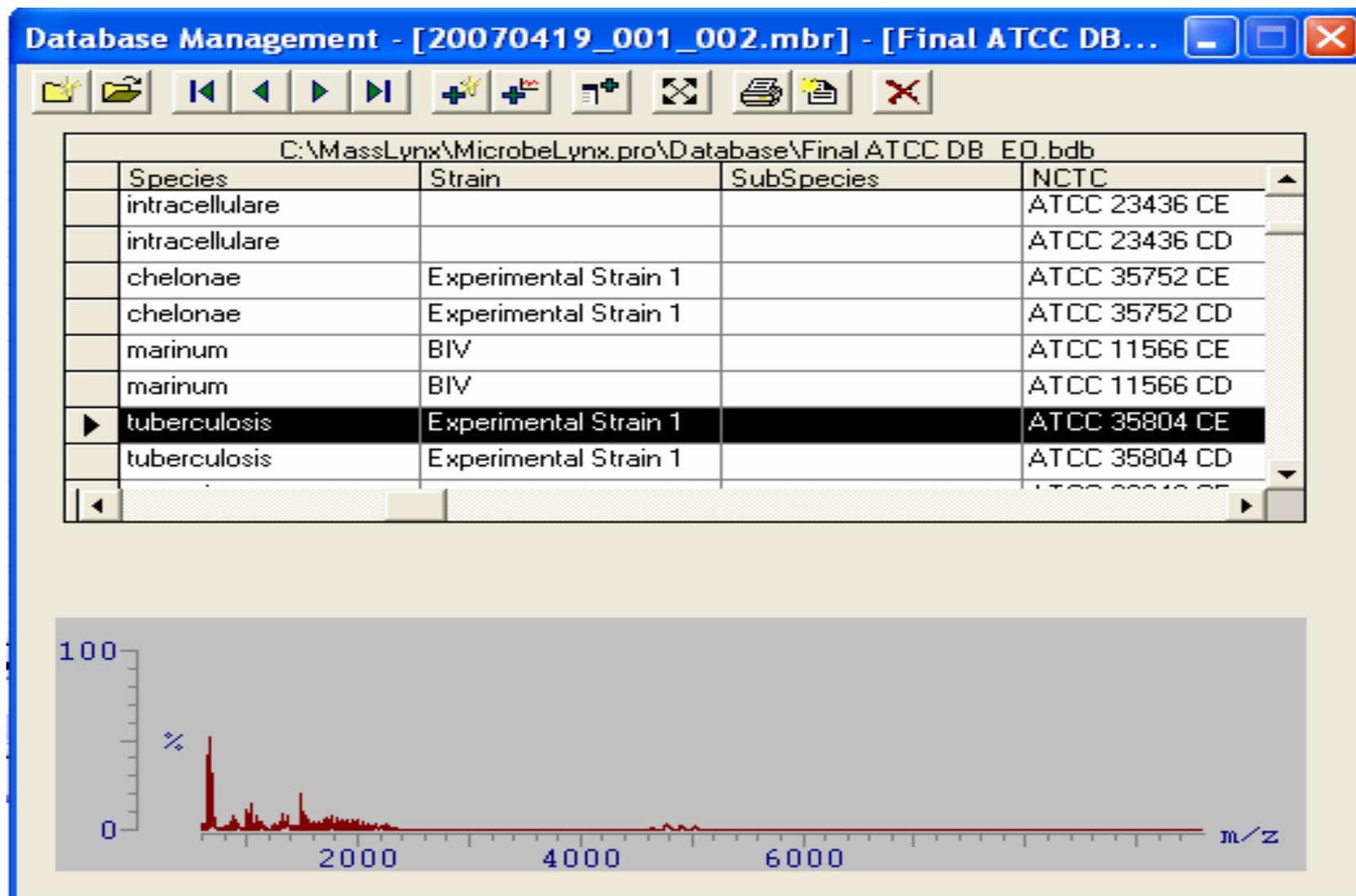
Figure 5.17

Result of a typical database generated search of the representative test organism: cell extract of *Mycobacterium tuberculosis* ATCC[®] 35804
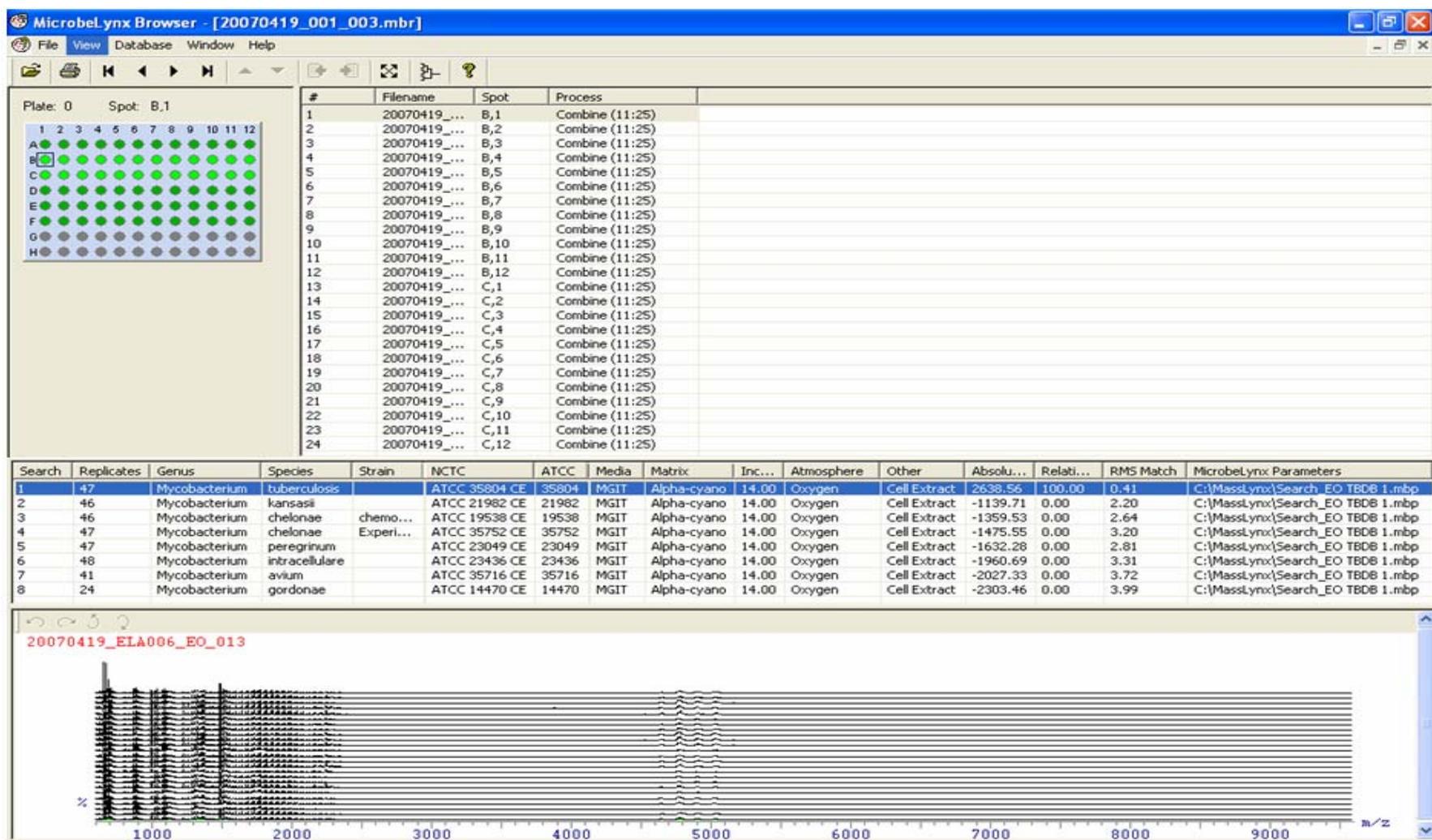
Figure 5.18

Result of a typical database generated search of the representative test organism: cell deposit of *Mycobacterium tuberculosis* ATCC[®]35804
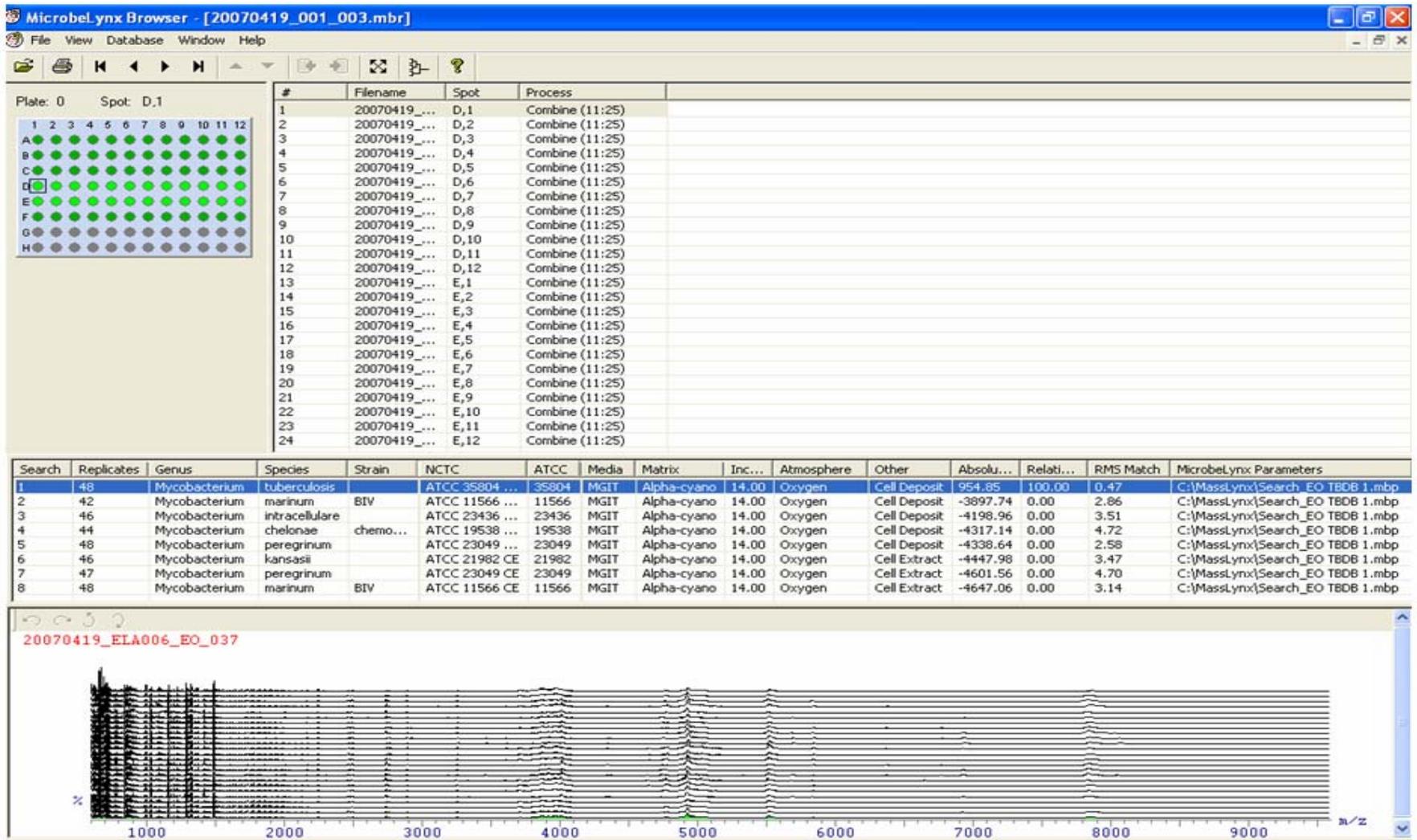
CHAPTER SIX

DISCUSSION

Introduction

The menace of tuberculosis, particularly in the immunocompromised patient, and generally to the larger society cannot be overemphasized. Emergence of multi drug resistant (MDR) and extensive drug resistant (XDR) strains of its etiologic agent, *Mycobacterium tuberculosis* in recent years has aggravated the already bad situation. Drug toxicity and treatment non-compliance sometimes leads to the emergence and proliferation of drug resistant strains in a population that had hither to been susceptible. In addition to this, the general slump in world economy has further lowered the already substandard poverty and literacy level that exists in most part of the world. These are but few of the situations that makes a grim situation gloomier.

Since the last millennia, scientists have put in tremendous efforts to combat the menace of tuberculosis. There had been concerted efforts in the area of research and development of new drugs, and as soon as it became possible, the genome of the type strain of the genus has been extensively explored in the quest to discover genetic information that maybe of value in conquering the causative organism; and consequently the disease. Early diagnosis, which has been established as a major cornerstone in the successful treatment and control of the disease has also had its fair share of scientific attention and research. This has led to enormous progress in the diagnostic area of Mycobacteriology. The last three decades have witnessed the gradual shift from the traditional laborious and time consuming solid media culture process to the much more rapid, less labor intensive semi-automated and fully automated liquid culture systems. To compliment this, a myriad of molecular techniques, most of them PCR (or related techniques) based have been developed.

Some of these techniques are commercially available as simplified, user friendly kits, while others requires specialized equipment that must be operated by trained personnel.

The MALDI-ToF technology is one of the areas being explored in the quest to find a rapid, sensitive and specific way of diagnosing tuberculosis. The identification and differentiation of members of the MTB complex from the non-tuberculous mycobacteria (NTM), many of which are emerging and potentially fatal pathogens of the immunocompromised host is another area being explored for the application of this technology.

The aim of this study (which is the first phase of a larger study) was to establish a database of mass spectra patterns of some selected ATCC® repository strains of *Mycobacterium*. Drawing from the relatively scanty knowledge available in literature, crucial experimental factors were determined, standardized and optimized. This paved the way for optimum spectrum generation, acquisition and processing. The result of this was that a database of mass spectra of the selected ATCC® strains was generated and validated using the same defined strains. The next phase of this study is to further explore the application of this technology in diagnostic Mycobacteriology by applying the database generated in the identification of clinical isolates of *Mycobacterium species*.

Our Findings:

6.1 What is new?

The virulence and infectivity of some members of the genus *Mycobacterium* requires that they are handled in a BSL3 facility. For safety purposes, all the strains of *Mycobacterium* used in this study were cultured and handled in the BSL3 facility. They were inactivated there before removal to the analytical laboratory for mass spectrum analysis. Two inactivating solutions were tested and found

to be effective as killing agents for all the 26 Mycobacterium strains included in this study. However, as the results show, solution A (a 50:50 ACN/H$_2$O mixture containing 4% TFA) allowed more clusters of ions (peaks) to be detected from the test organism (laboratory strain of *Mycobacterium smegmatis*) than solution B. Since the presence or absence of peaks is the major identification criteria used in whole cell MALDI-ToF bacterial analysis, it is a reasonable assumption that the more peaks detected, the greater the chances of correct identification and differentiation of organisms from each other. The fact that solution A allows more ions in the higher mass range (which are considered the more important ions which differentiate species) to be detected, especially from the cell deposit as shown in figures 5.1(a-c) further justifies its use as the inactivating solution of choice. Also, the better resolution of peaks provided by solution A as made more obvious in the comparative spectra generated by the MicrobeLynx^{TM} software (figure 5.2 (d), especially the last two peaks) further strengthens the confidence that the mass spectrum patterns included in the database was optimally generated. As far as we are aware from the literature, no other study has compared these two inactivating solutions, explored or explained their relevance and preference for mass spectrometry of *Mycobacteria*. Also, the very high numbers of correct identification of different test strains when used to challenge the database generated lends credence to the fact that the most advantageous inactivating solution of the two was chosen.

Of the numerous experimental factors that have been shown to influence the quality and reproducibility of mass spectra obtained when bacteria are analysed by MALDI-ToF mass spectrometry, the choice of matrix is of utmost importance (95). Various matrices have been tested and used with different analytes; of these, three are most commonly used by investigators for bacterial analysis. They are: alpha-cyano-4-hydroxy-cinnamic acid (α-Cyano or CHCA), 5-chloro-2-mercaptobenzothiazole (CMBT), and Sinapinic acid. Most of the investigators who had worked

on Maldi analysis of Mycobacteria have used α-Cyano (98, 107, & 108). In this study, two matrices were compared and one selected for use with the test organisms. Once again, to the best of our knowledge, no other study has compared both α-Cyano and CMBT as matrix for MALDI-ToF analysis of Mycobacteria. Our results confirm what other investigators have published in that α-Cyano is the preferred matrix to use in the MALDI-ToF analysis of Mycobacteria: figures 5.2 (a-d) shows this pictorially.

6.2   What contrasts?

As recorded by Hettick and his colleagues (98), no consensus exists amongst the various investigators as to the preferred material for use for the MALDI-ToF mass spectrometry: whether the whole cell deposit or the organic solvent cellular extract generates the most reproducible spectra remains a debatable issue. In this study, both were explored, compared and included in the database: the result of the pilot project shows that cell deposit had more ions at the higher mass range than the cell extract. This was found to be true of all 26 species of Mycobacterium included in this study. As explained in the previous chapters, the differentiating potentials of the higher mass range ions (ions with $>2000m/z$) makes the cell deposit more desirable than the cell extract for this phase of the study. These findings contrast markedly with the findings of Hettick and his group, who documented that "the analysis of cell extract produces data similar to the analysis of whole cells", and therefore used only cell extracts in their investigations.   Although a direct comparison of these two studies is not feasible due to differences in methodology, instrument and especially the number of species utilized, our current study has demonstrated consistently the advantageous difference of the cell deposit over the cell extract for most of the species of *Mycobacteria* tested. The data represented in Figures 5.3(a, c & d) demonstrates this fact when the laboratory strain of *Mycobacteria smegmatis* was used as test organisms. Furthermore, the glaring difference between

figures 5.6 and 5.7 corroborates this observation further. Also, our consistent observation that for closely related strains, a search of the database might put the second strain before the test strain for cell extract, but **not** for cell deposit (as shown in table5.1) further gives credence to this finding. For instance, Figures 5.10 and 5.11 explains this from another perspective: the differentiation of *M. avium* and *M. intracellulare* (members of the MAC complex) might be difficult if the spectrum of the cell deposit (figure 5.10) was not included. It must be noted that an attempt to identify Mycobacteria test organisms used in this study only by their cell extracts has not been carried out. However, as most search results shows, the identification and differentiation of *Mycobacterium* species only by their cell extract is a feasible possibility.

Attempts were made to obtain more ions with higher mass from the cell extract. One was by increasing and decreasing the polarity of the extracting solution (by varying the water content of the solution) or by increasing the temperature at which the extraction was conducted. In both cases, the resulting spectra, when compared to that obtained using the original parameter/condition, had negligible effects on the generation of the spectra for the strain used. We did not explore these ideas any further and the data generated were not detailed nor used in this thesis.

We conclude from this interesting and stimulating study that a unique database of mass spectra patterns of 26 repository strains of *Mycobacteria* representing 22 species has been generated. Included also are spectra of organisms representing 3 different genera that are phenotypically different but share the same acid-fast characteristics of the genus *Mycobacterium*. In addition, several 'control' spectra were included in the database and these are:

1. the spectra of un-inoculated MGIT medium with the α-Cyano matrix;
2. the mixture of the inactivating solution and α-Cyano matrix;

3. And, the spectra of the two quality assurance organisms used, namely the *Vibrio parahaemolyticus* (NCTC® 10903) and the *Micrococcus lylae* (NCTC® 11037) with their appropriate matrices. These organisms served as our internal QA step as previously explained.

The conditions under which the experiments were carried out insured that the spectra included in the database (though yet to be externally tested and clinically applied) are of good quality and are reproducible. The database has been challenged in-house using different test organisms with complete entries into the database as unknowns, and the results were excellent: the database differentiates easily between members of the MTB complex and non-tuberculous mycobacteria, and also between members of the same complex. It also differentiates very well between strains of the same species (especially with the cell deposit). The database generated is therefore recommended for testing in the next phase of the study, namely the clinical validation and application.

Future prospects

The database generated as it currently stands has the ability to recognize and differentiate between 22 species of Mycobacteria. This is more than the numbers of PCR primers currently available for the routine detection and identification of *Mycobacterium* species in the clinical laboratory. Although it is theoretically possible that the same range of species can be identified using a multiplex PCR technique, it is not very practical nor is it routinely done. The implication of this is that if the developed protocol is followed, the database generated can identify and differentiate (under experimental, not diagnostic conditions) more species than PCR and related techniques. It is therefore a realistic expectation that when the database is clinically validated and tested, it will contribute immensely to the diagnosis of tuberculosis and other mycobacterioses. It will also aid in the identification of emerging pathogens particularly amongst the non-tuberculous mycobacteria.

On-going work will determine the real value of the database: we intend to continue adding new spectra of clinical isolates of Mycobacterium to the database, especially those of multi-drug resistant strains (MDR's) and hopefully those of extreme drug resistant strains (XDR's). At present, the definition of such pathogenic strains relies on whether they are sensitive *in vitro* to the antibiotics used therapeutically. This therefore requires the primary culturing of the clinical sample followed by the sensitivity testing. We believe that a once-off identification step using the MALDI-ToF database will supersede these laborious methods and bring faster and more efficient diagnosis and therapy to the patient.

Ultimately, our clinical validation phase will determine whether the culture stage can be by-passed and *Mycobacterium* species identified directly from clinical specimens. This would be the ultimate success of the study: we would have developed a rapid, sensitive and specific way of diagnosing tuberculosis using MALDI-ToF MS technology.

Executive summary

It is a well established fact that the optimization and control of experimental variable will lead to a stable protocol for the MALDI-ToF analysis of bacteria. It has also been established that by following a clearly established protocol, reproducible spectra can be obtained (96-99). These profound statements were the banner under which this study was carried out: utmost care was employed to ensure compliance to the protocol developed, from the reconstitution and recovery of both test and quality assurance organisms, through their culture and inactivation (as applicable) and finally to the MALDI-ToF mass spectrometry data acquisition and analysis. Each step was checked and internally quality assured as described in the previous chapters. Any culture reagent, media or

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MALDI-ToF target plate over which any doubt existed was discarded, replaced or repeated as applicable. We would therefore propose the following document (appendix A) as a Standard Operating Procedure (SOP) to be followed by anyone who wants to develop a database for the MALDI-ToF identification of Mycobacterium. It remains the property of the authors and Synexa Life Sciences. Until its clinical validation of the database generated by the current study, the database must be considered as an experimental tool for the identification of the strains used in its generation.

APPENDIX A

Standard Operating Procedure:

Experimental Protocol for Preparation, Culture and Inactivation of Mycobacterial Strains for

MALDI-ToF Analysis:

1. From the lyophilized state, resuspend the entire cell pellets using 0.5ml of sterile Middlebrook 7H9 (M7H9) broth and inoculate into freshly prepared MGIT (with OADC but without PANTA) and 10ml of Middlebrook 7H9 broth in tissue culture flasks ('primary cultures'). Incubate in an ambient incubator at 37^OC for 7 days.

2. After 7 days, use 1ml of the above primary M7H9 broth culture to inoculate another set of fresh 10ml M7H9 broth to make the "first generation" culture.

3. Use approximately 1ml of the "first generation" culture to inoculate fresh MGIT prepared in the above manner.

4. Incubate in BACTEC^{TM} MGIT^{TM} 960 machine for 14 days.

5. After 14 days, vortex tubes lightly to resuspend organisms that may be growing on the sides of the tubes or may be 'clumped up' at the bottom of the tube.

6. Pour into sterile, screw-capped universal centrifuge tubes and rinse out the MGIT tubes used for the culture with approximately 7ml of sterile phosphate buffered solution (PBS).

7. Centrifuge at 3000rpm for 20 minutes.

8. Carefully decant and transfer cells to clean, safety-lock Eppendorf® tubes.

9. MiniSpin Eppendorf ® centrifuge at 1,300rpm for 5 minutes to further pack the cells and to remove excess fluid.

10. Add 1ml of inactivating solution (50:50 ACN: Water + 4% TFA) and vortex for approximately 2 minutes.

11. Centrifuge in MiniSpin Eppendorf ® centrifuge at 1,300rpm for 15 minutes.

12. Aspirate cell extracts into appropriately labeled safety-lock Eppendorf® tubes and transfer to the analytical laboratory.

13. Prepare MALDI-ToF target plates and include two QA organisms.

14. Process in the MALDI-ToF instrument.

APPENDIX B

Names and Culture Conditions of Quality Assurance Organisms

Genus	Species	Sub-specie	NCTC	Matrix	Incubation Period	Temperature ºC	Media	Atmosphere
Bacteriodes	*fragilis*		09343	Alpha-Cyano	24 hours	37	CBA	An-aerobic
Pseudomonas	*aeruginosa*		10332	Alpha-Cyano	24 hours	37	CBA	Oxygen
Escherichia	*coli*		09001	Alpha-Cyano	24 hours	37	CLED	Oxygen
Staphylococcus	*aureus*		13134	CMBT	24 hours	37	CBA	Oxygen
Bacillus	*licheniformis*		01097	CMBT	24 hours	37	CBA	Oxygen
Corynebacterium	*jeikieum*		11913	CMBT	24 hours	37	CBA	Oxygen
Vibrio	*Para-haemolyticus*	Kangawa negative	10903	Alpha-Cyano	24 hours	37	CBA	Oxygen
Micrococcus	*lylae*		11037	CMBT	24 hours	37	CBA	Oxygen

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