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Application of High Performance Liquid Chromatography for Identification of *Mycobacterium* spp

Carlos Adam Conte Junior,
César Aquiles Lázaro de la Torre,
Eduardo Eustáquio de Souza Figueiredo,
Walter Lilenbaum and Vânia M. Flosi Paschoalin

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1. Introduction

Since 1896, when Lehmann and Neumann described the bacterium responsible for causing tuberculosis and leprosy, about 150 species of *Mycobacterium* have been described. Except for *Mycobacterium leprae*, which does not grow *in vitro*, those species were classified in two distinct groups: species that belong to the *Mycobacterium tuberculosis* complex (MTC), and non-tuberculous mycobacteria (NTM) [1, 2]

Mycobacterium tuberculosis infects over one-third of the human population worldwide, causing nine million new cases of tuberculosis and two million deaths annually [3]. While members of the MTC cause more disease worldwide than any other bacteria [4], NTM are widespread in nature and, with some significant exceptions, are free-living saprophytes and opportunistic pathogens. Although considered to be non-pathogenic, NTM can pose a threat to humans, mainly in patients with underlying conditions such as AIDS or cancer, and there is an increasing awareness of their public-health importance, especially as nosocomial pathogens [5].

Some strategies employed to identify *Mycobacterium* spp. included observations of staining properties of bacilli, cultural morphology, biochemical tests, and, rarely, the inoculation of susceptible animals with live bacilli for observation of animal pathogenicity [6]. At the beginning of mycobacterial research identification the differentiation of species level

was performed by traditional culture methods based on phenotypic and biochemical characteristics. The principal disadvantage of this method is the time-consuming evaluation. Currently a genotypic evaluation is more preferred by the mycobacterial species [7]. Different species can display distinct antibiotic resistances and require different prescriptions for treatment. For this reason it is important to identify *Mycobacterium* species in a rapidly and accurately way [8, 9]

Complex high-molecular-weight β -hydroxyl fatty acids with a 22-or 24-carbon alkyl chain at the α -position are structural characteristics of mycolic acids (MAs), a type of fatty acid found in the *Mycobacterium* spp. cell wall. Several methods of fatty-acid analysis have indicated that MAs are species-or group-specific [10]. High-performance liquid chromatography (HPLC) analysis of MAs is a reliable method for the detection of mycobacteria, because of the rapid and reproducible nature of the method and because the MAs elution spectrum observed for each mycobacterial species has generally been found to be unique, except for two species (*M. bovis* and *M. tuberculosis*) that share the same spectrum pattern [11, 12]. The HPLC method has been considered a standard test for chemotaxonomic classification and rapid identification of *Mycobacterium* species by the Centers for Disease Control and Prevention (CDC) since 1990, and has been reported to be more than 96% accurate compared with DNA probe tests [6]. Even HPLC is considered one of the most reliable and cost-effective tools for the rapid identification of *Mycobacterium* spp. isolated in culture based on the presence of different MAs [13] and it was well described and standardized [14], the methodology could be affected by several factors and must be optimized in accordance with local laboratory capabilities in order to assure accurate diagnosis.

In this review are presented the procedures to saponification, extraction (chloroform), derivatization (p-bromophenacyl), separation (C18 column and a gradient of methanol and methylene chloride) and detection (ultraviolet spectrophotometer) of MAs. Also is explored the importance of built a pattern chromatogram library for successful identification of clinical samples based on comparison of the relative retention times (RRT) of the chromatogram patterns with those obtained from reference strains and with those available in external databases. HPLC is necessary for separation of MAs due to their large size and complexity that requires the use of different columns and solvents. Initial methods required manual interpretation of chromatograms with eventual development of automated systems.

2. *Mycobacterium* species and mycolic acids

The analysis of lipid fractions has contributed significantly to the knowledge of *Mycobacterium* species. The abundance of lipid constituents in mycobacterial cell walls made them classic candidates for early chemical investigations [6, 15]. MAs, 2-alkyl, 3-hydroxy long-chain fatty acids, are the hallmark of the cell envelope of *Mycobacterium tuberculosis* (Figure 1). They are found either unbound, extractable with organic solvents (esters of trehalose or glycerol) or esterifying the terminal pentaarabinofuranosyl units of arabinogalactan, the polysaccharide that, together with peptidoglycan, forms the insoluble cell wall skeleton [16]. Both forms

presumably play a crucial role in the remarkable architecture and impermeability of the cell envelope, also called the mycomembrane [17-19].

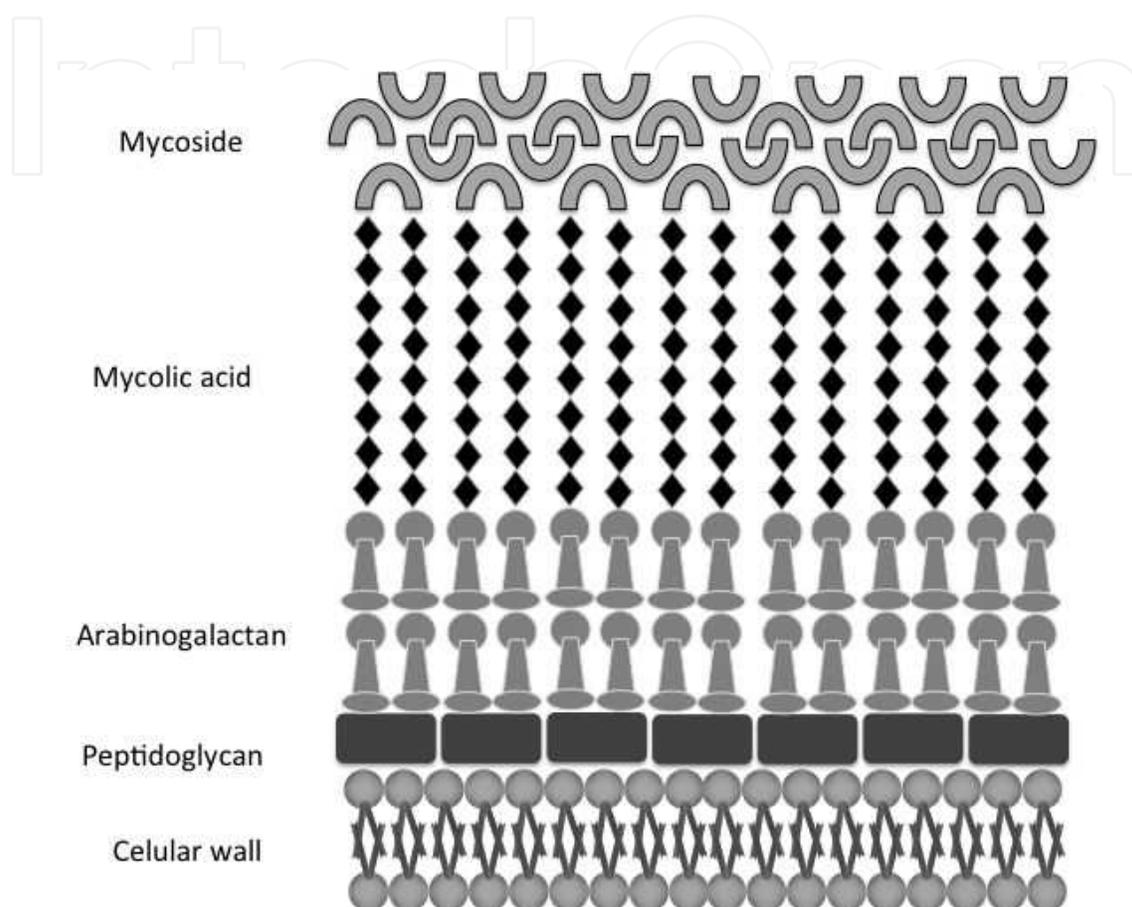


Figure 1. Diagrammatic presentation of mycobacterial cell wall and location of mycolic acids.

Structurally similar substances to MAs have been found in all mycobacterial species, with very few exceptions (e.g., *Corynebacterium amycolatum* and *Corynebacterium kroppenstedtii*). The identification of MAs structure has been addressed through the application of analytical techniques such as thin-layer chromatography (TLC), gas chromatography (GC), HPLC, mass spectrometry, and nuclear magnetic resonance spectroscopy. Based on their structural variability and complexity, MAs were largely used as taxonomic markers [17]. These large fatty acids contain a variety of functional groups and can vary in both qualitative and quantitative characteristics between species. This variety provides the basis for separation and identification of a large number of mycobacterial species using the HPLC.

3. High performance liquid chromatography methodology

Reverse-phase high-performance liquid chromatography of MAs esters has been demonstrated to be a rapid, reproducible, species-specific method for the identification of mycobacterial species. Also this method is relatively inexpensive and has been found to be more rapid alternative laboratory technique than the use of commercial nucleic acid probes [20]. Different methods have been developed for the detection of mycobacteria in clinical samples (e.g., blood, sputum) but they can also be applied to detection in other sources such as water [21] and milk [22]. Standard procedures to HPLC identification of mycobacterial species and most common steps used for different researchers are showed in the Figure 2 and Table 1, respectively.

Saponification	Fatty acid extraction	Stationary phase	Mobile phase/flow rate	Derivatization/detector	Ref.
25% KOH in 50% ethanol	Chloroform	C18	Chloroform (C) + methanol (M): 10 % C:90%M: 13 min 25% C:75%M: 14 min 70% C:30%M: 20 min Gradient elution: 0.6 mL/min	p-bromophenacyl/UV (254nm)	[13]
25% KOH in 50% methanol	Chloroform	C18	Methanol (M) + methylene chloride (MC): 98% M:2% MC: Initial 80% M:20% MC:1 min 35% M:65% MC: 10 min 98% M:2% MC: 10.5 98% M:2% MC: 12 min Gradient elution: 2.5 mL/min	p-bromophenacyl/UV (260nm)	[20]
50% aqueous potassium hydroxide Autoclaved: 1 h /121 °C	Chloroform	C18	Methanol (M) + methylene chloride (MC): 98% M:2% MC: Initial 85% M:15% MC:1 min 55% M:45% MC: 1.75 min 55% M:45% MC: 8.75 min 30% M:70% MC: 11.75 98% M:2% MC: 12 min Gradient elution: 2.5 mL/min.	4-bromomethyl- 6,7-dimethoxycoumarin/ FL (Emission: 418nm; Excitation: 240-420nm)	[26]
Methanolic potassium hydroxide	Chloroform	C18	Methanol-methylene chloride Gradient elution	p-bromophenacyl/UV (254nm)	[34]
25% of KOH in 50% ethanol	Chloroform	C18	Methanol (M) + methylene chloride (MC): 98% M:2% MC: Initial 80% M:20% MC:1 min	p-bromophenacyl /UV (254-260nm)	[35]

Saponification	Fatty acid extraction	Stationary phase	Mobile phase/flow rate	Derivatization/detector	Ref.
Autoclaved: 1 h /121 °C			35% M:65% MC: 10 min 98% M:2% MC: 10.5 98% M:2% MC: 12 min Gradient elution: 2.5 mL/min		
25% KOH in a water methanol (1:1) mixture. Autoclaved: 1 h /121 °C.	Chloroform	C18	Not referenced	p-bromophenacyl /UV	[36]
2 ml KOH 25% in 50% ethanol Autoclaved: 1 h /121 °C.	Chloroform	C18	Methanol (M) + methylene chloride (MC): 80% M:20% MC: Initial 60% M:40% MC:1 min 40% M:60% MC: 5.5 min 40% M:60% MC: 6 min 80% M:20% MC: 8 min Gradient elution: 3 mL/min	p-bromophenacyl /UV (254nm)	[37]
2 ml KOH 25% in methanol:H ₂ O (v:v) Autoclaved: 1 h /121 °C, 15 psi	Chloroform	C18	Methanol (M) + methylene chloride (MC): 98% M:2% MC: Initial 80% M:20% MC:1 min 35% M:65% MC: 2 min 98% M:2% MC: 17.5 min 98% M:2% MC: 20 min Gradient elution: 2 mL/min	p-bromophenacyl /UV (260nm)	[22]
25% KOH in 50% ethanol	Chloroform	C18	Chloroform (C) + methanol (M): 9 % C:91%M: Initial 70% C:30%M: 65 min Gradient elution: 2 mL/min	p-bromophenacyl /UV (254nm)	[38]
50% KOH in methanol solution	Chloroform	C18	Methanol (M) + 2-propanol (P): 60 % M: 40% P: Initial 60 % M: 40% P: 3 min 6 % M: 94% P: 21 min 30 % M: 70% P: 25 min Gradient elution: 1.5 mL/min	p-bromophenacyl /UV (260nm)	[25]

Table 1. Different chromatographic methods to *Mycobacterium* species identification from bacterial grown medium.

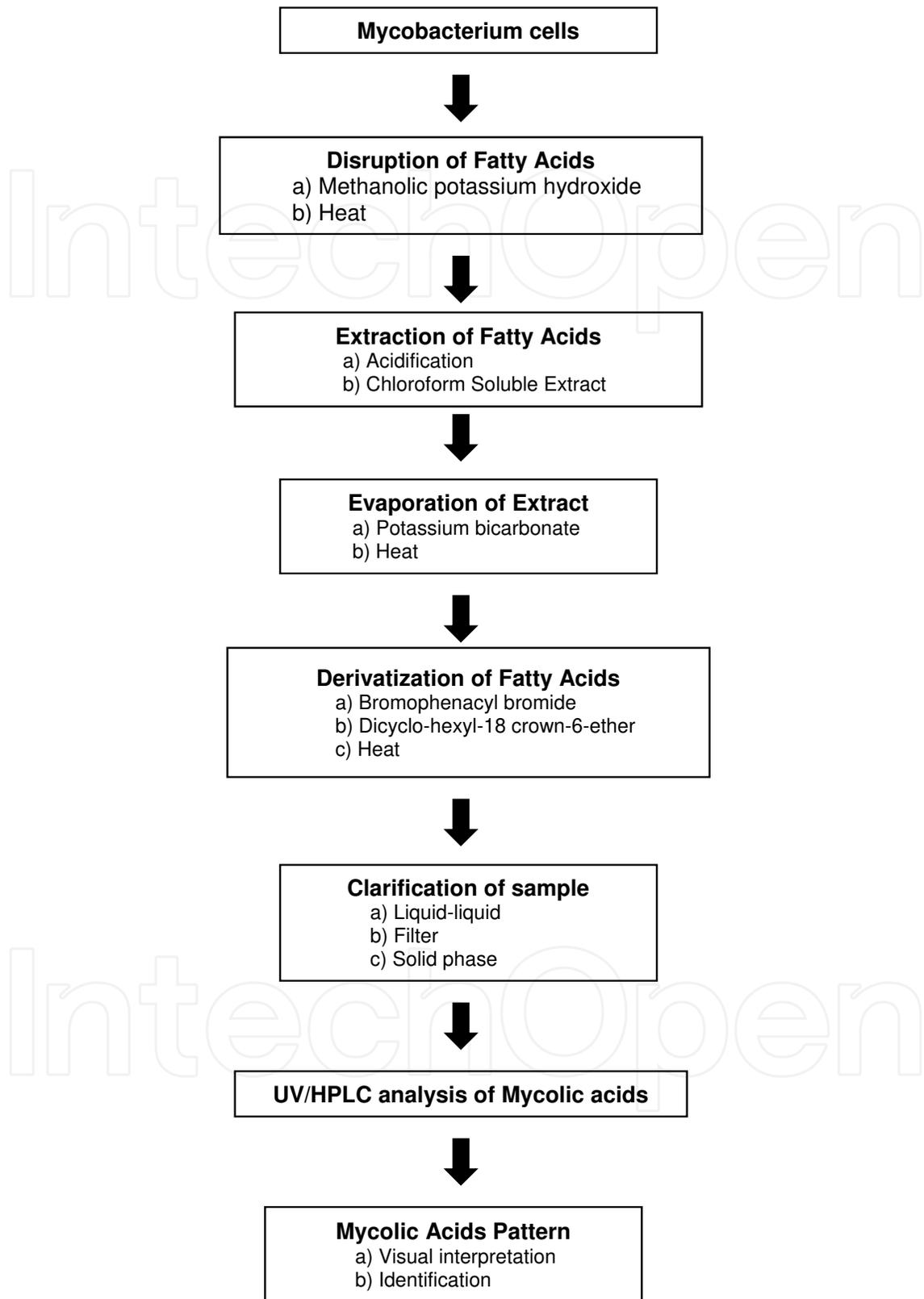


Figure 2. Flowchart for isolation and detection of mycolic acids. Adapted from [6].

3.1. Bacterial culture

HPLC still requires initial culture of isolates on solid medium before analysis. This can be problematic because the slow growth rate of mycobacteria delays full identification and leaves treating physicians with little useful information after the initial report of an acid-fast bacilli (AFB)-positive broth culture [23]. The identification is achieved when Mycobacteriae are grown under standardized culture medium conditions such as Lowenstein-Jensen (L-J) slant, which may be supplemented with additional growth factors for those strains of Mycobacteriae that are unable to grow on L-J. A carbol fuchsin/phenol or fluorochrome stain is performed to verify the presence of AFB. Another common solid medium used for mycobacterial species is the Middlebrook 7 H10 or 7 H11 at 35–37 °C. Currently available databases have been developed which incorporate mycobacterial species which require different growth conditions such as *Mycobacterium haemophilum* and *Mycobacterium marinum* (30 °C) [24]. According the Brazilian National Manual for the Laboratory Surveillance of Tuberculosis and other Mycobacteria [1], mycobacteria strains are cultured in L-J culture, except for *Mycobacterium bovis* that is grown in Stonebrink media, at 35 °C. Recently Buchan *et al.* [23] explored the use of broth culture for mycobacterial species such as an alternative for the solid medium and demonstrated a rapidly and accurately identification of mycobacteria to the species level from solid medium (7H11) or directly from broth culture such as Myco broth. It is important remark that culturing of *Mycobacterium tuberculosis* must be performed in special laboratorial conditions (Biosafety level 3) and follow and appropriate guidelines for the use and handling of pathogenic microorganisms [25].

3.2. Saponification

The autoclaving-saponification steps in the HPLC procedure is performed for two reasons: frees MAs and kills the mycobacteria, assuring laboratory safety. Also this step is important because it will determine the amount of MAs that will be extracted. MAs are covalently linked to the cell wall arabinogalactan matrix. Removal of the MAs requires saponification with potassium hydroxide (50 % w/v), which is often performed in an autoclave to accelerate the process and provide for the safety of laboratory personnel working with Biosafety Level III mycobacterial species. Once autoclaved, the organisms are killed by the procedure and the mycolic acids released from the cell wall [24]. A standard protocol for HPLC identification of mycobacteria of CDC suggest transfer 1–2 loops of bacterium culture to a glass tube (13 by 100 mm) and add 2 mL of methanolic saponification reagent (25% potassium hydroxide in 50% methanol). The tube is capped tightly, homogenised and autoclaved for 1 h at 121°C and 15 psi [14].

3.3. Extraction

MAs exist in the cell in two basic forms: covalently bound to the cell wall, and loosely associated with an insoluble matrix esterified to a variety of carbohydrate containing molecules. Treatment of intact cells with mixtures of chloroform and methanol is suitable for extracting the smaller quantity of non-covalently attached mycolate [16]. Once autoclaving has been completed, samples are cooled to room temperature, acidified, and extracted into chloroform.

Free MAs are extracted by acidifying with 1.5 ml of a 50% solution of concentrated HCl and H₂O (v/v) and 2 mL chloroform. The chloroform layer is dried under air at 80-100 °C, and 2 mg of potassium bicarbonate is added [14].

3.4. Derivatization

The preparation from extraction step is resuspended in 1.0 mL chloroform, and a derivatize reagent (*p*-bromophenacyl) is added. Derivatization is completed in a water bath at 80-100 °C for 20 min. Tubes are cooled, the mixture is acidified with 1 mL of the acidification solution (concentrated HCl and H₂O; 1:1, v/v), and 1 mL methanol is added. After that the solution is thoroughly mixed, the bottom chloroform layer is transferred to a glass tube and evaporated to dryness. Samples are resuspended in 50 µL methylene chloride before analysis.

Some experiences make done using fluorescence-labeling compounds. According to Butler and Guthertz [6] these compounds evolved as a follow: 4-bromomethyl-7-methoxycoumarin (Br-Mmc), performed in TLC analysis for detection of picomolar amounts; 4-bromomethyl-7-acetoxycoumarin (Br-Mac), suggested for femtomolar detection and finally 4-bromomethyl-6,7-dimethoxycoumarin and 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-1 and 4-bromomethyl-7-acetoxycoumarin. All fluorophores produced mycolic acid patterns similar to the patterns for *p*-bromophenacyl derivatives and were suggested for optimization of the method for clinical mycobacterial species identification. Jost *et al.* [26] reported the excellent analytical sensitivity of fluorescence (4-bromomethyl-6,7-dimethoxycoumarin) for the detection of MAs ester patterns and resulted in a substantial decrease in the time required to identify mycobacteria from cultures.

3.5. HPLC conditions

MAs are analyzed using a HPLC apparatus, in a gradient elution, and a UV detector set at 260 nm. Samples are separated in a C-18 reverse-phase column. The mobile phase is a mixture of methanol and methylene chloride in a flow rate of 2 mL min⁻¹. Authors performed some modifications in a CDC protocol for HPLC identification of MAs. Du *et al.* [9] tested a column with different dimensions (15.0 cm × 4.6 mm, 5 µm) and also a different elution program (run time 30 min and 1.5 mL min⁻¹ flow rate) from the CDC specifications [14]. However, they obtained chromatograms quite similar to those from the CDC protocol. On the other hand, Figueiredo *et al.* [22] use a C-18 column 33% taller than those used in the CDC protocol (7.5 cm), increased the run time to 20 min. With these changes they observed a superior resolution in an adapted protocol and could be an alternative to discriminate between species with homologous HPLC chromatogram patterns. Special careful must be taken when manual injection is performed. Its is recommended cleaned the syringe at least five times with HPLC-grade methylene chloride and the injection loop be cleaned one time with 1 mL of the mobile phase solvent; it is also recommended that a blank injection be used between samples when the prior MAs signal is high [27].

4. Identification of mycobacteria species

There is a wide range of structures and also concentrations of types or classes of MAs (α , methoxy, keto, epoxy mycolates, etc.) among mycobacterial species. The HPLC methodology is unable to separate all the homologous series of MAs, and for this reason the chemical composition of the chromatogram components could not be precisely identified. Although the individual mycolate cannot be identified, this is not necessary for identification of mycobacteria, since a species-specific chromatographic pattern is generated [10, 28].

In order to identify unknown mycobacteria specimens using HPLC, the laboratory maintains chromatograms of mycobacteria commonly seen in the laboratory. HPLC profiles of unknown mycobacteria are compared to the patterns contained in this spectral library. The chromatographic pattern for each strain is examined for differences in the heights for pairs of peaks. HPLC patterns are grouped according to species, and the values calculated for each ratio are combined, sorted in numerical order, and examined for their ability to discriminate species, using the range of the relative standard deviation (RSD) of the absolute retention times (ART) and the relative retention times (RRT). RRTs are adjusted by comparison with external mycobacterial MA peaks [29]. The *Mycobacterium intracellulare* mycolic acid fingerprint is usually used as a reference standard to help differentiate *Mycobacterium* spp.

The visual pattern recognition method employs only chromatographic criteria, although when available, other identification test results should be included in the decision-making processes. The initial step for identifying a species is determining the overall complexity and number of MA peak clusters. These clusters may consist of a few peaks or many peaks and are further defined as single-, double-, and triple- or multiple-peak clusters [6]. The amount of MA present is related to the amount of light emitted and the structure of the MA is related to the time of elution off the column. Pattern recognition is performed by visual comparison of sample results with MA patterns from reference species of known Mycobacteriae; however, a correct pattern of interpretation requires training. For that reason computer-assisted pattern recognition software, which utilizes retention time, peak width, and peak amount to provide a peak name which can then be compared to a library database, was developed [24].

5. Chromatogram profile database from *Mycobacterium* spp.

Due to the interpretation of chromatographic data can become tedious and time consuming for laboratories that process large numbers of samples, some studies recommend the construction of computer-based file (library) of *Mycobacterium* species. This library is to be used in conjunction with commercially available pattern recognition software packages like a Pirouette [20] or more recently, the Sherlock Mycobacterial Identification System (SMIS; MIDI, Inc.) has been developed for the rapid, computer-assisted identification of mycobacterial species based on the separation and quantification of MAs using HPLC technology [30].

Figueiredo *et al.* [22] grouped 35 strains of *Mycobacterium* species according a fingerprint library into three general patterns (single-, double- and triple-peak clusters) and divided in subgroups according to the chromatogram characteristics of MAs derivatives (Figure 3).

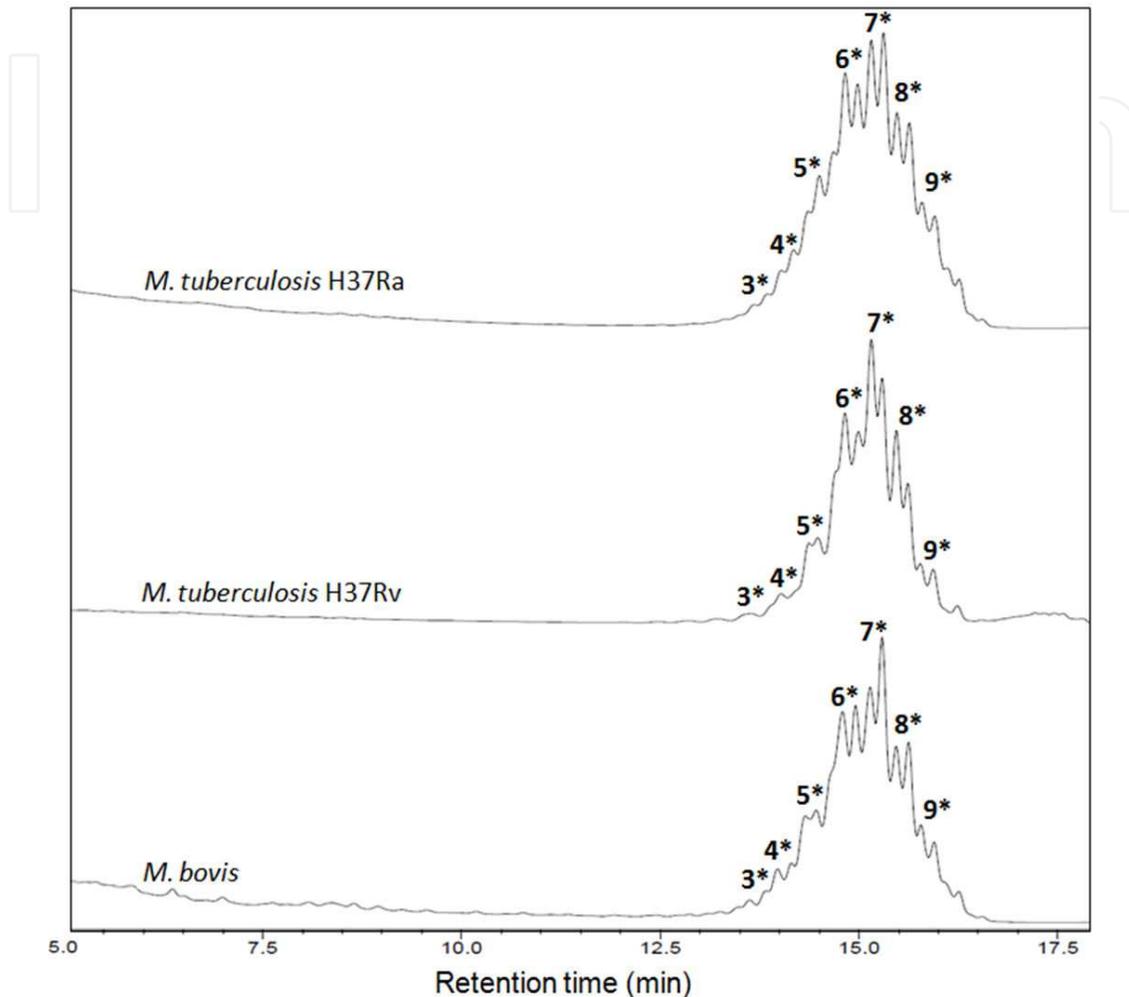


Figure 3. HPLC mycolic acid chromatograms from *Mycobacterium* reference strains, with single-cluster peak. *M. tuberculosis* H37Ra (ATCC 25177) and H37Rv (ATCC 27294) and *M. bovis* (ATCC 19210). *peaks showing a high degree of separation (appearing as a “double peak”), named according to [10]

5.1. Single-peak cluster patterns

Members of the MTC such as *Mycobacterium bovis* (Figure 4) and *Mycobacterium tuberculosis*, and others species such as *Mycobacterium asiaticum*, *Mycobacterium gordonae* chromotype I and *Mycobacterium kansasii* showed chromatogram patterns with a single, late-emerging peak cluster. *Mycobacteria* species within the same taxonomic groups, such as the *M. tuberculosis* complex species, show very similar chromatographic patterns, since they share the same MA structural types. A means to discriminate between the closely related species with similar HPLC chromatogram profiles could further distinguish the MAs that might be present in the same peak. Considerable heterogeneity exists within a particular class of MAs, considering

the chain length of individual acids (which can show a mixture of up to 100 structural isomers of α mycolates) and the potential range of heterogeneity in each species or subspecies [16].

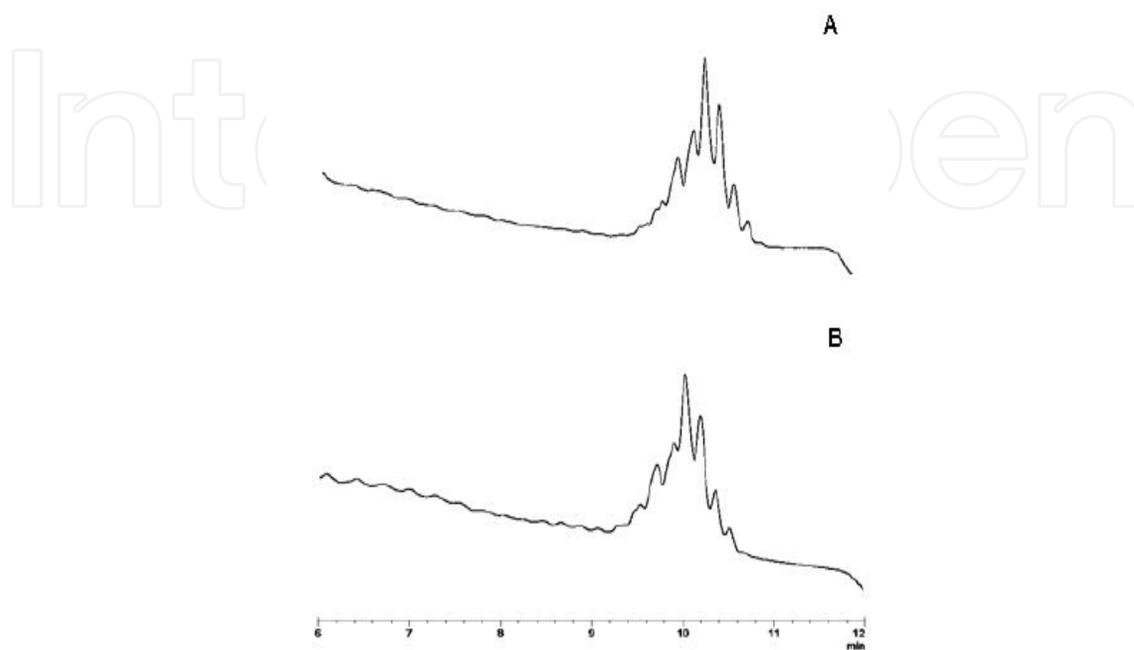


Figure 4. Representative reverse-phase HPLC chromatograms of bromophenacyl esters of mycolic acids from isolates of tissue fragments of Comparative Intradermal Tuberculin Test reactive cows: *M. bovis* ATCC 19210 (A) and *M. bovis* clinical isolates from bovines (B).

5.2. Double-peak cluster patterns

Mycobacterium chitae, *Mycobacterium porcinum* and *Mycobacterium agri* are representatives of this group that displays late-emerging and close-together clusters of peaks. *Mycobacterium fortuitum*, *Mycobacterium peregrinum* and *Mycobacterium smegmatis* are members of the *Mycobacterium fortuitum* complex and displayed very similar chromatogram patterns. Therefore, the HPLC results obtained for these species provided insufficient information to distinguish between them. The *Mycobacterium chelonae-Mycobacterium abscessus* taxonomic group has undergone several revisions following the identification of newly recognized species such as *Mycobacterium massiliense*, which was proposed based mainly on genotypic analysis. As expected for closely related species all the members of this group showed a single chromatogram pattern [31, 32].

5.3. Triple-peak cluster patterns

Mycobacterium simiae could be included in this group. The last group included *Mycobacterium chubuense*, *Mycobacterium obuense*, *Mycobacterium parafortuitum* and *Mycobacterium vaccae*, which showed early-peak clusters emerging before 10.0 min.

6. Application of high performance liquid chromatography

According to Figueiredo *et al.* [22] the identification of mycobacteria by HPLC is performed by comparing fingerprint patterns obtained from each clinical sample with those from the reference strains. The first criterion for identifying *Mycobacterium* spp. is to match the overall complexity and number of MA peak clusters: single, double and triple peaks. The second criterion is the range of time of elution between multiple peak groups, where the positions of peaks are determined as RRTs, adjusted by comparison to an external mycobacteria MA peak. To increase reliability, the relationships of peak heights of major diagnostic peaks are determined and compared to those from reference strains. Mondragón-Barreto *et al.* [33] describe the advantages of HPLC method to *Mycobacterium* identification but if results are unclear (problems are principally for inadequate HPLC reference patterns, the isolate should be analyzed using PCR-RFLP. Another interesting application to MAs identification by HPLC is the estimation of bacterial growth [27]. It was described a linear relationship between the total area under the MA chromatographic peaks of a culture of *Mycobacterium tuberculosis* and log CFU per milliliter, suggesting the possibility of using this results as a good estimator of mycobacterial growth.

7. Conclusion

HPLC procedure for MAs separation is a rapid, reproducible and easily way to *Mycobacteria* identification and can be executed by many laboratories, making this approach one of the most appropriate methods to distinguish among the species. A customized database, using locally adapted protocols, must be developed in order to obtain chromatogram spectra from reference strains in the new analytical conditions, accrediting the local methodology and allowing accurate analysis of clinical samples. Although HPLC equipment is too expensive for many laboratories, it is realize that this system is useful to MAs identification. It is recommended that the HPLC can be combined with other techniques like PCR as a confirmatory diagnosis for the identification of clinical isolates where the matching chromatogram fingerprints failed or were inconclusive in differentiating species within the same taxonomic group, such as the *M. tuberculosis* complex species.

Acronyms

AFB=Acid-fast bacilli

ART=Absolute retention time

CDC=Centers for Disease Control and Prevention

CFU=Colony forming unit

DNA=Deoxyribonucleic acid

GC=Gas chromatography

HPLC=High-performance liquid chromatography

L-J=Lowenstein-Jensen

MAs=Mycolic acids

MTC=*Mycobacterium tuberculosis* complex

NTM=Non-tuberculous mycobacteria

RRT=relative retention times

RSD=Relative standard deviation

SMIS=Sherlock Mycobacterial Identification System

TLC=Thin-layer chromatography

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Author details

Carlos Adam Conte Junior^{1*}, César Aquiles Lázaro de la Torre^{1,2},
Eduardo Eustáquio de Souza Figueiredo³, Walter Lilenbaum⁴ and
Vânia M. Flosi Paschoalin²

*Address all correspondence to: carlosconte@hotmail.com

1 Department of Food Technology, Faculty of Veterinary, Fluminense Federal University, Niterói, Rio de Janeiro, Brazil

2 Faculty of Veterinary Medicine, Universidad Nacional Mayor de San Marcos, San Borja, Lima, Peru

3 Department of Food and Nutrition, Federal University of Mato Grosso, Cuiabá/MT, Brazil

4 Department of Microbiology, Fluminense Federal University, Niterói, Rio de Janeiro, Brazil

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