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Targeted lipidomics of mycobacterial lipids and glycolipids

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Running Head: Targeted lipidomics

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Abstract

Decades of study have highlighted the richness and uniqueness of the repertoire of lipid and glycolipid families produced by mycobacteria. Many of these families potentially regulate host immune responses, in stimulatory or suppressive ways. Thus the global study of this repertoire in different genetic backgrounds or under model conditions of infection is gaining interest. Despite the difficulties associated with the specificities of this repertoire, the field of mass spectrometry-based lipidomics of mycobacteria has recently made considerable progress, particularly at the analytical level. There is still considerable scope for further progress, particularly with regard to the development of an efficient bioinformatics pipeline for the analysis of the large datasets generated. This chapter describes an HPLC-MS methodology allowing the simultaneous screening of more than 20 of the lipid families produced by mycobacteria and provides recommendations to analyse the generated data given the state of the art.

1. Introduction

Different analytical techniques are used to analyse lipids and glycolipids including thin layer chromatography (TLC), nuclear magnetic resonance (NMR) and mass spectrometry (MS), which have their strengths and weaknesses, as previously reviewed [1]. Nevertheless, if by lipidomic we mean the rapid and “global” analysis of the lipids and glycolipids that characterise a given system, mass spectrometry appears to be the method of choice because it provides sensitivity, resolution, high throughput and allows assessing the acylforms diversity of the lipid families.

Lipidomics approaches, which have benefited from the enormous progress of mass spectrometry, are today diverse and highly developed, in particular for the analysis of eukaryotic lipids [2]. These approaches share main steps: i) sample preparation (lipid extraction), ii) analytical measurement (MS-based detection), iii) data processing (lipid identification and quantification) and iv) data interpretation, which depend on biological questions and rely on more or less complex bioinformatics pipelines. The type of samples (biological fluids, tissue or cell culture) and the “targeted” versus “untargeted” nature of the study condition these different steps. Whether the study focuses on certain lipid families of the lipidome (targeted study) or, on the contrary, on the lipidome in a global way with the possible discovery of new lipids (untargeted study), will lead to favour specific methods for lipid extraction and MS devices according to their performance in terms of sensitivity, dynamic range, and resolution.

Mycobacteria produce a very diverse and specific repertoire of alkyl chain-containing molecules including lipids and glycolipids [3], lipoproteins [4] and lipoglycans, which are potent immunomodulators with key roles in host-pathogen interactions [5,6]. These molecules distribute differently within the two membranes of the mycobacteria’s envelope [7-10] (Fig. 1). The plasma membrane is mainly composed of phospholipids, phosphatidyl-*myo*-inositol di- and hexamannosylated (PIM₂ and PIM₆), triglycerides (TAG), lipoproteins, and lipoarabinomannan (LAM). The outer membrane, or mycomembrane, is made of an inner leaflet composed of the mycolic acids (MA) (up to 90 carbon-long fatty acids) that are covalently bound to the arabinogalactan, itself linked to the peptidoglycan. In contrast, the outer leaflet of the mycomembrane is not covalently bound to the inner leaflet and encloses, besides the LAM and lipoproteins, an impressive diversity of unique lipid and glycolipid families as illustrated by a few examples in Figure 1. Among these surface lipidic components, mycolyl esters such as trehalose mono- and dimycolates (TMM and TDM) are found for all mycobacteria, with variation in their mycolic acyl groups (alpha, methoxy or keto mycolic acids). In contrast,

phthiocerol dimycocerosate (PDIM), phenolic glycolipids (PGL) or sulfoglycolipids (SGL) are strain-specific lipid families. Each of these families exists as acylform variants, which number can reach several dozens. Finally, the bacillus surface is coated with a detergent-labile capsule layer essentially made of glycans and, to a lower extent, lipids and proteins [11]. Hence, similar to eukaryotes, mycobacteria also produce a repertoire of lipids that contains hundreds of molecular species [12].

Lipoproteins and lipoglycans, which are amphiphile macromolecules rather than lipids *per se*, are studied using specific extraction and analytical methods [13-16]. Hence, we will focus here on the so-called “extractible” mycobacterial lipids and glycolipids that are recovered by exposing mycobacteria pellets to organic solvents. This includes lipids from the cytoplasm, plasma membrane and outer leaflet of the mycomembrane. It is important to note that the mycolic acids of the mycomembrane’s inner leaflet are not included in the extractible lipid pool. Indeed, in the absence of saponification to cleave their linkage with the arabinogalactan, they remain associated to the insoluble material after exposure of mycobacteria with organic solvents.

The purification and analysis by TLC of many of these lipid families have been widely described [17] and is also detailed in previous editions of this protocol book [18]. Mass spectrometry-based detection methods relying on the use of MALDI-Tof or ESI/APCI-MS instruments have also been used for the analysis of MA [19,20], SGL [21-23], DIM [22], PIMs [24-26,23], PGL[23], TMM [27], TDM [28] and siderophores (mycobactin and carboxymycobactin families) [29] and might be favoured for the specific high throughput analysis of one of these lipid family. More recently, lipidomic methods have been developed to analyse the *M. tuberculosis* lipid repertoire in a more global way. This effort has faced the challenges associated with the unique and structurally diverse mycobacterial lipids, which prevented the direct application of analytical methods developed for eukaryotic lipids. Two main studies have described HPLC-(ESI)-Q-ToF approaches that differ in the reverse phase (C18) [30] versus normal phase (diol) [12] chromatographic method. While the first one separates lipids based on their acyl groups (by acylform), the second one separates lipids based on their polar head (by family), but both approaches provide a wide coverage of *M. tuberculosis* lipidome. Both studies also released a theoretical database listing the calculated m/z of adducts of the published *M. tuberculosis* lipid families, called Mtb LipidDB (<https://mrl.colostate.edu/mtb/>) [30] and MycoMass (<https://www.brighamandwomens.org/research/departments/rheumatology-immunology-allergy/moody-lab/overview>) [12], respectively.

If the *M. tuberculosis* lipidomic field has made significant progress it lags behind the lipidomics of eukaryotic lipids because it still suffers from several shortcomings: the lack of a MS/MS database that would allow Multiple Reaction Monitoring mode analysis for the identification of lipids, the poor availability of standards to achieve absolute quantification, and the lack of a straightforward bioinformatics pipeline for high-throughput data analysis, especially for unbiased and comparative analysis of large number of datasets.

Based on the previously published method that relies on diol chromatography coupled to (ESI)-MS [12], this chapter describes a lipidomic approach to perform the simultaneous screening of more than 20 of the main *M. tuberculosis* lipid families. This method has been successfully used in the past to characterise *M. tuberculosis* lipid profile in response to stress [31], to compare the lipid profile of mycobacteria strains [32] and also to detect mycobacterial lipid in tissues [32]. The mass spectrometer available in the environment of a given laboratory is a limiting and inflexible factor, given the cost of such an instrument. Thus, the parts of this chapter describing the analytical detection of mycobacterial lipids and the data analysis correspond to recommendations to implement this approach and ensure quality of the analyses rather than a wet-lab protocol.

2. Material

2.1 Glycolipid extraction from mycobacteria culture

1. Chemical fume hood
2. LC-MS-grade chloroform (CHCl_3) or dichloromethane (CH_2Cl_2) (*see Note 1*)
3. LC-MS-grade methanol (CH_3OH)
4. LC-MS-grade water.
5. Solvent evaporation system: centrifugal evaporator, Genevac or nitrogen stream
6. 50 mL polypropylene V-bottom tubes (Falcon type)
7. 50 mL screw centrifuge glass tubes (Pyrex quality) with PTFE coated screw caps
8. 250 mL round bottom flasks if using rotary vacuum evaporator as drying system or 15 mL-glass tubes if using stream-drying system to dry organic phases.

9. 500 µL Hamilton syringes
10. Pasteur glass pipettes
11. Rocking shaker
12. Nitrogen stream drying system
13. Microbalance
14. 2 mL-amber vials with PTFE screw caps

2.2 HPLC-MS analysis

1. LC-MS-grade hexane, 2-isopropanol, methanol, formic acid (CH_3COOH) and ammonium hydroxide (NH_4OH)
2. Solvent A: 70:30 hexane : isopropanol (v :v), 0.1% (v/v) formic acid, 0.05% (v/v) Ammonium hydroxide
3. Solvent B: 30:70 methanol : isopropanol, 0.1% (v/v) formic acid, 0.05% (v/v) Ammonium hydroxide
4. Diol (dihydroxypropyl group bonded phase) HPLC column (150 mm $\times$ 2 mm, 3 µm particles of 10 Å porosity) and a Diol guard column (3 µm $\times$ 4.6 mm) (*see Note 2*).
5. High-resolution mass spectrometer (*see Note 3*).

2.3 Targeted screening and comparative analysis

1. R software
2. msConvert ProteoWizard
3. MS database: Mtb LipidDB [30] (<https://mrl.colostate.edu/mtb/>) or MycoMass [12] (<https://www.brighamandwomens.org/research/departments/rheumatology-immunology-allergy/moody-lab/overview>)

3. Methods

3.1 Glycolipid extraction from mycobacteria culture

1. Transfer 50 mL of mycobacterial culture grown in the absence of detergent (*see Note 4*) into 50 mL polypropylene V-bottom tubes.
2. Centrifuge the tubes at 2000 g for 15 min at room temperature and discard the supernatant.
3. Wash the pellet twice with 10 mL of water by resuspending the bacteria and repeating step 2.
4. Resuspend the bacterial pellet in 1 mL of methanol.
5. Add internal lipid standard (*see Note 5*).
6. Transfer the methanolic bacterial suspension to 50 mL screw centrifuge glass tubes with PTFE coated screw caps containing 45 mL of 1:2 chloroform:methanol (v :v) (*see Note 6*).
7. Allow sterilisation overnight (*see Note 7*).
8. Shake tubes on a rocking shaker for about 1 h.
9. Pellet the bacteria at 2000 g for 15 min (or until a translucent supernatant is obtained), at room temperature.
10. Transfer and dry the supernatant in clean 250 mL-round bottom flask or in 15 mL-glass tubes depending on available evaporating system.
11. Resuspend the pellets in 45 mL of 1:1 chloroform:methanol (v:v) and repeat steps **8-10**. Pool the organic supernatants.
12. Resuspend the pellets in 45 mL of 2:1 chloroform:methanol (v:v) and repeat steps **8-10**. Pool the organic supernatants.
13. Dry the pooled organic supernatants using a centrifugal evaporator, gene-vac or under nitrogen stream in a chemical hood.
14. Resuspend the dried lipids in 1 mL of 1:1 chloroform:methanol (v:v), transfer to a pre-weighted 2 mL-amber glass vial with a Hamilton syringe and dry under a gentle nitrogen stream without heat.
15. Rinse with additional 1 mL of 1:1 chloroform:methanol (v:v), add to the 2 mL-amber glass vial with a Hamilton syringe and dry under a gentle nitrogen stream without heat.
16. Weight the dry lipids (*see Note 8*), resuspend at 2 mg/mL in 1:1 chloroform:methanol (v:v), store at -80°C if not analysed immediately (*see Notes 9*).

3.2 HPLC-MS analysis

3.2.a Set-up of the HPLC-MS system for targeted screening of mycobacterial lipids

1. Equilibrate the HPLC Diol column at room temperature with Solvent A at 0.15 mL/min for 30 min (*see Note 10*).
2. Establish the (m/z, RT) map of the lipid families of interest using your HPLC-MS instrument (*see Note 11*):
 - Inject purified/synthetic *M. tuberculosis* lipids (*see Note 12*) or reference *M. tuberculosis* lipid extract and apply the following gradient at room temperature and at a flow rate of 0.150 mL/min as follows (*see Note 13*) :

<i>Time (min)</i>	<i>A/B (%)</i>
0.0	100/0
10.0	100/0
17.0	50/50
22.0	50/50
30.0	0/100
35.0	0/100
40.0	100/0
44.0	100/0

- Map the respective *M. tuberculosis* lipids MS signals in your system. In the absence of MS/MS database, the assignment of MS signals to given lipid families relies on the validation of at least 2 of following criteria:

□ The lipids are detected at m/z matching the calculated m/z within the mass accuracy of the high-resolution mass spectrometer (as low as 1 ppm and never exceeding 10 ppm mass accuracy) and show the expected acylform profile when analysing purified lipid family or total lipid extract (*see Note 14*).

□ Observed retention time matches the retention time of standard (when available), i.e. synthetic or purified acylform of the lipid family (*see Note 12*).

□ The fragmentation pattern obtained by MS/MS confirms the building blocks that characterise the lipid family (*see Note 15*).

Table 1 lists the characteristics of the MS signal of the lipid families detected analysing the *M. tuberculosis* H37Rv lipid extract using the Diol HPLC-Agilent 6520 Accurate-Mass Q-Tof.

3. Ensure reproducibility and stability over time of the assigned MS signals by performing multiple and consecutive analyses of the same *M. tuberculosis* lipid extract. Alternate with blank runs (injection of 1:1 chloroform:methanol (v:v) or solvent A) to confirm absence of detection of carryover during the blank runs.

4. Store aliquots of the mycobacterial lipid mixture (purified/synthetic mycobacterial lipids or natural lipid extract) used for parameters optimisation and the associated datasets to control the response of the mass spectrometer before a new experiment and after mass spectrometer maintenance.

3.2.b Perform targeted screening of mycobacterial lipids in lipid extract of interest

1. Equilibrate the HPLC-MS diol column at room temperature with Solvent A at 0.15 mL/min for 30 min (*see Note 10*).

2. Ensure the cleanliness and the proper functioning of the system by analysing an alternation of blank samples (solvent used to solubilise the samples, *see* subheading 3.1 **step 14**) and lipid reference samples using the gradient described above (subheading 3.2.a, **step 2**).

3. Inject 10-20 µg of the lipid extract of interest resuspended in 10-20 µL of chloroform:methanol 1:1 (v:v) or solvent A (*see Note 13*). If needed, centrifuge samples at 2000 rpm for 5 min to remove potential insoluble residues before injection.
4. If semi-quantitative comparative analysis is the goal, determine the quantity of lipid extract to be injected for a linear response of the different lipid families to be screened and compared (*see Note 16*):
 - Inject a range from 0 to 20 µg of reference lipid extract.
 - Extract ion chromatograms for each lipid of interest and integrate area under the chromatograms.
 - Plot the curve of chromatogram area versus injected quantity of sample for the different lipid families of interest.
 - Determine the injection quantity range over which the majority of lipids show a linear response.

3.3 Targeted screening and comparative analysis

- Semi-automated screening of mycobacterial lipid families

Once the map of detected lipids is established routine screening of these families can be undertaken. For a moderate number of analyses, one of the easiest ways is to use the data acquisition and visualisation software associated to the spectrometer and to extract the ion chromatograms at the expected m/z for representative or all acylforms of the lipid families (*see Note 14*), with a tolerance within the mass spectrometer mass accuracy (1-10 ppm). It is often possible to pre-register a list of m/z for the simultaneous extraction of the ion chromatograms of all the lipids of interest.

- Automated lipid annotation

For a large number of samples such as biological fluids from patient cohorts or a mycobacteria library, it can be envisaged to set up a bioinformatic workflow for the extraction of all the chromatograms of the generated datasets using an appropriate peak picking algorithm and/or to carry out an automatic annotation with software integrating the available databases, MycoMass [12] or Mtb LipidDB [30] (*see Table 2 and Note 17*), such as the MS-LAMP software [33].

Several lipidomic data processing software packages have been developed for filtering, peak picking, peak integration and alignment, such as the online XCMS platform (<https://xcmsonline.scripps.edu>) [34], MZmine 2 [35] or MS-DIAL [36]. Each require converting the raw MS files in

mzXML format, which can be performed either with the data export module of the mass spectrometer data acquisition software (such as Agilent MassHunter) or can be carried out using msConvert ProteoWizard [37,38]. Processed data can then be exported toward data annotation software.

- Semi-quantitative comparison of mycobacterial lipid families present in studied samples

The area under the extracted-ion chromatogram of a given lipid species correlates with its abundance. However, translating this area in absolute quantity requires performing dose response curves using standards, which is still challenging for mycobacterial lipids due to the poor availability of standards. As well, differences in chromatograms areas between lipid classes cannot be translated into differences in absolute quantity without determining the response factor of each lipid class. Nonetheless, changes in the area under the chromatogram for a given lipid across samples of the same nature reflects changes in absolute quantity. Hence, the comparison of the lipid profiles obtained for different samples of the same nature allows highlighting changes in the relative proportion of the different lipid families without knowing their absolute value.

Normalization of the experiment is crucial to perform such semi-quantitative comparative analysis. Since the use of detergent in mycobacteria culture is not recommended, strong bacterial aggregation is observed and optical density of the cultures cannot be used for the normalisation of the comparative analysis of different mycobacterial strains (*see Notes 4*). Instead, comparative analysis can be normalized based on the weight of extracted bacteria pellet or on the amount of injected lipid extract (assuming that the same amount of lipids are extracted per bacillus for different strains) (*see Note 8*).

These considerations being said, the comparative analyses of samples should be performed as follows:

- 1- Add an internal standard(s) (*see Note 5*) to studied samples before lipid extraction to control the quality of the extraction process and normalisation.
- 2- Perform HPLC-MS analysis as above described by injecting the amount of lipid extract according to the chosen normalisation method (*see Notes 8 and 18*) and to the previously determined range of linear response of the studied lipid families (*see Note 16*).

3- Extract for each dataset the ion chromatograms at the expected m/z for representative or all acylforms of the lipid families (*see Note 14*), with a mass tolerance within the mass spectrometer mass accuracy (1-10 ppm), and integrate signals under the chromatogram curves.

4. Notes

1. Handle all organic solvents under the hood. Chloroform can be replaced by dichloromethane, which is less carcinogenic.
2. This approach was initially developed using an Agilent Technologies 6520 Accurate-Mass Q-ToF and a 1200 series HPLC system with a Varian MonoChrom Diol (dihydroxypropyl group bonded phase) column (150 mm $\times$ 2 mm, 3 μ m particles of 10Å porosity) and a Varian MonoChrom Diol guard column (3 μ m $\times$ 4.6 mm). Elution profile obtained with the Varian Diol column is presented in Table 1. This column is discontinued but equivalent Diol columns with the same characteristics are available (for example, Agilent or Princeton diol columns). Using equivalent Diol columns leads to similar order of elution of *M. tuberculosis* lipids families but with possible moderate variation in retention time.
3. Mass spectrometers show variable performance (sensitivity and dynamic range) and *M. tuberculosis* lipid families have different ionization properties. Hence, applying this approach to a different mass spectrometer might require the optimization of the ionization parameters of the instrument in order to allow the detection of the different lipid families. For information, this approach has been later successfully transferred on LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo) mass spectrometer coupled to a Dionex Ultimate 3000 HPLC system and to a Xevo G2 Qtof (WATERS) mass spectrometers (unpublished).
4. Traces of detergents alter lipidomic analyses by inducing important contaminant mass spectrometry signals and by suppressing the signal of relevant lipids. It is therefore necessary to take several precautions with regard to the culture media, the quality of the solvents and the appropriate use of plastic and glass materials.

Culture conditions: Mycobacteria are usually grown in standard Middlebrook 7H9 (Difco) or Sauton medium. It is recommended to avoid the use of detergent such as Tween, which is routinely employed to reduce the characteristic aggregation of mycobacteria in liquid media. Indeed, detergents

stick to the bacillus envelope and are recovered during lipid extraction by organic solvents. When growth monitoring by optical density measurement is required, it is preferable to use tyloxapol. Another alternative is to prepare a replicate culture in the presence of detergent in parallel with detergent-free cultures for each strain, in order to estimate their growth phase based on optical density.

Material: Material is also a source of contaminations. Methanol is compatible with plastic consumables but as soon as chloroform- or dichloromethane is used any contact with plastic consumables (pipette, tubes and caps) is strictly forbidden and therefore glass material must be used (pipette, syringe and tubes with PTFE caps). In addition, glassware washed with soap such as in common laboratory laundry is still a source of contaminations. Hence, attention has to be paid to clean the glassware twice with 1:1 chloroform:methanol before use. Polytetrafluoroethylene (PTFE) caps must be used with screw glass tubes.

Solvents: All solvents should be HPLC-grade and freshly prepared.

5. Addition of a known quantity of non-mycobacterial lipid standard, such as phosphatidylcholine for example, to the bacteria pellet allows controlling the overall lipid extraction process and to normalize comparative analyses. The intensity of the MS signal of the chosen standard should be within the intensity range of the analytes of interest. The determination of the response factor of the standard in the used LC-MS set up will determine the suitable amount of standard to be added.
6. The tip of the plastic pipette must not contact the chloroform:methanol solution when transferring the methanolic bacteria solution. All following steps of extraction protocol must be performed in a chemical hood
7. When manipulating live virulent *M. tuberculosis* strains, step 1-7 are performed in BSL3 facility. Contacting the bacterial pellet with chloroform:methanol mixture also aims at killing the bacteria before pursuing the protocol outside of the BSL3 facility. Successful sterilization relies on the ratio wet pellet / chloroform:methanol. From 50 mL of mycobacterium culture, recovered wet pellet is typically ≤ 1 mL. A ratio of 4 parts solvent to 1 part wet pellet (v/v) is expected to sterilize the sample overnight. In any case, when setting up this protocol, the sterilization conditions must be validated by a controlled experiment showing the absence of colony forming unit when plating the so-called "sterile" pellet on Petri dish. Once the sample is sterilized, spray the outside of the glass container with tuberculocidal agent and continue the protocol outside of the BSL3 facility in a chemical hood using glass pipettes or syringes and rinsed glassware as described in Note 4.

8. About 1-1.5 mg of dried lipids is usually recovered from 50 mL of log phase culture. If normalization of experiments is later carried out on the quantity of injected lipid extract, weight measurement must be as reliable as possible, which requires the complete evaporation of the solvents. Repeat weighing of the dried lipid film on a microbalance 20 min later and ensure a constant weight is reached otherwise desiccation time must be prolonged. Drying under vacuum might be necessary depending on the amount of lipid extract.
9. Best results are obtained with freshly prepared extracts. Lipid extracts should not be stored in a dry state. Oxidative degradation is slower in solutions (up to 1 year). Dichloromethane is more stable than chloroform for long-term storage, which should take place in a flammable storage laboratory freezer. Air and light should be avoided. To minimise the cleavage of ester lipids during long periods of storage, methanol can be replaced by 2-propanol in the solvent mixture.
10. HPLC solvents A and B should be freshly prepared and should not be stored. If (light) precipitates are observed when adding the additives to the solvent mixtures, 1 minute in a sonic bath can help the solubilisation. The HPLC system should be equipped with solvent resistant gaskets and solvent bottles with integrated air valve to avoid solvent evaporation. Sufficient volume of solvents should be prepared in order to analyse all the samples to be compared with the same batch of solvents and to avoid retention time variation.
11. For future screening of mycobacterial lipids in different samples or for different mycobacteria strains it is necessary to first establish a detection map (m/z , retention time) of these different families for a given HPLC-MS system. Mass spectrometers show variable performance (sensitivity and dynamic range) and *M. tuberculosis* glycolipid families have different ionization properties. Hence, applying this approach with a given mass spectrometer might require the optimization of the ionization parameters of the instrument in order to allow the detection of all lipid families. For information, initially set up using an Agilent 6520 (ESI)-Q-tof mass spectrometer, this approach has been later successfully transferred on a Thermo LTQ Orbitrap XL and a Waters Xevo G2 Qtof mass spectrometers (unpublished). Using the Agilent Technologies 6520 Accurate-Mass Q-ToF in full scan mode, recommended Dual ESI source and acquisition parameters are the following, as previously published [1]: gas temperature 325°C, 5 L/min drying gas flow, 30 psig nebulizer pressure, VCap 5,500 V. Spectra are collected from m/z 100 to 3,000 at 1 spectrum/s with continuous infusion of calibrants (m/z 121.050873 and 922.009798 in positive ion mode, m/z 112.985587 and 1033.98810 in negative ion mode).

12. Standards of mycobacterial lipids are currently poorly marketed. Polar Aventi mycolic acids are distributed by Sigma Aldrich and “*Mycobacterium tuberculosis*, Strain H37Rv, Total Lipids” (NR-14837) is provided by the BEI resources. Alternatives for MS signal optimisation are to directly work with the total lipid extract from *M. tuberculosis* culture to reproduce the lipid profile detailed in Table 1 or to contact laboratories with expertise in *M. tuberculosis* lipids purification or organic synthesis to obtain pure lipids for the optimization of individual lipid families detection.
13. The amount of lipids to be injected depends on the instrument sensibility. Agilent Technologies 6520 Accurate-Mass Q-ToF requires about 10 micrograms of *M. tuberculosis* total lipid extract to detect the different lipid families listed in Table 1. The same instrument shows a limit of detection of 10^{-12} to 10^{-9} mole injected when analysing purified lipid standards [1].
14. Each *M. tuberculosis* lipid family is composed of not one, but more often of several dozen acylforms that share a common polar head. Hence, the signal corresponding to a given lipid family is characterized by a series of molecular ions detected with m/z values that differ by 14.0156 a.m.u (CH_2 group), which elute within a narrow retention time window on diol chromatography. When performing the semi-quantitative comparison of the lipid profile of different samples, it is possible to perform the comparison using one representative acylform by lipid family, if the acylform profile of the lipid families is conserved between samples.
15. While MS/MS databases are not available for *M. tuberculosis* lipids, fragmentation profiles of several *M. tuberculosis* lipids families have been previously published [1].
16. When the goal is the semi-quantitative comparison of the lipid profiles of different strains (for example, WT, mutant and complemented strains) or of a same strain grown in different conditions, it is important to ensure that the injected quantity of lipid extract is within the linear response range of the lipids of interest. Indeed, the area under the chromatogram of a given molecular lipid species increases proportionally with its abundance until the signal saturates or even gradually extinguishes. Because each lipid family has its own response factor under given mass spectrometry settings, it is therefore important that the concentration of the studied lipid extract (i.e. the injected quantity) is suitable to assess changes in abundance for as many, if not all, lipid families as possible.

17. These databases represent a unique resource of information but it is important to keep in mind that they consist of lists of theoretical calculated m/z for the acylforms of the different mycobacterial lipid families described in the literature. The MycoMass was built to provide a tool to assist MS signals assignments or at least to propose possible formulas. As culture/infection conditions modulate the acylform profile of lipids, the list of listed acylforms is not limited to the published data but is sometimes extended in terms of acyl chain length and combination of fatty acids (**Table 2**). In addition, these databases do not contain retention time criteria, which are method- and therefore laboratory-dependent. Hence, a match between a detected m/z and an entry in these databases does not constitute, by itself, a definitive assignment. The use of these databases for the annotation of large HPLC-MS datasets currently requires an extensive manual curating to remove annotations that are incoherent with observed RTs. When using these databases on datasets generated using a different chromatographic separation, annotations of MS signals must be confirmed by MS/MS experiments. The time saved using database for the annotation of large MS datasets is to the user's appreciation.
18. When comparing the lipid profile of different strains, it is desirable to ensure that the cultures are in the same growth phase (log versus late phase). One replicate culture in the presence of detergent can be carried out in parallel with detergent-free cultures for each strain, in order to estimate their growth phase based on optical density measurement.

5. References

1. Jurowski K, Kochan K, Walczak J, Baranska M, Piekoszewski W, Buszewski B (2017) Analytical Techniques in Lipidomics: State of the Art. Critical reviews in analytical chemistry 47 (5):418-437. doi:10.1080/10408347.2017.1310613
2. Zullig T, Trotzmuller M, Kofeler HC (2020) Lipidomics from sample preparation to data analysis: a primer. Analytical and bioanalytical chemistry 412 (10):2191-2209. doi:10.1007/s00216-019-02241-y
3. Minnikin DE, Brennan PJ (2020) Lipids of Clinically Significant Mycobacteria. In: H. G (ed) Health Consequences of Microbial Interactions with Hydrocarbons, Oils, and Lipids. Handbook of Hydrocarbon and Lipid Microbiology. Springer, Cham, pp 1-76. doi:https://doi.org/10.1007/978-3-319-72473-7_7-1
4. Becker K, Sander P (2016) Mycobacterium tuberculosis lipoproteins in virulence and immunity - fighting with a double-edged sword. FEBS letters 590 (21):3800-3819. doi:10.1002/1873-3468.12273
5. Neyrolles O, Guilhot C (2011) Recent advances in deciphering the contribution of Mycobacterium tuberculosis lipids to pathogenesis. Tuberculosis 91 (3):187-195. doi:10.1016/j.tube.2011.01.002
6. Ray A, Cot M, Puzo G, Gilleron M, Nigou J (2013) Bacterial cell wall macroamphiphiles: pathogen-/microbe-associated molecular patterns detected by mammalian innate immune system. Biochimie 95 (1):33-42. doi:10.1016/j.biochi.2012.06.007

7. Chiaradia L, Lefebvre C, Parra J, Marcoux J, Burlet-Schiltz O, Etienne G, Tropis M, Daffe M (2017) Dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane. *Scientific reports* 7 (1):12807. doi:10.1038/s41598-017-12718-4
8. Hoffmann C, Leis A, Niederweis M, Plitzko JM, Engelhardt H (2008) Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proceedings of the National Academy of Sciences of the United States of America* 105 (10):3963-3967. doi:10.1073/pnas.0709530105
9. Zuber B, Chami M, Houssin C, Dubochet J, Griffiths G, Daffe M (2008) Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. *Journal of bacteriology* 190 (16):5672-5680. doi:10.1128/JB.01919-07
10. Bansal-Mutalik R, Nikaido H (2014) Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides. *Proceedings of the National Academy of Sciences of the United States of America* 111 (13):4958-4963. doi:10.1073/pnas.1403078111
11. Sani M, Houben EN, Geurtsen J, Pierson J, de Punder K, van Zon M, Wever B, Piersma SR, Jimenez CR, Daffe M, Appelmek BJ, Bitter W, van der Wel N, Peters PJ (2010) Direct visualization by cryo-EM of the mycobacterial capsular layer: a labile structure containing ESX-1-secreted proteins. *PLoS pathogens* 6 (3):e1000794. doi:10.1371/journal.ppat.1000794
12. Layre E, Sweet L, Hong S, Madigan CA, Desjardins D, Young DC, Cheng TY, Annand JW, Kim K, Shamputa IC, McConnell MJ, Debono CA, Behar SM, Minnaard AJ, Murray M, Barry CE, 3rd, Matsunaga I, Moody DB (2011) A comparative lipidomics platform for chemotaxonomic analysis of *Mycobacterium tuberculosis*. *Chemistry & biology* 18 (12):1537-1549. doi:10.1016/j.chembiol.2011.10.013
13. Becker K, Haldimann K, Selchow P, Reinau LM, Dal Molin M, Sander P (2017) Lipoprotein Glycosylation by Protein-O-Mannosyltransferase (MAB_1122c) Contributes to Low Cell Envelope Permeability and Antibiotic Resistance of *Mycobacterium abscessus*. *Frontiers in microbiology* 8:2123. doi:10.3389/fmicb.2017.02123
14. Laneelle MA, Nigou J, Daffe M (2015) Lipid and lipoarabinomannan isolation and characterization. *Methods in molecular biology* 1285:77-103. doi:10.1007/978-1-4939-2450-9_5
15. Parra J, Marcoux J, Poncin I, Canaan S, Herrmann JL, Nigou J, Burlet-Schiltz O, Riviere M (2017) Scrutiny of *Mycobacterium tuberculosis* 19 kDa antigen proteoforms provides new insights in the lipoglycoprotein biogenesis paradigm. *Scientific reports* 7:43682. doi:10.1038/srep43682
16. Nigou J, Gilleron M, Brando T, Puzo G (2004) Structural analysis of mycobacterial lipoglycans. *Applied biochemistry and biotechnology* 118 (1-3):253-267. doi:10.1385/abab:118:1-3:253
17. Slayden RA, Barry CE, 3rd (2001) Analysis of the Lipids of *Mycobacterium tuberculosis*. *Methods in molecular medicine* 54:229-245. doi:10.1385/1-59259-147-7:229
18. Lanelle M, Nigou J, Daff M (2005) Lipid and Lipoarabinomannan Isolation and Characterization. In: T. P, D. R (eds) *Mycobacteria Protocols*, vol 1285. Humana Press, New York, NY. doi:https://doi.org/10.1007/978-1-4939-2450-9_5
19. Portevin D, Sukumar S, Coscolla M, Shui G, Li B, Guan XL, Bendt AK, Young D, Gagneux S, Wenk MR (2014) Lipidomics and genomics of *Mycobacterium tuberculosis* reveal lineage-specific trends in mycolic acid biosynthesis. *MicrobiologyOpen* 3 (6):823-835. doi:10.1002/mbo3.193
20. Shui G, Bendt AK, Jappara IA, Lim HM, Laneelle M, Herve M, Via LE, Chua GH, Bratschi MW, Zainul Rahim SZ, Michelle AL, Hwang SH, Lee JS, Eum SY, Kwak HK, Daffe M, Dartois V, Michel G, Barry CE, 3rd, Wenk MR (2012) Mycolic acids as diagnostic markers for tuberculosis case detection in humans and drug efficacy in mice. *EMBO molecular medicine* 4 (1):27-37. doi:10.1002/emmm.201100185

21. Layre E, Paepe DC, Larrouy-Maumus G, Vaubourgeix J, Mundayoor S, Lindner B, Puzo G, Gilleron M (2011) Deciphering sulfoglycolipids of *Mycobacterium tuberculosis*. *Journal of lipid research* 52 (6):1098-1110. doi:10.1194/jlr.M013482
22. Jain M, Petzold CJ, Schelle MW, Leavell MD, Mougous JD, Bertozzi CR, Leary JA, Cox JS (2007) Lipidomics reveals control of *Mycobacterium tuberculosis* virulence lipids via metabolic coupling. *Proceedings of the National Academy of Sciences of the United States of America* 104 (12):5133-5138. doi:10.1073/pnas.0610634104
23. Larrouy-Maumus G, Puzo G (2015) Mycobacterial envelope lipids fingerprint from direct MALDI-TOF MS analysis of intact bacilli. *Tuberculosis* 95 (1):75-85. doi:10.1016/j.tube.2014.11.001
24. Gilleron M, Lindner B, Puzo G (2006) MS/MS approach for characterization of the fatty acid distribution on mycobacterial phosphatidyl-myo-inositol mannosides. *Analytical chemistry* 78 (24):8543-8548. doi:10.1021/ac061574a
25. Hsu FF, Turk J, Owens RM, Rhoades ER, Russell DG (2007) Structural characterization of phosphatidyl-myo-inositol mannosides from *Mycobacterium bovis* Bacillus Calmette Guerin by multiple-stage quadrupole ion-trap mass spectrometry with electrospray ionization. I. PIMs and lyso-PIMs. *Journal of the American Society for Mass Spectrometry* 18 (3):466-478. doi:10.1016/j.jasms.2006.10.012
26. Hsu FF, Turk J, Owens RM, Rhoades ER, Russell DG (2007) Structural characterization of phosphatidyl-myo-inositol mannosides from *Mycobacterium bovis* Bacillus Calmette Guerin by multiple-stage quadrupole ion-trap mass spectrometry with electrospray ionization. II. Monoacyl- and diacyl-PIMs. *Journal of the American Society for Mass Spectrometry* 18 (3):479-492. doi:10.1016/j.jasms.2006.10.020
27. Fujita Y, Naka T, Doi T, Yano I (2005) Direct molecular mass determination of trehalose monomycolate from 11 species of mycobacteria by MALDI-TOF mass spectrometry. *Microbiology* 151 (Pt 5):1443-1452. doi:10.1099/mic.0.27791-0
28. Fujita Y, Naka T, McNeil MR, Yano I (2005) Intact molecular characterization of cord factor (trehalose 6,6'-dimycolate) from nine species of mycobacteria by MALDI-TOF mass spectrometry. *Microbiology* 151 (Pt 10):3403-3416. doi:10.1099/mic.0.28158-0
29. Madigan CA, Cheng TY, Layre E, Young DC, McConnell MJ, Debono CA, Murry JP, Wei JR, Barry CE, 3rd, Rodriguez GM, Matsunaga I, Rubin EJ, Moody DB (2012) Lipidomic discovery of deoxysiderophores reveals a revised mycobactin biosynthesis pathway in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America* 109 (4):1257-1262. doi:10.1073/pnas.1109958109
30. Sartain MJ, Dick DL, Rithner CD, Crick DC, Belisle JT (2011) Lipidomic analyses of *Mycobacterium tuberculosis* based on accurate mass measurements and the novel "Mtb LipidDB". *Journal of lipid research* 52 (5):861-872. doi:10.1194/jlr.M010363
31. Galagan JE, Minch K, Peterson M, Lyubetskaya A, Azizi E, Sweet L, Gomes A, Rustad T, Dolganov G, Glotova I, Abeel T, Mahwinney C, Kennedy AD, Allard R, Brabant W, Krueger A, Jaini S, Honda B, Yu WH, Hickey MJ, Zucker J, Garay C, Weiner B, Sisk P, Stolte C, Winkler JK, Van de Peer Y, Iazzetti P, Camacho D, Dreyfuss J, Liu Y, Dorhoi A, Mollenkopf HJ, Drogaris P, Lamontagne J, Zhou Y, Piquenot J, Park ST, Raman S, Kaufmann SH, Mohny RP, Chelsky D, Moody DB, Sherman DR, Schoolnik GK (2013) The *Mycobacterium tuberculosis* regulatory network and hypoxia. *Nature* 499 (7457):178-183. doi:10.1038/nature12337
32. Layre E, Lee HJ, Young DC, Martinot AJ, Buter J, Minnaard AJ, Annand JW, Fortune SM, Snider BB, Matsunaga I, Rubin EJ, Alber T, Moody DB (2014) Molecular profiling of *Mycobacterium tuberculosis* identifies tuberculosinyl nucleoside products of the virulence-associated enzyme Rv3378c. *Proceedings of the National Academy of Sciences of the United States of America* 111 (8):2978-2983. doi:10.1073/pnas.1315883111

33. Sabareesh V, Singh G (2013) Mass spectrometry based lipid(ome) analyzer and molecular platform: a new software to interpret and analyze electrospray and/or matrix-assisted laser desorption/ionization mass spectrometric data of lipids: a case study from *Mycobacterium tuberculosis*. *Journal of mass spectrometry* : JMS 48 (4):465-477. doi:10.1002/jms.3163
34. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G (2006) XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Analytical chemistry* 78 (3):779-787. doi:10.1021/ac051437y
35. Pluskal T, Castillo S, Villar-Briones A, Oresic M (2010) MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC bioinformatics* 11:395. doi:10.1186/1471-2105-11-395
36. Tsugawa H, Cajka T, Kind T, Ma Y, Higgins B, Ikeda K, Kanazawa M, VanderGheynst J, Fiehn O, Arita M (2015) MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. *Nature methods* 12 (6):523-526. doi:10.1038/nmeth.3393
37. Adusumilli R, Mallick P (2017) Data Conversion with ProteoWizard msConvert. *Methods in molecular biology* 1550:339-368. doi:10.1007/978-1-4939-6747-6_23
38. Kessner D, Chambers M, Burke R, Agus D, Mallick P (2008) ProteoWizard: open source software for rapid proteomics tools development. *Bioinformatics* 24 (21):2534-2536. doi:10.1093/bioinformatics/btn323

Figure/Table Legends

Figure 1: Examples of unique lipids extracted by organic solvents from the mycobacterial envelope. Exposure of mycobacteria with organic solvents allows recovering the “extractable lipids” that compose the plasma membrane and the mycomembrane. AG: arabinogalactan, LAM: lipoarabinomannan, LM: lipomannan, LP: lipoprotein, MA: mycolic acids.

Table 1: List of detected mycobacterial lipid families and acylforms. Are listed the formula of the molecular ions of each acylform, the detected m/z of the indicated adduct, and their indicative retention times (subjected to changes depending on the Diol column brand). Detected m/z are listed for only one ion-mode. In positive ion-mode all lipid families are detected and $[M+NH_4]^+$ and $[M+H]^+$ adducts are the most commonly detected adducts. In negative ion mode, detected lipids are those that are naturally negatively charged $[M-H]^-$ such as the phosphated and sulphated lipids (phospholipids, PIM and sulfoglycolipids) while neutral glycolipids are either not detected or detected as $[M+HCOO]^-$ adducts depending on the MS instrument. Coverage of the repertoire of *M. tuberculosis* lipids will depend on the mass spectrometer’s performances. It is to note that TDM is absent of the list. TDM produced by *Mycobacterium* species is a family of very large (m/z centered ~ 2700 a.m.u) and hydrophobic acylforms, which are poorly ionising by electrospray. Therefore, TDM cannot be listed as routinely detected by HPLC-MS. TLC and MALDI-Tof are currently the most successful methods for TDM detection [28].

Table 2: Excerpt of the MycoMass database illustrating the provided data and their organisation, which follows the LIPIDMAPS classification.

Table 1

Lipid family	Formula	Ion-mode/Adduct	Calculated m/z	Indicative RT (min)
phthiocerol dimycocerosate PDIM A/A'	C87H172O5	pos/[M+NH4] ⁺	1315.3543	3.6
	C88H174O5	pos/[M+NH4] ⁺	1329.3699	
	C89H176O5	pos/[M+NH4] ⁺	1343.3856	
	C90H178O5	pos/[M+NH4] ⁺	1357.4012	
	C91H180O5	pos/[M+NH4] ⁺	1371.4169	
	C92H182O5	pos/[M+NH4] ⁺	1385.4325	
	C93H184O5	pos/[M+NH4] ⁺	1399.4482	
	C94H186O5	pos/[M+NH4] ⁺	1413.4638	
	C95H188O5	pos/[M+NH4] ⁺	1427.4795	
	C96H190O5	pos/[M+NH4] ⁺	1441.4951	
	C97H192O5	pos/[M+NH4] ⁺	1455.5108	
	C98H194O5	pos/[M+NH4] ⁺	1469.5264	
	C99H196O5	pos/[M+NH4] ⁺	1483.5421	
	C100H198O5	pos/[M+NH4] ⁺	1497.5577	
	C101H200O5	pos/[M+NH4] ⁺	1511.5734	
	C102H202O5	pos/[M+NH4] ⁺	1525.5891	
phthiocerol dimycocerosate PDIM B	C87H170O5	pos/[M+NH4] ⁺	1313.3387	
	C88H172O5	pos/[M+NH4] ⁺	1327.3543	
	C89H174O5	pos/[M+NH4] ⁺	1341.3700	
	C90H176O5	pos/[M+NH4] ⁺	1355.3856	
	C91H178O5	pos/[M+NH4] ⁺	1369.4013	
	C92H180O5	pos/[M+NH4] ⁺	1383.4169	
	C93H182O5	pos/[M+NH4] ⁺	1397.4326	
	C94H184O5	pos/[M+NH4] ⁺	1411.4482	
	C95H186O5	pos/[M+NH4] ⁺	1425.4639	
	C96H188O5	pos/[M+NH4] ⁺	1439.4795	
	C97H190O5	pos/[M+NH4] ⁺	1453.4952	
	C98H192O5	pos/[M+NH4] ⁺	1467.5108	
	C99H194O5	pos/[M+NH4] ⁺	1481.5265	
	C100H196O5	pos/[M+NH4] ⁺	1495.5421	
	C101H198O5	pos/[M+NH4] ⁺	1509.5578	
	C102H200O5	pos/[M+NH4] ⁺	1523.5734	

Table 1

mycolic acids	C81H160O4	neg/[M-H]-	1196.2244	3.6-3.7
	C82H162O4	neg/[M-H]-	1210.2400	
	C83H164O4	neg/[M-H]-	1224.2557	
	C84H166O4	neg/[M-H]-	1238.2713	
	C85H168O4	neg/[M-H]-	1252.2870	
	C87H172O4	neg/[M-H]-	1280.3183	
	C88H174O4	neg/[M-H]-	1294.3339	
triacylglycerols	C51H98O6	pos/[M+NH4]+	824.7706	3.7
	C53H100O6	pos/[M+NH4]+	850.7864	
	C54H102O6	pos/[M+NH4]+	864.8020	
	C55H104O6	pos/[M+NH4]+	878.8177	
	C56H108O6	pos/[M+NH4]+	894.8488	
	C57H108O6	pos/[M+NH4]+	906.8490	
	C57H110O6	pos/[M+NH4]+	908.8645	
	C58H112O6	pos/[M+NH4]+	922.8801	
	C59H112O6	pos/[M+NH4]+	934.8803	
	C60H116O6	pos/[M+NH4]+	950.9114	
	C61H116O6	pos/[M+NH4]+	962.9116	
	C61H118O6	pos/[M+NH4]+	964.9271	
	C62H120O6	pos/[M+NH4]+	978.9427	
	C63H122O6	pos/[M+NH4]+	992.9584	
diacylglycerols	C35H68O5	pos/[M+NH4]+	586.5410	3.8-3.9
	C36H70O5	pos/[M+NH4]+	600.5567	
	C37H70O5	pos/[M+NH4]+	612.5567	
	C37H72O5	pos/[M+NH4]+	614.5723	
	C38H74O5	pos/[M+NH4]+	628.5880	
	C39H74O5	pos/[M+NH4]+	640.5880	
menaquinone	C56H80O2	pos/[M+H]+	785.6231	3.9
	C56H82O2	pos/[M+H]+	787.6388	
	C56H80O2	pos/[M+NH4]+	802.6491	
	C56H82O2	pos/[M+NH4]	804.6648	
triglycosylated phenolic glycolipids (PGL)	C107H198O18	pos/[M+NH4]+	1789.491649	4
	C108H200O18	pos/[M+NH4]+	1803.507299	
	C109H202O18	pos/[M+NH4]+	1817.522949	

Table 1

	C110H204O18	pos/[M+NH4] ⁺	1831.538599	
	C111H206O18	pos/[M+NH4] ⁺	1845.554249	
	C112H208O18	pos/[M+NH4] ⁺	1859.569899	
	C113H210O18	pos/[M+NH4] ⁺	1873.585549	
	C114H212O18	pos/[M+NH4] ⁺	1887.601199	
	C115H214O18	pos/[M+NH4] ⁺	1901.616849	
	C116H216O18	pos/[M+NH4] ⁺	1915.632499	
	C117H218O18	pos/[M+NH4] ⁺	1929.648149	
	C118H220O18	pos/[M+NH4] ⁺	1943.663799	
	C119H222O18	pos/[M+NH4] ⁺	1957.679449	
	C120H224O18	pos/[M+NH4] ⁺	1971.695099	
glucose monomycolate alpha	C80H154O8	pos/[M+NH4] ⁺	1261.1982	
	C82H158O8	pos/[M+NH4] ⁺	1289.2295	
	C84H162O8	pos/[M+NH4] ⁺	1317.2608	
	C86H166O8	pos/[M+NH4] ⁺	1345.2921	
glucose monomycolate keto	C80H154O9	pos/[M+NH4] ⁺	1277.1931	
	C81H156O9	pos/[M+NH4] ⁺	1291.2088	
	C83H160O9	pos/[M+NH4] ⁺	1319.2401	
	C85H164O9	pos/[M+NH4] ⁺	1347.2714	
	C87H168O9	pos/[M+NH4] ⁺	1375.3027	
	C88H170O9	pos/[M+NH4] ⁺	1389.3183	
	C92H178O9	pos/[M+NH4] ⁺	1445.3809	
glucose monomycolate methoxy	C87H170O9	pos/[M+NH4] ⁺	1377.3183	
	C89H174O9	pos/[M+NH4] ⁺	1405.3496	
	C91H178O9	pos/[M+NH4] ⁺	1433.3809	
	C93H182O9	pos/[M+NH4] ⁺	1461.4122	
	C95H186O9	pos/[M+NH4] ⁺	1489.4435	
trehalose monomycolate alpha	C88H168O13	pos/[M+NH4] ⁺	1451.2823	
	C89H170O13	pos/[M+NH4] ⁺	1465.2980	
	C90H172O13	pos/[M+NH4] ⁺	1479.3136	
	C91H174O13	pos/[M+NH4] ⁺	1493.3293	
	C92H176O13	pos/[M+NH4] ⁺	1507.3449	
	C94H180O13	pos/[M+NH4] ⁺	1535.3398	
	C96H184O13	pos/[M+NH4] ⁺	1563.3711	

4.2

6.2-6.6

Table 1

trehalose monomycolate keto	C86H164O14	pos/[M+NH4] ⁺	1439.2459	
	C88H168O14	pos/[M+NH4] ⁺	1467.2772	
	C89H170O14	pos/[M+NH4] ⁺	1481.2929	
	C90H172O14	pos/[M+NH4] ⁺	1495.3085	
	C91H174O14	pos/[M+NH4] ⁺	1509.3242	
	C92H176O14	pos/[M+NH4] ⁺	1523.3398	
	C93H178O14	pos/[M+NH4] ⁺	1537.3555	
	C97H186O14	pos/[M+NH4] ⁺	1593.4181	
	C99H190O14	pos/[M+NH4] ⁺	1621.4494	
	C100H192O14	pos/[M+NH4] ⁺	1635.4650	
trehalose monomycolate methoxy	C89H172O14	pos/[M+NH4] ⁺	1483.3085	
	C91H176O14	pos/[M+NH4] ⁺	1511.3398	
	C93H180O14	pos/[M+NH4] ⁺	1539.3711	
	C95H184O14	pos/[M+NH4] ⁺	1567.4024	
	C97H188O14	pos/[M+NH4] ⁺	1595.4337	
	C98H190O14	pos/[M+NH4] ⁺	1609.4494	
	C99H192O14	pos/[M+NH4] ⁺	1623.4650	
	C100H194O14	pos/[M+NH4] ⁺	1637.4807	
	C101H196O14	pos/[M+NH4] ⁺	1651.4963	
	C102H198O14	pos/[M+NH4] ⁺	1665.5120	
	C103H200O14	pos/[M+NH4] ⁺	1679.5276	
diacyltrehalose	C52H98O13	pos/[M+NH4] ⁺	948.7345	5.7-6.3
	C53H100O13	pos/[M+NH4] ⁺	962.7502	
	C54H102O13	pos/[M+NH4] ⁺	976.7658	
	C55H104O13	pos/[M+NH4] ⁺	990.7815	
	C56H106O13	pos/[M+NH4] ⁺	1004.7971	
mycobactins	C46H75O10N5	pos/[M+Fe-2H] ⁺	911.4701	6.7-6.8
	C47H77O10N5	pos/[M+Fe-2H] ⁺	925.4858	
	C45H71O10N5	pos/[M+Fe-2H] ⁺	895.4388	
	C46H73O10N5	pos/[M+Fe-2H] ⁺	909.4545	
	C47H75O10N5	pos/[M+Fe-2H] ⁺	923.4701	
tetraacylated sulfoglycolipids	C132H254O20S	neg/[M-H] ⁻	2190.8506	6.7-7.2
	C133H256O20S	neg/[M-H] ⁻	2204.8663	
	C134H258O20S	neg/[M-H] ⁻	2218.8819	

Table 1

	C135H260O20S	neg/[M-H]-	2232.8976	
	C136H262O20S	neg/[M-H]-	2246.9132	
	C137H264O20S	neg/[M-H]-	2260.9289	
	C138H266O20S	neg/[M-H]-	2274.9445	
	C139H268O20S	neg/[M-H]-	2288.9602	
	C140H270O20S	neg/[M-H]-	2302.9758	
	C139H268O21S	neg/[M-H]-	2304.9551	
	C141H272O20S	neg/[M-H]-	2316.9915	
	C140H270O21S	neg/[M-H]-	2318.9708	
	C142H274O20S	neg/[M-H]-	2331.0071	
	C141H272O21S	neg/[M-H]-	2332.9864	
	C143H276O20S	neg/[M-H]-	2345.0228	
	C142H274O21S	neg/[M-H]-	2347.0021	
	C144H278O20S	neg/[M-H]-	2359.0384	
	C143H276O21S	neg/[M-H]-	2361.0177	
	C144H276O21S	neg/[M-H]-	2373.0177	
	C146H282O20S	neg/[M-H]-	2387.0697	
	C145H280O21S	neg/[M-H]-	2389.0490	
	C147H284O20S	neg/[M-H]-	2401.0854	
	C146H282O21S	neg/[M-H]-	2403.0647	
	C148H286O20S	neg/[M-H]-	2415.1010	
	C147H284O21S	neg/[M-H]-	2417.0803	
	C149H288O20S	neg/[M-H]-	2429.1167	
	C148H286O21S	neg/[M-H]-	2431.0960	
	C150H290O20S	neg/[M-H]-	2443.1323	
	C149H288O21S	neg/[M-H]-	2445.1116	
	C151H292O20S	neg/[M-H]-	2457.1480	
	C150H290O21S	neg/[M-H]-	2459.1273	
	C152H294O20S	neg/[M-H]-	2471.1636	
	C151H292O21S	neg/[M-H]-	2473.1429	
	C152H292O21S	neg/[M-H]-	2485.1429	
	C152H294O21S	neg/[M-H]-	2487.1586	
	C153H294O21S	neg/[M-H]-	2499.1586	
	C153H296O21S	neg/[M-H]-	2501.1742	

Table 1

	C154H296O21S	neg/[M-H]-	2513.1742	
	C154H298O21S	neg/[M-H]-	2515.1899	
	C155H298O21S	neg/[M-H]-	2527.1899	
	C155H300O21S	neg/[M-H]-	2529.2055	
	C156H300O21S	neg/[M-H]-	2541.2055	
	C156H302O21S	neg/[M-H]-	2543.2212	
	C157H302O21S	neg/[M-H]-	2555.2212	
	C157H304O21S	neg/[M-H]-	2557.2368	
	C158H304O21S	neg/[M-H]-	2569.2368	
	C158H306O21S	neg/[M-H]-	2571.2525	
	C159H306O21S	neg/[M-H]-	2583.2525	
	C159H308O21S	neg/[M-H]-	2585.2681	
	C160H308O21S	neg/[M-H]-	2597.2681	
	C160H310O21S	neg/[M-H]-	2599.2838	
	C161H312O21S	neg/[M-H]-	2613.2994	
	C162H312O21S	neg/[M-H]-	2625.2994	
	C162H314O21S	neg/[M-H]-	2627.3151	
	C163H316O21S	neg/[M-H]-	2641.3307	
	C164H318O21S	neg/[M-H]-	2655.3464	
	C165H320O21S	neg/[M-H]-	2669.3620	
	C166H322O21S	neg/[M-H]-	2683.3777	
	C167H324O21S	neg/[M-H]-	2697.3933	
	C168H326O21S	neg/[M-H]-	2711.4090	
	C169H328O21S	neg/[M-H]-	2725.4246	
	C170H330O21S	neg/[M-H]-	2739.4403	
	C171H332O21S	neg/[M-H]-	2753.4559	
	C172H334O21S	neg/[M-H]-	2767.4716	
	C173H336O21S	neg/[M-H]-	2781.4872	
N6-tuberculosinyladenosine	C30H45N5O4	pos/[M+H]+	540.3544	7-8
triacylated sulfoglycolipids	C102H196O18S	neg/[M-H]-	1740.4070	12.8
	C102H194O19S	neg/[M-H]-	1754.3862	
	C103H196O19S	neg/[M-H]-	1768.4382	

Table 1

	C105H202O18S	neg/[M-H]-	1782.4539	
	C106H204O19S	neg/[M-H]-	1812.4645	
	C108H208O18S	neg/[M-H]-	1824.5009	
	C108H208O19S	neg/[M-H]-	1840.4958	
	C109H210O19S	neg/[M-H]-	1854.5114	
	C110H212O19S	neg/[M-H]-	1868.5271	
	C111H214O19S	neg/[M-H]-	1882.5427	
	C112H216O19S	neg/[M-H]-	1896.5584	
	C113H218O19S	neg/[M-H]-	1910.5740	
	C114H220O19S	neg/[M-H]-	1924.5897	
	C116H224O18S	neg/[M-H]-	1936.6261	
	C115H222O19S	neg/[M-H]-	1938.6053	
	C116H222O19S	neg/[M-H]-	1950.6053	
	C116H224O19S	neg/[M-H]-	1952.6210	
	C117H224O19S	neg/[M-H]-	1964.6210	
	C117H226O19S	neg/[M-H]-	1966.6366	
	C118H226O19S	neg/[M-H]-	1978.6366	
	C118H228O19S	neg/[M-H]-	1980.6523	
	C119H228O19S	neg/[M-H]-	1992.6523	
	C119H230O19S	neg/[M-H]-	1994.6679	
	C120H232O19S	neg/[M-H]-	2008.6836	
diacylated sulfoglycolipids	C67H126O17S	neg/[M-H]-	1233.8643	22.1-22.7
	C67H128O17S	neg/[M-H]-	1235.8799	
	C68H130O17S	neg/[M-H]-	1249.8956	
	C69H130O17S	neg/[M-H]-	1261.8956	
	C69H132O17S	neg/[M-H]-	1263.9110	
	C70H132O17S	neg/[M-H]-	1275.9476	
	C70H134O17S	neg/[M-H]-	1277.9269	
	C71H136O17S	neg/[M-H]-	1291.9425	
	C72H136O17S	neg/[M-H]-	1303.9425	
	C74H142O16S	neg/[M-H]-	1317.9946	
	C73H140O17S	neg/[M-H]-	1319.9738	
	C74H142O17S	neg/[M-H]-	1333.9895	
	C75H142O17S	neg/[M-H]-	1345.9895	

Table 1

	C76H144O17S	neg/[M-H]-	1360.0051	
	C76H146O17S	neg/[M-H]-	1362.0208	
	C77H148O17S	neg/[M-H]-	1376.0364	
phosphatidylglycerol	C38H73O10P	neg/[M-H]-	719.4868	22.9-23.5
	C38H75O10P	neg/[M-H]-	721.5025	
	C39H77O10P	neg/[M-H]-	735.5181	
	C40H75O10P	neg/[M-H]-	745.4984	
	C40H77O10P	neg/[M-H]-	747.5181	
	C40H79O10P	neg/[M-H]-	749.5338	
	C41H79O10P	neg/[M-H]-	761.5338	
	C41H81O10P	neg/[M-H]-	763.5494	
	C42H79O10P	neg/[M-H]-	773.5338	
	C42H81O10P	neg/[M-H]-	775.5494	
	C42H83O10P	neg/[M-H]-	777.5651	
1-tuberculosinyladenosine	C30H46N5O4	pos/[M]+	540.3544	24-25
mannosyl phosphomycoketide	C38H77O9P	neg/[M-H]-	707.5196	24.5
cardiolipin	C71H134O17P2	neg/[M-H]-	1319.9024	24.9-25.9
	C71H136O17P2	neg/[M-H]-	1321.9180	
	C72H136O17P2	neg/[M-H]-	1333.9180	
	C72H138O17P2	neg/[M-H]-	1335.9337	
	C73H136O17P2	neg/[M-H]-	1345.9180	
	C73H138O17P2	neg/[M-H]-	1347.9337	
	C73H140O17P2	neg/[M-H]-	1349.9493	
	C74H138O17P2	neg/[M-H]-	1359.9337	
	C74H140O17P2	neg/[M-H]-	1361.9493	
	C74H142O17P2	neg/[M-H]-	1363.9650	
	C75H140O17P2	neg/[M-H]-	1373.9493	
	C75H142O17P2	neg/[M-H]-	1375.9650	
	C75H144O17P2	neg/[M-H]-	1377.9806	
	C76H142O17P2	neg/[M-H]-	1387.9650	
	C76H144O17P2	neg/[M-H]-	1389.9806	
	C76H146O17P2	neg/[M-H]-	1391.9963	
	C77H144O17P2	neg/[M-H]-	1401.9806	

Table 1

	C77H146O17P2	neg/[M-H]-	1403.9963	
	C77H146O17P2	neg/[M-H]-	1406.0119	
	C78H146O17P2	neg/[M-H]-	1415.9963	
	C78H148O17P2	neg/[M-H]-	1418.0119	
	C78H150O17P2	neg/[M-H]-	1420.0276	
	C79H148O17P2	neg/[M-H]-	1430.0119	
	C79H150O17P2	neg/[M-H]-	1432.0276	
	C79H152O17P2	neg/[M-H]-	1434.0432	
	C80H150O17P2	neg/[M-H]-	1444.0276	
	C80H152O17P2	neg/[M-H]-	1446.0432	
	C80H154O17P2	neg/[M-H]-	1448.0589	
	C81H152O17P2	neg/[M-H]-	1458.0432	
	C81H154O17P2	neg/[M-H]-	1460.0589	
	C81H156O17P2	neg/[M-H]-	1462.0745	
	C82H154O17P2	neg/[M-H]-	1472.0589	
	C82H156O17P2	neg/[M-H]-	1474.0745	
	C82H158O17P2	neg/[M-H]-	1476.0902	
	C83H156O17P2	neg/[M-H]-	1486.0745	
	C83H158O17P2	neg/[M-H]-	1488.0902	
	C83H160O17P2	neg/[M-H]-	1490.1058	
phosphatidyl-ethanolamines	C35H70O8NP	neg/[M-H]-	662.4766	25.7-26.7
	C36H72O8NP	neg/[M-H]-	676.4923	
	C37H72O8NP	neg/[M-H]-	688.4923	
	C37H74O8NP	neg/[M-H]-	690.5079	
	C38H74O8NP	neg/[M-H]-	702.5079	
	C35H66O8NP	neg/[M-H]-	704.5236	
	C39H74O8NP	neg/[M-H]-	714.5079	
	C39H76O8NP	neg/[M-H]-	716.5236	
	C39H78O8NP	neg/[M-H]-	718.5392	
	C40H78O8NP	neg/[M-H]-	730.5392	
	C40H80O8NP	neg/[M-H]-	732.5549	
	C41H80O8NP	neg/[M-H]-	744.5549	
	C41H82O8NP	neg/[M-H]-	746.5705	
	C42H84O8NP	neg/[M-H]-	760.5862	

Table 1

	C43H86O8NP	neg/[M-H]-	774.6018	
	C44H84O8NP	neg/[M-H]-	784.5862	
	C44H86O8NP	neg/[M-H]-	786.6018	
phosphatidylinositols	C41H79O13P	neg/[M-H]-	809.5186	26.2-27.4
	C42H81O13P	neg/[M-H]-	823.5342	
	C43H79O13P	neg/[M-H]-	833.5186	
	C43H81O13P	neg/[M-H]-	835.5342	
	C43H83O13P	neg/[M-H]-	837.5499	
	C44H83O13P	neg/[M-H]-	849.5499	
	C44H85O13P	neg/[M-H]-	851.5655	
	C45H85O13P	neg/[M-H]-	863.5655	
	C45H87O13P	neg/[M-H]-	865.5812	
	C46H87O13P	neg/[M-H]-	877.5812	
	C46H89O13P	neg/[M-H]-	879.5968	
lysophosphatidyl ethanolamines	C23H48O7NP	neg/[M-H]-	480.3096	30.7-31.9
	C24H50O7NP	neg/[M-H]-	494.3252	
	C25H52O7NP	neg/[M-H]-	508.3409	
lysophosphatidyl-inositols	C25H49O12P	neg/[M-H]-	571.2889	32.2
	C27H53O12P	neg/[M-H]-	599.3202	
	C28H55O12P	neg/[M-H]-	613.3359	
diacyl phosphatidylinositol dimannoside (PIM2Ac2)	C88H165O25P	neg/[M-H]-	1652.1304	25.6
	C90H169O25P	neg/[M-H]-	1680.1617	
	C91H171O25P	neg/[M-H]-	1694.1774	
monoacyl phosphatidylinositol trimannosides (PIM3Ac1)	C78H145O29P	neg/[M-H]-	1575.9536	32.1
	C80H149O29P	neg/[M-H]-	1603.9849	
monoacyl phosphatidylinositol dimannosides (PIM2Ac1)	C72H135O24P	neg/[M-H]-	1413.9008	32.1
	C74H139O24P	neg/[M-H]-	1441.9321	
phosphatidyl inositol dimannoside (PIM2)	C53H99O23P	neg/[M-H]-	1133.6241	32.5
	C56H105O23P	neg/[M-H]-	1175.6711	
monoacyl phosphatidylinositol hexamannoside (PIM6Ac1)	C96H175O44P	neg/[M-H]-	2062.1121	37.8
	C98H179O44P	neg/[M-H]-	2090.1434	
diacyl phosphatidylinositol hexamannoside (PIM6Ac2)	C114H209O45P	neg/[M-H]-	2328.3730	37.5
	C115H211O45P	neg/[M-H]-	2342.3887	

Table 1