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TITLE:

Analysis of the Lipid Composition of Mycobacteria by Thin Layer Chromatography

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SUMMARY:

A protocol is presented to extract the total lipid content of the cell wall of a wide range of mycobacteria. Moreover, extraction and analytical protocols of the different types of mycolic acids are shown. A thin-layer chromatographic protocol to monitor these mycobacterial compounds is also provided.

ABSTRACT:

Mycobacteria species can differ from one another in the rate of growth, presence of pigmentation, the colony morphology displayed on solid media, as well as other phenotypic characteristics. However, they all have in common the most relevant character of mycobacteria: its unique and highly hydrophobic cell wall. Mycobacteria species contain a membrane-covalent linked complex that includes arabinogalactan, peptidoglycan, and long-chains of mycolic acids with types that differ between mycobacteria species. Additionally, mycobacteria can also produce lipids that are located, non-covalently linked, on their cell surfaces, such as phthiocerol dimycocerosates (PDIM), phenolic glycolipids (PGL), glycopeptidolipids (GPL), acyltrehaloses (AT), or phosphatidil-inositol mannosides (PIM), among others. Some of them are considered virulence factors in pathogenic mycobacteria, or critical antigenic lipids in host-mycobacteria interaction. For these reasons, there is a significant interest in the study of mycobacterial lipids due to their application in several fields, from understanding their role in the pathogenicity of mycobacteria infections, to a possible implication as immunomodulatory agents for the treatment of infectious diseases and other pathologies such as cancer. Here, a simple approach to extract and analyze the total lipid content and the mycolic acid composition of mycobacteria cells grown in a solid medium using mixtures of organic solvents is presented. Once the lipid extracts are obtained, thin-layer chromatography (TLC) is performed to monitor the extracted compounds. The example

experiment is performed with four different mycobacteria: the environmental fast-growing *Mycolicibacterium brumae and Mycolicibacterium fortuitum,* the attenuated slow-growing *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), and the opportunistic pathogen fast-growing *Mycobacterium abscessus,* demonstrating that methods shown in the present protocol can be used to a wide range of mycobacteria.

INTRODUCTION:

Mycobacterium is a genus that comprises pathogenic and non-pathogenic species, characterized by having a highly hydrophobic and impermeable cell wall formed by their peculiar lipids. Specifically, the mycobacterial cell wall contains mycolic acids, which are α-alkyl and β-hydroxy fatty acids, in which the α-branch is constant in all mycolic acids (except for the length) and the β-chain, called the meromycolate chain, is a long aliphatic chain that may contain different functional chemical groups described along with the literature (α-, α'-, methoxy-, κ-, epoxy-, carboxy-, and ω-1-methoxy- mycolates), therefore producing seven types of mycolic acids (I–VII)[1]. Moreover, other lipids with unquestionable importance are also present in the cell wall of mycobacteria species. Pathogenic species such as *Mycobacterium tuberculosis,* the main causative agent of tuberculosis[2] produce specific lipid-based virulence factors such as phthiocerol dimycocerosates (PDIMs), phenolic glycolipid (PGL), di-, tri-, and penta-acyltrehaloses (DAT, TAT, and PAT), or sulfolipids, among others[3]. Their presence on the mycobacterial surface have been associated with the ability to modify the host immune response and therefore, the evolution and persistence of the mycobacterium inside the host[4]. For instance, the presence of triacylglycerols (TAG) has been associated with the hypervirulent phenotype of Lineage 2–Beijing sub-lineage of *M. tuberculosis,* possibly due to its capacity to attenuate the host immune response[5,6]. Other relevant lipids are lipooligosaccharides (LOSs) present in tuberculous and nontuberculous mycobacteria. In the case of *Mycobacterium marinum*, the presence of LOSs in its cell wall is related to sliding motility and the ability to form biofilms and interferes with recognition by macrophage pattern recognition receptors, affecting uptake and elimination of the bacteria by host phagocytes[7,8]. Additionally, the absence or presence of some lipids allows members of the same species to be classified into different morphotypes with virulent or attenuate profiles when interacting with host cells. For instance, the absence of glycopeptidolipids (GPL) in the rough morphotype of *Mycobacterium abscessus* has been associated with the ability to induce intraphagosomal acidification, and consequently cell apoptosis[9], unlike the smooth morphotype that possesses GPLs in their surface. Furthermore, the lipid content of the mycobacterial cell wall is related to the ability to modify the immune response in the host. This is relevant in the context of using some mycobacteria to trigger a protective immune profile against different pathologies[10–13]. It has been demonstrated, for example, that *Mycolicibacterium vaccae*, a saprophytic mycobacterium, which is currently in phase III clinical trials as an immunotherapeutic vaccine for tuberculosis, display two colonial morphotypes. While the smooth phenotype, that contains a polyester in its surface, triggers a Th2 response, the rough phenotype devoid of the polyester can induce a Th1 profile when it interacts with host immune cells[14]. The repertoire of lipids present in the mycobacterial cell not only depends on mycobacteria species, but also on the conditions of mycobacterial cultures: time of incubation[15,16] or composition of the culture medium[17,18]. In fact, changes in the culture medium composition affect the antitumor and immunostimulatory activity of *M. bovis* BCG and *Mycolicibacterium brumae in vitro*[17]. Moreover, the protective immune profile triggered by *M. bovis* BCG against *M. tuberculosis* challenge in mice models also depends on

the culture media in which *M. bovis* BCG grows[17]. These could then be related to the lipid composition of the mycobacteria in each culture condition. For all these reasons, the study of the lipid content of mycobacteria is relevant. A visual procedure to extract and analyze the lipid composition of the mycobacterial cell wall is presented.

PROTOCOL:

1. Extraction of the total non-covalent-linked lipids from mycobacteria (Figure 1)

1.1. Scratch 0.2 g of mycobacteria from a solid media and add to a glass tube with a polytetrafluoroethlene (PTFE) liner screw caps. Add a solution consisting of 5 mL of chloroform and 10 mL of methanol (chloroform:methanol, 1:2).

NOTE: When organic solvents are used, only glass recipient should be used. No plastic containers are allowed. Moreover, PTFE liner screw caps for bottles are needed.

CAUTION: Chloroform is a potentially toxic and extremely hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Methanol is a potentially toxic and extremely hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

1.2. Leave the tube in constant stirring overnight to extract non-covalent-linked lipids from the mycobacterial cell surface.

NOTE: If an orbital shaking platform is not available, constant stirring can be replaced by periodic manual stirring as frequently as possible.

1.3. Cover a glass funnel with a filter paper, filter the organic solvents, and collect them in a glass tube.

1.4. Use a nitrogen gas flux to evaporate the liquid phase in the tube. Fill the tube with nitrogen gas, cover and store it at 4 °C.

NOTE: Connect a glass Pasteur pipette to the stream of nitrogen gas to specifically evaporate the desired tube. Additionally, maintain the tube inside a dry block heater for tubes at 37 °C. When the solvent evaporates, fill the tube with nitrogen gas before closing it.

1.5. Add 15 mL of a solution of chloroform:methanol (2:1) to the cellular debris. Leave the tube in constant stirring overnight to extract non-covalent-linked lipids from the mycobacterial cell surface.

NOTE: If an orbital shaking platform is not available, constant stirring can be replaced by periodic manual stirring as frequently as possible.

1.6. Let the mixture rest for 1 h. With a Pasteur pipette, recover the organic solvents. Cover a glass funnel with a filter paper and filter the organic solvents and collect them in the same glass tube previously used in step 1.3. Use a nitrogen gas flux to evaporate the liquid phase in the tube. Fill the tube with nitrogen gas, close it and store it again at 4 °C.

2. Mycolic acid extraction by acid methanolysis (Figure 2A)

2.1. Add 2–5 mL of esterifying solution into a hermetic glass tube with a PTFE liner screw cap. Add 0.2 g of mycobacteria biomass into the glass tube.

NOTE: Esterifying solution is formed by mixing 30 mL of methanol, 15 mL of toluene, and 1 mL of sulfuric acid. Mycobacteria cells can be taken from solid cultures or, even from delipidated cells after performing extraction of total non-covalent-linked lipids from mycobacteria (remaining cells after filtering in step 1.6).

CAUTION: Toluene is a flammable and extremely hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Sulfuric acid is a corrosive and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

2.2. Mix the content by vortexing. Let the mixture stand inside a dry bath at 80 °C overnight.

2.3. Allow the tube to cool until it reaches the room temperature and then add 2 mL of n-hexane to the tube. Mix the contents by vortexing for 30 s and allow the tube to settle until two clear phases appear.

CAUTION: n-Hexane is a potential flammable, irritant, environmentally damaging, and extremely hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

2.4. Recover the upper phase corresponding to the n-hexane phase. Transfer it to a new tube.

2.5. Repeat the step 2.3. Recover the upper phase again and transfer it to the same tube used in step 2.4.

2.6. Evaporate the contents of the tube using a nitrogen gas flux. Fill the tube with nitrogen gas, close it, and store it at 4 °C.

3. Mycolic acid extraction by saponification and methylation (Figure 2B)

3.1. Scratch 0.2 g of mycobacteria from a solid media and add to a glass tube with a PTFE screw cap.

3.2. Add 2 mL of methanol-benzene solution (80:20) containing 5% potassium hydroxide. Mix

the contents by vortexing. Heat the mixture for 3 h at 100 °C.

CAUTION: Benzene is a flammable, carcinogenic, and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

3.3. Allow the tube to cool to room temperature. Add 20% sulfuric acid to acidify the samples to achieve pH = 1.

3.4. Add 3 mL of diethyl ether. Gently mix the contents by vortexing.

3.5. Let the two phases form by settling. Recover the diethyl ether phase and transfer to a new tube. Repeat the wash step for a total of three times.

3.6. Wash the diethyl ether extract with 2 mL of distilled water and transfer the upper part corresponding to the diethyl ether to a new tube. Repeat the wash step for a total of three times.

3.7. Add 2 g of anhydrous sodium sulfate over the diethyl ether extract to dry it.

3.8. Filter the suspension. Evaporate the content using a nitrogen gas flux.

3.9. To perform the methylation step, dissolve 3 g of N-nitroso-N-methyl urea in a precooled solution formed by 45 mL of diethyl ether and 9 mL of 40% KOH in distilled water.

CAUTION: N-nitroso-N-methylurea is a toxic, irritant, carcinogenic, and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

3.10. Agitate the mixture. Transfer the supernatant (diazomethane) to a new flask cooled in ice containing potassium hydroxide pellets (approximately 30 g).

NOTE: If the supernatant is not immediately used, it can be stored at -20 °C for a maximum of 1 h.

CAUTION: Potassium hydroxide pellets are an irritant and corrosive substance. This material must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Diazomethane is highly toxic and potentially explosive. It must be used in a laminar flow hood with safety glass wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

3.11. Add 2 mL of the ether solution containing diazomethane, obtained in step 3.10, into the dried diethyl ether extract that contains mycolic acids, obtained in step 3.8. Incubate for 15 min at room temperature.

3.12. Evaporate the suspension at 40 °C. Fill the tube with nitrogen gas, close it, and store the methylated lipids at 4 °C.

NOTE: Evaporate the diazomethane from the ether solution under the laminar flow hood, until the ether loses the yellow color.

4. Thin layer chromatography (TLC) analysis

4.1. Saturate the glass TLC chamber. To do this, cover one of the walls of the TLC chamber with a piece of filter paper and allow it to be in contact with the mobile phase composed by the mixture of solvents. Place the remaining volume of the solvent onto the bottom of the TLC chamber.

NOTE: The bottom of the TLC chamber must be covered by at least 1 cm of the mobile phase. In the present experiments, different mobile phases were used to develop the TLCs. They consisted of 85 mL of n-hexane plus 15 mL of diethyl ether; 100 mL of dichloromethane; 90 mL of chloroform, 10 mL of methanol, and 1 mL of water; 30 mL of chloroform, plus 8 mL of methanol, and 1 mL of water; 60 mL of chloroform, plus 35 mL of methanol, and 8 mL of water; 95 mL chloroform plus 5 mL of methanol; and 90 mL of petroleum ether (60–80 °C) plus 10 mL of diethyl ether.

NOTE: In the two-dimensional TLC, use n-hexane:acetone (95:5) in the first direction three times, and use a single development with toluene:acetone (97:3) in the second direction to analyze mycolic acid composition. To analyze PIMs, use chloroform:methanol:water (60:30:6) in the first direction once, and use chloroform:acetic acid:methanol:water (40:25:3:6) in the second direction. To analyze PDIM and AG, use petroleum ether (60–80 °C):ethyl acetate (98:2) in the first direction three times, and use a single development with petroleum ether (60–80 °C):acetone (98:2) in the second direction.

CAUTION: Diethyl ether is a potentially toxic and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Dichloromethane is a potentially toxic and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Petroleum ether is a potential flammable, environmentally damaging and extremely hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Acetic acid is a potential flammable and corrosive substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Ethyl acetate is a flammable and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective

eyewear, and nitrile gloves).

CAUTION: Acetone is a flammable and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

4.2. Close the TLC chamber to saturate it for at least 20 min. Meanwhile, dissolve the lipids present in the glass tube in 0.2–1 mL of chloroform.

NOTE: The volume used to dissolve the lipids can be modified depending on the desired or expected concentration of the sample.

4.3. Apply 10 µL of each suspension using a capillary glass tube directly on the TLC plate and let the sample dry for 5 min at room temperature.

NOTE: Samples must be applied at the bottom part of the plate leaving 1 cm on each side. Samples must be separated one from another for at least 0.5 cm. Once the sample is applied on the plate, tubes can be evaporated again with nitrogen gas and stored at 4 °C for further use.

4.4. Insert the plate into the saturated TLC chamber containing the mobile phase. Allow the mobile phase to run through the TLC.

NOTE: Any movement applied to the TLC chamber affects the running solvent on the plate and affects lipid mobility. In the case of performing two-dimensional TLC, two TLC chambers are required to contain both elution systems.

4.5. Remove the plate from the TLC chamber when the solvent reaches 1 cm distance from the upper end of the plate. Leave the plate under laminar flux until the silica is totally dried.

NOTE: In the case of analyzing the mycolic acid composition, repeat steps 4.4 and 4.5 two times more, until running the mobile phase three times over the TLC plate.

4.6. Reveal the plate with the required stain; heat the plate if required.

NOTE: In the present experiment, 15–20 mL of the following solutions were used to spray the TLC plates: 10% Molybdatophosphoric acid hydrate in ethanol until the plate is bright yellow, followed by heating the plate at 120 °C; 5% in ethanol and 10% α-naphthol in sulfuric acid followed by heating the plate at 120 °C; Molybdenum Blue reagent (1.3% molybdenum oxide in 4.2 M sulfuric acid) until phosphate bands appeared or 1% anthrone in sulfuric acid.

CAUTION: Molybdatophosphoric acid hydrate is a flammable and corrosive substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Ethanol is a potential flammable and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat,

protective eyewear, and nitrile gloves).

CAUTION: 1-Naphthol is a flammable, corrosive, and extremely hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Molybdenum Blue Spray Reagent is a corrosive, toxic, and extremely hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

REPRESENTATIVE RESULTS:

With the aim of showing a wide range of lipids present in different mycobacteria species, *M. bovis* BCG was selected as it is rough and slow-growing mycobacteria. The rough and fast-growing *M. fortuitum* and *M. brumae* were added in the procedure and, finally, the smooth morphotype of *M. abscessus* was also included. These four species permit us to visualize a broad spectrum of mycobacteria-derived lipids such as acyltrehaloses (AT), GPLs, PDIM, PGL, PIM, TDM, and TMM. Moreover, all four species have different mycolic acid patterns.

After performing the mycolic acid extraction protocols, lipid extracts were analyzed through 1D-TLC analysis using two different, equally valid, elution systems (**Figure 3A,B**). The first mobile phase (**Figure 3A**) was composed by n-hexane and diethyl-ether (85:15), and the plate was run three times. The second mobile phase consisted of 100% of dichloromethane and the plate was eluted once (**Figure 3B**). In both the elution systems, mycolic acids are located approximately in the middle of the TLC plate from the origin of sample application. As **Figure 3** shows, *M. brumae* only possesses type I mycolic acids, a mycolic acid present in all mycobacteria species. *M. bovis* BCG has type I and IV, *M. fortuitum* type I and V, and *M. abscessus*, type I and II mycolic acids profiles. Performing two types of methylation procedures permits us to confirm the presence of type V mycolic acid since type V mycolic acid is cleaved during the acid methanolysis procedure. As **Figure 3** shows, only after the saponification procedure was the spot corresponding to type V mycolic acid observed. After methanolysis, TLC showed the derived compounds from type V cleavage that migrated near the application point[19]. For neophyte researchers, 2D-TLC can allow for a complementary method to identify each mycolic acid type (**Figure 3C,D**). Mycolic acid extracts must be first run in an elution system formed by petroleum ether (60–80 °C) and acetone (95:5) three times. Then, the plate must be run in the second direction with a mobile phase formed by toluene and acetone (97:3). 2D-TLC combined with mass spectrometry (MS) has been used to identify and chemically characterize the functional groups of mycolic acids and has been used extensively to characterize mycolic acids[20–22]. Therefore, the mycolic acid pattern is one of the biochemical features of value in systematic mycobacterial evaluation in combination with other analyses due to shared mycolic acid patterns among different species.

After performing the above-mentioned procedure to extract the non-covalent linked lipids, different elution systems were selected in function of the polarity and size of the lipid profile found in mycobacteria cells. The ideal combination of solvents in the elution systems should enable to visualize the desired lipids in the middle zone of the TLC plate to facilitate their further purification, if desired. In **Figure 4**, TLC plates are ordered from the elution system that allows the most apolar lipids to be monitored (**Figure 4A**) to the elution system that

allows the most polar lipids to be visualized (**Figure 4E**).

Acyl glycerols (AG) and PDIMs are two of the most apolar lipids present in the mycobacterial cell wall and are easily visualized through 1D-TLC analyses using a mobile phase formed by petroleum ether:diethyl ether (90:10). **Figure 4** shows that AGs were present in *M. bovis* BCG, *M. fortuitum* and *M. brumae* but not in the smooth morphotype of *M. abscessus*. Although 1D-TLC suggested the presence of PDIM in *M. bovis* BCG and *M. fortuitum*, it was only corroborated in *M. bovis* BCG when 2D-TLC analysis was performed (**Figure 4B**). Altogether, these results demonstrate the importance of corroborating the presence of a mycobacterial compound by at least two different elution systems. Another interesting lipid to analyze in mycobacteria composition is PGL. In the chosen mycobacteria, PGL is only present in *M. bovis* BCG, and it is noticeable when TLC is eluted with the elution system consisting of chloroform and methanol (95:5) (**Figure 4**). Following the idea of visualizing more polar components, the elution system consisting of the mixture of 90:10:1 (chloroform:methanol:water) was used to monitor the presence of GPLs (**Figure 4D**), which are only present in *M. abscessus* smooth morphotype. In the same TLC: PGL, trehalose dimycolate (TDM), acyl trehaloses (AT), and trehalose monomycolate (TMM), can be also observed. PGL, GPLs, TDM, AT were also observed at the top of the plate when the elution system consisted of 30:8:1 (chloroform:methanol:water) (**Figure 4E**). TMM is located in the middle of the plate. TDM and TMM were clearly expressed in all mycobacteria studied. Despite phosphatidyl-inositol mannosides (PIMs) are observed at the bottom of the plate, the best elution system to analyze PIMs is 60:35:8 (chloroform:methanol:water) as shown in **Figure 5A**,**B**. While all sugar-containing lipids are revealed with anthrone (**Figure 5A**), PIMs contain phosphate groups that are specifically revealed with Molybdenum Blue reagent (**Figure 5B**). Similar to mycolic acids, AG, and PDIMs, PIMs can also be easily visualized through 2D-TLC analyses (**Figure 5C**). Moreover, in the case of analyzing mycobacteria that are able to synthesize LOSs, PIMs and LOSs would be differentiated using the same 2D elution system, as detailed in Ren et al.[8].

FIGURE AND TABLE LEGENDS:

Figure 1: Scheme of the procedure of extracting lipid content of mycobacteria grown on solid media. Main steps to decipher lipids present on mycobacteria cells.

Figure 2: Scheme of the procedure for extracting mycolic acid content of mycobacteria grown on solid media. Main steps to decipher mycolic acids present on mycobacteria cells using either (**A**) acid methanolysis or (**B**) saponification.

Figure 3: Representative results of lipid extraction from mycobacteria. Thin-layer chromatography (TLC) analysis of mycolic acids developed in (**A**) 85 mL of n-hexane, plus 15 mL of diethyl ether (three runs), and (**B**) 100 mL of dichloromethane. (**C**) Two-dimensional TLC analysis of mycolic acids extracted by acid methanolysis developed in 95:5 (n-hexane:acetone) (three runs) in the first direction and 97:3 (toluene:acetone) in the second direction. (**D**) Two-dimensional TLC analysis of mycolic acids from *M. fortuitum* extracted by saponification developed in 95:5 (n-hexane:acetone) (three runs) in the first direction and 97:3 (toluene:acetone) in the second direction. TLCs were revealed with 10% molybdatophosphoric acid hydrate in ethanol followed by heating the plate at 120 °C. *M. bovis* BCG Connaught (Line 1 and 1'); *M. fortuitum* (Line 2 and 2'); *M. abscessus* smooth

morphotype (Line 3 and 3') and *M. brumae* (Line 4 and 4'). 1–4 mycolic acids obtained by acid methanolysis and 1'–4' mycolic acids obtained by saponification. I, α-mycolates; II, α'-mycolates; IV, ketomycolates; V, epoxymycolates.

Figure 4: Representative results of lipid extraction from mycobacteria. (**A**) TLC analysis of acylglycerols (AG) and phthiocerol dimycocerosates (PDIMs) developed in 90:10 (petroleum ether (60–80 °C):diethyl ether). (**B**) Two-dimensional TLC analysis of PDIMs and AG developed in 98:2 (petroleum ether (60–80 °C):ethyl acetate) (three runs) in the first direction and 98:2 (petroleum ether (60–80 °C):acetone) in the second direction. (**C**) TLC analysis of phenolic glycolipid (PGL) developed in 95:5 (chloroform:methanol). (**D**) TLC analyses developed in 90:10:1 (chloroform:methanol:water) of PGL, glycopeptidolipids (GPL), trehalose dimycolate (TDM), acyl trehaloses (AT), and trehalose monomycolate (TMM). (**E**) TLC analysis of PGL, GPL, AT, TMM, and phosphatidyl-inositol mannosides (PIMs) developed in 30:8:2 (chloroform:methanol:water). **A–B–C** were revealed with 10% molybdatophosphoric acid hydrate in ethanol followed by heating the plate at 120 °C. **D–E** were revealed with 5% in ethanol of 10% α-naphthol in sulfuric acid and heated at 120 °C. Line 1: *M. bovis* BCG Connaught; Line 2: *M. fortuitum*; Line 3: *M. abscessus* smooth morphotype; Line 4: *M. brumae*.

Figure 5: Representative results of PIMs from mycobacteria. (**A–B**) TLC analysis of PIMs developed in 60:35:8 (chloroform:methanol:water). (**C**) Two-dimensional TLC analysis of PIMs developed in 60:30:6 (chloroform:methanol:water) in the first direction and 40:25:3:6 (chloroform:acetic acid:methanol:water) in the second direction. **A–C** were revealed with 1% anthrone in sulfuric acid followed by heating the plate at 120 °C. **B** was revealed with Molybdenum Blue reagent until phosphate bands appeared. Line 1: *M. bovis* BCG Connaught; Line 2: *M. fortuitum*; Line 3: *M. abscessus* smooth morphotype; Line 4: *M. brumae*.

DISCUSSION:

A simple protocol considered as the gold standard method for the extraction of noncovalently linked lipid compounds from the mycobacterial cell wall is presented. Further visualization by one- and two-dimensional TLCs from the extracted lipids of four different mycobacteria is shown.

Two consecutive combined mixtures of chloroform and methanol to recover the lipidic content of mycobacterial cells is the most widely used solvent mixture[23–29]. This mixture permits recovery of a wide range of apolar and polar lipids from the cells. Nevertheless, some other methods have been described in the literature to extract total or specific mycobacterial lipids, which have been recently reviewed by Hameed et al.[29]. For instance, the Folch method is one of the most widely used protocols developed to recover the total mycobacterial lipids from tissues[30] and has also been adapted to pure mycobacterial cultures. It consists of suspending mycobacterial cells in chloroform:methanol (1:2), followed by centrifugation and the addition of chloroform to obtain a ratio of 1:1. Finally, KCl is used to remove nonlipid components from the extract[31]. In parallel, other protocols have been developed to extract specific lipids. Slayden et al. used a mixture of chloroform:methanol plus acetone to specifically recover glycolipids such as TDM or TMM[32]. Altogether, published methods are based on exposing mycobacterial cells to different concentrations of solvents, mainly chloroform and methanol. Likewise, some salts are occasionally added to discard other cell

components present on the sample.

In addition to noncovalently linked lipids, mycolic acid extraction by two different procedures is also shown. While acidic methanolysis permits the easy extraction of mycolic acids with less hazardous reagents, the saponification procedure preserves the structure of all mycolic acid types, including type V mycolic acid, which is cleaved during the methanolysis procedures. Once the lipids are extracted, 1D- or 2D-TLC are standard methods to monitor them, and the assay utilized varies depending on the physicochemical characteristics of the lipids. The polarity and size of each molecule will determine the selection of the elution system needed, allowing for determination of the lipids that form part of the mycobacterium. One-dimensional TLC can be chosen when the retention factors (Rf) between mycobacterial lipids are different, while 2D-TLC facilitates visualization when different lipids share molecular weight and polarity characteristics. To facilitate the identification, purified lipids should be run in parallel to the extracted sample to compare similar Rf. The identification of a lipid can be achieved when it runs with the same Rf as that of the known purified control at least in two TLC systems (two different mobile phases). Purified lipids can be obtained from commercial suppliers or from mycobacterial research laboratories. Finally, the biochemical nature of the molecule indicates which stain can be used to reveal TLC plates. There are universal staining methods, such as phosphomolybdic acid which enables the visualization of any organic component to be visualized as it binds to carbon bonds. While others such as a-naphthol or anthrone provide specific colors to sugar residues, molybdenum blue specifically binds to phosphate residues.

The most important consideration to analyze the lipid content of mycobacteria is to avoid the use of plastic material throughout the procedures since the contact of organic solvents with traces of plastic can contaminate the samples and can be observed in the TLC plates. It is also relevant to consider that the culture medium composition used for mycobacteria cultivation, as well as temperature or days of incubation, can modify the lipid pattern of each mycobacterium, as previously described[16]. Mycobacteria grown on either liquid and solid media can be used to extract the non-covalent linked lipids or mycolic acids. When obtaining cells from liquid culture, they should be adequately filtered and dried to avoid the presence of liquid media in the sample. Moreover, when using mycobacteria from liquid media, bacteria must be properly and equally grown between experiments in order to obtain reproducible results over time. Moreover, mycobacterial cells can also be grown on pellicles, from which the most outermost lipids can be recovered using organic solvents and monitored by TLC, as we showed in the present article[17,33–36].

The main limitation of mycobacterial lipid extraction procedures remains the utilization of toxic solvents under safe conditions. The TLC procedure is less sensitive than other techniques, such as gas chromatography or high-performance liquid chromatography. Furthermore, TLC does not permit the quantification of samples, and further techniques need to be applied to identify the structure of the extracted compounds. For instance, nuclear magnetic resonance needs to be performed to distinguish lipid isomers. It is noteworthy that for describing the structure of a mycobacterial lipid for the first time, mass spectrometry or infrared spectroscopy are required. Thus, quantitative and qualitative analysis of lipid classes normally requires combinations of different extraction, derivatization, chromatographic, and detection methods, such as high- or ultra-performance liquid chromatography tandem mass

spectrometry and nuclear magnetic resonance spectroscopy[37–40]. Recent studies have demonstrated that using a single-step thin-layer chromatography-flame ionization detection technique permits the quantification and preliminary screening of mycolic acids in Actinobacteria[41]. Nevertheless, TLC is an extremely useful, timesaving, and cheap technique to screen and evaluate the lipidic composition of mycobacteria. Overall, the procedures presented here are highly versatile providing basic tools to analyze the most relevant feature of mycobacteria cells: its complex cell wall.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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642

Figure 1

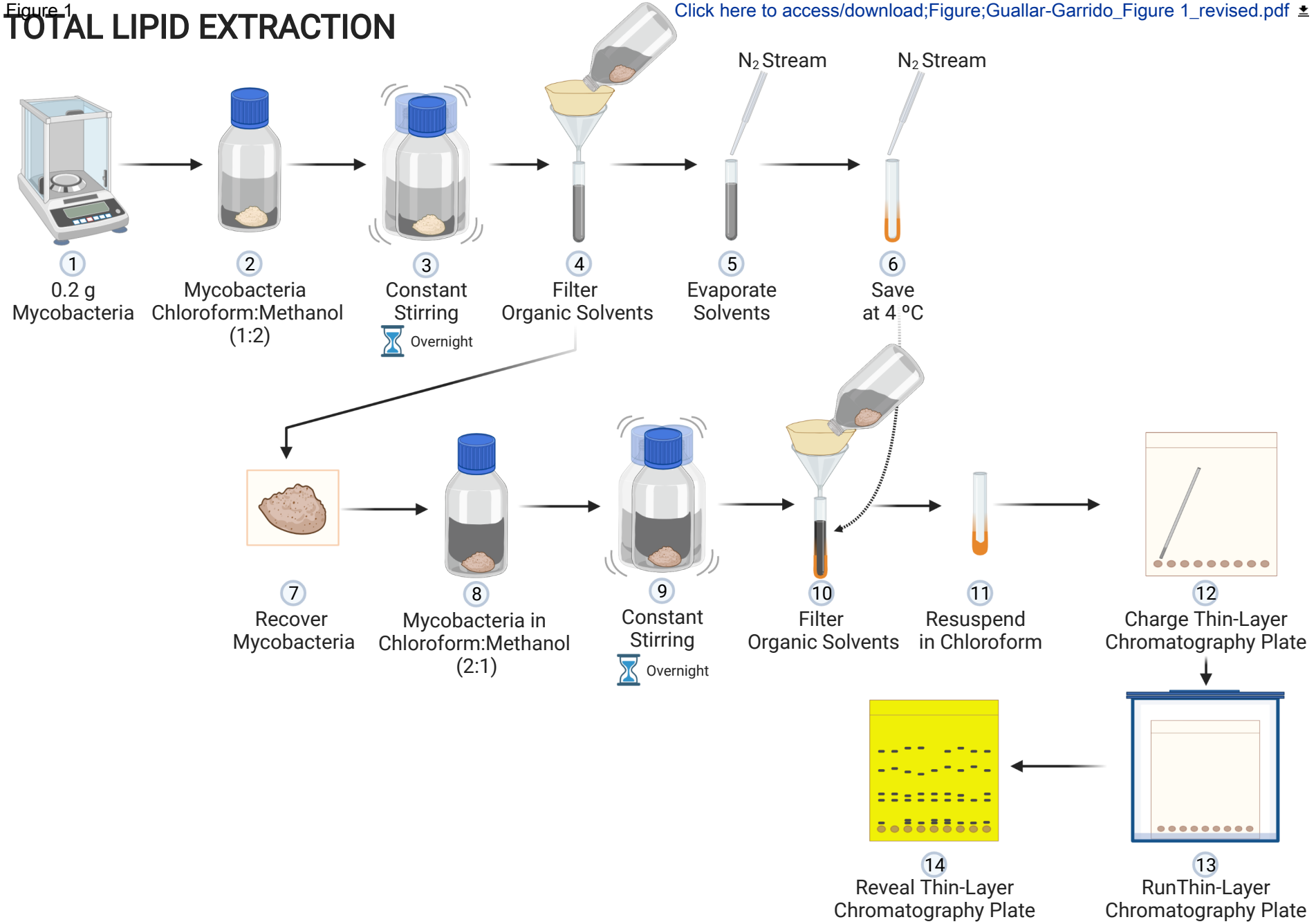
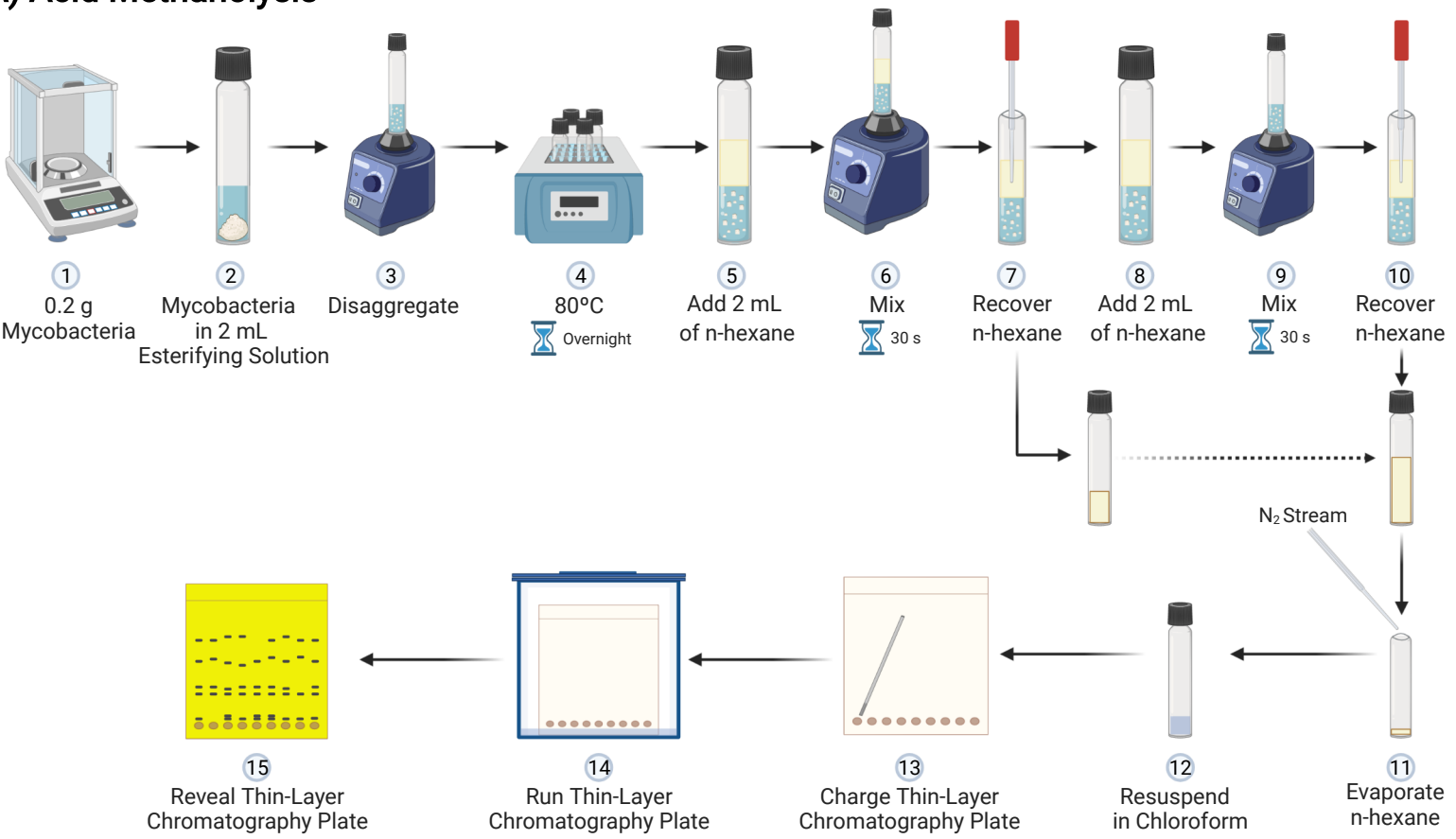


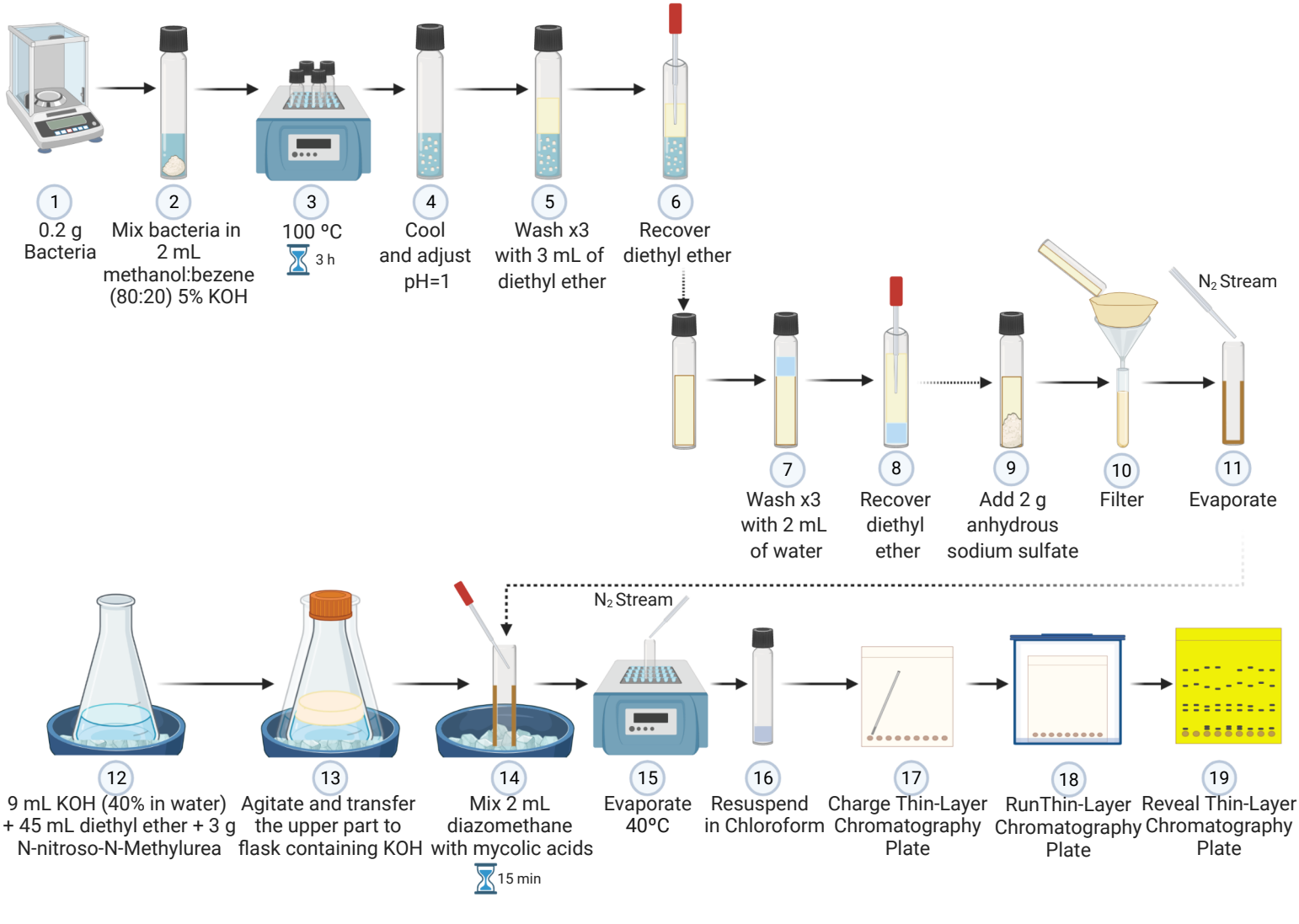
Figure 2

MYCOLIC ACID EXTRACTION

A) Acid Methanolysis



B) Saponification



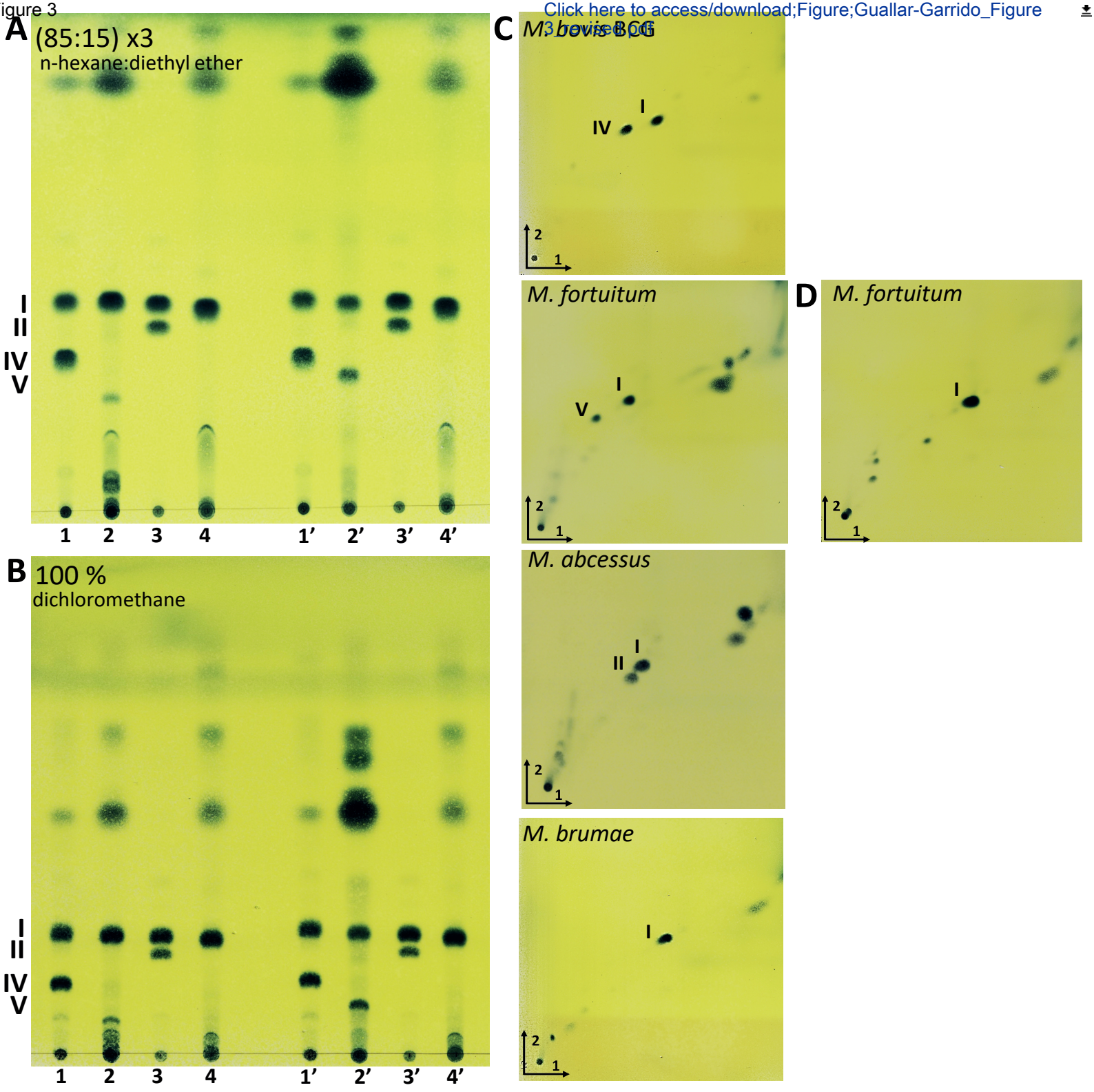


Figure 4

Click here to access/download;Figure;Guallar-Garrido_Figure 4_revised.pdf

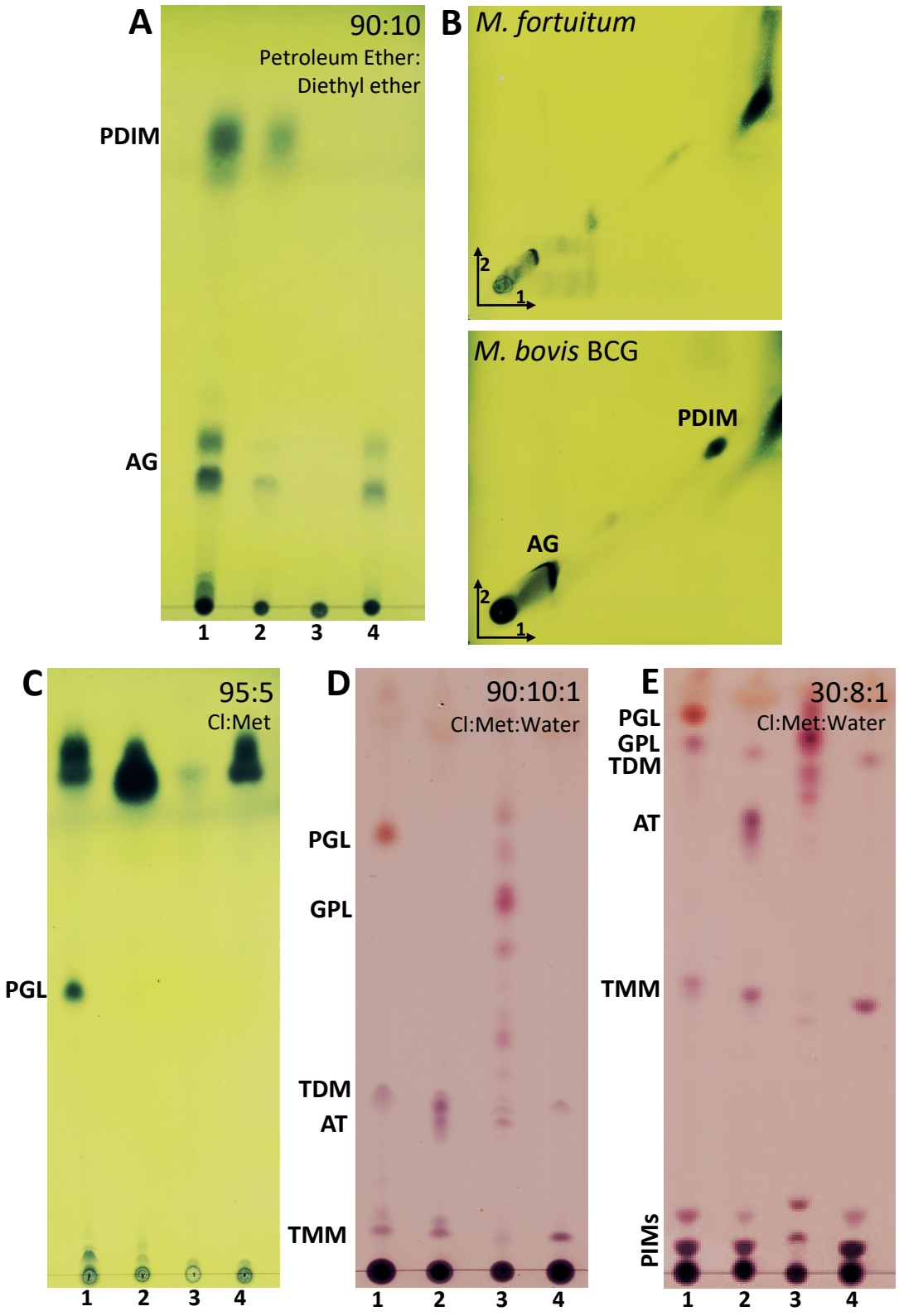


Figure 5

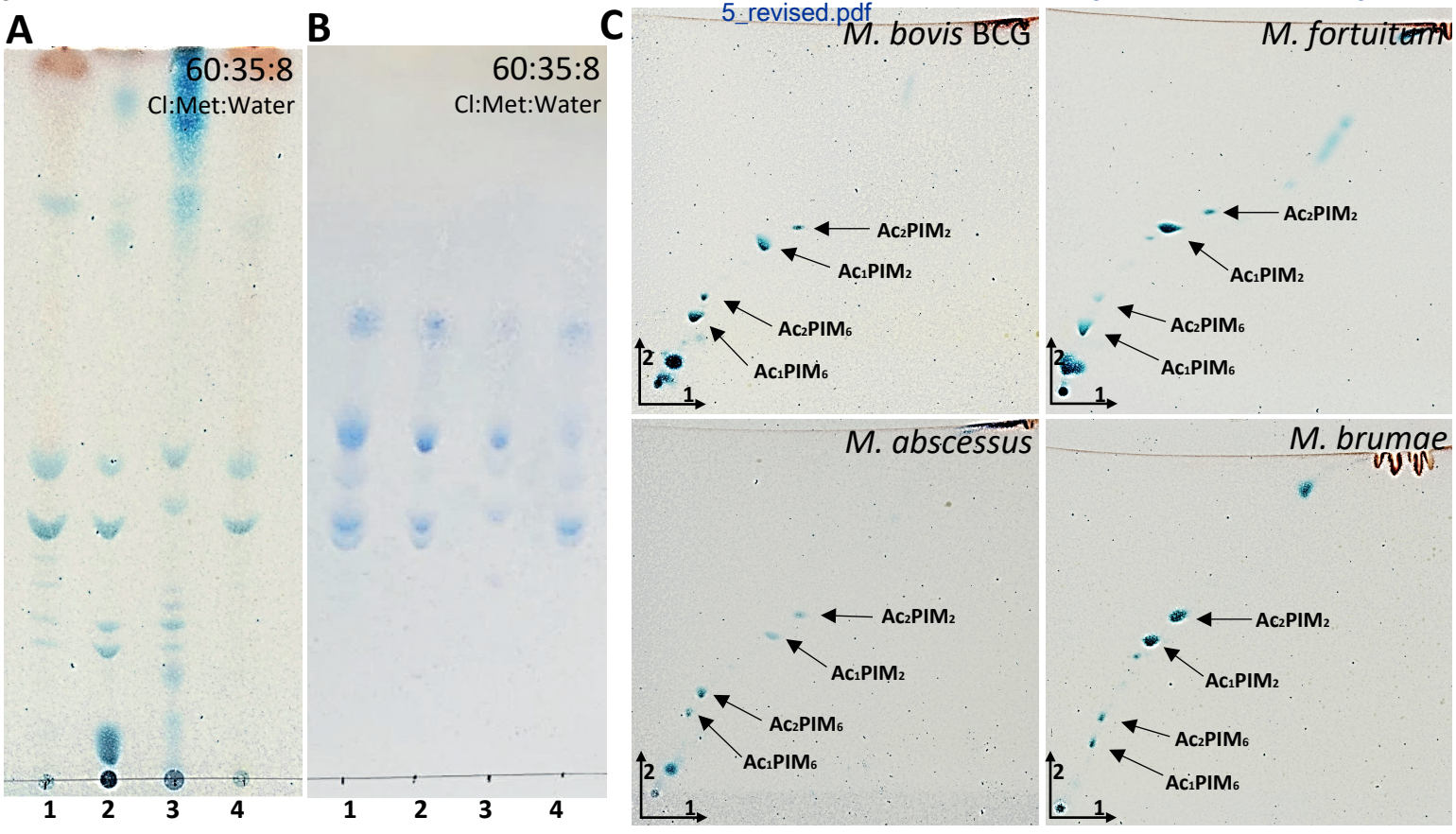


Table of Materials

Click here to access/download;Table of Materials;Guallar-Garrido_Table_Materials_v2.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Acetic Acid	Merck	100063	CAUTION. Anhydrous for analysis EMSURE® ACS,ISO,Reag. Ph Eur
Acetone	Carlo Erba	400971N	CAUTION. ACETONE RPE-ACS-ISO FOR ANALYS ml 1000
Anthrone	Merck	8014610010	Anthrone for synthesis.
Benzene	Carlo Erba	426113	CAUTION. Benzene RPE - For analysis - ACS 2.5 l
Capillary glass tube	Merck	BR708709	BRAND® disposable BLAUBRAND® micropipettes, intraMark
Chloroform	Carlo Erba	412653	CAUTION. Chloroform RS - For HPLC - Isocratic grade - Stabilized with ethanol 2.5 L
Dry block heater	J.P. Selecta	7471200	
Dicloromethane	Carlo Erba	412622	CAUTION. Dichloromethane RS - For HPLC - Isocratic grade - Stabilized with amylene 2.5 L
Diethyl ether	Carlo Erba	412672	CAUTION. Diethyl ether RS - For HPLC - Isocratic grade - Not stabilized 2.5 L
Ethyl Acetate	Panreac	1313181211	CAUTION. Ethyl acetate (Reag. USP, Ph. Eur.) for analysis, ACS, ISO
Ethyl Alcohol Absolute	Carlo Erba	4146072	CAUTION. Ethanol absolute anhydrous RPE - For analysis - ACS - Reag. Ph.Eur. - Reag. USP 1 L
Glass funnel	VidraFOC	DURA.2133148 1217/1	
Glass tube	VidraFOC	VFOC.45066A-16125	Glass tube with PTFE recovered cap
Methanol	Carlo Erba	412722	CAUTION. Methanol RS - For HPLC - GOLD - Ultragradient grade 2.5 L
Molybdatophosphoric acid hydrate	Merck	51429-74-4	CAUTION.
Molybdenum Blue Spray Reagent, 1.3%	Sigma	M1942-100ML	CAUTION.
n-hexane	Carlo Erba	446903	CAUTION. n-Hexane 99% RS - ATRASOL - For traces analysis 2.5 L
n-nitroso-n-methylurea	Sigma	N4766	CAUTION
Orbital shaking platform	DDBiolab	995018	NB-205L benchtop shaking incubator
Petroleum ether (60-80ºC)	Carlo Erba	427003	CAUTION. Petroleum ether 60 - 80°C RPE - For analysis 2.5 L
Sprayer	VidraFOC	712/1	
Sodium sulphate anhydrous	Merck	238597	
Sulfuric acid 95-97%	Merck	1007311000	CAUTION. Sulfuric acid 95-97%
TLC chamber	Merck	Z204226-1EA	Rectangular TLC developing tanks, complete L × H × W 22 cm × 22 cm × 10 cm
TLC plate	Merck	1057210001	TLC SilicaGel 60- 20x20 cm x 25 u
TLC Plate Heater	CAMAG	223306	CAMAG TLC Plat Heater III
Toluene	Carlo Erba	488551	CAUTION. Toluene RPE - For analysis - ISO - ACS - Reag.Ph.Eur. - Reag.USP 1 L
Vortex	Fisher Scientific	10132562	IKA Agitador IKA vórtex 3
1-naphthol	Sigma-Aldrich	102269427	CAUTION.

February 22, 2021

Dear Dr. Vineeta Bajaj,

Thank you for the thorough review of our paper and the opportunity to submit a revised version. We appreciate the editorial and reviewers' constructive comments regarding our manuscript (manuscript number: JoVE62368) which have been of great help and have greatly improved it over the previous version.

Our responses to his/her comments are detailed below (in red) together with the modified text in the revised manuscript (in blue).

We have, in essence, followed the recommendations of the editorial comments and the three reviewers, as detailed below. We have modified Figure 1 and we have added two new Figures (now Figures 3 to 5). According to reviewer 2 comments, the scheme has also been divided according to the protocols and improved with the addition of easily comprehensible icons.

With these changes to the manuscript and our answers to the reviewers' comments, I trust that you will now find the revised edition of our manuscript acceptable for publication in *JoVE.*

Sincerely,

Dr Esther Julián

RESPONSE TO EDITORIAL COMMENTS:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have accurately checked all spelling and grammar issues again.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have changed all personal pronouns of the text.

- Line 25: "*Moreover, we show how to extract and analyze the different types of mycolic acids*" was changed to "Moreover, extraction and analytical protocols of the different types of mycolic acids are shown".
- Line 26: "*We also provide a thin-layer chromatography analysis to monitor these mycobacterial compounds"* was changed to "*A thin-layer chromatographic protocol to monitor these mycobacterial compounds is also provided".*
- Line 44: "Here, we present a simple approach to extract and analyze the total lipid content and the mycolic acid composition of mycobacteria cells, growing in a solid medium, using mixtures of organic" was changed to "Here, a simple approach to extract and analyze the total lipid content and the mycolic acid composition of mycobacteria cells grown in a solid medium using mixtures of organic solvents is presented".
- Line 97: "We present here a visual procedure to extract and analyze the lipid composition of the mycobacterial cell wall" was changed to "A visual procedure to extract and analyze the lipid composition of the mycobacterial cell wall is presented".

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Teflon.

Thank you. We have delated all registered symbols.

4. Line 106/116/138/180: For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks.

Thank you for the correction. We have modified all abbreviations.

5. Line 110: Please elaborate the evaporation step. Is there any specific temperature? How is the tube filled with Nitrogen gas?

To elaborate and clarify step 1.4, we added the following to the revised manuscript. Thank you for the improvement; now, we think it is clearer.

"*NOTE: Connect a glass Pasteur pipette to the stream of nitrogen gas to specifically evaporate the desired tube. Additionally, the tube was maintained inside a dry block heater for tubes at 37°C. When the solvent is evaporated, fill the tube with nitrogen gas before close it.*".

6. Line113: For SI units, please use standard abbreviations when the unit is preceded by a numeral. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2.

Thank you for the correction. We modified line 130.

7. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted the steps of the protocol to be edited.

8. Please include any limitations of the technique in the Discussion.

We have included a new paragraph in the Discussion section to include the limitations of the technique:

"The main limitation of mycobacterial lipid extraction procedures remains the utilization of toxic solvents under safe conditions. The TLC procedure is less sensitive than other techniques, such as gas chromatography or high-performance liquid chromatography. Furthermore, TLC does not permit the quantification of samples, and further techniques need to be applied to identify the structure of the extracted compounds. For instance, nuclear magnetic resonance needs to be performed to distinguish lipid isomers. It is noteworthy that for describing the structure of a mycobacterial lipid for the first time, mass spectrometry or infrared spectroscopy are required. Thus, quantitative and qualitative analysis of lipid classes normally requires combinations of different extraction, derivatization, chromatographic and detection methods, such as high- or ultra-performance liquid chromatography tandem mass spectrometry and nuclear magnetic resonance spectroscopy [1–4]*. Recent studies have demonstrated that using a single-step thin-layer chromatography-flame ionization detection technique permits the quantification and preliminary screening of mycolic acids in Actinobacteria* [5]*. Nevertheless, TLC is an extremely useful, timesaving and cheap technique to screen and evaluate the lipidic composition of mycobacteria."*

9. Please do not use any abbreviations for journal titles and book titles. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

The references have been updated following the instructions of the journal.

RESPONSE TO REVIEWER 1

Manuscript Summary:

A disappointing paper that does not advance the subject. The aim of such a study must be to show methods that allow the analysis of the full range of mycobacterial lipids. It is therefore important to chose mycobacteria that express the important lipids. For example, the Abstract mentions PDIMs, DATs and PATs, but there's no sign of these in the Results. Similarly, lipoligosaccharides (LOSs) are very important and they are not even mentioned.

First, we want to note that Reviewer 1 is correct since our manuscript does not describe novel methods, as this methodology was described many decades ago. For instance, we have been using these methods for thirty years in our laboratory.

We would like to publish this procedure in JoVE because this journal enables visualization of the procedures, which will help neophyte researchers perform related experiments. The following sentence is included in the Objectives of the JoVE publication guidelines: “JoVE publishes novel methods, innovative application of existing techniques, and gold-standard protocols that enable a greater level of experimental transparency.”

We consider the extraction of lipids from the mycobacterial cell wall and their analysis by thin-layer chromatography to be a “gold-standard protocol”. There are many articles and book chapters, as Reviewer 3 comments, that describe this procedure. However, it has never been visualized in any journal.

The importance of the present article remains in the production of video instructions for this gold standard method. In other words, publication of this method in JoVE will increase the experimental transparency of these widely used and versatile gold-standard techniques **in a video**.

To visually demonstrate the extraction of lipids from mycobacteria, we selected four mycobacterial species that cover a wide range of interesting lipids as examples. We chose *M. bovis* BCG, *M. fortuitum*, *M. brumae* and the smooth colony variant of *M. abscessus*. These four species permit us to visualize a broad spectrum of mycobacteria-derived lipids, such as acyltrehaloses (AT), GPLs, PDIM, PGL, PIM, TDM and TMM.

Moreover, all four species have different mycolic acid patterns, including mycolic acid type V, which permits us to explain the different advantages of using acidic methanolysis or saponification procedures to obtain methyl esters.

Mycobacterial species are able to produce several lipids, some of which are species-specific or variant-specific within the same strain. It is not possible to include all mycobacterial lipids in this kind of article. Moreover, many elution systems have been described in the literature to analyze the presence of one specific lipid. For instance, TDM can be observed in both 90:10:1 chloroform:methanol:water and 30:8:1 chloroform:methanol:water, as shown in the article. Additionally, many staining techniques have been described that reveal specific chemical structures, such as those of sugars (anthrone or α-naphthol) or phosphor (Dittmer), to universal revelators such as phosphomolybdic acid.

We focused this article on the lipid extraction and analysis methods. To clarify what Reviewer 1 suggested and to make the manuscript easier for the readers, we added several references regarding the variety of mycobacterial lipids and their visualization by TLC.

The following statements are in response to the specific lipids mentioned by the reviewer:

- PDIM produced by *M. bovis* BCG is included in Figure 1. These lipids can be observed when the total mycobacterial lipid extract is charged in a TLC assay and exposed to petroleum ether:diethyl ether (90:10) as the elution system and was corroborated by 2D-TLC analyses that are included in the new version of the article. To clarify the presence of PDIM only in *M. bovis* BCG, we performed 2D-TLC experiments that clarified the presence of this lipid (new Figure 1). The following text was added.

  *Acyl glycerols (AG) and PDIMs are two of the most apolar lipids present in the mycobacterial cell wall and are easily visualized through 1D-TLC analyses using a mobile phase formed by petroleum ether:diethyl ether (90:10). Figure 4 shows that AGs were present in M. bovis BCG, M. fortuitum and M. brumae but not in the smooth morphotype of M. abscessus. Although 1D-TLC suggested the presence of PDIM in M. bovis BCG and M. fortuitum, it was only corroborated in M. bovis BCG when 2D-TLC analysis was performed (Figure 4*B*). Altogether, these results demonstrate the importance of corroborating the presence of a mycobacterial compound by at least two different elution systems."* (lines 346-353)

- PATs are exclusive lipids of the *M. tuberculosis* cell wall. We did not consider extracting *M. tuberculosis* lipids for safety reasons, as biosafety level 3 conditions were needed to grow these cells. As explained before, we selected four different mycobacteria that possess a wide range of lipids as representative results of the protocol described, which was the objective of our manuscript. To clarify that the described method can be applied to all the mycobacterial lipids of other species, we included new sentences in the Discussion section of the revised manuscript.

  "A simple protocol that is considered the gold standard method for extracting noncovalently bound lipid compounds from the mycobacterial cell wall is presented. Further visualization by one- and two-dimensional TLC assays from the extracted lipids of four different mycobacteria is shown.

  Two consecutive combined mixtures of chloroform and methanol to recover the lipidic content of mycobacterial cells are the most widely used [23-29]. This step permits recovery a wide range of apolar and polar lipids from the cells." (lines 422-428)

- As the Reviewer correctly asserted, lipooligosaccharides (LOSs) are important lipids in the mycobacterial cell wall and have not been mentioned in this article. Similar to other important lipids, such as phosphatidylglycerol, cardiolipin, phosphatidylserine, and phosphatidylethanolamine are considered essential for *M. tuberculosis* viability (Hameed, 2020). Following the Reviewer's suggestion, we have added a sentence in the Introduction section about LOS features, and we have explained in the Results section how 2D-TLC can differentiate PIM and LOSs in the species that produce LOSs.

  Other relevant lipids are lipooligosaccharides (LOSs) present in tuberculous and nontuberculous mycobacteria. In the case of Mycobacterium marinum, the presence of LOSs in its cell wall is related to sliding motility and the ability to form biofilms and interferes with recognition by macrophage pattern recognition receptors, affecting uptake and elimination of the bacteria by host phagocytes [7,8] (lines 71-76).

Similar to mycolic acids, AG and PDIMs, PIMs can also be easily visualized through 2D-TLC analyses (Figure 5C*). Moreover, in the case of analyzing mycobacteria that are able to synthesize LOSs, PIMs and LOSs would be differentiated using the same 2D elution system, as detailed in Ren et al.*[8].(lines 368-371).

We have also added new references supporting this information.

- *7. Szulc-Kielbik, I., Pawelczyk, J., Kielbik, M., Kremer, L., Dziadek, J., Klink, M. Severe inhibition of lipooligosaccharide synthesis induces TLR2-dependent elimination of Mycobacterium marinum from THP1-derived macrophages. Microbial Cell Factories. 16 (1), 217, doi: 10.1186/s12934-017-0829-z (2017).*
- *8. Ren, H. et al. Identification of the lipooligosaccharide biosynthetic gene cluster from Mycobacterium marinum. Molecular Microbiology. 63 (5), 1345–1359, doi: 10.1111/j.1365-2958.2007.05603.x (2007).*
- *22. Minnikin, D.E., Goodfellow, M. Lipid composition in the classification and identification of acid-fast bacteria. Society for Applied Bacteriology symposium series. 8, 189–256, at <https://europepmc.org/article/MED/7025224> (1980).*
- *23. Muñoz, M. et al. Occurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of Mycobacterium tuberculosis. FEMS Microbiology Letters. 157 (2), 251–259, doi: 10.1016/S0378-1097(97)00483-7 (1997).*
- *24. Daffé, M., Lacave, C., Lanéelle, M. -A, Gillois, M., Lanéelle, G. Polyphthienoyl trehalose, glycolipids specific for virulent strains of the tubercle bacillus. European Journal of Biochemistry. 172 (3), 579–584, doi: 10.1111/j.1432-1033.1988.tb13928.x (1988).*
- *25. Singh, P. et al. Revisiting a protocol for extraction of mycobacterial lipids. International Journal of Mycobacteriology. 3 (3), 168–172, doi: 10.1016/j.ijmyco.2014.07.008 (2014).*
- *26. L R Camacho , P Constant, C Raynaud, M A Laneelle, J A Triccas, B Gicquel, M Daffe, C.G. Analysis of the phthiocerol dimycocerosate locus of Mycobacterium tuberculosis. Evidence that this lipid is involved in the cell wall permeability barrier. Journal of Biological Chemistry. 8 (276) (2001).*
- *27. K R Dhariwal, A Chander, T.A.V. Alterations in lipid constituents during growth of Mycobacterium smegmatis CDC 46 and Mycobacterium phlei ATCC 354. Microbios. 16, 65–66 (1976).*
- *28. V Chandramouli, T.A.V. Effect of age on the lipids of mycobacteria. Indian Journal of Chest Diseases & Allied Sciences. 16, 1--207 (1974).*
- *29. Hameed, S., Sharma, S., Fatima, Z. Techniques to Understand Mycobacterial Lipids and Use of Lipid-Based Nanoformulations for Tuberculosis Management. NanoBioMedicine. (March), 1–517, doi: 10.1007/978-981-32-9898-9 (2020).*

Some of the methods are also inappropriate. M. fortuitum produces epoxymycolates, but these are degraded by acid methanolysis.

The reviewer is right. We showed the mycolic acid profile of *M. fortuitum* after a methanolysis procedure in which type V lipid was cleaved. To show how to visualize mycolic acid V extracted from *M. fortuitum*, we added the saponification procedure to the manuscript. Saponification is a procedure by which

epoxymycolates are not degraded, yet acid methanolysis is required to corroborate the presence of type V mycolic acid. Due to inherent characteristics of the saponification process, which include high toxicity and risk, it was avoided in the first version of the manuscript. We added the saponification procedure to the revised manuscript.

We have made the following changes:

- A new paragraph has been added to the Introduction section explaining what mycolic acids are (lines 57-62).

  Specifically, the mycobacterial cell wall contains mycolic acids, which are α-alkyl and β-hydroxy fatty acids, in which the a-branch is constant in all mycolic acids (except for the length) and the b-chain, called the meromycolate chain, is a long aliphatic chain that may contain different functional chemical groups described along with the literature (α-, α'-, methoxy-, κ-, epoxy-, carboxy- and ω-1-methoxy-mycolates), therefore producing seven types of mycolic acids (I-VII)[1].

- The saponification procedure was explained point by point in the protocol section (lines 173-224).

  3. Mycolic acid extraction by saponification and methylation (Fig. 2B)

  3.1. Scratch 0.2 g of mycobacteria from a solid media and add to a glass tube with a PTFE screw cap.

  3.2. Add 2 mL of methanol-benzene solution (80:20) containing 5% potassium hydroxide. Mix the contents by vortexing. Heat the mixture for 3 h at 100 °C.

  CAUTION Benzene is a flammable, carcinogenic and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

  3.2. Allow the tube to cool to room temperature. Samples were acidified by the addition of 20% sulfuric acid to achieve pH=1.

  3.3. Add 3 mL of diethyl ether. Gently mix the contents by vortexing.

  3.4. Let two phases form by settling. Recover the diethyl ether phase and transfer to a new tube. Repeat the wash step for a total of three times.

  3.6. Wash the diethyl ether extract with 2 mL of distilled water and transfer the upper part corresponding to the diethyl ether to a new tube. Repeat the wash step for a total of three times.

  3.5. Add 2 g of anhydrous sodium sulfate over the diethyl ether extract to dry it.

  3.6. Filter the suspension. Evaporate the content using a nitrogen gas flux.

  3.7. To perform the methylation step, dissolve 3 g of N-nitroso-N-methyl urea in a precooled solution formed by 45 mL of diethyl ether and 9 mL of 40% KOH in distilled water.

  CAUTION: N-nitroso-N-methylurea is a toxic, irritant, carcinogenic and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

  3.8. Agitate the mixture. Transfer the supernatant (diazomethane) to a new flask cooled in ice containing potassium hydroxide pellets.

  NOTE: whether the supernatant is not immediately used can be stored at -20ºC for 1 h as maximum.

  CAUTION: potassium hydroxide pellets are an irritant and corrosive substance. This material must be used in a laminar flow hood wearing appropriate person protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: Diazomethane is a highly toxic and potentially explosive. It must be used in a laminar flow hood with safety glass wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

3.9. Add 2 mL of the ether solution containing diazomethane, obtained in step 3.8, into the dried diethyl ether extract that contains mycolic acids, obtained in step 3.6. Incubate for 15 min at room temperature.

3.10. Evaporate the suspension at 40 ºC. Fill the tube with nitrogen gas, close it, and store the methylated lipids at 4 °C.

NOTE: evaporate the diazomethane from the ether solution under the laminar flow hood, until the ether loses the yellow color.

- Figure 1 has been modified to show 1D-TLC results in which *M. fortuitum* mycolic acids obtained by methanolysis and saponification are shown. The difference between the two procedures is now clear, increasing the value of the manuscript. Moreover, we carried out 2D-TLC assays to better differentiate the mycolic acid profile of all four mycobacteria. An explanation has also been added to the Representative Results section of the manuscript (lines 325-338).

  Performing two types of methylation procedures permits us to confirm the presence of type V mycolic acid since type V mycolic acid is cleaved during the acid methanolysis procedure. As Figure 3 shows, only after the saponification procedure was the spot corresponding to type V mycolic acid observed. After methanolysis, TLC showed the derived compounds from type V cleavage that migrated near the application point [19]. For neophyte researchers, 2D-TLC can allow for a complementary method to identify each mycolic acid type (Fig. 3C and 3D). Mycolic acid extracts must be first run in an elution system formed by petroleum ether 60-80°C) and acetone (95:5) three times. Then, the plate can be run in the second direction with a mobile phase formed by toluene and acetone (97:3). 2D-TLC combined with mass spectrometry (MS) has been used to identify and chemically characterize the functional groups of mycolic acids and has been used extensively to characterize mycolic acids [20–22]. Therefore, the mycolic acid pattern is one of the biochemical features of value in systematic mycobacterial evaluation in combination with other analyses due to shared mycolic acid patterns among different species.

- Finally, a paragraph in the Discussion section has also been written related to the mycolic acid extraction procedure (lines 441-451).

  In addition to noncovalently linked lipids, mycolic acid extraction by two different procedures is also shown. While acidic methanolysis permits the easy extraction of mycolic acids with less hazardous reagents, the saponification procedure preserves the structure of all mycolic acid types, including type V mycolic acid, which is cleaved during the methanolysis procedures.

  Once the lipids are extracted, 1D- or 2D-TLC are standard methods to monitor them, and the assay utilized varies depending on the physicochemical characteristics of the lipids. The polarity and size of each molecule will determine the selection of the elution system needed, allowing for determination of the lipids that form part of the mycobacterium. One-dimensional TLC can be chosen when the

retention factors (Rf) between mycobacterial lipids are different, while 2D-TLC facilitates visualization when different lipids share molecular weight and polarity characteristics.

- We have drawn a new version of the schemes, including both types of mycolic acid extraction procedures. Accordingly, new references have been added to the manuscript.

- *19. Secanella-Fandos, S., Luquin, M., Pérez-Trujillo, M., Julián, E. Revisited mycolic acid pattern of Mycobacterium confluentis using thin-layer chromatography. Journal of Chromatography B.* ***879****, 2821–2826, doi: 10.1016/j.jchromb.2011.08.001 (2011).*
- *20. Minnikin D. E., Dobson G., Parlet J. H., Datta A. K., Minnikin S. M., G.M. Analysis of mycobacteria mycolic acids. Topics in lipid research: from structural elucidation to biological function. (1986).*
- *21. Minnikin, D.E., Hutchinson, I.G., Caldicott, A.B., Goodfellow, M. Thin-layer chromatography of methanolysates of mycolic acid-containing bacteria. Journal of Chromatography A.* ***188*** *(1), 221–233, doi: 10.1016/S0021-9673(00)88433-2 (1980).*
- *22. Minnikin, D.E., Goodfellow, M. Lipid composition in the classification and identification of acid-fast bacteria. Society for Applied Bacteriology symposium series.* ***8****, 189–256, at <https://europepmc.org/article/MED/7025224> (1980).*

Single dimension TLC does not resolve all the components of PIMs and separate them from phosphatidylinositol (PI) and LOSs.

Since our four mycobacterial strains do not produces LOS lipids, we did not consider adding information about 2D-TLC assays to differentiate PIMs and LOSs. Nevertheless, following the reviewer suggestions and due to the relevance of LOSs, we carried out 2D-TLC assays to differentiate the PIM lipids from the four selected mycobacteria (new Figure 3). Moreover, we added an explanation in the Discussion section and a new reference in which the authors clearly show how to differentiate PIMs and LOSs in *Mycobacterium marinum* strains. This will help readers to differentiate the two lipid classes (lines 368-371).

Similar to mycolic acids, AG and PDIMs, PIMs can also be easily visualized through 2D-TLC analyses (Figure 5C). Moreover, in the case of analyzing mycobacteria able to synthesize LOSs, PIMs and LOSs can be differentiated using the same 2D elution system, as detailed in Ren et al. [8].

We also added an explanation of the procedure in the protocol section of the manuscript (lines 239-264).

In the two-dimensional TLC, n-hexane:acetone (95:5) was used in the first direction three times, and a single development with toluene:acetone (97:3) was used in the second direction to analyze mycolic acid composition.

To analyze PIMs, chloroform:methanol:water (60:30:6) was used in the first direction once, and chloroform:acetic acid:methanol:water (40:25:3:6) was used in the second direction.

To analyze PDIM and AG, petroleum ether (60-80°C):ethyl acetate (98:2) was used in the first direction three times, and a single development with petroleum ether (60-80°C):acetone (98:2) was used in the second direction.

CAUTION Diethyl ether is a potentially toxic and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION Dichloromethane is a potentially toxic and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION Petroleum ether is a potential flammable, environmentally damaging and extremely hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: acetic acid is a potential flammable and corrosive substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: ethyl acetate is a flammable and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

CAUTION: acetone is a flammable and hazardous substance. It must be used in a laminar flow hood wearing appropriate personal protective equipment (laboratory coat, protective eyewear, and nitrile gloves).

A new reference in this section has also been added.

8. *Ren, H. et al. Identification of the lipooligosaccharide biosynthetic gene cluster from Mycobacterium marinum. Molecular Microbiology. 63 (5), 1345–1359, doi: 10.1111/j.1365-2958.2007.05603.x (2007).*

As explained before, since the utility of 2D-TLC is not only for differentiating PIM from LOS lipids but also facilitates, for instance, the interpretation of the mycolic acid composition of the results, we also performed 2D-TLC analysis of the lipids from the four different species studied (Figure 3 of the revised manuscript)).

Previous methods for the analysis of mycobacterial lipids are not discussed.

Following the reviewer suggestions, the following paragraph is now included in the revised version of the article (lines 422-440):

"A simple protocol considered the gold standard method from the extraction of noncovalently linked lipid compounds from the mycobacterial cell wall is presented. Further visualization by one- and two-dimensional TLCs from the extracted lipids of four different mycobacteria is shown.

Two consecutive combined mixtures of chloroform and methanol to recover the lipidic content of mycobacterial cells is the most widely used solvent mixture [23–29]. This mixture permits recovery of a wide range of apolar and polar lipids from the cells. Nevertheless, some other methods have been described in the literature to extract total or specific mycobacterial lipids, which have been recently reviewed by Hameed et al. [29]. For instance, the Folch method is one of the most widely used protocols developed to recover the total mycobacterial lipids from tissues [30] and has also been adapted to pure mycobacterial cultures. It consists of suspending mycobacterial cells in chloroform:methanol (1:2), followed by centrifugation and the addition of chloroform to obtain a ratio of 1:1. Finally, KCl is used to remove nonlipid components from the extract [31]. In parallel, other protocols have been developed to extract specific lipids. Slayden et al. used a mixture of chloroform:methanol plus acetone to specifically recover glycolipids such as TDM or TDM [32]. Altogether, published methods are based on

exposing mycobacterial cells to different concentrations of solvents, mainly chloroform and methanol. Likewise, some salts are occasionally added to discard other cell components present on the sample.

Accordingly, the following references have been added to the revised manuscript:

- *23. Muñoz, M. et al. Occurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of Mycobacterium tuberculosis. FEMS Microbiology Letters. 157 (2), 251–259, doi: 10.1016/S0378-1097(97)00483-7 (1997).*
- *24. Daffé, M., Lacave, C., Lanéelle, M. -A, Gillois, M., Lanéelle, G. Polyphthienoyl trehalose, glycolipids specific for virulent strains of the tubercle bacillus. European Journal of Biochemistry. 172 (3), 579–584, doi: 10.1111/j.1432-1033.1988.tb13928.x (1988).*
- *25. Singh, P. et al. Revisiting a protocol for extraction of mycobacterial lipids. International Journal of Mycobacteriology. 3 (3), 168–172, doi: 10.1016/j.ijmyco.2014.07.008 (2014).*
- *26. L R Camacho , P Constant, C Raynaud, M A Laneelle, J A Triccas, B Gicquel, M Daffe, C.G. Analysis of the phthiocerol dimycocerosate locus of Mycobacterium tuberculosis. Evidence that this lipid is involved in the cell wall permeability barrier. Journal of Biological Chemistry. 8 (276) (2001).*
- *27. K R Dhariwal, A Chander, T.A.V. Alterations in lipid constituents during growth of Mycobacterium smegmatis CDC 46 and Mycobacterium phlei ATCC 354. Microbios. 16, 65–66 (1976).*
- *28. V Chandramouli, T.A.V. Effect of age on the lipids of mycobacteria. Indian Journal of Chest Diseases & Allied Sciences. 16, 1--207 (1974).*
- *29. Hameed, S., Sharma, S., Fatima, Z. Techniques to Understand Mycobacterial Lipids and Use of Lipid-Based Nanoformulations for Tuberculosis Management. NanoBioMedicine. (March), 1–517, doi: 10.1007/978-981-32-9898-9 (2020).*
- *30. Folch, J., Lees, M., Sloane Stanley, G.H. A simple method for the isolation and purification of total lipides from animal tissues. The Journal of biological chemistry. 226 (1), 497–509, doi: 10.1016/s0021-9258(18)64849-5 (1957).*
- *31. Pal, R., Hameed, S., Kumar, P., Singh, S., Fatima, Z. Comparative Lipidome Profile of Sensitive and Resistant Mycobacterium tuberculosis Strain. International Journal of Current Microbiology and Applied Sciences. 189–197, at <https://www.researchgate.net/publication/274255679_Comparative_Lipidome_Profile_of_Sensitive_and_Resistant_Mycobacterium_tuberculosis_Strain> (2015).*

Major Concerns:

Inadequate plan and execution.

The authors consider that, following the reviewer suggestions and comments, the manuscript has been considerably improved. We want to remark again that our aim is to help neophyte researchers perform these gold standard procedures. It is out of the scope of this manuscript to show in detail all the lipids present in the mycobacterial cell wall, which has already been reviewed in the literature.

Minor Concerns:

Many

RESPONSE TO REVIEWER 2

Manuscript Summary:

This describes a general method that is certainly of value. However, most of it has been used for well over 50 years, and as such seems to me to be short on detail of how it might be updated to take account of modern methods as well as TLC. By adding those methods, I think its value would be greatly increased. If it is left as it is, I think TLC needs to be included in the title.

Thank you for the recommendation. We have modified the title by adding TLC. Furthermore, we added a sentence in the Discussion section about the applicability of TLC together with other methods for the analysis of mycobacterial lipids, as indicated below. Accordingly, new references have also been added to the manuscript.

If I were going to write this procedure, I would want to add other analytical methods to make it much more valuable in 2020. Thus linking the products to lc-ms would add enormously to its value, and would, I think, almost certainly be what would be required to get the necessary detail. Even running NMR on the crude fractions has been reported many times in similar studies. At the moment it is difficult to make any assessment of the amounts of the different classes of compound present.

As we have explained to reviewer 1, the aim of the manuscript is to visualize the gold standard protocols to extract and visualize the lipid composition of mycobacteria to neophyte researchers. Although there are many articles and book chapters that describe these procedures. extraction using a mixture of solvents and TLC visualization have never been shown in a video in any journal.

In the revised manuscript, we explained what information is obtained via TLC analysis. Moreover, the limitations of TLC are included to explicitly mention the drawbacks of the technique and reinforce the utility of other analytical methods, such as liquid chromatography, high-performance liquid chromatography, nuclear magnetic resonance or mass spectrometry, to complement TLC limitations. We also mention the possibility of using a single-step thin-layer chromatography-flame ionization detection technique that permits the quantification of some of the lipids found in Actinobacteria, such as mycolic acids.

Accordingly, new references have been added in the revised manuscript.

The following paragraph was added to the Discussion section of the revised manuscript (Lines 474-488):

> *"The main limitation of mycobacterial lipid extraction procedures remains the utilization of toxic solvents under safe conditions. The TLC procedure is less sensitive than other techniques, such as gas chromatography or high-performance liquid chromatography. Furthermore, TLC does not permit the quantification of samples, and further techniques need to be applied to identify the structure of the extracted compounds. For instance, nuclear magnetic resonance needs to be performed to distinguish lipid isomers. It is noteworthy that for describing the structure of a mycobacterial lipid for the first time, mass spectrometry or infrared spectroscopy are required. Thus, quantitative and qualitative analysis of lipid classes normally requires combinations of different extraction, derivatization, chromatographic and detection methods, such as high- or ultra-performance liquid chromatography tandem mass spectrometry and nuclear magnetic resonance spectroscopy [37–40]. Recent studies have demonstrated that using a single-step thin-layer chromatography-flame ionization detection technique permits the quantification and preliminary screening of mycolic acids*

in Actinobacteria [41]. Nevertheless, TLC is an extremely useful, timesaving and cheap technique to screen and evaluate the lipidic composition of mycobacteria.""

37. *Butler, W.R., Guthertz, L.S. Mycolic acid analysis by high-performance liquid chromatography for identification of mycobacterium species. Clinical Microbiology Reviews.* ***14*** *(4), 704–726, doi: 10.1128/CMR.14.4.704-726.2001 (2001).*
38. *Teramoto, K., Suga, M., Sato, T., Wada, T., Yamamoto, A., Fujiwara, N. Characterization of Mycolic Acids in Total Fatty Acid Methyl Ester Fractions from Mycobacterium Species by High Resolution MALDI-TOFMS. Mass Spectrometry.* ***4*** *(1), A0035–A0035, doi: 10.5702/massspectrometry.a0035 (2015).*
39. *Sartain, M.J., Dick, D.L., Rithner, C.D., Crick, D.C., Belisle, J.T. 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 (2011).*
40. *Li, M., Zhou, Z., Nie, H., Bai, Y., Liu, H. Recent advances of chromatography and mass spectrometry in lipidomics. Analytical and Bioanalytical Chemistry.* ***399*** *(1), 243–249, doi: 10.1007/s00216-010-4327-y (2011).*
41. *Nahar, A., Baker, A.L., Nichols, D.S., Bowman, J.P., Britz, M.L. Application of Thin-Layer Chromatography-Flame Ionization Detection (TLC-FID) to Total Lipid Quantitation in Mycolic-Acid Synthesizing Rhodococcus and Williamsia Species. International Journal of Molecular Sciences.* ***21*** *(5), 1670, doi: 10.3390/ijms21051670 (2020).*

I think there are a number of issues that the Authors may want to address:

1. There is no real quantification of the method - thus no weights appear to be collected at any point. This means there is no idea of the total lipid concentration or the balance of bound/unbound.

Quantification of the samples is a limitation of the described technique, as we have explained before. It is not possible to know the exact amount of each compound. Nevertheless, in step 1.1, we have indicated the total mycobacterial lipid extraction, and in step 2.1, we indicated mycolic acid extraction and the weight of the cells scraped from the agar plate required for each procedure, as it is a regular step in our laboratory routine. We have also added the initial dry weight of mycobacteria to the scheme to clarify that step.

2. There appears to be no description in the text I have been asked to look at as to what the classes of mycolic acid I - V are.

Thank you for your feedback. We have added a description of mycolic acid characteristics. The following paragraph is included in the Introduction section of the revised manuscript (lines 57-62). Accordingly, new references related to mycolic acid characteristics have been added to the revised manuscript.

Specifically, the mycobacterial cell wall contains mycolic acids, which are α-alkyl and β-hydroxy fatty acids, in which the α-branch is constant in all mycolic acids (except for the length) and the β-chain, called the meromycolate chain, is a long aliphatic chain that may contain different functional chemical groups described along with the literature (α-, α'-, methoxy-, κ-, epoxy-, carboxy- and ω-1-methoxy-mycolates), therefore producing seven types of mycolic acids (I-VII)[1].

1. Watanabe, M. *et al.* Location of functional groups in mycobacterial meromycolate chains; the

recognition of new structural principles in mycolic acids. *Microbiology*. **148** (6), 1881–1902, doi: 10.1099/00221287-148-6-1881 (2002).

These are rather limited anyway as there are other classes that appear in slightly different places on TLC. Early workers in this area actually needed to use two dimensional TLC to resolve these.

Thank you for your comment. To clarify this point, we have addressed new experiments. As we have explained to reviewer 1, to improve the analysis and visualization of the mycolic acid profile of mycobacteria, we added another protocol for the extraction of mycolates, i.e., saponification (lines 173-224). In the new one-dimensional TLC figure, we show how the spots appear after each extraction procedure, which permits us to analyze the different profiles. Additionally, different elution systems for one-dimensional TLC were described in the manuscript to facilitate the interpretation of the results and decipher those types of mycolic acid that appear close together.

Moreover, following the reviewer suggestions and agreeing that the interpretation of TLC results can be a difficult step for beginners in chromatography, we also decided to include two-dimensional TLC to resolve the identification of mycolic acids with similar retention factors in one-dimensional TLC. Actually, two-dimensional TLC is a useful technique that is also required to differentiate other lipids Therefore, we found it important to include how two-dimensional TLC is performed in laboratories, increasing the value of the present manuscript.

A new paragraph has been included in the representative results and in the Discussion section of the revised manuscript (lines 325-338 and lines 441-451).

> *"Performing two types of methylation procedures permits us to confirm the presence of type V mycolic acid since type V mycolic acid is cleaved during the acid methanolysis procedure. As Figure 3 shows, only after the saponification procedure was the spot corresponding to type V mycolic acid observed. After methanolysis, TLC showed the derived compounds from type V cleavage that migrated near the application point [19]. For neophyte researchers, 2D-TLC can allow for a complementary method to identify each mycolic acid type (Fig. 3C and 3D). Mycolic acid extracts must be first run in an elution system formed by petroleum ether (60-80°C) and acetone (95:5) three times. Then, the plate must be run in the second direction with a mobile phase formed by toluene and acetone (97:3). 2D-TLC combined with mass spectrometry (MS) has been used to identify and chemically characterize the functional groups of mycolic acids and has been used extensively to characterize mycolic acids [20–22]. Therefore, the mycolic acid pattern is one of the biochemical features of value in systematic mycobacterial evaluation in combination with other analyses due to shared mycolic acid patterns among different species".*

> *"In addition to noncovalently linked lipids, mycolic acid extraction by two different procedures is also shown. While acidic methanolysis permits the easy extraction of mycolic acids with less hazardous reagents, the saponification procedure preserves the structure of all mycolic acid types, including type V mycolic acid, which is cleaved during the methanolysis procedures.*
>
> *Once the lipids are extracted, 1D- or 2D-TLC are standard methods to monitor them, and the assay utilized varies depending on the physicochemical characteristics of the lipids. The polarity and size of*

each molecule will determine the selection of the elution system needed, allowing for determination of the lipids that form part of the mycobacterium. One-dimensional TLC can be chosen when the retention factors (Rf) between mycobacterial lipids are different, while 2D-TLC facilitates visualization when different lipids share molecular weight and polarity characteristics."

3. It seems very strange to me to combine the total non-wall bound lipid and total MA arms of the scheme as is implied by the procedural spreadsheet, though I do accept that this may be common practice. These are not trivial experiments, so I think it is important to obtain the maximum information from each one. I would therefore hydrolyse the non-membrane bound mycolate (and other) esters and the bound ones separately - there is no guarantee that the derived MA mixtures are the same. There are also some reports of free mycolic acids in cells linked to biofilms. I think tis procedure would miss them. If the intention is that the arrow bringing the two arms together simply shows a common method rather than a combination of material, then I think the presentation could be clearer. I think it could be improved by noting additional points where TLC might be useful for comparison.

Thank you for your recommendation. To avoid misunderstanding the procedures, we modified the scheme by dividing both protocols. We have also made another protocol for the saponification procedure. Moreover, we added icons that make the procedures clearer. We think the revised manuscript is much more understandable than the original.

Regarding the mycolic acid recovery from biofilm samples, Ojha *et al.* described the presence of free mycolic acids in the extracellular matrix of *Mycobacterium smegmatis* (Ojha, 2010) and *M. tuberculosis* (Ojha, 2008) when both mycobacteria were grown as pellicles, implying the influence of liquid culture conditions on mycobacterial growth. Consequently, culture media conditions modify the lipid expression of mycobacterial species. Similarly, in a recently published article by our group, we also described the influence of culture medium composition on the antitumor and immunostimulatory effects of mycobacteria, especially *M. bovis* BCG and *M. brumae* (Guallar-Garrido, 2020). We have further demonstrated (Guallar-Garrido, unpublished results) that this difference in antitumor activity is related to the outermost cell wall composition. Otherwise, the expression of other lipids, such as glycerol monomycolate, has been related to growing mycobacteria as pellicles in liquid culture media (Layre, 2008). In that sense, we considered it necessary to explicitly mention in the Discussion section that culture media composition, culture conditions or days of incubation can modify the lipidic characteristics of mycobacteria. We have emphasized and added some references about this in the revised manuscript. Free mycolic acids, which are present on the extracellular matrix of mycobacterial species, can be easily removed from the mycobacterial surface. Ojha *et al*. (2008) exposed mycobacterial cells to methanol and petroleum ether to extract the most superficial mycobacterial lipids. Similarly, petroleum ether (40-60°C b.p.) was applied to *M. abscessus* growing in pellicles to extract trehalose polyphleates also present on the outermost part of mycobacterial cells, as shown in a paper by our group (Llorens-Fons, 2017). We consider that the specific analysis of the outermost layer of the mycobacteria is out of the scope of the present article; however, we have added a sentence in the Discussion section indicating that TLC is also used for the analysis of other types of extractions (lines 471-473) in the revised manuscript. Additionally, new references have been added to the revised manuscript.

"Moreover, mycobacterial cells can also be grown on pellicles, from which the most outermost lipids can be recovered using organic solvents and monitored by TLC, as we showed in the present article [17, 33–36]"

New references:

33. Ojha, A.K. *et al.* Growth of Mycobacterium tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. **69** (May), 164–174, doi: 10.1111/j.1365-2958.2008.06274.x (2008).
34. Ojha, A.K., Trivelli, X., Guerardel, Y., Kremer, L., Hatfull, G.F. Enzymatic hydrolysis of trehalose dimycolate releases free mycolic acids during mycobacterial growth in biofilms. *The Journal of biological chemistry*. **285** (23), 17380–9, doi: 10.1074/jbc.M110.112813 (2010).
35. Layre, E. *et al.* Mycolic Acids Constitute a Scaffold for Mycobacterial Lipid Antigens Stimulating CD1-Restricted T Cells. *Chemistry and Biology*. **16** (1), 82–92, doi: 10.1016/j.chembiol.2008.11.008 (2009).
36. Llorens-Fons, M. *et al.* Trehalose polyphleates, external cell wall lipids in Mycobacterium abscessus, are associated with the formation of clumps with cording morphology, which have been associated with virulence. *Frontiers in Microbiology*. **8** (JUL), doi: 10.3389/fmicb.2017.01402 (2017).

4. In practical terms, hexane is particularly toxic. Why can other hydrocarbons not be used? Heptane is much less toxic.

The toxicity of the current procedures is their main limitation, and alternative products must be found to obtain the same results with diminished risk. However, the toxicity of this protocol is diminished when working in a laminar flow cabinet and when wearing the appropriate personal protective equipment, including glasses, nitrile gloves and a laboratory coat. According to the reviewer argument, heptane is a less toxic and less volatile hydrocarbon than hexane but is still similar. To the best of our knowledge and according to information found in the literature, heptane seems to be a suitable substitute for n-hexane when running TLCs (link). However, we found a published article in which heptane was used to extract free mycolic acids (Kubota, 2020).

Replacement of n-hexane by heptane is an important consideration to have in mind. Future experiments will be carried out in our laboratory to decipher whether mycolic acid extraction efficiency with heptane is equally valid as that with n-hexane.

Reference:

- Kubota, M.; Iizasa, E.; Chuuma, Y.; Kiyohara, H.; Hara, H.; Yoshida, H. Adjuvant activity of Mycobacteria-derived mycolic acids. *Heliyon* **2020**, *6*, e04064, doi:10.1016/j.heliyon.2020.e04064.

5. 'stirring from time to time' in the Figure is a little imprecise. It is also nt a good idea to leave these esters for a very long time in chloroform, which is acidic and can lead to ester cleavage.

In the initial version of the manuscript, we decided to choose the expression of “stirring from time to time” for laboratories without an orbital shaking platform. However, “stirring from time to time” has been replaced by “constant stirring overnight”. Otherwise, the following NOTE has been added to the procedure.

> “*NOTE: If an orbital shaking platform is not available, constant stirring can be replaced by periodic manual stirring as frequently as possible.*”

We think this statement is clear in both the revised manuscript and the scheme.

7. Esterification not sterification and resuspend not ressuspend in scheme

Thank you for your feedback. These changes were made in the revised manuscript.

8. PTFE polytetrafluoro ethene or polytetrafluoro ethylene

Thank you for your feedback. This change has been made in the revised manuscript.

RESPONSE TO REVIEWER 3

Mycobacteria possesses unique sets of lipids crucial for their growth and pathogenesis. The authors have addressed a significant area of research through discussing the process of lipids extraction from Mycobacterium. However the process discussed is already been adapted frequently by various Mycobacterial research groups.

As we have explained to Reviewer 1, we want to remark that the aim of the manuscript is not to present a novel method since this methodology was described many decades ago, as Reviewer 3 highlights.

We propose to publish this procedure in JoVE because this journal enables visualization of the procedures and will help neophyte researchers perform related experiments. The following sentence is in the Objectives section of the JoVE publication guidelines: “JoVE publishes novel methods, innovative application of existing techniques, and gold-standard protocols that enable a greater level of experimental transparency.”

We consider that the extraction of lipids from the mycobacterial cell wall and its analysis by thin-layer chromatography is the “gold-standard protocol”. There are many articles and book chapters, as Reviewer 3 correctly comments, that describe this procedure. However, this procedure has never been visualized in any journal.

The importance of the present article is in the development of a video representation of this gold standard method. In other words, publication of this method in JoVE will increase the experimental transparency of these widely used and versatile gold-standard techniques **in a video**

Although mycobacteria lipid extraction is a procedure that has been modified and adapted through the last 5 decades to elucidate bacterial lipid composition, published methods are based on exposing mycobacterial cells to different solvent concentrations, mainly chloroform and methanol. Here, we show a method that enables the extraction of the total lipid content, which is exposed to different elution systems, allowing us to analyze a wide range of mycobacterial lipids.

Both extraction of total non-covalent-linked lipids from mycobacteria and mycolic acid extraction of mycobacteria cells is already known and published.

The importance of the present article is in showing methods that allow for the extraction and detection of all known mycobacterial lipids. In other words, this manuscript increases the experimental transparency of a widely used and versatile gold-standard techniques **in the form of a video**.

Additionally, there are several other process for the mycobacterial lipids extraction which may also be compared with the discussed process.

The reviewer is correct. We have explained the most commonly used procedure in the literature to extract mycobacterial lipids; however, there are other options. Following your recommendation, other processes that allow for extracting total or specific mycobacterial lipids are now included in the revised manuscript.

Please find enclosed the new paragraph included in the revised manuscript (lines 396-414).:

"A simple protocol considered the gold standard method from the extraction of noncovalently linked lipid compounds from the mycobacterial cell wall is presented. Further visualization by one- and two-dimensional TLCs from the extracted lipids of four different mycobacteria is shown.

Two consecutive combined mixtures of chloroform and methanol to recover the lipidic content of mycobacterial cells is the most widely used solvent mixture [23–29]. This mixture permits recovery of a wide range of apolar and polar lipids from the cells. Nevertheless, some other methods have been described in the literature to extract total or specific mycobacterial lipids, which have been recently reviewed by Hameed et al. [29]. For instance, the Folch method is one of the most widely used protocols developed to recover the total mycobacterial lipids from tissues [30] and has also been adapted to pure mycobacterial cultures. It consists of suspending mycobacterial cells in chloroform:methanol (1:2), followed by centrifugation and the addition of chloroform to obtain a ratio of 1:1. Finally, KCl is used to remove nonlipid components from the extract [31]. In parallel, other protocols have been developed to extract specific lipids. Slayden et al. used a mixture of chloroform:methanol plus acetone to specifically recover glycolipids such as TDM or TDM [32]. Altogether, published methods are based on exposing mycobacterial cells to different concentrations of solvents, mainly chloroform and methanol. Likewise, some salts are occasionally added to discard other cell components present on the sample.

Accordingly, the following references have been added to the revised manuscript:

23. *Muñoz, M. et al. Occurrence of an antigenic triacyl trehalose in clinical isolates and reference strains of Mycobacterium tuberculosis. FEMS Microbiology Letters. 157 (2), 251–259, doi: 10.1016/S0378-1097(97)00483-7 (1997).*
24. *Daffé, M., Lacave, C., Lanéelle, M. -A, Gillois, M., Lanéelle, G. Polyphthienoyl trehalose, glycolipids specific for virulent strains of the tubercle bacillus. European Journal of Biochemistry. 172 (3), 579–584, doi: 10.1111/j.1432-1033.1988.tb13928.x (1988).*
25. *Singh, P. et al. Revisiting a protocol for extraction of mycobacterial lipids. International Journal of Mycobacteriology. 3 (3), 168–172, doi: 10.1016/j.ijmyco.2014.07.008 (2014).*
26. *L R Camacho 1, P Constant, C Raynaud, M A Laneelle, J A Triccas, B Gicquel, M Daffe, C.G. Analysis of the phthiocerol dimycocerosate locus of Mycobacterium tuberculosis. Evidence that this lipid is involved in the cell wall permeability barrier. Journal of Biological Chemistry. 8 (276) (2001).*
27. *K R Dhariwal, A Chander, T.A.V. Alterations in lipid constituents during growth of Mycobacterium smegmatis CDC 46 and Mycobacterium phlei ATCC 354. Microbios. 16, 65–66 (1976).*
28. *V Chandramouli, T.A.V. Effect of age on the lipids of mycobacteria. Indian Journal of Chest Diseases & Allied Sciences. 16, 1--207 (1974).*
29. *Hameed, S., Sharma, S., Fatima, Z. Techniques to Understand Mycobacterial Lipids and Use of Lipid-Based Nanoformulations for Tuberculosis Management. NanoBioMedicine. (March), 1–517, doi: 10.1007/978-981-32-9898-9 (2020).*
30. *Folch, J., Lees, M., Sloane Stanley, G.H. A simple method for the isolation and purification of total lipides from animal tissues. The Journal of biological chemistry. 226 (1), 497–509, doi: 10.1016/s0021-9258(18)64849-5 (1957).*

31. *Pal, R., Hameed, S., Kumar, P., Singh, S., Fatima, Z. Comparative Lipidome Profile of Sensitive and Resistant Mycobacterium tuberculosis Strain. International Journal of Current Microbiology and Applied Sciences. 189–197, at <https://www.researchgate.net/publication/274255679_Comparative_Lipidome_Profile_of_Sensitive_and_Resistant_Mycobacterium_tuberculosis_Strain> (2015).*

I don't understand the reason of protocols of mycobacterial lipids extraction by the authors and its presentation as a research study.

We have submitted the manuscript as a “protocol” instead of a “research study” for publication in JoVE. According to JoVE requirements, there is a need to show representative results after applying the explained protocols. Thus, we selected four representative mycobacterial species that display a wide range of lipids, and we showed how to proceed to visualize them. However, it is not a research study. The description of the lipids shown here has already been described by ours in the past or by other authors.

There are several published articles, yet following are the references that may be checked –

- DOI: 10.1007/978-981-32-9898-9_18
  Hameed, S., Sharma, S., & Fatima, Z. (2020). Techniques to Understand Mycobacterial Lipids and Use of Lipid-Based Nanoformulations for Tuberculosis Management.
- PMID: 9921472.
  Besra GS. Preparation of cell-wall fractions from mycobacteria. Methods Mol Biol. 1998;101:91-107. doi:10.1385/0-89603-471-2:91
- PMID: 21285232.
  Sartain MJ, Dick DL, Rithner CD, Crick DC, Belisle JT. Lipidomic analyses of Mycobacterium tuberculosis based on accurate mass measurements and the novel "Mtb LipidDB". J Lipid Res. 2011;52(5):861-872. doi:10.1194/jlr.M010363
- PMID: 26786484
  Singh P, Sinha R, Tandon R, et al. Revisiting a protocol for extraction of mycobacterial lipids. Int J Mycobacteriol. 2014;3(3):168-172. doi:10.1016/j.ijmyco.2014.07.008
- PMID: 18770604.
  Vilchèze C, Jacobs WR. Isolation and analysis of Mycobacterium tuberculosis mycolic acids. Curr Protoc Microbiol. 2007;Chapter 10:. doi:10.1002/9780471729259.mc10a03s05
- PMID: 31624974
  Sharma S, Hameed S, Fatima Z. Lipidomic insights to understand membrane dynamics in response to vanillin in Mycobacterium smegmatis. Int Microbiol. 2020;23(2):263-276. doi:10.1007/s10123-019-00099-9
- PMID: 30863701
  Pal R, Hameed S, Kumar P, Singh S, Fatima Z. Understanding lipidomic basis of iron limitation induced chemosensitization of drug-resistant Mycobacterium tuberculosis. 3 Biotech. 2019;9(4):122. doi:10.1007/s13205-019-1645-4
- PMID: 30430296

Pal R, Hameed S, Fatima Z. Altered drug efflux under iron deprivation unveils abrogated MmpL3 driven mycolic acid transport and fluidity in mycobacteria. Biometals. 2019;32(1):49-63. doi:10.1007/s10534-018-0157-8

- PMID: 30018826.
  Pal R, Hameed S, Sabareesh V, Kumar P, Singh S, Fatima Z. Investigations into Isoniazid Treated Mycobacterium tuberculosis by Electrospray Mass Spectrometry Reveals New Insights into Its Lipid Composition. J Pathog. 2018;2018:1454316. Published 2018 Jun 19. doi:10.1155/2018/1454316
- PMID: 28955622
  Pal R, Hameed S, Kumar P, Singh S, Fatima Z. Comparative lipidomics of drug sensitive and resistant Mycobacterium tuberculosis reveals altered lipid imprints. 3 Biotech. 2017;7(5):325. doi:10.1007/s13205-017-0972-6

Thank you for the reference recommendations. They have been of value, and some of them have been added to the revised manuscript. We selected articles showing good TLC images of mycobacterial lipids. Nevertheless, we have also added some new references from classical articles in which all elution systems to visualize mycobacterial lipids by TLC are described.