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Chapter 5

Strategies Towards a Simpler Chromatographic Method for Sputum-based Diagnosis of Tuberculosis

Summary

Tuberculosis (TB) remains one of the most pressing diseases all over the world. Its late diagnosis adversely affects the patient and increases this global epidemic. In earlier work we have identified biomarkers for diagnosis of TB in sputum. Recently, we have successfully developed a serial combination of normal-phase liquid chromatography (NPLC) as sample clean-up prior to thermally assisted hydrolysis and methylation followed by gas chromatography-mass spectrometry (THM-GC-MS) in order to better separate the targeted biomarker compounds from the interfering matrix compounds, i.e. cholesterol. The method proved to be very powerful with a detection limit of 1×10^3 mycobacteria/mL and great potential for automation. However, this analytical strategy for detecting the responsible bacteria, *Mycobacterium tuberculosis*, is still very complex and demanding for developing countries. This chapter describes strategies for simplifying the chromatographic method for sputum-based diagnosis of TB. Three options are discussed: easier sample preparation (size-exclusion chromatography, SEC, and different phases for solid-phase extraction, SPE), simpler derivatization of the TB biomarkers (manual versus automatic THM) and cheaper and easier to operate detectors.

The results obtained indicate that SPE on diol-silica phases is a good substitute for LC sample clean-up, manual THM reaction can be performed in a conventional oven under optimized conditions, and the use of a flame ionization detector (FID) as an alternative detection system is possible. However, a major drawback of combining manual THM derivatization and GC-FID detection is the loss in overall sensitivity, which represents a significant setback for the early diagnosis of TB. Moreover, urine and serum matrices are also considered as future developments.

5.1 Introduction

Tuberculosis (TB) is still today one of the deadliest diseases in the world. Recent numbers from the World Health Organization (WHO) estimate that in 2015 there were 10.4 million new TB cases, among which 1.0 million were children, and 1.4 million people died from TB [1]. Even though the disease is treatable and curable, late diagnosis is the number one cause of these fatal statistics. The majority of TB cases occur in the so-called high-burden countries, which refers to the WHO list of countries that together have around 80% of arising TB cases [2]. Examples include low-income countries in particular in Africa and South Asia (i.e. South Africa, Mozambique, Vietnam, India, Bangladesh, Indonesia, etc.). In the fight against TB, in 2014 the World Health Assembly approved a new “End TB strategy”, endorsed by several governments, to end the global epidemic by 2035 [2]. This can be achieved through appropriate education on how to prevent and treat TB and by empowering research institutes all over the world.

Significant research efforts are needed since current methods for identifying the mycobacteria responsible for TB, *Mycobacterium tuberculosis* (MTB), lack sensitivity and specificity, which often leads to misdiagnosis and false-

positive results [3–5]. Our research group started this fight against TB in 2009 when Kaal and co-workers developed a thermally assisted hydrolysis and methylation-gas chromatography-mass spectrometry (THM-GC-MS) method for the direct identification of MTB in culture and sputum specimens [6]. In light of this, we further developed a 20-biomarker classification model, focusing mainly on the information from mycocerosates, to detect and differentiate cultured MTB [7,8]. However, in our studies we reported the presence of interfering compounds, such as cholesterol, that adversely affect the successful identification and detection of the characteristic mycocerosates markers. Therefore, we have successfully developed and validated a solid-phase extraction (SPE) clean-up method prior to THM-GC-MS analysis (SPE-THM-GC-MS) [9,10]. Yet, the separation of matrix interfering compounds eluting around the mycocerosates markers was insufficient, since very high levels of cholesterol-related compounds were still noted. For this reason we have recently developed a more efficient clean-up method by using an in-series combination of normal phase-liquid chromatography (NPLC) and THM-GC-MS [11]. This combination consists of three steps: an LC analysis, the collection of several fractions and their transfer from LC to GC-MS. The coupling of LC with GC-MS can be achieved either comprehensively (transfer of all LC fractions) or in a hyphenated mode (transfer of specific fractions only). Our NPLC×THM-GC-MS method used only two fractions and the detection limit of 1×10^3 mycobacteria/mL is ten times lower than that of microscopy (1×10^4 mycobacteria/mL) [11]. Even though the method is complex, it has a good potential for automation.

Parallel in time, other groups, such as O'Sullivan et.al [12,13], also used THM-GC-MS to detect MTB in solvent extracts from spiked and real TB positive sputum specimens and used a similar approach with SPE and molecular imprinting for sample pretreatment [14]. Also, new techniques have recently been introduced into the realm of TB diagnosis, namely MALDI-

TOF-MS for the direct identification of bacteria in complex samples [15–17] and multiplexed multiple reaction monitoring mass spectrometry (MRM-MS) for the detection of MTB peptides in exosomes [18]. Unfortunately, all these methods are very complex and expensive for developing countries and therefore a simpler, cheaper, easy to use, yet sensitive and specific alternative method is desired. In most developing countries microscopy is still the method of choice since it is cheap.

In this chapter, we describe several analytical strategies to simplify the TB diagnosis using GC as the detection technique of choice. Three options to do so are considered, (i) simplify the sample preparation step; (ii) perform a manual THM reaction and finally (iii) the choice between MS or FID as the detection system. We believe that ultimately a critical choice needs to be made, either one chooses the highest sensitivity and specificity that modern technology can provide, or opts for an affordable, easy to use, simpler but more laborious method. Furthermore, other types of matrices (as urine and serum) are also considered for future developments.

5.2 Materials and methods

5.2.1 Culture of mycobacteria

The *Mycobacterium tuberculosis* (MTB strain 124) was obtained from the Royal Tropical Institute (Amsterdam, The Netherlands). The strain was cultured in Middlebrook 7H9 enriched with OADC (oleic acid albumin dextrose and catalase) (BD Diagnostics, Detroit, MI, USA). Bacteria were harvested and divided into single suspensions as previously described [8,9]. Solid-phase experiments were performed by adding 10 μ L of the MTB suspension to 1 mL of sputum before the decontamination step (see section 5.2.4) [11].

5.2.2 Sputum specimens

For the spiking experiments, blank sputum samples were obtained from patients with lung infections other than TB attending the Academic Medical Centre (University of Amsterdam, The Netherlands).

5.2.3 Standards and reagents

A 25% tetra-methyl ammonium hydroxide (TMAH) solution in methanol was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Before using it for the THM-GC-MS analysis, the reagent was ten times diluted with deionized water obtained from a Satorius Arium 611 UV instrument (Satorius, Nieuwegein, The Netherlands). Cholesteryl oleate (Sigma-Aldrich, Schnellendorf, Germany) prepared with a concentration of 2 mg/mL in chloroform was used as a standard. Organic solvents (hexane, methanol, diethyl ether, ethyl acetate, isopropanol, chloroform and tetrahydrofuran stabilized with butylated hydroxytoluene) were purchased from Biosolve (Valkenswaard, The Netherlands).

5.2.4 Sample preparation for sputum specimens

Sputum specimens of 1 mL spiked or non-spiked with MTB 124 bacterial suspension were decontaminated in 15 mL polypropylene tubes (Greiner, Nürtingen, Germany) with an equal volume of 0.5 M NaOH + 0.05 M Na-citrate and 5 mg N-acetyl-l-cysteine (NALC)/mL as previously described in the literature [9]. In short: the samples were shaken for approximately 15 min, neutralized with seven volumes of 0.15 M sodium phosphate monobasic pH 4.0 and diluted with demineralized water to an end volume of 15 mL. The decontaminated sputum specimens were centrifuged for 45 min at 4000 rpm ($2700 \times g$) using a Hettich Universal 16 centrifuge (Hettich, Tuttlingen,

Germany). The supernatant was removed and the pellet was re-suspended in 0.75 mL of demineralized water. The resulting material was transferred to a 10 mL disposable Corning Pyrex glass tube (Corning B.V. Life Sciences, Amsterdam, The Netherlands), which had been cleaned thoroughly, using detergent brushing and deionized water, and heated for 1 hour at 300°C to remove traces of lipids [9,11]. The tube was then capped with a Teflon faced rubber liner screw cap and centrifuged for 30 min at 4000 rpm. The supernatant was removed with a disposable Pasteur pipette so that the total end volume of the samples was made 200 µL.

5.2.4.1 Hexane/methanol/water extraction of decontaminated sputum specimens

The extraction procedure used for decontaminated sputum was similar as previously described [11]. In brief: to each 200 µL of decontaminated spiked or non-spiked sputum, 1 mL of methanol and 1 mL of hexane were added. The mixture was then placed in a sonic water bath for 10 min and shaken for 1h in a horizontal position on a Vortex shaker. Subsequently, the mixture was centrifuged for 15 min at 4000 rpm. The hexane phase (upper layer) was harvested with a disposable glass Pasteur pipette and another 1 mL of hexane was added for a second extraction. The hexane extracts were combined and concentrated to 500 µL for SPE treatment.

5.2.4.2 Solid-phase extraction (SPE) strategies

Sample preparation using SPE for decontaminated sputum samples was performed with three types of 1 mL cartridges containing 100 mg of either silica (Phenomenex, Utrecht, The Netherlands), diol-silica (Agilent Technologies, Amstelveen, The Netherlands) or amino-silica (NH₂) (Phenomenex). The washing solution, the samples and the eluting solvents

were passed through the SPE cartridge by gravity. The SPE procedure for silica and diol-silica phase cartridges was very similar. In brief: the cartridge was conditioned by washing twice with 1 mL of hexane. Then 0.5 mL of the hexane extract from the sputum specimens was applied onto the cartridge, followed by 1 mL of hexane. Next, the cartridge was subsequently eluted with 1 mL of 2% diethyl ether in hexane, followed by 1 mL of 5% diethyl ether in hexane, 1 mL of 10% diethyl ether in hexane, 1 mL of 20% diethyl ether in hexane, 1 mL of 50 % diethyl ether in hexane, then 1 mL of methanol and finally with 1 mL of chloroform/methanol/water mixture (1: 2: 0.8, v/v/v). The run-through solution and the seven fractions were collected separately in auto-sampler vials.

In the case of SPE using NH₂-silica cartridges, a method that separates lipids in up to seven individual classes was applied (original conditions provided by Phenomenex and adapted from [19]). For this method the number of SPE cartridges used was optimized and this description is explained in section 5.3.1.2. The optimized SPE procedure was as follows; one cartridge was conditioned by washing twice with 0.5 mL of hexane. Then 0.25 mL of the hexane extract from the sputum specimens was applied. Next, the cartridge was subsequently eluted with 0.8 mL of hexane (fraction 4), followed by 2 times 1.5 mL of 7.5% ethyl acetate in hexane (fraction 7a and 7b) and finally with 1 mL of chloroform/methanol mixture (2:1, v/v) (fraction 8). The run-through solution and the four fractions were collected separately in auto-sampler vials.

All SPE fractions from the three procedures were evaporated to dryness under a stream of warm nitrogen and dissolved in 50 μ L of hexane. 20 μ L of each of the fractions were used for the THM-GC-MS analysis. “Dot plot” SPE-THM-GC-MS chromatograms were prepared in Excel (Microsoft Office 2010), as previously described [11].

5.2.5 Instrumentation

In the present experimental study, three chromatographic set-ups were used: an HPLC instrument to perform SEC, a GC-FID and a GC-MS. SEC analyses were performed on an Agilent 1100 Series instrument (Agilent, Waldbronn, Germany) with a quaternary pump (G1311A), a manual injection valve, a column oven (G1316A) and connected to a refractive index detector (RI) (G1362A). Two organic polymer columns, a 300 mm × 7.5 mm (Spherisorb, Waters) packed with 3 μm particles and 100 Å pore size and a 300 mm × 7.5 mm (Spherisorb, Waters) packed with 5 μm particles and 100 Å pore size were used at 30°C. The exclusion limit of the column set was 8 min and the total permeation time 18 min. SEC was performed at a flow rate of 1 mL/min running a program of 100% tetrahydrofuran (THF) stabilized with butylated hydroxytoluene (BHT) during 25 min under the control of Chemstation software.

The GC-FID experiments were performed on an Agilent 6890A Series (G1530A) instrument (Agilent) with a split/splitless injector (7683B) using Chemstation software. A 30 m × 0.25 mm I.D. CP-Sil 8 CB (5% phenylmethylpolysiloxane) column with a film thickness of 0.25 μm (Agilent, Waldbronn, Germany) was used. The injector was operated at 300°C in splitless mode (3 μL injection) and the FID was set at 320°C.

THM-GC-MS analyses were done on a Shimadzu GC-MS QP 2010 Plus system (Shimadzu, Den Bosch, The Netherlands). The system was equipped with a "Focus" XYZ robotic auto sampler and an Optic 4 Programmed Temperature Vaporizing (PTV) injector (GL Sciences, Eindhoven, The Netherlands). A 30 m × 0.25 mm I.D. InertCap 5MS/Sil (5% diphenyldimethylpolysilphenylene siloxane) column with a film thickness of 0.25 μm (GL Sciences, Eindhoven, The Netherlands) was used.

5.2.5.1 Comprehensive SEC×THM-GC-MS experiments

The hexane extract of MTB strain 124, equivalent to approximately 1×10^8 mycobacteria/mL, was used as a standard to establish the SEC×THM-GC-MS method. The sample was prepared using the same procedure described in section 5.2.4. 10 μ L of MTB 124 extract or spiked sputum extract was injected into the SEC system. The separation conditions were defined in the section above. As described in our previous work [11], in order to perform comprehensive SEC×THM-GC-MS analysis, adjacent fractions of 1 mL (collection window of 1 min) were manually collected, after a delay of 0.17 min, in auto-sampler vials. These fractions were evaporated to dryness under a stream of nitrogen and re-dissolved in 50 μ L of hexane. Then, the samples were transferred to the GC-MS system and 20 μ L were used for fully automated THM-GC-MS analysis (see below). “Dot plots” SEC×THM-GC-MS chromatograms were prepared in Excel (Microsoft Office 2010) [11].

5.2.5.2 THM-GC-FID/MS analysis: Manual vs. Automatic

Two derivatization modes for hydrolysis and methylation of lipids were used, the first route included a fully automated THM-GC-MS performed directly in the injector and the second route was a manual THM reaction performed in a vial heated in a laboratory oven.

The optimization of the manual THM reaction conditions using a “Design of Experiments” (DOE) approach is discussed in section 5.3.2. The operating conditions for the GC-MS analysis after manual THM and for the automatic THM-GC-MS experiments were identical to those used previously [6,7]. In brief, 20 or 25 μ L of sample were injected into the PTV injector at 40°C. The solvent was then quickly evaporated by programmed heating of the injector to 120°C. Next, the TMAH reagent was injected and the injector was heated to 450°C to perform pyrolysis with in situ methylation, resulting in the formation

of fatty acid methyl esters. The reaction products were transferred from the liner to the GC column for GC-MS analysis in the split mode (split ratio 1:8). The carrier gas used was Helium. The GC-oven program was set at an initial temperature of 60°C (held for 3 minutes), followed by a first temperature ramp of 20°C/min up to 100°C (held for 10 minutes) and a second temperature ramp of 5°C/min up to 300°C with a final holding time of 16 minutes. The MS used electron impact ionization at 70 eV and was operated in the full scan mode collecting spectra over the mass window from 60 to 500 amu at a rate of 5 Hz. GC-MS data were recorded using Shimadzu GCMS Solution software.

5.3 Results and discussion

Previously, we developed and validated a sample preparation method that focused on information from five biomarkers (compounds 16-20 in **Table 5.1**). This method used solid-phase extraction (SPE) on normal phase-silica as clean-up prior to THM-GC-MS analysis (SPE-THM-GC-MS) in order to better separate the interfering cholesterol-related compounds from the characteristic mycocerosate markers in sputum samples [9,10]. However, we still noted the presence of some interfering matrix compounds eluting closely to the target mycocerosate biomarkers, compounds 17-20 in **Table 5.1**.

Table 5.1 – Compounds identified as relevant for detecting MTB ^a using THM-GC-MS ^b [7].

| No. | Retention Time (min) | Name of compound | m/z ^d |
|-----|----------------------|--|------------------|
| 1 | 15.18 | Tetradecanoic acid, methyl ester (C14) | 74 |
| 2 | 31.23 | 9-Hexadecenoic acid, methyl ester | 83 |
| 3 | 31.40 | Hexadecanoic acid, methyl ester (C16) | 87 |
| 4 | 27.48 | 1-Nonadecene | 97 |
| 5 | 33.40 | Heptadecanoic acid, methyl ester (C17) | 74 |
| 6 | 34.94 | 9-Octadecenoic acid (Z)-, methyl ester | 69 |
| 7 | 35.30 | Octadecenoic acid, methyl ester (C18) | 298 |
| 8 | 36.01 | Octadecanoic acid, 10-methyl-, methyl ester (TBSA) | 312 |
| 9 | 40.48 | α -D-glucopyranoside, 2,3,4,6-tetra-O-methyl- α -D-glucopyranosyl 2,3,4,6-tetra-O-methyl- | 71 |
| 10 | 42.15 | Docosanoic acid, methyl ester (C22) | 354 |
| 11 | 45.21 | Tetracosanoic acid, methyl ester (C24) | 382 |
| 12 | 45.87 | Unknown fatty acid | 88 |
| 13 | 46.03 | Tetracosanoic acid, 2,4,6-trimethyl-, methyl ester (C27) | 101 |
| 14 | 46.20 | Tetracosanoic acid, 2,4,6,8-tetramethyl-, methyl ester (C28) | 101 |
| 15 | 46.90 | Pentacosanoic acid, methyl ester (C25) | 87 |
| 16 | 48.04 | Hexacosanoic acid, methyl ester (C26) | 410 |
| 17 | 48.79 | Hexacosanoic acid, 2,4,6-trimethyl-, methyl ester (C29) | 101 |
| 18 | 48.95 | Hexacosanoic acid, 2,4,6,8-tetramethyl-, methyl ester (C30) | 101 |
| 19 | 51.45 | Octacosanoic acid, 2,4,6,8-tetramethyl-, methyl ester (A) ^c (C32) | 101 |
| 20 | 51.61 | Octacosanoic acid, 2,4,6,8-tetramethyl-, methyl ester (B) ^c (C32) | 101 |

^a MTB, *Mycobacterium tuberculosis*.

^b THM-GC-MS, Thermally assisted hydrolysis and methylation-gas chromatography-mass spectrometry.

^c A and B C32 mycocerosates, two isomers of C32 mycocerosates.

^d The m/z values represent the characteristic ions used for quantification.

To better remove the interfering species, in our most recent work we described the successful in-series combination of NPLC with THM-GC-MS, where the LC step is used as a sample clean-up method prior to THM-GC-MS analysis (NPLC×THM-GC-MS) [11]. Furthermore, the NPLC×THM-GC-MS method provides information on the different mycocerosate precursors, e.g. the different phthiocerol dimycocerosates (PDIMs) [20], present in this type of sample matrix and, at the same time allows rapid and reliable quantification of the targeted compounds. The targeted compounds (numbers 17 to 20 in **Table 5.1**) are found in only two NPLC fractions and the set-up has a detection limit of 1×10^3 mycobacteria/mL.

The NPLC and THM-GC-MS are extremely selective and sensitive approaches but expensive and, more importantly, very complex for routine laboratories in developing countries. A true expert that can handle the instrumentation is required. Therefore, a simpler, cheaper, easy to use and possibly “on-location” method is highly desirable, albeit that it of course should provide reliable sensitivity and specificity for the early diagnosis of TB. Here, we describe three options to do so: (i) simplify the sample preparation step by either reducing the number of fractions with size-exclusion chromatography (SEC) or using other types of solid-phase extraction cartridges (as silica, diol-silica and NH_2 -silica) discussed in section 5.3.1; (ii) perform a manual THM reaction in a conventional oven prior to GC analysis, instead of the complex automatic THM, considered in section 5.3.2 and finally (iii) the choice between MS or FID as the detection system reflected in section 5.3.3. All samples described in the following sections were analyzed in triplicate and the matrix effect was studied by comparing culture MTB strain 124 with blank sputum and spiked sputum specimens with different amounts of MTB strain 124. Furthermore, the linearity of the described methods was evaluated with increasing spiking concentrations of MTB strain 124.

5.3.1 Sample Preparation strategies: a comparison study

5.3.1.1 Size-exclusion chromatography (SEC)

As reported in our most recent work, mycocerosates do not occur in the lipid extracts as free acids, but rather as esters bound to different long chain beta diols called phthiocerol dimycocerosates (PDMIs) [20–22]. This means that mycocerosates can be detected at multiple positions in an LC run depending on their PDIM lipid precursor [11]. In our NPLC×THM-GC-MS method, the characteristic biomarkers (the precursors of the compounds 17-20 in **Table 5.1**) were separated according to their polarities. The non-polar precursors were present in earlier fractions and the more polar species in one of the later fractions, ultimately resulting in the analysis of two fractions [11]. However, in order to make the LC step even simpler, SEC was investigated as an alternative clean-up method prior to THM-GC-MS analysis. This analytical technique can provide additional information on the size of the lipid precursors. Large molecules elute first from the column, while small molecules elute later. The selection of the pore size for SEC columns is made according to the molecular weight range of the targeted samples, with small particle sizes providing better resolution [23]. Therefore, the aim of the current series of experiments is to optimize the SEC set-up by separating the lipid complex of MTB according to their size and to elute the mycocerosate precursors in only one narrow fraction, while still achieving a clear separation between mycocerosates and cholesterol in the THM-GC-MS analysis. The set-up and the optimized operating conditions are summarized in section 5.2.5 and 5.2.5.1, respectively.

In the first series of experiments, cholesteryl oleate and the hexane extract of MTB strain 124, equivalent to 1×10^8 bacteria, were first analyzed in order to proof that SEC is capable of separating cholesterol esters, the main form of

occurrence of cholesterol in sputum, from the main target compounds. For this, 10 μL of each sample was individually injected into SEC and the fractions were collected as described before for subsequent THM-GC-MS analysis. The results are shown in **Figure 5.1**.

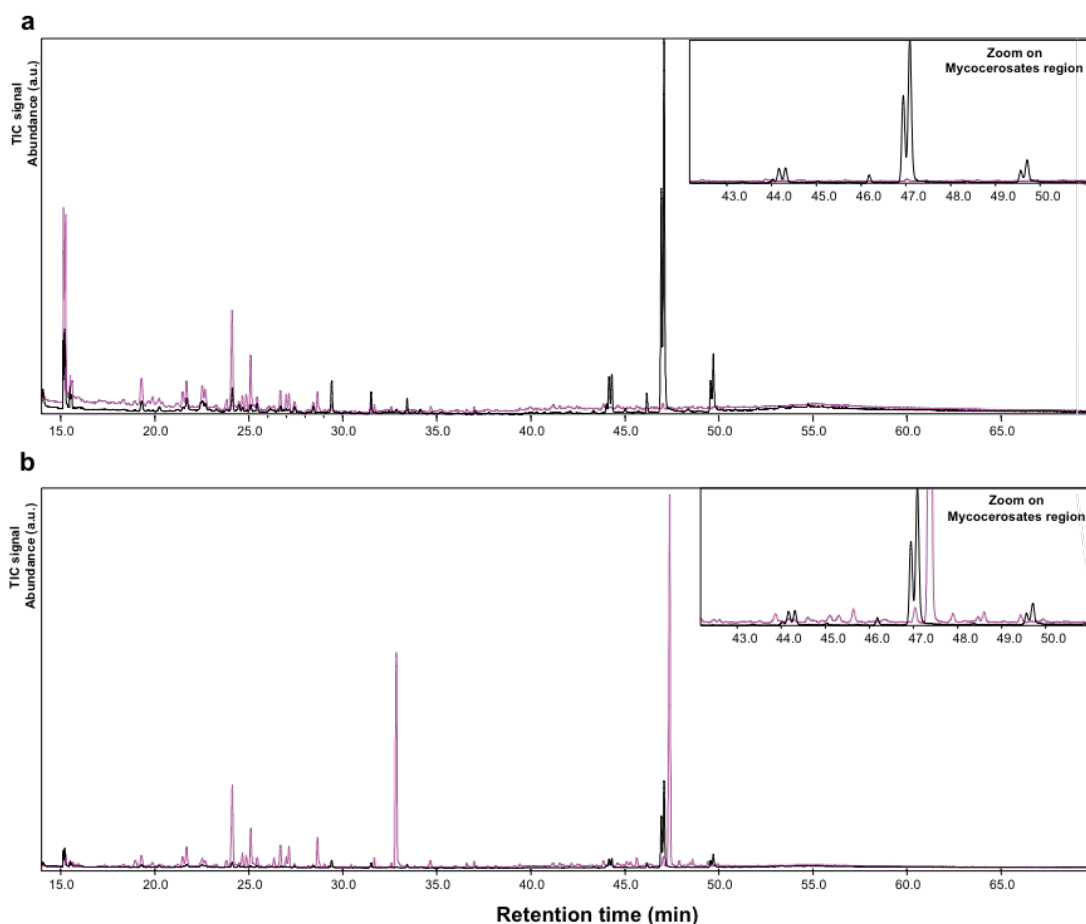


Figure 5.1 – Total ion current chromatograms of MTB strain 124 (hexane extract equivalent to approximately 1×10^8 bacteria, black line) and cholesteryl oleate (pink line) obtained with SEC \times THM-GC-MS of SEC fraction numbers 15 (a) and 16 (b). The inserts show the TIC zoomed in on the mycocerosates time window.

In this figure, the mycocerosates are separated from the interfering cholesterol. The characteristic biomarkers (the mycocerosates at around a retention time of 47 min) are mainly present in fraction number 15 whereas cholesterol is mostly in fraction 16. These preliminary results are comparable to the results reported with NPLC \times THM-GC-MS [11] and show the potential of using SEC

as a sample clean-up method for complex biological samples.

Encouraged by this, in the next set of experiments we spiked sputum specimens from different patients with lung infections other than TB (blank sputum) with MTB strain 124. The spiking was done with the hexane extract of MTB 124 at absolute amounts of 1×10^8 mycobacteria after sputum decontamination (see section 5.2.4) and the chromatogram is shown in **Figure 5.2**. Interestingly, we noticed a shift in elution time for cholesterol, from fraction 16 to fraction 17, while mycocerosates were only present in one fraction, fraction 15. This could be caused by the possible retention of the cholesterol molecules in the organic pores of the SEC columns by non-specific interactions and/or due to the poor choice of the solvent in the mobile phase. Nevertheless, these preliminary results might indicate the possible irreproducibility and non-repeatability of SEC for this type of matrices.

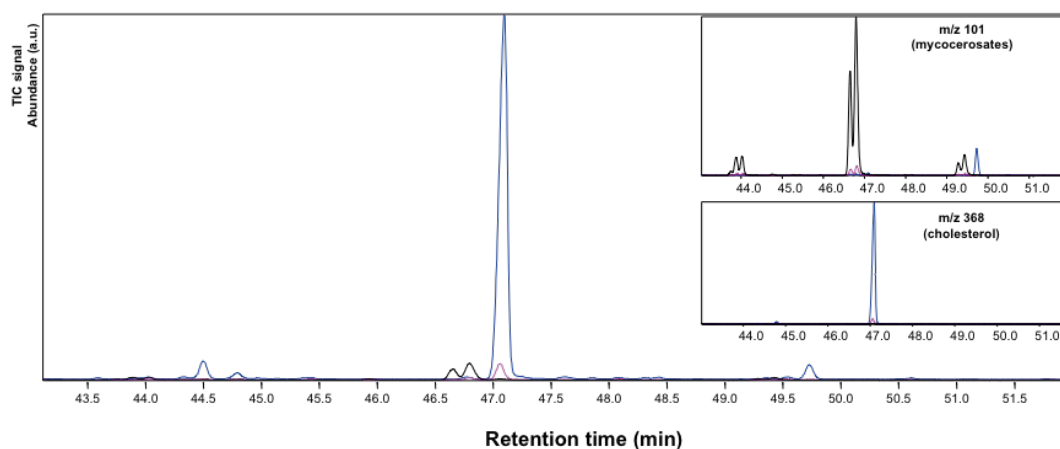


Figure 5.2 – Total ion current chromatograms (zoomed in on the mycocerosates time window) of SEC fractions 15 (black line), 16 (pink line) and 17 (blue line) obtained using SEC×THM-GC-MS of sputum samples spiked before decontamination at high levels of MTB strain 124. The inserts show the extracted ion chromatogram (m/z 101) for mycocerosates and for cholesterol (m/z 368).

A third and final series of experiments was meant to determine the detection limit (LOD) of the SEC×THM-GC-MS method. Blank sputum specimens were spiked with MTB strain 124 at levels ranging from 1×10^3 to 1×10^8

mycobacteria/mL. The lowest level of 1×10^3 mycobacteria/mL was selected because it represents the LOD of the comprehensive NPLC \times THM-GC-MS method described in our recent work [11] (chapter 4). The results, however, were not reproducible. The mycocerosates were only found at the highest spiked concentration level but now in fraction 12 (chromatograms shown in supplementary information section 5.7 **Figure 5.12**). Once more, these results could be due to the poor choice of the solvent or the organic SEC columns used. Perhaps a mobile phase of hexane and diethyl ether, as described in our previous work [11], and/or the use of silica SEC columns would be preferable. Overall, reliable results were not obtained by using SEC as a sample clean-up method prior to THM-GC-MS analysis for these complex samples, under the conditions described here. Therefore, we believe that it is unlikely that the SEC method can provide the very high degree of ruggedness needed for use of the technique in developing countries, and the idea of using SEC as a sample pretreatment method was abandoned.

5.3.1.2 Solid-phase extraction (SPE)

As explained previously, we developed and validated a sample preparation method using solid-phase extraction (silica-SPE) clean-up prior to THM-GC-MS analysis (SPE-THM-GC-MS) in order to better separate the interfering cholesterol-related compounds from the characteristic mycocerosate markers in sputum samples [9,10]. The method showed a good performance with a detection limit of 1×10^4 mycobacteria/mL, a sensitivity of 80% and a specificity of 98% [10]. However, we still noted cholesterol co-eluting or eluting closely to the mycocerosates (compounds 17-20 in **Table 5.1**), complicating in this way the interpretation of the results when lower levels of MTB are present in the sputum matrix. For this reason, we decided to investigate diol-silica SPE phases known for their selectivity for extraction of structurally similar molecules, their lower dependency on other species (e.g.

absorbs less water), better stability in terms of retention and more reproducible nature than bare-silica cartridges. However, a disadvantage is the weaker retention capacity of the diol groups [24]. We used a slightly adapted protocol as for the silica SPE experiments previously described [9,10]. In the first set of experiments, we spiked blank sputum specimens with an absolute amount of 1×10^6 mycobacteria/mL of the MTB strain 124. The results of these findings are shown in **Figure 5.3**, in a so-called “dot-plot”. In this “dot-plot”, the x-axis represents the number of the SPE fraction and the y-axis represents the compounds identified in our original 20 compounds model, **Table 5.1** [7]. The bubble size (area) reflects the absolute peak areas of the extracted fragment ions (m/z values in **Table 5.1**) obtained for each individual compound in the THM-GC-MS analysis. The graphs should be used for a quick assessment of the distribution and presence of the several compounds but not to compare different samples because the SPE conditions are sometimes varied.

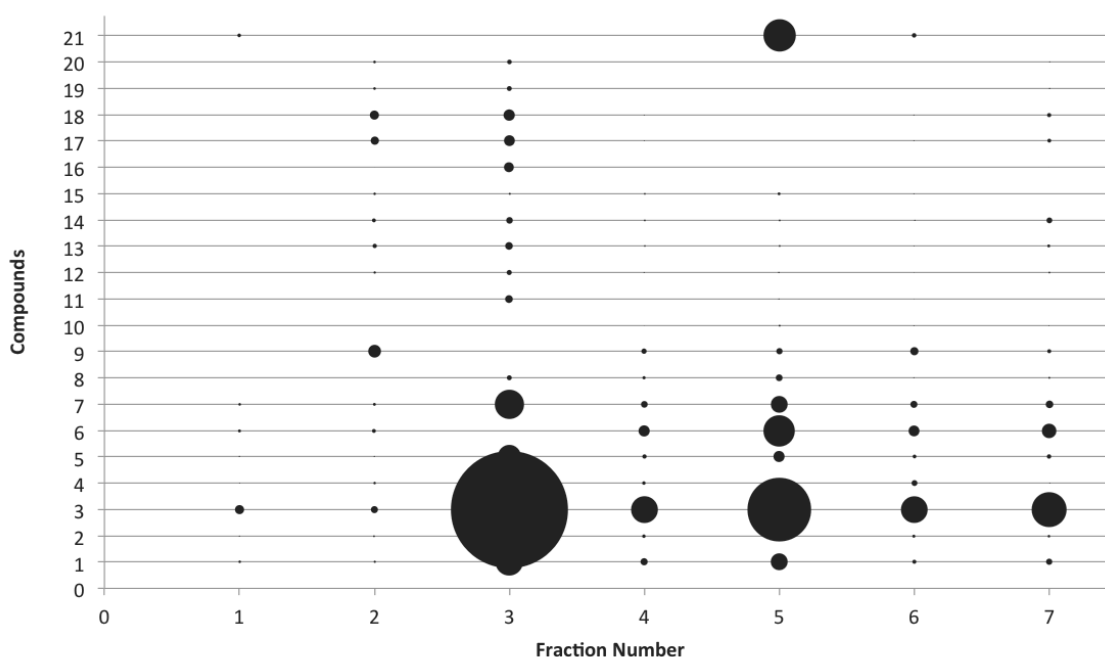


Figure 5.3 – Blank sputum specimen spiked with *M. tuberculosis* strain 124 (1×10^6 mycobacteria/mL), decontaminated, hexane extracted and analyzed with SPE-THM-GC-MS using a diol-silica SPE cartridge as sample clean-up. Cholesterol is represented as compound number 21. The numbers for compound identification are listed in **Table 5.1**. The dot areas represent the peak areas of the extracted fragments ions in **Table 5.1**.

As is clear from **Figure 5.3**, the target biomarkers were only present in three distinct SPE-diol fractions (numbers 2, 3 and 7), while cholesterol is present in other fractions (numbers 1, 5 and 6). Compared to our previous work [9], these results show the potential of using diol-silica SPE as a SPE clean-up method prior to THM-GC-MS analysis.

In order to establish a comparison between silica-SPE and diol-silica SPE, a next set of experiments was carried out to determine the detection limit. Blank sputum specimens were spiked with decreasing amounts of MTB strain 124 ranging from 1×10^3 to 1×10^6 mycobacteria/mL and analyzed with diol-silica SPE-THM-GC-MS. As explained above, the lowest concentration of 1×10^3 mycobacteria/mL was selected because it represents the LOD of the comprehensive NPLC \times THM-GC-MS method described in our recent work [11]. Unfortunately, even though the quality assurance procedure performed on the GC-MS (analyzing the MS tune file, injecting an GC alkane-mix and the MTB standard hexane extract equivalent to approximately 1×10^8 bacteria) indicated proper functioning of the instrument, poor results were obtained and the mycocerosates were only detected at the highest concentration level tested (1×10^6 mycobacteria/mL).

Before making a final conclusion on which solid phase method is the best, another SPE phase was considered. Amino-silica (NH_2) was investigated due to its more polar nature (hydrogen bonding) and a different selection of several organic solvents was used that allows the selective elution of different classes of lipids [25,26]. Initially we used a modified method from Phenomenex with three 100 mg SPE NH_2 cartridges (**Table 5.2**). The protocol consisted of 10 fractions with different elution solvents. All NH_2 -silica cartridges were conditioned with two portions of 0.5 mL of hexane. A summarized description is given in **Table 5.2**, with the elution solvents and the expected compounds in each fraction.

Table 5.2 – Summarized description of the original Phenomenex NH₂ protocol, using three cartridges with 10 fractions.

| Fraction number | Cartridge 1: Elution solvent | Cartridge 2: Elution solvent and steps | Cartridge 3: Elution solvent and steps | Expected compounds |
|------------------------|---|---|---|---|
| 1 | 1 mL of chloroform/2-propanol (2:1) | | | Neutral lipids |
| 2 | 1 mL of 2 % of acetic acid in diethyl ether | | | Fatty acids |
| 3 | 1 mL of methanol | | | Phospholipids |
| 4 | | Fraction 1 from cartridge 1 evaporated, reconstituted in 0.05 mL of hexane, applied on cartridge 2 and eluted with 1 mL of hexane | | Cholesteryl ester |
| 5 | | | Connect cartridge 2 above cartridge 3 and elute with 1.5 mL of 1% diethyl ether with 10% methylene chloride in hexane | Triglycerides |
| 6 | | | Keep cartridges 2 and 3 connected and elute with 3 mL of 5% ethyl acetate in hexane | Cholesterol |
| 7 | | Disconnect cartridge 2 | Elute only cartridge 3 with 1 mL of 15% ethyl acetate in hexane | Diglycerides |
| 8 | | | Elute only cartridge 3 with chloroform/methanol (2:1) | Monoglycerides and waxes in case of MTB |
| 9 | | Elute only cartridge 2 with 1 mL of chloroform/methanol (2:1) | | Waxes in case of MTB |
| 10 | | | Elute only cartridge 3 with 1 mL of chloroform/methanol (2:1) | Waxes in case of MTB |

Figure 5.4 shows the results obtained with the NH_2 fractionation of the hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^8 bacteria), where the mycocerosates and cholesterol are co-eluting in the same fractions. Moreover, the mycocerosates elute in multiple fractions. Therefore, the first next step was to reduce the number of fractions in which mycocerosates are eluting, preferably to only one or two. After this is optimized, the second step is to elute the mycocerosates and cholesterol in separate fractions.

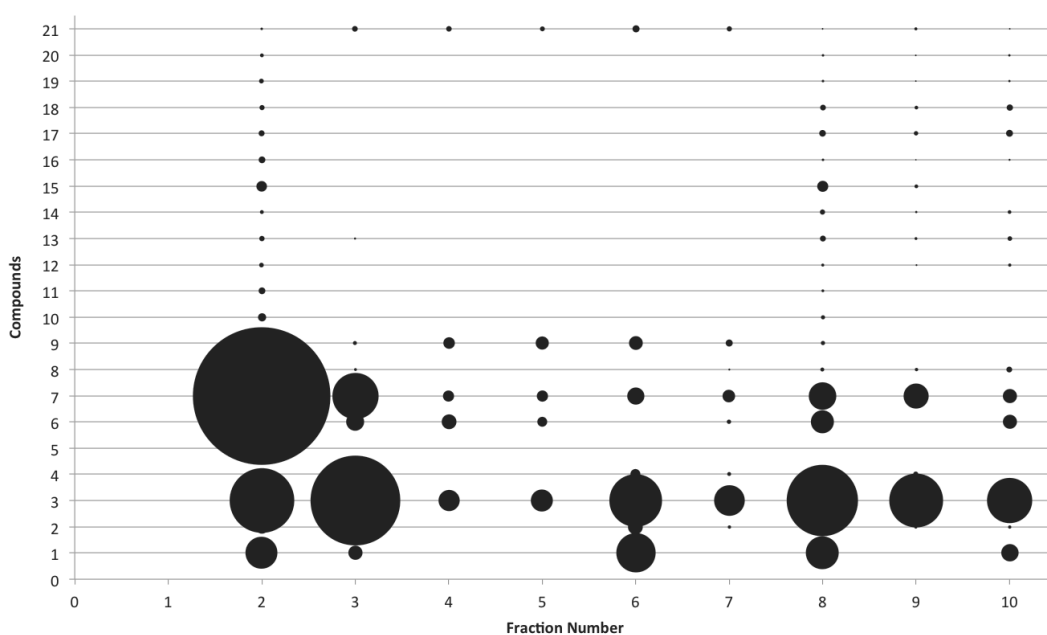


Figure 5.4 – SPE-THM-GC-MS analysis of the hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^8 bacteria) obtained using **three** NH_2 -silica SPE cartridges as sample clean-up. The SPE conditions are in **Table 5.2**. Cholesterol is represented as compound number 21. Other details as in **Figure 5.3**.

The experiments for reducing the number of fractions were carried out with decontaminated blank sputum, from patients with lung infections but not TB, spiked with $1 \mu\text{L}$ of the hexane extract of MTB strain 124 (equivalent to 1×10^8 bacteria).

The simplified SPE protocol is represented in **Table 5.3**. It now uses only two 100 mg NH₂-silica cartridges and yields four fractions. The cartridges were conditioned with two portions of 0.5 mL of hexane. The sample was applied on column 1 in 300 μL of hexane and further eluted as described in **Table 5.3**. We used the same fraction numbering in order to be comparable with the original Phenomenex NH₂ procedure from **Table 5.2**.

Table 5.3 – Summarized description of the altered SPE NH₂ protocol, using two cartridges with four fractions.

| Fraction number | Cartridge 1: Elution solvent | Cartridge 2: Elution solvent and steps | Cartridge 3: Elution solvent and steps |
|-----------------|--|---|---|
| 1 | 1 mL of chloroform/2-propanol (2:1). Fraction evaporated, reconstituted in 0.1 mL of hexane and applied on cartridge 2 | | Neutral lipids |
| 4 | | 0.9 mL of hexane | Cholesteryl ester |
| 7a | | 1.5 mL of 15% ethyl acetate in hexane | Diglycerides |
| 7b | | 1.5 mL of 15% ethyl acetate in hexane | Diglycerides, PDIMs |
| 8 | | 1 mL of chloroform/methanol (2:1) | Monoglycerides, PDIMs |

The results in **Figure 5.5** clearly show that the mycocerosates elute mainly in fraction 8. Even though cholesterol is also present in fraction 8, in the chromatograms these compounds are well separated (chromatograms shown in supplementary information section 5.7 **Figure 5.13**). We believe that this is possibly due to the choice of a different GC column, compared to the one we used in our previous work [11], namely a shorter column with a stationary phase with a slightly different polarity (5% diphenyl-dimethylpolysilphenylene siloxane).

Therefore, we conclude that the choice of the GC column can have a great impact on the GC-based diagnosis of MTB, especially when combined with the proper SPE sample clean-up method.

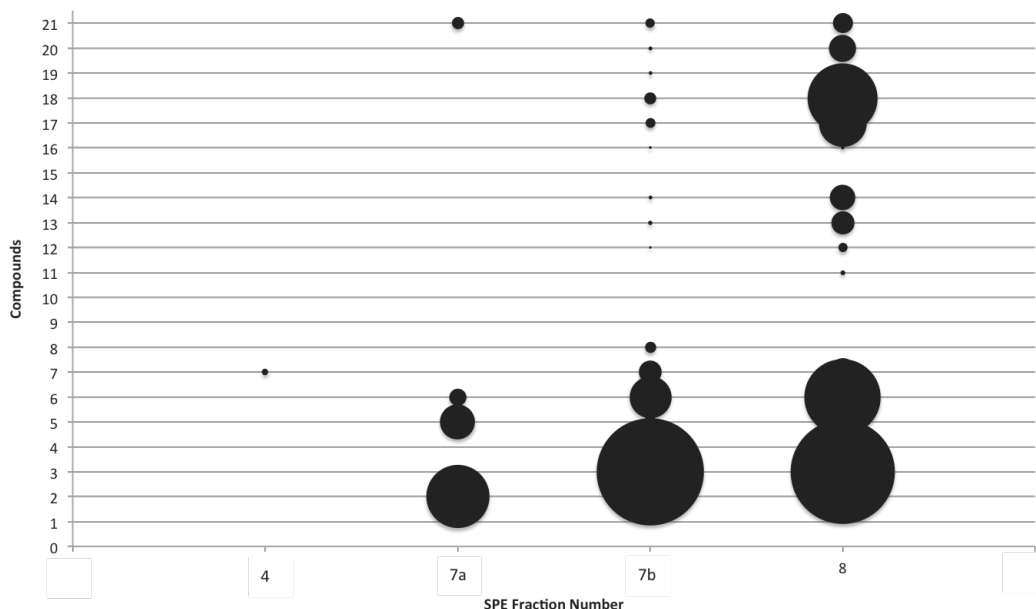


Figure 5.5 – Blank sputum specimen spiked with *M. tuberculosis* strain 124 (1×10^8 bacteria), decontaminated, hexane extracted and analyzed with SPE-THM-GC-MS using two NH_2 -silica SPE cartridges as sample clean-up. The SPE conditions are in **Table 5.3**. Cholesterol is represented as compound number 21. Other details as in **Figure 5.3**.

After successfully reducing the number of fractions to two and improving the GC separation we further investigated an even simpler method using only one NH_2 SPE cartridge. The experimental procedure, in terms of elution solvents and the number of fractions, was the same as described above. We noticed that by using only one cartridge, the target compounds are eluting in almost equal amounts in fraction 7a and fraction 8, while cholesterol is present in every fraction (**Figure 5.6**).

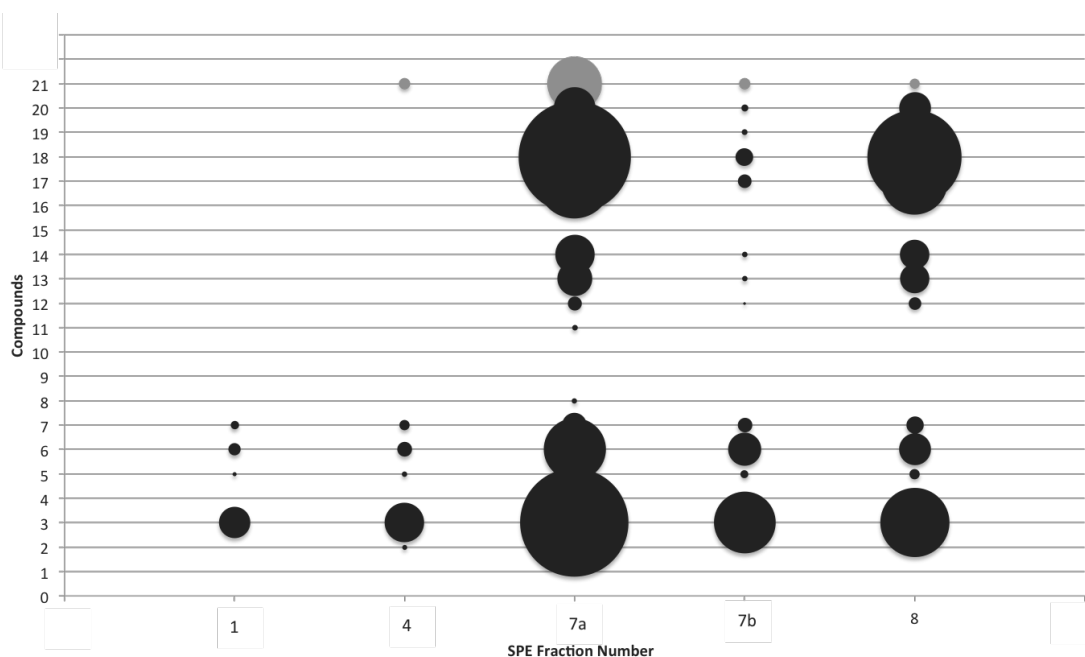


Figure 5.6 – Blank sputum specimen spiked with *M. tuberculosis* strain 124 (1×10^8 bacteria), decontaminated, hexane extracted and analyzed with SPE-THM-GC-MS using one NH_2 -silica SPE cartridge as sample clean-up. The SPE conditions are similar to those in **Table 5.3**. Cholesterol is represented as compound number 21 (grey dots). Other details as in **Figure 5.3**.

At this point, we decided to continue to use only one cartridge but change the elution solvent of fractions 7a and 7b in order to elute cholesterol, if possible, in only one fraction. To this end, we tested 10% and 7.5% of ethyl acetate in hexane. We concluded that 7.5% of ethyl acetate in hexane proved to be better than 10%, since cholesterol is then mainly present in fraction 7b where barely any mycocerosates are present (**Figure 5.7**). In addition, if fractions 7a and 8 are combined, the mycocerosates yield is enhanced. Although cholesterol could not be fully separated in one fraction that does not contain mycocerosates, most likely because of the similar polarity of this compound to that of the lipid precursors of mycocerosates, the GC chromatograms show these compounds fully separated (chromatograms shown in supplementary information section 5.7 **Figure 5.13**).

Nevertheless, we believe that SPE fractionation is still necessary for simplifying the complexity of these biological samples and that without it the GC-MS system could be severely compromised, particularly in terms of contamination of the MS detector.

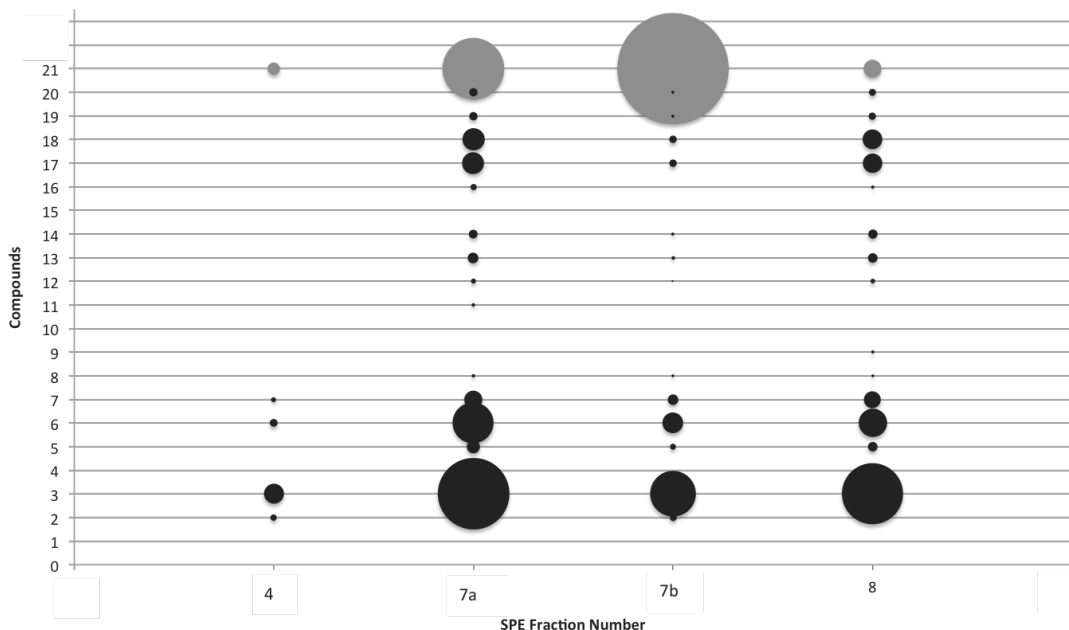


Figure 5.7 – Blank sputum specimen spiked with *M. tuberculosis* strain 124 (1×10^8 bacteria), decontaminated, hexane extracted and analyzed with SPE-THM-GC-MS using **one** NH_2 -silica SPE cartridge as sample clean-up (elution with 7.5% of ethyl acetate in hexane). The SPE conditions are similar to those in **Table 5.3**. Cholesterol is represented as compound number 21 (grey dots). Other details as in **Figure 5.3**.

Following up on this, a final series of experiments was conducted in order to determine the detection limit of the procedure using the optimum conditions presented in **Table 5.4**.

Table 5.4 – Optimized conditions for SPE (NH₂)-THM-GC-MS analyses, using one cartridge with four fractions.

| Fraction number | Elution solvent | Expected compound |
|-----------------|---|--|
| 4 | 0.25 mL of hexane | Cholesteryl ester |
| 7a+8 | 1.5 mL of 7.5% of ethyl acetate in hexane | PDIMs (Compounds 17-20 Table 5.1) |
| 7b | 1.5 mL of 7.5% of ethyl acetate in hexane | Cholesterol |
| 8 | 1 mL of chloroform/methanol (2:1) | PDIMs |

MTB strain 124 ranging from 1×10^3 to 1×10^7 mycobacteria/mL was used to spike blank sputum specimens for analysis with NH₂-silica SPE-THM-GC-MS. However, once again, the mycocerosate markers were only detected at the highest amount tested (1×10^7 mycobacteria/mL). Therefore, our conclusion of this study is that diol-silica SPE showed more promising results in terms of separating the mycocerosates and cholesterol in different fractions. Nevertheless, more studies could be done with diol-silica SPE cartridges on combining the mycocerosates eluting fractions (numbers 2, 3 and 7) in order to enhance their yield and potentially increase their detection at lower levels. However, regarding the detection limit, the silica SPE cartridge [9] gave much better results with a detection limit of 1×10^4 mycobacteria/mL compared to 1×10^6 mycobacteria/mL for the diol-silica SPE and 1×10^7 mycobacteria/mL for the NH₂-silica SPE. These higher detection limits for diol-silica and NH₂-silica could be explained by the weaker interactions between the mycocerosates and the diol and NH₂ groups of the SPE stationary phases, whereas in the bare silica cartridges the interaction is stronger. Due to the weaker interactions displacement of the PDIMs from the surface might occur by the vast excess of other compounds in the lipid extract. Even though, SPE is a simple, cheaper and easy method with a wide range of available phases that can be used in developing countries, the unexpected preliminary results described here are not conclusive and further investigations are needed in

terms of SPE procedure/conditions optimization.

5.3.2 Manual vs. Automatic THM-GC-MS: a comparison study

As mentioned previously, in our most recent work we developed an NPLC×THM-GC-MS method with a detection limit of 1×10^3 mycobacteria/mL for spiked sputum specimens [11].

However, since automatic THM-GC-MS analysis is complex and expensive for developing countries, a second route of simplifying the method is to perform a manual THM reaction prior to GC-MS analysis instead. Performing the derivatization reaction manually in a vial as compared to fully automated inside the liner of the GC injector requires the re-optimization of several reaction parameters. Kaal et al. started this investigation in 2008 [27] and concluded that some parameters, such as the thorough mixing of the sample and the TMAH reagent, a sufficiently high temperature for the reaction, and the use of freshly prepared TMAH solutions and its proper storage, are extremely important.

In the current study we used the Design of Experiments (DOE) methodology in order to optimize several parameters using a multi-variable approach in a time-efficient manner [28–31]. Moreover, the use of the DOE methodology reduces the risk of ending in a local optimum rather than the true optimum. The DOE approach eliminates the very time-consuming optimization of each parameter individually. The parameters selected here for inclusion in the DOE optimization were the sample volume, reagent volume, type of solvent used for the reagent, concentration of the reagent, time for the reaction and finally the reaction temperature. A summarized description is presented in **Table 5.5**. The DOE method created a list of 20 experiments to be done using a conventional oven prior to GC-MS analysis. For data evaluation we selected two criteria: the absolute peak areas of the targeted species (reflecting

sensitivity) and the ratio of the peak area of the target analytes versus that of the interfering compounds (reflecting selectivity). In the initial stage the design includes main effects only.

Table 5.5 – Parameters to optimize for manual THM reaction prior to GC-MS analysis in the Design of Experiments approach (DOE).

| Parameters | Ranges to analyze |
|-----------------------|---|
| Sample volume | 20-100 μL |
| Reagent volume | 20-100 μL |
| Reagent solvent | Methanol, water, water in isopropanol and methanol in isopropanol |
| Reagent concentration | 2.5-25% |
| Time of reaction | 0-60 minutes |
| Oven temperature | 80-120 $^{\circ}\text{C}$ |

The best conditions that gave the highest peak areas for mycocerosates, but low values for cholesterol, are shown in **Table 5.6**.

Table 5.6 – Optimized conditions for Manual THM-GC-MS analyses, using the DOE approach.

| Parameters | Optimized values |
|-----------------------|-------------------------|
| Sample volume | 55 μL |
| Reagent volume | 100 μL |
| Reagent solvent | Methanol in isopropanol |
| Reagent concentration | 7.5% |
| Time of reaction | 60 minutes |
| Oven temperature | 120 $^{\circ}\text{C}$ |

Now that these parameters were optimized, the next series of experiments was to determine the differences between the automatic THM reaction, performed in-situ in the PTV injector using the protocol summarized in section 5.2.5.2, and the manual THM reaction of the hexane extract of MTB strain 124. The optimized conditions for both methods are reviewed in **Table 5.7**. From the theoretical assessment presented here manual and automatic THM are expected to yield approximately the same sensitivity.

Table 5.7 – Comparison between the optimized parameters that impact sensitivity for manual and automatic THM-GC-MS analyses.

| | Parameters | Automatic THM | Manual THM |
|---|--|---------------------------------|--------------------|
| 1 | Sample volume | 20 μL | 55 μL |
| 2 | Reagent volume added | 25 μL | 100 μL |
| 3 | Solvent added after reaction (manual THM only, sample was completely evaporated) | n.a | 50 μL |
| 4 | Sample volume injected into the PTV | All 20 μL from row 1 | 25 μL |
| 5 | Effective volume transferred to column (split ratio 1:8) | 2.22 μL | 3.05 μL |
| 6 | Relative sensitivity | 1 | 1.375 |

n.a. – not applicable.

If the manual THM reaction would have the same results as automatic THM, i.e. similar lipid profile and the presence of mycocerosates in the correct amounts and ratios, it has great potential for use in developing countries. The chromatograms are depicted in **Figure 5.8** and clearly show the similarities of both methods.

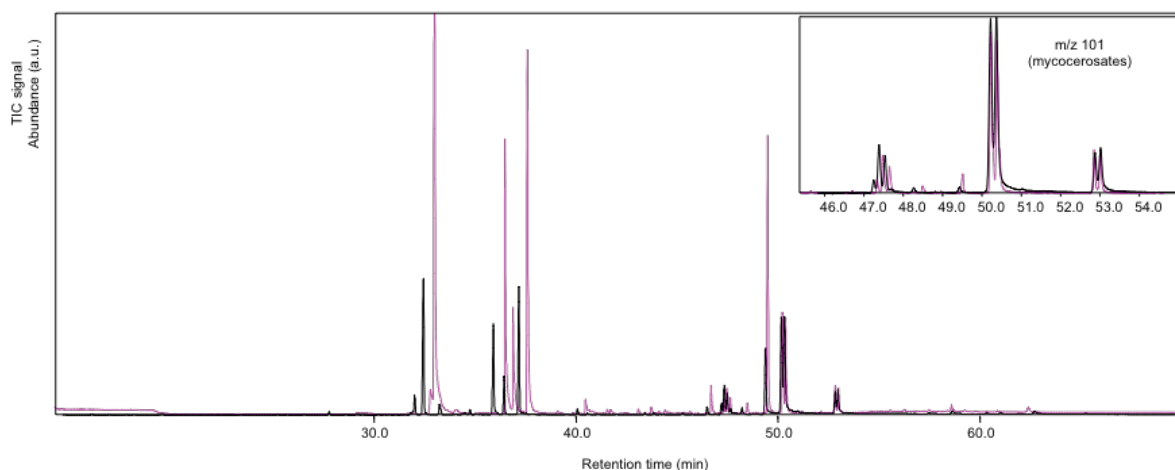


Figure 5.8 – Total ion current chromatograms of the THM reaction of the hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^8 bacteria) done automatically (pink line) and manually with the DOE optimized conditions (black line) obtained using GC-MS. The inserts show the extracted ion chromatogram (*m/z* 101) for mycocerosates.

Even though both approaches give similar results, it is important to determine their respective detection limit in order to take an informed decision of which method is preferable for the early diagnosis of TB. Therefore, a final series of experiments was conducted with decreasing amounts of the hexane extract of MTB strain 124. Unfortunately, we noted that this similar sensitivity is not always repeatable or reproducible and hence it can be difficult to make a final conclusion. Nevertheless in developing countries, where the complex instrumentation for automatic THM might not be available, manually performing the THM reaction with the conditions described in **Table 5.6** is quite acceptable.

5.3.3 GC detection: MS or FID?

The third and final option to simplify the GC-based TB diagnosis is the choice between detectors: either MS or FID. The MS has the power of truly identifying unknown molecules and allows using extracted or selected ion traces to improve selectivity and sensitivity. On the other hand the FID is a

simpler and cheaper universal detector that is easier to operate and is widely available. Our GC-FID experiments were done using manual THM (**Table 5.6**), simulating the laboratory conditions in developing countries. A description of the GC-FID method used is given in section 5.2.5. **Figure 5.9** compares the TICs of the hexane extract of MTB strain 124, equivalent to approximately 1×10^8 bacteria, analyzed with automatic THM-GC-MS (**Figure 5.9a**) and manual THM-GC-FID (**Figure 5.9b**).

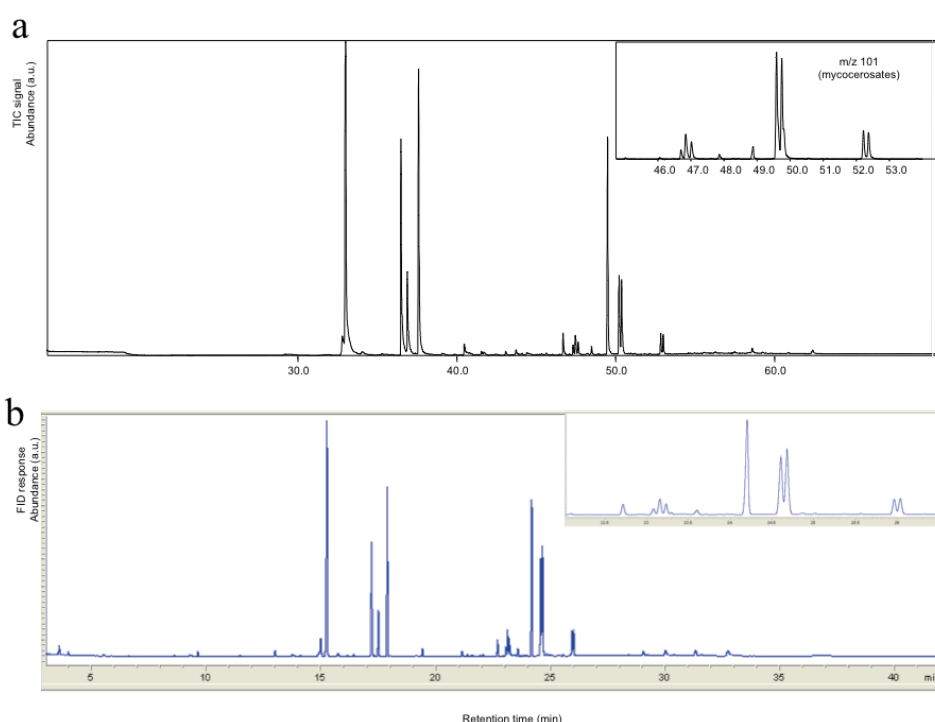


Figure 5.9 – GC chromatograms of the THM reaction of the hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^8 bacteria) done automatically on the GC-MS, with the insert showing the extracted ion chromatogram (m/z 101) for mycocerosates (**a**) and manually with the DOE conditions from **Table 5.6** on the GC-FID, with the insert showing the zoomed window on mycocerosates (**b**).

From the results it is clear that both methodologies, at least at high levels of mycobacteria, yield similar chromatograms. Therefore, we concluded that a detector as simple as the FID has great potential for the diagnosis of TB. However, without optimizing the GC-FID systems and its parameters (for

example the split/splitless mode, injection volume and/or column flow), one cannot conclude which detector is the best choice for detecting low levels of MTB. On the one hand the MS detector is able to positively confirm the identity of a molecule, on the other hand the FID is a simple detector that gives response to a wide range of compounds.

5.4 Conclusions

In the present contribution we describe several strategies for simplifying the GC-based diagnosis of TB in order to assist in the control of the disease. We first focused on simplifying the sample preparation step by investigating SEC and different SPE phases and concluded that even though silica SPE cartridges have a lower detection limit, diol-silica phases are better in separating the interfering compounds from the important mycocerosate biomarkers. Secondly, the THM derivatization reaction can be performed manually, instead of automatically in a PTV injector, since theoretically the relative sensitivity is very similar. Finally, the use of an FID detector is possible and gives acceptable results. However, the main disadvantage of using manual THM with GC-FID with a split/splitless type injector is that this combination is not suitable for detecting low levels of MTB unambiguously. Due to lack of mass spectral confirmation other compounds could be incorrectly identified as mycocerosates, hence compromising the early diagnosis of TB.

In the end, we believe that a critical choice needs to be made, either one chooses the highest sensitivity and specificity that modern technology can provide or one opts for an affordable, easy to use and simpler analytical method, but more laborious and less sensitive. This decision depends on ones needs, aims, possibilities and available instrumentation.

5.5 Future perspectives

5.5.1 Using simpler matrices (urine and serum) in combination with very selective GC-MS (GC-QTOF-MS) for the detection of Tuberculosis biomarkers

Simplifying the diagnosis of Tuberculosis has been one of the most pressing topics over the years. This urgency has been mostly caused by the invasive sample collection method that is used when the patient is suspected of having TB, but does not show signs of the disease. Sample collection directly from the lungs, i.e. induced sputum collection, can be difficult especially in the case of children. For these reasons, researchers have focused on other simpler matrices that are easier to collect, prepare and analyze. Brooks was the pioneer in 1987 by detecting TBSA in serum and cerebrospinal fluid with GC-ECD (electron-capture detector) [32], while more recently in 2008 Kim and co-workers have profiled for the first time phospholipids present in urine by nanoflow LC-ESI-MS-MS [33]. Their promising findings enabled the search for mycobacteria lipids in other body fluids as urine or serum that do not require complex sample preparation methods.

Some preliminary experiments were done focusing on using manual THM reaction followed by a typical GC-MS on urine samples from TB patients to monitor the presence of mycocerosates and TBSA (compounds 17-20 and 8 respectively in **Table 5.1**), in supplementary information section 5.7 **Figure 5.14**. Since TBSA is commonly present in other types of mycobacteria, we believe that the combination of TBSA with mycocerosates is needed to be sufficiently specific for the detection of MTB. The sample preparation method here consisted on a simple liquid-liquid extraction with hexane as the extraction solvent. The results show a very bad signal-to-noise (S/N) ratio that can be suppressing the signal from TBSA and mycocerosates. In order to test this hypothesis, the experiment was repeated with a much more selective GC-

MS, an accurate mass GC-Quadrupole (Q)-time-of-flight (TOF)-MS. The great advantage of the system is its high selectivity and the resulting high sensitivity. It can improve the S/N ratio by filtering out all other ions (of nominally identical mass yet different accurate mass) that are not of interest. In addition, GC-QTOF-MS allows reliable identification due to the high mass accuracy that comes from TOF, in which unknown compounds can be distinguished up to at least 4 decimal places after the comma [34,35].

The set-up used for the next series of experiments was a 7200B Series GC-QTOF-MS from JSB (partner of Agilent Technologies, Lelystad, The Netherlands). The instrument was operating with the conditions shown in **Table 5.8**.

Table 5.8 – GC-QTOF-MS instrumental conditions used.

| Parameters | Settings |
|------------|--|
| Column | DB-5ms Ultra Inert, 30m, 0.25 mm, 0.25 μm |
| Injector | 2 μL splitless injection at 350 $^{\circ}\text{C}$ |
| Oven | Same as in section 5.2.5.2 |
| QTOF | Ion source: EI at 230 $^{\circ}\text{C}$ Emission: 35.0 μA |
| MS | Mass range: 50 to 600 amu Acquisition rate: 5.00 spectra/s Acquisition time: 200 ms/spectrum |

Since the instrument uses a split/splitless injector it was not possible to perform automatic THM and so manual THM reaction was performed instead. As demonstrated above in **Table 5.7**, manual THM reaction gives similar results as automatic THM, so we believe that by using this very selective system the sensitivity will not be an issue.

First, an initial study with a diluted hexane extract of MTB strain 124 (equivalent to approximately 1×10^6 bacteria) was performed to establish the feasibility of using this type of technology. As evident from **Figure 5.10**, the S/N ratios have improved and the accurate mass of the characteristic fragments of the mycocerosates could be detected (101.0595 Da).

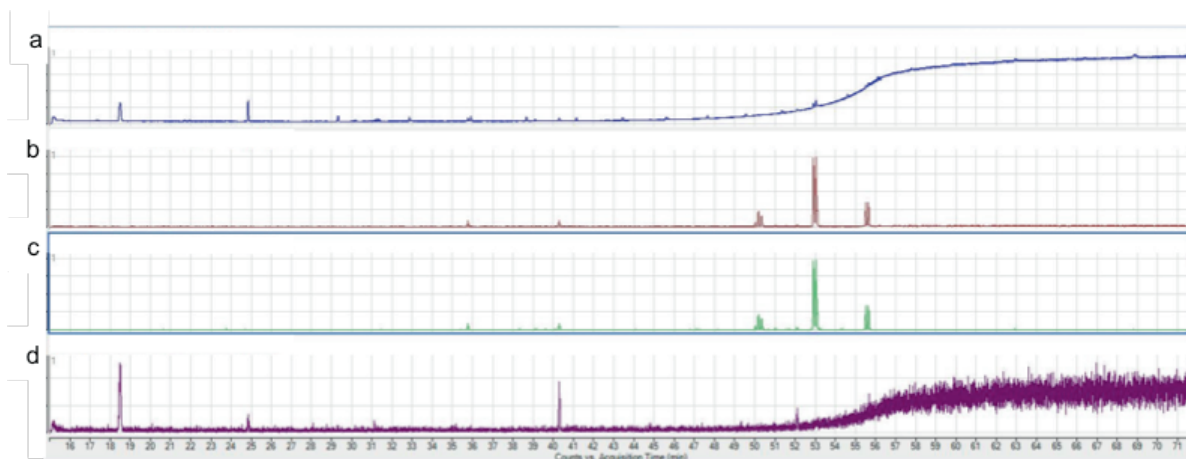


Figure 5.10 – Total ion current chromatograms of the diluted hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^6 bacteria) (a) obtained using manual THM reaction (DOE optimized conditions) followed by GC-QTOF-MS. Nominal (101) and accurate (101.0595) masses for mycocerosates are also shown (b) and (c) respectively, as well as the nominal mass for TBSA (312) (d).

Next, the urine samples previously tested (**Figure 5.14** in supplementary information section 5.7) were repeated using the GC-QTOF-MS instrument (**Figure 5.11**). Here, all three samples show the same lipid profile with different intensities. In addition, even though the samples were 100 times diluted (second analysis) the S/N ratio is significantly better.

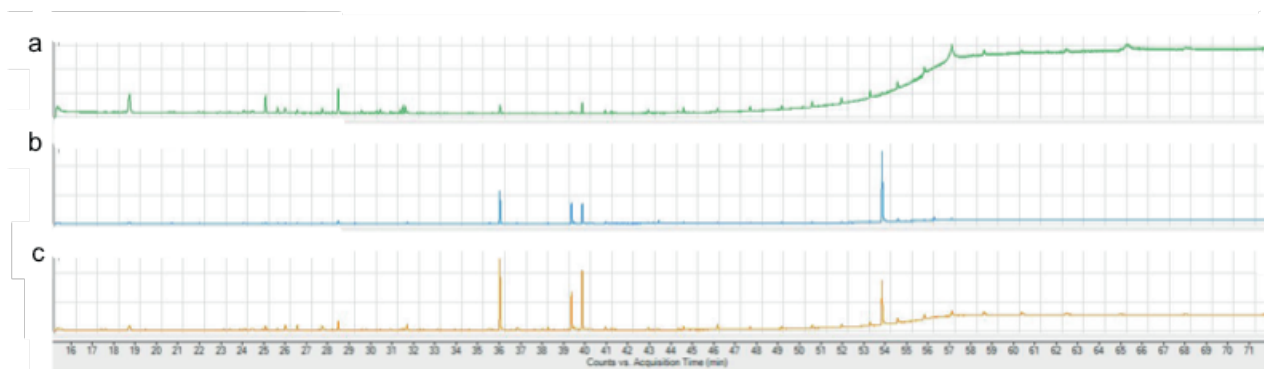


Figure 5.11 – Total ion current chromatograms of urine samples from three different TB patients (**a-c**) obtained using manual THM reaction (DOE optimized conditions) followed by GC-QTOF-MS.

Encouraged by this, we searched for the extracted ion 101 using nominal and accurate masses in order to prove the hypothesis that the compounds could be suppressed by the bad S/N ratio in nominal mass GC-MS, illustrated in **Figures 5.15** and **5.16** respectively (in supplementary information section 5.7). Despite the very promising results that this technology can provide, we confirmed that indeed these samples did not contain detectable levels of mycocerosates. However, if TBSA was plotted, we conclude that urine may contain an isomer of this compound. A compound with the same spectrum and accurate mass (312.3030 Da) was found yet its retention time is slightly different (in supplementary information section 5.7 **Figure 5.17**).

Finally, a serum sample from a TB patient was tested as an illustrative example of what other types of matrices could be analyzed, in supplementary information section 5.7 **Figure 5.18**. The sample was prepared in the same way as the urine samples. In this figure, the lipid profile is clearly different from that of urine as it is evident from the very intensive peaks and the presence of high amounts of cholesterol derived compounds. Unfortunately, neither mycocerosates nor TBSA were found.

Although the results were not as expected, the GC-QTOF-MS technique is very powerful in terms of selectivity due to the significant improvement of the

S/N ratio that, as a consequence, makes the technique more sensitive. However, we believe that the optimization of the QTOF-MS method, in terms of oven and ion source temperatures as well as MS voltages and resolution can be extremely important.

Furthermore, other GC-QTOF-MS methods for untargeted analysis [36] could be evaluated, such as Chemical Ionization (CI) similar to the EI approach, where a softer fragmentation takes place and most of the molecular ion stays intact. In contrast, for targeted analysis triple quadrupole mass spectrometry (QqQ) could be applied using multiple reaction monitoring (MRM) [37–39]. Operation of a triple quadrupole in MRM results in an extremely high selectivity and sensitivity.

A review on these mass separators as well as the MS operating modes have been fully discussed in Chapter 2.

5.6 Acknowledgments

Thanks to Daniela Peroni for her availability and help on working with the GC-QTOF-MS system and software at JSB Company (Lelystad, The Netherlands).

5.7 Supplementary information

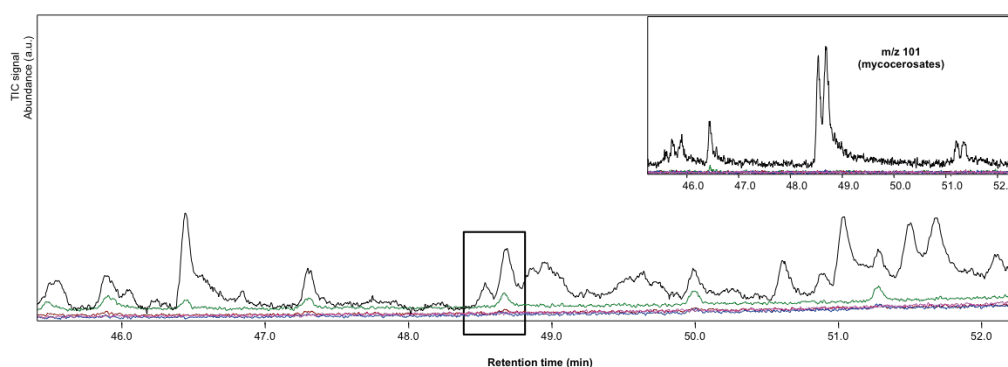


Figure 5.12 – Total ion current chromatograms (zoomed in on the mycocerosates time window) of SEC fractions 12 (black line), 13 (pink line), 14 (blue line), 15 (brown line) and 16 (green line) obtained using SEC×THM-GC-MS of sputum samples spiked before decontamination at very high levels of MTB strain 124 (1×10^8 mycobacteria/mL). The insert shows the extracted ion chromatogram (m/z 101) for mycocerosates.

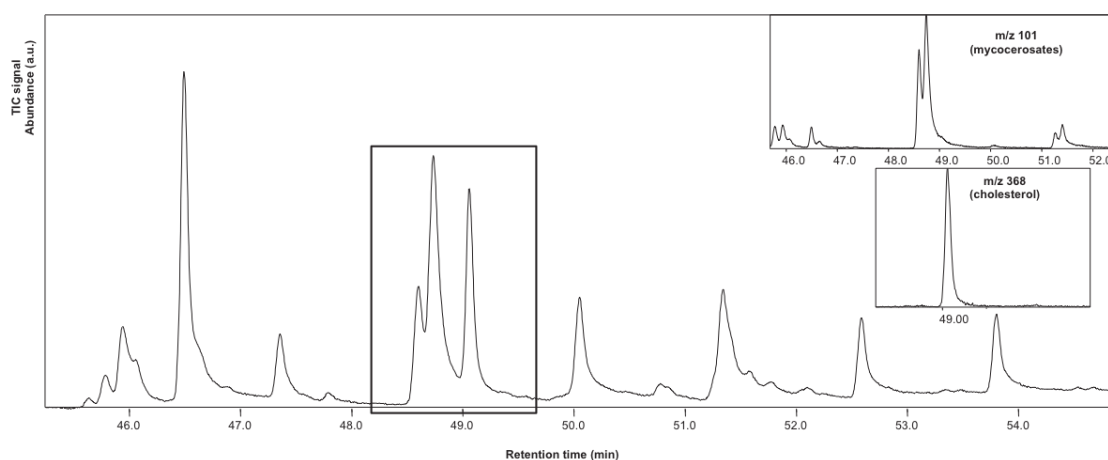


Figure 5.13 – Total ion current chromatograms (zoomed in on the mycocerosates time window) of NH₂-silica SPE fraction 8 obtained using NH₂ SPE-THM-GC-MS of sputum samples spiked before decontamination with MTB strain 124 (equivalent to 1×10^8 bacteria). The SPE protocol was as described in **Table 5.3**. The inserts show the extracted ion chromatogram (m/z 101) for mycocerosates and for cholesterol (m/z 368).

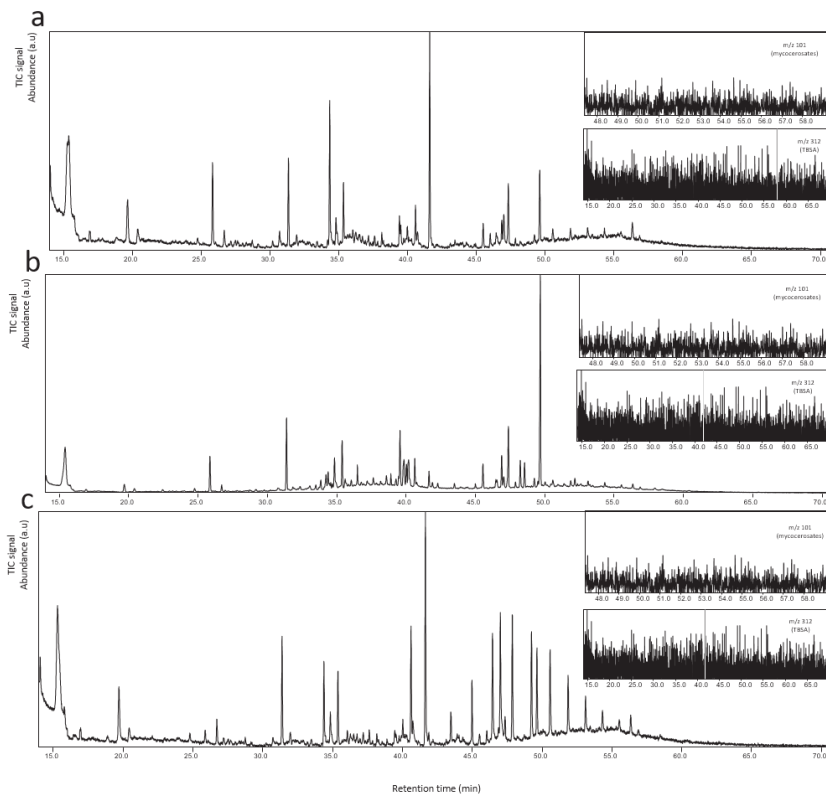


Figure 5.14 – Total ion current chromatograms of urine samples from TB patients (a-c) obtained using automatic THM reaction followed by GC-MS. The inserts show the extracted ion chromatograms (m/z 101) for mycocerosates (top insert) and (m/z 312) for TBSA (bottom insert).

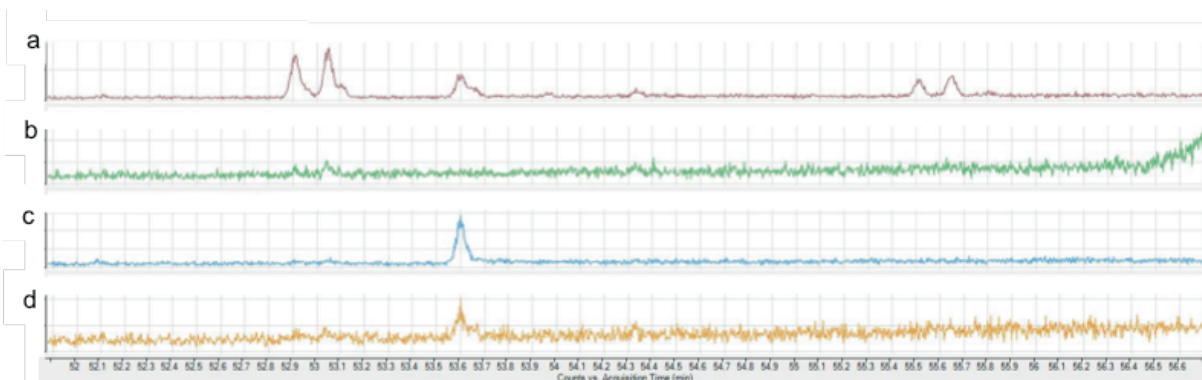


Figure 5.15 – Mycocerosates nominal mass (101) of the diluted hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^6 bacteria) (a) and urine samples from TB patients (b-d) obtained using manual THM reaction (DOE optimized conditions) followed by GC-QTOF-MS.

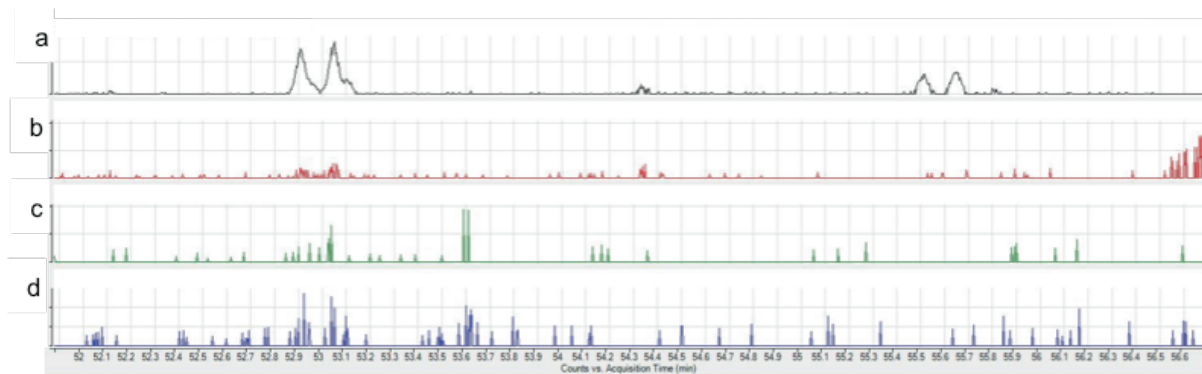


Figure 5.16 – Mycocerosates accurate mass (101.0595) of the diluted hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^6 bacteria) (a) and urine samples from TB patients (b-d) obtained using manual THM reaction (DOE optimized conditions) followed by GC-QTOF-MS.

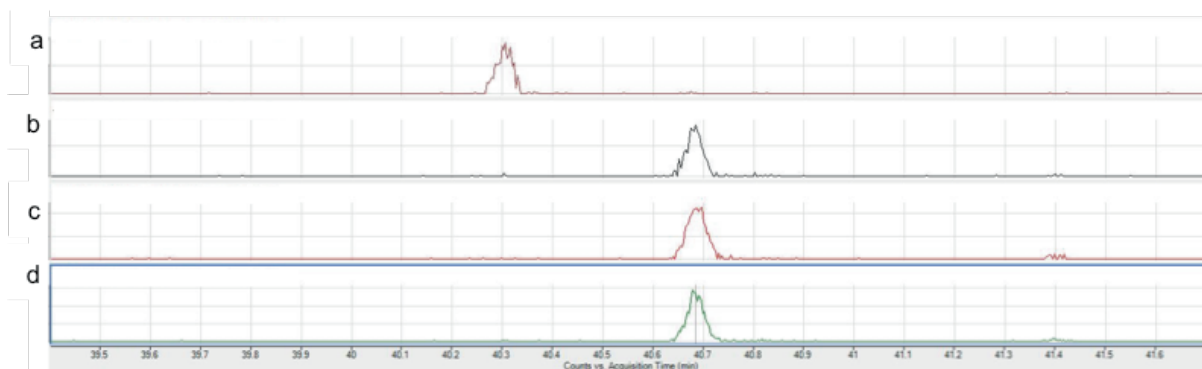


Figure 5.17 – TBSA accurate mass (312.3030) of the diluted hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^6 bacteria) (a) and urine samples from TB patients (b-d) obtained using manual THM reaction (DOE optimized conditions) followed by GC-QTOF-MS.

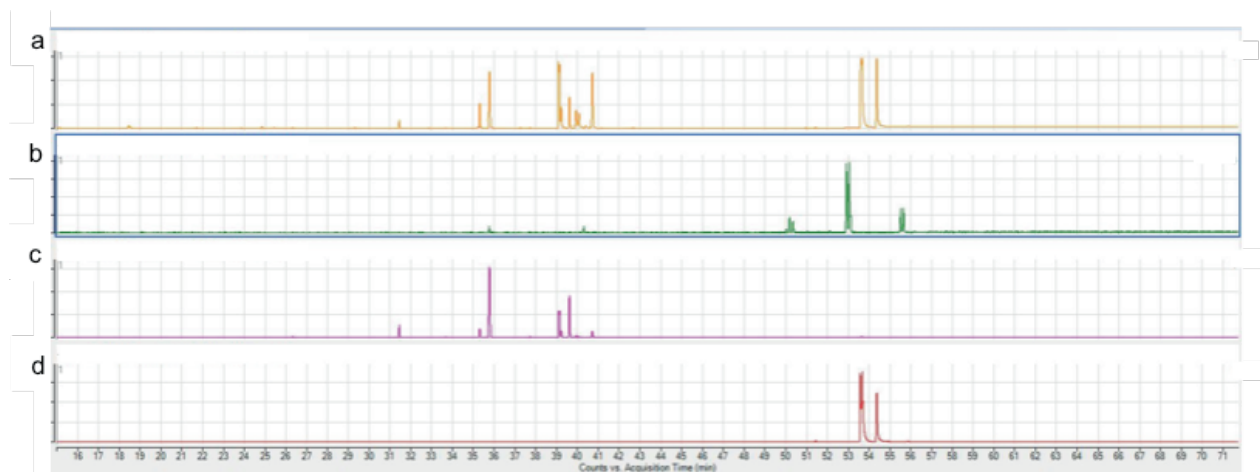


Figure 5.18 – Total ion current chromatograms of a serum sample from a TB patient (a) obtained using manual THM reaction (DOE optimized conditions) followed by GC-QTOF-MS. The nominal mass for mycocerosates (101) is shown for the diluted hexane extract of *M. tuberculosis* strain 124 (equivalent to approximately 1×10^6 bacteria) (b) and for the serum sample (c). Cholesterol derivative compounds are represented with a nominal mass of 368 (d).

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