Anish Zacharia Joseph, Anubhav Jain, Mukul Pachauri, Ajay Kumar, G.B.K.S Prasad, P.S. Bisen

ABSTRACT

AIM: The present study is focused on the identification of high abundant and low abundant biomarkers of Mycobacterium tuberculosis from serum specimen using Gas chromatography and Mass spectroscopy.

METHODS: The TB positive and negative sera were screened on the basis of sputum smear microscopy and the in house developed liposome based antibody detection kit. The lipid fraction was isolated from the collected sera and derivatized. In GCMS analysis, the derivatized lipid samples were analyzed through Gas Chromatograph Mass spectrometer (Shimadzu QP-2010 plus with Thermal Desorption system TD 20). Split or splitless mode of sample injection was performed to identify the high abundant and low abundant biomarkers of tuberculosis.

RESULTS: The study identified lipid biomarkers specific for M. tuberculosis in the serum of tuberculosis positive subjects. The study revealed that lipids like C_{21}H_{42}, C_{22}H_{38}O_2, C_{22}H_{34}, C_{26}H_{52}, C_{24}H_{48}O_2 was identified in the split mode of sample injection, whereas C_{28}H_{56}, C_{29}H_{52}, C_{30}H_{56}BrO_2, C_{31}H_{58}O_2, C_{32}H_{60}O_2S and C_{34}H_{70}N were identified in the splitless mode of sample injection. Under split injection mode of analysis, C_{24}H_{48}O_2 and C_{26}H_{52}O_2 were identified as low abundant lipids. The molecules like C_{30}H_{60}BrO_2, C_{31}H_{58}O_2S, C_{32}H_{60}O_2 and C_{34}H_{70}N were identified as low abundant lipids even after the splitless mode of analysis.

DISCUSSIONS: The GCMS analysis revealed the presence of lipid biomarkers of Mycobacterial tuberculosis in the circulation of selected tb positive sera samples. The study further identified the low abundant and high abundant biomarkers of tuberculosis. The detection of characterized low abundant biomarkers may help in identifying the disease in sputum smear negative cases.

Key words: Lipid biomarker; GCMS detection; VOC (volatile organic compound); MTB (Mycobacterium tuberculosis)

© 2016 The Authors. Published by ACT Publishing Group Ltd.

Joseph AZ, Jain A, Pachauri M, Kumar A, Prasad GBKS, Bisen PS. GCMS Based Detection of Lipid Biomarkers of Mycobacterium tuberculosis in the Serum Specimen. Journal of Respiratory Research 2016; 2(2): 47-55 Available from: URL: http://www.ghrnet.org/index.php/jrr/article/view/1630

INTRODUCTION

Mycobacterium tuberculosis is the causative pathogen of tuberculosis (TB) in more than 90% of the infected persons. These bacteria remain in a dormant state in granulomas for life with the ability to persist in host tissue referred to as latency, is central to the disease. The Mycobacterium tuberculosis possess cell wall lipid molecules which are responsible for the pathogenesis, virulence and invasiveness. Gas chromatography is being used for the analysis of cellular fatty acids and alcohols of Mycobacterial species. It is already known that isolates of Mycobacterial complex had a relatively high concentration of hexacosanoic acid (1-13%) and low level of tetracosanoic acid.
Enzymes are being used in the sample to enhance the microbial growth and generate higher volatiles in the head space of analysis tube. The fingerprint analysis of the volatile lipids in the enzyme enriched samples is normally used for the confirmation of diagnosis of tuberculosis. More than 100 volatile biomarkers of tuberculosis were detected in the head space in Mycobacterium avium subsp Paratuberculosis with variation in the release in different mycobacterium species. GC-MS based method was used to identify the VOC (volatile organic compound pattern) linked with the disease conditions. Nano Artificial NOSE (NA-NOSE) was developed to detect M. bovis infection in cattle based on the volatile lipid pattern. The other commonly used methods for the detection of Mycobacterium sp using VOC are gas chromatography with mass spectrometry (GCMS), proton transfer reaction mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS), laser spectrometry, ion mobility spectrometry (IMS) or differential ion mobility spectrometry (DMS). The highly stable hydrophobic waxes of cell wall components of Mycobacterium like Phthiocerol dimycocerosates (PDIMs) are also being explored for diagnostic purpose. Several analogues of major PDIMs such as C34 and C36 viz. phthiocerol, phthiodiolone, phthiocerolone, phthiotriol, mycolic acid and ester of phthiocol form mycocerates have been identified. The physical property of mycocerosic acid is analogous to the 2, 4, 6, trimethyl nonacosonate. Thermochemolysis GC-EI/MS have been employed for analyzing mycocerosic acid components of the phthiocerol dimycoceroter (PDIM) family of lipids to study for diagnosis of tuberculosis in sputum. Although several volatile lipid moieties specific for mycobacterium have been identified in the culture, and sputum of the infected subject. However, the present study is unique by itself in identifying high and low abundant lipid biomarkers of tuberculosis in the circulation of the tuberculosis infected subjects.

**MATERIALS AND METHODS**

**Ethical approval**

The study was approved by the Research Ethical Committee at the Gajra Raja Medical College in Gwalior, M.P. India. Written consent was obtained from all the studied patients for sample collection and subsequent analysis.

**Sample collection**

The sera samples were collected from tuberculosis patients (n = 10 samples) at a minimal volume of 0.5 mL from the primary health centre of Madhya Pradesh region of India. The patients were clinically diagnosed by sputum smear and culture with pulmonary tuberculosis. None of the patients had taken antibiotics before sampling. The sera samples of healthy cases were also collected from the same region (n = 10). For spiking experiments, sera specimens were obtained from patients with pulmonary infection (but free of TB), who were treated at Gajra Raja Medical College, Gwalior.

**Spiking of negative samples**

The spiking of negative sample with MTB is performed as per method described earlier with minor modifications having final concentration of 1×10^6 mycobacteria/mL sera.

**Extraction of lipids from serum**

Briefly, 1 mL of normal human serum was added to 10 mL of chloroform-methanol 2:1 (v/v). The mixture was agitated in a orbital shaker at 70 rpm for 12 hours. The organic extract was filtered through the 0.2 µ syringe filter and the lipid extract was evaporated to dryness. The lipids were derivatized for GCMS analysis.

**Culturing of M. tuberculosis**

*M. tuberculosis* strain H37Rv (ATCC27294) was cultured in a Lowenstein-Jensen agar culture medium (2×10 CFU/mL). The cells were then harvested by centrifugation (10,000 g×20 min×4°C). The cell pellet was isolated and washed with 100 mL of PBS at pH 7.2 followed by resuspending it in TNF buffer. The *M. tuberculosis* cells were heat inactivated and lyophilized.

**Extraction and isolation of lipid antigen(s) from M. tuberculosis**

Heat inactivated mycobacterial cell (5 g) was placed into a glass reagent bottle, and 100 mL of organic solvent of chloroform-methanol mixture (2:1) was added to it. This mixture was stirred at a temperature of 25°C for 60 minutes and filtered through Whatman number 1 filter paper. The organic phase was dried by evaporating the solvent in a rotary solvent evaporator at 45°C. Neutral lipids were removed from the dried mixture by adding 50 mL of chilled acetone while the mixture was vortexed for 10 min and then filtered through Whatman no. 1 filter paper. This step was repeated 3 times. The contents of the flask were filtered through Whatman filter paper no. 1, and the filtrate was discarded. The lipids present on the filter paper were dissolved with chloroform-methanol (2:1) and transferred to a round-bottom flask. The solvent was evaporated on a rotary evaporator under reduced pressure at 45°C. The crude preparation of lipid mixture was reconstituted in 10 mL of chloroform-methanol (2:1) and stored at a temperature of -20°C for further use.

**Derivatization**

The lipid samples were mixed with 20 µL of -obis (trimethylsilyl) -trifluoroacetamide (BSTFA) (Fluka). The samples are capped, wrapped with Teflon tape, and heated at 45°C for 12 hours to convert the targeted analytes to their trimethylsilyl derivatives. The samples were further reconstituted in hexane and GCMS profiling was performed.

**GCMS method**

The lipid samples were analyzed through Gas Chromatograph Mass spectrometer (Shimadzu QP-2010 plus with Thermal Desorption system TD 20). The column used was Rtx-5MS, cross bonded with 5% diphenyl and 95% dimethyl polysiloxane (30 m, 0.25 mm ID, 0.25 µm) and had minimal bleed even at a temperature range of 350°C-33°C. The column was conditioned with a temperature 150°C and injection temperature was optimized at 260°C. The carrier gas Helium was purged at a flow rate of 16.3 mL/min and 1.21 mL/ min flow was maintained in the column was conditioned in the column. The column oven was programmed at 150°C (hold time, 5 min) at rate of 15°C/min to 310°C (hold time 8 min). The sample injection was programmed at either split/splitless mode. The GC run time was optimized to 25 min. The analytes were analyzed through FTD detectors. The mass spectrum of the molecules was analyzed through CP 2010 plus programmed for data acquisition from 5-23.6 min. The ion source temperature was equilibrated to 230°C and the interface temperature was maintained at 280°C. The data acquisition was set a speed of 1,250 with a rise time of 0.5 sec. The mass fragmentation acquisition was set at a range of m/z 40– m/z 650. The mass spectrometric results were recorded.

**Data mining and logical analysis**

The mass spectrometry data were matched with the NIST Database, Wiley and the relative hits according to the retention time and mass fragmentation pattern was recorded. The data were compared with the lipid molecules of *Mycobacterium tuberculosis* cell lipids and further compared with the sera lipids of the healthy individuals. The molecules which were very specific to tuberculosis were used as biomarker.
RESULTS

The biomarkers of tuberculosis were identified by the sera samples of tuberculosis confirmed cases by GC-MS. Sera samples were extracted with organic solvents, lipid fraction was subjected to GCMS analysis and molecules were identified through mass spectrometric databases and respective controls like sera of healthy individuals, Mycobacterium tuberculosis cell lipids. The biomarkers specific for the tuberculosis infection were identified.

Identification of potential biomarkers of tuberculosis

The initial study identified and characterized the lipids of mycobacterium origin in the sera of the suspected individuals. The injection volume of the sample was only 1 μL. The sample was analyzed in the split mode of 1/10 ratio or split less mode. The mass spectrum of the individual lipid molecule separated in the Gas chromatograph and the mass spectrum of the individual molecule was determined. The molecules were identified using mass spectrometric databases.

The split mode of injection in GCMS analysis lead to the detection of C₄H₁₈O₃ lipids in TB positive sera, however, this lipid was not detected in the Mycobacterium tuberculosis cell wall extracts (Figure 1a, 1b) and Table 1 and 2. Another biomarker with a molecular formula C₇H₁₈O₉ was detected in the sera of the TB positive subjects. This molecule had a similar mass fragmentation pattern of hexopyranose. The Mycobacterium tuberculosis cell wall extract also showed the presence of a molecule with hexopyranoside unit in it (Figure 2a-d and Tables 1, 2). The sera of the TB positive cases showed the presence of higher alkane, C₃H₇N. This molecule had a similar mass fragmentation pattern of tetratriacontane lipids. These lipids are also found in the cell wall extract of Mycobacterium tuberculosis. The higher alkanes are found to be the building blocks of Phthiocerol lipids (Figure 3a-3d) and Table 1. The other higher alkane like C₉H₁₈ and C₈H₁₆ were also identified in the sera of the TB infected cases. These molecules serve as building blocks of phthiocerol lipids (Figure 4a-4d) and Table 1, 2; (Figure 5a- 5d) and Table 1, 2. To the best of our knowledge this is the first report of above mentioned biomarkers from tuberculosis sera hence considered as novel in nature. Another lipid C₇H₇N was detected in the sera of TB infected cases (Figure. 6a-d). This molecule had a close similarity with hexaacyclamine.

The splitless mode of injection in GCMS detected the presence of C₆H₉O₃= (Figure 7a, b). This molecule is similar to the mannoypuranose, however, the molecule was not detected in the lipid extract of Mycobacterium tuberculosis under the identical GCMS conditions. In splitless mode, biomarkers C₆H₉BrO (figure not shown), C₆H₉O₅ (figure not shown) and C₆H₉O₅S (figure not shown) were detected. In splitless mode of injection, molecules like C₆H₉O₅, C₆H₉N were identified. To the best of our knowledge this is the first report of these biomarkers in the sera of tuberculosis infected cases and the method is novel in isolating and identifying the low abundant and high abundant lipid biomarkers.

Identification of high abundant and low abundant biomarkers of tuberculosis

The high abundant and low abundant biomarkers of Mycobacterium tuberculosis were identified from the sample injected and analysis was performed under split and splitless mode. Under split mode of analysis the high abundant lipids like C₆H₉ and C₈H₉O lipids were identified at a level of 50.02% and 29.15%, respectively whereas low abundant molecules like C₆H₉O₅, C₆H₉N, C₆H₉S and C₆H₉O₅S were identified in the sera of the infected at a level of 0.76%, 1.46%, 2.98 and 4.74% of total lipid content (Table 3, 4). Under splitless mode injection, the molecules like C₆H₉O₅, C₆H₉O₅, C₆H₉O₇, C₆H₉ClO, C₆H₉O, C₆H₉O₅, C₆H₉BrO, C₆H₉O₅ and C₆H₉ClO were found in the sera of the infected at a level of 0.2%, 0.57%, 0.69%, 1.87%, 2.54%, 5.22%, 4.6%, 1.85%, 1.07% and 1.1%, respectively (Table 3 and Table 5). We identified the lipid biomarkers of Mycobacterial tuberculosis and further characterized its distribution in the circulation in the selected tb positive sera sample.

| Table 1 Mass spectrometric characteristics of Lipid Biomarkers of Tuberculosis |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| S.No | Molecular formula | Molecular weight | Database Hit | Mass spectrum figures |
| 1 | C₇H₁₈O₉ | 238 | 1-Heptadecene | 1a,b |
| 2 | C₇H₁₈O₉ | 540 | Hexopyranose | 2a-d |
| 3 | C₈H₁₆ | 478 | n-Tetratriacontane | 3a-d |
| 4 | C₈H₁₆ | 408 | n-Noracose | 4a-d |
| 5 | C₈H₁₆ | 618 | Tetratetracontane | 5a-d |
| 6 | C₈H₁₆ | 317 | 4-Hexacyclamine | 6a-d |
| 7 | C₈H₁₆ | 452 | Mannopyranose | 7a,b |

| Table 2 Lipid Biomarkers of tuberculosis in Biological specimens. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Molecule | Positive sera | M. tuberculosis cell extract | Negative sera |
| C₄H₁₈O₃ | + | - | - |
| C₅H₁₆O₅ | + | - | - |
| C₅H₁₆N | + | - | - |
| C₅H₁₆S | + | - | - |
| C₅H₁₆O₅S | + | - | - |

| Table 3 GCMS detection of Novel Biomarkers of tuberculosis under Split and Splitless mode of sample Injection. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Lipids identified | Split mode | Splitless mode of injections |
| C₆H₉S | + | - |
| C₆H₉O₇ | + | - |
| C₆H₉N | + | - |
| C₆H₉O₅ | + | - |
| C₆H₉BrO | + | - |
| C₆H₉O₅S | + | - |
| C₆H₉O₇S | + | - |

| Table 4 GCMS detection of High abundant and low abundant novel lipid biomarkers in split injection mode. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Lipids identified | High abundance lipids | Low abundance lipids |
| C₆H₉ | - | 1.46% |
| C₆H₉O₇ | - | 0.76% |
| C₆H₉N | 54.76% | - |
| C₆H₉O₅ | 2.98% | - |
| C₆H₉O₇S | 29.15% | - |

The lipids constituting less than 5% of the total lipid were included in low abundance lipids and the lipids constituting above 5% of the total lipids were high abundance lipids.

| Table 5 GCMS detection of High abundant and low abundant novel lipid biomarkers in splitless injection mode. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Lipids identified | High abundance lipids | Low abundance lipids |
| C₆H₉ | 45.14% | - |
| C₆H₉S | 11.22% | - |
| C₆H₉O₇ | 7.12% | - |
| C₆H₉BrO | - | 1.85% |
| C₆H₉O₅ | - | 0.76% |
| C₆H₉N | - | 0.69% |
| C₆H₉O₅S | - | 1.11% |
| C₆H₉O₇S | - | 4.66% |

The lipids constituting less than 5% of the total lipid were included in low abundance lipids and the lipids constituting above 5% of the total lipids were high abundance lipids.
Joseph AZ et al. GCMS based detection of biomarkers

Figure 1a and 1b. Mass spectral similarity of lipid molecule M1 with Mass spectral library NIST08. The molecule had a retention of 10.183 min and had 35 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C17H34. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 238.

Figure 2a and 2b. Mass spectral similarity of lipid molecule M2 with Mass spectral library Wiley 8. The molecule had retention of 10.275 min and had 28 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C21H52O6. The spectrum showed a base peak at m/z 204 and had a molecular weight of 540.

Figure 2c and 2d. Mass spectral similarity of lipid molecule M2 with Mass spectral library NIST08. The molecule had a retention of 15.4 min and had 33 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C27H32N2O7. The spectrum showed a base peak at m/z 288 and had a molecular weight of 528.
DISCUSSION

Tuberculosis (TB) remains one of the world’s deadliest communicable diseases with 9.6 million people fell ill and 1.5 million died in 2014 from the disease\(^\text{[12]}\).

In 2013, about 64% of the estimated 9 million people who developed TB were notified as newly diagnosed cases. This is estimated to have left about 3 million cases that were either not diagnosed, or diagnosed, but not reported to national TB programmes (NTPs)\(^\text{[12]}\). The present investigation relates to the identification of few lipid molecules of Mycobacterium sp. origin in the sera specimen of the tuberculosis infected cases. The metabolomics based approach was used to characterize and identify the biomarkers from sera specimen. Earlier studies revealed that Gas chromatography combined with mass spectrometry (GC/MS) could offer a more rapid test for the diagnosis of TB by detecting lipid markers of Mycobacterium tuberculosis in biological matrices like sputum, bone, and tissue. The present study showed the presence of long carbon chain molecules in the sera of infected subjects, however, none of these molecules have been reported in the sera or any other tissue specimen. Tuberculostearic acid (TBSA) lipid was widely studied as biomarker from culture and sputum specimens and other members of actinomycetes\(^\text{[13-20]}\). The other long chain carbon molecules detected in the tuberculosis positive sera was C\(_{34}\)H\(_{70}\), C\(_{29}\)H\(_{60}\), and C\(_{34}\)H\(_{90}\). The presence of these molecules could be attributed to presence of PDIMs. Gas chromatographic–mass spectrometric (GC–MS) analysis has proven to be successful for the TB diagnosis by detecting \(M.\) tuberculosis PDIMs biomarkers in sputum\(^\text{[9,21,22]}\). This PDIM has an exceptionally low polarity hence it is an advantage to separate these molecules from other host specific lipids. The lipid extract of TB positive sera showed the presence of hexopyranose units. The mannopyranose units were also detected in the sera of tuberculosis infected cases. In Mycobacterium, the lipoarabinomannan structure is composed of mannopyranosyl branches\(^\text{[22,23]}\). The presence of mannopyranose unit in the sera confirms the presence of Mycobacterium sp in the host. These lipids mainly produce the mycocerosic acid methyl esters C\(_{29}\), C\(_{30}\) and C\(_{32}\), with molecular masses of 452, 466, and 494 Da, respectively\(^\text{[23]}\). It was identified from the previous research that drug resistance has close influence on the PDIM biosynthesis\(^\text{[25,26]}\). The rifampicin mutation was found to have up regulation in the polyketide synthase genes which are involved in the phenolpthiocerol biosynthesis\(^\text{[27]}\). The present study identifies the PDIM lipids as high abundant biomarkers. These high abundant biomarkers are easily detected even at split mode of analysis. The splitless mode of detection identifies biomarkers like C\(_{14}\)H\(_{23}\)BrO, C\(_{11}\)H\(_{24}\)O\(_{2}\), C\(_{18}\)H\(_{44}\)O\(_{5}\), C\(_{14}\)H\(_{30}\)O\(_{3}\)S, C\(_{24}\)H\(_{39}\)N in the sera of infected cases. The lipids are high volatile and detected as low abundant molecules in the splitless mode of analysis. There are no reports available on these low abundant biomarkers in the circulation of tuberculosis infected cases.

The overall study concludes the presence of several biomarkers in the circulation of tuberculosis infections. These biomarkers are the truncated fragments of larger cell lipid rafts. Based on the availability of these lipids in the specimen, they have been further characterized as high abundant and low abundant lipids. The presence of these lipids can also be correlated with the state of infection like active infection and drug resistance. Hence these biomarkers could be explored further at various other diagnostic platform for detection of tuberculosis infection.
Figure 4a and 4b Mass spectral similarity of lipid molecule M4 with Mass spectral library Wiley8. The molecule had retention of 13.82 min and had 84 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C29H60. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 408.

Figure 4c and 4d Mass spectral similarity of lipid molecule M4 with Mass spectral library Wiley 8. The molecule had retention of 11.61 min and had 39 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C29H60O. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 424.

Figure 5a and 5b. Mass spectral similarity of lipid molecule M5 with Mass spectral library Nist08. The molecule had retention of 14.8 min and had 91 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C44H90. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 618.

Figure 5c and 5d. Mass spectral similarity of lipid molecule M5 with Mass spectral library Nist0 8. The molecule had retention of 12.8 min and had 45 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C44H90O. The spectrum showed a base peak at m/z 57.10 and had a molecular weight of 618.
Figure 6a and 6b. Mass spectral similarity of lipid molecule M6 with Mass spectral library Nist08. The molecule had retention of 13.05 min and had 15 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C22H39N. The spectrum showed a base peak at m/z 106.10 and had a molecular weight of 317.

Figure 6c and 6d. Mass spectral similarity of lipid molecule M6 with Mass spectral library Wiley8. The molecule had retention of 12.76 min and had 55 mass fragmentation peaks. The molecule had similar ring with side chains and had a mass fragmentation pattern of C44H90. The spectrum showed a base peak at m/z 106.10 and had a molecular weight of 317.

Figure 7a and 7b. Mass spectral similarity of lipid molecule M7 with Mass spectral library Nist08. The molecule had retention of 9.5 min and had 11 mass fragmentation peaks. This lipid had a similar mass fragmentation pattern of C18H44O5. The spectrum showed a base peak at m/z 73.05 and had a molecular weight of 452.
ACKNOWLEDGEMENT

The authors are thankful to Grand Challenges Canada (S4 025301) for financial support under bold ideas for humanity, and Vikrant Institute of Technology & Management, Gwalior, India for partial financial support. The authors also thank AIRF facility, JNU campus, New Delhi, India for GC-MS analysis.

CONFLICT OF INTERESTS

The authors declare that they do not have conflict of interests.

REFERENCES

1 Jantzen E, Tangen T, Eng J. Gas chromatography of mycobacterial fatty acids and alcohols: Diagnostic applications. *APMIS* 1989; 97: 1037-1045.
2 Treff P, Koehler H, Klepik K, Moebius P, Reinhold P, Schubert JK, Mieckisch W. Volatile Emissions from Mycobacterium avium subsp.paratuberculosis Mirror Bacterial Growth and Enable Distinction of Different Strains. *PloS One* 2013; 8: e76868.
3 Pavlou AK, Magana N, Jones JM, Brown J, Klatser P, Turner APF. Detection of *Mycobacterium tuberculosis* (TB) in vitro and in situ using an electronic nose in combination with a neural network system. *Biosensors and Bioelectronics* 2004; 20: 538-544.
4 Peleda N, Ionescub R, Nole P, Barash O,McCullom M, VerCauteren K, Koslowa M, Stahl R, Rhyian J, Haick H. Detection of volatile organic compounds in cattle naturally infected with *Mycobacterium bovis*. *Sensors and Actuators B* 2012; 171-172: 588-594.
5 Parkhart R, Kohler H, Tenorio EL, Meyer M, Becker G, Kimkowitz A, Reinhold P. Chronic intestinal Mycobacteria infection:discrimination via VOC analysis in exhaled breath and headspace of feces using differential ion mobility spectrometry. *J Breath Res* 2011; 5: 027103
6 Onwueme KC, Vos CJ, Zurita J, Ferreras JA, Quadren LEN. The distillation of methyl mycocerosates from Mycobacterium tuberculosis. *Chromatogr A* 2009; 1216: 6319.
7 Saad NR, McNearney R, Morgan GH. A method for the identification of *Mycobacterium tuberculosis* in sputum and cultures based on thermally assisted hydrolysis and methylation followed by gas chromatography–mass spectrometry. *J Chromatogr A* 2013; 1358: 171-177.
8 Mycobacterium tuberculosis. *Appl Environ Microbiol* 2005; 71: 5449-5453.
9 Minnikin DE, Bardwell MW, Corbett EL, McNerney R, Morgan GH. Development of sample cleanup methods for the analysis of *Mycobacterium tuberculosis* methyl mycocerosates and methyl mycocerin in sputum extracts by gas chromatography-mass spectrometry. *J Chromatogr B* 2013; 986-987: 135-42.
10 O'Sullivan DM, McNerney R, McNearney R, Mullens R, Paris K, Morgan G. Development and optimization of a gas chromatography/mass spectrometry method for the analysis of thermochemolytic degradation products of phthiocerol dimycolates and cord factor in human sputum. *J Chromatogr A* 2013; 1230: 161-166.
11 Drayson FK, Lewis JW, Polgar N. Experiments relating to phthiocerol. *Plas Dis* 2013; 27: 2374-2382.
12 Saad NR, McNearney R, Morgan GH. Development and optimization of a gas chromatography/mass spectrometry method for the analysis of mycobacterial fatty acids in sputum. *J Chromatogr B* 2013; 986-987: 135-42.
13 Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Drayson FK, Lewis JW, Polgar N. Experiments relating to phthiocerol. *Plas Dis* 2013; 27: 2374-2382.
14 Mayakova TI, Kuznetsova EE, Kovaleva MG, Plyusin SA. Gas chromatographic-mass spectrometric study of lipids and rapid diagnosis of *Mycobacterium tuberculosis*. *J Chromatogr B* 1995; 672: 133.
15 French GL, Chan CY, Cheung SW, Oo KT. Diagnosis of pulmonary tuberculosis by detection of tuberculostearic acid in sputum by using gas chromatography–mass spectrometry with selected ion monitoring. *J Infect Dis* 1987; 156: 356.
16 Odham G, Larsson L, Mardh PA. Demonstration of tuberculostearic acid in sputum from patients with pulmonary tuberculosis by selected ion monitoring. *J Clin Invest* 1979; 63: 813.
17 Larsson L, Odham G, Westerdahl G, Olsson B. Diagnosis of pulmonary tuberculosis by selected-ion monitoring: improved analysis of tuberculostearate in sputum using negative-ion mass spectrometry. *J Clin Microbiol* 1987; 25: 893.
18 Kaal E, Kolk AH, Kuijper S, Janssen H.G. A fast method for the identification of *Mycobacterium tuberculosis* in sputum and cultures based on thermally assisted hydrolysis and methylation followed by gas chromatography–mass spectrometry. *J Chromatogr A* 2009; 1216: 6319.
19 Nicoara SC, O'Sullivan DM, McNerney R, Corbett EL, Mutetwa R, Minnikin DE, Gilmour MA, Morgan GH. A study on offline and online methylation of tuberculostearic-acid biomarkers for tuberculosis. 29th Informal Meeting on Mass Spectrometry Book of Abstracts 2011; 87 ISBN: 978-88-98884-19-5.
20 Nicoara SC, Turner NW, Minnikin DE, Lee OY, O'Sullivan DM, McNerney R, Mutetwa R, Corbett LE, Morgan GH. Development of sample cleanup methods for the analysis of *Mycobacterium tuberculosis* methyl mycocerosates and methyl mycocerin in sputum extracts by gas chromatography-mass spectrometry. *J Chromatogr A* 2015; 1358-9: 171-177.
21 Nicoara SC, Turner NW, Minnikin DE, Lee OY, O'Sullivan DM, McNerney R, Mullens R, Paris K, Morgan G. Development and optimization of a gas chromatography/mass spectrometry method for the analysis of thermochemolytic degradation products of phthiocerol dimycolates and cord factor in human sputum. *J Chromatogr A* 2013; 1230: 161-166.
22 Saad NR, McNearney R, Morgan GH. Development and optimization of a gas chromatography/mass spectrometry method for the analysis of mycobacterial fatty acids in sputum. *J Chromatogr B* 2013; 986-987: 135-42.
23 Farhat MR, Shapiro BJ, Kieser KJ, Sultana R, Jacobson KR, Drayson FK, Lewis JW, Polgar N. Experiments relating to phthiocerol. *Plas Dis* 2013; 27: 2374-2382.
24 Saad NR, McNearney R, Morgan GH. Development and optimization of a gas chromatography/mass spectrometry method for the analysis of mycobacterial fatty acids in sputum. *J Chromatogr B* 2013; 986-987: 135-42.
Bisson GP, Mehaffy C, Broeckling C, Prenni J, Rifat D, Lun DS, Burgos M, Weissman D, Karakousis PC, Dobos K. Upregulation of the phthiocerol dimycocerosate biosynthetic pathway by rifampin-resistant, rpoB mutant *Mycobacterium tuberculosis*. *J Bacteriol* 2012; 194: 6441-6452.

Peer reviewer: Masoud Shamaei, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Masih Daneshvari University Hospital, Tehran, Iran.