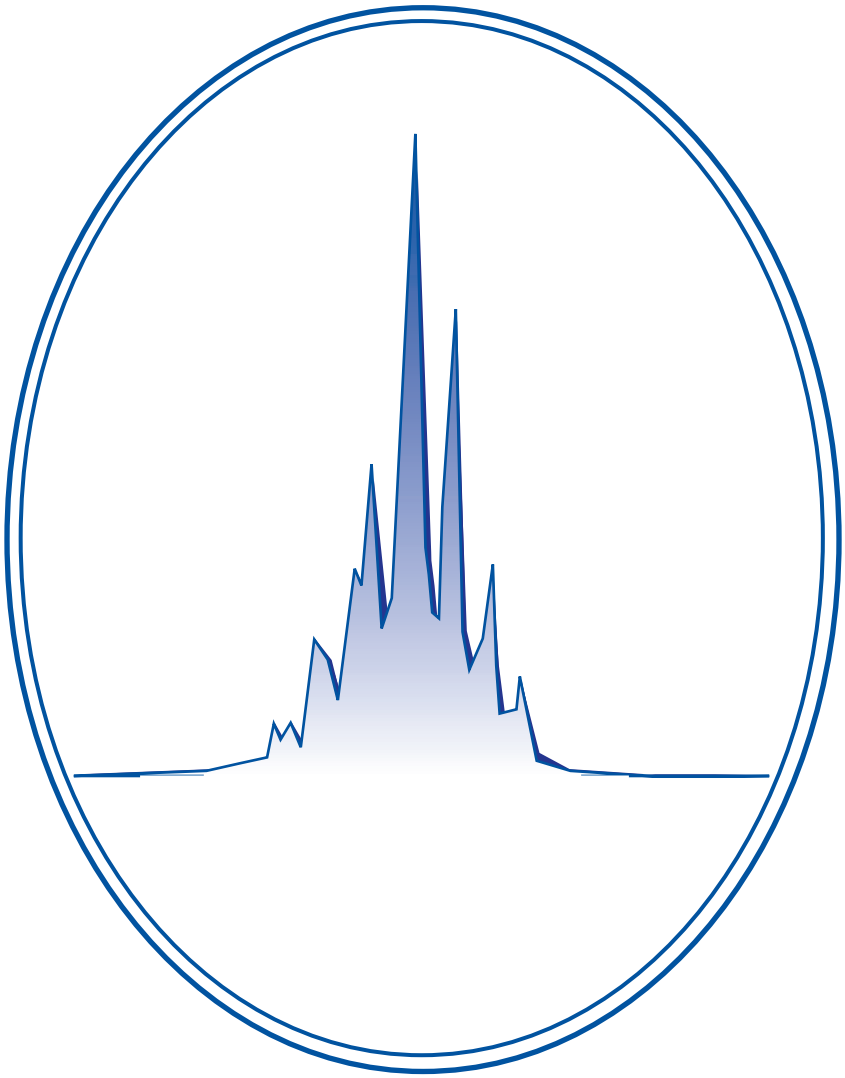
Standardized Method for HPLC Identification of Mycobacteria



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Standardized Method for HPLC Identification of Mycobacteria

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SAFETY

All work must be performed in at least a biosafety level-2 laboratory using biosafety level-3 practices[7, 15, 18]. Wear splatter-resistant clothing, eye protection, and gloves when handling reagents. Use a chemical fume cabinet[7, 25] when handling organic solvents. Observe local, state, and federal exposure limits for halogenated solvents[27]. Wear a thick glove when tightening caps to protect the hands in case of breakage.

PRINCIPLE

The objective of this method is to identify mycobacteria by analysis of mycolic acids[14, 16, 24], using high performance liquid chromatography (HPLC). A suspension of acid-fast bacteria is saponified to cleave the mycolic acids bound to the cell wall[3, 10]. Mycolic acids are then separated by acidification and extraction into chloroform. After conversion to ultraviolet(UV)-absorbing *p*-bromophenacyl esters[10] the mycolic acids are analyzed on a reverse-phase C18 column using HPLC[2-6,8, 9, 11, 13, 19, 21, 24, 26]. A gradient of methanol and dichloromethane (methylene chloride) generated by microprocessor-controlled pumps is used to separate the mycolic acid esters[3], which are detected with a UV spectrophotometer. Reproducible chromatographic patterns containing combinations of different diagnostic peaks[3, 4,12] are formed. Pattern recognition is by visual comparison of sample results with mycolic acid patterns from reference species of known mycobacteria. Correct pattern interpretation requires training. Computer-assisted pattern recognition technology[12] and high-sensitivity fluorescence detection[17] are being evaluated.

SPECIMENS

Reliable identification is achieved when mycobacteria are grown under standardized conditions. Accordingly, the cultures to be identified are grown on a Lowenstein-Jensen (L-J) slant[3]. The L-J medium may be supplemented with additional growth factors for those strains of mycobacteria unable to grow on L-J. Incubate the cultures at 35-37°C until sufficient growth for preparation of extracts is detected. Use the appropriate incubation temperature (30°C or 42°C) for mycobacteria unable to grow at 35-37°C. A carbolfuchsin/phenol or fluorochrome stain may be performed to verify the presence of acid-fast bacilli (AFB).

MATERIALS

A. Chemicals

Except where noted, chemicals and solvents must be American Chemical Society (ACS) reagent grade[20, 23]. Additionally, solvents must be HPLC grade (recommend filtered). Use ACS or United States Environmental Protection Agency (USEPA)-Type II or equivalent reagent-grade water (H_2O). The following chemicals are required:

1. Hydrochloric acid
2. Potassium hydroxide
3. Potassium bicarbonate
4. USEPA-registered, tuberculocidal, disinfectant (a phenolic compound is recommended)
5. Synthetic compounds

   High- and low-molecular weight internal standards (HMW-ISTD, and LMW-ISTD). (Note: Not ACS-reagent grade)

   The internal standards are available from the manufacturer:

   Ribi ImmunoChem Research, Inc.
   553 Old Corvallis Road
   Hamilton, MT 59840
   (406) 363-6214
   Product Nos. R-50 (1 mg) or R-51 (5 mg) (HMW-ISTD) and R-60 (1 mg) (LMW-ISTD).

6. Derivatization reagent

   p-bromophenylacyl bromide (0.1 mmol/ml), and dicyclohexyl-18-crown-6 ether (0.005 mmol/ml) in acetonitrile. (Note: Not ACS-reagent grade).

The premixed reagent is available from:

Pierce Chemical Company
P. O. Box 117
Rockford, IL 61105
1-800-874-3723
Product No. 48891

A kit containing the individual components is available from:

Alltech Associates, Inc.
2051 Waukegan Road
Deerfield, IL 60015
1-800-255-8324
Product No. 18036

7. Chloroform

   UV cutoff 245 nm, stabilized and packaged under nitrogen in a dark amber bottle or other opaque container (*see* Quality Control, *section* Solvent Purity Verification).

8. Dichloromethane (methylene chloride)

   UV cutoff 233 nm, stabilized and packaged under nitrogen in a dark amber bottle or other opaque container. (*see* Quality Control, *section* Solvent Purity Verification)

9. Methanol

   UV cutoff 210 nm.

B. Reagents for saponification, extraction, and derivatization of mycolic acids

1. Saponification reagent

Potassium hydroxide (KOH)	200 g
Reagent-grade water	400 ml
Methanol	400 ml

While stirring, **slowly** add the KOH to the water in a glass beaker, using an ice bath to cool the mixture. Continue stirring until the KOH has dissolved. When cooled, add the methanol. Store in a convenient container at ambient temperature until used.

Expiration: 1 year

2. Acidification reagent

Reagent-grade water	400 ml
Conc. hydrochloric acid (HCl)	400 ml

While stirring, **slowly** add 400 ml of conc. HCl to the water in a 1 L beaker.

Store in a convenient container at ambient temperature until used.

Expiration: 1 year

3. Potassium bicarbonate reagent

Potassium bicarbonate ($KHCO_3$)	4 g
Reagent-grade water	98 ml
Methanol	98 ml

Add the $KHCO_3$ to the 98 ml water in a suitable container. Stir until dissolved, then add the methanol and stir. Store at ambient temperature in a convenient container. If precipitation occurs, heat to $35^{\circ}C \pm 1^{\circ}C$ to re-solubilize before use.

Expiration: 1 year

4. Derivatization reagent

   Prepare and store as described in the manufacturer's instructions.

   Expiration: Two years from the date of manufacture of the oldest component.

5. Clarification reagent

   Mix 100 ml acidification reagent with 100 ml methanol. Store at ambient temperature in a convenient container.

   Expiration: 1 year

C. Sample diluent with internal standards

As a suggested starting point, add 4 mg of LMW-ISTD and 2 mg of HMW-ISTD to 50 ml dichloromethane in a 50 ml volumetric flask (8 and 4 µg/100 µl, respectively). Store at 4°C in a tightly capped dark amber bottle.

Expiration: 3 months

D. Equipment

1. Certified biological safety cabinet
2. Certified chemical fume cabinet
3. HPLC equipped with a C18 endcapped column with integral guard column, packed with 3 µm silica (4.6 mm x 7.5 cm) (Beckman/Altex kit, or an equivalent column); and a column oven. An inline filter and either a helium sparger or vacuum degasser is recommended for HPLC systems with low pressure pumps.
4. Autoclave
5. Heat block for 13 mm tubes (85-105°C) or vortex/heat/vacuum evaporator unit (85°C ± 5°C)
6. Evaporator air manifold for 13 mm tubes with blunt-ended needles or a vortex/heat/vacuum evaporator unit

7. Vortex-type mixer (multiple tube-type recommended)
8. Adjustable micropipette (20-200 µl)
9. Safety pipetting device

E. Supplies

1. Pasteur pipettes, 5 3/4", disposable glass
2. Borosilicate glass culture tubes (13 x 100 mm, new, defect-free, unwashed) with new, teflon-lined screw caps
3. Polyester fiber-tipped applicators
4. Inoculation loops (ca. 10 µl)
5. Dark amber glass reagent dispensing bottles; or 1.0 and 5.0 ml glass pipettes
6. Volumetric flasks, 50, 100 and 250 ml
7. Dark amber glass bottles with caps, 1 L, 50 and 100 ml
8. 200 µl tips for adjustable micropipette
9. Filtered dry air or nitrogen
10. pH paper (range pH 0 to pH 13) or blue litmus paper
11. Autosampler vials and caps

SAMPLE PREPARATION PROCEDURE

A. Cell harvesting procedure

1. Add 2.0 ml saponification reagent to a new 13 x 100 mm tube.
2. Remove a sample of bacteria from the L-J medium using a sterile polyester swab or transfer loop. An amount equivalent to 1-2 full loops is sufficient.
3. Add the bacteria to the tube and cap tightly with a new cap.
4. Mix vigorously for at least 20 sec.

B. Saponification and extraction procedure

1. Autoclave the tubes for a minimum of 1 h at 121°C, 15 psi; or heat for a minimum of 2 h at 100°C ± 5°C in a covered heat block. The tops of the tubes should be covered with aluminum foil to ensure adequate heat transfer for decontamination. Cool to ambient temperature or below.
2. Add 2.0 ml of chloroform.
3. Add 1.5 ml of acidification reagent. Cap tightly.
4. Vigorously mix the tubes for a minimum of 20 sec. Allow the layers to separate for 20-30 sec. If the bottom layer remains turbid, mix again for 30-60 sec. If still turbid, proceed.
5. Using a glass Pasteur pipette, remove the bottom (chloroform) layer (containing mycolic acids) and transfer to a new tube. Be careful not to transfer any of the upper (aqueous) layer. If necessary, samples may be capped and stored at 4-6°C overnight.
6. Use a heat block to evaporate the chloroform at 85-105°C under a stream of filtered dry air until the sample is thoroughly dry. Avoid excessive air pressure that could cause the sample to splatter. Alternatively, use a vortex/heat/vacuum evaporator to dry the sample.

C. Derivatization to *p*-bromophenacyl esters

1. Add 0.1 ml of potassium bicarbonate reagent to the dry sample.
2. Using a heat block, evaporate at 85-105°C under a stream of filtered dry air until thoroughly dry. Alternatively, use a vortex/heat/vacuum evaporator to dry sample.
3. Cool the sample to ambient temperature or below and add 1.0 ml of chloroform, followed by 50 µl of derivatization reagent.
4. Cap the tube tightly and mix vigorously for 30 sec.
5. Using a heat block, heat the sample at 85-105°C for a minimum of 20 min. After the first 30-60 sec of heating, check the volumes. If the volume in any tube appears to be less than 1 ml, cool the tube to ambient temperature or below. Adjust the chloroform to 1.0 ml, recap with a new cap and reheat the sample.

D. Clarification by liquid-liquid extraction

1. Cool the samples to ambient temperature or below and add 1 ml of clarification reagent. Recap.
2. Mix each tube vigorously for a minimum of 20 sec and allow the layers to separate (5-10 sec, minimum).
3. Remove the bottom (chloroform) layer with a glass Pasteur pipette and transfer to a new tube.

E. Completion and storage

1. Evaporate to dryness at 85-105°C under a stream of filtered dry air. Cap tightly.
2. Store the sample at 4-6°C in the dark until ready for analysis.

CHROMATOGRAPHIC SETUP

A. Quality control

Before starting HPLC analysis confirm that quality control specifications have been met (*see* section on Quality Control).

B. Gradient conditions

Gradient conditions were defined with a C-18, reverse phase analytical cartridge column, 4.6 mm x 7.5 cm, packed with 3 µm silica. Setup the instrument method to reproduce the following solvent flow conditions. Suggested instrument settings are summarized in the Appendices. See the manufacturer's instruction manual for specific instrument settings.

1. The solvent flow rate must be maintained at a constant flow of 2.5 ml/min until all samples have been analyzed.
2. The initial solvent mixture is 98% methanol (solvent A) and 2% methylene chloride (solvent B) (98:2, v/v).
3. During the first minute following injection the solvent mixture is changed to 80% A and 20% B using a linear gradient (elapsed time = 1 min).
4. During the next 9 min, using a linear gradient, the solvent mixture is changed to 35% A and 65% B (elapsed time = 10 min).
5. During the next 0.5 min the gradient is returned to 98% A and 2% B (elapsed time = 10.5 min).
6. Finally, the solvent mixture is held at 98% A and 2% B to equilibrate the column for a duration of 4.5 min (total elapsed time = 15 min).

C. Startup instructions

1. Turn on the chromatograph and the detector lamp 30 min before use or as recommended by the manufacturer.
2. Insure that the amount of solvent is adequate and that the waste collection container is in place.

3. Verify the column temperature is $35^{\circ}C \pm 1^{\circ}C$.
4. Prime the pumps.
5. Run the analysis method with or without the injection of a methylene chloride blank. Check for leaks, bubbles, and confirm the pump pressure to be within limits.

HPLC ANALYSIS

A. Sample preparation

1. Dissolve the sample in approximately 50-500 µl of sample diluent. The optimum amount of sample diluent is sample and system dependent. Sample concentration must be adjusted to produce a chromatogram with clearly detectable internal standards and mycolic acid peaks (which elute between the internal standards). For a chromatogram to be acceptable, a mycolic acid peak with 2-5% the height of the tallest mycolic peak must be detectable and all mycolic acid peaks must be on scale. Rapidly-growing mycobacteria may require more sample diluent. Poorly-growing mycobacteria may require less sample diluent.
2. Transfer the sample to an autosampler vial, if required, and label appropriately.

B. Analysis

1. Perform the instrument verification according to the criteria in the section on Quality Control.
2. Prepare the sample table and log in the samples. Load the samples into an autosampler tray. Samples may be individually analyzed using manual injection. The order of controls and samples should be as follows.
   a. Begin with a control set containing the negative and positive controls. (Optionally, include a carry-over control followed by a diluent blank.)
   b. Next, load the sample set. A positive control is added every 12-15 samples, and at the end of the sample set.
3. Inject and analyze the control set.
   a. Verify that the negative control pattern does not have any peaks in the region between the LMW-ISTD and HMW-ISTD (*see* Appendix C, Figure 1).
   b. Verify that the positive control pattern matches the pattern for *M. intracellulare*, ATCC 13950 (*see* Reference Patterns, Figure 13).

c. For the positive control, determine that a mycolic acid peak with 2-5% the height of the tallest mycolic peak is detectable.

d. If a peak-naming table is used to analyze the controls and samples (*see* Appendices), verify the peaks produced by the standards and the mycolic acid peaks with heights ≥ 2-5% of the tallest mycolic acid peak in the chromatogram are correctly named.

e. System performance can be determined by calculating peak symmetry, baseline noise, and the signal-to-noise ratio for a known amount of an analyte, such as the HMW-ISTD in the negative control. This can be done by using the system suitability software, if available, or by other calculation methods.[22]

f. If analysis of the control set demonstrates discrepancies, make corrections then rerun the controls.

4. When the controls are correct, inject the samples. Samples that yield unacceptable results must be rerun or reanalyzed after corrective measures are taken as described in the section on Quality Control.

5. Record and compare the beginning pressure of each run. If the pressure varies by more than approximately 10% of the previous day's starting pressure then take the appropriate corrective action (*see* Quality Control, *section* Instrument Operating Conditions).

6. Record separately the daily number of runs for the guard column, inline filter, and column. Record any adjustments or repairs on the sample log and on the equipment maintenance log.

INTERPRETATION OF RESULTS AND REPORTING

A. Sample specifications

1. If any sample produces a chromatogram with weak or off scale peaks, adjust the concentration and rerun the sample (*see* HPLC Analysis, *section* Sample Preparation).
2. If the HMW-ISTD in any sample does not elute within a mean retention time of approximately ± 0.15 min, determine cause of the drift, correct the problem, and rerun the sample (*see* Quality Control, *section* Procedure Controls).
3. If a positive control result is not satisfactory, correct the problem and rerun all samples following the last satisfactory positive control.
4. If a peak-naming table is used, verify proper naming of all sample peaks that correspond to peaks in the peak-naming table. Samples can be rerun after adjustment of the sample concentration or reanalyzed after adjustment of the peak-naming table. The peak-naming table in the method **must** be calibrated to properly name peaks.

B. Visual interpretation

Visual interpretation of chromatographic patterns has demonstrated an accuracy level of > 91%[8,26]. Before attempting to identify unknown mycobacteria, each laboratory must prepare a notebook containing chromatographic patterns derived from species whose identity has been verified either by genetic probe or other conventional methodology. (***Note**: Unknown strains must be analyzed with the same method and conditions as the reference samples and controls*). Results are interpreted visually by manually comparing the sample chromatogram with the laboratory reference pattern to determine a match.

1. Suggested approach for visual identification.
   a. Determine relative retention times (RRT) for all peaks in the chromatograms. Chromatographs can be setup to calculate RRT values automatically.

b. Separate chromatograms into groups of single, double, distinct triple cluster and multi-peak cluster patterns. **These groups can contain, but are not limited to the following organisms:**

Single	Double	Triple	Multi
M. asiaticum	*M. avium*	*M. simiae*	Other *Mycobacterium* species
M. bovis	*M. celatum*		
M. bovis var. BCG	*M. chelonae/ M. abscessus*		
M. gastri	*M. fortuitum/ M. peregrinum* group		
M. gordonae	*M. gordonae* (Chromatotype II)		
M. kansasii	*M. intracellulare*		
M. malmoense	*M. mucogenicum*		
M. marinum	*M. scrofulaceum*		
M. szulgai	*M. terrae* complex		
M. tuberculosis	*M. xenopi*		

c. Match chromatograms with those in the reference set having similar visual appearance and verify RRT values to determine species. The offset and overlay feature in chromatographic software may also be used. Patterns producing peaks before 3 min and ending about 6.5 min without any other peaks except the LMW-ISTD and HMW-ISTD indicate non-mycobacteria, with the exception of *Tsukamurella*. These species can be reported as "Not *Mycobacterium*" and/or referred to an appropriate laboratory for further identification.

d. If the unknown chromatogram matches a reference pattern, report the identification, and indicate "by HPLC". If a sample chromatogram does not match a reference pattern, indicate "not identified by HPLC". If unable to identify by HPLC use other identification methods, such as biochemical tests or genetic probes.

2. Single-cluster patterns may be easily confused and should be further evaluated using peak-height-ratios[3]. Several mycobacteria, including *M. tuberculosis, M. bovis* BCG, *M. kansasii, M. szulgai, M. gordonae, M. asiaticum, M. marinum,* and *M. gastri* form single clusters of late-eluting mycolic acid peaks that are

similar in appearance. The peaks involved in these clusters, numbered 1-9 by Butler, et al.[3], correspond to peaks named as B1-B9 by Glickman, et al.[12] Peak 5 in the Butler, et al. notation[3] corresponds to either of peaks $B5_a$ or $B5_b$ in the Glickman, et al. notation[12] (*see* Appendix A, Table IV). After confirming that the peaks have been correctly named, the flow diagram described by Butler, et al.[3] may be used to identify the sample.

3. Some mycobacteria produce HPLC chromatograms with two clusters of peaks. Examples include: *M. avium, M. intracellulare, M. scrofulaceum, M. gordonae* (Chromatotype II), *M. xenopi,* and *M. celatum*. These species may be evaluated using the peak height ratio method.[4] The peaks in the first eluting cluster, numbered 7-3 by Butler, et al.[4], correspond to peaks named A1-M3 by Glickman, et al.[12] The peaks in the second eluting cluster, designated 2-g by Butler, et al.[4], correspond to peaks B2-E2 by Glickman, et al.[12] After confirming the peaks have been correctly named, the flow diagram described by Butler, et al.[4] may be used to identify the sample.

QUALITY CONTROL

A. Instrument operating conditions

1. Maintain records of all service, maintenance intervals and calibration done.

   a. Service the chromatograph and verify it meets the manufacturer's specifications at least every 18 months.

   b. Re-calibrate the peak-naming table contained in the HPLC method file (if used) whenever the column is changed, or when changes are made in the fluid path of the chromatograph (*see* Appendix A, Table IV).

   c. Verify the accuracy of the peak-naming table periodically (if used).

   d. Verify injector carryover is within manufacturer's tolerance. This may be done as part of the periodic maintenance procedure, or as part of the control procedure when performing analyses (*see* Procedure Controls, *section* Carryover Control).

2. Verify the instrument function. Turn on the instrument 30 minutes before use. Check the solvent bottles, waste bottle, and the compressed air and helium (if used). Do not operate the chromatograph until any needed repairs or adjustments are performed.

   a. Verify that the room temperature is in the range of 60-75°F (15-26°C).

   b. Verify that the sparger or vacuum degasser (if used) is operating.

   c. Prime the pump(s) according to the manufacturer's procedure. Set conditions so that the solvent flow bypasses the column. Visually verify that the pump(s) show no evidence of "pulsing" at a high flow rate.

d. After stopping the pump redirect the solvent flow through the column according to the procedure for your machine, then increase the flow rate gradually to 2.5 ml/min.

e. Load the HPLC method file containing the specified gradient (*see* Appendix A, Tables I-V).

f. Periodically verify the pump(s) produce a constant flow, i.e. 25.0 ml ± 0.5 ml in 10.0 ± 0.1 min, while operating the gradient (using this suggested specification, retention time error due to pump inaccuracy is limited to ± 0.2 min at 10 min).

g. Verify the fluid path is free of any leaks.

h. Using the solvent conditions for the assay method the suggested tolerance for the system's pressure is 10% of the previous day's starting pressure.

i. Replace the inline filter and/or guard column if the day's starting pressure is, i.e. ≥ 150% of the initial pressure recorded when the component was installed, or if the total pressure exceeds the pressure limit recommended by the column manufacturer if available. The guard column should last approximately 250 injections.

j. If replacement of the inline filter and/or the guard column fails to bring the pressure into tolerance, replace the analytical column. The analytical column should last approximately 1000 injections.

k. Verify the column oven temperature is $35^{\circ}C \pm 1.0^{\circ}C$. Empirically, this specification limits retention time error due to temperature inaccuracy to ± 0.05 min.

l. Verify that the detector baseline is stable (no net baseline drift) while the chromatograph is operating under initial solvent conditions for the assay.

B. Procedure controls

1. As a suggested starting point add the internal reference standards (LMW-ISTD and HMW-ISTD) to all samples and controls at 8 µg/100 µl and 4 µg/100 µl, respectively, assuming a 5.0 µl injection. These standards are included in the sample diluent.

   a. The mean retention time (MRT) ± 0.15 min is calculated for the HMW-ISTD from 10 previous injections of the negative or positive derivatization controls with the same column. The MRT of the HMW-ISTD must be in the range from 8.5 to 11.0 minutes (machine and column dependent).

   b. The amount of internal standards in the injected sample must produce peak heights that are sufficient for detection and correct peak naming. Additionally, the amount of the LMW-ISTD must produce a peak height that exceeds the heights of its nearest neighbors.

2. A negative derivatization control is prepared from a non-mycolic acid-containing organism such as *Candida albicans* ATCC 60193 grown on L-J medium, and containing both the LMW-ISTD and the HMW-ISTD. The chromatogram produced from the negative derivatization control can be used to measure the performance of the HPLC system. System performance can be monitored automatically by the system suitability software, if available.

   a. Mycolic acic peaks should not be detected in the region between the LMW-ISTD and HMW-ISTD (*see* Appendix C, Figure 1).

   b. The HMW-ISTD in the negative control can be used to determine baseline noise and peak symmetry. The suggested calculated tolerance for peak symmetry is 0.7 to 1.3. Values outside this range may indicate a need to service the instrument.[22] Baseline noise can be calculated using a method where integration parameters (peak height and threshold) are set to minimum values. One-half minute ranges of retention time centered around 4, 6, and 8 min can be reanalyzed from the chromatogram file and the sum of the peak heights can be divided by the number of peaks integrated to yield an

average baseline noise value. Average baseline noise values can be compared to manufacturer's specifications or to previously determined average values to monitor system performance. Deviations in baseline noise may indicate a need to service the instrument.

3. A culture of *M. intracellulare* ATCC 13950 grown under standardized conditions can be processed with the specimens to serve as a positive control for the sample preparation procedure.

   a. The chromatogram pattern obtained for the positive control must match the chromatogram illustrated for the control strain (*see* Reference Patterns, Figure 13).

   b. The positive control should be run at the begining of each day's run, after every 12-15 samples, and at the end of the sample set.

   c. The positive control can also be used as an external chromatographic reference to confirm the accuracy of the peak naming table, if used. The mycolic acid peaks, the LMW-ISTD, and the HMW-ISTD reference peaks must be named correctly in every positive control sample. It may be necessary to adjust the initial peak height and threshold parameters in the method software so that all mycolic peaks are integrated (*see* Appendix A, Table III).

4. The carryover control is optional. A culture of *M. abscessus* ATCC 19977 or an equivalent *M. abscessus* strain serves to detect the presence of unwanted sample from a prior injection, i.e. carryover.

   a. The *M. abscessus* carryover control should be prepared so that its tallest mycolic acid peak has a height that is 50-100% of a full scale reading. Analyze a dicholoromethane blank sample immediately after the carryover control. Calculate carryover by dividing the height of tallest peak in the carryover control into the height of the corresponding peak in the blank. Carryover must not exceed the instrumentaton manufacturers specification.

   b. One carryover control per sample set is sufficient.

5. External chromatogram pattern controls. Each laboratory must establish an HPLC pattern notebook containing chromatographic patterns for cultures of mycobacteria grown under standardized conditions. The notebook must include chromatograms of the mycobacteria species that are to be identified using the HPLC method. Sample chromatograms are identified only if a match is obtained with a chromatogram in the HPLC pattern notebook. Samples prepared from cultures grown on media other than L-J must be compared with known reference cultures grown on the same medium.

C. Solvent Purity Verification

Chloroform and methylene chloride decompose in the presence of heat, light, and air to form hydrochloric acid, chloride ion, phosgene, and free chlorine[20]. The presence of these contaminants interferes with the mycolic acid assay, and can damage the chromatography column. Accordingly, these solvents can be tested to confirm the absence of breakdown products before use and at intervals, thereafter. Since chloroform breaks down more rapidly than methylene chloride, it can be tested more frequently. Do not use the solvents if any of the following tests are positive.

1. Reagents[20]

   a. Silver Nitrate reagent (for chloride determination)

Silver nitrate ($AgNO_3$)	4.25 g
Reagent-grade water	qs to 250 ml

   Place the $AgNO_3$ in a 250 ml volumetric flask. Bring to volume with H_2O. Store in a dark amber bottle at ambient temperature.

   Expiration: 1 year

   b. Potassium Iodide reagent (for free chlorine determination)

Potassium iodide (KI)	10 g
Reagent-grade water	qs to 100 ml

   Place the KI in a 100 ml volumetric flask. Bring to 100 ml with H_2O. Store in a dark amber bottle at ambient temperature.

   Expiration: 1 year

c. Sodium hypochlorite (for positive control)
Dispense 5.25% sodium hypochlorite (household bleach) into a dark amber bottle, and store at ambient temperature.

Expiration: 1 year

2. Procedure

a. To test for acid and chloride, shake approximately 17 ml of chloroform or 20 ml methylene chloride with 25 ml H_2O for a minimum of 1 min. Allow to separate, then draw off the upper (aqueous) phase.

(1) To test for acid, add a small piece of blue litmus or pH paper to 10 ml of the aqueous phase. For a negative test, the litmus paper should remain blue, or the pH should be in the neutral zone pH 6.0-8.0. If the test is negative, verify the proper function of the litmus or pH paper by adding a few drops of acidification reagent to cause a positive reaction.

(2) To test for chloride ion, add 0.25 ml of silver nitrate reagent to another 10 ml of aqueous phase. A negative reaction is indicated by the absence of turbidity (indicates no chloride ion present). If the test is negative, verify the reagent function by adding a few drops of 5.25% sodium hypochlorite (bleach) and mixing to cause formation of a precipitate, representative of a positive reaction.

b. To test for free chlorine, combine 10 ml of chloroform or methylene chloride with 10 ml H_2O containing 0.10 ml potassium iodide reagent. Shake for 1 min and allow to separate.

(1) A negative test is indicated by the lack of a purple tint in the bottom (aqueous) layer and indicates the absence of free chlorine.

(2) If the test is negative, verify the reagent function by adding a few drops of bleach to the mixture and shaking again to cause formation of a purple tint, representative of a positive reaction.

REFERENCE PATTERNS

The reference patterns presented in the following figures resulted from an interlaboratory study performed by members of the HPLC steering committee, using the standard method described in this document, and were based on a collection of specimens of known identity. The profiles shown are the median chromatograms inferred from a multivariate analysis of a set of specimens from each species.

Significant peaks are labeled with adjusted retention times (ART). In each chromatogram the LMW-ISTD has an ART of 3.257 and the HMW-ISTD has an ART of 9.419, and both are labeled as IS. Note that variations of the median chromatogram will occur in different laboratories, therefore the ART shown should not be used as benchmarks, rather as aids for comparison among species. These chromatograms are presented for reference only.

In order to identify unknown mycobacteria specimens, each laboratory must prepare a notebook containing chromatograms of mycobacteria commonly seen in their laboratory (*see* Interpretation of Results and Reporting, *section* Visual Interpretation). HPLC profiles of unknown mycobacteria must be compared to the patterns contained in the notebook.

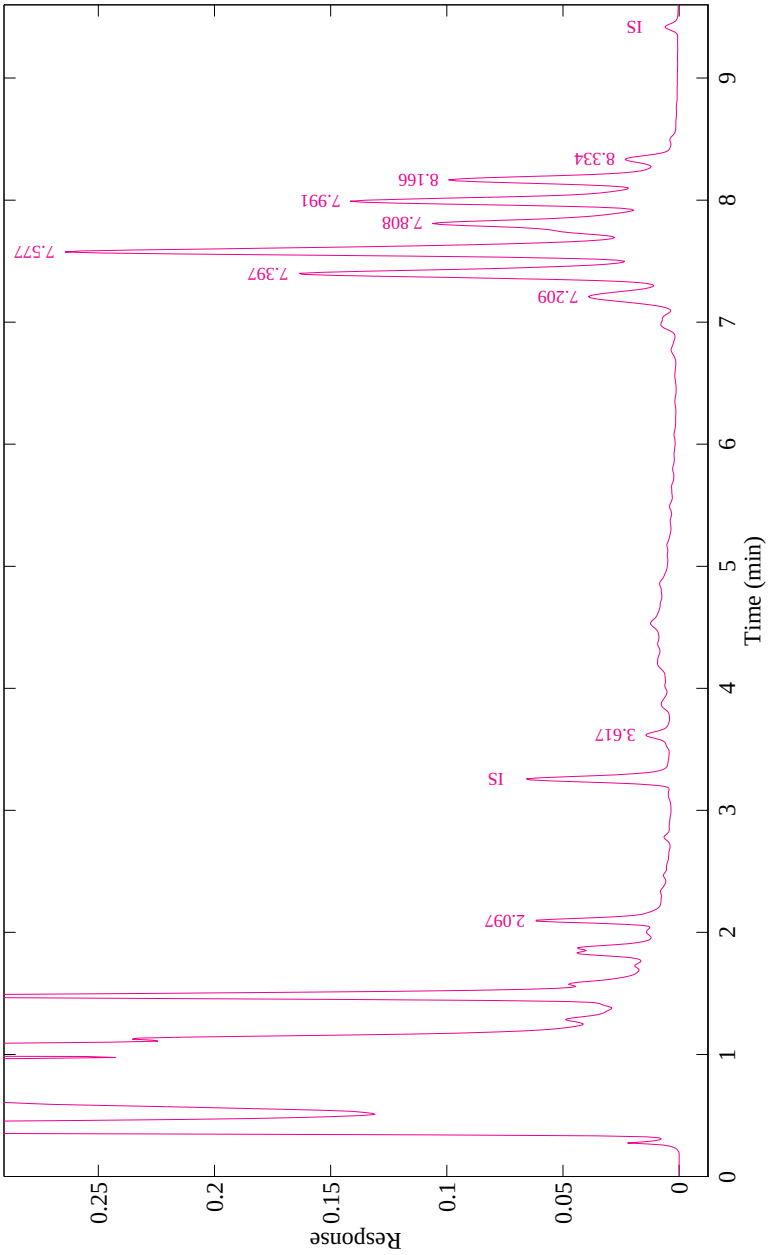


Figure 1. *M. asiaticum*

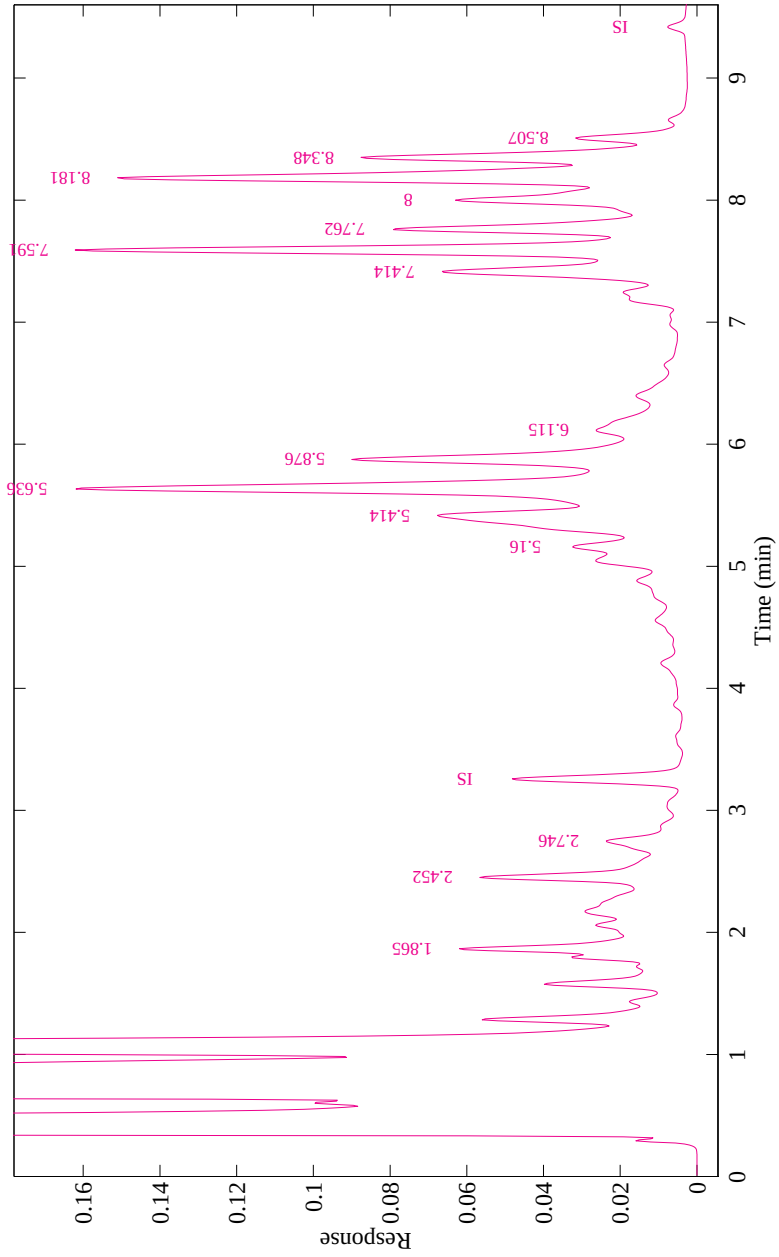
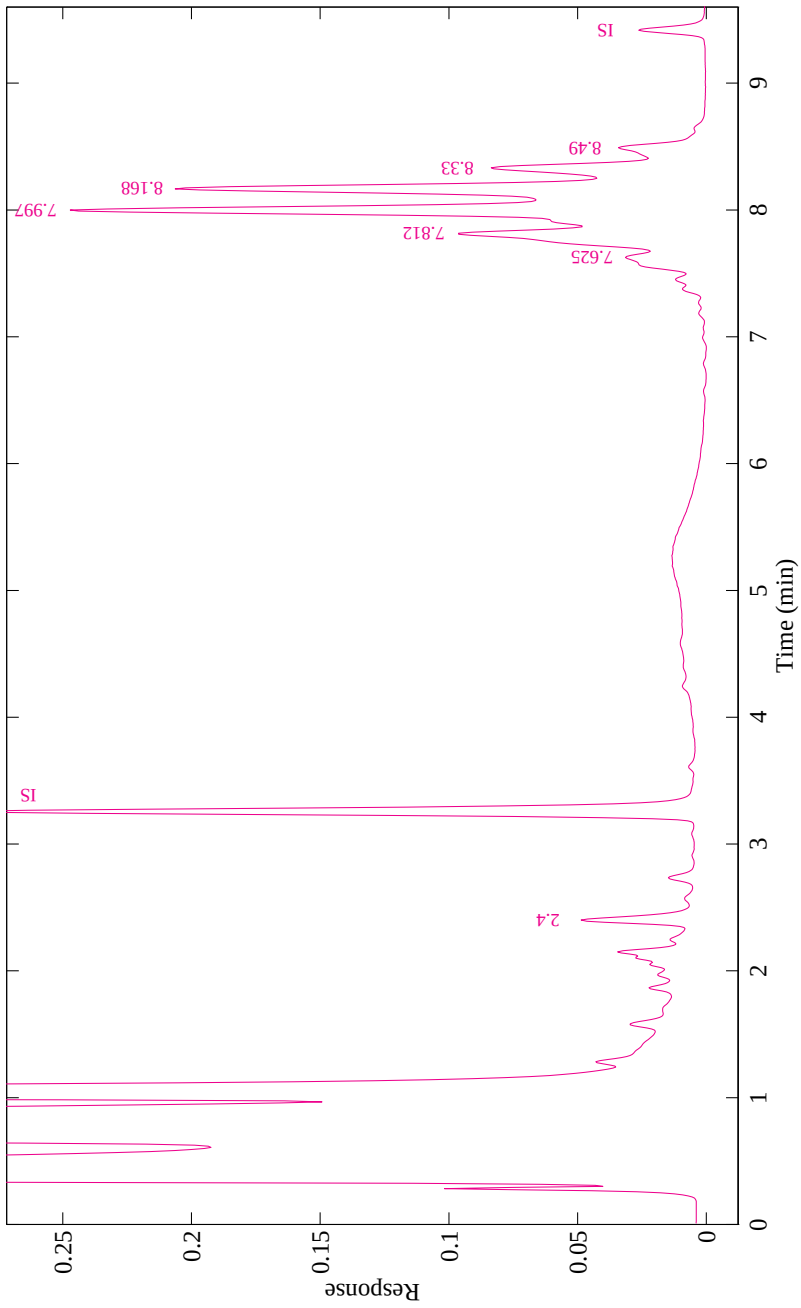


Figure 2. ***M. avium***

Figure 3. ***M. bovis***



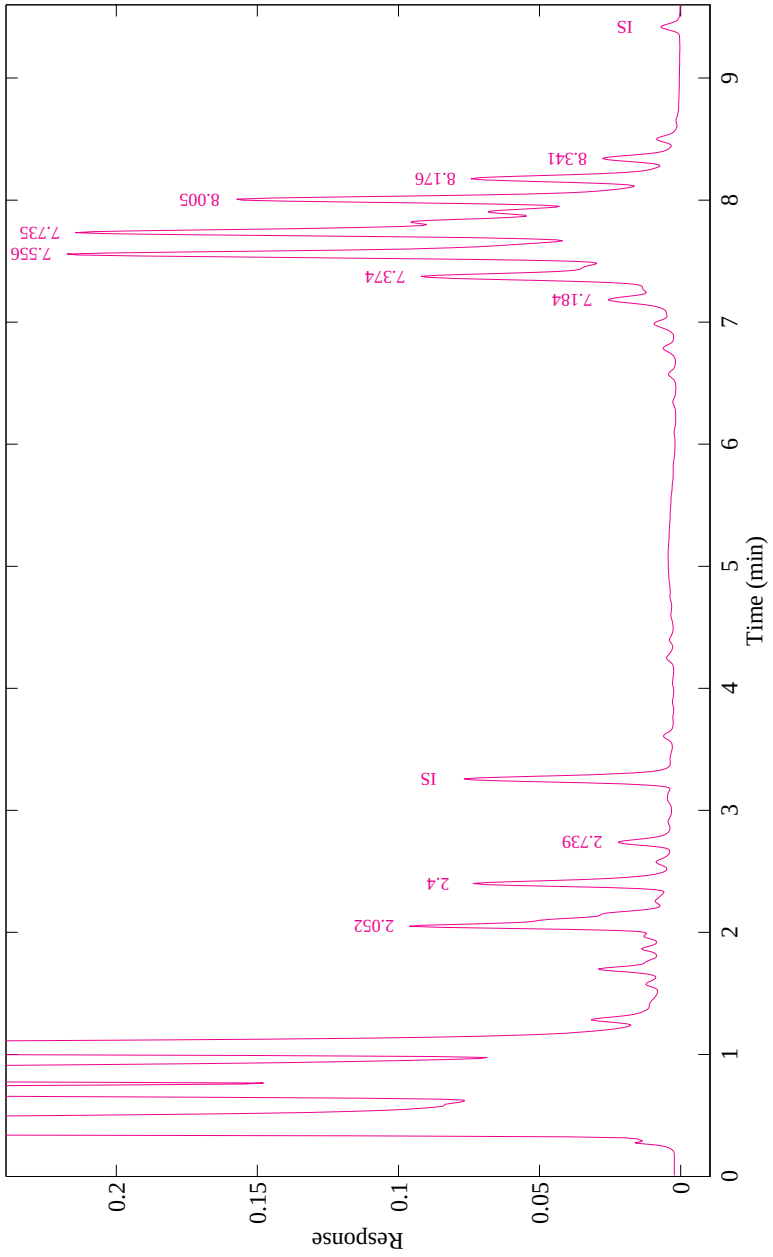


Figure 4. *M. bovis*, BCG

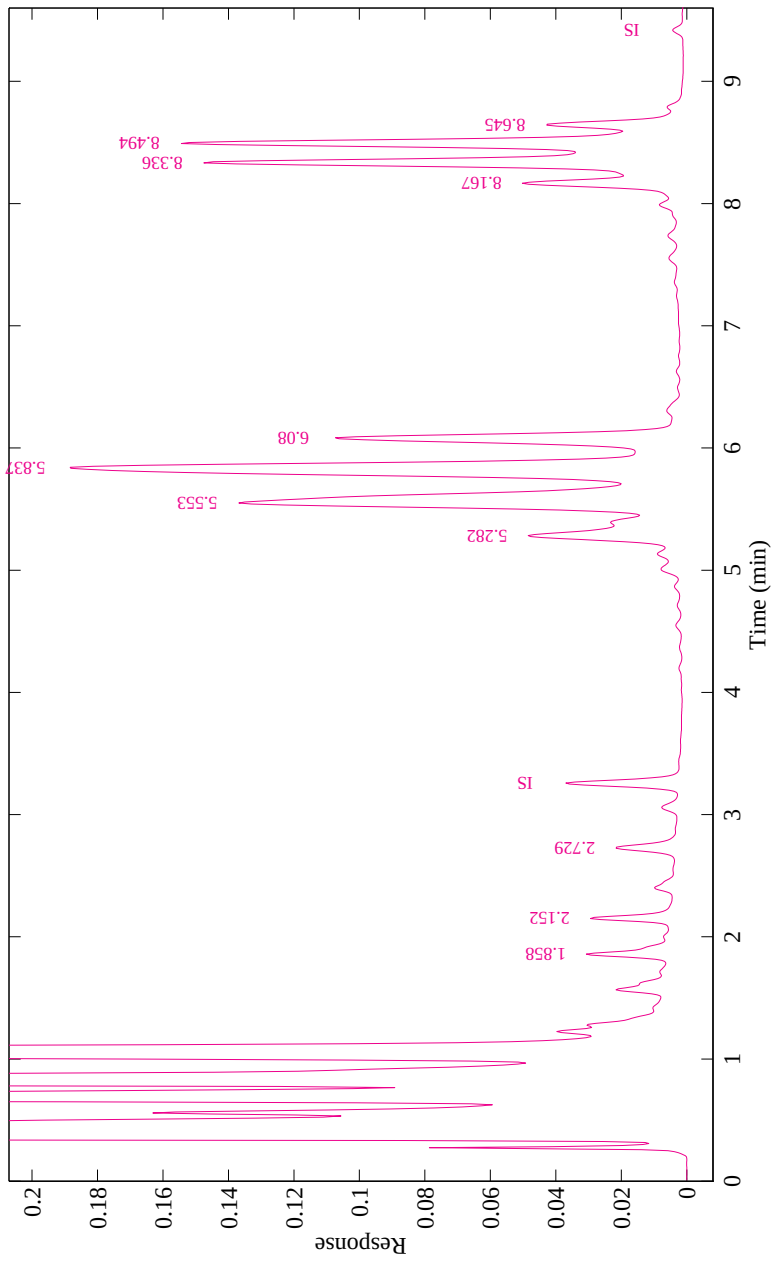


Figure 5. ***M. celatum***

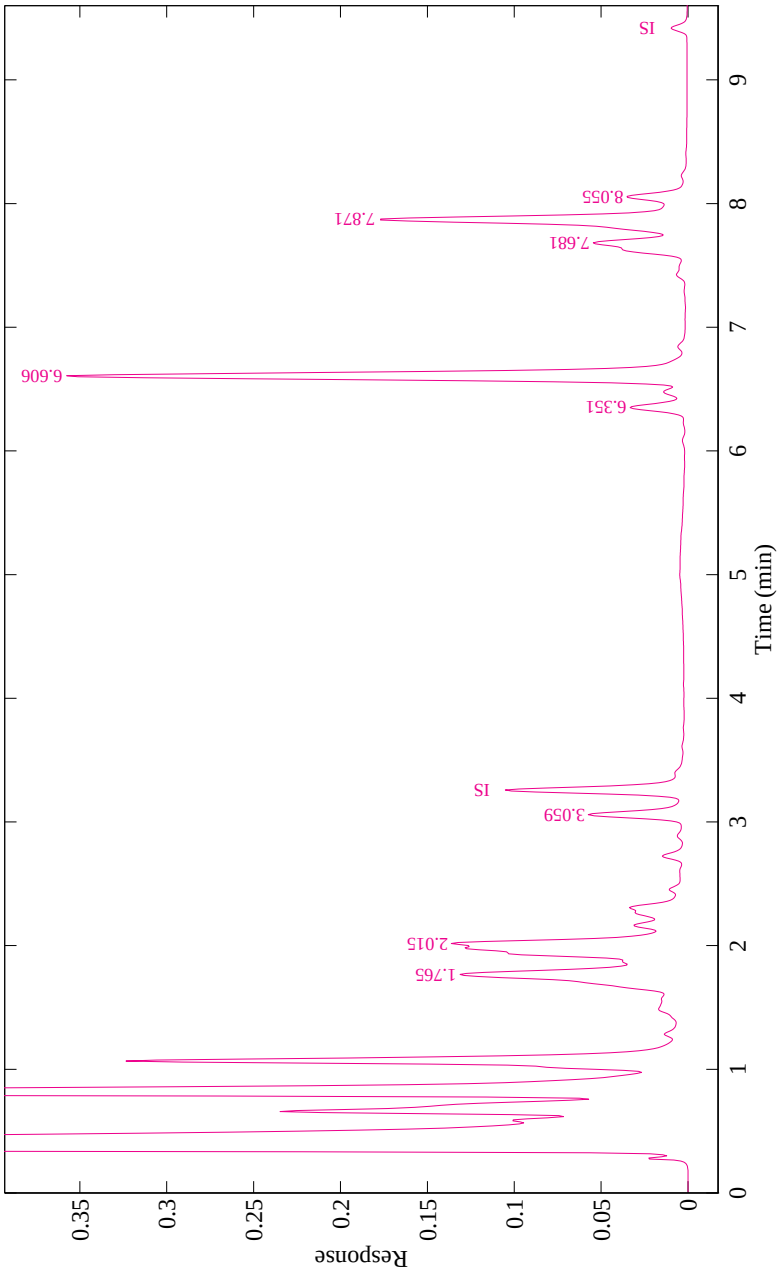


Figure 6. ***M. chelonae/M. abscessus***

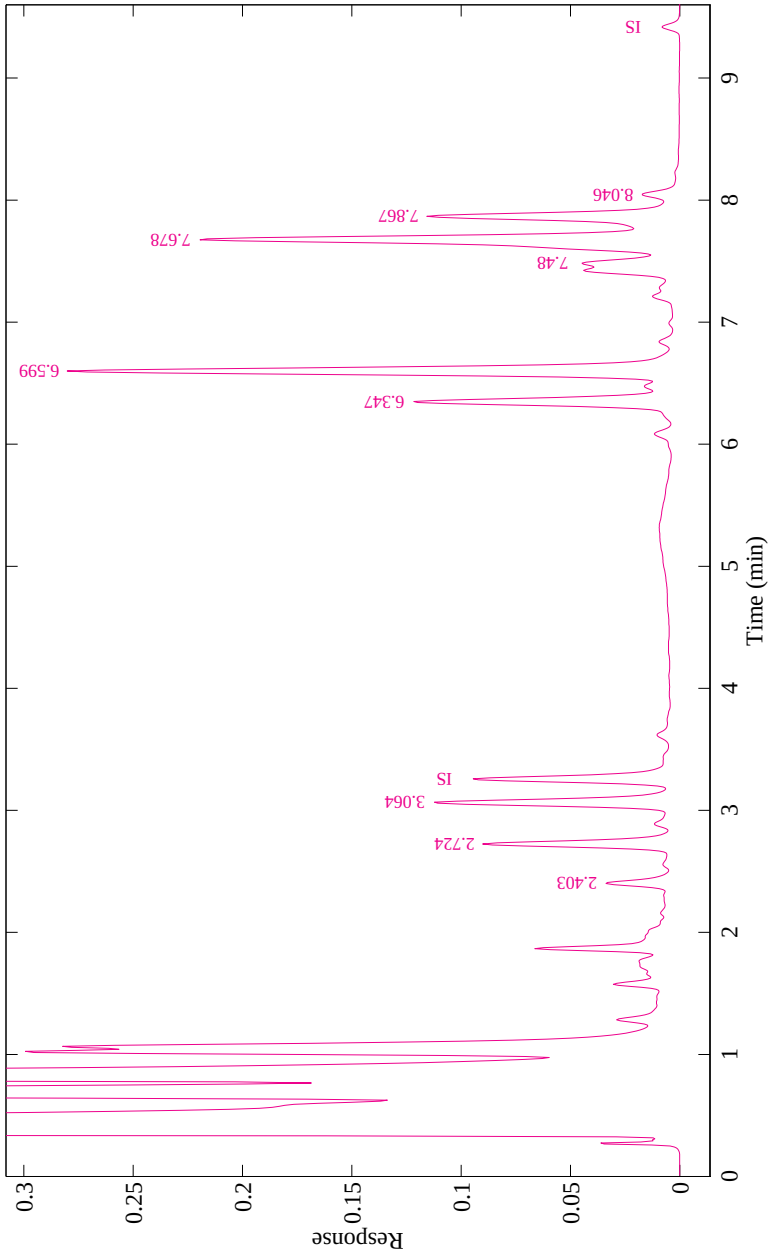


Figure 7. ***M. chelonae/M abscessus***

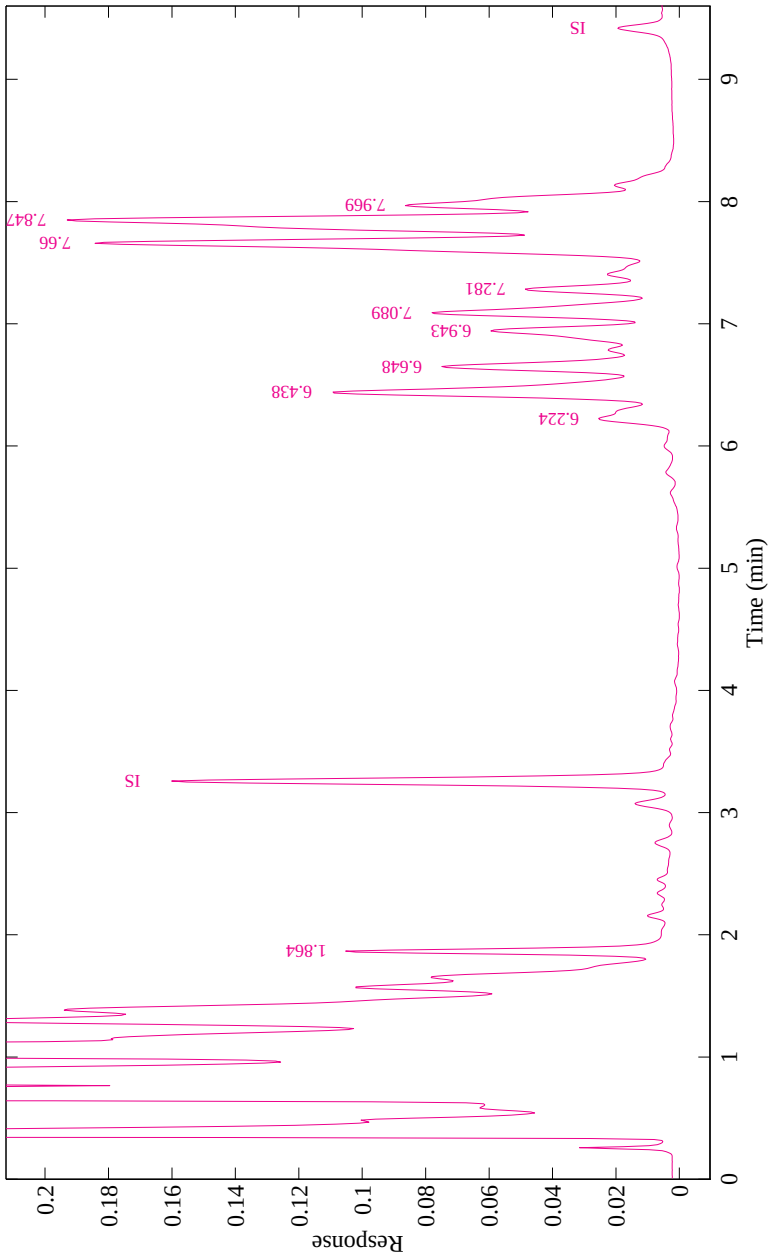


Figure 8. *M. fortuitum/M. peregrinum* group

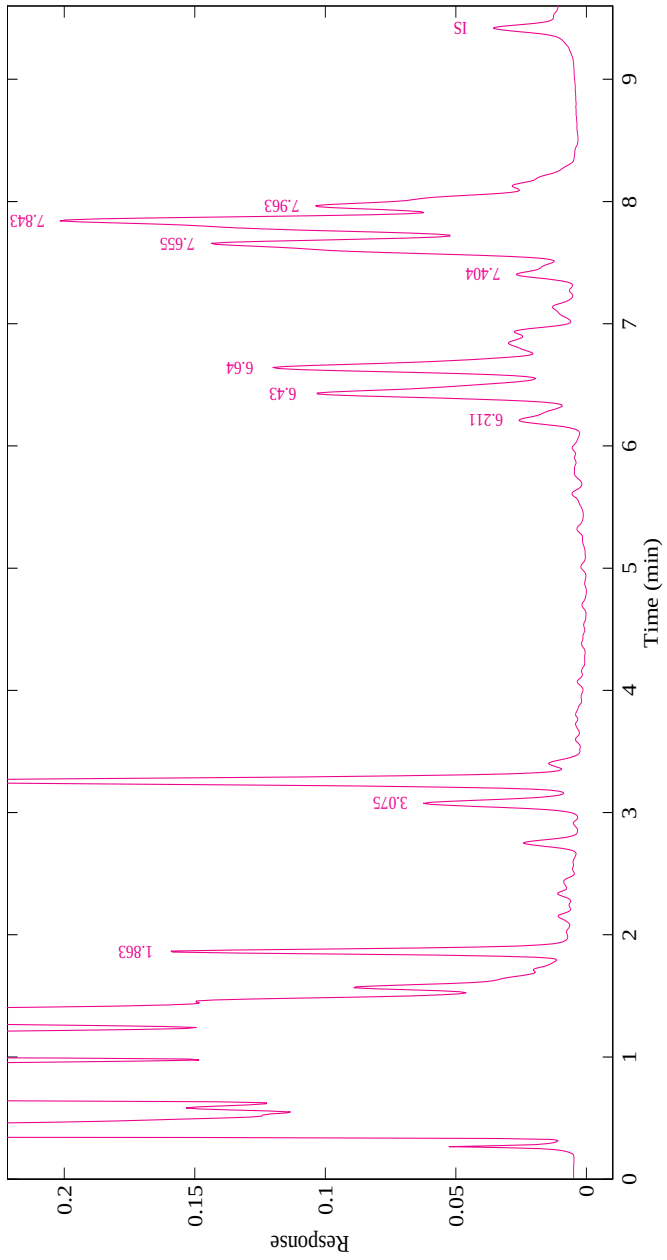


Figure 9. *M. fortuitum/M. peregrinum* group

Figure 10. ***M. gastri***

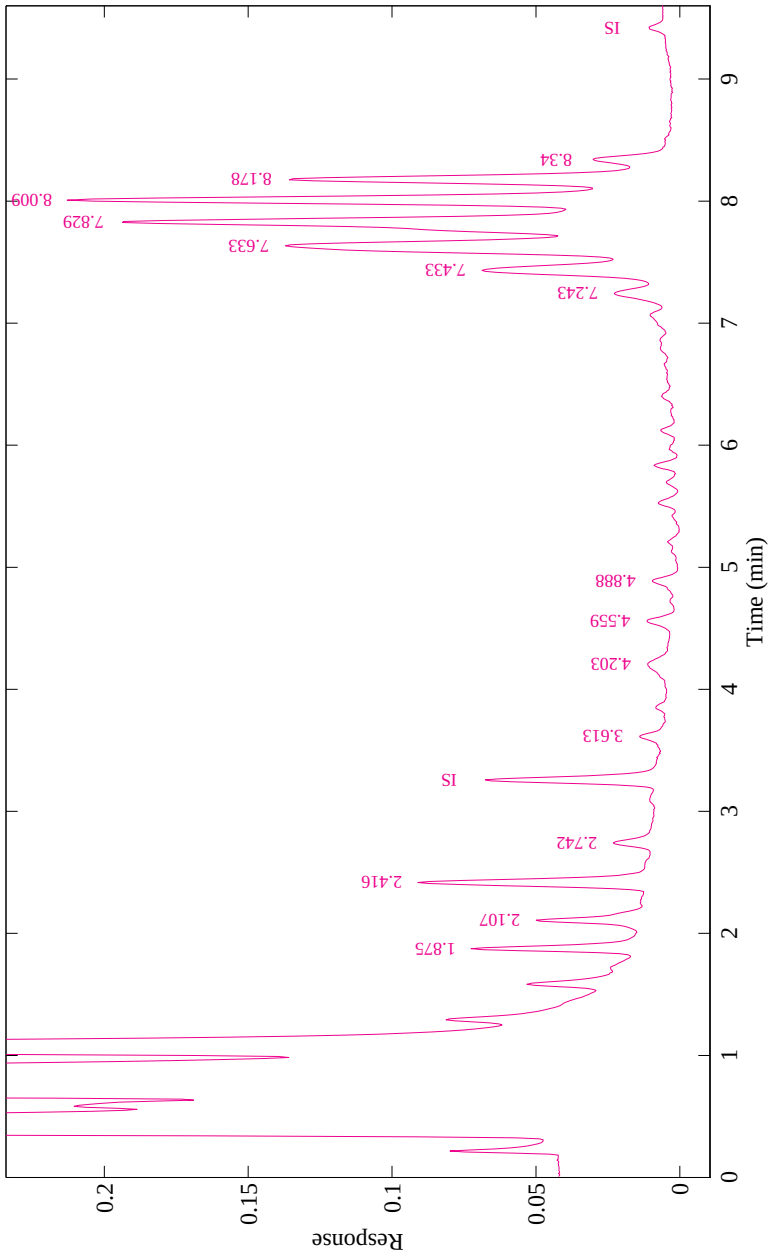
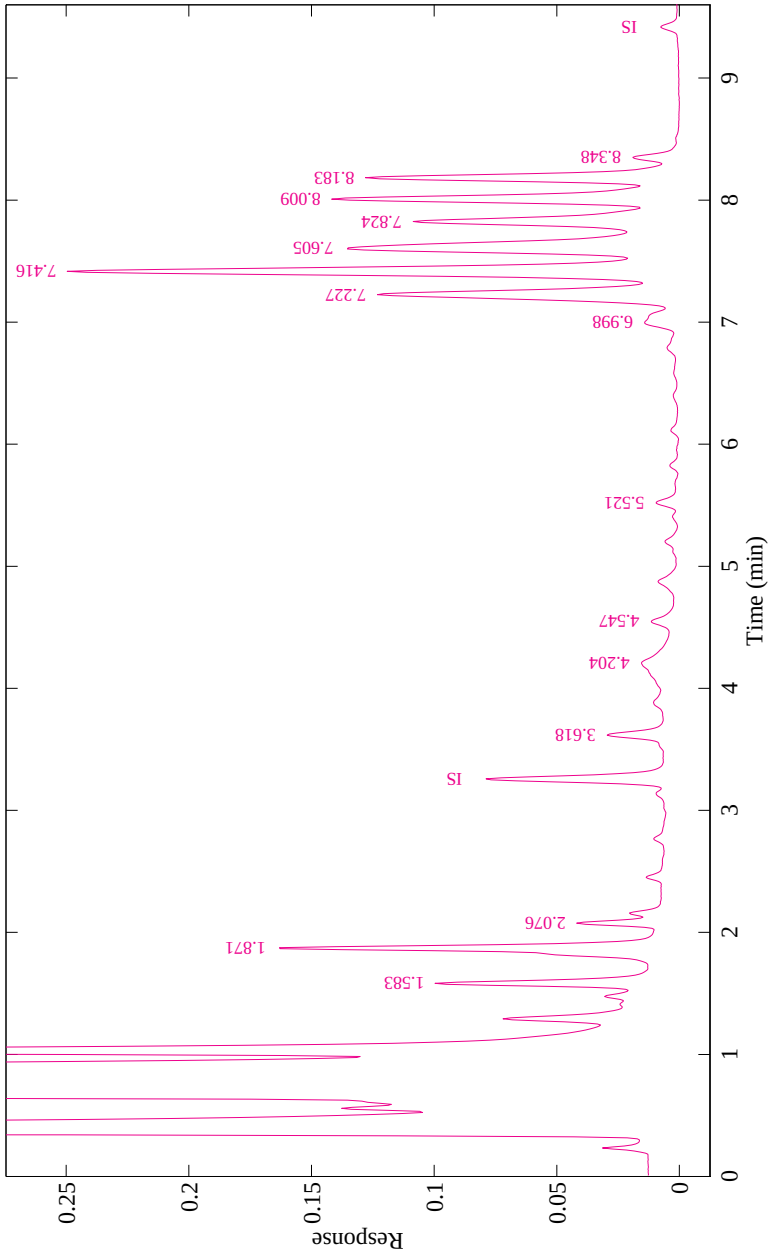


Figure 11. *M. gordonae*



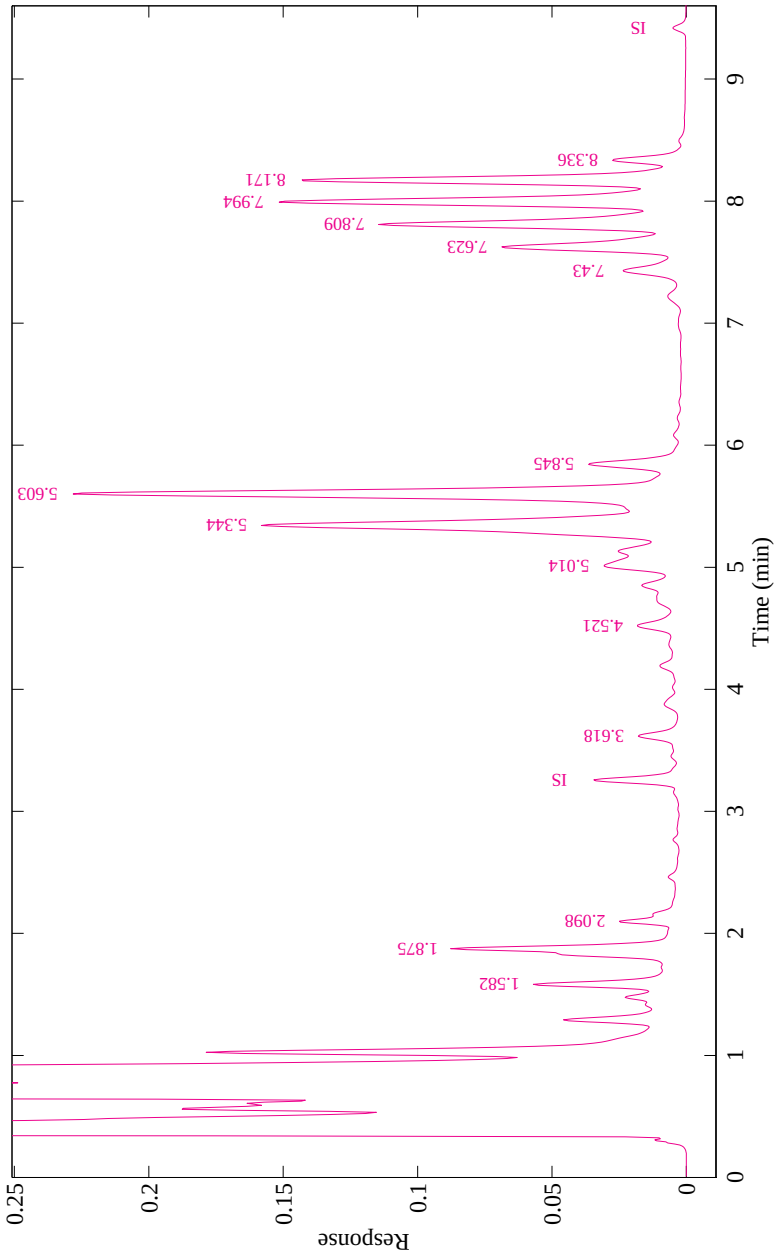


Figure 12. *M. gordonae* (Chromatotype II)

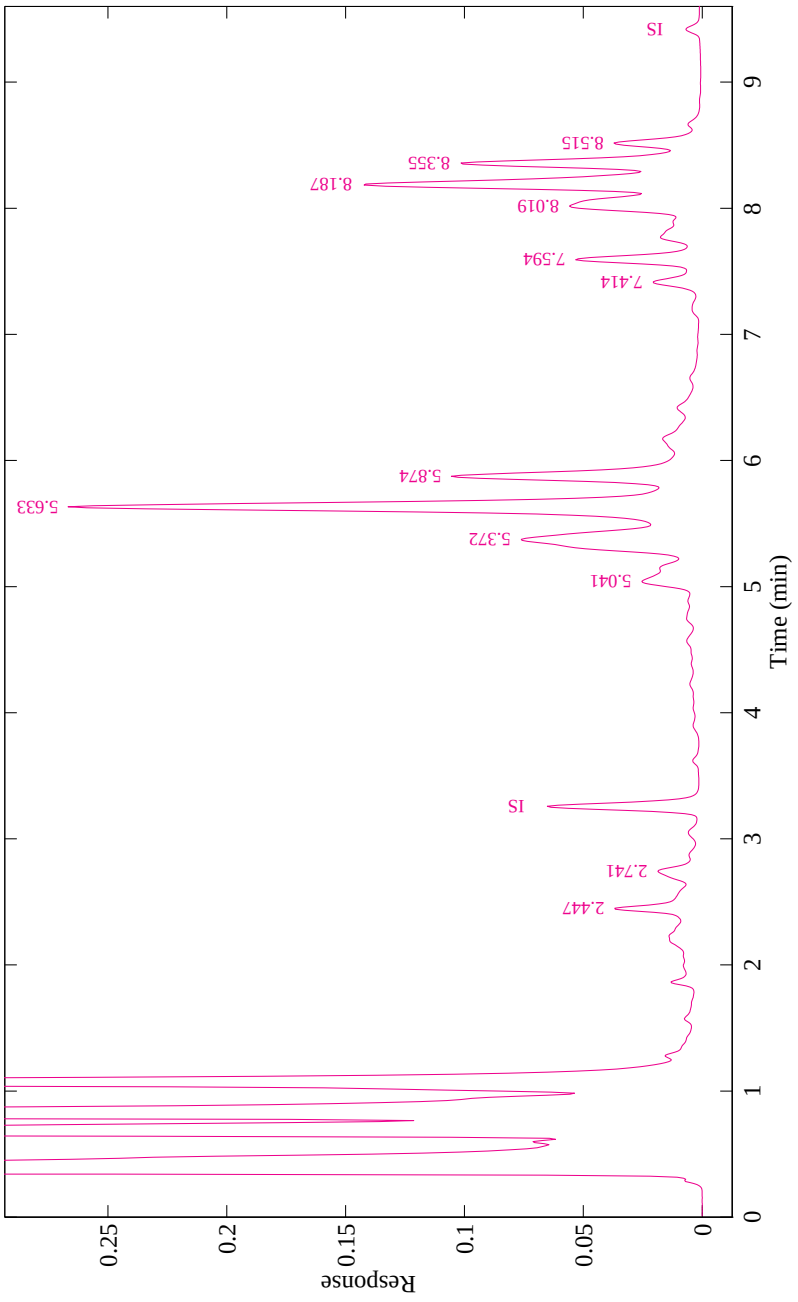


Figure 13. *M. intracellulare*

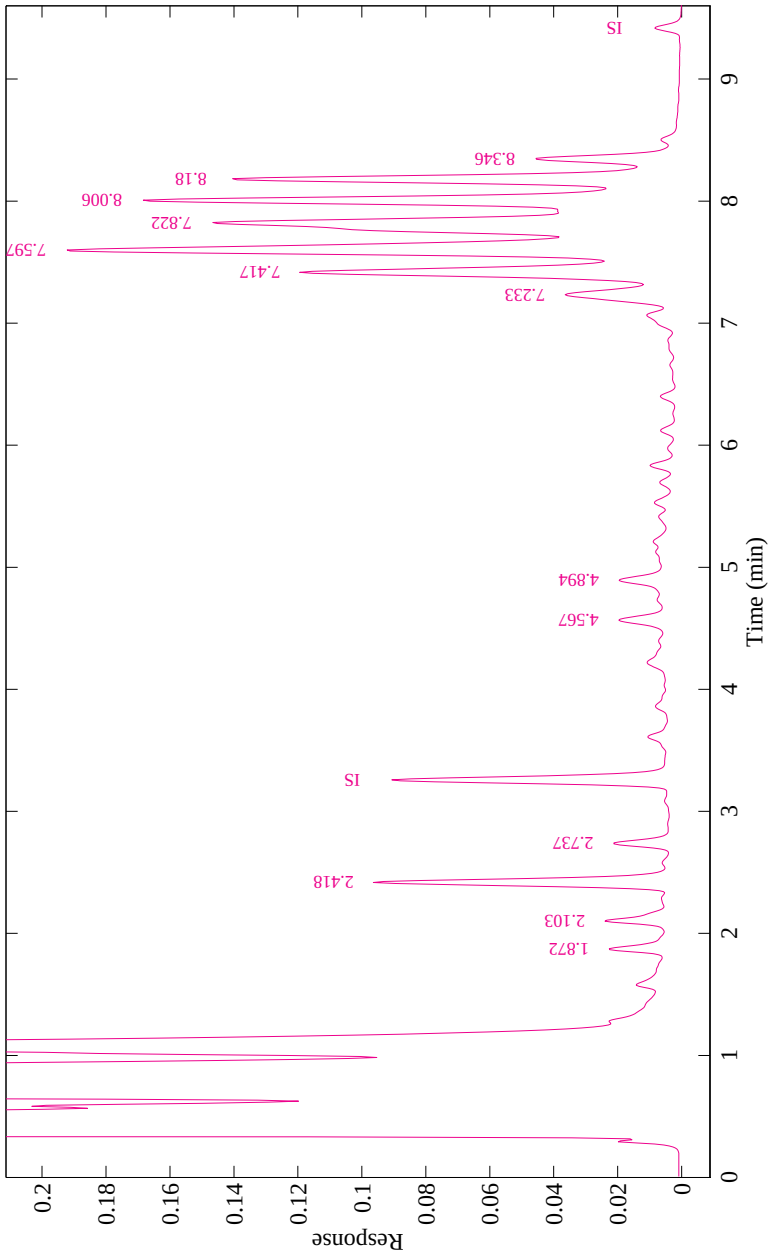


Figure 14. ***M. kansasii***

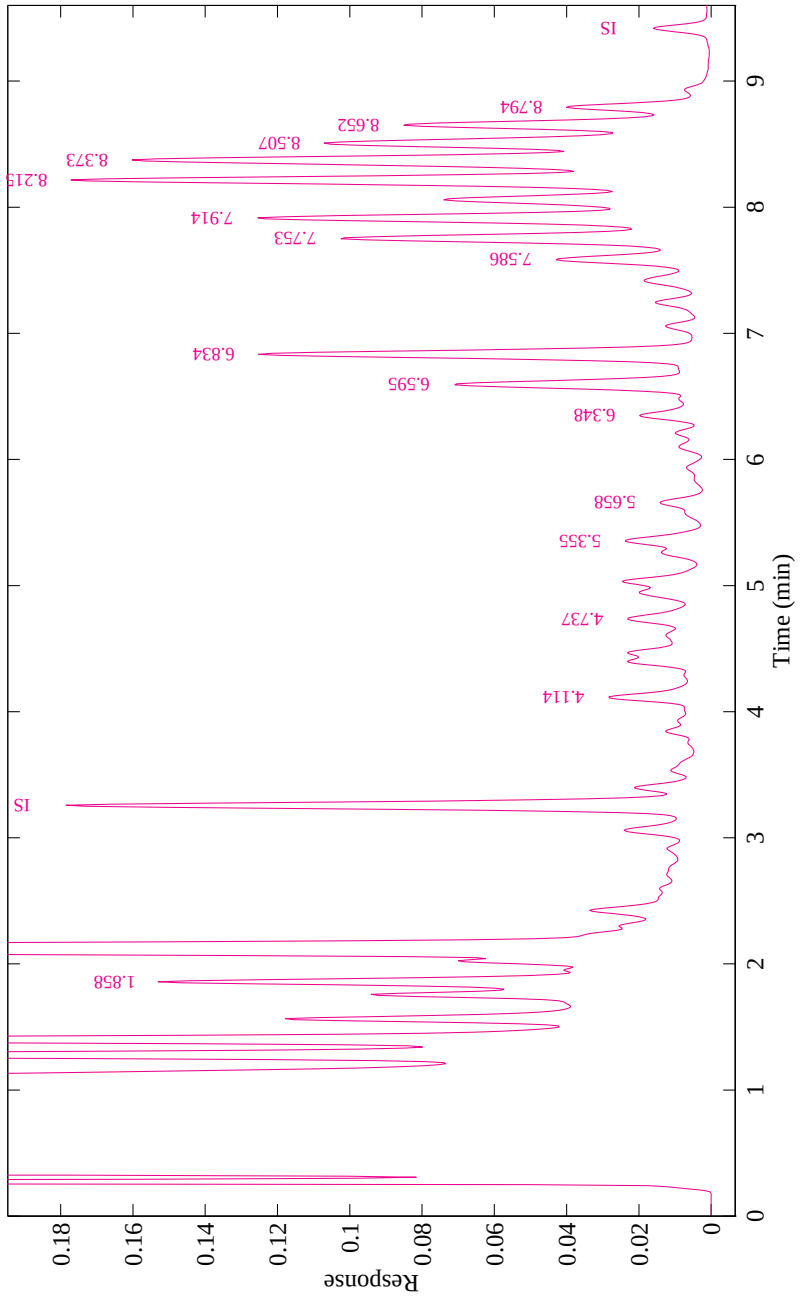


Figure 15. ***M. malmoense***

Figure 16. *M. marinum*

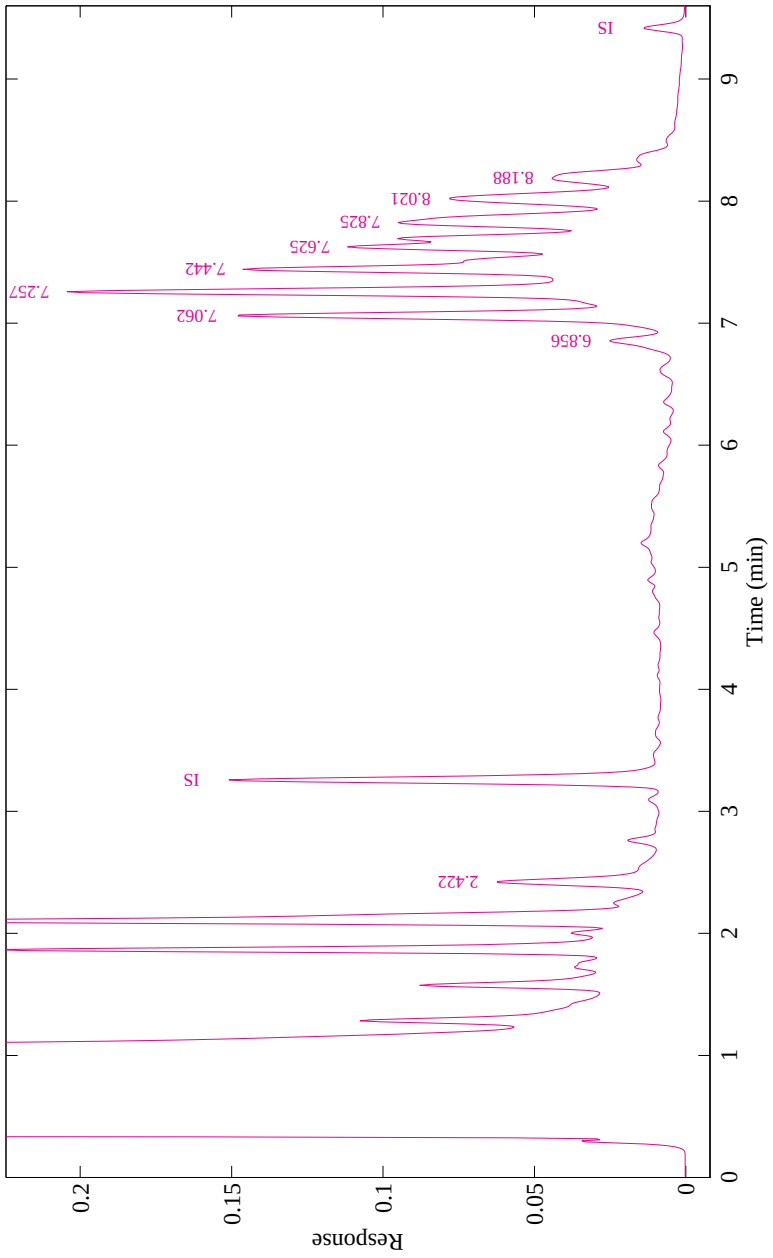
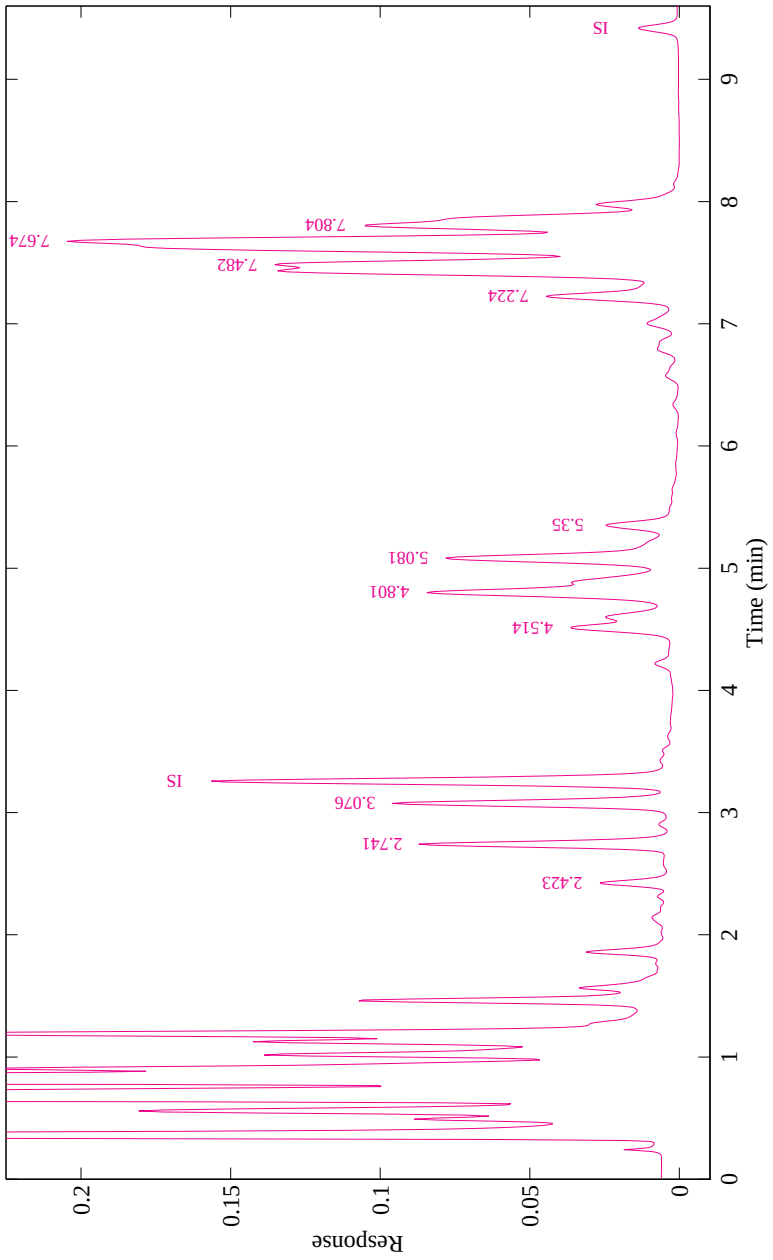


Figure 17. *M. mucogenicum*



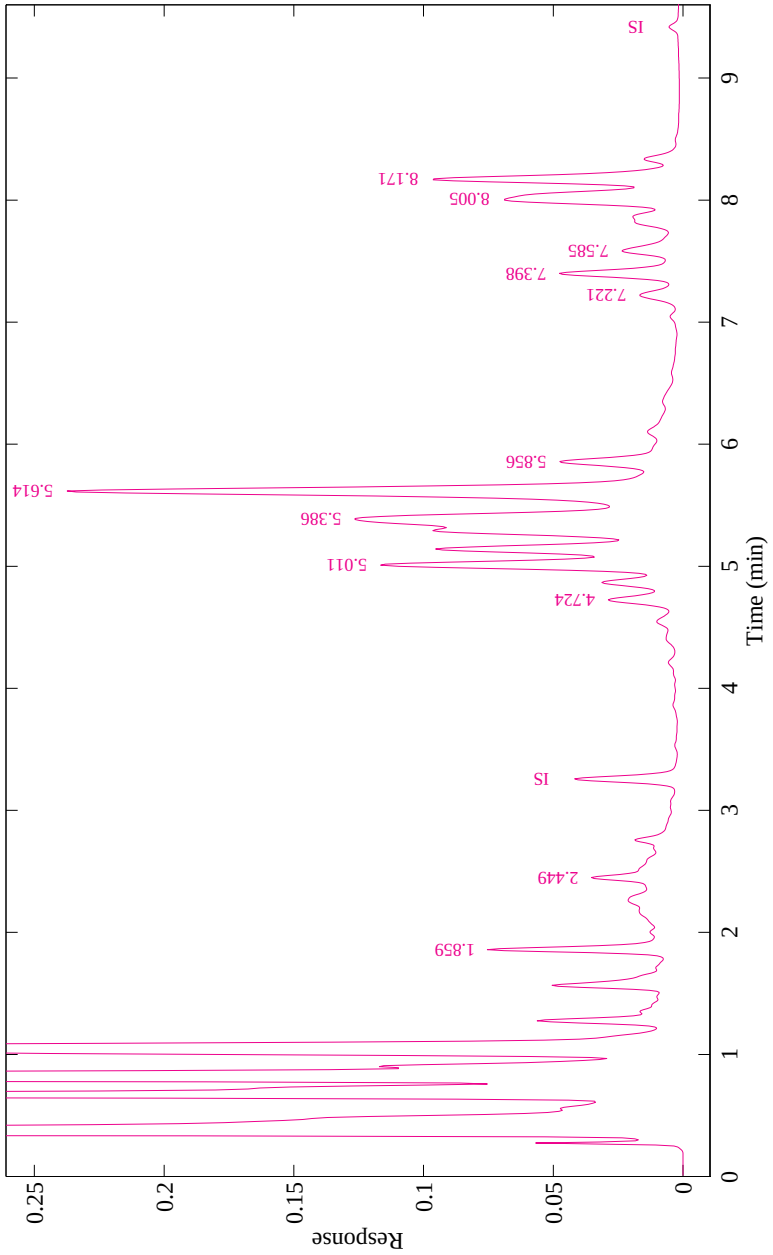
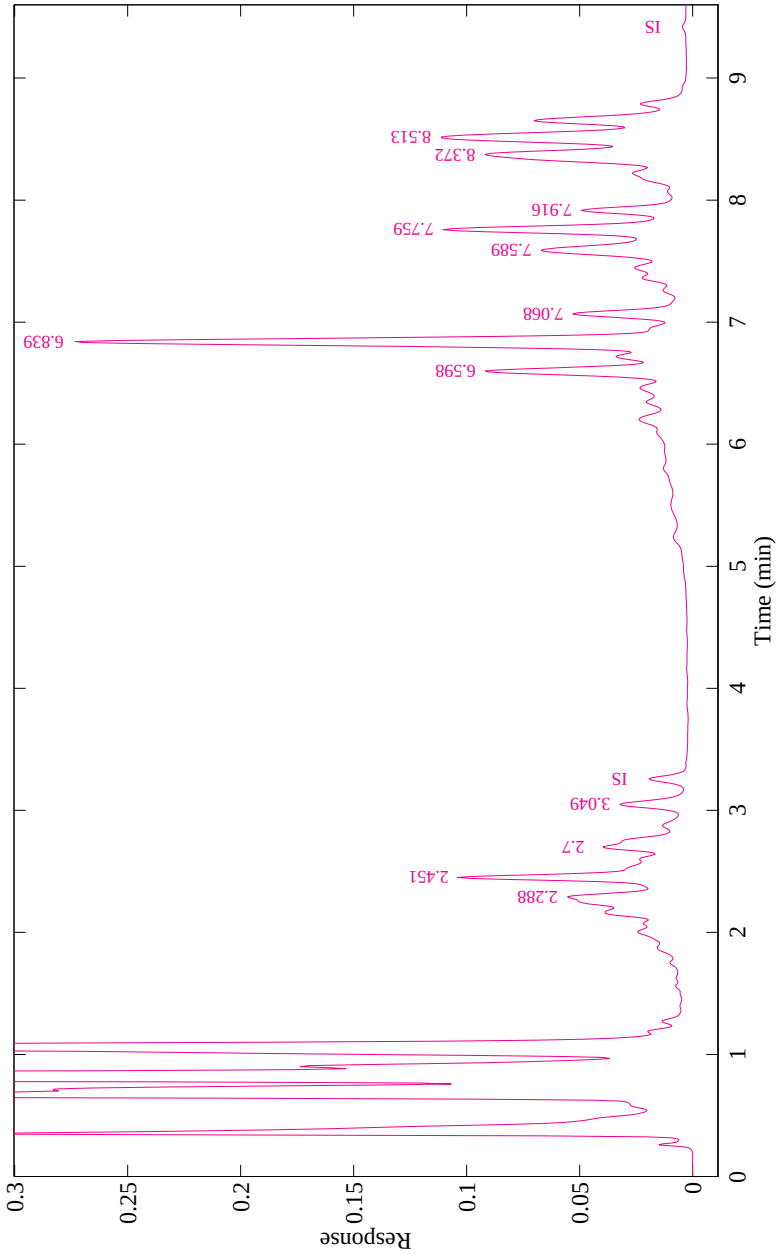


Figure 18. *M. scrofulaceum*

Figure 19. ***M. simiae***



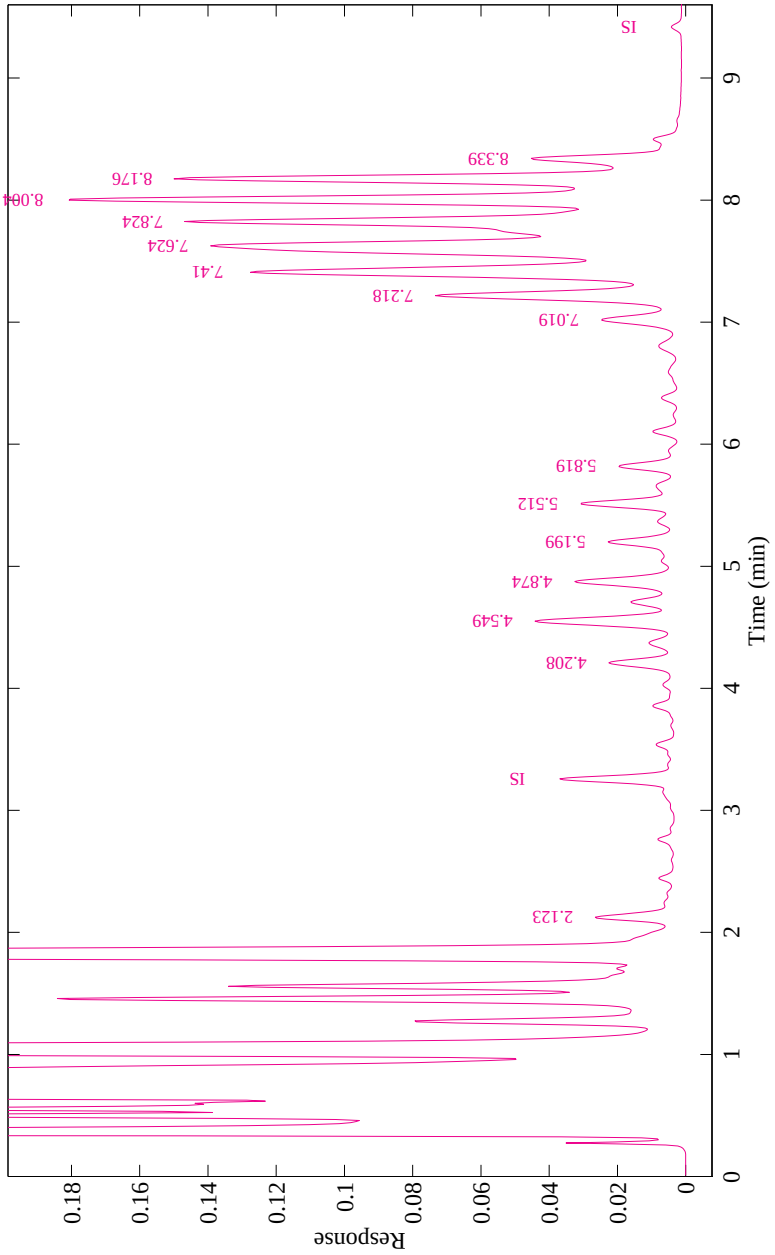


Figure 20. ***M. szulgai***

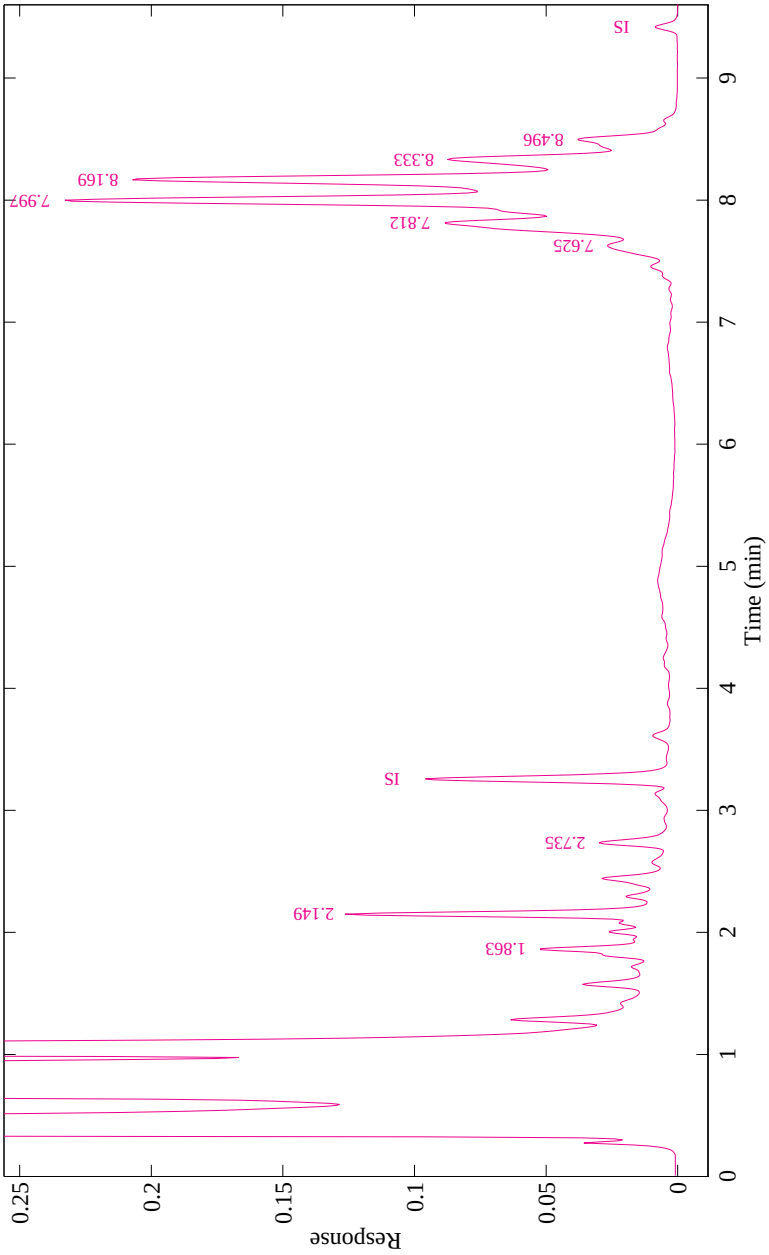
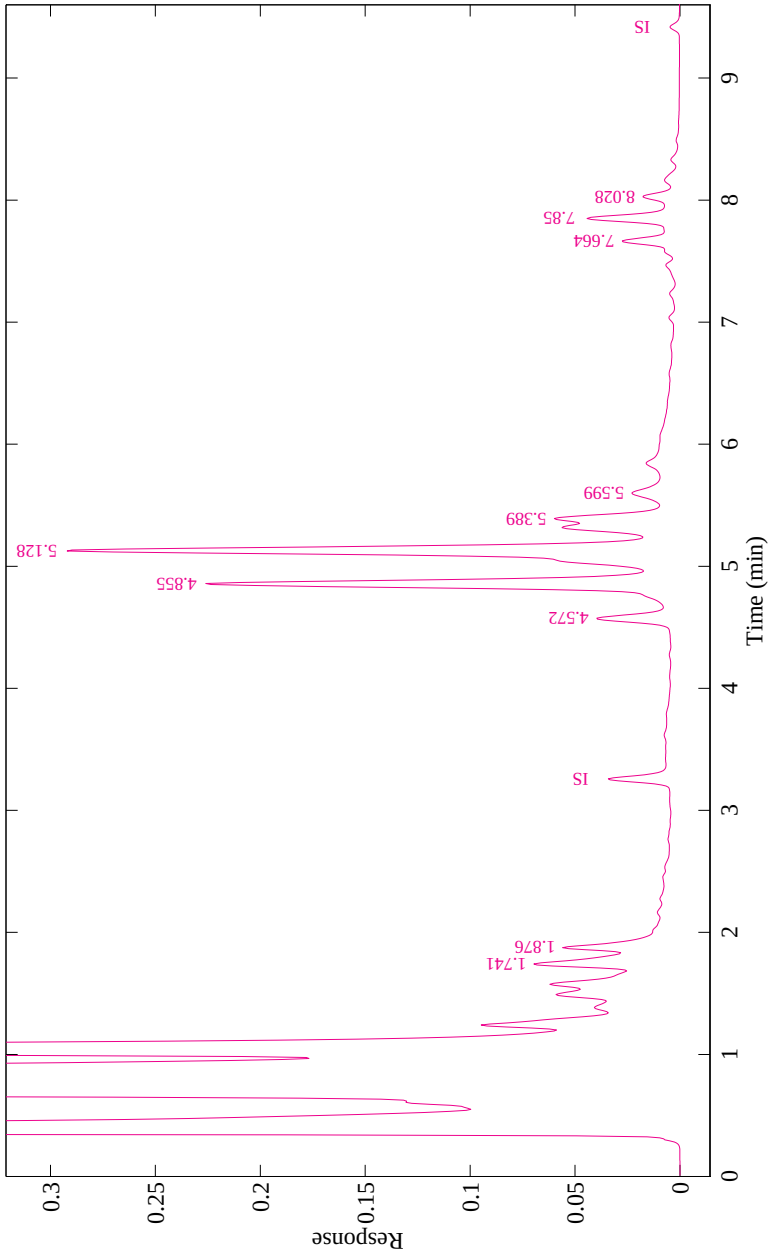


Figure 21. *M. tuberculosis*

Figure 22. *M. terrae* complex



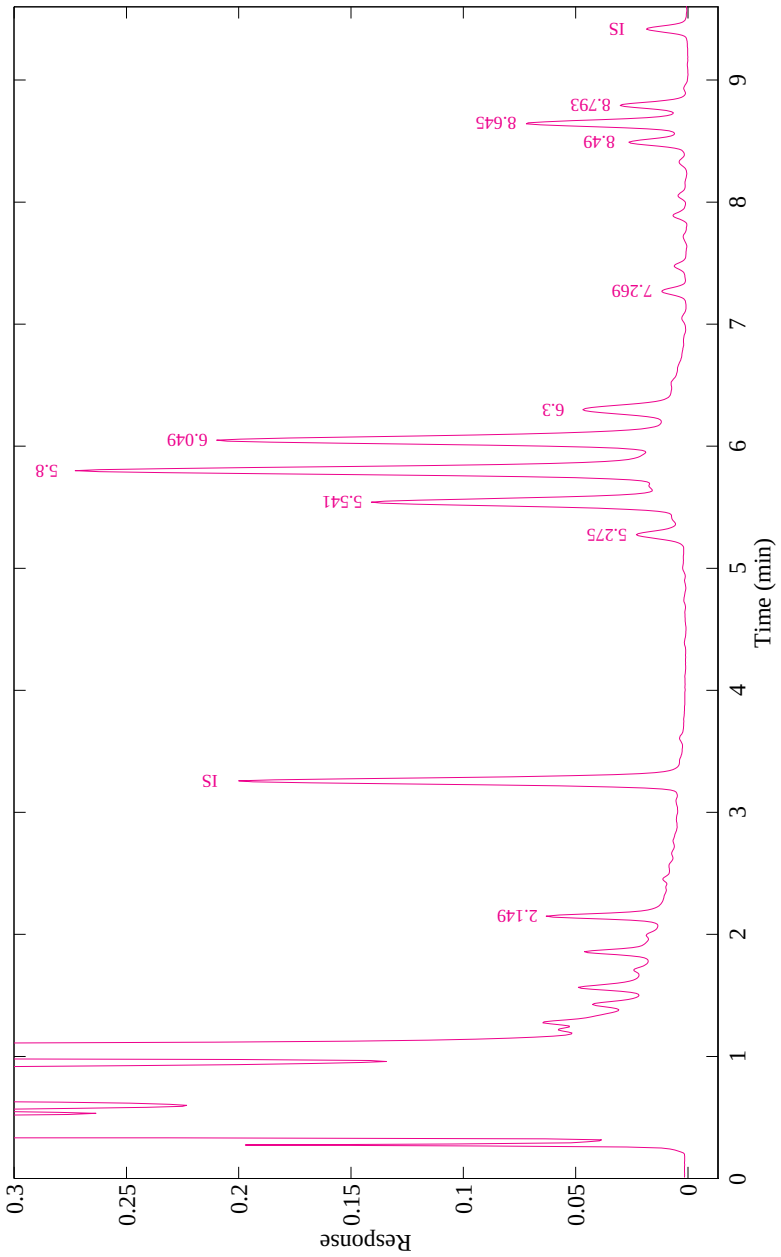


Figure 23. *M. xenopi*

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APPENDICES

Appendix A. HPLC Method File Setup

The following conditions have been established for stable separation of mycolic acids. Recommended instrument settings are summarized in Tables I through V.

Table I: Gradient Conditions[a]

Time (min)	Flow Rate (ml/min)	Percent Methanol (Solvent A)	Percent Methylene Chloride (Solvent B)
Initial	2.5	98	2
1.0	2.5	80	20
10.0	2.5	35	65
10.5	2.5	98	2
15.0[b]	2.5	98	2

a. The solvent concentrations are adjusted using a linear gradient.

b. The interval from 10.5 to 15 min is included to standardize the column wash time in order to re-equilibrate the column with approximately 20 void volumes of the initial solvent mixture.

Table II: Instrument Control Settings

Column Oven	Set point = 35°C	Low = 34°C	High = 36°C
Pressure Limits	Set point = None	Low = 600 psi	High = 3000 psi

Pump Compressibility Setting: Adjust to achieve 25.0 ± 0.5 ml in 10.0 ± 0.1 minutes when operating the gradient specified in Table I.

U.V. Detector Wavelength: Set point = 254 or 260 nm

Other Detector Settings: Use factory defaults.

Table III: Initial Integration Parameters[a]

Time(min)	Parameter	Setting	Comment
0.00	Suspend Integration	ON	
0.00	Initial Peak Width	0.2	Adjust so that peaks with heights ≥2-5% of the tallest mycolic peak in the chromatogram are integrated and named
0.00	Minimum Peak Height	0	
0.00	Minimum Peak Threshold	0.005	
1.2-1.4	Suspend Integration	OFF	Set the time to assure integration of the first calibrated peak
10.5	Suspend Integration	ON	Set the time to assure integration of the HMW-ISTD

a. Settings are for illustrative purposes only, and are representative of settings for Beckman System Gold chromatography software. Equivalent parameters and settings must be identified for instruments from other manufacturers.

Table IV: Peak Identification Table Settings

Peak Name	Time Reference\Standard (i.e., Must Find)	Window (min)[a]
S 1	NO	0.100
S 2	NO	0.100
S 3	NO	0.100
$S\ 4_a$	NO	0.100
$S\ 4_b$	NO	0.100
S 5	NO	0.100
$S\ 6_a$	NO	0.100
$S\ 6_b$	NO	0.100
S 7	NO	0.100
LMW-ISTD	YES	0.300
S 8	NO	0.100
S 9	NO	0.100
F 1	NO	0.200
F 2	NO	0.200
F 3	NO	0.200
F4	NO	0.200
A1	NO	0.100
A4	NO	0.100
A5	NO	0.100
M1	NO	0.150
M2	NO	0.150
M3	NO	0.150
M4	NO	0.150
M5	NO	0.150
M6	NO	0.150
B1	NO	0.150
B2	NO	0.150

Table IV: Peak Identification Table Settings (Continued)

Peak Name	Time Reference\Standard (i.e., Must Find)	Window (min)[a]
B3	NO	0.150
B4	NO	0.150
$B5_a$	NO	0.075
$B5_b$	NO	0.075
B6	NO	0.150
B7	NO	0.150
B8	NO	0.150
B9	NO	0.150
E1	NO	0.150
E2	NO	0.150
HMW-ISTD	YES	0.300

a. **The peak window suggested is a starting point only.** In this example integration is started at 1.2 to 1.4 min. Windows must be adjusted to produce reliable peak identification and prevent peak number reversals (i.e., an early peak reported after the adjacent later peak, caused by overlapping peak window settings).

Table V: Report Parameters (Order not important)

Peak Name[a]	Peak Height[a]	Relative Peak Height	Retention Time	Relative Retention Time	Symmetry (tailing)[a]	Signal-to-noise[b]

a. Only calibrated peaks may be reported. Calibrated peaks that are absent should be reported as 0.
b. If available.

Appendix B. Peak naming standard

If a peak naming table is included in the HPLC method it must label the diagnostic mycolic acid peaks[12], the LMW-ISTD, and the HMW-ISTD with their correct names according to their retention times. Correct naming of all peaks confirms that the retention times are within the desired tolerance. A new peak-naming table must be prepared each time the chromatography column is replaced, or whenever the fluid path of the instrument is modified.

1. An external calibration standard used to establish the peak-naming table and to verify the correct naming of the diagnostic peaks may be prepared by combining several species of mycobacteria. For example, approximately equal amounts of mycolic acids from *M. mucogenicum* ATCC 49651, *M. avium* ATCC 25291, *M. chelonae* ATCC 35752, *M. xenopi* ATCC 19250, and *M. malmoens*e ATCC 29571 may be combined to make a calibration mixture.
   a. The correct position and names of the diagnostic peaks in the calibration mixture can be verified by comparison with the reference chromatograms for each individual species in the mixture.
   b. The retention times derived from the composite chromatogram can be used to establish the peak-naming table. Once the peak-naming table has been defined, run each strain of the mixture and determine if the individual peaks have been correctly named.
   c. If the peaks are not correctly named in both the composite and the individual samples then adjust the peak-naming table for the expected retention time in the method to assure correct peak naming.
   d. The resulting calibration standard, with its labeled chromatogram may be used to periodically verify and correct the peak-naming table used in the HPLC method.

2. An alternate method for establishing the peak-naming table is to individually analyze the mycobacteria noted above. The individual chromatographic results can be combined to establish the peak-naming table. Correct naming of the peaks can be verified by reanalyzing the individual species.

Appendix C. Negative control

The negative control is *Candida albicans* ATCC 60193, a non-mycolic acid-containing organism grown under standardized conditions and analyzed with both standards.

Appendix D. Chromatograms of calibration species

Chromatograms shown in Figures 1-7 were prepared from extracts of cultures grown on L-J medium at 35°C following the standardized procedure. Chromatography was performed on a Hewlett Packard 1050 chromatograph equipped with a variable wavelength detector set at 260 nm. The column was the Beckman/Altex Part No 238370 operated at 35°C and 2.5 ml/min using the gradient stipulated in Table I. All peaks are named according to conventions described in Table IV.[12]

Figure 1. *Candida albicans* ATCC 60193

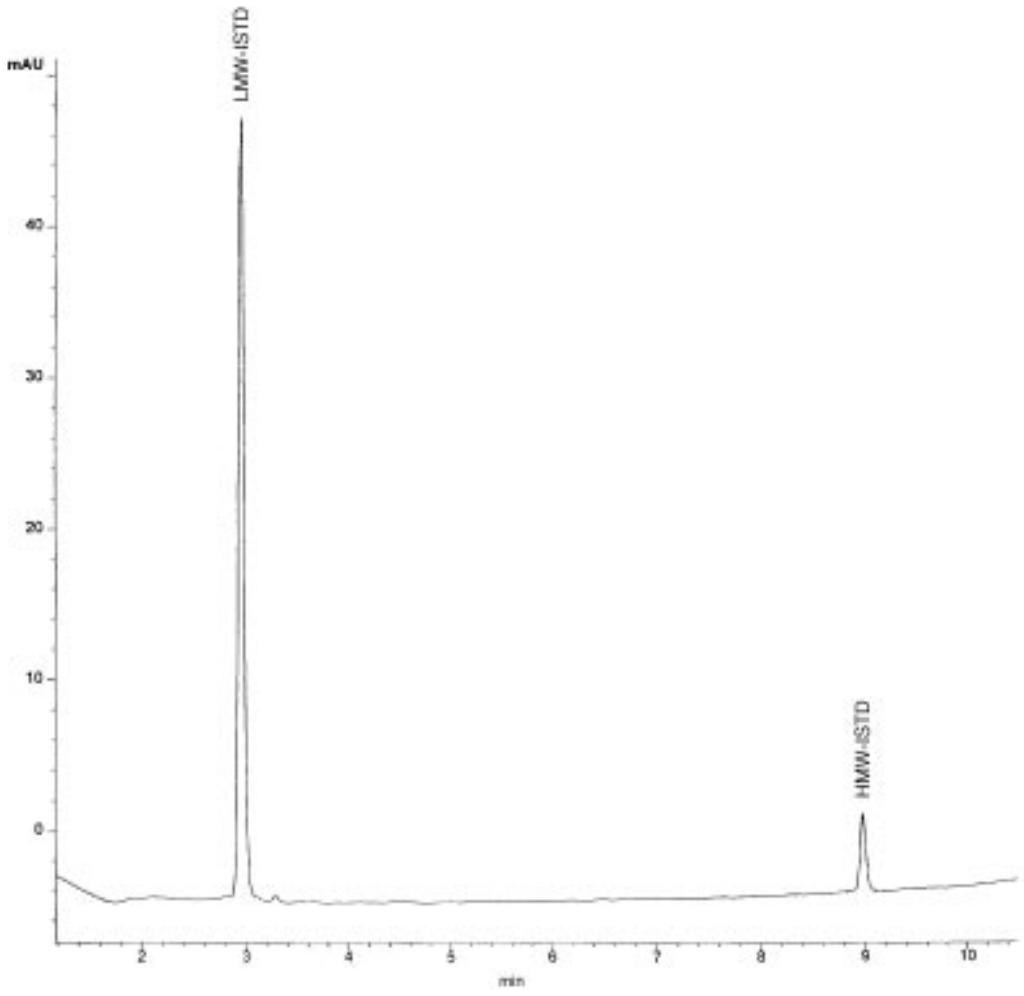


Figure 2. Calibration Mixture

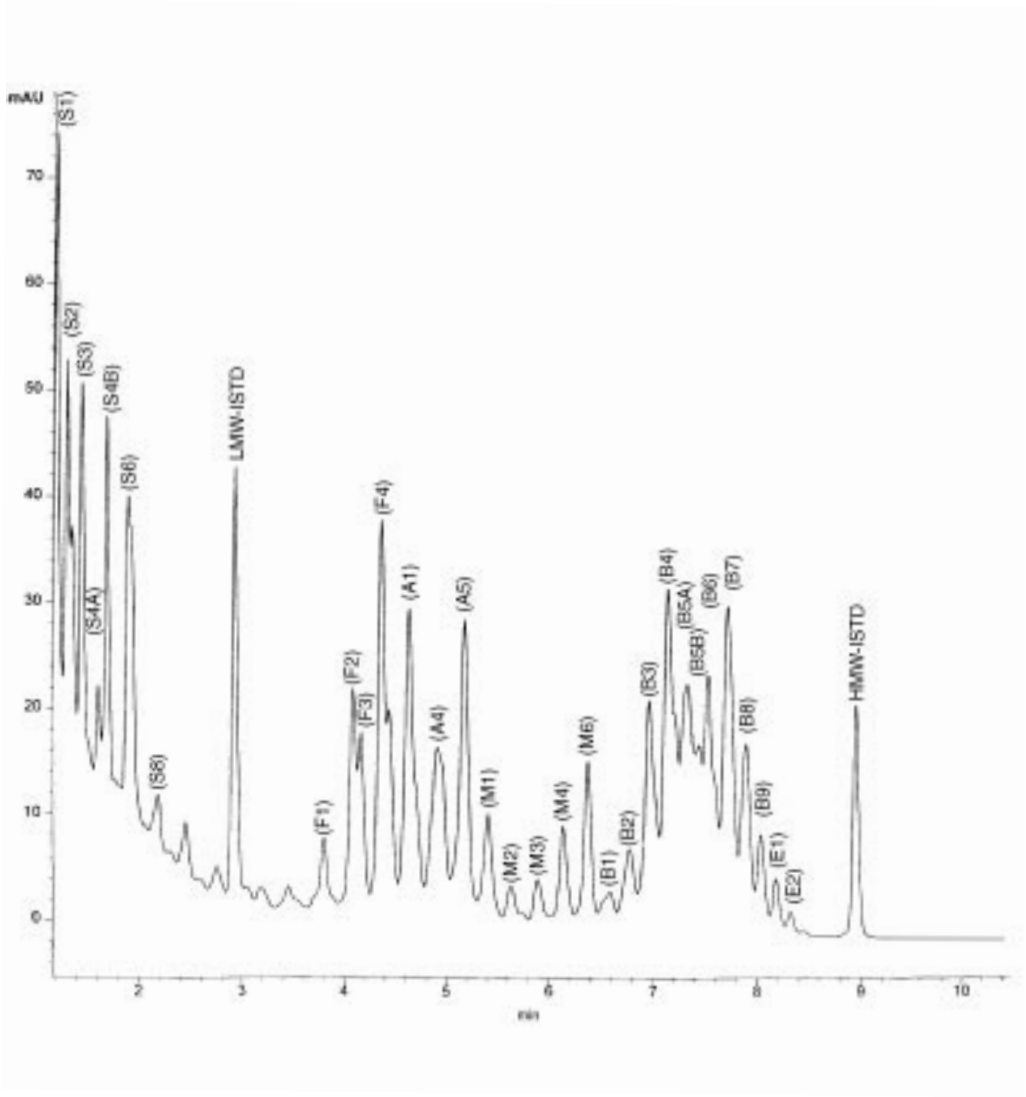


Figure 3. *Mycobacterium avium* ATCC 25291

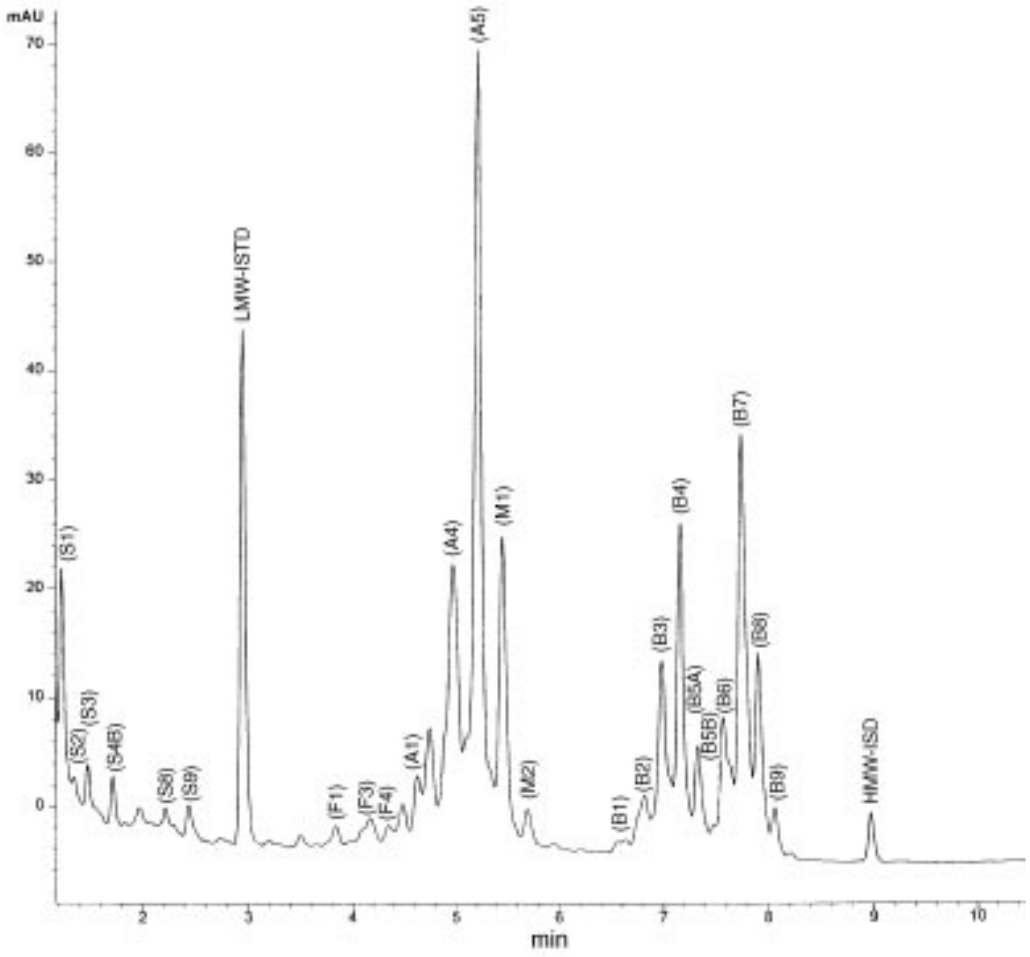


Figure 4. *Mycobacterium malmoense* ATCC 29571

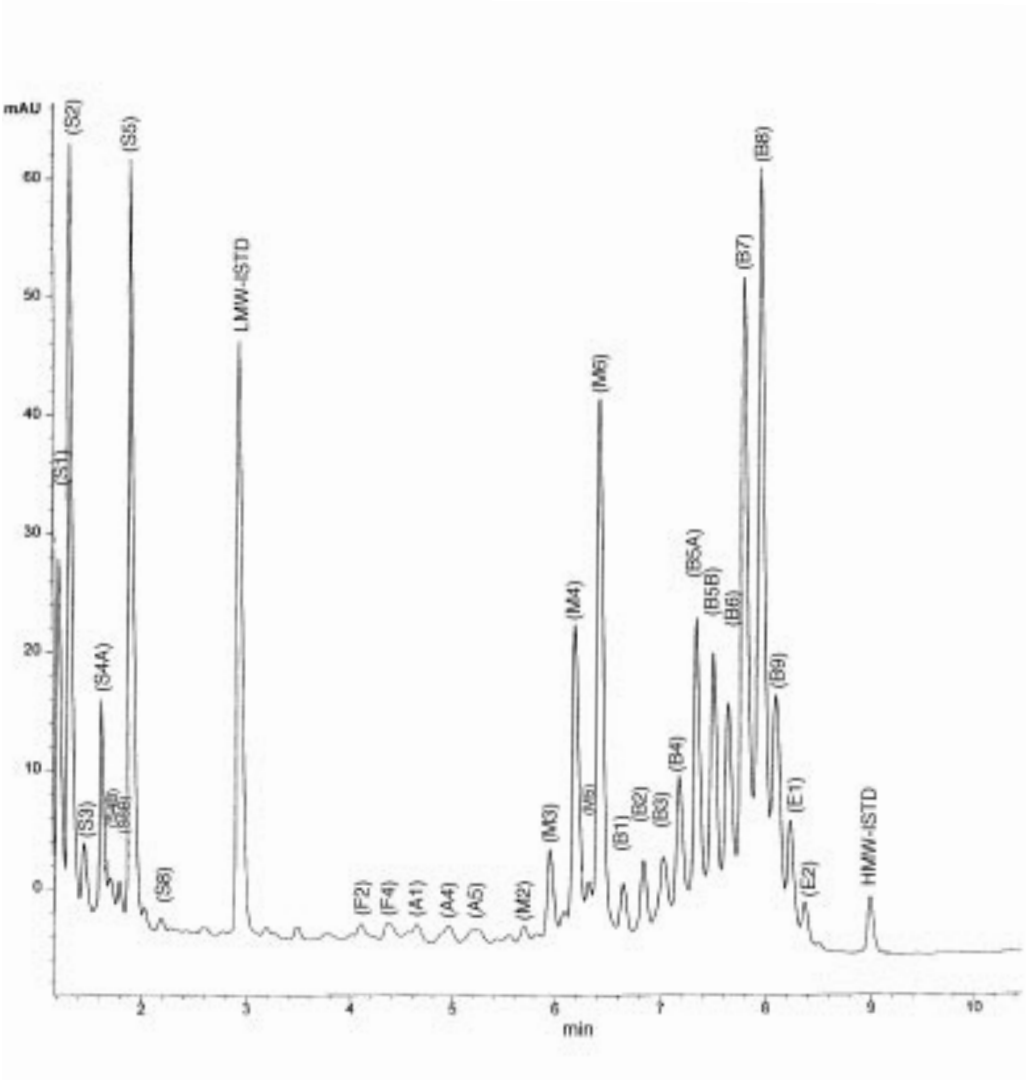


Figure 5. *Mycobacterium mucogenicum* ATCC 49651

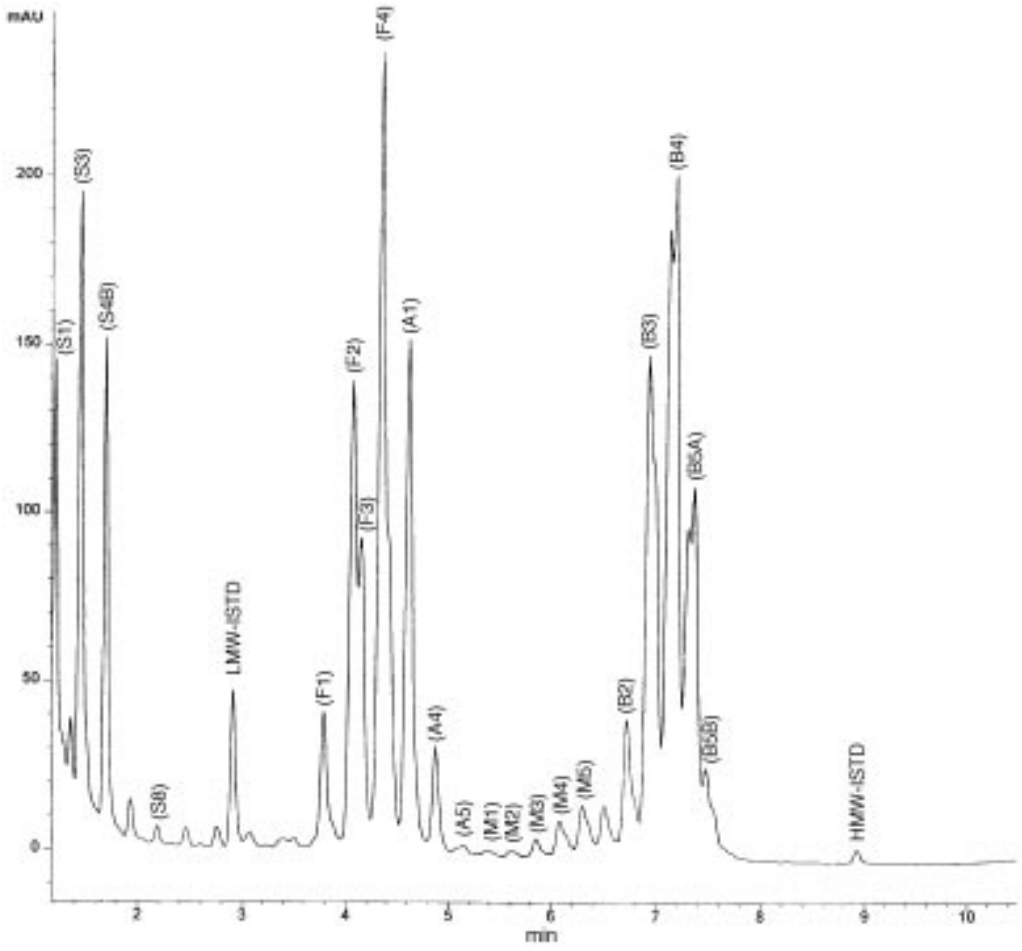


Figure 6. *Mycobacterium chelonae* ATCC 35752

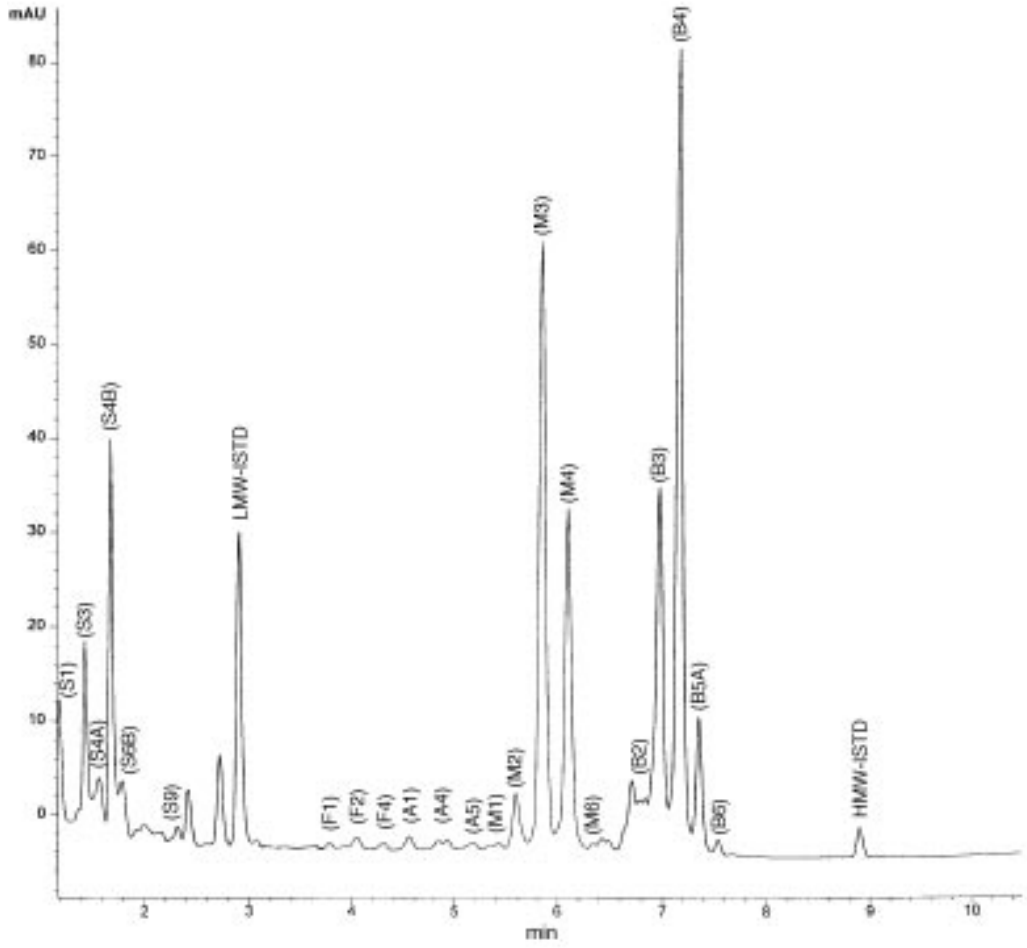
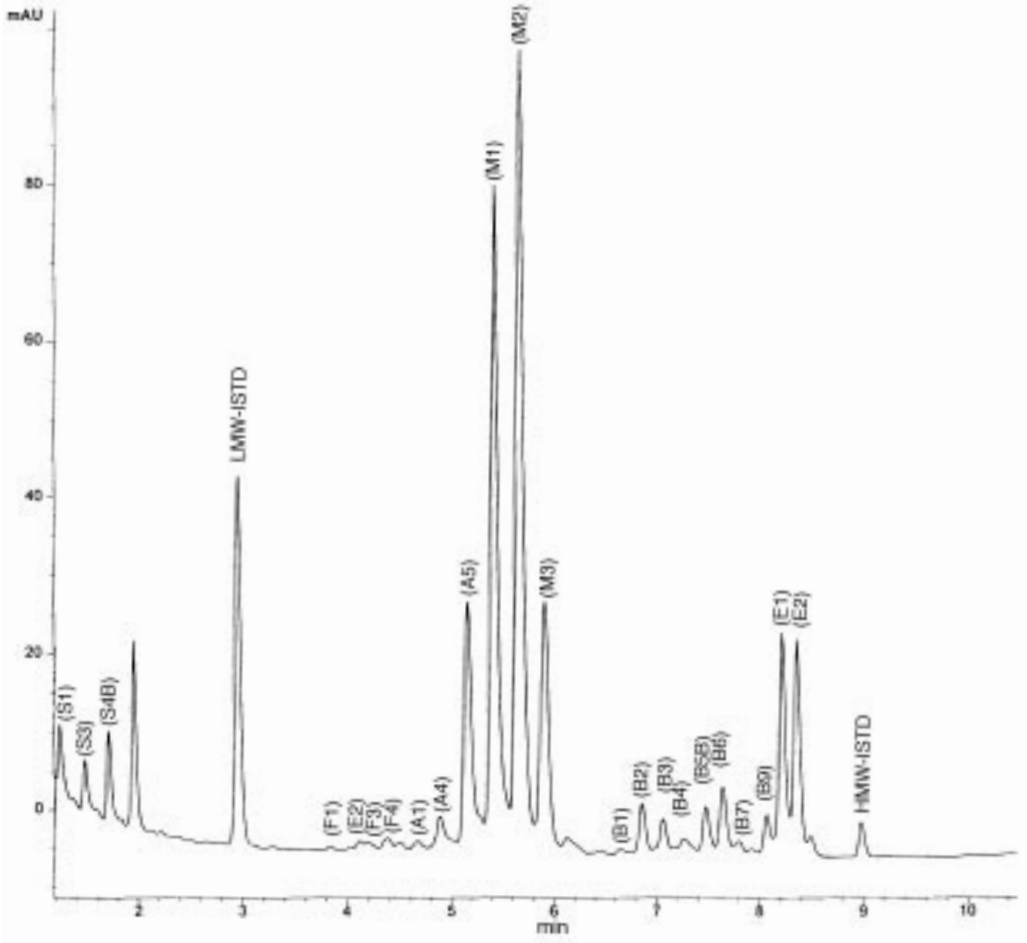


Figure 7. *Mycobacterium xenopi* ATCC 19250



Appendix E. Assaying mycolic acids directly from BACTEC culture media

Mycobacteria may also be identified from cultures grown in BACTEC 7H12B media[6], although the mycolic acid yield is significantly less than from solid media. **NOTE**: When identifying cultures grown on BACTEC culture media, results must be compared with chromatograms for reference strains that also were grown on BACTEC culture medium.

1. Materials

   a. Refrigerated centrifuge capable of attaining 3000 x g, with safety cups to fit 50 ml conical tubes.

   b. Vacuum multipurpose manifold and pump (or house vacuum) for Solid Phase Extraction units.

   c. Solid Phase Extraction (SPE) t C-18 units (500 mg capacity).

   d. Nylon-membrane syringe filter units (13 mm, 0.45 µm or smaller pore size).

   e. Disposable Luer-Lok syringes 5-10 ml and 20-23 ga 1 inch (or longer) Luer-Lok needles.

   f. Polypropylene centrifuge tubes 50 ml, screw-cap, conical.

2. Culture conditions

   The BACTEC cultures are incubated to a growth index of ≥ 50, and verified by acid-fast stain to contain AFB. Bottles positive for AFB are supplemented with 0.5 ml of oleic acid-albumin-dextrose-catalase[1] (OADC) enrichment, and re-incubated for another 5-7 days[6] before processing. Additional sample clean-up procedures (described below) are recommended to protect the chromatography column when samples are prepared from BACTEC cultures[9].

3. Sample harvesting procedure[6]

   a. Using a syringe, transfer the contents from the BACTEC bottle to a new 13 x 100 mm tube.

   b. Cap the tube and enclose it in a 50 ml centrifuge tube.

   c. Place the centrifuge tube into a centrifuge safety cup, and balance.

   d. Centrifuge at 4-10°C at 3000 x g for a minimum of 30 min.

   e. Without disturbing the pellet use a pipette to transfer the supernate into a container of tuberculocidal disinfectant. If the pellet is dislodged, retain some of the supernatant. (NOTE: observe applicable regulations concerning proper handling and disposal of radioactive materials).

   f. Add 2 ml of saponification reagent, cap the tubes tightly, and mix vigorously for at least 20 sec. Proceed to steps B and C of the standard extraction and derivatization procedure, and then to the solid-phase extraction procedure described below.

4. Solid-phase extraction[9]

   a. Prepare 80:20 v:v methanol:methylene chloride (allow 30 ml/sample).

   b. Condition the solid phase extraction column by passing 5 ml of the 80:20 mixture through it at ≤ 3 ml/min. A multi-position manifold is useful for holding the extraction units. Discard the effluent.

   c. Suspend the sample in 5 ml of 80:20 mixture and pass it into the column at ≤ 3 ml/min. Discard the effluent, which contains the unwanted material.

   d. Wash the column with 15 ml of 80:20 mixture at ≤ 3 ml/min. Discard the effluent.

e. Elute and collect the mycolic acid-containing sample with 3 ml methylene chloride at ≤ 3 ml/min.

f. Use a 13 mm nylon membrane, 0.45 µm or smaller pore size syringe filter to attached to a 5-10 ml syringe to filter the material into a fresh tube. Evaporate to dryness at 100°C ± 5°C under a stream of dry air. Cap the tube tightly. Store at 4-6°C in the dark until ready for analysis.

Appendix F. Automated identification software

Software packages are available to identify mycobacteria from chromatograms obtained using the standardized method for HPLC identification of mycobacteria. However, they have not been formally evaluated. If automated identification software is used, confirm the software identification as follows.

1. Verify that all significant peaks that are ≥ 2-5% of the height of the tallest mycolic acid peak between the LMW-ISTD and the HMW-ISTD in the sample chromatogram have been labeled and that all mycolic acid peaks in the external calibration standard mixture have been reported (*see* Appendix A, Table IV).

2. Compare the chromatogram for the sample against the reference chromatograms using the software package.

3. If the sample chromatogram agrees with one of the reference chromatograms, report the identification, indicate "by HPLC" and note the software package used.

4. If the species identified by the software package does not match any reference pattern, use the visual identification method described in this document.

5. If unable to identify by HPLC using a software or visual approach use other available methods.