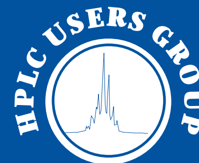
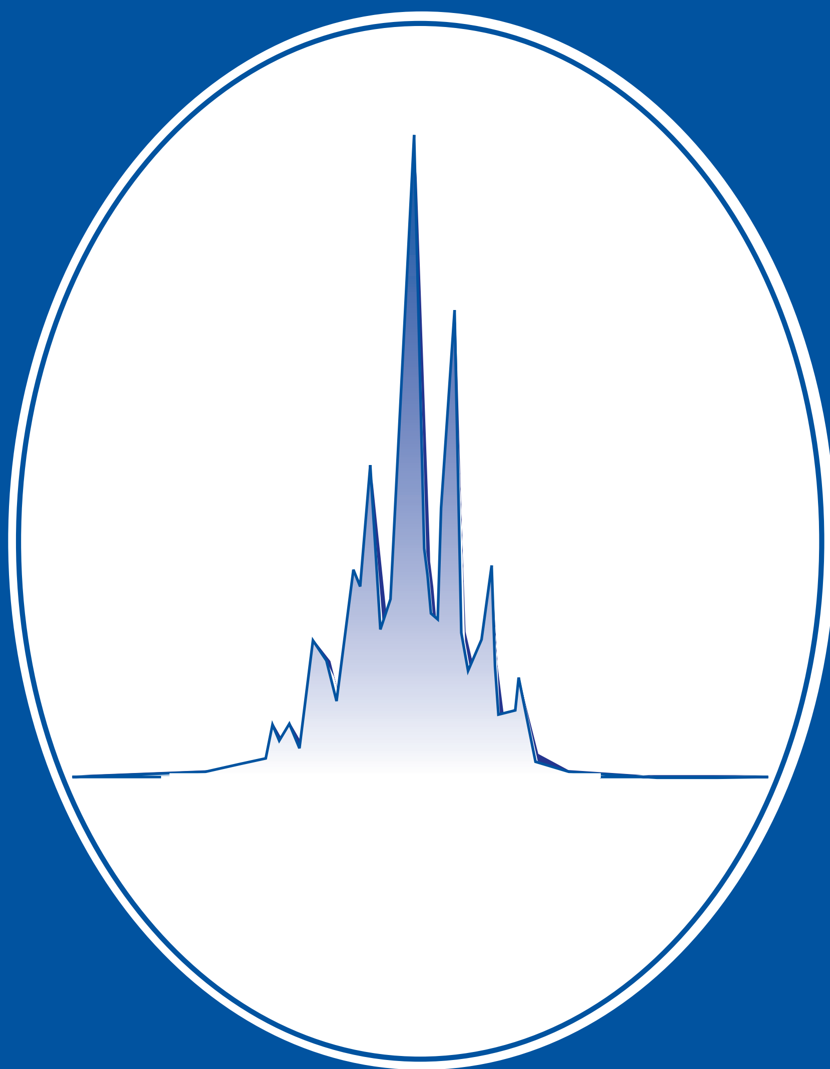


Mycolic Acid Pattern Standards for HPLC Identification of Mycobacteria



U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service

Mycolic Acid Pattern Standards for HPLC Identification of Mycobacteria

February 1999

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Preface

Contemporary clinical mycobacteriology activities have rapidly changed with the advent of new laboratory methods. Many methods are now available to reduce the time required to achieve test results. One of the rapid identification methods, developed at the Centers for Disease Control and Prevention (CDC) in 1985, is the use of High Performance Liquid Chromatography (HPLC) for the analysis of species-specific mycolic acids in the cell wall of mycobacteria.

In 1992, laboratorians who were using this method met in New Orleans, LA, and formed the HPLC Users Group. Their purpose was to enhance communication among mycobacteriologists using the new procedures and to achieve a mutual understanding of individual techniques. At this meeting a chairperson was named, and a newsletter was begun to disseminate information among users. Laboratorians with casual or active interest in the use of HPLC as a mycobacteria identification tool were invited to participate in the activities of the group.

The membership has now grown to more than 150 users. In addition to the group newsletter, *Peaks of Interest*, the HPLC Users Group has a website at:

http://www.userhome.com/hplcusersgroup/

and a listserv to which interested persons can subscribe by sending an email message to:

majordomo@userhome.com

and typing "**subscribe acid-fast-hplc**" in the body of the message. Additionally, the Steering Committee of the HPLC Users group has presented several joint HPLC workshops with the Association of Public Health Laboratories and the National Laboratory Training Network.

Informal surveys conducted within this membership revealed differences in techniques employed by users to achieve the same goal of identification. To address this problem, the chairperson nominated a steering committee of experts in the use of HPLC to guide the group and define collaborative studies which could standardize and improve the method.

Standardization became a priority of this committee. An extensive project involving five laboratories was undertaken to evaluate the status of the method. More than 350 well-characterized isolates, representing 23 species, were examined. Partial results from this study were recently published by the HPLC Users Group in cooperation with the CDC in a manual entitled, *Standardized Method for HPLC Identification of Mycobacteria*. The publication addresses the proper setup of the HPLC and use of this method. It can be downloaded from the CDC website, at:

http://www.cdc.gov/ncidod/publicat.htm

It is also available from the HPLC Users Group website at:

http://www.userhome.com/hplcusersgroup/user_index.html

This current document is also based on an interlaboratory study, designed to examine biological variation of strains for a species and its effect on the visual presentation of the HPLC

chromatogram. **Chromatograms illustrated in this manual were derived from the study isolates and do not represent all of the variation expected for the described species**. This manual is written primarily for laboratory personnel actively involved in the use of HPLC for identification of mycobacteria.

The HPLC Users Group Steering Committee is committed to validating HPLC as a dependable means of recognizing *Mycobacterium* species and reducing turnaround time for reporting identification test results. For this purpose both the Standardized Method manual and this manual should be used as guides for the laboratories involved in HPLC reference mycobacteriology work.

Acknowledgments

The editors wish to thank the following individuals for their valuable expertise during the preparation of this manual: Stephen Lapierre, Laboratoire de santé publique du Québec; Sher H. Chiu, Microbiological Services Division, Texas Department of Health; Yvonne Jang, Deborah F. Hanson, and Jean Todd, Microbial Diseases Laboratory, California Department of Health Services; and Donald A. Bonato and Frances Vadney, Tuberculosis Reference Laboratory, Veterans Affairs Medical Center, West Haven, CT.

Introduction

Presented in this document is a collection of high performance liquid chromatography patterns of mycolic acids derived from extracts of cell wall preparations of mycobacteria, which are used in the visual identification of target specimens. The chemotaxonomic potential of the mycolic acids for species identification of mycobacteria has been well documented. Extensive work with HPLC analysis resulted in a dramatic increase in usage by clinical laboratories. This popularity is due to the simplicity and reliability of the method and its ability to accurately identify mycobacteria from a single test.

Mycolic acid patterns presented in the following pages do not always present peaks that are chemically pure. Rather, each peak can be a composite of the different mycolic acid structural types present in the individual species. While it is not our purpose to chemically define the peaks, the gross patterns have been determined empirically to be species-specific.

However, laboratory workers who use this method quickly recognized that a species may produce HPLC patterns with subtle differences. A minor part of this variation in patterns is due to the heterogeneous nature of the chemically impure peaks that coelute. Most of the variation seems to be attributed to strain variation within a species or different species. In an effort to characterize this variation for a selected set of mycobacteria strains, an interlaboratory study was conducted.

A study of more than 350 strains, representing 23 species, was distributed to 5 laboratories for comparative analysis. Every effort was made to eliminate interlaboratory variation by optimizing all conditions of analysis. HPLC protocols were followed as outlined in the *Standardized Method for HPLC Identification of Mycobacteria* manual. The study was designed so that only validated, authentic cultures would be examined and analyzed. The study participants decided that isolates would not be accepted into the study based on a prior designation, not even reference culture collection isolates. Therefore, the identity of all the cultures were reconfirmed by available methods including genetic probes, biochemical tests, HPLC, and 16S rRNA sequencing when necessary.

Data files from each laboratory were exported from the respective chromatography software system as AIA files, a standard file interchange format for chromatographic data (and a subset of the netCDF file protocol). The collections of AIA files were sent to a single site for analysis (Infometrix, Inc., Woodinville, WA). After eliminating samples of inadequate response, the individual files were merged into one large database for subsequent data analysis. The HPLC profiles were aligned to a common standard, then representative patterns for each species were identified.

This publication illustrates the results of the study, shown as mycolic acid pattern standards (MAPS) for 23 of the most commonly encountered species of mycobacteria. All extracts were analyzed by HPLC with UV detection. Chromatographic figures shown in this document are represented on the horizontal scale by time (in minutes) and on the vertical scale by arbitrary response units.

Chromatogram Reproducibility

There are two issues of major importance for a chromatographic collection such as MAPS: intralaboratory and interlaboratory reproducibility. Within a laboratory, pattern reproducibility is critical to insure that samples are consistently identified over time. Interlaboratory variation must also be minimized for comparison of patterns between laboratories.

Pattern reproducibility is a concern because the correct visual interpretation of the pattern for species identification is directly related to the quality of the chromatogram. Comparisons of patterns can be made more reliable when the data have been adjusted according to relative retention time (RRT), usually by comparison to an internal standard peak. It is recommended that any laboratory using this document perform HPLC using the *Standardized Method for HPLC Identification of Mycobacteria* manual, which recommends the use of internal standards.

Most chromatography software packages have the ability to automatically compare the retention time (RT) of an eluting peak relative to such standards to calculate a RRT. Typically, relative values are expressed as a numerical value determined by using either a subtraction or division algorithm in the software. The tabular reports generated with such software include columns for both the RT and RRT. Relative times computed in this manner are more reproducible than are the real times.

However, the RRT calculations in these packages are applied only to the integrated peaks in the chromatograms. The chromatographic profiles themselves are not usually adjusted; some packages include an ability to synchronize or align chromatograms, but they do not allow the user to save the results for further analysis.

To generate this set of chromatograms for MAPS, we developed an additional alignment algorithm to adjust the retention time axis of the chromatographic patterns. This mathematical algorithm was used to align the peaks in each chromatographic pattern to the peaks in a pattern of a *Mycobacterium intracellulare* strain (ATCC 13950) which served as a reference standard. In the discussion that follows, the low and high molecular weight internal standard peaks are used to illustrate these alignment procedures.

First, the low (LMW) and high (HMW) molecular weight internal standard peaks in each sample were identified. Then, for each chromatogram, correction factors (CF) were determined for each internal standard by calculating the ratios of the internal standard retention times in the sample pattern to the corresponding internal standard times in the reference chromatogram.

$$CF = \frac{RT_{sample}}{RT_{reference}} \quad [1]$$

RT represents the real time of elution of either the LMW or HMW internal standards.

Next, a retention time fractional value (RTF) for the peak was calculated.

$$RTF_{peak} = \frac{RT_{peak} - RT_{LMW}}{[RT_{HMW} - RT_{LMW}]} \qquad [2]$$

This represents the fractional distance of the peak's RT measured from the RT of the LMW internal standard to the RT of the HMW standard.

The RT for each data point in a chromatographic pattern between the internal standards was then adjusted by a local correction factor (LCF).

$$LCF_{peak} = CF_{LMW} + RTF_{peak}(CF_{HMW} - CF_{LMW}) \qquad [3]$$

The value of this local factor lies between the internal standard correction factors and is proportional to the distance between the internal standard RT values.

Finally, the local correction factor is used to modify the RT of the peak and produce an adjusted retention time (ART).

$$ART_{peak} = \frac{RT_{peak}}{LCF_{peak}} \qquad [4]$$

Each data point in a sample pattern was adjusted according to this procedure. The result of this adjustment creates a new chromatographic profile for the sample in which the ART of the internal standard peaks becomes identical to those in the reference. Following the alignment, the positions of peaks between the internal standards should exhibit improved comparability to patterns of similar species.

Note that the ART is related to a RRT in that each uses the internal standard reference peak times for adjustments, but the time axis is expressed differently. Instead of a fractional time as in the RRT, an ART has a time axis for the resulting chromatographic profile which is in realistic time units (*i.e.*, those obtained from the main reference sample, such as the ATCC 13950).

Several plots of raw and adjusted patterns will help to demonstrate the results of alignment as used in this MAPS collection. First, intralaboratory comparison is shown in Figure 1. Although all of the patterns in this figure were generated in a single laboratory, there was considerable variation in retention times of the peaks making alignment of the patterns difficult.

After alignment of the patterns to the internal standards using the interpolation procedure discussed above, the intrinsic nature of the patterns becomes clear (Figure 2.). Because the RT adjustment was based on comparison to the times of the internal standard peaks, the times are referred to as ART-I.

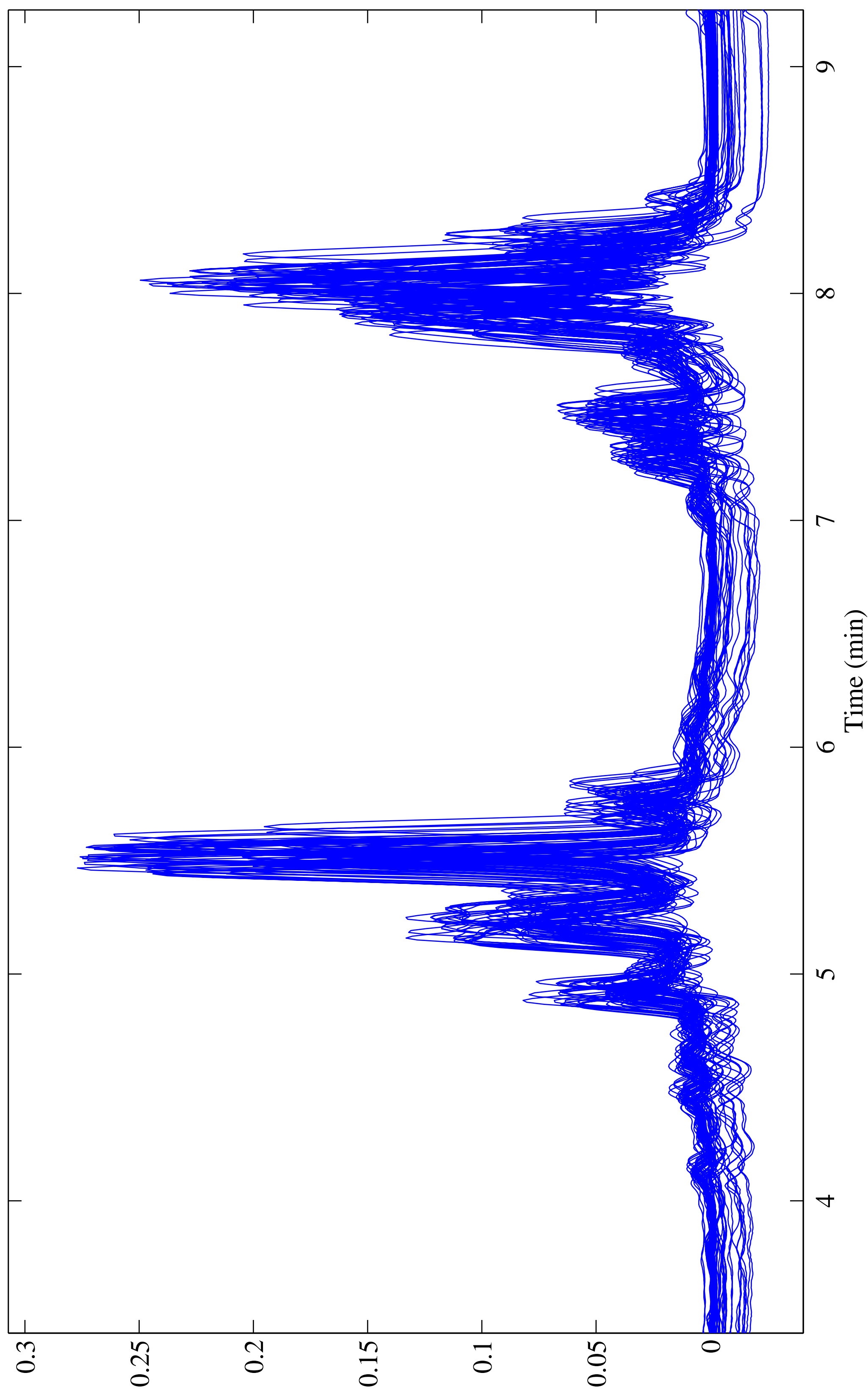


Figure 1. Patterns of 54 *Mycobacterium intracellulare* from samples analyzed in one laboratory

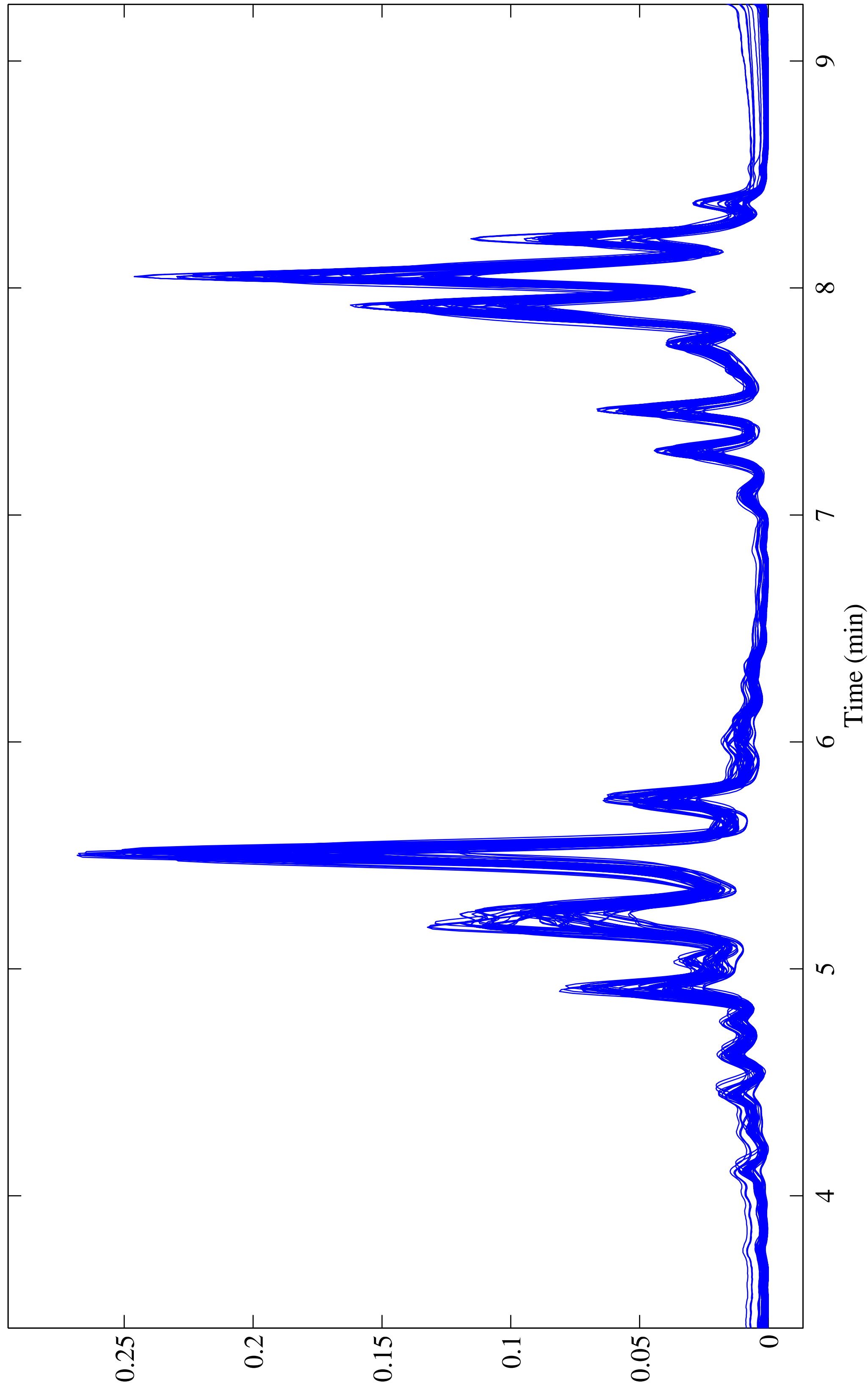


Figure 2. Patterns of 54 *Mycobacterium intracellulare* from samples analyzed in one laboratory, after alignment to the two internal standards (in ART-I units)

However, when comparing samples analyzed in different laboratories, an additional source of variation must be considered. We found that the alignment to internal standards was insufficient to allow unambiguous comparisons of patterns.

For example, profiles from analyses of *Mycobacterium simiae* in five laboratories are overlaid in Figure 3. The diverse ranges in retention times demonstrated for the peaks of this strain make interlaboratory comparison difficult. Following alignment to the two internal standards, the pattern of peaks becomes more clear, as shown in Figure 4. However, precise alignment of the patterns was not possible, an effect clearly associated with differences between laboratories. Recall that when the patterns from a single laboratory were compared, this slight imprecision practically disappeared (Figure 2.).

To improve the alignment, a different adjustment of the retention times was needed, beyond the alignment to internal standards. This additional step used the same alignment algorithm described earlier, but instead of comparing retention times of each chromatographic data point to the times of the internal standard peaks for the sample, the comparison was made to the times of selected mycolic acid peaks from a quality control (QC) sample run in the same batch. The standard method manual suggested that an analysis of a control strain (ATCC 13950) be performed after every 10 to 20 samples during the batch analysis; therefore, these analyses served as the QC samples.

The retention times of four prominent peaks in the ATCC 13950 QC samples—two in the first cluster and two in the second—were chosen for computation of the new correction factors. Again, the correction factors are derived from a ratio of retention times for these prominent peaks in the reference sample and the corresponding peaks in the QC samples (the LMW and HMW internal standards were not used in this computation).

The actual compounds which produced these four prominent peaks are not necessarily present in all samples. However, it was assumed that the variation in retention from one sample to the next in a small batch of samples is minimal. Therefore, the correction factors which are computed for the QC samples are also applied to each of the samples in the batch which were run close to a QC sample. The results of this extra alignment step on the *M. simiae* samples are shown in Figure 5. Because these corrections were calculated from peaks in an external standard, and not to internal standard peaks, they are referred to as ART-E (externally adjusted retention times).

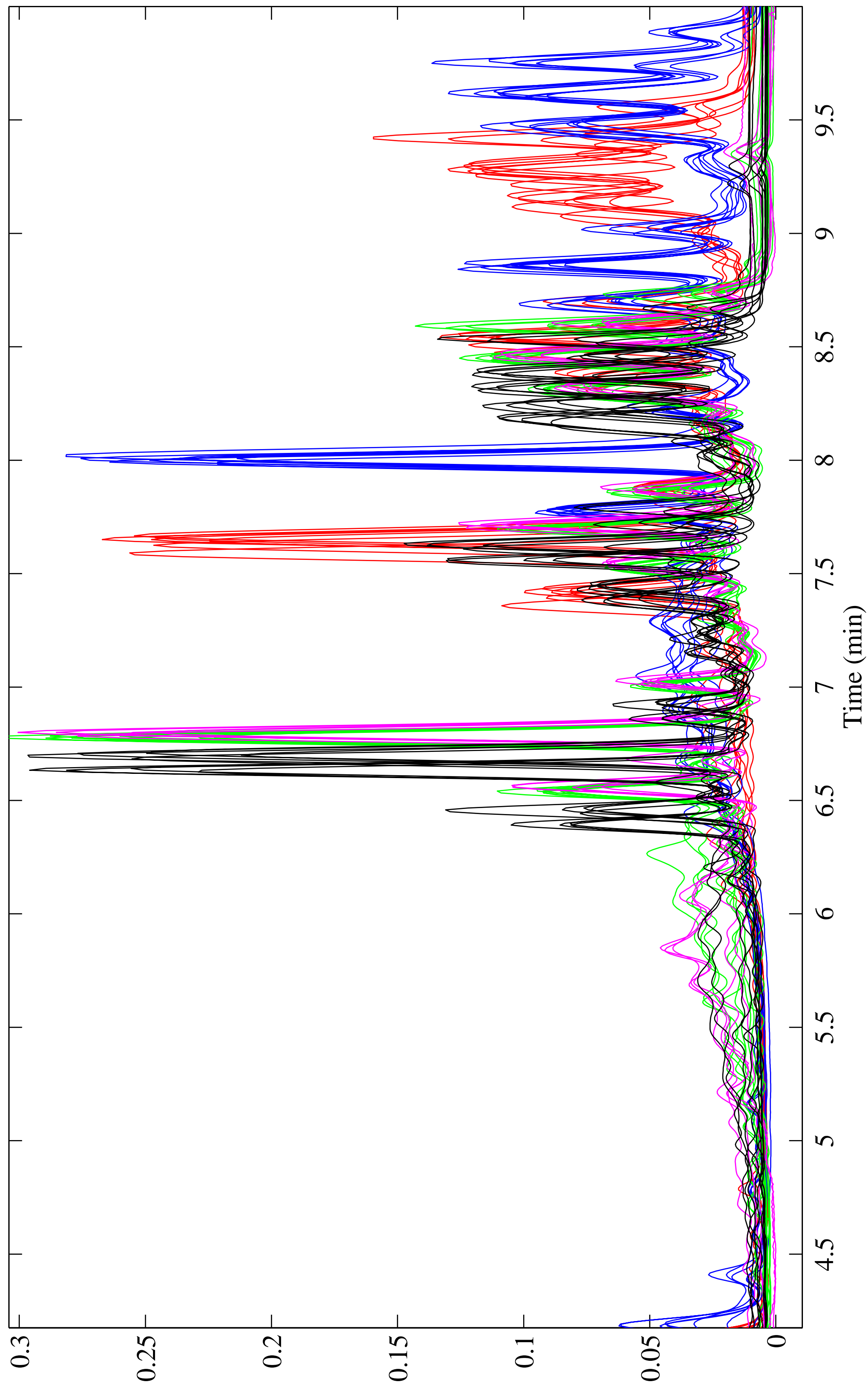


Figure 3. Patterns of 38 *Mycobacterium simiae* samples from 5 laboratories

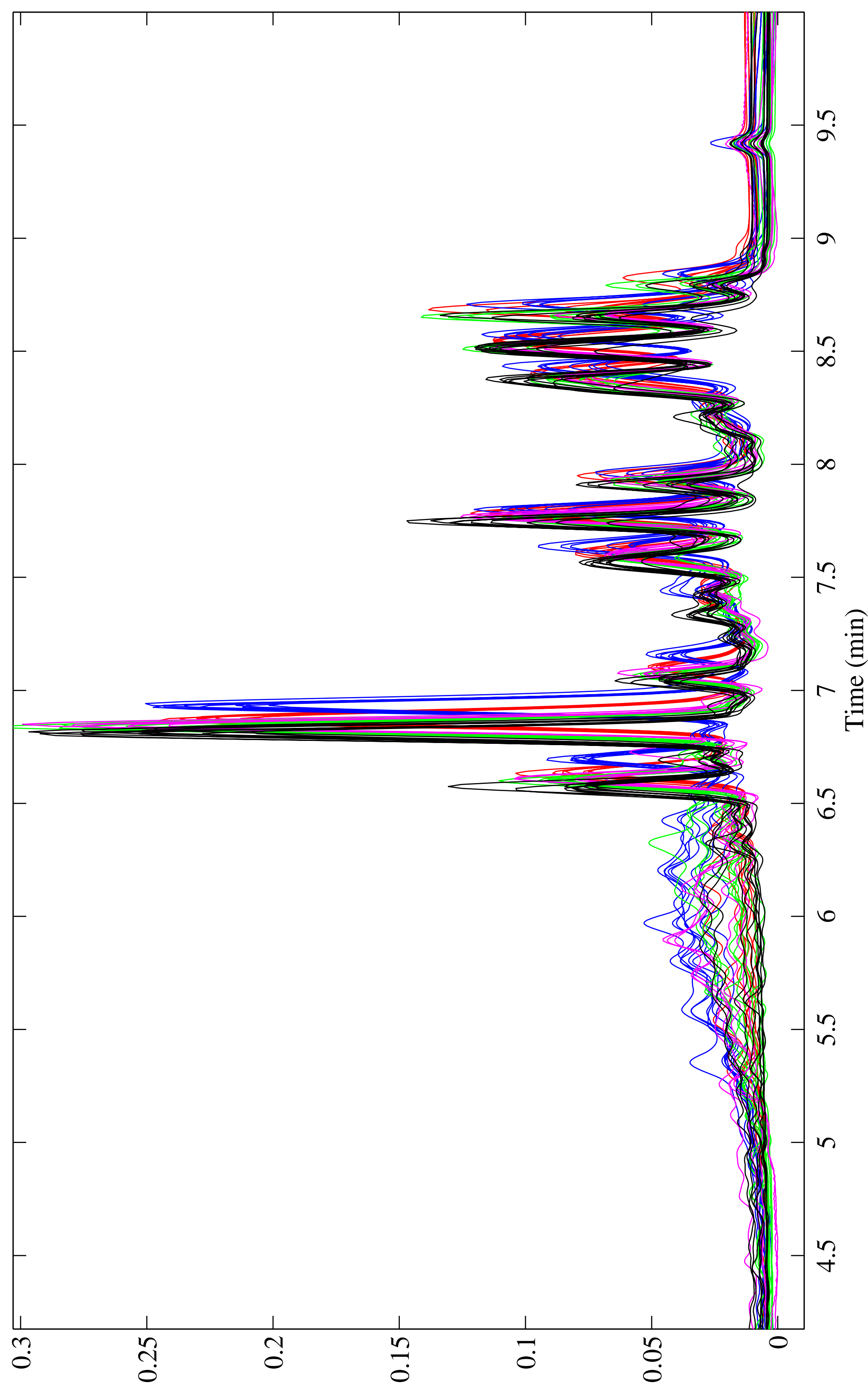


Figure 4. Patterns of 38 *Mycobacterium simiae* samples from 5 laboratories with alignment to internal standards (in ART-I units)

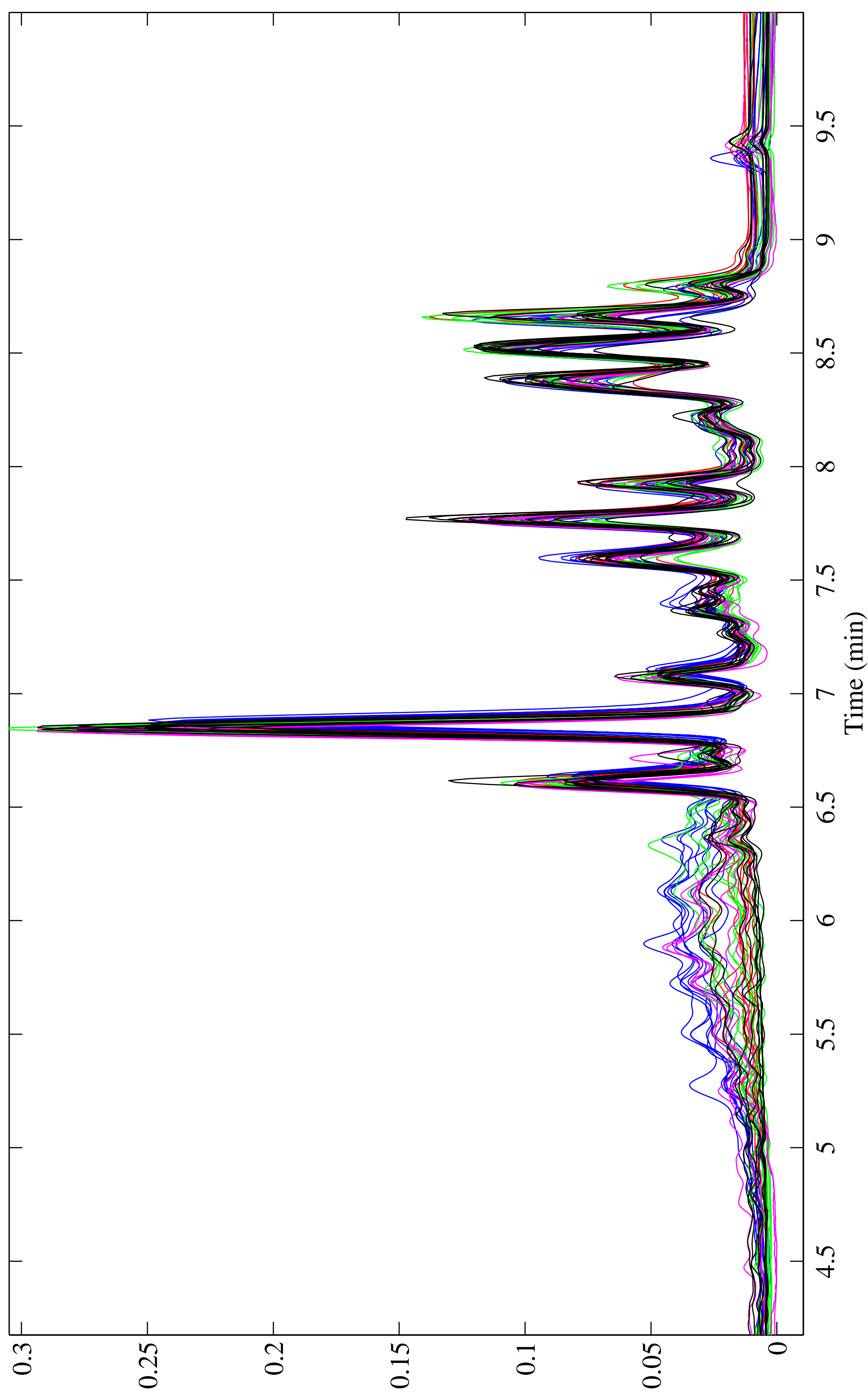


Figure 5. Patterns of 38 *Mycobacterium simiae* samples from 5 laboratories with alignment to the major peaks in the quality control samples (in ART-E units)

Chromatographic Patterns of Mycobacteria

Chromatographic variation within a species is demonstrated in the following chromatograms. The first pattern shown for each species represents the median strain as shown in the manual *Standardized Method for HPLC Identification of Mycobacteria*. However, for assistance in comparing the patterns of different species, each median strain pattern is overlaid with the pattern of *M. intracellulare*, ATCC 13950.

Observations

Some species demonstrate uniformity in both retention time and in relative height of key peaks (*e.g.*, *Mycobacterium gordonae*). Others are uniform only in retention times (*e.g.*, *Mycobacterium avium*) and show considerable variability in relative peak height. These examples appear to express a natural variability within a species.

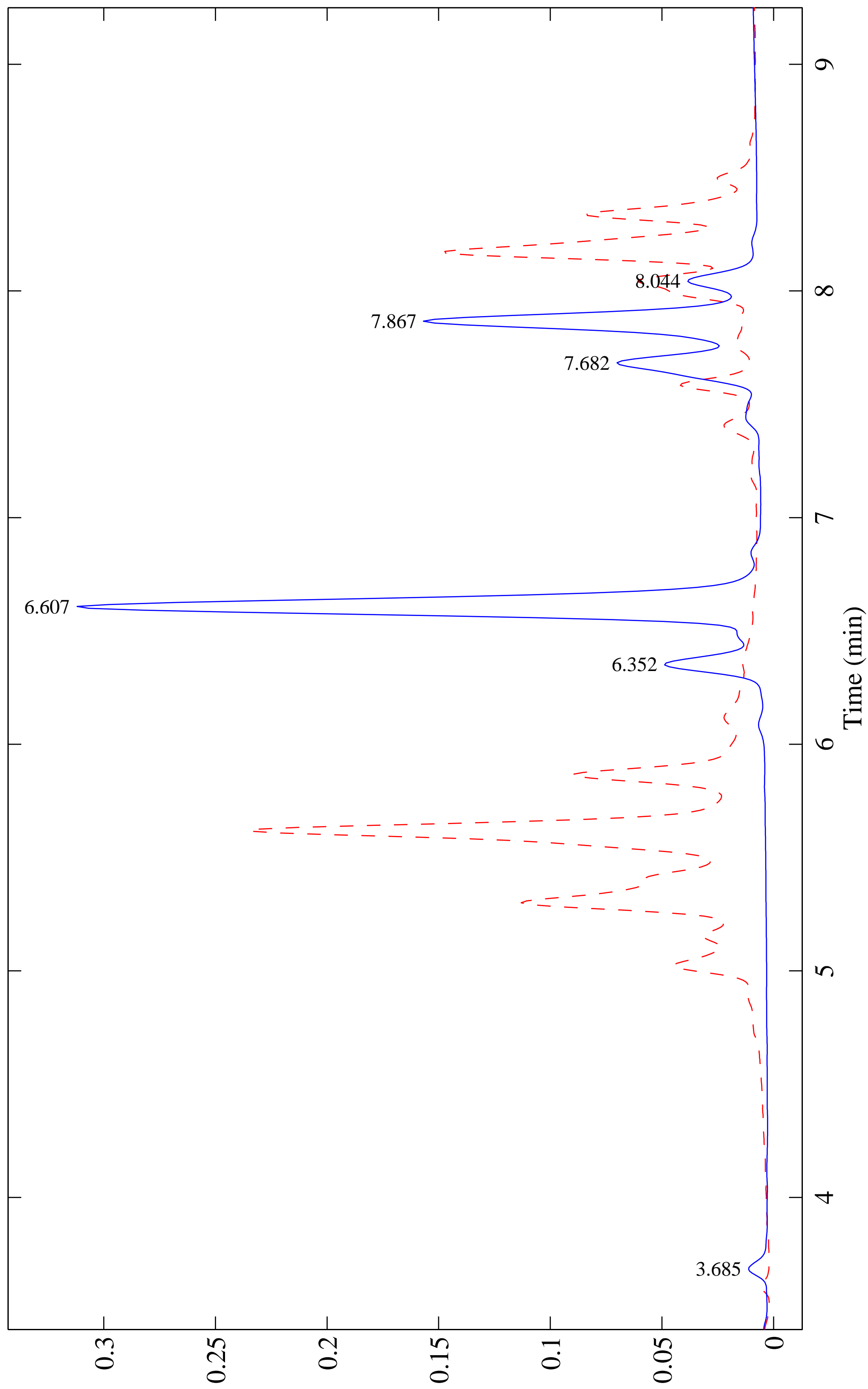


Figure 1. *Mycobacterium abscessus/Mycobacterium chelonae* group

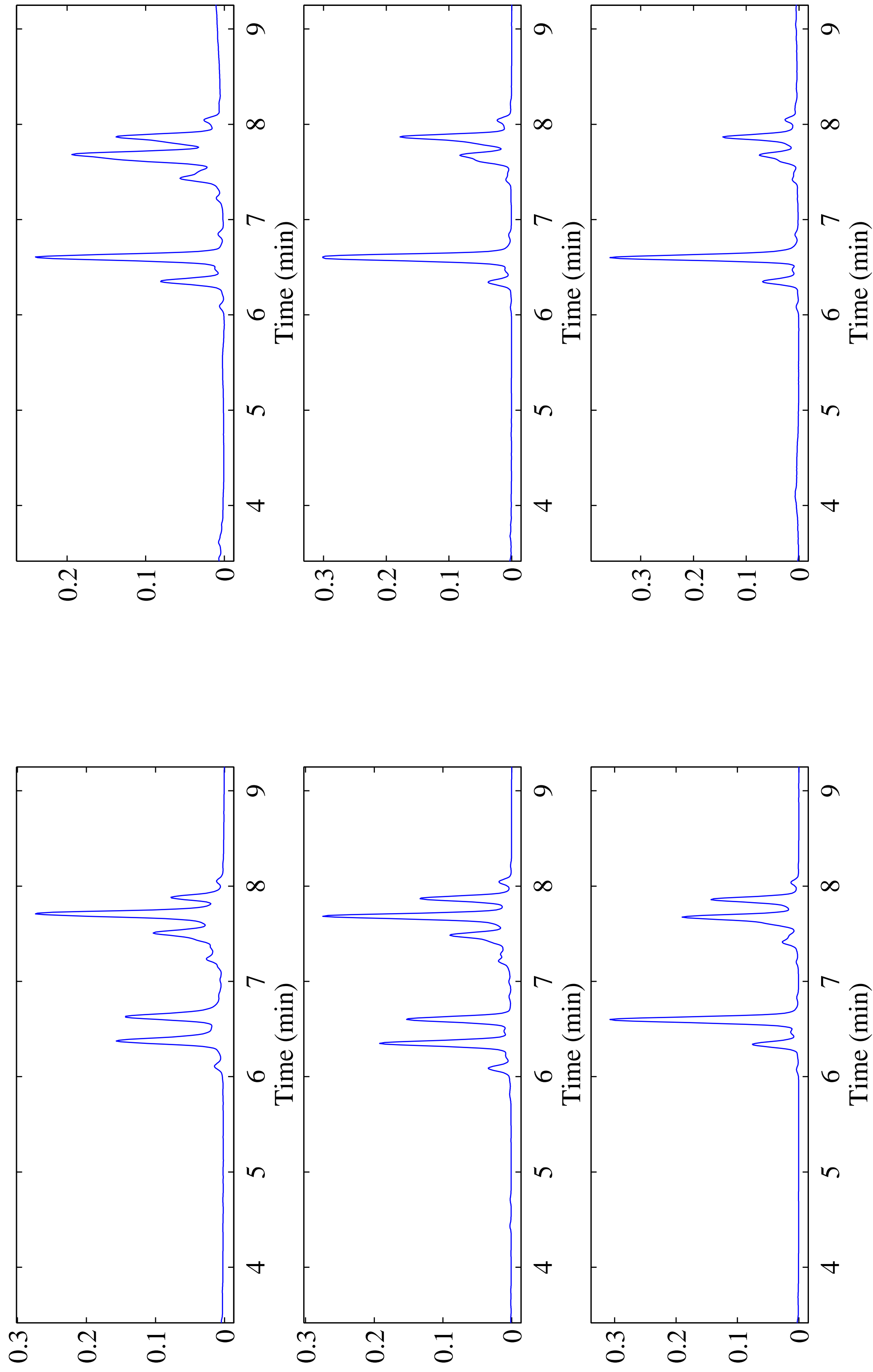


Figure 2. ***Mycobacterium abscessus/Mycobacterium chelonae*** **group variation**

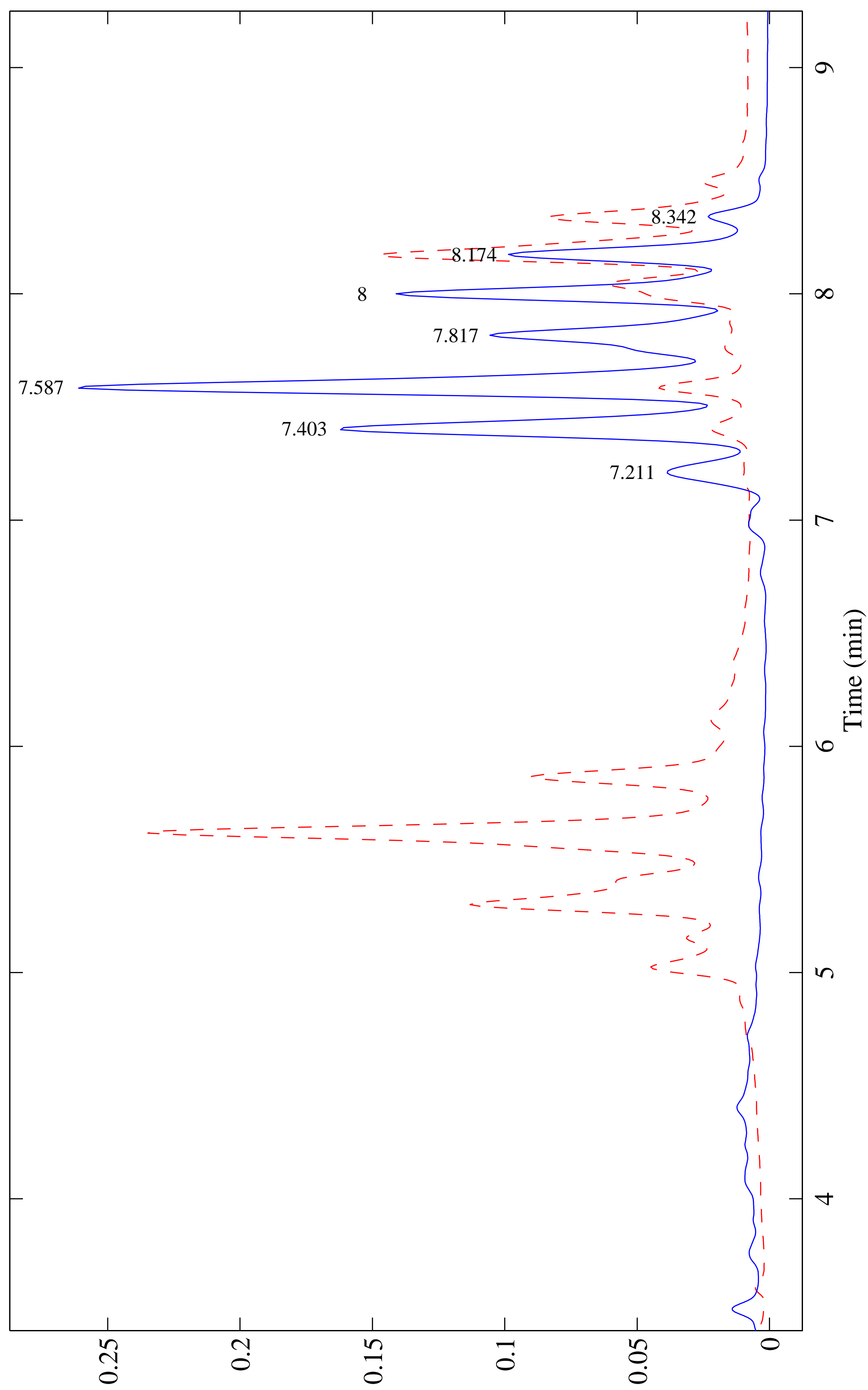


Figure 3. Mycobacterium asiaticum

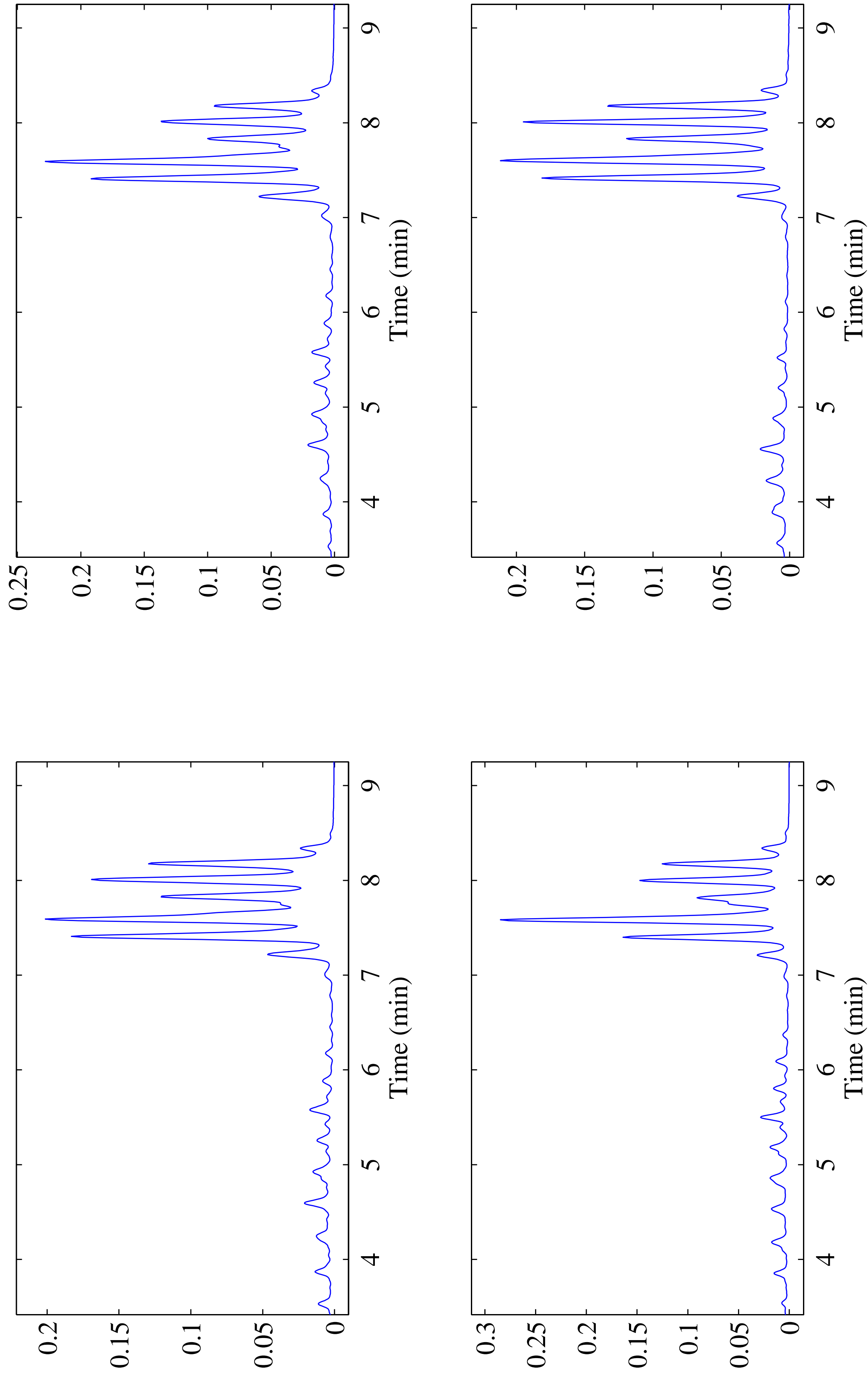


Figure 4. ***Mycobacterium asiaticum*** **variation**

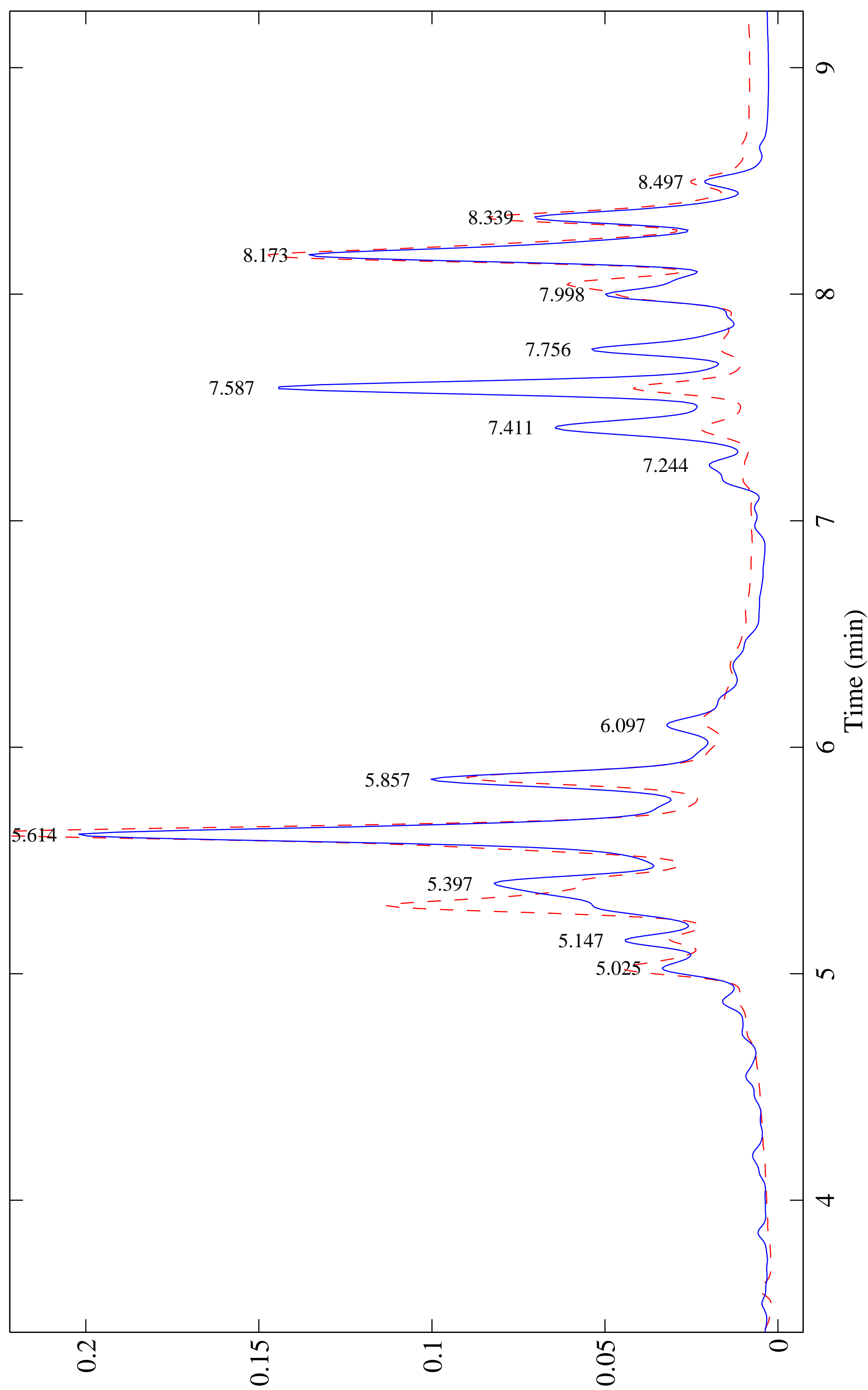


Figure 5. Mycobacterium avium

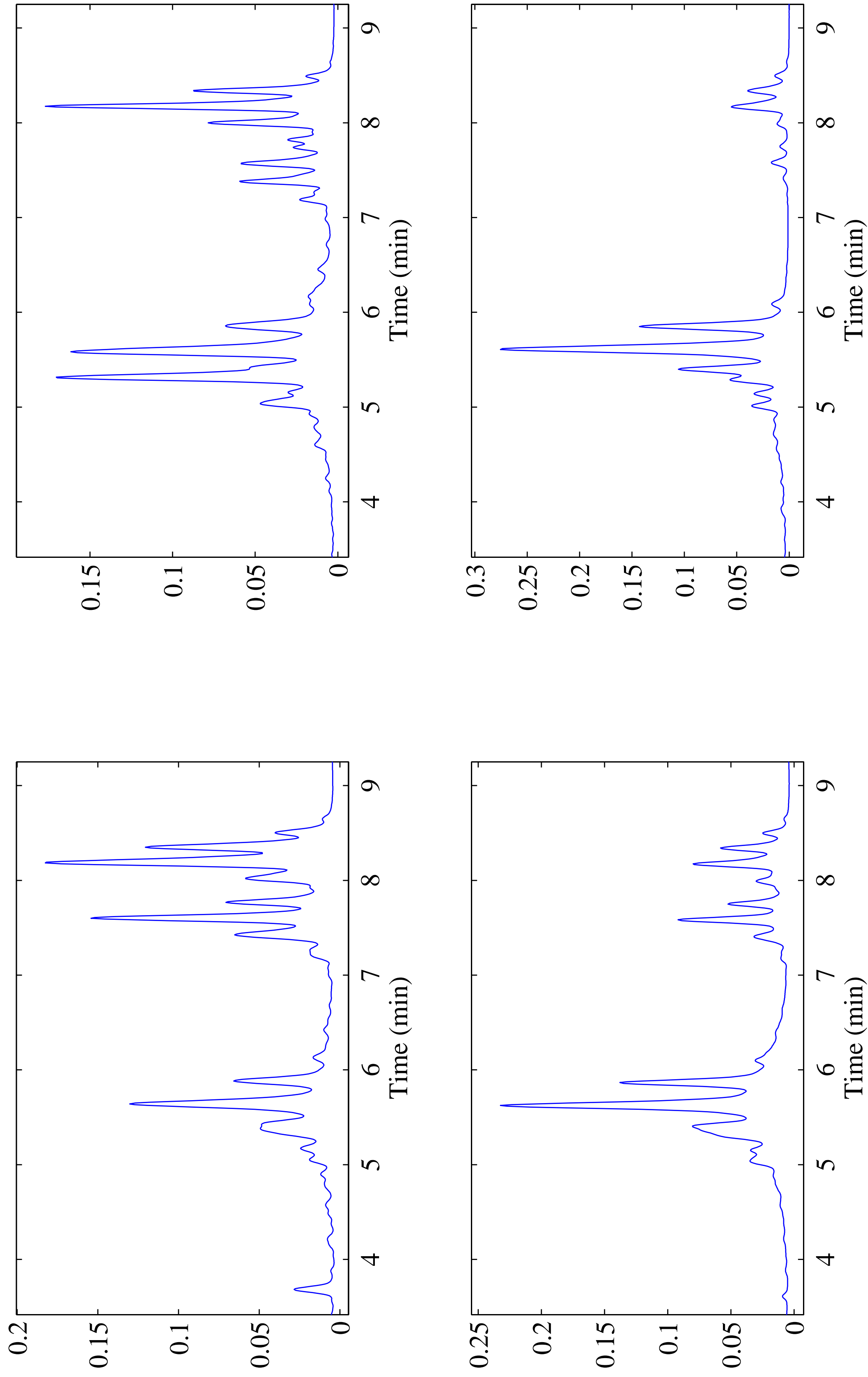


Figure 6. ***Mycobacterium avium*** **variation**

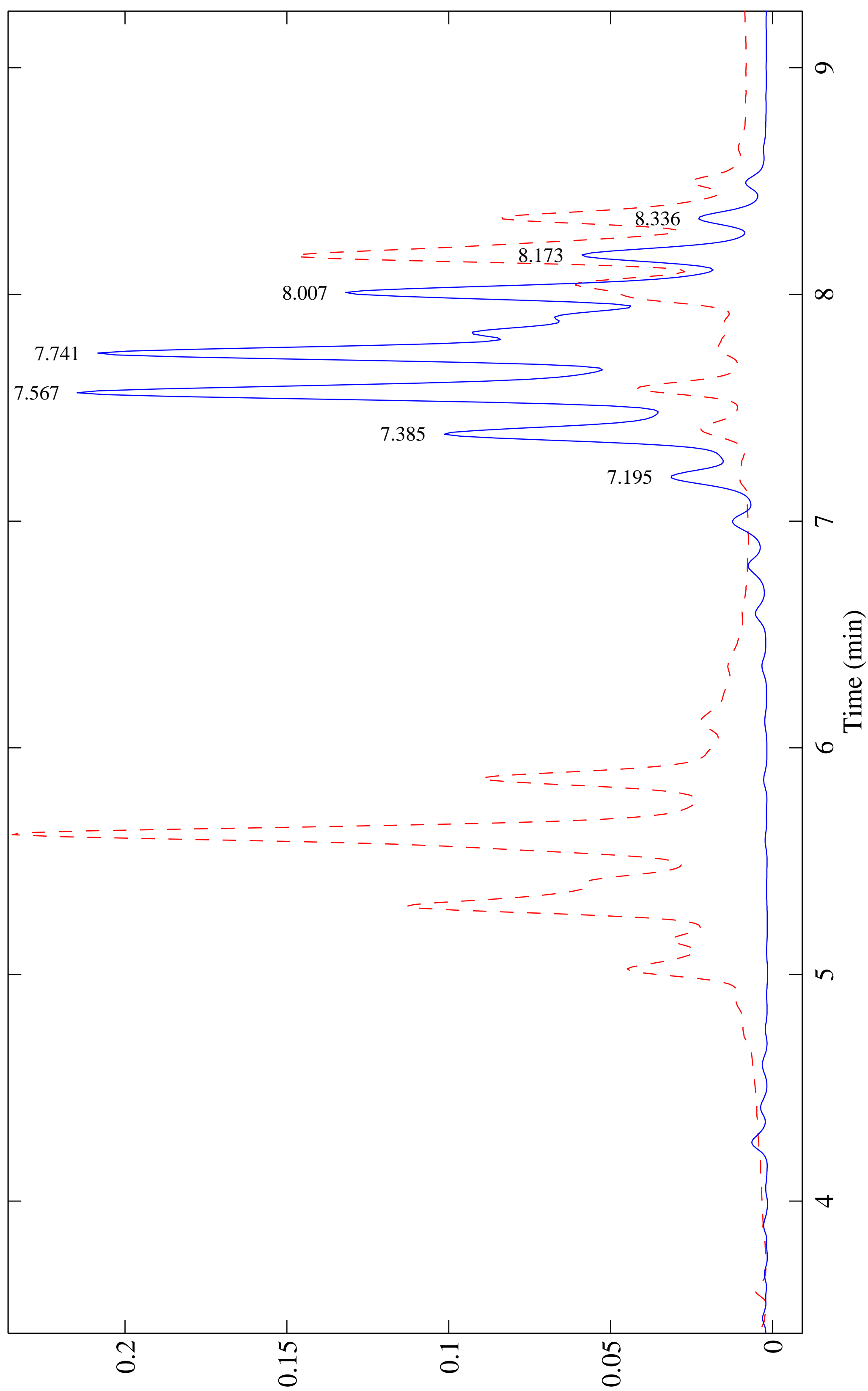


Figure 7. *Mycobacterium bovis* BCG

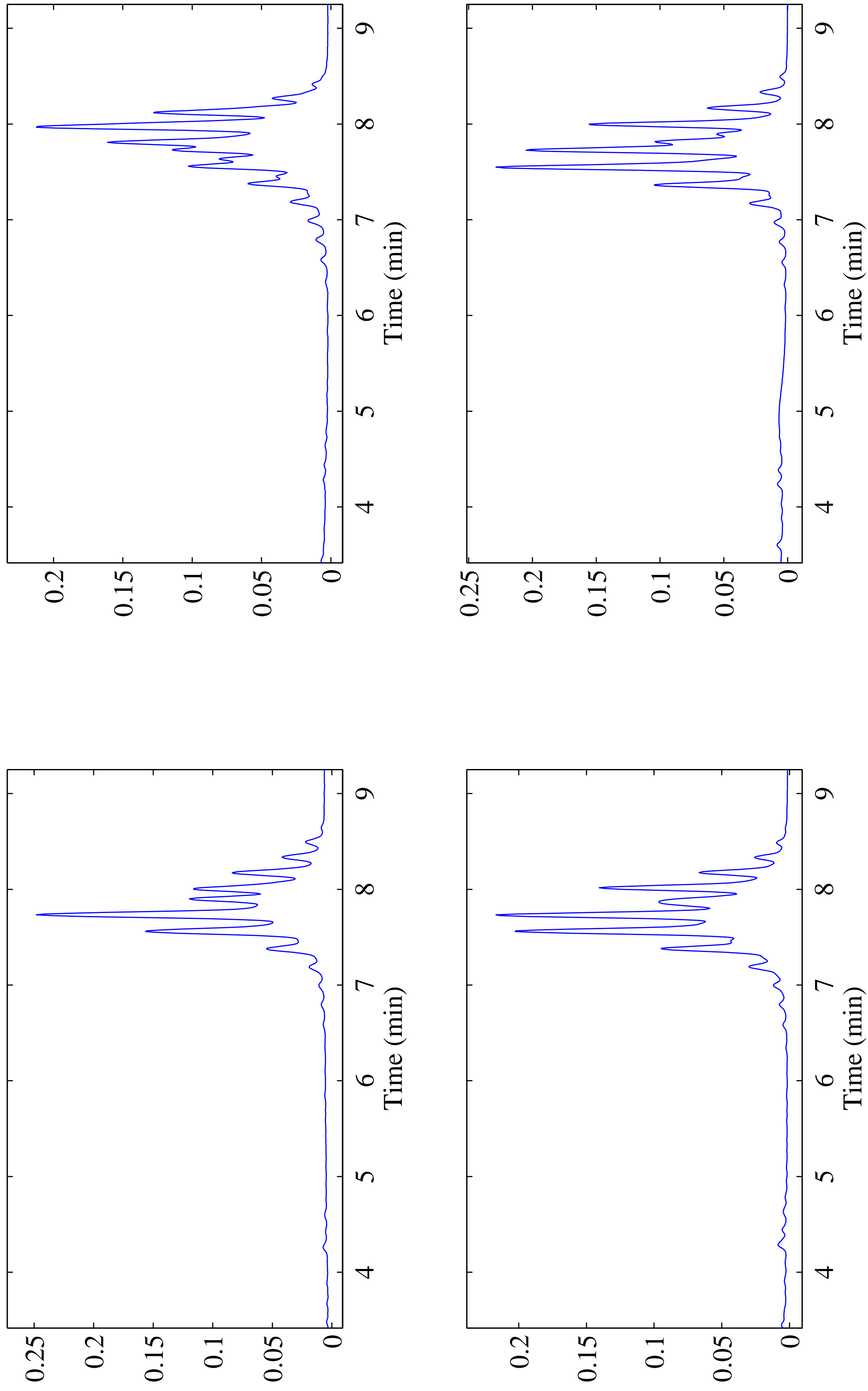


Figure 8. ***Mycobacterium bovis*** **BCG variation**

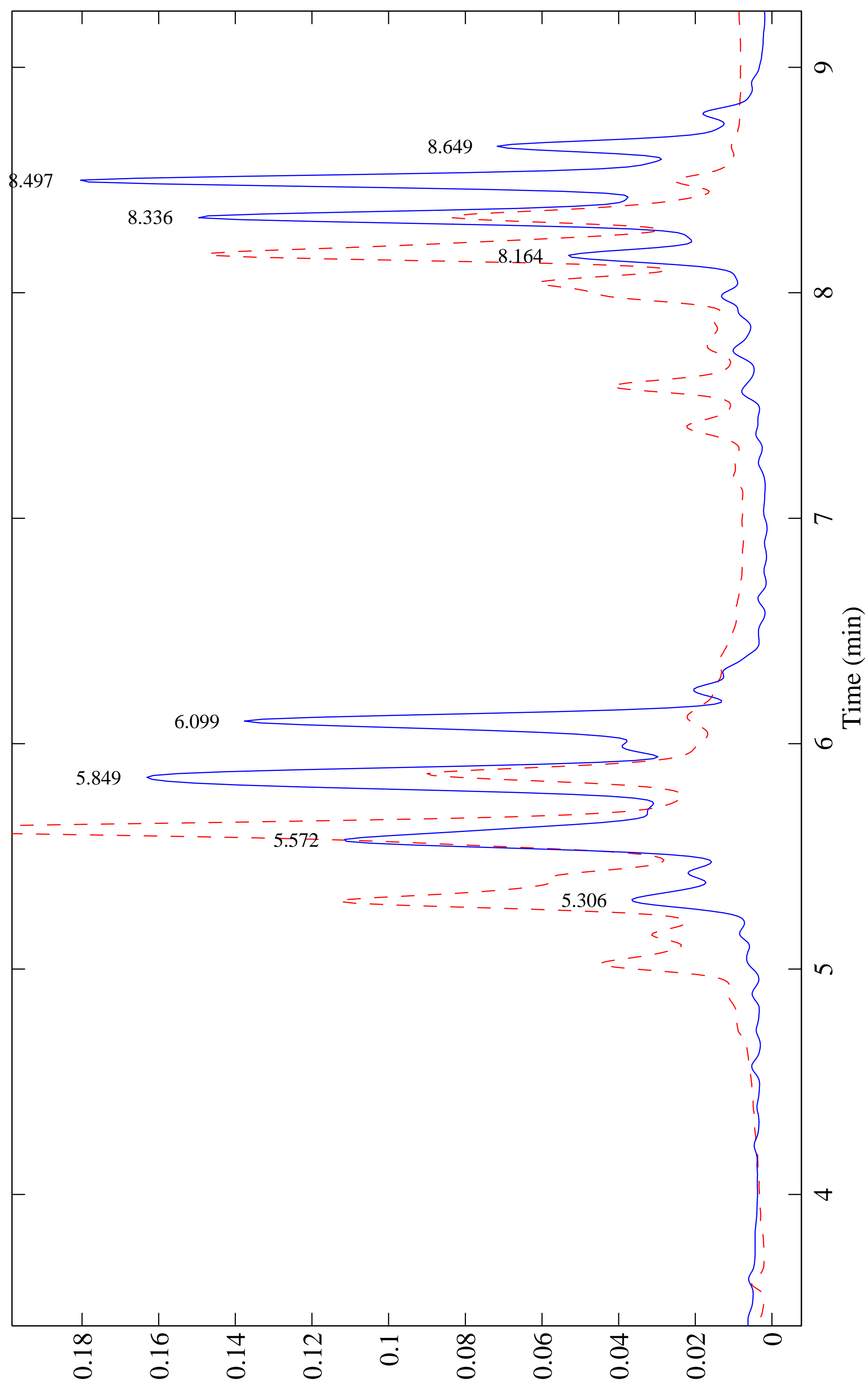


Figure 9. Mycobacterium celatum

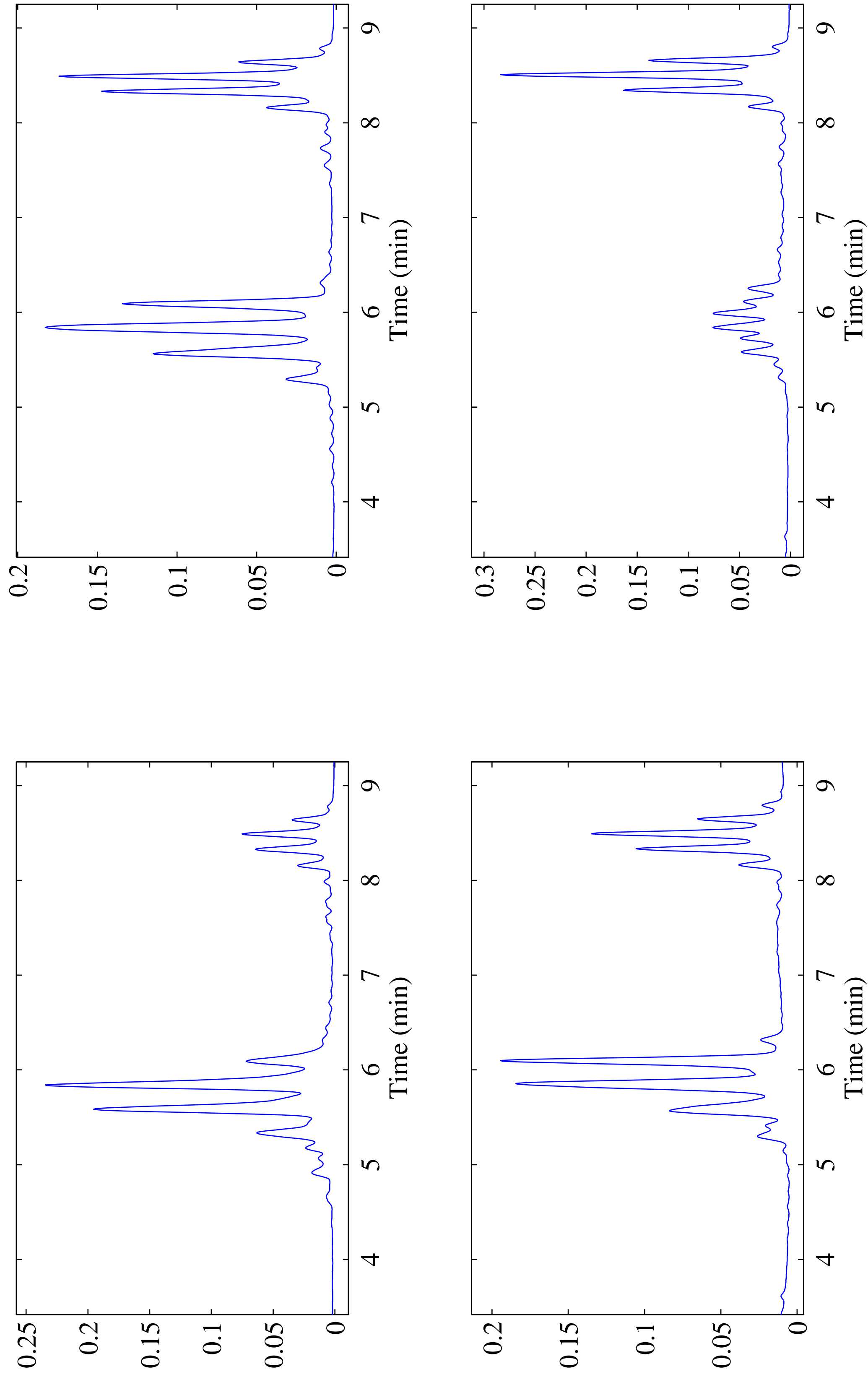


Figure 10. *Mycobacterium celatum* variation

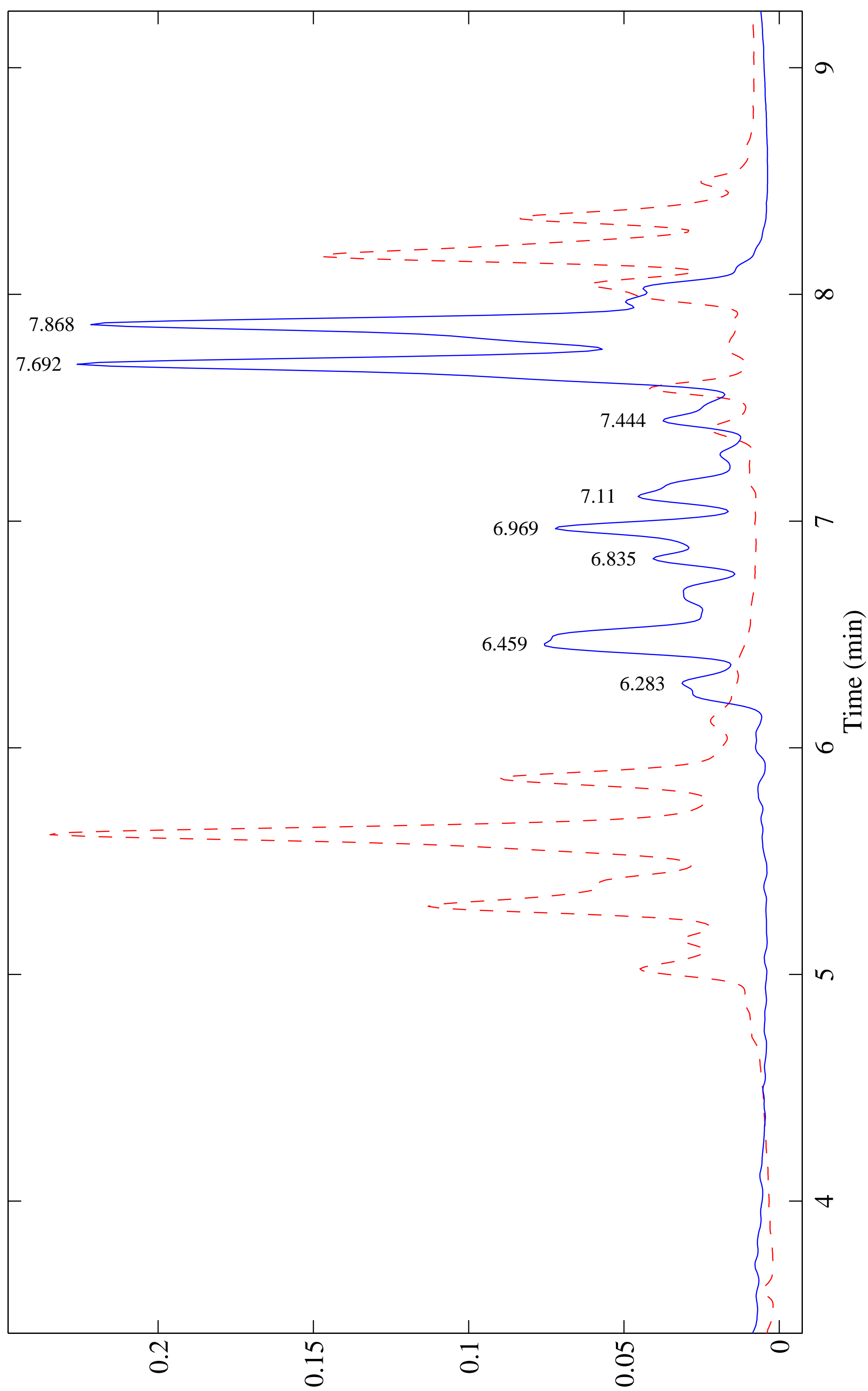


Figure 11. *Mycobacterium fortuitum/Mycobacterium peregrinum* group

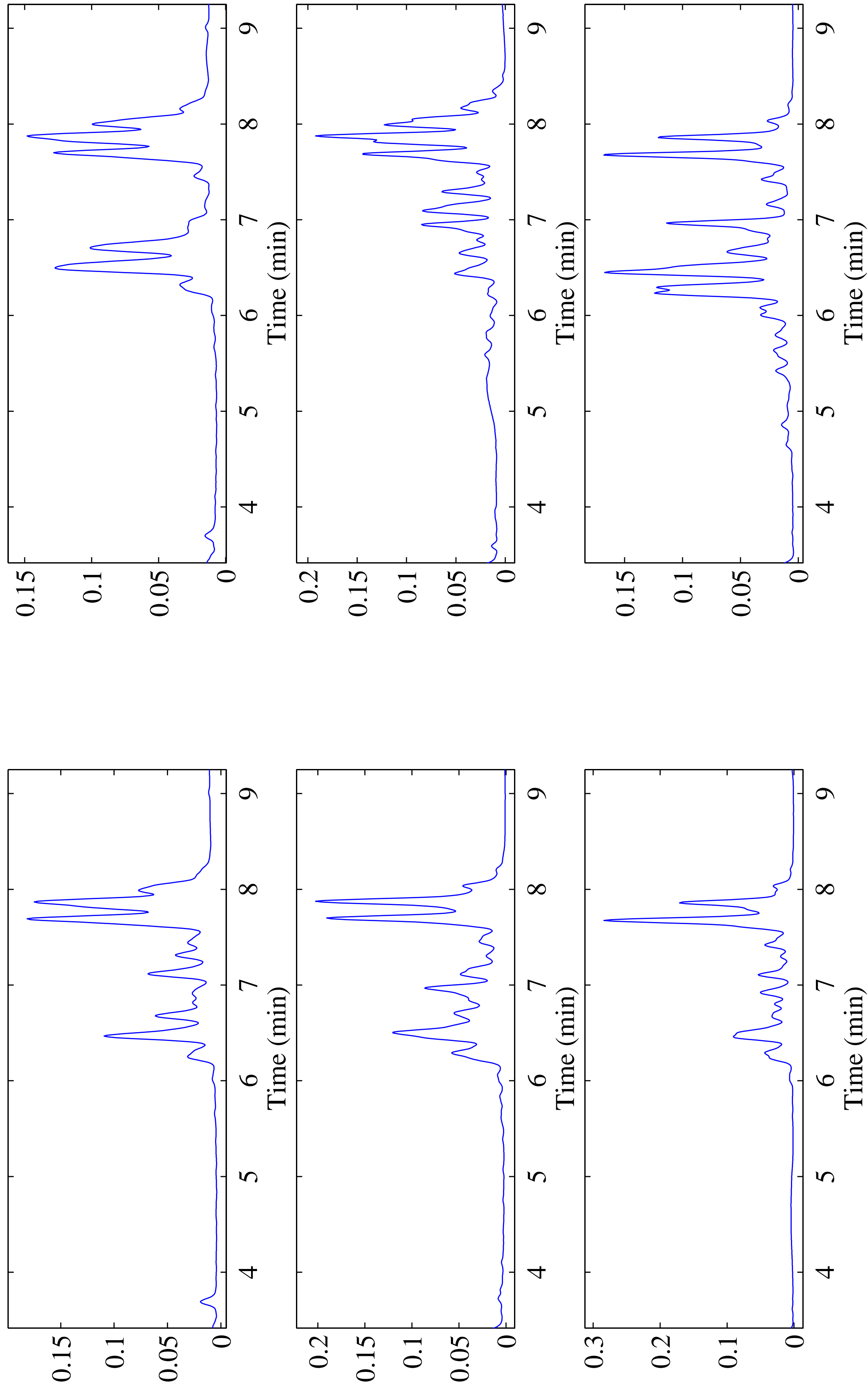


Figure 12. ***Mycobacterium fortuitum/Mycobacterium peregrinum*** **group variation**

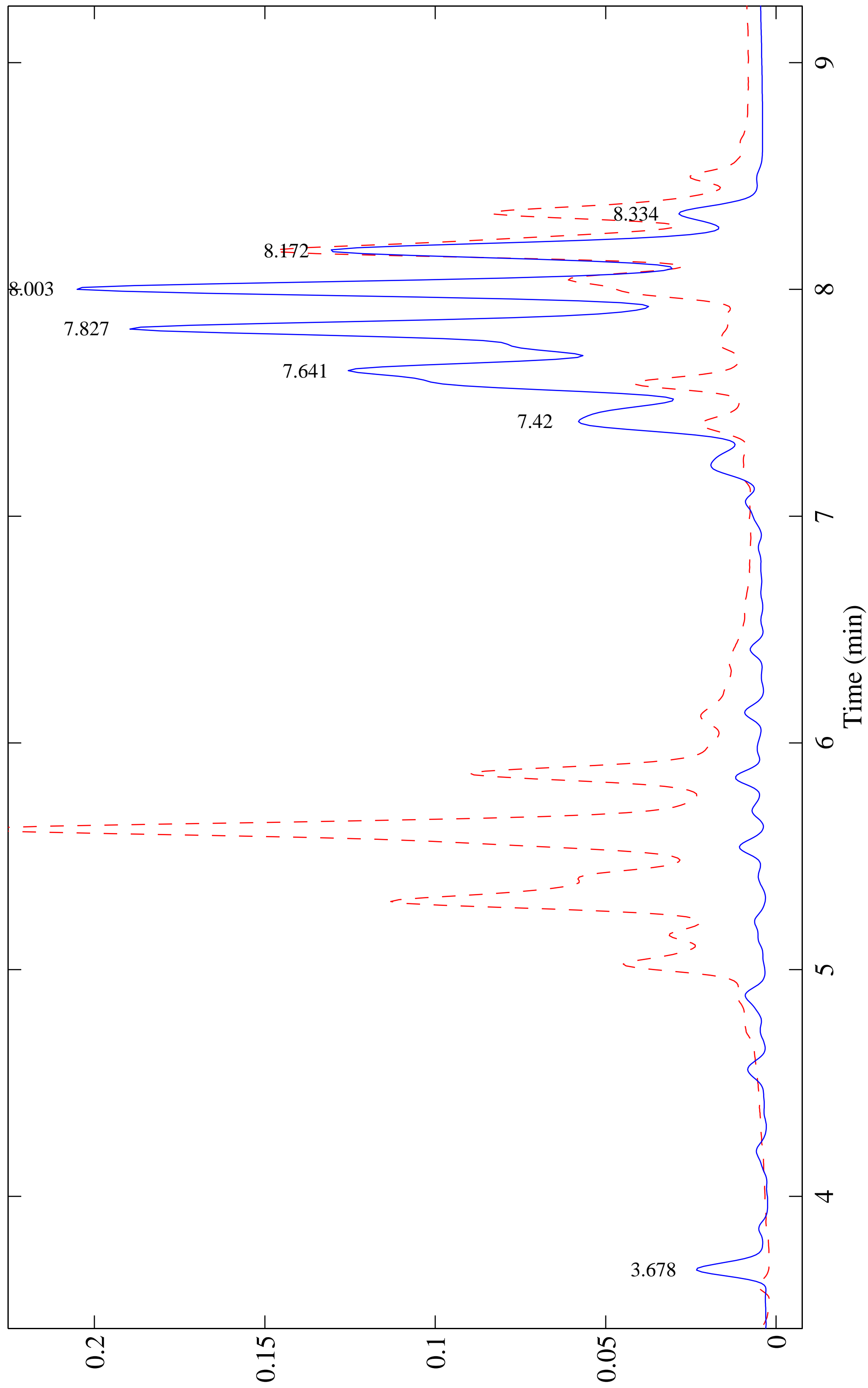
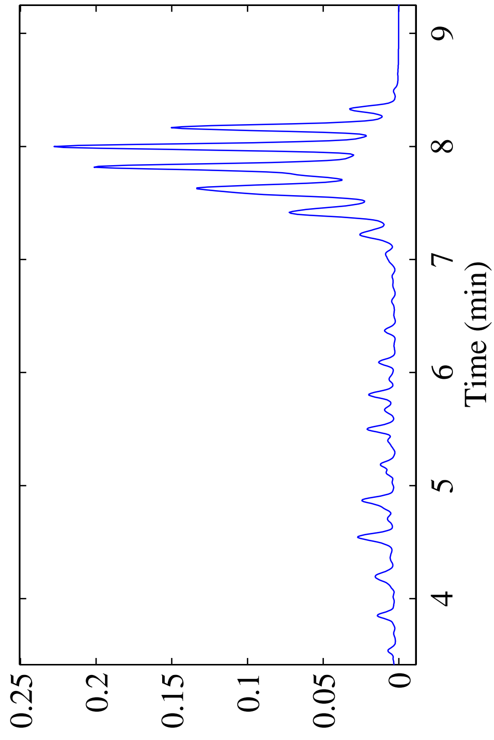
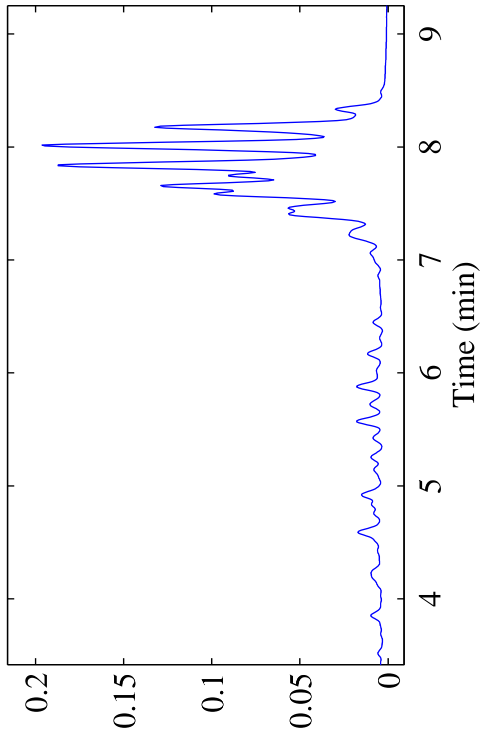


Figure 13. Mycobacterium gastri





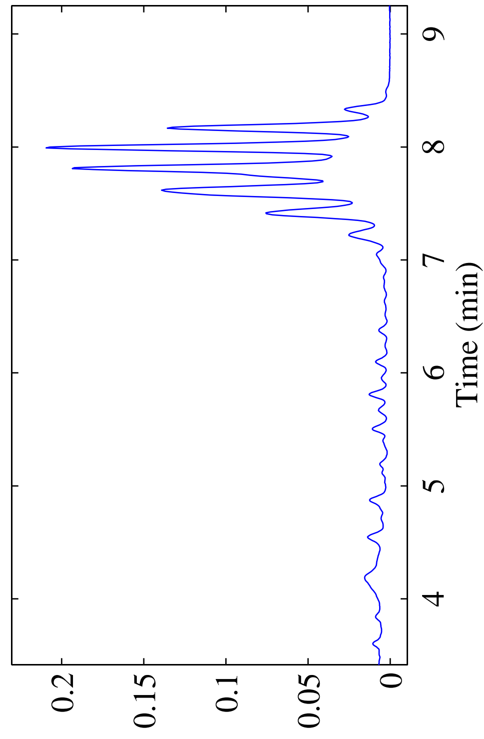


Figure 14. *Mycobacterium gastri* variation

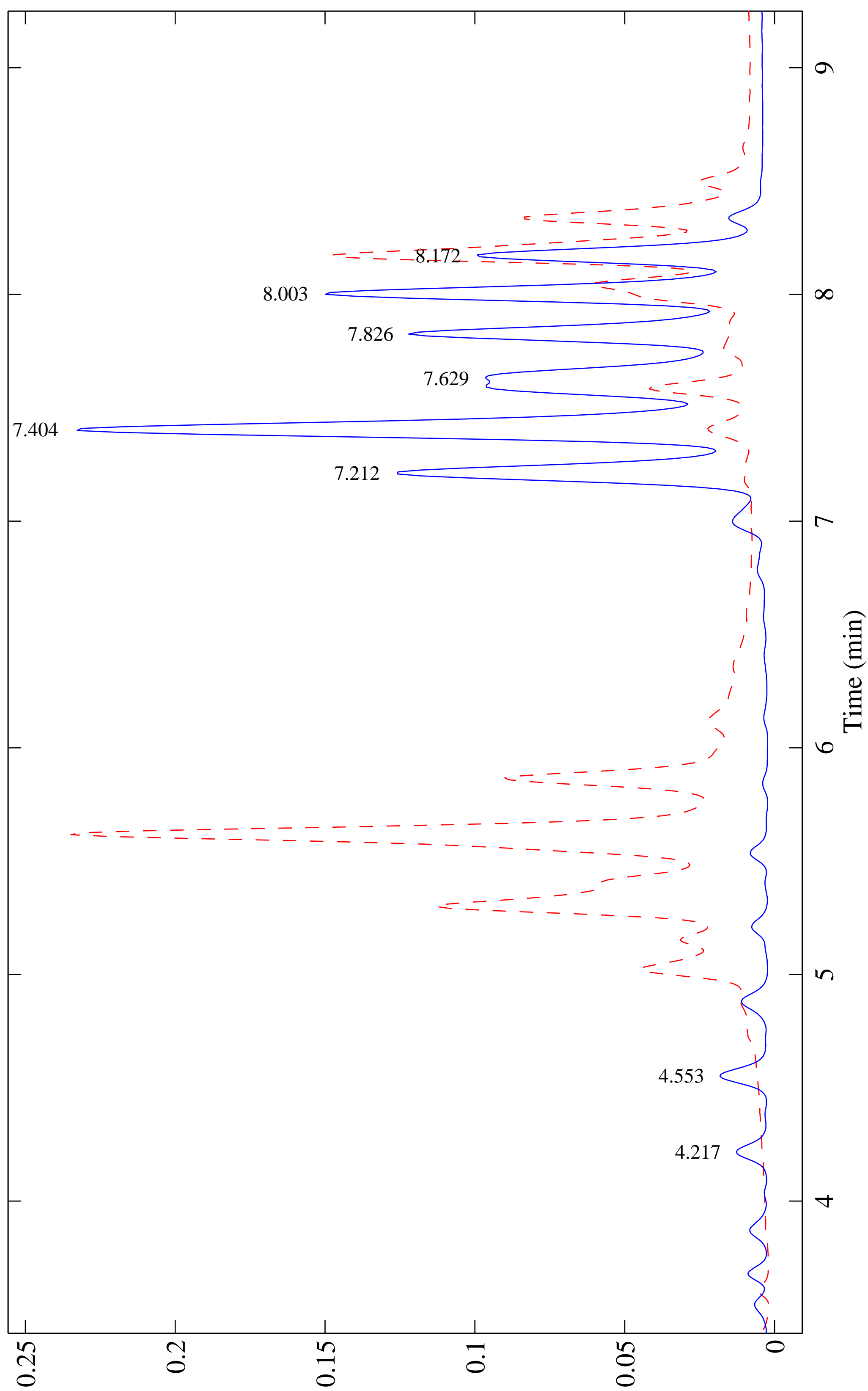


Figure 15. Mycobacterium gordonae

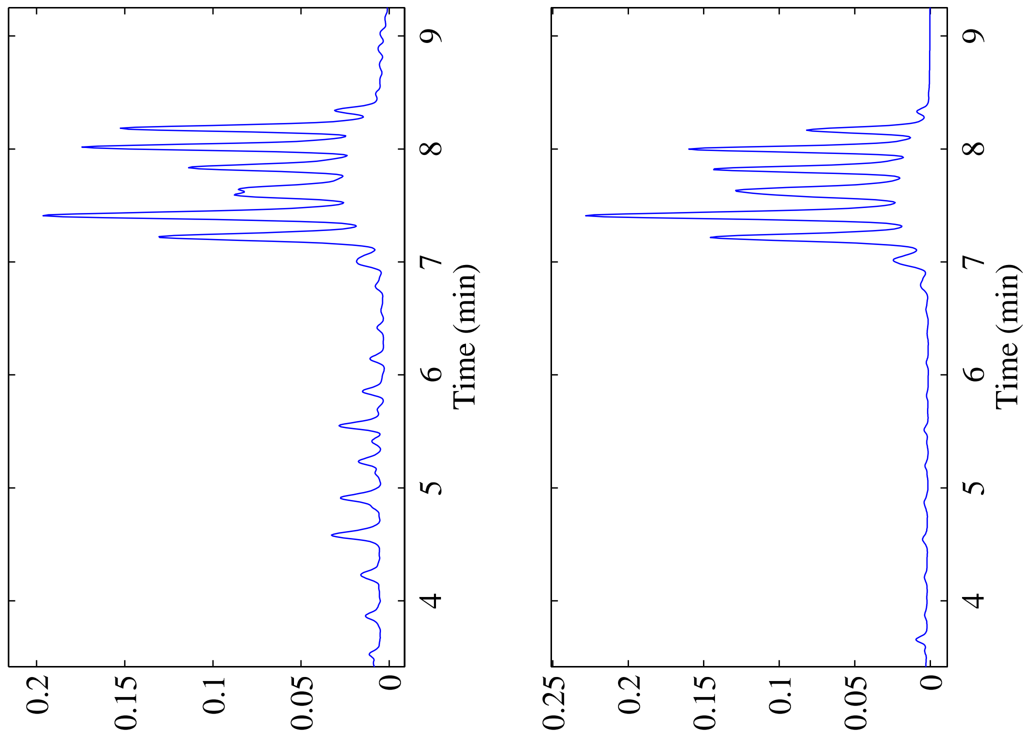


Figure 16. *Mycobacterium gordonae* variation

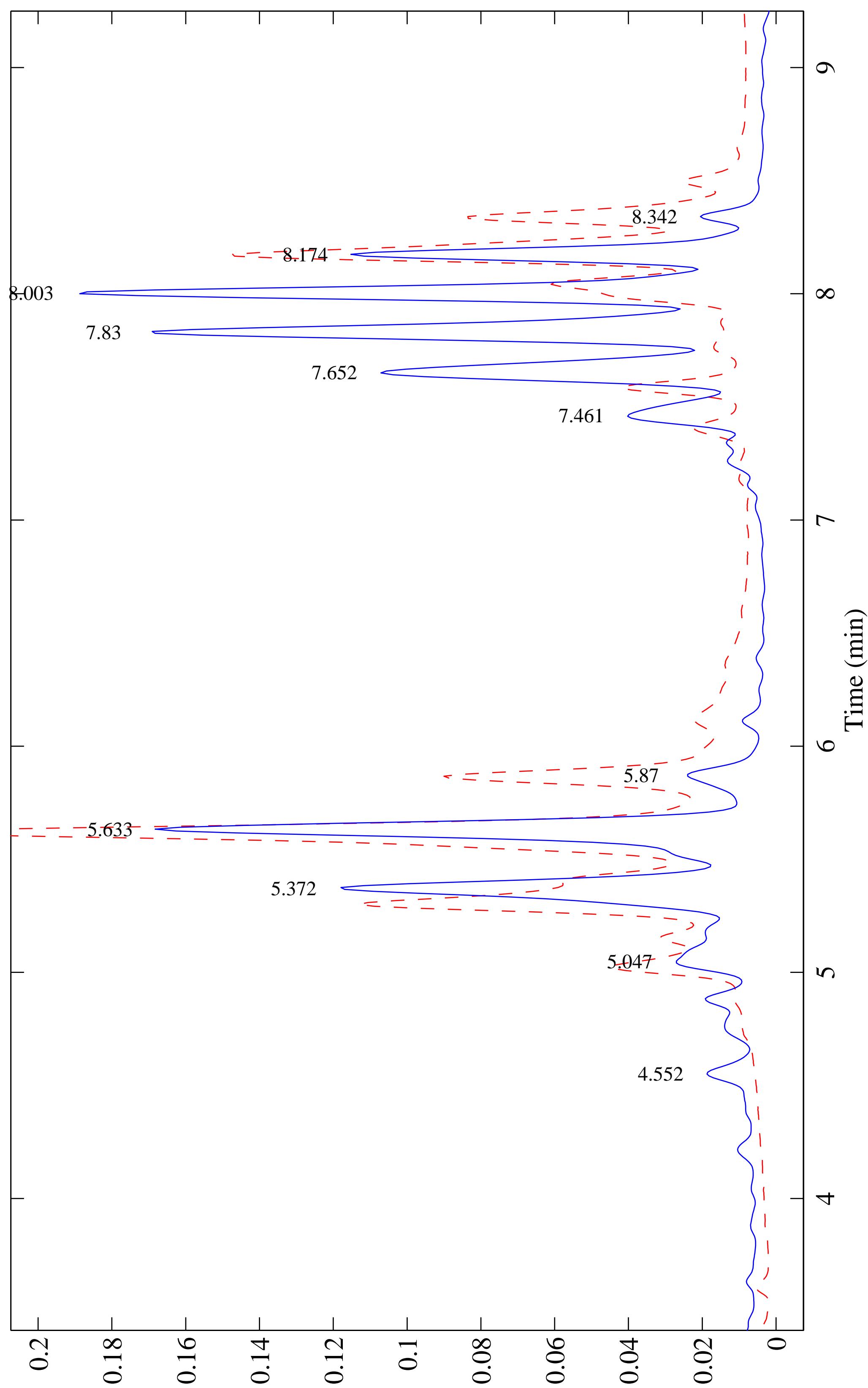


Figure 17. ***Mycobacterium gordonae*** **(Chromatotype II)**

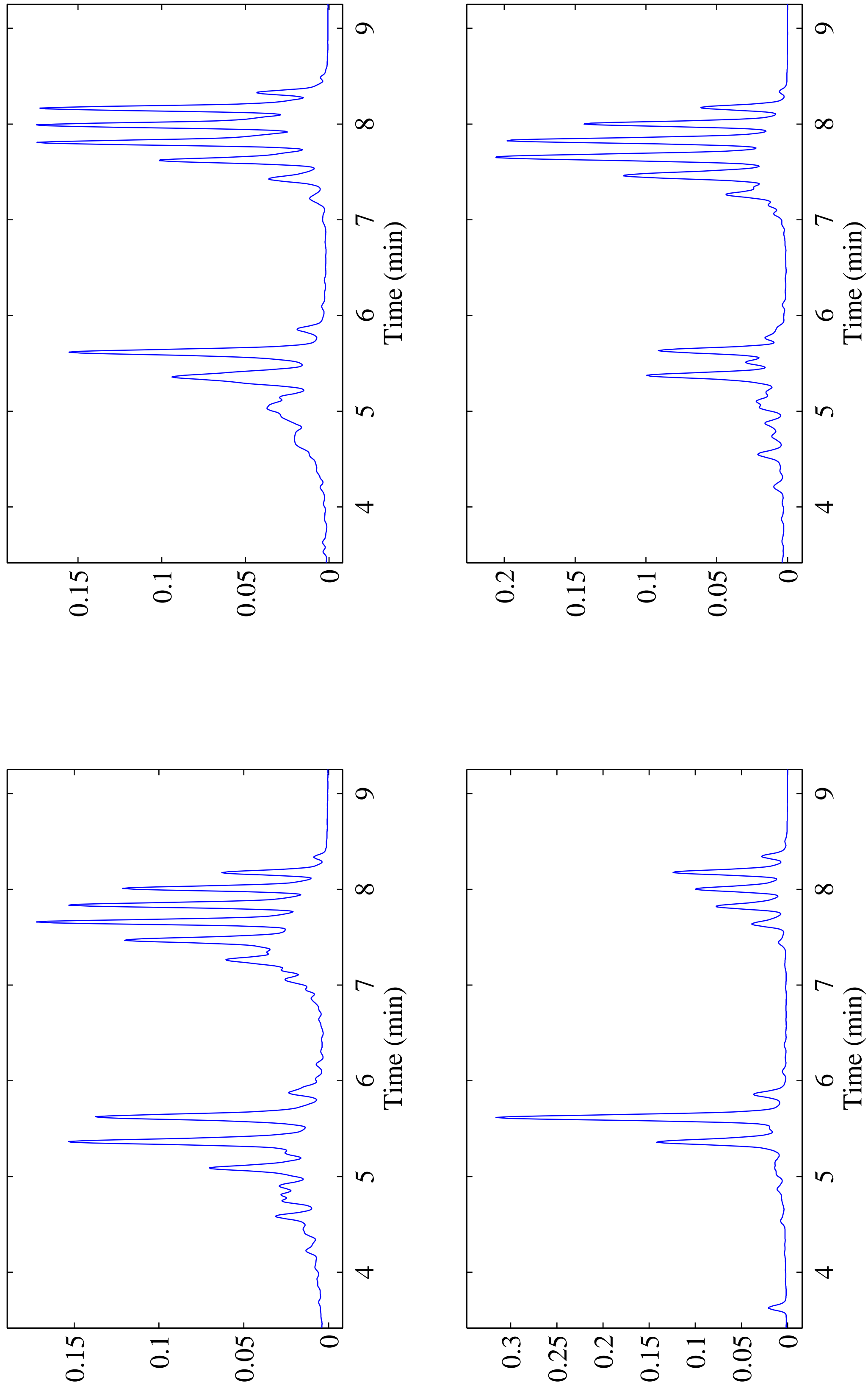


Figure 18. ***Mycobacterium gordonae*** **(Chromatotype II) variation**

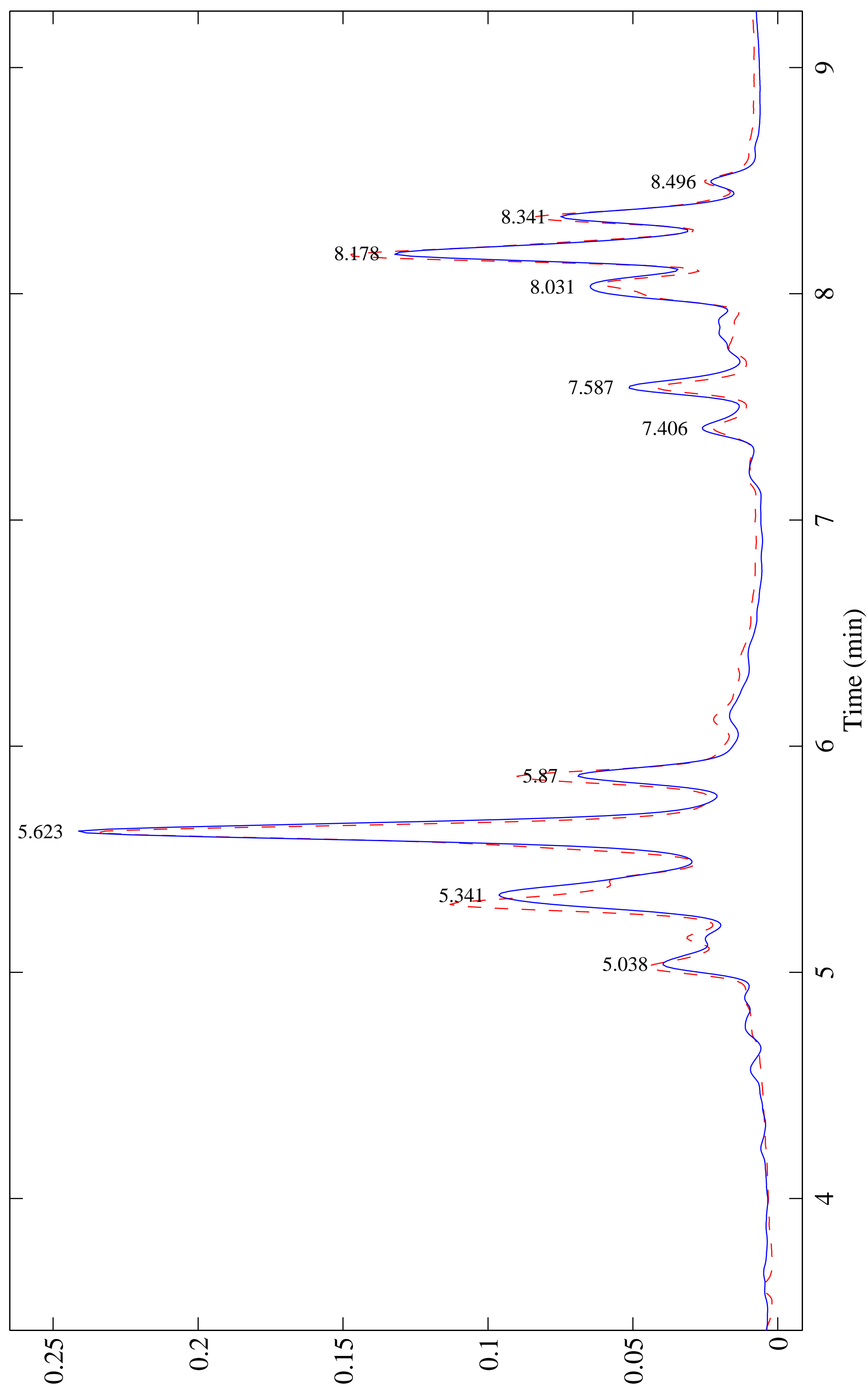


Figure 19. Mycobacterium intracellulare

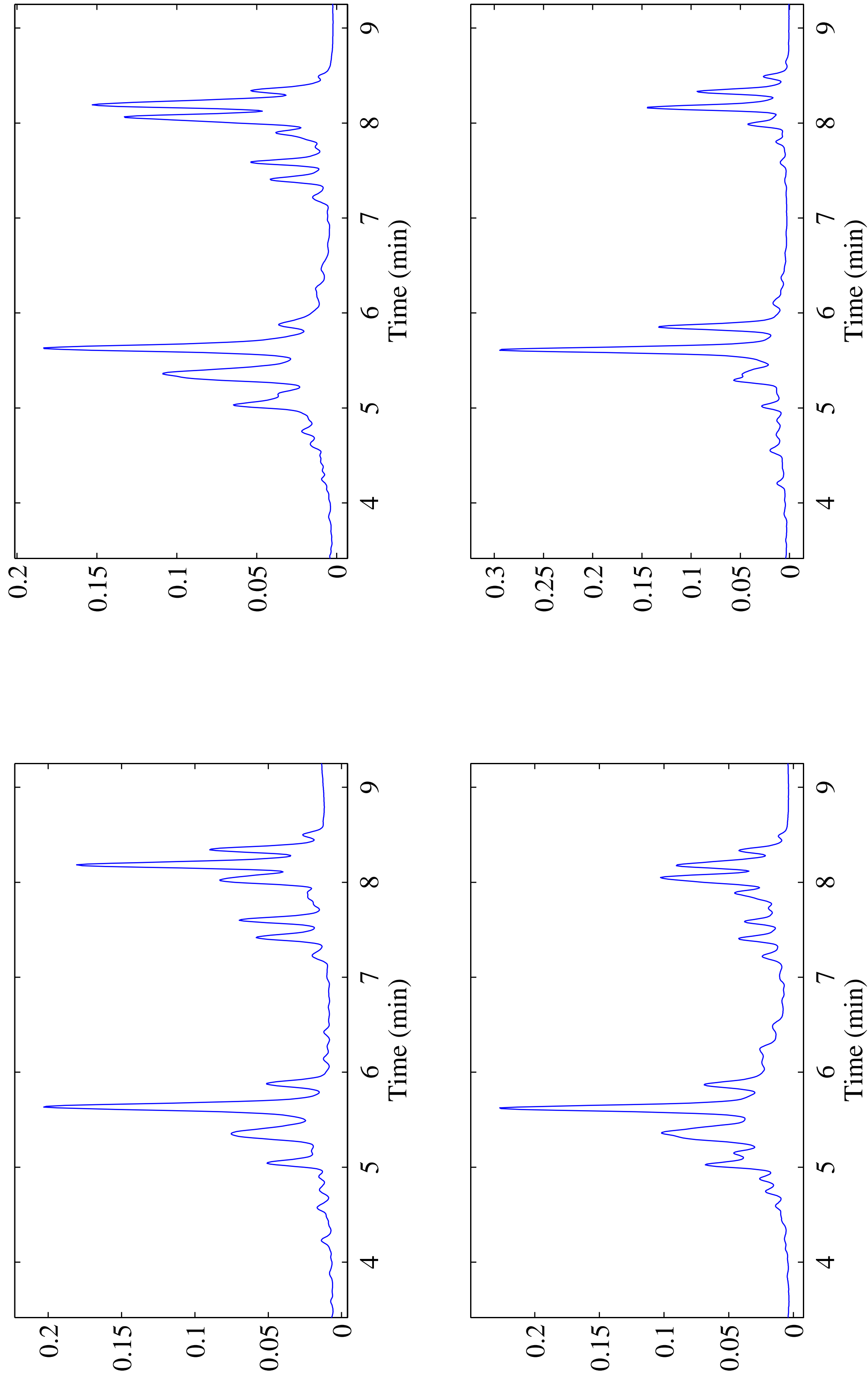


Figure 20. *Mycobacterium intracellulare* variation

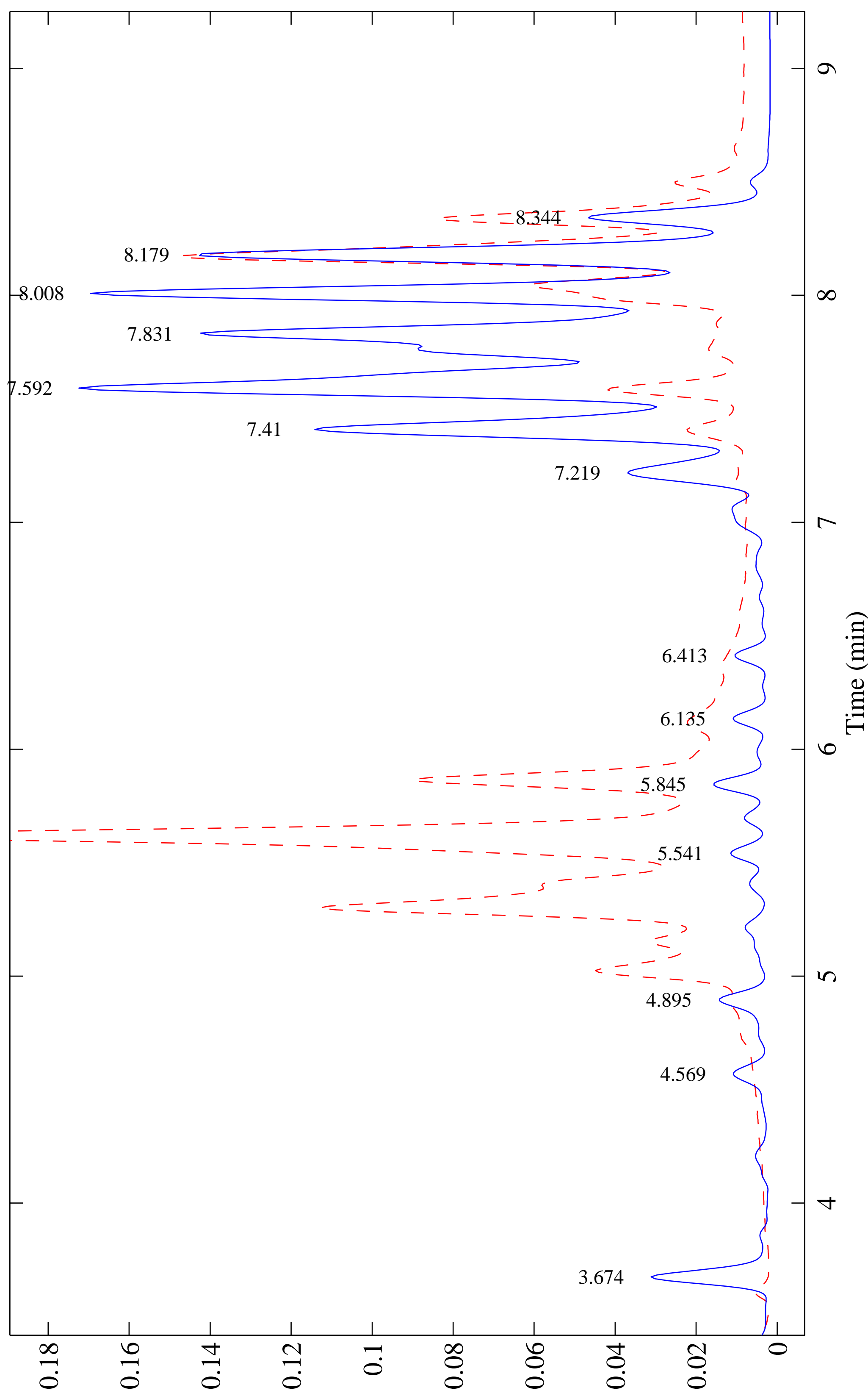


Figure 21. Mycobacterium kansasii

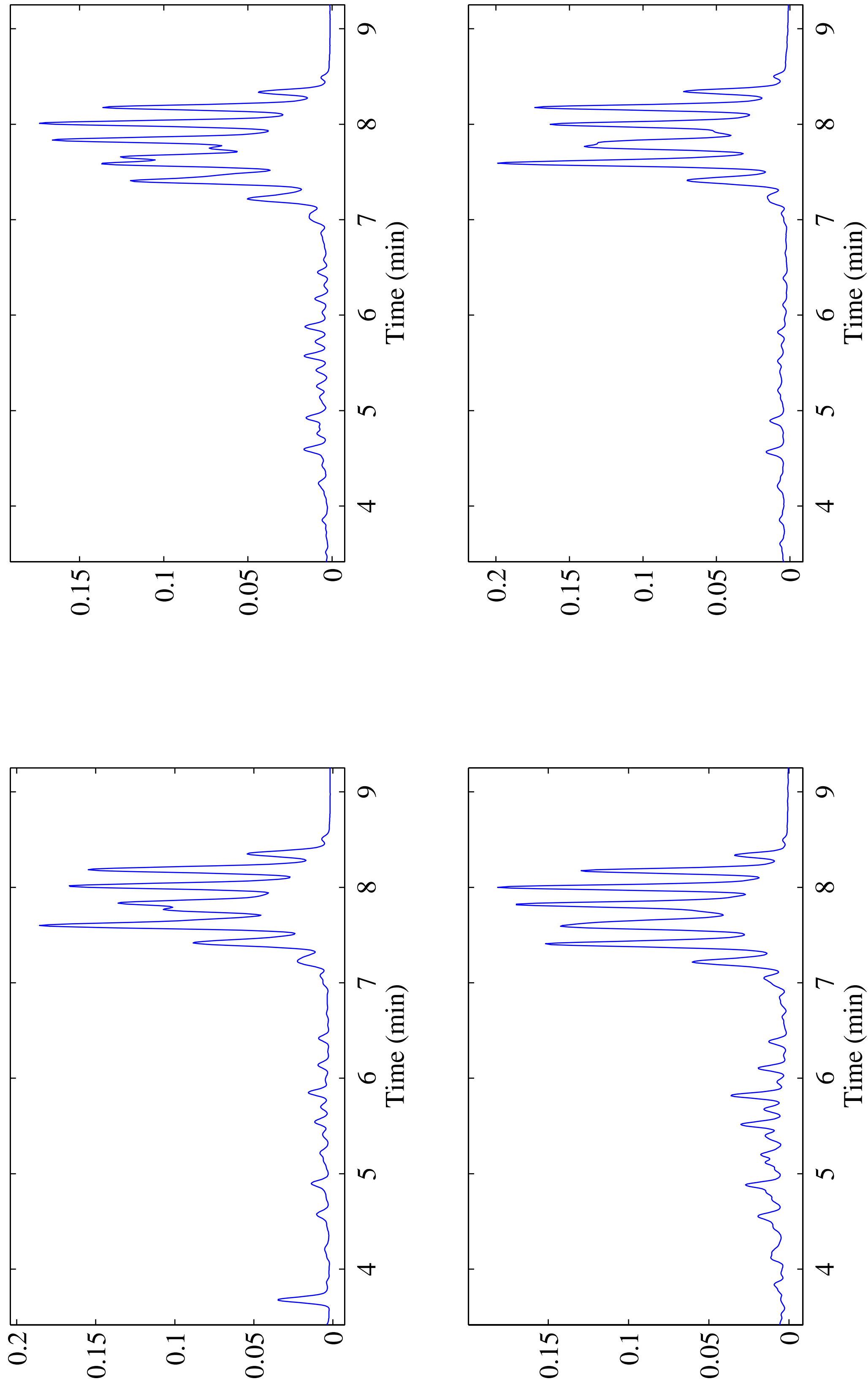


Figure 22. *Mycobacterium kansasii* variation

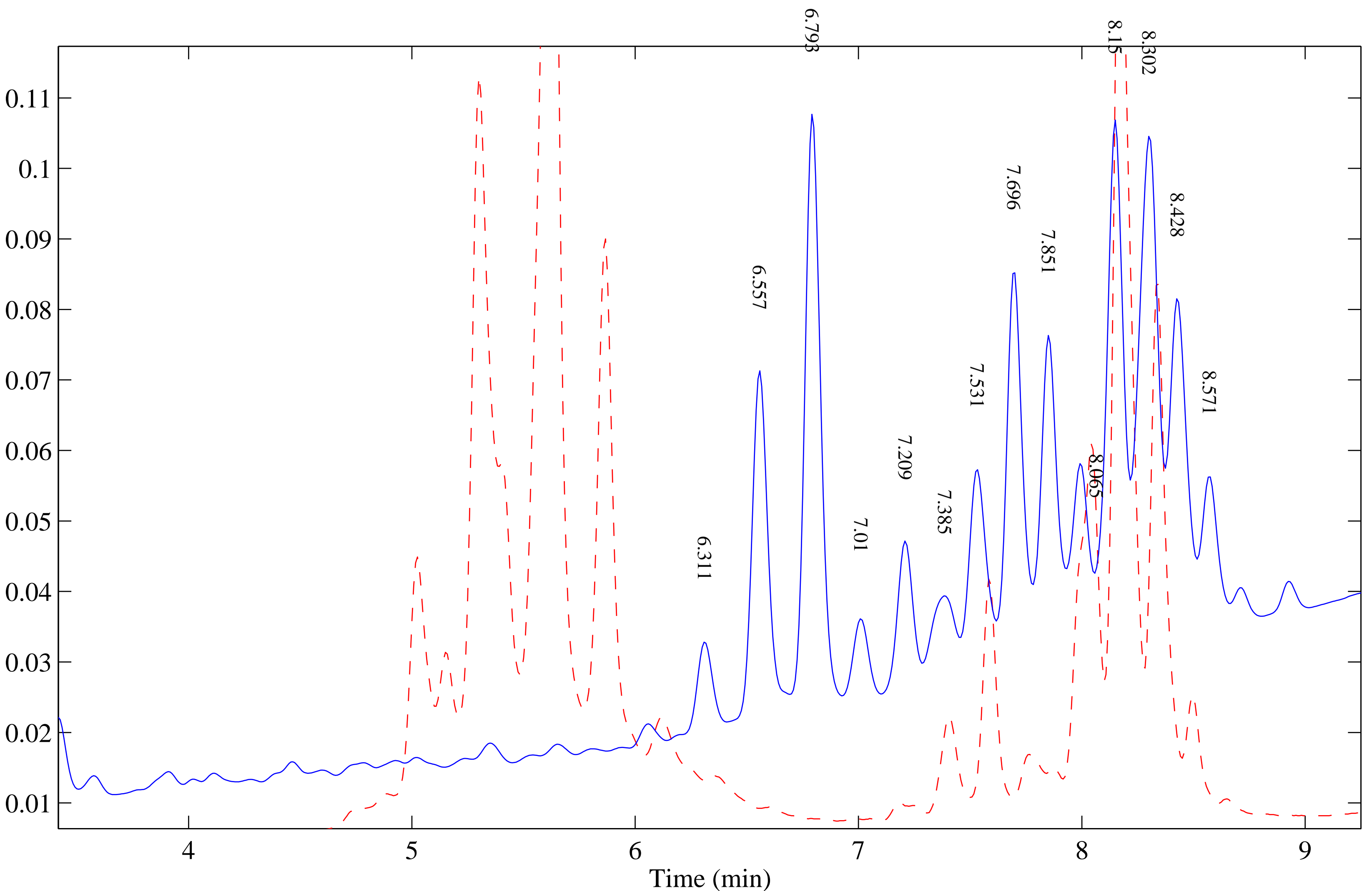


Figure 23. Mycobacterium malmoense

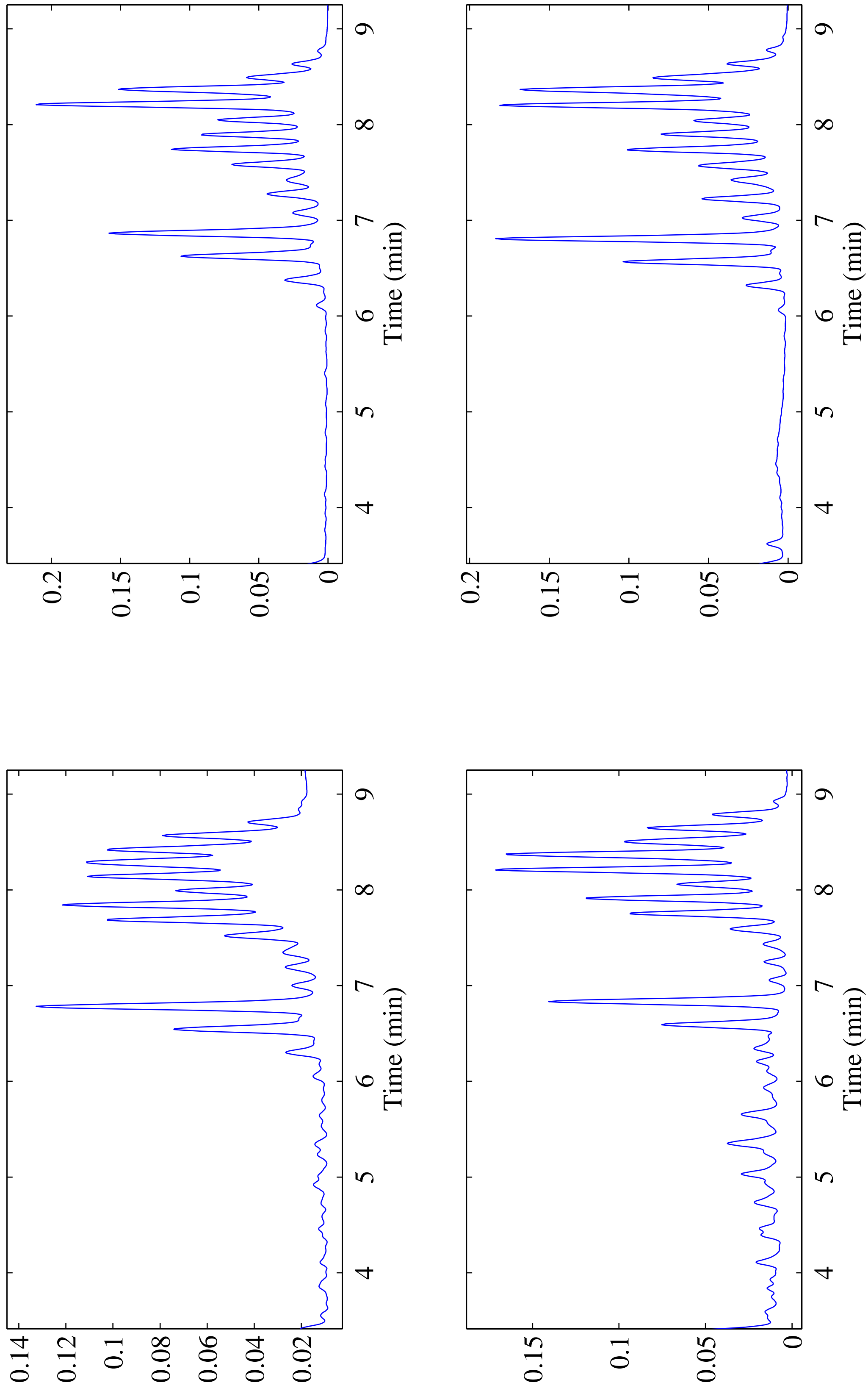


Figure 24. *Mycobacterium malmoense* variation

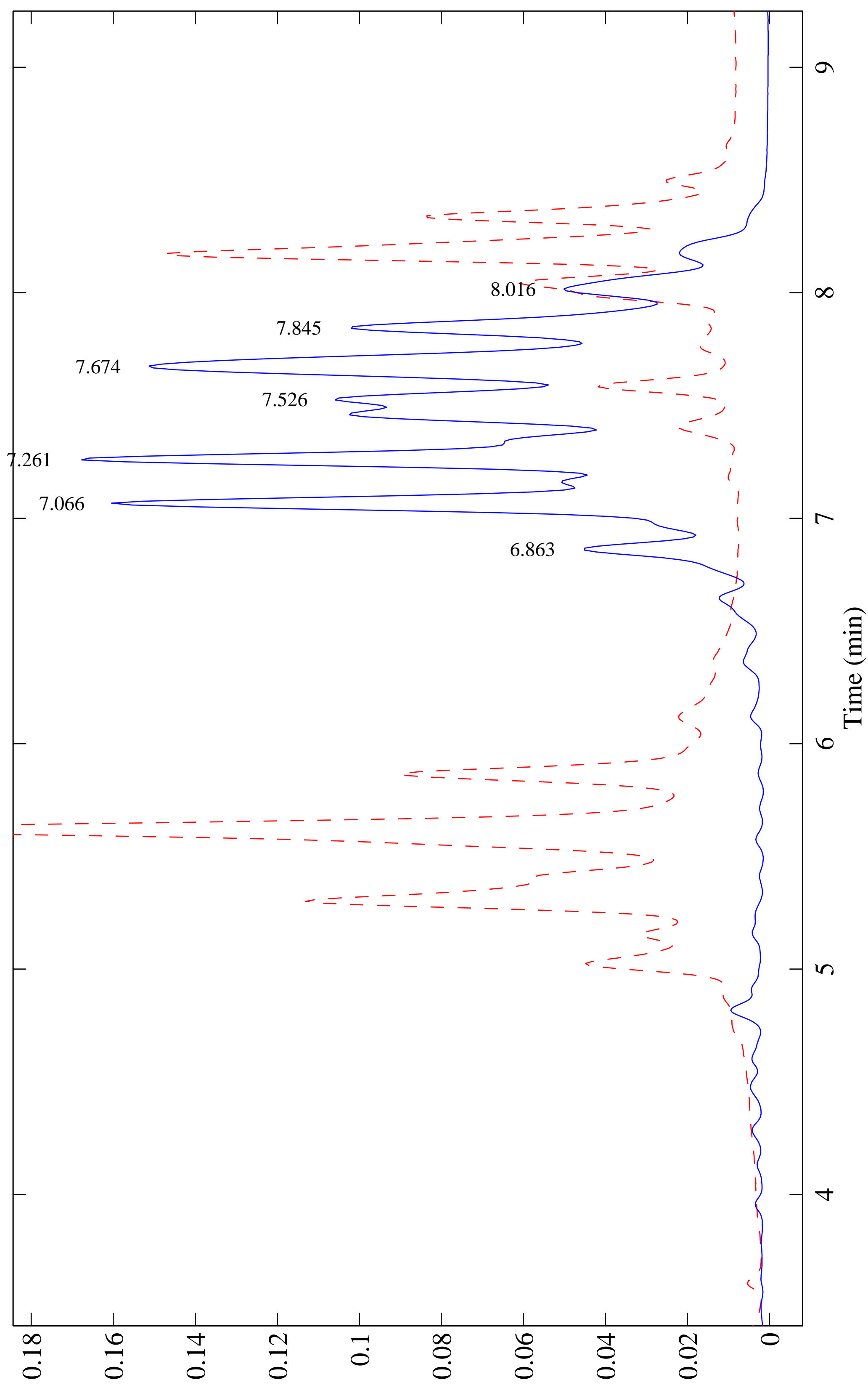


Figure 25. Mycobacterium marinum

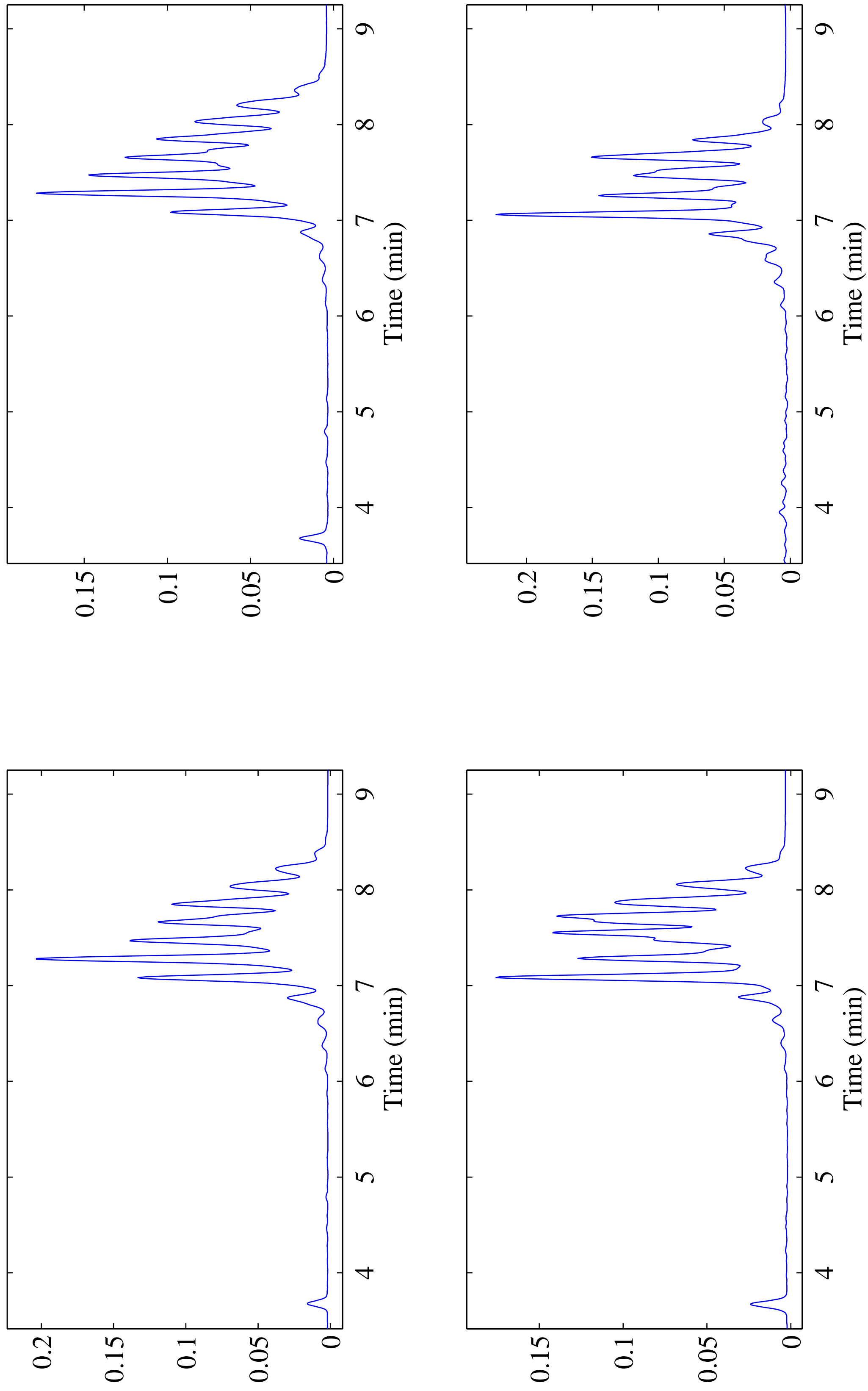


Figure 26. ***Mycobacterium marinum*** **variation**

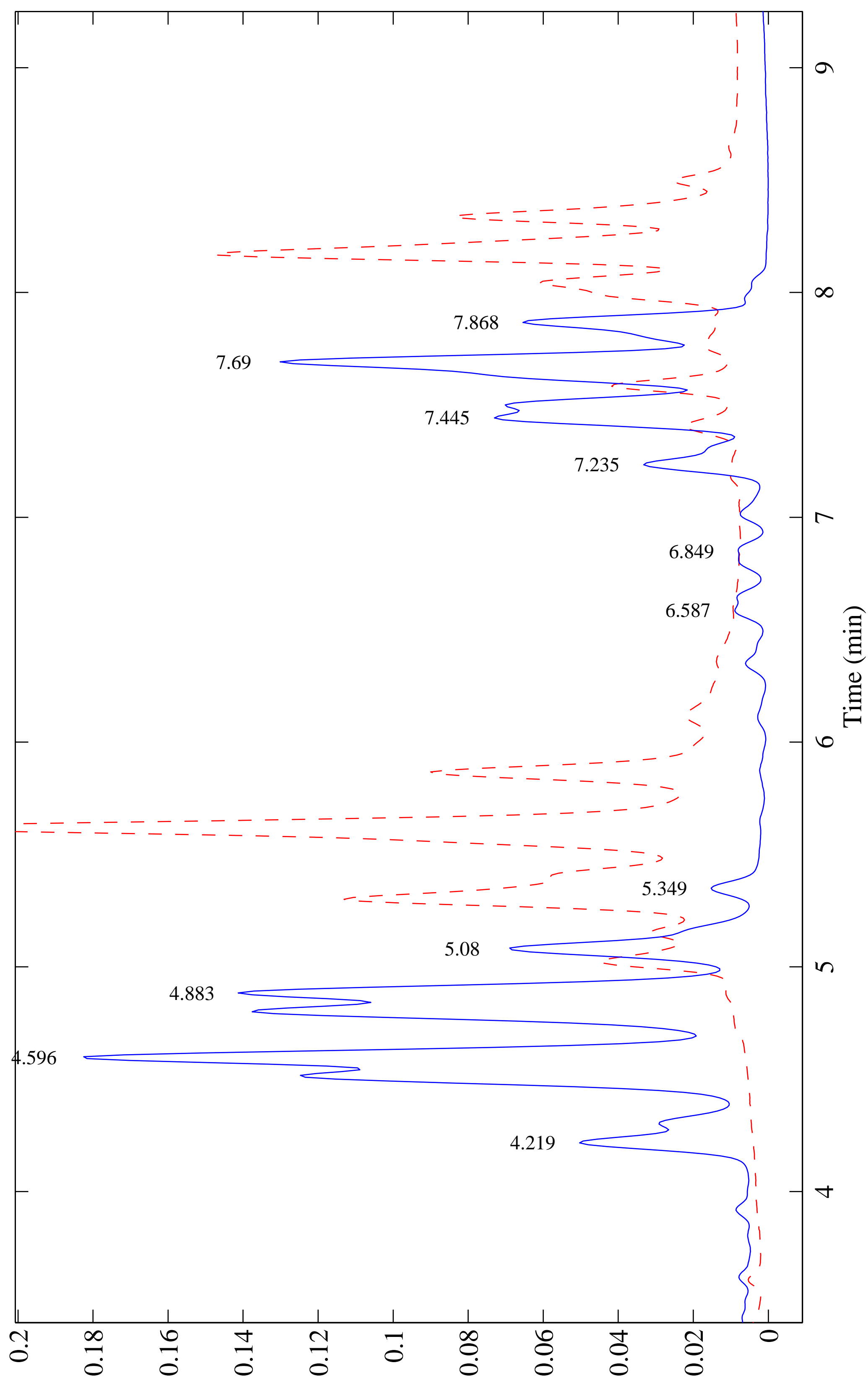


Figure 27. Mycobacterium mucogenicum

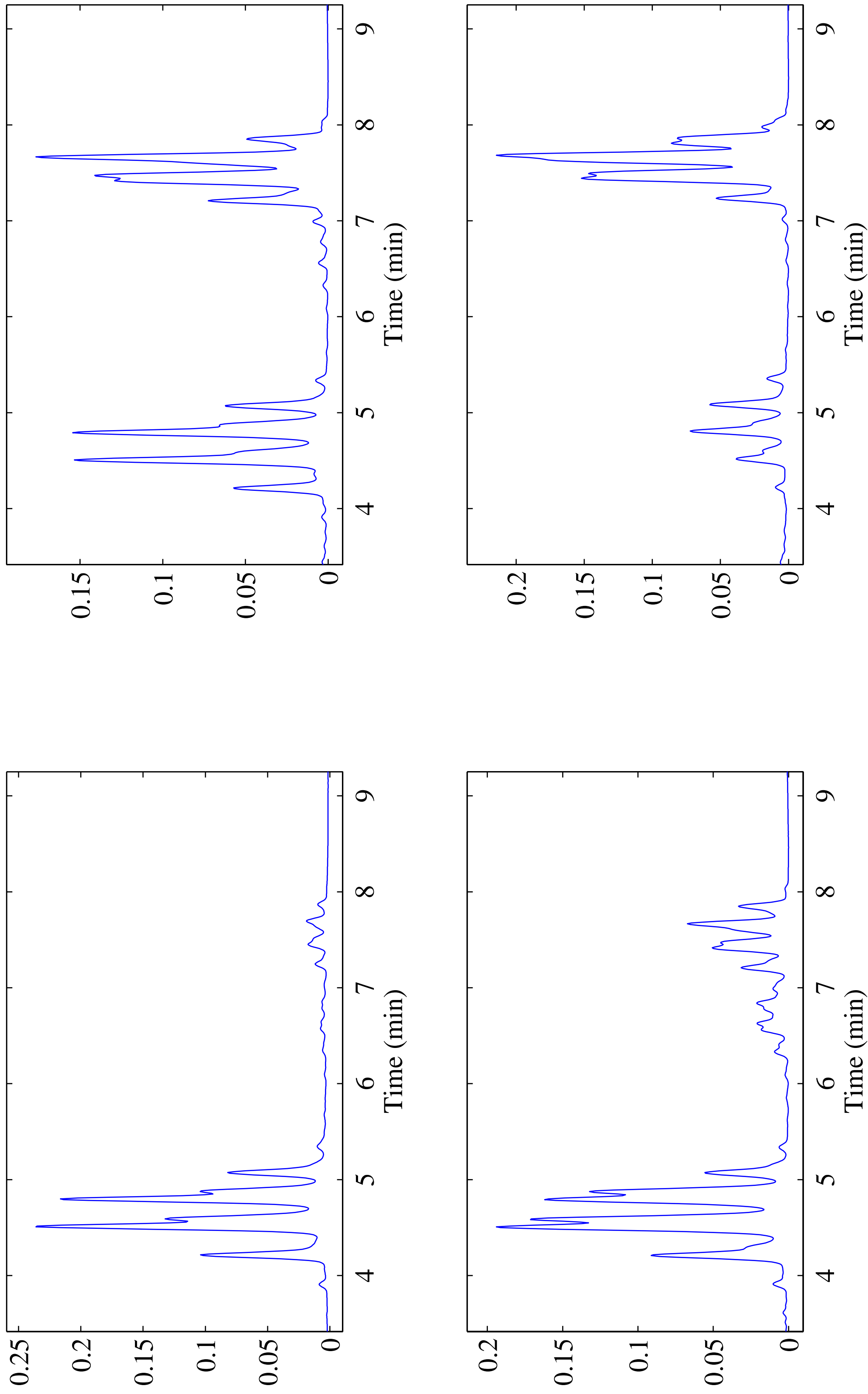


Figure 28. ***Mycobacterium mucogenicum*** **variation**

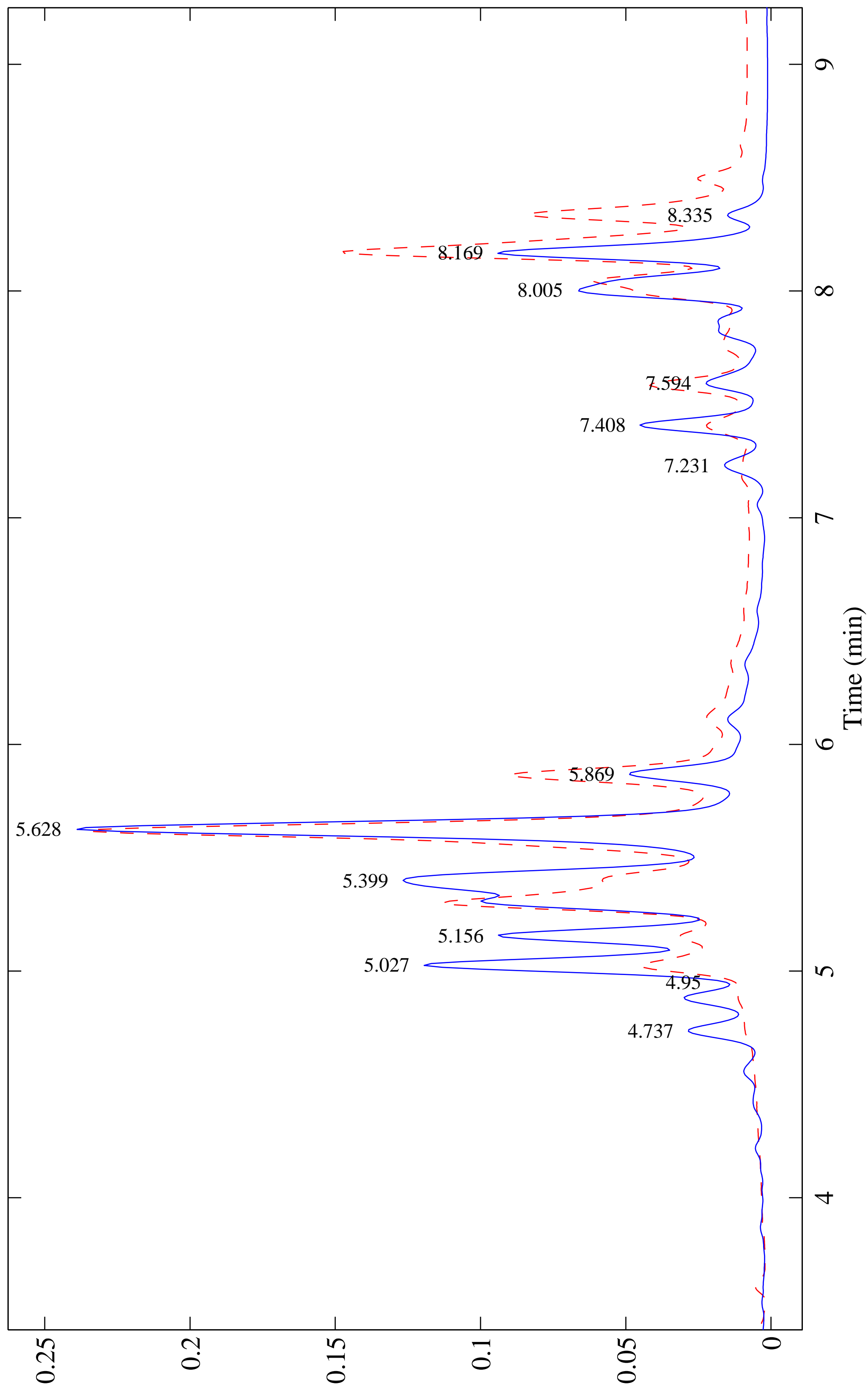


Figure 29. Mycobacterium scrofulaceum

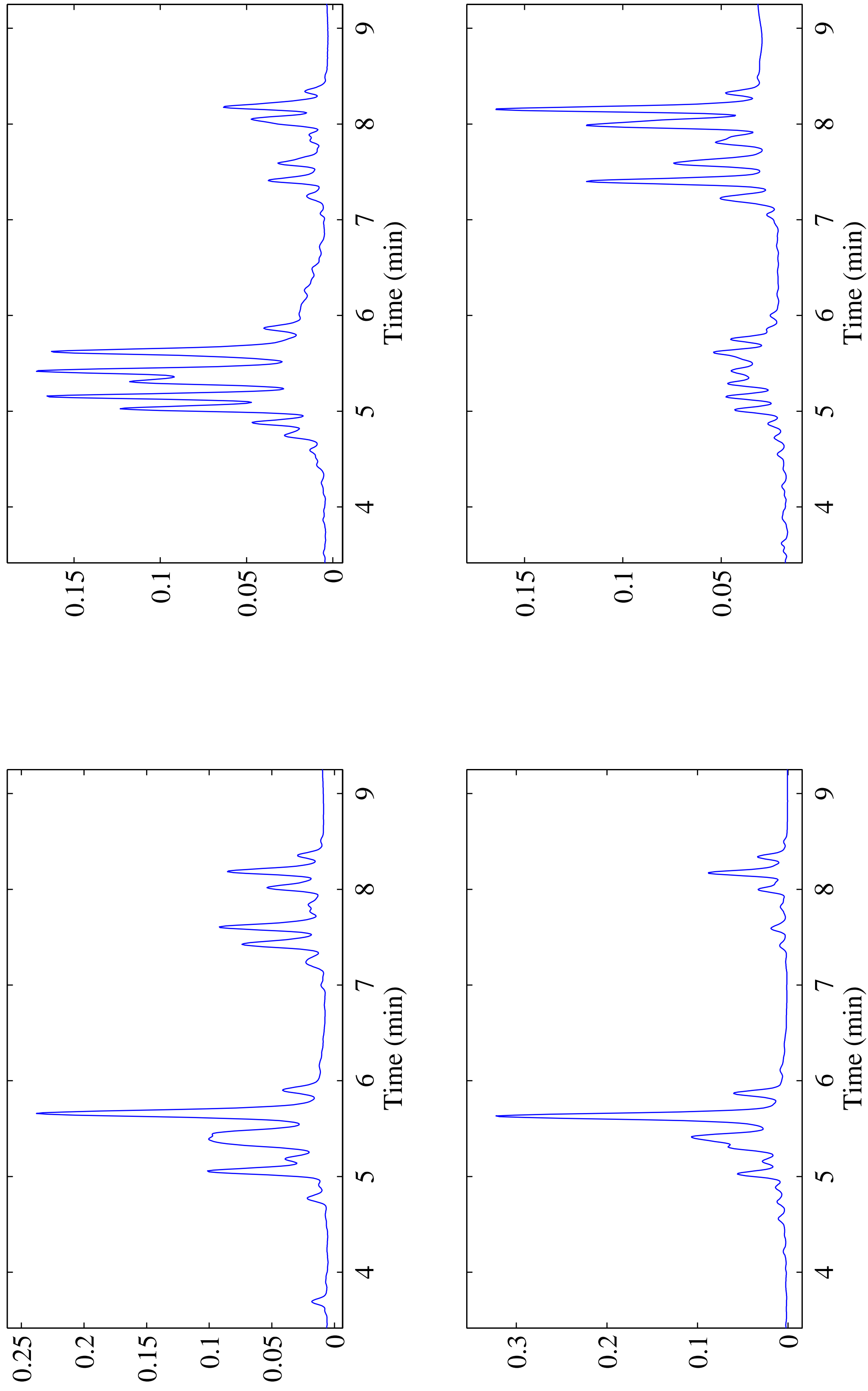


Figure 30. *Mycobacterium scrofulaceum* variation

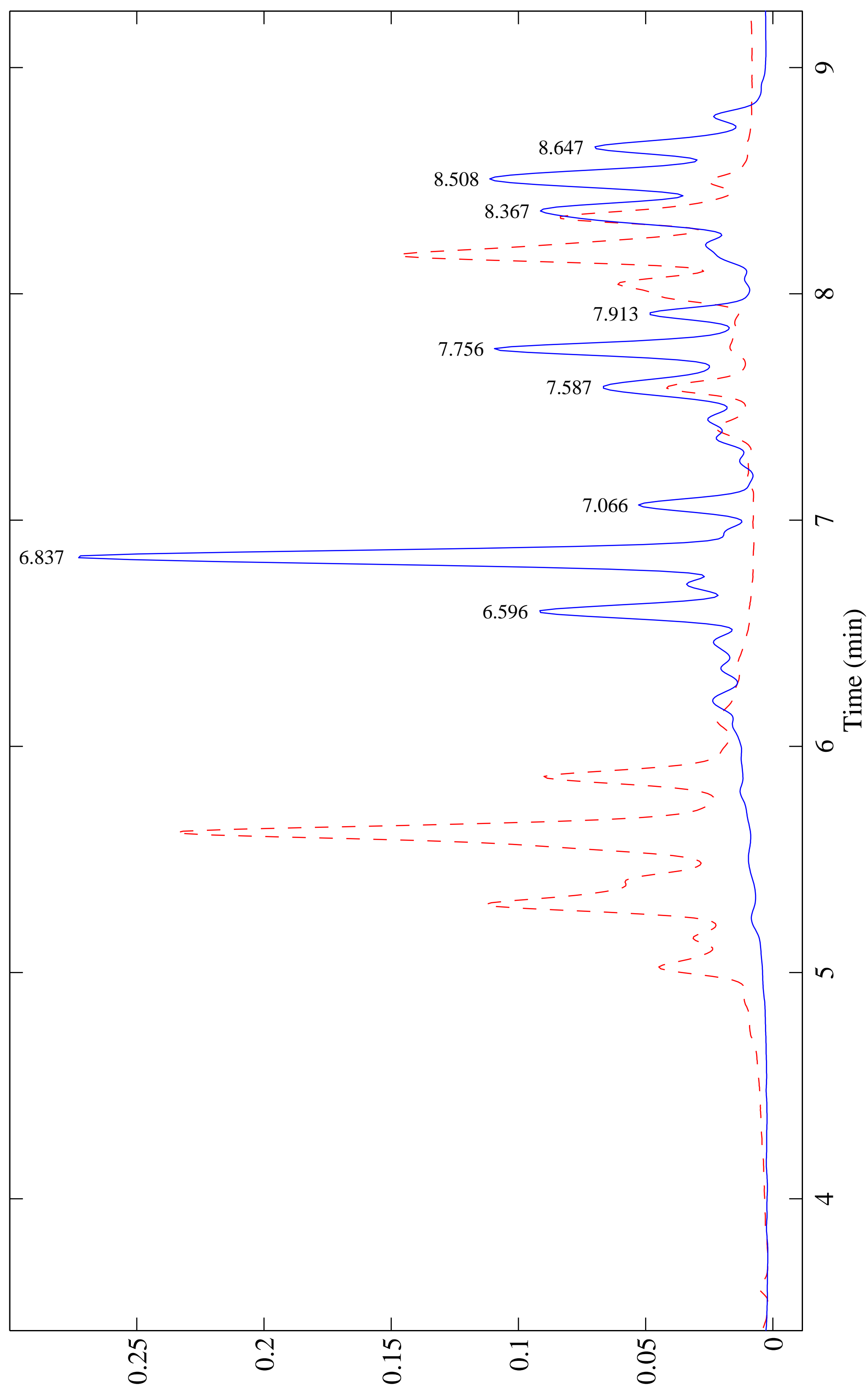


Figure 31. Mycobacterium simiae

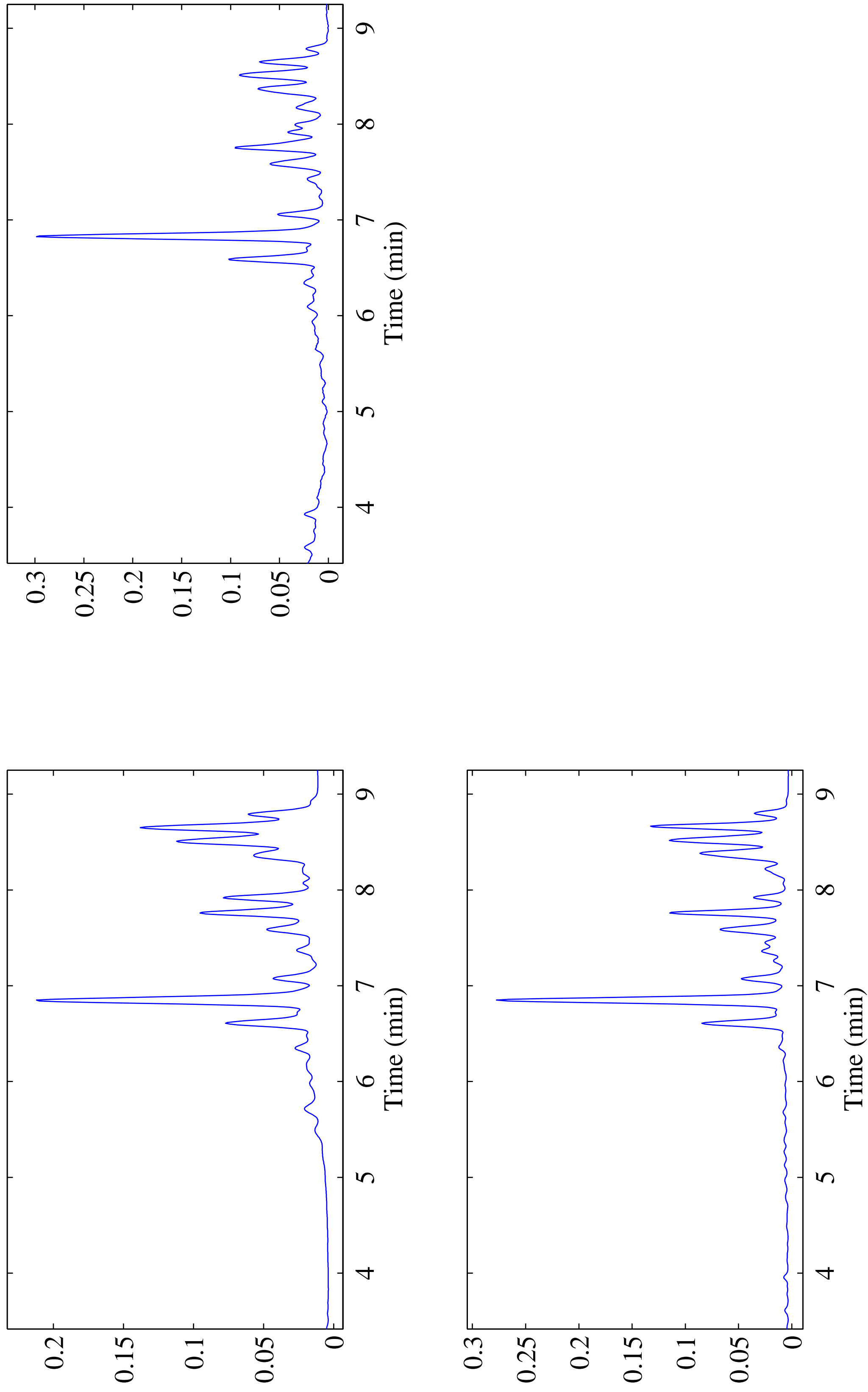


Figure 32. ***Mycobacterium simiae*** **variation**

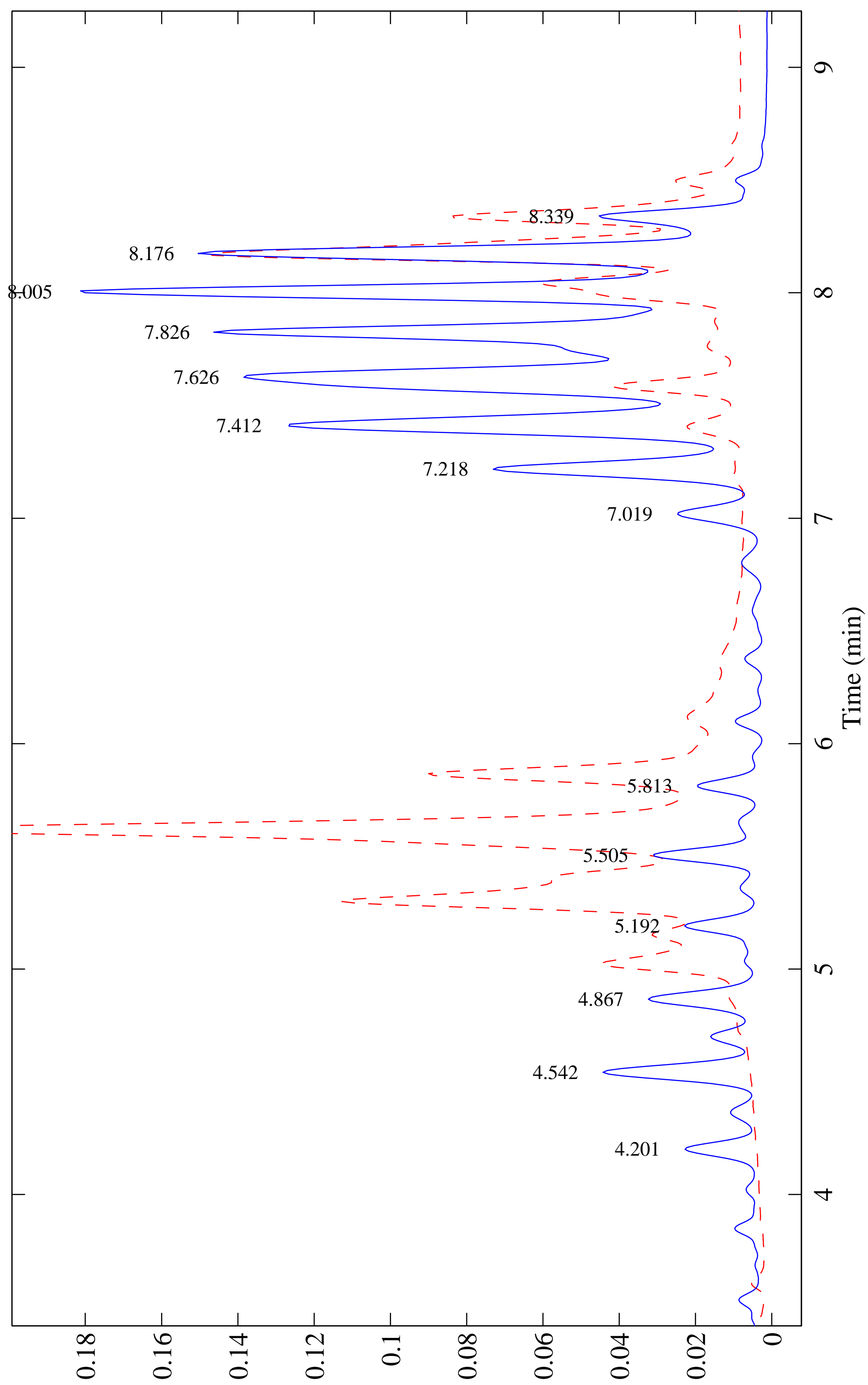


Figure 33. Mycobacterium szulgai

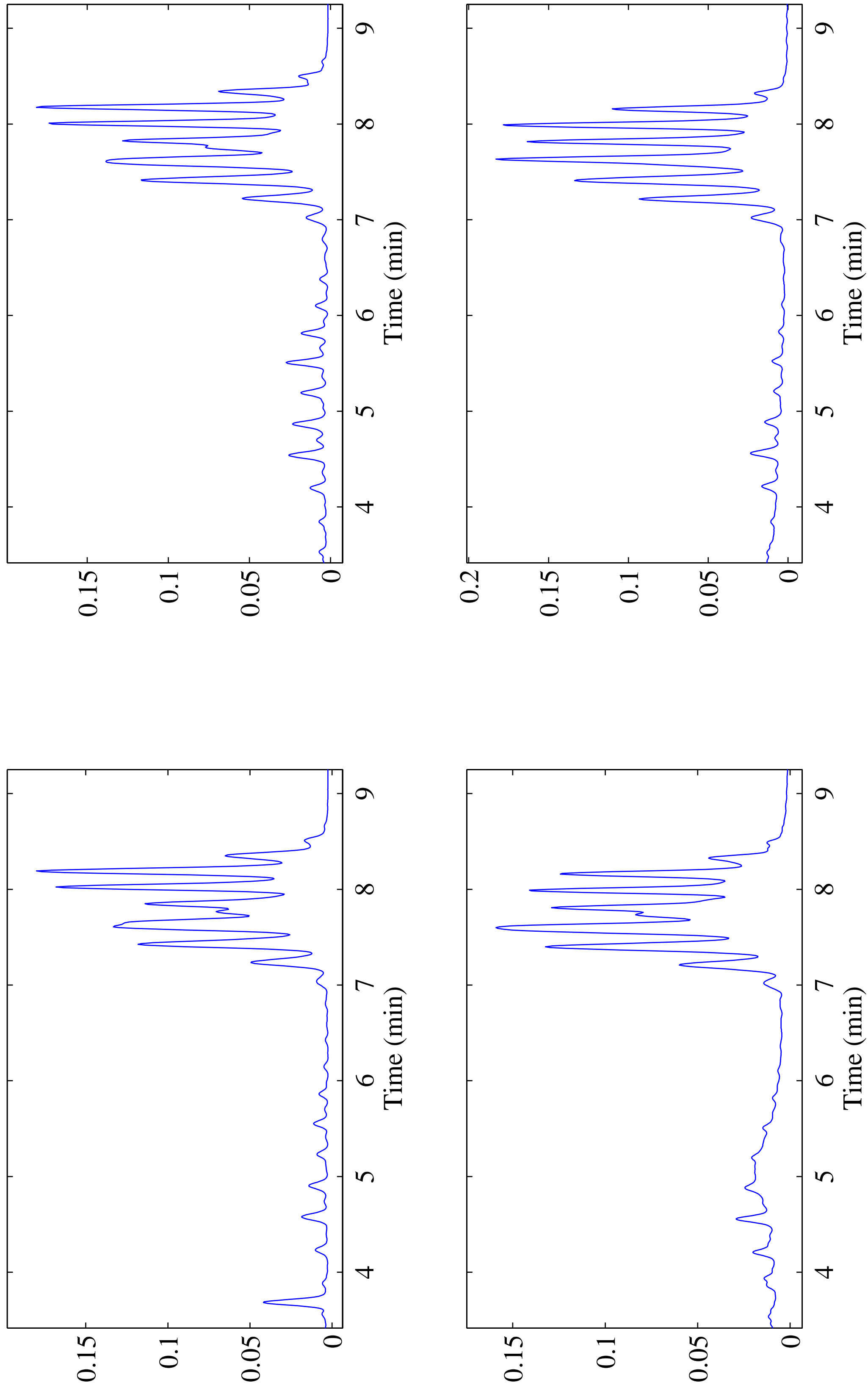


Figure 34. ***Mycobacterium szulgai*** **variation**

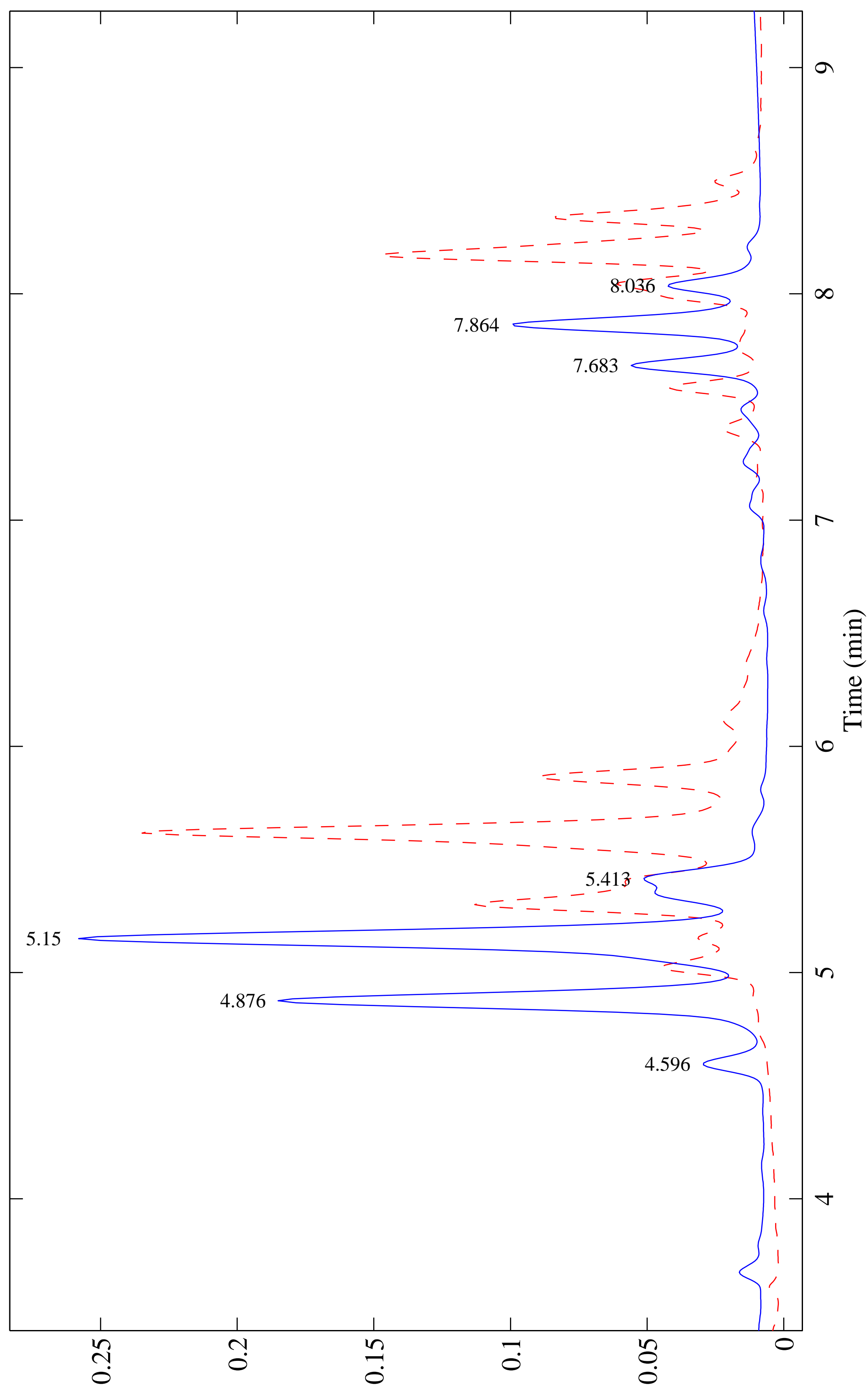


Figure 35. ***Mycobacterium terrae/Mycobacterium nonchromogenicum*** **group**

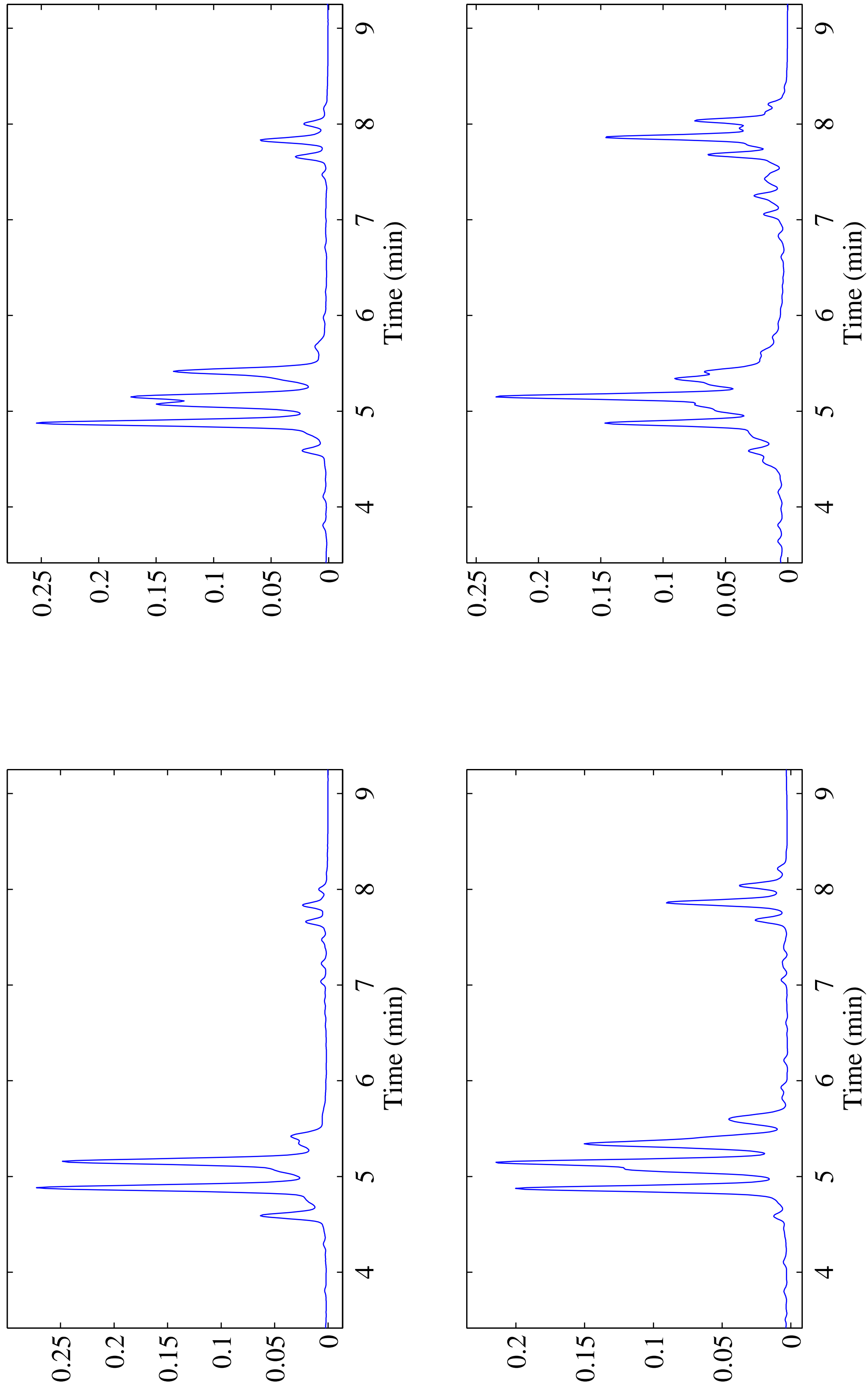


Figure 36. *Mycobacterium terrae/Mycobacterium nonchromogenicum* group variation

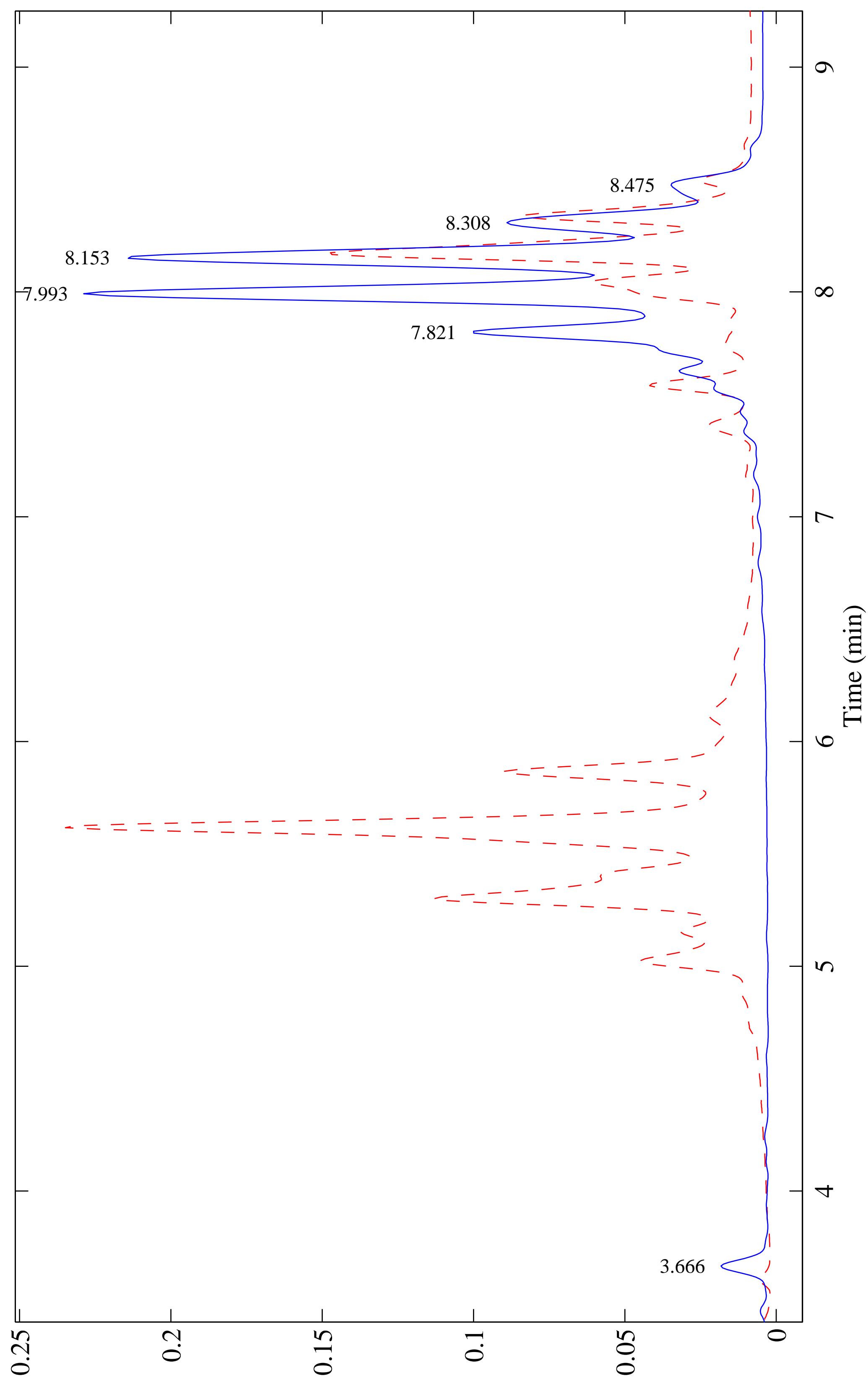


Figure 37. Mycobacterium tuberculosis complex (M. bovis)

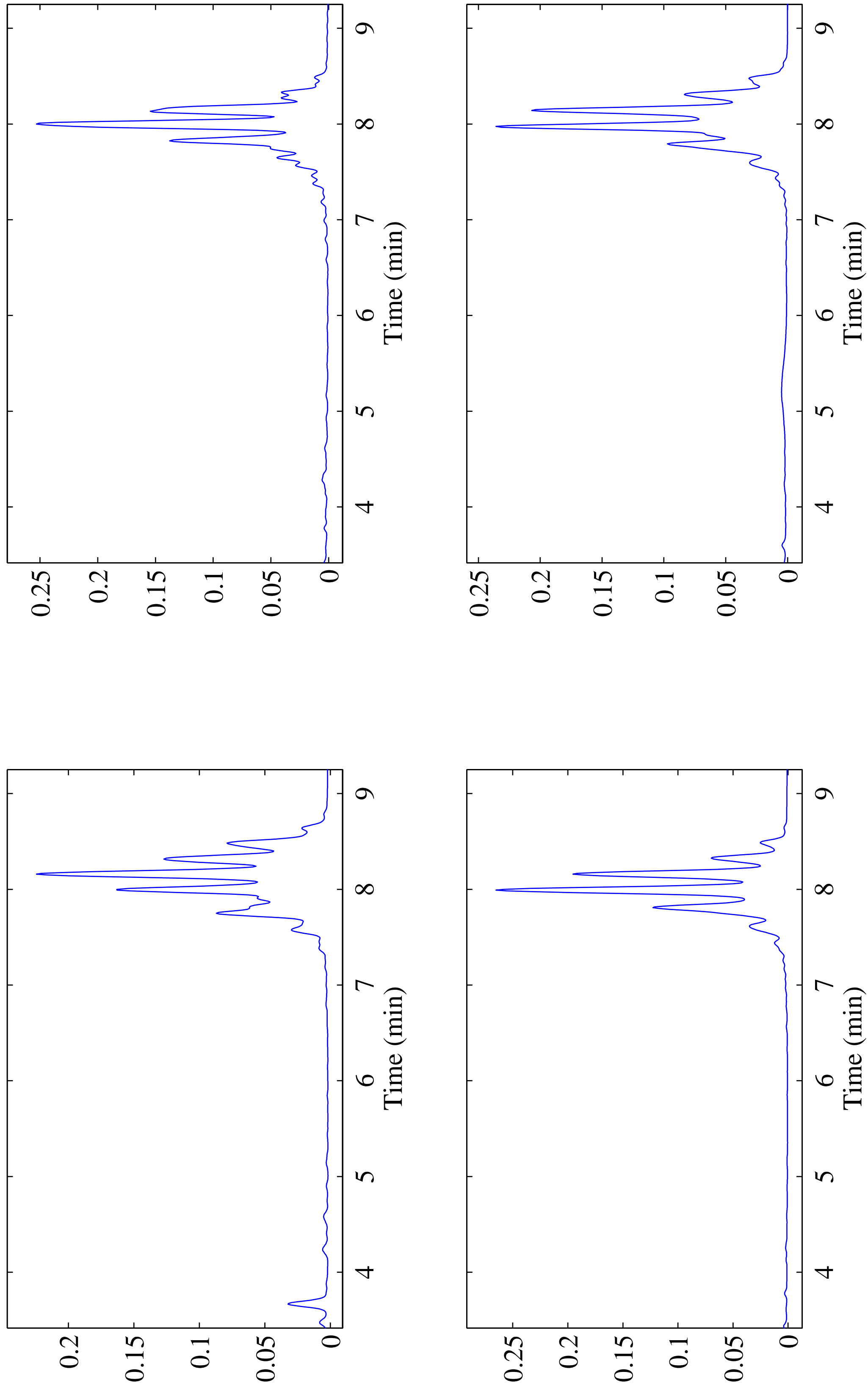


Figure 38. *Mycobacterium tuberculosis complex (M. bovis)* variation

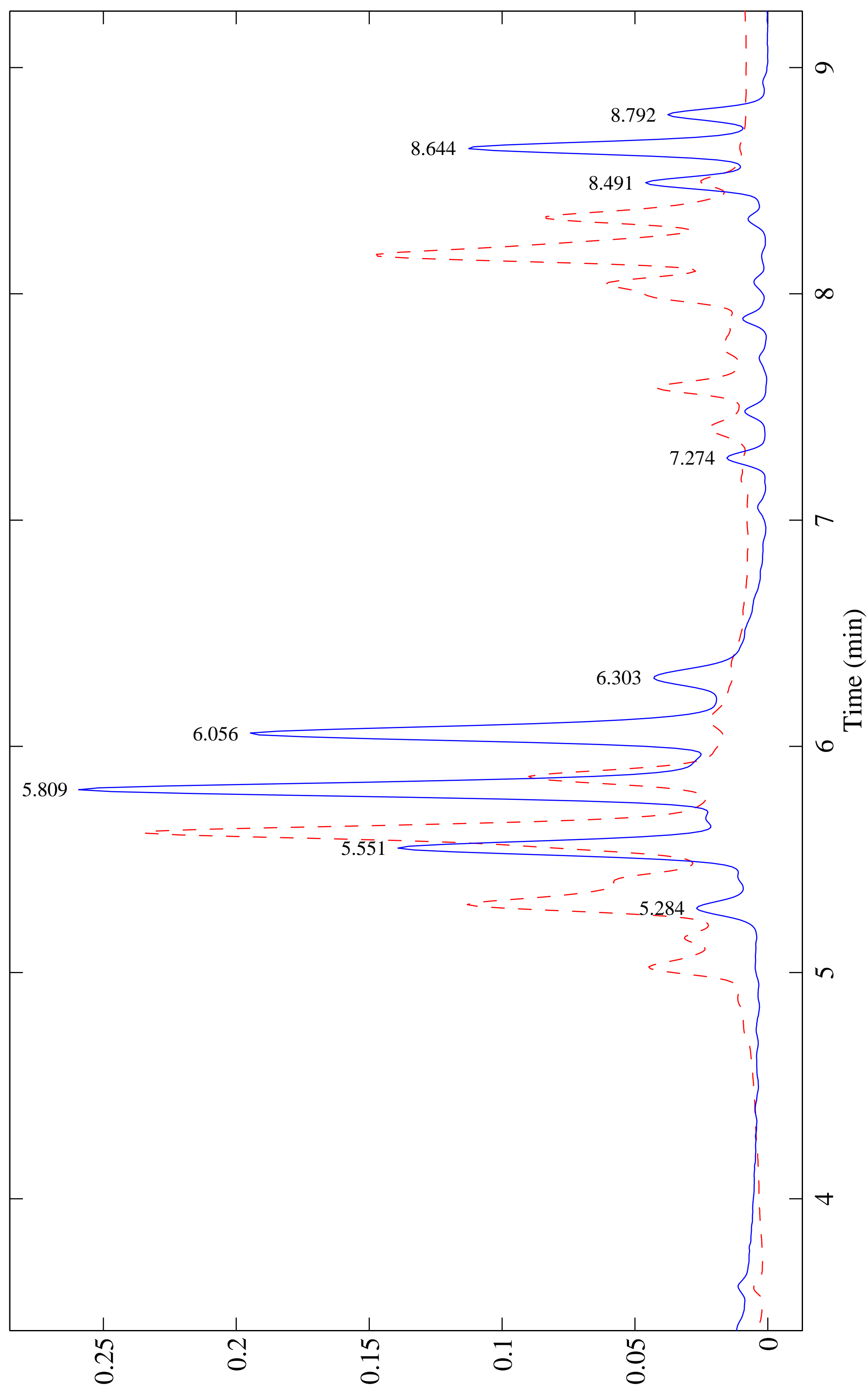
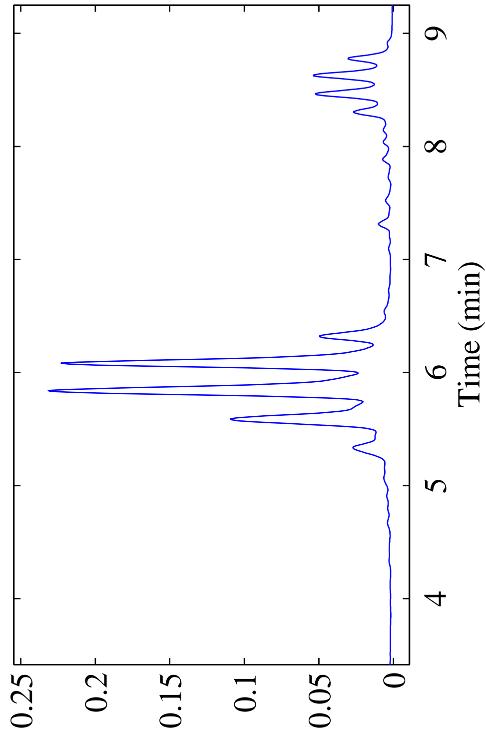
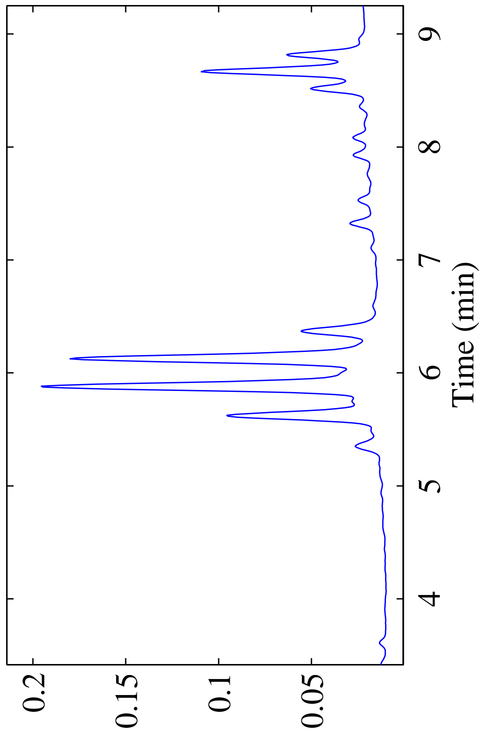


Figure 39. Mycobacterium xenopi





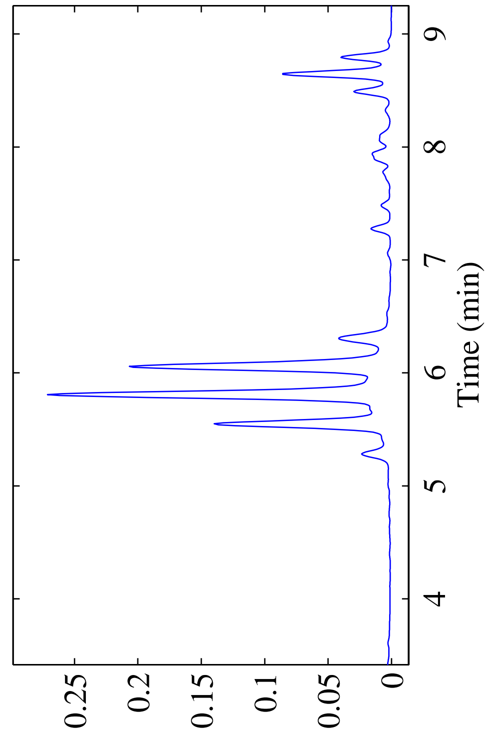


Figure 40. *Mycobacterium xenopi* variation

Chromatographic Comparisons

Many species of mycobacteria exhibit patterns which may be confusing. Not only do the chromatograms have peak clusters which may resemble those in another related species, but variations in patterns may occur among different laboratories and can even be expected to occur within a single laboratory over a period of time.

The following sets of patterns will help to demonstrate where the differences occur among profiles of otherwise similar species. For the plots in this chapter, the following colors—red, green, blue—apply to the species as listed in the plot caption. The ensuing figures are grouped roughly by a common visual criterion: the number of peak clusters in the chromatographic profile.

- Single Clusters
- Double Clusters
- Multi clusters
- Others

Members of the *Mycobacterium tuberculosis* complex used in this study included *M. tuberculosis* and *M. bovis*. Both of these species react to the commercial genetic probes designed for *M. tuberculosis*, are identical in DNA hybridization studies, in 16S rRNA gene sequence, and produce indistinguishable HPLC patterns. Respiratory infection caused by these species produce similar disease and as such are included in the annual statistics for *Reported Tuberculosis Cases in the United States*, a report published annually by CDC's Division of Tuberculosis Elimination. Because of the relative clinical significance of the species in this complex, we have presented a larger number of chromatographic comparisons including *M. tuberculosis*, compared to some of the other nontuberculous mycobacteria.

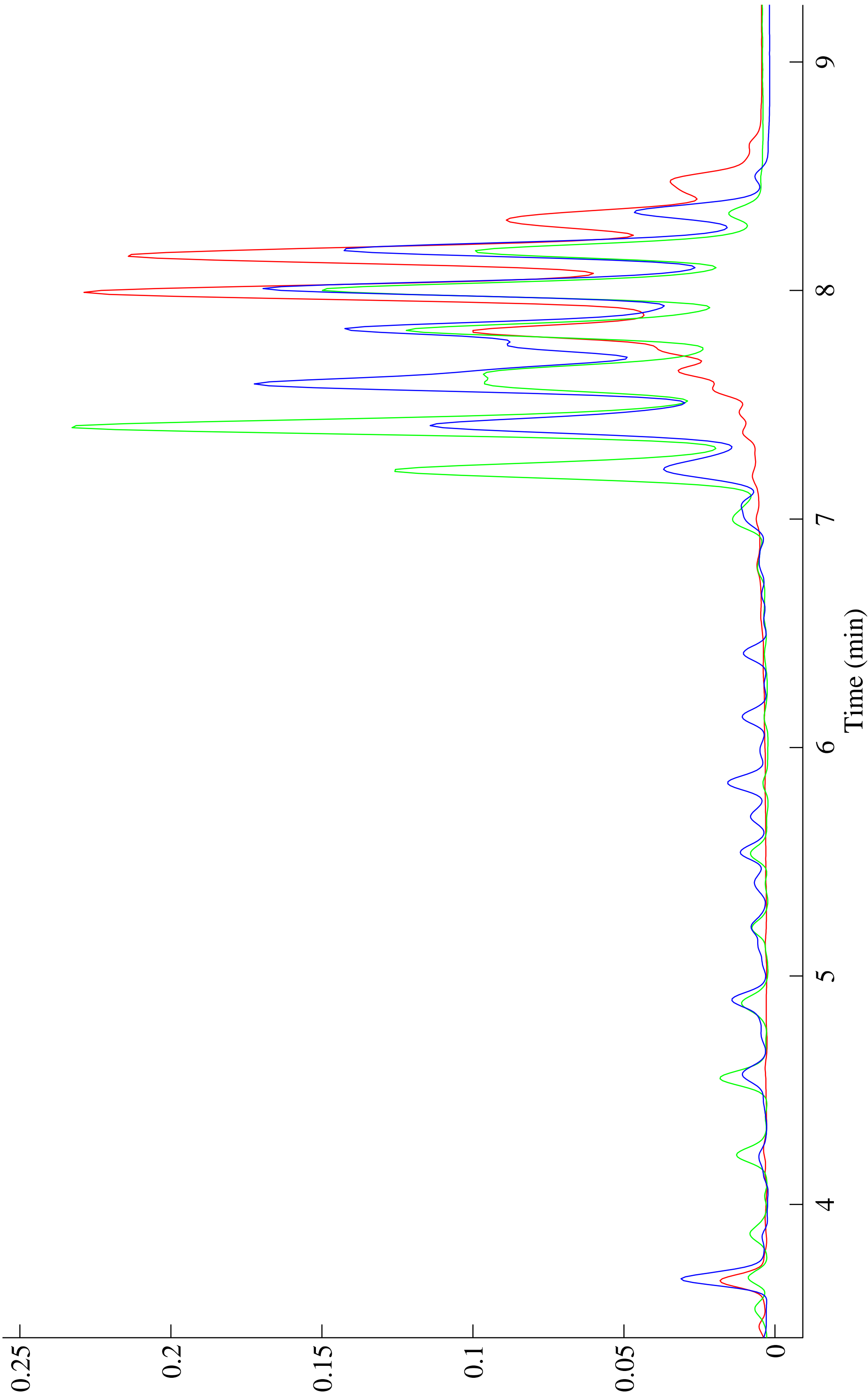


Figure 1. ***M. tuberculosis*****,** ***M. gordonae*****, and** ***M. kansasii***

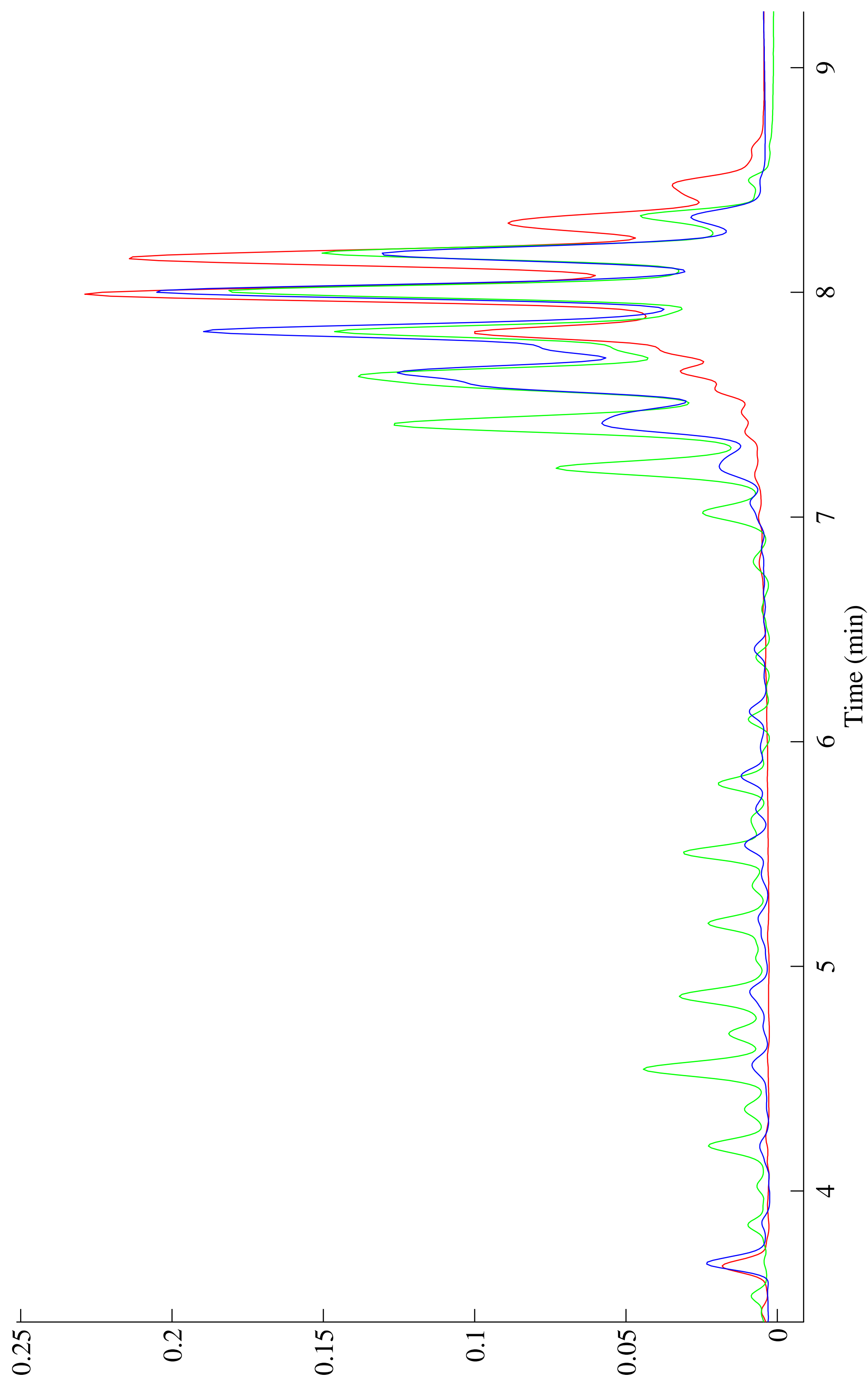


Figure 2. ***M. tuberculosis***, ***M. szulgai***, **and** ***M. gastri***

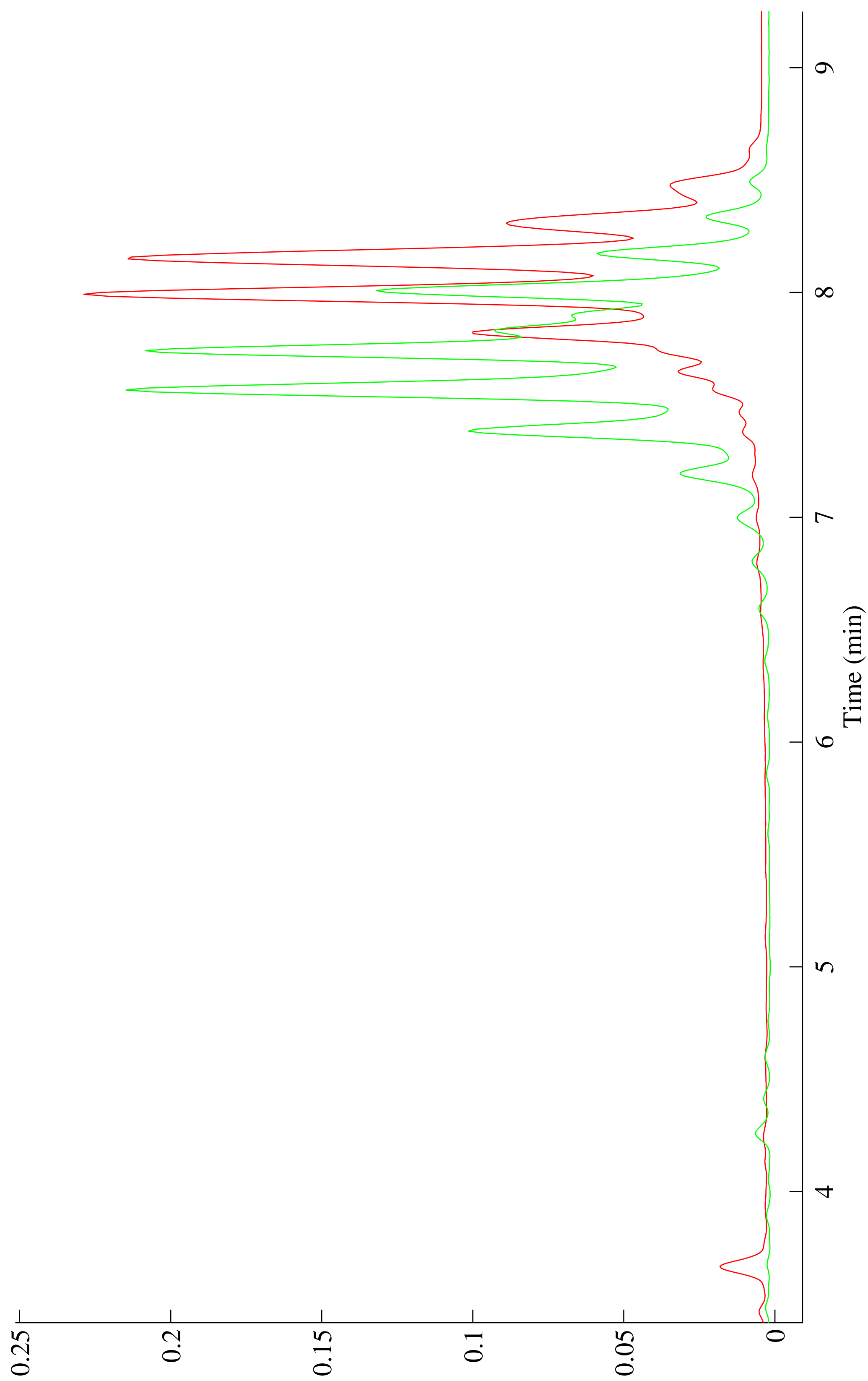


Figure 3. ***M. tuberculosis*** **and** ***M. bovis*** **BCG**

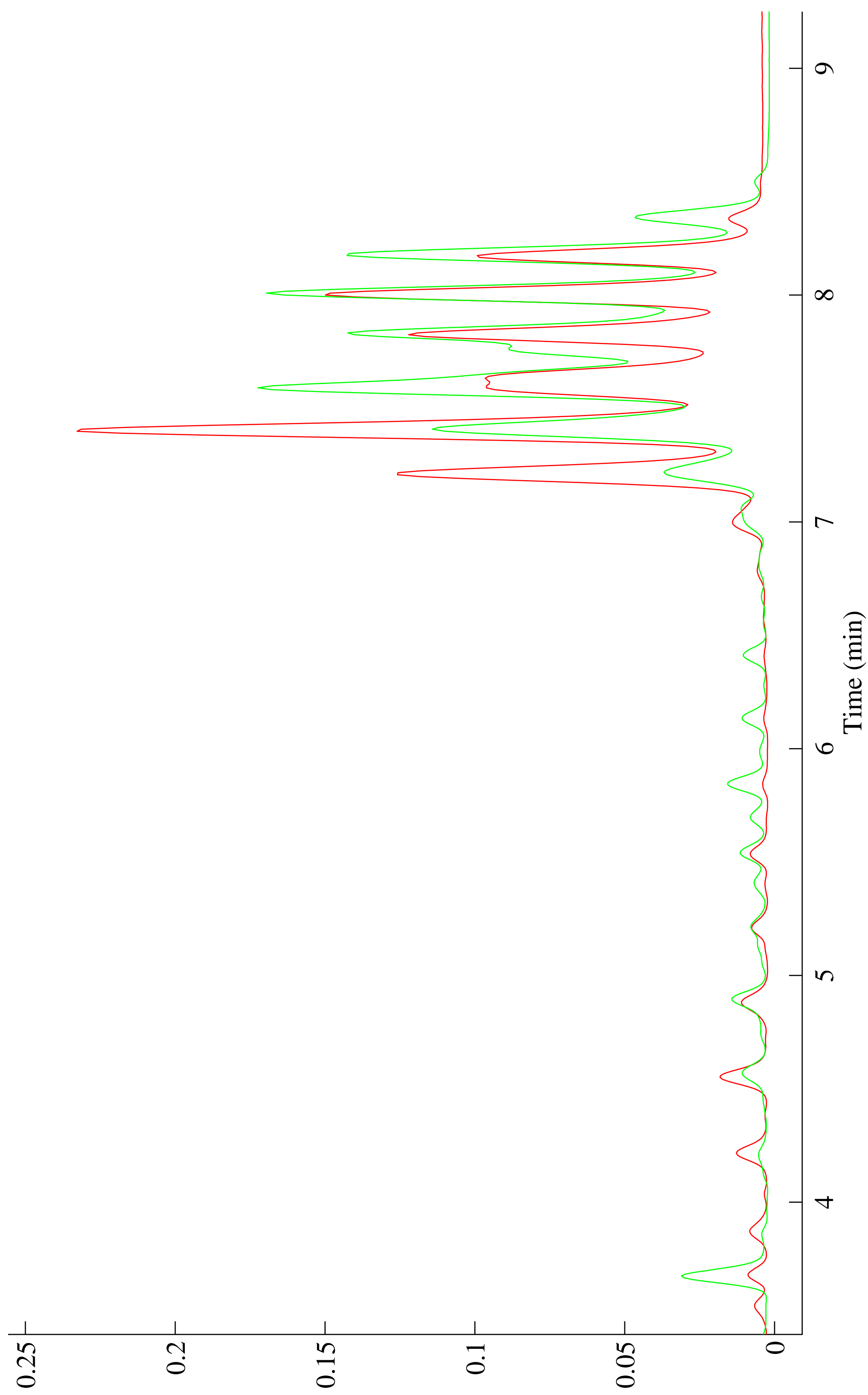


Figure 4. ***M. gordonae*** **and** ***M. kansasii***

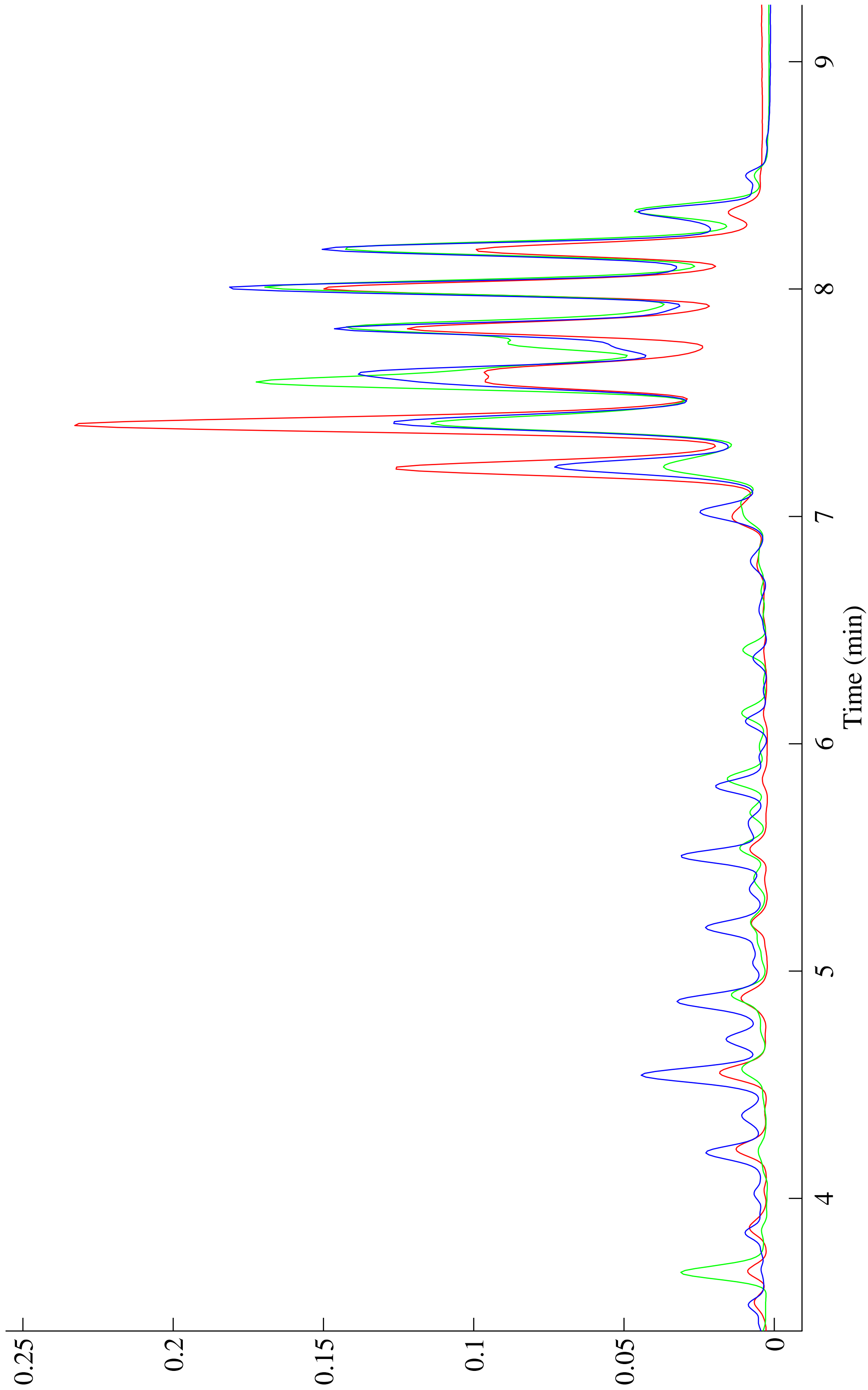


Figure 5. ***M. gordonae*****,** ***M. kansasii*****, and** ***M. szulgai***

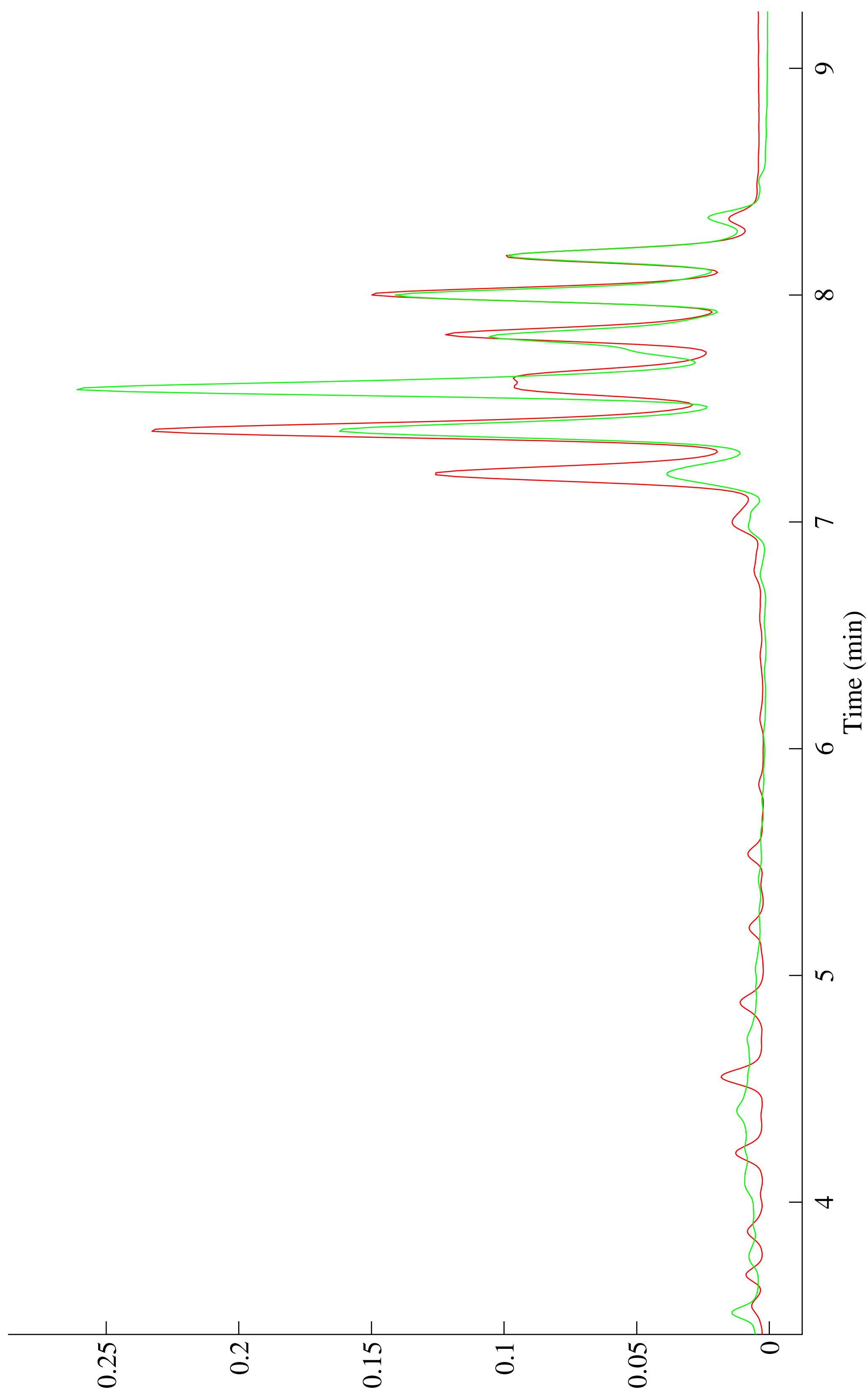


Figure 6. *M. gordonae* and *M. asiaticum*

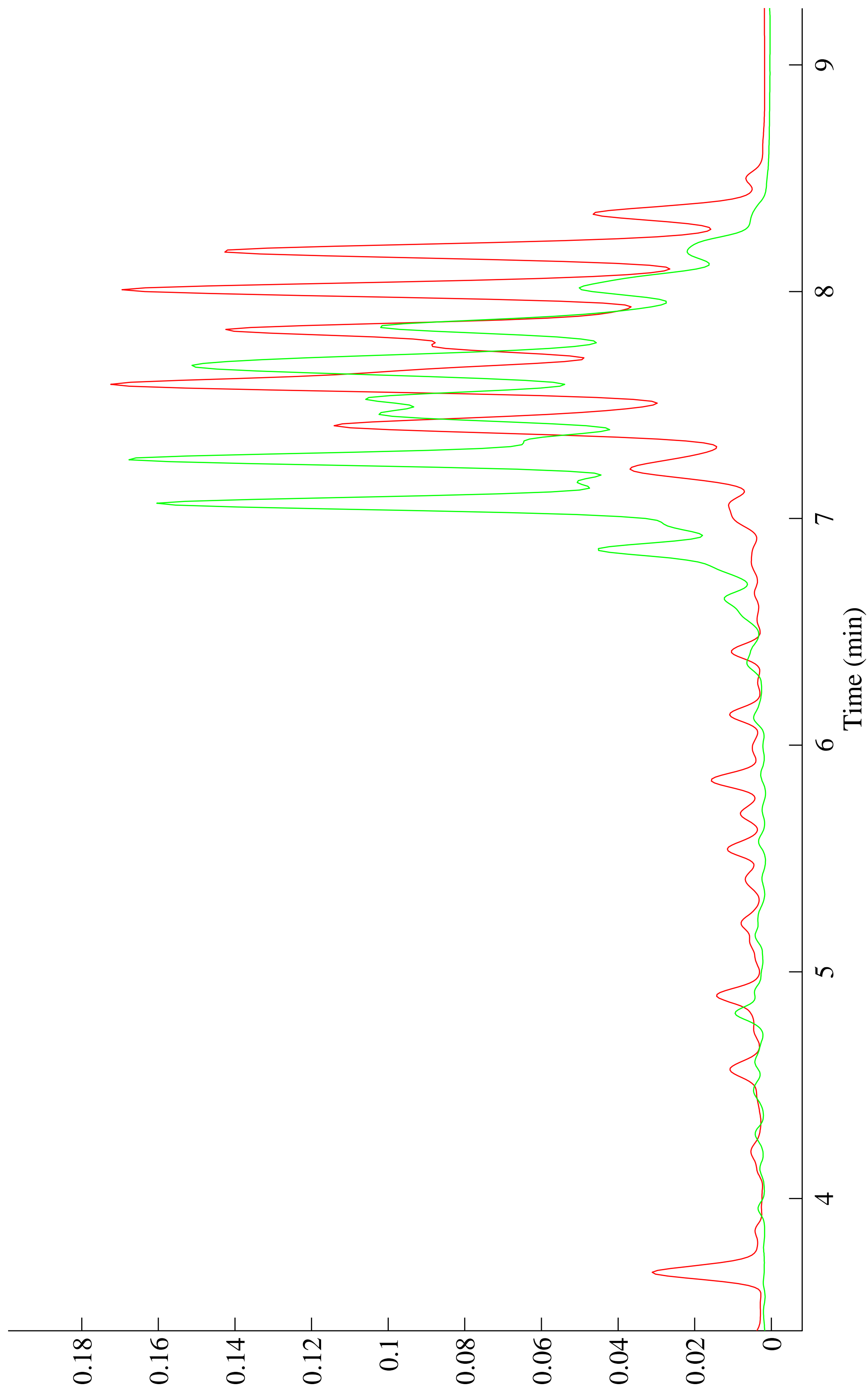


Figure 7. ***M. kansasii*** **and** ***M. marinum***

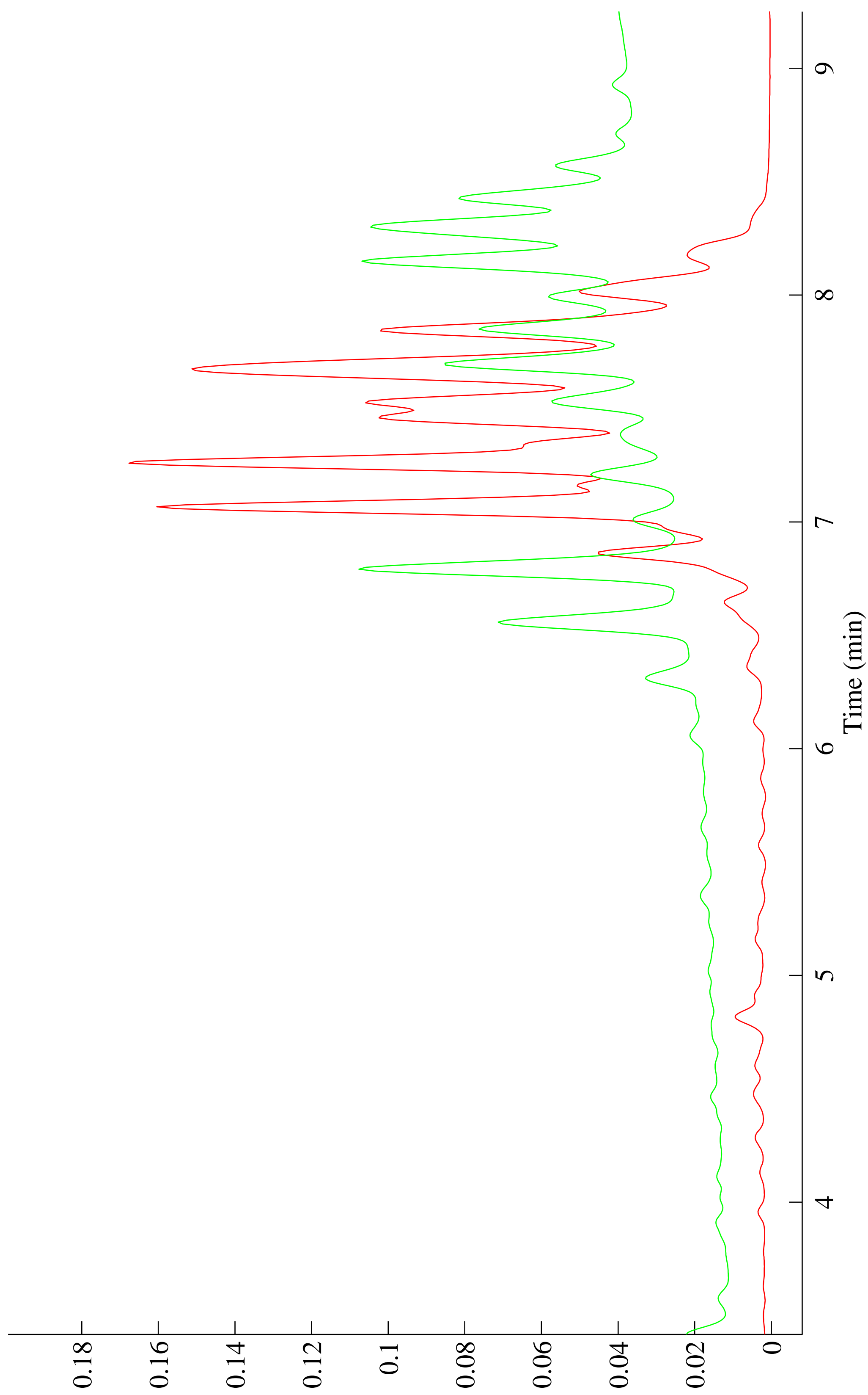


Figure 8. ***M. marinum*** **and** ***M. malmoense***

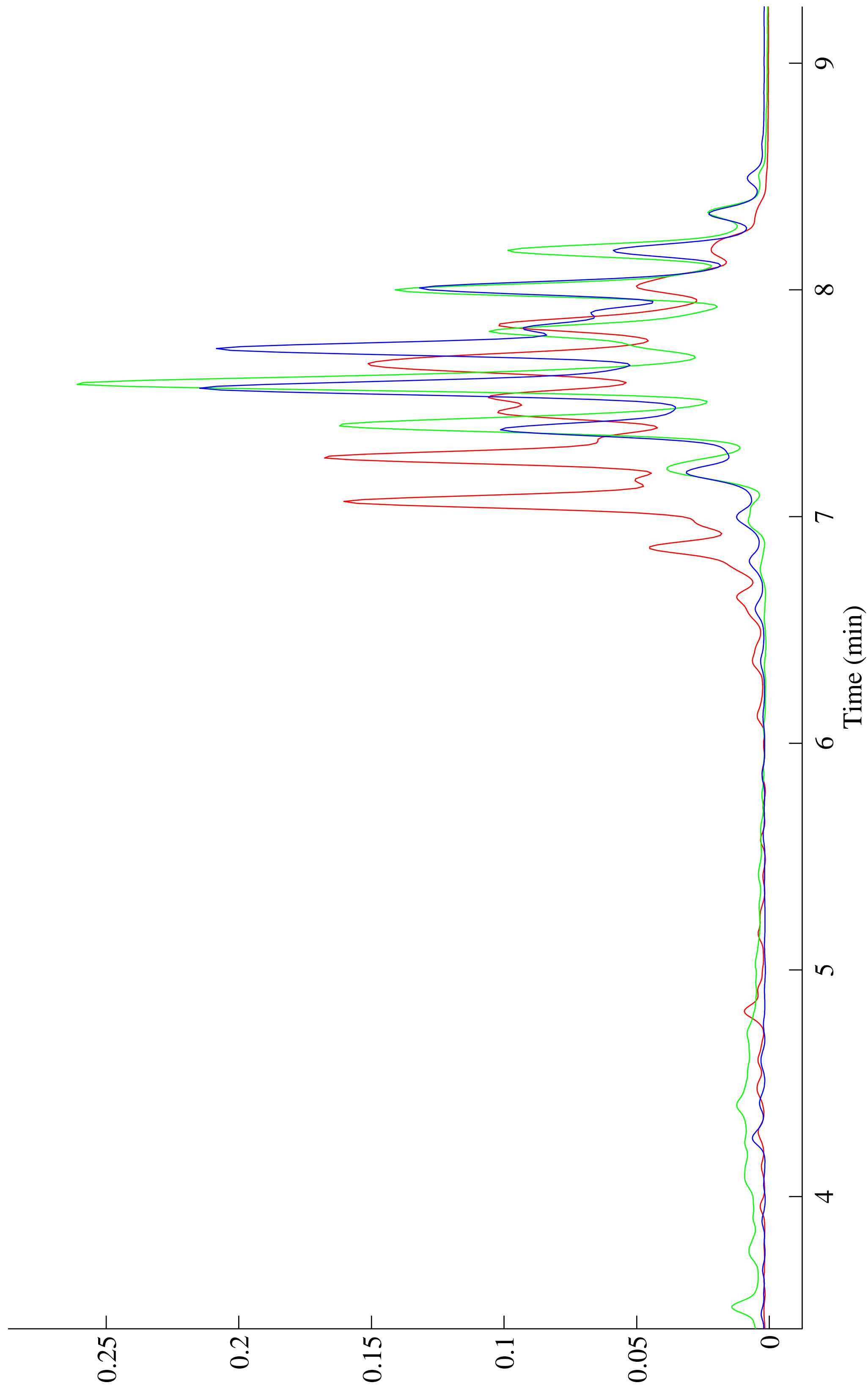


Figure 9. ***M. marinum*, *M. asiaticum*, and *M. bovis* BCG**

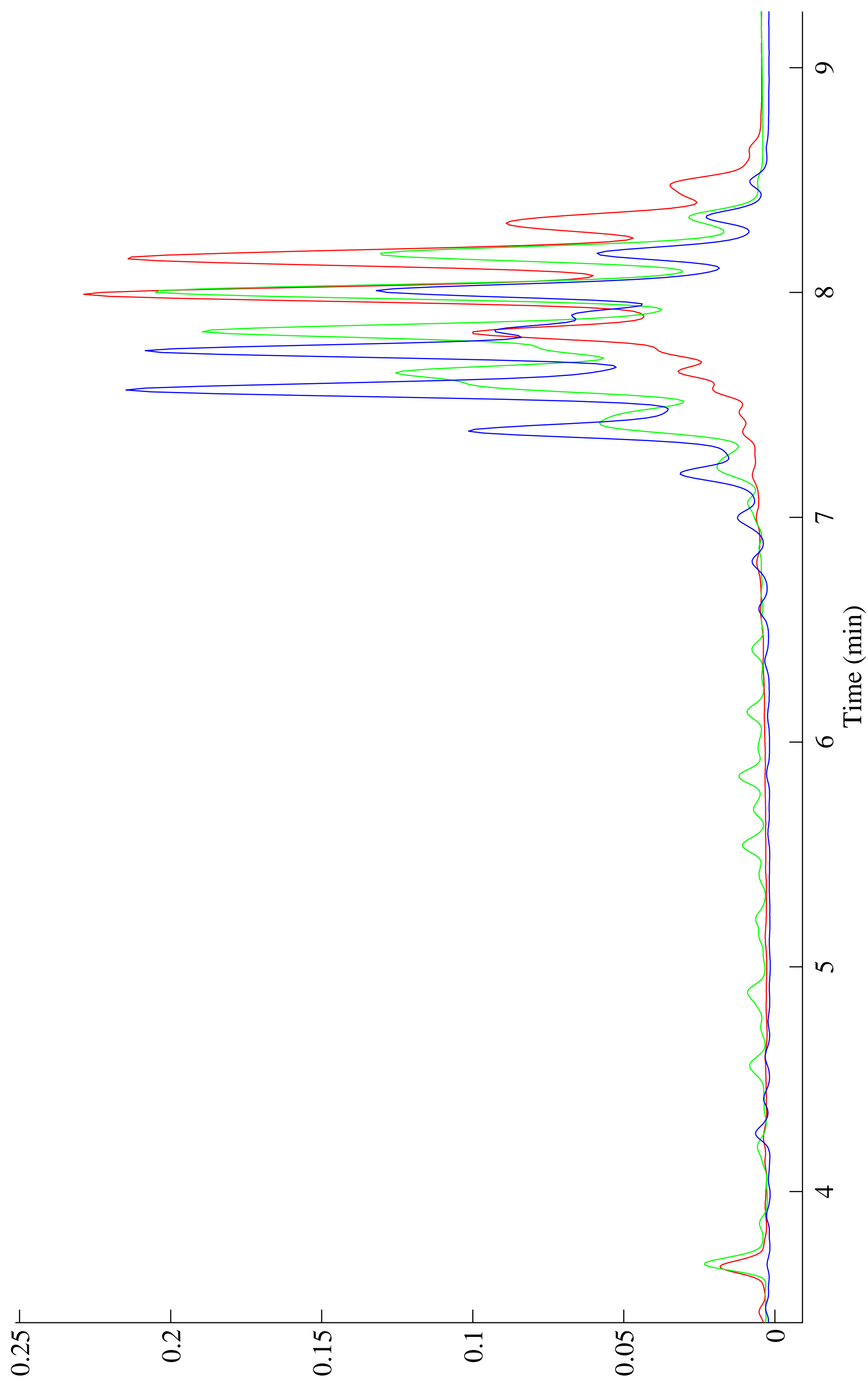


Figure 10. ***M. tuberculosis*, *M. gastri*, and *M. bovis* BCG**

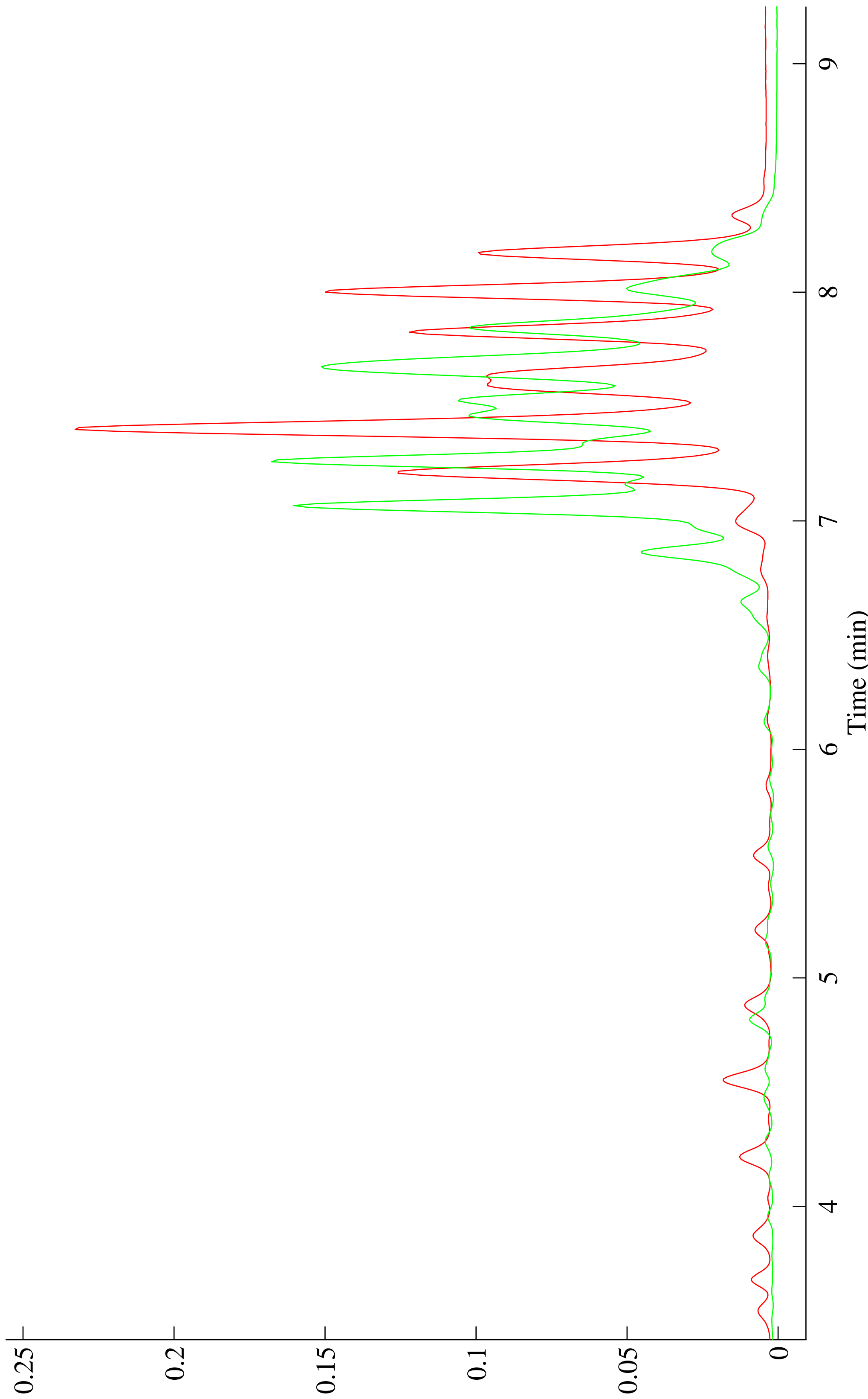


Figure 11. *M. gordonae* and *M. marinum*

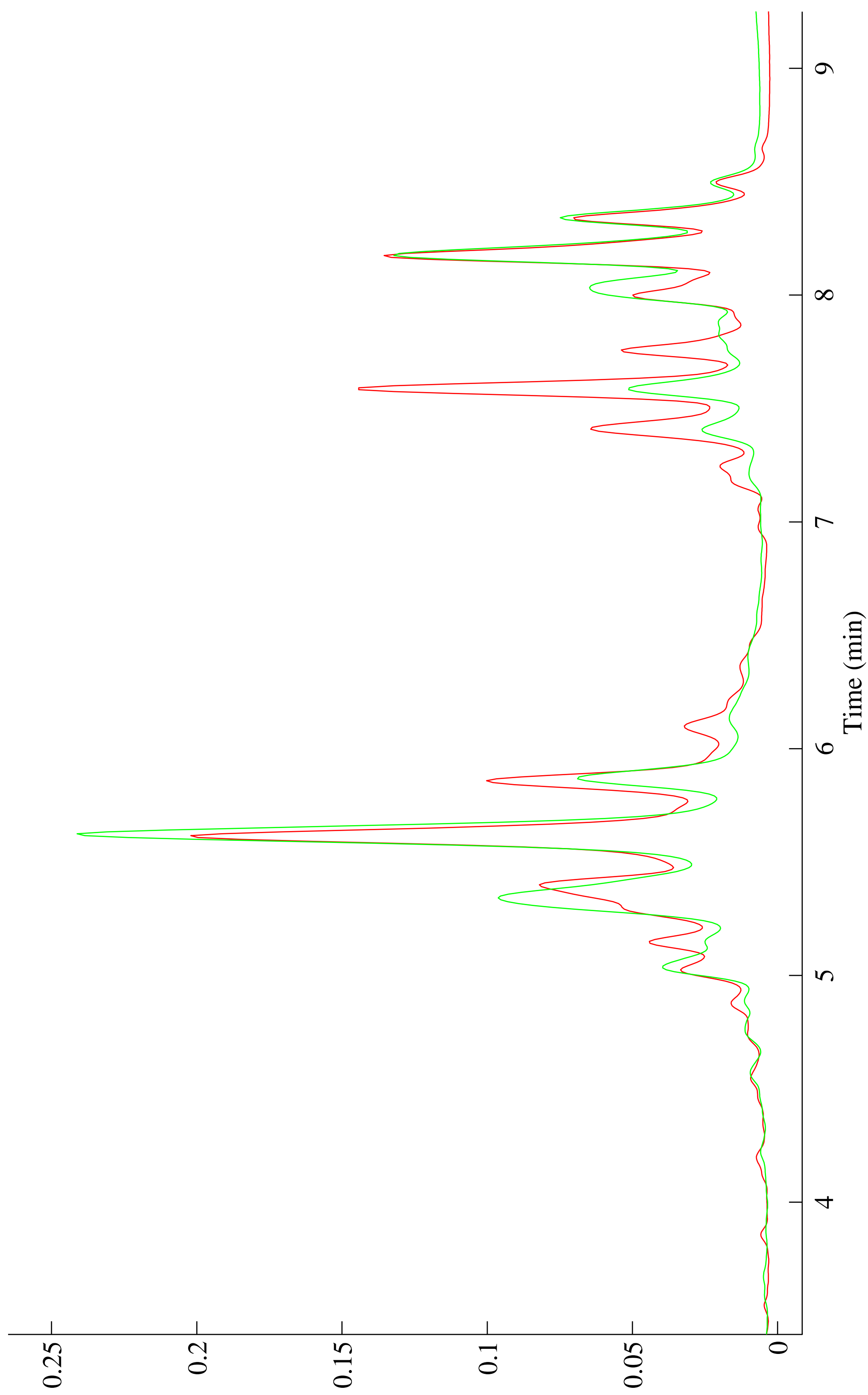


Figure 12. ***M. avium*** **and** ***M. intracellulare***

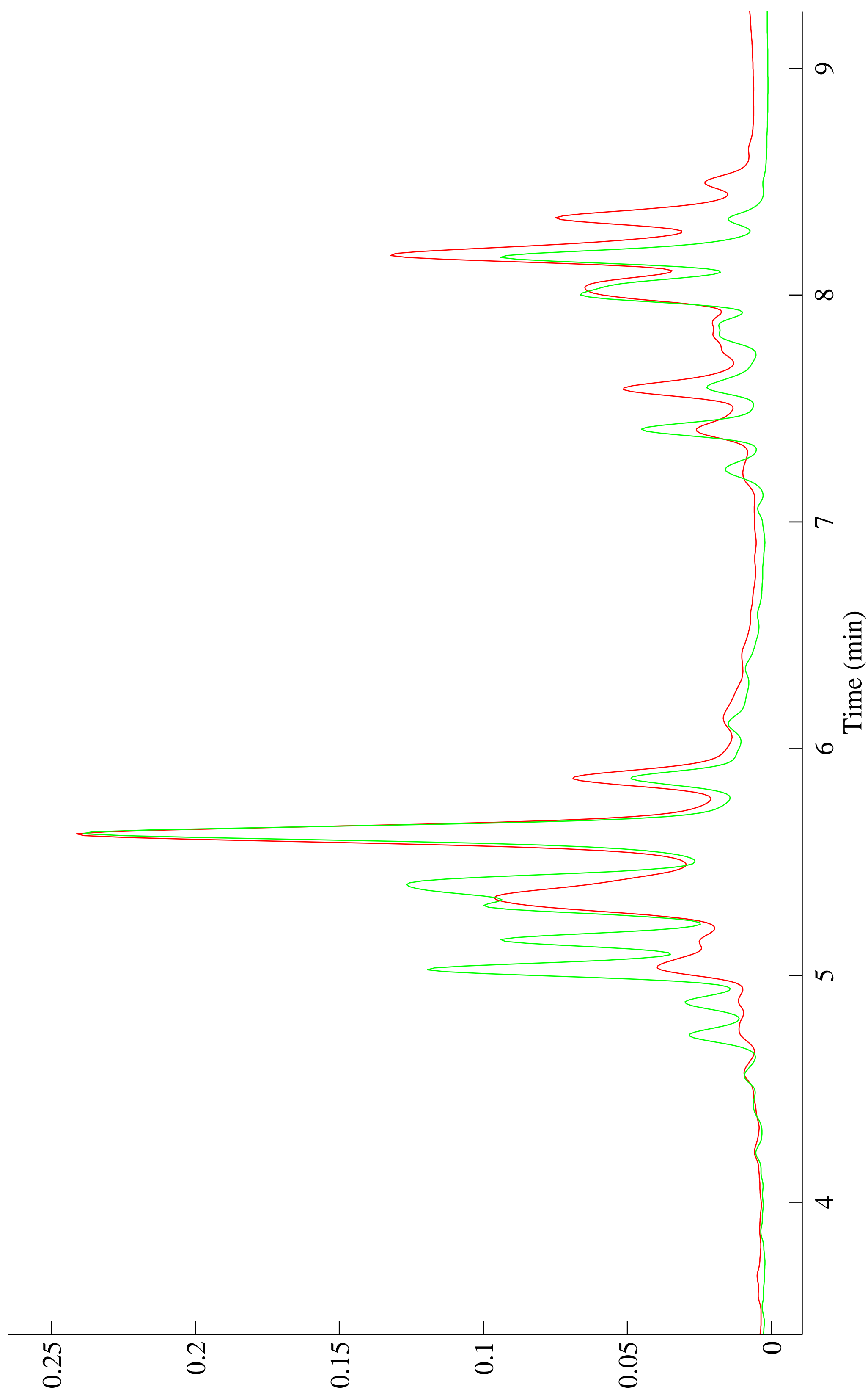


Figure 13. ***M. intracellulare*** **and** ***M. scrofulaceum***

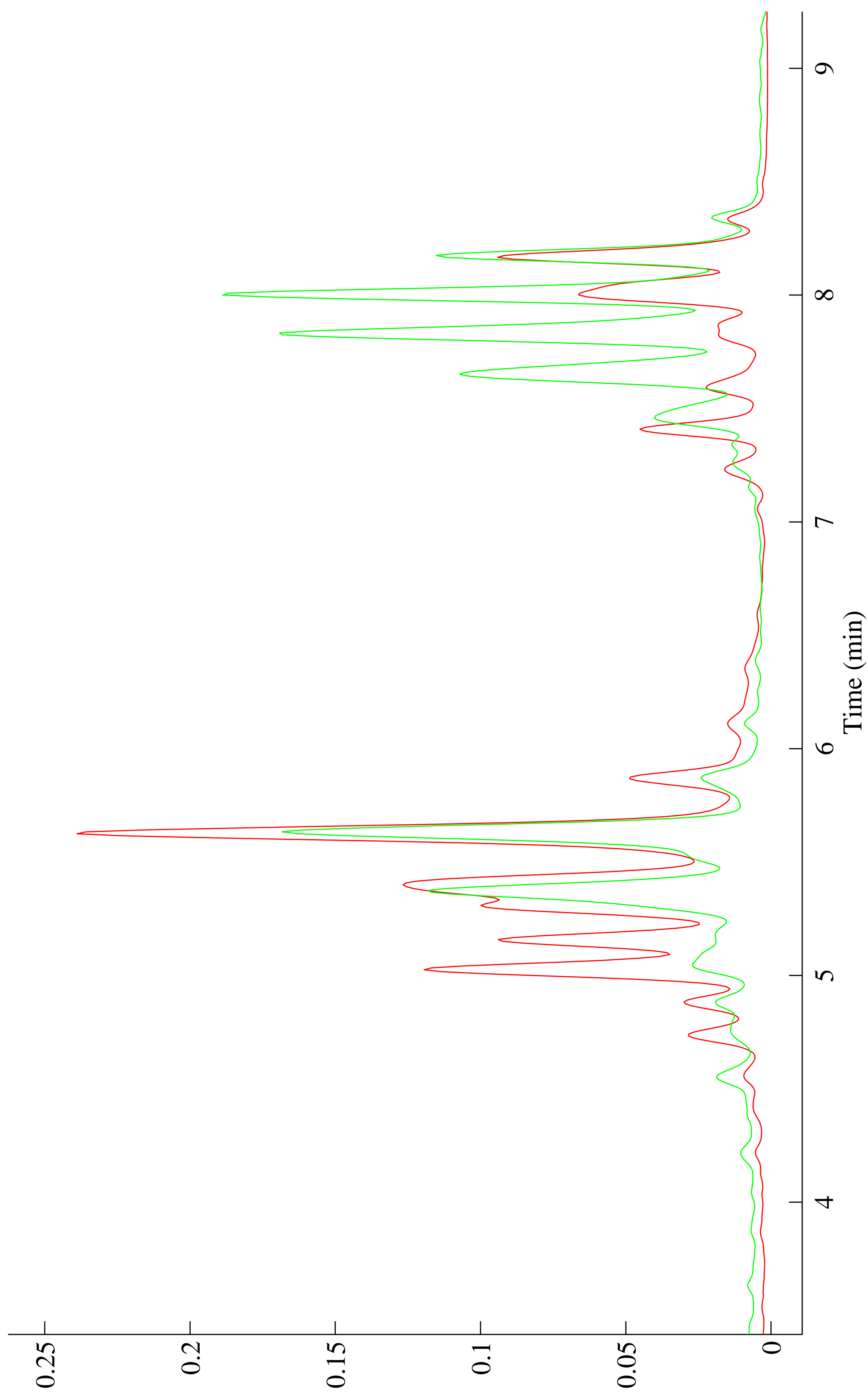


Figure 14. *M. scrofulaceum* and *M. gordonae* (II)

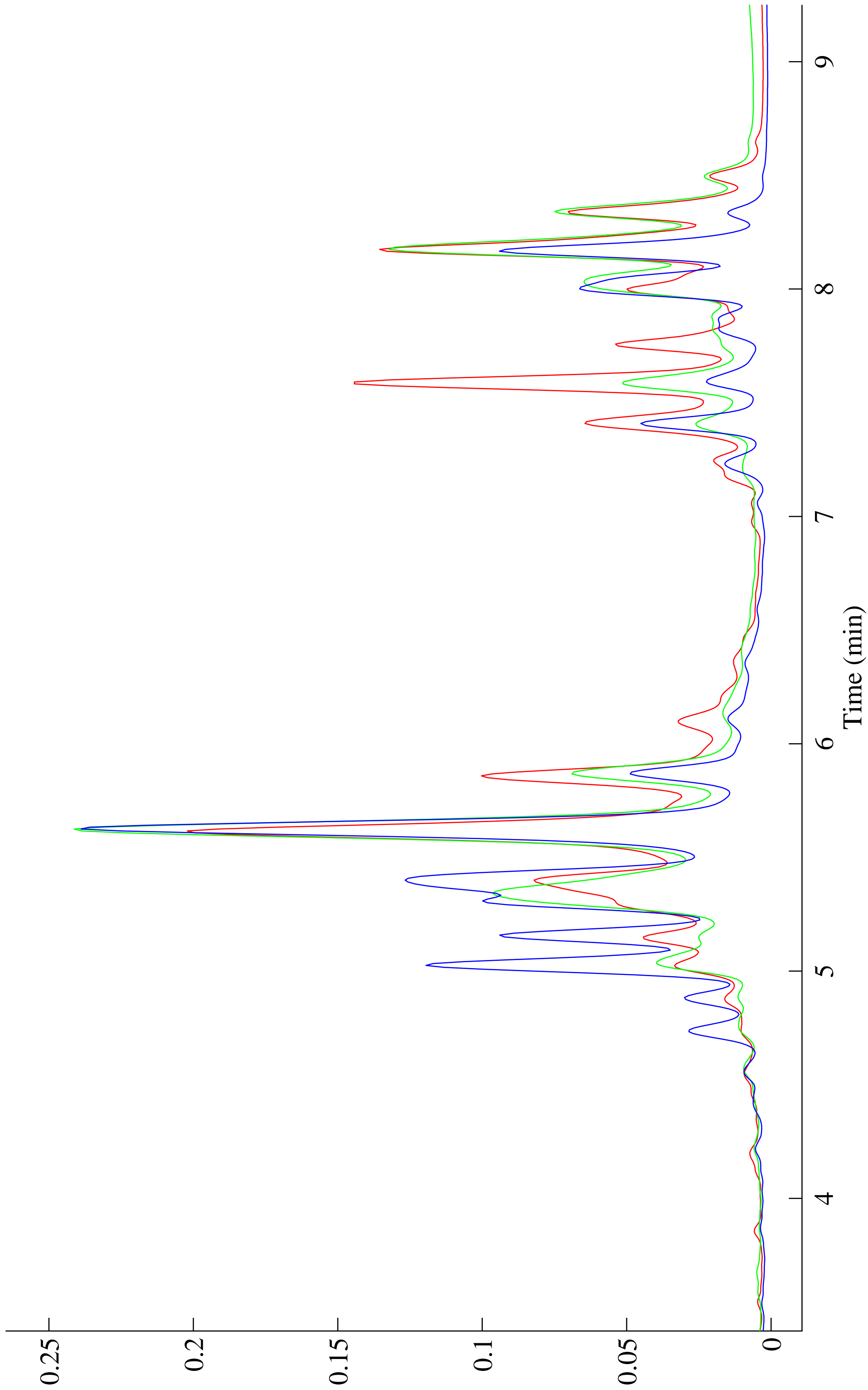


Figure 15. ***M. avium***, ***M. intracellulare***, **and** ***M. scrofulaceum***

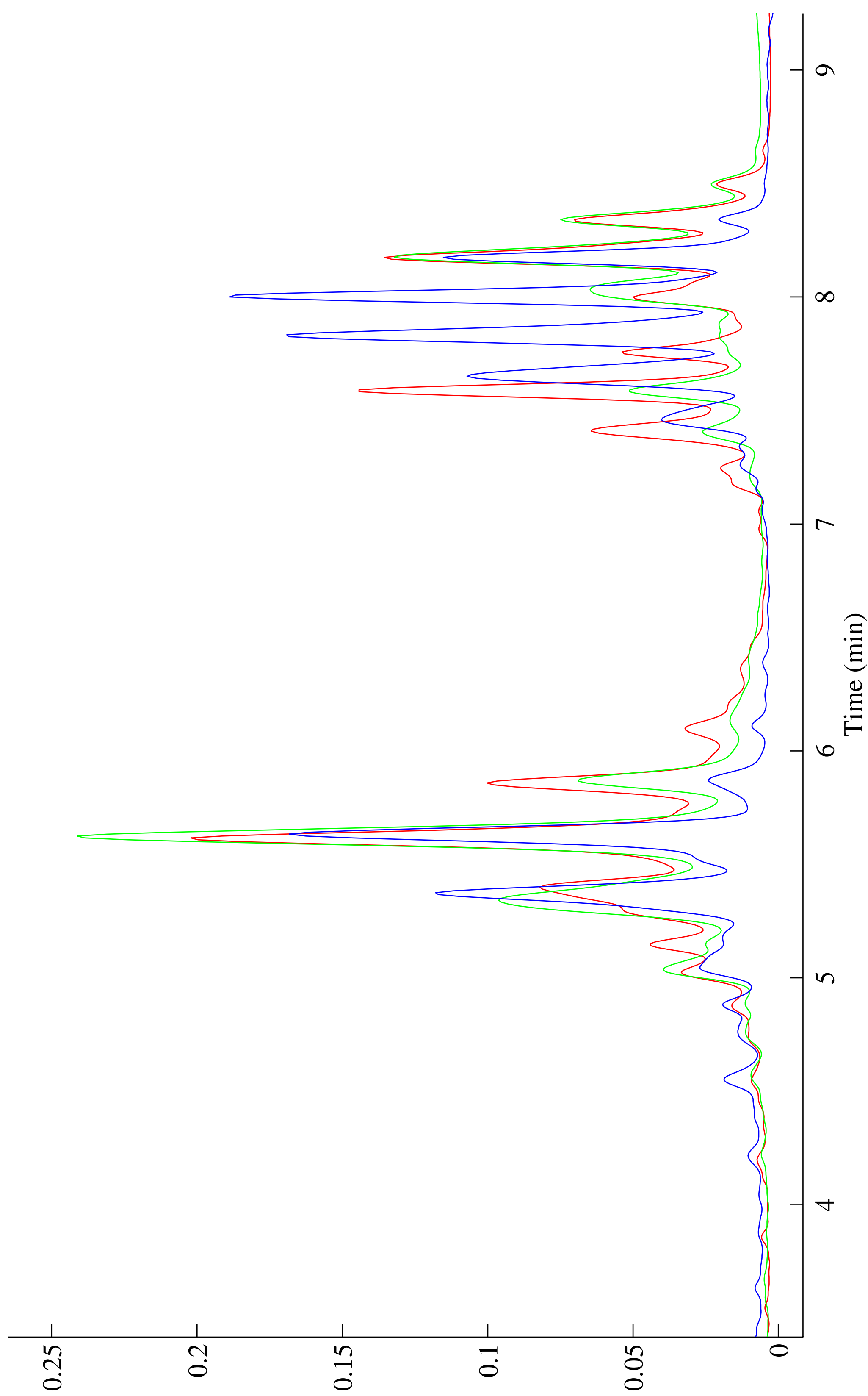


Figure 16. ***M. avium*, *M. intracellulare*, and *M. gordonae* (II)**

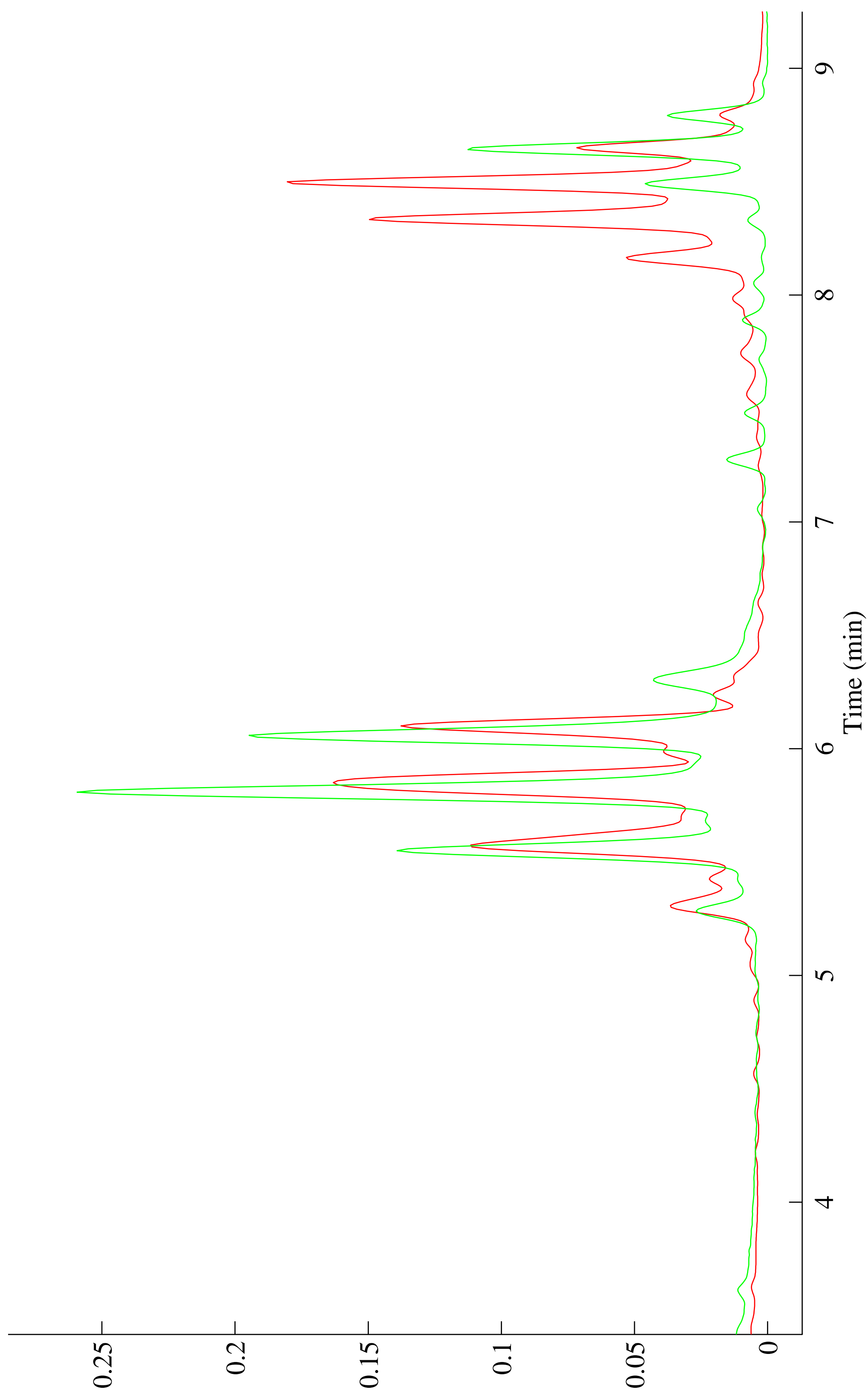


Figure 17. ***M. celatum*** **and** ***M. xenopi***

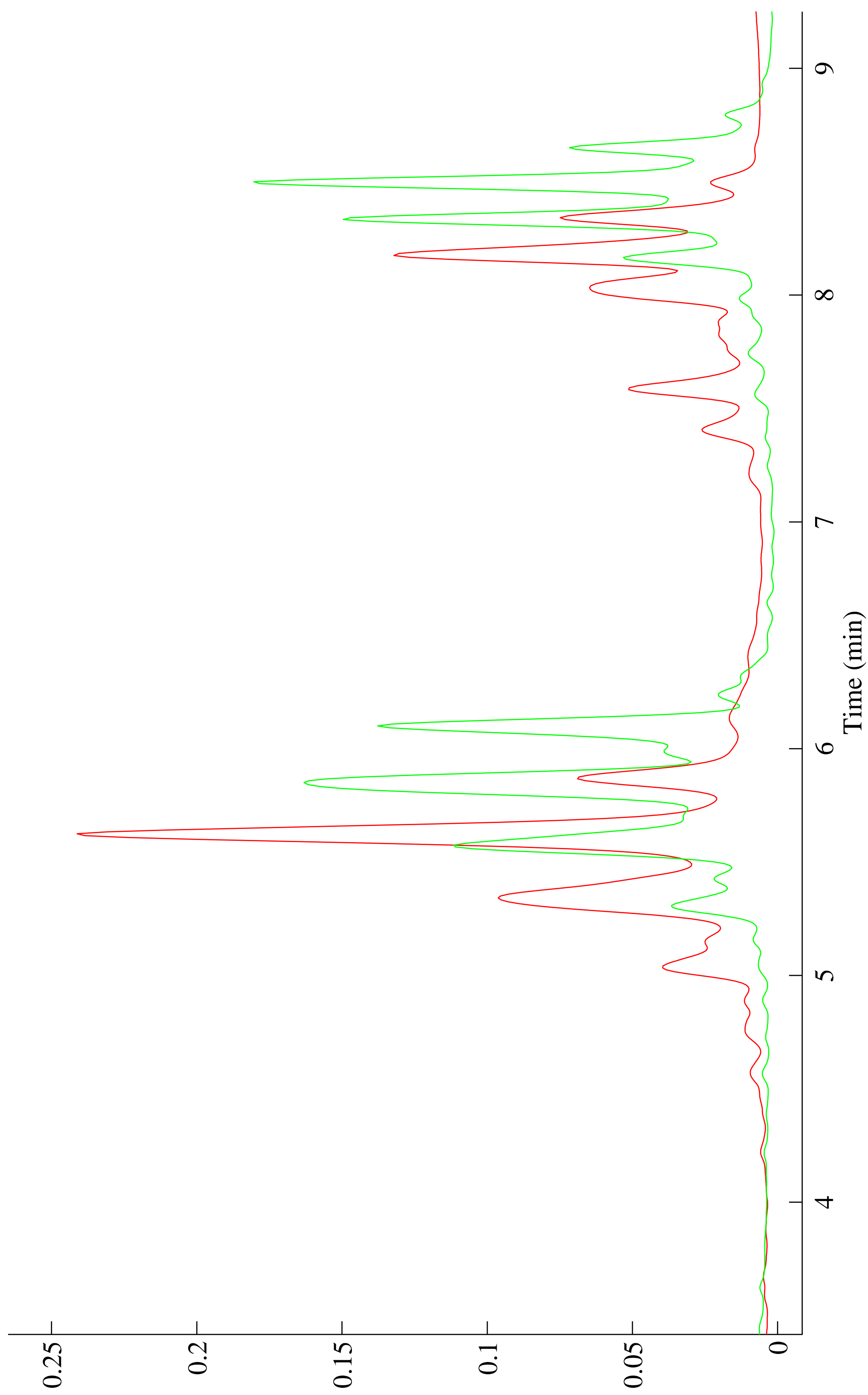


Figure 18. *M. intracellulare* and *M. celatum*

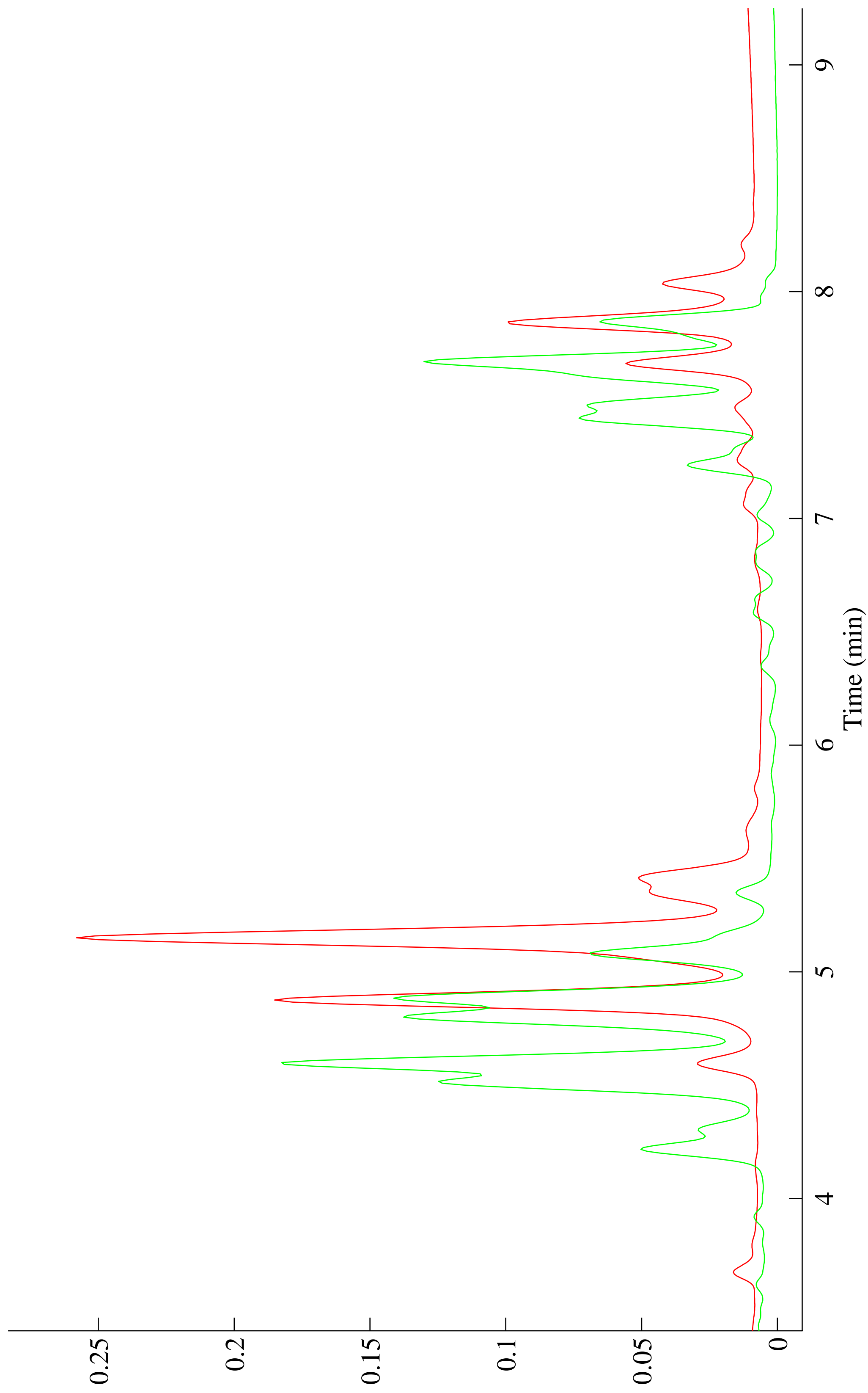


Figure 19. *M. terrae/M. nonchromogenicum group* and *M. mucogenicum*

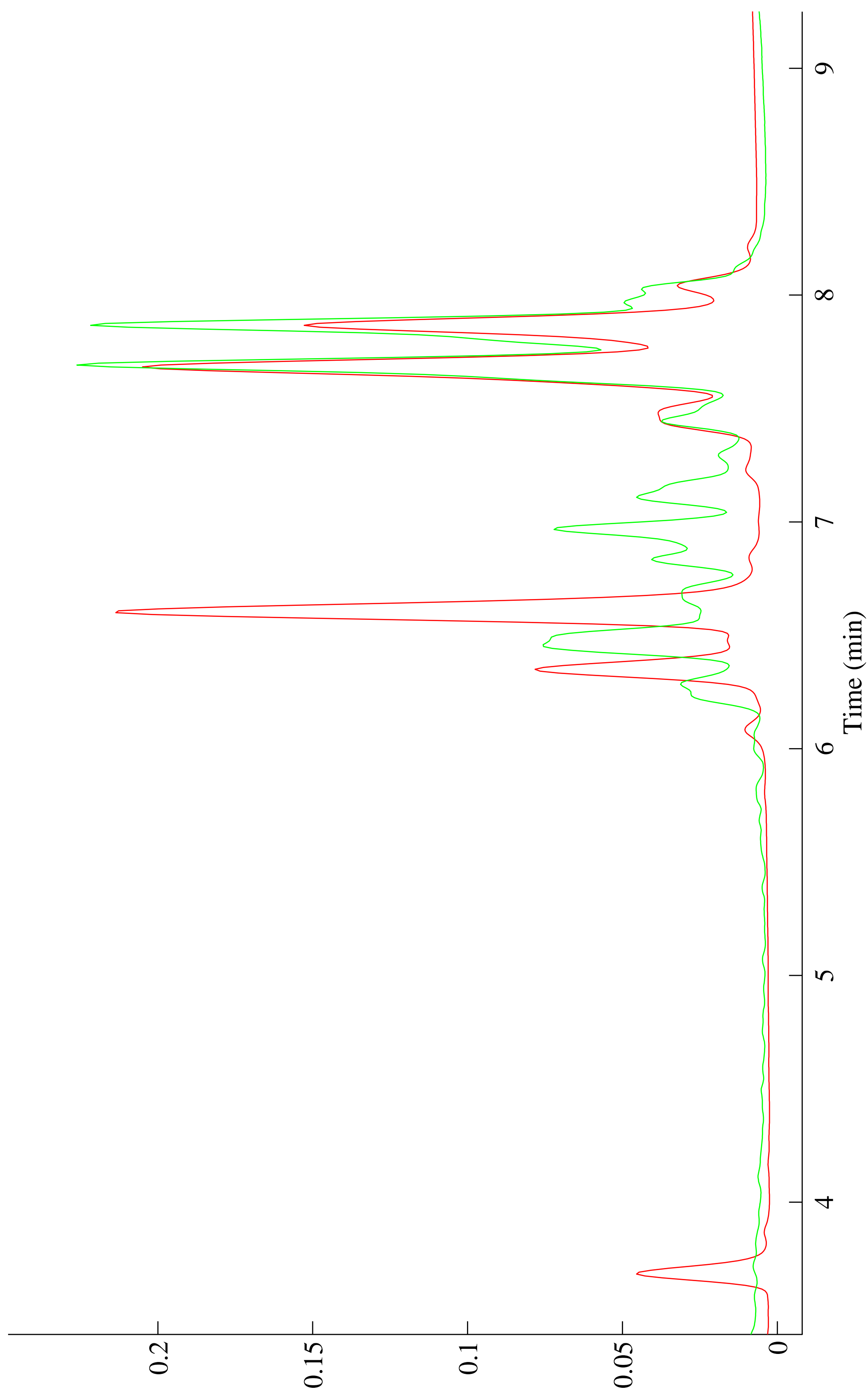


Figure 20. ***M. abscessus/M. chelonae group*** **and** ***M. fortuitum/M. peregrinum group***

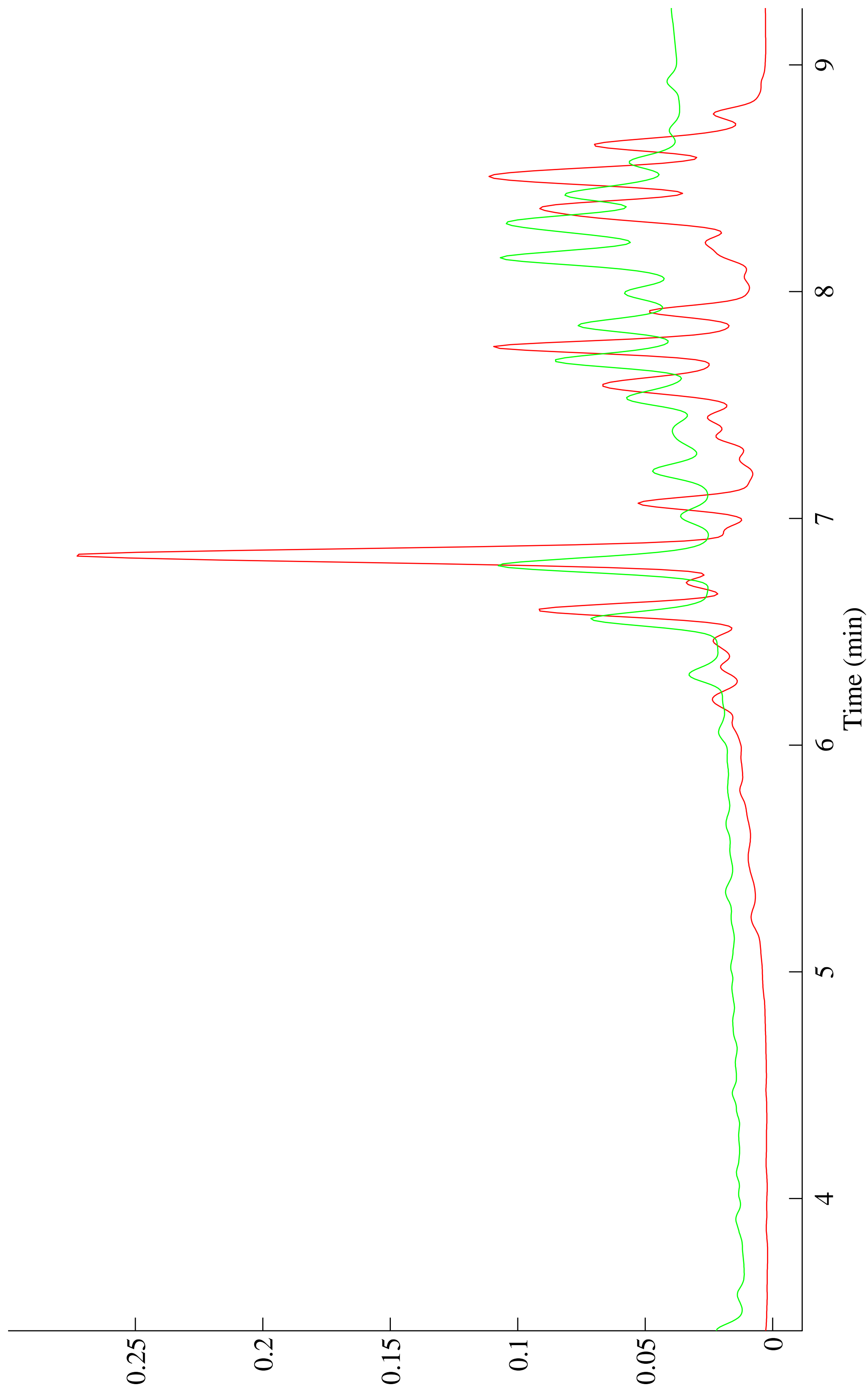


Figure 21. *M. simiae* and *M. malmoense*

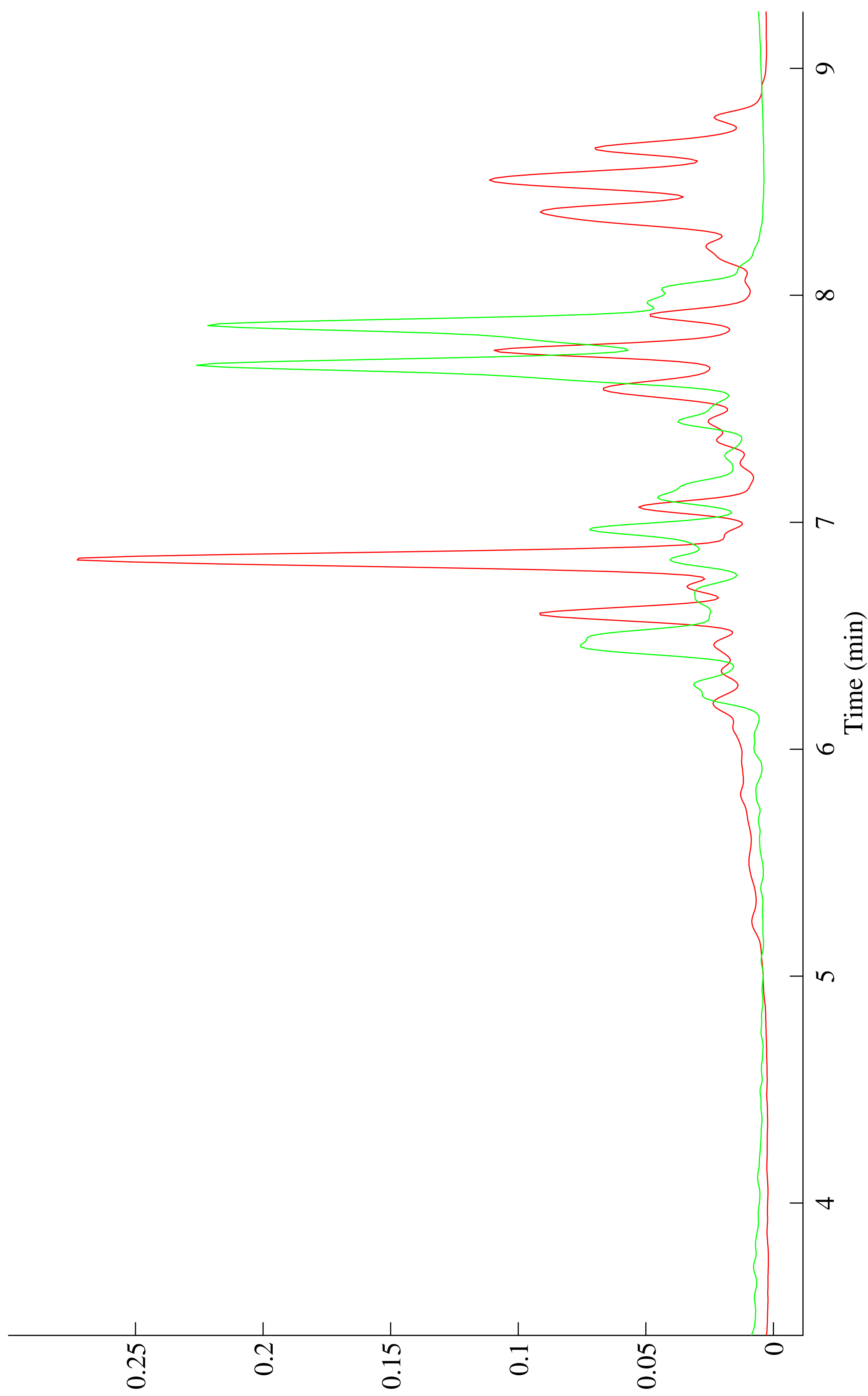


Figure 22. ***M. simiae*** **and** ***M. fortuitum/M. peregrinum*** **group**

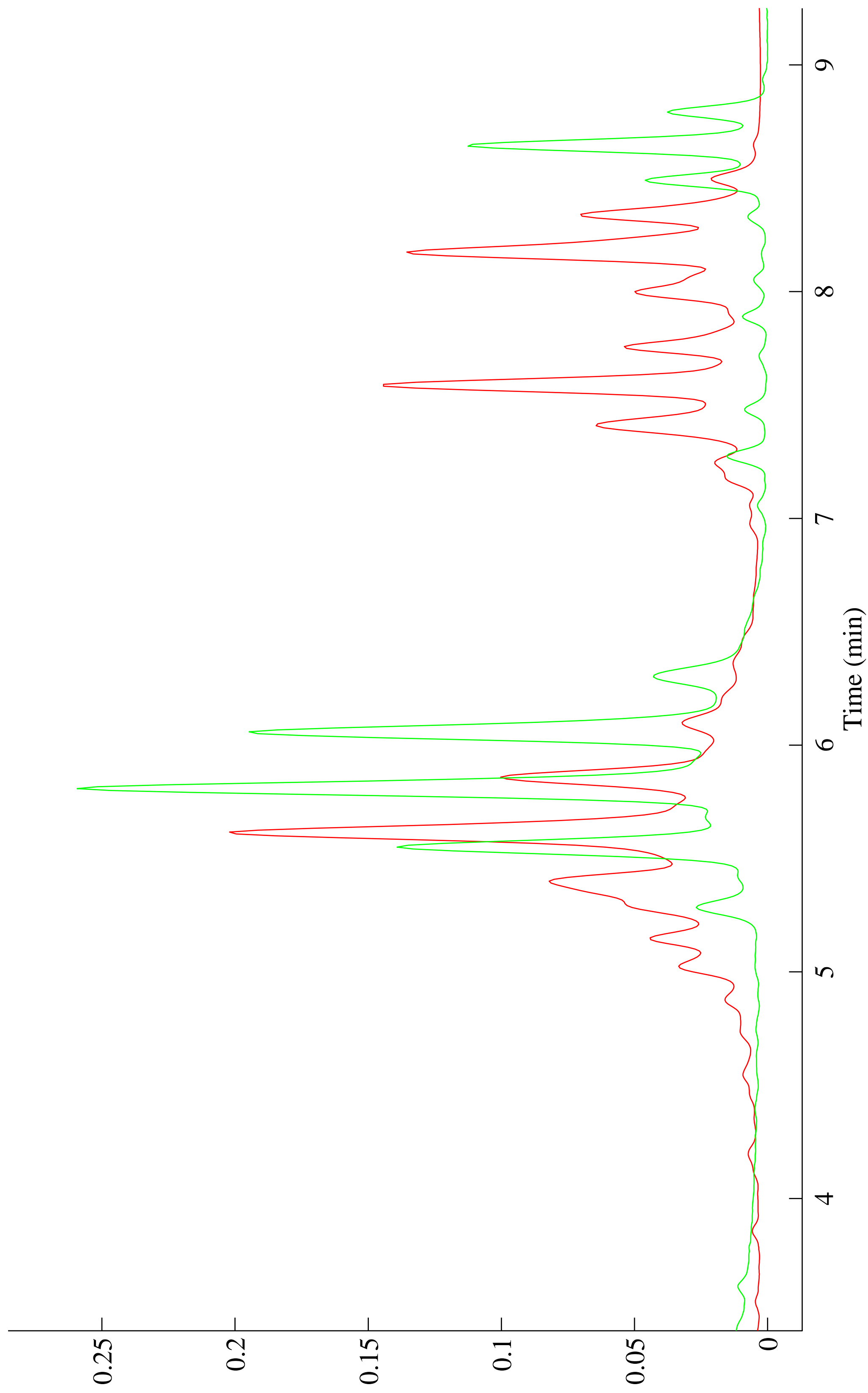


Figure 23. *M. avium* and *M. xenopi*

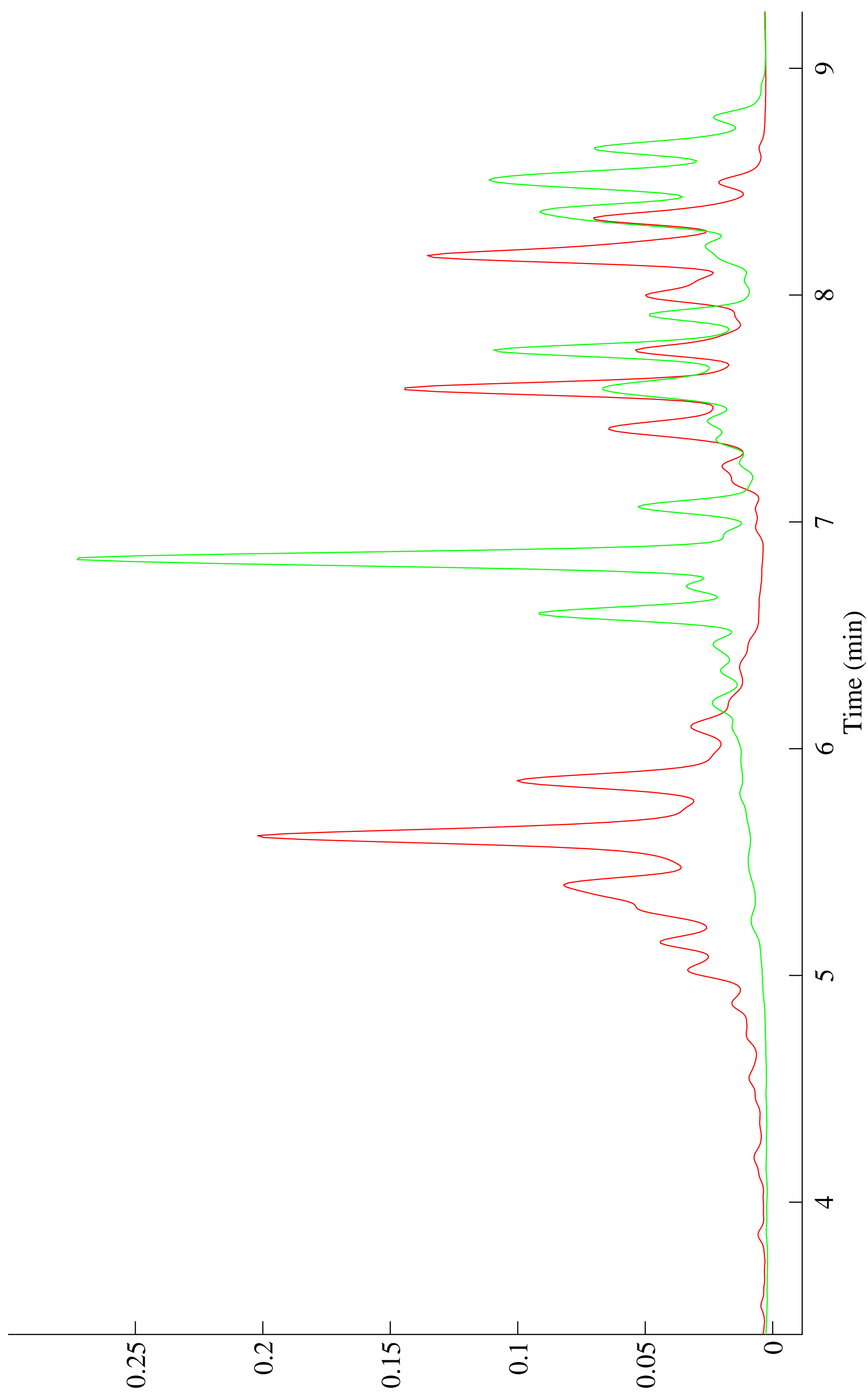


Figure 24. *M. avium* and *M. simiae*

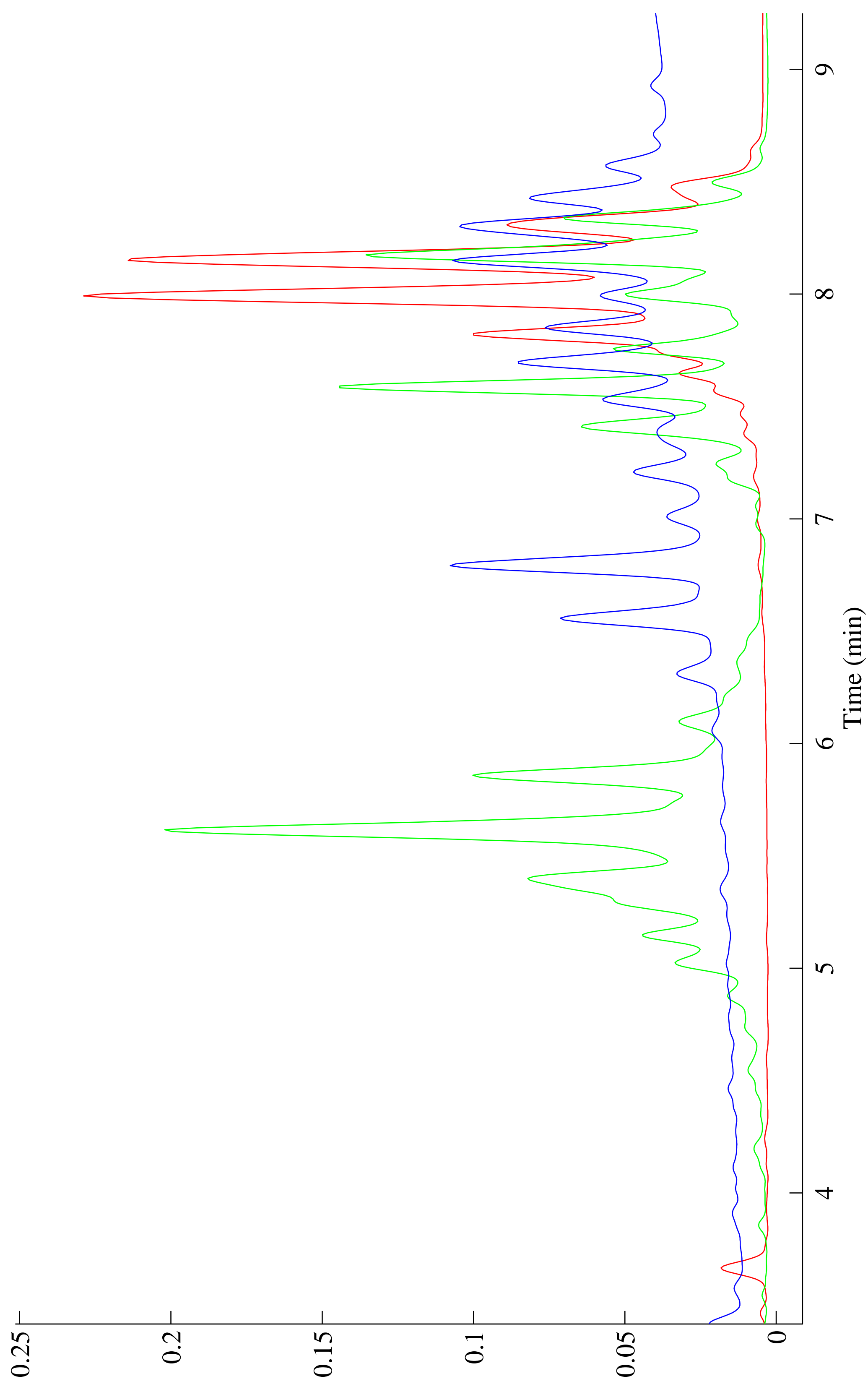


Figure 25. ***M. tuberculosis*, *M. avium*, and *M. malmoense***

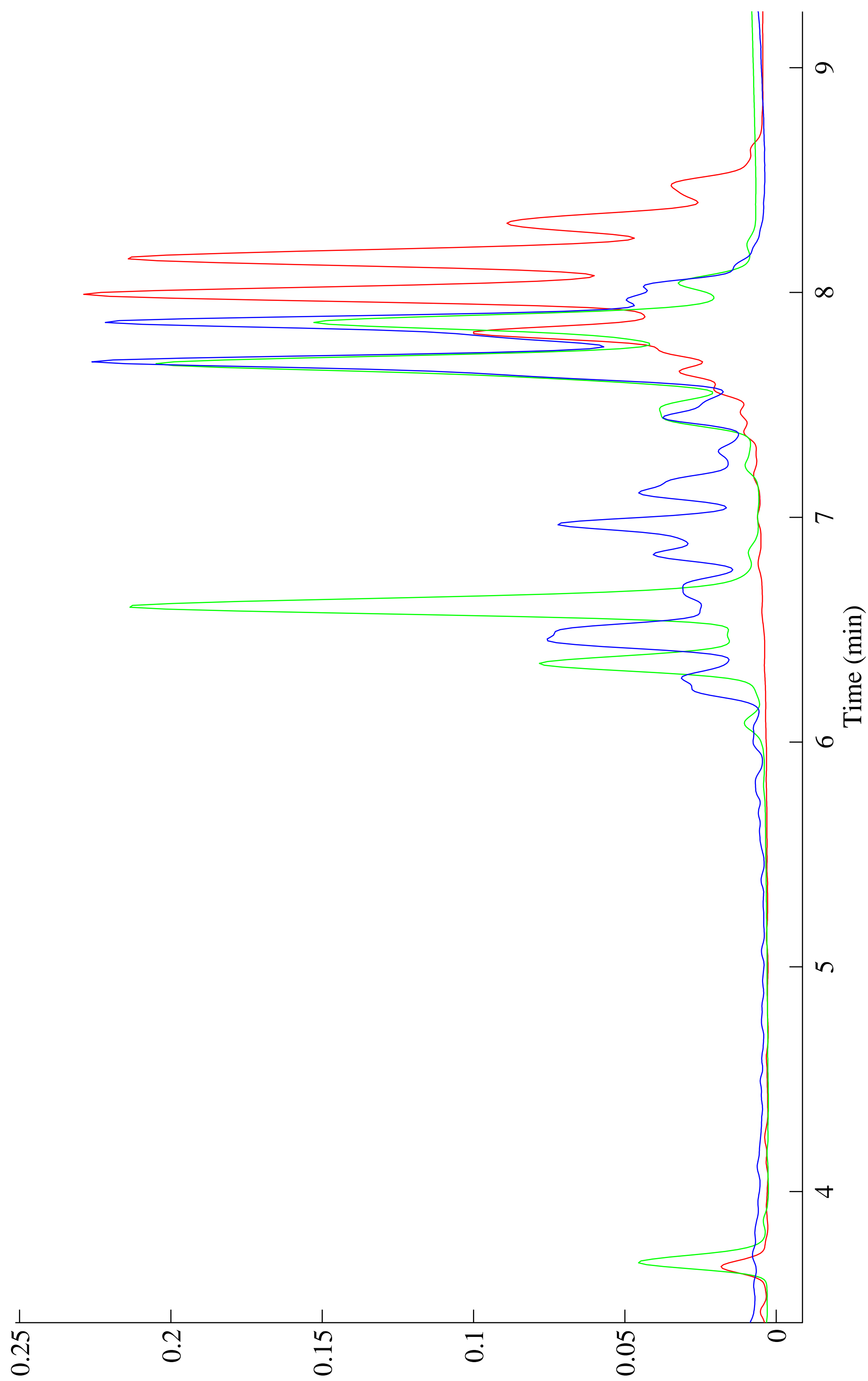


Figure 26. ***M. tuberculosis*, *M. abscessus/M. chelonae* group, and *M. fortuitum/M. peregrinum* group**

Commonly Used Chromatographic Terms

accuracy the degree of agreement between a value determined by an assay for a sample and the true value for that sample

adjusted retention time (ART) the time found by multiplying the RT of a given peak by a linear interpolation between the RRTs of designated reference peaks which bracket the given peak

analyte the target molecule to be detected by an assay

bandspreading the increase in volume of an injected sample caused by its travel through the column and chromatographic system

bonded phase the chemical function group bonded to the surface of the support matrix or stationary phase

chromatogram an electronic tracing of the signal from the detector versus time which is produced during a chromatographic run

continuous gradient elution a type of gradient elution in which the mobile phase composition is changed gradually over time or volume

detection limit the minimum amount of analyte that can be detected above the background noise under a given set of conditions

efficiency a standardized measure of the bandspreading of a column

eluent a mobile phase used to elute compounds from a chromatographic column

eluate the stream of mobile phase emerging from the column outlet

HPLC high performance liquid chromatography or high pressure liquid chromatography

isocratic a mobile phase composition which remains unchanged during the chromatographic run

mobile phase the fluid pumped through the column to elute the sample

mycolic acid α-branched-ß-hydroxy fatty acids contained in the cell walls of mycobacteria

precision the reproducibility of an assay such as that occurring with same day runs, runs between operators, or runs between instruments

regeneration a process of cleaning a column and returning it to its original chemical state

relative retention time (RRT) the time found by dividing (or subtracting) the RT of a given peak by the RT of a reference peak

resolution the measurement of the full degree of separation between two peaks, or the difference in retention divided by the average of the band widths

retention the time or volume between sample injection and the elution of a given peak

retention time (RT) the difference in time between the moment of injection and the time corresponding to the apex of a given peak

reverse phase chromatography the use of octadecyl phases bonded to silica or neutral polymeric beads

robust the ability of an assay to undergo small changes in particular operating parameters without affecting the performance

sensitivity the ability of an assay to detect small amounts of the target analyte

solvents the pure components of the mobile phase such as methanol or methylene chloride

specificity the ability of an assay to measure only the target analyte without interference by other molecules closely related to the target analyte

stationary phase the immobile phase material consisting of a solid support matrix and a bonded phase surface, which is packed into the column upon which the separation takes place

validation documented evidence that the analytical process will consistently give results that meet the intended purpose

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