

Metabolomics Analysis of Tuberculosis Drug Activity Using an Agilent 6545 Q-TOF LC/MS

Application Note

Metabolomics

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Abstract

Tuberculosis (TB) is both the leading cause of deaths due to an infectious disease and the leading cause of deaths due to a curable disease¹. However, drug resistance¹ is increasing while the pipeline of new drugs stagnates, and knowledge of existing drugs remains incomplete. Pyrazinamide (PZA) is a frontline TB drug¹ whose mechanism of action remains among the most poorly understood.

This Application Note presents a high-performance ion-pairing reversed-phase (IP-RP) Q-TOF LC/MS method that has enabled the biologically unbiased study of the impact of PZA on the *Mycobacterium tuberculosis* metabolome. Coupled with batch feature extraction and multivariate statistical analysis software, this workflow enabled the discovery of activity-specific metabolic changes that may help explain PZA's unique metabolic effects.



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Introduction

Pyrazinamide (PZA) is a frontline drug used in the treatment of tuberculosis (TB)¹. Unfortunately, widespread use has resulted in growing rates of PZA resistance, while knowledge of PZA's mechanism of action remains unresolved¹. Existing knowledge indicates that PZA is a prodrug that undergoes conversion to pyrazinoic acid (POA), the pyrazine analog of nicotinic acid, by the NAD salvage pathway-specific enzyme nicotinamidase (PncA)¹. Knowledge of the downstream biochemical consequences of POA production is incomplete. NAD biosynthesis and NAD-dependent reactions are two potentially direct, but understudied, biochemical targets of PZA. The reasons for this are both biological and experimental in nature due, in part, to limitations of existing analytical methodologies that have hindered broad and sensitive detection of cellular metabolites.

This Application Note presents a high-performance Q-TOF LC/MS method that enabled the biologically unbiased study of the impact of PZA on the *Mycobacterium tuberculosis* metabolome. In this method, the retention time reproducibility and compound separation of the IP-RP chromatography² combined with the high resolution and mass accuracy of the Agilent 6545 Q-TOF improved selectivity, analytical sensitivity, and reproducibility for metabolites in profiling experiments. Furthermore, the Agilent SWARM Autotune³ and Jet Stream (AJS)⁴ technologies were used to selectively enhance signals of smaller, labile metabolites and challenging phosphorylated metabolites by optimizing the ion transmission of a Q-TOF, and improving the efficiency of ion generation and sampling.

Experimental

LC/MS Metabolomics workflow

A Q-TOF LC/MS metabolomics workflow (Figure 1) was developed. This workflow includes the following steps:

1. Acquire high quality data from the tuberculosis cell extracts (three samples/biological group of six biological groups) using the Q-TOF LC/MS with an AJS source in negative ion mode.
2. Find metabolites from raw data using the Profinder⁵ Batch Targeted Feature Extraction algorithm with a user-created Agilent Personal Compound Database and Library (PCDL) file (.cdb) containing 111 metabolites.
3. Perform differential analysis using Mass Profiler Professional (MPP) (B.14.0), and map the differential metabolites to KEGG pathways to gain insight into the mechanistic details of PZA activity.

LC/MS Method

The ion-pairing reversed-phase (IP-RP) chromatography method² was used to achieve a wide coverage of metabolite classes and improve retention time reproducibility. The previous LC gradient² was slightly modified by holding 99 %B for an additional 2 minutes to ensure all coenzyme A (CoA) metabolites were eluted. LC/MS analyses were performed using an Agilent 1290 Infinity UHPLC system coupled to an Agilent 6545 Q-TOF with either Dual Electrospray (ESI) or Dual AJS source. The LC consisted of a binary pump with seal wash (G4220A), an autosampler (G4226A) with thermostat (G1330B), a thermostatted column compartment (G1316C), and an isocratic pump (G1310A) with a 100:1 splitter (G1607-60000) for reference mass addition. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution. Tables 1 and 2 summarize the optimized LC and MS conditions.

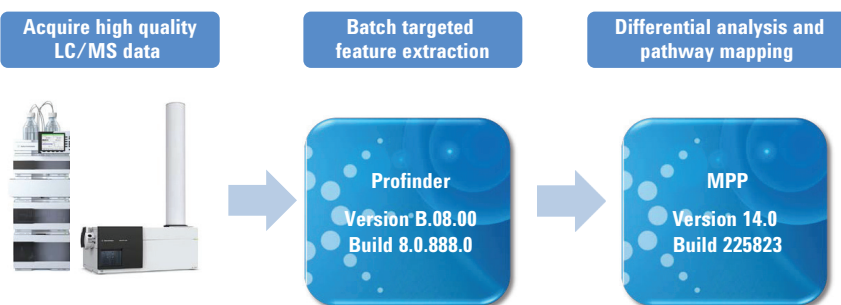


Figure 1. LC/MS metabolomics workflow for high quality data acquisition and analyses.

Preparation of metabolite standards and TB cell extracts

For analytical method optimization, all metabolite standards were prepared individually in either MeOH/H₂O (50/50) or MeOH (for CoA metabolites). The mixtures of metabolite standards were prepared before LC/MS analysis.

For the tuberculosis study, virulent *M. tuberculosis* cells were grown on hydrophilic Durapore membrane filters, and transferred to plates containing bacteriostatic or a bactericidal dose of PZA. In addition to the dose-response, experiments were controlled by altering the pH of the experimental media from pH 6.6 (wherein PZA has little or no activity) to pH 5.5 (wherein PZA is active). At various time points, filters were harvested for extraction. Metabolism was quenched by plunging the filters into 2:2:1 acetonitrile:methanol:water at -20 °C.

Quenched cells were lysed by bead beating in a Precellys homogenizer (Bertin Instruments), and lysates were sterile filtered twice and snap frozen on dry ice. At the time of analysis, samples were vortexed, centrifuged, and transferred to the HPLC vials for direct injection into the LC/MS system. Table 3 summarizes the sample information of six biological groups. Each sample was injected twice as technical replicates.

Table 1. LC parameters.

Agilent 1290 Infinity LC System	
Analytical column	Agilent ZORBAX Extend C18, 2.1 × 150 mm, 1.8 μm (p/n 759700-902)
Guard column	Agilent ZORBAX SB-C8, 2.1 mm × 30 mm, 3.5 μm (p/n 873700-936)
Column temperature	40 °C
Injection volume	5 μL
Autosampler temperature	4 °C
Mobile phase	A) 97 % water/3 % methanol containing 5 mM TBA and 5.5 mM acetic acid B) Methanol containing 5 mM TBA and 5.5 mM acetic acid
Seal wash solvent	Isopropanol : H ₂ O (1:1 v/v)
Flow rate	0.25 mL/min
Gradient	Time (min) %B 0 0 3.5 0 4 30 7.5 30 8 35 15 35 20 99 24 99 24.5 0
Stop time	24 minutes
Post time	5 minutes

Table 2. MS parameters.

Agilent 6545 Q-TOF	
Ion mode	Negative
Source	Agilent Dual ESI or AJS
Capillary voltage	3,500 V
Dry gas temperature	250 °C
Dry gas flow	13 L/min
Nebulizer pressure	35 psi
Sheath gas temperature	225 °C or 350 °C for AJS
Sheath gas flow	12 L/min
Nozzle voltage	2,000 V
Fragmentor	Optimized by SWARM Autotune technology
Skimmer	Optimized by SWARM Autotune technology
Oct 1 RF Vpp	Optimized by SWARM Autotune technology
MS range	60–1,600 <i>m/z</i>
MS acquisition rate	1.5 spectra/sec
Reference mass	62.0327 and 983.0347
Reference pump flow	0.5 mL/min
Reference delivery	Agilent 1100 isocratic pump with 100:1 splitter (p/n G1607-60000)
Instrument mode	Extended dynamic range (2 GHz)

Table 3. Summary of the sample information.

pH\Treatment	Untreated samples	PZA (bacteriostatic)	PZA (bacteriocidal)
pH 6.6 (little or no activity)	Control pH6.6 (triplicate)	PZA(s)_pH6.6 (triplicate)	PZA(c)_pH6.6 (triplicate)
pH 5.5 (activity)	Control pH5.5 (triplicate)	PZA(s)_pH5.5 (triplicate)	PZA(c)_pH5.5 (triplicate)

Results and Discussion

Agilent SWARM Autotune enables faster and easier optimization of ion transmission

Agilent SWARM autotune uses a robust multidimensional optimization algorithm to optimize up to 21 parameters simultaneously. This algorithm allows researchers to automatically optimize ion transmission 4x faster than previous Agilent Q-TOFs. The optimization options for small molecules include three mass ranges: 50–1,700 m/z , 50–750 m/z , and 50–250 m/z . For the lower mass ranges of 50–750 m/z and 50–250 m/z , ion optics can be optimized to preserve labile compounds that may otherwise suffer from low sensitivity due to unintended fragmentation. To determine the optimal tuning condition for analyzing TB cell extracts, we evaluated the signal responses for a set of metabolite standards in the mass ranges of 50–1,700 m/z and 50–750 m/z with fragile and stable ion tuning. Figure 2 shows the optimal analytical sensitivity obtained under three tuning conditions. It is evident that 50–750 m/z tuning provides better analytical sensitivity than the 50–1,700 m/z tuning, and that the fragile ion tuning further enhanced the smaller, labile metabolite signals. Thus, 50–750 m/z fragile ion tuning was chosen for subsequent analyses.

AJS Technology improves analytical sensitivity across a wide range of metabolites

AJS technology uses thermal gradient focusing ESI, and has demonstrated a substantial boost (5–15x) in analytical sensitivity for thermally stable molecules⁴. In this study, we assessed a set of endogenous metabolite standards including small labile ones. Figure 3 shows the signal responses of 17 representative metabolites obtained with either AJS or a conventional ESI source. AJS clearly provided better analytical sensitivity than the conventional ESI, especially for those challenging phosphorylated metabolites. We also found that signal enhancement is highly

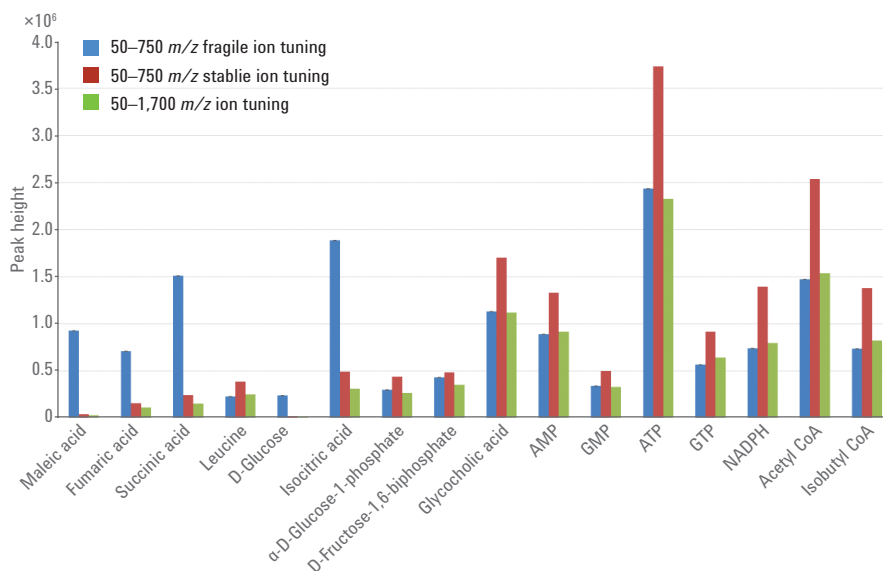


Figure 2. Easy optimization of ion transmission using Agilent SWARM Autotune algorithm and dual ESI.

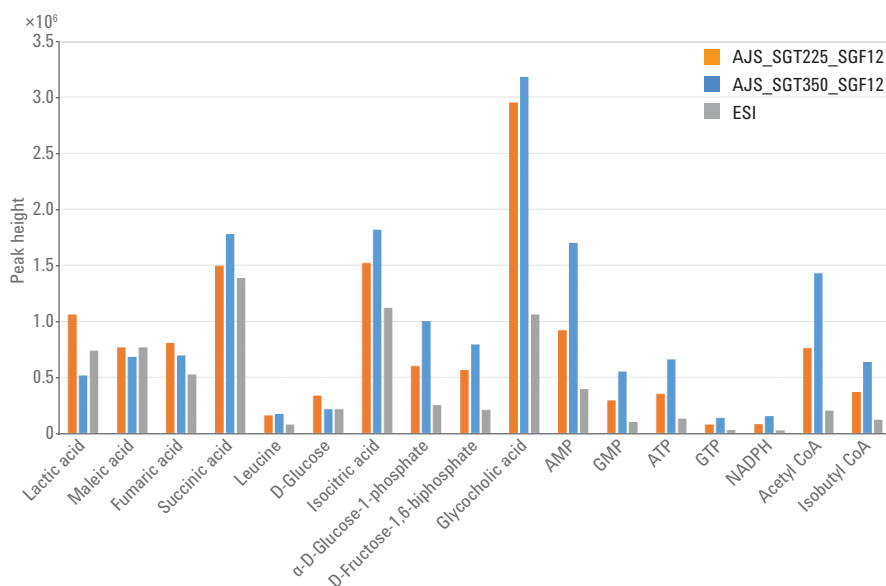


Figure 3. AJS enhances metabolite signals compared to a conventional ESI.

dependent on the thermal stability of a metabolite and sheath gas temperature. Labile metabolites such as lactic acid and maleic acid respond better at a lower sheath gas temperature (225 °C versus 350 °C). To maximize the analytical sensitivity for CoA and nucleotide metabolites, a sheath gas temperature of 350 °C was used for analyzing the cell extract samples.

The Agilent 6545 Q-TOF delivers accurate mass and isotope ratio measurement

Accurate mass measurement is critical for compound confirmation and molecular formula generation for unknown compounds, but is not sufficient by itself⁶. Isotope ratios provide essential orthogonal information that can greatly reduce the number of plausible molecular

formulas, and increase confidence in the result. To deliver excellent mass accuracy and accurate isotope ratio measurements, Agilent TOF-based mass spectrometers have incorporated several innovative hardware designs:

- A dual ion source for reference mass addition to ensure consistent real-time calibration of every spectrum
- A thermally stable flight tube to ensure a uniform flight path that resists variations in lab temperature
- Low-noise high-voltage power supplies to maintain precise voltages
- Proprietary ion pulser and detector ADC designs with thermal compensation circuitry to maintain stable mass accuracy

Figure 4 demonstrates the excellent mass accuracy of the Agilent 6545 Q-TOF. Mass errors of less than ± 1 ppm were achieved for 54 out of 64 mass measurements under four different tuning conditions. Based on the accurate mass measurements, the relative isotope abundance (RIA) ratios were further calculated from the same dataset. The isotopic ion abundance ratio (RIA) is defined as $M+1/M$ ($^{13}\text{C}_1/^{12}\text{C}$), where M represents the monoisotopic ion. Agilent MassHunter Qualitative Analysis software was used to calculate the theoretical RIA_{theo} values. The RIA errors were calculated as shown in Equation 1:

$$\text{RIA error (\%)} = 100 \times (\text{RIA}_{\text{exp}} - \text{RIA}_{\text{theo}}) / \text{RIA}_{\text{theo}}$$

Equation 1.

Figure 5 shows the RIA errors from the data acquired using extended dynamic range mode with the high resolution slicer. Overall, the RIA errors were below (10 %) for monoisotopic ions (M) with peak intensities in the range of $3.8 \times 10^3 \sim 3.5 \times 10^5$ (Figure 5A), and the first isotopic ions (M+1) with peak intensities in the range of $1.8 \times 10^2 \sim 4.3 \times 10^4$ (Figure 5B). The mean RIA error was -3.69% . Similar results were also obtained for the data acquired under the high-sensitivity slicer mode. These results

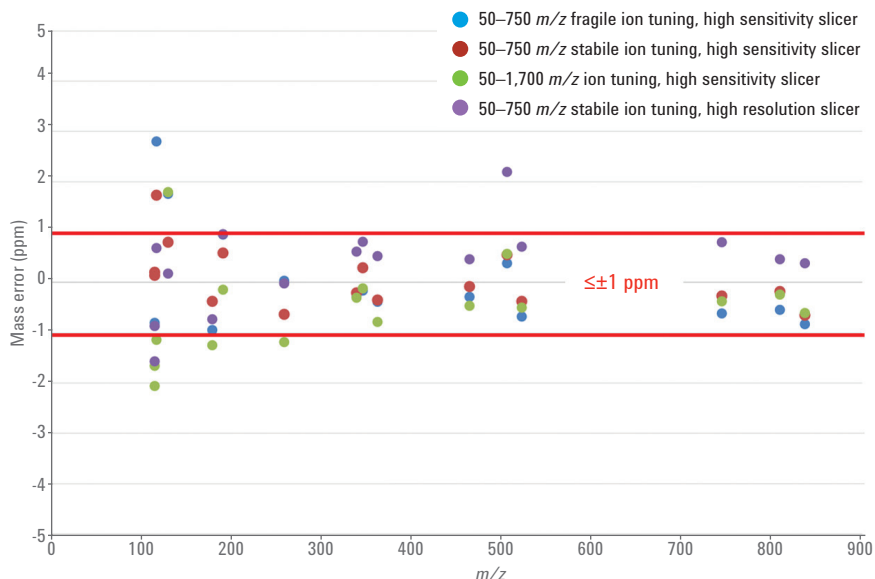


Figure 4. The Agilent 6545 Q-TOF provides excellent mass accuracy, with mass errors $< \pm 1$ ppm for 54 out of 64 mass measurements.

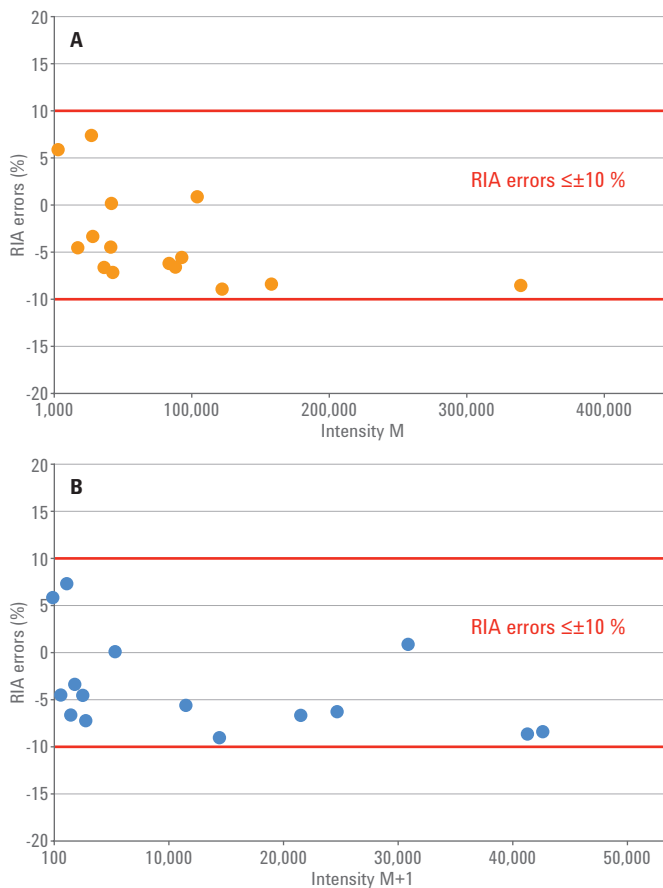


Figure 5. The Agilent 6545 Q-TOF delivers accurate relative isotope abundance measurements. A) Intensity M represents the peak height of the monoisotopic ion (^{12}C). B) Intensity M+1 represents the peak height of the isotopic ion (^{13}C).

clearly demonstrate that the 6545 Q-TOF delivers superior relative isotopic abundance measurements compared to an orbital-trapping FTMS⁷.

Data analysis

Agilent MassHunter Profinder – Batch Targeted Feature Extraction

Profinder was used for batch processing of the accurate mass LC/MS data. The Profinder Batch Targeted Feature Extraction provides a useful workflow for biological pathway-driven data analysis. In this study, Agilent Pathways to PCDL (B.07.00) software was used to create an Agilent PCDL file (.cdb)

from KEGG metabolic pathways specific to *M. tuberculosis* strain H37Rv. The created PCDL file was then edited in Agilent PCDL Manager software to remove and add some metabolites based on the study needs. The final database containing 111 metabolites was used as a target list for Batch Targeted Feature Extraction in MassHunter Profinder software. After batch targeted feature extraction, the results were reviewed and manually curated as necessary. Figure 6 illustrates that 73 out of 111 metabolites were found from the raw data. By selecting a metabolite, for example UDP-Glucose, in the Compound Groups panel, the Compound Details,

Chromatogram Results, and MS Spectrum Results for that metabolite are displayed. The compound-centric view in Profinder provides useful insight into the quality of the analytical data across different biological groups as well as the reproducibility of replicates within a sample group. The Profinder batch results can be exported as Compound Exchange Format files that could be imported into MPP (B.14.0) for statistical and pathway analyses.

Although targeted data mining is presented here, the same set of data can readily be analyzed using untargeted data mining with subsequent identification of novel metabolites.

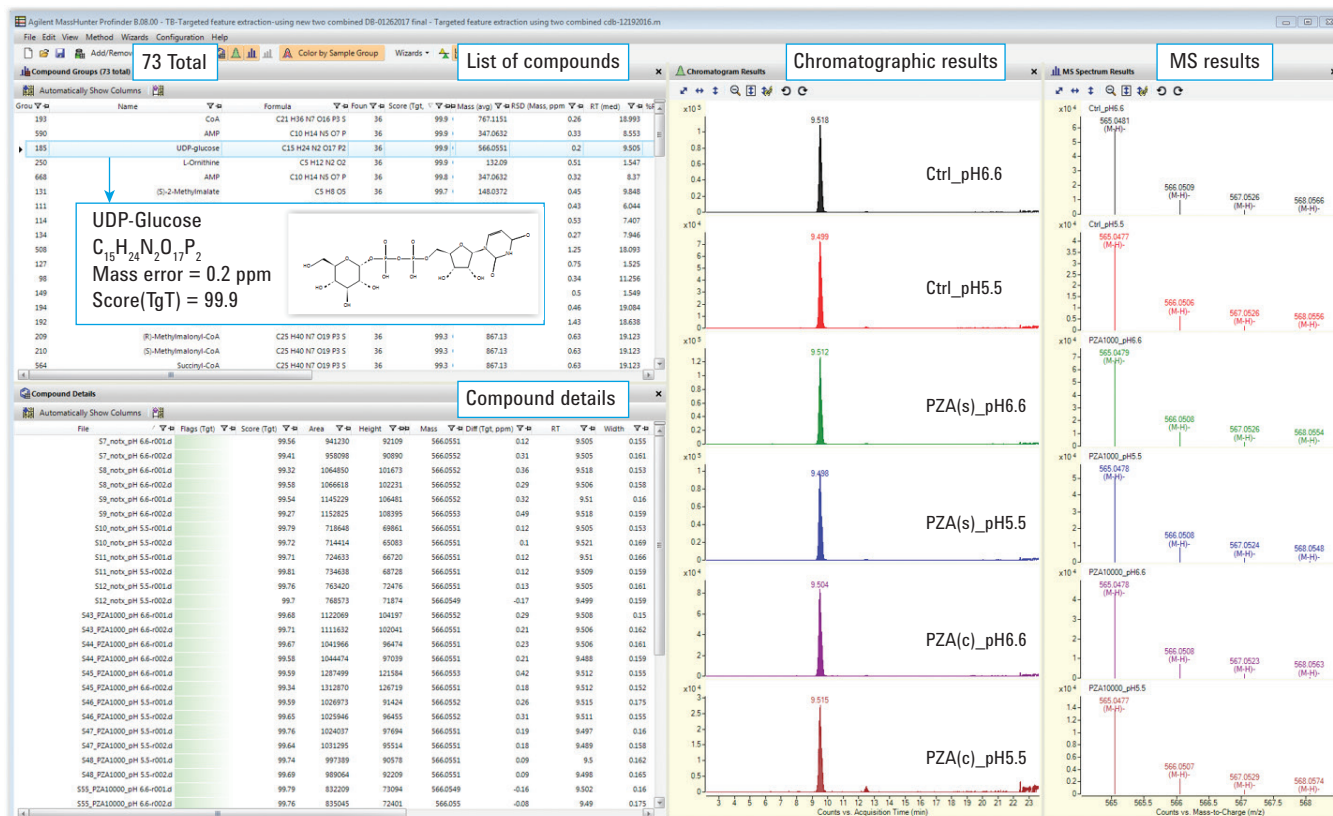


Figure 6. Targeted feature extraction results from the cell extracts in the control and PZA-treated biological groups.

MPP – differential analysis of PZA-specific metabolic changes

MPP is a powerful chemometrics software with many statistical tools that allow researchers to get biological insight from complex data faster and easier. Principal Component Analysis (PCA) is one of the most common unsupervised data analysis tools used to identify data patterns and quality. Figure 7 illustrates that PCA clearly detected the separation of biological groups with cell growth at two different pH conditions. Furthermore, PCA captures the overall variability in the metabolome of actively dividing cells taken from neutral pH in comparison with the lower variability of the nongrowing condition (pH 5.5).

A volcano plot provides a pair-wise comparison between two different biological groups where the results can be visualized by fold-change and significance simultaneously. In this study, PZA-treated samples showed dose-response efficacy at pH = 5.5, while at pH = 6.6 (bacteriostatic dose), efficacy was completely lost. To gain insight into PZA's mechanism of action, we performed differential analyses using MPP's Filter on Volcano Plot feature to compare the control versus PZA-treated samples at pH 5.5, as well as the PZA-treated samples with growth conditions at pH 5.5 versus pH 6.6. Figure 8 shows the volcano plot of the metabolites in the PZA(s)-treated samples versus the control at pH 5.5 with a cutoff of $p < 0.05$ and fold change (FC) > 1.5 . Fifty-four of the 73 metabolites displayed statistical significance, and 28 of them showed at least 1.5 fold changes in the abundance in the PZA(s)-treated samples compared to the control at pH 5.5. Using this approach, we were able to detect statistically significant metabolites among the treatments. For example, 25 differential metabolites were detected with a $p < 0.05$ and FC > 1.5 for the PZA(s)-treated group at pH 5.5 compared to that at pH 6.6.

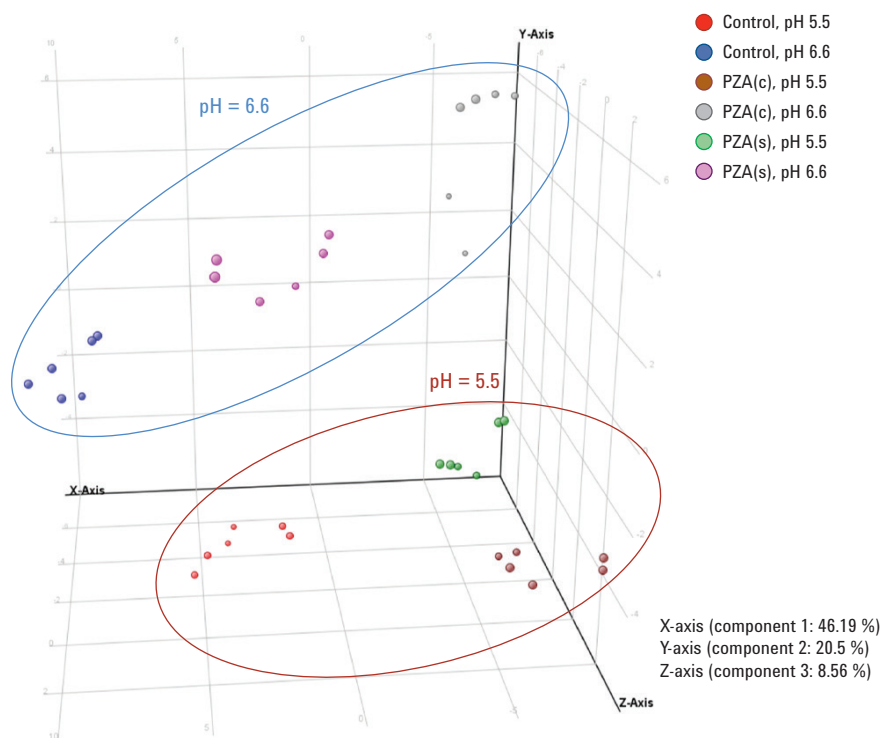


Figure 7. The PCA plot can clearly detect the separation of the treatment groups at two different pH conditions.

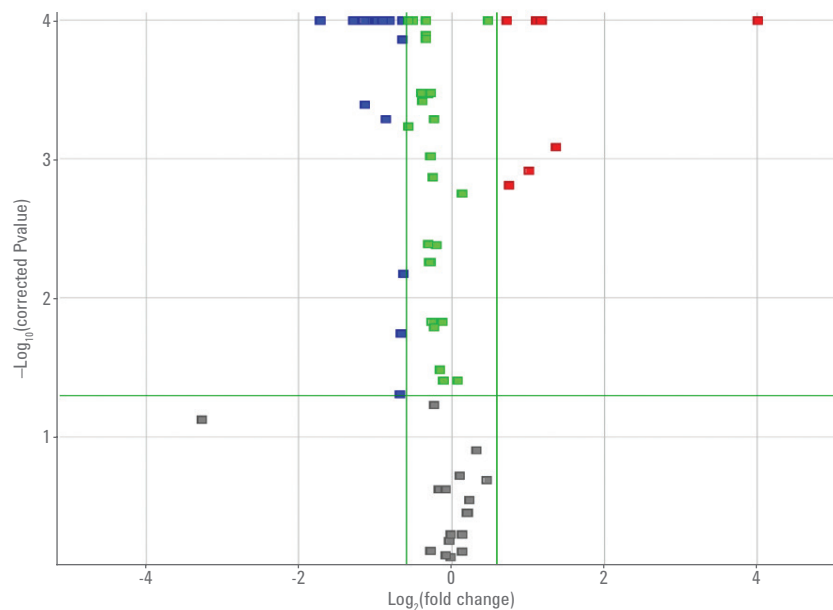


Figure 8. The volcano plot of the metabolites in the PZA-treated samples versus the control at pH 5.5. Red dots represent those metabolites whose abundances are higher in the PZA-treated samples than in the control ($P < 0.05$, FC > 1.5). Blue dots represent those metabolites whose abundances are lower in the PZA-treated samples than the control ($P < 0.05$, FC > 1.5).

Cluster analysis is a powerful method to organize entities and conditions in a dataset into clusters based on the similarity of their abundance profiles. Hierarchical clustering is one of the most widely used clustering techniques for unsupervised analysis of mass abundance data, in which the most similar abundance profiles are joined to form a

group. These are further organized in a tree diagram, until all data form a single group. Figure 9 shows the hierarchical clustering result performed on both conditions and entities. Each column represents a condition. In this case, a control or a treatment group, and each row represents an entity or a metabolite. The metabolites are displayed as a heat

map, and the relative abundance is indicated by color. The tree branching clearly distinguishes the metabolic profile of PZA-treated cells incubated at pH 5.5 from those incubated at pH 6.6. Consistent with PZA's selective activity at pH 5.5, we observed a grouping of PZA(s)-treated cells at pH 6.6 with those of the control samples.

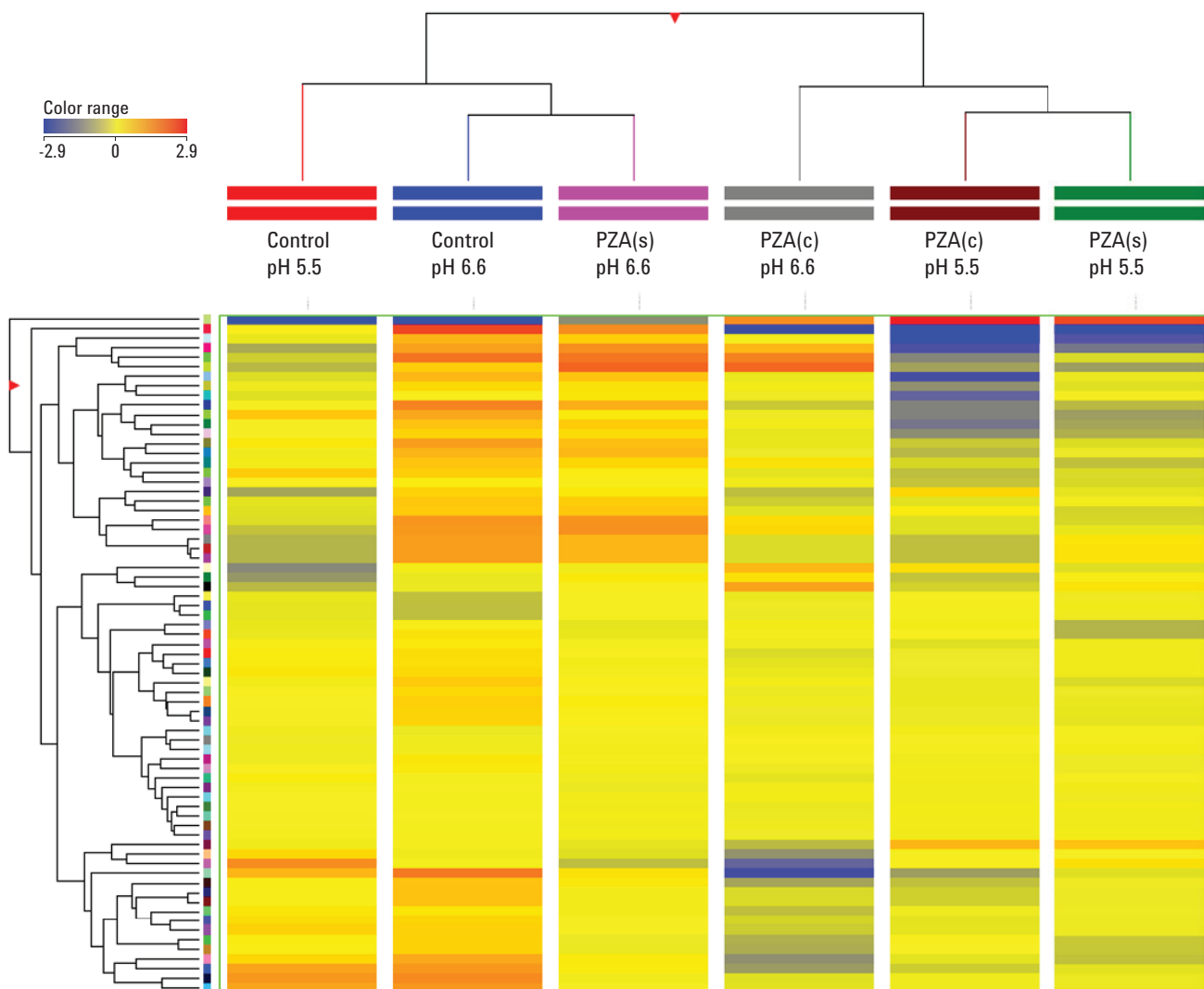


Figure 9. Hierarchical clustering on conditions and entities. The color range indicates the peak intensity of each metabolite for each condition. Metabolites with low intensity are shown in blue; those with intermediate intensity are shown in yellow, and those with high intensity are shown in red. The color range bar indicates the relative intensity of each metabolite.

Pathway Analysis using Pathway Architect in MPP

Pathway analysis was performed using Pathway Architect, providing a means for the visual analysis of experimental metabolite abundances. By mapping the differential metabolites and overlaying the abundance results onto curated

pathways, for example KEGG pathways, it is possible to view pathways enriched with metabolites. This facilitates a quick view of whether the pathway was significantly affected in response to the drug treatments or cell growth conditions. In this study, we found 23 pathways with at least five or more statistically

significant metabolites. Figure 10 shows the pantothenate and CoA biosynthesis pathway in which 15 metabolites were found. The individual heat strips adjacent to each metabolite are the normalized signal value.

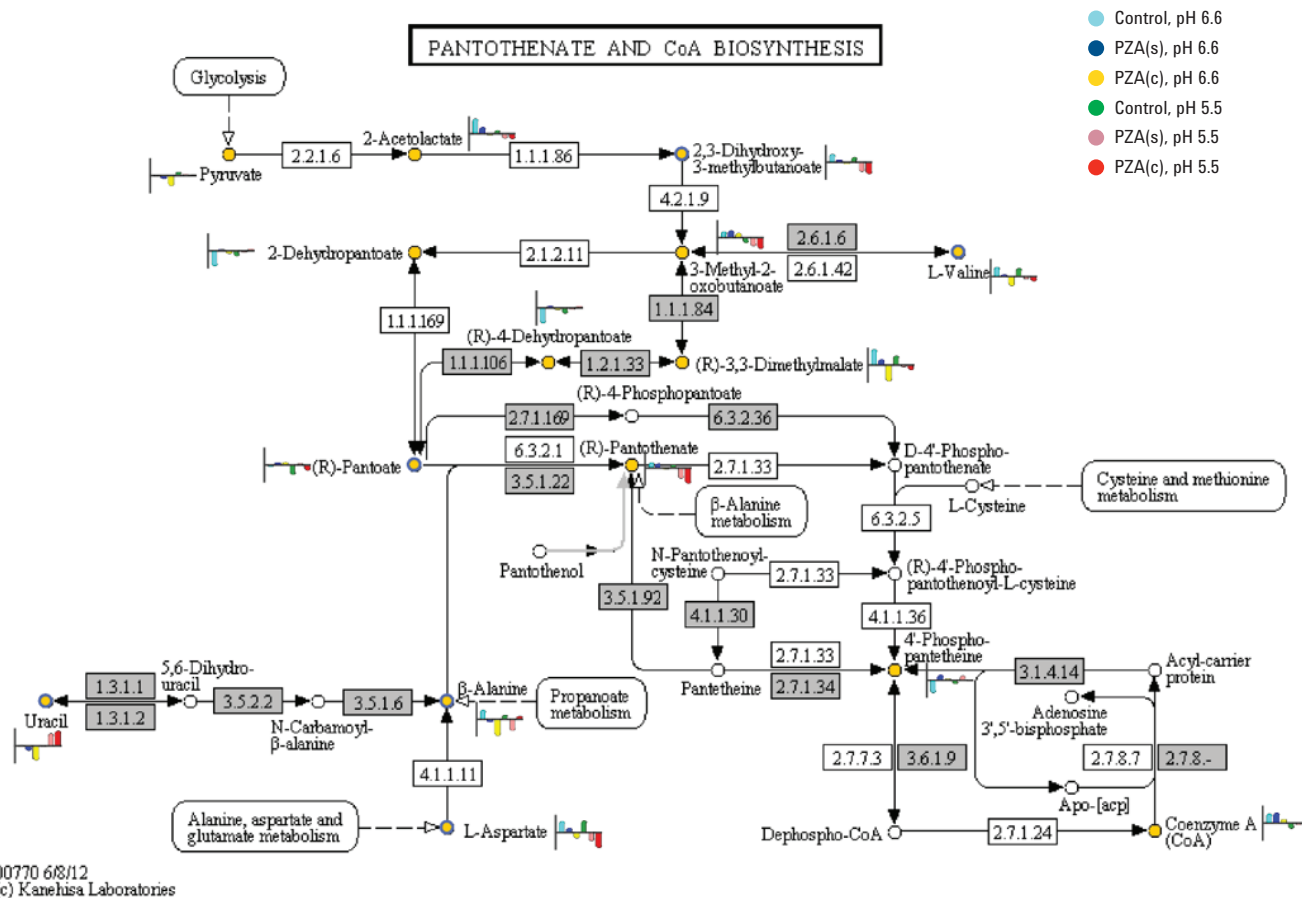


Figure 10. Fifteen significantly different metabolites were mapped to the pantothenate and CoA biosynthesis pathway.

Conclusions

Using a robust IP-RP Q-TOF LC/MS method and Agilent MassHunter Profinder and MPP software enabled the identification of a specific metabolic biosignature for PZA-treated *Mycobacterium tuberculosis*. The biosignature is derived from a broad survey of pathways. Importantly, the results were consistent with phenotypic data from reports in the scientific literature^{8,9}, and may help to explain PZA's unique metabolic effects.

References

1. Zhang, Y.; Mitchison, D. The curious characteristics of pyrazinamide: a review. *Int. J. Tuberc. Lung Dis.* **2003**, *7*(1), 6-21.
2. Dai, Y. Profiling of Endogenous Metabolites Using Time-of-Flight LC/MS with Ion-Pair Reverse Phase Chromatography, *Agilent Technologies Application Note*, publication number 5991-7802EN, **2017**.
3. 6545 Q-TOF Brochure, Agilent Technologies, publication number 5991-5468EN, **2015**.
4. Alex Mordehai, ASMS 2009, Poster number: MPM 331.
5. MassHunter Profinder: Batch Processing Software for High Quality Feature Extraction of Mass Spectrometry Data, *Agilent Technologies Technical Overview*, publication number 5991-3947EN, **2014**.
6. Kind, T.; Fiehn, O. Metabolomics database annotation via query of elemental compositions: Mass accuracy is insufficient even at less than 1 ppm. *BMC Bioinformatics* **2006**, *7*, 234.
7. Xu, Y.; *et al.* Evaluation of accurate mass and relative isotopic abundance measurements in the LTQ-Orbitrap Mass Spectrometer for Future Metabolomics Database Building. *Anal. Chem.* **2010**, *82*, 5490-5501.
8. Zhang, Y.; *et al.* Mechanisms of Pyrazinamide Action and Resistance. *Microbiol. Spectr.* **2013**, *2*(4), 1-12.
9. Gopal, P.; *et al.* Pyrazinamide resistance is caused by two distinct mechanisms: prevention of coenzyme A depletion and loss of virulence factor synthesis. *ACS Infect. Dis.* **2016**, *2*, 616-626.

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