# Characterization of Differential Metabolites in Bacteria Using a Q-TOF LC/MS Based Metabolomics Batch Data Analysis Workflow

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## Introduction

The growth of bacteria has been studied for more than a century. It was found that cells remain alive and metabolically active during the late stationary phase, but often respond differently to stimuli or stresses during the early part of stationary phase compared to the late stationary phase. Researchers hypothesize that one or more metabolites produced during the stationary phase may render a bacterium less viable in late stationary phase or, alternatively, that a protective metabolite may be lost during the progression of stationary phase. To our knowledge, there is no information available about the characteristics of those metabolites.

Here we present a Q-TOF LC/MS metabolomics approach to investigate differential metabolites of a bacterium in the early stationary versus late stationary phase. The Agilent suite of data processing software makes feature finding, statistical analysis, and identification easier. This enables rapid transformation of complex raw data into biologically relevant metabolite information.

## Experimental

### Sample preparation

Cell cultures were harvested either in early or late stationary phase, rinsed twice with phosphate-buffered saline to remove any residual extracellular media, and then rinsed with water to remove the buffer. Cold MeOH and 50% MeOH/H2O were used to extract metabolites from cell cultures. The extracts were filtered using a 3kDa Nanosep filter, then vacuum dried, and re-suspended in the water/acetonitrile (3:7 v/v) solvent for LC/MS analysis.

### Instrumentation

LC/MS Analysis was performed using an Agilent 1290 Infinity LC system coupled to either an Agilent 6230 TOF system or an Agilent 6550 iFunnel Q-TOF system. Tables 1 and 2 summarize the optimized LC and MS conditions.

Table 1. LC parameters

| Parameter | Agilent 1290 Infinity LC System |
|---|---|
| Analytical column | Cogent Diamond Hydride HPLC Column, 2.1 mm x 150 mm, 4 µm, 100 A (p/n 70000-15P-2), Microsolv Technology Corporation |
| Guard column | Agilent Zorbax SB-C8, 2.1 mm x 30 mm, 3.5 µm (p/n 827700-936) |
| Column temperature | 60 °C |
| Injection volume | 5 µL for profiling experiment on the Agilent 6230 TOF<br>10 µL for identification experiment on the Agilent 6550 Q-TOF |
| Autosampler temperature | 4 °C |
| Mobile phase for positive ion mode | A: 50% water/50% isopropanol containing 0.1% formic acid<br>B: 3% water/97% acetonitrile containing 0.1% formic acid |
| Mobile phase for negative ion mode | A: 50% water/50% isopropanol/0.025% formic acid and 5 µM EDTA<br>B: 10% water/90% acetonitrile/5 mM ammonium formate/5 µM EDTA, pH=7 |
| Flow rate | 0.6 mL/min |
| Gradient for positive ion mode | 0-1 min 97% B<br>1-15 min 97% B to 10 % B |
| Gradient for negative ion mode | 0-1 min 99% B<br>1-15 min 99% B to 20% B |
| Stop time | 15 min |
| Post time | 5 min |

Table 2. MS parameters

| Parameter | Agilent 6230 TOF system | Agilent 6550 Q-TOF system |
|---|---|---|
| Ion mode | Positive and Negative | Positive and Negative |
| Source | Agilent Dual ESI | Agilent Dual ESI |
| Capillary voltage | 3500 V (+/-) | 3500 V (+/-) |
| Dry gas temperature | 350°C | 200 °C |
| Dry gas flow | 10L/min | 15 L/min |
| Nebulizer pressure | 45 psi | 45 psi |
| MS range | 25-1600 m/z | 50-1700 m/z |
| MS acquisition rate | 2 spectra/sec | 3 spectra/sec |
| MS/MS range | NA | 25-1700 m/z |
| MS/MS acquisition rate | NA | 2 spectra/sec, Targeted MS/MS |
| Isolation width | NA | Medium (~4 m/z) |
| Collision energy | NA | 10-40 eV |
| Reference mass | Positive ion mode: 64.01577 and 922.009798<br>Negative ion mode: 68.9957 and 966.0007 | Positive ion mode: 64.01577 and 922.009798<br>Negative ion mode: 68.9957 and 1033.9881 |
| Reference pump flow | 0.5 mL/min (positive and negative ion modes) | 0.6 mL/min (positive ion mode)<br>0.5 mL/min (negative ion mode) |
| Reference delivery | Agilent 1100 isocratic pump with 100:1 splitter (p/n G1607-60000) | Agilent 1100 isocratic pump with 100:1 splitter (p/n G1607-60000) |
| Instrument mode | Extended dynamic range (2GHz) | Extended dynamic range (2GHz) |

## Experimental

### Data analysis software

- Agilent MassHunter Qualitative Analysis (Qual) B.07.00
- Agilent MassHunter Profinder B.06.00, service pack 1
- Agilent Mass Profiler Professional (MPP) B.13.0
- Agilent MassHunter Molecular Structure Correlator (MSC) B.07.00

## Results and Discussion

### LC/MS metabolomics workflow

A high-resolution Q-TOF LC/MS metabolomics workflow (**Figure 1**) was used for unbiased metabolomics study of a bacterium in the early and late stationary phases.

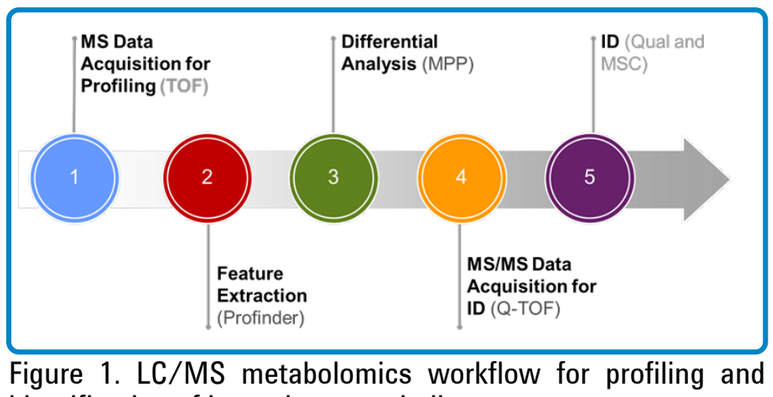


Figure 1. LC/MS metabolomics workflow for profiling and identification of bacterium metabolites.

### Metabolite profiling by TOF LC/MS

The difference in the metabolome of a bacterium in the early versus late stationary phase was assessed by performing metabolite profiling on cell extracts. **Figure 2** shows the metabolite profiling results of the cell extracts obtained in the negative ion mode.

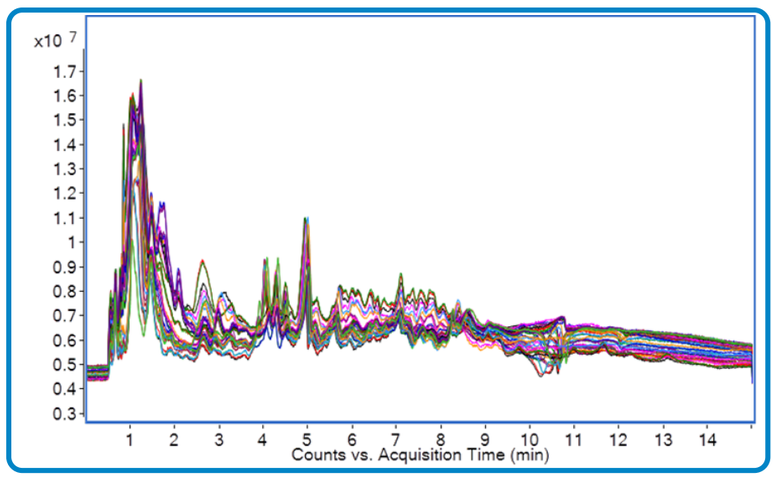


Figure 2. Thirty-eight overlaid total ion chromatograms (TICs) from 19 cell extracts in ESI negative ion mode. Each sample was injected twice.

### Batch data feature extraction using Agilent MassHunter Profinder

MassHunter Profinder software increases throughput using automated batch data processing. In this study, Profinder found 488 features from the positive ion data, and 623 features from the negative ion data (**Figure 3**) based on the user-defined compound filtering criteria and manual curation.

### Differential analysis using MPP

The resulting features were evaluated by MPP using the *Filter on Volcano Plot* algorithm with a cutoff of $P < 0.005$ and fold change (FC) > 2. For the positive ion data, 98 of the 488 features displayed statistical significance, and 57 of them were found to have higher abundances in the early stationary phase compared to the late stationary phase. Likewise, we detected 152 of 623 features that were significantly differential in the negative ion data (**Figure 4**), and 52 of them had higher abundance in the early stationary phase than in the late stationary phase (blue solid square dots).

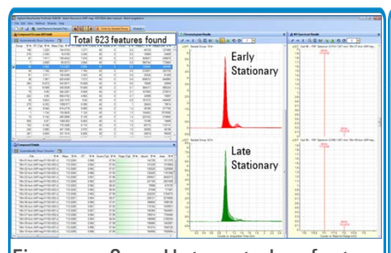


Figure 3. Untargeted feature extraction result of the negative ion MS data from the cell extracts in the early and late stationary phase.

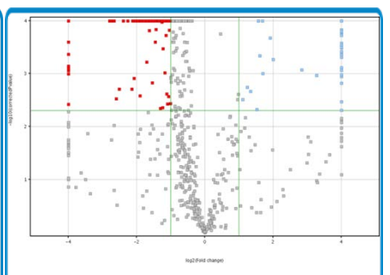

Figure 4. Volcano plot of the features in the early versus the late stationary phase (negative ion data).

## Results and Discussion

### Annotation using MPP

Differential features were annotated using the ID Browser tool in MPP and accurate mass database matches with 10 ppm mass tolerance. **Figure 5** shows the ID browser result for a set of differential features acquired in the negative ion mode.

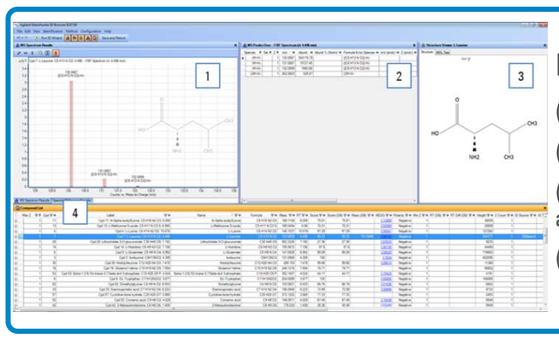


Figure 5
(1)MS spectrum
(2)Isotopic peaks
(3)Structure of the annotated compound
(4)Compound list

### Compound identification and software-assisted structure elucidation

Compound identification and structure elucidation were achieved using two approaches. The first approach uses MS/MS spectra matching. **Figure 6** shows a differential feature that was identified as nicotinic acid with high confidence. The mirror image plot (middle) showed excellent matches of the acquired sample spectrum (top) with Agilent-Metlin library standard spectrum (bottom).

When an acquired MS/MS spectrum had no matches to the Agilent-Metlin MS/MS library, the other approach using MSC software was employed. **Figure 7** shows the results by querying the PubChem database, structure hits were returned for all six compounds. The structure interpretation of the MS/MS spectrum for the m/z 187.1114 ion is shown in **Figure 8**. All fragment ions were labelled with the most plausible fragment structure proposed by MSC.


Figure 6. Compound structure identification using MS/MS spectra matching. Match score: 97 for nicotinic acid. CE=20 eV

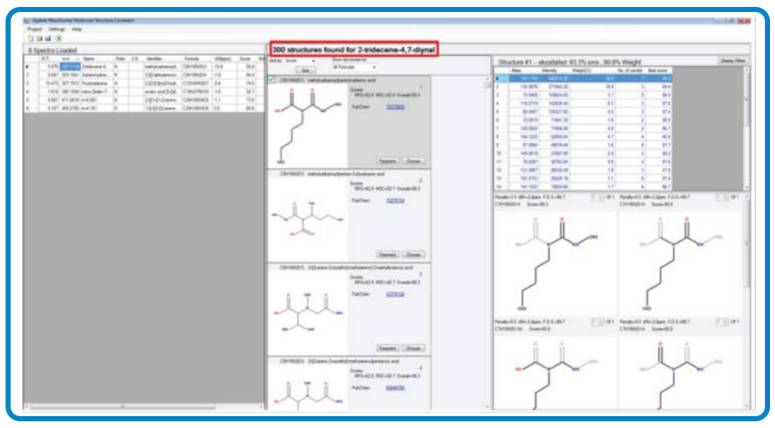


Figure 7. MSC results obtained by searching the web-based PubChem database.

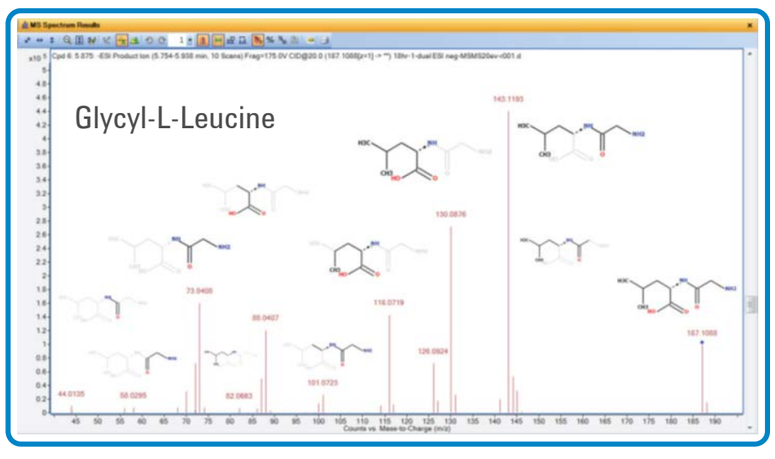


Figure 8. Structure interpretation of MS/MS spectrum for m/z 187.1088 ion using MSC and PubChem database

## Conclusions

An easy-to-use Q-TOF LC/MS based workflow is presented for a discovery metabolomics study of a bacterium. The Agilent software tools (Profinder, MPP, and MSC) enabled high efficiency and high quality feature extraction, statistical analysis, annotation, and identification. The results from this study revealed interesting bacterium metabolic variations in the early versus late stationary phase.