High-Throughput, High-Efficiency Metabolome Profiling Using the Agilent 6550 iFunnel Q-TOF LC/MS System

Application Note

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Abstract

The Agilent 6550 iFunnel Q-TOF LC/MS System (6550 Q-TOF) has been coupled with flow injection analysis (FIA) to provide profiling of small molecules in 96 biological extracts. This experiment demonstrates the superior performance of this platform, compared to the results produced with a previous generation Q-TOF instrument, resulting in the detection of an order of magnitude more ions. The increased sensitivity and resolution lead to a six to seven fold increase in coverage of the *E. coli* metabolome and more detailed pathway analysis.

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Introduction

Metabolomics is a field of research dedicated to the comprehensive characterization of small molecule metabolites in biological systems. Metabolic phenotypes emerge from the interplay of genomes with the environment and are thus best monitored at the level of metabolites. Metabolite concentrations are not only sensitive to perturbations that affect their biosynthesis but also generally respond to changes in the capacity of enzymes for which they are substrates. Therefore, they are diagnostic for regulatory events (for example, transcriptional or allosteric) that have a tangible effect on metabolic operation.

Metabolomics offers a unique opportunity to look at genotype-phenotype relationships, because the metabolome is closely tied to the genotype of an organism, its physiology, and its environment. It can be a useful tool for determining the phenotype resulting from genetic manipulation, such as gene deletions or insertions. Metabolomics can thus provide a means of predicting the function of unknown genes by comparison with the metabolic changes caused by the deletion or insertion of known genes.

The metabolic profiling of knockout experiments requires very high-throughput methods, as study size can quickly scale to several or tens of thousands of individual samples to fulfill the demand imposed by robust experimental designs. These methods must also detect and quantify hundreds of small molecule compounds simultaneously. Flow injection analysis (FIA) of samples on accurate-mass, high-resolving mass spectrometers (MS) is an effective way to maximize analytical throughput while allowing analyte discrimination in complex samples by mass-to-charge ratio. However, the MS platform must provide sufficient sensitivity and mass resolution to enable detection and quantification of very large numbers of metabolites present at low concentrations. The introduction of iFunnel technology in the Agilent 6550 iFunnel Q-TOF LC/MS System (6550 Q-TOF) provides metabolomics researchers with the lowest detection levels of any high resolution LC/MS instrument, enabling low femtogram-level sensitivity with high resolution (40,000 resolving power) and accurate-mass (< 1 ppm).

This application note describes a method that couples FIA with the 6550 Q-TOF to provide profiling of small molecules in > 1,400 biological extracts per day. The performance of the system has been compared to the results generated on a previous FIA Agilent 6520 Accurate-Mass Q-TOF LC/MS System (6520 Q-TOF) platform that analyzed more than 5,000 injections of chemically defined standards and *E. coli* cellular extracts obtained from miniscale cultivations.[1] The accurate mass and correlation analysis enabled high-confidence assignment of 400–800 ions to electrospray signals of metabolites listed in the genome-wide reconstruction of *E. coli* metabolism. The method described here illustrates the superior performance of the 6550 Q-TOF in this application, resulting in the detection of an order of magnitude more ions. This increased sensitivity and resolution led to a 6 to 7 fold increase in coverage of the *E. coli* metabolome. A screen with *E. coli* deletion mutants was run to demonstrate the superior sensitivity of the FIA 6550 Q-TOF platform in detecting ion signals from primary metabolism at high throughputs. The increased sensitivity was also used to enable MS/MS identification of compounds at concentrations as low as 0.006 µM.

Experimental

Standards and reagents

Eighty pure chemical standards, purchased from various suppliers, were mixed and diluted to span a concentration range of 0.006 µM to 50 µM. This mixture was used to optimize the analysis.

Instruments

This study was performed using an Agilent 1290 Series LC pump and Wellplate Autosampler coupled to an Agilent 6550 iFunnel Q-TOF LC/MS System equipped with a dual electrospray ion source operated in negative and positive mode. The instrument run conditions are given in Table 1.

Sample preparation

E. coli extracts were prepared by rapid centrifugation followed by extraction of the intracellular polar metabolome *in situ* with hot water.[1]

Table 1. FIA-Q-TOF run conditions.

FIA conditions	
Injection volume	1 µL
Flow rate	150 µL/min
HPLC Solvent	60 % isopropyl alcohol/40 % water 5 mM ammonium carbonate, pH 8.9 (v/v)
Autosampler needle wash solution	50 % isopropyl alcohol/50 % water (v/v)
Injection cycle parameters	Injection speed: 5 µL/s Wash speed: 5 µL/s Pre-injection delay: 500 ms Post-injection delay: 500 ms
Q-TOF MS conditions	
Ion mode	Negative ion mode, dual ESI
Drying gas temperature	325 °C
Drying gas flow	5 L/min (nitrogen)
Nebulizer	30 psig
Capillary voltage	3,500 V
Fragmentor voltage	360 V
Skimmer voltage	65 V
Oct RF Vpp	750 V
Acquisition parameters	MS mode 50–1,000 *m/z* acquisition 1.4 spectra/s 713.3 ms/spectrum 9,652 transients/spectrum
Q-TOF MS/MS conditions	
Mass filter mode	High resolution
Acquisition parameters	MS/MS mode 25–1,000 *m/z* acquisition 1.4 spectra/s

Results and Discussion

Analysis of pure standards

A mixture of 80 pure chemical standards was analyzed over a concentration gradient spanning from 0.006 µM to 50 µM. Extracted ion chromatograms (EICs) were produced according to known formulas and integrated. In brief, we estimate the Agilent 6550 Q-TOF decreased the lower limit of detection of the assay by a factor of 10 from the current limits of detection of 0.01 µM to 2.0 µM on the Agilent 6520 Q-TOF.[1]

Analysis of *E. coli* extracts

Analysis of 96 *E. coli* extracts on the 6550 Q-TOF produced ion counts that were 10 times higher than those previously obtained on the 6520 Q-TOF, but with similar noise. The combination of enhanced signal and resolution dramatically increased the number of ions detected by a factor of 11-fold (from 1,190 to 13,614 after background removal). The higher sensitivity of the 6550 Q-TOF enables detection of an impressive number of additional ions. The increase in the number of observable ions is clearly visible in the raw profiles (Figure 1). Ions that are only detectable in the shoulder of more intense ones on the 6520 Q-TOF are well resolved on the 6550 Q-TOF. Despite the 10-fold increase in detected features, the ion demography does not differ (Figure 2). As expected, ion counts (intensity) are on average one order of magnitude higher using the 6550 Q-TOF.

Metabolite coverage

A key issue for any biological application is coverage of known metabolites, which is the fraction of compounds that are expected to occur in an organism and are detectable by the instrument. The measured accurate mass of the entities was used to search the compounds currently listed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for *E. coli*. To date, the list contains 3,223 compounds. This catalogue contains a number of peptides, lipids, and antibiotics which are not expected to be present in this study. In both data sets, we searched for matching ions, including multiple adducts, isotopes, and frequently occurring neutral losses.[1] The mass tolerance was set to 0.001 atomic mass units (amu).

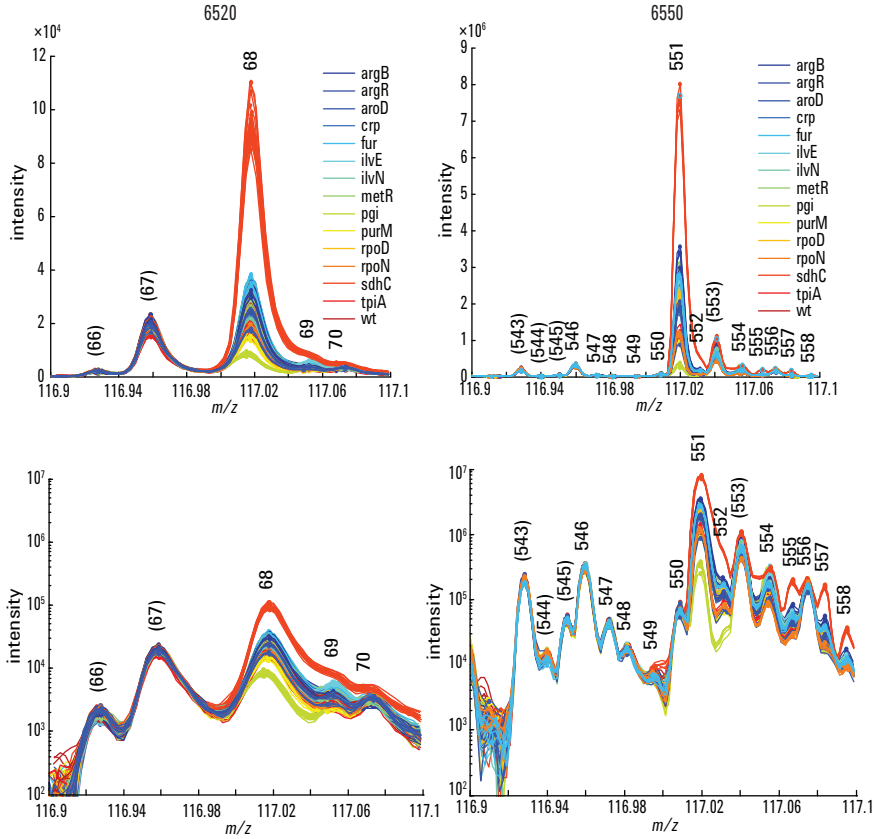

Figure 1. An example of raw profile data obtained with the Agilent 6550 iFunnel Q-TOF LC/MS System and the Agilent 6520 Accurate-Mass Q-TOF LC/MS Systems, using the flow injection analysis method. There is a dramatic increase in the number of observable ions captured with the Agilent 6550 and the ions are better resolved.

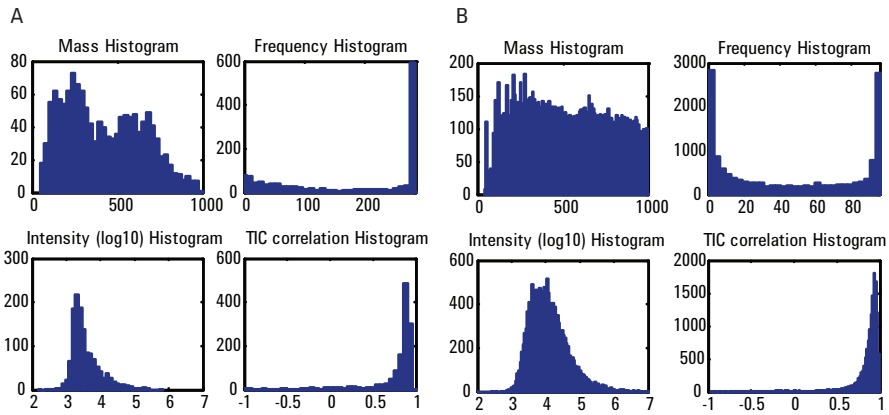

Figure 2. Ion statistics histograms show the demography of ions generated by the Agilent 6520 Q-TOF (A) and Agilent 6550 Q-TOF (B), illustrating the similarities between the two systems and the significantly higher ion counts achieved with the 6550 Q-TOF.

Overall, the fraction of ions that can be attributed to a known compound in the database using the 6550 Q-TOF is similar (around 11–12 %) to that determined using the 6520 Q-TOF. In absolute terms, however, about 1,600 unique compounds were potentially detected, which was 6–7 times more than what was detected with the 6520 Q-TOF (Table 2). Notably, this number includes isobaric compounds. In the absence of chromatographic separation, this method does not distinguish between them. Therefore, the number of 1,600 is for the best-case scenario. However, the method is sensitive to singular changes in one of the isomers if they account for a detectable fraction of the entire pool. In this case, the precise identity of the differential isomer has to be identified with another analysis such as LC/MS or MS/MS.

The difference in coverage between the two Q-TOF instruments for the metabolites of primary metabolism relevant in all cellular systems can be appreciated by visual inspection of the global KEGG metabolism maps (Figure 3). These maps do not contain all metabolites detected, just those in primary metabolism.

Table 2. Ion annotation as obtained with a mass tolerance of 0.001 amu using the KEGG Eco database.

	6520	6550
Ions		
total	1190	13614
annotated	130	1601
unknown	1060	12013
Compounds		
any score	305	1669
only top scores	253	1608

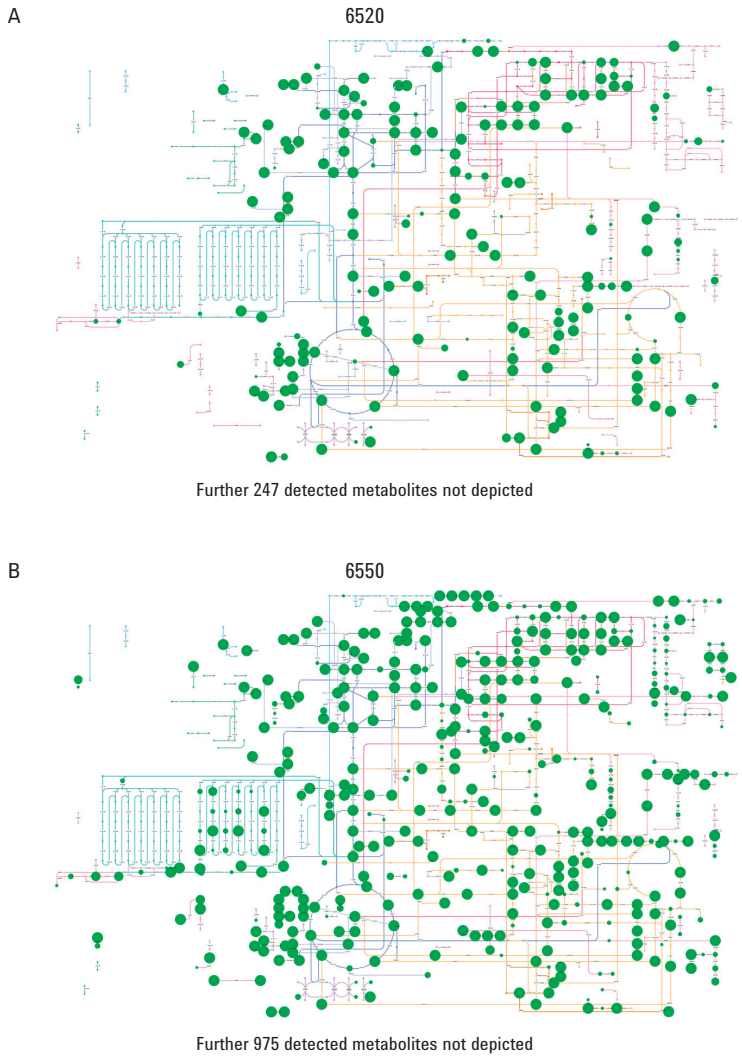

Figure 3. This map reports the compounds found by annotation and demonstrates six to seven times greater coverage with the Agilent 6550 Q-TOF (B) as compared to the 6520 Q-TOF (A). Dot sizes scale with the degree of confidence for the annotation.

Differential analysis

The 6550 Q-TOF was tested for its ability to detect the metabolic changes previously observed in a set of *E. coli* mutants with the 6520 Q-TOF. For all detectable ions and each mutant, a differential score (t-test) was calculated for the mutants versus the controls (wild-type), and examples are shown in Figure 4. In all tested cases, the top hits found using the 6550 Q-TOF contained the expected metabolites immediately upstream of the lesion.

Even though more compounds are detectable using the 6550 Q-TOF, there is no increase in the number of false positives. The statistical significance is comparable. These results generally confirm the excellent suitability of the FIA 6550 Q-TOF platform to screen for integrated metabolic responses or functional changes.

Compound identification using MS/MS

The sensitivity and quality of fragmentation spectra generated using the 6550 Q-TOF were assessed empirically by the capacity to correctly assign the identity of the compounds in our standard mix at low concentration, based on their MS/MS spectra. Product ion scans were collected by targeted FIA MS/MS with an inclusion list that included four different ions for each injection. Compounds were identified with MassHunter Qualitative Analysis Software (version B 04.00) using the **Find by Targeted MS/MS** function and searching against the Agilent METLIN Personal Compound Database and Library (PCDL).

Figure 4. Differential analysis with *E.coli* mutants demonstrates excellent suitability for integrated metabolic responses or functional changes. The size of a dot scales with the p-value, and its brightness with the fold change. Red=increased in the mutant. Green=decreased in the mutant. The arrows indicate the sites of metabolic lesions (upper: purM; lower: argB).

Initially, all compounds were fragmented at the highest concentration of 50 µM and tentatively identified. Virtually all compounds that were represented in the database could be successfully identified, either exactly or as a closely-related structural isomer. Fragmentation spectra on a dilution series ranging from 50 µM down to 0.006 µM were collected for four compounds: succinic acid, citric acid, ADP, and 6-P-gluconate. The 6550 Q-TOF produced informative MS/MS spectra at the lowest tested concentrations, surpassing the 6520 Q-TOF where peaks were not detected at a concentration of 0.1 µM. With some manual curation, the tested compounds were correctly annotated even at 0.006 µM.

Conclusions

The increase in sensitivity and resolution, and the outstanding mass accuracy of the Agilent 6550 iFunnel Q-TOF LC/MS System (6550 Q-TOF) directly translate into 10 times more reproducibly detectable ion features in this FIA Q-TOF method, 10 times higher ion counts, and significantly broader coverage of primary metabolism versus the Agilent 6520 Accurate-Mass Q-TOF LC/MS System. The end result is a more detailed pathway analysis. The increased sensitivity enables increased detection of mutants that were difficult to detect using the previous generation of Q-TOF instruments. For example, mutants that decrease a metabolic pool are more likely to be detected, as are regulatory mutants that result in small changes in metabolite concentrations. Screens of kinases, transcription factors, and inhibitors would immediately benefit from the advantages of the 6550 Q-TOF. The increased sensitivity also facilitates MS/MS identification of compounds, due to higher ion intensities.

Given the constraints of the method, which is meant for high throughput and does not include fractionation of the sample, it is unlikely that many more metabolites are present in these polar extracts. The 6550 Q-TOF thus enables a first step towards total metabolite coverage of an organism by providing dense coverage of known pathways. Areas of research limited by sample size due to the fact that cells must be cultivated in small quantities could benefit greatly from the increased sensitivity, resolution, and mass accuracy delivered by the 6550 Q-TOF.

Reference

1. Fuhrer *et al.* High-Throughput, Accurate Mass Metabolome Profiling of Cellular Extracts by Flow Injection-Time-of-Flight Mass Spectrometry. *Anal Chem*, **2011**, 83(18):7074-7080.

www.agilent.com/chem/QTOF

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