Introduction

A major challenge in metabolomics is achieving reproducible, robust chromatographic resolution with a single analytical LC/MS method for endogenous cellular metabolites, due to their diversity of chemical and physical properties. A more manageable chromatographic solution is to develop pathway-targeted analytical methods for smaller sets of metabolites. Building an optimized targeted LC/MS/MS method is a painstaking task requiring significant time and money to characterize a large number of chemical standards.

Agilent has developed a LC/MS/MS solution for >215 central carbon metabolites that provides robust, reproducible separation of acidic metabolites. The solution includes a curated database with retention times, optimized MS/MS acquisition parameters, and a data acquisition and analysis method. The Metabolomics dMRM Database and Method leverages the high sensitivity and wide dynamic range of the Agilent 6470 Triple Quadrupole LC/MS System, and the proven ionization enhancement capabilities of the Agilent Jet Stream Ionization Source [1].

This white paper illustrates the advantages of the Agilent Metabolomics dMRM Database and Method for enabling researchers to easily implement an already-optimized and straightforward targeted analytical method for central carbon metabolites.
**Solution Overview**

Many chromatographic approaches have been applied to metabolomics analysis, including reversed-phase, normal phase, and HILIC, but all are generally unsatisfactory due to poor selectivity, reproducibility, or retention of polar metabolites. To address these challenges, a highly reproducible and robust ion-pair reversed-phase (IP-RP) chromatographic method has been developed to provide separation of anionic and hydrophobic metabolites. The chromatographic method leverages the robustness of Agilent ZORBAX Extend C18 columns with the ion-pairing agent tributylamine (TBA). Normally, a nonvolatile quaternary amine would be used for ion pair chromatography, however, quaternary amines interfere with electrospray ionization. TBA is a volatile ternary amine amenable to electrospray ionization that functions as an ion-pairing reagent and facilitates reproducible retention of acidic metabolites. Nonacidic metabolites still maintain conventional reversed-phase selectivity in the presence of TBA. This ion-pairing LC/MS method enables simultaneous analysis of many metabolite functional classes including amino acids, citric acid cycle intermediates and other carboxylic acids, nucleobases, nucleosides, phosphosugars, and fatty acids.

**Instrumentation**

Coupling the Agilent 1290 Infinity Series UHPLC to an Agilent 6470 Triple Quadrupole LC/MS creates a sensitive and highly selective system for the detection of targeted metabolites (Figure 1). To achieve high sensitivity and selectivity, a triple quadrupole is operated in multiple reaction monitoring (MRM) mode. MRM Mode specifies for each compound a predetermined precursor ion and collision-induced product ion pair, which is termed a transition. In complex samples, a second transition is highly desirable, if possible, to increase confidence in compound identification. Including the chromatographic retention time of the transitions provides orthogonal confirmation of compound identification.

When analyzing many compounds in a single LC/MS analytical method, the number of concurrent MRM transitions increases, which reduces measurement (dwell) times and impacts the limits of detection (LODs). With retention time information for each compound, MRM transition lists can dynamically be created throughout an LC/MS run using a window around the expected retention times. In this way, compounds are only monitored while they are eluting from the LC, thus improving LODs and permitting more metabolites to be measured. In Agilent MassHunter Acquisition Software, using retention time windows is called dynamic MRM (dMRM) mode [2]. This solution uses a dMRM method to maximize LODs for central carbon metabolites.

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**Figure 1.** Agilent 1290 Infinity II UHPLC system with an Agilent 6470 Triple Quadrupole Mass Spectrometer.
This analytical method is intended for use with complex samples where matrix components may impact method performance. The LC/MS configuration for this solution includes a guard column and a switching valve configuration that permits backflushing of both the column and guard column following each analytical run. This very effectively clears potential matrix contaminants from the guard column, and provides consistent high-quality peak shapes and superior long-term retention time stability.

Methodology

The IP-RP chromatography provides effective separation of anionic and hydrophobic metabolites. This method uses a gradient between solvent A (97:3 water/methanol with 10 mM tributyramine + 15 mM acetic acid) and solvent B (methanol with 10 mM tributyramine + 15 mM acetic acid). We chose the Agilent ZORBAX Extend C18 column, as it is very stable at the pH used with this method. More detailed information about the chromatography and solvent preparation can be found in the Metabolomics Dynamic MRM Method Setup Guide (G6412-90002).

The Agilent advanced triple quadrupole Autotune program optimizes all noncompound-dependent instrument settings. Due to the different physiochemical properties of metabolites, there are specific mass spectrometer parameters that must be optimized for each compound to provide the best performance.

On the Agilent system, this includes fragmentor voltage, which optimizes transmission of the precursor ion into the mass spectrometer, and collision energy, which provides maximum intensity for a specific product ion. The Agilent Metabolomics dMRM Database provides optimized parameters for all metabolites. The choice of primary transitions, retention times, collision energies, and fragmentor voltages included in the database were developed in collaboration with the laboratory of Adam Rosebrock (University of Toronto), a leading group in the metabolomics field. These optimized parameters were the result of extensive analyses of chemical standards to generate results of the highest quality.

The database links to both MassHunter Acquisition Software and Agilent MassHunter Optimizer [3]. MassHunter Optimizer can be used to add additional metabolites to the database if needed. Users can select compounds from the database to rapidly create a customized analytical method for their specific needs. Figure 2 shows the MS QQQ Acquisition setup tab for controlling the Agilent 6400 Series Triple Quadrupole LC/MS system. The database contains metabolite information such as compound name, CAS number, and the optimized acquisition parameters for each transition. In addition, retention times with retention time windows are included for dMRM mode, which are valid if the user is implementing the same hardware configuration and LC method supplied with the database. Using the Agilent Database Browser (Figure 3), compounds with their optimized acquisition parameters can be selected for import into the acquisition method.
Figure 3. Database Browser view of the Metabolomics dMRM Database.
Key to this LC/MS analytical method are the compound retention times in the database. The retention times provide added confidence in assigning the MRM signals to the correct metabolite in a complex biological matrix, and enable dMRM acquisition. The retention time consistency of the IP-RP method allows narrow retention time windows, enabling the analysis of larger numbers of metabolites with better LODs.

A default acquisition method (.m) file is provided with the database for seamless implementation of the full dMRM method targeting all metabolites. The method file provides all of the required instrument parameters, many of which are essential to replicate the retention times specified in the database. The Agilent Jet Stream source settings provided in the analytical method were optimized for efficient ionization for the broad range of metabolites. The cycle time was chosen to maximize sensitivity, and ensure sufficient data points for reproducible chromatographic peak integration. Figure 4 shows the overlapped MRM chromatograms of a metabolite standard mixture acquired with the default acquisition method.

![Figure 4. Overlaid MRM chromatograms of more than 100 metabolite standards at 5 ng on-column.](image-url)
Data Analysis Workflow

The Metabolomics dMRM Database and Method includes a MassHunter Quantitative Analysis method for processing the LC/MS data. For large, multicompound batches, MassHunter Quantitative Analysis Software facilitates batch review of the data by including helpful features such as compound-at-a-glance data review (Figure 5), dynamically linked results, and parameterless integration algorithms.

This analytical method has been designed to enable relative comparison studies. The data analysis method could be quantitative if metabolite calibration curves were added by the user. In addition, the method, database, and workflow could be extended by the user to include spike-in controls and transitions for isotopically labeled internal standards to achieve better quantitation.

Results from the Metabolomics dMRM Database and Method are supported in Agilent comprehensive metabolomics workflows. To achieve this, a report is automatically generated (Figure 6) from MassHunter Quantitative Analysis Software for import into the Agilent multivariate analysis software, Agilent Mass Profiler Professional (MPP) [4]. The metabolite names, integrated peak abundances, and the chemical identifiers (CAS) are included in the import for multivariate sample comparisons as well as subsequent pathway analysis. The differential results can be used in the Pathway Architect module of MPP to identify affected pathways for biological interpretation (Figure 7). Pathway Architect supports three major sources of pathway content: WikiPathways, BioCyc/MetaCyc, and KEGG [5]. For increased biological understanding, the targeted metabolomics results can be combined with other omics data (proteomics, transcriptomics) for a multi-omics analysis within Pathway Architect.
Figure 6. Agilent MassHunter Quantitative Analysis Report generation and import into Agilent Mass Profiler Professional. A report is easily generated from the results of a large sample batch (A), and the results are imported into Mass Profiler Professional after the data source type is selected (B), and the location of the Quant report file is specified (C).

Figure 7. The Pathway Architect module of Agilent Mass Profiler Professional, showing the Yeast TCA pathway from KEGG. Metabolites matching experimental data are highlighted. Data are visualized on these pathways with heat strips indicating the normalized relative abundances between sample groups, in this case, yeast grown cultivated on acetate or glucose.
Conclusions

The Agilent Metabolomics dMRM Database and Method was developed for targeted LC/MS analysis of central carbon metabolites in complex biological matrices. The LC/MS method is optimized for an Agilent 1290 Infinity II UHPLC with an Agilent 6470 Triple Quadrupole Mass Spectrometer. The combined UHPLC-MS/MS method and acquisition database provides robust ion-pairing reversed-phase chromatography optimized for challenging acidic metabolites, a curated database including metabolite-specific LC/MS/MS transitions and retentions times, and a default data analysis method, which is supported in Agilent comprehensive metabolomics workflows.

The Metabolomics dMRM Database and Method provides rapid setup and excellent results, eliminating the need for analytical method development.

References


For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.