C146-E435

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Technical Report

Total Solution for Metabolomic Analysis of Endogenous Metabolites Using Exact Mass Database

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Abstract:

Metabolomics can target metabolites with a wide range of physical properties. Because of this, a comprehensive analysis of metabolites requires an analytical method and database suited to the target metabolites. This technical report describes the LC/Q-TOF exact mass database for endogenous metabolites and describes an example of applying this database as part of a total solution ranging from untargeted metabolomics on a Q-TOF LC-MS system to high-sensitivity widely targeted metabolomics on a triple quadrupole LC-MS system for analyzing metabolites in an iPS cell culture medium.

Keywords: Q-TOF, metabolites, database, metabolomics

1. Introduction

Metabolomics is a field of study that reveals differences between multiple samples through the comprehensive analysis of low-molecular-weight metabolites, such as amino acids and organic acids produced in the maintenance of biological functions. Performing a comprehensive analysis in metabolomics is considered easier than other omics fields, such as genomics and proteomics, because metabolomics involves a smaller number of targets. Since metabolite activity is more dynamic with respect to disease phenotypes, metabolomics was originally developed with the expectation it would prove effective in medical applications, such as searching for diagnostic markers in clinical samples or etiological analysis in animal models, but interest in metabolomics is growing, with the same techniques now being applied in food and industrial sectors to compare products between manufacturers and compare raw materials between sources.

In metabolomics, mass spectrometers are used to perform a comprehensive analysis of low-molecular-weight metabolites. In targeted metabolomics, which analyzes samples for a given group of target metabolites, triple quadrupole LC-MS and GC-MS systems are used. In untargeted metabolomics, which searches for unknown metabolites, Q-TOF and other high-resolution mass spectrometers (Fig. 1) are used.

Fig. 1 LCMS-9030 Q-TOF High-Performance Liquid Chromatograph Mass Spectrometer

2. Exact Mass Database for Endogenous Metabolites

Metabolomics can target metabolites with a wide range of physical properties. Because of this, a comprehensive analysis of metabolites requires an analytical method suited to the target metabolites.

The exact mass database for endogenous metabolites contains multiple "ready-to-use" methods for LC/Q-TOF that enable a comprehensive analysis of metabolites with a wide range of physical properties and do not require an investigation of analytical conditions for LC or MS. The database also lists pretreatment examples suited to each metabolite and sample. Hence, metabolomics analysis can be started without a difficult investigation of conditions. The database is an exact mass database developed based on primary metabolites, cell culture profiling, lipid mediators, short-chain fatty acids, and bile acids, all with proven performance in the LC/MS/MS method package series. The database contains retention times and exact mass information on a total of 470 metabolite components (including internal standard substances).

Table 1 Number of Registered Compounds

Method Name	Number of Registered Compounds
Primary metabolites	99
Culture medium components	96
Lipid mediators	214
Short-chain fatty acids	23
Bile acids	38

The database also supports the multi-omics analysis package (sold separately). By analyzing the results of targeted analysis with the multi-omics analysis package, metabolic mapping (Fig. 2), network analysis, and visualized comparisons between two groups can be easily performed.

1 Analytical & Measuring Instruments Division

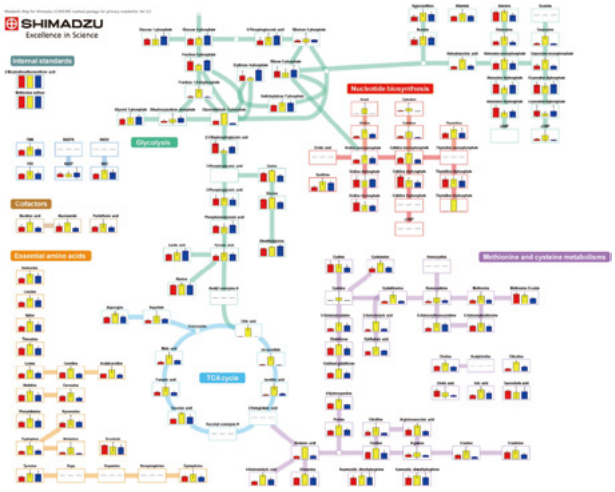

Fig. 2 Metabolic Mapping

The database also supports untargeted metabolomics. In contrast with targeted metabolomics, untargeted metabolomics produces massive amounts of data, hence data analysis is very challenging. Signpost MS™ (Reifycs Inc.) allows a user to easily perform all steps from peak-picking and alignment to statistical analysis (Fig. 3). Signpost MS supports principal component analysis, hierarchical clustering analysis, and scatter plotting (two-group comparison), and other types of statistical analysis commonly used in metabolomics. Using the database together with Signpost MS enables the entire range of process steps involved in untargeted metabolomics.

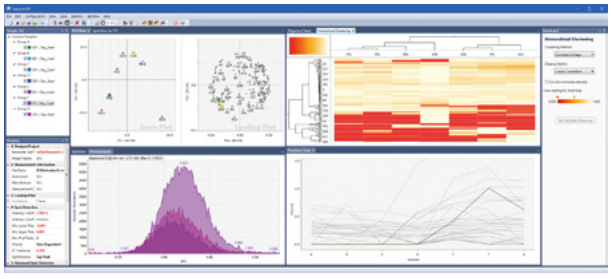

Fig. 3 Signpost MS Multivariate Analysis Software

3. Total Solution for Metabolomics

Normally, in metabolomics, after biomarker candidates and new compounds are discovered by untargeted analysis, they are validated by routine targeted analysis and widely targeted analysis that includes the newly discovered compounds. High-resolution mass spectrometers are often used for untargeted analysis, and triple quadrupole mass spectrometers, which are highly sensitive and offer high quantitative performance, are often used for routine targeted analysis and widely targeted analysis. Because of this, the method must be transferred for the transition from untargeted analysis to targeted analysis.

The LC conditions for each method included in the database are identical to the LC conditions in the method package series for triple quadrupole LC-MS systems, thereby facilitating smooth method transfer. In specific terms, when MRM transitions are created for metabolites discovered by untargeted metabolomics, these can simply be added to the corresponding method in the method package (Fig. 4).

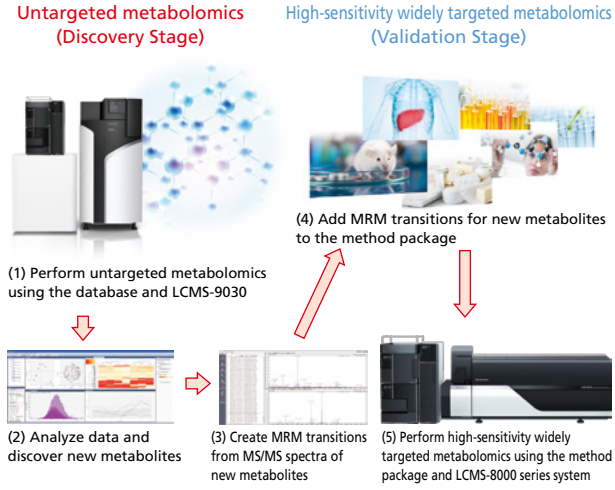

Fig. 4 Total Solution for Metabolomics

4. Culture Medium Analysis during iPS Cell Culturing

During the production of useful materials by fermentation or the manufacture of antibody drugs, culture media are monitored for pH, dissolved gas, carbon sources (glucose), nitrogen sources (glutamine), and other variables to optimize and control the culturing process. Culture components in the cell culture broth include a variety of compounds, not only glucose and glutamine but also vitamins and nucleic-acid related compounds as well as cell-secreted metabolites. Because of this, a comprehensive analysis of compounds found in the culture medium is expected to provide information that should be useful for investigating bioprocesses. An example of using the exact mass database for endogenous metabolites to monitor changes in culture supernatant components during iPS cell culturing is described below.

After seeding iPS cells, culture supernatant was sampled every 24 hours over the course of six-day culturing. Culturing conditions are shown in Table 2. Acetonitrile was added to the sampled culture supernatant to remove proteins. After the organic solvent precipitated, centrifuged supernatant was diluted 10 times with ultrapure water before analysis by the culture component method included in the database.

Table 2 Culturing Conditions

Cell line	: Feeder-free iPS cells 1231A3
Passage number	: 0P30
Seeding number	: 1.3×10^4 cells/well
Period	: 6 days
Medium	: AK02N
Cell substrate	: iMatrix (0.5 μg/cm^2)

Analysis of the culture supernatant detected 27 amino acids, vitamins, and other components included in the culture component method database. Fig. 5 shows some plotted results for component peak areas at each sampling time point. The plotted results show that kynurenine, ornithine, and alanine increased with culture time, and that tryptophan, arginine, and methionine decreased with culture time. Based on the variation of tryptophan and kynurenine, the kynurenine pathway (Fig. 6) in iPS cells is assumed to be changing during the culturing process.

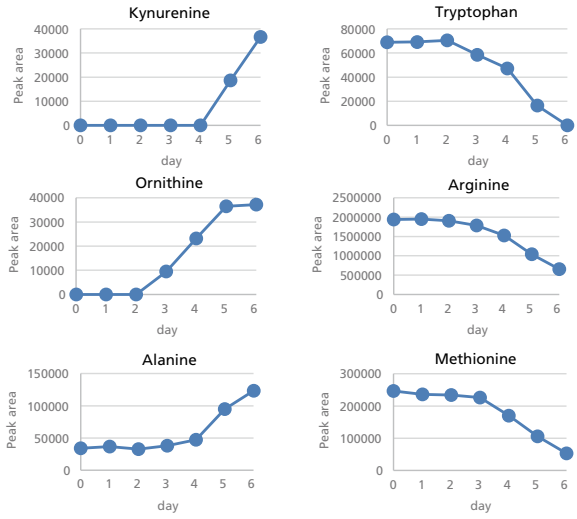

Fig. 5 Variation in Culture Supernatant Components over Course of Culturing

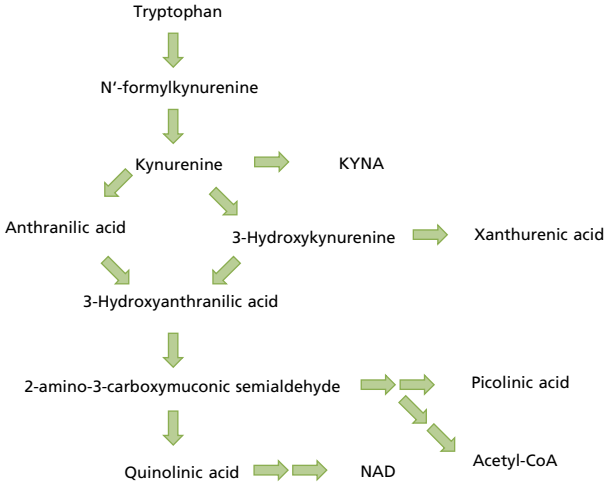

Fig. 6 Kynurenine Pathway

Next, untargeted analysis was performed to search for compounds showing quantitative variation that were not among components in the database. Signpost MS was used to analyze the data and search for unknown compounds that showed quantitative variation (Fig. 3). This analysis identified some components showing quantitative variation not present in the database.

Assuming the kynurenine pathway was changing in the iPS cells during the culturing process, based on the above analysis of components present in the database, it was inferred that kynurenine pathway metabolites other than tryptophan and kynurenine might also have varied over the course of the culturing process. Therefore, results obtained from Signpost MS were used to search for other possible kynurenine pathway metabolites showing quantitative variation. As a result, an unknown metabolite with a retention time of 5.25 min and *m/z* value 237.0870 was predicted to be N'-formylkynurenine. Fig. 7 shows the mass chromatogram and variation in peak area for this unknown metabolite.

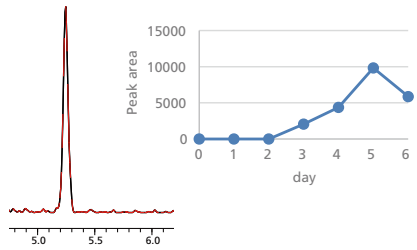

Fig. 7 Unknown Metabolite Showing Quantitative Variation over Course of Culturing

To confirm the unknown metabolite was N'-formylkynurenine, its MS/MS spectrum was acquired and compared to an MS/MS spectrum determined empirically. This comparison was performed using MS Workbook Suite by ACD/Labs. As shown in Fig. 8, the major fragment peaks for the unknown metabolite matched the fragment peaks in the empirical spectrum (red peaks indicate matched fragment peaks), with a divergence from theoretical values of no more than 1 mDa. As a result of the above findings, the unknown metabolite was predicted to be N'-formylkynurenine.

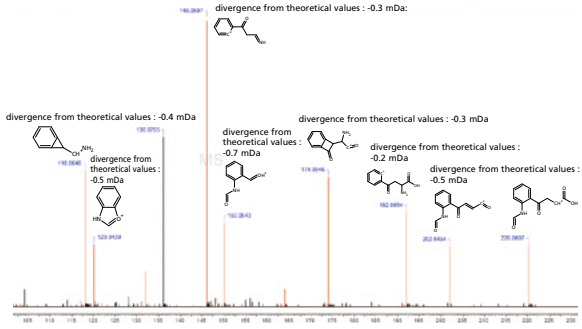

Fig. 8 Verification of Concordance with Theoretical MS/MS Spectrum

N'-formylkynurenine is an intermediate metabolite of tryptophan and kynurenine on the kynurenine pathway. Based on the above results, the concentration of tryptophan in the culture medium is believed to decrease over the course of culturing as tryptophan is taken up by cells in the culture medium, whereas the concentration of tryptophan metabolites N'-formylkynurenine and kynurenine in the culture medium is believed to increase over the course of culturing as cells secrete them into the culture medium (Fig. 9). The decrease in N'-formylkynurenine on day 6 of culture was probably caused by the depletion of tryptophan in the culture medium.

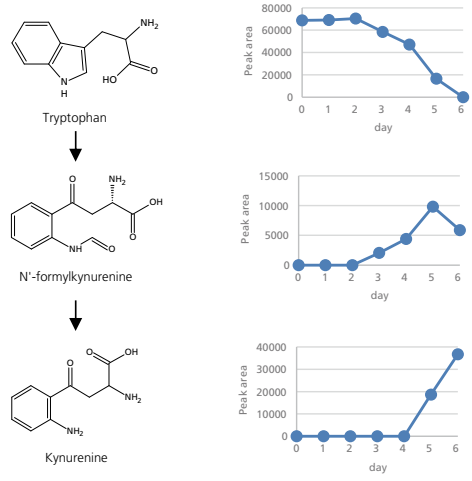

Fig. 9 Metabolic Variations in the Kynurenine Pathway

Next, a method was created for high-sensitivity analysis of major culture medium components and the newly discovered N'-formylkynurenine by triple quadrupole LC/MS. Major culture medium components were measured with high sensitivity using the LCMS-8000 series triple quadrupole LC-MS system and the cell culture profiling LC/MS/MS method package. Because the method package did not include N'-formylkynurenine, new MRM transitions for N'-formylkynurenine needed to be added to the analysis method. Since MRM transitions can be easily created from MS/MS spectral data acquired using LCMS-9030 (Fig. 10), MRM transitions used to identify N'-formylkynurenine were created from MS/MS spectral data and added to the cell culture profiling method (Fig. 11).

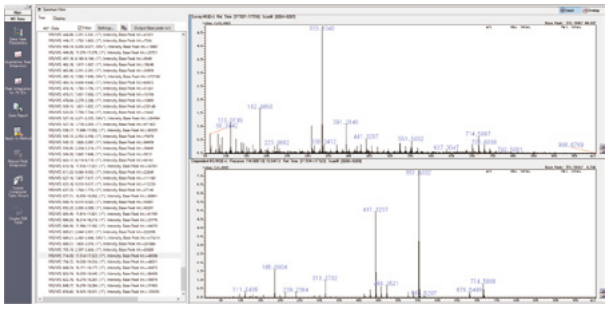

Fig. 10 Creating MRM Transitions

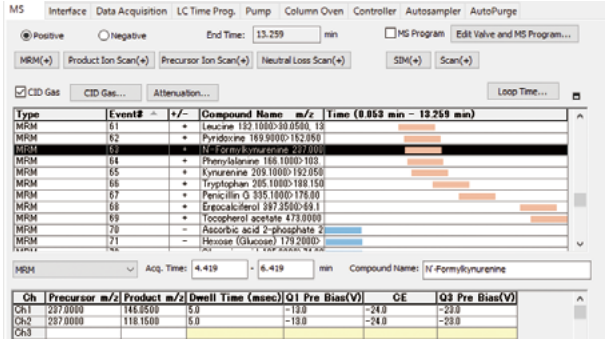

Fig. 11 Adding MRM Transitions to Existing Method

Using the created method and LCMS-8060 system, the iPS cell culture supernatant was analyzed for 96 target culture components, including N'-formylkynurenine. As shown in Fig. 12 and Fig. 13, the major culture medium components, including N'-formylkynurenine, were analyzed successfully.

* iPS cell cultures were performed by iPS Portal, Inc.

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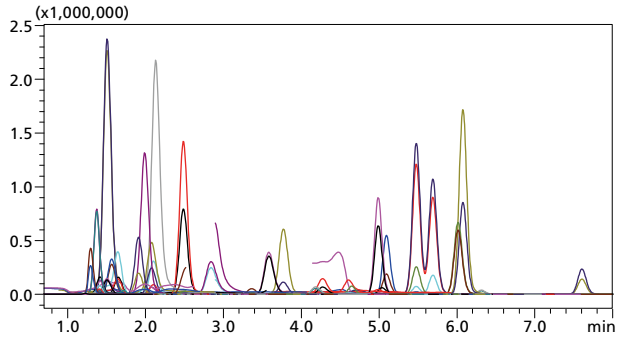

Fig. 12 MRM Chromatogram of iPS Cell Culture Supernatant

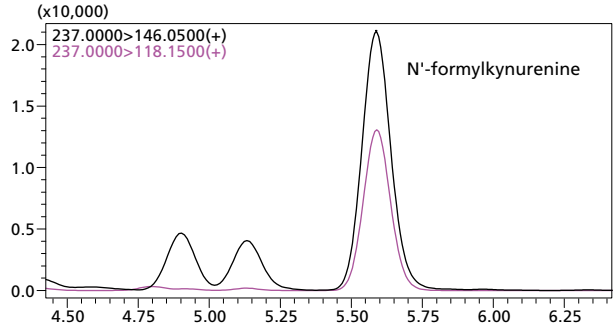

Fig. 13 MRM Chromatogram of N'-Formylkynurenine

5. Conclusions

The exact mass database for endogenous metabolites contains multiple methods for LC/Q-TOF analysis and enables the comprehensive analysis of metabolites with a wide range of physical properties. The database contains retention times and exact mass information for a total of 470 metabolite components (including internal standard substances) and provides an easy starting-point for metabolomics analysis. As shown in the above example involving N'-formylkynurenine, even metabolites not included in the database can be found with ease by skillfully applying information obtained by using the database for targeted metabolomics analysis. Transferring the method for high-sensitivity analysis of newly discovered metabolites on a triple quadrupole LC-MS system is also easy. Therefore, the database offers a total solution for steps ranging from untargeted metabolomics to high-sensitivity widely targeted metabolomics.

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