Simultaneous Analysis of Primary Metabolites by Triple Quadrupole LC/MS/MS

by Tsuyoshi NAKANISHI, Takako HISHIKI, Takayuki MORIKAWA, Mayumi KAJIMURA, and Makoto SUEMATSU

Abstract

We developed a "Primary metabolites LC/MS/MS method package" that enables the simultaneous measurement of 55 metabolites including those related to glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle as well as amino acids and nucleotides when used with a triple quadrupole mass spectrometer. In general, it is difficult to separate these highly polar metabolites by LC/MS coupled with normal reverse-phase chromatography because of their hydrophilic characteristics. Therefore in this method the separation of 55 high-polarity metabolites was made possible by adding an ion-pairing reagent to the mobile phase. Utilization of a triple quadrupole mass spectrometer with high sensitivity and selectivity for the simultaneous analysis of complex samples like cultured cell and tissue extracts is extremely effective. In this review we describe the features of the "Primary metabolites LC/MS/MS method package" and also introduce the simultaneous measurement of biological samples as an example.

Keyword: Metabolome, Metabolomics, Primary metabolites, Ion-pairing reagent, Triple quadrupole, LC/MS/MS

1. Introduction

"Oomics" analysis, which encompasses genomics and proteomics, is an area of research that advances understanding of biological function by the comprehensive and exhaustive analysis of huge amounts of biomolecular data. Metabolomics is one such "omics" analysis that focuses on groups of metabolites that have a more direct effect on biological function. Metabolomics is also pursued with the aim of complementing molecular information obtained through other "omics" analyses such as genomics and proteomics, as metabolites understood to be the final reactants of a living organism are created by the activity of a series of enzymes. Of these types of metabolites, the rapid analysis of changes in primary metabolites essential for biological activity such as the maintenance of biological function and cell differentiation and proliferation is an especially important part of metabolome analysis. There is a strong demand for the establishment of simple and reproducible methods of simultaneous quantitative analysis. There is a particularly apparent desire to evaluate quantitative changes in amino acids and nucleotides used in the synthesis of proteins and nucleic acids, and quantitative changes in metabolic pathways involved in energy production-known as central metabolic pathways-consisting of the glycolytic system, pentose phosphate cycle, and tricarboxylic acid (TCA) cycle. To date, methods of metabolome analysis have used GC/MS, LC/MS and CE/MS, but each of these techniques comes with problems such as a need for derivatization of target compounds, restrictions in terms of viable targets for analysis, or unstable retention times for compound separation. In general, when performing an analysis of highly hydrophilic metabolites using LC/MS, the separation achieved with a normal ODS column is often insufficient, and separation is frequently performed using either a pentfluorophenylpropyl (PFPP) column or an hydrophilic interaction chromatography (HILIC) mode that provide differing separation specificities. However, analysis of amino acids and nucleotides, and of metabolites involved in the central metabolic pathways consisting of the glycolytic system, pentose phosphate cycle, and TCA cycle requires a combination of analyses to be performed that comprised at least two of the above-mentioned separation modes. Another possible choice for separation of these kinds of hydrophilic compounds is the use of a reverse-phase column with addition of an ion-pairing reagent in the mobile phase. Introducing an ion-pairing reagent into the mobile phase consisting of a compound with a charged hydrophilic group and, frequently, a hydrophobic group in the form of an alkyl chain, is a method for improving retention times in a reverse-phase column. Also, recent years have seen the widespread use of multiple reaction monitoring (MRM) analysis with triple quadrupole mass spectrometers: a method that is capable of highly sensitive and highly selective detection for quantitative analysis. With the aim of performing simultaneous analysis of the above-mentioned primary metabolites, and of improving retention times and achieving the stable reproducibility offered by ion-pairing reagents, the authors have combined the use of ion-pairing reagents with a triple quadrupole mass spectrometer (Fig. 1) to develop an LC/MS/MS method package capable of the simultaneous analysis of a large number of highly polar metabolite components. This article provides an outline of the primary metabolites LC/MS/MS method package developed by the authors including its main features, and describes an example analysis of a biological sample using this method package.

Fig. 1 LCMS-8040 triple quadrupole mass spectrometer
2. Primary Metabolites LC/MS/MS Method Package

2.1 Outline of the Method Package

Primary metabolites are normally metabolites that are essential to the maintenance of cellular activity, and refers to compounds involved in energy production such as in central metabolic pathways, or the amino acids, nucleotides, lipids and other compounds that make up an organism. The LC/MS/MS method package developed by the authors targets 55 components among the above-mentioned types of primary metabolites including metabolites from the central metabolic pathways, amino acids, nucleotides and coenzymes, and incorporates analytical conditions for simultaneous analysis of these metabolites (Table 1). The use of an ion-pairing reagent is a distinctive feature of the LC separation, and by adding 10 mM of tributylamine to the mobile phase, the method developed by the authors can be used to retain hydrophilic metabolites. Compounds with a phosphate group are known to normally absorb to metal, so to mitigate this adsorption, a column with coated internal metal surfaces is used (metal free column, Mastro C18). The LC separation conditions and MS analysis conditions used by the method package for analysis of the target components are optimized in order to lessen the time used in investigating analysis conditions suitable for the target components. The primary metabolites LC/MS/MS method package also always assumes that its task will be to specifically find and compare metabolites present in different quantities in multiple samples, such as comparing changes in metabolite quantity between a normal model and disease model, and so includes analysis conditions for the use of two internal standard substances for normalizing results between different samples. Each parameter used in the MS analysis conditions is optimized individually for each of the 57 target components including the two internal standard substances. These parameters include MRM transition (Q1/Q3) and collision energies. Fig. 2 shows a mass chromatogram obtained from simultaneous analysis of a standard mixture made up of the 57 components. This mass chromatogram shows amino acids, are eluted first, followed by sugar phosphates and organic acids, then nucleotides and coenzymes in that order. As shown in Fig. 2, we confirmed that using tributylamine as the ion-pairing reagent achieved proper separation of high-polarity metabolites, including amino acids, organic acids, nucleotides, and sugar phosphates. To evaluate the retention time and peak area reproducibility of each peak, we also repeated the analysis (n = 3) using a reference standard mixture solution (5 µM). Reproducibility was confirmed to be sufficient as evidenced by a %RSD of <1% and <10% for almost all metabolite retention times and peak areas. A peak area %RSD of >10% was only observed with cysteine, nicotinamide dinucleotide, and the reduced form of nicotinamide adenine dinucleotide (NADH). These results are thought to be caused by sample degradation as each of these compounds is liable to be affected by oxidation.

Table 1  LC/MS analytical conditions

<table>
<thead>
<tr>
<th>HPLC (Nexera system)</th>
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<tbody>
<tr>
<td>Column</td>
<td>Mastro C18 (2.0 x 150 mm, 3 mm)</td>
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<tr>
<td>Mobile phase</td>
<td>A: 15 mM acetic acid, 10 mM tributylamine-water</td>
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<tr>
<td></td>
<td>B: Methanol</td>
</tr>
<tr>
<td>Gradient conditions</td>
<td>0% B (0.5 min) – 25% B (8.0 min) – 98% (12.0 – 15.0 min)</td>
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<tr>
<td></td>
<td>– 0% (15.1 – 20.0 min)</td>
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<tr>
<td>Flowrate</td>
<td>0.30 mL/min</td>
</tr>
<tr>
<td>Column temperature</td>
<td>40°C</td>
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</table>

<table>
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<tr>
<th>Mass spectrometer (LCMS-8040)</th>
<th></th>
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<tr>
<td>Ionization probe</td>
<td>ESI</td>
</tr>
<tr>
<td>Polarity</td>
<td>Negative</td>
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<tr>
<td>Analysis mode</td>
<td>MRM</td>
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<tr>
<td>Nebulizer gas</td>
<td>2.0 L/min</td>
</tr>
<tr>
<td>Drying gas</td>
<td>10 L/min</td>
</tr>
<tr>
<td>DL temperature</td>
<td>250°C</td>
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<tr>
<td>HB temperature</td>
<td>400°C</td>
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2.2 Analysis of a Biological Sample

2.2.1 Preparation of a Biological Sample

Next we describe an example simultaneous analysis of 55 components using the primary metabolites LC/MS/MS method package for samples of mouse heart and liver tissue. The method of pretreatment of the tissue extracts described below follows an already-reported method used for metabolome analysis. After quickly removing the organs from the mouse under anesthesia, each organ was frozen by immersion in liquid nitrogen. Frozen tissue was transferred to an Eppendorf tube and weighed, then methanol containing the internal standard substances L-methionine sulfone and 2-morpholinoethanesulfonic acid (MES) was added. The frozen tissue was crushed in a homogenizer or with beads, then protein and lipids were removed by chloroform / methanol extractions. The water layer containing the hydrophilic metabolites was then recovered and filtered. The recovered aqueous solution was freeze-dried and returned to solution with ultra-pure water. This aqueous solution was diluted as appropriate and simultaneous analysis performed with the triple quadrupole mass spectrometer (LCMS-8040). The sample injection volume used for this analysis was 3 µL, and a single analysis took 20 minutes. The LC separation conditions and MS analysis conditions were set according to the method package analysis conditions (Table 1).

2.2.2 Analysis of Biological Tissue

Fig. 3 shows the mass chromatograms obtained from simultaneous analysis for the 55 components in the mouse heart tissue and liver tissue extracts. With several metabolites, different results were obtained in different tissue types. Since no correction has been made for differences based on tissue weight or internal standard substance at this point, a normalization step is needed to accurately compare metabolite quantities between the different tissues. The comparison of changes in metabolite quantity in tissue extract and cultured cell extract samples normally necessitates some correction based on tissue weight or protein content. We corrected the peak area of each metabolite using the peak area of the internal standard substance, then incorporated the tissue weight measured at extraction with this corrected peak area result. Fig. 4 shows a comparison of the metabolite quantity per unit tissue weight present in the heart and liver tissue according to the above-described method. Out of the 55 components, Fig. 4 shows only those metabolites involved in the glycolytic system. That metabolites are present in different quantities in different organs is shown in Fig. 4 by the clear difference in metabolite quantity observed per unit tissue weight in tissue from the heart and liver. Although the above quantitative analysis compares the metabolites present in different organs taken from a single mouse, it can also be used to observe metabolite changes between normal and disease models for a biomarker discovery, and changes in metabolites in medically untreated and treated populations to evaluate therapy.
Fig. 3  Mass chromatograms of liver/heart tissue extracts

Fig. 4  Comparison of metabolites related to glycolysis between heart and liver tissue from a mouse

Comparison of the quantity of each metabolite per unit weight (mg) of heart and liver tissue

Glucose-1-Phosphate
Glucose-6-Phosphate
Fructose-6-Phosphate
Fructose-1,6-Diphosphate
Glyceraldehyde-3-phosphate
Dihydroxyacetonephosphate
Glycerol-3-Phosphate
1,3-Diphosphoglycerate
2,3-Diphosphoglycerate
2-Phosphoglycerate/3-Phosphoglycerate
Phosphoenolpyruvate
Pyruvate
Lactate

Fig. 4  Comparison of metabolites related to glycolysis between heart and liver tissue from a mouse
3. Changes in Metabolites in a Mouse Model of Cerebral Ischemia

3.1 Preparation of Brain Tissue from the Cerebral Ischemia Mouse Model

Section 2 outlined the primary metabolites LC/MS/MS method package and described an example analysis that used mouse heart and liver tissue. Section 3 will describe an example simultaneous analysis of multiple components within brain tissue samples taken from a cerebral ischemia mouse model in order to observe metabolite changes between a normal model and a disease model. The cerebral ischemia mouse model was created by inserting a nylon suture in the middle cerebral artery (MCA) to induce a middle cerebral artery occlusion (MCAO) and so reduce local cerebral blood flow. Brain tissue taken from a cerebral ischemia mouse model, in which a middle cerebral artery occlusion was induced for 60 minutes, was used as the sample in simultaneous analysis of metabolites. Perfusion was not performed after the period of occlusion, and an in situ freezing method was implemented to prevent as much metabolite degradation as possible during brain tissue extraction. When performing metabolome analysis, it is normally important to prepare the sample in a way that reduces metabolite degradation and allow an accurate evaluation of the quantity of metabolite present in the body. Methods of sample preparation that reduce metabolite degradation and have been reported to date include the above-mentioned in situ freezing method, and the focused microwave irradiation method. All such methods aim to allow for LC/MS analysis to provide a more accurate reflection of the quantities of metabolites in in vivo conditions by instantaneously stopping metabolic activity in cells and reducing metabolite degradation.

After using liquid nitrogen to freeze the removed brain, the tissue was separated into the left brain containing the cerebral ischemia, and the normal right brain. Fig. 5 shows HE-stained coronal sections of brain tissue from a control (normal) and cerebral ischemia model. The blue region in the figure corresponds to the ischemic region. Separate tissue extracts of left and right brains were prepared according to the method described in “2.2.1 Preparation of a Biological Sample” from both a normal model (control) and a disease model (cerebral ischemia) to be used in simultaneous analysis of multiple components with the primary metabolites LC/MS/MS method package.

Fig. 5 HE staining of the brain tissue section of a normal and a cerebral ischemia model

![Fig. 5](image)

Fig. 6 Mass chromatograms from the simultaneous analysis of a normal and a cerebral ischemia model

![Fig. 6](image)
3.2 Analysis of Brain Tissue from the Cerebral Ischemia Mouse Model

When cerebral infarction (MCAO) occurred, it caused reduced blood flow in a localized area of the brain. This caused glucose depletion and an insufficient supply of oxygen to the affected ischemic region, predictably reducing products of energy metabolism such as ATP. This is thought to lead to an associated substantial increase in AMP, which is the degradation product of ATP and ADP, in the ischemic region. Fig. 6 shows mass chromatograms obtained after simultaneous analysis (n = 4) for multiple components in brain tissue (left/right brains) from middle cerebral artery-occluded mice. A simple comparison of these mass chromatograms shows quantitative changes in some metabolites caused by the effects of ischemia. Also, while analysis was performed using brain tissue extracts from different mice, mass chromatograms from the normal mouse model and cerebral ischemia mouse model each displayed a similar pattern. This demonstrates the method package produced sufficiently reproducible results for the analysis of biological tissue extracts.

The graph in Fig. 7 summarizes the peak area ratio of normal areas to ischemic regions for metabolites present in different Quantities in the two types of tissue out of the 55 target components. As shown by the graph, metabolites linked to ATP and UTP energy metabolism are reduced in quantity in the left side of the brain containing the ischemic region. In contrast, the amount of AMP is greatly increased in the left side of the brain. These changes are believed to be associated with the reduced blood flow that occurred in the left side of the brain. In cerebral ischemia mouse models, NADH is also slightly increased in the ischemic region side of the brain. These results show the same metabolite distribution as observed in a previous report that used MALDI imaging for analysis of a mouse model of cerebral infarction (MCAO). The metabolite distribution observed with MALDI imaging in that report confirmed reduced ATP and ADP and increased AMP in the ischemic region as well as a slight increase in NADH, which supports in this case the suitability of using the primary metabolites LC/MS/MS method package for simultaneous analysis of multiple components. Consequently, the above results demonstrate that when a target of analysis is a large number of highly polar metabolites that are difficult to analyze simultaneously by normal LC/MS analysis, using the primary metabolites LC/MS/MS method package with an ion-pairing reagent is an extremely effective method for analyzing quantitative changes in these hydrophilic metabolites in vivo conditions. As introduced in this article, this method package is expected to be developed further for comparing quantitative changes in metabolite between normal and disease models with the objective of finding disease-causing irregularities in metabolic pathways.

4. Conclusion

In the previous sections, we described an example simultaneous analysis of a biological sample using the primary metabolites LC/MS/MS method package and confirmed that this method is effective for analyzing quantitative changes of metabolites in various tissue extracts. Although not described in this article, this method can also be used for analysis of cultured cell extracts and culture media components, and the method is anticipated to be further developed for quality control applications such as for simultaneous analysis of amino acids and organic acids in the food sector. This method package is expected to be compatible with a variety of different sample types. However, since it involves the addition of a high-concentration ion-pairing reagent to the mobile phase, using together with other modes of LC separation on the same instrument will be difficult. This LC/MS/MS system makes the simultaneous analysis of many different hydrophilic metabolites possible and because of this, the selection and introduction of new ion-pairing reagents is envisaged to become more important in the future. We also expect new analysis columns to appear and increase the number and types of compounds analyzed, such as PPFP columns that allow for analysis of some hydrophilic metabolites without use of ion-pairing reagents, or multi-mode columns with anion and cation exchange groups attached to the column carrier. Other methods of simultaneous quantitative analysis of an even larger number of components will no doubt be proposed in the future, and when that time comes, an important part of this technology will be the software used to support it. Since changes will need to be evaluated statistically between two or three different groups of metabolites, the development of analytical software capable of principal component analysis will also be beneficial. Also, due to the difficulty of evaluating changes in metabolites visually based solely on a list of peak area ratios, techniques such as cluster analysis among principal components, and the method is anticipated to be further developed when that time comes, an important part of this technology will be the software used to support it. Since changes will need to be evaluated statistically between two or three different groups of metabolites, the development of analytical software capable of principal component analysis will also be beneficial. Also, due to the difficulty of evaluating changes in metabolites visually based solely on a list of peak area ratios, techniques such as cluster analysis among multiple samples will also prove useful. In addition to analysis software and analysis tools being available commercially, such tools are also available through the Internet. We intend to use them according to our objectives.

Finally, we wish to express our sincere gratitude to Dr. Eiichiro Fukusaki of the Division of Advanced Science and Biotechnology, Graduate School of Engineering, Osaka University for his tremendous help in development of the primary metabolites LC/MS/MS method package.

![Fig. 7 Comparison of metabolites between a normal and a cerebral ischemia model](image-url)
References


