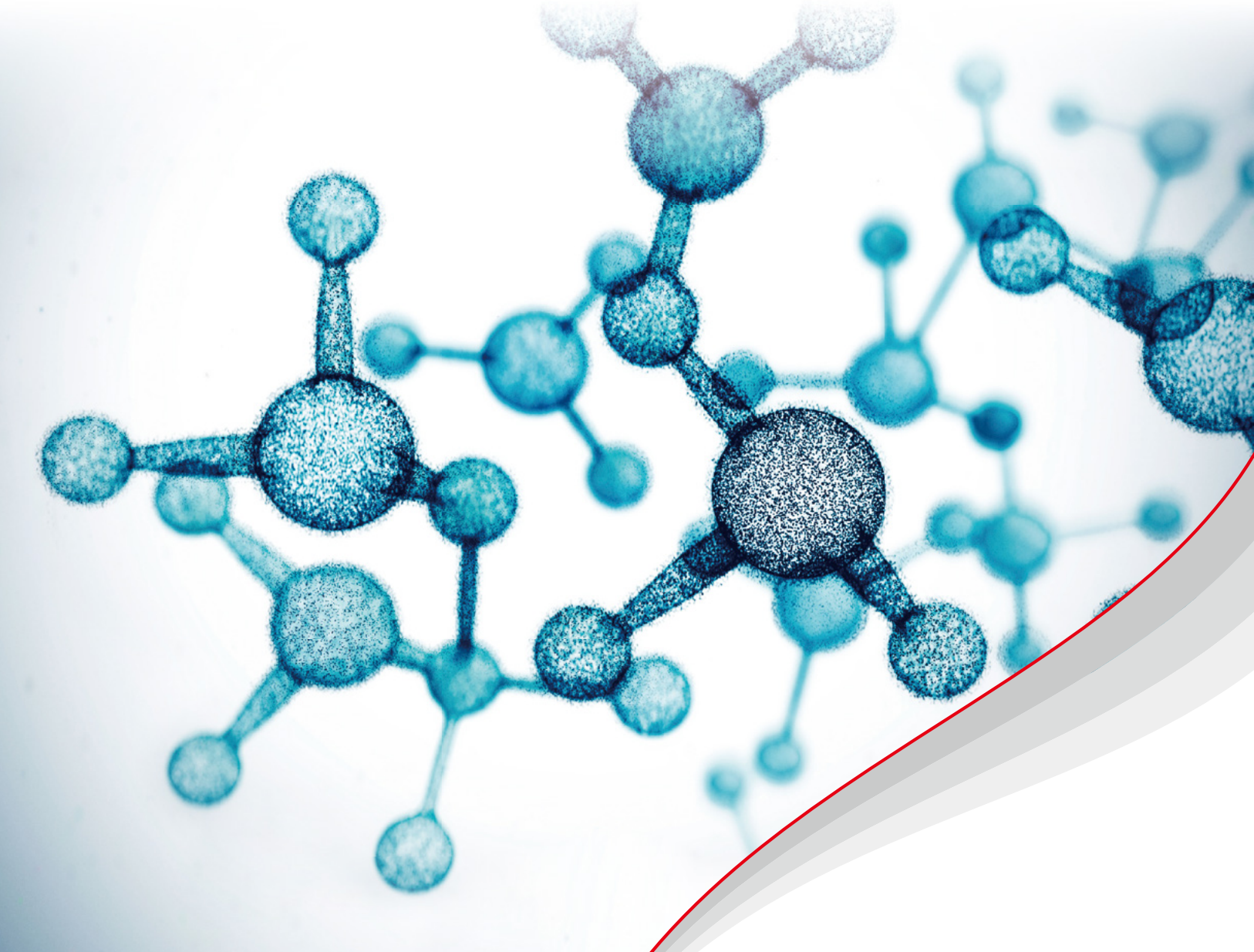
C10G-E103

SHIMADZU
Excellence in Science

Metabolic Pathway Analysis Solutions



Metabolic Pathway

Metabolic pathways are the chain of chemical and enzymatic reactions that occur within a cell in living organisms to support their life. They are a series of reaction pathways that include intermediates from reactants to products (Fig. 1). Some mediators are reversible, but an irreversible reaction at the beginning of a metabolic pathway determines the direction of the entire pathway. Regulation of critical metabolic pathways (e.g., glycolysis, the citric acid cycle, and the pentose phosphate cycle) maintains cellular homeostasis (Table 1).

Table 1 Location and Overview of Major Metabolic Pathways

Pathway	Location	Overview
Glycolytic pathway	Cytoplasm	Glucose is broken down into organic acids (e.g., pyruvate).
Citric acid cycle	Mitochondria	Acetyl-CoA is oxidized to produce energy.
Pentose phosphate cycle	Cytoplasm	The process of forming glyceraldehyde -3 phosphate from glucose -6 phosphate produces sugars essential for nucleic acid synthesis.
Gluconeogenesis	Liver and kidney	Glucose is produced from substances other than carbohydrates (e.g., pyruvate and glycerol).
Glycogen synthesis and degradation reactions	Liver and skeletal muscle	Glycogen, a storage body for glucose, is synthesized and degraded.
Synthesis and degradation of fatty acids	Synthesis in the cytosol and degradation in the mitochondria	The same metabolic pathways, including acyl-CoA and malonyl-CoA, proceed in opposite directions to synthesize and degrade fatty acids.

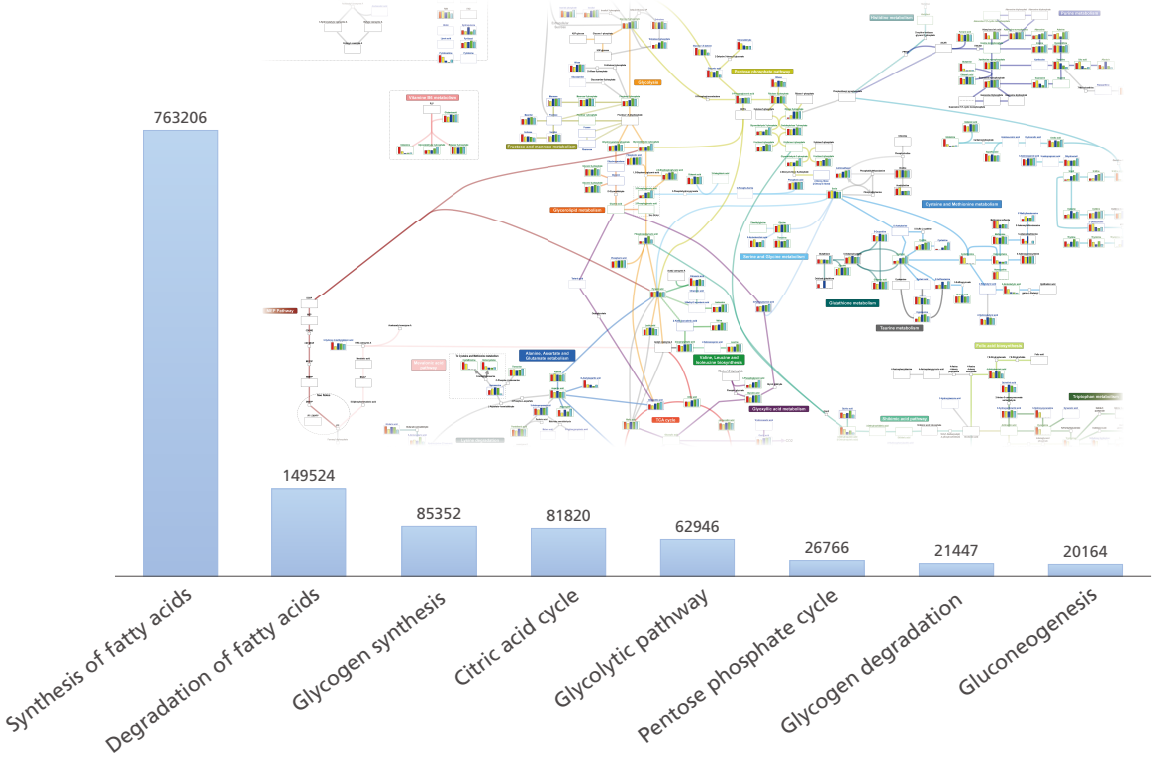


Fig. 1 Number of Published Documents for Each Metabolic Pathway (total number of patents and documents)

Metabolic Pathway Map

The peak area values of metabolites obtained from a gas chromatograph mass spectrometer or a liquid chromatograph mass spectrometer are shown as bar graphs. For each metabolite, the relative quantification value for each sample is shown as a bar graph, and the amount of change in the metabolite can be confirmed.

Applications

1. **Discovering differences in changes in metabolic pathways by change ratio among multiple experiments**
   It is possible to confirm metabolic changes in the growing environment and samples with genetic modifications.

2. **Confirming the reaction of metabolic enzymes**
   Changes can be made to enzymes to detect differences in metabolite levels.

3. **Identifying the unknown sample**
   It is possible to distinguish the sample group by the characteristic of the metabolite quantity.
   Example: Differences by type and/or production area

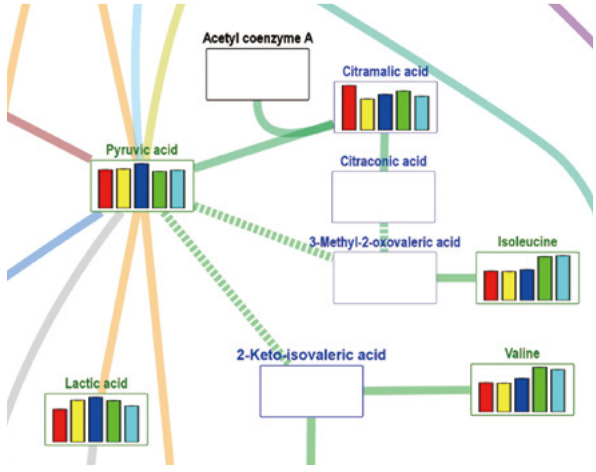


Fig. 2 Example of a Metabolic Pathway (enlarged view)

Multi-omics Analysis Package

Product

Multi-omics Analysis Package is software that automatically displays a large amount of mass spectrometry data obtained from metabolomics, proteomics, flux analysis, etc. on a metabolic map. It works with the various method package databases for metabolite analysis provided by Shimadzu to streamline metabolomics data analysis work. Intuitively visible data supports life sciences research, including drug discovery, functional foods, and bioengineering.

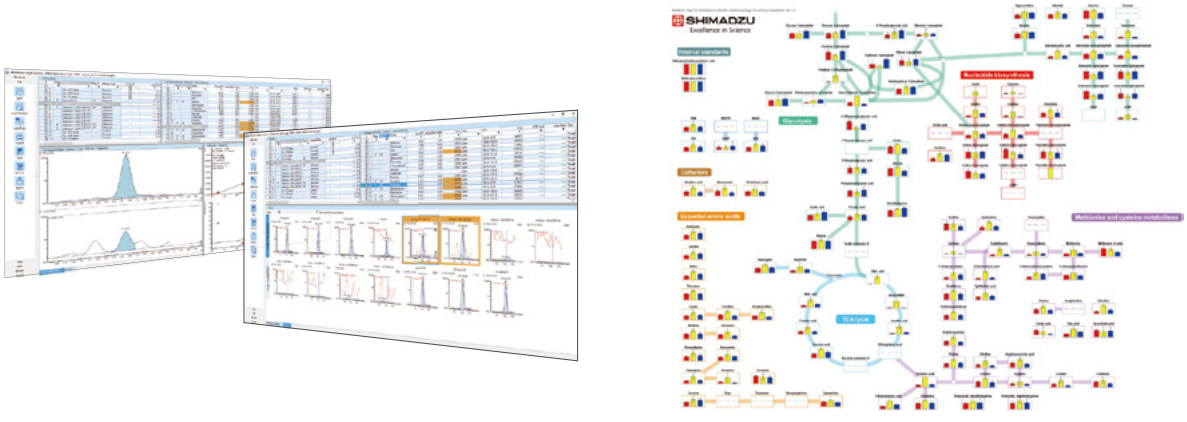


Fig. 3 LabSolutions Insight™ Analysis Projected onto Metabolic Map

Metabolic Map Analysis of 196 Eicosanoids

Application

The wide variety of metabolites in eicosanoid fatty acids complicates method development. For this reason, Shimadzu provides a 196-component eicosanoid assay with its "LC/MS/MS Method Package for Lipid Mediators Ver. 3."

benefits

A 196-component metabolic map analysis tool for "LC/MS/MS Method Package for Lipid Mediators Ver. 3" is available. By displaying quantitative differences in fatty acid metabolites on a metabolic map, the metabolic enzymes involved can be quickly analyzed.

■ Measurement

To a 30 µL sample, 300 µL of methanol containing 0.1% formic acid and 10 µL of an 18-component internal standard solution were added, and the mixture was stirred for about 3 minutes. After centrifugation, the supernatant was diluted 3 times with 0.1% formic acid water and added to the solid-phase extraction cartridge. The extract was dried and dissolved with 30 µL of methanol, and 5 µL was subjected to LC/MS analysis. Each sample was analyzed in triplicate.

■ Sample

Human plasma and serum

■ System Configuration

LC-MS System	: LCMS-8060 NX
Column	: Kinetex® C8 (2.1 mm I.D.×150 mm, 2.6 µm)

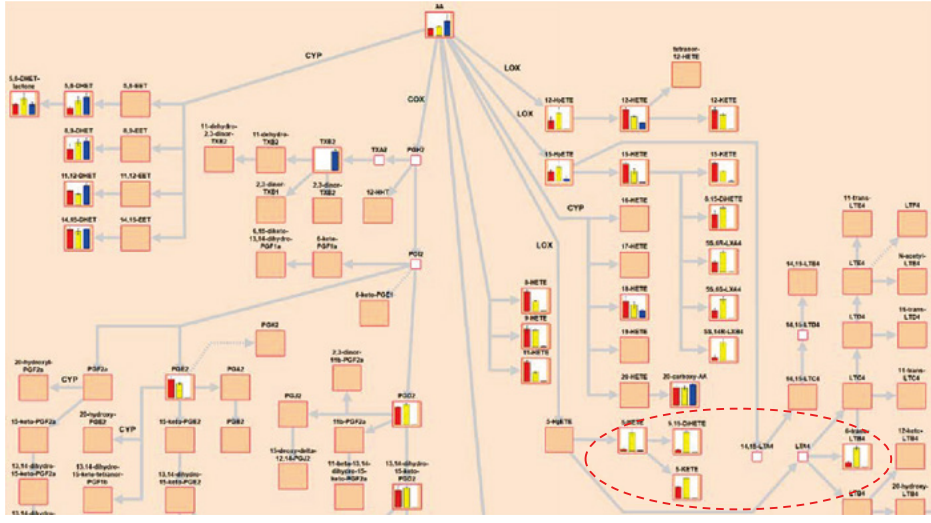


Fig. 4 Metabolic Map and Quantitative Profile of Arachidonic Acid Metabolites Detected in Human Plasma and Serum

■ Summary

An analysis tool that displays a metabolic map of these 196 components was developed. A total of 68 metabolites were detected in plasma and serum by the simultaneous analysis of 196 components. Using this analysis tool, it was easy to identify the enzymes involved in the detected metabolites.

LC/MS/MS Method Package for Lipid Mediators Ver. 3

Simultaneous analysis conditions for 214 components are included in this method package, with 196 lipid mediators and related substances derived from the arachidonic acid cascade and 18 parts of internal standards. All the compounds can be monitored in just 20 minutes.

Product

Differential Metabolite Analysis of Gene Mutant Flies Part 1

Application

A genetic mutation is a change in the DNA sequence that can be a substitution, insertion, or deletion. Genetic mutations can prevent proteins from being synthesized correctly or from functioning. Integrated analysis of metabolites and genomic information is fundamental research that may lead to drug development and personalized medicine for diseases. Therefore, it is necessary to study the effects of genetic mutations on metabolites.

benefits

- By using Multi-omics Analysis Package, measurement results obtained by GC/MS can be easily visualized.
- Statistical methods, such as principal component analysis, hierarchical clustering, volcano plots, and metabolic maps, are available to find sample differences.

■ Measurement

Fifty yellow fruit flies were used in the analysis. Of the 50 flies, 20 were wild-type and the remaining 30 were mutant flies. Five samples were prepared, consisting of 2 wild-type samples and 3 gene mutant samples. The fly samples were ground to homogenized mixtures.

Metabolites were extracted by adding methanol:water: chloroform (2.5:1:1) extract to the homogenized sample and shaking. After the aqueous phase was separated, methanol was removed by centrifugal concentration, and the remaining aqueous phase was dried in a lyophilizer. The dried samples were treated with methoxym derivatization using methoxyamine and trimethylsilyl (TMS) derivatization using N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA).

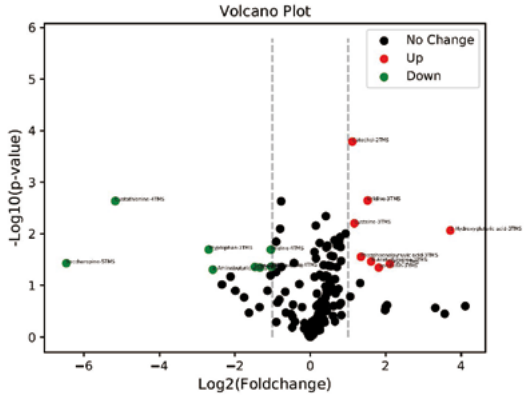


Fig. 5 Volcano Plot of Wild-Type and Mutant Yellow Drosophila

■ Summary

Metabolites of wild-type and mutant yellow fruit flies were analyzed on a GCMS-TQ8040 NX Gas Chromatograph Mass Spectrometer. The results of comprehensive metabolite detection were analyzed by principal component analysis, hierarchical cluster analysis, volcano plots, and metabolic maps using the Multi-omics Analysis Package. This enabled visualization of the metabolites whose genetic mutations caused the differences.

■ Sample

Yellow Drosophila (wild-type and mutant)

■ System Configuration

GC-MS System	: GCMS-TQ™8040 NX
Column	: DB5 (0.25 mm I.D.×30 m, 1 µm)
Carrier Gas	: Helium
Carrier Gas Control	: Linear Velocity

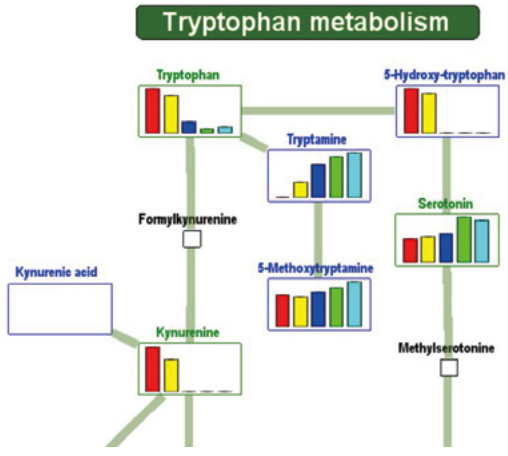


Fig. 6 Metabolite Map

Differential Metabolite Analysis of Gene Mutant Flies Part 2

Application

In the "Metabolic Difference Analysis of Gene Mutant Flies, Part 1", introduced in the previous section, metabolites of gene mutant fruit flies were measured using GC-MS and Smart Metabolites Database Ver. 2. In this example, the same fruit fly samples were measured using LC-MS and LC/MS/MS Method Package for Primary Metabolite Ver. 3, with the results from both LC-MS and GC-MS analysis projected onto a metabolic map and analyzed.

benefits

- Multi-omics Analysis Package enables easy visualization of the measurement results obtained by LC/MS and GC/MS.
- By integrating the results of LC/MS and GC/MS measurements and outputting them to a metabolic map, more metabolites can be displayed comprehensively.

■ Measurement

One hundred yellow fruit flies were used in the analysis. Of the 100 flies, 50 were wild-type and 50 were mutant. Twenty samples were prepared, consisting of 10 wild-type and 10 mutant samples. Fly samples were ground, pretreated according to the Metabolomics Pretreatment Handbook, and measured using the LCMS-8060NX and LC/MS/MS Method Package for Primary Metabolite Ver. 3.

■ Sample

Yellow Drosophila (wild-type and mutant)

■ System Configuration

LC-MS System	: LCMS-8060NX
Column	: Shim-pack™ GIST PFPP (2.1 mm I.D.×150 mm, 3 µm)

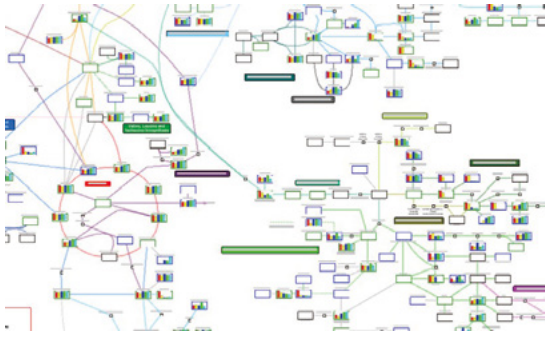


Fig. 7 Metabolic Pathway Map
(Integration of LC/MS and GC/MS Measurements)

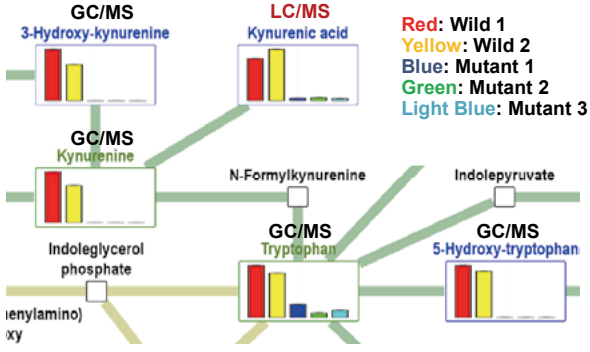


Fig. 8 Magnified View of the Metabolic Pathway Map

■ Summary

Metabolites from wild-type and mutant yellow fruit flies were analyzed by LC/MS. The results of comprehensive metabolite detection were analyzed using the Multi-omics Analysis Package. Statistical analysis methods, such as principal component analysis and volcano plots, enable the visualization of metabolites differentially expressed by genetic mutations. The results of GC/MS measurements were also projected onto a metabolic map to show metabolic pathways. Multi-omics Analysis Package enables the objective judgment of the results obtained using LC/MS.

LC/MS/MS Method Package for Primary Metabolite Ver. 3

This method package enables the user to start the analysis without having to perform complicated tasks such as determining the separation conditions required for LC/MS/MS analysis and optimizing the MS parameters for each compound.

Product

Homocysteine Measurement in Plasma

Application

Methionine is responsible for transporting selenium, an antioxidant mineral, and increases the antioxidant activity in the body. Since homocysteine can indicate the movement of methionine synthetase and methylenetetrahydrofolate reductase in methionine-related metabolic pathways, it is essential to establish a method for measuring homocysteine.

benefits

It is possible to trace analysis methods used in hospital labs (Meyer Children's Hospital, Mass Spectrometry, Clinical Chemistry, and Pharmacology Lab).

■ Measurement

To 100 µL of plasma, 10 µL of internal standard (d8-Homocysteine) and 20 µL of DTT were added and stirred. After 20 minutes of standing, 300 µL of acetonitrile containing 0.2 % formic acid was added. After centrifugation, it was measured using the LCMS-8040 in MRM mode.

■ Sample

Plasma

■ System Configuration

LC-MS System	: LCMS-8040
Column	: SUPELCO SIL LC-CN (3 mm I.D.×33 mm, 3 µm)

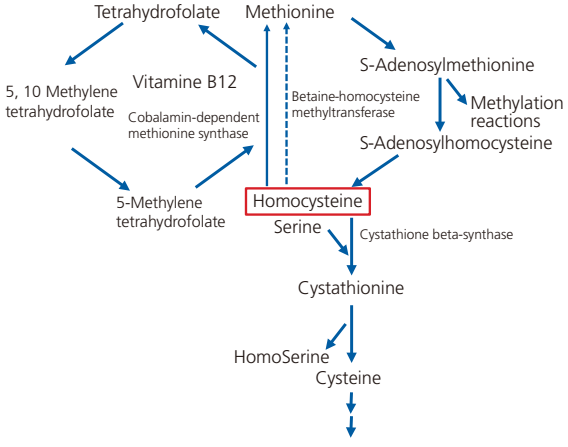


Fig. 9 Metabolic Pathways

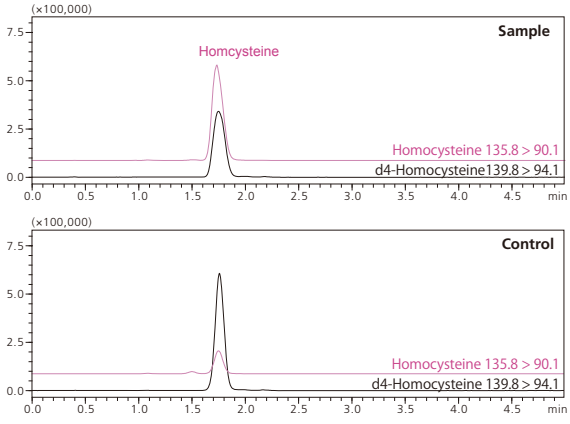


Fig. 10 Extracted Ion Chromatograms of Each Target Compound

■ Summary

The peak of homocysteine in plasma was easily detected. This system is for research purposes only and cannot be used for clinical diagnostic purposes. However, it is possible to confirm enzyme activity using this assay system.

Triple Quadrupole LC-MS/MS

LCMS-8040

This middle-class system offers high sensitivity exceeding that of general-purpose machines and has inherited the simple operation of conventional machines. The high speed and high sensitivity of the UHPLC system enable the study of metabolic pathways.

Product

Metabolic Analysis of Antibody-Producing Cells

Application

In recent years, many reports have shown the effects of variations in organic and inorganic components in culture fluids on antibody production and quality. Furthermore, it is known that organic and inorganic ingredients interact with each other in the culture medium and affect its dynamics. Therefore, it is necessary to analyze organic and inorganic components in the culture medium to understand their metabolism.

benefits

- Metabolic analysis combining organic and inorganic components is possible.
- Simple pretreatment enables simultaneous analysis of organic and inorganic components in culture media and supernatants.

Measurement

The Nexera™ X3 system and LCMS-8060NX were used to analyze the organic components in the culture medium. "LC/MS/MS Method Package for Cell Culture Profiling Ver. 3" was used as the analysis method. A total of 144 components related to cell metabolism, including medium components and secreted metabolites, were analyzed simultaneously using this method.

The ICPMS-2030 was used to analyze the inorganic components. The ICPMS-2030 assay was prepared by diluting the medium 20 times with one v/v% nitric acid. We analyzed Co, Cu, Fe, Mg, Mn, Mo, Ni, Se, and Zn, focusing on elements reported to affect antibody production.

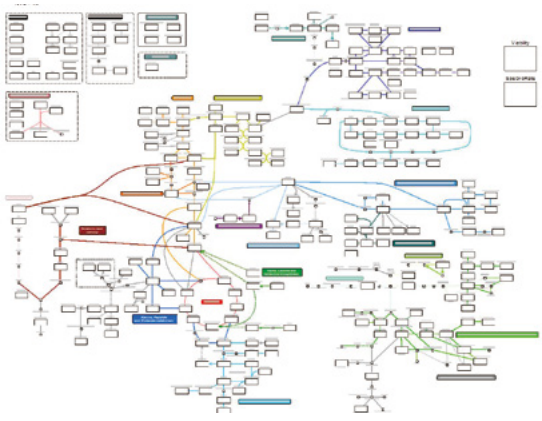


Fig. 11 Example of a White Map
(white map optimized for metabolic pathways in CHO cells)

Summary

By using LC-MS/MS (LCMS-8060NX) and ICP-MS (ICPMS-2030) to analyze the components in the culture supernatant, it will be possible to detect the details and metabolic pathways involved in antibody production. Using the results of the multi-component measurements, we identified the features and characteristic metabolic pathways associated with antibody production by analyzing the specific antibody production rate as the target variable.

Sample

Medium/culture supernatant of CHL-YN cell antibody producing strain

System Configuration

LC-MS System	: LCMS-8060NX
Column	: Shim-pack GIST PFPP (2.1 mm I.D.×150 mm, 3 µm)
ICP-MS System	: ICPMS-2030

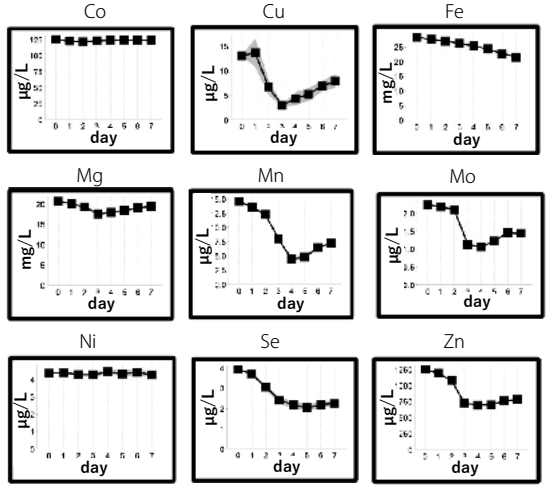


Fig. 12 Culture Supernatant Analysis Using ICP-MS

Triple Quadrupole LC-MS/MS

LCMS-8060NX

Only target ions can be efficiently introduced into the mass spectrometer with the newly developed focus electrode. Since contaminant components are removed, the effect of contamination is reduced, enabling stable analysis even for biological samples.

Product

Monitoring of Elemental Metals in Cell Culture Supernatants

Application

Antibody drugs are produced mainly by culturing CHO cells. Recently, it has been reported that cell metabolism and the primary structure of antibodies produced during culturing are affected by changes in the concentration of metallic elements in the culture medium. For example, the pathway of lactate metabolism in CHO cells changes in response to the attention of Cu in the culture medium, and the carbohydrate structure of the antibody changes in response to the Mn/Zn ratio in the medium. Therefore, to maintain the quality of antibody drugs, it is essential to monitor changes in the concentration of metallic elements in the culture medium over time.

benefits

- Using an atomic absorption spectrophotometer, cell culture supernatants can be analyzed by dilution-only pretreatment.
- By using an auto atomizer (AAC), which can easily switch between the flame method and the electric heating method, a wide range of concentrations of metallic elements can be easily analyzed from trace (ppb) to high (ppm) concentration.

■ Measurement

CHO cells were cultured in 125 mL culture flasks with shaking (120 rpm) for four days. After 1 mL of the initial culture was collected, 1 mL was collected every 24 hours. After centrifugation (5 min, 4°C) of each managed culture, the culture supernatant was dispensed into a separate tube to prepare the culture supernatant sample.

The AA-7000 Atomic Absorption Spectrophotometer, graphite furnace atomizer (automatic switching between the flame and electric heating), and autosampler were used for the measurement. Regarding the atomization method, we used the flame method for elements known to be highly concentrated in the culture solution (Mg, Zn) and the electric heating method for other trace elements (Cu, Mn, Co, Fe).

■ Sample

Media collected over time during cell culture

■ System Configuration

AA System	: AA-7000F/AAC
Autosampler	: ASC-7000

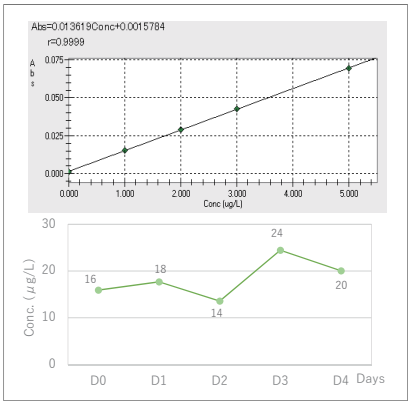


Fig. 13 Time Course of Cu Concentration, Which May Affect the Metabolic Pathway of Lactic Acid

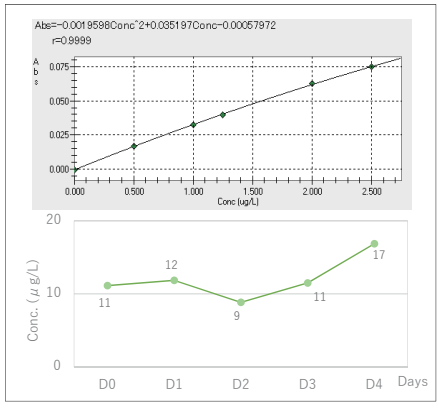


Fig.14 Time Course of Mn Concentration, Which is Believed to Affect Protein-Carbohydrate Structure

■ Summary

Concentrations of metal elements in culture supernatants were measured by atomic absorption spectrometry (flame and electric heating). It was possible to analyze a wide concentration range from several ppb to several ten ppm by simple pretreatment of the culture supernatant by dilution. These results indicate that the atomic absorption method applies to culture supernatants and culture media analysis.

Atomic Absorption Spectrophotometer

AA-7800 Series

It is possible to measure parts per million (ppm) by the flame method and parts per billion (ppb) by the electric heating method according to the analytical application. Both can measure various targets, from microanalysis to high-concentration analysis.

Product

Metabolomic Application to Microbial Breeding

Application

Metabolomics is an effective technology for understanding metabolic changes, including precursors, intermediates, and target substances, in microbial breeding. By better understanding the metabolic pathways related to substance production, it is expected to be applied to producing substances with high production efficiency. This application presents an example of LC/MS analysis of the changes in sulfur-containing metabolites when thiosulfate or sulfate is added to the sulfur source used for cysteine synthesis using cysteine-producing Escherichia coli.

benefits

It is possible to detect peaks mainly derived from amino acids, coenzymes, and nucleic acid-related compounds in the ion-pair method and from amino acids, organic acids, and nucleic acid-related compounds in the non-ion-pair process.

■ Measurement

After measuring the OD value of the recovered E. coli, the medium components and the bacterial bodies were rapidly separated by filter filtration. E. coli extract was prepared by crushing the isolated bacteria in methanol. LC/MS analysis was carried out by diluting methanol dissolved in ultrapure water after methanol was released in a concentrated centrifuge.

■ Sample

E. coli incubated for 3, 4, 5, 6, 7, 8, and 9 hours.

■ System Configuration

LC-MS Systems	: LCMS-8040 and LCMS-8050
Column	: RP columns

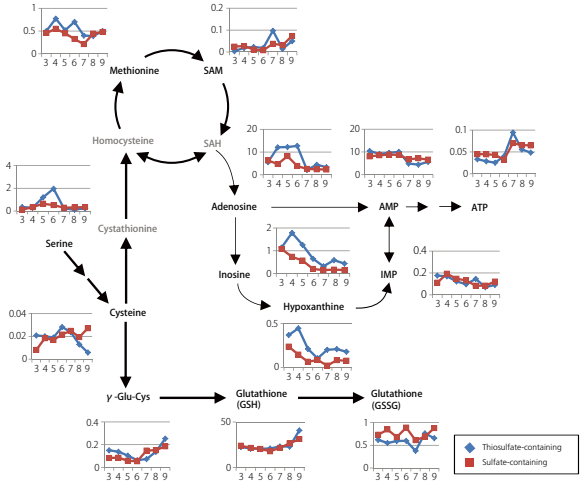


Fig. 15 Sulfur-Containing Metabolites in Escherichia Coli Culture

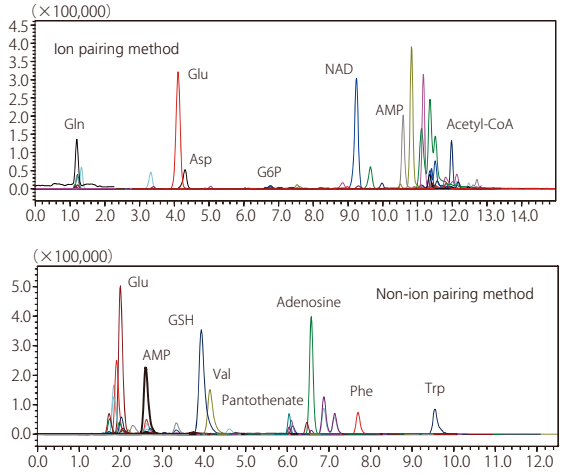


Fig. 16 MRM Chromatogram of Escherichia coli Extracts Cultured in Thiosulfate-Supplemented Medium

■ Summary

Variations in metabolites during cultivation were confirmed based on differences in sulfur sources. Cysteine decreased in thiosulfate-supplemented medium and increased in serine upstream of the metabolic pathway, especially around 6 hours when glucose was depleted. In addition, nucleosides (Adenosine, Inosine) were grown in the thiosulfate-supplemented medium. The metabolomics approach revealed that the different sulfur sources added to the medium affected the ability to produce sulfur-containing metabolites, including cysteine.

LC/MS/MS Method Package for Primary Metabolites Ver. 3

This method package enables the user to start the analysis without having to perform complicated tasks such as determining the separation conditions required for LC/MS/MS analysis and optimizing the MS parameters for each compound.

Product

^{13}C Metabolic Flux Analysis Using GC-MS

Application

In addition to measuring the number of metabolites (metabolome), the expression of enzyme genes (transcriptome) and the number of enzyme proteins (proteome) have been widely used to understand the intracellular metabolic state. Estimating intracellular metabolic flux (flux) has been attempted based on these results, but this is a difficult process because metabolite accumulation does not necessarily correlate with metabolic change. Intracellular metabolic flow is also affected by factors that are difficult to quantify, such as post-translational modification of proteins and allosteric control, which limits the estimation of in vivo metabolic status from enzyme gene expression and protein levels. To solve this problem, the ^{13}C metabolic flux analysis method, which measures the distribution of metabolic fluxes in cells, is helpful.

benefits

It provides a direct understanding of metabolism, such as the direction of intracellular metabolic reactions, branching ratios, and reaction rates. It reveals responses that are activated or inhibited.

Measurement

Wild Escherichia coli strain (Escherichia coli MG1655) was cultured aerobically in Sakaguchi flasks using 100 mL of M9 minimal medium supplemented with five g/L ^{13}C-labeled glucose ([1-^{13}C] glucose: [U-^{13}C]glucose=1:1). Cultures were sampled over time to determine cell concentration (OD_{600}) and media components. The cell concentration was measured by a spectrophotometer (UVmini-1240, Shimadzu Corporation) using turbidity at 600 nm (OD_{600}). To calculate the ^{13}C labeling fraction of protein-derived amino acids, 3 mL of mid-logarithmic growth phase (OD_{600} ~ 1) culture was collected in a Falcon tube and centrifuged (10,000 rpm, 10 min, 4°C) to make a bacterial pellet. Samples were stored at -80°C until use.

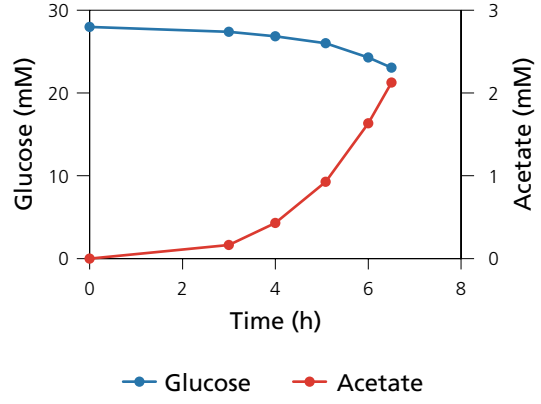


Fig. 17 Changes in the Concentrations of Glucose and Acetic Acid in the Medium

Summary

By performing a ^{13}C metabolic flux analysis, we obtained the flux distribution of the Escherichia coli central carbon metabolism pathway. This method is expected to be applied to the metabolic evaluation of microorganisms producing useful substances and disease cells such as cancer.

Sample

Escherichia coli wild strain

System Configuration

GC-MS System : GCMS-TQ8040
Auto Injector : AOC-20i

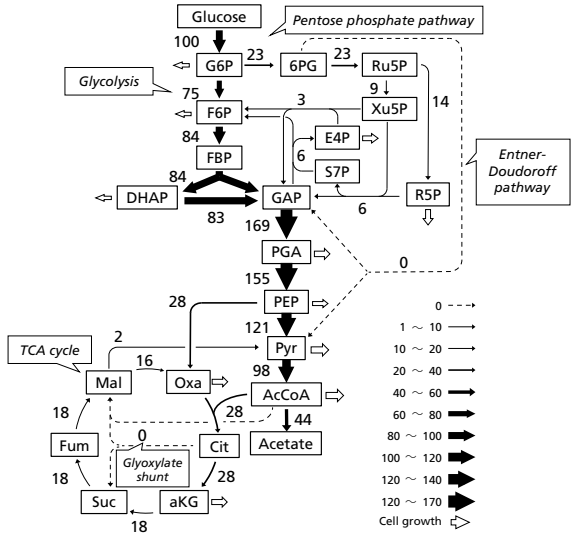


Fig. 18 Metabolic Flux Distribution in Growing Escherichia Coli

Triple Quadrupole GC-MS/MS

GCMS-TQ8040 NX

A stable ion source and a highly efficient collision cell enable analysis of a complex matrix (e.g., a biological sample) over a long period.

Product

Total Solution for Metabolomics with the Metabolite Precision Mass Database

Application

Metabolomics uses a mass spectrometer to analyze small molecule metabolites comprehensively. Triple quadrupole LC-MS and GC-MS are used for targeted metabolomics, where the metabolites to be analyzed are determined. Non-targeted metabolomics, such as the search for unknown metabolites, uses high-resolution mass spectrometers such as a Q-TOF LC-MS.

benefits

The Metabolite Precision Mass Database includes several "Ready to Use" methods for LC/Q-TOF to comprehensively analyze a wide range of metabolites (470 components) without needing LC or MS analysis.

■ Measurement

After the iPS cells were seeded, their culture supernatants were sampled every 24 hours and cultured for six days. The sampled culture supernatant was deproteinized by the addition of acetonitrile. After the organic solvent precipitation, the centrifugal supernatant was diluted ten times with ultrapure water and analyzed by the method of the medium component in this database.

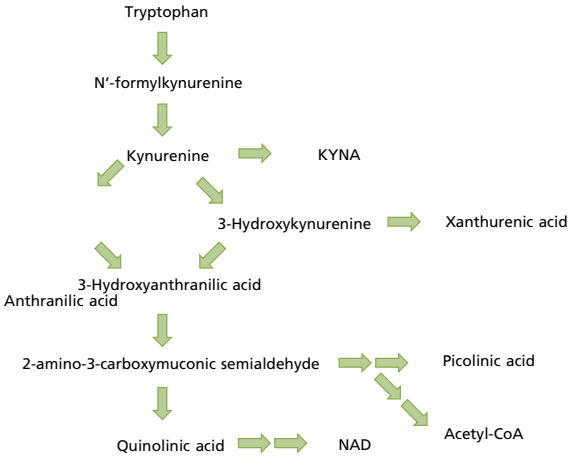


Fig. 19 Kynurenine Metabolic Pathway

■ Summary

The Metabolite Precision Mass Database contains several methods for LC/Q-TOF for comprehensive analysis of metabolites of a wide range of properties. It includes retention time and precise mass information of metabolites of 470 components (including internal standards) for easy metabolomics. As in the case of N'-formyl kynurenine, the search for metabolites not included in the database can be easily accomplished by using information obtained from targeted metabolomics utilizing the database. It also facilitates the transfer of methods for the analysis of newly discovered metabolites by triple quadrupole LC-MS, enabling a total solution from non-targeted metabolomics to highly sensitive wide-targeted metabolomics.

■ Sample

Culture medium of iPS cells

■ System Configuration

LC-MS System : LCMS-9030

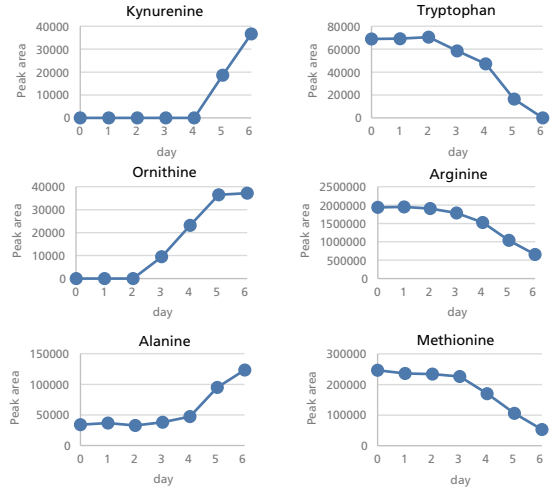


Fig. 20 Variation of Culture Supernatant Components with the Course of Culture

Quadrupole Time-of-Flight LC-MS/MS

LCMS-9030

This highly robust and sensitive instrument will contribute to developing metabolite applications. Patented technologies ensure high resolution and excellent mass measurement accuracy (MMA), supporting structure elucidation and identification of unknown compounds.

Product

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