C146-E323

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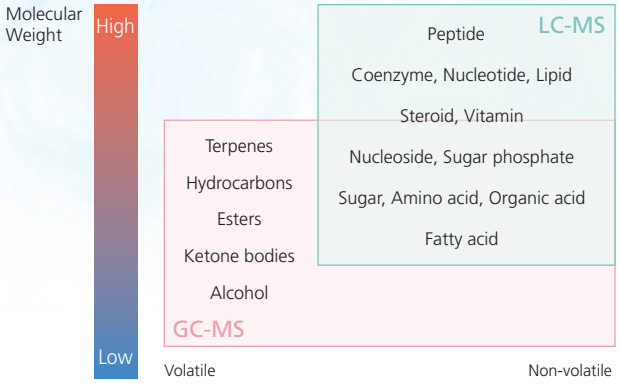
Pretreatment Procedure Handbook for Metabolites Analysis

Metabolomics and Mass Spectrometers

Quantitative Metabolomics

GC-MS(/MS) and LC-MS/MS are widely used for measuring the total amount of metabolites in a sample. Selection of the appropriate instrument to be used is based on the target compounds and the objective.



GC-MS

LC-MS

Instrument Features

GC-MS
Permits comprehensive measurement of several hundred compounds in a single measurement
Standard measurement methods with excellent robustness
Low installation cost
First-choice comprehensive measurement

LC-MS
Simple measurement of specific metabolites (up to 100 compounds)
Quick measurement, including pretreatment
Measurement of high-molecular weight non-volatile metabolites is possible
Ideal for efficient routine measurement of specific compounds

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Using GC-MS Analysis to Extract Metabolites from Blood Serum

Using LC-MS Analysis to Extract Metabolites from Blood Serum

Using GC-MS Analysis to Extract Metabolites from Blood Serum

To pretreat samples for GC-MS analysis, a mixture of water, methanol, and chloroform is first added to deproteinize the sample. Then water is added to separate the solution into two layers, from which the water layer is recovered. This method is used to extract most of the hydrophilic metabolites that are important for the primary metabolic pathways, such as the glycolytic pathway and TCA cycle.

1. List of Required Items

Consumables for Pretreatment

Item	Product Example
1.5 mL tube	Safe-Lock 1.5 mL (colorless)
Pipette	PIPETMAN P-100
Pipette tip	PP rack with 200 µL scale markings, (yellow)
Pipette	PIPETMAN P-1000
Pipette tip	System rack (PP) with 1000 µL scale markings, blue
Pipette	PIPETMAN P-20
Collection plate	Unirack S500-80AS
50 mL tube	Centrifuge tube 50 mL
50 mL tube stand	5410 4 way flipper blue
Graduated cylinder (for preparing extraction solvent)	PYREX Graduated cylinder 1000 mL
Graduated cylinder (for measuring extraction solvent)	PYREX Graduated cylinder 100 mL
Extraction solvent stock solution bottle	PYREX Medium shading bottle 1000 mL

Equipment

Equipment	Remarks
Vortex mixer	Able to handle 1.5 mL tubes
Heating shaker	Temperature-controllable to 37 °C and able to handle 1.5 mL tubes
Centrifuge	Able to handle 1.5 mL tubes and withstand up to 16,000 G torque
Centrifugal evaporator	Able to handle 1.5 mL tubes
Deep freezer	Able to cool to -80 °C or lower
Freeze dryer	Able to handle 1.5 mL tubes
Desiccator	Vacuum function not necessary
Sonicator	Able to handle 1.5 mL tubes
Electronic balance	With 1 mg or smaller scale markings

Reagents

Item	Remarks
Ultrapure water	LC/MS grade
Methanol	LC/MS grade
Chloroform	HPLC grade
Methoxyamine Hydrochloride	Source: Tokyo Chemical Industry, Sigma-Aldrich Japan, etc.
Pyridine	Special grade
N-Methyl-N-trimethylsilyltrifluoroacetamide	Source: GL Sciences, etc.
Hexane	HPLC grade
Acetone	HPLC grade
Internal standard substances	2-Isopropylmalate, etc.

Consumables for Analysis

Item	Recommended Products			
Vial	1	Target Screw vial (2 mL) (C4013-2)	2	Chromacol Screw vial (2 mL) (2-SV(A))
Cap Septum	1	Target Caps with septum (C4013-63W)	2	• Vial cap (221-34273-92) • Vial septum (221-34271-92)
Small volume glass insert	1	Target Small volume glass insert (150 µL) (GLC 4012-S530)	2	• Inserts. 2 mL Tapered Insert – Clear 1000 pcs (02-MTV) • Plastic 3 Prong Support Foot for Tapered Inserts 500 pcs (MTS-1)

Note: For vials, caps/septa, and small glass inserts, obtain number 1 or 2 (set) in the table.

Item	Recommended Products
Rinse solution vial	Vial 4 mL (221-34267-92)
Rinse solution vial cap	Vial cap 4 mL (221-34268-92)
Rinse solution vial septum	Vial septum 4 mL (221-34266-92)
Standard mixture of n-Alkanes	Custom Retention Time Index Standard (Restek 560295)
Column	• BPX5 30 m × 0.25 mm I.D. df = 0.25 µm (SGE 054101) • DB-5 30 m × 0.25 mm I.D. df = 1.00 µm (J&W 122-5033)

Note 1: The column is for analysis that uses the Smart Metabolites Database.
Chose one of the following:
Used for GCMS-TQ series systems: BPX5 is recommended (23 minute acquisition time)
Used for GCMS-QP2020/2010 series systems: DB-5 is recommended (37 minute or 67 minute acquisition time)
(Due to short acquisition time, BPX5 may result in inadequate separation for GC-MS analysis.)

Note 2: Use the consumables included in the consumables kit (225-20052-91), such as injection port septa and glass inserts. Use either split (225-20803-01) or splitless (221-48876-03) glass inserts selectively depending on the method.

Note 3: The example describes using a GCMS-TQ8040 system with the Smart Metabolites Database. For more information about the products, contact your Shimadzu sales representative.

2. Extracting Metabolites for GC-MS Analysis

1 Deproteination	• Serum is collected in a tube • Extraction solvent is added, and mixture is shaken • Centrifugation / Supernatant recovery
2 Extraction of hydrophilic metabolites	• Water addition (removal of highly hydrophobic compounds), mixing • Centrifugation / Supernatant recovery
3 Drying	• Centrifugal evaporation (methanol volatilization) • Lyophilization
4 Derivatization	• Add methoxy amine / pyridine solution (Methoxyamination) and shake it • Add MSTFA and shake it

This method*1 is used to extract mostly hydrophilic metabolites by adding an extraction solvent consisting of a mixture of water, methanol, and chloroform to deproteinize the liquid sample and then recover the water layer.

Proteins are denatured by adding a solution containing organic solvent to the blood serum. The denatured proteins are centrifuged and removed; subsequently, ultrapure water is added to further denature and remove any undissolved proteins. Deproteinization is essential for GC-MS analysis because proteins and other large molecules can interfere with analysis.

A centrifugal evaporator is used to evaporate the methanol and then the remaining sample is dried by freeze drying. A centrifugal evaporator is used because methanol contained in a solvent will prevent the solvent from freezing when cooled, making it impossible to use a freeze dryer.

After freeze drying, the sample is derivatized. Because only vaporized compounds are detected by GC-MS analysis, compounds that do not vaporize easily must be derivatized. Derivatization is performed in two stages by methoximation and trimethylsilylation (TMS).

*1: Serum metabolomics as a novel diagnostic approach for gastrointestinal cancer. (Ikeda A, Nishiumi S, Shinohara M, Yoshie T, Hatano N, Okuno T, Bamba T, Fukusaki E, Takenawa T, Azuma T, Yoshida M. *Biomed Chromatogr.* 2012 May;26(5):548–58. doi: 10.1002/bmc.1671. Epub 2011 Jul 20.)

1 Deproteination

Collect a 50 µL sample of blood serum in a 1.5 mL tube (Fig. 1). If an internal standard is used, add the internal standard to the tube. Be sure to select an internal standard substance that enables stable analysis and that is not otherwise present in the sample. Prepare an aqueous solution at an appropriate concentration (about 0.5 mg/mL in the case of 2-isopropylmalate), and add about 10 µL to the tube.

Add 250 µL of a solvent mixture containing water, methanol, and chloroform at a ratio of 1:2.5:1 (extraction solvent) to the tube. When the protein denatures, the tube contents turn a cloudy white color (Fig. 2). The extraction solvent can be prepared in large quantities in advance and stocked in 1-L reagent bottles, or other containers, at room temperature. The extraction solvent can also be added to the tube after premixing it with the internal standard solution in a 50 mL tube.

After mixing thoroughly with a vortex mixer (Fig. 3), heat the mixture to 37 °C and shake it for 30 minutes at about 1,200 rpm in a heated shaker (Fig. 4).

When finished shaking, centrifuge the mixture for three minutes at 4 °C and 16,000 G (Fig. 5).

The solution is separated into two layers, with the denatured protein precipitated to the boundary surface between the layers (Fig. 6). Obtain 225 µL of the supernatant by carefully inserting a pipette tip into the tube, so that the tip does not contact the precipitate or the chloroform layer, and place it in a new tube (Fig. 7).

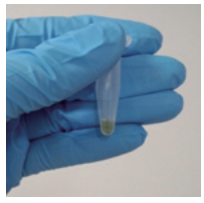


Fig. 1

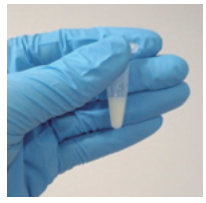


Fig. 2

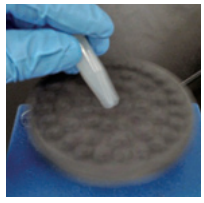


Fig. 3

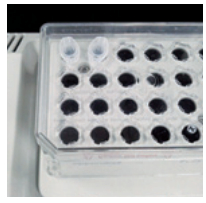


Fig. 4


Fig. 5

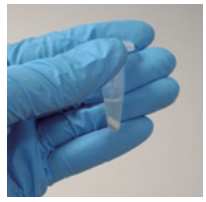


Fig. 6

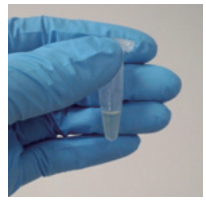


Fig. 7

2 Extraction of hydrophilic metabolites

Add 200 µL of ultrapure water to the new tube containing the recovered supernatant. Proteins still remaining in the solution are denatured, turning the solution a cloudy white color (Fig. 8). After mixing thoroughly in a vortex mixer (Fig. 9), centrifuge the mixture again for three minutes at 4 °C and 16,000 G. After centrifuging (Fig. 10), obtain 250 µL of the supernatant and place it in a new tube (Fig. 11).

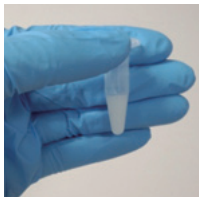


Fig. 8

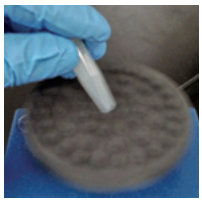


Fig. 9

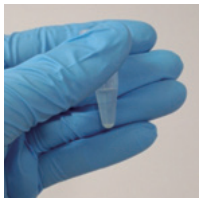


Fig. 10

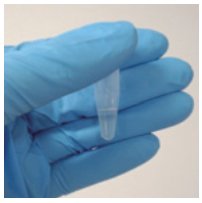


Fig. 11

Extracting Hydrophobic Metabolites

Many of the metabolites within most biological organisms are hydrophilic, such as amino acids, sugars, and nucleic acids. However, there are many important metabolites that exhibit hydrophobicity, such as phospholipids and sterols. Using this protocol, which is based on the Bligh–Dyer method, enables the extraction of hydrophobic metabolites. When the methanol, ultrapure water, and chloroform (2.5:1:1) extraction solvent is first added, adjust the quantity added so that the mixture does not separate into two layers. (For example, if 900 µL of the extraction solvent is added to 50 µL of blood serum, the solution will not separate into two layers.) Then add chloroform instead of ultrapure water. This causes the solution to separate into two layers, so use a Pasteur pipette or other appropriate means to collect sample from the lower chloroform layer.

3 Drying

Prepare a tube cap with punctured holes. After poking two or three small holes in the cap of the 1.5 mL tube using a syringe needle or other means, cut the cap from the tube using scissors (Fig. 12). Attach the cap to the tube containing the collected supernatant (Fig. 13). In this state, use the centrifugal evaporator to evaporate the methanol from the solution for 25 minutes (Fig. 14). If the solution contains a large amount of methanol, the methanol can prevent the solution from freezing when cooled, which makes it impossible to use the freeze dryer.

After evaporating for 25 minutes, place the tube in the deep freezer without changing the cap. Let it sit for about 15 minutes and then confirm that the solution is fully frozen. Finally, dry it in the freeze dryer (Fig. 15).

If the next process steps cannot be started immediately after freeze drying, store the sample in a desiccator at room temperature to prevent any moisture from collecting as it can interfere with the derivatization process.

Fig. 12

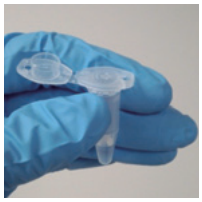


Fig. 13

Fig. 14

Fig. 15

4 Derivatization

Weigh the methoxyamine hydrochloride. Dissolve the weighed methoxyamine hydrochloride in pyridine to make a concentration of 20 mg/mL (Fig. 16). Prepare enough methoxyamine-pyridine solution for the given number of samples involved, assuming 80 µL is used per tube.

The methoxyamine hydrochloride may be difficult to dissolve in some cases. If undissolved residue is visible, use a sonicator or other means to ensure it is completely dissolved.

A solid substance with a white to whitish-yellow color will be clinging to the sample tube walls after freeze drying (Fig. 17). Add 80 µL of the 20 mg/mL methoxyamine-pyridine solution (Fig. 18) and mix it in the sonicator (about 20 minutes) until the residue is dispersed (Fig. 19). Any moisture contained in the sample will decrease derivatization efficiency, so be especially careful to prevent water from entering the sample (such as by wrapping the cap with Parafilm).

Heat and shake the sample in the heated shaker for 90 minutes at 30 °C and about 1,200 rpm (Fig. 20).

Then add 40 µL of MSTFA (Fig. 21) and heat and shake the sample in the heated shaker for 30 additional minutes at 37 °C and about 1,200 rpm.

If any residue remains, centrifuge at 16,000 G for three minutes (Fig. 22), collect the supernatant in a GC-MS vial, and use it for analysis (Fig. 23).

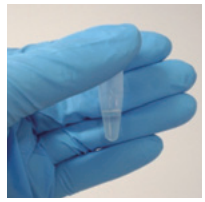


Fig. 16

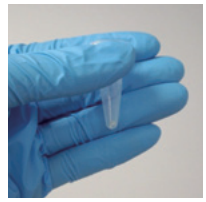


Fig. 17

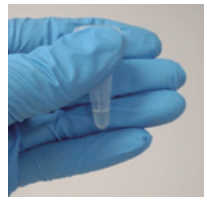


Fig. 18

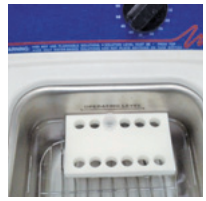


Fig. 19

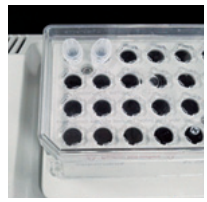


Fig. 20

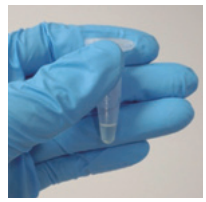


Fig. 21


Fig. 22


Fig. 23

Extracting Non-Serum Metabolites

This document describes how to pretreat blood serum, but metabolomics also involves samples other than blood serum. In addition to samples from animals, such as blood serum and urine, there are a wide variety of other metabolomic samples, such as plants and foods. The described protocol can be used for these samples as well.

However, in some cases, it may be necessary to search for more effective pretreatment methods, depending on the given sample and analytical objectives. Shimadzu offers applications for pretreatment in a variety of situations. For more information, visit the Shimadzu website or contact your Shimadzu sales representative.

Extracting Metabolites from Cell Cultures
Application data sheet No. 102
Analysis of Glycolysis Metabolites in Human Embryonic Stem Cells using GC-MS/MS (LAAN-J-MS-E102A)

Extracting Fatty Acids from the Edible Flesh of Saury
Application data sheet No. 86
Analysis of Fatty Acids in Food Using PCI-GC-MS/MS
(LAAN-J-MS-E086)

Direct Drying Method for Urease Treatment of Rat Urine
Application data sheet No. 60
Analysis of Metabolites in Rat Urine Using Scan/MRM via GC-MS/MS (1)
(LAAN-J-MS-E060)

Extracting Metabolites from Dried Tea Leaves
Technical report No. 1
Profiling of Japanese Green Tea Metabolites by GC-MS
(IMD-N-0360)

3. Analysis Using the Smart Metabolites Database

Smart Metabolites Database

Brochure: C146-E277

Samples were analyzed using a GCMS-TQ8040 system and the Smart Metabolites Database.

The Smart Metabolites Database is preregistered with methods containing GC condition settings and optimal scan and MRM measurement parameter settings for each compound. Retention indices for calculating predicted retention times are also registered in the database. Therefore, analysis can be started if standard samples for metabolites are not available.

Furthermore, by simply selecting the measurement compound from the compounds registered in the database, the database automatically creates measurement methods and data analysis methods for scan, SIM, or MRM modes, or combinations thereof.

This document describes using both scan and MRM methods to analyze metabolites extracted by the method indicated above. Only a portion of measurement parameters and measurement results for each analysis are indicated.

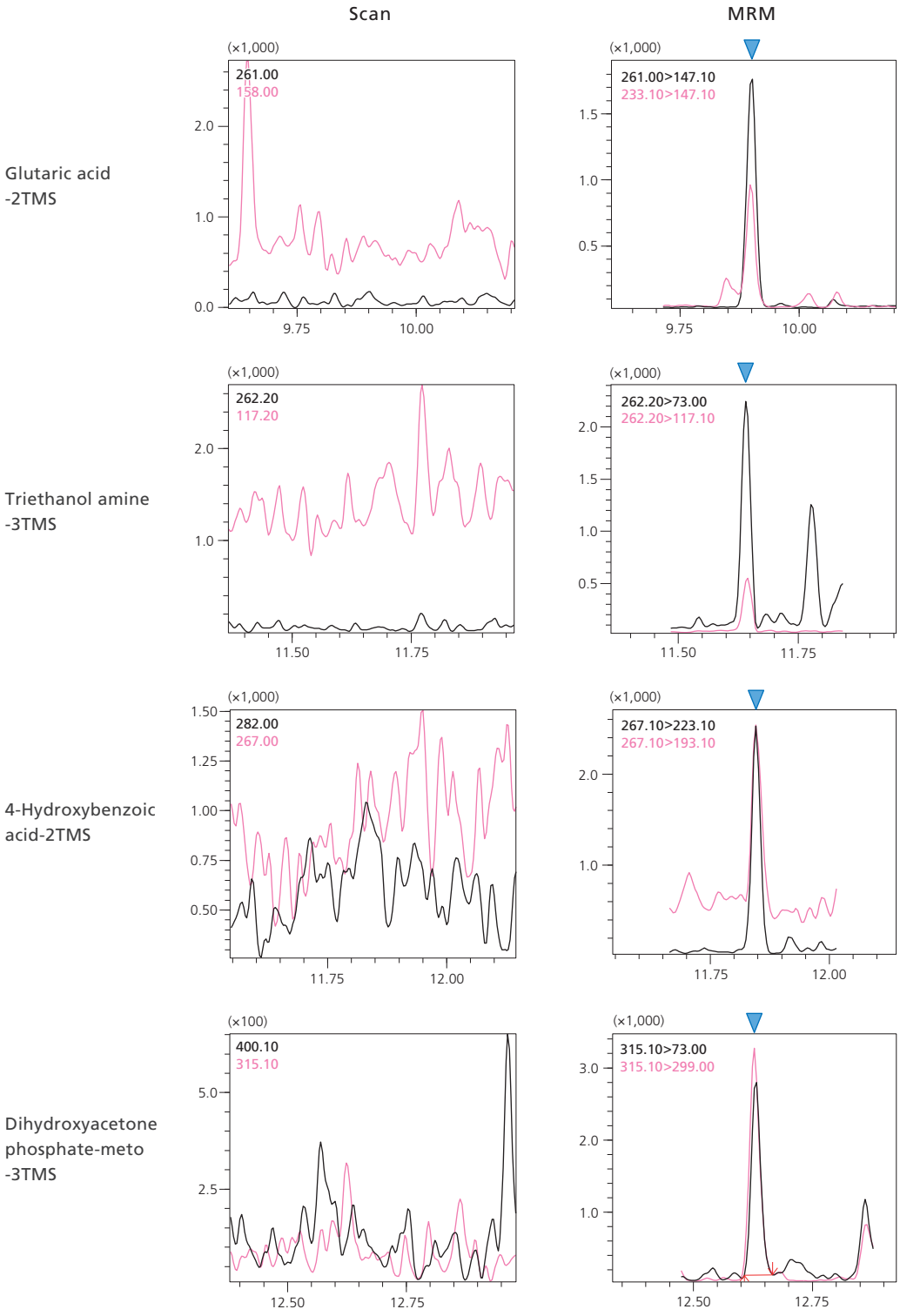
Measurement Parameters

GC Conditions	
Column	BPX5 (30 m × 0.25 mm I.D., df = 0.25 µm)
Insert	Split insert with wool (225-20803-01)
Injection unit temperature	250 °C
Oven temperature	60 °C (2 min) → 15 °C/min → 330 °C (3 min)
Injection mode	Split (30:1)
Carrier gas control	Linear velocity (39.0 cm/sec)
Injection volume	1 µL

MS Conditions	
Interface temperature	280 °C
Ion source temperature	200 °C
Measurement mode	Scan, MRM
Event time	0.3 sec

Measurement Parameters — Chromatogram Comparison of Scan and MRM Modes

MRM mode analysis is useful for metabolomic samples because they contain high levels of contaminants. The following shows a comparison of chromatograms obtained using scan and MRM modes from blood serum samples pretreated with an extraction solvent. The chromatograms show that for many compounds using the MRM mode tended to improve the peak shapes and sensitivity.



Triple Quadrupole Gas Chromatograph Mass Spectrometer

GCMS-TQ8040

Smart Technologies Boost Routine Analyses

Although measurement using GC-MS/MS is effective for measuring even trace amounts of a great variety of the chemical substances that can be found within a diverse range of samples, many parameter settings need to be made and the appropriate method files need to be created.
With the GCMS-TQ8040, the creation of complicated method files is automated, making possible to simultaneously perform high-sensitivity analysis of multiple components. This dramatically enhances the productivity of the system.

Smart Productivity

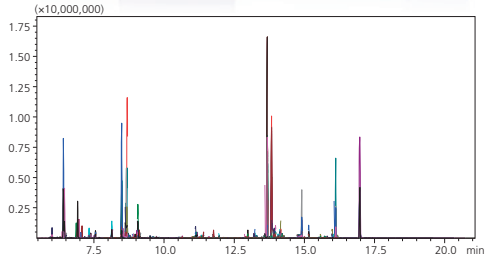
- Equipped with new firmware protocol
- Simultaneous high-sensitivity, high-precision analysis of a greater variety of compounds
- Twin Line MS system reduces the work required for changing columns

Smart Operation

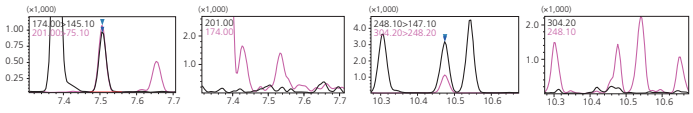
- Smart MRM offers automatic creation of optimal method
- Automatic search of the optimum transition
- AART function automatically adjusts retention times

Smart Performance

- Superior sensitivity realized through implementation of patented high-sensitivity ion source technology
- OFF-AXIS ion optical system greatly reduces noise
- Capable of high-sensitivity analysis even in single GC-MS mode



MRM analysis of metabolites in standard human plasma using smart metabolites database (TIC)



Comparison of mass chromatograms for metabolites in standard human plasma

Brochure: C146-E251

Gas Chromatograph Mass Spectrometer

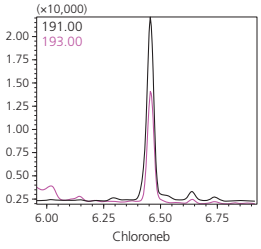
GCMS-QP2020

Delivering Smart Solutions

The GCMS-QP2020 is a top-of-the-line model that features a new turbomolecular pump with higher evacuation efficiency and is capable of achieving maximum sensitivity using a variety of carrier gases and analytical conditions.
In addition to providing mass spectra, it also helps achieve highly accurate qualitative analysis using a combination of three types of information.
The Smart SIM function and software for assisting multianalyte quantitative analysis dramatically improve the productivity of processes ranging from method creation to data acquisition and analysis.

Provides Higher Sensitivity and Reduces Operational Costs

The new large-capacity differential exhaust system offers improved exhaust performance when using hydrogen or nitrogen as the carrier gas, which ensures optimal MS status under a variety of carrier gas conditions.



Mass chromatograms of pesticides utilizing hydrogen as the carrier gas (5 ng/mL, SIM)

Dramatically Improves the Efficiency of Multicomponent Batch Analysis

The GCMS Insight software package supports everything from method creation to analysis. Smart SIM automatically creates SIM programs for measuring multiple components with high sensitivity and a dedicated data analysis program improves data processing efficiency.

Data processing program: LabSolutions Insight

Brochure: C146-E295

Using LC-MS Analysis to Extract Metabolites from Blood Serum

The following describes extracting metabolites for LC-MS analysis using an extraction solvent consisting of water, methanol, and chloroform. Size exclusion filtration is performed to prevent LC lines from clogging and interfering with the analysis.

1. List of Required Items

Consumables for Pretreatment

Item	Product Example
1.5 mL tube	Safe-Lock 1.5 mL (colorless)
Pipette	PIPETMAN P-100
Pipette tip	PP rack with 200 µL scale markings, (yellow)
Pipette	PIPETMAN P-1000
Pipette tip	System rack (PP) with 1000 µL scale markings, blue
Pipette	PIPETMAN P-20
Collection plate	Unirack S500-80AS
50 mL tube	Centrifuge tube 50 mL
50 mL tube stand	5410 4 way flipper blue
Solid phase extraction cartridge	Centrifugal filter 0.5 mL – 3K
Graduated cylinder (for preparing extraction solvent)	PYREX Graduated cylinder 1000 mL
Graduated cylinder (for measuring extraction solvent)	PYREX Graduated cylinder 100 mL
Extraction solvent stock solution bottle/ Mobile phase bottle	PYREX Medium shading bottle 1000 mL

Equipment

Equipment	Remarks
Vortex mixer	Able to handle 1.5 mL tubes
Heating shaker	Temperature-controllable to 37 °C and able to handle 1.5 mL tubes
Centrifuge	Able to handle 1.5 mL tubes and withstand up to 16,000 G torque
Centrifugal evaporator	Able to handle 1.5 mL tubes
Deep freezer	Able to cool to –80 °C or lower
Freeze dryer	Able to handle 1.5 mL tubes
Electronic balance	With 1 mg or smaller scale markings

Reagents

Item	Remarks
Ultrapure water	LC/MS grade
Methanol	LC/MS grade
Chloroform	HPLC grade
Formic acid	LC/MS grade
Acetic acid	LC/MS grade
Tributylamine	LC/MS grade
Internal standard substances	L-Methionine sulfone, 2-Morpholinoethanesulfonic acid

Consumables for Analysis

Item	Product Example
Vial	300 µL sample bottle (228-16850-91)
Cap	Cap black (228-15653-91)
Septum	Septum silicon rubber (221-26718-93)
Vial spacer	Spacer 300 µL (228-16873-91)
Column	Discovery HS F5 HPLC Column 2.1 mm I.D. × 150 mm L., 3 µm (Sigma-Aldrich 567503-U)

Note: In the given analysis example, the LCMS-8040 system with the Primary Metabolite LC/MS/MS Method Package Ver. 2 PFPP method was used for analysis. For more information about the products, contact your Shimadzu sales representative. Note that the Primary Metabolite LC/MS/MS Method Package Ver. 2 can be used with LCMS-8040, LCMS-8045 and LCMS-8050 systems as well.

2. Extracting Metabolites for LC-MS Analysis

1 Deproteination	• Serum is collected in a tube • Extraction solvent is added, and mixture is shaken • Centrifugation / Supernatant recovery
2 Extraction of hydrophilic metabolites	• Add ultrapure water and mix • Centrifugation / Supernatant recovery • Size exclusion filtration
3 Drying	• Centrifugal evaporation (methanol volatilization) • Lyophilization
4 Redissolve	• Bring to final volume in ultrapure water and transfer it to a vial.

The deproteinization operations are the same as for GC-MS analysis. First, denature proteins by adding a mixture containing ultrapure water, methanol, and chloroform (extraction solvent) to the blood serum.*2

Next, add ultrapure water to the supernatant to separate the solution into two layers. Hydrophilic metabolites are extracted by centrifuging and recovering the supernatant.

In LC-MS analysis, samples are exposed to a concentrated organic solvent environment during analysis. If proteins that were not fully deproteinized precipitate during analysis, it could cause line blockage that stops the analysis. To avoid that situation, size exclusion filtration is used after extraction to eliminate proteins.

Finally, use a centrifugal evaporator to evaporate the methanol and freeze dry the sample. The sample could also be directly saved in a deep freezer. Add ultrapure water to dissolve the sample before analysis.

*2: Development of a practical metabolite identification technique for non-targeted metabolomics. Ogura T, Bamba T, Fukusaki E. *J Chromatogr A.* 2013 Aug 2; 1301:73–9. doi: 10.1016/j.chroma.2013.05.054. Epub 2013 May 29

1 Deproteination

In a 1.5 mL tube add 50 µL sample of blood serum and 10 µL of an internal standard (Fig. 24). Select an internal standard substance that enables stable analysis and that is not otherwise present in the sample. Prepare an aqueous solution at an appropriate concentration (about 0.1 mg/mL in the case of methionine sulfone).

Add 900 µL of the extraction solvent mixture containing water, methanol, and chloroform, 1:2.5:1 to the tube. A large quantity of the extraction solvent can be made in advance and stored at room temperature. When the protein denatures, the tube contents turn a cloudy white color (Fig. 25).

After thoroughly vortexing (Fig. 26), heat the mixture to 37 °C and shake it for 30 minutes at 1,200 rpm (Fig. 27).

Centrifuge the mixture for three minutes at 4 °C and 16,000 G (Fig. 28). The denatured protein precipitates to the bottom of the tube (Fig. 29).

Carefully transfer 630 µL of the supernatant to a new tube. Do not allow the pipette tip to come in contact with the precipitate or chloroform layer (Fig. 30).

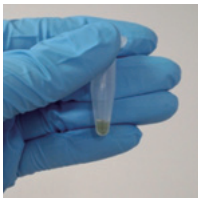


Fig. 24

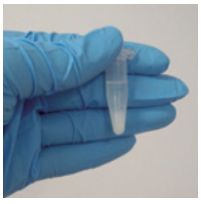


Fig. 25

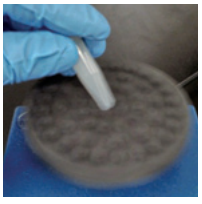


Fig. 26

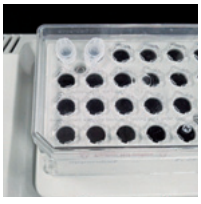


Fig. 27


Fig. 28


Fig. 29

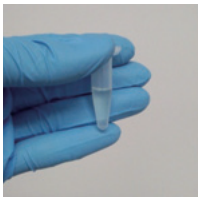


Fig. 30

2 Extraction of hydrophilic metabolites

Add 280 µL of ultrapure water to the new tube so the remaining proteins are denatured, turning the solution a cloudy white color (Fig. 31). After vortexing the mixture thoroughly, centrifuge the mixture for three minutes at 4 °C and 16,000 G. The denatured proteins precipitate to the boundary surface between the aqueous and organic layers (Fig. 32).

To remove the denatured proteins between the layers, attach the size exclusion filter to a clean tube (Fig. 33), add 500 µL of the supernatant to the top of the filter and close the cap. Centrifuge 60 minutes at 4 °C and 16,000 G (Fig. 34). After centrifuging, remove the filter from the tube.

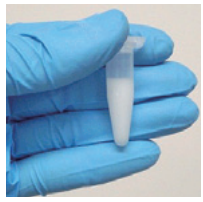


Fig. 31

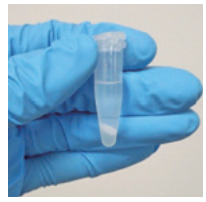


Fig. 32

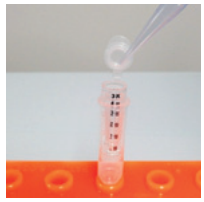
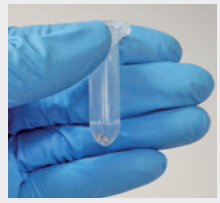


Fig. 33

Fig. 34

Extracting Metabolites from Tissue Fragments

If the sample is tissue fragment, add 5–10 mg of sample and extraction solvent to a 2 mL vial. Pulverize the sample using a ball from a ball mill and perform the same extraction, starting with the heated shaking.



3 Drying

Poke two or three small holes in the cap of the 1.5 mL tube and cut the cap from the tube (Fig. 35). Attach the cap to the tube containing the collected supernatant (Fig. 36). Use the centrifugal evaporator to evaporate the methanol from the solution for 25 minutes (Fig. 37).

Just as for GC-MS analysis, this step is performed to ensure the solution is completely frozen before freeze drying.

Place the tube in the deep freezer without changing the cap. After letting it sit for about 15 minutes and confirming that the solution is fully frozen, use the freeze dryer to freeze dry the sample (Fig. 38).

If the next process steps cannot be started immediately, store the dried sample in a deep freezer.

Fig. 35

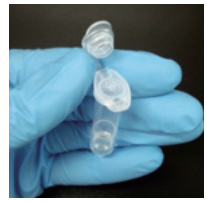


Fig. 36

Fig. 37

Fig. 38

4 Redissolve

After freeze drying, add 100 µL of ultrapure water to the tube (Fig. 39) and vortex the mixture. Dispense the solution into vials (Fig. 40) and analyze by LC-MS.

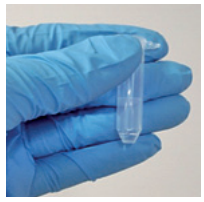


Fig. 39

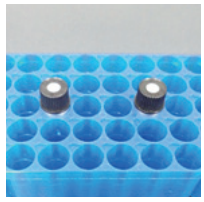


Fig. 40

LC-MS Application Information

LC-MS applications related to metabolomics are featured on the Shimadzu website. Please use the information as a reference.

Extracting Metabolites from Mouse Tissue
Application data sheet No. 49
Simultaneous Analysis of 97 Primary Metabolites By PFPP: Pentafluorophenylpropyl Column
(LAAN-J-LM-E018)

Application data sheet No. 42
Simultaneous Analysis of Hydrophilic Metabolites Using Triple Quadrupole LC/MS/MS
(LAAN-J-LM-E011)

3. Analysis Using the LC/MS/MS Method Package for Primary Metabolites Ver. 2

LC/MS/MS Method Package for Primary Metabolites Ver. 2

Brochure: C146-E227

Samples were analyzed using an LCMS-8040 system and the Primary Metabolite LC/MS/MS Method Package Ver. 2.

The Primary Metabolite LC/MS/MS Method Package Ver. 2 enables the simultaneous analysis of multi-components to be started without determining complicated separation parameters or optimizing MS parameters for each compound. The ease and simplicity of the LC/MS/MS Method Packages enhances laboratory workflow efficiency and productivity.

The method package includes a method for simultaneous analysis of amino acids and nucleotides (55 components) that are based on using ion pair reagents and PFPP methods for analyzing amino acids, organic acids, and bases (97 components). These enable the simultaneous analysis of multiple primary metabolite components based on the components targeted and the instrument environment used.

For a list of compounds that can be measured, refer to the Shimadzu bulletins LC-MS Application Data Sheet No. 42 (LAAN-J-LM-E011) and No. 49 (LAAN-J-LM-E018).

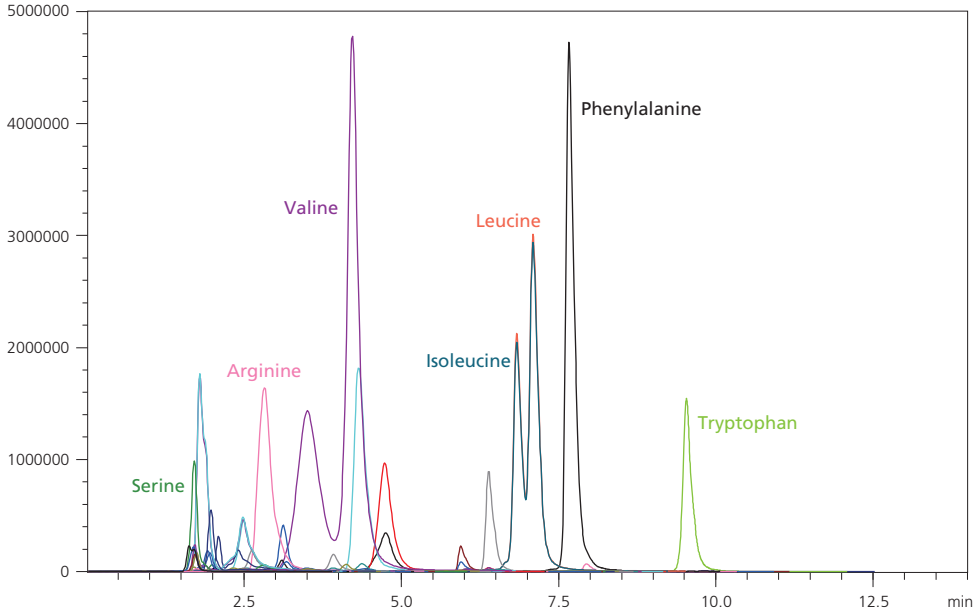
In this case, human blood serum pretreated as indicated above was analyzed using a method with a PFPP column. Measurement parameters and some of the chromatograms obtained are shown.

Measurement Parameters

HPLC Conditions	
Column	Discovery HS F5-3 (2.0 mm I.D. × 150 mm L., 3 µm)
Mobile phase A	0.1% formic acid/water solution
Mobile phase B	0.1% formic acid/acetonitrile
Time program	0%B (0–2.0 min) → 25%B (5.0 min) → 35%B (11.0 min) → 95%B (15.0–20.0 min) → 0%B (20.1–25.0 min)
Flow velocity	0.25 mL/min
Flow rate	5 µL
Oven temperature	40 °C

MS Conditions	
Ionization method	ESI (Positive / Negative)
Nebulizer gas flow rate	2.0 L/min
Drying gas flow rate	15.0 L/min
DL temperature	250 °C
Heat block temperature	400 °C

Measurement Results — Overlay of MRM Chromatograms



Methods with Ion Pair Reagents

The Primary Metabolite LC/MS/MS Method Package Ver. 2 includes methods that use ion pair reagents. These methods can be used to analyze sugar phosphates, nucleotides, and other hydrophilic compounds, making it ideal for analyzing pulverized tissue samples.

HPLC Conditions	
Column	Mastro C18 (2.0 mm I.D. × 150 mm L., 3 µm)
Mobile phase A	15 mmol/L Acetic acid 10 mmol/L TBA (Tributylamine)
Mobile phase B	Methanol
Time program	0%B (0–0.5 min) → 25%B (8.0 min) → 98%B (12.0–15.0 min) → 0%B (15.1–20.0 min)
Flow velocity	0.3 mL/min
Oven temperature	40 °C

MS Conditions	
Ionization method	ESI (Positive / Negative)
Nebulizer gas flow rate	2.0 L/min
Drying gas flow rate	15.0 L/min
DL temperature	250 °C
Heat block temperature	400 °C

High Performance Liquid Chromatograph Mass Spectrometer

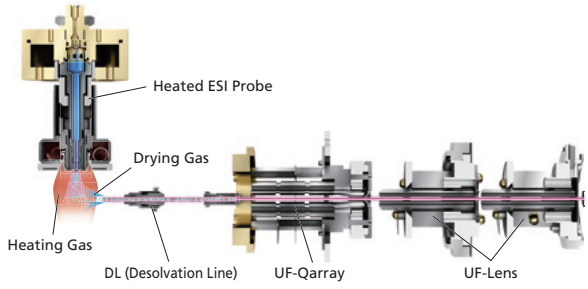
LCMS-8060

The LCMS-8060 is the latest model in the UFMS series of triple quadrupole mass spectrometers, which feature Shimadzu's patented UF Technologies that enable both the highest sensitivity and highest speed levels in the world. The LCMS-8060 can help improve data quality and throughput in a wide range of research fields, transforming current LC/MS/MS analysis.

CHANGES EVERYTHING

Sensitivity Highest Sensitivity

The LCMS-8060 features the newly developed UF-Qarray. By optimizing the ion guide and increasing ion sampling, the LCMS-8060 has achieved unprecedented sensitivity and robustness for a variety of measurement modes.



Speed Fastest Speed

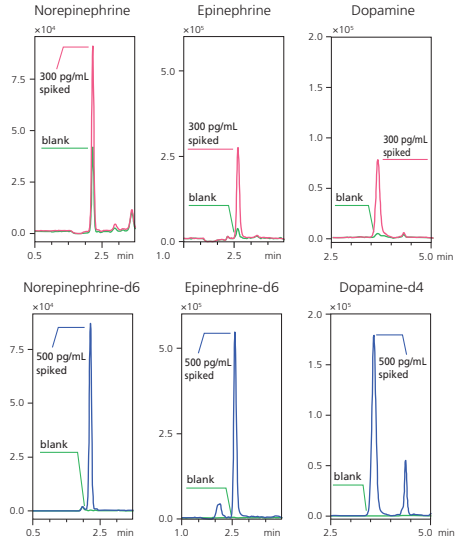
Shimadzu continues to have the fastest analysis speed on the market with minimal sensitivity loss. The LCMS-8060 has a scan speed of 30,000 u/sec, 5 msec ultrafast high-voltage polarity switching, and high-speed 555 ch/sec MRM acquisition.

Solutions Fusion of Sensitivity and Speed

The unstoppable combination of sensitivity and speed allows for a variety of opportunities for new applications, such as the analysis of ultra-trace components in biological samples, which have been difficult to detect. In addition, it improves measurement throughput and selectivity.

High Sensitivity Analysis of Catecholamines in Blood Plasma

Blood plasma matrix contains endogenous catecholamines, which can make it difficult to determine an LLOQ. The high-sensitivity of the LCMS-8060 enabled quantitating epinephrine, norepinephrine and dopamine in blood plasma samples without matrix interference. In quantitative analysis, a known concentration of a deuterated analyte is added to the sample prior to extraction to act as an internal standard. MRM chromatograms of a blood plasma sample are shown below.



Detection of Norepinephrine, Epinephrine and Dopamine and their deuterated internal standards in plasma

Brochure: C146-E286

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