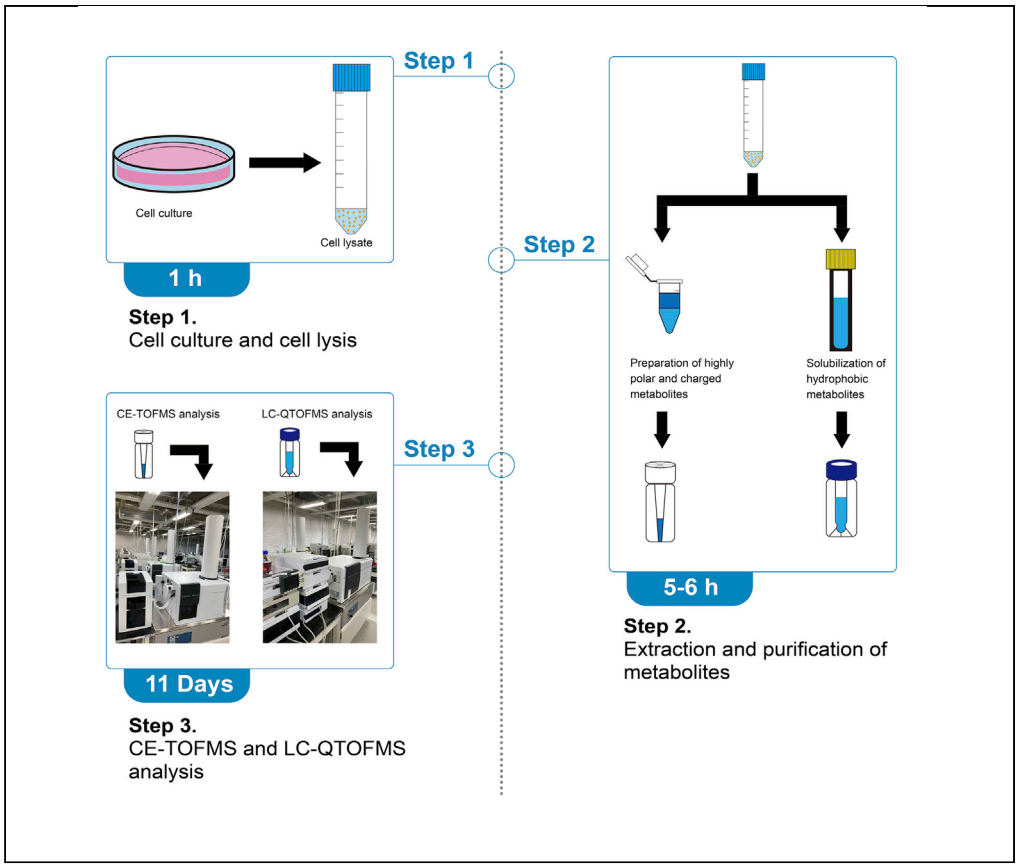
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Comprehensive metabolome analysis of intracellular metabolites in cultured cells



Capillary electrophoresis mass spectrometry (CE-MS) can measure the intracellular amount of highly polar and charged metabolites; liquid chromatography mass spectrometry (LC-MS) can quantify hydrophobic metabolites. A comprehensive metabolome analysis requires independent sample preparation for LC-MS and CE-MS. Here, we present a protocol to prepare for sequentially analyzing the metabolites from one sample. Here we describe the steps for breast cancer cell lines, MCF-7 cells, but the protocol can be applied to other cell types.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Ryuhei Kudo, Kaori Igarashi, Tomoyoshi Soga, Takamasa Ishikawa, Yasuhiro Saito

soga@sfc.keio.ac.jp (T.S.)
ishikawa@infinity-lab.jp (T.I.)
ysaito@ttck.keio.ac.jp (Y.S.)

Highlights

Extraction of cellular metabolites from adherent cells with reference metabolites

Extraction of both charged and hydrophobic metabolites from one sample

Comprehensive metabolome analysis by using CE-MS and LC-MS

Kudo et al., STAR Protocols 3, 101531
September 16, 2022
https://doi.org/10.1016/j.xpro.2022.101531

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Comprehensive metabolome analysis of intracellular metabolites in cultured cells

Ryuhei Kudo,[1,2] Kaori Igarashi,[1] Tomoyoshi Soga,[1,*] Takamasa Ishikawa,[1,2,3,*] and Yasuhiro Saito[1,4,*]

[1]Institute for Advanced Biosciences, Keio University, Tsuruoka, Yamagata 997-0052, Japan

[2]Infinity Lab Co., Ltd., Tsuruoka, Yamagata 997-0052, Japan

[3]Technical contact

[4]Lead contact

*Correspondence: soga@sfc.keio.ac.jp (T.S.), ishikawa@infinity-lab.jp (T.I.), ysaito@ttck.keio.ac.jp (Y.S.)
https://doi.org/10.1016/j.xpro.2022.101531

SUMMARY

Capillary electrophoresis mass spectrometry (CE-MS) can measure the intracellular amount of highly polar and charged metabolites; liquid chromatography mass spectrometry (LC-MS) can quantify hydrophobic metabolites. A comprehensive metabolome analysis requires independent sample preparation for LC-MS and CE-MS. Here, we present a protocol to prepare for sequentially analyzing the metabolites from one sample. Here we describe the steps for breast cancer cell lines, MCF-7 cells, but the protocol can be applied to other cell types.

BEFORE YOU BEGIN

Progress in mass spectrometry analysis enables understanding of comprehensive intracellular metabolism, called the metabolome, in various cell contexts, including cancer cells. Extensive metabolome analysis has demonstrated that deregulated cellular metabolism is a hallmark of cancer (Hanahan, 2022).

Capillary electrophoresis mass spectrometry (CE-MS) analysis has emerged as a powerful tool for comprehensive analysis of charged metabolites (Soga et al., 2003). CE separates charged metabolites based on their charge to size ratio and its separation capability exhibits extremely high because analytes are driven by the flat electroosmotic "plug flow". Overall, CE-MS is an excellent method for measuring various highly polar and charged metabolites, including structural isomers (Soga et al., 2006).

Liquid chromatography mass spectrometry (LC-MS) is also used for metabolome analysis. LC separates metabolites by the affinity between the mobile and stationary phases. LC-MS can analyze various metabolites independent of the charge of analytes. Thus, LC-MS analysis enables to detect diverse metabolites. However, LC-MS sometimes has a problem in the quantitation of metabolites due to ion suppression (Hirayama et al., 2014).

CE-MS has advantages as an analytical tool on metabolomics, such as high resolution for charged metabolites, small amount of sample consumption and very low running cost. Further, since CE-MS is little affected by ion suppression effects, it can provide better quantification accuracy compared with other analytical techniques.

CE-MS analysis can detect the metabolites such as amino acids, glycolysis metabolites, and nucleotides. However, some hydrophobic metabolites, such as phospholipids and fatty acids, are difficult to analyze by CE-MS. Therefore, CE-MS and LC-MS should be independently run to analyze the

intracellular metabolites, respectively in order to understand intracellular metabolism comprehensively. Unfortunately, the sample preparation processes for CE-MS and LC-MS analysis are not identical. Thus, researchers need to independently prepare samples for sequential analysis using both CE-MS and LC-MS systems.

Here, to solve the problem in sample preparation, we improved a protocol for sample preparation to sequentially analyze metabolites with capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) and liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOFMS). Our protocols enable one to explore comprehensive intracellular metabolites from the same sample in CE-TOFMS and LC-QTOFMS measurements.

Cell maintenance

Timing: 3–4 days

1. Cells are cultured in complete medium RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) until 80% confluent in a 10 cm dish.
2. Rinse cells with 7 mL of PBS (-).
3. Aspirate PBS (-).
4. Add 1 mL of 0.05% Trypsin.
5. Incubate cells at 37°C for 3–5 min.
6. Tap the side of the culture dish to detach cells from the plate completely.
7. Collect cells into a 15 mL collection tube using PBS (-) supplemented with 10% FBS.
8. Centrifuge the collection tube at 190 × *g* for 3 min.
9. Discard the supernatant.
10. Suspend cells with 2 mL of complete medium.
11. Take 10 μL of cell suspension into a new 1.5 mL tube and mix with 10 μL of 0.4% Trypan blue solution.
12. Transfer 10 μL of the mixture onto a cell counting chamber slide (Invitrogen).
13. Count cells with the Countess® II FL Automated Cell Counter (Invitrogen).
14. Spread 1 × 10^7 live cells in a 10 cm dish in triplicate.
15. Culture cells for two days at 37°C with 5% CO_2.

Note: Herein, we show an example with MCF-7 cells. If other cell lines are used, the culture condition such as medium, the number of cells for spreading, and the culture time till it reaches 80% confluency should be modified.

KEY RESOURCES TABLE

Caution: All solvents used are LCMS grade or higher.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
RPMI1640	Gibco	Cat#11875119
Fetal Bovine Serum	Biowest	Cat#S1400-500
Penicillin-Streptomycin	FUJIFILM Wako	Cat#168-23191
2.5 g/L Trypsin with 1 mM EDTA	Nacalai Tesque	Cat#32777-15
D-PBS, no calcium, no magnesium	Nacalai Tesque	Cat#14249-24
Trypan blue Solution, 0.4%	Invitrogen	Cat#T10282
D-Mannitol	Sigma-Aldrich	Cat#M4125
Methanol	FUJIFILM Wako	Cat#134-14523

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Acetonitrile	FUJIFILM Wako	Cat#018-19853
2-Propanol	FUJIFILM Wako	Cat#164-25533
Chloroform	FUJIFILM Wako	Cat#033-08631
Formic acid	FUJIFILM Wako	Cat#063-05895
Ammonium acetate	FUJIFILM Wako	Cat#019-02835
Ammonium formate	Kanto Chemical	Cat#01294-00
Methionine sulfone	Alfa Aesar	Cat#A17027
D-Camphor-10-sulfonic acid	FUJIFILM Wako	Cat#037-01032
Reserpine	Sigma-Aldrich	Cat#R0875
3-Aminopyrrolidine	Sigma-Aldrich	Cat#404624
Trimesate	FUJIFILM Wako	Cat#206-03641
Milli-Q water	Merck	n/a
Hexakis (2,2-difluoroethoxy) phosphazene	SynQuest Laboratories	Cat#8H79-3-02
Critical commercial assays		
API-TOF Reference Mass Solution Kit	Agilent	Cat#G1969-85001
Experimental models: Cell lines		
Human breast cancer cell line (MCF-7)	ATCC	Cat#HTB-22 RRID: CVCL_0031
Software and algorithms		
MasterHands 2.18.0.1	Metabolome Consortium, Keio University	n/a
MassHunter Workstation Data Acquisition B.02.01	Agilent	https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software/data-acquisition/acquisition-for-lc-ms
ChemStation B.04.03(16)	Agilent	https://www.agilent.com/en/product/software-informatics/analytical-software-suite/chromatography-data-systems/openlab-chemstation
MassHunter Workstation Data Acquisition B.08.00	Agilent	https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software/data-acquisition/acquisition-for-lc-ms
MassHunter Qualitative Analysis B.06.00	Agilent	https://www.agilent.com/en/product/software-informatics/mass-spectrometry-software/data-analysis/qualitative-analysis
Other		
CE-TOFMS system		
7100 Capillary Electrophoresis system	Agilent	Cat#G7100A
CE-MS adapter kit	Agilent	Cat#G1603A
CE-electrospray ionization (ESI)-MS sprayer kit	Agilent	Cat#G1607A
Dual sprayer ESI source	Agilent	Cat#G3251B
1260 Isocratic pump	Agilent	Cat#G1310B
1260 Degasser	Agilent	Cat#G1379B
6224 TOF mass spectrometer	Agilent	Cat#G6224A
Cooler	THOMAS	Cat#TRL-108H
Fused silica capillary (50 μm i.d.)	Polymicro Technologies	Cat#1068150017
COSMO (+) capillary (50 μm i.d.)	Nacalai Tesque	Cat#07584-44
LC-QTOFMS system		
1290 Binary pump	Agilent	Cat#G4220A
1260 Degasser	Agilent	Cat#G4225A
1290 Thermostatted column compartment	Agilent	Cat#G1316A
1290 Autosampler	Agilent	Cat#G4226A
1200 Autosampler thermostat	Agilent	Cat#G1330B
1100 Isocratic pump	Agilent	Cat#G1310A
6530 QTOF mass spectrometer	Agilent	Cat#G6530A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dust electrospray accessory	Agilent	Cat#G3251A
Acquity UPLC HSS T3 column (2.1 mm i.d. × 50 mm; 1.8 μm)	Waters	Cat#186003538
2 mL Screw vial	Agilent	Cat#5188-6535
250 μL Glass insert, deactivated	Agilent	Cat#5181-8872
Blue screw cap, PTFE/RS septa	Agilent	Cat#5185-5820
CE vial	Agilent	Cat#5190-3155
CE snap cap	Agilent	Cat#5042-6491
2.0 mL Glass jacket tube	FCR and Bio	Cat#JRD-1GS200
2.0 mL Glass jacket tube caps	FCR and Bio	Cat#GC2-1S(HI)
5-kDa cutoff filter	Millipore	Cat#UFC3LCCNB-HMT
Centrifuges	TOMY	Cat#EX-126
High-Speed Refrigerated Micro Centrifuge	TOMY	Cat#MX-305
Hybrid Refrigerated Centrifuge	TOMY	Cat#CAX-370
CentriVap Refrigerated Concentrator	Labconco	Cat#7310022
Glass tips	SHIBATA SCIENTIFIC TECHNOLOGY	n/a
Countess® II FL Automated Cell Counter	Invitrogen	Cat#AMQAF1000
Countess® cell counting chamber slide	Invitrogen	Cat#C10283
1 mL Syringe	TERUMO	Cat#SS-01T

MATERIALS AND EQUIPMENT

Caution: All solvents used are LCMS grade or higher.

Cell culture medium (complete medium)

Reagent	Final concentration	Amount
Heat-inactivated FBS	10%	50 mL
Penicillin/Streptomycin	100 IU/mL, 100 μg/mL	5 mL
RPMI1640	n/a	500 mL
Total	**n/a**	**555 mL**

Store at 4°C and prewarm up to 37°C before use.

5% mannitol solution

Reagent	Final concentration	Amount
D-Mannitol	5%	5 g
Milli-Q water	n/a	Up to 100 mL
Total	**n/a**	**100 mL**

Use a 100 mL-volumetric flask for mixing. Store at 4°C for up to 6 months.

Extraction methanol with reference metabolites

Reagent	Final concentration	Amount
10 mM Methionine sulfone solution	25 μM	25 μL
100 mM D-Camphor-10-sulfonic acid solution	25 μM	2.5 μL
1 mM Reserpine solution	1 μM	10 μL
Methanol	n/a	Up to 10 mL
Total	**n/a**	**10 mL**

Use a 10 mL-volumetric flask for mixing. Store at 4°C for up to 1 month.
One sample requires one mL of extraction methanol. Prepare an appropriate amount of extraction methanol, depending on the sample number.

External reference solution		
Reagent	Final concentration	Amount
100 mM 3-Aminopyrrolidine solution	200 μM	20 μL
10 mM Trimesate solution	200 μM	200 μL
Milli-Q water	n/a	Up to 10 mL
Total	**n/a**	**10 mL**
Use a 10 mL-volumetric flask for mixing. Store at 4°C for up to 1 month.		

5× standard metabolite mixture for cationic metabolite analysis (Group 1–5)		
Reagent	Final concentration	Amount
Metabolite stock solution	100 μM each	*
10 mM Methionine sulfone solution	200 μM	200 μL
100 mM 3-Aminopyrrolidine solution	200 μM	20 μL
Milli-Q water	n/a	Up to 10 mL
Total	**n/a**	**10 mL**
*The total volume of metabolites stock solution will differ depending on the group.		
The combinations of metabolite stock solutions are summarized in Table 1.		
Use a 10 mL-volumetric flask for mixing. Store at −30°C for up to one year after preparation.		

1× standard metabolite mixture for cationic metabolite analysis		
Reagent	Final concentration	Amount
5× metabolite stock solution (Group 1)	20 μM each	Equal amount
5× metabolite stock solution (Group 2)	20 μM each	Equal amount
5× metabolite stock solution (Group 3)	20 μM each	Equal amount
5× metabolite stock solution (Group 4)	20 μM each	Equal amount
5× metabolite stock solution (Group 5)	20 μM each	Equal amount
Total	**n/a**	
Store at 4°C for up to 1 week.		

5× standard metabolite mixture for anionic metabolite analysis (Group A–E)		
Reagent	Final concentration	Amount
Metabolite stock solution	100 μM each	*
100 mM D-Camphor-10-sulfonic acid solution	200 μM	20 μL
10 mM Trimesate solution	200 μM	200 μL
Milli-Q water	n/a	Up to 10 mL
Total	**n/a**	**10 mL**
*The total volume of metabolite stock solution will differ depending on the group.		
The combinations of metabolite stock solutions are summarized in Table 2.		
Use a 10 mL-volumetric flask for mixing. Store at −30°C for up to one year after preparation.		

1× standard metabolite mixture for anionic metabolite analysis		
Reagent	Final concentration	Amount
5× metabolite stock solution (Group A)	20 μM each	Equal amount
5× metabolite stock solution (Group B)	20 μM each	Equal amount
5× metabolite stock solution (Group C)	20 μM each	Equal amount
5× metabolite stock solution (Group D)	20 μM each	Equal amount
5× metabolite stock solution (Group E)	20 μM each	Equal amount
Total	**n/a**	
Store at 4°C for up to 1 week.		

Sheath solution for cation analysis in CE-TOFMS		
Reagent	Final concentration	Amount
Methanol	50%	50 mL
Milli-Q water	50%	50 mL
100 μM Hexakis (2,2-difluoroethoxy) phosphazene solution	0.1 μM	100 μL
Total	**n/a**	**100 mL**
Store at 4°C for up to 3 months.		

Sheath solution for anion analysis in CE-TOFMS		
Reagent	Final concentration	Amount
Methanol	50%	50 mL
Milli-Q water	50%	40 mL
50 mM Ammonium acetate solution	5 mM	10 mL
100 μM Hexakis (2,2-difluoroethoxy) phosphazene solution	0.1 μM	100 μL
Total	**n/a**	**100 mL**
Store at 4°C for up to 3 months.		

Mobile phase A solvent for LC-QTOFMS		
Reagent	Final concentration	Amount
Acetonitrile	60%	600 mL
Methanol	20%	200 mL
Milli-Q water	20%	200 mL
5 M Ammonium formate solution	5 mM	1 mL
Total	**n/a**	**1 L**
Degas the solvents by placing the bottles in an ultrasonic bath for 10 min. Store at 4°C for up to 3 months.		

⚠ CRITICAL: Do not use detergents to wash the solvent bottles.

Mobile phase B solvent for LC-QTOFMS		
Reagent	Final concentration	Amount
2-Propanol	100%	1,000 mL
5 M Ammonium formate solution	5 mM	1 mL
Total	**n/a**	**1 L**
Degas the solvents by placing the bottles in an ultrasonic bath for 10 min. Store at 4°C for up to 3 months.		

⚠ CRITICAL: Do not use detergents to wash the solvent bottles.

50× Reference stock solution for LC-QTOFMS		
Reagent	Final concentration	Amount
API-TOF Reference Mass Solution Kit 5 mM Purine (2.2 mL) 2.5 mM Hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazene (2.2 mL)100 mM Trifluoroacetic acid (2.2 mL)	220 μM Purine 110 μM Hexakis 4.4 mM Trifluoroacetic acid	Full amount (2.2 mL of each component)
95% Acetonitrile	n/a	Up to 50 mL
Total	**n/a**	**50 mL**
Use a 50 mL-volumetric flask for mixing. Store at 4°C for up to 6 months.		

⚠ CRITICAL: Do not use detergents to wash the solvent bottles.

1× Reference solution for LC-QTOFMS		
Reagent	Final concentration	Amount
Acetonitrile	95%	475 mL
Milli-Q water	5%	25 mL
50× Reference stock solution (see above)	440 nM Purin 220 nM Hexakis 8.8 μM Trifluoroacetic acid	1 mL
Total	n/a	500 mL
Store at 4°C for up to 6 months.		

⚠ CRITICAL: Do not use detergents to wash the solvent bottles.

STEP-BY-STEP METHOD DETAILS

An overview of the metabolite extraction is shown in Figure 1. Adherent cultured cells are detached from the culture dish by trypsinization and collected into a 15 mL tube. After centrifugation, cell pellets are lysed with methanol solution (steps 1–17). The crude lysate and cell debris mixture are treated for highly polar and charged metabolite analysis by CE-TOFMS (steps 18–60). The rest of the mix (200 μL in volume) is solubilized in chloroform to analyze hydrophobic metabolites by LC-QTOFMS (steps 61–89).

Metabolite extraction from adherent cultured cells

Timing: 1 h

This section describes how to collect cells. For example, we describe the procedure for breast cancer cell line MCF-7.

1. Culture cells until 80% confluent in a 10 cm dish.
2. Discard culture medium by aspiration.
3. Rinse the cells with pre-warmed PBS (-) once.
4. Add 1 mL of 0.05% trypsin-EDTA solution per 10 cm dish.
5. Incubate the dish at 37°C for approximately 5 min until the cells are detached from the culture dish.
6. Add 5 mL of PBS (-) supplemented with 10% FBS.
7. Transfer the cell suspension into a 15 mL tube (Figure 2A).
8. Suspend cells by pipetting.
9. Count the living cell number using an automated cell counter with trypan blue staining.
10. Calculate the total cell number in 6 mL of cell suspension.
11. Centrifuge at 190 × *g* for 3 min (Figure 2B).
12. Discard the supernatant by aspiration (Figure 2C).
13. Gently pour 5 mL of ice-cold 5% mannitol solution into the tube (Figure 2D).
14. Centrifuge at 190 × *g* for 3 min (Figure 2E).
15. Discard the supernatant by aspiration (Figure 2F).
16. Add 1 mL of ice-cold extraction methanol solution with internal reference metabolites (Figure 2G).
17. Suspend cells by vortexing and store the sample tubes at −80°C until use (Figure 2H).

Note: The concentrations of metabolites are normalized to cell number at step 60. Thus, cell numbers should be counted for all samples at step 9.

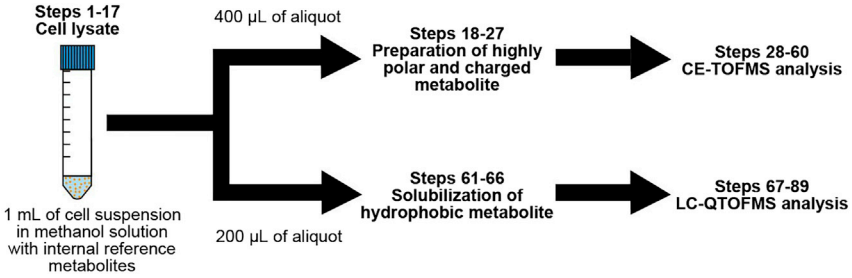


Figure 1. CE-TOFMS and LC-QTOFMS analysis overview

Note: In step 5, cells are examined using a phase-contrast microscope to confirm that all cells are entirely detached from the culture dish.

Note: If cells could not be collected well, refer to troubleshooting Problem 1.

⚠ CRITICAL: PBS should be eliminated from samples at step 12 because PBS affects the signal/noise ratio in mass spectrometry analysis.

Also, at step 13, mannitol solution should be added gently to keep cells in pellet form (Figure 2I).

Preparation of charged metabolites for CE-TOFMS analysis

Timing: 5 h

The cell suspension at step 17 contains proteins that increase CE-TOFMS noise. Therefore, additional preparation steps are needed, as previously described (Hirayama et al., 2009).

18. Transfer 400 µL of cell suspension (from step 17) into a 1.5 mL tube (Figure 3A).
19. Add 160 µL of Milli-Q water and 400 µL of chloroform into the tube and mix by vortexing (Figure 3B).
20. Centrifuge at 9,100 × *g* for 3 min at 4°C (Figure 3C).
21. Confirm that the phase is wholly separated (Figure 3D).
22. Transfer 400 µL of the supernatant from the methanol/water phase into the filter cassette (Millipore 5-kDa cutoff filter) (Figure 3E).
23. Centrifuge at 9,100 × *g* for 2 h at 20°C (Figure 3F).
24. Discard the filter cassette and dry the flow-through by centrifugal evaporation at 40°C for 2–3 h (Figure 3G).
25. Dissolve the dried pellet in 25 µL of the external reference solution and mix by vortexing (Figure 3H).
26. Transfer 7 µL of the sample into a CE vial (Figure 3I).
27. Store the vial at −80°C until measurement (Figure 3J).

Prepared metabolite analysis using CE-TOFMS analysis

Highly polar and charged metabolites are analyzed by using a CE-TOFMS system. CE-TOFMS is represented in Figure 4A. CE separates metabolites depending on the charge and molecular size. Then, TOFMS detects metabolites by monitoring the range of *m/z* values.

The CE-TOFMS measurement procedure is composed of two steps, cationic and anionic metabolite analyses (Soga et al., 2003, 2006, 2009). Cationic metabolites analysis measures positively charged metabolites such as amino acids, whereas anionic metabolites analysis detects negatively charged

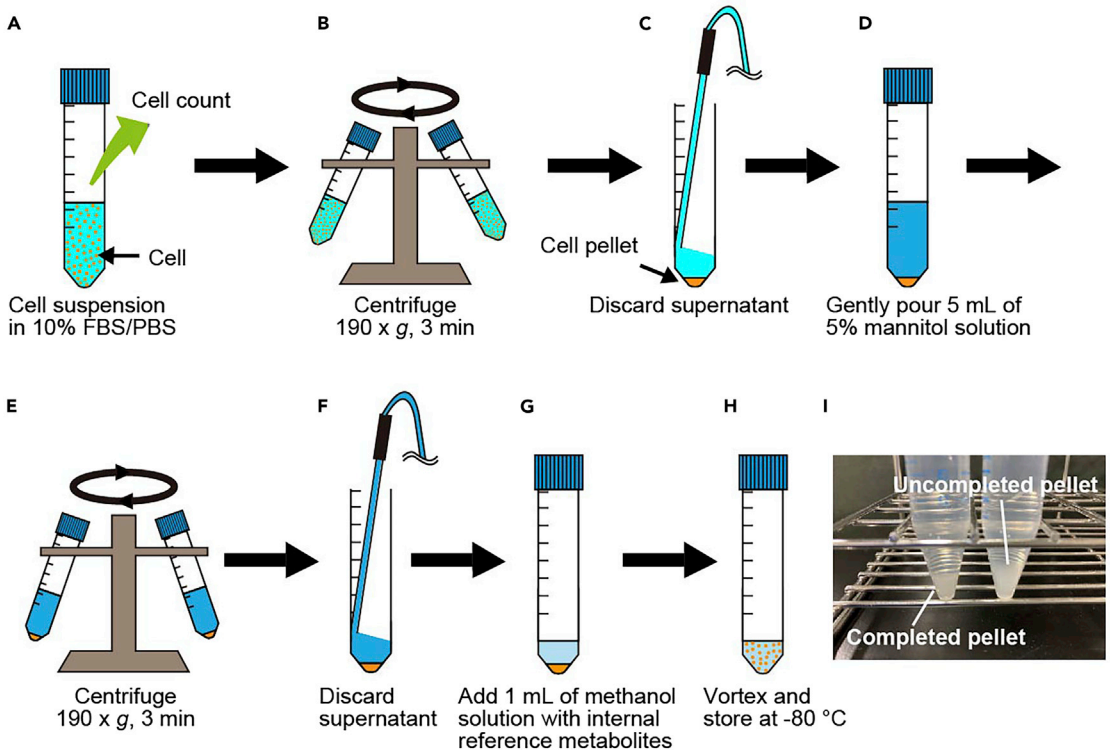


Figure 2. An overview of metabolite extraction

(A–H) Extraction steps.

(I) Representative image of cell pellet at step 13. Cells should be firmly pelleted (left). Image of incomplete pellet after subsequent centrifugation (right).

metabolites that are the metabolic intermediates in glycolysis, the pentose phosphate cycle, the TCA cycle, etc.

Next, the application profiles to run CE-TOFMS are shown. We have attached the settings file as supplementary file (Data S1 and S2 for cationic metabolite analysis and Data S3 and S4 for anionic metabolite analysis). Users can refer to all the parameters in the file. We review the key parameters that need to be confirmed below.

⚠ CRITICAL: To open the setting files Data S1, S2, S3, and S4, you need to install application software on your PC.

Cationic metabolite analysis

Timing: 2 days

28. Run ChemStation and MassHunter Workstation Data Acquisition B.02.01 on a PC.

Note: ChemStation is run to control the isocratic pump and CE, whereas MassHunter controls TOFMS.

29. Preparation of fused silica capillary (Polymicro Technologies).
    a. Cut the fused silica capillary to approximately 100 cm in length.
    b. Peel polyimide-coating at one end of the capillary. The coating should be removed 3–4 mm from the end of the capillary.

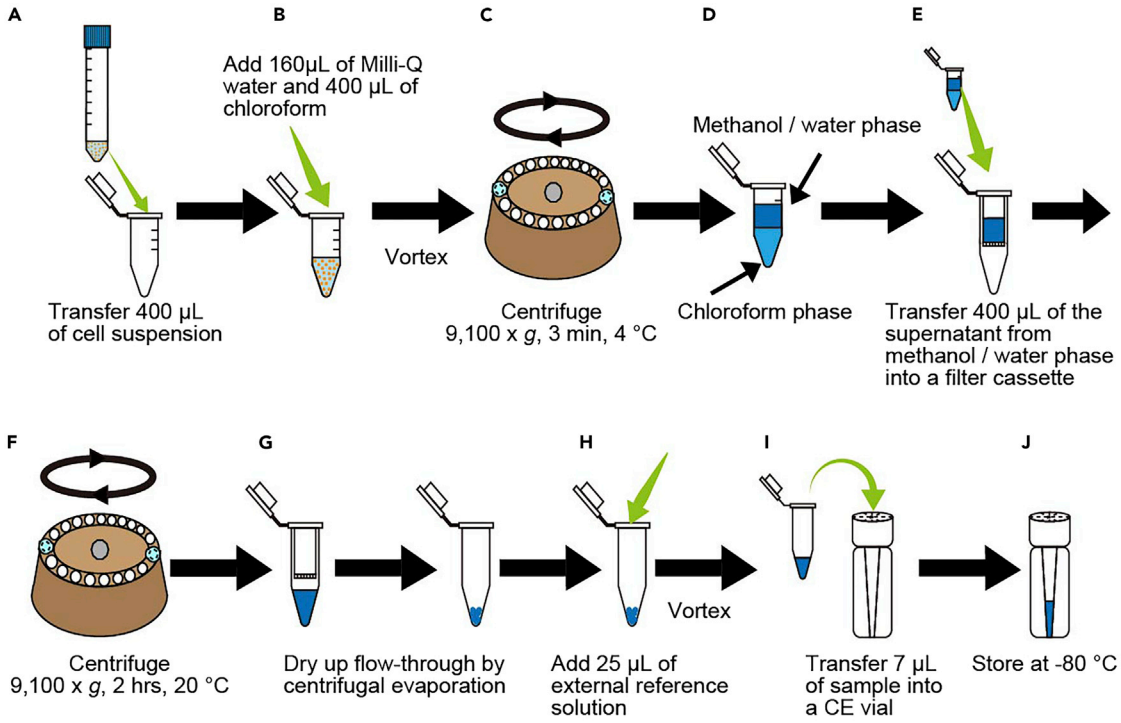


Figure 3. An overview of metabolite preparation

(A-J) Scheme of metabolite preparation.

c. Wipe off the peeled capillary with 50% 2-propanol/Milli-Q water.
d. Connect the capillary to the sprayer of TOFMS at the coating-free side, and the other side of the capillary to the CE cassette.
e. Run the Flush program with 1 M formic acid for 20 min.

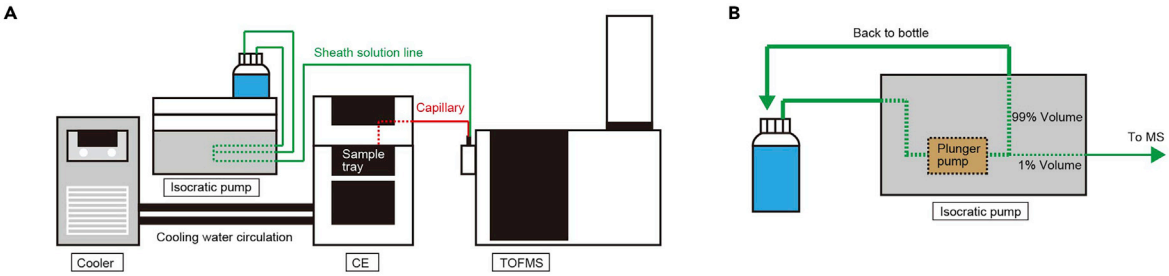
30. Prepare 80 mL of sheath solution (50% methanol containing 0.1 µM hexakis (2,2-difluoroethoxy) phosphazene) in a 100 mL bottle.
31. Connect the line of sheath solution to the isocratic pump.
32. Run the Purge program with the sheath solution at 5 mL/min flow rate for 3 min.

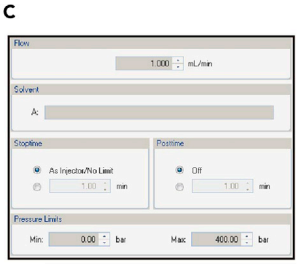
Note: The line connection between a sheath bottle and an isocratic pump is summarized in Figure 4B.

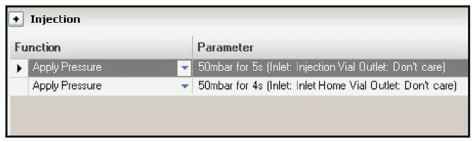
33. Change the flow rate of the sheath solution to 1 mL/min on ChemStation (Figure 4C).

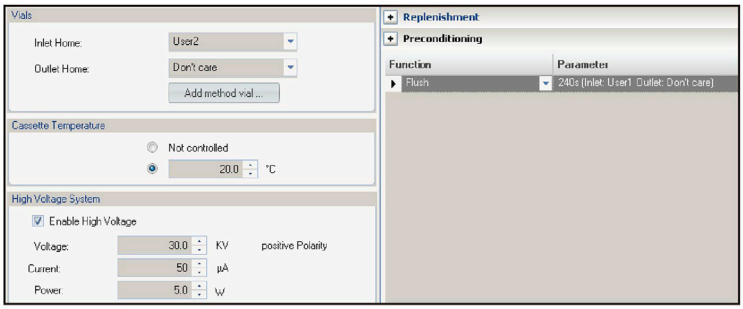
Note: One flow line goes into the MS equipment at a flow rate of 10 µL/min, and the other line goes to an original sheath bottle (Figure 4B).

34. Set up the CE method for cationic metabolites analysis in the ChemStation program.
    a. Inject the sample solution (from procedure step 26) at 50 mbar for 5 s (Figure 4D).
    b. Fill the capillary with 1 M formic acid as an electrolyte at 50 mbar for 4 s (Figure 4D).
    c. Keep the capillary temperature (Cassette Temperature) at 20°C.
    d. Set the parameters of the High Voltage System at 30 kV (Voltage), 50 µA (Current), and 5 W (Power) (Figure 4E).
    e. After running one sample, push out the capillary inside with 1 M formic acid washing buffer for 4 min to prevent contamination between samples (Figure 4E).
    f. Set the water bath temperature for cooling the sample tray at 4°C (Figure 4F).
    g. Run the following sample.

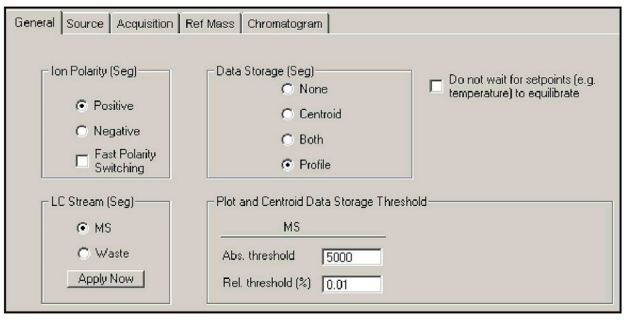

A
Sheath solution line
Capillary
Sample tray
Isocratic pump
Cooling water circulation
Cooler
CE
TOFMS
B
Back to bottle
99% Volume
To MS
Plunger pump
1% Volume
Isocratic pump

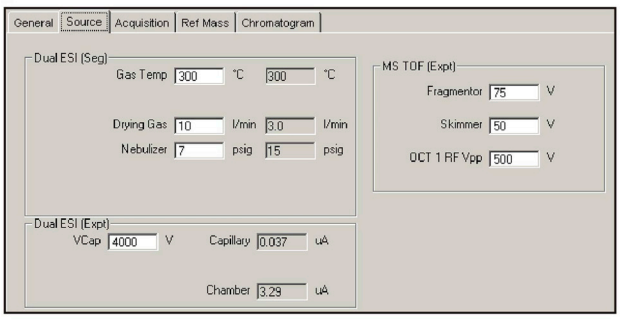

C
Flow
1.000 mL/min
Solvent
A:
Stoptime
Posttime
As Injector/No Limit
Off
Pressure Limits
Min: 0.00 bar
Max: 400.00 bar

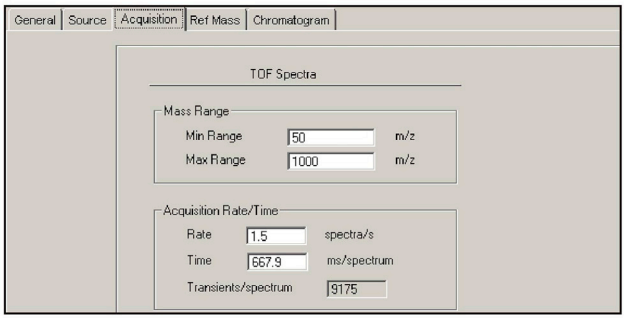

D
Injection
Function
Parameter
Apply Pressure
50mbar for 5s (Inlet: Injection Vial Outlet: Don't care)
Apply Pressure
50mbar for 4s (Inlet: Inlet Home Vial Outlet: Don't care)


E
Vials
Inlet Home:
User2
Outlet Home:
Don't care
Add method vial...
Cassette Temperature
Not controlled
20.0 °C
High Voltage System
Enable High Voltage
Voltage: 30.0 KV positive Polarity
Current: 50 µA
Power: 5.0 W
Replenishment
Preconditioning
Function
Parameter
Flush
240s (Inlet: User1 Outlet: Don't care)


F
SET POINT 4.0t
TEXT
UP
DOWN
SET


G
General
Source
Acquisition
Ref Mass
Chromatogram
Ion Polarity (Seg)
Positive
Negative
Fast Polarity Switching
Data Storage (Seg)
None
Centroid
Both
Profile
Do not wait for setpoints (e.g. temperature) to equilibrate
LC Stream (Seg)
MS
Waste
Apply Now
Plot and Centroid Data Storage Threshold
MS
Abs. threshold 5000
Rel. threshold (%) 0.01


H
General
Source
Acquisition
Ref Mass
Chromatogram
Dual ESI (Seg)
Gas Temp 300 °C 300 °C
Drying Gas 10 l/min 3.0 l/min
Nebulizer 7 psig 15 psig
MS TOF (Expt)
Fragmentor 75 V
Skimmer 50 V
OCT 1 RF Vpp 500 V
Dual ESI (Expt)
VCap 4000 V
Capillary 0.037 uA
Chamber 3.29 uA


I
General
Source
Acquisition
Ref Mass
Chromatogram
TOF Spectra
Mass Range
Min Range 50 m/z
Max Range 1000 m/z
Acquisition Rate/Time
Rate 1.5 spectra/s
Time 667.9 ms/spectrum
Transients/spectrum 9175

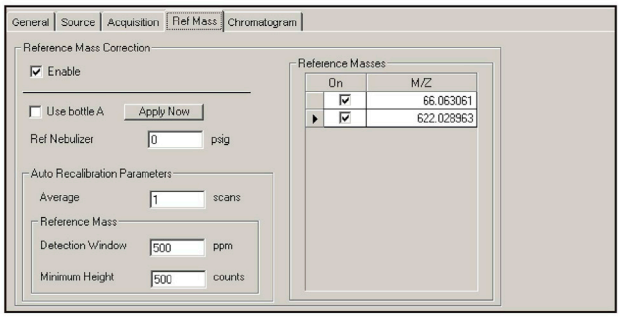

J
General
Source
Acquisition
Ref Mass
Chromatogram
Reference Mass Correction
Enable
Use bottle A
Apply Now
Ref Nebulizer 0 psig
Auto Recalibration Parameters
Average 1 scans
Reference Mass
Detection Window 500 ppm
Minimum Height 500 counts
Reference Masses
On
M/Z
66.063061
622.028963

Figure 4. Parameters for cationic metabolite analysis in CE-TOFMS analysis
(A) Cartoon representation of CE-TOFMS systems used in this protocol.
(B) Cartoon representation of line connections for sheath solution.
(C–E) Screenshots of ChemStation program.
(F) Water bath temperature.
(G–J) Screenshots of MassHunter program.

35. Set up the TOFMS method for cationic metabolites analysis in the MassHunter program.
    a. Check "Positive" at Ion Polarity (Seg) (Figure 4G).
    b. Set the Dual ESI (Seg) parameters at Gas Temp: 300°C, Drying Gas: 10 L/min, and Nebulizer: 7 psig (Figure 4H).
    c. Set the capillary voltage at 4,000 V (Dual ESI (Expt), VCap) (Figure 4H).
    d. Set the voltage parameters for the TOFMS setting (MS TOF (Expt)), the fragmentor, Skimmer, and OCT1 RFVpp voltages at 75 V, 50 V, and 500 V, respectively (Figure 4H).
    e. Set the Acquisition range (Mass Range) at Min Range *m/z* 50 and Max Range *m/z* 1000 (Figure 4I).
    f. Set the Acquisition Rate/Time at 1.5 spectra/s (Figure 4I).
    g. Perform Automatic recalibration of each acquired spectrum using reference masses of reference standards included in the sheath solution.
    h. Set Auto Recalibration Parameters as in Figure 4J.

Note: The ^{13}C isotopic ion of a protonated methanol dimer ($[2MeOH+H]^+$, *m/z* 66.063061) and Hexakis (2,2-difluoroethoxy) phosphazene ($[M+H]^+$, *m/z* 622.028963) are referenced as a lock mass for exact mass measurement (Figure 4J).

36. Create a worklist file in the MassHunter program for running CE-TOFMS.
37. Run standard mixture first to check the total ion chromatogram (TIC).

⚠ CRITICAL: If the TIC spectrum is not stable, refer to troubleshooting Problem 2.

38. Run the worklist program.
39. Check the graph of the current.

Note: The current should be kept around 35 μA for cationic metabolite analysis. If the current value shows inconsistency, refer to troubleshooting Problem 3 or Problem 4.

Note: Program to replace both the 1 M formic acid washing buffer and the 1 M formic acid electrolyte every ten samples. This program analyzes samples and a standard mixture from 272 compounds listed in Table 1. The reference compounds are used for the quantification of target metabolites.

Anionic metabolite analysis

Timing: 2 days

40. Run ChemStation and MassHunter Workstation Data Acquisition B.02.01 on a PC.

Note: ChemStation is run to control the isocratic pump and CE, whereas MassHunter controls TOFMS.

41. Prepare COSMO (+) capillary, which is chemically coated with a cationic polymer.
    a. Replace the storage solution with 50 mM ammonium acetate solution (pH 8.5) using a 1 mL syringe.

Table 1. List of metabolites targeted in CE-TOFMS in anionic mode (groups indicate the combination of metabolites)

Group 1		Group 2		Group 3		Group 4		Group 5	
Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time
Methionine sulfone	1.000	Methionine sulfone	1.000	Methionine sulfone	1.000	Methionine sulfone	1.000	Methionine sulfone	1.000
3-Aminopyrrolidine	0.377	3-Aminopyrrolidine	0.377	3-Aminopyrrolidine	0.377	3-Aminopyrrolidine	0.377	3-Aminopyrrolidine	0.377
S-Adenosylhomocysteine	0.738	Bis(3-aminopropyl)amine	0.371	Hexylamine	0.658	Muramate	0.921	Sepiapterin	1.492
S-Adenosylmethionine	0.603	Creatine	0.749	Oxidized glutathione	1.018	Kynurenine	0.830	Pseudopelletierine	0.670
3-Aminopropane-1,2-diol	0.623	Cyclohexylamine	0.653	gamma-Glutamylcysteine	1.069	S-Lactoylglutathione	1.148	Purine riboside	1.054
N-Acetylputrescine	0.715	Cysteine-glutathione disulphide	0.975	gamma-Glutamyl-2-aminobutyric acid	1.047	4-Methyl-5-thiazoleethanol	0.680	Pyridoxamine 5′-phosphate	0.881
Alliin	0.610	Diethanolamine	0.647	Homoserine	0.848	1-Methylhistamine	0.419	Serotonin	0.741
N1-Acetylspermine	0.475	2-Deoxystreptamine	0.513	Inosine	1.605	5-Methylcytosine	0.660	Thymidine	1.804
4-Amino-3-hydroxybutyrate	0.686	5′-Deoxyadenosine	0.816	Imidazole-4-acetate	0.678	3-Methyladenine	0.658	Thymine	1.805
gamma-Aminobutyric acid	0.651	Putrescine(1,4-Butanediamine)	0.402	Isoamylamine	0.633	Metformin	0.481	Uracil	1.801
Alanine	0.762	Benzamidine	0.649	Isobutylamine	0.602	Mannosamine	0.772	Pipecolate	0.866
N-Acetylglucosamine	1.803	N-alpha-Benzenolarginine ethylester	0.887	Glutathione	1.104	Nicotine	0.455	Purine	0.685
beta-Alanine	0.621	Betaine aldehyde	0.632	Glycine	0.702	Noradrenaline	0.764	Pyrazole	0.591
O-Acetylserine	1.054	Betaine	0.946	gamma-Guanidinobutyrate	0.700	Octopine	0.901	Picolinamide	0.868
N-Acetylornithine	0.807	Choline	0.582	Glycylglycine	0.708	Leucine	0.869	Pyridoxamine	0.505
Argininosuccinate	0.794	Cysteamine	0.544	Glucosamine	0.782	Lysine	0.584	Serine	0.840
1-Aminocyclopropane-1-carboxylate	0.749	Cadaverine	0.424	Glycerophosphorylcholine	1.771	1-Methyl-2-pyrrolidinone	1.792	Spermidine	0.386
1-Amino-1-cyclopentanecarboxylate	0.809	Creatinine	0.619	Glycylleucine	0.820	Alpha-Methylserine	0.872	Spermine	0.381
5-Aminolevulinate	0.676	Carnitine	0.720	Hydroxyproline	1.007	Methylguanidine	0.525	Sarcosine	0.798
6-Aminohexanoate	0.708	Castanospermine	0.774	Homocarnosine	0.586	N-Methylalanine	0.865	Symmetric dimethylarginine	0.661
Acetylcholine	0.644	3-Chloroalanine	1.081	Hypotaurine	1.501	1-Methylnicotinamide	0.623	Proline betaine	0.967
Amantadine	0.729	Canavanine	0.627	4-Hydroxymethylimidazole	0.592	N-Methylglutamate	1.056	Taurine	1.803
N-Acetylhistidine	0.828	beta-Cyanoalanine	1.177	Histamine	0.407	3-Methylhistidine	0.635	Threonine	0.884
L-alpha-Aminobutyric acid	0.813	1,3-Diaminopropane	0.376	5-Hydroxylysine	0.606	N6-Methyl-2′-deoxyadenosine	0.844	Tropine	0.681

(*Continued on next page*)

Table 1. *Continued*

Group 1		Group 2		Group 3		Group 4		Group 5	
Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time
4-(beta-Acetylaminoethyl) imidazole	0.714	N,N-Dimethylglycine	0.918	Histidinol	0.454	Methionine sulfoxide	0.986	Trimethylsulfonium	0.496
Arginine ethyl ester	0.526	2,3-Diaminopropionate	0.605	Hydroxyurea	1.797	S-Methylmethionine	0.618	Trigonelline	0.879
Agmatine	0.440	2,4-Diaminobutyrate	0.584	Isopropanolamine	0.595	3-Methoxytyramine	0.747	Tropinone	0.642
N8-Acetylspermidine	0.546	Dopamine	0.736	Guanine	0.702	Nicotinamide	0.626	Taurocyamine	1.801
N-Acetylglucosylamine	0.838	2′-Deoxycytidine	0.796	Guanosine	1.044	Nicotinamide ribotide	1.697	Urea	1.731
beta-Alanyl-L-lysine	0.572	N-alpha,N-alpha-Dimethylhistidine	0.702	Guanidinosuccinate	0.851	N6,N6,N6-Trimethyllysine	0.609	Uridine	1.807
5-Aminovalerate	0.680	N1,N8-Diacetylspermidine	0.865	Guanidoacetate	0.696	Ophthalmate	1.102	Pterin	0.857
Anserine	0.581	N1,N12-Diacetylspermine	0.641	Thyrotropin releasing hormone	0.907	Pyridoxal	0.742	Phenylalanylphenylalanine	0.920
O-Acetylcarnitine	0.765	N-gamma-Ethylglutamine	0.963	Glucosaminate	0.999	Leucyl-leucyl-tyrosine	1.001	O-Succinylhomoserine	1.858
Alanylalanine	0.783	Ectoine	0.796	Hypoxanthine	0.928	3-Methylguanine	0.710	Riboflavin	1.797
N-epsilon-Acetyllysine	0.960	Betonicine	1.095	6-Hydroxynicotinate	1.844	Lysinamide	0.483	Synephrine	0.758
Asymmetric dimethylarginine	0.650	Cytidine	0.815	Homocysteine	0.881	7-Methylguanine	0.695	Saccharopine	0.902
3-Aminoisobutyrate	0.663	Cystathionine	0.834	3-Hydroxyanthranilate	0.910	beta-Leucine	0.738	Tyrosine	0.953
N1-Acetylspermidine	0.539	Benzamide	1.793	3-Hydroxykynurenine	0.817	6-Methylaminopurine	0.689	Tyramine	0.700
N-Acetylvaline	1.847	Cysteine	0.947	Ibotenate	1.505	1-Methyladenosine	0.840	3,3′,5-Triiodothyronine	1.167
Thiamine	0.561	2′-Deoxyguanosine	0.962	beta-Imidazolelactate	0.747	5-Methyl-2′-deoxycytidine	0.823	Xanthine	1.600
Thiamine monophosphate	0.905	Cysteinylglycine	0.777	3-Iodotyrosine	1.008	5-Methyltetrahydrofolate	0.993	Urocanate	0.700
alpha-Aminoadipate	0.921	3,4-Dihydroxy-L-phenylalanine	0.978	Glutamic acid	0.918	Muscimol	0.657	Xanthosine	1.719
Adenine	0.644	Desthiobiotin	1.809	Glutamylglutamic acid	0.917	Ornithine	0.578	trans-Zeatin	0.813
4-Aminosalicylate	1.078	3,5-Diiodo-tyrosine	1.051	Glutamine	0.903	Melamine	0.644	Xanthopterin	1.097
Allantoin	1.799	5-Methylthioadenosine	0.850	Histidine	0.618	Methionine	0.901	Pyridoxine	0.730
5-Aminoimidazole-4-carboxamide ribotide	1.823	7,8-Dihydroneopterin	0.934	Homocystine	0.809	Methionine sulfoximine	0.712	Phosphorylcholine	1.691
2-Aminobenzimidazole	0.636	5,6-Dimethylbenzimidazole	0.678	Isoleucine	0.861	Phenylalanine	0.931	Tryptophan	0.926
Adenosine	0.831	Epinephrine	0.786	5-Hydroxy-3-indoleacetate	1.820	Proline	0.908	Valine	0.845
Anthranilate	0.891	7,8-Dihydrobiopterin	0.922	5-Hydroxytryptophan	0.950	5-Methoxytryptamine	0.743	Phenethylamine	0.656

(*Continued on next page*)

Table 1. *Continued*

Group 1		Group 2		Group 3		Group 4		Group 5	
Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time
Arginine	0.603	Carnosine	0.574	Indole-3-acetaldehyde	1.806	Melatonin	1.797	Trientine	0.357
Aspartic acid	0.967	Citrulline	0.928	n-Butyl Picolinate	0.878	N-Methylaniline	0.628	Tryptamine	0.704
Asparagine	0.880	2′-Deoxyinosine	1.498	Gramine	0.712	alpha-Methylbenzylamine	0.677	Tetrahydropalmatine	0.952
p-Aminobenzoate	0.814	Cytosine	0.615	2-Guanidinobenzimidazole	0.692	5-Methoxyindoleacetate	1.820	Trimethylamine N-oxide	0.559
3-Aminopropionitrile	0.541	gamma-Butyrobetaine	0.685	Phenylethanolamine	0.691	N-omega-Methyltryptamine	0.727		
Aniline	1.176	Cystine	0.923	Harman	0.729	Nornicotine	0.441		
2-Aminophenol	0.663	Dihydrouracil	1.801	Indole-3-ethanol	1.803	Octylamine	0.710		
		Benzimidazole	0.608	Indole-3-acetamide	1.799				
		2,4-Dimethylaniline	0.689	Indole-3-acetate	1.820				
				Isonicotinamide	0.621				

⚠ CRITICAL: COSMO (+) capillary has a storage solution inside. The storage solution must be replaced with 50 mM ammonium acetate solution.

b. Cut the capillary approximately 105 cm in length.
c. Peel polyimide coating at one end of the capillary.

Note: The coating should be removed 3–4 mm from the end of the capillary.

d. Wipe off the tip of the capillary with 50% 2-propanol/Milli-Q.
e. Connect the capillary to the sprayer of TOFMS at the coating-free side, and the other side of the capillary should be connected to the CE cassette.
f. Run Flush program with 50 mM ammonium acetate solution (pH 8.5) for 10 min.
g. Run Flush program with 50 mM ammonium acetate solution (pH 3.4) for 10 min.
h. Run Flush program with 50 mM ammonium acetate solution (pH 8.5) for 10 min.

42. Prepare 80 mL of sheath solution (5 mM ammonium acetate in 50% methanol containing 0.1 μM Hexakis (2,2-difluoroethoxy) phosphazene) in a 100 mL bottle.
43. Connect the line from sheath solution bottle to the isocratic pump.
44. Run the Purge program with the sheath solution at 5 mL/min flow rate for 3 min.

Note: The line connection between a sheath solution bottle and an isocratic pump is summarized in Figure 4B.

45. Change the flow rate to 1 mL/min for measurement in ChemStation program (Figure 5A).
46. Set up CE method for anionic metabolites analysis in ChemStation program.
    a. Keep the capillary temperature (Cassette Temperature) at 20°C (Figure 5B).
    b. Inject the sample solution at 50 mbar for 30 s (Figure 5B).
    c. Fill the capillary with 50 mM ammonium acetate solution (pH 8.5) at 50 mbar for 4 s (Figure 5B).
    d. Set the parameters of the High Voltage System at -30 kV (Voltage), 50 μA (Current), and 5 W (Power) (Figure 5B).
    e. After running one sample, wash the capillary with 50 mM ammonium acetate solution (pH 3.4) for 2 min (Figure 5C).
    f. Wash the capillary with 50 mM ammonium acetate solution (pH 8.5) for 5 min (Figure 5C).
    g. Set the temperature of the water bath for cooling the sample tray at 4°C (Figure 5D).
    h. Run a subsequent sample.
47. Set up the TOFMS method for anionic metabolite analysis in the MassHunt program.
    a. Check "Negative" at the section of Ion Polarity (Seg) (Figure 5E).
    b. Set the Dual ESI (Seg) parameters at Gas Temp: 300°C, Drying Gas: 10 L/min, and Nebulizer: 7 psig (Figure 5F).
    c. Set the capillary voltage (Dual ESI (Expt)) at 3,500 V (Figure 5F).
    d. Set the fragmentor, skimmer, and OCT1 RFVpp voltages at 100 V, 50 V, and 500 V, respectively in the TOFMS setting (MS TOF (Expt)) (Figure 5F).
    e. Set the Acquisition range (Mass Range) from Min Range *m/z* 50 to Max Range *m/z* 1,000 with an Acquisition Rate/Time of 1.51 spectra/s (Figure 5G).
    f. Perform Automatic recalibration of each acquired spectrum by using reference masses of reference standards.
    g. Set Auto Recalibration Parameters as in Figure 5H.

Note: The ^{13}C isotopic ion of deprotonated acetic acid dimer ($[2CH_3COOH\text{-}H]^-$, *m/z* 120.038339) and Hexakis (2,2-difluoroethoxy) phosphazene + deprotonated acetic acid (*m/z* 680.035541) are referenced as the lock mass for exact mass measurement (Figure 5H).

Figure 5. Parameters of CE-TOFMS method for cationic metabolite analysis

(A–C) Screenshots of ChemStation program.

(D) Water bath temperature.

(E–H) Screenshots of MassHunter program.

48. Create a worklist file in the MassHunter program for running CE-TOF/MS.
49. Run standard mixture first to check total ion chromatogram (TIC).

⚠ CRITICAL: If the TIC spectrum is not stable, refer to troubleshooting Problem 2.

Table 2. List of metabolites targeted in CE-TOFMS in cationic mode (groups indicate the combination of metabolites)

Group A		Group B		Group C		Group D		Group E	
Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time
D-Camphor-10-sulfonic acid solution	1.000	D-Camphor-10-sulfonic acid solution	1.000	D-Camphor-10-sulfonic acid solution	1.000	D-Camphor-10-sulfonic acid solution	1.000	D-Camphor-10-sulfonic acid solution	1.000
Trimesate	0.601	Trimesate	0.601	Trimesate	0.601	Trimesate	0.601	Trimesate	0.601
Acetyl Coenzyme A	0.852	Octanoate	1.022	Guanosine triphosphate	0.798	Inosine 3′,5′-cyclic monophosphate	1.068	Ribulose 1,5-diphosphate	0.685
Adenosine triphosphate	0.786	Cholate	1.342	Guanosine diphosphate	0.830	4-Oxopentanoate	0.882	Sedoheptulose 7-phosphate	0.888
Adenosine diphosphate	0.817	N-Carbamylglutamate	0.722	Glycocholate	1.383	Malonyl Coenzyme A	0.774	Saccharate	0.709
Adipate	0.703	Citicoline	1.334	m-Hydroxybenzoate	0.923	(Methylthio)acetate	0.831	Sorbitol 6-phosphate	0.866
2-Amino-3-phosphonopropionate	0.752	Reduced nicotinamide adenine dinucleotide	1.034	2-Hydroxyoctanoate	1.031	Nicotinamide adenine dinucleotide phosphate	0.906	Tiglate	0.887
N-Acetylglucosamine 6-phosphate	0.926	Nicotinamide adenine dinucleotide	1.410	3-Hydroxypropionate	0.838	Reduced nicotinamide adenine dinucleotide phosphate	0.799	2-Thiopheneacetate	0.903
Barbiturate	0.883	2-Deoxyribose 1-phosphate	0.819	3-Methylbutanoate	0.929	Nicotinate	0.875	Trehalose 6-phosphate	1.002
Carbamoylphosphate	0.662	Deoxycytidine monophosphate	0.880	Isobutyryl Coenzyme A	0.868	Nicotinic acid adenine dinucleotide	1.014	Threonate	0.923
Coenzyme A	0.833	2,5-Dihydroxybenzoate	0.901	Glucuronate	1.051	Orotidine 5′-monophosphate	0.755	Thymidine diphosphate glucose	0.990
Cytidine triphosphate	0.765	Flavin adenine dinucleotide	1.084	Glycolate	0.753	Phosphoribosyl pyrophosphate	0.674	3-Ureidopropionate	0.920
Cytidine 5′-monophosphate-N-acetylneuraminic acid	1.038	N-Formylaspartate	0.663	Glyoxylate	0.782	Isethionate	0.783	Pentanoate	0.920
Cytidine diphosphate	0.791	Citraconate	0.668	Glucose 1-phosphate	0.857	2-Oxoadipate	0.660	5-Oxoproline	0.901
N-Acetylneuraminate	1.222	Cytidine 2′,3′-cyclic phosphate	1.129	Glucosamine 6-phosphate	0.961	Lactate	0.826	Phosphonoacetate	0.644
Adenosine 3′,5′-diphosphate	0.734	Citramalate	0.664	Guanosine diphosphate mannose	1.024	Malate	0.628	Quinate	1.030
cis-Aconitate	0.594	Cysteine S-sulfate	0.817	Glycerate	0.843	2-Oxoisopentanoate	0.854	D-Ribose 5-phosphate	0.830
N-Acetylglutamate	0.738	Deoxycytidine triphosphate	0.762	Glyceraldehyde 3-phosphate	0.786	4-Methyl-2-oxopentanoate	0.899	D-Ribulose 5-phosphate	0.814
Adenylosuccinate	0.726	Deoxyuridine monophosphate	0.868	Glycerophosphate	0.769	Malonate	0.582	Succinate	0.631
Adenosine 5′-phosphosulfate	0.856	Deoxyuridine triphosphate	0.757	Gluconate	1.043	Methyl sulfate	0.676	Shikimate	1.034
2-Aminoethylphosphonate	1.094	Deoxyguanosine triphosphate	0.799	Galacturonate 1-phosphate	0.726	4-Methylthio-2-oxobutyrate	0.878	Tartrate	0.620

(*Continued on next page*)

Table 2. *Continued*

Group A		Group B		Group C		Group D		Group E	
Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time
1-Aminoethylphosphonate	1.048	Dihydroxyacetone phosphate	0.752	p-Hydroxyphenylacetate	0.975	2-Oxobutyrate	0.804	Taurocholate	1.384
N-Acetylmethionine	1.049	2,3-Diphosphoglyceric acid	0.655	3-Hydroxybutyrate	0.899	2-Oxoglutarate	0.625	Uridine diphosphate glucose	0.980
4-Acetylbutyrate	0.937	Deoxyadenosine diphosphate	0.812	2-Hydroxybutyrate	0.883	4-Oxohexanoate	0.926	Uridine diphosphate glucuronic acid	0.811
Adenosine diphosphate ribose	0.990	Deoxyadenosine monophosphate	0.909	2-Hydroxyisobutyrate	0.875	Phosphoenolpyruvic acid	0.630	Uridine diphosphate-N-acetylglucosamine	0.999
trans-Aconitate	0.580	Deoxyadenosine triphosphate	0.788	2-Hydroxyphenylacetate	0.946	Propionate	0.818	Uridine 5′-diphosphate	0.784
N-Acetylglucosamine 1-phosphate	0.895	Deoxycytidine diphosphate	0.785	2-Hydroxyglutarate	0.670	Pyruvate	0.740	Uridine 5′-monophosphate	0.878
N-Acetyl-beta-alanine	0.949	2′-Deoxyinosine triphosphate	0.781	Isocitrate	0.591	3-Phosphoglyceric acid	0.641	Uridine triphosphate	0.758
N-Acetylaspartate	0.708	Digalacturonate	0.897	Inosinic acid	0.895	2-Phosphoglyceric acid	0.645	Xanthylic acid	0.753
Adenosine diphosphate Glucose	1.013	2-Deoxyglucose 6-phosphate	0.856	Inosine diphosphate	0.808	6-Phosphogluconate	0.706	Deoxyuridine diphosphate	0.778
Butyrate	0.878	3′,5′-Cyclic deoxyadenosine monophosphate	1.128	Inosine triphosphate	0.779	Pantothenate	1.122	Xanthosine 5-triphosphate	0.711
Citrate	0.605	P1, P4-Di(adenosine-5′) tetraphosphate	0.829	Guanosine monophosphate	0.928	Phenyl phosphate	0.749	Succinyl Coenzyme A	0.783
Cysteine sulfinate	0.898	Ethanolamine phosphate	1.063	Cyclic Guanosine monophosphate	1.133	O-Phosphoserine	0.764	Prostaglandin F2alpha	1.319
Adenosine monophosphate	0.918	Fructose 6-phosphate	0.867	o-Hydroxyhippurate	0.857	Pimelate	0.728	4-Pyridoxate	0.975
Cyclic Adenosine monophosphate	1.150	Fructose 1,6-bisphosphate	0.708	3-(4-Hydroxyphenyl) propionate	1.015	3-Indoxyl sulfate	0.907	Syringate	1.003
3′-Adenosine monophosphate	0.885	Glucose 6-phosphate	0.875	trans-4-Hydroxy-3-methoxycinnamate	1.018	threo-beta-methylaspartate	0.964	Sinapate	1.075
Biotin	1.115	Cysteate	0.807	Glutarate	0.670	Mucate	0.720	Terephthalate	0.676
Cytidine monophosphate	0.892	o-Coumarate	0.981	p-Hydroxybenzoate	0.910	Methanesulfonate	0.683	Urate	0.954
trans-Cinnamate	0.959	Folate	0.855	4-Hydroxy-3-methoxymandelate	1.039	Orotate	0.871	Xanthurenate	0.763
Carbamoylaspartate	0.692	Dihydroorotate	0.907	Homovanillate	1.030	Phenylpyruvate	0.928	3-(2-Hydroxyphenyl) propionate	0.992
Allantoate	0.969	Fumarate	0.606	Hippurate	1.004	Porphobilinogen	1.142	2,3-Pyridinedicarboxylate	0.688

(*Continued on next page*)

Table 2. *Continued*

Group A		Group B		Group C		Group D		Group E	
Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time	Compound name	Relative migration time
N-Acetylleucine	1.069	2-Furoate	0.843	3-Hydroxy-3-methylglutarate	0.695	Prostaglandin E2	1.308	3-Phenylpropionate	0.969
N-Acetylmuramate	1.205	N-Formylmethionine	0.983	2-Hydroxy-4-methylpentanoate	0.966	2-Isopropylmalate	0.726	Pelargonate	1.054
Benzoylformate	0.879	2,4-Dihydroxypyrimidine-5-carboxylate	0.891	Itaconate	0.646	2-Oxooctanoate	0.967	Phthalate	0.699
Azelate	0.771	Hexanoate	0.956	Heptanoate	0.985	Phenaceturate	1.033	3-Phenyllactate	0.982
N-Acetylphenylalanine	1.075	Crotonate	0.854	2-Hydroxypentanoate	0.931	Dodecanoate	1.195	Pyrrole 2-carboxylate	0.849
Benzoate	0.877	Dodecanedioate	0.830	6-Hydroxyhexanoate	0.990			2-Quinolinecarboxylate	0.973
Benzylsuccinate	0.759	Decanoate	1.089	10-Hydroxydecanoate	1.117			Sebacate	0.792
p-Coumarate	0.973			4-Hydroxymandelate	0.984			Undecanoate	1.129
				Serine O-sulfate	0.816			5-Thymidylic acid	0.894
				3-Indolebutyrate	1.068			Deoxythymidine 5′-diphosphate	0.798
				o-Hydroxybenzoate	0.847			Thymidine 5′-triphosphate	0.773

50. Run the worklist program.
51. Check the graph of the current.

⚠ CRITICAL: The current should be kept at around 30 μA for analysis of anionic metabolites. If the current value shows inconsistency, refer to troubleshooting Problem 3 or Problem 4.

Note: Fifty mM ammonium acetate (both pH 3.4 and pH 8.5) washing buffer and 50 mM ammonium acetate (pH 8.5) electrolyte should be replaced every ten runs. Samples and 237 standard compounds listed in Table 2 can be analyzed. The reference metabolites will be measured for quantification and validation of target metabolites.

Data analysis of CE-TOFMS

Timing: 2 days

Raw data are processed by using MasterHands (Sugimoto et al., 2010). Each peak of metabolites is identified by matching *m/z* values and the normalized migration times of reference compounds.

Data processing comprises data conversion and peak integration from every raw data. The processed data are aligned, and a data matrix is yielded after filling missing peaks (Sugimoto et al., 2012).

52. MassHunter makes the files of each sample in the ".d extension" folder.
53. Convert each data .d folder to a .kiff extension by using a data conversion mode of MasterHands (Figure 6A).
54. Load all data .kiff files on the MasterHands program (Figure 6B).
55. Detect every peak with default options. Parameters for peak detection are S/N cutoff: 2 and valley (%): 50 (Figure 6C).
56. Determine the internal reference component peak and external reference component peak in all samples, as shown in Figure 6D.

Note: Internal reference components are methionine sulfone for cation and D-Camphor-10-sulfonic acid for the anion. External reference components are 3-Aminopyrrolidine for cationic metabolites and trimesate for anionic metabolites. Internal and external references are used to correct the migration time of metabolites in samples. Also, the area values of the internal references are used for the standardization of metabolites.

57. Run the alignment function to correct the migration time of the targeted metabolite among the samples.
58. Identify the signal peak by comparing with the peak of a mixed reference.

Note: If the signal peak is not clear, refer to troubleshooting Problem 5.

Note: On isobaric metabolites, refer to troubleshooting Problem 6.

59. Export a csv file for the calculation of concentration.
60. Calculate the normalized concentration of each metabolite in the sample by using the following formulas.

Metabolite concentration (μM)

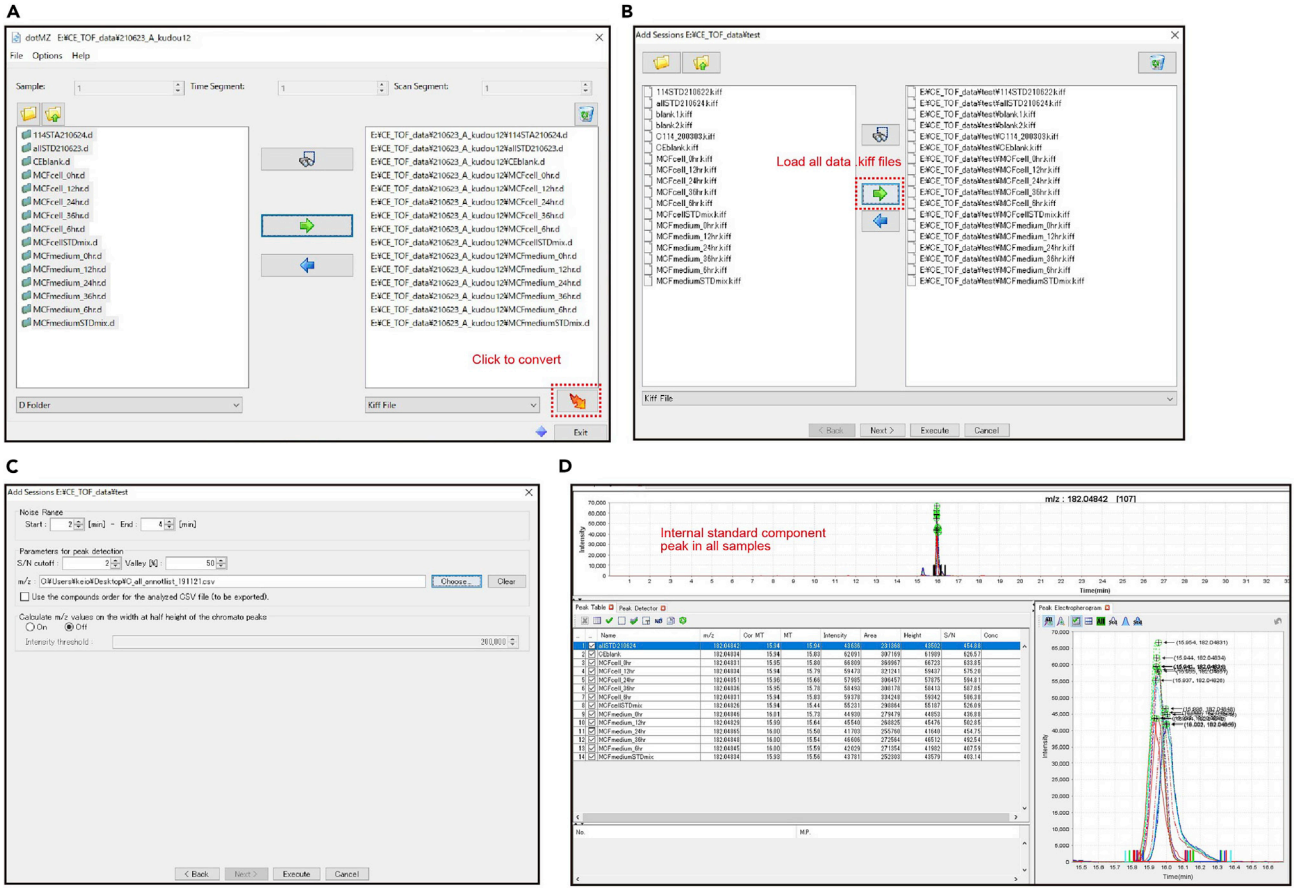


Figure 6. Screenshots of the MasterHands program for data processing

(A–D) The screenshot images of the MasterHands program.

$$= \frac{\frac{\text{Sample Area}}{\text{Sample ISArea}}}{\frac{\text{Standard Area}}{\text{Standard ISArea}}} \times 20\ \mu\text{M} \times \frac{25\ \mu\text{M}}{200\ \mu\text{M}} \quad \text{(Equation 1)}$$

$$\text{Normalized concentration}\left(\frac{\text{fmol}}{\text{cell}}\right) = \left(\text{metabolite concentration}\ (\mu\text{M}) \times \frac{1}{1000}\text{L}\right) \Big/ \text{cell number} \times 10^9 \quad \text{(Equation 2)}$$

Sample Area; Peak area of the targeting metabolite in a sample. Sample ISArea; Peak area of methionine sulfone or D-Camphor-10-sulfonic acid in a sample. Standard Area; Peak area of the targeting metabolite in 1× standard metabolite mixture for anionic or cationic metabolite analysis. Standard ISArea; Peak area of methionine sulfone or D-Camphor-10-sulfonic acid in 1× standard metabolite mixture for anionic or cationic metabolite analysis. 20 μM; standard concentration in 1× standard metabolite mixture for cationic or anionic metabolite analysis. 25 μM; the concentration of methionine sulfone or D-Camphor-10-sulfonic acid in extraction methanol with reference metabolites. 200 μM; the concentration of methionine sulfone or D-Camphor-10-sulfonic acid in 1× standard metabolite mixture for anionic or cationic metabolite analysis.

Solubilization of hydrophobic metabolites for LC-QTOFMS analysis

Timing: 1 h

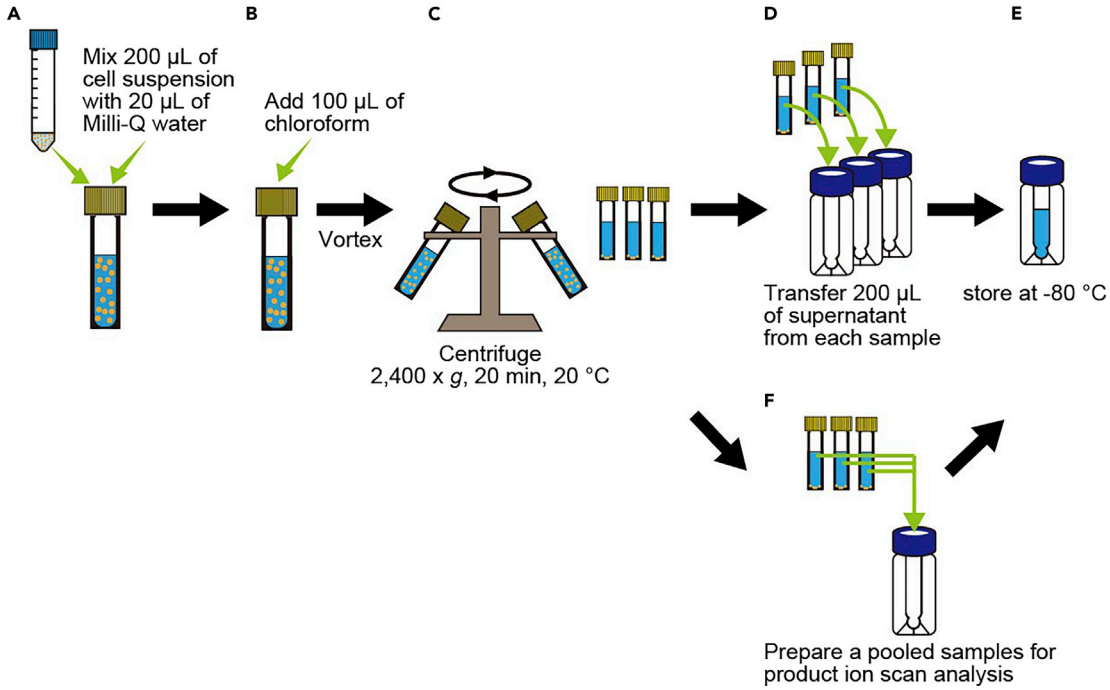


Figure 7. An overview of solubilization of hydrophobic metabolites for LC-QTOFMS analysis
(A–F) The schematic representation of pareparation steps for hydrophobic metabloites.

Whole lipid metabolites are extracted from biological materials (Bligh and Dyer, 1959). Briefly, hydrophobic metabolites are dissolved in chloroform and are analyzed by LC-QTOFMS system with a high-sensitivity full-scan mode. A pooled sample from each analyte is analyzed by LC-QTOFMS system with product ion scan mode (Calderón-Santiago et al., 2014; Spickett and Pitt, 2015).

61. Transfer a 200 µL volume of cell sample (from step 17) into a 2 mL glass jacket tube and add 20 µL of Milli-Q water (Figure 7A).
62. Add 100 µL of chloroform using a glass tip and mix by vortexing (Figure 7B).
63. Centrifuge at 2,400 × *g* for 20 min at 20°C using a swinging rotor centrifuge (Figure 7C).
64. Transfer 200 µL of the supernatant into a glass insert assembled in a glass vial (Figure 7D) and store samples at −80°C until measurement (Figure 7E).

Note: If the sample forms two layers, refer to troubleshooting Problem 7.

65. For product ion scan analysis, make a pooled sample by mixing 10–20 µL of each sample for measurement (Figure 7F).
66. Store samples at −80°C until measurement (Figure 7E).

⚠ CRITICAL: Glassware should be used for tips and tubes in the presence of chloroform to avoid plastic contamination.

Hydrophobic metabolite analysis by LC-QTOFMS

Timing: 2 days

The LC-QTOFMS system is represented as a cartoon in Figure 8A. Briefly, hydrophobic metabolites are separated on a reversed-phase LC column, and QTOFMS determines the molecular masses of major eluted species.

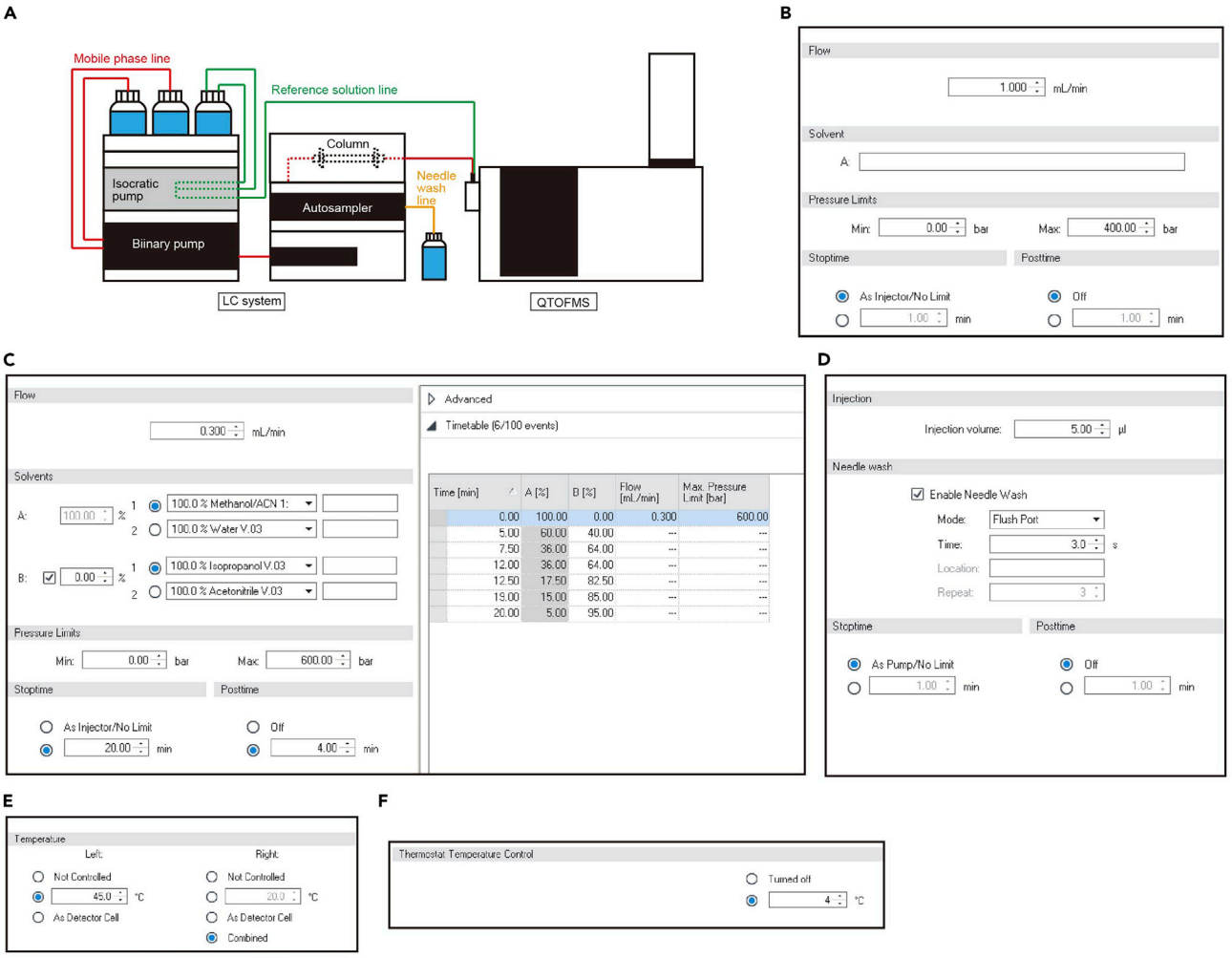


Figure 8. Parameters of the MassHunter program for LC analysis by full scan mode

(A) Cartoon representation of LC-QTOFMS systems used in this protocol.

(B–F) Screenshots of MassHunter program.

We have attached the setting file as supplementary file (Data S5, S6, S7, S8, S9, S10, S11, and S12). Users can refer to all the parameters in the file. We review the key parameters that should be confirmed in the following section.

⚠ CRITICAL: To open the setting files Data S5, S6, S7, S8, S9, S10, S11, and S12, you need to install application software on your PC.

67. Run MassHunter Data Acquisition B.08.00 Software on PC.

Note: All machine settings should be modified through this application program.

68. Prepare 1 L of mobile phase A solution (5 mM ammonium formate in acetonitrile-methanol-water (3:1:1)) and 1 L of mobile phase B solution (5 mM ammonium formate in 2-propanol) in a 1 L bottle.
69. Connect the mobile phase A solution line to the binary pump at channel A and the mobile phase B at channel B.

70. Run the Purge program at 5 mL/min flow rate for 3 min.
71. Prepare 100 mL of 2-propanol for needle wash solution in a 300 mL bottle and connect the needle wash line to the autosampler.
72. Prepare 80 mL of 1× reference solution in a 100 mL bottle and set it in the isocratic pump.
73. Run the Purge program with reference solution at a 5 mL/min flow rate for 3 min.
74. Change the flow rate of the reference solution to 1 mL/min (Figure 8B).
75. Set the column (Acquity UPLC HSS T3 C18 column 2.1 mm i.d. × 50 mm, 1.8 μm; Waters, Milford, MA) in LC system.
76. Set up the LC method.
    a. Set flow rate at 0.3 mL/min (Figure 8C).
    b. Prepare the gradient profiles of solvent B as described in Figure 8C (right panel).

    Note: The ratios of solvent B are 0% at 0 min, 40% at 5 min, 64% at 7.5 min, 64% at 12 min, 82.5% at 12.5, 85% at 19 min, and 95% at 20 min. Post time is set at 4 min (Figure 8C).

    c. Set the Injection volume of samples at 5 μL (Figure 8D).
    d. Set the column temperature (Temperature Left) at 45°C.
    e. Check "Combined" at Temperature Right (Figure 8E).
    f. Set the autosampler temperature (Thermostat Temperature Control) at 4°C (Figure 8F).
77. Set up QTOFMS method for full scan analysis.

    Note: Full scan analysis should be run by positive and negative modes. Thus, the QTOFMS setting should be prepared for positive and negative modes (Figure 9A). Meanwhile, product ion scan analysis should be run on only negative mode, but the method file should be prepared for each lipid class (Figure 9A). Here we show the case for the positive mode.

    a. Select positive at Ion Polarity (Seg) (Figure 9B).
    b. In Dual ESI (Seg) window, set the following parameters; Gas Temp: 350°C, Drying Gas: 10 L/min, and Nebulizer: 55 psig (Figure 9C).
    c. Set the capillary voltage (Dual ESI (Expt), VCap) at 3,500 V (Figure 9C).
    d. In MS TOF (Expt) section, set the Fragmentor, Skimmer, and Oct1 RFVpp voltages at 150 V, 90 V, and 500 V, respectively (Figure 9C).
    e. In the Spectral Parameters part, set the Acquisition range (Mass Range) at Min Range *m/z* 100 and Max Range *m/z* 1,700.
    f. Set the Acquisition Rate/Time at 1.5 spectra/s (Figure 9D).
    g. Perform Automatic recalibration by using two reference masses of reference standards, as shown in Figure 9E.

    Note: Positive mode: $[purine + H]^+$, *m/z* 121.050873 and [hexakis (1H,1H,3H-tetrafluoropropoxy) phosphazene + $H]^+$, *m/z* 922.009798. Negative mode: [trifluoroacetic acid - H]-, *m/z* 112.985587 and [hexakis (1H,1H,3H-tetrafluoropropoxy +trifluoroacetic acid - H]-, *m/z* 1033.988109

    h. Create a similar method file with negative mode.
78. Set up QTOFMS method for product ion scan analysis.
    a. Select negative mode in the Ion Polarity (Seg) part (Figure 10A).
    b. Input the Dual ESI (Seg) values as Gas Temp: 350°C, Drying Gas: 10 L/min, and Nebulizer: 55 psig (Figure 10B).
    c. Set the capillary voltage (Dual ESI (Expt)) at 3,500 V (Figure 10B).
    d. Input MS TOF (Expt) values as Fragmentor: 150 V; Skimmer: 90 V; and Oct1 RFVpp: 500 (Figure 10B).
    e. In the Acquisition tab, check "Auto MS/MS (Seg) mode" and input Mass Range values as Min Range *m/z* 100 and Max Range *m/z* 1,100 in both MS and MS/MS sections (Figure 10C).

f. Set the Acquisition Rate/Time at 1.5 spectra/s for MS and at 2 spectra/s for MS/MS (Figure 10C).
g. Select "Narrow (*m/z* 1.3)" in Isolation Width for MS/MS (Figure 10C).
h. In the Precursor Selection I tab, set 3 Max Precursors Per Cycle (Figure 10D).
i. In the Precursor Threshold part, set Abs. Threshold as 10,000 counts (Figure 10D).
j. Change the collision energy value in the Collision Energy tab depending on the phospholipid class.
k. Check "Use Fixed Collision Energies" and enter the value of the Collision Energy column listed in Table 3.

Note: We show the example of phosphatidic acid in Figure 10E. The collision energy should be optimized depending on the instruments used as shown in the following section "optimization of collision energy for product ion scan".

Note: The precursor *m/z* of each phospholipid in Figure 10F is summarized in Table 3.

l. Perform Automatic recalibration by using two reference masses of reference standards (shown below, Figure 10G).

Note: Negative mode: [trifluoroacetic acid - H]$^-$, *m/z* 112.985587 and [hexakis (1H,1H,3H-tetrafluoropropoxy +trifluoroacetic acid - H]$^-$, *m/z* 1033.988109

m. Create method files for each class of phospholipids so that the Product ion scan can be executed for each category.

Note: In total, six methods should be created (for phosphatidylserine, phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol).

79. Create a worklist in the MassHunter program.
80. Run the worklist program.

Note: Samples are analyzed in independent runs in positive and negative modes using the conditions for full scan analysis. The pooled sample is analyzed six times in negative mode using product ion scan analysis.

Note: Check the column pressure during measurement. If the column pressure increases more than 50 mbar from the start, refer to troubleshooting Problem 8.

Optimization of collision energy for product ion scan

Timing: 1 day

The collision energy should be optimized depending on the instruments. Next, we describe the step of collision energy optimization for product ion scan. The optimization of collision energy is performed in each phospholipid class using five phospholipid standards. The standards are listed in Table 4.

81. Dissolve the set-up standards in methanol at 10 μM final concentration.
82. Transfer each standard solution into LC vials.
83. Run LC-QTOFMS with the collision energy in the range between 10 eV and 50 eV.
84. Check the intensity of the evaluation spectrums listed in Table 4.
85. Select the collision energy that shows high intensity in all evaluation spectrums.

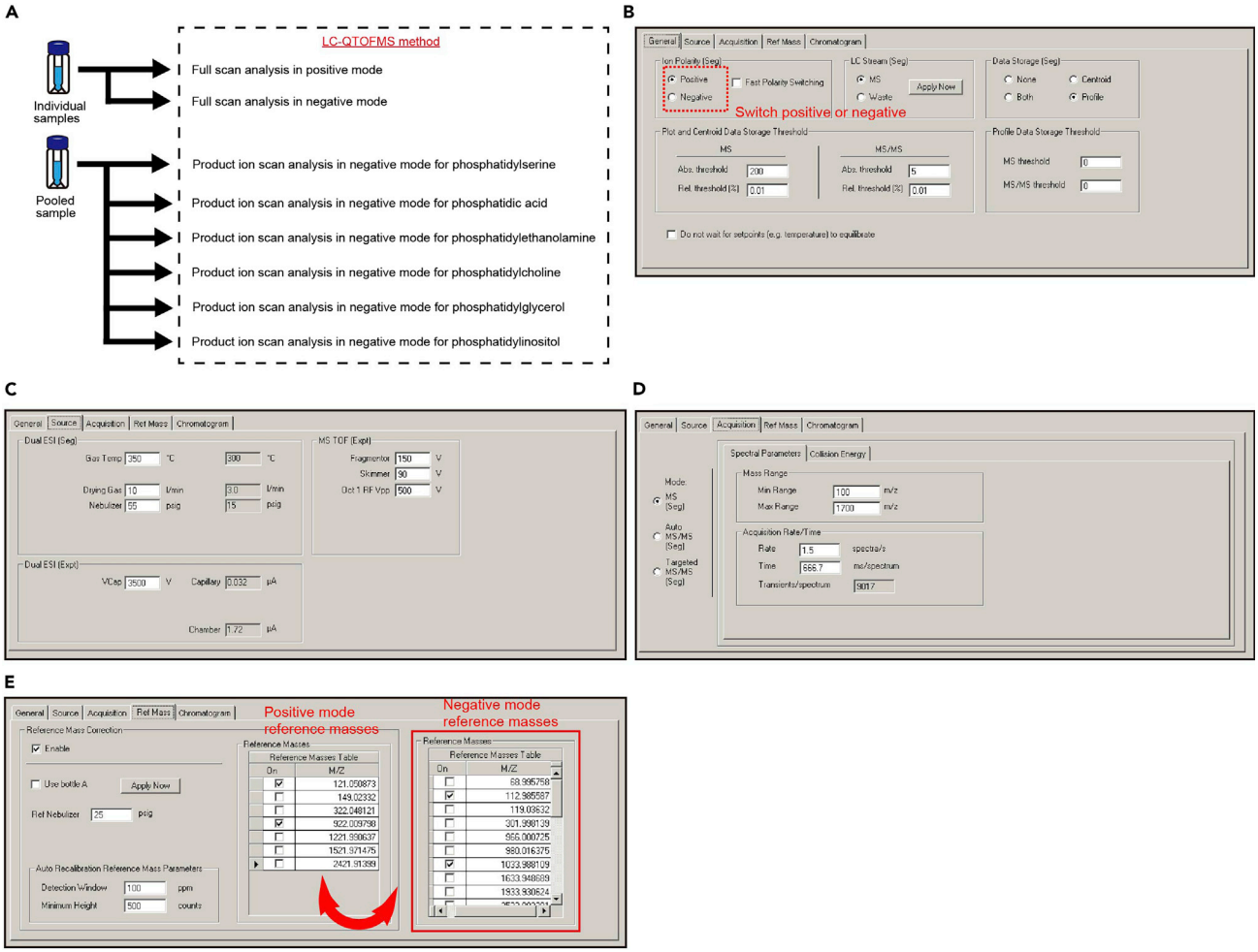


Figure 9. The parameters for the QTOFMS method for product ion scan analysis

(A) Schematic representation of the method's files prepared in this protocol.

(B–E) Screenshots of the MassHunter program.

Data analysis for LC-QTOFMS in full scan

Timing: 2 days

Data analysis of LC-QTOFMS data is the same way as analyzing the data of CE-TOFMS except for the calculation of metabolite concentration (Refer to steps 52–59). The retention times of LC-QTOFMS is shown as the two-dimensional map (Figure 11A).

The amount of hydrophobic metabolite is normalized with cell number.

Data analysis for LC-QTOFMS in product ion scan to identify the phospholipid class

Timing: 1 day

Product ion scan analysis data are imported into the MassHuter Qualitative Analysis software to identify the class of fatty acids with respect to phospholipid. The phospholipid is evaluated by *m/z* value and the presence of fatty acid-derived peaks. The MassBank data identifies the phospholipid class (Horai et al., 2010; Taguchi et al., 2005; Taguchi and Ishikawa, 2010).

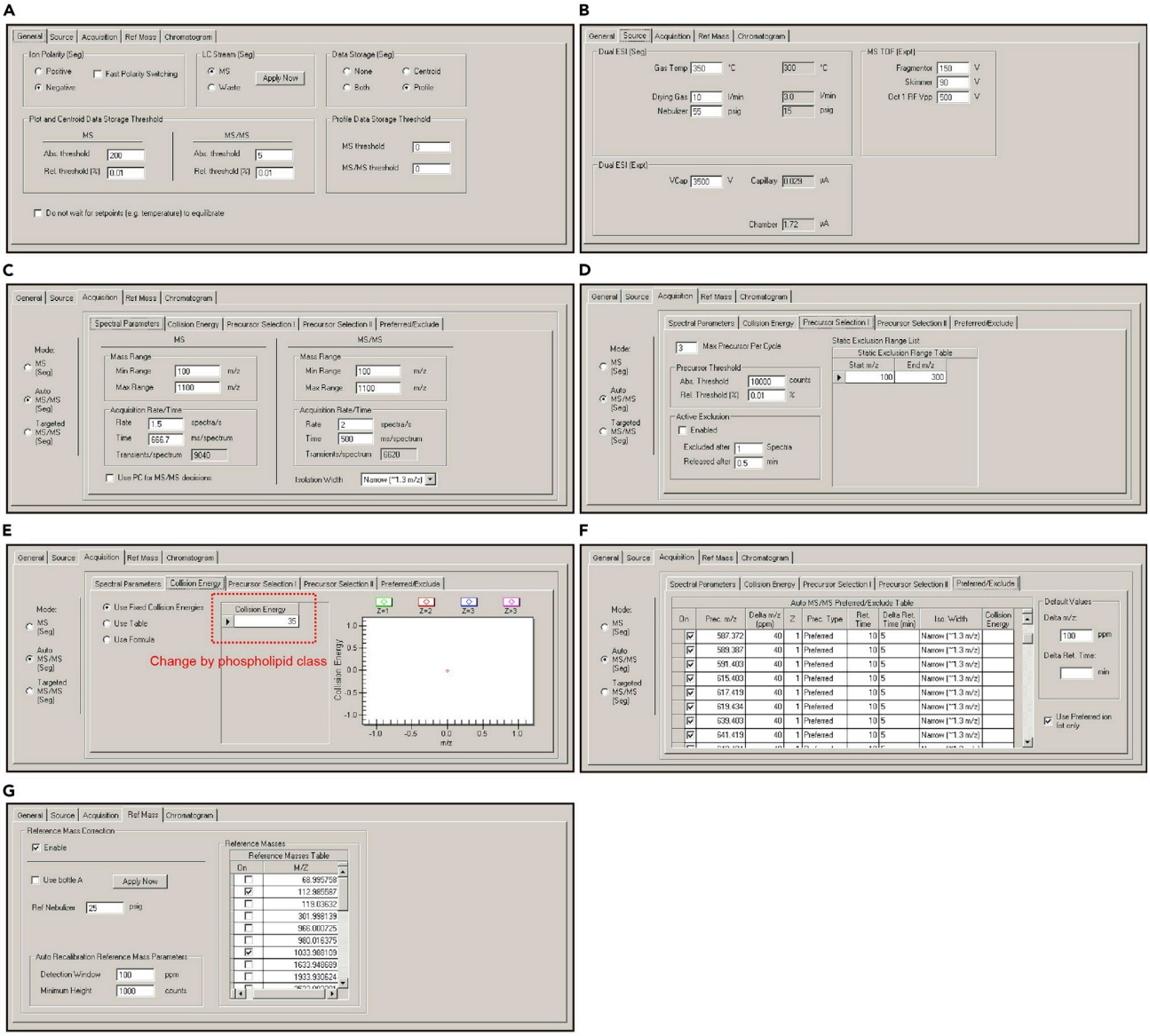


Figure 10. The parameters for the QTOFMS method

(A–G) Screenshot of the MassHunter program.

Note: For the analysis of pooled samples, a data folder ''.d extension'' is yielded by MassHunter. The data folder is processed by MassHunter Qualitative Analysis software.

86. Display Fragment mass spectra (Figure 11B).
87. Check the peak of neutral loss and polar head-derived spectrum.

Note: The spectrum *m/z* is summarized in Table 5.

88. Check the peak of the fatty acid spectrum.

Note: The spectrum *m/z* is summarized in Table 6.

Table 3. List of collision energies for phospholipid identification and the precursor *m/z*

Phospholipid class	Phosphatidylserine (PS)		Phosphatidic acid (PA)		Phosphatidylethanolamine (PE)		Phosphatidylcholine (PC)		Phosphatidylglycerol (PG)		Phosphatidylinositol (PI)	
Collision energy	22		35		35		38		40		44	
Precursor *m/z*	PS(28:2)	674.404	PA(28:2)	587.372	PE(28:2)	630.414	PC(28:2)	718.466	PG(28:2)	661.409	PI(28:2)	749.425
	PS(28:1)	676.42	PA(28:1)	589.387	PE(28:1)	632.43	PC(28:1)	720.482	PG(28:1)	663.424	PI(28:1)	751.44
	PS(28:0)	678.435	PA(28:0)	591.403	PE(28:0)	634.445	PC(28:0)	722.498	PG(28:0)	665.44	PI(28:0)	753.456
	PS(30:2)	702.435	PA(30:2)	615.403	PE(30:2)	658.445	PC(30:2)	746.498	PG(30:2)	689.44	PI(30:2)	777.456
	PS(30:1)	704.451	PA(30:1)	617.419	PE(30:1)	660.461	PC(30:1)	748.513	PG(30:1)	691.456	PI(30:1)	779.472
	PS(30:0)	706.466	PA(30:0)	619.434	PE(30:0)	662.477	PC(30:0)	750.529	PG(30:0)	693.471	PI(30:0)	781.487
	PS(32:4)	726.435	PA(32:4)	639.403	PE(32:4)	682.445	PC(32:4)	770.498	PG(32:4)	713.44	PI(32:4)	801.456
	PS(32:3)	728.451	PA(32:3)	641.419	PE(32:3)	684.461	PC(32:3)	772.513	PG(32:3)	715.456	PI(32:3)	803.472
	PS(32:2)	730.466	PA(32:2)	643.434	PE(32:2)	686.477	PC(32:2)	774.529	PG(32:2)	717.471	PI(32:2)	805.487
	PS(32:1)	732.482	PA(32:1)	645.45	PE(32:1)	688.492	PC(32:1)	776.545	PG(32:1)	719.487	PI(32:1)	807.503
	PS(32:0)	734.498	PA(32:0)	647.466	PE(32:0)	690.508	PC(32:0)	778.56	PG(32:0)	721.503	PI(32:0)	809.519
	PS(34:6)	750.435	PA(34:6)	663.403	PE(34:6)	706.445	PC(34:6)	794.498	PG(34:6)	737.44	PI(34:6)	825.456
	PS(34:5)	752.451	PA(34:5)	665.419	PE(34:5)	708.461	PC(34:5)	796.513	PG(34:5)	739.456	PI(34:5)	827.472
	PS(34:4)	754.466	PA(34:4)	667.434	PE(34:4)	710.477	PC(34:4)	798.529	PG(34:4)	741.471	PI(34:4)	829.487
	PS(34:3)	756.482	PA(34:3)	669.45	PE(34:3)	712.492	PC(34:3)	800.545	PG(34:3)	743.487	PI(34:3)	831.503
	PS(34:2)	758.498	PA(34:2)	671.466	PE(34:2)	714.508	PC(34:2)	802.56	PG(34:2)	745.503	PI(34:2)	833.519
	PS(34:1)	760.513	PA(34:1)	673.481	PE(34:1)	716.524	PC(34:1)	804.576	PG(34:1)	747.518	PI(34:1)	835.534
	PS(34:0)	762.529	PA(34:0)	675.497	PE(34:0)	718.539	PC(34:0)	806.592	PG(34:0)	749.534	PI(34:0)	837.55
	PS(36:7)	776.451	PA(36:7)	689.419	PE(36:7)	732.461	PC(36:7)	820.513	PG(36:7)	763.456	PI(36:7)	851.472
	PS(36:6)	778.466	PA(36:6)	691.434	PE(36:6)	734.477	PC(36:6)	822.529	PG(36:6)	765.471	PI(36:6)	853.487
	PS(36:5)	780.482	PA(36:5)	693.45	PE(36:5)	736.492	PC(36:5)	824.545	PG(36:5)	767.487	PI(36:5)	855.503
	PS(36:4)	782.498	PA(36:4)	695.466	PE(36:4)	738.508	PC(36:4)	826.56	PG(36:4)	769.503	PI(36:4)	857.519
	PS(36:3)	784.513	PA(36:3)	697.481	PE(36:3)	740.524	PC(36:3)	828.576	PG(36:3)	771.518	PI(36:3)	859.534
	PS(36:2)	786.529	PA(36:2)	699.497	PE(36:2)	742.539	PC(36:2)	830.592	PG(36:2)	773.534	PI(36:2)	861.55
	PS(36:1)	788.545	PA(36:1)	701.513	PE(36:1)	744.555	PC(36:1)	832.607	PG(36:1)	775.549	PI(36:1)	863.566
	PS(36:0)	790.56	PA(36:0)	703.528	PE(36:0)	746.571	PC(36:0)	834.623	PG(36:0)	777.565	PI(36:0)	865.581
	PS(38:7)	804.482	PA(38:7)	717.45	PE(38:7)	760.492	PC(38:7)	848.545	PG(38:7)	791.487	PI(38:7)	879.503
	PS(38:6)	806.498	PA(38:6)	719.466	PE(38:6)	762.508	PC(38:6)	850.56	PG(38:6)	793.503	PI(38:6)	881.519
	PS(38:5)	808.513	PA(38:5)	721.481	PE(38:5)	764.524	PC(38:5)	852.576	PG(38:5)	795.518	PI(38:5)	883.534
	PS(38:4)	810.529	PA(38:4)	723.497	PE(38:4)	766.539	PC(38:4)	854.592	PG(38:4)	797.534	PI(38:4)	885.55
	PS(38:3)	812.545	PA(38:3)	725.513	PE(38:3)	768.555	PC(38:3)	856.607	PG(38:3)	799.549	PI(38:3)	887.566
	PS(38:2)	814.56	PA(38:2)	727.528	PE(38:2)	770.571	PC(38:2)	858.623	PG(38:2)	801.565	PI(38:2)	889.581
	PS(38:1)	816.576	PA(38:1)	729.544	PE(38:1)	772.586	PC(38:1)	860.639	PG(38:1)	803.581	PI(38:1)	891.597
	PS(38:0)	818.592	PA(38:0)	731.56	PE(38:0)	774.602	PC(38:0)	862.654	PG(38:0)	805.596	PI(38:0)	893.612
	PS(40:8)	830.498	PA(40:8)	743.466	PE(40:8)	786.508	PC(40:8)	874.56	PG(40:8)	817.503	PI(40:8)	905.519
	PS(40:7)	832.513	PA(40:7)	745.481	PE(40:7)	788.524	PC(40:7)	876.576	PG(40:7)	819.518	PI(40:7)	907.534
	PS(40:6)	834.529	PA(40:6)	747.497	PE(40:6)	790.539	PC(40:6)	878.592	PG(40:6)	821.534	PI(40:6)	909.55
	PS(40:5)	836.545	PA(40:5)	749.513	PE(40:5)	792.555	PC(40:5)	880.607	PG(40:5)	823.549	PI(40:5)	911.566
	PS(40:4)	838.56	PA(40:4)	751.528	PE(40:4)	794.571	PC(40:4)	882.623	PG(40:4)	825.565	PI(40:4)	913.581

(*Continued on next page*)

Table 3. ***Continued***

Phospholipid class	Phosphatidylserine (PS)		Phosphatidic acid (PA)		Phosphatidylethanolamine (PE)		Phosphatidylcholine (PC)		Phosphatidylglycerol (PG)		Phosphatidylinositol (PI)	
Collision energy	22		35		35		38		40		44	
	PS(40:3)	840.576	PA(40:3)	753.544	PE(40:3)	796.586	PC(40:3)	884.639	PG(40:3)	827.581	PI(40:3)	915.597
	PS(40:2)	842.592	PA(40:2)	755.56	PE(40:2)	798.602	PC(40:2)	886.654	PG(40:2)	829.596	PI(40:2)	917.612
	PS(40:1)	844.607	PA(40:1)	757.575	PE(40:1)	800.617	PC(40:1)	888.67	PG(40:1)	831.612	PI(40:1)	919.628
	PS(40:0)	846.623	PA(40:0)	759.591	PE(40:0)	802.633	PC(40:0)	890.686	PG(40:0)	833.628	PI(40:0)	921.644
	PS(42:8)	858.529	PA(42:8)	771.497	PE(42:8)	814.539	PC(42:8)	902.592	PG(42:8)	845.534	PI(42:8)	933.55
	PS(42:7)	860.545	PA(42:7)	773.513	PE(42:7)	816.555	PC(42:7)	904.607	PG(42:7)	847.549	PI(42:7)	935.566
	PS(42:6)	862.56	PA(42:6)	775.528	PE(42:6)	818.571	PC(42:6)	906.623	PG(42:6)	849.565	PI(42:6)	937.581
	PS(42:5)	864.576	PA(42:5)	777.544	PE(42:5)	820.586	PC(42:5)	908.639	PG(42:5)	851.581	PI(42:5)	939.597
	PS(42:3)	868.607	PA(42:3)	781.575	PE(42:3)	824.617	PC(42:3)	912.67	PG(42:3)	855.612	PI(42:3)	943.628
	PS(42:2)	870.623	PA(42:2)	783.591	PE(42:2)	826.633	PC(42:2)	914.686	PG(42:2)	857.628	PI(42:2)	945.644
	PS(42:1)	872.639	PA(42:1)	785.607	PE(42:1)	828.649	PC(42:1)	916.701	PG(42:1)	859.643	PI(42:1)	947.659
	PS(42:0)	874.654	PA(42:0)	787.622	PE(42:0)	830.664	PC(42:0)	918.717	PG(42:0)	861.659	PI(42:0)	949.675
	PS(44:7)	888.576	PA(44:7)	801.544	PE(44:7)	844.586	PC(44:7)	932.639	PG(44:7)	875.581	PI(44:7)	963.597
	PS(44:6)	890.592	PA(44:6)	803.56	PE(44:6)	846.602	PC(44:6)	934.654	PG(44:6)	877.596	PI(44:6)	965.612
	PS(44:5)	892.607	PA(44:5)	805.575	PE(44:5)	848.617	PC(44:5)	936.67	PG(44:5)	879.612	PI(44:5)	967.628
	PS(44:2)	898.654	PA(44:2)	811.622	PE(44:2)	854.664	PC(44:2)	942.717	PG(44:2)	885.659	PI(44:2)	973.675
	PS(44:1)	900.67	PA(44:1)	813.638	PE(44:1)	856.68	PC(44:1)	944.733	PG(44:1)	887.675	PI(44:1)	975.691
	PS(44:0)	902.686	PA(44:0)	815.654	PE(44:0)	858.696	PC(44:0)	946.748	PG(44:0)	889.69	PI(44:0)	977.706

(A:B) A; The number of carbon in fatty acids, B; The number of double bond in fatty acids.

Table 4. List of lipid standards used for the collision energy optimization

Phospholipid class	Set-up standard	Evaluation spectrum		
Phosphatidylserine (PS)	PS(12:0/13:0)	Neutral loss spectrum [*m/z* 549.3562]	Fatty acid(12:0) [m/z 199.1704]	Fatty acid(13:0) [m/z 231.1860]
	PS(14:0/14:0)	Neutral loss spectrum [*m/z* 591.4031]	Fatty acid(14:0) [m/z 227.2017]	
	PS(17:0/14:1)	Neutral loss spectrum [*m/z* 631.4344]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(14:1) [m/z 225.1860]
	PS(17:0/20:4)	Neutral loss spectrum [*m/z* 709.4814]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(20:4) [m/z 303.2330]
	PS(21:0/22:6)	Neutral loss spectrum [*m/z* 789.5440]	Fatty acid(21:0) [m/z 325.3112]	Fatty acid(22:6) [m/z 327.2330]
Phosphatidic acid (PA)	PA(12:0/13:0)	Polar head spectrum [m/z 152.9958]	Fatty acid(12:0) [m/z 199.1704]	Fatty acid(13:0) [m/z 231.1860]
	PA(14:0/14:0)	Polar head spectrum [m/z 152.9958]	Fatty acid(14:0) [m/z 227.2017]	
	PA(17:0/14:1)	Polar head spectrum [m/z 152.9958]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(14:1) [m/z 225.1860]
	PA(17:0/20:4)	Polar head spectrum [m/z 152.9958]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(20:4) [m/z 303.2330]
	PA(21:0/22:6)	Polar head spectrum [m/z 152.9958]	Fatty acid(21:0) [m/z 325.3112]	Fatty acid(22:6) [m/z 327.2330]
Phosphatidylethanolamine (PE)	PE(12:0/13:0)	Polar head spectrum [m/z 140.0118, 196.038]	Fatty acid(12:0) [m/z 199.1704]	Fatty acid(13:0) [m/z 231.1860]
	PE(14:0/14:0)	Polar head spectrum [m/z 140.0118, 196.038]	Fatty acid(14:0) [m/z 227.2017]	
	PE(17:0/14:1)	Polar head spectrum [m/z 140.0118, 196.038]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(14:1) [m/z 225.1860]
	PE(17:0/20:4)	Polar head spectrum [m/z 140.0118, 196.038]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(20:4) [m/z 303.2330]
	PE(21:0/22:6)	Polar head spectrum [m/z 140.0118, 196.038]	Fatty acid(21:0) [m/z 325.3112]	Fatty acid(22:6) [m/z 327.2330]
Phosphatidylcholine (PC)	PC(12:0/13:0)	Neutral loss spectrum [*m/z* 620.4297]	Fatty acid(12:0) [m/z 199.1704]	Fatty acid(13:0) [m/z 231.1860]
	PC(14:0/14:0)	Neutral loss spectrum [*m/z* 662.4766]	Fatty acid(14:0) [m/z 227.2017]	
	PC(17:0/14:1)	Neutral loss spectrum [*m/z* 702.5079]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(14:1) [m/z 225.1860]
	PC(17:0/20:4)	Neutral loss spectrum [*m/z* 780.5549]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(20:4) [m/z 303.2330]
	PC(21:0/22:6)	Neutral loss spectrum [*m/z* 860.6175]	Fatty acid(21:0) [m/z 325.3112]	Fatty acid(22:6) [m/z 327.2330]
Phosphatidylglycerol (PG)	PG(12:0/13:0)	Polar head spectrum [m/z 227.0326]	Fatty acid(12:0) [m/z 199.1704]	Fatty acid(13:0) [m/z 231.1860]
	PG(14:0/14:0)	Polar head spectrum [m/z 227.0326]	Fatty acid(14:0) [m/z 227.2017]	
	PG(17:0/14:1)	Polar head spectrum [m/z 227.0326]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(14:1) [m/z 225.1860]
	PG(17:0/20:4)	Polar head spectrum [m/z 227.0326]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(20:4) [m/z 303.2330]
	PG(21:0/22:6)	Polar head spectrum [m/z 227.0326]	Fatty acid(21:0) [m/z 325.3112]	Fatty acid(22:6) [m/z 327.2330]
Phosphatidylinositol (PI)	PI(12:0/13:0)	Polar head spectrum [m/z 241.0119]	Fatty acid(12:0) [m/z 199.1704]	Fatty acid(13:0) [m/z 231.1860]
	PI(16:0/16:0)	Polar head spectrum [m/z 241.0119]	Fatty acid(16:0) [m/z 255.2330]	
	PI(17:0/14:1)	Polar head spectrum [m/z 241.0119]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(14:1) [m/z 225.1860]
	PI(17:0/20:4)	Polar head spectrum [m/z 241.0119]	Fatty acid(17:0) [m/z 269.2486]	Fatty acid(20:4) [m/z 303.2330]
	PI(21:0/22:6)	Polar head spectrum [m/z 241.0119]	Fatty acid(21:0) [m/z 325.3112]	Fatty acid(22:6) [m/z 327.2330]

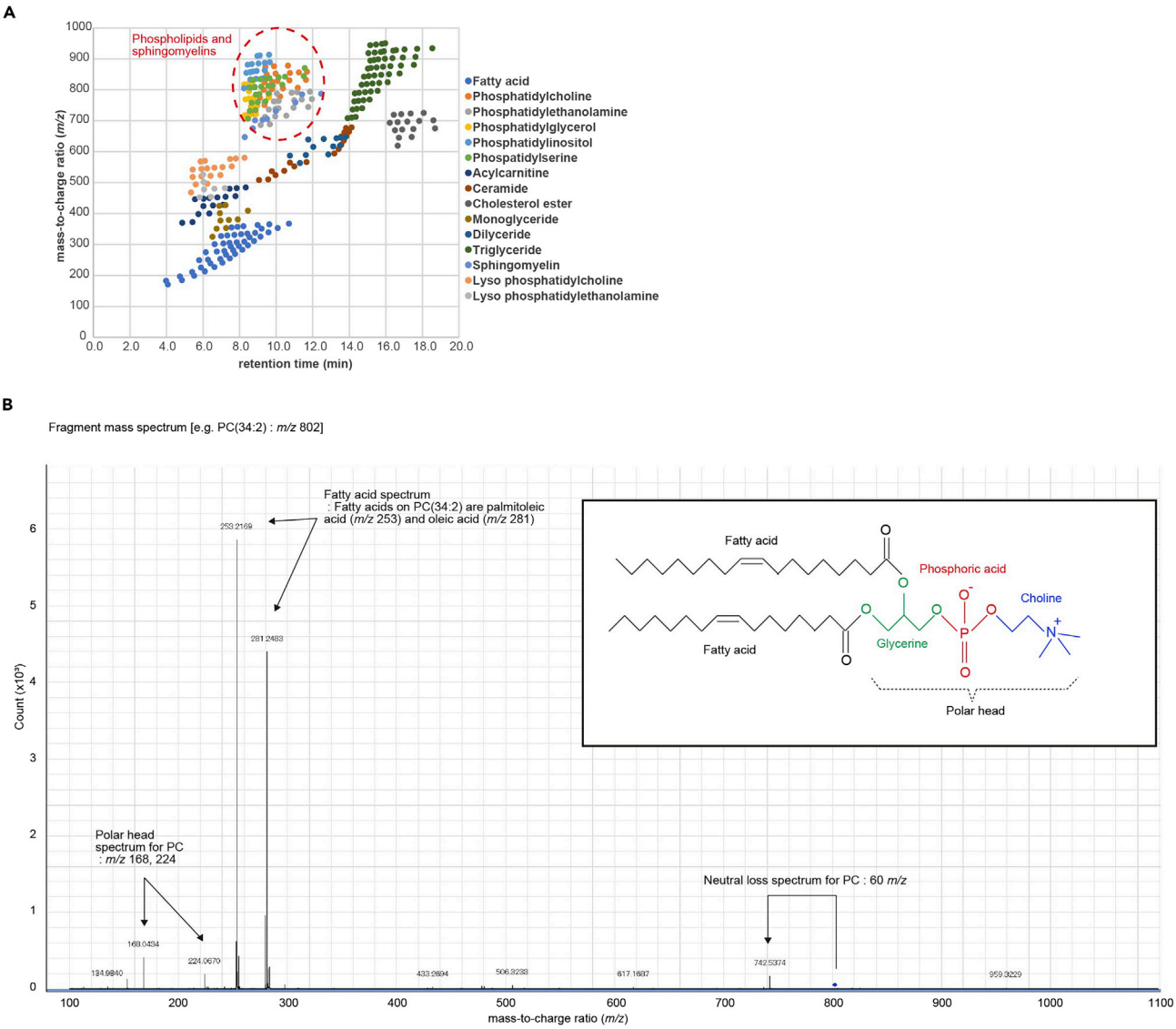


Figure 11. Example of LC-QTOFMS data

(A) The retention time of lipid classes.

(B) Mass spectrum data.

89. Identify the phospholipid metabolites based on the characters from steps 87 and 88.

EXPECTED OUTCOMES

We performed three independent experiments with three biological replicates. One representative dataset is shown in Table 7 from CE-TOFMS and Table 8 from LC-QTOFMS. Although 817 metabolites were targeted in this CE-TOFMS and LC-QTOFMS analysis, we consistently detected 378 metabolites from the MCF-7 cell lysate under our experimental condition. The number of detected metabolites and the metabolite quantity could be different depending on the experimental conditions. For instance, the cell number applied to the experiments, cell lines, and culture conditions will affect the results.

Also, to understand the extraction efficiency of metabolites from the conventional method (Adam et al., 2013) and from our method, we compared the concentration of metabolites that are detected

Table 5. The peak *m/z* list of neutral loss and polar head-derived spectrum

Phospholipid class	Phosphatidylserine (PS)	Phosphatidic acid (PA)	Phosphatidylethanolamine (PE)	Phosphatidylcholine (PC)	Phosphatidylglycerol (PG)	Phosphatidylinositol (PI)
Neutral loss *m/z*	-87.032	Not detected	Not detected	-60.0211	Not detected	Not detected
Polar head *m/z*	Not detected	152.9958	140.0118, 196.038	168.0431, 224.0693	227.0326	241.0119

by CE-TOFMS (Figure 12A). Although we noticed that some metabolites were specifically detected through either our method or conventional methods, 148 metabolites were commonly detected in both protocols by CE-TOFMS analysis. Meanwhile, the number of the detected metabolites were coincident in LC-QTOFMS analysis (Figure 12B and Table 9).

QUANTIFICATION AND STATISTICAL ANALYSIS

To examine the statistical significance of the data, we have used the web program "MetaboAnalyst" (https://www.metaboanalyst.ca). Refer to the original website for instructions.

LIMITATIONS

We provide a protocol for sequential analysis of intracellular metabolites using both CE-TOFMS and LC-QTOFMS. The detection of metabolites by MS highly depends on the actual intracellular concentration. Therefore, some metabolites could not be detected by our analysis methods.

Table 6. List of the peak *m/z* of the fatty acid spectrum

FA	*m/z*	FA	*m/z*	FA	*m/z*	FA	*m/z*	FA	*m/z*	FA	*m/z*
10:0	171.1391	14:3	221.1547	19:0	297.2799	22:3	333.2799	25:3	375.3269	28:3	417.3738
10:1	169.1234	14:4	219.1391	19:1	295.2643	22:4	331.2643	25:4	373.3112	28:4	415.3582
10:2	167.1078	14:5	217.1234	19:2	293.2486	22:5	329.2486	25:5	371.2956	28:5	413.3425
10:3	165.0921	15:0	241.2173	19:3	291.233	22:6	327.233	25:6	369.2799	28:6	411.3269
10:4	163.0765	15:1	239.2017	19:4	289.2173	22:7	325.2173	25:7	367.2643	28:7	409.3112
10:5	161.0608	15:2	237.186	19:5	287.2017	22:8	323.2017	25:8	365.2486	28:8	407.2956
11:0	185.1547	15:3	235.1704	20:0	311.2956	23:0	353.3425	26:0	395.3895	29:0	437.4364
11:1	183.1391	15:4	233.1547	20:1	309.2799	23:1	351.3269	26:1	393.3738	29:1	435.4208
11:2	181.1234	15:5	231.1391	20:2	307.2643	23:2	349.3112	26:2	391.3582	29:2	433.4051
11:3	179.1078	16:0	255.233	20:3	305.2486	23:3	347.2956	26:3	389.3425	29:3	431.3895
11:4	177.0921	16:1	253.2173	20:4	303.233	23:4	345.2799	26:4	387.3269	29:4	429.3738
11:5	175.0765	16:2	251.2017	20:5	301.2173	23:5	343.2643	26:5	385.3112	29:5	427.3582
12:0	199.1704	16:3	249.186	20:6	299.2017	23:6	341.2486	26:6	383.2956	29:6	425.3425
12:1	197.1547	16:4	247.1704	20:7	297.186	23:7	339.233	26:7	381.2799	29:7	423.3269
12:2	195.1391	16:5	245.1547	20:8	295.1704	23:8	337.2173	26:8	379.2643	29:8	421.3112
12:3	193.1234	17:0	269.2486	21:0	325.3112	24:0	367.3582	27:0	409.4051	30:0	451.4521
12:4	191.1078	17:1	267.233	21:1	323.2956	24:1	365.3425	27:1	407.3895	30:1	449.4364
12:5	189.0921	17:2	265.2173	21:2	321.2799	24:2	363.3269	27:2	405.3738	30:2	447.4208
13:0	213.186	17:3	263.2017	21:3	319.2643	24:3	361.3112	27:3	403.3582	30:3	445.4051
13:1	211.1704	17:4	261.186	21:4	317.2486	24:4	359.2956	27:4	401.3425	30:4	443.3895
13:2	209.1547	17:5	259.1704	21:5	315.233	24:5	357.2799	27:5	399.3269	30:5	441.3738
13:3	207.1391	18:0	283.2643	21:6	313.2173	24:6	355.2643	27:6	397.3112	30:6	439.3582
13:4	205.1234	18:1	281.2486	21:7	311.2017	24:7	353.2486	27:7	395.2956	30:7	437.3425
13:5	203.1078	18:2	279.233	21:8	309.186	24:8	351.233	27:8	393.2799	30:8	435.3269
14:0	227.2017	18:3	277.2173	22:0	339.3269	25:0	381.3738	28:0	423.4208		
14:1	225.186	18:4	275.2017	22:1	337.3112	25:1	379.3582	28:1	421.4051		
14:2	223.1704	18:5	273.186	22:2	335.2956	25:2	377.3425	28:2	419.3895		

Table 7. Raw data of CE-TOFMS analysis

Cation mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
Methionine sulfone	108601	1	156.654	200	127022	1	351.896	25
3-Aminopyrrolidine	173498	1.59757	853.469	20	170013	1.33845	702.501	0
1,3-Diaminopropane	10778	0.09924	88.502	20	0	0	0	0
1-Amino-1-cyclopentanecarboxylate	26405	0.24314	171.103	20	0	0	0	0
1-Aminocyclopropane-1-carboxylate	14459	0.13314	85.0193	20	0	0	0	0
1-Methyl-2-pyrrolidinone	20105	0.18513	2.9963	20	0	0	0	0
1-Methyladenosine	20614	0.18981	324.825	20	0	0	0	0
1-Methylhistamine	20445	0.18826	630.57	20	0	0	0	0
1-Methylnicotinamide	21399	0.19704	255.712	20	0	0	0	0
2,3-Diaminopropionate	10956	0.10088	101.269	20	0	0	0	0
2,4-Diaminobutyrate	13755	0.12666	138.863	20	0	0	0	0
2,4-Dimethylaniline	47158	0.43423	235.5	20	0	0	0	0
2-Aminobenzimidazole	37413	0.3445	578.137	20	0	0	0	0
2-Aminophenol	18877	0.17382	121.951	20	0	0	0	0
2′-Deoxycytidine	10193	0.09386	104.923	20	0	0	0	0
2′-Deoxyguanosine	5759	0.05303	86.8939	20	0	0	0	0
2′-Deoxyinosine	668	0.00615	2.07972	20	0	0	0	0
2-Deoxystreptamine	14482	0.13335	219.801	20	0	0	0	0
2-Guanidinobenzimidazole	30225	0.27831	507.838	20	0	0	0	0
3,3′,5-Triiodothyronine	1442	0.01328	36.9643	20	0	0	0	0
3,4-Dihydroxy-L-phenylalanine	9729	0.08958	102.245	20	0	0	0	0
3,5-Diiodo-tyrosine	9764	0.08991	273.231	20	0	0	0	0
3-Aminoisobutyrate	17797	0.16388	171.109	20	576	0.00453	6.32787	0.06918
3-Aminopropane-1,2-diol	12152	0.1119	16.9636	20	0	0	0	0
3-Aminopropionitrile	8917	0.08211	21.9698	20	0	0	0	0
3-Chloroalanine	8329	0.07669	90.1357	20	0	0	0	0
3-Hydroxyanthranilate	10862	0.10002	73.5533	20	0	0	0	0
3-Hydroxykynurenine	12661	0.11658	57.3483	20	0	0	0	0
3-Iodotyrosine	9904	0.0912	234.304	20	0	0	0	0
3-Methoxytyramine	23421	0.21566	280.935	20	0	0	0	0
3-Methyladenine	19530	0.17983	292.417	20	0	0	0	0
3-Methylguanine	13214	0.12167	167.156	20	0	0	0	0
3-Methylhistidine	16838	0.15504	240.425	20	20103	0.15826	284.879	2.55191
4-(beta-Acetylaminoethyl)imidazole	30870	0.28425	195.619	20	0	0	0	0
4-Amino-3-hydroxybutyrate	13228	0.1218	184.448	20	0	0	0	0
4-Aminosalicylate	7354	0.06772	39.0457	20	0	0	0	0
4-Hydroxymethylimidazole	17048	0.15698	126.558	20	0	0	0	0
4-Methyl-5-thiazoleethanol	21647	0.19933	339.406	20	0	0	0	0
5,6-Dimethylbenzimidazole	45301	0.41713	679.911	20	0	0	0	0
5-Aminoimidazole-4-carboxamide ribotide	5105	0.04701	73.1035	20	0	0	0	0
5-Aminolevulinate	12415	0.11432	205.518	20	0	0	0	0
5-Aminovalerate	11084	0.10206	18.0067	20	0	0	0	0
5′-Deoxyadenosine	15718	0.14473	164.557	20	0	0	0	0
5-Hydroxy-3-indoleacetate	4420	0.0407	35.601	20	0	0	0	0
5-Hydroxylysine	13068	0.12033	124.983	20	0	0	0	0
5-Hydroxytryptophan	11228	0.10339	46.5629	20	0	0	0	0
5-Methoxyindoleacetate	8887	0.08183	47.5635	20	0	0	0	0
5-Methoxytryptamine	30372	0.27967	227.324	20	0	0	0	0
5-Methyl-2′-deoxycytidine	14247	0.13119	178.75	20	0	0	0	0
5-Methylcytosine	15523	0.14294	220.563	20	0	0	0	0
5-Methyltetrahydrofolate	4311	0.0397	92.5287	20	0	0	0	0

(*Continued on next page*)

Table 7. *Continued*

Cation mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
5-Methylthioadenosine	16926	0.15585	333.545	20	2431	0.01914	42.0981	0.30699
6-Aminohexanoate	21740	0.20018	147.713	20	0	0	0	0
6-Hydroxynicotinate	5120	0.04715	21.9807	20	0	0	0	0
6-Methylaminopurine	15589	0.14354	241.896	20	0	0	0	0
7,8-Dihydrobiopterin	4881	0.04494	69.5483	20	0	0	0	0
7,8-Dihydroneopterin	1963	0.01808	27.3308	20	0	0	0	0
7-Methylguanine	15487	0.1426	216.444	20	0	0	0	0
Acetylcholine	49921	0.45967	834.034	20	2825	0.02224	59.5072	0.12096
Adenine	15634	0.14396	216.565	20	615	0.00484	8.91129	0.09075
Adenosine	12717	0.1171	251.364	20	2001	0.01575	57.4576	0.35694
Agmatine	18178	0.16738	477.298	20	0	0	0	0
Alanine	11443	0.10537	50.469	20	370188	2.91436	1547.05	67.5215
Alanylalanine	17000	0.15654	111.22	20	0	0	0	0
Allantoin	2354	0.02168	4.48819	20	0	0	0	0
Alliin	3615	0.03329	33.8627	20	0	0	0	0
alpha-Aminoadipate	12767	0.11756	146.371	20	1076	0.00847	7.29866	0.18014
alpha-Methylbenzylamine	31891	0.29365	171.795	20	0	0	0	0
Alpha-Methylserine	14402	0.13261	136.28	20	0	0	0	0
Amantadine	57580	0.5302	97.6767	20	0	0	0	0
Aniline	20174	0.18576	106.964	20	0	0	0	0
Anserine	26092	0.24026	188.35	20	0	0	0	0
Anthranilate	20379	0.18765	186.522	20	0	0	0	0
Arginine	18009	0.16583	261.222	20	148390	1.16822	2148.65	19.7641
Arginine ethyl ester	32242	0.29688	221.009	20	0	0	0	0
Argininosuccinate	5556	0.05116	40.9811	20	8174	0.06435	79.4783	3.14461
Asparagine	9880	0.09098	48.7106	20	95297	0.75024	728.28	23.4889
Aspartic acid	9737	0.08966	56.7178	20	602303	4.74172	3006.34	138.013
Asymmetric dimethylarginine	20879	0.19225	128	20	1539	0.01212	13.0368	0.15755
Benzamide	20472	0.18851	2.43975	20	0	0	0	0
Benzamidine	31390	0.28904	187.81	20	0	0	0	0
Benzimidazole	32044	0.29506	307.124	20	0	0	0	0
beta-Alanine	11662	0.10738	60.4387	20	5253	0.04136	28.1188	0.99873
beta-Alanyl-L-lysine	29346	0.27022	1041.49	20	0	0	0	0
beta-Cyanoalanine	7731	0.07119	28.0671	20	0	0	0	0
beta-Imidazolelactate	9405	0.0866	5.32701	20	0	0	0	0
Betaine	15932	0.1467	16.6544	20	16588	0.13059	22.6885	2.22545
Betaine aldehyde	1407	0.01296	8.33977	20	0	0	0	0
beta-Leucine	32862	0.30259	210.511	20	0	0	0	0
Betonicine	14416	0.13274	66.0057	20	0	0	0	0
Bis(3-aminopropyl)amine	19727	0.18165	966.533	20	0	0	0	0
Cadaverine	16846	0.15512	442.152	20	0	0	0	0
Canavanine	14488	0.13341	91.6639	20	0	0	0	0
Carnitine	20121	0.18527	357.165	20	40357	0.31772	810.484	4.28711
Carnosine	21258	0.19574	190.945	20	615	0.00484	6.92543	0.06443
Castanospermine	15122	0.13924	221.301	20	0	0	0	0
Choline	37399	0.34437	877.594	20	16452	0.12952	282.857	0.94027
Citrulline	12189	0.11224	158.623	20	5549	0.04369	48.1162	1.06079
Creatine	16319	0.15027	233.204	20	272684	2.14675	4169.08	33.9345
Creatinine	16838	0.15504	240.906	20	5035	0.03964	68.0564	0.63915
Cyclohexylamine	33542	0.30886	157.818	20	0	0	0	0
Cystathionine	12177	0.11213	31.1805	20	69246	0.54515	192.839	12.9347

(*Continued on next page*)

Table 7. *Continued*

Cation mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
Cysteamine	703	0.00647	8.04798	20	0	0	0	0
Cysteine	8469	0.07798	56.461	20	0	0	0	0
Cysteine-glutathione disulphide	8857	0.08156	97.1951	20	0	0	0	0
Cysteinylglycine	8653	0.07968	45.7525	20	0	0	0	0
Cystine	9099	0.08378	91.2353	20	0	0	0	0
Cytidine	11172	0.10287	202.615	20	1989	0.01566	44.1441	0.38338
Cytosine	11448	0.10541	127.008	20	0	0	0	0
Desthiobiotin	20640	0.19005	25.1037	20	0	0	0	0
Diethanolamine	18710	0.17228	26.9344	20	4331	0.0341	6.40602	0.49478
Dihydrouracil	2932	0.027	4.69428	20	0	0	0	0
Dopamine	14859	0.13682	60.8615	20	0	0	0	0
Ectoine	21783	0.20058	107.341	20	0	0	0	0
Epinephrine	16354	0.15059	90.6987	20	0	0	0	0
gamma-Aminobutyric acid	13401	0.1234	143.028	20	1419	0.01117	17.4098	0.21716
gamma-Butyrobetaine	25134	0.23143	441.989	20	7439	0.05856	108.096	0.63263
gamma-Glutamyl-2-aminobutyric acid	11933	0.10988	98.0031	20	0	0	0	0
gamma-Glutamylcysteine	1436	0.01322	1.41538	20	825	0.00649	1.49795	0.23971
gamma-Guanidinobutyrate	16210	0.14926	349.454	20	1245	0.0098	25.0314	0.16417
Glucosaminate	9276	0.08541	85.0534	20	0	0	0	0
Glucosamine	11549	0.10634	106.109	20	0	0	0	0
Glutamic acid	11835	0.10898	105.784	20	1208422	9.51349	6287.02	239.874
Glutamine	10202	0.09394	81.9366	20	245679	1.93415	1780.28	59.873
Glutamylglutamic acid	12251	0.11281	115.865	20	0	0	0	0
Glutathione	3572	0.03289	52.8797	20	1120472	8.82109	18047.8	267.606
Glycerophosphorylcholine	6287	0.05789	33.0457	20	216854	1.70722	1054.88	73.7257
Glycine	9770	0.08996	41.1955	20	124135	0.97727	638.233	29.8088
Glycylglycine	10872	0.10011	77.3651	20	565	0.00445	3.44619	0.11108
Glycylleucine	32795	0.30198	231.552	20	0	0	0	0
Gramine	44151	0.40654	430.63	20	0	0	0	0
Guanidinosuccinate	11615	0.10695	167.28	20	0	0	0	0
Guanidoacetate	12949	0.11923	125.667	20	0	0	0	0
Guanine	10299	0.09483	93.0404	20	0	0	0	0
Guanosine	11118	0.10237	184.505	20	0	0	0	0
Harman	53007	0.48809	295.479	20	0	0	0	0
Hexylamine	30381	0.27975	266.45	20	0	0	0	0
Histamine	20224	0.18622	573.497	20	0	0	0	0
Histidine	15383	0.14165	261.631	20	45025	0.35447	730.298	6.63555
Histidinol	20496	0.18873	536.909	20	0	0	0	0
Homocarnosine	21597	0.19887	130.888	20	0	0	0	0
Homocysteine	1044	0.00961	13.3133	20	0	0	0	0
Homocystine	2942	0.02709	60.47	20	0	0	0	0
Homoserine	16443	0.15141	170.35	20	0	0	0	0
Hydroxyproline	11239	0.10349	92.0858	20	187538	1.47642	1838.83	35.3606
Hydroxyurea	3492	0.03215	1.67798	20	0	0	0	0
Hypotaurine	6809	0.0627	22.6713	20	31111	0.24493	113.969	11.4789
Hypoxanthine	14792	0.13621	76.9304	20	1107	0.00872	6.00993	0.2611
Ibotenate	6033	0.05555	17.083	20	0	0	0	0
Imidazole-4-acetate	12844	0.11827	74.4118	20	0	0	0	0
Indole-3-acetaldehyde	1044	0.00961	5.58875	20	0	0	0	0
Indole-3-acetamide	7939	0.0731	15.8674	20	0	0	0	0
Indole-3-acetate	5232	0.04818	34.68	20	0	0	0	0

(*Continued on next page*)

Table 7. *Continued*

Cation mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
Indole-3-ethanol	7871	0.07248	39.1391	20	0	0	0	0
Inosine	7466	0.06875	47.1525	20	0	0	0	0
Isoamylamine	33916	0.3123	380.978	20	0	0	0	0
Isobutylamine	26819	0.24695	31.607	20	0	0	0	0
Isoleucine	33314	0.30676	185.196	20	137350	1.08131	1139.9	9.47564
Isonicotinamide	12017	0.11065	42.115	20	0	0	0	0
Isopropanolamine	14488	0.13341	85.3717	20	0	0	0	0
Kynurenine	16109	0.14833	85.786	20	1238	0.00975	5.92357	0.16427
L-alpha-Aminobutyric acid	16799	0.15469	138.07	20	10757	0.08469	93.3443	1.38794
Leucine	33380	0.30736	169.319	20	214336	1.68739	1745.68	14.8703
Leucyl-leucyl-tyrosine	32080	0.29539	474.017	20	0	0	0	0
Lysinamide	16388	0.1509	182.346	20	0	0	0	0
Lysine	11823	0.10887	205.965	20	40612	0.31972	615.335	5.61054
Mannosamine	12091	0.11133	124.384	20	0	0	0	0
Melamine	15002	0.13814	83.4036	20	0	0	0	0
Melatonin	18005	0.16579	51.5109	20	0	0	0	0
Metformin	27310	0.25147	419.778	20	0	0	0	0
Methionine	17761	0.16354	198.152	20	11661	0.0918	180.922	1.94774
Methionine sulfoxide	12069	0.11113	69.2051	20	1470	0.01157	6.34146	0.26034
Methionine sulfoximine	12282	0.11309	28.1354	20	0	0	0	0
Methylguanidine	22321	0.20553	32.5873	20	0	0	0	0
Muramate	12116	0.11156	47.7215	20	0	0	0	0
Muscimol	11077	0.102	76.6075	20	0	0	0	0
N,N-Dimethylglycine	13231	0.12183	95.4371	20	446	0.00351	3.54098	0.07205
N1,N12-Diacetylspermine	29576	0.27234	625.771	20	0	0	0	0
N1,N8-Diacetylspermidine	52400	0.4825	658.194	20	0	0	0	0
N1-Acetylspermidine	28357	0.26111	824.153	20	634	0.00499	28.9381	0.04779
N1-Acetylspermine	38473	0.35426	387.643	20	0	0	0	0
N6,N6,N6-Trimethyllysine	18197	0.16756	244.319	20	1930	0.01519	31.5332	0.2267
N6-Methyl-2′-deoxyadenosine	18734	0.1725	365.099	20	0	0	0	0
N8-Acetylspermidine	25084	0.23097	622.373	20	0	0	0	0
N-Acetylglucosamine	5676	0.05226	17.4114	20	0	0	0	0
N-Acetylglucosylamine	3494	0.03217	12.0228	20	0	0	0	0
N-Acetylhistidine	15373	0.14155	141.181	20	0	0	0	0
N-Acetylornithine	15627	0.14389	102.393	20	0	0	0	0
N-Acetylputrescine	25774	0.23733	193.408	20	718	0.00565	8.82581	0.05954
N-Acetylvaline	12788	0.11775	32.6724	20	0	0	0	0
N-alpha,N-alpha-Dimethylhistidine	15351	0.14135	103.186	20	0	0	0	0
N-alpha-Benzenolarginine ethylester	32623	0.30039	213.255	20	0	0	0	0
n-Butyl a-Picolinate	44829	0.41279	448.353	20	0	0	0	0
N-epsilon-Acetyllysine	17602	0.16208	108.005	20	0	0	0	0
N-gamma-Ethylglutamine	20671	0.19034	107.962	20	0	0	0	0
Nicotinamide	13247	0.12198	41.056	20	1203	0.00947	6.47141	0.19411
Nicotinamide ribotide	5763	0.05307	70.4151	20	0	0	0	0
Nicotine	22935	0.21119	227.463	20	0	0	0	0
N-Methylalanine	18147	0.1671	140.533	20	0	0	0	0
N-Methylaniline	29956	0.27584	160.399	20	0	0	0	0
N-Methylglutamate	11790	0.10856	110.076	20	0	0	0	0
N-omega-Methyltryptamine	39146	0.36046	424.37	20	0	0	0	0
Noradrenaline	8042	0.07405	78.822	20	0	0	0	0
Nornicotine	23620	0.21749	380.261	20	0	0	0	0

(*Continued on next page*)

Table 7. *Continued*

Cation mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
o-Acetylcarnitine	27117	0.24969	215.557	20	42472	0.33437	296.934	3.34777
O-Acetylserine	10105	0.09305	64.7388	20	0	0	0	0
Octopine	15561	0.14329	134.846	20	0	0	0	0
Octylamine	56494	0.5202	330.518	20	0	0	0	0
Ophthalmate	12696	0.11691	134.781	20	1413	0.01112	19.26	0.24706
Ornithine	15740	0.14493	269.324	20	18430	0.14509	276.142	2.90136
o-Succinylhomoserine	3742	0.03446	11.3009	20	0	0	0	0
Oxidized glutathione	12489	0.115	185.731	20	96056	0.75622	1128.94	12.2817
p-Aminobenzoate	11621	0.10701	129.13	20	0	0	0	0
Phenethylamine	35442	0.32635	199.17	20	0	0	0	0
Phenylalanine	25480	0.23462	238.484	20	89326	0.70323	651.882	7.81715
Phenylalanylphenylalanine	31454	0.28963	256.907	20	0	0	0	0
Phenylethanolamine	12956	0.1193	125.883	20	0	0	0	0
Phosphorylcholine	7115	0.06552	28.9157	20	228726	1.80068	729.161	68.7125
Picolinamide	13180	0.12136	27.1881	20	0	0	0	0
Pipecolate	22650	0.20856	122.136	20	0	0	0	0
Proline	19807	0.18238	130.217	20	533833	4.20268	5328.17	63.066
Proline betaine	24352	0.22423	292.34	20	0	0	0	0
Pseudopelletierine	29591	0.27247	352.246	20	0	0	0	0
Pterin	12249	0.11279	144.318	20	0	0	0	0
Purine	10621	0.0978	52.391	20	0	0	0	0
Purine riboside	10954	0.10086	50.8492	20	0	0	0	0
Putrescine(1,4-Butanediamine)	14333	0.13198	84.6245	20	3054	0.02404	10.5491	0.47538
Pyrazole	11478	0.10569	1.87126	20	0	0	0	0
Pyridoxal	13908	0.12807	129.352	20	0	0	0	0
Pyridoxamine	19958	0.18377	236.676	20	0	0	0	0
Pyridoxamine 5′-phosphate	7300	0.06722	73.1081	20	0	0	0	0
Pyridoxine	22672	0.20876	231.165	20	0	0	0	0
Riboflavin	6425	0.05916	42.9915	20	0	0	0	0
Saccharopine	12572	0.11576	92.9444	20	0	0	0	0
S-Adenosylhomocysteine	15927	0.14666	147.049	20	915	0.0072	10.8268	0.10526
S-Adenosylmethionine	17668	0.16269	312.263	20	12345	0.09719	151.91	1.47787
Sarcosine	12444	0.11458	53.9788	20	2711	0.02134	14.3894	0.46566
Sepiapterin	7019	0.06463	25	20	0	0	0	0
Serine	10808	0.09952	51.8644	20	80522	0.63392	487.298	18.6766
Serotonin	4751	0.04375	19.6046	20	0	0	0	0
S-Lactoylglutathione	3924	0.03613	81.7333	20	0	0	0	0
S-Methylmethionine	3492	0.03215	73.0662	20	0	0	0	0
Spermidine	23071	0.21244	978.018	20	4826	0.03799	206.217	0.5191
Spermine	27182	0.25029	521.695	20	314	0.00247	9.76974	0.02514
Symmetric dimethylarginine	22655	0.20861	132.312	20	1052	0.00828	10.2652	0.09925
Synephrine	22716	0.20917	249.078	20	0	0	0	0
Taurine	2378	0.0219	11.6183	20	12002	0.09449	34.1693	9.12115
Taurocyamine	3327	0.03064	4.05824	20	0	0	0	0
Tetrahydropalmatine	51163	0.47111	346.294	20	0	0	0	0
Thiamine	17299	0.15929	212.401	20	2069	0.01629	34.8808	0.25564
Thiamine monophosphate	6497	0.05982	193.347	20	0	0	0	0
Threonine	11825	0.10888	112.615	20	47394	0.37312	476.606	8.62928
Thymidine	3360	0.03094	12.3348	20	3090	0.02433	10.5246	1.96569
Thymine	4660	0.04291	9.49305	20	0	0	0	0
Thyrotropin releasing hormone	14317	0.13183	158.163	20	0	0	0	0

(*Continued on next page*)

Table 7. *Continued*

Cation mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
trans-Zeatin	19419	0.17881	271.457	20	0	0	0	0
Trientine	10055	0.09259	475.761	20	0	0	0	0
Trigonelline	14905	0.13725	137.079	20	0	0	0	0
Trimethylamine N-oxide	26592	0.24486	162.189	20	0	0	0	0
Trimethylsulfonium	29505	0.27168	118.133	20	0	0	0	0
Tropine	44524	0.40998	767.092	20	0	0	0	0
Tropinone	21766	0.20042	214.311	20	0	0	0	0
Tryptamine	22086	0.20337	215.396	20	0	0	0	0
Tryptophan	19185	0.17666	129.201	20	21711	0.17092	102.673	2.49168
Tyramine	16316	0.15024	166.355	20	0	0	0	0
Tyrosine	11805	0.1087	59.0469	20	41965	0.33038	267.52	7.13169
Uracil	2523	0.02323	3.99483	20	0	0	0	0
Urea	7112	0.06549	2.14436	20	28296	0.22276	12.9282	8.5041
Uridine	2862	0.02635	8.60262	20	0	0	0	0
Urocanate	11348	0.10449	80.2361	20	0	0	0	0
Valine	22313	0.20546	28.7308	20	161509	1.2715	287.923	15.6823
Xanthine	6181	0.05691	3.45983	20	0	0	0	0
Xanthopterin	5375	0.04949	37.2959	20	0	0	0	0
Xanthosine	6662	0.06134	28.4502	20	0	0	0	0
Anion mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
D-Camphor-10-sulfonic acid solution	2070948	1	2065.06	200	2430129	1	1170.75	25
Trimesate	693404	0.33482	432.183	20	711625	0.29283	653.071	0
(Methylthio)acetate	37823	0.01826	1.29713	20	0	0	0	0
10-Hydroxydecanoate	298043	0.14392	236.907	20	0	0	0	0
1-Aminoethylphosphonate	45662	0.02205	20.8552	20	0	0	0	0
2,3-Diphosphoglyceric acid	59401	0.02868	182.126	20	49692	0.02045	93.2925	1.72715
2,3-Pyridinedicarboxylate	78972	0.03813	74.9123	20	0	0	0	0
2,4-Dihydroxypyrimidine-5-carboxylate	84281	0.0407	10.8124	20	0	0	0	0
2,5-Dihydroxybenzoate	46524	0.02247	30.5861	20	0	0	0	0
2-Amino-3-phosphonopropionate	60901	0.02941	215.361	20	0	0	0	0
2-Aminoethylphosphonate	48516	0.02343	21.1855	20	0	0	0	0
2-Deoxyglucose 6-phosphate	51477	0.02486	62.8782	20	0	0	0	0
2′-Deoxyinosine triphosphate	46276	0.02235	29.5263	20	0	0	0	0
2-Deoxyribose 1-phosphate	70070	0.03383	69.5118	20	0	0	0	0
2-Furoate	32162	0.01553	32.2872	20	0	0	0	0
2-Hydroxy-4-methylpentanoate	199178	0.09618	238.923	20	0	0	0	0
2-Hydroxybutyrate	101140	0.04884	47.7853	20	6229	0.00256	4.25128	0.13121
2-Hydroxyglutarate	65852	0.0318	64.1486	20	2984	0.00123	6.25909	0.09143
2-Hydroxyisobutyrate	123140	0.05946	83.5754	20	0	0	0	0
2-Hydroxyoctanoate	311599	0.15046	232.919	20	0	0	0	0
2-Hydroxypentanoate	155941	0.0753	101.568	20	16184	0.00666	13.3379	0.22111
2-Hydroxyphenylacetate	130487	0.06301	17.4221	20	0	0	0	0
2-Isopropylmalate	253781	0.12254	544.999	20	0	0	0	0
2-Oxoadipate	54565	0.02635	35.9808	20	0	0	0	0
2-Oxobutyrate	27945	0.01349	5.18207	20	0	0	0	0
2-Oxoglutarate	63547	0.03068	11.8159	20	41110	0.01692	4.93097	1.45734
2-Oxoisopentanoate	103095	0.04978	64.9412	20	9235	0.0038	4.98566	0.19334
2-Oxooctanoate	190368	0.09192	171.854	20	0	0	0	0

(*Continued on next page*)

Table 7. *Continued*

Anion mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
2-Phosphoglyceric acid	62520	0.03019	102.502	20	0	0	0	0
2-Quinolinecarboxylate	45187	0.02182	80.8978	20	0	0	0	0
2-Thiopheneacetate	48338	0.02334	6.18799	20	0	0	0	0
3-(2-Hydroxyphenyl)propionate	141411	0.06828	51.6781	20	0	0	0	0
3-(4-Hydroxyphenyl)propionate	72356	0.03494	23.1443	20	0	0	0	0
3′,5′-Cyclic deoxyadenosine monophosphate	50936	0.0246	78.1506	20	0	0	0	0
3′-Adenosine monophosphate	66539	0.03213	128.891	20	0	0	0	0
3-Hydroxy-3-methylglutarate	185124	0.08939	54.195	20	9672	0.00398	5.06624	0.11131
3-Hydroxybutyrate	59006	0.02849	24.2202	20	15663	0.00645	10.7278	0.56553
3-Hydroxypropionate	32299	0.0156	10.1215	20	0	0	0	0
3-Indolebutyrate	80608	0.03892	99.2682	20	0	0	0	0
3-Indoxyl sulfate	71977	0.03476	112.595	20	0	0	0	0
3-Methylbutanoate	59278	0.02862	48.9233	20	0	0	0	0
3-Phenyllactate	142863	0.06898	118.583	20	0	0	0	0
3-Phenylpropionate	118308	0.05713	50.5161	20	0	0	0	0
3-Phosphoglyceric acid	74290	0.03587	107.282	20	95921	0.03947	141.919	2.99795
3-Ureidopropionate	42123	0.02034	23.5783	20	0	0	0	0
4-Acetylbutyrate	107067	0.0517	81.7831	20	4744	0.00195	5.59486	0.0944
4-Hydroxy-3-methoxymandelate	77445	0.0374	49.48	20	0	0	0	0
4-Hydroxymandelate	45644	0.02204	58.7715	20	0	0	0	0
4-Methyl-2-oxopentanoate	130958	0.06324	97.7738	20	19945	0.00821	38.283	0.32448
4-Methylthio-2-oxobutyrate	21159	0.01022	5.5989	20	0	0	0	0
4-Oxohexanoate	95021	0.04588	77.1872	20	0	0	0	0
4-Oxopentanoate	78455	0.03788	48.7016	20	6127	0.00252	5.11079	0.16638
4-Pyridoxate	505901	0.24428	473.924	20	0	0	0	0
5-Oxoproline	68222	0.03294	56.8235	20	92833	0.0382	197.128	2.89906
5-Thymidylic acid	85606	0.04134	141.229	20	1930	7.94E-04	7.23869	0.04618
6-Hydroxyhexanoate	93752	0.04527	56.7295	20	0	0	0	0
6-Phosphogluconate	70807	0.03419	89.3383	20	8825	0.00363	8.25329	0.26544
Acetyl Coenzyme A	21772	0.01051	104.353	20	1027	4.23E-04	8.00279	0.01558
Adenosine 3′,5′-diphosphate	58844	0.02841	261.429	20	0	0	0	0
Adenosine 5′-phosphosulfate	32757	0.01582	80.9667	20	0	0	0	0
Adenosine diphosphate	68646	0.03315	186.617	20	711463	0.29277	1896.21	23.0996
Adenosine diphosphate Glucose	42444	0.02049	104.711	20	10486	0.00431	14.541	0.52635
Adenosine diphosphate ribose	41448	0.02001	82.1456	20	0	0	0	0
Adenosine monophosphate	91830	0.04434	197.182	20	162721	0.06696	473.372	5.10403
Adenosine triphosphate	60750	0.02933	96.204	20	1652177	0.67987	1469.42	58.3457
Adenylosuccinate	34117	0.01647	230.314	20	6104	0.00251	50.7809	0.38117
Adipate	76704	0.03704	163.691	20	0	0	0	0
Allantoate	47697	0.02303	59.7751	20	0	0	0	0
Azelate	182592	0.08817	335.436	20	2213	9.11E-04	6.44836	0.02582
Barbiturate	14819	0.00716	10.5841	20	0	0	0	0
Benzoate	73783	0.03563	3.6678	20	0	0	0	0
Benzoylformate	80560	0.0389	63.6983	20	0	0	0	0
Benzylsuccinate	141552	0.06835	211.84	20	0	0	0	0
Biotin	67448	0.03257	20.1619	20	0	0	0	0
Butyrate	25497	0.01231	27.4839	20	905	3.72E-04	1.82973	0.07562
Carbamoylaspartate	74998	0.03621	45.9743	20	5590	0.0023	7.79491	0.1588
Carbamoylphosphate	1070	5.17E-04	6.34947	20	0	0	0	0
Cholate	191838	0.09263	249.495	20	0	0	0	0
cis-Aconitate	325633	0.15724	168.38	20	40407	0.01663	27.8439	0.36377

(*Continued on next page*)

Table 7. *Continued*

Anion mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
Citicoline	37125	0.01793	65.9938	20	18337	0.00755	22.4221	1.05231
Citraconate	308841	0.14913	236.434	20	0	0	0	0
Citramalate	224353	0.10833	164.582	20	0	0	0	0
Citrate	96068	0.04639	190.98	20	383373	0.15776	484.361	8.37068
Coenzyme A	25134	0.01214	122.341	20	22517	0.00927	47.6891	0.44523
Crotonate	14968	0.00723	15.4086	20	0	0	0	0
Cyclic Adenosine monophosphate	63593	0.03071	95.1069	20	0	0	0	0
Cyclic GMP	55809	0.02695	89.5522	20	0	0	0	0
Cysteate	58069	0.02804	164.153	20	0	0	0	0
Cysteine S-sulfate	44816	0.02164	127.061	20	0	0	0	0
Cysteine sulfinate	36418	0.01759	32.4626	20	0	0	0	0
Cytidine 2′,3′-cyclic phosphate	59229	0.0286	68.0731	20	0	0	0	0
Cytidine 5′-monophosphate-N-acetylneuraminic acid	37640	0.01818	103.91	20	0	0	0	0
Cytidine diphosphate	68268	0.03296	172.739	20	49584	0.0204	152.365	1.50975
Cytidine monophosphate	75213	0.03632	216.622	20	18465	0.0076	83.4524	0.53637
Cytidine triphosphate	49342	0.02383	163.07	20	140956	0.058	329.554	5.59127
Decanoate	169344	0.08177	89.9226	20	3823	0.00157	1.8815	0.0481
Deoxyadenosine diphosphate	61415	0.02966	184.94	20	2113	8.70E-04	10.4051	0.0733
Deoxyadenosine monophosphate	51835	0.02503	145.114	20	478	1.97E-04	7.7379	0.01965
Deoxyadenosine triphosphate	44385	0.02143	95.2843	20	3652	0.0015	7.20524	0.19965
Deoxycytidine diphosphate	53392	0.02578	160.244	20	1354	5.57E-04	4.11723	0.05403
Deoxycytidine monophosphate	76892	0.03713	286.781	20	1885	7.76E-04	8.53904	0.05223
Deoxycytidine triphosphate	51602	0.02492	285.659	20	2518	0.00104	11.25	0.09727
Deoxyguanosine triphosphate	36272	0.01751	47.0937	20	0	0	0	0
Deoxythymidine 5′-diphosphate	53069	0.02563	69.6549	20	6122	0.00252	8.56314	0.23138
Deoxyuridine diphosphate	156315	0.07548	221.064	20	0	0	0	0
Deoxyuridine monophosphate	67569	0.03263	53.3081	20	0	0	0	0
Deoxyuridine triphosphate	51064	0.02466	146.52	20	0	0	0	0
Digalacturonate	54730	0.02643	163.147	20	0	0	0	0
Dihydroorotate	77454	0.0374	40.9505	20	0	0	0	0
Dihydroxyacetone phosphate	45712	0.02207	32.4038	20	6879	0.00283	6.59642	0.32689
Dodecanedioate	342548	0.16541	579.985	20	0	0	0	0
Dodecanoate	221941	0.10717	57.7904	20	22826	0.00939	2.9558	0.21912
D-Ribose 5-phosphate	84895	0.04099	58.8597	20	0	0	0	0
D-Ribulose 5-phosphate	57841	0.02793	44.9202	20	9795	0.00403	9.2975	0.37759
Ethanolamine phosphate	50930	0.02459	30.7608	20	150764	0.06204	100.316	6.30672
Flavin adenine dinucleotide	19255	0.0093	48.8623	20	0	0	0	0
Folate	24874	0.01201	105.656	20	0	0	0	0
Fructose 1,6-bisphosphate	74627	0.03604	91.5152	20	42156	0.01735	41.4869	1.28207
Fructose 6-phosphate	51734	0.02498	54.2963	20	0	0	0	0
Fumarate	64820	0.0313	83.9208	20	138174	0.05686	212.544	5.97754
Galacturonate 1-phosphate	72862	0.03518	67.4762	20	0	0	0	0
Gluconate	101028	0.04878	28.4994	20	42615	0.01754	6.02435	1.08413
Glucosamine 6-phosphate	68071	0.03287	74.4815	20	0	0	0	0
Glucose 1-phosphate	124706	0.06022	117.191	20	263190	0.1083	143.04	7.07923
Glucose 6-phosphate	77594	0.03747	95.7963	20	15096	0.00621	13.8251	0.42946
Glucuronate	73587	0.03553	39.7974	20	6047	0.00249	3.35025	0.17507
Glutarate	88113	0.04255	57.3431	20	20618	0.00848	60.596	0.49852
Glyceraldehyde 3-phosphate	28939	0.01397	16.6245	20	0	0	0	0

(*Continued on next page*)

Table 7. *Continued*

Anion mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
Glycerate	76411	0.0369	3.11645	20	53766	0.02212	1.51771	1.4991
Glycerophosphate	52737	0.02547	59.8484	20	116851	0.04808	155.405	5.0182
Glycocholate	176086	0.08503	278.066	20	0	0	0	0
Glycolate	18973	0.00916	2.52179	20	12855	0.00529	1.68013	1.39033
Glyoxylate	5339	0.00258	3.39906	20	0	0	0	0
Guanosine diphosphate	54727	0.02643	178.418	20	68607	0.02823	204.14	3.09883
Guanosine diphosphate mannose	46866	0.02263	108.656	20	9214	0.00379	12.6587	0.41886
Guanosine monophosphate	60255	0.0291	172.75	20	36573	0.01505	77.5412	1.40399
Guanosine triphosphate	42205	0.02038	113.505	20	284222	0.11696	378.541	15.3741
Heptanoate	139627	0.06742	189.71	20	0	0	0	0
Hexanoate	92525	0.04468	84.1138	20	1766	7.27E-04	4.48984	0.04066
Hippurate	100824	0.04868	20.0852	20	0	0	0	0
Homovanillate	50479	0.02437	3.39325	20	0	0	0	0
Inosine 3′,5′-cyclic monophosphate	59040	0.02851	57.9412	20	0	0	0	0
Inosine diphosphate	55036	0.02658	29.8395	20	0	0	0	0
Inosine triphosphate	49074	0.0237	63.549	20	0	0	0	0
Inosinic acid	65137	0.03145	64.0893	20	36773	0.01513	55.2567	1.37916
Isethionate	63110	0.03047	65.3417	20	6869	0.00283	6.85426	0.23189
Isobutyryl Coenzyme A	147027	0.071	612.085	20	1689	6.95E-04	9.90897	0.02447
Isocitrate	85118	0.0411	58.5673	20	13972	0.00575	10.1779	0.39902
Itaconate	128657	0.06212	133.331	20	0	0	0	0
Lactate	86588	0.04181	28.017	20	3658070	1.5053	569.631	129.723
Malate	143500	0.06929	134.568	20	1009460	0.41539	816.845	19.6825
Malonate	178478	0.08618	28.9694	20	17466	0.00719	4.58842	0.20849
Malonyl Coenzyme A	13805	0.00667	110.217	20	0	0	0	0
Methanesulfonate	49717	0.02401	9.74922	20	0	0	0	0
Methyl sulfate	71608	0.03458	111.561	20	0	0	0	0
m-Hydroxybenzoate	49834	0.02406	17.3277	20	0	0	0	0
Mucate	94017	0.0454	142.286	20	0	0	0	0
N-Acetylaspartate	81707	0.03945	285.591	20	926353	0.38119	3770.95	24.1544
N-Acetyl-b-alanine	77239	0.0373	90.5429	20	0	0	0	0
N-Acetylglucosamine 1-phosphate	74280	0.03587	245.004	20	27669	0.01139	186.022	0.7936
N-Acetylglucosamine 6-phosphate	73115	0.03531	201.018	20	12038	0.00495	21.3708	0.35077
N-Acetylglutamate	107275	0.0518	300.784	20	10624	0.00437	30.7182	0.21099
N-Acetylleucine	215759	0.10418	364.376	20	0	0	0	0
N-Acetylmethionine	100753	0.04865	92.5031	20	2435	0.001	3.05062	0.05149
N-Acetylmuramate	76045	0.03672	117.118	20	0	0	0	0
N-Acetylneuraminate	80392	0.03882	152.072	20	4086	0.00168	5.90035	0.10828
N-Acetylphenylalanine	169599	0.08189	159.224	20	0	0	0	0
N-Carbamylglutamate	71725	0.03463	106.342	20	0	0	0	0
N-Formylaspartate	83114	0.04013	124.501	20	0	0	0	0
N-Formylmethionine	84512	0.04081	187.877	20	0	0	0	0
Nicotinamide adenine dinucleotide	55885	0.02699	88.6554	20	217272	0.08941	197.092	10.92
Nicotinamide adenine dinucleotide phosphate	15813	0.00764	42.2824	20	9405	0.00387	64.2259	0.50201
Nicotinate	86369	0.04171	39.8704	20	0	0	0	0
Nicotinic acid adenine dinucleotide	29846	0.01441	40.7463	20	0	0	0	0
o-Coumarate	79223	0.03825	69.1078	20	0	0	0	0
Octanoate	146675	0.07083	72.1389	20	0	0	0	0
o-Hydroxybenzoate	221295	0.10686	91.5255	20	0	0	0	0
o-Hydroxyhippurate	128342	0.06197	201.978	20	0	0	0	0

(*Continued on next page*)

Table 7. *Continued*

Anion mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
O-Phosphoserine	68495	0.03307	149.467	20	1961	8.07E-04	5.45888	0.061
Orotate	92638	0.04473	13.6058	20	0	0	0	0
Orotidine 5′-monophosphate	72484	0.035	200.19	20	0	0	0	0
P1, P4-Di(adenosine-5′) tetraphosphate	96888	0.04678	166.226	20	0	0	0	0
Pantothenate	132711	0.06408	173.175	20	110509	0.04547	84.3413	1.77407
p-Coumarate	109635	0.05294	86.3833	20	0	0	0	0
Pelargonate	172491	0.08329	82.8325	20	9401	0.00387	4.27215	0.11611
Pentanoate	51101	0.02468	48.4519	20	0	0	0	0
Phenaceturate	134193	0.0648	115.369	20	0	0	0	0
Phenyl phosphate	56293	0.02718	47.0647	20	0	0	0	0
Phenylpyruvate	73339	0.03541	25.8216	20	0	0	0	0
Phosphoenolpyruvic acid	61600	0.02974	105.785	20	11510	0.00474	33.7357	0.42175
Phosphonoacetate	53452	0.02581	36.9539	20	1906	7.84E-04	1.8862	0.07597
Phosphoribosyl pyrophosphate	45882	0.02216	90.7655	20	24868	0.01023	227.339	1.24693
Phthalate	286409	0.1383	276.676	20	784	3.23E-04	1.62549	0.00583
p-Hydroxybenzoate	71588	0.03457	22.9075	20	0	0	0	0
p-Hydroxyphenylacetate	43222	0.02087	4.80186	20	0	0	0	0
Pimelate	106521	0.05144	261.765	20	0	0	0	0
Porphobilinogen	42948	0.02074	20.1923	20	0	0	0	0
Propionate	5552	0.00268	3.32208	20	0	0	0	0
Prostaglandin E2	2831	0.00137	2.37609	20	0	0	0	0
Prostaglandin F2alpha	217072	0.10482	151.312	20	0	0	0	0
Pyrrole-2-carboxylate	40051	0.01934	73.7674	20	0	0	0	0
Pyruvate	33194	0.01603	4.368	20	103312	0.04251	11.4357	7.20015
Quinate	114540	0.05531	61.3174	20	0	0	0	0
Reduced nicotinamide adenine dinucleotide	1486	7.18E-04	6.90647	20	14309	0.00589	20.8968	20.5149
Reduced nicotinamide adenine dinucleotide phosphate	30163	0.01456	176.413	20	22035	0.00907	147.228	2.18165
Ribulose 1,5-diphosphate	46180	0.0223	26.7805	20	2257	9.29E-04	2.76081	0.10413
Saccharate	90272	0.04359	218.308	20	2591	0.00107	6.03739	0.06115
Sebacate	267701	0.12926	412.74	20	0	0	0	0
Sedoheptulose 7-phosphate	53585	0.02587	26.3591	20	6737	0.00277	10.4367	0.28071
Serine O-sulfate	65502	0.03163	139.343	20	0	0	0	0
Shikimate	60909	0.02941	34.2617	20	0	0	0	0
Sinapate	60339	0.02914	11.8988	20	0	0	0	0
Sorbitol 6-phosphate	62885	0.03037	80.2711	20	0	0	0	0
Succinate	117611	0.05679	62.8797	20	262608	0.10806	275.709	4.38362
Succinyl Coenzyme A	23334	0.01127	136.141	20	0	0	0	0
Syringate	48464	0.0234	41.5378	20	0	0	0	0
Tartrate	96613	0.04665	222.089	20	0	0	0	0
Taurocholate	149574	0.07222	305.834	20	0	0	0	0
Terephthalate	93362	0.04508	102.458	20	648	2.67E-04	2.52836	0.01479
threo-beta-methylaspartate	62719	0.03029	68.9291	20	4590604	1.88904	1545.17	155.938
Threonate	88421	0.0427	26.1921	20	171584	0.07061	26.2632	4.13429
Thymidine 5′-triphosphate	55305	0.02671	134.833	20	10800	0.00444	19.7476	0.40518
Thymidine diphosphate glucose	28764	0.01389	72.028	20	5040	0.00207	7.77126	0.3733
Tiglate	25517	0.01232	29.868	20	0	0	0	0
trans-4-Hydroxy-3-methoxycinnamate	72640	0.03508	55.4875	20	0	0	0	0
trans-Aconitate	85377	0.04123	63.6769	20	0	0	0	0
trans-Cinnamate	102622	0.04955	67.8311	20	0	0	0	0

(*Continued on next page*)

Table 7. *Continued*

Anion mode								
Sample Name	Standard sample				cell_sample			
Annotation Name	Area	Rel Area	S/N	Conc	Area	Rel Area	S/N	Conc
Trehalose 6-phosphate	43300	0.02091	83.1575	20	0	0	0	0
Undecanoate	198940	0.09606	224.463	20	0	0	0	0
Urate	44211	0.02135	52.5018	20	5025	0.00207	14.3811	0.24215
Uridine 5′-diphosphate	57808	0.02791	81.3783	20	205459	0.08455	247.283	7.57211
Uridine 5′-monophosphate	62777	0.03031	93.0759	20	90941	0.03742	107.983	3.08631
Uridine diphosphate glucose	55184	0.02665	248.603	20	156548	0.06442	260.859	6.04386
Uridine diphosphate glucuronic acid	51107	0.02468	214.066	20	544872	0.22422	3535.34	22.714
Uridine diphosphate-N-acetylglucosamine	47136	0.02276	146.291	20	1997172	0.82184	3321.34	90.2698
Uridine triphosphate	58712	0.02835	185.598	20	560888	0.23081	1148.52	18.3017
Xanthosine 5-triphosphate	52729	0.02546	85.1987	20	0	0	0	0
Xanthurenate	60797	0.02936	210.458	20	0	0	0	0
Xanthylic acid	49941	0.02412	177.905	20	1053	4.33E-04	5.96725	0.04492

We have showed the CE-TOFMS and LC-QTOFMS protocols for the Agilent's instruments. The protocol for CE and LC could be applied to the non-Agilent's instruments and MS parameters can be used to the Agilent instruments. If users have no access to the Agilent MS instruments, the parameters for MS must be optimized by users.

The CE-TOFMS analysis uses the migration time and *m/z* of the targeting metabolites. Users might encounter misidentification problem if the metabolites have the identical values of both migration time and *m/z*.

The LC-QTOFMS analysis enabled the identification of fatty acid molecular formulas. However, it could not reveal the fatty acid acyl chain structure (i.e., double bonds positions and carbon branching).

This protocol enables to screen intracellular metabolites including charged metabolites and hydrophobic metabolites. This protocol requires several minutes to collect cells. Thus, the results of quickly metabolized pathway such as glycolysis might be affected by the trypsinization steps. The cell collection steps should be changed as described in troubleshooting (Problem 9) if needed.

TROUBLESHOOTING

Problem 1

Cells could not be wholly collected in steps 6–8.

Potential solution

Use a fine-tipped pipette (e.g., micro tip) to add PBS (-) supplemented with 10% FBS to the culture dish and then pipette several times.

Problem 2

The total ion chromatogram (TIC) becomes unstable at steps 37 and 49 (Figure 13A).

Potential solution

Adjust the length of the capillary from the tip of the sprayer. In general, the capillary length from the tip is 2–3 mm (Figure 13B).

Table 8. Raw data of LC-QTOFMS analysis

Positive mode				Negative mode			
Sample Name	Cell_sample			Sample Name	Cell_sample		
Annotation Name	Area	Rel Area	S/N	Annotation Name	Area	Rel Area	S/N
Reserpine	6120085	1	2164.42	Reserpine	331200	1	213.229
AcylCarnitine(12:0)	0	0	0	FA(10:0)	0	0	0
AcylCarnitine(12:1)	0	0	0	FA(11:0)	0	0	0
AcylCarnitine(14:0)	1218639	0.19912	313.264	FA(11:1)	0	0	0
AcylCarnitine(14:1)	152179	0.02487	44.9152	FA(12:0)	0	0	0
AcylCarnitine(16:0)	1615823	0.26402	356.207	FA(12:1)	0	0	0
AcylCarnitine(16:1)	416720	0.06809	92.2423	FA(13:0)	0	0	0
AcylCarnitine(18:0)	234292	0.03828	39.3742	FA(13:1)	0	0	0
AcylCarnitine(18:1)	907532	0.14829	170.686	FA(14:0)	22330	0.06742	12.5765
AcylCarnitine(18:2)	0	0	0	FA(14:1)	0	0	0
AcylCarnitine(18:3)	0	0	0	FA(15:0)	8374	0.02528	4.14105
AcylCarnitine(20:0)	13837	0.00226	4.9607	FA(15:1)	0	0	0
AcylCarnitine(20:1)	65031	0.01063	20.6633	FA(16:0)	403443	1.21813	48.9669
AcylCarnitine(20:2)	0	0	0	FA(16:1)	14904	0.045	8.2181
AcylCarnitine(20:3)	0	0	0	FA(16:2)	0	0	0
AcylCarnitine(20:4)	0	0	0	FA(16:3)	0	0	0
AcylCarnitine(20:5)	0	0	0	FA(17:0)	14227	0.04296	5.09682
AcylCarnitine(22:0)	0	0	0	FA(17:1)	0	0	0
AcylCarnitine(22:1)	0	0	0	FA(18:0)	519706	1.56916	69.6128
AcylCarnitine(22:2)	0	0	0	FA(18:1)	119863	0.36191	34.1763
AcylCarnitine(22:3)	0	0	0	FA(18:2)	4302	0.01299	3.90072
AcylCarnitine(22:4)	0	0	0	FA(18:3)	0	0	0
AcylCarnitine(22:5)	0	0	0	FA(18:4)	0	0	0
AcylCarnitine(22:6)	0	0	0	FA(19:0)	0	0	0
AcylCarnitine(24:0)	0	0	0	FA(19:1)	0	0	0
AcylCarnitine(24:1)	0	0	0	FA(19:2)	0	0	0
AcylCarnitine(24:2)	0	0	0	FA(20:0)	10323	0.03117	5.49507
Cer(d18:1–14:0)	20100	0.00328	12.7958	FA(20:1)	45056	0.13604	33.52
Cer(d18:1–14:1)	0	0	0	FA(20:2)	0	0	0
Cer(d18:1–14:2)	0	0	0	FA(20:3)	0	0	0
Cer(d18:1–15:0)	25555	0.00418	8.19519	FA(20:4)	8796	0.02656	7.55476
Cer(d18:1–15:1)	0	0	0	FA(20:5)	0	0	0
Cer(d18:1–15:2)	0	0	0	FA(21:0)	0	0	0
Cer(d18:1–16:0)	630746	0.10306	213.087	FA(21:1)	0	0	0
Cer(d18:1–16:1)	68746	0.01123	43.4461	FA(22:0)	3827	0.01155	3.38445
Cer(d18:1–16:2)	0	0	0	FA(22:1)	15370	0.04641	18.5285
Cer(d18:1–17:0)	11618	0.0019	3.00215	FA(22:2)	0	0	0
Cer(d18:1–17:1)	0	0	0	FA(22:3)	0	0	0
Cer(d18:1–17:2)	0	0	0	FA(22:4)	0	0	0
Cer(d18:1–18:0)	79074	0.01292	17.5826	FA(22:5)	0	0	0
Cer(d18:1–18:1)	13715	0.00224	6.76465	FA(22:6)	22817	0.06889	20.4612
Cer(d18:1–18:2)	0	0	0	FA(23:0)	0	0	0
Cer(d18:1–19:0)	0	0	0	FA(23:1)	0	0	0
Cer(d18:1–19:1)	0	0	0	FA(24:0)	5629	0.017	4.90336
Cer(d18:1–19:2)	0	0	0	FA(24:1)	12226	0.03691	17.2274
Cer(d18:1–20:0)	100307	0.01639	14.6672	FA(24:2)	0	0	0
Cer(d18:1–20:1)	0	0	0	FA(24:3)	0	0	0
Cer(d18:1–20:2)	0	0	0	FA(24:4)	0	0	0
Cer(d18:1–21:0)	8129	0.00133	0.84006	FA(24:5)	0	0	0
Cer(d18:1–21:1)	0	0	0	FA(24:6)	0	0	0
Cer(d18:1–21:2)	0	0	0	PC(28:0)	3286628	9.92339	627.361

(*Continued on next page*)

Table 8. *Continued*

Positive mode				Negative mode			
Sample Name	Cell_sample			Sample Name	Cell_sample		
Annotation Name	Area	Rel Area	S/N	Annotation Name	Area	Rel Area	S/N
Cer(d18:1–22:0)	372714	0.0609	90.8663	PC(28:1)	0	0	0
Cer(d18:1–22:1)	0	0	0	PC(28:2)	0	0	0
Cer(d18:1–22:2)	0	0	0	PC(30:0)	5541847	16.7326	1207.78
Cer(d18:1–23:0)	128953	0.02107	12.4601	PC(30:1)	3557336	10.7407	18.252
Cer(d18:1–23:1)	0	0	0	PC(30:2)	0	0	0
Cer(d18:1–23:2)	0	0	0	PC(32:0)	2561638	7.73441	40.0901
Cer(d18:1–24:0)	1075292	0.1757	153.697	PC(32:1)	8670185	26.1781	311.672
Cer(d18:1–24:1)	3483162	0.56914	571.666	PC(32:2)	1918197	5.79166	118.254
Cer(d18:1–24:2)	0	0	0	PC(32:3)	0	0	0
Cer(d18:1–25:0)	52857	0.00864	4.03039	PC(32:4)	0	0	0
Cer(d18:1–25:1)	129005	0.02108	5.14433	PC(34:0)	384892	1.16211	15.1603
Cer(d18:1–25:2)	0	0	0	PC(34:1)	1.1E+07	33.509	322.334
Cer(d18:1–26:0)	311383	0.05088	31.2003	PC(34:2)	5087001	15.3593	432.004
Cer(d18:1–26:1)	2177802	0.35585	663.193	PC(34:3)	292628	0.88354	73.0115
Cer(d18:1–26:2)	0	0	0	PC(34:4)	0	0	0
Cer(d18:1–27:0)	0	0	0	PC(34:5)	0	0	0
Cer(d18:1–27:1)	0	0	0	PC(34:6)	0	0	0
Cer(d18:1–27:2)	0	0	0	PC(36:0)	0	0	0
Cer(d18:1–28:0)	0	0	0	PC(36:1)	3611332	10.9038	193.295
Cer(d18:1–28:1)	0	0	0	PC(36:2)	7351836	22.1976	259.496
Cer(d18:1–28:2)	0	0	0	PC(36:3)	1368420	4.1317	186.256
Cer(d18:1–29:0)	0	0	0	PC(36:4)	577624	1.74403	279.514
Cer(d18:1–29:1)	0	0	0	PC(36:5)	155709	0.47014	38.2774
Cer(d18:1–29:2)	0	0	0	PC(36:6)	0	0	0
Cer(d18:1–30:0)	0	0	0	PC(36:7)	0	0	0
Cer(d18:1–30:1)	0	0	0	PC(38:0)	0	0	0
Cer(d18:1–30:2)	0	0	0	PC(38:1)	0	0	0
Cer(d18:1–31:0)	0	0	0	PC(38:2)	2422092	7.31308	128.321
Cer(d18:1–31:1)	0	0	0	PC(38:3)	404543	1.22145	29.6536
Cer(d18:1–31:2)	0	0	0	PC(38:4)	238593	0.72039	14.0597
Cer(d18:1–32:0)	0	0	0	PC(38:5)	728947	2.20093	106.451
Cer(d18:1–32:1)	0	0	0	PC(38:6)	127387	0.38462	6.83106
Cer(d18:1–32:2)	0	0	0	PC(38:7)	0	0	0
Cho-ester(14:0)	0	0	0	PC(40:0)	0	0	0
Cho-ester(14:1)	0	0	0	PC(40:1)	0	0	0
Cho-ester(16:0)	0	0	0	PC(40:2)	0	0	0
Cho-ester(16:1)	0	0	0	PC(40:3)	0	0	0
Cho-ester(18:0)	0	0	0	PC(40:4)	0	0	0
Cho-ester(18:1)	67426	0.01102	15.1505	PC(40:5)	0	0	0
Cho-ester(18:2)	25593	0.00418	10.9646	PC(40:6)	15358	0.04637	3.91674
Cho-ester(18:3)	0	0	0	PC(40:7)	128500	0.38798	12.9371
Cho-ester(20:0)	0	0	0	PC(40:8)	0	0	0
Cho-ester(20:1)	0	0	0	PC(42:0)	0	0	0
Cho-ester(20:2)	0	0	0	PC(42:1)	0	0	0
Cho-ester(20:3)	7348	0.0012	3.46509	PC(42:2)	0	0	0
Cho-ester(20:4)	13215	0.00216	9.52257	PC(42:3)	0	0	0
Cho-ester(20:5)	0	0	0	PC(42:4)	0	0	0
Cho-ester(22:0)	0	0	0	PC(42:5)	0	0	0
Cho-ester(22:1)	0	0	0	PC(42:6)	0	0	0
Cho-ester(22:2)	0	0	0	PC(42:7)	0	0	0
Cho-ester(22:3)	0	0	0	PC(42:8)	0	0	0

(*Continued on next page*)

Table 8. *Continued*

Positive mode				Negative mode			
Sample Name	Cell_sample			Sample Name	Cell_sample		
Annotation Name	Area	Rel Area	S/N	Annotation Name	Area	Rel Area	S/N
Cho-ester(22:4)	0	0	0	PC(44:0)	0	0	0
Cho-ester(22:5)	0	0	0	PC(44:1)	0	0	0
Cho-ester(22:6)	9888	0.00162	3.276	PC(44:2)	0	0	0
DG(28:0)	0	0	0	PC(44:3)	0	0	0
DG(28:1)	0	0	0	PC(44:4)	0	0	0
DG(30:0)	0	0	0	PC(44:5)	0	0	0
DG(30:1)	0	0	0	PC(44:6)	0	0	0
DG(32:0)	0	0	0	PC(44:7)	0	0	0
DG(32:1)	49917	0.00816	17.6131	PC(44:8)	0	0	0
DG(32:2)	0	0	0	PE(28:0)	0	0	0
DG(34:0)	0	0	0	PE(28:1)	0	0	0
DG(34:1)	331910	0.05423	48.6002	PE(28:2)	0	0	0
DG(34:2)	0	0	0	PE(30:0)	0	0	0
DG(34:3)	0	0	0	PE(30:1)	0	0	0
DG(36:0)	0	0	0	PE(30:2)	0	0	0
DG(36:1)	24279	0.00397	7.24028	PE(32:0)	0	0	0
DG(36:2)	52901	0.00864	11.9197	PE(32:1)	1567834	4.7338	1287.29
DG(36:3)	0	0	0	PE(32:2)	432850	1.30691	236.674
DG(36:4)	0	0	0	PE(32:3)	0	0	0
DG(36:5)	0	0	0	PE(32:4)	0	0	0
LPC(14:0)	840201	0.13729	235.651	PE(34:0)	0	0	0
LPC(14:1)	0	0	0	PE(34:1)	4659241	14.0678	535.549
LPC(16:0)	1.2E+07	1.9008	1762.4	PE(34:2)	5545212	16.7428	853.716
LPC(16:1)	1023904	0.1673	209.961	PE(34:3)	109513	0.33066	55.5831
LPC(18:0)	7725366	1.2623	127.09	PE(34:4)	0	0	0
LPC(18:1)	6258590	1.02263	800.274	PE(34:5)	0	0	0
LPC(18:2)	103411	0.0169	17.4787	PE(34:6)	0	0	0
LPC(18:3)	0	0	0	PE(36:0)	0	0	0
LPC(20:0)	132090	0.02158	11.246	PE(36:1)	8394277	25.345	306.544
LPC(20:1)	796226	0.1301	217.41	PE(36:2)	1E+07	30.6059	619.872
LPC(20:2)	70399	0.0115	4.3341	PE(36:3)	1407766	4.2505	135.186
LPC(20:3)	69212	0.01131	5.8979	PE(36:4)	1336561	4.03551	303.049
LPC(20:4)	159178	0.02601	33.2824	PE(36:5)	169454	0.51164	22.1604
LPC(20:5)	0	0	0	PE(36:6)	0	0	0
LPC(22:0)	32996	0.00539	15.8616	PE(36:7)	0	0	0
LPC(22:1)	89163	0.01457	23.1135	PE(38:0)	0	0	0
LPC(22:2)	16432	0.00268	5.74761	PE(38:1)	0	0	0
LPC(22:3)	0	0	0	PE(38:2)	3826429	11.5532	142.242
LPC(22:4)	0	0	0	PE(38:3)	1650332	4.98289	44.1084
LPC(22:5)	44447	0.00726	7.89694	PE(38:4)	5320398	16.064	893.481
LPC(22:6)	67649	0.01105	19.7264	PE(38:5)	2791047	8.42707	283.288
LPE(14:0)	0	0	0	PE(38:6)	415205	1.25364	11.1696
LPE(14:1)	0	0	0	PE(38:7)	3660507	11.0523	175.735
LPE(16:0)	584945	0.09558	131.644	PE(40:0)	0	0	0
LPE(16:1)	143188	0.0234	28.2896	PE(40:1)	0	0	0
LPE(18:0)	1425202	0.23287	106.86	PE(40:2)	0	0	0
LPE(18:1)	1262151	0.20623	265.643	PE(40:3)	0	0	0
LPE(18:2)	0	0	0	PE(40:4)	684763	2.06752	29.2355
LPE(18:3)	0	0	0	PE(40:5)	1856052	5.60402	210.146
LPE(20:0)	0	0	0	PE(40:6)	708171	2.1382	13.5679
LPE(20:1)	0	0	0	PE(40:7)	935896	2.82577	43.0027

(*Continued on next page*)

Table 8. *Continued*

Positive mode				Negative mode			
Sample Name	Cell_sample			Sample Name	Cell_sample		
Annotation Name	Area	Rel Area	S/N	Annotation Name	Area	Rel Area	S/N
LPE(20:2)	0	0	0	PE(40:8)	0	0	0
LPE(20:3)	0	0	0	PE(42:0)	0	0	0
LPE(20:4)	49625	0.00811	11.626	PE(42:1)	0	0	0
LPE(20:5)	0	0	0	PE(42:2)	0	0	0
LPE(22:0)	0	0	0	PE(42:3)	0	0	0
LPE(22:1)	0	0	0	PE(42:4)	0	0	0
LPE(22:2)	0	0	0	PE(42:5)	0	0	0
LPE(22:3)	0	0	0	PE(42:6)	0	0	0
LPE(22:4)	0	0	0	PE(42:7)	0	0	0
LPE(22:5)	0	0	0	PE(42:8)	0	0	0
LPE(22:6)	0	0	0	PE(44:0)	0	0	0
MG(14:0)	0	0	0	PE(44:1)	0	0	0
MG(14:1)	0	0	0	PE(44:2)	0	0	0
MG(16:0)	0	0	0	PE(44:3)	0	0	0
MG(16:1)	0	0	0	PE(44:4)	0	0	0
MG(18:0)	0	0	0	PE(44:5)	0	0	0
MG(18:1)	0	0	0	PE(44:6)	0	0	0
MG(18:2)	0	0	0	PE(44:7)	0	0	0
MG(18:3)	0	0	0	PE(44:8)	0	0	0
MG(20:0)	0	0	0	PG(28:0)	0	0	0
MG(20:1)	0	0	0	PG(28:1)	0	0	0
MG(20:2)	0	0	0	PG(28:2)	0	0	0
MG(20:3)	0	0	0	PG(30:0)	0	0	0
MG(20:4)	0	0	0	PG(30:1)	0	0	0
MG(20:5)	0	0	0	PG(30:2)	0	0	0
MG(22:0)	0	0	0	PG(32:0)	0	0	0
MG(22:1)	0	0	0	PG(32:1)	236329	0.71355	5.19275
MG(22:2)	0	0	0	PG(32:2)	116942	0.35309	35.4174
MG(22:3)	0	0	0	PG(32:3)	0	0	0
MG(22:4)	0	0	0	PG(32:4)	0	0	0
MG(22:5)	0	0	0	PG(34:0)	0	0	0
MG(22:6)	0	0	0	PG(34:1)	1020812	3.08216	35.7689
Phthalate	0	0	0	PG(34:2)	570594	1.72281	21.1714
SM(d30:0)	0	0	0	PG(34:3)	0	0	0
SM(d30:1)	103001	0.01683	52.6662	PG(34:4)	0	0	0
SM(d30:2)	0	0	0	PG(34:5)	0	0	0
SM(d32:0)	0	0	0	PG(34:6)	0	0	0
SM(d32:1)	1E+07	1.64065	1797.31	PG(36:0)	0	0	0
SM(d32:2)	0	0	0	PG(36:1)	314418	0.94933	36.8302
SM(d34:0)	1.7E+07	2.74437	203.744	PG(36:2)	964341	2.91166	107.634
SM(d34:1)	7.2E+07	11.7957	1534.69	PG(36:3)	105513	0.31858	36.4915
SM(d34:2)	7290306	1.19121	755.405	PG(36:4)	50864	0.15357	9.11893
SM(d34:3)	0	0	0	PG(36:5)	0	0	0
SM(d34:4)	0	0	0	PG(36:6)	0	0	0
SM(d36:0)	0	0	0	PG(36:7)	0	0	0
SM(d36:1)	6160659	1.00663	140.628	PG(38:0)	0	0	0
SM(d36:2)	0	0	0	PG(38:1)	0	0	0
SM(d36:3)	0	0	0	PG(38:2)	0	0	0
SM(d36:4)	0	0	0	PG(38:3)	0	0	0
SM(d36:5)	0	0	0	PG(38:4)	0	0	0
SM(d36:6)	0	0	0	PG(38:5)	73411	0.22165	13.6037

(*Continued on next page*)

Table 8. *Continued*

Positive mode				Negative mode			
Sample Name	Cell_sample			Sample Name	Cell_sample		
Annotation Name	Area	Rel Area	S/N	Annotation Name	Area	Rel Area	S/N
SM(d38:0)	0	0	0	PG(38:6)	0	0	0
SM(d38:1)	7766104	1.26895	686.813	PG(38:7)	0	0	0
SM(d38:2)	0	0	0	PG(40:0)	0	0	0
SM(d38:3)	0	0	0	PG(40:1)	0	0	0
SM(d38:4)	0	0	0	PG(40:2)	0	0	0
SM(d38:5)	0	0	0	PG(40:3)	0	0	0
SM(d38:6)	0	0	0	PG(40:4)	0	0	0
SM(d38:7)	0	0	0	PG(40:5)	0	0	0
SM(d40:0)	0	0	0	PG(40:6)	0	0	0
SM(d40:1)	1.5E+07	2.48524	174.299	PG(40:7)	308322	0.93092	123.239
SM(d40:2)	9595623	1.56789	1365.03	PG(40:8)	35424	0.10696	15.5472
SM(d40:5)	0	0	0	PG(42:0)	0	0	0
SM(d40:6)	0	0	0	PG(42:1)	0	0	0
SM(d40:7)	0	0	0	PG(42:2)	0	0	0
TG(40:0)	39423	0.00644	12.78	PG(42:3)	0	0	0
TG(40:1)	12297	0.00201	3.61233	PG(42:4)	0	0	0
TG(40:2)	0	0	0	PG(42:5)	0	0	0
TG(42:0)	334509	0.05466	65.7328	PG(42:6)	0	0	0
TG(42:1)	142476	0.02328	54.222	PG(42:7)	0	0	0
TG(42:2)	23069	0.00377	6.02978	PG(42:8)	0	0	0
TG(44:0)	1694576	0.27689	59.2724	PG(44:0)	0	0	0
TG(44:1)	1645315	0.26884	277.347	PG(44:1)	0	0	0
TG(44:2)	153268	0.02504	46.1945	PG(44:2)	0	0	0
TG(44:3)	0	0	0	PG(44:3)	0	0	0
TG(46:0)	3725825	0.60879	17.3381	PG(44:4)	0	0	0
TG(46:1)	8667112	1.41618	354.602	PG(44:5)	0	0	0
TG(46:2)	1658235	0.27095	869.965	PG(44:6)	0	0	0
TG(46:3)	75936	0.01241	11.9065	PG(44:7)	0	0	0
TG(46:4)	0	0	0	PG(44:8)	0	0	0
TG(48:0)	4373254	0.71457	7.60083	PI(28:0)	0	0	0
TG(48:1)	2.2E+07	3.67379	6546.26	PI(28:1)	0	0	0
TG(48:2)	1.1E+07	1.79186	4413.6	PI(28:2)	0	0	0
TG(48:3)	735182	0.12013	104.453	PI(30:0)	0	0	0
TG(48:4)	31098	0.00508	1.79825	PI(30:1)	0	0	0
TG(50:0)	2009808	0.3284	5.7706	PI(30:2)	0	0	0
TG(50:1)	2.9E+07	4.77398	22.7422	PI(32:0)	438339	1.32349	6.9875
TG(50:2)	4E+07	6.47126	5593.84	PI(32:1)	2869478	8.66388	293.268
TG(50:3)	5516897	0.90144	1097.92	PI(32:2)	537822	1.62386	257.714
TG(50:4)	263042	0.04298	7.93143	PI(32:3)	0	0	0
TG(50:5)	65756	0.01074	4.92656	PI(32:4)	0	0	0
TG(52:0)	448996	0.07336	48.2629	PI(34:0)	404014	1.21985	75.7419
TG(52:1)	1.2E+07	2.00095	9.14742	PI(34:1)	6048782	18.2632	863.087
TG(52:2)	6.4E+07	10.458	6558.56	PI(34:2)	2569867	7.75926	700.804
TG(52:3)	1.9E+07	3.07815	3689.8	PI(34:3)	0	0	0
TG(52:4)	1314595	0.2148	96.2114	PI(34:4)	0	0	0
TG(52:5)	375766	0.0614	43.9892	PI(34:5)	0	0	0
TG(52:6)	250844	0.04099	34.2242	PI(34:6)	0	0	0
TG(54:1)	2233081	0.36488	87.016	PI(36:0)	0	0	0
TG(54:2)	2.5E+07	4.15271	153.465	PI(36:1)	5188815	15.6667	83.0638
TG(54:3)	2.5E+07	4.08451	3527.55	PI(36:2)	4078819	12.3153	777.072
TG(54:4)	2980341	0.48698	245.766	PI(36:3)	668154	2.01737	214.158

(*Continued on next page*)

Table 8. *Continued*

Positive mode				Negative mode			
Sample Name	Cell_sample			Sample Name	Cell_sample		
Annotation Name	Area	Rel Area	S/N	Annotation Name	Area	Rel Area	S/N
TG(54:5)	1167113	0.1907	31.1022	PI(36:4)	1689015	5.09968	902.815
TG(54:6)	819716	0.13394	79.6556	PI(36:5)	202068	0.61011	129.743
TG(54:7)	293225	0.04791	19.7509	PI(36:6)	0	0	0
TG(56:1)	403918	0.066	73.5573	PI(36:7)	0	0	0
TG(56:2)	4602105	0.75197	507.493	PI(38:0)	0	0	0
TG(56:3)	9809032	1.60276	484.317	PI(38:1)	0	0	0
TG(56:4)	1619560	0.26463	38.6498	PI(38:2)	1911444	5.77127	611.37
TG(56:5)	1281543	0.2094	70.1104	PI(38:3)	2272930	6.86271	423.674
TG(56:6)	1801026	0.29428	288.427	PI(38:4)	5464269	16.4984	1000.1
TG(56:7)	708025	0.11569	56.9611	PI(38:5)	2716954	8.20336	1082.97
TG(56:8)	170139	0.0278	43.3769	PI(38:6)	655540	1.97929	271.06
TG(56:9)	0	0	0	PI(38:7)	0	0	0
TG(58:10)	0	0	0	PI(40:0)	0	0	0
TG(58:7)	706485	0.11544	23.7444	PI(40:1)	0	0	0
TG(58:8)	349762	0.05715	30.9797	PI(40:2)	0	0	0
TG(58:9)	36263	0.00593	2.68883	PI(40:3)	0	0	0
				PI(40:4)	730078	2.20434	90.5259
				PI(40:5)	725579	2.19076	192.861
				PI(40:6)	513874	1.55155	206.793
				PI(40:7)	0	0	0
				PI(40:8)	0	0	0
				PI(42:0)	0	0	0
				PI(42:1)	0	0	0
				PI(42:2)	0	0	0
				PI(42:3)	0	0	0
				PI(42:4)	0	0	0
				PI(42:5)	0	0	0
				PI(42:6)	0	0	0
				PI(42:7)	0	0	0
				PI(42:8)	0	0	0
				PI(44:0)	0	0	0
				PI(44:1)	0	0	0
				PI(44:2)	0	0	0
				PI(44:3)	0	0	0
				PI(44:4)	0	0	0
				PI(44:5)	0	0	0
				PI(44:6)	0	0	0
				PI(44:7)	0	0	0
				PI(44:8)	0	0	0
				PS(28:0)	0	0	0
				PS(28:1)	0	0	0
				PS(28:2)	0	0	0
				PS(30:0)	83356	0.25168	34.7328
				PS(30:1)	0	0	0
				PS(30:2)	0	0	0
				PS(32:0)	0	0	0
				PS(32:1)	1441913	4.3536	391.649
				PS(32:2)	0	0	0
				PS(32:3)	0	0	0
				PS(32:4)	0	0	0
				PS(34:0)	0	0	0

(*Continued on next page*)

Table 8. ***Continued***

Positive mode				Negative mode			
Sample Name	Cell_sample			Sample Name	Cell_sample		
Annotation Name	Area	Rel Area	S/N	Annotation Name	Area	Rel Area	S/N
				PS(34:1)	3660367	11.0518	175.735
				PS(34:2)	1533010	4.62865	301.558
				PS(34:3)	0	0	0
				PS(34:4)	0	0	0
				PS(34:5)	0	0	0
				PS(34:6)	0	0	0
				PS(36:0)	0	0	0
				PS(36:1)	1.1E+07	33.5258	695.254
				PS(36:2)	2128756	6.4274	115.509
				PS(36:3)	175388	0.52955	30.0263
				PS(36:4)	48298	0.14583	6.29707
				PS(36:5)	0	0	0
				PS(36:6)	0	0	0
				PS(36:7)	0	0	0
				PS(38:0)	0	0	0
				PS(38:1)	615239	1.85761	10.9937
				PS(38:2)	1169508	3.53112	198.388
				PS(38:3)	526351	1.58922	57.6168
				PS(38:4)	229737	0.69365	9.7968
				PS(38:5)	79305	0.23945	13.0384
				PS(38:6)	45316	0.13682	13.1199
				PS(38:7)	0	0	0
				PS(40:0)	0	0	0
				PS(40:1)	0	0	0
				PS(40:2)	281379	0.84957	24.1054
				PS(40:3)	50466	0.15237	2.41169
				PS(40:4)	508341	1.53485	86.5848
				PS(40:5)	635314	1.91822	119.418
				PS(40:6)	652582	1.97036	22.91
				PS(40:7)	128398	0.38768	11.8395
				PS(40:8)	0	0	0
				PS(42:0)	0	0	0
				PS(42:1)	0	0	0
				PS(42:2)	233770	0.70583	10.1473
				PS(42:3)	0	0	0
				PS(42:4)	0	0	0
				PS(42:5)	0	0	0
				PS(42:6)	0	0	0
				PS(42:7)	0	0	0
				PS(42:8)	0	0	0
				PS(44:0)	0	0	0
				PS(44:1)	0	0	0
				PS(44:2)	0	0	0
				PS(44:3)	0	0	0
				PS(44:4)	0	0	0
				PS(44:5)	0	0	0
				PS(44:6)	0	0	0
				PS(44:7)	0	0	0
				PS(44:8)	0	0	0

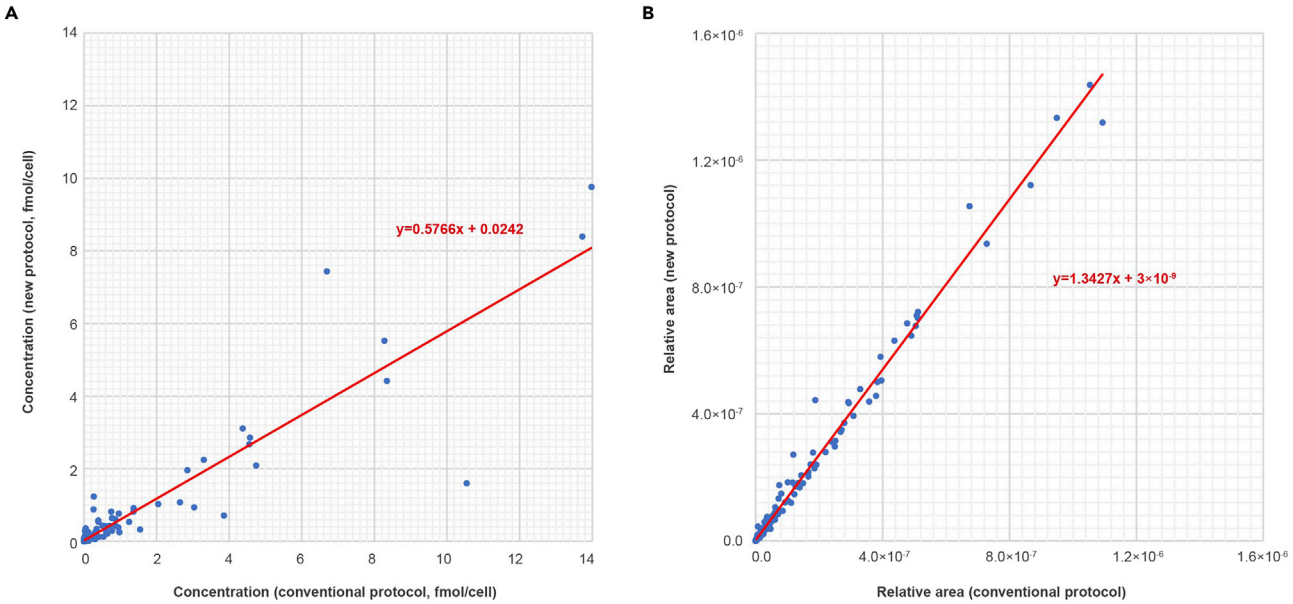


Figure 12. Comparative analysis of the metabolome data

(A and B) Comparative analysis of the data from a new protocol and a conventional protocol in CE-TOFMS (A) or LC-QTOFMS (B) analysis. Each dot indicates the average value of concentration (A) or relative area (B) from 9 biological replicates from three independent experiments.

Problem 3

The current value does not show a constant pattern at steps 39 or 51 as shown in Figure 13C.

Potential solution

There are two possible causes:

- The capillary is scratched or cracked. In this case, replace the capillary with a new one.
- The ion concentration is too high in the sample. In this case, dilute the sample for analysis.

Problem 4

The current value gradually decreases through the run at steps 39 or 51.

Table 9. Summary of CE-TOFMS and LC-QTOFMS data

	CE-TOFMS		LC-QTOFMS	
	Conventional protocol	New protocol	Conventional protocol	New protocol
Number of detections	153	160	218	218
Number of original detections	5	12	0	0
Original detection metabolites	o-Hydroxybenzoate	Hypoxanthine	N/A	N/A
	Fructose 6-phosphate	Citrulline		
	Adenosine diphosphate ribose	Cytidine		
	Pyridoxine	Adenylosuccinate		
	Heptanoate	gamma-Glutamylcysteine		
		Adenosine		
		Deoxythymidine 5′-diphosphate		
		Deoxyadenosine diphosphate		
		Deoxycytidine diphosphate		
		Acetyl Coenzyme A		
		Isobutyryl Coenzyme A		
		Deoxyadenosine monophosphate		

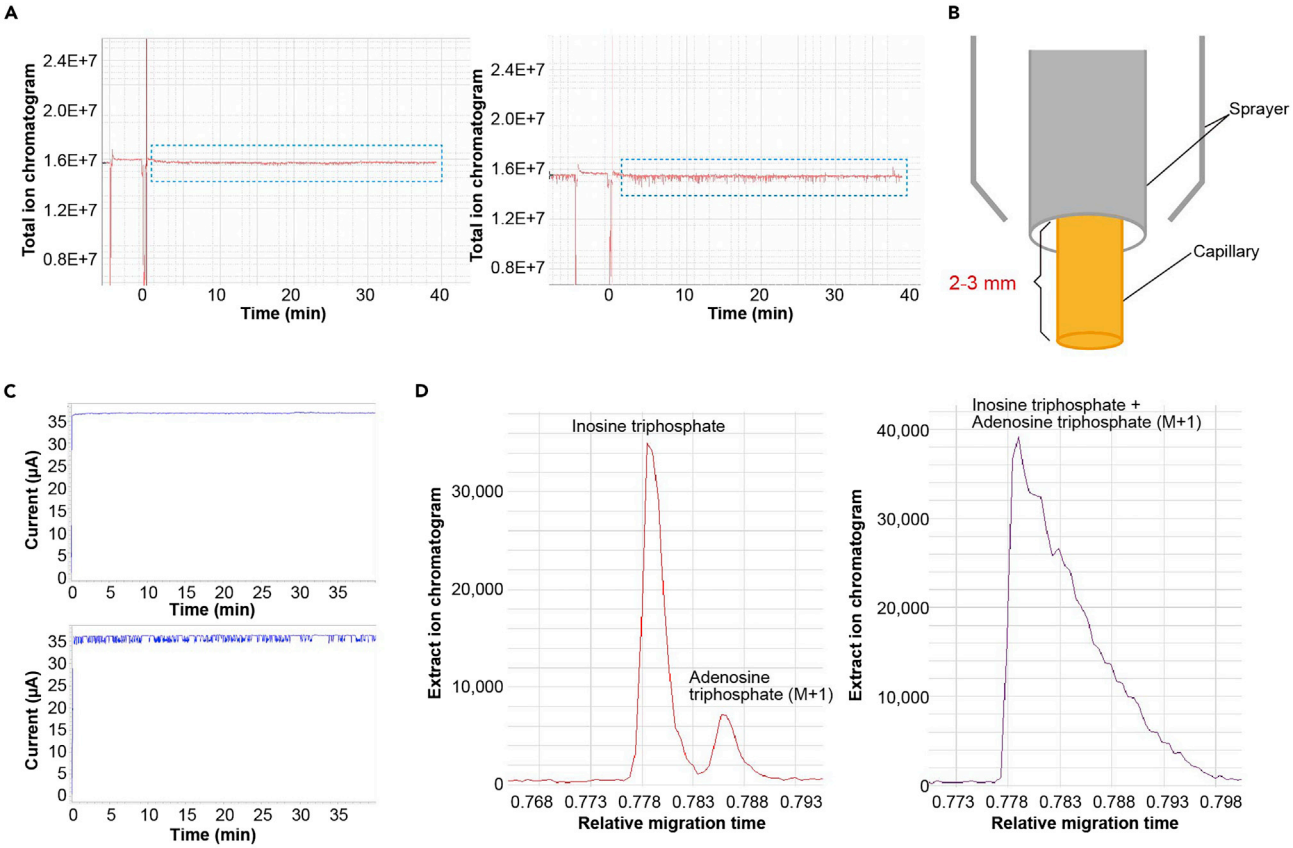


Figure 13. Troubleshooting

(A) Example of a total ion chromatogram (TIC). Ideal TIC (left) and unstable TIC (right).

(B) Cartoon representation of capillary position.

(C) Example of a current graph. The stable current pattern (top) and the unstable current pattern (bottom).

(D) Example of the signal peak. The signal peaks of inosine triphosphate (ITP) and adenosine triphosphate (M+1) (ATP M+1) are separated (left) and are not separated (right).

Potential solution

Protein absorption in the capillary may be involved. Replace the capillary with a new one.

Problem 5

In the anion analysis mode in CE-TOFMS, the shape of the chromatogram peak is poor (step 58).

Potential solution

PBS contamination in the sample is the cause of this problem. Completely remove the supernatant in step 12.

Problem 6

The signals from the isobaric metabolites are not separated in CE-MS analysis as shown in Figure 13D (step 58).

Potential solution

We have provided the list of the isobars that require attention (Table 10). Dilution of the analytes will improve the signal separation.

Table 10. The list of the isobars that may need to be confirmed in data analysis

Mode	Compound name (isotope)	*m/z*	Relative migration time
Cation	Diethanolamine	106.0863	0.647
	gamma-Aminobutyric acid (M+2)	106.0773	0.651
Cation	Homoserine	120.0655	0.848
	Valine (M+2)	120.0930	0.845
Anion	Nicotinate	122.0248	0.875
	Benzoate (M+1)	122.0329	0.877
Anion	4-Methyl-2-oxopentanoate	129.0557	0.899
	5-Oxoproline (M+1)	129.0387	0.901
Anion	Quinate	191.0561	1.030
	N-Acetylmethionine (M+1)	191.0577	1.049
Anion	Phenaceturate	192.0666	1.033
	Quinate (M+1)	192.0595	1.030
Anion	4-Hydroxy-3-methoxymandelate	197.0455	1.039
	Gluconate (M+2)	197.0577	1.043
Anion	Sorbitol 6-phosphate	261.0381	0.866
	Fructose 6-phosphate (M+2)	262.0325	0.867
Anion	Inosine triphosphate (ITP)	506.9725	0.779
	Adenosine triphosphate (ATP) (M+1)	506.9918	0.786

Problem 7

In step 64, the sample forms two layers instead of one.

Potential solution

Add an appropriate amount of methanol and vortex, and centrifuge again.

Problem 8

The column pressure is higher than 50 bar from the start at step 80.

Potential solution

This problem is caused by a clogged column. Pump 2-propanol through the column for several hours. If this problem is not solved, use a new column.

Problem 9

The concentrations of metabolites that is catabolized fast such as glycolytic intermediates are not reproducible among experiments.

Potential solution

Metabolic enzymes are active in the steps of cell collection (steps 1–17). Collect cells by keeping cells below 4°C. If the problem is not solved, the steps 1–17 should be replaced with the alternative protocol as describe below.

- Culture cells until 80% confluent in a 10 cm dish with extra dishes for cell counting.
- Discard cell culture medium by aspiration.
- Gently pour 7 mL of ice-cold 5% mannitol solution onto the dish.
- Gently swell the dish.
- Discard mannitol solution by aspiration.
- Rince the cells with mannitol solution twice.
- Add 1 mL of ice-cold extraction methanol with internal reference metabolites onto the dish.
- Incubate the dish at 25°C for 10 min.
- Transfer the 1 mL of extraction methanol into 1.5 mL tube and store the sample tubes at −80°C until use.

- Trypsinize and collect cells from the extra dishes for cell counting.
- Count the cell number with trypan blue staining.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yasuhiro Saito (ysaito@ttck.keio.ac.jp).

Materials availability

This study did not generate any unique reagents.

Data and code availability

We provide set-up files for CE-TOFMS and LC-QTOFMS in supplemental information (Data S1, S2, S3, S4, S5, S6, S7, S8, S9, S10, S11, and S12). Also, we provide the raw data of CE-TOFMS and LC-QTOFMS in Tables 7 and 8.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101531.

ACKNOWLEDGMENTS

We thank Dr. Masaru Tomita for supporting us on this project. This work is supported by funding from the Shonai Industrial Promotion Center (T.I. and Y.S.) and research funds from the Yamagata Prefectural Government and the City of Tsuruoka. The research of Y.S. is supported by a Grant-in-Aid for Scientific Research (C) JSPS KAKENHI grant number 20K07620, The Naito Foundation, The Princess Takamatsu Cancer Research Fund, The Kobayashi Foundation for Cancer Research, The Takeda Science Foundation, and The Sumitomo Foundation.

AUTHOR CONTRIBUTIONS

R.K., K.I., T.S., T.I., and Y.S. designed and interpreted experiments and cowrote the paper. R.K. and T.I. performed CE-TOFMS and LC-QTOFMS analyses.

DECLARATION OF INTERESTS

R.K. is an employee of Infinity lab Co., Ltd., and T.I. is a president of the Infinity lab Co., Ltd.

REFERENCES

Adam, J., Yang, M., Bauerschmidt, C., Kitagawa, M., O'Flaherty, L., Maheswaran, P., Özkan, G., Sahgal, N., Baban, D., Kato, K., et al. (2013). A role for cytosolic fumarate hydratase in urea cycle metabolism and renal neoplasia. Cell Rep. *3*, 1440–1448. https://doi.org/10.1016/j.celrep.2013.04.006.

Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can J Biochem Physiol *37*, 911–917. https://doi.org/10.1139/o59-099.

Calderón-Santiago, M., Priego-Capote, F., Galache-Osuna, J.G., and Luque de Castro, M.D. (2014). Analysis of serum phospholipid profiles by liquid chromatography-tandem mass spectrometry in high resolution mode for evaluation of atherosclerotic patients. J. Chromatogr. A *1371*, 154–162. https://doi.org/10.1016/j.chroma.2014.10.052.

Hanahan, D. (2022). Hallmarks of cancer: new dimensions. Cancer Discov. *12*, 31–46. https://doi.org/10.1158/2159-8290.Cd-21-1059.

Hirayama, A., Igarashi, K., Tomita, M., and Soga, T. (2014). Development of quantitative method for determination of γ-glutamyl peptides by capillary electrophoresis tandem mass spectrometry: an efficient approach avoiding matrix effect. J. Chromatogr. A *1369*, 161–169. https://doi.org/10.1016/j.chroma.2014.10.007.

Hirayama, A., Kami, K., Sugimoto, M., Sugawara, M., Toki, N., Onozuka, H., Kinoshita, T., Saito, N., Ochiai, A., Tomita, M., et al. (2009). Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. Cancer Res. *69*, 4918–4925. https://doi.org/10.1158/0008-5472.Can-08-4806.

Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., Ojima, Y., Tanaka, K., Tanaka, S., Aoshima, K., et al. (2010). MassBank: a public repository for sharing mass spectral data for life sciences. J. Mass Spectrom. *45*, 703–714. https://doi.org/10.1002/jms.1777.

Soga, T., Baran, R., Suematsu, M., Ueno, Y., Ikeda, S., Sakurakawa, T., Kakazu, Y., Ishikawa, T., Robert, M., Nishioka, T., and Tomita, M. (2006). Differential metabolomics reveals ophthalmic acid as an oxidative stress biomarker indicating hepatic glutathione consumption. J. Biol. Chem. *281*, 16768–16776. https://doi.org/10.1074/jbc.M601876200.

Soga, T., Igarashi, K., Ito, C., Mizobuchi, K., Zimmermann, H.P., and Tomita, M. (2009). Metabolomic profiling of anionic metabolites by capillary electrophoresis mass spectrometry. Anal. Chem. *81*, 6165–6174. https://doi.org/10.1021/ac900675k.

Soga, T., Ohashi, Y., Ueno, Y., Naraoka, H., Tomita, M., and Nishioka, T. (2003). Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. J. Proteome Res. *2*, 488–494. https://doi.org/10.1021/pr034020m.

Spickett, C.M., and Pitt, A.R. (2015). Oxidative lipidomics coming of age: advances in analysis

of oxidized phospholipids in physiology and pathology. Antioxidants Redox Signal. *22*, 1646–1666. https://doi.org/10.1089/ars.2014.6098.

Sugimoto, M., Kawakami, M., Robert, M., Soga, T., and Tomita, M. (2012). Bioinformatics tools for mass spectroscopy-based metabolomic data processing and analysis. Curr. Bioinf. *7*, 96–108. https://doi.org/10.2174/157489312799304431.

Sugimoto, M., Wong, D.T., Hirayama, A., Soga, T., and Tomita, M. (2010). Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. Metabolomics *6*, 78–95. https://doi.org/10.1007/s11306-009-0178-y.

Taguchi, R., Houjou, T., Nakanishi, H., Yamazaki, T., Ishida, M., Imagawa, M., and Shimizu, T. (2005). Focused lipidomics by tandem mass spectrometry. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. *823*, 26–36. https://doi.org/10.1016/j.jchromb.2005.06.005.

Taguchi, R., and Ishikawa, M. (2010). Precise and global identification of phospholipid molecular species by an Orbitrap mass spectrometer and automated search engine Lipid Search. J. Chromatogr. A *1217*, 4229–4239. https://doi.org/10.1016/j.chroma.2010.04.034.