Protocol

Measurement of Metabolome Samples Using Liquid Chromatography–Mass Spectrometry, Data Acquisition, and Processing

Tomáš Pluskal^{1,2,3} and Mitsuhiro Yanagida¹

¹*G0 Cell Unit, Okinawa Institute of Science and Technology Graduate University (OIST), Onna-son, Kunigami, Okinawa 904-0495, Japan*

We present a protocol for metabolomic sample measurement using hydrophilic interaction chromatography (HILIC) combined with high-resolution Orbitrap mass spectrometry (MS). We also introduce a raw data processing method using MZmine 2 software, and include a list of 111 metabolite peaks (with their *m/z* values and retention times) previously identified in metabolome samples using this method.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

Reagents

Acetonitrile (HPLC-grade or better) (100%)

Ammonium carbonate (10 mM, adjusted to pH 9.3 with ammonium hydroxide) (HPLC-grade or better)

H₂O (distilled, HPLC-grade or better)

Mixture of pure metabolite standards

Samples from Protocol: **Preparation of Intracellular Metabolite Extracts from Liquid *Schizosaccharomyces pombe* Cultures** (Pluskal et al. 2016)

Equipment

High-performance liquid chromatography (HPLC) system coupled to a high-resolution mass spectrometry (MS) detector (e.g., Orbitrap [Thermo Fisher Scientific])

The MS detector should be equipped with an electrospray ionization (ESI) interface.

MZmine 2 software (version 2.10) (Pluskal et al. 2010a)

Other software packages or tools can be used for feature detection, such as XCMS2/XCMS Online (Benton et al. 2008; Tautenhahn et al. 2012), MAVEN (Melamud et al. 2010), or mzMatch (Scheltema et al. 2011).

SeQuant ZIC-pHILIC HPLC column (150×2.1 mm, 5-μm particle size) (Merck Millipore)

²Present address: Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142.

³Correspondence: pluskal@wi.mit.edu

From the Fission Yeast collection, edited by Iain M. Hagan, Antony M. Carr, Agnes Grallert, and Paul Nurse.

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Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot091561

METHOD

1. Determine the optimal conditions for the MS detector and ESI interface by analyzing pure metabolite standards by direct infusion. Use both negative and positive ionization modes. Operate the MS detector in a full scan mode with a 100–1000 m/z scan range.

For the Orbitrap detector, these initial parameters can be used: spray voltage 3 kV (negative ESI) or 3.5 kV (positive ESI), capillary temperature 300°C, sheath gas (N_2) flow rate 35 arbitrary units, auxiliary gas (N_2) 5 arbitrary units.

2. Develop an HPLC method using the ZIC-pHILIC column. Use 100% acetonitrile as mobile phase A and 10 mM ammonium carbonate (pH 9.3) as mobile phase B. Set the elution profile to gradient elution from 80% A (20% B) to 20% A (80% B) in 30 min at the flow rate of 100 μ L/min. Follow the gradient with a washing phase (e.g., 5 min of 80% B flow) and equilibration phase (e.g., 10 min of 20% B flow).

A mixture of several pure metabolites dissolved in H_2O in $\sim 10 \mu M$ to 1 mM concentrations (depending on the metabolite) can be used as a standard to optimize and routinely check the LC conditions (Fig. 1).

3. Analyze your samples using liquid chromatography–mass spectrometry (LC–MS).

The sample injection volume can be initially set to 1 μ L. Larger injection volumes are possible, but peak shapes may become distorted by injecting a large volume. Each sample should be injected at least twice, once for analysis in negative ionization mode and once in positive ionization mode. If the MS detector allows

FIGURE 1. Raw LC–MS data in negative (upper panel) and positive (bottom panel) ESI modes. Data were obtained using a 1- μ L injection of a mixture of metabolites. All were dissolved in H_2O at various concentrations. A total molar amount of each injected metabolite is indicated for each peak.

tandem MS (MS/MS) analysis, we recommend collecting fragmentation spectra for as many peaks as possible, e.g., using automatic data-dependent precursor selection.

4. Process the raw MS data using MZmine 2 software and export the resulting peak areas into a table.

We present an example MZmine 2 workflow in Table 1.

TABLE 1. Workflow and parameters for processing of Orbitrap data using MZmine 2 software

1) Project/set preferences	<i>m/z</i> value format	4 decimals
	Retention time value format	1 decimals
	Intensity format	1 decimal with exponent
2) Raw data methods/raw data file import	Filename	Select all raw data files
3) Raw data methods/peak detection/mass detection	Mass detector	Exact mass
	Noise level	1E3 for negative mode data, 5E3 for positive mode data
	MS level	1
	Mass list name	Mass list MS1
4) Raw data methods/peak detection/FTMS shoulder peaks filter	Mass list	Mass list MS1
	Mass resolution	60000
	Peak model function	Lorentzian extended
	Suffix	filtered
	Mass list	Mass list MS1 filtered
5) Raw data methods/peak detection/chromatogram builder	Min time span (min)	0.1
	Min height	1E4
	<i>m/z</i> tolerance	0.001 <i>m/z</i> or 10 ppm
	Suffix	Chromatograms
	Suffix	Smoothed
6) Peak list methods/peak detection/smoothing	Filter width	5
7) Peak list methods/peak detection/chromatogram deconvolution	Suffix	Deconvoluted
	Peak resolver	Local minimum search
	Chromatographic threshold	85%
	Search minimum in RT range	0.1
	Minimum relative height	1%
	Minimum absolute height	1E4
	Min ratio of peak top/edge	2
	Peak duration range (min)	0–20
	Name suffix	Deisotoped
	<i>m/z</i> tolerance	0.02 <i>m/z</i> or 20 ppm
8) Peak list methods/isotopes/isotopic peaks grouper	Retention time tolerance	0.1 (absolute)
	Monotonic shape	No
	Maximum charge	2
	Representative isotope	Most intense
9) Peak list methods/alignment/join aligner (run separately for negative ionization mode and positive ionization mode data)	Peak list name	Positive mode or negative mode
	<i>m/z</i> tolerance	0.001 <i>m/z</i> or 5 ppm
	Weight for <i>m/z</i>	10
	Retention time tolerance	0.5 (Absolute)
	Weight for RT	10
	Require same charge state	No
	Require same ID	No
	Compare isotope pattern	No
10) Peak list methods/gap filling/same RT and <i>m/z</i> range gap filler	Name suffix	Gap-filled
	<i>m/z</i> tolerance	0.001 <i>m/z</i> or 5 ppm
11) Peak list methods/normalization/standard compound normalizer	Name suffix	Normalized
	Normalization type	Weighted contribution of all standards
	Peak measurement type	Peak area
	<i>m/z</i> vs. RT balance	10
	Standard compounds	PIPES (301.053 <i>m/z</i> at 12.1 min in negative mode, 303.067 <i>m/z</i> at 12.1 min in positive mode) and HEPES (237.091 <i>m/z</i> at 8.4 min in negative mode, 239.105 <i>m/z</i> at 8.4 min in positive mode)
12) Peak list methods/export/import/export to CSV file	Filename	Choose final file name
	Field separator	,
	Export common elements	All fields
	Export identity elements	All fields
	Export data file elements	All fields

TABLE 2. List of 111 metabolites identified in *S. pombe* extracts with their *m/z* values and retention times. All retention times in this table were verified by analyzing pure standards. (Adapted from Pluskal et al. 2010b, with permission of The Royal Society of Chemistry.)

	Ionization mode	<i>m/z</i> (theoretical)	Retention time (min)	Name	Ion formula
Nucleotides	neg	346.0558	10.7	AMP	C ₁₀ H ₁₃ N ₅ O ₇ P ⁻
	neg	426.0221	12.3	ADP	C ₁₀ H ₁₄ N ₅ O ₁₀ P ₂ ⁻
	neg	505.9885	13.5	ATP	C ₁₀ H ₁₅ N ₅ O ₁₃ P ₃ ⁻
	neg	328.0452	5.8	3'-5'-cAMP	C ₁₀ H ₁₁ N ₅ O ₆ P ⁻
	neg	322.0446	12.8	CMP	C ₉ H ₁₃ N ₃ O ₈ P ⁻
	neg	402.0109	14.2	CDP	C ₉ H ₁₄ N ₃ O ₁₁ P ₂ ⁻
	neg	481.9772	15.3	CTP	C ₉ H ₁₅ N ₃ O ₁₄ P ₃ ⁻
	neg	362.0507	13.6	GMP	C ₁₀ H ₁₃ N ₅ O ₈ P ⁻
	neg	442.0171	14.9	GDP	C ₁₀ H ₁₄ N ₅ O ₁₁ P ₂ ⁻
	neg	521.9834	16.1	GTP	C ₁₀ H ₁₅ N ₅ O ₁₄ P ₃ ⁻
	neg	323.0286	12.1	UMP	C ₉ H ₁₂ N ₂ O ₉ P ⁻
	neg	402.9949	13.6	UDP	C ₉ H ₁₃ N ₂ O ₁₂ P ₂ ⁻
	neg	482.9613	14.8	UTP	C ₉ H ₁₄ N ₂ O ₁₅ P ₃ ⁻
	pos	786.1644	8.5	FAD	C ₂₇ H ₃₄ N ₉ O ₁₅ P ₂ ⁺
	neg	347.0398	12.5	IMP	C ₁₀ H ₁₂ N ₄ O ₈ P ⁻
	pos	664.1164	11.4	NAD ⁺	C ₂₁ H ₂₈ N ₇ O ₁₄ P ₂ ⁺
	pos	666.1320	10.5	NADH	C ₂₁ H ₃₀ N ₇ O ₁₄ P ₂ ⁺
	pos	744.0827	14.1	NADP ⁺	C ₂₁ H ₂₉ N ₇ O ₁₇ P ₃ ⁺
	pos	746.0984	14.7	NADPH	C ₂₁ H ₃₁ N ₇ O ₁₇ P ₃ ⁺
Nucleosides, nucleobases	pos	136.0618	6.2	Adenine	C ₅ H ₆ N ₅ ⁺
	pos	268.1040	5.9	Adenosine	C ₁₀ H ₁₄ N ₅ O ₄ ⁺
	neg	282.0844	9.2	Guanosine	C ₁₀ H ₁₂ N ₅ O ₅ ⁻
	neg	267.0735	7.5	Inosine	C ₁₀ H ₁₁ N ₄ O ₅ ⁻
	neg	111.0200	5.3	Uracil	C ₄ H ₃ N ₂ O ₂ ⁻
	pos	244.0928	8.6	Cytidine	C ₉ H ₁₄ N ₃ O ₅ ⁺
	neg	151.0261	7.6	Xanthine	C ₅ H ₃ N ₄ O ₂ ⁻
	neg	283.0684	8.3	Xanthosine	C ₁₀ H ₁₁ N ₄ O ₆ ⁻
	pos	282.1197	10.8	1-Methyladenosine	C ₁₁ H ₁₆ N ₅ O ₄ ⁺
	pos	298.1146	6.4	1-Methylguanosine	C ₁₁ H ₁₆ N ₅ O ₅ ⁺
Methylated nucleosides	pos	312.1302	5.6	Dimethyl-guanosine	C ₁₂ H ₁₈ N ₅ O ₅ ⁺
	pos	768.1225	11.4	Coenzyme A	C ₂₁ H ₃₇ N ₇ O ₁₆ P ₃ S ⁺
	pos	810.1330	10.1	Acetyl-CoA	C ₂₃ H ₃₉ N ₇ O ₁₇ P ₃ S ⁺
Coenzymes	pos	912.1647	12.9	HMG-CoA	C ₂₇ H ₄₅ N ₇ O ₂₀ P ₃ S ⁺
	pos	175.1190	22.8	Arginine	C ₆ H ₁₅ N ₄ O ₂ ⁺
Amino acids	neg	131.0462	11.7	Asparagine	C ₄ H ₇ N ₂ O ₃ ⁻
	pos	134.0448	11.9	Aspartate	C ₄ H ₈ NO ₄ ⁺
	pos	176.1030	12.8	Citrulline	C ₆ H ₁₄ N ₃ O ₃ ⁺
	neg	141.0670	10.4	Ectoine	C ₆ H ₉ N ₂ O ₂ ⁻
	pos	148.0604	11.4	Glutamate	C ₅ H ₁₀ NO ₄ ⁺
	pos	147.0764	11.9	Glutamine	C ₅ H ₁₁ N ₂ O ₃ ⁺
	pos	156.0768	11.3	Histidine	C ₆ H ₁₀ N ₃ O ₂ ⁺
	neg	130.0874	8.0	Isoleucine ^a	C ₆ H ₁₂ NO ₂ ⁻
	neg	130.0874	7.5	Leucine	C ₆ H ₁₂ NO ₂ ⁻
	neg	145.0983	21.7	Lysine	C ₆ H ₁₃ N ₂ O ₂ ⁻
	pos	150.0583	8.1	Methionine	C ₅ H ₁₂ NO ₂ S ⁺
	neg	131.0826	19.5	Ornithine	C ₅ H ₁₁ N ₂ O ₂ ⁻
	pos	166.0863	6.8	Phenylalanine	C ₉ H ₁₂ NO ₂ ⁺
	pos	116.0706	9.5	Proline	C ₅ H ₁₀ NO ₂ ⁺
	neg	128.0353	6.5	Pyroglutamic acid	C ₅ H ₆ NO ₃ ⁻
	pos	277.1394	12.7	Saccharopine	C ₁₁ H ₂₁ N ₂ O ₆ ⁺
	pos	106.0499	12.4	Serine	C ₃ H ₈ NO ₃ ⁺
	pos	120.0655	11.0	Threonine	C ₄ H ₁₀ NO ₃ ⁺
	pos	205.0972	8.2	Tryptophan	C ₁₁ H ₁₃ N ₂ O ₂ ⁺
	pos	182.0812	9.5	Tyrosine	C ₉ H ₁₂ NO ₃ ⁺
	pos	118.0863	9.2	Valine	C ₅ H ₁₂ NO ₂ ⁺
	neg	116.0717	11.8	5-Aminovalerate	C ₅ H ₁₀ NO ₂ ⁻
	pos	385.1289	10.6	S-Adenosyl-homocysteine	C ₁₄ H ₂₁ N ₆ O ₅ S ⁺
	pos	399.1445	13.8	S-Adenosyl-methionine	C ₁₅ H ₂₃ N ₆ O ₅ S ⁺

(continued)

TABLE 2. Continued

	Ionization mode	m/z (theoretical)	Retention time (min)	Name	Ion formula
Methylated amino acids	pos	118.0863	8.0	Betaine (trimethyl-glycine) ^b	C ₅ H ₁₂ NO ₂ ⁺
	pos	203.1503	18.9	Dimethyl-arginine	C ₈ H ₁₉ N ₄ O ₂ ⁺
	pos	175.1441	18.4	Dimethyl-lysine	C ₈ H ₁₉ N ₂ O ₂ ⁺
	pos	189.1598	19.8	Trimethyl-lysine	C ₉ H ₂₁ N ₂ O ₂ ⁺
	pos	146.1176	3.3	Leucine methyl ester	C ₇ H ₁₆ NO ₂ ⁺
	pos	162.0761	7.7	Glutamate methyl ester	C ₆ H ₁₂ NO ₄ ⁺
	pos	144.1019	7.9	Dimethyl-proline	C ₇ H ₁₄ NO ₂ ⁺
Acetylated amino acids	pos	189.1234	12.2	N ₂ -acetyl-lysine	C ₈ H ₁₇ N ₂ O ₃ ⁺
	pos	189.1234	9.8	N ₆ -acetyl-lysine	C ₈ H ₁₇ N ₂ O ₃ ⁺
	pos	175.1077	12.4	N-acetyl-ornithine	C ₇ H ₁₅ N ₂ O ₃ ⁺
	pos	217.1295	11.9	N-acetyl-arginine	C ₈ H ₁₇ N ₄ O ₃ ⁺
	pos	190.0710	11.0	N-acetyl-glutamate	C ₇ H ₁₂ NO ₅ ⁺
	pos	198.0873	6.6	N-acetyl-histidine	C ₈ H ₁₂ N ₃ O ₃ ⁺
Redox compounds	pos	230.0958	11.5	Ergothioneine	C ₉ H ₁₆ N ₃ O ₂ S ⁺
	pos	308.0911	11.2	Glutathione (GSH)	C ₁₀ H ₁₈ N ₃ O ₆ S ⁺
	pos	613.1592	14.6	Oxidized glutathione (GSSG)	C ₂₀ H ₃₃ N ₆ O ₁₂ S ₂ ⁺
Sugars	neg	237.0616	10.7	2-Keto-3-deoxyoctonate	C ₈ H ₁₃ O ₈ ⁻
	neg	307.0842	7.5	Ribulose (boron complex) ^c	C ₁₀ H ₁₆ BO ₁₀ ⁻
	neg	341.1089	13.0	Trehalose	C ₁₂ H ₂₁ O ₁₁
Sugar phosphates	neg	229.0119	12.5	Ribose-5-phosphate	C ₅ H ₁₀ O ₈ P ⁻
	neg	388.9445	16.3	5-Phosphoribose-1-diphosphate (PRPP)	C ₅ H ₁₂ O ₁₄ P ₃ ⁻
Nucleotide-sugars	neg	259.0224	13.8	Glucose-6-phosphate ^d	C ₆ H ₁₂ O ₉ P ⁻
	neg	259.0224	13.0	Fructose-6-phosphate	C ₆ H ₁₂ O ₉ P ⁻
	neg	338.9888	15.5	Fructose-1-6-diphosphate	C ₆ H ₁₃ O ₁₂ P ₂ ⁻
	neg	606.0743	12.2	UDP-acetyl-glucosamine	C ₁₇ H ₂₆ N ₃ O ₁₇ P ₂ ⁻
	neg	565.0477	13.3	UDP-glucose	C ₁₅ H ₂₃ N ₂ O ₁₇ P ₂ ⁻
Ferrichrome	neg	604.0699	15.1	GDP-glucose	C ₁₆ H ₂₄ N ₅ O ₁₆ P ₂ ⁻
	neg	686.3115	5.5	Deferri-ferrichrome	C ₂₇ H ₄₄ N ₉ O ₁₂
Vitamins	neg	739.2230	4.4	Ferrichrome	C ₂₇ H ₄₁ FeN ₉ O ₁₂
	pos	245.0954	5.2	Biotin	C ₁₀ H ₁₇ N ₂ O ₃ S ⁺
	pos	220.1179	5.1	Pantothenate	C ₉ H ₁₈ NO ₅ ⁺
	pos	170.0812	5.2	Pyridoxine	C ₈ H ₁₂ NO ₃ ⁺
TCA cycle	pos	377.1456	5.5	Riboflavin	C ₁₇ H ₂₁ N ₄ O ₆ ⁺
	neg	145.0142	12.1	2-Oxoglutarate	C ₅ H ₅ O ₅ ⁻
	neg	191.0197	15.0	Citrate	C ₆ H ₇ O ₇ ⁻
	neg	115.0037	12.7	Fumarate	C ₄ H ₃ O ₄ ⁻
Others	neg	133.0142	12.8	Malate	C ₄ H ₅ O ₅ ⁻
	pos	162.0761	11.9	2-Aminoadipate	C ₆ H ₁₂ NO ₄ ⁺
	pos	146.0924	12.0	4-Guanidinobutyrate	C ₅ H ₁₂ N ₃ O ₂ ⁺
	neg	275.0174	14.8	6-Phospho-gluconate	C ₆ H ₁₂ O ₁₀ P ⁻
	neg	337.0555	12.5	AICAR	C ₉ H ₁₄ N ₄ O ₈ P ⁻
	neg	151.0612	9.4	Arabitol	C ₅ H ₁₁ O ₅ ⁻
	neg	147.0299	12.0	Citramalate	C ₅ H ₇ O ₅ ⁻
	neg	131.0350	11.0	Glutaric acid	C ₅ H ₇ O ₄ ⁻
	neg	171.0064	11.9	Glycerol-phosphate	C ₃ H ₈ O ₆ P ⁻
	pos	258.1101	11.7	Glycerophosphocholine	C ₈ H ₂₁ NO ₆ P ⁺
	pos	123.0553	4.7	Nicotinamide	C ₆ H ₇ N ₂ O ⁺
	pos	290.1347	10.3	Ophthalmic acid	C ₁₁ H ₂₀ N ₃ O ₆ ⁺
	neg	166.9751	14.6	Phosphoenolpyruvate	C ₃ H ₄ O ₆ P ⁻
	neg	184.9857	14.0	Phospho-glyceric acid	C ₃ H ₆ O ₇ P ⁻
	neg	165.0193	10.1	Phthalic acid	C ₈ H ₅ O ₄ ⁻
	neg	175.0612	8.5	Propylmalate	C ₇ H ₁₁ O ₅ ⁻
	neg	191.0561	9.0	Quinic acid	C ₇ H ₁₁ O ₆ ⁻

^aPeaks of leucine and isoleucine often overlap, thus requiring extra caution during data processing.^bNote that betaine and valine have the same chemical formula and, therefore, mass.^cPentose sugars analyzed by the ZIC-pHILIC LC-MS method often appear in complexes with boron atoms. The source of the boron is unknown.^dNote that glucose-6-phosphate and fructose-6-phosphate have the same mass and close retention times, so their peaks may overlap.

5. Identify metabolites found in the processed data table using m/z values and MS/MS fragmentation.

For ZIC-pHILIC LC-based detection, reference metabolites are listed in Table 2.

The identification step can also be performed directly within the MZmine 2 software, using the “Custom database search” module.

See Troubleshooting.

TROUBLESHOOTING

Problem (Step 5): Some ions are retained in the ZIC-pHILIC column, leading to the appearance of various adducts in the MS data (e.g., ATP+Mg²⁺) which compromise reproducibility.

Solution: The column can be briefly washed with high-concentration salt (e.g., 500 mM ammonium carbonate) after each sequence, or even after each sample run. Be certain that the column outlet is directed to waste during washing, not to the MS detector! For a more thorough regeneration of the initial column conditions, consult the product manual.

DISCUSSION

Development of chromatographic and mass spectrometric protocols often requires a considerable investment of labor, even for experienced operators. The protocol detailed here for measurement of metabolomic samples is based on liquid chromatography separation on a HILIC column and detection with an Orbitrap detector, and is only one of many possible LC–MS setups that can be applied. Other separation and detection methods, such as gas chromatography MS (GC–MS), capillary electrophoresis MS (CE–MS), or nuclear magnetic resonance (NMR), are available. Our recommendation of the current protocol is based on its application and validation in multiple published studies, and its good long-term stability and reproducibility. It is optimal for measurement of highly polar metabolites such as ATP. In contrast, it is not suitable for the separation of sugar isomers (such as glucose/fructose/mannose, etc.).

ACKNOWLEDGMENTS

We thank Dr. Koji Nagao for his assistance at the initial stage of LC–MS method development, Dr. Yoshiya Oda for suggesting the HPLC method based on ZIC-pHILIC and ammonium carbonate, and Dr. Steven D. Aird for editing the manuscript. We acknowledge the generous funding and support of Okinawa Institute of Science and Technology Promotion Corporation and Okinawa Institute of Science and Technology Graduate University.

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Cold Spring Harbor Protocols

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Cold Spring Harb Protoc; doi: 10.1101/pdb.prot091561

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