

Oct 02, 2024

JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Polar HILIC-Z

DOI

<https://dx.doi.org/10.17504/protocols.io.kxygxydwkl8j/v1>

Katherine Louie¹, Suzanne Kosina², Thomas Harwood¹, Meghana Faltane¹, Marie Lynde¹, Benjamin Bowen^{1,2}, Trent Northen^{1,2}

¹Lawrence Berkeley National Laboratory, Joint Genome Institute, Berkeley, CA, United States;

²Lawrence Berkeley National Laboratory, Environmental Genomics and Systems Biology Division, Berkeley, CA, United States

JGI/LBNL Metabolomics ...



Katherine B. Louie

Lawrence Berkeley National Laboratory, Joint Genome Institut...

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.kxygxydwkl8j/v1>

Protocol Citation: Katherine Louie, Suzanne Kosina, Thomas Harwood, Meghana Faltane, Marie Lynde, Benjamin Bowen, Trent Northen 2024. JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Polar HILIC-Z. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.kxygxydwkl8j/v1>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: September 26, 2024

Last Modified: October 02, 2024

Protocol Integer ID: 108480

Keywords: metabolomics, JGI, HILIC, LBNL, Joint Genome Institute, LC-MS, Thermo Orbitrap, Berkeley Lab, Environmental Metabolite Atlas, jgi metabolomic, lbnl metabolomic, metabolomic, mass spectrometry dataset, consistent outcomes across mass spectrometry dataset, confident annotation of metabolite, environmental metabolite atlas, thermo orbitrap mass spectrometer with esi source, polar metabolite, primary metabolite, metabolite, thermo orbitrap mass spectrometer, chromatography, ms esi method, using normal phase chromatography, normal phase chromatography, thousands of compound standard, fragmentation spectra

Funders Acknowledgements:

The work conducted by the U.S. Department of Energy Joint Genome Institute (<https://ror.org/04xm1d337>), a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy

Grant ID: Contract No. DE-AC02-05CH11231

The work conducted by ENIGMA – Ecosystems and Networks Integrated with Genes and Molecular Assemblies

(<http://enigma.lbl.gov>), a Science Focus Area Program at Lawrence Berkeley National Laboratory is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research and used resources of the National Energy Research Scientific Computing Center, a Department of Energy Office of Science User Facility

Grant ID: Contract No. DE-AC02-05CH11231

Abstract

This protocol describes the standard LC-MS/MS ESI method developed at Lawrence Berkeley National Laboratory (LBNL) by JGI Metabolomics and EGSB's Northern Lab to analyze polar metabolites (e.g. amino acids, nucleic acids, sugars, organic acids, primary metabolites, etc.) using normal phase chromatography (HILIC-Z) coupled to a Thermo Orbitrap Mass Spectrometer with ESI source. This robust method of detection is easily reproduced and adapted onto similar LC-MS/MS systems to achieve consistent outcomes across mass spectrometry datasets, foster inter-lab and inter-experiment comparability, and enable effective data integration and analysis. Since its inception in 2018, this method has been used to analyze tens of thousands of experimental samples, with many of these datasets publicly available in the MassIVE data repository^{1,2}. This method has also been used to run thousands of compound standards to collect characteristic retention time, *m/z* and fragmentation spectra. This forms the basis of Berkeley Lab's Environmental Metabolite Atlas (EMA), a database used for confident annotation of metabolites and publicly available through GNPS/GNPS2³.

Overview

- 1
- This protocol describes the standard LC-MS/MS ESI method developed at Lawrence Berkeley National Laboratory (LBNL) by JGI Metabolomics and EGSB's Northern Lab to analyze polar metabolites (e.g. amino acids, nucleic acids, sugars, organic acids, primary metabolites, etc.) using normal phase chromatography (HILIC-Z) coupled to a Thermo Orbitrap Mass Spectrometer with ESI source.

Instrumentation / Equipment

2

Mass Spectrometry Instrumentation

	A	B	C	D
	Mass Spectrometer	Source / Probe	ESI needle (calibration)	ESI needle (running samples)
	Thermo Q Exactive HF Orbitrap (QE-HF)	Thermo Ion Max API Source w/ H-ESI II probe	32G Metal Needle High Flow (OPTON-53010, Thermo)	32G Metal Needle High Flow (OPTON-53010, Thermo)
	Thermo Q Exactive Orbitrap (QE)	Thermo Ion Max API Source w/ H-ESI II probe	32G Metal Needle High Flow (OPTON-53010, Thermo)	32G Metal Needle High Flow (OPTON-53010, Thermo)
	Thermo Orbitrap Exploris 120 (Exp120)	Thermo OptaMax NG™ API source w/ H-ESI probe	35G Metal Needle Low Flow, 50 um ID (OPTON-30139)	32G Metal Needle High Flow, 100 um ID (OPTON-30694)
	Thermo Orbitrap IQ-X Tribrid (IQX)	Thermo OptaMax NG™ API source w/ H-ESI probe	35G Metal Needle Low Flow, 50 um ID (OPTON-30139)	32G Metal Needle High Flow, 100 um ID (OPTON-30694)

Table 1. Mass spectrometer and source.

Note: Other Thermo Orbitrap mass spectrometers may also be used (e.g. ID_X, Exploris 240, Astral, etc) with appropriate source, ESI needle and modified acquisition parameters.

3

UHPLC System

A	B	C	D
Module Name	Module Type	Part Numbers	Alternative Part Numbers
1290 Infinity DAD*	Diode Array Detector	G4212A	G7115A, G7117BR
1200 Infinity Series TCC	Column Compartment	G1316A	G7116B
1290 Infinity Sampler	Autosampler	G4226A	G7167B - Multisampler w/ thermostat
1290 Infinity Thermostat	Autosampler Thermostat	G1330B	G7167B - Multisampler w/ thermostat
1290 Infinity Bin Pump	Binary Pump	G4220A	G7120A

Table 2. Agilent 1290 Infinity UHPLC Modules and type.

* Optional

Note: To use Agilent LC systems in line with Thermo Orbitrap mass spectrometers, communication / compatibility requires either installation of Chromeleon software, or a contact closure board (for QE-HF or QE) or Universal Interface Box (UIB) (for IQX, IDX, and Exploris models), as well as specialized software packages and cables. Information, manuals and installation procedures are available from Agilent and Thermo.

4 UHPLC Column information

A	B
Column name	InfinityLab Poroshell 120 HILIC-Z
Part #	#683775-924
Manufacturer	Agilent
Column chemistry	HILIC-Z, zwitterionic
Inner Diameter (ID)	2.1 mm
Length	150 mm
Particle size	2.7 µm
Pore size	100 Å
Max pressure	600 bar



A	B
pH range	2-12
Max temperature	80 °C @ pH 7; 35 °C @ pH 2-12

Table 3. UHPLC column information.

5 Chemicals / solvents

A	B
Chemicals / solvents	Product Number
acetonitrile (LC-MS grade)	AX0156, Sigma
water (LC-MS grade)	9831-03, VWR
ammonium acetate (LC-MS grade)	73594, Sigma
acetic acid (glacial, ≥99.7%)	JT9515-3, VWR
methylene-di-phosphonic acid (medronic acid)	64255, Sigma
methanol (LC-MS grade)	MX0486, Sigma

Table 4. Chemicals and solvents. These are used to prepare mobile phase and resuspend extracts. For solvents, other LC-MS grade products can also be used. For chemicals, high purity compounds of analytical grade or listed as suitable for mass spectrometry can be used.

LC-MS/MS Method Parameters

6 LIQUID CHROMATOGRAPHY

A	B
Mobile Phase A	99.8% H ₂ O and 0.2% acetic acid, w/ 5 mM ammonium acetate and 5 μM methylene-di-phosphonic acid
Mobile Phase B	99.8% 95:5 v/v ACN:H ₂ O and 0.2% acetic acid, w/ 5 mM ammonium acetate

Table 5. Mobile phase composition for HILIC. Sufficient mobile phase for all injections of a sample set are prepared prior to starting a run. To prepare Mobile Phase B, ammonium

acetate is first dissolved in water prior to adding acetonitrile and other components (for solubility).

Column

InfinityLab Poroshell 120 HILIC-Z, 2.1 × 150 mm, 2.7 μm, 100 Å (Agilent, #683775-924)

Column Temperature 40 °C

Autosampler Temperature 4 °C

	A	B	C	D	E
	Time (min)	Flow (mL/min)	%A	%B	minutes for segment
	0	0.45	0	100	
	1	0.45	0	100	1
	11	0.45	11	89	10
	15.75	0.45	30	70	4.75
	16.25	0.45	80	20	0.5
	18.5	0.45	80	20	2.25
	18.6	0.45	0	100	0.1
	21	0.45	0	100	2.4

Table 6. Mobile phase gradients for HILIC. Each segment is a linear gradient to the new mobile phase composition.

Typically, depending on the length of the lines connecting the ESI needle through to the autosampler, the first 0.3-0.8 minutes of the run does not contain signal from the injected sample (void volume). This is then followed by a large peak (solvent front) comprised of metabolites that do not retain on the column. Signals acquired during this time window are typically not used in analysis. Also, signals acquired in the time window following final isocratic elution (here, between 18.5 to 21 minutes during column re-equilibration), are also not used in analysis.

Salty samples: When analyzing samples that still have a lot of salt in the extract, diverting flow to waste for the first 1 minute (void volume containing the most salt) may help keep the ESI needle and source cleaner throughout the run and preserve data quality.

7 MASS SPECTROMETRY

Source settings

A	B	C	D	E
Parameter	QE-HF	QE	Exp120	IQX
Sheath Gas Flow Rate (au)	55	55	50	50
Auxillary Gas Flow Rate (au)	20	20	10	10
Sweep Gas Flow Rate (au)	2	2	1	1
Spray Voltage (V) - POS	3000	3000	3500	3500
Spray Voltage (V) - NEG	3000	3000	2500	2500
Capillary Temperature (°C)	400	400	325	325
Vaporizer Temperature (°C)	N/A	N/A	300	300
S-Lens RF Level (%)	50	50	70	50

Table 7. ESI source settings. These settings are used with these specific Orbitrap models. Other mass spectrometers will need these source settings adjusted to achieve similar results.

- au = arbitrary units

MS1 Settings

A	B	C	D	E
Parameter	QE-HF	QE	Exp120	IQX
Microscans	1	1	1	1
Resolution	60,000	70,000	60,000	60,000
AGC Target	3e6	3e6	Standard (100%)	1e5
Maximum IT (ms)	100	100	Auto	118
Scan range (m/z)	70 - 1050	70 - 1050	70 - 1050	70 - 1050
Spectrum data type	Centroid	Centroid	Centroid	Centroid

Table 8. MS1 scan settings. Full MS spectra are collected in both positive and negative ionization modes. These settings are used with these specific Orbitrap models. Other mass spectrometers will need these source settings adjusted to achieve similar results.

Collection time: 21 minutes

MS2 Settings

	A	B	C	D	E
	Parameter	QE-HF	QE	Exp120	IQX
	Microscans	1	1	1	1
	Resolution	15,000	17,500	15,000	15,000
	AGC Target	1e5	1e5	Standard (100%)	5e4
	Maximum IT (ms)	50	50	Auto	22
	Loop count	2 (or 4)	2 (or 4)	4	N/A
	Cycle time	N/A	N/A	N/A	0.8 sec
	MSX count	1	1	1	1
	TopN	2 (or 4)	2 (or 4)	4	10-15
	Exclusion duration (sec)	7 - 10	7 - 10	4	5
	Stepped Collision Energies (eV)	10, 20, 40 (or 20, 50, 60)	10, 20, 40 (or 20, 50, 60)	10, 20, 40 (or 20, 50, 60)	10, 20, 40 (or 20, 50, 60)

Table 9. MS2 scan settings for collecting fragmentation data. Stepped and then averaged collision energies of 10, 20, 40 eV and/or 20, 50, 60 eV. A full MS1 scan is followed by "N" MS2 scans of the most intense precursor ions (TopN), excluding those precursors already fragmented in the previous time period (exclusion duration).

Source and acquisition settings listed here are the settings used for standard LC-MS/MS runs. Depending on experimental details and goals, these can be adjusted (e.g. instrument resolution, customization of data-dependent MS2, scan range, etc) as needed.

Sample Vial Preparation

8 Quality Control (QC) Mix

This is a custom formulation of compounds with annotated *m/z*, retention time (RT), and MS2 spectra dissolved in 100% MeOH. Compounds are representative of the metabolite classes detected using this LC-MS/MS method, as well as *m/z* and retention time ranges.

Usage: QC injections are interspersed throughout the LC-MS run to monitor instrument performance (calibration, intensity, retention time, etc.) as well as adjust compound retention times between runs.

	A	B	C	D	E	F	G	H
	Compound	Formula	Monoisotopic mass	Adduct (POS)	m/z (POS)	Adduct (NEG)	m/z (NEG)	Expected RT
	1-methyladenosine	C ₁₁ H ₁₅ N ₅ O ₄	281.1124	[M+H] ⁺	282.1197	[M-H] ⁻	280.1051	10.78
	2,4-dihydroxypteridine	C ₆ H ₄ N ₄ O ₂	164.0334	[M+H] ⁺	165.0407	[M-H] ⁻	163.0261	1.27
	2'-deoxyadenosine	C ₁₀ H ₁₃ N ₅ O ₃	251.1018	[M+H] ⁺	252.1091	[M-H] ⁻	250.0946	2.23
	2'-deoxyguanosine	C ₁₀ H ₁₃ N ₅ O ₄	267.0968	[M+H] ⁺	268.1040	[M-H] ⁻	266.0895	6.87
	2-hydroxyphenylacetic acid	C ₈ H ₈ O ₃	152.0473	[M+H] ⁺	153.0546	[M-H] ⁻	151.0401	1.62
	2-oxovaleric acid	C ₅ H ₈ O ₃	116.0473	[M+H] ⁺	117.0546	[M-H] ⁻	115.0401	4.45
	4-coumaric acid	C ₉ H ₈ O ₃	164.0473	[M+H] ⁺	165.0546	[M-H] ⁻	163.0401	1.51
	4-guanidinobutanoic acid	C ₅ H ₁₁ N ₃ O ₂	145.0851	[M+H] ⁺	146.0924	[M-H] ⁻	144.0779	13.86
	4-methoxyphenylacetic acid	C ₉ H ₁₀ O ₃	166.0630	[M+H] ⁺	167.0703	[M-H] ⁻	165.0557	1.07
	5-methylcytosine	C ₅ H ₇ N ₃ O	125.0589	[M+H] ⁺	126.0662	[M-H] ⁻	124.0516	4.42
	5-oxo-proline	C ₅ H ₇ N ₃ O ₃	129.0426	[M+H] ⁺	130.0499	[M-H] ⁻	128.0353	11.65
	ABMBA (2-amino-3-bromo-5-methylbenzoic acid)	C ₈ H ₈ BrNO ₂	228.9738	[M+H] ⁺	229.9811	[M-H] ⁻	227.9666	1.20

	A	B	C	D	E	F	G	H
	abscisic acid	C ₁₅ H ₂₀ O ₄	264.13 62	[M+H] +	265.14 34	[M-H]-	263.12 89	1.15
	acetylcholine	C ₇ NH ₁₆ O ₂ ⁺	146.117 6	[M] ⁺	146.117 6	[M-2H]-	144.10 30	1.96
	adenine	C ₅ H ₅ N ₅	135.05 45	[M+H] +	136.06 18	[M-H]-	134.04 72	2.56
	adenosine	C ₁₀ H ₁₃ N ₅ O ₄	267.09 68	[M+H] +	268.10 40	[M-H]-	266.08 95	3.09
	alanine	C ₃ H ₇ N O ₂	89.047 7	[M+H] +	90.055 0	[M-H]-	88.04 04	13.41
	alpha-glucose	C ₆ H ₁₂ O 6	180.06 34	[M+H] +	181.07 07	[M-2H]2-	89.070 7	5.06
	alpha-ketoglutaric acid	C ₅ H ₆ O ₅	146.02 15	[M+H] +	147.02 88	[M-H]-	145.01 42	14.51
	arginine	C ₆ H ₁₄ N 4O ₂	174.111 7	[M+H] +	175.119 0	[M-H]-	173.10 44	16.94
	asparagine	C ₄ H ₈ N ₂ O ₃	132.05 35	[M+H] +	133.06 08	[M-H]-	131.04 62	14.37
	aspartic acid	C ₄ H ₇ N O ₄	133.03 75	[M+H] +	134.04 48	[M-H]-	132.03 02	16.13
	benzoic acid	C ₇ H ₆ O ₂	122.03 68	[M+H] +	123.04 41	[M-H]-	121.02 95	1.27
	betaine	C ₅ H ₁₂ N O ₂ ⁺	118.08 63	[M] ⁺	118.08 63	[M-2H]-	116.071 7	7.91
	caffeic acid	C ₉ H ₈ O ₄	180.04 23	[M+H] +	181.04 95	[M-H]-	179.03 50	3.22
	caffeine	C ₈ H ₁₀ N 4O ₂	194.08 04	[M+H] +	195.08 77	[M-H]-	193.07 31	0.88
	carnitine	C ₇ H ₁₆ N O ₃ ⁺	162.112 5	[M+H] +	162.112 5	[M-H]-	160.09 79	13.29
	cis-4-hydroxy-proline	C ₅ H ₉ N O ₃	131.05 82	[M+H] +	132.06 55	[M-H]-	130.05 10	13.67
	citrulline	C ₆ H ₁₃ N 3O ₃	175.09 57	[M+H] +	176.10 30	[M-H]-	174.08 84	15.09
	creatine	C ₄ H ₉ N ₃ O ₂	131.06 95	[M+H] +	132.07 68	[M-H]-	130.06 22	13.39

	A	B	C	D	E	F	G	H
	cysteic acid	C3H7N O5S	169.00 45	[M+H] +	170.011 8	[M-H]-	167.99 72	14.54
	cytidine	C9H13N 3O5	243.08 55	[M+H] +	244.09 28	[M-H]-	242.07 82	6.93
	cytosine	C4H5N3 O	111.043 3	[M+H] +	112.05 05	[M-H]-	110.03 60	4.83
	deoxycytidine	C9H13N 3O4	227.09 06	[M+H] +	228.09 79	[M-H]-	226.08 33	5.59
	deoxyuridine	C9H12N 2O5	228.07 46	[M+H] +	229.08 19	[M-H]-	227.06 73	1.88
	ectoine	C6H10N 2O2	142.07 42	[M+H] +	143.08 15	[M-H]-	141.06 70	12.50
	fumaric acid	C4H4O 4	116.011 0	[M+H] +	117.018 2	[M-H]-	115.00 37	16.31
	gamma-aminobutyric acid	C4H9N O2	103.06 33	[M+H] +	104.07 06	[M-H]-	102.05 61	14.39
	glutamic acid	C5H9N O4	147.05 32	[M+H] +	148.06 04	[M-H]-	146.04 59	15.94
	glutamine	C5H10N 2O3	146.06 91	[M+H] +	147.07 64	[M-H]-	145.06 19	14.31
	glutaric acid	C5H8O4	132.04 23	[M- H+2Na]+	177.08 58	[M-H]-	131.03 50	12.60
	guanosine	C10H13 N5O5	283.09 17	[M+H] +	284.09 89	[M-H]-	282.08 44	8.57
	homoserine	C4H9N O3	119.05 82	[M+H] +	120.06 55	[M-H]-	118.051 0	13.65
	hypoxanthine	C5H4N4 O	136.03 85	[M+H] +	137.04 58	[M-H]-	135.03 12	3.10
	inosine	C10H12 N4O5	268.08 08	[M+H] +	269.08 80	[M-H]-	267.07 35	5.43
	isoleucine	C6H13N O2	131.09 46	[M+H] +	132.101 9	[M-H]-	130.08 74	9.71
	jasmonic acid	C12H18 O3	210.12 56	[M+H] +	211.132 9	[M-H]-	209.11 83	1.04
	lactic acid	C3H6O3	90.031 7	[M+H] +	91.039 0	[M-H]-	89.024 4	5.06

	A	B	C	D	E	F	G	H
	leucine	C ₆ H ₁₃ N O ₂	131.09 46	[M+H] +	132.101 9	[M-H]-	130.08 74	9.32
	lysine	C ₆ H ₁₄ N 2O ₂	146.10 55	[M+H] +	147.112 8	[M-H]-	145.09 83	17.01
	maltose	C ₁₂ H ₂₂ O ₁₁	342.11 62	[M+Na] +	365.10 54	[M-H]-	341.10 89	14.07
	mannitol	C ₆ H ₁₄ O 6	182.07 90	[M+H] +	183.08 63	[M-H]-	181.071 8	9.53
	mannosamine	C ₆ H ₁₃ N O ₅	179.07 94	[M+H] +	180.08 66	[M-H]-	178.07 21	14.52
	methionine	C ₅ H ₁₁ N O ₂ S	149.05 10	[M+H] +	150.05 83	[M-H]-	148.04 38	10.44
	MRFA (Met- Arg-Phe-Ala)	C ₂₃ H ₃₇ N ₇ O ₅ S	523.25 77	[M+H] +	524.26 50	[M-H]-	522.25 04	13.61
	N-acetyl- aspartic acid	C ₆ H ₉ N O ₅	175.04 81	[M+H] +	176.05 53	[M-H]-	174.04 08	14.82
	N-acetyl- glutamic acid	C ₇ H ₁₁ N O ₅	189.06 37	[M+H] +	190.07 10	[M-H]-	188.05 64	15.16
	N-acetyl- mannosamine	C ₈ H ₁₅ N O ₆	221.08 99	[M+Na] +	244.07 92	[M-H]-	220.08 27	7.15
	N-alpha- acetyl-lysine	C ₈ H ₁₆ N 2O ₃	188.116 1	[M+H] +	189.12 34	[M-H]-	187.108 8	15.13
	n-butylamine	C ₄ H ₁₁ N	73.089 1	[M+H] +	74.096 4	[M-H]-	72.081 9	4.17
	nicotinamide	C ₆ H ₆ N ₂ O	122.04 80	[M+H] +	123.05 53	[M-H]-	121.04 07	1.22
	nicotinic acid	C ₆ H ₅ N O ₂	123.03 20	[M+H] +	124.03 93	[M-H]-	122.02 48	5.63
	ornithine	C ₅ H ₁₂ N 2O ₂	132.08 99	[M+H] +	133.09 72	[M-H]-	131.08 26	17.04
	phenylacetic acid	C ₈ H ₈ O ₂	136.05 24	[M+H] +	137.05 97	[M-H]-	135.04 52	5.88
	phenylalanine	C ₉ H ₁₁ N O ₂	165.07 90	[M+H] +	166.08 63	[M-H]-	164.07 17	8.98
	pipecolic acid	C ₆ H ₁₁ N O ₂	129.07 90	[M+H] +	130.08 63	[M-H]-	128.07 17	10.97
	proline	C ₅ H ₉ N O ₂	115.06 33	[M+H] +	116.07 06	[M-H]-	114.05 61	10.92

	A	B	C	D	E	F	G	H
	pyridoxine	C ₈ H ₁₁ N O ₃	169.07 39	[M+H] +	170.08 12	[M-H]-	168.06 66	2.16
	raffinose	C ₁₈ H ₃₂ O ₁₆	504.16 90	[M+H] +	505.17 63	[M-H]-	503.16 18	15.53
	rhamnose	C ₆ H ₁₂ O 5	164.06 85	[M+H] +	165.07 57	[M-H]-	163.06 12	2.80
	ribose	C ₅ H ₁₀ O 5	150.05 28	[M+H] +	151.06 01	[M-H]-	149.04 55	2.75
	salicylic acid	C ₇ H ₆ O ₃	138.03 17	[M+H] +	139.03 90	[M-H]-	137.02 44	2.20
	serine	C ₃ H ₇ N O ₃	105.04 26	[M+H] +	106.04 99	[M-H]-	104.03 53	14.31
	shikimic acid	C ₇ H ₁₀ O 5	174.05 28	[M+H] +	175.06 01	[M-H]-	173.04 55	13.41
	succinic acid	C ₄ H ₆ O 4	118.02 66	[M+H] +	119.03 39	[M-H]-	117.019 3	9.92
	sucrose	C ₁₂ H ₂₂ O ₁₁	342.11 62	[M+Na] +	365.10 54	[M-H]-	341.10 89	13.45
	syringic acid	C ₉ H ₁₀ O 5	198.05 28	[M+H] +	199.06 01	[M-H]-	197.04 55	1.59
	taurine	C ₂ H ₇ N O ₃ S	125.01 47	[M+H] +	126.02 19	[M-H]-	124.00 74	12.16
	threonine	C ₄ H ₉ N O ₃	119.05 82	[M+H] +	120.06 55	[M-H]-	118.051 0	13.49
	thymidine	C ₁₀ H ₁₄ N ₂ O ₅	242.09 03	[M+H] +	243.09 75	[M-H]-	241.08 30	1.60
	thymine	C ₅ H ₆ N ₂ O ₂	126.04 29	[M+H] +	127.05 02	[M-H]-	125.03 57	1.26
	trans-4- hydroxy- proline	C ₅ H ₉ N O ₃	131.05 82	[M+H] +	132.06 55	[M-H]-	130.05 10	13.25
	trans- cinnamic acid	C ₉ H ₈ O ₂	148.05 24	[M+H] +	149.05 97	[M-H]-	147.04 52	1.15
	trehalose	C ₁₂ H ₂₂ O ₁₁	342.11 62	[M+Na] +	365.10 54	[M-H]-	341.10 89	14.21
	tryptophan	C ₁₁ H ₁₂ N O ₂	204.08 99	[M+H] +	205.09 72	[M-H]-	203.08 26	10.16

	A	B	C	D	E	F	G	H
	uracil	C ₄ H ₄ N ₂ O ₂	112.02 73	[M+H] +	113.03 46	[M-H]-	111.020 0	1.39
	uridine	C ₉ H ₁₂ N ₂ O ₆	244.06 95	[M+H] +	245.07 68	[M-H]-	243.06 23	2.89
	urocanic acid	C ₆ H ₆ N ₂ O ₂	138.04 29	[M+H] +	139.05 02	[M-H]-	137.03 57	9.35
	valine	C ₅ H ₁₁ N ₂ O ₂	117.079 0	[M+H] +	118.08 63	[M-H]-	116.071 7	11.12
	vanillic acid	C ₈ H ₈ O ₄	168.04 23	[M+H] +	169.04 95	[M-H]-	167.03 50	1.54
	vanillin	C ₈ H ₈ O ₃	152.04 73	[M+H] +	153.05 46	[M-H]-	151.04 01	0.86
	xanthine	C ₅ H ₄ N ₄ O ₂	152.03 34	[M+H] +	153.04 07	[M-H]-	151.02 61	2.73
	xanthosine	C ₁₀ H ₁₂ N ₄ O ₆	284.07 57	[M+H] +	285.08 30	[M-H]-	283.06 84	9.78

Table 10. Representative QC Mix for polar HILIC-Z. For each compound, observed adduct in positive and negative mode are listed as well as the observed retention time using this LC-MS/MS method.

- **Note:** Some compounds are only detected in a single polarity. Most ionize well at a concentration of 25 µM.

9 Blank

100% MeOH only (or other solvent matching the resuspension solvent of experimental samples).

Usage: Blank injections are interspersed between each sample injection to monitor background and minimize carryover (e.g. compounds "caught" in the system from the previous injection and detected in the next injection) between samples.

10 Internal Standard (ISTD) mix

A custom mixture of isotopically labeled (and/or non-biological/synthetic) compounds. These are added (at a specific concentration) to each sample prior to running LC-MS (typically during resuspension).

Usage: (1) Similar to QC mix, injections of ISTD mix only are interspersed throughout the LC-MS run to monitor instrument performance (calibration, intensity, retention time, etc.) as well as adjust compound retention times between runs. (2) Every experimental sample is also resuspended in solvent containing ISTDs. Since these are present in every sample at the same concentration, these can be used to assess individual sample

injection properties, including changes in retention time (e.g. due to sample pH, clogging), failed injections, or intensity variations (e.g. due to matrix effects, source fouling, or other factors). Additionally, since concentrations are known, an approximation of concentration for the same compound found in an experimental sample (not isotopically labeled) can be estimated based on ratio.

A	B	C	D	E	F	G	H	I	J	K
Compound	Formula	Concentration	Monoisotopic mass	Adduct (POS)	m/z (POS)	Adduct (NEG)	m/z (NEG)	Expected RT	Part number	Notes
alanine (U - ¹³ C, ¹⁵ N)	[¹³ C] ₃ H ₇ [¹⁵ N] ₂ O ₂	32.5 μM	93.0548	[M+H] ⁺	94.0620	[M-H] ⁻	92.0475	13.41	767964, Sigma	
arginine (U - ¹³ C, ¹⁵ N)	[¹³ C] ₆ H ₁₄ [¹⁵ N] ₄ O ₂	11 μM	184.1199	[M+H] ⁺	185.1272	[M-H] ⁻	183.1127	16.94	767964, Sigma	
asparagine (U - ¹³ C, ¹⁵ N)	[¹³ C] ₄ H ₈ [¹⁵ N] ₂ O ₃	9.5 μM	138.0610	[M+H] ⁺	139.0683	[M-H] ⁻	137.0537	14.37	767964, Sigma	
aspartic acid (U - ¹³ C, ¹⁵ N)	[¹³ C] ₄ H ₇ [¹⁵ N] ₂ O ₄	26 μM	138.0480	[M+H] ⁺	139.0552	[M-H] ⁻	137.0407	16.13	767964, Sigma	
cysteine (U - ¹³ C, ¹⁵ N)	[¹³ C] ₃ H ₇ [¹⁵ N] ₂ O ₂ S	10 μM	125.0268	[M+H] ⁺	126.0341	[M-H] ⁻	124.0196	Not detected	767964, Sigma	Not detected, oxidizes to cystine
cystine (U - ¹³ C, ¹⁵ N)	[¹³ C] ₆ H ₁₂ [¹⁵ N] ₂ O ₄ S ₂	10 μM	248.0380	[M+H] ⁺	249.0453	[M-H] ⁻	247.0308	16.9	N/A	Not added but a byproduct of cysteine (in amino acid mix)



	A	B	C	D	E	F	G	H	I	J	K
	glutamic acid (U - ¹³ C, ¹⁵ N)	[¹³ C] 5H9[¹⁵ N] O4	21 μM	153.0670	[M+H] ⁺	154.0742	[M-H] ⁻	152.0597	15.94	767964, Sigma	
	glutamine (U - ¹³ C, ¹⁵ N)	[¹³ C] 5H10[¹⁵ N] 2O3	10 μM	153.0800	[M+H] ⁺	154.0873	[M-H] ⁻	152.0727	14.31	767964, Sigma	
	glycine (U - ¹³ C, ¹⁵ N)	[¹³ C] 2H5[¹⁵ N] O2	24 μM	78.0358	[M+H] ⁺	79.0430	[M-H] ⁻	77.0285	14.1	767964, Sigma	
	histidine (U - ¹³ C, ¹⁵ N)	[¹³ C] 6H9[¹⁵ N] 3O2	2.5 μM	164.0807	[M+H] ⁺	165.0880	[M-H] ⁻	163.0734	14.88	767964, Sigma	
	isoleucine (U - ¹³ C, ¹⁵ N)	[¹³ C] 6H13[¹⁵ N] O2	10.5 μM	138.1118	[M+H] ⁺	139.1191	[M-H] ⁻	137.1045	9.71	767964, Sigma	
	leucine (U - ¹³ C, ¹⁵ N)	[¹³ C] 6H13[¹⁵ N] O2	22.5 μM	138.1118	[M+H] ⁺	139.1191	[M-H] ⁻	137.1045	9.32	767964, Sigma	
	lysine (U - ¹³ C, ¹⁵ N)	[¹³ C] 6H14[¹⁵ N] 2O2	9.5 μM	154.1197	[M+H] ⁺	155.1270	[M-H] ⁻	153.1124	17.01	767964, Sigma	
	methionine (U - ¹³ C, ¹⁵ N)	[¹³ C] 5H11[¹⁵ N] O2S	4.5 μM	155.0649	[M+H] ⁺	156.0721	[M-H] ⁻	154.0576	10.44	767964, Sigma	
	phenylalanine (U - ¹³ C, ¹⁵ N)	[¹³ C] 9H11[¹⁵ N] O2	8.5 μM	175.1062	[M+H] ⁺	176.1135	[M-H] ⁻	174.0989	8.98	767964, Sigma	
	proline (U - ¹³ C, ¹⁵ N)	[¹³ C] 5H9[¹⁵ N] O2	9.5 μM	121.0771	[M+H] ⁺	122.0844	[M-H] ⁻	120.0699	10.92	767964, Sigma	
	serine (U - ¹³ C, ¹⁵ N)	[¹³ C] 3H7[¹⁵ N] O3	14 μM	109.0497	[M+H] ⁺	110.0570	[M-H] ⁻	108.0424	14.31	767964, Sigma	



A	B	C	D	E	F	G	H	I	J	K
threonine (U - ¹³ C, ¹⁵ N)	[¹³ C] 4H9[¹⁵ N] O3	14 μM	124.0687	[M+H] ⁺	125.0760	[M-H] ⁻	123.0614	13.49	767964, Sigma	
tryptophan (U - ¹³ C, ¹⁵ N)	[¹³ C] 11H12[¹⁵ N] 2O2	10 μM	217.1209	[M+H] ⁺	218.1281	[M-H] ⁻	216.1136	10.16	767964, Sigma	
tyrosine (U - ¹³ C, ¹⁵ N)	[¹³ C] 9H11[¹⁵ N] O3	6.5 μM	191.1011	[M+H] ⁺	192.1084	[M-H] ⁻	190.0938	11.86	767964, Sigma	
valine (U - ¹³ C, ¹⁵ N)	[¹³ C] 5H11[¹⁵ N] O2	14.5 μM	123.0928	[M+H] ⁺	124.1001	[M-H] ⁻	122.0855	11.12	767964, Sigma	
mannitol (U - ¹³ C)	[¹³ C] 6H14 O6	10 μg/mL	188.0992	[M+H] ⁺	189.1064	[M-H] ⁻	187.0919	9.53	ALD-030, Omicron Biochemicals	
trehalose (U - ¹³ C)	[¹³ C] 12H22 O11	10 μg/mL	354.1565	[M+Na] ⁺	377.1457	[M-H] ⁻	353.1492	14.44	TRE-002, Omicron Biochemicals	
adenine (U - ¹⁵ N)	C5H5[¹⁵ N]5	4 μg/mL	140.0397	[M+H] ⁺	141.0469	[M-H] ⁻	139.0324	2.56	NLM-6924, Cambridge Isotope Labs	
hypoxanthine (U - ¹⁵ N)	C5H4[¹⁵ N]4O	3 μg/mL	140.0267	[M+H] ⁺	141.0339	[M-H] ⁻	139.0194	3.1	NLM-8500, Cambridge Isotope Labs	
uracil (U - ¹³ C, ¹⁵ N)	[¹³ C] 4H4 O2[¹⁵ N]2	2 μg/mL	118.0348	[M+H] ⁺	119.0420	[M-H] ⁻	117.0275	1.39	CNLM-3917, Cambridge Isotope Labs	
inosine (U - ¹⁵ N)	C10H12[¹⁵ N]4O5	5.5 μg/mL	272.0689	[M+H] ⁺	273.0762	[M-H] ⁻	271.0616	5.43	NLM-4264, Cambridge	

A	B	C	D	E	F	G	H	I	J	K
									Isotope Labs	
cytosine (13C2, 15N3)	C2[13C]2H5[15N]3O	5 µg/mL	116.0411	[M+H] ⁺	117.0483	[M-H] ⁻	115.0338	4.83	492108, Sigma	
guanine (U - 15N)	C5H5[15N]5O	2 µg/mL	156.0346	[M+H] ⁺	157.0419	[M-H] ⁻	155.0273	6.27	NLM-6926, Cambridge Isotope Labs	
thymine (U - 13C, 15N)	[13C]5H6[15N]2O2	2.5 µg/mL	133.0538	[M+H] ⁺	134.0610	[M-H] ⁻	132.0465	1.26	CNLM-6945, Cambridge Isotope Labs	
2-amino-3-bromo-5-methylbenzoic acid (ABMBA) (Br-nat)	C8H8BrNO2	1 µg/mL	228.9738	[M+H] ⁺	229.9811	[M-H] ⁻	227.9666	1.2	631531, Sigma	

Table 11. Representative ISTD Mix used for resuspension. For each compound, observed adduct in positive and negative ion mode are listed as well as the observed retention time using this LC-MS/MS method. Concentrations listed are the typical concentrations used in this protocol.

- For the Sigma amino acid mix (#767964, Sigma), the final concentration varies for each compound between lots, but is usually very similar between lots. Average concentration is ~15 µM for all compounds across the Sigma amino acid mix.
- For stable isotope labeling studies with 13C, typically only the 13C-15N amino acid mixture and ABMBA (synthetic and contains bromine - isobaric) are used as internal standards. Similar adjustments are made for other types of stable isotope labeling experiments based on type of labeling and overall experimental design.

11 Experimental Sample Vial

Samples typically consist of metabolite extracts (or a compound standard at a specified concentration) resuspended in solvent, usually 100% MeOH, and containing a mixture of isotopically labeled internal standards (see example ISTD mix above).

Usage: To profile metabolites in a sample and/or annotate the retention time, ionization characteristics (m/z for an adduct) and fragmentation spectra of a compound.

LC System Preparation

- 12 To prepare the LC, column compartment (40 °C) and autosampler temperatures (4 °C) are set and monitored until stable. The LC binary pump is typically prepared by purging 100% mobile phase A, 50/0 mobile phase A/B, then 100% B, each for 7 minutes at a flow rate of 5 mL/min. A flow rate of 0.45 mL/min of 100% B is maintained while the UHPLC system is checked for leaks or clogs. Backpressure is monitored until stable at ~130 bar. The HILIC column is equilibrated by performing 3-10 injections using the method gradients provided in Table 6.

Mass Spectrometer Preparation

- 13 Prior to data acquisition, the mass spectrometer is calibrated using standard calibration procedures available in the Thermo XCalibur operating software. ESI needle position is optimized relative to the source to achieve stable and acceptable ion intensity levels.

Calibration procedure for QE and QE-HF. Here, calibration is performed in positive mode with Pierce™ LTQ Velos ESI Positive Ion Calibration Solution (#88323, Thermo Scientific) and negative mode with Pierce™ Negative Ion Calibration Solution (#88324, Thermo Scientific) using direct injection from a syringe pump. Standard calibration is then followed by a custom low mass calibration procedure to ensure compounds near 100 m/z are also well-calibrated.

Calibration procedure for Exp120 and IQX. Here, calibration is performed in both positive and negative ionization mode with Pierce™ FlexMix Calibration Solution (#A39239, Thermo Scientific) using direct injection from a syringe pump and insertion of the low-flow ESI needle into the source housing. Custom low mass calibration procedures are not necessary using these systems. The system can be re-calibrated periodically during data acquisition using an automated point calibration (EasyIC) with the internal calibrant compound fluoroanthene. Prior to starting an LC-MS/MS run for data collection, the low-flow needle is replaced by the high-flow needle.

Note: For the IQX, an auto-calibration option can be performed using the Auto-ready Ion Source. This uses a less concentrated calibration mix (Pierce™ FlexMix Calibration Solution for Auto-Ready Mass Spectrometers, #A51739, Thermo Scientific) and eliminates the need to switch between low- and high-flow ESI needles.

LC-MS/MS Data Collection

- 14 In a typical LC-MS/MS run, an injection volume of 2–3 μL for each sample is used. Each sample is run in positive and negative ionization mode, with an injection blank of 100% methanol interspersed between each sample, replaced by an ISTD mix interspersed every 3 samples and a QC mix every 9–15 samples. Sample injection order is randomized between groups of replicate 1, then replicate 2, etc. Prior to starting a full experimental run, at least 4 injection blanks and several QC and ISTD injections are performed to ensure column and system equilibration and to verify that data is being acquired as expected. ISTD mix compounds are regularly monitored throughout LC-MS runs to assess drops in intensity, retention time shifts or increases in m/z ppm error, and performing the appropriate cleaning, re-calibration, maintenance or other troubleshooting as needed.

Protocol references

1 - <https://massive.ucsd.edu/>

2 - Eric W. Deutsch, Attila Csordas, Zhi Sun, Andrew Jarnuczak, Yasset Perez-Riverol, Tobias Ternent, David S. Campbell, Manuel Bernal-Llinares, Shujiro Okuda, Shin Kawano, Robert L. Moritz, Jeremy J. Carver, Mingxun Wang, Yasushi Ishihama, Nuno Bandeira, Henning Hermjakob, Juan Antonio Vizcaíno, The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition, *Nucleic Acids Research*, Volume 45, Issue D1, January 2017, Pages D1100–D1106, <https://doi.org/10.1093/nar/gkw936>

3 - Wang, M., Carver, J., Phelan, V. *et al.* Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nat Biotechnol* **34**, 828–837 (2016). <https://doi.org/10.1038/nbt.3597>