Oct 08, 2024

JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Nonpolar C18

DOI
dx.doi.org/10.17504/protocols.io.261ge5mzjg47/v1

Katherine B. Louie[1], Suzanne Kosina[2], Thomas Harwood[1], Meghana Faltane[1], Marie Lynde[1], Benjamin P. Bowen[1,2], Trent Northen[1,2]

[1]Lawrence Berkeley National Laboratory, Joint Genome Institute, Berkeley, CA, United States;
[2]Lawrence Berkeley National Laboratory, Environmental Genomics and Systems Biology Division, Berkeley, CA, United States

JGI Metabolomics Reposit…

Katherine B. Louie
Lawrence Berkeley National Laboratory, Joint Genome Institut...

OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.261ge5mzjg47/v1

Protocol Citation: Katherine B. Louie, Suzanne Kosina, Thomas Harwood, Meghana Faltane, Marie Lynde, Benjamin P. Bowen, Trent Northen 2024. JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Nonpolar C18. **protocols.io https://dx.doi.org/10.17504/protocols.io.261ge5mzjg47/v1**

Protocol status: Working
We use this protocol and it's working

Created: October 01, 2024

Last Modified: October 08, 2024

Protocol Integer ID: 108806

Keywords: metabolomics, JGI, LBNL, Joint Genome Institute, LC-MS, Thermo Orbitrap, Berkeley Lab, Environmental Metabolite Atlas, C18, nonpolar, reverse phase, Northen, GNPS, MassIVE

Funders Acknowledgement:
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 Northen Lab to analyze non-polar metabolites (e.g. secondary metabolites, etc) from sample extracts using reverse phase chromatography (C18) 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 2014, 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/GNPS2[3].

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 Northen Lab to analyze analyze non-polar metabolites (e.g. secondary metabolites, etc) from sample extracts using reverse phase chromatography (C18) 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. IDX, 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

	A	B	C	D
	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	ZORBAX Eclipse Plus C18, Rapid Resolution HD
	Part #	#959757-902
	Manufacturer	Agilent
	Column chemistry	C18
	Inner Diameter (ID)	2.1 mm
	Length	50 mm
	Particle size	1.8 µm
	Pore size	95 Å
	Max pressure	1200 bar
	pH range	2-9
	Max temperature	40 °C @ pH 6-9; 60 °C @ pH 2-6

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
	methanol (LC-MS grade)	MX0486, Sigma

	A	B
	formic acid (for mass spectrometry, ca. 98%)	94318, Fluka, Honeywell Research Chemicals

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	99.9% H2O and 0.1% formic acid
	Mobile Phase B	99.9% acetonitrile and 0.1% formic acid

Table 5. Mobile phase composition for C18. Sufficient mobile phase for all injections of a sample set are prepared prior to starting a run.

Column

Agilent ZORBAX Eclipse Plus C18, Rapid Resolution HD, 2.1 x 50 mm, 1.8 µm, 95 Å (Agilent, 959757-902); max pressure 1200 bar

Column Temperature 60 °C

Autosampler Temperature 4 °C

	A	B	C	D	E
	Time (min)	**Flow (mL/min)**	**%A**	**%B**	**minutes for segment**
	0	0.40	100	0	
	1	0.40	100	0	1
	8	0.40	0	100	7
	9.5	0.40	0	100	1.5
	10.5	0.40	100	0	1
	11.5	0.40	100	0	1

Table 6. Mobile phase gradients for C18. 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 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)	80 - 1200	80 - 1200	80 - 1200	80 - 1200

	A	B	C	D	E
	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: 12 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. Pre-made mixes can also be used (e.g. AOAC QuEChERS QC Spike Mix, #31999, Restek).

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**
	2'-deoxyadenosine	C10H13N5O3	251.1018	[M+H]+	252.1091	[M-H]-	250.0946	1.02
	glutaric acid	C5H8O4	132.0423	[M-H+2Na]+	177.0858	[M-H]-	131.0350	1.05
	xanthosine	C10H12N4O6	284.0757	[M+H]+	285.0830	[M-H]-	283.0684	1.42
	2-oxovaleric acid	C5H8O3	116.0473	[M+H]+	117.0546	[M-H]-	115.0401	1.45
	phenylalanine	C9H11NO2	165.0790	[M+H]+	166.0863	[M-H]-	164.0717	1.46
	thymidine	C10H14N2O5	242.0903	[M+H]+	243.0975	[M-H]-	241.0830	2
	tryptophan	C11H12N2O2	204.0899	[M+H]+	205.0972	[M-H]-	203.0826	2.21
	vanillic acid	C8H8O4	168.0423	[M+H]+	169.0495	[M-H]-	167.0350	2.64
	caffeine	C8H10N4O2	194.0804	[M+H]+	195.0877	[M-H]-	193.0731	2.7
	2-hydroxyphenyl acetic acid	C8H8O3	152.0473	[M+H]+	153.0546	[M-H]-	151.0401	2.83
	4-coumaric acid	C9H8O3	164.0473	[M+H]+	165.0546	[M-H]-	163.0401	2.93
	benzoic acid	C7H6O2	122.0368	[M+H]+	123.0441	[M-H]-	121.0295	3.39
	salicylic acid	C7H6O3	138.0317	[M+H]+	139.0390	[M-H]-	137.0244	3.42
	4-methoxyphenylacetic acid	C9H10O3	166.0630	[M+H]+	167.0703	[M-H]-	165.0557	3.56
	ABMBA (2-amino-3-bromo-5-methylbenzoic acid)	C8H8BrNO2	228.9738	[M+H]+	229.9811	[M-H]-	227.9666	4.77
	chenodeoxycholate	C24H40O4	392.2927	[M+H]+	393.2999	[M-H]-	391.2854	5.81
	enoxolone	C30H46O	470.339	[M+H]+	471.346	[M-H]-	469.332	6.61

	A	B	C	D	E	F	G	H
		4	6		9		3	
	9-cis-retinoic acid	C20H28O2	300.2089	[M+H]+	301.2162	[M-H]-	299.2017	7.35
	sarsasapogenin	C27H44O3	416.3290	[M+H]+	417.3363	[M-H]-	415.3218	8.16
	nigericin	C40H68O11	724.4762	[M+H]+	725.4834	[M-H]-	723.4689	8.56

Table 10. Representative QC Mix for C18. 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**

	A	B	C	D	E	F	G	H	I	J	K
	alanine (U - 13C, 15N)	[13C]3H7[15N]O2	32.5 µM	93.0548	[M+H]+	94.0620	[M-H]-	92.0475	0.34	767964, Sigma	
	arginine (U - 13C, 15N)	[13C]6H14[15N]4O2	11 µM	184.1199	[M+H]+	185.1272	[M-H]-	183.1127	0.34	767964, Sigma	
	asparagine (U - 13C, 15N)	[13C]4H8[15N]2O3	9.5 µM	138.0610	[M+H]+	139.0683	[M-H]-	137.0537	0.34	767964, Sigma	
	aspartic acid (U - 13C, 15N)	[13C]4H7[15N]O4	26 µM	138.0480	[M+H]+	139.0552	[M-H]-	137.0407	0.34	767964, Sigma	
	cysteine (U - 13C, 15N)	[13C]3H7[15N]O2S	10 µM	125.0268	[M+H]+	126.0341	[M-H]-	124.0196	*Not detected*	767964, Sigma	Not detected, oxidizes to cystine
	cystine (U - 13C, 15N)	[13C]6H12[15N]2O4S2	10 µM	248.0380	[M+H]+	249.0453	[M-H]-	247.0308	0.34	N/A	Not added but a bypr oduct of cysteine (in amino acid mix)
	glutamic acid (U - 13C, 15N)	[13C]5H9[15N]O4	21 µM	153.0670	[M+H]+	154.0742	[M-H]-	152.0597	0.34	767964, Sigma	
	glutamine (U - 13C, 15N)	[13C]5H10[15N]2O3	10 µM	153.0800	[M+H]+	154.0873	[M-H]-	152.0727	0.34	767964, Sigma	
	glycine (U - 13C, 15N)	[13C]2H5[15N]O2	24 µM	78.0358	[M+H]+	79.0430	[M-H]-	77.0285	0.34	767964, Sigma	
	histidine (U - 13C, 15N)	[13C]6H9[15N]3O2	2.5 µM	164.0807	[M+H]+	165.0880	[M-H]-	163.0734	0.34	767964, Sigma	
	isoleucine (U - 13C, 15N)	[13C]6H13[15N]O2	10.5 µM	138.1118	[M+H]+	139.1191	[M-H]-	137.1045	0.78	767964, Sigma	Retains on C18
	leucine (U - 13C, 15N)	[13C]6H13[15N]O2	22.5 µM	138.1118	[M+H]+	139.1191	[M-H]-	137.1045	0.78	767964, Sigma	Retains on C18
	lysine (U - 13C, 15N)	[13C]6H14[15N]2O2	9.5 µM	154.1197	[M+H]+	155.1270	[M-H]-	153.1124	0.34	767964, Sigma	
	methionine (U - 13C, 15N)	[13C]5H11[15N]O2S	4.5 µM	155.0649	[M+H]+	156.0721	[M-H]-	154.0576	0.39	767964, Sigma	

	A	B	C	D	E	F	G	H	I	J	K
	phenylalanine (U - 13C, 15N)	[13C]9H11[15N]O2	8.5 µM	175.1062	[M+H]+	176.1135	[M-H]-	174.0989	1.77	767964, Sigma	Retains on C18
	proline (U - 13C, 15N)	[13C]5H9[15N]O2	9.5 µM	121.0771	[M+H]+	122.0844	[M-H]-	120.0699	0.34	767964, Sigma	
	serine (U - 13C, 15N)	[13C]3H7[15N]O3	14 µM	109.0497	[M+H]+	110.0570	[M-H]-	108.0424	0.34	767964, Sigma	
	threonine (U - 13C, 15N)	[13C]4H9[15N]O3	14 µM	124.0687	[M+H]+	125.0760	[M-H]-	123.0614	0.34	767964, Sigma	
	tryptophan (U - 13C, 15N)	[13C]11H12[15N]2O2	10 µM	217.1209	[M+H]+	218.1281	[M-H]-	216.1136	2.34	767964, Sigma	Retains on C18
	tyrosine (U - 13C, 15N)	[13C]9H11[15N]O3	6.5 µM	191.1011	[M+H]+	192.1084	[M-H]-	190.0938	0.70	767964, Sigma	Retains on C18
	valine (U - 13C, 15N)	[13C]5H11[15N]O2	14.5 µM	123.0928	[M+H]+	124.1001	[M-H]-	122.0855	0.34	767964, Sigma	
	mannitol (U - 13C)	[13C]6H14O6	10 µg/mL	188.0992	[M+H]+	189.1064	[M-H]-	187.0919	0.34	ALD-030, Omicron Biochemicals	
	trehalose (U - 13C)	[13C]12H22O11	10 ug/mL	354.1565	[M+Na]+	377.1457	[M-H]-	353.1492	0.34	TRE-002, Omicron Biochemicals	
	adenine (U - 15N)	C5H5[15N]5	4 µg/mL	140.0397	[M+H]+	141.0469	[M-H]-	139.0324	0.34	NLM-6924, Cambridge Isotope Labs	
	hypoxanthine (U - 15N)	C5H4[15N]4O	3 µg/mL	140.0267	[M+H]+	141.0339	[M-H]-	139.0194	0.59	NLM-8500, Cambridge Isotope Labs	Retains on C18
	uracil (U - 13C, 15N)	[13C]4H4O2[15N]2	2 µg/mL	118.0348	[M+H]+	119.0420	[M-H]-	117.0275	0.34	CNLM-3917, Cambridge Isotope Labs	
	inosine (U - 15N)	C10H12[15N]4O5	5.5 µg/mL	272.0689	[M+H]+	273.0762	[M-H]-	271.0616	1.02	NLM-4264, Cambridge Isotope Labs	Retains on C18
	cytosine (13C2, 15N3)	C2[13C]2H5[15N]3O	5 µg/mL	116.0411	[M+H]+	117.0483	[M-H]-	115.0338	0.34	492108, Sigma	

	A	B	C	D	E	F	G	H	I	J	K
	guanine (U - 15 N)	C5H5 [15N]5 O	2 µ g/m L	156. 034 6	[M+ H]+	157.0 419	[M-H] -	155.0 273	0.34	NLM-6926, Cambridge Isotope La bs	
	thymine (U - 13C, 15N)	[13C]5 H6[15 N]2O2	2.5 µ g/m L	133. 053 8	[M+ H]+	134.0 610	[M-H] -	132.0 465	0.83	CNLM-694 5, Cambrid ge Isotope Labs	Retains on C18
	2-amino-3-bromo-5-methylbenzoic acid (ABMBA) (Br-nat)	C8H8 BrNO2	1 µ g/m L	228. 973 8	[M+ H]+	229.9 811	[M-H] -	227.9 666	4.77	631531, Sigma	Retains on C18

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.
- Since this specific mix is optimized for HILICZ chromatography, when using this C18 method, note that only some of these compounds retain on the column beyond the void volume / solvent front @ ~0.34 min. Check column "Expected RT".

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 (60 °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 0.40 mL/min of 100% B is maintained while the UHPLC system is checked for leaks or clogs. Backpressure is monitored until stable. 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