



Feb 26, 2025

JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Lipids

DOI

<https://dx.doi.org/10.17504/protocols.io.yxmvm9x45l3p/v1>

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JGI/LBNL Metabolomics ...



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Protocol Citation: Katherine Louie, Meghana Faltane, Marie Lynde, Benjamin Bowen, Trent Northen 2025. JGI/LBNL Metabolomics - Standard LC-MS/MS ESI Method - Lipids. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.yxmvm9x45l3p/v1>

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Protocol status: Working

We use this protocol and it's working

Created: November 15, 2024

Last Modified: February 26, 2025

Protocol Integer ID: 112212

Keywords: metabolomics, JGI, LBNL, Joint Genome Institute, LC-MS, Thermo Orbitrap, Berkeley Lab, Environmental Metabolite Atlas, C18, nonpolar, reverse phase, Northen, GNPS, MassIVE, lipid, carotenoid, galactolipids, algae, pigment, triglyceride, GNPS2, ESI, betaine lipids, SQDG, DGTS, MGDG, DGDG, PC, phospholipid, triacylglyceride, jgi metabolomic, lbnl metabolomic, mass spectrometry dataset, gnps2 mass spectrometry analysis, mass spectrometry, consistent outcomes across mass spectrometry dataset, galactolipids such as mgdg, thermo orbitrap mass spectrometer with esi source, thermo orbitrap mass spectrometer, betaine lipids such as dgt, lipids this protocol, galactolipid, lipid, other lipid, betaine lipid, ms esi method, reverse phase chromatography, using reverse phase chromatography, like compounds from sample extract, thousands of experimental sample, sample extract, jgi, esi source, carotenoid, mgdg

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

Abstract

This protocol describes the standard LC-MS/MS ESI method developed at Lawrence Berkeley National Laboratory (LBNL) by JGI Metabolomics to analyze lipids (e.g. TAGs, DAGs, SQDG, betaine lipids such as DGTS, galactolipids such as MGDG and DGDG, glycerophospholipids such as PC, PE, PS, etc), carotenoids, pigments, and other lipid-like compounds 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 2015, this method has been used to analyze thousands of experimental samples, with many of these datasets publicly available in the MassIVE data repository^{1,2} and analyzed through the GNPS/GNPS2 mass spectrometry analysis hubs^{3,4}.

Overview

- 1 This protocol describes the standard LC-MS/MS ESI method developed at Lawrence Berkeley National Laboratory (LBNL) by JGI Metabolomics to analyze lipids (e.g. TAGs, MGDG, DGDG, SQDG, DGTS, glycerophospholipids such as PC, PE, PS, etc) and lipid-like compounds 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
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

A	B
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
isopropanol (LC-MS grade)	BJ34965, VWR
ammonium acetate (LC-MS grade)	73594, Sigma
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% 60:40 water:acetonitrile and 0.1% formic acid with 5 mM ammonium acetate
Mobile Phase B	99.8% 90:10 isopropanol:acetonitrile, 0.1% water and 0.1% formic acid with 5 mM ammonium acetate

Table 5. Mobile phase composition for Lipid C18. Sufficient mobile phase for all injections of a sample set are prepared prior to starting a run. For mobile phase B, dissolve ammonium acetate in water prior to adding the remaining solvents. For mobile phase A, degas before running on the LC to prevent bubbles in the line. This can be performed by sonicating the prepared buffer in a water bath for several minutes (uncapped, topped with foil) until minimal gas bubbling is observed.

Column

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

Column Temperature 🌡 55 °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
	3	0.40	45	55	2
	11	0.40	20	80	8
	16	0.40	0	100	5
	17.5	0.40	0	100	1.5
	18.5	0.40	100	0	1
	20	0.40	100	0	1.5

Table 6. Mobile phase gradients for C18 lipid. 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 18.5-20 minutes during column re-equilibration), are also not used in analysis.

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	3000	3500
Spray Voltage (V) - NEG	3000	3000	3000	2500
Capillary Temperature (°C)	400	400	350	300
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)	132-1500	132-1500	132-1500	132-1500
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: 20 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%)	1.25e4
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 formulation of compounds with annotated m/z , retention time (RT), and MS2 spectra dissolved in 100% MeOH or 3:3:4 IPA:ACN:MeOH. Compounds are representative of the metabolite classes detected using this LC-MS/MS method, as well as m/z and retention time ranges. Internal standard mixes can also serve as QC mixes.

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.

9 Blank

3:3:4 IPA:ACN: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 (µg/mL) in stock	Monoisotopic mass	Adduct (POS)	m/z (POS)	Adduct (NEG)	m/z (NEG)	Expected RT	Part number	Notes
TG 15:0-18:1(d7)-15:0	C51H89d7O6	55	811.7646	[M+NH4] ⁺	829.7985			13.06	330707, SPLASH LIPIDOMIX Mass Spec Standard, Avanti	not detected in NEG



	A	B	C	D	E	F	G	H	I	J	K
										Polar Lipids	
	DG 15:0- 18:1(d 7)	C36 H61d 7O5	10	587. 550 6	[M+ NH 4]+	605 .58 44			8.3 9	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	not detecte d in NEG
	PC 15:0- 18:1(d 7)	C41H 73d7 NO8 P	160	752 .60 61	[M+ H]+	753 .613 4	[M+ HC OO] -	797. 604 3	7.6 8	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	PE 15:0- 18:1(d 7)	C38 H67d 7NO 8P	5	710. 559 1	[M+ H]+	711. 566 4	[M- H]-	709 .551 9	6.8 8	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	Lyso PC 18:1(d 7)	C26 H45d 7NO7 P	25	528 .39 21	[M+ H]+	529 .39 93	[M+ HC OO] -	573 .39 03	3.8 7	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	Lyso PE 18:1(d 7)	C23 H39d 7NO7 P	5	486 .34 51	[M+ H]+	487 .35 24	[M- H]-	485 .33 79	3.6 8	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	Chol Ester 18:1(d 7)	C45 H71d 7O2	350	657. 644 1	[M+ NH 4]+	675 .67 79			13. 6	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti	not detecte d in NEG



	A	B	C	D	E	F	G	H	I	J	K
										Polar Lipids	
	SM d18:1- 18:1(d 9)	C41H 72d9 N2O 6P	30	737. 639 7	[M+ H] ⁺	738 .64 70	[M+ HC OO] -	782 .63 79	6.9 5	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	PS 15:0- 18:1(d 7)	C39 H67d 7NO1 0P	5	754 .54 90	[M+ H] ⁺	755 .55 63	[M- H] ⁻	753 .541 7	5.7 6	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	PG 15:0- 18:1(d 7)	C39 H68d 7O10 P	30	741. 553 7	[M+ NH 4] ⁺	759 .58 76	[M- H] ⁻	740 .54 65	5.7 5	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	PI 15:0- 18:1(d 7)	C42 H72d 7O13 P	10	829 .56 98	[M+ H] ⁺	830 .57 70	[M- H] ⁻	828 .56 25	5.5 2	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	PA 15:0- 18:1(d 7)	C36 H62d 7O8P	7	667. 516 9	[M+ NH 4] ⁺	685 .55 08	[M- H] ⁻	666 .50 97	6.1 4	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti Polar Lipids	
	MG 18:1(d 7)	C21H 33D7 O4	2	363 .33 66	[M+ H] ⁺	364 .34 39			4.0 4	330707, SPLASH LIPIDOM IX Mass Spec Standar d, Avanti	not detecte d in NEG

	A	B	C	D	E	F	G	H	I	J	K
										Polar Lipids	
	Cholesterol (d7)	C27H ₃₉ O ₇ D7	100	393.3988	[M+H-H ₂ O]	376.3955			6.12	330707, SPLASH LIPIDOMIX Mass Spec Standard, Avanti Polar Lipids	not detected in NEG

Table 10. Representative ISTD Mix used for lipid resuspension (330707, Splash Lipidomic Mass Spec Standard, Avanti Polar Lipids). 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.

- This lipid mixture contains a variety of lipids observed using this C18 lipid LC-MS/MS method (although not all will be detected or not in both polarities). Concentrations given are the stock concentration of each compound in methanol (as sold by the manufacturer).
- Typical usage of this mix is spiking in **8 µL mix for every 150 µL resuspension volume** (~20x dilution from the starting concentration).
- Store the mix at -20 °C as many lipids in this mix (as well as samples!) are not stable at higher temperatures (including 4 °C) and will degrade quickly over the course of a week at room temperature.
- Additional isotopically labeled lipids can be added to this mix to evaluate other lipid classes (e.g. DGTS, SQDG).
- For stable isotope labeling studies with ¹³C or ¹⁵N, this lipid mix is appropriate because labeling is only with ²H/deuterium. For ²H labeling experiments, a different mix containing ¹³C and/or ¹⁵N labeled lipids can be used or a custom mixture. 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 3:3:4 IPA:ACN:MeOH for lipids**, 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

- To prepare the LC, column compartment (55 °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, 50:50 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, then switched to 50:50 mobile phase A:B for 1-2 minutes, then 100% A for at least 5 minutes prior to starting the run. During this time, switching between mainpass and bypass is helpful to ensuring the switching valve is clean and equilibrated with each mobile phase composition. Backpressure is monitored until stable. The C18 column is equilibrated by performing 3-10 injections using the method gradients provided in Table 6.

Note: To avoid potential clogs, it's recommended to not switch directly from 100% A to 100% B mobile phase and vice versa, but adjust more gradually using a 50:50 composition between them first.

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 3:3:4 IPA:ACN:MeOH interspersed between each sample, replaced by an ISTD mix interspersed every 3 samples and a QC mix (if applicable) 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

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