ADNI Alzheimer's Disease Neuroimaging Initiative

Lipidomic analysis by UPLC-QTOF mass spectrometry

West Coast Metabolomics Center
Genome Center, University of California, Davis, CA, USA

Contents

1. Instruments:

- Agilent 1290 UHPLC-6530-QTOF
- Agilent 1290 UHPLC-6550-QTOF
- Pipettes calibrated following SOP006_2003
- Ultrasonicator

2. Chemicals and consumables

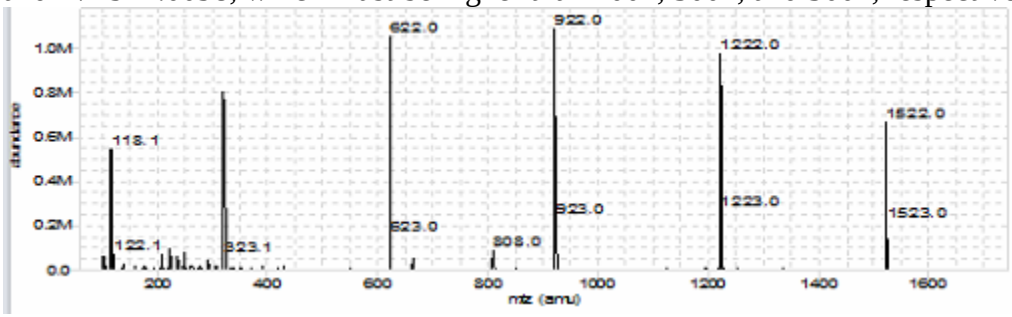
- Waters Acquity CSH C18 2.1x10 0mm 1.7 µm Column
- Waters Acquity VanGuard CSH C18 1.7 µm Pre-column
- Pipettes calibrated following SOP006_2003
- Ultrasonicator
- Agilent Tune Mix: G1969-85000
- Acetonitrile: J.T. Baker LC/MS Grade, 4 L (9829-03)
- Formic Acid: Fluka Mass Spec Grade (94318-250mL-F)
- Ammonium Formate: Fluka, Mass Spec Grade (70221-25G-F)
- Ammonium Acetate: Sigma, Mass Spec Grade (A7330-500G)
- Isopropanol: LC/MS grade (Fluka 34965-2.5L)
- Agilent 0.17ID (green) metal tubing: 90 cm 5065-9963 and 20 cm (5065-9931)
- Red Agilent Peek Tubing 5 meters (0.13 ID) (5042-6461)
- Plastic Agilent Connectors (for peek tubing) (0100-1516)
- Stainless Steel Agilent Fitting (5062-2418)

3. Procedure:

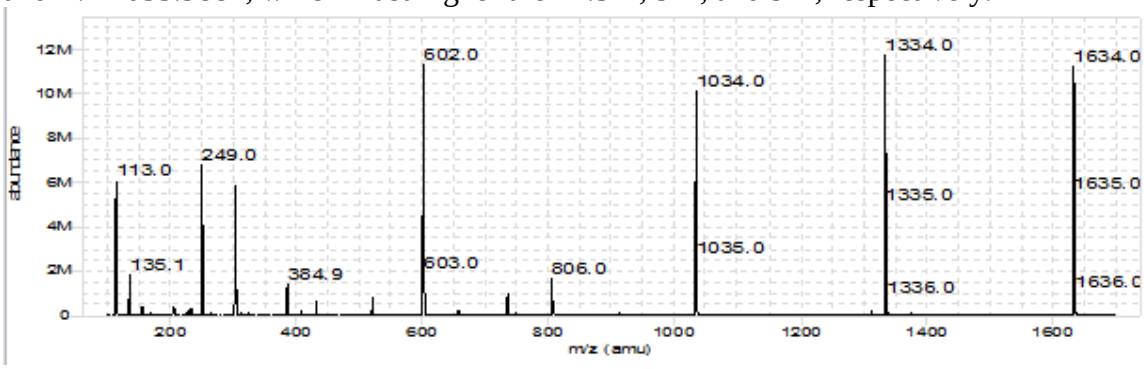
3.1 Pre-run procedures

3.1.1 Instrument tuning (Instrument in Tune mode)
a. Use "Standard Tune" before each run of 300 sample batch.
b. Use the "Tuning Solution" (see preparation of solutions below) for the instrument tuning.

c. The mixture for the instrument tuning must be prepared fresh at the beginning of each 300 sample batch.
d. Print the tune report from the standard tune.
- In ESI(+), check the profile of the calibrant and the intensity of ions *m/z* 322.0481; *m/z* 622.0290; and *m/z* 922.0098, which must be higher than 400k, 500k, and 500k, respectively.



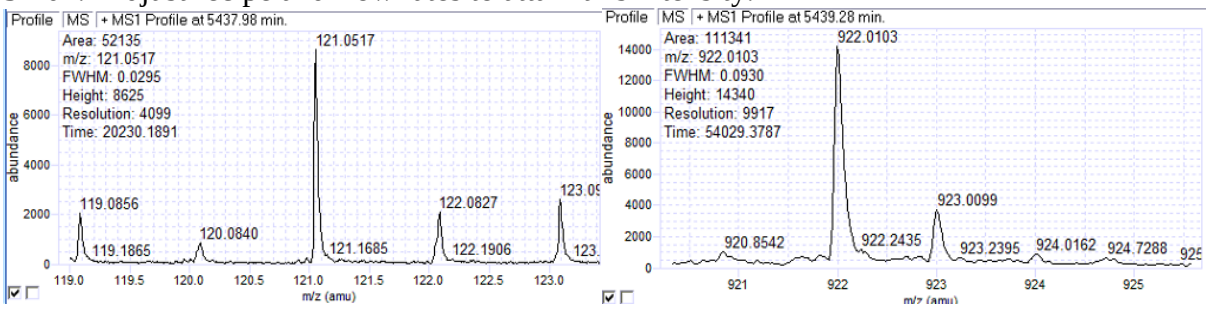
- In ESI(–), check the profile of the calibrant and the intensity of ions *m/z* 301.9981; *m/z* 601.9790; and *m/z* 1033.9881, which must higher then 4.5M, 9M, and 9M, respectively.



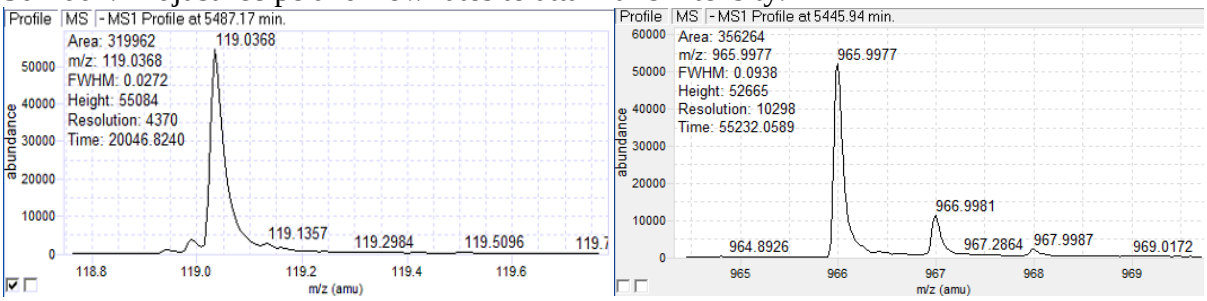
e. If the intensity of even one of the selected ion is below this value clean the ion source and repeat the instrument tuning.
3.1.2 Check Reference ions (Instrument in Acquisition mode)

a. Use the "Reference Ion Mass Solution" (see preparation of solutions below) for mass correction during the analyses (lock mass).
b. The mixture for the reference ion solution must be prepared fresh at the beginning of each 300 sample batch.
c. Check the following reference ions:

- In ESI(+), check the intensity of ions m/z 121.0509 and m/z 980.0164, which should be between 5-20k. Adjust recipe and flow rates to attain this intensity.



- In ESI(–), check the intensity of ions *m/z* 119.0363 and *m/z* 966.0007, which should be between 50-100k. Adjust recipe and flow rates to attain this intensity.



3.2 New column installation

a. Purge the pumping system of any old buffers and connect the inlet of the column to the injector outlet. Attach the outlet line, but allow flow to go into a beaker instead of to the ion source of mass spectrometer.
b. Flush column with 85% Mobile Phase A (see preparation of solution below) with a pump flow rate at 0.6mL/min over 30 minutes.
c. Switch to 99% mobile phase B and flush the column for 30 minutes.
d. When the mobile phase is flowing freely from the column outlet, stop the flow and attach the column outlet to the ion source of mass spectrometer.
e. Switch back to 85% Mobile Phase A and monitor the backpressure until a steady value is achieved. Expected values are between 500-550 bar at the start of the injection.
f. Perform 8 "No Injections" and monitor the backpressure on the first and last sample injected.
NOTE: Use a new column after ~1000 sample injections. The UPLC column must be coupled to a VanGuard pre-column. The VanGuard pre-column is replaced after ~330 sample injections. The number of injections (both solvents and plasma samples) is recorded by an operator in a folder created for each acquisition.

3.3 Preparation of solutions

a. Preparation of Tuning Solution

- 88.5 mL acetonitrile
- 1.5 mL H_2O
- 10 mL Agilent Low Concentration ESI Tuning Mix
- 5 µL 322 Reference Ion (sonicate before use)
- Degas by sonication for 5 min
- 100 mL will typically last months

b. Preparation of Reference Mass Solution

- 95 mL acetonitrile
- 5 mL H_2O
- 200 µL 5 mM 921 Reference Ion (sonicate before use)
- 250 µL 10 mM Purine Reference Ion (sonicate before use)
- Degas by sonication for 5 min

c. Mobile phase A Positive Mode (60:40 ACN:water + 10 mM Ammonium Formate + 0.1% Formic Acid)

1. Pre-rinse three times 1 L glass bottle with pure acetonitrile
2. Measure exactly 600 mL of acetonitrile in a pre-rinsed graduated cylinder and add them to the 1 L glass bottle.
3. Measure exactly 400 mL of MilliQ water in a pre-rinsed graduated cylinder and add them to the 1 L glass bottle.
4. Add 1 mL formic acid
5. Weight 0.630 g of ammonium formate and add them to the glass bottle
6. Sonicate for 10 min at room temperature until all the ammonium formate is dissolved.
7. 1 L will last for around 200 samples
8. Combine multiple batches into one 4L bottle
9. 3L of mobile phase A will be enough to analyze 1/3 of a batch (~330 samples) including re-injections.

d. Mobile phase B Positive Mode (90:10 IPA:ACN + 10 mM Ammonium Formate + 0.1% Formic Acid)

1. Add 1mL H2O to a 1L glass bottle
2. Add 1mL Formic Acid to the same 1L glass bottle
3. Add 0.630g Ammonium Formate to the same 1L glass bottle
4. Gently shake 1L glass bottle to dissolve as much Ammonium Formate as possible
5. Add exactly 900mL LC/MS grade isopropanol
6. Add exactly 100mL LC/MS grade acetonitrile
7. Sonicate for 10 min at room temperature.

e. Mobile phase A Negative Mode (60:40 ACN:water + 10 mM Ammonium Acetate)

1. Pre-rinse three times 1 L glass bottle with pure acetonitrile
2. Measure exactly 600 mL of acetonitrile in a pre-rinsed graduated cylinder and add them to the 1 L glass bottle.
3. Measure exactly 400 mL of LC/MS grade water in a pre-rinsed graduated cylinder and add them to the 1 L glass bottle.
4. Weight 0.770 g of ammonium acetate and add them to the glass bottle
5. Sonicate for 10 min at room temperature until all the ammonium formate is dissolved.
6. 1 L will last for around 200 samples
7. Combine multiple batches into one 4L bottle
8. 3L of mobile phase A will be enough to analyze 1/3 of a batch (~330 samples) including re-injections.

f. Mobile phase B Negative Mode(90:10 IPA:ACN + 10 mM Ammonium Acetate)

1. Add 1mL H2O to a 1L glass bottle
2. Add 1mL Formic Acid to the same 1L glass bottle
3. Add 0.770g Ammonium Acetate to the same 1L glass bottle
4. Gently shake 1L glass bottle to dissolve as much Ammonium Formate as possible
5. Add exactly 900mL LC/MS grade isopropanol
6. Add exactly 100mL LC/MS grade acetonitrile

7. Sonicate for 10 min at room temperature.

3.4 Pre-run sequence

a. Before starting the run inject the following:

1. 3 x "No sample Injection"
2. 3 x Blank sample injection (methanol
3. 3 x Biorec plasma injection

b. For the QC-mix, monitor the retention time, intensity, *S*/*N*, mass accuracy, and peak width (FWHM) of particular analytes (**Table 1**). Use the MassHunter Qualitative Analysis software for data processing. The acceptable ranges of the parameters are stored at <D>:\\<MassHunter\Methods\TEDDY methods>\TEDDY_QC-mix_default.XLS.

c. If those criteria are not met, the following actions should be considered:

(*i*) Replace the VanGuard pre-column and/or the UPLC column (if retention time shift >±2.5% and/or peak width expressed as FWHM increased >20%);

(*ii*) Clean the ion source (if intensity of particular analytes <80%);

(*iii*) Re-tune the mass spectrometer (mass accuracy of particular analytes >10 ppm).

Table 1 Analytes of the QC-mix solution

Common Name	Formula	MS1 m/z	RT (min)
CE (22:1) [M+Na]+ iSTD	C49H86O2	729.652	11.727
CE (22:1) [M+NH4]+ iSTD	C49H86O2	724.6966	11.727
Ceramide C17 [M+H]+ iSTD	C35H69NO3	552.535	5.948
Ceramide C17 [M+H-H2O]+ iSTD	C35H69NO3	534.5245	5.948
Ceramide C17 [M+Na]+ iSTD	C35H69NO3	574.517	5.948
Cholesterol d7 [M-H2O+H]+ iSTD	C27H39D7O	376.3955	4.787
CUDA (pos) iSTD [M+H]+	C19H36N2O3	341.2799	0.774
DG (12:0/12:0/0:0) [M+Na]+ iSTD	C27H52O5	479.3707	4.248
DG (12:0/12:0/0:0) [M+NH4]+ iSTD	C27H52O5	474.4153	4.248
DG (18:1/2:0/0:0) [M+Na]+ iSTD	C23H42O5	421.2925	3.162
DG (18:1/2:0/0:0) [M+NH4]+ iSTD	C23H42O5	416.3371	3.162
LPC (17:0) [M+H]+ iSTD	C25H52NO7P	510.3554	1.827
LPE (17:1) [M+H]+ iSTD	C22H44NO7P	466.2928	1.346
MG (17:0/0:0/0:0) [M+H]+ iSTD	C20H40O4	345.2999	3.038
MG (17:0/0:0/0:0) [M+Na]+ iSTD	C20H40O4	367.2819	3.038
MG (17:0/0:0/0:0) [M+NH4]+ iSTD	C20H40O4	362.3265	3.038
PC (12:0/13:0) [M+H]+ iSTD	C33H66NO8P	636.4596	3.502
PE (17:0/17:0) [M+H]+ iSTD	C39H78NO8P	720.5538	6.263
SM (17:0) [M+H]+ iSTD	C40H81N2O6P	717.5915	5.053
Sphingosine (d17:1) [M+H]+ iSTD	C17H35NO2	286.2741	1.04
TG (17:0/17:1/17:0) [M+Na]+ d5 iSTD	C54H97D5O6	874.7877	10.997

TG (17:0/17:1/17:0) [M+NH4]+ d5 iSTD	C54H97D5O6	869.8323	11.006

Table 2. Internal Standards for Negative mode

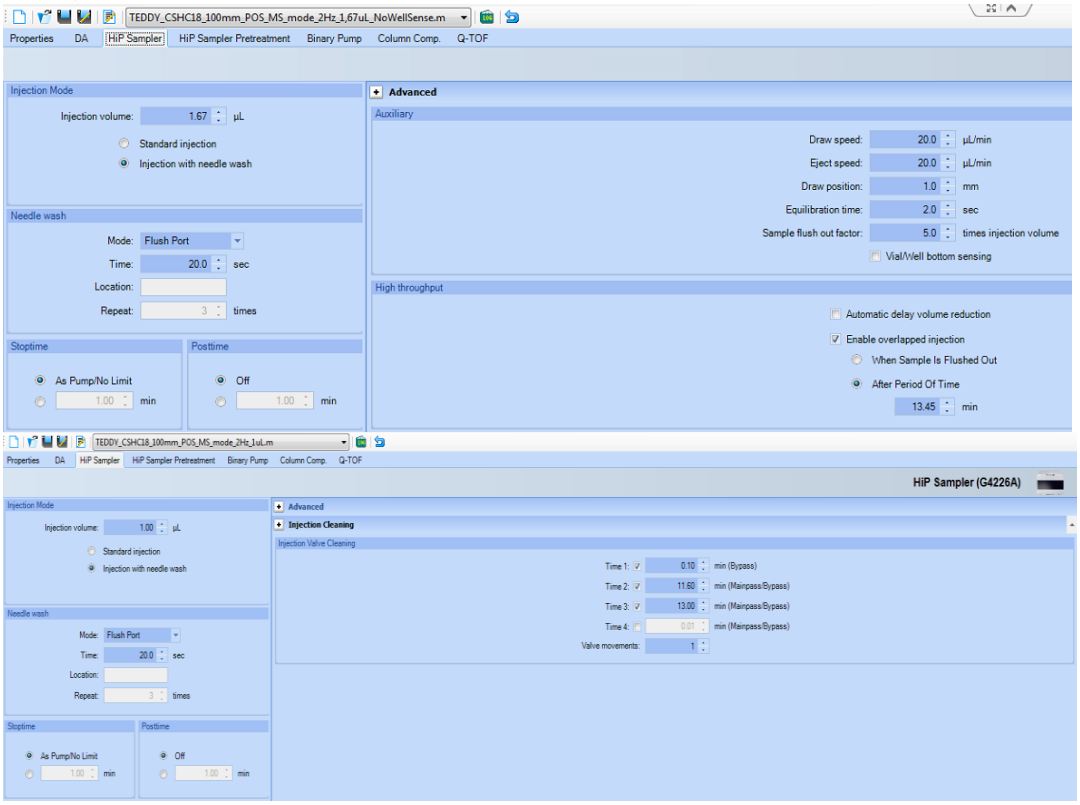
COMMON NAME	FORMULA	MS1 M/Z	RT (MIN)
CERAMIDE (D18:1/17:0) [M+CL]- ISTD	C35H69NO3	586.5	6.114
CERAMIDE (D18:1/17:0) [M+HAC-H]- ISTD	C35H69NO3	610.54	6.106
CUDA [M-H]- ISTD	C19H36N2O3	339.26	0.66
FA (16:0)-D3 [M-H]- ISTD	C16H29D3O2	258.25	2.4
LPC (17:0) [M+HAC-H]- ISTD	C25H52NO7P	568.36	1.912
LPE (17:1) [M-H]- ISTD	C22H44NO7P	464.28	1.43
MAG (17:0/0:0/0:0) [M+HAC-H]- ISTD	C20H40O4	403.31	3.135
PC (12:0/13:0) [M+HAC-H]- ISTD	C33H66NO8P	694.47	3.61
PE (17:0/17:0) [M-H]- ISTD	C39H78NO8P	718.54	6.422
PG (17:0/17:0) [M-H]- ISTD	C40H79O10P	749.53	5.225
SM (D18:1/17:0) [M+HAC-H]- ISTD	C40H81N2O6P	775.6	5.199

NOTE: Compare the profile of citrate plasma from a previously acquired sequence to that of a pre-run sequence. The variation within the TIC intensity must be <±15%.
NOTE: The backpressure should be within the range 500–580 bar at the beginning of each run [elution at 40% of the mobile phase (B)] and should not exceed the range 850–1000 bar [elution at 99% of the mobile phase (B)].
NOTE: If the initial backpressure is in the range of 580–725 Barr, switch LC flow to "Bypass" if pressure decreases change the Needle Seat and Seat Capillary. If pressure does not decrease, change the Rotor Seal and/or Sample Needle. If the initial backpressure is still high then replace the VanGuard pre-column. If pressure is still high replace Column.

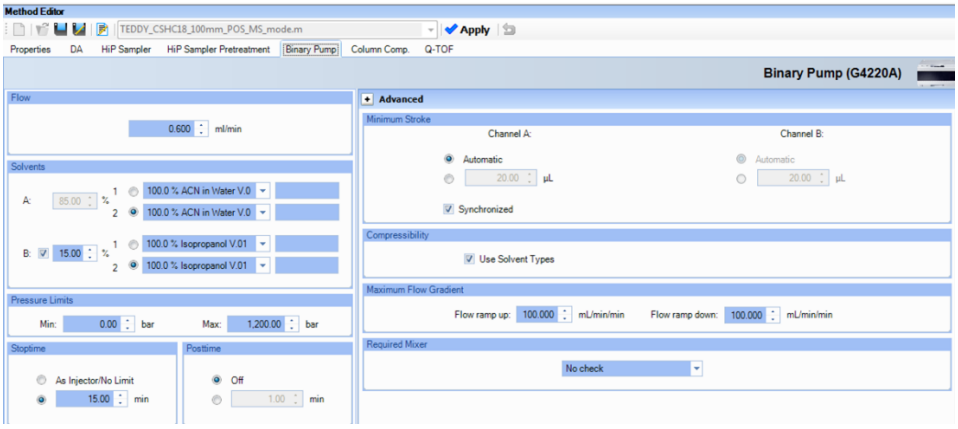
3.5 Lipid analysis method

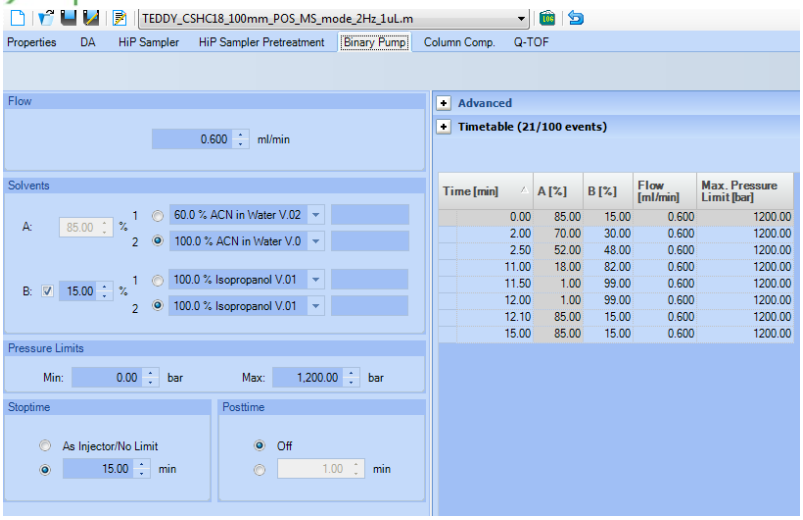
a. There are four different methods for lipid analysis, under the folder
<D>:\\<MassHunter\methods\
- Positive ion mode: CSH_Pos_Centroid_1.67µL.m
- Negative ion mode: CSH_neg_acetate_BWW_04202015.m

b. The autosampler, separation and column parameters for the lipid analysis method are as shown below:
- Autosampler: The only difference between Negative and Positive modes is the injection volume. Positive mode injects 1.67uL of sample, and negative mode injects 5uL of sample.

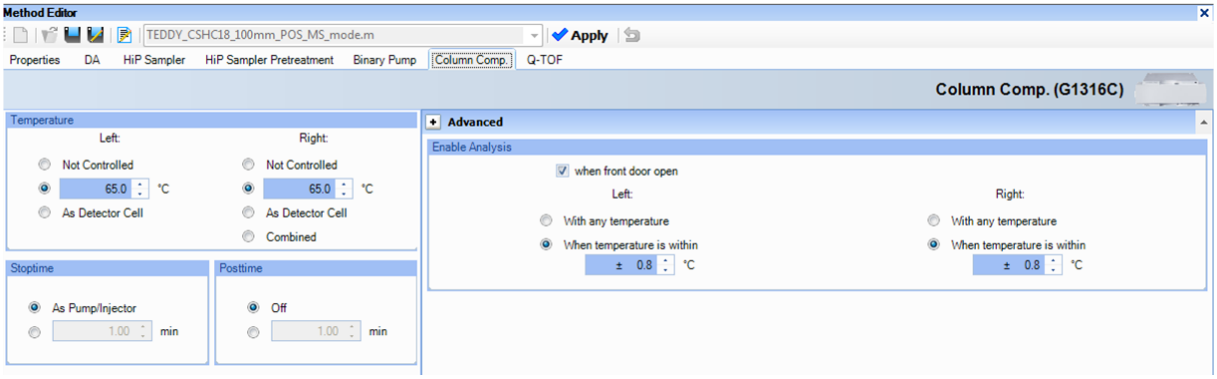


- Binary Pump Parameters:





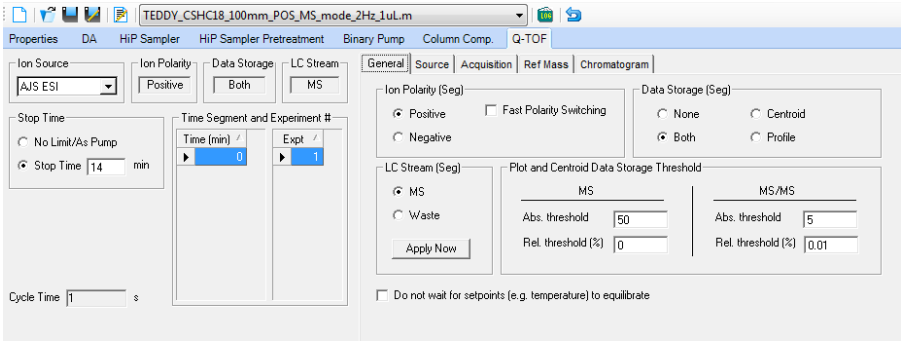
- Column manager



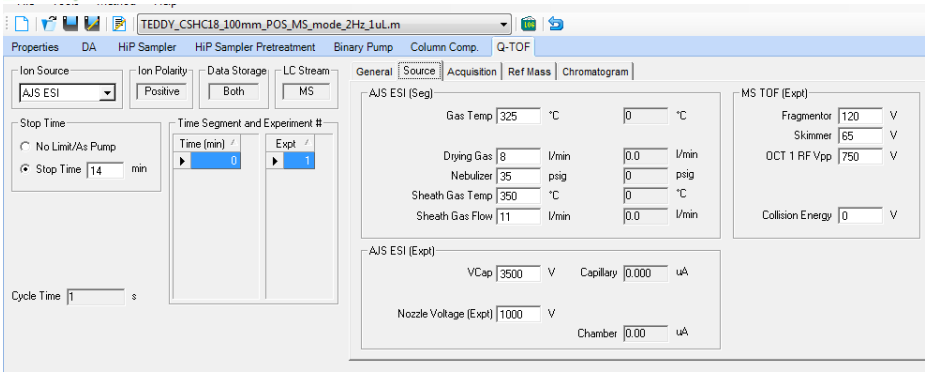
The MS conditions are the following:

3.5.1 Positive ion mode

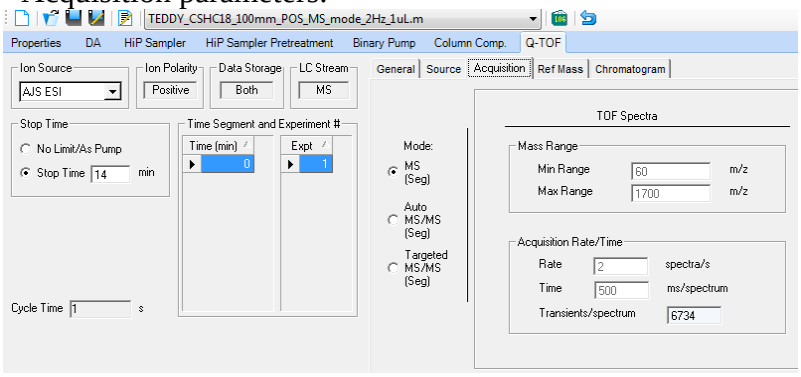
- General parameters



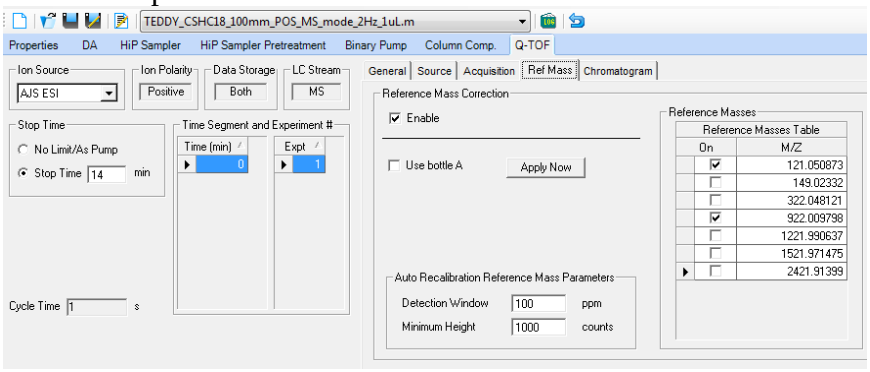
- Source parameters



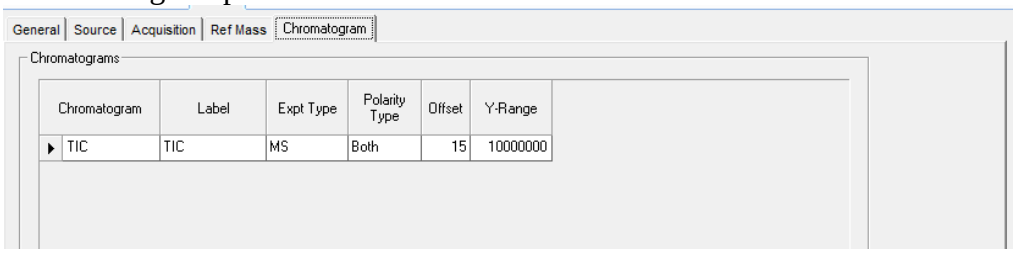
- Acquisition parameters:



- Ref Mass parameters



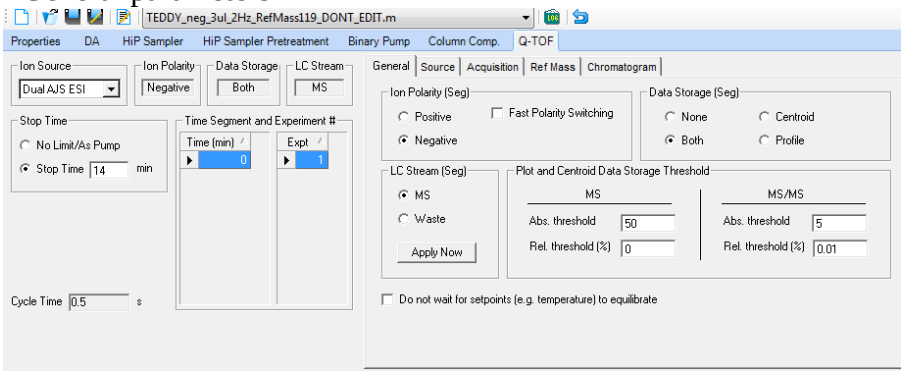
- Chromatogram parameters:



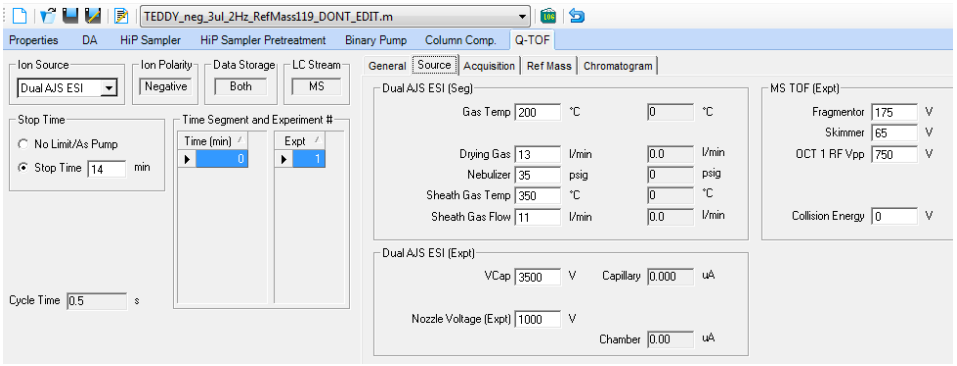
3.5.2 Negative ion mode

The parameters that vary from the positive mode are the following:

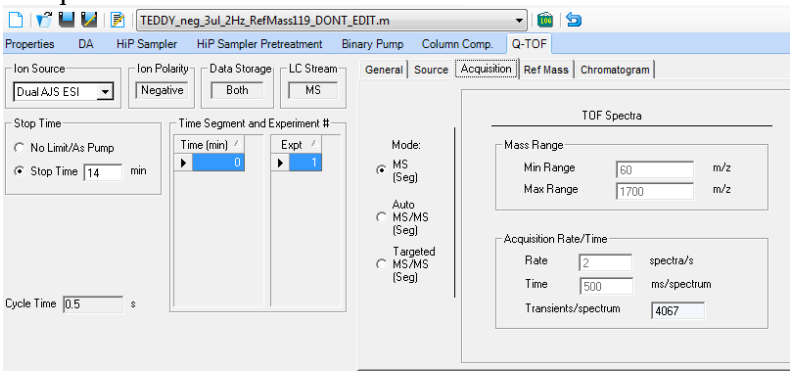
- General parameters



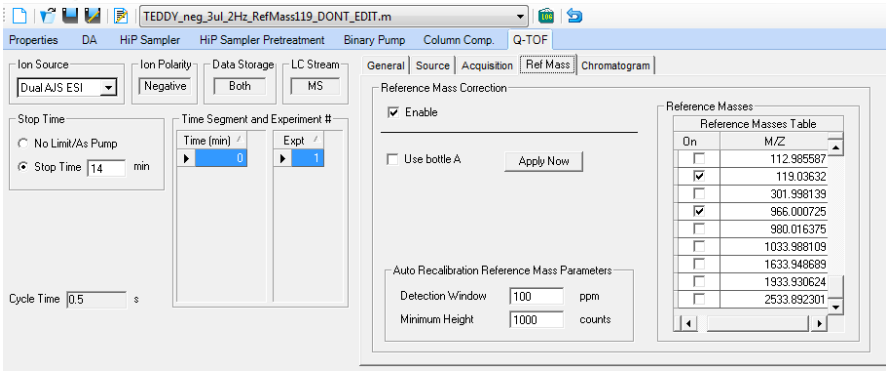
-Source Parameters:



-Acquisition Parameters:



- Reference Mass parameters



3.6 Column storage

Use this procedure to avoid precipitation mobile-phase buffers on the column and in the system.
a. Flush column with 50% acetonitrile by setting the pump flow rate to 0.1 mL/min and increase the flow rate to 0.6 mL/min over 5 min; keep the column at this flow rate for 10 min.
b. Flush column with 100% acetonitrile by setting the pump flow rate to 0.1 mL/min and increase the flow rate to 0.6 mL/min over 5 min; keep he column at this flow rate for 10 min.
c. Remove the column from the system.
d. Store the column in the box until the next batch analysis. Add the story usage of the column.

4. Problems

In order to avoid cross-contaminations and artifact formation, disposable consumables are used (Eppendorf plastic tubes, plastic pipette tips)

5. Disposal of waste

Chemicals are disposed into appropriate bottles in lab 2.157 under the fume hood before monthly disposal collection. Glass vials and consumables are collected into the plastic bags and stored under the fume hood in lab 2.157 before monthly disposal. Other GC-TOF waste (rubber seals, O-rings etc.) can be disposed into regular waste.

6. Data Processing of Lipidomics data using MassHunter (Agilent)

1. Open all samples in MassHunter Qualitative Analysis
2. Close all sample that will not be processed (ie bad original injection)
    a. Delete the samples that will not be processed from the folder
3. Open MassHunter Quantitative Analysis
    a. Go to file → New Batch
    b. Create a name and select all samples and add to batch
4. Open method (current is Positive Mode DP 08.20.2015)
    a. In qualitative analysis, have samples open
    b. Go through each internal standard and do an EIC

   c. Compare retention time (RT) for the internal standards between method file and samples
   d. Adjust RT as necessary for iSTDs by time segment (Update → Shift Retention Time)
   e. Go through the method and look at the peaks, adjusting as necessary for an early BioRec or qc sample.
   f. Save method for the batch
5. Click exit and apply+analyze the method to the batch.
6. Once batch has been analyzed, check a later BioRec/QC in the batch and go through peaks again (View→Compounds-at-a-Glance)
7. If happy with the peaks, then export the results (File→Export→Export Table). Save as a .csv table.
8. Go through the initial export and backfill all missing values.
9. Export results as .csv file.

Excel tabs:
Raw: Re-label first worksheet as "Raw"

Matched: Copy the data from the "Raw" worksheet. Insert 10 columns and 7 rows. Sort result by Identifier. Copy the template from *Pos template 1.1* excel file. In J14, set the formula as =A14=L14, then drag down to the end of the compound list. Check to make sure all values are true for positive mode.

Sorted: Copy the data from Matched. Copy the MZ and RT data from results (Column L and M) to the template (Column F and G). Create these columns:

Blank Average	Fold1	Sample average	Sample min	Sample Max	Fold2	Stdev	%CV
=average(blanks)	$=\frac{Avg._{sample}}{Avg_{blank}}$	=average(samples)	=min(samples)	=max(samples)	$=\frac{\max_{sample}}{Avg_{blank}}$	=stdev(samples)	$=\frac{Stdev_{sample}}{Avg_{sample}}$

Reduced: Copy data from sorted. Sort all data, except iSTDs. Delete all data with fold2 < 10. Also delete all data with a sample average <500 (for negative) and <1000 (for positive).

Check: Copy data from reduced tab. Sort left to right to group the biorecs/QCs together. Get %CV for biorecs/QC. Investigate all compounds that have a %CV > 20%. If peaks look inconsistent with results, then reprocess the data. Otherwise, make a note indicating that the results match the data.
Final: Copy from check. Paste over rows that were fixed.

Combined: Copy data from final tab. Add a row below each set of compounds that are adducts of one another. Combine identifier, annotation, and mz with =concatenate(A24," _", A25) changing

column for each. For annotation and InChi key, take value from one row above. For retention time, average all adduct RTs together. Do this for all rows, the copy and paste values. Ex:

Identifier	Annotation	InChi key		Batch m/z	Batch RT
10.34_640.60	CE (16:1)	[M+NH4]+	HODJWNWCVNUPAQ-FSAOOAOSSA-N	640.6032	10.495
10.33_645.56	CE (16:1)	[M+Na]+	HODJWNWCVNUPAQ-FSAOOAOSSA-N	645.5581	10.493
10.34_640.60 _10.33_645.56	CE (16:1)	[M+NH4]+ _[M+Na]+	HODJWNWCVNUPAQ-FSAOOAOSSA-N	640.6032 _645.5581	10.494

Then delete individual adduct rows.

Submit: Copy data from combined sheet. Format the data (colors). Replace all blank cells with value of 1. Fill in the info for the study.

Data were then normalized. "Natural" batches were first considered based upon run time intervals. Five batches were selected that have the biggest time gap among them. A T-test was used on QC's between neighbor batches. If T-test failed to show a group difference between these two batches, those two batches were then merged, providing more power for LOESS fitting. Data were normalized using R with QC (BIOREC plasma) – based LOESS normalization (span = 0.25) after normalizing the batches together (see above). Performance was validated by random cross-validation using 20 QCs (BIOREC plasma) as a test set. When multiple adducts exist for a single compound, the adduct with the lowest CV of the QCs was selected for inclusion. Positive and Negative ion mode data were combined into a single file.

About the Authors

This document was prepared by staff members at the West Coast Metabolomics Center. For more information, please contact Dinesh Kumar Barupal, PhD by email at dinkumar@ucdavis.edu.