Issued: 06-24-2013 Valid from: 06-24-2013	Validity area: UC Davis Genome Center, Metabolomics Core and Research Laboratories
Responsible: Bill Wikoff	Secondary: Carlos Leon, Brian DeFelice
This SOP supersedes: *Lipidomic Analysis by UPLC-QTOF MS* lipids 10102012	approved: Oliver Fiehn

Lipidomic analysis by UPLC-QTOF mass spectrometry

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 (ACN): 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 –Aldrich,Mass Spec Grade (73594-25G-F)
- Isopropanol (IPA): LC/MS grade (Fluka 34965-2.5L)
- Bioreclamation IVT Normal Human Plasma Sodium EDTA lot No BRH599015
- 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)

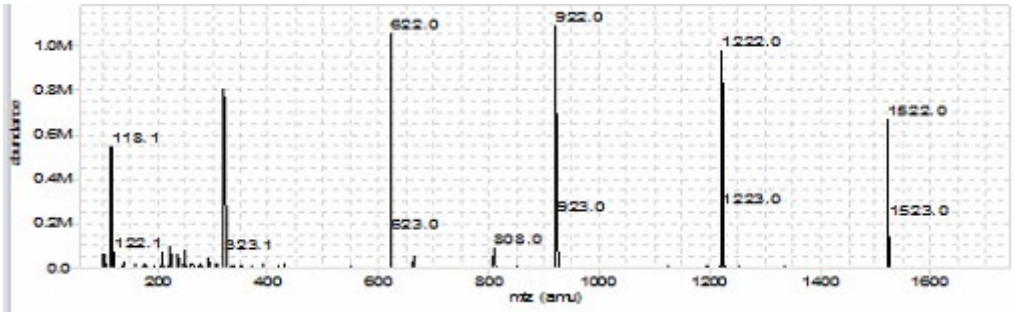
a. Use “Standard Tune” before each run of 300 sample batch.

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.

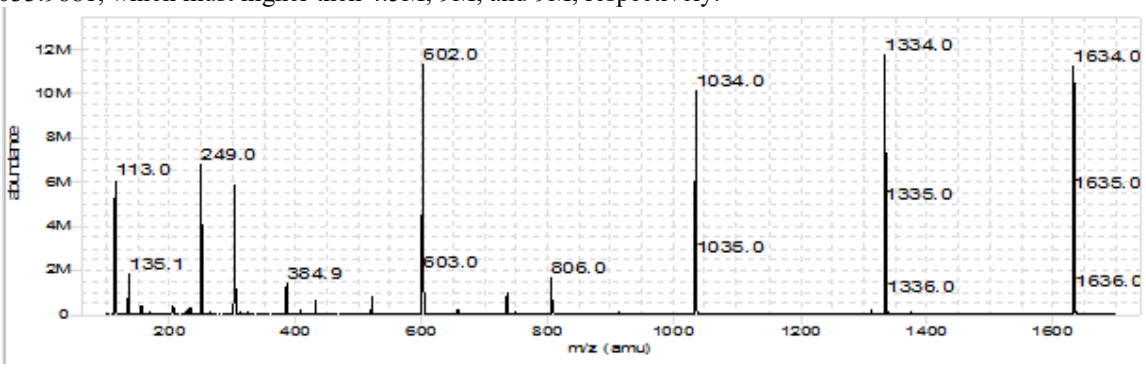
d. Print the tune report from the standard tune.

- 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.

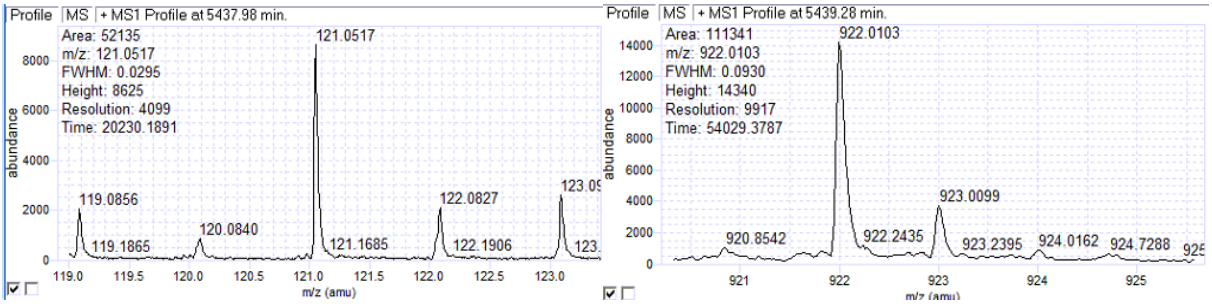


e. If the intensity of even one of the selected ion is below this value clean the ion source and repeat the instrument tuning.

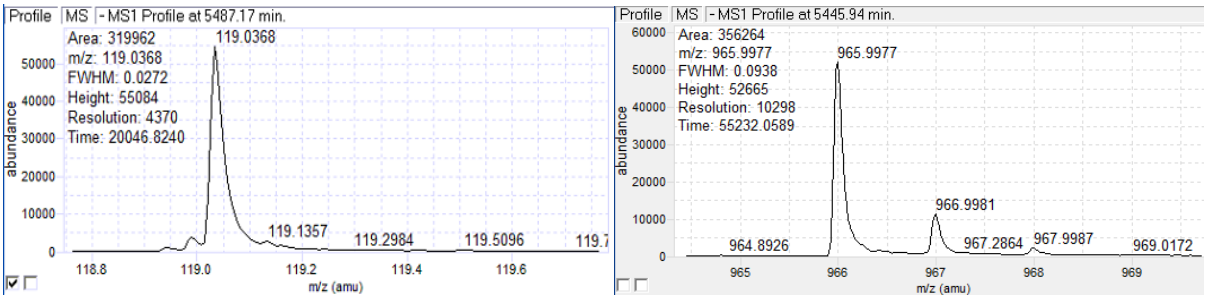
3.1.2 Check Reference ions (Instrument in Acquisition mode)

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* 922.0098, 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 buffer-containing mobile phases and connect the inlet end of the column to the injector outlet.
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.

c. When the mobile phase is flowing freely from the column outlet, stop the flow and attach the column outlet to the ion source of the mass spectrometer.
d. Gradually increase the flow rate 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.
e. Monitor the backpressure until a steady values is achieved.
f. Stop the flow and flush column with mobile phase (A) and (B) (see preparation of solutions below) at a ratio of 50:50 by setting the pump flow rate to 0.1 mL/min and increase the flow rate to 0.6 mL/min over 5 min.
g. Monitor the backpressure until a steady values is achieved. For a new column a value of backpressure should be 500–550 bar at the beginning of the injection (elution at 40% of the mobile phase (B), see preparation of solutions below).
h. Inject 5 blank (methanol). Record the lowest and highest value of backpressure for the first and the last sample injected.
f. Inject 10 X Bioreclamation plasma samples. Record the lowest and highest value of backpressure for the first and the 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 (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 (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 B for negative ion mode** (90:10 IPA:ACN + 10 mM ammonium acetate).
(*Only mobile B is changed for negative mode. Mobile Phase A is kept same as positive mode*.)

1. Add 1mL H2O to a 1L glass bottle
2. Add 0.770g ammonium acetate to the same 1L glass bottle
4. Gently shake 1L glass bottle to dissolve ammonium acetate
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. 1× "No sample injection"
2. 5× Blank sample injection (methanol)
3. 2× QC-mix injection
4. 2× Bioreclamation 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 (positive ion mode)

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 Analytes of the QC-mix solution (negative ion mode)

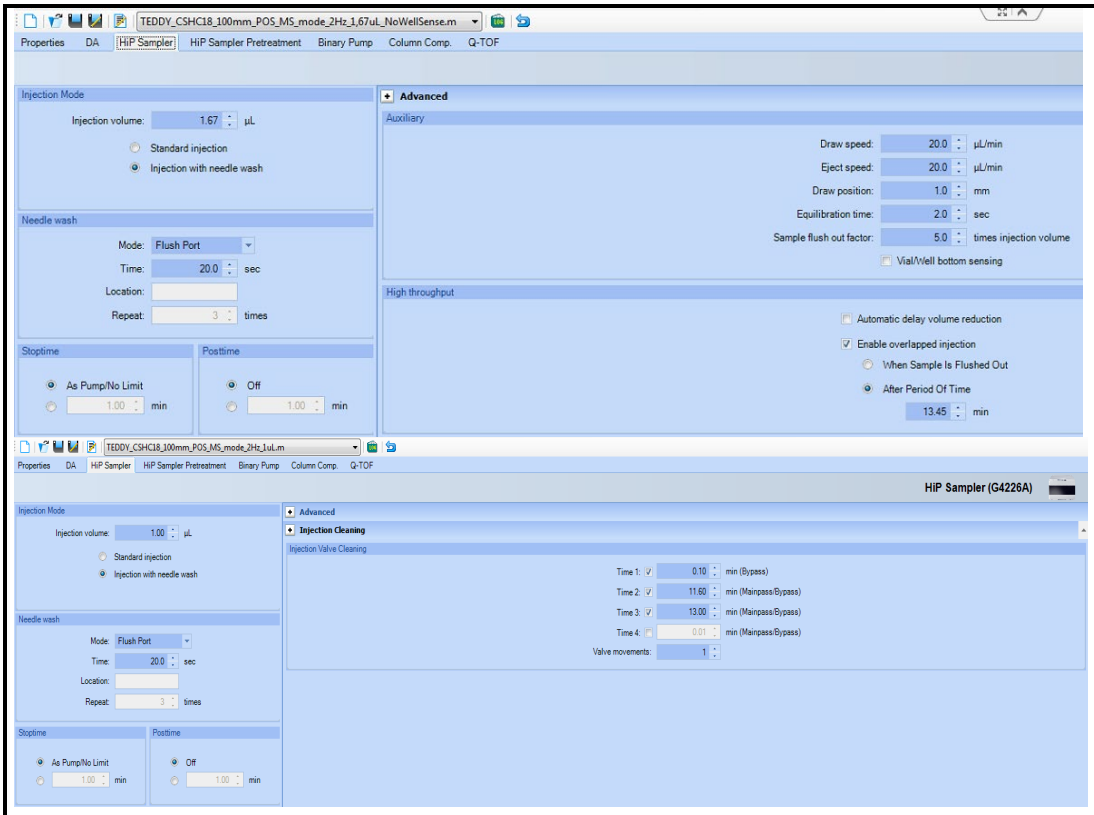
Common Name	Formula	MS1 m/z	RT (min)
Ceramide (d18:1/17:0) [M+Cl]$^-$	C35H69NO3	586.4961	5.90
Ceramide (d18:1/17:0) [M+HAc-H]$^-$	C35H69NO3	610.5409	5.90
CUDA [M-H]$^-$	C19H36N2O3	339.2644	0.57
Palmitic acid (16:0)-d3 [M-H]$^-$	C16H29D3O2	258.2515	2.40
LPC (17:0) [M+HAc-H]$^-$	C25H52NO7P	568.3609	1.81
LPE (17:1) [M-H]-	C22H44NO7P	464.2770	1.31
MAG (17:0/0:0/0:0) [M+HAc-H]$^-$	C20H40O4	403.3054	3.04
PC (12:0/13:0) [M+HAc-H]$^-$	C33H66NO8P	694.4662	3.53
PE (17:0/17:0) [M-H]$^-$	C39H78NO8P	718.5394	6.16
PG (17:0/17:0) [M-H] $^-$	C40H79O10P	749.5347	5.07
SM (d18:1/17:0) [M+HAc-H]$^-$	C40H81N2O6P	775.5985	5.06

NOTE: Compare the profile of Bioreclamation 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 bar, 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 analytical column.

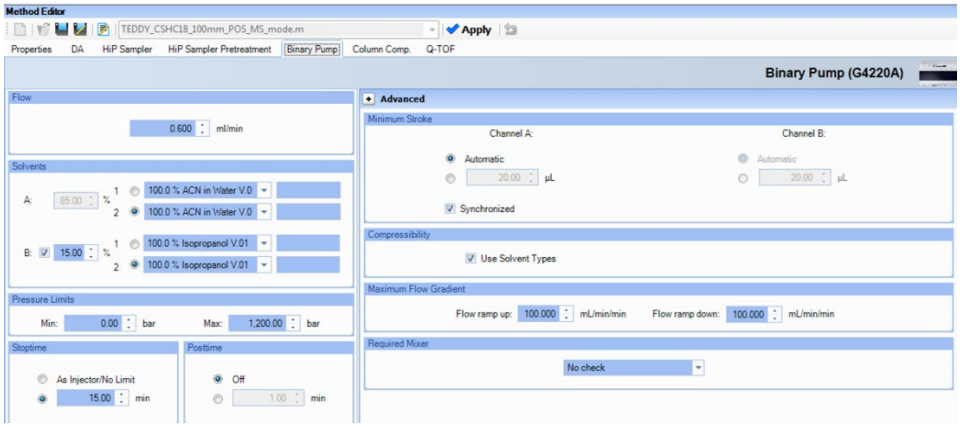
3.5 Lipid analysis method

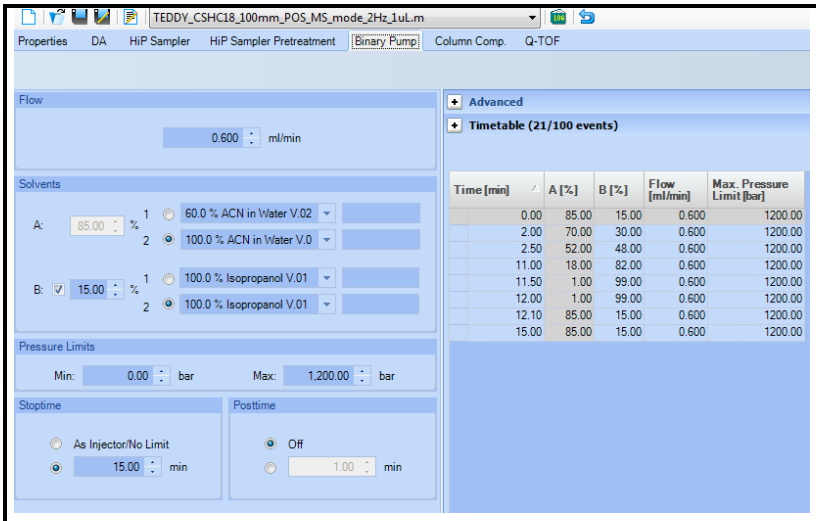
a. There are four different methods for lipid analysis, under the folder <D>:\\<MassHunter\methods\TEDDY methods>\:
- Positive ion mode: TEDDY_CSHC18_100mm_POS_MS_mode_2Hz_1,67uL_NoWellSense.m
- Negative ion mode: TEDDY_neg_5uL_2Hz_RefMass119_DONT_EDIT.m
- Positive ion MSMS mode: TEDDY_CSHC18_100mm_POS_MSMS _mode_1,67uL_2Hz.m
- Negative ion MSMS mode: TEDDY_MSMS negative mode_2Hz_5uL_119Ref_2.m

b. The autosampler, separation and column parameters for the lipid analysis method are as shown below:
- The only differences between Negative and Positive modes are the injection volume and mobile phase B. Positive mode injects 1.67uL of sample, and negative mode injects 5uL of sample. Mobile Phase B for negative ion mode has ammonium acetate as a modifier with exactly same organic solvent composition.

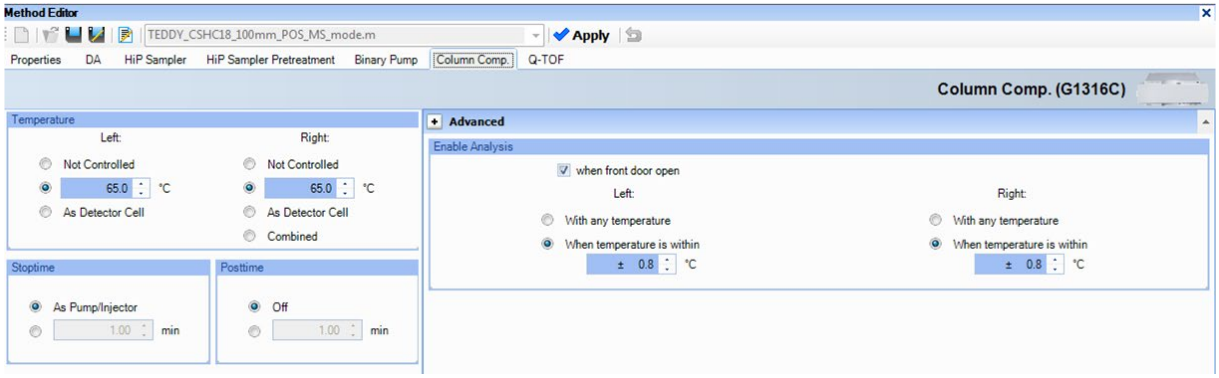


- Binary Pump Parameters:

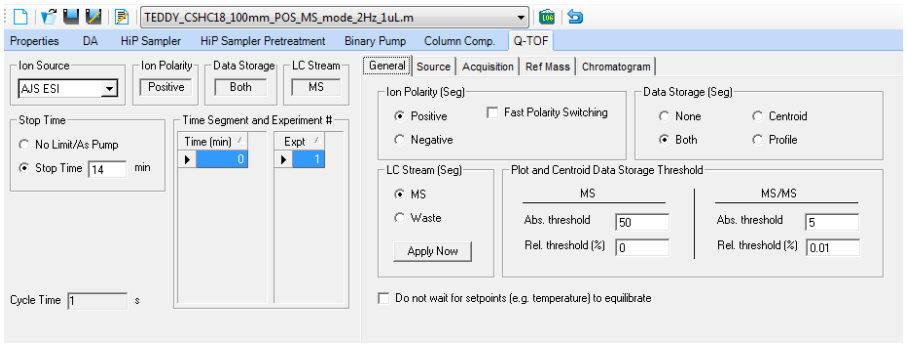




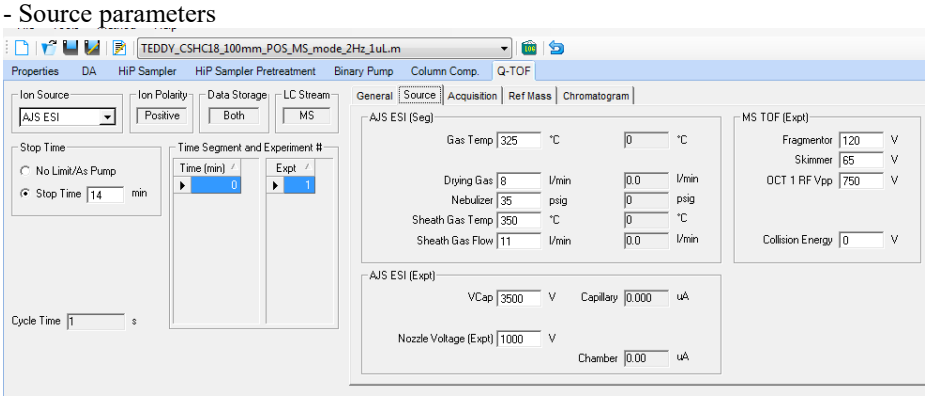
- Column manager



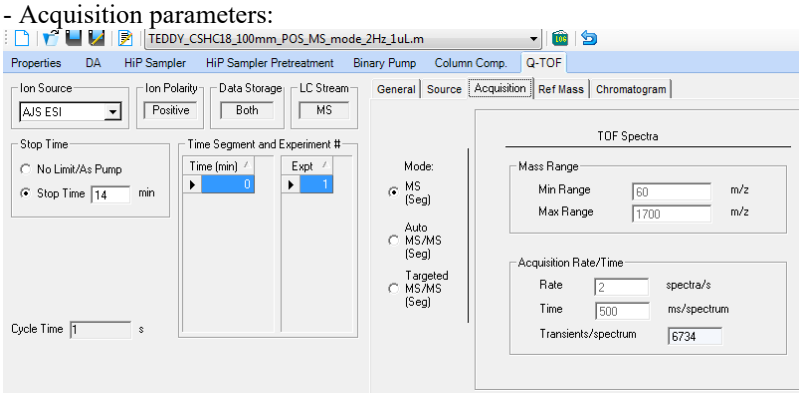
The MS conditions are the following:
3.5.1 Positive ion mode
- General parameters



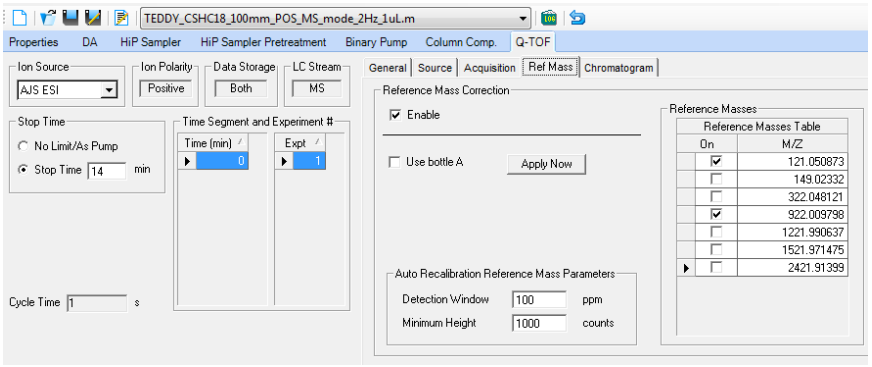
- Source parameters



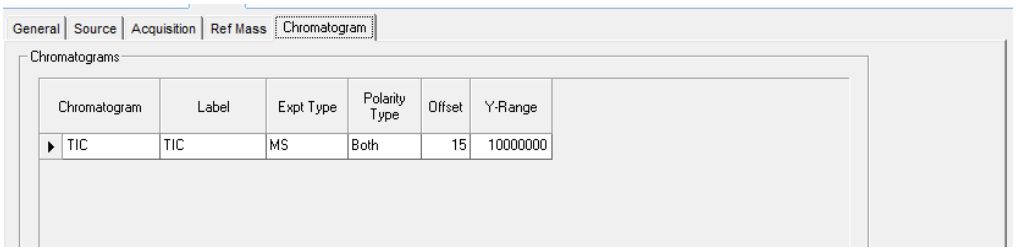
- Acquisition parameters:



- Ref Mass parameters



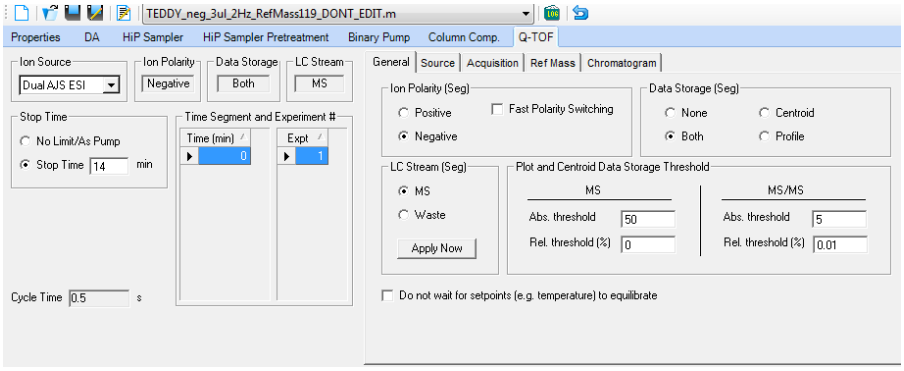
- Chromatogram parameters:



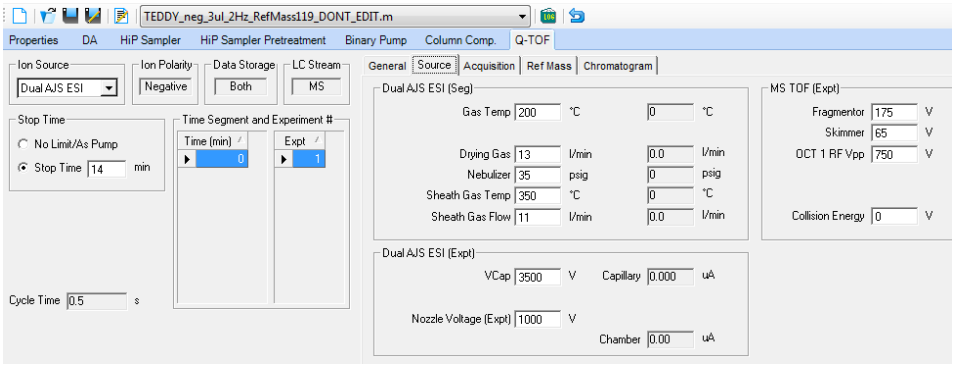
3.5.2 Negative ion mode

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

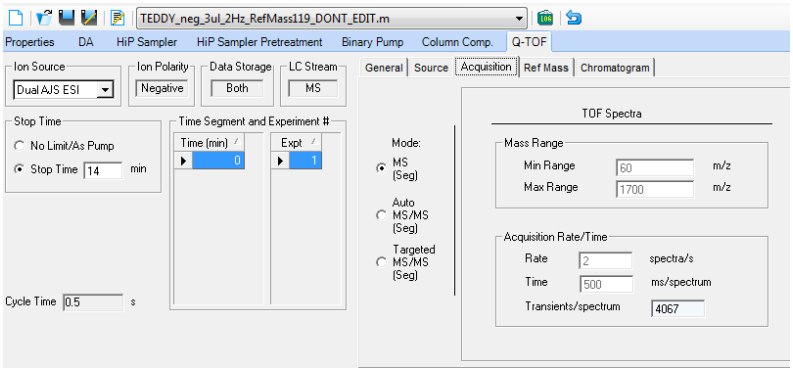
- General parameters



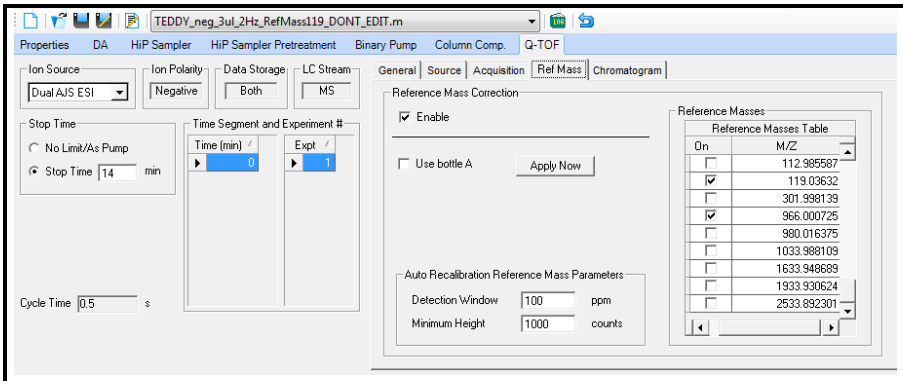
-Source Parameters:



-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.