Issued: 07-26-2019 Valid from: 07-26-2019	Validity area: UC Davis Genome Center, Metabolomics Core and Research Laboratories
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This SOP supersedes: new	Approved: Oliver Fiehn

1. Instruments

- Thermo Scientific Q Exactive mass spectrometer
- Thermo Scientific Vanquish UHPLC Systems
- Ultrasonicator
- VWR VX-2500 Multi-tube vortexer
- Eppendorf Centrifuge 5424

2. Chemicals and consumables

- Kinetex PFP column (1.7 µm, 2.1 mm X 30 mm) (00A-4476-AN)
- Security Guard ULTRA Holder, for UHPLC Columns 2.1 to 4.6mm ID (AJ0-9000)
- SecurityGuard™ ULTRA Cartridges UHPLC PFP 2.1mm ID Columns, 3/Pk (AJ0-8787)
- Pipettes
- Pierce™ LTQ ESI Positive Ion Calibration (88322)
- Pierce™ Negative Ion Calibration Solution (88324)
- Acetonitrile: Optima LC/MS Grade Fisher Chemical (A955-4, 4L)
- Water: Optima LC/MS grade Fisher Chemical (W6-4, 4 L)
- Formic Acid: Fluka Mass Spec Grade (F0507, 250 mL)

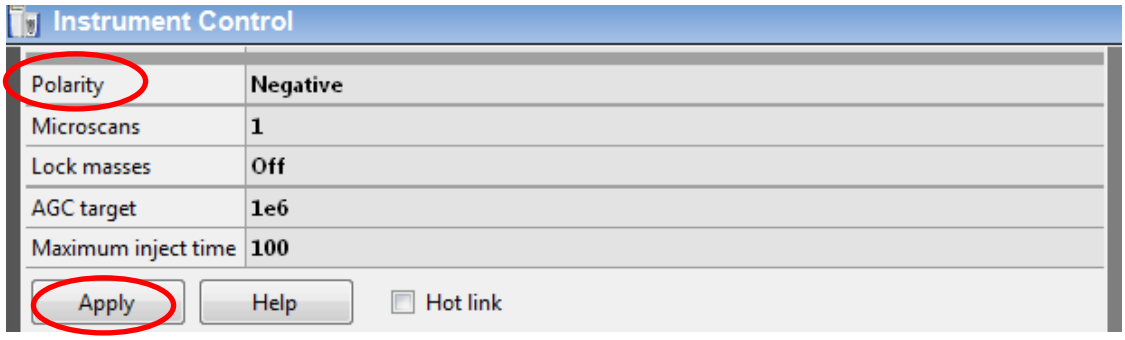
3. Procedure

3.1 Pre-run procedures

3.1.1 Instrument calibration

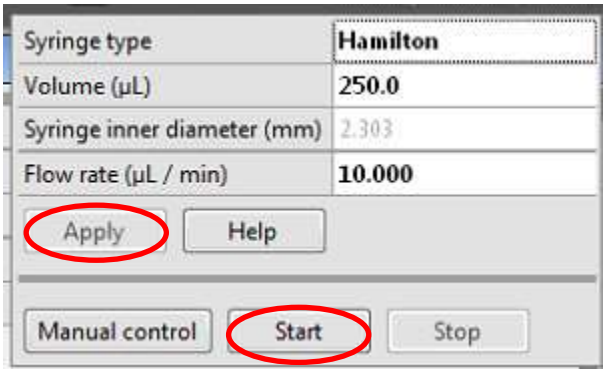
a. Click on Tune icon  . Go to **File** > **Load tune file**. (i.e. Polyphenol_Tune_07122019.mstune)

b. Select appropriate polarity and click **Apply**.

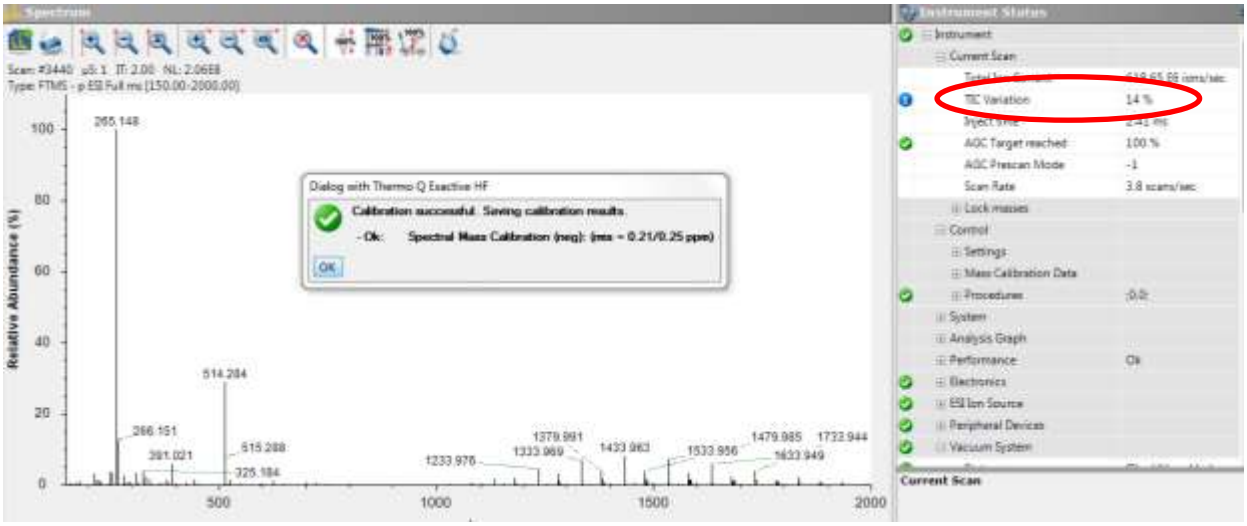


c. Use the Thermo Scientific Pierce ESI Negative Ion Calibration Solution for negative mode calibration and Pierce™ LTQ ESI Positive Ion Calibration for positive mode calibration. Load the ready use reference standard solution into a syringe and inject directly into the ion source using syringe pump and designated peek tubing.

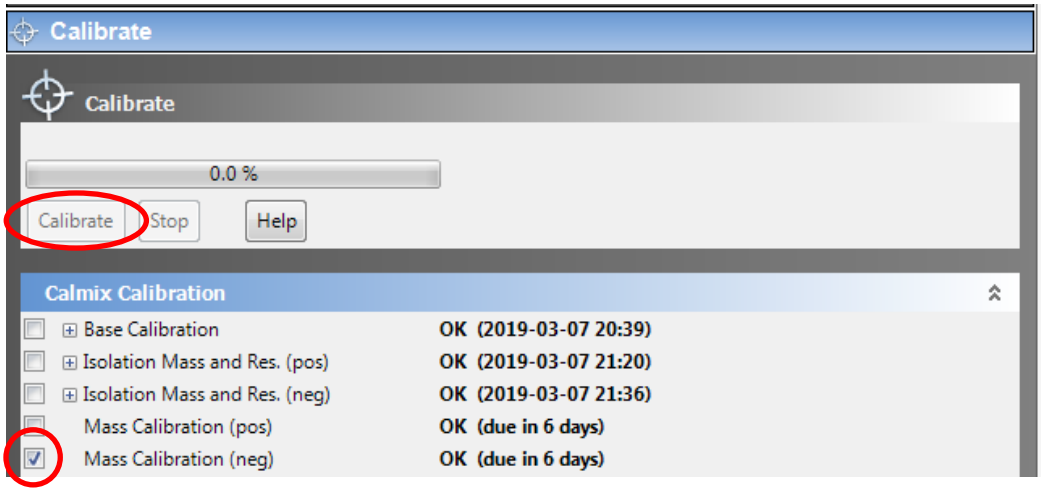
d. Click on syringe pump settings  . Enter flow rate (µL/minutes) (i.e. 5, 10, etc), then click **Apply** and **Start** to begin the calibration procedure.



e. In (-) mode, check the profile of the calibrant and ions of *m/z* 265.148 (SDS), *m/z* 514.284 (sodium taurocholate), and Ultramark 1621. The TIC variation should be less than 15%. If the TIC variation is higher than 15%, clean the ion source and repeat the instrument calibration.

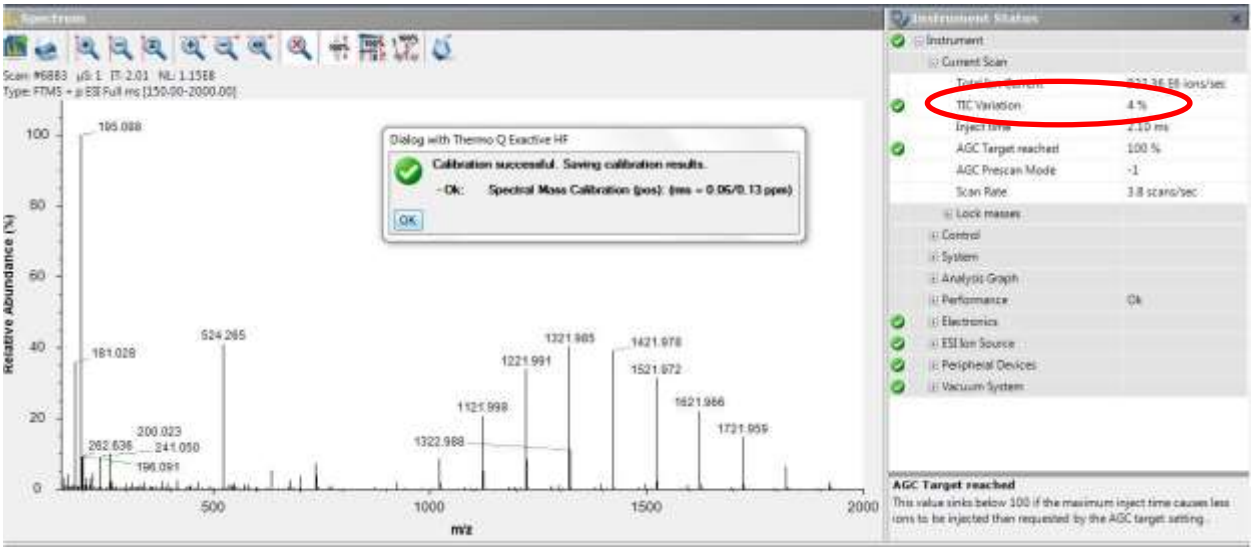


f. Once the signal intensity has stabilized, check **Mass calibration (neg)** and click **Calibrate**. The instruments should be re-calibrated weekly.



g. Repeat steps b-d for positive mode calibration.

h. In (+) mode, check the profile of the calibrant and ions of m/z 195.088 (Caffeine), m/z 524.265 (MRFA) and Ultramark 1621.



i. If the TIC variation is higher than 15%, clean the ion source and repeat the instrument calibration.

3.1.1 Column equilibration

a. Click on Thermo Xcalibur icon  .

b. From the menu bar, click  for main menu or  for sequence setup.
c. To take control of the UHPLC module, click **Status** and then **Direct Control** (bottom).
d. This will direct you to the pump module page.
e. Purge the system by clicking **More Options**. Make sure appropriate eluent is selected (i.e.A1, B1 or A2, B2). Click 100% on line A and purge for 3 minutes at 3 mL/minutes with 50:50 = (acetonitrile: water). Repeat for line B.
f. Then purge line A with mobile phase (A) and line B with mobile phase (B) for 3 minutes at 3 mL/minutes to remove acetonitrile and water.
g. Place the column with guard column into the column compartment. Equilibrate the column with mobile phase (A) and mobile phase (B) at ratio of 93:7 (starting condition) by setting the pump flow rate at 0.1 mL/minutes; increase the flow rate to 0.4 mL/minutes gradually over 5 minutes. Leave at this flow rate for 30 minutes.

3.2 Preparation of solutions

a. **Mobile phase A** (100% LC/MS grade water + 0.1% Formic Acid)
1. Pre-rinse three times 1 L glass bottle with pure acetonitrile.
2. Add exactly 1000 mL LC/MS grade water.
3. Add 1 mL formic acid to the a 1 L glass bottle.
4. Sonicate for 10 minutes at room temperature.

b. **Mobile phase B** (100% ACN + 0.1% Formic Acid)
1. Pre-rinse three times 1 L glass bottle with pure acetonitrile.
2. Add exactly 1000 mL LC/MS acetonitrile.
3. Add 1 mL formic acid to the a 1 L glass bottle.
4. Sonicate for 10 minutes at room temperature.

c. Resuspension solvent
1. Prepare 100 mL of 7%ACN in water in a graduate flask.
2. Aliquot 100 uL of hippuric acid-d5 iSTD (100 ppm), 100 uL of quercetin-d3 iSTD (1000 ppm), and 200 uL for all other iSTDs (100 ppm) (See Table 1 & 2) into a 20 mL volumetric flask.
3. Bring to the final volume of the solution to 20 mL with 7%ACN in water.
4. Sonicate for 10 minutes.
5. Store at 4°C.

d. Preparation of samples for analysis
1. Sonicate resuspension solvent for 5 minutes and swirl gently before use.
2. Resuspend samples in 100 µL of the resuspension solvent depending on sample concentration.
3. Vortex sample for 30 seconds.
4. Sonicate samples for 5 minutes.
5. Centrifuge for 2 minutes at 16,100 rcf.
6. Transfer supernatant to LC-MS micro insert in an amber vial.
7. Cap and load into auto sampler.

3.3 Run sequence setup

a. From the menu bar, click  for sequence setup.
b. Create run list on excel spreadsheet and save as .csv.
c. From the menu bar, chose **File** > **Import sequence** > **.csv file**
d. After running the pre-run sequence (3 x blank injection), inject the following:
   i) Method Blank
   ii) BioRec EDTA plasma/ NIST SRM3291
   iii) Pool samples
   iv) 10 samples
   v) Repeat steps i-iv

Table 1 Internal standards for positive mode

Name	Concentration in Resuspension Solvent (ppm or ug/mL)	MS1 (*m/z*)	RT (min)
Hippuric acid-d5 iSTD	0.5	185.097	0.76
Caffeine-d10 iSTD	1	204.144	0.77
(−)Epigallocatechin Gallate-d3/d4 iSTD	1	462.111	1.44
trans-Cinnamic Acid -d5 iSTD	1	154.091	3.01
trans-Resveratrol-d4 iSTD	1	233.111	3.03
Daidzein-d4 iSTD	1	259.09	3.16
Quercetin-d3 iSTD	5	306.069	3.92
Genistein-d4 iSTD	1	275.085	4.32
Apigenin-d5 iSTD	1	276.092	4.62
2-hydroxyfluorene-d9 iSTD	1	192.136	5.39
CUDA iSTD	1	341.28	5.71
Reserpine-d9 iSTD	1	618.337	7.58

Table 2 Internal standards for negative mode

Name	Concentration in Resuspension Solvent (ppm or ug/mL)	MS1 (*m/z*)	RT (min)
Hippuric acid-d5 iSTD	0.5	183.081	0.76
(−)Epigallocatechin Gallate-d3/d4 iSTD	1	460.095	1.37
trans-Cinnamic Acid -d5 iSTD	1	152.075	2.95
trans-Resveratrol-d4 iSTD	1	231.095	2.97
Daidzein-d4 iSTD	1	257.075	3.11
Quercetin-d3 iSTD	5	304.053	3.86
Genistein-d4 iSTD	1	273.07	4.27
Apigenin-d5 iSTD	1	274.076	4.59
2-hydroxyfluorene-d9 iSTD	1	190.121	5.38
CUDA iSTD	1	339.265	5.71
Betulinic acid-d3 iSTD	1	458.372	6.18
Reserpine-d9 iSTD	1	616.322	7.54

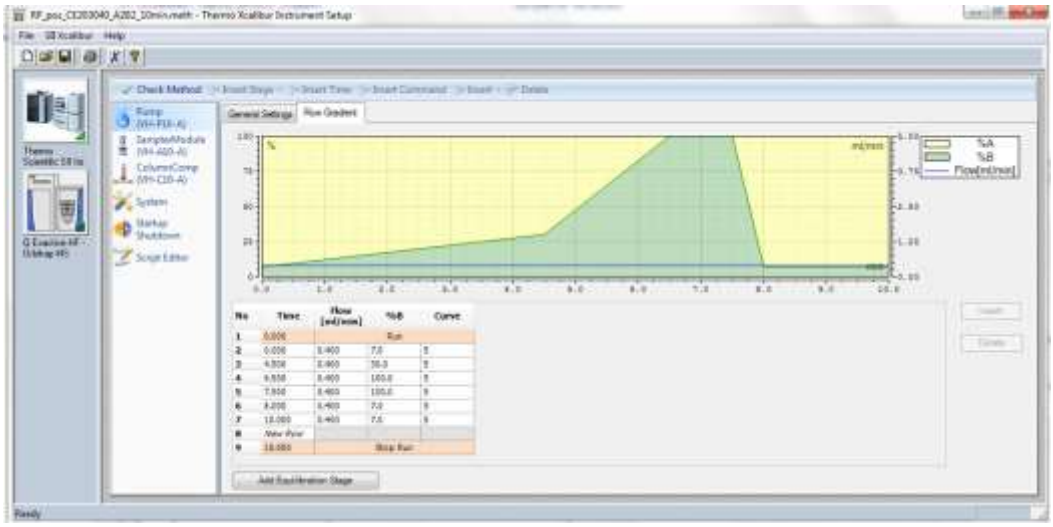
3.4 Reverse Phase analysis method

a. The methods for reverse phase analysis are located under the folder C:\Xcalibur\methods\2019\CORE\Polyphenol

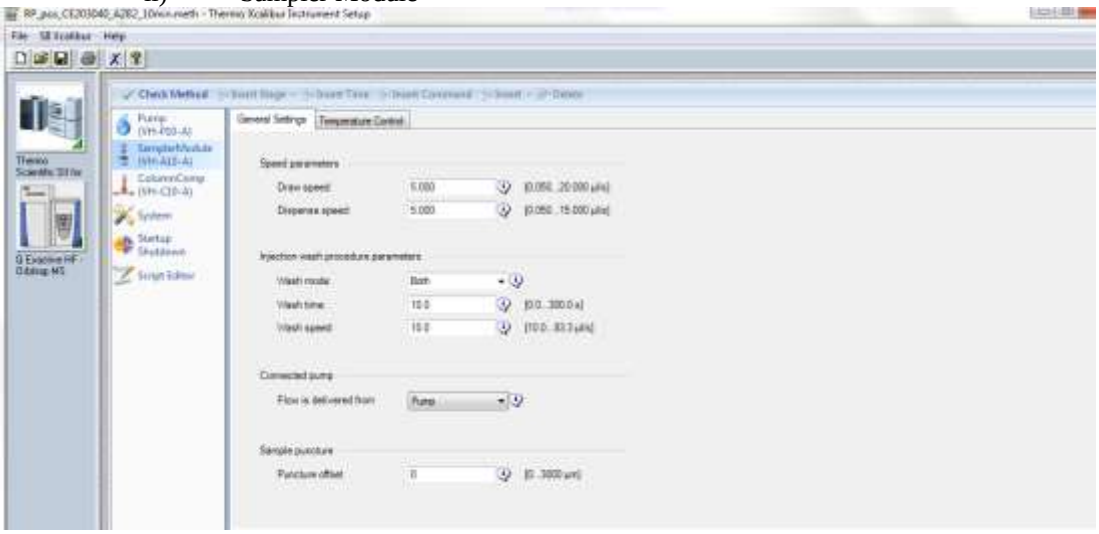
Positive ion mode: RP_pos_CE203040_A2B2_10min
Negative ion mode: RP_neg_CE203040_A2B2_10min
Polarity switching mode: RP_pos_neg_CE203040_A2B2_10min

b. The acquisition method for the reverse phase analysis method is as shown below:
3.4.1 **Positive ion mode**

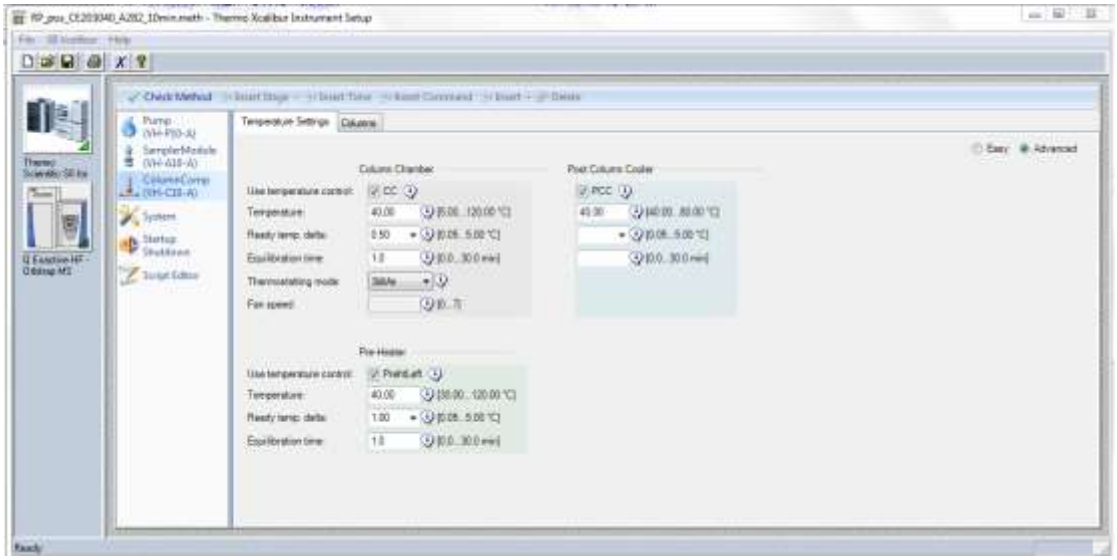
i) Pump


Check Method
Insert Stage
Insert Time
Insert Command
Insert
Delete
Pump (WI-P10-A)
SamplerModule (WI-A00-A)
ColumnComp (WI-C00-A)
System
Markup Shutdown
Script Editor
General Settings
Flow Gradient
%A
%B
Flow[ml/min]
No
Time
Flow [ml/min]
%B
Curve
Run
Stop Run
Add Equilibration Stage
Ready

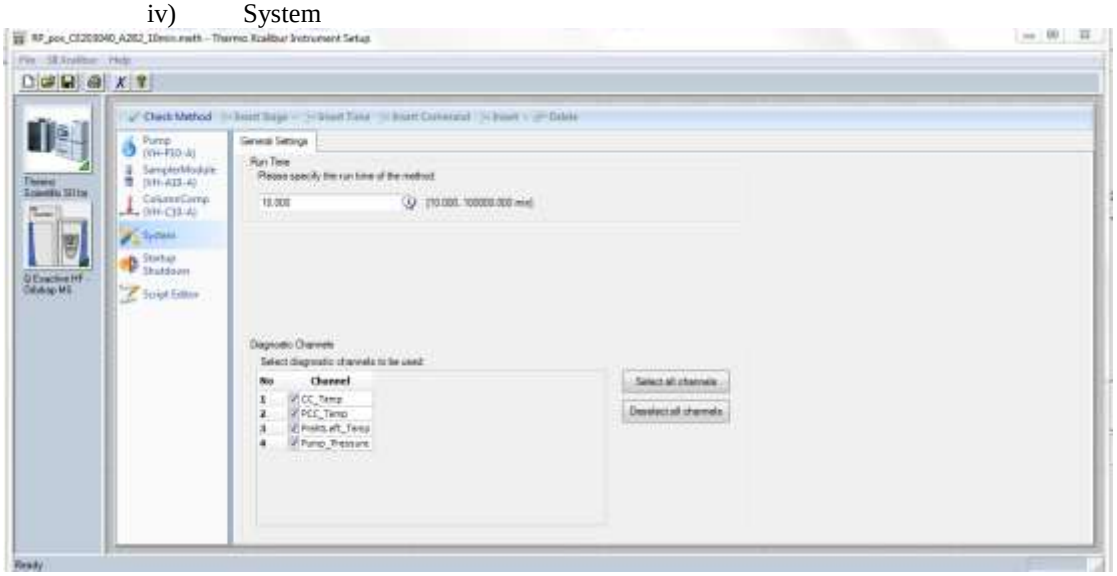
ii) Sampler Module



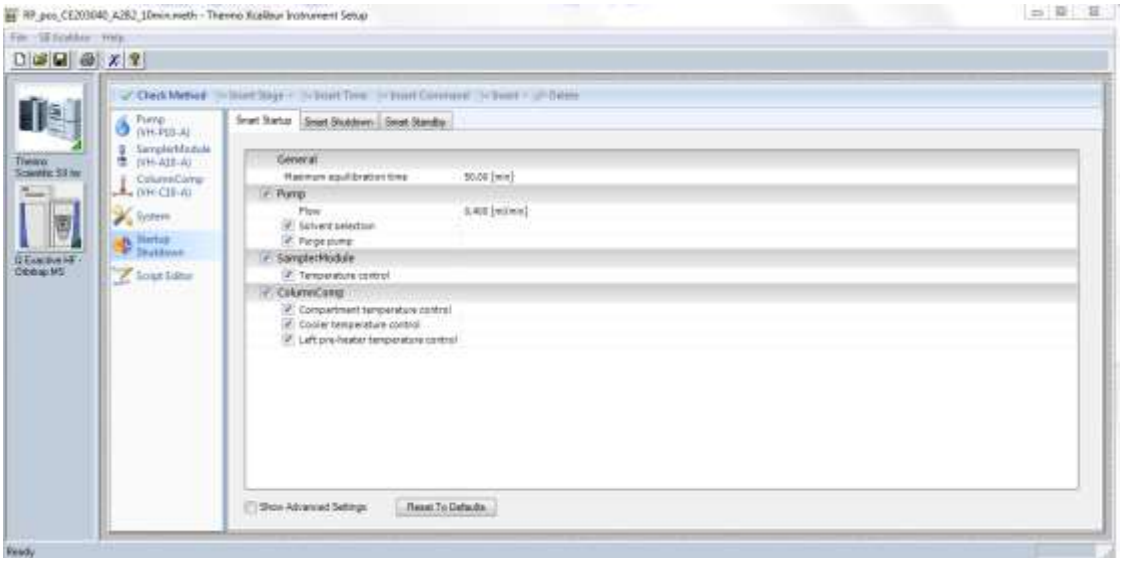
iii) Column Compartment



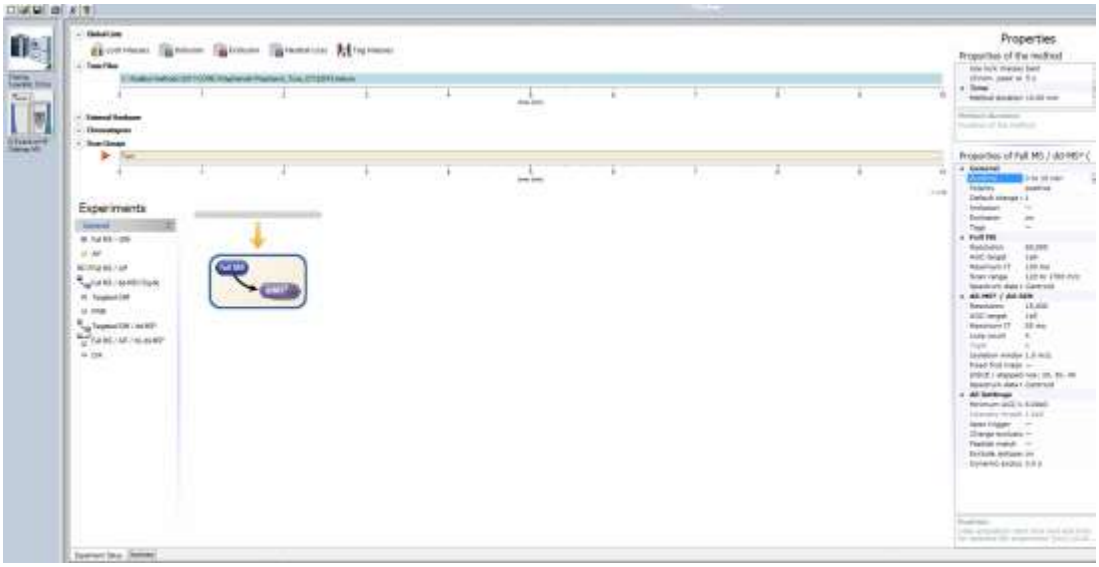
iv) System



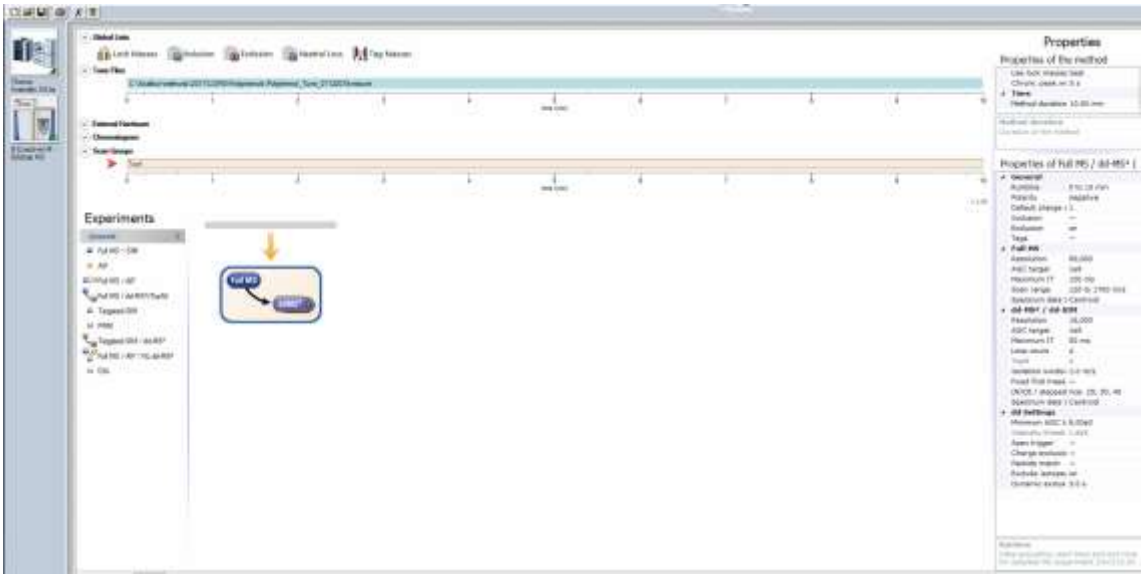
v) Startup Shutdown



The **MS** conditions are the following:



3.4.2 **Negative ion mode**
The parameters that vary from the positive mode are the following:



3.5 Column storage

Use this procedure to avoid precipitation mobile phase additives on the column and in the system.
a. Flush column with 50:50 (acetonitrile:water) by setting the pump flow rate to 0.1 mL/minutes and increase the flow rate to 0.4 mL/minutes over 5 minutes; keep the column at this flow rate for 10 minutes.
b. Flush column with 100% acetonitrile by setting the pump flow rate to 0.1 mL/minutes and increase the flow rate to 0.4 mL/minutes over 5 minutes; keep the column at this flow rate for 10 minutes.
c. Remove the column from the system.
d. Store the column in the box until the next batch analysis.