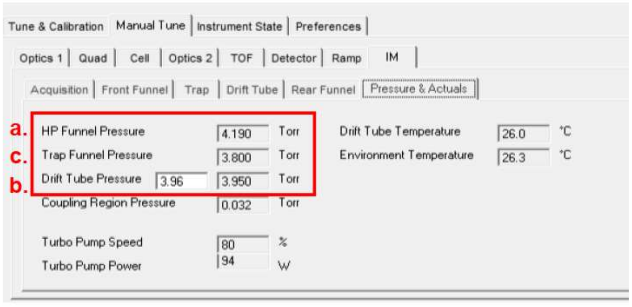
Preparing for experiments with Agilent 6560:

1. Switch on HPLC modules (if they are not showing green light). Do this even if you are not using HPLC.
2. Switch on high purity gas on gas kit and check that you have correct gas connected for your experiments (N_2 or He).
3. Switch on HP funnel gas.
4. Switch on PC and start MassHunter Acquisition software.
5. Switch instrument on and let it equilibrate for 15 to 30 min.
6. Record gas cylinder pressures and vacuum readings in log book. If there is not enough gas for you measurements contact Elina or Johanna.
7. If you use direct infusion for your measurements, start to rinse infusion line with MeOH.
8. If you don´t need calibration, go directly to Measuring Mass Spectrum.

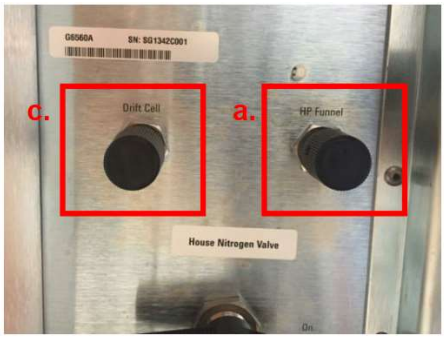
Calibrating Agilent 6560 for mass accuracy:

Note: Calibration can be done only using N2 as drift gas.

1. Perform the calibration with the same ESI-source that will be used to the measurements (dualESI for direct infusion and AJS dualESI for HPLC).
2. In MassHunter Acquisition software (brown icon) go to *Tune* context.
3. Check the pressures for source and drift region from Manual tune / IM / Pressures and actuals. They should be following for N2:
    a. *HP Funnel* pressure 4.0-5.0 Torr (Turn HP funnel knob clockwise to increase)
    b. *Drift Tube* Pressure 3.95 ± 0.3 Torr
    c. *Trap Funnel* Pressure 0.1-0.15 Torr below drift tube pressure (Turn Drift Cell knob clockwise to increase difference between Drift tube and Trap funnel pressures).

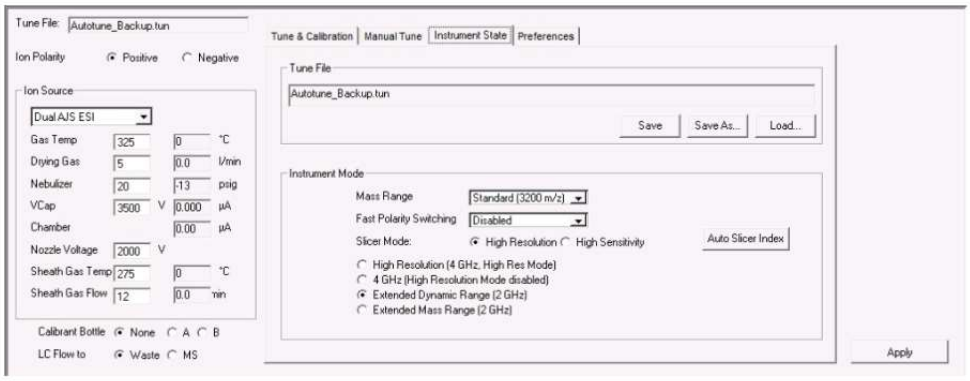


Tune Settings

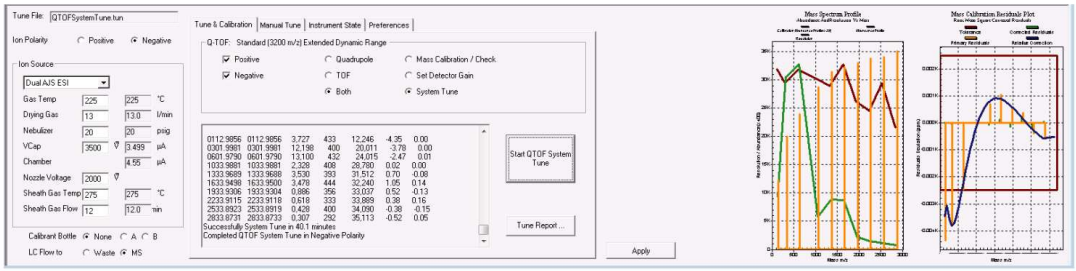


Pressure Control

4. Attach peek tubing coming from instrument to sprayer.
5. Download correct calibration file and add parameters for SWARM calibration at instrument state slide. Note: this sets mass range to your measurements! Typically following are used:
    - *Standard mass range (3200 m/z)*
    - *High Resolution*
    - *Extended Dynamic Range (2GHz)*



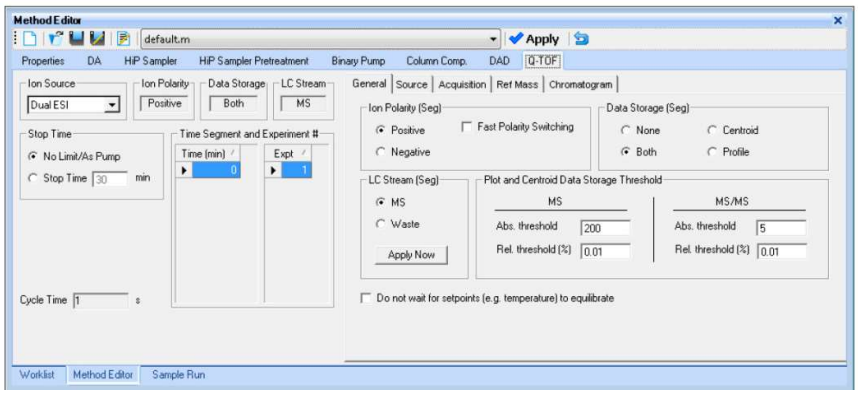
6. Go to tune and calibration slide and select *Positive* and/or *Negative* polarization, *TOF* and *Mass calibration / Check.*



7. Check that you have enough calibrant in bottle B, switch it on and start calibration when instrument is ready (showing green icons).
8. Check that the calibration is ok and save the calibration.

Measuring mass spectrum:

1. In MassHunter Acquisition software (brown icon) go to acquisition context.
2. Check that instrument ready and running.
3. Infuse your sample by direct infusion (flow rates 1-5 ul/min) or by HPLC. Please note: for direct infusion and HPLC runs different ESI-sources are used.
4. Download your method file and modify parameters in Method Editor. Save your modified method file with new name in your folder at *Data D:/MassHunter/Methods/User*



5. Start experiment using Sample Run or Worklist. Save your data in your own folder in *Data D:/MassHunter/Data/User*
    a. If you are using direct infusion, rinse the sprayer and infusion line after experiment with at least one full syringe of solvent you used in sample and one full syringe of methanol
    If you use HPLC remember to equilibrate column and change solvent as advised in column instructions. If you don´t use column on following day, please wash the column, fill it with storage solvent and remove it from the instrument.

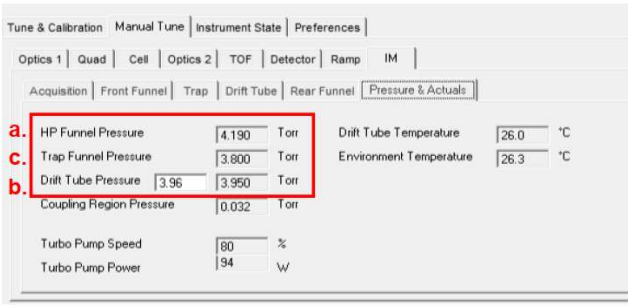
Instructions for running IM-MS experiment using N_2 drift gas:

1. Switch drift gas valve in gas kit to high purity gas.
2. Tune and calibrate instrument like normally using appropriate mass range, polarity and options for your application.
3. Go in *Tune* context → *Manual Tune* → *IM* → *Pressures and Actuals* check that with N_2:
    a. *HP Funnel* pressure is 4.0-5.0 Torr (typically 4.3 Torr, Turn HP funnel clockwise to increase)
    b. *Drift Tube Pressure* is 3.95 ± 0.3 Torr (typically 3.95 Torr, automatically adjusted)
    c. *Trap Funnel Pressure* is 0.1-0.15 Torr below that (typically 3.8 Torr, To increase pressure difference between Drift tube and Trap funnel turn *Drift Cell knob* to clockwise, to decrease it turn counterclockwise.).

If necessary, adjust them by using *Drift Cell valve* and *HP Funnel valve* in instrument front panel. Follow them during your measurements in Actuals window (Drift tube and trap funnel shown).

NOTE 1 : changes are extremely slow!!! Change in small increments!

NOTE 2: With He different values are used (see later)!



Tune Settings

Pressure Control

4. In *Acquisition* context open suitable IM tune file (for example MicroESI_IMMS.m) for your application. Modify parameters according to your needs and save it to your folder by new name.

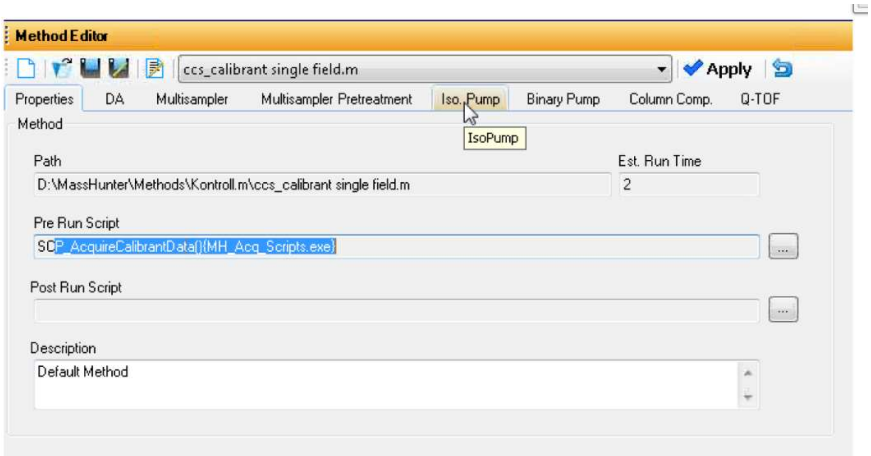
5. In *Method Editor* go to *QTOF* → *Advanced Parameters and Tune Values.* Tune following parameters according to your application:

Parameters	Labile molecules	**Small molecules**	Intact Proteins
High Pressure Funnel RF	150	150	200
Trap Funnel RF	50	120	200
Trap Entrance Grid Delta	7	7	12
Trap Exit Grid 2 Delta	10	10	15
Drift Tube Entrance Voltage	**1574**	**1574**	**1574**
Drift Tube Exit Voltage	217	224	224
Rear Funnel RF	120	90	200
Rear Funnel Exit	**38**	**45**	**45**
Rear Funnel Entrance	**210.5**	**217.5**	**217.5**
IM Hex Delta	9	9	9
Collision Cell Delta Delta	2	2	2
IBC Delta Delta	0	0	0

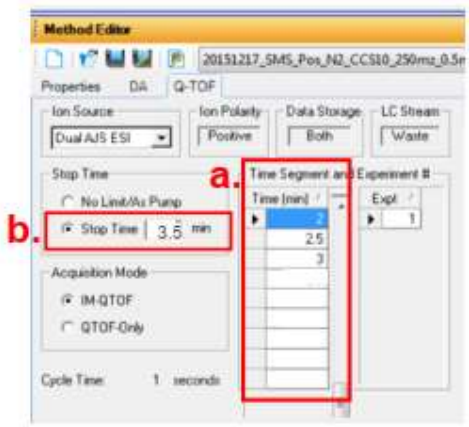
Especially important is Trap Funnel RF, which should be tuned according to your sample. In a few cases, heating/fragmentation can occur after the drift tube. If fragments occur at the same drift time as the analyte, this is indicative of post drift tube fragmentation. To reduce post drift tube fragmentation reduce: collision cell delta, IBC delta delta and IM Hex delta. All of these will have a negative impact on IM-MS resolution, as ions are slowed down post drift-separation, and the diffusion leads to a spread of the ion packet. Keep at least bold ones in set values.

6. Select correct acquisition parameters for you experiment (max drift time, multiplexing, trap fill time)
7. Run the sample by using the method you just modified and saved.
8. Open data in IM-MS Browser (see separate guide for IM-MS browser).
9. If you want to calculate accurate CCS values use stepped field method:

    a. Measure first IM-MS for ES tuning mix using stepped-field setup. For to run ES calibrant use script shown below.

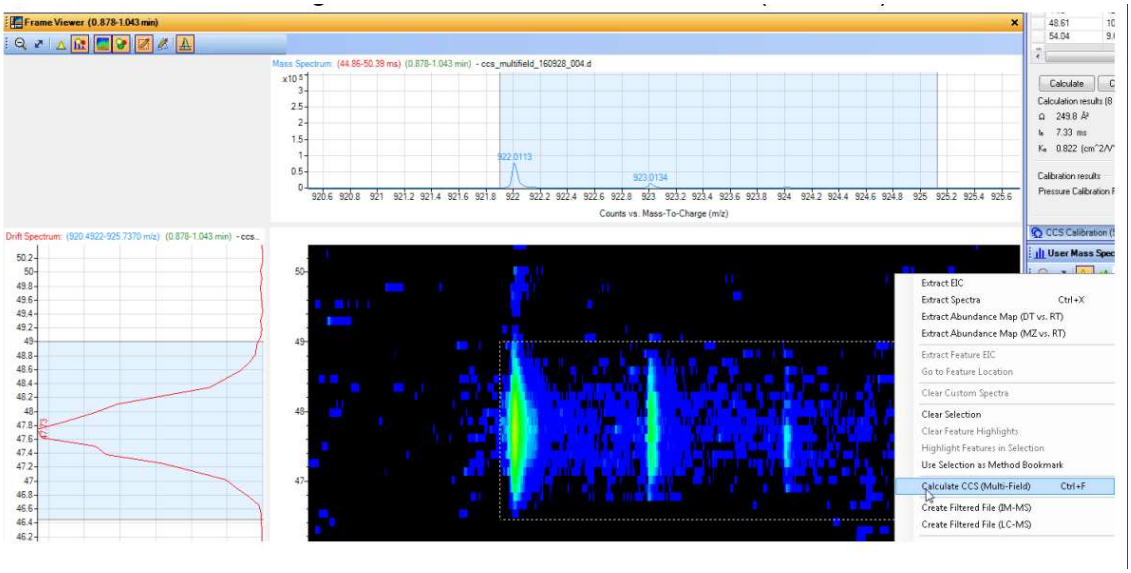


b. Add 7 time segments in method file having increasing *Drift Tube Entrance* voltage (1074, 1174, 1274, 1374, 1474, 1574 and 1674) and keep other parameters same as in single field measurement. Remember to adjust the stop time according to your stepped-field method.



Time Sequence	Time (min)	Drift Tube Entrance (V)	Drift Tube Exit (V)	Rear Funnel Entrance (V)	Rear Funnel Exit (V)
1	0	-1074	-224	-217.5	-45 *
2	0.5	-1174	-224	-217.5	-45
3	1	-1274	-224	-217.5	-45
4	1.5	-1374	-224	-217.5	-45
5	2	-1474	-224	-217.5	-45
6	2.5	-1574	-224	-217.5	-45
7	3	-1674	-224	-217.5	-45

c. Check CCS values for ES tuning mix ions in IMMS-browser. Perform Multifield calibration selecting frames from one field. Add values in Excel file located at desktop. If CCS values for ES tuning mix ions are within 3% compared to literature values, you can proceed to Stepped-field measurements with sample.



d. Measure your sample using same Stepped-field method and calculate CCS values in IMMS-Browser.

Instructions for running IM-MS experiment using He drift gas:

NOTE: Instrument cannot be tuned / calibrated using He as drift gas! Perform tuning / calibration using N2.

1. Switch drift gas valve in gas kit to high purity gas.
2. Open He flow from gas cylinder needle valve.
3. Switch in drift gas kit to He and close needle valve for N_2.
4. In acquisition, open method file using He as drift gas (for example: Tunemix_Pos_3200mz_He_highmass_stepfieldCCS_RF125V_Final.m) and save it with new name.
5. Pressures in drift cell region should be following when He is used:
    a. *HP Funnel* pressure is 4.0-5.0 Torr (typically 4.3 Torr, Turn HP funnel clockwise to increase)
    b. *Drift Tube Pressure* is 3.95 ± 0.3 Torr (typically 3.95 Torr, automatically adjusted)
    c. *Trap Funnel Pressure* is 0.23 Torr below that (typically 3.72 Torr, Turn Drift Cell to clockwise to increase difference between Drift tube and Trap funnel pressures).
6. With He maximum drift tube entrance voltage is 1000V. Please use following voltages in your single-field experiments:

Parameters with He	**V**
High Pressure Funnel RF	180
Trap Funnel RF	125
Drift Tube Entrance Voltage	**800**
Drift Tube Exit Voltage	133
Rear Funnel RF	**120-150**
Rear Funnel Entrance	**130**

7. In Stepped-field experiments with He use following voltages for segments: 563, 615, 667, 719, 771, 824 and 876 V.