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Version 3

My protocol V3 V.3

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Protocol status: Working

We use this protocol and it's working

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Abstract

For calibration. Cleaning. Setting the instrument

Before start

Check that tray Y1-2-3 in the sampler is always empty.

Each pump has 3 lines (A, B and C). Each bottle has two hoses corresponding to the two pumps. Check the labels are correct.

When preparing new solvent: Write your name, date, and composition of the solvent mix in the bottle.

Always purge the instrument when switching solvents. (Degass is automatic).

Check room temperature is around 18 degrees.

Cleaning of MS is performed once a month.

Calibration of MS is performed every 2 weeks.

Do NOT touch the cone of the MS analyser with gloves, use tweezers!

Setting Up Software for HPLC

- 1 Open the software suite (XCalibur).
- 2 Double-click Sequence Setup to open general control panel
- 2.1 Go to Status. Click Thermo Scientific (name of HPLC system). The data of the instrument (the control modules) will appear in one corner of the screen.
- 2.2 Click "Direct Control" and press OK. The window will maximise and allow control of the HPLC system.
- 3 Open Column Chamber menu and activate it (Green box checked). It takes 1 h to stabilise and reach the temperature in the full length of the column (40 degrees).
- 4 Open the Orbitrap console. It will happen automatically when turning on the PC. It displays a black screen with commands. Nothing is input here.
- 5 Double-click X-Tune to start control of the Orbitrap instrument.
- 6 Double-click "In" module from the general control panel.
- 6.1 Open a method. Check the total runtime and calculate the total volume of solvent required for the run. Consider the total number of samples and the the volume of each of the mobile phases (A and B).
- 7 Check the column and pump to be used for the experiment. The instrument has 2 pumps (top and bottom). Bottom pump is connected to left column (C18 column). Top pump is connected to right column (HILIC). One column works with each pump.
- 8 Open "Bottom Pump" menu and click Eluent Selection. Check that both pumps A and B are working with line A (you should see A1 and B1).
- 9 Click Purge: ON. Set to solvent composition 50% A and 50% B. Purge the lines to assure stability of the instrument and no air bubbles. It lasts 5 minutes.



- 10 Set the flow to 0.1 mL/min 50% A 50% B. This must increase gradually until conditions of the method are reached.
- 11 Check the pressure and register it in your Excel File. Shorter tubbings, and higher temperatures will lower the pressure. Higher polarity and viscosity of solvents will rise the pressure.
- 12 Click the crane button on the top menu. Go to Pump 1 module and press Acquisition ON. This way you can see the actual pressure in the screen to check the stabilisation.
- 13 Open Column Compartment L menu. pay extra attention to the letter L. Around 10 minutes are necessary to reach the initial conditions of the method.
- 14 Click the crane button on the top menu. Go to pump module 1 and press acquisition OFF. The sequence cannot start if the acquisition is ON.

Setting Up Software for MS (in X-Tune window)

- 15 Turn MS on by clicking the arrow button ("play button").
- 16 Click the valve button on top of the screen once to switch the flow to the MS instrument. (You will see position 2 in the window next to the instrument). Position 1 sends flow to waste. You should read the message 1→6 next to the valve message indicating that the flow goes to the MS. Position 1→2 indicates it goes to waste.
- 16.1 (to turn off after measurement: first turn the flow to waste (position 1→2 should be read next to valve button) and then turn off MS.
- 17 Check all the MS parameters are set to ready (green boxes) and that they match with the initial conditions of the method.

Cleaning MS source

- 18 Click the Stop button to Turn the instrument OFF to cool down 30 minutes before starting the process.
- 19 Remove the source by lifting the two metal handles and pushing it towards you very carefully.





- 20 Unscrew the 3 bolts with the dedicated screwdriver (first drawer next to the computer) and remove the cone carefully. Check out it isn't HOT.
- 21 Unscrew and remove the transfer tube with the dedicated accessory in the first drawer. Once it is removed the instrument is safe.
- 22 Cover the bare MS instrument with the plastic dedicated cover to avoid dirt into the MS analyser.
- 23 Prepare around 500 mL of a mixture of 50% MeOH (HPLC grade) and 50% MilliQ water + 10% FA (Recipe: 250 mL MeOH + 250 mL water + 50 mL FA in a beaker).
- 24 Put the transfer tube in a cylinder (25 mL) and fully cover it with the solution from step 20.
- 25 Put the sweep cone into the beaker and fully cover it with the solution
- 26 Remove the waste tube and cover it with the remaining solution. Add MeOH if it isn't enough volume.
- 27 Cover the cylinder and the beaker with aluminium foil.
- 28 Sonicate all the parts for 30 minutes.
- 29 Open the oil pump valve for 30 minutes by turning the black ring tip and aligning it with the two metal dots on the metal plate. After that, close it by turning the ring 90 degrees (to close position).
- 30 Rinse thoroughly all parts with MilliQ water to remove all the FA.
- 31 Fill the cylinder and beaker with MilliQ water. Sonicate for 15-30 minutes.
- 32 Rinse all 3 parts with methanol (HPLC)
- 33 Fill the cylinder and beaker with methanol (HPLC). Sonicate for 15-30 minutes.





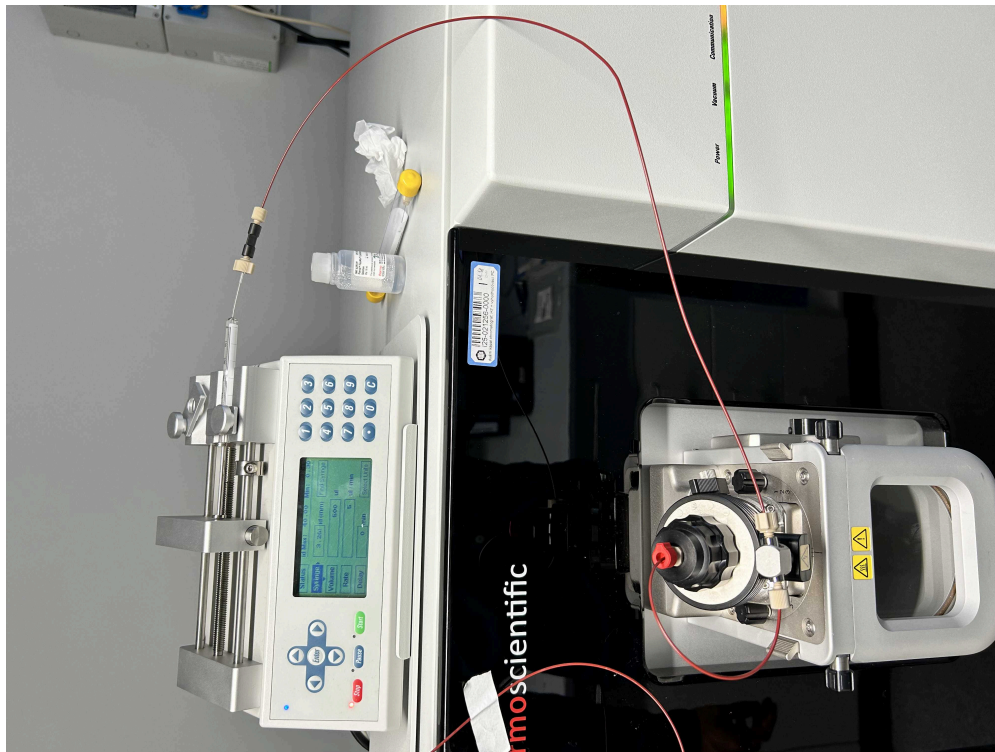
- 34 Rinse with MeOH (LC MS grade)
- 35 Clean the parts with lint free swabs or tissue soaked in MeOH or mixture of MeOH and water.
- 36 Reassemble the parts. The waste tube into the source first, Then the transfer tube, this is very difficult, move in the position applying gentle force until it fits. And then the cone and screw the three bolts. Finally the source and attach with the metallic clips.



Calibration

- 37 Insert the low flow needle. Check the tip is slightly pointing outside through the glass window of the source. Check this with the flat red accessory in the drawer. (It should look flat on the surface of the accessory to assure ideal distance of needle tip to the source). The needle has a letter L on top. To check needle tip length unscrew the two black rings on the source (see picture Step 40). To change the needle only two black parts are unscrewed. To set the length, unscrew the whole module with the metallic ring and the needle attached.
- 38 Screw again the two black accessories after inserting the low-flow needle
- 39 Change the setting to low flow from the right side of the source using the metallic accessory (unscrew and move the selector from medium to low).
- 40 Check MS-tune software that play arrow is ready to click.
- 41 Fill the syringe (Labeled MS calibration in the drawer) with around 500 uL of calibration mix. pay attention to the label of the mix. there are two flasks.
- 42 Check the syringe is free of air by pushing slightly and releasing a few drops.
- 43 Insert the dedicated tubing and fittings (Labeled MS calibration in the drawer). (Picture)





Final setting of the instrument after inserting tubing, fittings and syringe with mix.

- 44 Check that the metallic wedge of the syringe is against the instrument metallic part of the place to hold the syringe. This will allow the instrument to push the plunger correctly.
- 45 Turn MS ON on X-Tune
- 46 Set current flow to 5 $\mu\text{L}/\text{min}$ and press Get Defaults. Wait until default values are reached.
- 47 Set/Check the valve position to 1 $\rightarrow$ 2
- 48 Click Syringe ON. then Syringe OFF, then ON and set flow to 5 μL . Use the "prime" button to accelerate the flow. Check for any leaks in the tubing.
- 49 Define scan range from 100-2000



- 50 Click the button with a chromatogram drawing on bottom-right side of the screen and set mass to 1322 to obtain the EIC.
- 51 Check RSD value is less than 15%
- 52 Pour the remaining mix into the dedicated flask in the drawer after the mass is detected in the system.
- 53 Open the calibration menu. Set Type: Orbitrap Mass & System. Mode: Check + Calibrate if required. Mode: Positive.
- 54 Click Start. Wait until process is completed. Watch out for aborted process or failures. Collect the information in pdf files.
- 55 Repeat steps 49-50 but in Negative mode.
- 56 Unscrew the tubing and fittings and store the syringe.
- 57 After calibration. Stow away the long red tubing, empty the syringe and stow it.
- 58 Unscrew the left side tubing. Change the needle to the normal flow one by unscrewing the two black parts (ring and the one on top).
- 59 Re-connect the short tubing to the right side of the instrument and re-connect the tubing coming from the instrument to the left-side of the metallic connector in the picture. (Picture Step 40).
- 60 Change the syringe to high flow and check the free length of the tip with the red accessory in the drawer.
- 61 To do this: unscrew the whole module from the outer larger back ring.
- 62 Remove the whole module including three black parts and the needle.
- 63 Remove the first 2 black accesories. This will allow to handle the rotation of the needle



- 64 Turn the whole module upside down and carefully hold the lower part to allow rotation only of the needle.
- 65 Insert the red accessory on the tip of the needle
- 66 Rotate the needle clockwise until the tip of the needle goes beyond the edge of the red accessory
- 67 Rotate counterclockwise to match the tip of the needle to the base of the red accessory, this will set the perfect length.
- 68 Reassemble the whole module: Insert the two black small parts and reinsert the whole module. Adjust tightly.
- 69 change the instrument to medium flow setting with the metallic accessory on the right side of the source.
- 70 If the process fails: change the mix, or check for leaks, wait until all temperatures are reached (75C for oven).



Creating a sequence

- 71 Modify the file from the creation_sequence.py script with the path, method and volume
- 72 Save as .csv
- 73 Import the sequence from the Import Sequence Menu