Manual HPLC3 WATERS-PDA996

Sjef Boeren 2006.12.15 12:58:21 +01'00'

MAKE SURE NEVER TO: 1. pump particles or air through the column.
2. inject when the injector is dry.
3. inject solutions containing particles.
4. apply large (sudden) pressure drops over the column.

Ad.1.: Filter eluent trough 0.2 um filter when you use solid buffer components. Make sure the degasser is on and purge the pump thoroughly. Flush eluent with argon gas or sonicate when necessary.
Ad.2.: Pump eluent through the injector and inject a suitable solvent in injector before injecting onto the column.
Ad.3.: Centrifuge all samples (and filtrate when necessary).
Ad.4.: Increment/decrement the flow rate and %B in small steps (0.2 ml/min resp. 20%).

Always:
- Clean injector with a suitable solvent when you stop. Leave the injector in the 'inject' position when you stop.
- Use uncontaminated eluents. When using water, refresh it daily.
- Keep solvent reservoir bottles and the solvent waste barrel as much closed as possible. Organic solvents are volatile and can be toxic.
- Make up the eluent (e.g. add TFA) in the fume cupboard.

1. Startup.

Switch on the main power on the power extension block. This will switch on the degasser, computer and monitor. Separately, switch on the pump + controller. When it does not switch on, the fuse is blown (this sometimes happens when you switch on the main power too fast). This fuse is the leftmost one located under the HPLC pump in that funny box. Reset it when necessary.

2. Purging the pump. For this manual it is assumed that you use eluents A and B. A and B should be replaced when you use different eluents in all of the following text.

Open the prime (THIS IS ESSENTIAL, handle to the right, see figure).
Check whether the waste barrel is NOT full yet. If so, change it.
Purge some clean water behind the pump seals with the plastic syringe.
Use the pumpcontroller to elute eluents by:
push direct, **insert flow 1.0 ml/min** at the flow field, **enter**, **insert 50% at the %A field (Enter) and 50% at the %B field, Enter, Enter, Enter**. Wait 1 min to wet the pump.
The pump is now working, pressure should be below 100 psi since the prime is open. Check! The solvent should now be flowing through the prime outlet. Check!

Push Home.

Enter: flow (ml/min):	**5**	**Enter**
%A:	**50**	**Enter**
%B:	**50**	**Enter**

%A + %B should always be 100%. In the following the %B only will be mentioned.
Decrease the %B to 10%. Check whether all air bubbles are out of the water inlet tube. If not, force them. **Increase the %B to 90% (always stepwise) and make sure that also the methanol inlet tube is free of airbubbles. Increase the %B to 100% and decrease the flow to 2 ml/min.** Eluent should now equable be flowing through the prime outlet. If not, then there is an airbubble in the pump itself. Force it out by: connect a large plastic syringe to the inlet manifold valve (see figure), open it, fill the syringe with some eluent and push it back into the pump. Close the inlet manifold valve.

Decrease the flow to 0.1 ml/min (stepwise).

Close the prime. Increase the flow to .2 ml/min (keep the percentage of B at 100%). The pressure should now go up since eluent is flowing through the column and the detector. The pressure should stay below 300 psi.
Push Set Up. Move the cursor to the 'High' field. **Enter the high pressure limit** value for the column that you use (2000 psi for a glass column, 3000 psi for a metal column).
Push Direct. Stepwise increase the flow to 0.6 ml/min. MAKE SURE THE PRESSURE DOES NOT REACH 1000 psi, if so, decrease the flow and wait until the pressure drops.
Observe the detector outlet. When the system is perfectly OK, no airbubbles should be visible. ***Do not continue until this is obtained****.*
Increase the flow to 0.8 ml/min for glass columns, 1.0 ml/min for metal columns.
Write down the column name + its pressure.

3. Switching the pump to its initial conditions.
Decrease the %B in steps of 20% in ca. 1 min to the value you want to start the injection with (generally 5%) using the pump controller as described above. Do not inject ntil the column is equilibrated to these conditions (generally 10 min.).

4. Switch the detector on.

5. Initialization of the computer program.
At this moment you should be watching to the windows 'Desktop'.
5.1. **Double Click the Millenium32 Login icon**.
Wait until the Millennium Login dialogue box appears.
5.2. **Click the 'Login' button.**

Enter: User Name: ? (see info HPLC)
Press TAB
Password: ?
Press ENTER

When the program is activated, the taskbar at the bottom of the monitor screen contains a button named 'Message Center', which (enlarged) will show error messages when they occur.
All the icons in the login window will now be lit indicating that they are active. The different parts of the HPLC program can be accessed from the login window but also from the Project window.

The Project window can be regarded as the central window of the HPLC program. Move to the Project window by:
Select a project from the Project drop down list.
5.3. **Double click the 'Browse Project' icon, click OK.**
The top lane of each window shows the name of the project, the person logged in and the name of the window itself. The toolbar shows possible actions while the line of tabs below the toolbar can be used to select what information the table will show. Put the cursor on top of an icon and holding still will reveal the name of an icons function in the toolbar. All windows have an icon with a question mark on it. Clicking this icon followed by clicking somewhere in the window will result in a text balloon giving extra information about the item you clicked upon.
Except for the central Project window, each window also has a 'Wizard' icon. The 'Wizard' can guide you through that windows function. Additional help can be found under 'Help'.

To perform an injection you will have to instruct the instrument what to do. Click the 'Methods' tab. All methods available are shown. Open a 'Method Set' by double clicking on the name of a method set (named 'Method Set' in the 'Type' column). This shows what is inside each method set. A 'Method Set' contains:

A. An 'Instrument method' (e.g. HPLC_INSTR_MTH) specifying the pump settings (e.g. your gradient) as well as the detector settings (for a PhotoDiodeArray system this is the wavelength range, the resolution and the scan rate).
B. A 'Processing method' (e.g. HPLC_PROC_MTH) which contains variables needed for baseline calculation like: how steep should the signal rise to be regarded as the start of a peak.
C. A 'Report method' (e.g. HPLC_REPORT) describing the way you want the results to be plotted on paper.
D. Maybe an 'Export method' that describes how to write the data to a file enabling you to read data into another software package.

Exit the Method Set Editor.

Next to the (central) 'Project' window there are: the 'QuickSet' window where the actual measurements are performed, the 'Review' window to have a closer look at your data and the 'Preview' window to see what your data will look like when printed.

5.4. Go to the 'QuickSet' (=measurement) window by **click**ing on **the blue 'QuickSet' icon** in the Project window or on the 'Run Samples' icon in the Login window.
5.5. **Select Instrument Method 'Baseline' from the instrument method drop down box.**
5.6. **Click 'Monitor'** in the Instrument Control pane. *Select: Options, 'Customize Channels'. Enter the desired wavelength and time window in the PDA pane. Click 'OK'.*
Observe the baseline as long as necessary.

Now you have three signals, which give you an idea of the status of the system.
A: the pressure. Using a new column the pressure is approximately 800 psi (eluting with methanol at 0.8 (glass column) or 1 ml/mm (metal column)). For older columns the pressure may be a little bit higher.
B: the detector outlet. When the system is perfectly OK, no air bubbles should be visible.
C: the detector signal. If air bubbles are still present, the deflection is unstable (for PDA observe both the spectrum and the chromatogram).

Air bubbles decrease the sensitivity drastically. Try removing air bubbles in the detector by blocking the outlet line with your fingers when there are any in the detector.

Wait until the system is OK (pressure + detector outlet + detector deflection). Contact Sjef when the pressure does not drop below 1000 (glass column) respectively. 1500 (metal column) bar.

5.7. Stop monitoring the baseline by **clic**king **the red injector** icon.

6. Injecting.
6.1. Move the cursor to the 'Method Set / Report Method' field in the table of the 'Quick Set' window and **select the 'method set'** that has been set up for you by an experienced user or use the 'Wizard' (toolbar) to make your own Method Set.
Enter into the table: a 'SampleName' and its 'Run Time'. Other sample identifiers are optional. Method Set 'Meet' does not contain any pump settings so that you can use this method for isocratic measurements.
Select 'Run and Process' or 'Run and report' (righthandside, above the table).
Check whether all settings are OK (PUMP settings!).
Select this run by clicking its number in the leftmost field.
6.2. Load your sample into the injection loop.
6.3. Click the green injector. Wait until the message 'Injector Wait' appears. Start the acquisition by injecting with the injector.
6.4. For the next injection: click on the next line in the 'Sample Name' field and change the name. Select the new line and proceed as under 4.2.

7. Integration.
In the Project window, the table can show names of data in different ways (by selecting the tabs). Data can be viewed by ‘Sample Sets’ (e.g. give one global name to 25 samples in a autosampler tray), Injections (one name for each injection since each sample can be injected more then one time) or Channels (when there are several detectors each injection will result in more than one chromatogram). Once a PDA Channel is processed (a chomatogram has been extracted, a baseline is calculated, peaks are integrated) spectra + retention times of peaks can be compared to a library yielding additional data that can be found under the ‘Results’ tab.

Go to the ‘Review’ window by:
- Bring the ‘Project’ window to the front. Click the ‘Channels’ tab just above the table.
  Double clicking on a channel name or
- Select a channel (click on its name) and click the yellow/white ‘Review’ icon in the
  Project window or
- Double click the ‘Review Data’ icon in the ‘Login’ window, select ‘Channels’, select a run
  and press the ‘Review’ button.

A. When you have used 'Run only' you can integrate the run by:
Select 'File, Open, Method Set, name, Open. Click the ‘Apply Method Set’ icon. You will now see a integrated chromatogram in the chromatogram display. Save the chromatogram + integration settings by clicking 'File, Save, Result. Continue as described under B.

B. When you have used 'Run and Process' or 'Run and Report' your run will have been integrated already (if your general method set has been set up properly). Click the 'Results' tab in the 'Project' window, select your run(s), double click it or click the ‘Review’ icon.

When you have specified more then one wavelength in your PDA 'Instrument Method' you can observe more then one chromatogram at the same time by clicking the ‘Overlay’ icon.
When you have measured more then one detector for an injection you can select them all before starting review and click the ‘Overlay’ icon.
The sensitivity can of course be increased by drawing a box around the desired area with the mouse.
When you are not satisfied with the way the baseline is calculated then click the ‘Processing Method Wizard’ and follow the instructions or directly change PeakWidth, Threshold, MinimumArea or MinimumHeight and click the ‘Integrate’ icon.
Peak area’s can be seen by selecting the ‘Peaks’ tab at the bottom of the ‘Review’ window.
PDA only: The contours in the contour plot can be adjusted by right clicking in the Contour plot, properties. Especially changing from linear distribution to Exponential distribution is helpful for observing small peaks better. PDA Spectra can also be viewed changing the Contour plot to SpectrumIndex plot.

Users of the old HPLC software will have noticed an extra option in the ‘Save’ list. When you save as: - result = integrated chromatogram with spectral data when calculated (purity, match data).
- chromatogram = extracted chromatogram, one wavelength is saved. This chromatogram can be seen in the ‘Channel view’.
Compare: can be used with 2D data (= single chromatograms) from the ‘Channel view’ only. Comparing chromatograms from the diode array can therefore only be done by saving the chromatogram first with ‘Save as, Chromatogram’.

8. Printing results.
All graphs from the ‘Review’ window can be printed as you see them on the screen by selecting a graph (click on it), click 'File, Print preview, Print.
Printing can also be performed automatically (method A) or semi-automatically (method

B) when you have a proper 'Report Method'. Let an experienced user install one for you.
When you have a proper 'Processing Method' and a 'Report Method' you can make a hardcopy by:

A. Select 'Run and Report' in the 'Quick Set' window before you inject.
This will automatically extract a chromatogram, calculate peak area's and print the result.
B. Select a run in the Project window using the 'Channel' tab and perform the calculation in the Review window as described above under 5. Save the result by clicking 'File, Save, Result'. Proceed with C.
C. Select a result of a run in the Project window using the 'Results' tab and click the 'Preview' icon.
Select the appropriate 'Report Method' and observe whether this is really the way you want to have it printed. Press the 'Print' tool.

9. How to quit.
Switch off the detector, clean the injector.

Push 'Direct' on the pump controller twice. Increase the %B stepwise to 100% using steps of 20% in ca. 1 min. Wait 15 min. Decrease the flow rate **stepwise** to 0 ml/min.

When you are **using TFA or salts** in your eluent then:
Open the prime and pump 20 ml 100% H2O (e.g. at 5 ml/min) to clean the pump. Decrease the flow to 0 ml/min. Close the prime.

Close all windows by clicking: 'File, Exit/log out'.
Close 'Windows' by clicking the 'Start' button, Shutdown. Switch off the pump controller and the main power on the power extension block.

10. When you are in a hurry.
It is possible to make an injection and let the equipment "automatically" clean the column and switch off the detector lamp. Do this only when you really now how to do it.
In the Quick Set Control window:
a. Change under 'Options' the **operating mode** from 'Single-inject' to **'Sample Set'**.
b. **Add a new line** into the 'Sample Loading Table' as you normally do.
c. Again **add a new line** to the 'Sample Loading Table' and **select: Method set = 'StopFrom?', Function = 'Condition column' and insert: Run time = 30 min.**
d. **Select both lines**.
e. Make sure that the injector is in the 'load' position. **Click** the **'Run Sample Set'** control button in the 'Instrument Control' pane. Enter a name for the Sample Set when asked to.
Click on: Run Selected in the next appearing window. Wait until the "injector wait" message appears and **inject** your sample.
Clean the injector, switch off the monitor (nothing else) and run home.

11. Questions.
Just ask (chromatographic problems) or try to find the answer in the manuals or (better) use the 'Help' option in the program (software problems). Ofcourse, improvements to this manual are welcome.

Sjef