Manual HPLC-4 Alliance-UV

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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 (20% of the normal flow rate resp. 20% B).

Always:
- 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.
- Clean the manual injector with a suitable solvent when you stop. Leave the injector in the 'load' position when you stop.

Normally, Bottle A = Water, B = Methanol, C = variabel, D = variabel. When you do not use Water/Methanol, then use bottles C and D.

1. Check the volume of the 2 bottles with wash solvent at the top of of the pump. The first marked 'Injector' is used to wash the autosamplers needle. This is normally filled with 1 ml/l TFA in water. The second one marked 'Pump' is used to wash the pumps seals. This one is normally filled with 10% methanol in water. When they contain less then 100 ml, replace them.

Switch on the main power of the pump, computer and monitor.
Switching the pump on will start the 'Startup diagnostics' which will take a few minutes. The pump screen will show: Initializing (software, needle + syringe, carousels, solvent manager) and some pump seal wash liquid will be pumped behind the seals. When ready, all 5 socalled 'screen keys' (Develop methods .. to .. Log in the bottom of the screen) in the 'Main' screen are lit.

The needle wash wash drain has to be flushed first.
Press the 'Diag'(nostics) button, Prime Seal Wash, Start, wait 0.5 minute, press Halt, Close.
Press the 'Prime NdlWsh' (Needle Wash) button, Start and wait until it is finished, Close. Press the 'Exit' button.
Press the 'Menu/Status' button to go to the 'Status' screen.
In the Status screen, Move the cursor to the '**degasser**' field, press the Enter button and select '**Continuous**', press Enter'.

Move the cursor to the composition field and set it to **0%A 100%B 0%C 0%D**. Set the flow to **0.010 ml/min. Wait 2 minutes to wet the pump**. Check wether the waste barrel is NOT full yet. If so, change it.
2. **Prime the pump**. Press the 'Direct Function' screen key in the Status screen. Select the **Wet Prime** option, then press OK. In the 'Wet Prime' dialog box, keep the initial settings when you do not change bottles, press OK. When you have to change bottle B then change it now, and wet prime for 3 minutes. The pump will now prime solvent B. Shake the filters in the reservoirs to remove any bubbles that may have formed.When finished, change the composition to 100% A and use 'wet prime' again. When you have to change bottle A then change it now, and wet prime for 3 minutes instead of 1 minute. When your method will use solvent A, then purge the autosampler syringe now so that it is filled with eluent A by:
Press 'Direct functions', select 'Purge injector', Enter, Enter, Enter. Inspect the autosampler's syringe for airbubbles. When present, repeat the last purge step while gently tapping against the side of the syringe.

Repeat the priming step for bottles C and D when you use them. When your method will use solvent C, then purge the autosampler syringe after priming solvent C so that it is filled with solvent C by: Press 'Direct functions', select 'Purge injector', Enter, Enter, Enter. Inspect the autosampler's syringe for airbubbles. When present, repeat the last purge step while gently tapping against the side of the syringe.

When finished priming all 4 solvents, change the composition to 100% B or D (depending on what you will use) and increase the flow to 0.05 ml/min. The pump is now working, the pressure should be below 200 psi at 0.05 ml/min. Check!
Set the temperature of the sample trays to the temperature you want in the 'Sample' field or keep it off when you can use it at room temperature.

3. Switch the detector(s) on when not on yet.

4. Initialization of the computer program.

At this moment, on the computer you should be watching the Millenium32 main screen. If not, then:

4.1. Double Click the Millenium32 Login icon.
Wait until the Millennium Login dialogue box appears.
4.2. 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.
4.3. **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. STD_INSTR_MTH) specifying the pump settings (e.g. your gradient) as well as the detector settings (the wavelengths + measurement sensitivity).
B. A 'Processing method' (e.g. STD_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. STD_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 'Run Samples' 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.

4.4. Check the 2487 UV detector if it is finished heating up + calibrating when you had to switch it on. When it does not give any errors then go to the ‘Run Samples’ (=measurement) window by **click**ing on **the blue ‘Run Samples’ icon** in the Project window or on the ‘Run Samples’ icon in the Login window.
4.5. **Select Instrument Method ‘Meet_instr_mth’ from the instrument method drop down box in the bottom of the screen.** Instrument Method ‘Meet_instr_mth’ does not contain any pump settings so that the pump will remain pumping at the present setting.
4.6. **Click the 'Monitor' button** in the Instrument Control pane. Observe the baseline.

Now you have three signals, which give you an idea of the status of the system.

A: the pressure. Using a new 2 mm metal column the pressure is approximately 150 psi (eluting with methanol at 0.05 ml/min). For older columns the pressure may be a little bit higher, for wider columns the pressure will be lower.

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.

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). **When the system is perfectly OK, no airbubbles should be visible. Do not continue until this is obtained.** Contact Sjef when the pressure does not drop below 400 psi.

5. Switching the pump to its initial conditions.

Increase the flow (keep the percentage of B at 100%) by clicking the 'change flow' button in the 'Run samples' window of the computer software: enter flow: 0.200 ml/min, select: 'Ramp for' and insert 2 min. Insert 'Pressure limits: low = 0, high = 3000 psi for a metal column, 2000 psi for a glass column. Click: Apply. The pressure should now go up since the flow is increasing, eluent is flowing through the injector, column and the detector(s). MAKE SURE THE PRESSURE DOES NOT REACH 1000 psi, if so, decrease the flow and wait until the pressure drops.

Decrease the %B in steps of 20% in ca. 1 min to the value you want to start the injection with (generally 0-10%) or again use the 'Ramp for' option as described above. Wait until the column is equilibrated to these conditions (generally 10 to 15 min.).
Stop monitoring the baseline by **clic**king **the red injector** icon.

6. Injecting.

6.1. Select the ‘Single’ tab. **Enter a ‘SampleName’, ‘Vial’ number, the 'Injection volume', ‘Run Time’ and select the ‘method set’** that has been set up for you by an experienced user or use the ‘Develop Methods’ button to make your own Method Set. Other sample identifiers are optional.
Select ‘Run and Process’ (righthandside, above the table).
Check whether all settings are OK (proper vial number, PUMP settings!). Put the manual injector in the load position if it is not already.
6.2. Put a vial in the right position: open the sample compartment, press the 'A' screen key (pump display) for position 1-24 when necessary and put a vial in the right place. When you would like to use the manual injector instead of the autosampler, then put a vial containing water into position and load your sample into the injection loop.
6.3. Click the ‘Inject’ button. When you use the manual injector, then it is easiest to observe the pump valves (second page of the pumps 'Status' screen), wait until the autosampler injects which happens when valve 1 is opened and, at the same time, inject with the manual injector.
6.4. For the next injection: Wait until the run is finished or click the red injector. Proceed as under 6.1.

6.5 Using the autosampler to inject several samples one after another.
a) Select the ‘Samples’ tab. Fill in the ‘SampleName’, ‘Vial’ number, the 'Injection volume' and ‘Run Time’

and select the ‘method set’ for as many lines as you have samples + one extra. The last run should be used to clean the column + the pump, decrease the flow and to switch off the lamp of the Waters 2487 UV detector. In the last line, select a suitable cleaning method (e.g. ‘Clean pump and lamp off) in the ‘Method Set’ field. Change its function in the ‘Function’ field from ‘Inject Samples’ to ‘Condition Column’, use a run time of 10 min (at 13 min. the lamp is shut off).
b) Select all runs by clicking and dragging the mouse over their numbers.
c) Click the Green Injector button.
d) Give your sample set a name, and select ‘run selected’ in the last window.
Remark: about 6 ul wil remain in the vial because it cannot be injected when socalled ‘total recovery’ vials are used.

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 120 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 wavelengths measured each injection will result in more than one chromatogram). Once a Channel is processed (a baseline is calculated, peaks are integrated) it can also be saved as a result. All results 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 ‘Integrate’ 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 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.

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.

The pump must be TFA and salt free when it is stopped. Click the 'change flow' button in the 'Run samples' window. Stepwise bring the pump to elute 0.2 ml/min 100% Methanol to clean the pump and the column. Elute 100% Methanol for at least 10 minutes. Stepwise decrease the flow to 0 ml/min.
When you used TFA or buffer, clean the pump seals by using: Go to the main screen by pressing the 'Menu/status' button on the pump, select 'Diag'(nostics), press 'Prime SealWsh, Start, wait for about 0.5 min, press 'Halt', 'Close'.

Switch off the lamp of the detector(s): Waters 2487: Monday - Thursday: run method ‘Lamp off’,
Waters 2487: Friday: switch it off.
ABi: switch it off (lamp cannot be switched off from within the software).

Clean the manual injector when you used it and put it back into the load position.
Close all windows by clicking: 'File, Exit/log out'.
Close 'Windows' by clicking the 'Start' button, Shutdown.
Switch off the pump, computer monitor and the computer.

10. 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.

Have many happy hours working with this equipment,

Sjef