Fundamentals on operation of Waters LC and control software

Waters China Ltd
Hong Kong Office

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Content – Hardware part

- Introduction to Waters LC and detectors
  - Arc HPLC
  - H-Class (QSM)
  - I-Class
- Care and use of Waters LC modules
  - Routine operation and maintenance
  - Solvent consideration
- Sample preparation considerations
- Introduction to Waters LC column selection
  - Chemistry selection
  - Method development consideration
- Method transfer considerations

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Content – Software part

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Waters CDS	Empower 3	MassLynx 4.1/4.2
Empower™ 3 MassLynx®	▪ Configuration – Data management – System management ▪ Data Acquisition – Acquity Console – Inlet methods – Sample set ▪ Data Review – Auto and manual integration – Calibration curve ▪ Exporting data ▪ Report Generation	▪ Overview of MassLynx ▪ Data Acquisition ▪ Data Review – TargetLynx ▪ Data management with MassLynx ▪ Waters Data Converter

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Introduction to Waters LC

Waters™

Arc HPLC	H-Class (QSM)	I-Class (BSM)
		
9500 PSI HPLC	15000 PSI UPLC	18000 PSI UPLC

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Arc HPLC

Waters™

- Quaternary HPLC pump system
  - 9500 PSI @ 5mL/min flow
  - Dual flow path design – max uptime and optional flexibility
- FTN style injection
  - Temp control 4 – 40°C
  - Max 50µL default injection
  - 96 × 2mL glass vials in two plates
- Column heater
  - Temp control 20 – 65°C
  - Max fit 3 columns

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Acquity H-Class UPLC

Waters™

- Quaternary UPLC pump system
  - 15000 PSI max output @ 1 mL/min flow
  - 12000 PSI @ 2mL/min flow
- FTN style injection
  - Temp control 4 – 40°C
  - Max 10µL default injection
  - 96 × 2mL glass vials in two plates
- Column heater
  - Temp control 20 –90°C
  - 150mm column with single guard column

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Acquity I-Class UPLC

Waters™

- Binary UPLC pump system
  - 18000 PSI max output @ 1mL/min
  - 9000 PSI @ 2mL/min flow
  - Default reversed phase config
- FTN style injection
  - Temp control 4 – 40°C
  - Max 50µL default injection
  - 96 2mL glass vials in two plates
- Column heater
  - Temp control 20 –90°C
  - 150mm column with single guard column

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Tubing Size, µL/foot and Usage

Waters™

Component Systems---Pump to Injector **(250 µL per foot)**	**0.040” ID**
Component Systems---Pump Plumbing **(0.020” ID - 62 µL per foot)**	**0.020” ID**
Most Alliance Tubing **(0.009” ID - 12 µL per foot)**	**0.009” ID**
Acquity Classic **(0.005” ID - 3.5 µL per foot)**	**0.005” ID**
H-Class System **(0.004” ID – 2.5 µL per foot)**	**0.004” ID**
I-Class System **(0.003” ID – 1.3 µL per foot)**	**0.003” ID**

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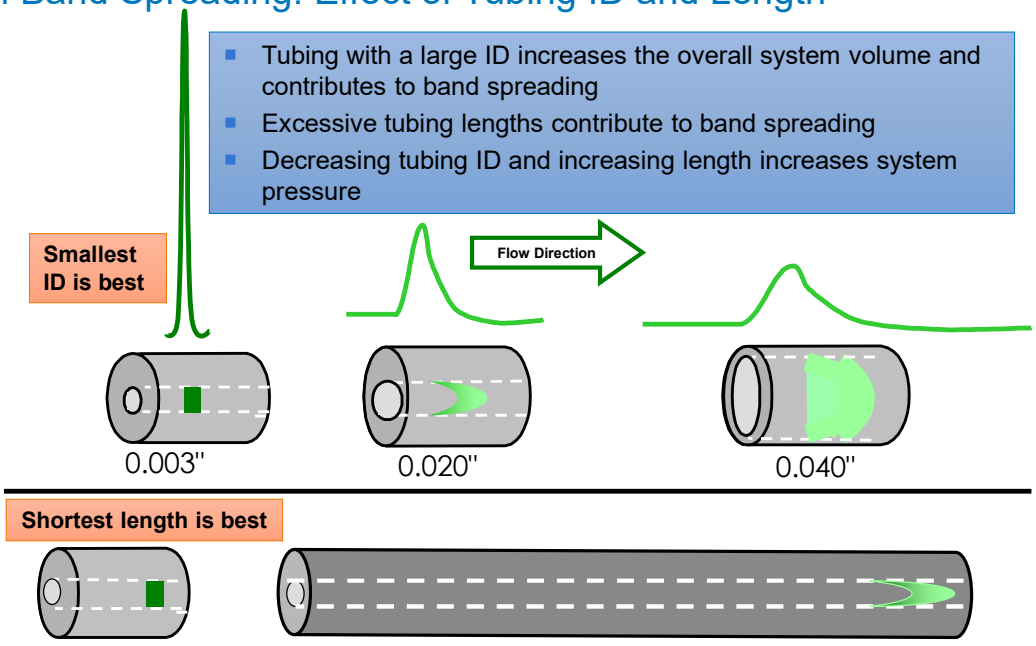
System Band Spreading: Effect of Tubing ID and Length

Waters™

- Tubing with a large ID increases the overall system volume and contributes to band spreading
- Excessive tubing lengths contribute to band spreading
- Decreasing tubing ID and increasing length increases system pressure



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Differentiating between HPLC and UPLC

Waters™

HPLC	UPLC
▪ Pressure <9500 PSI or 655 bar	▪ Pressure >15000 PSI or 1034 bar
▪ Large diameter tubings	▪ Small diameter tubings
▪ Wide band spread	▪ Tight band spread
▪ Delay volume > 200µL	▪ Delay volume within 100µL
▪ Can run with high flow rates (≥ 1 mL/min)	▪ Generally low flow rates (<0.8 mL/min)

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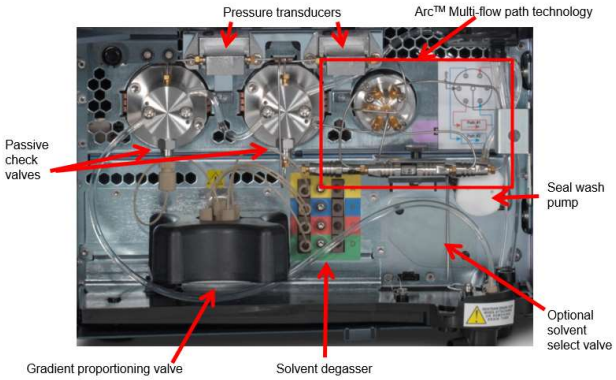
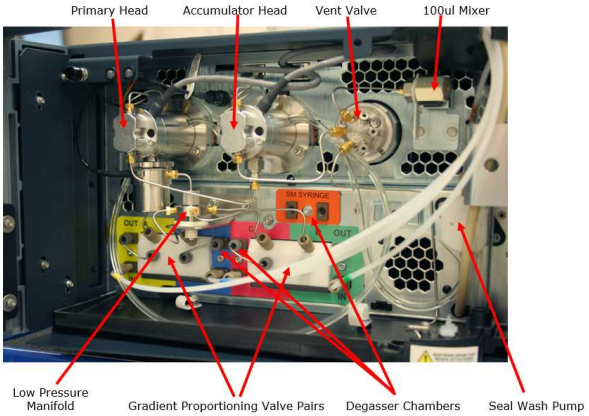
Detailed view – Solvent Manager, Quaternary

Waters™

- QSM-R on Arc HPLC
- For Arc HPLC path 1 is 675µL and path 2 can configure with optional mixer



- QSM on H-Class



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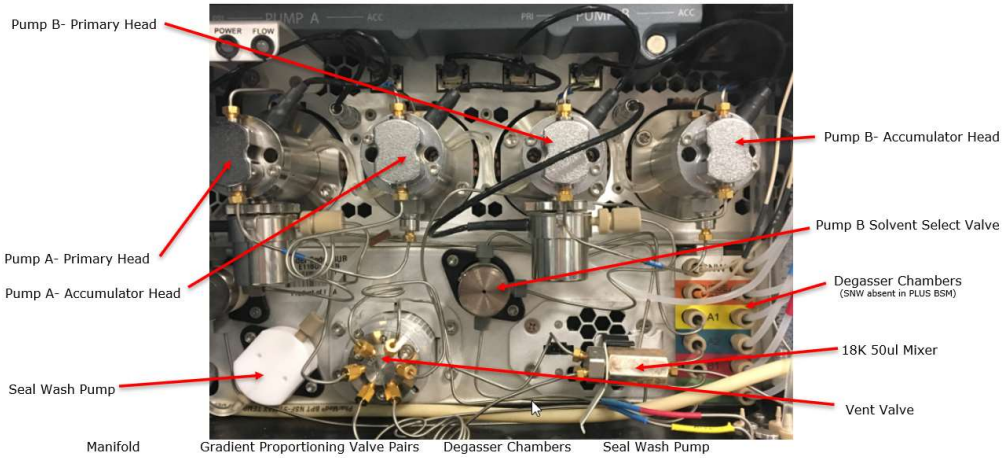
Detailed view – Solvent Manager, Binary

Waters™

- BSM on I-class
- Pair of pump heads working in parallel



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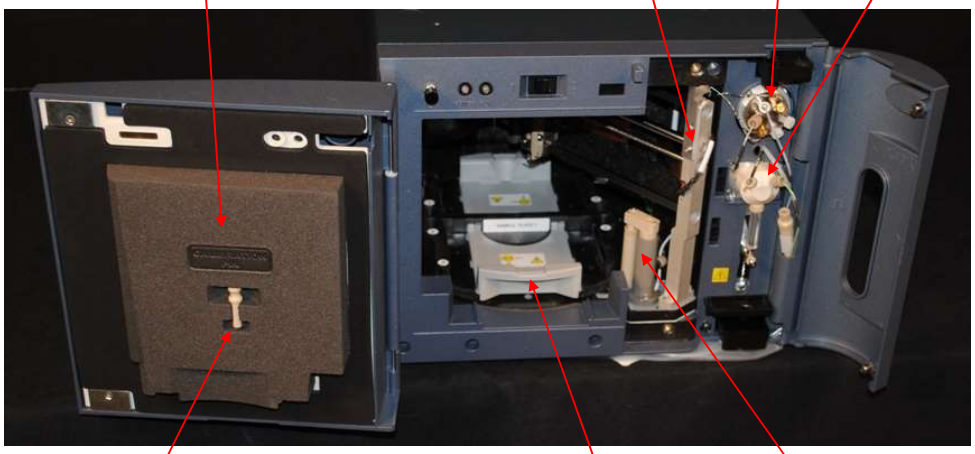
Detailed view – Flow Through Needle (FTN) Sample Manager

Waters™



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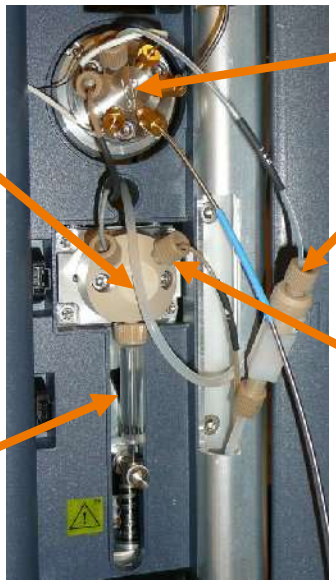
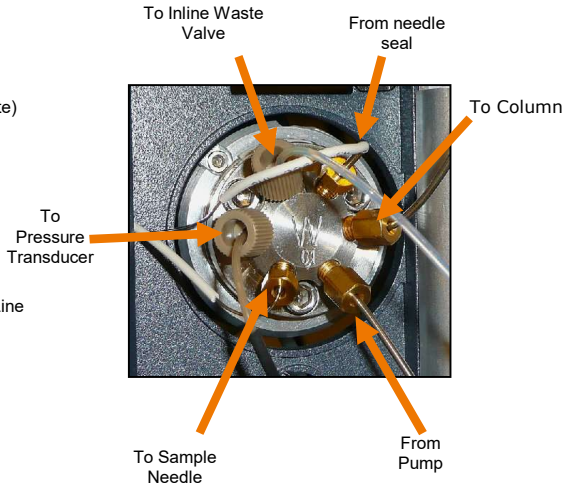
Flow Through Needle (FTN) Sample Manager

Waters™





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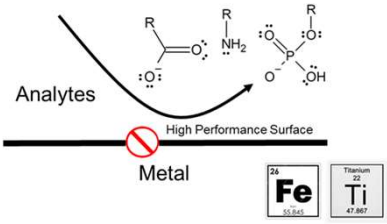
Waters recommended vials for SM-FTN

Waters™

- Routine use – use general clear glass or amber coloured vials
- When volume of samples are low, use reduced volume vials
  - Total Recovery vials
  - 300µL PP vials
- When dealing with troublesome analytes, consider HPS coated vials for maximum recovery

MaxPeak HPS



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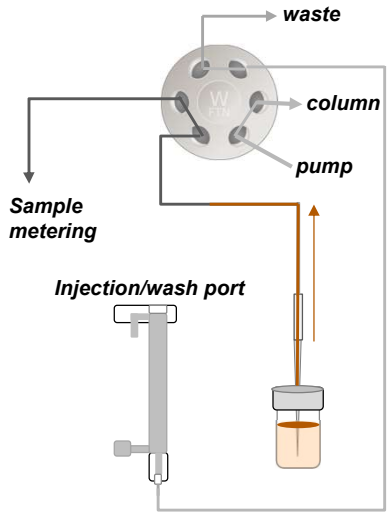
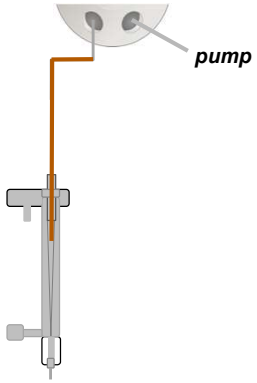
FTN Injection Mechanism

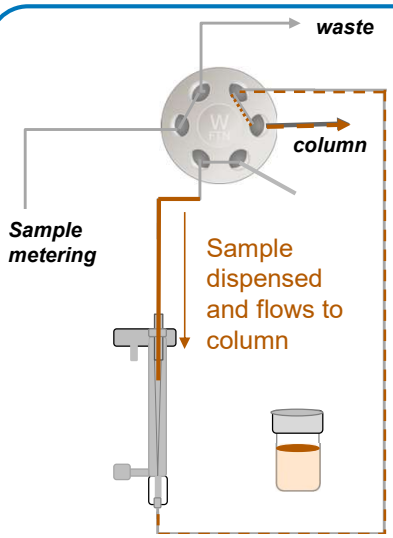
Waters™



Step 1 – Sample is aspirated into needle by the metering syringe



Step 2 – Needle moves to inject/wash port. Pre-inject wash is performed before the needle makes the high pressure seal for injection.



Step 3 – Flow from the pump is directed through needle, taking sample to the column.

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Flow Through Needle: Key Characteristics

Waters™

- Multiple needle and loop options, typically has a wider injection range
  - Arc HPLC injection volume range: 0.1-50 µL standard configuration
  - UPLC injection volume range: 0.1-10 µL standard configuration
  - Can go up to 1000 µL with extensions (not recommended for UPLC)
- Needle in flow path design
  - No wasted sample; all volume sample injection
  - Interior of needle flushed with gradient for lowest carryover performance
- Wide range of plates and vials
  - FTN does not have a piercing needle, so some adhesive/foil plate caps can lead to clogging/blocking of needle
- Simplest mechanism for easiest usage

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Column management – Arc HPLC

Waters™

- Forced air column heater
- Fit 3 columns
  - 1x 250mm
  - 1x 150mm, 1x 50mm
- Temp control 20 – 65°C
- Optional column selection valve for quick column switching (3 selections)
- Clip column onto holder with space between ferrule and column body
- Column connection with finger tight fitting, no tools required.

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Column management – Acquity UPLC CH-A

Waters™

- Temperature range: From 5°C Above Ambient to 90°C
- 2.1 to 4.6 mm, up to 150 mm with filter or guard column
- Active pre-heating with finger tight fitting
  - Improves RT stability
  - Reduces backpressure

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Column management – Acquity UPLC CM-30A

Waters™

- Column manager with possibility of using 4 x 50mm or 2x 150mm long columns
- Programmable column switching (max 4 selections)
- Temp control 4 – 90°C, two independent temperature zone
- Use Active Pre-Heaters for best UPLC peak results

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Tightening Procedure for 3-Piece Active Pre-Heater

Waters™

- Loosen the Stainless Steel Cap Nut from Gold Fitting
- Finger tighten the Gold Fitting with ferrule into column detail
- Engage the column hardware, finger tighten column onto Gold Fitting
- Finger tighten the stainless steel Cap Nut onto Gold Fitting

Pictured Above: Active Pre-Heater prior to modifications made for "Plus" Instruments

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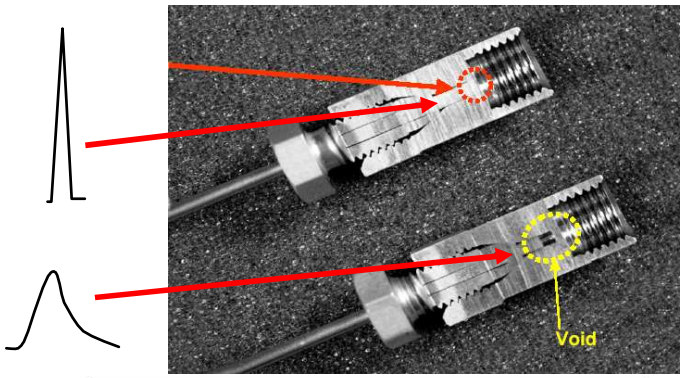
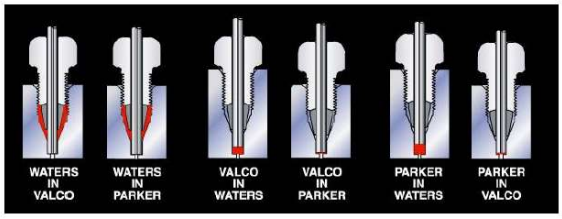
Dead volume - fixed fittings

Waters™

- Improper fittings results in void or dead volumes
- Causes bad peaks, shouldering peaks, leakages
- Waters ferrule depth is not the same with other vendors
- Fixed fittings are generally not reusable cross vendors





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Waters LC detectors

Waters™

Main focus

- 2998/Acquity eλ – PDA, 3D UV
- 2489 – TUV, dual 2D UV
- 2475 – FLR
- 2414 – RI
- 2424 – ELS

Discuss next time

- QDa (SQ-MS)

Waters 2998/Acquity eλ Detector

Waters™

- Both 2998 and Acquity eλ supports 3D UV data collection
  - 2998 on HPLC, Acquity eλ on UPLC
- 190 – 800 nm , 1.2 – 12 nm bandwidth
- 80Hz data collection
- New lamps warranty: 2000 hours or 1 year
- Under Empower control, supports one (1) 3D channel + Eight (8) 2D channels data collection simultaneously
- Flowcell not interchangeable between 2998 and Acquity eλ
- Analytes must have chromophore
- Best to keep responses below 1AU

2998 PDA

Waters™

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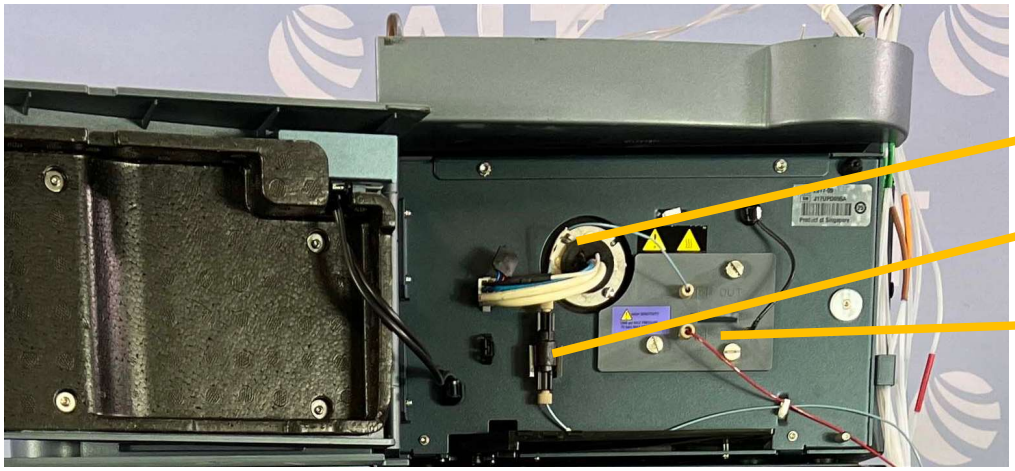
Acquity PDA

Waters™



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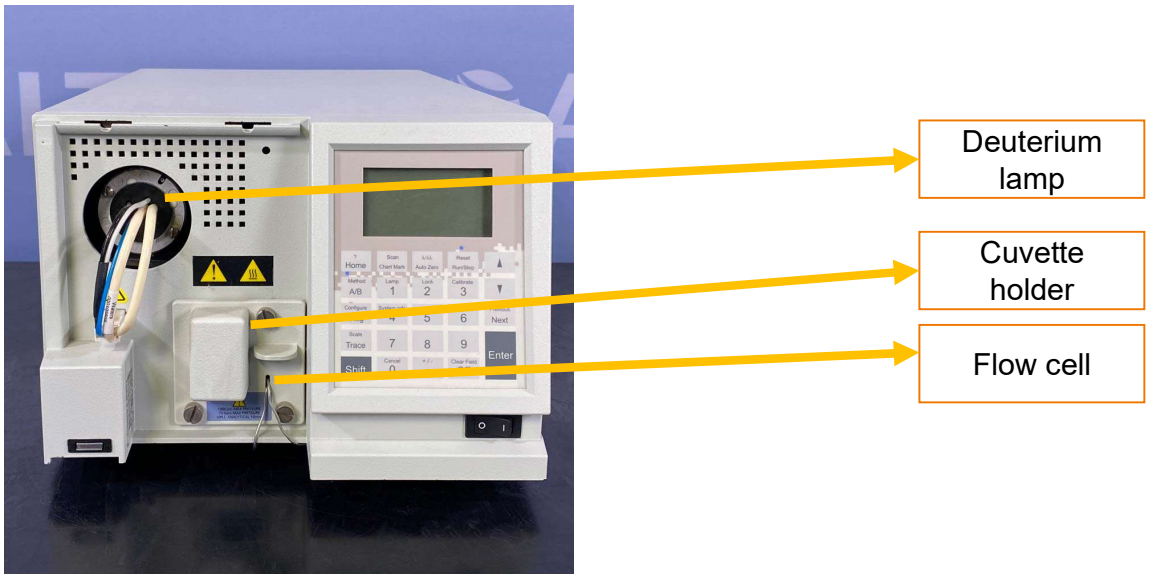
Waters 2489 dual channel UV detector

Waters™



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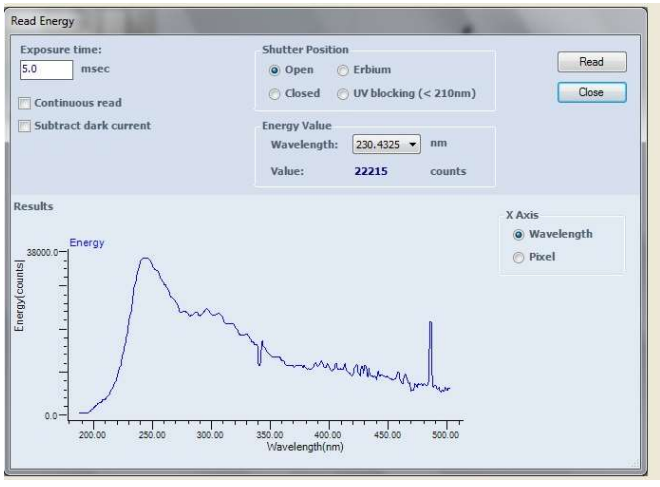
Read lamp energy

Waters™

- Good lamp + clean flow cell + good bench optics will produce value >20,000 at 230nm
- Must turn on lamp for 60 minutes before performing test
- Run 0.5mL/min Methanol through flow cell when testing
- WKB68243
- If reading is <20,000 then change lamp and try again
- If new lamp still gives low reading, clean flow cell or call for service
- WKB48267



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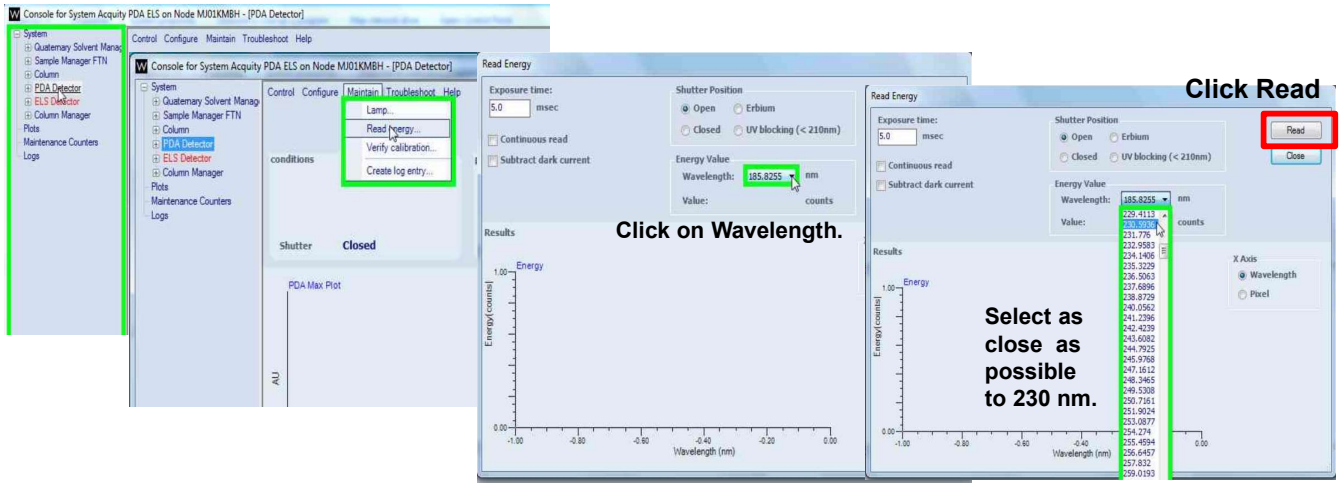
Read lamp energy

Waters™



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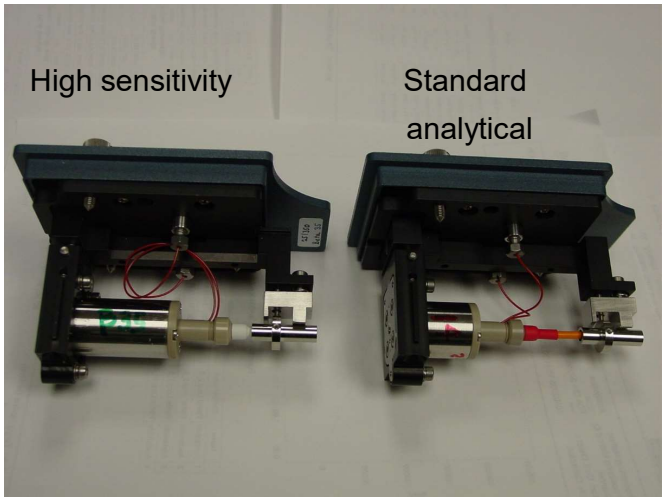
High sensitivity flow cell option

Waters™

- High sensitivity flow cell path length is 25mm vs standard analytical at 10mm
- Theoretically provides 2.5X sensitivity
- Will reduce resolution between closely eluted peak due to increased system volume and larger bandspread now
- Very expensive >HK$40,000



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Waters 2475 Fluorescence Detector

Waters™

- Excitation: 200 ~ 890nm
- Emission: 210 ~ 900 nm
- Up to four 2D channels or one 3D channel
  - 10 points/s in single wavelength mode
  - 1 point/s in 3D mode
- Illumination by Xenon lamp
  - Warrantied at 2000 hours or 1 year
- Flow cell:
  - 13µL
  - 145PSI or 10 bar. Limit to below 5mL/min, or check backpressure when using
  - Expensive, > HK$45000

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Waters 2475/Acquity FLR operation consideration

Waters™

- Waters FLR can operate in Emission or Energy mode
- WKB118420

- Emission mode
  - Is normalized to standard water reference
  - Peak response not usually affected by gain settings
  - Compensate variation from degradation of bench optics and lamp
  - Allows more comparable result with other 2475 or Acquity FLR
  - WKB193270

- Energy mode
  - Is the direct output from PMT
  - Influenced by gain settings
  - Usually used with legacy established methods
  - WKB29076

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Waters 2475 FLR Detector

Waters™

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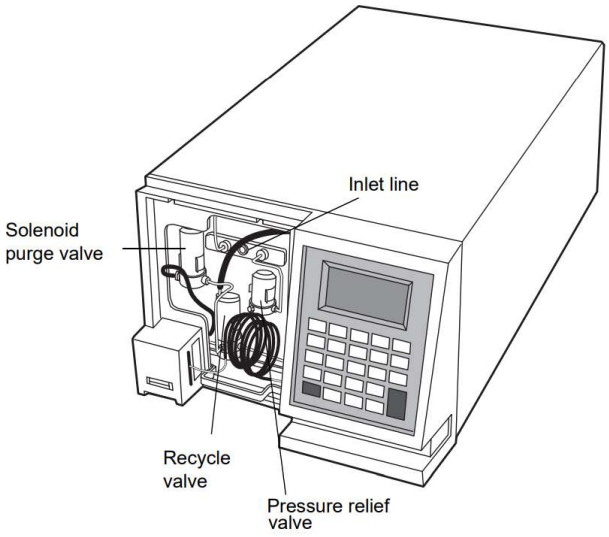
Waters 2414 Refractive Index Detector

Waters™

- Detection is based on a change of optical refraction in sample cell compared to reference cell
- Generally useful for most UV transparent analytes
  - Sugars, alcohols, fatty acids etc
- Used only with isocratic method
  - Cannot run gradient, must use premixed mobile phase
  - Changing gradient, back pressure causes undesirable baseline drift
- Generally wider peak width, less sensitive and selective



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Waters 2414 Refractive Index Detector

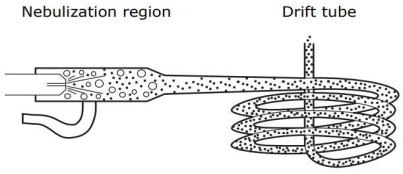
Waters™

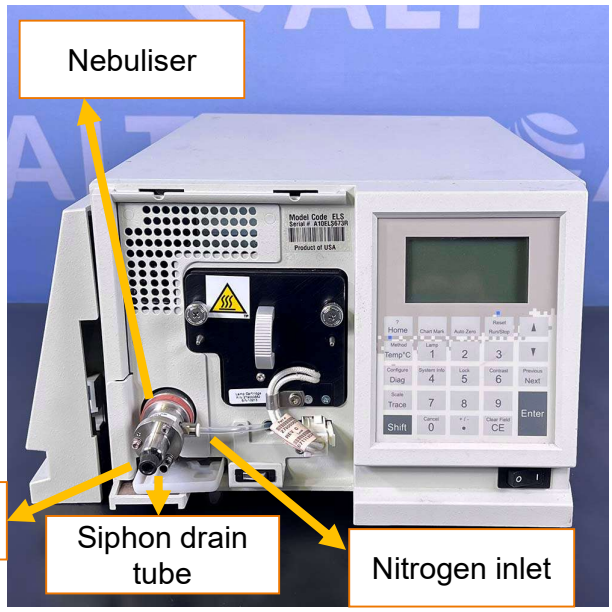
- Flow cell backpressure rated at 100PSI
  - Further protected by pressure relieve valve
  - Contains reference and sample cell in one unit
  - Reference path content not change during sample acquisition
- Flow rate: 0.1 to 10.0 mL/min
- Internal oven: 30 to 55 °C
  - Oven takes a long time to reach set temperature
  - Turn on when reach lab if to be used later

Waters 2424 Evaporative Light Scattering Detector

Waters™

- Detection based on light scattering of very fine solid particles
  - Not gaseous
  - Nitrogen helps to blow dry the mobile phase
- Operation principle:
  - Nebulization
  - Desolvation / Evaporation of the mobile phase
  - Detection: diffusion chamber





Waters 2424 operation consideration

Waters™

- Evaporative Light Scattering Detector is almost a universal detector and is destructive
- Detection of light scattered from particles of analyte formed by nebulization and evaporation of the mobile phase
- Compounds have to be less volatile than mobile phase
- The sensitivity of the ELSD (nanogram level, low ppm level) is better than the refractive index detector, and can be used with gradient elution
- Non-linear calibration curve
- Use of LCMS compatible MP is highly recommended
- Siphone drain tube usually requires a column of liquid for ELS to perform best

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Waters™

Routine Operation Considerations

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Turning on PC and starting Empower 3

Waters™

- If all LC and computer are turned off, switch on PC first
- Login to Windows and wait for 5 minutes
  - Empower 3 will then be operational because it takes time to start Oracle database
- After logged into Windows, turn on all LC modules
- Switching on 2998/2489/2475/2414 from powered down state will ignite lamps
- All modules will initialise and perform startup check
- If your PC is running MassLynx as acquisition software, just turn on PC, switch on modules, and run MassLynx after logging into Windows

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General LC operation – shutdown operation

Waters™

- Flush buffered mobile phase from system and detector with purified water
  - For Binary Pump, reserve either A1 or A2 for purified water
  - For Quaternary Pump, reserve A for purified water
  - Run separate method for flushing pathway and column
    - E.g. 0.5mL/min 90:10 water:methanol for 20 minutes, 50:50 water:methanol for 20 minutes, then 100% methanol for 10 minutes
    - Substitute methanol with acetonitrile is possible
    - Consult user manual for your column for best storage solvent
    - Can maintain pump at very low flow (0.02 mL/min) if will be using on next day
- Turn off column heating
- Store flow cell in methanol or acetonitrile
- Turn off if going for a long holiday
- Cap the inlet and outlet

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Solvent manager – usage considerations

Waters™

- All mobile phase sinkers must be immersed in mobile phase at all time
- Mobile phase bottles must be covered appropriately at all time
- Prime all solvent lines and ensure no bubbles in it
  - Solvent refresh: >3 minutes
  - Solvent changeover: ≥5 minutes
- Use proper seal wash – 90% water + 10% methanol/isopropyl alcohol
  - Set frequency to 2 – 5 mins
  - This is compatible for all reversed phase methods
- Prime seal wash to remove air bubbles. Use syringe to pull seal wash solvent through when necessary
- Online degasser need time to draw vacuum, turn on Solvent manager upon arriving in lab to reduce startup wait time

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Solvent manager – usage considerations

Waters™

- Respect pH range of instrument, 1 – 12.5
  - Do not use mineral acids (HCl, HF, HNO_3) and strong bases (NaOH, KOH) as mobile phase
  - H_2SO_4 can be used at very low conc., ~ 0.005N
  - Final operational pH must be compatible with your LC column
- Use Type 1 water for preparing aqueous buffers
  - Replace aqueous mobile phases daily or at least weekly
  - If water is your mobile phase, replace daily
- Use only HPLC grade organic solvents or above
  - Or ACS grade if not available
- When using salts, use HPLC, LCMS or ACS grade
  - Generally highest quality that you can acquire, of reputable source
  - Concentrated buffer (< 0.1M) can be used but generally please avoid

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Solvent manager – Recommended solvents/modifiers

Waters™

- Waters HPLC/UPLC System recommended solvents
  - Methanol, Acetonitrile, Isopropanol and their water mixtures
  - THF (replace all PEEK tubings with Stainless steel, THF/Hexane modification kit, p/n 205000464)
- Sample diluents (in addition to the solvents listed above)
  - Dimethyl sulfoxide (DMSO)
  - Dimethylformamide (DMF)
- Additives / Modifiers
  - 0.2% formic acid, 0.1% trifluoroacetic acid (TFA), 0.1% triethyl amine (TEA), phosphate buffer, ammonium bicarbonate, ammonium hydroxide, ammonium acetate, Ethylenediaminetetraacetic acid (EDTA)
- Cleaners
  - Phosphoric acid (~30%), Sodium hydroxide (~1M)
    - Ask before using these

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Solvent manager – usage considerations

Waters™

- Filter all buffered mobile phase through 0.2 µm membrane filter or risk clogging your LC and column
  - Filtering also degas them
- When preparing isocratic premixed aqueous organic mobile phase, degas before loading onto the LC
- Degas mobile phase before use produce flatter baseline – sonication or vacuum filtration
- Don't top up aqueous mobile phase, especially during acquisition
- Store mobile phases in clean bottle
  - For buffered mobile phase, consider amber bottles to reduce microbial growth
  - Label all mobile phase properly, including content and date prepared
  - Cover exposed mouth or openings properly

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Solvent manager – filtration set

Waters™

- Using filtered mobile phase is very important
- Laziness will cost you instrument downtime and column lifetime
- Filtration will also degass your mobile phase and improves chromatographic performance

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Sample manager – common issues

Waters™

- Issues are generally physical or LC method related issues
- Physical
  - Bent needle: misalignment when drawing sample, vial inserts, user ignorance
  - Blunt needle: due to thick septum or non-preslit caps
  - Lost prime in solvent tubing serving FTN: trapped air in tubings
  - Worn injection valve: high usage or corrosive solvent
  - Syringe leak: high usage or corrosive solvent
- Method related
  - Carryover due to insufficient wash time/bad solvent choice
  - Wrong solvent in purge
  - Sample too concentrated

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SM–FTN – solvent consideration and tubing management

Waters™

- SM–FTN served with two solvent tubing:
  - Sample manager purge : 90% water + 10% methanol
  - Sample manager wash : 50% methanol
- Sample manager purge solvent line for auto dilution and hydraulic action of SM–FTN
  - Usually 90% aqueous
  - When using HILIC, use initial condition ratio in gradient table
- Sample manager wash for washing exterior of sampling needle
  - Usually 50% methanol, choose a solvent that will dissolve your sample/analytes readily
- Both solvent tubings must be filled and without air bubbles for reliable performance

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Sample Manager – extending lifetime of needle

Waters™

- Use preslit caps
  - 186000305
- Do not use crimp style GC-caps – too thick to puncture
- Do not use vial inserts, they will damage needle
  - For low volume sample, consider 300 µL PP vials 186002639
  - Or consider Total Recovery vial 186000384C
- Do not user buffered solution for washing needle.
- Using low concentration of acid such as 0.1% FA is fine

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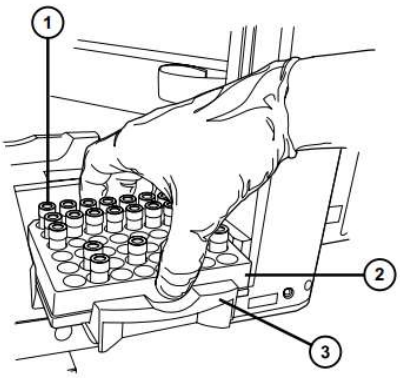
Sample Manager – loading sample plates

Waters™

- When required to load or remove sample plates, pull the sample tray handle out to do so
- Failure to do so may result in hitting autosampler arm, bending needle or misalignment

Figure 2–14: Loading sample plate onto sample tray



1 Well position A,1

2 Forward edge of sample plate

3 Sample tray handle

Sample Manager FTN usage consideration

Waters™

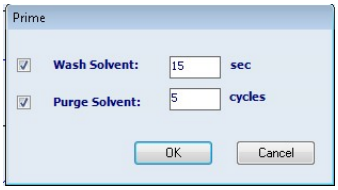
- Temp control 4 – 40°C, commonly used at 10°C when temperature control is required
  - Advise not to cool for extended time, use refrigerator for sample storage
- Do not overtighten caps
- Injection more than 50 µL on Arc HPLC is possible by installing larger extension loop
- Air gap can be used when injecting very large or small volume to protect sample during injection and reduce sample dispersion
- Arc HPLC control column temperature through SM-FTN page

Sample Manager FTN usage consideration

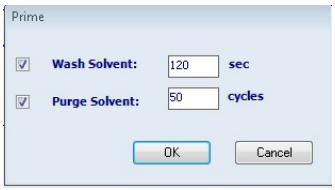
Waters™

- Priming Recommendations →
- Selecting Wash Solvents
  - Sample should be very soluble in the wash solvent
  - Purge solvent
    - does not contact sample
    - should be similar to initial mobile phase conditions, 10% MeOH is very commonly used
    - should not contain additives

To Refresh Solvents



For New Solvents



51

Alternative to APH/Arc Fingertight fitting

Waters™

- APH are expensive, >HK$8000
- Improper handling of APH results in stucked ferrule, bent and deformed tubing
- Use PEEK tubings, Arc HPLC can use 0.010 or 0.005” i.d. tubings
- H-Class and I-Class must use stainless steel tubings for >6000 PSI application
- Use either fixed ferrule tubings or other finger tight types
- Waters sell MarvelX UPLC tubing finger tight to 18,000 PSI and reusable for over 100 times

MarvelX UPLC tubing

52

Acquity In-line filter – protects your precious columns

Waters™

- Houses a 0.2µm frit – effectively trap any particulates in mobile phase or sample that may otherwise clog columns
- Protects column from premature failure due to overpressure
- Frits are easily replaceable and cost ≤ HK$100
  - May need to replace nut also when frit cannot be separated from it
- Each in-line filter are configured for that brand of column, using on other brand may result in leakages or bad peak shape

Waters 2998/Acquity eλ Detector operation consideration

Waters™

- Flow cells are very expensive, >HK$40,000
- Inject only filtered samples and use only filtered mobile phases
  - Clogged flow cell or outlet tubing will result in increased back pressure causing flow cell to break
  - Filter all mobile phase or sample with 0.2µM membrane filter
  - When using concentrated buffer MP, ensure the salt do not precipitate out when switching to high organic which may cause blockages
- Flow cell will withstand 1000 PSI back pressure

Waters 2998/Acquity eλ Detector operation consideration

Waters™

- Powering on instrument using front switch will always turn lamp on
- Understand which wavelength is the cutoff of your MP or modifier
  - E.g., TFA ~210nm, Acetate ~ 240nm, Methanol ~205 nm
- Collect sufficient points within a peak for best peak shape
  - 25-50 points per peak
- Check lamp energy regularly
- Turn off lamp after completing sample sets
- Flush buffered mobile phase from PDA flow cell after use
- Store PDA flow cell in Methanol or ACN
  - For long holiday, cap inlet and outlet

55

Cleaning PDA/TUV flow cell

Waters™

- Dirty flow cell issues:

Lowered intensity	High baseline noise
Baseline fluctuations	Calibration failure

- General cleaning procedure
  - Connect system with union
  - Flush system and flow cell with water, 30 mins
  - Prime one or all line with magic mix (25% each: MeOH, IPA, ACN, Water w/ 0.1% FA)
  - Set column temperature to 40 °C and route flow path through column heater/manager
  - Set flow to 0.2 mL/min, 60 mins
  - Flush system and flow cell with water until neutral, set flow 0.2 ~ 0.5 mL/min or do not exceed 1000 PSI
  - Setup system and try run
  - If blockage of flow cell suspected, try backflushing
  - WKB48267

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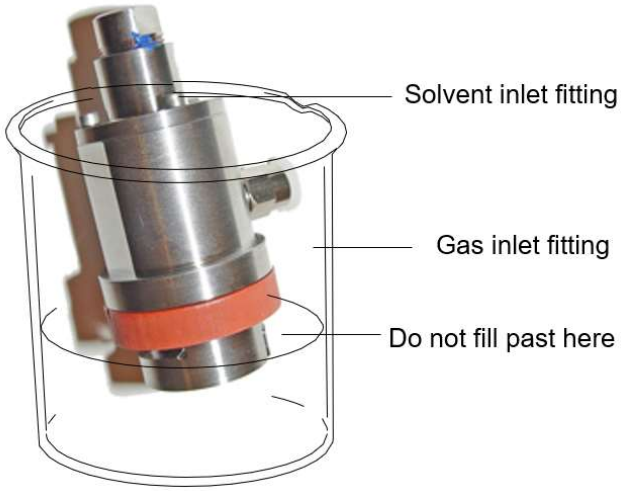
Waters 2424 operation consideration

Waters™

- Nebuliser is generally set to cooling temperature to prevent introducing too many particles or analytes into the detection chamber
- After completing samples, run 50% methanol to clean the nebulizer
- Blow dry the ELS detector for another 1 hour with nitrogen
- If blockage in nebulizer is suspected, remove and sonicate with 50% methanol



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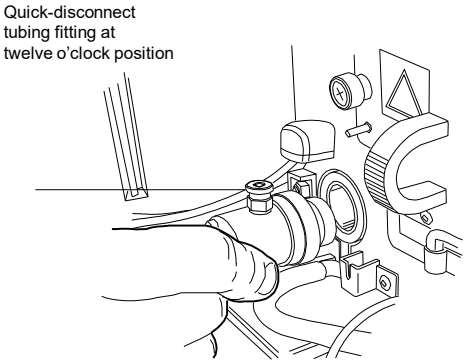
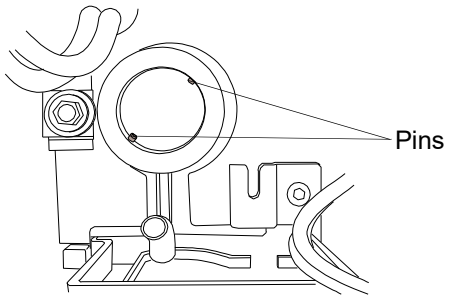
Waters 2424 – removing nebuliser

Waters™



Removing the nebulizer:



Pins inside the desolvation chamber

58

Waters™

Using Empower 3 for data acquisition and data analysis

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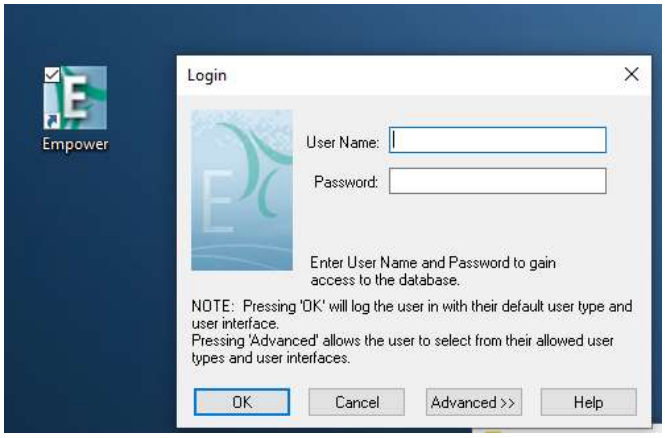
Empower 3 – login

Waters™

- Look for shortcut on desktop or Start menu
- Empower 3 requires password to unlock
  - Username: system
  - Password: manager
- Do not change password, if you forgot it and call for repair service, Waters will charge for that
- If you rebooted the computer, wait for 5 minutes before login because Oracle database is starting in background



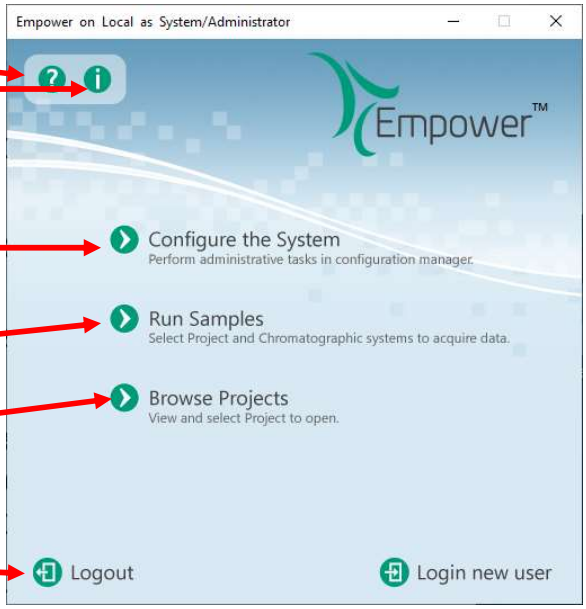
 60

60

Empower 3 – main screen

Waters™

- Empower Help files
- Empower information
- Administration tasks
- Data Acquisition and instrument control
- Data review and analysis
- Exit from Empower



 61

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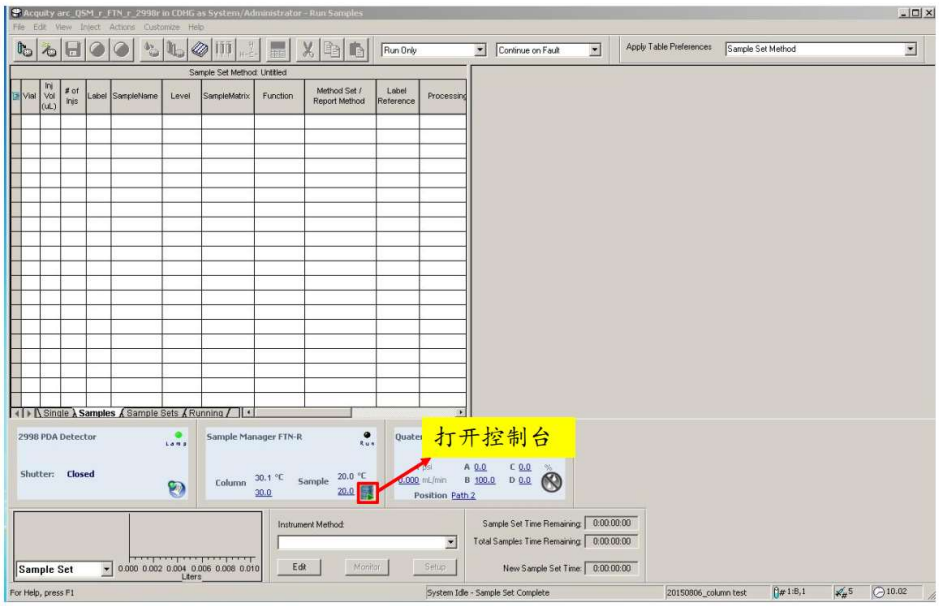
Empower 3 – Bringing up console

Waters™



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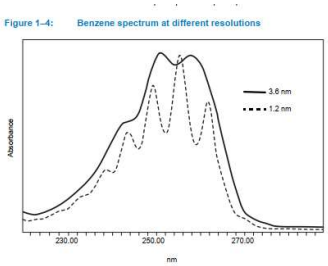
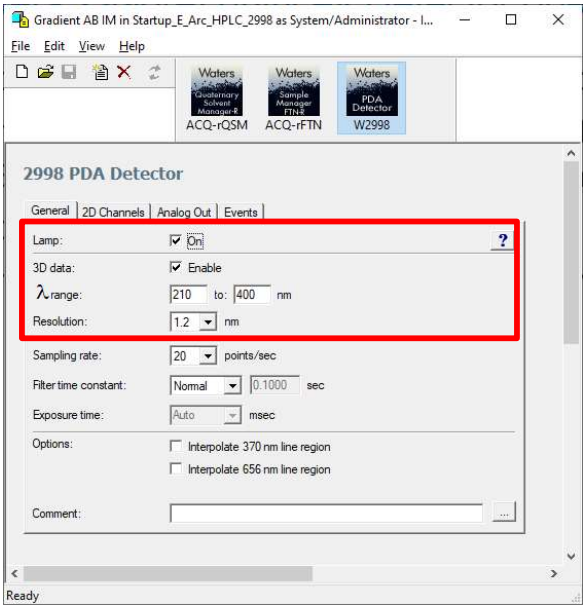
Waters 2998/Acquity eλ Detector operation consideration

Waters™

- Always check Lamp On box for acquisition method
  - Uncheck Lamp On for shutdown method
- Collect 3D data saves your time to rerun sample at specific wavelength and can provide spectra information
  - Use desired range, or check literature
  - Use highest resolution for best quality data

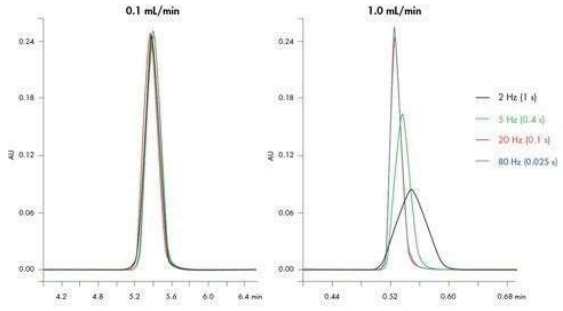


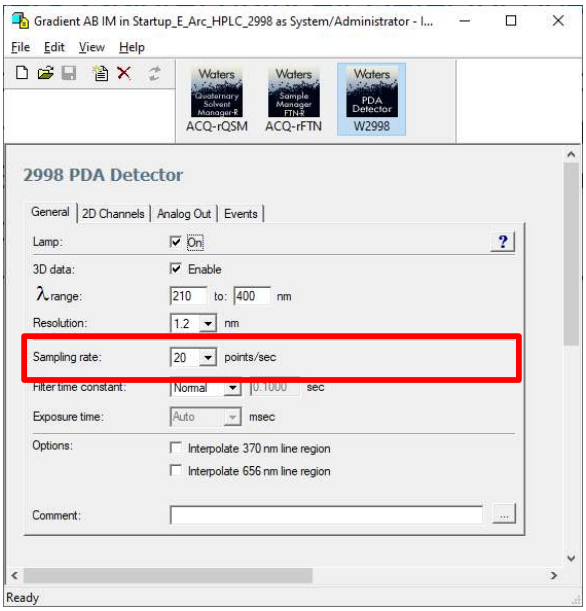


Waters 2998/Acquity eλ Detector operation consideration

Waters™

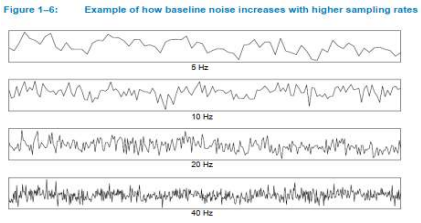
- Use sampling rate to maximise SN ratio and collecting sufficient points across a peak for confident quantitation
  - Around 20 – 30 points per peak required
  - HPLC methods use 10 or 20 pts/sec
  - UPLC methods use 20 or 40, 80 when using ballistic gradients





Waters 2998/Acquity eλ Detector operation consideration

- Use sampling rate to maximise S/N ratio and collecting sufficient points across a peak for confident quantitation
  - Around 25 – 50 points per peak required
  - HPLC methods use 10 or 20 pts/sec
  - UPLC methods use 20 or 40, 80 when using ballistic gradients
- Using sampling rate that is too high may result in more noise, decreasing S/N ratio



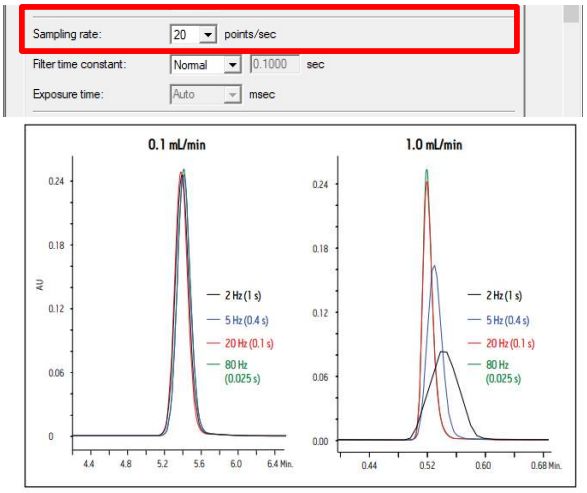
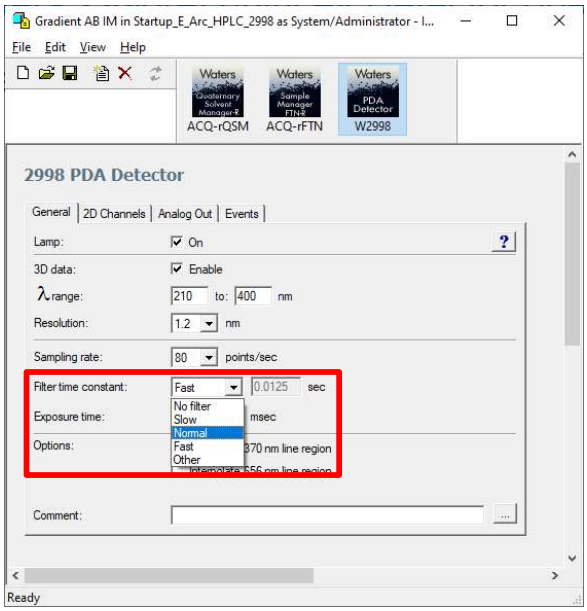


Figure 1. Impact of different detector settings on the peak shape for acenaphthene. The number in parenthesis is the filter time constant, in seconds.

65

Waters 2998/Acquity eλ Detector operation consideration

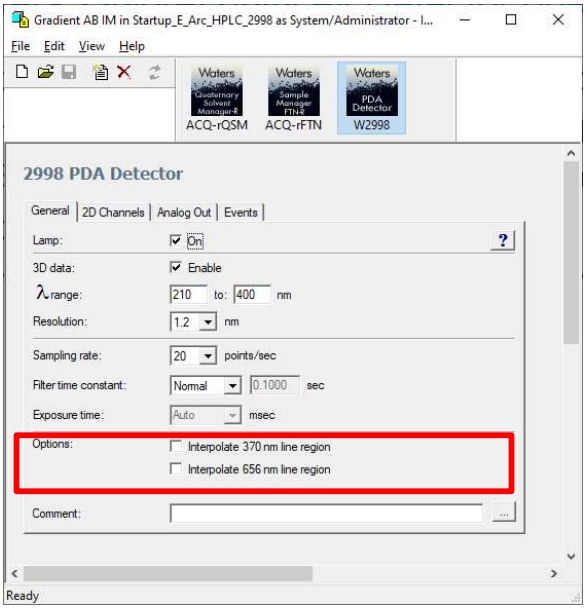
- Filter time constant
  - Noise reduction calculation performed in instrument
    - Default is normal
    - Can turn off
    - Does not affect peak area
  - For max resolution, use Fast
  - For max sensitivity, use Normal
  - When set to Fast, baseline noise greatly reduced but will shorten or broaden peaks
  - When set to Slow, removes less baseline noise, peak shape maintained narrower, small peaks more difficult to pick out from baseline
- WKB90261 and Tip207



66

Waters 2998/Acquity eλ Detector operation consideration

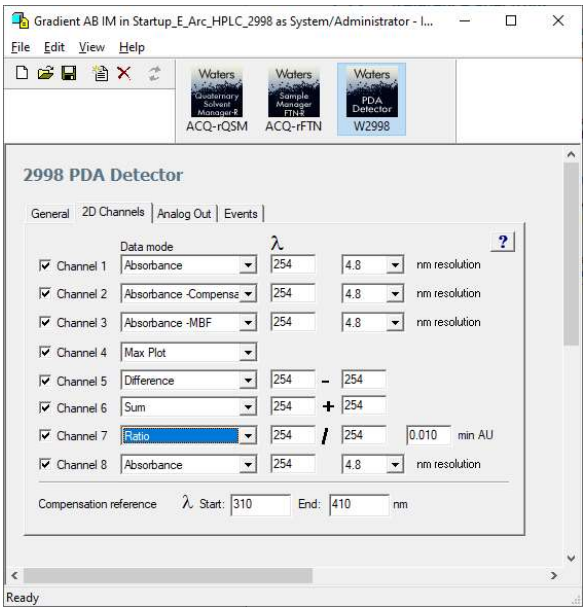
- D2 lamp produced peak at 370 and 656 nm
- Enabling will tell detector to ignore signal from these diodes
- When you disable this parameter, the detector reports the signal from the photodiode at 656 nm
- Disable this parameter only if you are working with compounds that absorb in the 656-nm range
- WKB44903



67

Waters 2998/Acquity eλ Detector operation consideration

- Max eight other 2D channels
- These functions are available
  - Absorbance
  - Absorbance –Compensation
  - Absorbance –MBF
  - Max Plot
  - Difference
  - Sum
  - Ratio
- Resolution of 4.8nm works for many analytes in both HPLC & UPLC
  - Optimisable further for sensitivity and S/N ratio



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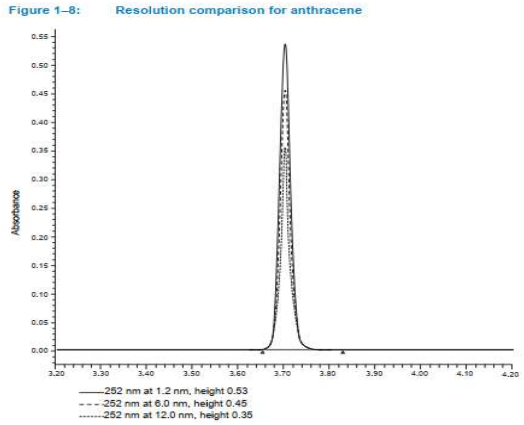
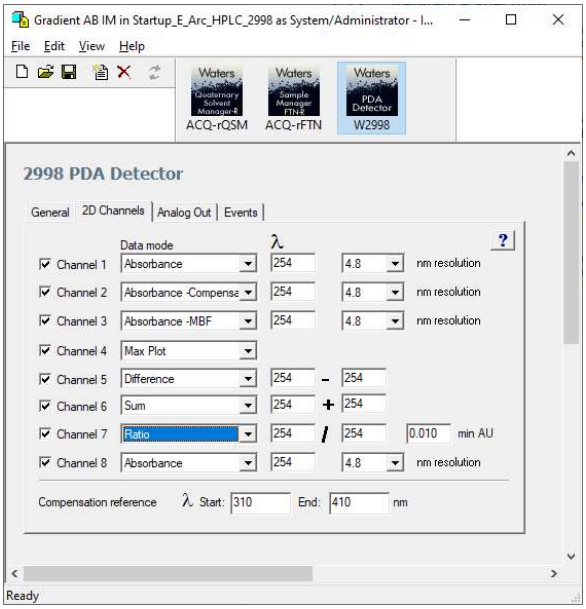
Waters 2998/Acquity eλ Detector operation consideration

Waters™

- If monitoring at analyte λmax, higher bandwidth generally reduced peak height





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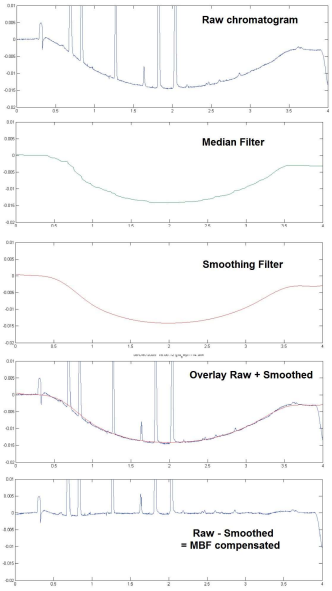
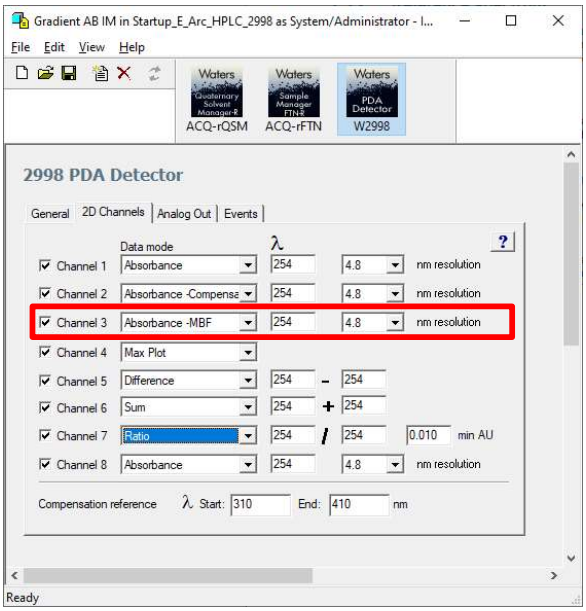
Waters 2998/Acquity eλ Detector operation consideration

Waters™



- WKB67349



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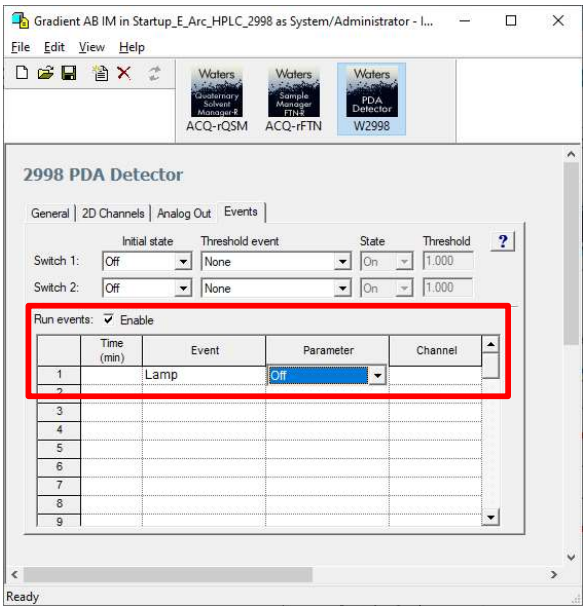
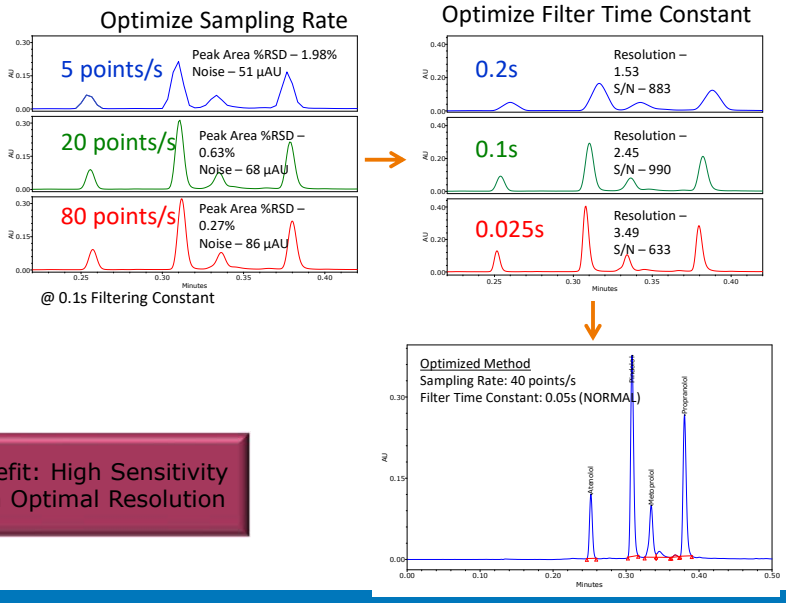
Waters 2998/Acquity eλ Detector operation consideration

- Usually will add a Lamp Off event
- The Time is your actual run time + 1 minute
  - i.e. if your acquisition time is 10 mins, set to 11 mins
- In the event of Empower disconnected from the running instrument, the 11th minute will trigger a lamp off
- This protects the lamp and detector
- But if the time was not changed when sample was required to run pass 11th minute, no meaningful data will be acquired



ACQUITY UPLC®
Independent Sampling Rates and Filter Time Constants

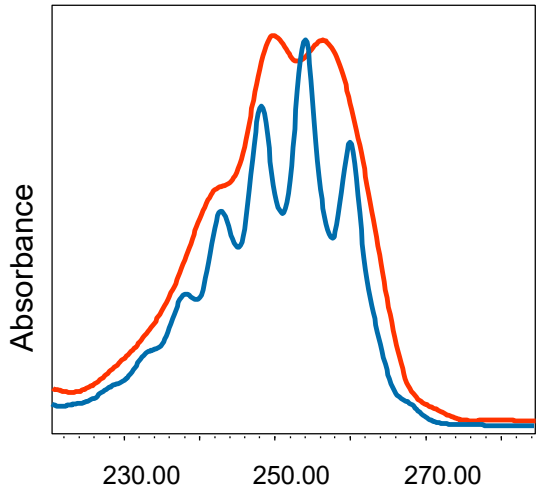


Benefit: High Sensitivity with Optimal Resolution

UV Spectra Resolution (Bandwidth) Effects on Peak Identifcation

Waters™

- High spectral resolution leads to easier ID of components
- Lower resolutions shift UV maxima
- High Confidence in Spectral Homogeneity Checks (Peak Purity)
- High confidence in UV/Spectral Matching



Spectral Resolution
1.2 nm vs. 3.6 nm

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Waters 2475/Acquity FLR principle and consideration

Waters™

- Most molecule do not fluoresces, derivatisation may be required to detect certain compounds
  - E.g. amino acid, aflatoxins etc
- Principle: in a dark environment, excite everything with some light, then measure emitted light
- Fluorescence is proportional to :
  - Source intensity i.e. more intense source = larger signal
  - Volume of flow cell i.e. longer path length means more photons captured = larger signal
- High temperature decreases fluoresce intensity
- Response is only linear at low concentration, analyte dependent
- Normalisation of FLR needs to be performed regularly so to ensure results are comparable. WKB193270

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Waters 2475/Acquity FLR operation consideration

Waters™

- Waters FLR detectors flow cell can only withstand 500 PSI back pressure
- Must ensure FLR is the last in system connection
- FLR flow cells are very expensive, ~HK$45,000
- Warmup FLR for one hour before starting data acquisition
- When Xenon lamp is operating, temperature is very high and will cause burns when mishandled
- If 2475 failed to normalise during startup, try flushing flow cell clean and then run again
- Clean water and fresh mobile phase produce cleaner baseline and better S/N ratio
- FLR PMT will get saturated at very high concentration

75

Waters 2475/Acquity FLR usage consideration

Waters™

- Saturation of PMT, due to:
  - Incorrect gain setting
  - Analyte concentration too high
- Use timed segment gain settings
- Dilute sample
- Use another excitation wavelength
- WKB17612

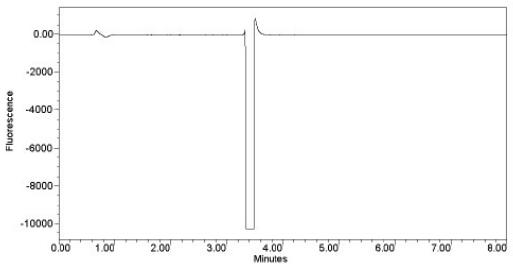

Figure 3-19 Gain Set too High (Fluorescence Pinned to –9999.9 EU)

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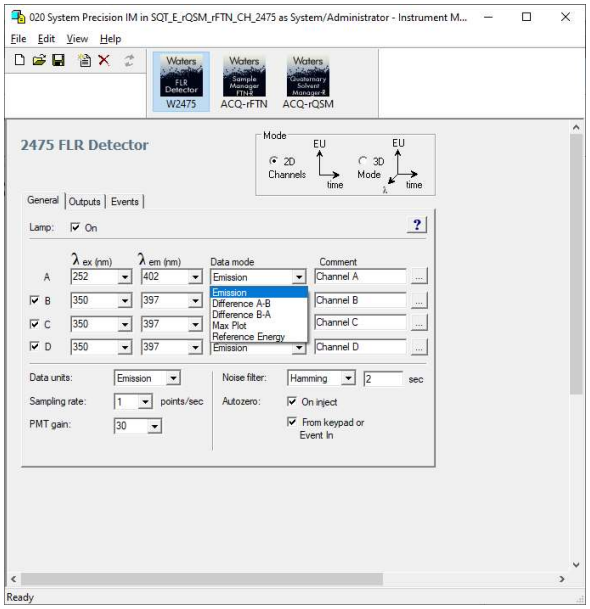
Waters 2475/Acquity FLR operation consideration

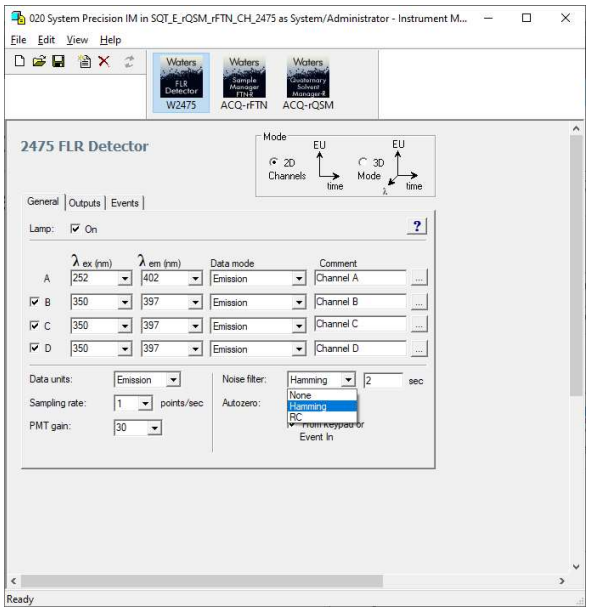
- When operating in 2D mode, 4 channels are available
- Only a single 3D mode is available
- 2D and 3D cannot be simultaneously used in acquisition
- Supports the following data mode in 2D:
  - Emission
  - Difference A-B
  - Difference B-A
  - Max Plot
  - Reference Energy



77

Waters 2475/Acquity FLR operation consideration

- Noise filter:
  - None
  - Hamming, digital
  - RC, analog
- For general purpose, Hamming filter and 1 sec is used
- Effect of:
  - Lower time constant
    - Remove less baseline noise
    - Small peaks hard to discriminate from baseline noise
    - Narrower peaks
  - Higher time constant
    - Remove more baseline noise
    - Broader and shorter peaks



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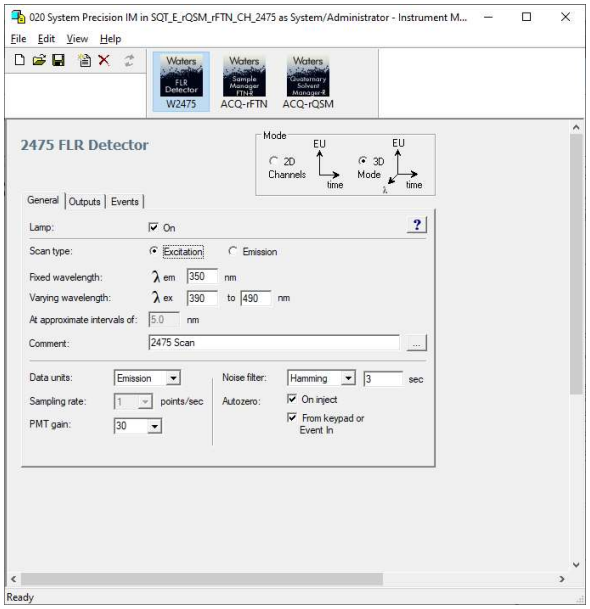
Waters 2475/Acquity FLR operation consideration

Waters™

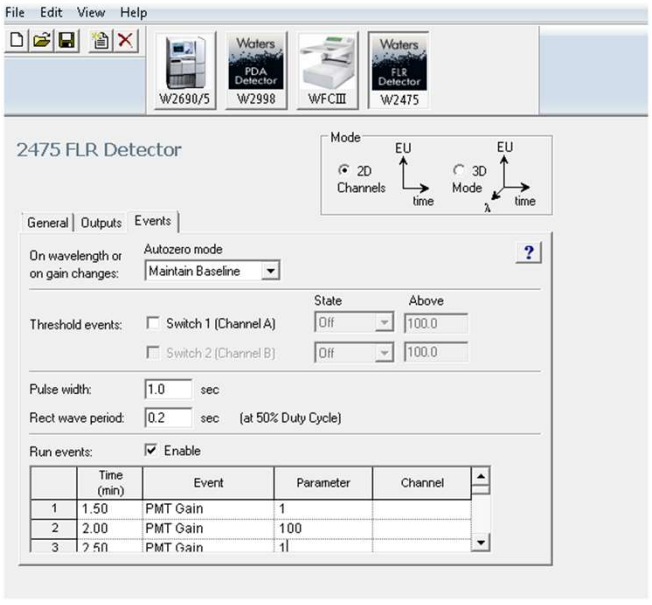
- Under 3D mode, acquisition scan type can be configured as Excitation or Emission
- The instrument will then perform a scan in the other mode across the specified wavelength range
- PMT gain depends on your analyte and concentration, adjust as required
- Noise filter hamming and 3 sec enough for general use



Waters 2475/Acquity FLR operation consideration

Waters™

- Events should be enabled for turning off lamp if computer lost connection with running instrument
  - Add an event of run time + 5 mins to turn off lamp
- Can also be used for optimizing gain across detection window
- WKB15462

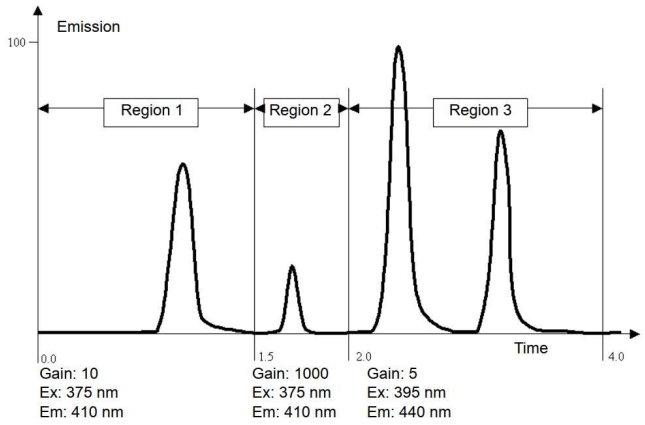


Waters 2475/Acquity FLR operation consideration

Waters™

- Ample time should be allowed between changing of gain value to stabilize baseline
- Peaks that are too closed cannot be used with time segment, compromise is needed

Gain optimized chromatogram:



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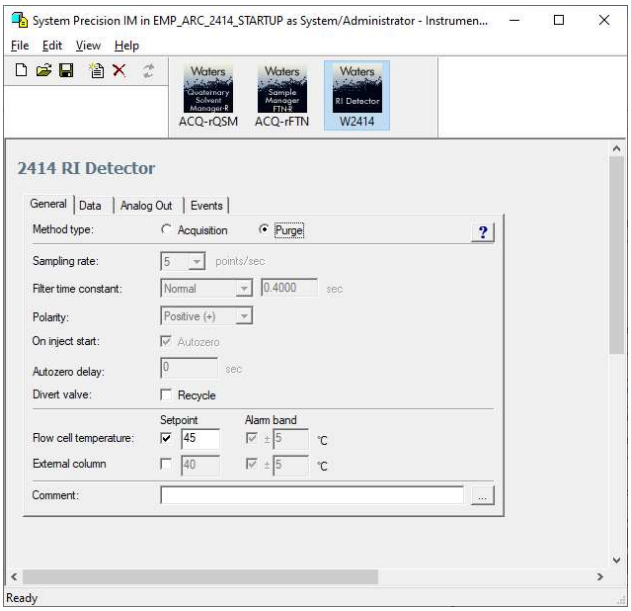
2414 Detector - General

Waters™

- Method type can be acquisition or purge
- When selected to use purge, RI purge valve will open
- Mobile phase will flow through both reference and sample cell
- Used when conditioning the entire system
- All other functions will be locked out
- Unless mobile phase is very precious, recycle valve can be unchecked



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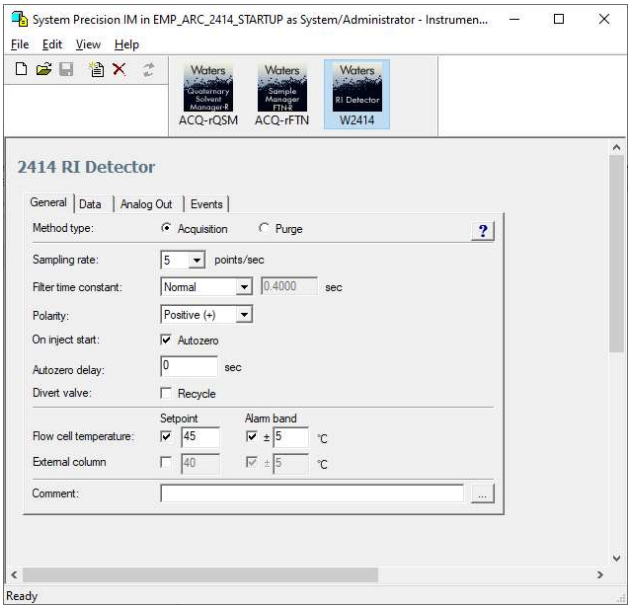
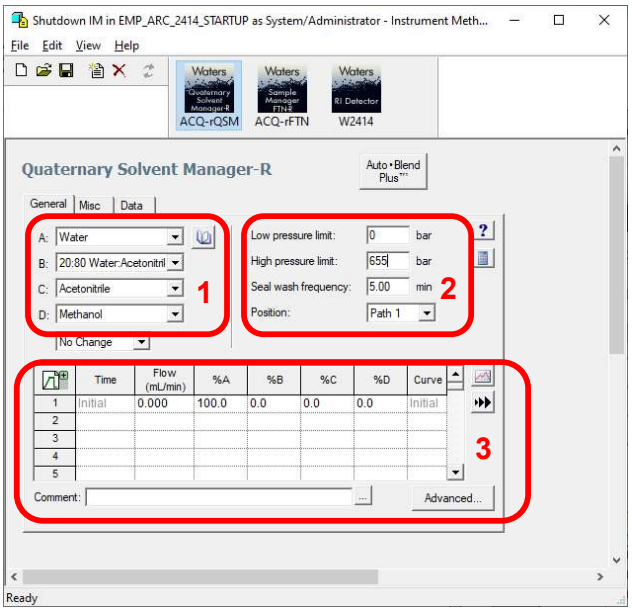
Waters 2414 RI Detector

- When method type is in acquisition mode, all settings can be altered
- Sampling rate use 5 or 10, method dependent
- Filter time constant use Normal for most application
- Polarity will affect peaks upright positioning, if they come out as negative peaks then change to negative
- Should always autozero on inject start
- Recycle off
- Set target flow cell temperature, usually 40 is fine
- External column temperature is not required to set here



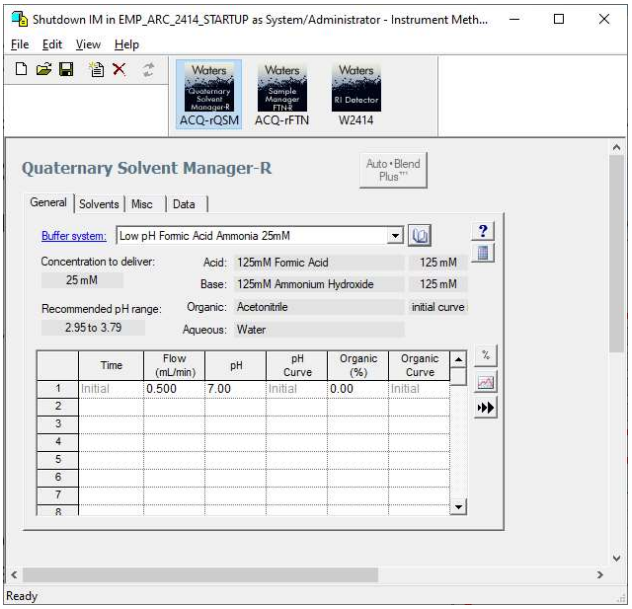
Arc HPLC QSM-R - General

- 1. Solvent information only, no actual physical performance affected
- 2. Set pressure limit according to your column
  - HPLC column: generally ~6000 PSI
  - UHPLC column: generally up to 9000 PSI
  - Other specialty column: Check user manual
- 3. Gradient timetable, set according to desired gradient
  - Gradient curve available for method transfer or delicate control of gradient profile
  - Comment for information
  - Do not adjust values in Advanced tab



Arc HPLC QSM-R – General/Auto Blend

- Arc HPLC QSM can be programmed to perform automatic mixing of mobile phase to produce different concentration of salt and pH
- Useful for unattended method development or column screening
- Refer to 720006557 application note



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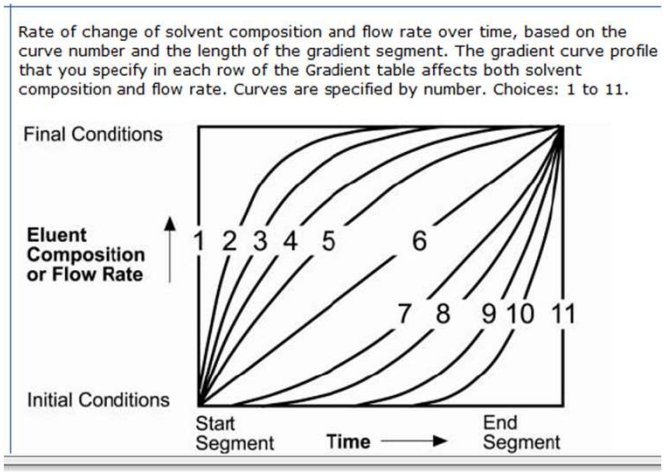
Arc HPLC QSM-R – Gradient curve

- Generally useful for delicate method development
- When used, transferring method to another LC may be challenging
- 6 is always linear ramp
- 1 is immediate ramp at beginning
- 11 is immediate ramp at end
- WKB3489



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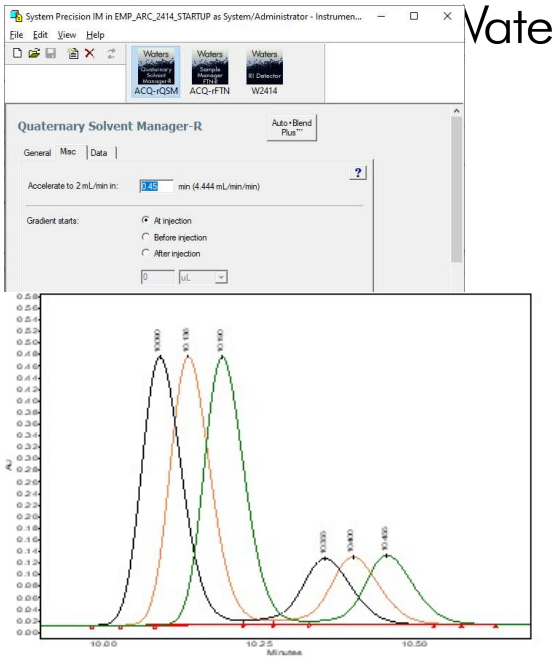
Arc HPLC QSM-R – Gradient SmartStart

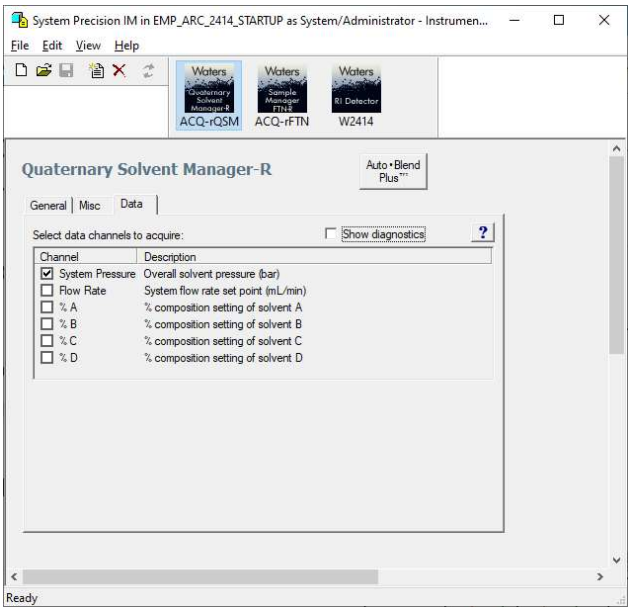
- Do not adjust accelerate speed
- Gradient starts is beneficial for method transfer
  - At injection: system inject samples and starts gradient
  - Before injection: system starts gradient and then injects, peaks eluted earlier
  - After injection: system gradient pauses after injection, then begin gradient
- Does not adjust gradient table, less impact on regulatory standpoint



87

Arc HPLC QSM-R – Data

- Keep System Pressure data is always recommended
  - For diagnostic use
  - For keeping track of column backpressure
- Keeping too much data is not recommended, will cause too many channels to be recorded

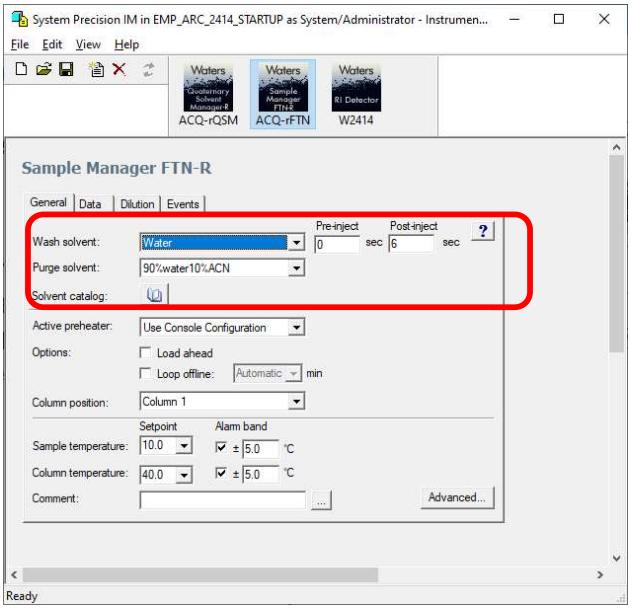


88

Arc HPLC FTN-R – General

Waters™

- Wash Solvent
  - Choose something that will dissolve all your analytes
  - 50% methanol is a good choice
  - Can choose to wash needle before it sit into seal, or after sitting and inject the samples
  - Wash longer for sticky samples
- Purge solvent fills the syringe, part of hydraulics in injection system
- Selecting whichever solvent will not impact system performance, only information only

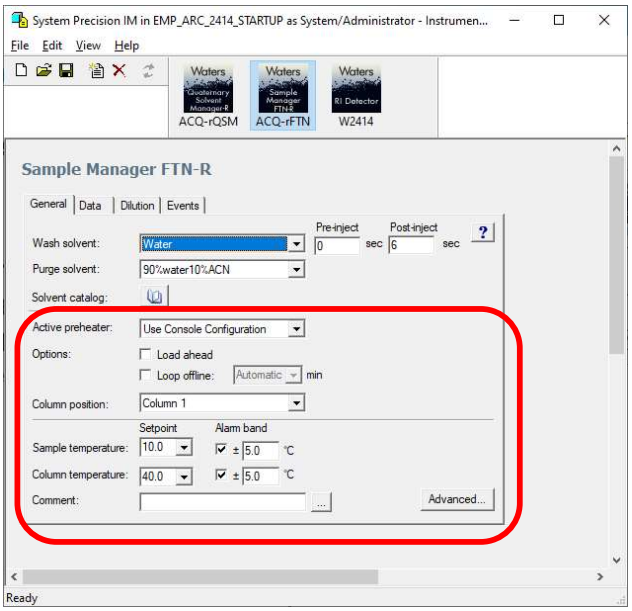


89

Arc HPLC FTN-R – General

Waters™

- Active preheater – generally not available in Arc HPLC config
- Loop ahead and loop offline used for high throughput, don't use for ordinary LC analysis
- Column position for choosing Column if system configured with column selection valve
- Sample compartment temperature can be controlled between 4 and 40 ºC
- Column temperature configurable depending on column compartment
  - CH/C 20 – 65°C
  - CH-A 20 – 90°C
  - Alarm band is optional

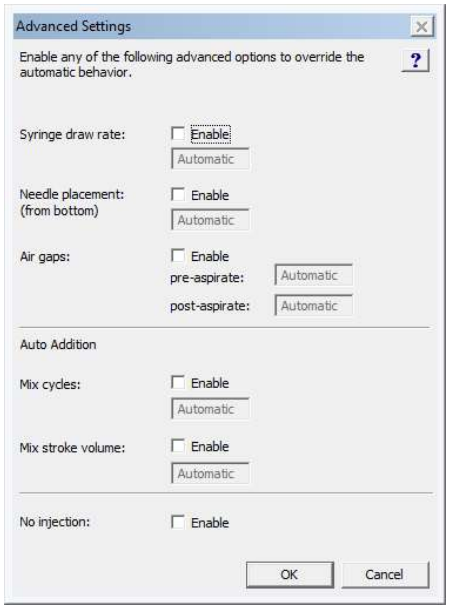


90

Arc HPLC FTN-R – General/Advanced

Waters™

- Do not adjust settings related to:
  - Syringe draw rate
  - Needle placement
  - Air gaps
  - Unless you know what you are trying to do
  - This carries a risk to contaminating entire injection system and bending needle
- Auto Addition can be used if needed to perform online mixing of samples and reagent
- No injection can be used to diagnose system or injection carryover. This option allows for recording of data

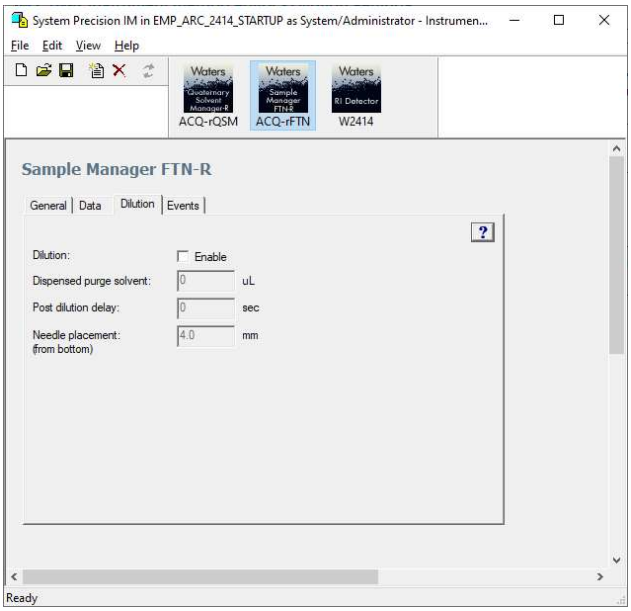


91

Arc HPLC FTN-R – Dilution

Waters™

- Arc HPLC FTN-R can perform online dilution
- Recommended to dilute offline



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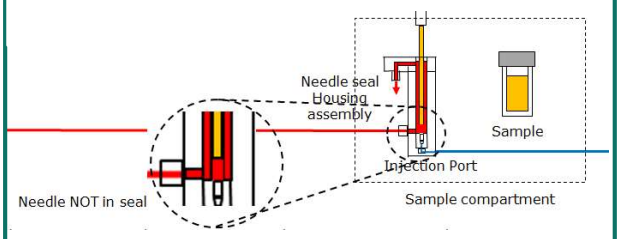
FTN – Wash Mechanisms

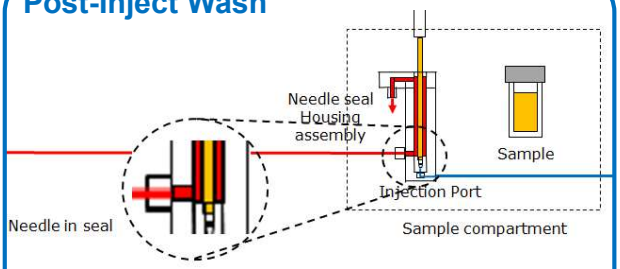
Waters™

Pre-Inject Wash



- Exterior of needle is washed
- Needle **NOT** in the seal, **NOT** under pressure during washing
- Wash solvent flows from bottom of port and exits through top
- Occurs before injection is started

Post-Inject Wash



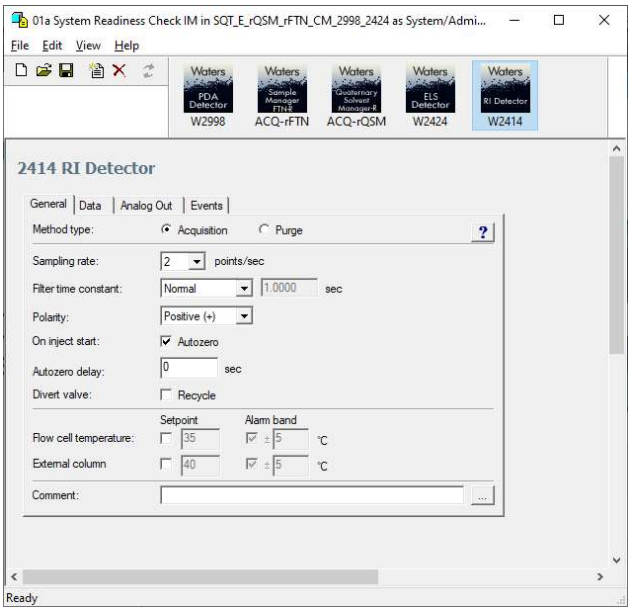
- Exterior of needle is washed
- Needle is in the seal at high pressure during washing
- Wash solvent flows from bottom of port and exits through top
- Occurs while injection is running

93

2414 Refractive Index Detector

Waters™

- Method type:
  - Acquisition: acquire data
  - Purge: do not acquire data, will purge reference cell only. Choose this for unattended instrument conditioning method
- Sampling rate:
  - Choose between 2, 5 and 10
  - Aim between 25 to 50 points across peak
  - Usually HPLC methods use 5
  - UHPLC methods use 10



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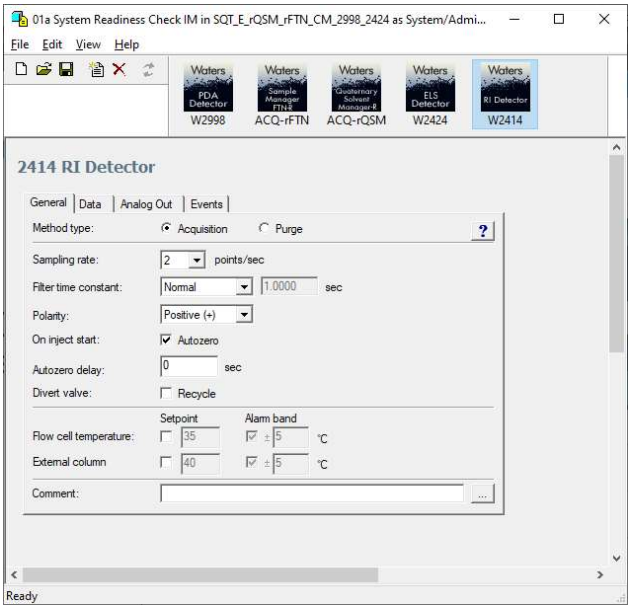
2414 Refractive Index Detector

Waters™

- Filter time constant:
  - Normal is fine
  - Other settings requires optimization
  - RI methods usually too long to make it worthwhile to optimise this setting
- Polarity:
  - Positive or negative
  - If all peaks came out as negative peaks, then switch polarity. Vice versa
- Autozero:
  - Should be checked
- Divert valve to Recycle:
  - Generally not used to rid the possibility of contaminating mobile phase reservoir

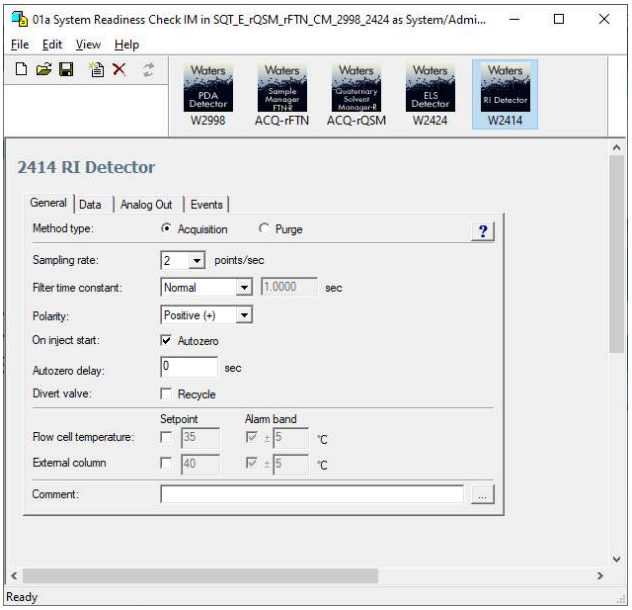


95

2414 Refractive Index Detector

Waters™

- Flow cell temperature:
  - Generally do not affect peak shape
  - Use 40°C or 5 °C above ambient
- External column
  - Not used
- Comment
  - Type in notes for reminder, will not affect acquisition

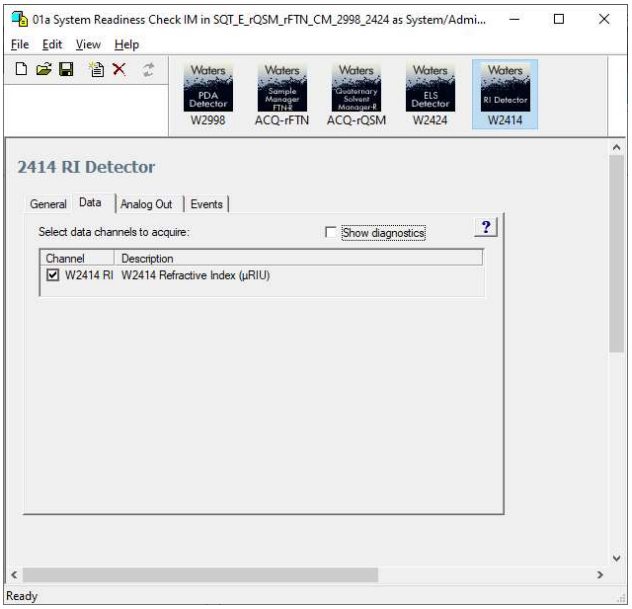


96

2414 Refractive Index Detector

Waters™

- Data channel
  - Must be checked, else no data points recorded

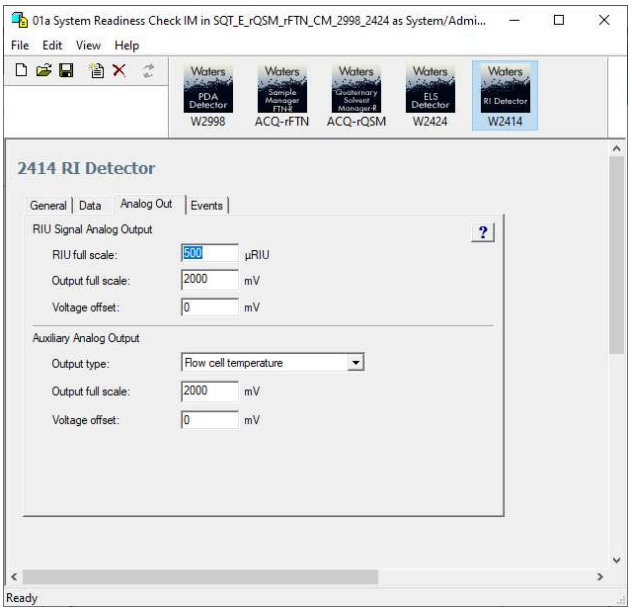


97

2414 Refractive Index Detector

Waters™

- Analog out is not usually configured
- Can be safely ignored for most of the time

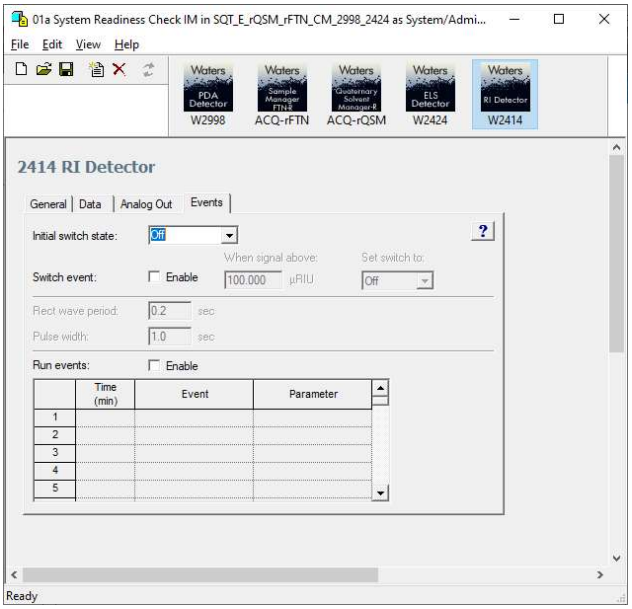


98

2414 Refractive Index Detector

Waters™

- Events control is beneficial when running for extended time and unattended shutdown of instrument is required
- Generally not configured for most routine operation

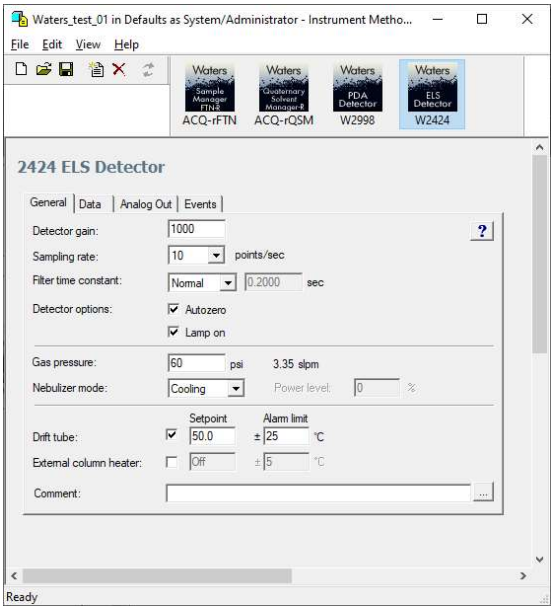


99

2424 Evaporative Light Scattering Detector

Waters™

- Detector gain
  - 1 ~ 1000
  - Will amplify peak and noise
  - Start with 200
- Sampling rate
  - 1 ~ 80Hz
  - HPLC method use 10 or 20 Hz
  - No significant improvement over 40 Hz
- Filter time constant
  - Normal is sufficient for general use
- Detector options:
  - Autozero is usually selected
  - Lamp on for acquisition, uncheck for shutdown method

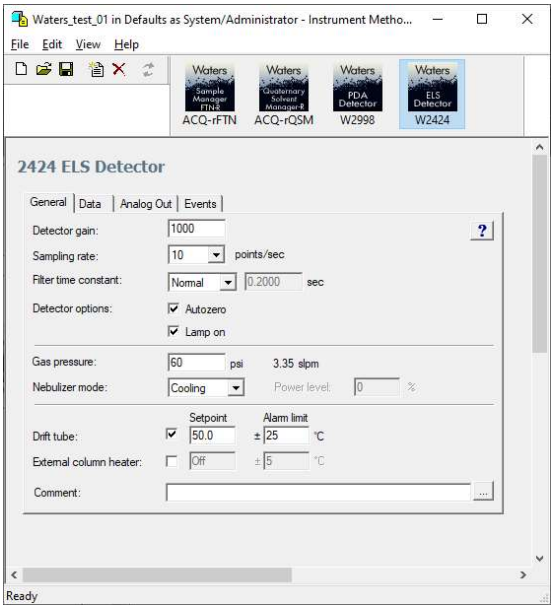


100

2424 Evaporative Light Scattering Detector

Waters™

- Gas pressure
  - 20 ~ 60 PSI, or off
  - Have an effect on peak intensity
  - Generally starts with 40 PSI
- Nebuliser mode
  - Cooling, heating or off
  - Use only cooling
- Drift tube temperature
  - 5 to 100 ºC
  - Higher temperature reduce baseline noise and peak intensity as this parameter is destructive
  - Alarm limit default at 25 ºC is sufficient

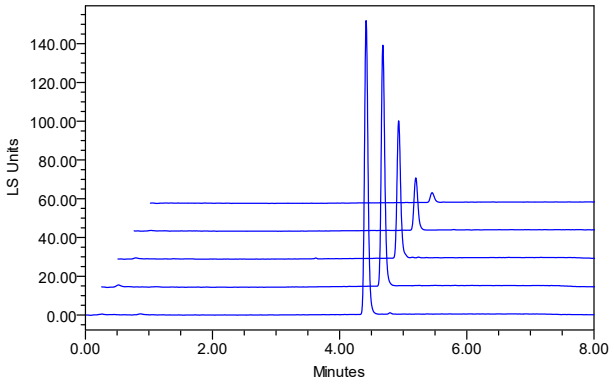


101

2424 Evaporative Light Scattering Detector

Waters™

- Impact of drift tube temperature on the detection of dibutyphtalate
  - Above 45°C, DBP is nearly not detected
  - At 30°C, the baseline noise is 50% higher than at 45°C, but the response is 30 times greater
  - The best sensitivity is obtained with the lowest temperature value



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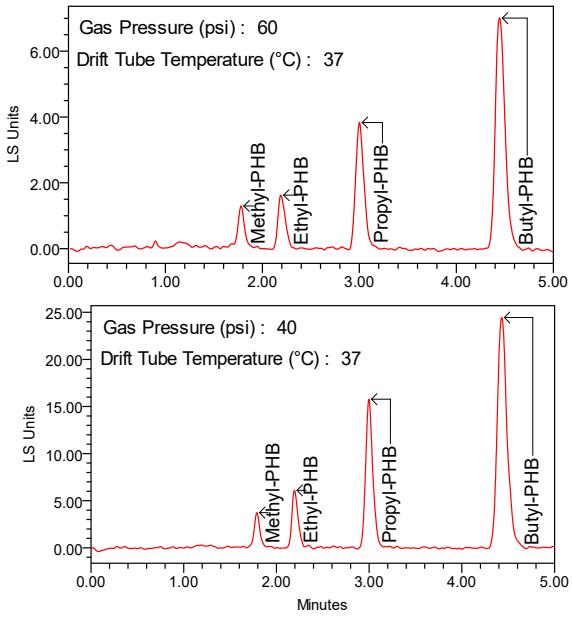
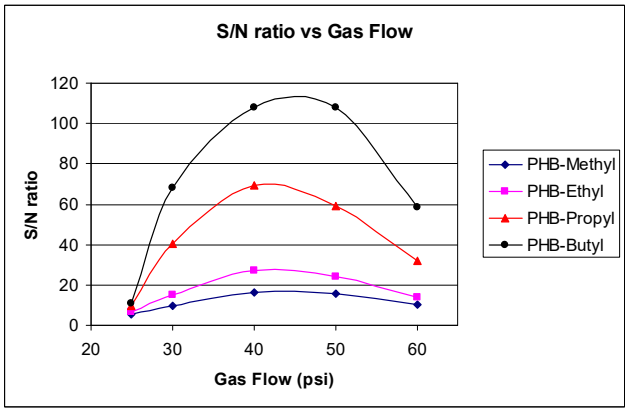
2424 Evaporative Light Scattering Detector

Waters™

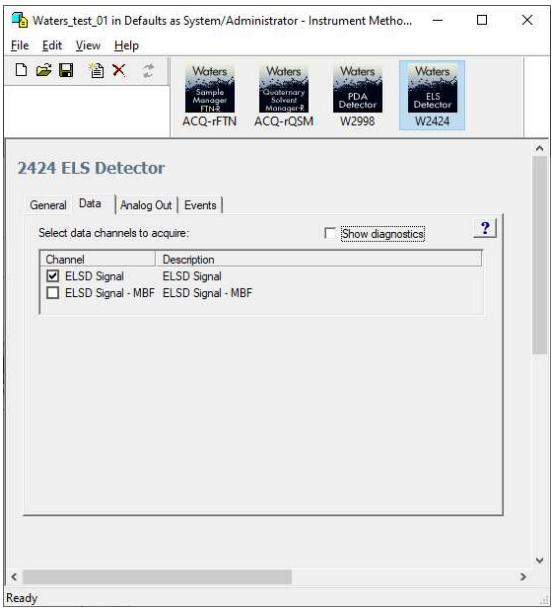




2424 Evaporative Light Scattering Detector

Waters™

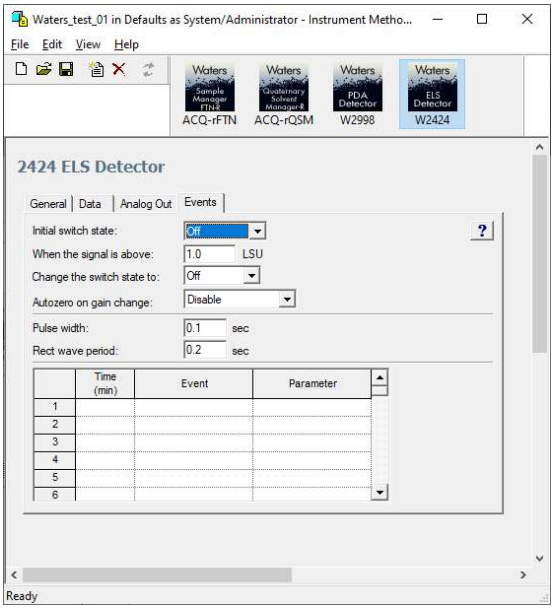
- ELSD signal
  - Must be checked for recording signal
- ELSD signal - MBF
  - Similar to PDA MBF feature
  - Will smoothen the baseline drift so in the final result the baseline will appear as straight baseline
  - Will likely lost data of very small peaks



2424 Evaporative Light Scattering Detector

Waters™

- Events page is useful for time segmented gain adjustments
  - Some analytes concentration may be very high
  - Good to reduce gain to prevent oversaturating PMT
  - Likewise, gain can be increased to boost signal of less intense peak
  - Can also set Lamp to off
  - Can also set Gas Pressure to off
  - Both used for switching off ELSD after completing run



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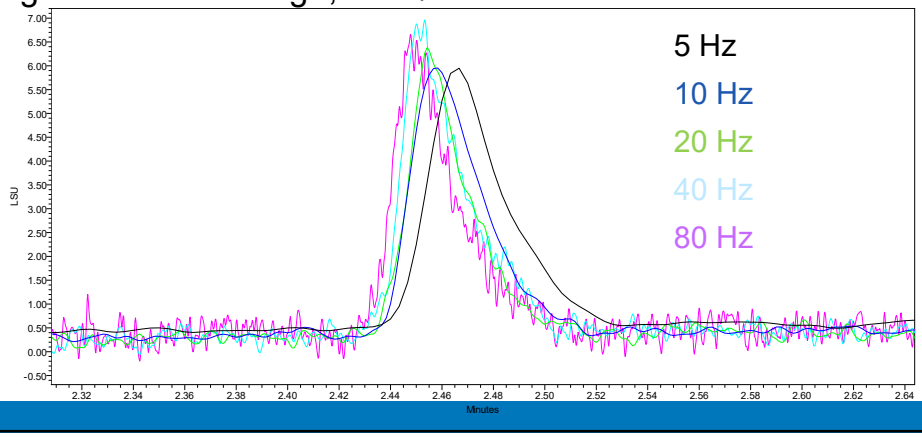
Sampling rate and Filter Time constants

Waters™

- General advice:
  - For UPLC application set filter time constant to “fast”
  - For sampling rate aim at 15-20 datapoints across the peak

- When sampling rate is set too high, the s/n will decrease:



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General troubleshooting rule on LC hardware

Waters™

- First: Only change one thing at a time
- Second: Only call it a problem if it occurred twice
- Third: Put it back if it doesn't fix the issue
- Fourth: Throw bad parts away
- Fifth: Maintain a logbook for your LC system and write down important things
- Following these will reduce further downtimes – first time right

- John W. Dolan
  - https://www.chromatographyonline.com/view/rules-thumb
  - J.W. Dolan, LC•GC6(4), 304–308 (1988).

107

General LC operation consideration

Waters™

- Avoid adjusting pH of buffer after adding organics
  - Presence of organic affect pH
- Never top up buffer reservoir
  - Risk shifting pH and introducing debris into flowpath
- Use new column for LC method development
- If a column was used for ion-pairing methods, don't use on other methods
  - IP reagents will never ever be washed from column
- It is riskier to use column across multiple LC methods
- Not all C18 columns are equivalent
  - Use column coach to find out more about column selectivity
  - https://find.waters.com/ColumnCoach/about
- https://www.chromatographyonline.com/view/seven-things-avoid-liquid-chromatography-laboratory-1
- J.W. Dolan, LCGC North Am. 33(1), 18–22 (2015).

108

General LC operation consideration

Waters™

- Equilibrate and condition column properly
- Run blanks to prove system is clean before running samples
- It may be beneficial to run few prime injections

- https://www.chromatographyonline.com/view/seven-things-avoid-liquid-chromatography-laboratory-1
  - J.W. Dolan, LCGC North Am. 33(1), 18–22 (2015).
- https://www.chromatographyonline.com/view/baker-s-dozen
  - J.W. Dolan, LCGC North Am. 23(9), 1008–1012 (2005).

109

General LC column usage consideration

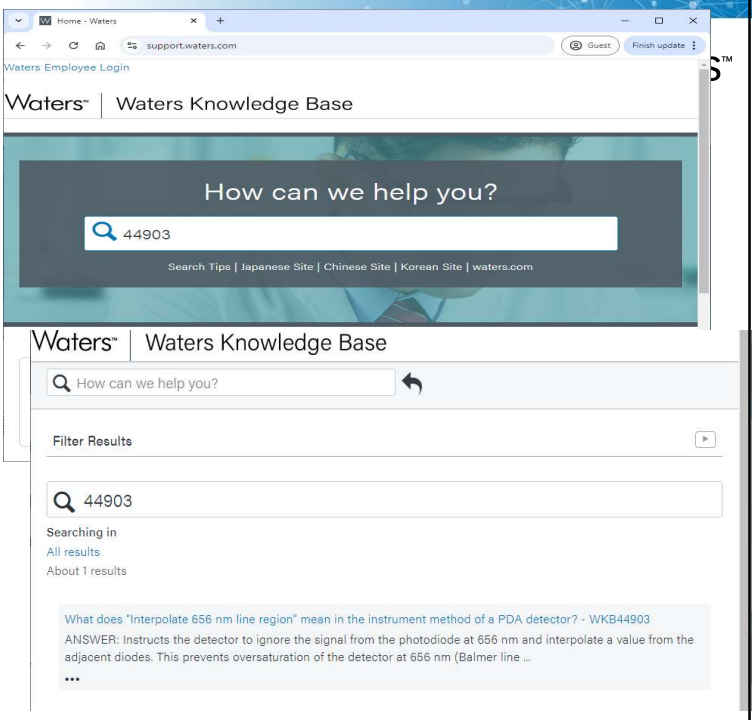
Waters™

- One column, one method
- In-line filters protect column from clogging effectively
- Clean column during idling
- Use temperature control – even for room temperature methods
- All column have void volume, make analytes elute after that
- Inject clean samples
- Use highest quality mobile phase available
- When validating methods, think of alternatives
- https://www.chromatographyonline.com/view/lc-columns-top-10-list-1
  - J.W. Dolan, LCGC North Am.21(3), 262–266 (2003).

110

Using Waters Knowledge Base

- Support.waters.com
- No need to signup/login
- If you have WKB (we call it KCS internally) article number, enter it in search box without WKB
- Or search with keywords
- Will return solution to general issues not available through Empower/MassLynx help files
- Contact us if your issue cannot be found on



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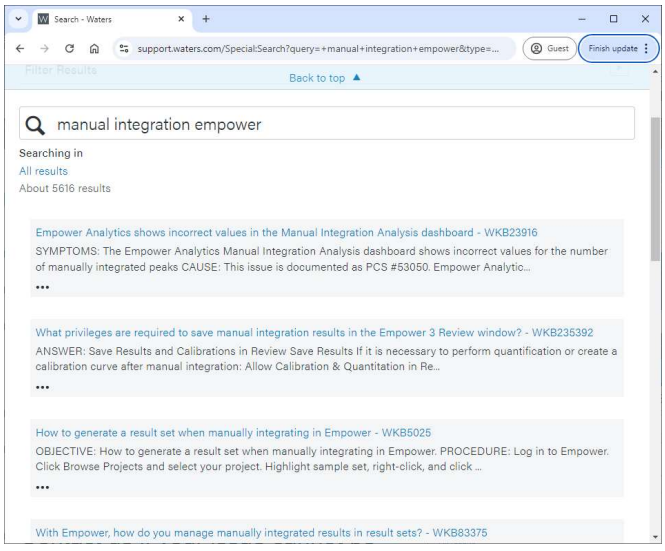
Using Waters Knowledge Base

Waters™

- Support.waters.com
- Searching with keywords works as well
- WKB articles cover informatics, instruments and consumables



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Waters™

Sample preparation – A very brief introduction

113

Why Do Sample Preparation?

Waters™

- Transfer analytes from matrix into a solution to be analyzed
- Remove matrix interferences
  - Increase signal to noise
  - Reduce variability in analytical results
  - Increase column lifetime
  - Reduces system downtime
  - Reduce chromatogram complexity
- Concentrate analyte
  - To meet the low detection limit
  - To stay within the sensitivity range of the available instrument

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Sample preparation consideration

Waters™

- Ideally, HPLC/UPLC sample injections should be:
  - Clean, without visible solid particulates
    - Physical particulates will clog system
  - Optimal concentration to not contaminate instrument
    - Must account for matrix composition
  - Sample solvent must be compatible with mobile phase and system
    - Injecting neat strong solvent is generally advised against in Reversed Phase systems
  - Injection volume optimal for system and method
    - Not exceeding injection volume or consider the impact of injecting very large volumes

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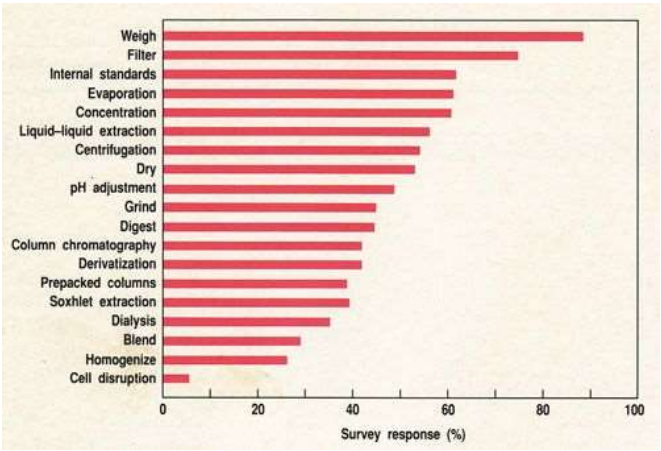
Common ways to prepare samples

Waters™

- Two major sample categories
  - Solid
  - Liquid
- At minimum (solids):
  - Grind
  - Weigh
  - Extract with solvent
  - Sonication
  - Filter through 0.22µm, OR, centrifuge/spin down
- https://www.chromatographyonline.com/view/overview-sample-preparation



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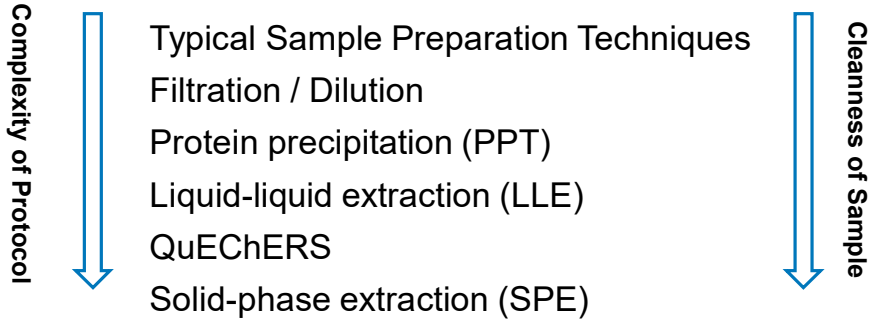
Sample Preparation Techniques: Which One?

Waters™



Objectives:
Simplest technique
Fastest preparation procedure
Cleanest extracts

"Good Enough" Sample Preparation

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Better ways to produce cleaner samples

Waters™

- Although you will get cleaner samples, these methods are:
  - More time consuming
  - Requires method development
  - Requires more consumables and equipment
  - Provides batch processing capability

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Waters Oasis HLB SPE Products

Waters™

- Comes in both cartridges and well plate elution format
- Suitable for automated equipment or manual workflows

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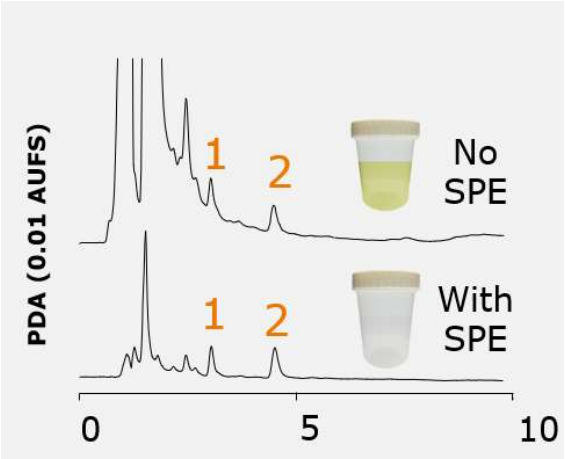
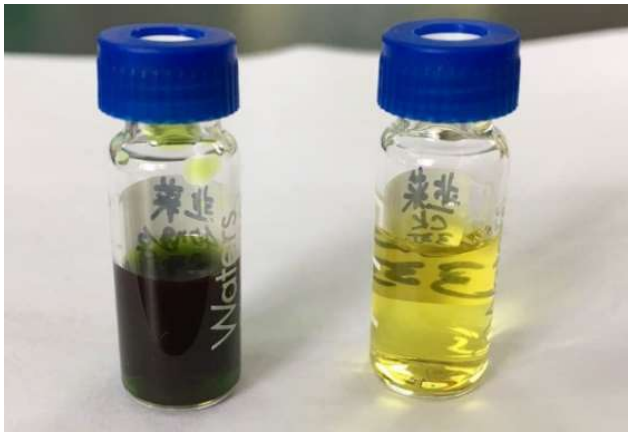
Some examples

Waters™

- General SPE will remove coloured contaminants and other matrix interferences



- Oasis PRiME effectively removed most coloured contaminants



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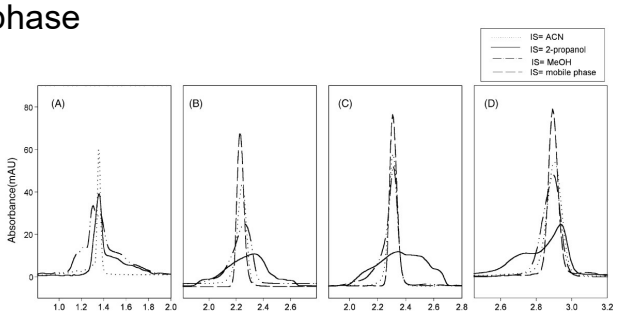
Sample dilution consideration for LC analysis

Waters™

- Best to dilute in initial composition of mobile phase
- Avoid injecting in 100% organic solvent
  - Usually result in peak shape issue due to strong solvent effect
  - Right figure shows typical effect
- Sample concentration of around 1mg/mL is a good starting point, adjust when necessary to achieve good signal intensity
- For liquid samples, consider 100 or 1000 times dilution first



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Waters™

Method transfer and development – A very brief introduction

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Method transfer - Introduction

Waters™

- Why method transfer?
  - Adapting from official methods (ChP, GB, USP etc)
  - Reproducing method/results from other entity or published works
  - Obsolete column
  - Updated requirements, including accuracy and speed
- System where methods originate from are usually different from what you have
- System = LC + Column + Solvent + sample

123

Method transfer – criteria to consider

Waters™

- Validated method with example chromatograms
- Properties of donor and target system
  - Dwell volume and Mixer differences
  - Temperature control module, e.g. forced air or convection
  - Gradient shapes, i.e. if gradient curve was used
  - Detector settings, e.g. data rate, wavelength, bandwidth etc
- Preparation of mobile phase, online or offline mixing
- Steepness of gradient change, especially in UPLC methods
- Column chemistry, not all C18 are the same
- If transferring method from very old papers, consider modernisation

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Required Information: Original Method, Results & Criteria

Waters™

- Column
  - Chemistry (ligand, particle size, brand)
  - Dimensions
- Conditions
  - Mobile phase
  - Flow rate
  - Gradient profile, including re-equilibration
  - Column temperature
- Sample
  - Diluent
  - Concentration
  - Injection volume
  - Molecular weight
- Chromatogram
  - Number of analytes
  - Retention required
  - Resolution required
- Quantitation
  - Limit of detection
  - Limit of quantitation
  - Linear dynamic range
  - Accuracy
  - Precision

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Method transfer – desirable outcomes

Waters™

- Desirable outcomes from successful transfer:
  - No “missing” peaks
  - Equal or greater resolution between all critical pairs
  - Equal or greater signal to noise ratios
  - Equal or better precision for retention times, peak areas, peak heights and peak “shape” metrics (tailing, asymmetry, width)
  - Equal or greater dynamic range – upper and lower limits of quantitation meet or exceed current requirements
  - Order of elution is the same
  - Run time and cycle time are equal or faster
  - Solvent consumption is equal or less

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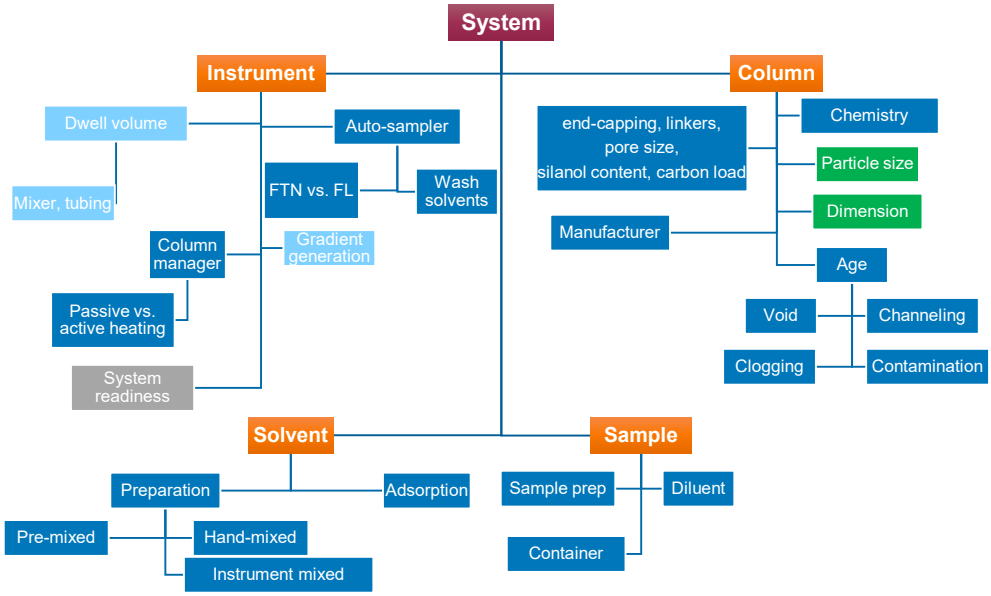
Method transfer - Sources of System differences



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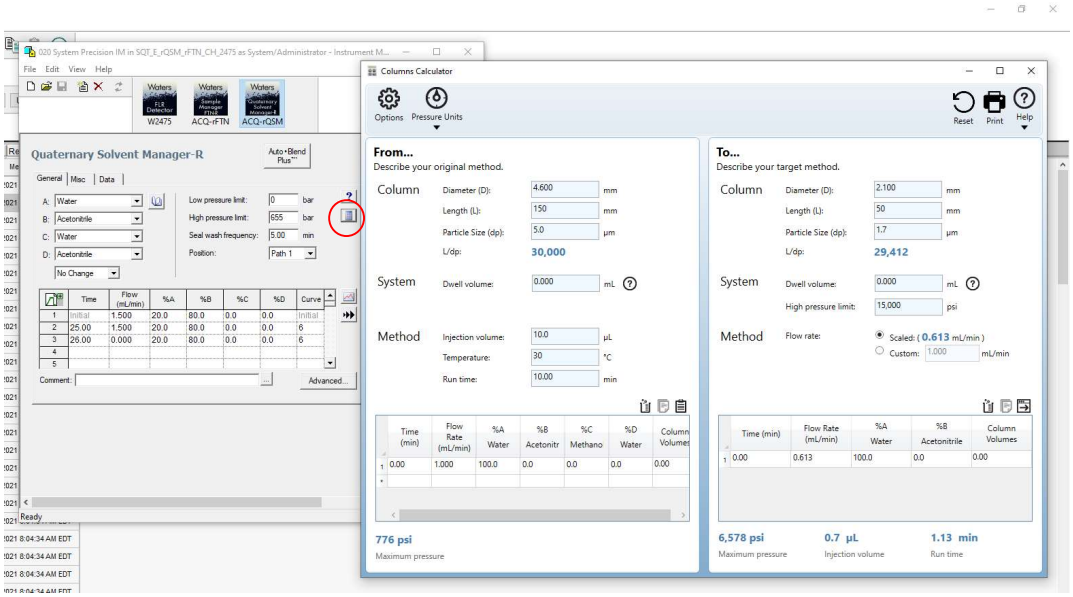
Empower 3 column calculator



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Empower 3 column calculator

Waters™

- Column calculator comes with Empower 3
- Can be used to scale down or up a method
- Will take into account dwell volume of different systems
- Preferably using column of same chemistry in both systems
  - E.g. transferring Xbridge C18 from HPLC into Acquity BEH C18 in UPLC, same selectivity
- Use Waters Column Coach for looking for Waters column that provides comparable selectivity to your current column
  - https://find.waters.com/ColumnCoach/about

Method development

Waters™

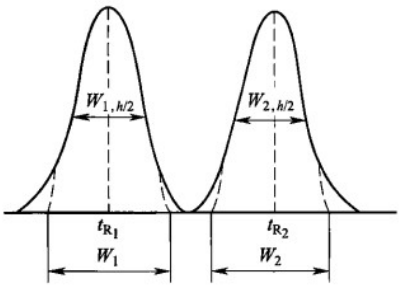
- Why method development?
  - New product
  - Need a higher throughput LC method
  - Obsolete of column or previous LC system
  - End of life of LC method
- Core issue of LC method: resolution of all peaks in a chromatogram
  - What is resolution?
  - How much is enough?
  - Which factors will affect it?

$$R=\frac{2\times(t_{R_2}-t_{R_1})}{W_1+W_2} \text{ 或 } R=\frac{2\times(t_{R_2}-t_{R_1})}{1.70\times(W_{1,h/2}+W_{2,h/2})}$$

式中 t_{R_2} 为相邻两色谱峰中后一峰的保留时间；

t_{R_1} 为相邻两色谱峰中前一峰的保留时间；

W_1、W_2 及 $W_{1,h/2}$、$W_{2,h/2}$ 分别为此相邻两色谱峰的峰宽及半高峰宽，见下图。



Resolution equation

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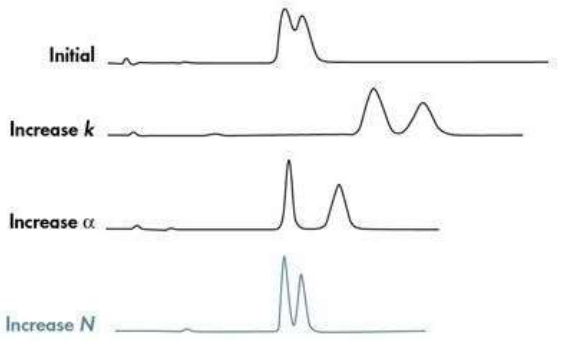
- k_2: Retentivity
  - Chemical factor
  - Different mobile phase (MP), pH, composition of MP, temperature
- α : Separation factor
  - Chemical factor
  - Different stationary phase will improve resolution, or even change elution order
  - Derivatisation
- N: Efficiency
  - Theoretical plates
  - Physical/mechanical factor
  - Related to size of column and particles

$$R_s = \left(\frac{\sqrt{N_2^*}}{4}\right)\left(\frac{\alpha^* - 1}{\alpha^*}\right)\left(\frac{k_2^*}{1 + k_2^*}\right)$$



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Method development – Other criteria

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- Sensitivity
- On-column loading
- Speed/throughput requirement
- Solvent consumption
- Ease of usage
- Column robustness/lifetime

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Method development strategy

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- Look for reference, e.g. published papers or official methods
  - Must be soluble in aqueous or general reversed phase solvents
  - If there are no references usable, try scouting method
- General steps:
  - Scouting with 50 or 100 mm column
  - Run with standard first
  - Water/Acetonitrile is a good choice, adjust pH or use modifiers if required
  - Adjust k' for best retention
  - Adjust α for optimal selectivity
  - Consider different column length for best efficiency

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Beneficial information during method development

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- Analyte solubility
- Number of analytes/peaks to be chromatographed
- Chemistry of analyte
- Type of detection required
- The effect of pH
- Detection level
- Matrix effect

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Method development – choice of particles

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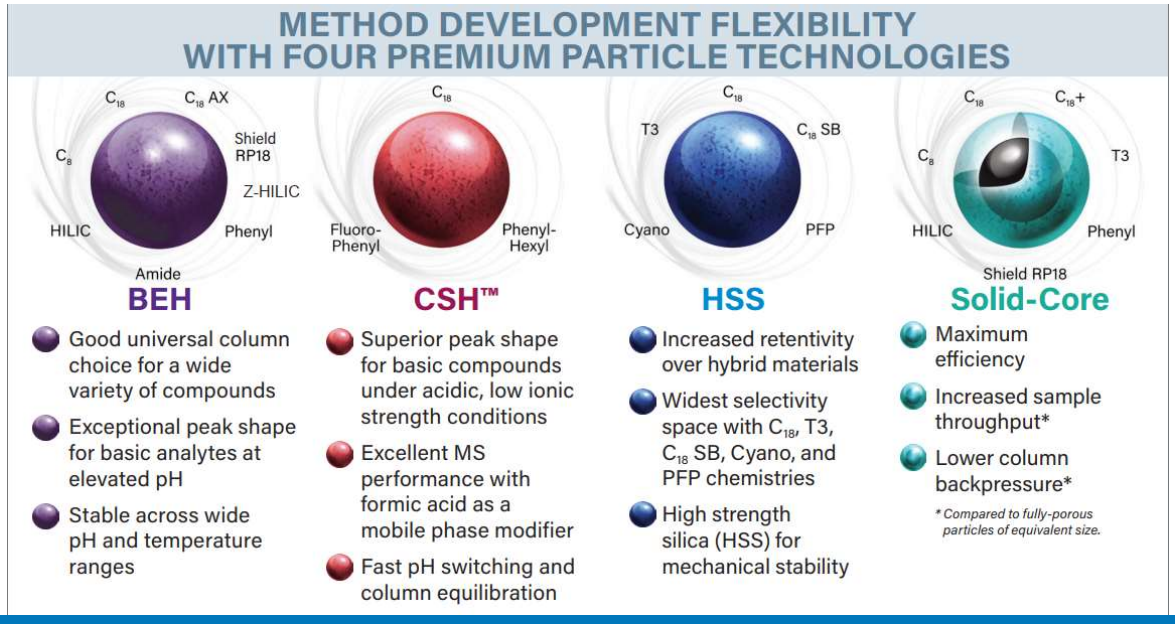

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Method development – choice of chemistry

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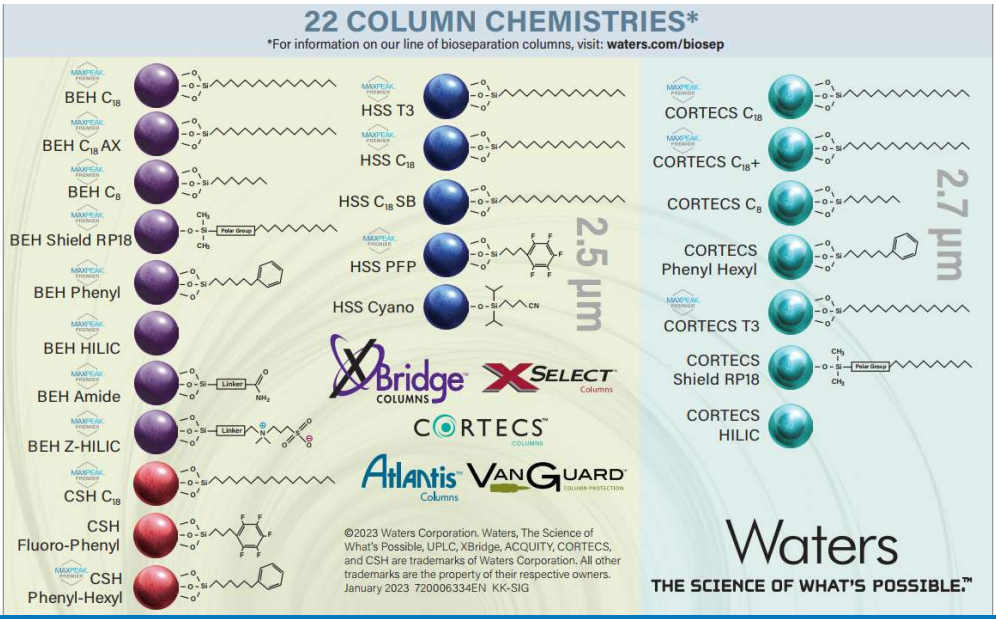

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Method development- choosing the optimal column length

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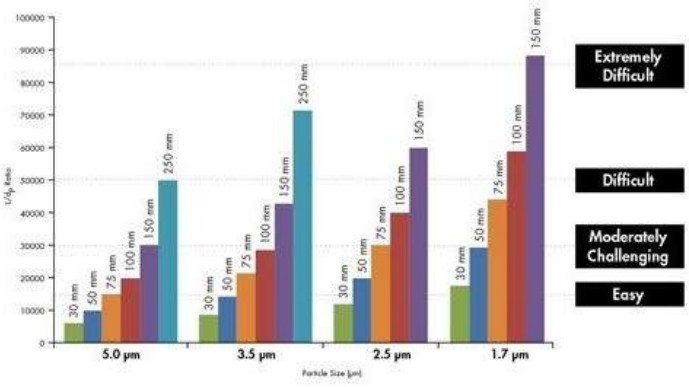

- L/d_p ratio is particularly useful when trying to determine which particle size packing material and column length may be necessary for a given application
- Easy – Simple assay, < 5 components
- Moderately challenging <10 components
- Difficult, general impurity profiling
- Extremely difficult, metabolites identification, toxicology screening

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Method development- choosing the optimal column length

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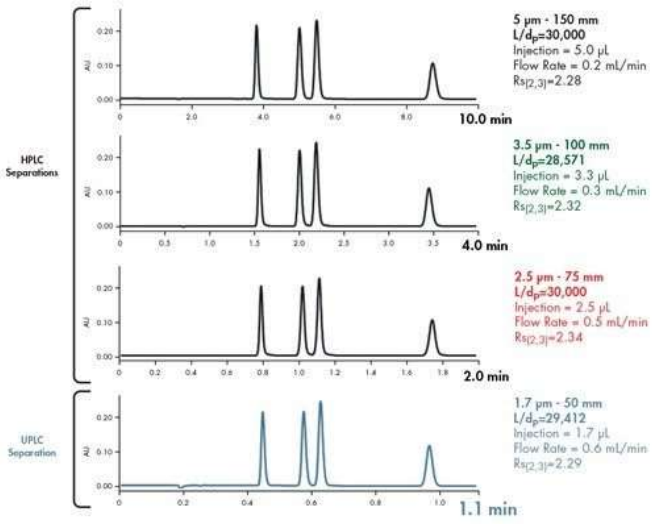

- Key take-away:
  - Moving into UHPLC territory makes a great difference
  - Further cost savings and exceptional performance possible with UPLC
  - The key decision is throughput and economical consideration

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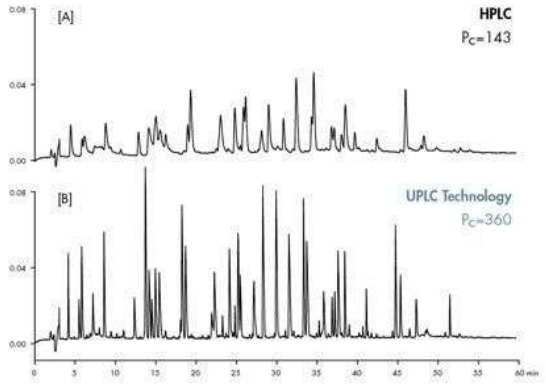
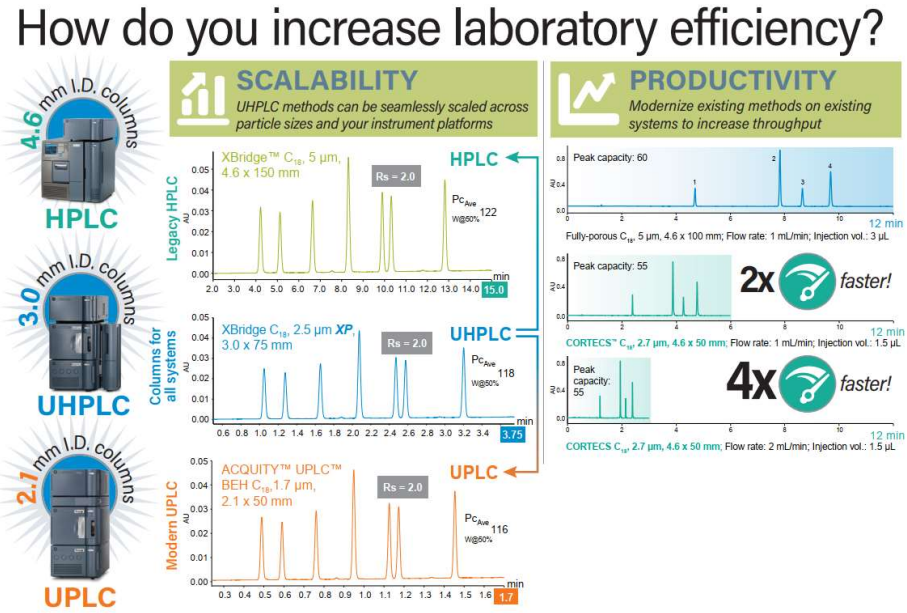


- Peak capacity (k') is a better measurement of gradient method performance
- Definition:
  - Theoretical number of peaks that can be separated in a given gradient time
  - See diagram to right for comparison between

Method transfer for efficiency gain

Waters™



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