

# **Interfacing SFC to various detectors with emphasis on cold electrospray mass spectrometry & achieving the sensitivity, robustness, as well as dynamic range needed for a variety of applications**

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# The value of SFC

- Reverse phase (RP) separations cannot do it all!
  - For polar molecules without much lipophilicity (often pivotal in chemistry, biology, and biomedical efforts), normal phase (NP) often is most versatile column chromatography approach
  - The potential biology / biomedical need for NP separations may be similar in size to current RP usage for DMPK efforts → Science is telling us we must isolate & measure biomarkers!
- A strong case can be made that SFC is the best way to perform normal phase (NP) chromatography
  - Both NP-LC and SFC can be used for very challenging NP separations, often producing similar separations with the same columns, **but:**
  - While similar outcomes can be achieved, there are significant differences between NP-LC and SFC
  - The primary differences are in the productivity and detection

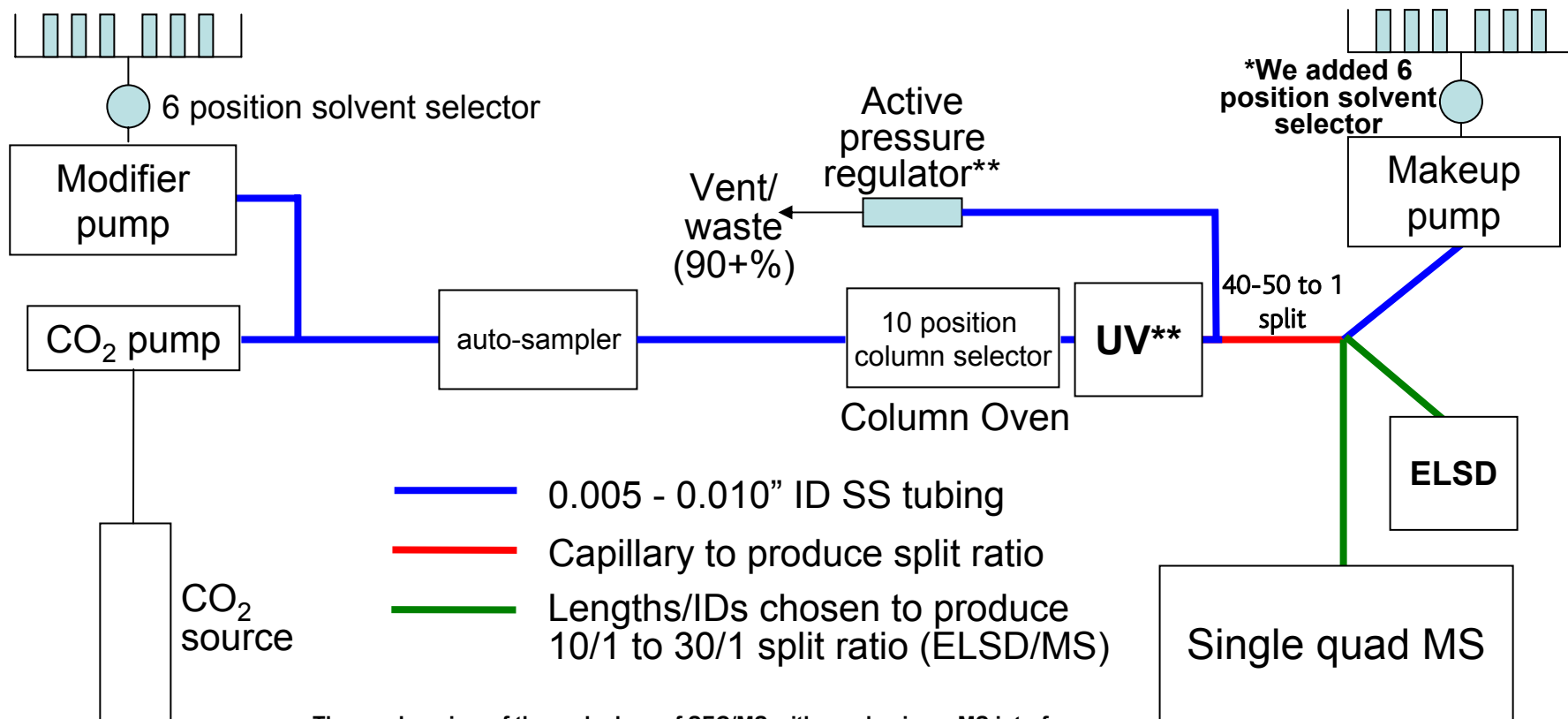
# The value of SFC: productivity

- SFC is much faster than NP-LC because the optimum eluent velocities are much higher (& van Deemter curves flatter)
- Greater analyte solubility in SFC eluent often allows a larger range of mass load than NP-LC
- Perhaps most important – generic gradients in SFC allow rapid method development and focused gradients can achieve stacked injection speeds while simultaneously improving the separation relative to isocratic operation
- We perform many NP-LC & SFC separations and see the sum of the benefits:
  - Repeated run metrics combining  $R_s$ /unit time (3x) & mass load (>2x) suggest SFC is 7 fold more productive (average) than NP-LC
  - Simultaneously, SFC requires only one third the method development time
- **Still, in some regards (trace analysis), SFC is only as good as detectors that can be used effectively with it!**

# The value of SFC: detection

- While SFC is a clear productivity winner in terms of the NP separations, it remains mixed at best with regard to key detection approaches\*
  - UV: noisy & noise raises detection limit ( $\geq 10x$ )\*\*
  - ELSD: ditto, but much higher T and less  $N_2$  can help\*\*\*
  - MS: SFC works better than NP-LC with hexane, but still poor compared RP-LC
    - Particularly poor with most desirable ionization: electrospray\*\*\*\*
    - Cold electrospray (ESI) is noisy and sensitivity is down 100x or more relative to RP-LC\*\*\*\*
    - APCI works better than ESI when APCI heated to high temperatures\*\*\* but produces unwanted fragments and covers limited chemical space
- **In order to reach its full potential, SFC needs to be on par with RP-LC using the 3 detection approaches above**

# Most common detector interfacing approach for SFC: our initial approach for a generic SFC system



**Thorough review of the early days of SFC/MS with emphasis on MS interface:**  
M.T. Combs, M. Ashraf-Khorassani, L.T. Taylor *J. Chromatogr. A* 785, 1997, 85.

**Traditional split interface SFC/MS set up:**

P.J.R. Sjoberg, K.E. Markides *J. Chromatogr. A* 785, 1997, 101.  
T. Baker, J.D. Pinkston *J. Am. Soc. Mass Spectrom.* 9, 1998, 498.  
D.G. Morgan, K.L. Harbol, N.K. Kitrinis *J. Chromatogr. A* 800, 1998, 39.  
M. Garzotti, M. Hamdan, *J. Chromatogr. B* 770, 2002, 53.  
B. Bolanos, et.al. *Int. J. Mass Spectrom.* 238, 2004, 85.

**SFC/ELSD split interface:**

J.D. Pinkston, TR Baker, *J. Am. Soc. Mass Spectrometry*, 9, 1998, 498.  
Z Wang, *Int. Labmate*, Jan 2007, 12-13.  
P Carraud, M Dreux et.al., *J. Chromatogr.*, 1987, 404, 95.

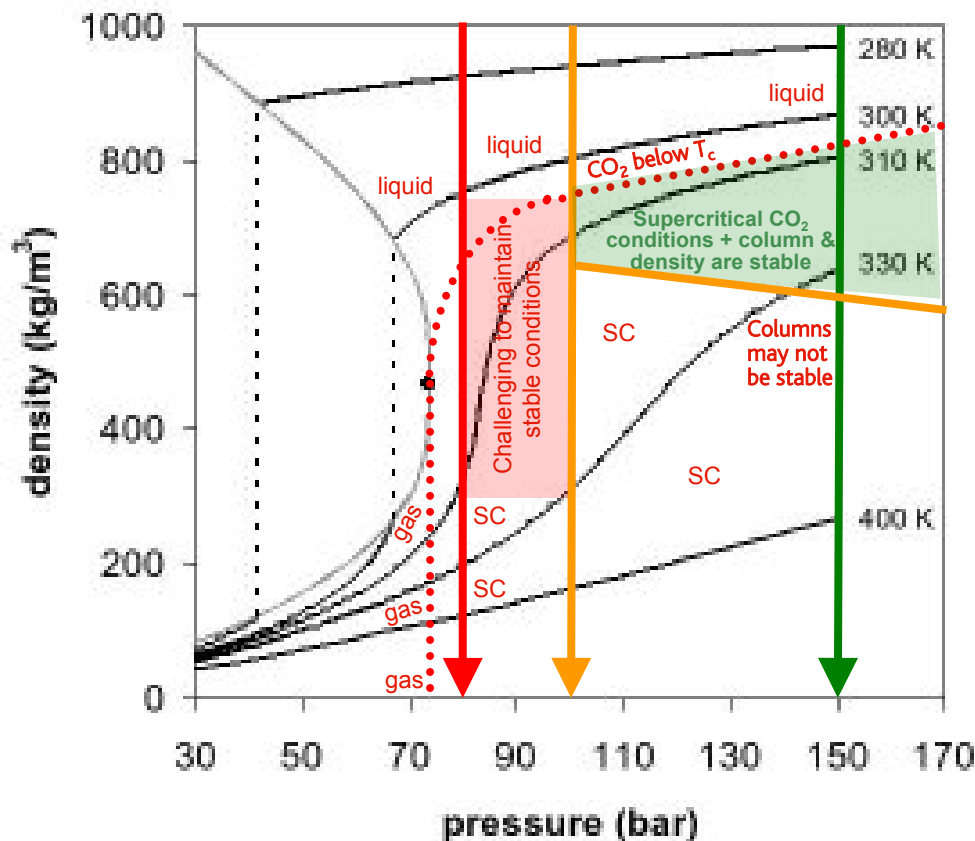
\*Solvent & buffer choice for make up  
should drive sensitivity

5

\*\*UV noise driven by density (RI) changes caused  
by BPR → pressure & density are important!!!  
TA Berger, BK Berger, *J. Chromatogr. A*, 2011,  
1218, 2320-2326.

# The importance of operating pressure: making the case for 150+ bar at column exit and UV

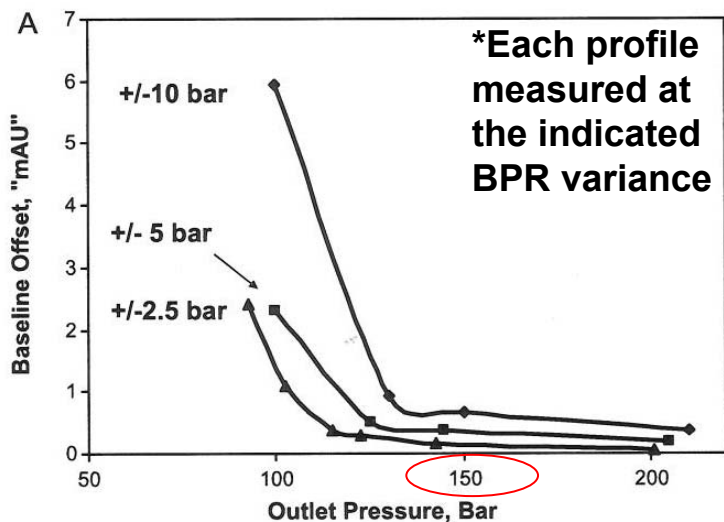
## CO<sub>2</sub> density versus pressure



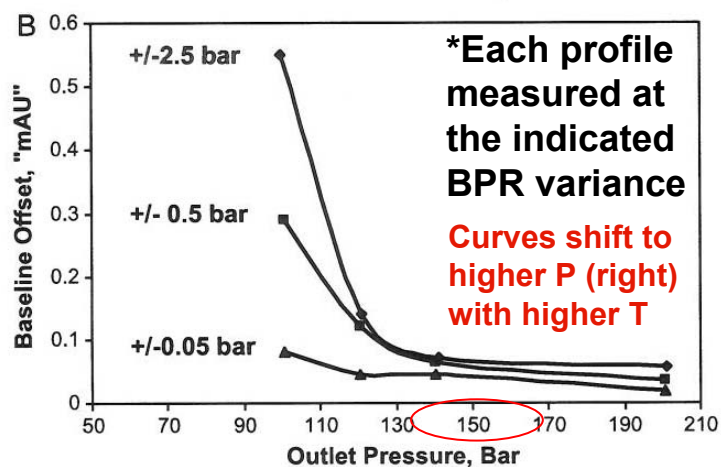
..... Phase boundary

- Some consider 80-100 bar post column to be sufficient for SFC operation
- However, density is highly temperature dependent at 80-100 bar (3°C change can result in 2x density change) which results in a high variability in retention times (RTs)
- ±2°C is as well as we can expect to control temperature
- UV noise also driven by density changes caused by BPR cycling (pressure changes)\*
- Do we really need active BPRs?\*
- **Operation at 150+ bar reduces UV noise\* as well as density & RT variation across full temperature range where columns are known to be stable (≤60°C) → choose 150+ bar!**

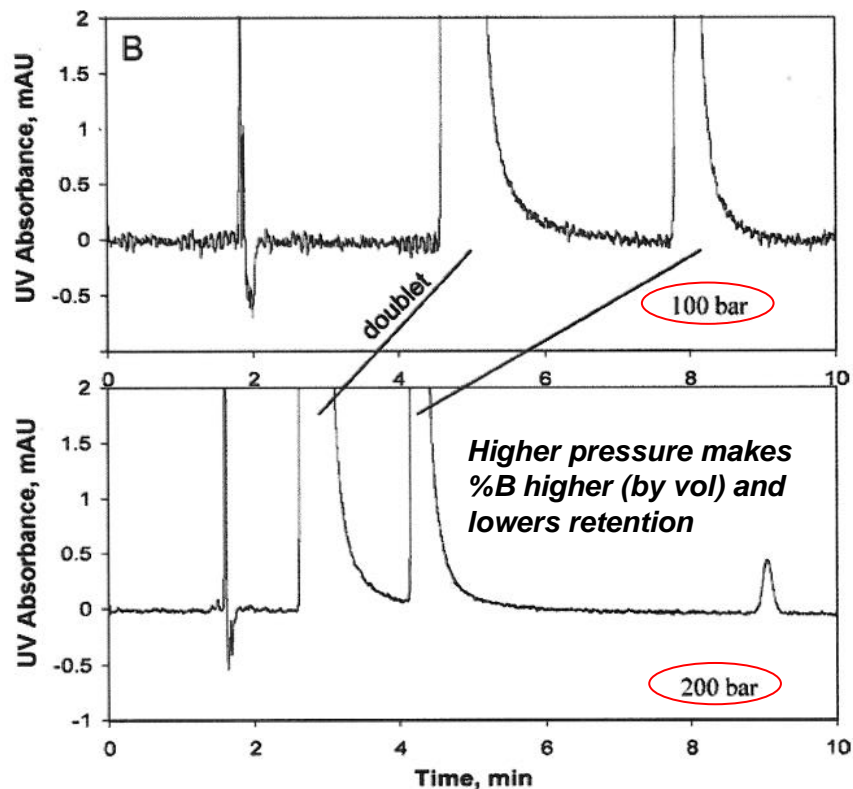
# The importance of operating pressure: with focus on UV detection – noise problem appears solved



- BPR noise flattens out above 130 bar (left)\*
- Even with small BPR variance, it's important to have sufficient pressure (right)\*
- **Do we need active BPR?\***



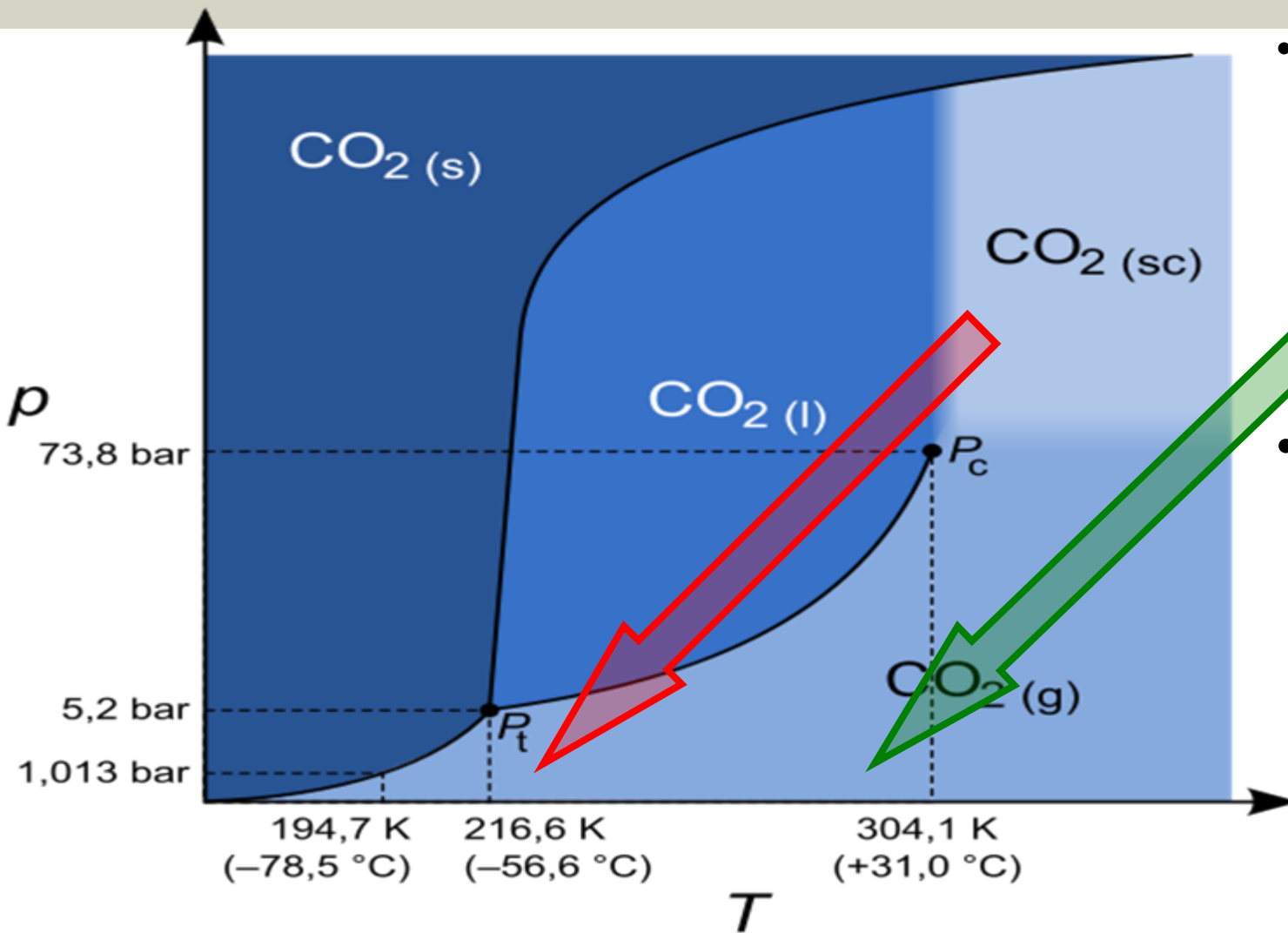
40°C



# Challenges of interfacing SFC to MS (& ELSD): hard to understand the seemingly contradictory data

- **Splitting flow & using make up solvent (classic approach)**
  - Despite eluent being mostly gas at AP, full flow (1-5 mL/min) into the source (ESI, APCI) hasn't worked well (especially ESI: high background, low response)
    - Sample blown away?
    - Lower flow, 5-50  $\mu\text{L}/\text{min}$  alcohol from column seems to provide better sensitivity
  - Conventional Wisdom: Use APCI & make up flow (200-400  $\mu\text{L}/\text{min}$ ) of alcohol improves signal stability and sensitivity (via dilution of amine buffer?)
- **CO<sub>2</sub> is different (not as inert as N<sub>2</sub>)**
  - The use of flow injection (FI) on a LC/MS is not a viable approach toward tuning / optimizing make up solvent composition
  - Presence / absence of buffer does not correlate well with SFC sensitivity
  - FI/MS under LC/MS conditions (identical to SFC except no CO<sub>2</sub>) often suggests acetonitrile as most sensitive make up solvent
  - In the presence of CO<sub>2</sub>, alcohols for make up flow usually provide better sensitivity (MS & ELSD)
  - SFC sensitivity seems to correlate with physical properties (viscosity), not chemical properties (sensitivity trend: IPA > EtOH > MeOH > ACN)
  - **Perhaps the real issue is phase separation upon expansion of CO<sub>2</sub>**

# Avoiding phase separation: a working hypothesis for ELSD & MS interfacing with SFC



- Going to atmospheric pressure the usual way (near T<sub>c</sub>) results in cold CO<sub>2</sub> liquid causing phase separation
- Increasing temperature allows expansion without phase separation (SC → gas, SC already gas like)

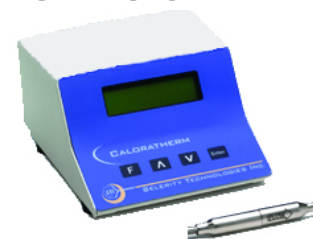
Starting expansion pressure will define temperature needed

9

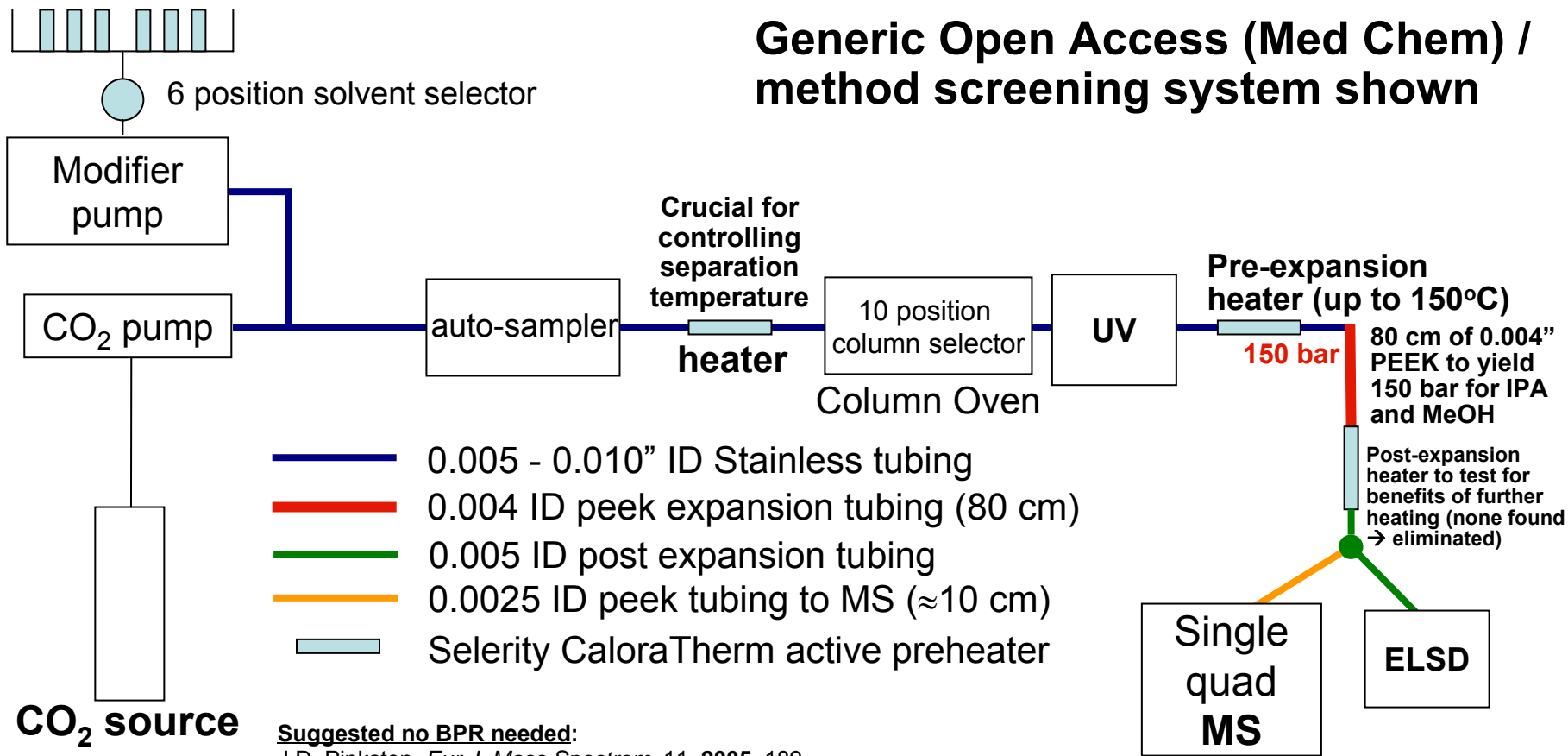
Again, choosing column pressure is an impactful decision

# Addressing the phase separation hypothesis to improve noise and sensitivity in ELSD & MS

- Need to actively heat flowing eluent stream (gradients) → Selerity CaloraTherm
- Need improve sample utilization
  - All eluent to detectors instead of majority of sample to waste via BPR
- Need to minimize pressure variation from BPR
- Proposed solution: combine preheating with fixed restrictor (instead of BPR)\* held at 150 bar\*\*



# An alternative interface of SFC to detectors



**Suggested no BPR needed:**

J.D. Pinkston, *Eur.J. Mass Spectrom.* 11, 2005, 189.

**Full flow into heated APPI, ESI, APCI MS sources with good results:**

R.A. Coe, J.O. Rathe, J.W. Lee *J. Pharm. Biomed. Anal.* 42, 2006, 573.

**Noted importance of temperature for MS interface:**

F Sadoun, H. Virlizier, P.J. Arpino *J. Chromatogr.* 647, 1997, 351.

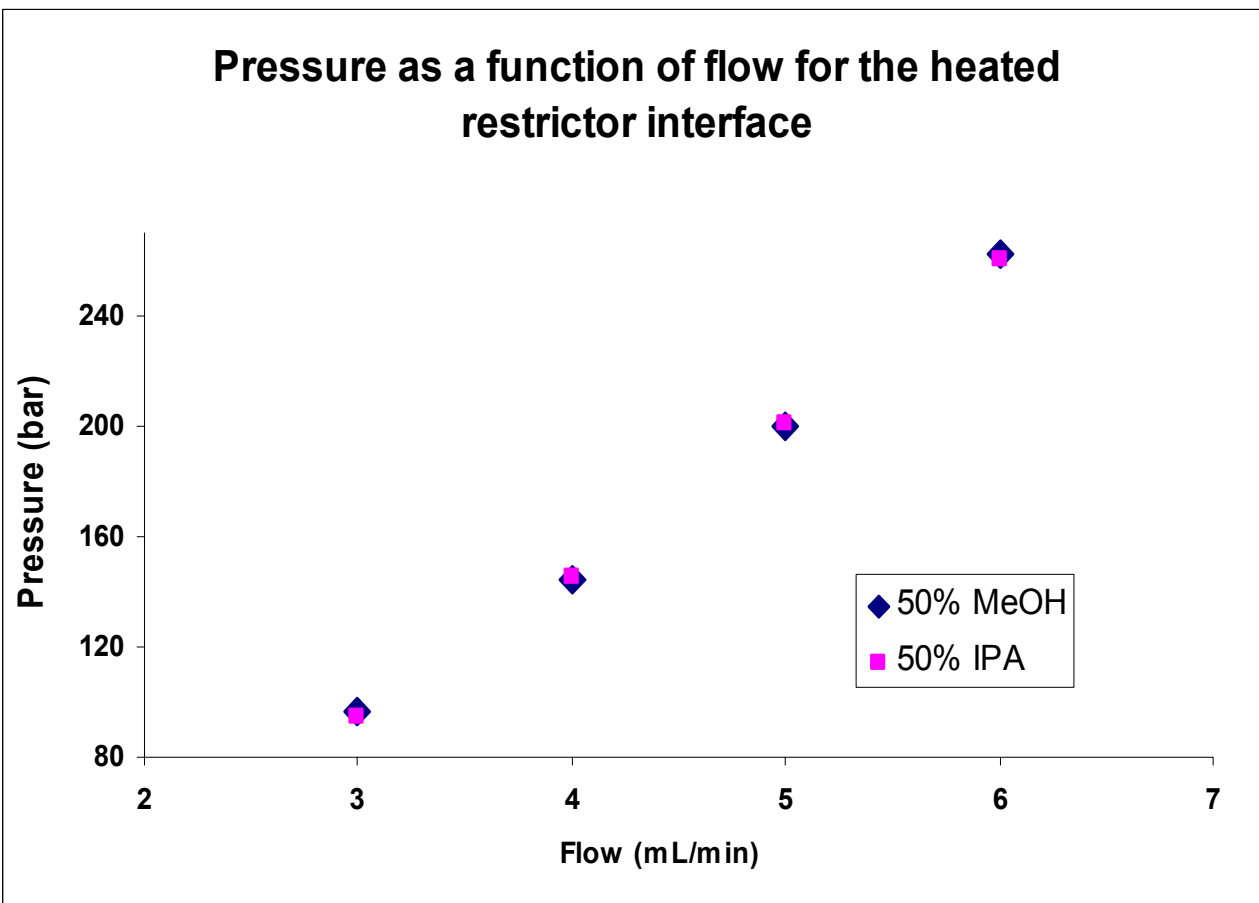
**For high sensitivity bioanalysis, eliminate UV / ELSD and replace MS with triple quad MS/MS (full flow from restrictor into MS source)**

# Initial characterization of fixed restrictor

- Initially thought it would be more complicated than it turned out to be because we started with conditions far from optimal (pressure and temperature too low)
- Anticipated that multiple fixed restrictors would be required to adapt to:
  - Different modifier viscosities
  - Different percentages of modifier
- Turned out to be much simpler because optimal expansion conditions (temperature, pressure) occur where the CO<sub>2</sub> / modifier mixture is supercritical and defining pressure is easier to achieve than initially expected
- Won't bother showing all the ineffective conditions

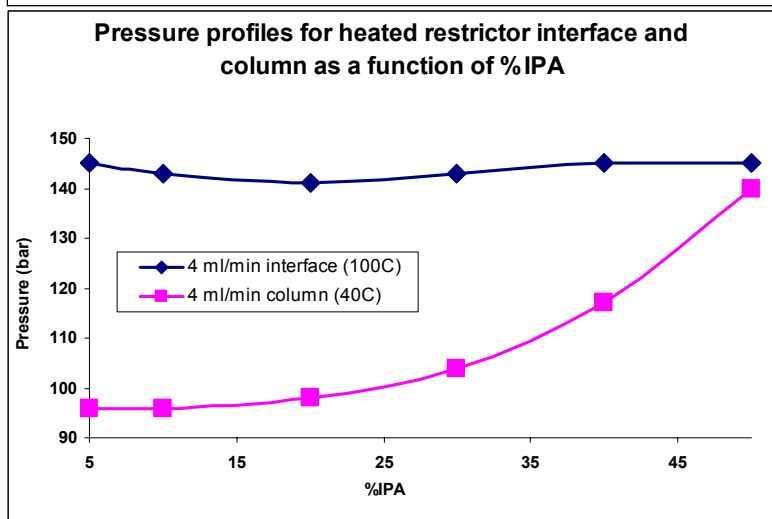
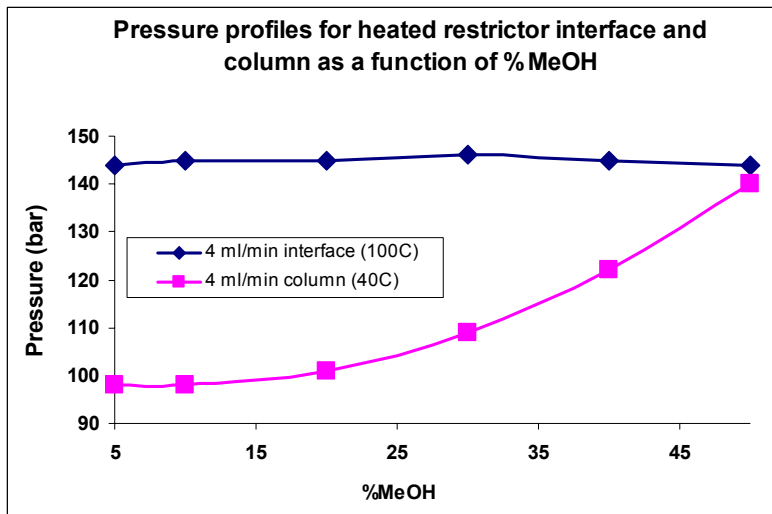
# Characterization of fixed restrictor

Pressure as a function of flow for the heated restrictor interface



- 0.004" ID PEEK tubing – 80 cm long with eluent entering at 100°C
- 4 ml/min very close to optimum velocity for most separations and gives target pressure of 150 bar
- Does not follow Darcy's law (turbulent flow), which is not surprising given (CO<sub>2</sub> SC) high Reynolds number ( $Re = 10^4 - 10^5$ )

# Characterization of fixed restrictor

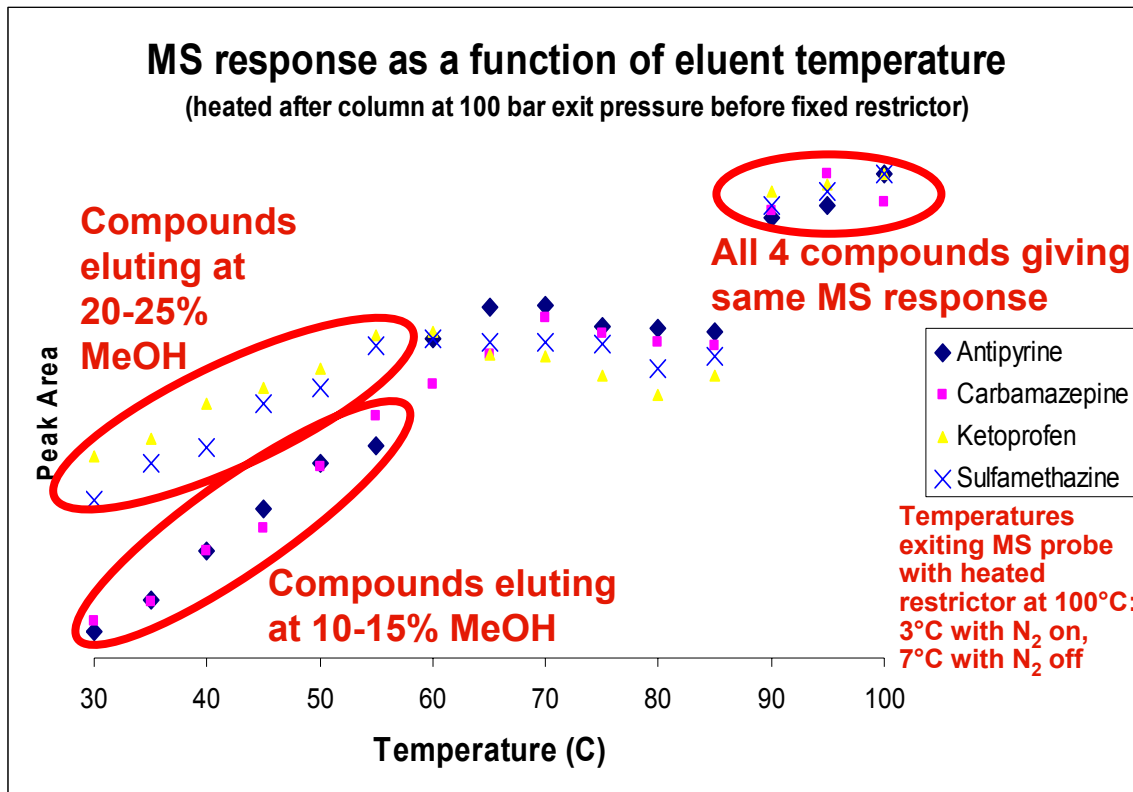


- Data shown for column 150 mm in length with 3  $\mu\text{m}$  particles without MS & ELSD nebulizers\* (note: we use only 100-150 mm columns with 3 or 5  $\mu\text{m}$  particles)
- Restrictor pressure constant for all ordinary modifiers (MeOH, EtOH, IPA) across normal modifier range (5-50%)
- Variation in restrictor pressure is due to pump pulsing (not observed with column as it acts as a pulse dampener)
- At 100°C and pressure  $\approx 150$  bar, ordinary SFC eluent behaves in supercritical like manner
- Under ordinary separation conditions in column (35-60°C), eluent does not behave in supercritical like manner
- Conclusion: if operating at fixed flow (4 ml/min chosen), a single fixed restrictor can be employed for all other conditions\*\*

# Initial characterization of heating

- Initially thought it would be simpler than it turned out to be because we increased temperature and immediately saw improvement at relatively low temperatures
  - Used CO<sub>2</sub> refrigeration data suggesting 80 bar to AP results in 40°C drop (temperature drop much bigger)
  - Started with post column pressure too low
    - 80 bar data not sufficiently reproducible
    - 100 bar data reproducible at low column temperature (35-40°C), but we frequently go up to 60°C and needed still higher pressure
- Ultimately found we needed to go to even higher temperature
  - Got Selerity to make a higher temperature version of CaloraTherm
- Started with our biggest initial objective → understand MS
  - To prove that issues with MS interfacing are physical (phase changes, i.e. CO<sub>2</sub> is inert gas) and not chemical (CO<sub>2</sub> can be reactive)

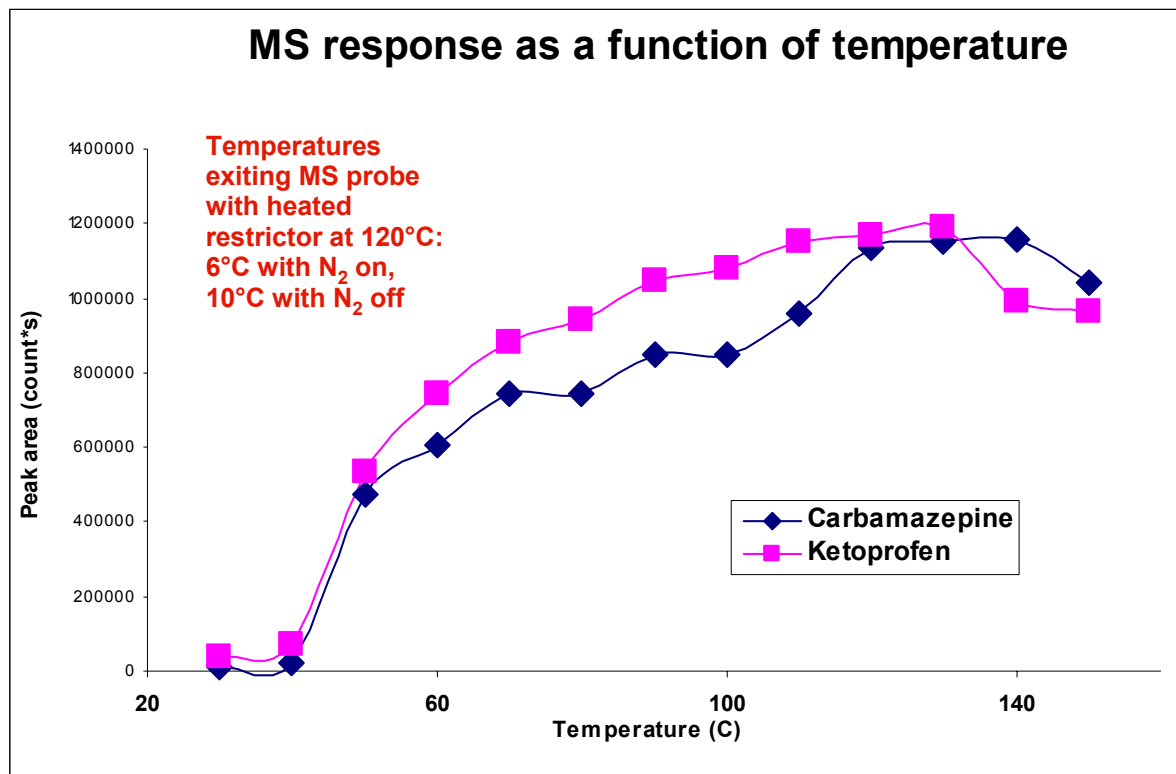
# Characterization of heating using MS detection



- 4 compounds with concentration normalized to give same MS response (FI/MS) at apparent SFC flow rates for MeOH (gradient)
- If SFC/MS conditions can be found where all 4 give same MS peak area, then CO<sub>2</sub> may be inert gas
- If MS peak area follows viscosity, then we have confirmation data
- Indeed, CO<sub>2</sub> appears to be inert ( $\geq 90^\circ\text{C}$ ) and MS sensitivity does follow viscosity ( $< 60^\circ\text{C}$ )!

Data strongly supports phase separation during expansion to AP hypothesis, but still begs the question: What happens at higher temperature? Optimum conditions not yet found...

# Characterization of heating: higher T using MS detection

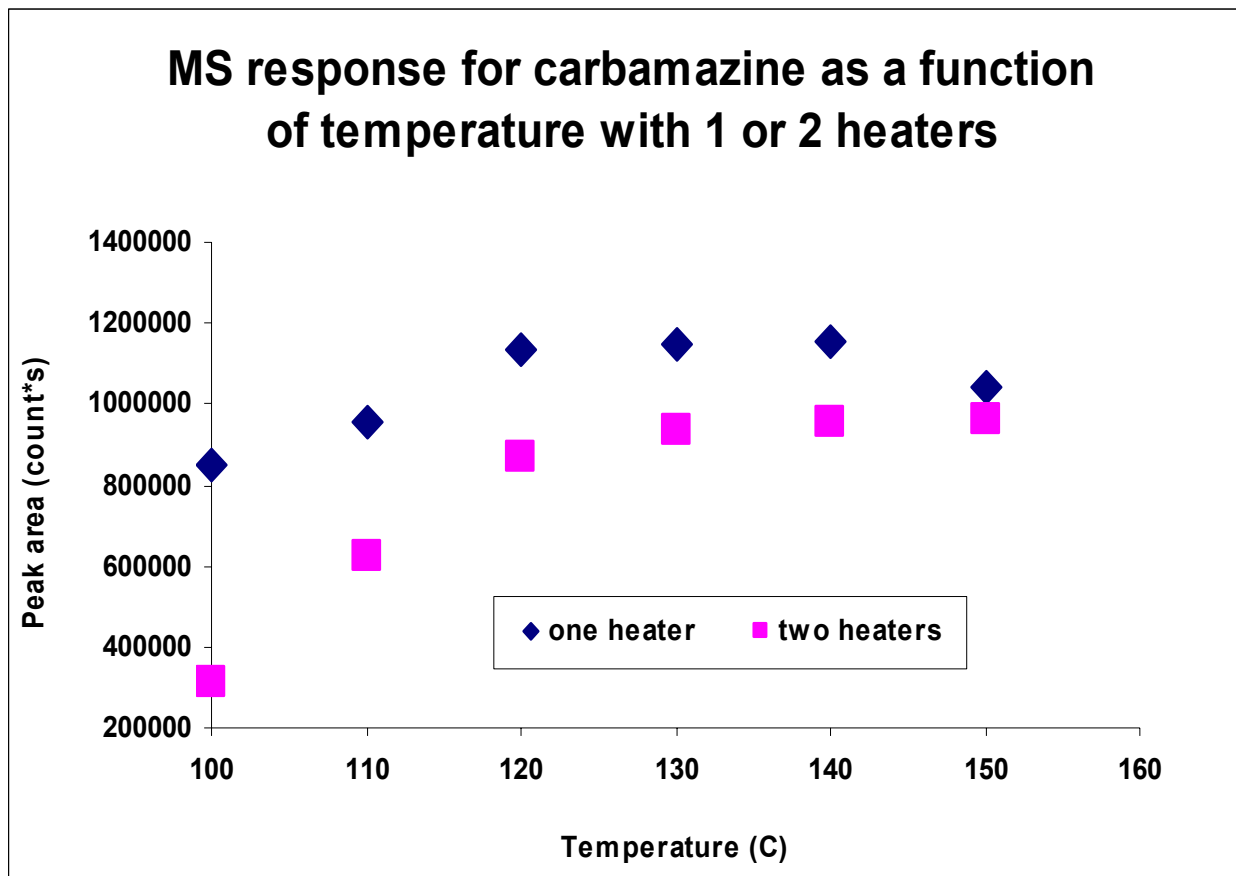


There is some small but significant benefit in using the higher temperature version of the CaloraTherm heater

- 2 of the previous 4 compounds with concentration normalized but now at 150 bar
- Apparent maximum in MS response 130°C
- Shape rise in response seems to occur at lower T when starting from higher P and gives a wide acceptable range of temperatures to operate
- Same viscosity trend seen at lower temperatures

# Characterization of heating: 2 heaters

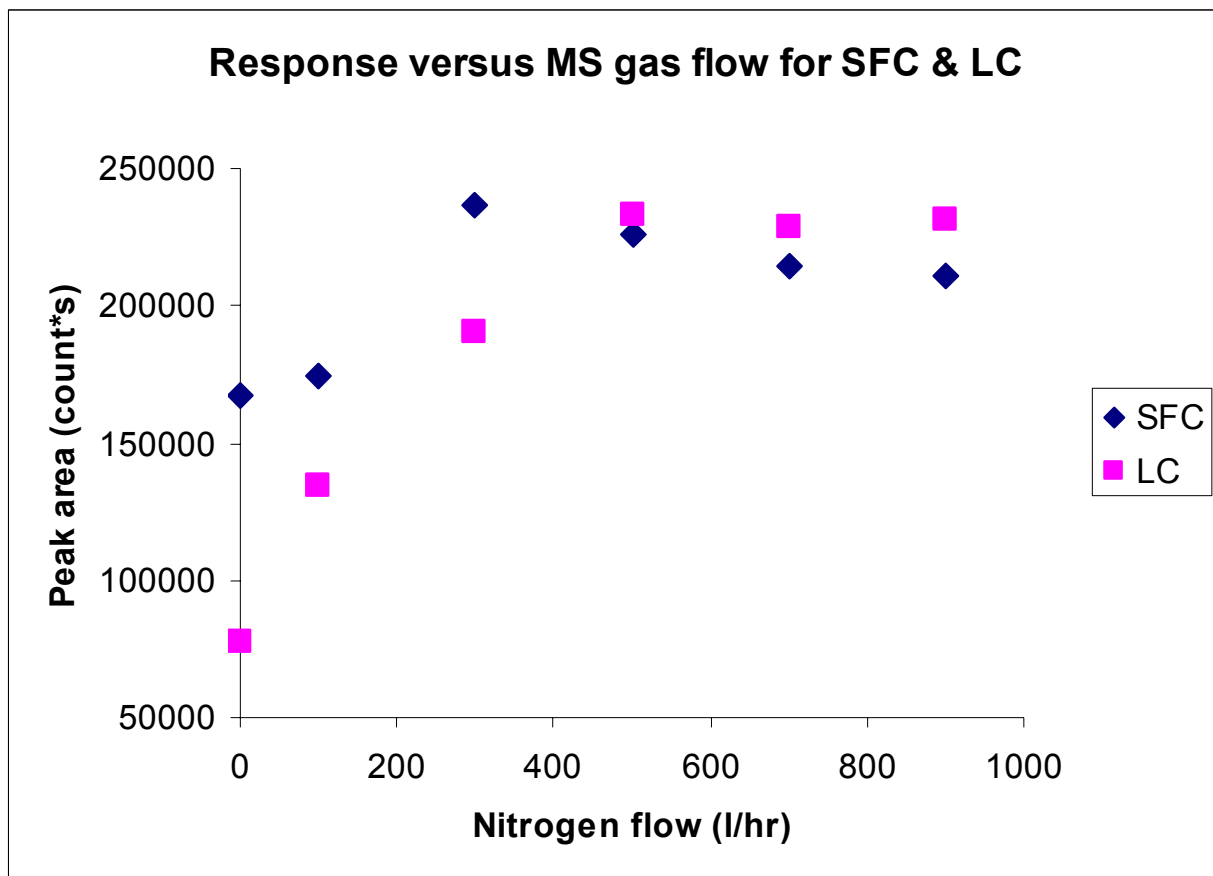
## One pre-CO<sub>2</sub> expansion, one post expansion



Using MS detection

- Many combinations of temperatures tested
  - First heater low, second higher
  - First high, second lower
  - Positive and negative fixed offsets
  - Both the same
- Selected data that best represents overall picture (both same):
  - In all cases, one heater works better than two
  - In all cases, heating before expansion works better than after
- Differences are even bigger at lower T

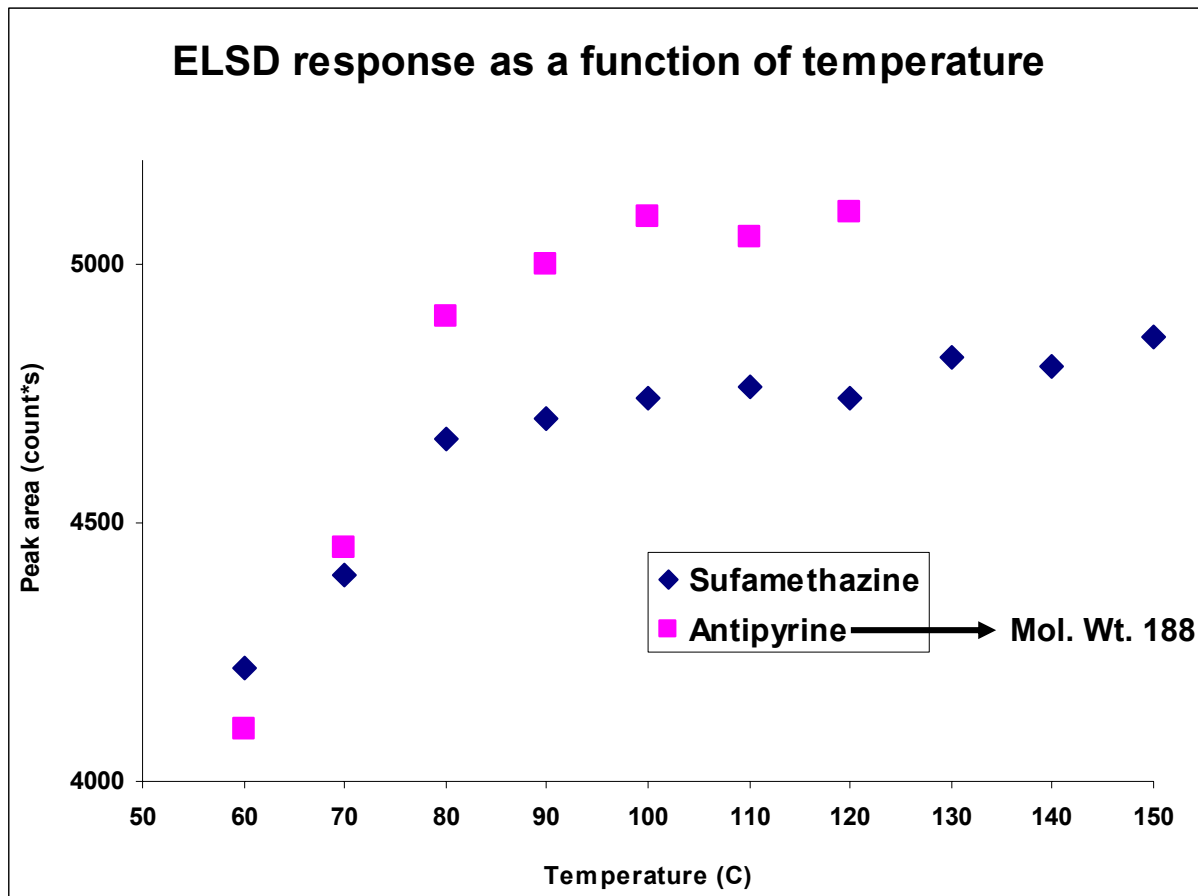
# Characterization of heating: need for N<sub>2</sub> with MS (in addition to 20 l/hr CO<sub>2</sub>)



**Gas load from SFC CO<sub>2</sub> helps nebulization and allows slightly less N<sub>2</sub> flow**

- Sulfamethazine SFC/MS (w/ 150 bar fixed restrictor 120°C) and RP-LC/MS (200 µl/min into source)
- Both using Waters 3100 MS
- Optimum 500 l/hr for RP-LC/MS lowered to 300 l/hr for SFC/MS
- Less effect on SFC/MS at lower flow → 20 l/hr CO<sub>2</sub> already doing some but not all nebulization

# Characterization of heating: ELSD



- 150 bar at column exit and “normal” ELSD conditions for RP-HPLC (T & P for N<sub>2</sub> in ELSD)
- Higher T helps with benefits leveling off above 100°C
- At still higher T, particle size of low mol. wt. compounds are smaller than can be seen by ELSD (still observed by MS)
- Greater tolerance of lower T at ELSD nebulizer while maintaining sensitivity

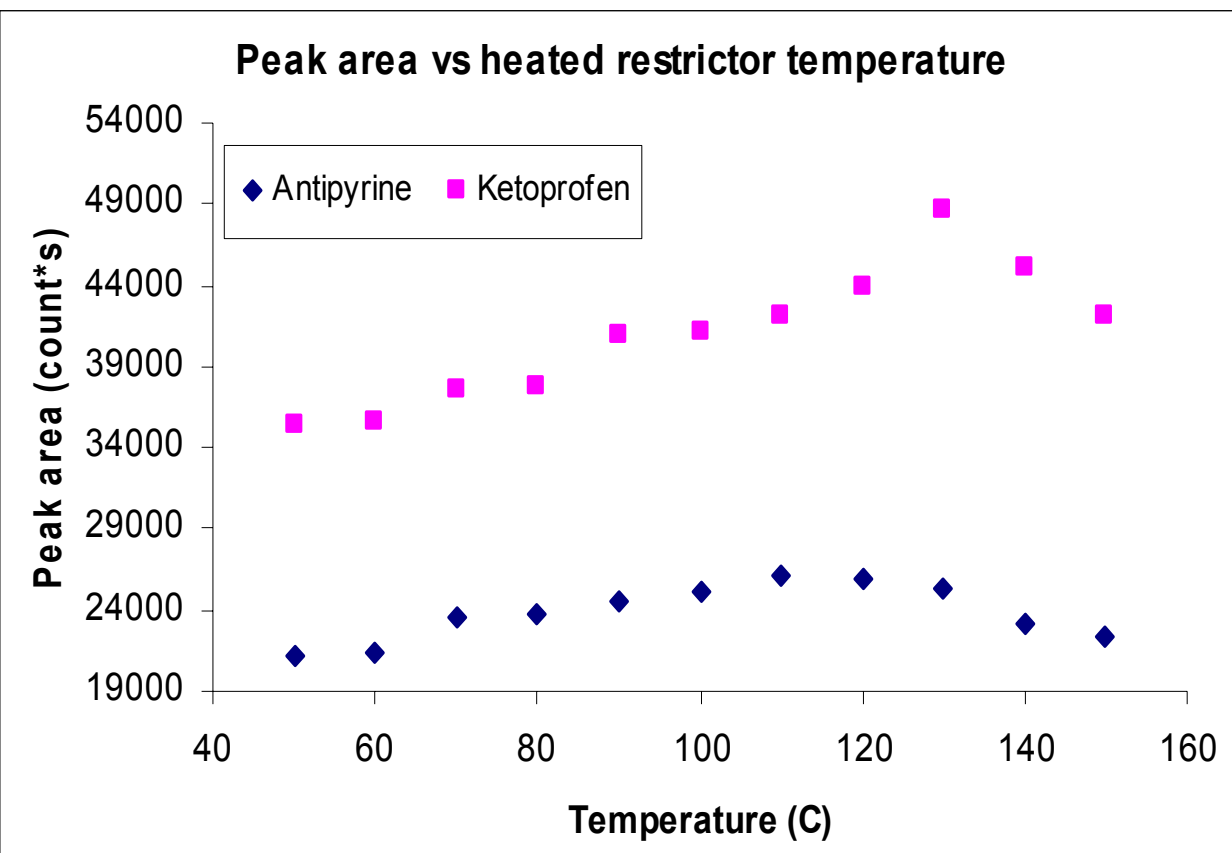
Waters 2424 ELSD optimal settings:

RP-HPLC – N<sub>2</sub> pressure 60 psi – nebulizer temperature 60°C

SFC (heated restrictor @120°C) – N<sub>2</sub> pressure 50 psi – nebulizer temperature 35°C

# Characterization of heating: ELSD

## second iteration with ELSD optimized for SFC



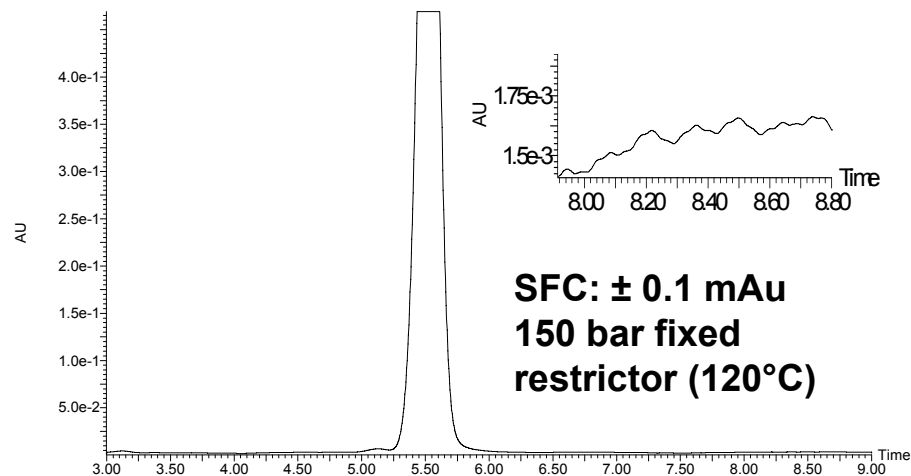
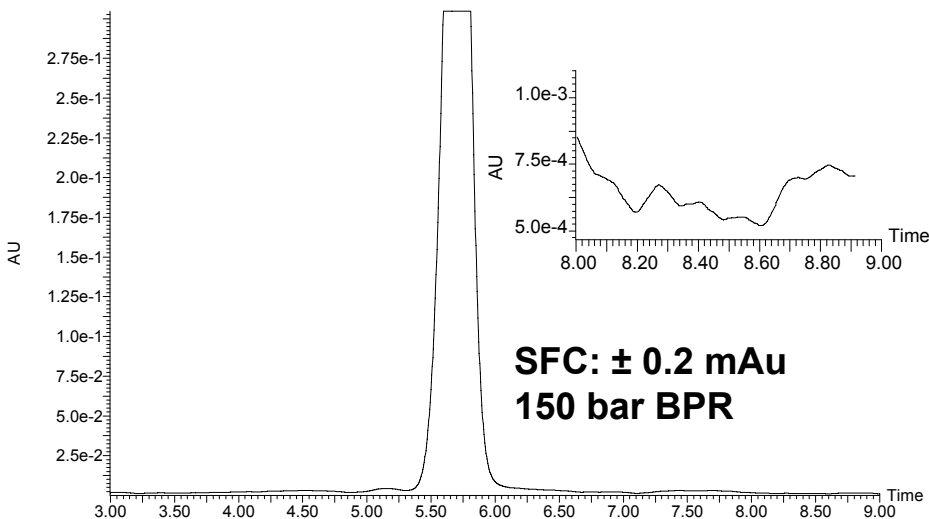
Initial impression: ELSD with SFC looks surprisingly good relative to ELSD with RP-HPLC (more examples later)

- 150 bar at column exit and new SFC based ELSD conditions ( $N_2$  pressure 50 psi – nebulizer temperature 35°C)
- Useable fixed restrictor temperature extended upward to match ELSD and MS optimal conditions
- No more loss of low mol. wt. compounds in ELSD
- Added benefit: much greater sensitivity – antipyrine up 5 fold & dynamic range  $10^3$

# Critical examples comparing SFC with both interfaces and RP-HPLC with all 3 detectors

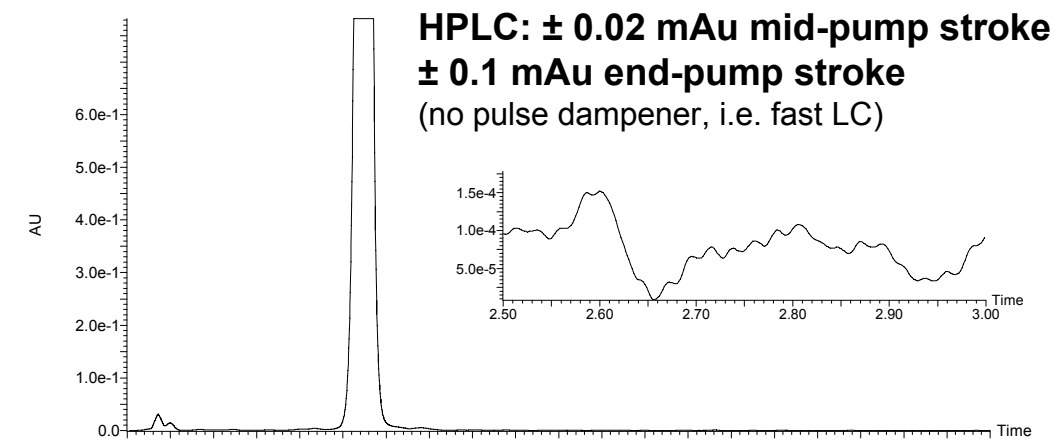
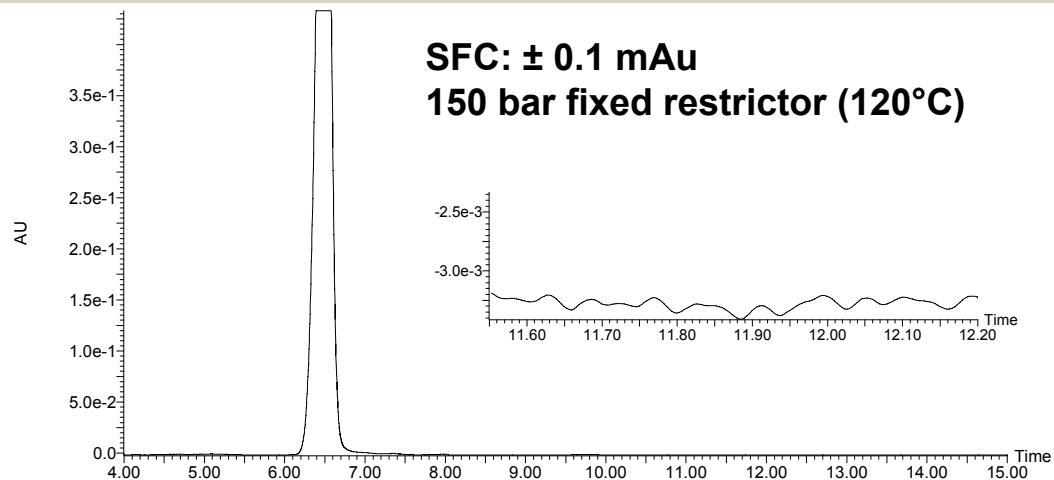
- UV, MS, and ELSD compared for:
  - SFC with traditional split / makeup interface
  - SFC with heated fixed restrictor interface
  - HPLC performed in the usual ways
- Emphasis placed on sensitivity, noise, and dynamic range
- Goals:
  - Compare BPR / split interface with heated fixed restrictor interface for SFC
  - Establish recommended conditions for heated fixed restrictor interface
  - Compare SFC with RP-HPLC to evaluate if SFC detection is on par with RP-HPLC

# UV detection: BPR & fixed restrictor



- Sulfamethazine SFC (Waters/Thar FDM & 2998 PDA)
- Tested at 150 bar with BPR and fixed restrictor
- Noise  $\pm 0.2$  mAu with BPR and  $\pm 0.1$  mAu fixed restrictor
- Noise due to RI change still 4x higher than Berger data at 200 bar\*
- Perhaps 200-300 bar pressure needed to get to lowest noise levels possible\*

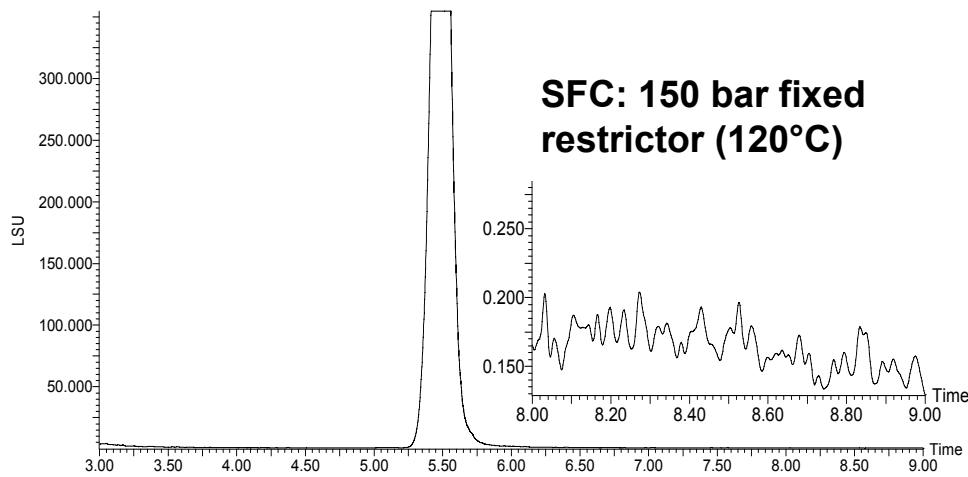
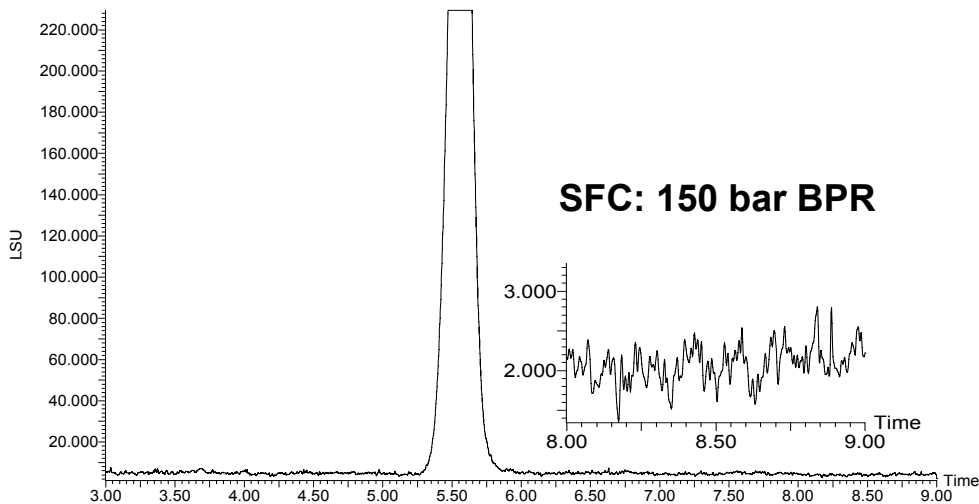
# UV detection: SFC & RP-LC



**SFC and fast RP-LC nearly equivalent for UV detection**  
**Slower pulse dampened LC can go to 5x lower conc**

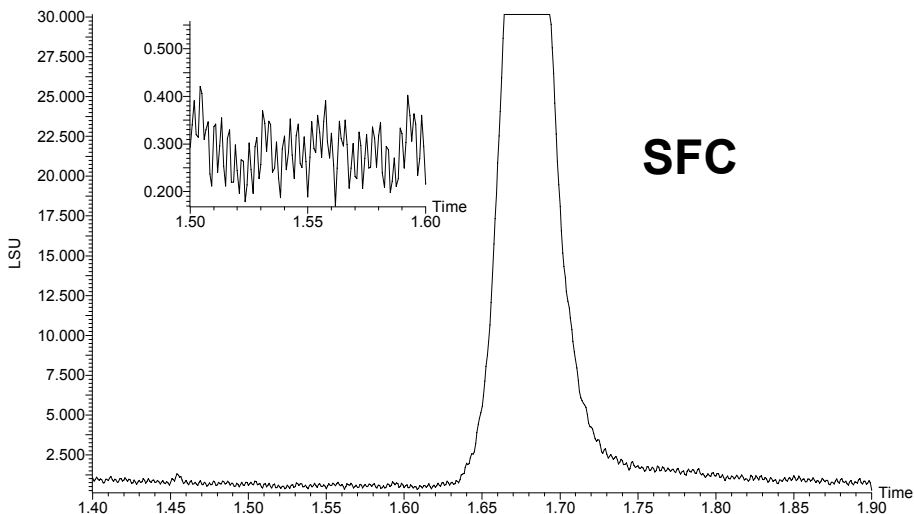
- Sulfamethazine SFC (Waters/Thar FDM & 2998 PDA @150 bar-fixed restrictor w/ high pressure cell) and RP-LC (Waters 1525 & 2998 PDA @AP w/ low pressure cell)
- Filter: 1 s for both
- Cell 10 mm and 9.3  $\mu$ l for both
- Noise due to RI change still 5x higher for SFC compared to LC with pulse dampener
- LC pump pulse noise (fast LC, no dampener) about the same amplitude as SFC RI change noise, but lower frequency
- LC pulse noise can be removed (but costs time)

# ELSD detection: BPR & fixed restrictor

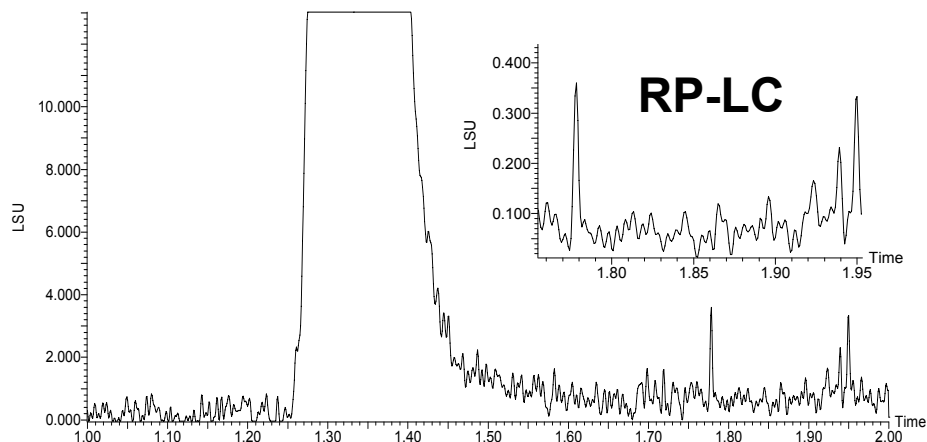


- Sulfamethazine SFC (Waters/Thar FDM & 2424 ELSD)
- Tested at 150 bar with BPR and fixed restrictor
- 20x less noise for fixed restrictor
- 2x greater sensitivity for fixed restrictor

# ELSD detection: SFC & RP-LC



**SFC**



**RP-LC**

- Sulfamethazine SFC (Waters/Thar FDM & 2424 ELSD heated fixed restrictor and RP-LC (Waters 1525 & same 2424 ELSD)
- Filter: 1 s for both
- Noise roughly 1.5x higher for RP-LC
- Sensitivity (response ratio) 3-5x higher for SFC
- Dynamic range  $10^3$  for SFC and  $10^2$  for RP-LC
- ELSD clearly works better with SFC relative to RP-LC

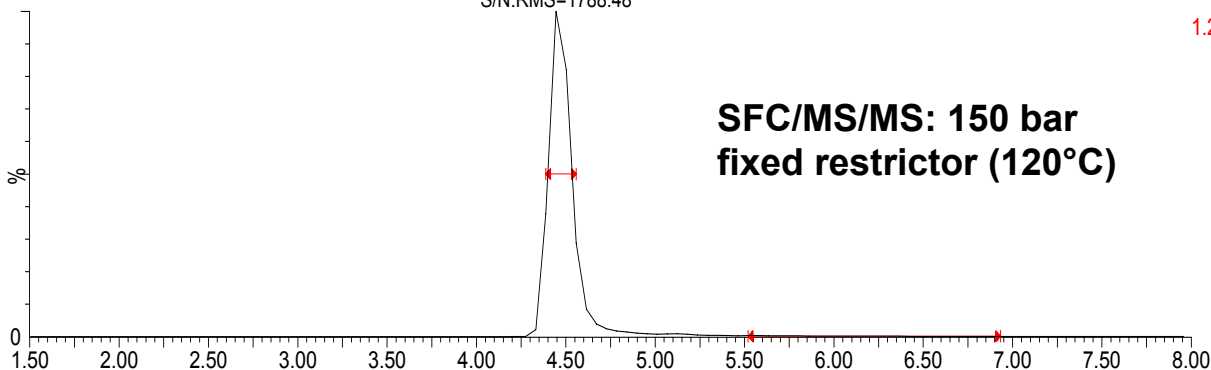
# MS detection: BPR & fixed restrictor

rat-223-plasma

S/N:RMS=1788.48

1: MRM of 2 Channels ES+  
TIC  
1.28e6

SFC/MS/MS: 150 bar  
fixed restrictor (120°C)

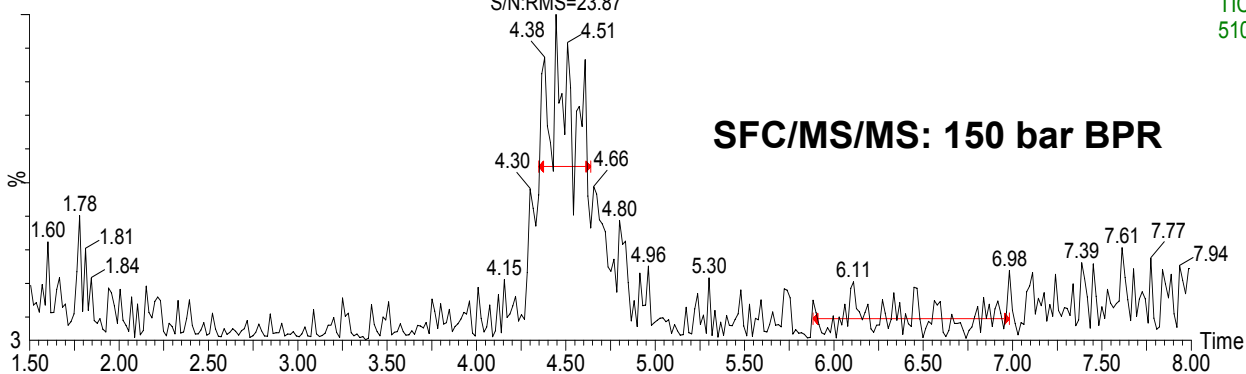


plasma-223-A4b

S/N:RMS=23.87

1: MRM of 2 Channels ES+  
TIC  
510

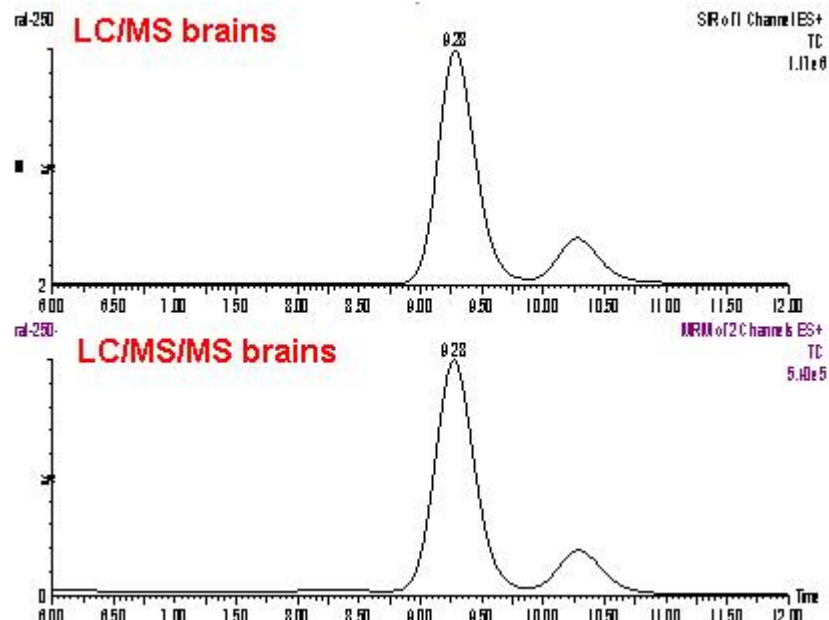
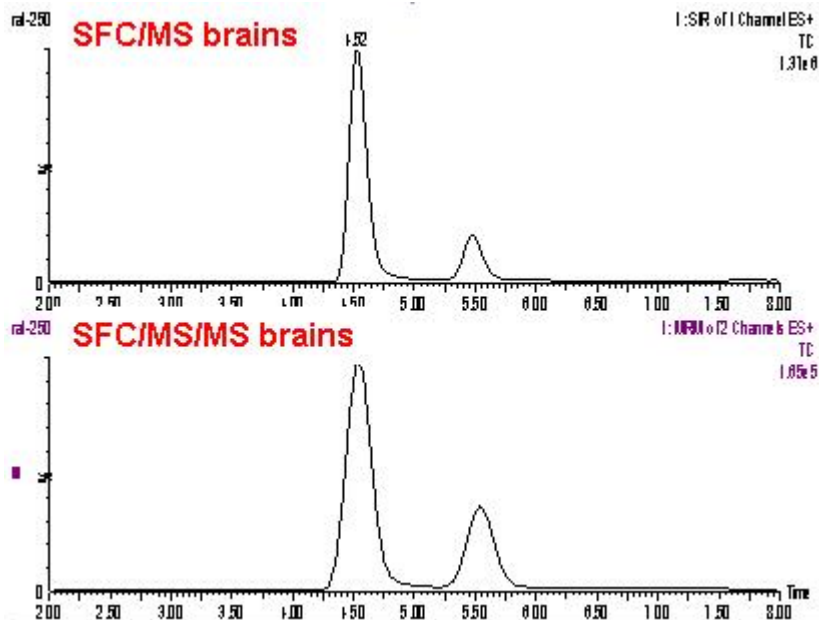
SFC/MS/MS: 150 bar BPR



**Heated fixed restrictor (full 4 ml/min flow into MS) dramatically improves SFC/MS performance relative to BPR split interface**

- Plasma bioanalysis of drug at 40 mg/ml with Waters/Thar FDM & Quattro Premier XE (MS/MS)
- Automatic S/N calculator says 74x difference in S/N (seems optimistic)
- Raw data says peak height 2000x higher with fixed restrictor

# MS detection: SFC & RP-LC for chiral bioanalysis



- 2 samples each from 44 animals + half of all samples were serial diluted 3 times to ensure no detector saturation for a total of 210 samples each run 4 times. LC and SFC used same MS/MS. Peak shape and response were steady throughout the 840 analyses. Concentrations vary from 40 to 90 ng/ml.
- SFC: S/N 2-3k with MS > MS/MS
- LC: S/N 6-9k with MS/MS > MS (LC vs. SFC = 100% MeOH vs. CO<sub>2</sub>/IPA?)
- Animal to animal variation in enantiomer ratio <2%
- Brain to plasma (same animal) variation in enantiomer ratio ≤1%
- SFC is on par with RP-LC with respect to using MS detection

# Summary: comparison of both SFC with BPR / split & heated fixed restrictor detector interfaces with RP-LC

- **UV detection**

- SFC: fixed restrictor provides lower noise (2x @ 150 bar) than BPR but column pressure seems to be most important
- RP-LC: still has 5x lower noise and detection limits (needed for relatively few applications)
- Is this a reason to take SFC column exit pressures to 200-300 bar? Maybe so for some applications where  $>10^3$  dynamic range needed (another reason to go UHP-SFC)

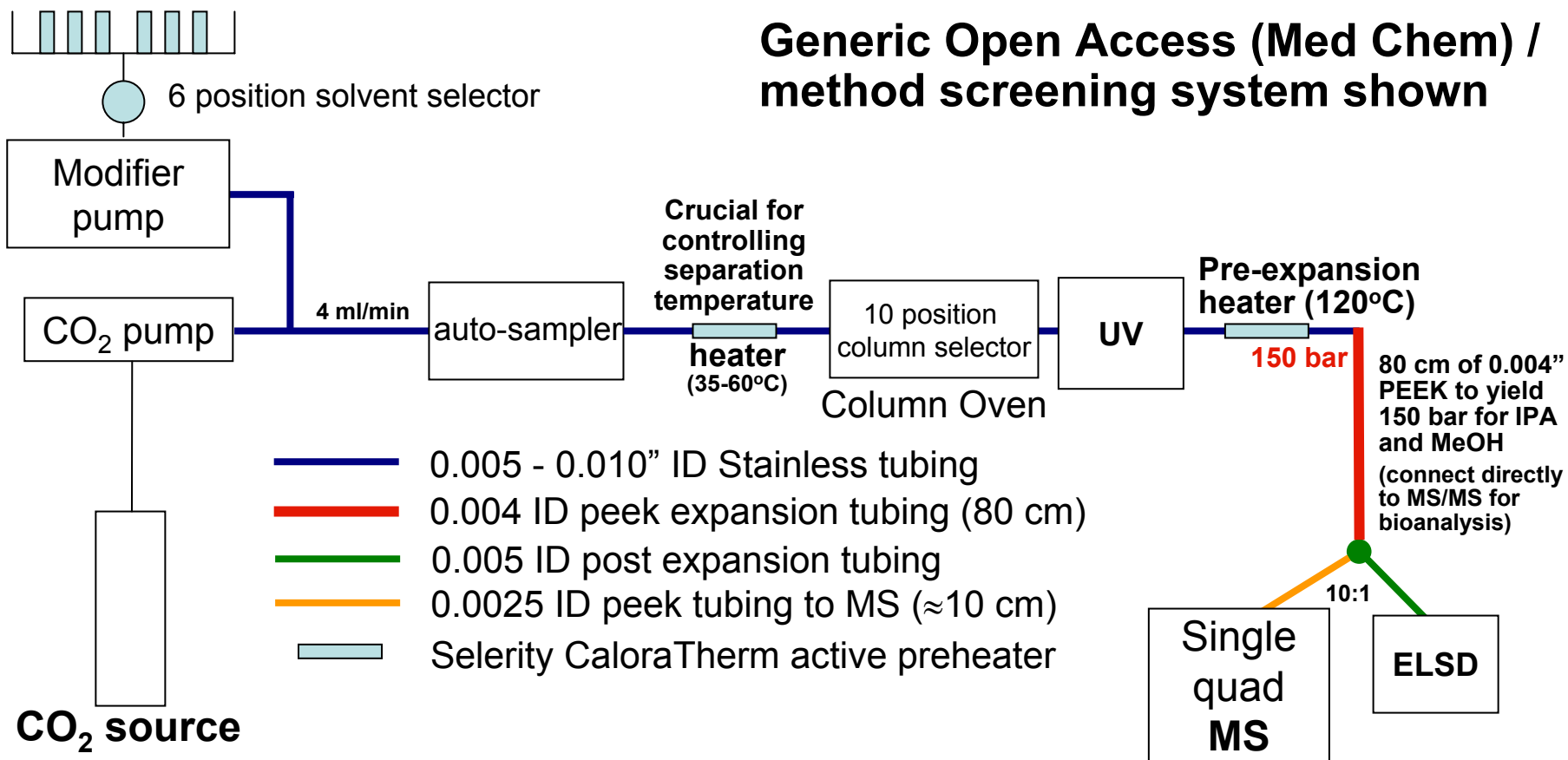
- **ELSD**

- SFC: fixed restrictor clearly provides lower noise plus higher sensitivity and dynamic range than SFC with BPR or RP-LC

- **MS**

- SFC: fixed restrictor provides much higher (ca.  $\geq 10^2$ ) sensitivity than BPR
- SFC provides equivalent results to RP-LC at concentrations  $>100$  pg/ml but still needs to be proven at lower concentrations

# Our current “standard” in SFC detector interfacing



**Suggested no BPR needed:**

J.D. Pinkston, *Eur. J. Mass Spectrom.* 11, 2005, 189.

**Full flow into heated APPI, ESI, APCI MS sources with good results:**

R.A. Coe, J.O. Rathe, J.W. Lee *J. Pharm. Biomed. Anal.* 42, 2006, 573.

**Noted importance of temperature for MS interface:**

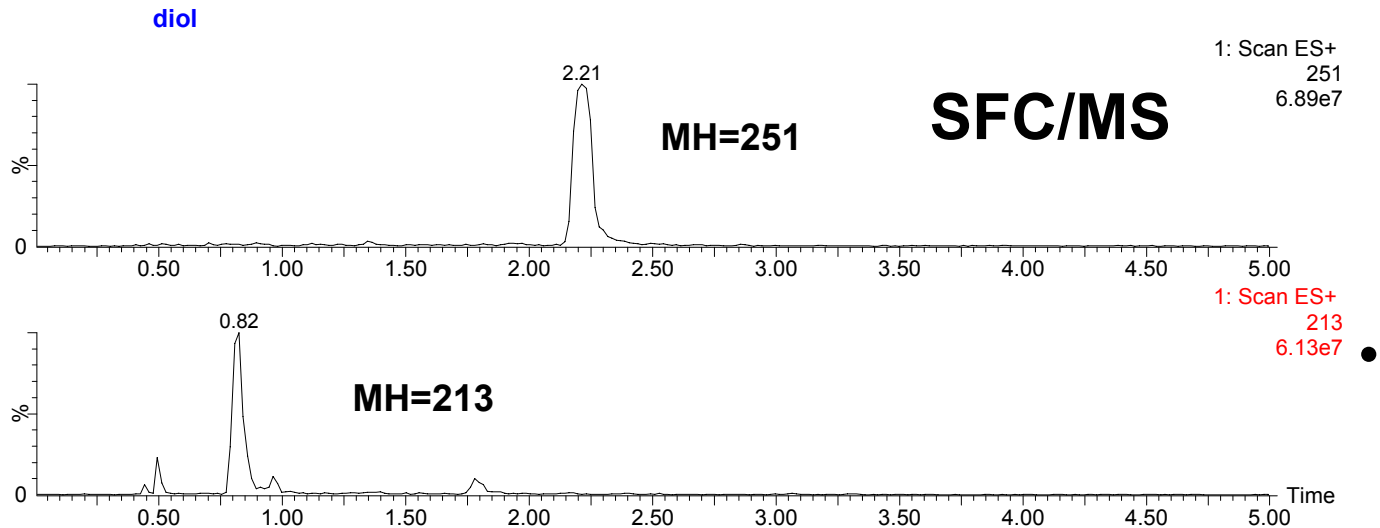
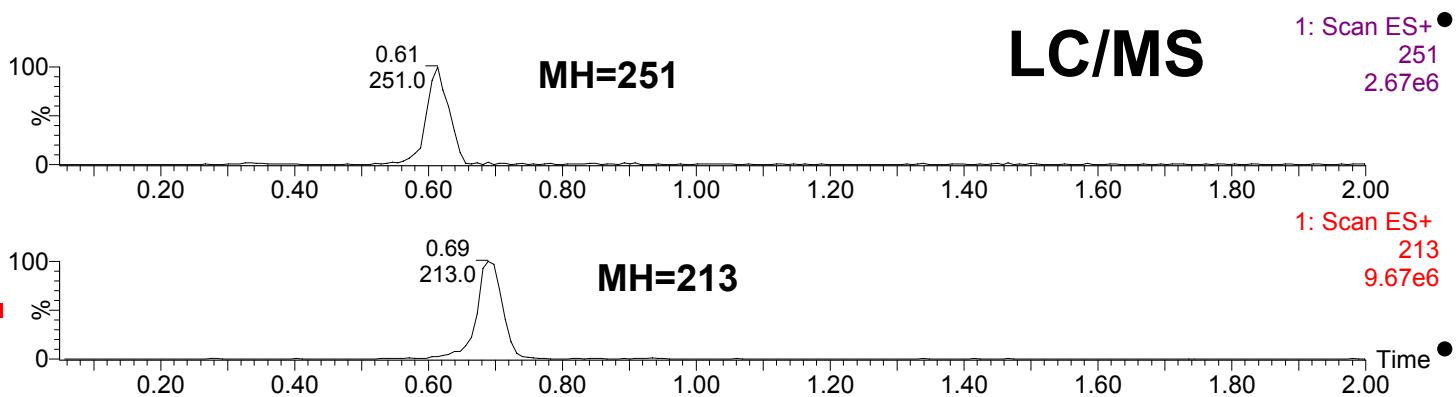
F Sadoun, H. Virlizier, P.J. Arpino *J. Chromatogr.* 647, 1997, 351.

**For high sensitivity bioanalysis, eliminate UV / ELSD and replace MS with triple quad MS/MS (full flow from restrictor into MS source)**

# Highlight of new application in SFC: Open Access (OA) SFC/UV/ELSD/MS

- To gain efficiency, complementary capabilities, and greater capacity, we have deployed OA-SFC/UV/ELSD/MS
  - True orthogonal separation option for Med Chem support (TLC with awesome detectors)
  - Still has broad overlap with RP-LC/UV/ELSD/MS for Med Chem support, thereby providing added capacity for routine reaction monitoring
  - Also opens up chiral method development and ee measurement to “everyone”
  - 3 achiral column choices & 7 for chiral (6 modifier / buffer options)
- Using the detector interfacing techniques described herein and recent software releases, SFC/UV/ELSD/MS is ready for prime time in providing immediate gratification in the above applications

# Orthogonal SFC separations can be highly complementary to the frequently used RP-LC

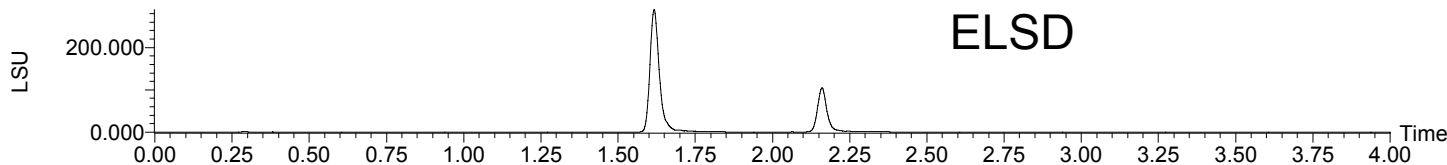
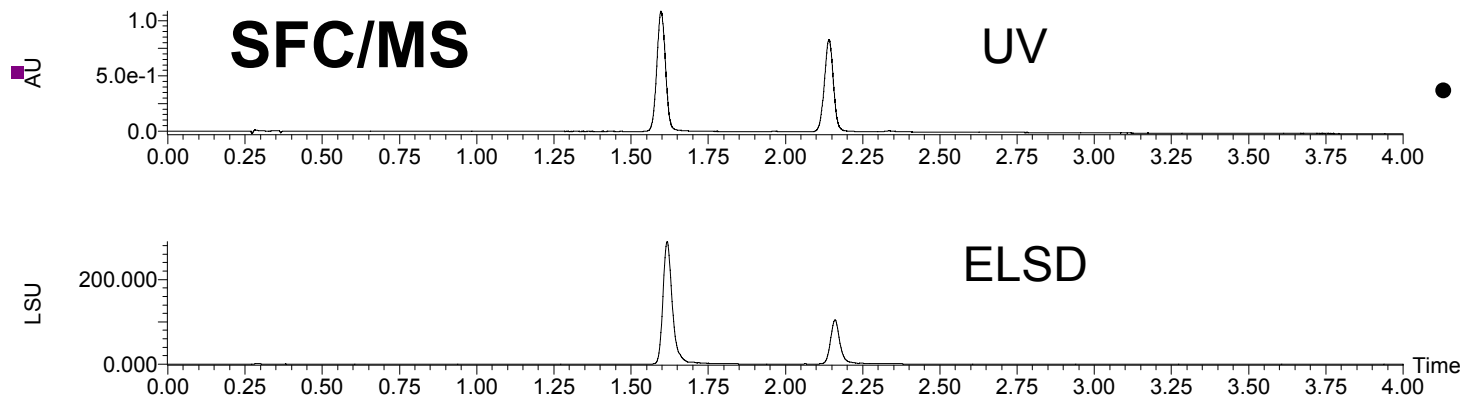
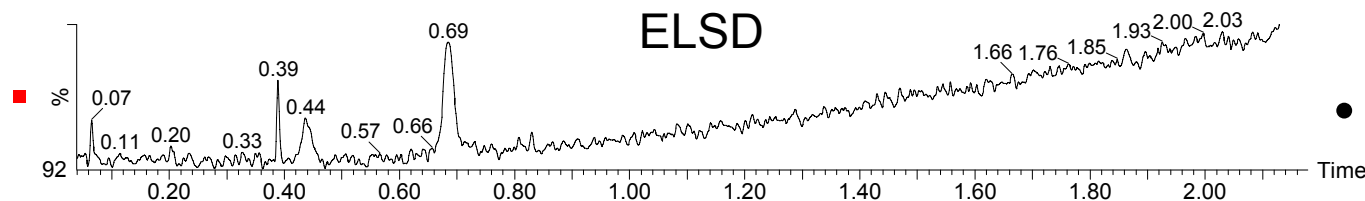
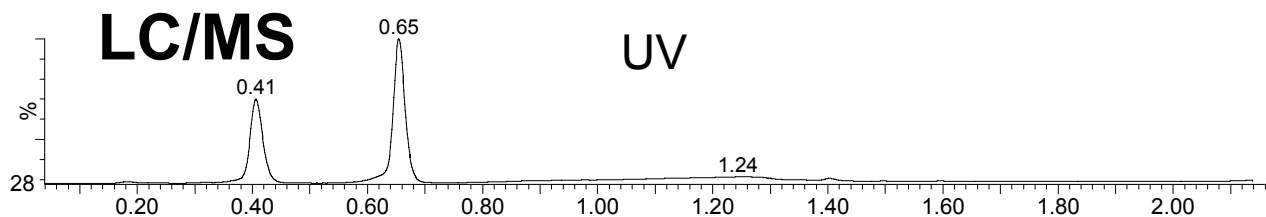


Truly orthogonal SFC approach can separate starting material and products that RP-LC can't

These SFC methods also are aligned with preparative scale methods allowing immediate purification

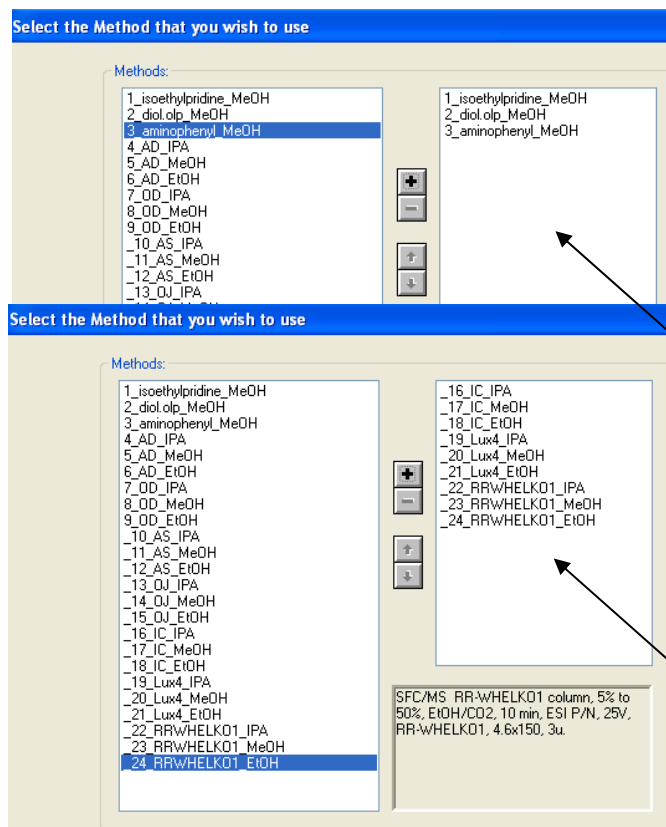
MS used in this application due to lack of chromophore

# OA-SFC/UV/ELSD/MS can provide similar information as OA-LC/UV/ELSD/MS



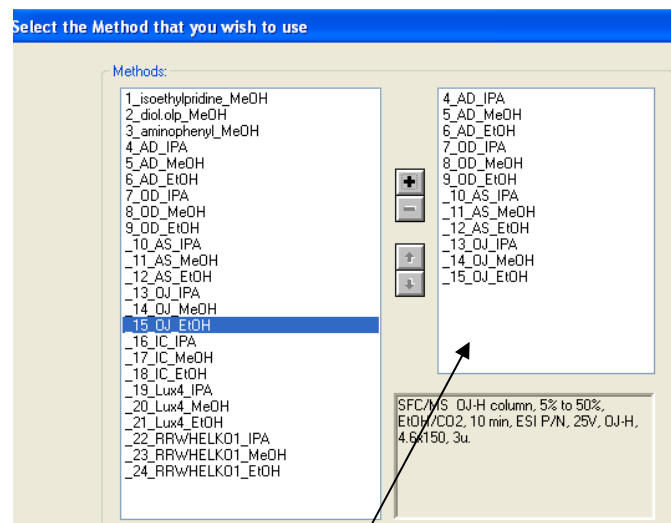
- Chromatograms showing starting material and product (reaction progress)
- Essentially same data with either approach except reverse elution order (TLC-like)
- Note the improved quality of ELSD with SFC!

# Chiral screening of many methods on a single sample login (MassLynx / OpenLynx SCN 798)



Method set for achiral analysis

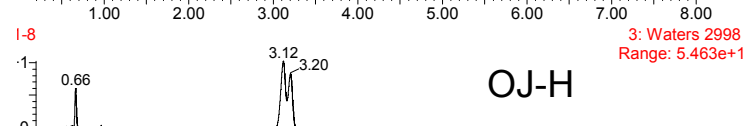
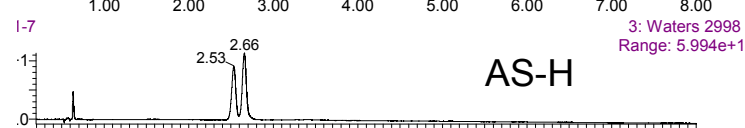
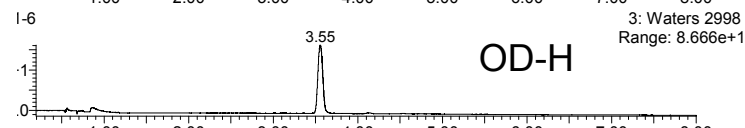
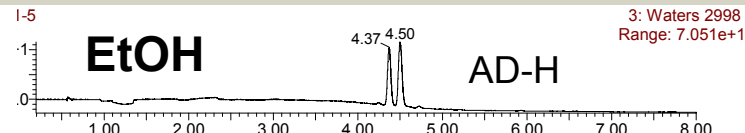
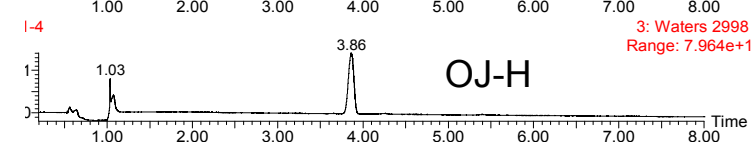
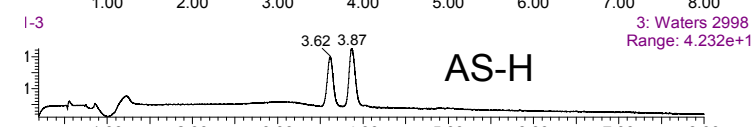
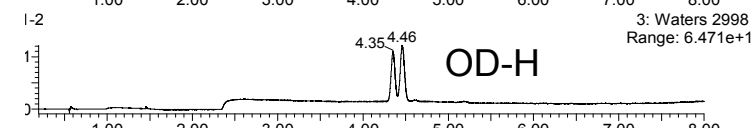
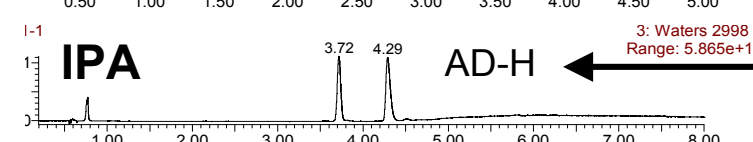
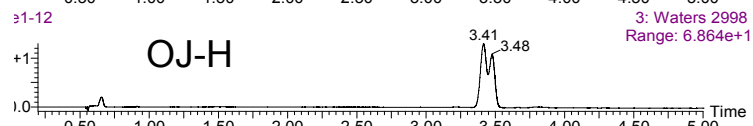
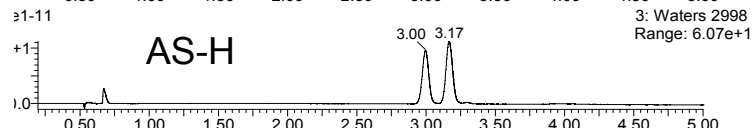
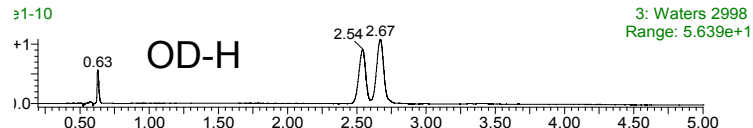
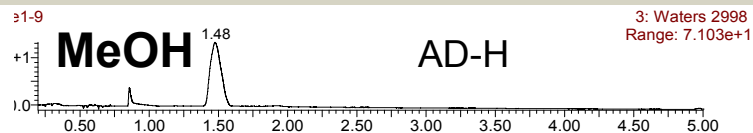
Second set of methods for chiral column screening if first set don't work



First set of methods for chiral column screening

## New software makes method screening easy!

# Screening chiral conditions for preparative method development (4 x 3)



Screening 4 columns and 3 solvent gradients showed AD-H with IPA gives a useful separation

Scaled preparative version of same method was immediately used to resolve 10g on same day

OA-SFC/UV/ELSD/MS is a viable screening approach for preparative work