High Performance Liquid Chromatography

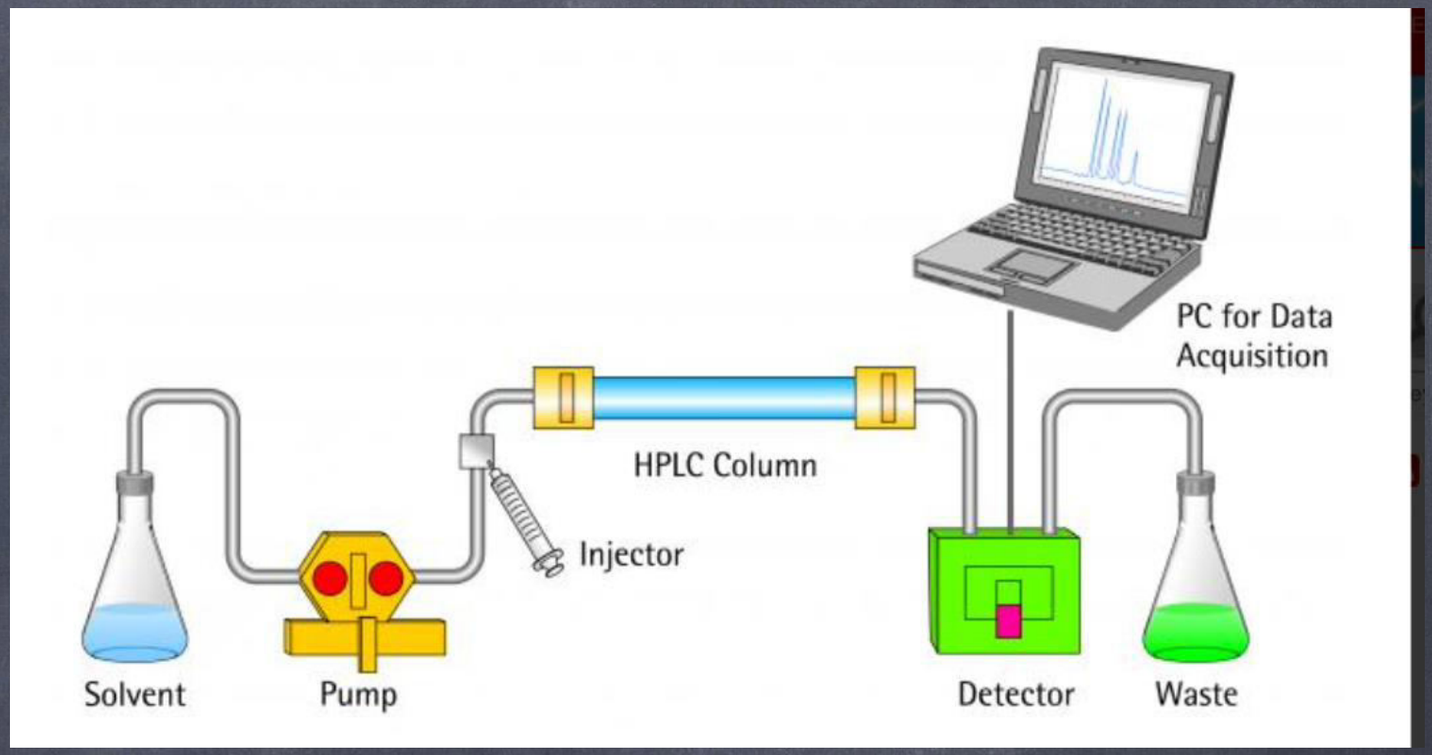
Lis Smith Alder Hey Children's Hospital
Nana Ghansah Great Ormond Street Children's Hospital

HPLC

HPLC is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, or ion-exchange processes, depending upon the type of stationary phase used

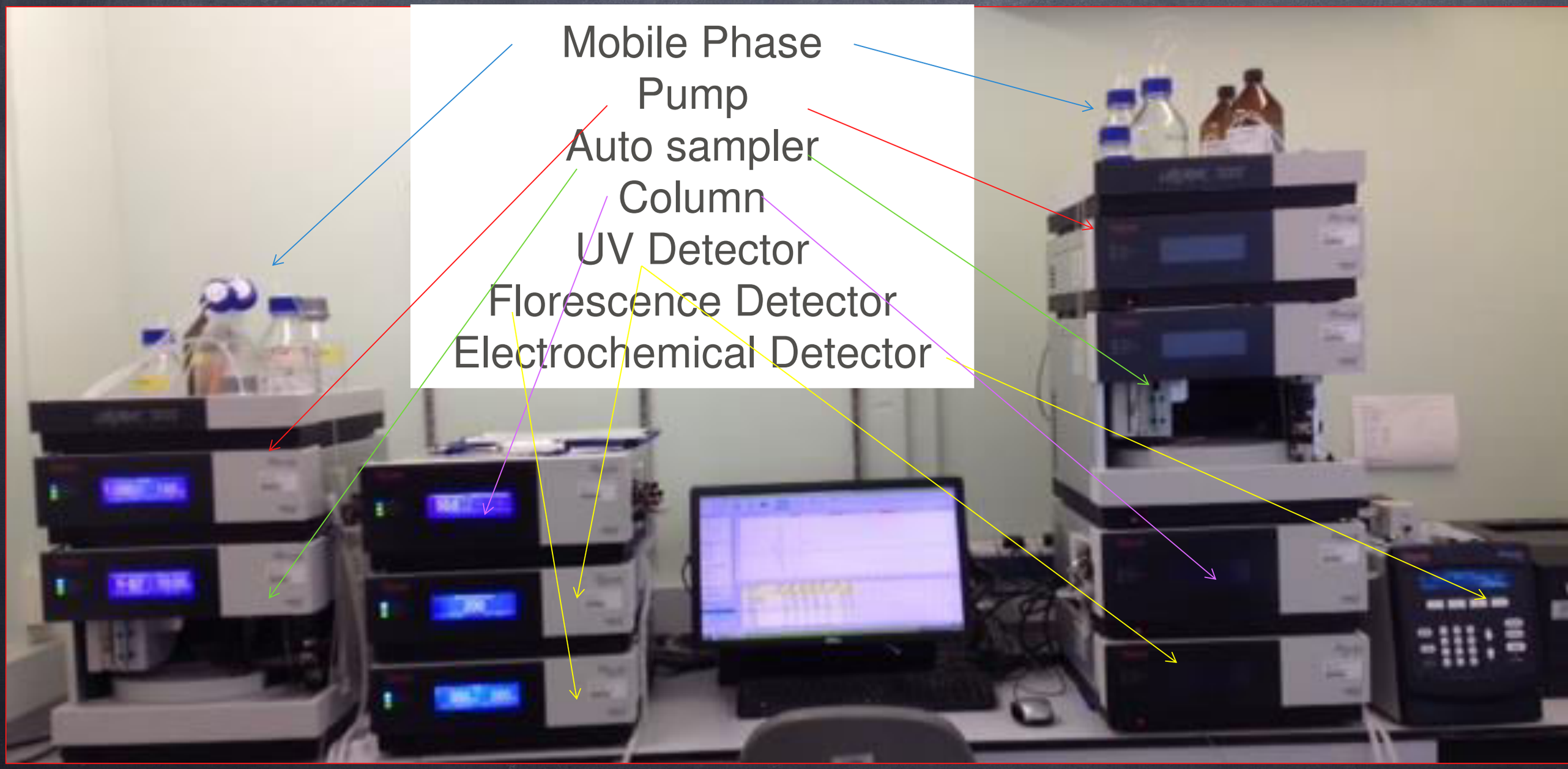
Overview of HPLC

- Solvent (Mobile Phase)
- Pump
- Auto sampler
- Column (Stationary Phase)
- Detector – UV, Florescence, Electrochemical ECD



Waters Alliance

Thermo Ultimate 3000



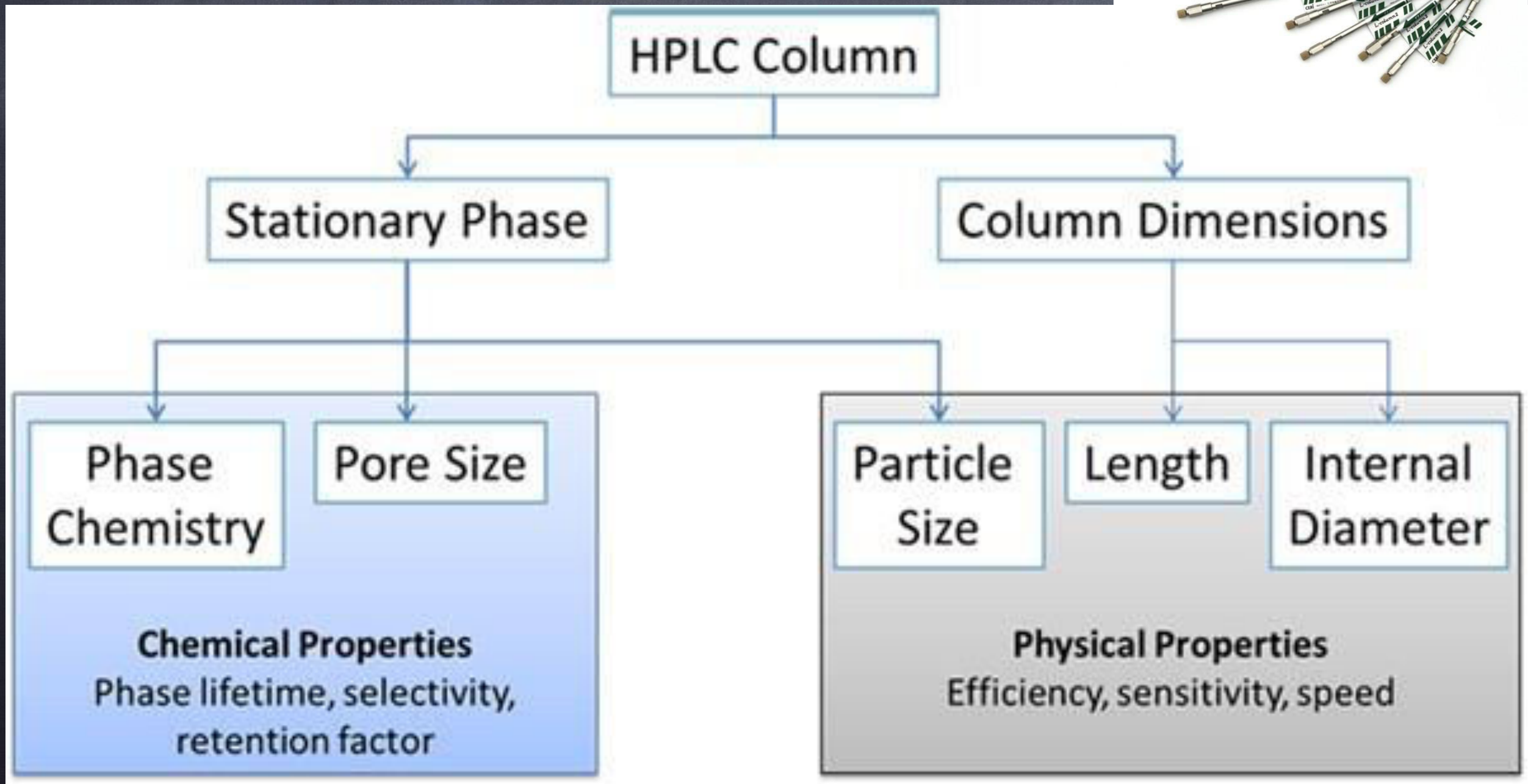
Why UHPLC ?

- Small particle size (<2 μm) results in higher theoretical plate numbers for more resolution and/or faster separations.
    - smaller particles cause less dispersion (band broadening)
    - flow rate can be increased with less loss of efficiency compared to larger particles
    - the chromatographic efficiency, N, is directly proportional to the ratio of column length and particle diameter, L/d
- This means that column length can be shorter without losing resolution
- Faster analyses using higher flow rates and shorter column
- Narrower columns are often used which also reduces eluent consumption
    - smaller volumetric flow for same linear flow down the column
      1ml/min on 4.6mm i.d. = 0.21ml/min on 2.1mm i.d.

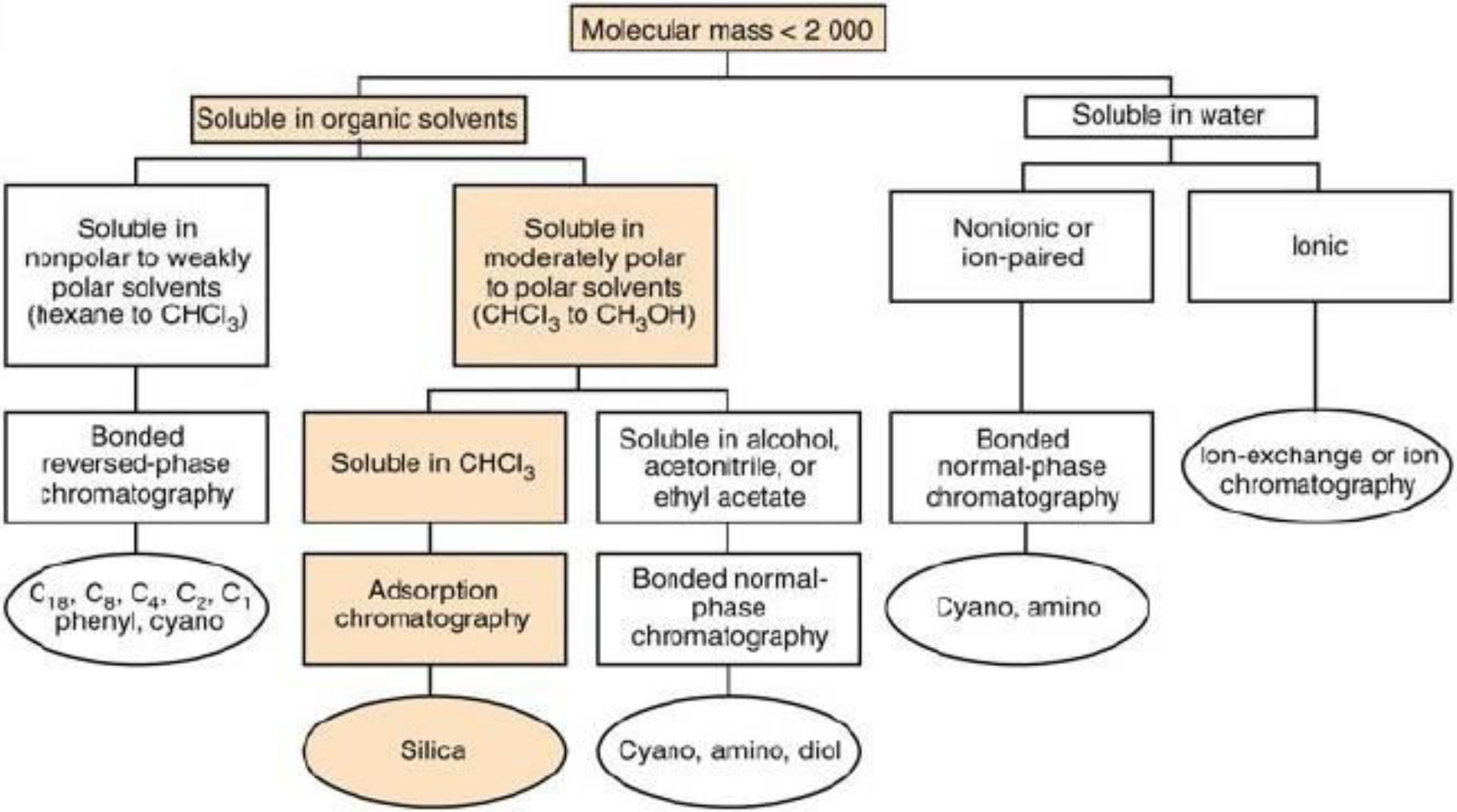
HPLC vs UHPLC

Traditional HPLC	UHPLC
Pressures up to 300bar 4000psi	Pressures up to 1400bar 20,000psi
Longer column larger particle size	Shorter Column Smaller particle size
Longer run times	Shorter run times = higher throughput
Uses more mobile phase	Uses less Mobile Phase
Cheap	Expensive
Often bail out with High Pressure	Prone to Blockages

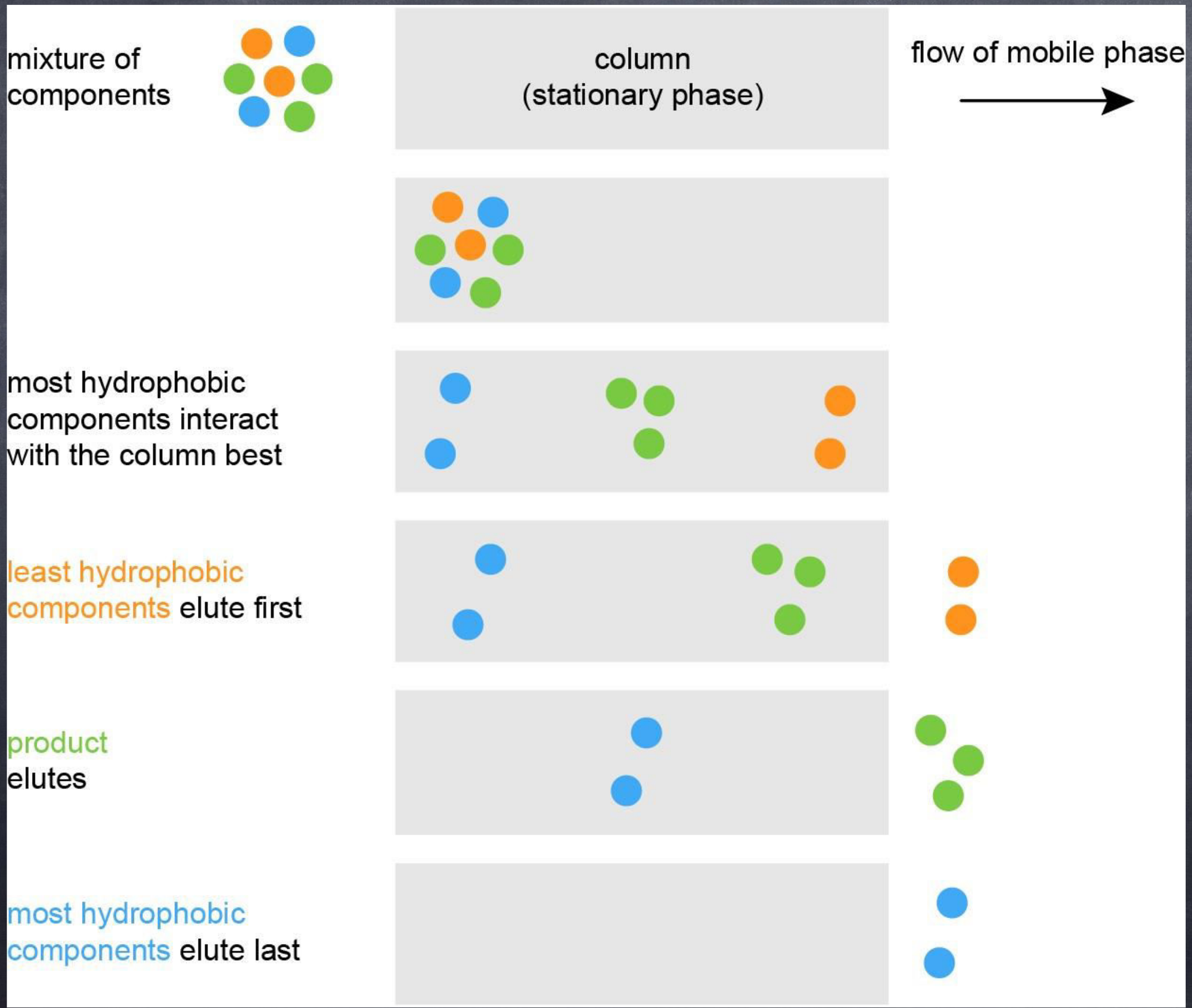
Column Selection



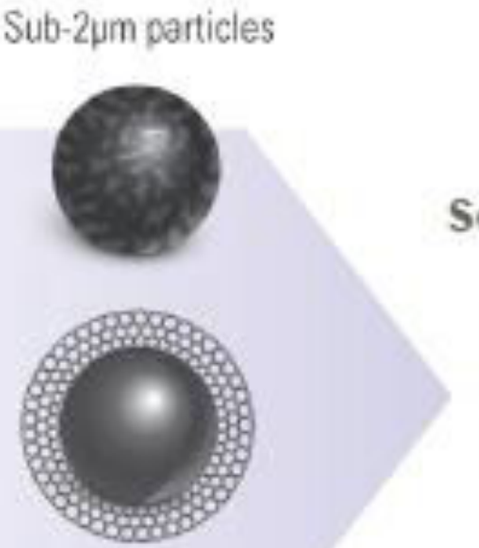
Separation based on selection of column material

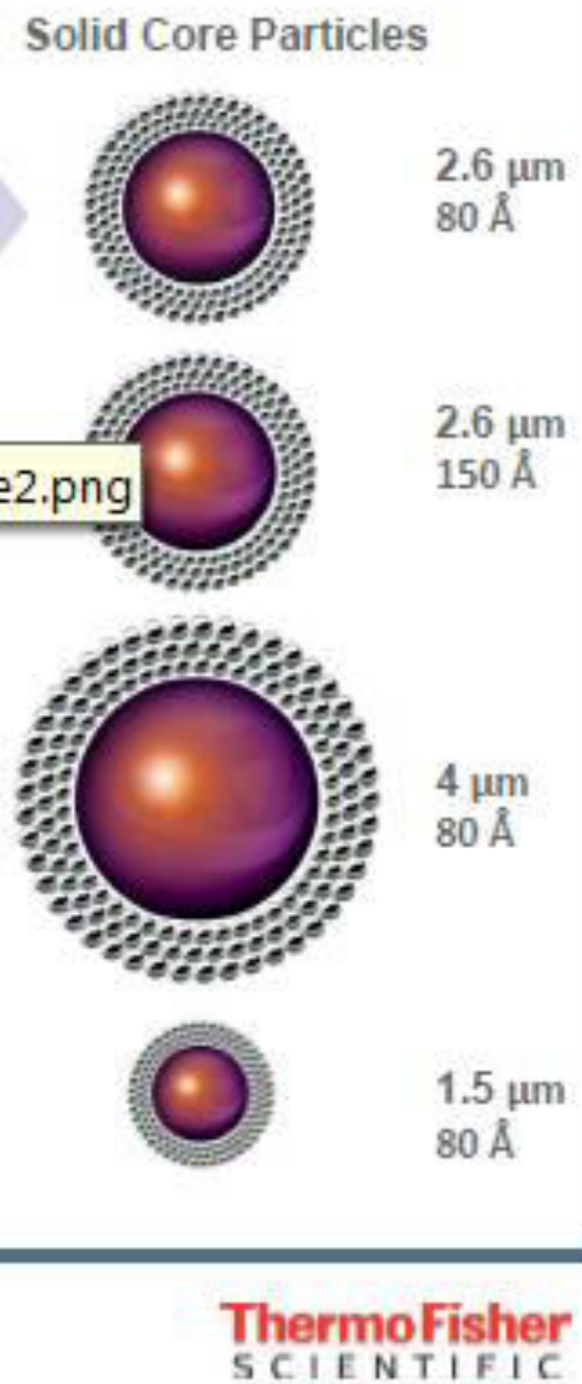


Reverse Phase Chromatography

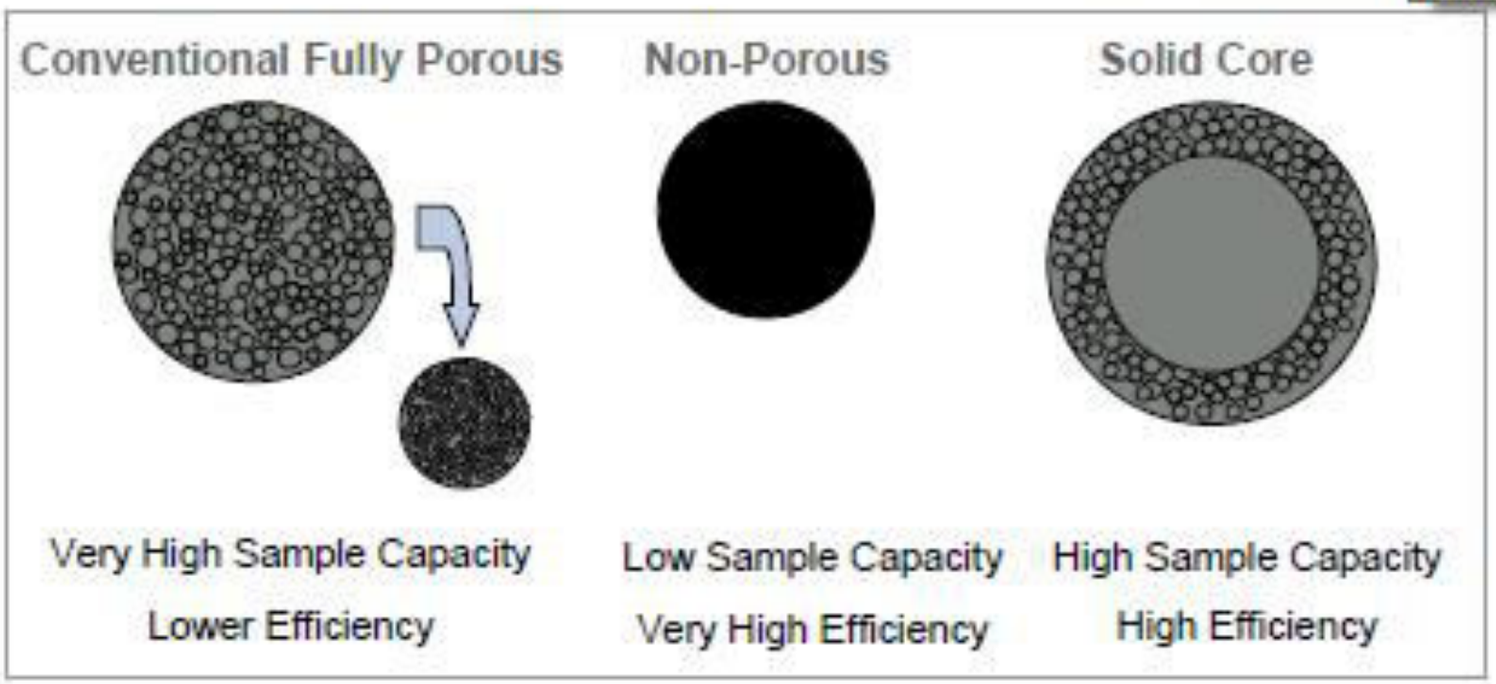


What type of Particle to Chose?





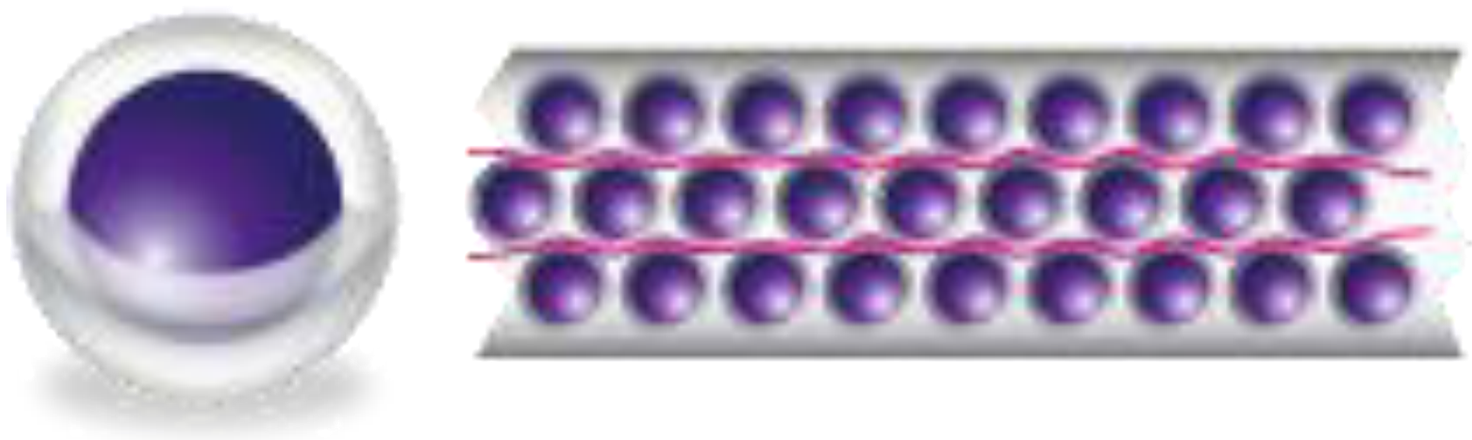
particle_evolution-page2.png

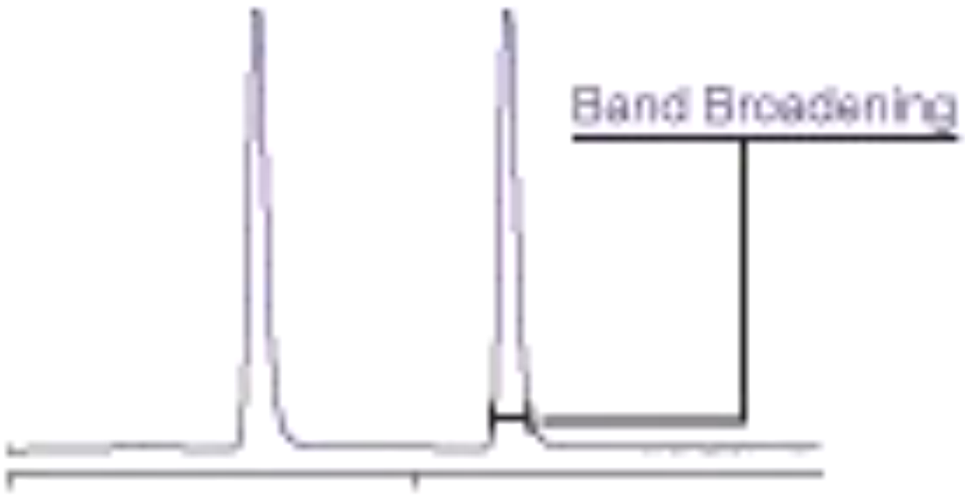


ThermoFisher SCIENTIFIC

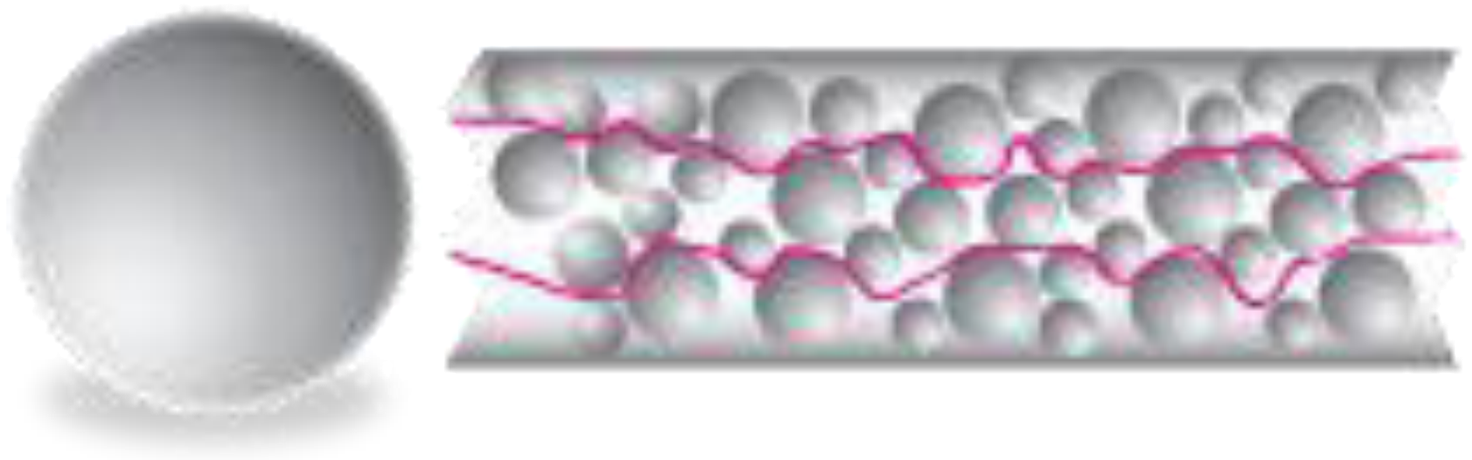
Eddy Diffusion

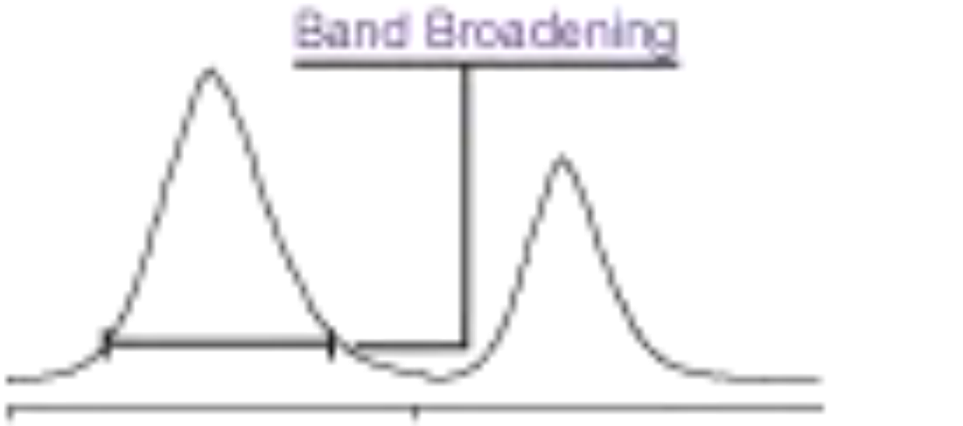
Kinetex Core-Shell



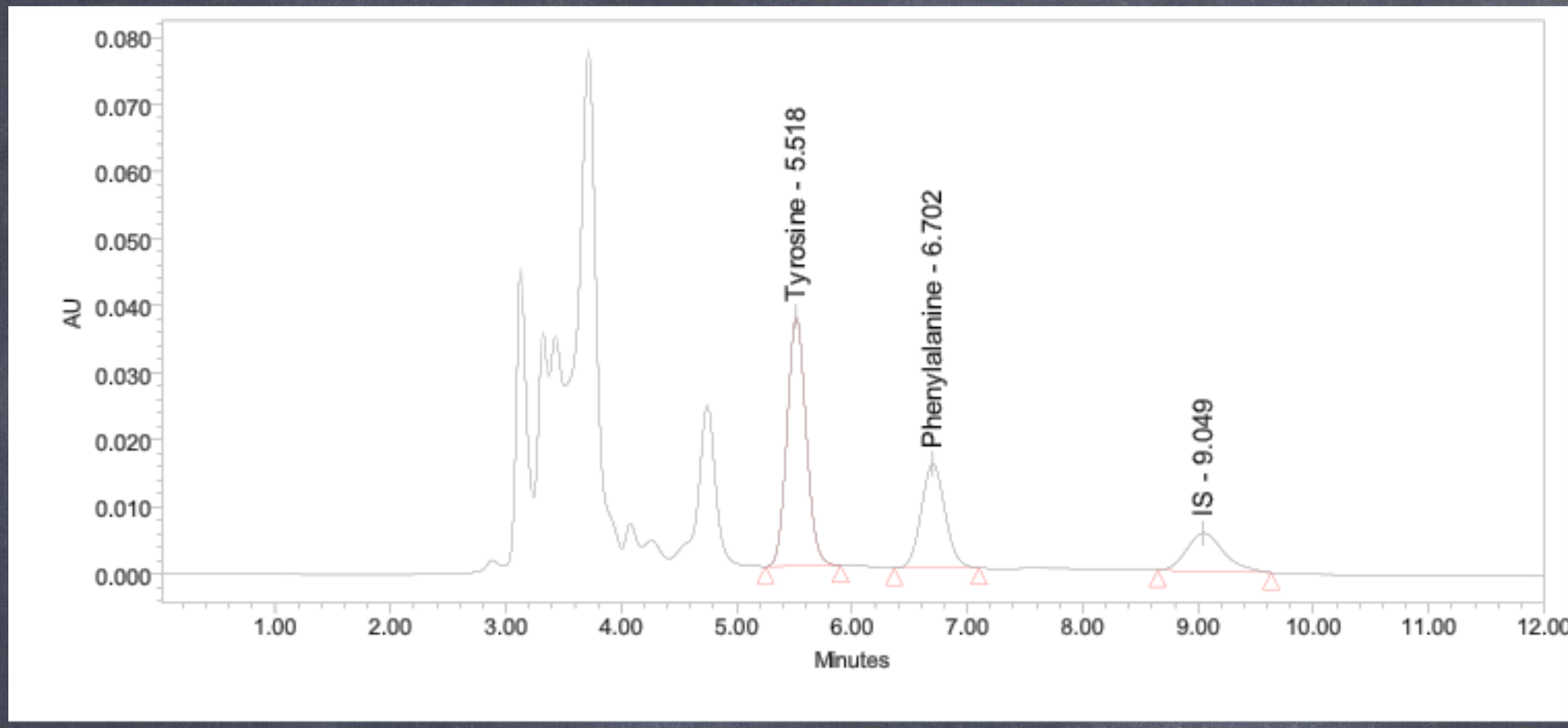


Fully Porous



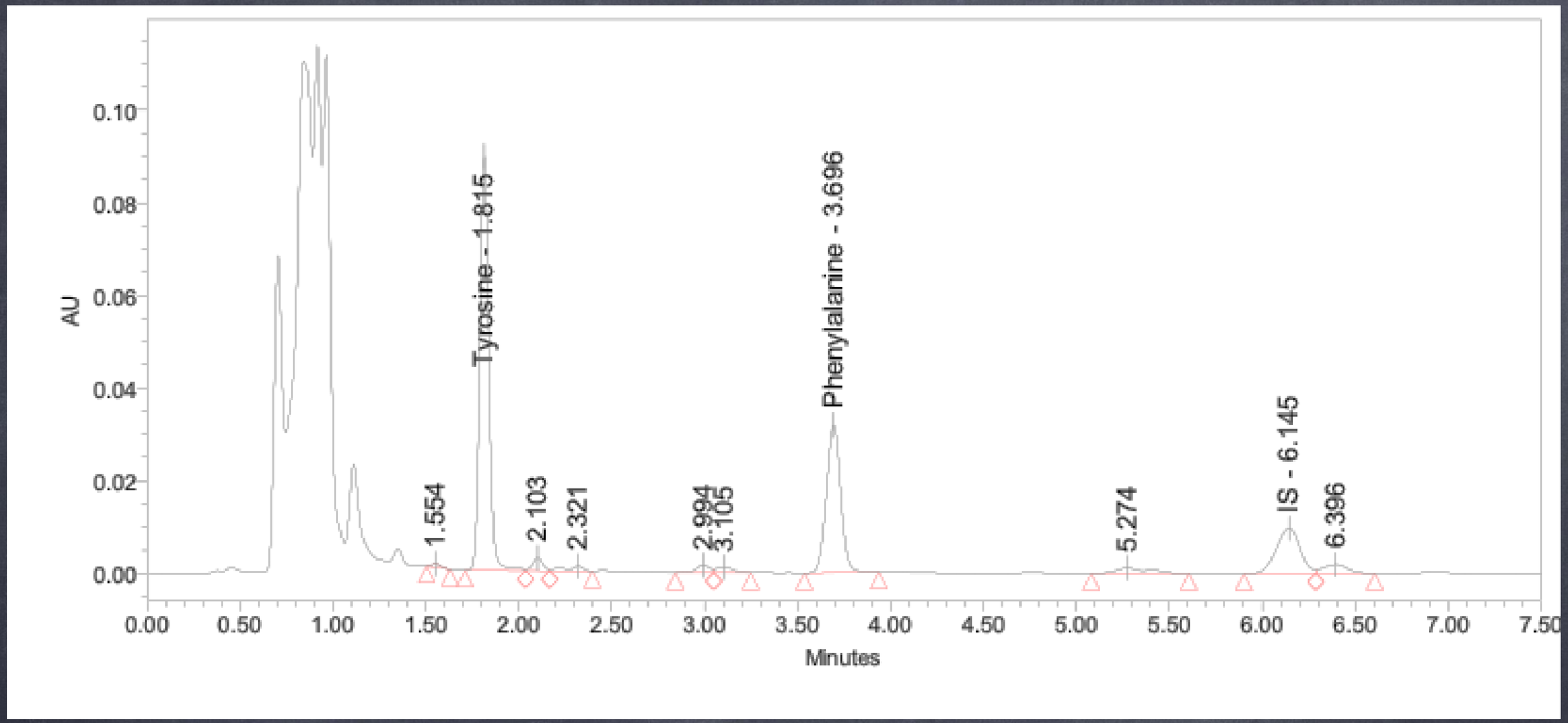


Plasma Phenylalanine + Tyrosine



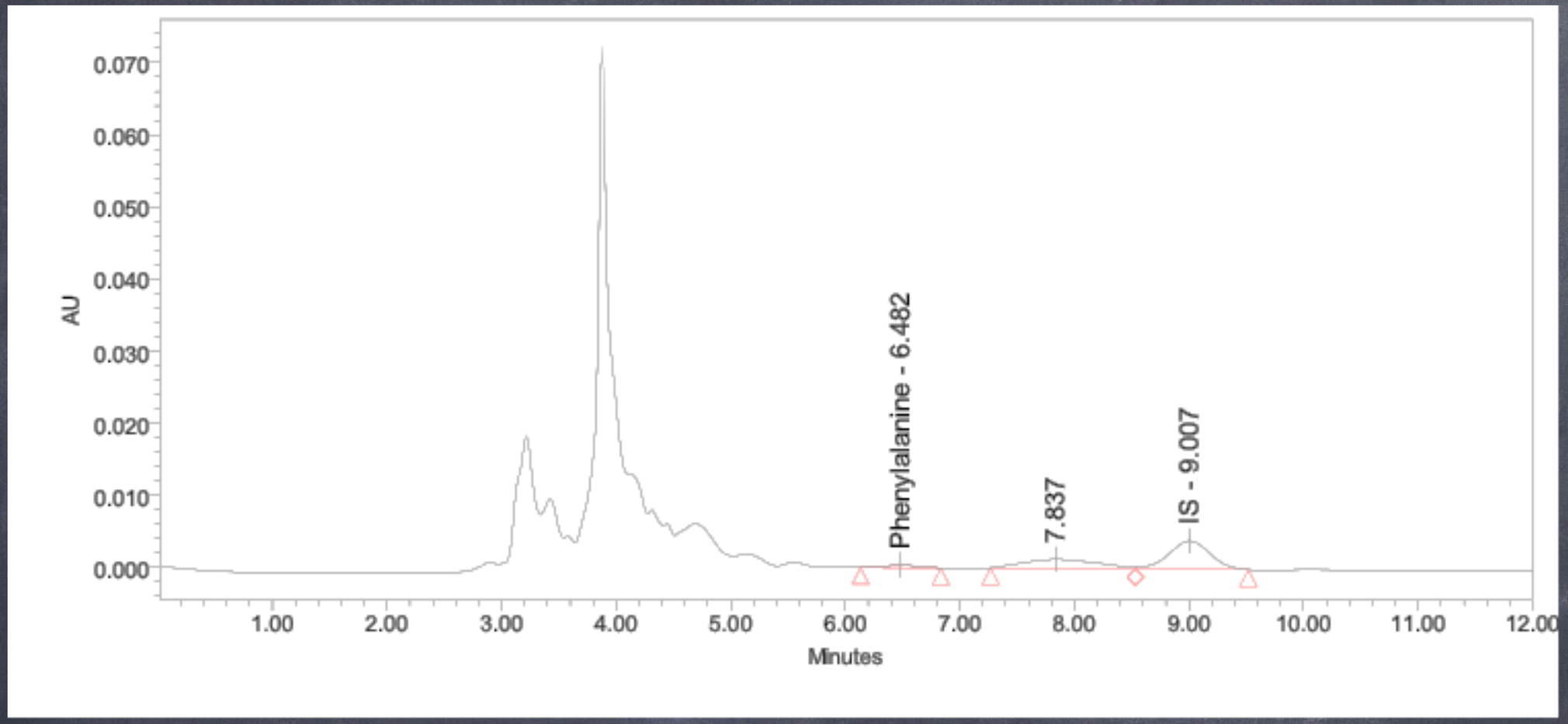
- Partisil column 250mm x4.6 10um Temperature 25°C
- Mobile phase 60mL Acetonitrile &2mL 70%perchloric Acid in 1L Deionised water
- 100uL plasma & 200uL 10%perchloric Acid -mix & spin
- 200uL supernatant & 200uL internal std methyl phenylalanine in mobile phase
- Flow 1.0ml/min injection volume 10uL

Plasma Phenylalanine + Tyrosine



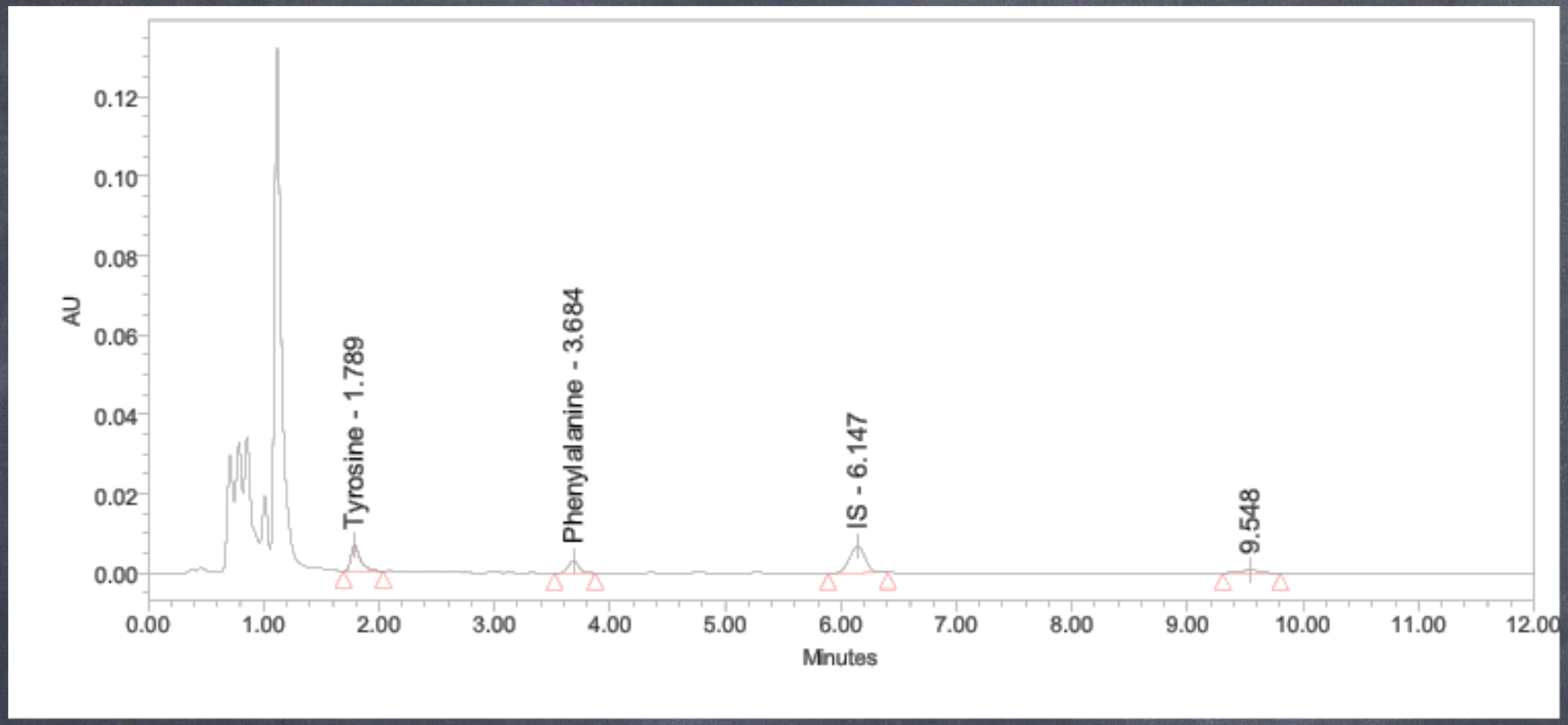
- Phenomenex Kinetex C18 2.6um 100mm x4.6
- Temperature 50°C use a guard column

Blood Spot Phenylalanine



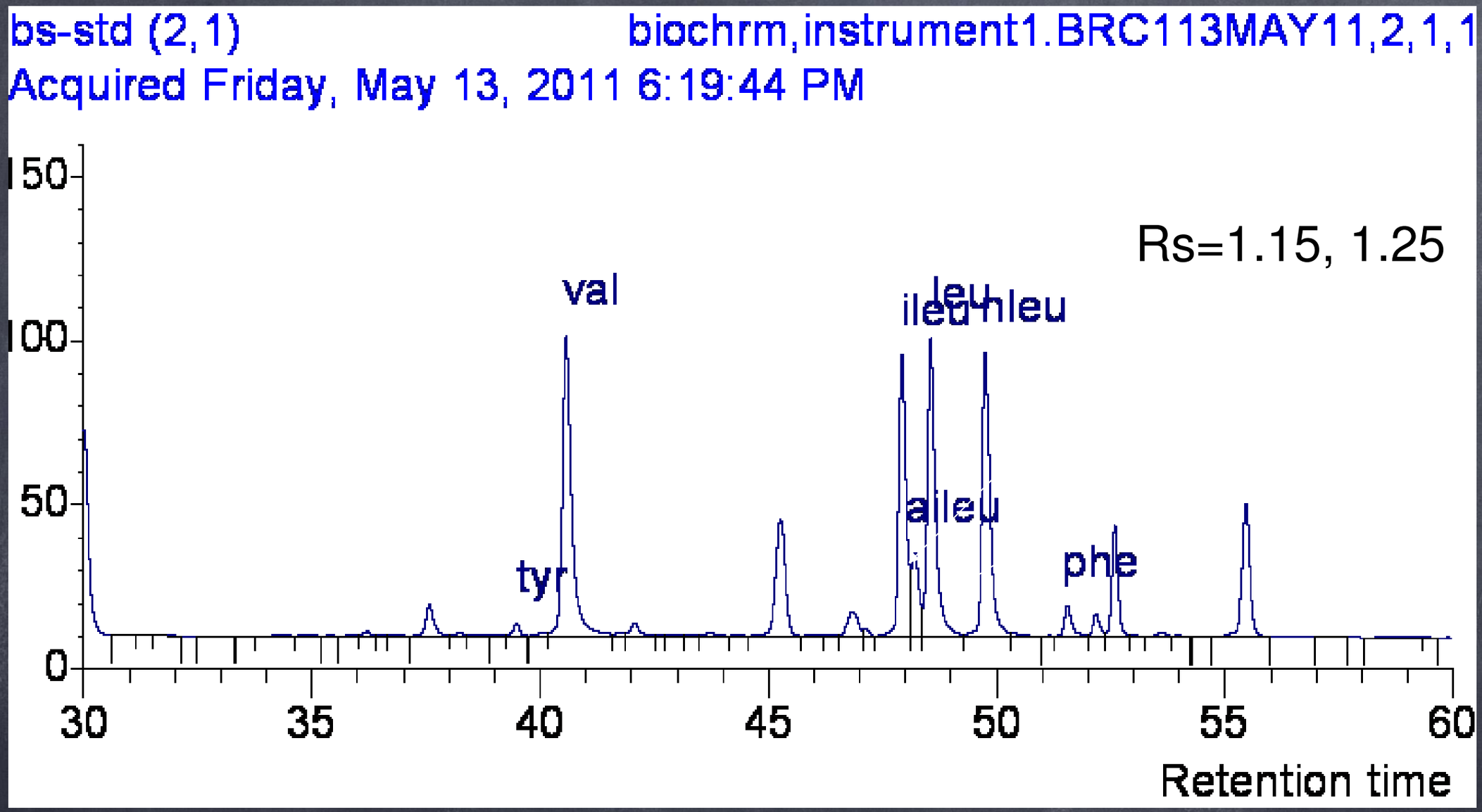
- Punch 2 blood spots
- Add 150uL 70%Ethanol /internal Std
- Mix for 1 min
- Inject 10uL elutent onto Partisil 250x4.6 10u column

Blood Spot Phenylalanine



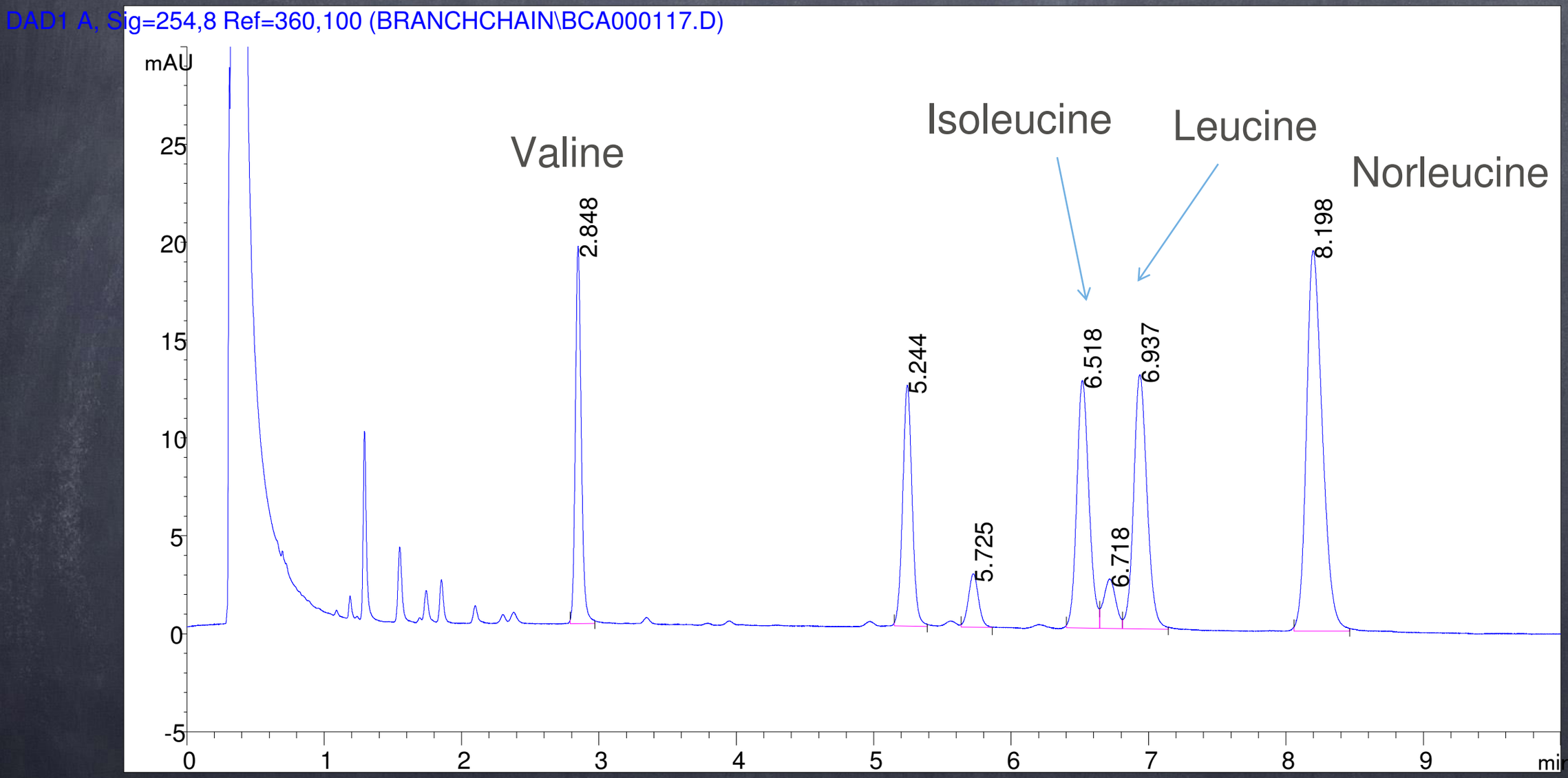
- Punch 2x blood spots
- Add 150uL 80% ACN mix for 1min & spin
- Take 100uL of supernatant & dry under nitrogen at 37°C for 2mins
- Reconstitute with 100uL Internal Std
- Inject 10uL onto Kinetex 100x4.6 2.6um temp 50°C flow 1.4mL

Current LC method – Blood Spot Standard



Column: Waters Picotag **4µm, 3.9 x 300 mm** Temperature: 46ºC
Flow rate: 1ml/min Injection volume: 20µl
Mobile Phase A: Sodium Acetate buffer adjusted to pH6.55 with acetic acid
Mobile Phase B: Acetonitrile/Methanol / Water (50:15:35)

A = 79 %
B= 21 %
COLUMN = ECLIPSE PLUS 2.1, 150MM X 1.8UM



Flow rate = 0.95 ml/min (pressure = 1100 bar)

Whether you are using HPLC or UHPLC the troubleshooting remains the same

Each of the following items need to be optimised in order to generate a satisfactory chromatogram

- Mobile phase composition
- Bonded phase chemistry
- Column and packing dimensions
- Injection volume
- Sample pre-treatment and concentration
- Mobile phase flow rate
- Column temperature
- Detector parameters

Good Housekeeping

- Use HPLC grade solvents for mobile phase
- Equilibrate column well before use
- Use a guard column to prolong the life of the column
- Never leave the lamp or ECD on without mobile phase going through the system
- Flush the system with 50% methanol after use
- Record daily maintenance & running pressures for each assay
- Record any instrument or assay problems and actions taken to resolve the issues.

Good Housekeeping

- Replace solvents regularly- composition may change
- Filter solvents-remove- particulate matter could damage components
- Degas solvents-removal of dissolved gases from the mobile phase helps to prevent bubble formation, lead to loss of prime

Trouble shooting

- High Pressure
- Low Pressure
- Poor Chromatography

High Pressure

- High Organic content of Mobile Phase-try slowly increasing flow when column first installed
- ?Blocked guard column-change filter
- ? Blocked column – try reversing column
- Does the pressure drop when the column is removed?
- Yes - try replacing column
- No - ?blockage in tubing – replace tubing…make sure you replace like for like otherwise chromatography will be effected!

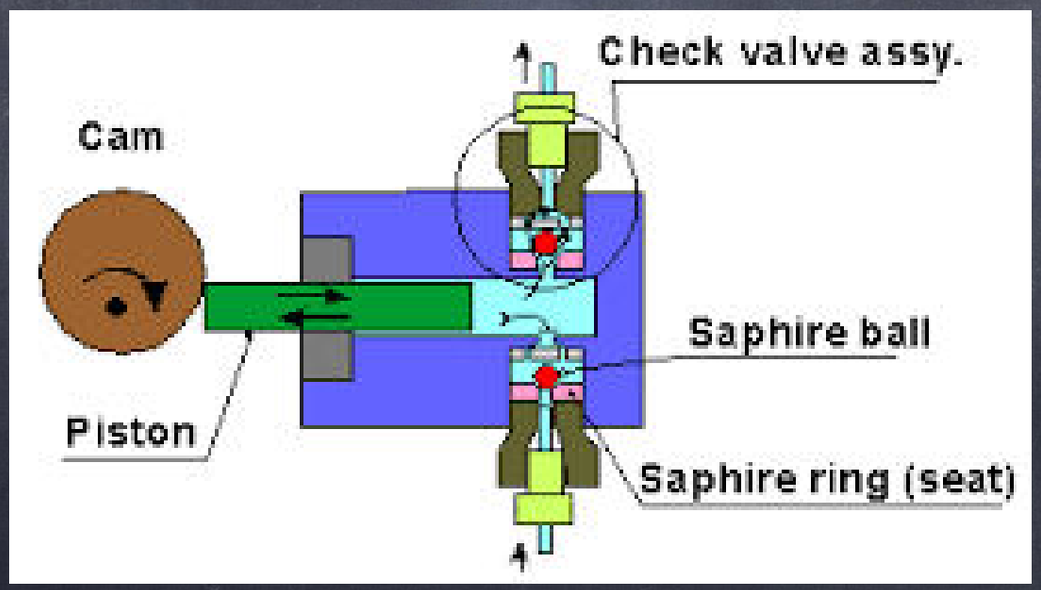
Low Pressure

- Look for leaks
- Is the column installed properly?
- Is the prime valve open?
- Lost Prime -sonicate check valves

(make sure re-installed correctly)

Check valves

Pump Head

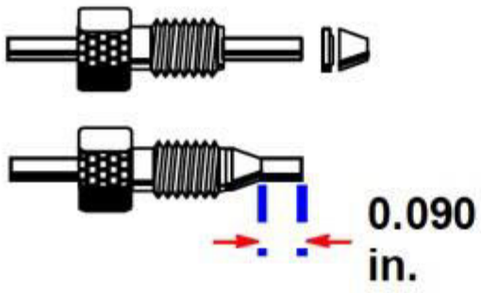


Poor Chromatography

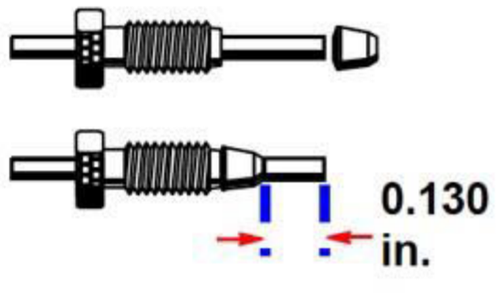
- Be Systematic
- Check Mobile Phase + Solvent line
- Check Column + Column Temperature
- Check sample vial + sample preparation
- Check Tubing and column fittings –void/dead volumes

Dead Volumes

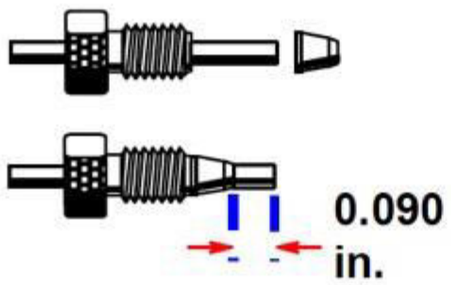
Swagelok



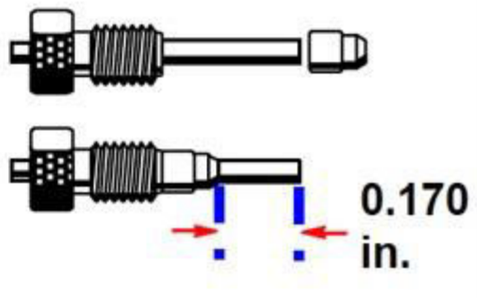
Waters



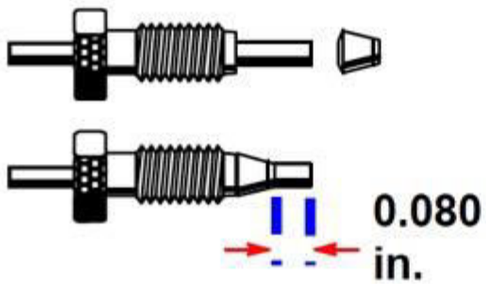
Parker



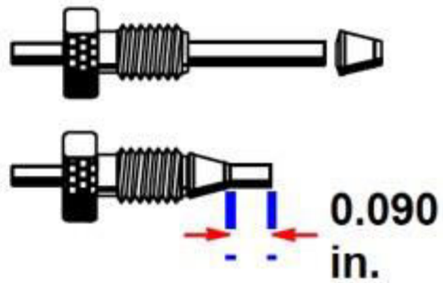
Rheodyne

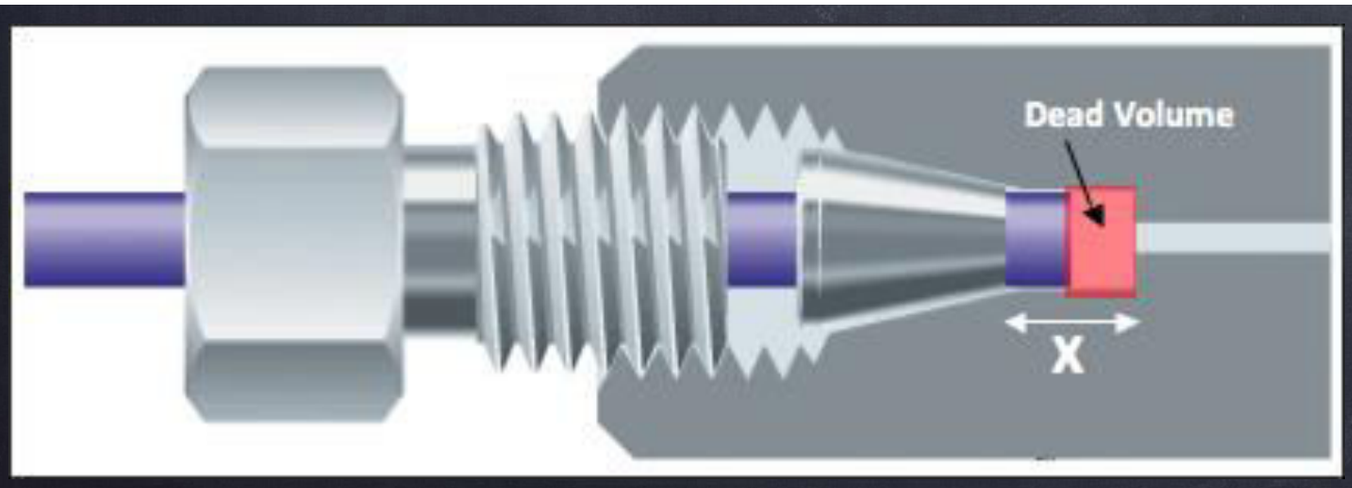


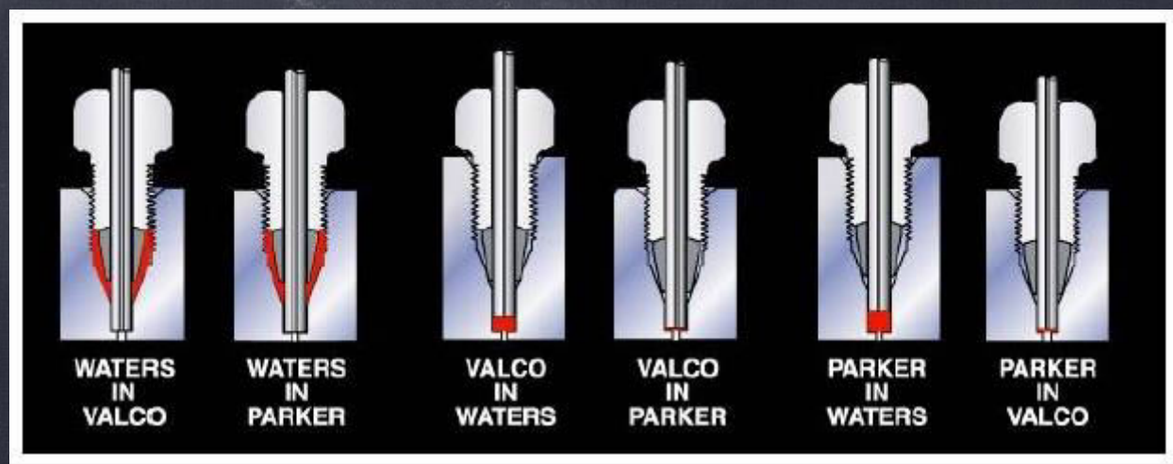
Valco

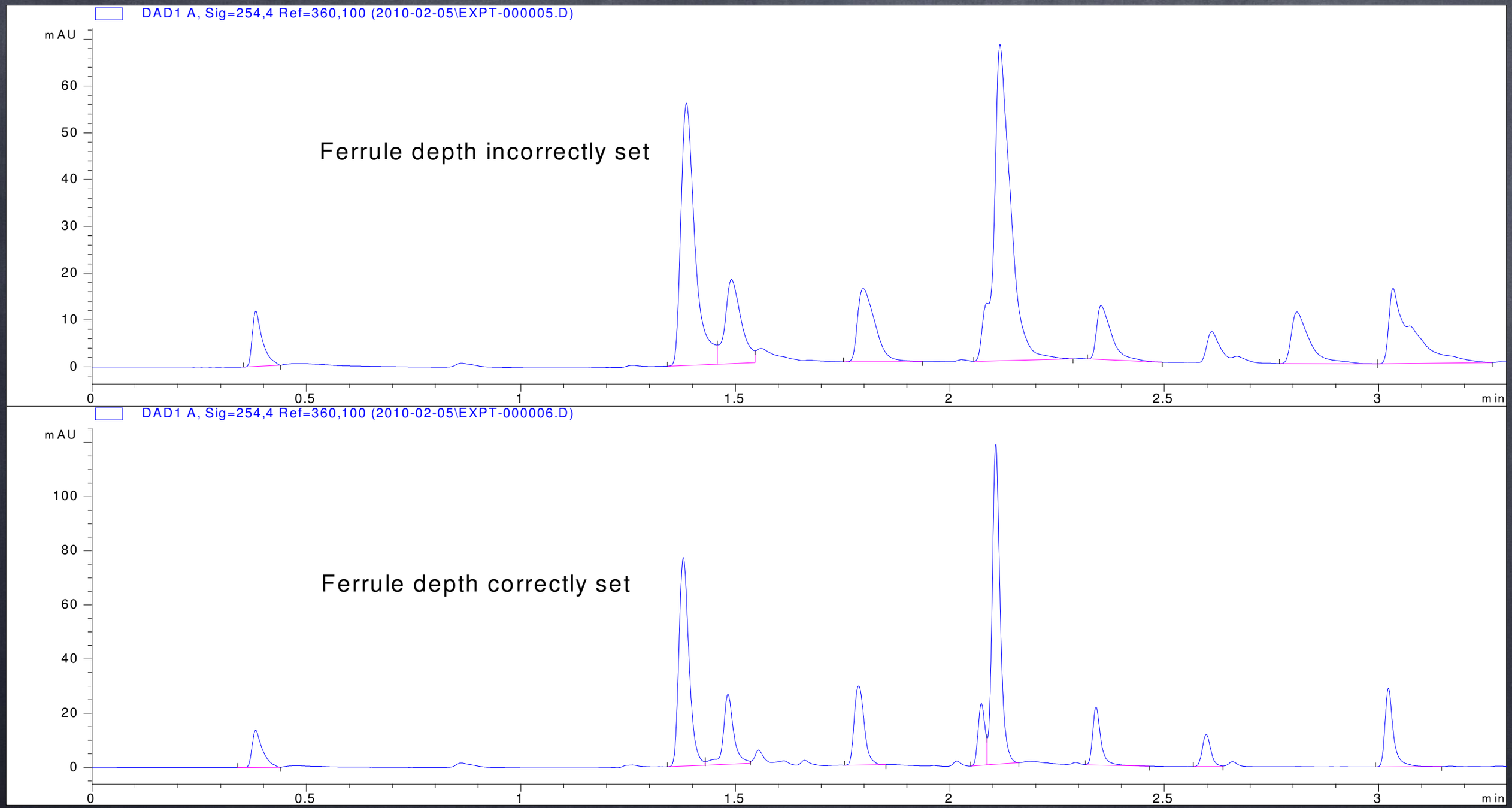


Uptight

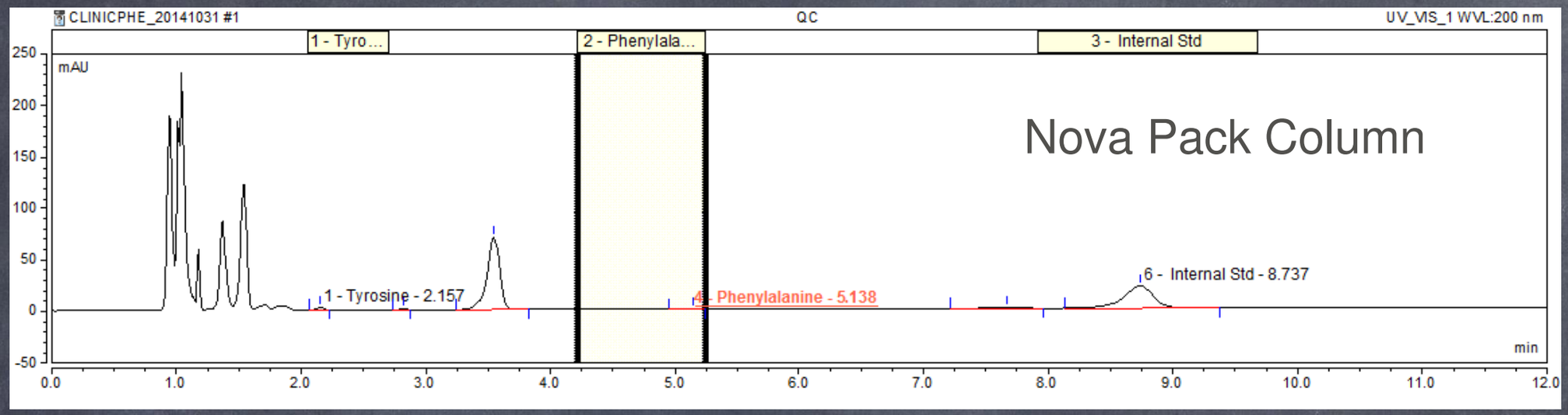


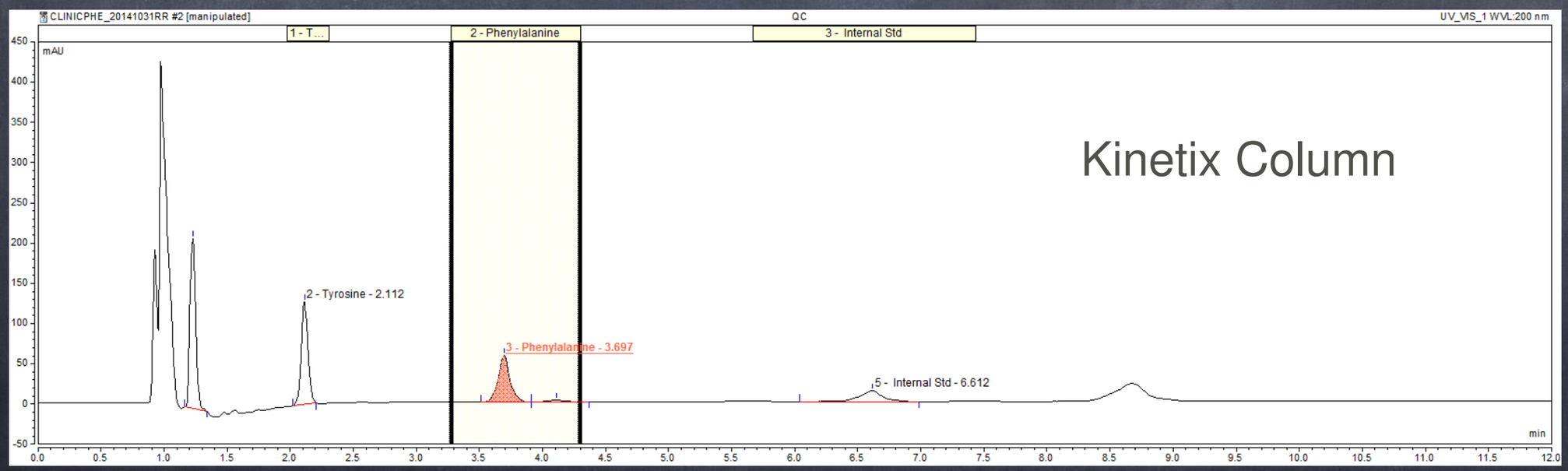





DAD1 A, Sig=254,4 Ref=360,100 (2010-02-05\EXPT-000005.D)
mAU
60
50
40
30
20
10
0
Ferrule depth incorrectly set
0
0.5
1
1.5
2
2.5
3
min
DAD1 A, Sig=254,4 Ref=360,100 (2010-02-05\EXPT-000006.D)
mAU
100
80
60
40
20
0
Ferrule depth correctly set
0
0.5
1
1.5
2
2.5
3
min

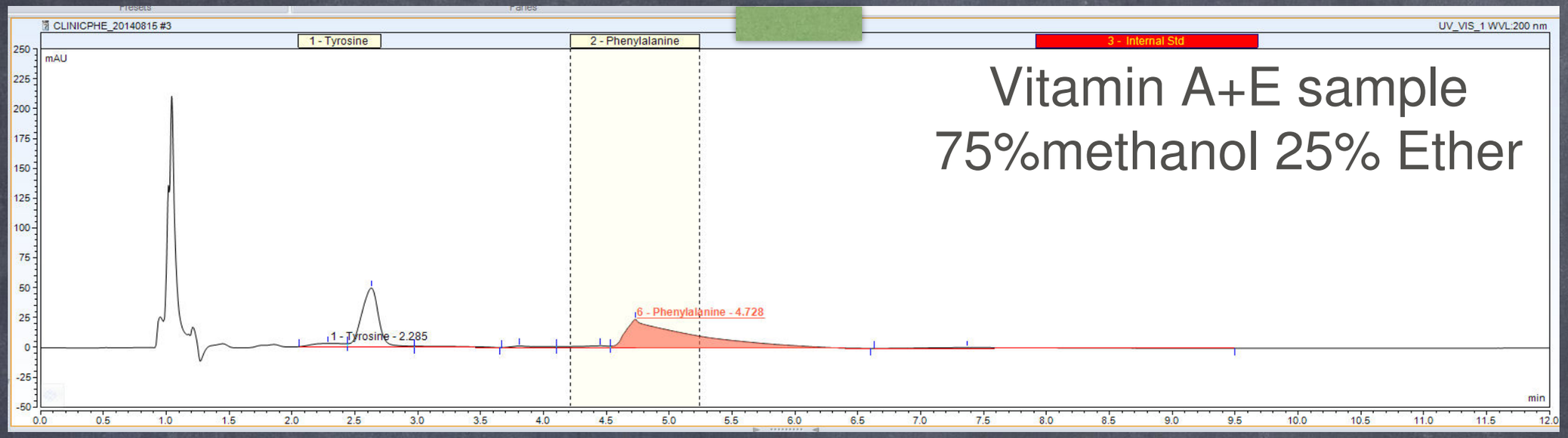
Plasma Phenylalanine + Tyrosine problem

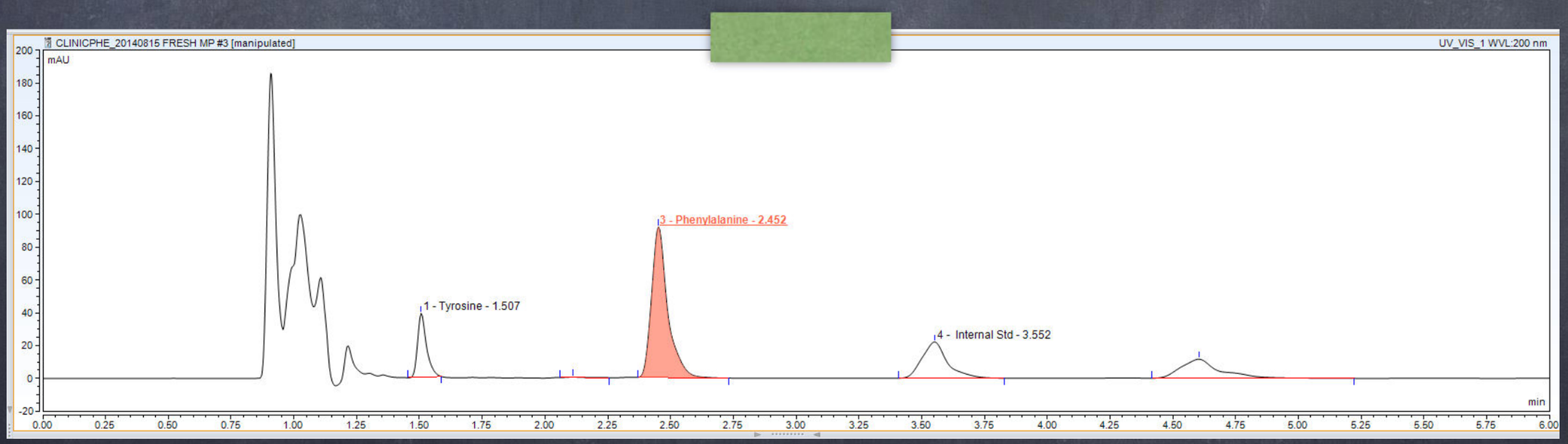




Wrong Column!

Plasma Phenylalanine + Tyrosine problem

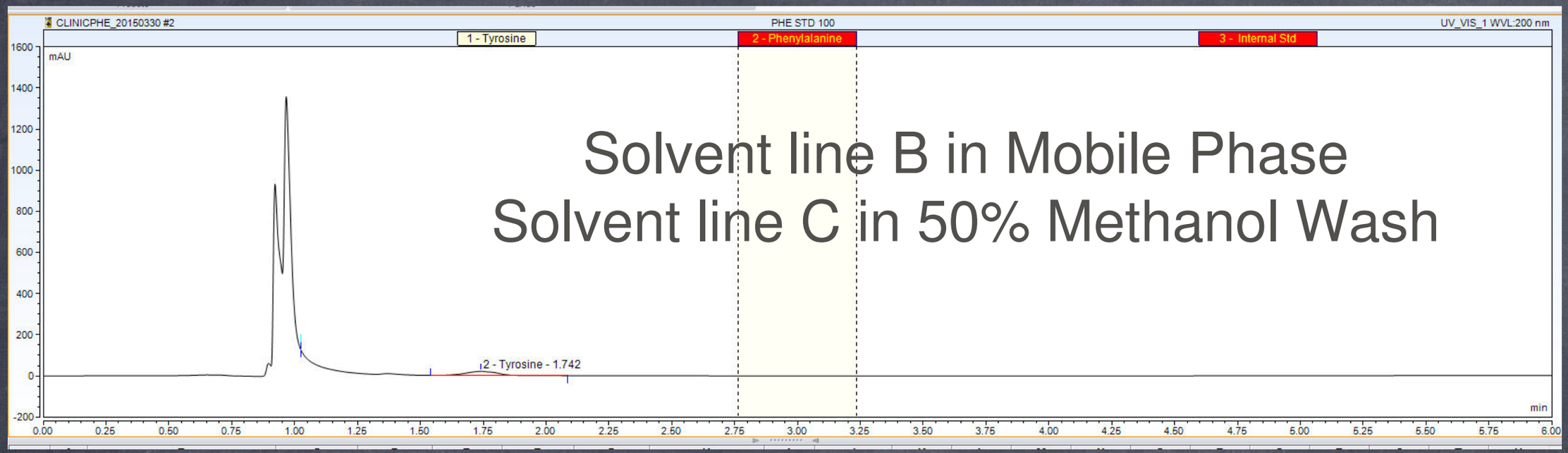


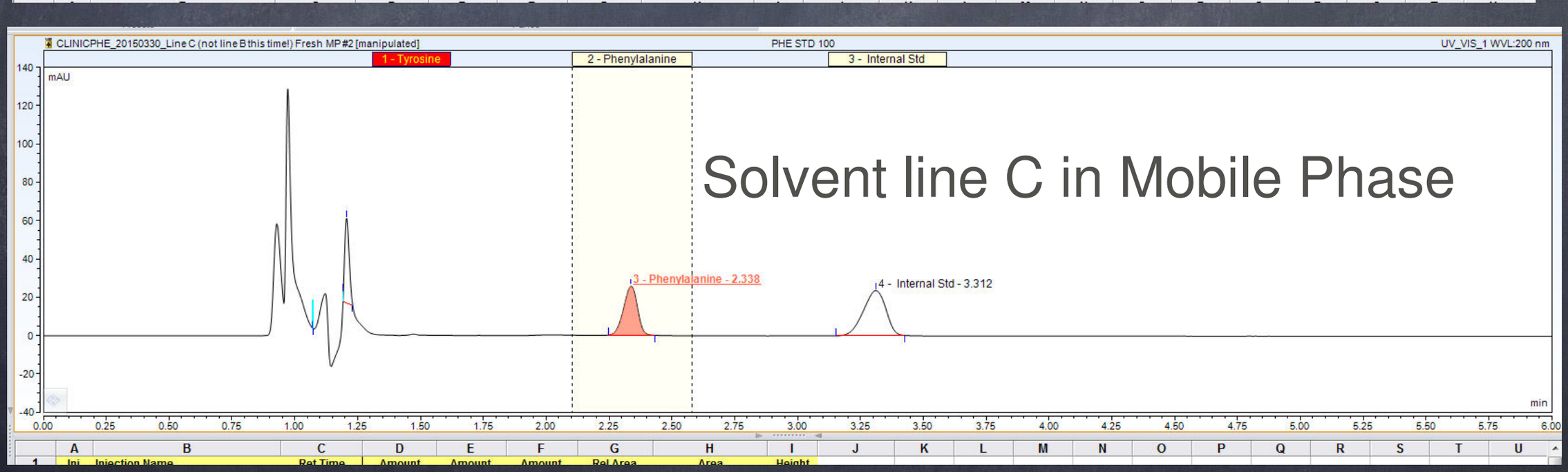


Clue: No Internal Standard!

Problem: Wrong sample vial injected

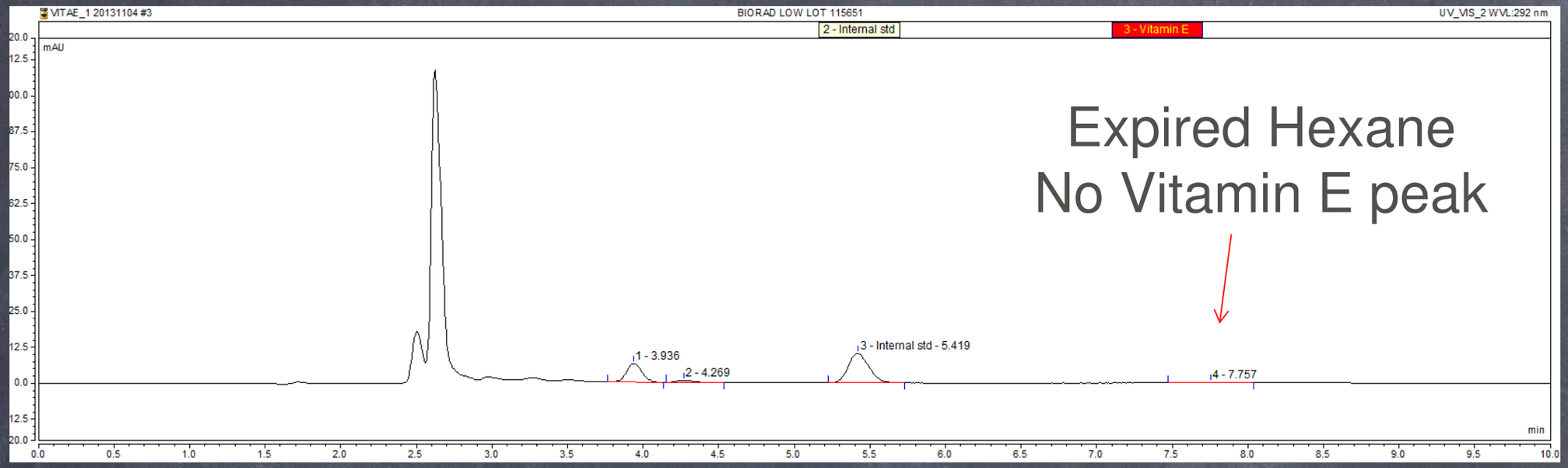
Plasma Phenylalanine + Tyrosine problem

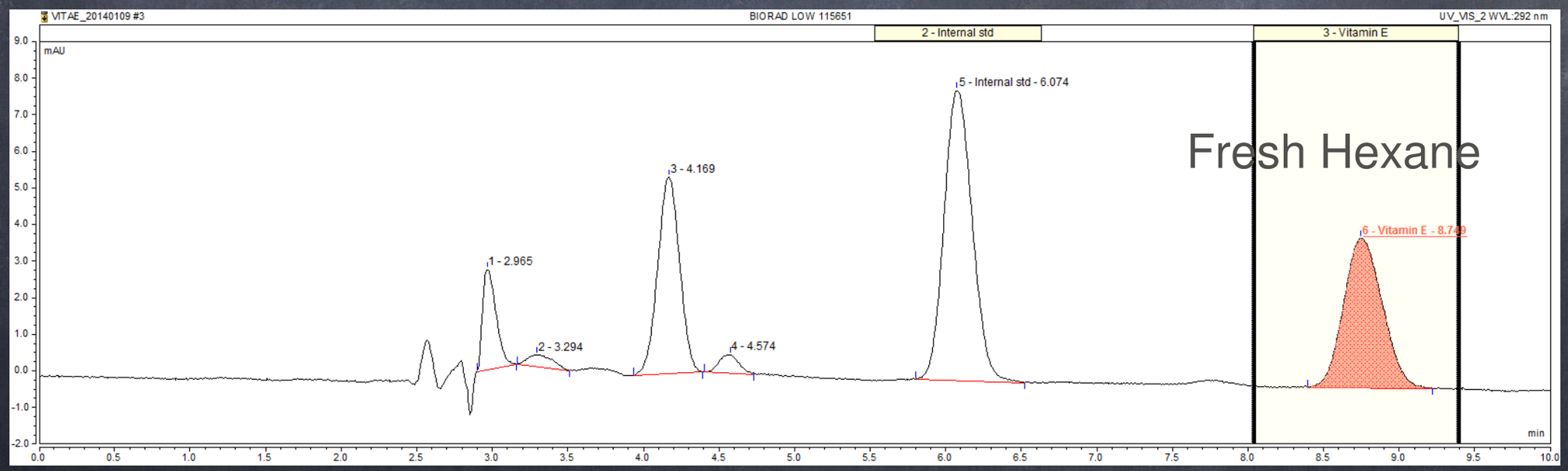




Wash line (50% Methanol)
and MP lines switched

Disappearing Peaks!
Vitamin A + E

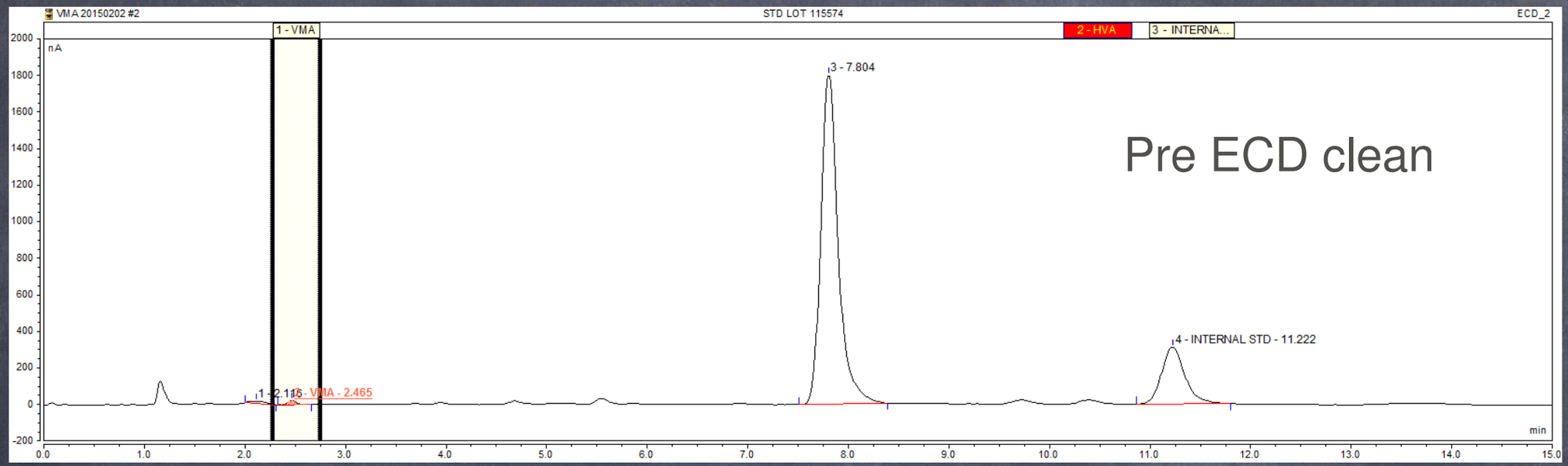


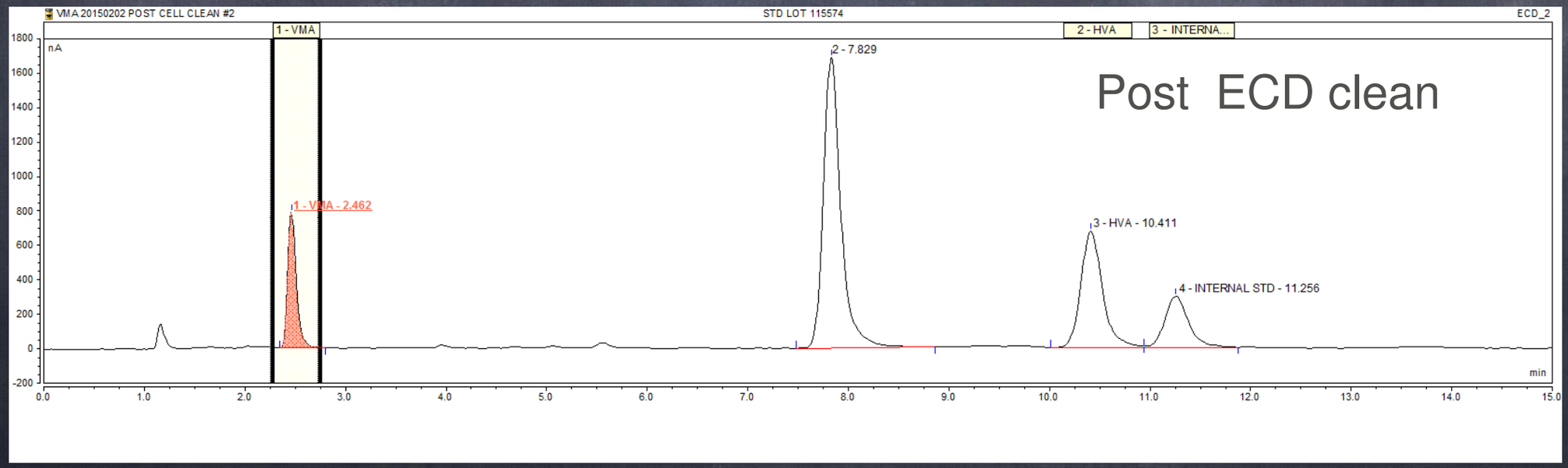


Sample Preparation -Expired Hexane!

More Disappearing Peaks!

VMA + HVA



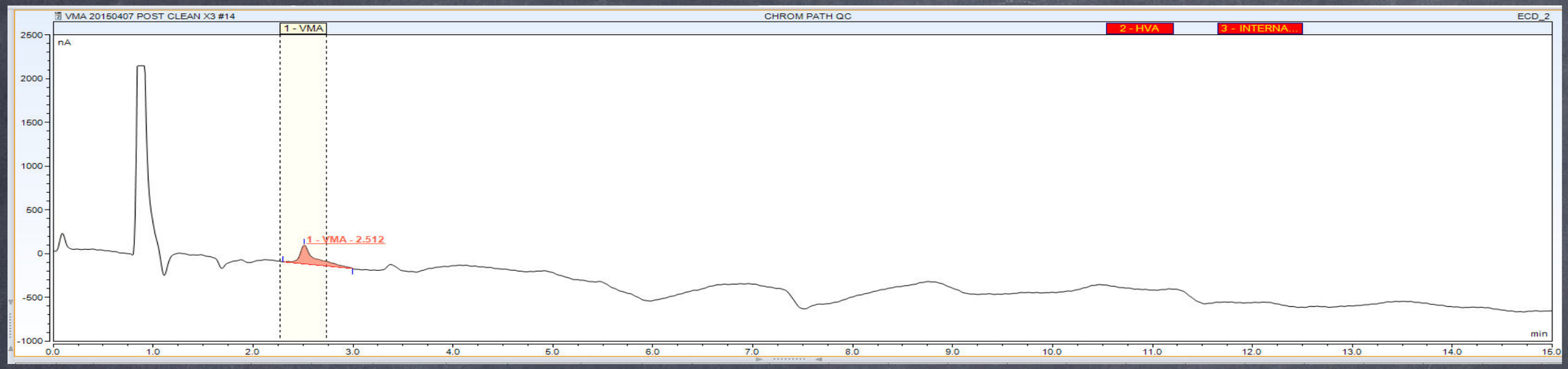


ECD needs cleaning

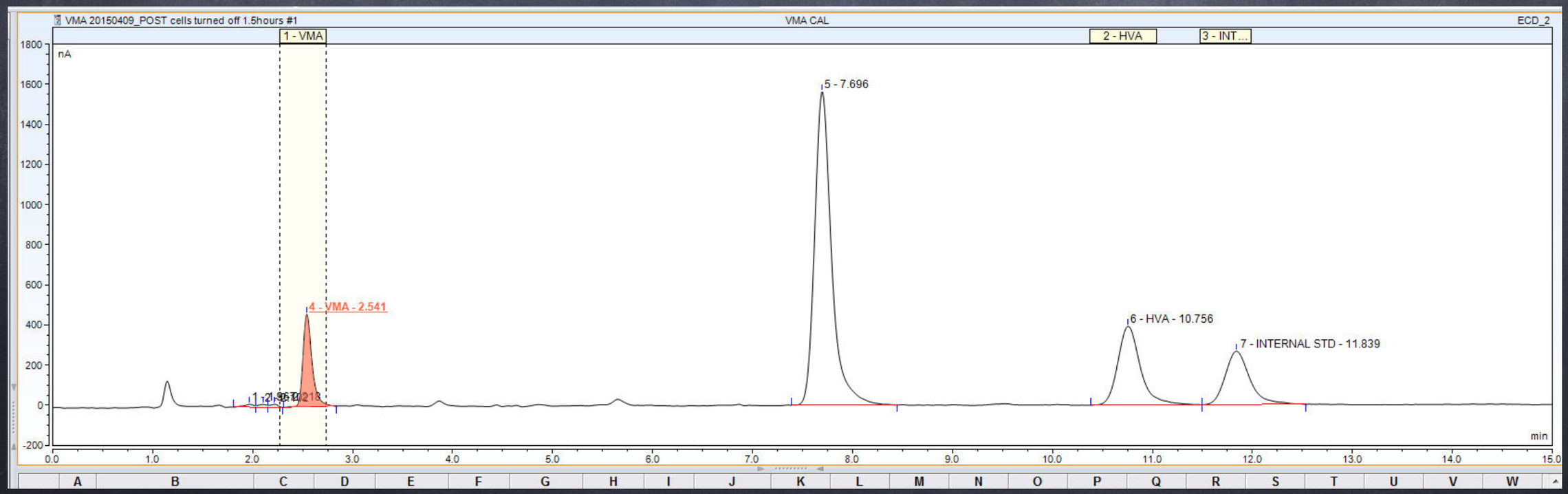
Cleaning ECD

- Cell 2
- +1000mv for 3min
- -500mv for 3mins
- +1000mv for 3min
- Turn cell off for 10minutes with mobile phase running through
- Turn cell back on

Is this ECD Dead? (VMA + HVA)



ECD cells turned off for 1.5hrs with MP still flowing

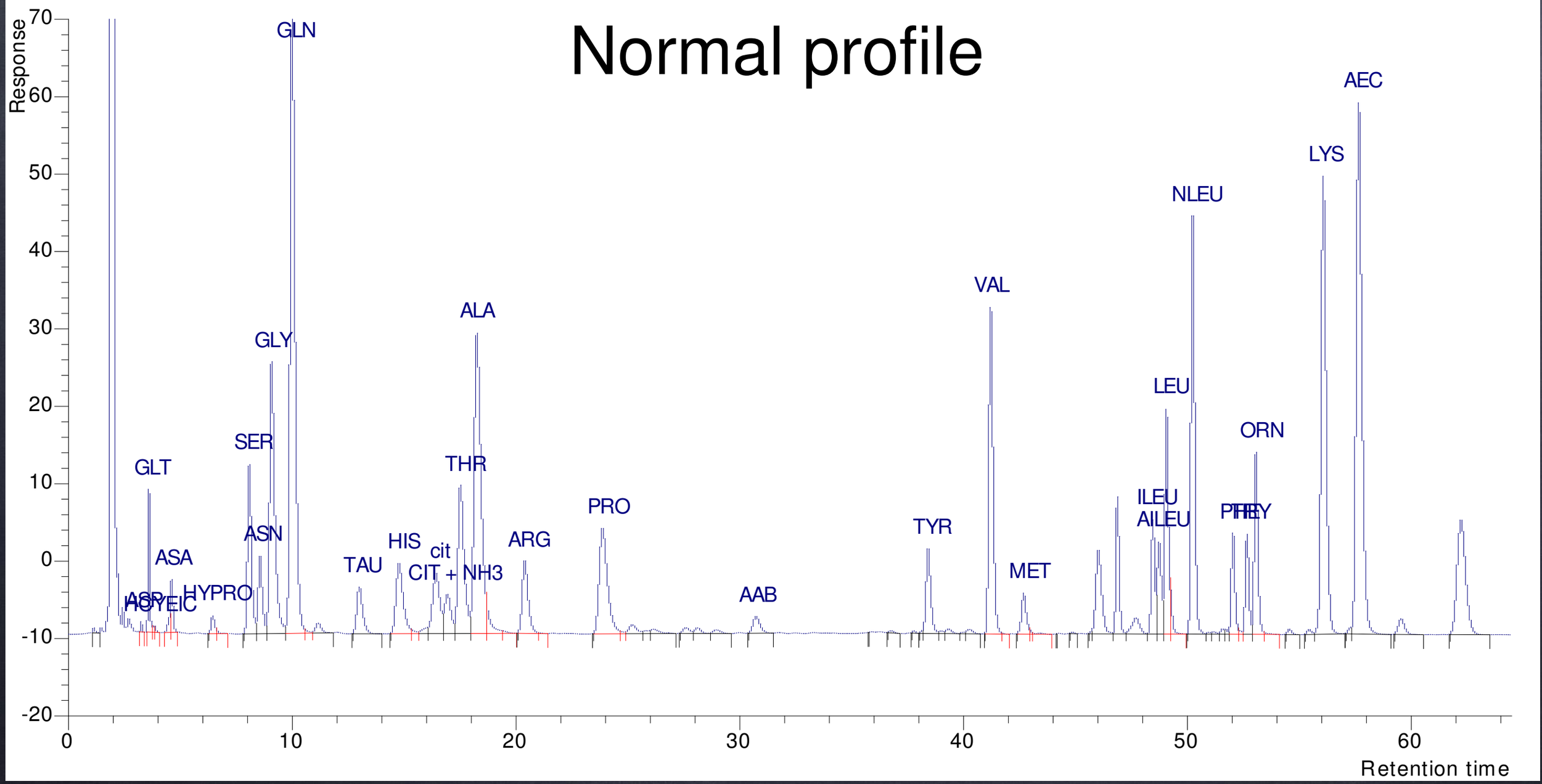


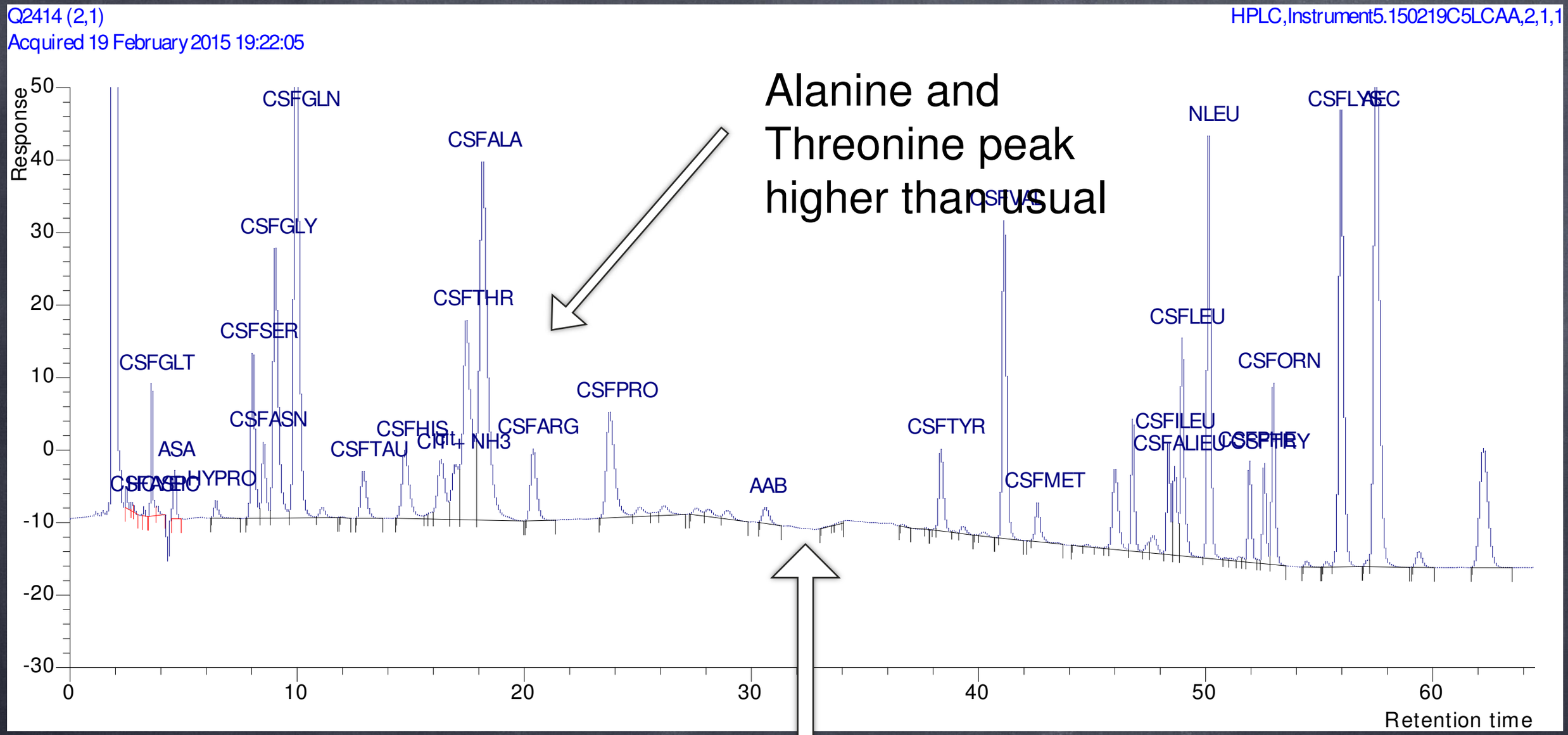
Column: Waters Picotag **4µm, 3.9 x 300 mm** Temperature: 46ºC
Flow rate: 1ml/min Injection volume: 20µl
Mobile Phase A: Sodium Acetate buffer adjusted to pH6.55 with acetic acid
Mobile Phase B: Acetonitrile/Methanol / Water (50:15:35)

Q2414 (2,1)
Acquired 13 February 2015 17:40:27

HPLC,Instrument5.150213C5L,2,1,1

Normal profile

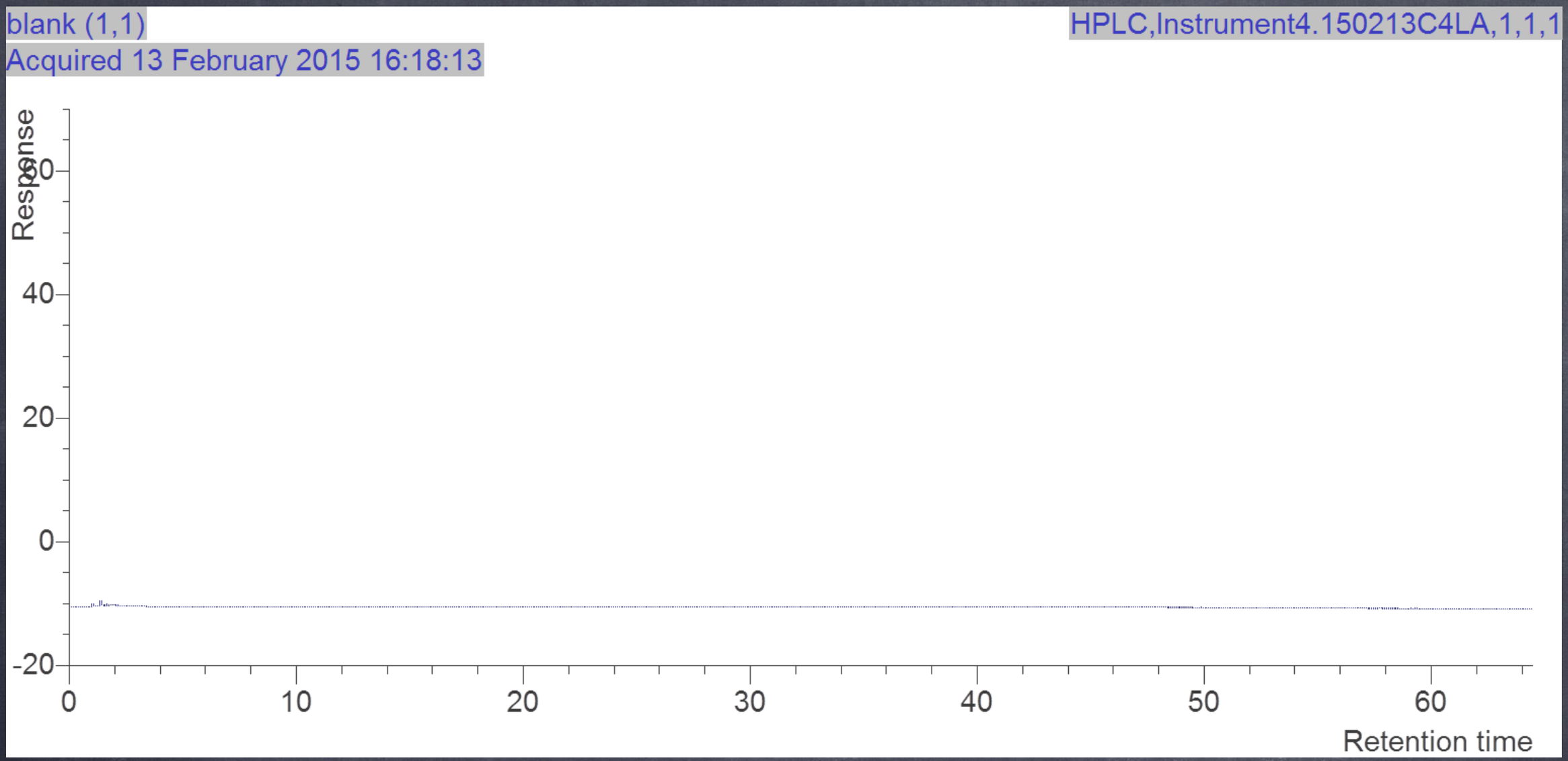




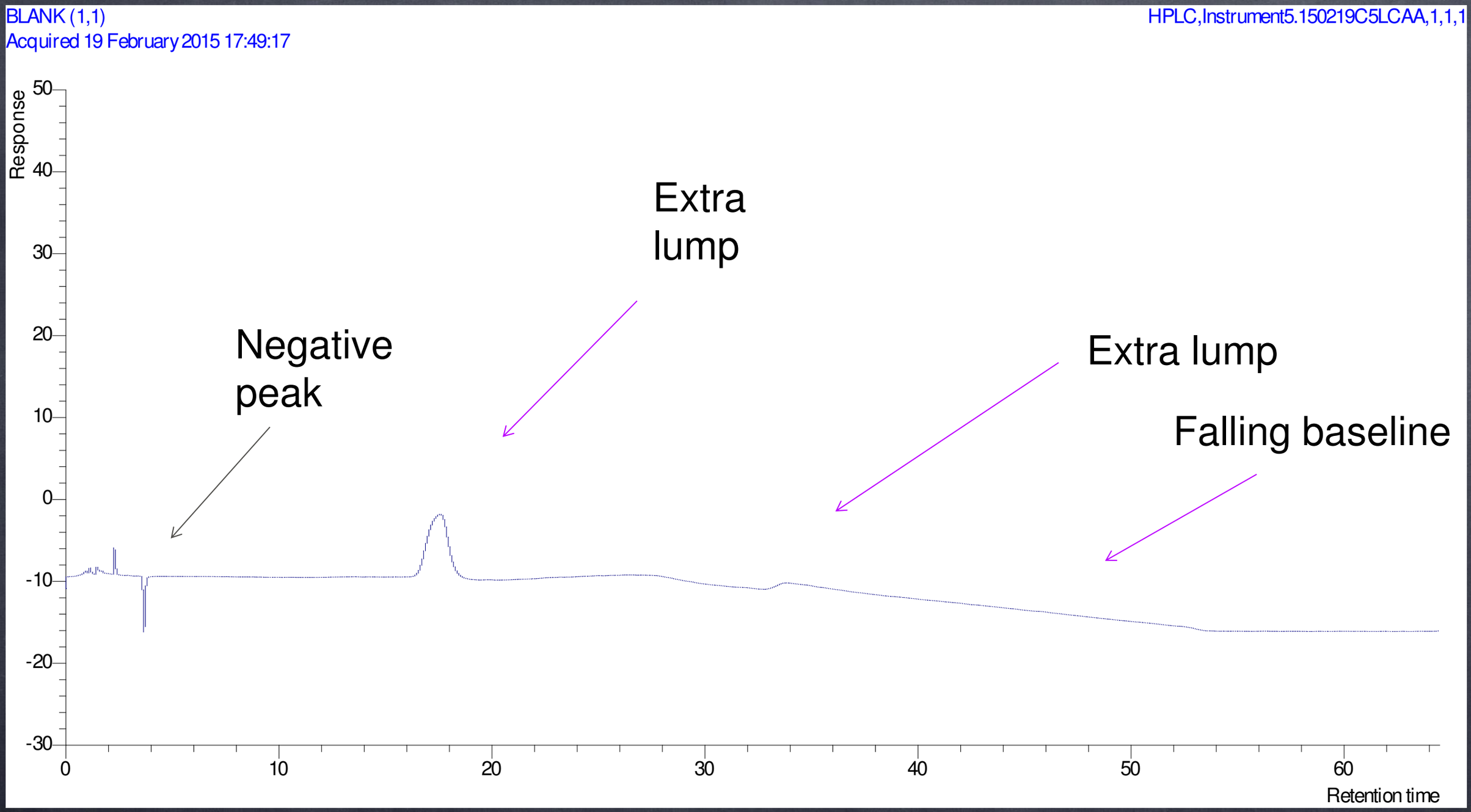
Alanine and Threonine peak higher than usual

? Contamination, Check blank

Sloping baseline

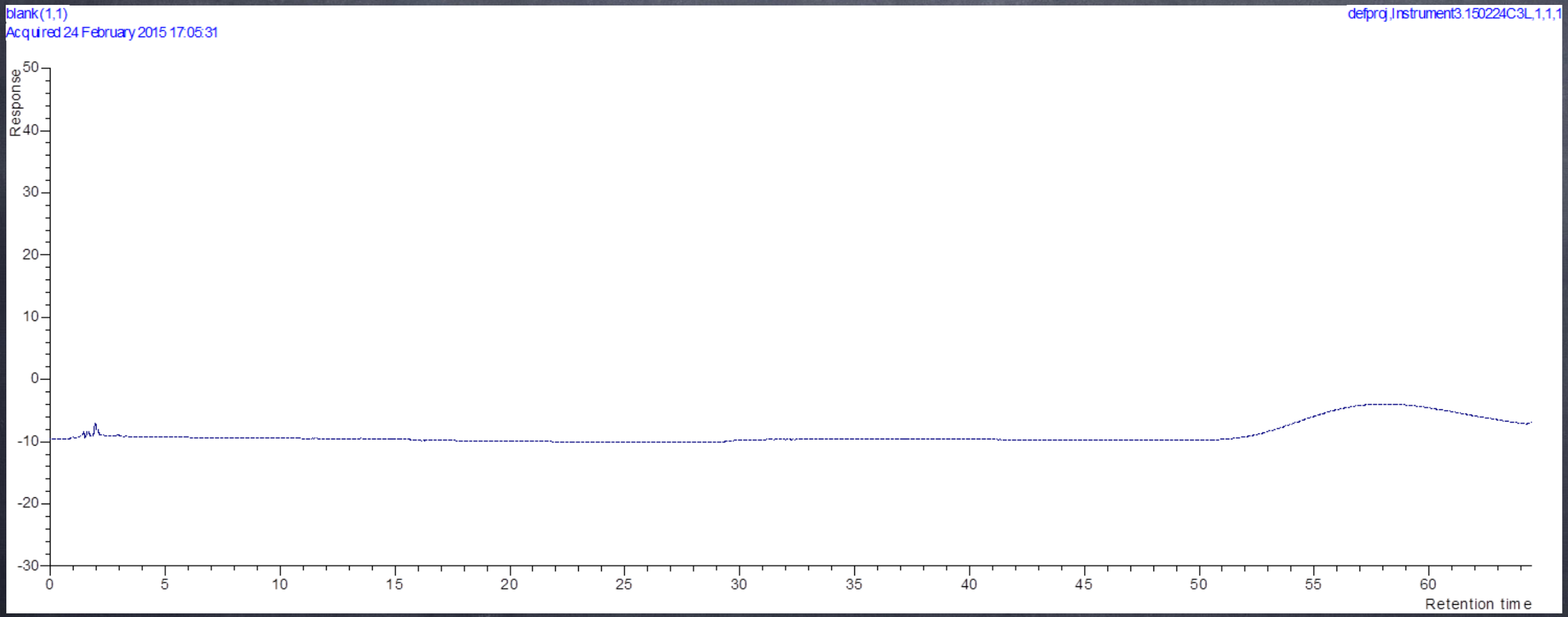


Blank on working system , flat straight baseline



Problem contamination in system ? Buffer A
? Buffer B, Needle wash, poor house keeping ,Millipore water system

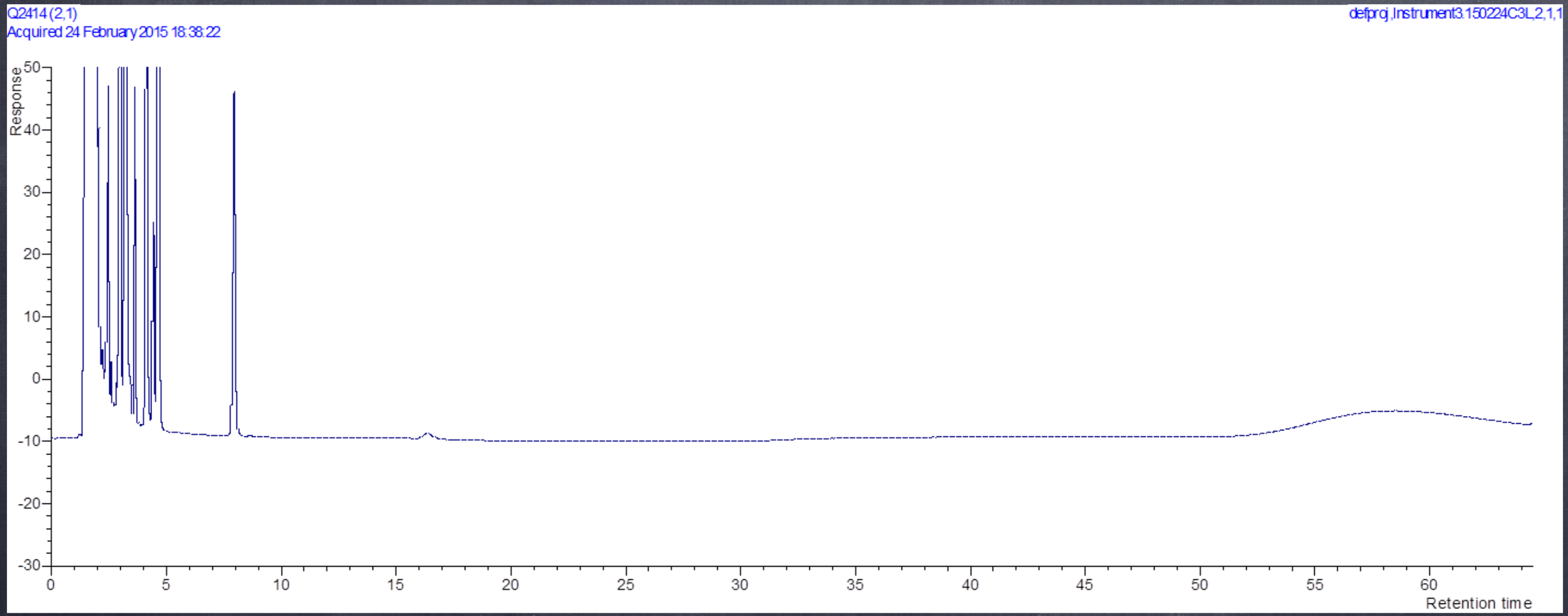
On this occasion check blank



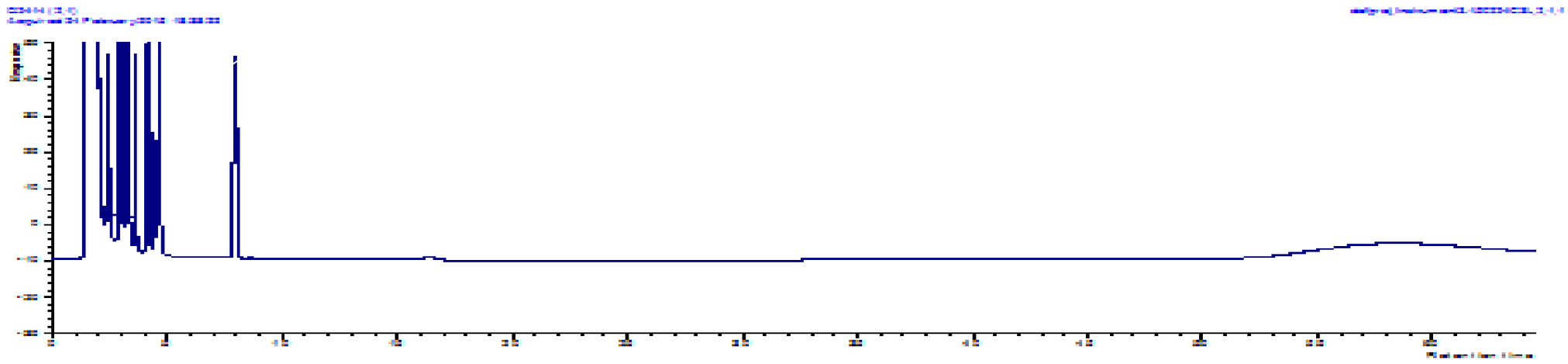
Blank sample run with HPLC gradient – not straight as slide ?

Check samples

Amino acid QC sample / where are peaks?



Phase organic buffer, wash sample off column

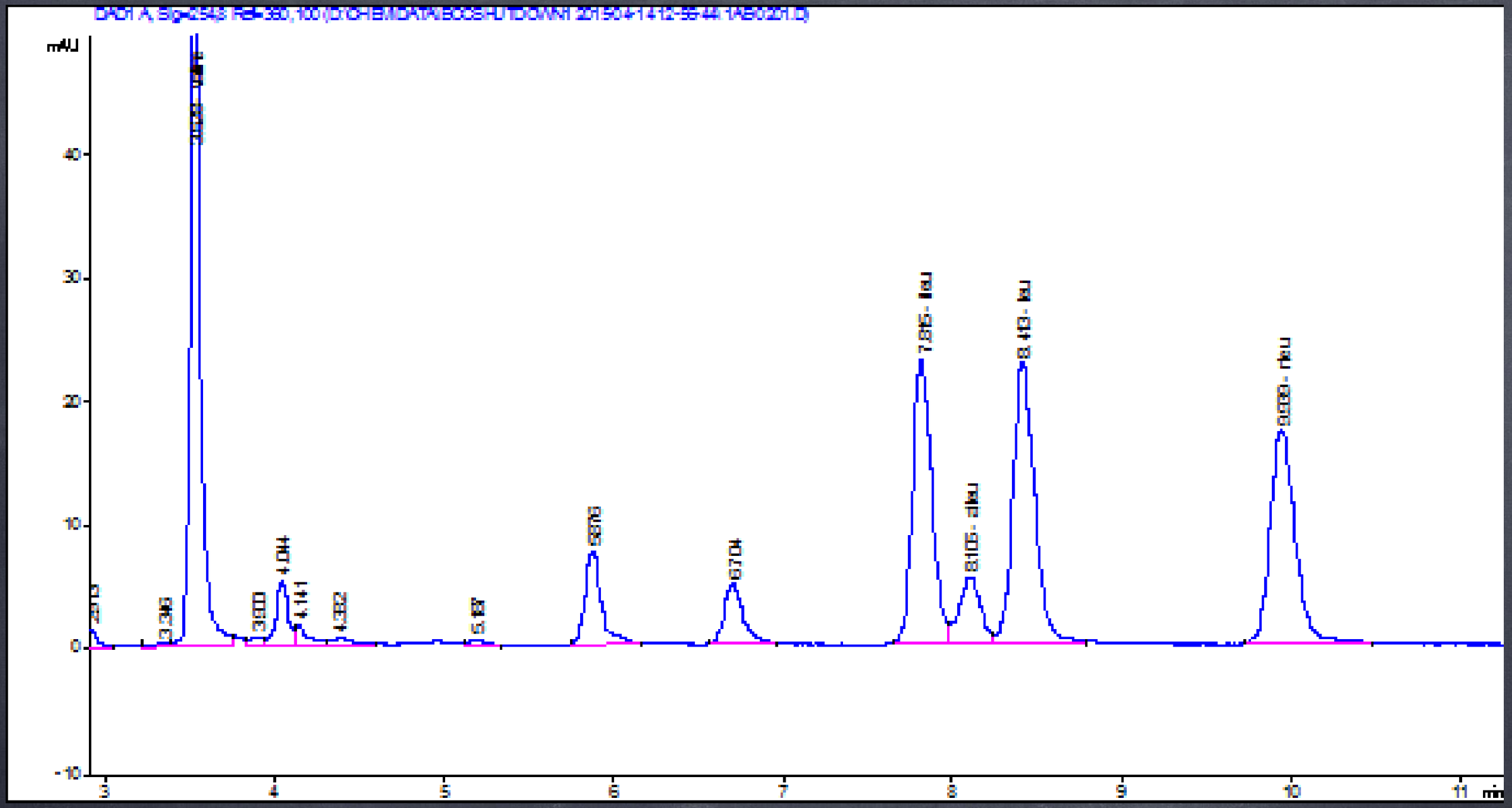


Troubleshooting

Has anything change ?

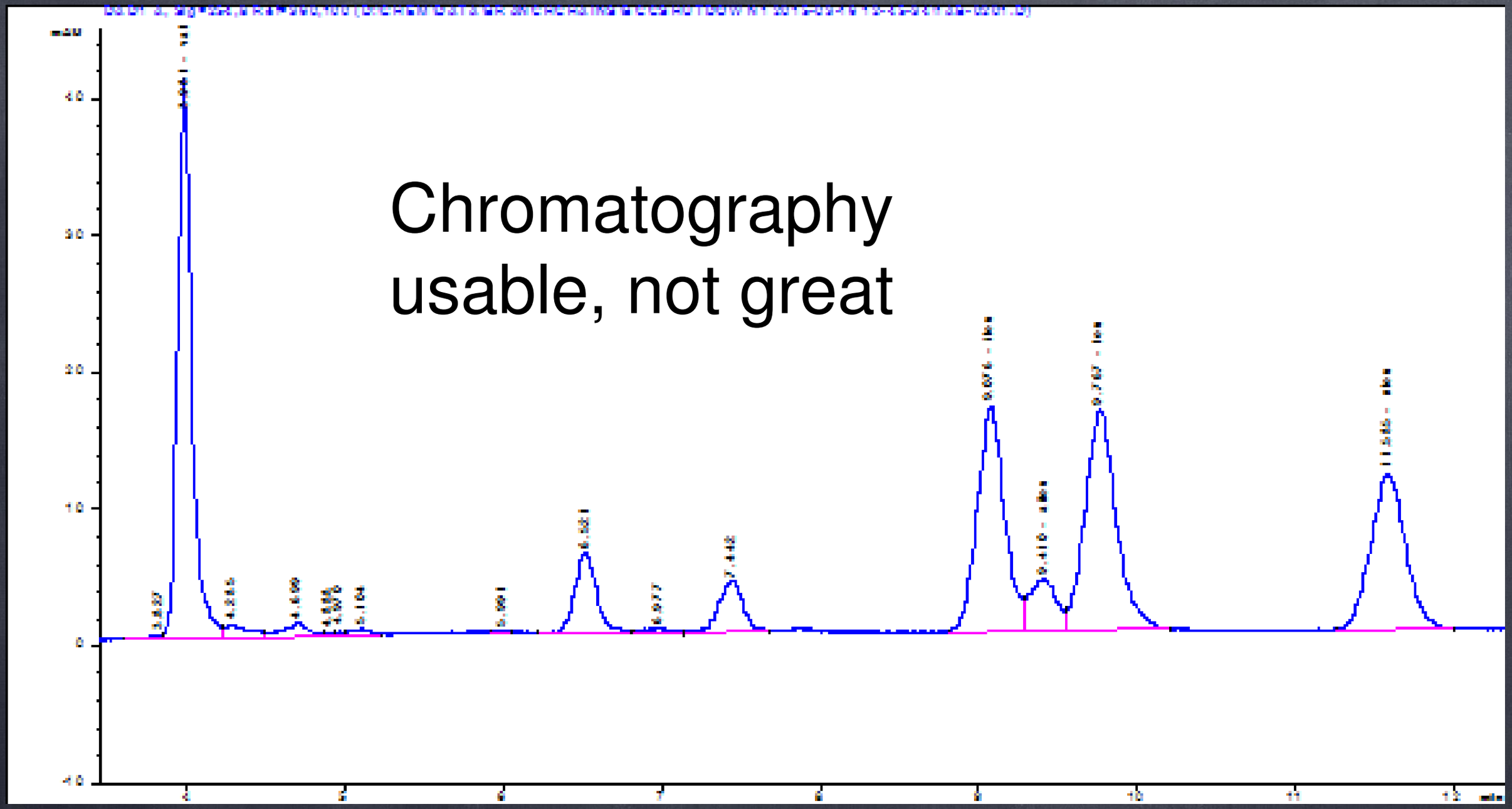
- Initial conditions 100% buffer A psi usually 1800 psi, on this run 1600 psi
- Buffers changed on day of run
- Other machine chromatography fine with same buffer
- Therefore Problem with that particular HPLC Machine ?
- Possible cause ?

- Answer
- Buffer A and buffer B line switched

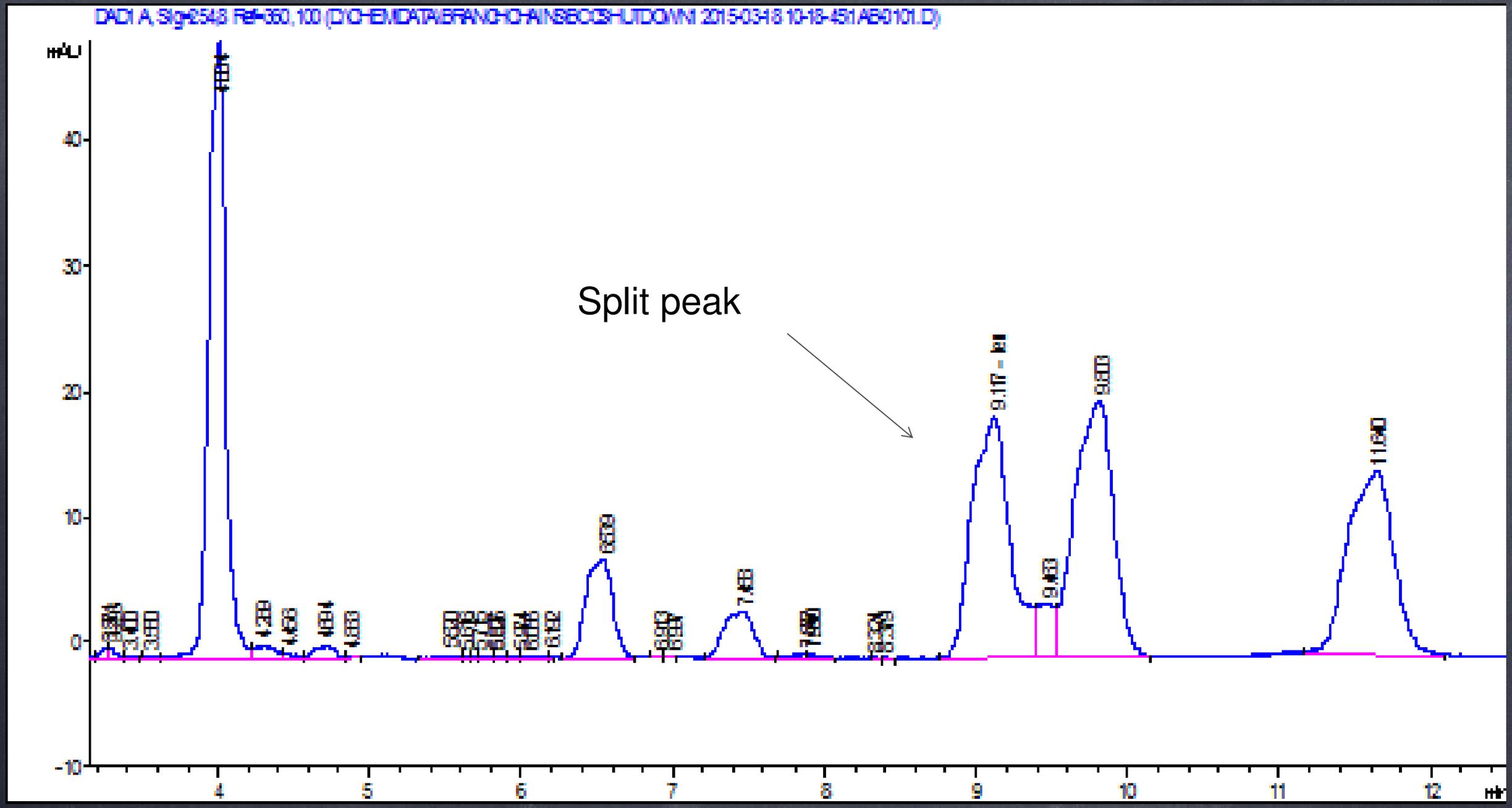


A = 79 %
B = 21 %
COLUMN = POROSHELL EC-C18 2.7µm, 2.1 x 150mm
BLOOD SPOT STD

UHPLC MSUD Method



A = 79 %
B = 21 %
COLUMN = POROSHELL EC-C18 2.7µm, 2.1 x 150mm
BLOOD SPOT STD



Poor chromatography ? split peaks why ? High pressure

Answer

Several problems, poor fitting connections= void volume, dirty frit ,column aged

Change column !

Prevention is better than cure!