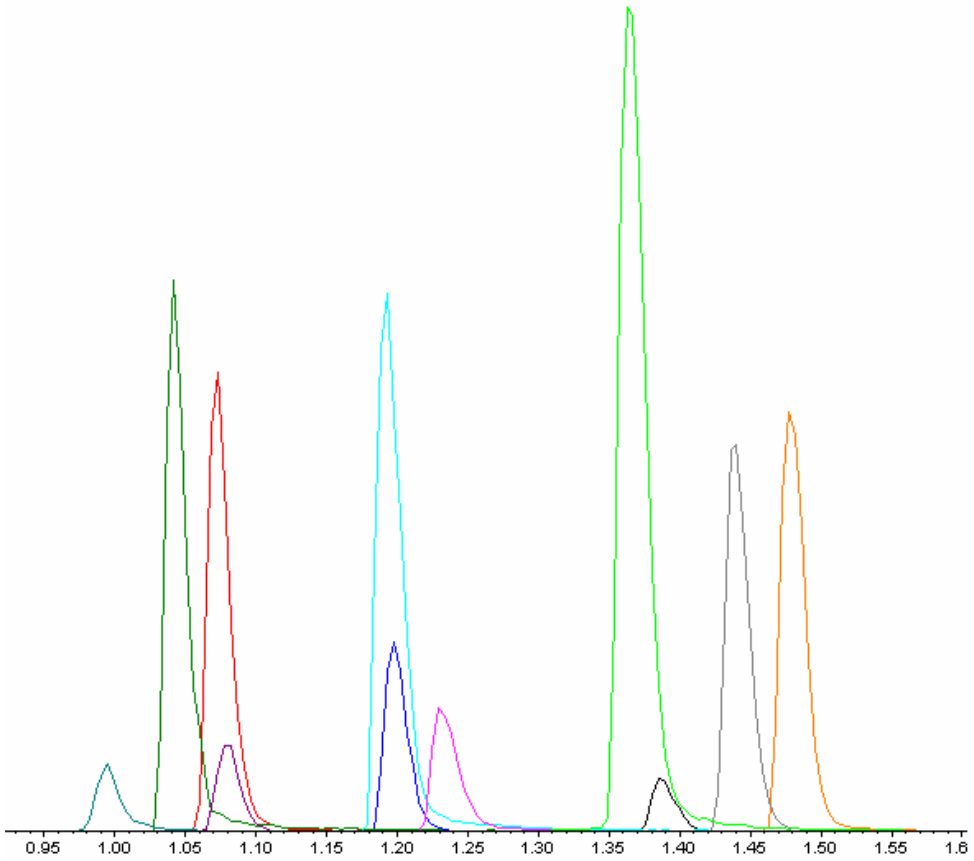
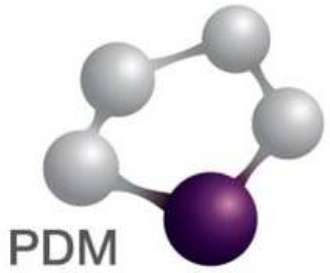
Improved Sensitivity, Selectivity and Robustness through Trace Enrichment on UHPLC-MS/MS

Daniela Fraier

Bioanalytical Group - Pharmacokinetics, Dynamics & Metabolism



Content

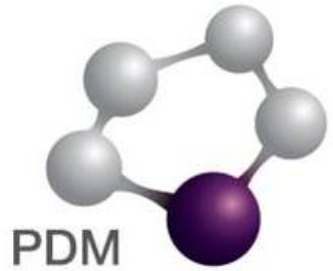


- Background
  - Bioanalysis at Pfizer
- Opti-Trap EXP™* evaluation
  - System setup
  - Applications
    - Dry Blood Spot (DBS)
    - Brain µ-Dialysis
    - Small peptide analysis
  - Robustness
- Conclusions

* U.S. and Foreign Patents Pending

Pfizer

Bioanalysis at Pfizer



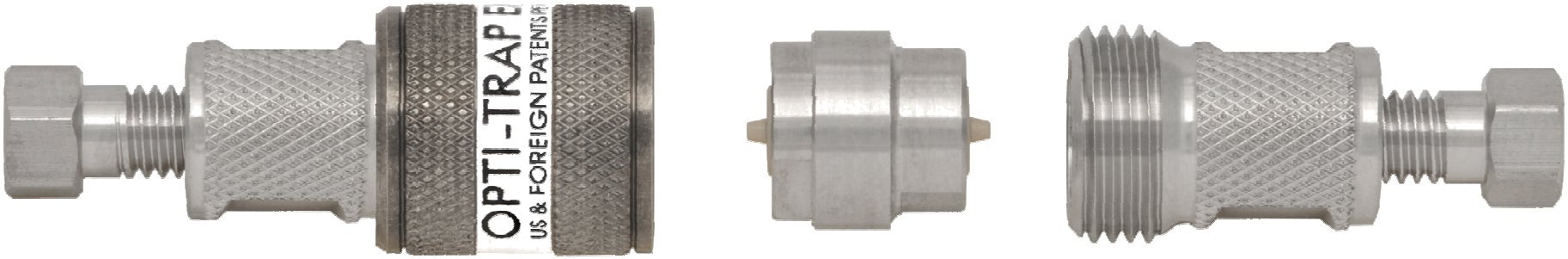
- Supplies the bioanalytical solutions essential to bring compounds from early discovery through candidate nomination (CAN) & clinical phase.

- Developed a rational approach to deal with the physicochemical diversity of a large number of compounds and a wide range of biological matrices (e.g. plasma, blood, urine, tissues, etc.).

- Harness the synergies between general bioanalysis expertise and biomarker specialist knowledge to solve quantitation issues associated with challenging compound chemistries.

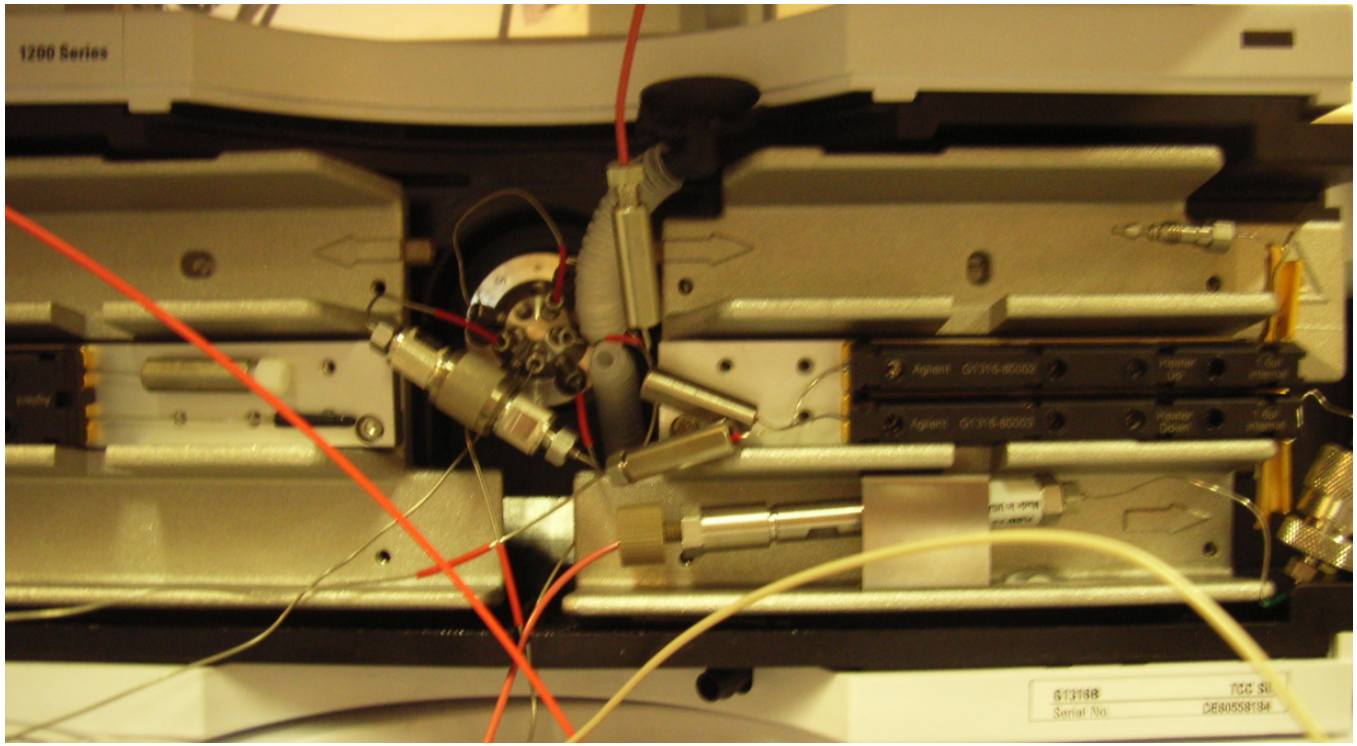
- Evaluate and implements novel analytical technologies and methodologies.

Opti-Trap EXP™ Evaluation



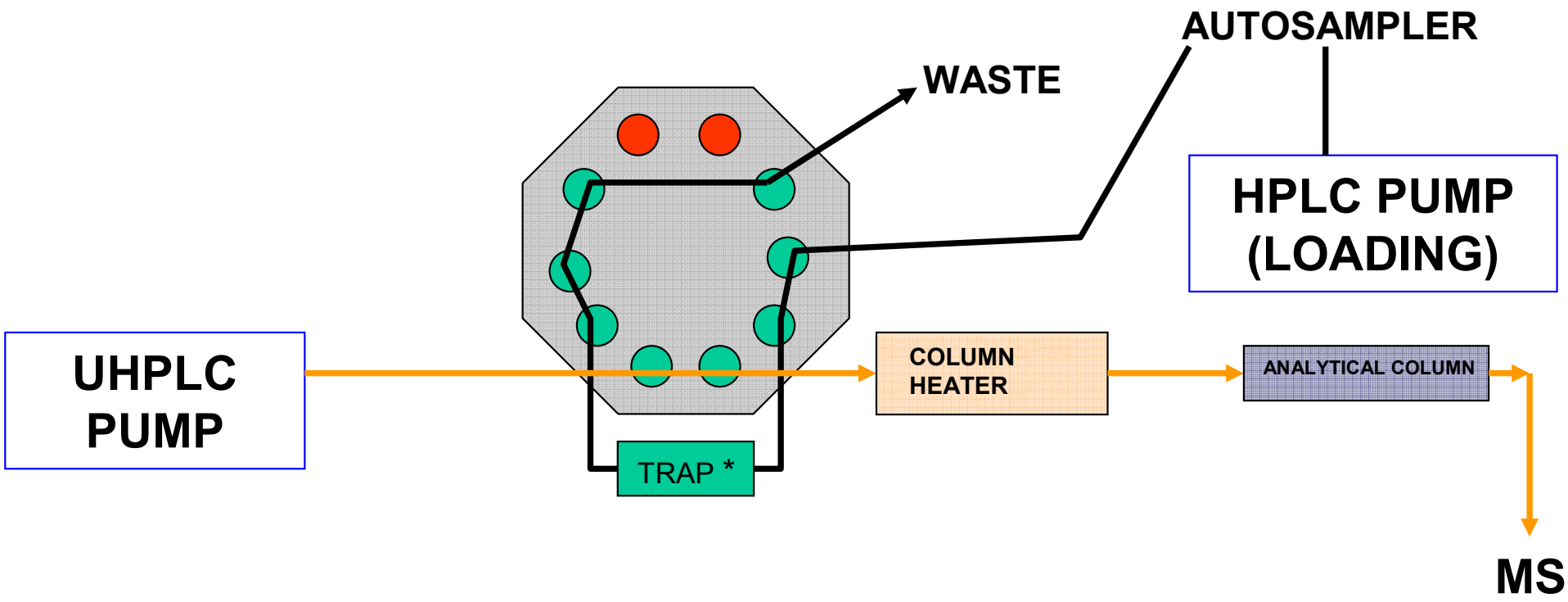
(U.S. and Foreign Patents Pending)

System Setup



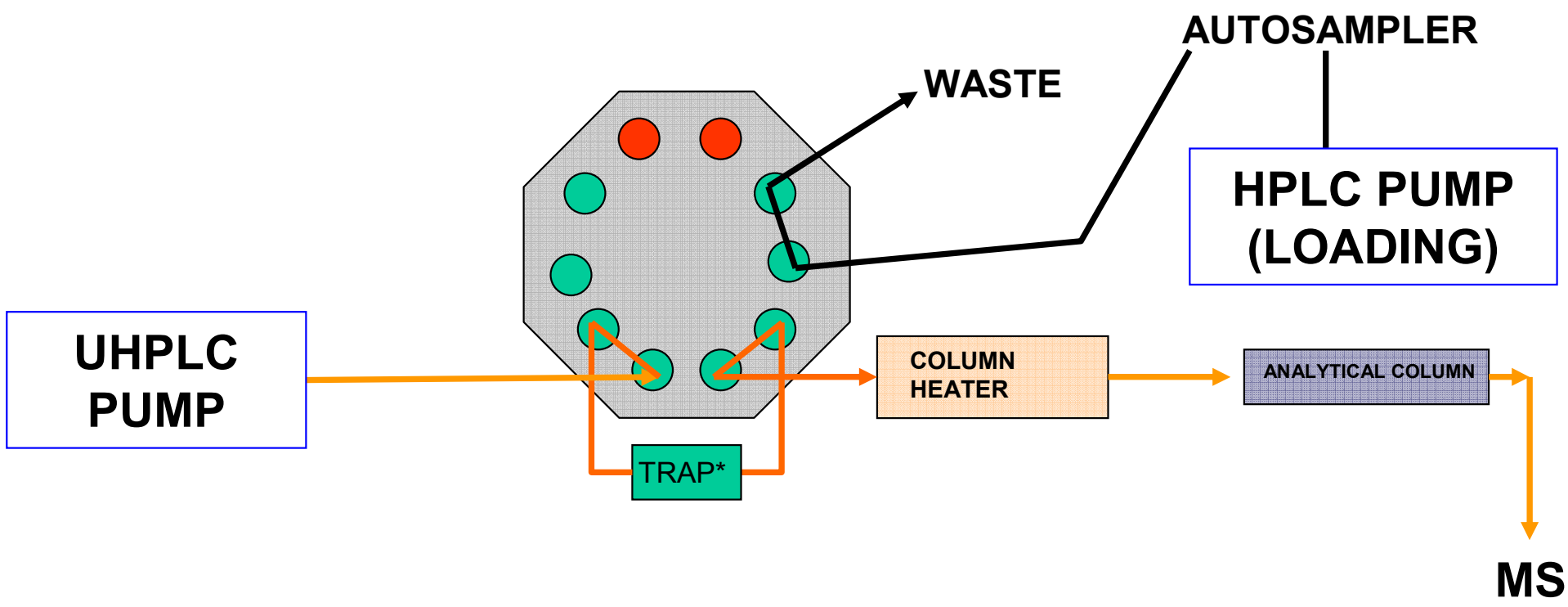
- The Opti-Trap EXP™ was installed in the integrated column selection valve of the Agilent 1200RR thermostated column compartment

System Setup: 10-Port Valve (Load)



* Opti-Trap EXP™ (U.S. and Foreign Patents Pending)
* Halo™ C18, 4.6 x 5mm (100µL bed volume)
* Designed to work with UHPLC up to 15,000 psi

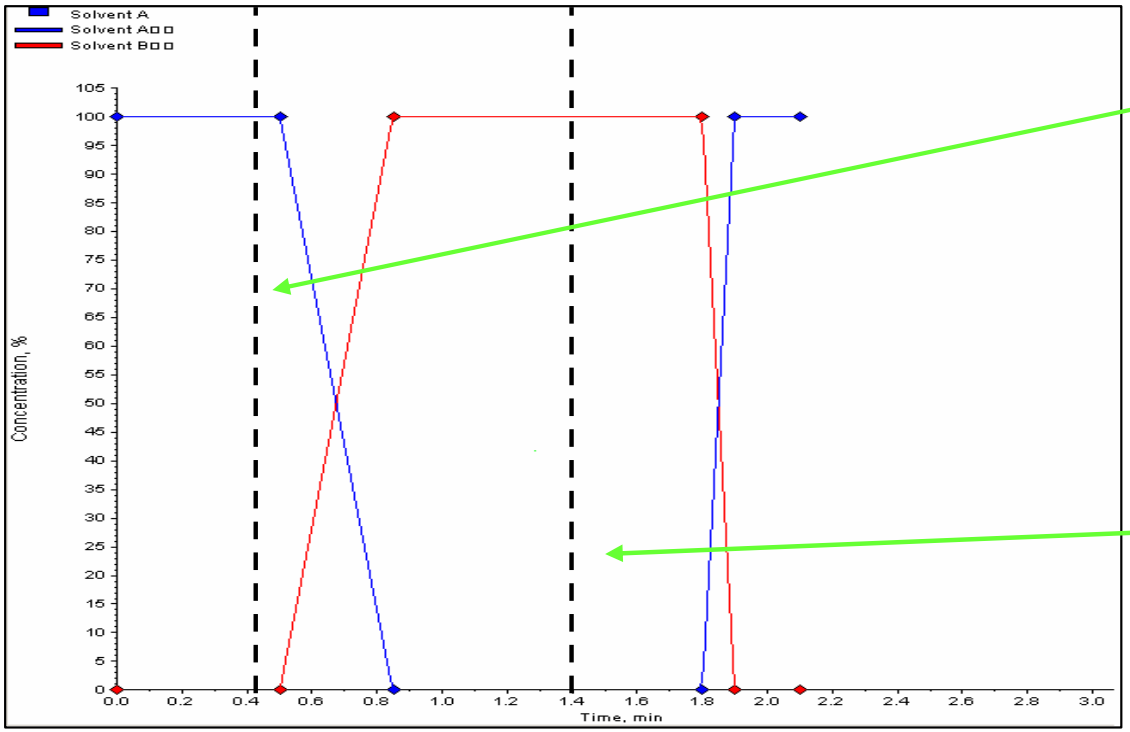
System Setup: 10-Port Valve (Elute)



* Opti-Trap EXP™ (U.S. and Foreign Patents Pending)

System Design

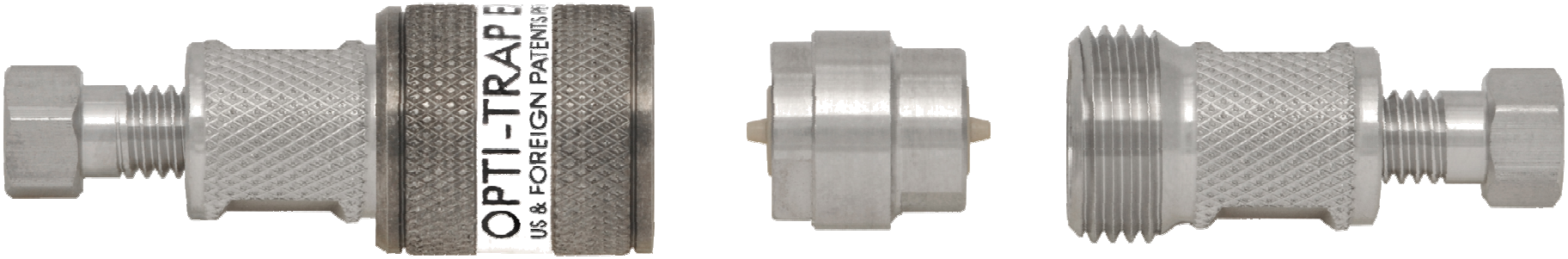
- Trapping column loaded in aqueous mobile phase and back flushed onto an Eclipse XDB-C18 3 x 50 mm,1.8µ column.
- “Generic” rapid gradient from 10% methanol (mobile phase A) to 90 % (mobile Phase B) run through the analytical column to elute the analytes of interest.



Trap in line with analytical column

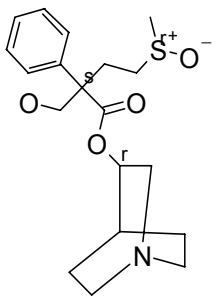
Trap diverted to waste

Applications



(U.S. and Foreign Patents Pending)

DBS: The Test Mixture

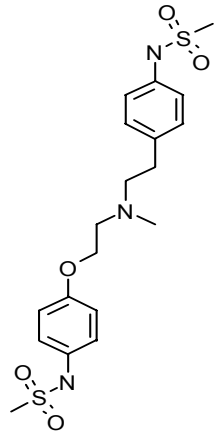


UK-112166

pKa 8.6/4.9

cLogP -0.05

Mw 365

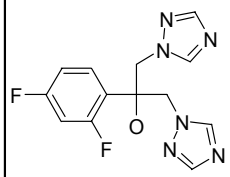


Dofetilide

pKa 7 / 9.2

cLogP 1.56

Mw 441

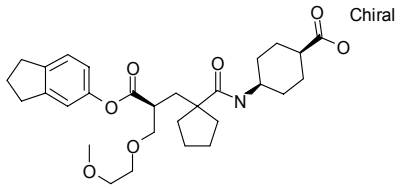


Fluconazole

pKa 4.5 / 5.3

cLogP -0.11

Mw 306

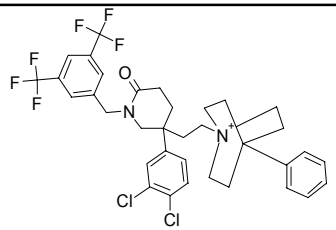


Candoxatril

pKa 4.5

cLogP 2.94

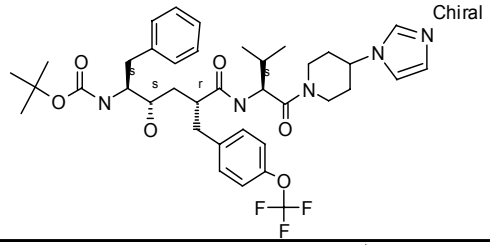
Mw 515



UK-258300

pKa NA, Mw 682

cLogP 4.2

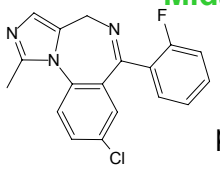


UK-141495

pKa 6.1

cLogP 3.94

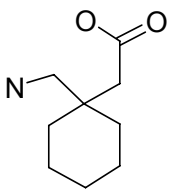
Mw 730



Midazolam

pKa 5.59

cLogP 3.93, Mw 325

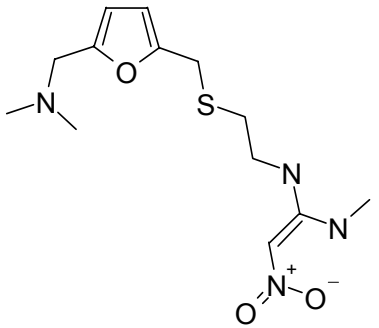


Gabapentin

pKa 3.7

cLogP 1.19

Mw 171

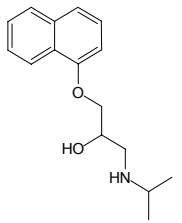


Ranitidine

pKa 8.18/2.28

cLogP 1.28

Mw 314

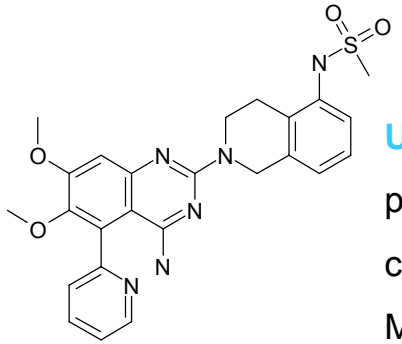


Propanolol

pKa 13.84/9.14

cLogP 3.1

Mw 259



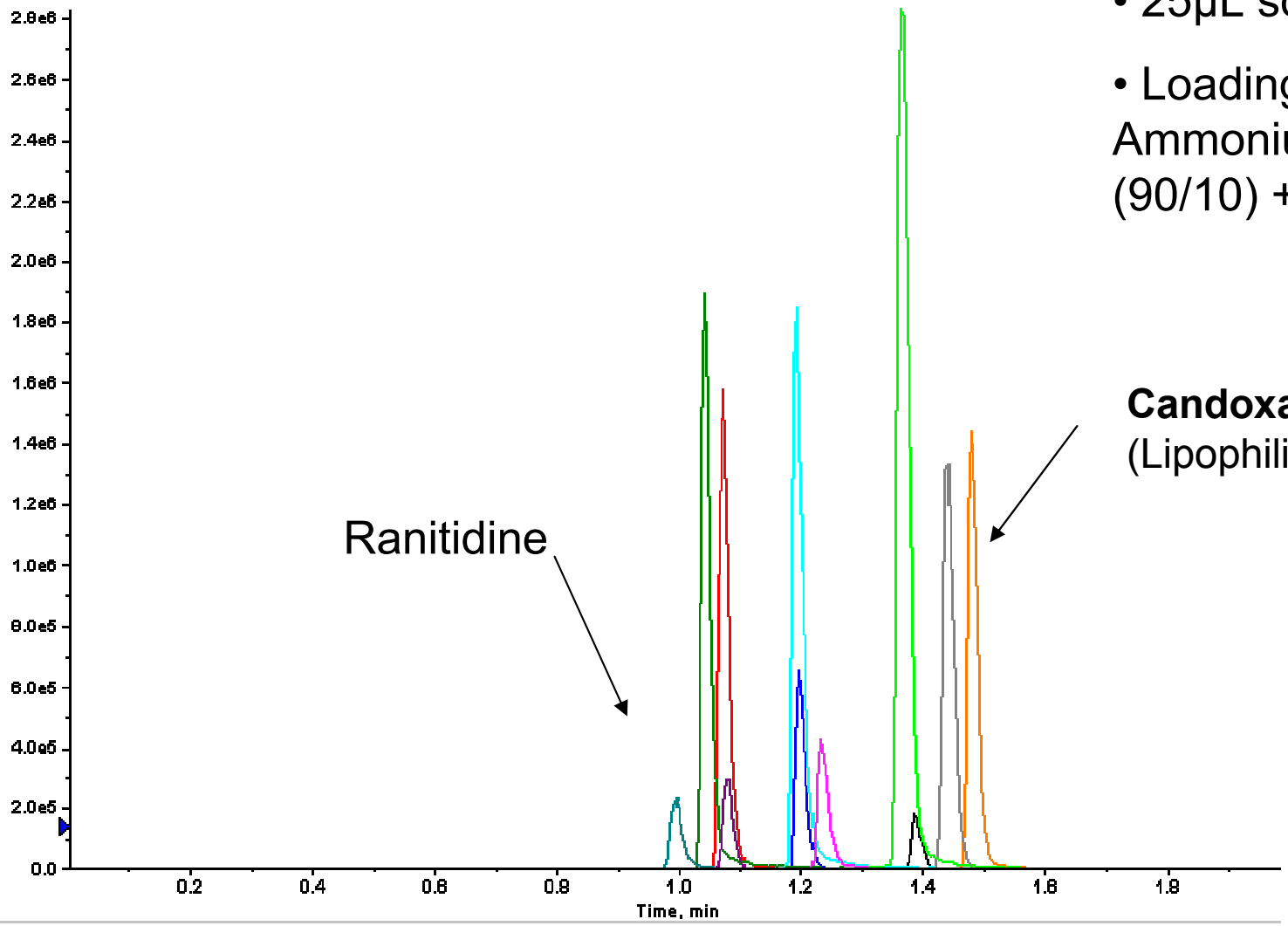
UK-338003

pKa 10.32/9.29

cLogP 0.69

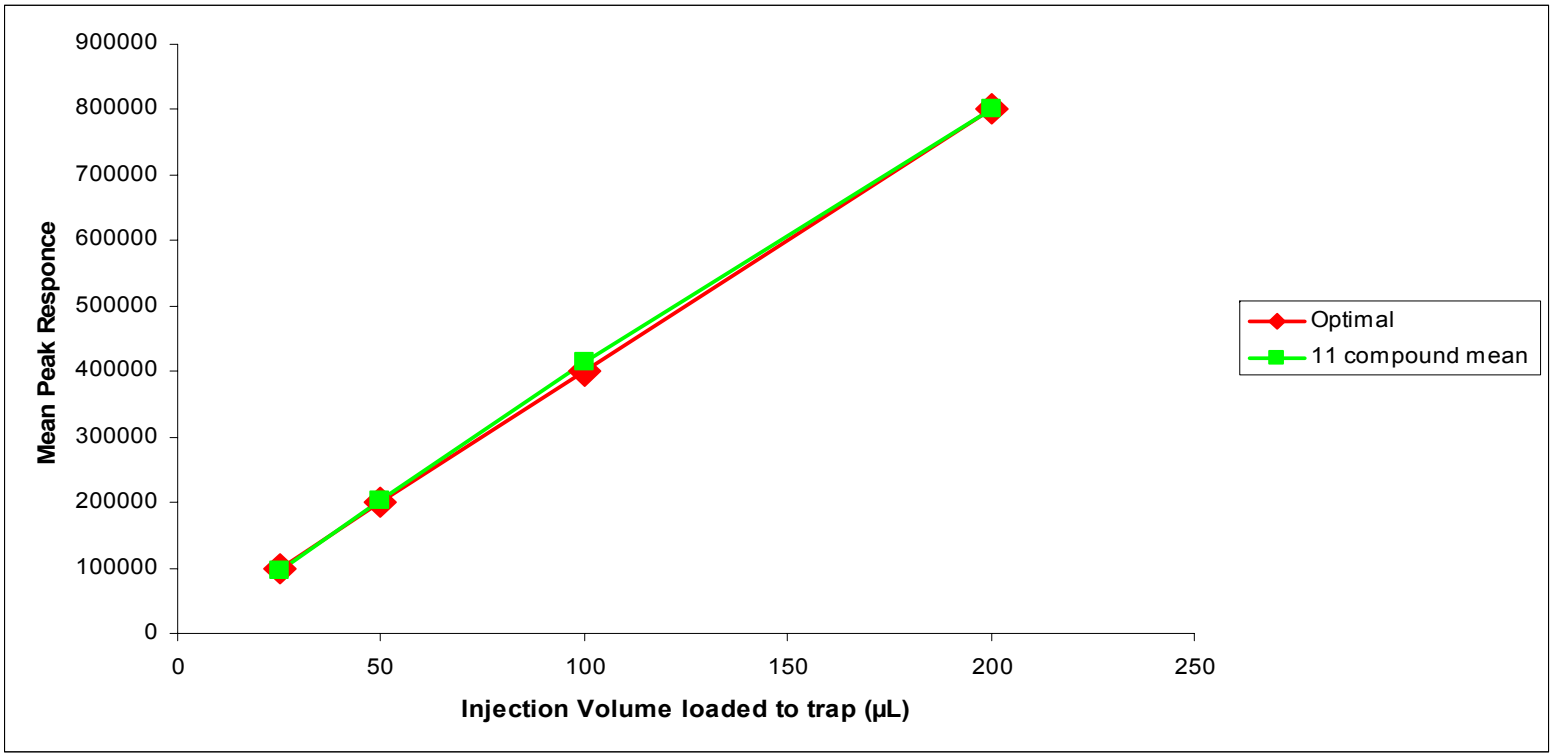
Mw 506

DBS: Example Chromatogram



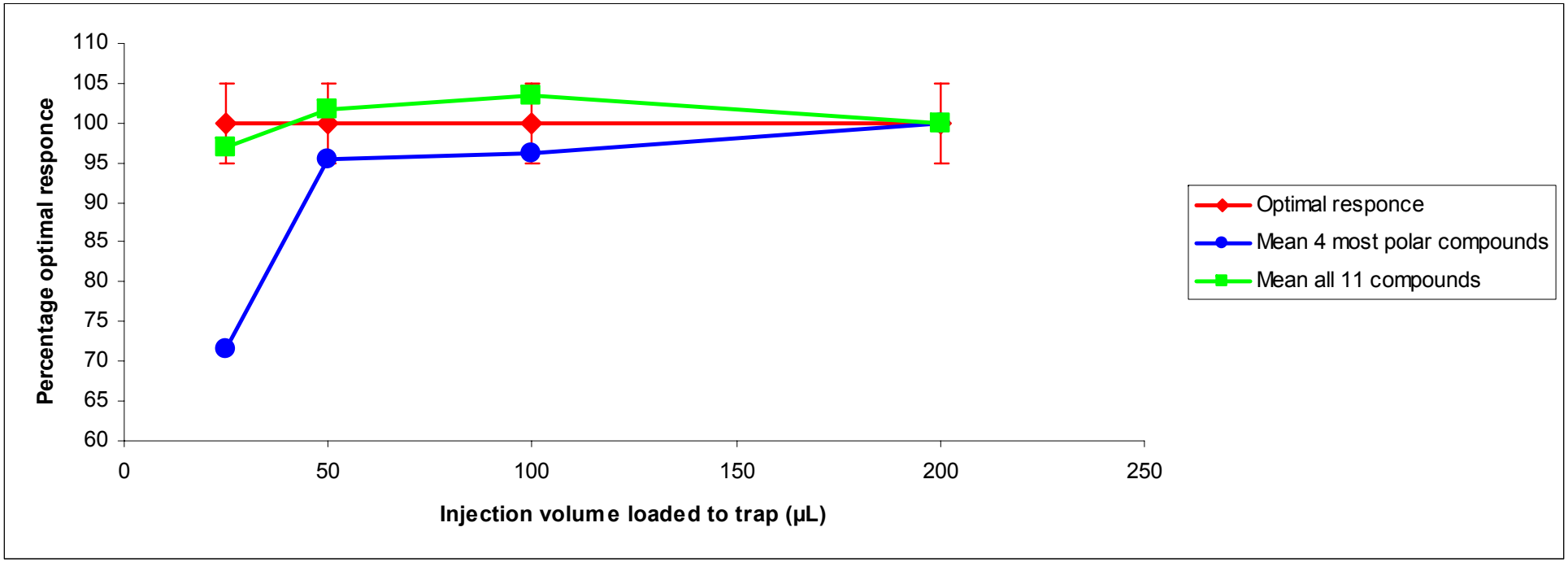
- 25µL solution injection
- Loading Mobile Phase: 2mM Ammonium Acetate/MeOH (90/10) + 0.1% TFA

DBS: Opti-Trap EXP™ Loading Capacity



- Enables larger injection volumes than conventional setup (linear increase in sensitivity up to 200µL injected).
- Reconstitution solvent and loading mobile phase can be optimised for retention purposes.

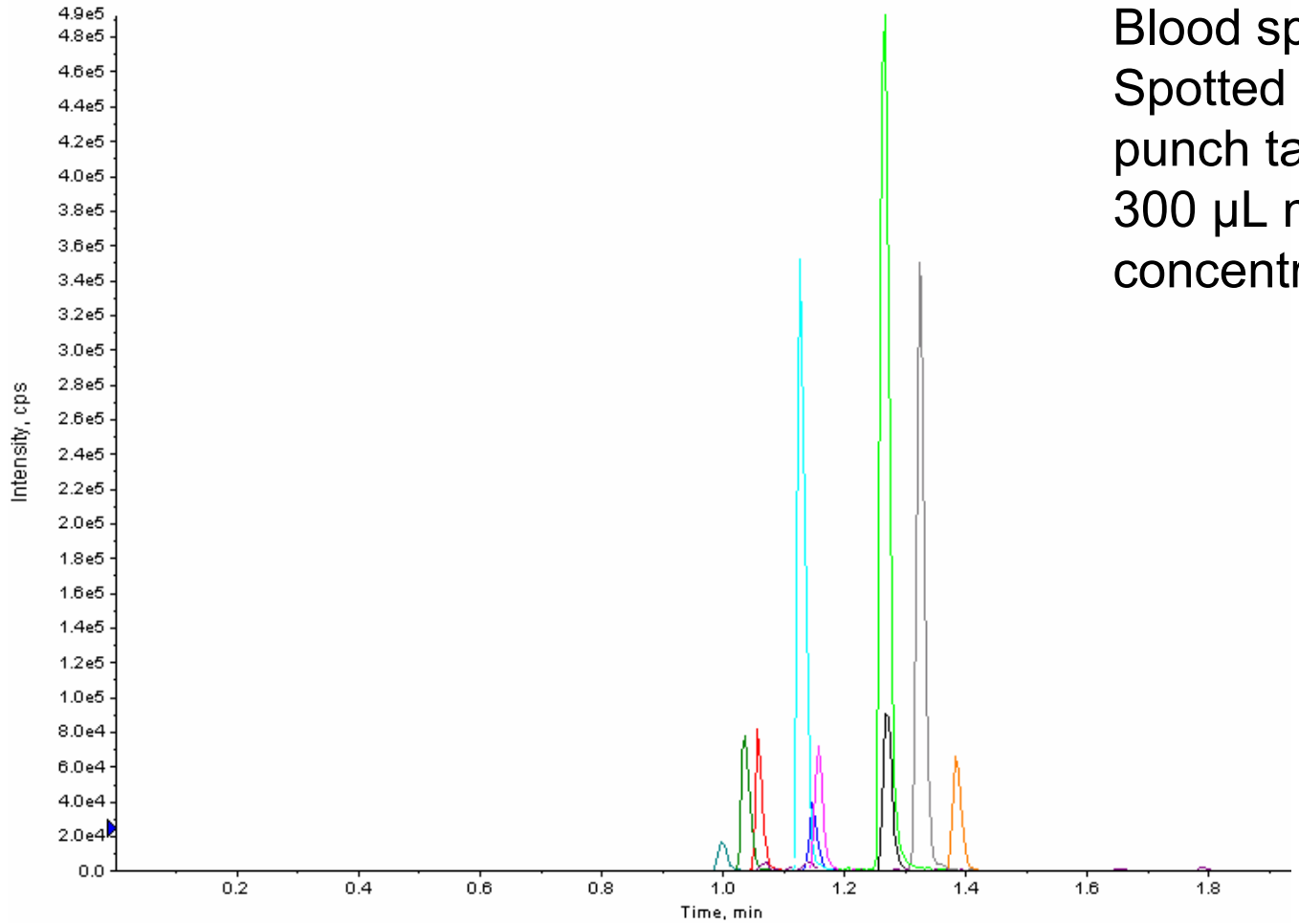
DBS: Polar Compounds



- Data was plotted based on the response as a ”% optimal response”.
- The very polar compounds gave an exponential increase of response with increased loading volume rather than a linear increase.
- Overall, polar compounds displayed better sensitivity with larger load volumes.

DBS: Sample Extract

Good peak shape and retention



Blood spiked at 100 ng/mL. Spotted to dry, 3mm (~2.8 µL) punch taken and extracted into 300 µL methanol. Final sample concentration ~ 1ng/mL.

DBS: Matrix Effects

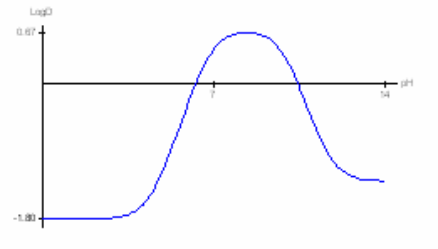
Matrix effect (% recovery)

	Flucon	UK-112166	Dofetalide	Candoxatril	UK258300	UK-141495	Ranitidine	Gabapentin	UK338003	Midazolam	Propranolol
DBS	77.5	111.5	105.9	79.9	85.5	86.4	99.2	105.2	66.5	87.7	58.3
DBS Paper	79.8	112.6	108.5	89.5	92.5	86.2	99.4	105.8	64.8	89.9	58.5
Blood PPT	72.7	95.9	91.0	85.4	143.5	92.4	93.1	50.8	75.9	82.3	75.5

- Matrix effect was looked at for all the compounds in the DBS extraction mix.
- Matrix effects from the blood on the DBS card, the card itself and PPT fresh blood (25µL blood with 275µL Methanol) were investigated.
- Most of the compounds gave a matrix effect that was no greater than usually reported experimental errors (±20%).
- Those compounds giving greater matrix effects were still not unacceptably large.

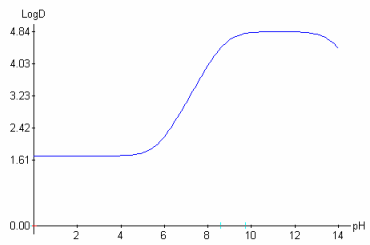
Brain μ-Dialysis:
In vitro Recovery of "Sticky CPDs"

Compound A



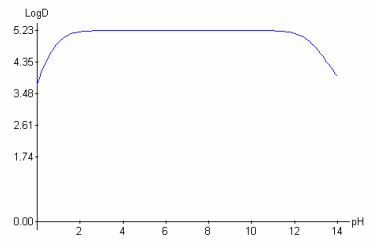
Mw 353.4
cLogP 0.7
pKA 9.99/6.89

Compound B



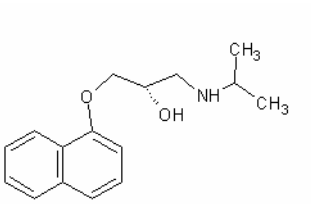
Mw 488.3
cLogP 4.84
pKA 13.97/9.08

Compound C



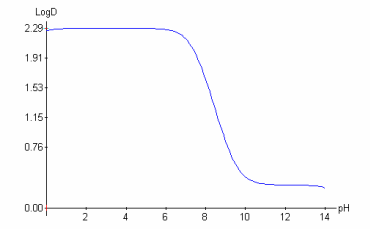
Mw 413.5
cLogP 5.29
pKA 12.77/0.52

Propanolol (IS)



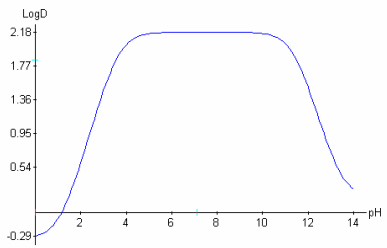
Mw 259.3
cLogP 2.9
pKA 12.84/9.5

Compound D



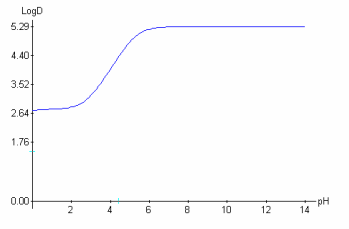
Mw 476.5
cLogP 2.29
pKA 14.92/7.42

Compound E

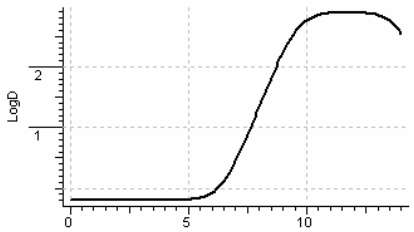


Mw 357.4
cLogP 2.18
pKA 11.46/3.66

Compound F

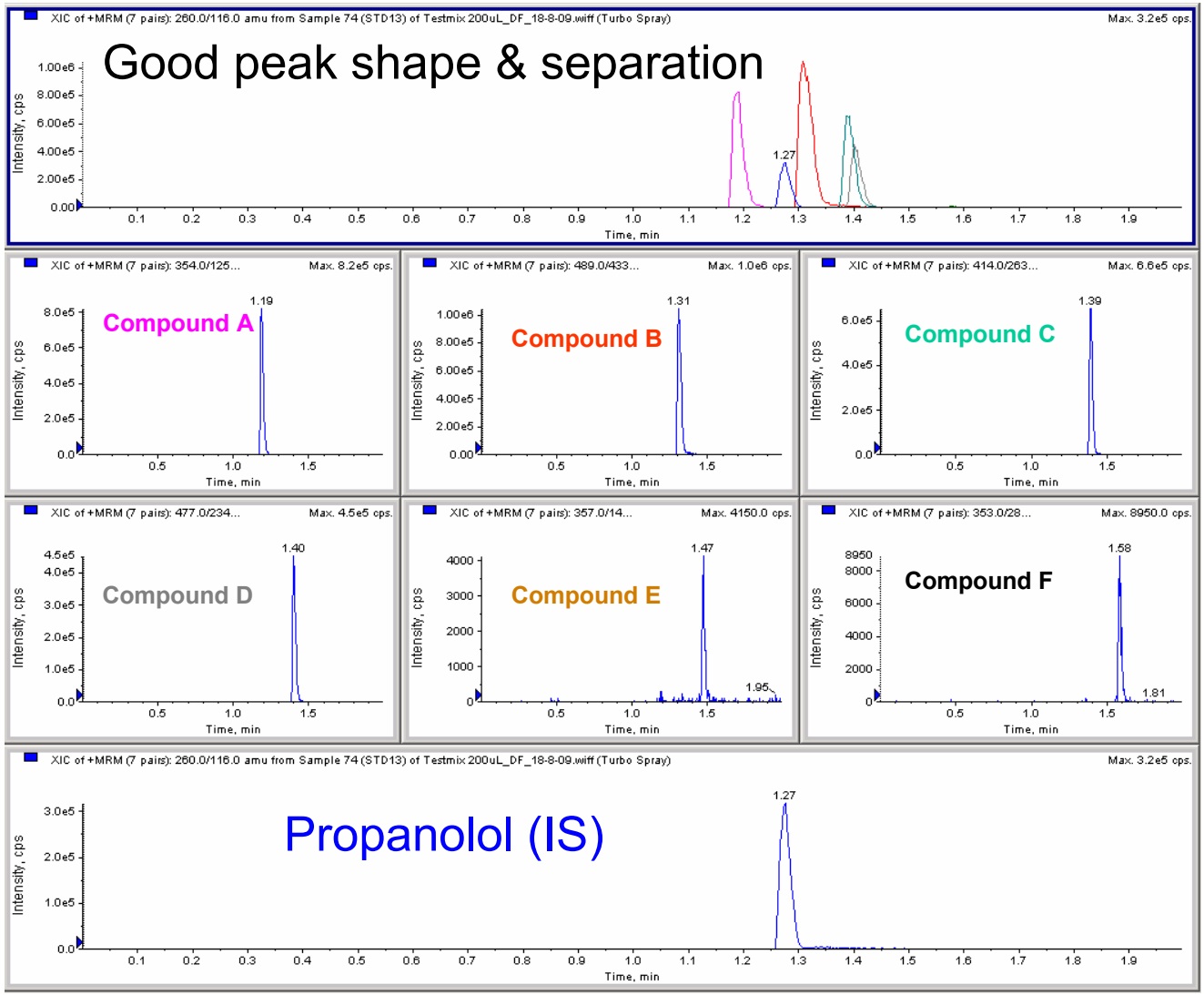


Mw 352
cLogP 5.29
pKA 4.85



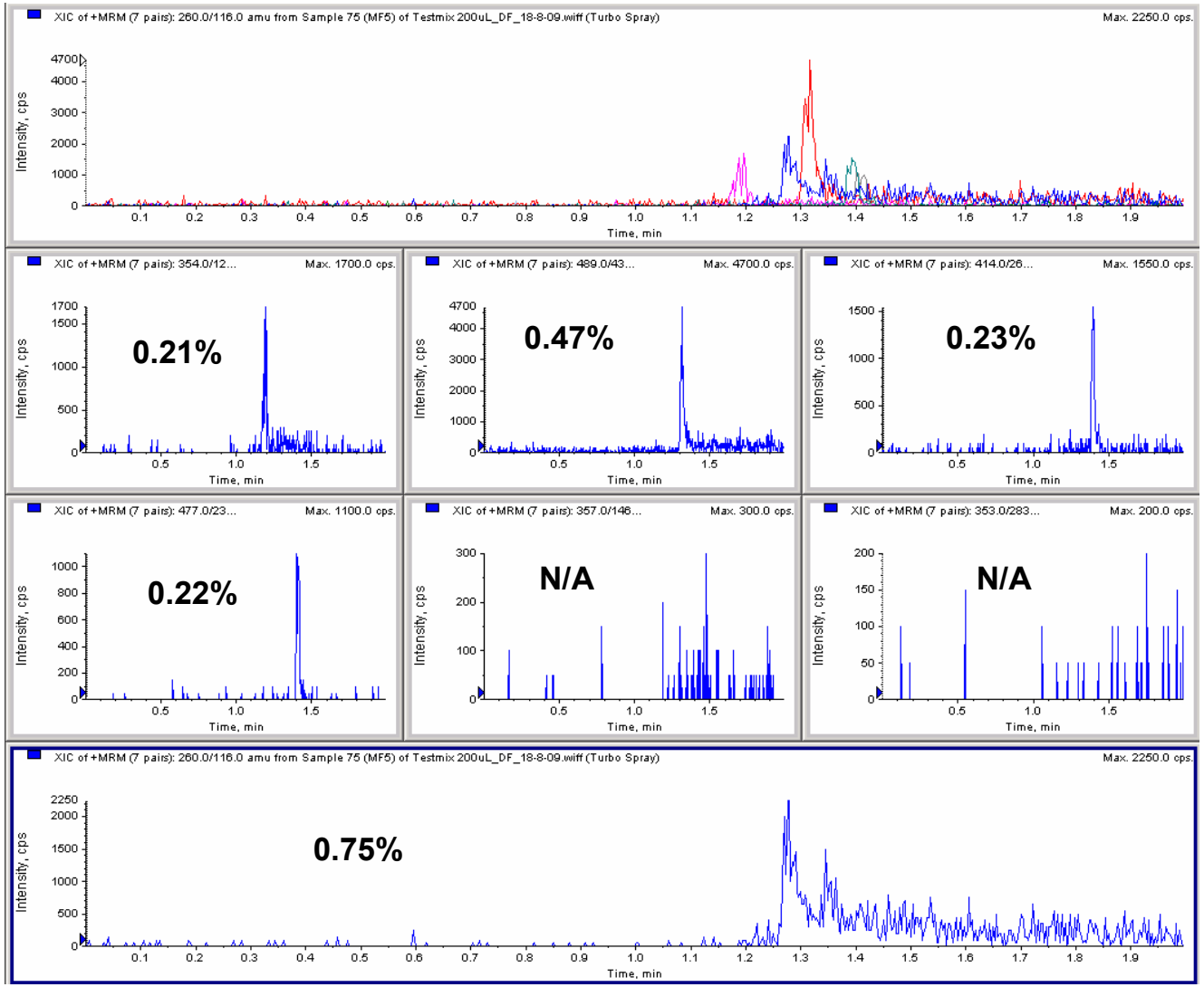
Brain µ-Dialysis: Example Chromatogram

- 5 µL µ-dialysis sample (CSF) diluted with 200 µL reconstitution solvent.
- 100 µL injected onto the system.



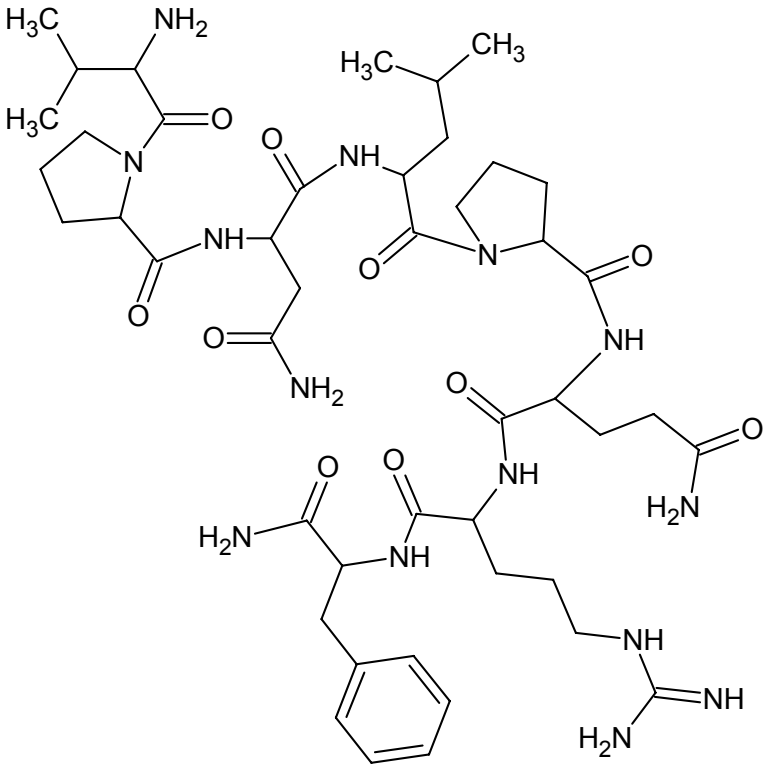
Brain μ-Dialysis: System Carry-over

- Blank matrix injection following top standard.
- Strong solvent wash flushed through the trap during previous sample data collection.



Small Peptide Assays (4-10 amino acids)

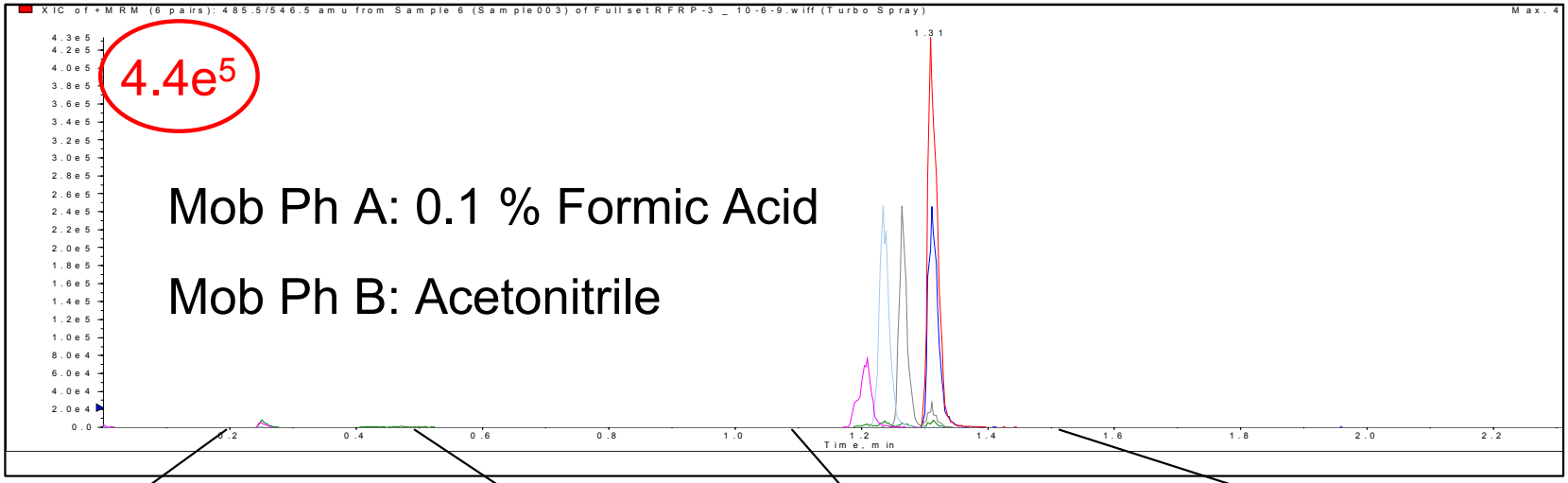
- In blood the octapeptide RFRP-3 (RFamide-related peptide-3) degrades to a variety of smaller peptides (the hexapeptide, the pentapeptide and the tetrapeptide).
- Assay requirement to simultaneously quantify the 8,6,5 and 4 amino acid peptides.
- High sensitivity (sub ng/mL) required due to low expected levels.

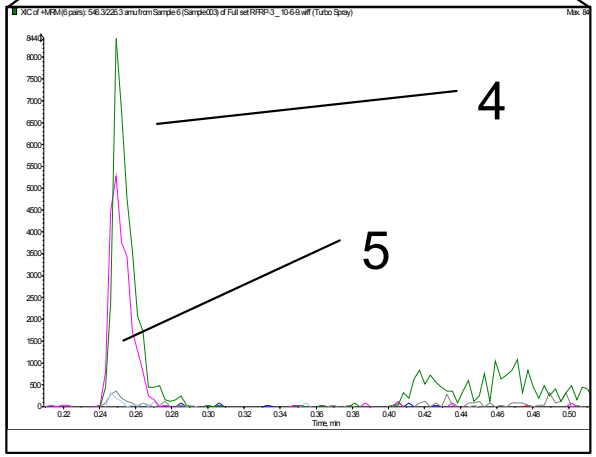


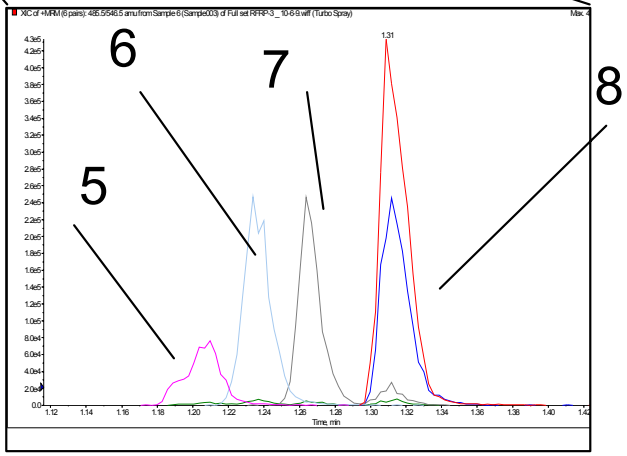
Standard UHPLC:

- The 8, 7 and 6 amino acid peptides well retained, 5 partially retained (with a % coming off at solvent front) and 4 not retained.
- Signal at $4.4e^5$ for RFRP-3 for 100 ng/mL solution.
- The best analytical conditions for RFRP-3 assay shown in next slide.

Small Peptide Assays: Standard UHPLC



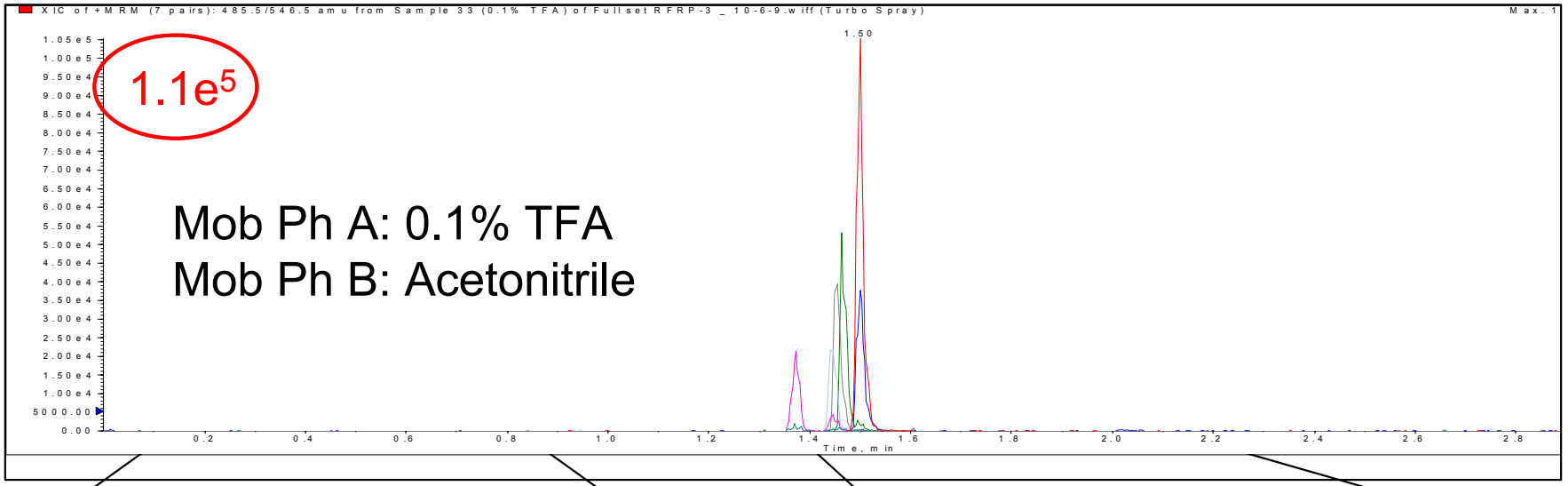


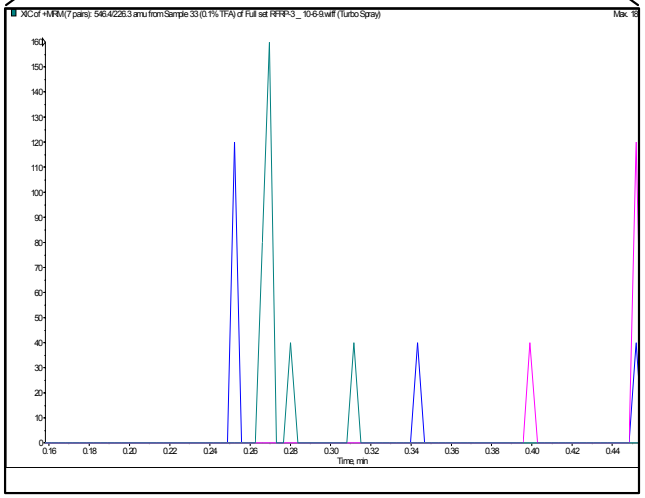


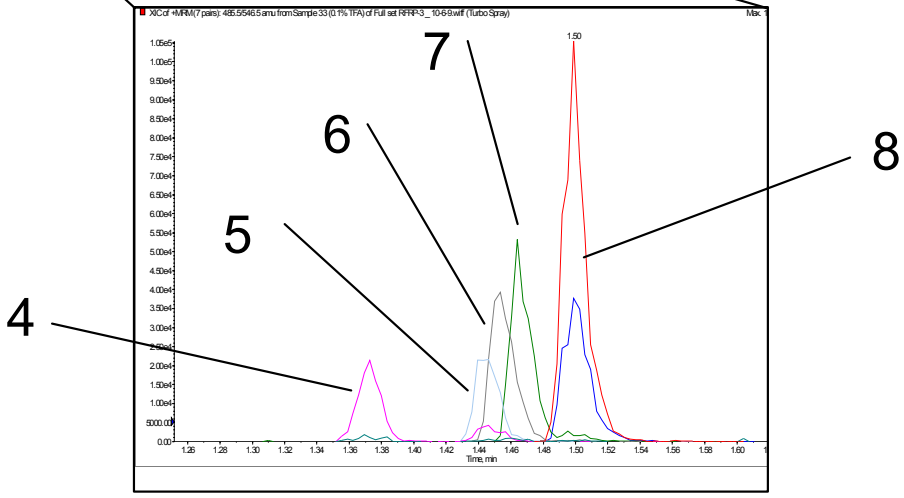
Small Peptide Assays: Ion Pairing

- Mobile phase altered to include 0.1% TFA to aid retention of the smaller peptides.
- The 8, 7, 6, 5 and 4 amino acid peptides all retained and separated.
- However large drop in signal intensity due to inclusion of TFA in mobile phase.
- Signal at 1.1 $e^{5.}$
- The best analytical conditions for RFRP-3 assay shown in next slide.

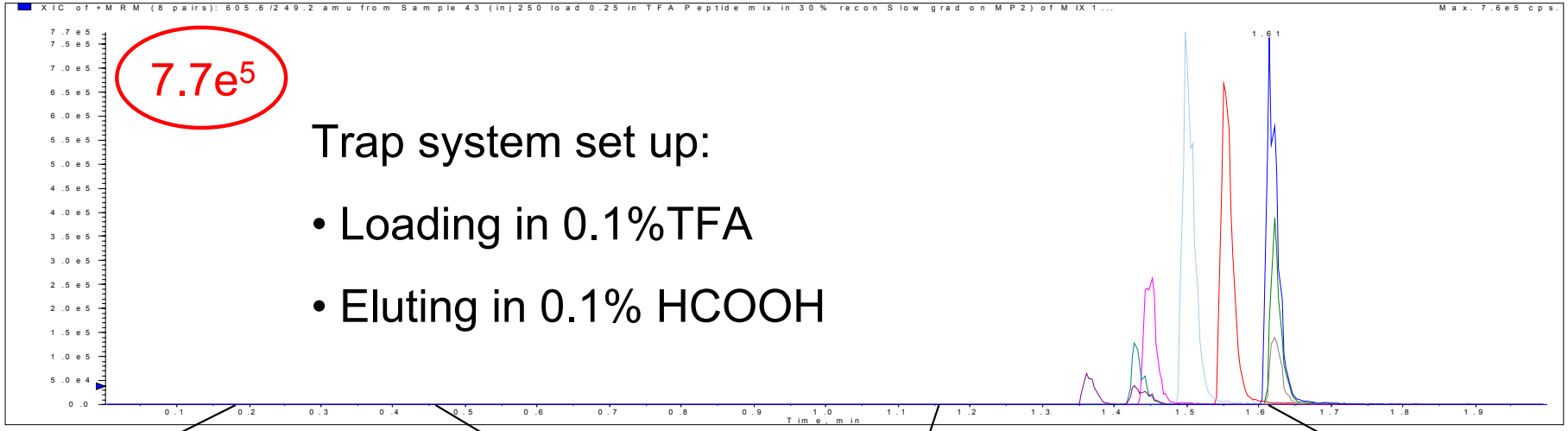
Small Peptide Assays: Ion Paired Standard UHPLC





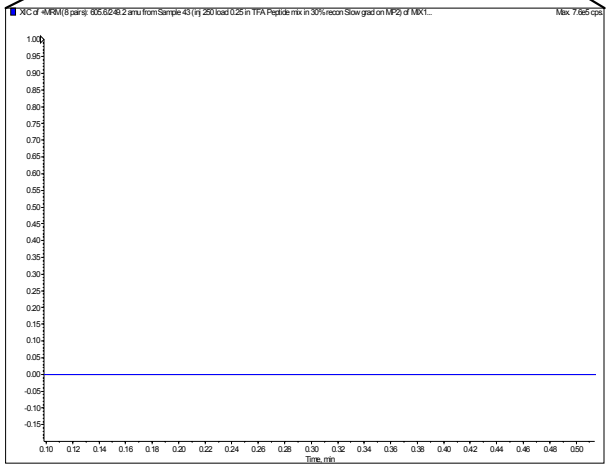


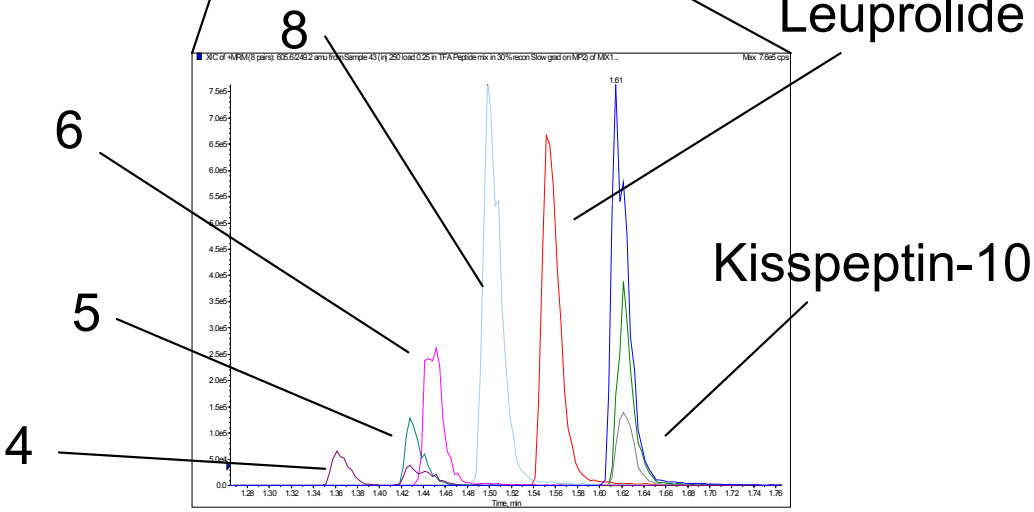
Small Peptide Assays: Ion Paired Trapping UHPLC



Trap system set up:

- Loading in 0.1%TFA
- Eluting in 0.1% HCOOH





Small Peptide Assays: Trapping System

- The 8, 6, 5 and 4 amino acid peptides were all retained.
- Signal at $7.7e^{5.}$
- With the TFA not pumping into MS, the signal intensity did not suffer and in fact due to:
  - a) the ability to wash away some of the protein injected into the system; and
  - b) the greater linearity of loading volume on the trap;

  signal intensity from the same extract concentration almost doubled.
- Additionally the endogenous peptide kisspeptin-10 and the modified peptide leuprolide were also retained.

Robustness

- All the work to date has been performed using a single Halo™ C18, 4.6 x 5mm Opti-Trap EXP™.
- We have not experienced issues of leaking from the trap and no notable drop in retention, sensitivity or peak performance from the trap.
- Additionally the UHPLC column has shown no notable increase in backpressure or deterioration of peak shape after injection of ~ 5000 biological samples, the majority of which dirty methanol crashed blood or ACN crashed plasma samples.
- Previous work performed using the Eclipse XDB-C18 3 x 50 mm,1.8µ column UHPLC columns on the same systems has shown then to be prone to blocking and over pressuring after injection of multiple PPT samples.
- The use of the Opti-Trap EXP™ enables routine PPT sample preparation, also desirable for retaining the most polar analytes such as metabolites which could be lost when performing sample extraction.

Conclusions

- A variety of significant gains can be made by integrating the Opti-Trap EXP™ technology into a UHPLC system.
- The UHPLC system can be set up as a generic system to capture all analytes of interest (including more polar metabolites) or can be optimised for a single component by varying the loading mobile phase and the chemistry of the trap cartridge packing material.
- The loading mobile phases can carry additives that you wouldn't use with MS due to causing ion suppression (e.g. TFA, derivatisers, etc.).
- Dirty sample extracts (e.g. PPT blood and plasma) can be directly injected without fear of blocking the narrow bore sub 2-micron particle column.
- Column lifetime is greatly increased (ca 5000 samples introduced into the system with no need to replace either the trap cartridge or the analytical column).

Pfizer

Acknowledgements

- Julian Haynes (PDM, Pfizer)
- Paul Turnpenny (PDM, Pfizer)
- Pooja Sood (University of Leicester)
- Mike J.E. Stephens (Optimize Technologies Inc.)
- Douglas W. Ford (Optimize Technologies Inc.)
- Hashim Javaid (Crawford Scientific Limited)