Sample preparation strategies for low concentration biomarker assays in human plasma

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10th EBF YSS
17-May-2024

Agenda

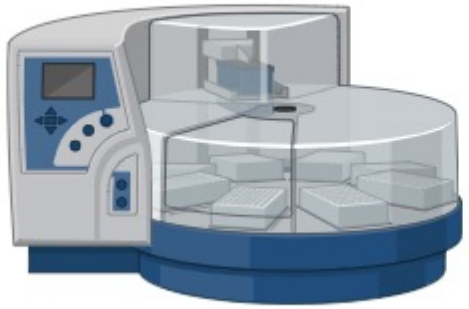
- Background / Objectives
- Antibodies screening
- LC-MS method optimization
- IC optimization on Kingfisher
- Troubleshooting
- MS model comparison
- Future Perspectives

Background / Objectives

- Some NN projects require the quantification of peptide biomarkers.
- No LBA method could be used due to cross reactivity with Novo Nordisk (NN) compounds.
- This peptide biomarker are often found in the picomolar range (LLOQ ~1 pM).
- IC-LC-MS/MS needed to achieve the required specificity and LLOQs and used in a high throughput environment.
- Current IC protocols needed to be optimized.

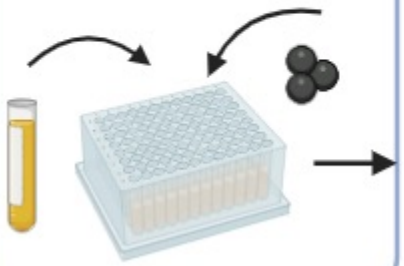
Immunocapture	LC	MS
Antibody selection	Gradient	Source parameters
Sample volume	Flow rate	Compound dependent parameters
Buffer	Injection volume	Instrument model
Wash steps	Mobile phase composition	Scheduled ionization
Elution volume	Analytical column	Resolution

Original Protocol



Reagent transfer

- 50 µL Plasma
- 10 µL MB
- 150 µL IS in BSA



Immunocapture

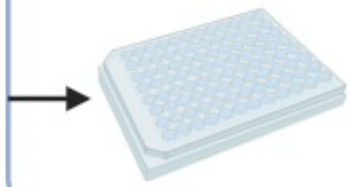
Direct capture
Fast speed
30 min 37°C

Wash
3x 300 µL PBS

Elute
200 µL
ACN:MQ 1%FA

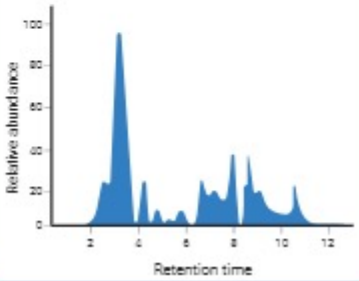
Dilution

- Centrifuge
- Transfer 100 µL to clean plate
- Dilute 100 µL water



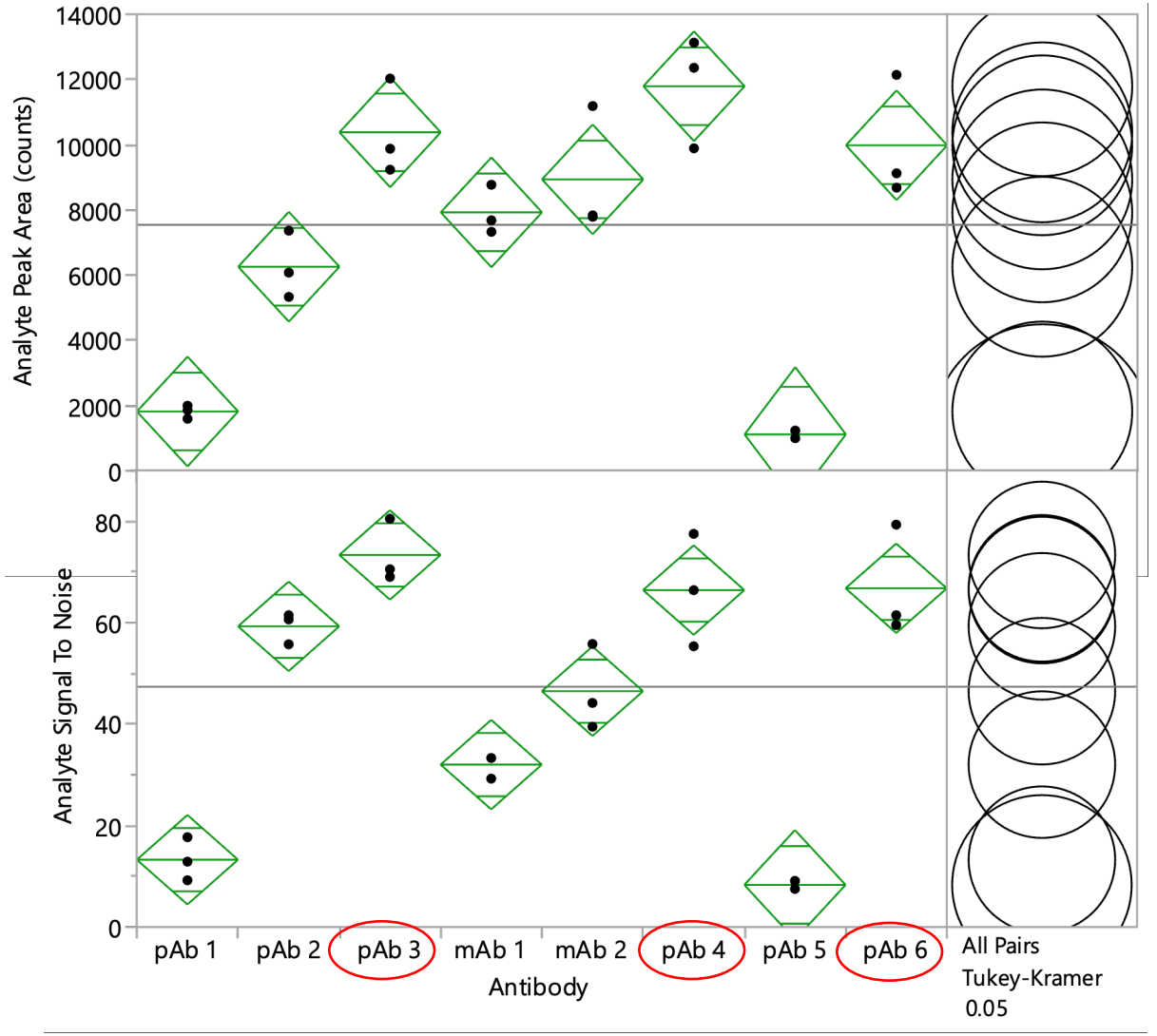
LC-MS/MS

- Sciex 6500+
- Peptide CSH C18 column



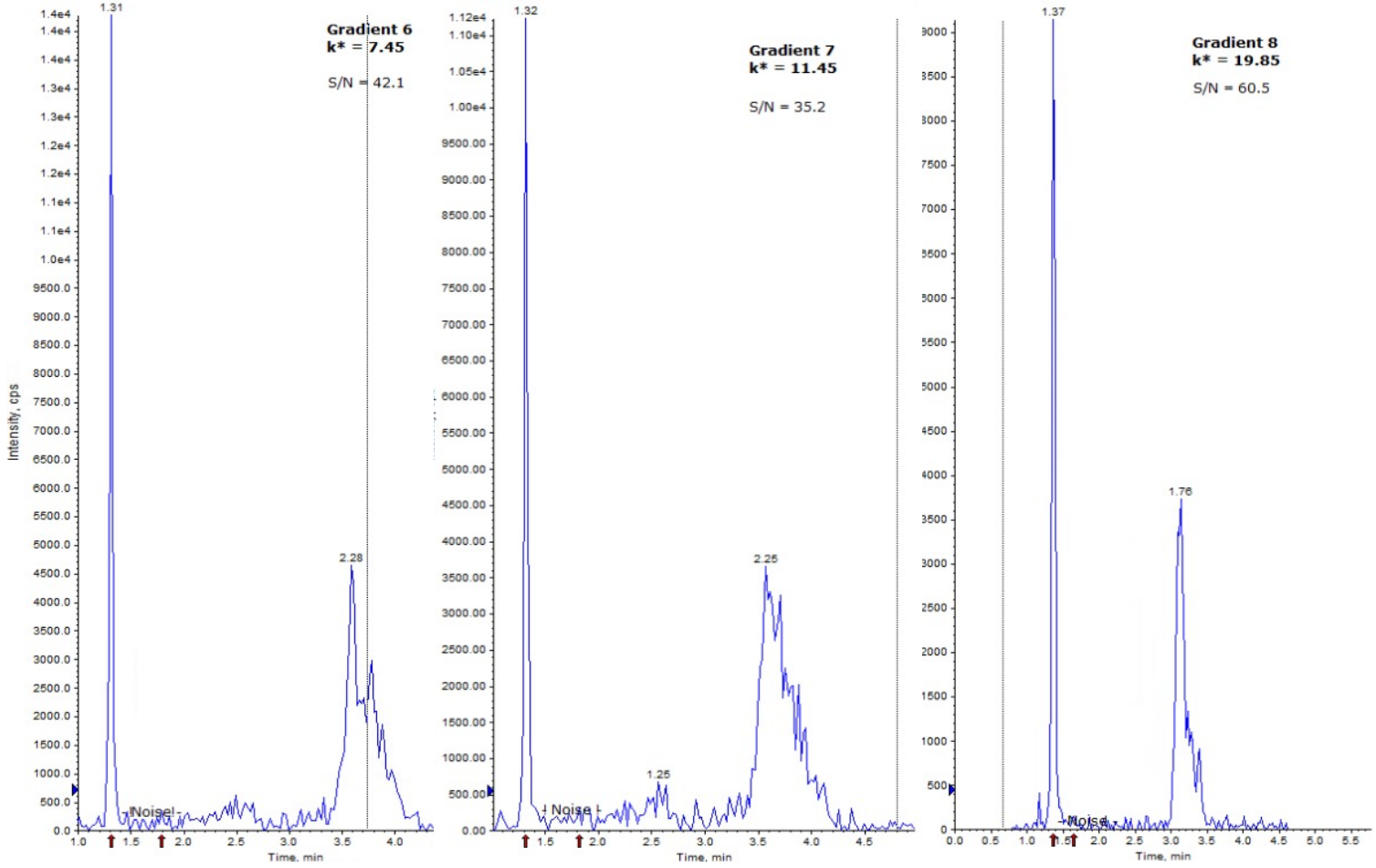
Antibody screening

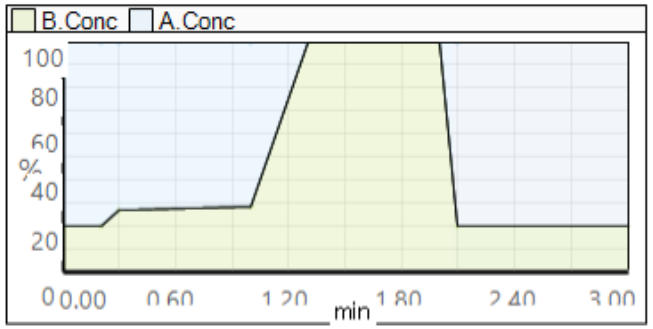
- 8 antibodies were selected according to their efficacy in LBA assays.
- Antibodies coated directly into Dynabeads MyOne Tosylactivated magnetic beads.
- Final concentration: 40 µg AB/mg magnetic beads.
- 50 µL of human plasma with 6.0 nM Biomarker
- Area and S/N evaluated

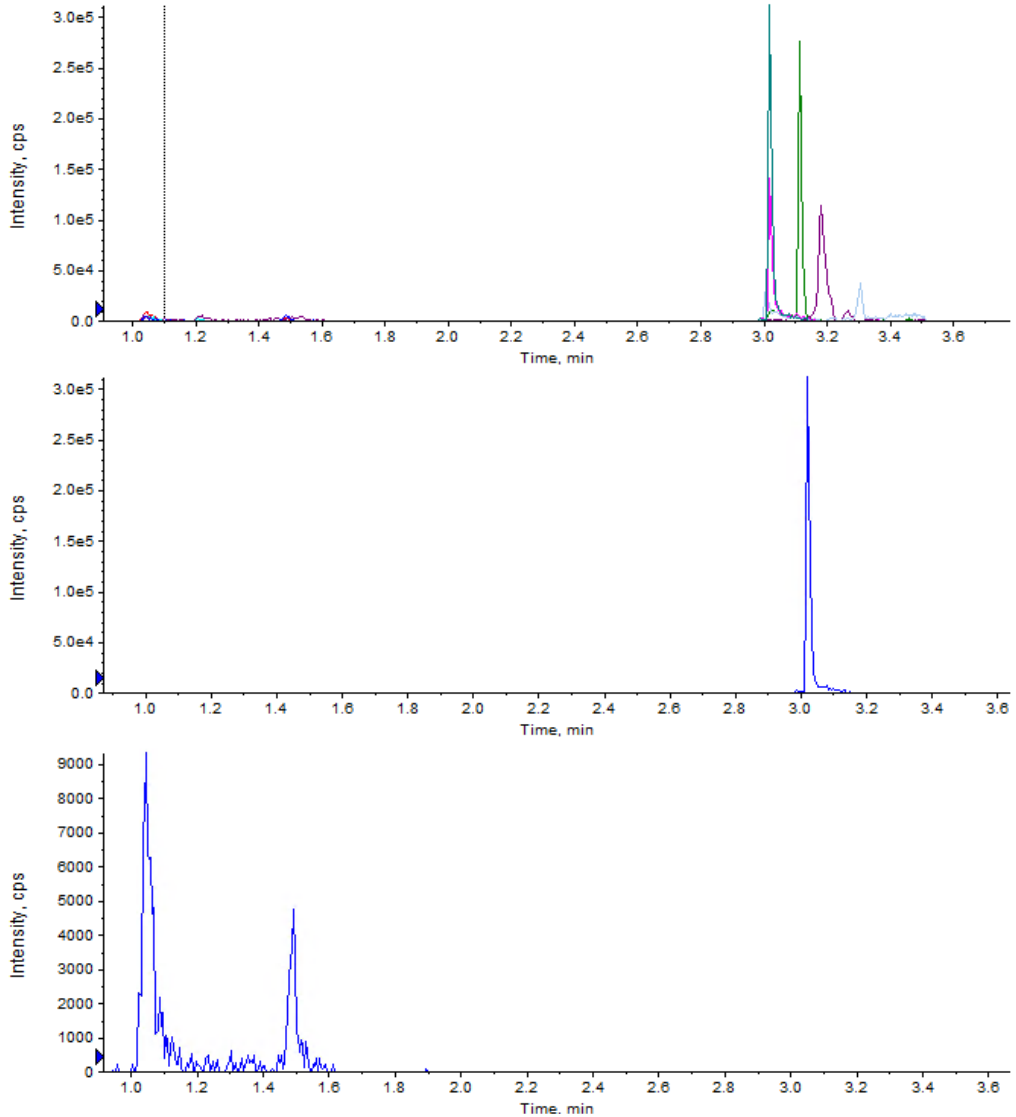


LC gradient optimisation

- Gradient was optimized while keeping optimal values of 1<k*<20.
- Optimal S/N obtained at k* ~20
- Flowrate optimised from 0.2 to 0.5 mL/min.
- What about higher flow rates?
- Pressure ~600 bar at 0.5 mL/min with full porous columns
- Biozen peptide XB-C18 core-shell allowed low pressure ~450 at 0.7 mL/min







TIC

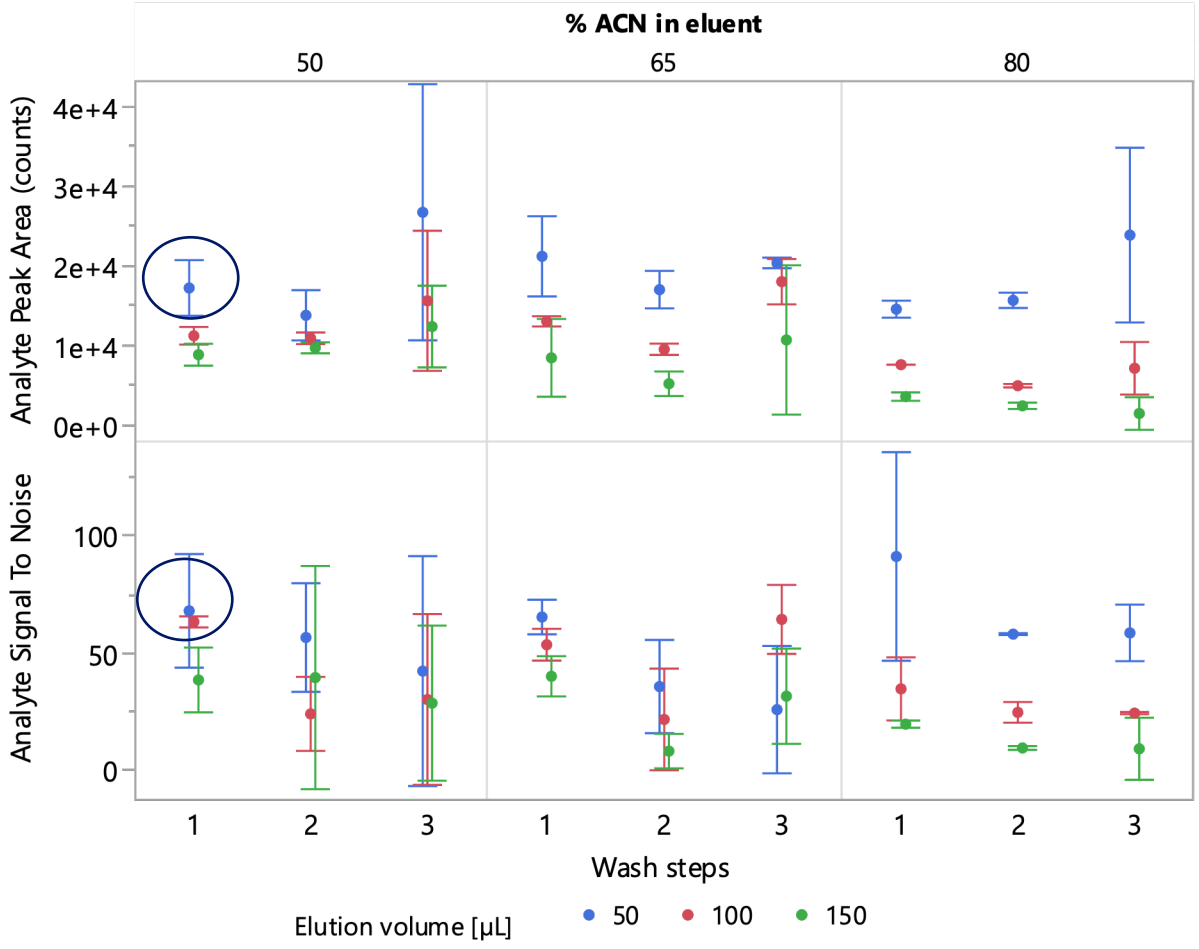
NN compound

Peptide biomarker

Initial Kingfisher optimization

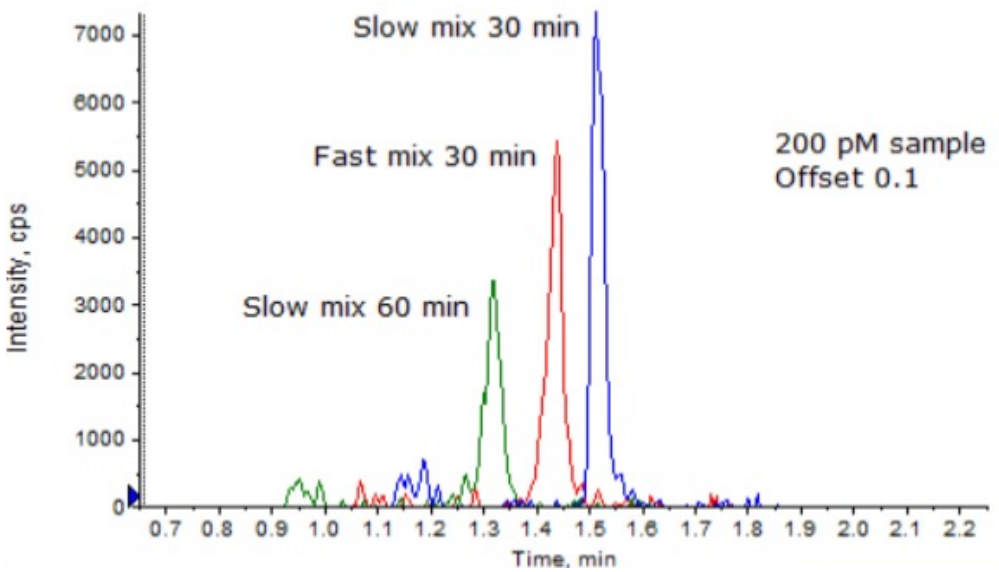
The hypotheses were the following:

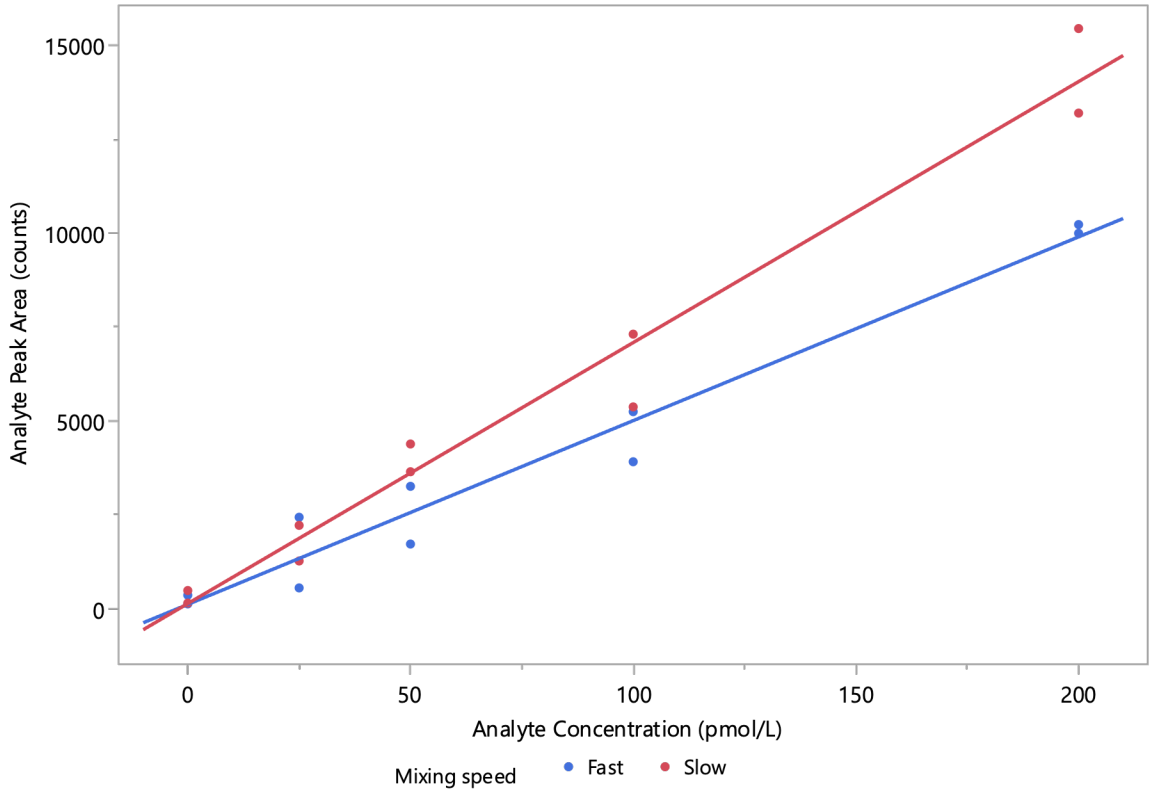
- Lower percentages of ACN should be able to elute our analyte from the magnetic beads while improving peak shape considering that it elutes with percentages of ACN ~25% during LC.
- Lower elution volume would increase peak intensity to ~2x.
- Increased elution volume would improve recovery from magnetic beads.
- Magnetic beads may be lost during multiple washing steps and 1 washing step could be enough to provide a clean sample.



Kingfisher binding optimization

- Fast speed for 30 min (current setup)
- Slow speed for 30 min (speed change)
- Slow speed for 60 min (speed and time change)

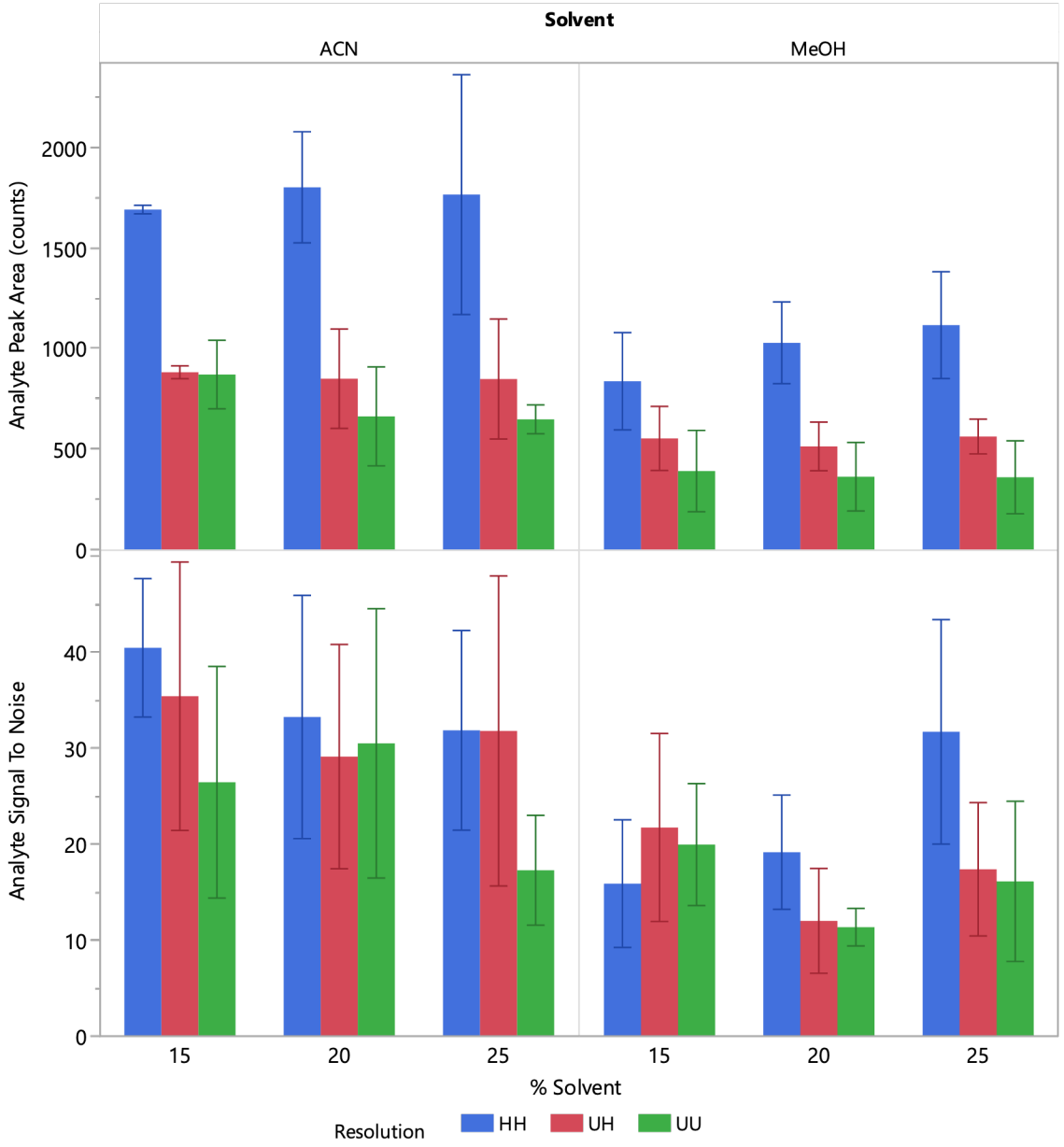




Lower recovery at high speeds and longer times probably due to partial precipitation of the analyte

High resolution mode

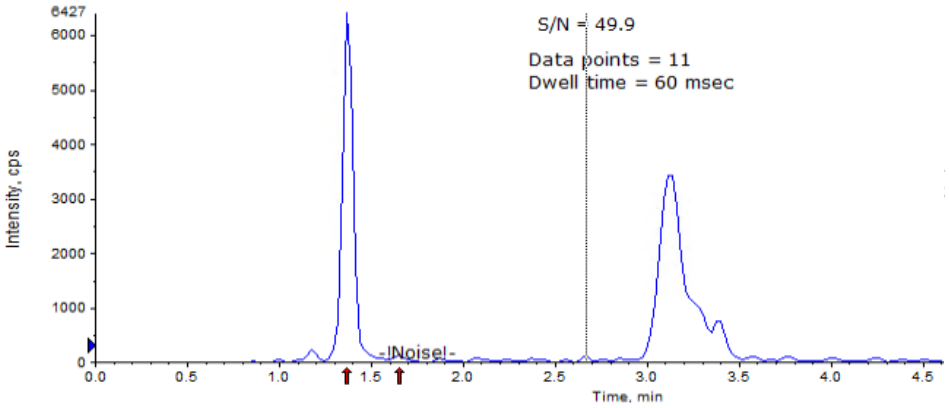
- High resolution mode was tested on Q1 and Q3, considering that it may further improve S/N.
- Three combinations were tested on both quadrupoles: high-high (HH), unit-high (UH), and unit-unit (UU) on a 25 pM solution
- High resolution on both quadrupoles improved signal intensity and S/N

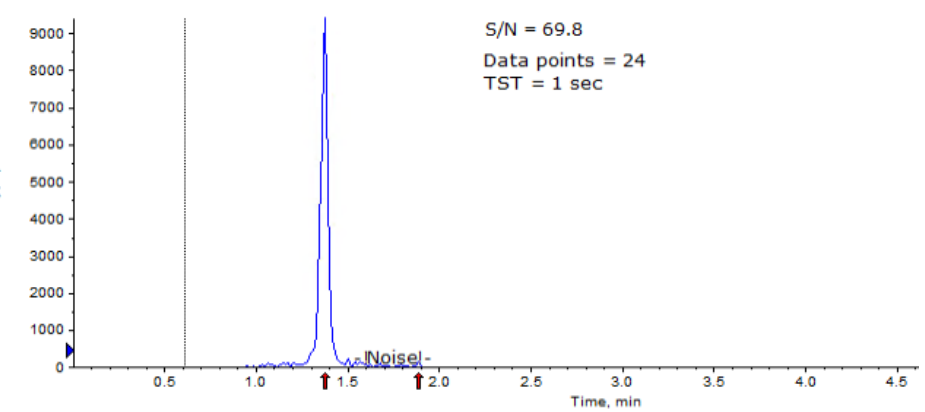


Scheduled MRM

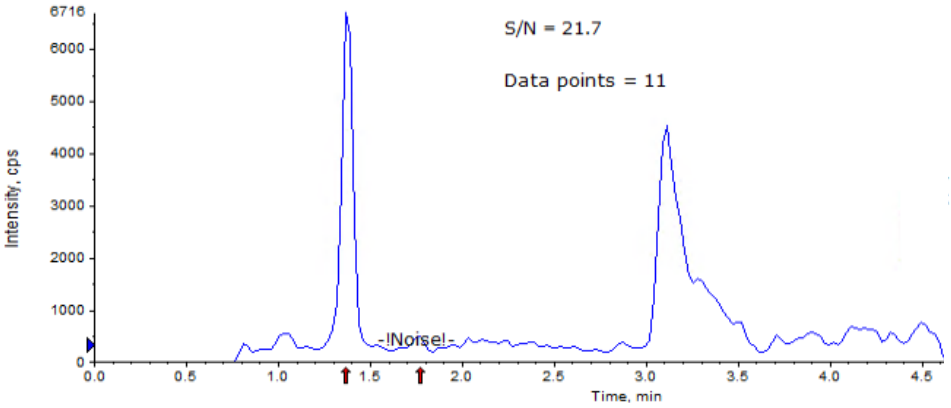
- SMRM method was compared against regular MRM to optimize the dwell time, data points across peak and S/N
- Target scan time (TST) = 1 sec
- Scan window = 60 sec

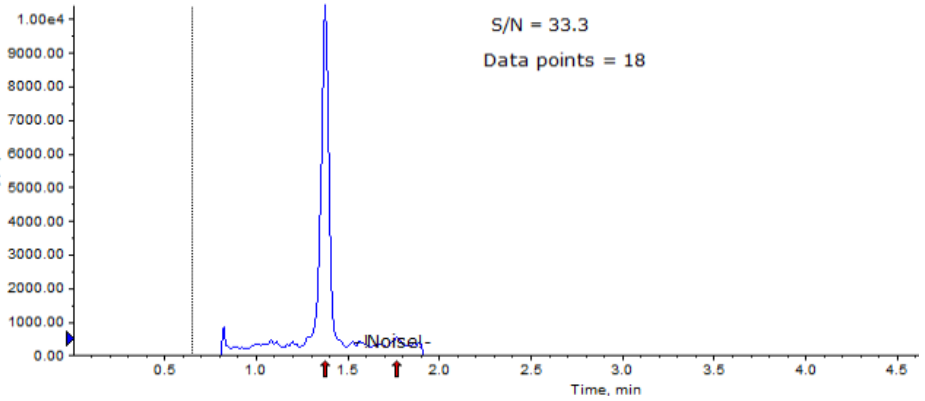
Quantifier





Qualifier



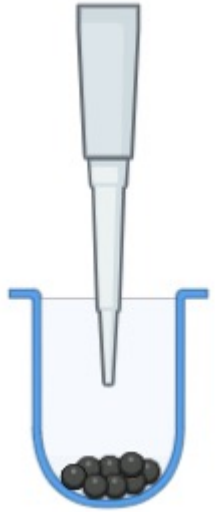


Mean	Standard Deviation	%CV
4842.400000	633.306569	13.078361
8495.366667	324.287902	3.817233

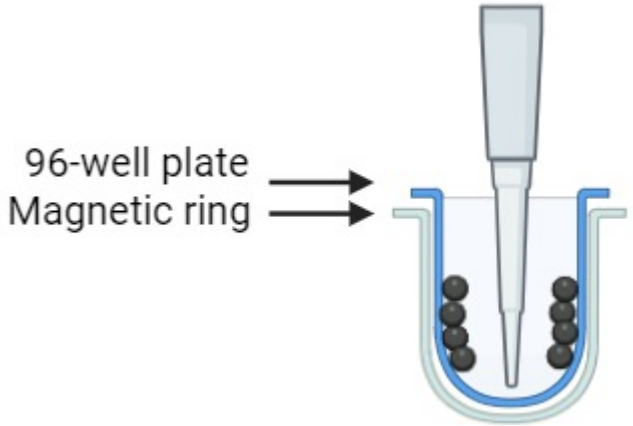
Troubleshooting

- Pressure increase was observed in a batch of 50 injections from 200 to 600 bar and finally column was clogged (>1000 bar).
- MB residues at the bottom of the plate was the cause of the issue
- 3 approaches were used to minimize the risk of MB entering the LC:
  - IC method on Kingfisher was modified to include post collection step after each process
  - Use of magnetic ring instead of centrifugation after elution
  - Use of 140 µL 384 well plates and modifying needle stroke from 52 to 50 mm

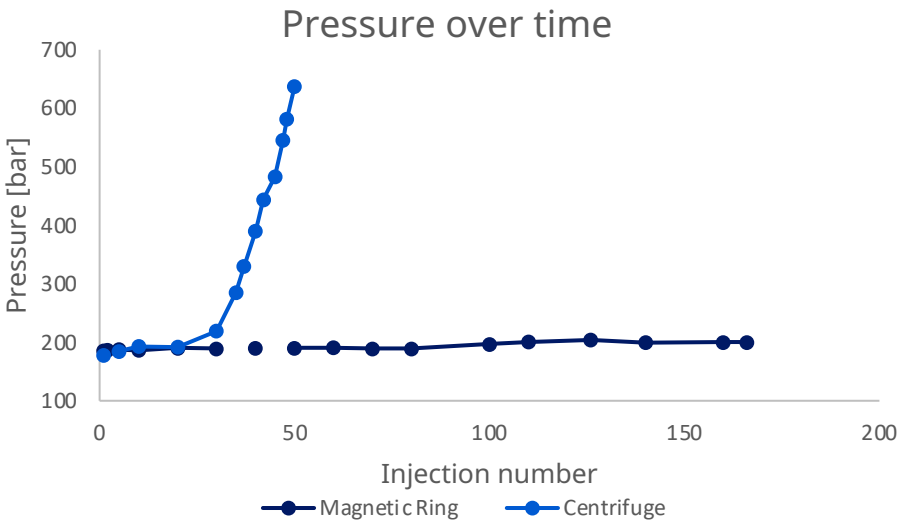
Magnetic-Ring Stand (96 well)



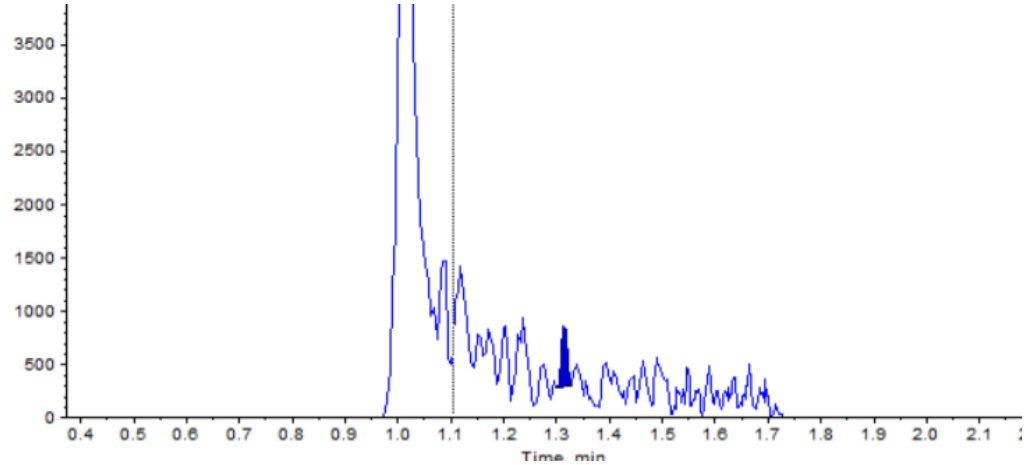
96 well plate drawing after centrifugation



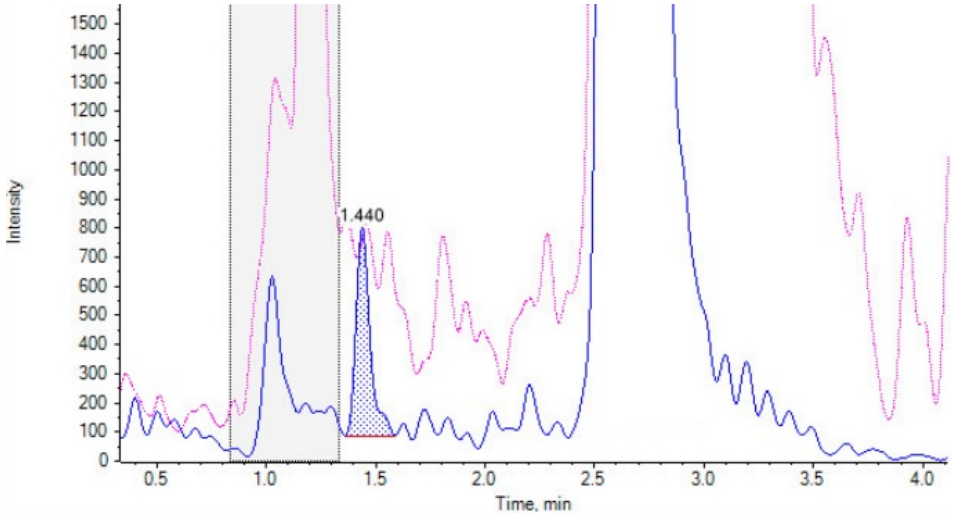
96 well plate drawing on the magnetic ring



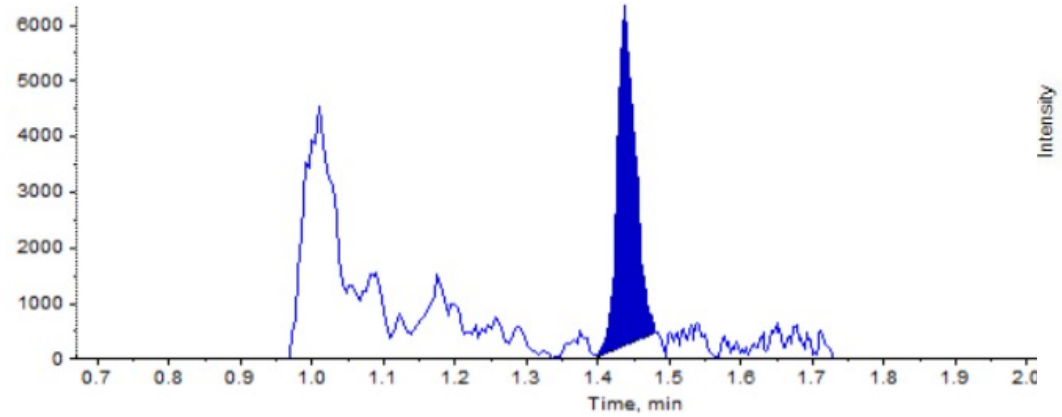
Mass spectrometer comparison: Sciex 6500+ vs Sciex 7500



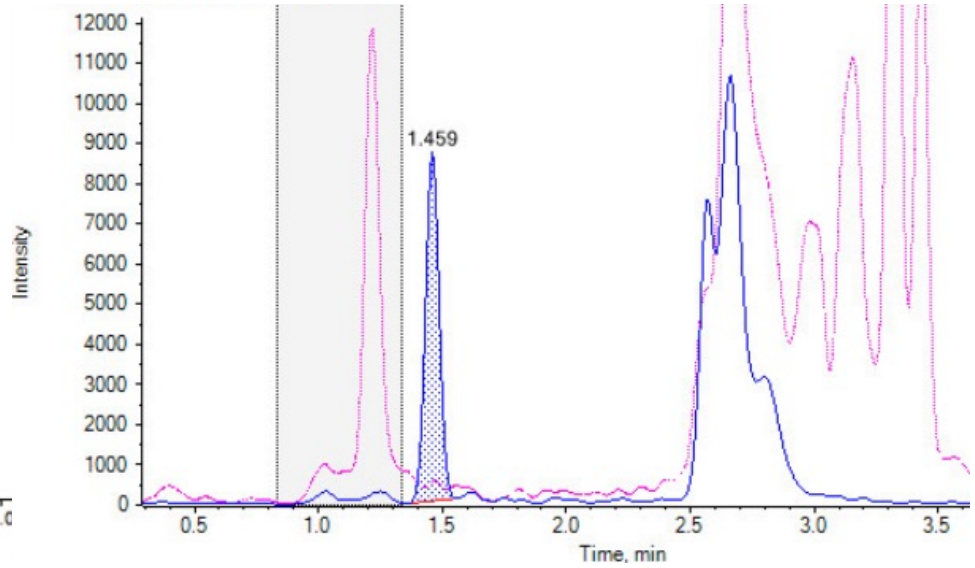
1.0 pM sample on a Sciex 6500+



1.0 pM sample on a Sciex 7500

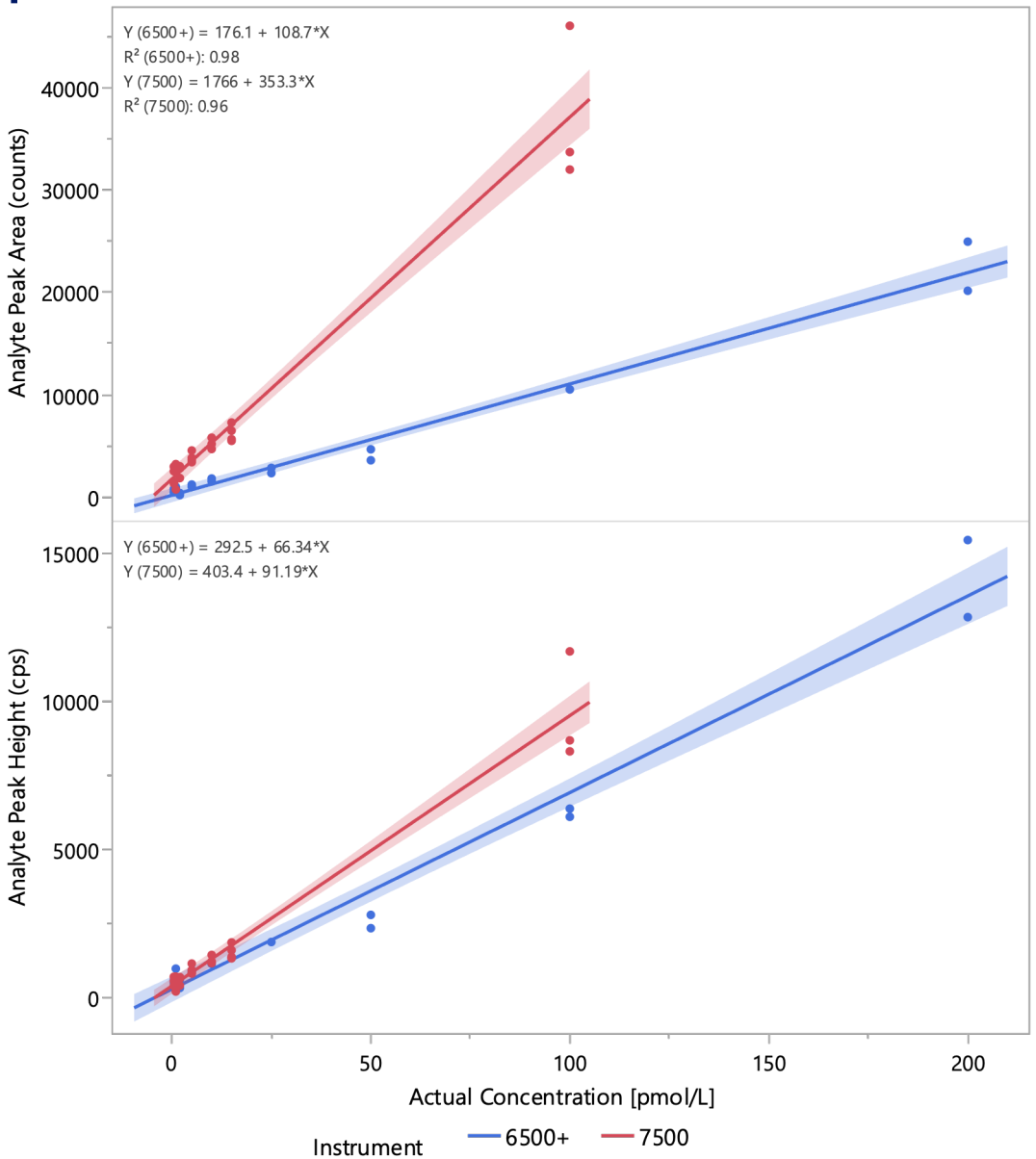


100 pM sample on a Sciex 6500+

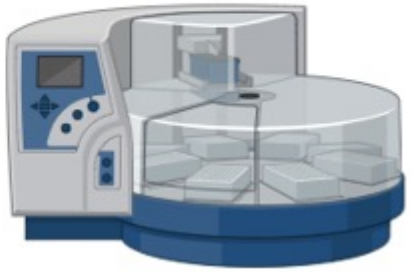


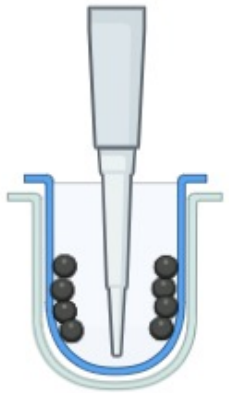
100 pM sample on a Sciex 7500

Linear range comparison



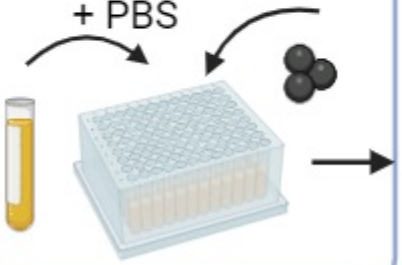
Optimized Protocol





Reagent transfer

- 250 µL Plasma
- 20 µL MB
- 150 µL IS in BSA + PBS



Immunocapture

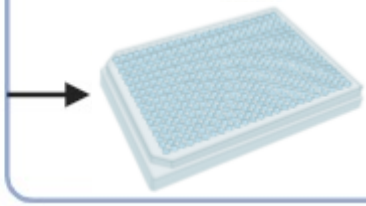
Direct capture
Slow speed
30 min 37°C

Wash
1x 300 µL PBS

Elute
100 µL
ACN:MQ 25:75
+1%FA

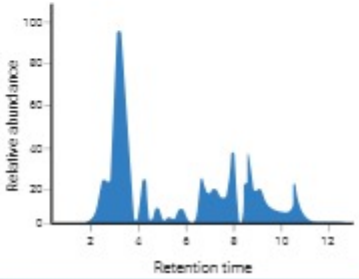
Transfer

- Magnetic ring
- Transfer 100 µL to clean 384 well plate



LC-MS/MS

- Sciex 7500
- Biozen Peptide XB- C18 column



Summary and future perspectives

- IC optimization such as sample volume, binding speed, eluent volume and composition in addition to MS parameters like high resolution MRM and SMRM and new MS technology allowed us to detect and quantify our peptide biomarker in human plasma.

- Use of polyclonal antibodies with LC separation allowed to separate our biomarker from other endogenous compounds in plasma and NN compounds.

- Future applications of the method include its use in clinical trials to clarify the mode of action of several NN compounds.

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