Utilizing high-resolution mass spectrometry to improve the sensitivity of a therapeutic protein assay

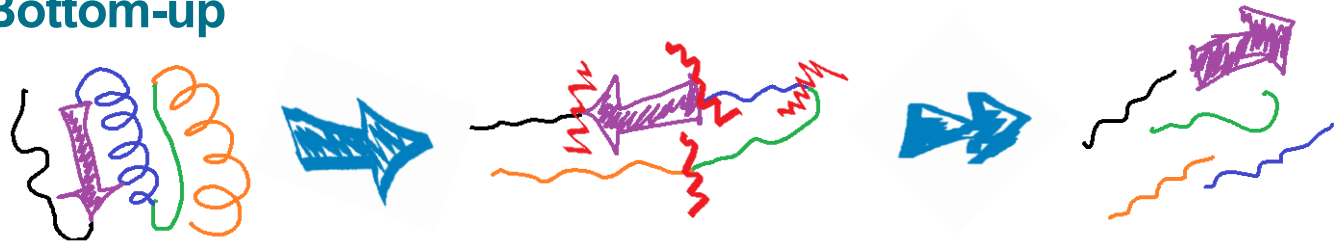
Drug Development Solutions

Szabolcs Szarka, PhD

EBF Cyberconnect Events:
Focus Workshop: Peptides & Proteins with (LC-)MS
17th June 2021

Protein LC-MS

- **Bottom-up**



  - Proteins are denatured
  - Digested with the appropriate protease enzyme into peptides
  - Peptides are analysed by LC-MS
  - Specific – unique peptide selection x mass selective detection
  - Sensitive
- **Optional protein and/or peptide level clean-up**
- **Triple quadrupole platform**
  - Sensitive
  - Robust

Therapeutic Protein Quantitation

- **Bottom-up LC-MS/MS assay**
  - Lys-C digestion
- **Platform: Waters Acquity UPLC – Sciex 6500 (QqQ)**
- **Validated in human plasma**
- **Supported SAD PK study**

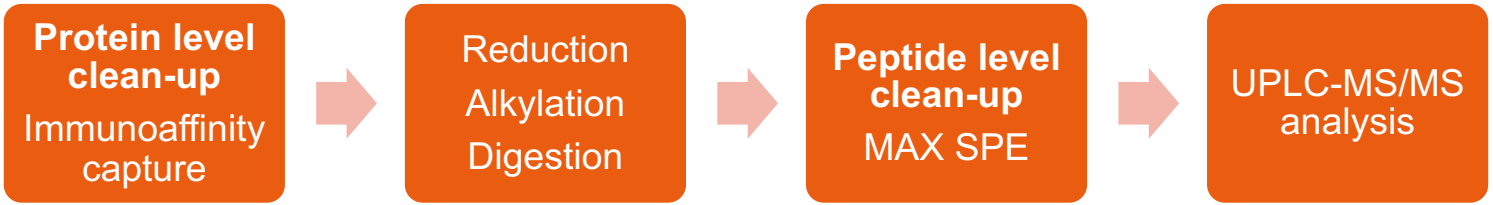
- **Assay sensitivity not sufficient to fully characterize PK**

The Challenge

Improve assay sensitivity

How to improve assay sensitivity?

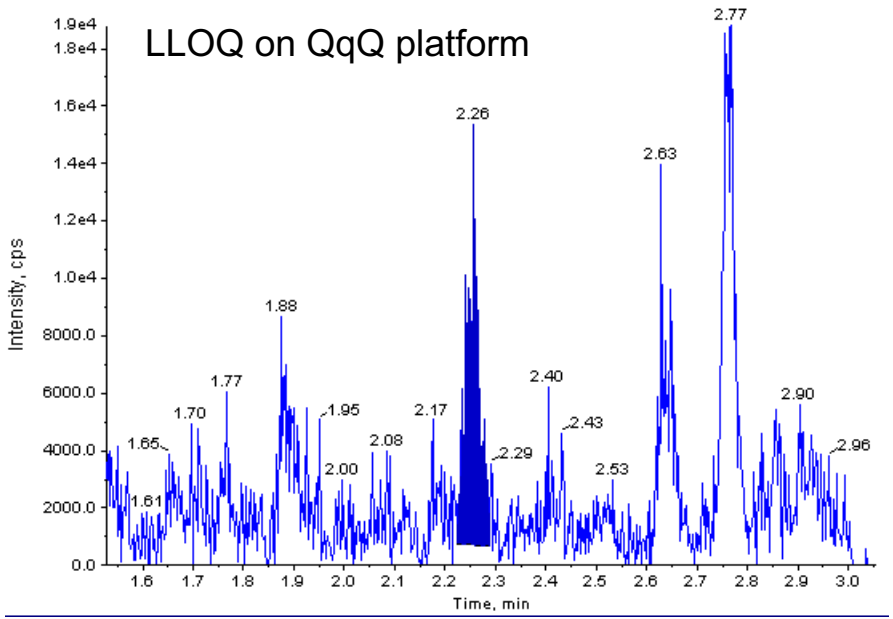
- **Why not LBA?**
  - Target protein is different from endogenous isoform by **only 3 amino acids**
  - Endogenous concentration **~500x** higher
- **Change extraction protocol?**
  - Complex extraction



  - Fully optimized
  - Maximum recovery at each step
  - Challenging method development

How to improve assay sensitivity?

- **Change MS platform?**



High background noise

↓

Can we minimize noise?

↓

Improve selectivity

↓

High-resolution MS

High-resolution MS method development

HRMS method development

- ~~Sample preparation~~
- ~~UPLC parameters~~
- **Sciex 6600 TripleTOF (Q-ToF)**
- **Ion source settings**
  - Optimisation similar to unit resolution instruments (e.g. QqQ)

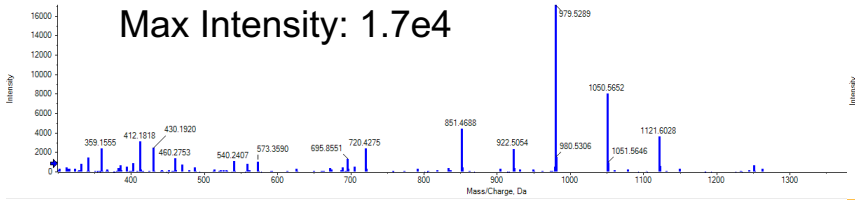
- **HRMS specific aspects**
  - Enhanced ion mode
  - Isolation window
  - Summing ion responses

HRMS method development

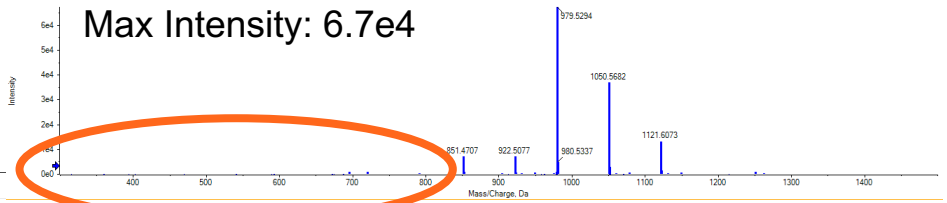
- **Enhanced ion mode**
  - High-resolution high sensitivity MRM function

Select target mass (m/z) → **Stored and focused in collision cell** → **Response in the target m/z region is enhanced** → **Improved sensitivity**

Non-Enhanced MS Spectrum



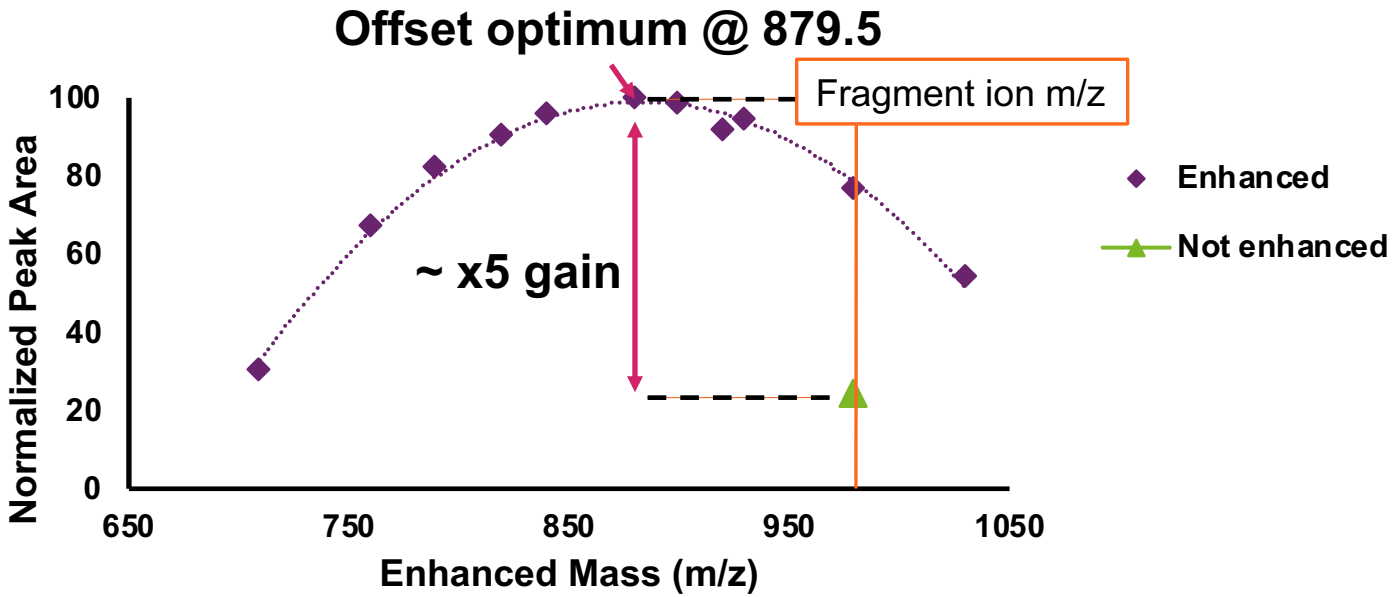
Enhanced MS spectrum



Ion responses outside of the enhanced region are suppressed

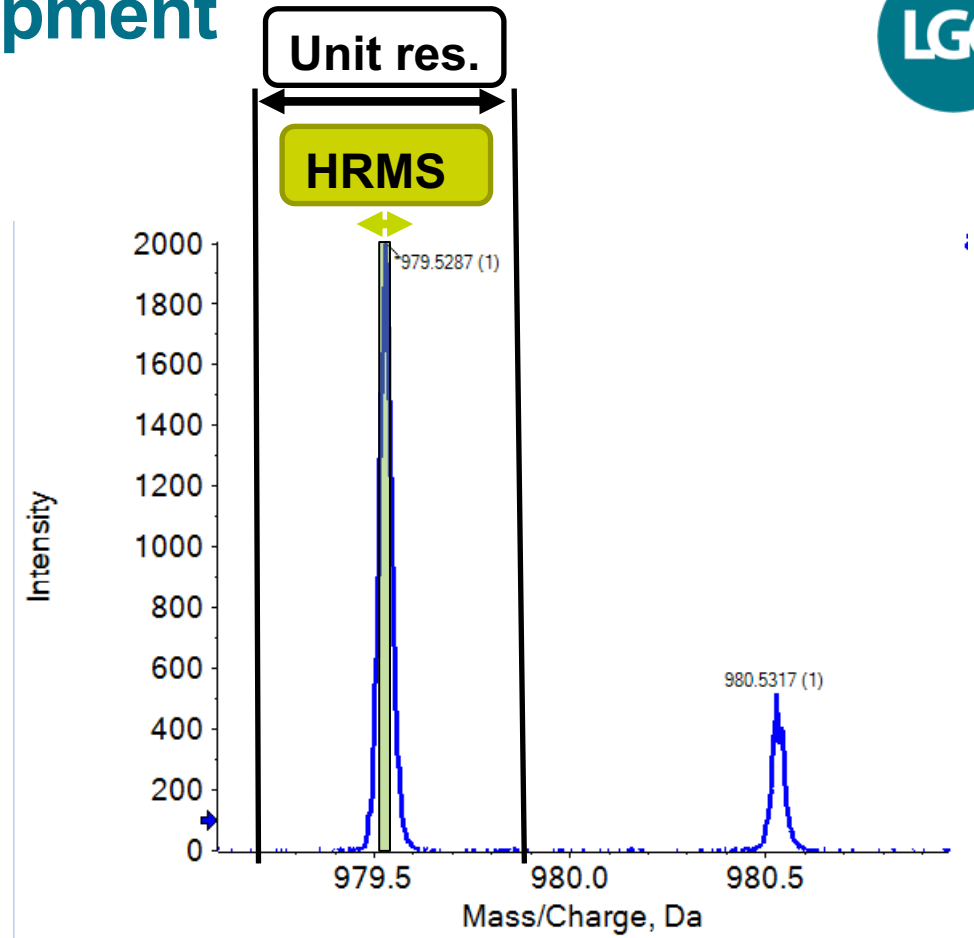
HRMS method development

- **Enhanced ion mode**
  - Select the quantifier fragment ion mass to enhance signal (979.5)
  - Optimum is not always on target mass, optimisation needed



HRMS method development

- **Accurate mass extraction window**
  - typically ~0.7 Da on unit resolution QqQ, not changed normally
  - HRMS narrow extraction window
  - Post-acquisition data processing
  - Extraction window size adjustable

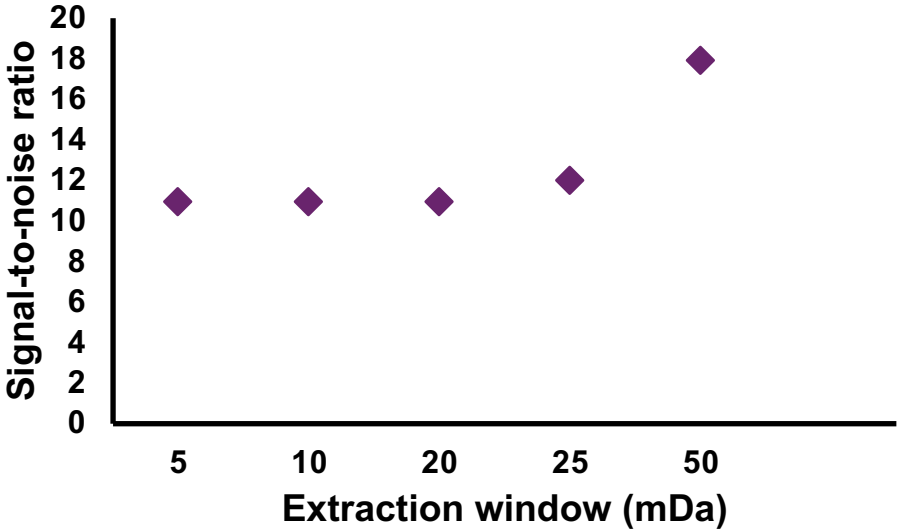


HRMS method development

- **Accurate mass extraction window**
  - Suggested to assess different window sizes
  - Too narrow → low and variable response and S/N
  - Too wide → suboptimal selectivity

- S/N acceptable down to 5 mDa

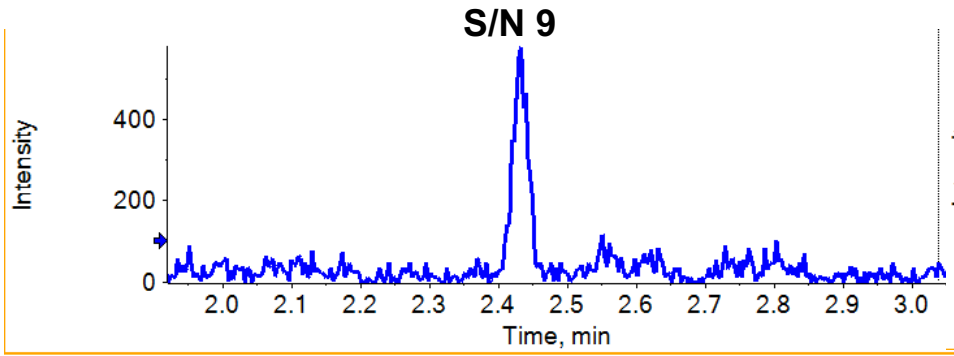
25 mDa was selected



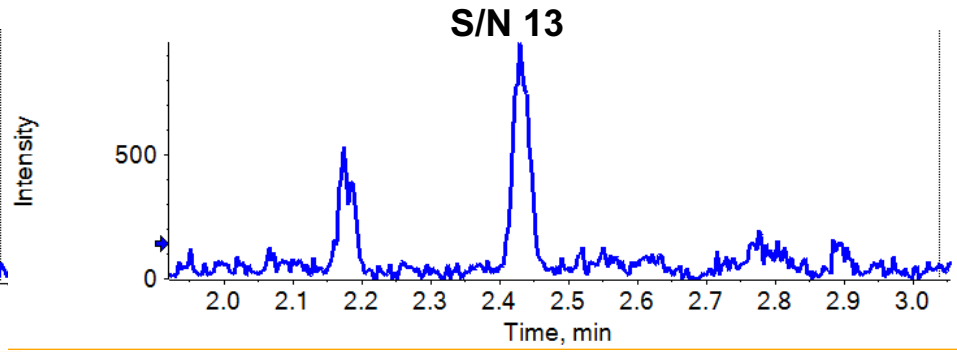
HRMS method development

- **Summing multiple fragment ion responses**
  - May improve S/N on high-resolution platforms

Single fragment ion



2 fragment ions summed



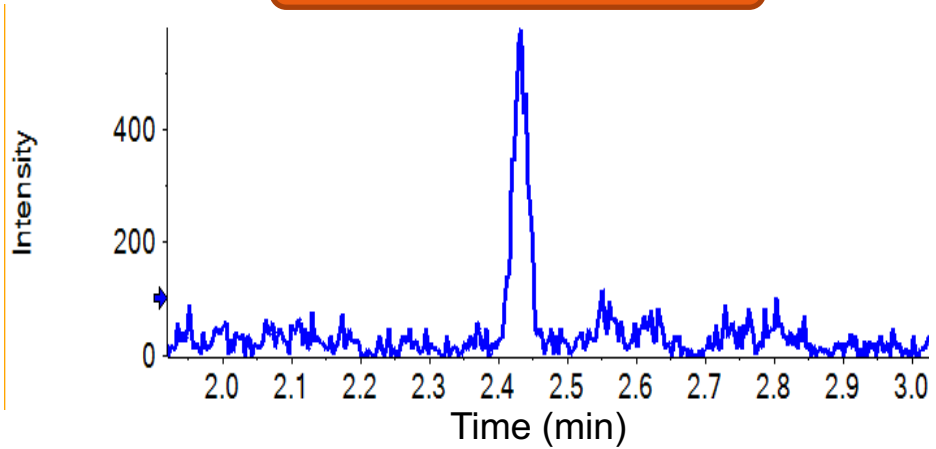
Summing was not used

- No significant S/N improvement
- Interference at 2nd fragment ion

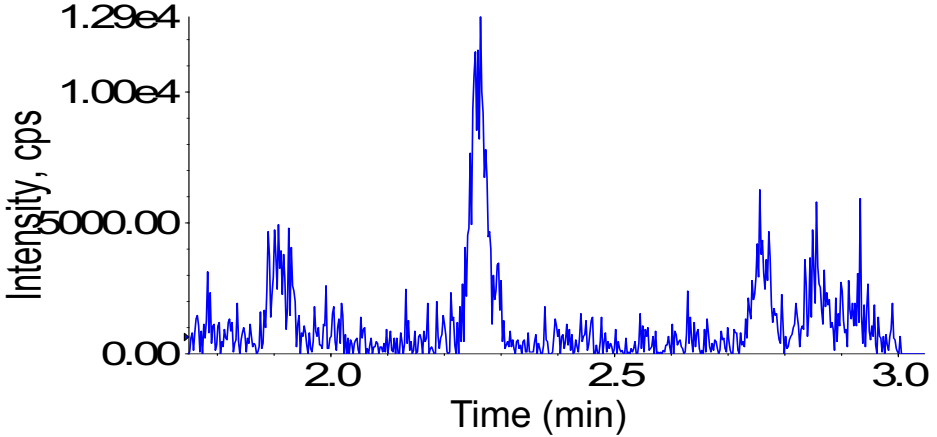
HRMS method development

- Assay sensitivity Q-ToF vs QqQ

20 ng/mL Q-ToF



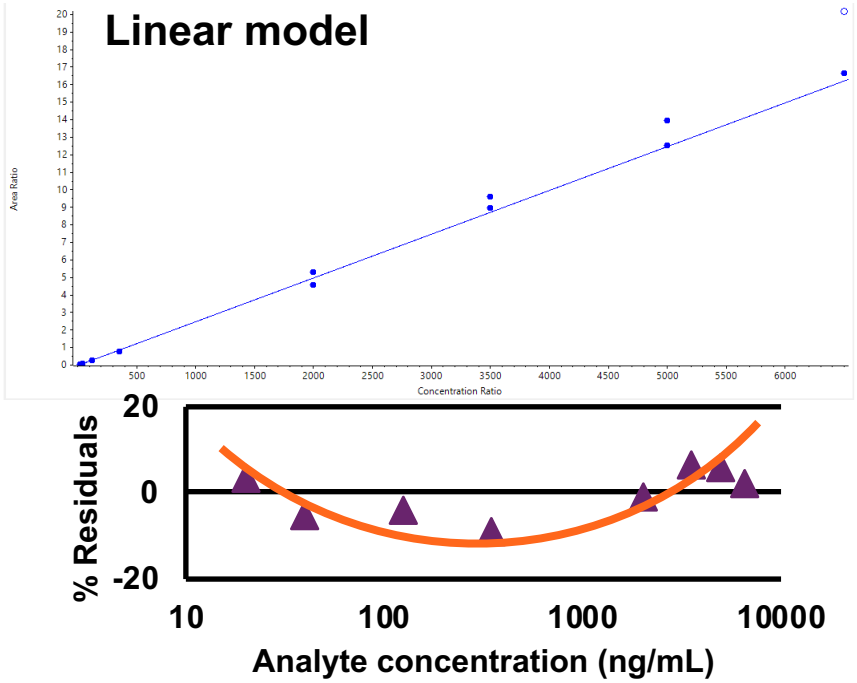
100 ng/mL QqQ

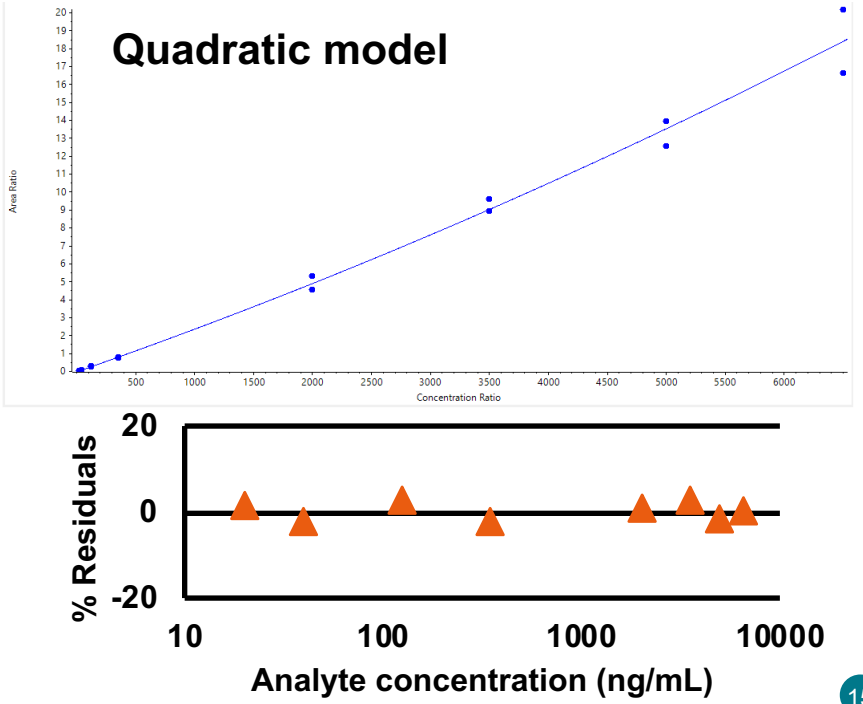


Sensitivity improved by 5-fold on Q-ToF

HRMS assay performance

- Linearity assessment at 20-6500 ng/mL





HRMS assay performance

- **Precision and accuracy (n=6)**

QC level	Conc. (ng/mL)	Mean (ng/mL)	%CV	%RE
LLOQ	20	22.6	**2.4**	**12.5**
Low	60.4	56.7	**7.9**	**-6.2**
Medium [a]	2500	2558	**3.5**	**2.3**
High	4875	5010	**6.6**	**2.8**

[a] n=5, one datapoint excluded as statistical outlier

%CV and %RE < 15%

Summary

- HRMS platform provided 5-times higher sensitivity
- Optimal enhanced ion mass setting is 100 Da off from fragment ion mass → optimisation suggested
- 25 mDa extraction window → acceptable S/N, robustness
- Summing 2 fragment ion response → no further improvement of S/N
- Method developed successfully
- Validation is ongoing

Special Thanks!
Jason Pembroke
Rob Wheller

Sponsor

Thank you.

Szabolcs Szarka, PhD
Principal Scientist, Drug Development Solutions, LGC
Email: Szabolcs.Szarka@lgcgroup.com