Strategies for the Analysis of Therapeutic Peptides in Biofluids by LC-MS/MS

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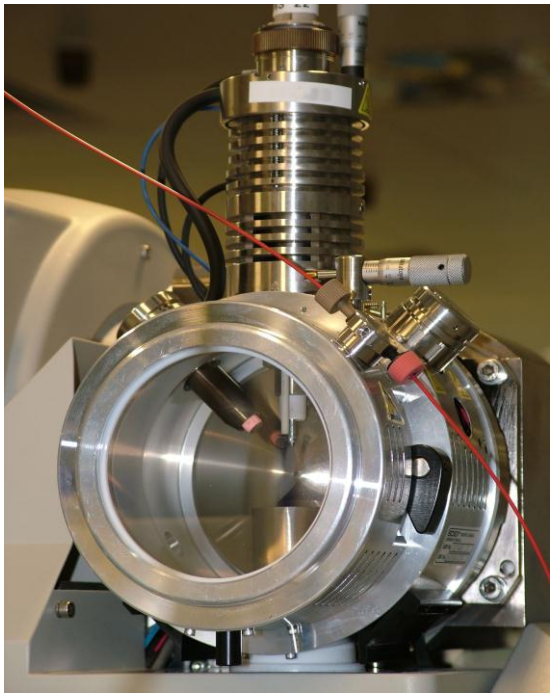
Outline

- Sample Preparation
- Chromatography
- Detection
- General Strategies
- Examples
- New Approaches
- Summary



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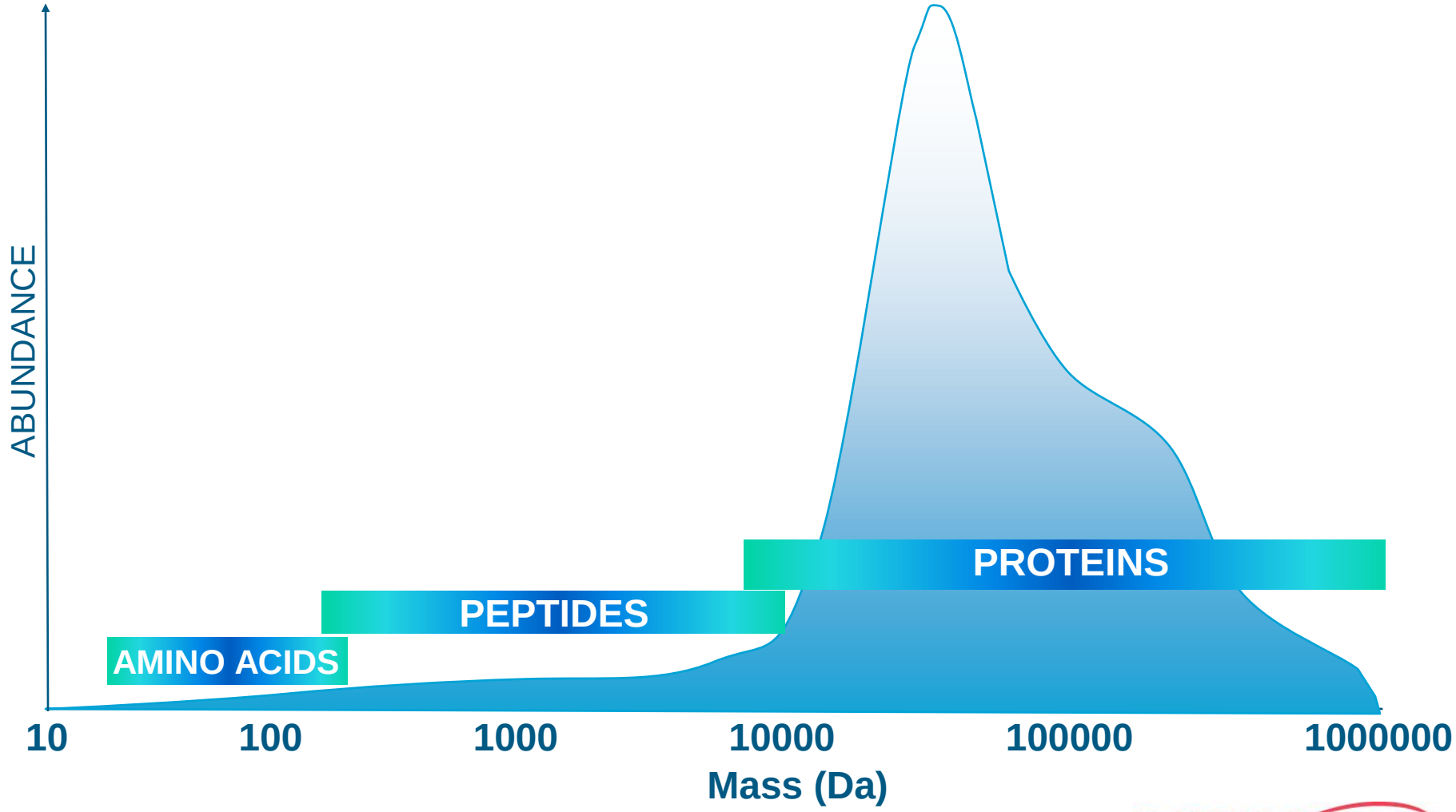
Introduction



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Introduction

- Working definition of Peptide vs Protein
  - Peptide consists of <50 amino acid residues
    - Homogenous
    - Usually <10 kDa
    - May be synthetic
    - May contain non-natural amino acids
  - Protein consists of >50 amino acid residues
    - Non-homogenous
- The chemical and physical properties of both peptides and proteins will be determined by the composition and relative abundance of the constituent amino acids.

Sample Preparation

- Adsorption:
  - Low adsorption polypropylene storage vessels
  - Preparation of standards in matrix
  - Using inhibitors or competitors e.g. surfactants
  - Using aqueous and organic modifier mix to keep peptides in solution, e.g. acetonitrile, methanol and iso-propanol
- Elimination of peptide - protein binding (changing the structure of the protein)
  - Guanadine HCl
  - Urea
  - Organic solvent
  - Trifluoroacetic acid
  - Phosphoric acid

Sample Preparation

- Reverse Phase (SPE)
  - Use of polymeric sorbents
- Ion-exchange (SPE)
  - Mixed mode polymeric sorbents such as WCX, MCX (cation ex.) MAX (anion ex.)
  - Pore size of 40-80 Å allows elimination of large proteins at the sample preparation stage
  - Very efficient at removing salts

Chromatography

- Reverse Phase Chromatography
  - The most widely used
  - Efficient mode of separation
  - Can distinguish peptides with minor structural differences
  - Compatible with electrospray MS
- Mechanism:
  - The peptide adsorbs on to the reverse phase sorbent and is desorbed from the surface at a critical organic modifier concentration
  - Combination of adsorption/desorption and partitioning

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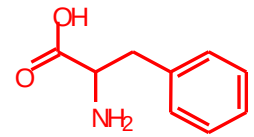
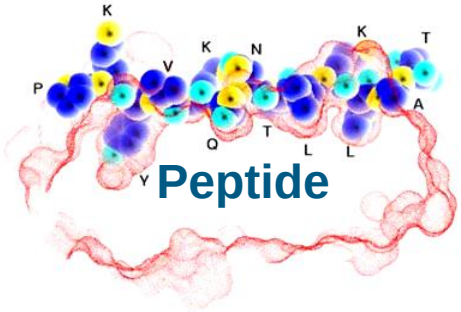
Chromatography



Amino acid

PARTITIONING ADSORPTION

SIZE



Peptide

HPLC Particle

C18 Brush Border

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Chromatography

- Particle size
  - Smaller particles give greater efficiency
- Pore size
  - Must be big enough to allow peptides to penetrate
  - Typical pore sizes for peptide separation - 120 to 300 Å
- Column length
  - Rate of gradient more important than column length
- Isocratic or gradient elution?
  - Gradient elution preferred
  - Typical gradient <10% change in acetonitrile.

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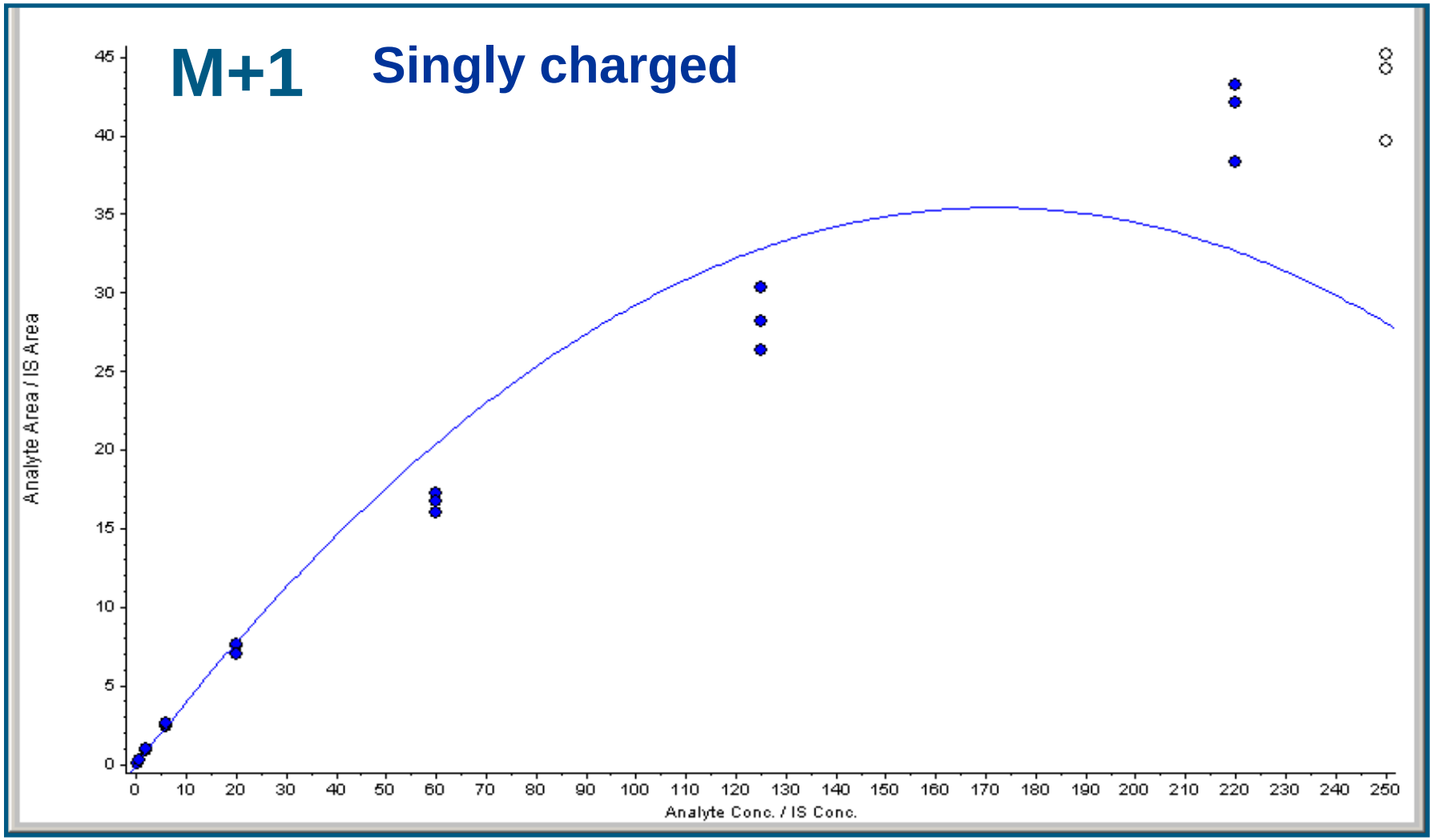
Chromatography

- Effect of Ionic Modifiers
  - TFA is the most common modifier for peptides
    - Strong ion pair reagent:
      - protonates silanols
      - eliminates secondary effects
    - Suppresses MS sensitivity
    - Reducing concentration from 0.1% to 0.01% reduces this effect
  - Formic and acetic acid may be used to increase MS sensitivity (fine balance between chromatography and MS sensitivity)
- Require more inert (end capped columns) e.g. RP-18 shield, C18-peptide
- Neutral peptides will chromatograph better
- Higher charged peptides will show greater MS sensitivity

Detection

- Analysis of peptides by electrospray ionisation
  - Electrospray is suitable for polar compounds
  - Electrospray is a concentration dependent technique and sensitivity can be increased by running narrower columns: 2.1 mm and below with lower flow rates.
  - Multiple charging enables larger masses to be analysed, but compromises sensitivity through charge distribution

Variable Ionisation



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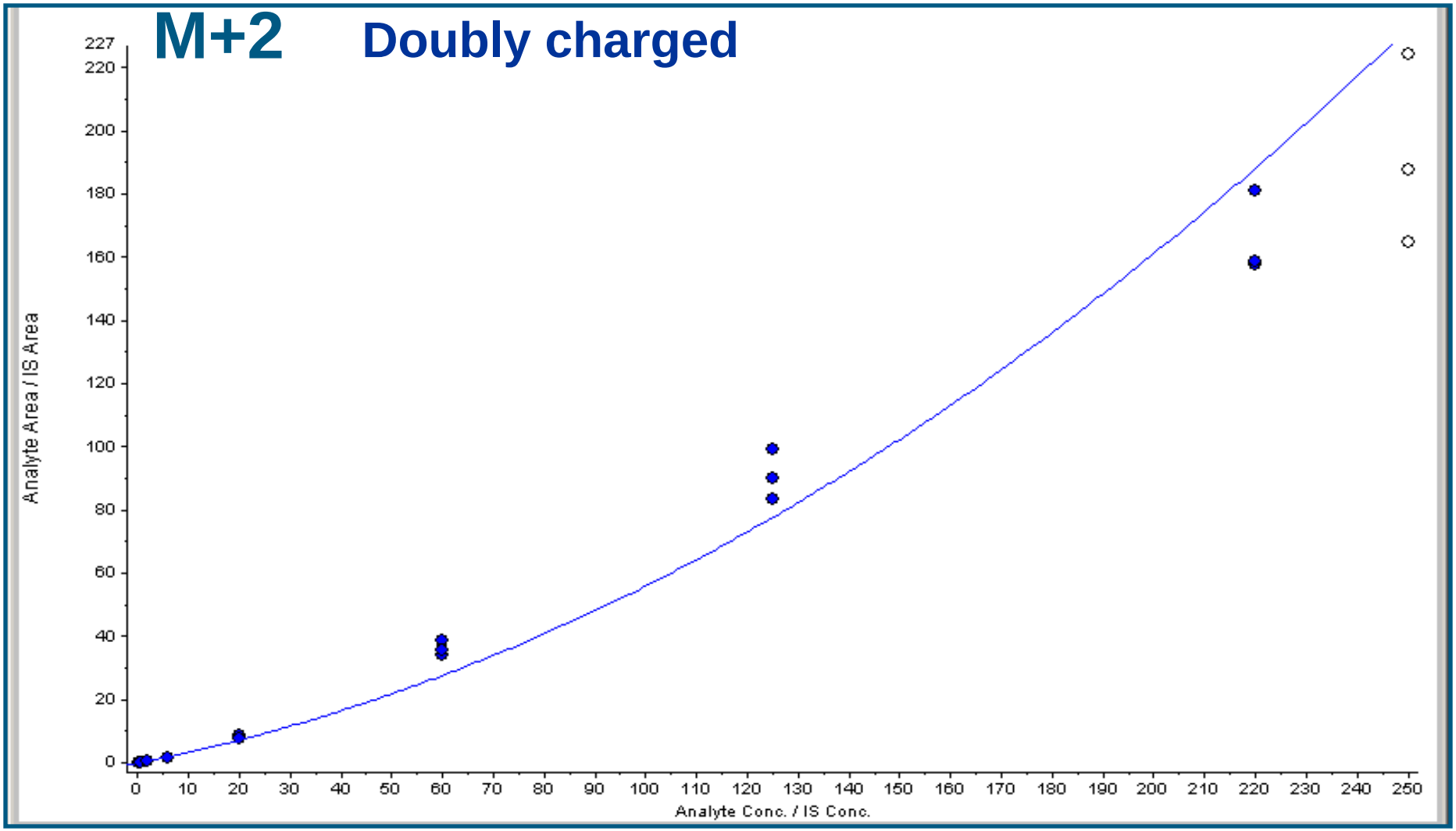
Variable Ionisation



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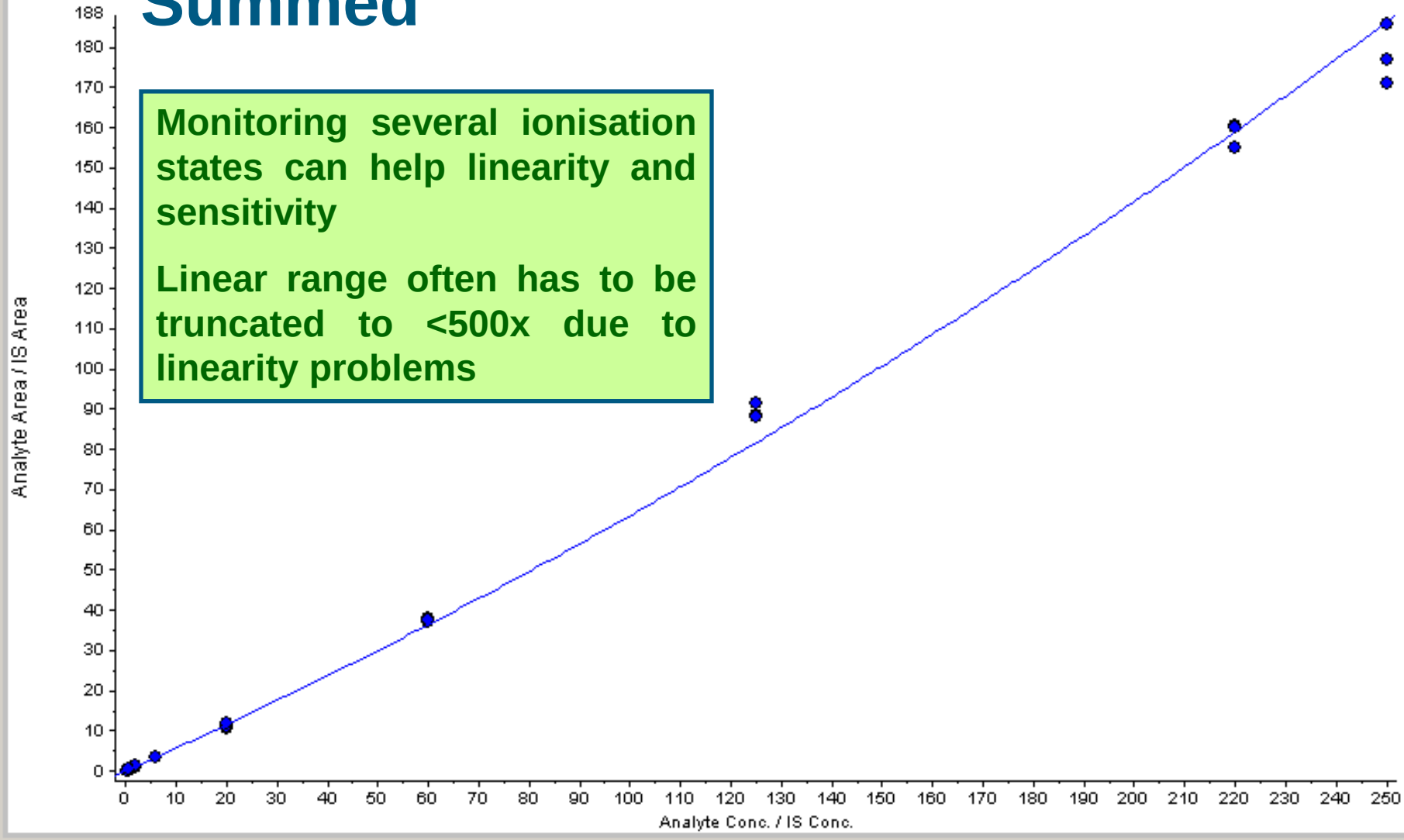
Variable Ionisation

Summed

Monitoring several ionisation states can help linearity and sensitivity

Linear range often has to be truncated to <500x due to linearity problems



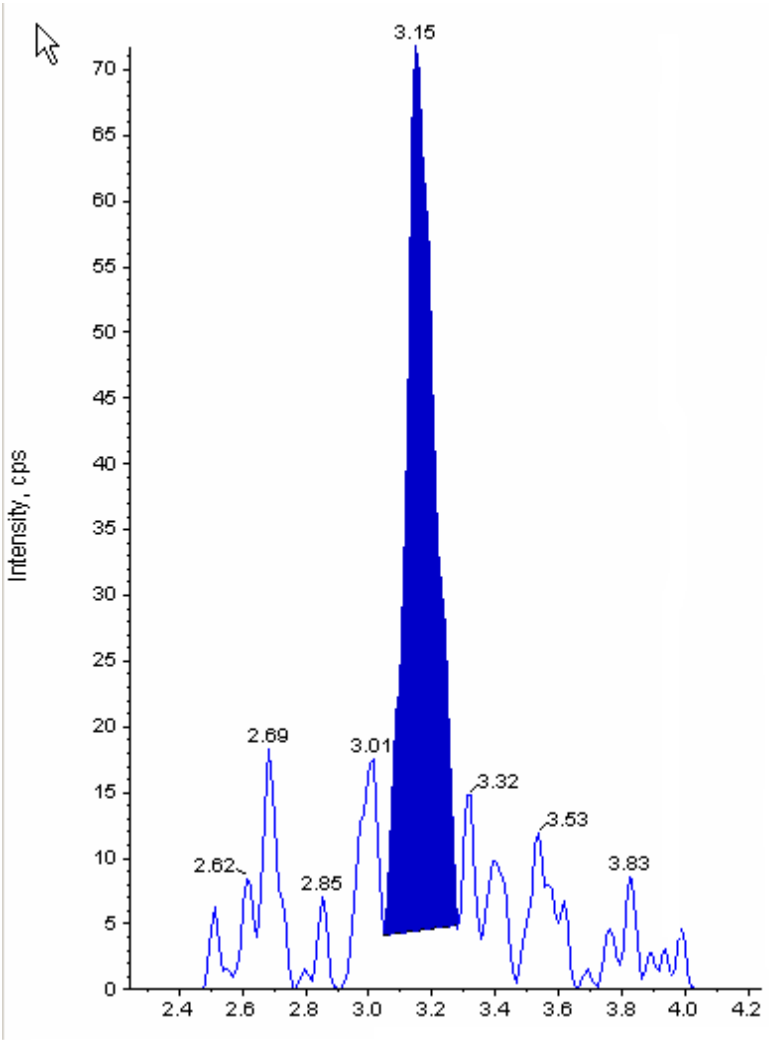
Detection

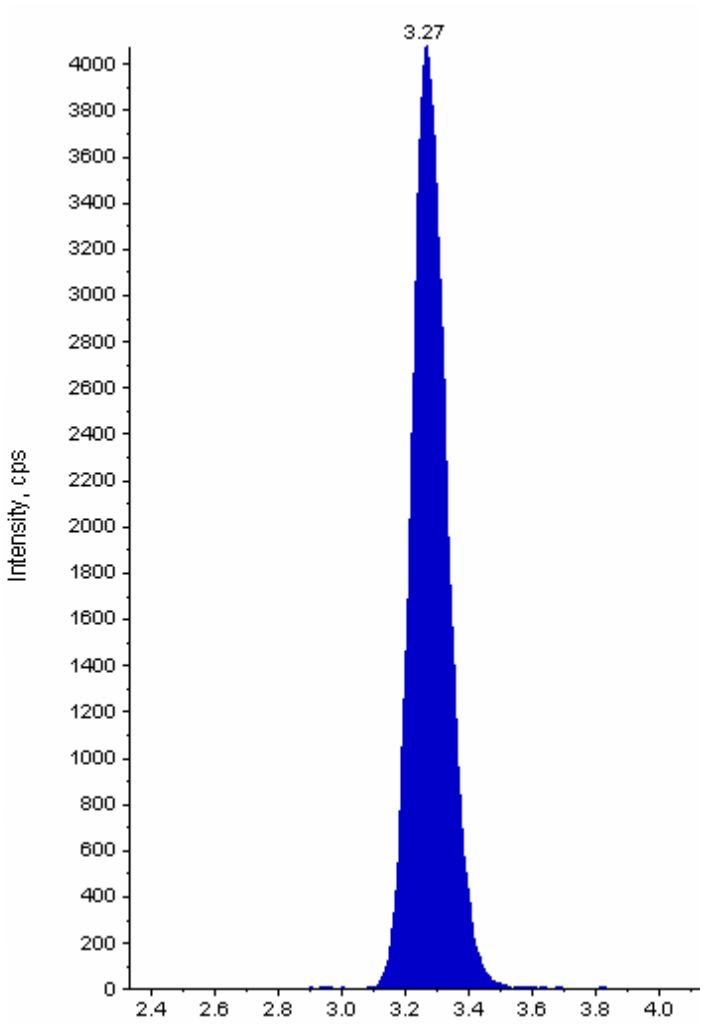
- Peptides can be difficult to fragment under collision induced dissociation (CID) within the quadrupole instrument
  - All or nothing
- Collision cell pressure (amount of collision gas) is more critical than the collision energy of the ions to increase the efficiency of the desired fragmentation

General Strategies

- Small peptides
  - Small peptides (up to approx. 3 kDa)
  - TFA often used in place of solvent precipitation - denaturation step prevents protein binding
    - Many peptides are insoluble in high levels of methanol or acetonitrile
  - Off-line SPE using mixed mode ion-exchange
  - Sample concentration under temperature and nitrogen
  - Use of RP or HILIC UPLC

General strategies





LLOQ 5 ng/mL

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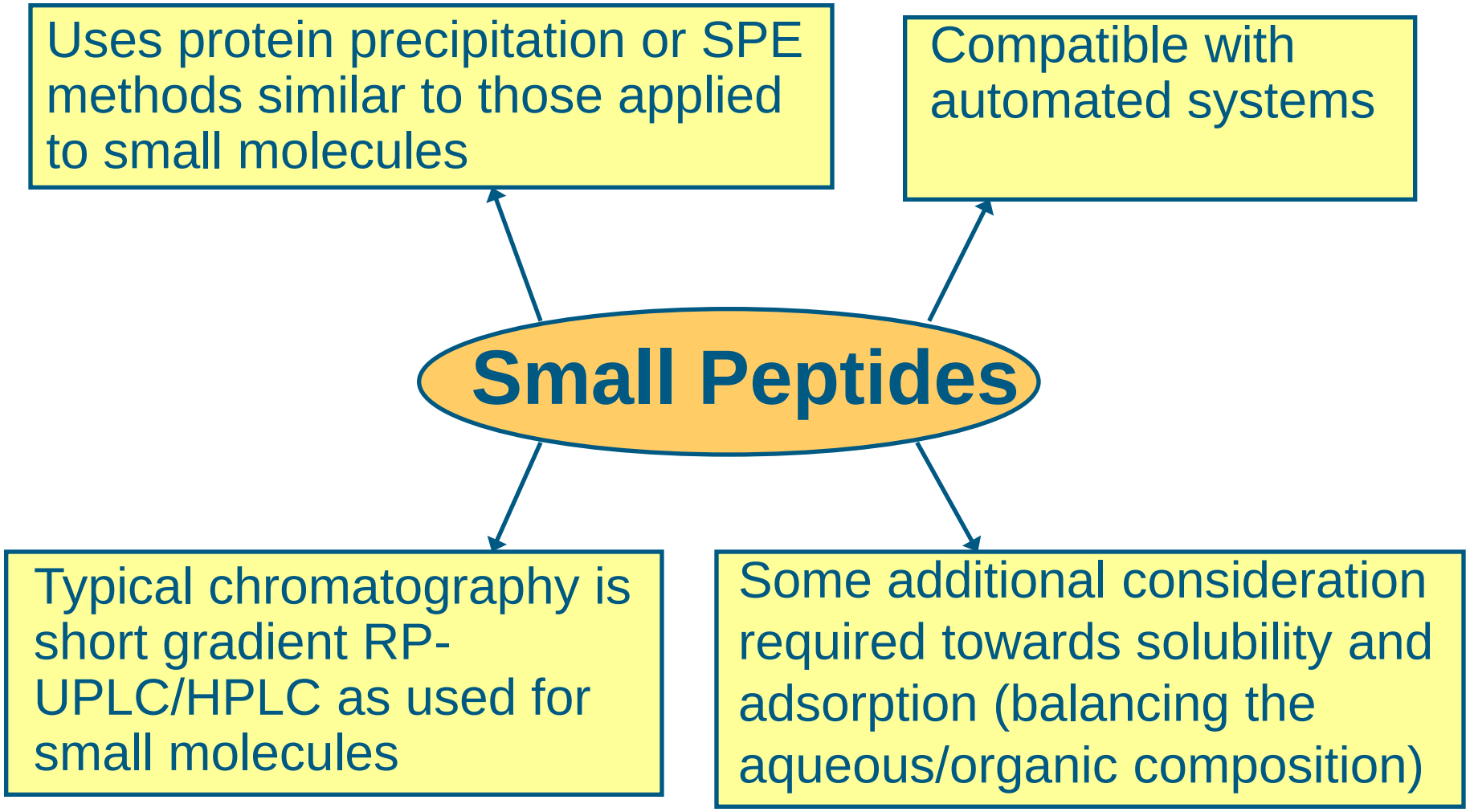
Approach to Small Peptides



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Why the need for a second approach?

- Insufficient sensitivity for larger peptides
  - Multiple charge states mean lower sensitivity and greater need for high sample and loading volumes
  - Greater sample and loading volumes requires additional cleanup
- Insufficient cleanup for larger peptides
  - Larger peptides are often quite polar and are difficult to separate from endogenous contaminants using conventional approaches

General Strategies

- Mid-mass range peptides
  - 3 to 10 kDa
  - Off-line SPE using mixed mode ion-exchange
  - No concentration of sample by the use of temperature or pressure
    - Peptides are susceptible to adsorption
  - On-line SPE
    - aid sample concentration, maximise sample loading and further clean-up
  - Analytical column with reverse phase TFA gradient

General strategies

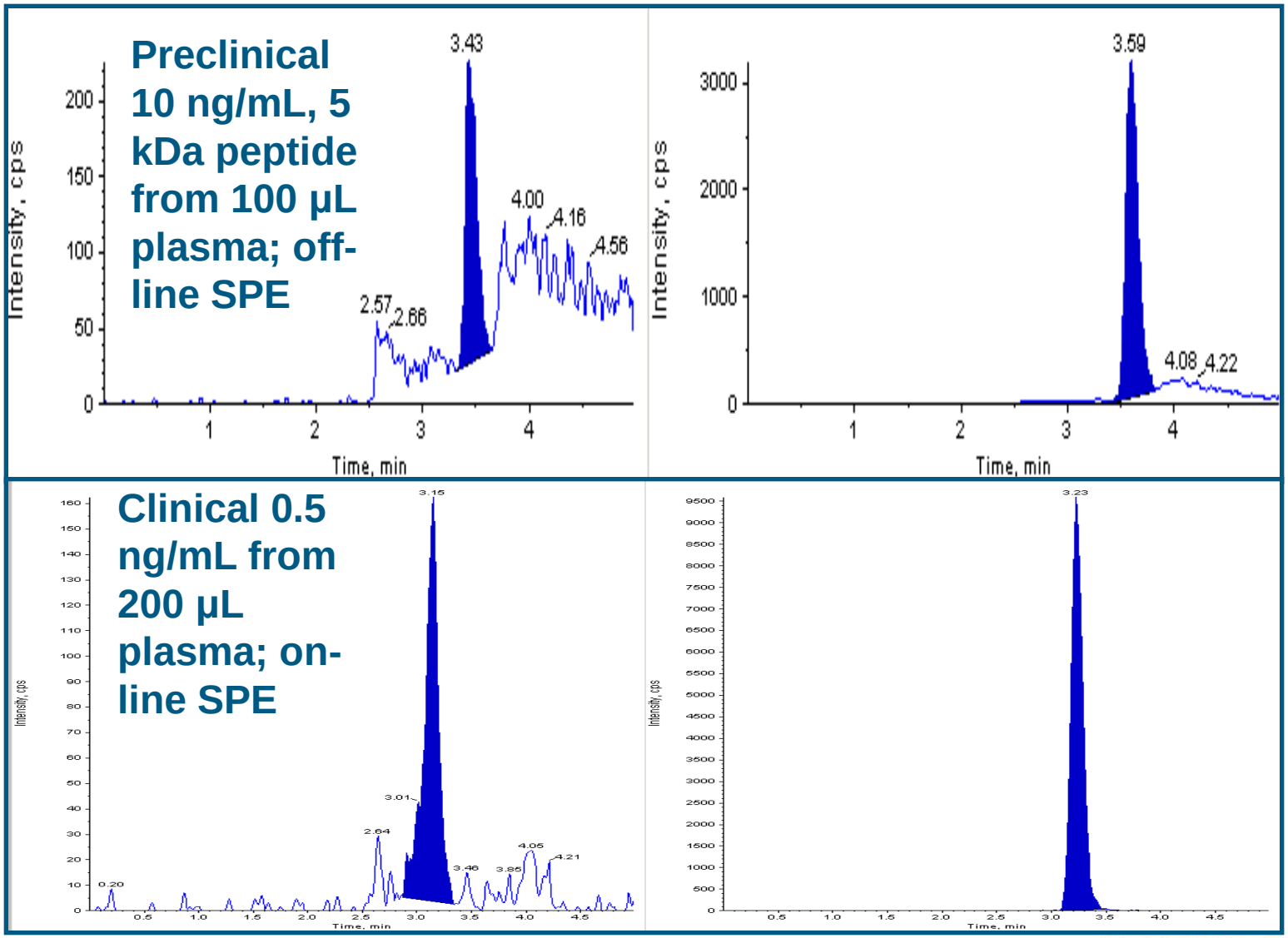
Protein binding is common & is resolved by:

- Incubation with guanidine prior to SPE
- Incubation with 10% phosphoric acid prior to SPE
- Protein precipitation with 10% TFA

Ensure that aqueous:organic mixture is optimised

- Keep peptide in solution
- Prevent adsorption to vessels

5 kDa Peptide in Plasma



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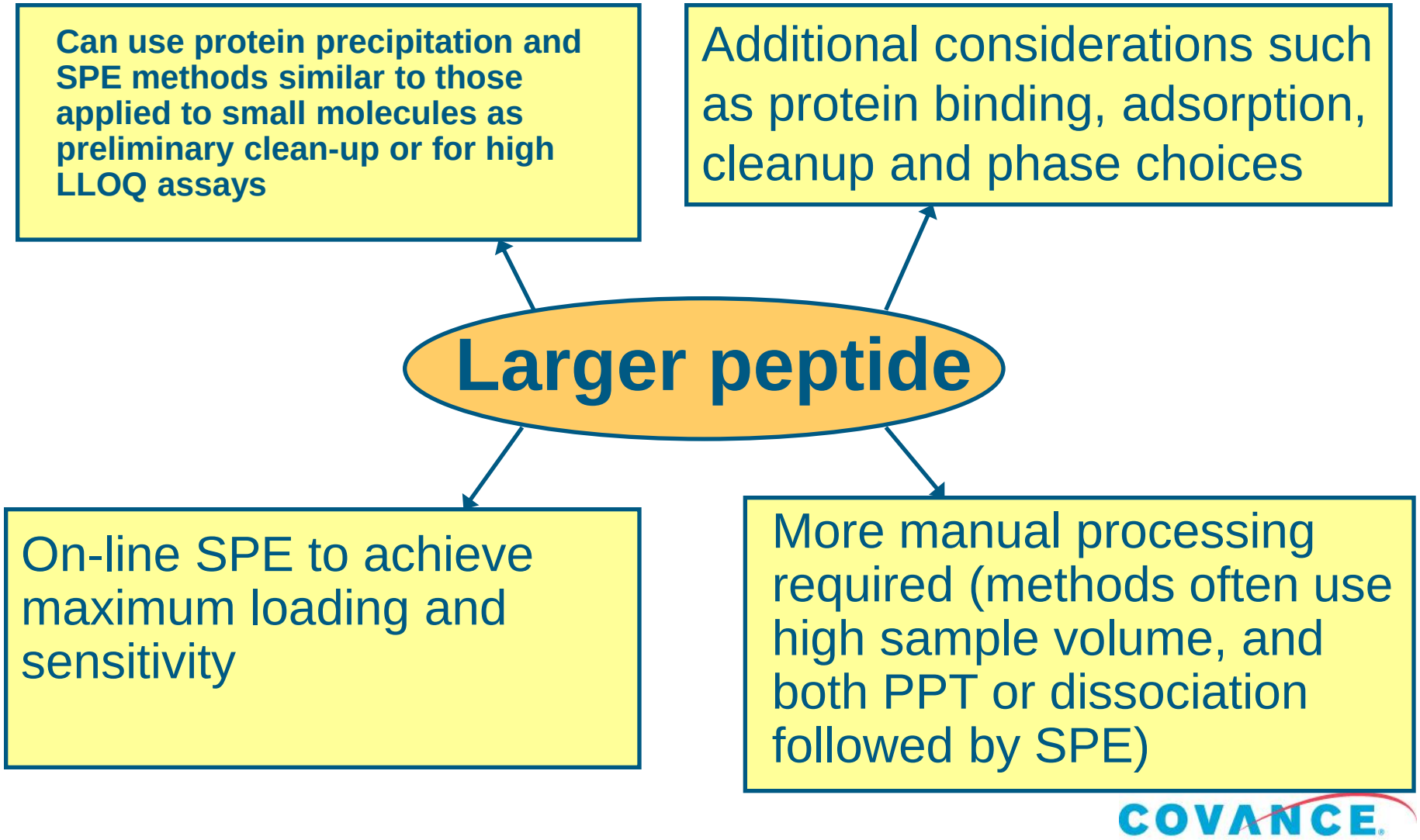
Approach to Larger Peptides



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Large Peptides/Proteins

- Recent increase in large biomolecules has led to an interest in LC-MS/MS approaches for quantification
- Immunoassay is limited by the requirement for, and the selectivity of antibodies
- LC-MS/MS allows sensitive and selective quantification of small peptides, however, sensitivity for larger molecules is compromised by many multiple charge states forming
- Therefore tryptic digestion is used, followed by analysis of ‘proteotypic’ surrogate peptide sequence(s).

General strategies

- Proteins
  - Proteins (>10 kDa)
  - Digest the peptide with trypsin at arg and lys residues
  - Identify the smaller characteristic peptide fragments (surrogate peptide)
  - Quantitate using the surrogate peptide from the target polypeptide by LC-MS/MS - treat as ‘approach 1 or 2’!

Proteolytic Digestion and Surrogate Peptides

Denature pure protein standard and digest

↓

Infuse on MS/MS system

↓

Look for predicted surrogate peptides

(typical masses 500-3000, usually M+2 ions formed)

↓

Develop LC-MS/MS method for chosen surrogate

↓

Apply method to quantify plasma samples

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Tryptic Digestion of Lysozyme

Mol. Wt 14.3 kDa.

Denature 500 µL sample

Abundant Protein Depletion
(Oasis HLB)

Digest

Surrogate peptide

Cleanup by manual SPE (Oasis HLB)

Dilute out Eluate with 0.1% TFA in water containing 0.01% Triton X-100

Load onto Symbiosis system

Choice of internal standard

Optimising denaturation

Choice of surrogate peptide

Optimise cleanup

Optimise Symbiosis

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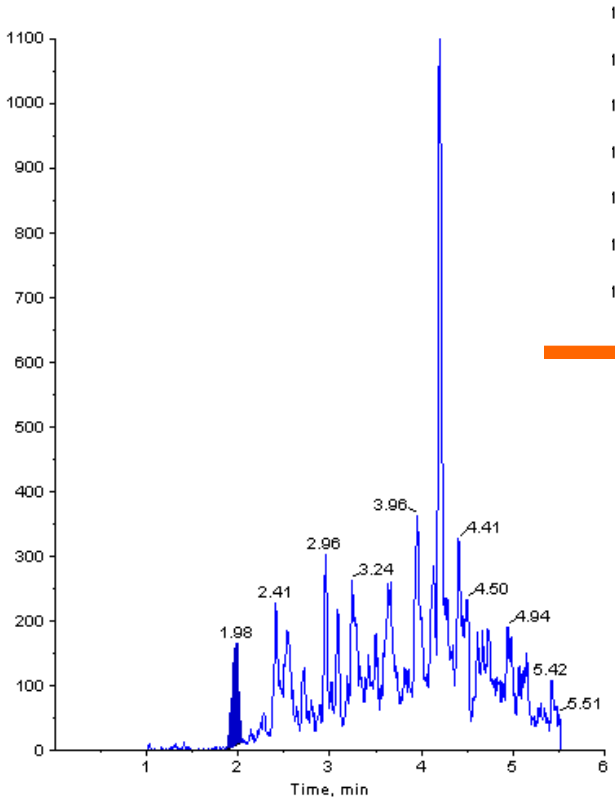
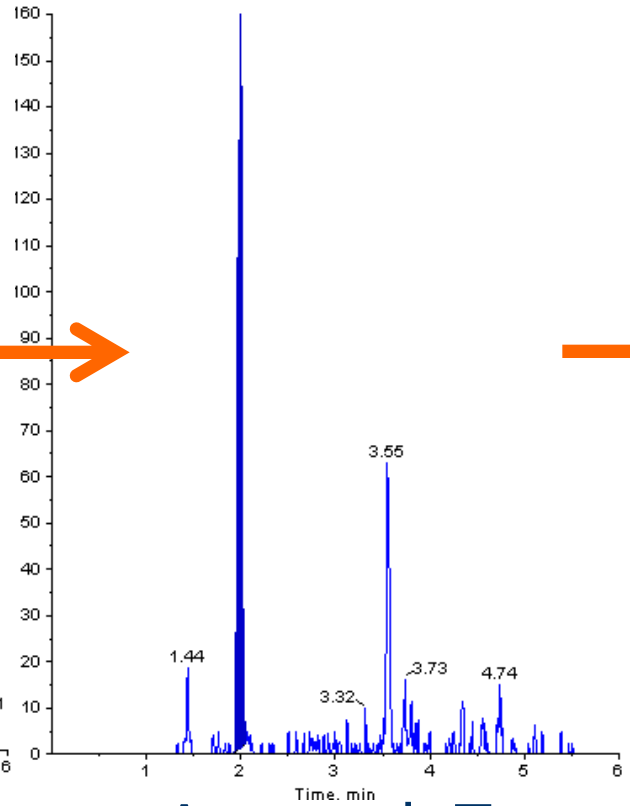
Refinement of Method for Lysozyme in Plasma



Approach One

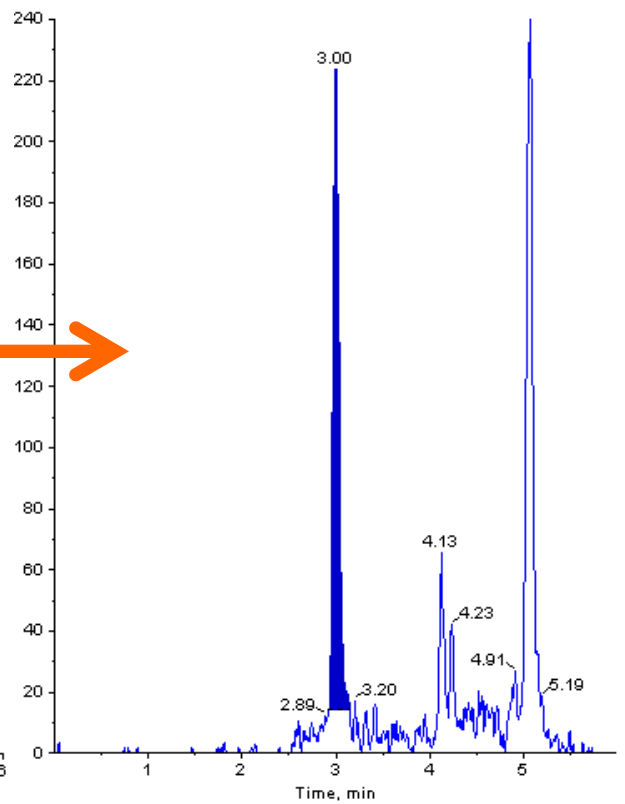
Initial method 5000 ng/mL (350 nM)



Approach Two

Protein Depletion

500 ng/mL (35 nM)



Approach Three

Symbiosis (larger volume extracted)

50 ng/mL

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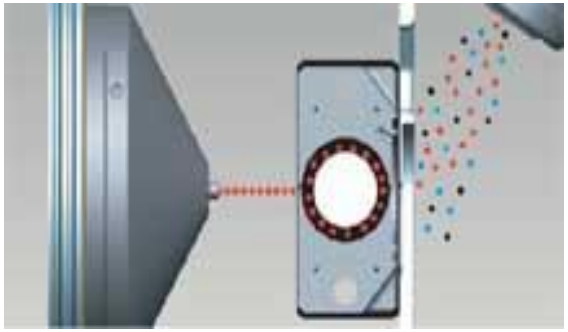
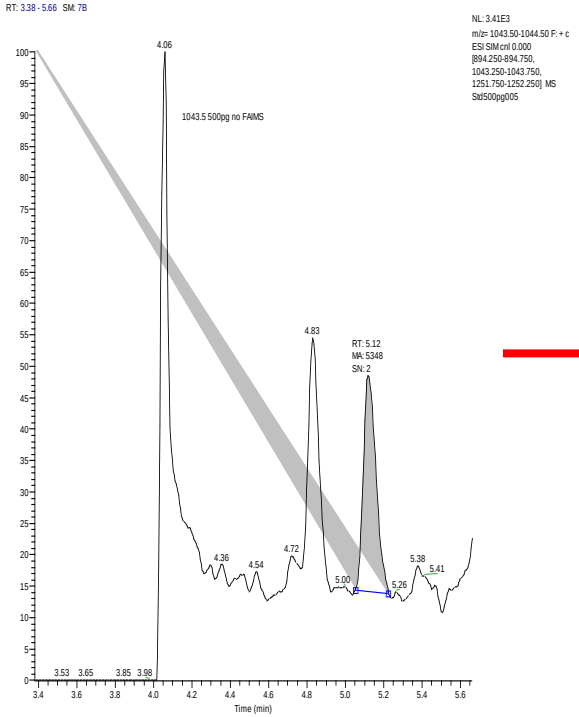
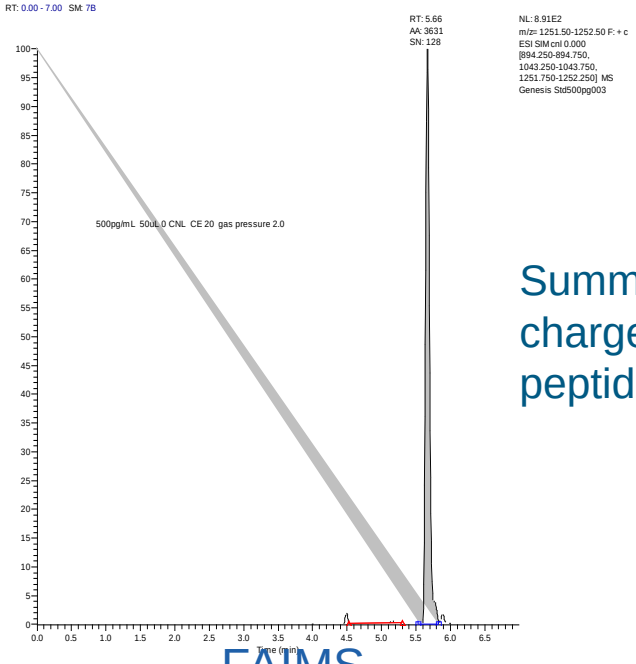
High Field Asymmetric Waveform Ion Mobility Spectrometry



- Acts as an ion filter between the source and MS:
  - Removes singly charged interferences
    - *eg lipids, plasticisers*
  - Allows polycharged ions through, increasing specificity



No FAIMS



FAIMS

Summing +5, +6 and +7 charge states of a 6.2 kDa peptide

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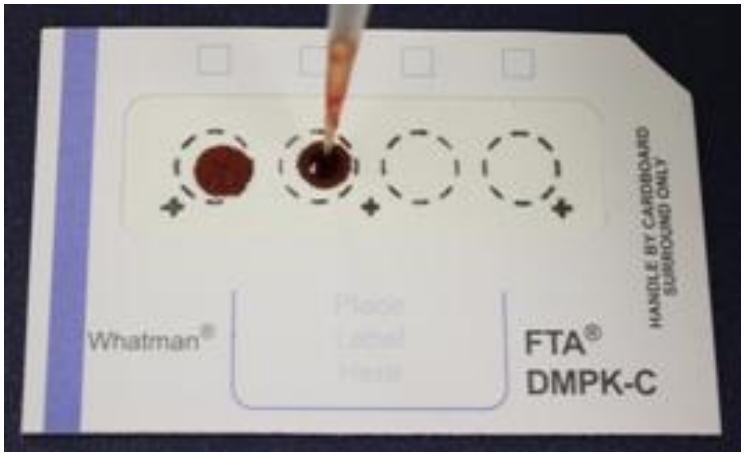
Potential benefits of DBS for peptides

- Peptides often degrade or protein bind in liquid samples
  - Proteolytic degradation
  - Enzymic modification
- Blood spot cards are treated with a variety of surfactants and enzyme inhibitors to lyse cells and inhibit enzymes
  - Whatman FTA DMPK-A and B
    - (Treated cards)
  - Whatman FTA DMPK C
    - (Untreated cards)
- DBS sampling may prevent degradation



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Challenges for DBS of Peptides

- Achieving 10 ng/mL sensitivity (same as the plasma LLOQ) with only a fraction of the equivalent sample volume from a blood spot punch.
  - 20 µL spot approximate diameter 7mm
  - 4 mm punch equivalent to only 6.5 µL volume (65 pg ramoplanin)
- Achieving recovery of the peptides from the bloodspot cards without compromising their solubility
  - Balancing aqueous and organic composition may be critical
- Combating the usual challenges of peptides, including adsorption and poor MS/MS fragmentation

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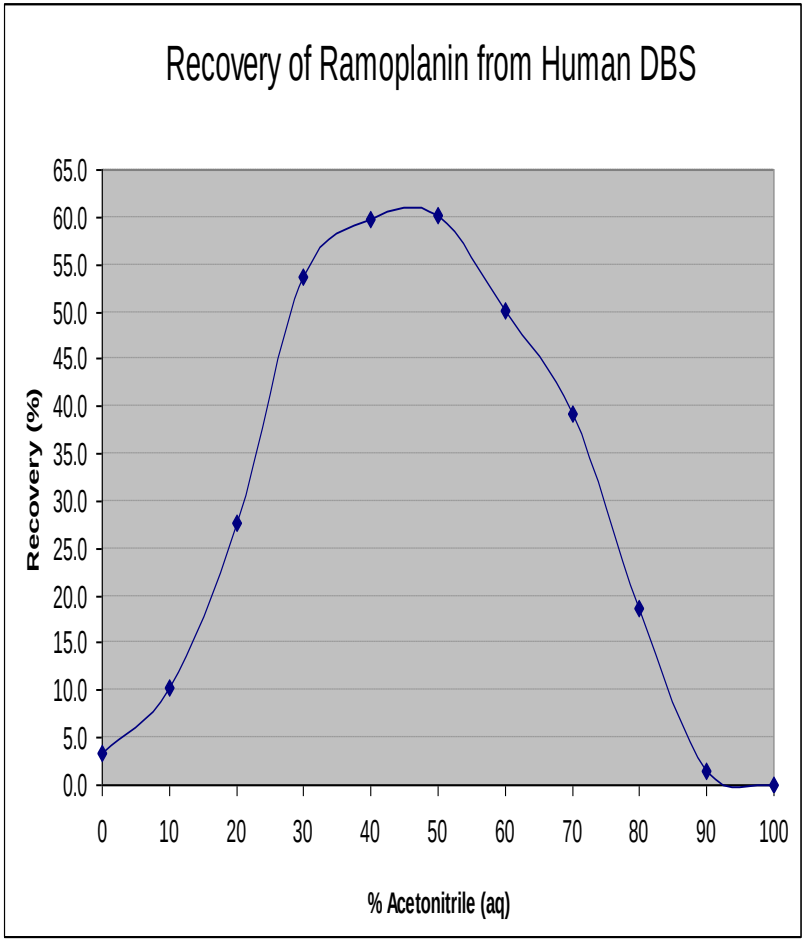
Results of Optimisation

- 4 mm punch of sample added to 1 mL 96-well plate.
- 100 µL of water: acetonitrile (50:50 v/v) containing 1000 ng/mL Teicoplanin added (blanks receive blank solvent instead).
- Stand plate for 20 minutes then vortex mix for 90 minutes.
- Centrifuge plate and remove 60 µL supernatant to 1 mL well plate.
- Add 40 µL 10 mM ammonium formate + 0.2% formic acid.
- Cap plate, vortex mix, centrifuge and inject (50 µL)



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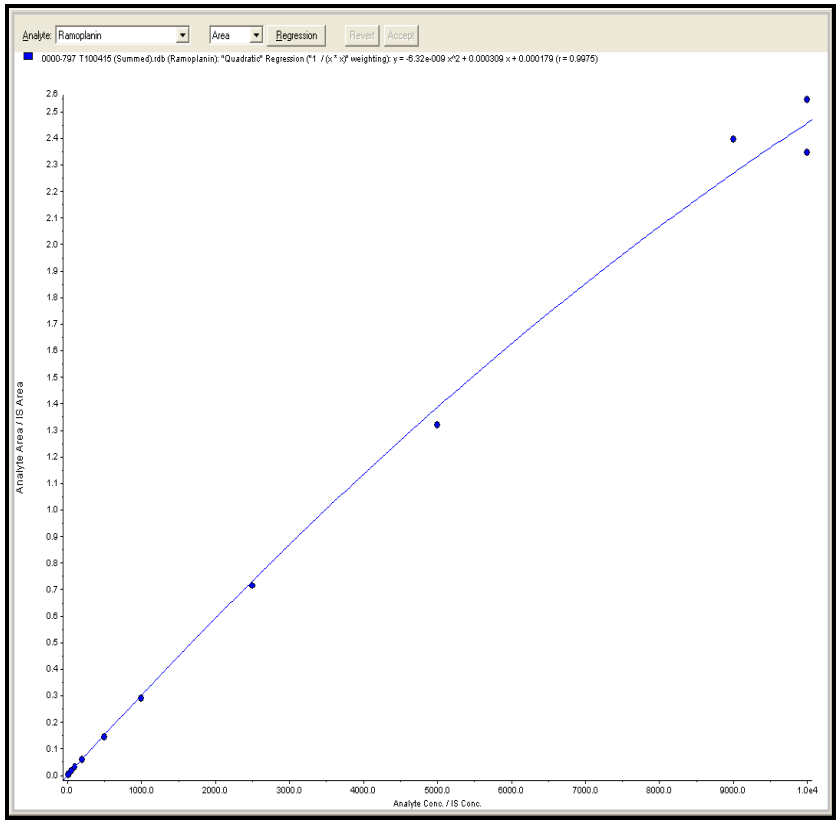
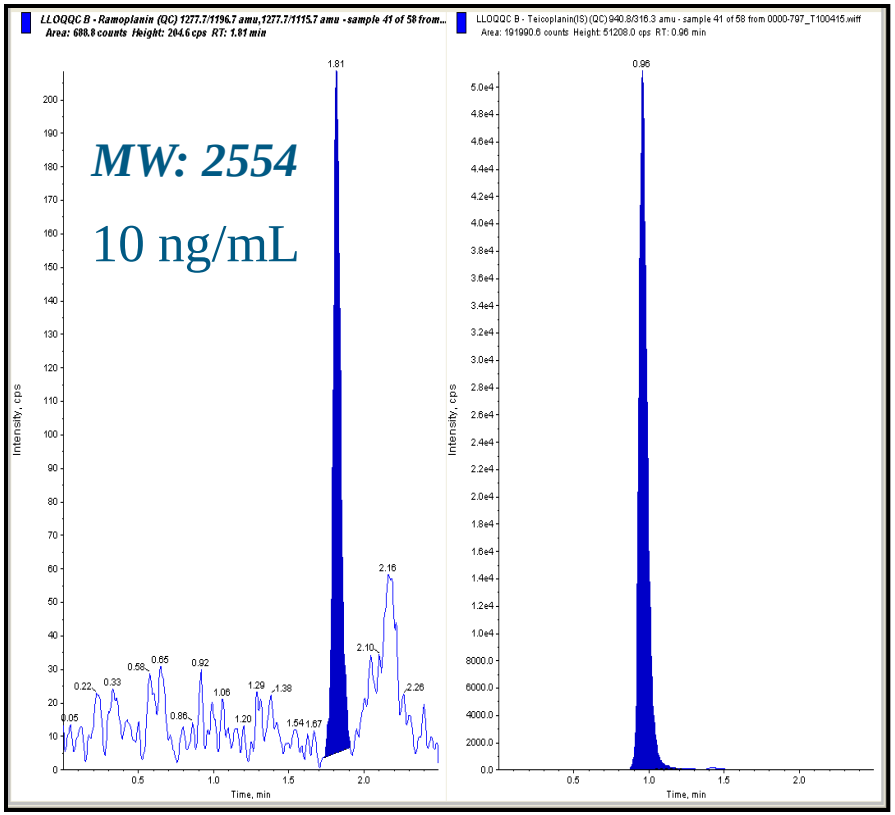
DBS of Ramoplanin





Calibration Curve 10-10000 ng/mL

Validation of a bioanalytical method for the quantification of a therapeutic peptide, ramoplanin, in human dried blood spots using LC-MS/MS. Matthew F. Ewles, Phillip E. Turpin, Lee Goodwin, David M. Bakes; Biomedical Chromatography: *In Press*

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Summary

- Sample Preparation
  - Very different from small molecules
  - Binding, solubility and adsorption effects can all be significant
  - Use of mixed mode ion exchange
- Chromatography
  - Important considerations: column chemistry, ionic modifier, flow rate, gradient rate and column temperature
- The best analytical combination:
  - Mixed mode ion exchange followed by reverse phase chromatography (Analysis based upon 2D separation)
  - HILIC chromatography for small peptides
- Detection
  - Electrospray for quantitative work, but there are limitations with fragmentation, charge distribution and selectivity with increasing mass

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