Quotient Bioresearch

Challenges in validating protein and peptide LC-MS/MS methodologies

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Quotient Bioresearch

Introduction

- Quotient Bioresearch's BioMS™ Platform
- Sensitivity
- Tryptic Digestion Efficiency
- Test Materials
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Quotient Bioresearch's BioMS™ Platform

- Quotient Bioresearch's BioMS™ platform has been used widely over recent years
- Quotient Bioresearch has significant experience of regulated (GxP) bioanalysis of both proteins and peptides
- Small molecule LC-MS/MS acceptance criteria has been applied to numerous peptides & proteins in a range of biofluids & tissues using uHPLC coupled to a Sciex API5000 or API5500

Protein or Peptide?

- An amino acid chain, measureable by LC-MS/MS without Tryptic digest, is treated as a peptide

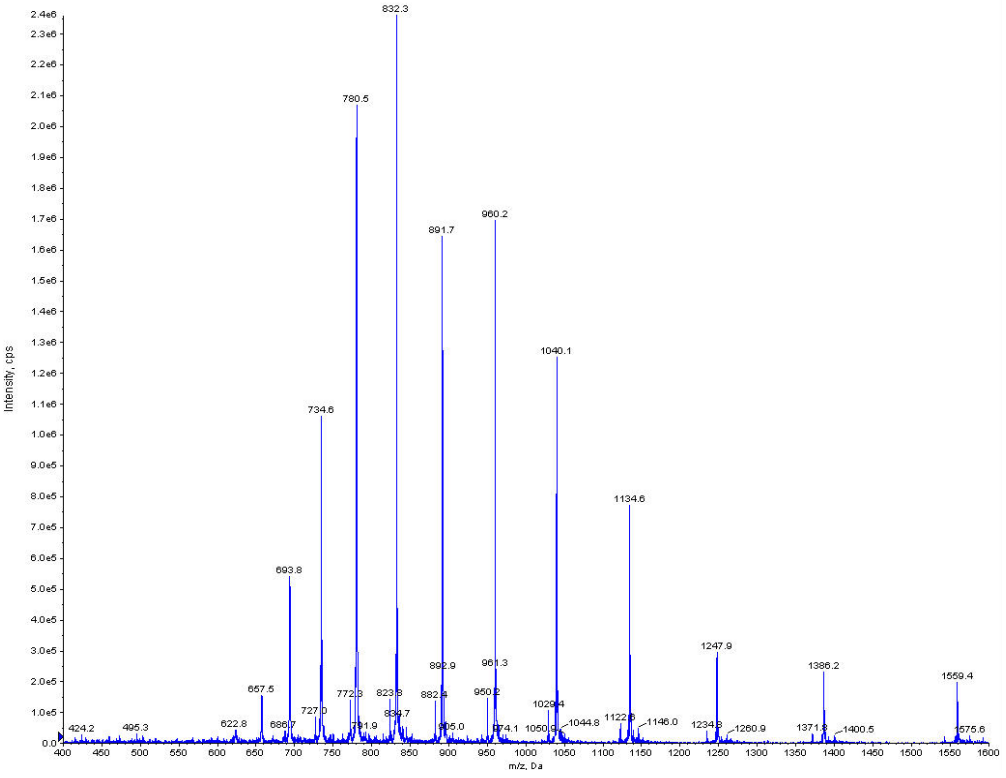
- An amino acid chain, only measureable by LC-MS/MS following Tryptic digest, is treated as a protein

Sensitivity; Peptides & Proteins

- Standard a acceptance criteria is that peaks at the LLOQ must be at least 5x the response from blank samples
- Sensitivity of proteins & peptides by LC-MS/MS is perceived to be a significant problem
- Absolute concentrations (ng/mL) are inherently lower than that of small molecules due to the molar concentration of the analytes; 1 mg of protein will generate fewer ions than 1 mg of small molecule
- Proteins may also require a Tryptic digest, which may result in relatively low yields

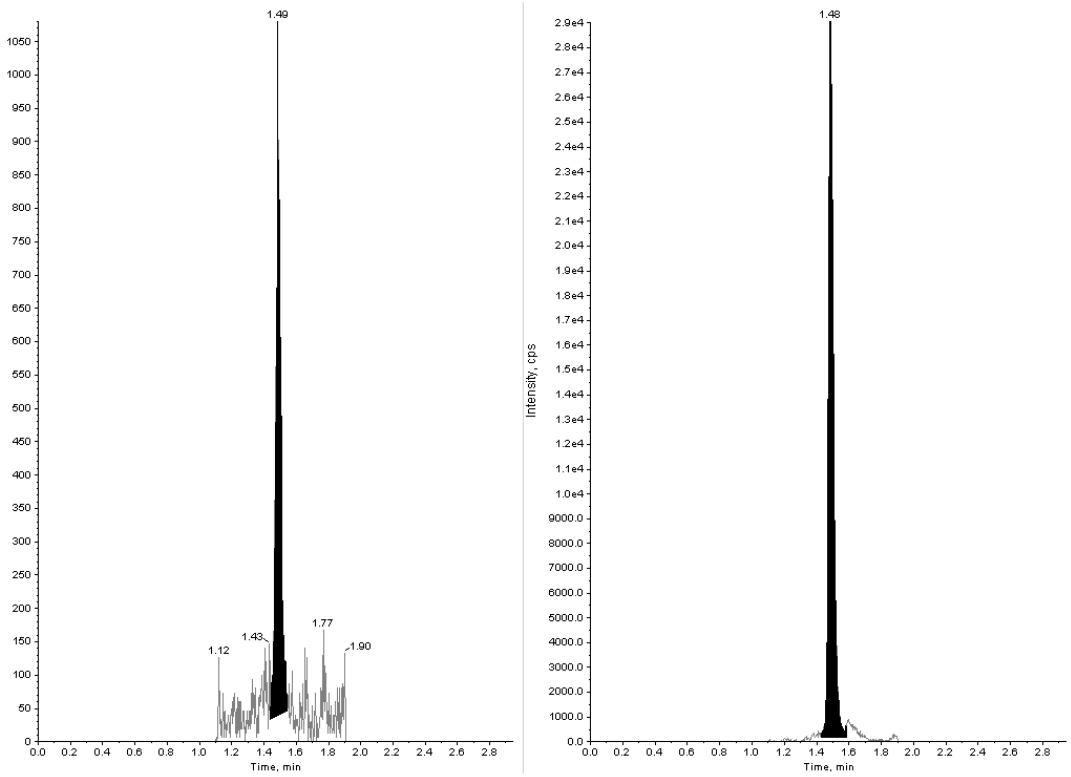
Sensitivity; Peptides & Proteins

- Peptides become multiply charged in the ion source of the mass spectrometer
- LC-MS/MS response is divided between several charge states
- It is possible, although not always advantageous, to sum multiple ions



Sensitivity: LLOQ's

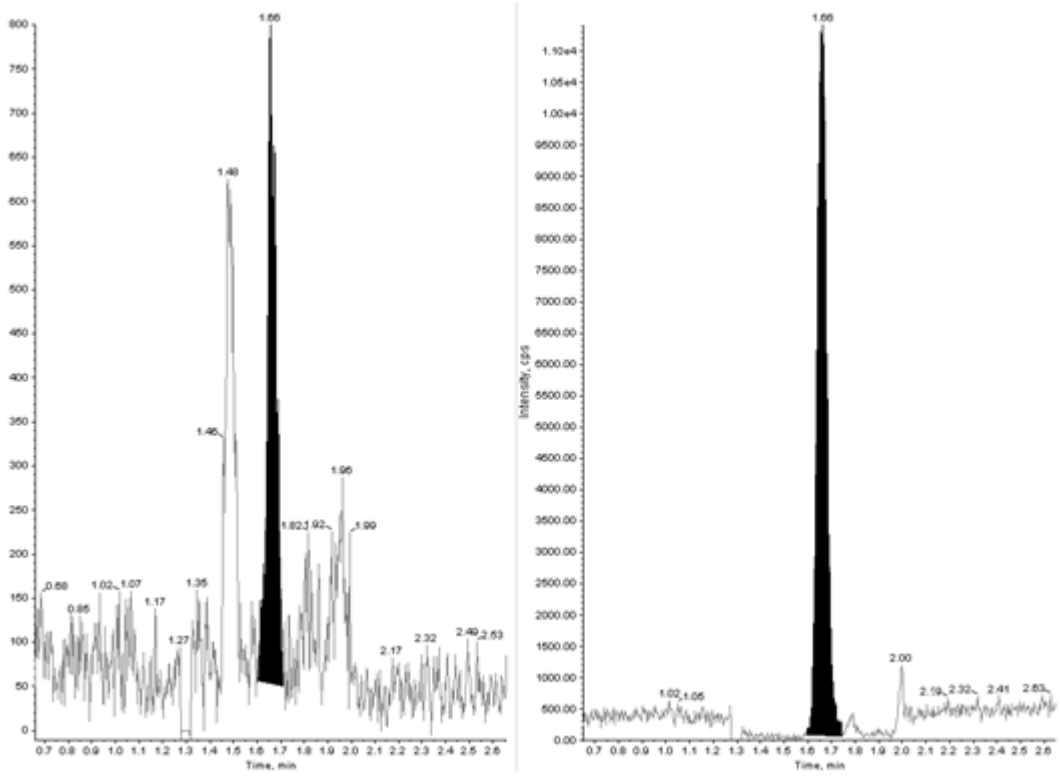
- LLOQ's have been reduced with the use of uHPLC
- Aim for LLOQ peaks with a signal to noise ratio of >10:1
- Quotient Bioresearch have successfully quantified peptides at 0.1 ng/mL



Sensitivity: LLOQ's

- Quotient Bioresearch have quantified proteins at 1.0 ng/mL

- Resolution of uHPLC chromatography allows baseline separation of peptides with similar retention times



Tryptic Digestion; Proteins

- Current mass spectrometers are able to measure mass charge ratios of up to 2700 *m/z*

API4000 / 4000 QTrap	API 5000 / 5500	API5500 QTrap	Waters Xevo
2700 *m/z*	1250 *m/z*	1000 *m/z*	2048 *m/z*

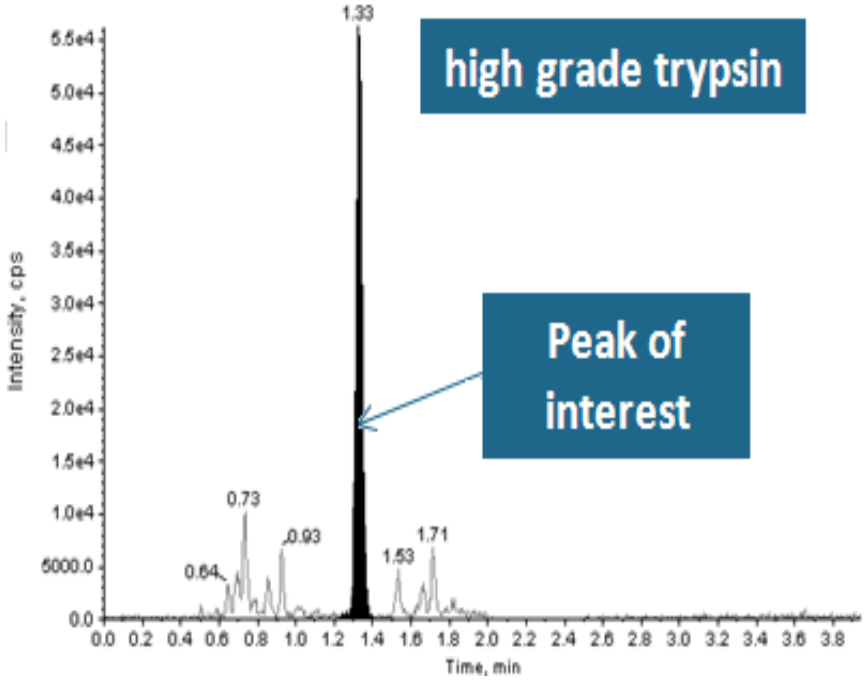
- Digestion by Trypsin or Chymotrypsin is used to form signature peptides with a *m/z* ratio within the dynamic range of the mass spectrometer

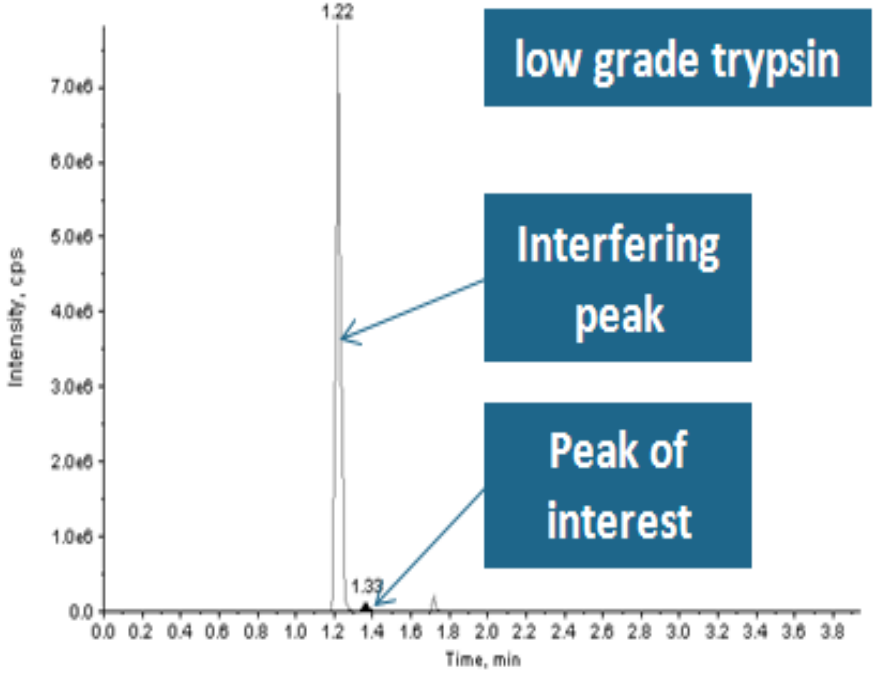
- Unique signature peptides selected based on sensitivity and more importantly specificity

- Quotient Bioresearch recommend measurement of only one well characterised and specific signature peptide per analyte

Tryptic Digestion; Proteins

- Vital to optimise digestion conditions at an early stage;
  - Time to completion
  - Required enzyme concentration
  - Enzyme type & source/quality





Test Materials; Peptides & Proteins

- Short peptides can be synthesised relatively easily & quickly, although it is not always feasible to synthesise very large quantities of test materials

- Purity of test materials is a common problem; even specially synthesised peptide/proteins can sometimes yield a test material with a low purity

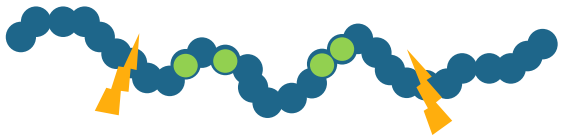
- Proteins should not be quantified against peptide calibration curves;
  - Absolute concentrations will be effected by Tryptic digest
  - This approach may be suitable for endogenous proteins or pharmacodynamic assays

Internal Standards; Peptides

- Like small molecule analysis, the use of a labelled internal standard is vital for accurate and reproducible analysis by LC-MS/MS
- Labelled peptide internal standards (Peptide IS) are available and behave well
- Peptide IS can only be chosen once the initial optimisation of analyte *m/z* transitions has been made and the charge state of the analyte is known
- It is vital to insure significant mass difference between analyte peptide and IS peptides

Internal Standards; Proteins

- Synthesis if entire labelled proteins is not usually feasible
- Prohibitively expensive and difficult to synthesise
- Extended peptides are feasible and will offer some compensation for protein digestion efficiency in most cases



Labelled Tryptic peptide compensates for digestion efficiency

Internal Standards; Proteins

- Quotient Bioresearch's BioMS™ platform typically measures protein analytes vs. peptide internal standards

- Careful consideration must be given to the point in the sample extraction that Tryptic digestion occurs;
  - Digestion post extraction gives cleaner extracts but higher variability
  - Digestion pre-extraction gives lower variability but may lead to interfering peaks at the retention time of interest

- Introduction of a labelled peptide post digestion is an absolute minimum; this should still compensate for volumetric differences in sample preparation and LC-MS/MS variability

Stability

- Proteins and peptides are often susceptible to biological breakdown
- Sequential cleavage of amino acids form either the C or N terminal is a commonly observed problem in biological matrix
- Stability can be improved by addition of specific stabilisers and by handling samples at low temperatures
- Will be discussed further by Richard Kay

Binding

- Peptides and proteins will often adhere to experimental equipment
- Should be considered as soon as possible and investigated during he development phase
- Can be a problem in solvent reference samples (e.g. SST or other reference solutions)
- Bovine Serum Albumin (BSA) may be used in excess to prevent binding of proteins and peptides to experimental materials

Sample Preparation

- Sample preparation should be optimised in the same way as any LC-MS/MS small molecule method
- Reference peptides (for protein assays) are useful for early method development experiments
- Protein recoveries may be increased by a reduction / alkylation step prior to Tryptic digest (i.e incubation with DTT (dithiothreitol) for *ca*. 60 minutes)
- Quotient Bioresearch have not seen any reduction in uHPLC column lifetimes even with complex peptide digests following protein precipitation extraction

Precision & Accuracy; Peptides & Proteins

- Standard small molecule criteria is achievable (i.e. ±15% accuracy & precision & ±20% at the LLOQ)

Typical validation data from a peptide (4.3 kDa), using labelled peptide IS

	LLOQ QC	LOW QC	MED QC	HIGH QC
	0.5 ng/mL	1.25 ng/mL	12.5 ng/mL	200 ng/mL
Intra-run Mean	0.536	1.43	12.1	189
Intra-run SD	0.0303	0.107	0.553	7.81
Intra-run %CV	5.7	7.5	4.6	4.1
Intra-run %Bias	7.2	-4.7	-3.2	-5.5
n	6	6	6	6
Intra-run Mean	0.560	1.56	12.6	200
Intra-run SD	0.0296	0.166	0.455	3.54
Intra-run %CV	5.3	10.6	3.6	1.8
Intra-run %Bias	12.0	4.0	0.8	0.0
n	6	6	6	6
Intra-run Mean	0.48	1.45	12.6	200
Intra-run SD	0.0326	0.0678	0.479	5.47
Intra-run %CV	6.8	4.7	3.8	2.7
Intra-run %Bias	-4.0	-3.3	0.8	0.0
n	6	6	6	6
Inter-run Mean	0.525	1.48	12.4	196
Inter-run SD	0.0449	0.127	0.516	7.88
Inter-run %CV	8.6	8.6	4.2	4.0
Inter-run %Bias	5.0	-1.3	-0.8	-2.0
n	18	18	18	18
n	18	18	18	18

Additional Considerations

- Performed in addition to standard (GxP) small molecule validation criteria on a case by case basis

- Quotient Bioresearch's BioMS™ platform includes a number of additional validation experiments
  - Matrix effect on digestion
  - Matrix effect on ionisation
  - Trypsin/Chymotrypsin optimisation

- Synthesis and use of an analyte peptide reference standard (when quantifying proteins) is vital
  - Enables accurate assessment of digestion efficiency, matrix effects & recovery
  - Useful LC-MS/MS diagnostic tool (non-extracted SST)

Conclusions

- Small molecule GxP LC-MS/MS procedures and guidelines are valid
- A well developed protein or peptide LC-MS/MS assay should meet traditional small molecule criteria
- It is vital to select specific & sensitive characteristic peptides and internal standards
- Additional care needs to be taken to insure any digestion step is well controlled
- Careful method development and investigation of stability and binding is critical