QUANTITATIVE LC-MS/MS
Analysis of proteins and peptides

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MASS SPECTROMETRY

Overview

- Introduction: Quantitative approach to protein and peptide analysis
- Background: Instrumentation, workflow
- Assay development: Step by step
- Quantitative LC-MS/MS assay: method, results
- Common issues:
  - Matrix effect
  - Internal standard (IS)
  - Method validation
  - Analyte stability

Absolute quantitation – targeted analysis

- Quantitation on peptide level

Proteolytic digest

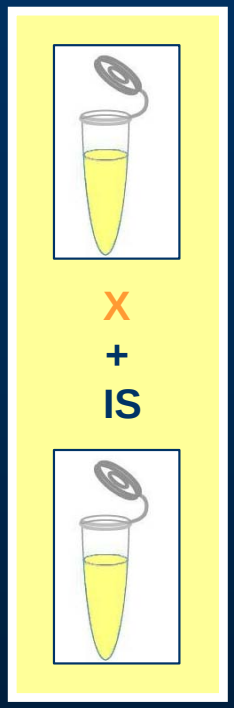
(Trypsin, chymotrypsin, AspN etc.)

- Know what you are going to quantify:
  - well characterized protein
  - protein ID via LC-IT-MS/MS
  - *in silico* digest based on theoretical AA sequence

Sample

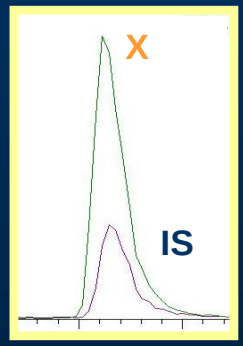
- Protein – unlimited size (gel, solution, biological matrix)
- Peptides – ideally 6-12 aa (up to 30-40 aa); may be phosphorylated
- Biological matrices:
  - plasma, serum, erythrocytes
  - cerebrospinal fluid (CSF)
  - urine
  - bile
  - cell culture media
  - plant and animal tissues (e.g. leaf, brain, liver)

Analysis and data processing overview

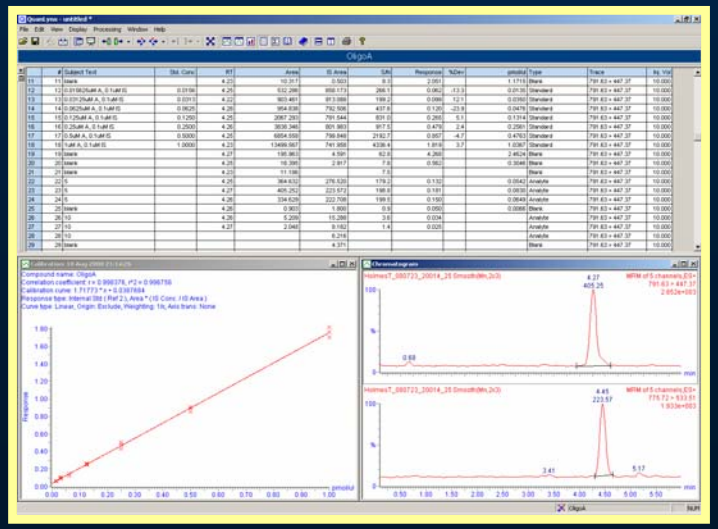


A) Analysis workflow

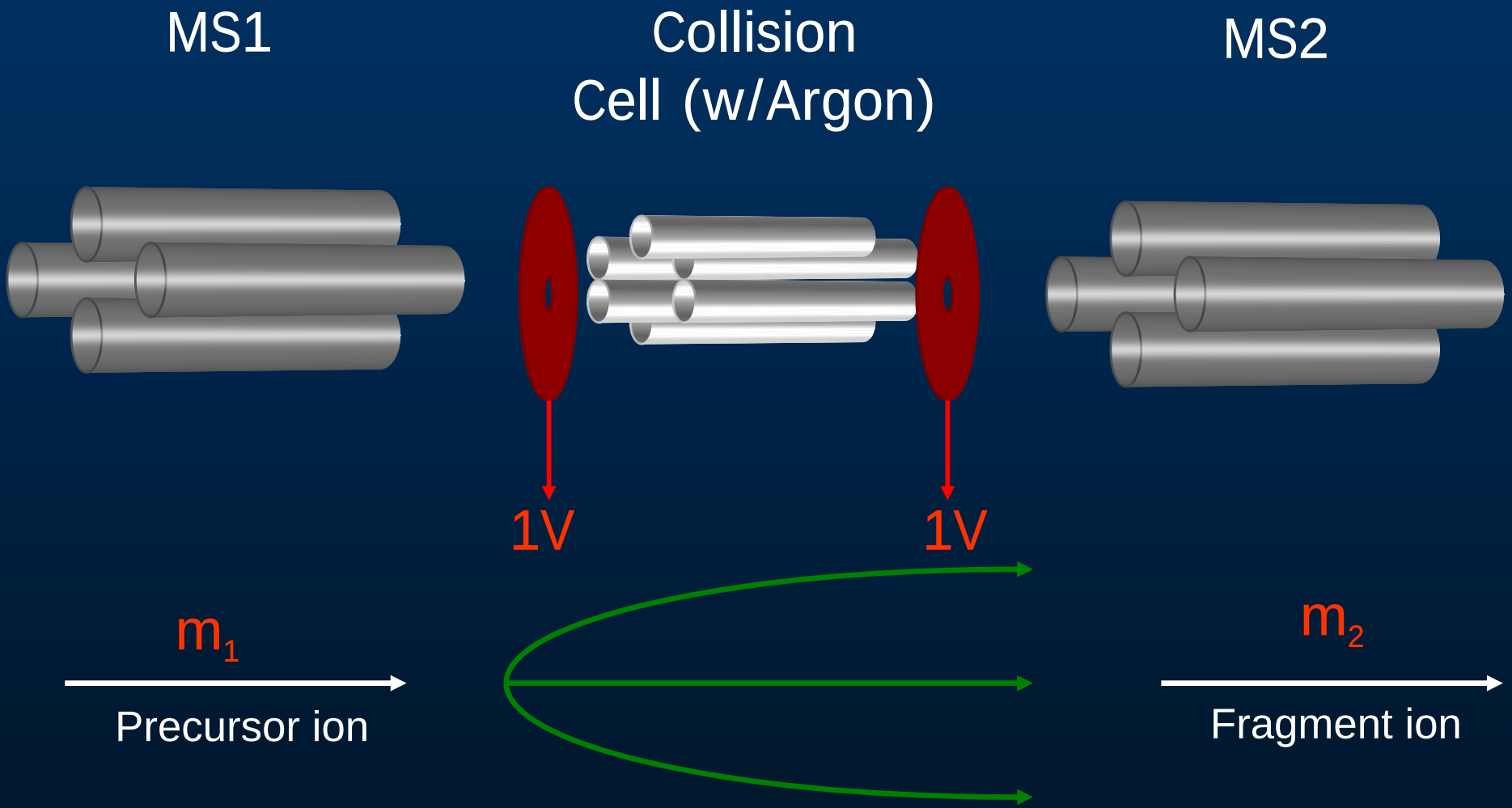
Sample Prep + digest → HPLC → Q1 → Collision cell → Q3 →



B) Data processing



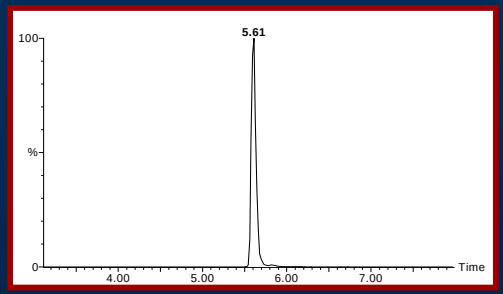
Triple quadrupole analyzer

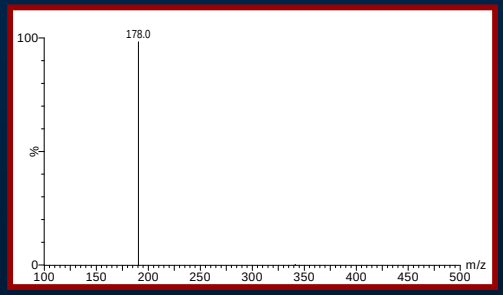


Scheme from Water Quattro Premiere Training

Advantages of SRM scanning mode

- MS/MS provides higher sensitivity and selectivity, enabling
  - Less extensive sample preparation
  - Greater sensitivity via increased selectivity
  - Use of shorter HPLC columns
  - Use of shorter run times and higher sample throughput
- Translates into time and effort savings, plus a more sensitive method





Before we start – checklist

Questions:

- What is the protein of interest? (MW, sequence, PTMs)
- Number of peptides included in the assay?
- Sample types? (matrices)
- Desired/required LLOQ and calibration range?
- Standards availability? (purified protein, peptides and labeled peptides)
- Purpose of developing the assay? (preliminary studies, confirmation of findings from different methods, publication)
- Available funding and timelines?

Peptide selection rules

Main rules:

- 6-12 AA optimal (up to 30-40aa)
- No chemically reactive residues (Trp, Met, Cys)
- No ragged ends (2xR; RK; 2xK)
- No potential PTM
- unique sequence

Also consider:

- Preferably containing P (dominant cleavages)
- If MS/MS data already available than look for peptides giving high intensity fragment ions of m/z higher than precursor ion (noise reduction)
- R in P proximity (potential missed tryptic cleavage)
- Select multiple peptides for each protein whenever possible

Protein X - fragment

Digest Table

#	Pos	Mass	pI	Peptide
1	1213.50	9.50	MAAVAALQLGLR
2	543.62	9.79	AAGLGR
3	828.93	9.79	APASAAWR
4	473.57	9.47	SVLR
5	1464.69	12.30	VSPRPGVAWRPSR
6	1082.15	5.99	CGSSAAEASATK
7	3203.69	4.03	AEDDSFLQWVLLLIPVTAFGLGTWQVQR
8	174.20	9.75	R
9	146.19	8.75	K
10	332.40	8.75	WK
11	1157.33	4.53	LNLIAELESR
12	1719.07	4.14	VLAEPVPLPADPMELK
13	1018.18	8.59	NLEYRPVK
14	273.34	9.72	VR
15	792.86	6.73	GCFDHSK
16	939.16	6.10	ELYMMPR

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#	Pos	Mass	pI	Peptide
17	816.97	5.50	TMVDPVR
18	374.40	6.10	EAR
19	2654.93	5.32	EGGLISSSTQSGAYVVTPFHCTDLGK
20	146.19	8.75	K
21	714.78	5.97	VNPETR
22	274.32	8.75	QK
23	1515.74	4.14	GQIEGEVDLIGMVR
24	618.69	6.00	LTETR
25	1326.43	4.53	QPFVPENNPER
26	911.98	8.76	NHWHYR
27	804.92	4.37	DLEAMAR
28	2744.06	4.37	ITGAEPIFIDANFQSTVPGGPIGGQTR
29	487.60	9.72	VTLR
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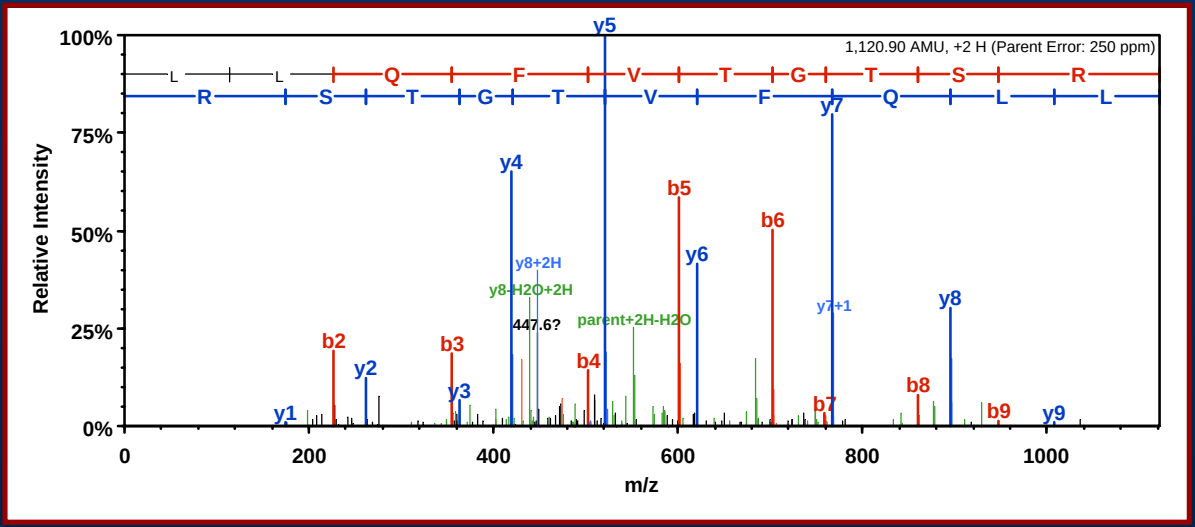
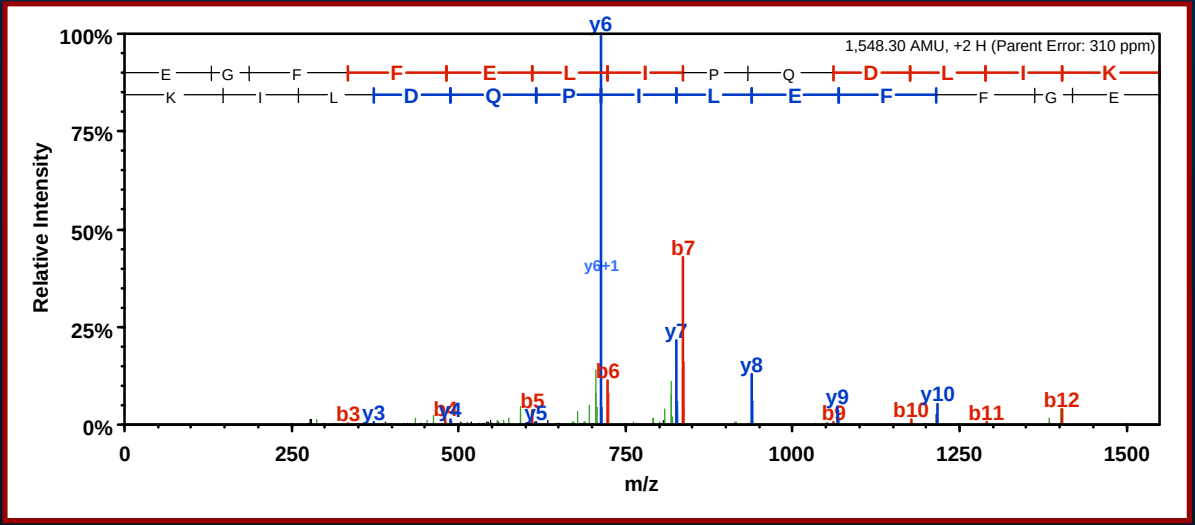
Peptide selection – "good spectrum"



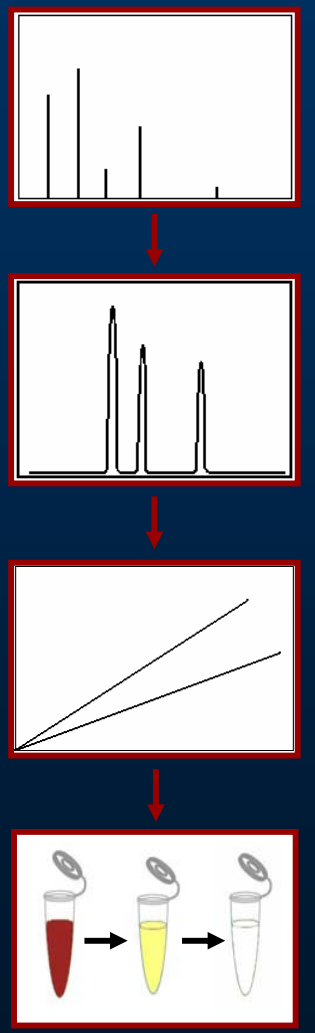
- Excellent sequence coverage
- High dispersion of energy
- Good for peptide ID



- Good sequence coverage
- Low dispersion of energy
- Good for quantitation

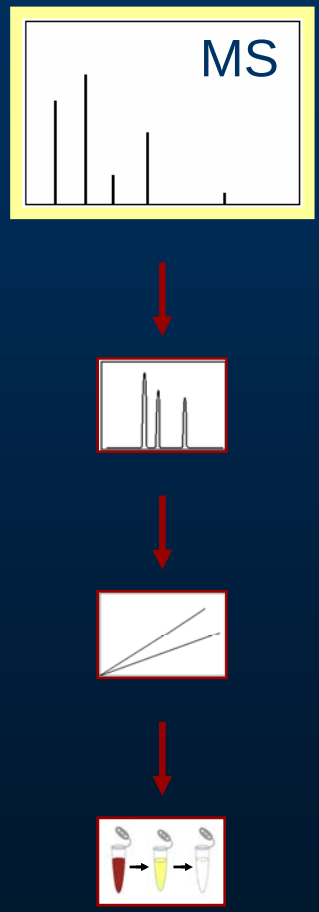
Assay development outline

- Mass spectrometry (MS)
- Liquid chromatography (LC)
- LC-MS/MS method optimization and characterization
- Sample preparation



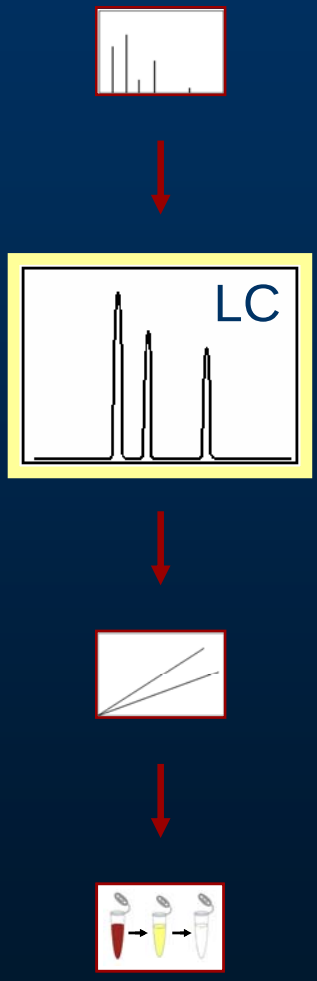
Method development - mass spectrometry

- Acquisition of MS and MS/MS spectra for standard solution of the peptide
  - 10-50µM,
  - direct infusion
- Precursor ion – fragment ion MS parameters optimization
  - most efficient ionization; precursor ion preferably +2
  - most efficient fragmentation; fragment ions preferably with m/z higher than precursor ion
- Method set up and test run
  - Choose three precursor ion – fragment ion pairs for each peptide



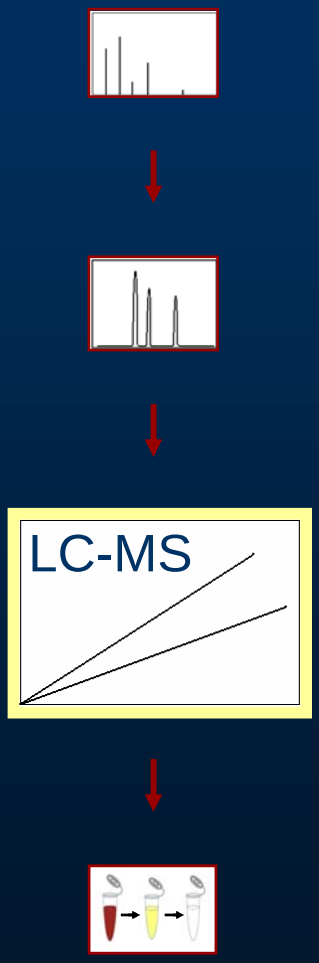
Liquid chromatography

- HPLC column
  - RP type column – C18
  - Retention
  - Separation – usually no need to have a base peak separation
  - Gradient elution
  - MS compatible solvents and buffer modifiers (0.1% FA in water, 0.1% FA in acetonitrile)
  - Shortest runs possible (usually 4-8 min per injection)



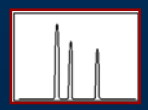
LC-MS method optimization & characterization

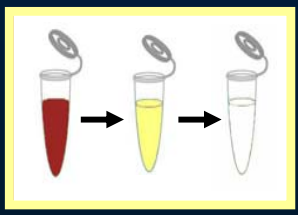
- Linear calibration curve
- Limit of detection (LOD)
  - 3:1 signal-to-noise ratio
- Limit of quantitation (LOQ)
  - 10:1 signal-to-noise ratio
- Sample matrix interferences
  - analysis of blank sample matrix spiked with pure standard
- Carryover
- Stability of the analyte



Sample preparation

- Objectives:
  - Isolating analyte from matrix
  - Removing contaminants, desalting
  - Concentrating analyte if necessary
  - Reconstituting in appropriate LC-MS compatible reagent
- Extraction methods:
  - Prior to proteolytic digest
    - Protein precipitation
      - Methanol
      - Acetonitrile
    - Immunoprecipitation
  - After proteolytic digest
    - Solid Phase Extraction (SPE)
      - C18
      - Ion exchange (e.g. SCX)
  - Combination of the above

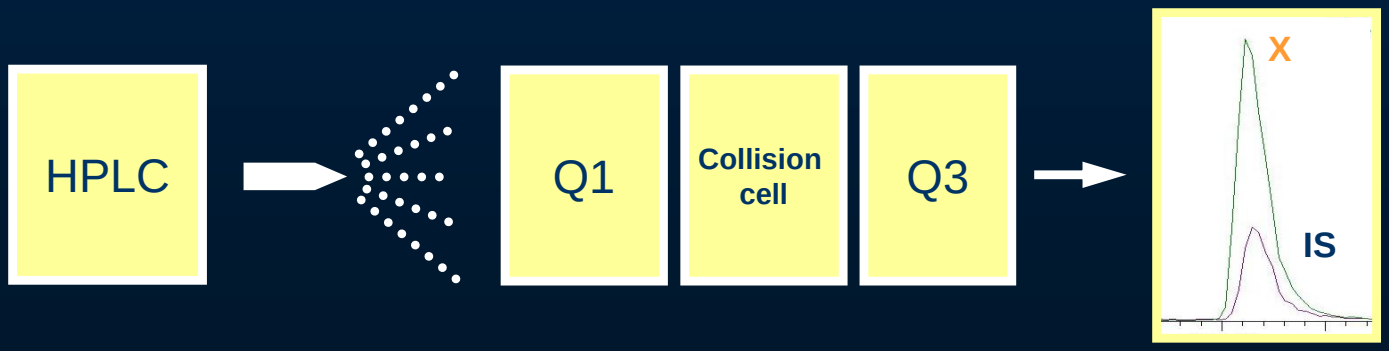





Quantitative LC-MS/MS assay

Each set of analyzed samples contains the following:

- 6-8 point calibration curve sets (in triplicate).
  A calibration curve set is usually run at the beginning, at the end, and once or more during the sample set.
- 1 QC per 10 samples (in triplicate)
- n# of samples (in triplicate)
- blank injections if necessary



Results – QuanLynx report

Analyte and IS area

Signal-to-noise ratio

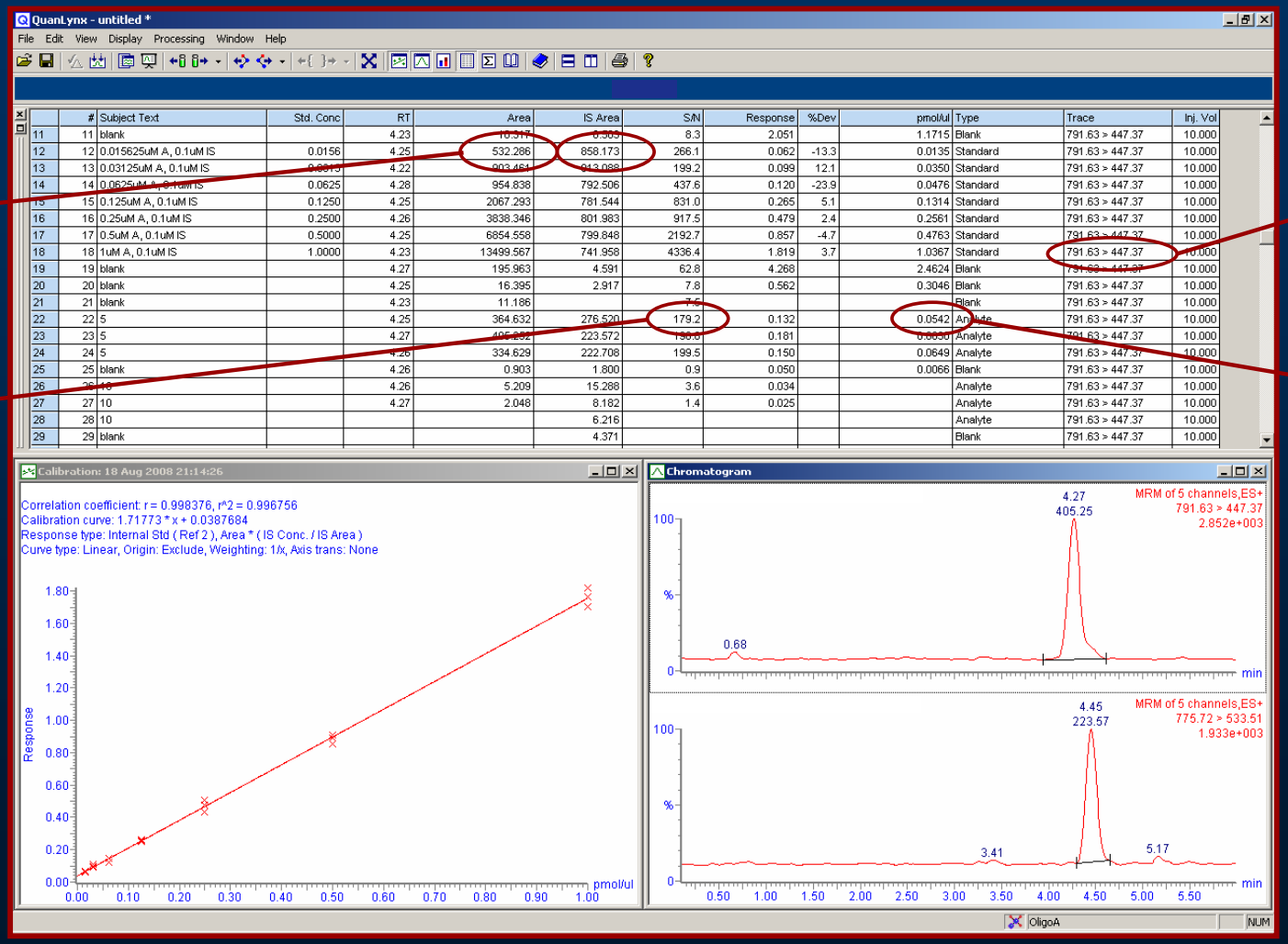
Calibration curve

SRM trace

Calculated concentration

Analyte

IS

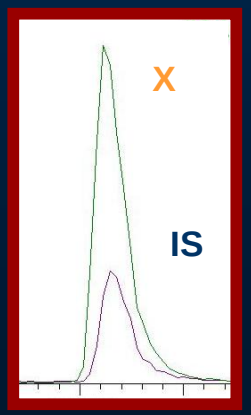


Most common challenges

- Internal standard (IS)
- Matrix effects
- Method validation

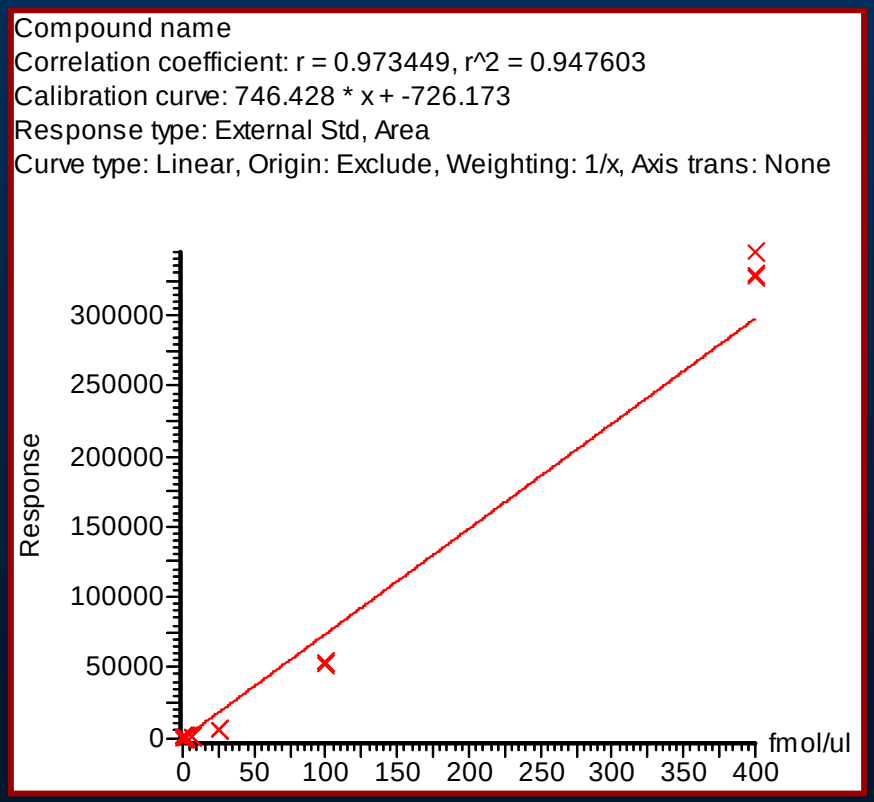
Internal standard – to use or not to use?

- Benefit of IS:
  - If the analyte and IS suffer the same losses and the same effects in the matrix, matrix effects and sample losses cancel when we take the ratio of IS to analyte
- IS compensates for common analyte losses:
  - **Analyte adsorption on surfaces**
  - Extensive sample manipulation
  - Degradation
  - Evaporation
  - Autosampler variability

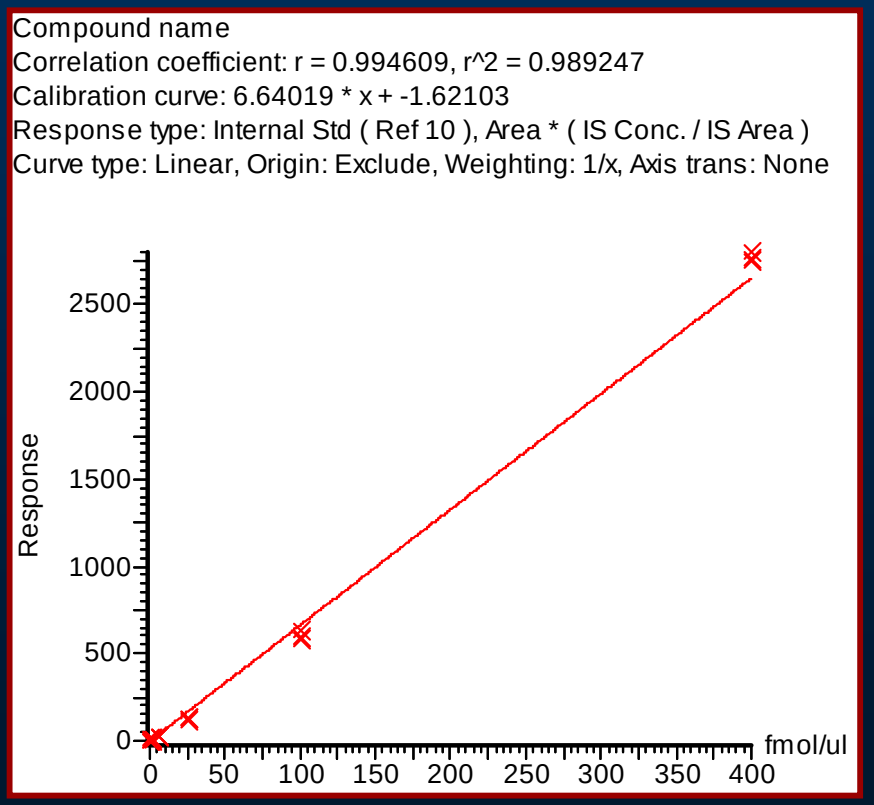


Peptide adsorption on the surface

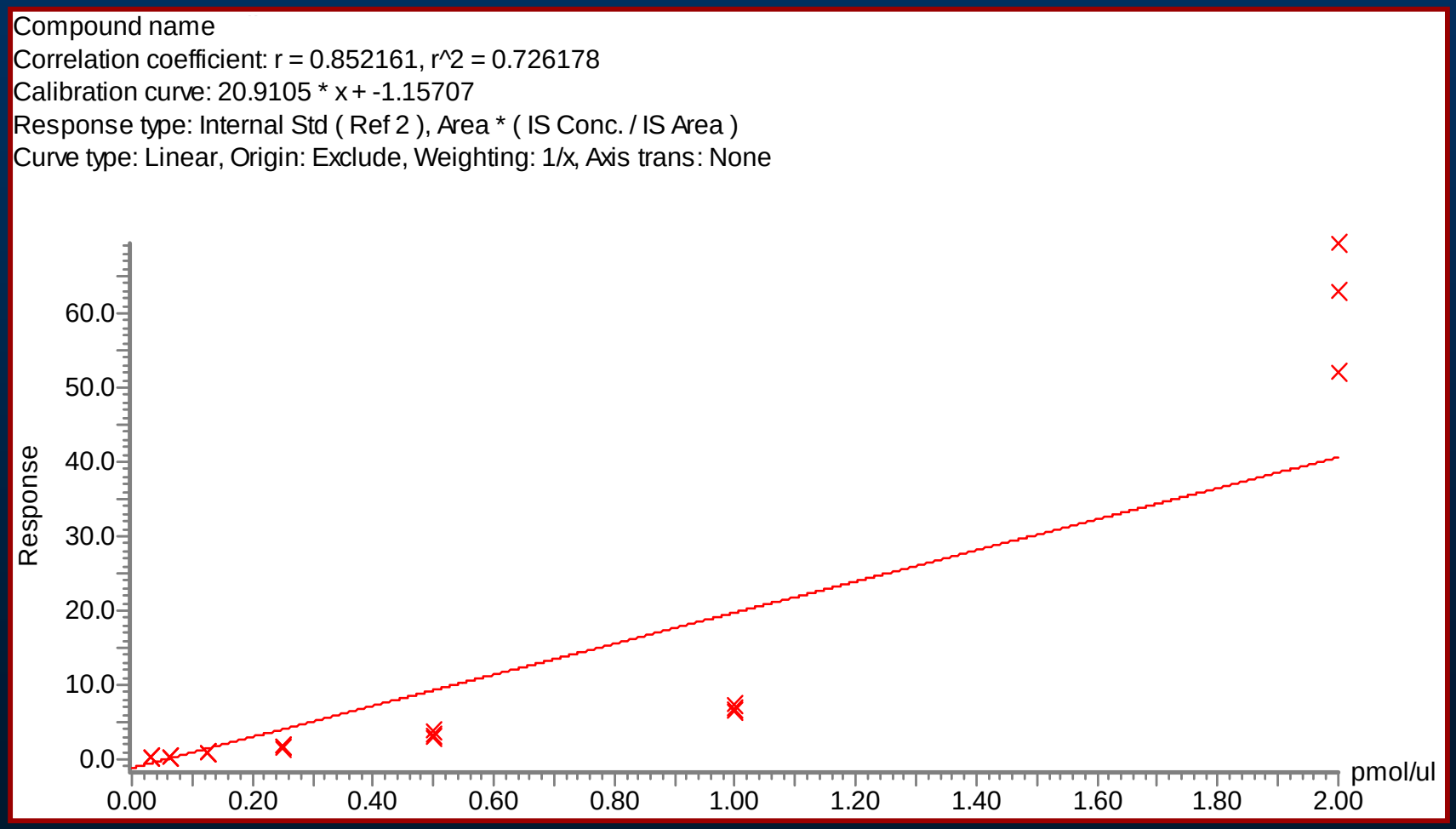
No IS



With IS



Extreme example



Tricks to improve calibration curve linearity

- Isotopically labeled internal standard
- Sample buffer modification:
  - Sample matrix
  - Diluted sample matrix
  - Addition of neutral protein
  - Higher organic solvent content

Matrix effects

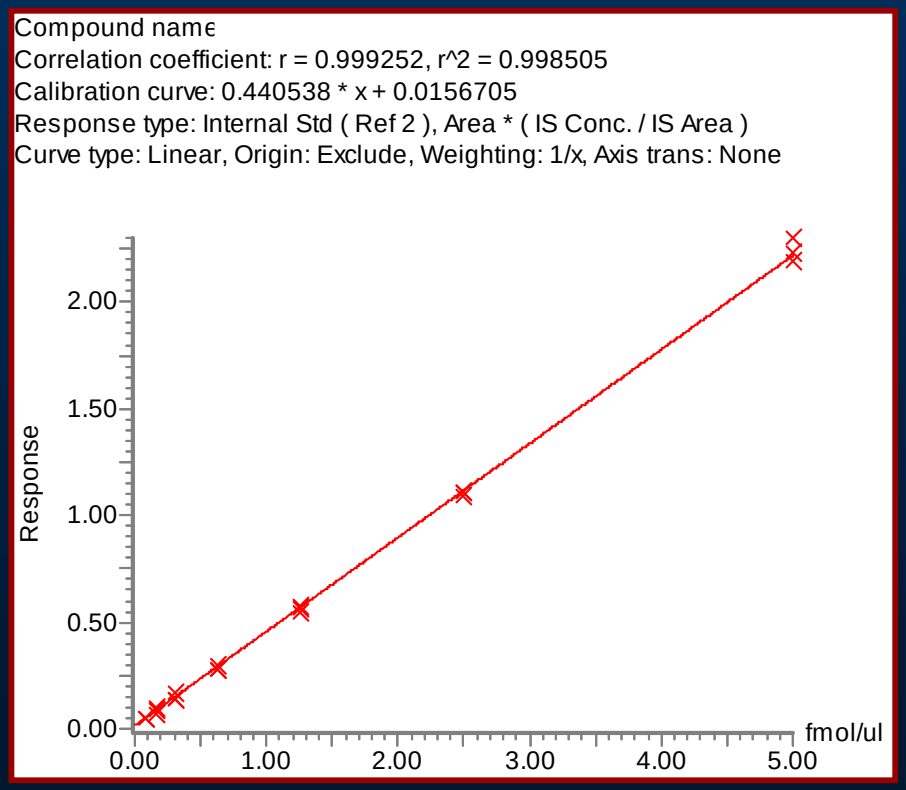
- Matrix effects:
  - Ion suppression
  - Interferences from metabolites
  - Signal enhancement
- Assessment of matrix effect:
  - Post-column infusion of analyte
  - Comparison of analyte in matrix-free solution vs. spiked blank matrix – extraction efficiency assessment
- Minimizing matrix effects:
  - Use isotopically labeled internal standard
  - Use fragment ions of m/z higher than precursor ion
  - Generate “cleaner” extract
  - Optimize HPLC method

Method Validation

- Detection capability:
  - LOD – signal to noise ratio 3:1
  - LOQ – signal to noise ratio 10:1
- Calibration curve – linear dynamic range
- Precision and accuracy
- Selectivity
- Specificity
- Stability of analyte (and matrix):
  - Short-term, long-term
  - Low and high concentrations
  - Analyte in sample solvent and in raw matrix
  - Dry extract, reconstituted standard/extract
  - Freeze/thaw cycles

Analyte stability

With IS



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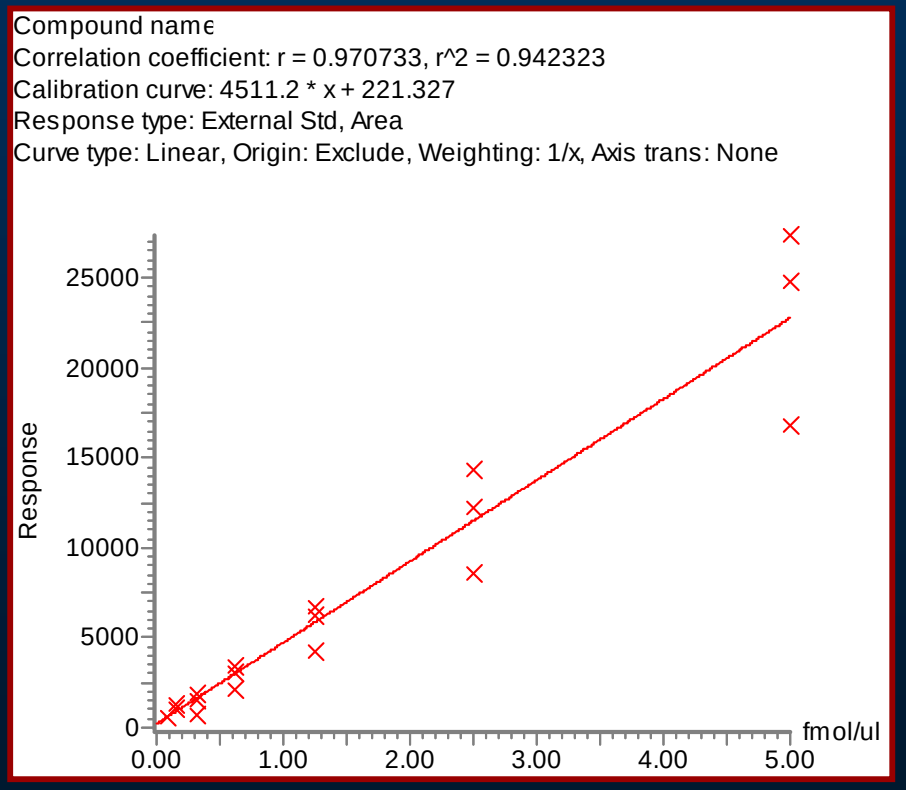

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2. Trace Quantitative Analysis by Mass Spectrometry by Robert K. Boyd, Cecilia Basic, Robert A. Bethem
3. LC/MS: A practical User's Guide by Marvin McMaster
4. Quantitative Proteomics by Mass Spectrometry by Salavatore Sechi
5. www.ionsource.com
6. SUMS website: mass-spec.stanford.edu

Acknowledgements

Stanford University Mass Spectrometry Staff

- Allis Chien
- Chris Adams
- Pavel Aronov
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