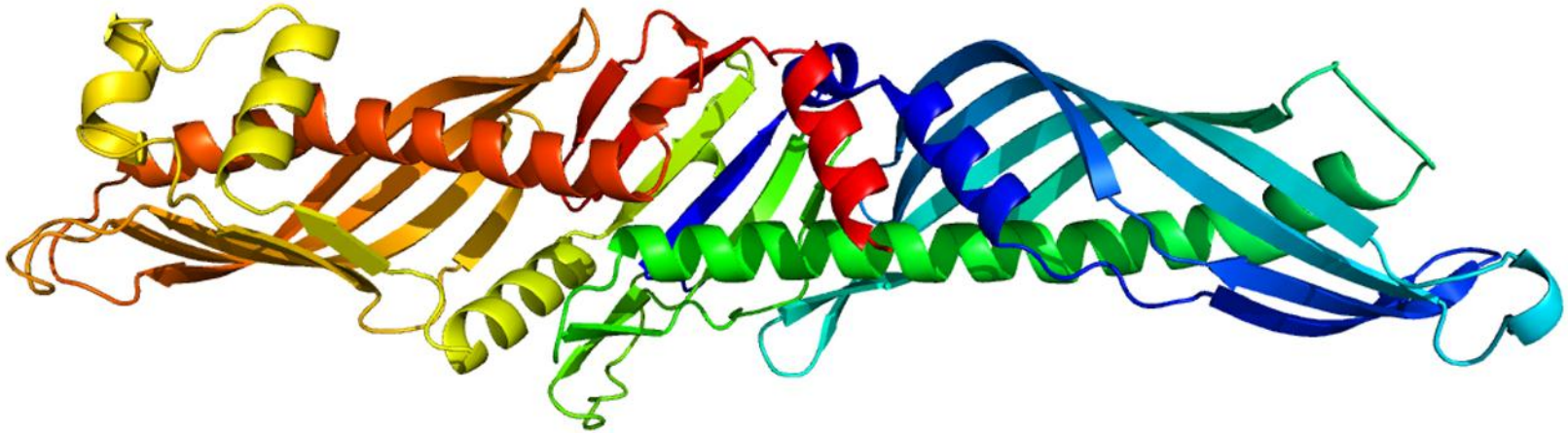
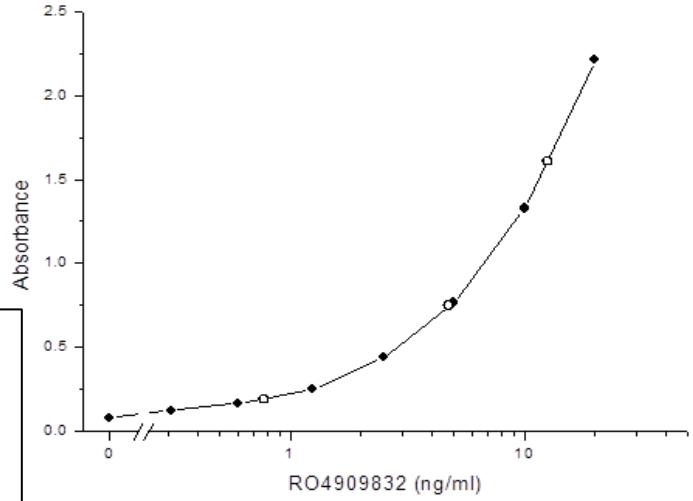
Comparison of quantitative assays for a large protein: ELISA vs. LC-MS/MS

A. Guenzi, G. Fischer, N. Justies, B. Lausecker

F. Hoffmann - La Roche Ltd., Basel, Switzerland



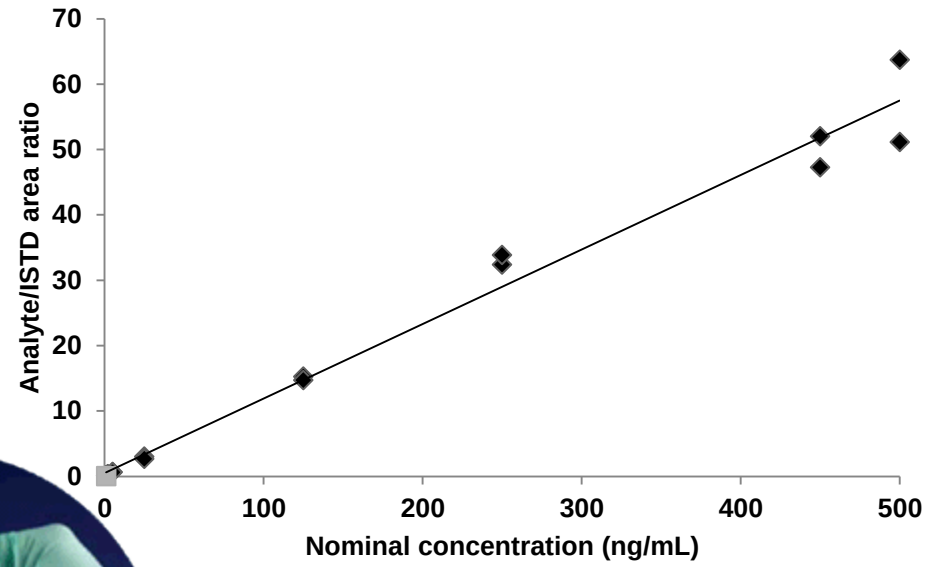
ELISA



ELISA (proteins and peptides)

- **ADVANTAGES:**
  - **Highly sensitive**
  - **Quick and easy to perform**
  - **Inexpensive instrumentation and material**
  - **If anti-idiotypic Abs used, only 'active' is detected**
- **DISADVANTAGES:**
  - **Need for very specific antibodies: animal immunization, selection and purification of reagents**
    - **For small peptides specific Abs may be difficult to find**
  - **Small differences in analyte sequence may require development of new reagents**
  - **Batch-to-batch variability of reagents**
  - **Each ELISA method needs multiple optimizations**
  - **Each species needs optimization (cyno≠rat≠man≠…)**
  - **Each matrix needs optimization (plasma≠serum≠…)**
  - **Enzyme/substrate reaction is short term so read as soon as possible**

LC-MS/MS


Analyte/ISTD area ratio
70
60
50
40
30
20
10
0
0
100
200
300
400
500
Nominal concentration (ng/mL)

LC-MS/MS (proteins and peptides)

- **ADVANTAGES:**
  - **Highly sensitive**
  - **Quick and easy to perform**
  - **Multi-analyte assays possible**
  - **Species-independent**
  - **Only ‘total’ detectable**
- **DISADVANTAGES:**
  - **Multiply charged ions may reduce sensitivity**
  - **Need for digestion (proteins) to ‘simplify’ to peptides**
  - **Is a signature peptide formed during digestion?**
  - **Lipophilicity of peptides**
  - **Wide range of concentrations possible (from pg/mL to µg/mL)**
  - **Mass range of MS instruments**

ELISA and LC-MS/MS data complement each other, they are not in competition

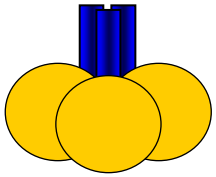
CASE STUDY:

Analysis of a protein (300 kDa including lipidation)

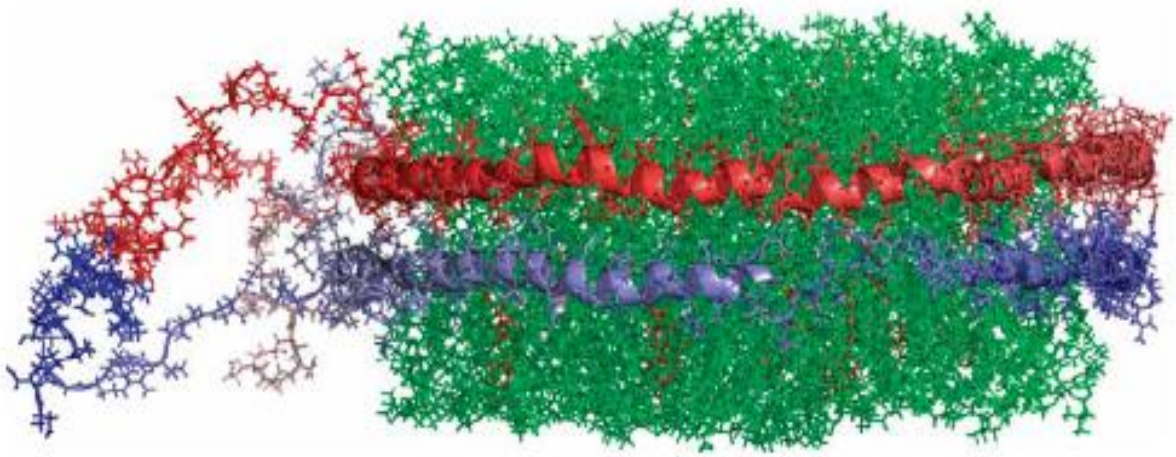
by ELISA and LC-MS/MS

The protein

A complex, trimeric fusion protein (150 kDa)

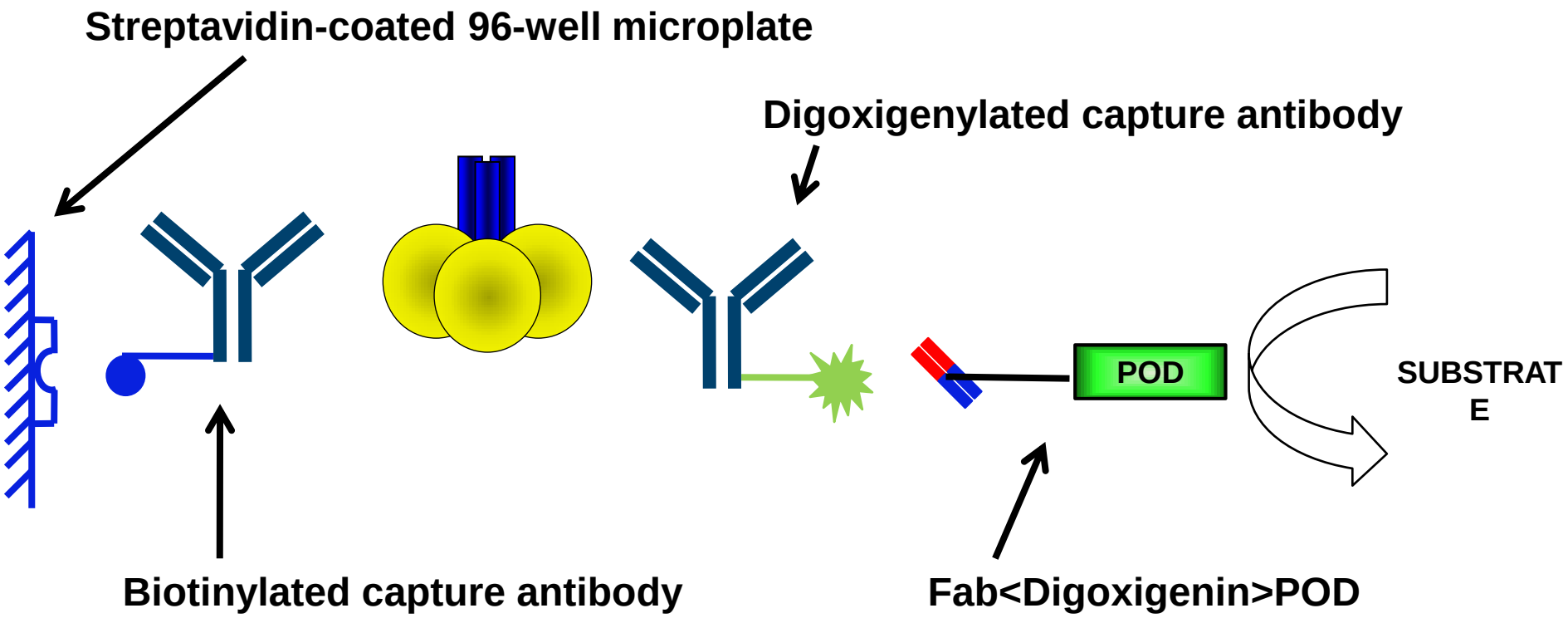


containing phospholipids



Molecular weight: 300 kDa

ELISA (PK) assay



Validation results of the ELISA cynomolgus plasma

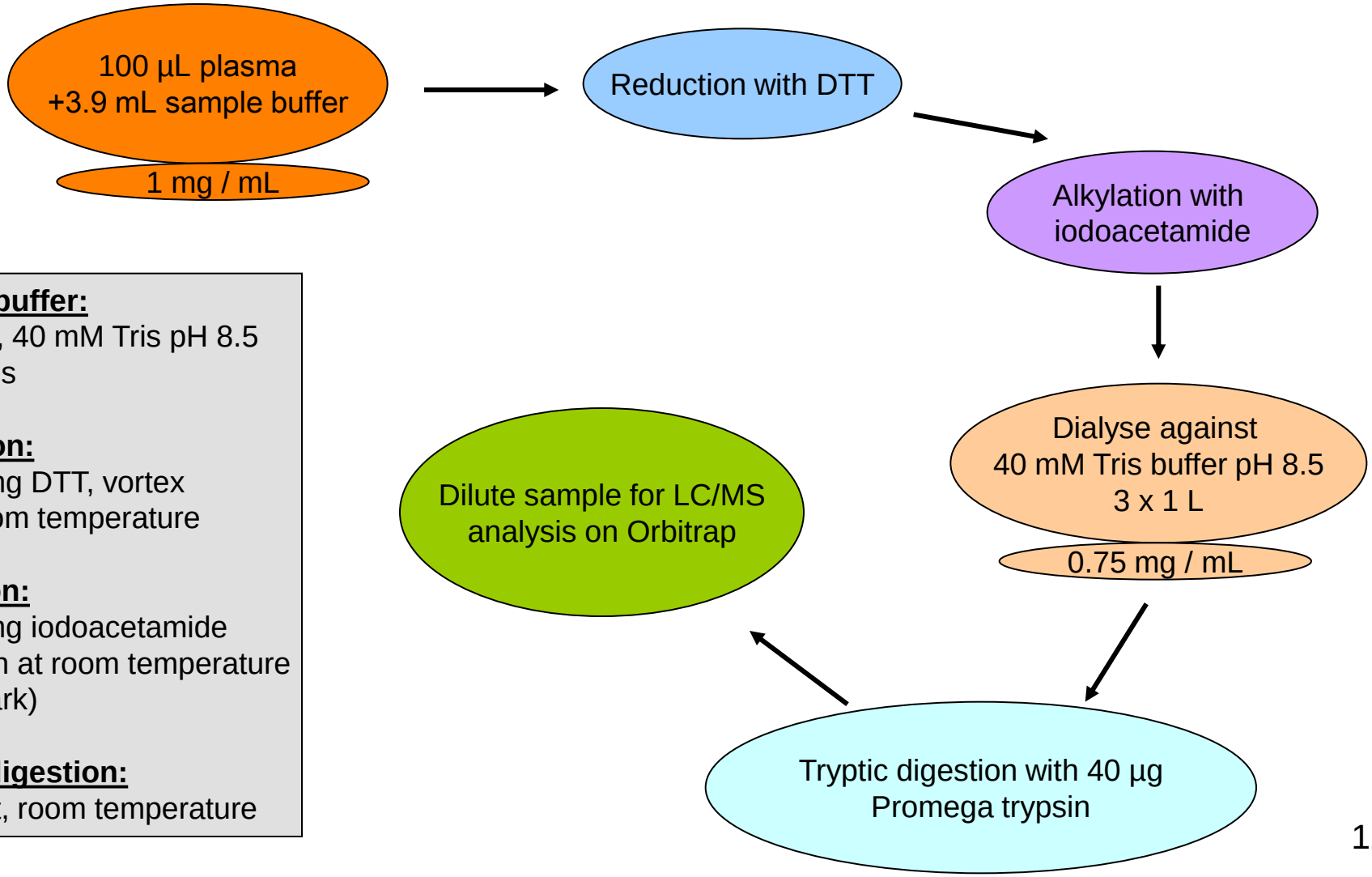
- **Minimum required dilution (MRD):** **1%**
- **Validated range (in 100% plasma):** **1.02 to 65.0 µg/mL**
- **Validated range (in assay buffer):** **10.2 to 650 ng/mL**
- **Precision and accuracy (intra-assay):** **<9.2% and 89.9 – 101.3%**
- **Precision and accuracy (inter-assay):** **<4.7% and 99.9 – 111.2%**
- **Selectivity, specificity:** **within Guidance criteria**
- **Dilution linearity and parallelism:** **no hook effect up to 6.50 mg/mL**
- **Assay signal stability:** **readout immediately after addition of SDS**

- **During application (routine analysis of studies) the ELISA-measured concentrations did not fit with other pharmacodynamic observations**

- **The possibility of exploiting for quantitative purposes the results obtained by proteomics studies were considered**

An orthogonal analytical method (LC-MS/MS) able to confirm the ELISA data was developed

Starting point for LC-MS assay: Proteomics qualitative procedure



Sample buffer:
8 M urea, 40 mM Tris pH 8.5
2% Chaps

Reduction:
Add 15 mg DTT, vortex
1 h at room temperature

Alkylation:
Add 60 mg iodoacetamide
vortex 1 h at room temperature
(in the dark)

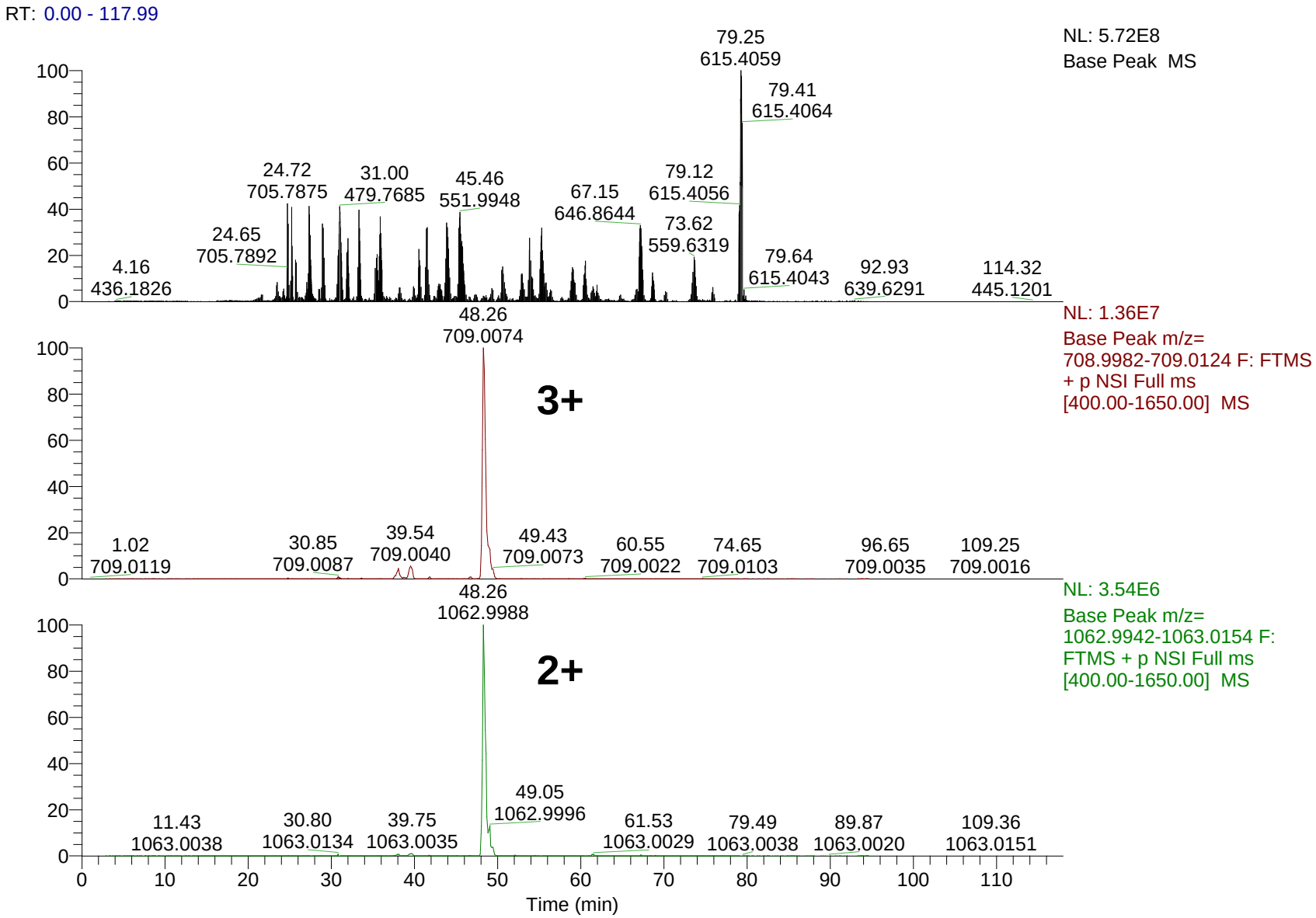
Tryptic digestion:
overnight, room temperature

Starting point for LC-MS: Identification of signature peptide

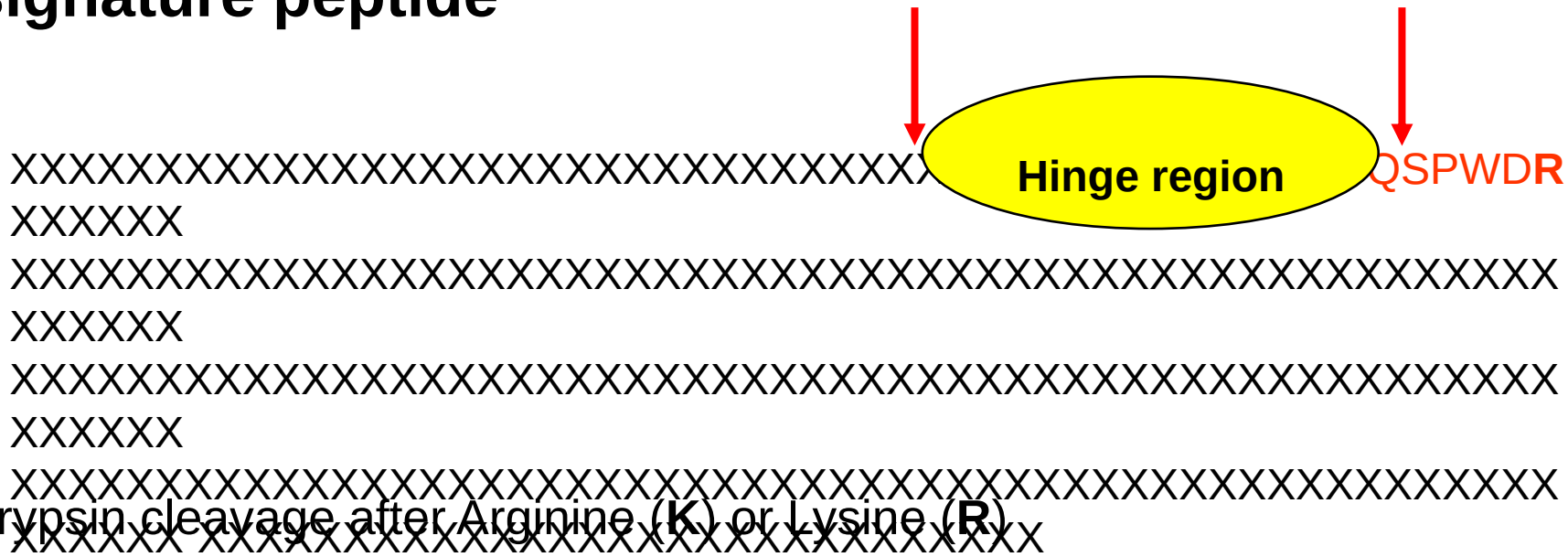
- **Samples containing proteins (e.g., plasma) are digested to peptides with trypsin. Peptides are selected as surrogates of the protein by the following criteria:**

- **The fragmentations (transitions) are unique for one single protein**
- **The peptide contains no Cys, Met or other commonly modified residues**
- **The peptides lie in the mass range 800 – 2000 MW**

MS analysis of protein in plasma (proteomics)



Assay development LC-MS/MS signature peptide



Trypsin cleavage after Arginine (**K**) or Lysine (**R**)

Elemental Composition: **C91 H139 N26 O33**

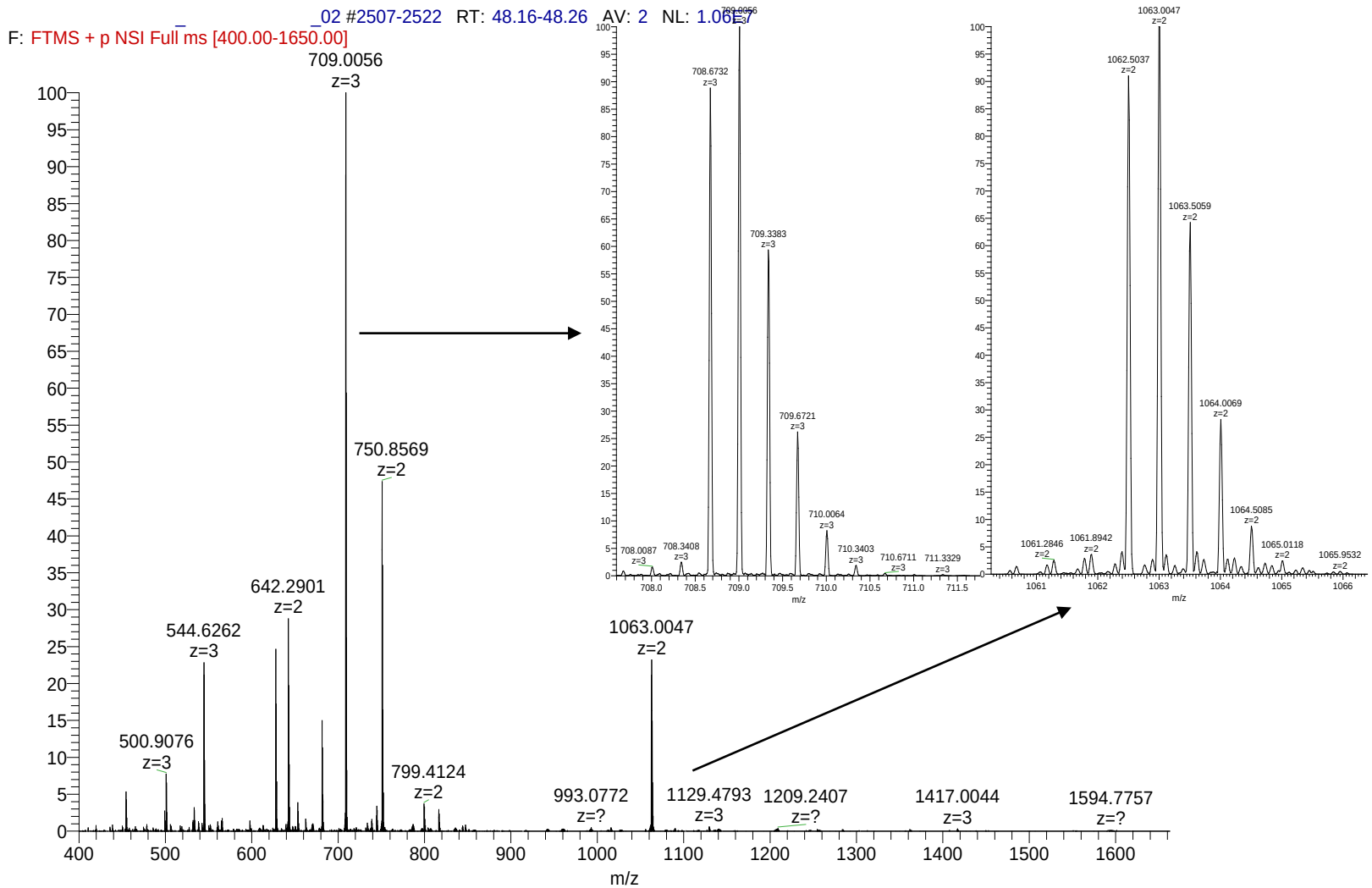
Parent Charge: **2+**

⇒ Monoisotopic M/Z: **1062.50326**

Parent Charge: **3+**

⇒ Monoisotopic M/Z: **708.67126**

Full ion scan of the signature peptide

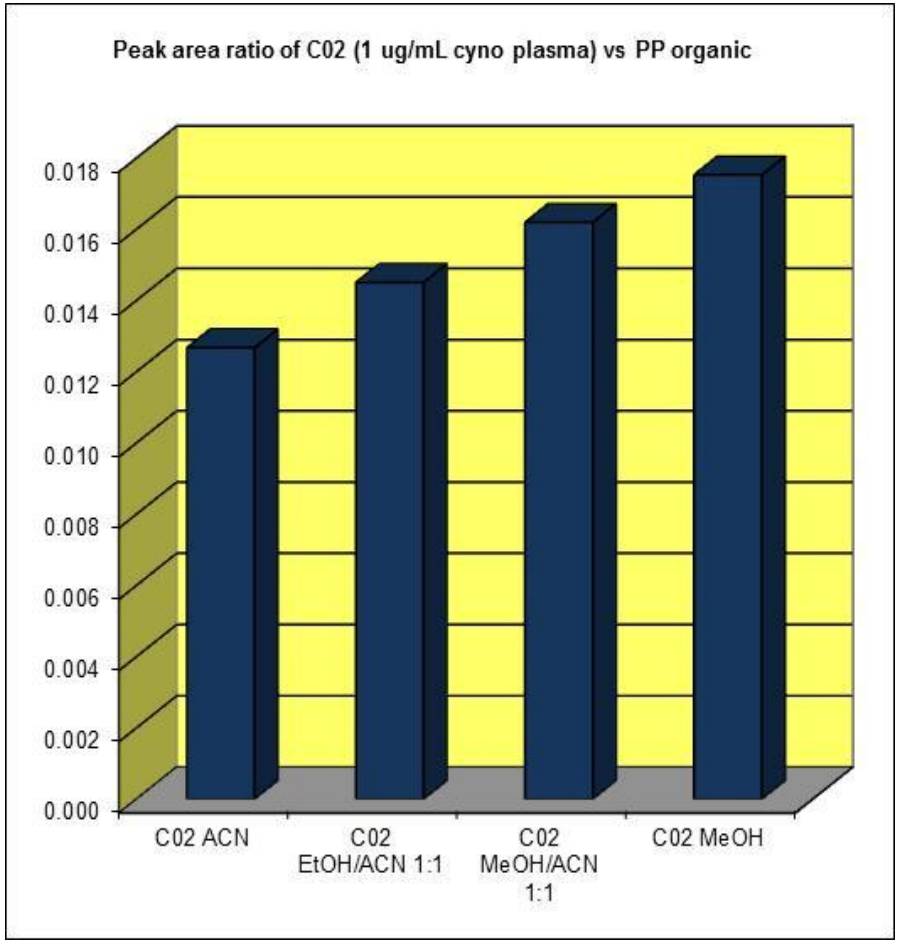


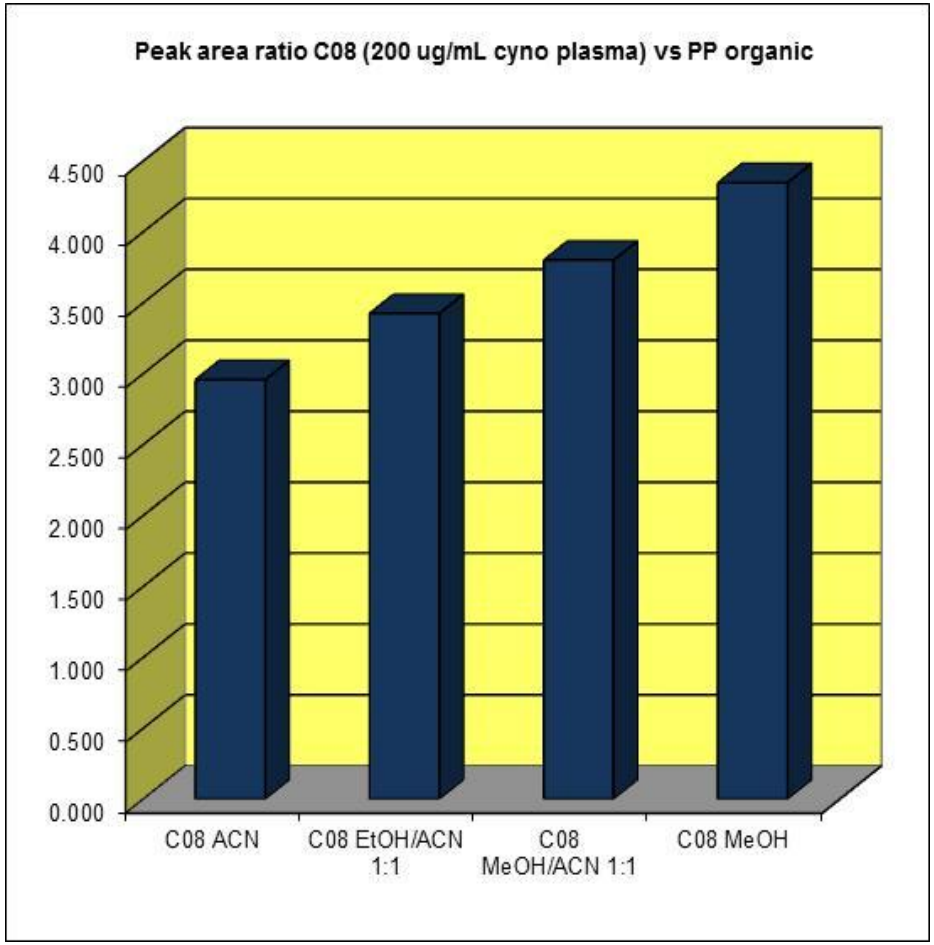
Multidimensional optimization of the LC-MS/MS assay

Optimization of the following parameters

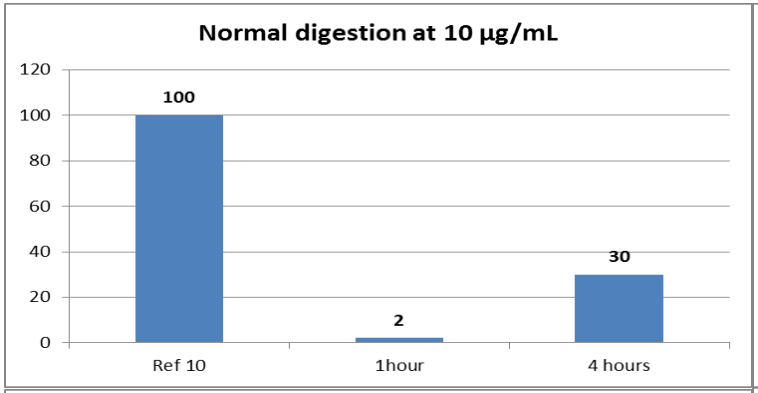
- **Volume of plasma**
- **Volume of buffer**
- **Trypsin concentration**
- **Protease-protein (mainly albumin) ratio**
- **Precipitating solvent**
- **Duration of incubation**
- **Chromatographic conditions**
- **Addition of internal standard**

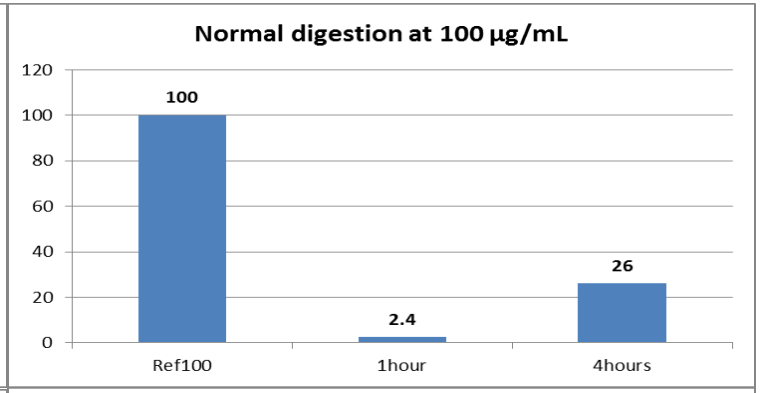
Example: choice of precipitating solvent in the preliminary step

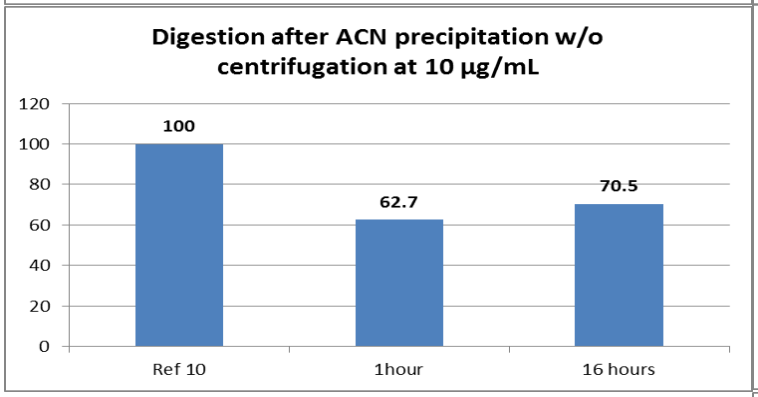


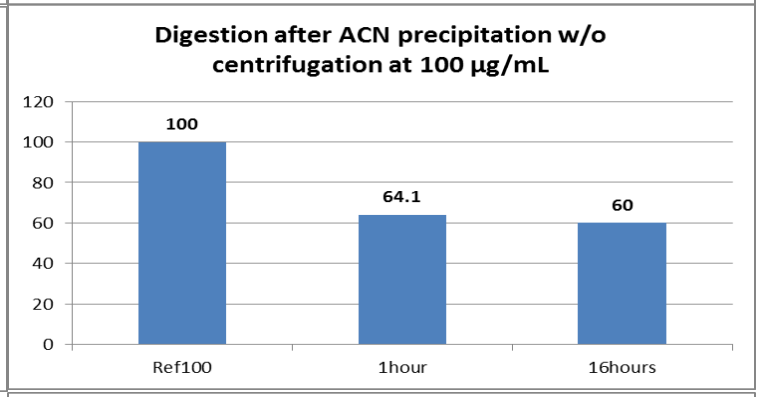


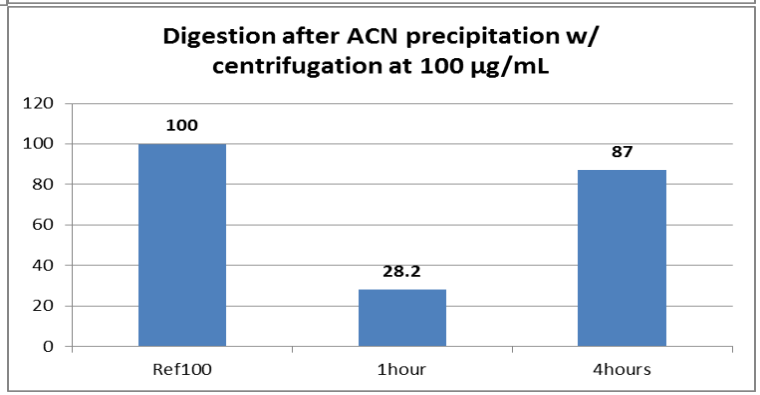
Example: protein precipitation parameters









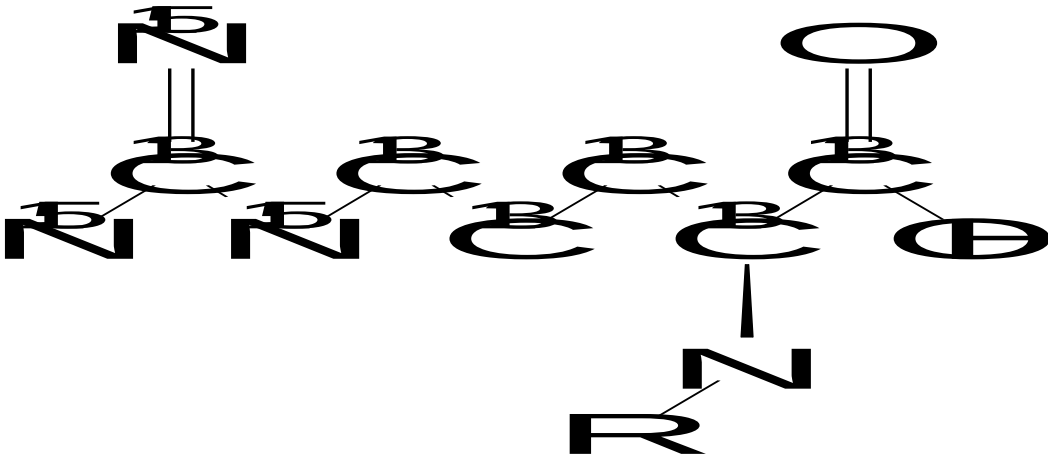


CONCLUSION

⇒ Plasma albumin has an effect on digestion efficiency: it must be eliminated by precipitation followed by centrifugation

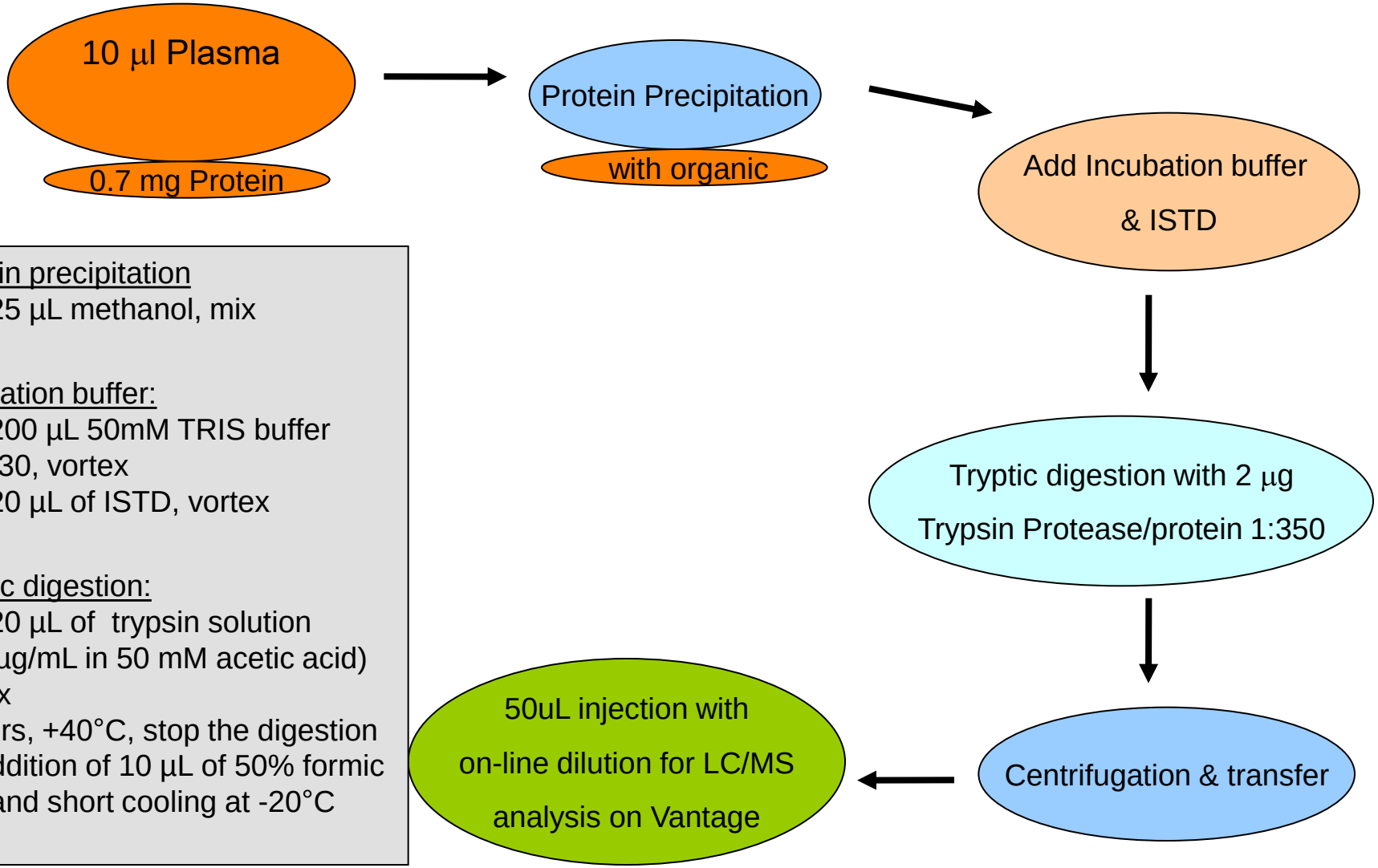
Internal standard

- **Terminal arginine residue labeled with multiple ^{13}C and ^{15}N**



- **ISTD added before digestion does not compensate for losses during the initial protein precipitation and for digestion yield**

Cyno plasma: final sample preparation



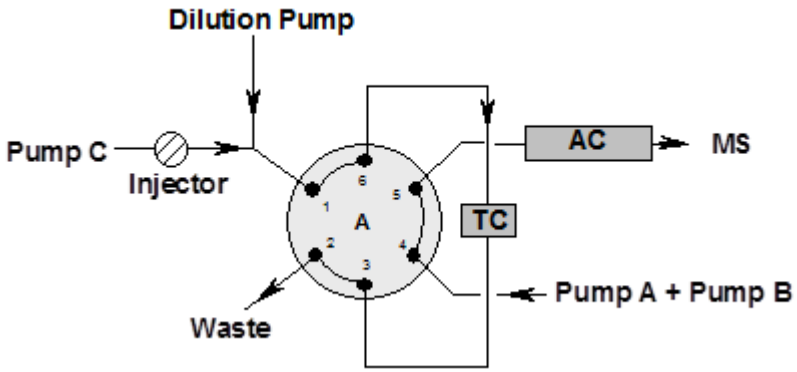
<u>Protein precipitation</u>
Add 25 µL methanol, mix

<u>Incubation buffer:</u>
Add 200 µL 50mM TRIS buffer pH 8.30, vortex
Add 20 µL of ISTD, vortex

<u>Tryptic digestion:</u>
Add 20 µL of trypsin solution (100 µg/mL in 50 mM acetic acid)
Vortex
2 hours, +40°C, stop the digestion via addition of 10 µL of 50% formic acid and short cooling at -20°C

LC-MS/MS of the signature peptide

- **The traditional approach in our group (column-switching with on-line solid-phase extraction) was followed**



- **50 µL injection volume (1.8 µL plasma-equivalents)**
- **Pump C to dilution pump flow-rate ratio 1:6.6**
- **Gradient elution with acetonitrile-water-formic acid on the analytical column (Supelco Ascentis Express C18, 2.0 (i.d.) x 50 mm)**
- **Detection on a Thermo TSQ Vantage EM triple quad with H-ESI source**

Validation results of the LC-MS/MS assay cynomolgus plasma

- **Validated range (plasma):** **1.00 to 200 µg/mL**
- **Precision and accuracy (intra-assay):** **<7.9% and 100.0 – 106.7%**
- **Precision and accuracy (inter-assay):** **<7.9% and 92.5 – 105.6%**
- **Selectivity, specificity:** **within Guidance criteria**
- **Dilution linearity:** **validated up to 1:100**
- **Matrix effect:** **compensated by SLIS, ca. 0.92**
- **Extraction recovery:** **between 93.5 and 97.8 (SLIS)**

It does not cover digestion!

Chromatograms

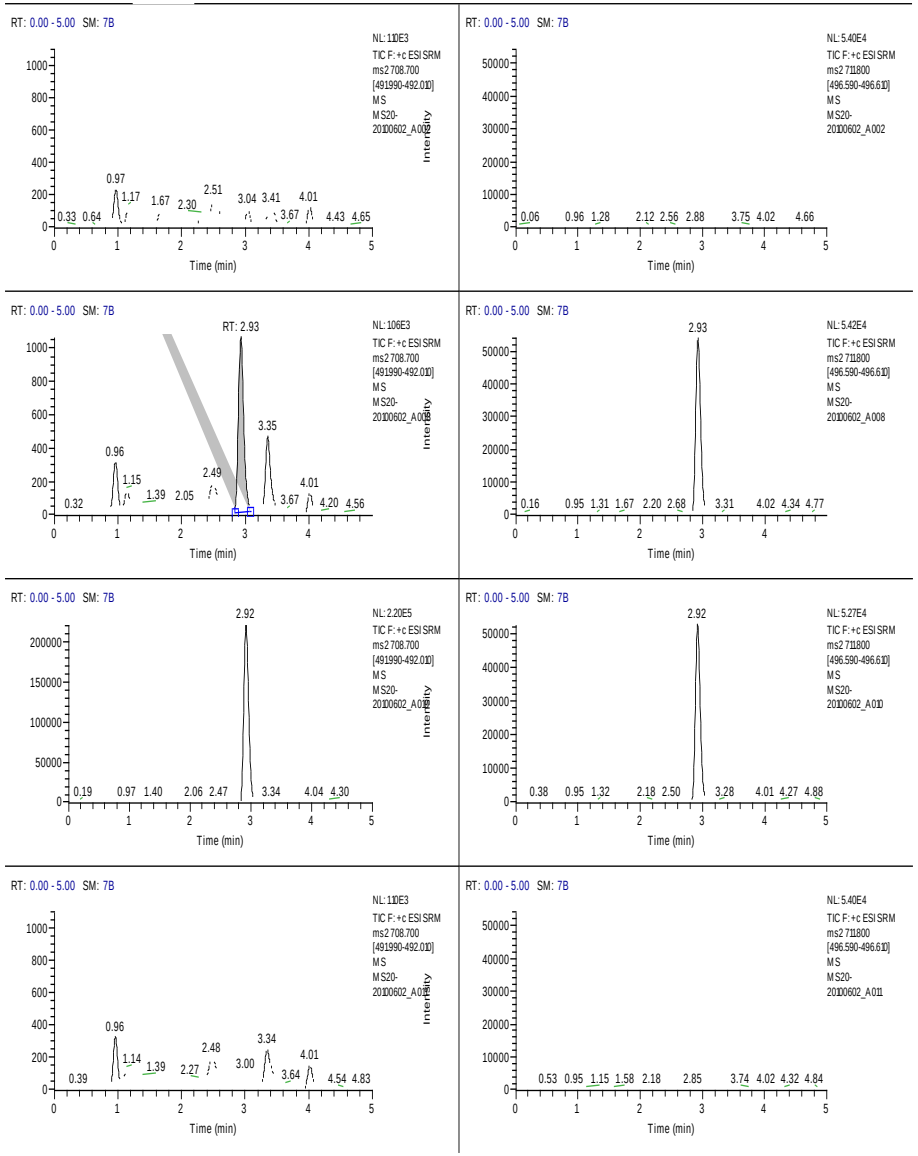
D:\Xcalibur\...\MS20-2(008 02-Jun-2010 12:29:18 PM C01

Blank Cyno plasma

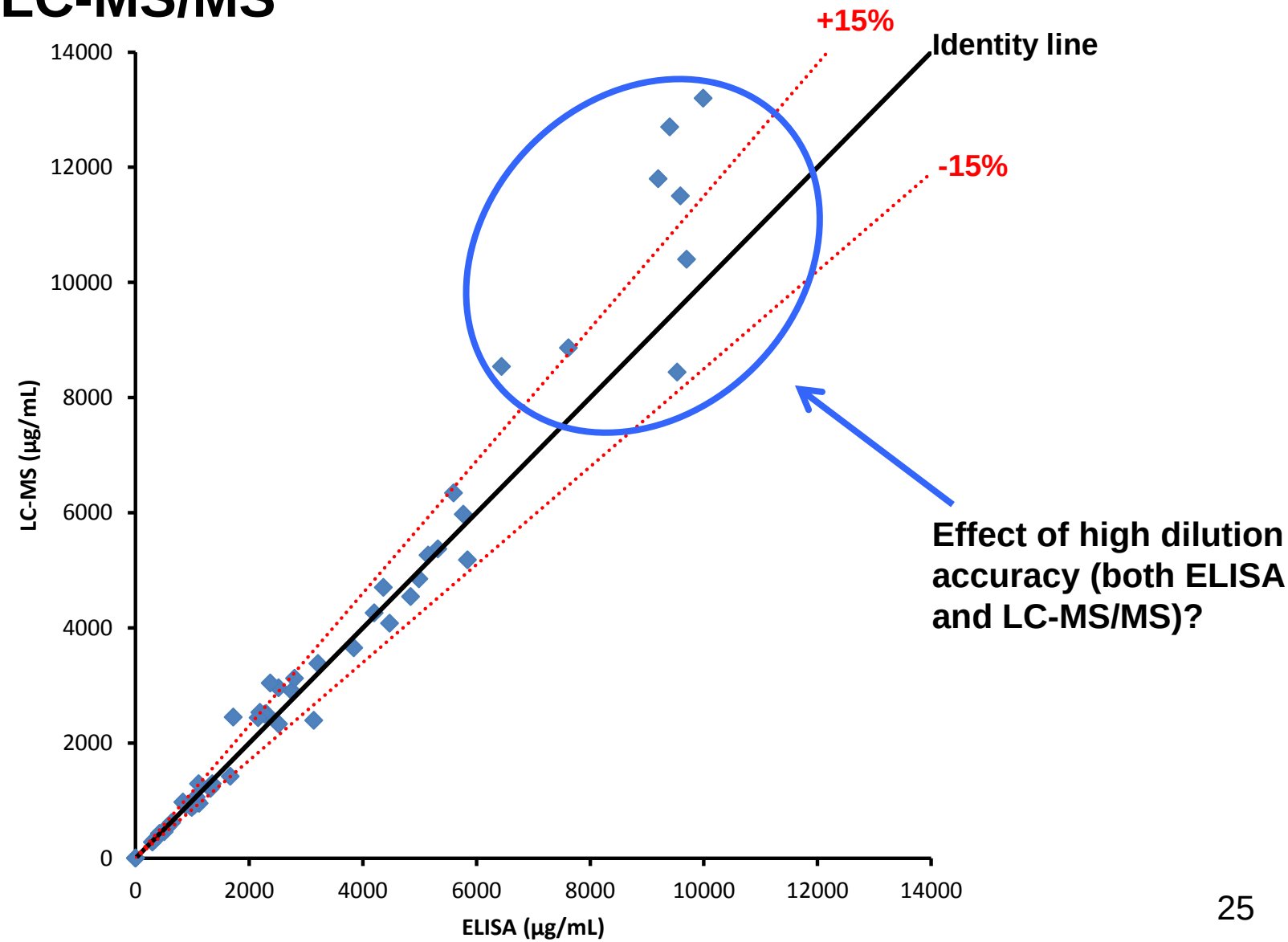
LLOQ= 1 µg/mL

ULOQ = 200 µg/mL

Blank after ULOQ (carry-over test)



Cross-validation results ELISA vs. LC-MS/MS



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We Innovate Healthcare