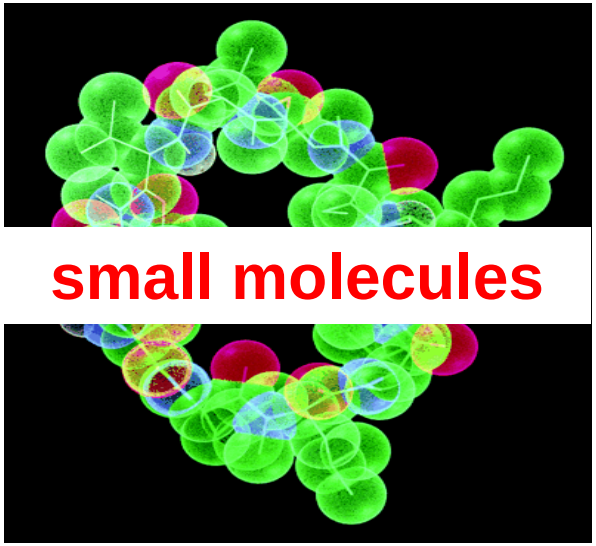
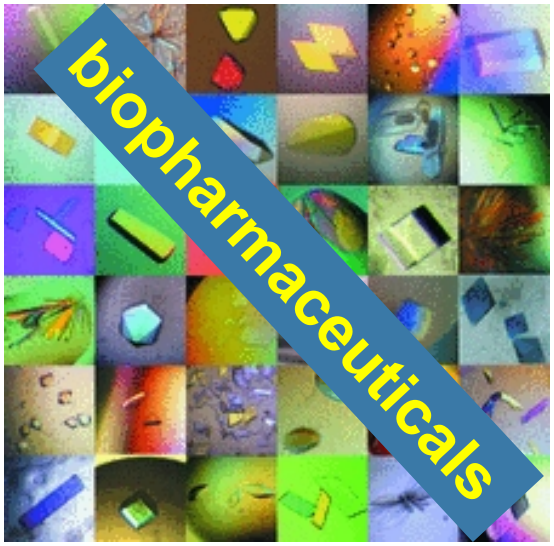
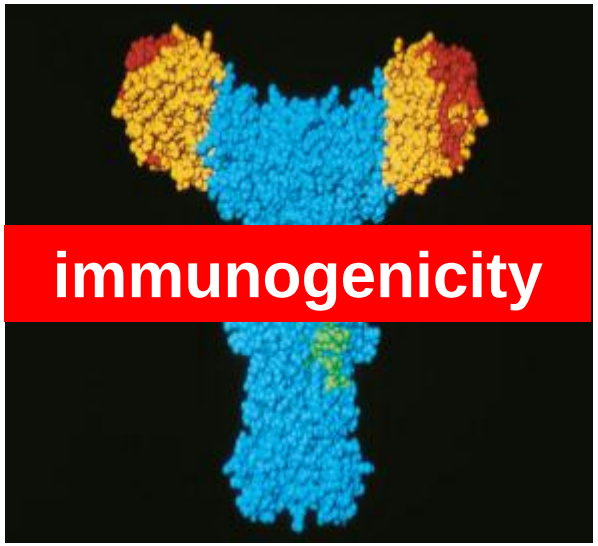
Bioanalytical LC-MS of monoclonal antibody therapeutics

William D van Dongen, product manager pharma bioanalysis



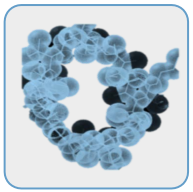




40 years experience in bioanalysis

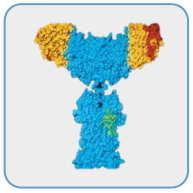
Bioanalytical Expertise and Technology

BIOPHARMACEUTICALS



SMALL MOLECULES

BIOMARKERS:
-Cytokines
-Hormones



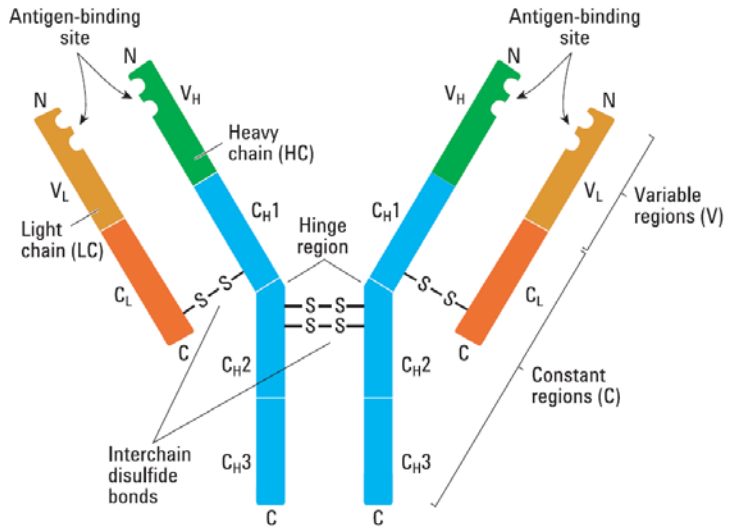
IMMUNOGENICITY:
-Anti-drug antibodies

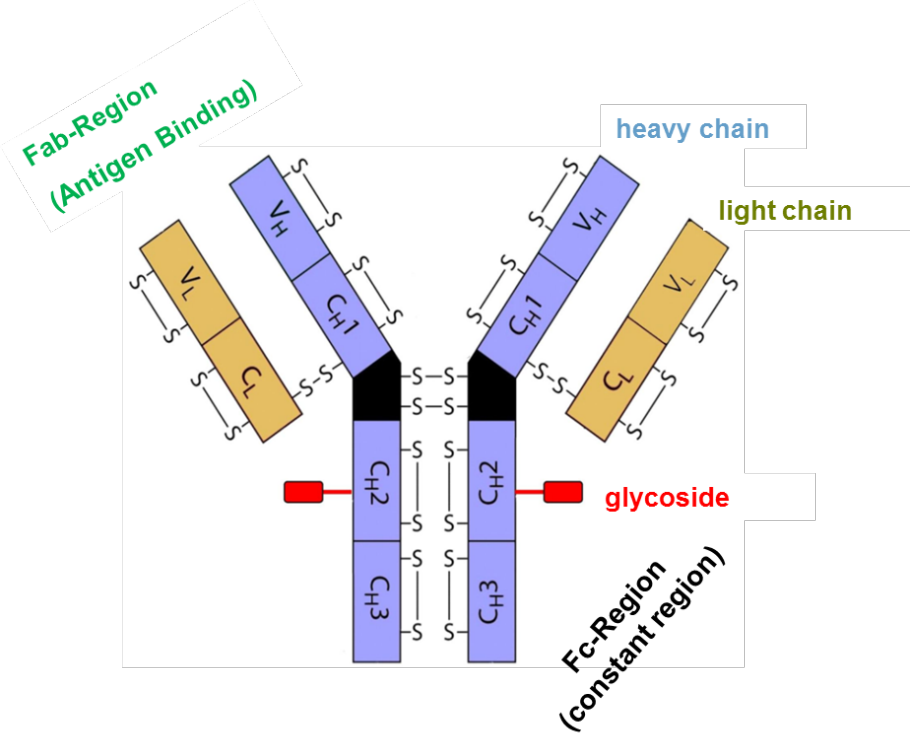
UPLC-MS
XEVO-TQS
API6500
API4000
Q-Exactive
LTQ-Orbitrap
nano-UPLC

Ligand Binding Assays
ELISA
RIA
Luminex
Mesa Scale
Discovery
Biacore

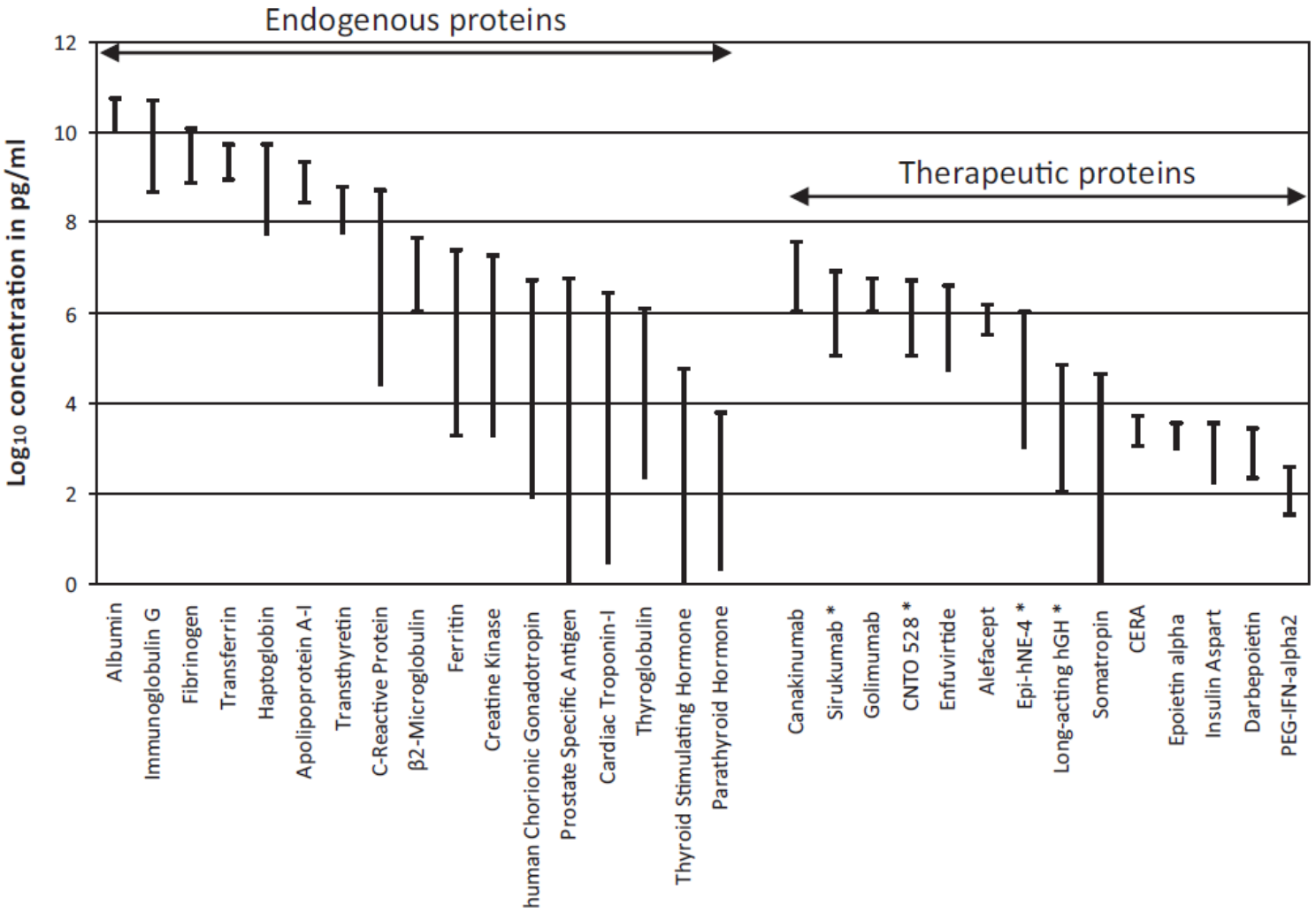
Monoclonal antibody therapeutics

- approximately 30 therapeutic mAbs
- marketed in the United States and Europe
- variety of indications
- sales in the US alone ***$18.5 billion*** in 2010



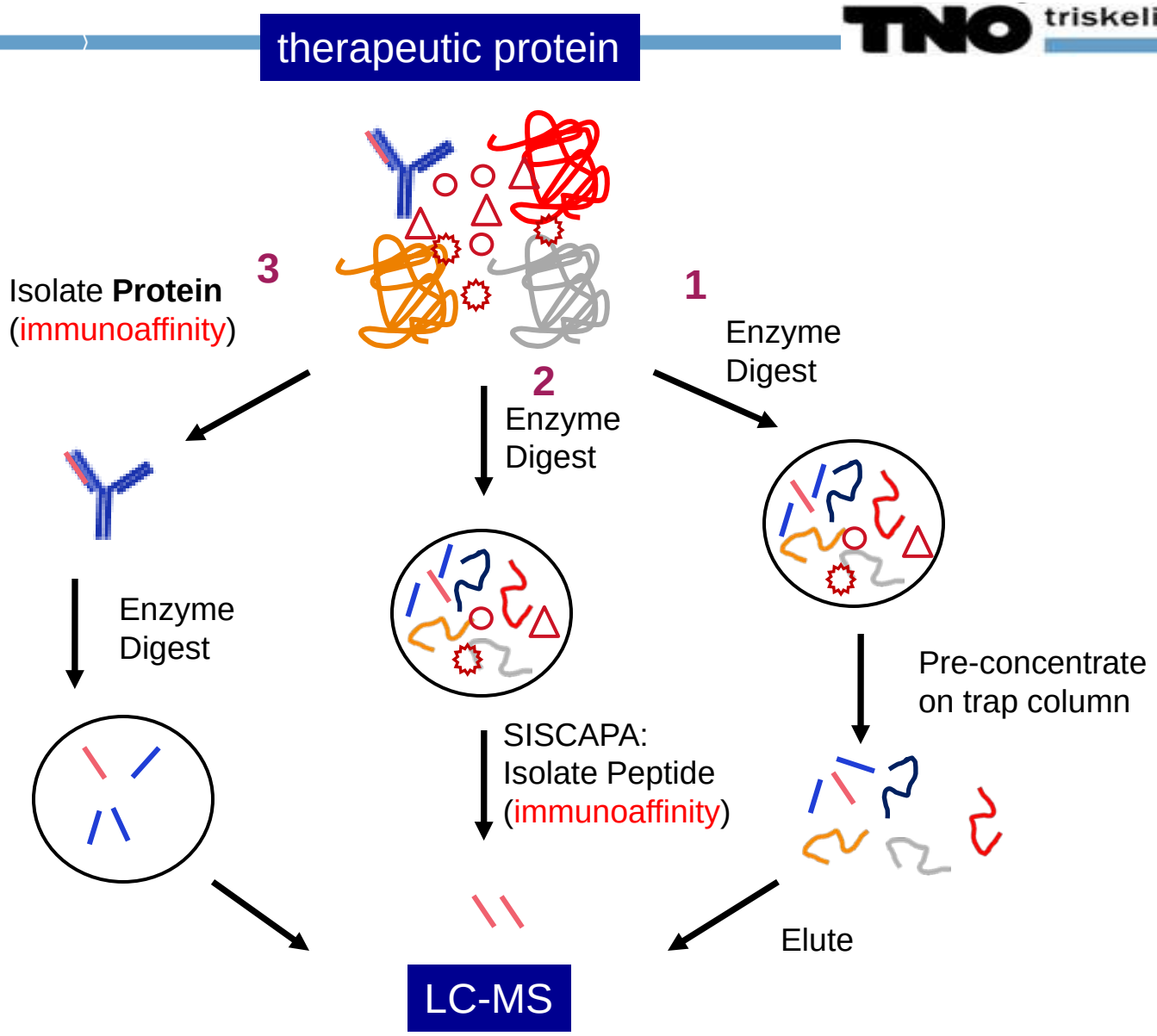


Therapeutic proteins: big molecules, low levels, low numbers



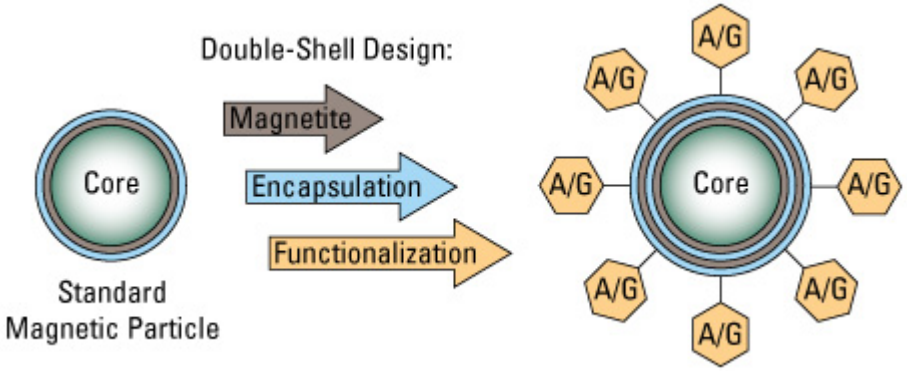
Irene van den Broek, Wilfried M.A. Niessen and William D. van Dongen: Journal of Chromatography B, 929 (2013) 161– 179

Therapeutic proteins: too large for MS quantitation

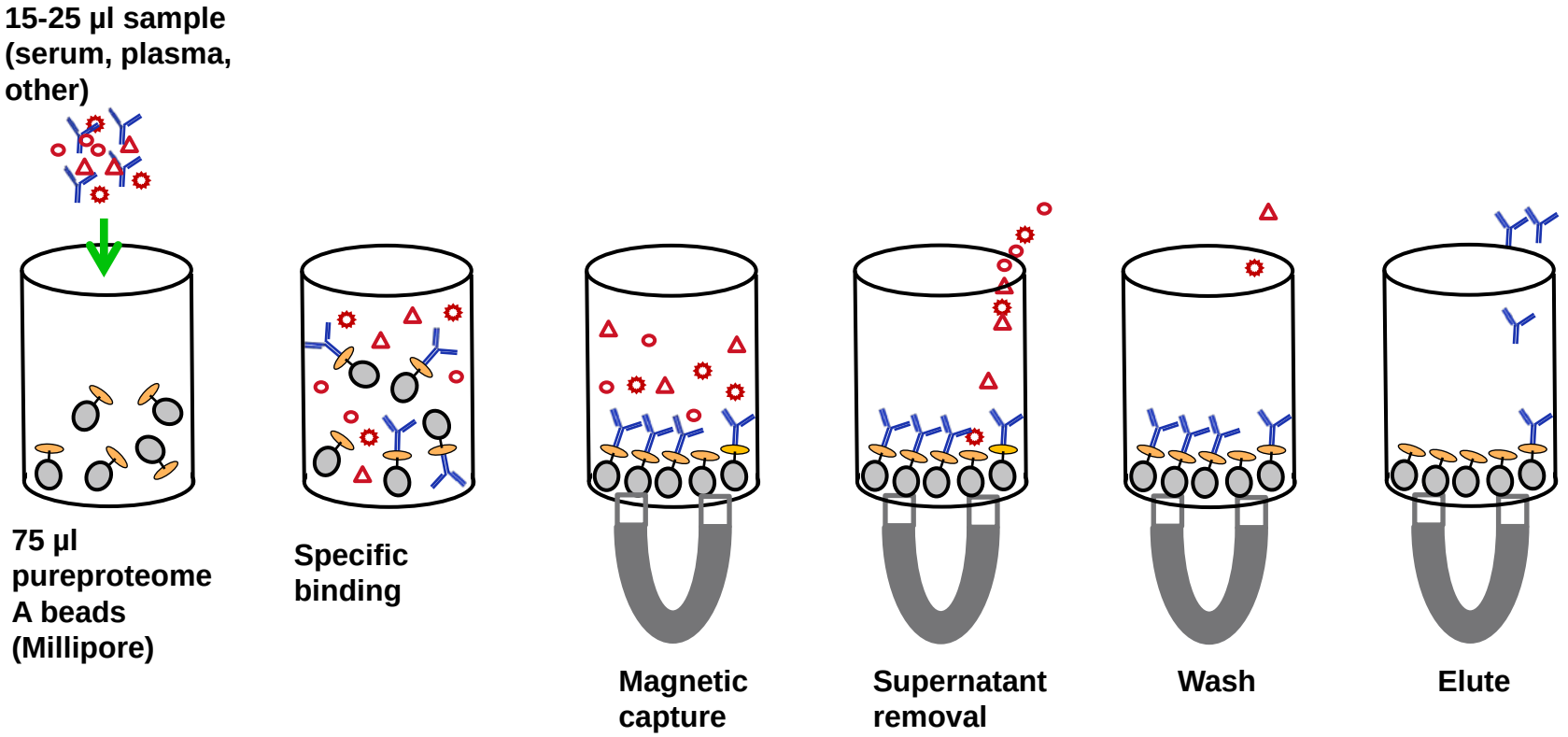


Sample clean-up and enrichment: immunocapture

- Protein A (PureProteome™, Millipore)
- Fc region of IgG from a variety of species. Can be used to purify classes, subclasses, and fragments of immunoglobulins as well as for isolation of immune complexes.
  - human IgG1, IgG2, IgM, IgA, IgE
  - mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgE



Sample clean-up and enrichment: immunocapture



Digestion: cleaves after K/R

AGCLFGIL**K**FNDTSDFAA**R**SFGGAIL

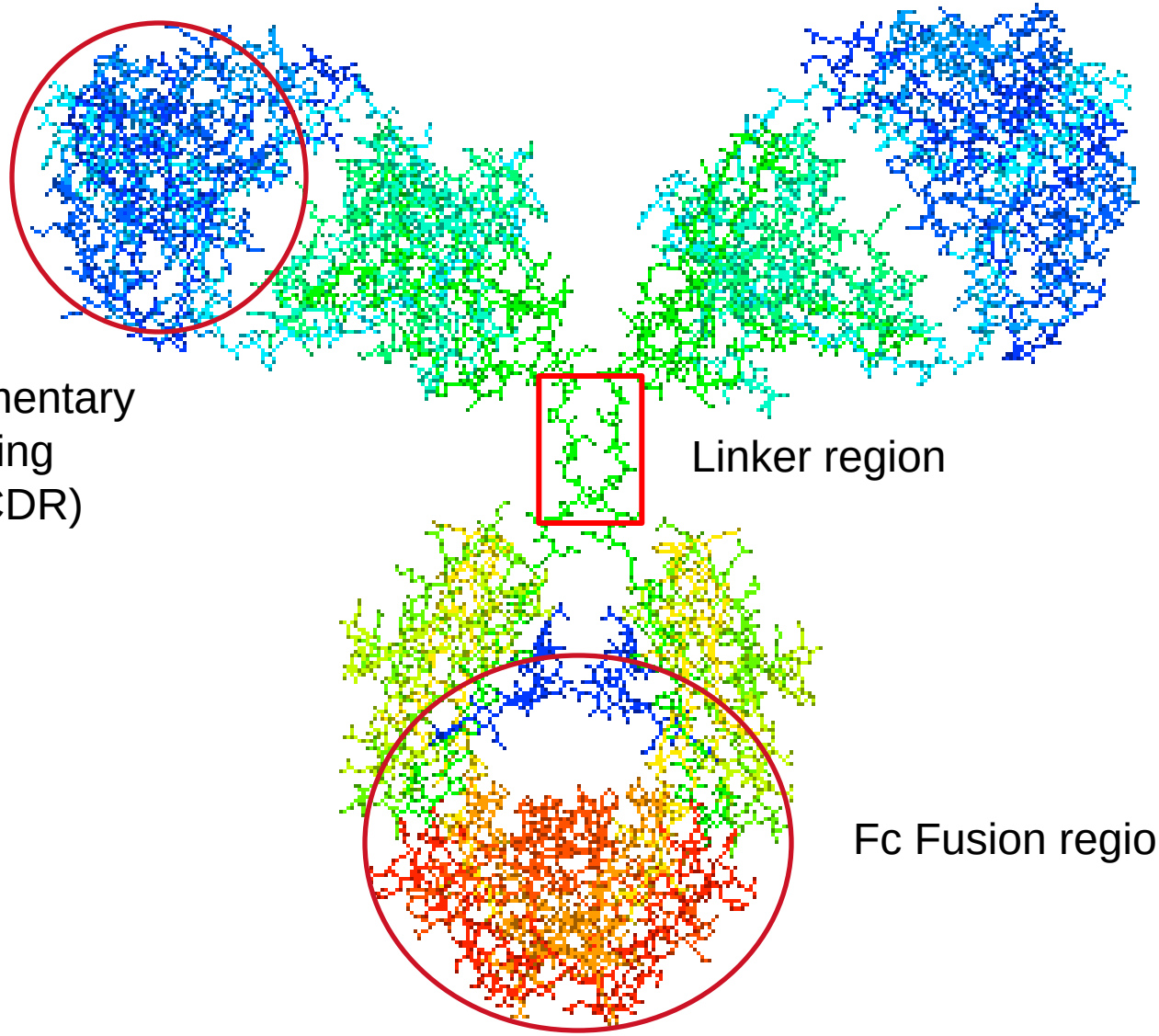
↓

AGCLFGIL**K** FNDTSDFAA**R** SFGGAIL

aim for 100% digestion at a plateau

- Add 10 µl 1M Tris pH 8.5 (including IS) to eluate
- Add 12 µl 1% Rapigest - Vortex
- Add 6 µl 1M DTT in milliQ water - Vortex
- Incubate at 37 °C for 1 hour, 1000 rpm
- Add 35 µl 0.5 M iodoacetamide in 100 mM ammonium bicarbonate pH 8 - Vortex
- Incubate at ambient temperature in the dark for 30 minutes
- Add 3 µl 1M DTT in milliQ water
- Incubate at 37°C for 20 minutes, 1000 rpm
- Add 70 µl 80 µg/300µl trypsin in 100 mM ammonium bicarbonate pH 8
- **Incubate at 37°C for 2.5 hours, 1000 rpm**
- Add 18 µl 5% TFA in acetonitrile
- Incubate at 37°C for 1 hour, 1000 rpm
- Analyze with LC-MS/MS

Signature peptide selection



Complementary determining region (CDR)

Linker region

Fc Fusion region

Example: LC-MS infliximab in mouse serum

Compound

Remicade (infliximab): 144190,3 g/mol

Remicade Heavy chain [2]:

EVKLEESGGGLVQPGGSMKLSCVASGFIFSNHWMNWVRQSPEKGLEWVAEIRSKSINSATHYAES
VKGRFTISRDDSKSAVYLQMNSLRTEDTGVYYCSRNYYGSTYDYGQGTTLTVSXASTKGPSVFPL
APSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNHKPSNTKVDKRVEPKSPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Remicade Light chain [2]:

DILLTQSPAILSVSPGERVSFSCRASQFVGSSIHWYQQRTNGSPRLLIKYASESMSGIPSRFSGS
GSGTDFTLSINTVESEDIADYYCQQSHSWPFTFGSGTNLEVKRTVAAPSVFIFPPSDEQLKSGTAS
VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEV
THQGLSSPVTKSFNRGEC

Conserved region: blue
variabele regions: in red
CDR regions: green/bold/underlined.
Unidentified amino acid residue: X
Signature peptides: BOXED

KR Tryptic cleavage sites

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Example: LC-MS infliximab in mouse serum

species (taxid nr)	signature peptide				
	universal "Furlong" VVSVLTVLHQDWLNGK human IgG-based mAb and Fc-fusion protein candidates		**universal "new"** SLSLSPGK human IgG-based mAb and Fc-fusion protein candidates		**"unique" infliximab** DILLTQSPAILSVSPGER
	IgG1,3,4 (**L**8)	IgG2 (**L**8→**V**)	IgG1-3 (**P**6)	IgG4 (**P**6→**L**)	for infliximab
 (10090)	+	+	+	+[a]	-/+[b]
 (10116)	+	+	+	+	+
 (9986)	+	+	+	+	+
 (10140)	+	+	+	+	+
 (9615)	+	+	+	+	+
 (9541)	+	+	-[c]	+	+
 (9544)	+	+	-[c]	+	+
 (9606)	-	-	-	-	+

[a]1 hit: peptide present in zinc finger protein, partial [Mus musculus], however, tryptic peptide cannot be formed since no K/R precedes signature peptide.
[b]several hits: peptide present in N-terminal immunoglobulin light/heavy chain variable region [Mus musculus], it is anticipated that tryptic peptide will not be formed, since no K/R precedes peptide.
[c]1 hit: peptide, in meta IgC, it is anticipated that tryptic peptide will be formed, since K precedes peptide.

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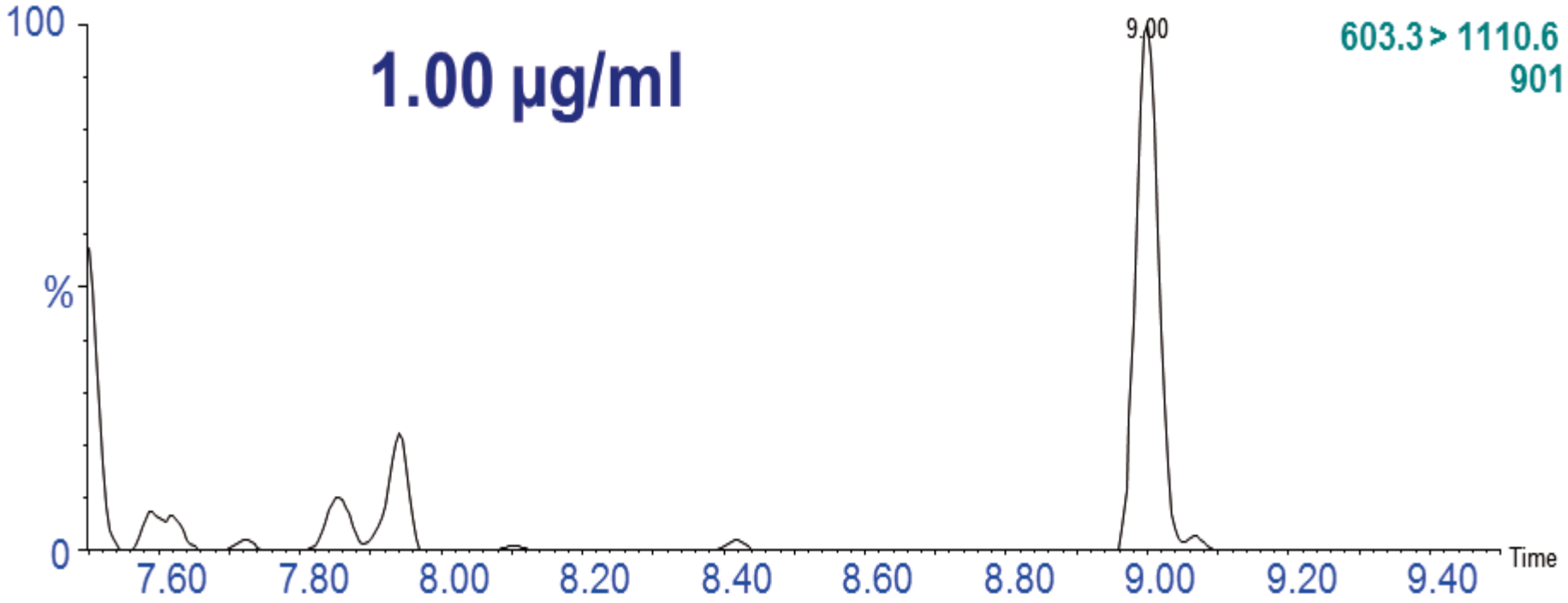
Universal “Furlong” signature peptide

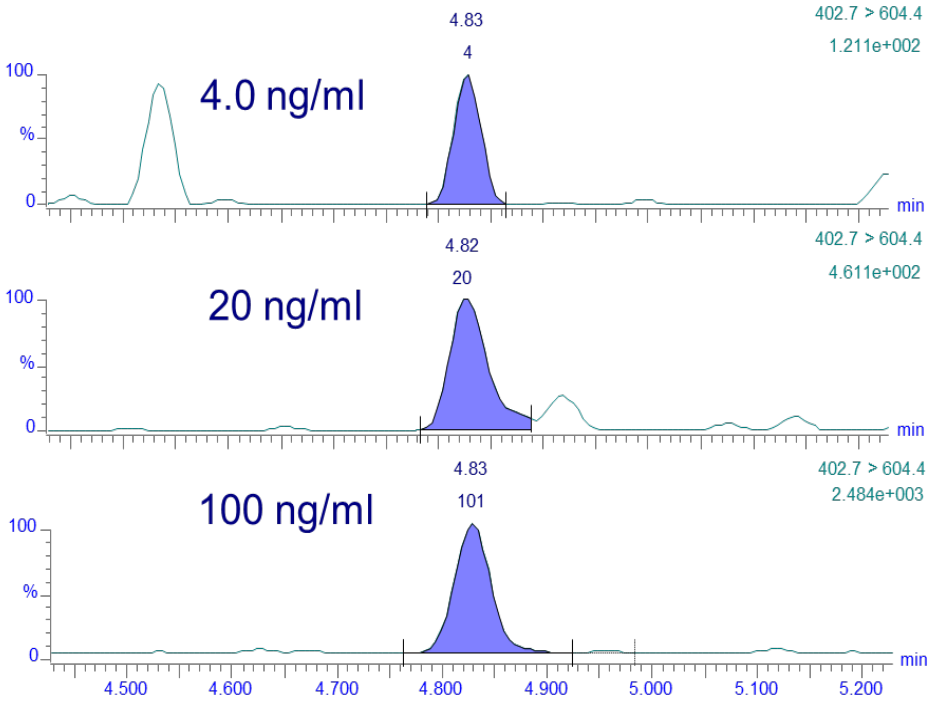
Furlong et al., Biomed.Chromatogr. (2012) 1024–1032.

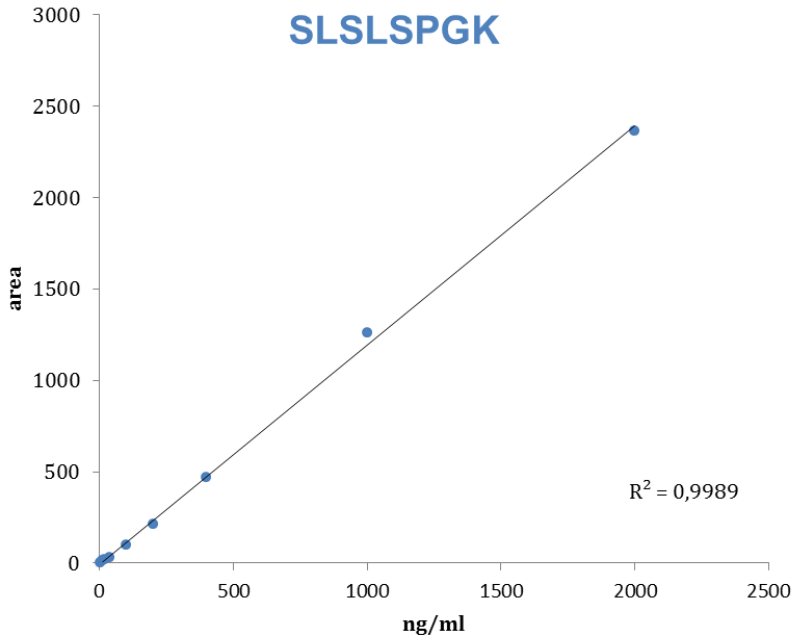
UPLC-MS/MS SRM (m/z 603 → 1110) chromatograms of signature peptide VVSVLTVLHQDWLNGK of tryptic digested infliximab in rat serum extract



Universal “new” Fc peptide

UPLC-MS/MS SRM (m/z 402 → 604) chromatograms of signature peptide SLSLSPGK of tryptic digested infliximab in rat serum extract.





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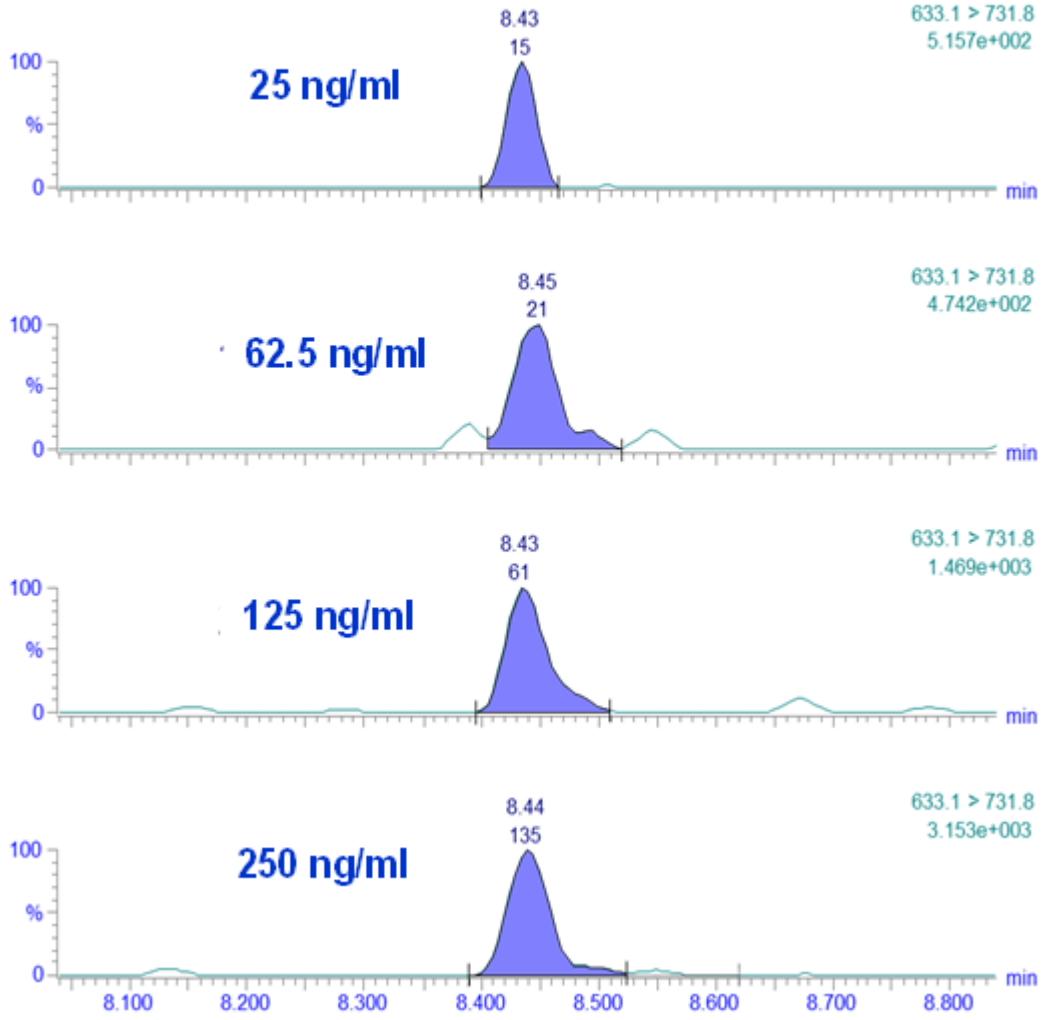
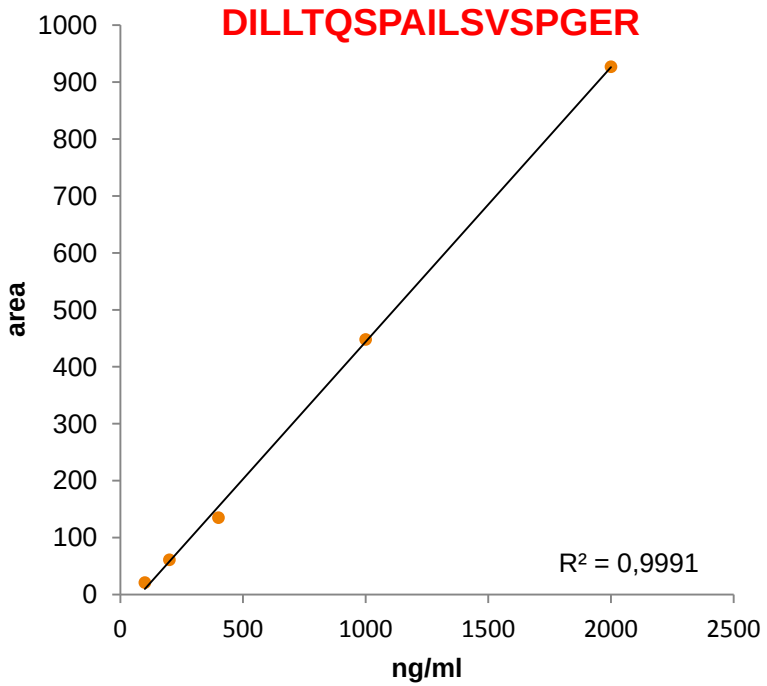
Unique signature peptide

UPLC-MS/MS SRM (m/z 633 → 731) chromatograms of unique signature peptide DILLTQSPAILSVSPGER of tryptic digested infliximab in rat serum extract.





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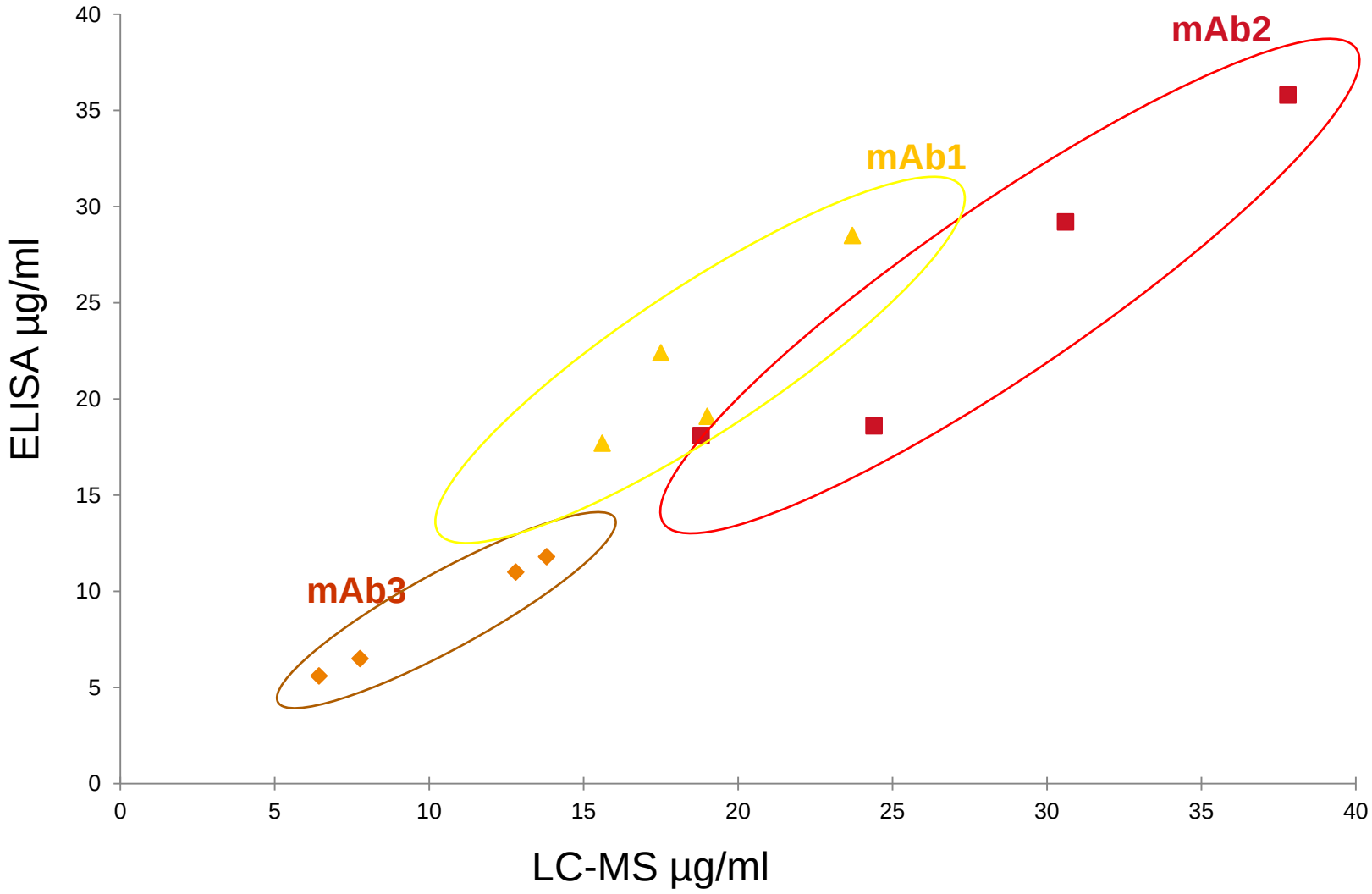
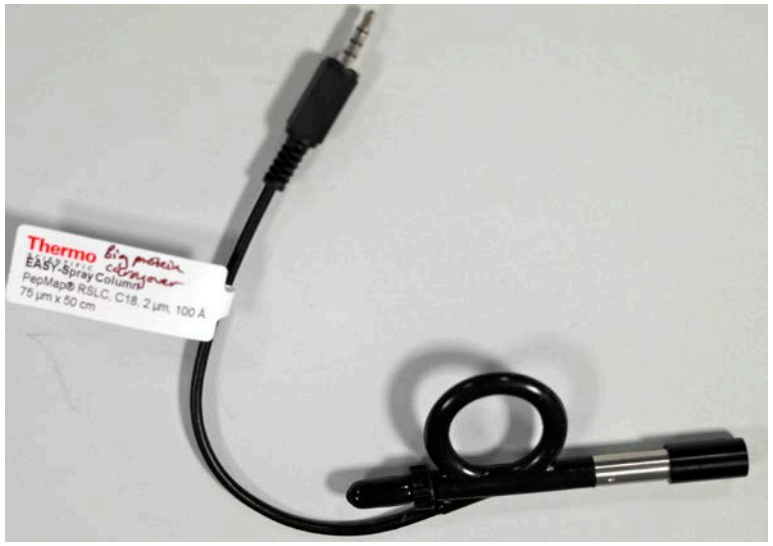
Results: LC-MS vs. ELISA



- Nano-LC-MS (easyspray-Q-Exactive): 10-200 time increase in sensitivity
- Target sensitivity sub ng/ml of therapeutic proteins



Nano-LC-HRMS

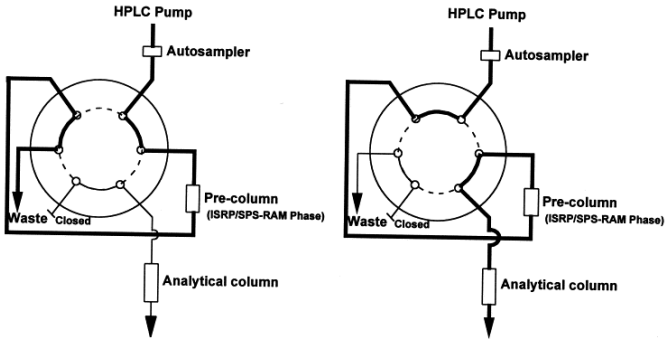
Sample prep:

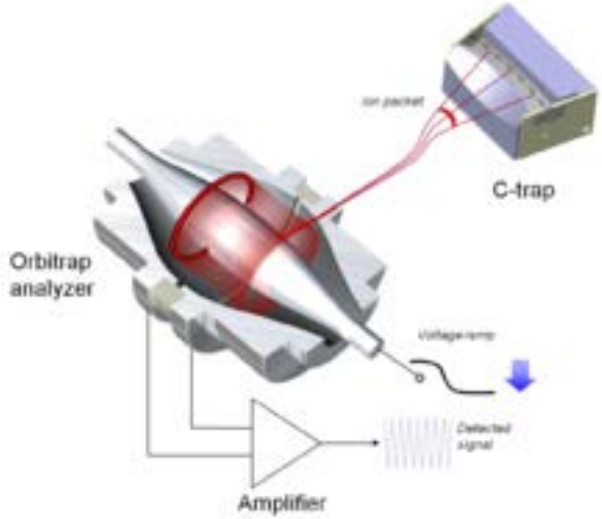
- Magnetic bead protein A extraction

LC-MS method:

- **Trap**: Pre-column: Acclaim® PepMap 100, 75 µm x 2 cm, nanoViper, C18, 3µm, 100Å
- **EasySpray:** Pepmap® 50 µm x 15 cm, RSLC C18, 2µm, 100Å
- ESI+
- **Orbitrap**: QExactive

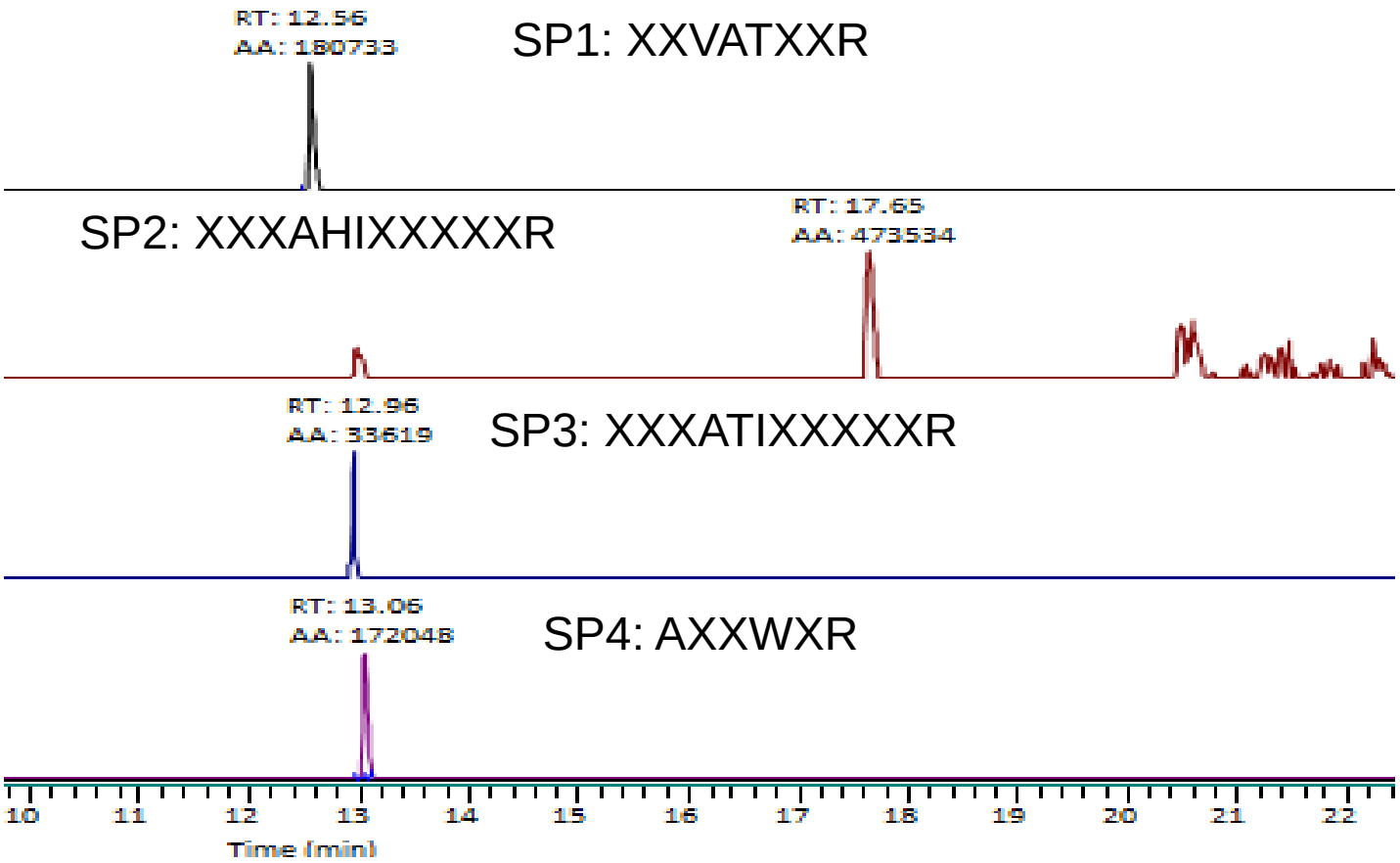
- Sample volume: 15 µL
- Injection volume: 10 µL





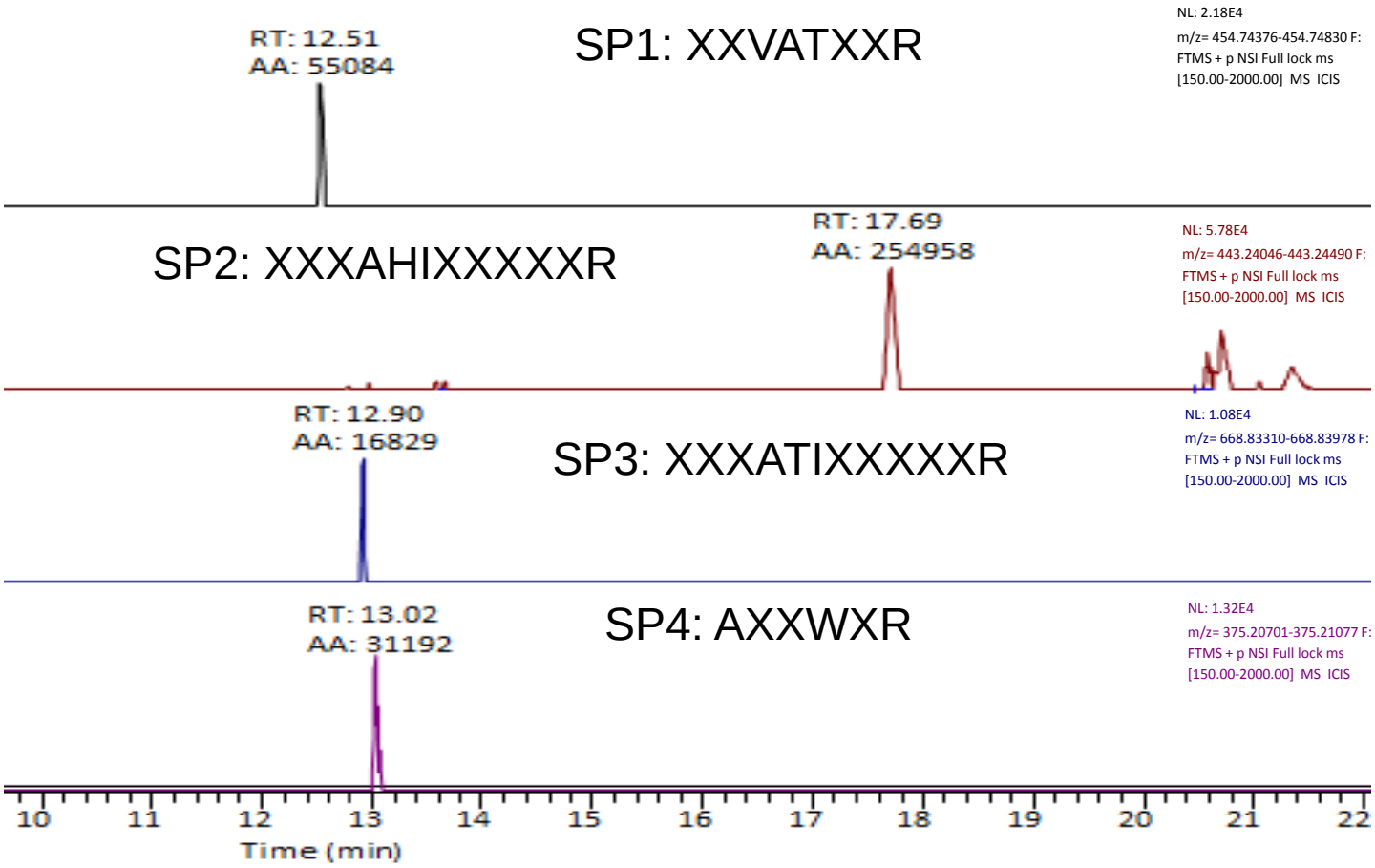
Nano-LC-HRMS: results

400 pg/ml (7.0 fM) of therapeutic protein in mouse serum



Nano-LC-HRMS: results

100 pg/ml (1.7 fM) of therapeutic protein in mouse serum



Nano-LC-HRMS

Performance of EasySpray-Q Exactive system is not optimal:

Lifetime nanoLC column + spray needle:

- 30-50 injections:
- Signal failure
- Black deposition on spray needle.

Trouble-shooting:

- by-pass non-analyte eluate to waste
- needle:
  - clean needle
  - adapt spray voltage parameter
  - avoid droplet formation at needle tip
- sample clean-up
  - replace TFA with formic acid
  - additional step (3rd): may result in loss of analyte

Carry-over

- 1-2%
- only slowly declines after further blank injections
- caused by injection system (also observed for cafeïne)

nanoLC implementation is essential for *sub ng/ml* sensitivity!

Conclusions

LC-MS bioanalysis of therapeutic proteins at low level ng/ml is feasible

Method development is time consuming (3-6 weeks):

- Signature peptide selection
  - Universal vs. unique
  - Synthesis of SIL peptides for IS (several weeks)
- Sample prep: Apply immuno precipitation when feasible
  - Protein A/G or anti-huFc for mAbs
  - Drug target/antigen for other
- Digestion: optimize to 100 % digestion
  - Especially when no SIL-protein is available

Nano-LC - MS:

- One commercial “plug and play” system was tested
- It is anticipated that several weeks of optimization and troubleshooting is required

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Review

Bioanalytical LC–MS/MS of protein-based biopharmaceuticals

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ABSTRACT

Biotechnology increasingly delivers highly promising protein-based biopharmaceutical candidates to the drug development funnel. For successful biopharmaceutical drug development, reliable bioanalytical methods enabling quantification of drugs in biological fluids (plasma, urine, tissue, etc.) are required to generate toxicokinetic (TK), pharmacokinetic (PK), and bioavailability data. A clear observable trend is that liquid chromatography coupled to (tandem) mass spectrometry (LC–MS(/MS)) is more and more replacing ligand binding assays (LBA) for the bioanalytical determination of protein-based biopharmaceuticals in biological matrices, mainly due to improved selectivity and linear dynamic ranges. Practically all MS-based quantification methods for protein-based biopharmaceuticals traditionally rely on (targeted) proteomic techniques and include "seven critical factors": (1) internal standardization, (2) protein purification, (3) enzymatic digestion, (4) selection of signature peptide(s), (5) peptide purification, (6) liquid chromatographic separation and (7) mass spectrometric detection. For this purpose, the variety of applied strategies for all "seven critical factors" in current literature on MS-based protein quantification have been critically reviewed and evaluated. Special attention is paid to the quantification of therapeutic monoclonal antibodies (mAbs) in serum and plasma since this is a very promising and rapidly expanding group of biopharmaceuticals. Additionally, the review aims to predict the impact of strategies moving away from traditional protein cleavage isotope dilution mass spectrometry (PC-IDMS) toward approaches that are more dedicated to bioanalysis.