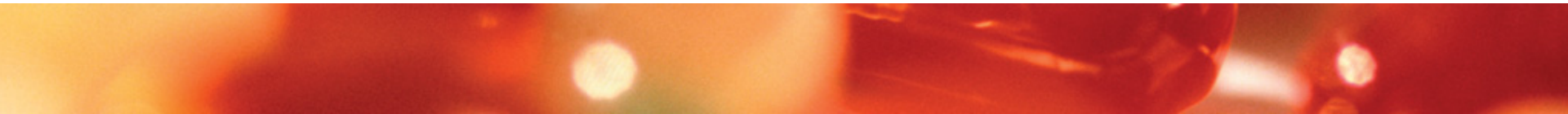
Chip-based LC-MS methods for sensitive determination of mAbs in serum

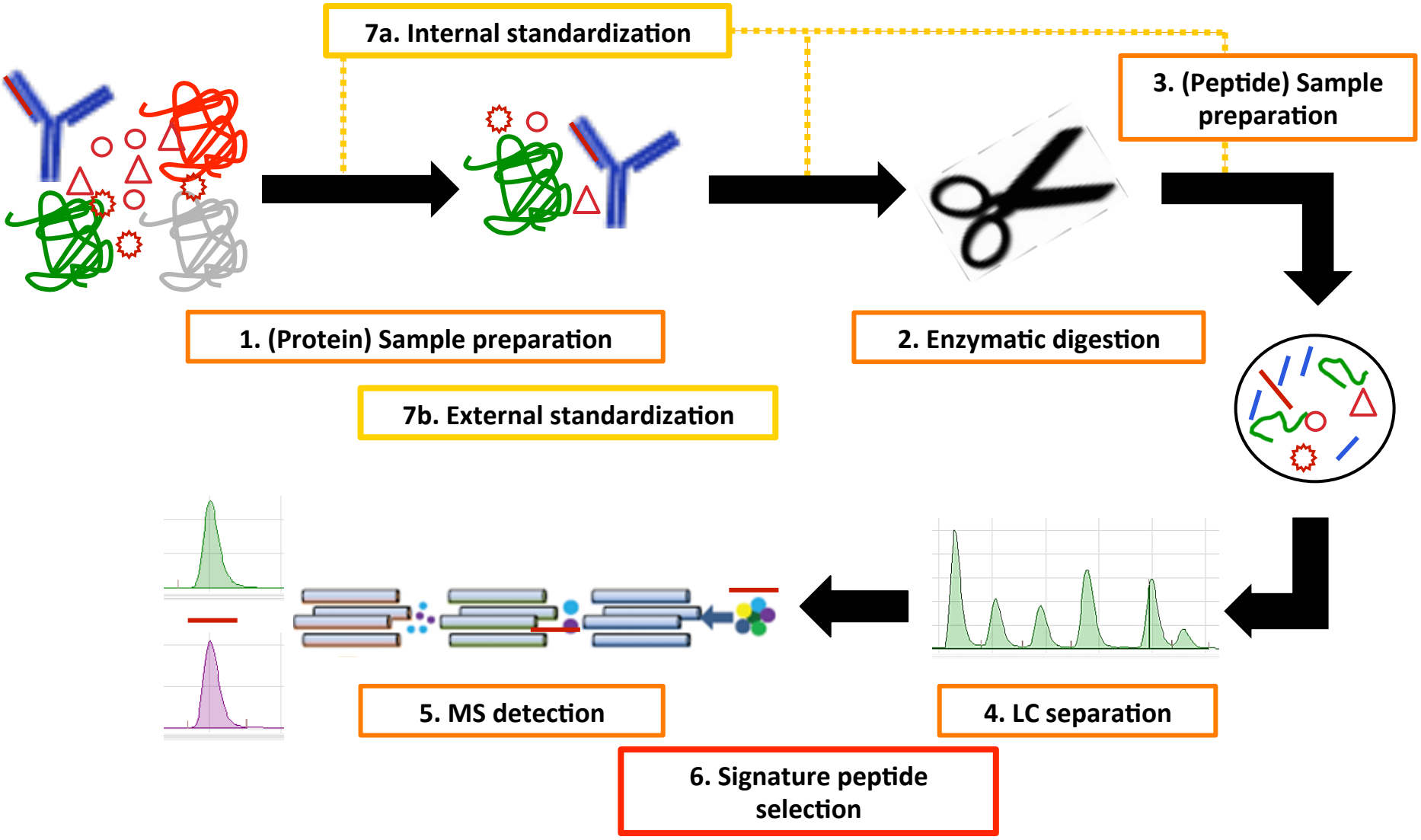
William D van Dongen, PhD
Product Manager Bioanalysis
william.vandongen@tno.triskelion.nl

40 years experience in bioanalysis



Bioanalytical LC-MS of therapeutic proteins: **7 Critical Factors**

TNO triskelion bv



I van den Broek, WMA Niessen and WD van Dongen, *J Chromatogr B* 929 (2013) 161-179

Bioanalytical LC-MS of therapeutic proteins: Limitations

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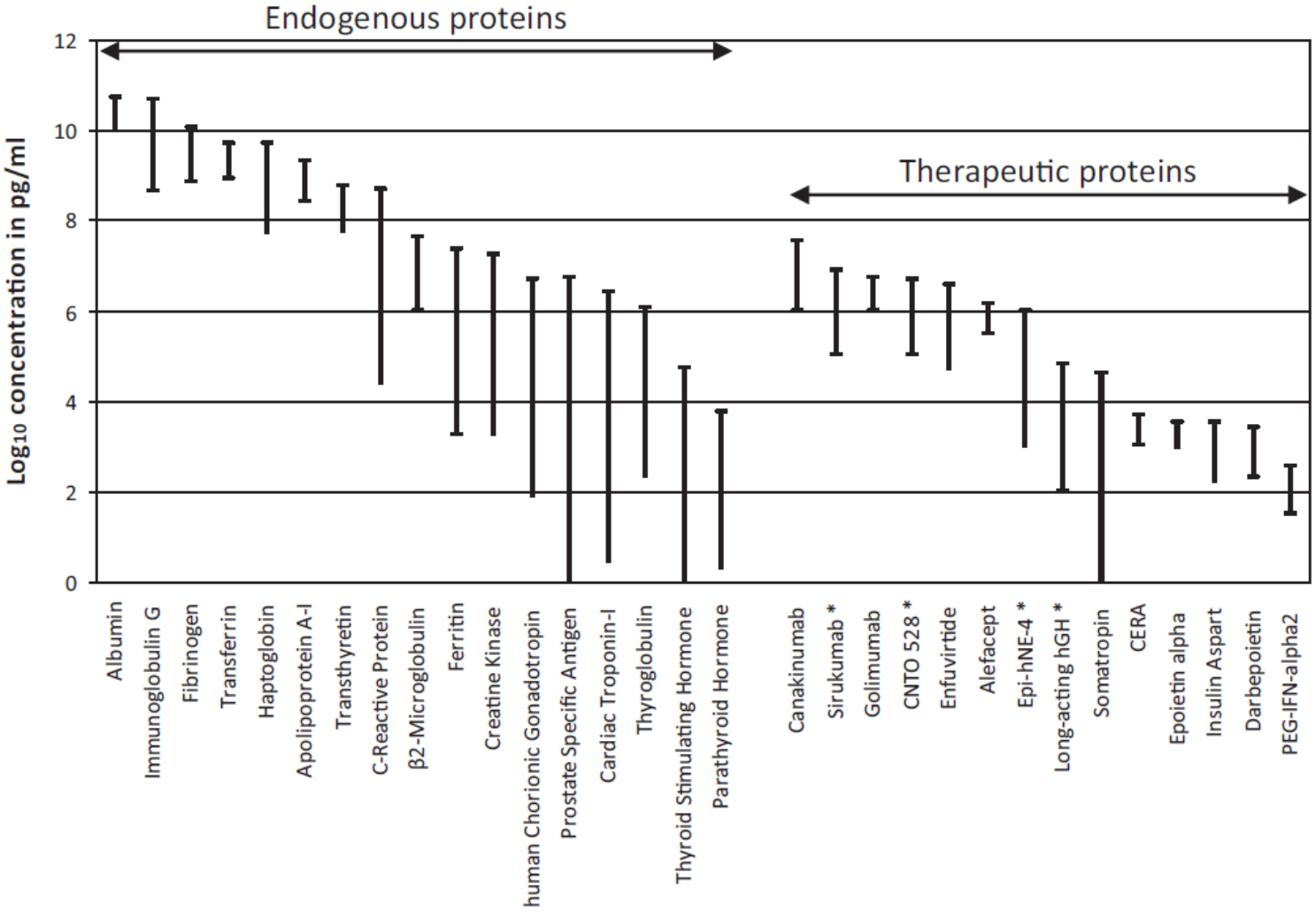
Therapeutic proteins: big molecules, low levels, low numbers



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

Bioanalytical LC-MS of therapeutic proteins

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Limitations

- Complex biological matrix
  - High protein (IgG) background in plasma/serum
- Enzymatic (tryptic) digestion:
  - time consuming reducing throughput
  - decreases reproducibility
  - Sensitivity loss:
    - sample dilution (5-10*)
    - only small piece (1-2%) is analyzed: signal reduction

Solutions

- Efficient targeted sample prep (factor 1+3)
- Complete and robust enzymatic digestion (factor 2)
- LC-MS (factors 4+5)
  - Triple quad vs. HRMS
  - Efficient ionization: nano-Spray
- Careful selection of “sensitive” signature peptide (factor 6)
  - Preferably several SPs for sensitivity optimization

Bioanalytical LC-MS of therapeutic proteins: 7 Critical Factors

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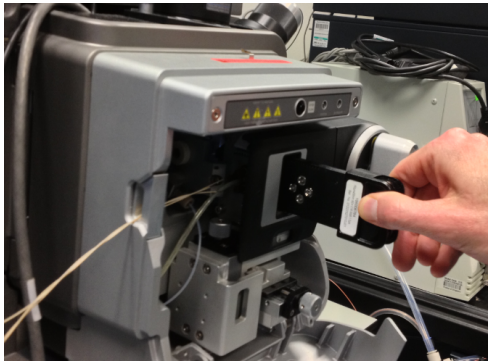
1. Protein Sample preparation	**Immuno precipitation**
2. Enzymatic digestion	100% and robust [Journal Proteome Res 12 (**2013**) 5760]
3. (Peptide) Sample preparation	Optional
4. LC separation	**micro/nano LC**
5. MS detection	**micro/nano ESI**
6. Signature peptide selection	**BLAST** **SP** selection based on sensitivity
7a. Internal standardization	@ protein or peptide level [J Chromatogr B 893-894 (**2012**) 1]
7b. External standardization	Ref material spiked to plasma



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ANALYTES

TNO triskelion bv

Name peptide	origin	[M+H]---$^{+}$	[M+2H]---$^{2+}$	[M+3H]---$^{3+}$	[M+4H]---$^{4+}$
Peptide ASG	other	697.35	349.18	233.12	175.09
IYPTNGYTR	Trastuzumab	1084.54	542.76	362.19	271.89
SLSLSPGK	Infliximab	788.45	394.73	263.49	197.87
FTISADTSK	Trastuzumab	969.49	485.25	323.84	243.13
Peptide EFV	other	1337.71	669.36	446.58	335.18
DTYIHWVR	Trastuzumab	1089.55	545.28	363.85	273.14
Peptide AIG	other	759.41	380.21	253.81	190.61
Peptide YAG	other	570.29	285.65	190.77	143.33
DILLTQSPAILSVSPGER	Infliximab	1896.04	948.53	632.69	474.77
VVSVLTVLHQDWLNGK	Infliximab	1808.01	904.51	603.34	452.76

Trastuzumab (trade names Herclon, Herceptin): monoclonal antibody that interferes with the HER2/neu receptor. Its main use is to treat certain breast cancers

Infliximab (trade name Remicade) is a chimeric monoclonal antibody against tumour necrosis factor alpha (TNF-α) used to treat autoimmune diseases.

- Concentration peptides: 50 ng / ml (direct injection)
- Concentration peptides: 0.5-2000 pg/ml (with trapping)
- Concentration mAbs: 1-10000 ng/ml (protein level)

EXPERIMENTAL

Columns: 150 µm x 50 mm iKey™

- Peptide BEH C18 130Å 1.7µm
  - wide usable pH range, low pH stable, and low column bleed
- Peptide CSH C18 130Å 1.7µm
  - low concentration of positive charges for FA- or TFA mobile phases
- HSS T3 130Å 1.8µm
  - aqueous mobile phase compatible for polar and non-polar compounds.

TVM Trap Column

- 300 µm x 50 mm M-Class Trap Symmetry C18 100Å 5µm

EXPERIMENTAL

- Column temperature: 40°C
- Eluent A: 0.1% formic acid (FA) in MilliQ water
- Eluent B: 0.1% FA in acetonitril (ACN)
- Injection volume: 0.2 – 5 µl (direct injection)
- Injection volume: 5-20 µl (trapping)
- Acquity M-Class flow: 3 µl/min
- Trapping flow: 30 µl/min
- Gradient:

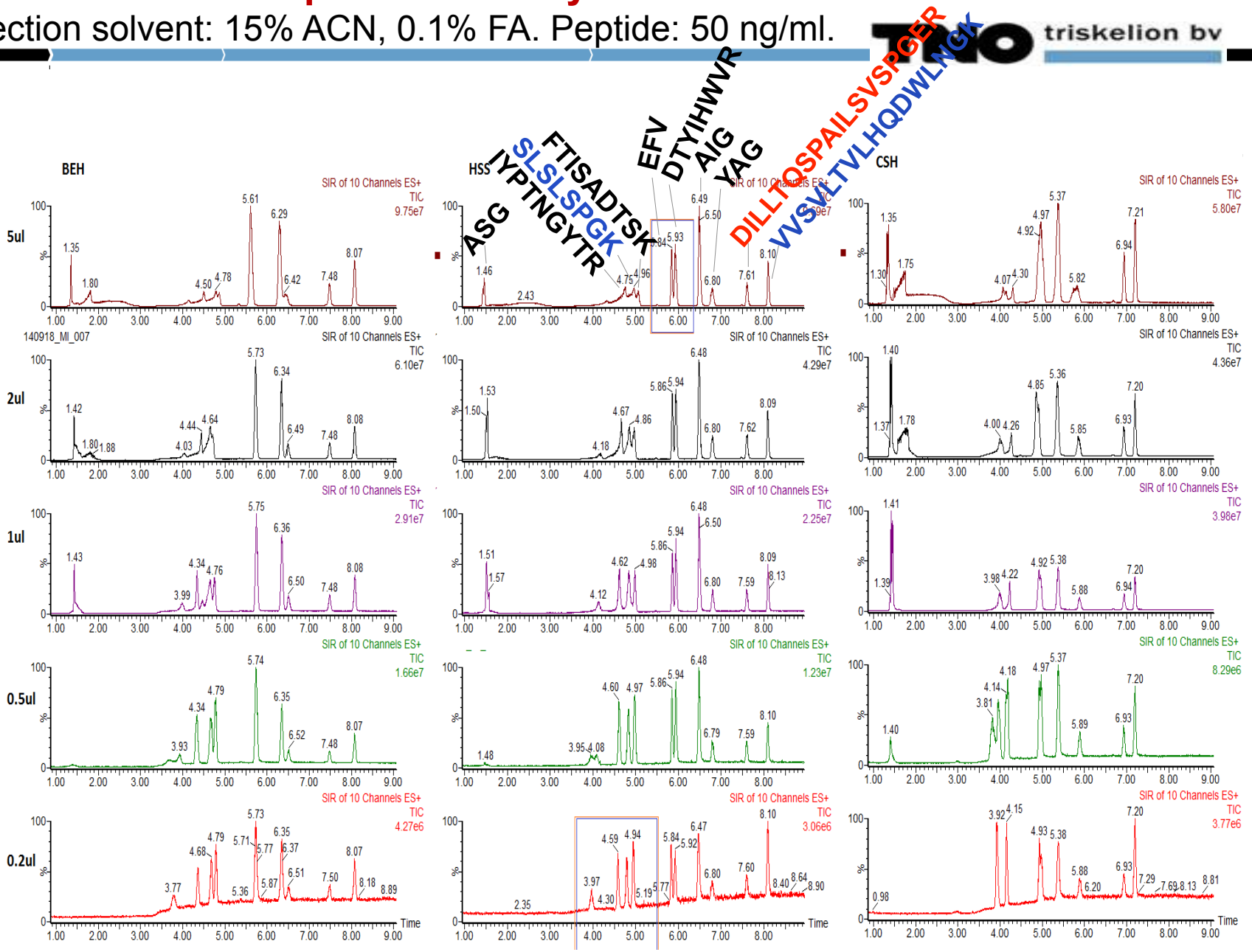
Time (min)	% A	% B
-1.5 (trap)	100	0
0	95	5
10	50	50
10,5	15	85
12	15	85
12,5	95	5
15	95	5

- MS: Xevo-TQS

Optimisation: Comparison of iKey columns

injection solvent: 15% ACN, 0.1% FA. Peptide: 50 ng/ml.

triskelion bv



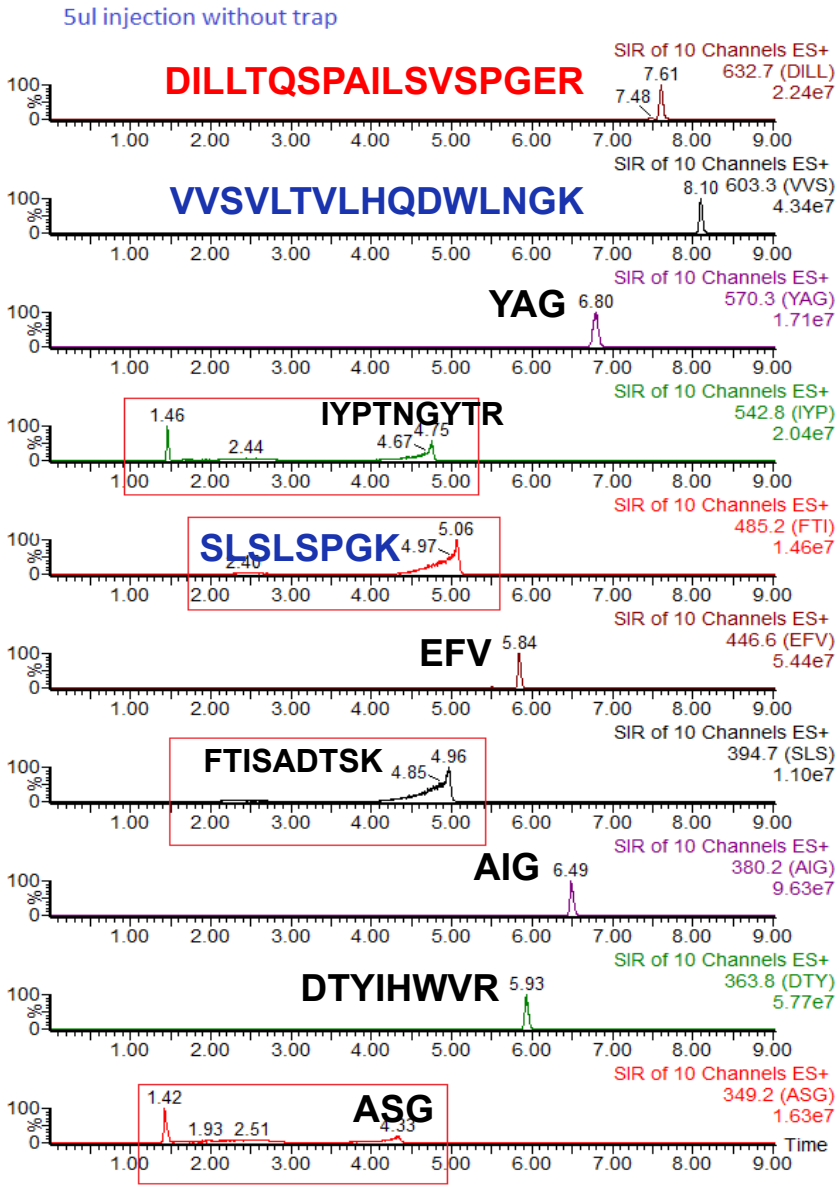
Trap Column: ACQUITY UPLC M-Class

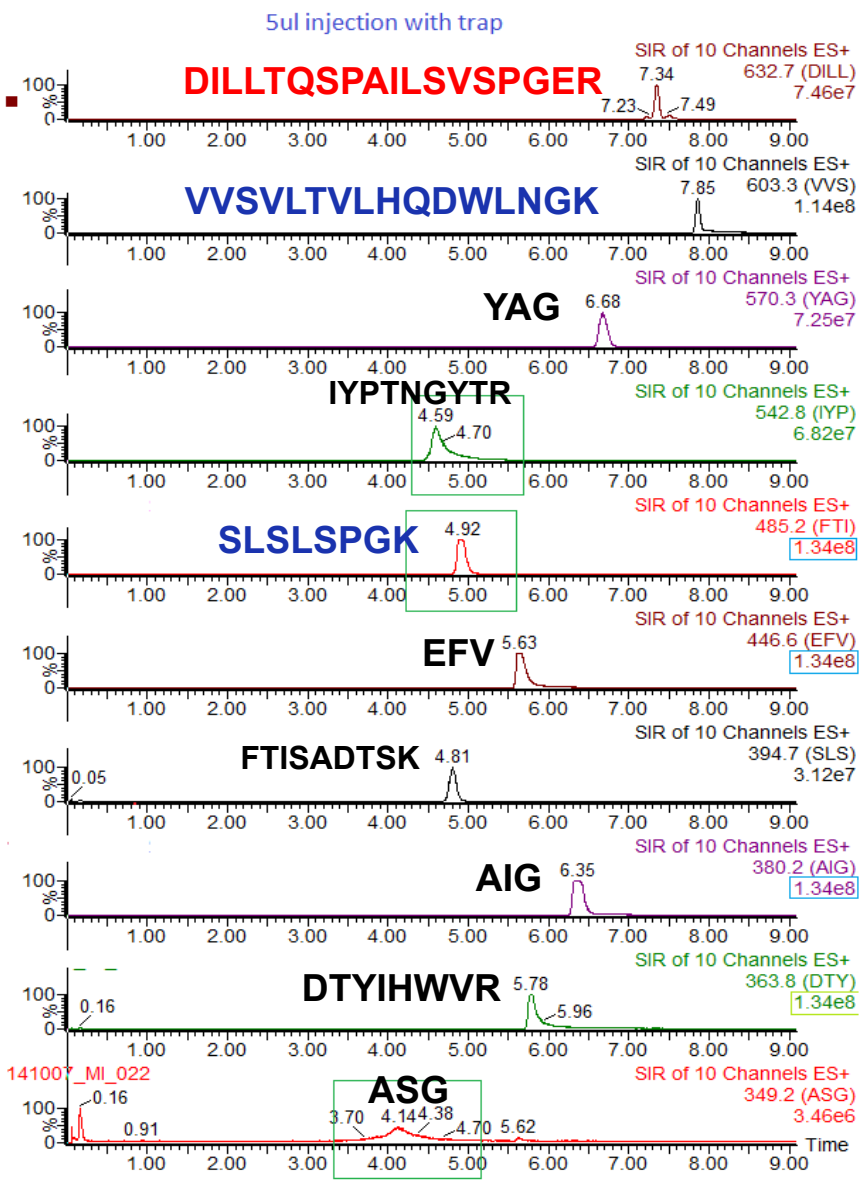
TVM Trap Symmetry C18;

100Å; 5µm; 300 µm x 50 mm

Optimisation: TVM Trap Column prior to analytical column

injection solvent: 15% ACN, 0.1% FA. Peptide: 50 ng/ml.





Infliximab (trade name Remicade) is a chimeric monoclonal antibody against tumour necrosis factor alpha (TNF-α) used to treat autoimmune diseases.

ionKey after optimisation using peptide standards
Sensitivity SLSLSPGK: 0.5 pg/ml
Sensitivity VVSVLTVLHQDWLNGK: 2.0-5.0 pg/ml
Sensitivity DILLTQSPAILSVSPGER: 5.0 pg/ml

A peptide sensitivity of **0.5-5 pg/ml** corresponds to **0.5-5 ng Infliximab/ml** biological matrix using TNO Triskelion standard protein A purification protocol.
Factor 100 is lost protein cleavage
Factor 10 is lost during sample preparation

See poster at this conference:
Optimization of LC and MS parameters for the detection of signature peptides using IonKey-MS
A.J. Kleinnijenhuis, M. Ingola, J.H. Toersche, F.L. van Holthoon, R.C. Bas, W.D. van Dongen

Sample clean-up and enrichment

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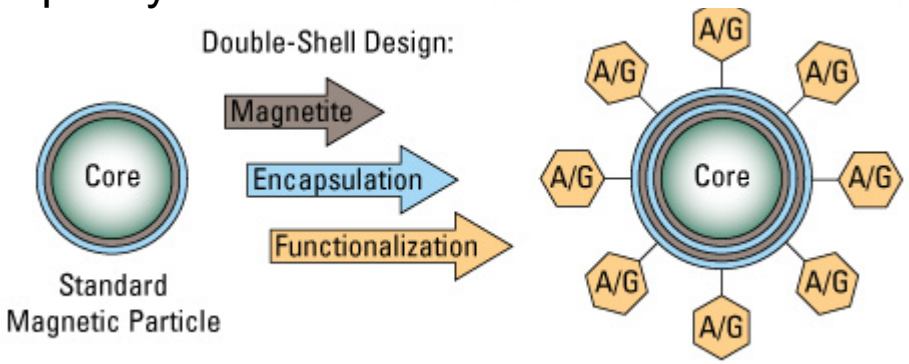
PureProteome™ Magnetic Beads for Immunocapture

- **Protein A** (PureProteome™, Millipore) is a 56 kDa surface protein originally found in the cell wall of the bacterium *Staphylococcus aureus*.
- Protein A binds immunoglobulins. 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

- Reduce complexity of the sample by mAb enrichment
- After binding, washing and elution => sample digestion



pilot LC-MS infliximab in rat serum

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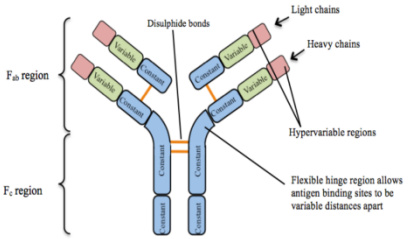


Compound

Remicade (infliximab): 144190,3 g/mol (K)(R) Tryptic cleavage sites

Remicade Heavy chain [2]:

EV(K)LEESGGGLVQPGGSM(K)LSCVAS**GFIFSNHWMN**WVR(Q)SPE(K)GLEWV**AEIR(S)(K)SINSATH**YAES
VKG(R)FTIS(R)DDS(K)SAVYLQMNSLRTEDTGVYYC**SRNYYGSTYD**YGQGTTLTVSXAST(K)GPSVFPL
APSS(K)STSGGTAALGCLV(K)DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL
GTQTYICNVNH(K)PSNT(K)VDK(R)VEP(K)SP(K)SCD(K)THTCPPCPAPELLGGPSVFLFPP(K)PKDTLMIS(R)
TPEVTCVVVDVSHEDPEV(K)FNWYVDGVEVHNA(K)T(K)P(R)EEQYNSTYR**VVSVLTVLHQDWLNG(K)**EYK
C(K)VSN(K)ALPAPIE(K)TIS(K)A(K)GQP(R)EPQVYTLPPS(R)DELT(K)NQVSLTCLV(K)GFYPSDIAVEWESNG
QPENNY(K)TTPPVLDSDGSFFLYS(K)LTVD(K)S(R)WQQGNVFSCSVMHEALHNHYTQ(K)**SLSLSPG(K)**

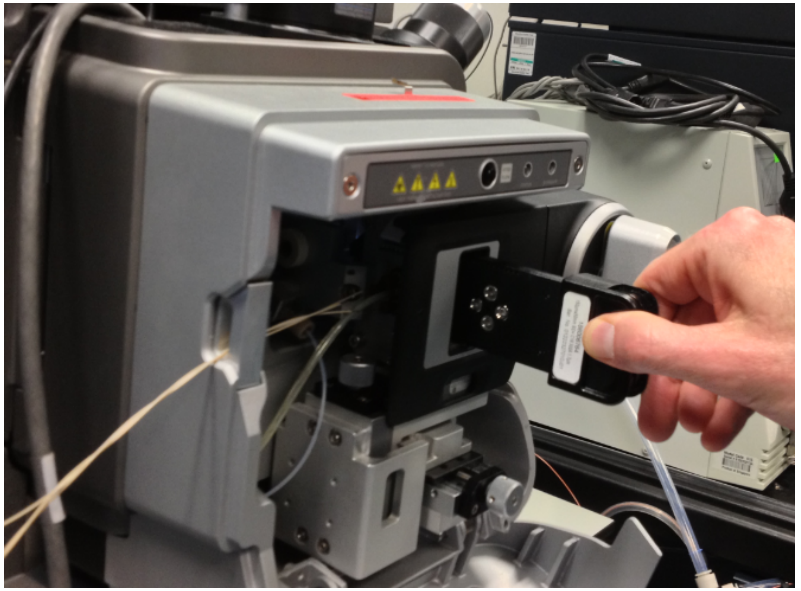
Remicade Light chain [2]:

DILLTQSPAILSVSPGE(R)VSFSC(R)A**SQFVGSS**IHWYQQ(R)TNGSP(R)LLI**(K)YASESMSGI**PS(R)FSGS
GSGTDFTLSINTVESEDIADYYC**QQSHSWPFTFGS**GTNLEV(K)TVAAPSVFIFPPSDEQL(K)SGTAS
VVCLLNNFYP(R)EAKVQW(K)VDNALQSGNSQESVTEQDS(K)DSTYSLSSTLTLS(K)ADYE(K)H(K)VYACEV
THQGLSSPVT(K)SFN(R)GEC

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

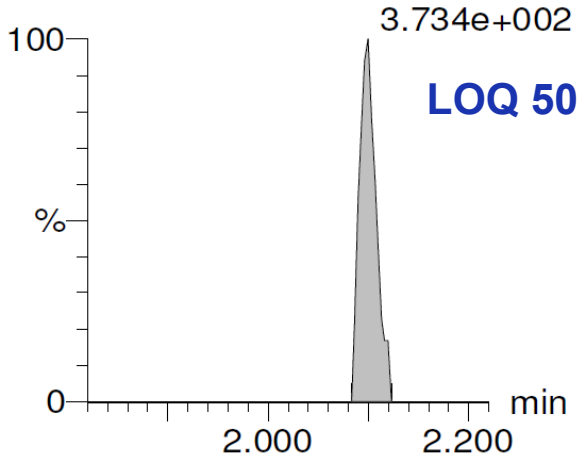
BLAST= Basic Local Alignment Search Tool
Compares protein sequences to sequence databases

- Rat serum spiked with Infliximab at 0, 0.01, 0.02, 0.1, 0.2, 0.5, 1.0, 10 µg per ml
- Sample preparation: Immunocapture and digestion
- UPLC MS/MS analysis: - Xevo-TQS
- **M-Class + Ion-Key Xevo-TQS**



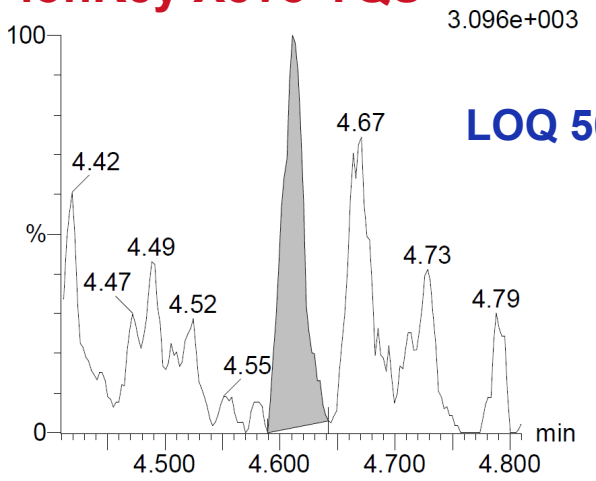
DILLTQSAPAILSVSPGER

UPLC Xevo-TQS

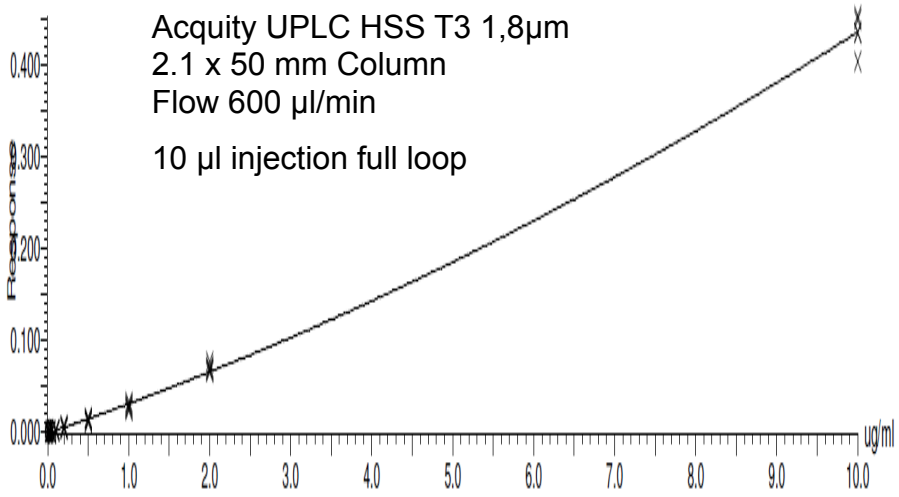


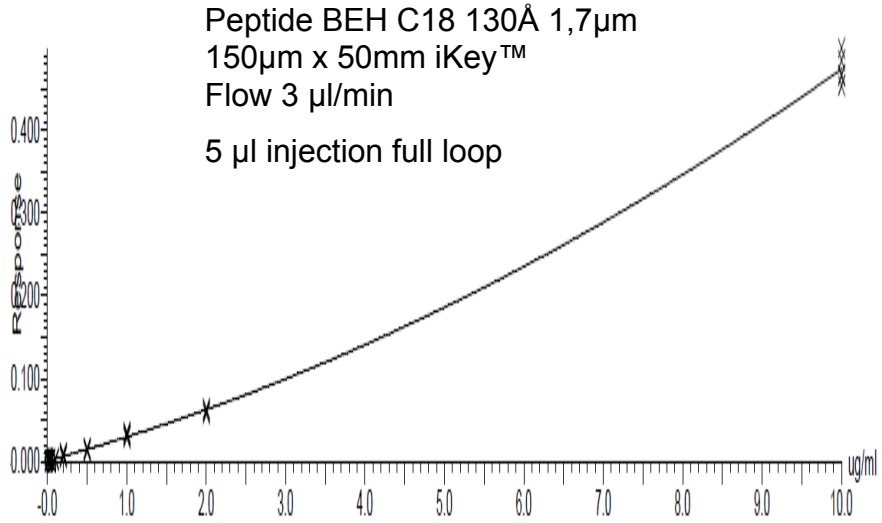
LOQ 50 ng/ml

ionKey Xevo-TQS



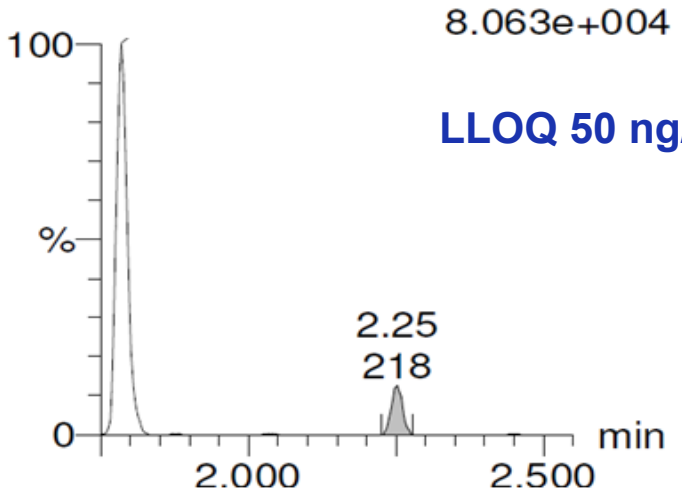
LOQ 50 ng/ml





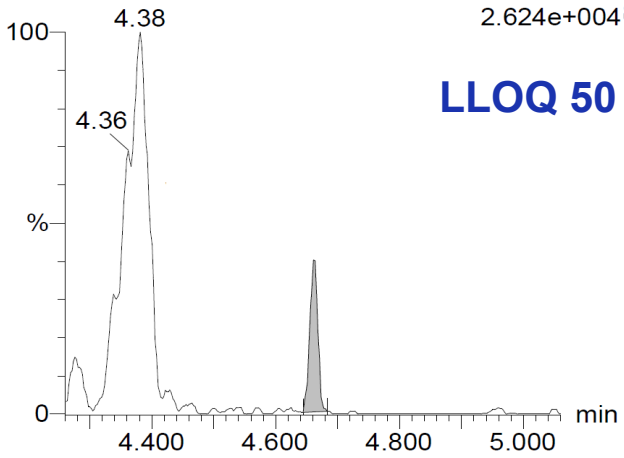
VVSVLTVLHQDWLNGK

UPLC Xevo-TQS



LLOQ 50 ng/ml

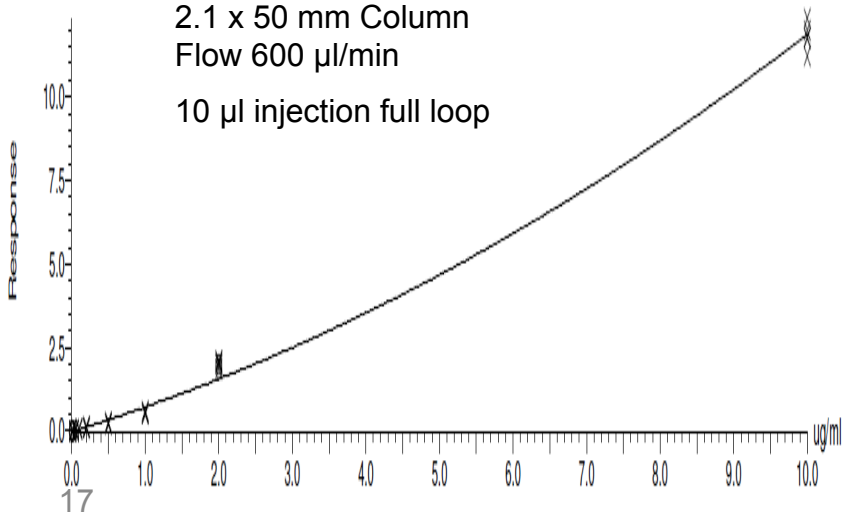
ionKey Xevo-TQS



LLOQ 50 ng/ml

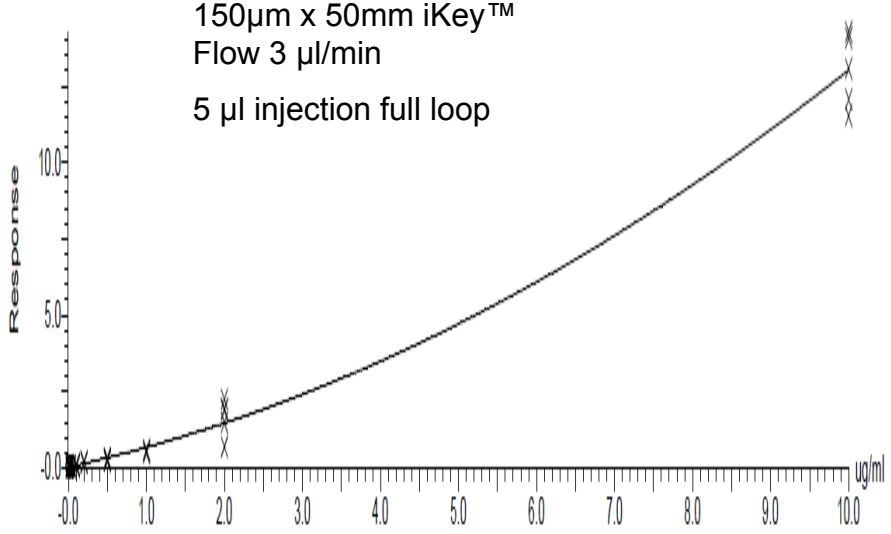
Acquity UPLC HSS T3 1,8µm
2.1 x 50 mm Column
Flow 600 µl/min

10 µl injection full loop



Peptide BEH C18 130Å 1,7µm
150µm x 50mm iKey™
Flow 3 µl/min

5 µl injection full loop

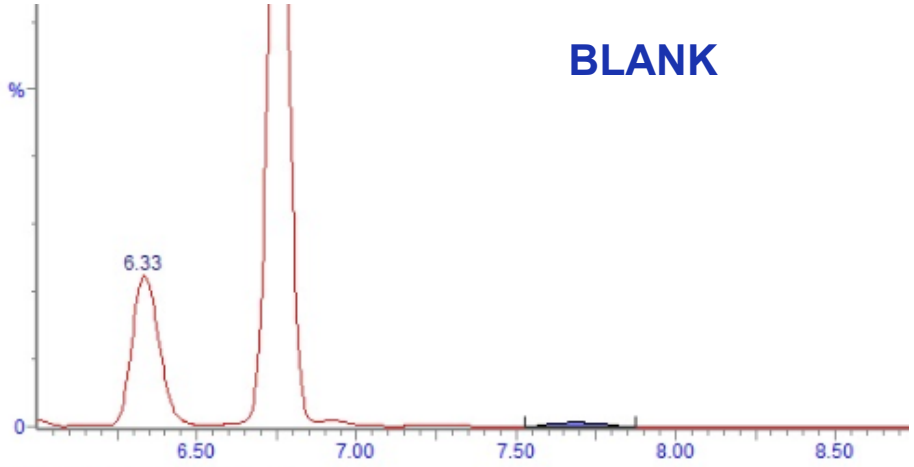


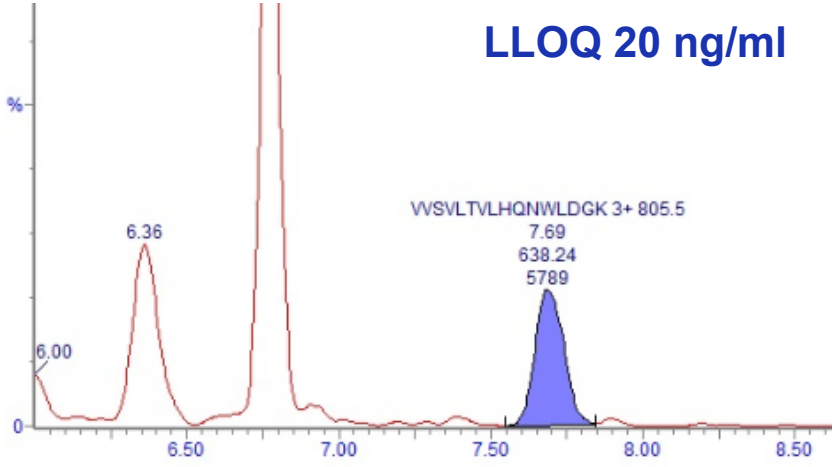
Trap Column: ACQUITY UPLC M-Class

TVM Trap Symmetry C18;

100Å; 5µm; 300 µm x 50 mm

VVSVLTVLHQDWLNGK



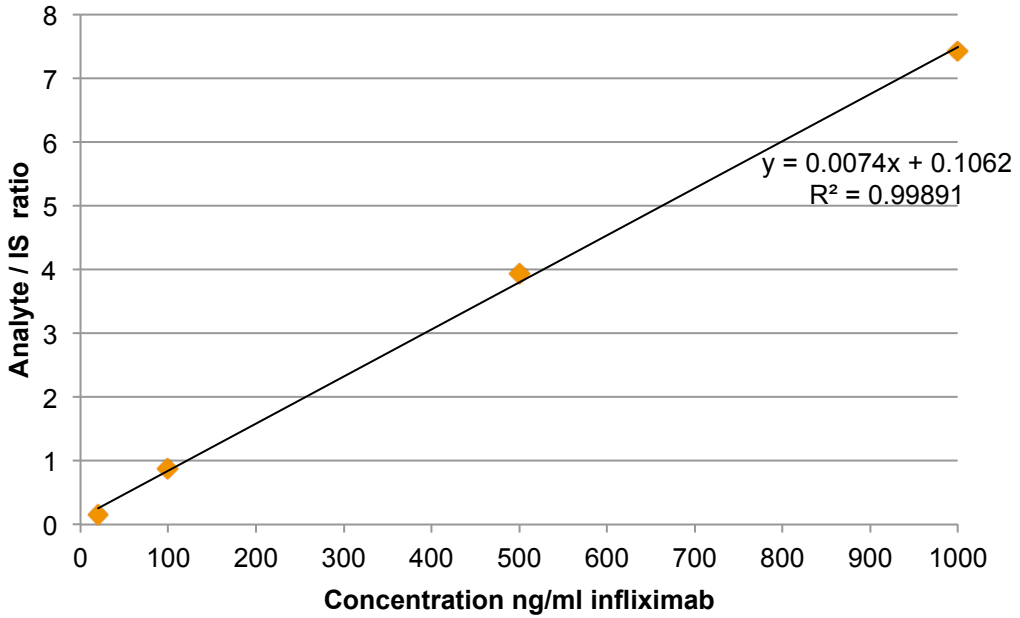


ionKey

Column: 150 µm x 50 mm iKey™ HSS T3 130Å 1.8µm, Flow 3 µl/min

TVM Trap Column: 300 µm x 50 mm M-Class Trap Symmetry C18 100Å 5µm

20 µl injection



20 ng/ml @ mAb level vs. potentially 0.5-5.0 ng/ml: Critical Factors

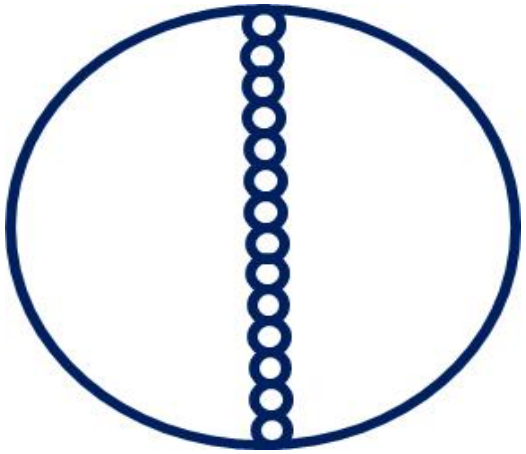
TNO triskelion bv

Differences ionKey vs conventional

- **Sensitivity approx. 10 times better for ionKey (several users)**
  - We find **no** difference (**5** µl inj. ionKey vs. **10** µl inj. conventional)
  - factor **2.5** (including trap **20** µl inj. ionKey vs. **10** µl inj. conventional)

- **Suppresion effects (pre-EBF workshop Waters 17-11-2014)**
  - Approx. factor **25** less with ionKey due to more efficient ionization

- **Column dimensions ionKey (150 µm) vs 2.1 mm :**



2100 µm

Δ surface area = factor **196** (concentration)

20 ng/ml @ mAb level vs. potentially 0.5-5.0 ng/ml: 7 Critical Factors

TNO triskelion bv

How to obtain full potential (1 ng/ml mAb in serum) of micro LC-MS?

It's all about sample prep. (and avoiding the need of it)

1. Protein Sample preparation

Removal of matrix effects:

2. Enzymatic digestion →

- Change reduction agent: TCEP vs. DTT
- Change alkylation: iodoacetic acid (volitile) vs. iodoacetamide
- Tryptic digestion on bead (allowing to wash of DTT + iodoacetamide)

3. (Peptide) Sample preparation →

- Injection volume
- Additional extraction, eg micro-elution plates

4. LC separation

5. MS detection

6. Signature peptide selection

7a. Internal standardization

7b. External standardization

Conclusions

- LLOQ LC-MS infliximab for 2 signature peptides (DILLTQSAPAILSVSPGER + VVSVLTVLHQDWLNGK) is 50 ng/ml in rat serum extracts

- Calibration curves showed acceptable linearity in range 50-10,000 ng/ml in rat serum extracts

- Application of ionKey UPLC-MS/MS with trapping column allows for LLOQ of 20 ng/ml (VSVLTVLHQDWLNGK) in rat serum extracts.
  - Improvement is anticipated after lowering matrix in extracts

- Use of ionKey requires thinking in different dimensions

Acknowledgement

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