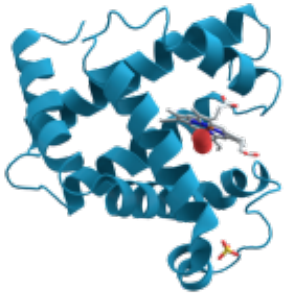
The usefulness of LC-MS as a platform for protein quantification

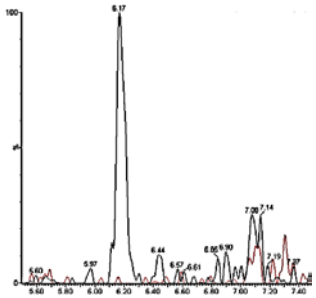
-

from theory to practice



Nico van de Merbel

20 November 2013



Content

From theory…

- LC-MS and LBA
- Quantitative aspects

…to practice

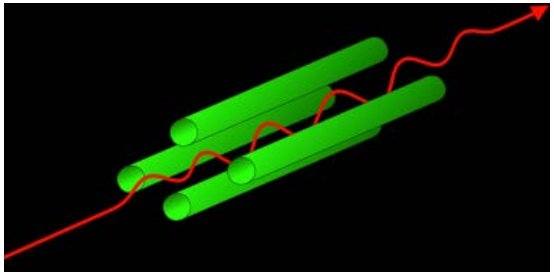
- Selectivity
- Accuracy and precision
- Multiplexing
- Structural information

LC-MS and LBA

Useful aspects of both platforms:

LC-MS:

- selectivity (no cross-reactivity)
- accuracy and precision (direct measurement, internal standardization)
- multiplexing
- structural information
- generic potential (no dependence on critical reagents)

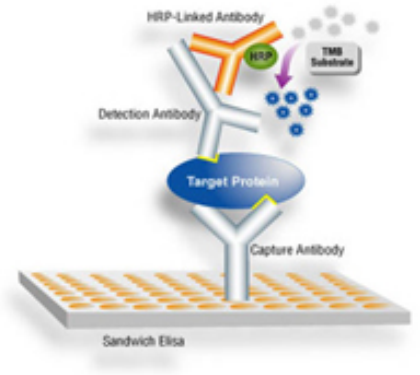


LC-MS and LBA

Useful aspects of both platforms:

LBA:

- sensitivity (sub-pg/mL level)
- high throughput
- ease of operation
- correlation with biological integrity / activity



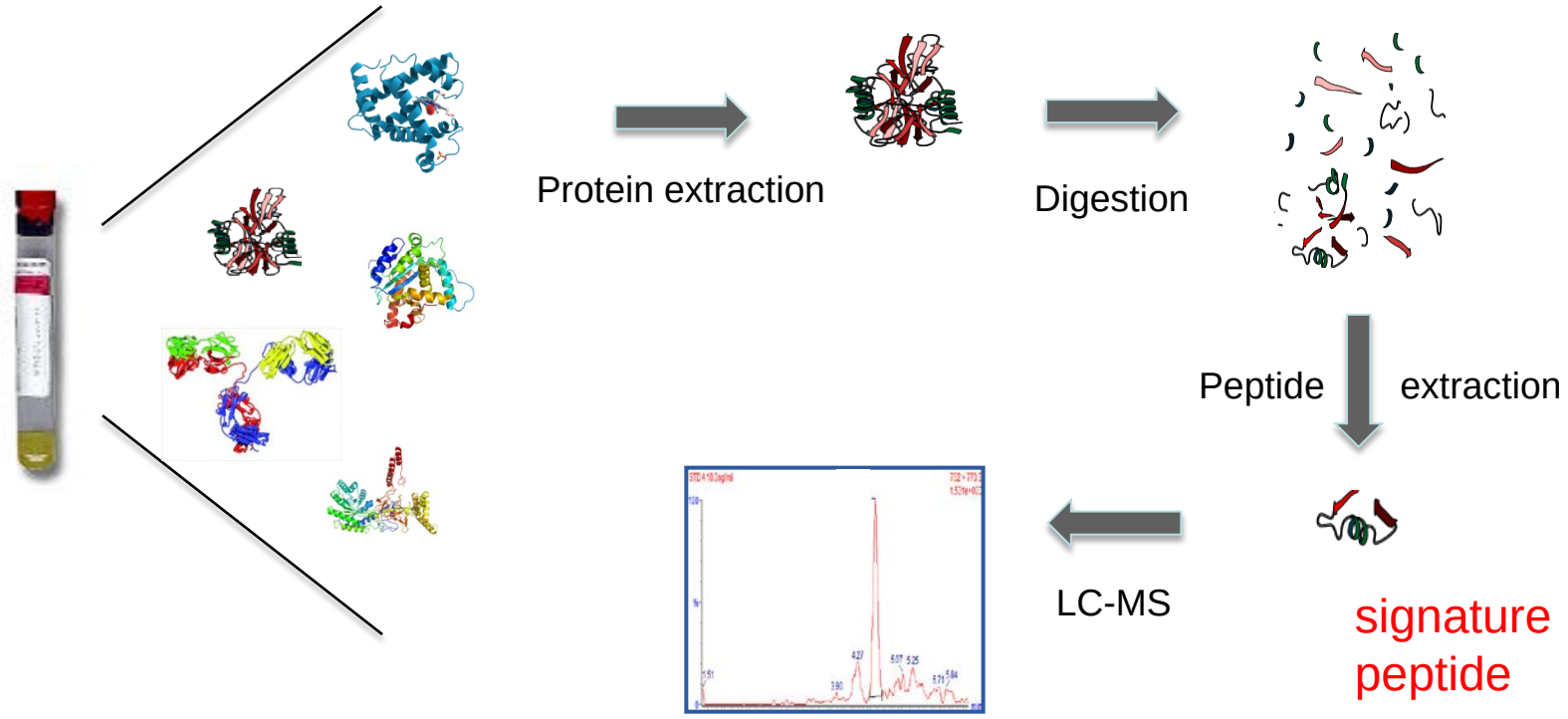
Quantitative aspects

What is “the concentration” of a protein?

Chemical concentration:

- Primary structure integrity
- Changes after e.g. amino acid oxidation
- Does not depend on 3D structure
- Determined by LC-MS, but only of (small) part of the total protein

Quantitative aspects

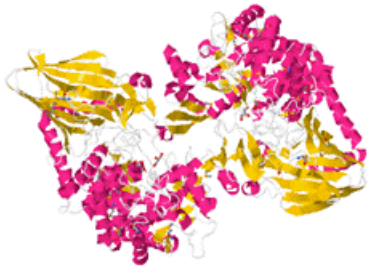


Quantitative aspects

MGVRHPPCSH	RLLAVCALVS	LATAALLGHI	LLHDFLLVPR	ELSGSSPVLE	ETHPAHQQGA
SRPGPRDAQA	HPGRPRAVPT	QCDVPPNSRF	DCAPDKAITQ	EQCEARGCCY	IPAKQGLQGA
QMGQPWCFFP	PSYPSYKLEN	LSSSEMGYTA	TLTRTTPTFF	PKDILTLRLD	VMMETENRLH
FTIKDPANRR	YEVPLETPRV	HSRAPSPLYS	VEFSEEPFGV	IVHRQLDGRV	LLNTTVAPLF
FADQFLQLST	SLPSQYITGL	AEHLSPLMLS	TSWTRITLWN	RDLAPTPGAN	LYGSHPFYLA
LEDGGSAHGV	FLLNSNAMDV	VLQPSPALSW	RSTGGILDVY	IFLGPEPKSV	VQQYLDVVGY
PFMPPYWGLG	FHLCRWGYSS	TAITRQVVEN	MTRAHFPLDV	QWNDLDYMDS	RRDFTFNKDG
FRDFPAMVQE	LHQGGRRYMM	IVDPAISSSG	PAGSYRPYDE	GLRRGVFITN	ETGQPLIGKV
WPGSTAFPDF	TNPTALAWWE	DMVAEFHDQV	PFDGMWIDMN	EPSNFIRGSE	DGCPNNELEN
PPYVPGVVGG	TLQAATICAS	SHQFLSTHYN	LHNLYGLTEA	IASHRALVKA	RGTRPFVISR
STFAGHGRYA	GHWTGDVWSS	WEQLASSVPE	ILQFNLLGVP	LVGADVCGFL	GNTSEELCVR
WTQLGAFYPF	MRNHNSLLSL	PQEPYSFSEP	AQQAMRKALT	LRYALLPHLY	TLFHQAHVAG
ETVARPLFLE	FPKDSSTWTV	DHQLLWGEAL	LITPVLQAGK	AEVTGYFPLG	TWYDLQTVPI
EALGSLPPPP	AAPREPAIHS	EGQWVTLPAP	LDTINVHLRA	GYIIPLQGPG	LTTTESRQQP
MALAVALTKG	GEARGELFWD	DGESLEVLER	GAYTQVIFLA	RNNTIVNELV	RVTSEGAGLQ
LQKVTVLGVA	TAPQQVLSNG	VPVSNFTYSP	DTKVLDICVS	LLMGEQFLVS	WC

Recombinant human alpha-glucosidase:

LC-MS response obtained is determined by *chemical* integrity of signature peptide (1.2% of intact protein)



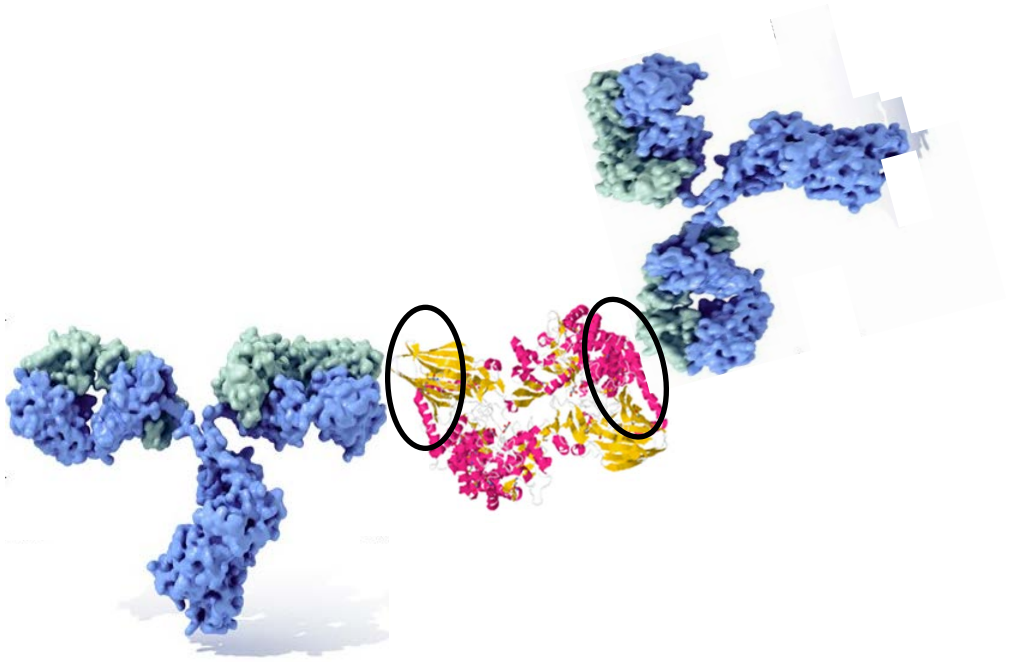
Quantitative aspects

What is “the concentration” of a protein?

Biological concentration:

- Primary, secondary, tertiary and quarternary structure integrity
- Depends on 3D structure
- Determined by LBA, based on part(s) of the total protein
- Might or might not correlate with active form of the protein

Quantitative aspects

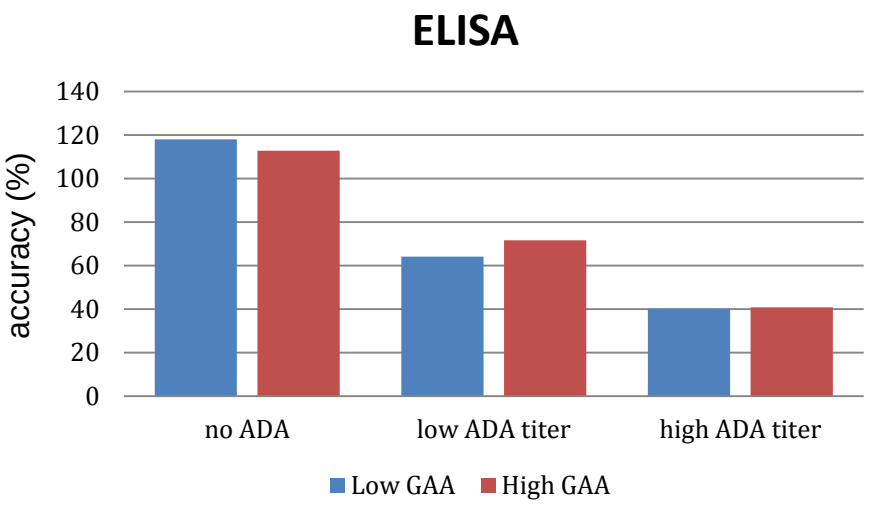


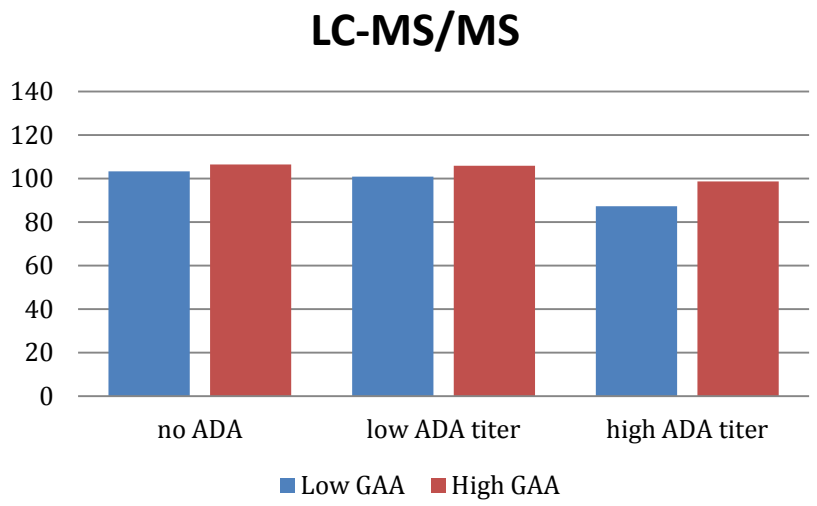
Recombinant human alpha-glucosidase:

ELISA response obtained is determined by *biological* integrity of two different parts of the protein

Quantitative aspects

LBA: free/immune-reactive concentration
digestion/LC-MS: total protein concentration





Quantitative aspects

- Because of its highly complex structure “the” concentration of a protein does not exist
- It depends on what part of the protein molecule one is looking at and by which technique
- LC-MS measures a (small) part of the molecule and will give a chemical concentration
- LBA measures a binding event and will give a biological concentration – or immune-reactivity
- These may not necessarily be similar for the same sample

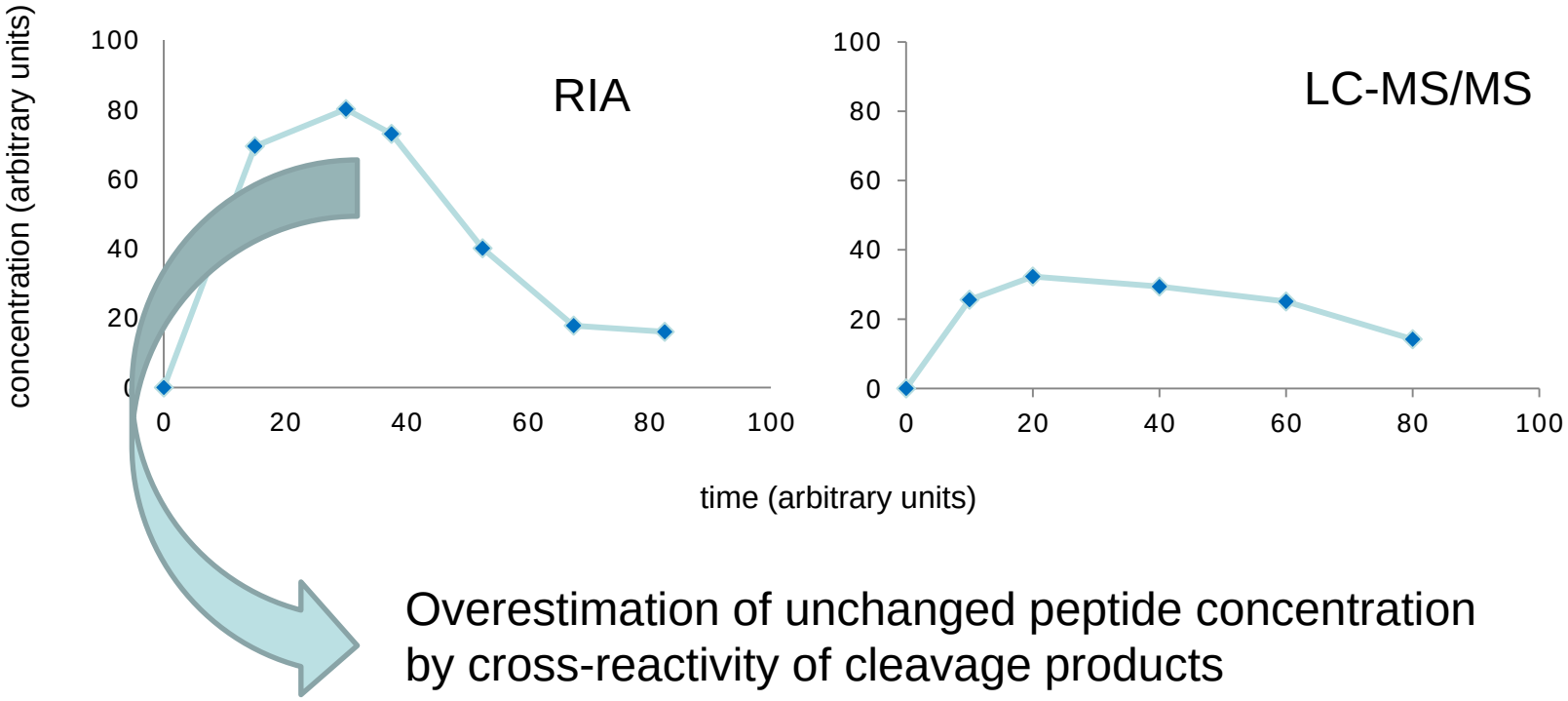
Selectivity

Cross-reactivity

Proprietary peptide

- molecular mass 5 kDa
- in vivo converted to several cleavage products
- existing competitive radioimmunoassay
- new LC-MS/MS method of intact peptide

Selectivity



Overestimation of unchanged peptide concentration by cross-reactivity of cleavage products

Accuracy and precision

Criteria

- Performance expected to be better for (antibody-free) LC-MS than for LBA
- EBF topic team proposes to start with conservative approach based on current acceptance criteria for macromolecules with LBA

M. Knutsson et al, Bioanalysis (2013) 5(18), 2211-14

Accuracy and precision

Recent experience

- 8 antibody-free methods
- SIL-protein and SIL-peptide IS
- digestion only, protein extraction, peptide extraction
- 6 analysts
- 3 types of MS

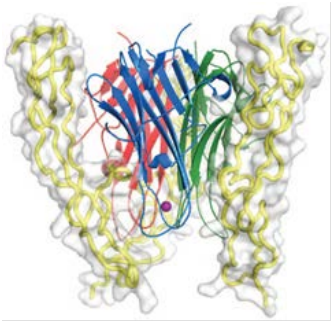
	LOW-HIGH		LLOQ	
	CV(%)	bias (%)	CV(%)	bias (%)
<1 ng/mL	5.1	1.5	2.6	0.1
1-10 ng/mL	3.0-3.2	1.0-2.1	5.4	3.2
10-100 ng/mL	0.8-10.1	1.2-7.6	7.0-11.7	4.2-10.4
100-1000 ng/mL	2.7-8.4	0.9-8.0	3.1-6.4	3.2-5.3
1-10 µg/mL	1.6-3.8	3.2-9.4		
10-100 µg/mL	1.3-6.2	0.8-8.0		
>100 µg/mL	0.5-3.4	1.3-5.8		

Multiplexing

(1) Quantification of multiple proteins

Tumor necrosis factor Related Apoptosis Inducing Ligand (TRAIL) variants

- recombinant trimeric metalloprotein in development for cancer treatment
- different receptor-specific variants available
- 168 amino acids per monomer
- molecular mass: 58 kDa



Multiplexing

Wild type:

VRERGPQ RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI LLMKSARNSC
WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH EASFFGAFLV G

4C7 specific:

VRERGPQ RVAAHITGTR **R**RSNTLSSPN SKNEKALG**I**K INSWESSR**R**G HSFLSNLHLR
NGELVIHEKG FYYIYSQTYF RFQEEIKE**R**T **H**NDKQMVQYI YKYT**D**YPDPI LLMKSARNSC
WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH EASFFGAFLV G

Multiplexing

Wild type:

VRERGPQ RVAAHITGTR GRSNTLSSPN SKNEKALGRK INSWESSRSG HSFLSNLHLR
NGELVIHEKG FYYIYSQTYF RFQEEIKENT KNDKQMVQYI YKYTSYPDPI LLMKSARNSC
WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH EASFFGAFLV G

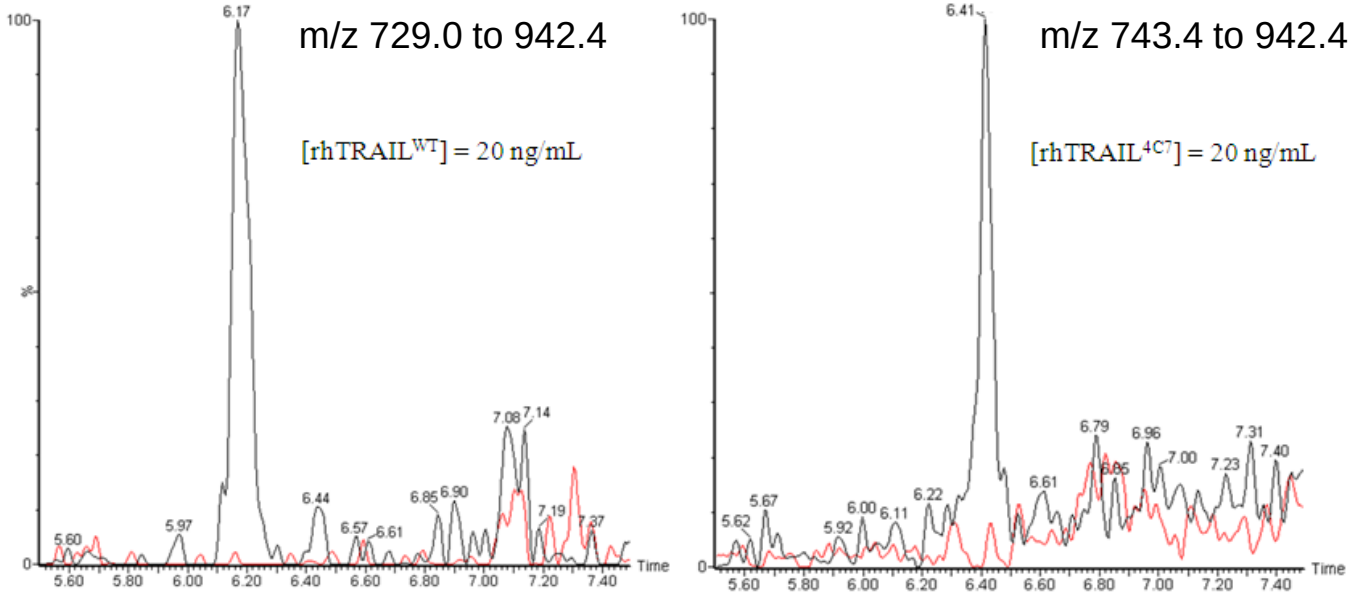
4C7 specific:

VRERGPQ RVAAHITGTR **R**RSNTLSSPN SKNEKALG**I**K INSWESSR**R**G HSFLSNLHLR
NGELVIHEKG FYYIYSQTYF RFQEEIKE**R**T **H**NDKQMVQYI YKYT**D**YPDPI LLMKSARNSC
WSKDAEYGLY SIYQGGIFEL KENDRIFVSV TNEHLIDMDH EASFFGAFLV G

Selection of distinguishable signature peptides

Multiplexing

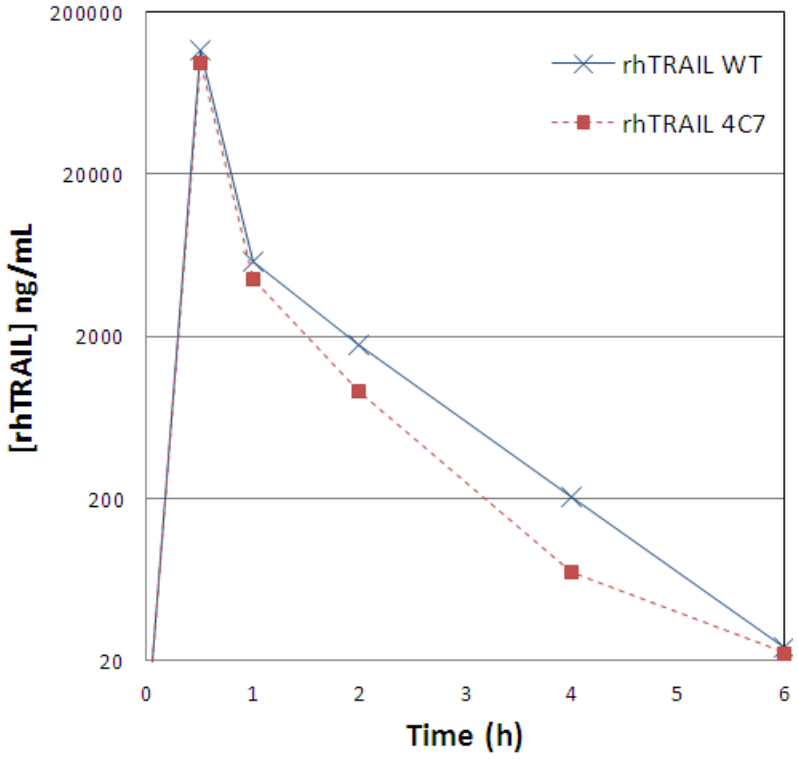
protein extraction by immobilized metal affinity chromatography (IMAC), 2-hour tryptic digestion, oxidation of signature peptides



Multiplexing

Application:

TK study in mice
(5 mg/kg i.p. of each TRAIL variant)

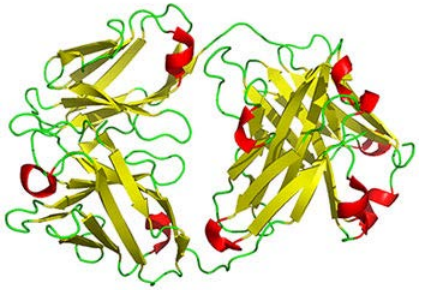


Multiplexing

(2) Quantification of multiple parts of a protein

Trastuzumab

- recombinant humanized monoclonal antibody for treatment of breast cancer
- 1328 amino acids in four polypeptide chains (two heavy, two light)
- molecular mass: 146 kDa



Multiplexing

Light chain:

DIQMTQSPSS LSASVGDRVT IT**CRASQDVN** **TAVA**WYQQKP GKAPKLLIY**S** **ASFLYS**GVPS
RFSGSRSGTD FTLTISSLQP EDFATYYC**QQ** **HYTTPPT**FGQ GTKVEIKRTV AAPSVFIFPP
SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT
LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC

Heavy chain:

EVQLVESGGG LVQPGGSLRL SCAASGFNIK **DTYIH**WVRQA PGKGLEWVA**R** **IYPTNGYTRY**
ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSR**WG** **GDGFYAMDY**W GQGTLVTVSS
ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS
GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG
PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQY**N**
STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE
MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

Bold = complementarity determining region (CDR)

Multiplexing

Light chain:

DIQMTQSPSS LSASVGDRVT IT**CRASQDVN** **TAVA**WYQQKP GKAPKLLIY**S** **ASFLYS**GVPS
RFSGSRSGTD FTLTISSLQP EDFATYYC**QQ** **HYTTPPT**FGQ GTKVEIKRTV AAPSVFIFPP
SDEQLKSGTA SVVCLLNNFY PREAKVQWKV DNALQSGNSQ ESVTEQDSKD STYSLSSTLT
LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC

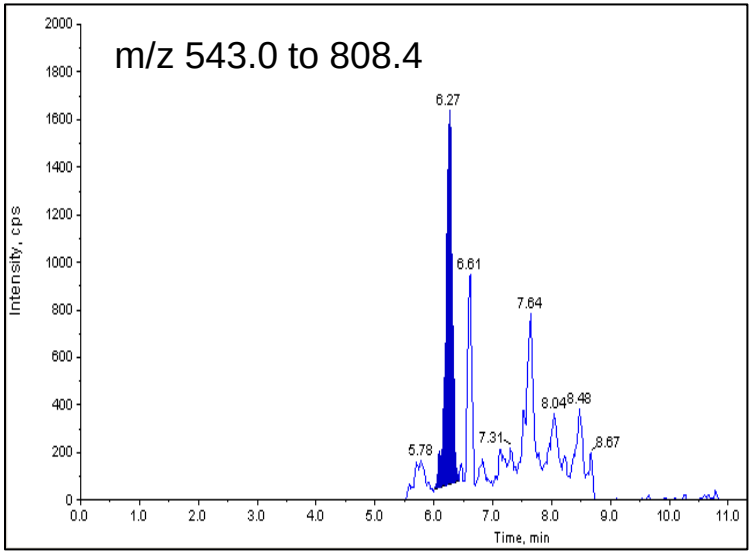
Heavy chain:

EVQLVESGGG LVQPGGSLRL SCAASGFNIK **DTYIH**WVRQA PGKGLEWVA**R** **IYPTNGYTRY**
ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSR**WG** **GDGFYAMDY**W GQGTLVTVSS
ASTKGPSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS
GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG
PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQY**N**
STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ VYTLPPSREE
MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK

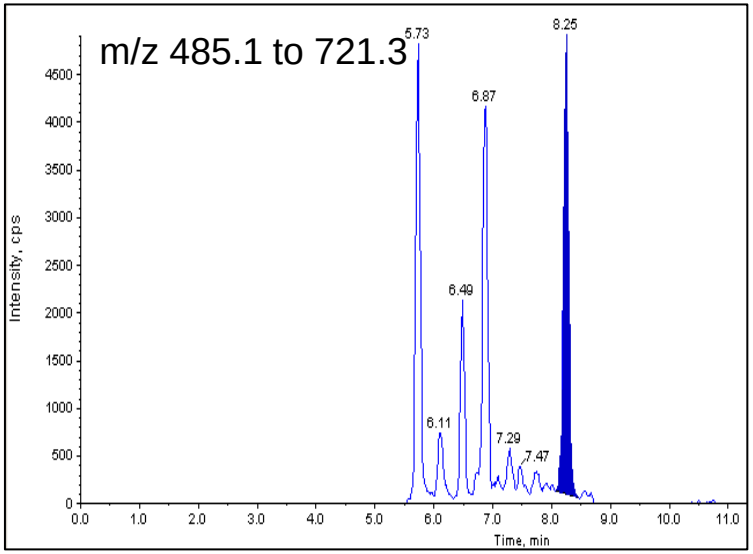
Bold = complementarity determining region (CDR)

Multiplexing

3-hour tryptic digestion of untreated plasma, direct injection of digest



IYPTNGYTR (500 ng/mL)

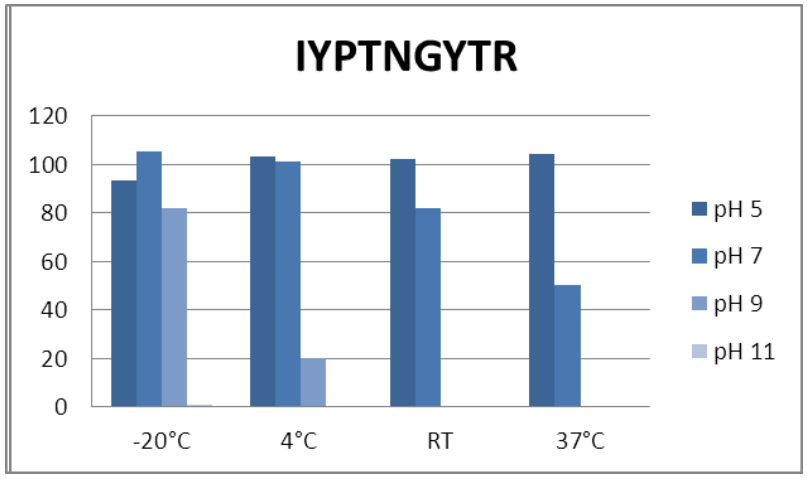


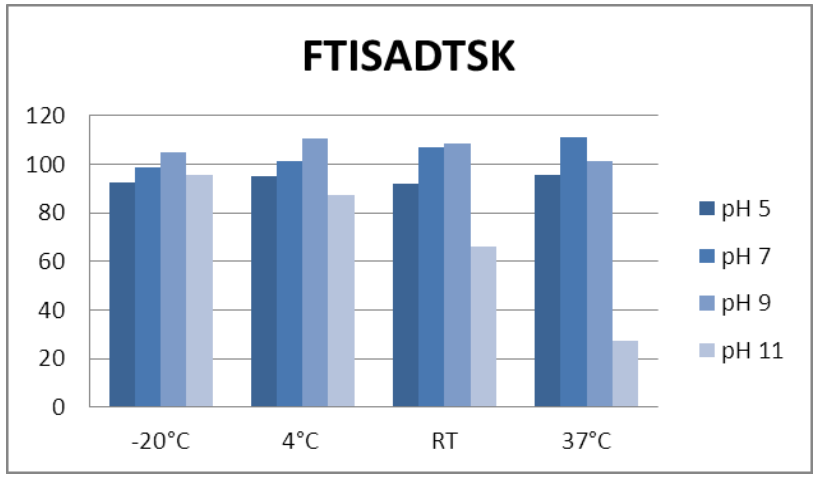
FTISADTSK (500 ng/mL)

Multiplexing

Application:

In vitro stress test (68-h) of trastuzumab peptides in plasma digest at different conditions

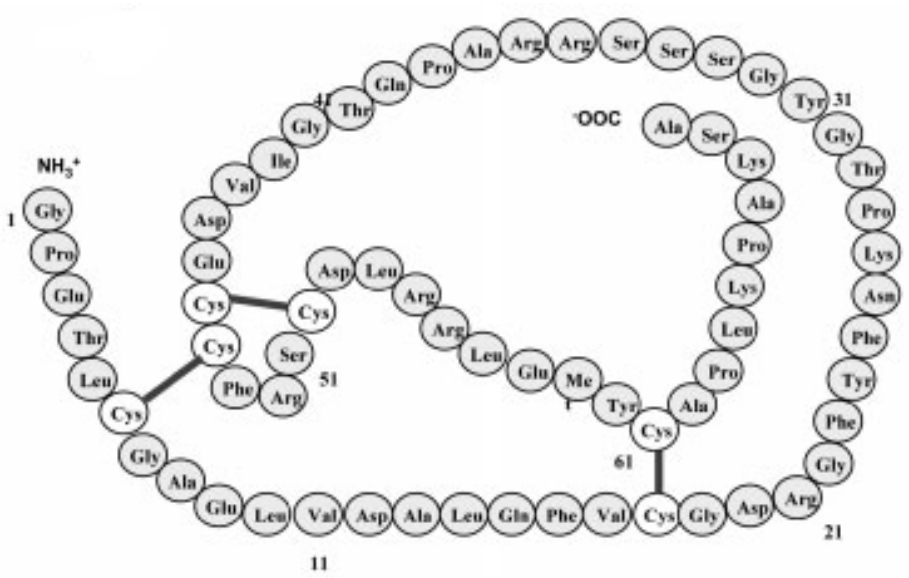




Structural information

Insulin-like Growth Factor-1 (IGF-1)

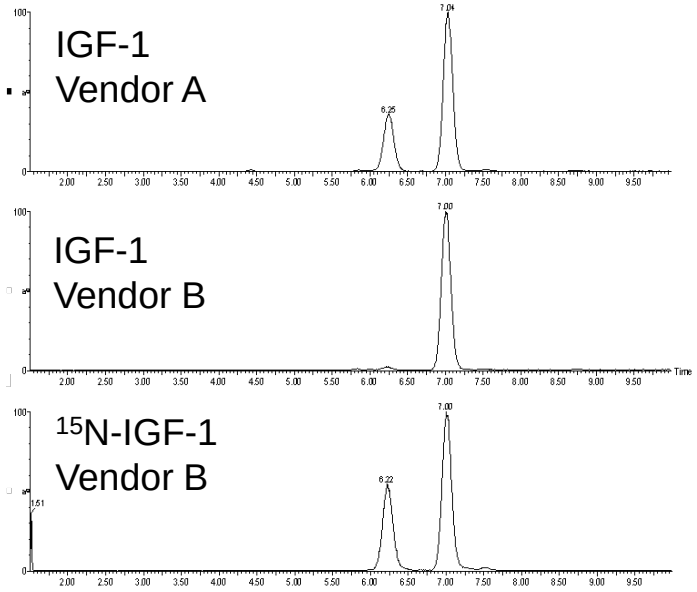
- (plasma) biomarker for growth deficiencies
- 70 amino acids, three intra-molecular disulfide bonds
- molecular mass: 7.5 kDa



Structural information

LC-MS/MS chromatograms

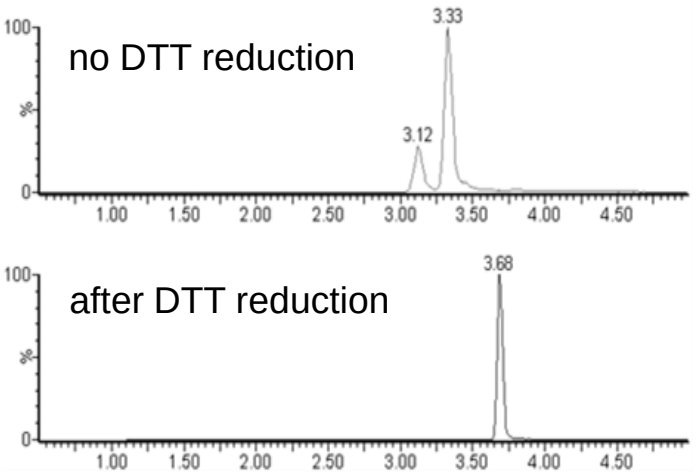
- test solutions
- 8-min reversed-phase gradient
- positive-mode ESI
- m/z 957.3 – 957.3 (IGF-1)
- m/z 968.9 – 968.9 (^{15}N-IGF-1)
- $[M+8H]^{8+}$



Structural information

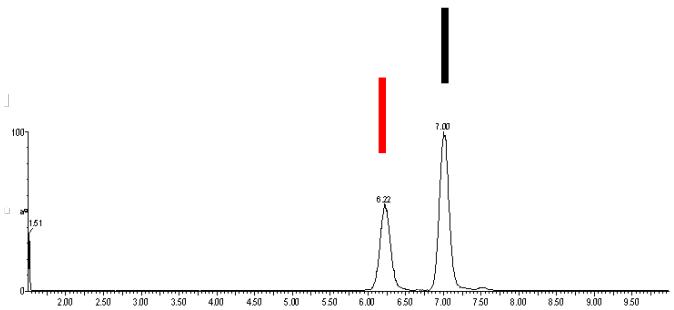
LC-MS/MS chromatograms

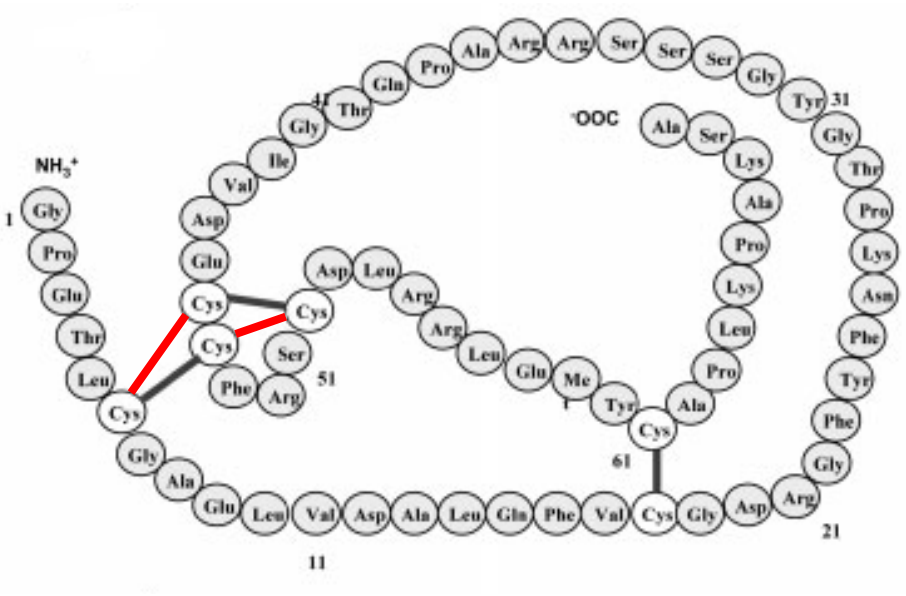
- test solutions IGF-1 (vendor A)
- 5-min reversed-phase gradient
- positive-mode ESI
- m/z 957.3 – 957.3 (before)
- m/z 957.7 – 957.7 (after)
- $[M+8H]^{8+}$



Structural information

Separate peaks probably originate from two steric isomers formed by different intra-molecular disulfide arrangements





Conclusion

LC-MS/MS has several advantageous properties for protein quantification, making it a useful addition to the toolkit

- Separation power
- Typically better accuracy and precision
- Multiplexing potential
- Structural information

Acknowledgements

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