Functional Genomics and Proteomics
National Centre for Biomolecular Research
Faculty of Science Masaryk University

CEITEC

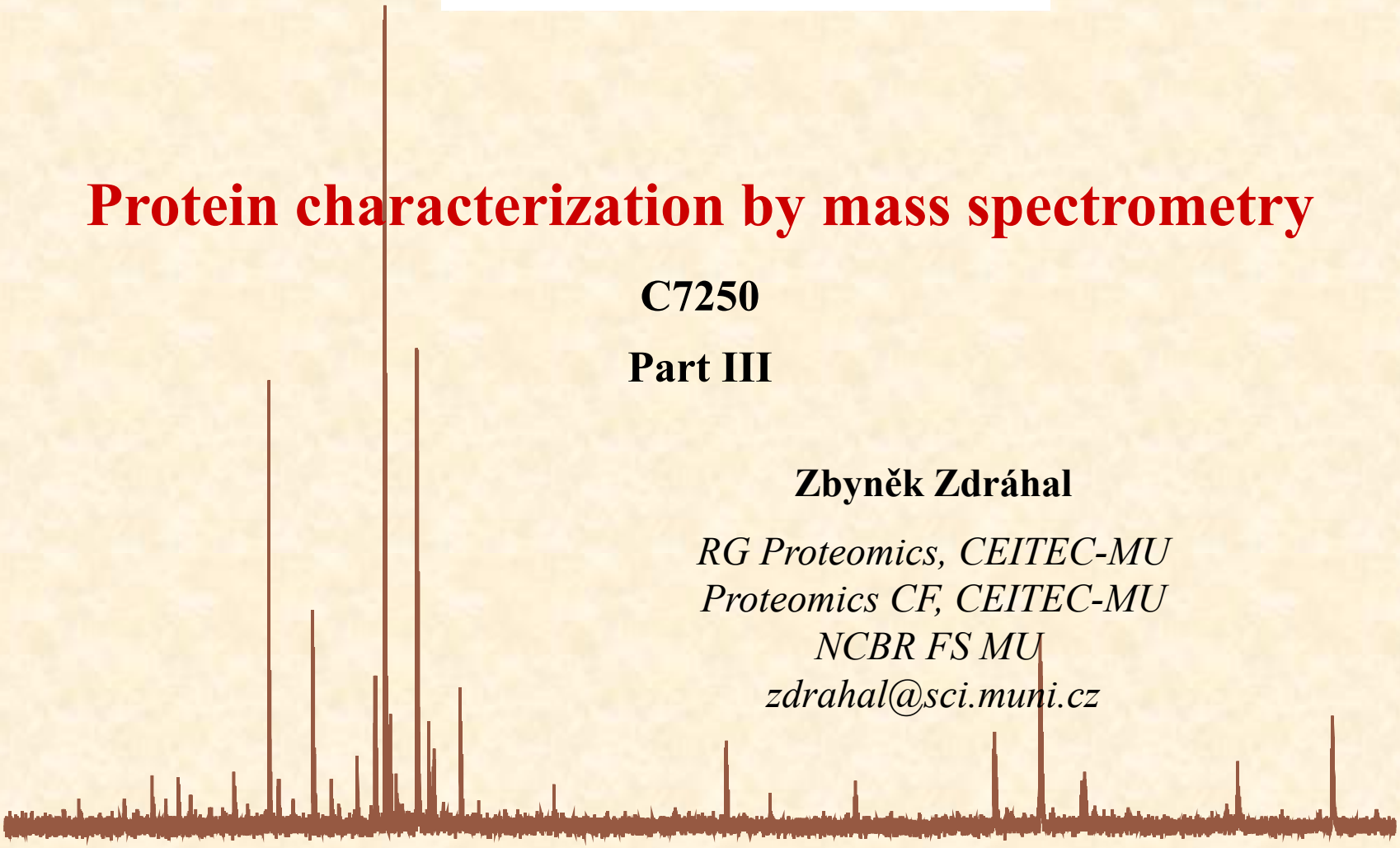
Protein characterization by mass spectrometry

C7250

Part III

Zbyněk Zdráhal

RG Proteomics, CEITEC-MU
Proteomics CF, CEITEC-MU
NCBR FS MU
zdrahal@sci.muni.cz



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Characterization of Protein Modifications

Why?

PTM type number > **400**

number of PTM sites ≈ **90 000** (detected experimentally)

≈ **230 000** (prediction)

(SwissProt, per ≈ 530 000 proteins)

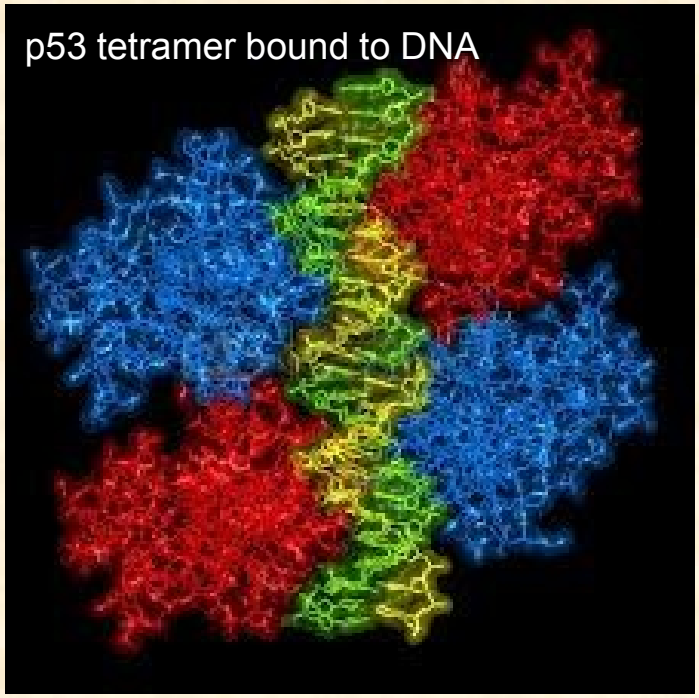
G. A. Khoury et al., Sci. Rep. 1, 90; (2011); http://selene.princeton.edu/PTMCuration

...PTMs are known to act alone and in combination **to regulate nearly all aspects of protein function**...

...Post-translational modifications (PTMs) occur on nearly all proteins. Many domains within proteins are modified on multiple amino acid sidechains by diverse enzymes to create a myriad of possible protein species. **How these combinations of PTMs lead to distinct biological outcomes is only beginning to be understood...**

A. P. Lothrop, M. P. Torres, S. M. Fuchs, FEBS Letters. 587 (2013) 1247–1257

Protein p53



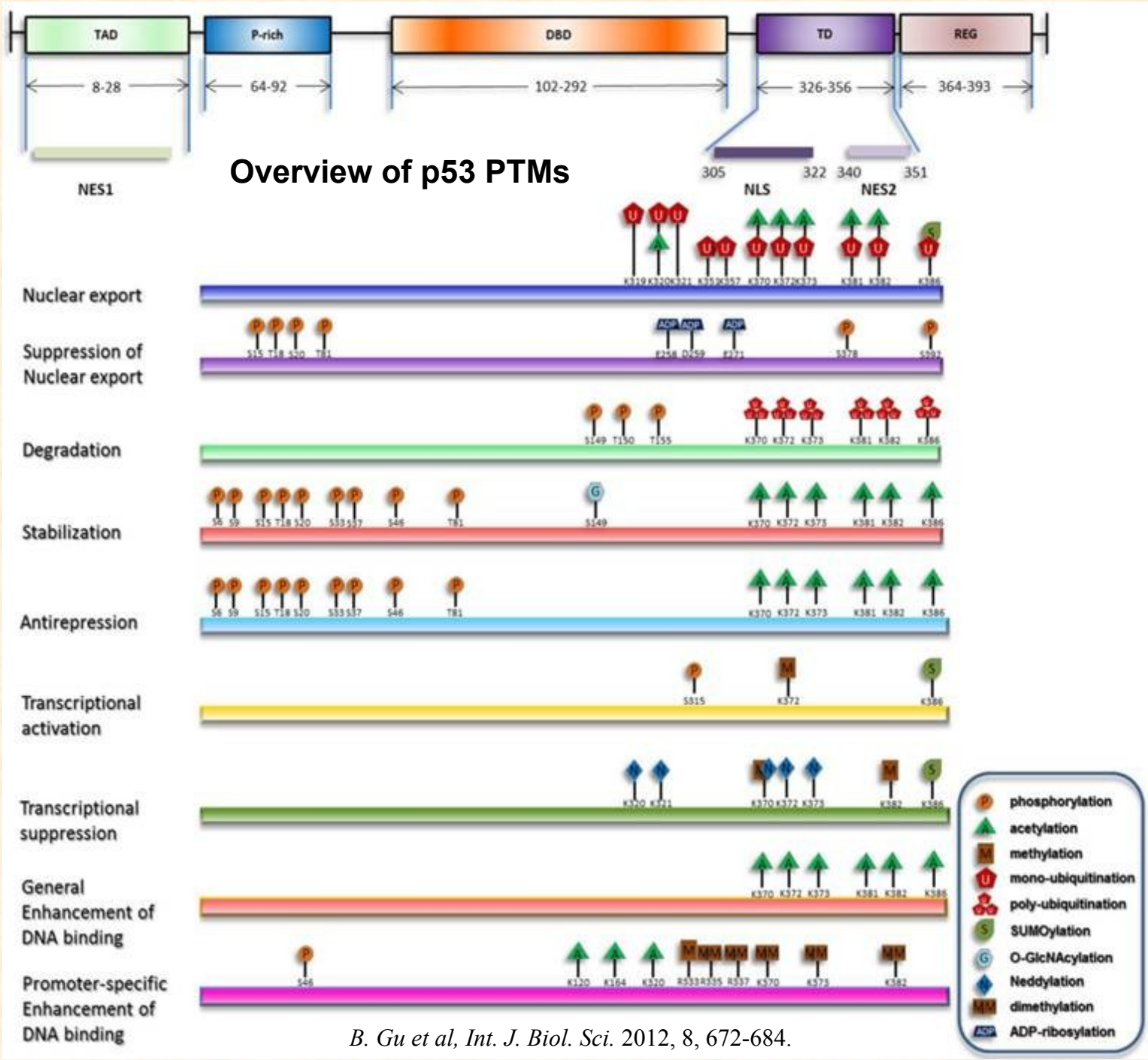
p53 exerts **irreplaceable anti-neoplastic functions** at homeostasis and thus is considered to be '**the guardian of the genome**‘.

p53 is able to coordinate a regulatory network that supervises and responds to a variety of stress signals:

- DNA damage
- aberrant oncogenic activation
- telomere erosion
- ribosomal stress
- loss of cell-cell or cell-matrix adhesion
- hypoxia

Mutations of p53 or **disruptions** of p53 **coordination**,
to a lesser extent, can disturb the normal physiological balance,
if genome disarrangement reaches a critical value **it leads to cancer**

B. Gu et al, Int. J. Biol. Sci. 2012, 8, 672-684.

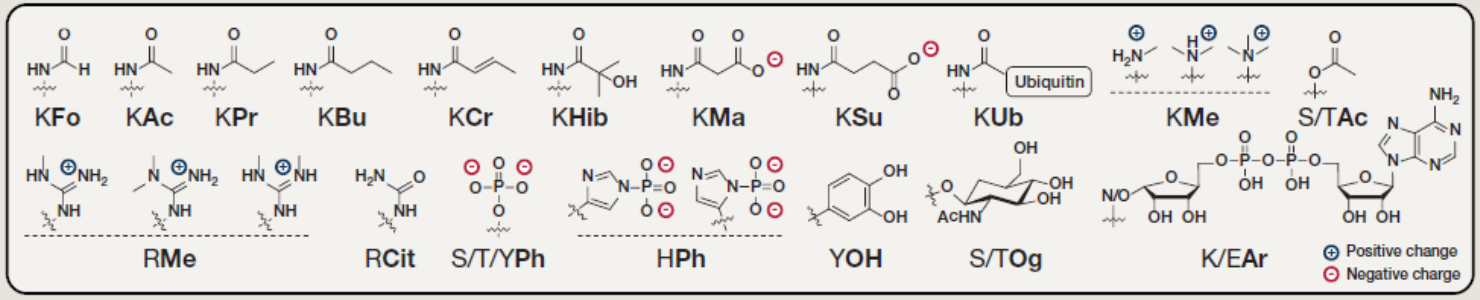


B. Gu et al, Int. J. Biol. Sci. 2012, 8, 672-684.

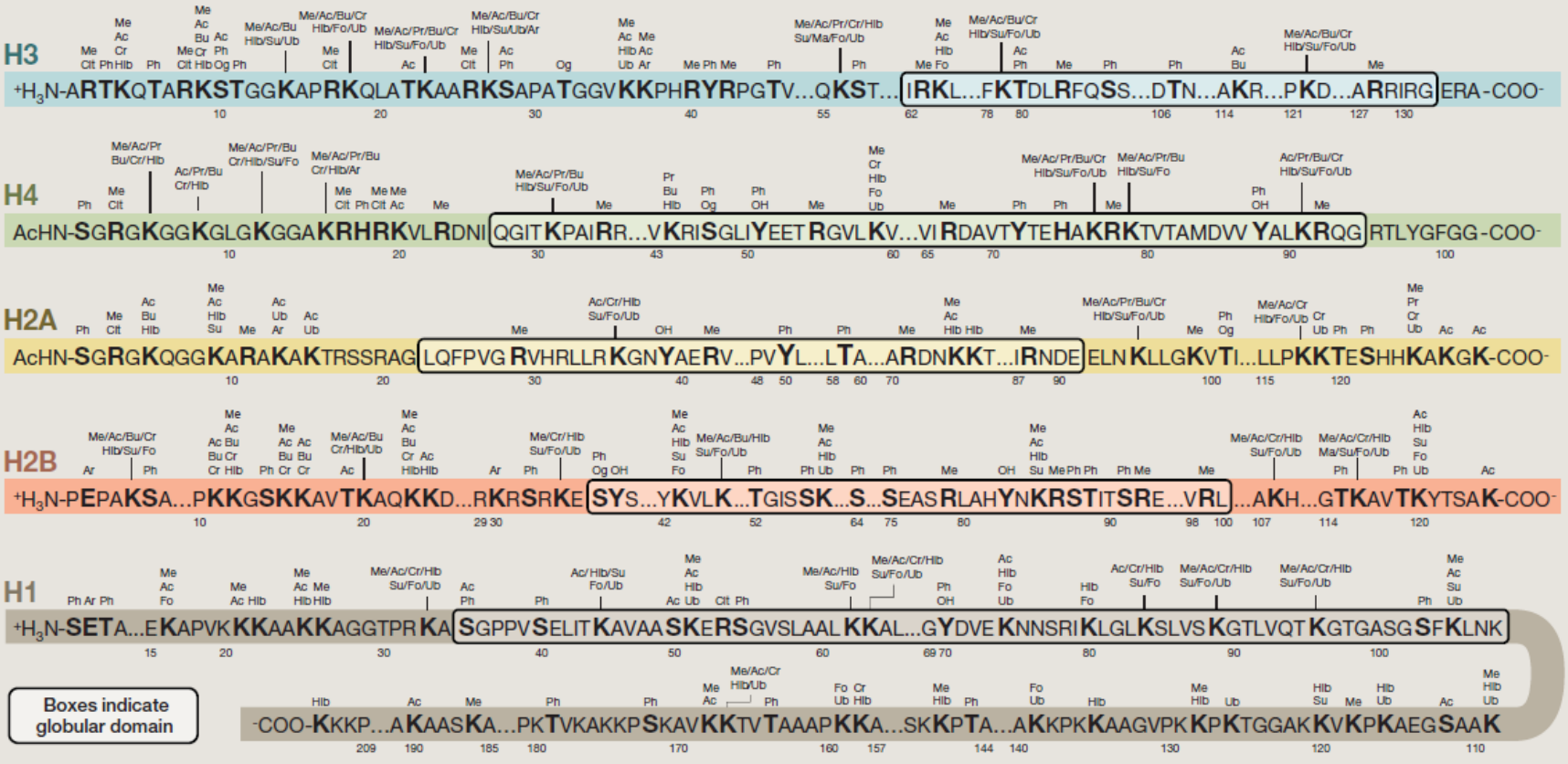
SnapShot: Histone Modifications

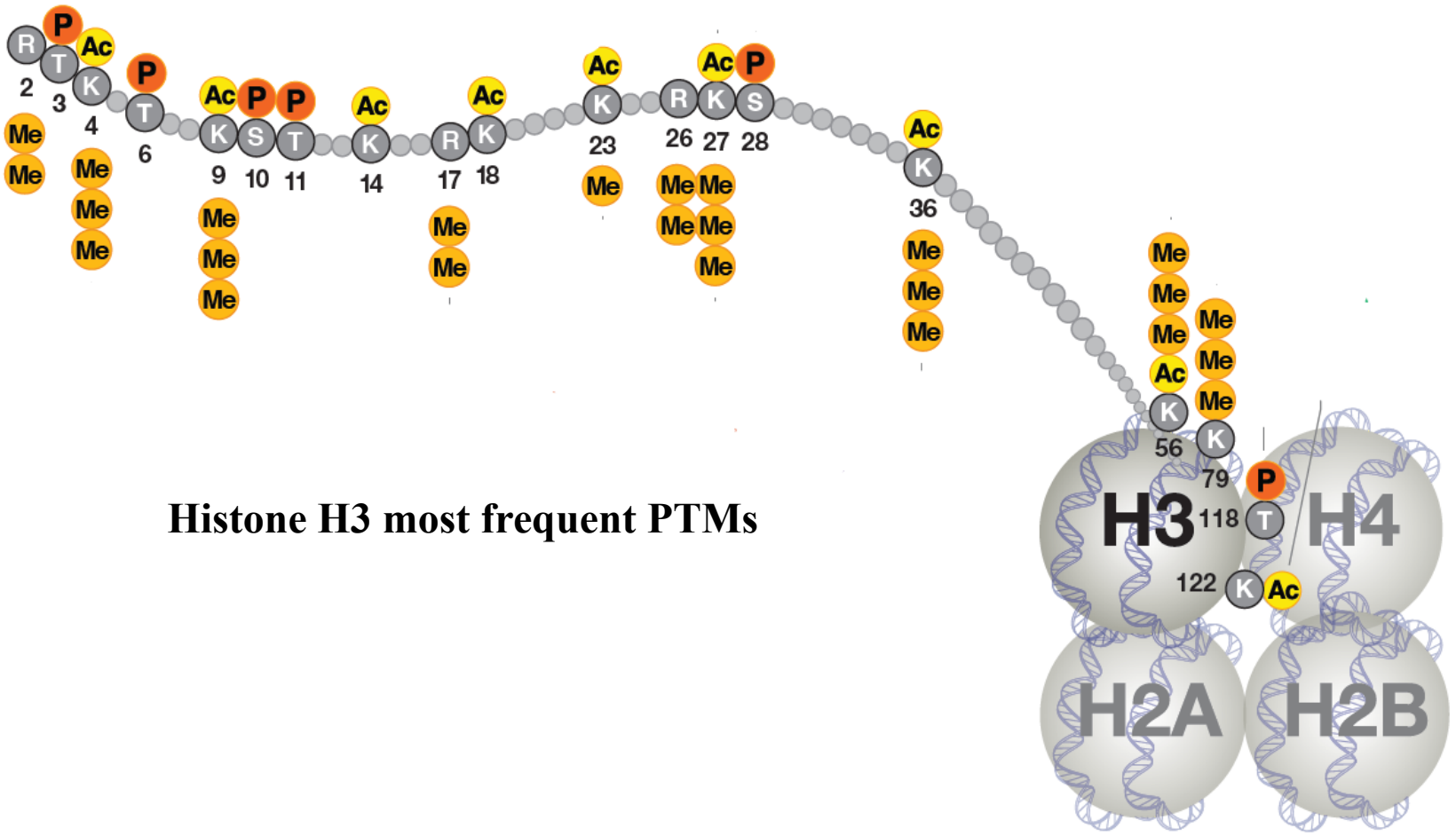
He Huang,[1] Benjamin R. Sabari,[2] Benjamin A. Garcia,[3] C. David Allis,[2] and Yingming Zhao[1]
[1]Ben May Department of Cancer Research, The University of Chicago, Chicago, IL 60637, USA
[2]Laboratory of Chromatin Biology and Epigenetics, The Rockefeller University, New York, NY 10021, USA
[3]Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA 19104, USA

Cell



Me	Methylation (K, R)
Ac	Acetylation (K, S, T)
Pr	Propionylation (K)
Bu	Butyrylation (K)
Cr	Crotonylation (K)
Hib	2-Hydroxyisobutyrylation (K)
Ma	Malonylation (K)
Su	Succinylation (K)
Fo	Formylation (K)
Ub	Ubiquitination (K)
Cit	Citrullination (R)
Ph	Phosphorylation (S, T, Y, H)
OH	Hydroxylation (Y)
Og	O-GlcNAcylation (S, T)
Ar	ADP ribosylation (K, E)





Histone H3 most frequent PTMs

Potential of MS in analysis of modifications

- **Type**
- **Site localization**
- **Site occupancy**

demands

MS
"screening"
detailed characterization
of individual modification

×

Western blot
detection of PTM type
localization of selected single modification

Specific staining of gels
detection of PTM type without site localization
(phospho, glyco proteins)

Modification groups:

- **mutation** (AA replacement)
- **chemical**
- **posttranslational**

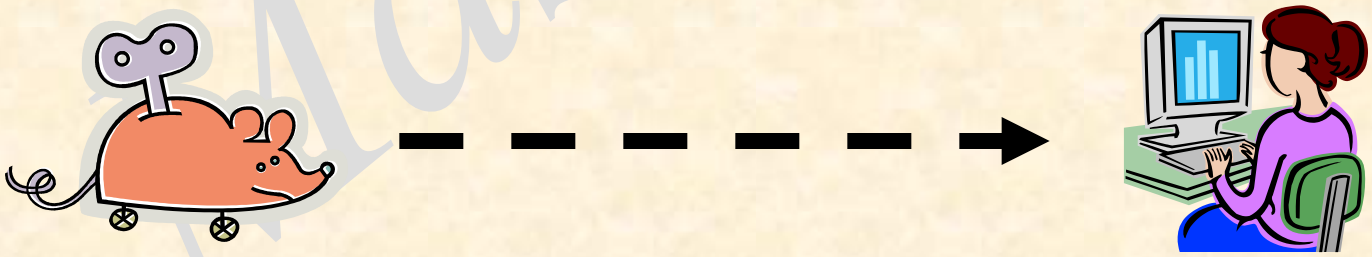
Overview of PTMs and prediction SW tools :

DeltaMass - https://www.abrf.org/delta-mass

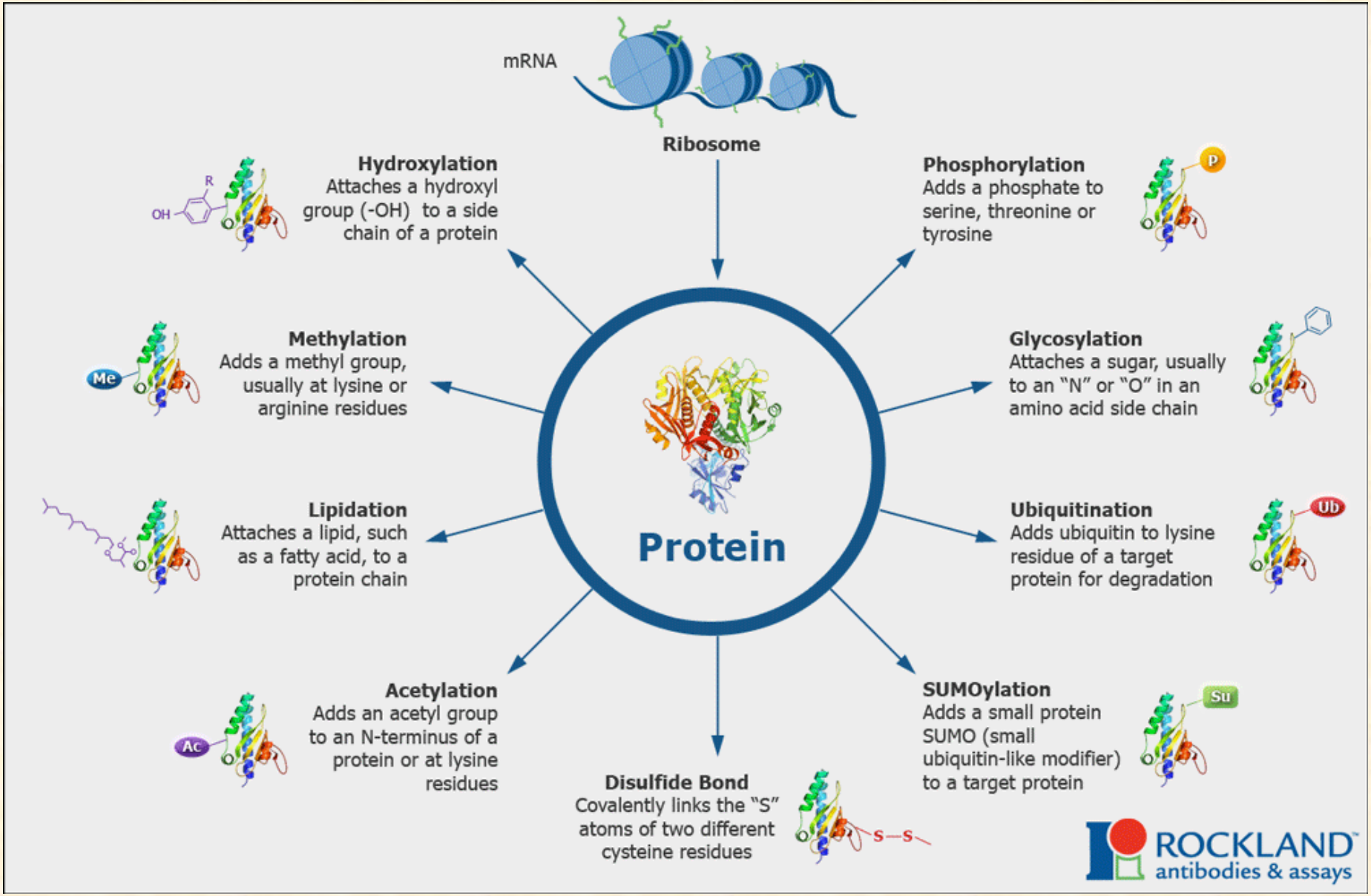
ExPASy - *http://www.expasy.org/proteomics/post-translational_modification*

„Chemical modifications:

- wanted modifications *(carbamidomethylation Cys, N –terminal acetylation, **quantification tags**, etc.)*
- unwanted modifications *(Met oxidation, deamidation N → D during sample preparation, drug adducts, etc.)*



Common Posttranslational Modifications



Common Posttranslational Modifications

Amines (K/N-terminus)	Methylation	+14.0269	Formylation	+28.0104
	Acetylation	+42.0373	Lipoic acid	+188.3147
	Farnesylation	+204.3556	Myristoylation	+210.3598
	Biotinylation	+226.2994	Palmitoylation	+238.4136
	Stearoylation	+266.4674	Geranylgeranylation	+272.4741

Acids & amides (E/D/Q/N)	Pyroglutamic acid (Q)	-17.0306	Deamidation (Q/N)	+0.9847
	Carboxylation (E/D)	+44.0098		

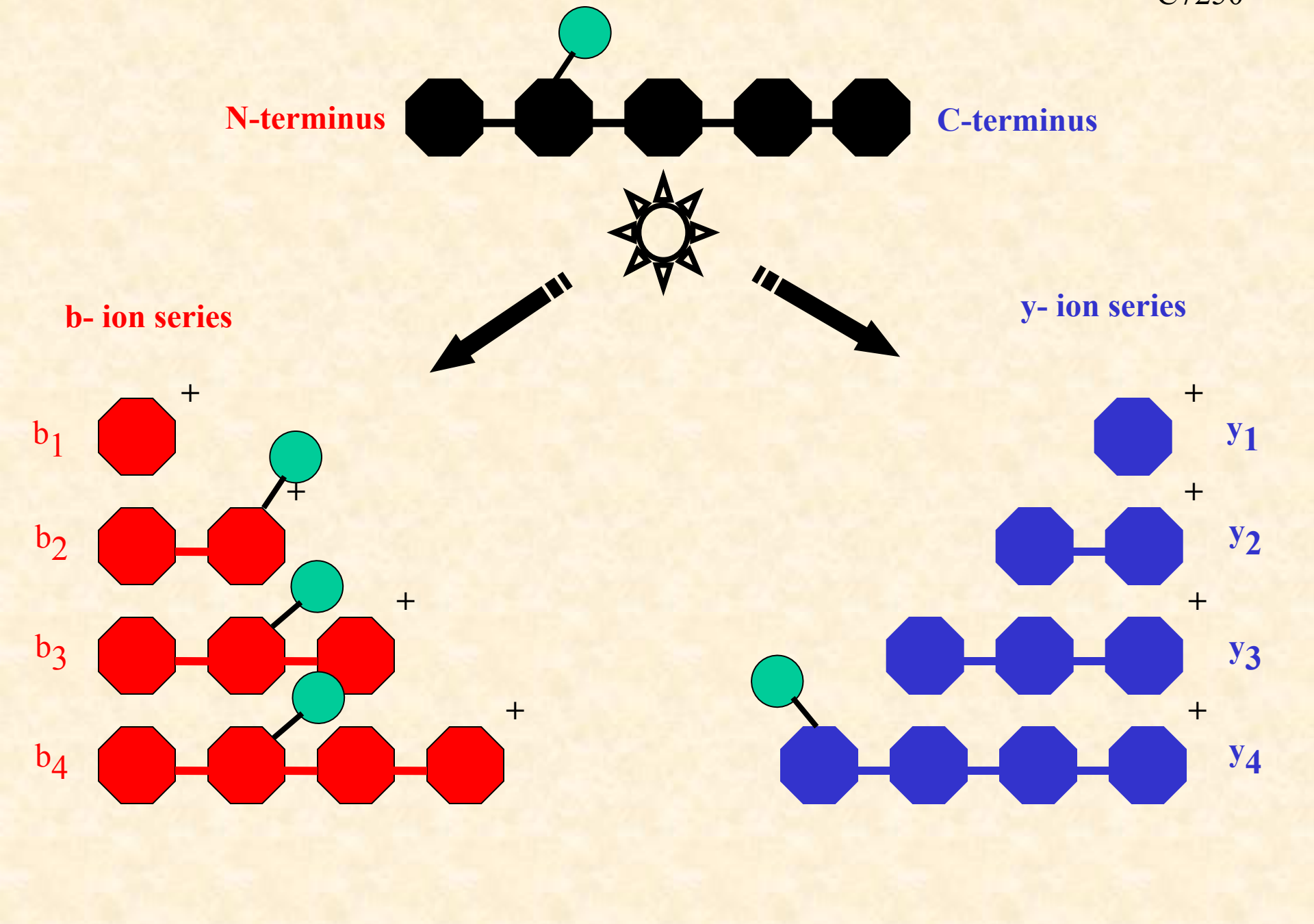
Hydroxyl groups (S/T/Y)	**Phosphorylation**	+79.9799	Sulphation	+80.0642

Carbohydrates (S/T/N)	Pentoses	+132.1161	Deoxyhexoses	+146.1430
	Hexosamines	+161.1577	Hexoses	+162.1424
	N-acetylhexosamines	+203.1950	Sialic acid	+291.2579

Further details e.g.: http://themedicalbiochemistrypage.org/protein-modifications.php

MS/MS fragmentation of peptides

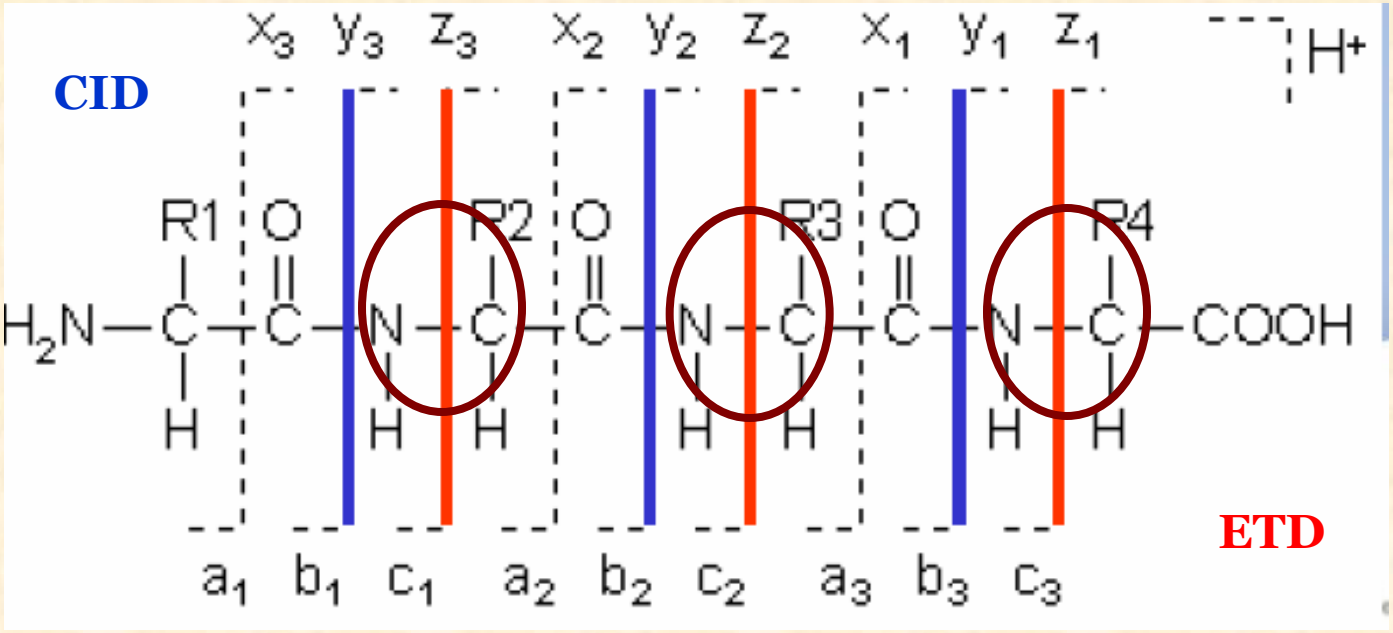
repetition


N-terminus
C-terminus
b- ion series
y- ion series
b1
b2
b3
b4
y1
y2
y3
y4

CID *vs* **ETD**

b, y *c, z*

C-terminus ***(z- series)***



N-terminus ***(c-series)***

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Characterization of mutations

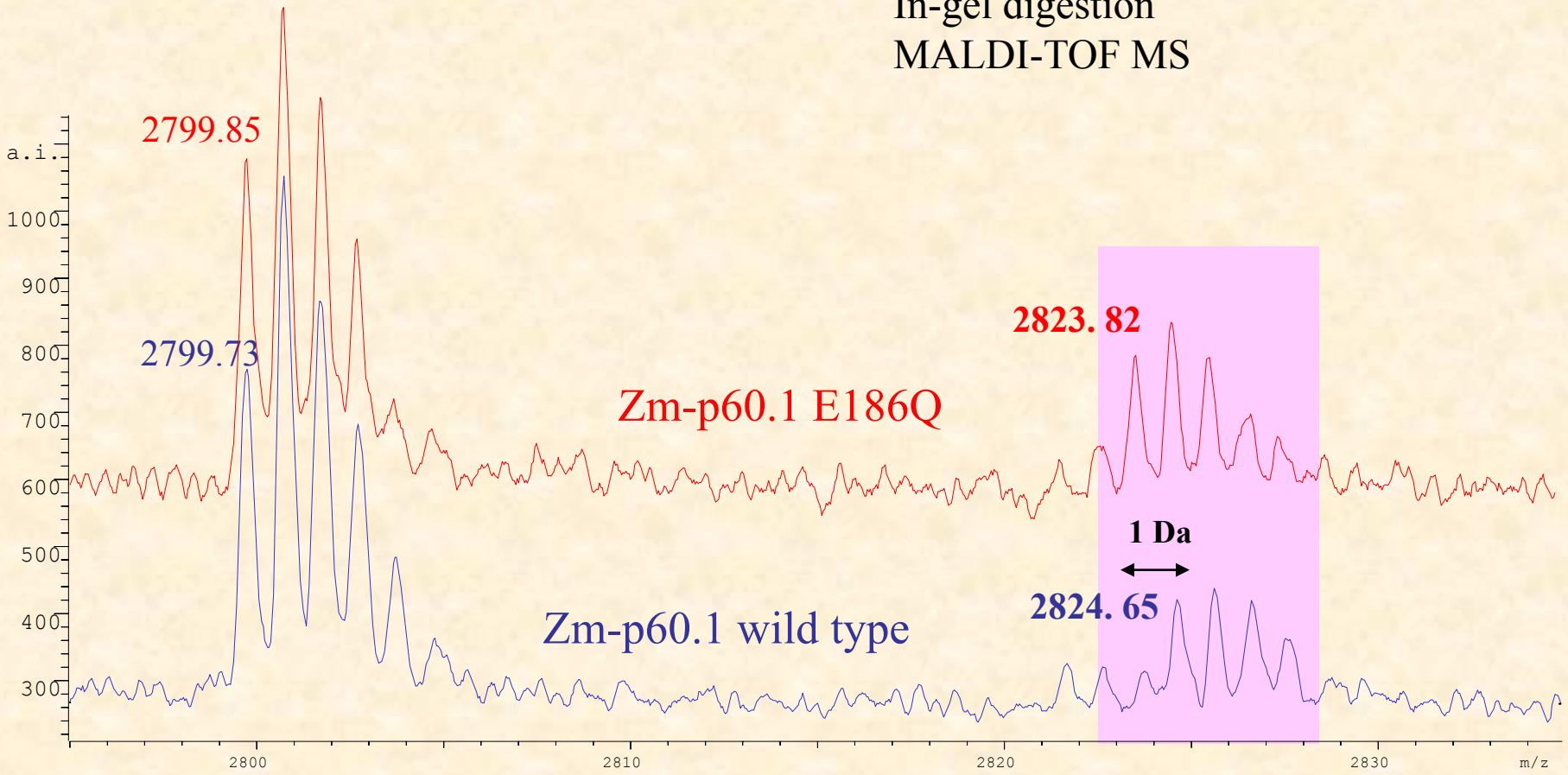
Confirmation of AA replacement at peptide level

Protein: Zm-p60 (position 186)

wild type 179 - 203	**NWLTFNEPQTFTSFSYGTGVFAPGR**	**2824**
E186Q	**Q**	**2823**

peptide mass difference: **- 1 Da**

In-gel digestion
MALDI-TOF MS



Identification of AA replacement

Protein detected in two variants in on 2-DE spot

Initial information:

Detection of two variants of tryptic peptide with mass difference: **–14 Da**

. . .
DEEELQKENVKNTASLTGK**ITLSVTQSKPETGEVIGVFESIQPSDTDLGAK**VPKDVKIQG
. . .

MALDI-MS confirmation of tryptic peptide mass difference (2 proteases)
localization of sequence region with the change

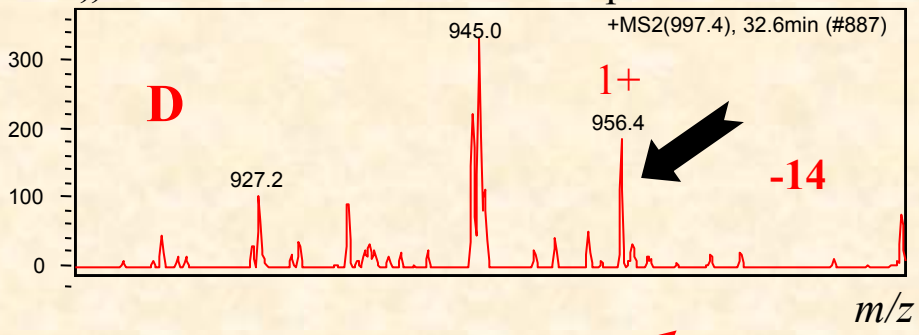
MALDI-PSD ambiguous results

LC-MS/MS mutation D/E in position 210 confirmed

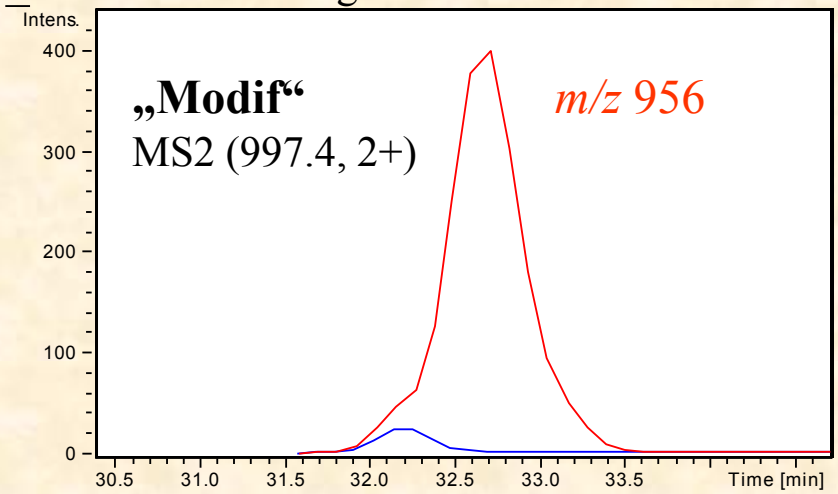
. . .
DEEELQKENVKNTASLTGK**ITLSVTQSKPETGEVIGVFESIQPSDTDLGAK**VPKDVKIQG
. . .

MS/MS of peptide LSVTQSKPXTGEVIGVFES, MW 2006.0 (1992.0)

„Modif“ - detail of MS/MS spectrum



EIC chromatogram

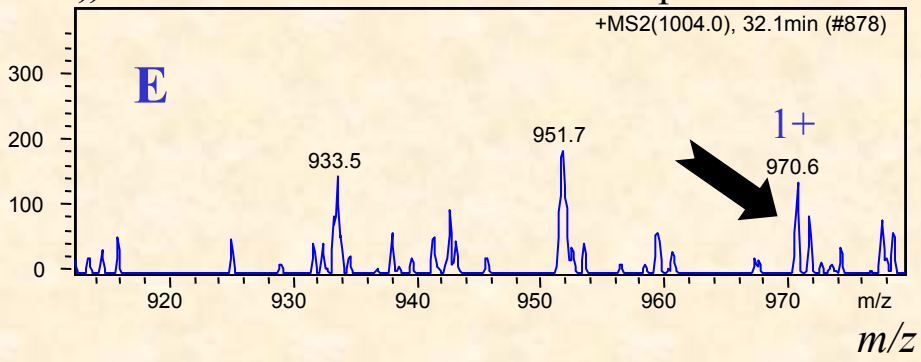


suggestion

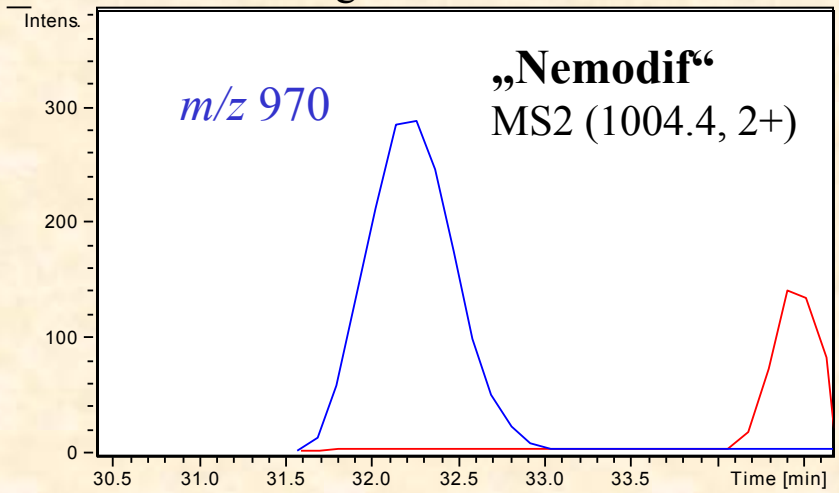
Ion b_9 LSVTQSKPX

D / E

„Nemodif“ – detail of MS/MS spectrum



EIC chromatogram



Rt (min)

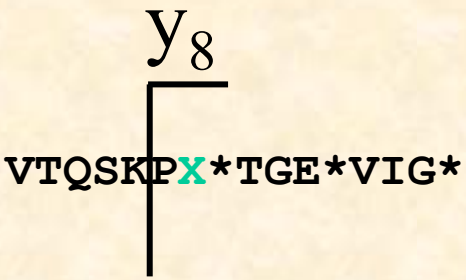
Confirmation of D in position 210 by methylation and LC-MS/MS

after methylation *exchange of H for CH3 in each carboxyl group*

↓

mass increase ***14 Da/group***

pouze D, E a C-terminal

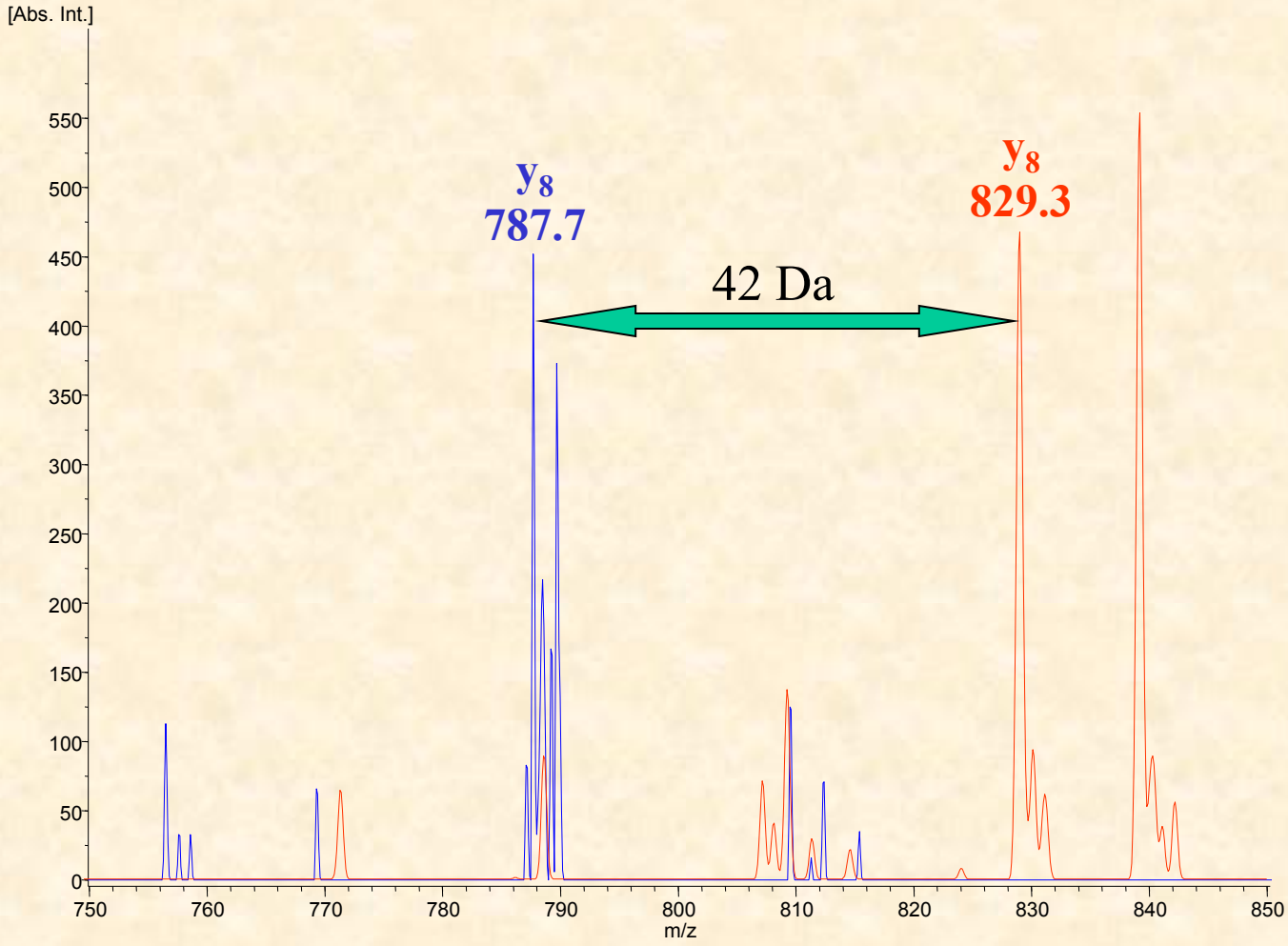


Δ = 42 Da

y_8 nederiv *m/z* 787.2 pro D

y_8 methyl *m/z* 829.3 pro D

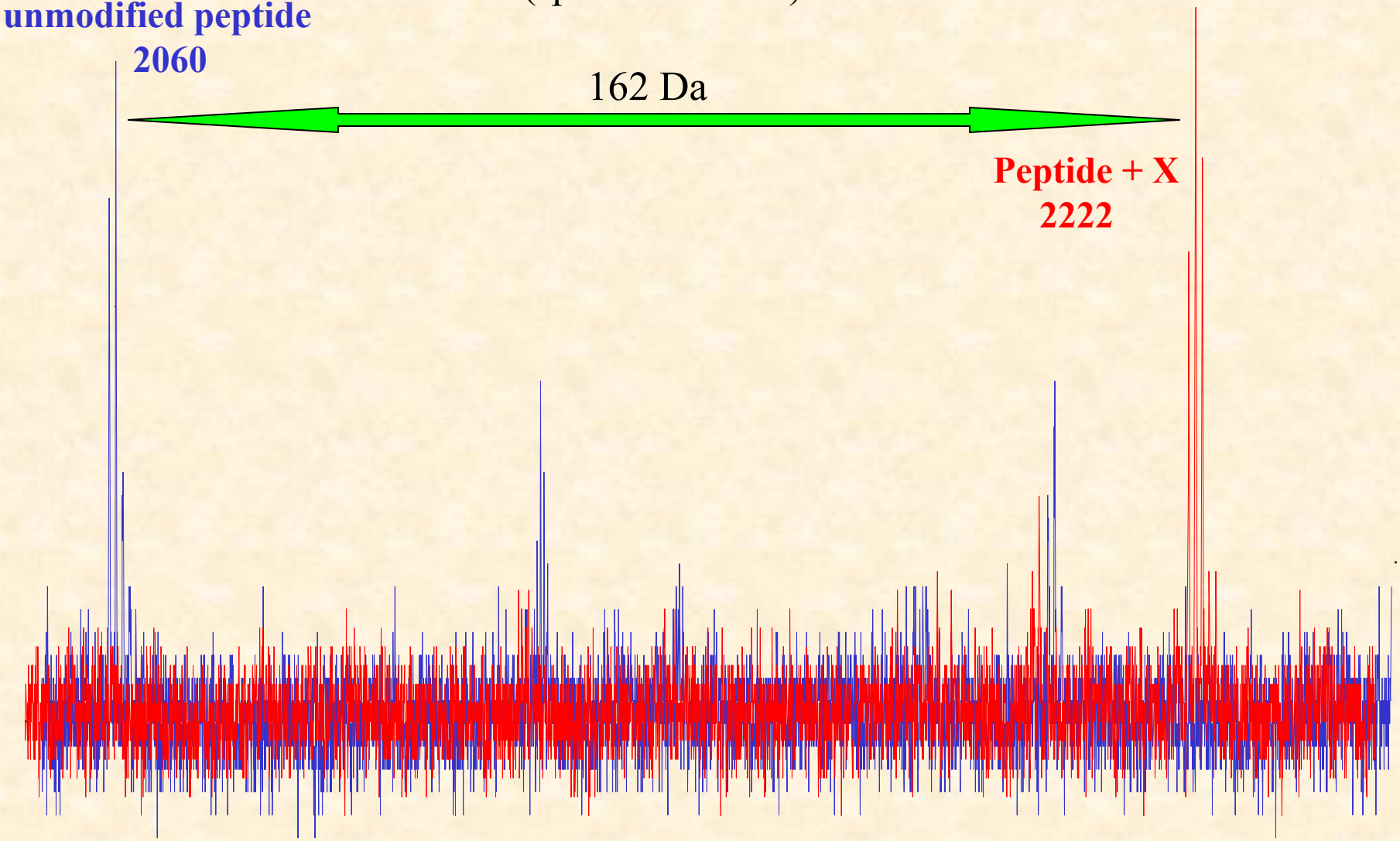
MS/MS spectrum of peptide VTQSKPXTGEVIG
before and after methylation



Characterization of modifications „chemical“

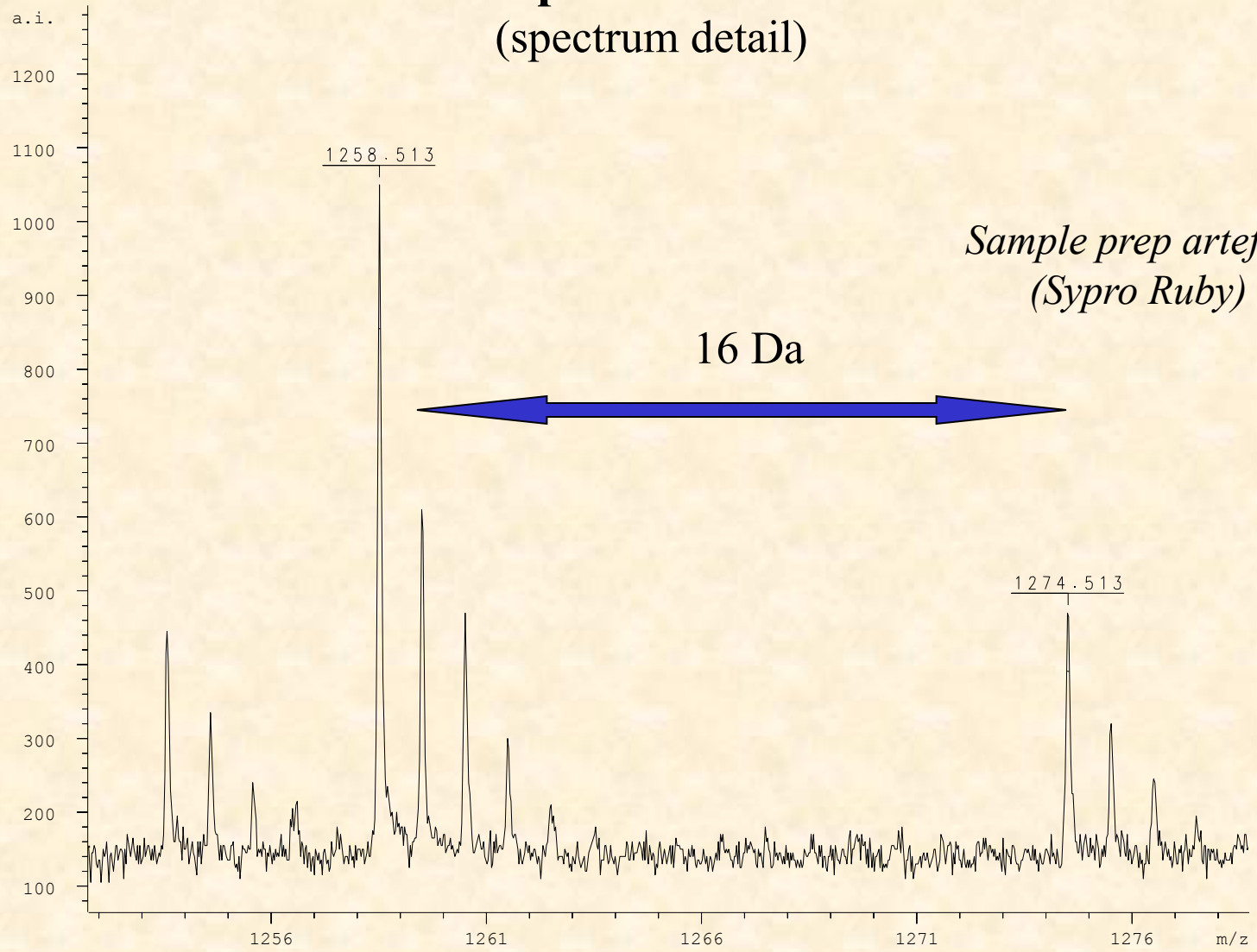
MALDI–MS spectrum of tryptic digests before a after modification

(spectrum detail)



MALDI–MS spectrum – Met oxidation

(spectrum detail)



Sample prep artefact (Sypro Ruby)

Protein identification – MS/MS data (Mascot)

1. gi|15803837 **Mass:** 13532 **Score:** 487 **Queries matched:** 5
50S ribosomal protein L14 [Escherichia coli O157:H7]

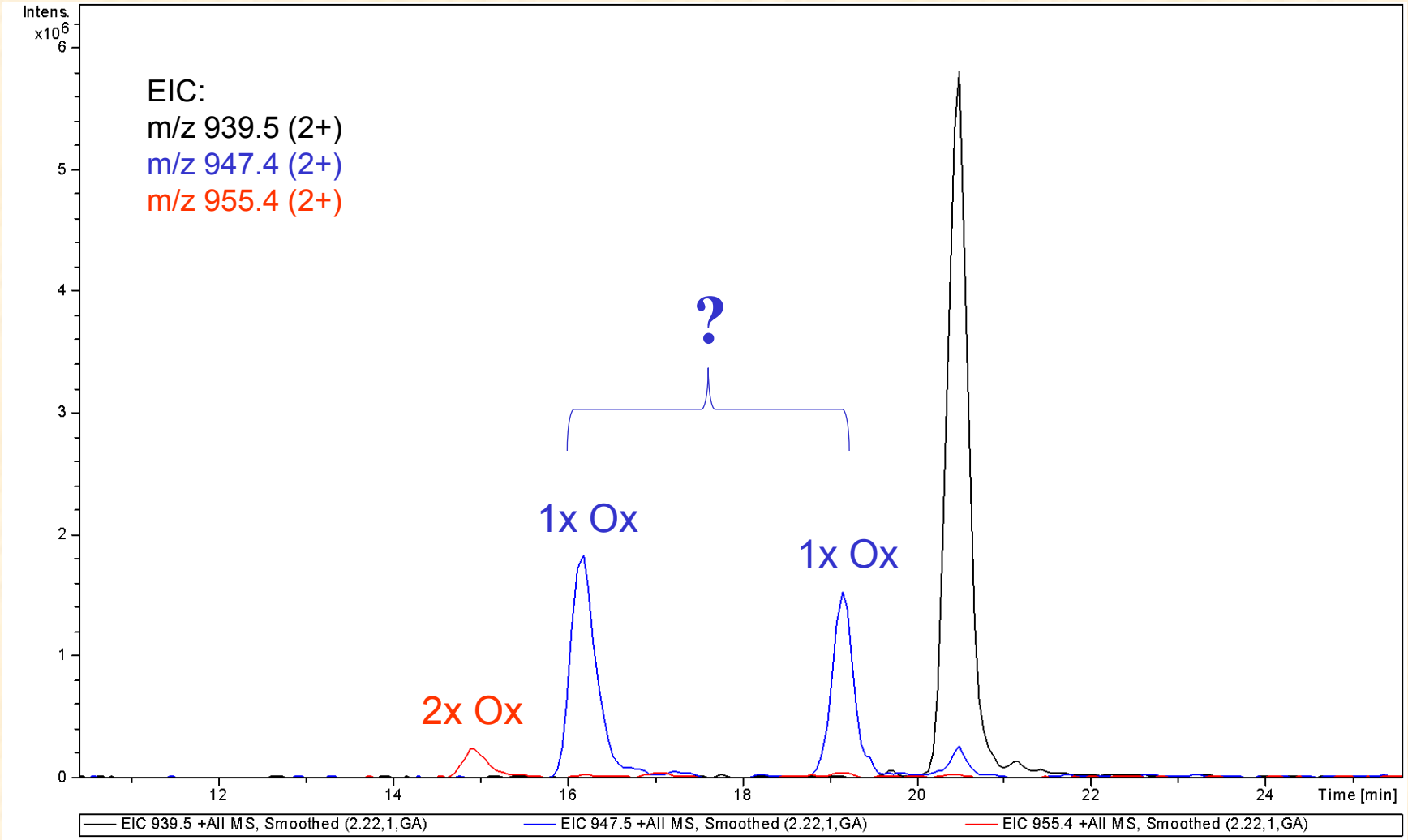
Observed	*Mr(expt)*	*Mr(calc)*	*Delta*	*Miss*	*Score*	*Peptide*
.....						
939.45	1876.89	1876.88	0.02	0	(125)	MIQEQTMLNVADNSGAR
947.44	1892.87	1892.87	-0.01	0	159	MIQEQTML<u>M</u>NVADNSGAR + Oxidation (M)
947.45	1892.88	1892.87	0.01	0	(147)	<u>M</u>IQEQTMLNVADNSGAR + Oxidation (M)
955.45	1908.89	1908.87	0.03	0	(118)	<u>M</u>IQEQTML<u>M</u>NVADNSGAR + 2 Oxidation (M)
.....						

Δ *m/z* 8 (16) ……. 16 (32)

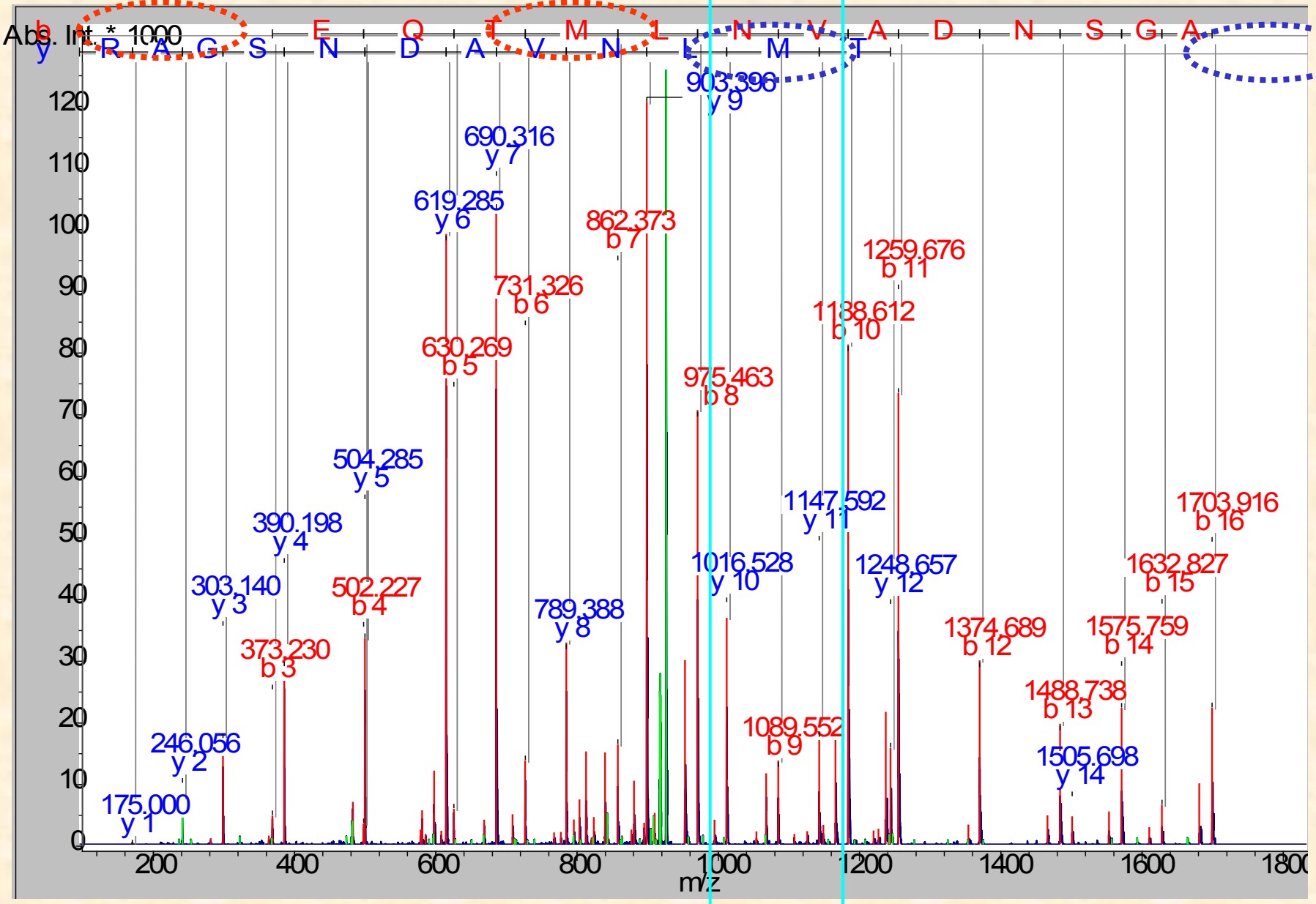
(Petra P. Vz. 3, 080821)

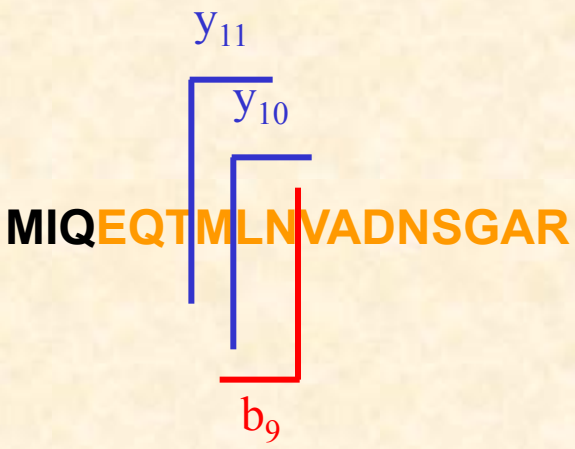
LC-MS/MS

C7250



MS/MS spectrum of unmodified peptide - **MIQEQTMLNVADNSGAR**





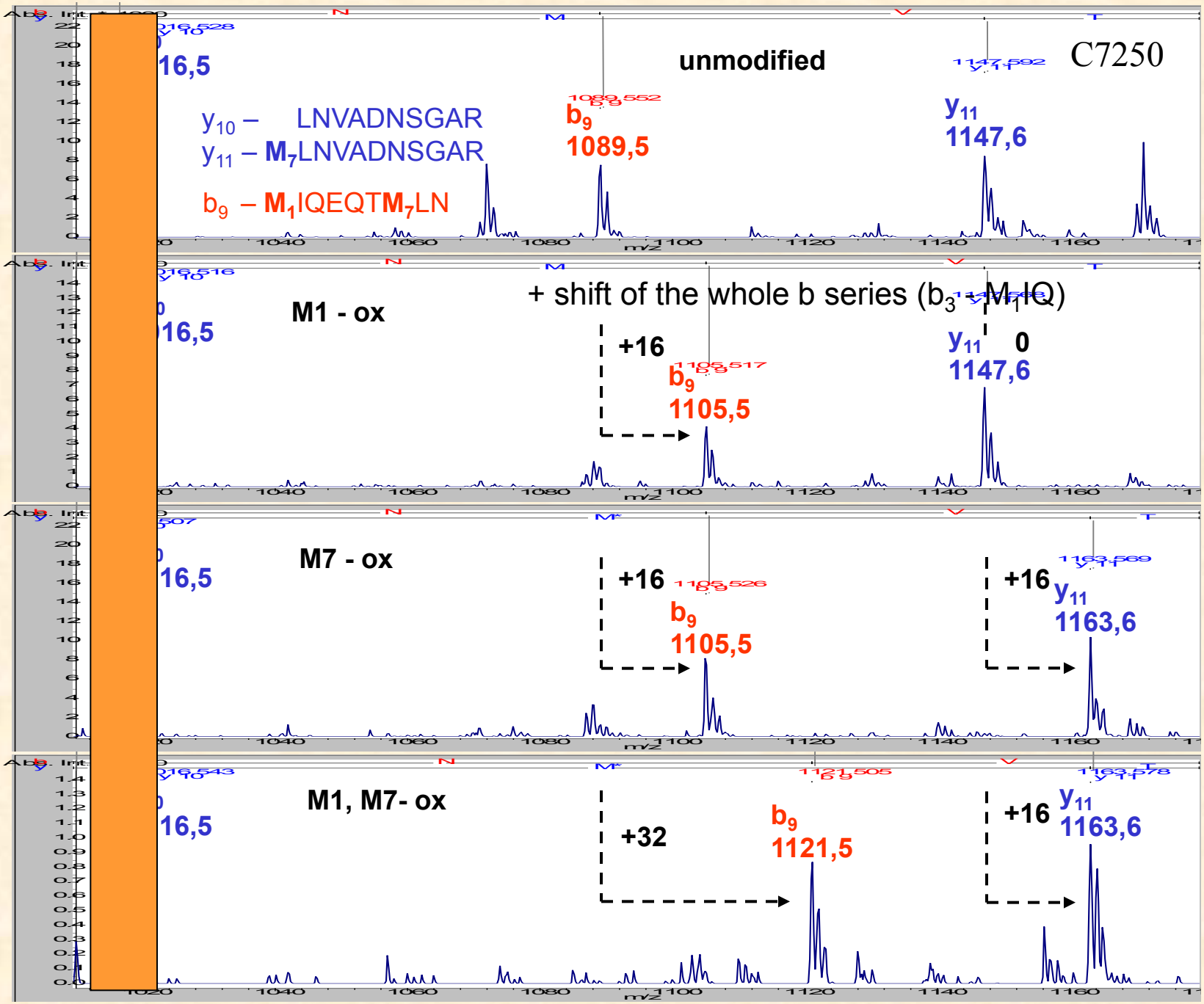
b_9 – **M_1**IQEQT**M_7**LN

y_{10} – LNVADNSGAR

y_{11} – **M_7**LNVADNSGAR

Shifts in m/z of selected fragments for individual $M_{(ox)}$ peptide forms

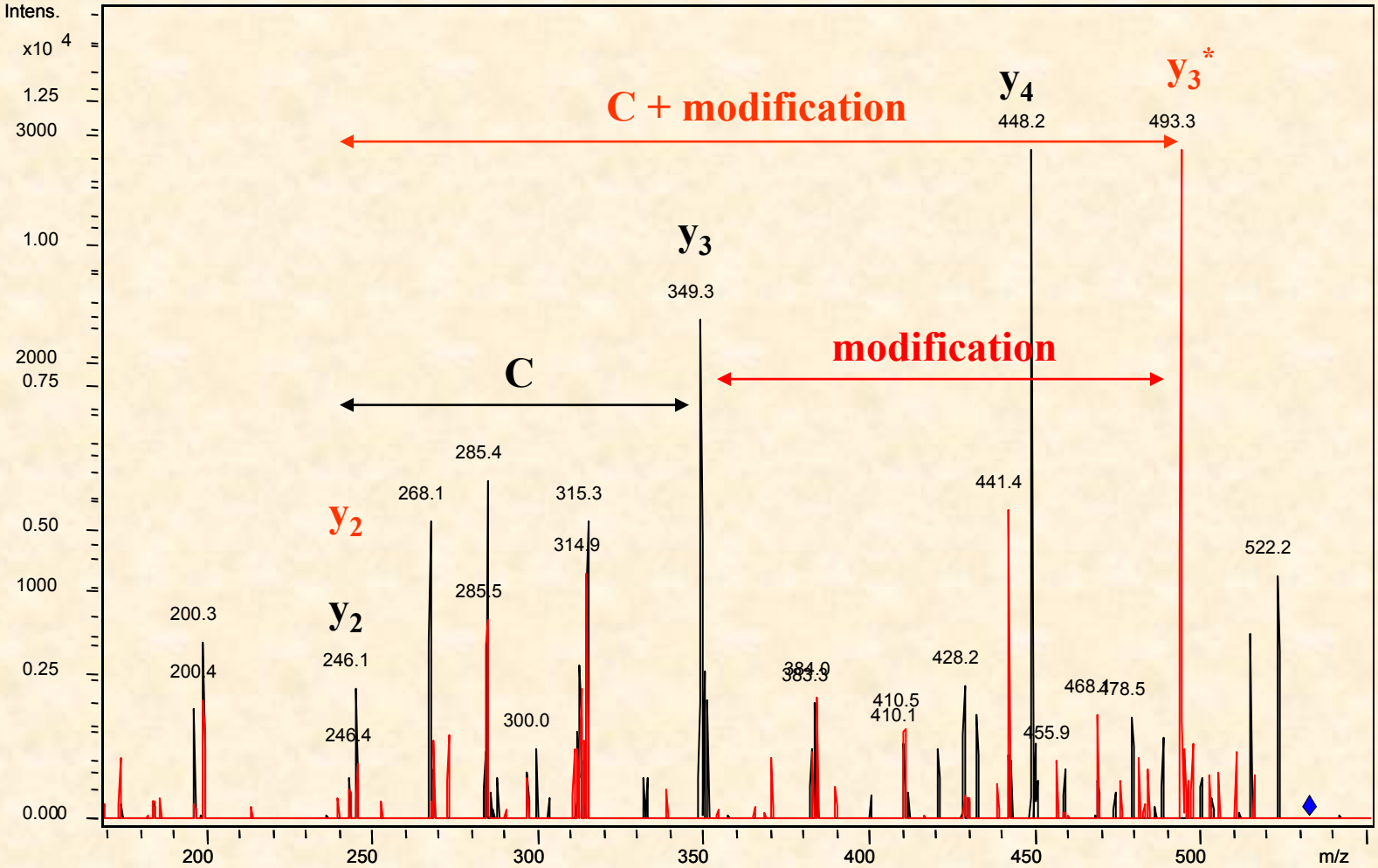
	no	*1 Ox*	*1 Ox*	*2 Ox*
		M_1	**M_7**	**both**
y_{10}	0	0	0	0
y_{11}	0	0	16	16
b_9	0	16	16	32


unmodified
C7250
y10 – LNVADNSGAR
y11 – M7LNVADNSGAR
b9 – M1IQEQTM7LN
b9
1089,5
y11
1147,6
M1 - ox
+ shift of the whole b series (b3 - M1IQ)
+16
b9
1105,5
y11
1147,6
0
M7 - ox
+16
b9
1105,5
+16
y11
1163,6
M1, M7- ox
+32
b9
1121,5
+16
y11
1163,6

LC-MS/MS spectrum (detail)

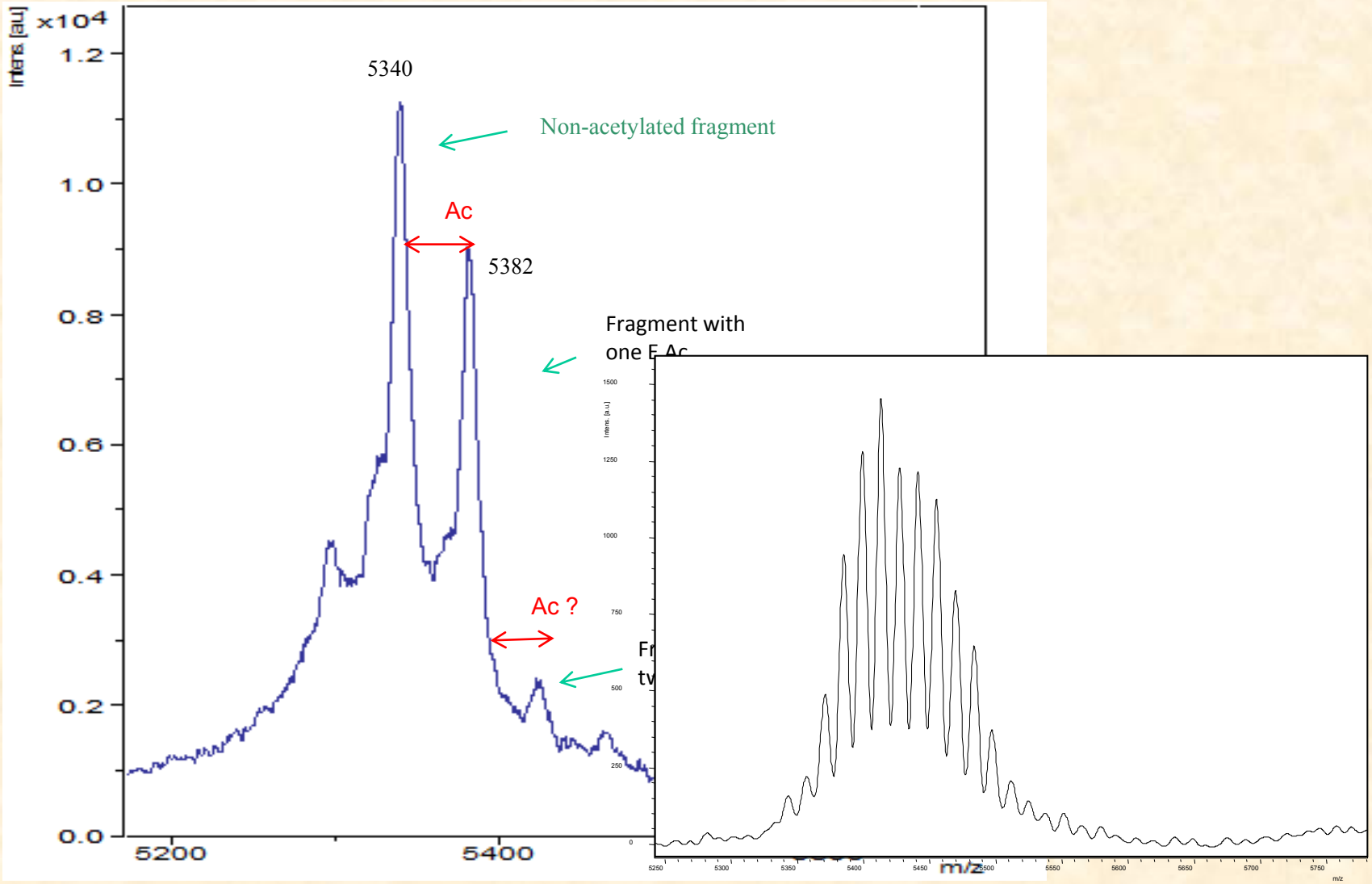
Confirmation of modification of Cys

…VC*AR



Fryčák P. et al., J. Mass Spectrom., ***40*** *(10), 1309-1318 (2005)*

Histone H3, detection of acetylation *(in-vitro)*
MALDI-MS digestu (spectrum detail)



C7250

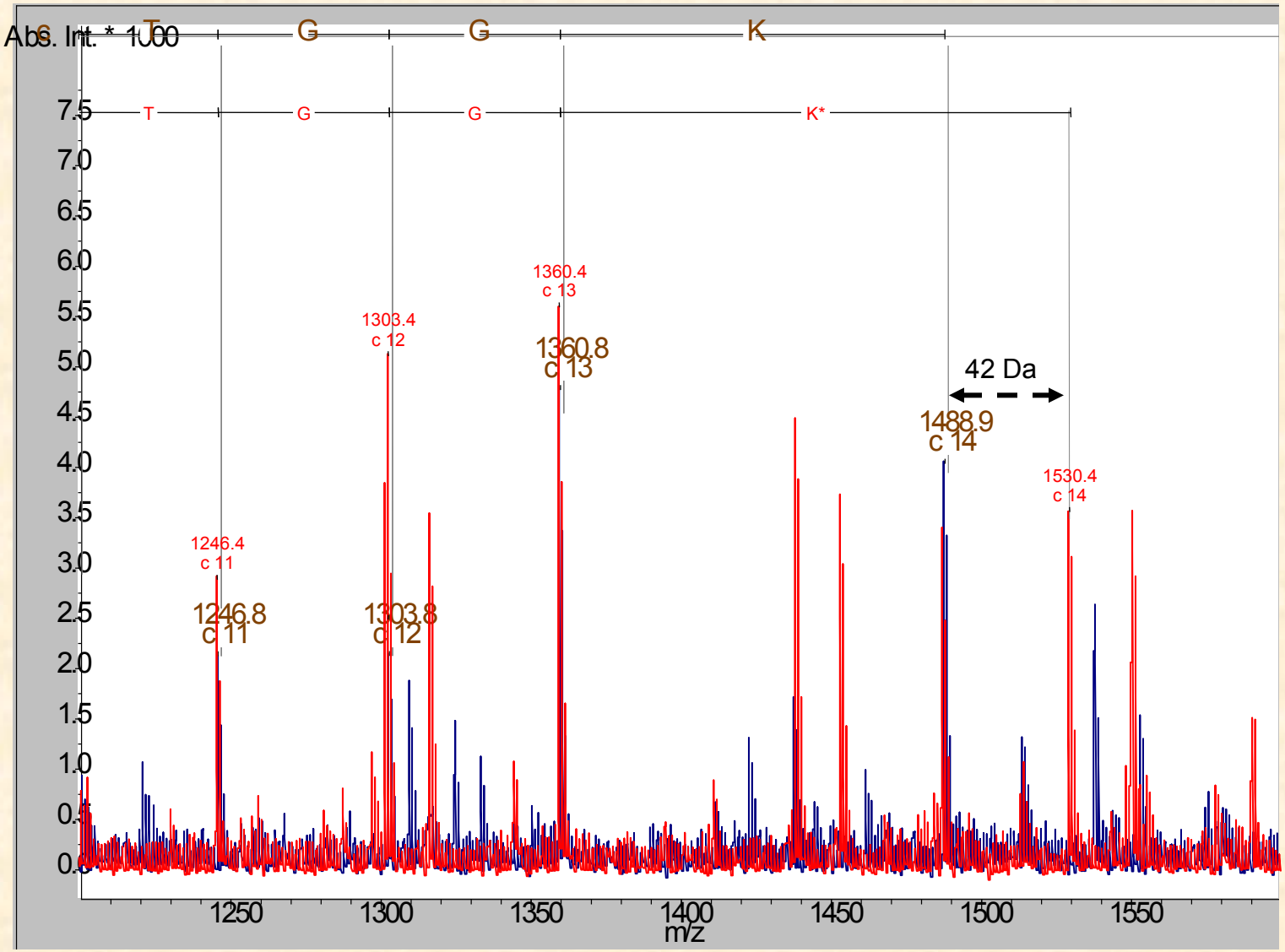
Histone H3, localization acetylace K(14)

MALDI-ISD MS (spectrum detail)

c_{14}

c_{13}

ARTKQTARKSTGGKAPR



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Characterization of modifications „posttranslational “

Phosphorylation

Protein Phosphorylation is of Fundamental Importance in Biological Regulation

cca 10-30% of all proteins are phosphorylated

- S, T, Y 1800 : 200 : 1 ???
- H ???

*Whereas phosphorylation of **serine, threonine or tyrosine** results in the formation of **a phosphoester linkage**, phosphorylation of **histidine** residues occurs **on nitrogen atoms**, producing a phosphoramidate bond. Phosphohistidines have a large standard free energy of hydrolysis making them **the most unstable** of any known phosphoamino acid.*

Klumpp et al, *Eur. J. Biochem*. **269**, 1067-1071 (2002)

Phosphorylation sites db: ***http://www.phosphosite.org/homeAction.do***

http://phospho.elm.eu.org

Phospho.ELM version 9.0 (September 2010) contains 8,718 substrate proteins from different species covering 3,370 tyrosine, 31,754 serine and 7,449 threonine instances.

Phosphorylation

9 aminoacid, which might be phosphorylated

serine (Ser) > threonine (Thr) > tyrosine (Tyr)

histidine (His)

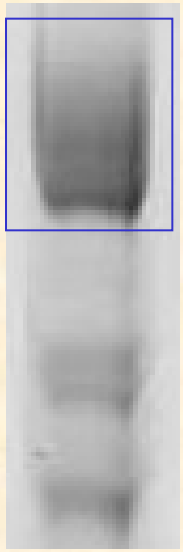
aspartic acid (Asp), glutamic acid (Glu)

lysine (Lys), arginine (Arg), cysteine (Cys)

Phosphoproteome - troublemaker

- most of signaling proteins are low abundant
- proteins are usually in several phospho variants
- proteins can be easily dephosphorylated during sample preparation by phosphatases present in sample
- signal suppression in MS
  - preferential ionization of unmodified peptides (*only small portion out of total peptide content is phosphorylated; low ionization efficiency of phosphopeptides)*

enrichment necessary



Sample preparation

- phosphostatus stabilization
  - **phosphatase inhibitors** *(as soon as possible)*
  - **denaturation** *(e.g. FASP - lysis in SDT buffer)*
- phosphopeptide(protein) enrichment
  - **TiO_2** *(or other metal oxides, MOAC – „metal oxide affinity chromatography“)*
  - **IMAC** („*immobilized metal affinity chromatography*“)
  - **SCX** resp. **SAX** or **HILIC** *(„ion exchange or hydrophilic interaction chromatography“)*
  - ***immunoprecipitation*** *by specific antibody*

I.L. Batalha, Trends in Biotechnology 30 (2), 100-110 (2012)

MS analysis

other types of MS/MS fragmentation

CID

ETD (ECD)

electron transfer (capture) dissociation

HCD

higher-energy collision dissociation

EThcD

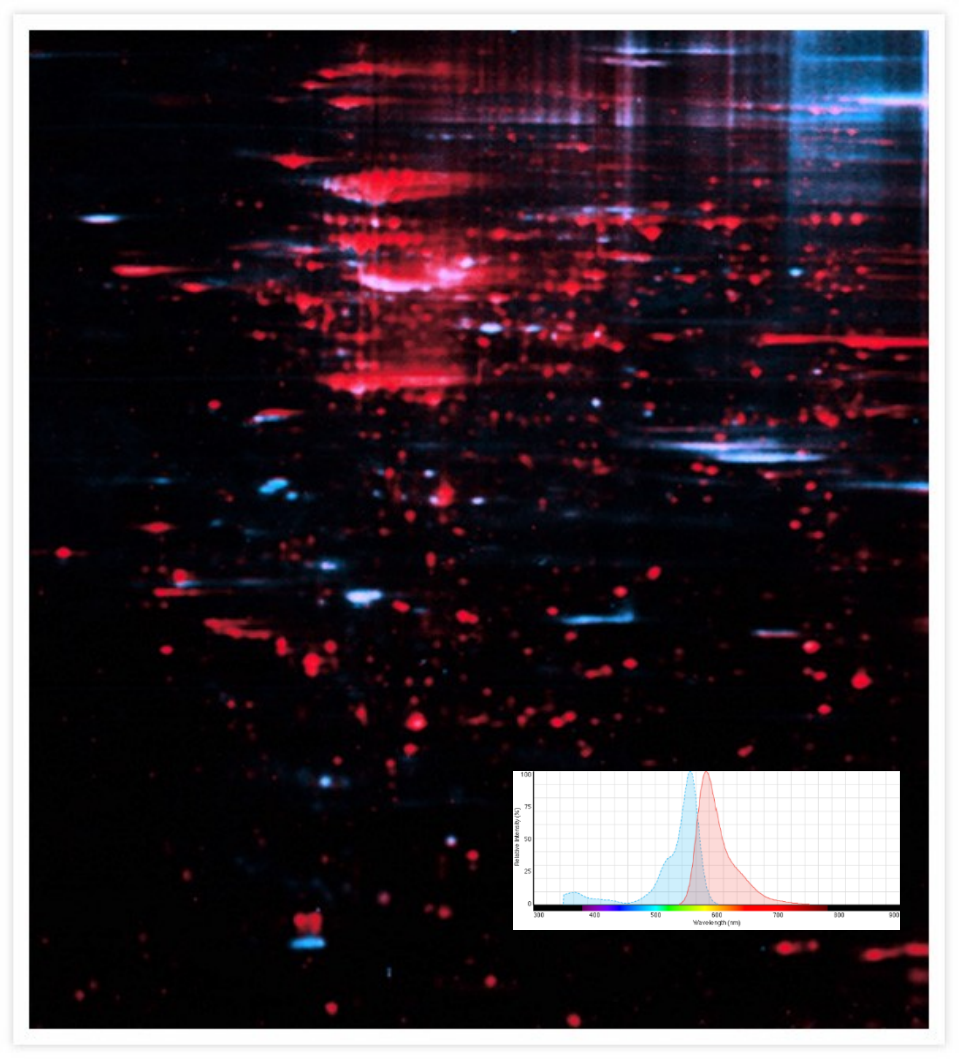
electron-transfer/higher-energy collision dissociation

Frese at al., J. Proteome Res., 12, 1520−1525 (2013)

neutral loss scan (different variants)

precursor scan

Specific staining of phosphoproteins, 2D GE



phosphoproteins
(Pro-Q Diamond , **blue**)
proteins
(SYPRO Ruby, **red**).

alternatives
Metabolic tagging by ^{32}P
radioactivity measurement

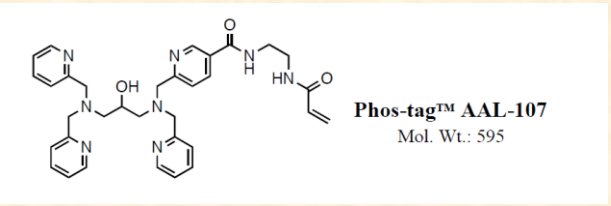
immunoblotting

phosphatase treatment
phosphoproteins display a basic shift in their pI after the dephosphorylation.
comparison 2D gels

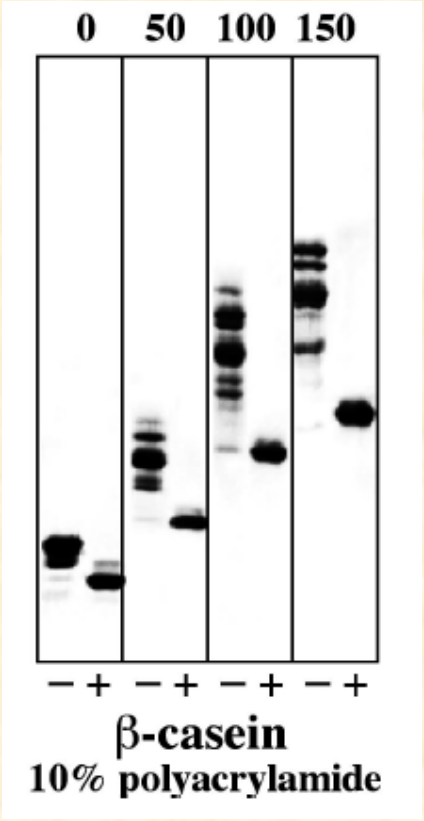
(https://www.lifetechnologies.com/order/catalog/product/P33300)

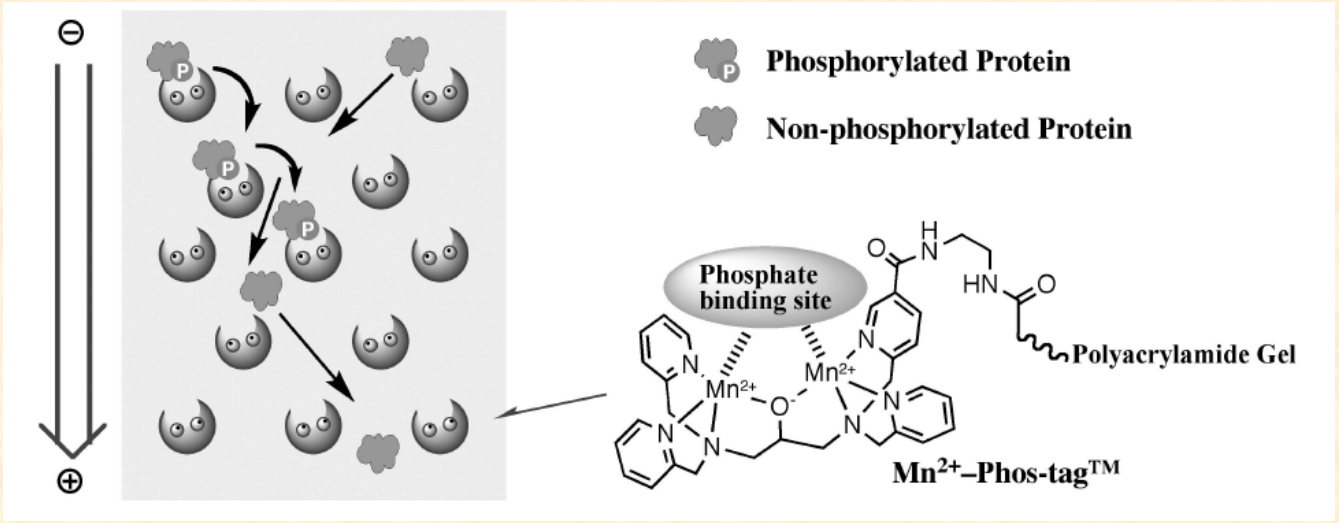
Mobility Shift Detection of Phosphorylated Proteins

SDS-PAGE using an Phos-tagTM complex with two manganese(II) ions
Mn2+–Phos-tagTM™



c(Mn^{2+}) μM





– : Phosphorylated proteins (octa-, penta-, and di-phosphorylated, respectively)

+ : Dephosphorylated proteins (AP-treated proteins)

A commercially available β-casein contains partially dephosphorylated proteins.

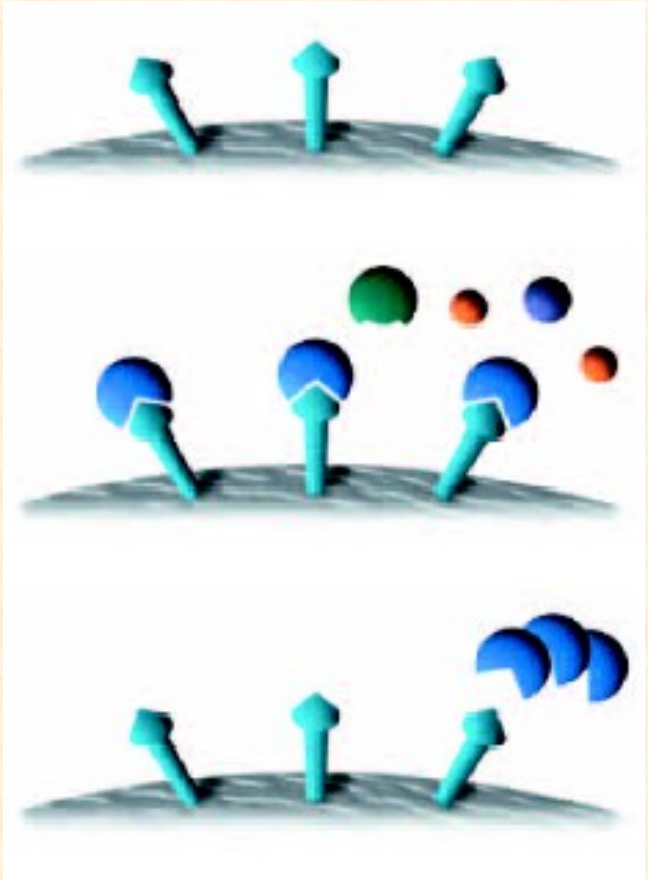
E. Kinoshita et al., *Molecular & Cellular Proteomics*, *5*, 749-757 (2006)

Immobilized metal affinity chromatography (IMAC)

charging

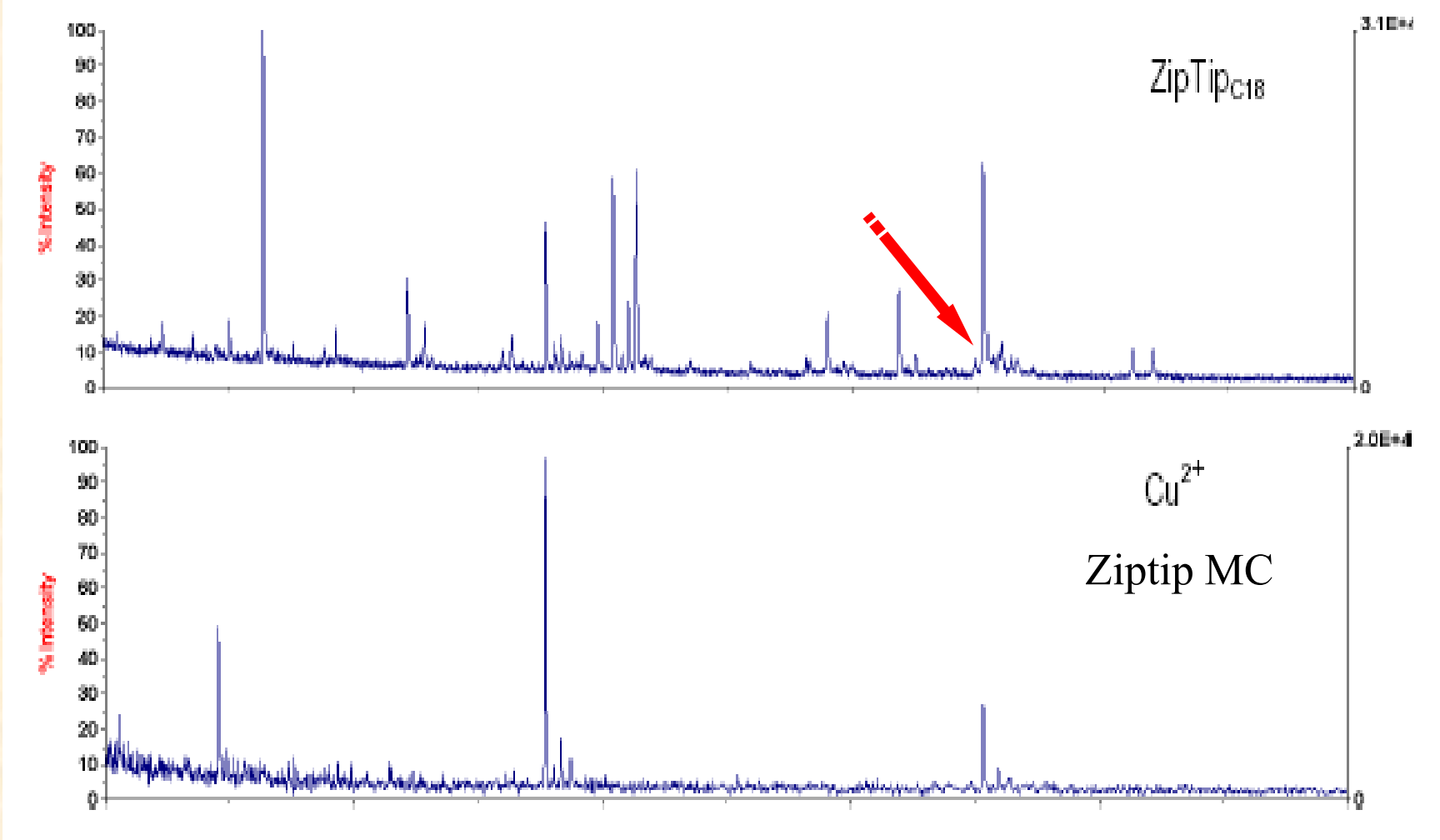
specific binding

eluce



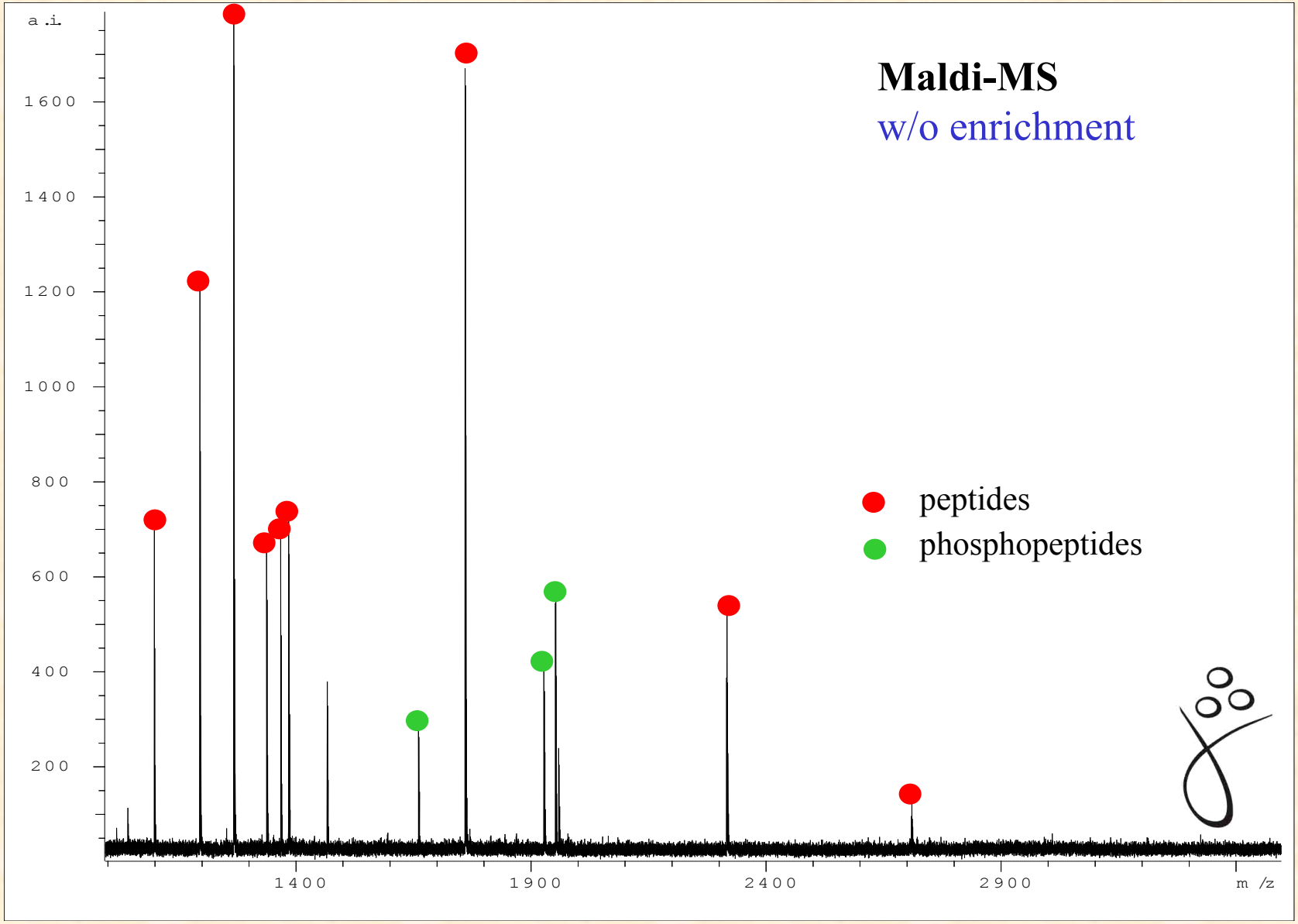
(Cu^{2+}, Fe^{3+}, Ga^{3+})

IMAC enrichment of -Casein phosphopeptides (1 pmol of tryptic digest)



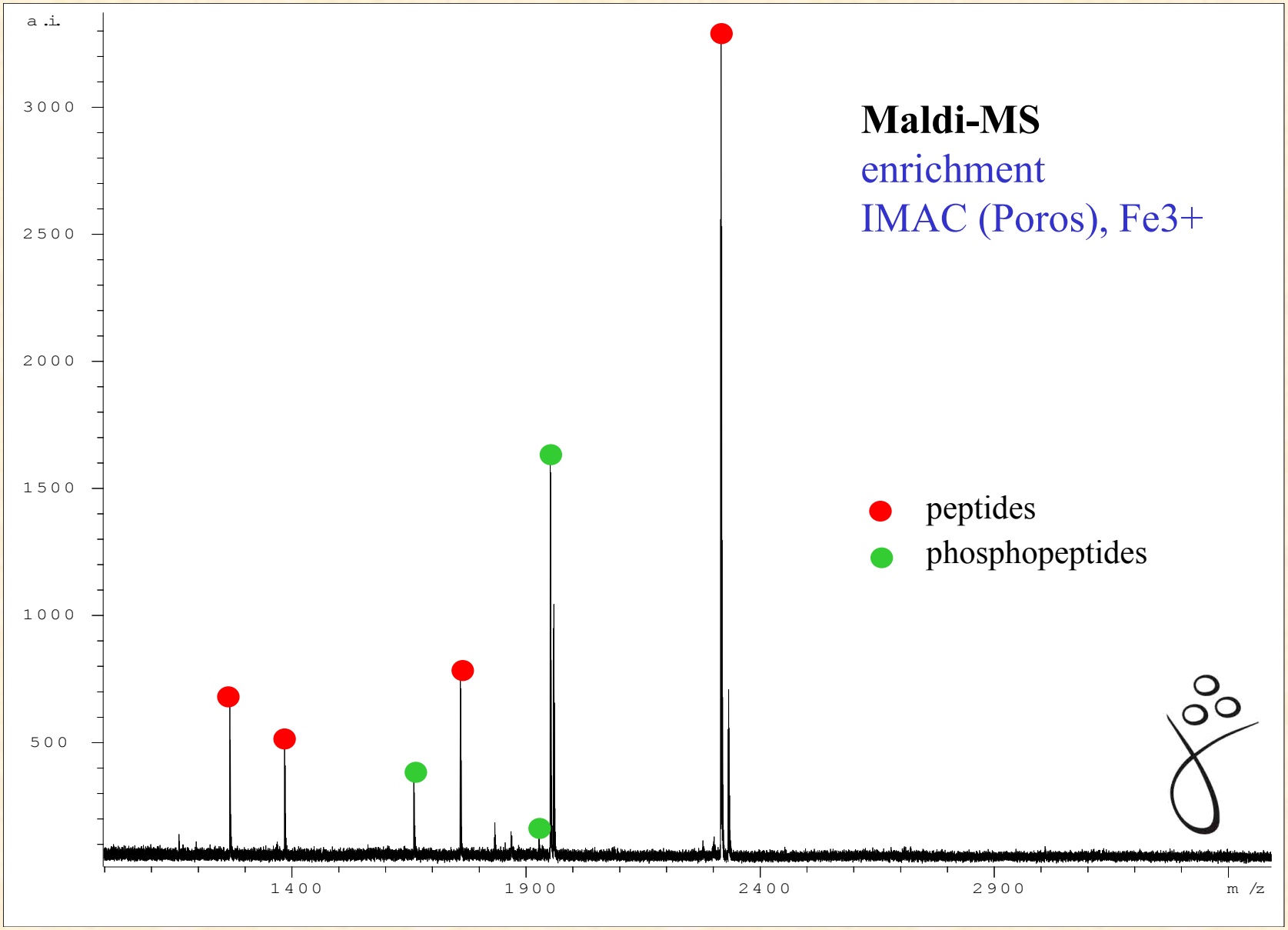
Technical note - Millipore

Casein (1 ug) after tryptic digestion

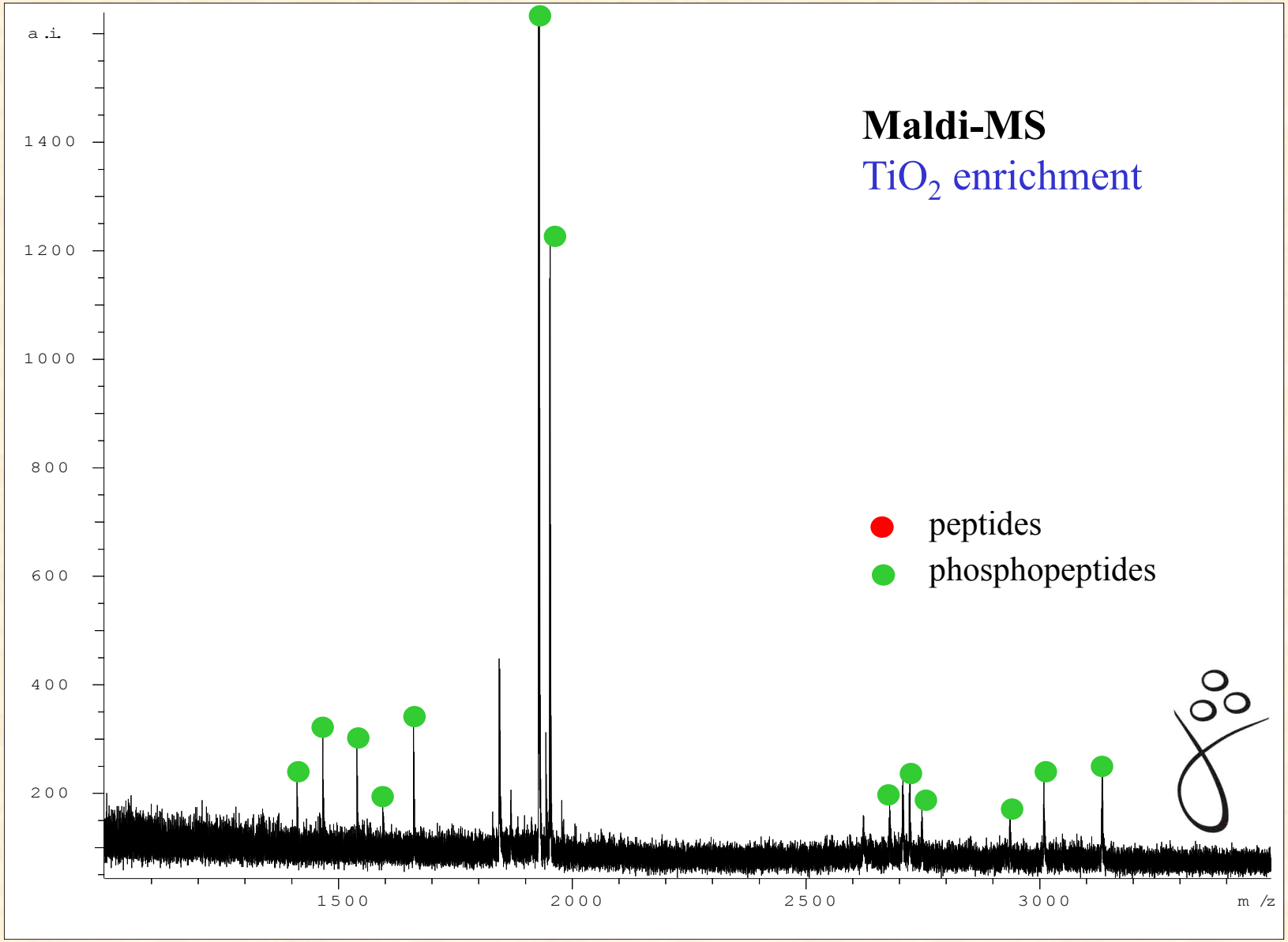


C7250

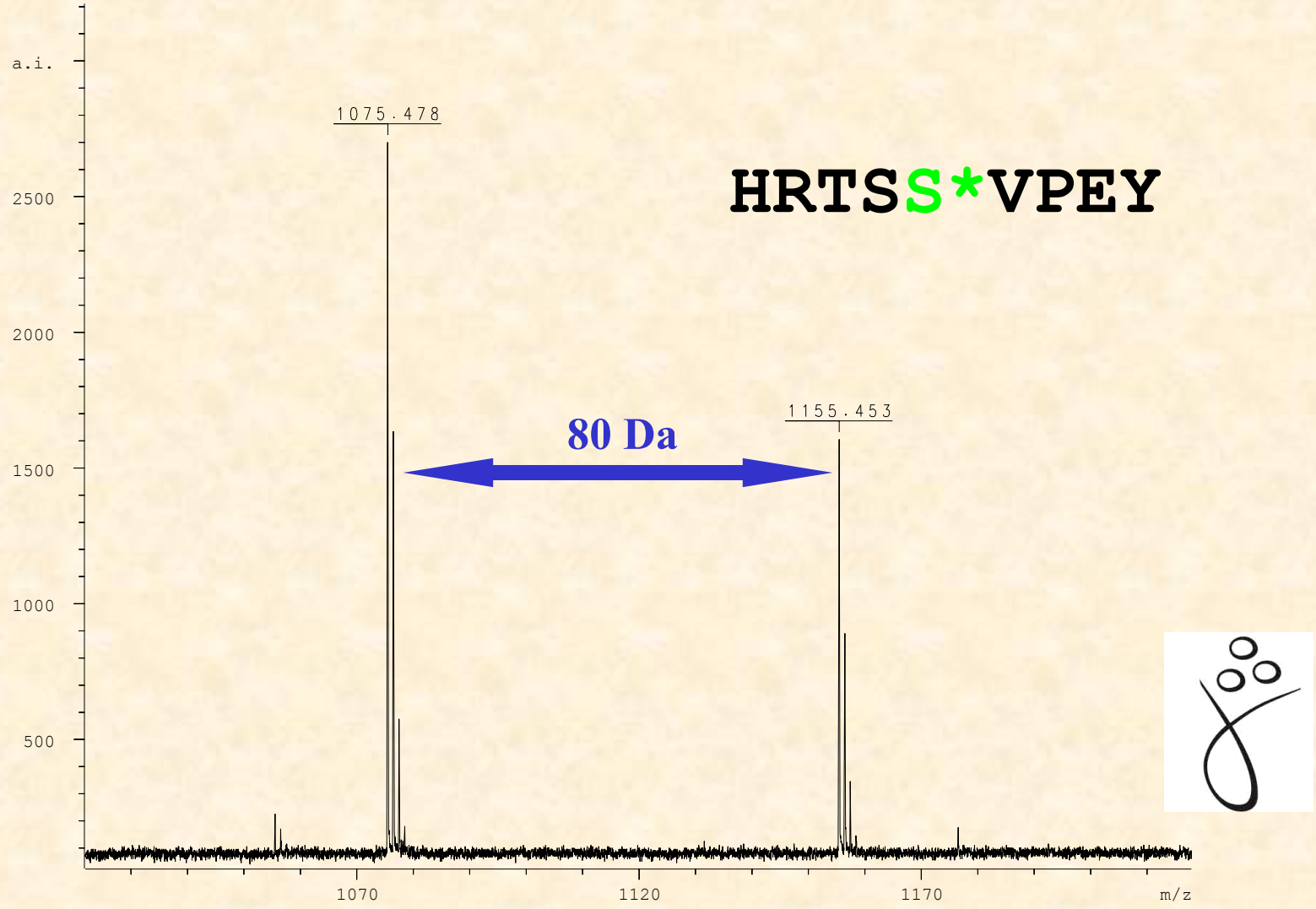
Casein (1 ug) after tryptic digestion



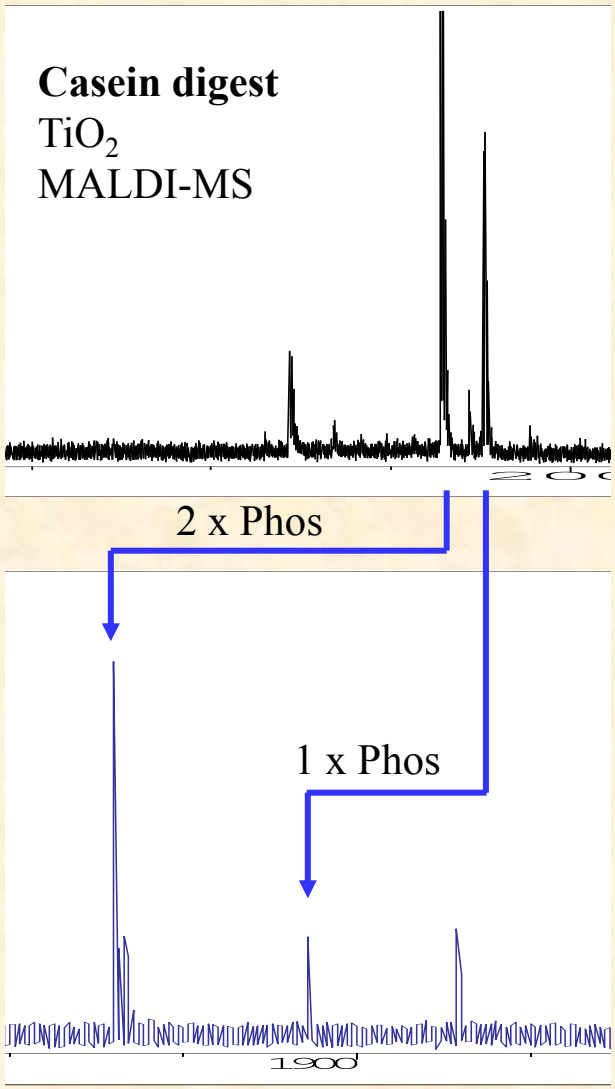
Casein (1 ug) after tryptic digestion



MALDI–MS spectrum of peptide w/o and with phosphorylation

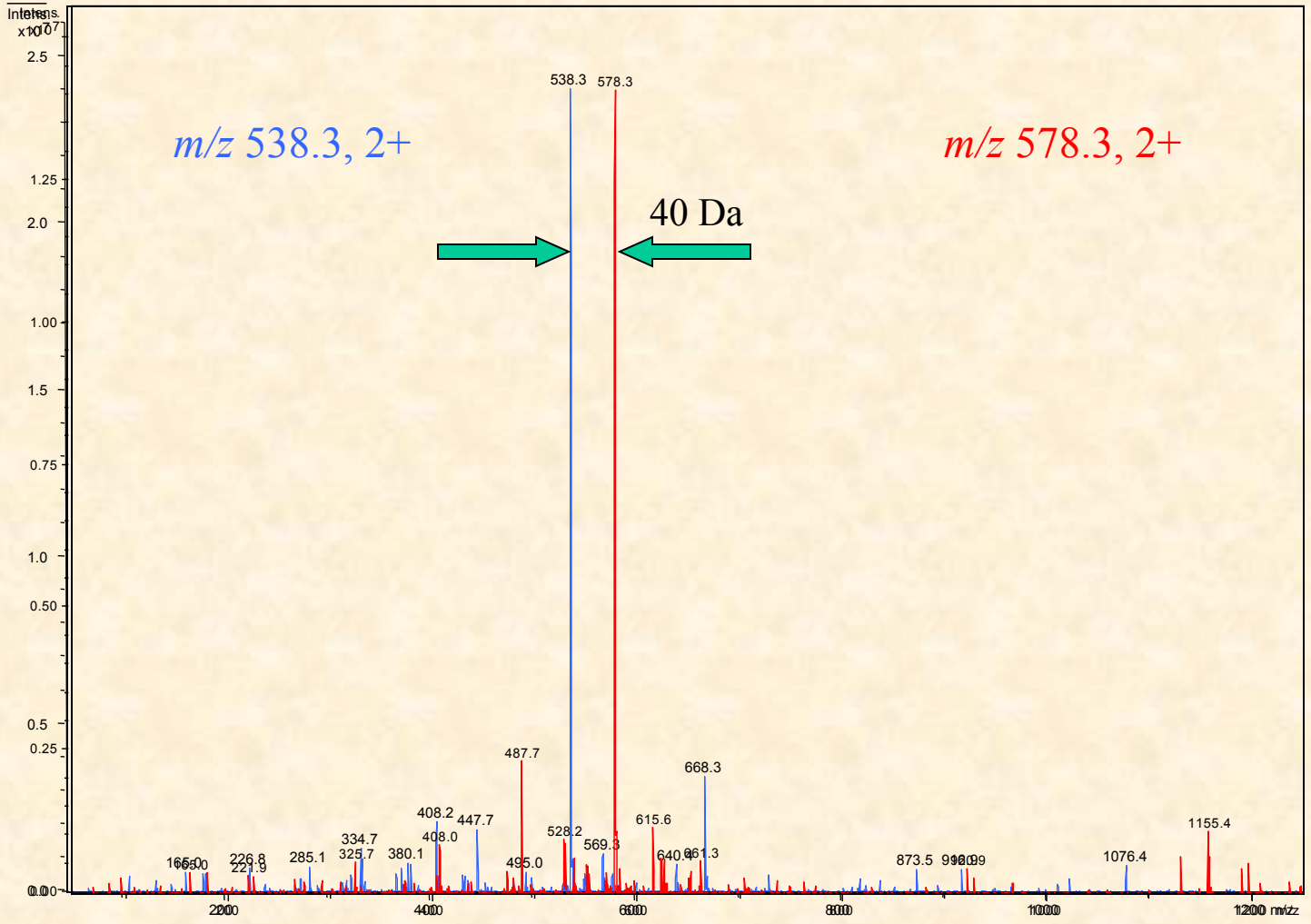


Confirmation of phosphorylation by alkaline phosphatase



ESI–MS (IT) spectrum of peptide w/o and with phosphorylation

positive mode



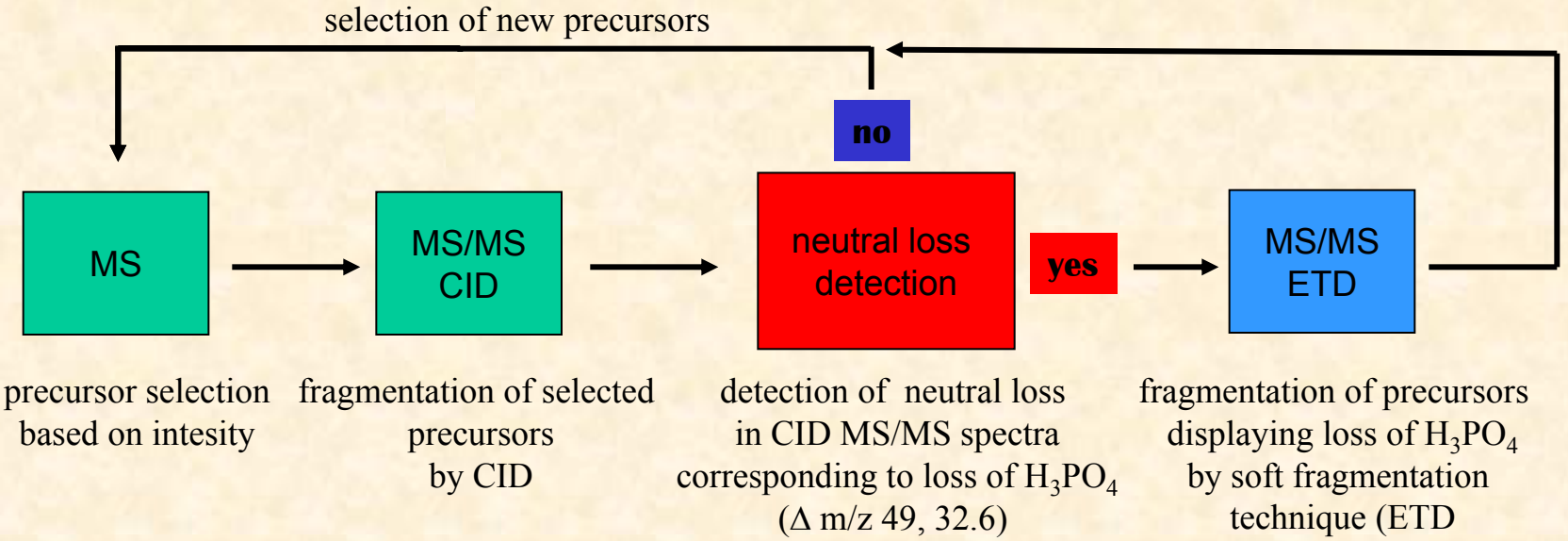
Characterization of Histone H4 phosphorylation by Aurora B kinase

(K. Šedová)

Experimental procedure

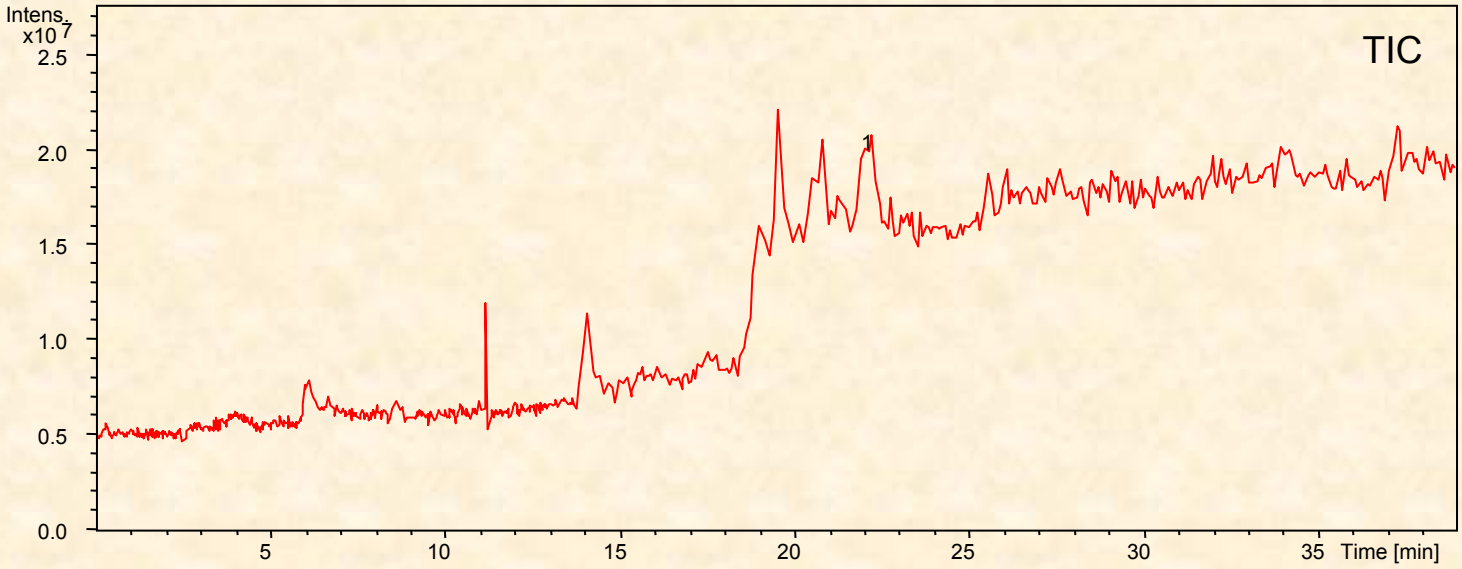
- protein phosphorylation *in vitro*
- tryptic digestion
- TiO_2 phosphoenrichment
- LC-MS/MS analysis – neutral loss scan (ETD) in ion trap

design of MS analysis

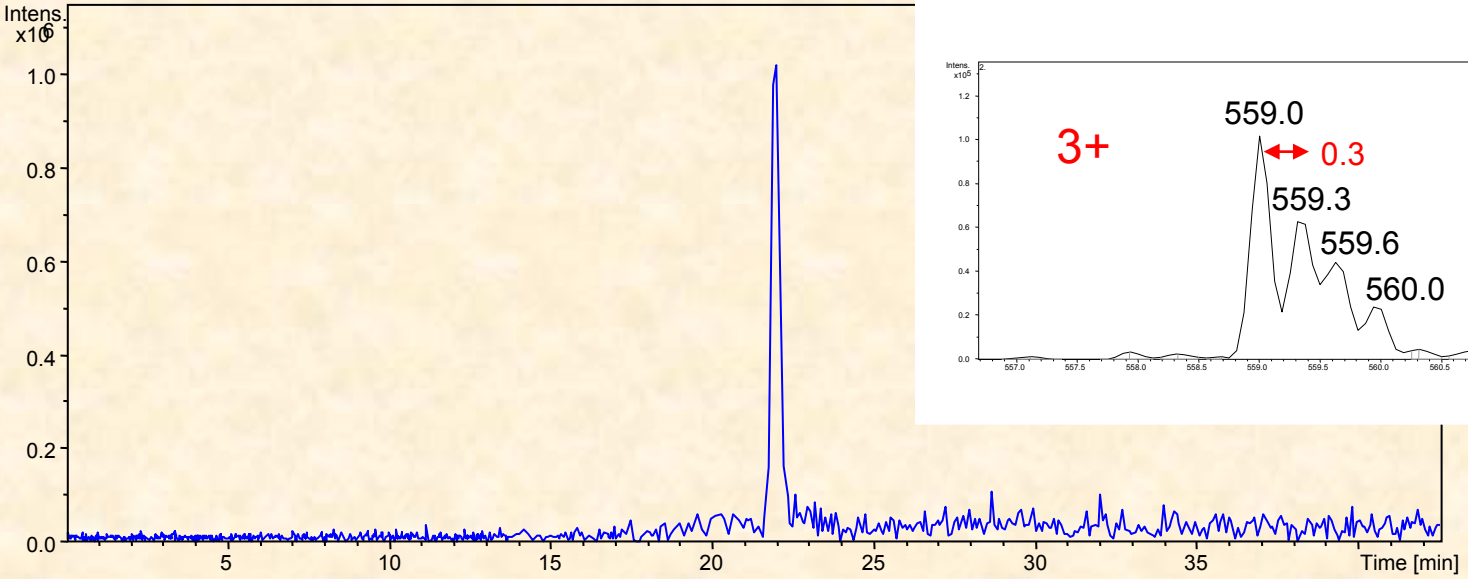


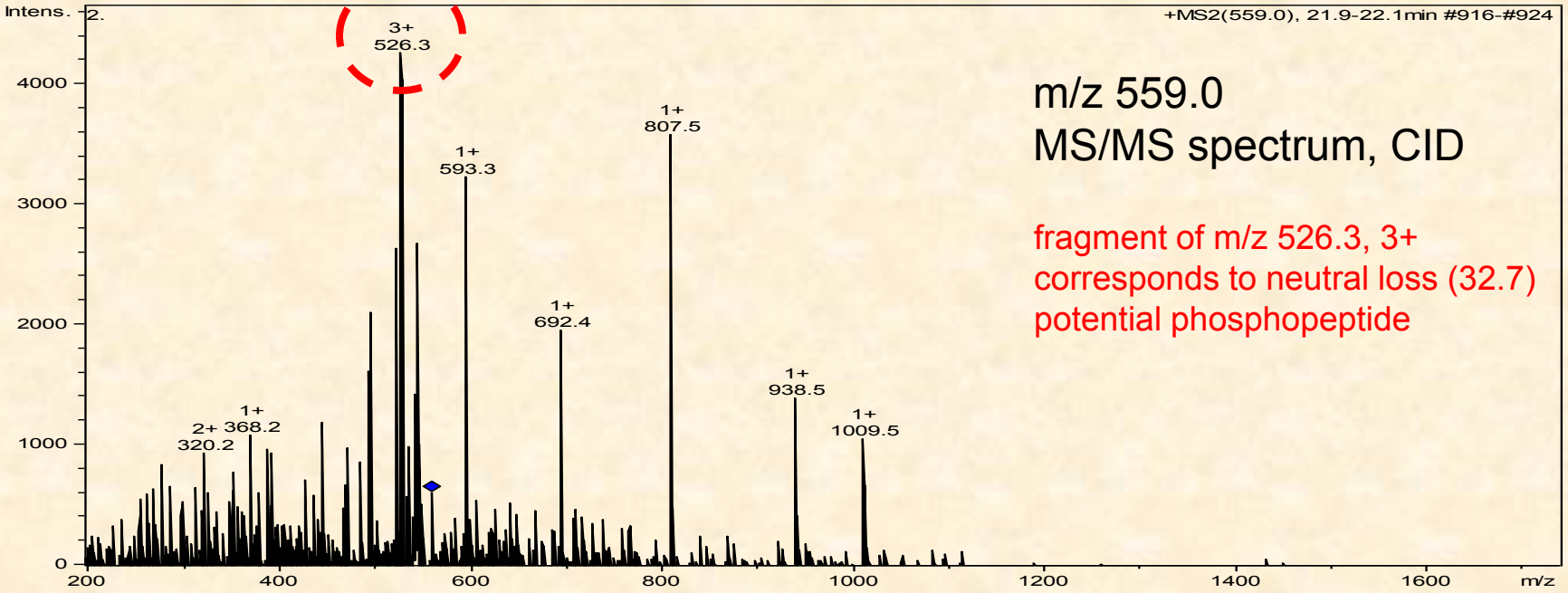
C7250

LC-MS chromatogram



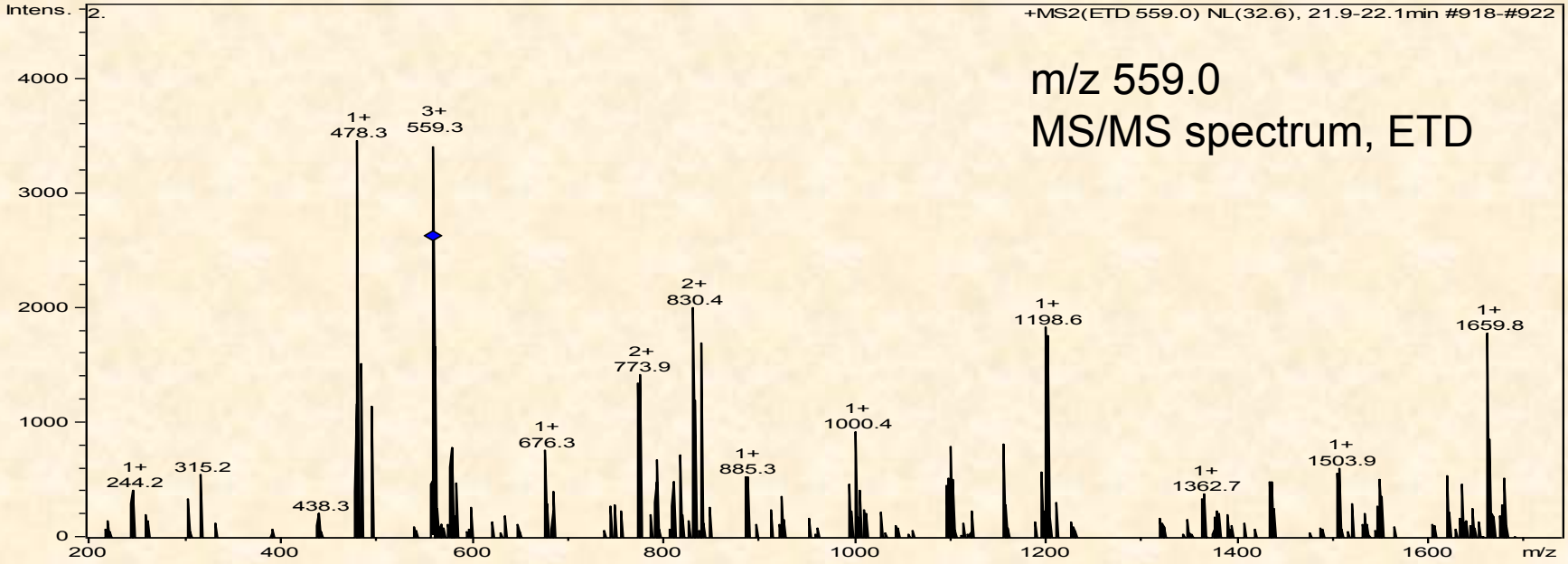
EIC chromatogram of selected ion - m/z 559.0





m/z 559.0
MS/MS spectrum, CID

fragment of m/z 526.3, 3+
corresponds to neutral loss (32.7)
potential phosphopeptide



m/z 559.0
MS/MS spectrum, ETD

Identification of phosphopeptide by database searching
MS/MS Ion Search (MASCOT)

C7250

m/z	Charge	RT (min)	Expect Mr
559.0	3+	22.063	1674.0

MASCOT

- gi|223582 **Mass:** 11230 **Score:** 74 **Queries matched:** 2
  histone H4

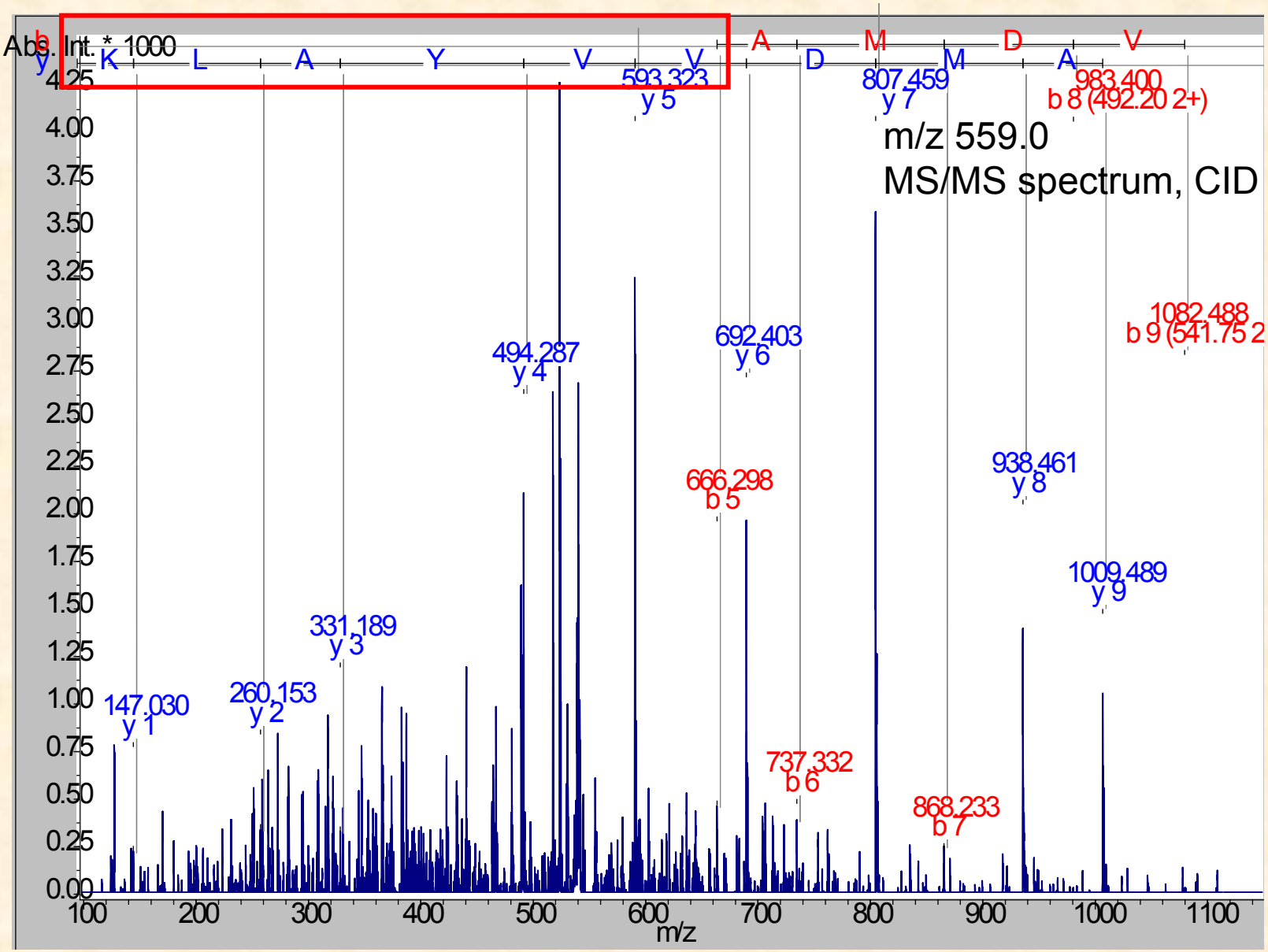
Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide	
558.98	1673.93	1673.86	0.076	2	(23)	7.8	1	K.RK<u>T</u>VTAMDVVYALK.R + Phospho (ST)	CID
558.98	1673.93	1673.86	0.076	2	75	5e-05	1	K.RK<u>T</u>VTAMDVVYALK.R + Phospho (ST)	ETD

Modifications: Optional: Phospho (ST)
Search Parameter: Charge=2+ and 3+, MS Tol.:0.500000 Da, MSMS Tol.:0.500000 Da, Trypsin
Mascot 2.2.03, NCBInr NCBInr_20081101.fasta

Biotools

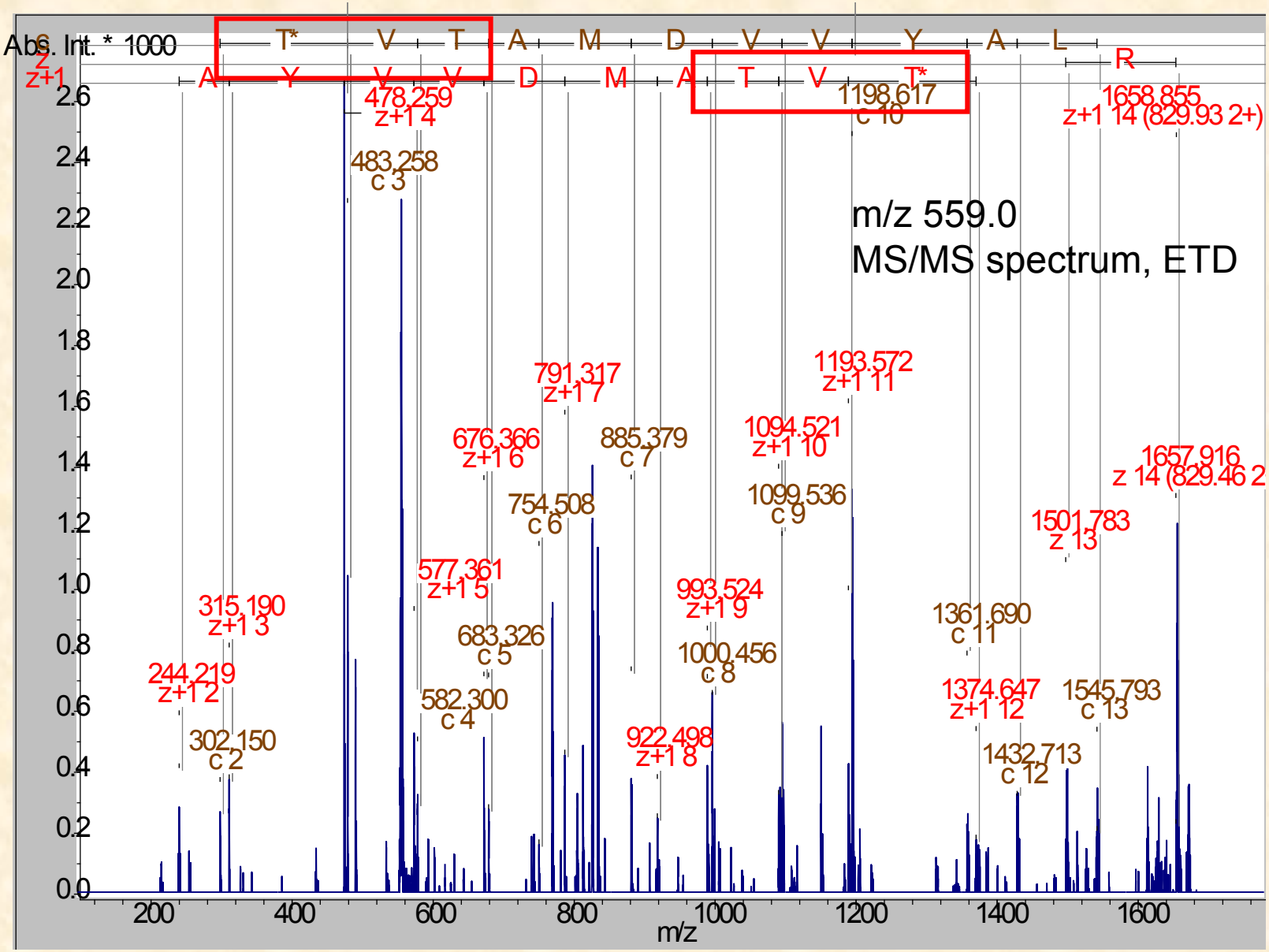
	Score (Biotools)	**Score (Mascot)**
CID		
RK**T**VTAMDVVYALK	520	23
RKTV**T**AMDVVYALK	518	22
ETD		
RK**T**VTAMDVVYALK	6149	**75**
RKTV**T**AMDVVYALK	774	47

RKTVTAMDVVYALK C7250



based on CID MS/MS data localization of phosphorylation is not possible

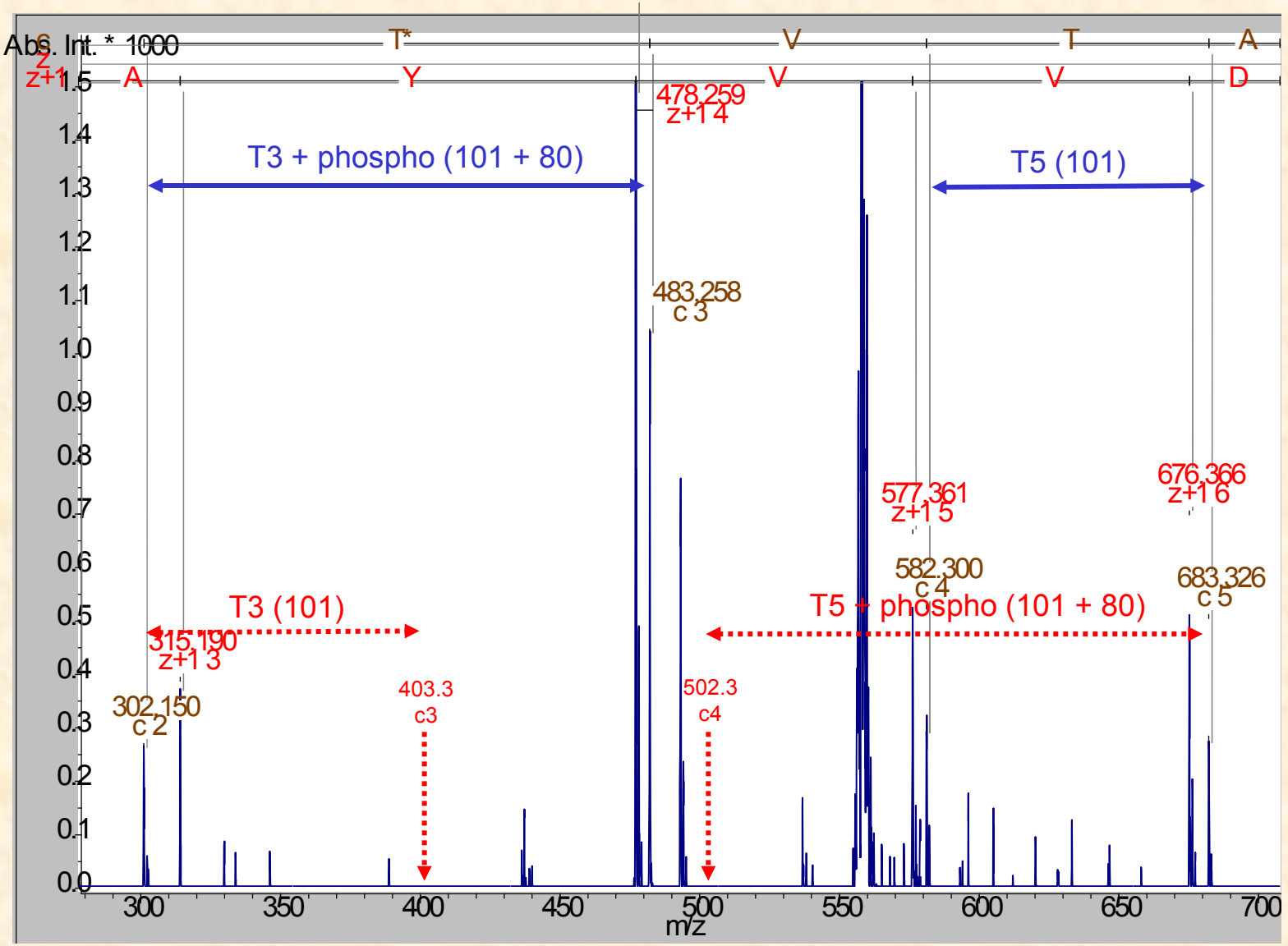
RKTVTAMDVVYALK C7250



based on ETD MS/MS data phosphorylation is reliably assigned to T(3)

C7250

T(3) x T(5)


Abs. Int. * 1000
T*
V
T
A
A
Y
V
V
D
478.259
z+1 4
T3 + phospho (101 + 80)
T5 (101)
483.258
c 3
577.361
z+1 5
676.366
z+1 6
582.300
c 4
683.326
c 5
T3 (101)
T5 + phospho (101 + 80)
315.190
z+1 3
302.150
c 2
403.3
c3
502.3
c4
m/z

C7250

TABLE 1 | Phosphopeptide/phosphotprotein enrichment methodologies.

Enrichment method	Description	Advantage	Disadvantage	References
Immunoaffinity enrichment	Use of antibodies directed against pTyr, pSer, pThr, and more recently against the surrounding consensus sequences for pSer/pThr.	Highly specific.	Low efficiency, high cost, use of different antibodies for different phosphorylation motifs.	Stokes et al., 2012
Immobilized metal affinity chromatography (IMAC)	Negatively charged phosphate groups on the phosphorylated amino acids interact with positively charged metal ions such as Ni^{2+}, Fe^{3+}, Ga^{3+}, Zr^{4+}, and Ti^{4+} that are chelated with silica or agarose through nitriloacetic acid or iminodiacetic acid.	Good for both phosphoproteins and phosphopeptides. When used with peptides, it can enrich mono- and multiple phosphorylated peptides.	Tends to bind strongly to monophosphorylated peptides, which makes it difficult for elution. Non-specific binding of acidic peptides can occur.	Fila and Honys, 2012
Metal oxide affinity chromatography (MOAC)	Similar to IMAC, the phosphate groups on the amino acids interact with positively charged metal oxides, e.g., titanium or zirconium that acts as anchoring molecules to trap phosphopeptides through the formation of multi-dentate bonds.	Good for both phosphoproteins and phosphopeptides. When used with peptides, it can enrich mono- and multiple phosphorylated peptides.	Tends to binds strongly to multiple phosphorylated peptides, which makes it difficult for elution. Nonspecific binding of acidic peptides can occur.	Gates et al., 2010
Phos-Tag chromatography,	Uses 1,3-bis[bis(pyridine-2-ylmethyl)amino]propan-2-olato dizinc(II) complex as a selective phosphate binding tag in aqueous solution at neutral pH.	Increased sensitivity due to complete deprotonation of phosphoproteins/ phosphopeptides at neutral pH. Elution at the physiological pH allow for protein activity and functional analysis.	Mainly used to confirm the phosphorylation state in relatively pure proteins, but not with complex mixtures.	Kinoshita et al., 2006
Prefractionation by strong cation exchange (SCX) and strong anion exchange (SAX)	In SCX, tryptic peptides often carry a charge of +2, except for phosphopetides with a net charge of +1, making them elute early in the chromatography. SAX retains phosphor-peptides, allowing separation based on the number of phosphorylated residues.	Used for fractionation of highly complex mixtures, it can be performed on-line with mass spectrometry.	Similar degree of unspecific binding as IMAC and MOAC.	Leitner et al., 2011
Hydrophilic interaction liquid chromatography (HILIC)	Phosphopeptides with polar phosphate groups are strongly retained on the HILIC stationary phase resulting in separation from non-phosphorylated species.	Good for both phosphoproteins and phosphopeptides. When used with peptides, it can enrich mono- and multiple phosphorylated peptides.	Similar degree of unspecific binding as IMAC and MOAC.	(Yang et al., 2013)
Electrostatic repulsion hydrophilic interaction chromatography (ERLIC)	ERLIC is a variation of HILIC using electrostatic repulsion as an additional phase to adjust selectivity by varying pH or organic solvents.	Good for both phosphoproteins and phosphopeptides. When used with peptides, it can enrich mono- and multiple phosphorylated peptides.	Similar degree of unspecific binding as IMAC and MOAC.	Gan et al., 2008
Hydroxyapatite chromatography	It takes advantage of the strong interaction between positively charged hydroxyapatite and phosphate ions.	Good for fractionating mono-, di-, tri-, and multi-phosphorylated peptides when using gradient of a phosphate buffer.	Developed with phosphoprotein standards, not tested with complex samples.	Marmone et al., 2010

Li et al., Front. Plant Sci., 6, 430 (2015)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FOX
Western Pacific
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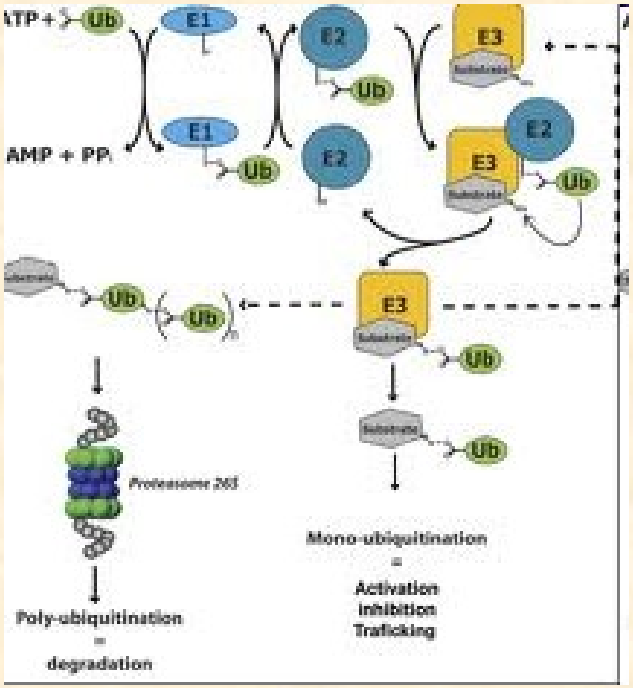
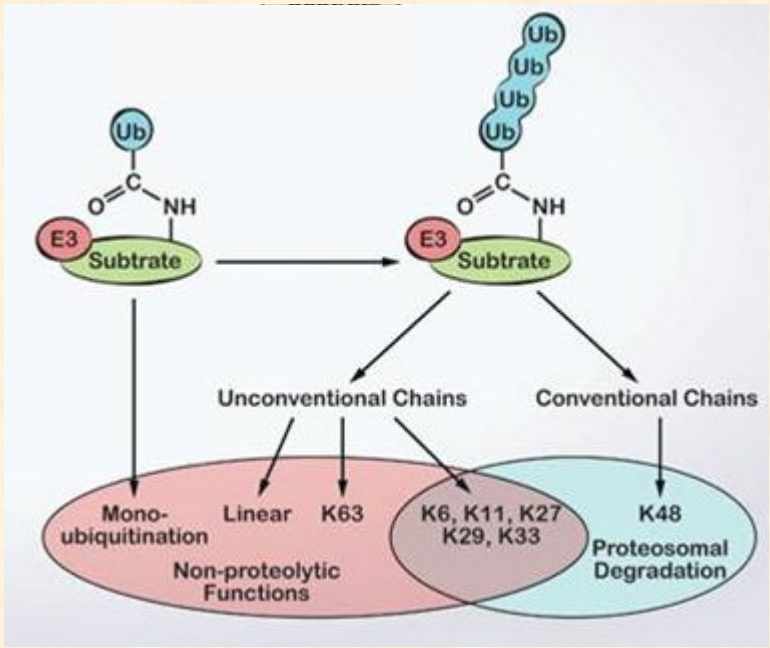
AIRLINERS.NET

Ubiquitination

Ubiquitination is an enzymatic, protein post-translational modification (PTM) process in which the carboxylic acid of the terminal glycine from the **di-glycine motif** in the activated ubiquitin forms an amide bond to the epsilon amine of the lysine in the modified protein.

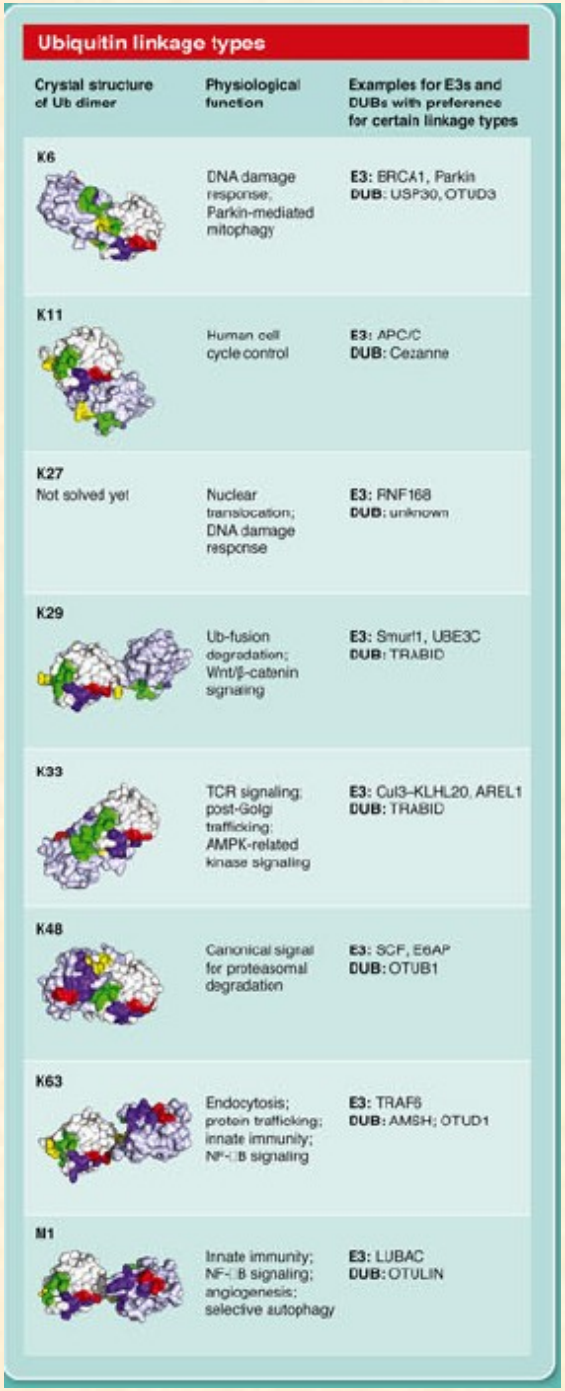
Protein ubiquitination regulates many cellular processes including transcription, endocytosis, cell cycle control, signal transduction, stress response, DNA repair as well as **proteasomal-mediated degradation**

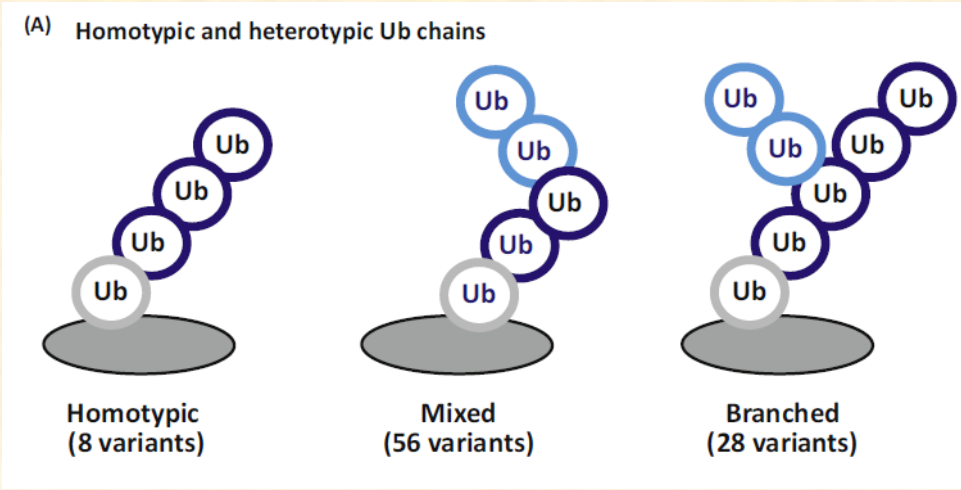




S. Liu, Z.J. Chen , Cell Research (2011) ***21****:6–21*

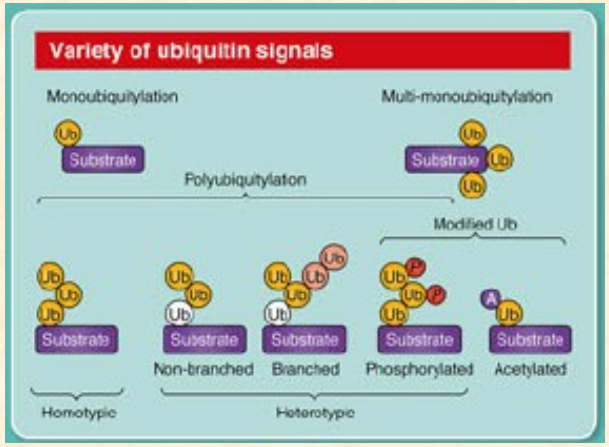
Ubiquitination





The complex Ub code contains numerous variants of homotypic and heterotypic (mixed or branched) chains. Based on the eight possible linkages (M1, K6, K11, K27, K29, K33, K48, and K63) between two Ub moieties, **at least 92 different Ub chain types exist**.

Stolz A. et al., Trends in Cell Biology, 28 (1), 1-3 (2018)

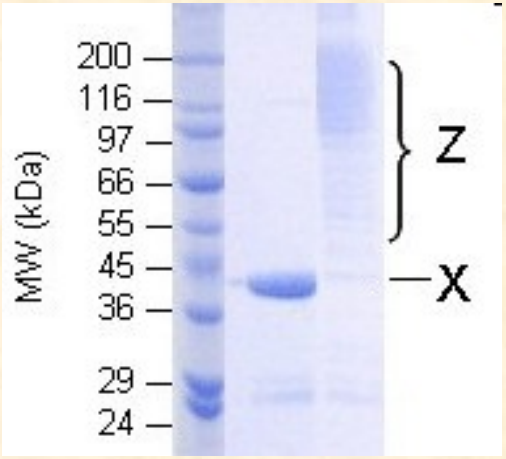


Akutsu M. et al., J. Cell Sci., 129, 875-880 (2016)

ubiquitin – protein 8.5 kDa (76 AA)

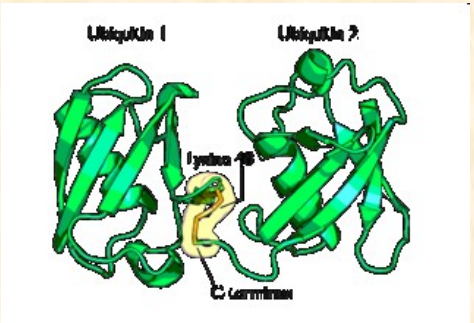
MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGK
QLEDGRTLSDYNIQKESTLHLV LRLRGG

K



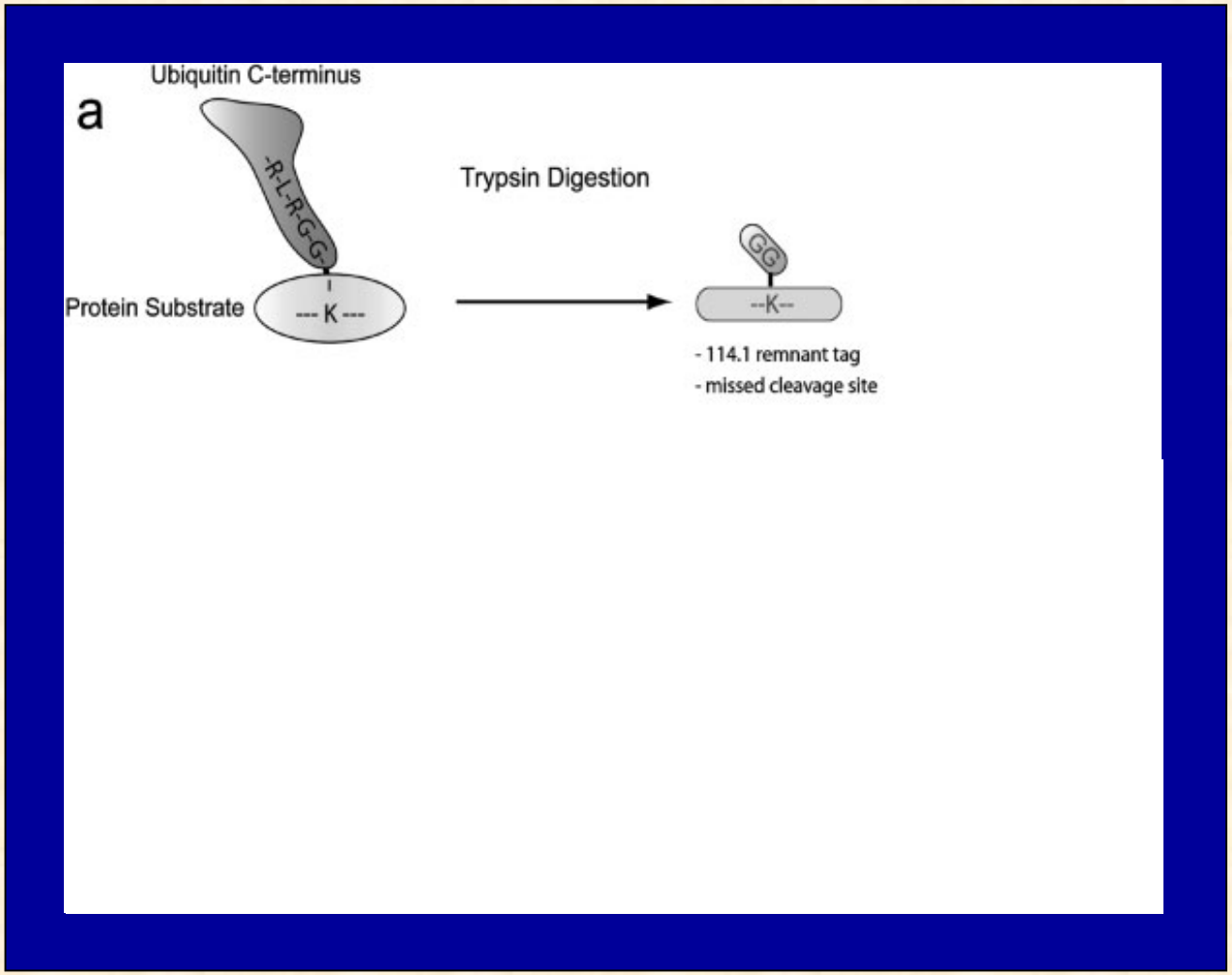
heterogeneity of modified forms

- **localization of modified AA sites**
- **determination of polyubiquitin crosslinks**



The most studied polyubiquitin chains - lysine48-linked - target proteins for destruction

Strategy of ubiquitinated site analysis



N.J. Denis, Proteomics 2007, *7,* 868–874

False-positive identification or K(91) is really ubiquitinated ???

BBAP monoubiquitylates histone **H4 at lysine 91** and selectively modulates the DNA damage response."
Yan Q., Dutt S., Xu R., Graves K., Juszczynski P., Manis J.P., Shipp M.A.
Mol. Cell 36:110-120 (2009)

Histone H4 (trypsin)

```
  1   SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
 51   YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF
101   GG
```

Mascot

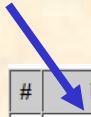
79 –100	658.6059	2630.3944	2630.4003	-2.23	0	45	0.0013	1	R.KTVTAMDVVYALKRQGRTLYGF.G + UBI_dT (K)

All matches to this query

Score	Mr(calc)	Delta	Sequence
[redacted]	2630.4003	-0.0059	[redacted]
	2630.4003	-0.0059	
0.3	2630.3792	0.0152	KSAPAPKKGSKKAVTKAQKKD

False-positive identification or K(91) is really ubiquitinated ???

K(1) is not ubiquitinated

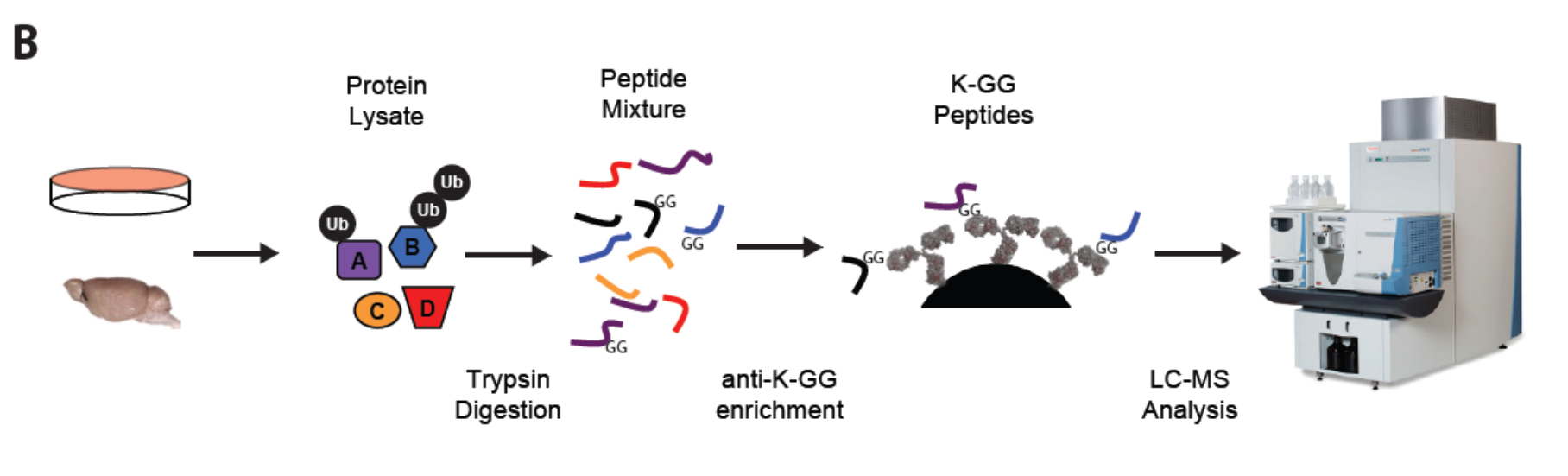


#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	*129.1022*	65.0548	112.0757	56.5415			K							22
2	*230.1499*	115.5786	213.1234	107.0653	212.1394	106.5733	T	2503.3126	1252.1599	2486.2860	1243.6467	2485.3020	1243.1547	21
3	*329.2183*	165.1128	312.1918	156.5995	311.2078	156.1075	V	2402.2649	1201.6361	2385.2384	1193.1228	2384.2543	1192.6308	20
4	*430.2660*	215.6366	413.2395	207.1234	412.2554	206.6314	T	2303.1965	1152.1019	2286.1700	1143.5886	2285.1859	1143.0966	19
5	*501.3031*	251.1552	484.2766	242.6419	483.2926	242.1499	A	2202.1488	1101.5780	2185.1223	1093.0648	2184.1383	1092.5728	18
6	632.3436	316.6754	615.3171	308.1622	614.3330	307.6702	M	2131.1117	1066.0595	2114.0852	1057.5462	2113.1011	1057.0542	17
7	*747.3706*	374.1889	730.3440	365.6756	729.3600	365.1836	D	2000.0712	1000.5392	1983.0447	992.0260	1982.0607	991.5340	16
8	846.4390	423.7231	829.4124	415.2098	828.4284	414.7178	V	1885.0443	943.0258	1868.0177	934.5125	1867.0337	934.0205	15
9	945.5074	473.2573	928.4808	464.7441	927.4968	464.2520	V	1785.9759	893.4916	1768.9493	884.9783	1767.9653	884.4863	14
10	1108.5707	554.7890	1091.5442	546.2757	1090.5601	545.7837	Y	1686.9075	843.9574	1669.8809	835.4441	1668.8969	834.9521	13
11	1179.6078	590.3075	1162.5813	581.7943	1161.5973	581.3023	A	1523.8441	762.4257	1506.8176	753.9124	1505.8336	753.4204	12
12	1292.6919	646.8496	1275.6653	638.3363	1274.6813	637.8443	L	1452.8070	726.9071	1435.7805	718.3939	1434.7964	717.9019	11
13	1534.8298	767.9185	1517.8032	759.4053	1516.8192	758.9132	K	1339.7229	670.3651	1322.6964	661.8518	1321.7124	661.3598	10
14	1690.9309	845.9691	1673.9043	837.4558	1672.9203	836.9638	R	1097.5851	549.2962	1080.5585	540.7829	1079.5745	540.2909	9
15	1818.9895	909.9984	1801.9629	901.4851	1800.9789	900.9931	Q	941.4839	471.2456	924.4574	462.7323	923.4734	462.2403	8
16	1876.0109	938.5091	1858.9844	929.9958	1858.0004	929.5038	G	813.4254	407.2163	796.3988	398.7030	795.4148	398.2110	7
17	2032.1120	1016.5597	2015.0855	1008.0464	2014.1015	1007.5544	R	756.4039	378.7056	739.3774	370.1923	738.3933	369.7003	6
18	2133.1597	1067.0835	2116.1332	1058.5702	2115.1492	1058.0782	T	600.3028	300.6550			582.2922	291.6498	5
19	2246.2438	1123.6255	2229.2172	1115.1123	2228.2332	1114.6202	L	499.2551	250.1312					4
20	2409.3071	1205.1572	2392.2806	1196.6439	2391.2965	1196.1519	Y	386.1710	193.5892					3
21	2466.3286	1233.6679	2449.3020	1225.1547	2448.3180	1224.6626	G	223.1077	112.0575					2
22							F	166.0863	83.5468					1

based on hte MS/MS data is not possible to decide K(91)ubi or C-terminal GG

Characterization of ubiquitinations using immunoprecipitation

Scheme of experiment



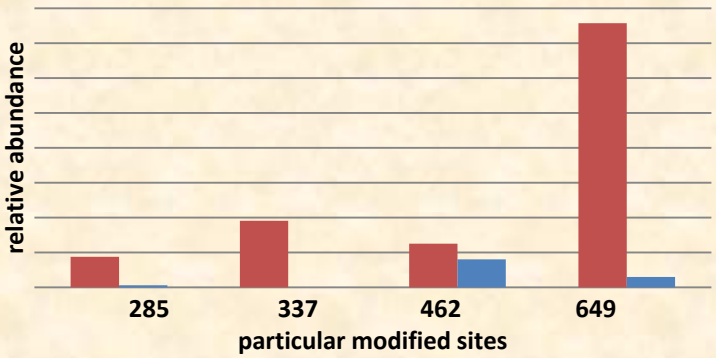
D.Bustos, MCP, 2012 11: 1529-1540

Ubiquitination

Semiquantitative assessment of site occupancy

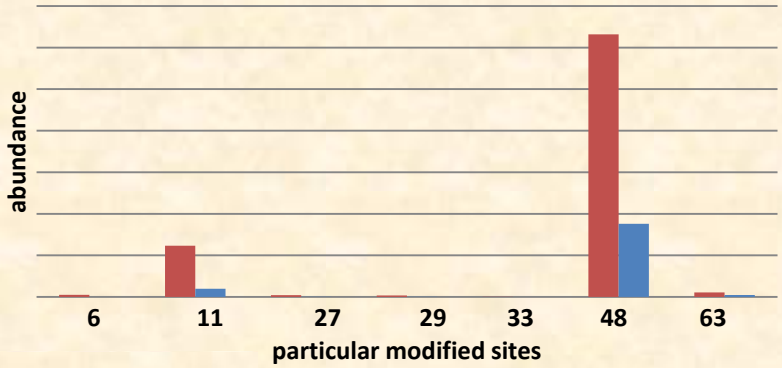
site occupancy of individual Ubi sites

sample *vs* *control*



site occupancy within polyubiquitin chains

sample *vs* *control*



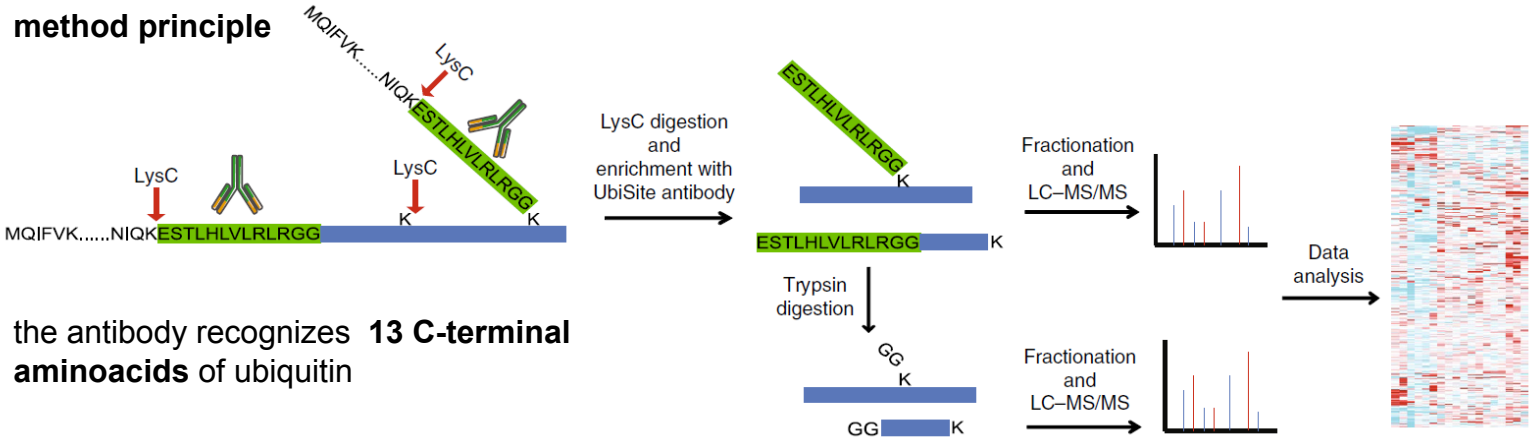
de Groot R. E.A. et al., Sci. Signal., 7 (317), ra26 (2014)

Characterization of ubiquitinations using immunoprecipitation II

UbiSite antibody

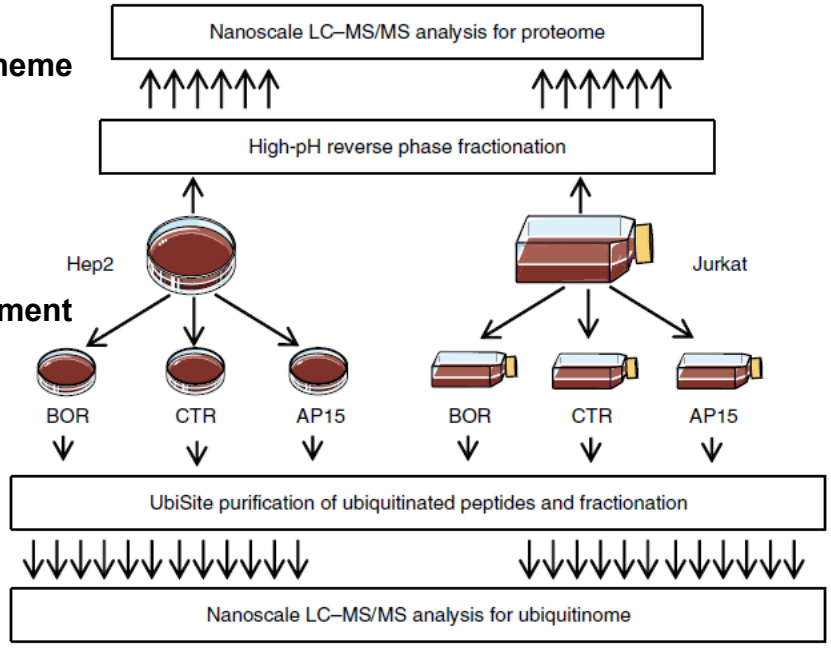
C7250

method principle



the antibody recognizes **13 C-terminal aminoacids** of ubiquitin

experimental scheme



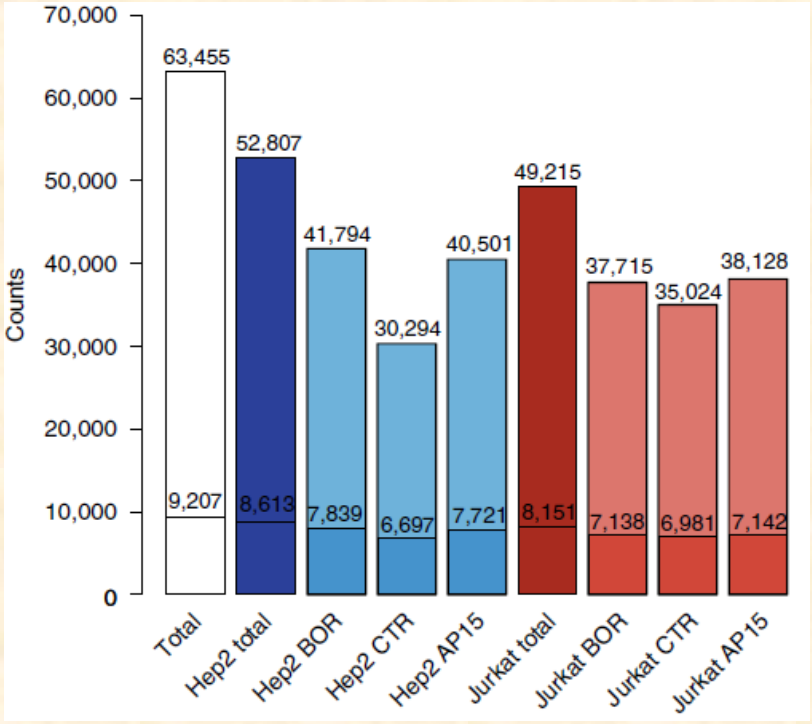
two human cell lines
Hep2
Jurkat

proteasomal inhibitors treatment
BOR - bortezomib
AP15 - b-AP15

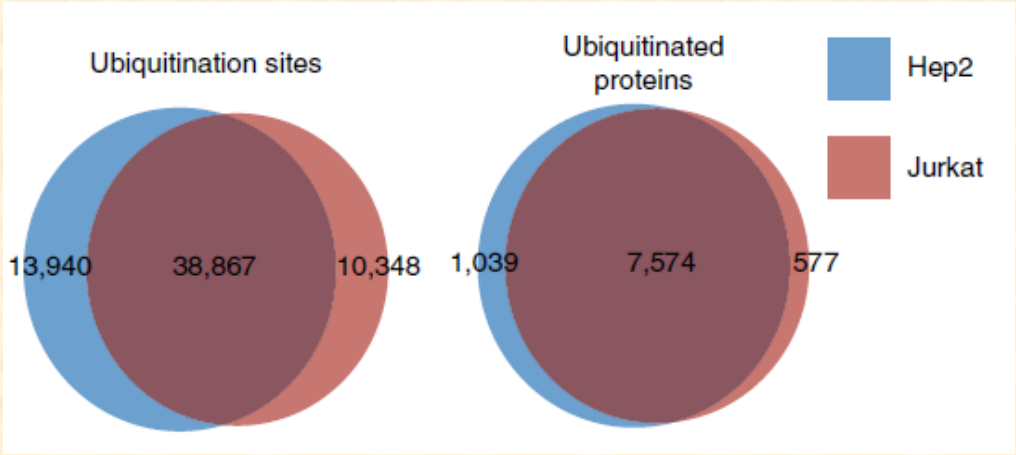
Nat. Struct. Mol. Biol., 25 (July), 631–640, (2018)

Characterization of ubiquitinations using immunoprecipitation II

UbiSite antibody



Numbers of ubiquitination sites and ubiquitinated proteins identified in the two cell lines (*n* = 3 independent biological replicates).
Numbers on the top of bars indicate identified ubiquitination sites; numbers within bars indicate identified proteins.



Overlap of ubiquitination sites and ubiquitinated proteins between Hep2 and Jurkat cells.
Numbers indicate the number of identified ubiquitination sites (left) or proteins (right).

Nat. Struct. Mol. Biol., 25 (July), 631–640, (2018)

Ubiquitin-like proteins

Table 1. Ubl's and Their E1 and E2 in Human and Budding Yeast

	proteins in *H. sapiens*			proteins in *S. cerevisiae*		
family	Ubl	E1	E2	Ubl	E1	E2
SUMO	SUMO1, SUMO2, SUMO3, SUMO4[a]	UBA2/SAE1	UBC9	Smt3	Uba2/Aos1	Ubc9
NEDD8	NEDD8	UBA3/NAE1	UBC12, UBE2F	Rub1	Uba3/Ula1	Ubc12
ATG8	LC3A, LC3B, LC3B2, LC3C, GABARAP, GABARAPL1, GATE-16[a]	ATG7	ATG3	Atg8	Atg7	Atg3
ATG12	Atg12	ATG7	ATG10	Atg12	Atg7	Atg10
URM1	URM1	UBA4	–	Urm1	Uba4	–
UFM1	UFM1	UBA5	UFC1	–	–	–
FAT10	FAT10	UBA6	UBE2Z[b]	–	–	–
ISG15	ISG15	UBA7	UBCH8[b]	–	–	–

[a]SUMO5 and GABARAPL3 were not included in this table as they are likely pseudogenes. [b]UBE2Z and UBCH8 can also work with ubiquitin.

DOI: 10.1021/acs.chemrev.6b00737
Chem. Rev. 2018, 118, 889–918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E19
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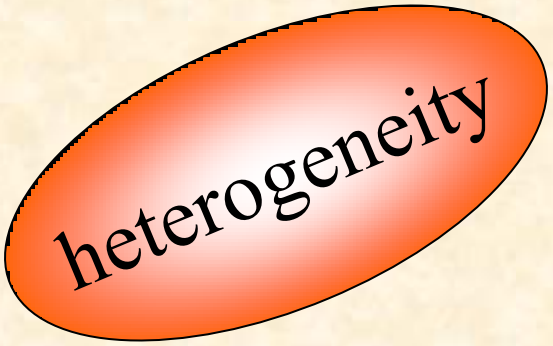
Glycosylation

one of the most common post-translational modifications of proteins in eukaryotic cells.

involved in a wide range of biological functions such as receptor binding, cell signaling, immune recognition, inflammation, and pathogenicity.

basic types of glycans:

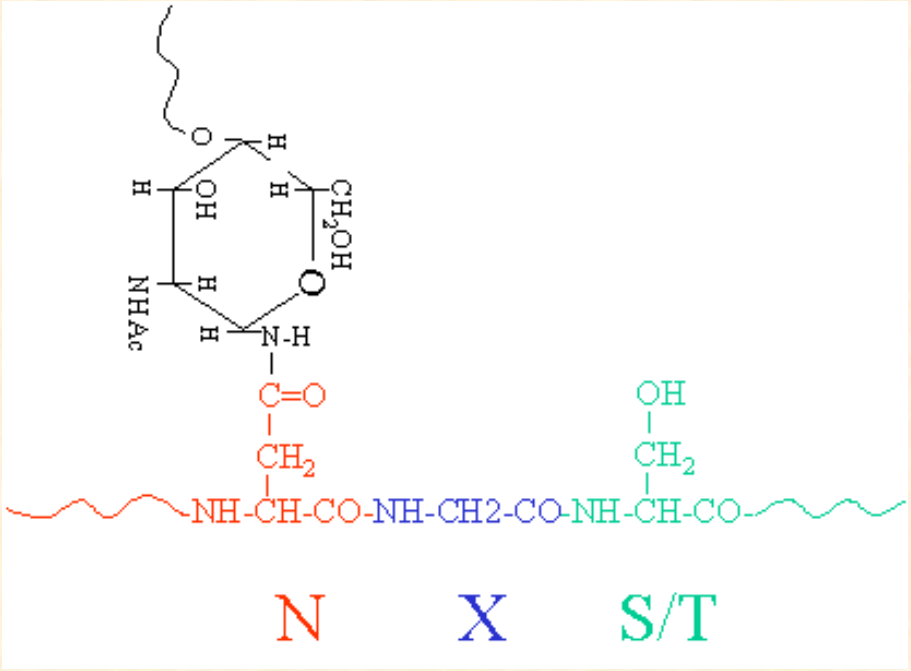


- **N-linked**
- **O-linked**
- **GPI anchors**
  - **C-linked**
  - **glykace**

Variation in the degrees of saturation at available glycosylation sites results
in heterogeneity in the mass and charge of glycoproteins

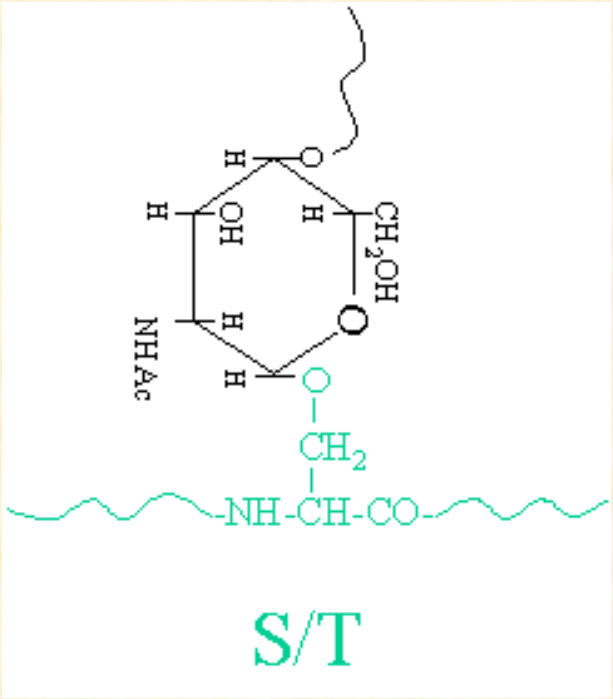
Signal Supression

http://www.uniprot.org/manual/carbohyd



N - linked

O - linked



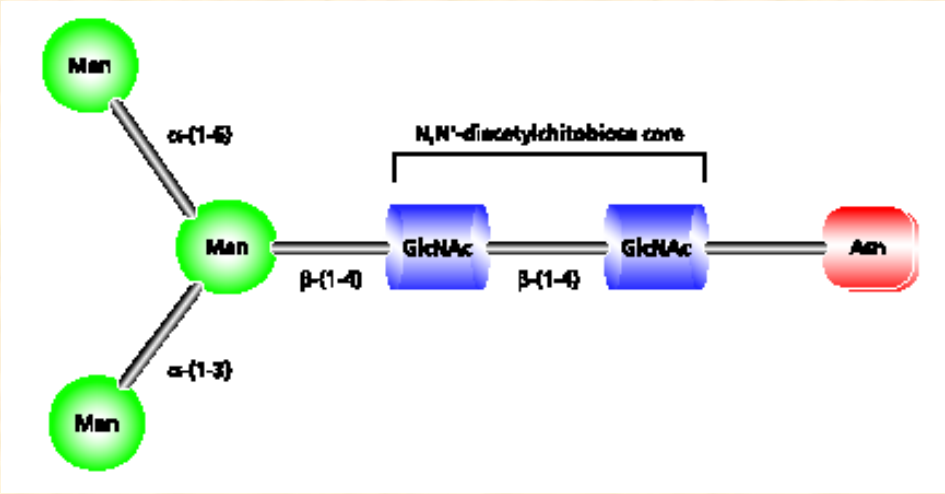
N-linked glycosylations

glycans are attached to the protein backbone via an amide bond to an asparagine *during protein synthesis*

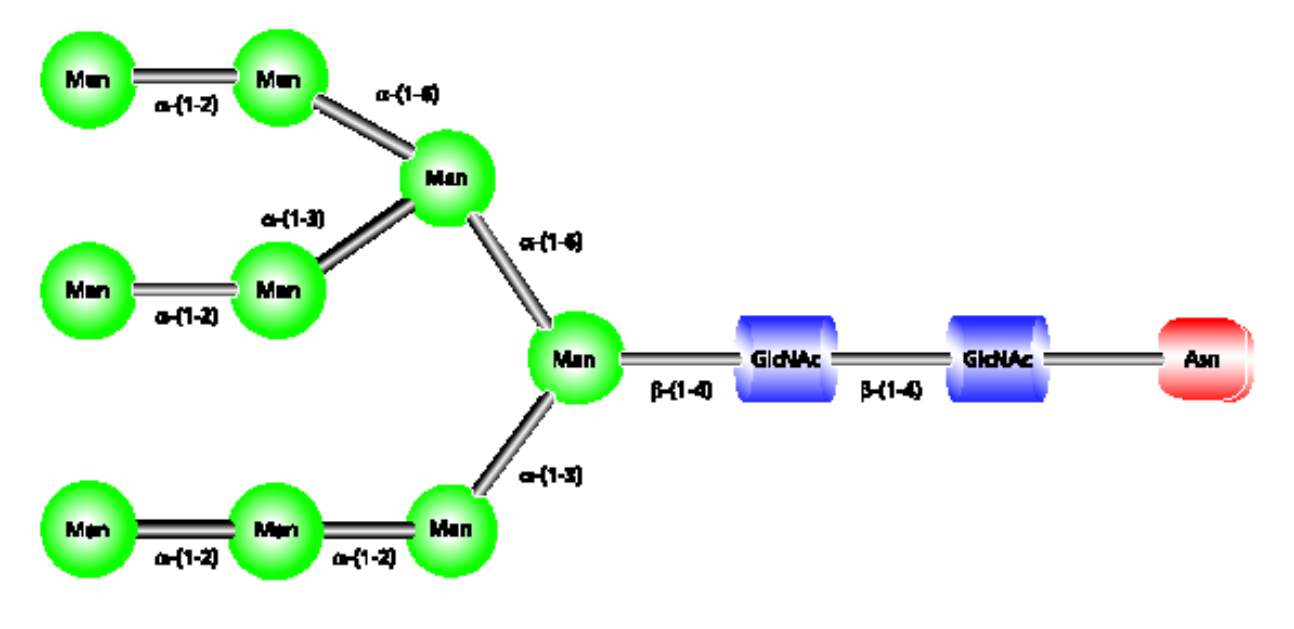
N-X-S(T) **X** not to stand for **P**

subtypes:

- **High-mannose**
- **Hybrid**
- **Complex**



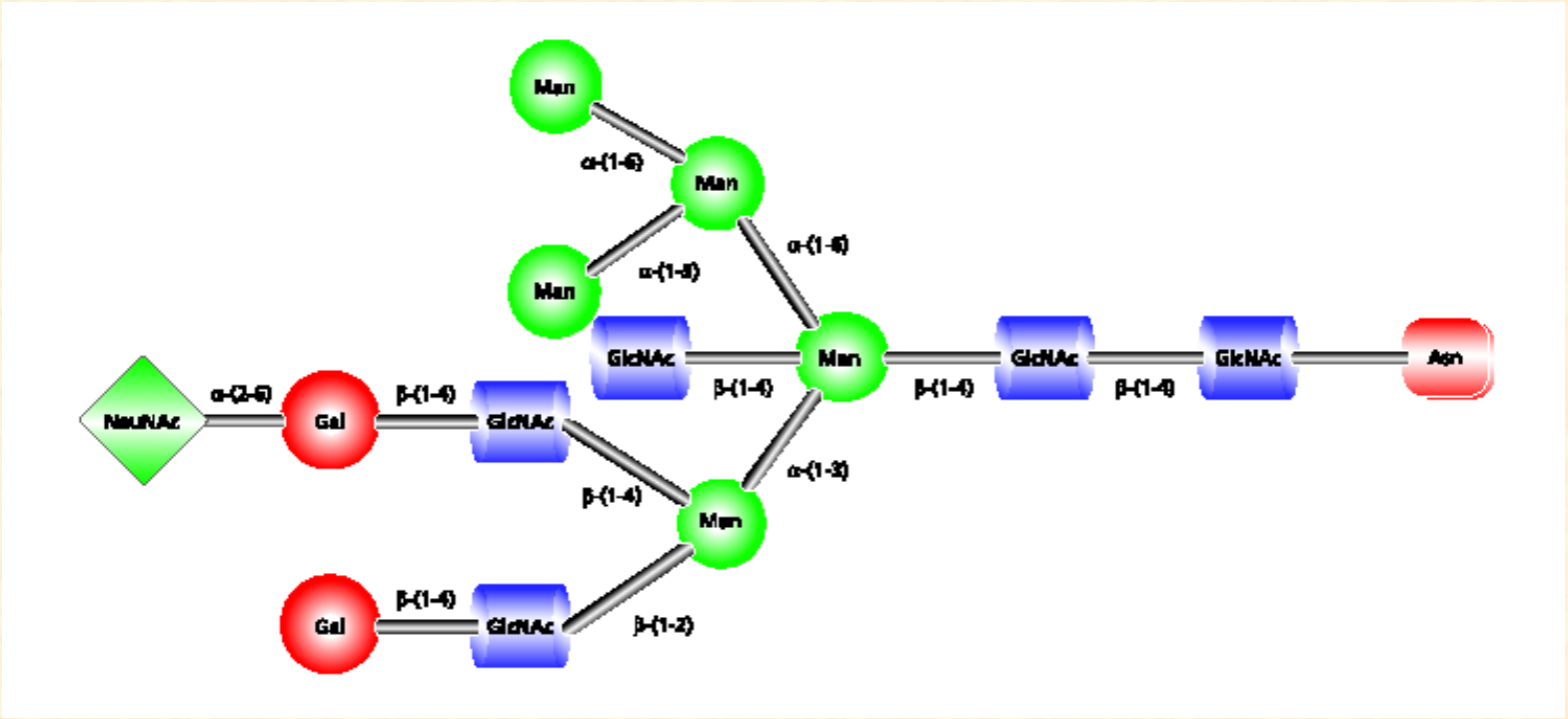
N-linked:High-mannose subtype



Man β-D-Mannose

GlcNAc β-D-*N*-Acetylglucosamine

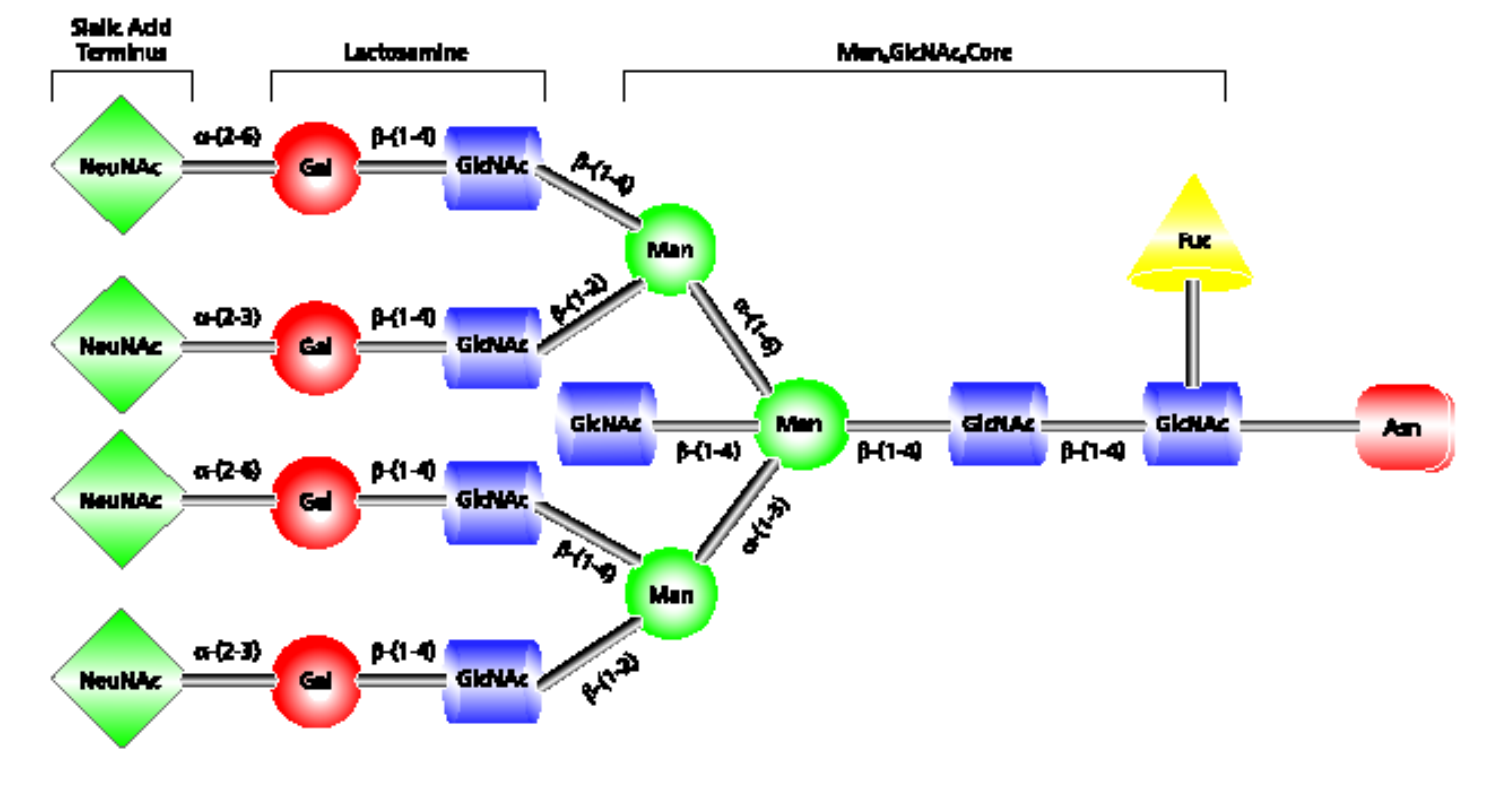
N-linked: Hybrid subtype



Gal β-D-Galactose

NeuNAc α-*N*-Acetylneuraminic acid (Sialic Acid)

N-linked: Complex subtype

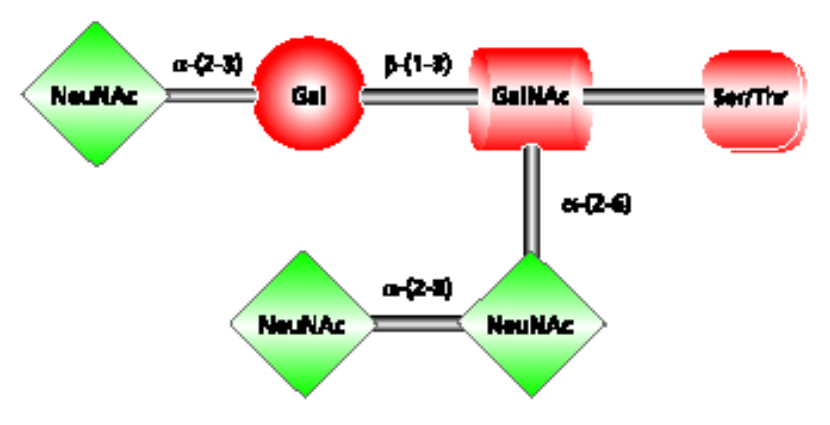


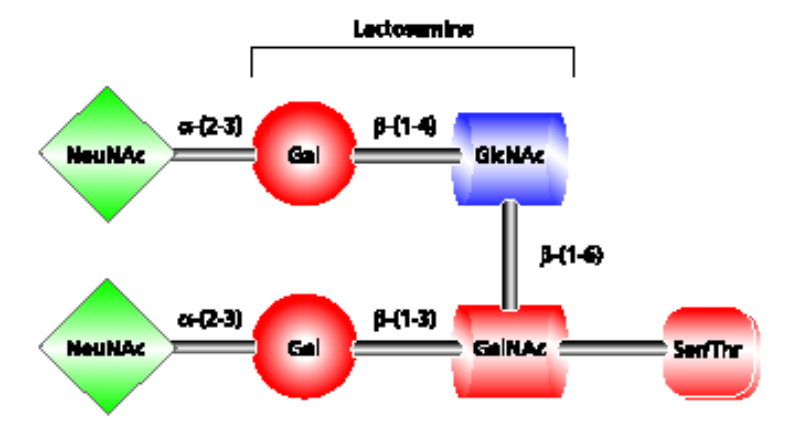
Fuc α-L-Fucose

O-linked glycosylations

glycans are linked via the hydroxyl group of serine or threonine

examples:

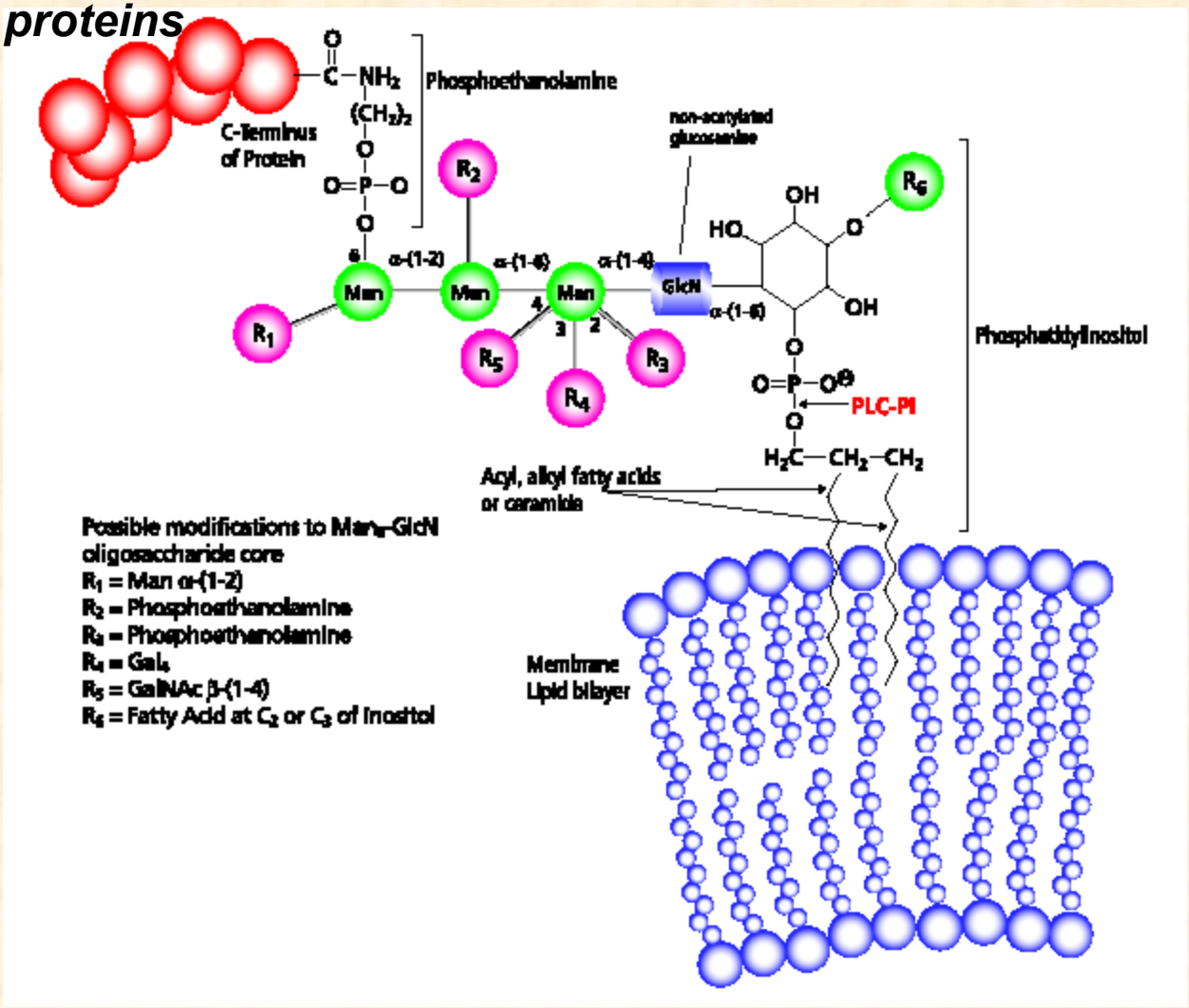




β-D-*N*-Acetylgalactosamine

GPI (glycosylphosphatidylinositol) anchors

anchors are linked via C-terminus, membrane bound proteins



Characterization of glycoproteins

- specific detection of glycosylated proteins
- protein identification
- site localization
- determination of glycan structure

Specific detection of glycosylated proteins

Pro-Q Emerald 300 - glyco only

Sypro Ruby - all

alternative detection techniques:

colorimetric detection

fluorescence detection

specific enrichment:

affinity chromatography

(lectins,

m-Aminophenylboronic Acid)

Deglycosylation

chemical:

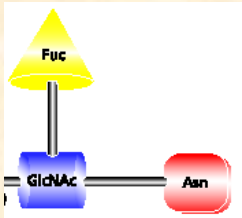
Hydrazinolysis

Hydrazine hydrolysis has been found to be effective in the complete release of unreduced O- and N-linked oligosaccharides.

Alkaline β-Elimination - only O-linked (some exceptions)

Trifluoromethanesulfonic Acid - glycan destruction

enzymatic:



PNGase F N-linked, all removed, if not

PNGase A N-linked, all removed, if not

Endoglycosidase H N-linked, cleaves after first

Endoglycosidase F1, F2, F3 N-linked, cleave specifically with respect to glycane structure

O-Glycosidase O-linked. all removed

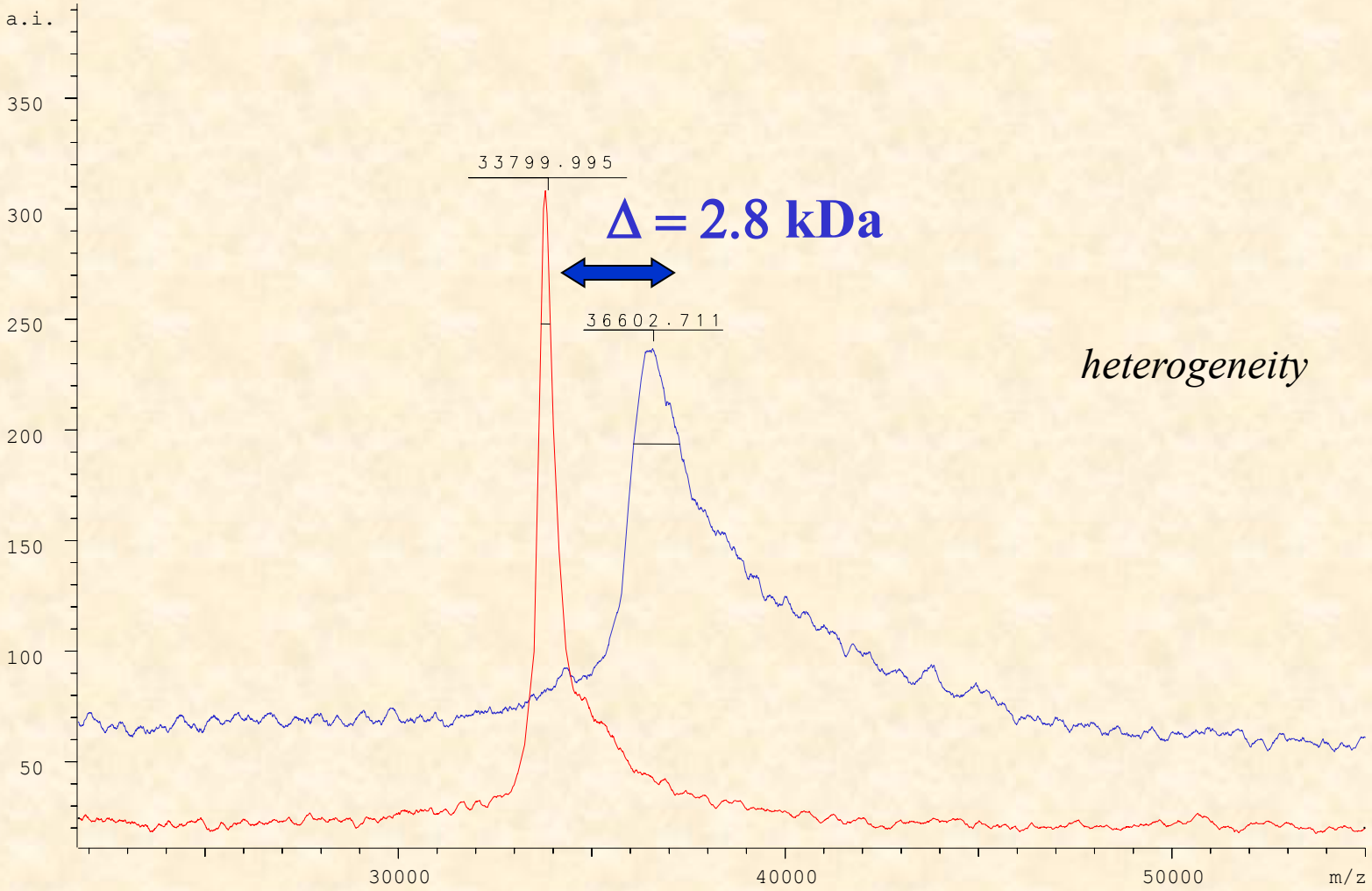
β-galactosidase cleaves before

...

Site assigment, glycan structure elucidation

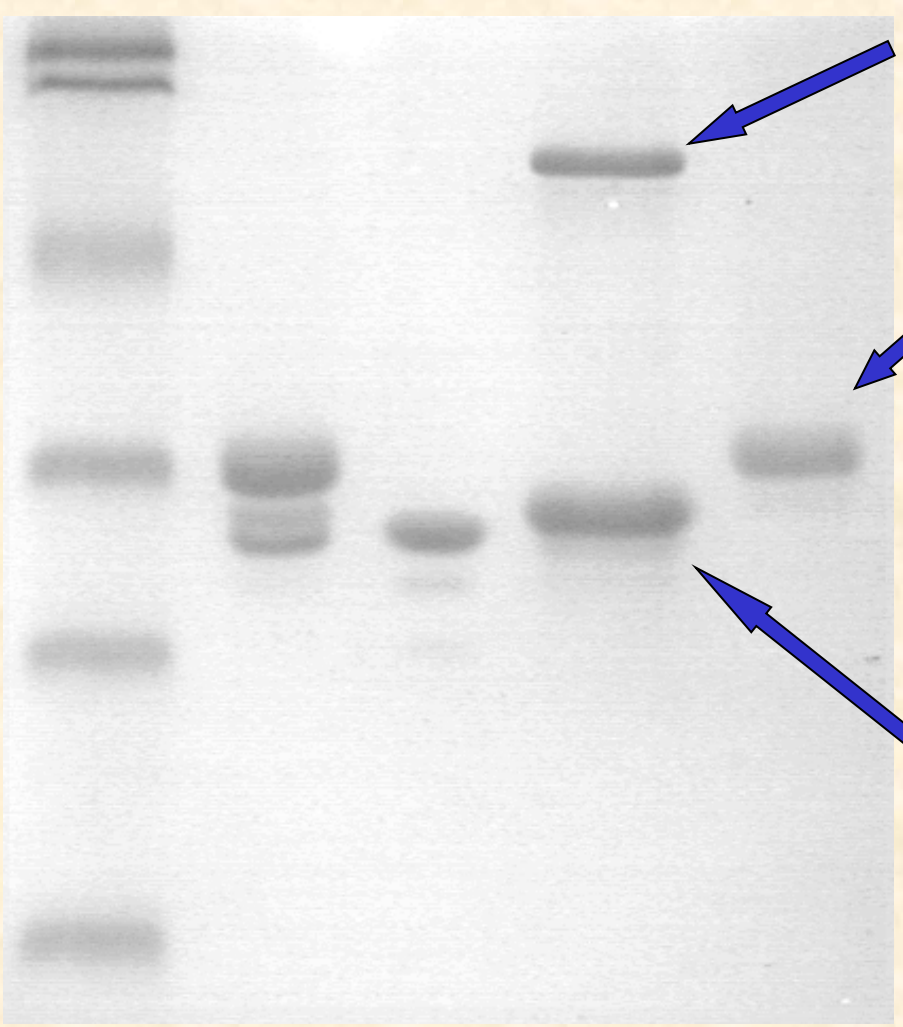
- **glycosylation „only“ at S or T** (O-linked)
  NXS(T) (N-linked)
  prediction of potential glyco site
- **combination of MS and MS/MS techniques**
- **separation of glycoproteins or glycopeptides**
- **deglycosylation strategies**
- **glycan derivatisation**

MALDI-MS spectrum of glycosylated and non-glycosylated protein
size of glycan part



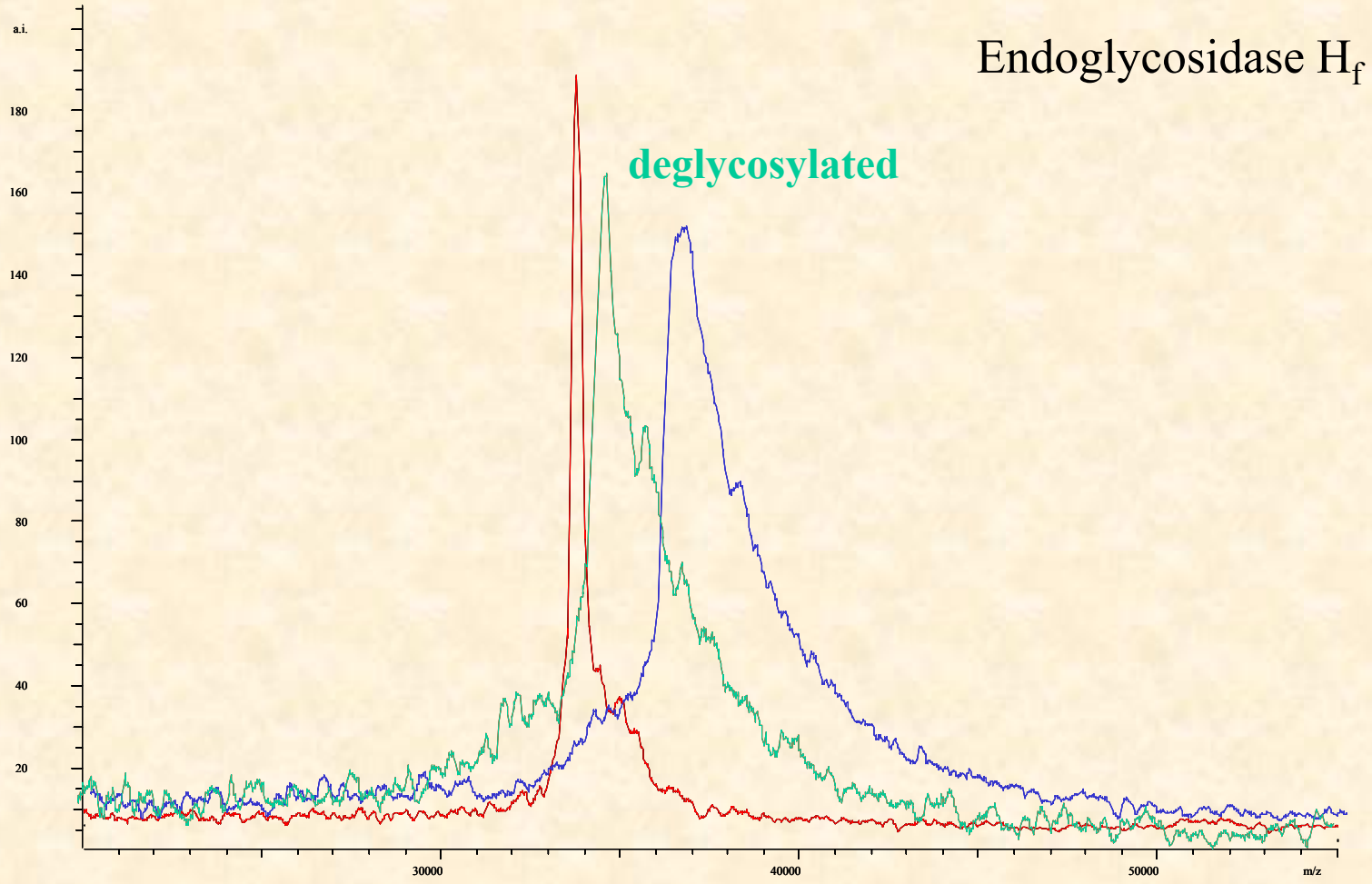
1D GE of protein before and after deglycosylation

confirmation of glycosylation



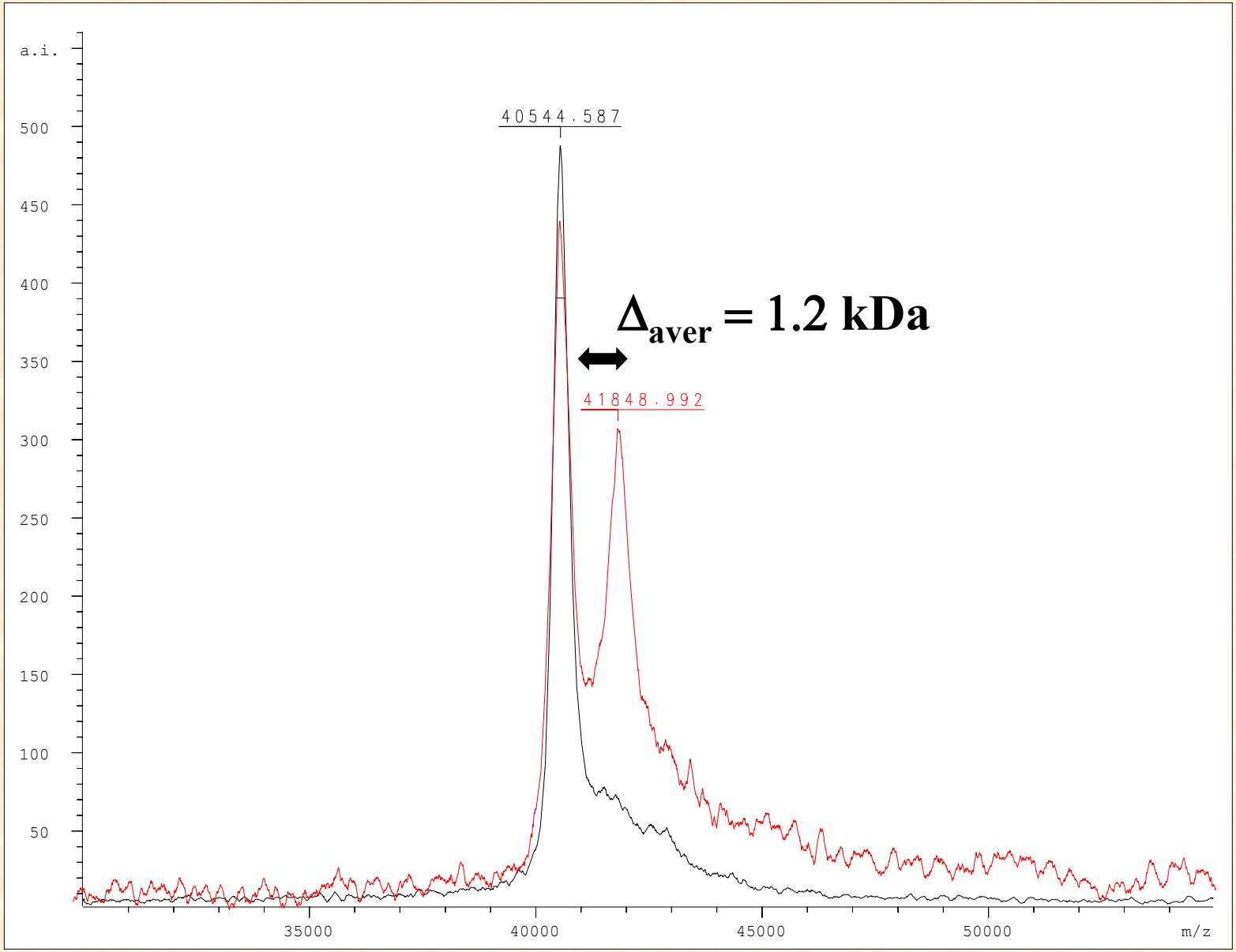
MALDI-MS spectrum of deglycosylated protein

confirmation of glycosylation



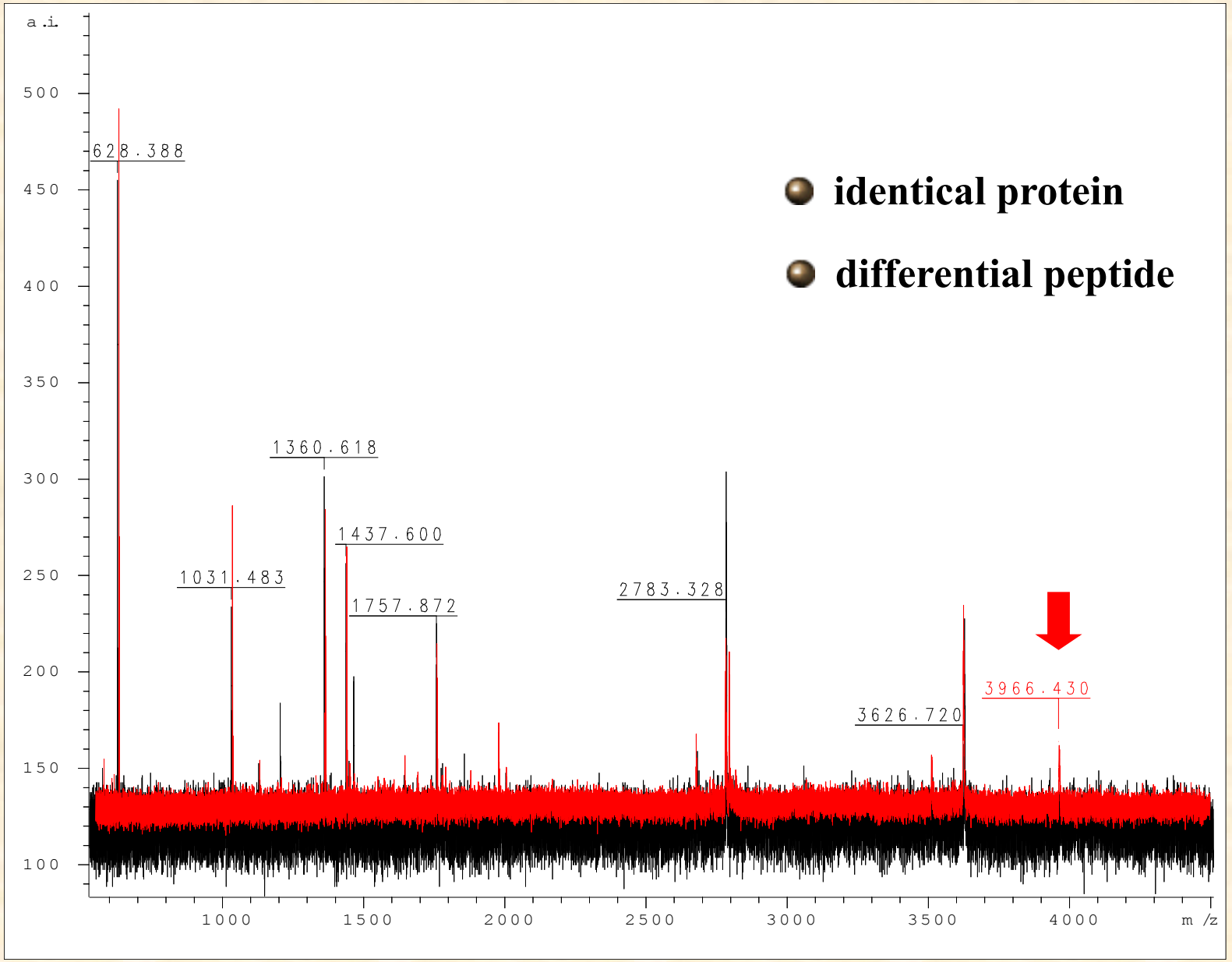
MALDI-MS of intact protein

C7250

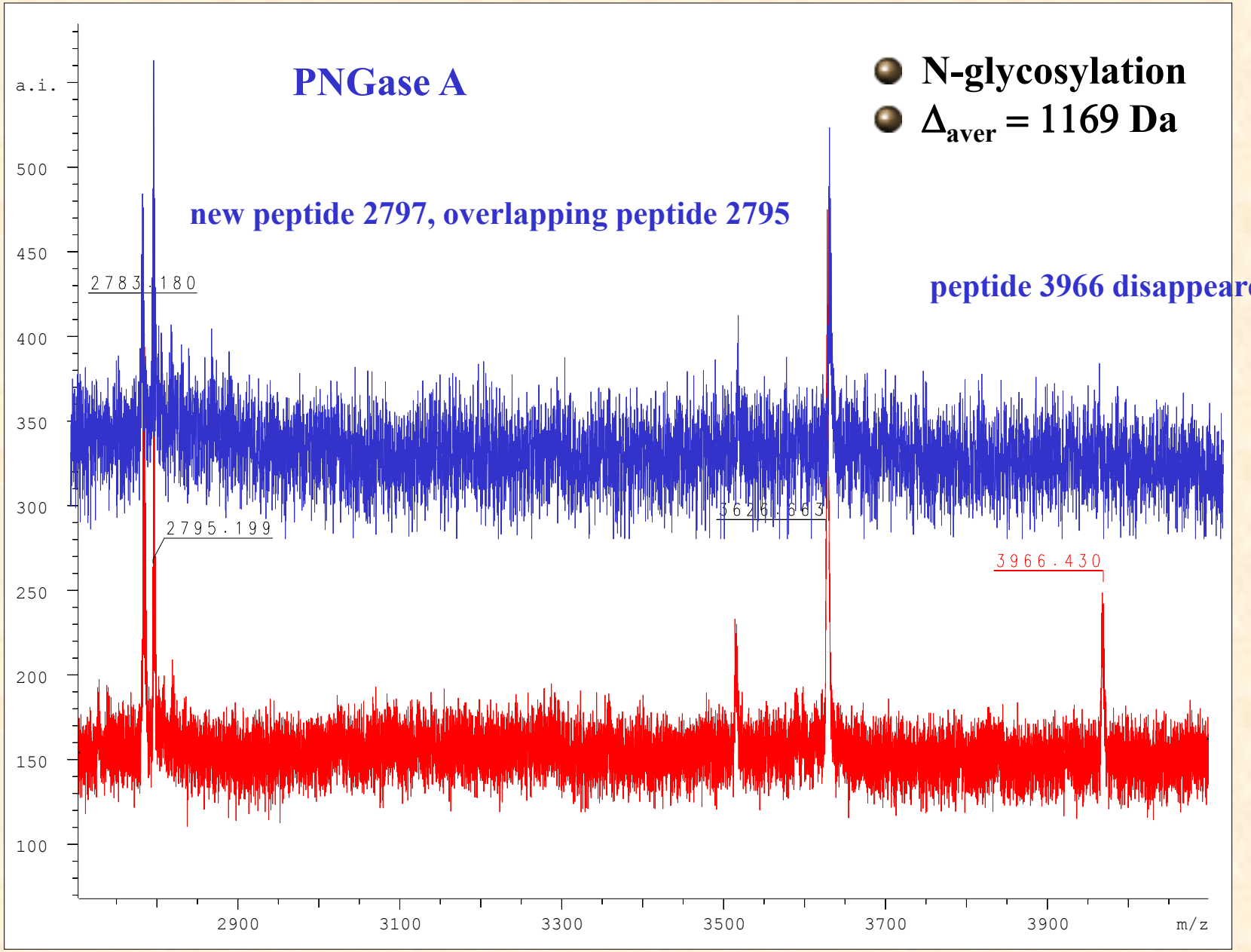


MALDI-MS of tryptic digests

C7250

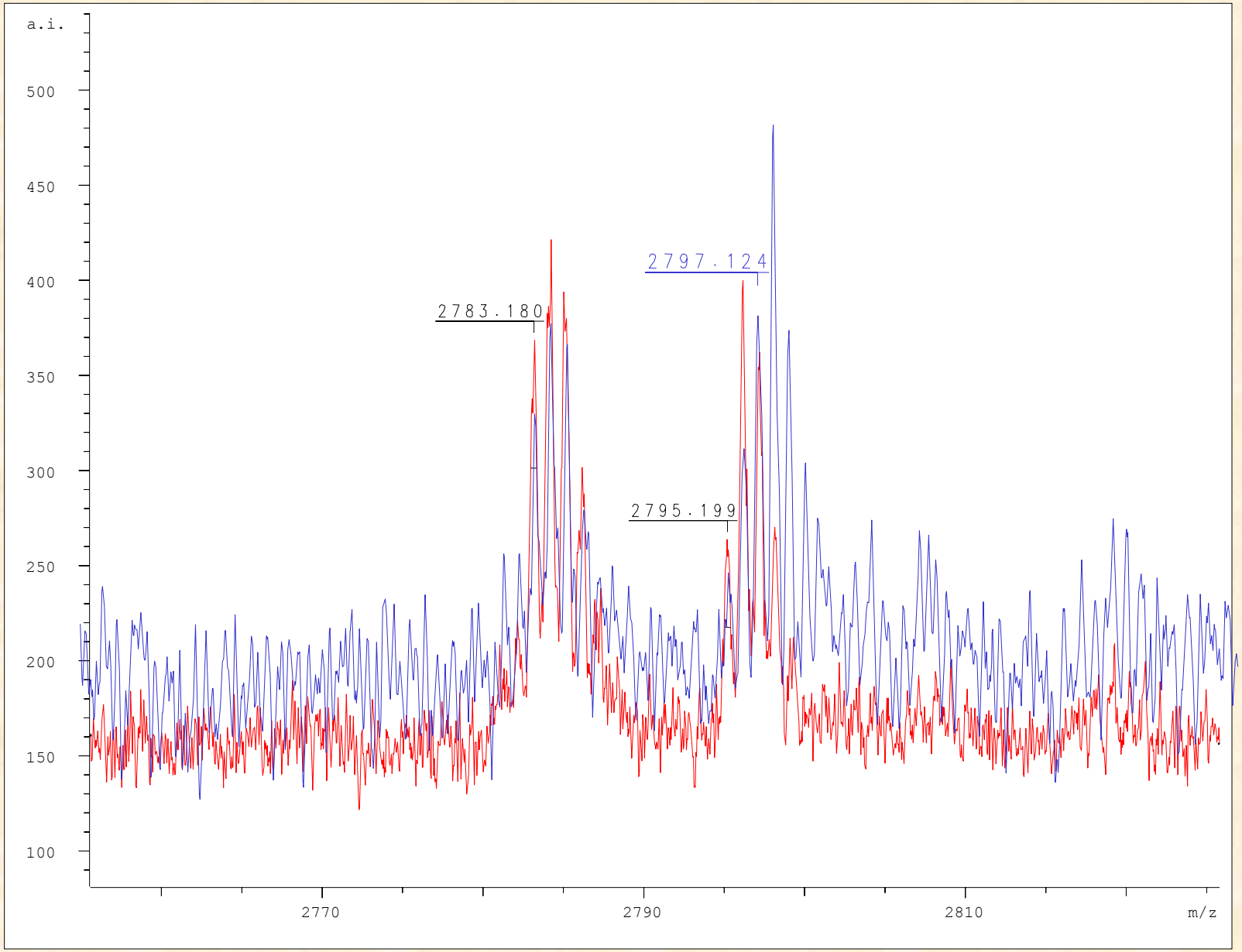


Detail of spectra of protein digest before and after deglycosylation



C7250

Detail of spectra of protein digest before and after deglycosylation



Summary

tryptic peptide - 2796 Da …PHIF**D**YSGS… ,
N is transformed to D during deglycosylation by PNGase A

original sequence is …PHIF**N**YSGS… (mass 2795 Da)

Peptide was also confirmed by LC-MS/MS analysis (original one was not found in sample before deglycosylation)

Glycan mass 1170 Da corresponds to already reported glycan
xylose+fucose+3*mannose+2*N-acetylglukosamin

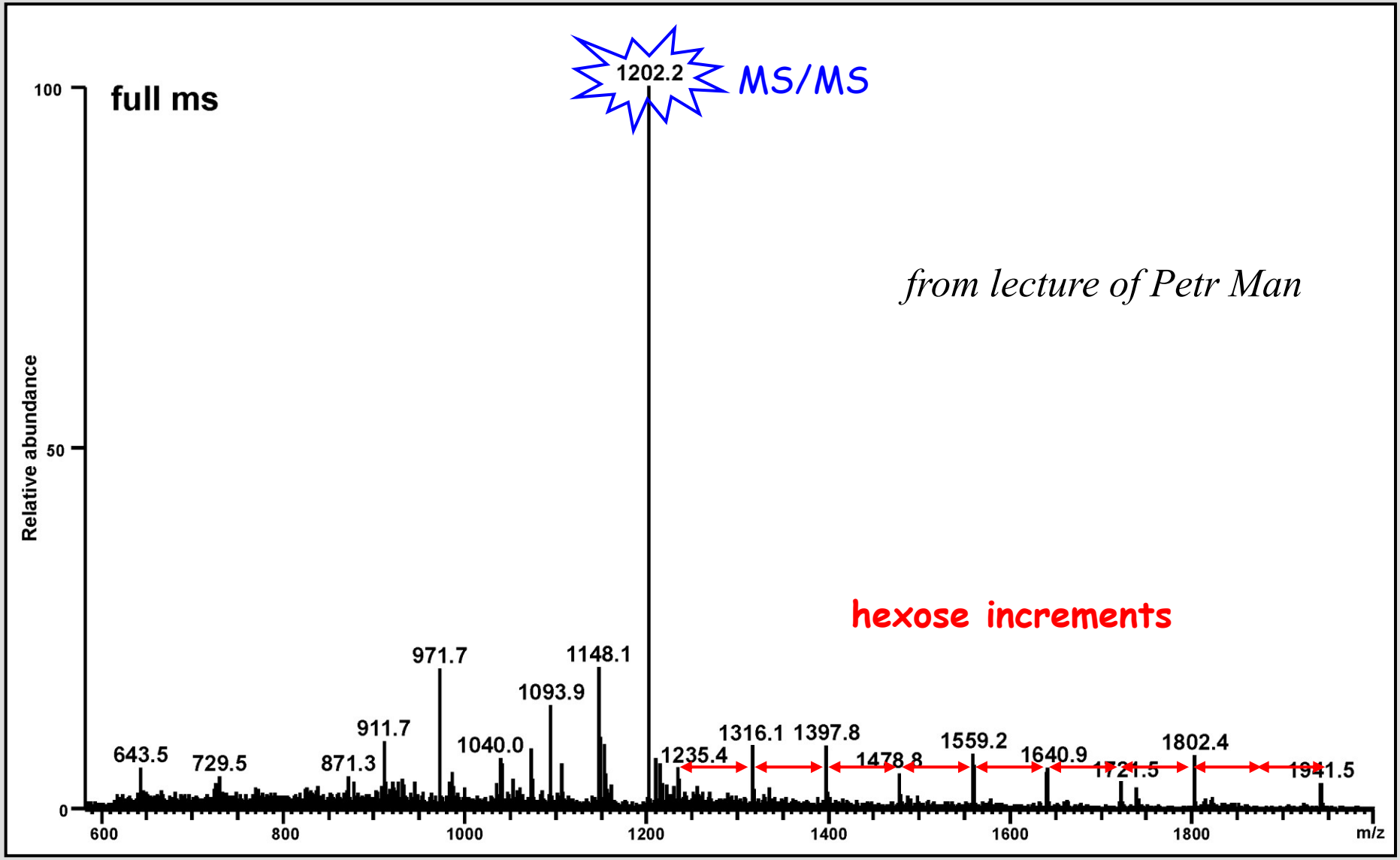
So glycan structure was not confirmed by MS/MS.

…missing parts have potential N-glycosylation sites...

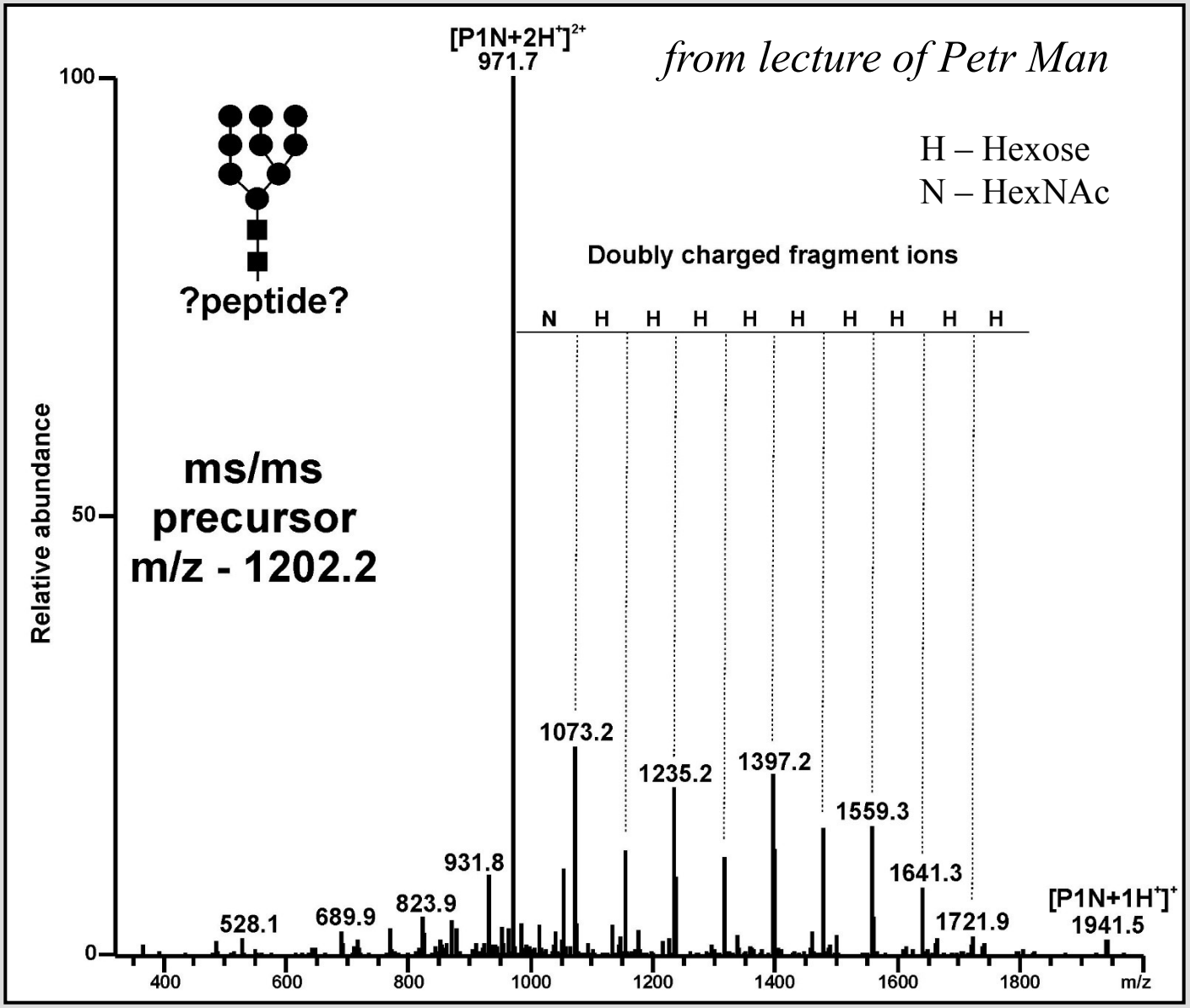
MLRNVCPVLILLIIGATAQDPTDVGEAFANVEWSVAELKRV
LVMGVPRDCGELFLSGQNHSGVYNIYPYKDSLLPVS
AYCDMETDGGGWTVFQRRGQFGNPVYYFYKKWA
DYAHGFGDPAKEYWLGNNVLHALTSDKAMSLRIE
KNHSLETLTAEYSVFKVASEEEYFKINVGGYIGSK
GSDAFSIANGSMFTASDQDHDTYTNNCAVEFKG
AWYTSCHGSNLNGLNLNGEHPSYADGIEWSAR
GGSTGLYYYSYPNVEMKVRDAHFISRVADGRAS

from lecture of Petr Man

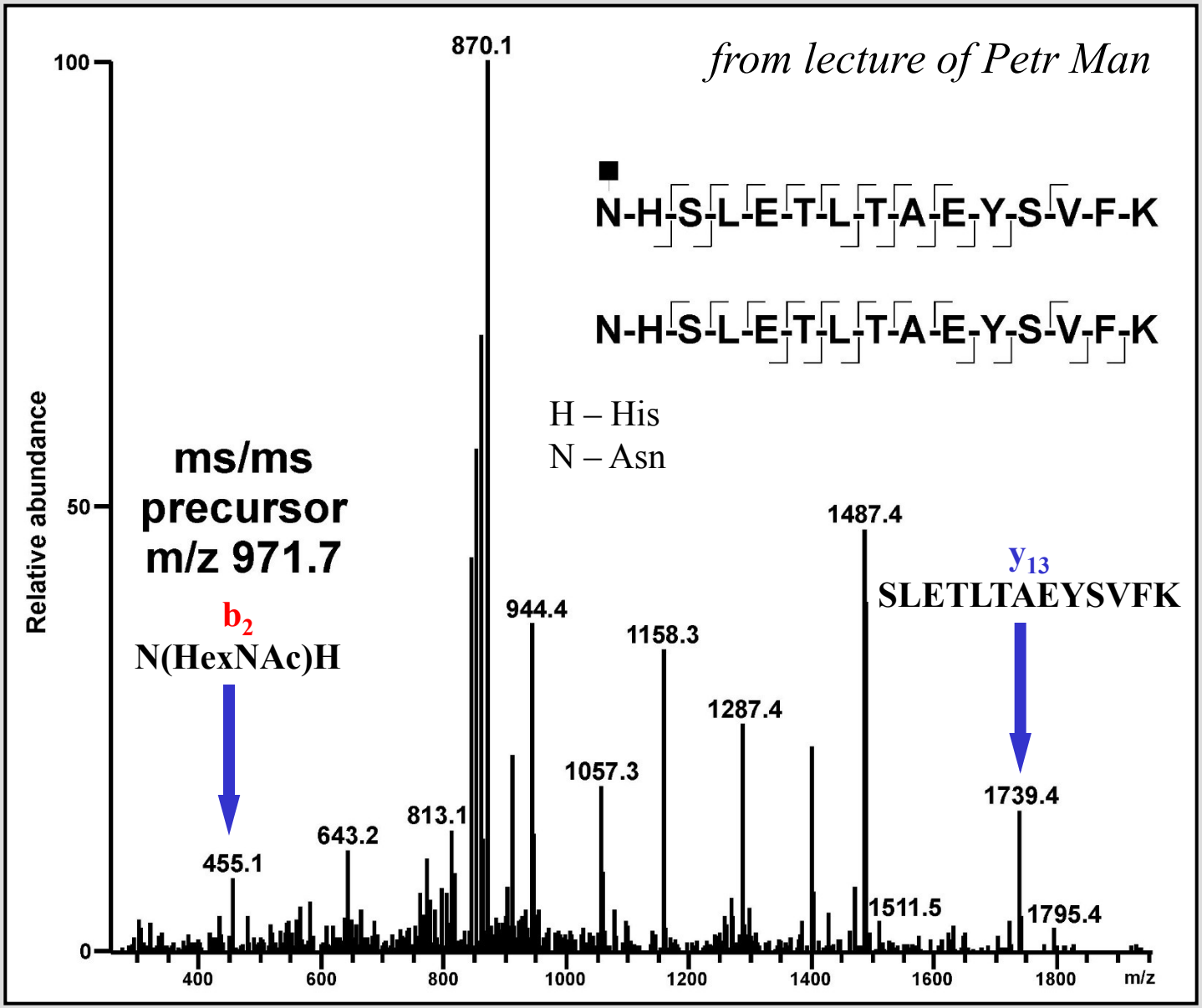
... glycopeptide...



MS/MS from 1202.2 - – glycopeptide, type of glycan identified



MS/MS from 971.7 – peptide with one HexNAc – site of glycosylation identified



MLRNVCPVLILLIIGATAQDPTDVGEAFANVEWSVAELKRV
LVMGVPRDCGELFLSGQNHSGVYNIYPYKDSLLPVS
AYCDMETDGGGWTVFQRRGQFGNPVYYFYKKWA
DYAHGFGDPAKEYWLGNNVLHALTSDKAMSLRIE
KNHSLETLTAEYSVFKVASEEEYFKINVGGYIGSK
GSDAFSIANGSMFTASDQDHDTYTNNCAVEFKG
AWYTSCHGSNLNGLNLNGEHPSYADGIEWSAR
GGSTGLYYYSYPNVEMKVRDAHFISRVADGRAS

from lecture of Petr Man

MALDI-MS spectrum of ribonuclease B

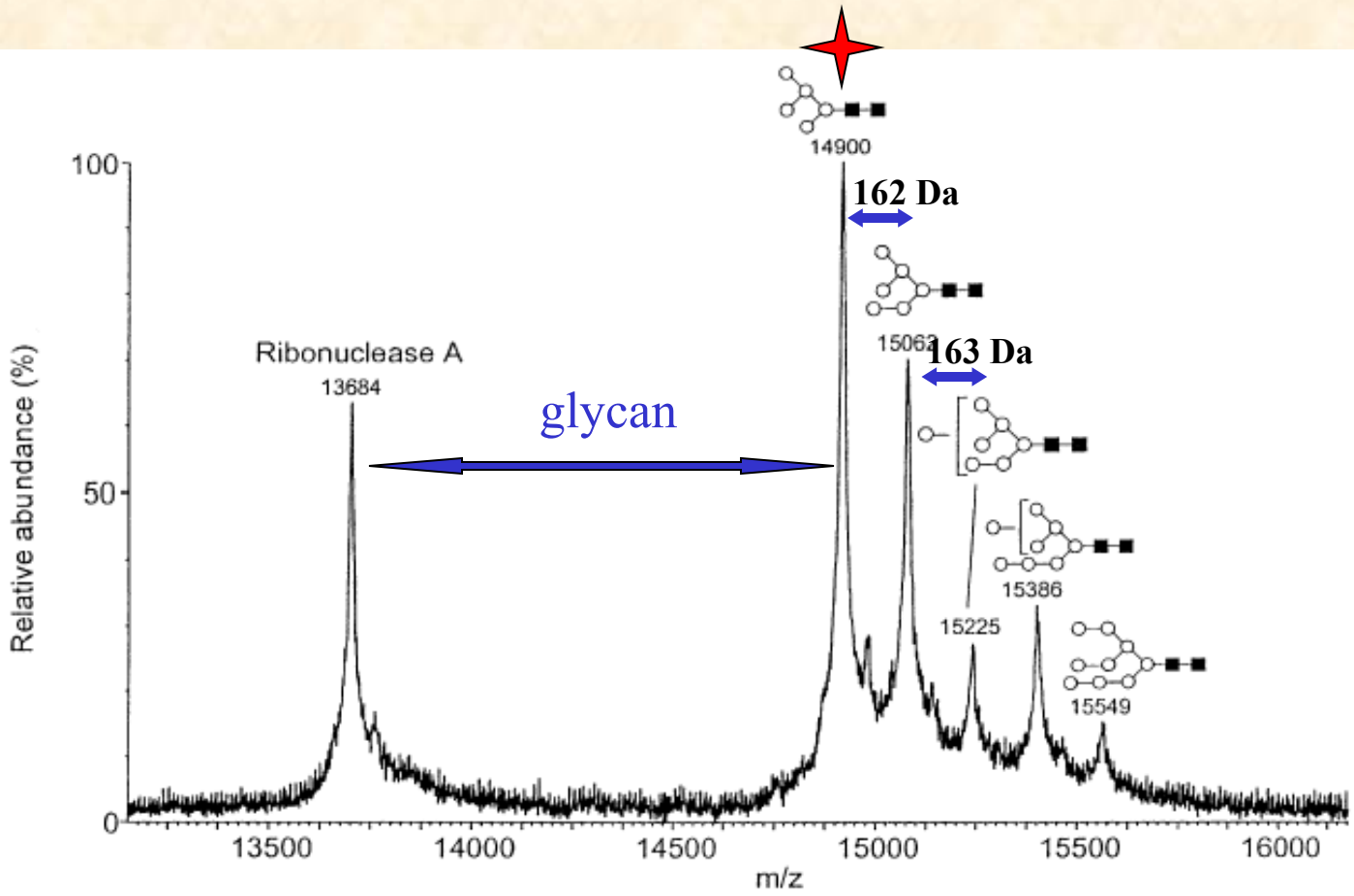


Fig. 2. Positive ion linear MALDI mass spectrum of a mixture of ribonuclease A (unglycosylated) and B (glycosylated) recorded from 4-HCCA with a Micromass TofSpec 2E mass spectrometer. The structures of the five high-mannose glycans attached to the protein to form ribonuclease B are shown. Key to symbols: (■) GlcNAc, (○) mannose.

D.J. Harvey / International Journal of Mass Spectrometry 226 (2003) 1–35

MSn of ribonuclease B glycans (AP-MALDI-IT MS) C7250

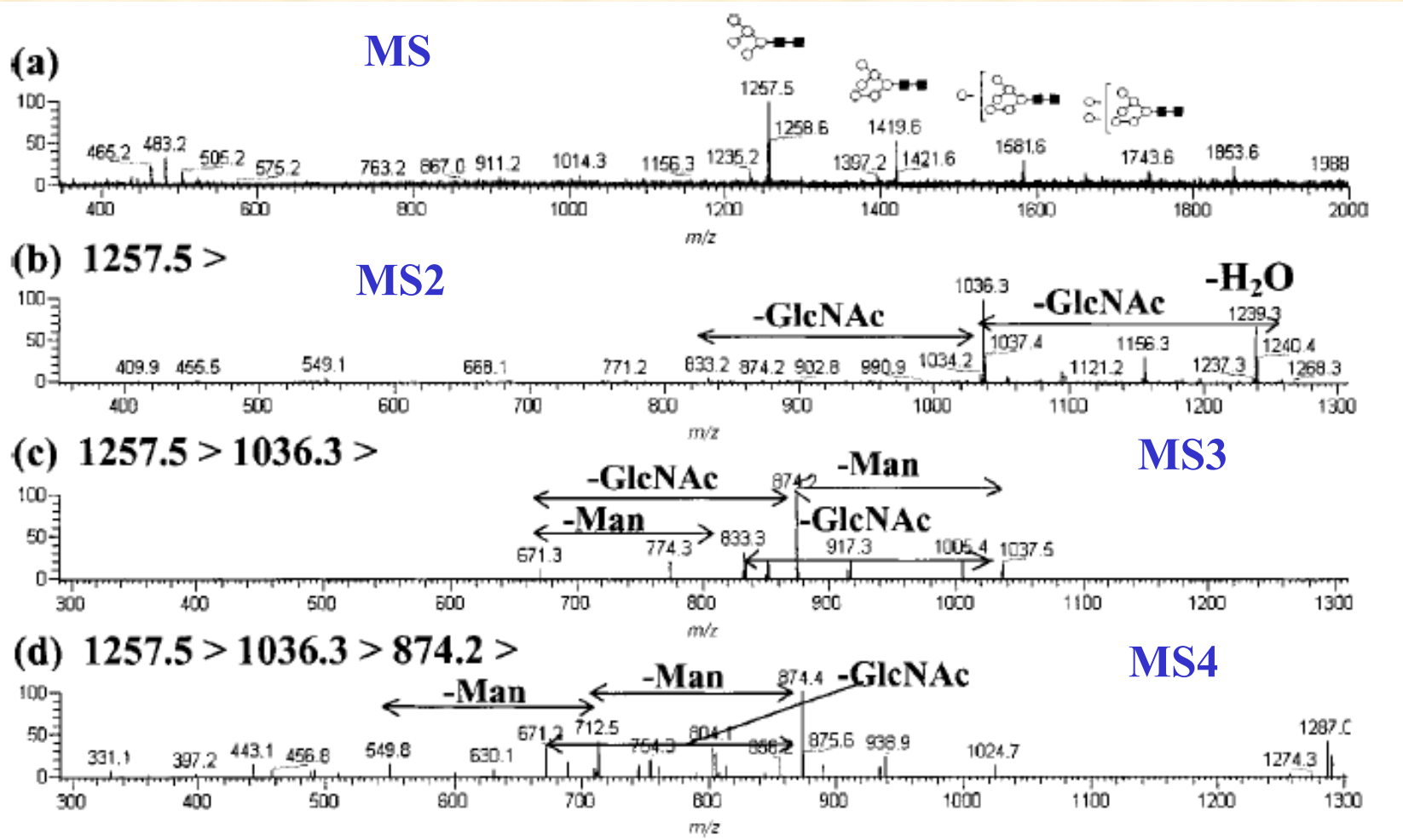
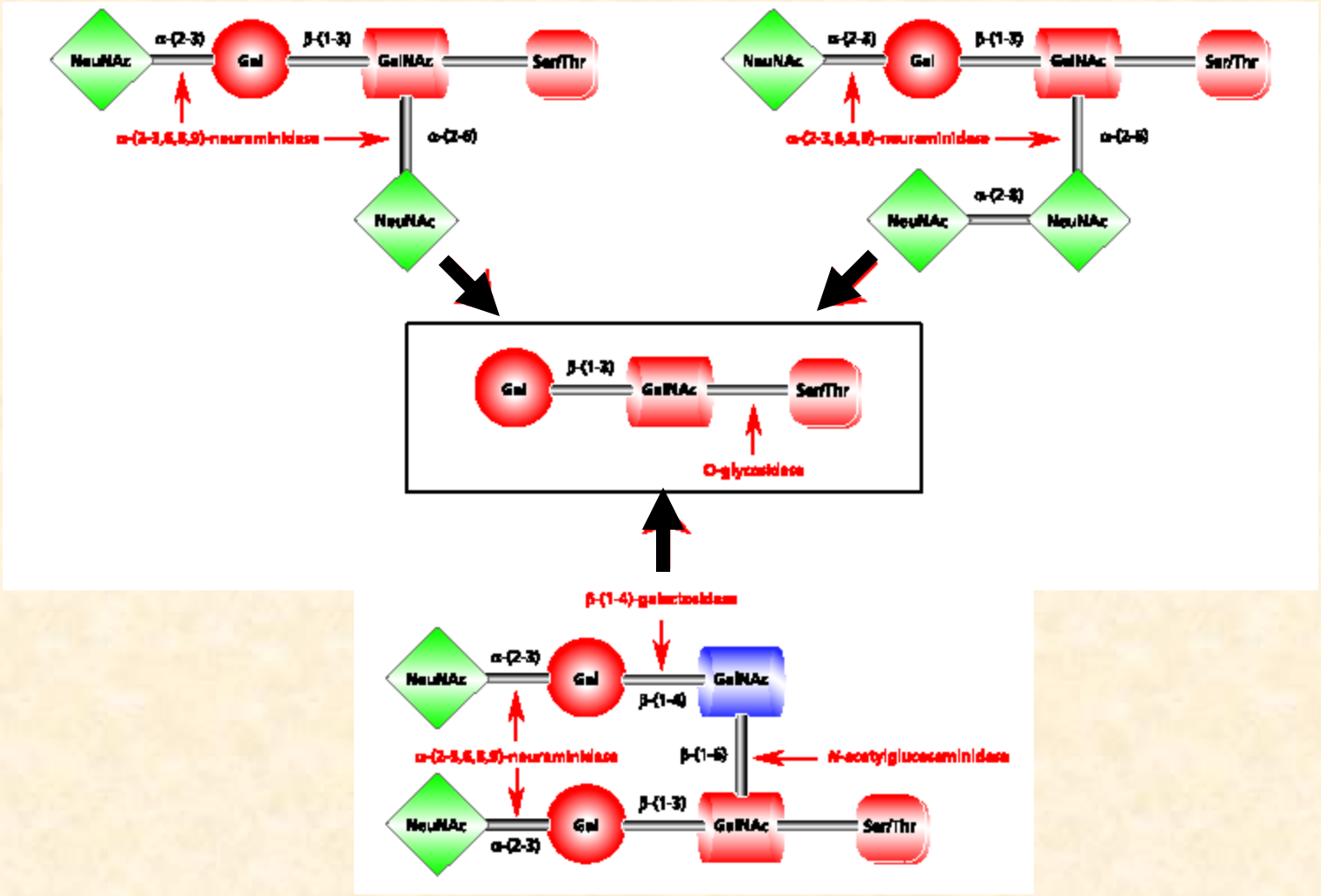


Figure 6. MS^4 analysis of ribonuclease-B glycans from a HABA matrix. (a) MS spectrum of underivatised glycan solution (500 pmol); (b) MS/MS spectrum of $(Man)_5(GlcNAc)_2$ (*m/z* 1257.5); (c) MS^3 spectrum of $(Man)_5GlcNAc$ (*m/z* 1036.3); and (d) MS^4 spectrum of $(Man)_4GlcNAc$ (*m/z* 874.2).

C. S. Creaser, J. C. Reynolds and D. J. Harvey *Rapid Commun. Mass Spectrom.* 2002; **16**: 176–184

Combination of deglycosylation enzymes



Sequential deglycosylation by different enzymes (MALDI-MS)

C7250

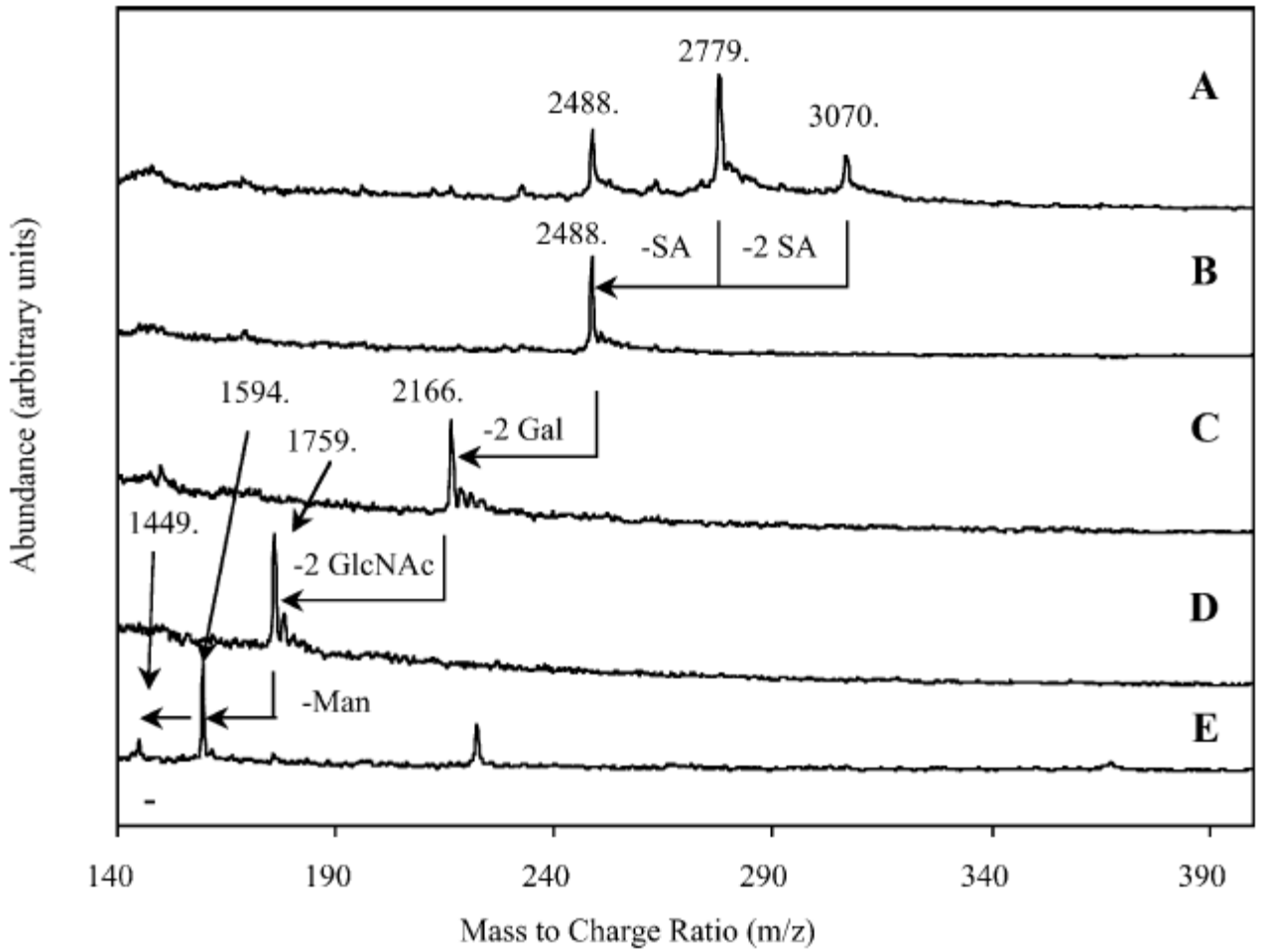


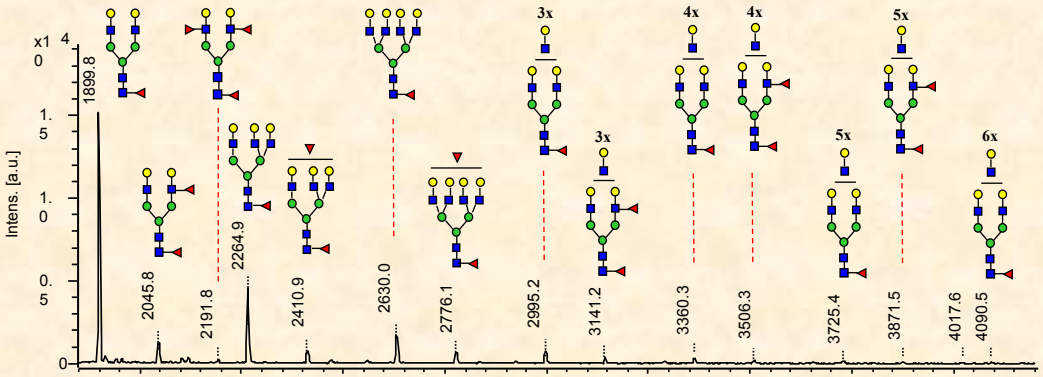
Fig. 2. MALDI-MS analysis of fraction T9 (A), after the digestion of fraction T9 with sialidase S (B), followed by β1-4 galactosidase (C) and then by β1-2-*N*-acetylglucosaminidase digestion (D). MALDI-MS analysis of fraction T9 after treatment with α1-6-fucosidase and α-mannosidase (E).

L.P. Kotra et al. / Bioorganic Chemistry 30 (2002) 356–370

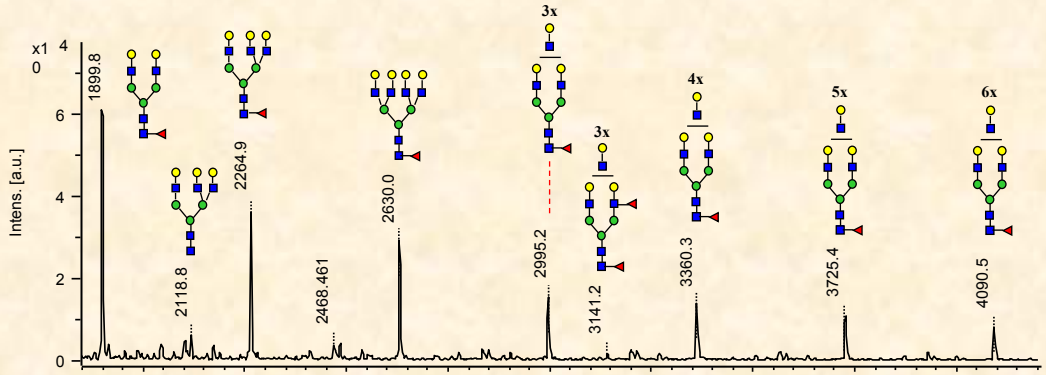
Glycan profiling and structural analysis of glycans

C7250

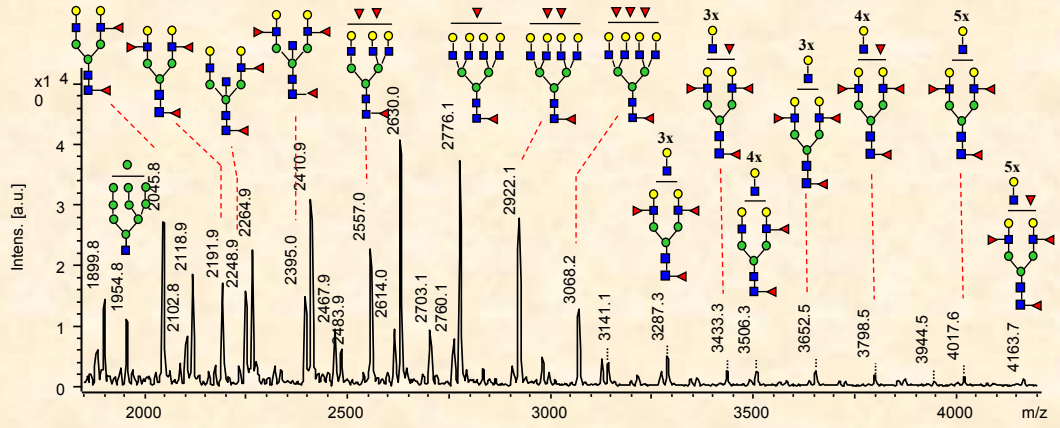
*Lattová E. et al., J. Proteome Res., **15** (8), 2777-2786 (2016)*



NSCLC - Bronchoalveolar Carcinoma



Bronchoalveolar Adenocarcinoma



Large Cell Carcinoma

MALDI-TOF-MS spectra of N-glycans after desialylation

Man; Gal; GlcNAc; Fuc

And this is the end