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Current state and perspectives of proteomics– are we heading in the right direction?

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Why proteomics?

- *understand biological processes?*
- *understand the mechanism behind diseases?*
- *discover biomarkers?*
- *challenge the technology?*

Mass spectrometry essential in proteomics

Two decades ago almost no instruments available

Now

Protein identification and sequencing

- *MALDI TOF and TOF/TOF's*
- *Iontraps*
- *Q-TOF's*
- *Orbitraps*

Single Reaction Monitoring / Multiple Reaction Monitoring

- *Triple quadrupoles or TOF's*

Proteomics levels

Expression proteomics

Which gene products are expressed, when and how much

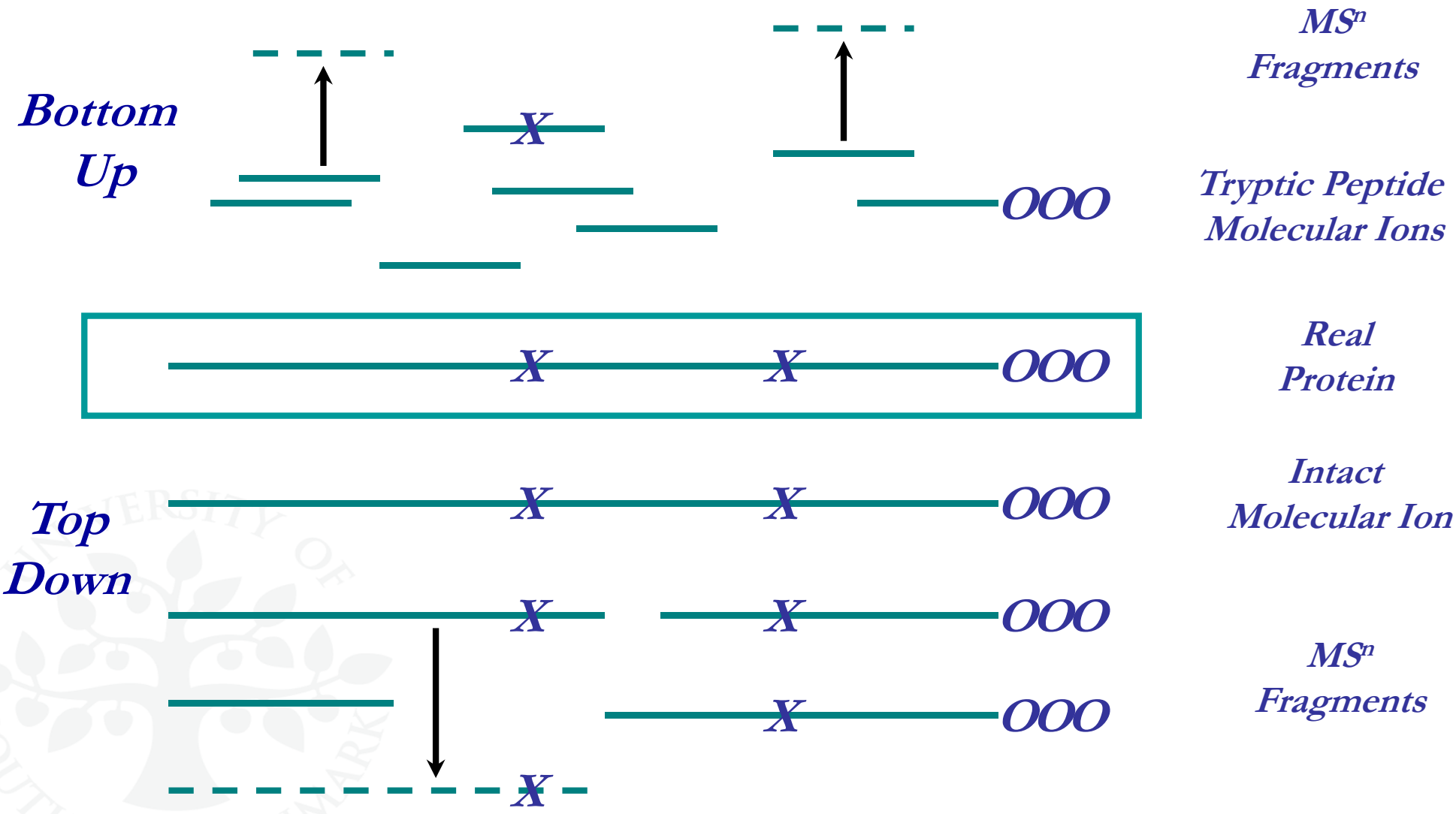
Modification specific proteomics ”modificomics”

Which variants are present of each protein, when and how much?

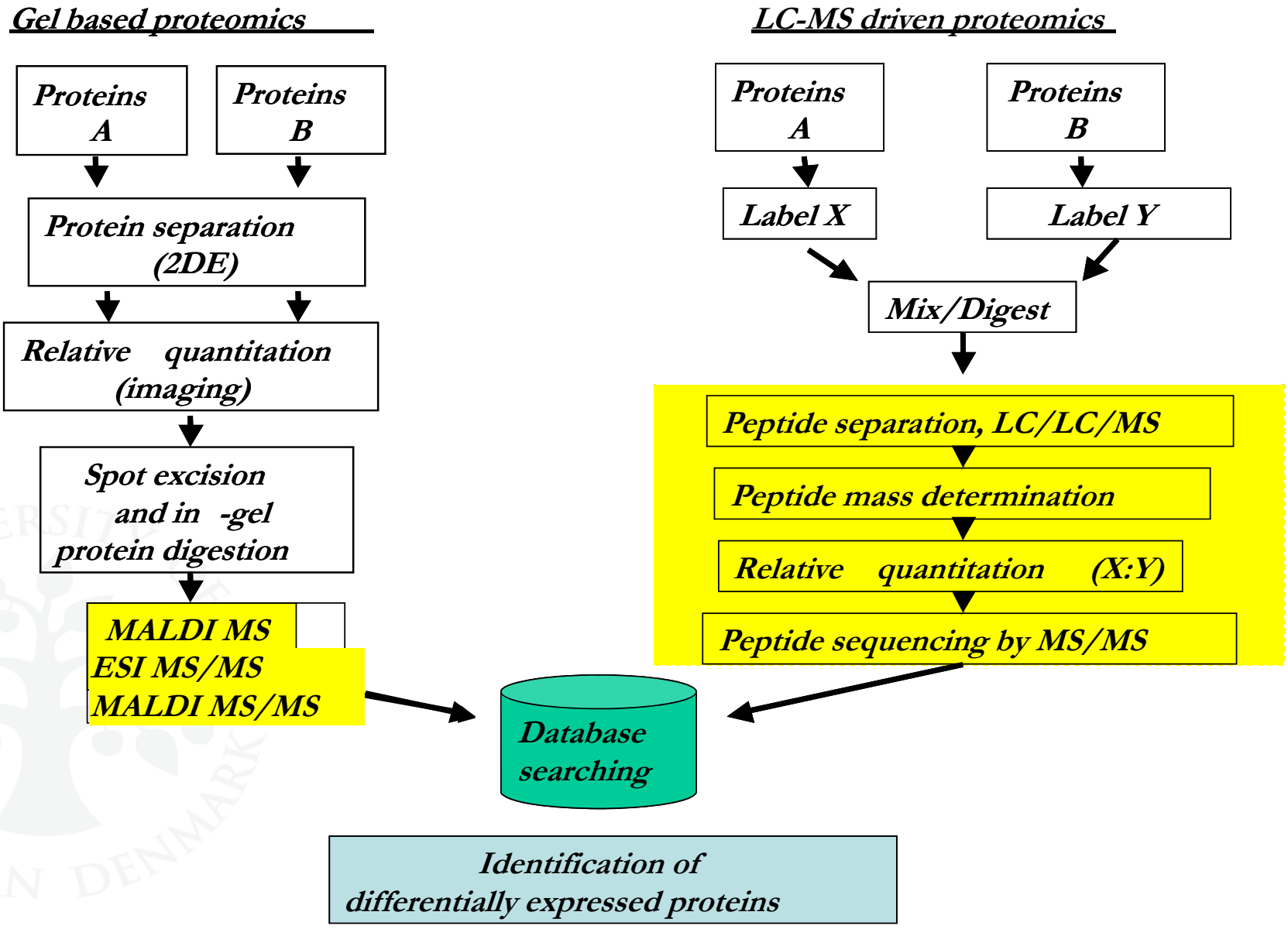
Interactomics, Cell map proteomics

Who interacts, when and where

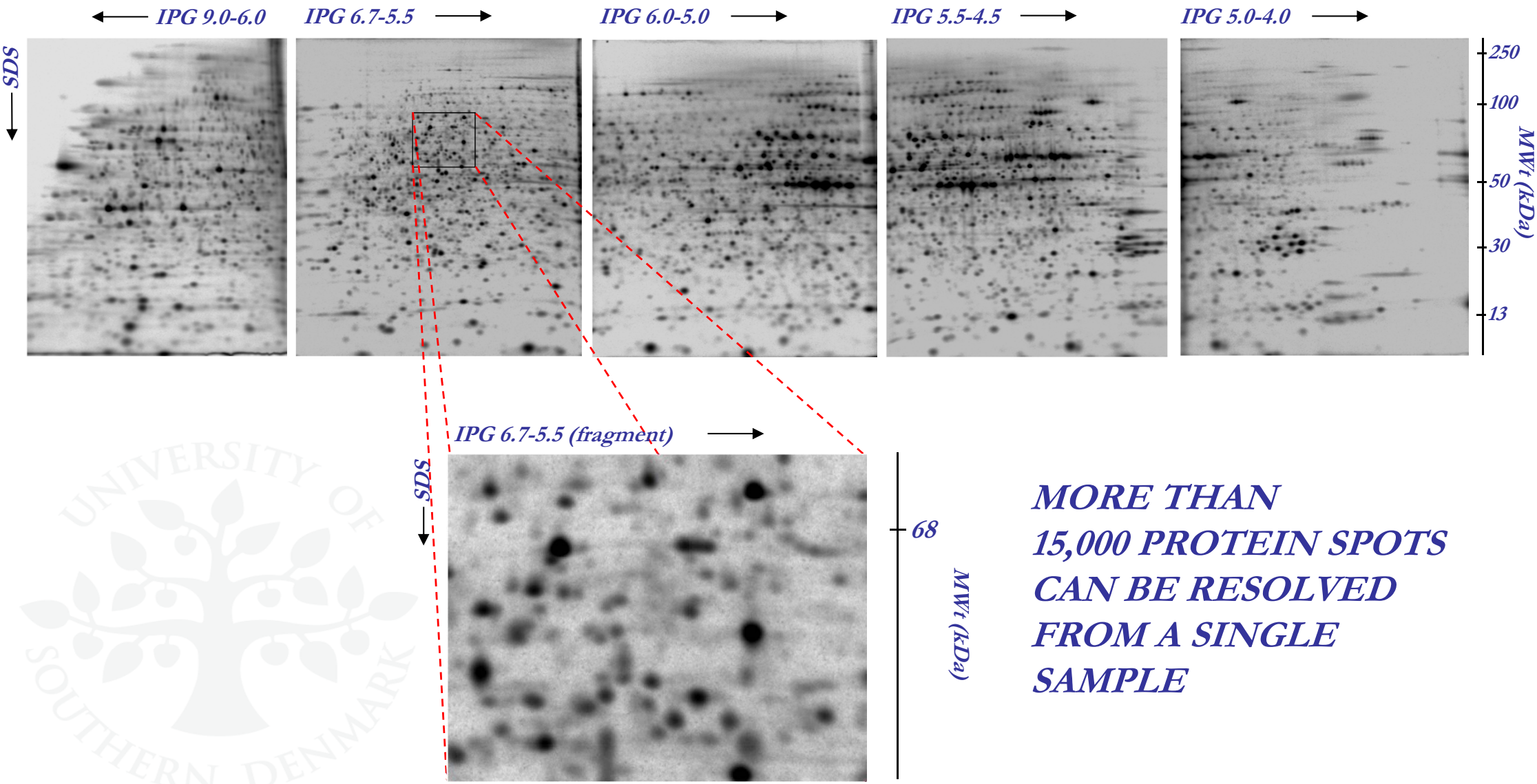
Top-Down vs Bottom-Up



Strategies for expression proteomics

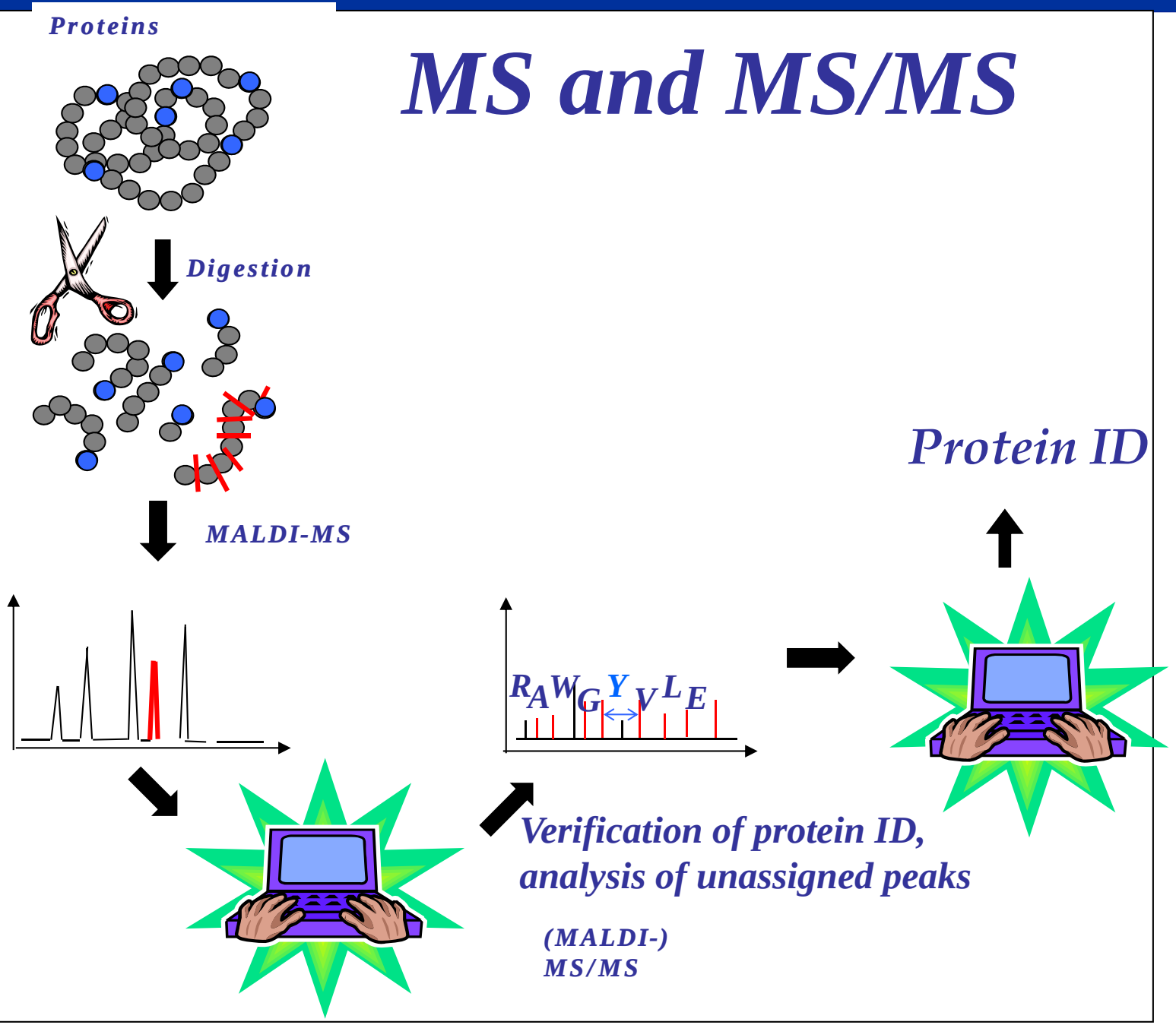


DATABASE OF [^{35}S]-METHIONINE LABELLED HeLa CELLS



MORE THAN 15,000 PROTEIN SPOTS CAN BE RESOLVED FROM A SINGLE SAMPLE

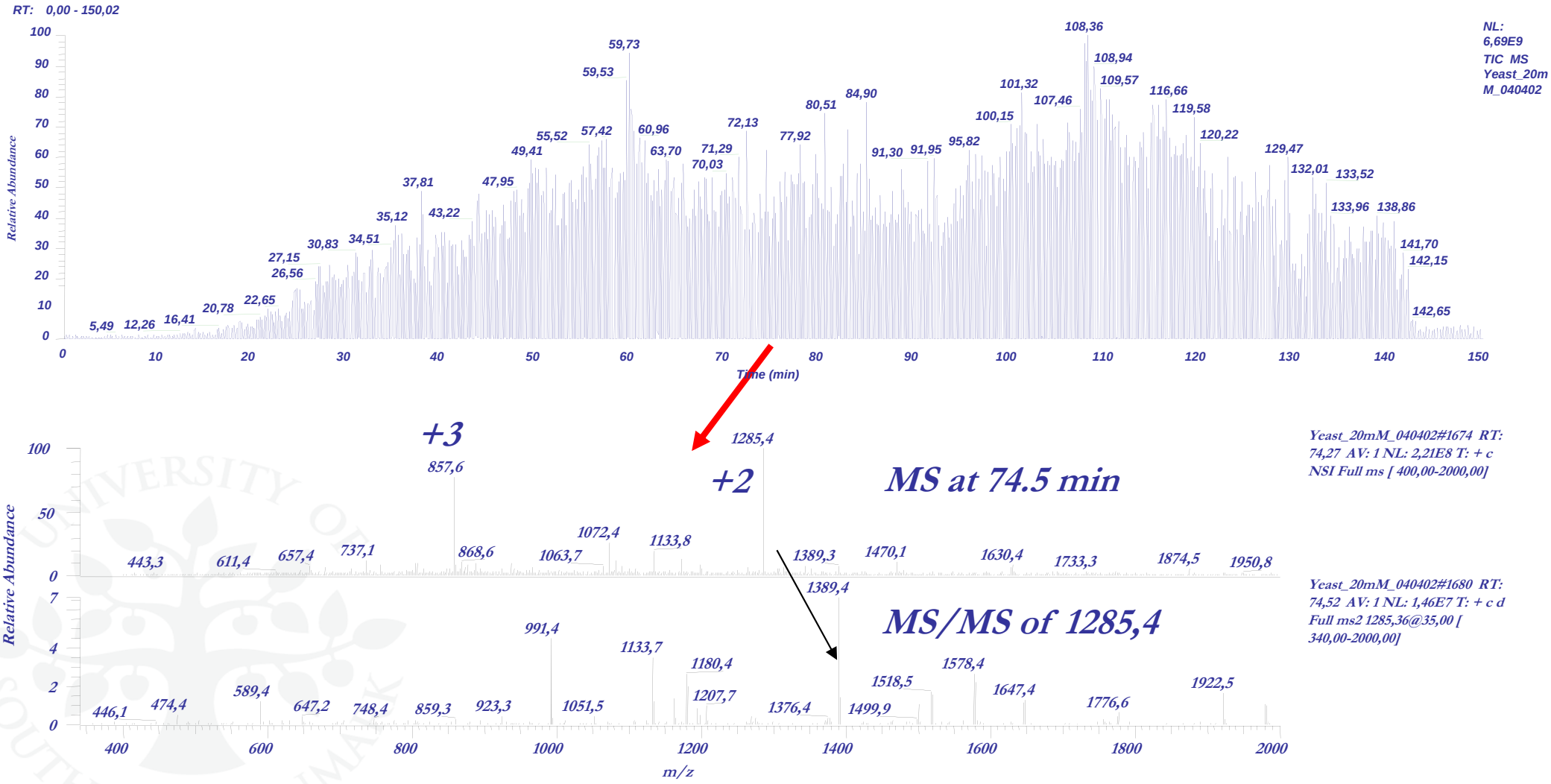
MS and MS/MS


Proteins
Digestion
MALDI-MS
Protein ID
R A W G Y V L E
Verification of protein ID,
analysis of unassigned peaks
(MALDI-)
MS/MS

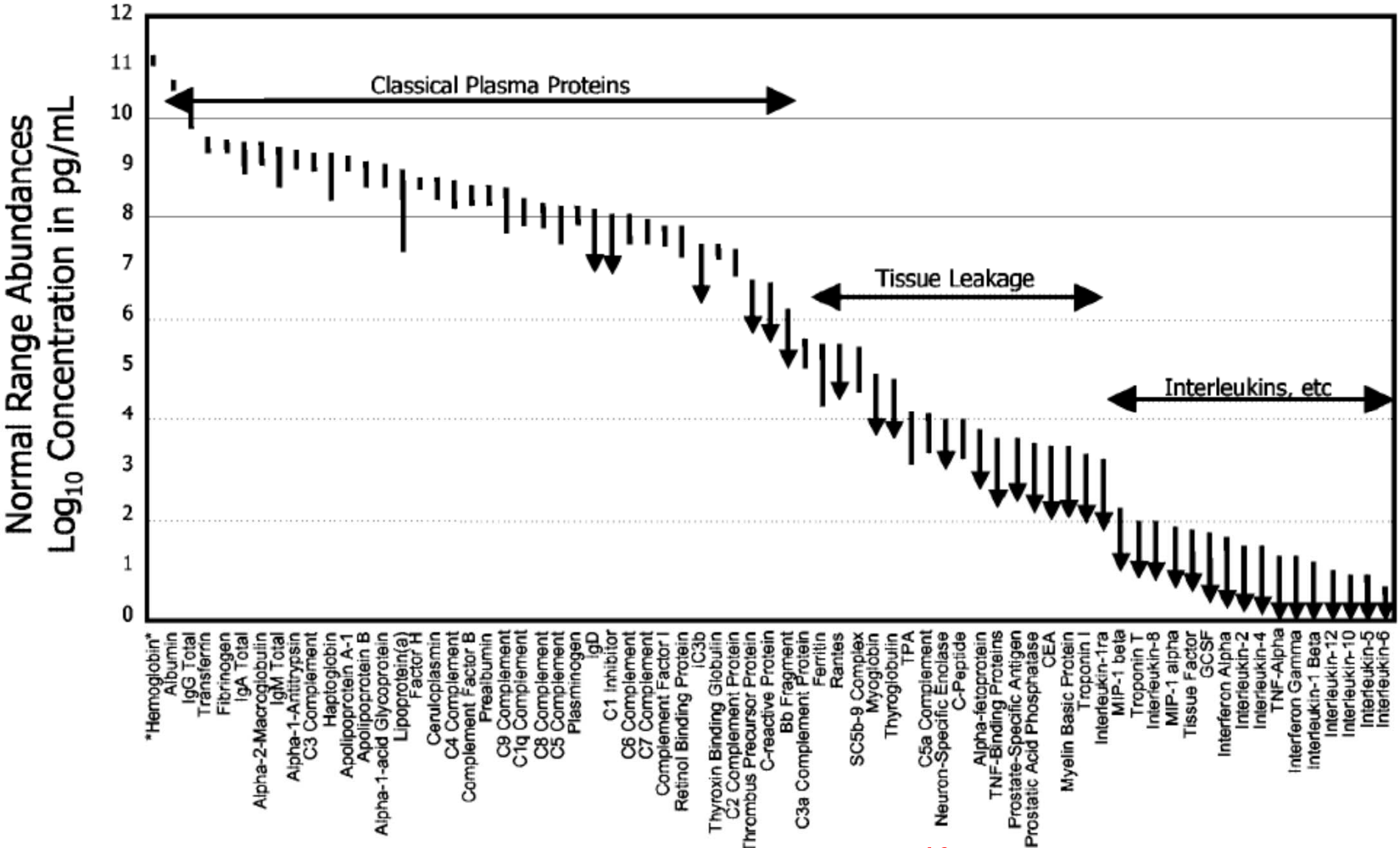
Nanoscale LC/LC-MS/MS (MUDPIT)

Typically 1000-2000 proteins claimed identified in one run (24-40 hours). How many are correct?

E:\LC_Packings\...\Yeast_20mM_040402 05.04.2002 08:22:44



Biomarker discovery – clinical proteomics



Dynamic range - 10^{10}

Problems with traditional data-dependent MS/MS: under-sampling

Journal of proteome research

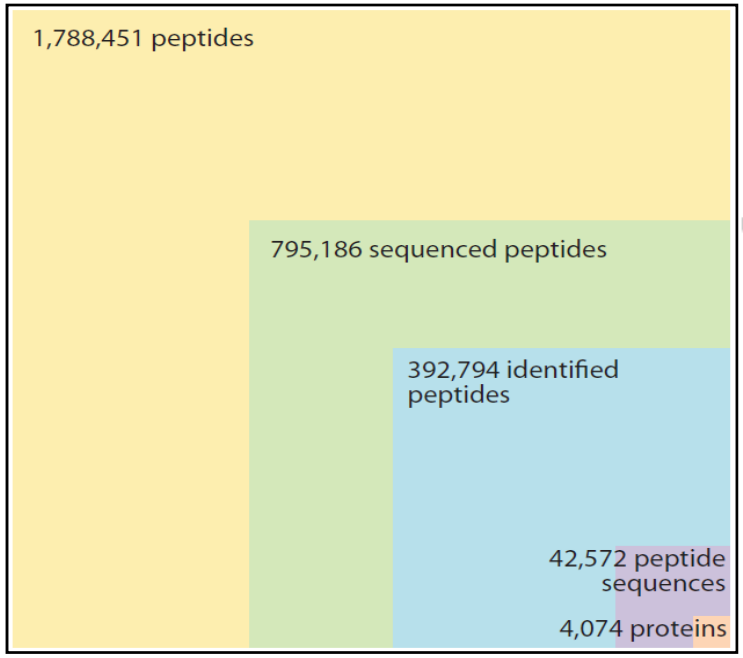
ARTICLE

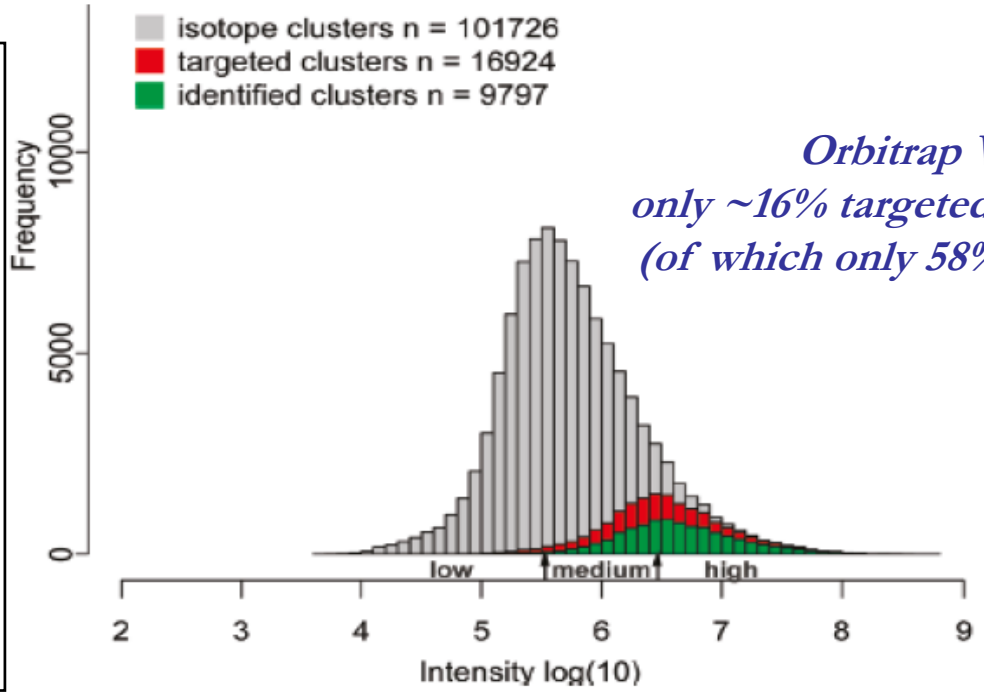
pubs.acs.org/jpr

More than 100,000 Detectable Peptide Species Elute in Single Shotgun Proteomics Runs but the Majority is Inaccessible to Data-Dependent LC–MS/MS

Annette Michalski, Juergen Cox, and Matthias Mann*

Department for Proteomics and Signal Transduction at the Max-Planck Institute of Biochemistry, Martinsried, Germany





Orbitrap Velos:
only ~16% targeted for MS/MS...
(of which only 58% identified...)

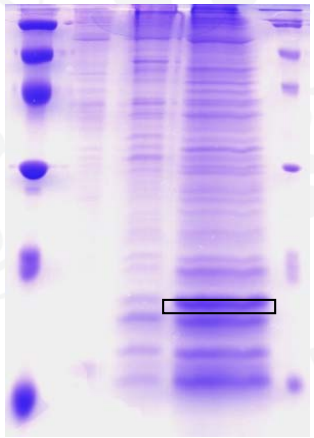
Michalski *et. al.*, *J. of Prot. Res.*, **2011**, *10*, 1785-93.

Our preferred analytical strategy for LC-MS

Step 1.
Parasite production

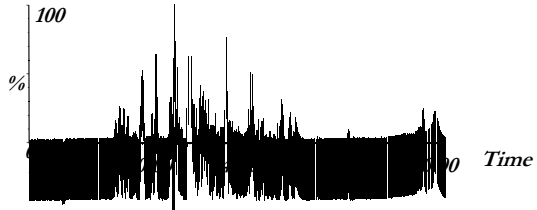
Step 2.
Protein extraction

Step 3.
SDS-PAGE

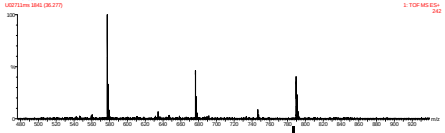


Step 4
In-gel digestion

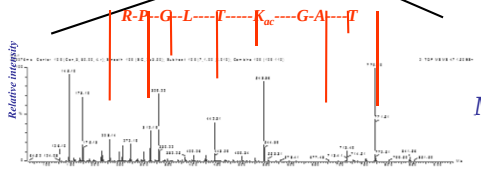
Step 5 LC-MS/MS



Chromatogram



MS spectrum



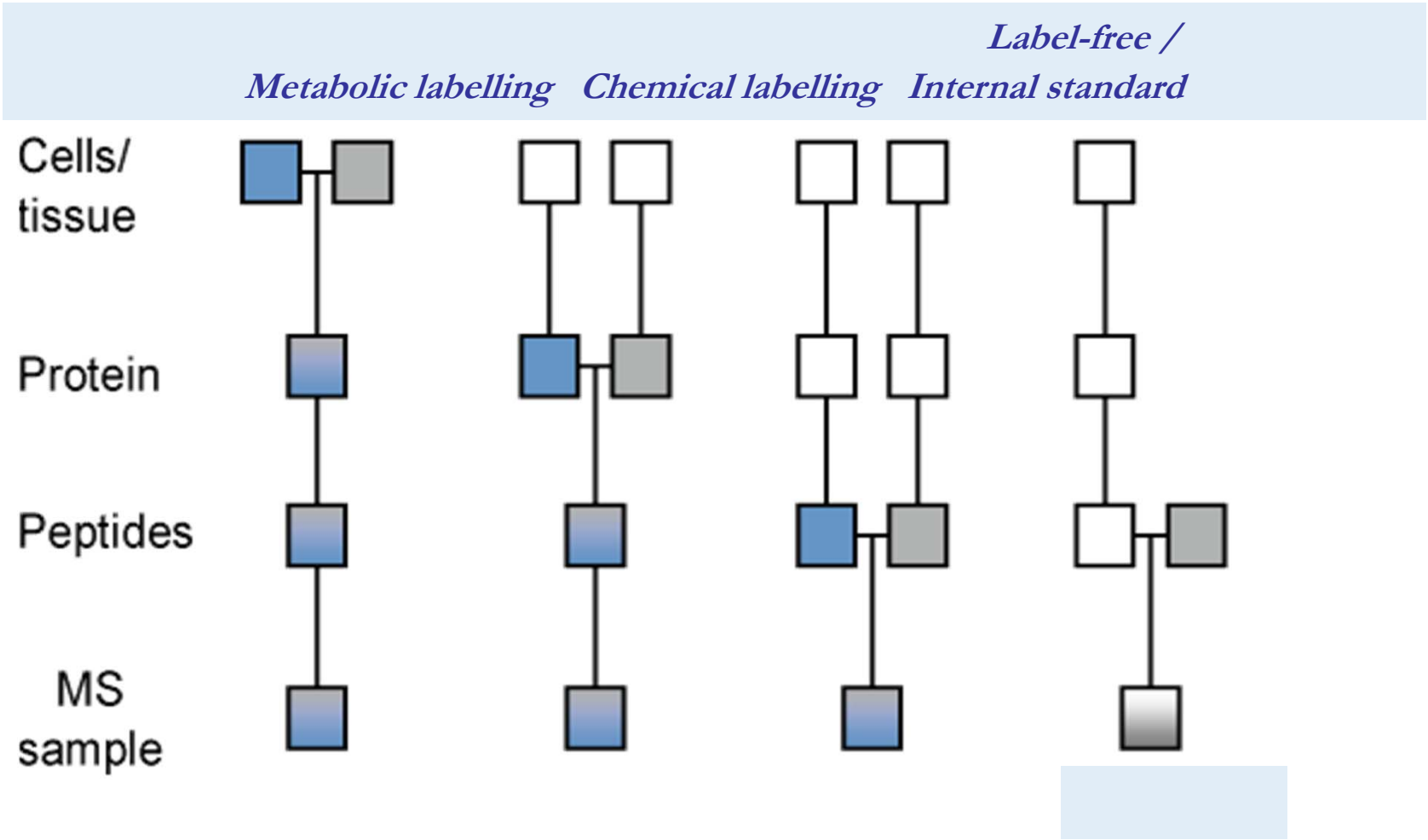
MS/MS spectrum

Step 6.
Comparative search against protein database

Step 7.
Manual validation of search result

Step 8.
Organization and presentation of data

Error sources in quantitative approaches



Incorporation of the stable isotope occurs early in the protocol, so fewer sources of relative quantitation errors exist downstream

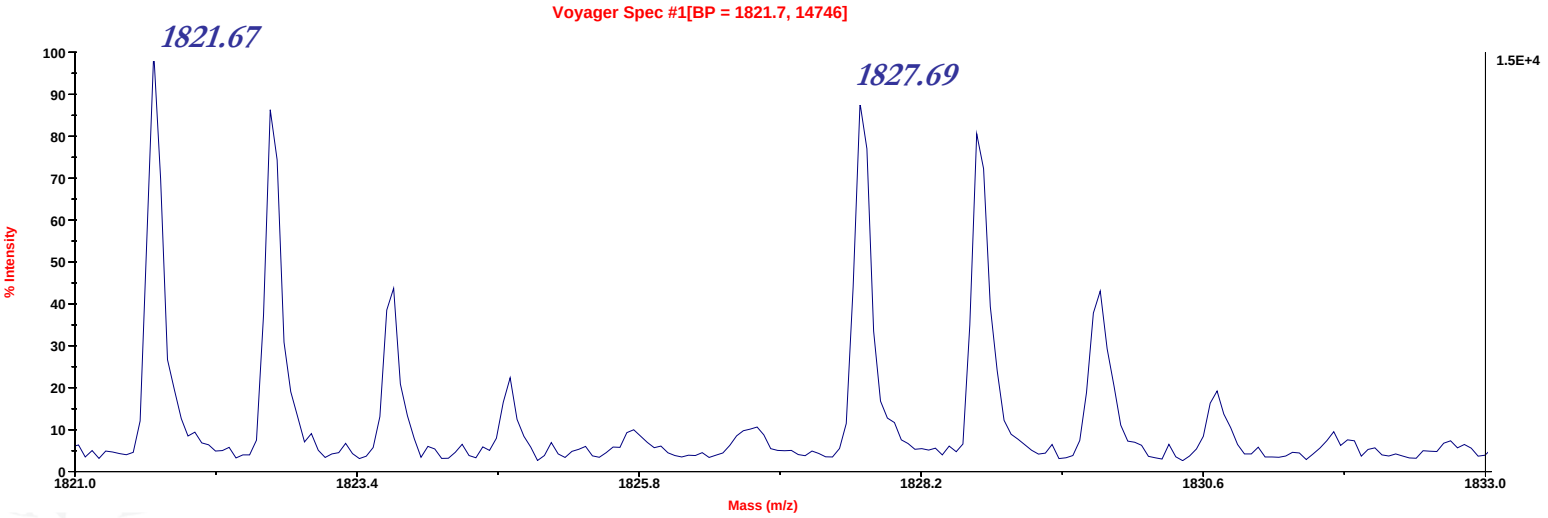
(but you also do not notice what you loose...)

Quantitation with Stable Isotopes

Advantages of stable isotope labeling (^{13}C; ^{15}N; ^{2}H):

- ***peptide pair is chemically very similar***
- ***sample combined during/before preparation***
- ***analyzed in the same MS experiment***

Measurement of relative intensities:

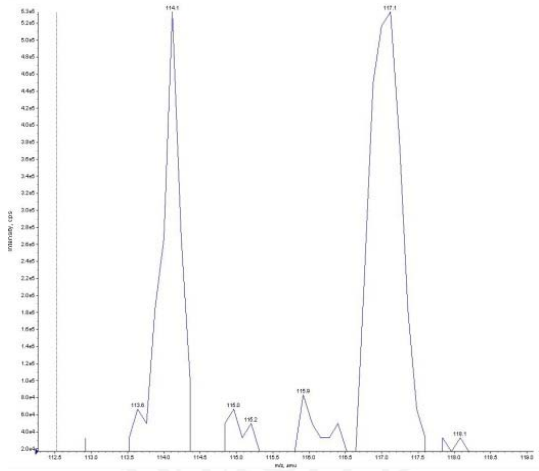


Examples of labeling reagents

SILAC (Stable Isotope Labeling by Amino acids in cellculture) 15N, 13C, 2H	Cell level	MS based quantification
ICAT (Isotope Codded Affinity Tag)	Protein level	
Mass Tag, SPITC, CAF, ^{18}O, dimethyl, ICPL	Peptide level Peptide level	
iTRAC, TMT, DiArt	Peptide level	MS/MS based Quantification

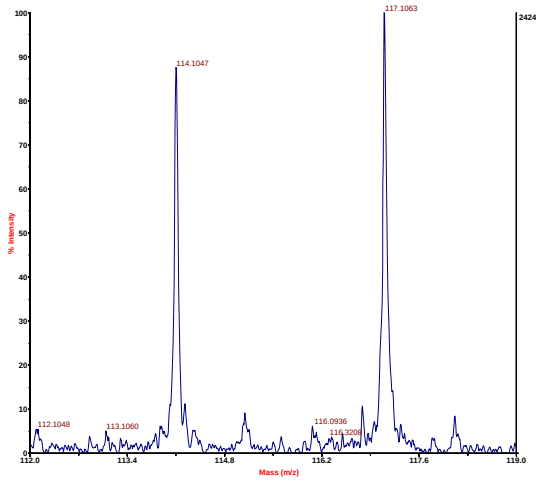
Quality of the reporter ion when using iTRAQ

R = 200 - 400



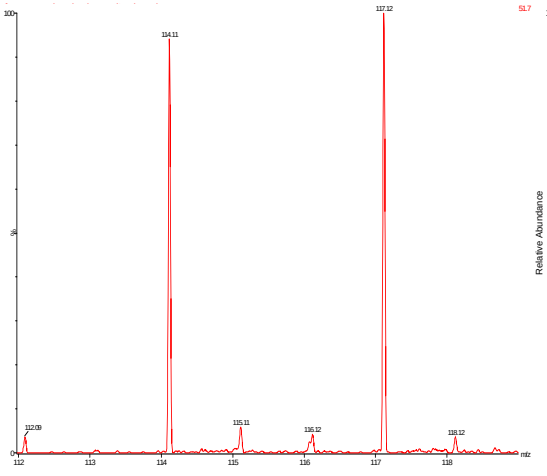
Q-TRAP

R = 1600



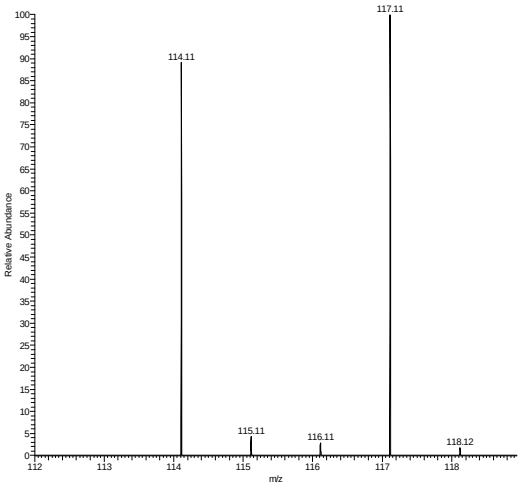
4700 TOF/TOF

R = 3800



Q-TOF

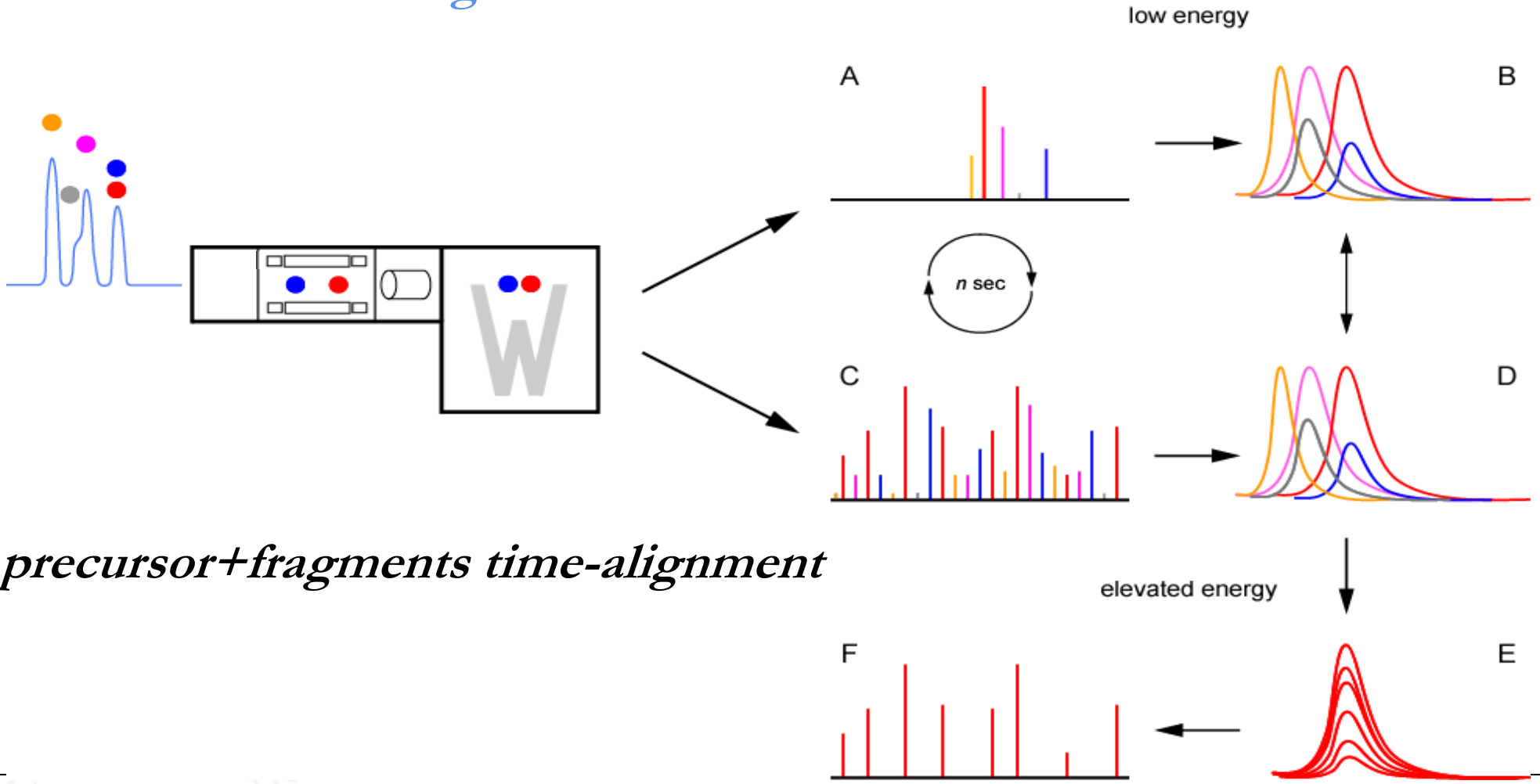
R = 15600



Orbitrap

Classical MS/MS versus MS^E

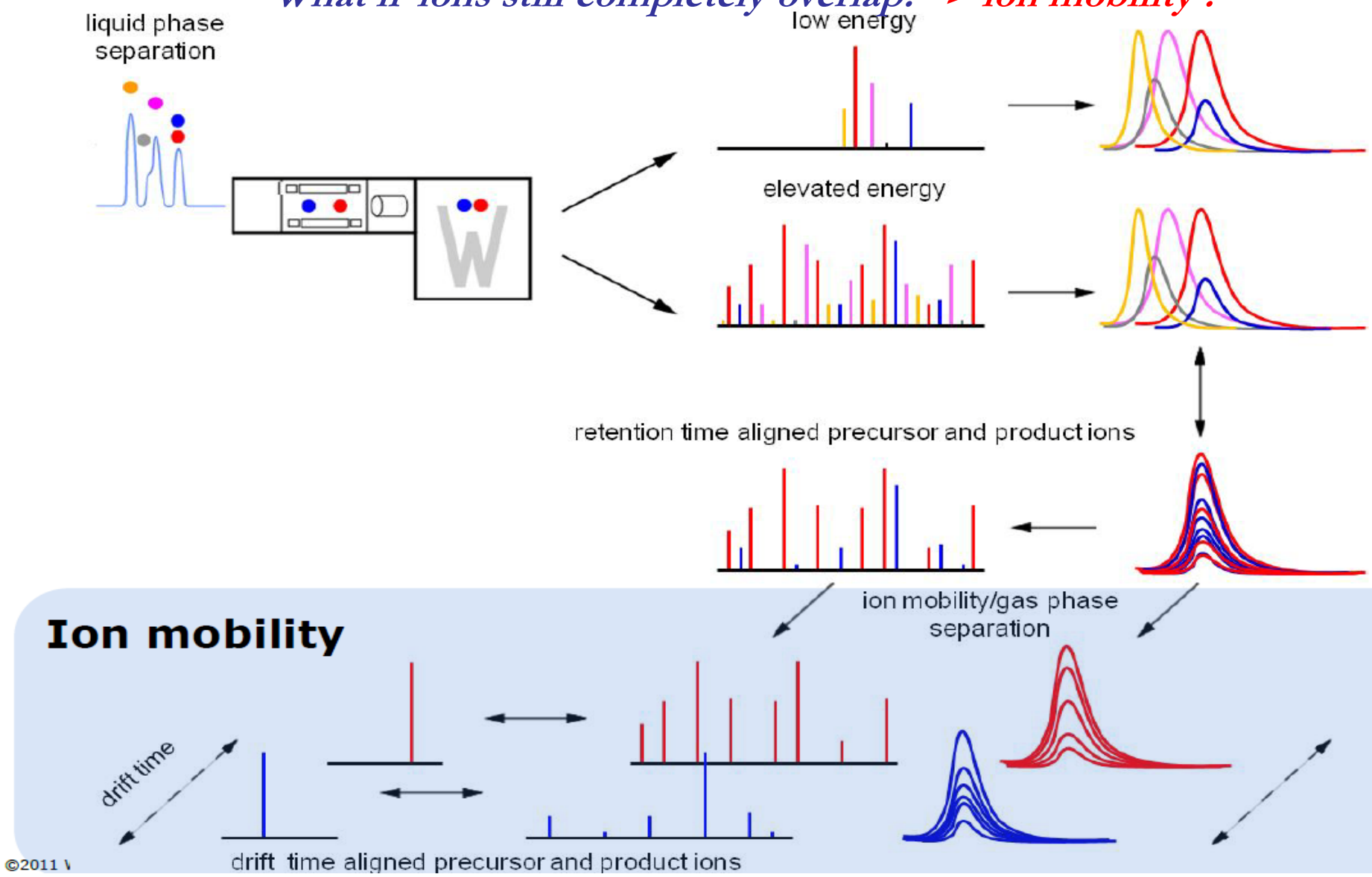
alternate scanning



precursor+fragments time-alignment

Latest developments: HD-MS^E

What if ions still completely overlap? -> ion mobility !



Pro's and con's of label-free quantitation

Advantages:

- ***Relatively simple and cheap***
- ***Applicable to any sample***
- ***Excellent quantitative dynamic range (only limited by instrument used)***

Disadvantages:

- ***Requires very well standardized sample handling***
- ***Potentially time-consuming with many samples / replicate runs***
- ***<u>With increasing sample complexity, instruments are simply not fast enough:</u>***

  accurate quantitative information (peak intensity/area) is lost when instrument switches to MS/MS mode (problem with duty cycle – 'points over the peak')

  A trade off between accurate MS quantitation and peptide identification...

2DE case story: Castor bean (*Ricinus communis*)

Organel enrichment

Nogueira FCS, Domont GB, Campos FAP

University Federal de Rio de Janerio, unpublished

- *60% of storage are TAG;*
- *TAG, ~ 90% ricinoleate (12-hydroxy-oleate);*
- *Industrial importance, Biodiesel;*
- *Genome Project approx. 400Mpb (JCVI & TIGR);*
- *Residue(cake) / Toxins / Alergens;*
- *Ricin;*

2S Albumin.

ANALYSIS OF PROTEINS PRESENT IN CASTOR BEAN PLASTIDS

A

Seeds

Endosperm were dissected from the seed;

Endosperm

Homogenization Buffer (Hepes, pH 7.5);
Maceration and filtration;
1st centrifugation;
2nd centrifugation;

2nd Pellet: Fraction I

1st Pellet

Fraction I: fraction rich in organeles.
Resuspended in Homogenization Buffer;
Percoll density gradient centrifugation;

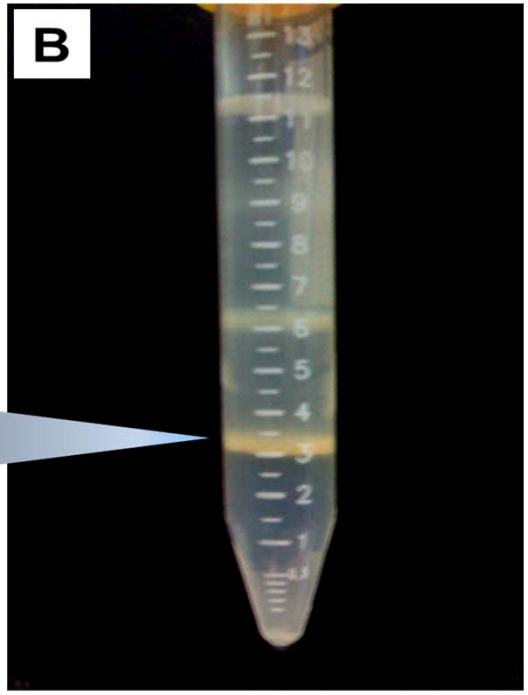
Fraction II: Isolated Plastids

Protein extraction with Pyridine Buffer and precipitation with 10% TCA in Acetone.

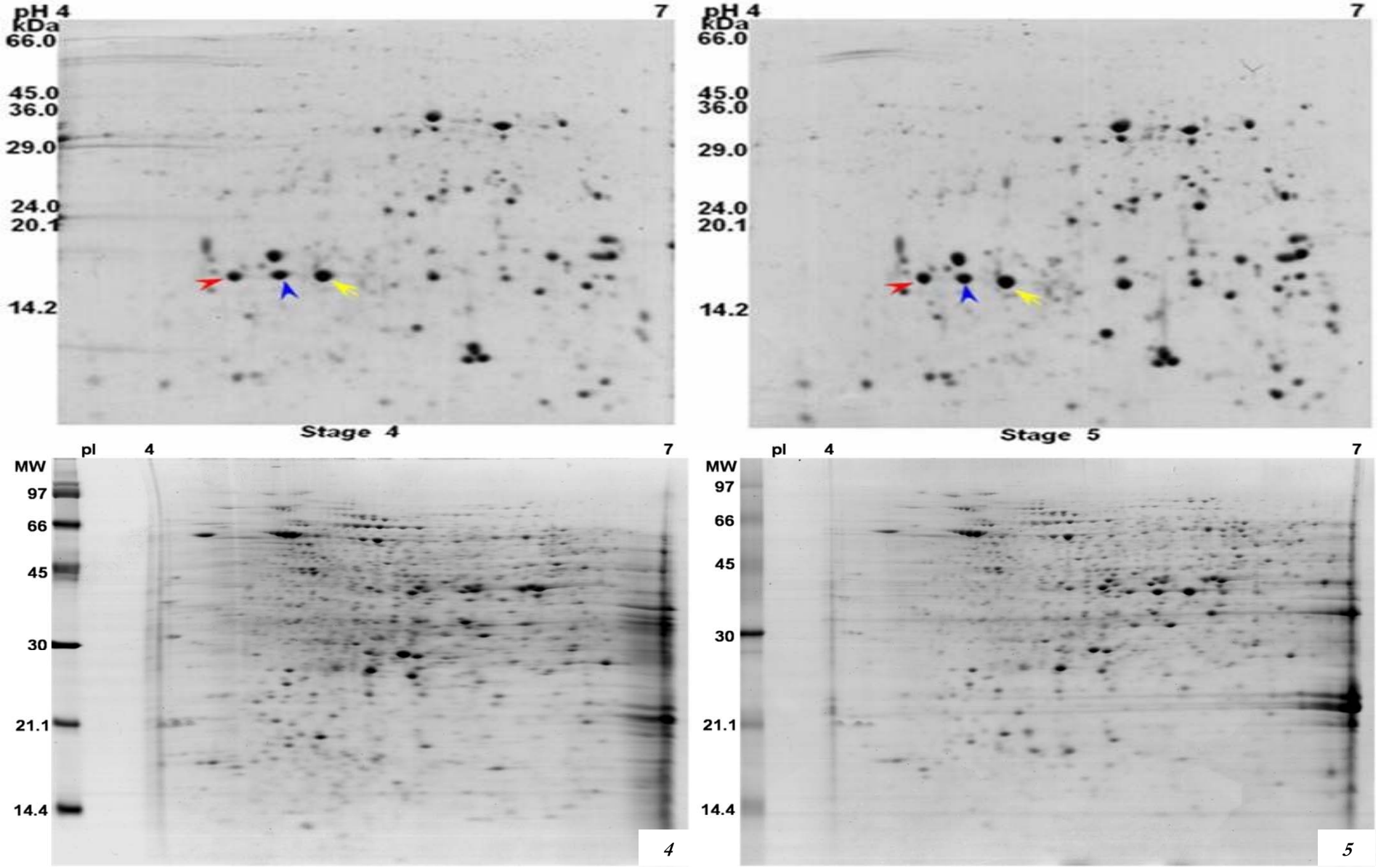
Electrophoresis sample

PROTOCOL FOR ISOLATION OF PLASTIDS

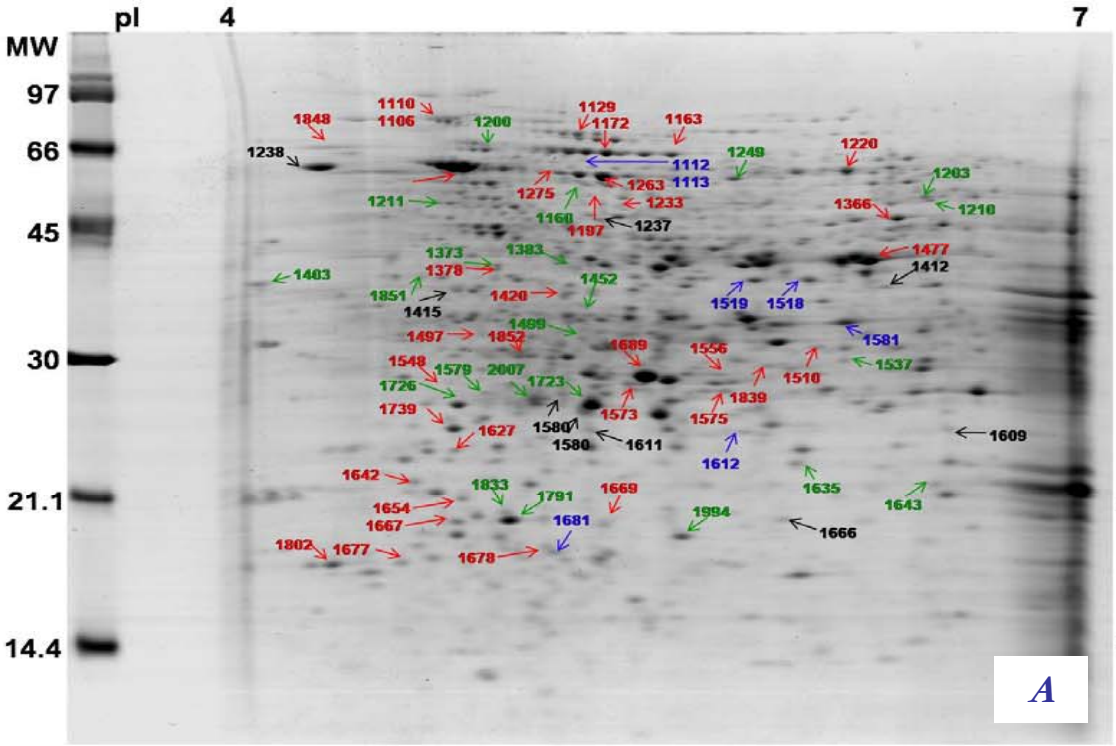
B

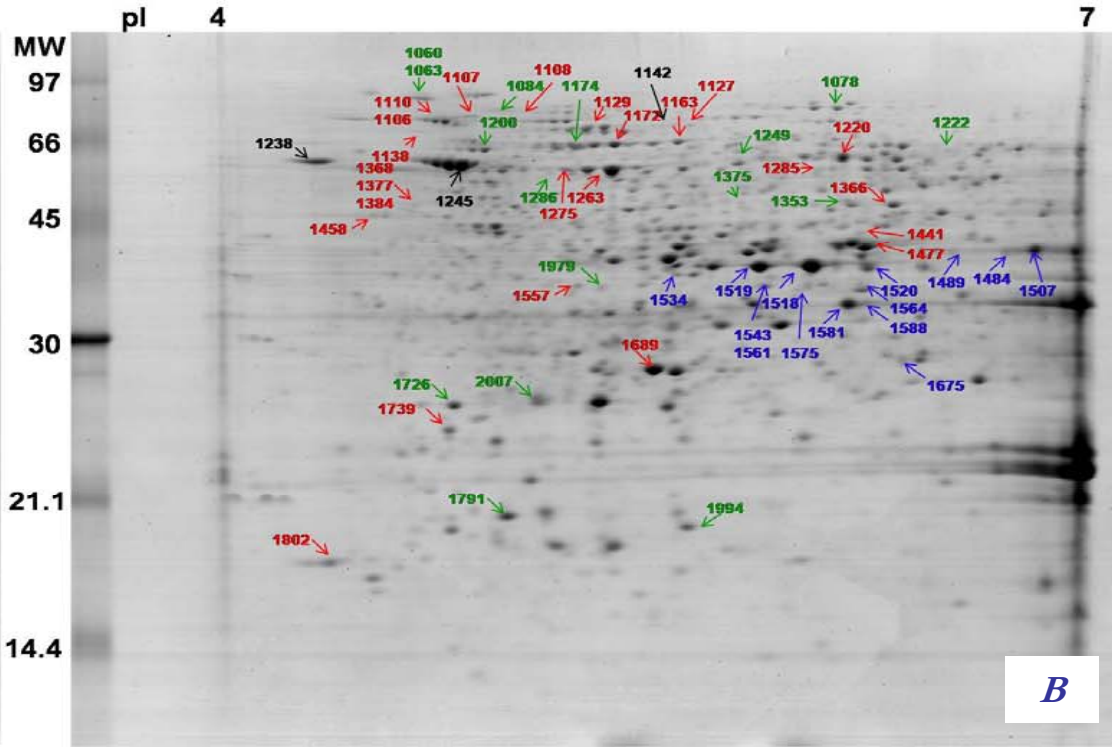


2-DE OF ENDOSPERM AND PLASTID RICH FRACTION Of CASTOR BEANSEED IN DEVELOPMENT STAGES 4 AND 5



2-DE OF PLASTDS RICH FRACTION OF SEED ENDOSPERM IN DEVELOPMENT STAGE 4 (A) AND 5 (B)





GREEN: plastids proteins

RED: plastid and mitochondria proteins

BLUE: storage proteins

BLACK: other proteins

2DE case story: Isoforms

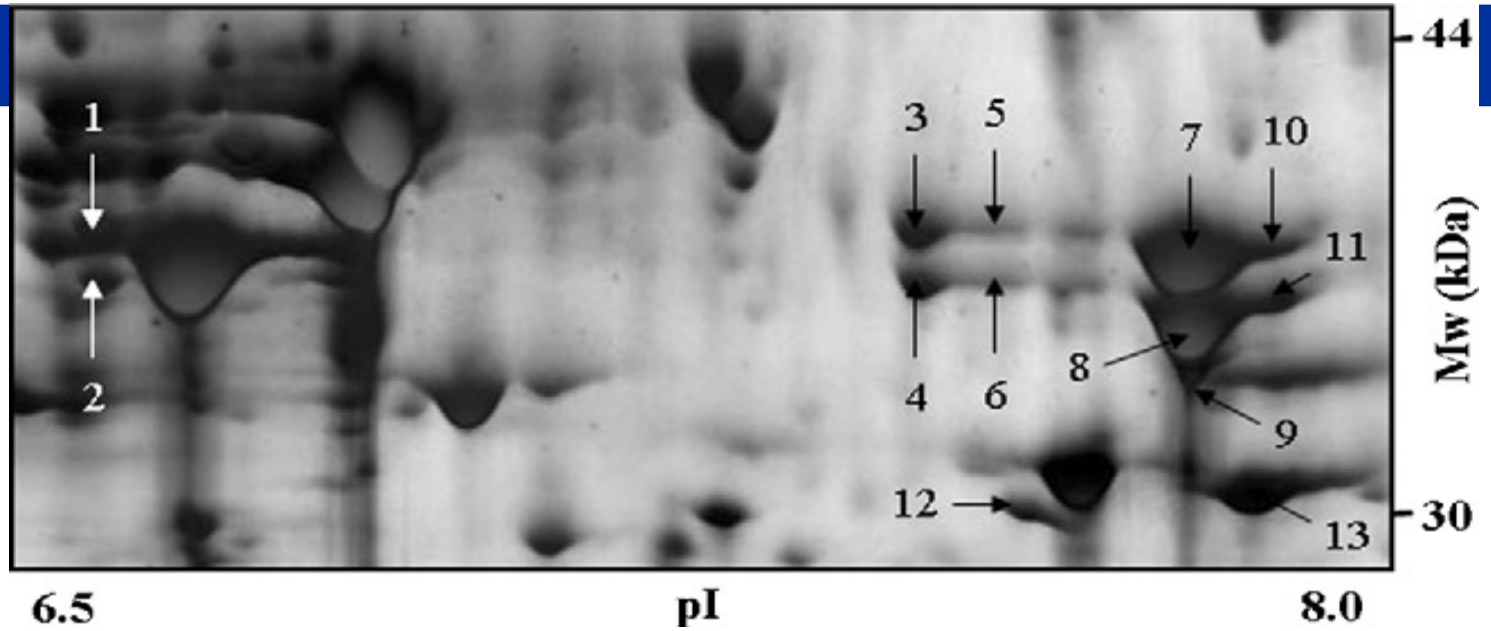
Barley peroxidase isozymes

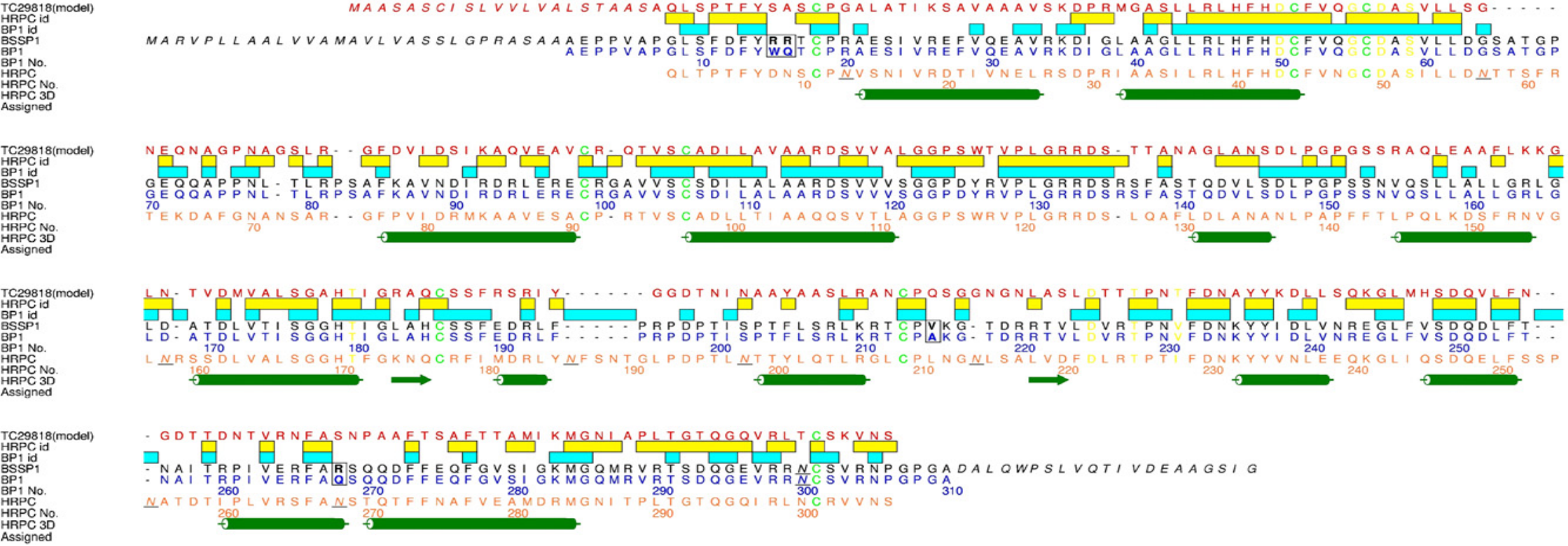
Expression and post-translational modification in mature seeds as identified by two-dimensional gel electrophoresis and mass spectrometry

Sabrina Laugesen, Kristian Sass Bak-Jensen, Per Hägglund, Anette Henriksen, Christine Finnie, Birte Svensson Peter Roepstorff

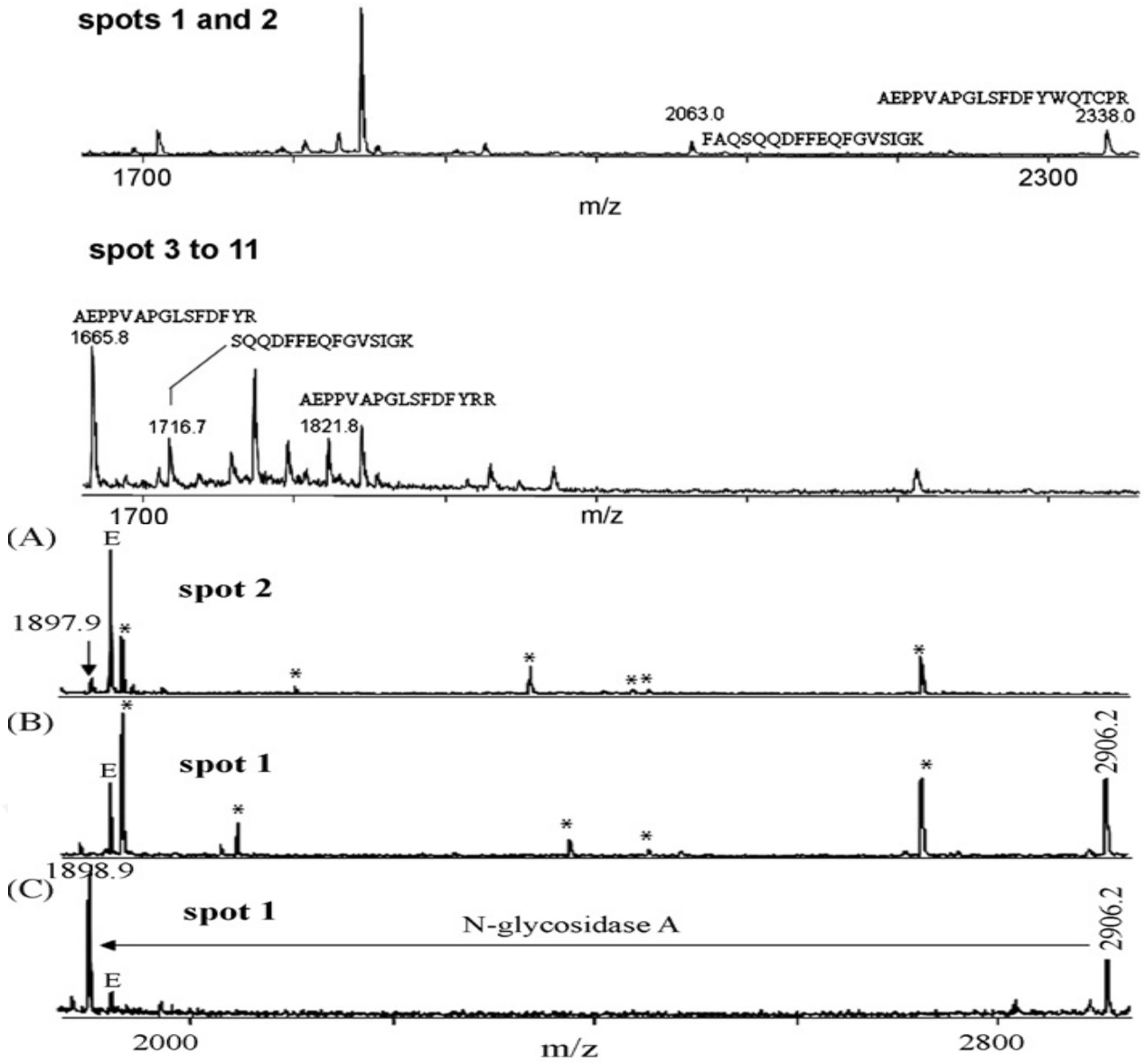
International Journal of Mass Spectrometry 268 (2007) 244–253

Barely peroxidase isozymes





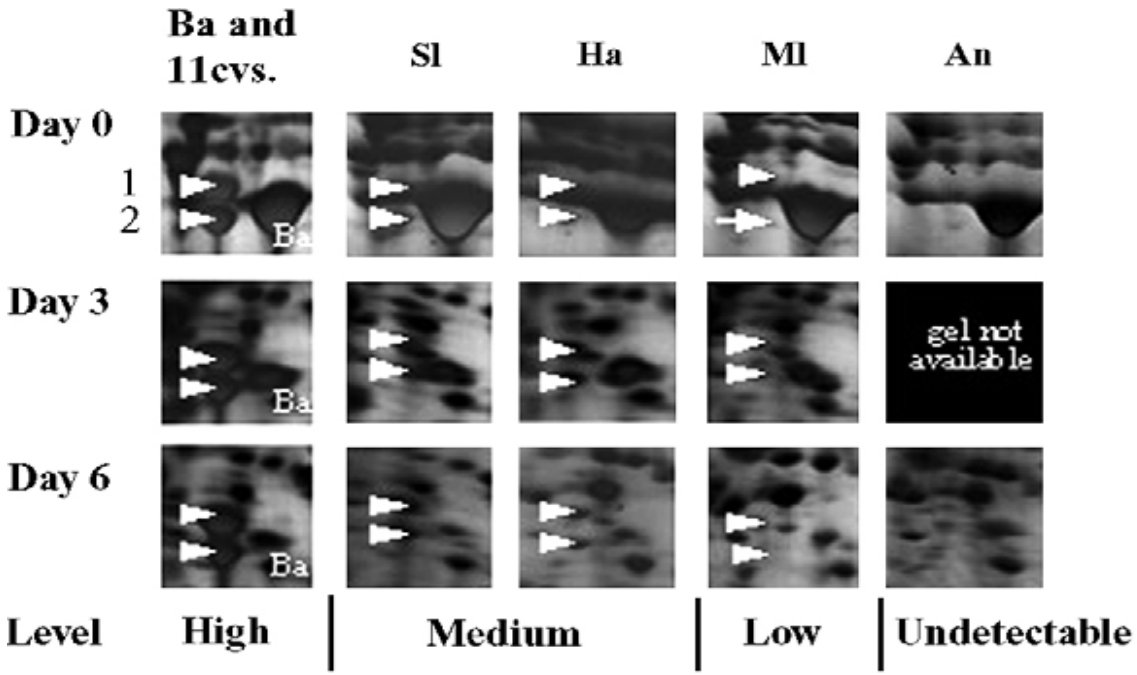
Barley peroxidase isozymes. Identification of sequence variants and glycosylation

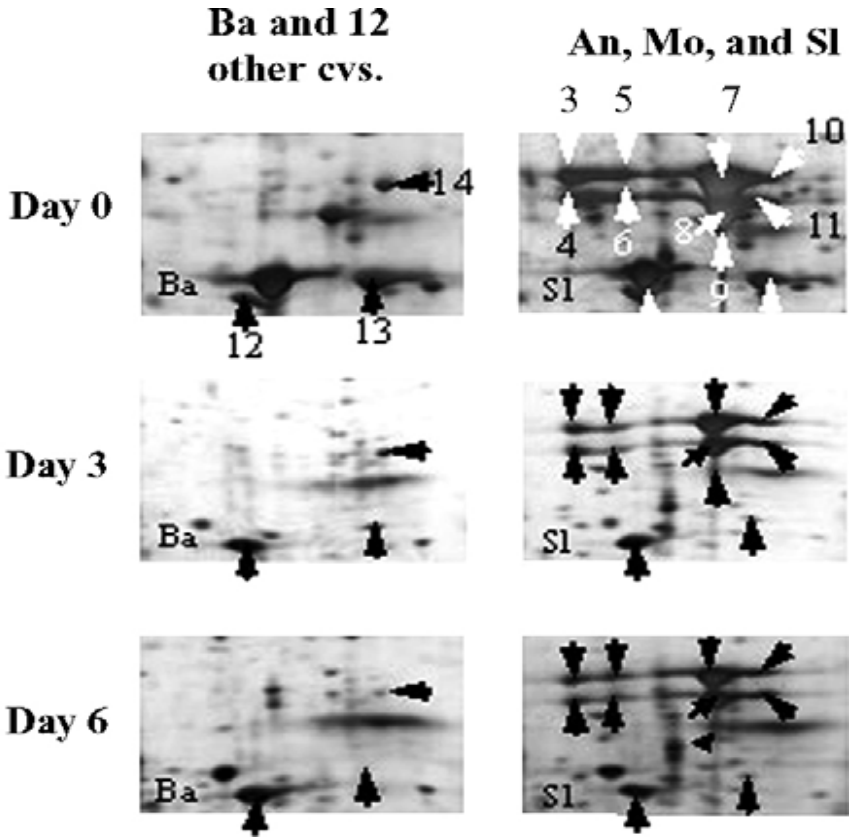


Peaks at m/z 2063.0 and 2338.0 (top) correspond to BP1 tryptic peptides residues 266–283 and 1–20, respectively. These two peptides are modified in BSSP1 Peaks at m/z 1665.8 and 1821.8 correspond to BSSP1 N-terminal peptide in which 15ArgArg16 replaced TrpGln in BP1. The peak at m/z 1716.7 corresponded to the tryptic peptide 269–283 where Glu268 in BP1 was replaced by arginine in BSSP1 yielding a cleavage site for trypsin.

Glycosylation is the difference between upper and lower spots

Barley peroxidase isozymes: Variation during germination and between cultivars



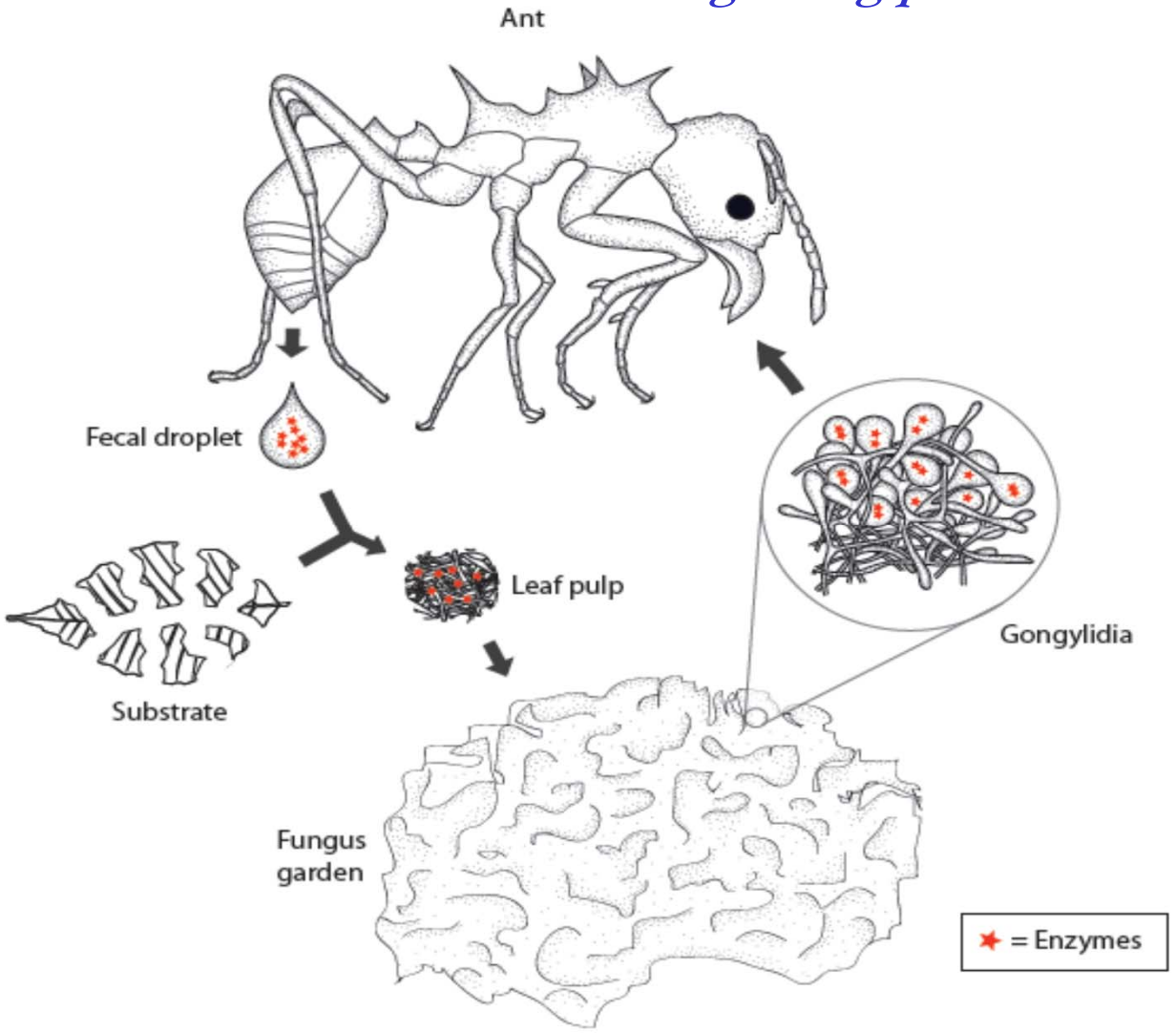


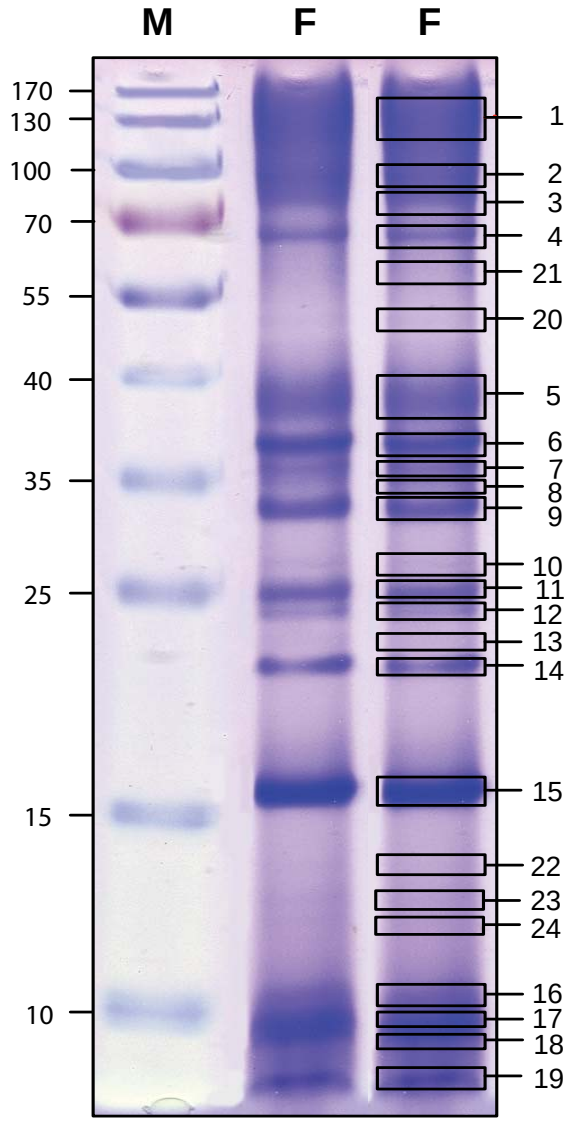
Monitoring of three barley seed peroxidases during germination of 16 cultivars. BP1 in spots 1 and 2, BP1 in spot 14, BSSP1 in 9 spots (3–11), and TC29818 in spots 12 and 13 at day 0 were overdeveloped during silver staining, thus the real level of spots 14 and 3–11 is roughly constant throughout germinating.

The top row contains the glycosylated and the lower row the non-glycosylated forms.

An, Annabell; Ba, Barke; Ha, Harrington; Ml, Meltan; Mo, Morex; Sl, Sloop.

leaf-cutting ant and fungi have convergently evolved cell wall degrading pectinase complexes

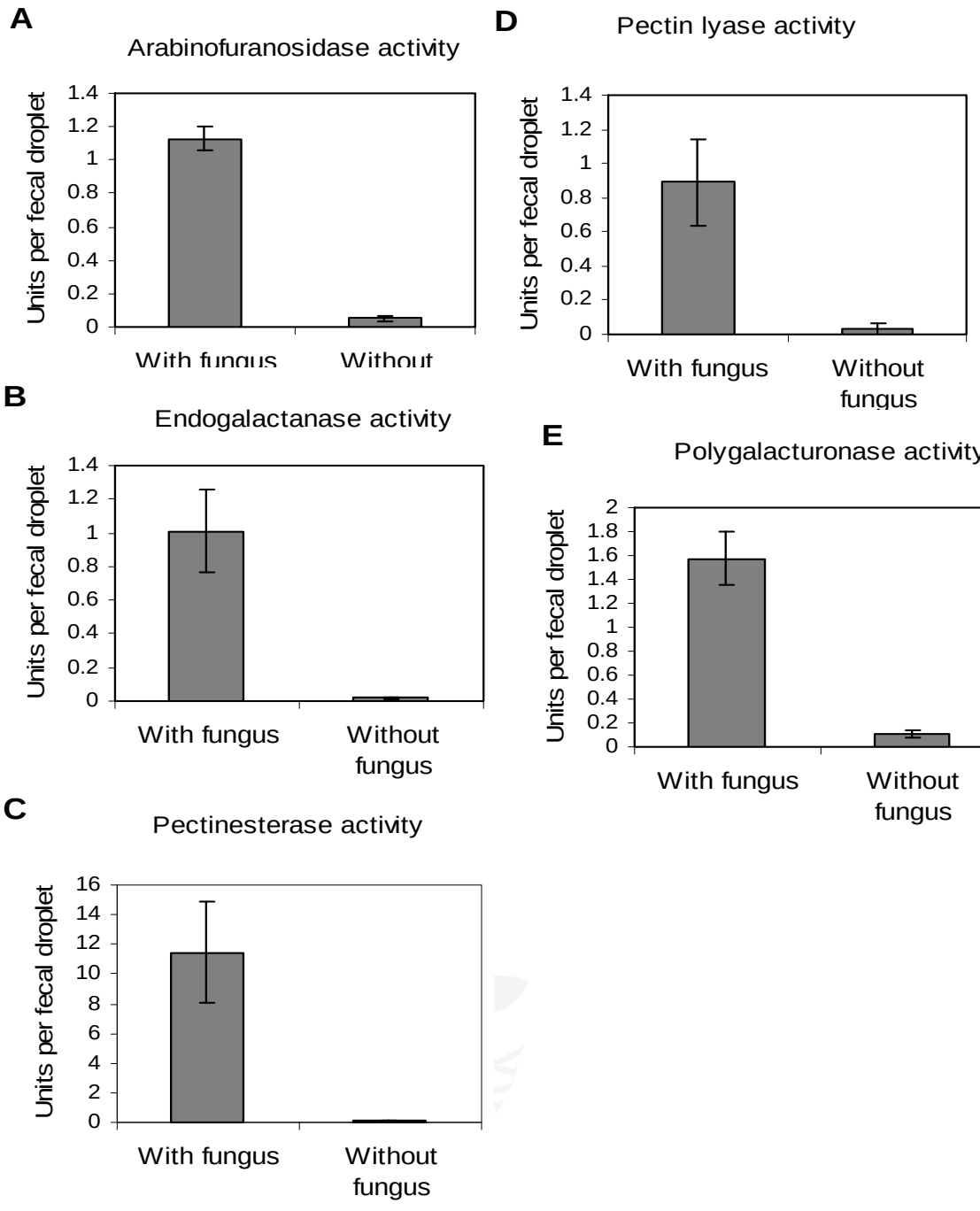




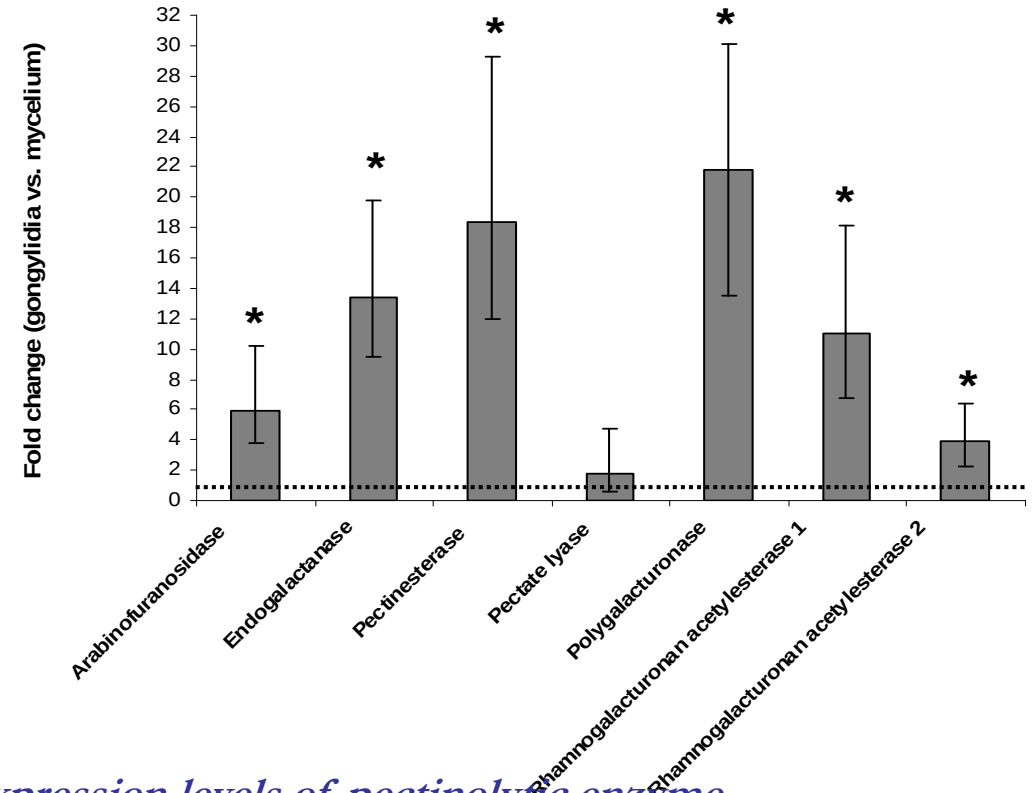
Morten Schiøtt, Adelina Rogowska-Wrzesinska and Jacobus J. Boomsma, BMC Biol. 2010 Dec 31;8(1):156

Fragmentation	Mode	Fragments
CID collisionally induced dissociation	Collisions with neutrals, All MSMS instruments	*b, y, a* *Weakest bonds first*
IRMPD IR multiphoton dissociation	Collisions with IR photons, ICR Cell	*b, y, a* *Weakest bonds first*
ECD Electron capture dissociation and ETD Electron transfer dissociation	Electron capture, ICR Cell Electron transfer Ion trap, orbitrap	*c*, $z^{\bullet}$, *a* *Any bond, except next to P*

Protein	Predicted function	Mass spectrometry data	Sequence data	Band[¤]	MW (Da)
FDP1	Arabinofuranosidase	FPGGNNJENTVDQR GD[YQ/QY]JPSTJPSSTGTVFWSVVR NDJASAJAEVG[SP/PS]FWR PEDFAANTYTFR STAJPNAJHVVJAPSR VESAAGEAAFFTGJER YFE[W/GE]YAAJSTNNP/PNDJFG	FPGGNNLENTVDQR GDQYIPSTLPSSTGTVFWSVVR NDIASALAEVGPSFWR NEDFFAANTYTFR STALPNALHVVIPTGR VESAAGEAAFMTGLER YFEGEYAAISTNPNDIFG	4	69139
FDP2	Endogalactanase	DJDGJNTQJFTYTR GAVTPFEEJJHNHGA[L/I/N] GWFSSJANJESSGR	DLDGLNTQIFTYTR GAVTPFEELLHNHGAN GADFSSLANLESSGR	7	37416
FDP3	Pectin esterase	GQAYFGGNTJ[QR/GVK] GAGWVTASGR GYJEGATDFJFGQR NNQATJQFGJDAGQAGSDDASGTJR NTFGVGSQAJAJSQYGDR QAYFGGNTJGVK	GQAYFGGNTLGVK GAGWVTASGR GYIEGATDFIFGQR NNQATIQFGLDAGQAGSDDASGTLR NTFGVGSQAIALSQYGDR QAYFGGNTIGVK	6 + 7 + 8 + 9 + 10 + 18 + 19 + 22	37571
FDP4	Pectate lyase	JVJJSGNJSGDAVVR VJNENNVJJR	IVLLSGNLSGDAVVR VLNENNVIIR	7 + 8	
FDP5	Polygalacturonase	VAVN(1710)…(1206)TGTWNWSNJK	VAVNCGVGSCTGTWNWSNLK	6	37055
FDP6	Rhamnogalacturonan acetylesterase-1 (Rhamnogalacturonase)	PDNLWVNGEJGAGPR DWFGHNDGJSGAVDNMQJR FVGYAQTAASR VNDAJAGR	PDNIWVNGEIGAGPR VIEFGHNDGLSGAVDNGRED FVGYAQTAASR VNDAIAGR	10 + 16 + 17 + 18 + 19	26946
FDP7	Rhamnogalacturonan acetylesterase-2	FVTYAQSJGJR	FVTYAQSLGLR	18	26467



Activities of different Pectinolytic enzymes



Expression levels of pectinolytic enzyme genes in gongylidia relative to gongylidia-free mycelium measured with quantitative real time PCR

Proteomics levels

Expression proteomics

Which gene products are expressed, when and how much

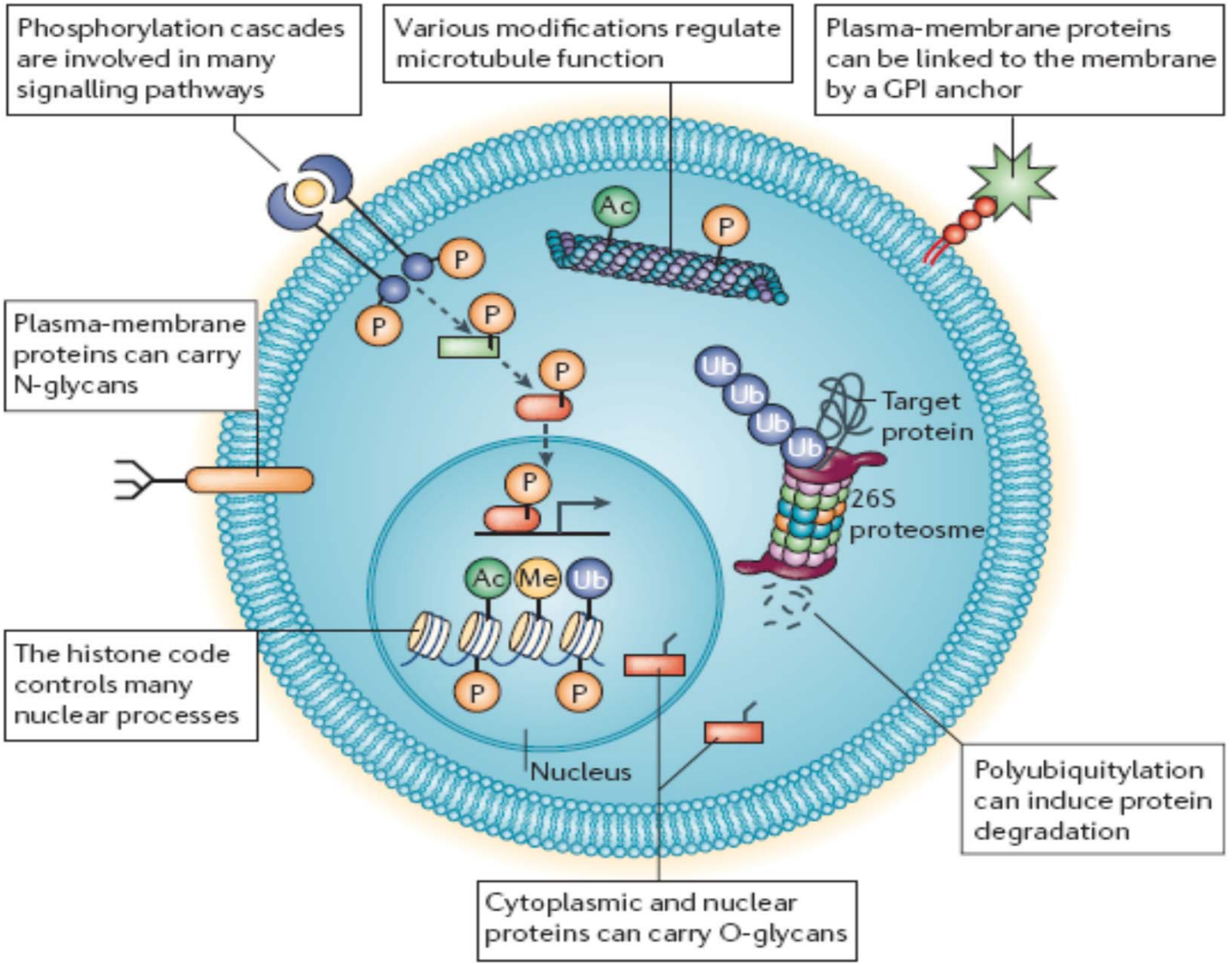
Modification specific proteomics ”modificomics”

Which variants are present of each protein, when and how much?

Interactomics, Cell map proteomics

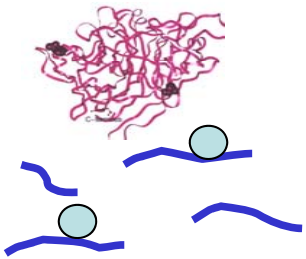
Who interacts, when and where

Post-translational modifications

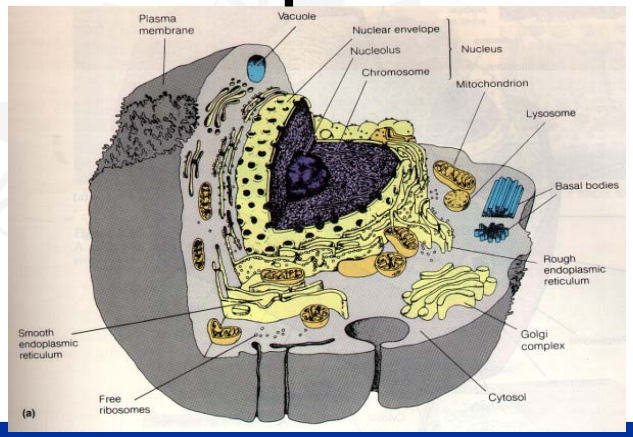


Modification-specific proteomics

Optimized sample preparation for PTM-peptides

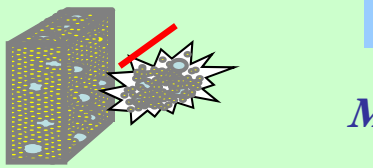


- ***Organelle/complex purification, and/or***
- ***PTM protein enrichment, and/or***
- ***PTM peptide enrichment***

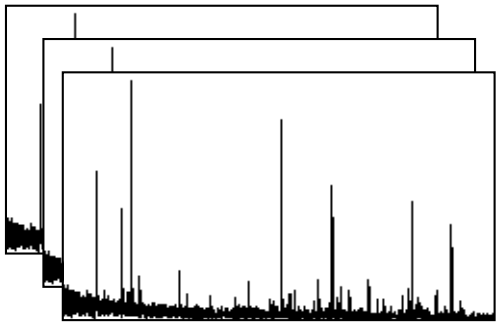


MS data acquisition

MS (Δm)
MS/MS sequencing
MS/MS neutral loss
MS/MS diagnostic ions
Multistage MS



MALDI MS/MS
ESI MS/MS



Mass spectra

Computational data analysis and data mining

PTM assignments

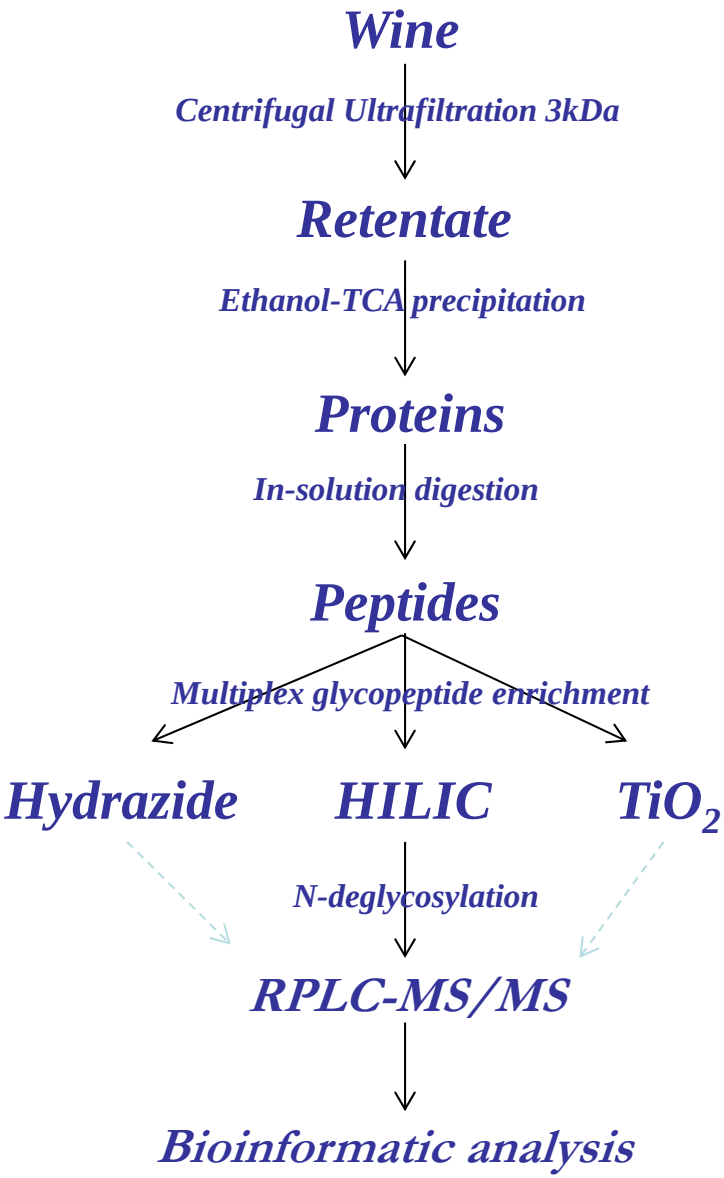
Validation

PTM function

Case story: Protein glycosylation

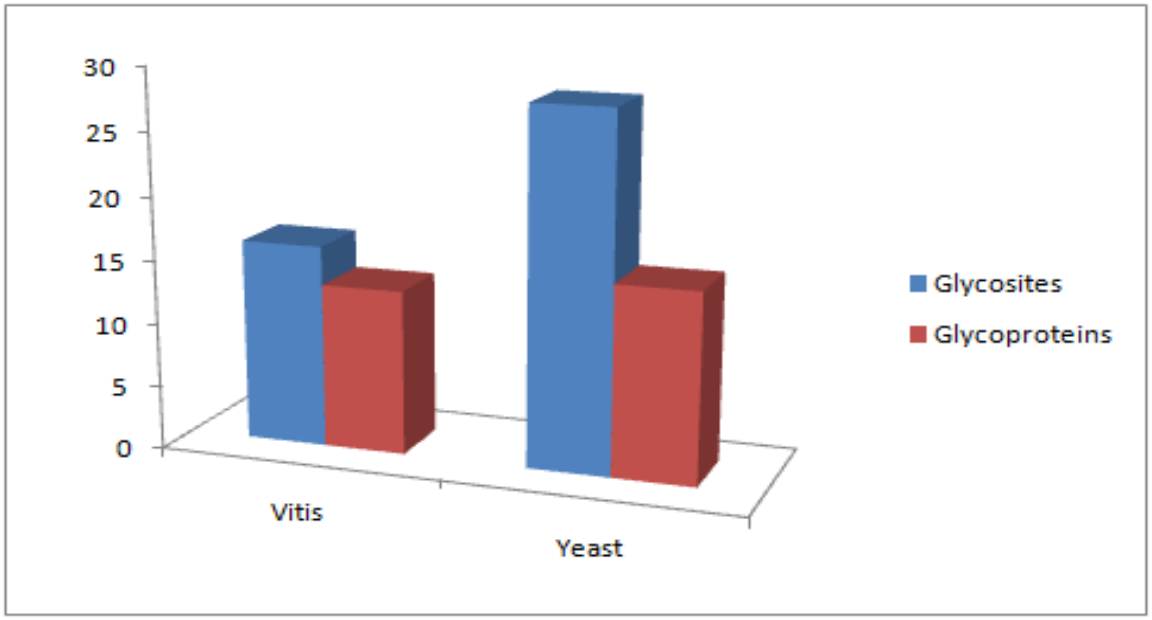
Glycoproteomic profile in wine: a 'sweet' molecular renaissance
Giuseppe Palmisano, Donato Antonacci, and Martin R. Larsen
J. Proteome Research, 2010 9, 6184

N-linked glycoprotein analysis in wine



Palmisano G. et al. JPR 2010

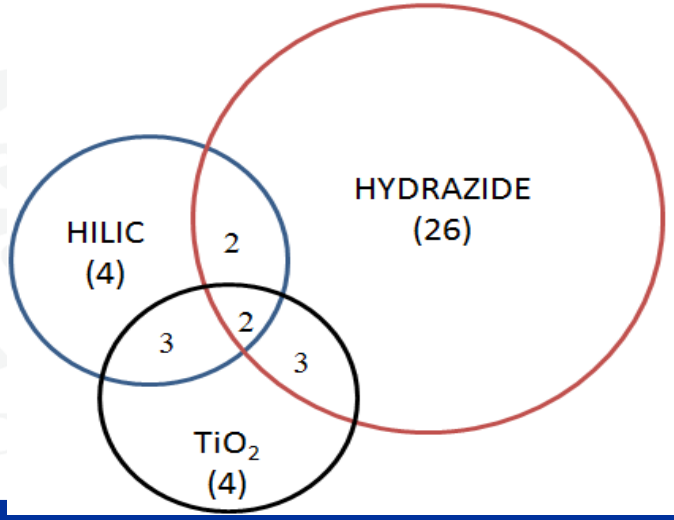
Identified glycoproteins

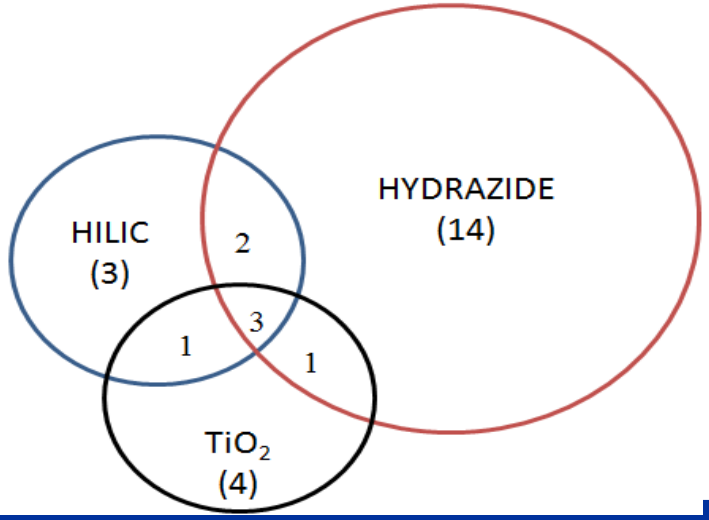


28 glycoproteins
44 glycosylation sites

N-linked Glycosites

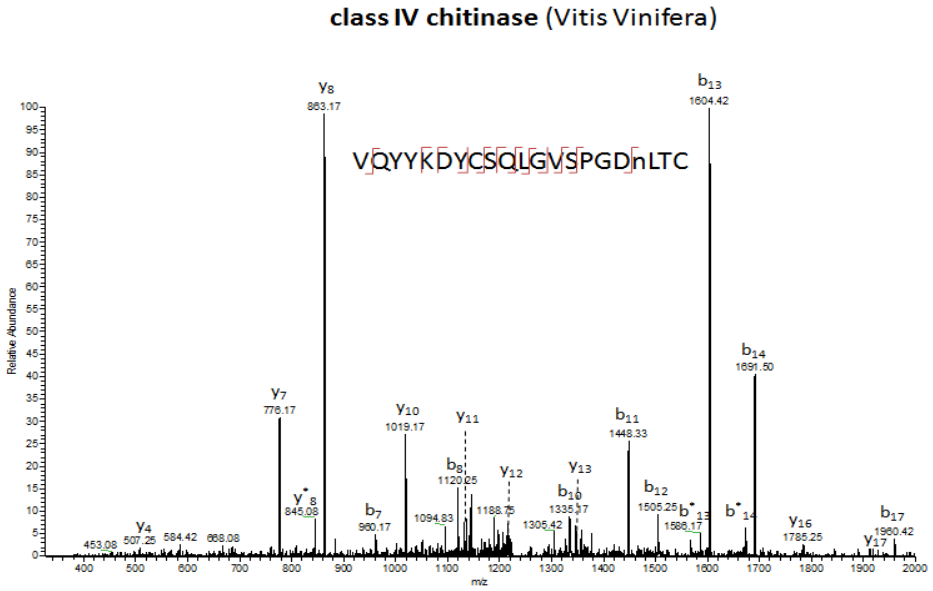
N-linked Glycoprotein

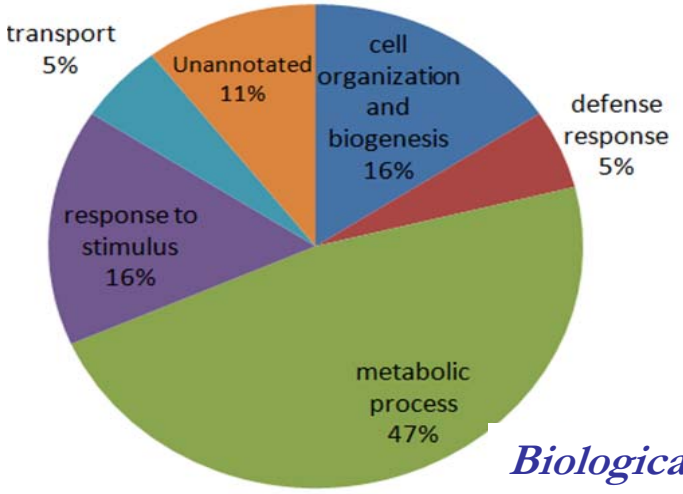




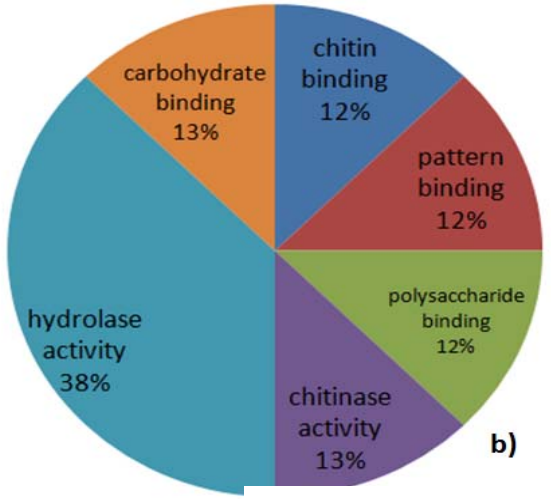
Biological and Molecular functions

Vitis Vinifera



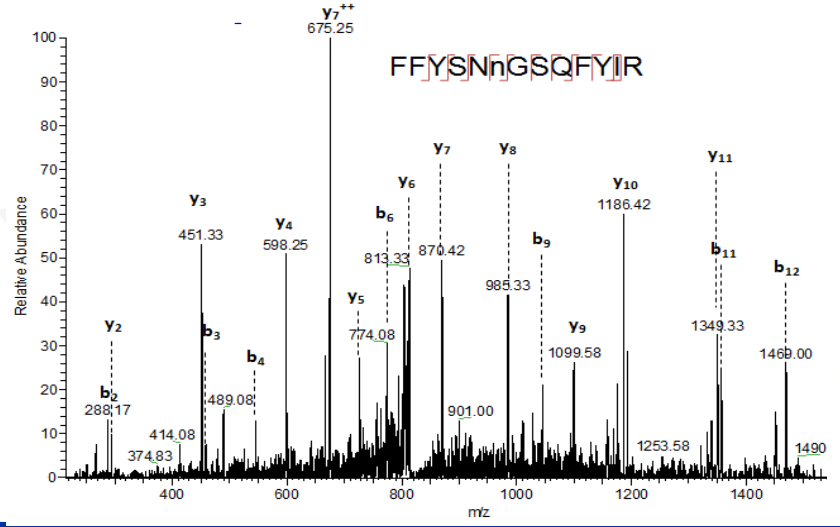


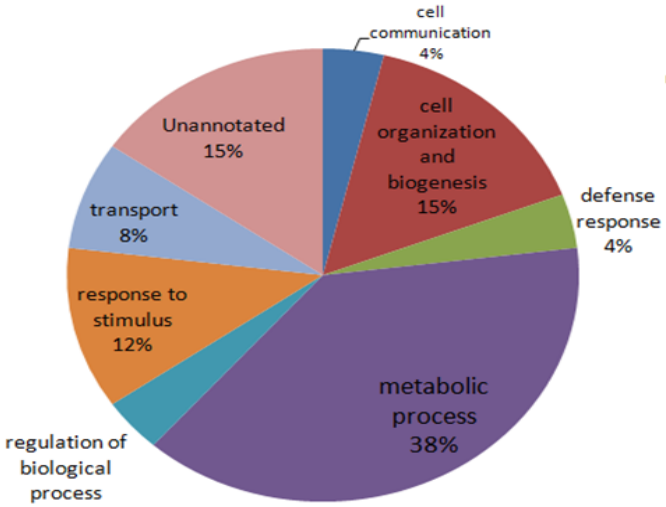
Biological processes



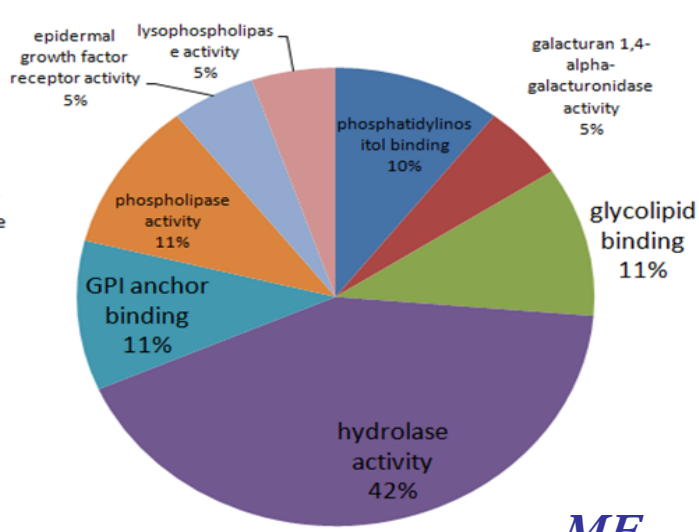
Molecular Function

Saccharomyces Cervisiae





BP



MF

Grape glycoproteins in wine

•*Class IV Chitinase and class IV endochitinase (PR-3 family) are enzymes that cleave chitin;*

-are constitutively expressed in plants and enhanced by pathogen infection amd stress conditions;

•***Thaumatin-like proteins (TLP) (PR-5 family)** in wine **with three different MW16, 22, 40kDa ;***

-PsTL1 (Pyrus serotina thaumatin-like protein 1) glycosylated 32-kDa protein

•*Vacuolar Invertase 1 (GIN1) convert the sucrose into glucose and fructose;*

-express during plant development, growth, during biotic and abiotic conditions;

-GIN1 is more expressed in the grape berry pericarp compared to the isogene GIN2;

-GIN1 with 12 potential N-linked glycosylation sites.

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New anti-

Relaxnews

Monday, 22 November 20

Does a nice Bord
headache, stuffy
miserable ailmen
are well documen
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allergies.

For eight percent
of wine can produ
symptoms. While
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Monday November 29th 2010

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Print edition

Allergy to wine

The oenophile's lament

An explanation for a most unfortunate condition

Nov 25th 2010 | from PRINT EDITION

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ONE of life's sadder statistics is that about 8% of people get sneezy and stuffy-headed after drinking wine. This mild allergic reaction is often blamed on preservative chemicals called sulphites, but they are responsible for only an eighth of cases. The reason for the rest is obscure. Giuseppe Palmisano of the University of Southern Denmark, however, thinks he knows the answer.

As he and his colleagues report in the *Journal of Proteome Research*, the culprits are glycoproteins—compounds composed, as their name suggests, of sugar and protein. That is not a complete surprise. Glycoproteins are implicated in several other allergies. But Dr Palmisano thinks he has identified the ones specific to wine.

To do so he started with a cheeky little chardonnay, treated it with ice-cold trichloroacetic acid and ethanol to precipitate any glycoproteins, then digested those glycoproteins into smaller molecules called peptides that can be analysed by mass spectroscopy. He screened the results against a database of known allergenic proteins. Three stood out. One is similar to allergenic proteins found in latex and pears. Another looks like a second latex protein and an olive protein, both known allergens. The third resembles one of the most rampant allergens of them all, a ragweed protein that causes hay fever.

Whether winemakers will be able to act on this knowledge is moot. But it might be possible to tweak the production process to reduce the presence of the allergens. In any case,

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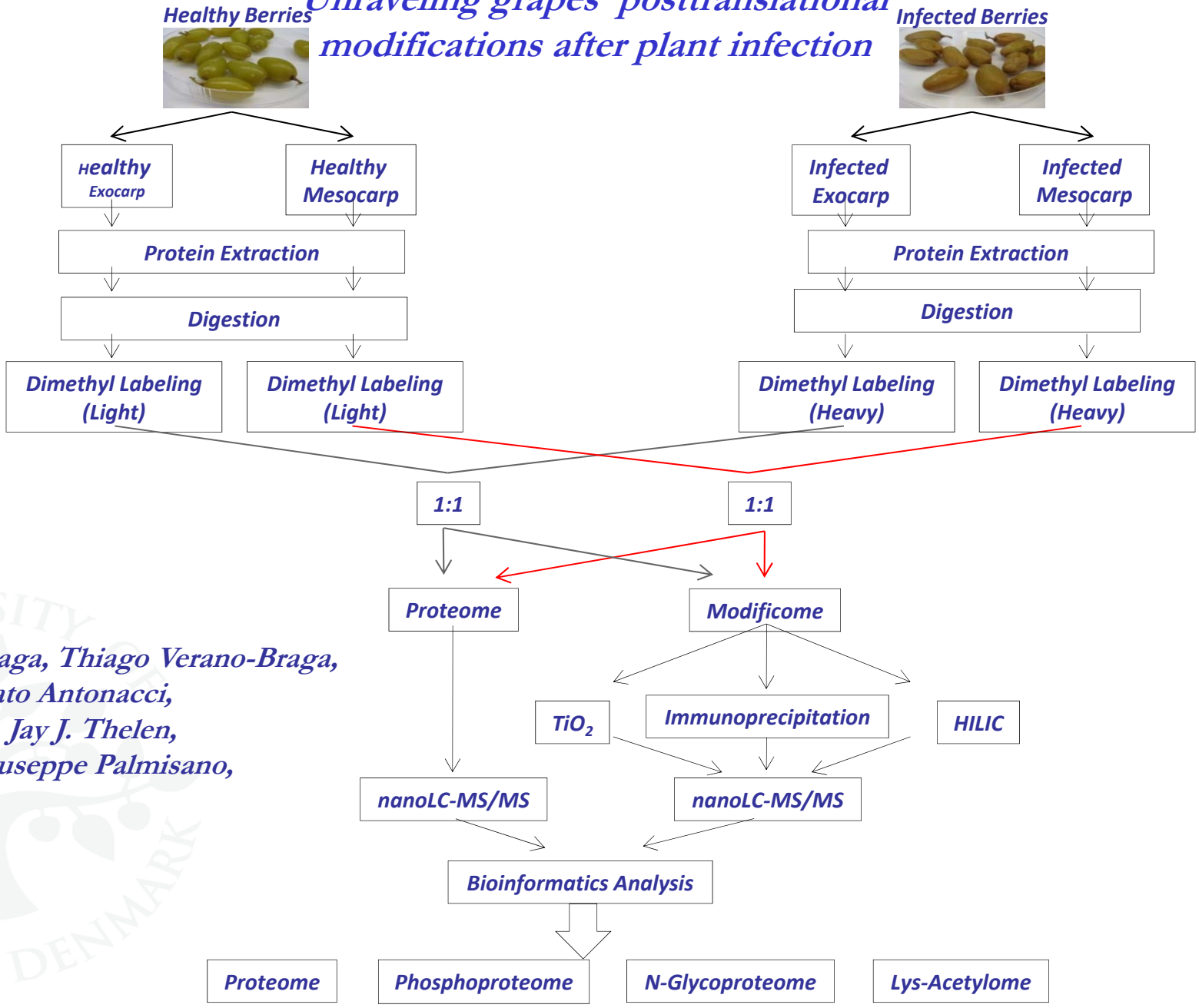
TIME NE

Unraveling grapes' posttranslational modifications after plant infection



Marcella N. Melo-Braga, Thiago Verano-Braga, Ileana R. León, Donato Antonacci, Fábio C.S. Nogueira, Jay J. Thelen, Martin R. Larsen, Giuseppe Palmisano,
MCP just accepted

Protein glycosylation, phosphorylation and acetylation

Comparison between the total number of high confidence proteins, peptides, phosphopeptides, glycopeptides and acetylated peptides from berries.

Sample	Proteins*	Phosphopeptides	Glycopeptides**	Acetylated Peptides
Exocarp	2025	263	567	10
Mesocarp	1907	436	665	18

Proteins containing at least 2 detected peptides. **Deamidated peptides containing the recognition sequence motif N-X-S/T/C for N-glycosydase A.

Comparison between the total number of high confidence proteins, peptides, phosphopeptides, glycopeptides and acetylated peptides identified in infected berries using microorganism protein database.

Sample	Taxonomic Group	Proteins*	Phosphopeptides	Glycopeptides**	Acetylated Peptides
Exocarp	Bacteria	25	19	ND	ND
Mesocarp	Bacteria	121	28	ND	ND
Exocarp	Fungi	31	26	21	ND
Mesocarp	Fungi	105	29	26	ND

Proteins containing at least 2 detected peptides. **Deamidated peptides containing the recognition sequence motif N-X-S/T/C for N-glycosydase A. ND, not determined.

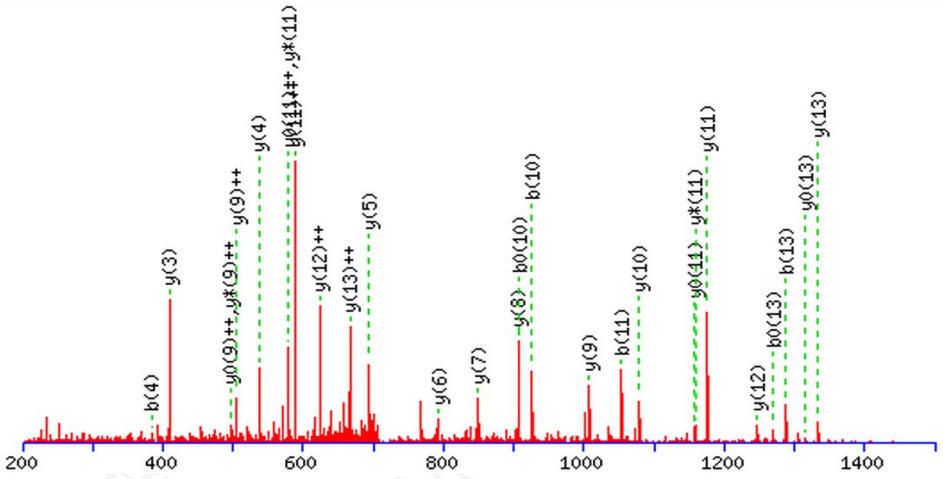
Modifications we have looked at:

Phosphorylation: Ser, Thr, Tyr, His*
Glycosylation: Asn, Ser, Thr*
Oxidation: Met, Cys, Trp
Methylation: Lys, Arg, His
Acylation: Lys, N-terminal
Hydroxylation: Pro, Asp
Carboxylation: Glu*
Pyroglutamate formation: N-terminal Gln
Nitration: Tyr*
Halogenation (Br, Cl): aromatic amino acid residues
Carbonylation, many residues

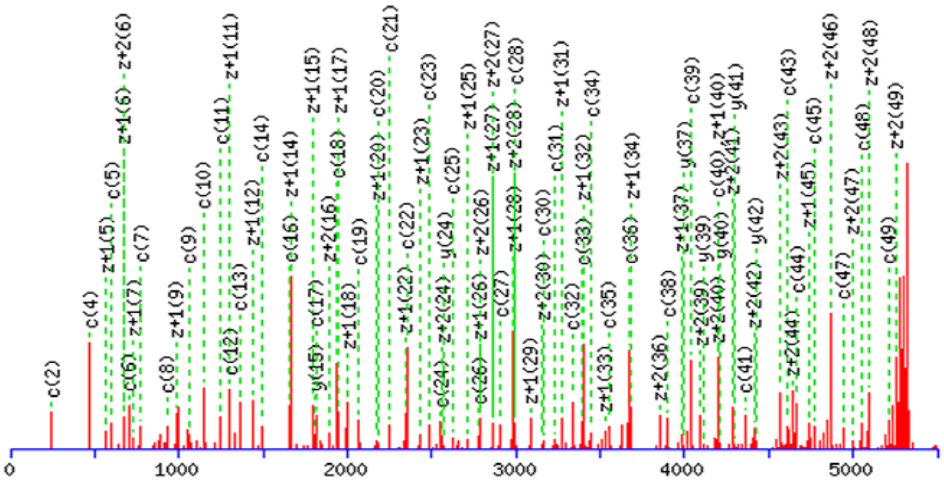
Most of these (except*) are stable under MS conditions and do not cause suppression

Top or middle down proteomics needed to unravel modification codes
Histone H3 tail and possible modifications

Young et al. Cell. Mol. Life Sci. (2011)



CID bottom-up

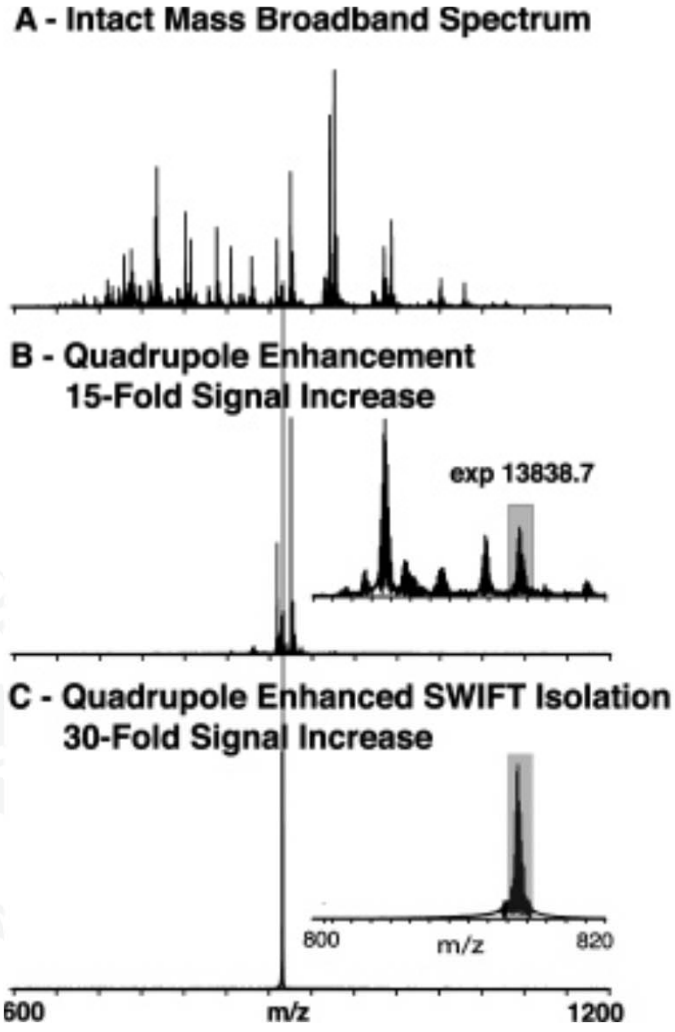


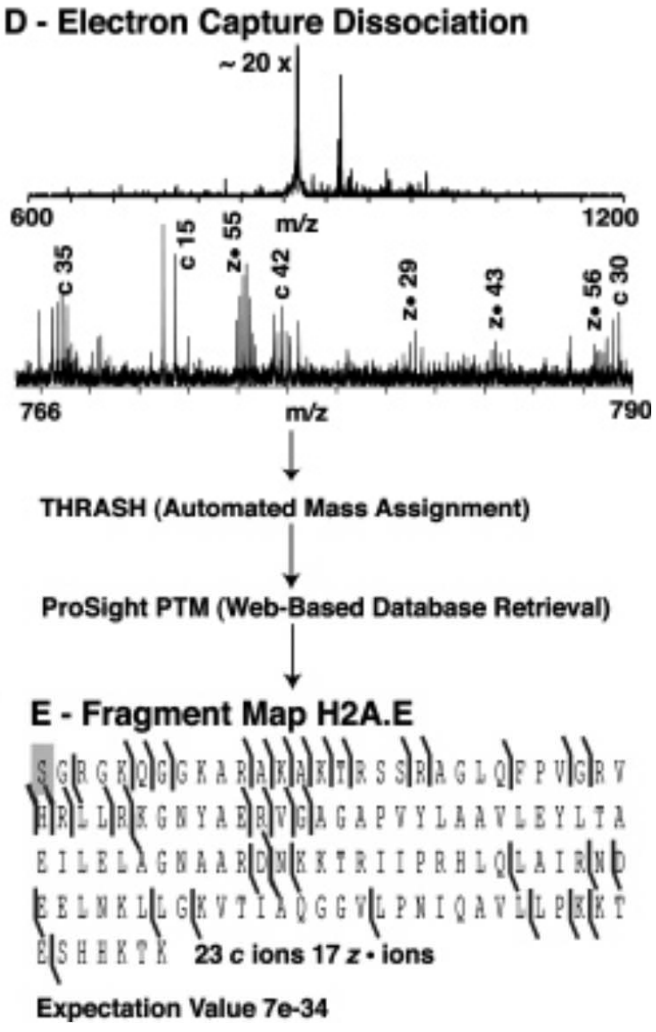
ETD middle-down

Precise Characterization of Human Histones in the H2A Gene Family by Top Down Mass Spectrometry,

Michael T. Boyne II,James J. Pesavento,Craig A. Mizzen and Neil L. Kelleher, Journal of Proteome Research 2006, 5, 248-253

custom built 8.5 T Quadrupole-enhanced Fourier Transform Mass Spectrometer (Q-FTMS)

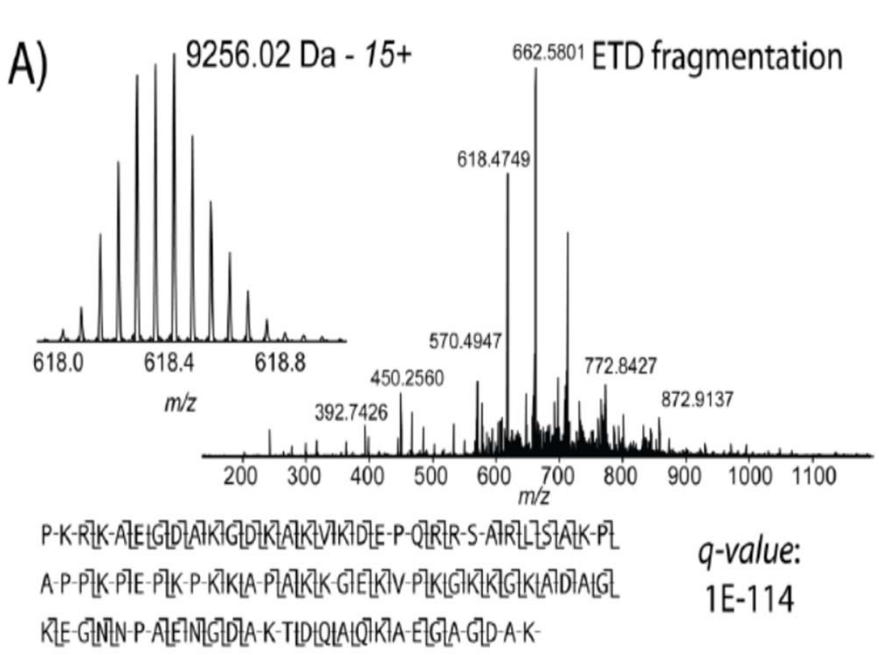


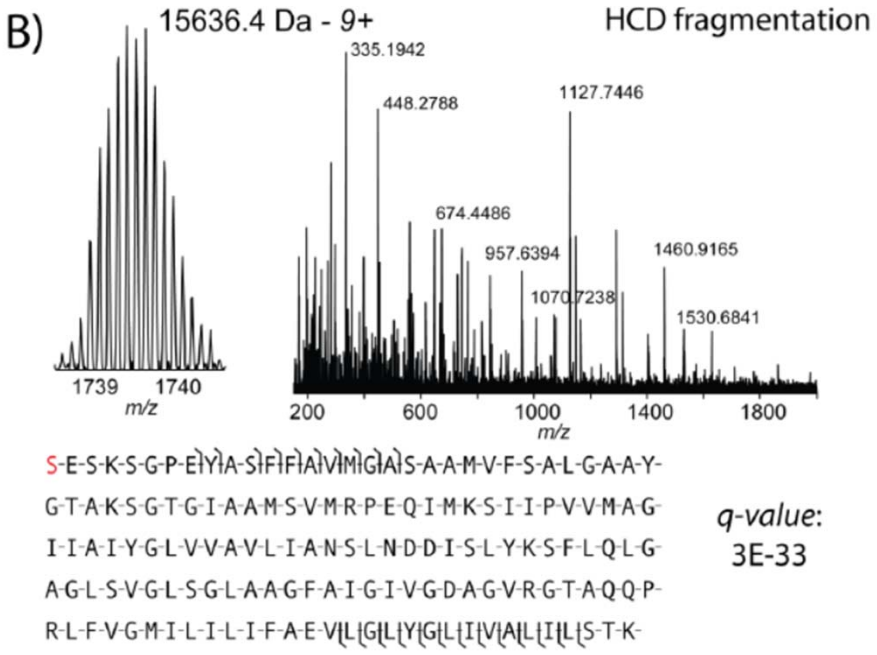


Evaluation of the Compact High-Field Orbitrap for Top-Down Proteomics of Human Cells

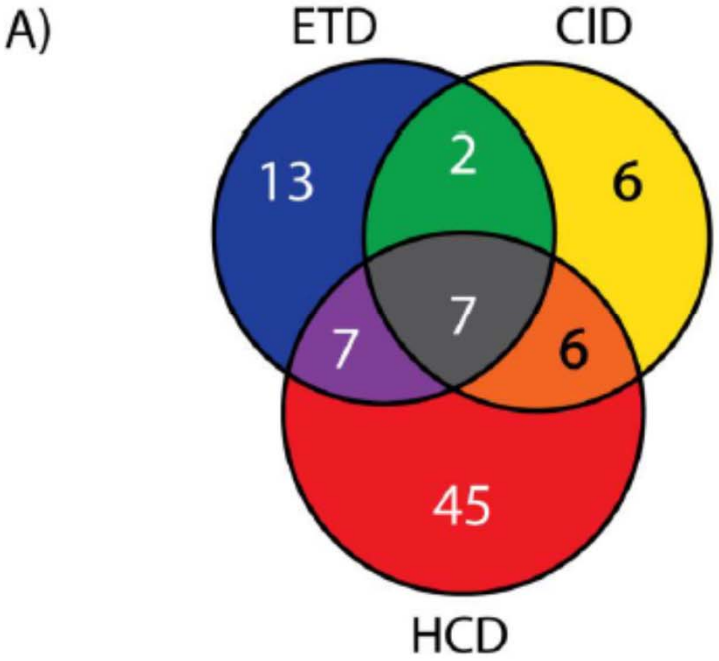
Dorothy Rose Ahlf, Philip Daniel Compton, John C. Tran, Bryan P Early, Paul Martin Thomas, and Neil L. Kelleher. *J. Proteome Research, just accepted*

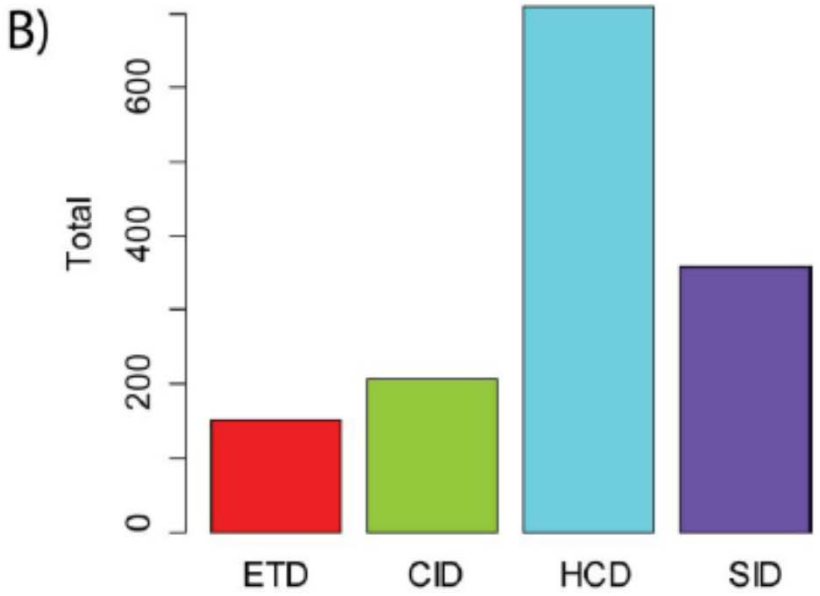
Top down proteomics on an orbitrap elite mass spectrometer





Identifications as function of fragmentation method





Application of this platform to H1299 human lung cancer cells resulted in the unambiguous identification of 690 unique proteins and over 2000 proteoforms identified from proteins with intact masses <50 kDa.

Proteomics levels

Expression proteomics

Which gene products are expressed, when and how much

Modification specific proteomics ”modificomics”

Which variants are present of each protein, when and how much?

Interactomics, Cell map proteomics

Who interacts, when and where

Concepts used for protein interaction and conformational studies by mass spectrometry

Affinity fishing (DNA/RNA-interacting proteins)

Identifying interaction partners

Surface plasmon resonance combined with MS

Identifying interaction partners and interfaces and binding constants

Cross linking/surface labeling

Identifying interaction partners and interfaces and conformation

Deuterium exchange combined with MS

Identifying interaction interfaces, conformation changes and binding constants

Selected Reaction Monitoring / Multiple Reaction Monitoring

The MS western blotting instrument

Why use SRM?

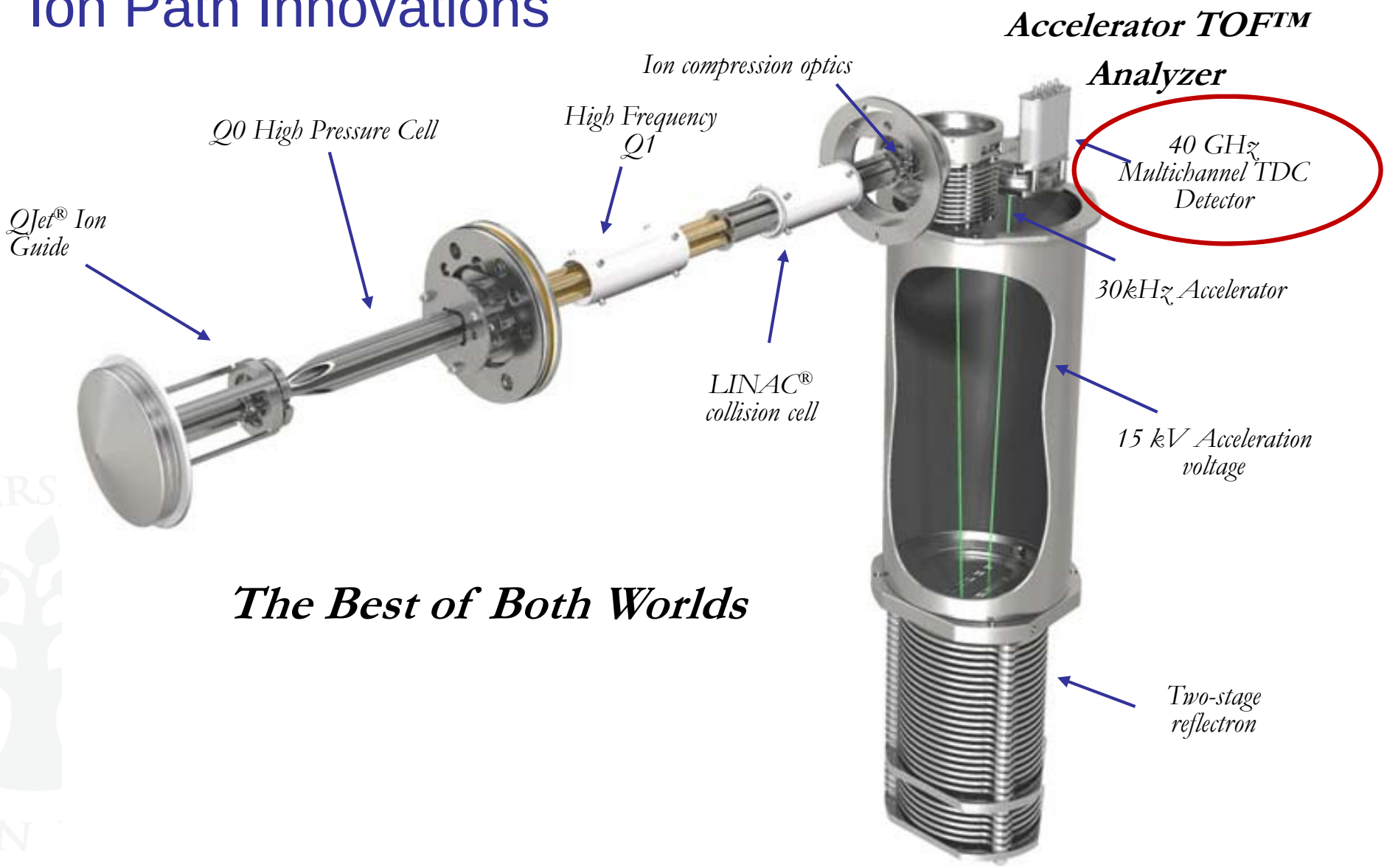
- *It is a targeted and selective validation tool!*
- *With SRM is it possible to quantify many target peptides in one experiment ("multi western blotting").*
- *Using internal standards one can get absolute concentrations of target peptides*
- *The reproducibility is better then usual discovery experiments and even between labs*
- *It is possible to tune each transition and thereby increasing sensitivity*
- *Good dynamic range for detecting peptides at different concentrations (four orders of magnitude).*

EASY-nLC™

TSQ Vantage

AB SCIEX TripleTOF™ 5600 System

Ion Path Innovations



The Best of Both Worlds

Future needs

Improved software for

- *de novo sequencing*
- *Assignment of PTM's*

Improved separation techniques for

- *Chromatographic protein separation compatible with MS for top down proteomics*

New or improved enrichment techniques

- *for organell enrichment*
- *for PTM's, e.g. oxidation, acylation etc.*

Cheaper and more robust instrumentation

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Protein Research Group December 2011