**Proteomics Standards Research Group (sPRG)**

www.abrf.org/sprg

# ABRF-sPRG2011 study: development and characterization of a comprehensive standard for analysis of post-translational modifications


***Proteomics Standards Research Group (sPRG)***

# sPRG

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Susan T. Weintraub *University of Texas HSC*

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# Corporate Collaboration

**Sigma-Aldrich** – *intact proteins*
Kristin Rolwes, Shantanu Roychowdhury, George Lipscomb

**Thermo Fisher Scientific** – *synthetic peptides*
Rainer Gebhart, Georgi Videnov, Joel Louette, Manuela Schaffrath

**Michrom Bioresources**– *STTR grant application for future collaborations*
Kerry Nugent

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# Rationale

• Characterization of PTMs and post-translationally regulated cellular processes is one of the major reasons for proteomics to stay

**Post-translational modifications of proteins:**

• Major role in regulation of cellular processes.
• Analysis of PTM sites is a major challenge.
• Every PTM poses characteristic analytical difficulties.
• New techniques and approaches are emerging.
• More efficient PTM characterization may open new landscapes for the sudy of biology in health and disease.
• What works best?
•How to get better?
•How to expend the toolkit?

# Background

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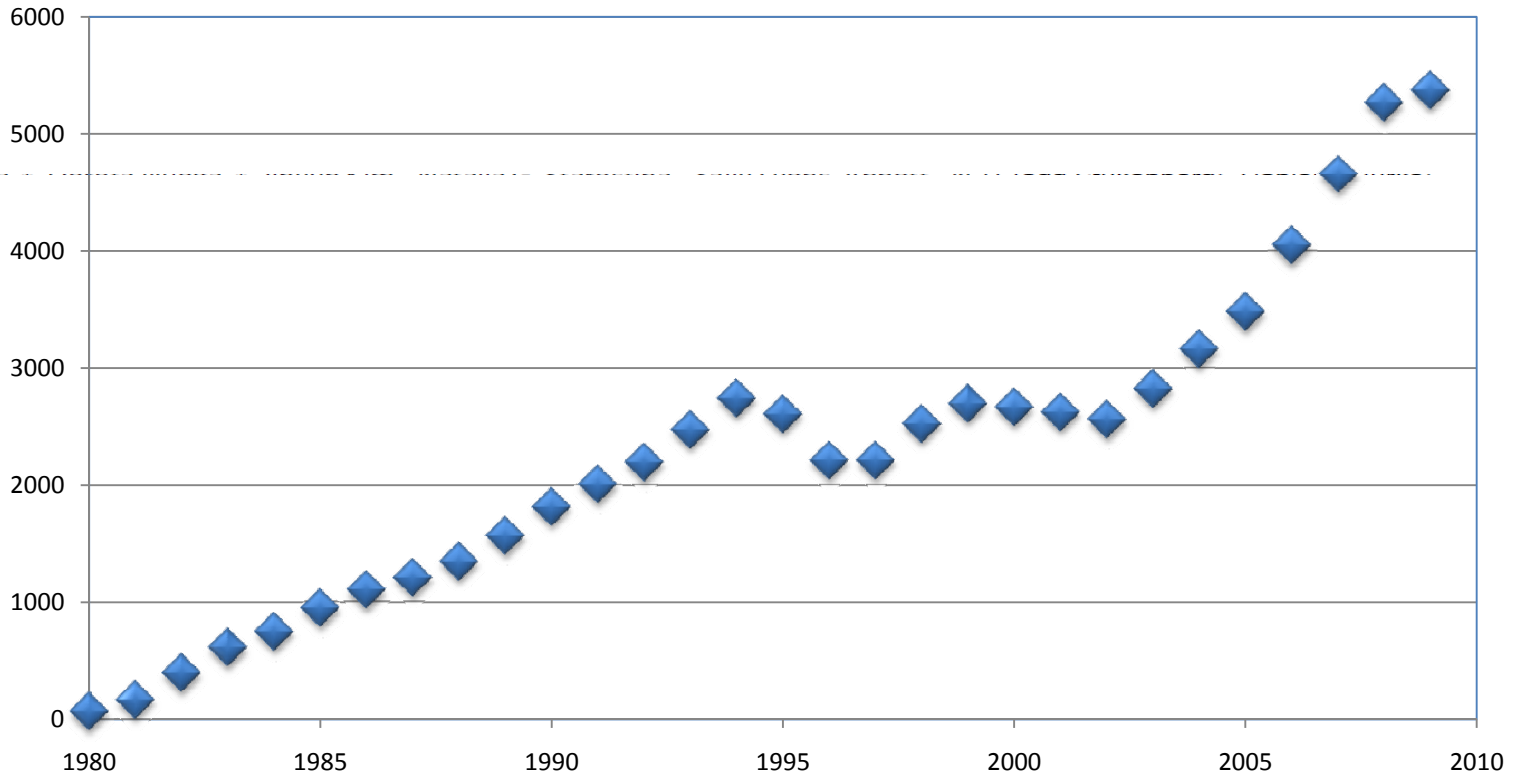
**PubMed # of Publications ("PTM" or "post-translational modification" or "post-translational protein modification“)**




Fine Mapping of Posttranslational Modifications of the Linker Histone H1 from *Drosophila melanogaster*

Ana Villar-Garea, Axel Imhof*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# Background

**PRG 2003 Study**

2 synthetic phosphopeptides and 2 digested proteins

**sPRG 2007 Study**-- phosphopeptide standard ver 1.0

(mixture of seven protein digests)

**sPRG 2010 Study** – phosphopeptide standard ver 2.0

(23 synthetic phosphopeptides (mono-, di-, tri-, tetra-) including 2 peptides from PRG 2003 in the background of equimolar digest of six proteins

**iPRG 2010 Study** – Informatic Evaluation of Phosphopeptide Identification and Phosphosite Localization

**sPRG 2011 Study** – PTM standard (phosphorylation, acetylation, sulfation, nitration, methylation)

# Background: sPRG2010 take home messages

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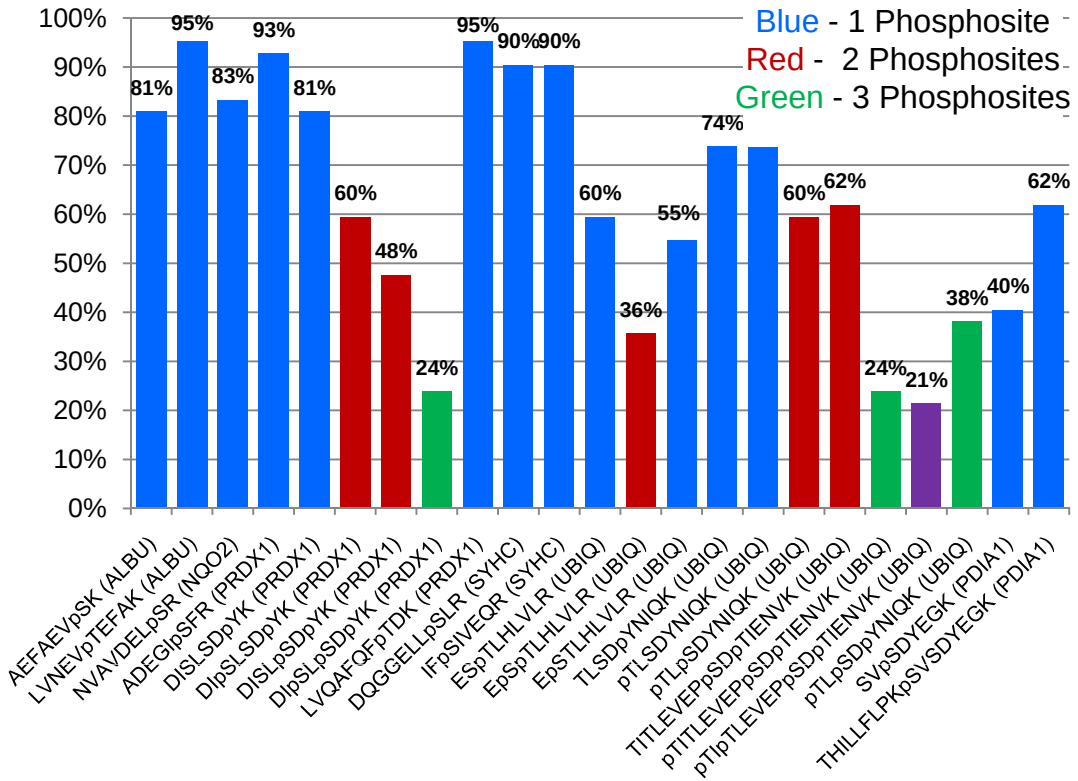
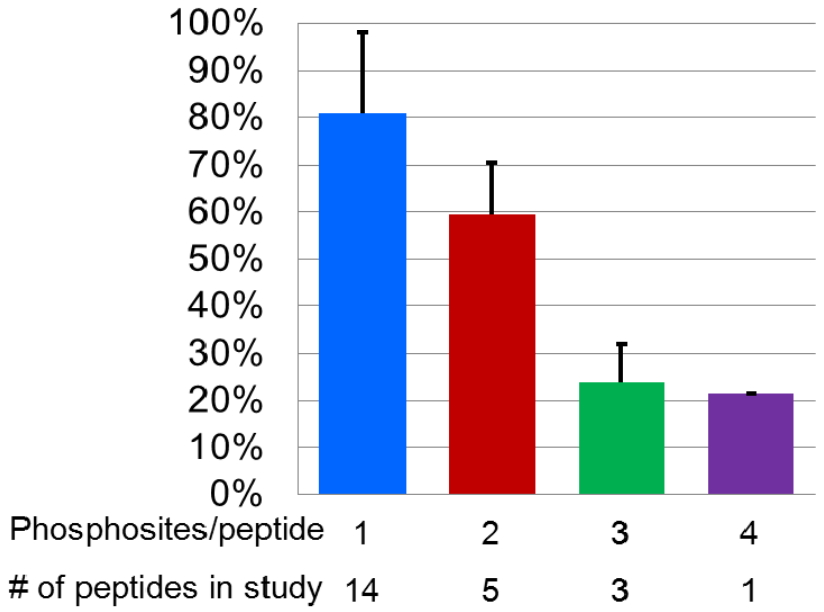
| | | 2010 | | 2003 | |
|---|---|---|---|---|---|
| Protein | Sequence | # of times ID | % ID | # of times ID | % ID |
| PDIA1_BOVIN | SVpSDYEGK (PDIA1) | 17 | 40% | 8 | 15% |
| PDIA1_BOVIN | THILLFLPKpSVSDYEGK (PDIA1) | 26 | 62% | 8 | 15% |

Phosphopeptide identification rate for 2003 and 2010 sPRG studies.




Success of Detection by Number of Phosphosites per Peptide

# Background: iPRG2010

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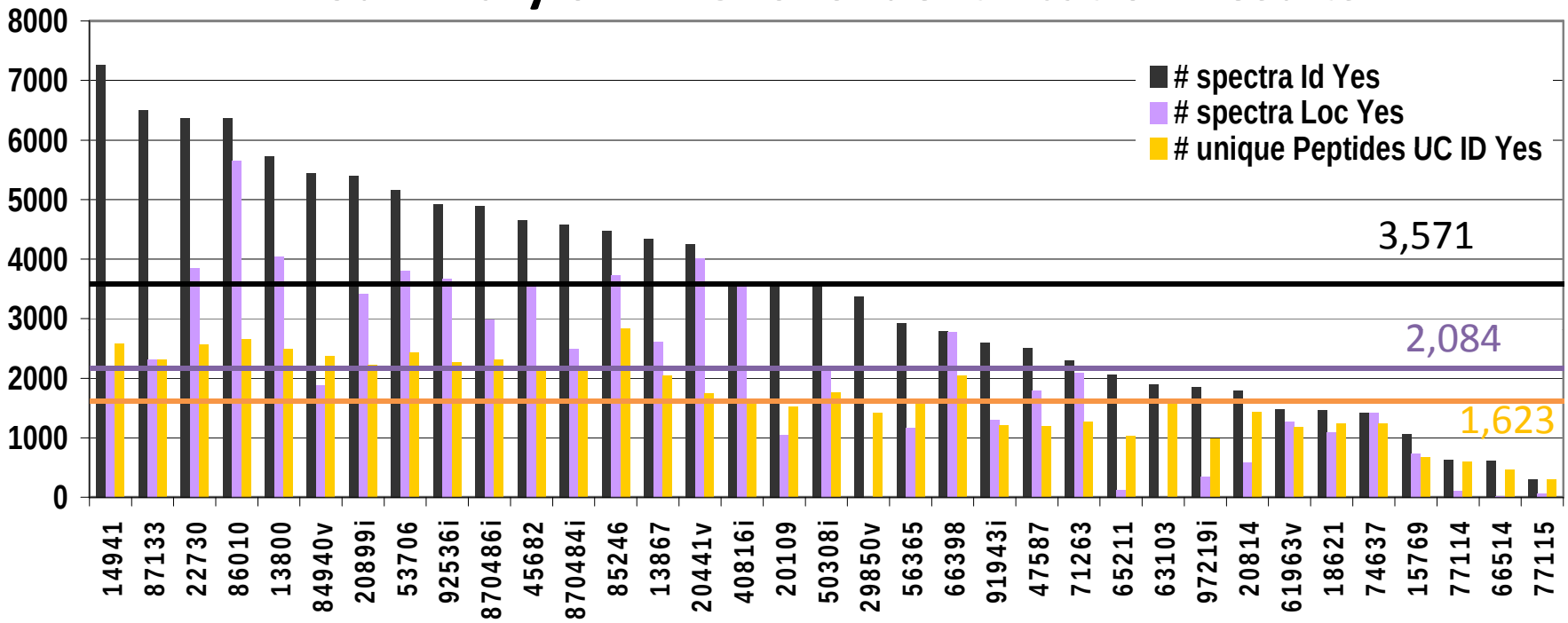
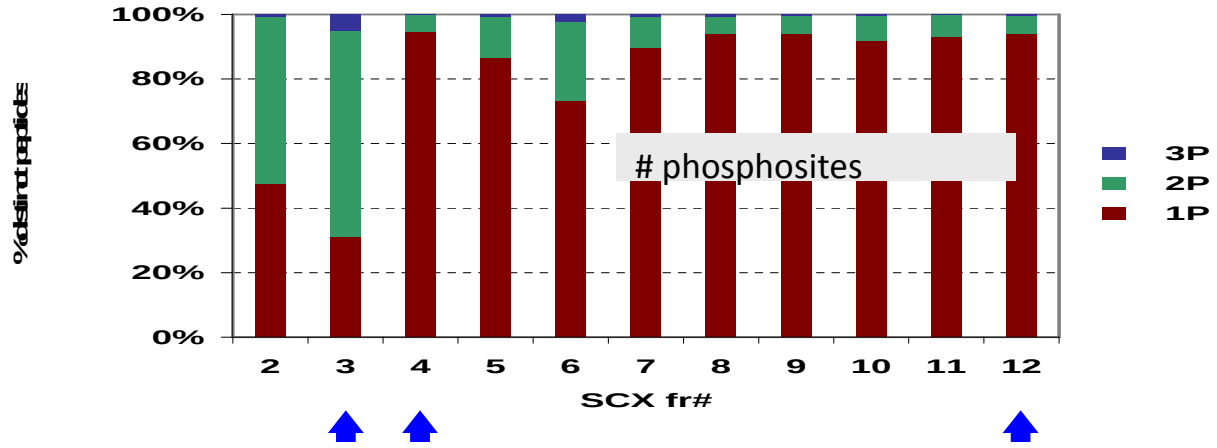
## Summary of iPRG2010 Identification Results




Courtesy of iPRG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# sPRG 2011 Study Objectives

**Goals:**

To develop a readily available standard for:

•assessment of a laboratory's ability to detect an array of PTMs in a complex proteomic sample,

•development of new approaches for characterization of post-translationally modified proteins,

•generate guidelines for effectual analysis of the selected modifications.

**Study Design:**

• To establish collaborative partnership with commercial companies to enable the study and to secure prospective commercialization of the standard.

•To allocate two years for the study instead of the typical 1-year long RG study time frame.

# sPRG 2011 Study Design

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**Thermo**

*Synthetic peptides*

~30 O-phosphopeptides (S,T, and Y):
~15 multi-phospho (di, tri, & tetra)
***including positional isomers***

5 sulfotyrosine
5 nitrotyrosine
5 acetylated Lys
5 monomethylated Arg
5 monomethylated Lys
5 dimethylated Lys
5 trimethylated Lys
3 asymmetric dimethylated Arg
3 symmetric dimethylated Arg

QC

**sPRG2011 standard**

QC

**Sigma**

*Intact Proteins*

ALBU PDIA1
PRDX1 UBIQ
NQO2 SYHC

SPE cleanup

Tryptic digestion

**sPRG**

# sPRG 2009 Sample Development

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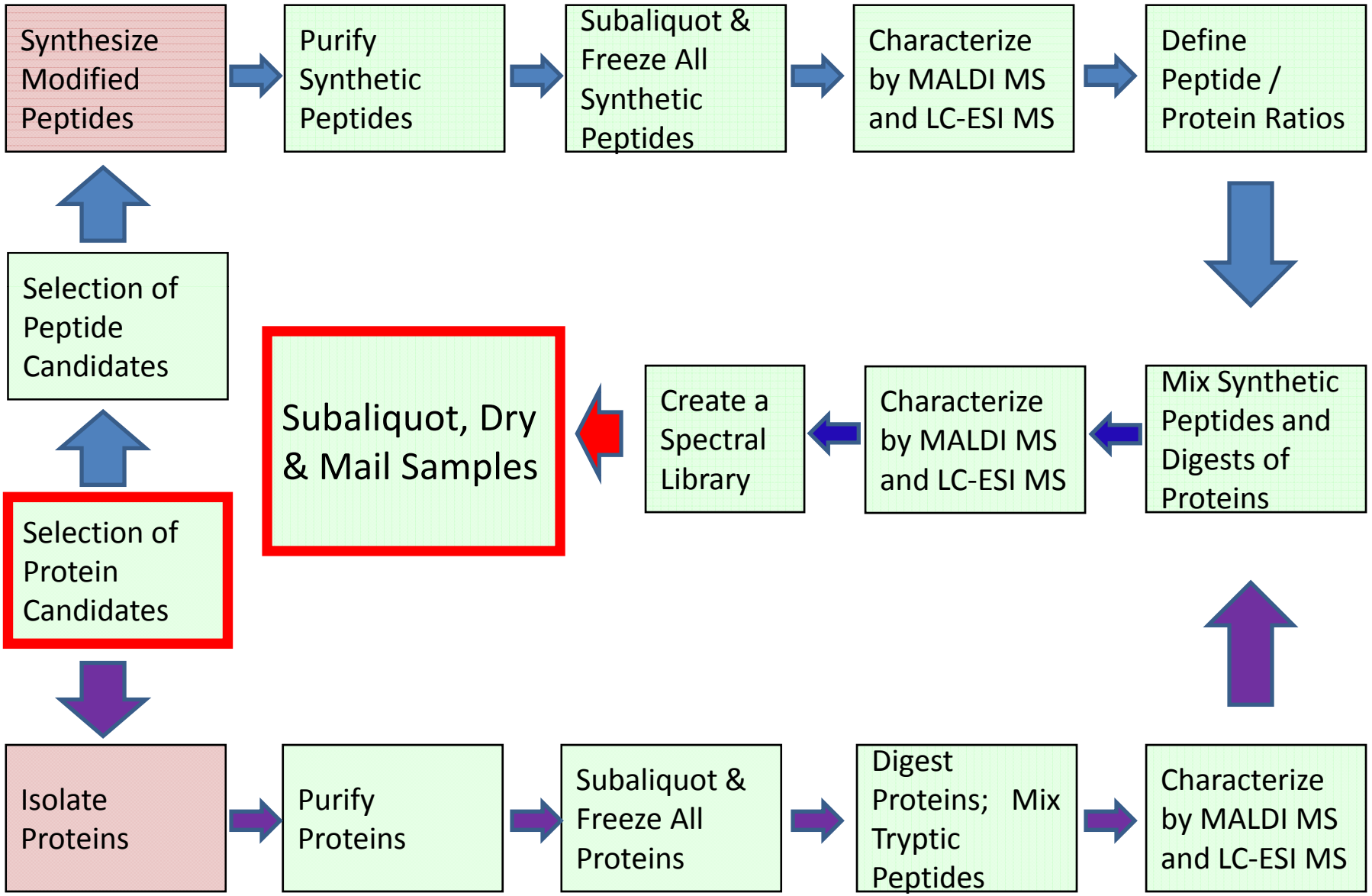


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# Preliminary Observations

## "Bonus" proteins

**Protein Standards Used:**

1. Histidyl-tRNA synthetase (human)
2. Peroxiredoxin 1 (human)
3. Protein disulfide isomerase (bovine)
4. Quinone reductase 2 (human)
5. Serum albumin (Human)
6. Ubiquitin (human)

**Proteins Detected:**

✓Histidyl-tRNA synthetase (human)
✓Peroxiredoxin 1 (human)
✓Protein disulfide isomerase (bovine)
✓Quinone reductase 2 (human)
✓Serum albumin (Human)
✓Ubiquitin (human)
*Apolipoprotein A-I (bovine)*
*Calmodulin*
*Tropomyosin alpha chain (human)*
*Cytochrome b5 (bovine)*
*Glyceraldehyde 3-phosphate dehydrogenase (E. coli)*
*Senescence marker protein-30 (bovine)*
…

# “Bonus” peptides. Synthesis byproducts.

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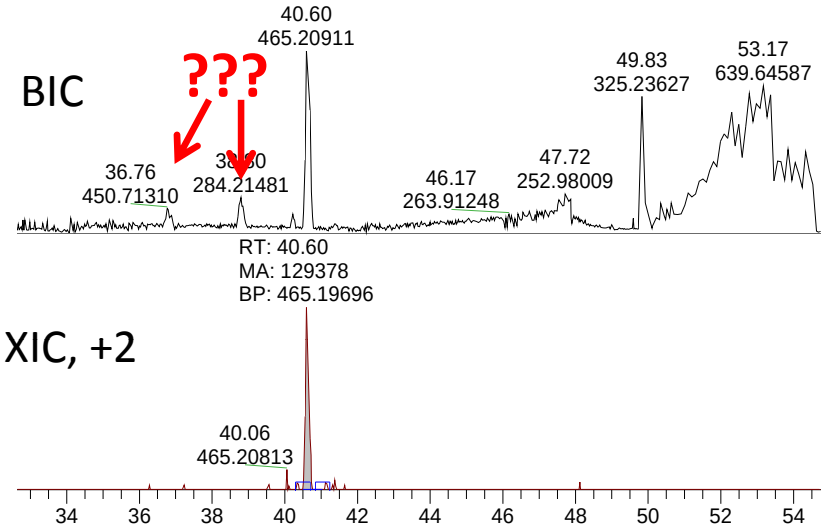
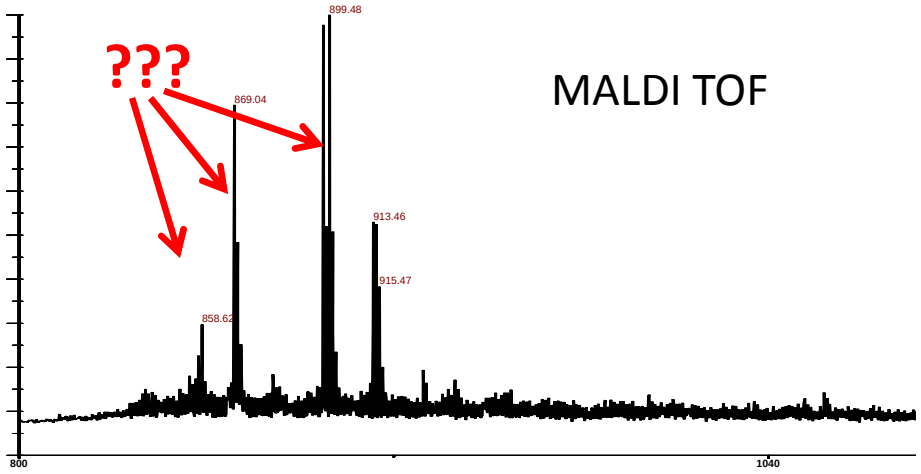
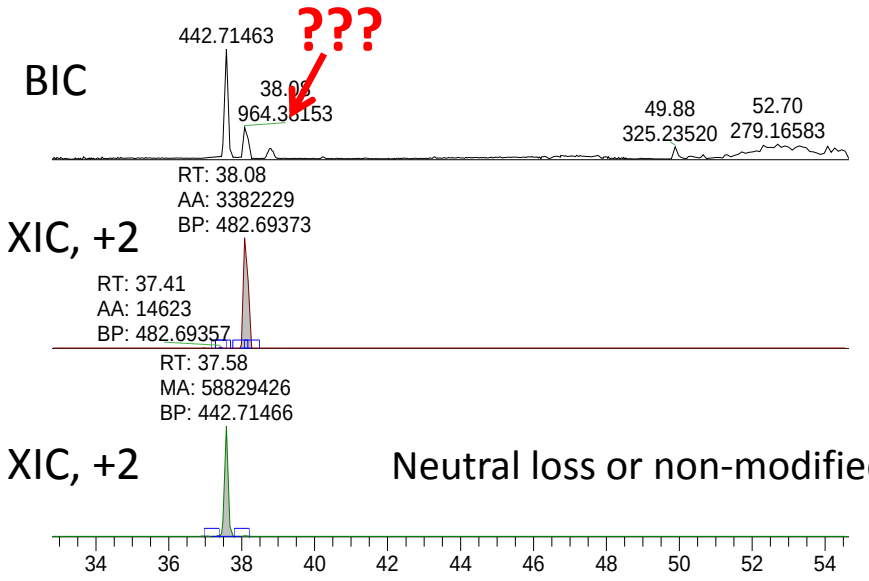
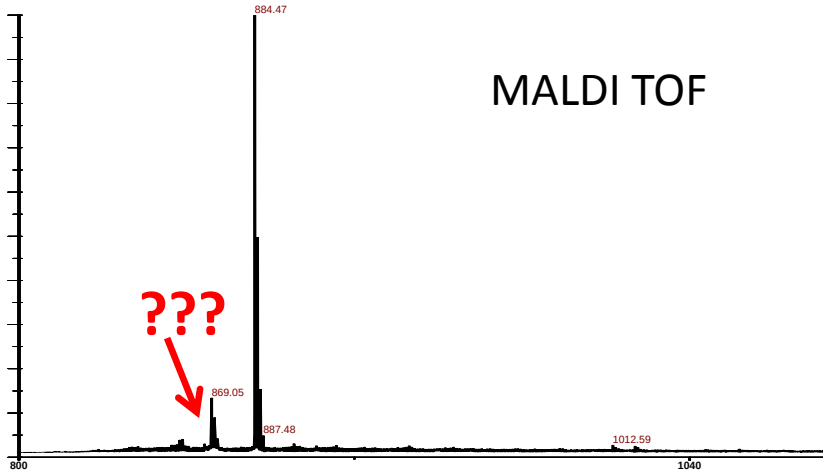



SVSDnYEGK ; MW 928.37738; m/z 465.1965 (z=2)




SVSDsYEGK , MW 963.3491; m/z 482.6824 (z=2)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# Instrument Platforms Used by the sPRG to Validate the Sample and Individual Sample Constituents

**NanoLC ESI MS:**

nLC – LTQ Orbitrap (Thermo)
nLC – LTQ Orbitrap Velos (Thermo)
nLC – LTQ (Thermo)
QQQ TSQ Vantage (Thermo)

**MALDI MS:**

Axima TOF/TOF (Shimadzu)
AB4700 TOF/TOF (AppliedBio)
AB4800 TOF/TOF (AppliedBio)

**Fragmentation Modes**

CID, HCD, ETD

CID, PSD

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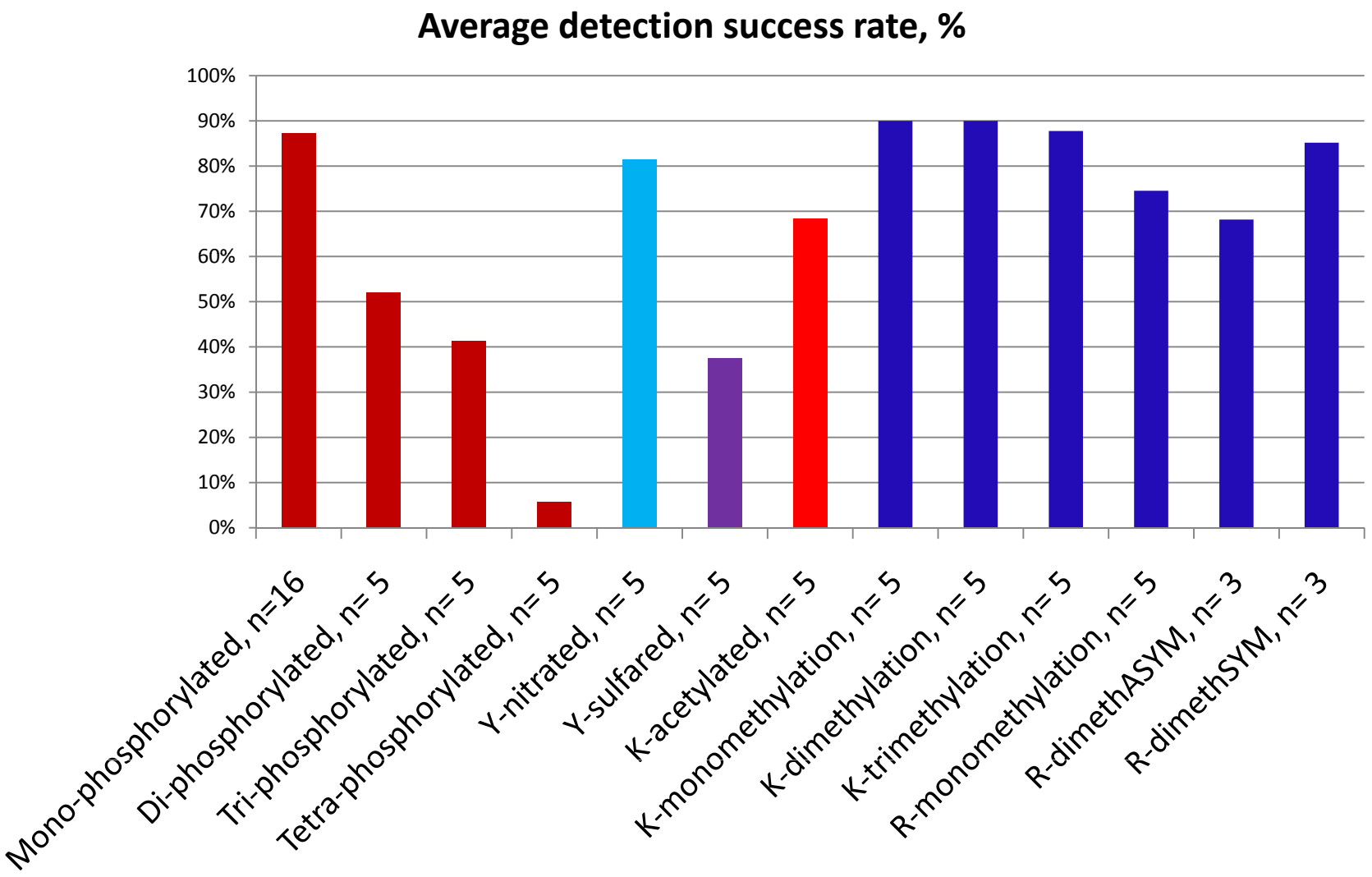
# Results of the Initial Sample Validation by the sPRG

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# Preliminary Observations. Multiply Phosphorylated Peptides.

- Difficult to detect without any enrichment
- Often elute in broad peaks
- Poor ionization efficiency
- Predominant neutral loss in MS2. Poor CID fragmentation efficiency
- Some detected as ion species corresponding to partially dephosphorylated forms possibly due to in-source decay
- Additional use of alternative to CID fragmentation modes and multistage activation CID fragmentation was helpful
- Additives to the sample mixture (EDTA, phosphate, citrate, etc) helped improve the MS signal for some peptides
- Optimization of LC separation conditions and MS data acquisition settings helped improve detection sensitivity for closely eluting  isobaric positional isomers and sulfopeptides
- RT prediction and site localizition algorithms might be helpful

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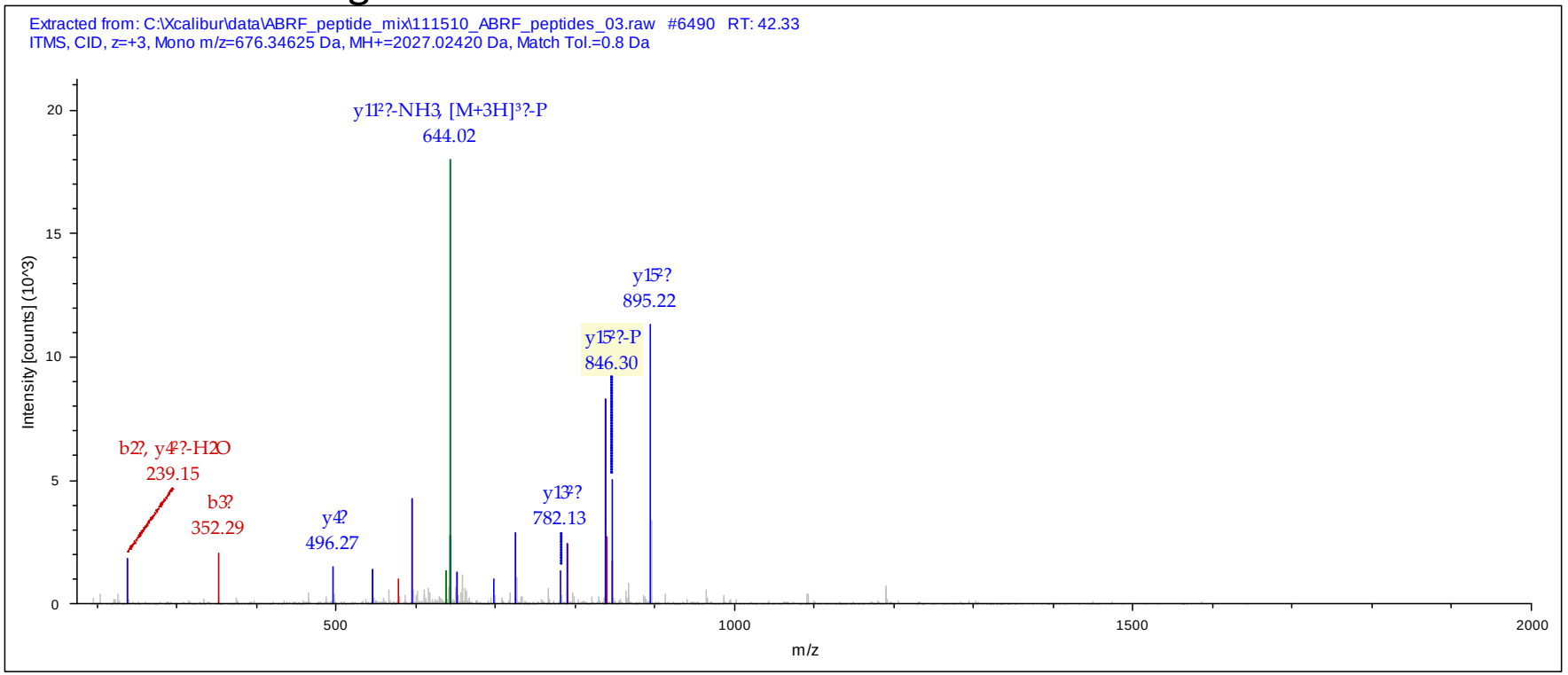
# Alternative Fragmentation Modes: CID, z = 3

Pros:

- Nearly 100% efficient for non-neutral loss phosphorylations, and >50% efficient for neutral loss phosphorylations
- Same collision voltage is used for all peptides

Cons:

- Many phosphorylations show loss of 98, sometimes making assignment of the site difficult



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# Alternative Fragmentation Modes: ETD , z = 3

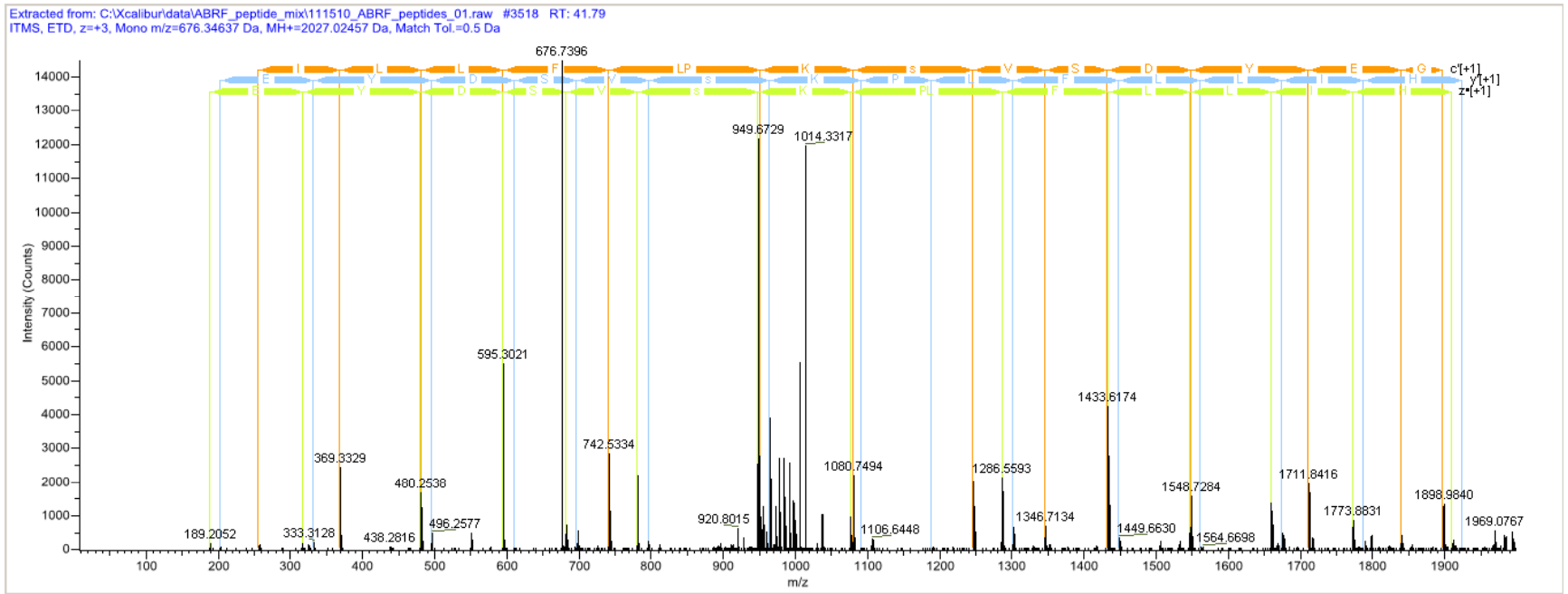
Pros:

- Does not cleave phosphorylation

Cons:

- Efficiency ~30% maximum for observed phosphorylations (% of ions that are assignable as b and y-type ions / total estimated ions)

T⌈H⌋I ⌋L⌋L⌋F⌋L P⌋K⌋**s**⌋V⌋S⌋D⌋Y⌋E⌋G⌋K



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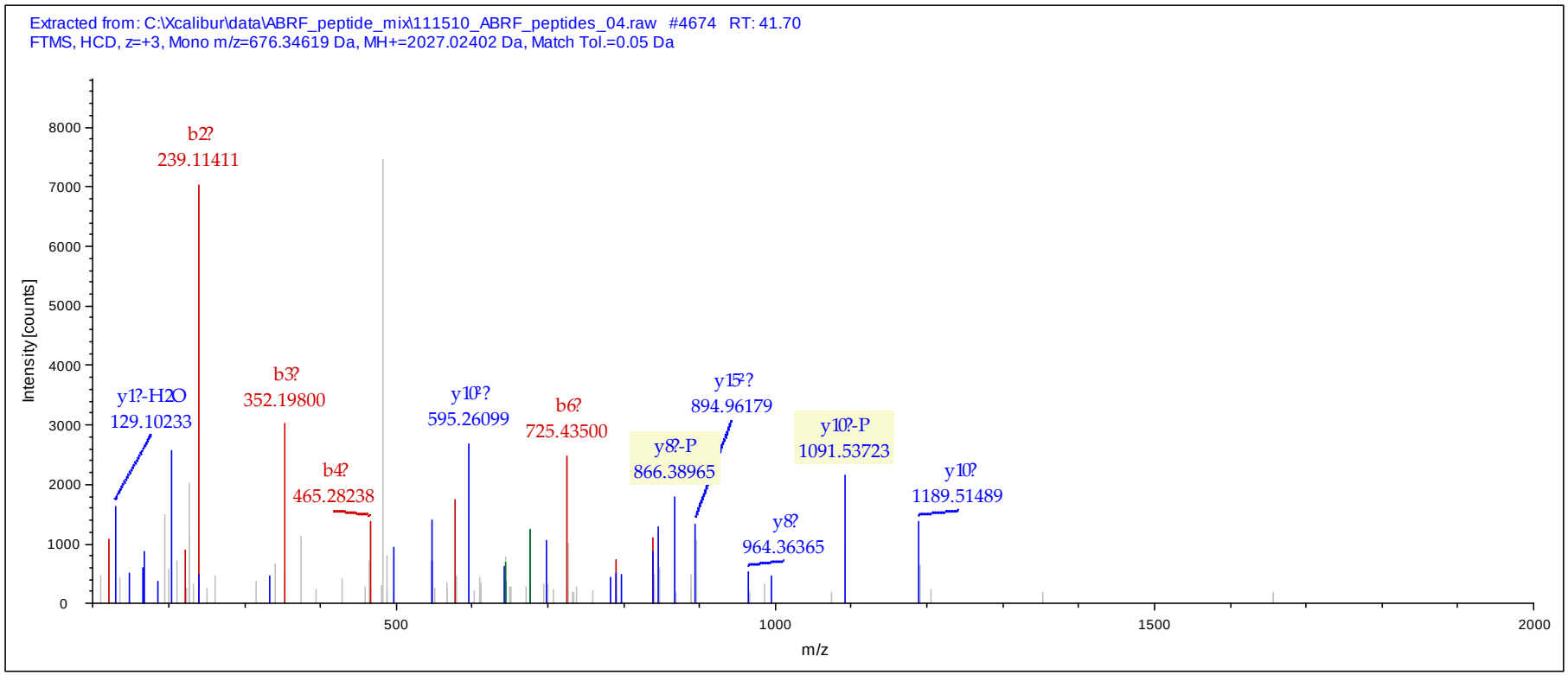
# Alternative Fragmentation Modes: HCD, z = 3

Pros:

- Can be used to produce transitions for triple quadrupole work

Cons:

- Hard to get the optimal energy for each different peptide, either leaving behind undissociated precursor or producing second generation product ions which are no usable in a database search



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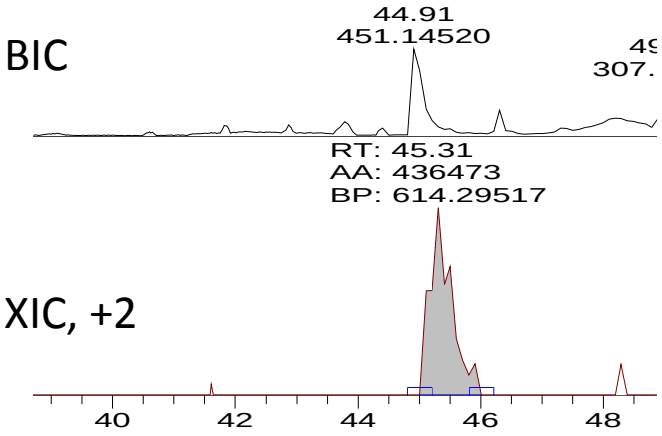
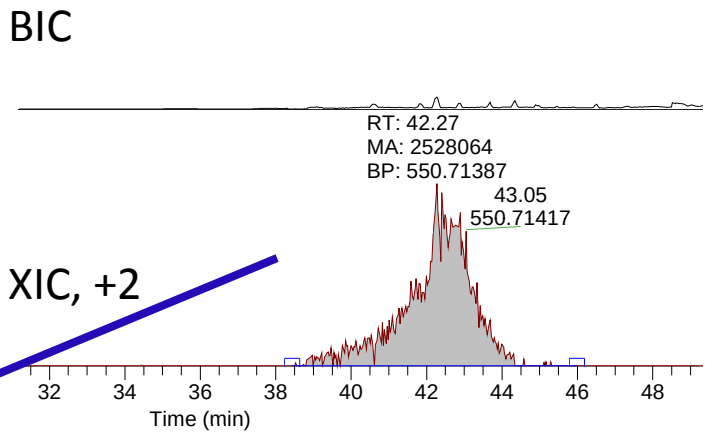
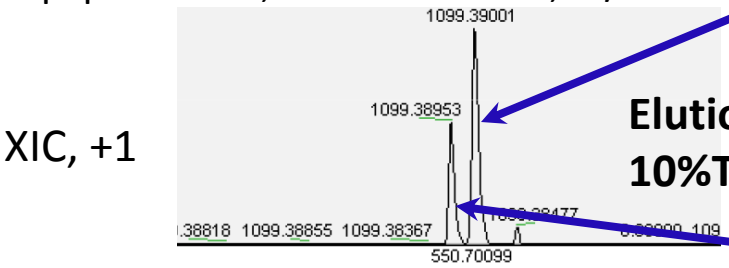
# Multiply Phosphorylated Peptides: Peak Broadening, Co-elution of Positional Isoforms, Low Signal



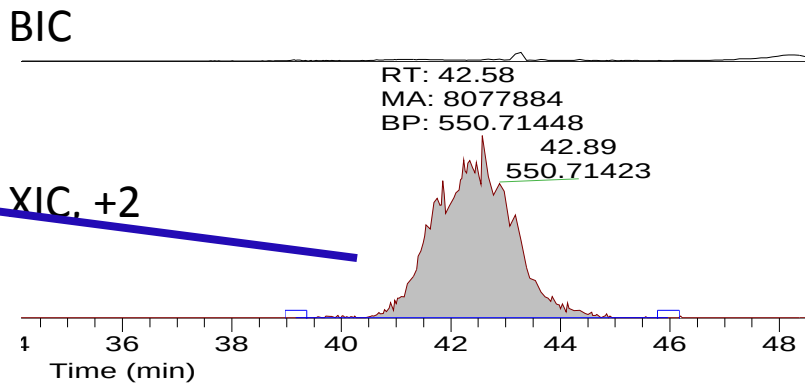

EpSpTLHLVLR ; MW 1226.5461; m/z 614.2809 (z=2)




DIpSLpSDpYK ; MW 1179.3539; m/z 590.6848

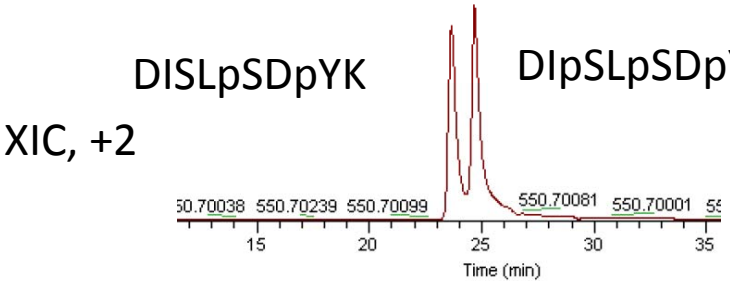



**Elution with 10%TFE**




DISLpSDpYK

DIpSLpSDpYK




DISLpSDpYK; MW 1099.3876; m/z 550.7016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# Sulfotyrosine-containing peptides

- Predominant neutral loss peaks
- Poor fragmentation efficiency
- Intense Na and K adducts
- Elution times and *m/z* values for sulfo- and phospho-isopeptides are very close but possible to differentiate with higher mass accuracy, optimized separation conditions and RT prediction
- Some sulfopeptides reveal several isobaric LC-MS1 peaks with close RTs

# Sulfated Peptides: Neutral Loss

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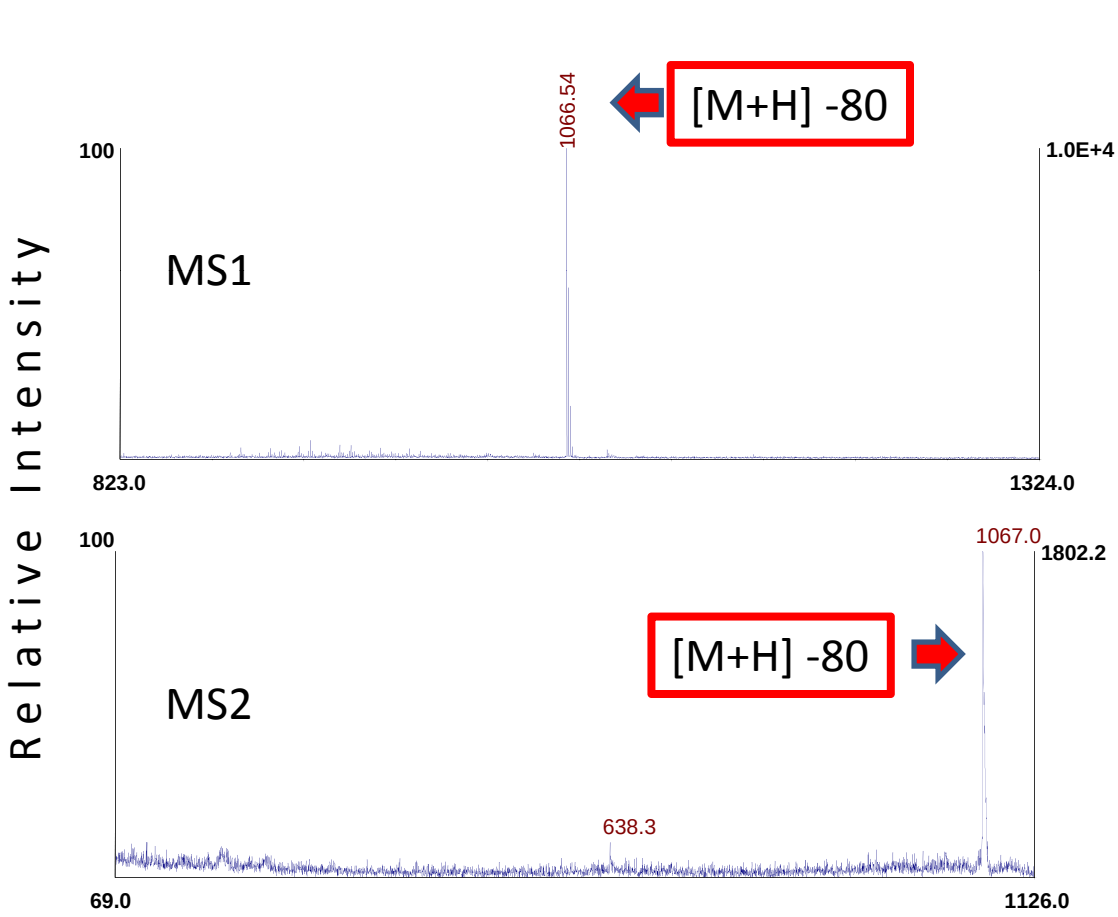
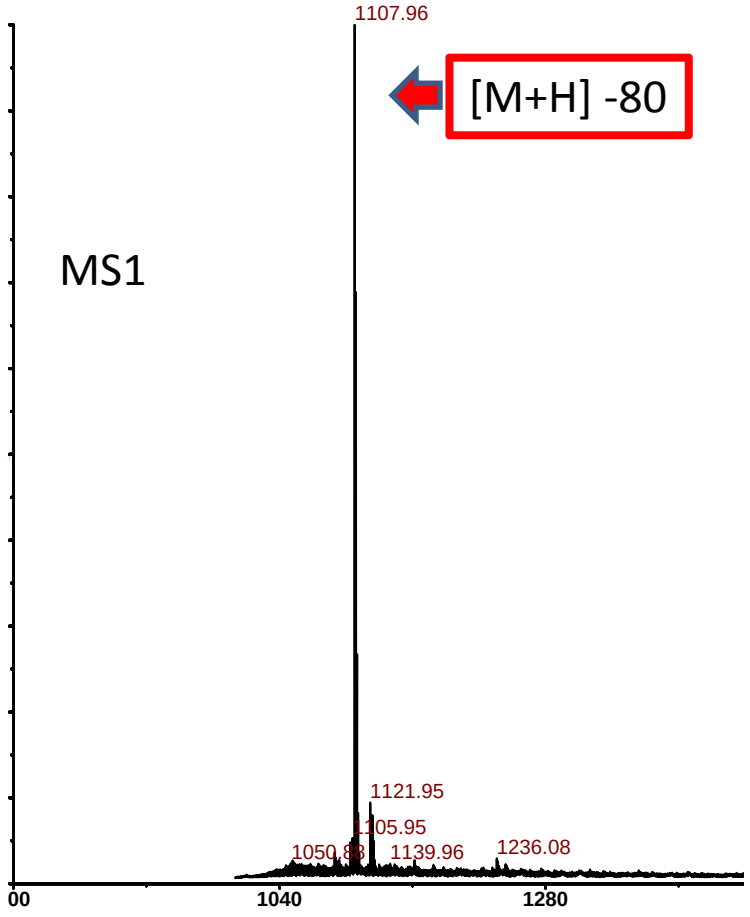
MALDI TOF/TOF




TVIDsYNGER; m/z 1146.47




TIAQDsYGVLK ; m/z 1187.56

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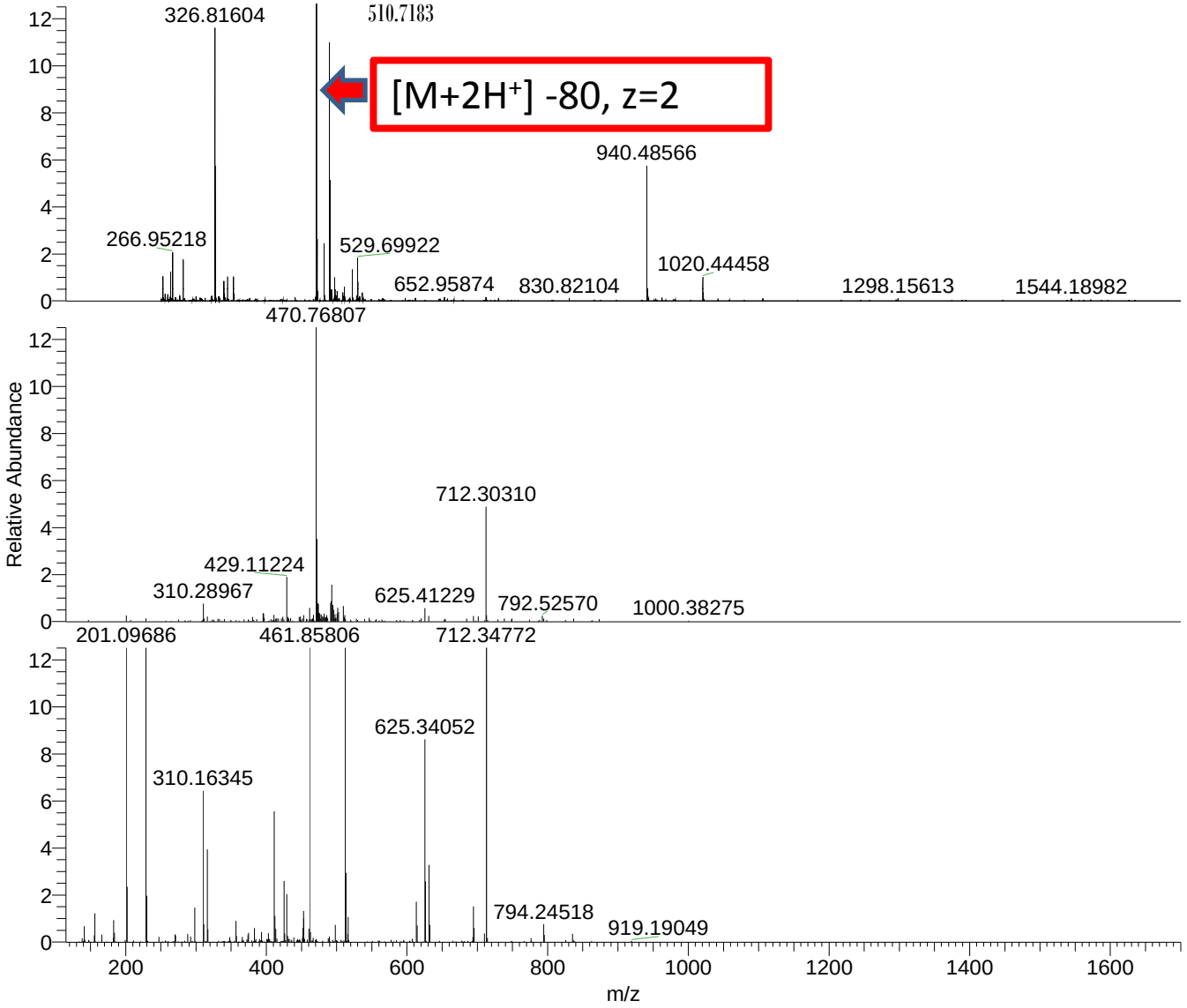
# Sulfated Peptides: Neutral Loss and Poor Fragmentation

MS1

MS2 on the sulfopeptide (m/z 510.71, z=2)

MS2 on the non-modified peptide (m/z 470.74, z=2)



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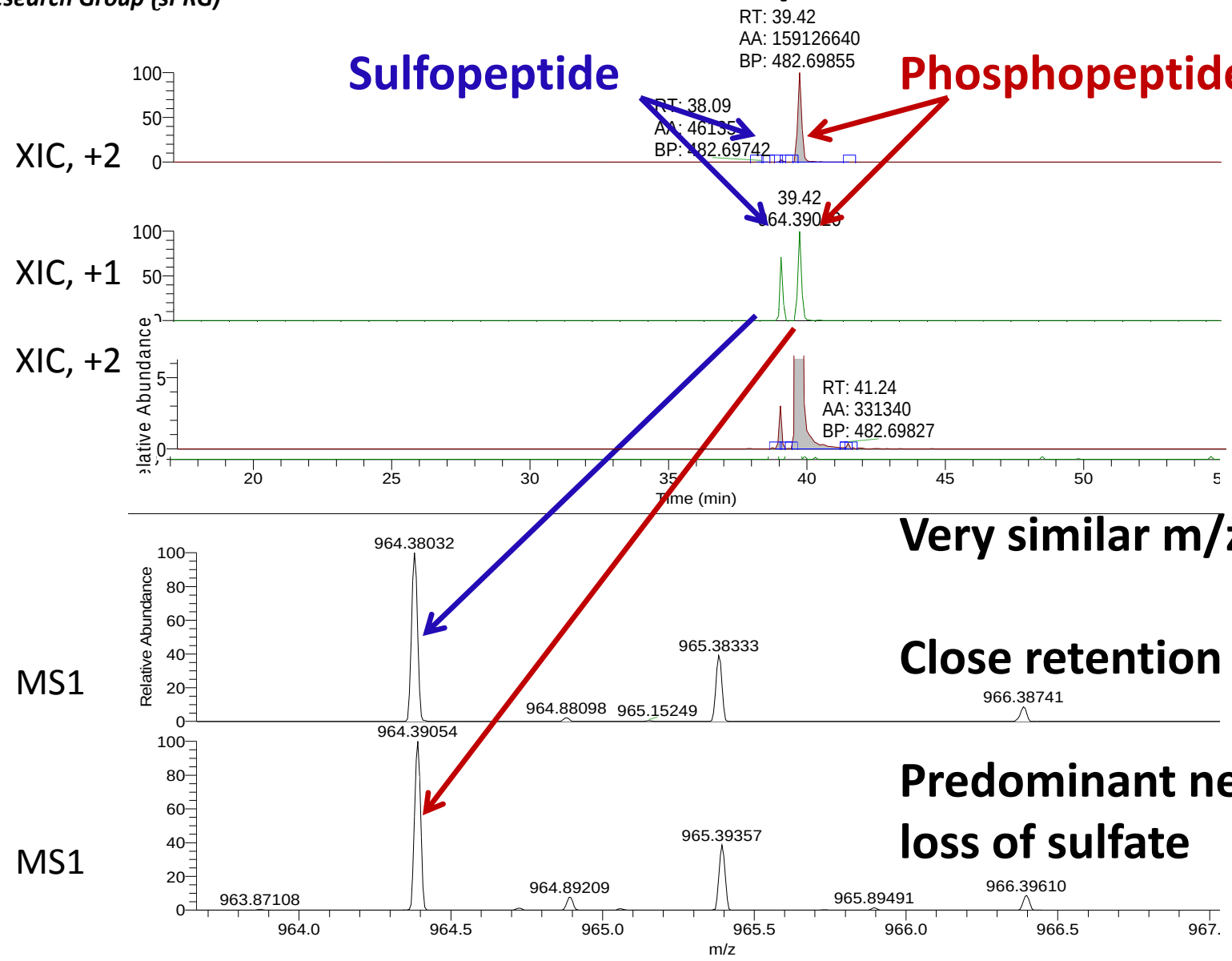
# Sulfated and Phosphorylated Peptides




**Very similar m/z values;**

**Close retention times;**

**Predominant neutral loss of sulfate**

# Nitro-Tyr, Acetyl-Lys, and Methyl-Lys/Arg

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- **Nitro-Tyr**
  Intense neutral loss ions and Na and K adducts (not as pronounced as in sulfotyrosine peptides)

- **Acetyl-Lys** Some result in low intensity signal

- **Methyl-Lys and Arg**

o Close elution or co-elution of mono-, di-, and tri-methylated peptides (shallower gradients are helpful)

o Co-elution of symDIMETH-R and asymDIMETH-R peptides on C18

o Some symDIMETH-R and asymDIMETH-R peptides were indistinguishable in CID and ETD spectra

o Some DIMETH-R peptides demonstrate predominant neutral loss in MS2 spectra;

# Differentially modified peptide isoforms

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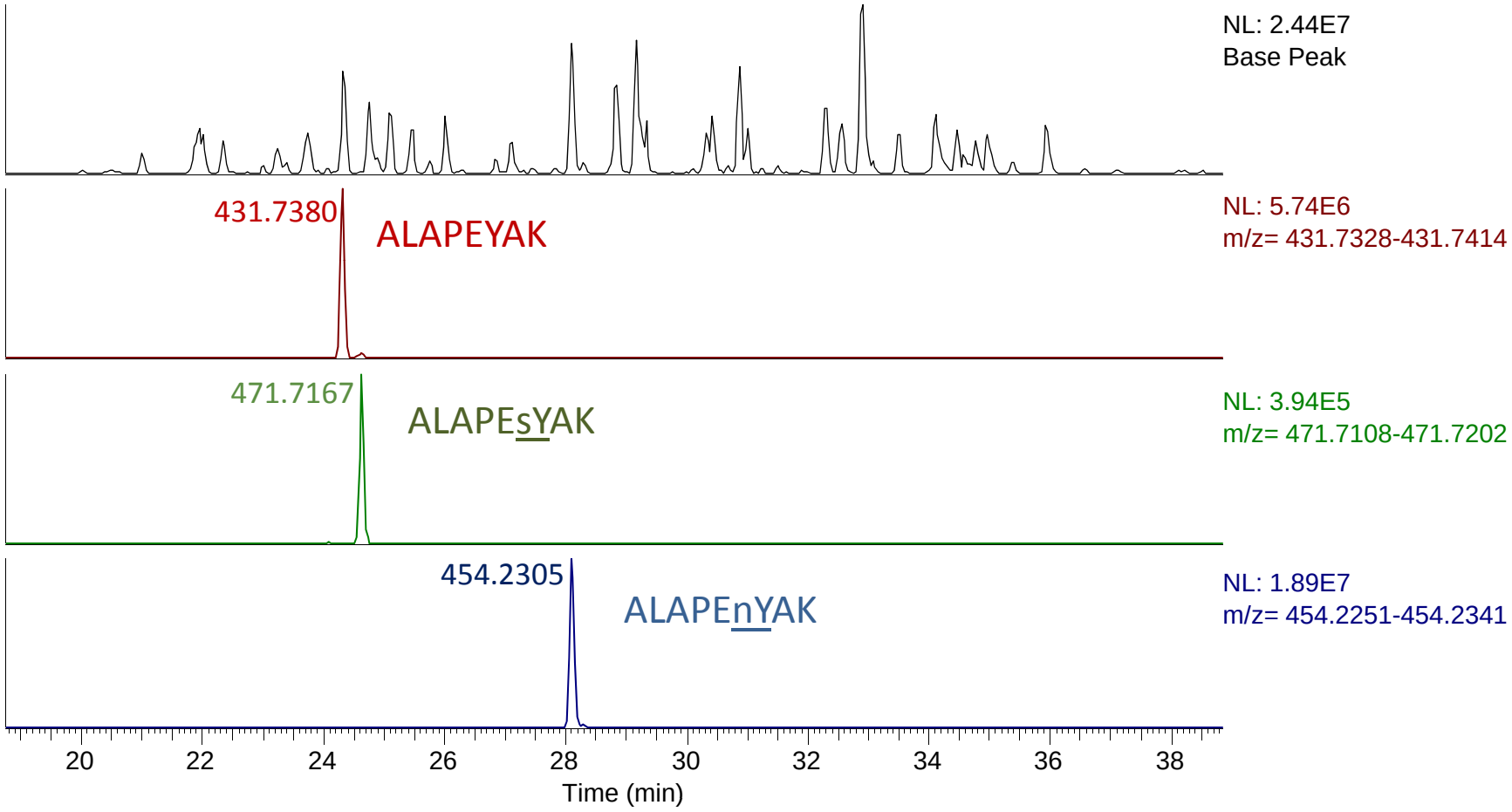

# Differentially modified peptide isoforms

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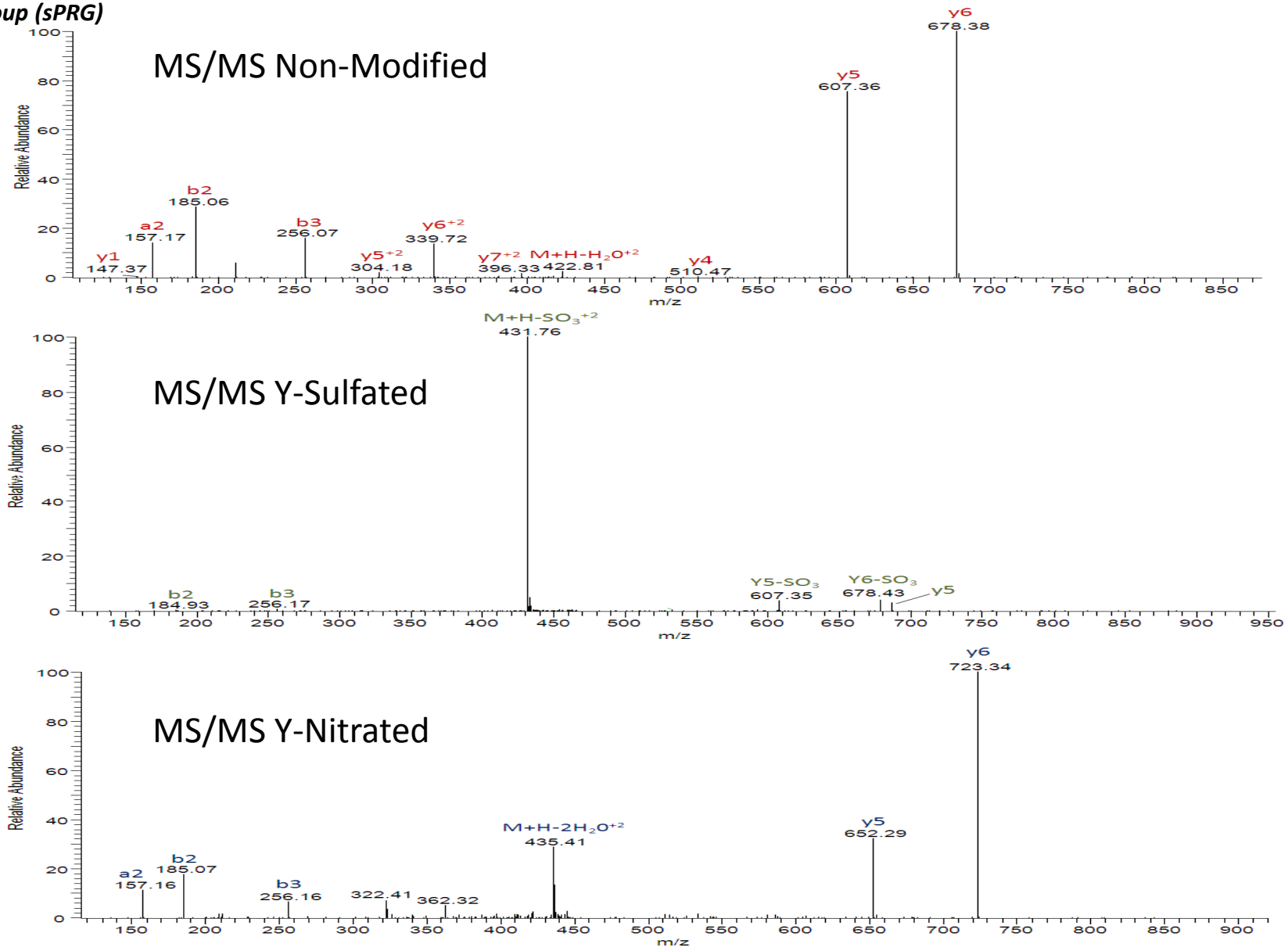

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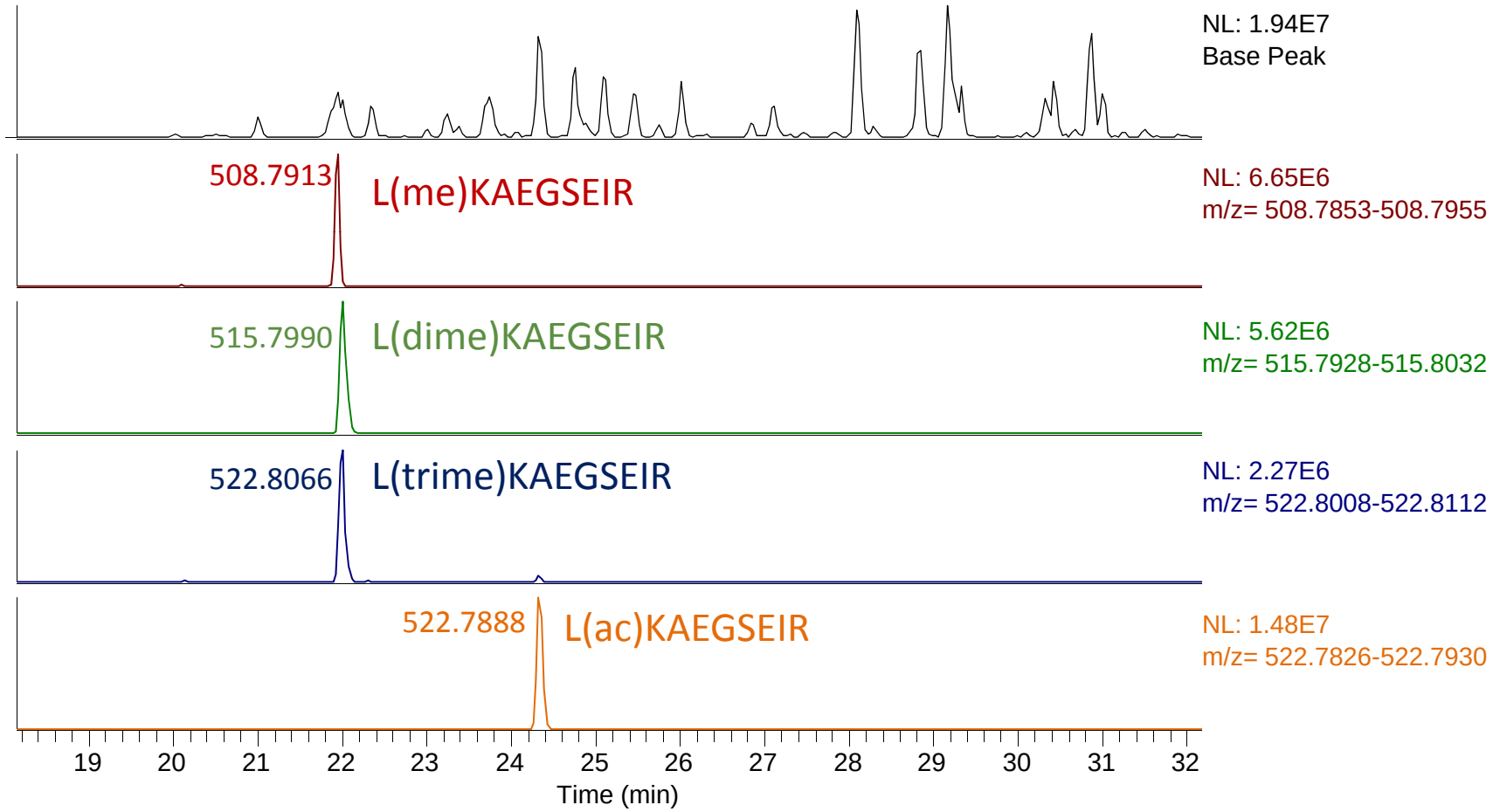
# Methylated Peptides



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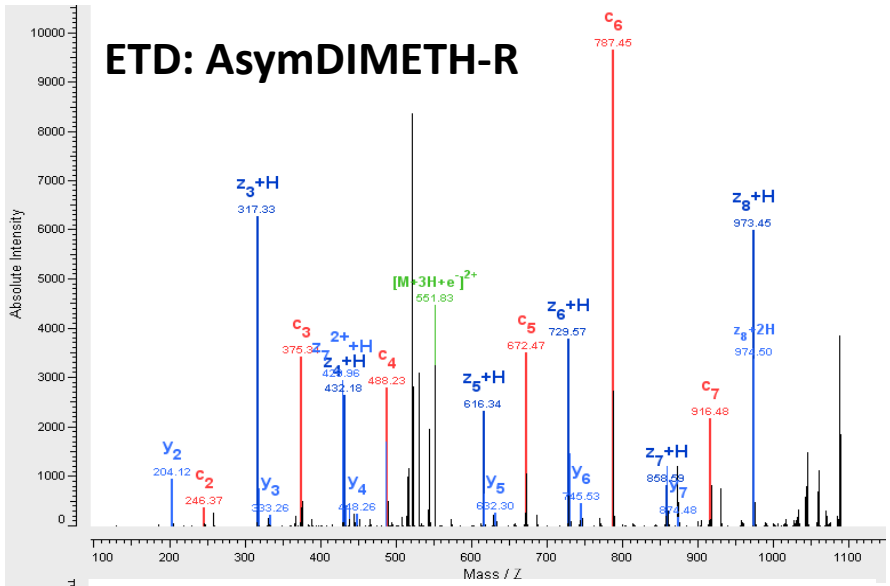
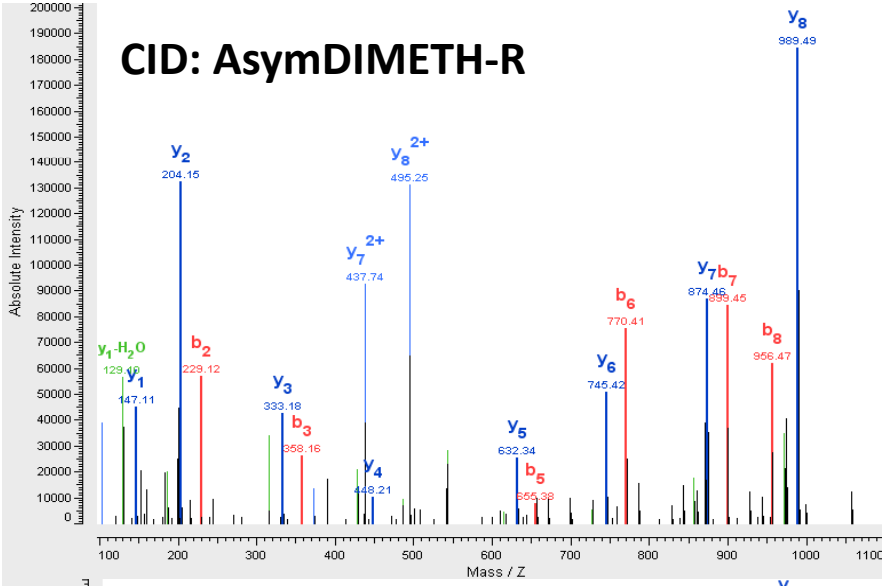
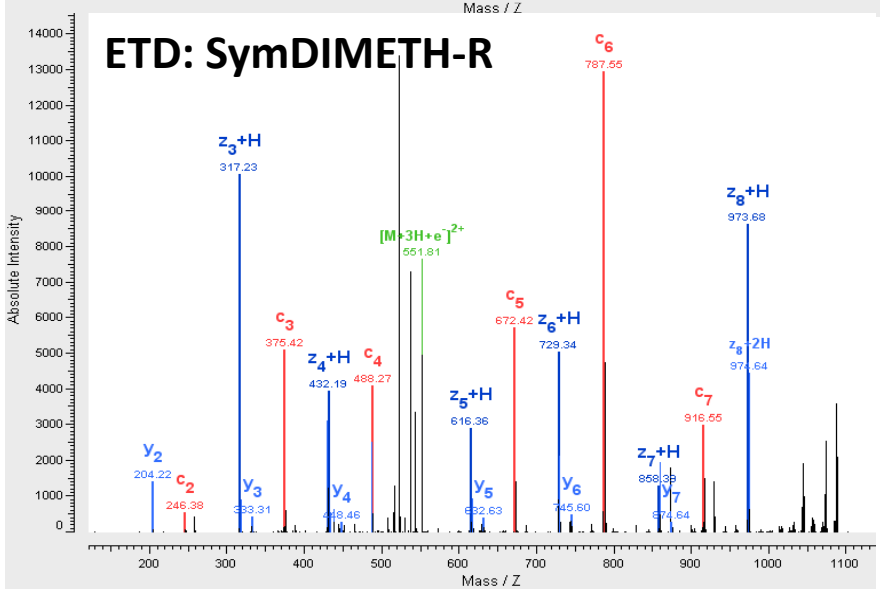
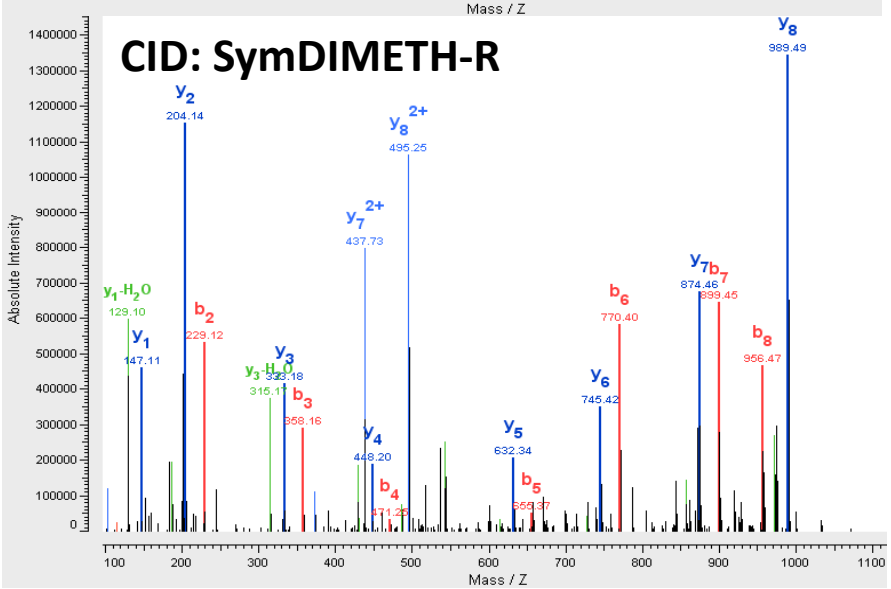
# R-Dimethylated Peptides: Co-elution and Similarity of Fragmentation Patterns



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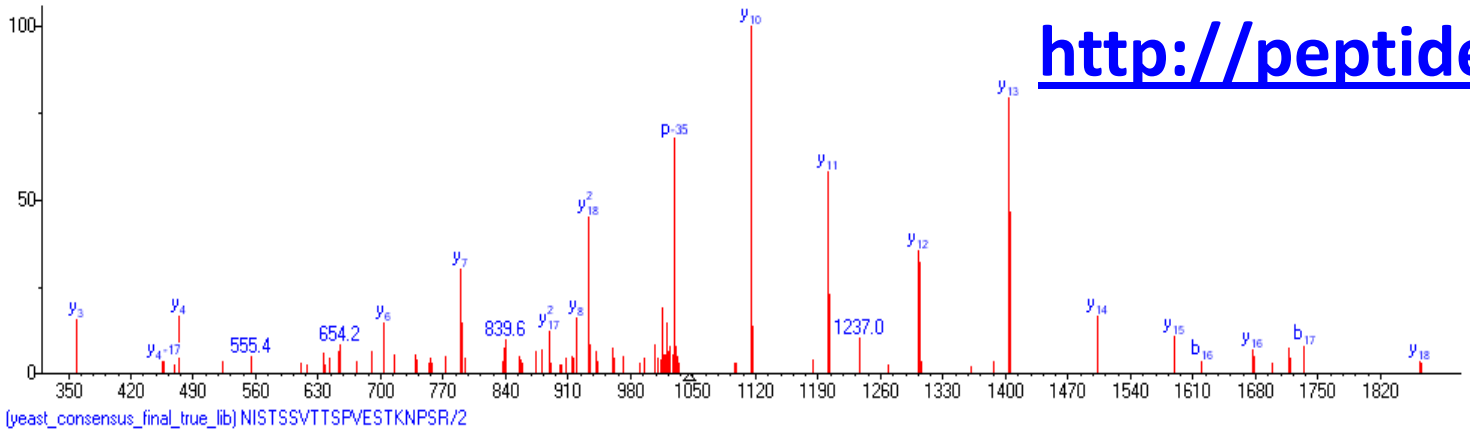
# A Spectral Library for the sPRG 2011 Standard is Being Created

## NIST Libraries of Peptide Tandem Mass Spectra

:: Biochemical Reference Data for Protein and Proteome Analysis




**http://peptide.nist.gov/**

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Goals of this project

- To build and maintain high-quality reference libraries of peptide tandem mass spectra
- To distribute the libraries for general use and software development purposes

Purpose for this site

1. Download access to the libraries
2. On-line access to the library content by protein accession/name or peptide sequence
3. Access to the latest NIST software
4. Links to related, non-NIST projects


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# Standard Stability Study

**For a standard to be of general utility to the mass spectrometry community it must be stable upon transportation and storage. sPRG will test the following:**

Stability of freeze dried standard

with time/ temperature of storage

Stability of standard upon reconstitution in recommended reagents

an array of different common reconstitution reagents

will be tested and stability with time/ storage

***Issue:*** *a method to disentangle the stability of the standard from instrument performance will be established. This is necessary to ensure fluctuations in instrumentation performance do not confound measurements made within longitudinal studies.*


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# sPRG 2011 Study Announcement and Call for Study Participants

Participate in the sPRG2011 Study and have fun with characterizing more PTMs and refining your analytical approaches!!!

sPRG2011Standard@gmail.com

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# Acknowledgements


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**ABRF**

**Sigma-Aldrich:**
Kristin Rolwes
Shantanu Roychowdhury
George Lipscomb

**Thermo Fisher Scientific**
Rainer Gebhart
Georgi Videnov
Joel Louette
Manuela Schaffrath

**Michrom Bioresources:**
Kerry Nugent


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# Questions for the audience

• Should we distribute the sample releasing all information about mixed constituents ?

OR

• Should we rather run the study in two stages:

1. send the sample without providing any specific information about peptide sequences and proteins and collect data (similar to PRG studies);

2. provide all information and request reanalysis of acquired data and sample leftovers?

• Would it be helpful to provide the standard in two formulations: with and without addition of background digests?


*Proteomics Standards Research Group (sPRG)*

Please Visit Our Website www.abrf.org ,
Click on Research Groups then sPRG

Questions, Ideas or Interested in Joining Us?

Contact Alexander Ivanov (sPRG Chair)
aivanov@hsph.harvard.edu

Participate in the sPRG2011 Study and have fun with PTMs (**PTMomics or Modificomics** ☺)!!!
sPRG2011Standard@gmail.com