ETH
Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

Institute of Molecular Systems Biology

Pinpointing phosphorylation sites using Selected Reaction Monitoring and Skyline

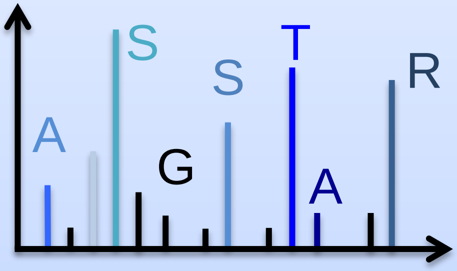
Christina Ludwig

group of Ruedi Aebersold, ETH Zürich

The challenge of phospho-site assignment

Peptides

MS/MS spectra

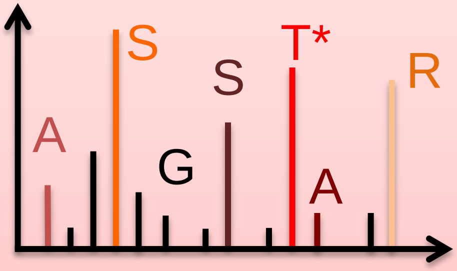


ASGSTAR

peptide identification

Phospho-peptides

MS/MS spectra



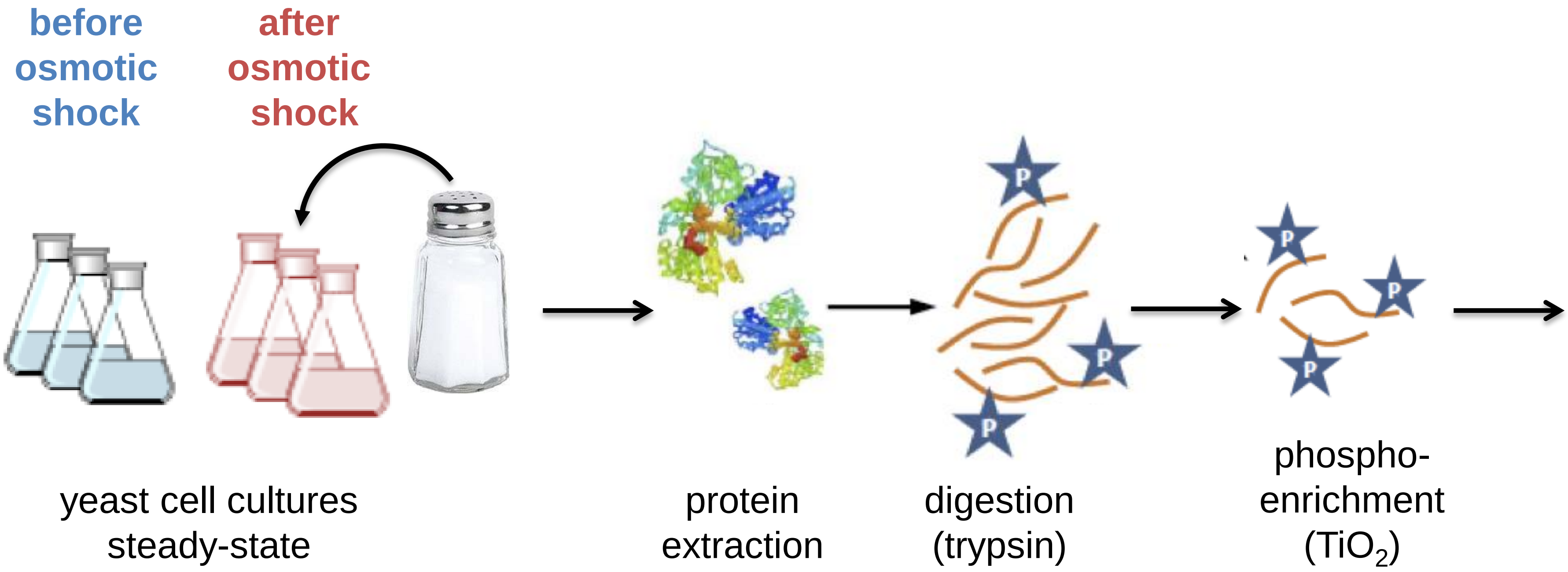
AS*GS*T*AR

phospho-peptide identification

ASGST*AR

phospho-site assignment

The case study: Phosphorylation changes upon osmotic shock



before osmotic shock

after osmotic shock

yeast cell cultures
steady-state

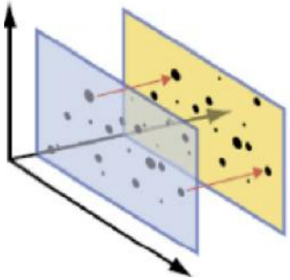
protein
extraction

digestion
(trypsin)

phospho-
enrichment
(TiO_2)

The case study: Phosphorylation changes upon osmotic shock

shotgun
LC-MS/MS
(LTQ-Orbi)



label-free
quantification

phosphorylation results table

phospho-peptide sequence	before osmotic shock	after osmotic shock	ratio
YGGH**S[p]**MSDPGTTYR	367889	90293	0.25
KG**S[p]**MADVPK	562301	574839	1.02
SASAV**S[p]**LPAK	305	3992	13.1
…			

Ambiguities and missing values in discovery-driven data sets

Gpd2 = Glycerol-3-phosphate dehydrogenase 2

Peptide sequence	**phospho-site**	**PepP probability**	**before osmotic shock A**	**before osmotic shock B**	**before osmotic shock C**	**after osmotic shock A**	**after osmotic shock B**	**after osmotic shock C**	**ratio**	**Ttest**
S[p]DSAVSIVHLK	**[S70]**	0.9881	0	4567890	355678	432123	0	0	0.18	#DIV/0!
SD**S[p]**AVSIVHLK	**[S72]**	0.9999	5320300	4670960	4236830	0	1042920	1154030	0.23	3.0E-03
SDSAV**S[p]**IVHLK	**[S75]**	0.9961	1915980	1574980	1120860	510526	1309220	0	0.59	2.3E-01
S[p]DSAV**S[p]**IVHLK	**[S70],[S75]**	0.9788	834567	0	590897	0	890753	0	1.25	#DIV/0!
SD**S[p]**AV**S[p]**IVHLK	**[S72],[S75]**	0.9888	966789	1056789	997654	0	780864	697650	0.73	1.0E-02

Gpd1 = Glycerol-3-phosphate dehydrogenase 1

Peptide sequence	**phospho-site**	**PepP probability**	**before osmotic shock A**	**before osmotic shock B**	**before osmotic shock C**	**after osmotic shock A**	**after osmotic shock B**	**after osmotic shock C**	**ratio**	**Ttest**
S**S[p]**SSVSLK	**[S23]**	0.9671	0	0	789098	0	987539	0	1.25	#DIV/0!
SS**S[p]**SVSLK	**[S24]**	0.9979	6086840	5087770	4586845	0	22236600	18536600	3.88	2.0E-03
SSS**S[p]**VSLK	**[S25]**	0.9995	2345001	0	2678960	5754910	4796671	5196380	2.09	5.5E-03
SSSSV**S[p]**LK	**[S27]**	0.9889	2377960	227652	2146060	2806640	0	2502570	1.68	3.1E-01
SS**S[p]**SV**S[p]**LK	**[S24],[S27]**	0.9991	6744910	5888620	4656130	20481500	16751200	12117300	2.85	1.3E-02

- Are all identified phospho-sites truly occurring in the sample?
- Do the obtained quantitative results reflect true biological changes?

Targeted phospho-proteomics using SRM and Skyline ←

(Phospho)-SRM workflow carried out in Skyline

(phospho)-peptide selection

transition selection

SRM measurement

crude synthetic heavy
phospho-peptides
(JPT technologies)

Gpd1

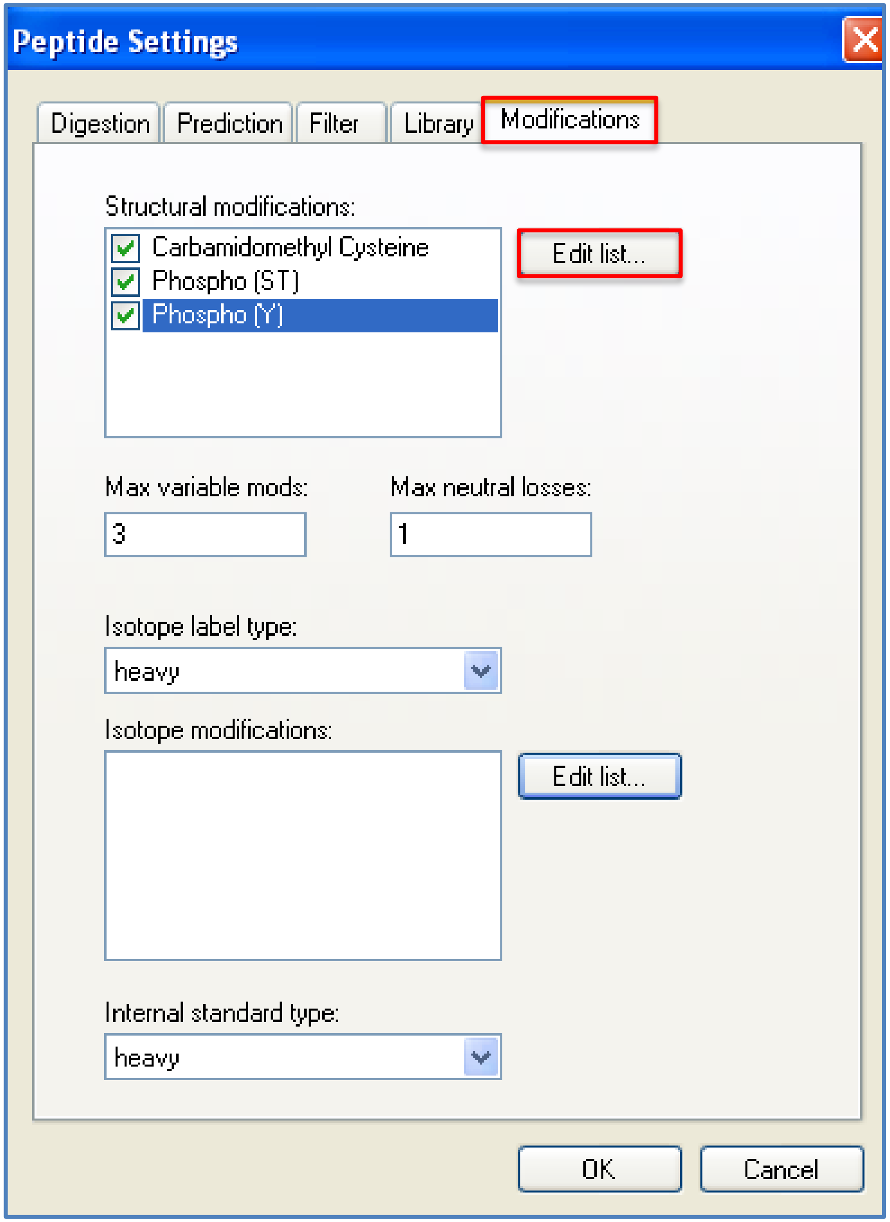
peptide sequence	phospho-site
S[p]SSSVSLK	**[S22]**
S**S[p]**SSVSLK	**[S23]**
SS**S[p]**SVSLK	**[S24]**
SSS**S[p]**VSLK	**[S25]**
SSSSV**S[p]**LK	**[S27]**

Gpd2

peptide sequence	phospho-site
S[p]DSAVSIVHLK	**[S70]**
SD**S[p]**AVSIVHLK	**[S72]**
SDSAV**S[p]**IVHLK	**[S75]**

Getting phospho-peptides into Skyline

(phospho)-
peptide
selection



Structural modifications:

- ☑ Carbamidomethyl Cysteine
- ☑ Phospho (ST)
- ☑ Phospho (Y)

Max variable mods: 3

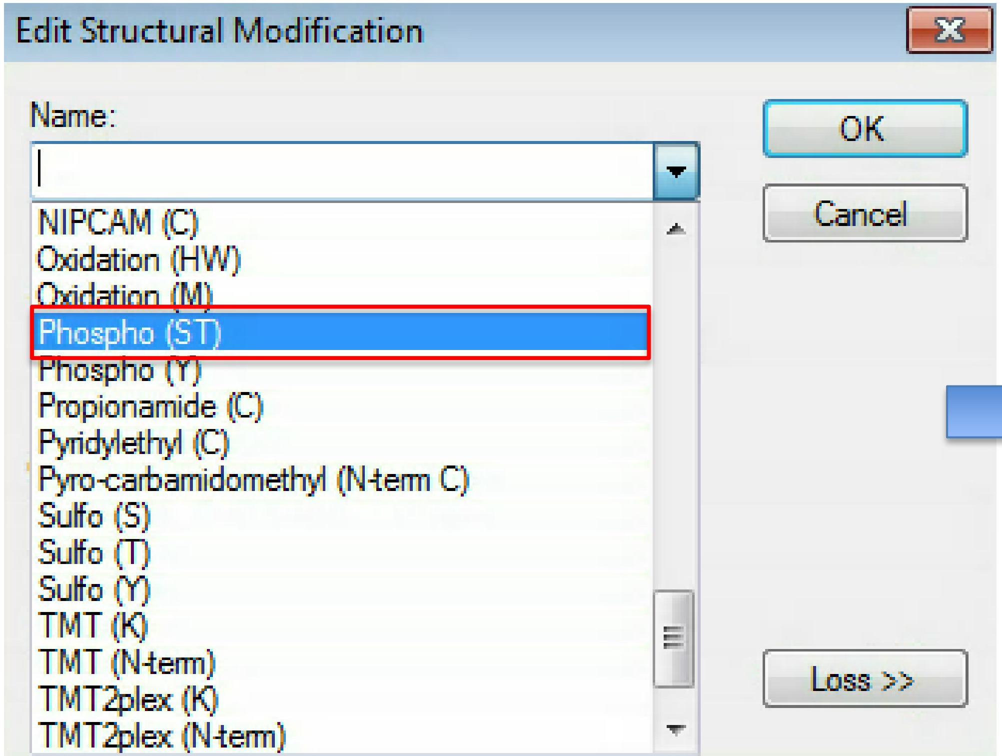
Max neutral losses: 1

Isotope label type: heavy

Isotope modifications:

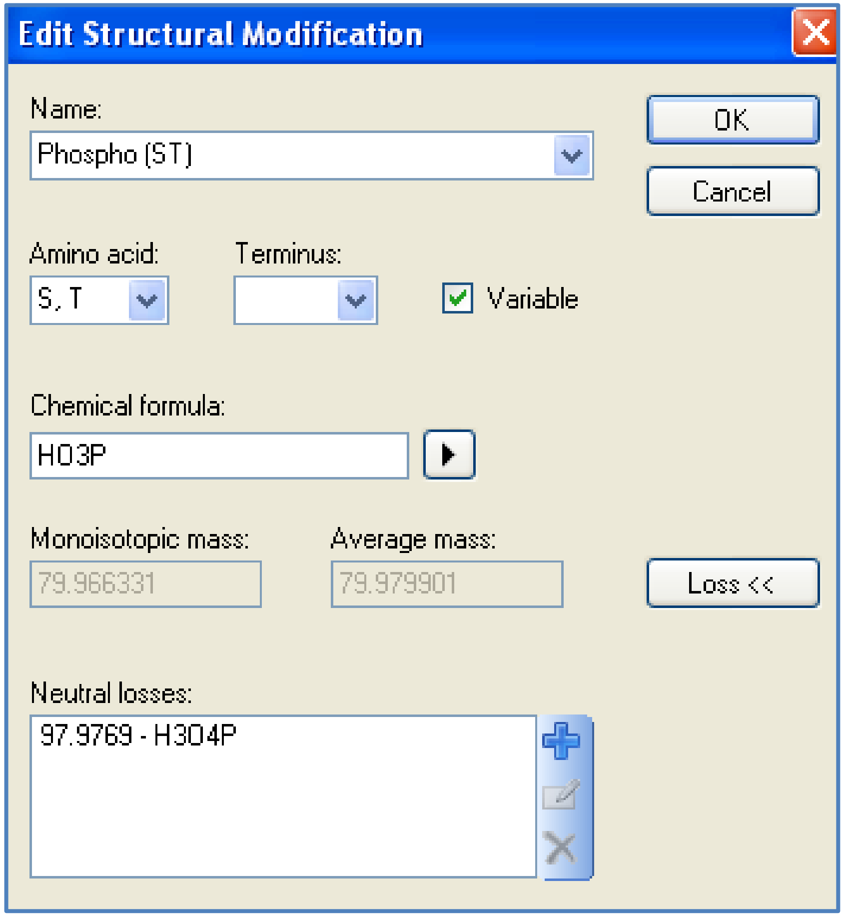
Internal standard type: heavy

Getting phospho-peptides into Skyline



Name:

NIPCAM (C)
Oxidation (HW)
Oxidation (M)
Phospho (ST)
Phospho (Y)
Propionamide (C)
Pyridylethyl (C)
Pyro-carbamidomethyl (N-term C)
Sulfo (S)
Sulfo (T)
Sulfo (Y)
TMT (K)
TMT (N-term)
TMT2plex (K)
TMT2plex (N-term)



Name: Phospho (ST)

Amino acid: S, T

Terminus:

☑ Variable

Chemical formula: H03P

Monoisotopic mass: 79.966331

Average mass: 79.979901

Neutral losses:

97.9769 - H3O4P

All Uniprot modifications available

(Phospho)-SRM workflow carried out in Skyline

(phospho)-peptide selection

transition selection

SRM measurement

automatic selection of
the y-ion and b-ion series (> 300 Da)
using the transition filter settings
in Skyline

🟥 shared transitions

🟩 unique transitions

peptide sequence	phospho-site	precursor m/z	y10 b1 [m/z]	y9 b2 [m/z]	y8 b3 [m/z]	y7 b4 [m/z]	y6 b5 [m/z]	y5 b6 [m/z]	y4 b7 [m/z]	y3 b8 [m/z]	y2 b9 [m/z]	y1 b10 [m/z]
S[p]DSAVSIVHLK	[S70]	622.31	🟩 1076.62 -	🟩 961.59 -	🟥 874.56 370.06	🟥 803.52 441.1	🟥 704.45 540.17	🟥 617.42 627.20	🟥 504.27 740.29	🟥 405.27 839.35	🟥 - 976.41	🟥 - 1089.50
SD**S[p]**AVSIVHLK	[S72]	622.31	🟥 1156.59 -	🟥 1041.56 -	🟥 874.56 370.06	🟥 803.52 441.1	🟥 704.45 540.17	🟥 617.42 627.20	🟥 504.27 740.29	🟥 405.27 839.35	🟥 - 976.41	🟥 - 1089.50
SDSAV**S[p]**IVHLK	[S75]	622.31	🟥 1156.59 -	🟥 1041.56 -	🟩 954.53 290.10	🟩 883.49 361.14	🟩 784.42 460.20	🟥 617.42 627.20	🟥 504.27 740.29	🟥 405.27 839.35	🟥 - 976.41	🟥 - 1089.50

(Phospho)-SRM workflow carried out in Skyline

(phospho)-peptide selection

transition selection

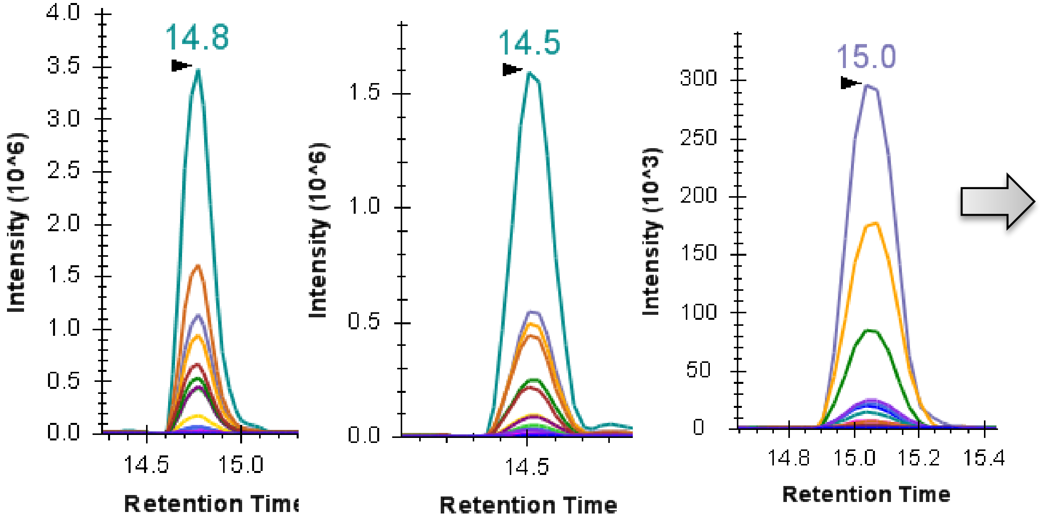
SRM measurement

→ of synthetic peptides

Targeting of synthetic phospho-peptides for Gpd2

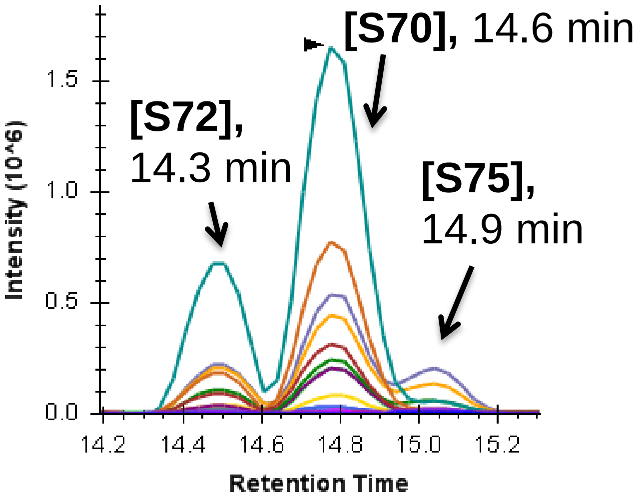
SRM measurement

individual measurements of synthetic peptides



[S70] **[S72]** **[S75]**

peptide mixture



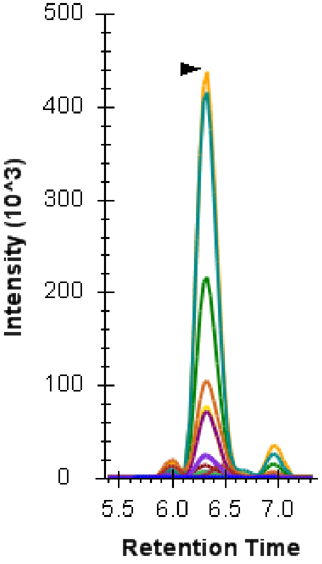
all phospho-peptide forms
are separable
in retention time

Targeting of synthetic phospho-peptides for Gpd1

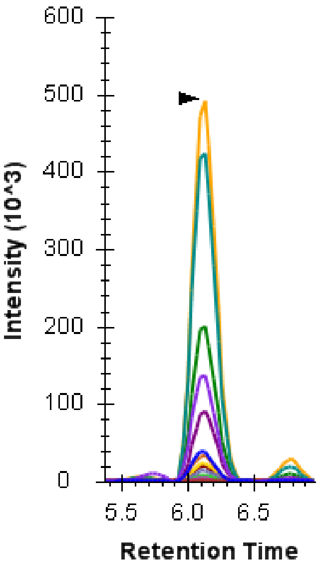
SRM measurement

individual measurements of synthetic phospho-peptides

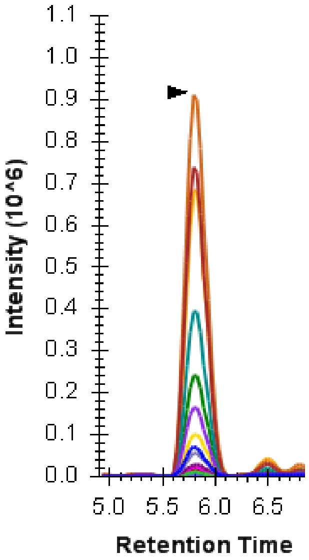
[S22]



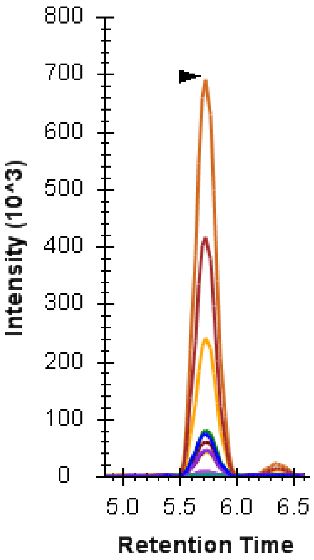
[S23]



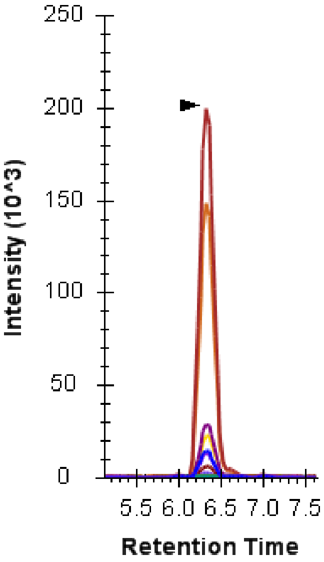
[S24]



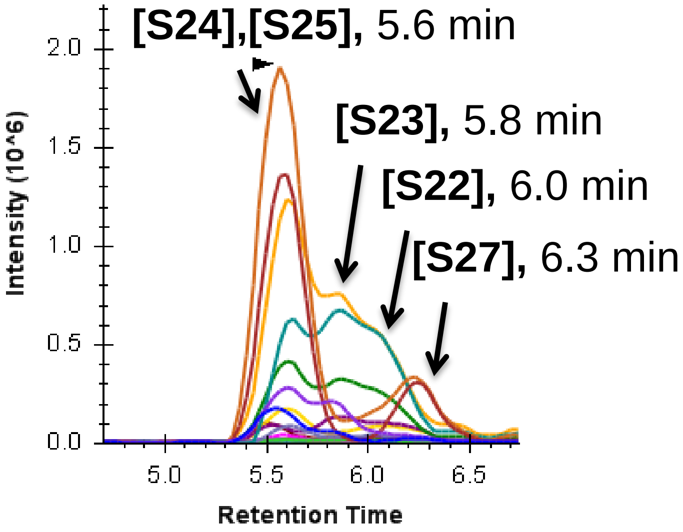
[S25]



[S27]



peptide mixture



[S24],[S25], 5.6 min

[S23], 5.8 min

[S22], 6.0 min

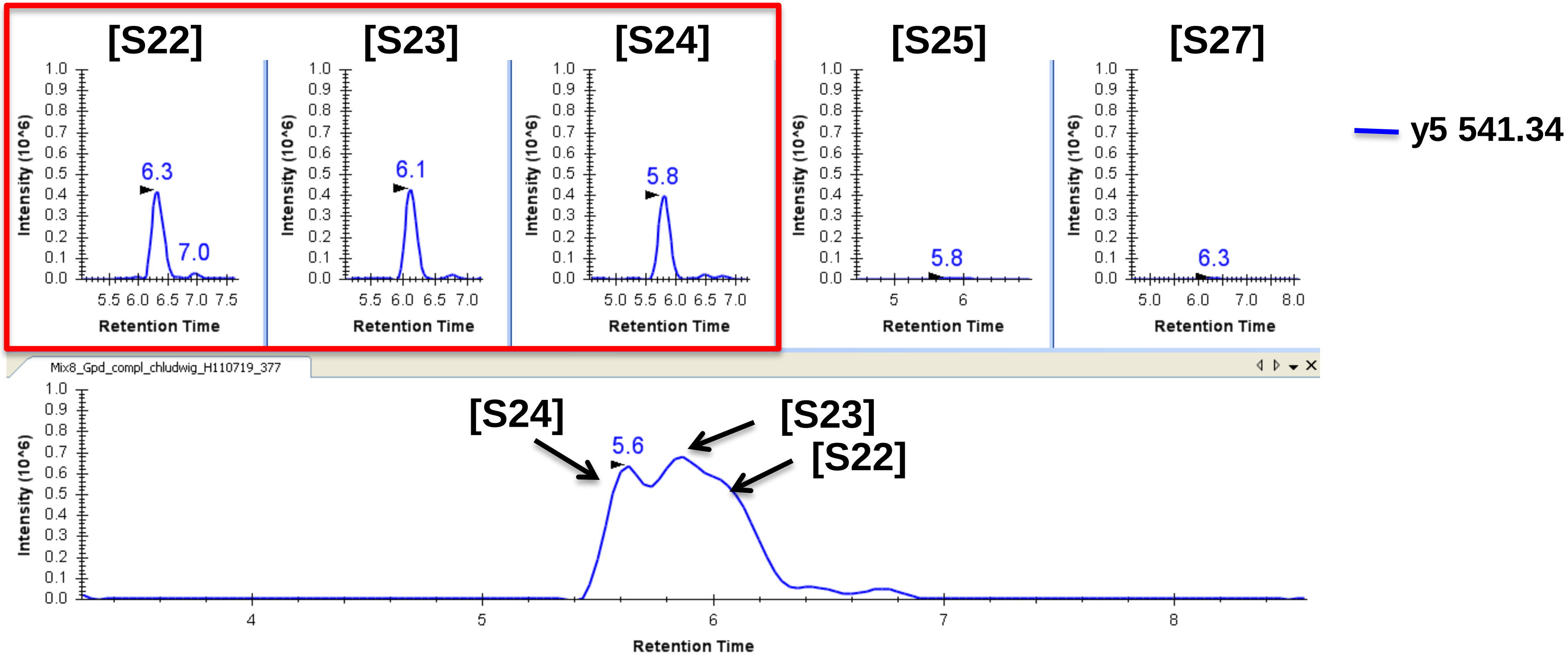
[S27], 6.3 min

phospho-peptide forms **[S24]** and **[S25]** are **not separable** in retention time.
Other peptide forms are strongly overlapping but distinguishable.

Discrimination of [S24] [S25] despite co-elution

SRM measurement

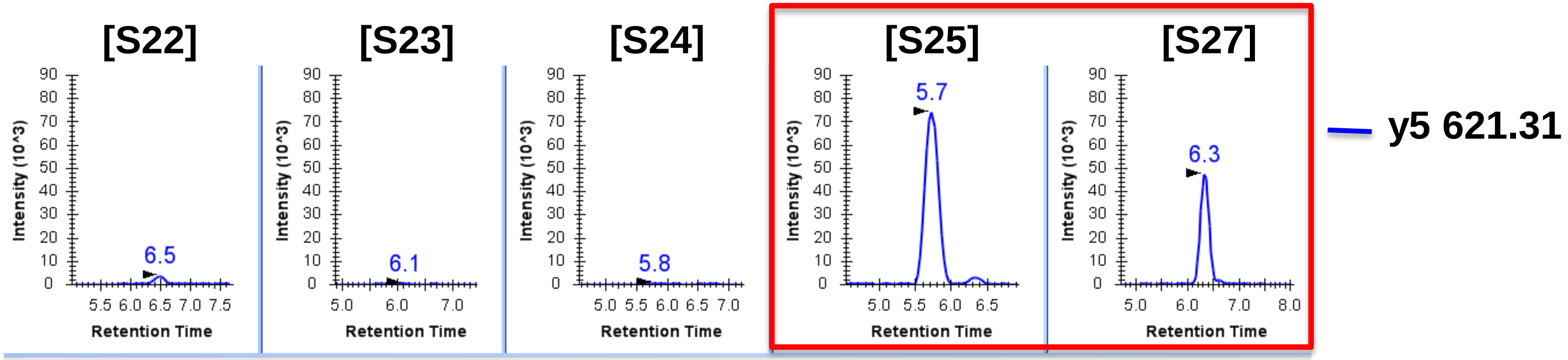
peptide sequence	phospho-site	precursor m/z	y7 [m/z]	y6 [m/z]	y5 [m/z]	y4 [m/z]	y3 [m/z]	rt [min]
S[p]SSSVSLK	[S22]	437.70	707.40	628.38	541.34	454.31	355.24	6.0
S**S[p]**SSVSLK	[S23]	437.70	795.37	628.38	541.34	454.31	355.24	5.8
SSS[p]SVSLK	**[S24]**	**437.70**	**795.37**	**708.43**	**541.34**	**454.31**	**355.24**	**5.6**
SSSS[p]VSLK	**[S25]**	**437.70**	**795.37**	**708.43**	**621.31**	**454.31**	**355.24**	**5.6**
SSSSV**S[p]**LK	[S27]	437.70	795.37	708.43	621.31	534.28	435.21	6.3

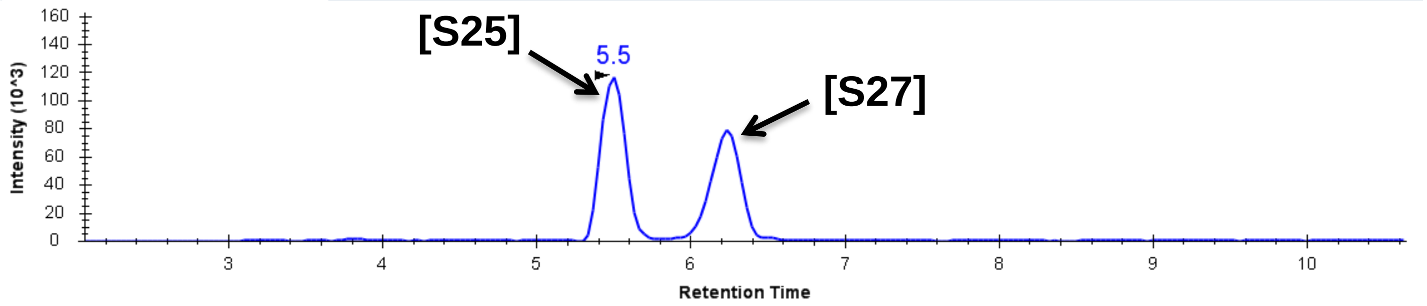


Discrimination of [S24] [S25] despite co-elution

SRM measurement

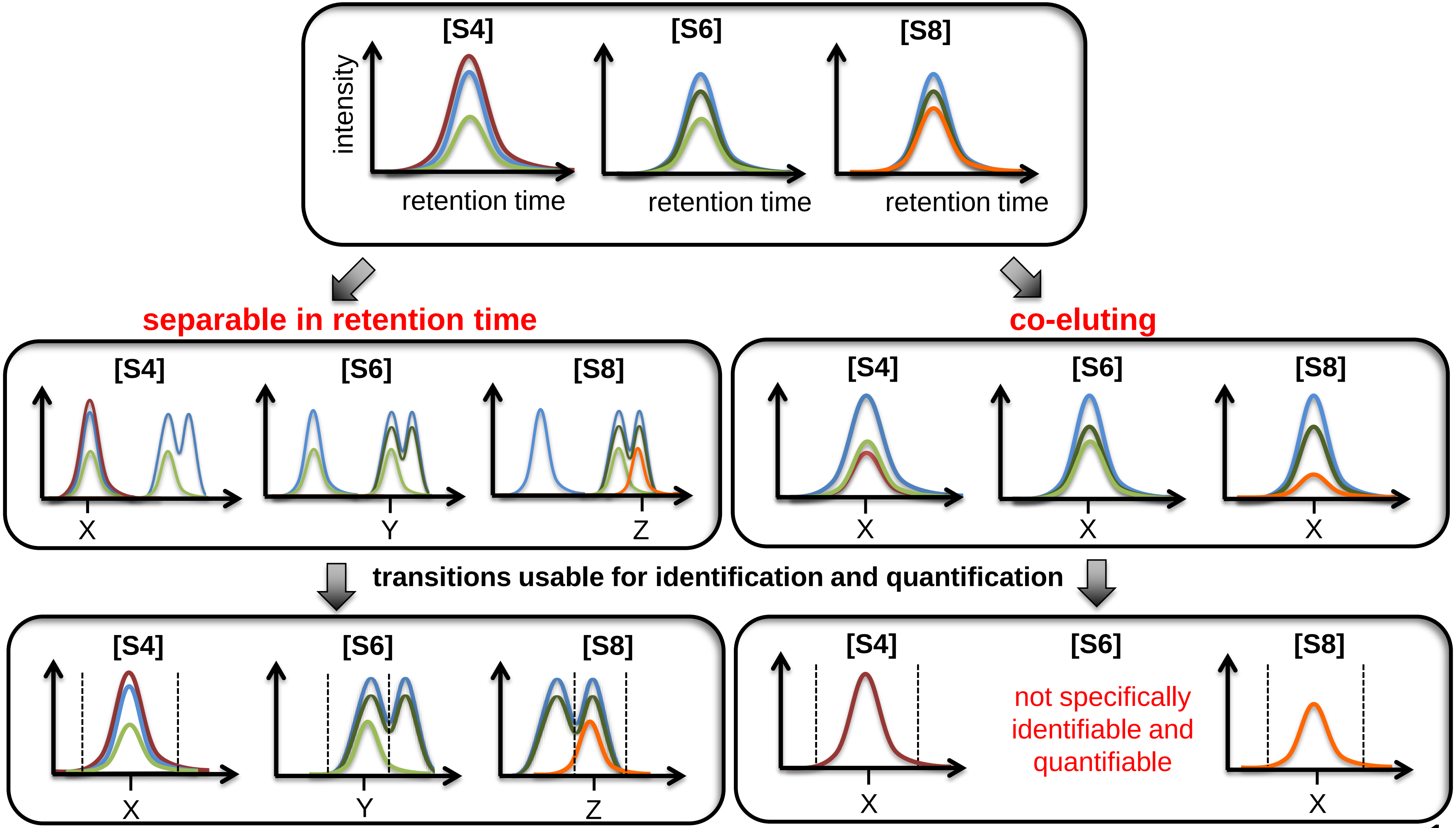
peptide sequence	phospho-site	precursor m/z	y7 [m/z]	y6 [m/z]	y5 [m/z]	y4 [m/z]	y3 [m/z]	rt [min]
S[p]SSSVSLK	[S22]	437.70	707.40	628.38	541.34	454.31	355.24	6.0
S**S[p]**SSVSLK	[S23]	437.70	795.37	628.38	541.34	454.31	355.24	5.8
SSS[p]SVSLK	**[S24]**	**437.70**	**795.37**	**708.43**	**541.34**	**454.31**	**355.24**	**5.6**
SSSS[p]VSLK	**[S25]**	**437.70**	**795.37**	**708.43**	**621.31**	**454.31**	**355.24**	**5.6**
SSSSV**S[p]**LK	[S27]	437.70	795.37	708.43	621.31	534.28	435.21	6.3





Retention time information is important for pinpointing phospho-sites

Phospho-peptide: XXX[**S4**]X[**S6**]X[**S8**]XX



separable in retention time

co-eluting

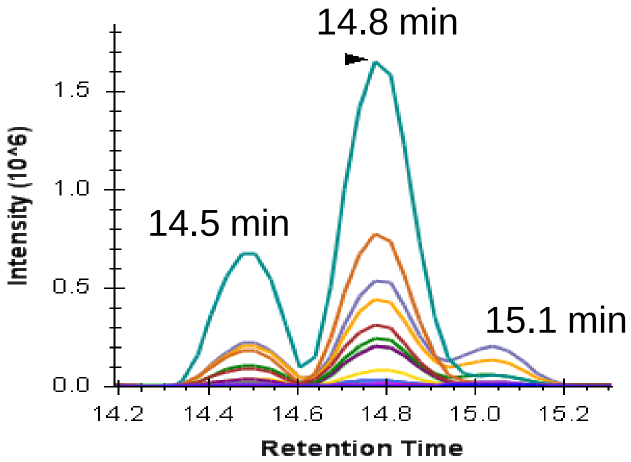
transitions usable for identification and quantification

not specifically identifiable and quantifiable

The “iRT” concept implemented into Skyline

The problem:

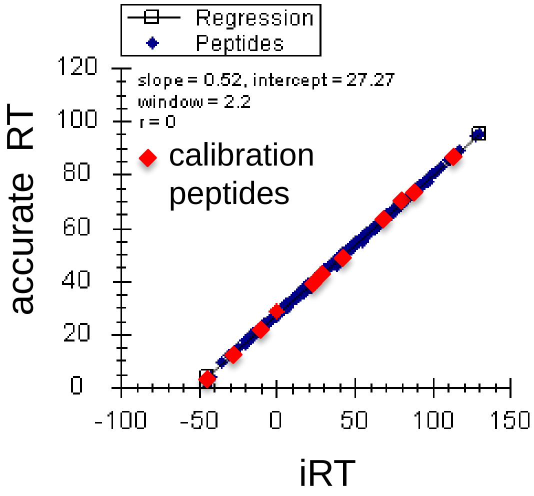
Accurately measured empirical retention times are **dependent** on the setup of the currently used **chromatographic system**



**Consequence in practice:
retention times need to be determined over and over again**

The solution:

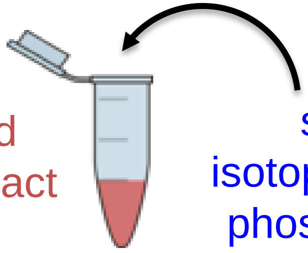
Usage of a **set of calibration peptides** to normalize all peptide retention times to a **dimensionless “iRT value”**



**Consequence in practice:
once an iRT value is determined, the respective peptide can be scheduled and identified on any LC system**

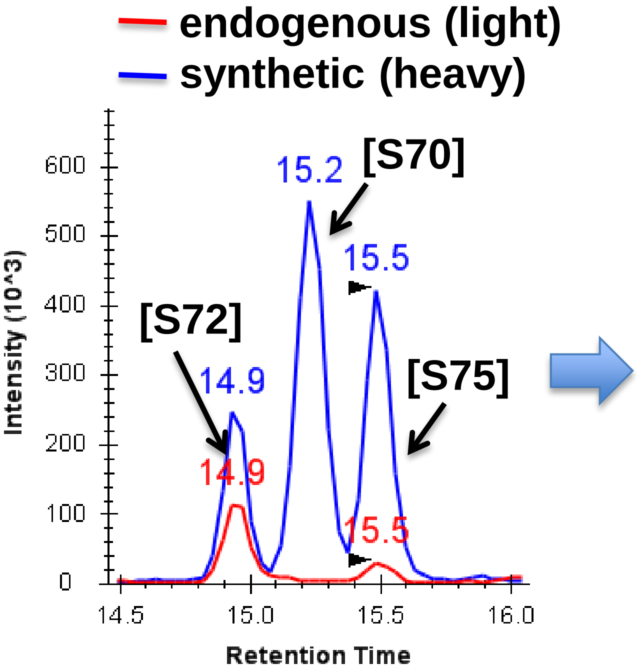
Escher C. *et al.*, Proteomics, **2012**, 12, 1111-1121
Monday AM, Poster 624, Escher C. *et al.*

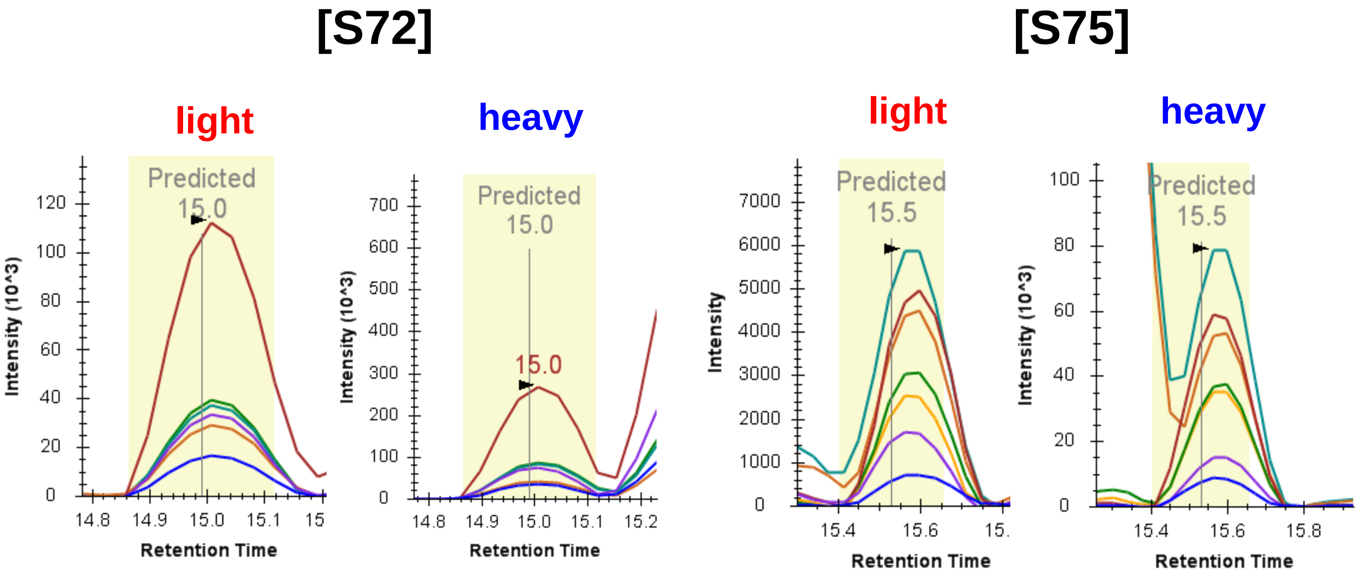
Quantification of endogenous phospho-peptides – Gpd2



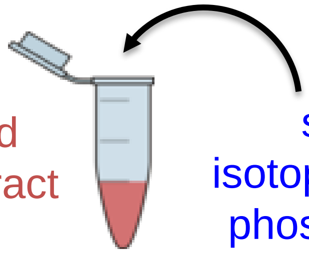
phospho-enriched
yeast total cell extract

synthetic,
isotopically-labeled
phospho-peptides



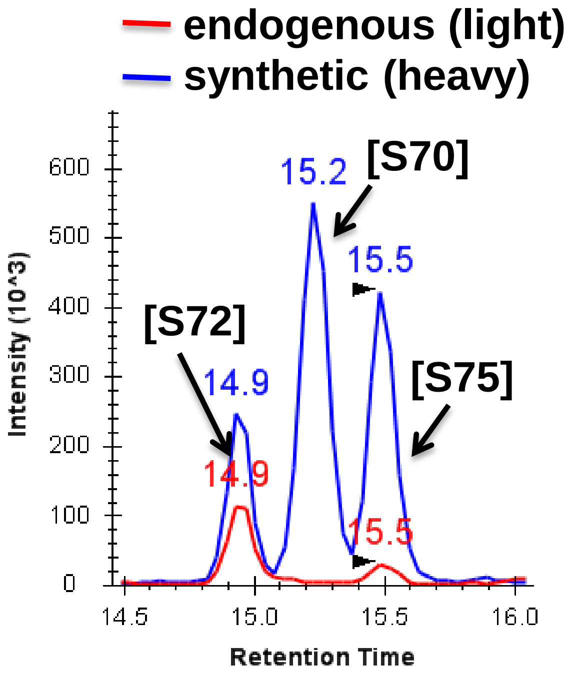


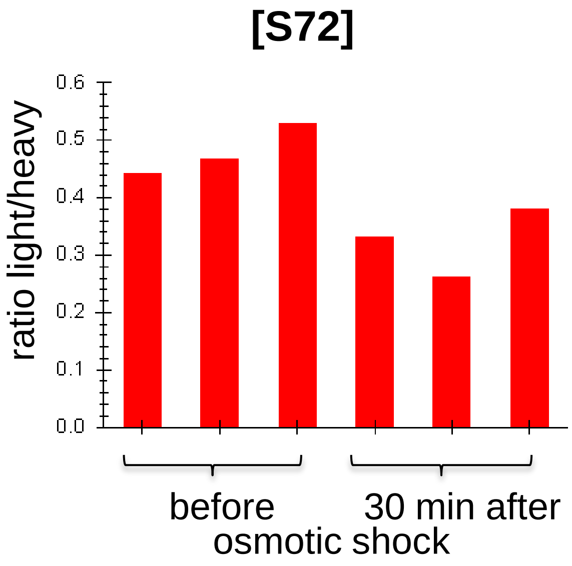
Quantification of endogenous phospho-peptides – Gpd2

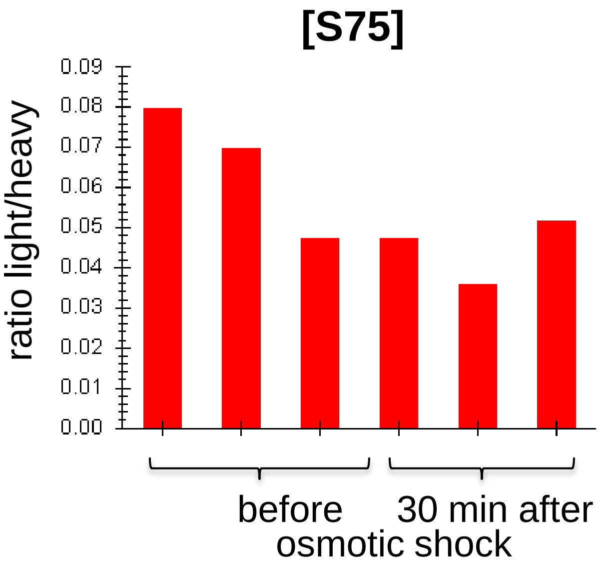


phospho-enriched
yeast total cell extract

synthetic,
isotopically-labeled
phospho-peptides



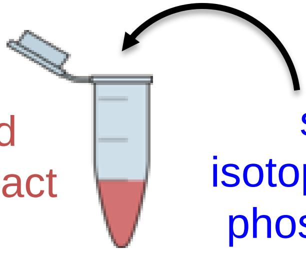




1.5-fold down-regulation
(t-test p-value = 0.01)

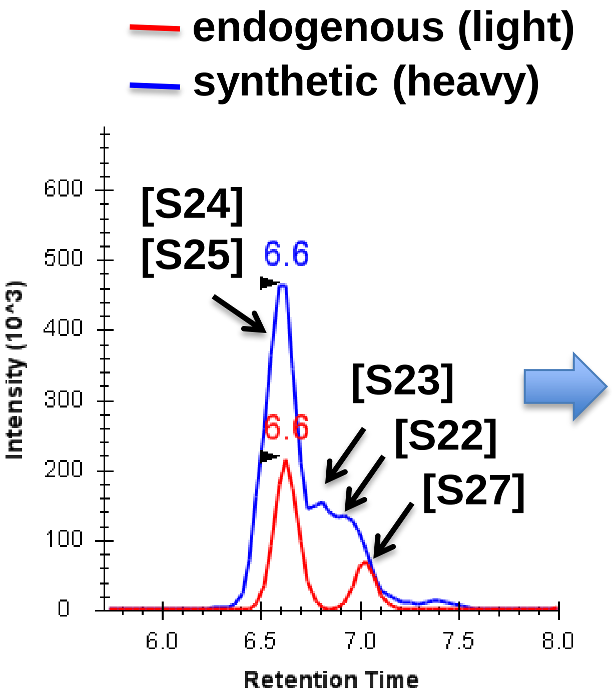
1.7-fold down-regulation
(t-test p-value = 0.01)

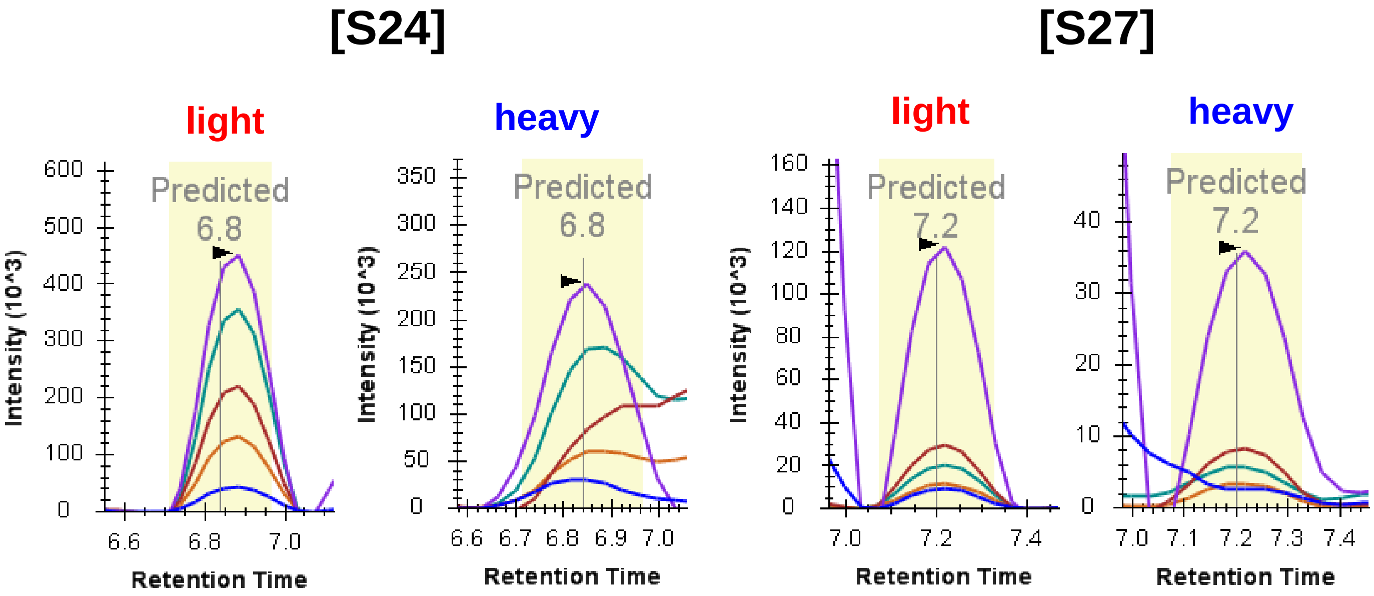
Quantification of endogenous phospho-peptides – Gpd1



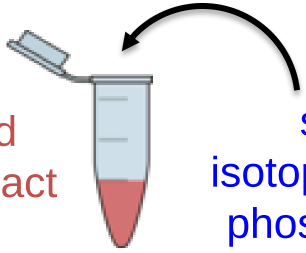
phospho-enriched
yeast total cell extract

synthetic,
isotopically-labeled
phospho-peptides



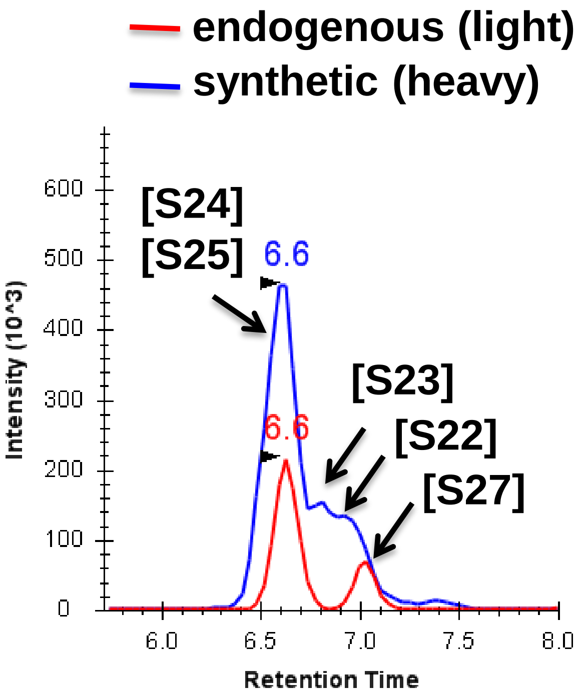


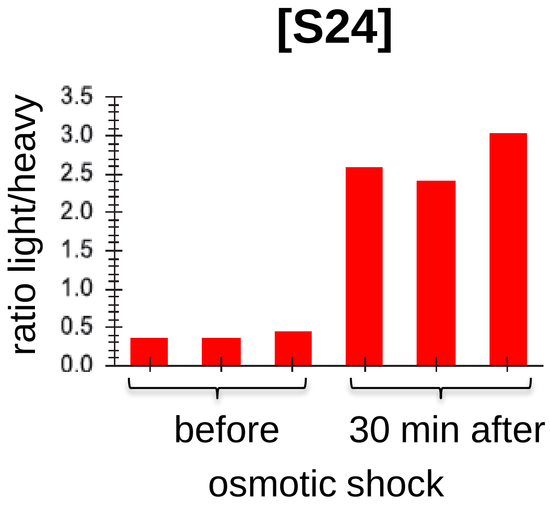
Quantification of endogenous phospho-peptides – Gpd1

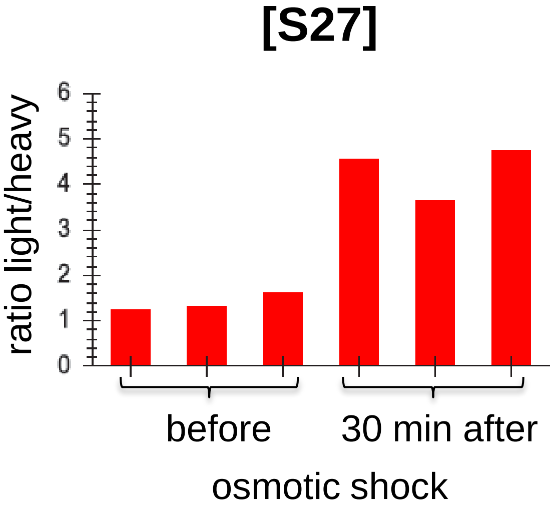


phospho-enriched
yeast total cell extract

synthetic,
isotopically-labeled
phospho-peptides





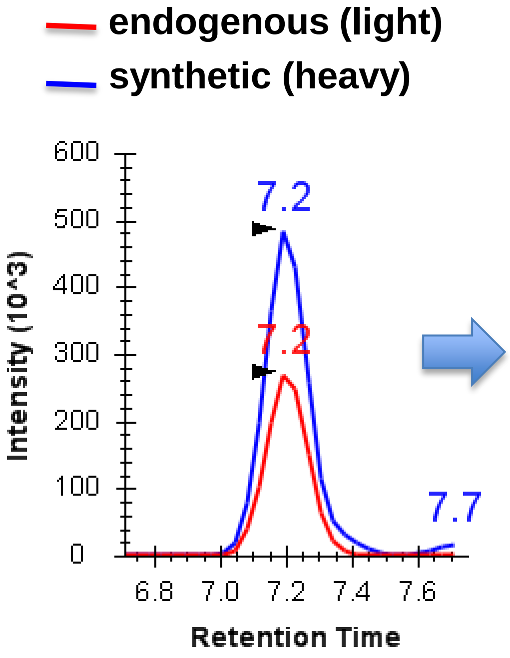


7.2-fold up-regulation
(t-test p-value = 2.1E-4)

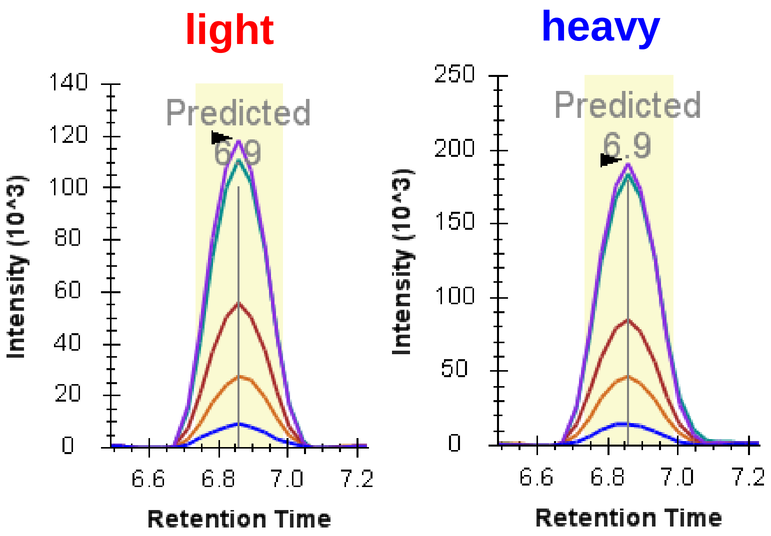
3.2-fold up-regulation
(t-test p-value = 2.4E-4)

Quantification of doubly phosphorylated peptide – Gpd1

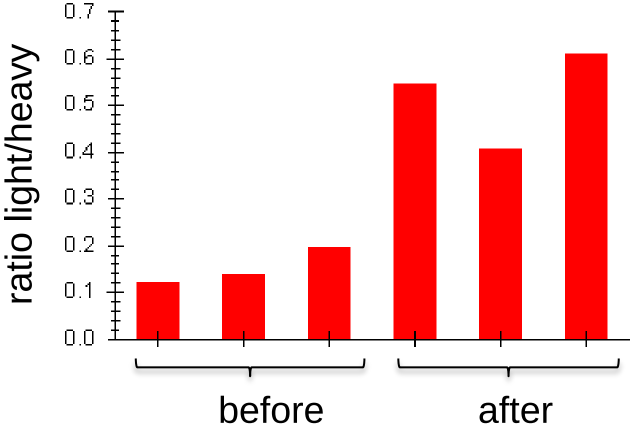
SS**S[p]**SV**S[p]**LK **[S24,27]**



[S24,S27]



[S24,S27]



osmotic shock

3.5-fold up-regulation
(t-test p-value = 4.4E-4)

Take-home messages

- **Targeted analysis of phosphorylation using SRM provides quantitative data of high quality, accuracy and reproducibility**

  Requirement: A priori knowledge

- **Specific phosphorylation-sites can be pinpointed with high confidence**

  Requirement: Learn the chromatographic behavior of phospho-peptide forms from synthetic reference peptides

- **Application of the iRT concept improves acquisition scheduling and peptide identification**

  Requirement: Consistent use of retention time calibration peptides

Thank you

Collaborators

→ Ana Paula Oliveira
→ Paola Picotti

TSQ-support

→ Mariette Matondo
→ Nathalie Selevsek

Ruedi Aebersold and the whole Aebersold lab

Skyline

→ Brendan MacLean
→ Alana Killeen

BIOGNOSYS
PROTEOMICS – NEXT GENERATION

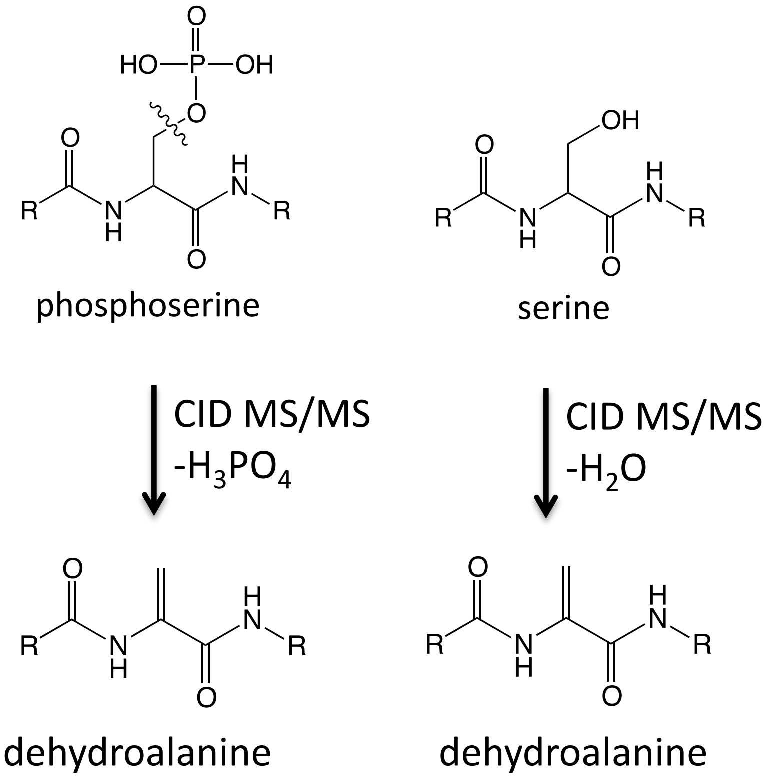
→ Lukas Reiter
→ Oliver Rinner

jpt
Innovative Peptide Solutions

YeastX
Towards an Understanding of Nutrient Signaling and Metabolic Operation

Further complications for a phospho-analysis with SRM: Neutral Loss H_3PO_4

1. Loss of H_3PO_4 = -98 Da



phosphoserine

serine

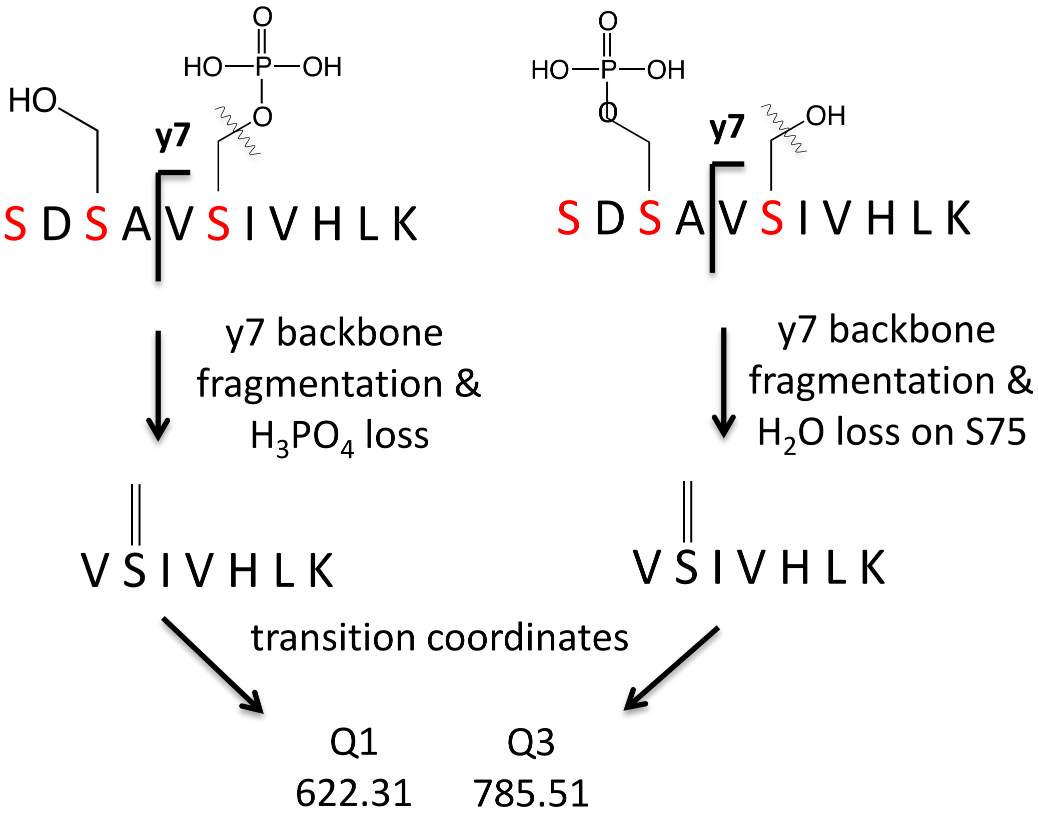
CID MS/MS
-H_3PO_4

CID MS/MS
-H_2O

dehydroalanine

dehydroalanine

example:



S D S A V S I V H L K

y7 backbone fragmentation & H_3PO_4 loss

y7 backbone fragmentation & H_2O loss on S75

V S I V H L K

transition coordinates

Q1
622.31

Q3
785.51

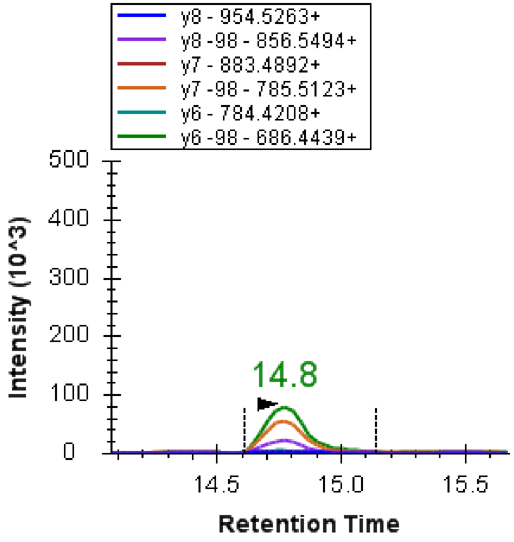
Conclusion

For peptides comprising **several possibly phosphorylated residues** phospho-site **assignments** based exclusively on **H_3PO_4-neutral loss fragment ions** can be **erroneous**, because their occurrence can also be due to a water-loss of a non-phosphorylated serine residue.

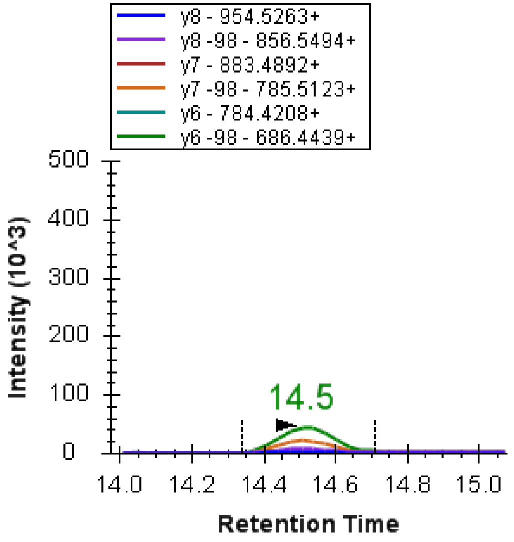
example for H_3PO_4 (-98 Da) phosphate loss interference

peptide sequence	Phos-pho-site	pre-cursor m/z	y10 y10-H_3PO_4 b1 b1-H_3PO_4 [m/z]	y9 y9-H_3PO_4 b2 b2-H_3PO_4 [m/z]	y8 y8-H_3PO_4 b3 b3-H_3PO_4 [m/z]	y7 y7-H_3PO_4 b4 b4-H_3PO_4 [m/z]	y6 y6-H_3PO_4 b5 b5-H_3PO_4 [m/z]	y5 y5-H_3PO_4 b6 b6-H_3PO_4 [m/z]	y4 y4-H_3PO_4 b7 b7-H_3PO_4 [m/z]	y3 y3-H_3PO_4 b8 b8-H_3PO_4 [m/z]	y2 y2-H_3PO_4 b9 b9-H_3PO_4 [m/z]	y1 y1-H_3PO_4 b10 b10-H_3PO_4 [m/z]	rt[min]
S[p]DSAVSIVHLK	[S70]	622.31	1076.62 - - -	961.59 - - -	874.56 - 370.06 252.09	803.52 - 441.1 343.12	704.45 - 540.17 442.19	617.42 - 627.20 529.23	504.27 - 740.29 642.31	405.27 - 839.35 741.38	- - 976.41 878.44	- - 1089.50 991.52	14.6
SD**S[p]**AVSIVHLK	[S72]	622.31	1156.59 1058.61 - -	1041.56 943.53 - -	874.56 - 370.06 252.09	803.52 - 441.1 343.12	704.45 - 540.17 442.19	617.42 - 627.20 529.23	504.27 - 740.29 642.31	405.27 - 839.35 741.38	- - 976.41 878.44	- - 1089.50 991.52	14.3
SDSAV**S[p]**IVHLK	[S75]	622.31	1156.59 1058.61 - -	1041.56 943.53 - -	954.53 856.55 290.10 -	883.49 785.51 361.14 -	784.42 686.44 460.20 -	617.42 - 627.20 529.23	504.27 - 740.29 642.31	405.27 - 839.35 741.38	- - 976.41 878.44	- - 1089.50 991.52	14.9

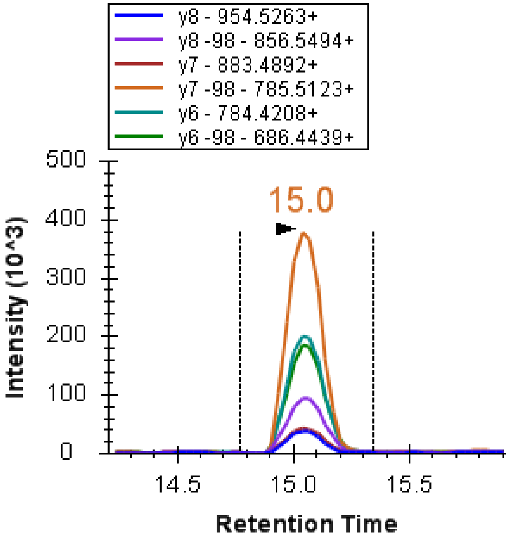
[S70]



[S72]

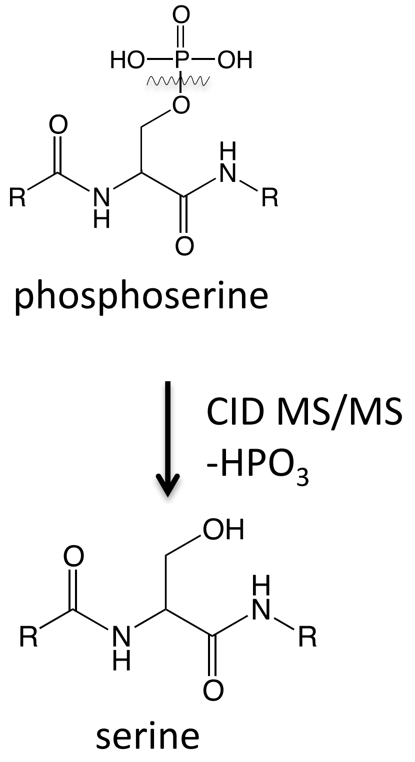


[S75]



Further complications for a phospho-analysis with SRM: Neutral Loss HPO_3

2. Loss of HPO_3 = -80 Da

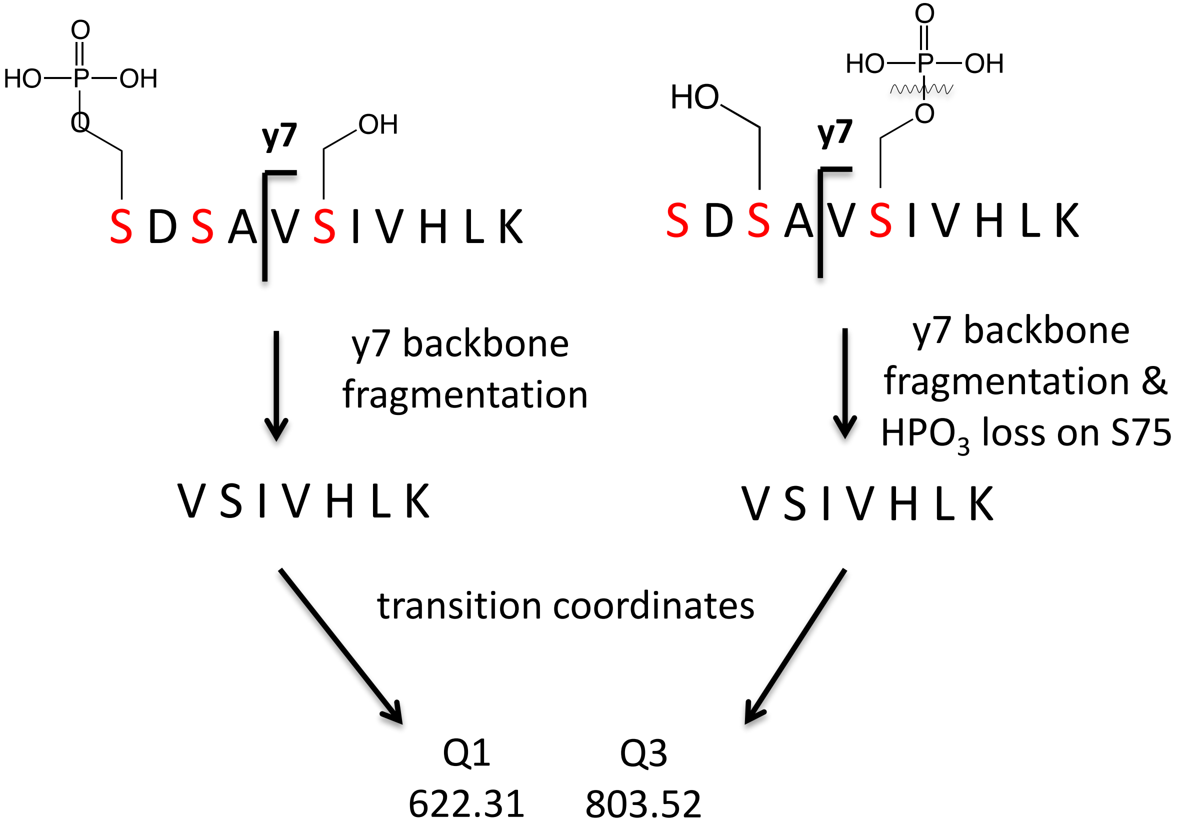


phosphoserine

CID MS/MS
-HPO_3

serine

example:



y7 backbone fragmentation

y7 backbone fragmentation & HPO_3 loss on S75

V S I V H L K

V S I V H L K

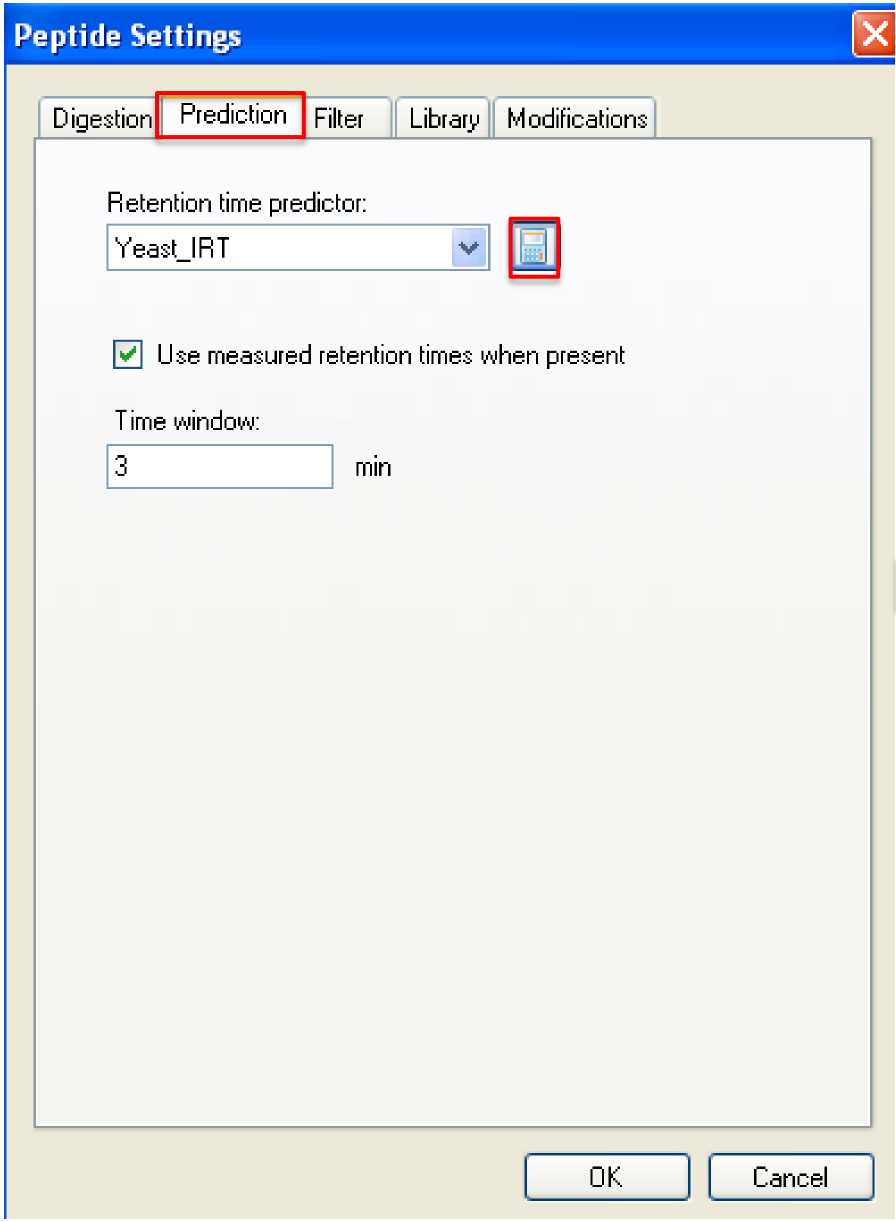
transition coordinates

Q1	Q3
622.31	803.52

Conclusion

For peptides comprising **several possibly phosphorylated residues** phospho-site **assignments** based exclusively on **fragment ions NOT carrying the phosphate group** can be **erroneous**, because their occurrence can also be due to a neutral loss of HPO_3.

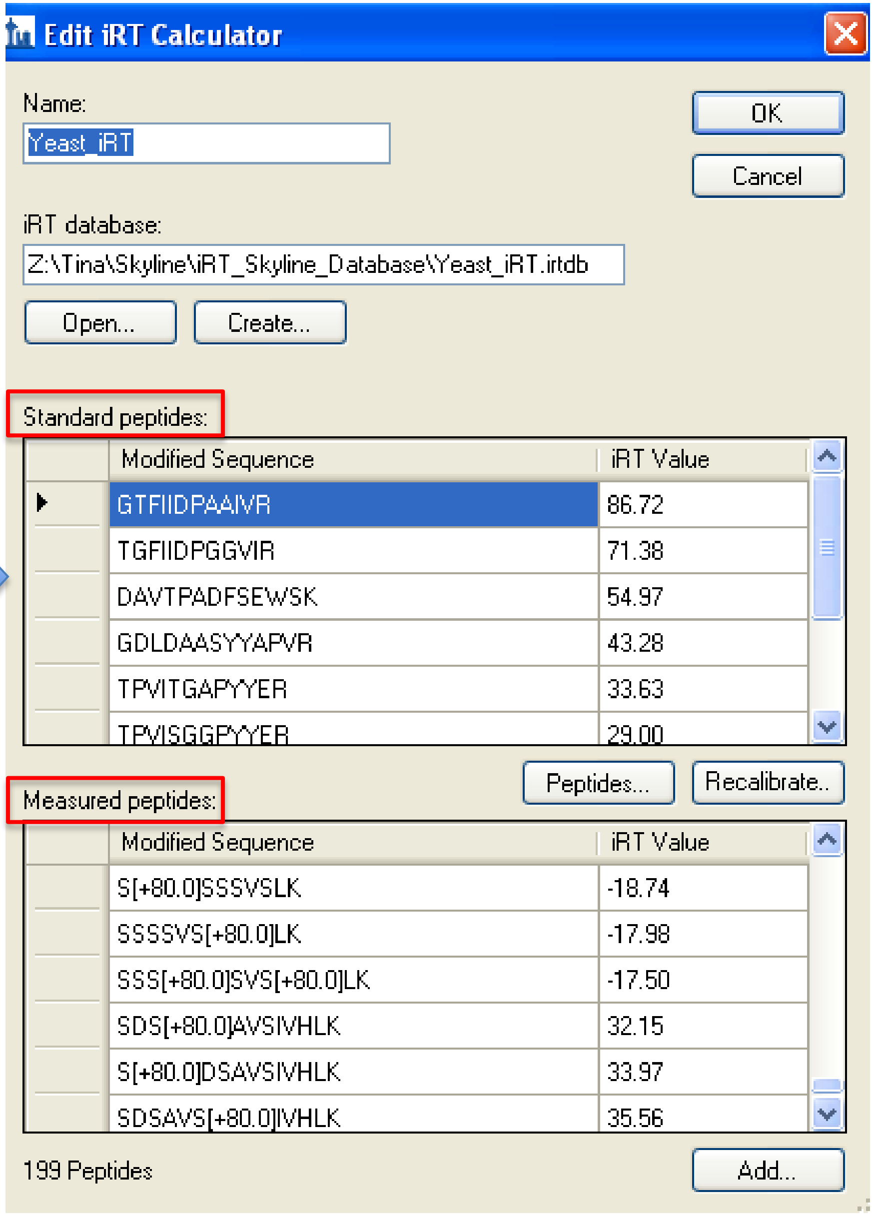
The “iRT” concept implemented into Skyline



Retention time predictor: Yeast_IRT

☑ Use measured retention times when present

Time window: 3 min



Name: Yeast_iRT

iRT database: Z:\Tina\Skyline\iRT_Skyline_Database\Yeast_iRT.irtdb

Standard peptides:

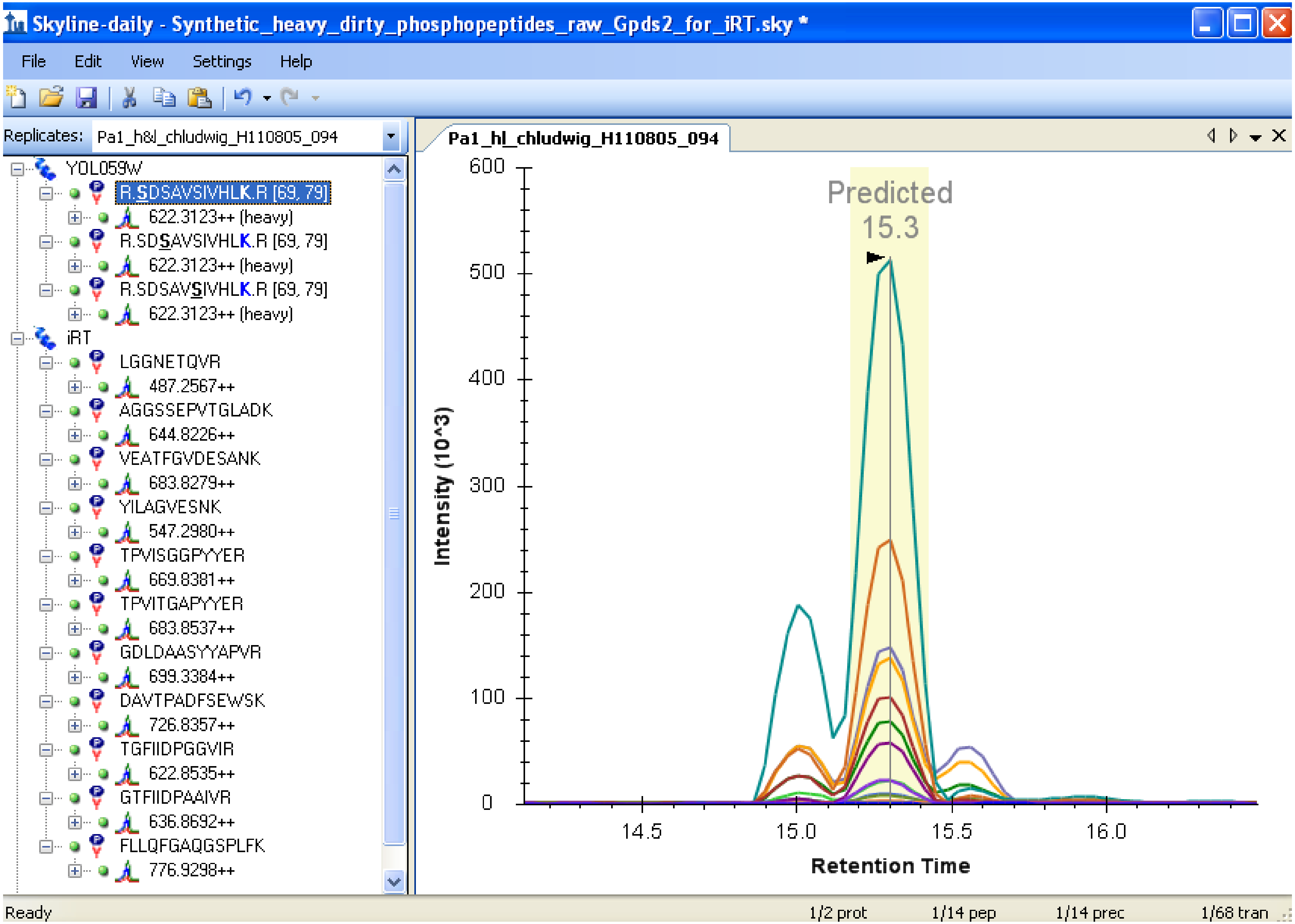
Modified Sequence	iRT Value
GTFIIDPAAIVR	86.72
TGFIIDPGGVIR	71.38
DAVTPADFSEWSK	54.97
GDLDAASYYAPVR	43.28
TPVITGAPYYER	33.63
TPVISGGPYYER	29.00

Measured peptides:

Modified Sequence	iRT Value
S[+80.0]SSSVSLK	-18.74
SSSSVS[+80.0]LK	-17.98
SSS[+80.0]SVS[+80.0]LK	-17.50
SDS[+80.0]AVSIVHLK	32.15
S[+80.0]DSAVSIVHLK	33.97
SDSAVS[+80.0]IVHLK	35.56

199 Peptides

The “iRT” concept implemented into Skyline



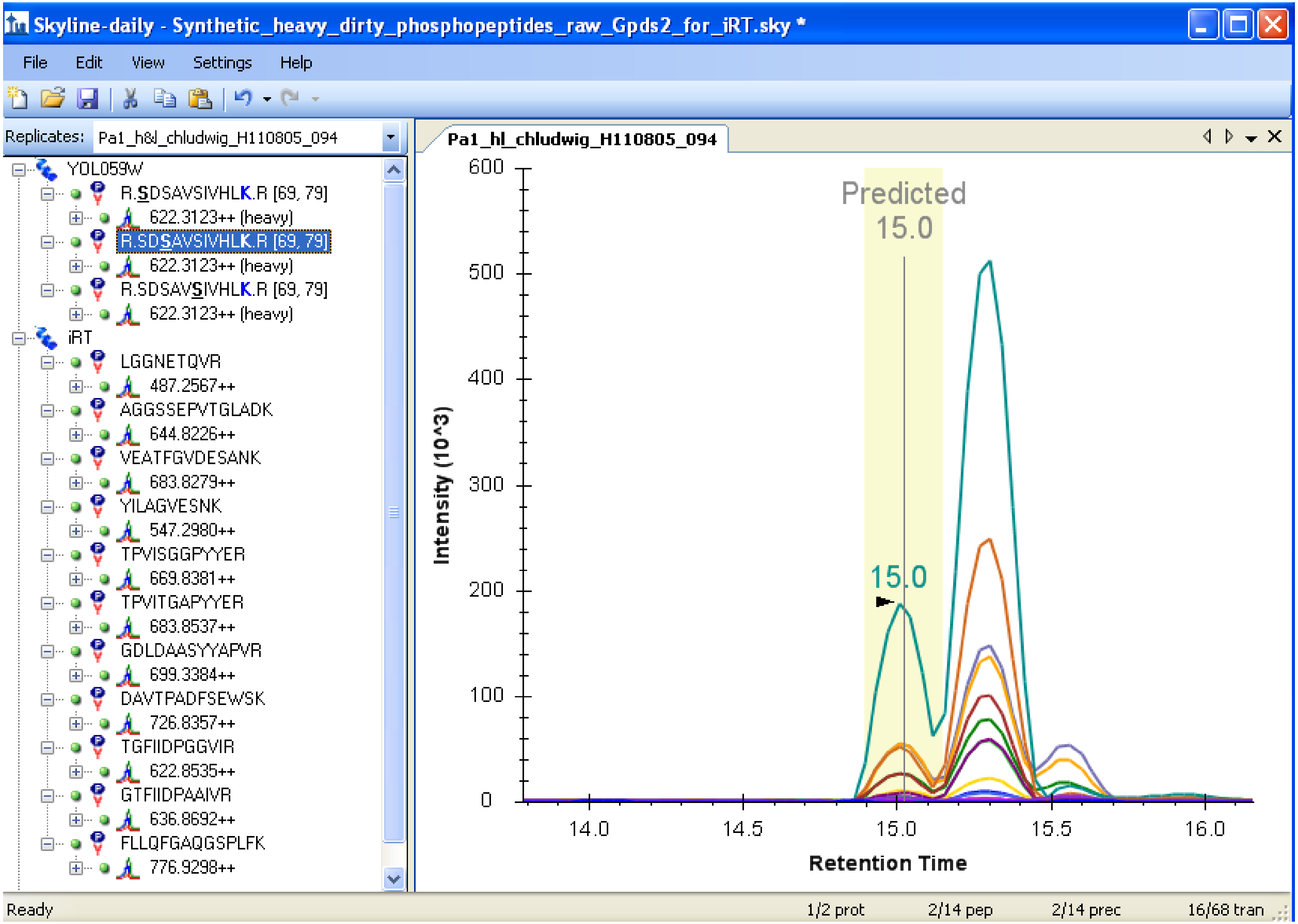
Skyline-daily - Synthetic_heavy_dirty_phosphopeptides_raw_Gpds2_for_iRT.sky *

Replicates: Pa1_h&l_chludwig_H110805_094

- YOL059W
  - R.SDSAVSIVHLK.R [69, 79]
    - 622.3123++ (heavy)
  - R.SDSAVSIVHLK.R [69, 79]
    - 622.3123++ (heavy)
  - R.SDSAVSIVHLK.R [69, 79]
    - 622.3123++ (heavy)
- iRT
  - LGGNETQVR
    - 487.2567++
  - AGGSSEPVTGLADK
    - 644.8226++
  - VEATFGVDESANK
    - 683.8279++
  - YILAGVESNK
    - 547.2980++
  - TPVISGGPYYER
    - 669.8381++
  - TPVITGAPYYER
    - 683.8537++
  - GDLDAASYYAPVR
    - 699.3384++
  - DAVTPADFSEWSK
    - 726.8357++
  - TGFIIDPGGVIR
    - 622.8535++
  - GTFIIDPAAIVR
    - 636.8692++
  - FLLQFGAQGSPLFK
    - 776.9298++

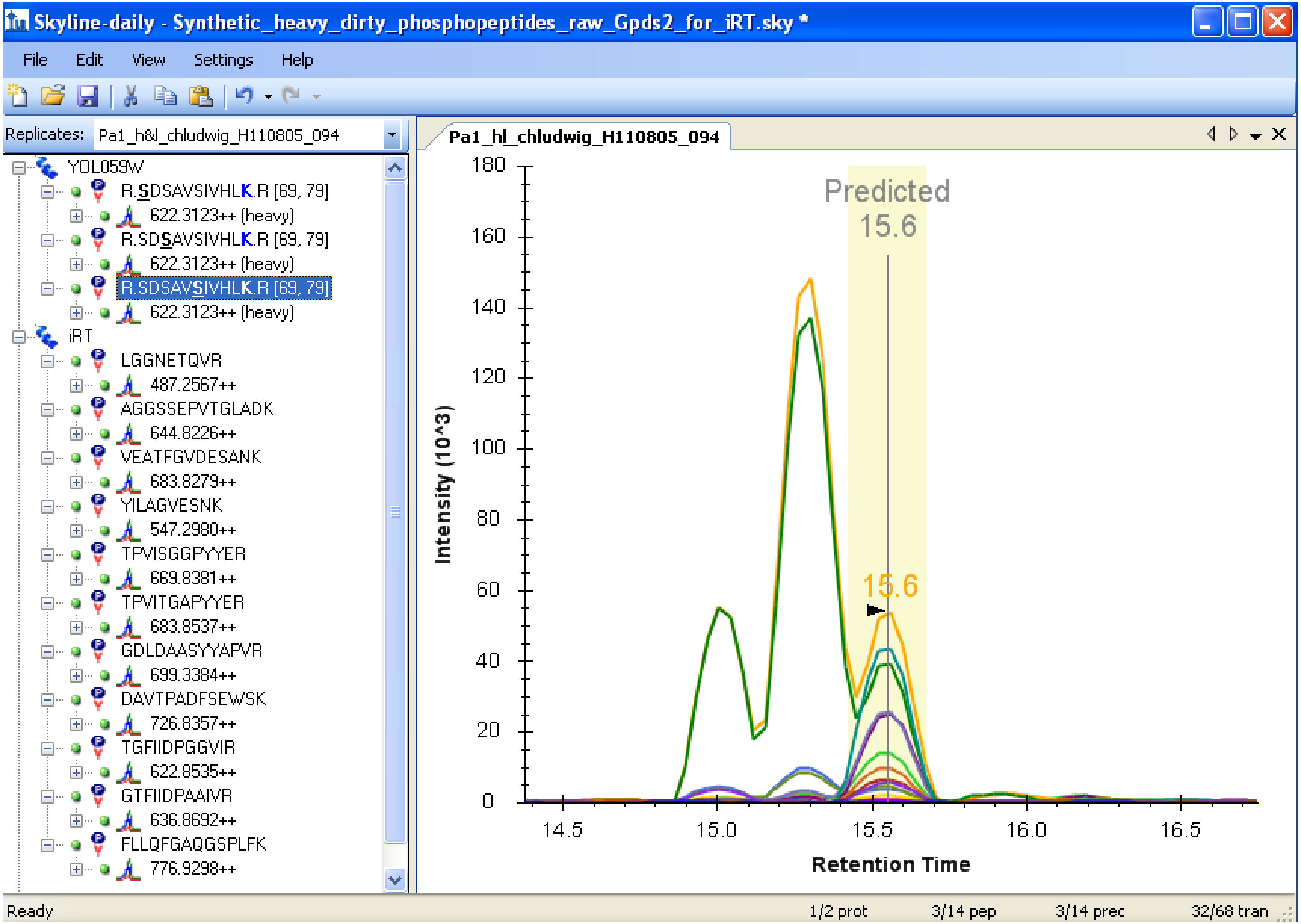
Ready 1/2 prot 1/14 pep 1/14 prec 1/68 tran

The “iRT” concept implemented into Skyline



- YOL059W
  - R.SDSAVSIVHLK.R [69, 79]
    - 622.3123++ (heavy)
  - R.SDSAVSIVHLK.R [69, 79]
    - 622.3123++ (heavy)
  - R.SDSAVSIVHLK.R [69, 79]
    - 622.3123++ (heavy)
- iRT
  - LGGNETQVR
    - 487.2567++
  - AGGSSEPVTGLADK
    - 644.8226++
  - VEATFGVDESANK
    - 683.8279++
  - YILAGVESNK
    - 547.2980++
  - TPVISGGPYYER
    - 669.8381++
  - TPVITGAPYYER
    - 683.8537++
  - GDLDAASYYAPVR
    - 699.3384++
  - DAVTPADFSEWSK
    - 726.8357++
  - TGFIIDPGGVIR
    - 622.8535++
  - GTFIIDPAAIVR
    - 636.8692++
  - FLLQFGAQGSPLFK
    - 776.9298++

The “iRT” concept implemented into Skyline



Replicates: Pa1_h&l_chludwig_H110805_094

YOL059W
R.SDSAVSIVHLK.R [69, 79]
622.3123++ (heavy)
R.SDSAVSIVHLK.R [69, 79]
622.3123++ (heavy)
R.SDSAVSIVHLK.R [69, 79]
622.3123++ (heavy)
iRT
LGGNETQVR
487.2567++
AGGSSEPVTGLADK
644.8226++
VEATFGVDESANK
683.8279++
YILAGVESNK
547.2980++
TPVISGGPYYER
669.8381++
TPVITGAPYYER
683.8537++
GDLDAASYYAPVR
699.3384++
DAVTPADFSEWSK
726.8357++
TGFIIDPGGVIR
622.8535++
GTFIIDPAAIVR
636.8692++
FLLQFGAQGSPLFK
776.9298++

Ready 1/2 prot 3/14 pep 3/14 prec 32/68 tran