A Novel Mixed Mode Chromatography Method for the Isolation and Separation of Phosphopeptides

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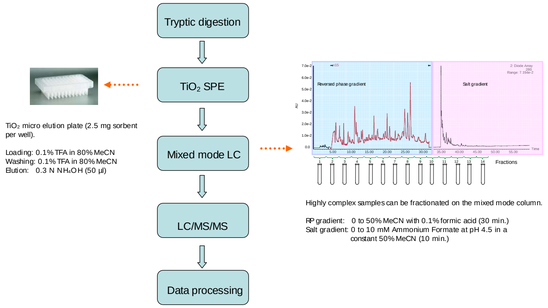
INTRODUCTION

IMAC and TiO_2 type of affinity chromatography is widely used to isolate phosphopeptides. However, some non-phosphopeptides (*e.g.*, acidic peptides) also bind to the affinity sorbent; the degree of non-specific binding increases with increased sample complexity. We present an improved phosphopeptide enrichment/separation method that combines TiO_2 and a prototype mixed mode (reversed-phase and strong cation exchange) liquid chromatography media prior to tandem mass spectrometry analysis.

- Phosphopeptides are weakly retained on the mixed mode LC column due to the negatively charged phosphate. A reversed-phase gradient elutes phosphopeptides according to the number of phosphate groups and their hydrophobicity.
- Non-phosphorylated peptides retain strongly on the mixed mode LC column due to ionic interaction. A rapid shallow salt gradient is used to elute these peptides.
- The isolation of phosphopeptides are further improved by processing complex samples using a TiO_2 SPE device prior to the mixed mode LC separation.

METHODS

Scheme 1. The work flow of enriching and separating phosphopeptides from a complex protein digest for LC/MS/MS analysis



Highly complex samples can be fractionated on the mixed mode column.

RP gradient: 0 to 50% MeCN with 0.1% formic acid (30 min.)
Salt gradient: 0 to 10 mM Ammonium Formate at pH 4.5 in a constant 50% MeCN (10 min.)

- **Instrument and software**

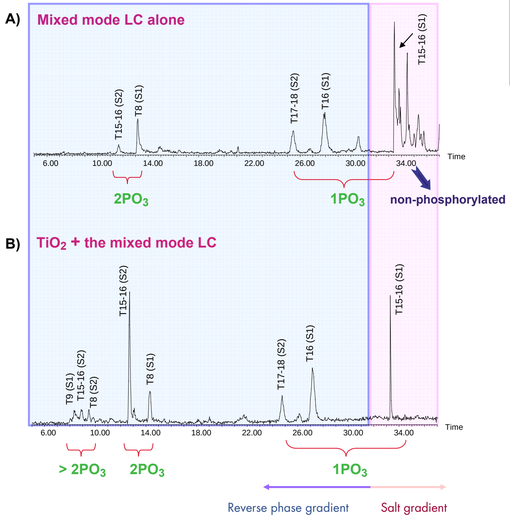
Waters nanoACQUITY™ UPLC with 75 µm nanoACQUITY column
Waters Micromass® QTof API US Mass Spectrometry
Waters Alliance HPLC 2795
Waters Micromass® ZQ Mass Spectrometry

MassLynx 4.0 software is used to control LC/MS experiments
Protein Global Server v. 2.2 for data processing and database search

RESULTS

- **Separation of phosphopeptides using the mixed mode LC**

LC/MS (+)



Alpha-casein S1
T16 VPQLEIVPNpSAEER
T15-16 YKVPQLEIVPNpSAEER
T8 DIGpSEpSTEDQAMEDIK
T9 QMEAEpSIpSpSpSpSEEIVPNpSVEQK

Alpha-casein S2
T15-16 EQLpSTpSEENSKK
T17-18 TVDMEpSTEVFTKK
T3 NTMEHVpSpSpSEESIIpSQETYK
T8 NANEEEYSIGpSpSpSEEpSAEVATEEVK

Figure 1. Alpha casein tryptic peptides were separated using A) the mixed mode LC column alone and B) the mixed mode LC column after TiO_2 SPE extraction.

- *Most phosphopeptides are separated and eluted under a reverse phase gradient (0—50% MeCN); while other peptides are retained unless a salt gradient is applied.*
- *The elution order of phosphopeptides is determined by their charge and hydrophobicity; the early eluting phosphopeptides contain more negatively charged phosphate groups than the later ones.*
- *TiO_2 SPE was used before the mixed mode LC separation to improve the detection of phosphopeptides, especially the singly phosphorylated peptides which retain more strongly on the mixed mode column.*

- **Acidic peptides (pI < 4.5) are separated from phosphopeptides**

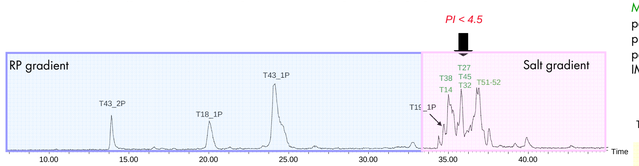


Figure 2. A yeast enolase tryptic digest contains four synthetic phosphopeptides (see Table 1) are separated using the mixed mode LC. Three out four phosphopeptides are eluted during a reversed phase gradient while the non-phosphopeptides are eluted during a salt gradient. Acidic peptides that have pI less than 4.5 are labeled in green.

Mixed mode column: acidic peptides are separated from phosphopeptides; these acidic peptides are problematic for IMAC type of affinity separation.

Table 1.

Phosphopeptide Description	Sequence	$[M+H]^+$	$[M+2H]^{2+}$
T18_1P	NVPLpIYK	813.39	407.20
T18_3p	HLADLpSEK	863.40	432.21
T43_1p	VNQGGpTJLSESK	1368.68	684.84
T43_2P	VNQGGTLpSDEpSK	1448.64	724.83

- **Complex Sample (Yeast YPD Cytosol Tryptic Digest)**

Yeast sample preparation:
Yeast (YPD) was grown in dextrose. At the log phase after 3 hr, alpha factor was added. The cytosolic fraction of the yeast was digested using trypsin, followed by enrichment using TiO_2 SPE and the mixed mode LC fractionation (14 fractions were collected).

LC/MS/MS instrumentation:
LC: nanoLC (Waters nanoACQUITY) equipped with a 75 µm nanoACUITY column
MS: QTOF (API US) MS
Data Dependent Acquisition was performed on ions with (+1, +2, +3, +4) charges.

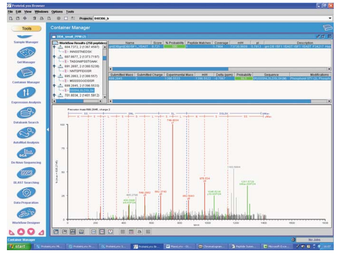
Data Processing and search → Protein Links Global Server V 2.2 and Mascot

Results
A total of 70 non-redundant phosphopeptides with unique phosphorylation sites were identified using the yeast database. More than one third of the identified peptides are multiply phosphorylated (Table 2).

Table 2. All the listed phosphopeptides have scores > 20 from Mascot search. The search parameters used are: fixed modification with carbamidomethyl on Cysteine, variable modification with N-Acetyl on protein, Methionine oxidation and Phosphorylation on S, T, Y.

Protein Name	Swiss Accession #	Peptide MW	Ion Charge	Peptide Sequence (Phosphorylation Sites)	# of Phosphorylation
EMI2_YEAST	(Q04409)	995.4113	2+	SFENLHK + N-Acetyl (Protein)	1
HXKG_YEAST	(P17709)	982.3797	1+	SFDDLHK + N-Acetyl (Protein)	1
YG22_YEAST	(P53235)	2986.3848	3+	ALLELLDDSPVTPGETRPAYDGYEASK	1
IXR1_YEAST	(P33417)	751.3517	1+	VPELSK	1
ASK2_YEAST	(P35734)	1920.7027	2+	LPDSSDEDTGSEAVPSR	2
IXR1_YEAST	(P33417)	751.3517	1+	VPELSK	1
HUL5_YEAST	(P53119)	1223.5240	2+	DNVLLYVTK	1
IXR1_YEAST	(P33417)	1170.4917	2+	EESRLNDTK	1
SMF1_YEAST	(P38925)	626.2676	1+	EALSK	1
YHM4_YEAST	(P38788)	2666.9058	3+	EENAEEDDESEWSDDEPEVVR	2
TPS3_YEAST	(P38426)	1802.7505	2+	VCSPSQEASASSSISASR	1
YBF1_YEAST	(P34217)	1396.5524	2+	SSQNLSLDSLSK	2
ZEO1_YEAST	(Q08245)	1294.5442	2+	NEATPEAEQVK	1
EF3A_YEAST	(P16521)	1619.5317	2+	ELGDAYVSSDEEF	2
PUF6_YEAST	(Q04373)	1697.6321	2+	ISIGSSDEESELSK	2
YNQ6_YEAST	(P53890)	1256.5285	2+	SGGSTPLDSQTK	1
ABP1_YEAST	(P15891)	1451.6462	2+	EPVKTPSPAPAAK	2
ZEO1_YEAST	(Q08245)	1296.5598	2+	EQAEASQDNLK	1
ZEO1_YEAST	(Q08245)	2573.0934	3+	EQAEASQDNLKNEATPEAEQVK	2
G3P2_YEAST	(P00358)	1453.6813	2+	TASGNIIPSSTGAAK	1
BAF1_YEAST	(P14154)	2066.8317	2+	ADDEEDLSDENQPELR	1
GLY1_YEAST	(P37303)	1570.6511	2+	SESTEVDVDGNAIR	1
ENT5_YEAST	(Q03769)	3368.2749	3+	MNNNDNGSDDDDEFGDFQSETSPDTTAPK	1
TOM22_YEAST	(P49334)	3795.3629	3+	ASATNNDYVDDEDDSDSDFEDEFDENETLLDR	2
UBP1_YEAST	(P25037)	3153.2869	3+	INVEDQLTTSSEEDVPAPPNYAR	3
FKBP4_YEAST	(Q06205)	3418.267	3+	GEYYNQDNNDGLEEDEFSEQEQADVPKR	2
RCC1_YEAST	(P21827)	3257.2003	3+	EQLKDMDADDSSDDEDGDLNELESTPAK	2
NU159_YEAST	(P40477)	2742.1561	3+	LPETPSEDGSVVEEEAQKSPGK	2
T2FB_YEAST	(P41896)	3291.2652	3+	SGNISGDEVLSQEEEVFDGNDIENNETK	2
ISR1_YEAST	(Q06098)	1369.6279	2+	NDLDVLETPR	1
EF3A_YEAST	(P16521)	1747.6266	2+	KELGDAYVSSDEEF	2
TPS3_YEAST	(P38426)	3751.39	3+	YSQQFQDNFRDTSEDDIDSDLETDATKK	3
NAB4_YEAST	(P15891)	2963.1521	3+	SSDNEDFNDYGDDKPTTTEEVKK + N-Acetyl (Protein)	2
SUB2_YEAST	(Q07478)	2899.182	3+	SHEGEEDLLEYSDMEQEIQDASK + N-Acetyl (Protein)	1
LSB5_YEAST	(P25369)	3486.3102	3+	YDISESSFEESLSSYQPSTSINPFGDHNK	3
IFSA2_YEAST	(P22301)	2982.1684	3+	SDEIHTFETADAGSSATVPMQCSALR + N-Acetyl (Protein)	1
SODC_YEAST	(P00445)	2792.1813	3+	FEQASESEPTTVSVEAGAGNPNAER	1
RCC1_YEAST	(P21827)	3257.2003	3+	EQLKDMDADDSSDDEDGDLNELESTPAK + Oxidation (M)	2
RPN8_YEAST	(P00449)	2392.9672	3+	VSDDSESESGDKEATAPLIQR	2
SIS1_YEAST	(P25294)	3361.4332	3+	SASSSPTYPEEETVQVNLPVSLEDLFVGK	3
UBP1_YEAST	(P34223)	1643.7807	2+	LGSPIPGESSPAEVPK	1
YK28_YEAST	(P36114)	2663.9459	3+	DSSNLEFDEEDEEMDGFTLLHPGK + Oxidation (M)	2
FKBP3_YEAST	(P38911)	3812.2982	3+	NPDFEDDDFLGGDFDEDEIDEESESEEEEEEEK	2
HXKG_YEAST	(P17709)	982.3797	1+	SFDDLHK + N-Acetyl (Protein)	1
NU159_YEAST	(P40477)	1347.5707	2+	DGYYSGSEISVR	1
PIK1_YEAST	(P39104)	1742.7780	2+	SSTPTSPPDLIDPIK	2
CYS3_YEAST	(P31373)	1748.7406	2+	QSSPTANPIGTYEYSR	1
NCPR_YEAST	(P16603)	832.3367	1+	YLSPGAK	1
BBC1_YEAST	(P47068)	1304.5901	2+	DLPERPSPETK	1
EF1B_YEAST	(P32471)	4201.5967	3+	ADEFDSFPAASAAAAEEEEDDVDLFGSDDEEADAEAEK	1
IF43_YEAST	(P12962)	3114.3652	3+	GSGEDDEEETITTPTSTVPVATAQETLK	2
IF43_YEAST	(P12962)	3322.4200	3+	KGSGEDDEEETITTPTSTVPVATAQETLK	2
ENO1_YEAST	(P00924)	1367.6697	2+	VNQIGTLSESIK	1
SIS1_YEAST	(P25294)	3281.4668	3+	SASSSPTYPEEETVQVNLPVSLEDLFVGK	2
YBB7_YEAST	(P34216)	3330.4653	3+	SGSSSVNMAPQSVRDDVELPETLEER	2
WEB1_YEAST	(P38968)	1321.6278	2+	VPSLVATSESPR	1
TPS3_YEAST	(P38426)	3015.3375	3+	SSSVTSASSNGSLPQEAQINGSFLQR	2
TPS3_YEAST	(P38426)	3671.4237	3+	YSQQFQDNFRDTSEDDIDSDLETDATKK	2
DPH5_YEAST	(P32469)	2817.1330	2+	DVANDQEYFKPAAWVPPTEDDSQE	1
SUB2_YEAST	(Q07478)	2899.1820	2+	SHEGEEDLLEYSDMEQEIQDASK + N-Acetyl (Protein)	1
YEMO_YEAST	(P40019)	5251.0537	4+	SENNGTVEDSESDMDDAKLDALMGNEGEEEEDDLAEIDTSNIITSGR + 2 Oxidation (M)	2
MDG1_YEAST	(P53885)	2600.1530	3+	SFSQEVVELPDSESETQQVNK	1
GYS2_YEAST	(P27472)	1394.6472	2+	YARPLSVPGSPR	2
YG22_YEAST	(P53235)	3308.4205	3+	SSETSPDSTPAPSAPASTNAPTMNKETSPEEK	1
RLA1_YEAST	(P05318)	2286.8035	2+	EEEEAKEESQDDMGFGLFD + Oxidation (M)	1
RLA2_YEAST	(P05319)	4462.8430	4+	LAAVPAAGPASAGGAAAASGDAAAEEEKEEEAAEESQDDMGFGLFD + Oxidation (M)	1
VPS74_YEAST	(Q06385)	1770.7057	2+	AGSSDTSSIHSSANNTK	1
IF2B_YEAST	(P09064)	3820.7080	3+	AGLDNVDAESKEQTPSANSSSQQEVGLPYSELLSR	2
TREA_YEAST	(P32356)	3117.3157	3+	RLSSLSEPNDPFSNAEVYYGPPTDPR	2
YKF4_YEAST	(P35732)	3691.5196	3+	NNYNYYQTQNGQEQQGPNQGVAQHSEDSQQK	1

An example of database search result (a screen shot from PLGS)



Ion with m/z of 699.28 was identified using PLGS data processing and database search. A screen capture shows the ID of this phosphopeptide collected in fraction # 5.

Mascot search result for the same ion

```
YBF1_YEAST Mass: 73788 Score: 49 Queries matched: 1

(P34217) Hypothetical 73.8 kDa protein in SAS3-SEC17 intergenic region

Query    Observed    Mr(expt)    Mr(calc)    Delta    Miss    Score    Expect    Rank    Peptide

55       699.2845    1396.5544   1396.5524   0.0021   0       49       0.00033   1       SSSNLSLSDSLSK + 2 Phospho (ST)
```

Discussion

- Multiply phosphorylated peptides gave low confidence scores due to their low ionization efficiency and the complexity of the fragment ions.
- Many phosphopeptides with good MS/MS signals were not identified (no match). Further optimization of the search parameter is needed to identify these peptides.

CONCLUSION

- Affinity chromatography (IMAC or TiO_2) alone is not sufficient for isolating phosphopeptides from highly complex samples. A combination of TiO_2 SPE and the mixed mode LC separation/fractionation improves the overall phosphopeptide selectivity and reduces the sample complexity for LC/MS/MS analysis and data processing.
- The advantage of using the mixed LC column is to further separate phosphopeptides and non-phosphorylated peptides (mostly acidic peptides) *via* a combination of ionic and reversed-phase interactions. Also phosphopeptides with similar charge properties are further separated by their hydrophobicity.

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