PHOSPHOPEPTIDE ENRICHMENT METHOD DEVELOPMENT USING MICRO SCALE HIGH AFFINITY SOLID PHASE EXTRACTION

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OVERVIEW

- A novel phosphopeptides enrichment method has been developed using micro scale solid phase extraction device.
- The enrichment method is selective, robust, and reproducible.
- Sample loading with the displacer (under the trade name Enhancer™) further improves the selectivity.
- The performance of phosphopeptide enrichment is evaluated with phosphopeptide standards and alpha-casein digest.

INTRODUCTION

Phosphorylation is one of the most common post-translational protein modifications in living cells and its investigation is a key interest in proteomics. However, the analysis of phosphorylation in proteins and peptides by mass spectrometry is challenging due to their low abundance and low ionization efficiency. Phosphopeptides are often suppressed in comparison to the unphosphorylated species when measured in complex mixtures. Therefore, it is critical to selectively enrich phosphopeptides prior to mass spectrometry analysis. In these experiments, we show the enrichment method using Waters® MassPREP™ Phosphopeptide Enrichment Kit. The robustness and reproducibility of the method are tested using the micro scale solid phase extraction device packed with high affinity metal oxide sorbent. Reduction of non-specific acidic peptides binding is achieved by addition of Enhancer™. This is especially useful for highly complex mixture. The performance of Waters MassPREP™ Phosphopeptide Enrichment Kit is compared to immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO_2).

EXPERIMENTAL METHOD

1. Sample Preparation

- Waters MassPREP™ Phosphopeptide standard (Table 1) is mixed with MassPREP™ Enolase digestion standard in 1:1, 1:10, 1:50 and 1:100 molar ratio in loading solution(0.5% TFA in 80% acetonitrile).
- Tryptic digested alpha-casein derived from bovine milk (Sigma) was prepared in 2µg/µL.

Phosphopeptide	Sequence	$[M+H]^+$	$[M+2H]^{2+}$
T18_1P	NVPLpYK	813.39	407.20
T19_1P	HLADLpSK	863.40	432.21
T43_1P	VNQIGpTLSESIK	1368.68	684.84
T43_2P	VNQIGTLpSEpSIK	1448.64	724.83

Table 1. Amino acid sequence and m/z of the four phosphopeptide standards

2. Instrumentation

Waters nanoACQUITY UPLC® and Q-TOF Premier™
Waters Alliance 2795 and PDA996 and ZQ™ Single Quadrupole MS
Waters MALDI micro MX

3. Methods

nanoLC-MS:
Analytical column Atlantis dC18, 3.5µm, 75µmx100mm
Trapping column Symmetry C18, 5µm, 180µmx20mm
Mobile phase A: 0.1% Formic acid in 100% water
Mobile phase B: 0.1% Formic acid in 100% acetonitrile
Gradient: 2% - 60%B
Injection volume: 2µL, partial loop, Flow rate: 300nL/min.

MALDI on reflectron positive and negative mode
MassPREP™ MALDI Matrix DHB
2% Phosphoric acid as a matrix additive

4. Enrichment protocol (the SPE plate is operated using a vacuum manifold.)

Condition : 200µL water
↓ Vacuum manifold settings: 7-12in. Hg
Equilibrate : 200µL 0.5% TFA in 80% MeCN
↓ Vacuum
Load :200µL of sample in 0.5% TFA in 80% MeCN or in 50mg/mL Enhancer™ in 0.5% TFA in 80% MeCN
↓ Gravity (no vacuum on)
Wash 1 : 200µL 0.5% TFA in 80% MeCN
↓ Vacuum
Wash 2 : 200µL water
↓ Vacuum
Elute : 200µL 100mM Diammonium Phosphate in 20% MeCN or 2% Triethylamine in 20% MeCN

*Figure 1. **Waters MassPREP™ Phosphopeptide Enrichment Kit** includes a µElution plate packed with affinity sorbent, MassPREP™ Enhancers, MassPREP™ Enolase digestion standard with Phosphopeptide Standard.*

RESULTS

NanoLC-MS

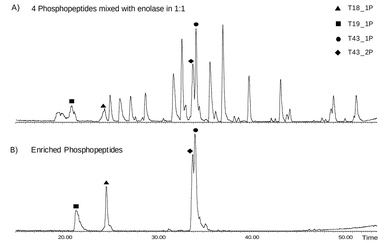


Figure 2. NanoLC/MS analysis of A) Four phosphopeptides with enolase in 1:1 molar ratio without enrichment (10 pmol). B) The same sample processed using MassPREP™ Phosphopeptides Enrichment Kit. The four phosphopeptides were selectively enriched. The LC injection amount was 500 fmol.

MALDI (LD +)

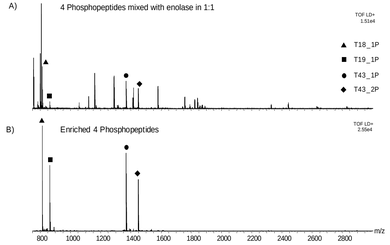


Figure 3. MALDI-TOF analysis of A) Phosphopeptide standards with enolase in 1:1 molar ratio without enrichment (10 pmol). B) The same sample enriched using Waters MassPREP™ Kit. Approximately 2 pmol of sample was spotted onto the MALDI target.

MALDI (LD +)

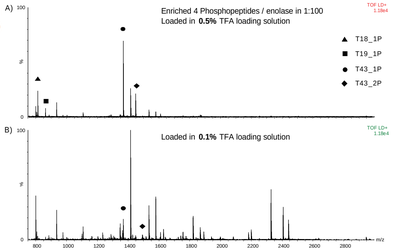


*Figure 4. Improved selectivity using high TFA in the loading solution. A) 0.5% TFA in 80% acetonitrile was used to load 4 phosphopeptides mixed with enolase in **1:100** molar ratio (10 pmol phosphopeptides with 1nmol enolase peptides). B) 0.1% TFA in 80% acetonitrile was used to load the same sample.*

NanoLC-MS

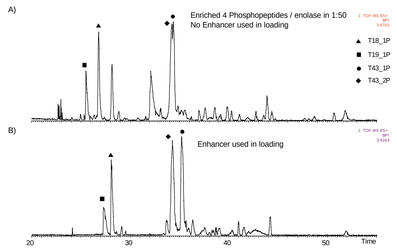


*Figure 5. **Enhancer™ improves the selectivity of the MassPREP™ kit.** A) Four phosphopeptides mixed with enolase in 1:50 molar ratio enriched without Enhancer™ (5 pmol phosphopeptides with 250 pmol of enolase peptides). B) The same sample enriched with 50mg/mL Enhancer™ in loading. Binding from acidic peptides were reduced.*

MALDI (LD +)

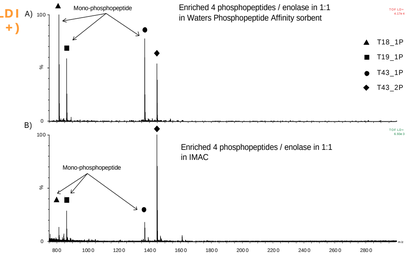


*Figure 6. **MassPREP™ Phosphopeptide Affinity sorbent vs. IMAC (NTA-Fe III)** A) Four phosphopeptides mixed with enolase enriched in Waters Phosphopeptide Affinity sorbent. B) The same sample enriched in IMAC. Di-phosphopeptide was recovered better than mono-phosphopeptides. The same amount of sample was used for both affinity sorbent (10 pmol of phosphopeptides mixed with 10 pmol of enolase peptides).*

MALDI (LD -)

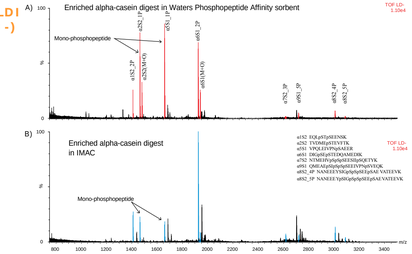


*Figure 7. **MassPREP™ Phosphopeptide Affinity sorbent vs. IMAC.** The enriched phosphopeptides are color coded. A) Alpha-casein digest (loaded 10µg) enriched using MassPREP™ Kit. B) The same sample enriched using IMAC. Singly phosphorylated peptides showed weaker affinity binding towards IMAC than multiple phosphorylated peptides.*

NanoLC-MS

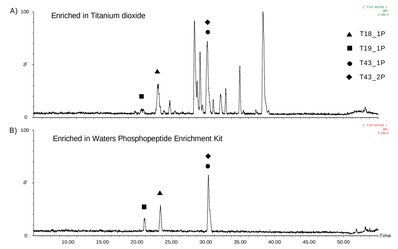
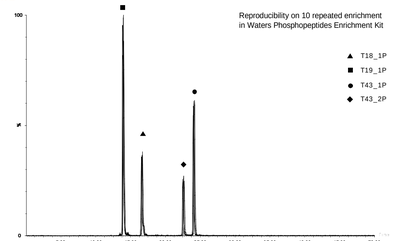


*Figure 7. **MassPREP™ Phosphopeptide Affinity sorbent vs. TiO_2** A) 4 phosphopeptides (10 pmol) mixed with enolase peptide (100 pmol) enriched in TiO_2 sorbent packed in µelution plate. B) The same sample enriched in Waters MassPREP™ Kit. Extensive binding from acidic peptides was observed with TiO_2, but not with MassPREP™ Phosphopeptide Affinity SPE device (no Enhancer™ was used).*

LC-MS



n=10	T19p	T18p	T43pp	T43p
Peak area % RSD	7	5	8	8

Figure 8. Overlaid of SICs from the phosphopeptides from 1st, 4th, 7th, and 10th measurement (400 pmol loaded). The % RSD of the peak area shows that the kit is highly reproducible.

CONCLUSIONS

- Waters MassPREP™ Phosphopeptide Enrichment Kit offers high selectivity and reproducibility for phosphopeptide isolation.
- The micro scale solid phase extraction device allows fast and robust phosphopeptide enrichment.
- The Enhancer™ chemical further improves the selectivity towards phosphopeptides in complex mixture by reducing non-specific binding.
- Singly phosphorylated peptides are enriched better using MassPREP™ phosphopeptide affinity sorbent than IMAC.
- Higher selectivity is observed using MassPREP™ phosphopeptide affinity sorbent than TiO_2.

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