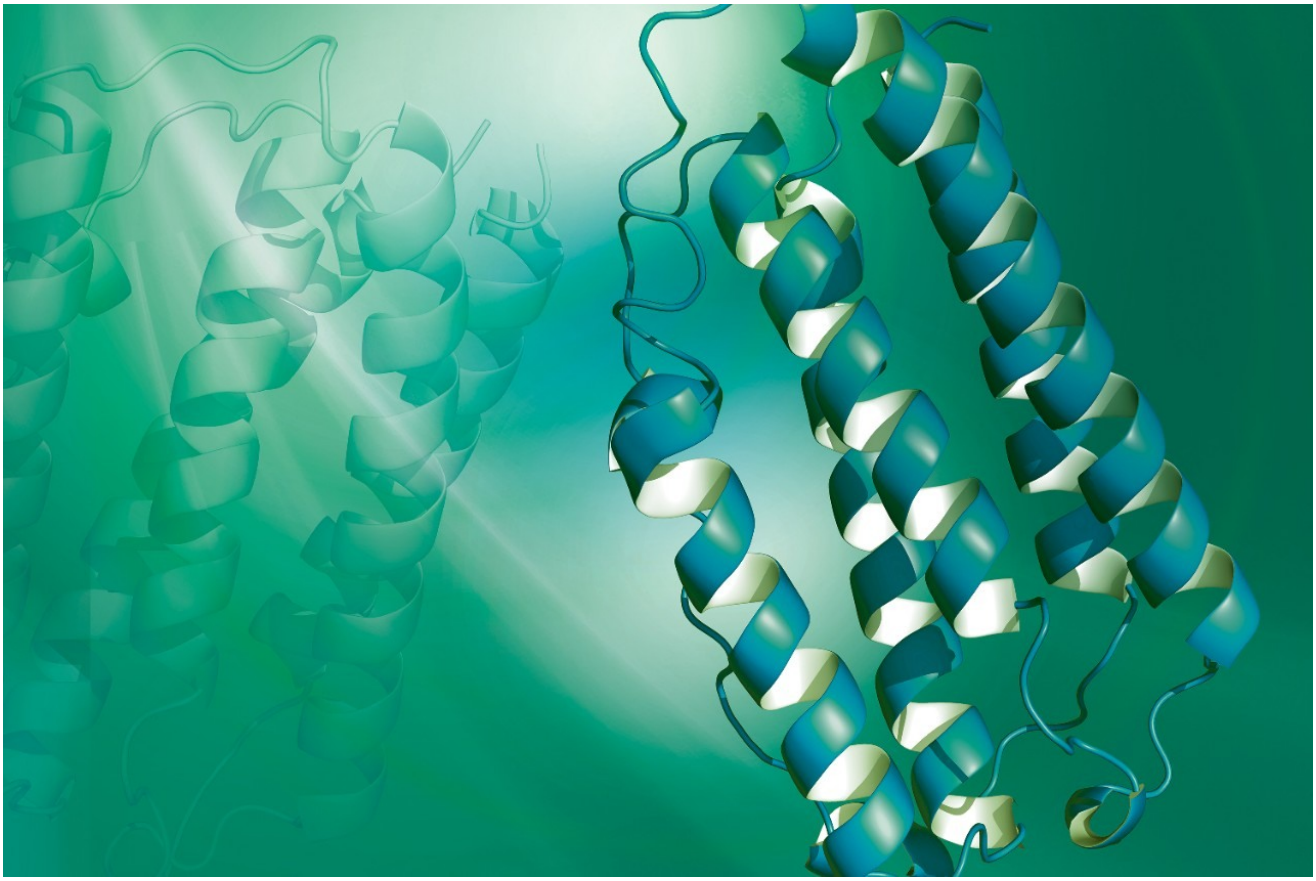
Waters™

應用手冊

Phosphopeptide Enrichment Method Using High Affinity Solid-Phase Extraction µElution Plate

Ying Qing Yu, Joomi Ahn, Ananya Dubey, Martin Gilar

Waters Corporation



Abstract

This application note illustrates the use of a Phosphopeptide Enrichment Kit that consists of an affinity extraction µElution plate, and a unique chemical reagent under the trade name of Enhancer, which is shown to improve the selectivity of the affinity solid-phase extraction (SPE) towards phosphopeptides for highly complex biological samples.

Introduction

The reversible phosphorylation of serine, threonine and tyrosine, is one of the most important post-translational modifications involved in various cellular functions. Identification of phosphorylation sites by mass spectrometry is challenging due to the low abundance of phosphopeptides and their limited ionization efficiency. Therefore, it is critical to selectively enrich the phosphopeptides prior to MS analysis. In this study we illustrate the use of a Phosphopeptide Enrichment Kit that consists of an affinity extraction µElution plate, and a unique chemical reagent under the trade name of Enhancer, which is shown to improve the selectivity of the affinity solid-phase extraction (SPE) towards phosphopeptides for highly complex biological samples. Also, a vial of phosphopeptide standard is included in the kit as a control sample to validate the affinity extraction method. MALDI-ToF MS and nano-scale LC-MS were used to evaluate the performance of the affinity solid-phase extraction device. Recommended protocol for the affinity SPE µElution plate, and sample preparation for MS analysis are included in the application.

Experimental

Materials Included in the Kit

- MassPREP Phosphopeptide Enrichment µElution Plate (P/N 186003820 < https://www.waters.com/nextgen/us/en/shop/application-kits/186003820-massprep-phosphopep-enrich-elute-plate.html>): A 96 well µElution plate packed with phosphopeptide affinity sorbent.
- MassPREP Enolase/Phosphopeptide Standard (P/N 186003286 < https://www.waters.com/nextgen/us/en/shop/standards--reagents/186003286-massprep-enolase-digest-with-phosphopeptides-mix.html>): A standard peptide sample of yeast enolase digest mixed with 4 synthetic phosphopeptides is included for method optimization (see Table 1). Concentration is 1 nmol/vial.
- MassPREP Enhancer (P/N 186003821): A chemical reagent to improve the selectivity of the Waters affinity

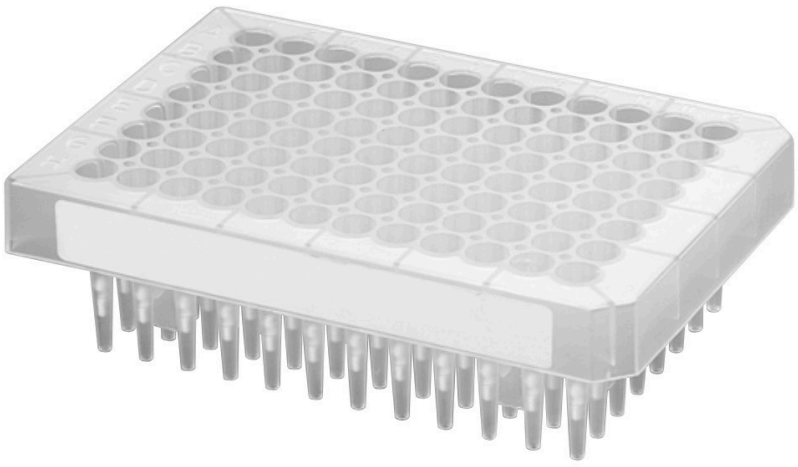
enrichment procedure. Enhancer reagent concentration is 500 mg/vial.

Phosphopeptide Description	Sequence	$[M+H]^+$	$[M+sH]^{2+}$
T18_1P	NVPL(pY)K	813.39	407.20
T19_1P	HLADL(pS)K	863.40	432.21
T43_1P	VNQIG(pT)LSESIK	1368.68	684.84
T43_2P	VNQIGTL(pS)E(pS)IK	1448.64	724.83

Table 1. Amino acid sequences and the mass to charge ratios of the four synthetic phosphopeptides (MassPREP Phosphopeptide Standards P/N 186003286).

Affinity SPE µElution Plate Protocol

The µElution plate is operated using a vacuum manifold (The extraction plate vacuum manifold (Waters P/N 186001831 <https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/186001831-extraction-plate-manifold-for-oasis-96-well-plates.html> is not included in this kit).



Vacuum Manifold Settings: 7 to 12 in. Hg

General Protocol

Condition the well Condition the wells with 200 µl of Milli-Q water first, then 200 µl of MeOH.

Prepare the sample Solubilize the sample in 0.2 to 0.5% TFA in 80% MeCN solution, the final volume is between

200 to 400 µl. For highly complex samples, load the sample in the appropriate solution containing between 50 to 100 mg of Enhancer solution[a].

Sample loading Load the sample into each well (loading capacity for each well is ~100 µg), and let the gravity pull the sample through the well (no need to turn on the vacuum for this step), collect the breakthrough solution using a collection plate. It takes about 15 to 25 minutes.

Washing the well Wash the wells with 200 µl 0.2-0.5% TFA in 80% MeCN (repeat this step if necessary). Wash again using 200 µl Milli-Q water.

Elution Elute with 200 to 400 µl of 100 mM diammonium phosphate (pH~8)b or 2% (v/v) triethylamine (pH~11)[b].

Lyophilization Neutralize the eluent and lypholize the eluent for further analysis.

[a] *Enhancer solution preparation: First prepare a solution of 0.2% TFA in 80% MeCN, 19.8% water (v/v) that is used to reconstitute the Enhancer powder. Weigh 100 mg of Enhancer and mix it with 1 ml (or 2 ml) of this dilution solution to make a final concentration of 100 mg/ml (or 50 mg/ml).*

[b] *We observed that diammonium phosphate eluent recovers multiply phosphorylated peptides slightly better than triethylamine. However, diammonium phosphate must be removed prior to MS analysis due to its ion-suppression effects*.

MALDI MS Analysis

If triethylamine is used to elute the enriched phosphopeptides, the lyophilized sample can be directly analyzed via MALDI MS after reconstituting the sample with appropriate solvent. No additional sample cleanup prior to MS analysis is required since triethylamine is a volatile reagent that is eliminated during lyophilization). However, for the diammonium phosphate eluted phosphopeptides, ZipTip like devices are needed to remove diammonium phosphate that suppresses MALDI ion signals. The following procedure can be used as a guideline for removing excess diammonium phosphate prior to MALDI Tof MS analysis.

1. Reconstitute the lyophilized phosphopeptides with Milli-Q water.

2. Follow the recommended manufacturing procedure to extract the peptides if ZipTip µC_{18} is the choice of clean up device.

3. Prepare a 20 mg/ml solution of 2,5-dihydroxide benzoic acid (DHB) (e.g., MassPREP DHB MALDI Matrix, P/N 186002333) using pure Ethanol, with 1% H_3PO_4 (v/v) mixed in the DHB solution.

4. Mix the ZipTip cleaned sample with DHB matrix in 1:1 ratio, and spot 1 µl directly onto the MassPREP MALDI

target. Dry the droplet in ambient temperature.

5. Proceed with MALDI MS analysis (see Figure 1). Instrument used: Waters MALDI micro MX.

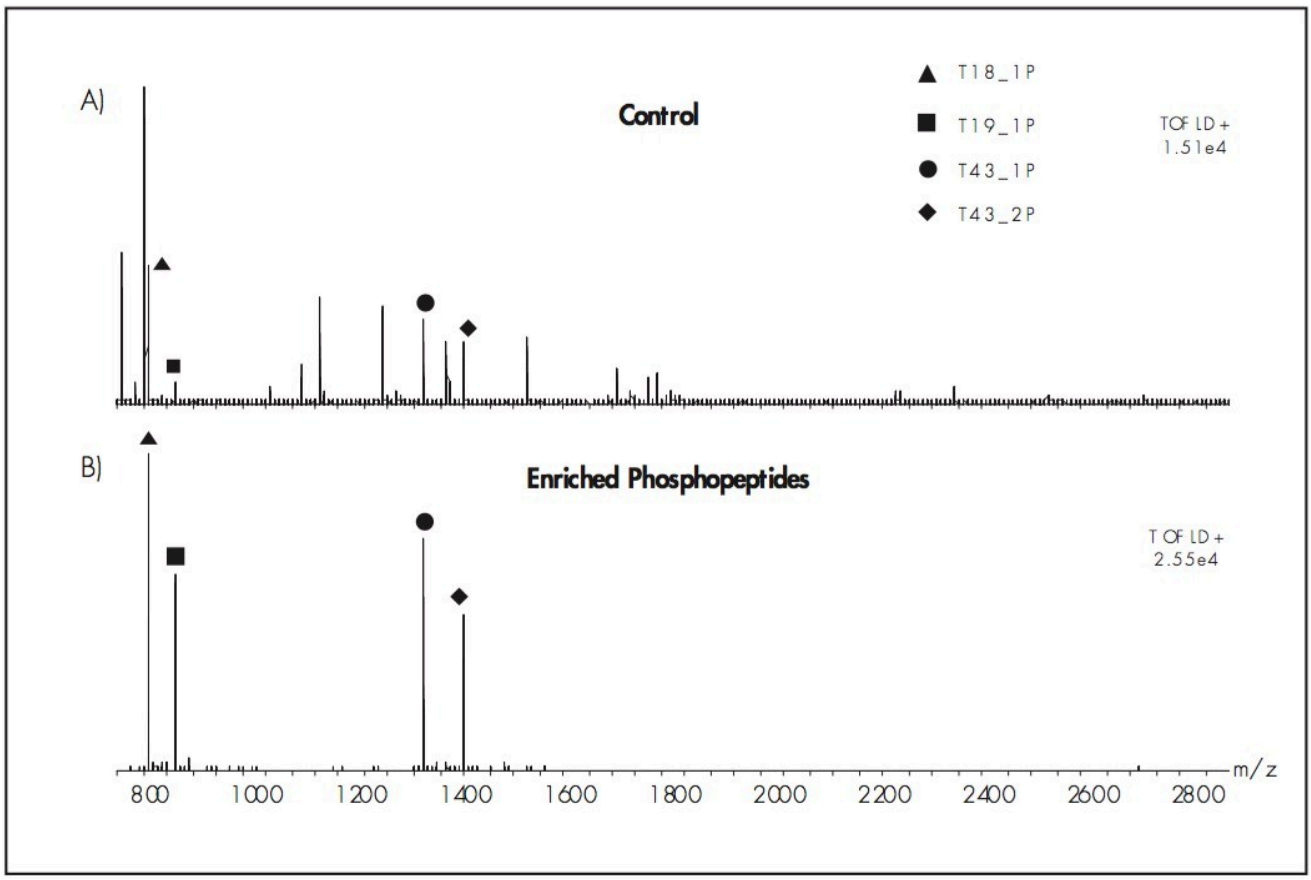


Figure 1. (A) MALDI-ToF MS spectrum of a control sample that was made with MassPREP Enolase Digest peptides mixed with 4 synthetic peptides in 1:1 molar ratio (Table 1). The amount spotted is 2 pmol.
(B) The same sample that was enriched using the affinity µElution plate.
The diammonium phosphate was used to elute the phosphopeptides. ZipTip µC_{18} tip was used to remove diammonium phosphate from the sample. Highly selective enrichment of the 4 phosphopeptides was observed.

Reversed-phase nanoLC-MS Analysis

1. Prepare a solution composed of 10-25 mM diammonium phosphate and 10-25 mM EDTA in MillQ-water.

2. Solubilize the lyophilized phosphopeptides in this solution. (The combination of diammonium phosphate and EDTA was used to minimize the loss of phosphopeptides due to exposure to potential metal surfaces in the LC System fluid path.) A trap column (e.g Symmetry C_{18}, nanoACQUITY UPLC Trap: Part Number 186002841 < https://www.waters.com/waters/gpl.htm?page=info&ProductName=nanoACQUITY&id=9618&retid=5269>)

can to used to remove excess diammonium phosphate and EDTA from the isolated phosphopeptides prior to MS analysis.

LC Conditions

UPLC system:	nanoACQUITY System
Trapping column:	Waters Symmetry C_{18}, 5 µm, 180 µm x 20 mm
Trapping mode:	5 µl/min for 3 minutes (100% aqueous)
nanoACQUITY column:	Waters Atlantis dC_{18}, 3 µm, 5 µm x 100 mm
Solvent A:	0.1% formic acid in 100% Milli-Q water
Solvent B:	0.1% formic acid in 100% acetonitrile
Flow rate:	300 nl/min
Gradient:	2%-40% B, 1% B per minute
Injection volume:	2 µl, full loop

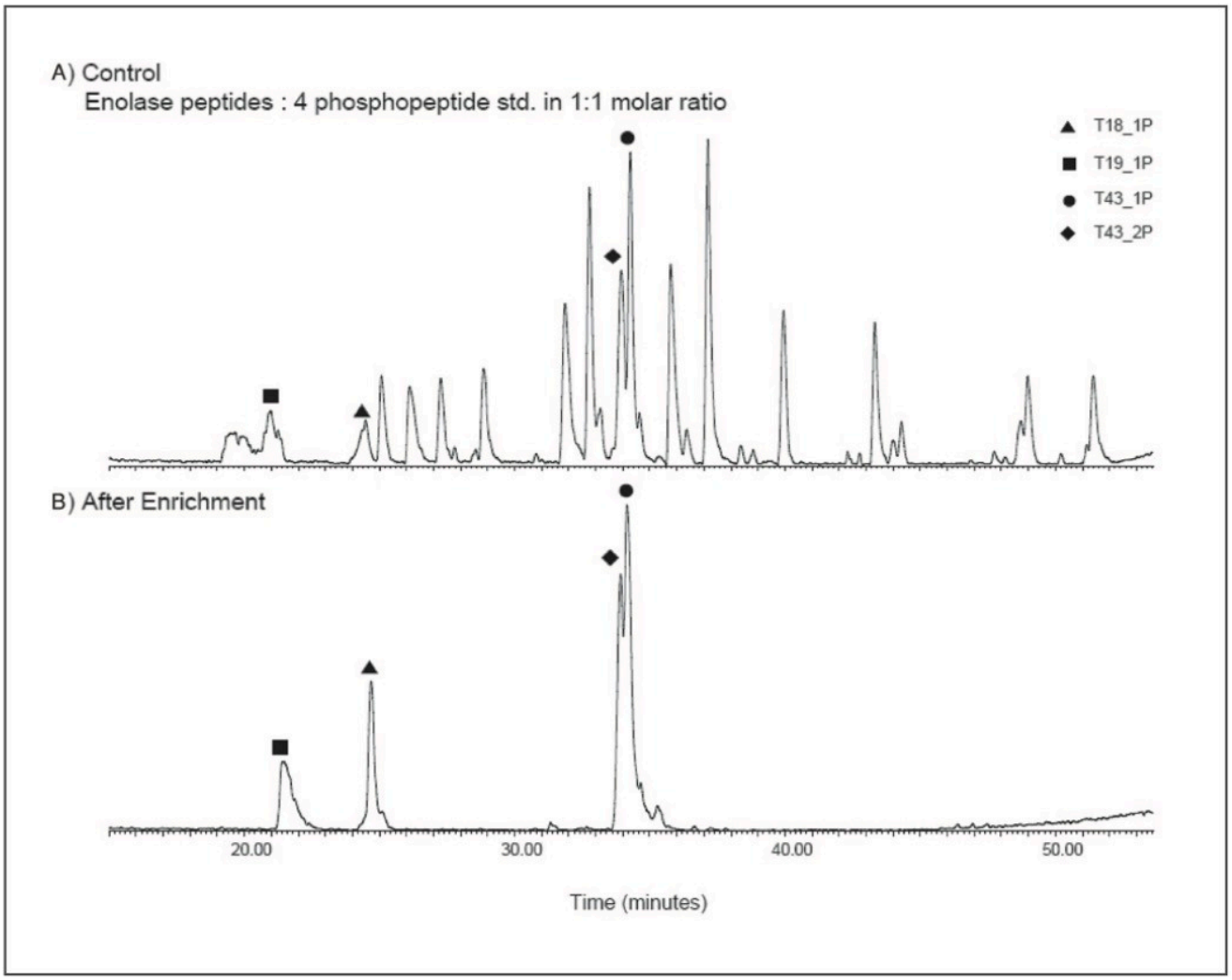


Figure 2. Nano LC-MS analysis of: (A) Control sample (before affinity enrichment) that is made of 1:1 MassPREP Enolase/Phosphopeptide Standard (Table 1). Total sample injected onto the 75 µm I.D. nanoACQUITY UPLC C_{18} Column is 250 fmol.

(B) The four phosphopeptides are selectively enriched. The phosphopeptides are marked with symbols. Total sample injected on nanoLC Column is 250 fmol. A trap column is used to remove the excess EDTA in the sample.

Results and Discussion

Enhancer Improves the Selectivity of the Affinity SPE

Figure 3 illustrates the use of Waters Enhancer (50 mg/ml) Reagent in the loading solution and subsequent improvement in the selectivity of the affinity extraction. The Enhancer can selectively displace acidic peptides without removing phosphopeptides from the µElution Plate and improves the selectivity towards

phosphopeptides.

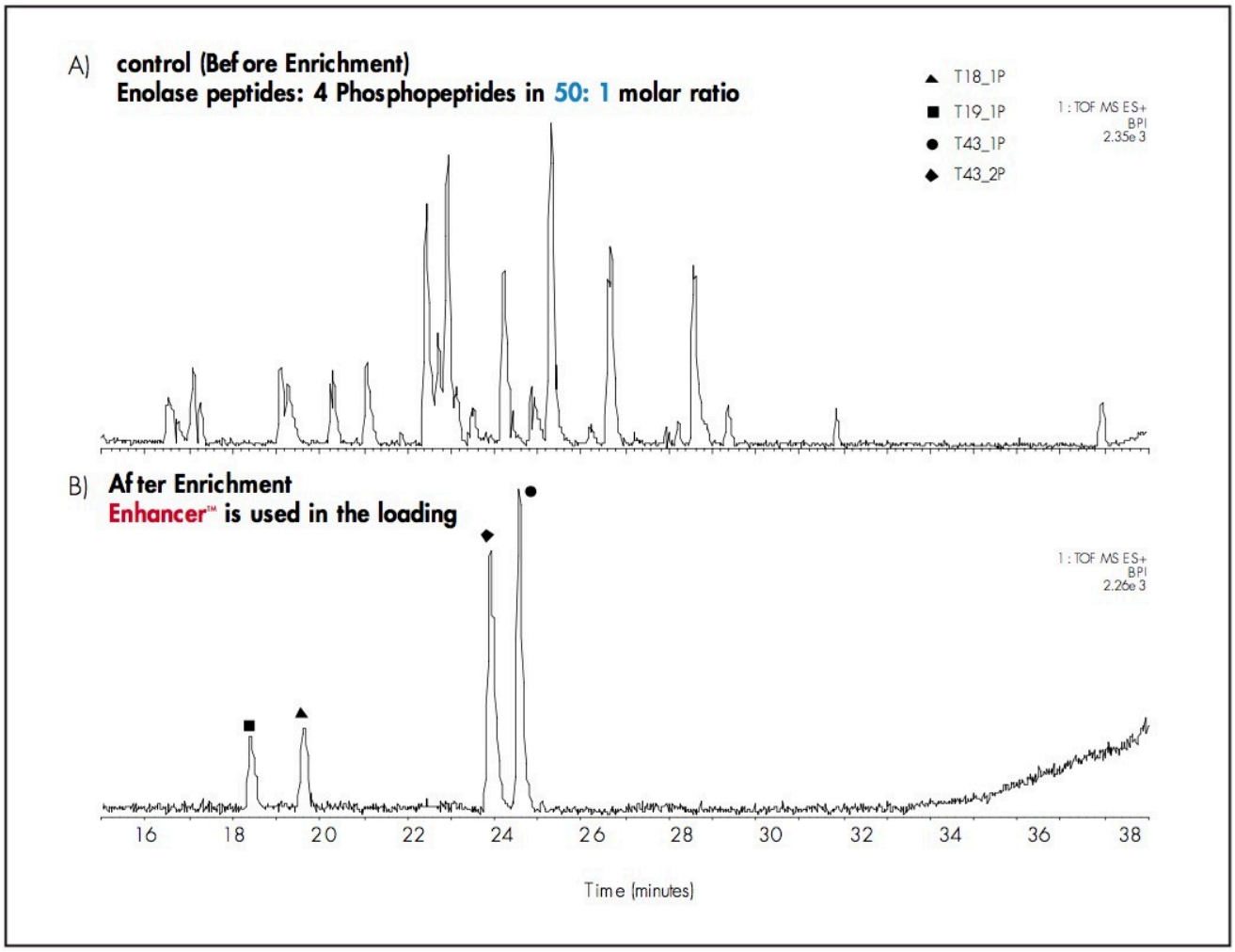


Figure 3. (A) A control sample that is made of MassPREP Enolase digest peptides mixed with the 4 phosphopeptide standards in 50 to 1 molar ratio (250 fmol enolase:5 fmol phosphopeptide standards). The non-phosphorylated enolase peptides are dominant in the LC-MS chromatogram; the 4 phosphopeptides are not detected due to their low abundance.

(B) Affinity enriched phosphopeptides. Enhancer solution, 50 mg/ml in 80% MeCN with 0.2% TFA, is used to reconstitute the sample. All nonphosphorylated enolase phosphopeptides were removed. Superior selectivity was observed. The LC-MS setup is similar as in Figure 2; the estimated amount phosphopeptide loaded is about 250 fmol

Conclusion

- Waters MassPREP Phosphopeptide Enrichment Kit is an enabling tool for isolation of phosphopeptides from complex biological samples.
- The affinity µSPE device offers highly selective enrichment of phosphopeptides, ease of use and better performance than IMAC-based kits.
- A phosphopeptide standard is included in the kit and allows users to validate and optimize experimental conditions.

Featured Products

- ACQUITY UPLC M-Class System <https://www.waters.com/134776759>

720002179, August 2007