ApplicationNOTE

Waters

ENHANCED *nano*LC/MS ANALYSIS OF PHOSPHOPEPTIDES

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Introduction

The reversible phophorylation of serine, threonine, and tyrosine is one of the most frequent and significant posttranslational modifications involved in a variety of cellular functions. The identification of phosphorylated peptides by LC-MS is a challenging task. It is in part due to the relative low abundance of phosphopeptides; and their low ionization efficiency in positive ESI-MS ionization mode due to the presence of negatively charged phosphate groups. The problem is further exacerbated by the phosphopeptides ability to form complexes with metals such as Fe (III) or Al (III) accumulated in the LC systems.[1] Thus, the amount of phosphopeptides eluted from the LC system may be less than the amount injected, especially for multiply phosphorylated species. It is difficult to identify and eliminate metal ions sources since they may be present in common LC solvents. It has been reported that adding chelating agents such as EDTA into the sample before LC injection can disrupt the formation of phosphopeptide-metal complexes and improve the phosphopeptide detection and method reproducibility.[1] This document demonstrates how EDTA additive was utilized for successful nanoLC/MS analysis of femtamole amounts of phosphopeptides on Waters nanoACQUITY UPLC™ and Q-Tof Premier™ system.

nanoLC/MS Setup for Phosphopeptide Analysis.

ESI-MS (Direct Infusion)

ESI source conditions were optimized for phosphopeptide detection (see the right panel) using MassPREP™ phosphopeptide standards available from Waters (PN186003285). Table 1 lists the sequences of the four synthetic phosphopeptides along with their singly and doubly charged ion masses. The peptides were solubilized in a formic acid,acetonitrile and water solution (0.1/50/49.9; v/v) to 100 fmol/ µl. Figure 1 shows a spectrum collected over one minute. All four phosphopeptides including the doubly phosphorylated peptide T43_2P were clearly observed.

Table 1. Amino acid sequences and the mass to charge ratios of the four synthetic phosphopeptides *(MassPREP™ Phosphopeptide Standards PN 186003285).*

Phosphopeptide Description	Sequence	[M+H]+	$[M+2H]^{2+}$
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

ESI MS conditions:

Ion mode:	ESI (+)
Capillary voltage:	3.8 kV
Source Temperature:	90 °C
Sample Cone Voltage:	38 V
Cone Gas Flow:	30 L/Hr
Nano Gas Flow:	0.1 L/Hr
Collision Energy:	4 V (Ar)
Scan Time:	2.4 Sec.
Inter Scan Time:	0.1 Sec.
Detection mode:	V mode
Lock Mass Spray:	Glu-Fib (m/z785.84)

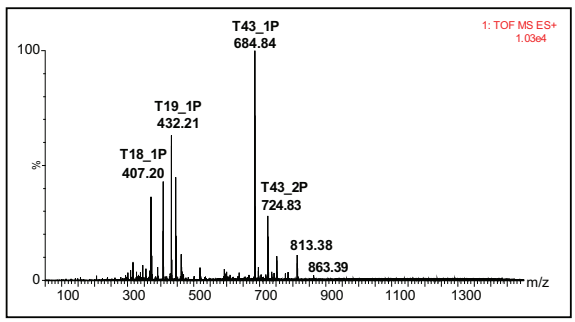

Figure 1. ESI/MS spectrum of the Waters MassPREP™ phosphopeptide standards. The doubly protonated phosphopeptide ions are labeled.

Sample Preparation for LC/MS

Before LC injections, phosphopeptide samples were prepared in 50 mM diammonium phosphate solution $(NH_4)2HPO_4$ buffered to pH 9, containing 25 mM EDTA.Phosphopeptides are typically acidic, therefore, they solubilize well in basic pH solutions. EDTA is added to chelate residual metal ion contaminants. Combination of alkyline pH with the EDTA additive improves the detection of phosphopeptides and the reproducibility of LC chromatograms.

A nanoACQUITY UPLC™ Trapping Column is used to retain peptides while removing the excess EDTA (see left panel).

nanoLC Conditions

nanoACQUITY UPLC™	
Trapping Column:	Symmetry® C_{18}, 5 µm, 180 µm x 20 mm.
Trapping mode:	5 µl/min for 3 minutes (100% aqueous).
nanoACQUITY Column:	BEH, 1.7µm, 75µm 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% – 50% B, 1% B per minute
Injection volume:	2 µl, full loop

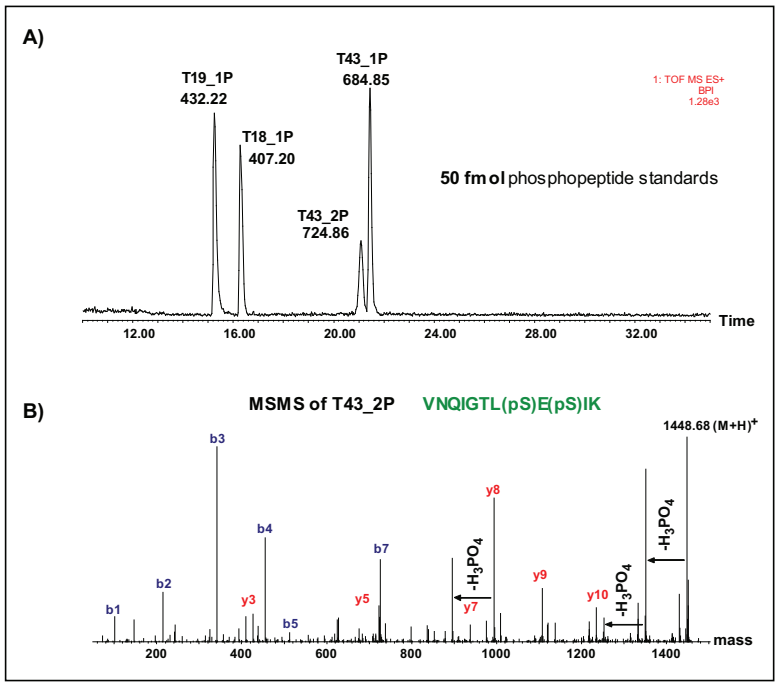

Figure 2. A) nanoLC/MS base peak chromatogram of a 50 fmol injection of MassPREP™ phosphopeptide standards. All four peptides were observed. B) An example of MS/MS fragmentation on the doubly phosphorylated peptide T43_2P was shown, loss of both H_3PO_4 groups (-98 Da) was observed along with y and b ions. The spectrum has been deconvoluted using the Maxium Entropy 3 Algorithm (MaxEnt 3) in MassLynx™ software version 4.0.

Conclusions

- Direct infusion of mono and doubly phosphorylated peptides using the Q-Tof Premier™ MS shows no loss due to ion suppression on the multiply phosphorylated peptide standards.
- Mono and multiply phosphorylated peptides were successfully analyzed using Waters nanoACQUITY UPLC™ system. Addition of diammonium phosphate and EDTA to the sample improves the recovery of phosphopeptides and the reproducibility of the LC/MS method.
- Excellent peak shape and separation were achieved using Waters nanoACQUITY UPLC™ 1.7µm particle BEH Technology C_{18} column.
- Low fmol amounts of phosphopeptides were detected with Q-Tof Premier™ system in both ESI, LC/MS and LC/MS/MS modes. Good sensitivity and high mass accuracy were achieved.

Reference

Rapid Commun. Mass Spectrom. 2005; 19: 2747-2756. *Formation of phosphopeptide-metal ion complexes in liquid chromatography/electrospray mass spectrometry and their influence on phosphopeptide detection.*

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