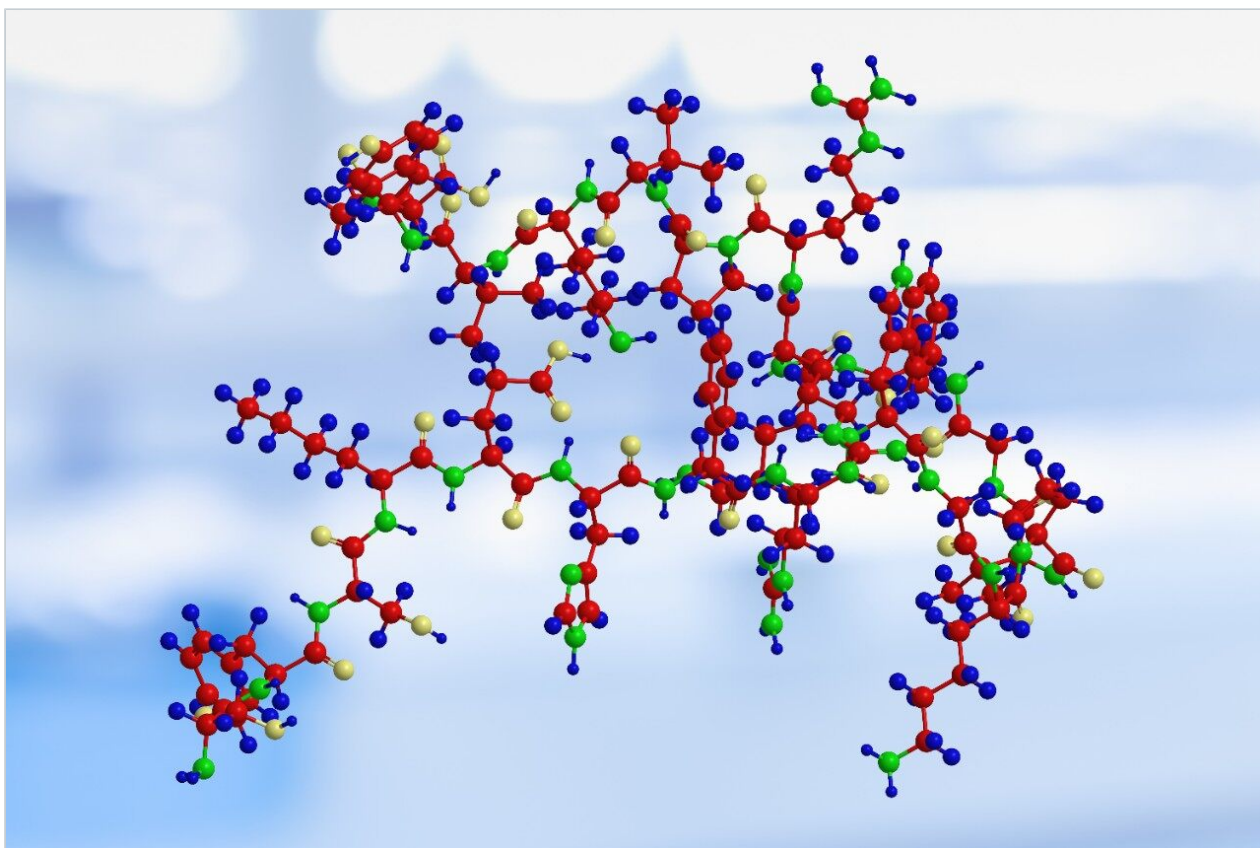


## Enhanced *nano*LC-MS Analysis of Phosphopeptides

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Ying Qing Yu, Iain Campuzano, Martin Gilar

Waters Corporation



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### Abstract

This document demonstrates how EDTA additive was utilized for successful *nano*LC-MS analysis of femtomole amounts of phosphopeptides on Waters nanoACQUITY UPLC and Q-ToF PREMIER System.

## Benefits

Good sensitivity and high mass accuracy

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## Introduction

The reversible phosphorylation 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.<sup>1</sup> 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.<sup>1</sup> This document demonstrates how EDTA additive was utilized for successful *nano*LC-MS analysis of femtomole amounts of phosphopeptides on Waters nanoACQUITY UPLC and Q-ToF PREMIER System.

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## Experimental

### *nano*LC-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 (p/n: [186003285](https://www.waters.com/nextgen/us/en/shop/standards--reagents/186003285-massprep-phosphopeptide-) <  
<https://www.waters.com/nextgen/us/en/shop/standards--reagents/186003285-massprep-phosphopeptide->

[standard-enolase.html](#) ). 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/  $\mu$ L. Figure 1 shows a spectrum collected over one minute. All four phosphopeptides including the doubly phosphorylated peptide T43\_2P were clearly observed.

Phosphopeptide Description	Sequence	[M+H] <sup>+</sup>	[M+2H] <sup>2+</sup>
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: 186003285).

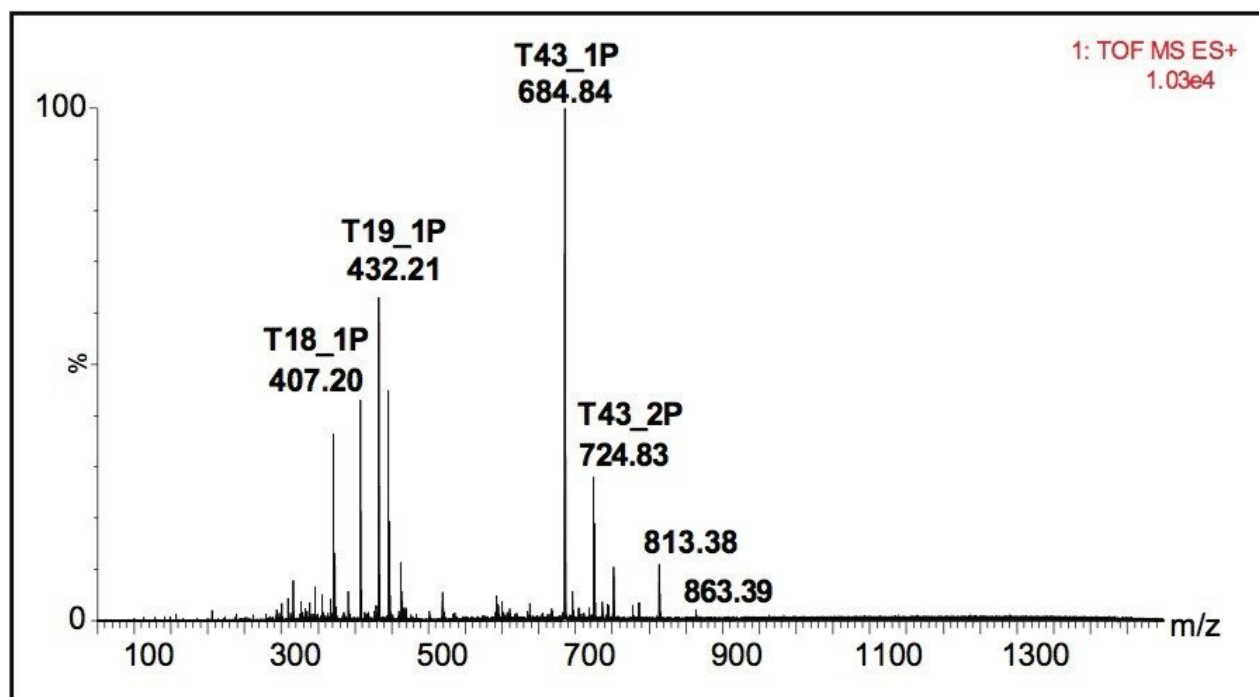


Figure 1. ESI-MS spectrum of the Waters MassPREP phosphopeptide standards. The doubly protonated phosphopeptide ions are labeled.

## ESI MS Conditions

Ion mode:	ESI+
Capillary voltage:	3.8 kV
Source temp.:	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 ( <i>m/z</i> 785.84)

## Sample Preparation for LC-MS

Before LC injections, phosphopeptide samples were prepared in 50 mM diammonium phosphate solution (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> 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 alkaline 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 <sub>18</sub> , 5 µm, 180 µm x 20 mm.
Trapping mode:	5 µl/min for 3 minutes (100% aqueous)
Column:	nanoACQUITY UPLC BEH, 1.7 µm, 75 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% – 50% B, 1% B per minute

Injection volume:

2  $\mu$ l, full loop

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## Results and Discussion

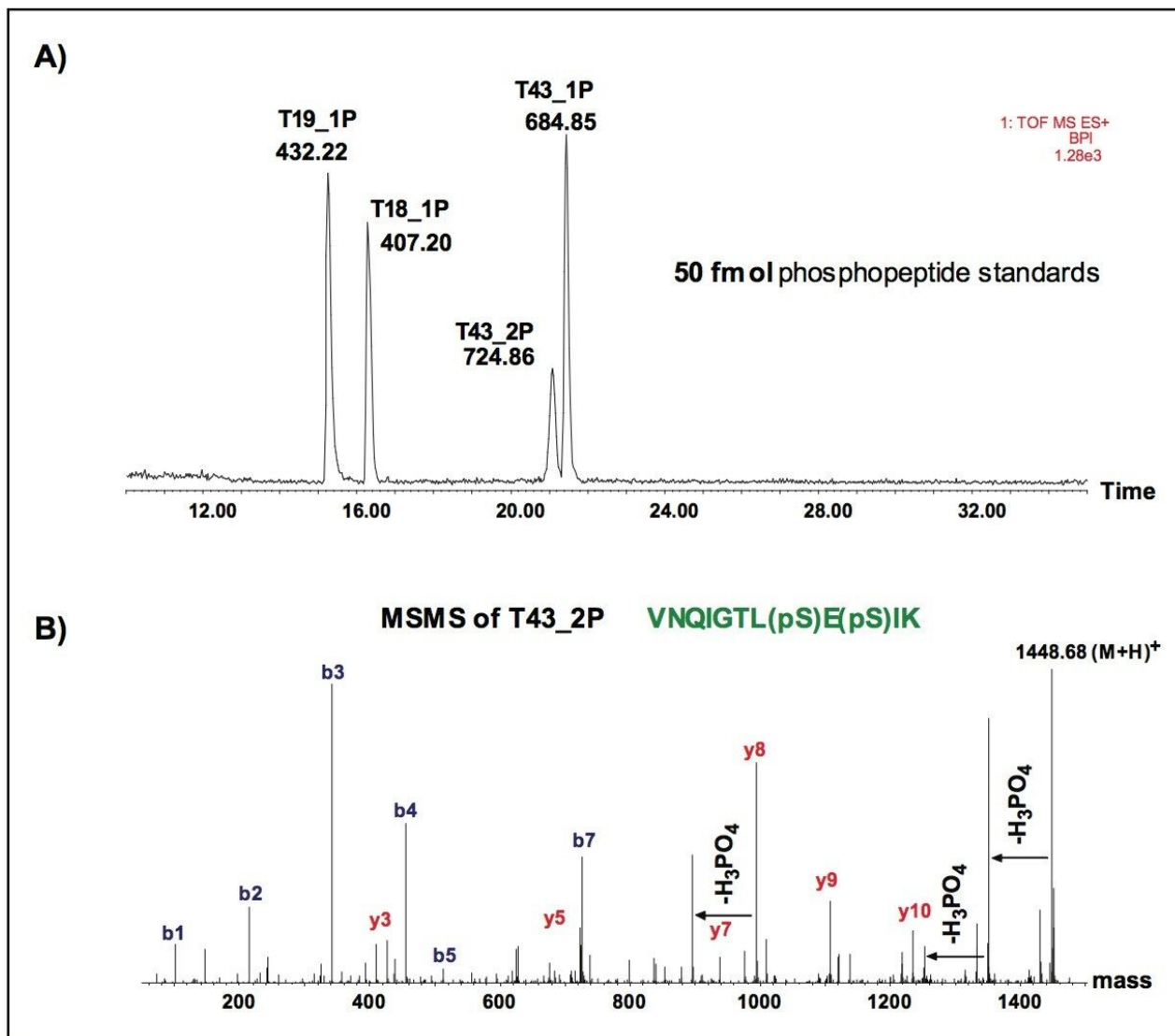


Figure 2. A) nanoLC-MS base peak chromatogram of a 50 fmol injection of MassPREP phosphopeptide standards. All four peptides were observed. B) An example of MS/MS fragmentation on the doubly phosphorylated peptide T43\_2P was shown, loss of both H<sub>3</sub>PO<sub>4</sub> groups (-98 Da) was observed along with y and b ions. The spectrum has been deconvoluted using the Maximum Entropy 3 Algorithm (MaxEnt 3) in MassLynx Software version 4.0.

## Conclusion

- 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 1.7  $\mu\text{m}$  particle BEH Technology C<sub>18</sub> 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

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## References

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

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