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アプリケーションノート

A Novel Strategy for the Analysis of Phosphopeptides, Coupling N-Terminal Peptide Derivatization and HPLC Separation with Mass Spectrometry

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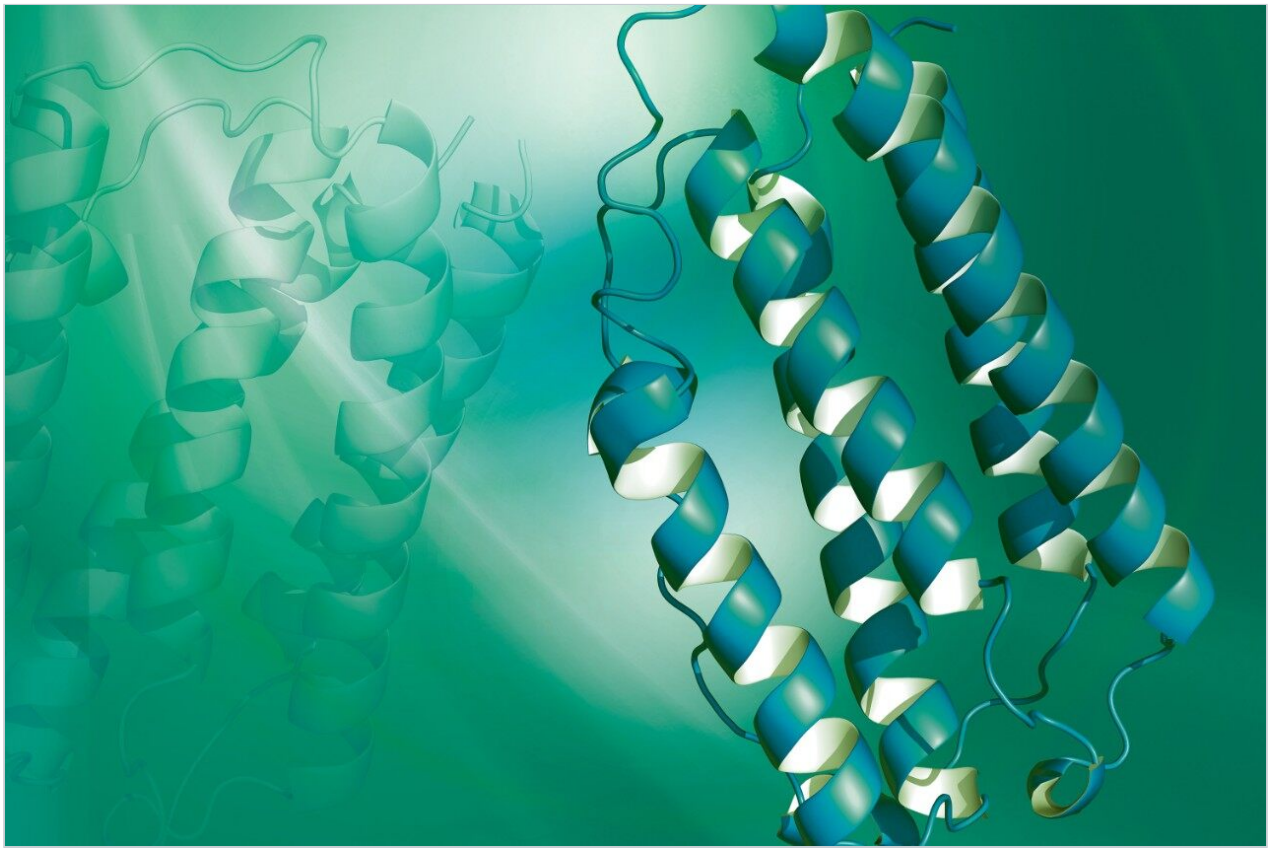


Abstract

- Here we present an N-terminal derivatization approach, using TMPP-Ac-Osu that improves the HPLC performance for small hydrophilic phosphopeptides.
- This approach is compatible with LC-MS and LC/MALDI.
- The chromatographic enhancement was evaluated by on-line nanoscale HPLC coupled with Electrospray on a QTof Premier.
- LC/MALDI-MS/MS analysis was also investigated. Both MS/MS on a QTof Ultima MALDI and PSD MX on a MALDI micro MX were used.
- The use of TMPP-Ac-Osu derivatization results in a fixed charge on the N-terminus of the peptide, directing fragmentation for CID and PSD experiments.

Introduction

Protein phosphorylation is one of the most common post-translational modification occurring in mammalian systems and is especially important in a number of key biological processes, such as intracellular signalling, facilitating the method of action of several key hormones.

Numerous strategies for studying phosphorylation have been described; several incorporating trypsin digestion with subsequent analysis by LC-MS and MS/MS. However, the analysis of small hydrophilic phosphopeptides by LC-MS/MS techniques is particularly challenging due to the poor retention characteristics of these phosphopeptides on reverse phase media. During the HPLC experiment they are often ‘lost’, either by not binding to trapping columns during the loading step often employed in nano LC experiments or by eluting in the void volume for direct loading LC experiments. In addition the analysis of these species by either Electrospray (ESI) or Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) is complicated by their low molecular weight.

In this work we describe the N-terminal derivatization of small phosphopetides using N-Tris (2,4,6-trimethoxyphenyl) phosphonium-acetic acid N-hydroxysuccinimide ester (TMPP-ac-OSu). This derivatization chemistry improves phosphopeptide retention on reverse phase materials and increases the mass of peptides by 572 Da whilst also enhancing fragmentation in the MS/MS mode for singly charged peptides.[1,2]

The Waters nanoACQUITY UPLC System, Waters Micromass QTof Premier and MALDI micro MX Mass Spectrometers.

Experimental

Sample Preparation

- Experiments were performed on 4 synthetic phosphopeptides, with the following sequences: -

  NDRSpE (MW 699.2225 Da)

  DSpT (MW 401.0835 Da)

  GHNSpLK (MW 734.3113 Da)

  SNEDYpR (MW 862.2858 Da)

- Individual phosphopeptides were solubilized and subsequently diluted to 100 pmol/µL in 0.5 M 4-Methylmorpholine buffer/80% Acetonitrile.
- A TMPP derivatization solution (Waters, Milford, MA) was diluted in acetonitrile and added to this phosphopeptide solution at a molar ratio of 33:1.

- Samples were incubated at room temperature for 30 minutes followed by a 100-fold dilution with 25% Acetonitrile/0.1% TFA solution.

Chromatography And Mass Spectrometry

Nanoscale LC-MS

Phosphopeptide samples were analyzed on the QTof Premier using nanoscale LC-MS. Chromatographic separation was achieved using a Waters NanoEase Atlantis dC_{18} (100 mm x 75 µM) analytical column, using a gradient from 5% to 85% acetonitrile over 45 minutes.

125 fmol of each TMPP modified phosphopeptide were injected.

All data was acquired on the QTof Premier in continuum mode over the *m/z* ranges 50-1990 in the W-Optics mode of operation.

Instrument resolution was better than 17,500 FWHM.

LC-MALDI Conditions

- The matrix used for MALDI experiments was α-cyano-4-hydroxycinnamic acid matrix (Waters, MA).
- 2 µL of individual peptide solutions were injected and separated by reverse phase chromatography using a Waters NanoEase Atlantis C_{18} column 150 mm x 75 µm at a flow rate of 200 nL/min split from 8 µ L/min
- Mobile phase A (95% H_2O, 0.1% TFA), mobile phase B (95% ACN, 0.1% TFA)
- Gradient: initial conditions 5% B changed to 40 % B over 45 minutes.
- The entire flow from the HPLC column was spotted onto a 96 well MALDI target plate using a Waters 2700 MS spotting device
- Each MALDI target position had 30 seconds of HPLC eluent applied, equivalent to 100nL. Through a separate capillary, α -cyano-4-hydroxycinnamic acid, 2.5 mg/mL (1/1 v/v MeCN/0.1 % aqueous TFA) was co-deposited at a rate of 1.8 µL/min.

QTof Ultima MALDI

- Data were acquired in positive ion mode.
- Selected ions were fragmented by collision induced dissociation (CID) to provide product ion (MS/MS) data.
- Polyethylene Glycol (PEG) was used for an external multi-point calibration.

MALDI micro MX

- Data were acquired in positive ion mode using automated software control.

- In reflectron MS mode, a digest of alcohol dehydrogenase (ADH) was used to generate a multi-point external calibration.
- In PSD MX mode, data were acquired in parallel mode[3] and calibrated using PSD fragments generated from polyproline (P14R) (Sigma, St Louis, MO); $[MH]^+$= 1533.8582 Da.

Data Processing

MALDI QTof data processing

- MS/MS spectra were deisotoped using MaxEnt3 (MaxEnt Solutions, UK) and *de novo* sequencing was performed using MassSeq Software (Waters, Manchester, UK).

PSD MX data processing

- PSD data were smoothed, background subtracted and centroided.
- Fragment ions were matched to their precursor ions using a deconvolution algorithm, implemented in MassLynx. A peaklist (pkl) was generated for each sample.

Results and Discussion

Evaluation of derivatization on peptide trapping & separation using HPLC

a) LC/ESI-MS

- Both unmodified and TMPP modified phosphopeptides were analyzed by LC/ESI-MS. The unmodified species were not bound to the trapping column and therefore were not detected by the mass spectrometer. In contrast, TMPP modified phosphopeptides were retained on the reverse phase trapping column and were subsequently separated on the analytical column. Chromatograms are shown in Figure 1.

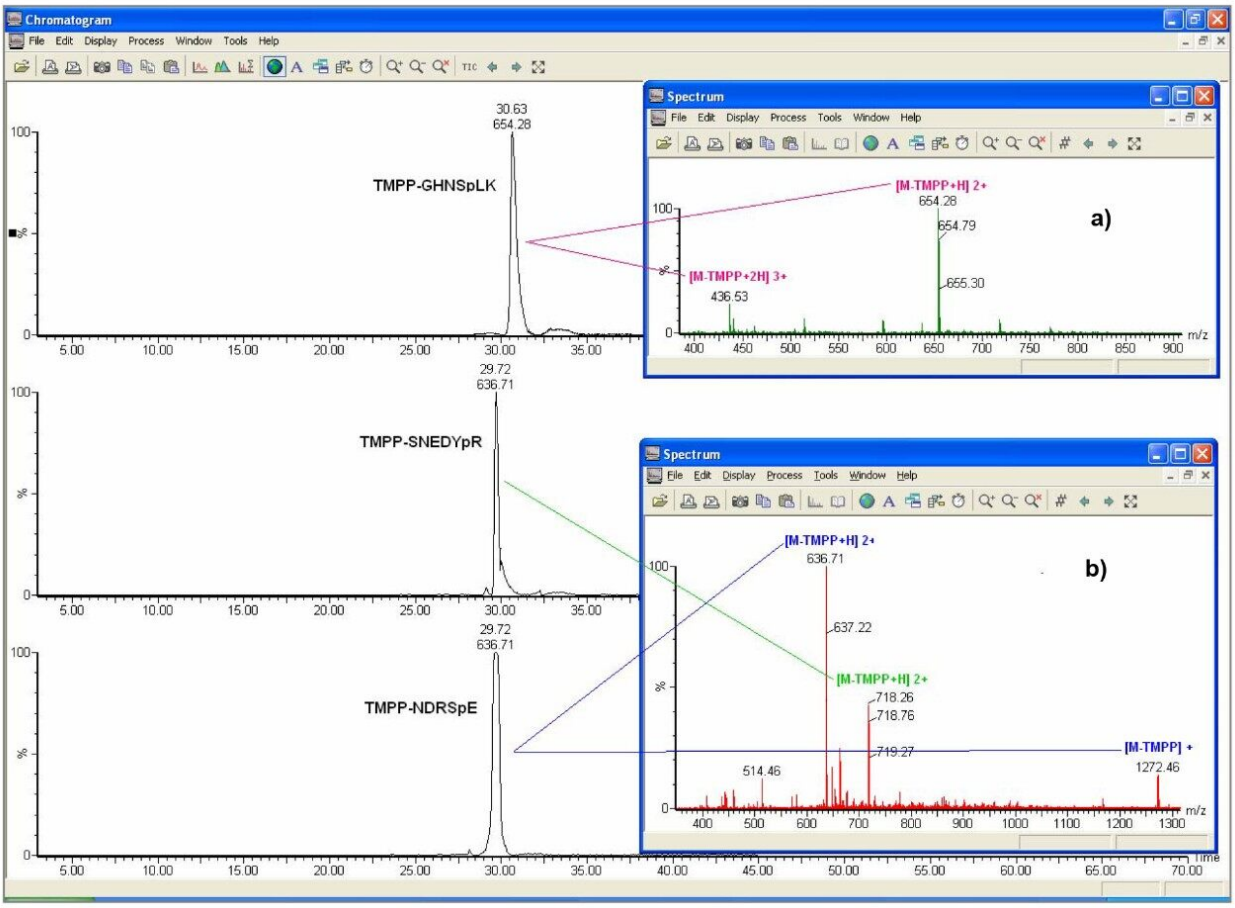


Figure 1. Reconstructed mass chromatograms of TMPP-GHNSpLK, TMPP-SNEDYpR and TMPP-NDRSpE. Inset are the mass spectra obtained for the different phosphopeptide species. a) MS spectrum at 30.6 mins. b) MS spectrum at 29.7 mins.

- It can be seen from these data that TMPP-NDRSpE and TMPP-SNEDYpR have co-eluted with a chromatographic retention time of 29.7 minutes. TMPP-GHNSpLK is resolved from the other phosphopeptides and elutes after 30.6 minutes.
- TMPP-DSpT was not well resolved under the HPLC conditions used and gave a broader chromatographic peak after approximately 30 minutes.

LC/MALDI-MS

- Modified phosphopeptides were also analyzed using off-line HPLC coupled to MALDI-MS and MALDI-MS/MS. Figure 2 shows the MS spectra recorded from fractions collected at different retention times, all TMPP modified phosphopeptides were detected.

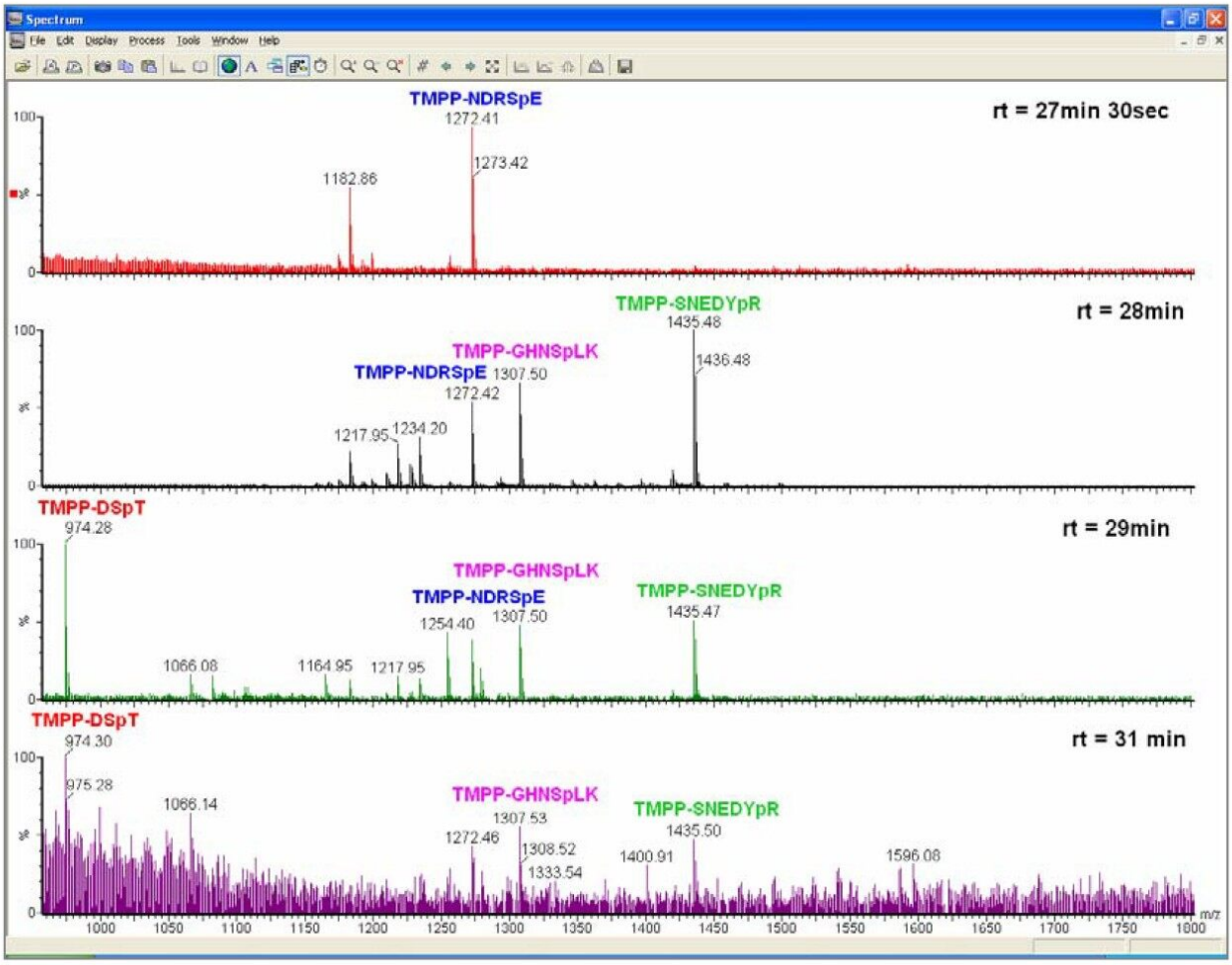


Figure 2. The MS spectra recorded from fractions collected at different retention times, all TMPP modified phosphopeptides were detected.

The order of elution is slightly different in the LC/ESI and LC/MALDI experiments. The range of retention times between species was similar in both experiments. Reasons for the difference in chromatography observed are the slightly different chromatographic conditions used. In the LC/ESI experiment the aqueous mobile phase has 1% formic acid, whereas in the LC/MALDI experiment the aqueous mobile phase was 0.1% TFA.

Effect of TMPP derivatization on MALDI MS/MS

- To illustrate the effect of TMPP derivatization on MALDI MS/MS fragmentation patterns, the derivatized peptides TMPP-NDRSpE and TMPP-GHNSpLK were analyzed using low energy CID on a QTof Ultima MALDI Mass Spectrometer and using parallel PSD on a MALDI micro MX mass spectrometer.
- The fragmentation data for these peptides are shown in Figures 3 and 4 respectively.

- The effect of TMPP derivatization on peptide fragmentation patterns has been previously described.[1,2] These earlier studies have shown that the fragmentation is more predictable, compared to the native, un-modified peptide.

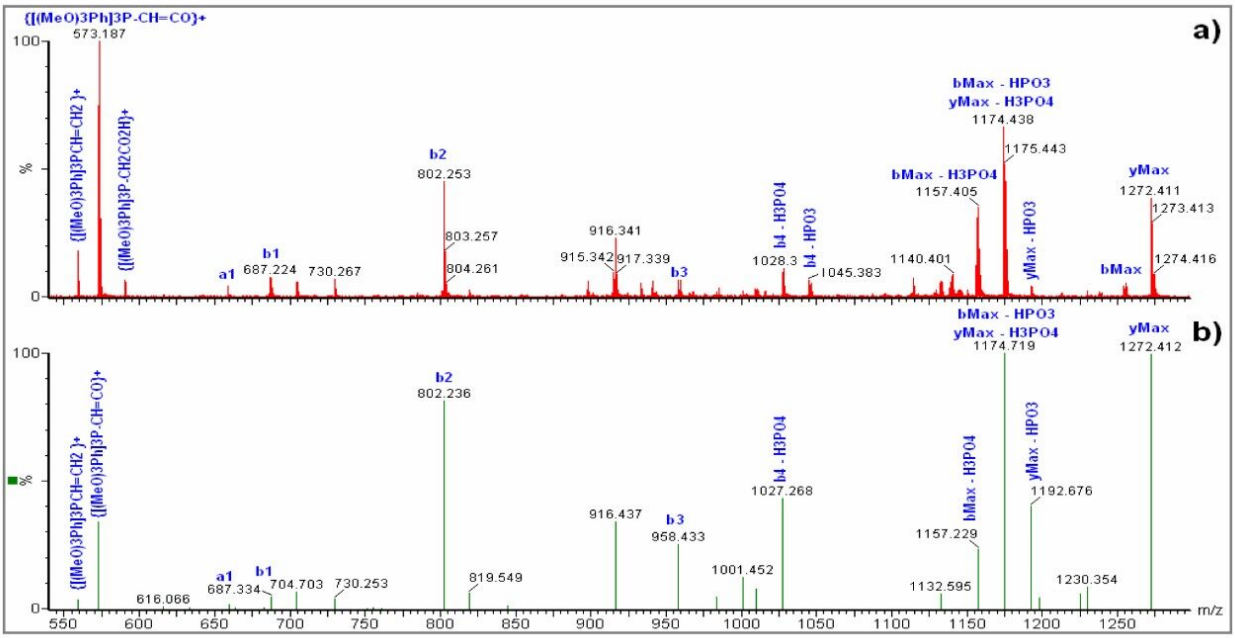
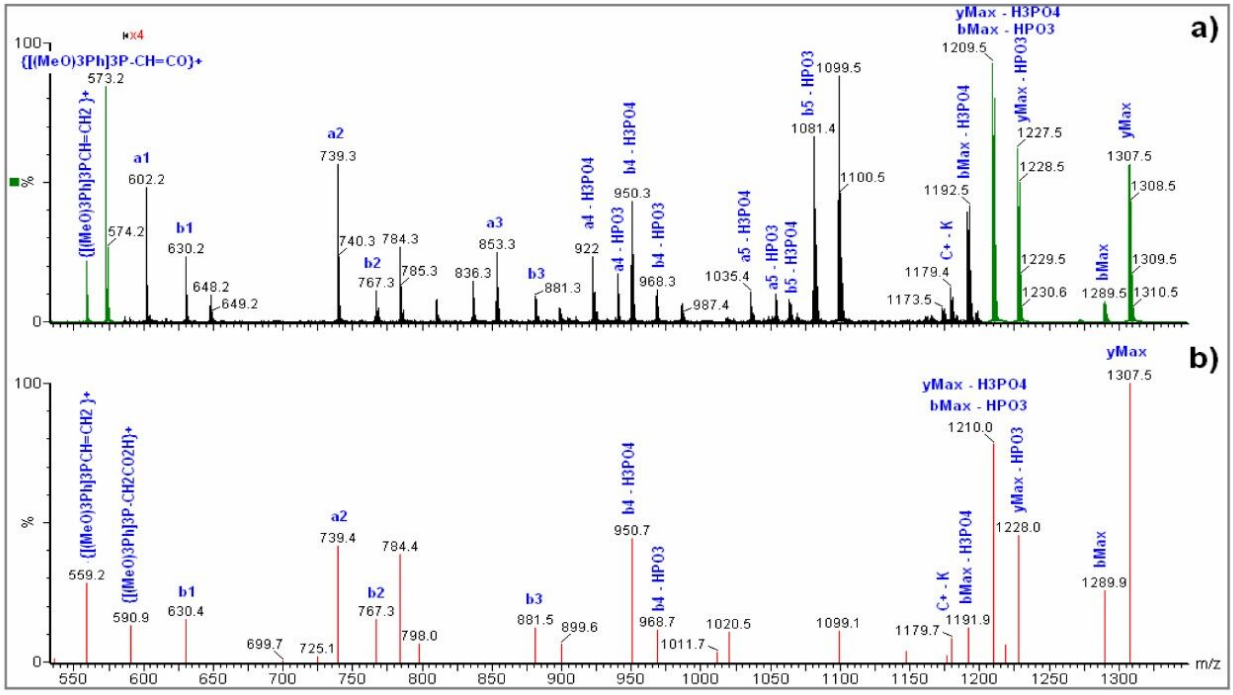


Figure 3. MS/MS spectra of TMPP-NDRSpE a) MALDI-QTof, b) MALDI-PSD MX.



MXFigure 4. MS/MS spectra of TMPP-GHNSpLK a) MALDI-QTof, b) MALDI-PSD MX.

- The fragment ions observed from tryptic peptides, N-terminally modified, with TMPP are exclusively $*a_n$ or $*b_n$ type ions. This fragmentation pattern is observed, due to the charge localization on the N-terminal TMPP group.
- In the MS/MS spectra of TMPP modified peptides, peptide fragment ions are observed between 573.1898 Da and the precursor ion mass. The 573.1898 Da ion is the M^+ ion of the TMPP reagent.
- As internal rearrangement is minimized, noise in product ion spectra from TMPP-derivatized peptides is extremely low.

Conclusion

- The derivatization of peptides using TMPP-Ac-OSu is a quick and simple reaction.
- In line with previous experience, low molecular weight hydrophilic phosphopeptides did not bind to reverse phase trapping columns, and this precluded their analysis by nanoscale LC-MS.
- Derivatization of the phosphopeptides with TMPP greatly improvedthe retention of these small hydrophilic peptides on trapping columns, and thus made nanoscale LC-MS studies possible.

- Analysis and interpretation of MALDI-MS/MS spectra from singly charged ions was simplified through TMPP modification of peptides.
- Future studies will look at enhancing the sensitivity of this analytical strategy and the analysis of endogenous phosphopeptides from biological sources.

References

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