

Detecting Challenging Post Translational Modifications (PTMs) using CESI-MS

Sensitive workflow for the detection of PTMs in biological samples from small sample volumes

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Overview

Who Should Read This: Senior Scientists, Lab Directors and Proteomics researchers.

Focus: Detection of Post Translational Modifications (PTMs) using capillary electrospray ionization (CESI) in combination with accurate MS detection.

Goals: Detect challenging PTMs such as asparagine deamidation, aspartate isomerization, arginine citrullination, and phosphorylated peptide isomers in biological samples where sample volume is limited and the complete depletion of a sample needs to be avoided to allow multiple analyses from the same sample.

Problem: Traditional LC-MS methods struggle to identify and quantify aspartate and iso-aspartate isomers separately, as they have the same mass and similar fragmentation patterns using CID or HCD. Phosphorylated peptides are often polar and elute early in traditional LC analyses. Positional isomers of phosphorylated peptides are identical in mass and may have very similar fragmentation patterns, which makes the identification of individual modification sites by traditional LC-MS approaches.

Results: The Sciex CESI 8000 system in combination with accurate MS detection was able to detect and quantitate aspartate and iso-aspartate isomers and citrullinated proteoforms in biological extracts. In addition positional isomers of mono-phosphorylated peptides were successfully separated allowing unambiguous identification and quantification of individual protein modification sites.

Key Challenges:

- Separation and detection of phosphopeptide isomers.
- Separation and detection of aspartate and iso-aspartate isomerization in proteins.
- Detection of arginine citrullination.



Key Features:

- Direct combination of capillary electrophoresis with mass spectrometry enabling the charge based separation of peptides from minute (<20 nL) injection volumes.
- The separation and detection of mono-phosphorylated peptide isomers, sites of citrullination and other challenging PTMs in a single analysis of biological samples.

Experimental Design

Sample Preparation: Histone H1 proteins were extracted from a human placenta according to Lindner et al.,¹ solubilized in 5 mM ammonium bicarbonate buffer (pH 8.0) and digested for 1 h at 37° C using endoproteinase Arg-C at a ratio of 1:20. Core histones were extracted from blood cells and fractionated by reversed-phase HPLC according to Helliger et al.² The histone H4 fraction was dissolved in 10 mM sodium phosphate buffer (pH 7.8) and digested for 8 h at 37° C using endoproteinase Glu-C at a ratio of 1:10. Resulting peptides were desalted using Perfect Pure C-18 pipet tips (Eppendorf, Austria). The growth of SILAC labeled yeast strains, protein extraction thereof, and subsequent sample preparation was performed as described elsewhere.³ All samples were lyophilized, re-solubilized in 50 mM ammonium acetate buffer (pH 4.0), and stored at -20° C until analysis.

CESI-MS methods. For CESI-MS analysis a CESI 8000 equipped with a bare or neutrally coated fused-silica capillary was coupled to a Thermo Scientific Q Exactive Plus or LTQ Orbitrap XL ETD mass spectrometer.⁴ Prior to each analysis the CESI system was rinsed with background electrolyte (BGE) of 0.1 M acetic acid or 0.1% (v/v) formic acid for 3 min at 50 psi. The sample was injected by applying a pressure (5 psi for 20 s equivalent to 17 nL) followed by an injection plug of BGE (5 psi for 5 s). The separations were performed at +30 kV with a 2 stage pressure profile (0 - 10 min at 1 psi and 10 - 52 min

at 1.5 psi). The Q Exactive Plus mass spectrometer was operating in data dependent mode and set to switch between MS and MS/MS acquisition. The full scan survey spectra was acquired with a resolution of $R = 70,000$. To generate MS/MS spectra the ten highest precursors were selected for higher energy collision dissociation (HCD). Product ion spectra were acquired with a resolution of $R = 35,000$ (unassigned singly charged and peptides with a charge state higher than +7 were excluded).

Separation of Phosphorylated Peptides

It is known that capillary zone electrophoresis (CZE) is able to separate peptides with identical amino acid composition but different sequences as long as the sequence variation causes a variation in the pKa values of the corresponding peptides. Such a change can be caused by the inclusion of a phosphate residue at different positions on the peptide. This was demonstrated by

the identification of a number of phosphopeptide isomers in a test of a fraction of metabolically labeled peptides obtained after Lys-C cleavage from two yeast strains (Figure 1). In figure 1 three different phosphorylated sites were separated and identified in a digest sample.

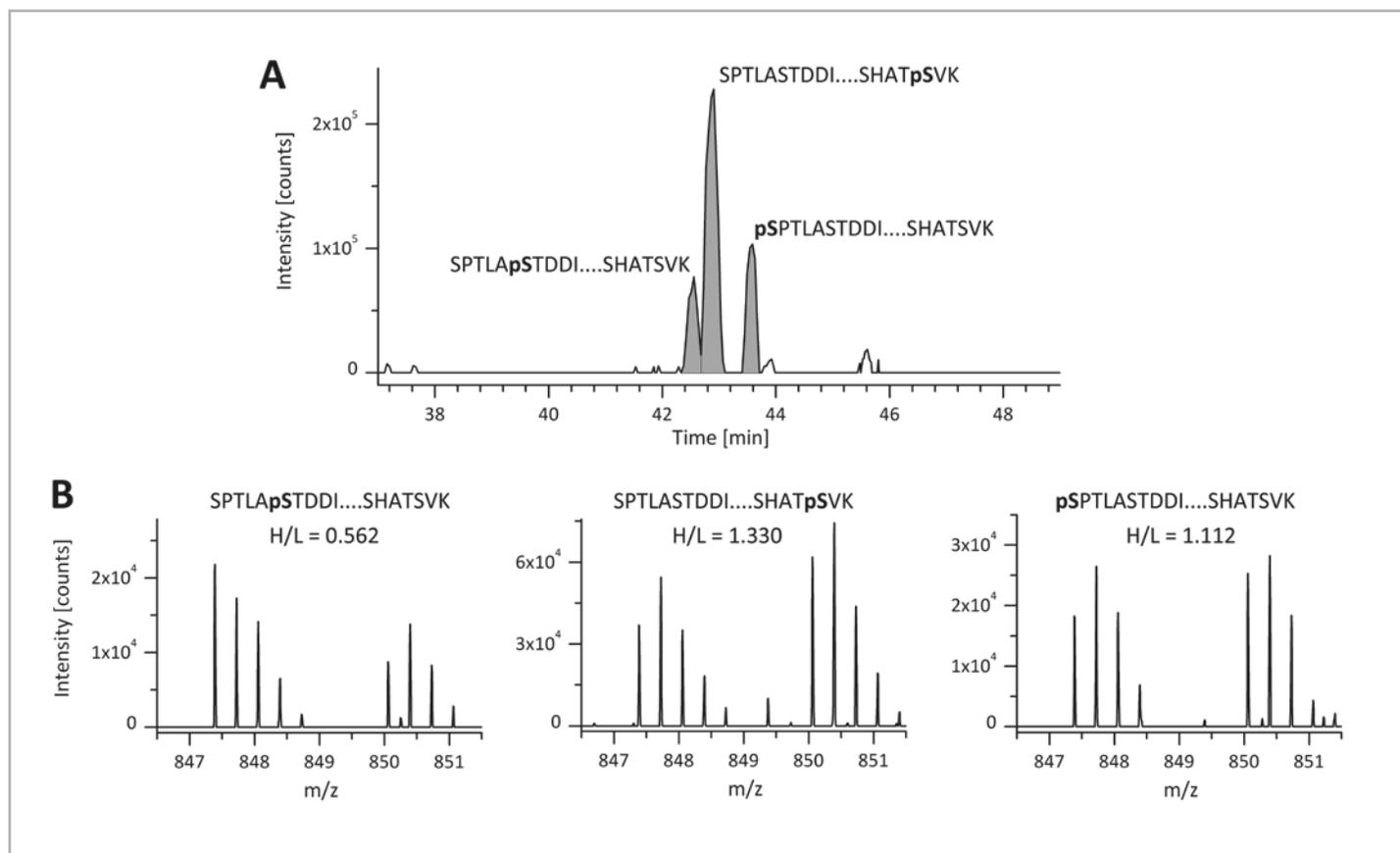


Figure 1. A) CESI-MS separation of 3 mono-phosphopeptide isomers of SPTLASTDDINSASASVNSHATSVK peptide detected in a fraction of a Lys-C cleaved yeast sample. B) Quantification spectra and H/L ratios of the three SILAC labeled phosphopeptide pairs.

Identification of deamination products in vivo

CESI-MS was used to investigate the in vivo deamidation rate of histone H1.0, which is known to be deamidated at position 3.⁴ H1 histones were extracted from human placenta and digested with Arg-C, which preferentially cleaves after arginine residues. This highly efficient enzyme allows very short reaction times which in turn prevent generation of artificial deamidation products. The sample was then analyzed by CESI-MS using

a neutral capillary and MS/MS spectra were obtained and searched against the IPI-human database. Figure 2 shows how CESI-MS is capable of separating Asp /iso-Asp modifications as well as acetylated and non-acetylated forms of the same N-terminal peptide TENSTSAPAAKPK. Quantification revealed 85% Asp and only 15% iso-Asp present in this sample.

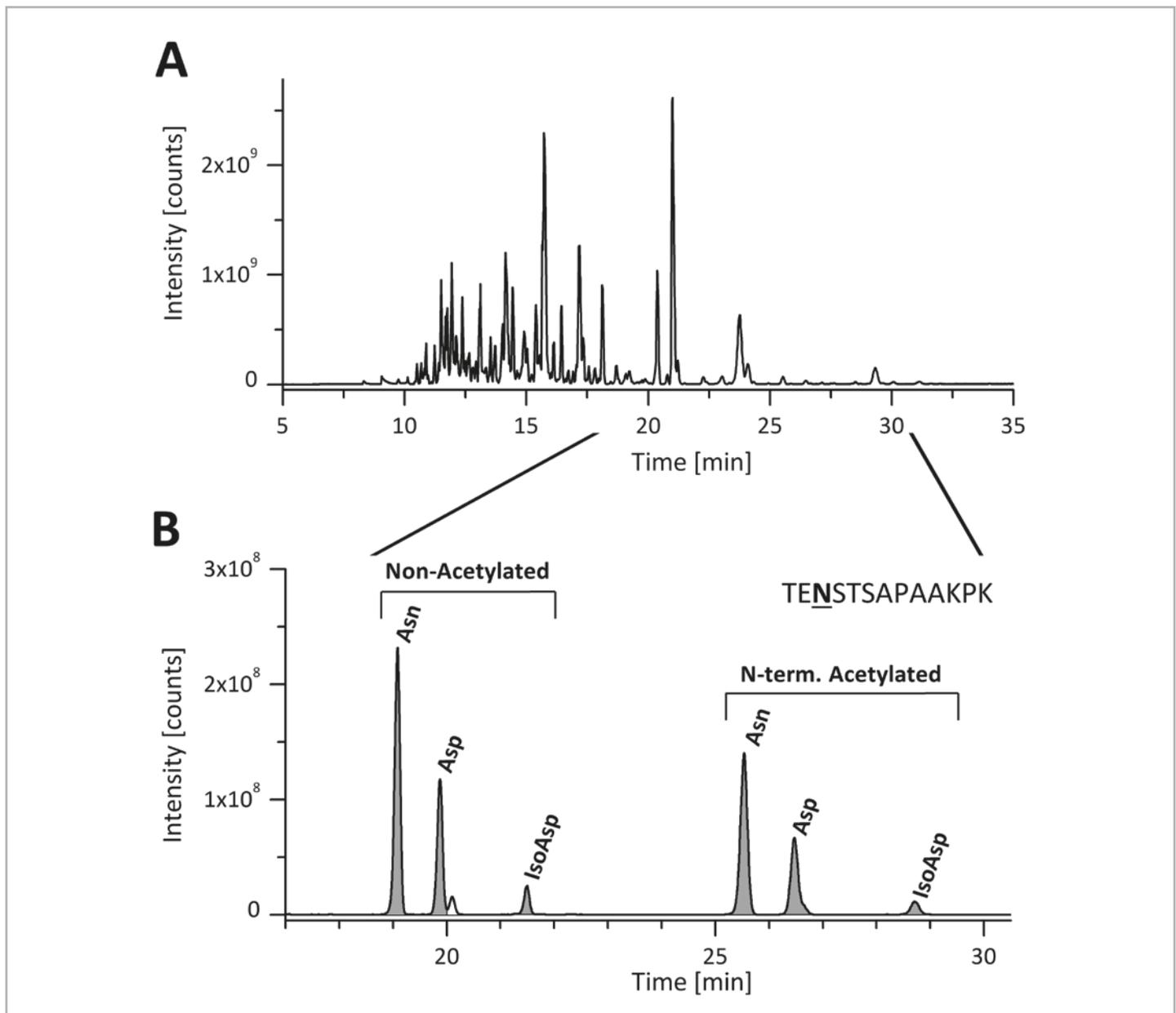


Figure 2. CESI-MS separation using a neutrally coated capillary (BGE 0.1 M acetic acid) of human placenta H1 histones digested with endoproteinase Arg-C. (A) Base peak electropherogram and (B) extracted ion electropherogram of deamidated and acetylated forms of the N-terminal peptide TENSTSAPAAKPK.

Identification of citrullinated sites

When H4 histones originating from human blood cells were analyzed in their intact form heterogeneity causing mass differences of just +1 Da were found that could not be assigned either to acetylation nor methylation (Figure 3). To confirm if this mass shift was caused by deamidation the proteins were cleaved with endoproteinase Glu-C.⁴ Using CESI-MS analysis with a neutral capillary, 8 peptide sequences covering 89.2% of histone H4 were identified. The reason for the heterogeneity was found to be located within the first 24 amino acids of H4. MS/MS analysis identified the peptide 1-24 to be N-terminally acetylated and dimethylated at Lys-20. A second peak exhibiting the same isotope distribution for a 6+ charge but with a mass difference of roughly 1 Da was also identified as peptide 1-24, however, citrullinated at position 3 (Figure 4). As Glu-C was used as the cleaving enzyme, deamidation could be excluded⁴ but as similar shifts in mass and migration time can also occur when a positively charged arginine residue is post translationally converted into the uncharged non-coded amino acid citrulline this modification was attributed to citrullination.⁴ If both citrullinated and non citrullinated peptides coelute, the m/z signal from the citrullinated peptide will fall within the isotopic cluster of the un-citrullinated peptide hindering identification and so the separation achieved by CESI-MS is essential for the identification of this post translational modification in both the intact and digested sample.

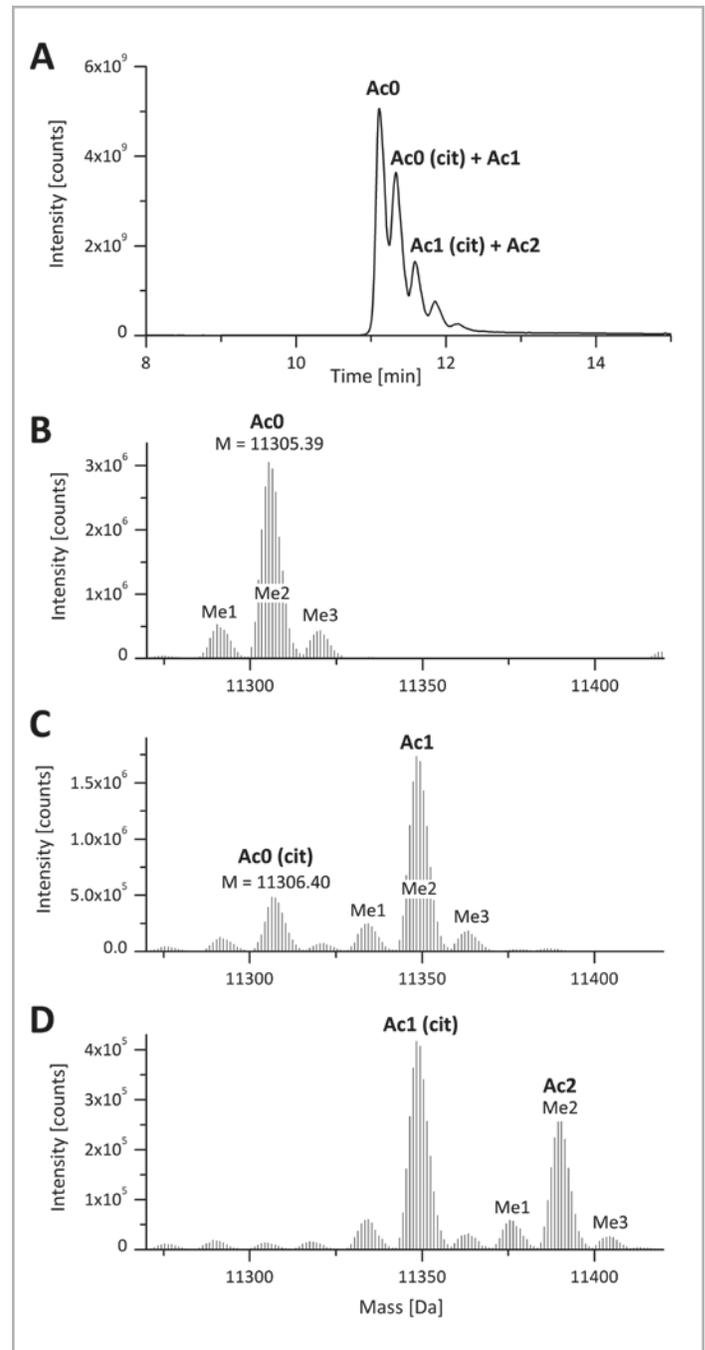


Figure 3. CESI-MS separation using a neutrally coated capillary (BGE, 0.1 M acetic acid) of intact citrullinated and non-citrullinated core histone H4. (A) Total ion electropherogram and (B-D) Mass deconvoluted spectra of individual H4 peaks.

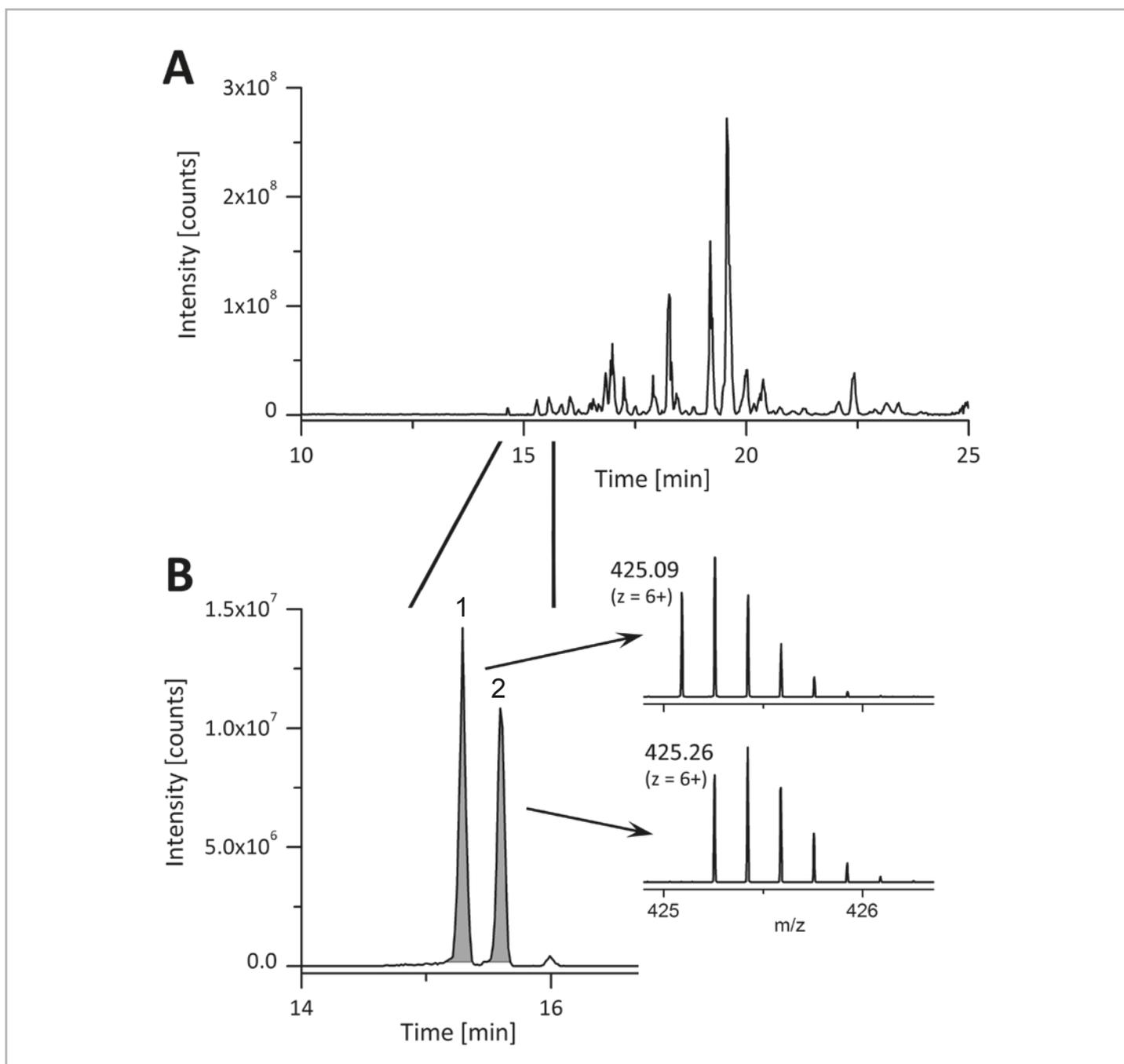


Figure 4. CESI-MS separation using a neutrally coated capillary with a BGE of 0.1 M acetic acid of peptides from human H4 histones extracted from human blood cells and digested with endoproteinase Glu-C. (A) Base peak electropherogram. (B) Extracted ion electropherogram of peak 1 (m/z 425.09) the 6+ charged peptide 1-24 (N-terminally acetylated, di-methylated at Lys-20) and of peak 2 (m/z 425.26; N-terminally acetylated, di-methylated at Lys-20 and citrullinated at Arg-3).

Further Information

For further information on this topic we would like to refer readers to the full scientific publication on which this application note is based.⁴

References

1. H. Lindner, B. Sarg, B. Hoertnagl, W. Helliger, The microheterogeneity of the mammalian H1(0) histone. Evidence for an age-dependent deamidation, *J. Biol. Chem.* 273 (1998) 13324–13330.
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