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Exploiting charge differences for the analysis of challenging post-translational modifications by capillary electrophoresis-mass spectrometry

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ABSTRACT

Reversed-phase high-performance liquid chromatography (RP-HPLC) in combination with mass spectrometry (MS) is typically employed for mapping modifications in proteins and peptides. Here we applied a low-flow capillary electrophoresis (CE) –electrospray ionization interface coupled to Orbitrap mass spectrometers to analyze challenging modifications such as asparagine deamidation, aspartate isomerization, arginine citrullination, and phosphopeptide isomers. We achieved excellent resolution of asparagine (Asn), aspartic acid (Asp) and isoaspartic acid (iso-Asp) containing peptides using a synthetic peptide mixture. The migration order in CE enabled a clear assignment of *in vitro* deamidation/isomerization sites in a protein standard mixture of intermediate complexity (48 proteins) as well as the determination of the *in vivo* deamidation rate of histone H1.0 directly in a crude nuclear protein fraction. Besides these well-known modifications citrullination, a post-translational modification which changes the positively charged guanidinium group of arginine to the uncharged ureido group of citrulline, was investigated. Applying CE-MS for fast and sensitive analyses of various post-translational modifications of intact and enzymatically digested histone H4, we were able to detect a variety of citrullinated proteoforms. MS/MS analysis with electron transfer dissociation (ETD) fragmentation identified the presence of deiminated Arg at position 3 and 17 of histone H4. Moreover, based on CE-MS, isobaric mono-phosphorylated peptides obtained in the course of a kinase activity study were separated and individual positional isomers quantified.

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1. Introduction

Currently, the most frequently employed technique for the analysis of deamidation products is mass spectrometry [1–4]. MS identification of deamidated peptides is relatively straightforward as deamidation adds 0.984 Da to the mass of a molecule. Collision-induced dissociation (CID) MS/MS spectra can usually reveal the exact position of deamidated Asn even in the presence of several Asn residues in the peptide sequence. Analytical techniques for the identification and quantification of iso-Asp and the elucidation of degradation pathways are much more challenging primarily because Asp and iso-Asp have identical mass and formal charge. CID is inefficient for distinguishing Asp and iso-Asp due to low specificity and unreliability [5]. A recent advance is the application of electron-capture dissociation (ECD) and electron transfer dissociation (ETD) [6,7]. These fragmentation methods produce reporter ions (c + 57 and z-57), which are unique to iso-Asp [2,8–10]. However, for large scale iso-Asp analysis using shotgun proteomics strategies, many of the initial hits based on reporter ions have been found to be false positives. The difficulty is mainly due to the low peak intensities for both reporter ions. When iso-Asp, Asp and Asn co-elute, the intensities of the reporter ions are further reduced and are not sufficient for unambiguous assignment. Moreover, not all peptides are amenable for ETD fragmentation, such as short and low-charged peptide ions. Therefore, a reliable pre-separation is an important prerequisite for the analysis of deamidation products, as it adds complementary information before MS analysis. Typical reversed phase HPLC conditions may lead to erroneous assignment, because the separation of the deamidation products is often poor and the elution order depends on the peptide sequence as well as on the chromatographic conditions [11]. Different chromatographic strategies have been developed for the detection

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of iso-Asp in proteins. Hao et al. described the use of electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) for selective separation of deamidated peptides from rat liver tissue [12]. They identified 302 unique N-deamidated peptides, of which 20 were identified *via* all three deamidation-related products and 70 of which were identified *via* two of them. It was shown, that ERLIC effectively separates the structural isomers, however, further optimization is needed before it can be used efficiently with complex samples. Iso-Asp formation in monoclonal antibodies was studied utilizing specific digestion of the antibody with IdeS protease followed by hydrophobic interaction chromatography (HIC) separation [13]. Breaking the intact antibody into two smaller domains afforded better chromatographic separation and quantitation of iso-Asp containing species, which elute earlier than the Asp-containing peptide. However, this method cannot be used for complex samples. For a structure-based prediction of Asp and iso-Asp sites in antibody variable regions, Sydow et al. used RP-HPLC applying a Polaris C18-Ether column [14]. Deamidated species could be separated from the corresponding unmodified peptide, however, a more detailed assignment was not shown.

A further analytical challenge is the identification of citrullinated peptides. Citrullination is a post-translational modification converting protein arginine residues in the non-standard amino acid citrulline [15]. The conversion adds 0.984 Da to the mass of the intact molecule, which is the same mass increase as observed during deamidation. At physiological pH, arginine has a positive charge due to the guanidinium group, whereas citrulline is neutral. The conversion is catalyzed by a family of calcium-binding enzymes, called peptidylarginine deiminases (PAD) and is thought to alter protein structure, function and distribution. Recently, citrullination has become an area of significant interest, because of its suspected role in various pathological conditions, such as rheumatoid arthritis, multiple sclerosis and Alzheimer's disease [16]. The separation of citrullinated peptides from the corresponding non-citrullinated peptides is crucial for unambiguous distinction by MS.

Capillary electrophoresis, which allows separations based on mass to charge ratio of molecules, can be successfully applied to study post-translationally modified peptides and proteins. The change of net charge introduced by deamidation and citrullination as well as the slight differences between the pK_as of Asp and iso-Asp makes CE particularly well suited for this kind of analysis [17–19]. With recent developments in interfacing CE to electrospray ionization (ESI)-MS, CE has shown its application in protein and peptide analyses with its superior sensitivity and separation efficiency [20–25]. A new separation and ionization technology called CESI, which integrates the high efficiency of CE with ESI, relies on a separation capillary with a porous tip acting as nanospray emitter [26]. It has been demonstrated that this interface is able to work at flow rates less than 10 nL/min, which reduces ion suppression and improves sensitivity [27–31]. This CE-MS coupling was evaluated successfully for use in peptide and protein analysis, and was shown to be able to identify the localization of posttranslational modifications on antibodies and histones in a highly sensitive manner [32–35].

As there are few reports on CE-MS regarding its ability for the analysis of deamidation products [36–38] and none of citrullinated peptides, we systematically investigated CESI-MS for separation and differentiation of Asp and iso-Asp products due to Asn deamidation: i, a synthetic peptide mixture consisting of Asn, Asp and iso-Asp containing decapeptides was used to examine the migration behavior on CE; ii, a protein standard mixture of medium complexity (48 proteins) was digested with trypsin under different conditions to study the *in vitro* deamidation products and the reliability of the CE-MS method; iii, our method was applied to a crude nuclear protein fraction in order to directly evaluate the *in vivo* deamidation rate of histone H1.0.

The CE-MS method was also able to characterize arginine citrullination both on intact histone H4 and on peptides obtained after protease cleavage. The capability of CE to separate deimination- and deamidation products impressively shows how slight charge differences can be exploited for a successful MS analysis of even multiply modified proteoforms. For this reason, CE-MS was used as complementary approach for the quantification of mono-phosphorylated isobaric peptides, still a very challenging task in the proteomics field, as side specific product ions are often weak or not even detectable [39,40]. If such positional isomers cannot be separated, an unambiguous assignment of site-specific phosphorylation effects is problematic, a quantification often impossible. In the course of a kinase activity study isobaric mono-phosphorylated peptides were identified as potential substrates and therefore, the CE-MS approach was evaluated for their identification and quantification. As a result, a successful separation and quantification of individual positional isomers could be demonstrated.

2. Materials and methods

2.1. Materials

Trypsin (mass spectrometry grade) was obtained from Promega (Mannheim, Germany); 1,4 dithiothreitol was purchased from Biomol (Hamburg, Germany); and iodoacetamide from GE Healthcare (Vienna, Austria). Deamidated and acetylated forms of the histone H1.0 peptide with a sequence TENSTSAPAA were synthesized by Sigma Genosys (Cambridge, UK). The Universal Proteomics Standard (UPS1) containing 48 human proteins (5 pmoles each), endoproteinases Arg-C and Glu-C (sequencing grade), and all other chemicals were purchased from Sigma-Aldrich (Vienna, Austria). Water was purified with a Millipore Milli-Q Academic water purification system (Vienna, Austria).

2.2. Sample preparation

Proteins present in the Universal Proteomics Standard (UPS1) were solubilized in 10 μL ammonium bicarbonate buffer (10 mM, pH 8.0), reduced with 2 μL dithiothreitol (50 mM) at 56 °C for 30 min, and alkylated with 2 μL iodoacetamide (200 mM) at room temperature for 20 min. Proteins were digested at 37 °C for 18 h or 70 h using trypsin at a ratio of 1:20 (w/w). Histone H1 proteins were extracted from a human placenta according to Lindner et al. [41], 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. [42]. 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 PerfectPure 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 [29]. All samples were lyophilized, re-solubilized in 50 mM ammonium acetate buffer (pH 4.0), and stored at −20 °C until analysis.

2.3. Capillary electrophoresis – mass spectrometry

For CE-MS analysis a CESI 8000 (Sciex, Brea, CA) equipped with a bare or neutrally-coated fused-silica capillary with a porous tip acting as nanospray emitter (Sciex, Brea, CA) (total length: 90 cm, i.d.: 30 μm, o.d.: 150 μm) was coupled *via* an ESI module to a Thermo Scientific Q Exactive Plus or LTQ Orbitrap XL ETD (Bremen, Germany). The neutral coating manufactured by Sciex comprises two layers, a hydrophobic coating and a hydrophilic polyacrylamide coating. The lower hydrophobic coating protects siloxanes

from hydrolysis whereas the upper hydrophilic polyacrylamide coating eliminates the electroosmotic flow and inhibits surface interactions. Prior to each analysis the system was rinsed with background electrolyte (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 of 5 psi for 20 s (17 nL) followed by an injection plug of BGE (5 psi for 5 s). The separation was performed at +30 kV with pressure applied at the capillary inlet. The pressure profile was: 0–10 min, 1 psi; 10–52 min, 1.5 psi. The flow rate was determined to be approximately 10 nL/min using the neutrally-coated and 30 nL/min using the bare fused-silica capillary, respectively.

2.4. Mass spectrometry data acquisition

The Q Exactive Plus mass spectrometer was operating in data dependent mode to switch between MS and MS/MS acquisition. Survey full scan MS spectra were acquired with a resolution of R = 70,000 at an AGC target of 3e6 in profile mode. To generate MS/MS spectra the ten highest precursors were selected for higher energy collision dissociation (HCD) applying a stepped normalized collision energy of 28.0 and 30.0. Fragments were scanned with a resolution of R = 35,000 at an AGC target of 5e5. Dynamic exclusion was set to 10 s. Unassigned, singly charged and peptides with charge higher than +7 were excluded from HCD fragmentation. For peptide fragmentation using the LTQ Orbitrap XL ETD mass spectrometer ETD and CID were used. Settings were as described elsewhere [33].

2.5. Data analysis and protein identification

Proteome Discoverer version 1.2.0.208 (ThermoScientific) with search engine Sequest was used for data analysis. Raw data obtained by CE-MS from UPS1 and histone H1 samples were searched against the IPI human database version 3.86 (91,519 entries) to which 48 sequences of the Universal Proteomics Standard were added. The following settings were applied: Enzyme for protein cleavage was trypsin; two missed cleavages were allowed. Variable modifications were carbamidomethylcysteine; oxidation of methionine and deamidation of asparagine and glutamine. For data obtained from human histone H4 analyses the enzyme for protein cleavage was set to endoproteinase Glu-C and the variable modification deamidation of arginine was added. Precursor mass tolerance was set to 10 ppm. The fragment mass tolerance was 50 mmu for Q Exactive data and 0.8 Da for LTQ Orbitrap XL ETD data. False discovery rate (FDR) was set to 1%.

3. Results and discussion

In vitro and *in vivo* deamidation of proteins and peptides is a remarkable modification reaction as it occurs spontaneously and it is usually not caused by enzymes. It may significantly influence biological activity in both directions; it can be decreased or increased. Deamidation follows a rather complex mechanism *via* a succinimide intermediate with a release of ammonia. Hydrolytic cleavage of this cyclic imide provides two reaction products, peptides with Asp and iso-Asp instead of Asn (for more details see Ref. [18])

Regarding the chemical characteristics of the reaction products, the Asn containing peptide has the highest isoelectric point and therefore the highest electrophoretic mobility at acidic conditions. Due to significant differences in the pK_a values of Asp (pK_a = 3.9) and iso-Asp (pK_a = 3.1) and considering preliminary studies [43], we expected also differences in their individual electrophoretic mobilities, sufficient for an acceptable separation of all three peptides.

3.1. Synthetic peptides containing deamidated residues

For further *in vivo* studies on histone H1 modifications we were interested in the even more challenging analysis of doubly-modified peptides, acetylated and deamidated. To find appropriate conditions for the unambiguous identification and quantification we analyzed 6 synthetic peptides (TENSTSAPAA, TEDSTSAPAA, TEisoDSTSAPAA, Ac-TENSTSAPAA, Ac-TEDSTSAPAA Ac-TEisoDSTSAPAA). These peptides comprise the N-Terminus of the human histone H1.0, which is known to be both, N-terminally acetylated and age-dependently deamidated at position 3 [41].

In a first attempt to separate the synthetic peptide mixture a bare fused-silica capillary and 10% (v/v) acetic acid (pH 2.26) as BGE were applied. As can be seen in Fig. 1A, using these initial conditions a clear separation of all six peptides was not achievable, which was especially true for the N-terminally acetylated peptides. Due to the lack of positively charged groups no significant charge difference between these three peptides at this low pH conditions occurred. However, baseline separation could be accomplished when changing the BGE to 0.1% formic acid (pH 2.7) as this higher pH generates a sufficient net charge difference between the peptides (Fig. 1B). As expected, the peptide containing the asparagine (TENSTSAPAA) migrated fastest, followed by the deamidated form with the aspartyl residue (TEDSTSAPAA) and finally the deamidated form containing the isoaspartyl residue (TEisoDSTSAPAA). The acetylated forms generally migrated slower than the non-acetylated peptides but shared the same order of appearance.

An unambiguous identification of the isoaspartyl residue containing peptide was performed *via* ETD fragmentation. As can be seen in Fig. 1D, the characteristic z_8^+-57 fragment ion that appears during the fragmentation process is specific for iso-Asp but not for Asp (Fig. 1C). The possibility to differentiate peptides on the basis of the z-57 fragment ion is commonly known but the success strongly depends on the suitability of the peptide for ETD fragmentation. Mostly long and/or highly charged peptides are proper targets for this type of fragmentation and even in this case the reporter ion intensity is often too low and varies depending on the position of the deamidated amino acid. In addition to that, mass spectrometers are frequently not equipped with ETD. Therefore, an important step forward in the analysis of deamidation products is an efficient separation strategy that enables differentiation on the basis of migration time rather than signature ions.

3.2. Deamidation products in a medium complex peptide mixture

Several studies investigated the factors that influence deamidation and isomerization rates in proteins with the result that both processes depend on pH, temperature, the neighboring C-terminal amino acid, and the tertiary structure. Deamidation rates increase at neutral to alkaline pH and at higher temperatures. Exactly those conditions are commonly used for sample preparation in bottom-up proteomics because trypsin and other enzymes used to cleave proteins operate best at a pH of around 8 and at 37 °C. Therefore, sample preparation is prone to generate artificial deamidation products. To investigate this in more detail and to collect data from a more complex sample, we digested the Universal Proteomics Standard, a mixture consisting of 48 human proteins with trypsin for 18 h and 70 h and analyzed the resulting peptides with CE-MS.

Based on the previously used CE conditions (bare fused-silica capillary, 0.1% (v/v) formic acid as BGE) and an EOF of approximately 30 nL/min a maximum separation time of about 20–25 min can be calculated (total capillary volume ~650 nL). For more complex samples longer separation times allow a better separation of individual peptides and increase in this way the identification rate substantially. For this reason, the bare fused-silica capillary was replaced by a neutral capillary that enables separations up

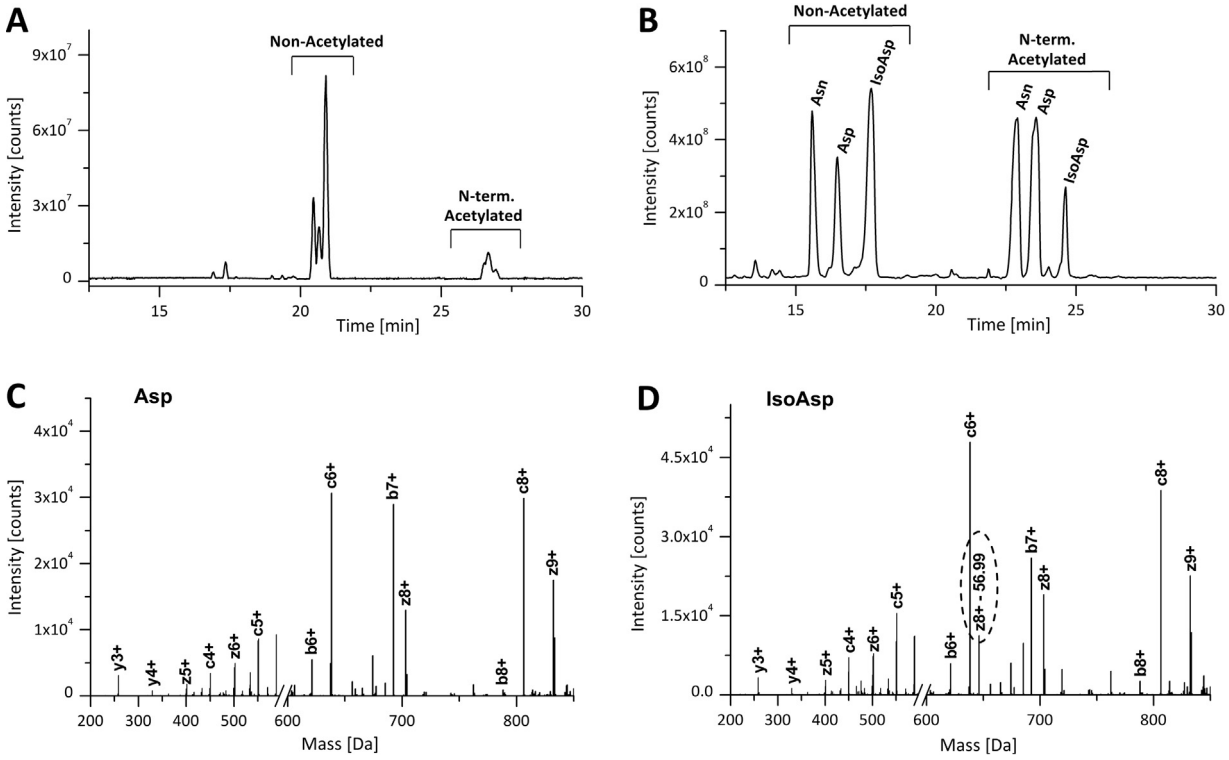


Fig. 1. CESI-MS separation of 6 synthetic peptides covering deamidated and acetylated forms of the N-terminal peptide TENSTSAPAA of the human histone H1.0. CESI-MS separation using (A) 10% (v/v) acetic acid and (B) 0.1% (v/v) formic acid as BGE. (C) ETD spectra obtained from the aspartate containing peptide TEDSTSAPAA. (D) ETD spectra obtained from the isoaspartate containing peptide TEisoDSTSAPAA. CESI-MS conditions: Bare fused silica capillary; BGE, 0.1% (v/v) formic acid; MS instrument, Thermo Scientific LTQ Orbitrap XL ETD. All other conditions as described in the Method section.

to 100 min depending on the pressure applied at the capillary inlet (flow rate 4–10 nL/min when applying 0.5–1.5 psi pressure). In total, the CE-MS analysis of the two digests yielded a total of 928 identified peptide sequences. A set of 390 peptides contained at least one Asn and roughly one quarter was indeed found to be deamidated. The number of deamidated peptides increased from 58 to 95 when elongating the digestion step from 18 h to 70 h, and also the proportion of deamidated products increased with a simultaneous decrease of their non-deamidated counterparts.

It has already been described that the formation of the cyclic succinimide intermediate proceeds more quickly if the Asn is followed by a small residue such as glycine or serine, whereas amino acids with bulky, hydrophobic side chains reduce the intermediate formation [44]. We can confirm that the glycine increases the susceptibility for deamidation of asparagines, since 29% of the deamidated peptides identified contained the Asn-Gly motif. Other amino acids increase the deamidation rate less significantly (data not shown). In this context it is worth highlighting that all deamidated peptides, regardless of the motif, showed the same separation pattern. In all cases we found the same migration order, the Asn containing peptide was followed by the Asp and finally by the iso-Asp containing peptide (Fig. 2A). On the basis of the migration times we were able to calculate the separation selectivity of the Asp and iso-Asp containing peptides to be $\alpha = 1.067$ (SD = 0.018). Peptides with the Asn located at the N-terminus deviate from this tendency and exhibit increased separation selectivity of $\alpha = 1.196$ (SD = 0.015) (Fig. 2B). We assume that this difference is caused by an interaction of the carboxylic side chain of the aspartic/isoaspartic amino acid with the N-terminal amino group, leading to a zwitterionic structure which reduces the overall charge of the peptide. As can be read off the extracted ion electropherograms (Fig. 2A and B) the Asp and iso-Asp moieties correlate to the equilibrium constants of the two possible cleavage reactions, from succinimide intermediate to the aspartyl peptide (22%) and succinimide intermediate to the isoaspartyl peptide (78%), respectively [45]. Indeed, the quantification of 30 deamidated peptides, which was straight forward due to the excellent separation, revealed an allocation of 23% for the aspartyl and 77% (SD = 5%) for the isoaspartyl peak, respectively (see supplemental Table S-1). The method enables an efficient separation even of di-deamidated forms. The peptide ANALLANGVELR, for example, was found to be deamidated at position 2 and 7 to a different extent (Fig. 2C). The mono-deamidated forms from Asn-7 were found to be the main deamidation products, the minor di-deamidated forms arose from Asn-2. This result further illustrates the efficiency of the CE-MS technique to reproducibly separate and quantify deamidation products.

3.3. *Deamidation products* in vivo

A pronounced deamidation *in vivo* arises particularly in long-lived proteins (*e.g.*, crystallins from the eye lens and beta-amyloid in the brain) and may drastically alter protein structure and function [46–48]. A broad spectrum of organisms possess an enzyme called PIMT (protein L-isoaspartyl/D-aspartyl O-methyltransferase) which is able to reduce the levels of iso-Asp *via* a repair mechanism. The enzyme catalyzes the transfer of a methyl group from S-adenosylmethionine to iso-Asp to form isoaspartate-methyl-ester that converts back to Asp [10,18]. The repair function for PIMT *in vivo* was verified by the observation that a reduction of the PIMT activity dramatically increases the level of iso-Asp-containing proteins [49,50].

We were interested to apply CE-MS to investigate the *in vivo* deamidation rate of histone H1.0, which is known to be deamidated at position 3 [41]. Therefore, H1 histones were extracted from human placenta and digested with Arg-C, which preferentially cleaves after arginine residues although hydrolysis proceeds

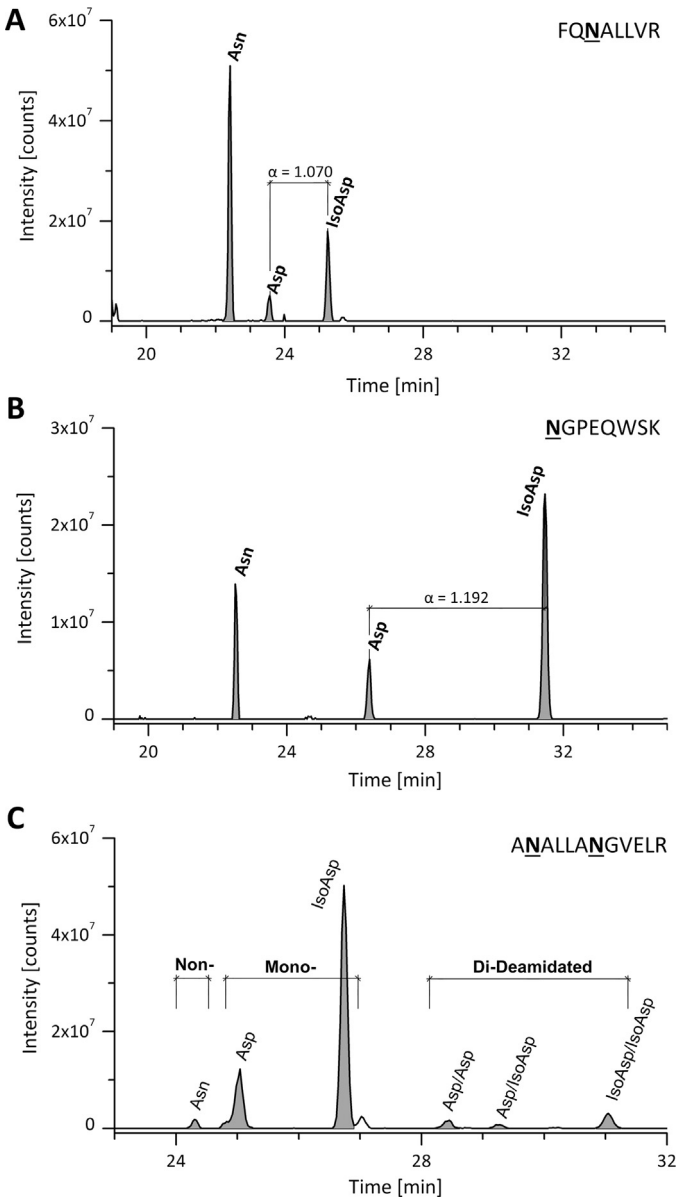


Fig. 2. Extracted ion electropherograms of deamidated peptides present in the Universal Proteomics Standard. (A) The peptide FQNALLVR exhibit the deamidated Asn within the peptide sequence, (B) the peptide NGPEQWSK at the N-terminus. (C) The peptide ANALLANGVELR was found to be di-deamidated. CESI-MS conditions: Neutrally coated capillary; BGE, 0.1 M acetic acid; MS instrument, Thermo Scientific Q Exactive Plus.

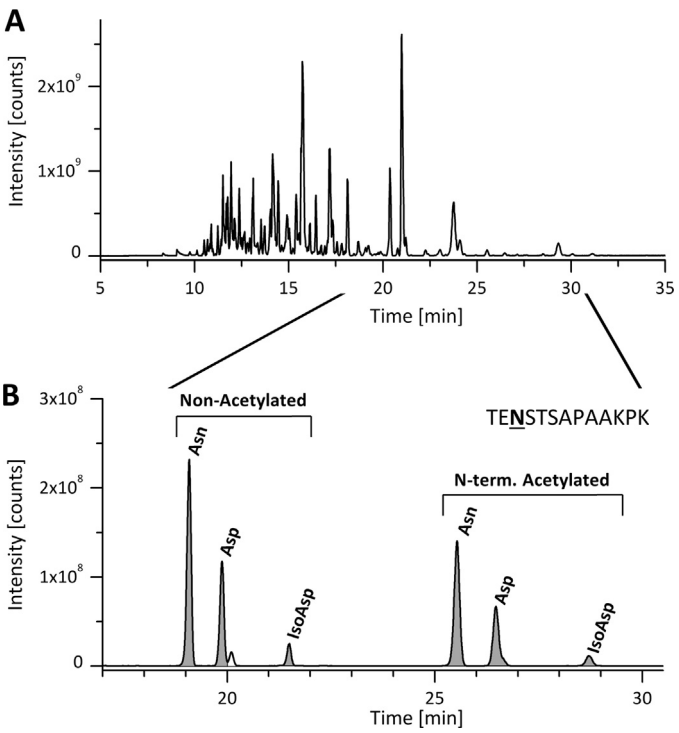


Fig. 3. CESI-MS separation of human placenta H1 histones digested with endoproteinase Arg-C. (A) Base peak electropherogram and (B) extracted ion electropherogram of deamidated and acetylated forms of the N-terminal peptide TENSTSAPAAKPK of the human histone H1.0. CESI-MS conditions: Neutrally coated capillary; BGE, 0.1 M acetic acid; MS instrument, Thermo Scientific Q Exactive Plus.

to a minor degree in most Lys-containing substrates. This highly efficient enzyme allows very short reaction times which in turn prevent generation of artificial deamidation products. The sample was analyzed on a neutral capillary and MS/MS spectra obtained were searched against the IPI-human database. Fig. 3 shows the extracted ions of the N-terminal peptide TENSTSAPAAKPK and its modified forms. The Asn containing peptides showed the highest intensity. This was true for the N-terminally acetylated and the unacetylated proteoforms. The quantification revealed 85% Asp and only 15% iso-Asp, which is in stark contrast to the previous results of solely artificially (*in vitro*) generated deamidation products in the protein standard mixture. Therefore, we investigated the *in vitro* deamidation rate of the synthetic decapeptide TENSTSAPAA, which comprises the N-Terminus of the human histone H1.0. The ratio of the Asp/iso-Asp containing peaks strongly correlated to the equilibrium constants of the two spontaneous reactions (29% Asp and 71% iso-Asp; see supplemental Fig. 1). We conclude, therefore, that *in vivo* the PIMT repair mechanism shifts the ratio from about 1:4 towards 6:1, thereby reducing the amount of iso-Asp to one-fifth. Our investigations clearly show that CE-MS is well suited to separate and quantify Asp/iso-Asp containing peptides in a reliable way and provides a very potent alternative strategy to ETD fragmentation.

3.4. Identification of citrullinated proteoforms

When evaluating the potential of CESI-MS for the characterization of core histone subtypes and their modifications, we were able to demonstrate quite recently that CESI-MS is a very powerful technique not only for the characterization of different acetylation and methylation sites on the peptide level but also for the analysis of the acetylation and methylation status of the intact protein [33]. When H4 histones originating from human blood cells were analyzed in the same way, we found a remarkable heterogeneity that could be assigned neither to acetylation nor methylation. Fig. 4A shows that histone H4 was resolved into 3 main peaks and mass deconvolution of corresponding protein peaks revealed a mass difference of +1 Da (Fig. 4B and C). The mass shift as well as the decrease in migration time (less positively charged) resembles the properties of deamidation products.

As there are evidences in the literature that Asn-25 can be deamidated *in vitro* [51,52], the protein was cleaved with endoproteinase Glu-C, which cleaves amongst others after Asp-24. Using a neutral capillary 8 peptide sequences covering 89.2% of histone H4 were identified (supplemental Table S-2 and Table S-3). No evidence was found for a deamidation of Asn-25 as peptides 25–52 and 25–53 yielded single peaks. The reason for the heterogeneity was found to be located within the first 24 amino acids of H4 (SGRGKGGKGLGKGGAKRHRKVLRD). MS/MS analysis applying ETD

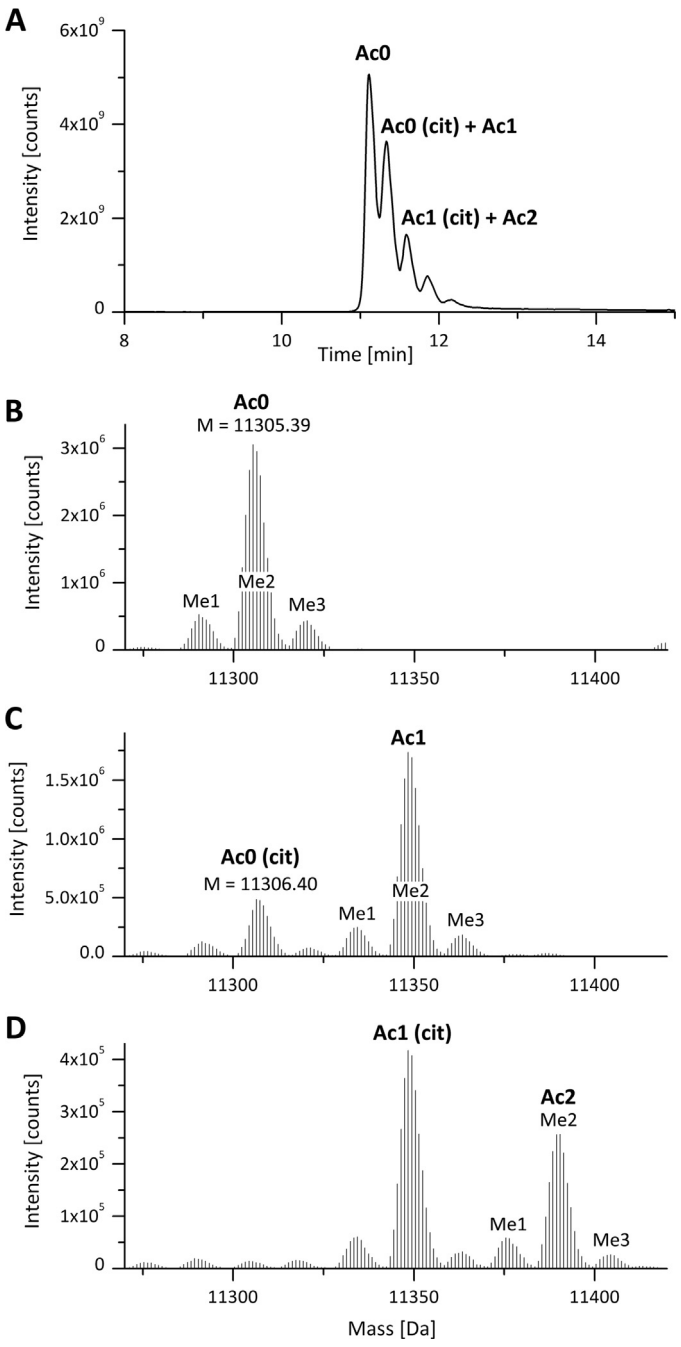


Fig. 4. CESI-MS analysis of intact citrullinated and non-citrullinated core histone H4 using a neutral capillary. (A) Total ion electropherogram and (B-D) Mass deconvoluted spectra of individual H4 peaks (1–3). Average mass of histone H4 calculated for Ac0Me2: 11305.39 Da; average mass of the citrullinated Histone H4 Ac0Me2: 11306.40 Da.

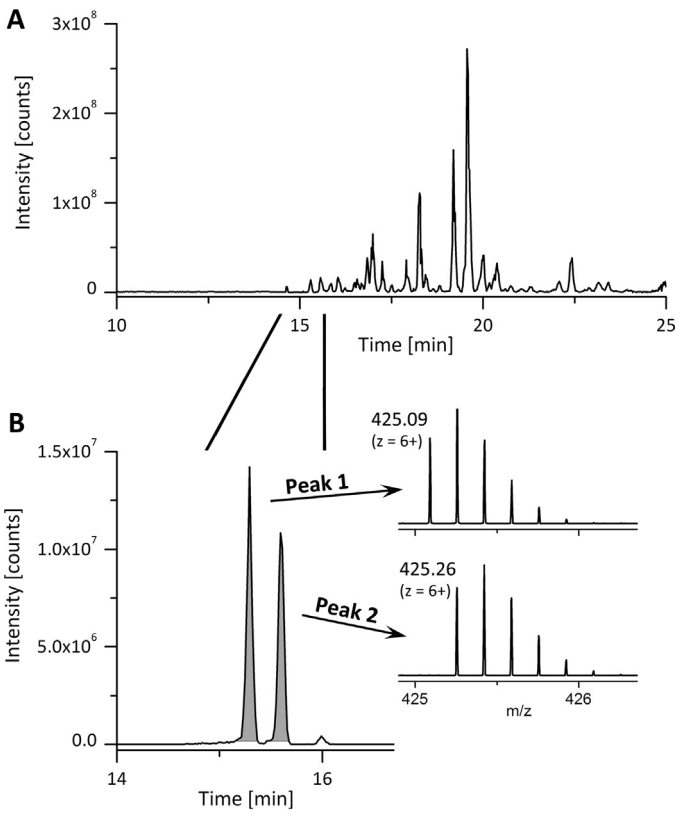


Fig. 5. CESI-MS separation of human H4 histones extracted from human blood cells and digested with endoproteinase Glu-C. (A) Base peak electropherogram. (B) Extracted ion electropherogram of peak 1 (*m*/*z* 425.09) the 6+ charged peptide 1-24 (N-terminally acetylated, di-methylated at Lys-20) and of peak 2 (*m*/*z* 425.26; N-terminally acetylated, di-methylated at Lys-20 and citrullinated at Arg-3). CESI-MS conditions: Neutrally coated capillary; BGE, 0.1 M acetic acid; MS instrument, Thermo Scientific Q Exactive Plus.

fragmentation identified the peptide 1-24 N-terminally acetylated and di-methylated at Lys-20. The extracted ion electropherogram and isotope distribution for the 6+ charge state (*m*/*z* 425.09) is shown in Fig. 5B. Manual inspection of the data revealed a second peak exhibiting the same isotope distribution for a 6+ charge state at *m*/*z* 425.259, indicating a mass difference of roughly 1 Da between the two peaks.

As Glu-C was used as cleaving enzyme deamidation as possible explanation could be excluded. Similar shifts in mass and migration time should also occur when a positively charged Arg residue is posttranslationally converted into the uncharged non-coded amino acid citrulline. Database search adding citrullinated arginine revealed that peak 2 contains peptide 1-24 di-methylated at Lys-20 and citrullinated primarily at Arg-3 but also on Arg-17 (see supplemental Table S-3). Manual inspection gave evidence for the presence of a di-citrullinated peptide as well, but due to its small amount the spectral quality was insufficient for unambiguous identification (data not shown).

The clear separation of the citrullinated peptide from the arginine containing peptide is important as the +0.984 Da mass shift caused by citrullination is close to that of a single neutron +1.008 Da. When both peptides coelute, the *m*/*z* signal from the citrullinated peptide will fall within the isotopic cluster of the un-citrullinated peptide. This causes an apparent change in the isotopic cluster, but in conventional bottom-up proteomics it will hinder a successful identification of the PTM [53]. At the peptide level, citrullinated peptides can often be separated by RP-HPLC as well because of an increase in hydrophobicity caused by this modification [54]. In contrast to RP-HPLC, however, separation at the level of intact proteins is possible only by CE. The deconvoluted average mass of the 3 main peaks shown in Fig. 4 revealed that CE allows the unambiguous identification of the following proteoforms: (i) un-acetylated (peak Ac0), (ii) un-acetylated citrullinated and mono-acetylated (peak Ac0 cit + Ac1) and (iii) mono-acetylated citrullinated and di-acetylated forms (peak Ac1 cit + Ac2) (Fig. 4B–D). The non-, mono-, di-, and tri-methylated forms were observed to co-migrate with the different acetylated/citrullinated states. The selectivity of this separation is not only leading to less complex MS spectra but enables the assignment of nearly isobaric tri-methyl and acetyl modifications without the requirement of high-resolution mass spectrometry as they can be confidently distinguished because of their different

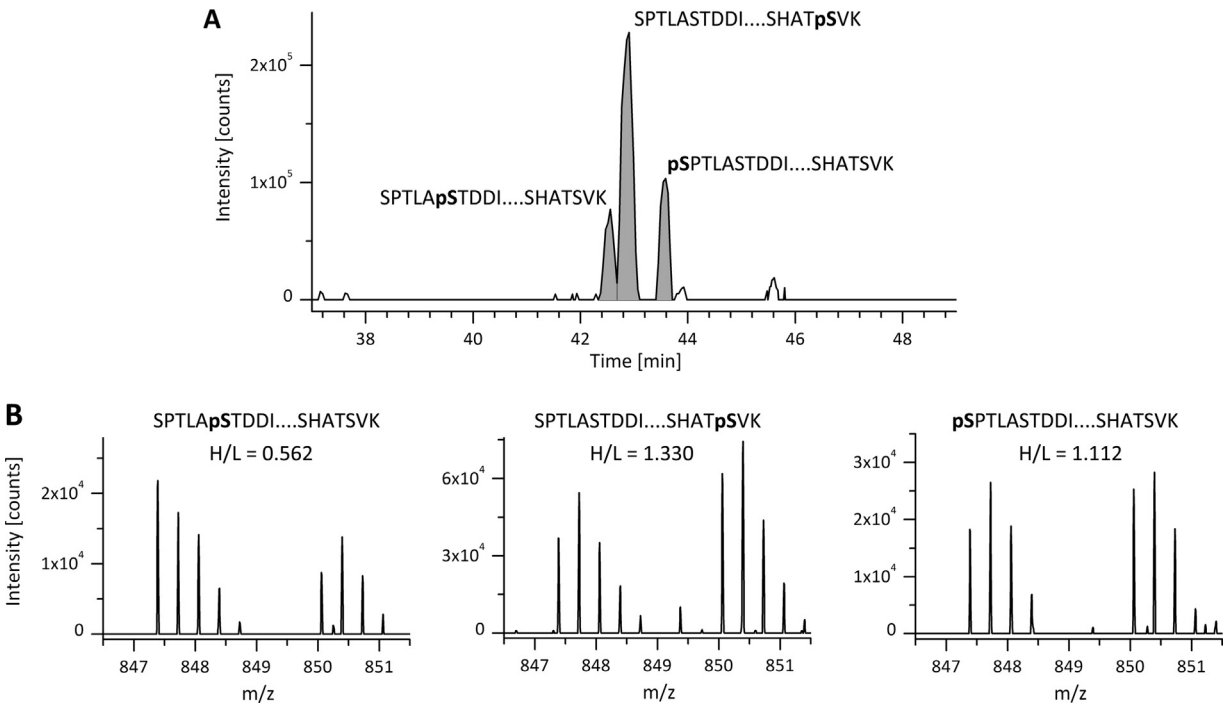


Fig. 6. CESI-MS separation of phosphopeptide isomers. (A) Extracted ion electropherogram of the peptide: SPTLASTDDINSASASVNSHATSVK mono-phosphorylated on three different sites. (B) Quantification spectra and H/L ratios of the three SILAC labeled phosphopeptide pairs.

migration time. It should be noted in this context that we have never observed deamidated histone H4. Very harsh conditions (*e.g.*, during Edman degradation) seem to be necessary to induce deamidation of Asn-24.

3.5. Phosphorylated peptide isomers

It is known that CZE is able to separate peptides with identical amino acid composition but with different sequence provided the sequence variation causes a variation in pKa values of the corresponding peptides. This can be induced, *e.g.* by local differences in hydrophobicity, conformation, extent of hydration and even more importantly by interaction between charged moieties (for review, see [55,56]). For this reason, variation of buffer pH and concentration and the addition of organic solvents, *e.g.* TFE can be used to optimize resolution [39,57]. It was further shown that the number and position of adjacent charges present either at the amino terminus or in the interior of the peptide may selectively alter the overall charge of those peptides [58]. This effect is nicely demonstrated in Fig. 2 showing that the resolution of Asp/iso-Asp containing peptides significantly increased when deamidation occurs at the N-terminus.

Similar effects can be expected by the introduction of charged phosphate groups. Depending on the primary structure of the peptides, the slight differences in the overall charge can be exploited for the separation of positional isomers of mono-phosphorylated peptides and in turn for an unambiguous identification and quantification of particular modification sites by MS/MS, still a challenging task in the proteomics field.

This is demonstrated with a number of phosphopeptide isomers identified in a fraction of metabolically labeled peptides obtained after Lys-C cleavage from two yeast strains. The complex peptide sample was pre-fractionated by RP-HPLC and the fractions of interest were analyzed by CE-MS using a neutral capillary (for method details, see [29,59]). Among other interesting candidates as potential kinase substrates, six phosphopeptide isomers were identified that differ in their migration time by at least 0.4 min (see supplemental Table S4). Five phosphopeptide isomers were found to be mono-phosphorylated showing two phosphorylation sites. One peptide (SPTLASTDDINSASASVNSHATSVK) showed even three different phosphorylation sites. The individual isobaric phosphopeptides were mutually clearly separated. The lower electrophoretic mobility was consistently found for the isomer with the phosphate group bound to a serine or threonine residue being in close vicinity to a basic residue or the N-terminus. As an example, the CE-MS analysis of the three isomers of the above mentioned peptide is shown in Fig. 6A, the peptide phosphorylated on Ser-6 shows the highest mobility, followed by the peptide modified at Ser-23 close to the C-terminal Lys-25. The peptide with the phosphorylated N-terminal serine exhibits the lowest electrophoretic mobility. It should be noted that also other structural effects could selectively influence the migration behavior; however, further and more extended studies are necessary to verify such relationships.

Based on the ability of CZE to separate the three positional isomers an unambiguous identification of the corresponding phosphorylation site along with a reliable quantification of all three mono-phosphorylated peptides was achieved (Fig. 6B, ratios ranging from H/L = 0.562 to 1.330).

4. Conclusion

In this work, we demonstrate that capillary electrophoresis combined with mass spectrometry can be used effectively for the analysis of peptides containing aspartate and isoaspartate as main reaction products occurring during *in vivo* and *in vitro* deamidation. Due to the reduced net charge introduced by deamidation as well as the slight differences between the pKa of the Asp and iso-Asp isomers, a reliable separation and identification based on CID or HCD fragmentation can be obtained. Based on the consistent separation pattern, the method described provides a powerful alternative strategy to ETD fragmentation and its inherent limitations. We could further demonstrate that our method enables the separation of citrullinated proteoforms from intact histone H4, because each citrullination event causes the loss of one positive

charge, which in turn lowers the overall charge. Moreover, even slight differences in the overall charge of analytes can be exploited for their separation, as shown for the successful separation of positional isomers of mono-phosphorylated peptides. In this way the unambiguous identification and quantification of individual modification sites was possible by CE-MS. It is important to note in this context that phosphopeptide isomers can be separated by chromatographic methods, however, at a low success rate, especially for longer peptides. Also CE-MS is not a generally applicable method, however, it is an interesting and promising alternative to increase the identification rate of such positional isomers. Moreover, advantages of the CE-MS technique used are high separation efficiency and very low flow rates, leading to reduced ion-suppression and very high sensitivity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.01.086.

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