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## Exploring the proteome by CE-ESI-MS

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# Chapter 1

## **Ultra-Low flow ESI-MS for improved ionization efficiency in phosphoproteomics**

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## Abstract

The potential benefits of ultra-low flow ESI ionization for the analysis of phosphopeptides in proteomics was investigated. Firstly, the relative flow dependent ionization efficiency of non-phosphorylated vs. multiplyphosphorylated peptides was characterized by infusion of a 5 synthetic peptide mix with none to four phosphorylation sites at flowrates ranging from 4.5 to 500 nL/min. Most importantly, similarly to what was found earlier by Schmidt et al., it has been verified that at flow rates below 20 nL/min the relative peak intensities for the various peptides show a trend toward an equimolar response, which would be highly beneficial in phosphoproteomic analysis. As the technology to achieve liquid chromatography separation at flow rates below 20 nL/min is not readily available a sheathless CE-ESI-MS strategy based on the use of a neutral separation capillary was used to develop an analytical strategy at flowrates as low as 6.6 nL/min. An inline preconcentration technique, namely transient isotachopheresis (t-ITP) to achieve efficient separation while using larger volume injections (37% of capillary thus 250 nL) was incorporated to achieve even greater sample concentration sensitivities. The developed t-ITP-ESI-MS strategy was then used in a direct comparison with nano-LC-MS for the detection of phosphopeptides. The comparison showed significantly improved phosphopeptide sensitivity in equal sample load and equal sample concentration conditions for CE-MS while providing complementary data to LC-MS, demonstrating the potential of ultra-low flow ESI for the analysis of phosphopeptides in liquid based separation techniques.

# 1 Introduction

The introduction of modern ESI sources[1-4], and subsequent hyphenation of liquid chromatography[5] (LC) and capillary electrophoresis[6-8] (CE) to mass spectrometry (MS) are probably the most important events in the history of analytical/bio-analytical sciences over the last twenty years. Currently ESI-MS is the core of multiple proteomics workflows, which implies the use of a broad range of LC systems, columns and separation modes over a wide range of flow rates. ESI is indeed compatible with different flow rates, but the technique is inherently optimal at low flow. In early days coupling to HPLC (then > mL/min) was realized through splitting[9]. With the development of nano-ESI[10] and nano-LC such advantages of low flow rates as reduced ion-suppression and improved ionization efficiency became commonly accepted[11-15]. Schmidt et al.[13] have systematically studied the effects of flow rates on the ion signal for a model compound mixture and demonstrated that the ionization bias towards neurotensin, which is commonly observed at usual flow rates, was greatly reduced at flow rates below 20 nL/min. While this is a very important bottleneck for various kinds of MS applications, the mentioned ionization bias is especially problematic in the field of phosphoproteomics, where MS analysis suffers from low ionization efficiency of phosphorylated peptides on top of the already low natural abundance of phosphorylations. Despite much discussion and research on the reasons for the low phosphopeptide ionization efficiency[16-19], improved ionization efficiency only shows a concentration sensitive increase of signal, which results in an equal increase of phospho and non-phospho peptide signal and does not improve the detection of phosphopeptides specifically. For this reason, off-line enrichment techniques have been developed to compensate for the generally high limits of detection in phosphopeptide analysis, but such techniques, while they remain very powerful for large-scale proteomic studies, are costly and labor intensive[20]. Additionally, as any step of an analytical workflow, especially when affinity reactions are involved, they may introduce biases and losses depending on the solid phase material chosen. Another way to potentially improve the compatibility of common workflows for phosphorylated peptides is to work directly on the ESI process where a homogenization of the ionization efficiencies could potentially advance the analysis of phosphopeptides and phosphoproteomics in general. As previously demonstrated, working on the magnitude of the flow rates, rather than or in addition to various enrichment procedures, upstream from the ionization process could represent a very valuable path to explore. Considering LC-based technologies first, which are today the preferred separation strategies in proteomics, it is not an easy task to conduct LC separations at flow rates

below 20 nL/min with satisfactory robustness and high peak efficiencies. In this context, capillary electrophoresis (CE) strategies [21-25] in combination with the ESI sources capable of maintaining a stable spray at such low flow rates[10, 14, 26] may constitute a very interesting option.

Therefore, we have investigated the effect of ultra-low flow ESI for the mass spectrometric detection of phosphopeptides. An ESI interface based on the design of Moini et al.[27] was used to achieve stable ESI spray at flow rates below 10 nL/min. The effects of ultra-low flow on the ionization efficiency of a model peptide with up to 4 phosphorylation sites was studied under hydrodynamic infusion conditions. As a proof of principle, a typical model sample for phosphoproteomics of relatively low complexity was studied. The tryptic digest of bovine milk was analyzed by sheathless CE-ESI-MS using a neutrally coated capillary and the integration of transient-isotachopheresis (tITP) as the sole sample concentration step, without any of the commonly used off-line phosphopeptide sample pre-concentration techniques. Subsequently, the developed strategy was used for the identification of phosphopeptides in a skimmed milk digest sample on an ion trap and compared to results obtained with a “more traditional” phosphoproteomic nano-LC-MS method.

## **2 Materials and Methods**

### **2.1 Chemicals**

All chemicals used were of analytical reagent grade and obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) otherwise stated specially. A 13 amino acid peptide was designed (YQTYPIYASYHLR) with four incorporated tyrosine moieties allowing for its synthesis in a non to tetra-phosphorylated form using a previously reported method[28]. Purity after synthesis was determined with HPLC-UV with the consecutive phosphorylation states being 100%, 100%, 64.7%, 49.9% and 60.2% pure respectively. The measured purities were used for compensation when making stock solutions of the individual synthetic peptides. Powdered bovine milk was purchased locally. All buffers and solutions were prepared in nano-pure water from an Alpha-Q Millipore system (Amsterdam, The Netherlands).

## 2.2 Sample preparation

Dry milk powder (20 mg) was resuspended in 5 mL 50 mM TEAB (triethylammonium bicarbonate buffer, pH 8.0, Fluka) containing 0.1% of RapiGest (Waters, Milford, MA). The cysteines were reduced with 2 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride) for 45 min at 60 °C and subsequently alkylated with 4 mM MMTS (S-methyl methanethiosulfonate, Fluka) for 30 min at room temperature. Proteins were then digested with trypsin overnight at 37 °C (Sequencing grade modified trypsin, Promega, Madison, WI) using a 50:1 (protein:trypsin) ratio. RapiGest was cleaved and removed from the sample according to the manufacturer's protocol. Aliquots containing 1 mg of the digested sample were lyophilized and stored at -20 °C prior to use.

## 2.3 Capillary Electrophoresis

All CE and infusion experiments were performed using a PA 800 plus capillary electrophoresis (CE) system from Beckman Coulter (Brea, CA, USA), which was equipped with a temperature controlled sample tray, capillary cooling liquid and a power supply able to deliver up to 30 kV. Both neutrally coated and bare fused silica capillaries were used depending on the requirements per experiment. The neutral capillary coating was a bi-layer with the outer surface consisting of polyacrylamide, currently in development by Beckman Coulter (Brea, CA, USA).

The BGE and LE consisted of 10% acetic acid and ammonium acetate (pH = 4 and various ionic strengths) respectively. Injection volumes were calculated using the Poiseuille equation and a fluid viscosity of 1.04 cP.

## 2.4 CE-ESI-MS sheathless interface

The detection end of the separation capillary was etched using hydrofluoric acid, creating a porous section of approximately 3 cm following a previously described method[26, 27]. The resulting spray tip has an outer diameter around 40  $\mu\text{m}$  without having a tapered internal diameter. The etched portion of the capillary was inserted into a stainless steel housing containing a retractable head to protect the protruding tip (about 5 mm). The stainless steel housing can be automatically filled with a conductive buffer to close the separation circuit and/or apply the ESI voltage or ground when required. A retractable head was placed around the stainless steel housing as protection of the spray tip. The complete housing was fitted in a custom mount (Beckman Coulter, Brea, CA) to fit the mass spectrometer, which included an x-y-z platform to allow for position optimization. Generally the spray tip was placed coaxially to the MS entrance at a distance ranging from 2 to 5 mm. The positioning of the tip with respect to the MS entrance was optimized by hydrodynamically and/or electrophoretically infusing a test mixture and following the response (intensity and ratio's). The produced interface presented an ability to produce stable ESI sprays at ultra-low flow rates. During long runs, to insure a good electrical contact, the conductive buffer contained in the stainless steel cylinder was continuously refreshed by applying a small pressure (1psi) on the vial containing the conductive buffer. This resulted in a consistently stable spray and current for all runs, whatever their duration.

## 2.5 nano-LC

Reverse phase separation of peptides from milk digest was performed on a Ultimate 3000 LC RSLC nano-LC system (Dionex, Sunnyvale, CA). Sample was injected onto a C18 trapping column (Acclaim PepMap100: 100  $\mu\text{m}$ ×2 cm, 5  $\mu\text{m}$ , 100 Å, Dionex). After 2 min washing with 2.0% MeCN, 0.1% FA at 300 nL/min, following valve switching, the sample was separated on a C18 nano column (Acclaim PepMap RSLC 75  $\mu\text{m}$ ×15 cm nanoViper, 2  $\mu\text{m}$  100 Å, Dionex) by a piece-linear gradient (5-40 min – 2-10%; 40-78 min – 10-30%; 78-83 min – 30-70%; 83-85 min – 70-90%; 85-90 min – 90% of mobile phase B, where B was 95% MeCN, 0.1% FA) at a constant flow rate 300 nL/min.

## 2.6 Mass Spectrometry

All initial infusion and method optimization experiments were performed on a MaXis 4G UHR-TOF mass spectrometer from Bruker Daltonics (Bremen, Germany) at 1 Hz



acquisition frequency. All tandem MS experiments were performed on an amaZon speed ETD ion trap instrument from Bruker Daltonics. MS scan was within a range of 300–1500 m/z with ion focus on 800 m/z. The ten most abundant multiple charged ions of an MS spectrum were selected for MS/MS analysis by collision-induced dissociation using helium as the collision gas with a precursor threshold of 10000 counts. In case of observation of a neutral loss fragment (26.7; 32.7; 38.6; 40.0; 49.0; 58.0; 65.0; 80.0; 98.0 Da) the multistage activation of this fragment was automatically triggered (fragment only mode). The masses corresponding to the fragmented ions were dynamically excluded for 0.1 min from further MS<sub>n</sub> analysis in the nano-LC-MS method. No exclusion was applied in the CE-MS strategy due to strongly varying peakwidths throughout the separation.

For the coupling of the sheathless CE sprayer to the mass spectrometer, a specially designed sprayer mount in combination with the Bruker nano spray shield was used. Generally, stable spray for positive ionization was achieved between -750 and -1500 V ESI Voltage, which was dependent on the distance between the sprayer tip and the MS entrance. Drying gas was set to 2 L/min (nitrogen) while the source temperature was set to 180 °C.

The coupling of the nano-LC to the ion trap mass spectrometer was performed via a Bruker CaptiveSpray ionization (CSI) source (Bremen, Germany). Stable spray for positive ionization was obtained at -1300 V ESI Voltage with the source temperature at 150 °C and the drying gas (nitrogen) set to 3 L/min.

## **2.7 Viscosity and flow rate measurement.**

Generally, ESI voltage may cause an ultra-low flow in bare fused capillaries due to the combination of ESI suction and EOF in the spray tip during infusion experiments. Therefore, the flow rates at ultra-low pressures needed to be accurately determined for the infusion experiments performed in bare fused capillaries. A 30 µm i.d. × 150 µm o.d. × 100 cm bare fused capillary with a porous spray tip was filled hydrodynamically with BGE. The non-phosphorylated synthetic peptide dissolved in BGE was then continuously introduced hydrodynamically into the capillary while the ESI voltage was applied to the sprayer until the peptide was detected by the mass spectrometer. The absolute flow rate was determined by the capillary volume divided by the mobilization time. As some diffusion takes place the mobilization time was taken to be the time at half height between the moment the peptide was detected and the maximum signal intensity.

The combined ESI suction and EOF effect reduced exponentially at increased pressure; therefore the effect became negligible above 10 nL/min flow rates (1.4 psi pressure for infusion experiments). All higher flow rates were calculated from the BGE viscosity, which was measured with a previously reported method[29]. Briefly, in a CE-UV configuration, the capillary was filled with the studied BGE. Subsequently a short water plug (< 1% of total capillary volume) was hydrodynamically introduced in the capillary. The zone was then mobilized hydrodynamically until detection of the water plug by UV. Viscosity ( $\eta$ ) was calculated from the mobilization time applying the Hagen- Poiseuille law:

$$\eta = dc^2\Delta Pt/(32L^2) \quad (1)$$

Where  $dc$  is the internal capillary diameter,  $\Delta P$  the mobilization pressure,  $t$  the mobilization time, and  $L$  the length of the capillary. The flow rates were then calculated from the Poiseuille equation (2) taking the applied pressure and the experimentally determined BGE viscosity into account.

$$V = (\pi/128)dc^4((\Delta Pt)/(\eta L)) \quad (2)$$

## 2.8 Data analysis

Peak lists were generated from the raw spectra files using ESI Compass for amaZon 1.3 Data Analysis V4 SP4 (Bruker Daltonics, Bremen, Germany) with an autoMSn method allowing 2000 compounds with an intensity threshold of 1000 counts and a 0.5 min retention time window, and exported as Mascot Generic Files (MGF). These files were searched against the bovine protein database (containing 27254 records) using the Mascot search algorithm (Matrix Science). The parameters of the search were: fixed modifications – methylthio(C); variable modifications – oxidation(M), phospho (STY); trypsin missed cleavages – 2; MS tolerance (with # 13C=1) - 0.5 Da; MS/MS tolerance - 0.5 Da.

## 3 Results and Discussion

### 3.1 Characterization of ionization behavior of phosphopeptides at ultra-low flow rates

In phosphoproteomics the frequency of incidence of protein phosphorylation defines the phosphopeptide concentrations in a conventional protein digest. Moreover, playing a key regulatory function in intracellular signaling these phosphopeptides are short lived and far from abundant. Consequently, the presence of very high concentrations of non-phosphorylated peptides can hamper phosphopeptide analysis due to co-elution or co-migration and subsequent ion-suppression under ESI conditions. Although the obvious solution for the reduction of ion-suppression of phosphopeptides is improved separation power, the generally low ionization efficiency of multiply phosphorylated peptides, even under non-suppressed conditions, is problematic. In previous investigations of the ESI process,[13, 14, 26, 30] it was shown that the application of ultra-low flow rates in nano-ESI can have significant advantages with regard to ion suppression effects and ionization efficiencies. For this reason it was important to investigate the effects of strongly reduced flow (<30 nL/min) on the ionization efficiency of peptides with extensively varying phosphorylation states.

To this end, a synthetic peptide containing four tyrosine moieties for the incorporation of zero to four phosphorylations was designed to serve as a model in a low flow infusion study of the phosphopeptide ionization process. The C-terminal amino acid was chosen to be an arginine to emulate the characteristics of peptides resulting from tryptic digestion, which is currently the most commonly used protein digestion strategy. These peptides were dissolved as a mix in BGE (10% acetic acid) in equimolar concentrations before direct hydrodynamically driven infusion. An initial infusion experiment showed a clear change in signal profile between infusion at high flow rates (>100 nL/min) and low flow rate (~10 nL/min) (Figure 1-1) warranting further investigation.

To track the changes in relative ionization for the model peptides, an infusion experiment was designed and performed as follows. After hydrodynamic filling of the capillary the pressure was varied from 0.2 psi to 60 psi in steps of 0.2 to 10 psi with continuous MS detection. At each tested flowrate, the ESI voltage was optimized for spray stability and for the highest summed signal intensity of the  $[M+2H]^{2+}$  and  $[M+3H]^{3+}$  ions of the non-phosphorylated model peptide. Although each pressure was applied for two minutes, only 1.8 minute intervals were integrated for data analysis, allowing 0.2 minutes interval

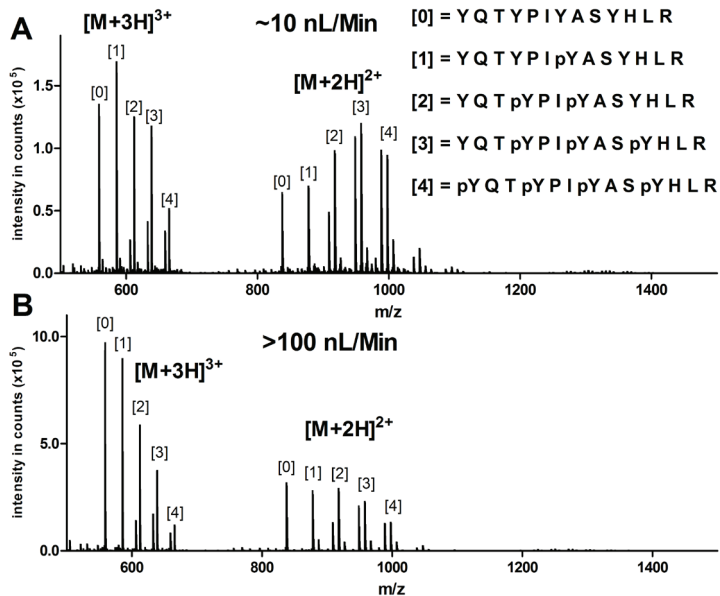


Figure 1-1: Typical mass spectra of the model phosphopeptide mix infusion at flows below 10 nL/min and above 100 nL/min. Only double and triple charged species are observed and the peaks are labeled with a number corresponding to their number of phosphorylations. Additional masses can be observed corresponding to a -18 Da mass difference from the di- to tetra-phospho form. These masses are due to impurities after synthesis.

after pressure increase for spray stabilization. The peak intensities of the  $(M+2H)^{2+}$  and  $(M+3H)^{3+}$  were summed and thus reported as the total signal at each flow rate for each respective peptide. For all 5 infused peptides a stable signal could be observed within the two minute windows. Investigating the signal intensities at flow rates below 100 nL/min (Figure 1-2A), it could be discerned that the evolution of the signal intensity as a function of the flow rate was not equal for all phosphopeptides. To better assess differences in behavior for each phosphopeptide present in the mixture, the ratio of its intensity with respect to the non-phosphorylated was plotted (Figure 1-2B). The ratio plot clearly shows that the ionization bias observed at conventional flow rates ( $> 50$  nL/min) is significantly reduced at ultra-low flow rates. If only the most extreme case corresponding to the tetra-phosphorylated peptide is considered, it can be observed that its intensity only corresponds to less than 20% of the one of the non-phosphorylated peptide at flow rates above 50 nL/min while it accounts for more than 60% below 10 nL/min. Keeping in mind the work of Busnel et al.[26] and Marginean et al. [14] who studied ionization efficiency at ultra-low flow rates, it was shown that the detection sensitivity for all phosphorylated peptides is strongly enhanced when working at flow rates below 30 nL/min. The respective increase is dependent on the number of phosphorylation

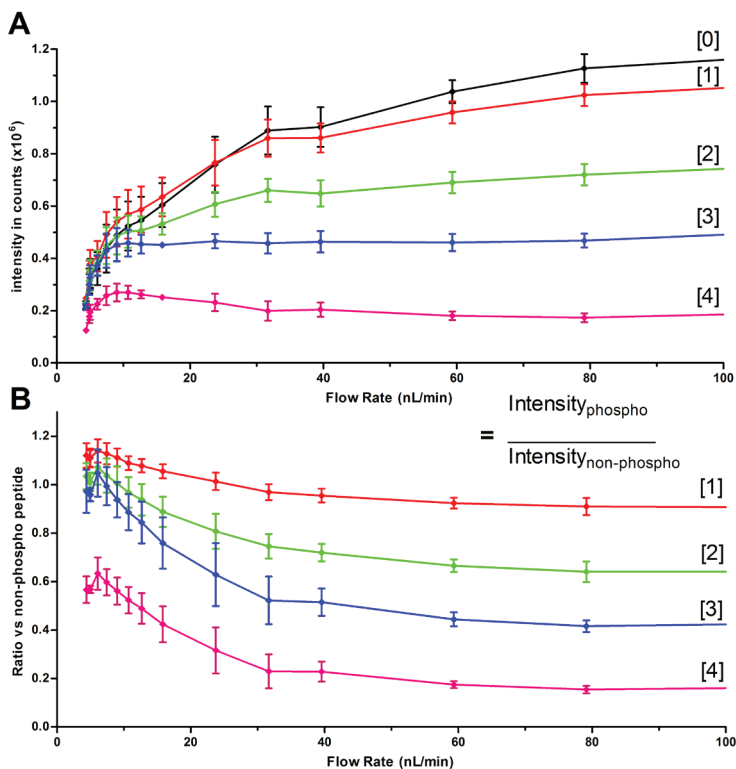


Figure 1-2: (A) Evolution of peak intensity of model phosphopeptides below 100 nL/min; (B) Signal intensity ratio as a function of the non-phosphorylated peptide. Experimental conditions: Bare fused silica capillary with porous tip, total length 30  $\mu\text{m}$  i.d.  $\times$  150  $\mu\text{m}$  o.d.  $\times$  100 cm; infusion of phosphopeptide mix at equimolar 5  $\mu\text{M}$  concentration in 10% acetic acid. Mass spectrometry; capillary voltage was optimized for each flowrate (-1050 to -1275 V); detection range 300-2900 m/z, other experimental conditions described in Materials and Methods. (Black) non-phosphorylated [0], (Red) mono-phosphorylated [1], (green) di-phosphorylated [2], (Blue) tri-phosphorylated [3], (Pink) tetra-phosphorylated [4]

sites and therefore the largest increase in relative sensitivity was found for the tetra-phosphopeptide (Factor 4). This confirms, as previously shown by Schmidt et al. [13], the existence of an ESI regime (flow rate region below 30 nL/min) where common ionization bias can be reduced to such a large extent that the MS detection shows a trend toward an equimolar response[31]. Although the peptides were infused in equal concentration, the signal intensity of the tetra-phosphorylated peptide did not equal that of the other four model peptides. Nevertheless, it experienced the greatest increase in relative signal intensity.

After having assessed with an infusion-based approach the potential impact of lowering the flow rate on the ionization efficiency of different phosphopeptides, the next step was to understand to which extent we could take advantage of this behavior in an ultra-low flow separation system.

## 3.2 Neutral capillary coating in sheathless tITP-ESI-MS

### 3.2.1 Optimization of analyte stacking

Our infusion experiments demonstrated phenomena, which are of particular importance for the analysis of phosphopeptides. To improve total peptide coverage in a complex sample, however, an analytical strategy which combines ESI under ultra-low flow with an efficient separation technique has to be found. Regrettably, there are just a few separation techniques which are compatible with the flow rates required to achieve close-to-equimolar ionization (<10 nL/min). Although nano-splitting has been applied in some studies to achieve these very low flow rates in liquid chromatography[32], this is a far from common practice as it can easily result in band broadening and additional/unwanted dead volumes. Porous layer open tubular (PLOT) columns, for example, can efficiently operate at flows rates of a similar order of magnitude (~20 nL/min)[33]. Currently, these columns require in-house manufacturing as they are not commercially available, and their operation is challenging because of their extreme dimensions (10 µm ID and up to several meters long) and the nano splitting pumps required to produce these flowrates. Only CE separation using neutrally coated capillaries aided by hydrodynamic pressure produces a straightforward approach to achieve excellent separation while maintaining consistent flows below 15 nL/min.

When implementing CE in neutrally coated capillaries as the separation strategy, as opposed to the use of bare fused capillaries, no EOF is produced and the flow required for electrospray has to be produced by the application of a slight hydrodynamic pressure at the capillary inlet. Although it has now been shown that ultra-low flow ESI can result in a significant increase of sensitivity, CE, as compared to LC based techniques, has a significant limitation with regard to loadability. Additionally, as phosphorylations and therefore phosphopeptides are generally only present in complex samples at ultra-low concentrations, the use of larger volume injections would be greatly beneficial. As a rule of thumb, in traditional CZE separations, the sample plug is usually limited to about 1 % of the total capillary volume because larger sample volumes result in broad peaks and subsequently reduced resolution. To increase loadability in CE while maintaining high efficiency capabilities, transient-isotachopheresis (t-ITP) [34] and other stacking strategies have been developed. Significant increases of sensitivity can be achieved as sample volumes up to 50% of the total capillary volume can be injected while maintaining high efficiency and satisfactory resolution. Depending on the volume of the used separation capillary, varying degrees of analytical resolution and loadabilities can be achieved.

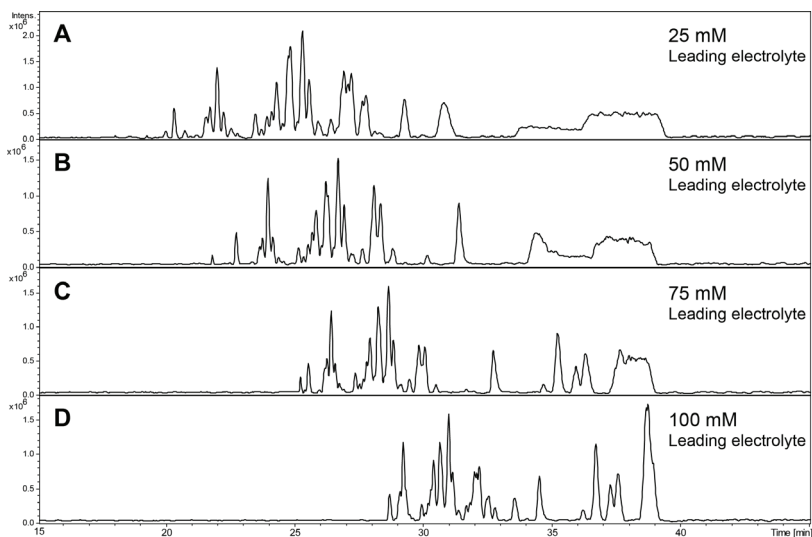


Figure 1-3: Neutral capillary t-ITP-MS analysis of 2,5 ng milk digest (37% capillary fill, 30 psi for 60 s) Base Peak electropherogram. Mass spectrometry; ESI voltage of -1050V; detection range 300-2900 m/z, other experimental conditions described in Materials and Methods (A) sample dissolved in 25 mM leading electrolyte (LE) (B) sample dissolved in 50 mM leading electrolyte (C) sample dissolved in 75 mM leading electrolyte (D) sample dissolved in 100 mM leading electrolyte

Unfortunately, the use of an ITP strategy can result in great losses in resolution. Firstly, the capillary length used to achieve electrophoretic resolution is reduced as a significant portion of the capillary is used to load the sample of interest. Secondly, the composition and concentration of leading electrolyte (LE) has a great influence on the level of stacking and the degree of resolving power in an ITP strategy. As phosphopeptides generally have a low isoelectric point due to their highly acidic phosphate moieties, it could be expected that high concentrations of LE are required for optimal stacking. To evaluate the required concentration of LE in our ultra-low-flow sheathless t-ITP-CE-ESI-MS system, a digest of bovine milk was taken as a model sample. Bovine milk is a biological sample of medium complexity with a large proportion of the protein content corresponding to caseins. These 4 caseins ( $\alpha$ S1,  $\alpha$ S2,  $\beta$  and  $\kappa$ ) have abundant phosphorylations and make bovine milk an ideal model sample to optimize phosphoproteomic workflows.

To achieve a significant loading capacity, the sample volume was set to 250 nL (37% of the total capillary volume). Figure 1-3 shows the varying levels of stacking and of analytical resolution achieved in the scope of the optimization of the stacking conditions. LE ionic strengths were varied from 25 mM to 100 mM while separation was performed with 0.8 psi of pressure applied at the inlet to hydrodynamically induce flow in the capillary to achieve a stable ESI spray. This hydrodynamic pressure was determined empirically during the infusion experiments. A separation voltage of 25 kV was chosen to achieve

optimal separation while maintaining a separation current below 6  $\mu$ A. As determined by using the last peak in the electropherogram as a marker, complete stacking was achieved at 100 mM ionic strength (Figure 1-3D). The effective separation window was reduced to only 10 minutes as a result of the high concentration of LE and the short capillary length (63%) left for actual preconcentration/separation. Although the reduced resolution is not detrimental to the ionization efficiency as the low flow ESI process has shown minimal ionization suppression for phosphopeptides, the mass spectrometric analysis of complex samples could be significantly hampered at low analytical resolution as tandem mass spectrometry of many co-eluting compounds is difficult, mainly due to technical limitations in the number of MS<sub>n</sub> events per time unit.

### **3.2.2 Tandem mass spectrometry identification of phosphopeptides with t-ITP separation followed by sheathless-CZE-MS**

The above described separation strategy was applied to analyze the bovine milk digest sample. To further assess the potential of the ultra-low flow CE-ESI-MS method for the analysis of phosphopeptides, 5 ng of milk digest was loaded on both CE and nano-LC systems and the results obtained from a merge of 4 technical replicates with both approaches were compared. The mass spectrometric method was optimized for phosphoproteomic fragmentation which included the secondary fragmentation of any neutral loss ion related to a phosphopeptide. The fragmentation of this neutral loss compound does not result in a full MS<sub>3</sub> spectrum but in a cumulative MS<sub>2</sub> plus MS<sub>3</sub> spectrum. As both CE and LC do not appear to provide resolution for peptides with the same peptide backbone but with varying phosphorylated sites, peptides identified by the MASCOT search with equal backbones but varying phosphorylated sites were assigned as one unique peptide. Table 1-1 clearly shows the advantages of the CE-MS phosphoproteomic strategy with regard to minute sample amounts as 13 phosphopeptides could be determined, including di-phosphorylated peptides while only 5 phosphorylated peptides were identified by the nano-LC based approach.

Although the absolute sensitivity of the CE-MS strategy is undeniable, an argument can be made for the capability of the nano-LC system to load larger amounts of sample onto the system. In this context, a larger amount of material (50 ng) was additionally loaded on the nano-LC-MS system (2.5  $\mu$ L of a sample at 20 ng/ $\mu$ L). The merged data of 4 technical replicates of this significantly higher sample load only yielded four additional phosphopeptides as compared to the 5 ng sample load, while not resulting in detection of multiply phosphorylated species. Although only three phosphopeptides detected by



analysis characteristics	no. of phosphopeptides	1P	2P	unique phosphopeptides
5 ng of milk digest tITP-MS	12	10	2	5
5 ng of milk digest nano-LC-MS	5	5	0	1
50 ng of milk digest nano-LC-MS	9	9	0	3

Table 1-1: Comparison of the detected phosphopeptides from a bovine milk digest. The t-ITP-MS method consisted of 0.8 psi pressure for 60 min (6.7 nL/min) with 25 kV separation and 1100 V ESI voltage. The nano-LC-MS method consisted of a 300 nL/min water/acetonitrile gradient for 90 min. Other experimental conditions are described in the Materials and Methods. The peptide numbers are a result of a merge of multiple analyses of the same sample (n = 4). Only phosphopeptides with a peptide score above 25 were included in the table.

nano-LC-MS were unique for their separation technique, it does indicate some form of complementarity to the two techniques.

The difference in phosphopeptide detection between CE-MS and nano-LC-MS can certainly be explained by an accumulation of multiple factors. Naturally, the previously discussed increase in ionization efficiency at low flowrates is of great influence. Secondly, the nature of the separation strategy could be a contributing factor as number of the detected peptides are highly polar and therefore interact only poorly with the used stationary phases. Subsequently, it is indeed possible that a portion of the peptides detected by CE-MS were not retained on the trap-column before the chromatographic separation. As the CE-MS strategy did not contain a trapping protocol, all compounds in the sample had indeed the potential to reach the detector.

Reversely, while the ionization process in sheathless CE-ESI-MS at ultra-low flow rates is very favorable to the MS detection of multi-phosphorylated peptides, it is also very likely that a number of phosphopeptides remain undetermined due to the intrinsic nature of the CE separation mechanism. As highly phosphorylated peptides can indeed present a ultra-low pI, they can be negatively charged, even at pH 2.2, and therefore migrate toward the inlet of the capillary rather than toward the MS. As a consequence, it is likely that a portion of the phosphoproteome was missed by the considered CE-based analytical strategy. Consequently, a complementary CE strategy to the one presented here will have to be developed to achieve comprehensive phosphoproteomic analysis, if CE is to be used as the standard method in future investigations.

## 4 Conclusions

In this study, the potential of ultra-low flow CE-ESI-MS was investigated for phosphoproteomic analysis. The influence of the ESI flow rate on the ionization efficiency of a number of synthetic peptides showed a significant increase in phosphopeptide ionization at ultra-low flow rates. The flowrate could be decreased to such an extent that a near equimolar ESI response was approached for flowrates below 15 nL/min.

Applying the knowledge of ultra-low flow ESI and combining it with CE capabilities, a sheathless t-ITP-CE-ESI-MS strategy was developed using an ammonium acetate buffer at pH 4.0 as a leading electrolyte. Although the nature of the stacking process reduces the electrophoretic resolution compared to conventional CZE, unprecedented sensitivities could be achieved in the detection of phosphopeptides. When compared to nano-LC-MS, the proposed strategy was superior in both absolute (equal sample amount loaded) and concentration sensitivity (equal sample concentration). The developed CE-MS strategy was able to identify 2 multi-phosphorylated peptides from the sample whereas the nano-LC-MS was only able to identify mono-phosphorylated peptides, even in the high sample load analysis. Moreover, the use of ultra-low flow ionization also shows improved ionization of pSerine peptides as the doubly phosphorylated peptides (supplementary information) were both pSerine peptides, the pSer and pThr moieties are more labile than the investigated pTyr. Therefore, the stability of pSer and pThr at ultra-low flow ionization need to be investigated when considering it as a strategy in quantitative phosphoproteomics.

In conclusion it was shown that ultra-low flow ESI greatly increases the detection sensitivity for multi-phosphorylated peptides in mass spectrometric analysis and that this feature can be translated into a sheathless CE-ESI-MS platforms strategy. Although a sheathless CE-ESI-MS strategy was applied to achieve separation at the required flowrates (<10 nL/min), in principle any liquid based separation strategy (also LC or CEC) could be applied in concert with ultra-low flow ESI. As Schmidt et al.[13] have shown that the observed effect was also present for a 50% methanol solution it can be concluded that ultra-low flow ESI should also be greatly beneficial to conventional RP-LC-MS strategies using organic modifier based separation.

As this report was a proof of principle investigation for mass spectrometric analysis in phosphoproteomics, no sample pretreatment and preconcentration techniques were investigated for further improvement of phosphoproteomics analysis. Therefore, ultra-

low flow LC separation techniques sample pretreatment techniques that are compatible with the applied CE-MS strategy should be the target of future investigation for improved phosphoproteomic work flows.

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