# Compact Quantitative Proteomics Workflow Combining SILAC Labeling, Chromatographic Pre-fractionation and CESI-MS with a Neutral Capillary Surface

- ***Fully automated reverse-phase chromatography fractionation and CESI-MS analyses***
- ***Increased coverage of modified peptides without sample enrichment***
- ***Reduced workload due to the single in-solution digestion***
- ***Ultralow sample consumption (c.a. 40nL) per analysis***

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## Overview

Capillary electrophoresis coupled with mass spectrometry is a powerful combination of a high-performance liquid phase separation technique and a versatile detection method, providing excellent selectivity, high sensitivity and structural information. CESI is the combination of electrospray ionization (ESI) with capillary electrophoresis (CE) in a single dynamic process. In this work, an ultra-low flow CESI approach in combination with reversed-phase liquid chromatography pre-fractionation was applied for quantitative proteomics. Proteins were extracted from SILAC (stable isotope labeling by amino acids in cell culture) labeled and unlabeled yeast strains, mixed and enzymatically digested in solution. The resulting peptides were pre-fractionated using chromatography and the fractions were analyzed by CESI-MS using a neutral surface capillary. A total of 28,538 peptides were identified corresponding to 3,272 quantified proteins. CESI-MS measurement was performed under ultra-low flow conditions (<10 nL/min) to obtain the highest separation efficiency with the neutral surface capillary. The CESI-MS approach applied also proved to be a powerful method for identification of low-abundance modified peptides within the same sample without the need for further enrichment. Using the CESI-MS approach, 1,371 phosphopeptides were successfully identified, 49 of which were found to be differentially regulated in the 2 yeast strains. Apart from the 33,854 unique peptides found using this method, 8,106 acetylated, phosphorylated, deamidated or oxidized peptide forms were also identified. This technical note is based on previously published results.[1]

## Introduction

Quantitative proteomics recently has gained a high level of interest and is considered an essential tool in molecular biology and biomedical sciences. This trend has been facilitated by the rapid development of high-resolution mass spectrometers enabling fast and sensitive identification of proteins relevant to biological processes. The basic workflow of quantitative proteomics using a technique such as SILAC comprises the following steps: (i) stable isotope labeling of proteins or peptides; (ii) enzymatic digestion of these proteins into peptides; (iii) separation of peptides and (iv) mass spectrometry detection and analysis. In most instances, a multi-dimensional separation strategy is included in the proteomic workflows because of the large number of proteins and cleaved peptides in the sample. The most commonly used techniques in the first dimension are sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), off-gel isoelectric focusing (IEF) and LC-based strategies such as ion exchange, hydrophilic interaction (HILIC) or reverse-phase chromatography.[2-5] The latter is mostly used in acidic separation conditions as a second separation dimension. Benefits of these methods include MS compatibility with the solvents used for separation, and the ability to easily tune the separation conditions to deal with the complexity of the sample and the scan speed of the MS instrument. However, this method is less suitable for hydrophilic peptides, which can be lost during the pre-column wash step, and phosphopeptides, which may undergo ion suppression due to co-eluting peptides.[6,7] In order to

avoid these problems, efforts have been made to couple capillary electrophoresis (CE) with mass spectrometry to utilize the proven high separation efficiency of CE for the separation of modified proteins and peptides of any size.[8-15]

Over the last decades, numerous interfaces have been designed and developed to enable efficient CE-MS coupling.[16-17] The sheath flow interface enables ESI voltage contact through a constant flow of sheath liquid applied hydrodynamically or electrokinetically.[18-20] A modified version of this interface is the "liquid junction."[21] Sheathless interfaces usually apply a steel needle at the terminus of the separation capillary to assure closure of the electric circuit for both the CE and the ESI processes[22] using conductive materials to coat the emitter tip.[23, 24] The flow rate toward the MS unit is influenced by the electroosmotic flow and/or the applied pressure in the system. CESI represents an advanced version of sheathless sprayers, utilizing a separation capillary with a porous tip acting as a nanospray tip.[25] The main advantage of using CESI is the capability to operate at low nanoliter flow rates (<10 nL/min) resulting in decreased ion suppression and overall improved sensitivity.[26-28] In analogous work, CESI-MS was successfully applied for discovery of post-translational modifications on antibodies and histones with high sensitivity.[29-33]

In this study, CESI-MS resulted in the highly sensitive quantitative analysis of the yeast proteome. Extracted proteins from SILAC labeled and unlabeled yeast strains were mixed and digested enzymatically. Following digestion, the resulting peptides were first fractionated with reverse-phase chromatography and then analyzed by CESI-MS using a neutral surface capillary column. CESI-MS data were analyzed to identify any post-translational modifications, primarily phosphorylated peptides (including phosphorylation sites) and other modifications such as acetylation, deamidation and oxidation.

## Materials and methods

**Chemicals:** Dithiothreitol was purchased from Biomol (Hamburg, Germany) and iodoacetamide from GE Healthcare (Vienna, Austria). Yeast growth media was from Sunrise Science Products (CSM−His, −Arg, −Lys). $^{13}C_6$ $^{15}N_2$-L-Lysine and Endoproteinase Lys-C from Lysobacter enzymogenes and all other chemicals were purchased from Sigma-Aldrich (Vienna, Austria).

**Cell culture:** MBY4 yeast strain was used (MATα leu2-3, 112 ura3-52 his3-200 trp1-901 lys2-801 suc2-9, vps4:TRP1)1 for all experiments. Isogenic yeast ESCRT mutants (vps4Δ, pRS413 were compared to the corresponding wild type (WT) cells (vps4Δ, pRS413-VPS4).

**In-solution protein digestion:** Cleared cell lysates (1.5 mg of extracted yeast proteins) were TCA-precipitated and washed twice with acetone. The precipitated protein pellet was resuspended in ammonium bicarbonate (100 mM, pH 8.0). Proteins were reduced with dithiothreitol (5 mM) at 56 °C for 30 min and alkylated with iodoacetamide (18 mM) at room temperature for 20 min. Proteins were digested overnight at 37 °C by adding Lys-C at 1:75 ratio (protease/protein).

**Reverse-phase chromatography fractionation:** In-solution digested peptides were loaded on a Beckman Gold HPLC system (Beckman Coulter, Brea, CA) and fractionated by reverse-phase chromatography using an EC 250/4.6 Nucleosil 120-3 µm C18 column (Machery-Nagel, Düren, Germany). Digested yeast proteins (1.4 mg) were eluted within 2 h using a constant flow rate of 0.5 mL/min. Eluents were 0.1% trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in 85% acetonitrile (solvent B). The gradient started at 4% solvent B for 14.5 min and increased to 60% solvent B in 90 min, up to 100% B in 4 min, and was held at 100% B for 11.5 min.

**Fraction collection:** Collection started 5 min after injection at 0.5 min intervals for 80 min and 1 min intervals for another 22 min. In total, 182 fractions were collected and then lyophilized and stored dry at -20 °C. Prior to capillary electrophoresis, the peptides were dissolved in 15 µL of 50 mM ammonium acetate (pH 4.0).

**Capillary electrophoresis:** The CESI 8000 Plus High Performance Separation-ESI Module (SCIEX, Brea, CA) was used with a 100 cm long 30 µm i.d. (150 µm o.d.) neutral surface capillary with an integrated 3 cm long porous tip, serving both as separation capillary and electrospray emitter. The CESI capillary was coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, San Jose, CA) by inserting the porous segment into the sprayer interface.[1] A second capillary was used to supply the sprayer housing with conductive liquid in order to provide electrical contact. 10% (v/v) acetic acid was used both as background electrolyte (BGE) and conductive liquid for the emitter. Prior to capillary electrophoresis, both the separation and the conductive liquid capillaries were rinsed with fresh buffer. The sample was introduced by applying 5 psi pressure for 50 s (40 nL injection volume), followed by a plug of BGE (5 psi for 5 s). The applied electric voltage was +30 kV with a simultaneous pressure of 1 psi for 60 min at the capillary inlet resulting in an approximate flow rate of 10 nL/min.

## Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

**Mass spectrometry:** The mass spectrometer (LTQ Orbitrap XL, Thermo Scientific) was used in data-dependent mode to switch between MS and MS/MS acquisition. Survey full scan MS spectra were acquired in the Orbitrap with a resolution of R = 60,000 (at m/z = 400) in profile mode after accumulation to an automated gain control (AGC) target value of $1 \times 10^6$ in the linear ion trap. MS/MS spectra were obtained in the linear ion trap (LTQ) using collision induced dissociation (CID). The 6 most intense precursors were sequentially selected for MS/MS fragmentation. Parameters applied for fragmentation were as follows: minimum signal required 1000; isolation width (m/z) 2.0; activation time 30 ms; normalized collision energy 35.0 and activation Q of 0.250. MS/MS spectra were acquired in centroid mode with an AGC target value of $1 \times 10^4$ and 100 ms maximum ionization time, respectively. Dynamic exclusion was set to 15 s.

**Data analysis and quantification:** Proteome Discoverer version 1.4.0.288 (Thermo Scientific) and MaxQuant version 1.3.0.5 were used for data analysis. Raw data obtained by CESI-MS were searched against a yeast ORF database downloaded from the SGD Saccharomyces Genome Database (www.yeastgenome.org; 6,627 entries, last modified February 3, 2011).

## Results

The aim of this study was to test the application of ultra-low flow capillary electrophoresis mass spectrometry coupling for SILAC-based quantitative proteomics with a special focus on specific post-translational modifications. Protein extracts of 2 isogenic yeast strains (a heavy-lysine labeled wild-type and a non-labeled mutant) were mixed in a 1:1 ratio and enzymatically digested by Lys-C in solution. The resulting peptides were then fractionated by reverse-phase chromatography. To circumvent losing the hydrophilic peptides, which otherwise poorly interact with the reverse-phase material, the separation was initiated in low organic isocratic mode (3% acetonitrile) and continued by gradient elution up to 85% acetonitrile with a total separation time of 120 min. Highest UV absorbance was observed between 55 and 70 min, suggesting that these fractions contained the greatest number of peptides. The collected 182 fractions were then analyzed with CESI-MS employing a neutral surface capillary column. 40 nL sample was introduced into the capillary, corresponding to ~6% of the total column volume. Please note that re-dissolving the LC fractions in 15 µL and using 2 µL aliquots, 325 injections could be performed with 40 nL injection volumes.

### *CESI−MS analysis of the yeast proteome*

CESI-MS analysis of all 182 fractions with subsequent database search using the Proteome Discoverer software was performed and resulted in 33,656 identified peptides (modified forms not included). Of these peptides, 28,536 were quantified, corresponding to ~85% quantification rate. The remaining non-quantified 5,120 peptides included: 2,254 (44%) peptides with no lysine in their sequence (837 C-terminal and 1,417 non-specifically cleaved peptides); 1,124 (22%) peptides with non-unique protein sequence and 1,742 (34%) peptides, which were not quantified due to low signal intensity or overlapping peptide isotopic distributions.

The largest number of peptides was found in fractions 55–150 and approximately 86% of all quantified peptides were in these fractions. Hydrophilic peptides contributed approximately 7% (fractions 1–54), and very hydrophobic peptides accounted for approximately 8% (> fraction No 150). In addition, 3,429 proteins were identified with at least 2 unique peptides and 3,272 proteins were quantified with at least 2 unique peptides and 2 peptide H/L ratios.

High efficiency was observed during the RP chromatographic separation of early and middle eluting peptide fractions, while slightly reduced separation efficiency was obtained for hydrophobic peptides in the later fractions. In total, 55% of all peptides were quantified in a single fraction and 29% in 2 other reactions, which indicated excellent separation efficiency. Only 5% of peptides, mainly hydrophobic, were quantified in more than 5 fractions. The total time required to analyze all 182 fractions was 215 h (the MS data acquisition time was 182 h). The proteins identified with absolute cellular protein abundances were compared to literature data,[35] and the CESI-MS approach identified nearly all high-abundance proteins (>$10^4$ copies per cell), as shown in Figure 1. Also, a large number of medium- and low-abundance proteins (<$10^4$ copies per cell) were identified. Please note that CESI-MS was even able to identify very low-abundance proteins. These results clearly indicate that the CESI-MS approach is quite powerful for high sensitivity protein identification. These results also highlight the multidimensional fashion of chromatographic pre-fractionation and CESI-MS analysis.

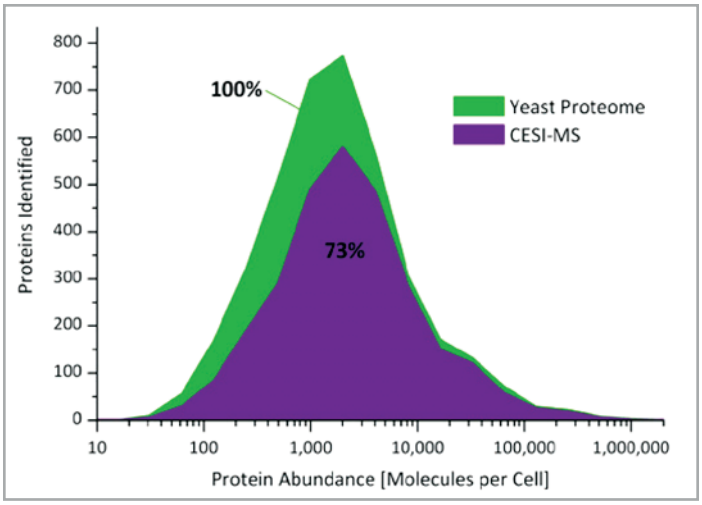


**Figure 1.** The ability of CESI-MS to identify proteins at various abundance levels.

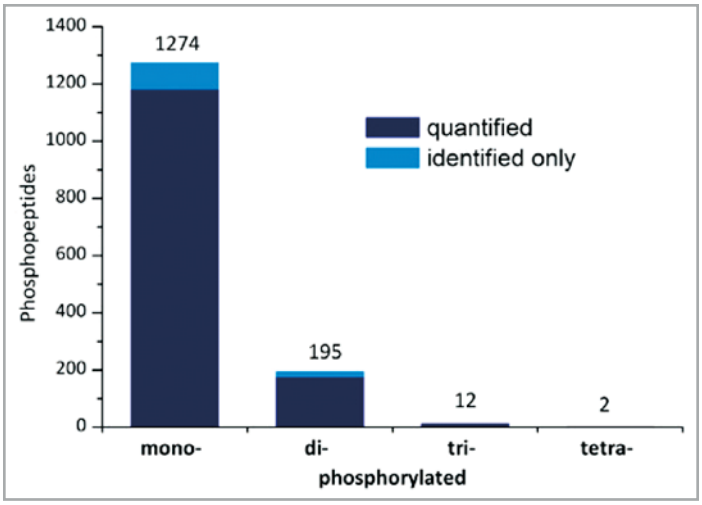


**Figure 2.** Quantification of phosphopeptides by CESI-MS.

## *Analysis of phosphopeptides*

Additional database searches of the obtained mass spectra were performed using 3 different search engines (Sequest, Mascot and Andromeda) to identify phosphorylation levels. As a result, 1,483 phosphorylated peptides, identified by at least 2 of the search programs, were selected for further investigations. As depicted in Figure 2, detailed data analysis revealed the presence of 1,274 mono-, 195 di-, 12 tri- and 2 tetra-phosphorylated peptides with a total of 1,371 peptides quantified. 1,127 modification sites were assigned with >95% accuracy according to localization scores calculated with Proteome Discoverer and MaxQuant software. The number of phosphopeptides identified by CESI-MS was rather high, especially bearing in mind that no enrichment strategy was used. This phenomenon can be explained by the greatly reduced ion suppression inherent to CESI at very low flow rate conditions of 10 nL/min. On the other hand, phosphopeptides migrated significantly slower than most of the regular peptides present in the fractions because of their reduced net charge at the pH of the background electrolyte.

Light/heavy (L/H) ratios of phosphopeptides, the proteins corresponding to them and the change in phosphorylation level are depicted in Figure 3. According to the results, 50 peptides were found to be variably abundant in heavy and light labeled yeast strains, and 16 phosphopeptides were not significantly regulated when their expression level was corrected by the corresponding protein expression. On the other hand, an additional set of 15 peptides became significantly regulated for the same reason.

## *Analysis of other post translational modifications*

The CESI-MS data sets were also searched for the presence of PTMs such as acetylated, deamidated and oxidized peptides. This additional database search revealed the existence of 6,623 modified peptides (Figure 4). A large number of peptides was found containing 1 (3,860) and 2 (403) deamidated asparagines. Moreover, 900 proteins were found to be co-translationally and 153 peptides post-translationally acetylated on N-terminal and lysine residues, respectively. Please note that acetylation of these amino groups lowers the net charge of these peptides causing lower electrophoretic mobility. Consequently, most of these acetylated peptides appeared at the higher migration time range of >30 min, similar to that of phosphopeptides, as shown in Figure 5. The separation of acetylated and phosphorylated peptides from their non-modified counterparts at a region where only a few solute molecules migrate enables high sensitivity identification of even very low abundant peptides.

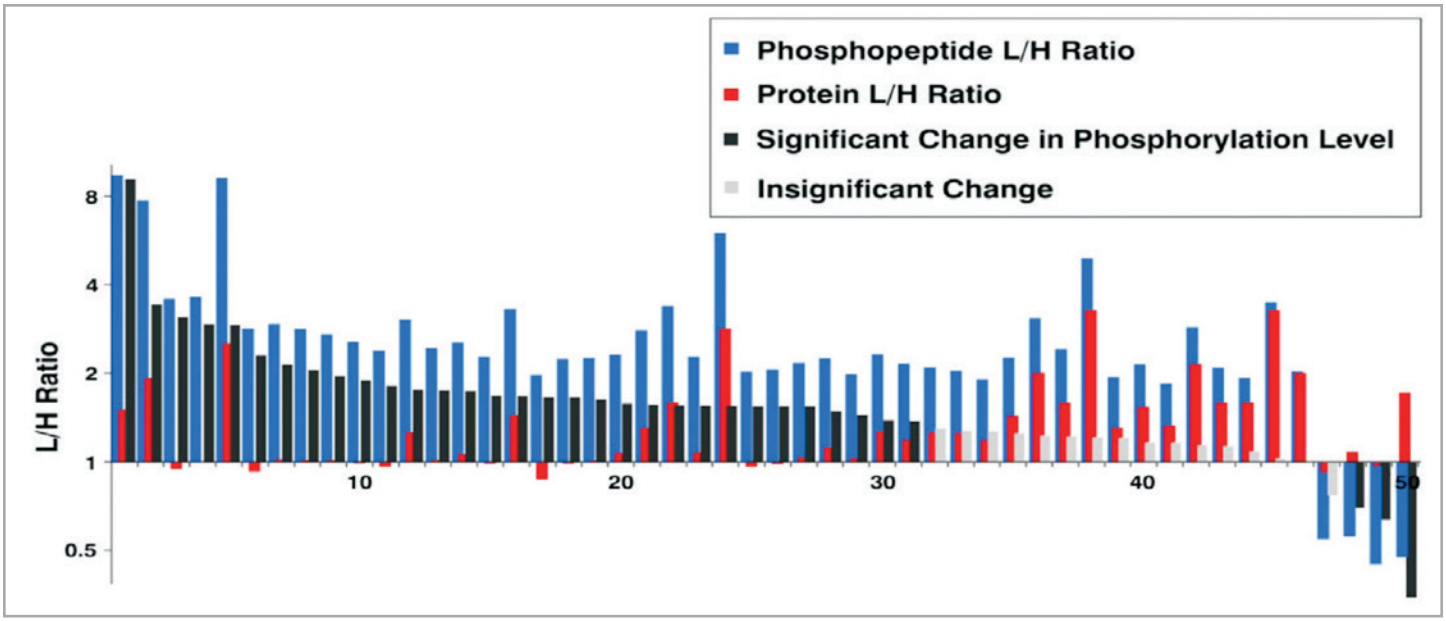


**Figure 3.** Light/heavy (L/H) ratios of phosphopeptides, the proteins corresponding to them and the change in phosphorylation level.

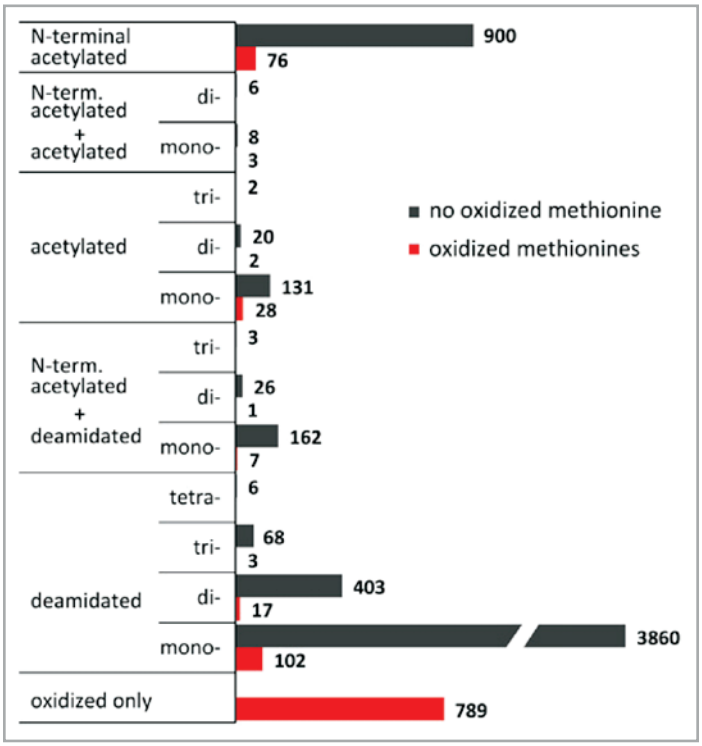


**Figure 4.** Modified peptides (except phosphopeptides) found in the CESI-MS data set of the 182 LC fractions analyzed.

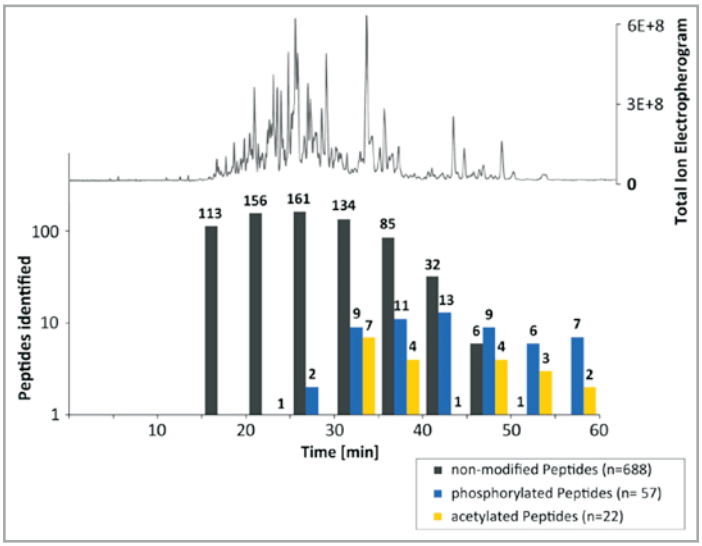


**Figure 5.** CESI-MS separation of peptides also showing the intensity and the particular time of quantification in chromatography fraction. Black bars, 688 non-modified peptides; yellow bars, 22 acetylated peptides; blue bars, 57 phosphorylated peptides.

## Conclusions

A novel proteomic analysis strategy combining reverse-phase chromatography pre-fractionation and ultra-low flow CESI-MS analysis for relative quantification of SILAC labeled yeast strains has been presented. A very large number of phosphopeptides and other modified peptides (e.g., acetylated, deaminated, etc.) were also identified and quantified without the need for any sample enrichment strategies.

Some of the substantial benefits this novel approach offers are as follows:

1. The workload of the method is strongly reduced due to the single in-solution digestion step.
2. Both the reverse-phase chromatography and CESI-MS analyses steps were fully automated.
3. Chromatography pre-fractionation helped to decrease the complexity of the sample for subsequent CESI-MS analysis without the need for any additional sample cleanup.
4. Because of the small sample consumption of CESI-MS (c.a. 40 nL), the samples can be easily reanalyzed or stored for later use.

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