# Sensitive Identification of Phosphopeptides in Brain Tissue using 2D-NanoLC-ESI-MS$^n$

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## Introduction

One of the most important post-translational modifications is phosphorylation of serine, threonine or tyrosine residues. Detection of phosphorylation sites by mass spectrometry in proteins extracted from biological material is complicated by low abundance, low stoichiometry, and poor ionization of phosphopeptides [1]. In this work, a biocompatible nano liquid chromatography (LC) system, Ettan™ MDLC, was used for separating tryptic peptides from brain tissue by cation exchange (SCX) to enrich the phosphopeptides followed by reversed-phase chromatography (RPC). The phosphopeptides were detected by neutral loss MS.

## Methods

Mouse brain tissue was trypsin digested and analysed using Ettan MDLC (Amersham Biosciences) coupled to a Finnigan LTQ linear ion trap (Thermo ElectronCorp.). 40 µg of sample was injected onto a 2.1 * 250 mm SCX column (BioBasic, Thermo Electron) and eluted with a linear salt gradient (A: 20 mM Citric acid, 25% $CH_3CN$, B: A+ 1 M $NH_4Cl$) where fractions were collected (Fig.1). The fractions were injected onto a trap column (Zorbax, Agilent, 0.3*5 mm) and RPC separation was performed on a 0.075 * 150 mm Zorbax column (Agilent). Two sets of trap/separation columns
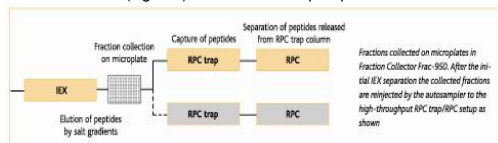


**Fig 1.** The MDLC off-line configuration.

The MS method consisted of a cycle combining one full MS scan with three MS$^2$ events (25% collision energy) followed by a MS$^3$ event (35% collision energy) that was triggered upon detection of -98, -49, or -32.7 Daltons from the precursor (neutral loss of phosphoric acid, charge states 1+, 2+, and 3+). Dynamic exclusion duration was set to 30 s. The MS$^2$ and/or MS$^3$ spectra from all the runs were searched using TurboSEQUEST protein identification software (Thermo Electron). Modifications were set to allow for the detection of oxidized Met (+16), carboxyamidomethylated Cys (+57), phosphorylated Ser, Thr, and Tyr (+80), and dehydrated Ser and Thr (−18). The peptide matches were filtered based on cross-correlation scores (Xcorr) of 1.5, 2, and 2.5 for charge states 1+, 2+, and 3+ , respectively.

1. Ficarro, S. B. *et al*. Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae. Nat. Biotechnol.* **20**, 301–305 (2002).
2. Beausoleil, S. A. *et al*. Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. USA* **101**, 12130–12135 (2004).

## Results

The neutral loss MS method was optimised for single dimension RPC separation. The detection limit was then shown to be less than 1 fmol for a phosphopeptide in a protein digest standard. In this work another separation dimension was added to the system to both increase the chromatographic resolution in the system and to concentrate the phosphopeptides [2] by SCX.
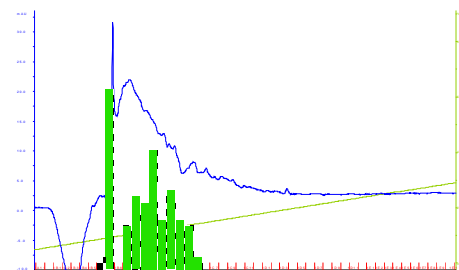


**Fig 2.** UV trace from SCX separation. The green bars indicate the relative amount of phosphopeptides that were identified in the fractions.

The SCX separation of the tryptic digest can be seen in Fig. 2. The phosphopeptides eluted between 8-17% $NH_4Cl$ and were limited to 11 fractions of totally 31 analysed. Fraction, b7, contained most of the identified phoshopeptides, namely 15 peptides. Most of the phosphopeptides were only found in one fraction which indicates that the size of the fractions (collected every 30 second) correlated well with the peak width.
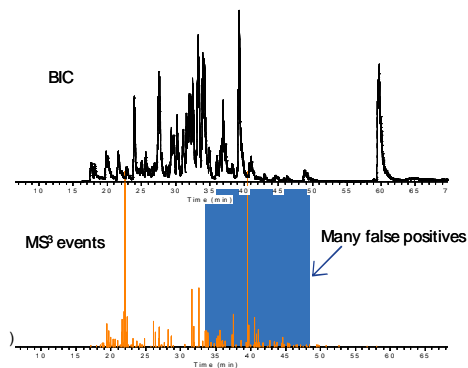


**Fig 3.** Base peak ion chromatogram from fraction b10 and all MS$^3$ events.

The TurboSEQUEST searches resulted in some false positives, often due to incorrectly assigned charge states for peptides eluting late in the RPC run, see Fig. 3. Some examples of peptides that were found to be phosphorylated in both MS$^2$ and MS$^3$ database searches are shown in Fig 4. The neutral loss ion is apparent in the MS$^2$ spectrum and the peptide is sequenced from the MS$^3$ spectrum. Some of the identified ohosphopeptides are shown in Table 1.
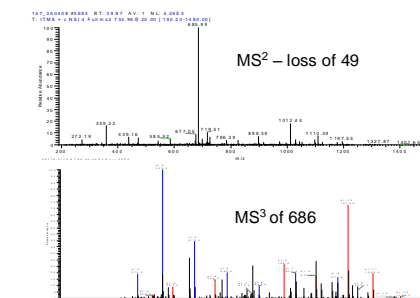


**Fig 4.** MS$^2$ and MS$^3$ spectra from ApSGQAFELILSPR (from Stathmin 1)

The developed strategy for confident analysis of phosphopeptides in complex mixtures is summarised below:

*1. 2D LC (SCX/RPC)*
*2. MS$^3$ on all peptides that loses phosphoric acid (neutral loss)*
*3. TurboSequest searches on all MS$^3$ spectra (-18@ST)*
*4. Manual confirmation of charge state and that neutral loss dominates MS/MS spectra.*
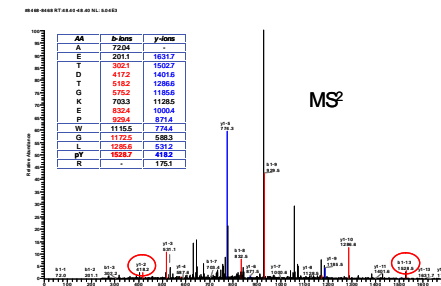*5. Further confirmation by MS$^2$ searches of +80@STY*



**Fig 5.** A tyrosine-phosphorylated peptide originating from seryl-aminoacyl-tRNA synthetase 2, Xcorr: 2.6

**Table 1.** Some of the phosphopeptides that were identified in both MS$^3$ and MS$^2$ using TurboSEQUEST.

| Protein | Sequences[a] | MH+ | Xcorr[b] | SCX fraction |
|---|---|---|---|---|
| Microtubule associated protein 1B | p**T**PEEGGYSYBSEK | 1570 | 4.3 | b6 |
| | KEpSGKEETPEVTK | 1386 | 3.4 | c2, c3 |
| | ADpSRESLKPATK | 1284 | 3.2 | c2 |
| | CYTTEKK(p)ST(p)SEAR | 1539 | 2.8 | c3 |
| Similar to Microtubule-associated protein 2 | VDHGAEK(p)DTDDK(p)ST(p)SR | 1492 | 4.3 | b10 |
| | VAIIPpTPPKSPATPK | 1558 | 3.6 | c1, c2 |
| | DKVTDG3KpSPEK | 1386 | 3.4 | c2 |
| myristoylated alanine rich protein C substrate | LSGFpSFK | 767 | 1.8 | b7 |
| | LSGFpSRKK | 895.5 | 2.9 | b11 |
| | VNGDApSPAAAEPGAK | 1337 | 3 | b7 |
| Stathmin 1, leukemia associated phosphoprotein | DLpSLEEIQK | 1057 | 2.7 | b7 |
| | ApSGQAFELILSPR | 1371 | 3.9 | b7 |
| | SKEp(p)SPDFRLpSPPK | 1510 | 4.2 | b10,b11 |
| | RApSGQAFELILSPR | 1527 | 3.9 | b11 |
| MARCKS-like protein | LSGLpSFK | 733 | 1.6 | b7 |
| | LSGLpSFKR | 896 | 2.9 | b11 |
| | AAApTPESQEPOAK | 1309 | 3 | b7 |
| | GDVTAEEAACApSPAK | 1355 | 4.3 | b7 |
| | GEVAPKEpTPK | 1038 | 3.4 | b10 |
| | GEVAPKEpTPKK | 1166 | 2.3 | c2 |
| SFB2_MOUSE Scaffold attachment factor B2 | APTAALpSPEPODSK | 1394 | 3.9 | b7 |
| Erythrocyte protein band 4.1, isoform 1 | RSEAEEGEVR(p)THp(p)TK | 1571 | 3.9 | c4 |

a) (p) refers to an uncertain phosphorylation site.
b) Xcorr lowest value 1.5, 2, 2.5 for charge states 1+, 2+, and 3+ respectively

Phosphorylated tyrosine does not lose phosphoric acid in the MS, i.e. it cannot be detected by neutral loss. Instead, the MS$^2$ spectra was studied. The sequence information is less in MS$^2$ compared to MS3 because of the lower collision energy. Despite this, some probable tyrosine-phosphorylated peptides were identified. A MS$^2$ spectra from a tyrosine-phosphorylated peptide is shown in Fig. 5.

The total number of proteins identified in the 31 fractions were approximately 5000, of which about 40 proteins (50 peptides) were found to be phosphorylated. Considering that about 10% of the proteins are phosphorylated, 500 phosphoproteins could theoretically be identified. For more identifications, prefractionation of the proteins should be performed before 2D LC on the peptides. Compared with the 1D LC method 10 times more phosphorylations were found.

## Conclusion

At least 50 phoshorylated peptides originating from 40 proteins were detected in the brain tissue with high confidence, many of the phosphorylation sites had not earlier been reported of in the lietrature.

Ten times more phosphorylated peptides were found using 2D LC compared to 1D LC and with higher confidence.

Prefractionation of the proteins will be performed to increase the number of identifications.

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