Sensitive Identification of Phosphopeptides in Brain Tissue using 2D-NanoLC-ESI-MSn

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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, EttanMDL, 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% CH3CN, B: A+ 1 M NH4Cl) where fractions were collected (Fig.1). The fractions were injected onto a trap column (Zorbax, Agilent, 0.35*5 mm) and RPC separation was performed on a 0.075 * 150 mm Zorbax column (Agilent). Two sets of trap/separation columns were used for the analytical separations for both RPC and SCX separations. The SCX separation of the tryptic digest can be seen in Fig. 2. The phosphopeptides eluted between 8-17% NH4Cl, and most of the identified phosphopeptides, 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.

The developed strategy for confident analysis of phosphopeptides in complex mixtures is summarised below: 1. 2D LC (SCX/RPC) 2. MSn on all peptides that loses phosphoric acid (neutral loss) 3. TurbosQuEST searches on all MSn spectra (18BST) 4. Manual confirmation of charge state and that neutral loss dominates MS/MS spectra. 5. Further confirmation by MS2 searches of +80@STY

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 [4] by SCX.

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

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Fig 3. Base peak ion chromatogram from fraction b10 and all MSn events.

The TurboQUEST 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 MS2 and MS3 database searches are shown in Fig. 4. The neutral loss ion is apparent in the MS3 spectrum and the peptide is sequenced from the MS2 spectrum. Some of the identified phosphopeptides are shown in Table 1.

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<th>Table 1. Some of the phosphopeptides that were identified in both MS2 and MS3 using TurboQUEST.</th>
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<td><strong>Protein</strong></td>
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Phosphorylated tyrosine does not lose phosphoric acid in the MS, i.e. it cannot be detected by neutral loss. Instead, the MS2 spectra was studied. The sequence information is less in MS2 compared to MS3 because of the lower collision energy. Despite this, some probable tyrosine-phosphorylated peptides were identified. A MS2 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 be phosphorylated. For more identifications, prefractonation 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 phosphorylated peptides originating from 40 proteins were detected in the brain tissue with high confidence, many of the phosphorylation sites had not been earlier reported in the literature.

Ten times more phosphorylated peptides were found using 2D LC compared to 1D LC and with higher confidence. Prefractonation of the proteins will be performed to increase the number of identifications.