Protein phosphorylation plays an important role in various cellular mechanisms, including signal transduction, cell cycle, and metabolism; therefore, interest in phosphoproteome analysis continues to increase. It is estimated that ~1/3 of all proteins in eukaryotes are phosphorylated. Although shotgun LC-MS/MS is a well-established technique in phosphoproteomics, the analysis of phosphopeptides is hindered by the low concentrations at which these biological compounds are present compared to their unmodified counterparts; as a result of this, there is a need for higher sensitivity and improved selectivity prior to analysis to improve detectability. Here, we report a workflow that uses a 2D RP/RP MS/MS approach for profiling the phosphoproteome of mouse brain from TiO², phosphoprotein samples, followed by nEUI-PLC/MS/MS validation for high-throughput targeted quantitation of phosphopeptides.

**METHODS**

**LC/MS Systems Conditions**
- nanoACQUITY UPLC® with 2D Technology SYNAPT™ G2-S HDMS® were used for phosphoprotein profiling.
- 1D nanoACQUITY UPLC®/XEVO™ TQ-SMS were used for targeted MRM quantitation of phosphopeptides.

**LC/MS Conditions - Phosphoproteome Profiling**
- **Resolution Mode**
  - 8 MS/MS functions
  - 50 ms MS survey scan
  - Resolution 10,000
- **DDA Acquisition**
  - First Dimension: 1D nanoACQUITY UPLC®
    - Mobile phase A: 0.1% formic acid in water
    - Mobile phase B: acetonitrile
    - Source temperature: 70 ºC
    - Cone voltage: 30 V
    - Mass Spectrometry Parameters:
      - Source temperature: 70 ºC
      - Cone voltage: 30 V
      - Mass window: 1.8 uL/min
    - Column: 180 um x 2 cm Symmetry C18 (5 um)
    - Flow rate: 2 uL/min
    - Subgradient: 5 to 45% B in 36 min. 50 min acquisition
- **Second Dimension**
  - Mobile phase A: 20 mM ammonium formate pH 10.0
  - Mobile phase B: acetonitrile
  - Source temperature: 70 ºC
  - Cone voltage: 30 V
  - Mass Spectrometry Parameters:
    - Source temperature: 70 ºC
    - Cone voltage: 30 V
    - Mass window: 1.8 uL/min
  - Column: 180 um x 2 cm Symmetry C18 (1.8 um)
  - Flow rate: 350 nL/min
- **LC/MS Conditions - Targeted MRM Quantitation**
  - Chromatographic Separation:
    - Trappeing column: 100 uL x 2 Symmetry C18 (5 um)
    - Separation column: 75 um x 15 cm I.D. T3 C18 (3.8 um)
  - Mobile phase A: 0.1% formic acid in water
  - Mobile phase B: 100% acetonitrile in water
  - Source temperature: 70 ºC
  - Cone voltage: 30 V
  - Mass Spectrometry Parameters:
    - Source temperature: 70 ºC
    - Cone voltage: 30 V
    - Mass window: 1.8 uL/min
  - Column: 180 um x 2 cm Symmetry C18 (1.8 um)
  - Flow rate: 350 nL/min
  - Subgradient: 4.7 to 9.4% ACN
  - Resolution 10,000
  - Maximum of 200 kDa
  - Nearest adjacent precursor exclusion
  - MRM transitions were predicted from the product ion mass spectrum.

**RESULTS**

The phosphoproteome of healthy mouse brain was profiled using a hybrid 2D DDA mass spectrometer with enhanced/optimized nanoACQUITY G2-S HDMS. The depth of phosphoproteome coverage was analyzed using peptide phosphosummary scores from enriched phosphopeptides identified with high confidence. The optimal time window was determined to be the 2D RP/RP separation, as well as the optimization of the fast DD parameters.

**CONCLUSION**

The suitability of this method for profiling the phosphoproteome healthy mouse brain was successfully demonstrated. 795 proteins were identified with a 0.2% false discovery rate at the peptide level, and 152 unique phosphopeptides were quantified with a 2042-fold increase of biological interest in mouse brain to be made.

**References**


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