# プロテオームサンプルからのリン酸化ペプチドのオンライン濃縮

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## INTRODUCTION

リン酸化ペプチドは一般的には二酸化チタンやIMAC吸着剤を用いてオフラインで濃縮されている。オフラインであるために自動化は簡単ではなく、そのため再現性に問題が生じる可能性がある。本研究では、オンライン希釈法を用いたリン酸化ペプチドの酸化金属による自動濃縮システムで測定を行った。オンライン希釈法と組み合わせることで、溶出バッファーのpHおよび有機溶媒濃度に関わらず、容易に効率的なトラッピングと分離を維持することができる。本システムではリン酸化ペプチドのターゲット分析を行うことも、非リン酸化ペプチドと濃縮されたリン酸化ペプチドの両方を一度に網羅的に分析することも可能である。

## METHODS

**LC/MS System**: nanoACQUITY UPLC® / SYNAPT™ G2 HDMS™

**Columns:**
**Enrichment: 180 µm x 2 cm Alumina B, 20-40 µm particles**
**Trapping: 180 µm x 2 cm Symmetry C18 (5 µm)**
**Separation: 75 µm x 15 cm HSS T3 $C_{18}$ (1.8 µm)**

**Gradient:**
**Method Optimization: 7 to 40% B in 20 min at 500 nL/min**
**Peptide Library: 5 to 40% B in 45 minutes at 500 nL/min**
**BSM Eluent A: 0.1% formic acid in water**
**BSM Eluent B: 0.1% formic acid in acetonitrile**
**ASM Eluent: 0.34% TFA in 90/10 water/acetonitrile**

**Sample Preparation, Enrichment, and Release:** Waters MassPREP™ *E.coli* and Enolase with Phosophopeptide digestion standards were utilized. All samples were prepared using the ASM Eluent. Enriched samples were released from the alumina column using either 1M glycolic acid or 2% $^{v}/_{v}$ TEA prepared in the ASM eluent.

**Online Dilution:** To maximize sample recovery on the trap column, an aqueous flow was delivered with the BSM pump and mixed with the enriched fraction prior to trapping. Typical dilution ratios ranged from 1 to 7 µL/min from the ASM combined with 17 µL/min from the BSM.

**MS Data processing :** $MS^E$ and $HDMS^E$ data were processed and searched with ProteinLynx Global Server (PLGS 2.5.2) with Identity$^E$ informatics.

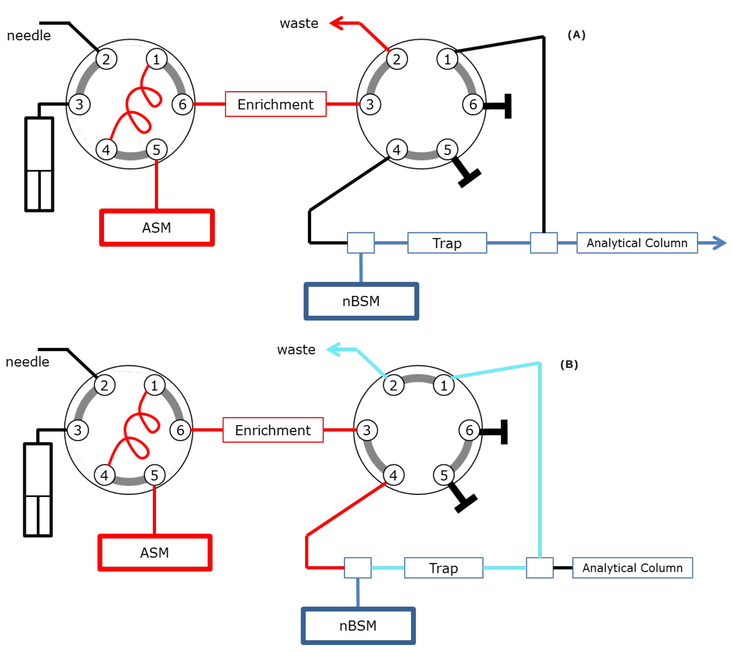


*Figure 1. Fluidic layout of the online enrichment system with (A) and without online dilution (B).*

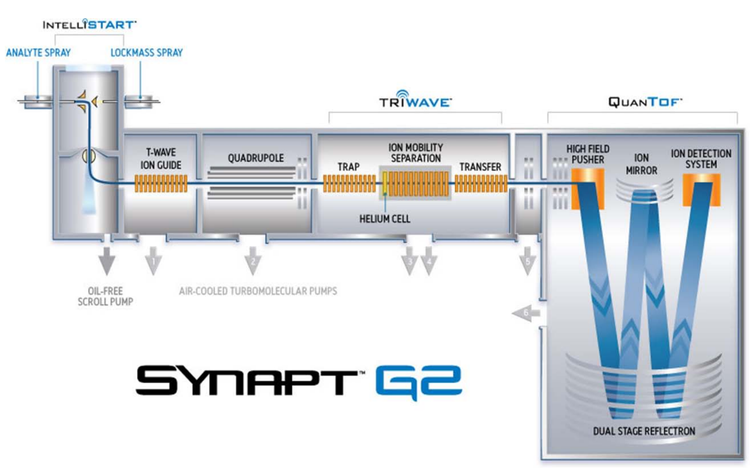


*Figure 2. Instrument schematics of the Waters Synapt G2 mass spectrometer.*

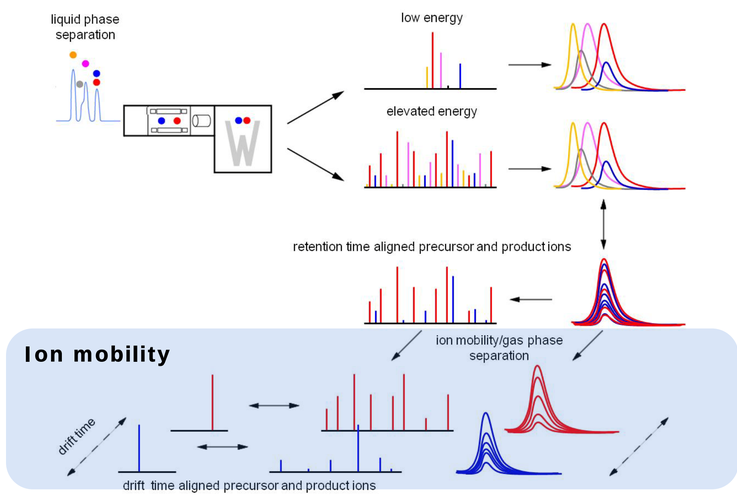


*Figure 3. Alignment of low energy and elevated energy ions by LC retention time (top, $MS^E$) and also with the orthogonal dimension of separation by mobility (bottom, $HDMS^E$).*

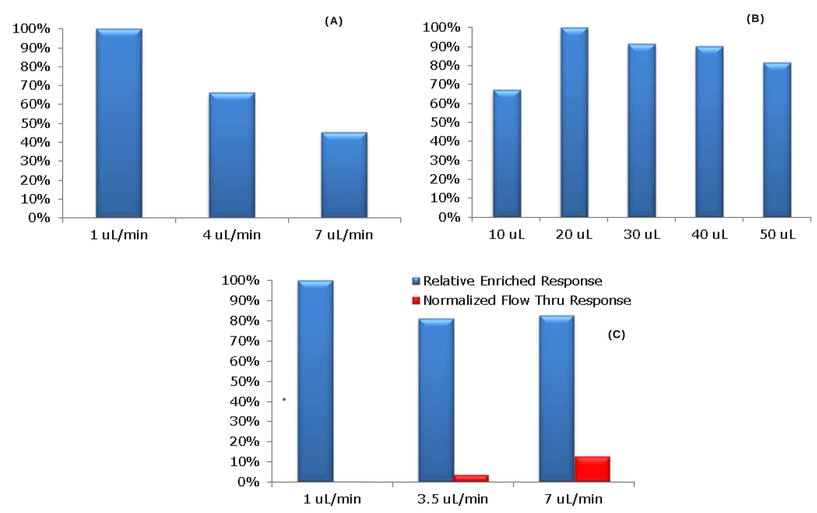


*Figure 4. Normalized phosphopeptide response as a function of elution buffer delivery rate (A), elution buffer volume (B), and sample enrichment flow rate (C). Included with the sample enrichment flow rate is a normalized response for the amount of phosphopeptide that was not enriched.*

## RESULTS

The fluidic layout for the phosphopeptide enrichment system with online dilution is depicted in Figure 1. This configuration allows multiple options for sample loading, washing, and elution. This includes sample enrichment while diverting the flow thru to waste (A) or trapping the flow thru (B) for subsequent analysis. The online dilution prior to trapping allows organic concentrations as high as fifty percent in the sample and elution buffers. This configuration allows for either targeted phosphopeptide experiments or complete analysis of the non-phosphorylated and phosphorylated portions of the sample. The instrument schematic for the Synapt G2 is depicted in Figure 2. Fragmentation occurs in the transfer region of the Triwave device, after the ion mobility separation, so that precursors and fragments share the same drift time. An overview of the $MS^E$ and $HDMS^E$ process is shown in Figure 3. While both techniques utilize chromatographic peak shape and retention time for the alignment of precursor and product ions, only $HDMS^E$ provides an orthogonal mode of separation which ultimately increases specificity. Several parameters were studied to optimize the enrichment and elution of the system. Utilizing a slow elution flow rates (Figure 4A) and washing with a moderate volume (Figure 4B) was consistent with previously reported findings[1]. Phosphopeptide break thru was observed at higher sample loading flow rates (Figure 4C). The system was evaluated using a simple enolase digest (Figure 5) as well as phosphopeptides spiked into a whole cell lysate digest of *E.coli* (Figure 6). In both cases, good recovery and selectivity was observed.

The performance of the system was evaluated by spiking in a complex library containing up to several thousand phosphopeptides into the *E.coli* cell lysate digest. The sample was enriched and subsequently analyzed using $HDMS^E$ (Figure 7). Examining a single ion series spanning a two minute window results in five primary peaks (Figure 7 inset). When examining the same ion, combining drift and chromatographic separation, we can observe up to 8 different species. Each of these peaks represents a unique phosphopeptide.

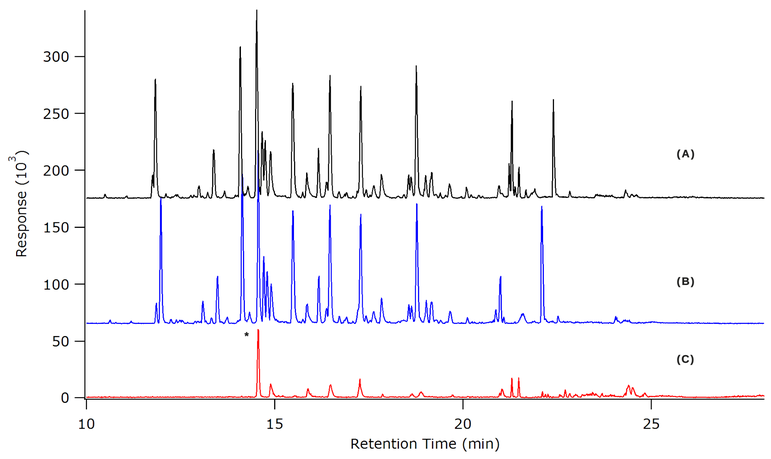


*Figure 5. Comparison of the chromatograms obtained for the enolase digest standard when no enrichment column is inline (A), the enrichment column flow thru (B), and the isolated phosphopeptide separation (C). The phosphopeptide is highlighted with an '*'.*

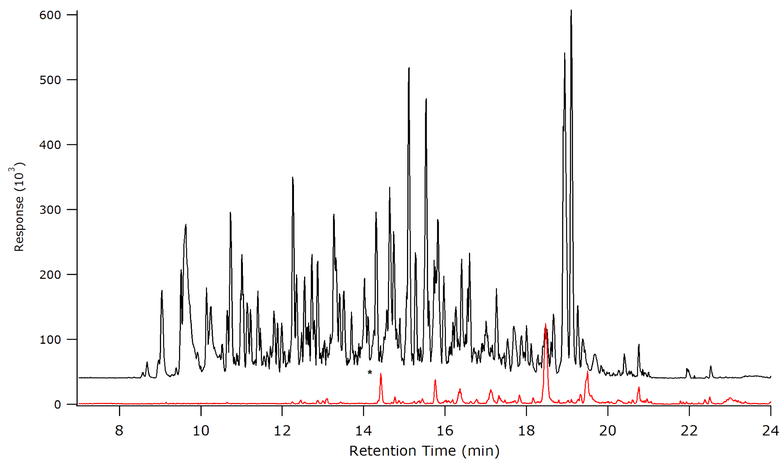


*Figure 6. Chromatogram obtained for the enrichment of the enolase phosphopeptide spiked into an E.coli whole cell lysate digest. The flow thru (black), and enriched (red) separations are depicted. The phosphopeptide is highlighted with an '*'.*

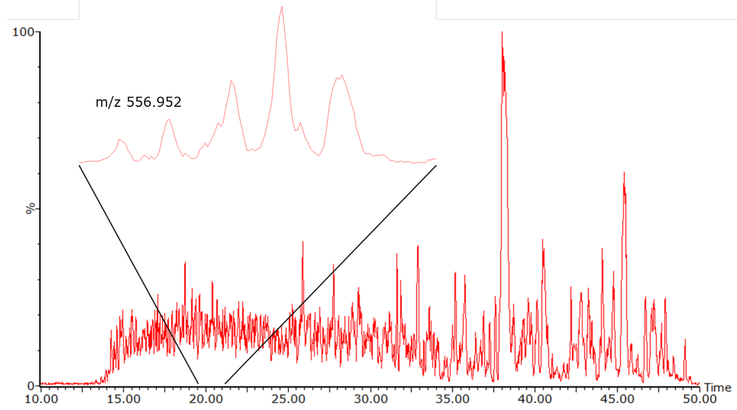


*Figure 7. Enriched chromatogram obtained for a complex phosphopeptide library spiked into E.coli. An ion series centered at m/z 556.952 is highlighted spanning a two minute window of the separation.*

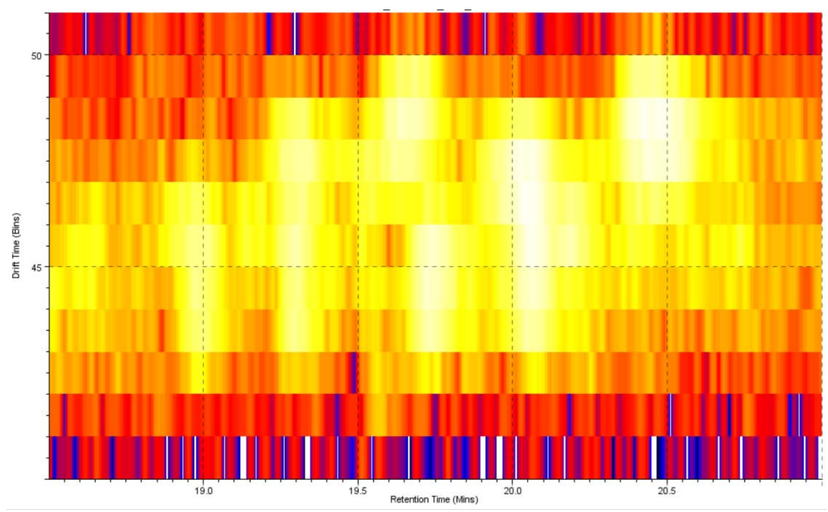


*Figure 8. Ion mobility separation for the selected ion series highlighted in Figure 7. There are eight distinct peaks eluting within the two minute window corresponding to different phosphopeptide species.*

## CONCLUSIONS

- リン酸化ペプチド濃縮を、溶出バッファーのpHと有機溶媒濃度をトラップカラムへの保持に適した組成にするためのオンライン希釈と組み合わせた
- 本システムは複雑な混合物のターゲット分析と網羅的分析の両方を可能にする
- イオンモビリティー機能は特異性を向上させ、複雑なリン酸化ペプチド混合物の分析を容易にする

## References

1. ***Schlosser, Andreas, et. al. Anal. Chem. 2005, 77(16):5243-5250.***


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