Published in final edited form as:

Anal Chem. 2018 September 18; 90(18): 10889–10896. doi:10.1021/acs.analchem.8b02397.

Improved Sensitivity and Separations for Phosphopeptides using Online Liquid Chromatography Coupled with Structures for Lossless Ion Manipulations Ion Mobility-Mass Spectrometry

Christopher D. Chouinard[#], Gabe Nagy[#], Ian K. Webb, Tujin Shi, Erin S. Baker, Spencer A. Prost, Tao Liu, Yehia M. Ibrahim, and Richard D. Smith^{*}

Biological Sciences Division, Pacific Northwest National Laboratory, Richland, Washington 99352, United States

[#] These authors contributed equally to this work.

Abstract

Phosphoproteomics greatly augments proteomics and holds tremendous potential for insights into the modulation of biological systems for various disease states. However, numerous challenges hinder conventional methods in terms of measurement sensitivity, throughput, quantification, and capabilities for confident phosphopeptide and phosphosite identification. In this work, we report the first example of integrating structures for lossless ion manipulations ion mobility-mass spectrometry (SLIM IM-MS) with reversed-phase liquid chromatography (LC) to evaluate its potential for addressing the aforementioned challenges. A mixture of 51 heavy-labeled phosphopeptides was analyzed with a SLIM IM module having integrated ion accumulation and long-path separation regions. The SLIM IM-MS provided limits of detection as low as 50–100 pM (50–100 amol/ μ L) for several phosphopeptides, with the potential for significant further improvements. In addition, conventionally problematic phosphopeptide isomers could be resolved following an 18 m SLIM IM separation. The 2-D LC-IM peak capacity was estimated as ~9000 for a 90 min LC separation coupled to an 18 m SLIM IM separation, considerably higher than LC alone and providing a basis for both improved identification and quantification, with additional gains projected with the future use of longer path SLIM IM separations. Thus, LC-SLIM IM-MS offers great potential for improving the sensitivity, separation, and throughput of phosphoproteomics analyses.

Graphical Abstract

^{*}Corresponding Author: Phone: 509-371-6576; rds@pnnl.gov.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the [ACS Publications website](https://pubs.acs.org/doi/10.1021/acs.anal-chem.8b02397) at DOI: 10.1021/acs.anal-chem.8b02397.

Lists of target phosphoproteins and 51 heavy-labeled phosphopeptides, LC-SLIM IM-MS extracted ion chromatograms, and figures of 1-D LC separation, SLIM IM-MS of phosphosite isomers, and examples of achievable LOD (PDF)

The authors declare no competing financial interest.

Phosphorylation is one of the most biologically significant protein post-translational modifications (PTMs), leading to differences in protein folding, enzyme activity, and facile modulation of important cellular signaling pathways.^{1–4} As such, numerous mass spectrometry (MS)-based methods have been developed to characterize the phosphoproteome. Conventional bottom-up approaches utilize liquid chromatographic (LC) separations of phosphopeptides coupled to tandem MS strategies, and particularly multiple reaction monitoring (MRM)- or parallel reaction monitoring (PRM)-based approaches, to confidently identify and quantify these peptides.^{5–10} However, these methods suffer from major limitations in throughput, sensitivity, and the ability to distinguish peptide phosphosite isomers.^{5–10} Low throughput stems from the extended chromatographic times (often >1h) required for sufficient separation as well as their achievable peak capacity, while sensitivity is challenged by the relatively low abundance of the phosphopeptide analytes in comparison with their unmodified counterparts.^{11–14} Because of the varied biological significance of phosphosite “localization variant”^{15–17} isomers, their reproducible separations are critical, but LC separations are often insufficient.^{17,18} Furthermore, the labile nature of these PTMs means that ergodic dissociation methods such as collision induced dissociation (CID) and higher energy collisional dissociation (HCD) often cause phosphate loss prior to production of peptide sequence fragments, greatly hindering the ability to identify the phosphorylation site.^{4,19} More recently developed dissociation techniques, including ultraviolet photodissociation (UVPD), electron capture dissociation (ECD), and electron transfer dissociation (ETD), have shown promise for improving phosphoproteomic sequence coverage. UVPD fragments peptide ions when chromophores, naturally present in the peptide backbone or aromatic side chains, absorb a high-energy photon emitted from a nanosecond-scale laser pulse.^{20–22} Although this technique has been demonstrated to minimize loss of labile PTMs, including phosphorylations, implementation in commercial instrumentation has thus far been limited.²² Electron-based activation methods such as ECD²³ and ETD¹⁴ cause cleavage of N-C_α bonds in the peptide backbone to form c- and z-type fragment ions and have been shown to nearly eliminate PTM losses.^{24–26} However, these methods are limited by slow reaction rates, requiring extended activation times, and low efficiency due to charge-state-dependency, which limit their utility for phosphopeptides that frequently have lower overall charge states in positive mode.²⁷ Importantly, these methods are significantly slower or less sensitive than direct (i.e., intact) phosphopeptide detection, and for all methods unless the phosphopeptide isomers can be effectively separated, the ability for quantification is substantially degraded.

Ion mobility-mass spectrometry (IM-MS) is a promising and rapid alternative for proteomics analysis.^{28,29} In contrast to relatively lengthy solution-phase LC separations (minutes to hours), IM separations occur in the gas-phase typically on a time scale of tens to hundreds of milliseconds. Because of its speed, IM also couples well with chromatography and fast acquisition mass spectrometers (particularly time-of-flight) for tandem LC-IM-MS,

providing overall improvements in peak capacities.^{30–34} Furthermore, IM has shown tremendous potential for separation of structurally similar biomolecules, including structural isomers and other conformers.^{35,36} However, IM presents its own limitations, primarily in the limited resolution afforded by current commercial instrumentation and the poor sensitivity attributed to its relatively low duty cycle and constraints on the size of ion populations that can be used in each IM separation.

The recently developed structures for lossless ion manipulations ion mobility (SLIM IM) platform has demonstrated significant improvements in these regards.³⁷ SLIM operate by moving ions within extended ion paths created by electric fields generated using voltages applied to large arrays of electrodes patterned using photolithography. Initial SLIM IM efforts utilized a linear, constant field arrangement, analogous to drift tube-based instruments (DT-IM),³⁸ but more recently, SLIM from our laboratory has been implemented using traveling waves (TW). TW-based IM separations allow for modest voltage requirements (<100 V TW amplitudes) in comparison with DT-IM instruments that can require several thousand volts be applied, with progressively higher requirements for longer drift tubes. Furthermore, TW voltages are not influenced by the length of the separation device, paving the way toward longer path IM separations and thus tremendous potential increases in resolution.^{37–47} Several additional capabilities have also been developed with SLIM to enable improvements in the analysis of biological samples. First, SLIM allow for ion turns and movement around corners without loss of IM resolution, in contrast to the straight path utilized in conventional IM platforms, allowing for separations over long serpentine paths (>10 m) without increasing the instrumental footprint. Second, an ion switching mechanism at the end of the serpentine ion path can either route ions to the time-of-flight mass spectrometer for mass analysis or divert ions back to the beginning of the serpentine ion path for additional passes.³⁹ This can allow essentially infinite separation path lengths and has been demonstrated to provide significantly improved resolution for previously intractable isomers using multipass path lengths of >100 m.^{39–43} Finally, compression ratio ion mobility programming (CRIMP) has been developed for the repetitive “squeezing” of several TW bins into a single bin, effectively allowing the compression of a spatially wide peak or distribution of peaks into a narrower, higher intensity peak or peaks, without significant loss of resolution.^{44,45} In addition, a portion of the SLIM module itself can function as a large ion accumulation region. In comparison with the traditional trapping and ion injection methods (such as with an ion funnel trap), accumulation of ions within a SLIM module allows for greatly extended trapping regions, allowing ion populations in excess of a billion ions to be accumulated and providing the basis for significantly improving sensitivity and the limits of detection especially for lower abundance species.^{46,47}

In this work, we utilize the aforementioned capabilities of ultrahigh resolution long path SLIM IM separations and accumulation of large ion populations to benefit targeted phosphoproteomics analysis. In addition, we report the first coupling of SLIM with online nanoUPLC separations to further improve sensitivity and peak capacity.

EXPERIMENTAL CONDITIONS

Phosphopeptide Target Samples.

The peptide standards consisted of 51 heavy-labeled phosphopeptides purchased from New England Peptide, Inc. (Gardner, MA); the complete list, including the modified sequence that indicates the location of the phosphorylated serine or threonine, is shown in Table S1. The standard mixture (30% acetonitrile, 0.5% formic acid) was spiked into a complex *Shewanella oneidensis* microbial proteome digest matrix (0.1 $\mu\text{g}/\mu\text{L}$) at 12 concentration points: 1 pM, 5 pM, 10 pM, 50 pM, 100 pM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, and 1 μM . All solvents were purchased from Sigma-Aldrich (St. Louis, MO).

Direct Introduction SLIM IM-MS Operation.

The TW-based SLIM IM-MS system has been described previously.³⁹ Briefly, samples were introduced by a syringe pump for nESI at 0.3 $\mu\text{L}/\text{min}$ with +3 kV applied. Ions traveled through a stainless steel heated capillary inlet (130 °C), a high pressure ion funnel (~10 Torr), and an ion funnel trap (IFT; ~2.30 Torr) before entering the SLIM IM module maintained at a slightly positive pressure relative to the IFT (~2.35 Torr). For these experiments, the IFT was not used for trapping, but instead, the exit grid was used as a gate to allow for timed injection (e.g., 1 s) of ions into the SLIM IM module for accumulation.⁴⁶ The SLIM IM module consisted of two separate TW regions (9 and 4.5 m, respectively) with a total path length of 13.5 m. Ion accumulation was achieved by blocking ions at the interface between the two TW regions. This method has previously been shown to allow accumulation of $>10^9$ ions.^{46,47} Following accumulation in the first 9 m region, ions underwent a separation in the second region before entering the Agilent 6224 TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). Ions could also be diverted using an ion switch⁴¹ back into the beginning of the serpentine ion path for additional separation in the second 13.5 m pass, providing a 18 m total IM path length. The TW conditions were maintained at 25 V_{pp} amplitude, 200 m/s speed, and 15 V guard bias voltage. The confining RF was maintained at 360 V_{pp} and 900 kHz. Conditions were optimized for LC-SLIM IM and consisted of 2-s SLIM module ion accumulation period followed by the 18 m IM separation; under these conditions, the total acquisition time was ~ 2.9 s. SLIM IM-MS instrument control was achieved and direct infusion data was processed using custom developed software. Data acquisition was performed using a U1084A ADC digitizer (Acqiris, Switzerland) and custom developed software. Target analyte mobilities (i.e., arrival times) were determined based on these direct infusion results.

LC-SLIM IM-MS Operation.

Online reversed-phase nanoLC with SLIM IM-MS was performed using a Waters nanoAcquity UPLC system with Waters C18 column (100 μm i.d. $\times$ 10 cm, packed with 1.7 μm BEH particles) maintained at 40 °C. Mobile phase composition was: A) water (0.5% formic acid), B) acetonitrile (0.5% formic acid). The gradient employed was as follows: 0 min, 0.5% B, 0.500 $\mu\text{L}/\text{min}$ in; 11 min, 0.5% B, 0.500 $\mu\text{L}/\text{min}$; 11.5 min, 0.5% B, 0.400 $\mu\text{L}/\text{min}$; 13 min, 0.5% B, 0.400 $\mu\text{L}/\text{min}$; 13.5 min, 5% B, 0.400 $\mu\text{L}/\text{min}$; 63.5 min, 30% B, 0.400 $\mu\text{L}/\text{min}$; 65 min, 95% B, 0.500 $\mu\text{L}/\text{min}$; 80 min, 95% B, 0.500 $\mu\text{L}/\text{min}$; 83 min, 0.5% B, 0.500 $\mu\text{L}/\text{min}$. Samples were maintained at 4 °C prior to injection; injection volume was 8

μ L. LC control was performed using the Waters MassLynx software suite. For comparison, the same samples were analyzed using similar chromatographic conditions with an established SRM method on a Thermo Scientific TSQ Vantage QQQ (Thermo Scientific, San Jose, CA).^{48,49}

Raw LC-SLIM IM-MS and LC-SRM data were analyzed with Skyline software.^{50,51} Ion intensities/peak areas for the SLIM IM-MS data were calculated using the mobility predictor feature of Skyline,⁵² which filters analytes based on arrival times from a target list. Target analyte mobilities (arrival times) were previously determined based on the direct infusion results, as noted above. Resolving power values for m/z and mobility filtering were set to 8000 and 50, respectively.

RESULTS AND DISCUSSION

Initial Refinement of Parameters for Online LC-SLIM IM-MS Analyses.

Prior to performing online LC-SLIM IM-MS analysis, a representative phosphopeptide standard mixture (25 nM/peptide) was directly infused to initially optimize for instrument sensitivity, mobility separation, and analysis speed. Specifically, SLIM IM module accumulation time (ranging from 1 to 5 s) and SLIM IM separation path length (4.5 m or greater) were evaluated for their effects on sensitivity and separation, respectively, with the additional goal of providing sufficient sampling across chromatographic peaks. First, the relative intensities for the target peptides were compared for SLIM IM module accumulation times of 1, 2, 2.5, 3, and 5 s followed by a SLIM IM separation in the second region. With each increase in accumulation time, measured peak areas increased proportionally, while peak width also increased due to the mechanism of spatially accumulating ions within the SLIM IM module (Figure 1A). Longer accumulation time (>3 s) also resulted in minor phosphate loss (<10%) at this relatively high concentration, an observation we tentatively attribute to space charge effects with extended ion accumulation to the TW amplitudes used in this step. It was further noted that increasing SLIM IM module accumulation time from 1 to 2 s provided significant gains in signal for several of the peptides present at lower abundance (Figure 1B). Many of these peptides were undetected with only 1 s of SLIM accumulation, while the signal/noise (S/N) exceeded the limit of detection following >2 s of accumulation. Although even higher intensities and improved S/N were further observed with increased SLIM IM module accumulation times (>2 s), this would ultimately hinder the chromatographic sampling rate. For example, a 5 s SLIM module accumulation time (and subsequent separation time) would only permit ~ 2–3 data points to be sampled across a 15–20 s wide chromatographic peak. As such, a 2 s SLIM module ion accumulation period was chosen as a reasonable compromise for these initial online LC-SLIM IM experiments; however, we note that we expect that significant further improvements are likely with the use of alternative ion accumulation conditions, as well as conducting this step in parallel to the separation of the previously accumulated ion population.

Next, several phosphopeptide isomer pairs were evaluated for their IM separation after 4.5 m of separation, followed by additional passes (providing total separation path lengths of 18 m, 31.5 m, etc.). These isomer pairs differed only in the specific location of the phosphorylated serine. For example, two isomers not resolved after 4.5 m of separation (Figure 2A) were

nearly baseline resolved (Figure 2B) with an additional 13.5 m of separation (18 m total separation path). Further improved separation was observed for longer separations (>31.5 m). However, this also resulted in decreased chromatographic peak sampling and some “lapping” by higher mobility species of the lower mobility species (due to the relatively wide mobility range). As such, an 18 m total separation length was utilized for this initial study. With the 2 s SLIM module ion accumulation step and an 18 m IM separation, each acquisition lasts ~ 2.9 s and, as noted above, in the future can be reduced by conducting the separation and accumulation events in parallel using dedicated regions of the SLIM for each process. Average peak widths for this chromatographic gradient were ~ 1.5–2.0 s, which resulted in sampling of approximately 6–7 IM separations/peak. SLIM IM peak capacity using this method was calculated through a previously described method⁵³ to be ~100. Briefly, our mobility range for the observed heavy-labeled phosphopeptides was ~ 300 ms (125 to 425 ms) with peak widths at full width half-maximum (fwhm) to be ~ 3 ms, resulting in a calculated peak capacity of ~ 100 for our 18 m SLIM IM separations. Arrival times were determined for all target peptides (Table S2) to aid in identification and filtering of LC-SLIM IM-MS results. We note that improvements to peak capacities may also be achievable using CRIMP,^{44,45} particularly in conjunction with the use of larger ion populations; however, this was beyond the scope of these initial studies.

Targeted Online LC-SLIM IM-MS Analysis.

Phospho-peptide mixtures ranging in concentration from 1 pM to 1 μ M/peptide spiked into *S. oneidensis* proteome digest matrix (0.1 μ g/ μ L) were injected in triplicate for online LC-SLIM IM-MS analysis. The primary focus was to achieve the lowest limits of detection for each peptide in conjunction with separation of phosphorylation site isomers while maximizing chromatographic sampling. A chromatographic overlay for all 51 peptides is shown in Figure 3.

In contrast to conventional LC-MS/MS methods for targeted proteomics in which retention time, precursor mass, and fragment ion(s) are used to identify and quantitate target peptides, the LC-SLIM IM-MS approach utilized retention time, high-resolution SLIM IM arrival time, and high-resolution m/z measurements. The IM filtering feature of the Skyline program produces extracted ion chromatograms (XICs) for a target peptide list based on both accurate mass and IM arrival time. We expect IM filtering to be analogous and complementary to the use of fragment ion filtering as effectively practiced in MS/MS methods. This IM filtering significantly improved the S/N ratio for the targeted peptides in comparison with a simple m/z -based filtering. Figure 4A shows a m/z -based XIC for LGTGFPNTP₁LDKQK²⁺ (m/z 810.897), while Figure 4B demonstrates this S/N improvement based on IM-filtering at an arrival time of 322 ms. The target peak, with retention time at ~37.2 min, has considerable interference from matrix components at a similar m/z . However, the high-resolution SLIM IM separation permits the desired phosphopeptide to be resolved from these interferences.

This same strategy of simultaneous m/z and IM filtering was applied to all 51 target peptides across the range of concentrations analyzed, and these results were compared with a standard targeted triple quadrupole MS-based LC-SRM method, and generally considered

the most sensitive of MS-based approaches.^{48,49} In several cases, use of LC-SLIM IM-MS provided improved limits of detection (LOD) for the target peptides. See the Supporting Information for LOD values for both LC-SLIM IM-MS and LC-SRM methods for all 51 heavy-labeled phosphopeptides. Additionally, retention times, most intense m/z ion observed, and arrival times, are provided in the Supporting Information for all peptides analyzed. It is important to note that, while multiple charge state species were observed for each phosphopeptide, we only chose the ones that were highest in intensity to so as provide lowest achievable LOD values. As an example of achievable D from our mobility filtering strategy, please see the Supporting Information for the target peptide GSDASpGQLFHGR²⁺ (m/z 661.282) at concentrations ranging from 50 pM to 5 nM. Additionally, our LC-SLIM IM-MS method showed a 20-fold improvement in LOD for this peptide over the LC-SRM method. The identity of the peptide was further supported by the isotopic pattern (Figure S1). The resulting mobility-filtered intensities resulted in a linear dynamic range of approximately four orders of magnitude (Figure 5A). A minor deviation in linearity is noted at higher concentrations (i.e., >10 nM for peptide GSDASpGQLFHGR²⁺) due to saturation of the mass spectrometric ion abundances, a result of the much larger than typical ion populations introduced with 2 s SLIM module accumulation time. However, an algorithm previously designed for use with LC-DTIMS-MS data⁵⁴ is being further developed for LC-SLIM IM-MS to correct for the saturation effects and potentially improve linear dynamic range. The improvements in S/N and LOD for the target peptide are shown to be a result of removal of a chromatographically coeluting matrix interference at nearly identical m/z but different mobility. However, this component is well-resolved from the target in the high-resolution SLIM IM separation (Figures 5B and C), allowing it to be “filtered out”. This mobility filtering strategy provides an advantage over LC-SRM, where an interference may coelute with the target peptide, thus hindering its detectability. Furthermore, the observed gain in sensitivity can also be attributed to our ability to accumulate large ion populations (2 s) in the SLIM device, providing improved ion statistics. This combination of gains in resolution from the 18 m SLIM IM separation, and thus mobility separating out our desired peptides of interest from background interferences, in conjunction with the ability to accumulate large ion populations, increases our achievable sensitivity. We would like to stress that there remains tremendous future potential in optimizing our in-SLIM accumulation conditions (varying the TW frequency and amplitude during accumulation) as well as conducting ion accumulation and IM separation steps in parallel to further increase our gains in sensitivity or speed.

Improved Separation of Isomeric Phosphopeptides.

Another major limitation in targeted LC-MS/MS approaches is the difficulty in determining the site of labile PTMs such as phosphorylation. Specifically, ergodic MS/MS approaches (e.g., collision induced dissociation, CID) often cause loss of the phosphorylation modification before peptide backbone cleavages, obscuring its location. This SLIM IM-MS method has been demonstrated as an alternative capable of improving sensitivity through accumulation of massive ion populations without causing significant PTM loss. As such, confidence in their identification can be greatly increased by augmenting exact mass information with high resolution IM data. The target peptide mixture contained several phosphosite isomers resolvable with SLIM IM-MS. Figure 6 shows target peptide isomers

from the human RBP1 protein, TPS(p)SEEIS(p)-PTKFPGLYR²⁺ (*m/z* 999.976) and TPS(p)SEEIS(p)PTK²⁺ (*m/z* 632.283), where the phosphorylated serine is located at either the third or eighth residue. After an 18 m SLIM IM, these isomers were readily separated. (For 1-D LC reversed-phase separation of these phosphosite isomers, see Supporting Information.) The specific identity of the isomers can be ascertained by analysis of their individual standards. This potential, for high-resolution phosphopeptide isomer separation, provides an attractive and much faster alternative to chromatographic separations, where these isomers may be unresolved. Specifically, this SLIM IM-MS-based approach is able to separate these phosphorylation site isomers without sacrificing detection of the other heavy-labeled phosphopeptides. Furthermore, by coupling online reversed-phase LC with SLIM IM, we are able to achieve a 2-D LC-SLIM IM peak capacity of ~9000 (~100 for SLIM, and ~90 for LC), far surpassing any single-stage chromatographic method or conventional IM separations approach, but also significantly lower than should be achievable as the LC stage can be capable of achieving peak capacities of >300, while even greater improvements should be feasible with longer path SLIM IM separations.

CONCLUSIONS

Targeted phosphoproteomics applications are often challenged by measurement throughput, sensitivity, and ability to separate/identify phosphorylation site isomers. In this work, the first example of SLIM IM-MS coupled with online LC demonstrated improvements for the detection of phospho-peptides compared to LC-SRM, notably the ability of SLIM IM-MS to separate phosphopeptide isomers. Adequate chromatographic sampling was achieved (6–7 spectra/peak) with an approximate 2-D LC-SLIM IM peak capacity of ~9000 provided by a 90 min LC analysis coupled to 18 m SLIM IM-MS separation. This far surpasses the peak capacity achievable with LC alone and provides an enormous improvement in throughput over methods that involve 2D LC or fractionation followed by additional LC-MS/MS measurements. The initially applied SLIM IM module demonstrated the trapping of initial ion populations in excess of 10⁸ ions compared to ~10⁶ in conventional IM-MS, providing limits of detection as low as 50 pM (50 amol/μL) for some of the target peptides in this work. Furthermore, 45 of the 51 heavy-labeled phosphopeptides were detected between the concentrations of 50 pM to 10 nM (see Supporting Information for LOD values). We speculate that the undetected peptides are due to their greater hydrophilicity and thus poor retention during reversed-phase LC separations; we envision exploring alternative (e.g., HILIC-based) separations for applications. The linear dynamic range was observed to be approximately four orders of magnitude when extracted ion chromatograms were filtered by SLIM arrival time, effectively removing bias from coeluting species with nearly identical *m/z*. In addition, this SLIM IM-MS method was shown to be gentle enough to retain phosphorylation site information such that phosphorylation site isomers could be resolved with an 18 m SLIM IM separation. Finally, we note that practically every aspect of this initial work is subject to potential significant further gains in performance, and particularly the SLIM IM resolution; studies are underway using a new SLIM IM module design that allows for concurrent ion accumulation and separation to further improve the duty cycle and thus chromatographic peak sampling. We also are continuing our efforts to optimize the in-SLIM accumulation process through, e.g., varying the TW amplitude/frequency and

accumulation periods as well as the use of CRIMP to enable the effective use of much larger ion populations. This LC-SLIM IM-MS method shows tremendous potential in targeted, as well as simultaneously for broad discovery-based, proteomics analyses. Work is underway to develop SLIM IM-MS/MS methods that provide greater IM resolution and also permit it mobility and fragmentation information and filtering to be used in a phosphoproteomics-based workflow.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Portions of this research were supported by grants from the National Institute of General Medical Sciences (P41GM103493) and National Cancer Institute Clinical Proteomic Tumor Analysis Consortium (U24CA210955) at Pacific Northwest National Laboratory. This work was performed in the W.R. Wiley Environmental Molecular Sciences Laboratory (EMSL), a DOE national scientific user facility at the Pacific Northwest National Laboratory (PNNL). PNNL is operated by Battelle for the DOE under contract DE-AC05-76RL0 1830.

REFERENCES

- (1). Hunter T *Cell* 2000, 100, 113–127. [PubMed: 10647936]
- (2). Cohen P *Trends Biochem. Sci* 2000, 25, 596–601. [PubMed: 11116185]
- (3). Mann M; Jensen ON *Nat. Biotechnol* 2003, 21, 255–261. [PubMed: 12610572]
- (4). Molina H; Horn DM; Tang N; Mathivanan S; Pandey A *Proc. Natl. Acad. Sci. U. S. A* 2007, 104, 2199–2204. [PubMed: 17287340]
- (5). Gupta MK; Jung JW; Uhm SJ; Lee H; Lee HT; Kim KP *Proteomics* 2009, 9, 4834–4836. [PubMed: 19810035]
- (6). Gerber SA; Rush J; Stemman O; Kirschner MW; Gygi SP *Proc. Natl. Acad. Sci. U. S. A* 2003, 100, 6940–6945. [PubMed: 12771378]
- (7). Schmidt A; Gehlenborg N; Bodenmiller B; Mueller LN; Campbell D; Mueller M; Aebersold R; Domon B *Mol. Cell. Proteomics* 2008, 7, 2138–2150. [PubMed: 18511481]
- (8). Lange V; Picotti P; Domon B; Aebersold R *Mol. Syst. Biol* 2008, 4, 222. [PubMed: 18854821]
- (9). Addona TA; Abbatiello SE; Schilling B; Skates SJ; Mani DR; Bunk DM; Spiegelman CH; Zimmerman LJ; Ham AJ; Keshishian H; Hall SC; Allen S; Blackman RK; Borchers CH; Buck C; Cardasis HL; Cusack MP; Dodder NG; Gibson BW; Held JM; Hiltke T; Jackson A; Johansen EB; Kinsinger CR; Li J; Mesri M; Neubert TA; Niles RK; Pulsipher TC; Ransohoff D; Rodriguez H; Rudnick PA; Smith D; Tabb DL; Tegeler TJ; Variyath AM; Vega-Montoto LJ; Wahlander A; Waldemarson S; Wang M; Whiteaker JR; Zhao L; Anderson NL; Fisher SJ; Liebler DC; Paulovich AG; Regnier FE; Tempst P; Carr SA *Nat. Biotechnol* 2009, 27, 633–641. [PubMed: 19561596]
- (10). Peterson AC; Russell JD; Bailey DJ; Westphall MS; Coon J *Mol. Cell. Proteomics* 2012, 11, 1475–1488. [PubMed: 22865924]
- (11). Beausoleil SA; Jedrychowski M; Schwartz D; Elias JE; Villen J; Li J; Cohn MA; Cantley LC; Gygi SP *Proc. Natl. Acad. Sci. U. S. A* 2004, 101, 12130–12135. [PubMed: 15302935]
- (12). Pinsky MWH; Uitto PM; Hilhorst MJ; Ooms B; Heck AJR *Anal. Chem* 2004, 76, 3935–3943.
- (13). Grimsrud PA; Swaney DL; Wenger CD; Beauchene NA; Coon JJ *ACS Chem. Biol* 2010, 5, 105–119. [PubMed: 20047291]
- (14). Syka JE; Coon JJ; Schroeder MJ; Shabanowitz J; Hunt DF *Proc. Natl. Acad. Sci. U. S. A* 2004, 101, 9528–9533. [PubMed: 15210983]
- (15). Chi A; Huttenhower C; Geer LY; Coon JJ; Syka JE; Bai DL; Shabanowitz J; Burke DJ; Troyanskaya OG; Hunt DF *Proc. Natl. Acad. Sci. U. S. A* 2007, 104, 2193–2198. [PubMed: 17287358]

- (16). Cunningham DL; Sweet SMM; Cooper HJ; Heath JK J. Proteome Res. 2010, 9, 2317–2328. [PubMed: 20225815]
- (17). Langlais P; Mandarino LJ; Yi ZJ Am. Soc. Mass Spectrom. 2010, 21, 1490–1499.
- (18). Singer D; Kuhlmann J; Muschket M; Hoffmann R Anal. Chem 2010, 82, 6409–6414. [PubMed: 20593796]
- (19). Palumbo AM; Reid GE Anal. Chem 2008, 80, 9735–9747. [PubMed: 19012417]
- (20). Robinson MR; Taliaferro JM; Dalby KN; Brodbelt JS J. Proteome Res. 2016, 15, 2739–2748. [PubMed: 27425180]
- (21). Mayfield JE; Robinson MR; Cotham VC; Irani S; Matthews WL; Ram A; Gilmour DS; Cannon JR; Zhang YJ; Brodbelt JS ACS Chem. Biol 2017, 12, 153–162. [PubMed: 28103682]
- (22). Fort KL; Dyachenko A; Potel CM; Corradini E; Marino F; Barendregt A; Makarov AA; Scheltema RA; Heck AJ Anal. Chem 2016, 88, 2303–2310. [PubMed: 26760441]
- (23). Zubarev RA; Kelleher NL; McLafferty FW J. Am. Chem. Soc 1998, 120, 3265–3266.
- (24). Riley NM; Coon JJ Anal. Chem 2018, 90, 40–64. [PubMed: 29172454]
- (25). Sweet SMM; Bailey CM; Cunningham DL; Heath JK; Cooper HJ Mol. Cell. Proteomics 2009, 8, 904–912. [PubMed: 19131326]
- (26). Wiesner J; Prensler T; Sickmann A Proteomics 2008, 8, 4466–4483. [PubMed: 18972526]
- (27). Compton PD; Strucl JV; Bai DL; Shabanowitz J; Hunt DF Anal. Chem 2012, 84, 1781–1785. [PubMed: 22182179]
- (28). Mason EA; McDaniel EW Transport Properties of Ions in Gases; Wiley: New York, 1988.
- (29). McLean JA; Ruotolo BT; Gillig KJ; Russell DH Int. J. Mass Spectrom. 2005, 240, 301–315.
- (30). Liu X; Plasencia M; Ragg S; Valentine SJ; Clemmer DE Briefings Funct. Genomics Proteomics 2004, 3, 177–186.
- (31). Valentine SJ; Plasencia MD; Liu X; Krishnan M; Naylor S; Udseth HR; Smith RD; Clemmer DE J. Proteome Res. 2006, 5, 2977–2984. [PubMed: 17081049]
- (32). Baker ES; Livesay EA; Orton DJ; Moore RJ; Danielson WF; Prior DC; Ibrahim YM; LaMarche BL; Mayampurath AM; Schepmoes AA; Hopkins DF; Tang K; Smith RD; Belov ME J. Proteome Res. 2010, 9, 997–1006. [PubMed: 20000344]
- (33). Baker ES; Burnum-Johnson KE; Jacobs JM; Diamond DL; Brown RN; Ibrahim YM; Orton DJ; Piehowski PD; Purdy DE; Moore RJ; William F; Danielson I; Monroe ME; Crowell KL; Slys GW; Gritsenko MA; Sandoval JD; LaMarche BL; Matzke MM; Webb-Robertson B-JM; Simons BC; McMahon BJ; Bhattacharya R; Perkins JD; Robert L; Carithers J; Strom S; Self SG; Katze MG; Anderson GA; Smith RD Mol. Cell. Proteomics 2014, 13, 1119–1127. [PubMed: 24403597]
- (34). Burnum-Johnson KE; Nie S; Casey CP; Monroe ME; Orton DJ; Ibrahim YM; Gritsenko MA; Clauss TR; Shukla AK; Moore RJ; Purvine SO; Shi T; Qian W; Liu T; Baker ES; Smith RD Mol. Cell. Proteomics 2016, 15, 3694–3705. [PubMed: 27670688]
- (35). Ibrahim YM; Shvartsburg AA; Smith RD; Belov ME Anal. Chem 2011, 83, 5617–5623. [PubMed: 21692493]
- (36). Shvartsburg AA; Creese AJ; Smith RD; Cooper H Anal. Chem 2010, 82, 8327–8334. [PubMed: 20843012]
- (37). Ibrahim YM; Hamid AM; Deng L; Garimella SV; Webb IK; Baker ES; Smith RD Analyst 2017, 142, 1010–1021. [PubMed: 28262893]
- (38). Webb IK; Garimella SV; Tolmachev AV; Chen TC; Zhang X; Norheim RV; Prost SA; LaMarche B; Anderson GA; Ibrahim YM; Smith RD Anal. Chem 2014, 86, 9169–9176. [PubMed: 25152066]
- (39). Deng L; Ibrahim YM; Baker ES; Aly NA; Hamid AM; Zhang X; Zheng X; Garimella SVB; Webb IK; Prost SA; Sandoval JA; Norheim RV; Anderson GA; Tolmachev AV; Smith RD ChemistrySelect 2016, 1, 2396–2399. [PubMed: 28936476]
- (40). Deng L; Ibrahim YM; Hamid AM; Garimella SV; Webb IK; Zheng X; Prost SA; Sandoval JA; Norheim RV; Anderson GA; Tolmachev AV; Baker ES; Smith RD Anal. Chem 2016, 88, 8957–8964. [PubMed: 27531027]

- (41). Deng L; Webb IK; Garimella SVB; Hamid AM; Zheng X; Norheim RV; Prost SA; Anderson GA; Sandoval JA; Baker ES; Ibrahim YM; Smith RD *Anal. Chem* 2017, 89, 4628–4634. [PubMed: 28332832]
- (42). Zheng X; Deng L; Baker ES; Ibrahim YM; Petyuk VA; Smith RD *Chem. Commun. (Cambridge, U. K.)* 2017, 53, 7913–7916.
- (43). Wojcik R; Webb IK; Deng L; Garimella SV; Prost SA; Ibrahim YM; Baker ES; Smith RD *Int. J. Mol. Sci* 2017, 18, 183.
- (44). Garimella SV; Hamid AM; Deng L; Ibrahim YM; Webb IK; Baker ES; Prost SA; Norheim RV; Anderson GA; Smith RD *Anal. Chem* 2016, 88, 11877–11885. [PubMed: 27934097]
- (45). Garimella SV; Ibrahim YM; Tang K; Webb IK; Baker ES; Tolmachev AV; Chen TC; Anderson GA; Smith RD *J. Am. Soc. Mass Spectrom.* 2016, 27, 1128–1135. [PubMed: 27052738]
- (46). Deng L; Garimella SVB; Hamid AM; Webb IK; Attah IK; Norheim RV; Prost SA; Zheng X; Sandoval JA; Baker ES; Ibrahim YM; Smith RD *Anal. Chem* 2017, 89, 6432–6439. [PubMed: 28497957]
- (47). Deng L; Ibrahim YM; Garimella SV; Webb IK; Hamid AM; Norheim RV; Prost SA; Sandoval JA; Baker ES; Smith RD *Anal. Chem* 2016, 88, 10143–10150.
- (48). Shi T; Fillmore TL; Sun X; Zhao R; Schepmoes AA; Hossain M; Xie F; Wu S; Kim JS; Jones N; Moore RJ; Pasa-Tolic L; Kagan J; Rodland KD; Liu T; Tang K; Camp DG, 2nd; Smith RD; Qian WJ *Proc. Natl. Acad. Sci. U. S. A* 2012, 109, 15395–15400. [PubMed: 22949669]
- (49). Shi T; Sun X; Gao Y; Fillmore TL; Schepmoes AA; Zhao R; He J; Moore RJ; Kagan J; Rodland KD; Liu T; Liu AY; Smith RD; Tang K; Camp DG, 2nd; W. J. Qian J. *Proteome Res.* 2013, 12, 3353–3361. [PubMed: 23763644]
- (50). MacLean B; Tomazela DM; Shulman N; Chambers M; Finney GL; Frewen B; Kern R; Tabb DL; Liebler DC; MacCoss MJ *Bioinformatics* 2010, 26, 966–968. [PubMed: 20147306]
- (51). Schilling B; Rardin MJ; MacLean BX; Zawadzka AM; Frewen BE; Cusack MP; Sorensen DJ; Bereman MS; Jing E; Wu CC; Verdin E; Kahn CR; MacCoss MJ; Gibson BW *Mol. Cell. Proteomics* 2012, 11, 202–214. [PubMed: 22454539]
- (52). Pratt B; Thompson JW; Baker ES; MacCoss MJ; MacLean B In *65th Conference on Mass Spectrometry and Allied Topics*; Indianapolis, IN, 2017.
- (53). Merenbloom SI; Bohrer BC; Koeniger SL; Clemmer DE *Anal. Chem* 2007, 79, 515–522. [PubMed: 17222015]
- (54). Bilbao A; Gibbons BC; Slys GW; Crowell KL; Monroe ME; Ibrahim YM; Smith RD; Payne SH; Baker ES *Int. J. Mass Spectrom.* 2018, 427, 91–99.

Figure 1.

Arrival time distributions for ESI of a 25 nM target peptide solution showing: (A) TELISpVSEVHPSR²⁺ (m/z 772.373) with SLIM IM module accumulation times of 1, 2, 2.5, 3, and 5 s, and (B) YLSFTpPPEKDGFPSPGTPALNAK²⁺ (m/z 1213.082) with SLIM IM module accumulation times of 1, 2, and 3 s, demonstrating increased S/N.

Figure 2.

Arrival time distributions for target phosphopeptide isomers TPSpSEEISPTK^{2+} and TPSSEEISpPTK^{2+} after (A) 4.5 and (B) 18 m of separation, demonstrating improved resolution for longer path SLIM IM separations.

Figure 3.
Overlay of selected ion chromatograms for the 51 targeted heavy-labeled phosphopeptides in a *S. oneidensis* proteome digest matrix.

Figure 4. Extracted ion chromatograms for target LGTGFNPNTp-LDKQK²⁺ (m/z 810.897), which elutes at 37.2 min, both (A) without SLIM IM-filtering and (B) with SLIM IM filtering at 322 ms.

Figure 5.

(A) Log/log plot of IM filtered intensity vs concentration for target peptide GSDASpGQLFHGR²⁺ (*m/z* 661.282), demonstrating approximately 4 orders of linear dynamic range. The improvement in S/N is primarily a result of IM filtering, in which a coeluting interference at very similar *m/z* is filtered out from the target peak (arrival time 246 ms) at (B) 1 nM and (C) 50 pM.

Figure 6. Arrival time distributions following 18 m SLIM IM separation for 5 nM concentrations of the target singly phosphorylated peptide isomer pairs: (A) TPS(p)SEEIS(p)PTKFPGLYR²⁺ (m/z 999.976) and (B) TPS(p)SEEIS(p)PTK²⁺ (m/z 632.283), where the two phosphoserine residue sites are indicated by red.