

Identification and Quantitation of Phosphopeptide Positional Isomers using Trapped Ion Mobility Spectrometry and PASEF



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Introduction

Deep characterization and quantitative analysis of proteins and their modifications as they occur post translationally is critical to understand signaling pathways and aberrant disease states. PTMs exist as a common chemical moiety that selectively modify a small repertoire of amino acids. Mass spectrometers, by definition, measure molecular mass and as such detection of PTMs is often trivial but site localization and quantitation is not. It is now common to identify tens of thousands of phosphopeptides in a single shot LCMS run, but the percentage of those phosphopeptides occurring as positional isomers is unknown. Here we show that Trapped Ion Mobility Spectrometry uniquely demonstrates site localization and quantitation of hundreds of phosphopeptide positional isomers that would otherwise remain uncharacterizable by all other current technologies. We apply the approach to both antibody-based enrichments as well as metal affinity protocols.

Methods

Enrichments of digested human cell lysates were performed by both IMAC and CST PTMScan® Phospho-Tyrosine Rabbit mAb (P-Tyr-1000). The LC was a Bruker nanoElute using an IonOpticks 25cm C18 column at a flow

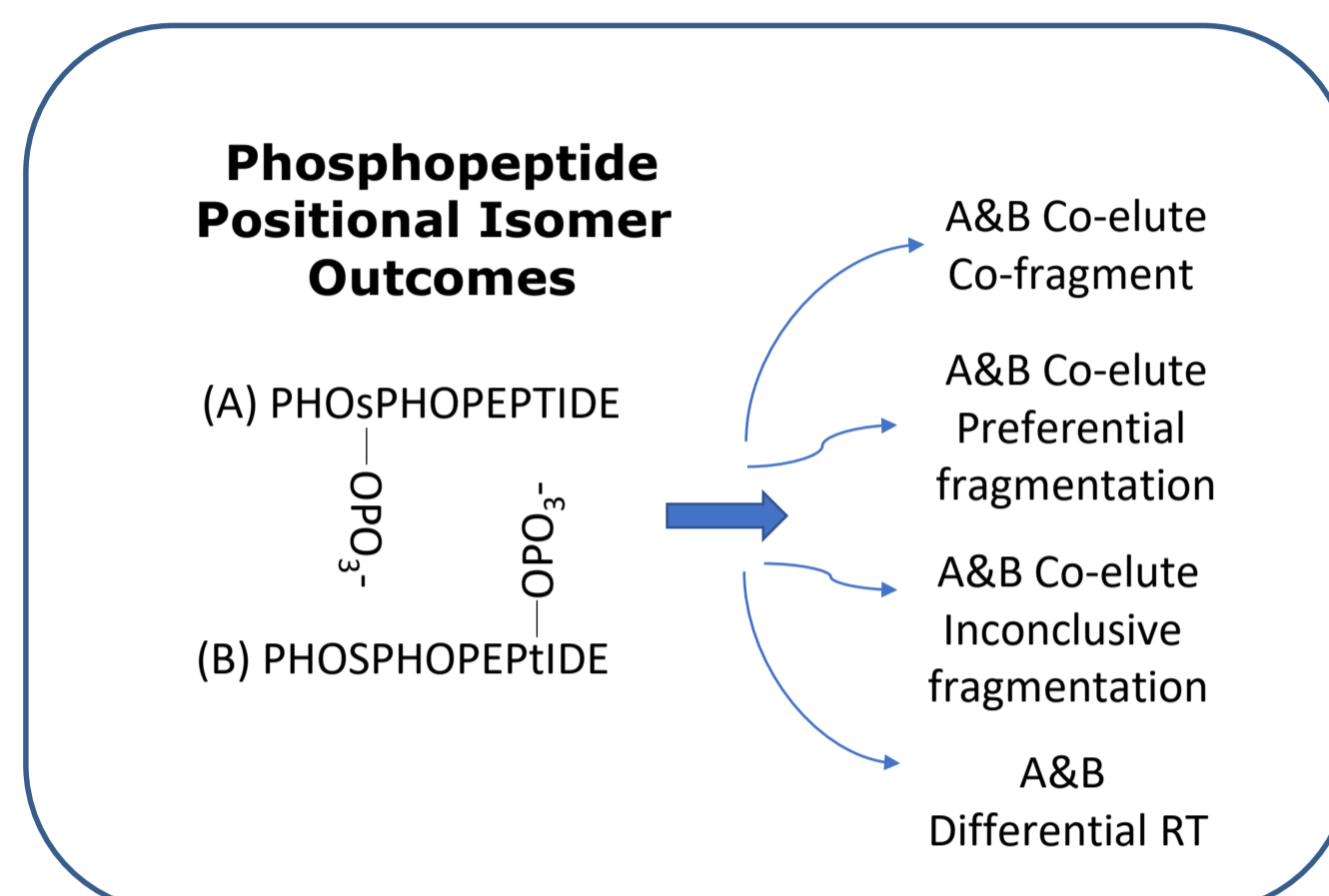


Figure 1. A general schema of some of the outcomes in dealing with phosphopeptide positional isomers where the isobaric modified peptides in many instances result in unobserved and unquantifiable outcomes.

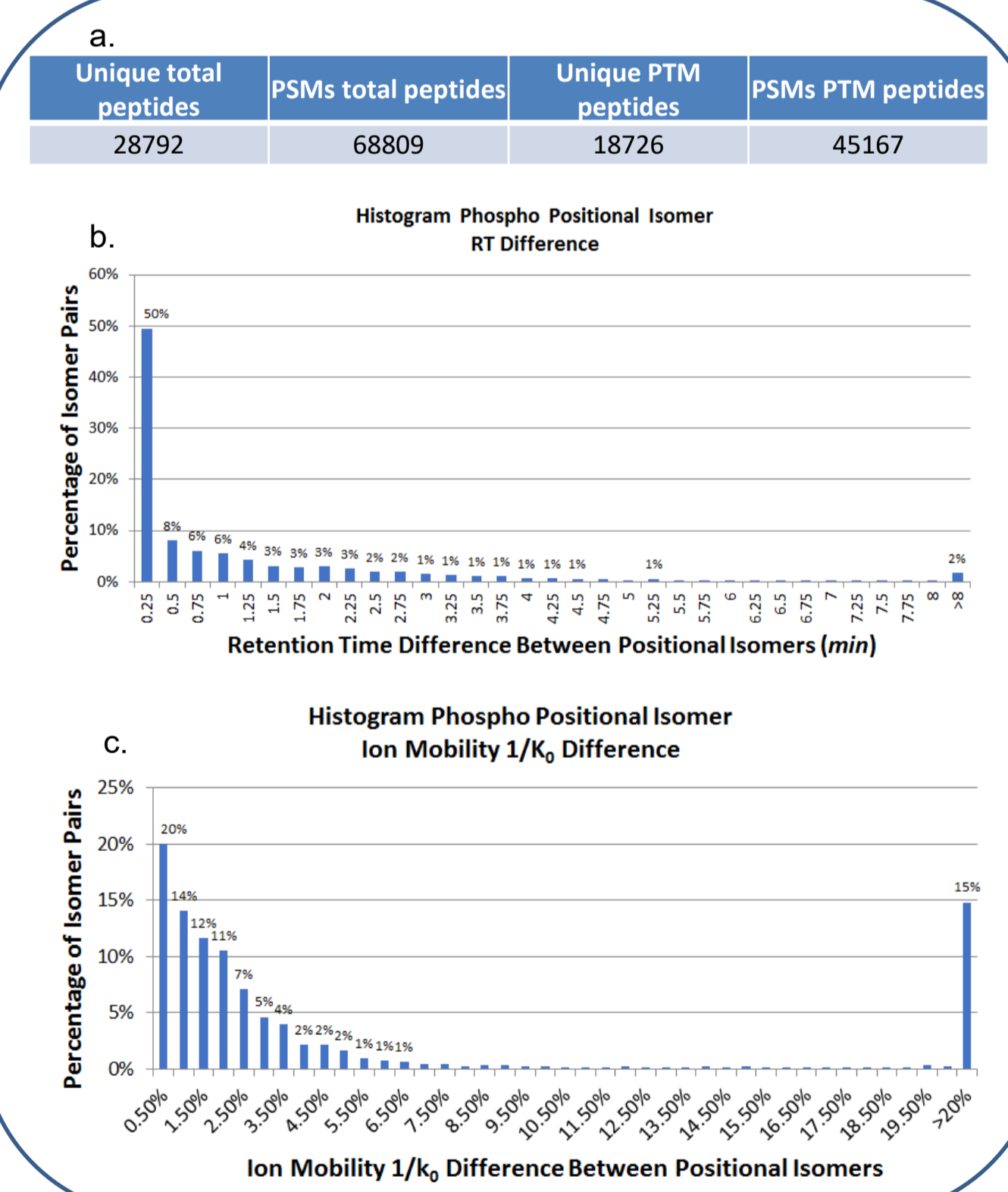


Figure 2. (a) IMAC enrichment of 100ugram digest WCL resulted in the observation of >45K modified PSMs and >18.5K unique modified peptides. Of the unique modified peptides >6,300 were identified as positional isomer pairs and >4,900 had positional determinate fragment ions to be called correctly. (b) Histogram distribution of the 4900 phosphopeptide positional isomers as they deviated in LC retention time. (c) Histogram distribution of the same subset in percent mobility (true CCS) change where a change of ~2% allows distinction in an extracted ion mobilogram.

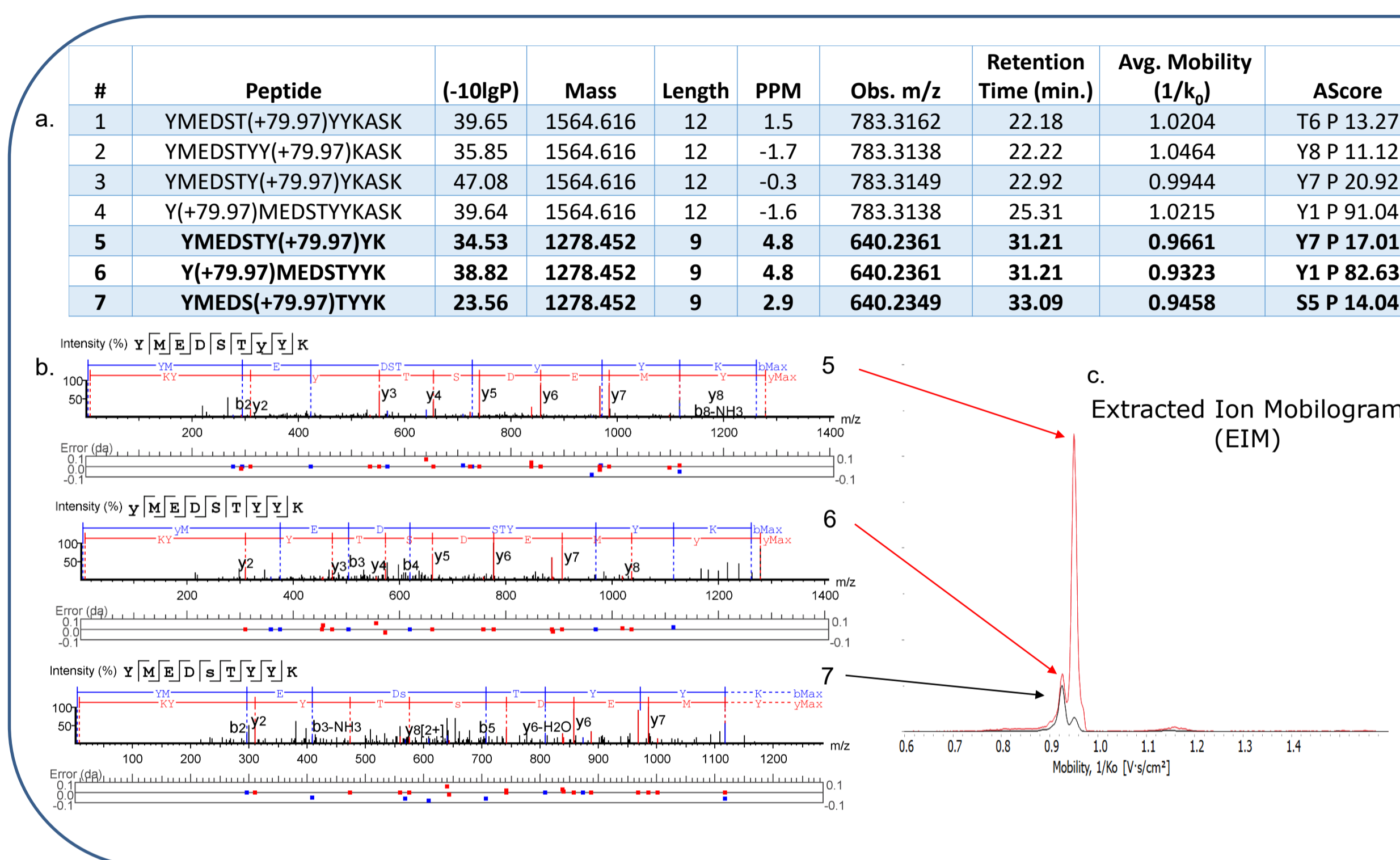


Figure 3. (a) 7 different phosphorylated forms at two different isobaric masses. All peptides have a positional determinate fragment ion and the site localization is confident as reflected in the high AScore. Note that the retention time difference in most instances renders quantitation by EIC null. (b) MSMS spectra for peptides 5-7 where peptides 5 and 6 co-elute in time but have differences in mobility (CCS) such to provide clean MSMS afforded by tims. (c) An extraction ion mobilogram is performed at different retention times and mobility (CCS) terms allowing quantitation on co-eluting phosphopeptide positional isomers. Quantitation on all three phosphopeptide positional isomers can be done because the mobility (CCS) term is generated in addition to MS and MSMS and used as an analyte attribute.

rate of 400nL/min. The mass spectrometer was a timsTOF Pro set to acquire data in a parallel accumulation serial fragmentation (PASEF) workflow. All data was searched using PEAKS X, which has the unique capabilities to 1) decipher chimeric MSMS spectra and 2) require a positional determinate fragment ion for irrefutable assignment of phospho-groups.

Results

Single shot 90-minute gradients of IMAC enriched and pY-1000 enriched lysates identified more than 18,500 and 1,100 unique PTM modified peptides, respectively. The percentage of phosphorylated forms between these pools was greater than 90%. Using PEAKS X and the positional determinate feature we asked how many of the phosphopeptides exist as positional isomers. For the IMAC enriched data set some 6315 were

identified as positional isomers and 4902 had the required positional determinate fragment ions. In the case of pY-1000 enriched pool some 329 phosphopeptides were identified as positional isomers and 293 contained positional determinate fragment ions. Collectively, this suggests that in DDA workflows some 25-30% of all the identified phosphopeptides exist as positional isomers. As described in Figure 1 it is easy to discern the presence of the site-specific fragment ions but not the absence which as our data suggests is surprisingly common. Next we plotted histograms of the positional isomers we identified within our datasets. Specifically Figure 2b describes the observation that of the 4900 phosphopeptide positional isomers identified, 50% of them elute within a 15 second time window. Extracted ion chromatograms (EICs) are commonly used to quantitate the abundance of peptide forms but cannot be performed on co-eluting and overlapping peptides of the same molecular mass.

be resolved and therefore not quantified. Within the same data set we also plotted the distribution of mobility (CCS) percent differences (Figure 2c) where 43% of the phosphopeptide positional isomers exist with a gas phase confirmation divergent enough to resolve them by performing a Extracted Ion Mobilogram (EIM) as shown in Figure 3c. Digging deeper into the datasets it becomes clear that in many instances many more than just phosphopeptide positional isomer pairs exist, as demonstrated in Figure 3. In these examples 7 phosphopeptides representing 2 isobaric masses and the same primary sequence suggest the localization 5 different phospho groups within the same single primary sequence. In this example the peptides often co-elute rendering EIC uninformative, but it's true that of the seven peptides the combination of RT and mobility (CCS) allowed us to quantitate 5 of the 7 where RT alone would only be capable of 2.

Conclusions

- Phosphopeptide positional isomers are extremely common in phosphopeptide enriched samples
- Positional isomers are hard to identify in the absence of positional determinate fragment ions
- RT distinction of phosphopeptide positional isomers from a large pool was typically less than 15 seconds
- TIMS and true CCS provides an analyte attribute to perform identification and quantification of positional isomers otherwise not possible.



timsTOF Pro