Data Independent Analysis with Data Dependent Isolation Widths

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INTRODUCTION

As a data independent acquisition (DIA) approach, SWATH™-MS methods have been showing great potential to reliably quantify a wide range of different species, with higher reproducibility in both peptide extraction and quantitation than those obtained with data dependent acquisition methods (DDA)[1,2]. The information dependent acquisition (IDA) strategy selects the precursor ions based on the MS1 spectra to fragment, resulting in the inherent stochastic sampling which leads to missing data points and lack of confidence in the quantification. DIA methods such as SWATH™ acquisition provide a means to analyze all of the precursor ions in a designed m/z range, independent of MS1 selection. Using MS/MS fragment ions to quantify the compounds provides unbiased measurements of peptide ions which show reduced potential for interference and thus higher data quality for more accurate quantitation.

So far many SWATH™ acquisition-related studies have used a wide Q1 precursor isolation window (e.g. 25 Da[3]) cycling through the whole mass range, balancing specificity (Q1 window widths) and sensitivity (accumulation time in collecting each MSMS spectrum), and making it possible for analyzing peptides with a broad m/z range in a LC timescale. Recent advances have shown over the past years the potential for variable Q1 widths. Although these methods improve the quality of the data the desire is to have a single method which does not require potential optimization of Q1 isolation widths.

The complex SWATH scans are processed by the extraction of fragment ions from the data in the form of XIC's. Narrower precursor isolation window widths reduce the complexity of MSMS spectra simplifying data processing, and could potentially provide higher specificity and selectivity, however, at the expense of reduced MSMS accumulation time which could affect the detection quality. Ideally, to enhance quantitative proteomics we need to achieve IDA Q1 isolation widths with unbiased measurements and maintain sufficient sensitivity and cycle time to provide reproducible quantitation.

In this presentation we will discuss the performance of SWATH™ acquisition at narrow Q1 isolation widths comparable to MS1-based analysis in measurements of both peptide identification and quantitation.

MATERIALS AND METHODS

Sample Preparation: Trypsin digested *E.coli* cell lysate from Waters were used, with dilution to concentrations of 500 ng/µL in solvent containing 98/2 water/acetonitrile (v/v) with 0.1% formic acid.

Chromatography: The peptide samples were analyzed using the Eksigent nanoLC-Ultra® system combined with the cHiPLC® system in trap-elute configuration. In each run, 1 µL of sample was loaded onto the trap column (200 µm x 0.5 mm ChromXP™ C18-CL, 3 µm, 120 Å) and washed for 10 min at 2 µL/min, then eluted from a nano cHiPLC column (75 µm x 15 cm ChromXP™ C18-CL, 3 µm, 120 Å) with a buffer gradient of 5% to 30% acetonitrile (0.1% formic acid) in 60 min.

Mass Spectrometry: The peptide samples eluted from the nanoLC column were sprayed using the NanoSpray® Source and analyzed in either IDA or SWATH™ acquisition mode in a TripleTOF® 6600 system (AB SCIEX, concord, ON, Canada). The data in SWATH™ acquisition were collected at four different precursor isolation widths, 25 amu, 12.5 amu, 6.2 amu and 3 amu, respectively. Each experiment was conducted at a constant Q1 width stepping across a mass range from 400 Da to 1000 Da, with accumulation time of each single MSMS scan (100-2000 Da) adjusted from 100 ms to 20 ms accordingly to keep the cycle time fixed around 4 s.

Data Processing: A spectral library of peptides was generated from processing either the IDA data or the SWATH data from the sample of interest with ProteinPilot™ V4.5 software. With the library, the SWATH data were processed and the spectra of confidently identified species were extracted in a research version of PeakView® software where automated peak detection, transitions/peptides selection, and area extraction were performed.

Results

1. Peptide Extraction

Table 1. Comparison of peptide extraction with four Q1 isolation window sizes in SWATH™ acquisition

Q1 Window Size (amu) X Number of Windows	# of Peptides Extracted[b,c]	Peptides at Intersection[d]	Peptide Extraction Reproducibility
3 amu[a] X 200	6891±12	6055	87.9
6.2 amu X 96	6188±157	5066	81.9
12.5 amu X 48	5911±128	4907	83.0
25 amu X 24	4979±71	4021	80.8

a) Evaluation of the Q1 window shapes in SWATH™ acquisition: Square windows can be achieved with widths of as small as 2 amu applying to a broad mass range.
b) The peptide extraction was controlled with a false detection rate (FDR) threshold of 1%.
c) The variations are based on standard deviations of three replicates.
d) The intersection is calculated from the number of peptides extracted in all 3 replicates.

- More peptides were extracted as narrowing the Q1 isolation widths.
- The 3 amu Q1 window gave better reproducibility in peptide extraction than the larger windows.

Why better peptide extraction in 3 amu? ⟹ ***Less interference improves specificity !***

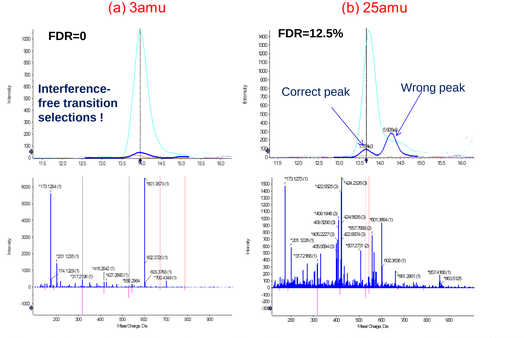

Figure 1. Re-constructed chromatogram of transitions generated from the peptide, GQNEDQNVGIK 3+, and the corresponding MSMS spectra acquired at Q1 widths of (a) 3 amu showing a clean isolation of the XIC data and (b) 25 amu indicating a transition ion with m/z in the same XIC extraction window as the target transition. Actually it is from a different peptide with a similar retention time and a m/z in the same 25 amu isolation window. As isolated in 3 amu window this peptide can be excluded from the target peptide window.

2. Peptide Quantitation

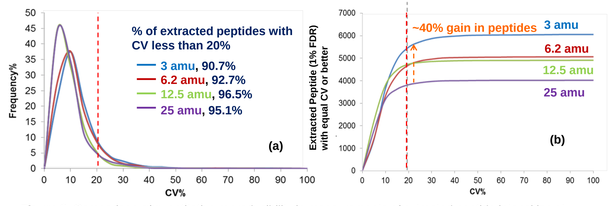

Figure 2. Comparison of quantitative reproducibility in measurements of extracted peptide intensities at different Q1 window widths.

- At 3 amu Q1 windows, more than 90% of extracted peptides were quantified with CV% of 20% or better.
- The results indicate that the quantitative quality is not affected by the reduced MSMS accumulation time when small Q1 isolation windows are applied.

CV% dependence on the peptide intensities at Q1 width = 3amu

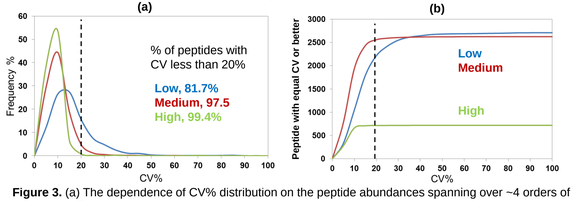

Figure 3. (a) The dependence of CV% distribution on the peptide abundances spanning over ~4 orders of magnitude. The peptides are sorted by intensities into three bands, as presented with blue, red and green traces corresponding to low (< 10^5), medium (10^5-10^6) and high (> 10^6) intensities respectively.
(b) Cumulative plot of peptides vs. CV% in three intensity bands, showing the numbers of peptides in each intensity band.

- More than 80% of low-intensity peptides show good quantitation reproducibility with CV% of 20% or better at small Q1 widths of 3 amu.

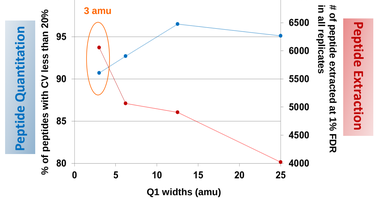

Figure 4. A summarized illustration of the impact of Q1 isolation window sizes on the peptide extraction and quantitation. It clearly demonstrates the improved peptide extraction with smaller Q1 widths applied without compromising the quantitative reproducibility.

3. Peptide Identification and Quantification from a Single SWATH File

Using a simple method of exporting the 3amu SWATH data the output was searched using ProteinPilot ™ v4.5 Beta. The search results were then processed again using the SWATH application in Peakview® software. This resulted in **6057±151** peptides being identified at a 1% FDR in SWATH with the intersection of 5083 (84%) peptides in three replicates. The %CV of the quantitative data is shown below for this data.

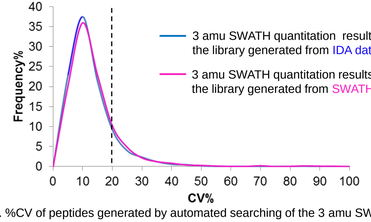

Figure 5. %CV of peptides generated by automated searching of the 3 amu SWATH data followed by quantitative measurements (pink trace), consistent with the results using peptides extracted from the IDA library (blue trace).

CONCLUSIONS

1. **The current TOF system and methods enable us to run DIA analysis (SWATH™ acquisition) at small Q1 widths of 3 amu which is comparable to those in MS1-based DDA analysis.**
2. **As expected, narrow precursor isolation windows reduce the complexity of MSMS spectra and provide better selectivity and specificity, thus significantly improving the coverage of peptides confidently extracted.**
3. **The peptide quantitation quality is not affected along with the increases of the peptides detection coverage.**
4. **The application of small precursor isolation window sizes extends the power of SWATH™ technique on routine proteomic analysis, by providing an improved peptide extraction with good quantitative reproducibility.**
5. **Minimizing Q1 windows allows for a single SWATH analysis for both identification as well as quantification.**

REFERENCES

1. Kang, Y. et al. Poster presentation at ASMS (2013)
2. Domon, B. et al. Nat Biotechnol. (2010): 28, 710-721
3. Gillet, L. C. et al. Mol. Cell. Proteomics (2012): 11, O111.016717

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