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Research Article

EVALUATION OF SWATH-MS BASED QUANTIFICATION FOR ITS ACCURACY AND CONSISTENCY ACROSS CONCENTRATIONS OF SPIKED-IN PEPTIDES

Ajay Bhat[1, 2], Dipankar Malakar[3] and Shantanu Sengupta[1, 2*]

[1]*Genomics and Molecular Medicine, CSIR-Institute of Genomics and Integrative Biology, Mathura Road, New Delhi 110020, India*
[2]*Academy of Scientific & Innovative Research (AcSIR), CSIR- Institute of Genomics and Integrative Biology, Mathura Road Campus, New Delhi 110020, India*
[3] *Sciex, 121 Udyog Vihar, Gurugram, Haryana 122015, India*

Abstract: Mass spectrometry based proteomics have emerged as an important tool for studying different aspects of biological networks. However, the major challenges in this field are reproducibility, accuracy and sensitivity for detecting and quantifying low abundant proteins. We have previously reported that sequential window acquisition of all theoretical fragment ion spectra (SWATH–MS) holds the ability to quantitate larger number of low abundant proteins as compared to isobaric tag for relative and absolute quantitation (iTRAQ). Here we provide a detailed evaluation of the quantification performance of SWATH-MS for its precision and reproducibility in quantifying peptides of different abundance using β-galactosidase as a reference protein. This study demonstrates that SWATH-MS can reproducibly quantitate peptides as low as 2.5 fmoles. The coefficient of variation was below 13% at 2.5 fmoles while it was < 8% for concentrations above 25 fmoles clearly indicating good reproducibility between replicates. Thus, our analysis further supports the strength of this method to quantitate low abundant proteins.

Key words: SWATH; ; Coefficient of variation; Low abundant proteins.

Introduction

With the advent of high throughput mass spectrometers, proteomics has emerged as a fascinating tool to study proteins in a global context (Aebersold and Mann, 2003; Brewis and Brennan, 2010; Shiio and Aebersold, 2006; Wasinger et al., 2013; Xie *et al.*, 2011; Yates *et al.*, 2009; Domon and Aebersold, 2006). The two major approaches widely used in this field are Discovery proteomics and Targeted proteomics. Discovery proteomics (shotgun or un-targeted proteomics) involves initial MS scan of peptides, followed by the selection of peptides above a pre-determined intensity for fragmentation (Domon and Aebersold, 2006; Domon and Aebersold, 2010; Law and Lim, 2013). In this data dependent acquisition method (DDA) the fragments generated are mapped to corresponding peptides using different algorithms (Marcotte, 2007; Nesvizhskii, 2007). On the other side, targeted approach employs data in-dependent method of acquisition (DIA) where pre-selected peptides are identified and quantified (Picotti and Aebersold, 2012). Targeted approach is better than the discovery based approach in terms of its reproducibility and accuracy (Law and Lim, 2013; Picotti and Aebersold, 2012) but unlike discovery proteomics it is not a method of choice for a larger set of proteins. This problem is circumvented using SWATH-MS, a recently developed data independent acquisition method that combines the advantages of both Targeted and Discovery

Corresponding Author: **Shantanu Sengupta**
E-mail: shantanus@igib.res.in

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approaches (Gillet *et al.,* 2012). It neither depends on abundance of the fragments nor the pre-knowledge of the precursor ions for fragmentation. SWATH-MS mode allows the fragmentation of all the masses by dividing the entire mass range in consecutive m/z windows (Gillet *et al.,* 2012). Identification of the fragments generated by SWATH-MS depends on spectral library created through a data dependent acquisition method. However, sequential window and data independent mode of acquisition makes SWATH comparable to MRM for its specificity and sensitivity (Gillet *et al.,* 2012).

In an earlier study we compared SWATH-MS with iTRAQ, a widely used labeled method of MS based quantification and reported that SWATH-MS has the advantage of quantifying a larger percentage of low abundant proteins (below 60 ppm) compared to iTRAQ (Basak *et al.,* 2015).This ability of SWATH to quantify low abundant proteins can prove to be of tremendous value in the field of biomarker discovery. But this method has not been evaluated in detail for its consistency and accuracy in terms of quantifying peptides at very low concentrations. Gillet et.al had earlier reported that SWATH can quantify a wide range of spiked-in peptides and found that the limit of detection was in the attomole range (Gillet *et al.,* 2012). They also calculated the coefficient of variation (CV) at 47 fmol and found it to be below 15%. In this study using beta- galactosidase (beta-gal) as a model protein, we undertook a detailed analysis of SWATH-MS for it reliability and precision in quantifying various concentrations (1 fmol-250 fmol) of peptides in the background of complex peptides (tryptic digest of yeast lysate) and found that even at concentrations as low as 2.5 fmol the CV is below 10%.

Materials and Methods

Materials

Yeast media components, DTT (dithiothreitol), IAA (Iodoacetamide), ammonium formate and formic acid were procured from Sigma (U.S.A.). Modified trypsin (sequencing grade, V511), was procured from Promega. Pre-digested beta-galactosidase, nano-LC column (Chromo XP 3C-18, 0.075× 150 mm, 3 µm) was procured from Sciex (USA). The nanospray picotip was purchased from New Objective (USA). LC-MS grade water and acetonitrile were procured from J.T.Baker (USA). All other chemicals used were of analytical grade.

Yeast protein digestion and spiking of beta-galactosidase

Yeast lysate was prepared by bead-beating method as described previously (Maity *et al.,* 2014) and isolated protein was reduced with 25mM DTT for 30 minutes at 56°C and the cysteine's were blocked by 55mM IAA at room temperature in dark for 15-20 minutes. Digestion of protein was performed using modified trypsin (Promega, V511) at 37°C, for 16-18 hrs (Basak *et al.*, 2015; Maity *et al.*, 2014) .Yeast lysate of about 20ng/µl was spiked with different concentrations of pre-digested beta-galactosidase (0.1fmol/µl, 0.25 fmol/µl, 0.5 fmol/µl, 11fmol/µl, 2.5 fmol/µl, 5 fmol/µl and 25 fmol/µl in 0.1% formic acid) and 10µl of this mixture was loaded on column with two injections of each.

LC condition and SWATH-MS acquisition

Samples were analyzed on a TripleTOF5600 (Sciex, USA) system coupled to an Eksigent NanoLC-Ultra 2Dplus system. Ten µl of each sample were loaded on the trap column at a flow rate of 2.5µl/min for 20 minutes and eluted from the analytical column at a flow rate of 250nl/min with the following gradient: Solvent B (100% ACN, 0.1% FA) was increased from 5 to 10% in first 10 minutes. Then buffer B was ramped up to 40% for the next 50 minutes. In the next 10 minutes %B was increased up to 50% and reached 90% within 2 minutes and was at 90% for another 9 minutes for column washing. Finally, the column was re-equilibrated by solvent A (100% water, 0.1% FA) for 9 minutes. MS was specifically operated in a "create swath" mode where it was set to allow a quadruple resolution of 25 Da/mass selections. Using an isolation width of 26 Da (25 Da of optimal ion transmission efficiency 1 Da for the window overlap), a set of 36 overlapping windows was constructed covering the mass range 350–1250 Da. The collision energy for each window was determined based on the appropriate collision energy set automatically

with a spread of 5eV. The total duty cycle was of 3.3 s (3.2 s total for stepping through the 36 isolation windows- 0.1 s for the optional survey scan).

Data analysis

The raw .wiff files generated were analyzed by Multi-Quant2.1 for quantification of different peptide fragments. The linearity was assessed through standard curve generated by plotting the average area of the intensity at each concentration subtracted from the background (containing no beta-gal peptide but only yeast peptides) versus concentration of the peptides loaded. The experimental concentration from each sample was calculated from the regression analysis of the standard curves. The accuracy of the quantification was calculated as: (Calculated concentration/Expected concentration) ×100. The % CV was calculated from the average and the standard deviation of the intensity.

Results and Discussion

Reproducibility of the SWATH-MS method was first assessed by comparing the Total Ion chromatogram across different technical replicates for sample spiked with 250 pmol of beta-gal (Fig. 1), which clearly shows a perfect overlay of chromatogram. We have previously demonstrated the possible strength of SWATH in quantifying low abundant proteins (Basak *et al.*, 2015). In this study, we have evaluated this method for robustness and precision at different concentrations of peptides. We have taken three different peptides (VDEDQPFPAVPK, IDPNAWVER, and GDFQFNISR) of beta–gal across different retention time for quantification (Fig. 2). In the background of yeast extract the intensities of the XIC (Extracted ion chromatogram) of the peptide fragment VDEDQPFPAVPK (998.48) of beta-gal at different concentration is shown in Fig. 3. To further check the robustness of this method at different concentrations of peptides, we assessed the linearity of this method for different range of peptides. The linear correlation (r2) between the concentration and the intensity of the peptides measured (Fig 4) for the entire concentration range (1-250 fmol) was greater than 0.99. Even the linear correlation for the lower range (1fmol to 10 fmol) was approximately 0.99 for all the peptides. Further, from the linearity curve we determined the accuracy of quantification by calculating the experimental concentration of peptides. We found that the quantification using SWATH above 5 fmol of beta was 86% accurate and with minor standard deviation (±0.5-9%) across different peptides, whereas average accuracy across different peptides in 2.5 fmol of beta-gal was 77% with standard deviation of ±6% (Fig. 5).However, for 1 fmol beta-gal accuracy was lowest (73%) with a large standard deviation (±30%) within different peptides. Thus, SWATH could quantitate upto 2.5 fmol with 77% accuracy and quantification was consistent for different peptides of beta-gal at this concentration.

As label free LC-MS based quantitation greatly depends on reproducibility of intensities across different replicates(Patel et al., 2009; Zhu

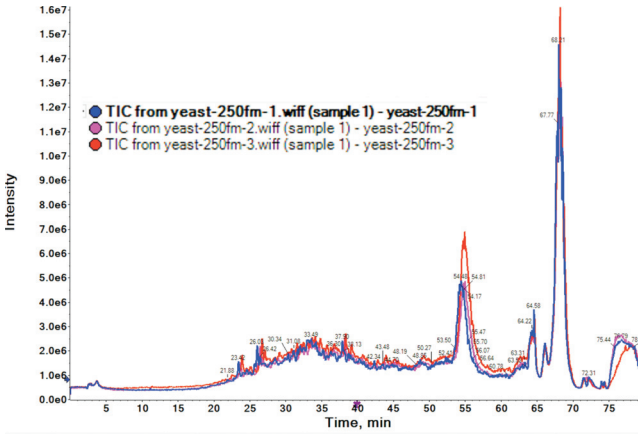

Figure 1: **TIC of three replicates of sample containing yeast peptides spiked with 250 fmol of beta-gal peptides**

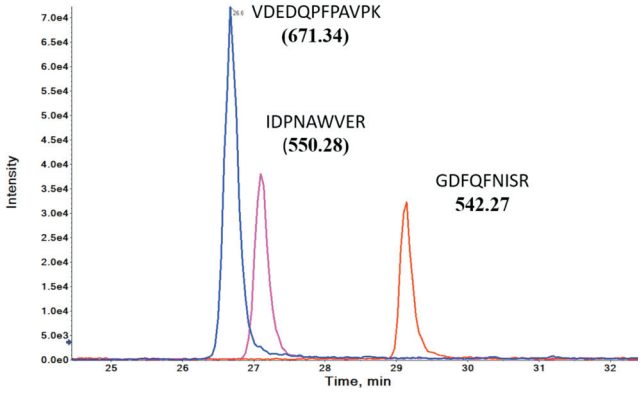

Figure 2 : **XIC of three peptides of beta-gal (250 fmol) chosen for quantification**

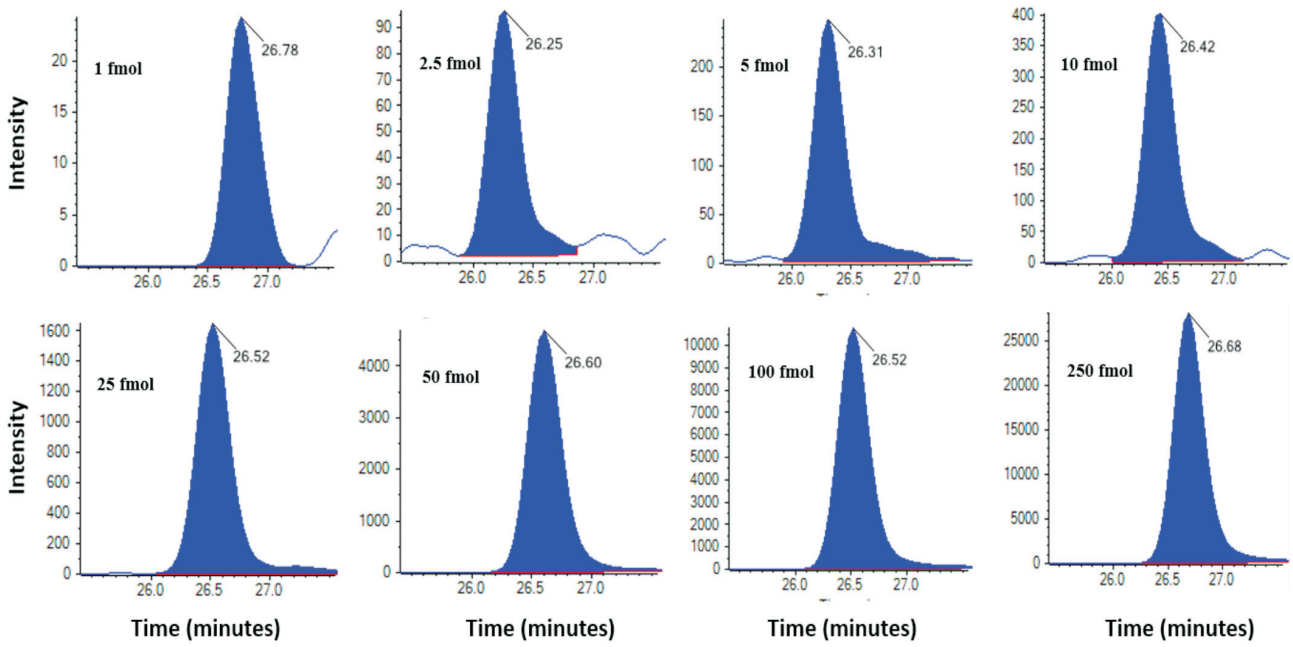

Figure 3: **XIC of a peptide fragment VDEDQPFPAVPK (m/z=998.48) of beta-gal at different concentration**

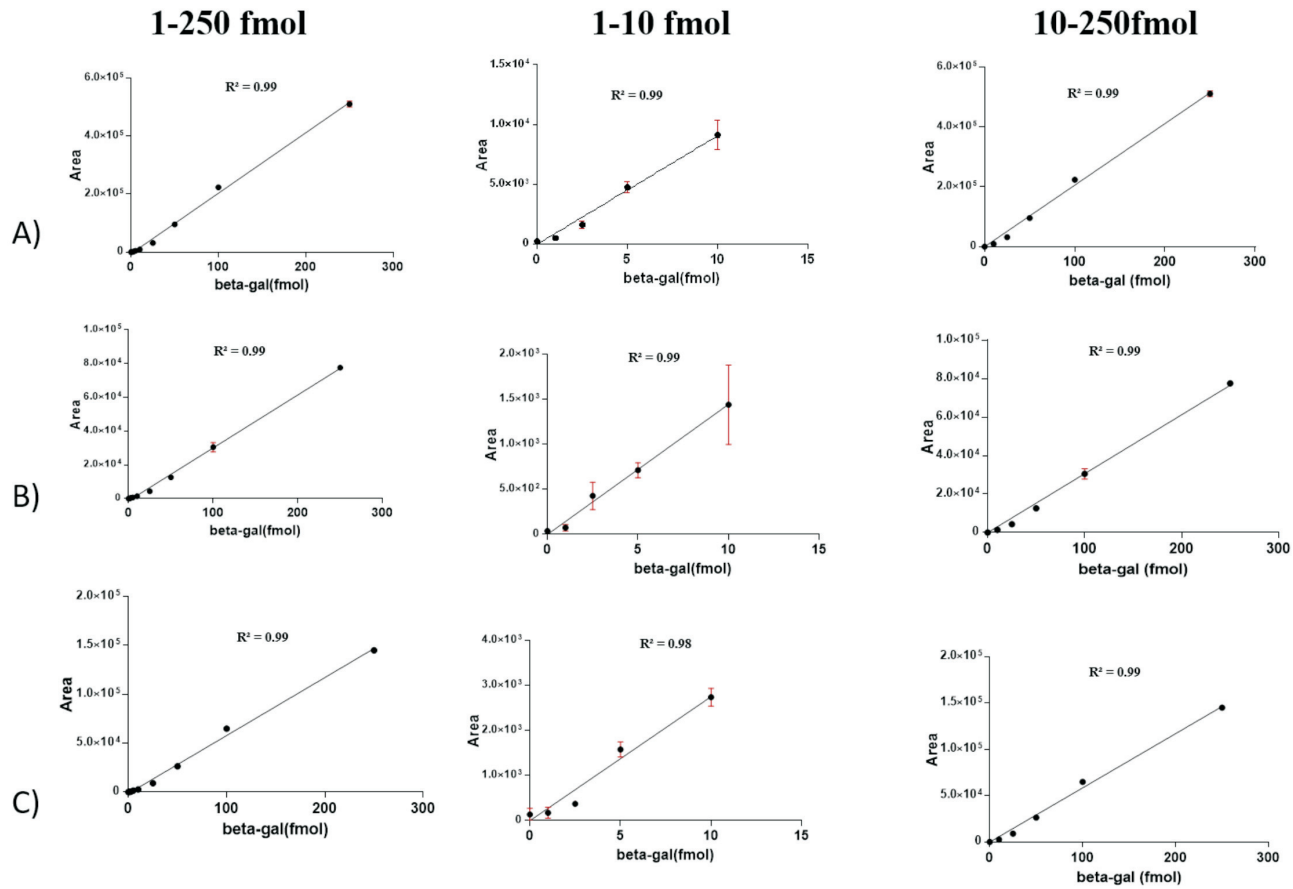

Figure 4: **Area of different fragments A) 775.44 B) 998.49 C) 511.27 of VDEDQPFPAVPK of beta galactosidase spiked in different concentration with equal yeast digest (left column for 1-250 fmole, middle column for 1-10 fmole and right column for 10-250 fmoles of beta-gal)**

et al., 2010), we further calculated the coefficient of variation across different replicates for all the peptide concentrations (Fig. 6). We found that % CV among different replicates of beta gal was lowest (<8%) in peptides with higher concentrations (25-250 fmol) while for peptides with lower concentration (2.5–10 fmol) it was between 8% – 12.4%. However, the CV was about 30% for 1 fmol of beta-gal. Thus, our results indicate that SWATH could reproducibly quantitate peptides with concentrations as low as 2.5 fmol even in the presence of a complex background matrix.

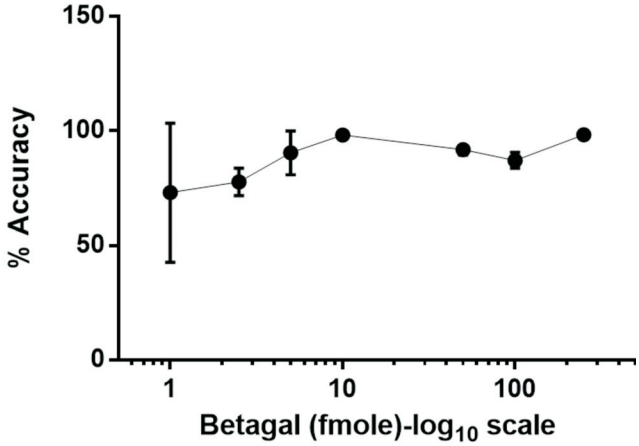

Figure 5: **Accuracy in quantification of different peptide (VDEDQPFPAVPK, IDPNAWVER, GDFQFNISR) of beta-gal at different concentration**

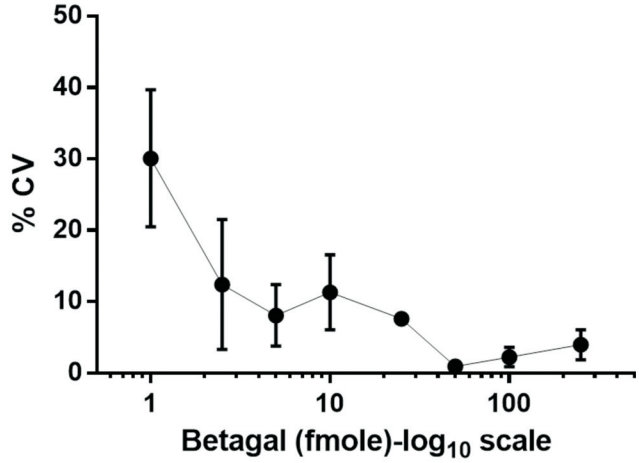

Figure 6: **Average %CV of quantification within replicates of different peptides (VDEDQPFPAVPK, IDPNAWVER, GDFQFNISR) of beta-gal at different concentration**

Conclusion

In conclusion, our study has demonstrated the depth of quantification of SWATH with respect to its reliability and accuracy. This study have further strengthened our previous observation that SWATH could be a vital tool for quantifying low abundant proteins

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Abbreviations

LC-MS, Liquid chromatography-mass spectrometry; SWATH, Sequential window acquisition of all theoretical fragment ion spectra; iTRAQ, Isobaric tag for relative and absolute quantitation; DTT, Dithiothreitol; DDA, Data dependent method of acquisition; IDA, Information dependent acquisition; MRM, Multiple reaction monitoring; IAA, Iodoacetamide; CAN, Acetonitrile; FA, Formic acid; TIC, Total Ion Chromatogram; XIC, Extracted ion chromatogram; CV, Coefficient of variation; Beta-gal, Beta-galactosidase.

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