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Article

Discovery Proteomics and Absolute Protein Quantification Can Be Performed Simultaneously on an Orbitrap-Based Mass Spectrometer

Taufika Islam Williams,* Cara Kowalchyk, Leonard B. Collins, and Benjamin J. Reading

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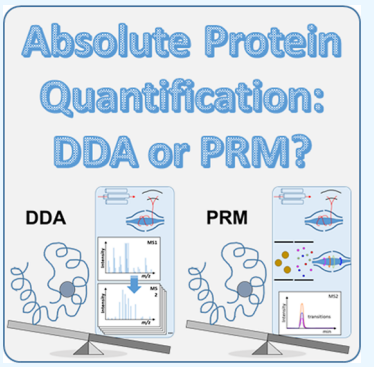
ABSTRACT: Mass spectrometry (MS) has steadily moved into the forefront of quantification-centered protein research. Protein cleavage isotope dilution MS is a proven way for quantifying proteins by using an isotope-labeled analogue of a peptide fragment of the parent protein as an internal standard. Parallel reaction monitoring (PRM) has become the *go-to* approach for such quantification on an Orbitrap-based instrument as it is assumed that the instrument sensitivity is enhanced. We performed a comparative study on data-dependent acquisition (DDA) and PRM-based workflows to quantify egg yolk protein precursors or vitellogenins (VTGs) Aa, Ab, and C in striped bass (*Morone saxatilis*). VTG proportions serve as a developmental measure of egg quality, possibly changing with the environment, and have been studied as an indicator of the health of North Carolina stocks. Based on single-factor analysis of variance comparisons of mean VTG amounts across fish from the same sample groupings, our results indicate that there is no statistical difference between MS1-based and MS2-based VTG quantification. We further conclude that DDA is able to deliver both discovery data and absolute quantification data in the same experiment.



INTRODUCTION

Mass spectrometry (MS) has served as a formidable analytical platform for the discipline of proteomics, cataloging proteins at an unprecedented scale and scope. Two major areas of MS-based proteomics have emerged: (1) discovery-based or "shot-gun" workflows that aim to capture as comprehensive a snapshot of the sample proteome as technologically feasible and (2) targeted workflows—a natural progression from discovery proteomics—that focus on the acquisition of additional, quantitative details on highly specific spaces of the sample proteome. The Orbitrap mass spectrometer has become indispensable for discovery proteomics, but creation of innovative quantification applications is also possible.[1]

Central to discovery proteomics is the data-dependent acquisition or DDA experiment. For a traditional DDA setup, an extracted/cleaned proteomic sample is proteolytically digested, chromatographed, and introduced into a high-resolution/accurate mass (HR/AM) instrument for mass analysis. Our most commonly used acquisition method for the Orbitrap-based Q Exactive HF-X automatically selects the "top-*N*" most abundant precursor ions from a full MS spectrum for subsequent MS/MS fragment analysis, where *N* is usually a value ≤40. Precursors may be fragmented only once, but the high mass resolving power of the QE-HFX, up to 240k at m/z 200, allows highly confident peptide identification from a single MS2 spectrum.[2] A well-optimized DDA workflow[3] then returns to the MS1 mode at regular intervals to interrogate newly chromatographed peptides. Regularly spaced acquisition of precursor ion data points creates integratable chromatographic peaks based on the MS1 scans. In perfecting the DDA experiment, we not only achieve unbiased global proteome interrogation of complex biological matrices but also retrieve quantitative information of precursor peptides.[3,4]

The typical absolute quantification utilizes parallel reaction monitoring (PRM)[5−8] in an Orbitrap-based instrument or a multiple reaction monitoring (MRM)/selected reaction monitoring (SRM)[9−12] in triple quadrupole (QqQ) platforms. In both types of mass spectrometers, precursor ions are selected for fragmentation in each experiment cycle. The major difference in fragmentation data is that while the QqQ aims to focus on a few pre-selected transitions per precursor, the Orbitrap-based platform[2,13−16] measures all transitions for that precursor over a mass range. Given the significant benefits that HR/AM instruments have brought to the table for discovery proteomics, Coon and co-workers[7] endeavored to leverage highly accurate mass measurement capabilities in the Orbitrap-based instrument in the targeted proteomics space. Coon *et al.* directly compared quantitative performance between an Orbitrap-based instrument and a triple quadrupole instrument with the conclusion that the PRM experiment yielded superior quantification overall. The experimental approach was to

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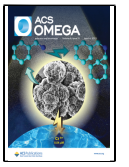

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emulate QqQ operation with the Orbitrap-based instrument for direct comparison, but this does not take advantage of its high mass resolving power. PRM has similar multiplexing capability to MRM but offers superior mass resolving power for both precursor and product ions. Despite being quite powerful, neither PRM nor MRM[9−11] is suitable for discovery-based applications. Of note is that DDA can be interlaced with a PRM experiment such that the instrument time not dedicated to the interrogation of specific target peptides can be applied toward global DDA. Notably, even such "hybrid" DDA/PRM analyses do not offer the depth of proteome coverage as afforded by traditional DDA due to acquisition time constraints.

The research question presented in this paper relies on isotope dilution MS (IDMS)[17] to compare MS1- and MS2-based quantification strategies. The use of stable isotope-labeled (SIL) internal standards has been staple in MS for normalizing a signal. Absolute proteomic quantification using stable isotopic peptides[18] entails spiking known concentrations of synthetic, heavy isotopologues of natural target peptides (from target proteins). Having essentially the same chemical properties, two peptide ions of an isotope pair are simultaneously introduced into a HR/AM mass spectrometer upon chromatographic co-elution and are unequivocally distinguished by their mass difference. IDMS normalizes the final mass spectrometric output by accounting for the variation at every stage of mass analysis, from initial ionization through subsequent ion transmission and fragmentation events.

The quantification of vitellogenin (VTG) proteins in striped bass (*Morone saxatilis*) ovaries presented a good test case to compare the results obtained in DDA and PRM experiments. Striped bass is an important recreational and commercial fish species native to North Carolina; however, there is limited natural replenishment in the Tar, Neuse, and Cape Fear rivers. The basis of population failure is unclear, rendering it important to understand how egg characteristics play a role in adaptations of striped bass early life stages, especially in environments of differing salinities. VTG proportions can serve as a developmental measure of egg quality, possibly changing with the environment.[19−21]

We employed DDA and PRM to quantify VTGs Aa, Ab, and C in striped bass ovaries to determine the effectiveness of each data acquisition strategy as absolute quantification platforms. Specifically, we intended to determine if a DDA experiment can provide absolute quantification of surrogate peptides from target proteins using precursor ions or if quantification using product ions in a PRM experiment is required. The nature of MS1 and MS2 data acquisition for DDA and PRM in an Orbitrap-based instrument leads us to hypothesize that both workflows would yield indistinguishable results.

MATERIALS AND METHODS

Chemicals and Materials. Optima LC/MS Grade water (Fisher catalog no. W6-4), Optima LC/MS Grade ACN (Fisher catalog no. A955-4), 1 M Tris-HCl, pH 8.0 (Fisher catalog no. BP1758-100), 1 M Tris-HCl, pH 7.5 (Fisher catalog no. BP1757-100), Pierce Standard LC/MS Grade BSA Protein Digest (Thermo Scientific catalog no. 88341), and Pierce Standard HeLa Protein Digest (Thermo Scientific catalog no. 88329) came from Thermo Fisher Scientific. Formic acid (Fluka catalog no. 94318-50ML) was obtained from Fluka. Dithiothreitol (Bio-Rad catalog no. 161-0611), urea (Bio-Rad catalog no. 161-0730), and iodoacetamide (Bio-Rad catalog no. 163-2109) were purchased from Bio-Rad. Zwittergent 3-16 detergent (Calbiochem catalog no. 693023) was obtained from Calbiochem. Calcium chloride, Technical Grade (Sigma catalog no. 222313-25G), came from Sigma. Trypsin GOLD, MS Grade (Promega catalog no. V528A), was purchased from Promega. Vivacon-500, 30 kDa MWCO filters (Sartorius catalog no. VN01H22ETO) came from Sartorius.

Striped Bass Ovary Biopsy. All experiments in the present work were approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC) protocol 13-041-A. Working in conjunction with North Carolina Wildlife Resource Commission and North Carolina Division of Marine Fisheries personnel during the annual spawning season (April to May 2018−2019), samples of eggs as ovarian biopsies were obtained from female striped bass (Table 1).[22]

Table 1. Striped Bass Samples and Their Origin[a]

sample identification	origin of striped bass (sample grouping)	salinity (ppt or parts per thousand)
CFR River no. 090 CFR River no. 100 CFR River no. 011 CFR River no. 015 CFR River no. 093 CFR River no. 057	Cape Fear River	high (5−25)
CFR Estuary no. 014 CFR Estuary no. 087	Cape Fear Estuary	high (5−25)
Neuse River no. 001 Neuse River no. 034 Neuse River no. 020 Neuse River no. 031 Neuse River no. 078 Neuse River no. 063 Neuse River no. 017	Neuse River	intermediate (0.5−5.5)
Neuse Estuary no. 157 Neuse Estuary no. 082 Neuse Estuary no. 073 Neuse Estuary no. 063	Neuse Estuary	intermediate (0.5−5.5)
RNK River no. 104 RNK River no. 074 RNK River no. 065 RNK River no. 080 RNK River no. 175 RNK River no. 170 RNK River no. 114 RNK River no. 052	Roanoke River	low (0−0.5)
ALB Estuary no. 158 ALB Estuary no. 103 ALB Estuary no. 067 ALB Estuary no. 002	Albemarle Estuary	low (0−0.5)

[a]Working in conjunction with North Carolina Wildlife Resource Commission and North Carolina Division of Marine Fisheries personnel during the annual spawning season (April to May 2018−2019), samples of eggs in the form of ovarian biopsies were obtained from female striped bass.

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The waters sampled for striped bass and their salinities are as follows:

1. High-salinity system (5−25 parts per thousand [ppt]): Cape Fear River (Cape Fear River Estuary)
2. Intermediate-salinity systems (0.5−5.5 ppt): Neuse/Tar River (Pamlico Sound)
3. Low-salinity system (0−0.5 ppt): Roanoke River (Albemarle Sound).

Pulse-DC boat electrofishing was used with two dip-netters located on the front of the boat to collect striped bass of all sizes. Fish were handled quickly and carefully to reduce stress but without MS-222 sedation. All fish were weighed (kg) and measured for total length (mm), and fin clips were taken for parentage-based genetics analyses. Water temperature and salinity were tested using a YSI (Yellow Springs Instruments) water quality sampling and monitoring meter and recorded upon arrival at each sample location, as salinity was a key factor in the hypotheses of egg characteristic adaptations.

Ovary biopsies were collected in accordance with Harrell and colleagues.[22] Briefly, a 3 mm diameter catheter was inserted 2−3 in. into the urogenital pore at a 30−45° angle, and mouth pipette suction was applied to draw egg samples into the catheter tube. The eggs were then divided *via* a pipette and transferred to the corresponding pre-labeled Nalgene 2 mL cryogenic vials and recorded in data sheets. Egg samples for proteomics analysis were immediately placed on dry ice. The samples were photographed to determine the ovary stage (13−15 h Bayless or post-vitellogenic stage oocytes are desired), transported on ice to North Carolina State University, and stored at −80 °C prior to proteomics analysis, as described elsewhere.[20,23,24] Digested protein samples were analyzed using *traditionally semiquantitative*, non-targeted proteomics approaches through DDA, as well as the more selective, *traditionally quantitative* protein cleavage isotope dilution MS (PC-IDMS) through PRM experiments.

SIL Peptide Internal Standards. The initial discovery proteomics experiments were performed to identify peptides appropriate for quantifying VTGs Aa, Bb, and C. The criteria for selection included peptide uniqueness compared to proteins available in the striped bass protein database (see the Data Analysis by Proteome Discoverer section), overall peptide length between 8 and 16 amino acids, and complete chromatographic separation from isobaric co-eluting peptides. SIL internal standard peptides were synthesized by New England Peptide (Gardner, MA) (Table 2) for absolute quantification work reported here and were characterized extensively elsewhere for absolute quantification of VTGs.[19] In an exploratory work, serial dilution experiments were performed with a representative sample of striped bass ovarian tissue and SIL standard peptides for VTGs Aa, Ab, and C to determine the appropriate spike-in amount of standards for absolute VTG quantification (Supporting Information Figure S1). SIL peptide addition was performed at the proteolytic digestion step of the proteomics sample preparation workflow (*vide infra*).

Table 2. Selected Surrogate Peptides Used for Quantifying VTGs Aa, Ab, and C

VTG (UniProt accession no.)	sequence of surrogate (SIL) peptide	precursor $[M + 2H]^{2+}$
Vtg Aa (A5GXQ1_MORAM)	H_2N-TEGLQEALL$\hat{\hat{K}}$-OH	551.3111 (light)
	$\hat{\hat{K}}$ = lysine ($^{13}C_6$)	554.3212 (heavy)
	10 residues (MW: 1107.59)	
Vtg Ab (A5GXQ2_MORAM)	H_2N-IATALVDTFAVA$\hat{\hat{R}}$-OH	674.3852 (light)
	$\hat{\hat{R}}$ = arginine ($^{13}C_6$)	677.3952 (heavy)
	13 residues (MW: 1353.74)	
Vtg C (A5GXQ3_MORAM)	H_2N-YFQATTLGLPLEIS$\hat{\hat{K}}$-OH	840.964 (light)
	$\hat{\hat{K}}$ = lysine ($^{13}C_6$)	843.9741 (heavy)
	15 residues (MW: 1686.93)	

Filter-Aided Sample Preparation. Dounce-homogenized ovary samples were thawed and diluted with Tris-buffered saline (20 mM Tris-HCl pH 8.0, 150 mM NaCl, and 2 mM $CaCl_2$) to a final protein concentration of 1 mg/mL. Protein concentrations of the digests were obtained using a NanoDrop at A280 (Thermo Scientific, Wilmington, DE). A modified filter-aided sample preparation (FASP) protocol was used to prepare ovary samples from each fish.[25−27] Briefly, samples were incubated for 30 min at 56 °C with 15 μL of 50 mM dithiothreitol or DTT (in 0.1 M Tris-HCl pH 8.0) per 200 μL (200 μg) of the sample to reduce disulfide bonds. Proteins were denatured by adding 200 μL of 8 M urea in 0.1 M Tris-HCl, pH 8.0, to each sample. This was followed by the transfer of each sample onto a separate Vivacon 500 30 kDa molecular weight cutoff or MWCO filter (Sartorius Stedim Biotech, Goettingen, Germany); the filtration units were centrifuged at 12,000*g* for 15 min at 21 °C. In an effort to ensure protein denaturation, an additional 200 μL of 8 M urea in 0.1 M Tris-HCl pH 8.0 was added to each sample on MWCO filters. On-filter alkylation was performed by adding 64 μL of 200 mM iodoacetamide or IAA (50 mM final concentration) to the second aliquot of 8 M urea in the sample MWCO filters. The samples were incubated in the dark at 37 °C for 1 h and then centrifuged at 12,000*g* for 15 min at 21 °C. Each filter was washed three times with 100 μL of 2 M urea/10 mM $CaCl_2$, followed by centrifugation (after each wash) for 10 min at 12,000*g*. This was proceeded by three washes with 100 μL of 0.1 M Tris-HCl pH 7.5, followed by centrifugation (after each wash) for 10 min at 12,000*g*. All flow-through solutions were discarded up to this point. Following a change to fresh collection tubes for each MWCO filtration unit, modified trypsin, freshly prepared in 0.1 M Tris-HCl pH 7.5, was added to each sample at an enzyme-to-protein ratio of 1:5. This trypsin solution contained one SIL internal standard surrogate peptide (at known concentrations) for each VTG of interest. After overnight digestion at 37 °C, trypsinization was quenched with 50 μL of 0.001% Zwittergent 3-16 (Calbiochem, La Jolla, CA)/1% formic acid, and tryptic peptides were collected by centrifugation at 12,500*g* for 15 min at 21 °C. A second quench/elution step was carried out with 400 μL of 0.001% Zwittergent 3-16 (Calbiochem, La Jolla, CA)/1% formic acid, and tryptic peptides were collected by centrifugation at 13,000*g* for 30 min at 21 °C to maximize tryptic peptide recovery. The samples were dried using a SpeedVac (Thermo Fisher Scientific, San Jose, CA) and then stored at −20 °C until nano-LC−MS/MS analyses.[19]

Nano-LC−MS/MS Analysis. All samples were analyzed in triplicate (technical replicates) by both DDA-based and PRM-based nano-LC−MS/MS workflows. The samples were reconstituted to a protein concentration of 0.2 μg/μL using mobile phase A or MPA (98/2/0.1% water/acetonitrile/formic acid). Two microliters of each sample was subjected to online desalting and reversed-phase nano-LC separation ("trap and

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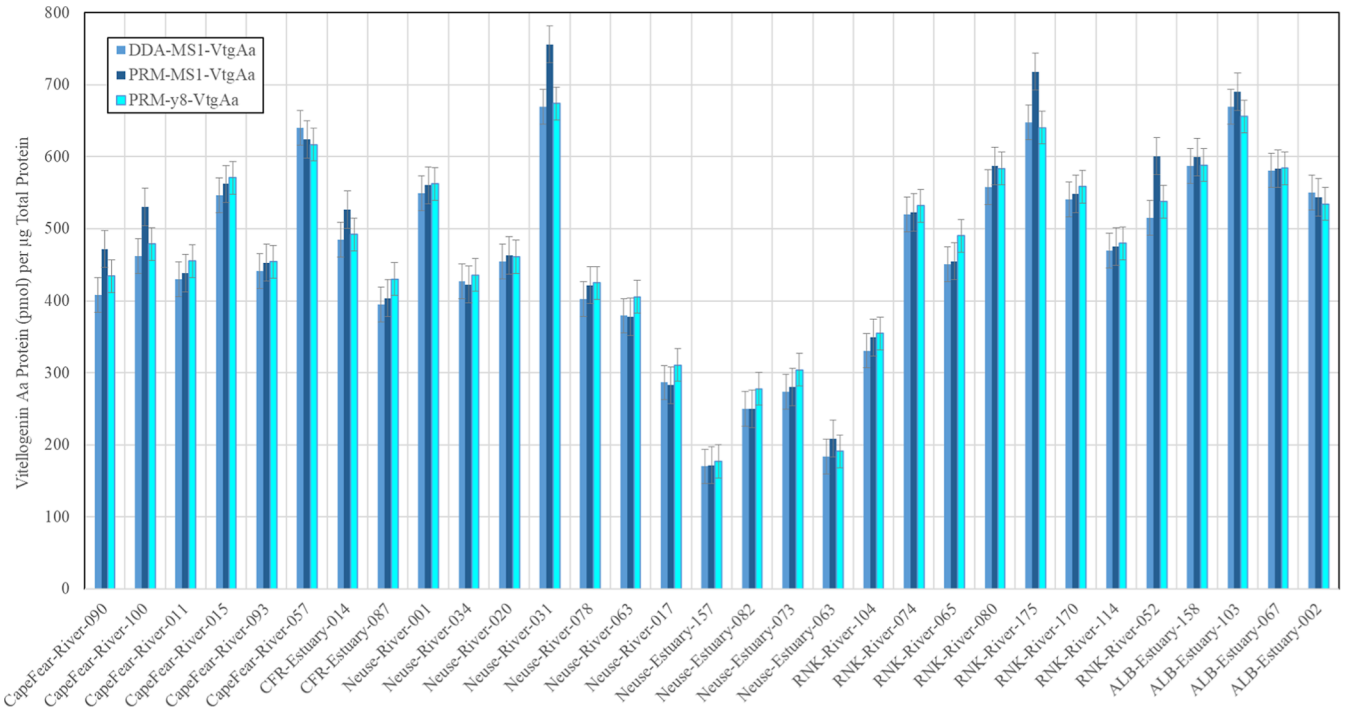

Figure 1. Absolute quantification of VTG Aa in striped bass: DDA and PRM analyses.

elute" configuration) on a Thermo EASY nano-LC 1000 system coupled to a Q-Exactive high-field X mass spectrometer with an EASY-spray ion source (Thermo Scientific, Bremen, Germany). Reversed-phase separation was performed on a 25 cm (2 μm particle size, 100 Å pore size, and 75 μm diameter) PepMap C18 column from Thermo Fisher Scientific (San Jose, CA). The trap column was a 2 cm (3 μm particle size, 100 Å pore size, and 75 μm diameter) Acclaim PepMap C18 column which is also from Thermo Fisher Scientific (San Jose, CA). Analytical separations were run on a nano-flow pump at 300 nL/min, initially maintaining 5% MPB.

The nano-LC−MS/MS data were collected using a DDA method for discovery and targeted proteomics and a PRM method for targeted proteomics only. In the DDA analyses, a flow rate of 300 nL/min was set, along with an initial condition of 5% mobile phase B or MPB (2/98/0.1% water/acetonitrile/formic acid). The % MPB was increased to 25% over 105 min, followed by a ramp to 40% over 15 min. The gradient was then steeply ramped to 95% MPB over 1 min and maintained at 95% mobile phase B for 17 min to wash the column. The column was then re-equilibrated at 5% MPB prior to the next run. The DDA method used to analyze the samples involved a full-MS top 40 data-dependent MS/MS (dd-MS/MS) analysis, performed with the following parameters: spray voltage of +1800 V, capillary temperature of 275 °C, funnel RF level of 40 V, 400−2000 m/z scan range, 120k MS resolving power, 3×10^6 MS AGC target, MS max IT 100 ms, 7.5k MS/MS resolving power, 1×10^5 MS/MS AGC target, 18 ms MS/MS max IT, 1.5 m/z isolation window, 27 normalized collision energy, and a dynamic exclusion of 20 s. Unassigned and +1 charges were excluded from selection for MS/MS, and peptide match was set to "preferred".

PRM analyses applied a flow rate of 300 nL/min, along with an initial condition of 5% MPB. The % MPB was increased to 40% over 120 min. The gradient was then steeply ramped to 100% MPB over 1 min and maintained at 100% mobile phase B for 6 min to wash the column. There was a subsequent sharp drop to 100% MPA over 1 min, and the column was maintained under those conditions for 10 min. The column was then re-equilibrated at 5% MPB prior to the next run. The PRM method used to analyze the samples involved the following parameters: spray voltage of +1800 V, capillary temperature of 275 °C, funnel RF level of 40 V, 500−900 m/z scan range, 120k MS resolving power, 3×10^6 MS AGC target, MS max IT 100 ms, 15k MS/MS resolving power, 2×10^5 MS/MS AGC target, 50 ms MS/MS max IT, 1.5 m/z isolation window, 12 loop count, and 27 normalized collision energy. Unassigned and +1 charges were excluded from selection for MS/MS.

Data Analysis by Proteome Discoverer. Raw nano-LC−MS/MS files were analyzed using Proteome Discoverer 2.2 (Thermo Scientific, San Jose, CA) with the Sequest HT search engine. In database searching, a composite FASTA file—consisting of the six open reading frame (ORF)-translated striped bass genome (Reading *et al.*, NCBI ID: 10722), six ORF-translated ovary transcriptome (GenBank: SRX007394),[28] and target VTG sequences VTG Aa, VTG Ab, and VTG C (GenBank accession DQ020120.1, DQ020121.1, and DQ020122.1, respectively)[21,28,29]—was used in the interrogation of experimental samples, as well as to perform protein identifications (and subsequent VTG quantifications) from experimental nano-LC−MS/MS data. Sequences corresponding to human keratins and porcine trypsin were also included in the database. The data were searched using the following parameters: trypsin as the enzyme which performs *in silico* digestion of the target database proteins at arginine (R) and lysine (K) residues, fixed carbamidomethyl modification of cysteine residues, variable oxidation of methionine, variable deamidation of asparagine and glutamine, maximum of two missed cleavages, 5 ppm precursor tolerance, and 0.02 Da MS/MS tolerance. Data were filtered at a 1% peptide FDR using the percolator node. The

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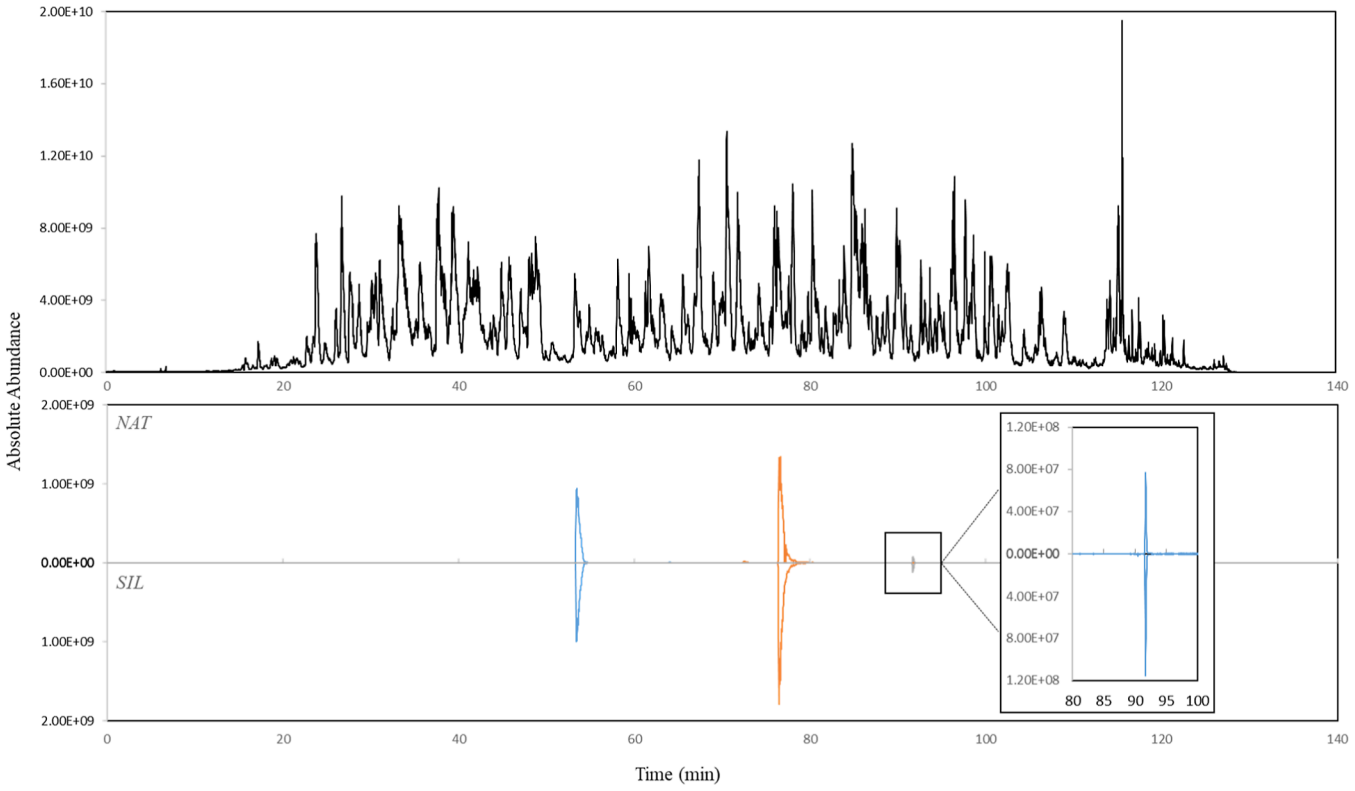

Figure 2. DDA-MS1—TIC with extracted ion chromatograms for $[M + 2H]^{2+}$ NAT and SIL peptides from target VTGs Aa, Ab, and C. The ovarian biopsy sample came from striped bass no. 52 from the Roanoke River. The DDA nano-LC−MS/MS run no. 3 is shown here.

"precursor ions quantifier" node was used to perform quantification by peak area for fold-change analysis.

Data Analysis by Skyline and Statistical Work. Data from DDA and PRM nano-LC−MS/MS experiments were imported into Skyline v.19.1.0.19336 where reproducible co-elution of native and SIL peptides along with their respective transitions was used to confirm the presence of target peptides. Peak integration was manually verified and adjusted in order to ensure consistent integration across injections. Data were exported into Excel and the peak area and abundance ratios of NAT-to-SIL peptides were multiplied by the amount of each SIL peptide to obtain absolute quantities in picomoles (pmol) of VTG protein/μg of total protein (See Figure 1 for VTG Aa data). Dispersion is reported as standard error of the mean. Tabulated quantification data for all the three VTGs is given in Supporting Information S05−S07. Graphs for VTG Ab and C are provided in the Supporting Information (S08−S09).[19] Other nano-LC/MS data were evaluated using Skyline, such as retention times, points across peaks, mass accuracy, and apex peak heights.

Single-factor analysis of variance (ANOVA) was performed using the "ANOVA: single factor" function in the "Data Analysis" add-in package of Microsoft Excel software. The sample groups, degrees of freedom, *F*-values, and *p*-values are shown in Supporting Information (S10). The α values was set to 0.05.

RESULTS AND DISCUSSION

The traditional PC-IDMS-based, bottom-up proteomics workflow for protein quantification in biological samples using MS involves multiple analytical steps. First is to reproducibly identify digested peptides that can serve as representatives or surrogates for proteins of interest using DDA experiments. Once a consistently detectable set of peptides is found, the second step involves synthesis of SIL analogues for absolute quantification of target proteins. Many times, as in the present study, full proteome coverage is still desired, so the third step still applies DDA on study sample protein digests. Since the ultimate goal is absolute quantification of specific proteins, a fourth step using PRM acquisition is performed. Our experiments followed this pattern, whereby surrogate peptides for target VTGs had been identified in prior work,[19] full proteome interrogation was desired in experiments by applying a long, discovery-based DDA analysis, and a shorter, targeted PRM analysis was performed expecting more precise quantification of surrogate peptides for absolute quantification of target VTGs.

The quantification results for VTG Aa are shown in Figure 1 (refer to Supporting Information S08−S09 for data on VTGs Ab and C) as calculated from precursor and y8 product ions using PRM and precursor ions using DDA MS experiments. Tabulated quantification data for all the three VTGs is provided in Supporting Information S05−S07. The amounts ranged from 169.9 ± 0.4 to 669.6 ± 8.2 pmol/μg total protein (DDA-MS1), 171.8 ± 0.7 to 755.9 ± 17.9 pmol/μg total protein (PRM-y8 or y8 ion from PRM), and 177.1 ± 1.9 to 673.9 ± 5.1 pmol/μg total protein (PRM-MS1) for VTG Aa; 561.3 ± 1.0 to 2469.4 ± 5.1 pmol/μg total protein (DDA-MS1), 601.8 ± 16.8 to 2559.5 ± 4.8 pmol/μg total protein (PRM-y8), and 534.0 ± 6.2 to 2549.6 ± 41.1 pmol/μg total protein (PRM-MS1) for VTG Ab; and 10.4 ± 0.1 to 47.8 ± 0.1 pmol/μg total protein (DDA-MS1), 10.7 ± 0.1 to 44.5 ± 1.3 pmol/μg total protein (PRM-y8), and 10.4 ± 0.1 to 47.4 ± 0.2 pmol/μg total protein (PRM-MS1) for VTG C. The representative chromatograms displaying total ion chromato-

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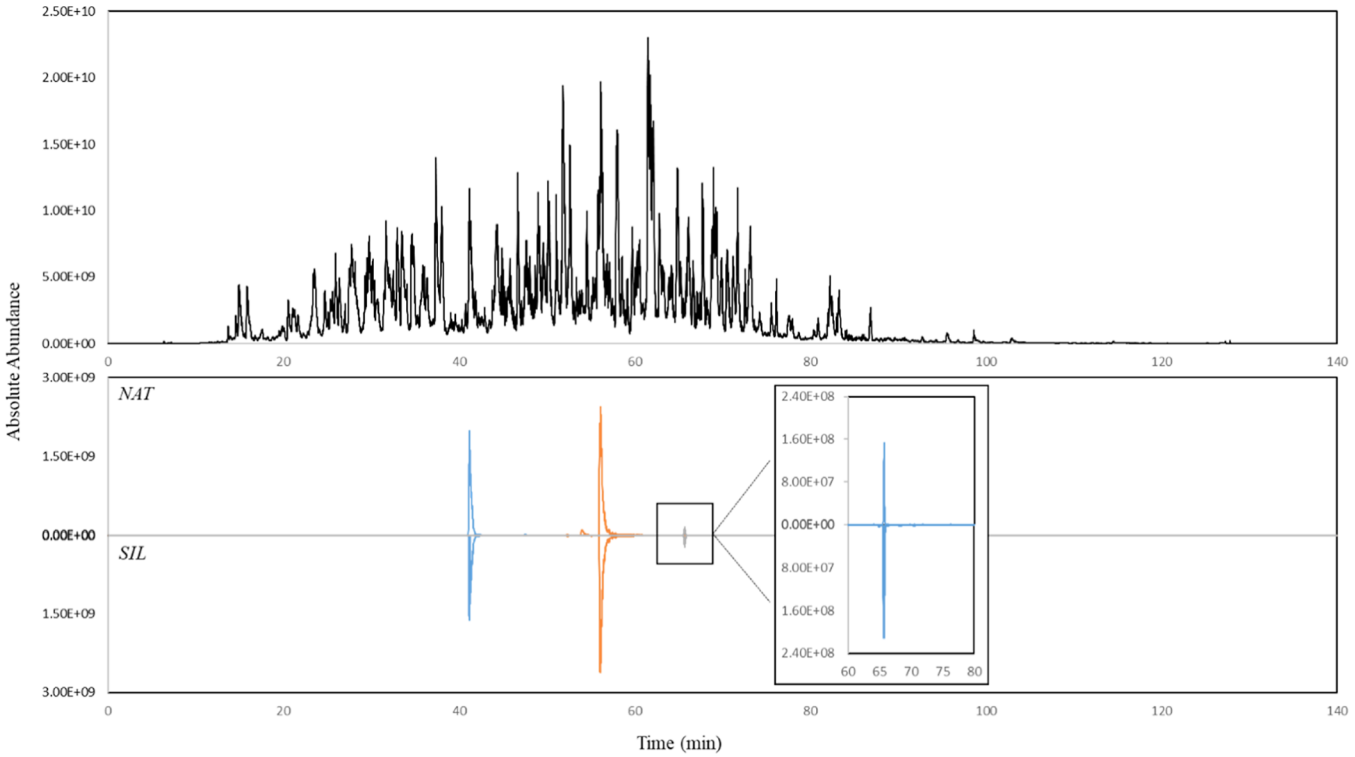

Figure 3. PRM (PRM-MS1)—TIC with extracted ion chromatograms for $[M + 2H]^{2+}$ NAT and SIL peptides from target VTGs Aa, Ab, and C. The ovarian biopsy sample came from striped bass no. 52 from the Roanoke River. The PRM nano-LC−MS/MS run no. 3 is shown here.

Table 3. Comparison of Points across a Peak for DDA and PRM (SIL)[a]

peptide	DDA-MS1 avg	DDA-MS1 σ	PRM-MS1 avg	PRM-MS1 σ	PRM-y8 avg	PRM-y8 σ
TEGLQEALLK (VtgAa)	83	28	91	18	92	15
IATALVDTFAVAR (VtgAb)	92	41	128	43	136	38
YFQATTLGLPLEISK (VtgC)	30	16	58	14	60	12

[a]Top-*N* of 40 was used in the MS method, so the top 40 most intense precursors were selected for MS2. However, every precursor is still captured by the full scan (MS1), which is what is being used for quantification. The number of data points obtained for a precursor ion chromatographic peak is sufficient in a DDA analysis to obtain accurate integrations. Cycle time dictates the number of precursor data points obtained. As long as the cycle time is short enough to acquire 10 points or more for a precursor ion,[31] peak integration is reliable. The higher number of data points acquired in the PRM analysis is due to a shorter experiment cycle between precursor scans. However, as long as an acceptable number of data points are collected in either case, the final integrated areas will be comparable.

grams (TICs) and extracted ion chromatograms for NAT and SIL peptide precursors (MS1) of target VTGs acquired by DDA and PRM are shown in Figures 2 and 3, respectively. The MS2 data of the same used for peptide identification is described in Figures S02−S04 in the Supporting Information. These example data were selected from the ovarian biopsy of striped bass no. 52 from the Roanoke River (nano-LC−MS/MS replicate no. 3).

The limit of detection (LOD) and limit of quantification (LOQ) for each VTG were calculated in a prior experiment using the PRM method by injecting a calibration series of the SIL peptides spiked into 200 μg of the striped bass ovary protein matrix (Supporting Information S01). The LOD and LOQ results for each peptide were 0.27 pmol/μg total protein and 0.81 pmol/μg total protein for VTG Aa, 0.36 pmol/μg total protein and 1.1 pmol/μg total protein for VTG Ab, and 0.44 pmol/μg total protein and 1.3 pmol/μg total protein for VTG C, as shown in Supporting Information S10. The LOD for each peptide was calculated according to the method of Miller and Miller,[30] and the LOQ was established as 3× LOD. The lowest calculated amounts for the NAT VTG peptides were 169.9 pmol/μg total protein for VTG Aa, 561.3 pmol/μg total protein for VTG Ab, and 10.4 pmol/μg total protein for VTG C using the DDA-MS1 method (Supporting Information S05−S07). The lowest calculated amounts for the NAT VTG peptides were 171.8 pmol/μg total protein for VTG A, 534.0 pmol/μg total protein for VTG Ab, and 10.4 pmol/μg total protein for VTG C using the PRM-MS1 method (Supporting Information S05−S07). Lastly, the lowest calculated amounts for the NAT VTG peptides were 171.8 pmol/μg total protein for VTG A, 601.8 pmol/μg total protein for VTG Ab, and 10.7 pmol/μg total protein for VTG C using the PRM-y8 method (Supporting Information S05−S07).

In both of the PRM and DDA acquisition methods described here, the instrument was required to make a full mass range scan in a given cycle time window. The time allotted for a full acquisition cycle included specific maximum times to perform a full mass range scan to detect and measure precursor ions, isolate precursor ions, produce product ions, and make mass measurements of product ions. Individual precursor isolation and product ion mass scans continued until the allotted time for the full acquisition experiment expired.[2] In

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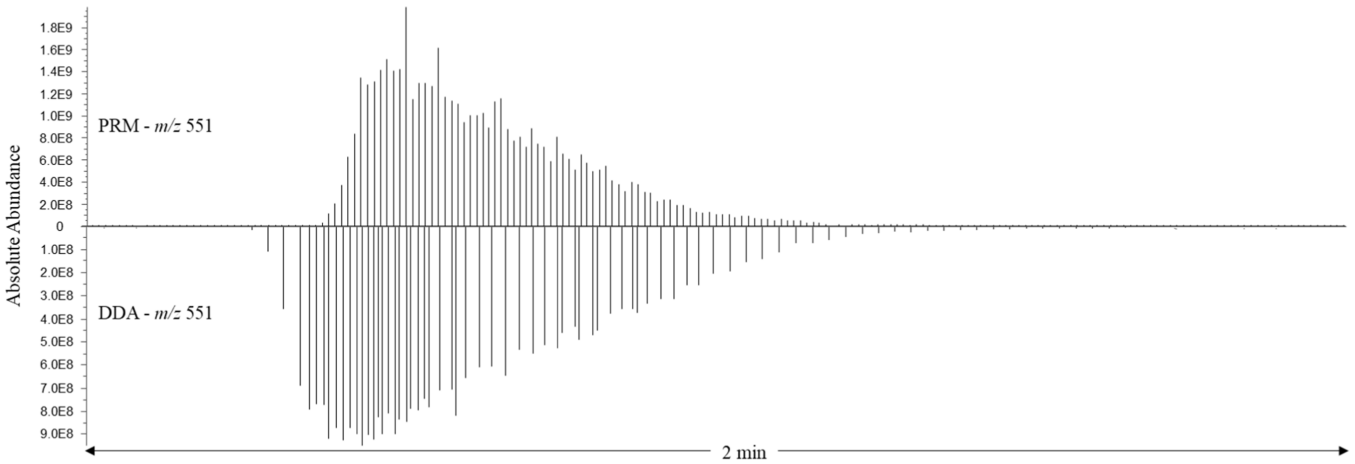

Figure 4. Spacing of MS1 events in PRM-MS1 and DDA-MS1 analyses for doubly charged $[M + 2H]^{2+}$ peptide TEGLQEALLK from VTG Aa. The ovarian biopsy sample came from striped bass no. 52 from the Roanoke River. The PRM and DDA nano-LC−MS/MS run no. 3 is shown here.

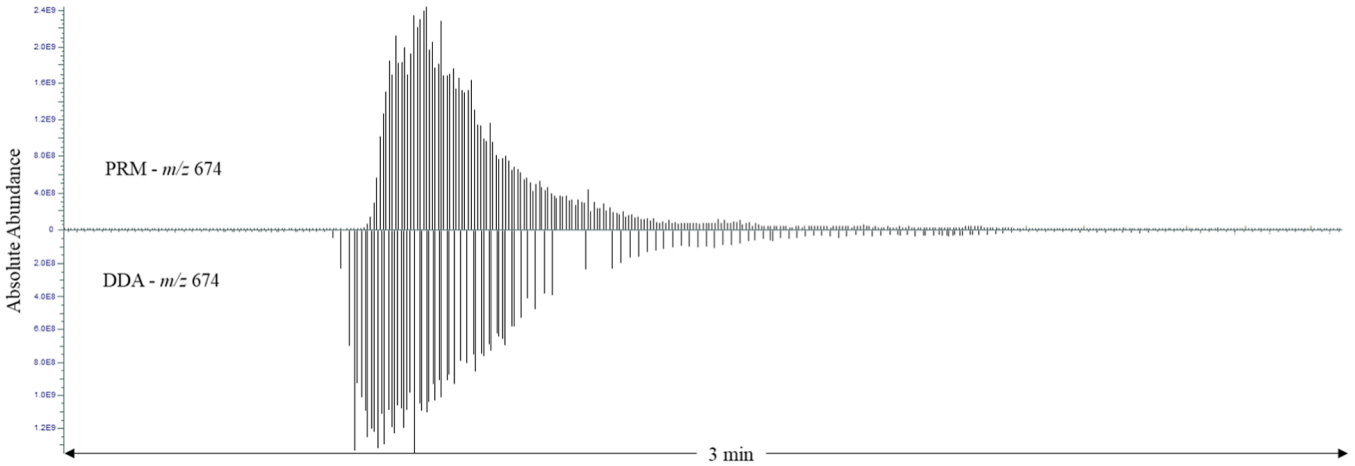

Figure 5. Spacing of MS1 events in PRM-MS1 and DDA-MS1 analyses for doubly charged $[M + 2H]^{2+}$ natural peptide IATALVDTFAVAR from VTG Ab. The ovarian biopsy sample came from striped bass no. 52 from the Roanoke River. The PRM and DDA nano-LC−MS/MS run no. 3 is shown here.

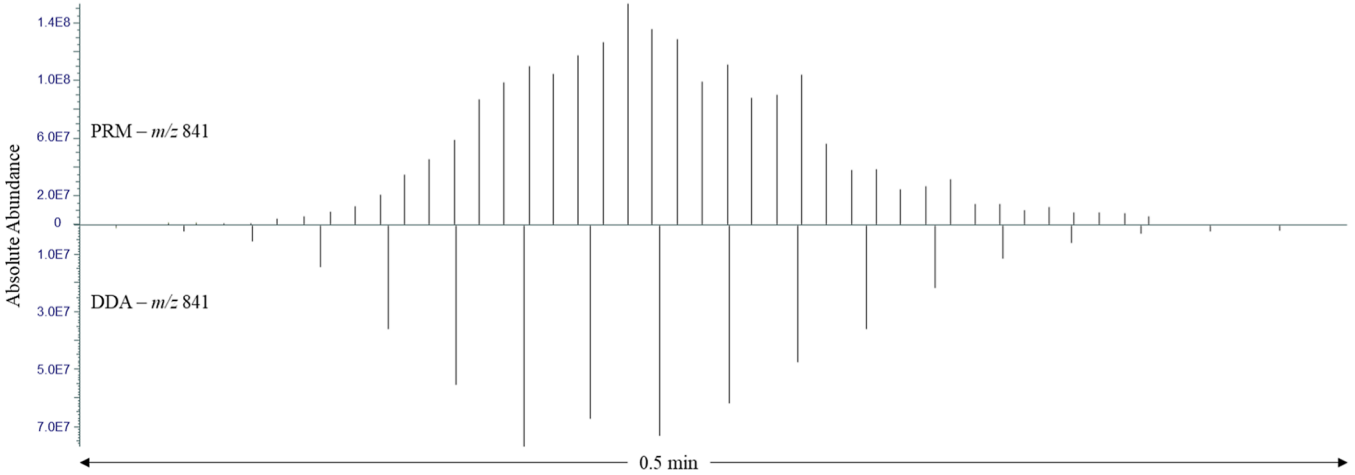

Figure 6. Spacing of MS1 events in PRM-MS1 and DDA-MS1 analyses for doubly charged $[M + 2H]^{2+}$ natural peptide YFQATTLGLPLEISK from VTG C. The ovarian biopsy sample came from striped bass no. 52 from the Roanoke River. The PRM and DDA nano-LC−MS/MS run no. 3 is shown here.

our DDA experiment, the method required a precursor ion scan approximately every 1.54 s, while the PRM experiment required a precursor ion scan approximately every 0.7 s. Both PRM and DDA techniques provided regular collection of data points and simultaneous detection of NAT and SIL VTG peptides. Table 3 shows the average number of data points

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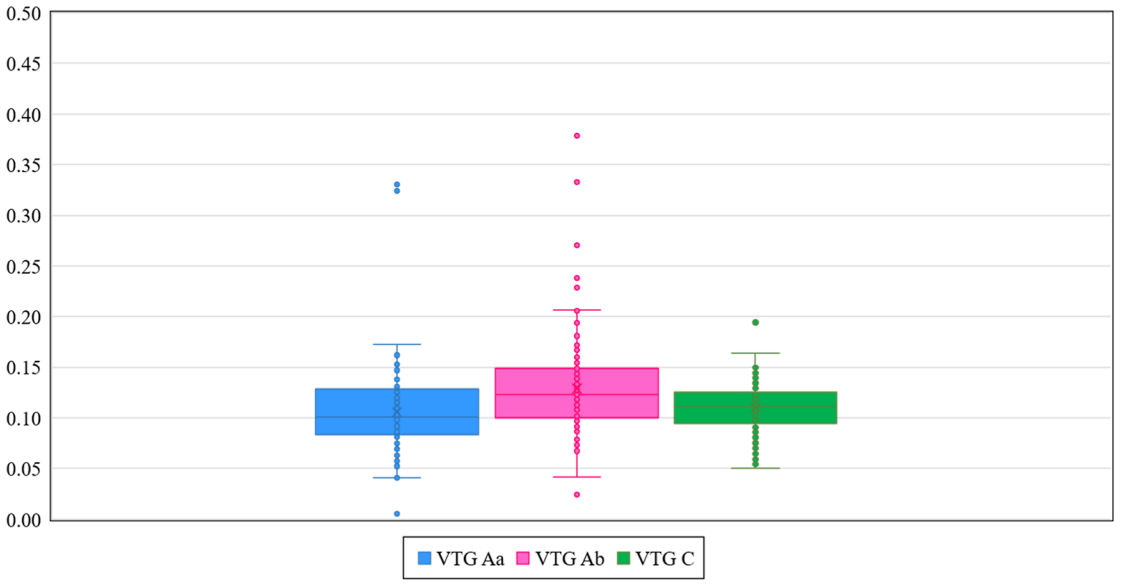

Figure 7. SIL peptide peak area ratio PRM-y8/DDA-MS1. This ratio shows the relative reduction in the number of ions actually detected for the y-8 ion vs the precursor ion. There is not a *1-to-1 precursor-to-product* ion conversion in the fragmentation process in any mass spectrometer.

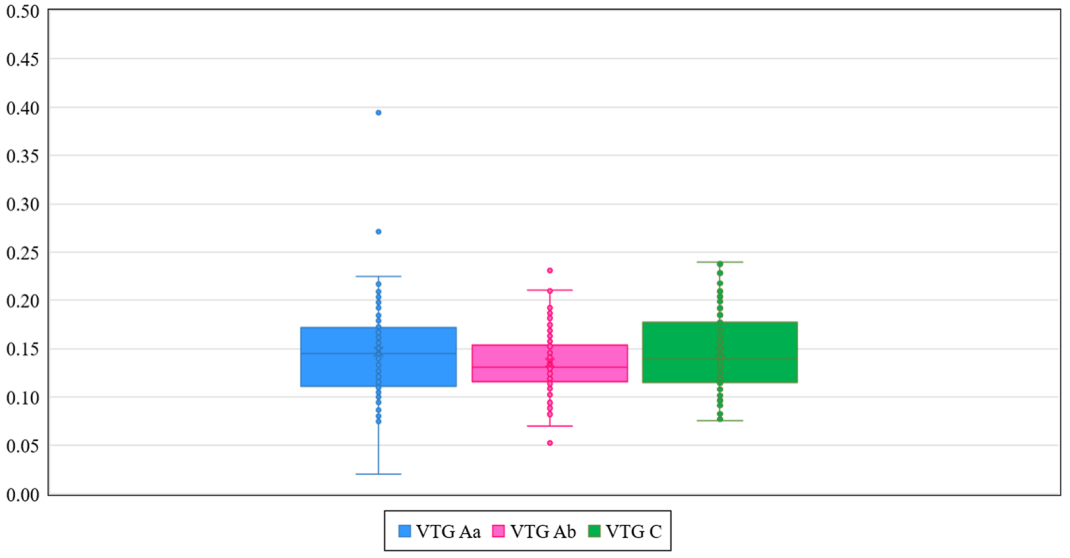

Figure 8. SIL peptide precursor ion abundance (peak height) ratio PRM-y8/DDA-MS1. This ratio shows the relative reduction in the number of ions actually detected for the y-8 ion *vs* the precursor ion. There is not a 1-to-1 precursor-to-product ion conversion in the fragmentation process in any mass spectrometer.

across chromatographic peaks for the SIL VTG peptides as collected in the PRM and DDA experiments.

The average number of data points across all samples is shown. The SIL peptide from VTG Ab displayed the widest chromatographic peak in DDA-MS1 and showed the most data points with an average of 92 ± 41. The VTG Aa SIL peptide displayed the next widest chromatographic peak in DDA-MS1, showing an average of 83 ± 28 points across the peak. The SIL peptide from VTG C had the narrowest peak with an average of 30 ± 16 points. PRM analyses yielded more points per peptide chromatographic peak for both precursor (MS1) and y8 product ions. For PRM precursors, we observed 128 ± 43 points across the peak for the VTG Ab peptide, 91 ± 18 points across the peak for the VTG Aa peptide, and 58 ± 14 points across the peak for the VTG C peptide. For PRM product ion y8, the numbers were similar to those obtained for PRM-MS1 (Table 3). If we use the liberal benchmark of 10 data points required for accurate chromatographic peak integration,[31] then both PRM and DDA techniques provided a sufficient number of data points to accurately integrate each VTG peptide peak, whether derived from MS1 or MS2 scans.

Closer inspection of the acquired data points shows a difference in spacing in time between DDA and PRM. Figures 4−6 show traces of VTG peptides in the "stick mode" in which the individual data point intensities across peptide peaks are revealed. These data were also collected from the ovarian biopsy sample that came from striped bass no. 52 (replicate no. 3) from the Roanoke River. The presence of irregularly spaced points in DDA chromatograms is due to the mass spectrometer taking precursor scans before the specified 1.54 s requirement. This occurs when too few precursor ions that exceed the abundance threshold for MS2 are selected in a full scan to

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require the full experiment time of 1.54 s to complete all of the MS2 scans. In this way, the full experiment time of 1.54 s stands as an upper limit of time between precursor scans but does not force the mass spectrometer to wait the full 1.54 s when the automatically selected list of precursors has been interrogated. The PRM acquisition method, however, forces a full scan at a specified experiment time limit of 0.7 s to ensure regular measurement of NAT and SIL peptide precursors in the targeted m/z list. The total number of data points collected with both DDA and PRM show that either technique can reliably provide enough points to accurately describe the NAT and VTG peptide chromatographic peaks.

The timing of MS2 scans was different between PRM and DDA, but both methods still produced confirmatory amino acid sequence data to identify NAT and SIL VTG peptides. Because the PRM method forced the mass spectrometer to take a MS2 scan for each of the targeted precursors during every 0.7 s experiment cycle, chromatographic traces of product ions could be recorded. There were fewer MS2 data points for the VTG peptides using the DDA method because the precursor ion was added to an exclusion list for 20 s after an MS2 scan was completed. Exclusion lists are commonly used in DDA to acquire as many MS2 scans as possible on unique precursors, whereas PRM forces MS2 acquisition only for targeted precursors. Both acquisition techniques, however, ensured that NAT and SIL VTG peptide identities could be confirmed with at least one MS2 scan.

Quantification of the target VTG surrogate peptides was achieved by integrating the chromatographic peaks (peak area) and calculating the peak ratios of the NAT and SIL precursor ions (Figure 1 and Supporting Information S05−S09). Because the same amount of a given SIL peptide was spiked into all samples, we compared average SIL peptide areas from all samples between PRM and DDA results. Figure 7 shows box and whisker plots of the ratio of PRM-y8 peak areas to DDA-MS1 peak areas. Clearly, the PRM-y8 average peak areas are lower due to the expected decrease in total number of product ions obtained after collision-induced dissociation. The apex peak heights were also compared, as shown in Figure 8. In contrast, the peak area and peak height ratios of precursor ions differed far less between PRM-MS1 and DDA-MS1 (Supporting Information S11−S12). The notable difference (in PRM-MS1 *vs* DDA-MS1) lies with the VTG C peptide, but the higher peak apex acquired by PRM is easily explained by chromatographic peak compression due to the sharper gradient purposefully used in the PRM method. Even so, the abundance advantage given by the narrower VTG C peak in the PRM-MS1 method was only ∼1.5 times the abundance measured by DDA-MS1 in the peak-area-based analysis (Supporting Information S11), and it was about 2 times that of the intensity-based analysis (Supporting Information S12). The shorter gradient and peak compression of VTG C described earlier yielded no advantage for our purpose of quantification. ANOVA (Supporting Information S13) confirmed that there was no statistical difference between the final VTG Aa, VTG Ab, and VTG C amounts measured across the sample groups regardless of the data acquisition method employed.

CONCLUSIONS

Bottom-up or shotgun proteomics workflows achieve protein identification by analysis of peptide fragments generated by proteolytic digestion of intact proteins, and a mainstay of these workflows is MS1-based quantification. Given an internal standard, signal response factors (counts/mol) allow accurate quantification for all proteins tested.[32] The PC-IDMS technique is well established in QqQ-based acquisitions, and we have shown that quantification is achieved with MS1 acquisition in an FTMS-based instrument.

The simplicity and sensitivity of the method, coupled with the widespread availability of tandem mass spectrometers, make the PC-IDMS strategy a highly useful procedure for measuring the levels of proteins and post-translational modifications directly from cell lysates. In QE-based instruments such as the one employed in these studies, the PRM workflow is typically used for PC-IDMS experiments, with the instrument time being specifically devoted to target peptides. We have demonstrated that a traditional DDA experiment using a PC-IDMS workflow provides detailed discovery data and absolute quantification data in a single nano-LC−MS/MS run. Single-factor ANOVA was performed on sample group mean values calculated from the respective MS method results, and there was no significant difference observed. The statistically indistinguishable results we obtained for DDA-based and PRM-based analyses rely on the key observation that the precursor ion measurement in both experiments—the bedrock of MS1 quantification—is performed in essentially the same manner. Ongoing research will allow confident quantification of VTGs in striped bass ovaries, but the data acquisition strategies detailed here will also be extended to other quantification projects.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c07614.

Standard curve for VTG SIL peptide chromatographic response; Extracted Ion Chromatograms (EICs) for NAT and SIL VTG Aa, DDA, and PRM analyses. No smoothing algorithms were applied; Extracted Ion Chromatograms (EICs) for NAT and SIL VTG Ab, DDA, and PRM analyses. No smoothing algorithms were applied; Extracted Ion Chromatograms (EICs) for NAT and SIL VTG C, DDA, and PRM analyses. No smoothing algorithms were applied; Absolute quantification of VTG Aa in striped bass: tabulated DDA and PRM analyses; Absolute quantification of VTG Ab in striped bass: tabulated DDA and PRM analyses; Absolute quantification of VTG C in striped bass: tabulated DDA and PRM analyses; Absolute quantification of VTG Ab in striped bass: graphical DDA and PRM analyses; Absolute quantification of VTG C in striped bass: graphical DDA and PRM analyses; LOD and LOQ for target VTGs determined by PRM-MS1 analyses; SIL peptide peak area ratio PRM-MS1/DDA-MS1; SIL peptide precursor ion intensity (peak height) ratio PRM-MS1/DDA-MS1; and Single-factor ANOVA comparison of final VTG amounts found using DDA-MS1, PRM-MS1, and PRM-y8 analyses. The results from all technical replicates were included in this analysis. Degrees of freedom (df) equals N−1. Alpha was set to 0.05 for p-value threshold. All calculated F-statistics were lower than the critical Fthreshold, and all p-values were above 0.05.(PDF)

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AUTHOR INFORMATION

Corresponding Author

Taufika Islam Williams − *Molecular, Education, Technology, and Research Innovation Center (METRIC), North Carolina State University, Raleigh, North Carolina 27606, United States; Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States;* orcid.org/0000-0002-5838-4647; Phone: 919-513-7791; Email: Taufika_Williams@ncsu.edu

Authors

Cara Kowalchyk − *Marine Fisheries, Department of Environmental Quality, Raleigh, North Carolina 27603, United States*

Leonard B. Collins − *Molecular, Education, Technology, and Research Innovation Center (METRIC), North Carolina State University, Raleigh, North Carolina 27606, United States*

Benjamin J. Reading − *Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina 27695, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c07614

Notes

The authors declare no competing financial interest.

The raw data can be found at PanoramaWeb (NCSU-METRIC): 20230216 - DDA-PRM paper on Striped Bass VTGs.

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