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Comparing SRM and SWATH Methods for Quantitation of Bovine Muscle Proteomes

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Supporting Information

ABSTRACT: Mass spectrometry (MS) has become essential for efficient and accurate quantification of proteins and proteomes and, thus, a key technology throughout all biosciences. However, validated MS methods are still scarce for meat quality research applications. The objective of this work was to develop and compare two targeted proteomic methods, namely, selected reaction monitoring (SRM) and sequential window acquisition of all theoretical spectra (SWATH), for the quantification of 11 bovine muscle proteins that may be indicators of meat color. Both methods require evaluation of spectra from proteotypic and quantotypic peptides, and we here report our evaluation of which peptides and MS parameters are best suited for robust quantification of these 11 proteins. We observed that the SRM approach provides better reproducibility, linearity, and sensitivity than SWATH and is therefore ideal for targeted quantification of low-abundance proteins, while the SWATH approach provides a more time-efficient method for targeted protein quantification of high-abundance proteins and, additionally, supports the search for novel biomarkers.

KEYWORDS: *label-free quantitation, SRM, SWATH, Bos taurus, proteomics, meat color*

INTRODUCTION

In the postgenome era, a wide range of mass spectrometry (MS)-based proteome analysis methods have been developed, which provide valuable insight into proteins and their expression levels directly from complex tissue and body fluid samples.[1,2] The exact identification and quantitation of proteins are essential for a better understanding of biological processes in health and disease. Within food sciences, exact protein assays are invaluable for the development of new food safety and quality measures for protein-based foods like meat and milk.[3] Moreover, precise quantification of specific proteins in tissues and body fluids provides valuable insights and validation of potential biomarkers for, e.g., disease stages.[4]

Selected reaction monitoring (SRM), sometimes also termed multiple-reaction monitoring (MRM), is currently the method of choice for sensitive protein analyses. SRM is suited to quantification of attomolar to millimolar amounts of a specific protein within a body fluid[5] or equivalent to measuring in the range of 50 to >1 million copies of a specific protein within a cell.[6] SRM methods rely on using a triple-quadrupole (QQQ) mass spectrometer as a dual mass filter, to allow passage and analyses of only predefined targeted proteotypic peptides, by specifically selecting precursor ions in Q1 and their specific fragment ions in Q3 as predefined mass to charge (m/z) values.[7] The signal intensities of SRM transitions (precursor/fragment ion pairs) of the unique peptide can be monitored over time and are efficient as surrogate measures of the quantity of a specific protein. Because of its high sensitivity, reproducibility, and broad dynamic range, SRM has become a powerful tool employed in absolute and relative quantification across multiple biological samples, especially in the area of biomarker research, but its widespread application is limited by a relatively low throughput. Depending on sample type and instrument methods, only 10−50 proteins can be quantified within each analytical run of a complex sample.

More recently, with the introduction of ultrafast scanning high-resolution Q-TOF instruments, the sequential window acquisition of all theoretical spectra (SWATH) has been presented as a novel SRM-like analysis method but based on a data-independent acquisition (DIA) strategy.[8] SWATH-MS presents a new and faster alternative to SRM-MS, and like SRM-MS, SWATH-MS operates by collecting time-resolved data from peptides and their fragments, to support identification and quantification of specific proteins within complex tissue sample. SWATH-MS can theoretically collect all MS/MS fragment ion spectra for all precursor ions within a complex sample by using stepped m/z windows and is therefore primarily used for biomarker discovery, with parallel and consistent detection of 30000−40000 peptides from 4000 to 5000 targeted proteins and across large sets of samples.[8,9]

Both SRM and SWATH methods for quantification of human proteins have been developed at an amazing pace over the past decade, and today, we have open access to accurate and validated MS methods for every known human protein.[10,11] However, such MS-based methods are species- and tissue-specific because the linearity and limit of quantification of any given protein and peptide strictly depend

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Table 1. Characteristics of the Quantotypic Peptides Used in SRM and SWATH Methods

protein	peptide[a]	peptide sequence	molecular weight	Q3 ions (SRM)	Q3 ions (SWATH)
triosephosphate isomerase	TPI1-1	VVLAYEPVWAIGTGK	1602.8	2y12; 2y11; 2y9	2y12; 2y11; 2y10; 2y9; 2y7
	TPI1-2	SNVSDAVAQSAR	1204.2	2y9; 2y8; 2y5	2y9; 2y8; 2y7; 2y6; 2y5
	TPI1-3	TATPQQAQEVHEK	1466.5	3y7; 3y6; 3y5	3y9; 3y8; 3y7; 3y6; 3y5
L-lactate dehydrogenase A chain	LDHA-1	VTLTHEEEACLK	1372.5	3y7; 3y6; 3y4	3y9; 3y8; 3y7; 3y6; 3y4
	LDHA-2	QVVDSAYEVIK	1250.4	2y9; 2y8; 2y7	2y9; 2y8; 2y7; 2y6; 2y5
	LDHA-3	DLADEVALVDVMEDK	1661.8	2y9; 2y7; 2y6	2y10; 2y9; 2y8; 2y7; 2y6
fructose-bisphosphate aldolase A	**ALDOA-1**	IGEHTPSSLAIMENANVLAR	2123.4	3y9; 3y8; 3y7	3y9; 3y8; 3y7; 3y6; 3y5
	ALDOA-2	VNPCIGGVILFHETLYQK	2031.4	3y9; 3y8; 3y7	3y9; 3y8; 3y7; 3y6; 3y5
peroxiredoxin-6	**PRDX6-1**	LPFPIIDDK	1057.2	2y8; 2y7; 2y6	2y8; 2y7; 2y6; 2y5; 2y4
	PRDX6-2	DLAIQLGMLDPAEK	1513.7	2y9; 2y6; 2y5	2y9; 2y8; 2y7; 2y6; 2y5
	PRDX6-3	LSILYPATTGR	1191.3	2y8; 2y7; 2y6	2y10; 2y9; 2y8; 2y7; 2y6
glycerol-3-phosphate dehydrogenase [NAD(+)]	**GPD1-1**	IVGGNAAQLAHFDPR	1565.7	3y7; 3y6; 3y5	3y9; 3y8; 3y7; 3y6; 3y5
	GPD1-2	LGIPMSVLMGANIANEVADEK	2172.5	2y14; 2y13; 2y12	2y14; 2y13; 2y12; 2y9; 2y8
	GPD1-3	NIVAVGAGFCDGLGFGDNTK	1955.1	3y7; 3y6; 3y5	3y10; 3y9; 3y7; 3y6; 3y5
peroxiredoxin-2	**PRDX2-1**	QVTINDLPVGR	1211.3	2y9; 2y7; 2y5	2y9; 2y7; 2y6; 2y5; 2y4
	PRDX2-2	GLFVIDGK	848.0	2y6; 2y5; 2y4	2y7; 2y6; 2y5; 2y4
	PRDX2-3	AHVGKPAPEFQATAVVDGAFK	2140.4	3y11; 3y8; 3y7	3y11; 3y9; 3y8; 3y7; 3y6
phosphoglucomutase-1	**PGM1-1**	IDNFEYSDPVDGSISR	1813.9	2y11; 2y10; 2y8	2y11; 2y10; 2y8; 2y7; 2y6
	PGM1-2	LSGTGSAGATIR	1090.2	2y10; 2y8; 2y5	2y11; 2y10; 2y8; 2y6; 2y5
	PGM1-3	YDYEEVEAEGANK	1516.5	2y9; 2y8; 2y7	2y10; 2y9; 2y8; 2y7; 2y6
heat shock cognate 71 kDa	**HSPA8-1**	DAGTIAGLNVLR	1199.3	2y8; 2y7; 2y6	2y10; 2y8; 2y7; 2y6; 2y4
	HSPA8-2	STAGDTHLGGEDFDNR	1691.6	3y9; 3y8; 3y4	3y9; 3y8; 3y7; 3y5; 3y4
	HSPA8-3	SQIHDIVLVGGSTR	1481.6	2y10; 2y8; 2y7	2y11; 2y7; 2y6; 2y5; 2y4
pyruvate kinase	**PKM2-1**	LDIDSPPITAR	1197.3	2y8; 2y7; 2y6	2y9; 2y8; 2y7; 2y6; 2y5
	PKM2-2	NTGIICTIGPASR	1302.5	2y9; 2y8; 2y5	2y11; 2y9; 2y8; 2y7; 2y5
	PKM2-3	GDYPLEAVR	1019.1	2y6; 2y5; 2y4	2y8; 2y7; 2y6; 2y5; 2y4
glycogen phosphorylase, muscle form	**PYGM-1**	HVIDQLSSGFFSPK	1561.7	3y8; 3y7; 3y6	3y9; 3y8; 3y7; 3y6; 3y5
	PYGM-2	HLQIIYEINQR	1426.6	3y6; 3y5; 3y4	3y8; 3y7; 3y6; 3y5; 3y4
	PYGM-3	DFNVGGYIQAVLDR	1566.7	2y10; 2y7; 2y6	2y11; 2y10; 2y9; 2y7; 2y6
superoxide dismutase [Cu−Zn]	**SOD1-1**	HVGDLGNVTADK	1225.3	2y11; 2y10; 2y8	2y11; 2y10; 2y8; 2y7; 2y6
	SOD1-2	GGNEESTK	820.8	1y6; 1y5; 1y4	1y7; 1y6; 1y5; 1y4
	SOD1-3	TGNAGSR	661.6	1y6; 1y5; 1y4	1y6; 1y5; 1y4

[a]The peptides marked in bold type were used for quantification analysis in experiment 2.

on the complexity of the given biological sample. The development of MS-based protein quantification for many other species, including those of farm animals, is lagging severely behind what is available for human studies. Thus, such MS methods must be developed specifically for every farm animal species, to become available also for future studies of food and farm animal health.[12] Within food analyses, SRM methods are so far applied to quantify only already know allergens in complex food products.[13,14] For farm animal health studies, SRM methods have so far been presented only for the analyses of 20 bovine host response proteins,[15,16] and none of these proteins are of immediate relevance for, e.g., supporting meat quality studies. Our lab has spent a long time developing MS approaches to support analyses of farm animal proteins,[3,17] and we recognize the need to develop SRM and SWATH technology to farm animal research.

Hence, the aim of this paper was to develop and compare two alternative quantification methods, namely, SRM and SWATH, to quantify a subset of 11 bovine muscle proteins in a background of the complete bovine proteome. These 11 proteins are key indicators of muscle growth, development, and oxidative defense and have previously been suggested to be useful as predictors of meat color and other meat quality traits;[18−20] thus, the ultimate aim of this study was to make these methods applicable for other laboratories studying meat and muscle proteomes. To evaluate the applicability of SRM versus SWATH for meat research, we here present two parallel experiments: (1) a study of technical variation of SRM versus SWATH for detection of 11 specific muscle proteins from a representative complex cow muscle proteome and (2) a study of quantitative linearity, demonstrated by relative quantification of these 11 proteins in a serial dilution of a cow muscle proteome spiked with increasing ratios of *Escherichia coli* proteomes.

MATERIALS AND METHODS

Materials. Muscle samples were collected from *Longissimus dorsi* of a young Danish Holstein bull (9 months old, ∼210 kg) after postmortem storage of the carcass at 4 °C for 3 days. An *E. coli* BL21-Gold (DE3) strain was obtained from BioNordika (Herlev, Denmark). All of the mobile phase solvents (LC-MS grade) and other chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

Protein Extraction. Muscle samples (200 mg) were homogenized in 1 mL of ice-cold TES buffer [10 mM Tris, 1 mM EDTA, and 0.25 M sucrose (pH 8.0)] using a tissue lyser (Qiagen, Valencia, CA). The homogenate was centrifuged at 10000*g* and 4 °C for 20 min. The 11

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proteins in this study are all sarcoplasmic proteins that can be readily and consistently extracted without the addition of detergent or chaotropic agents.[21] The supernatant (sarcoplasmic proteome extract) was frozen at −20 °C until further analysis.

For the *E. coli* proteome sample, a scrape of BL21-Gold (DE3) was inoculated and cultured in 2× TY medium in 1 L, shaking overnight at 37 °C. Bacteria were harvested after centrifugation at 5000*g* and 4 °C for 10 min and resuspended in 50 mM Tris and 500 mM NaCl (pH 8.0) before being lysed by sonication at 100% power in an ice bath for 5 × 20 s using a sonicator from Fisher Scientific (Pittsburgh, PA). The lysed cells were centrifuged for 20 min at 8000*g* and 4 °C, and the supernatant was removed and stored at −20 °C. The protein concentration was analyzed in all protein extracts, according to the manufacturer's manual, using the Pierce BCA Protein Kit (Thermo Scientific, Pittsburgh, PA) with bovine serum albumin (BSA) as the standard.

Protein Digestion. Muscle protein (200 μg) and *E. coli* protein (200 μg) samples were prepared and digested separately. Essentially, protein extracts containing 200 μg of proteins were reduced with 10 mM dithiothreitol (56 °C for 45 min), followed by alkylation by 55 mM iodoacetamide at room temperature for 30 min in the dark, and diluted four times with 50 mM ammonium bicarbonate before 2 μg of trypsin was added [1:100 (w/w)] (Sigma-Aldrich). Digestions were incubated for 16 h at 37 °C. All samples were filtered through 10 kDa cutoff centrifugal filters (VWR International, West Chester, PA) at 10000*g* for 10 min.

Selection of Targeted Quantotypic Peptides. Proteotypic peptides of 11 targeted proteins (Table 1) were chosen from the list of unique peptides based on the FASTA sequence in UniProt (http://www.uniprot.org/) and ranked by the suitability score of each tryptic peptide in the bovine database of PeptideAtlas (http://www.peptideatlas.org/). This atlas provides a collection of previously reported peptide and protein LC-MS data, from our previous MS studies of bovine proteins and proteome experiments.[17] We further used the additional peptide selection criteria, as described in detail by Brownridge et al.,[22] as follows. (1) preferred peptide length of 7−20 amino acids, (2) avoid glycosylation sites, and (3) avoid Arg-Pro and Lys-Pro cleavage sites. Two or three peptides for each protein were selected as final targets to quantify, as presented (Table 1).

Selection of Transitions for SRM Experiments. Skyline 3.7 software was used to generate transitions (precursor/fragment ion pairs) *in silico* and to evaluate and select the best detected proteotypic peptides for each targeted protein. Skyline is freely available from http://skyline.ms/. The iterative selection and evaluation process included the five following steps. (1) For each targeted peptide, five series of y-ions (generally 400 < m/z < 800) were selected *in silico* and exported to build MS methods. (2) LC-MS data were acquired using the primary selections. (3) Manual editing of acquired LC-MS data supported selection of the three most intense transitions. (4) Optimal collision energy (CE) and declustering potential (DP) values were selected for each transition after testing rolling values at steps of 4 and 15 V, respectively. (5) A final Skyline method with the scheduled retention time for each transition was thereafter used to acquire the SRM analyses of the 11 selected proteins within the muscle proteome samples.

SRM Assay. For experiment 1, which aimed to test the technical variation of SRM data, the digested muscle sample was diluted to 0.25 μg/μL with 0.1% (v/v) formic acid in water. For experiment 2, which aimed to evaluate the linearity of quantification of 11 muscle proteins, digested muscle proteome samples (0.5 μg/μL) were combined with digested *E. coli* proteome samples, to produce sequential dilution ratios of 1:9, 2:8, 4:6, 6:4, 8:2, and 10:0 of *Bos taurus* muscle proteome in an *E. coli* proteome background. Samples were analyzed with a 6500 QTRAP LC-MS system equipped with an IonDrive Turbo V Source and an Eksigent NanoLC 415 system (AB/Sciex, Framingham, MA) controlled by Analyst 1.6.3 software. For all of the LC-SRM experiments, samples of 4 μL were loaded onto a YMC-Triart C18 trap column (0.5 mm × 5.0 mm, 3 μm particles, YMC, Kyoto, Japan) in 0.1% (v/v) formic acid in water for 3 min at a flow rate of 10 μL/min in line with the resolving Eksigent ChromXP C18CL column (0.3 mm × 150 mm, 3 μm particles, Eksigent of AB/Sciex) and eluted using a 55 min gradient from 97% buffer A (0.1% formic acid in water) and 3% buffer B [0.1% formic acid in acetonitrile (ACN)] to 20% buffer A and 80% buffer B at a flow rate of 5 μL/min. For MS parameters, the following settings were used: ion spray voltage of 5000 V, curtain gas of 30 psi, ion source gas 1 of 25 psi, and a 1 s targeted scan time in positive scheduled MRM mode, based on observing the actual RTs for every peptide and then monitoring within a 4 min window around the observed RTs.

DDA Acquisition for Assembly of a Bovine Spectral Library. For building a spectral library, five independent LC-MS/MS analyses of bovine muscle, liver, and lymphocyte samples were performed on a TripleTOF 6600 instrument (AB/Sciex) using data-dependent acquisition (DDA). To support retention time alignment between individual data files, synthetic indexed retention time (iRT) peptides (Biognosys, Schlieren, Switzerland) were spiked into all digested samples at a 1:25 ratio (v/v) within each tryptic digest of 1 μg/μL protein, and 5 μL samples were analyzed by a TripleTOF 6600 mass spectrometer coupled with a DuoSpray Ion Source and an Eksigent NanoLC 415 system (AB/Sciex) controlled by Analyst TF 1.7.1. Peptides were separated by a YMC-Triart C18 trap column (0.5 mm × 5.0 mm, 3 μm particles, YMC) running 0.1% (v/v) formic acid and 2% ACN in water at a flow rate of 10 μL/min for 3 min and then eluted onto an analytical column (0.3 mm × 150 mm, 3 μm particles, YMC). An 87 min gradient from 95% A (0.1% formic acid in water) and 5% B (0.1% formic acid in ACN) to 20% buffer A and 80% buffer B at a flow rate of 5 μL/min was applied for chromatography. The DDA mode was acquired with a high-resolution TOF-MS scan over a mass range of m/z 400−1250 with an accumulation time of 0.25 s, followed by product ion scans of a maximum of 30 candidate ions within each cycle, collecting MS data in high-sensitivity mode, using the following settings: floating ion spray voltage of 5500 V, curtain gas of 25 psi, ion source gas 1 of 10 psi, CE of 10 V, and DP of 90 V.

SWATH-MS (DIA acquisition) Assay. For experiment 1, the digested muscle sample was diluted to 1 μg/μL with buffer A. For experiment 2, both muscle and *E. coli* digests were prepared at a concentration of 1 μg/μL and mixed to provide increasing muscle proteome:*E. coli* proteome ratios of 1:9, 2:8, 4:6, 6:4, 8:2, and 10:0. Finally, iRT peptides were spiked into each sample in a ratio of 1:25 (v/v). Totals of 5 μg (experiment 1) and 8 μg (experiment 2) of peptide material were injected for each SWATH acquisition on the same TripleTOF 6600 mass spectrometer coupled with an Eksigent NanoLC 415 system (AB/Sciex) as used for DDA acquisition. The chromatographic parameters were identical to those of DDA analysis described above, with the exception that a 57 min gradient was used for SWATH acquisition. SWATH-MS analysis was performed in a looped product ion mode, with a set of 100 consecutive windows across a range of m/z 400−1250 range, using an m/z 1 window overlap, e.g., 399.5−406.5, 405.5−412.5, etc. The accumulation time was set to 0.25 s for the TOF-MS scan and 25 ms for the product ion scan in high-sensitivity mode, resulting in a total cycle time of 2.8 s.

Creation of a SWATH-MS Library from DDA Files. A spectral library was created from five combined DDA data files from a selection of healthy bovine liver, lymphocyte, and muscle tissues analyzed on a TripleTOF 6600 mass spectrometer. An overview of samples and MS data quality of the library DDA files is presented in Table S1.

To create the library, raw *.wiff* files were searched using ProteinPilot 5.0.1 interrogating the UniProt *B. taurus* reference proteome (http://www.uniprot.org/, downloaded November 15, 2017; 24000 protein entries) to which the iRT peptide sequences were added. The following search parameters were set for ProteinPilot searches: fixed modification, Cys alkylation = iodoacetamide; digestion, trypsin; instrument, TripleTOF 6600 mass spectrometer; search effort, thorough ID; confidence score, >0.05, which equals 1% FDR at the peptide level. The generated *.group* file was imported into Skyline 3.7 and used as the reference ion library for SWATH data processing.

Data Processing for SRM and SWATH-MS. Skyline 3.7 was also used to process both SRM and SWATH data files. The three best

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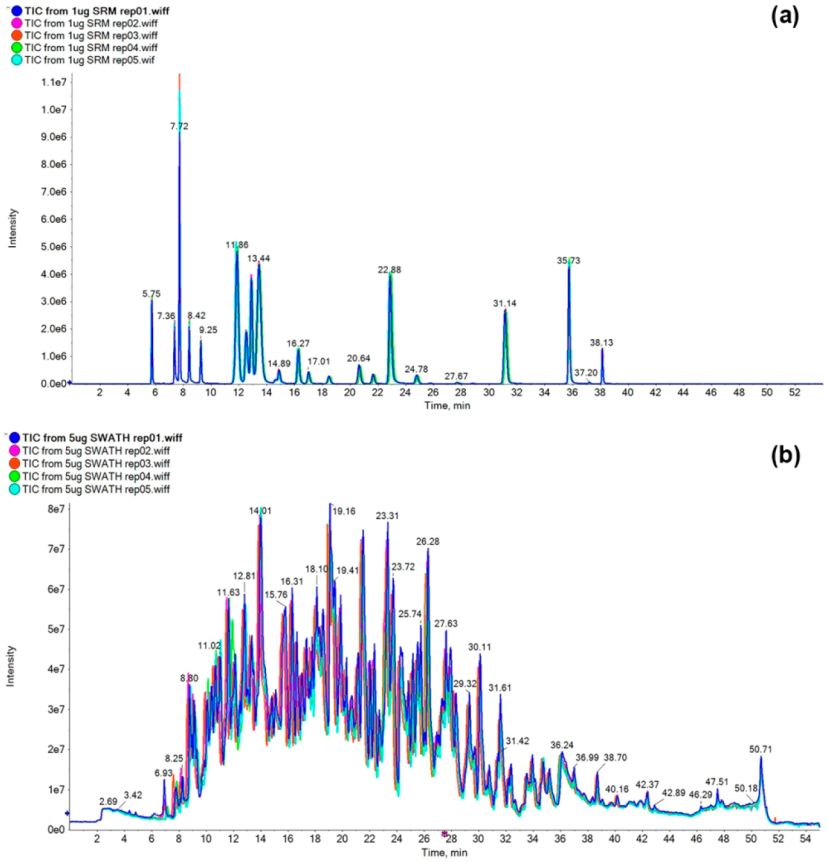

Figure 1. Overlaid TICs of a digested bovine muscle proteome sample measured as five technical replicates. (a) SRM data acquired by QTRAP 6500 instruments. (b) SWATH data acquired by a TripleTOF 6600 instrument.

transitions of each precursor were targeted for SRM experiments, while the five best transitions were selected for each peptide analyzed within the SWATH-MS data. For SRM analysis, after raw data had been imported into Skyline software, all peptide and fragment signals were manually checked, peak picking was refined, and a custom-defined report containing protein name, peptide sequence, total peak area, and retention time was generated. For processing of SWATH data in Skyline, the appropriate settings for peptide and transition were changed as follows: MS/MS filtering, DIA; product mass analyzer, TOF; isolation scheme, SWATH (VW 100); resolving power, 30000. Then the query parameters of iRT peptides were imported into Skyline, to make a linear regression over the measured retention times. SWATH data files were interrogated against the spectral library; peptide and fragment signals were manually checked, and peak picking was refined essentially as described for SRM data processing. Finally, a custom-defined report containing protein name, peptide sequence, total peak area, and retention time was generated and exported for further statistical analyses of protein abundance based on the transition peak area as described below.

Statistical Analysis. The sum of the total transition peak areas of each precursor peptide ion was used for quantification. The coefficients of variation (CVs) of individual peak areas and RT in experiment 1 were calculated from five replicate injections. In experiment 2, the intensities of each peptide were averaged by triplicate samples, and the simple linear regression analysis was performed via the data processing software SPSS 22.0 (IBM). The CVs of total intensities were calculated from triplicate injections. Then, an independent *t* test was performed for each serial dilution sample between SRM and SWATH analysis, while one-way analysis of variance was performed for all serial dilution samples analyzed by SRM and SWATH. Furthermore, the actual relative fold values of every single peptide were calculated on the basis of comparing increasing ratios of the digested muscle proteome within the total samples (20%/10%, 40%/10%, 60%/10%, 80%/10%, and 100%/10%, correlating to 2-, 4-, 6-, 8-, and 10-fold increases of muscle protein to background proteome, respectively).

RESULTS

Two parallel experiments were undertaken to evaluate and compare the efficiency of SRM and SWATH methods for fast and reproducible quantification of 11 selected muscle proteins that may be relevant as meat quality markers. These are all cytoplasmic proteins, which can be consistently prepared from muscle without the use of detergents or chaotropic agents.[21] The 32 selected peptides, representing the 11 muscle proteins analyzed in both experiments, are summarized in Table 1. Protein quantification was based on three proteotypic peptides per protein, with the exception of ALDOA, for which only two peptides were found to be suitable for consistent MS detection.

Experiment 1: Comparing SRM- and SWATH-Based Detection of 11 Targeted Muscle Proteins. To obtain the optimal quantification sensitivity, we undertook dedicated screening of optimal transitions and MS parameters for each proteotypic peptide, as explained in detail in Materials and Methods. The specific MS parameters (e.g., CE, DP, and targeted retention time) applied in the final SRM methods are listed in Table S2. Table 1 shows all of the objective transitions detected in SRM and SWATH modes. Essentially, SRM and SWATH shared mostly the same optimal Q3 fragment ions from each of the targeted peptides. Moreover, the optimal charge states of parent peptides were the same for both SRM and SWATH; e.g., for 10 of the 32 total peptides, the Q3 signals were optimally detected as triply charged ions, because

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of their relatively large mass. These include ALDOA-1 and GPD1-3 with molecular weights (MWs) of ~2000 Da; 20 of the total 32 peptides were optimally detected as doubly charged Q3 ions, and the two short peptides, SOD-2 and SOD-3, could be detected only as singly charged Q3 ions. Compared with only the three best transitions selected with the SRM method, SWATH-MS has no limitation on the number of transitions, but for the sake of consistent and comparable quantification across samples, we selected and monitored the five most intense transitions from each peptide, with the exceptions of the short peptides of PRDX2-2 (octapeptide), SOD1-2 (octapeptide), and SOD1-3 (heptapeptide), for which only four suitable y-fragment ions were consistently detected.

Figures S1–S4 present the extracted transition peaks of each peptide in each of the five replicated SRM and SWATH-MS samples studied in experiment 1. Essentially, most of the transitions were detected consistently in both SRM and SWATH-MS; in addition, the relative signal intensities of the detected peptides were also consistent. As shown in Figures S1 and S2, the targeted transitions from the same peptides had highly consistent peak shapes and overlapping fragment clusters. Most of the peptides had the desirable MS signal with unique and sharp peaks, such as TPI1-1, TPI1-2, and TPI1-3. However, for peptides PRDX2-3 and PRDX6-2, low signal-to-noise ratios (S/N) were observed. For SOD1-2, only the y6 ion was detected, and for SOD1-3, none of the targeted transitions could be detected in any of the samples, not even with an increased ($\leq$4 μg) sample load on the QTRAP instrument (data not shown). Therefore, SOD1-3 was excluded for further SRM analysis in the current work. As for the SWATH approach, the detectability of peptides was quite similar to that of SRM (Figures S3 and S4), where PRDX6-2, PRDX2-3, SOD1-2, and SOD1-3 were detected with low S/N. Moreover, ALDOA-2 was also a challenge to detect with the SWATH approach, despite its robust detection by SRM. It was, however, notable that confounding fragment ions not belonging to the targeted peptides were also detected and extracted from the SWATH data, causing a higher background noise in SWATH data for, e.g., ALDOA-1, GPD1-1, and PRDX2-1.

Consistent MS data acquisition is crucial for quantification of multiple proteins across large numbers of replicate samples and for comparable quantification across different biological samples, which can have very different background proteomes. One relevant example is when monitoring a protein within a raw meat sample versus within differently processed meat products, which typically will contain components from several food species. For this purpose, we assessed the reproducibility of our SRM and SWATH methods for detection of the validated transitions across five technical replicates of digested bovine muscle samples. Figure 1 shows the total ion chromatogram (TIC) of both SRM and SWATH data extracted by Peakview 2.2 software, clearly demonstrating identical chromatographic profiles across the five replicated data. In Figure 1a, only the peaks of targeted transitions are presented in the TICs acquired in SRM mode. We observed 20 sharp peaks (mostly with a peak width of <0.5 min), all corresponding to the peptides of high ion intensities. The apparently missing peptides are those with weaker MS signals, which are however clearly observed by magnifying the signal intensity *Y*-axes (Figures S1 and S2). Unlike SRM, the TICs of SWATH-MS are too complex to allow direct peptide information (Figure 1b); hence, SWATH-MS data must be extracted from spectral library searches, as explained in Materials and Methods.

As displayed in Figure 2, the CVs (%) of integrated peak area and RT for all of the targeted peptides were calculated on

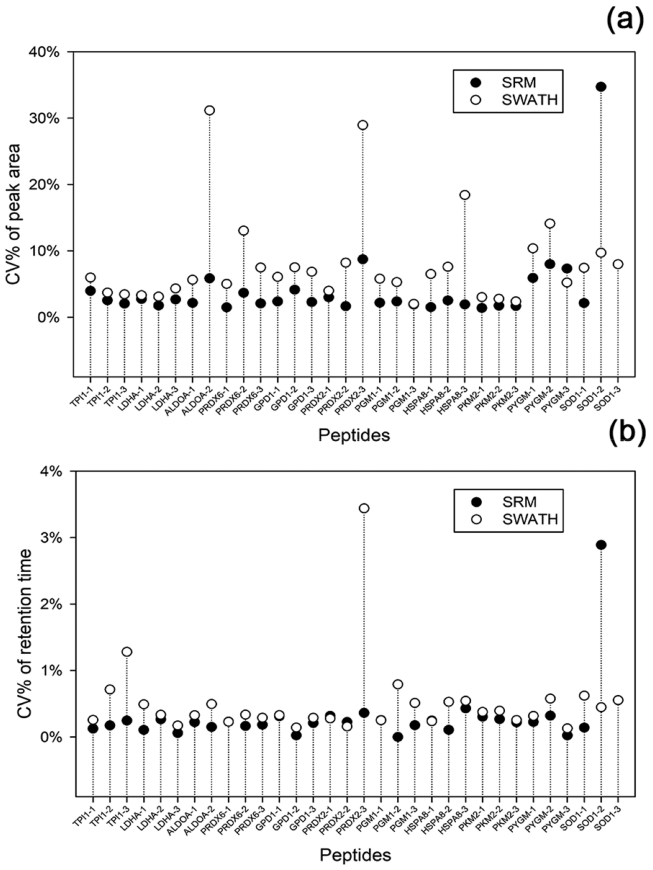

Figure 2. CV values of 32 targeted proteotypic peptides measured as five technical replicates. CV values of (a) total peak areas of all targeted transition and (b) retention times for each quantotypic peptide acquired by SRM and SWATH.

the basis of five technical replicates. Figure 2a presents an impressive reproducibility obtained by SRM assays, where 97% of the peptides are detected with CVs of total peak areas well below 10%, and in fact below 5% for 80% of the peptides. In comparison, the SWATH-based assays were a little less consistent, with 81% of the peptides detected with CVs of <10% and three peptides (ALDOA-2, PRDX2-3, and HSPA8-3) detected with CVs of >15%. With respect to RT stability, excellent reproducibility was observed in both approaches, with the majority of peptides with CVs of <1% presented in Figure 2b, which clearly demonstrates that SRM provides a reproducibility that is better than that of SWATH and that variation mainly depends on the accuracy of the LC system.

Experiment 2: Linearity and Sensitivity of SRM- and SWATH-Based Quantification. To further assess the quantification performance of SRM and SWATH-MS, we mixed the digested bovine muscle proteome sample with the digested *E. coli* proteome sample, to study the robustness of quantification of these 11 targeted proteins within an increasingly dominating and complex proteome background.

As shown in Figures 3 and 4, SRM allowed remarkably higher total peak area detection for most of the peptides

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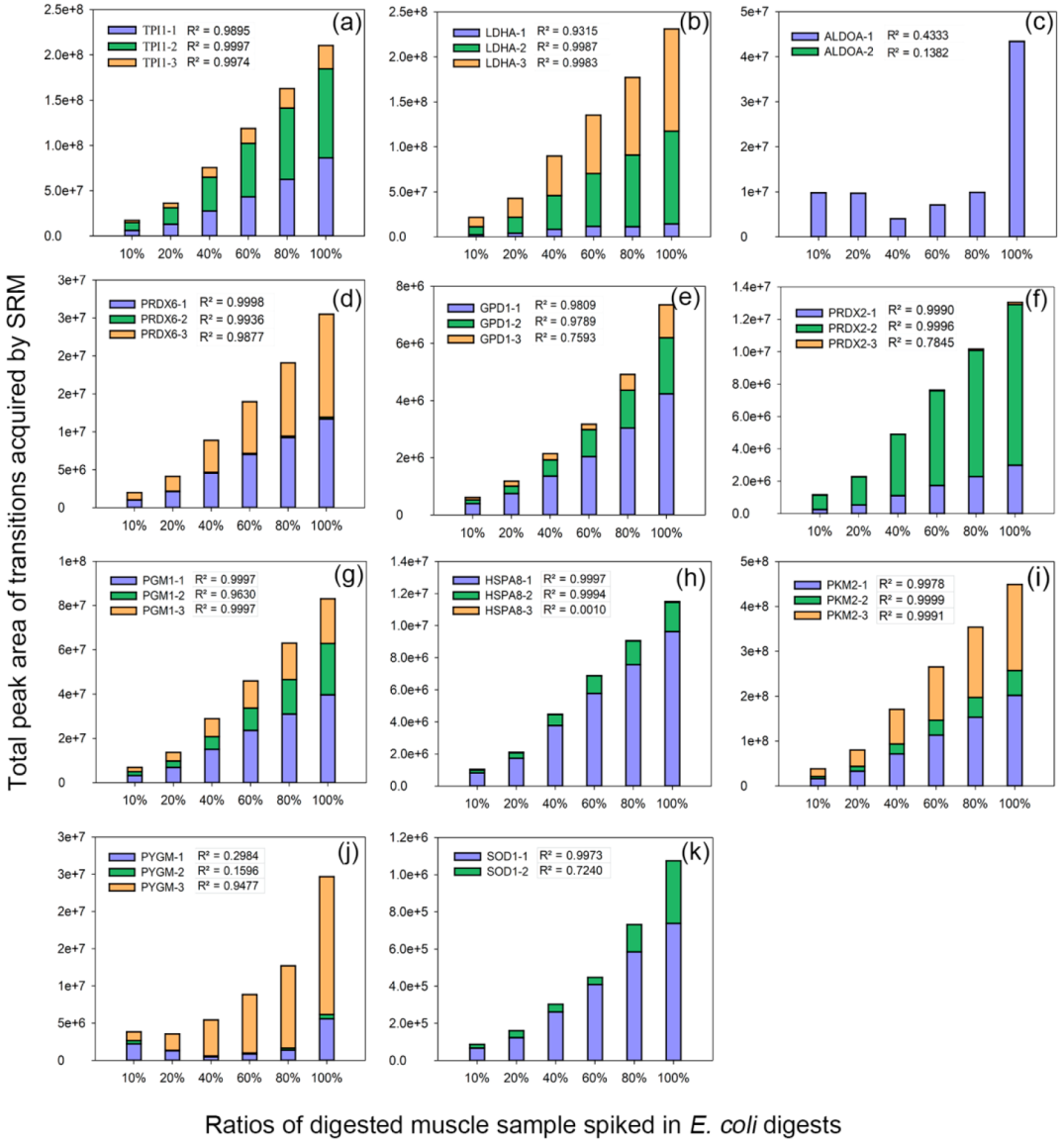

Figure 3. Linearity of bovine proteomes diluted with an *E. coli* proteome background, presenting total peak areas of each measured peptide from 11 targeted proteins, (a) TPI1, (b) LDHA, (c) ALDOA, (d) PRDX6, (e) GPD1, (f) PRDX2, (g) PGM1, (h) HSPA8, (i) PKM2, (j) PYGM, and (k) SOD1, acquired by SRM methods. The R^2 values of simple linear regression for each peptide were calculated and are marked next to the respective peptides.

compared to SWATH. Furthermore, considering that total injected amount was 2 μg in SRM compared to 8 μg in SWATH, this suggests that SRM is approximately 40-fold more sensitive based on total ion intensities for detection of bovine muscle proteins, even when measured within an artificial complex background.

We designed a serial dilution of muscle proteins (from 10 to 100%) to determine the linearity of SRM and SWATH-MS methods. In general terms, the measured total ion intensities of both assays demonstrated excellent linearity between loaded amounts and the detected peak areas, signifying the robustness of the assays we developed for these 11 muscle proteins (Figures 3 and 4). For TPI1, LDHA, PGM1, and PKM2 proteins, all three selected peptides for each protein were detected with excellent MS signal intensity and perfect linearity (R^2 > 0.93) in both SRM and SWATH (Figures 3a,b,g,i and 4a,b,g,i). Furthermore, several peptides, TPI1-2, PGM1-1, and PKM2-2, determined by SRM had excellent R^2 values (0.9997−0.9999). For GPD1, PRDX2, and HSPA8, only two peptides from each of these proteins that presented ideal linearity (R^2 > 0.96) for quantification were observed in both methods (Figures 3e,f,h and 4e,f,h). Peptide PRDX6-2, despite its excellent linearity (R^2 = 0.9936), was not suitable for quantification due to a low SRM signal intensity compared to those of its sibling peptides, PRDX6-1 and PRDX6-3 (Figures 3d and 4d). As shown in Figures 3j and 4j, for peptide PYGM-1, SWATH actually allowed a better linearity of detection, with an R^2 value of 0.9632 in comparison with the R^2 value of 0.2984 determined by SRM. This is attributed to inconsistent peak detection for these peptides in SRM mode, caused by unstable RT observed for PYGM-1. Similar observations also apply for ALDOA-1 (Figures 3c and 4c).

The two most consistently detected peptides from each of the 11 protein targets were used for relative quantification analysis, except for ALDOA and SOD1, for which only one peptide was found to be suitable for consistent detection and

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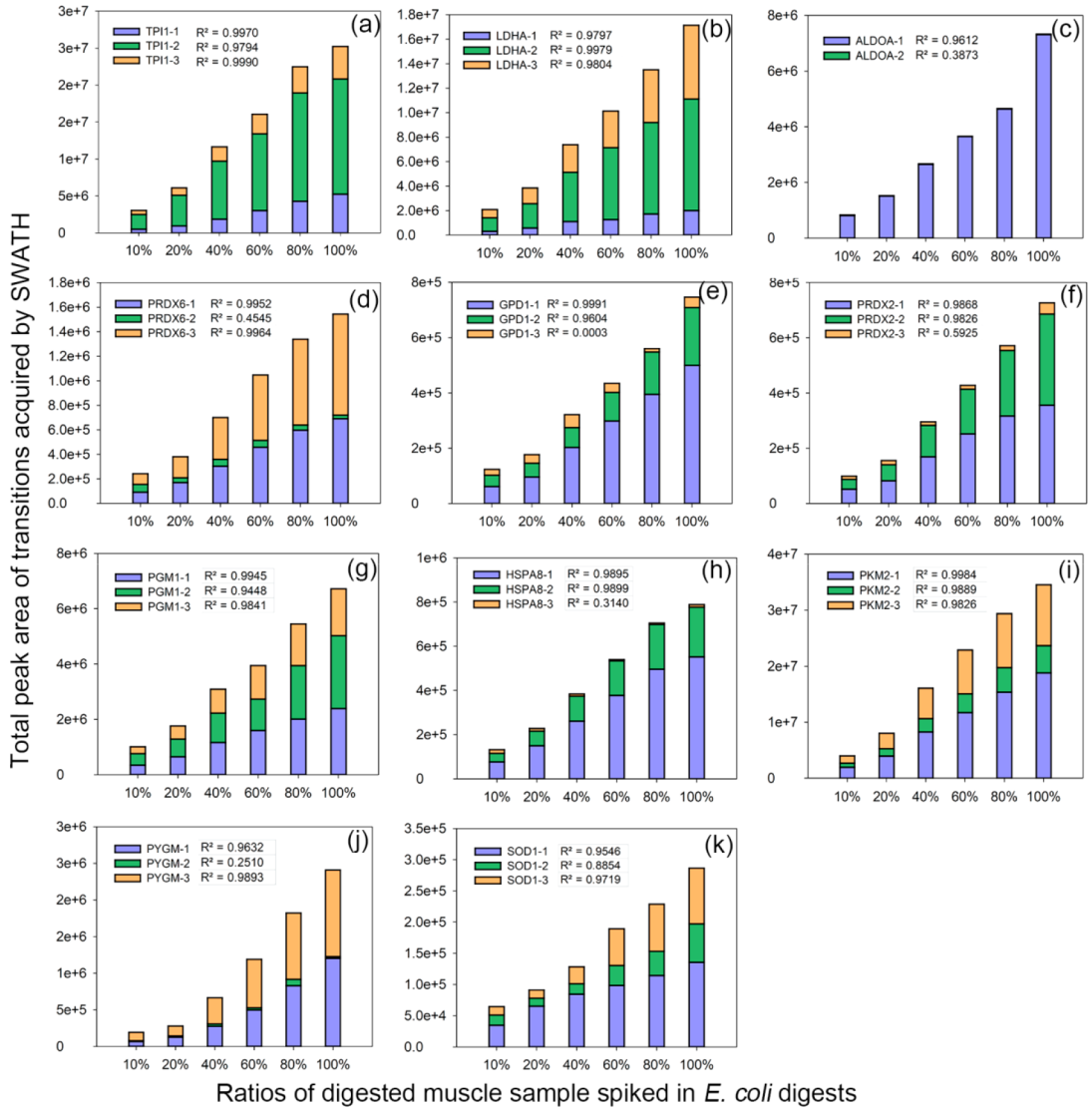

Figure 4. Linearity of bovine proteomes diluted with an *E. coli* proteome background, presenting total peak areas of each measured peptide from 11 targeted proteins, (a) TPI1, (b) LDHA, (c) ALDOA, (d) PRDX6, (e) GPD1, (f) PRDX2, (g) PGM1, (h) HSPA8, (i) PKM2, (j) PYGM, and (k) SOD1, acquired by SWATH-MS. The R^2 values of simple linear regression for each peptide were calculated and are marked next to the respective peptides.

quantification. These 20 quantotypic peptides of 11 proteins are marked in Table 1.

The CVs of total peak areas measured for each targeted peptide and different serial dilution samples (from 10 to 100%), based on triplicate injections in both SRM and SWATH, are listed in Table S3. The average values are based on all quantotypic peptides, thus, excluding the nine inconsistently observed peptides, as listed in Table S3, and demonstrated in Figure 5. This figure clearly shows that CVs depend on the abundance of the targeted proteins. It is clear that CVs decline with an increased protein abundance for both SRM and SWATH. Furthermore, SRM has CVs consistently lower than those of SWATH, especially for the diluted samples containing only 10% muscle proteome ($P < 0.05$), while targeting high-abundance proteins (e.g., the 60, 80, and 100% muscle proteome samples) demonstrated that SWATH delivers very robust quantitative stability with a CV of <6%.

Ideally, two sibling peptides should demonstrate parallel fold changes, to consistently quantify the parent protein. In Figure 6, we demonstrate that this was indeed the case with the linearly increasing relative abundance of muscle proteins in the serial dilution of muscle/*E. coli* samples described above. Panels a, b, d, and e of Figure 6 present the relative fold changes calculated for every individual proteotypic peptide from nine muscle proteins (ALDOA and SOD1 were excluded from this analysis), while panels c and f of Figure 6 demonstrate the fold changes observed on the basis of averaged total ion intensities of two sibling peptides by SRM and SWATH analysis. Figure 6 clearly confirms that a serial dilution of muscle/*E. coli* proteomes (20, 40, 60, 80, and 100%, compared to the 10% muscle sample) can be quantified as 2-, 4-, 6-, 8-, and 10-fold increasing ion intensities, respectively. This was most clear for eight of the nine proteins presented in Figure 6, demonstrating that the experimental fold differences closely adhered to the theoretical fold values in both SRM and SWATH data, with the exception of PYGM, where poor linearity of one peptide (PYGM-1) reflects the fact that quantification can indeed be achieved by a single quantotypic peptide per protein. Furthermore, with theoretical fold changes of 2 (20%/10%) and 4 (40%/10%), observed averaged fold

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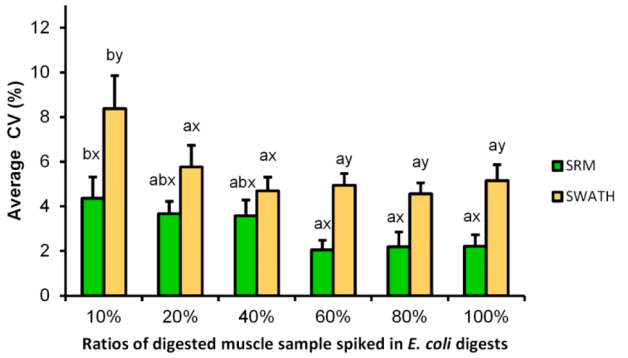

Figure 5. Average CV values of 23 targeted proteotypic peptides, (excluding nine peptides due to their inconsistent peak detection) depend on the MS method (SRM vs SWATH) as well as the relative protein abundance. The columns marked with different "a" and "b" superscripts refer to significantly different ($P < 0.05$) CVs in low- vs high-abundance protein samples and calculated individually for SRM and SWATH data, respectively. Columns marked with different "x" and "y" superscripts are significantly different ($P < 0.05$) for SRM and SWATH.

differences of 2 (20%/10%) and 4 (40%/10%) were perfectly identical to the theoretical values (Figure 6c,f). However, it is noteworthy that the determined fold changes in SWATH data generally underestimated the quantity compared to the theoretical values, while fold changes were slightly overestimated using SRM measurements. In addition, for theoretical fold changes of 6 (60%/10%), 8 (80%/10%), and 10 (100%/10%), the observed values drifted farther than did those of SRM. Even though SWATH lacks the excellent accuracy of SRM, its quantification ability is still impressive and in most cases, in fact, comparable to that of SRM.

DISCUSSION

We designed and examined SRM and SWATH methods for detection and quantification of 11 muscle proteins, which from our previous studies are hypothesized to correlate to the color development of *longissimuss lumborum*, *psoas major*, and *semitendinosus* from Chinese *Luxi* yellow cattle.[18,19] Moreover, several of these targeted proteins, e.g., TPI1, HSPA8, PRDX6, and SOD1, have also been reported to be indicators of meat tenderness.[20,23] However, the correlations between these proteins and meat quality are likely both species- and muscle-specific.[24] The 11 proteins we present here participate in multiple biological pathways, including energy metabolism, cellular stress, and oxidative stress; thus, developing accurate and efficient quantification methods for these proteins is an important step toward exploring the complex mechanisms of how muscle metabolism is correlated to variations in meat quality.

It is well documented that sensitivity of SRM methods mainly depends on optimization of peptide- and transition-specific MS parameters.[7] In our case, we also found that selecting appropriate proteotypic peptides is crucial as a first step toward successful SRM-based proteome quantification. Essentially, according to our current results, most peptides with 10−20 amino acids were easily observed with excellent MS signals, while larger and smaller peptides, e.g., ALDOA-2 (20 amino acids; MW = 2031.4), PRDX2-3 (21 amino acids; MW = 2140.4), and SOD1-3 (7 amino acids; MW = 661.6), could not support consistent detection, neither in SRM nor in SWATH analysis. Because y_3-y_{n-1} ions provide the best evidence for specific peptide detection,[25] short peptides must be critically observed before selecting them as proteotypic targets. While others have suggested that 5−10[26] and 7−20[16] amino acids are optimal peptide lengths for proteotypic peptides, our study suggests preferentially selecting peptides of 10−20 amino acids to achieve optimal MS intensity.

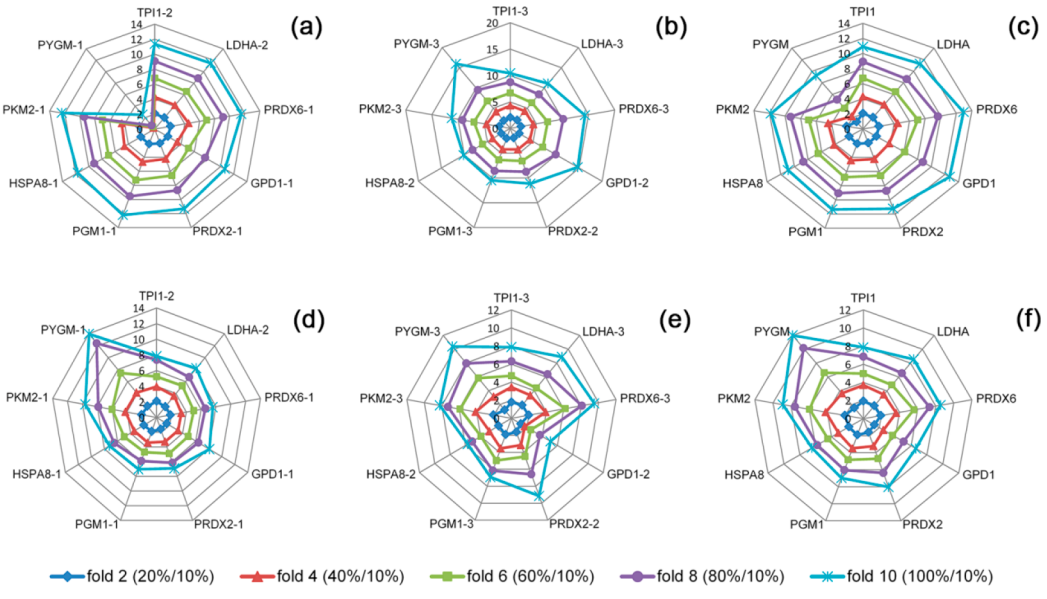

Figure 6. Relationships between observed and theoretical fold changes of nine targeted proteins (excluding ALDOA and SOD1) acquired by SRM and SWATH. The relative fold changes were (a and b) calculated for a single peptide or (c) averaged between two peptides in SRM analysis. Panels d−f show the counterparts acquired by SWATH. The vertical labels on the radar mean theoretical fold values. The symbols with different colors signify the observed fold values measured in mixed samples with a linearly increasing 20, 40, 60, 80, and 100% muscle protein content within the *E. coli* proteome sample, using the 10% muscle proteome sample as the reference.

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J. Agric. Food Chem. 2019, 67, 1608−1618

Despite SRM's status as the gold standard method for protein quantification in complex mixtures, the multiple steps needed for selection and validation of optimal peptides, transitions, MS parameters, and retention times render SRM pipelines as time-consuming, compared to SWATH. Despite the fact that processing and validation of SWATH data are more time-consuming than for SRM data, all in all, SWATH was a more time-efficient approach for the method development we present in this study.

It is well-known that tryptic peptides with 2+ and 3+ charge states are most consistently observed in the range of m/z 400−800;[27,28] hence, this is the predefined filter criterion we used for y-ion selection in the Skyline methods. We observed parallel constellations of charge states for optimal Q3 ions in SRM and SWATH-MS, likely reflecting the fact that both MS instruments used in this work, namely, QTRAP and TripleTOF for SRM and SWATH, respectively, are equipped with the same type of electrospray ionization (ESI) interface. This phenomenon reflects the fact that relative y- and b-ion abundances depend on the type of ion source as well as on the ionization conditions used and that such fragmentation bias is similar under both default and optimized CE conditions.[29] Furthermore, previous studies also reported the fragmentation patterns were similar between MS instruments with different mass analyzers, e.g., Triple TOF and Q Exactive HF (Thermo Scientific, Bremen, Germany),[30] or different fragmentation modes, e.g., collision-induced dissociation (CID) and high-energy CID.[31]

The supplementary figures clearly demonstrate that some targeted peptides had excellent MS signals with sharp transition peaks and high S/N while others were poorly detected. In fact, there are many reasons why peptides evade detection in MS, with factors such as poor chromatographic elution, degradation of peptides, missed cleavage sites, and ion suppression being the most common. The strong hydrophobicity of peptides PRDX6-2, PRDX2-3, and ALDOA-2 could well be the reason we failed to detect strong and reproducible MS signals for these peptides (Table 1). Also, because of their highly hydrophobic nature, these peptides elute at a late RT and may even adhere to the column or to other hydrophobic peptides and, thus, appear with weak and inconsistent MS signals.[32] Compared to SRM, SWATH presented some interfering peaks in the background that we could not avoid entirely, because SWATH operates by acquiring all MS/MS information for all co-eluting peptides within complex biological samples. This highlights the need to validate SWATH methods specifically for every targeted peptide within every biological sample type and to exclude peptides that cannot contribute to robust quantification of the given protein.

Our technical replicate analyses revealed excellent reproducibility in both SRM and SWATH analyses. TICs of SRM and SWATH data were perfectly overlapping and well in line with similar quantitative performance data from SWATH-MS of mouse cell lysate samples.[33] Despite a larger technical variation of peak area being observed in SWATH than in SRM, we still observed 81% of peptides with CVs of <10% acquired by SWATH-MS, which is comparable to SWATH quantification of the *Saccharomyces cerevisiae* proteome (76% of peptides with CVs of <10% for $n = 4$).[34] The quantitative methods with CVs of <10% are usually considered stable enough for complex biological samples, which clearly outperform many enzyme-linked immunosorbent assay-based assays, for example, the commercial ELISA/LUMINEX kits that report CVs of <20%.[35]

In experiment 2, we simulated the well-known challenge of analyzing and quantifying specific proteins across different tissues and, e.g., disease states where expression profiles of individual proteins and proteomes are highly variable and often not even well described. This application is for example relevant for detection of specific proteins within diverse and complex samples like processed food and feed samples, where the limit of detection strongly depends on the sample matrix. The complex background we created in experiment 2 did not compromise the sensitivity of SRM and SWATH methods in the range we tested. Moreover, SRM detection exhibited a sensitivity increased 40-fold versus that of SWATH in our study. This is to be expected, given the wider isolation window of precursor ions used in SWATH mode compared to SRM that runs at unit resolution. Gillet et al. found that SRM appears to be 10 times more sensitive than SWATH using serial dilution of isotopically labeled peptides spiked into yeast tryptic digests.[8]

Linearity over a large abundance range is essential for precise relative and absolute quantification. Huang et al. reported that the total ion intensities were proportional to sample loading amounts ($R^2 = 0.95$) in a SWATH study of a complex mouse cell lysate.[33] Our results demonstrate the linearity ($R^2 > 0.9$) of quantification for 23 of the 32 selected peptides with both SRM and SWATH applications. It is notable that the proteotypic peptides with poor linearity are detected with low signal intensity and reproducibility (CVs of >15% and S/N of <6) for both SRM and SWATH. These peptides include, e.g., ALDOA-2, PRDX2-3, and HSPA8-3, which are not optimal surrogates for targeted protein quantification.

After comparing the relative fold differences of nine muscle proteins, we found that using two quantotypic peptides per protein for calculation would reduce the dependence on poor ionization or otherwise suboptimal peptide performance. Furthermore, we observed underestimated fold ratios compared with theoretical values acquired by SWATH, which implies that ion suppression occurred in the Q-TOF instrument, which is a well-known challenge with complex samples, where multiple ions co-elute from the HPLC instrument, leading to matrix-based ion suppression.[36] In spite of this, the results of relative quantification in our study proved that both SRM and SWATH approaches had robust quantification accuracy for nine muscle proteins, over a 10-fold protein dilution range, with optimal precision for relative fold changes of <4-fold. Nakamura et al. performed a comparison on absolute quantification of drug-metabolizing enzymes and transporters in several human tissue microsomes by SRM, SWATH, and HR-SRM. They concluded that SWATH enables large-scale multiplex absolute protein quantification while retaining a quantitative capability similar to that of SRM or HR-SRM.[37] Until recently, a multilaboratory evaluation from 11 sites worldwide demonstrated that SWATH-MS can consistently detect and reproducibly quantify >4000 proteins from HEK293 cells across laboratories.[9] Another recent example is ref 38 in which Shan et al. used SWATH to observe 48 proteins that were significantly correlated to guinea pig retina emmetropization and subsequently validated and confirmed these observations by independent high-resolution SRM-MS. Such studies support the confidence in SWATH-MS as a reproducible and high-throughput quantification method and, as such, a promising alternative to SRM.

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Overall, our study clearly shows that for nine of the 11 analyzed proteins, both SWATH-MS and SRM-MS provide equally robust quantification, but we also clearly see that for these muscle proteins, SRM provided a detection method that was approximately 40-fold more sensitive than that of SWATH-MS. Thus, SRM should be preferred when analyzing low-abundance proteins, while SWATH-MS provides a time-efficient method and would be the method of choice for discovery of novel markers because a larger panel of proteins can be studied within each sample.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b05459.

DDA data quality of five bovine tissue samples searched by ProteinPilot for building the ion library for SWATH data processing, optimal MS parameters (retention time, DP, and CE) acquired for SRM analysis, CV values of total peak areas measured for each quantotypic peptide by SRM and SWATH in experiment 2, and extracted transition peaks of quantotypic peptides of all 11 targeted proteins in meat samples analyzed by SRM and SWATH (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

MS, mass spectrometry; SRM, selected reaction monitoring; SWATH, sequential window acquisition of all theoretical spectra; MRM, multiple-reaction monitoring; *m/z*, mass to charge; DIA, data-independent acquisition; CE, collision energy; DP, declustering potential; DDA, data-dependent acquisition; iRT, indexed retention time; CV, coefficients of variation; MW, molecular weight; S/N, signal-to-noise ratio; TIC, total ion chromatogram; CID, collision-induced dissociation.

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