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Peptide Selection for Accurate Targeted Protein Quantification via a Dimethylation High-Resolution Mass Spectrum Strategy with a Peptide Release Kinetic Model

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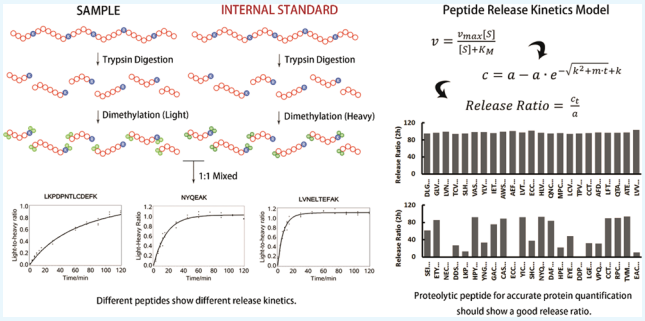
ABSTRACT: A crucial step in accurate targeted protein quantification using targeted proteomics is to determine optimal proteotypic peptides representing targeted proteins. In this study, a workflow of peptide selection to determine proteotypic peptides using a dimethylation high-resolution mass spectrum strategy with a peptide release kinetic model was investigated and applied in peptide selection of bovine serum albumin. After specificity, digestibility, recovery, and stability evaluation of tryptic peptides in bovine serum albumin, the optimal proteotypic peptide was selected as LVNELTEFAK. The quantification method using LVNELTEFAK gave a linear range of 1−100 ppm with the coefficient greater than 0.9990, and the detection limit of bovine serum albumin in milk was 0.78 mg/kg. Compared with the proteotypic peptides selected by Skyline, the method showed a better performance in method validation. The workflow exhibited high comprehensiveness and efficiency in peptide selection, facilitating accurate targeted protein quantification in the food matrix, which lack protein standards.



1. INTRODUCTION

Accurate quantification of individual protein, or a group of proteins, is crucial for studies of biochemical systems, such as food and food consumer as well as for the quality and safety control of food. Moreover, modeling studies necessary for understanding the complex interplay of the system (food or consumer) components or interplay between components of two systems (food and consumer) require accurate absolute quantitative information.[1,2] Mass spectrometry-based quantitative proteomics in combination with stable isotope-labeling is an advanced technology leading a systematical and quantitative analysis of protein profiles.[3−5] For absolute quantitative analysis, the protein standard absolute quantification (PSAQ) method has been developed using full-length isotope-labeled proteins as internal standards.[6,7] Considering the high cost and complicated processing in synthesis of isotopic protein standards, an absolute quantification (AQUA) method has been developed using stable isotope-labeled peptides as internal standards.[8,9] Targeted proteomics is quantitative proteomics based on the AQUA method, in which the quantification of the target protein relies on the optimal proteolytic peptides that have quantification characteristics similar to the target protein.[10,11] As a result, selection of proteotypic peptides is a crucial step in targeted proteomics determining the quantification accuracy of target proteins.[12,13]

In recent years, several strategies have been built for proteotypic peptide selection in targeted proteomics.[14,15] The enhanced signature peptide (ESP) predictor is a computational method to predict high-responding peptides of target proteins without experimental data and the high-responding peptides are regarded as the optimal proteotypic peptides.[16] PeptidePicker, as a software package, provides a scientific workflow to process and integrate the information from different online data sources for selecting the optimal signature peptides of target proteins.[17] Additionally, selection of optimal proteotypic peptides that relies on experimental data has been developed using in vitro-synthesized proteins and Skyline software.[18,19] Most of these strategies are based on the following major principles: peptides should (i) be unique in the assay matrix, (ii) have a good response in the mass spectrometer, and (iii) have a specific and stable fragmentation pattern. It is worth mentioning that an incomplete proteolytic peptide release could result in the inaccurate quantification of targeted protein.[20] So proteolytic digestion is an important factor affecting the accuracy of protein quantification, which should be considered in the selection of optimal proteotypic peptides as well.

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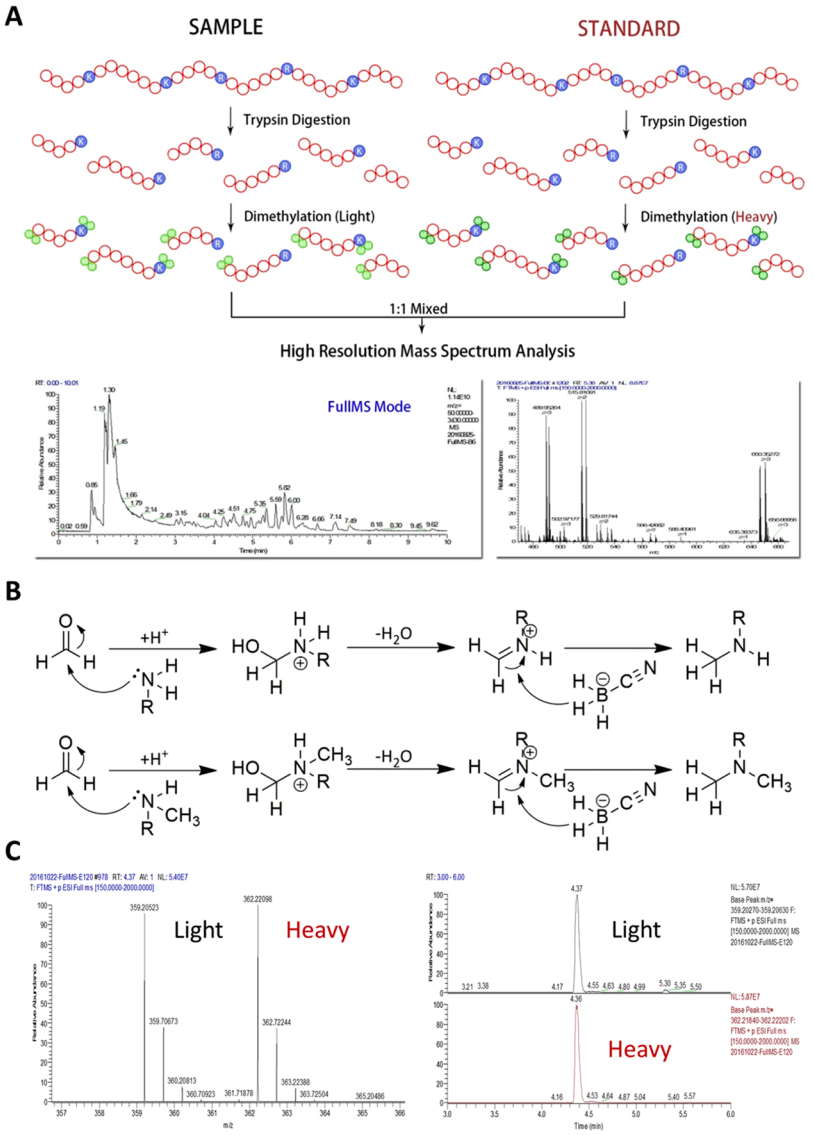

Figure 1. Schematic overview of the dimethylation high-resolution mass spectrum strategy. (A) A schematic of peptide screening and quantification method included tryptic digestion, dimethylation, and high-resolution mass spectrum acquisition. High-resolution mass spectrum analysis was performed on UHPLC-quadrupole-orbitrap mass spectrometry with the full MS/dd-MS2 (TopN) mode, which was able to acquire major parent ions and the related daughter ions. (B) Reaction pathway of dimethylation. Dimethylation was the combination of a nucleophilic addition reaction on formaldehyde and an imine reduction reaction using sodium cyanoborohydride. (C) Two levels of dimethylation labeling. The light label was reacted with formaldehyde, and the heavy label was reacted with isotopic formaldehyde.

Sequencing grade modified trypsin is an important digestion enzyme of targeted proteomics that specifically cleaves peptide chains at the carboxyl side of lysine or arginine, unless either is followed by proline.[21] Ideally, the specificity of trypsin guarantees that 1 M targeted protein is able to be hydrolyzed into equimolar characteristic peptides to accurately quantify the target protein. But it has been demonstrated that the cleavage sites surrounded by neutral residues could be quickly cut while those with neighboring charged residues or proline residue could be slowly cut.[22] This means that not all tryptic cleavage sites could be completely hydrolyzed and not all peptides could be completely released from target protein in the actual application of trypsin. In recent researches, kinetic parameters of sequencing grade modified trypsin have been modified and reported according to the Michaelis−Menten equation.[21] However, the protein degradation kinetics could not represent the release kinetics of proteolytic peptides and different peptides have different release kinetic parameters under the specific digestion condition.[23,24]

In this article, a kinetic equation of peptide release was developed, which is able to describe the peptide release kinetics during tryptic digestion and evaluate the release ratio of fully tryptic peptides for proteotypic peptide selection. On the basis of the peptide release kinetic model, a comprehensive workflow of peptide selection for accurate targeted protein quantification was developed using a dimethylation high-resolution mass spectrum strategy, considering the specificity, digestibility, recovery, and stability of tryptic peptides. To simplify the description, we used bovine serum albumin (BSA) in raw bovine milk as an example. One part of the strategy was establishment of the peptide quantification method, which is based on high-resolution mass spectrometry (UHPLC-Quadrupole-Orbitrap) as well as dimethylation labeling and proteomics database (Uniprot, http://www.uniprot.org/). The

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Table 1. Parameters in Multiple Reaction Monitoring (MRM) Mode[a]

peptides	CS (z)	precurser ion (m/z)	product ion (m/z)	cone voltage (V)	collision energy (eV)	fragmentation pattern
LVNELTEFAK-L	2+	610.4	393.3/1078.6*	20/30	30/25	y3/y9
LVNELTEFAK-H	2+	616.4	399.3/1084.6*	20/30	30/25	y3/y9
YLYEIAR-L	2+	478.3	651.4/764.4*	20/20	20/20	y5/y6
YLYEIAR-H	2+	481.3	651.4/764.4*	20/20	20/20	y5/y6
QTALVELLK-L	2+	535.8	813.5/914.6*	20/20	20/23	y6/y7
QTALVELLK-H	2+	541.8	819.6/920.6*	20/20	20/23	y6/y7
AEFVEVTK-L	2+	489.8	504.3/879.5*	20/20	20/20	y4/y7
AEFVEVTK-H	2+	495.8	510.3/885.5*	20/20	20/20	y4/y7
LGEYGFQNALIVR-L	2+	754.4	274.2/328.2*	20/20	35/30	y2/b3
LGEYGFQNALIVR-H	2+	757.4	274.2/334.2*	20/20	40/30	y2/b3

[a]*Quantitative ion.

other part was peptide selection via specificity, digestibility, recovery, and stability of peptides. Finally, method validation of proteotypic peptides for quantification of bovine serum albumin was performed and compared to that determined by Skyline.

2. RESULTS AND DISCUSSION

2.1. Peptide Screening and Quantification. Fully tryptic peptides of BSA (UniprotKB #P02769) were screened using targeted proteomics following the schematic shown in Figure 1A. The first step is tryptic digestion, during which the primary sequence of target protein was hydrolyzed into specific peptides with the lysine or arginine at the C-terminal. Next, to produce the isotopic interior label of all specific peptides, dimethylation was reacted on the free amino of both lysine residue and N-terminal of specific peptides.[25] Because every tryptic peptide has a free amino in the N-terminal, all of them have no less than one marked site, guaranteeing the application of dimethylation labeling in peptide selection. Dimethylation labeling in this method has two levels (Figure 1C): the light label with a mass increase of 28.031 Da using formaldehyde and the heavy label with a mass increase of 34.063 Da using isotopic formaldehyde. After trypsin digestion and dimethylation labeling, the peptide solution of BSA with the light label and the heavy label was, respectively, analyzed by a UHPLC-Q-Orbitrap mass spectrometer with full MS/dd-MS^2 (TopN) mode.

The raw files were further processed by Proteome Discoverer 2.1 (Thermo) with the Sequest algorithm. The advanced parameters of the Sequest algorithm were set at precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.02 Da, the static modifications of carbamidomethyl and dimethylation, and the dynamic modifications of acetyl and oxidation. However, not all fully tryptic peptides could be determined by Proteome Discoverer 2.1. Combining with the Xcalibur (Thermo), all fully tryptic peptides with 5−21 amino acids except for peptides VLASSAR and DAIPENLPPLTADFAEDK were identified considering the modifications of carbamidomethyl, dimethylation, and oxidation. Accurate mass of all of these peptides was determined using a high-resolution mass spectrum and is shown in Table S1.

Quantification of tryptic peptides with a light label in samples relied on the light-to-heavy ratio in which the heavy isotopic label was the heavy-labeled homologous peptides from the BSA standard. After the isometric mixing of the sample peptide solution with the light label and standard peptide solution with the heavy label, the mixture was analyzed using a quadrupole-orbitrap mass spectrometer in full MS mode. The identification information of all 49 detected peptides is shown in Table S1, including precursor charge, modifications, retention time, and response value. According to the quantification results of different peptides, different peptides have significantly different quantification values of BSA in raw bovine milk (shown in Figure S2). It was an issue that which peptide was the proteotypic peptide undertaking the accurate quantification of targeted protein (Table 1).

2.2. Peptide Release Kinetics. According to the experimental data of each peptide, fitting digestion curves and corresponding residues of 46 tryptic peptides are shown in Figure S3. Among them, the release rate of 6 peptides such as YNGVFQECCQAEDK or ECCHGDLLECADDR was too slow so their release kinetics curves were not applicable to eq 3. The calculation result of the other 40 peptides converged after several iterations and residues of all fitting points were no more than 20%, which showed a good fitting degree in release kinetic curves using the peptide release kinetics model. What's more, the Michaelis constant (K_M) and maximum reaction rate (v_{max}) of 40 peptides in BSA are shown in Table S2. A first-order kinetic equation had been reported to fit the kinetic curve of peptide release.[23] Compared with the first-order kinetic equation, our kinetic model showed a better fitting degree (Figure 2C). Additionally, this kinetic model could be used to predict the peptide release content at a specific digestion time, especially the ideal content at the end of the digestion, which could evaluate the release ratio and digestion properties of each fully tryptic peptide (as described in Section 3.5.2).

2.3. Peptide Selection. Peptide selection bases on the peptide quantification method. For peptide selection, we attempted to screen peptides via specificity, digestibility, recovery, and stability to determine the proteotypic peptides, which should (i) be unique in the assay matrix, (ii) be completely hydrolyzed in tryptic digestion, (iii) be stable for the entire experiment, and (iv) have a good recovery in peptide quantification.

The specificity of tryptic peptides was evaluated by the hit number and alignment score (Table 2). As shown in Figure 2A, the alignment score correlated well with peptide length (R = 0.9933), whereas the hit number was determined by the specificity of peptide in the whole database, which had a negative correlation with the peptide length (R = −0.4902). In UniProtKB and Swiss-Prot database, 16 fully tryptic peptides of BSA had no hits and they were marked as H grade, which were considered as the unique peptides in the whole proteome. In addition, 26 fully tryptic peptides of BSA had less than 10 hits, which were marked as M grade. The most common hits

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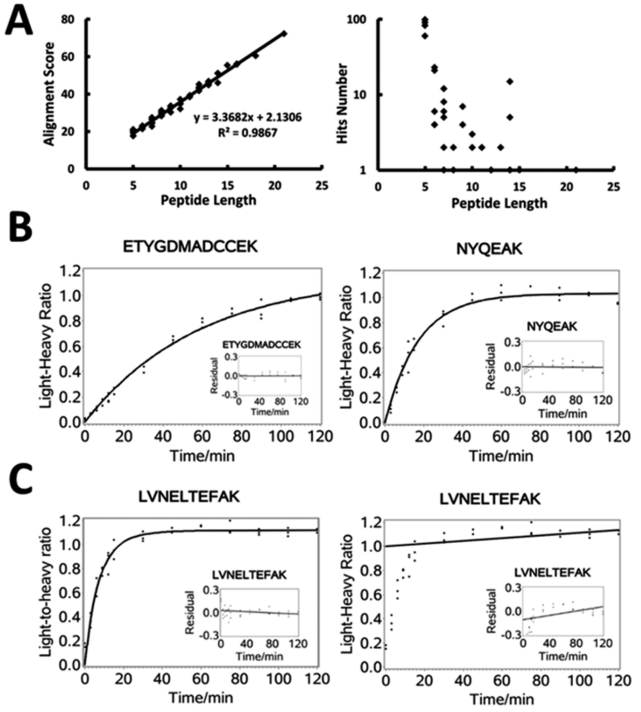

Figure 2. Selection of proteolytic peptides. (A) The relationship between the hit number and peptide length ($R = -0.4902$) and the correlation between the alignment score and peptide length ($R^2 = 0.9867$). (B) Fitting digestion curves and residual plots of ETYGDMADCCEK and NYQEAK. (C) Fitting release kinetic curves and residual plots of peptide LVNELTEFAK using our model and the first-order kinetic model.

reported were serum albumin in *Ovis aries* (sheep), *Capra hircus* (goat), *Sus scrofa* (pig), *Equus caballus* (horse), *Homo sapiens* (human), and so on. After the confirmation of absence of proteins from other species, the peptides marked as M grade could be considered as well. All of the tryptic peptides with five amino acids were marked as L grade, which suggested that fully tryptic peptides with more than five amino acids showed a better performance in specificity as proteotypic peptides.

The digestibility of tryptic peptides was determined using the tryptic hydrolysis ratio, which was calculated from the fitting digestion equation of each peptide. As shown in Figure 2B, the peptide ETYGDMADCCEK was not completely hydrolyzed within 120 min and according to the fitting equation, 70% of this peptide could be hydrolyzed from protein at the time point of 120 min and it might be completely hydrolyzed after 48 h of digestion. It is consented that the efficiency of trypsin rapidly reduces after 12 h, which indicates that ETYGDMADCCEK can never be completely hydrolyzed. Peptides NYQEAK and LVNELTEFAK were the other two peptides that showed good performance in tryptic digestion. According to their fitting curves, NYQEAK could be completely hydrolyzed within 100 min, while LVNELTEFAK, within 60 min. Tryptic hydrolysis ratios of all detected peptides are shown in Table 2; 17 fully tryptic peptides were marked as H grade as their tryptic hydrolysis ratios at the time point of 120 min were equal to 100 ± 5%. It was considered that the molality of these peptides could be similar to the real molality of target protein after proper digestion.

The recovery rate (RR) of tryptic peptides was determined using a spiked BSA standard in dimethylation and raw milk matrix (Table 2). In dimethylation, the light-to-heavy ratio of all detected peptides ranged from 84.28 to 106.07%, except for the peptides QEPER and LGEYGFQNALIVR. The differences in the light-to-heavy ratio of different peptides may result from different ionization rates between light and heavy-labeled peptides. The RR of tryptic peptides in dimethylation that ranged from 90 to 105% had a similar ionization rate in light and heavy-labeled peptides. In the raw milk matrix, the RR of some detected peptides could be influenced by the matrix. For example, RR in the matrix of peptide GLVLIAFSQYLQQCPF-DEHVK was 523.13%, which had matrix interference in the channel of the light label. As for AEFVEVTK, the RR in the matrix was 34.18% and the matrix interference of this peptide was in the channel of the heavy label. In general, 24 fully tryptic peptides were marked as H grade as their recovery rate ranged from 90 to 105% in both dimethylation and the matrix, which guaranteed that these peptides could eliminate errors from dimethylation and assay matrix.

The stability of tryptic peptides was evaluated by relative standard deviation (RSD) shown as reproducibility. In Table 2, the reproducibility of most peptides in dimethylation and in raw milk matrix was good and less than 10%. Peptides having poor reproducibility were considered to have a long length or have matrix interference, such as GLVLIAFSQYLQQCPF-DEHVK, having a long length and CCAADDK having matrix interference in the channel of the light label. In general, 26 fully tryptic peptides were marked as H grade, for their reproducibility in dimethylation and the matrix was both less than 5%.

According to the results shown in Table 2, peptide LVNELTEFAK accomplished H grade in specificity, digestibility, recovery, and stability. Therefore, peptide LVNELTE-FAK was chosen as proteotypic peptide of BSA in accurate quantification. The peptides that accomplished H grade in digestibility, recovery, and stabilit, but M grade in specificity could also be considered if the assay matrix did not show interference from other species, such as YLYEIAR, AWSVAR, HLVDEPQNLIK, and QTALVELLK.

2.4. Method Validation. The proteotypic peptide LVNELTEFAK were used to quantify BSA in raw bovine milk. On the basis of the proteotypic peptide, the concentration of bovine serum albumin in tenfold-diluted raw bovine milk was 16.93 ± 0.80 mg/kg, similar to the BSA contents reported by Indyk et al.[26] Method validation of proteotypic peptides in BSA quantification was reported as follows.

The internal standard method was used to quantify BSA using proteotypic peptides as markers. Calibration curves were obtained by the relationship between the light-to-heavy ratio and the concentration of the BSA standard in the range of 1−100 ppm, with 10 ppm heavy-labeled BSA standard as the internal standard. The calibration curve with the linear regression equation and correlation coefficient of proteotypic peptide is shown in Figure 3A. Good linearity of proteotypic peptides ($R^2 > 0.9990$) was achieved over concentration levels ranging from 1 to 100 ppm. Standard curves of proteotypic peptides selected by Skyline are shown in Figure S4.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined as the concentration of the BSA standard, where the signal-to-noise (S/N) ratio of peptides reached 3:1 and 10:1, respectively. The spectrum chromatograms of proteotypic peptide are shown in Figure 3B. LOD and LOQ of proteotypic peptide shown in Table 3 were 0.78 and 2.59 mg/kg in raw bovine milk, respectively.

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Table 2. Specificity, Digestibility, Recovery, and Stability of Fully Tryptic Peptides with 5−21 Amino Acids in Peptide Selection (n = 3)[a]

		specificity			digestibility			recovery			stability		
number	sequence	hit number	alignment score	grades	hydrolysis ratio in standard (%)	hydrolysis ratio in matrix (%)	grades	recovery rate in dimethylation (%)	recovery rate in matrix (%)	grades	reproducibility in dimethylation (%)	reproducibility in matrix (%)	rrades
1	SEIAHR	21	22.3	L	69.01	61.77	L	95.08	104.55	H	8.54	2.92	M
2	DLGEEHFK	2	29.5	M	91.41	94.69	M	92.29	100.52	H	4.01	3.07	H
3	GLVLIAFSQYLQQCPFDEHVK	1	72.3	M	100.52	97.04	H	101.18	523.13	L	30.29	49.72	L
4	LVNELTEFAK	0	34.6	H	100.76	99.19	H	98.70	93.47	H	2.59	4.39	H
5	TCVADESHAGCEK	0	44.8	H	102.99	94.40	M	93.08	90.33	H	2.47	1.33	H
6	SLHTLFGDELCK	1	41.8	M	98.70	94.75	M	97.34	93.23	H	3.49	3.09	H
7	VASLR	99	17.6	L	99.39	98.38	H	98.63	98.69	H	2.00	1.64	H
8	ETYGDMADCCEK	1	45.2	M	68.48	85.58	L	91.75	94.54	H	3.04	3.42	H
9	QEPER	83	20.2	L	ND	ND	ND	149.50	296.62	L	45.50	40.17	L
10	NECFLSHK	0	30.3	H	0.00	0.00	L	88.72	104.15	M	19.99	13.53	L
11	DDSPDLPK	1	28.6	M	39.03	27.10	L	92.16	104.52	H	3.96	7.26	M
12	LKPDPNTLCDEFK	0	46.4	H	20.90	12.43	L	86.66	84.69	L	2.40	2.74	H
13	YLYEIAR	6	27.4	M	101.47	98.48	H	100.39	101.42	H	1.96	2.87	H
14	HPYFYAPELLYYANK	1	55.4	M	83.08	91.88	L	99.75	179.91	L	11.56	6.15	L
15	YNGVFQECCQAEDK	1	51.1	M	18.97	33.39	L	89.16	89.56	M	5.97	5.92	L
16	GACLLPK	1	24.8	M	41.66	75.52	L	93.81	97.21	H	3.05	1.86	H
17	IETMR	91	21.0	L	100.01	95.64	H	98.84	100.24	H	1.91	1.75	H
18	VLASSAR	1	22.7	M	ND	ND	ND	ND	ND	ND	ND	ND	ND
19	CASIQK	4	22.3	M	95.97	88.35	L	93.77	88.72	M	2.48	1.34	H
20	AWSVAR	6	22.7	M	99.55	98.96	H	96.79	99.20	H	2.33	2.14	H
21	AEFVEVTK	0	28.2	H	99.22	101.04	H	95.26	34.18	L	1.90	2.02	H
22	LVTDLTK	5	24.4	M	100.32	97.71	H	95.15	88.55	M	2.22	1.88	H
23	ECCHGDLLECADDR	15	51.1	L	8.12	0.00	L	89.56	119.84	L	7.21	11.25	L
24	ADLAK	99	17.6	L	28.25	92.90	L	88.90	103.35	M	3.48	6.70	M
25	YICDNQDTISSK	0	43.1	H	100.81	91.99	M	95.25	91.57	H	3.12	2.22	H
26	ECCDKPLLEK	3	37.1	M	84.24	101.34	L	84.28	83.83	L	5.42	3.48	M
27	SHCIAEVEK	0	32.5	H	40.99	37.45	L	89.21	124.27	L	5.31	5.69	M
28	DAIPENLPPLTADFAEDK	0	60.4	H	ND	ND	ND	ND	ND	ND	ND	ND	ND
29	NYQEAK	4	23.1	M	100.44	92.81	M	94.80	91.88	H	2.32	2.02	H
30	DAFLGSFLYEYSR	0	45.6	H	98.42	83.72	L	93.29	86.24	M	6.45	15.00	L
31	HPEYAVSVLLR	2	38.4	M	76.69	21.85	L	95.45	125.21	L	6.49	5.80	M
32	EYEATLEECCAK	0	43.5	H	33.75	48.14	L	92.04	95.93	H	3.03	3.54	H
33	DDPHACYSTVFDK	0	46.9	H	0.02	0.00	L	93.83	96.17	H	3.13	4.97	H
34	HLVDEPQNLIK	2	39.2	M	101.09	96.85	H	97.14	93.20	H	2.44	1.90	H
35	QNCDQFEK	0	31.2	H	98.45	95.36	H	90.61	87.33	M	2.88	2.96	H
36	LGEYGFQNALIVR	2	44.8	M	99.32	32.05	L	132.10	167.25	L	85.34	62.16	L
37	VPQVSTPTLVEVSR	5	46.0	M	100.70	31.05	L	103.79	90.50	H	5.88	5.61	M
38	CCTKPESER	0	33.7	H	96.62	89.64	L	91.45	87.38	M	5.85	4.75	M
39	MPCTEDYLSLILNR	1	51.1	M	97.32	95.58	H	92.71	108.15	M	7.43	2.72	M
40	LCVLHEK	12	26.5	L	101.11	94.05	M	89.88	87.27	M	2.86	2.30	H
41	TPVSEK	23	21.4	L	97.09	94.82	M	96.92	94.09	H	5.97	2.95	M

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Table 2. continued

number	sequence	specificity			digestibility			recovery			stability		
		hit number	alignment score	grades	hydrolysis ratio in standard (%)	hydrolysis ratio in matrix (%)	grades	recovery rate in dimethylation (%)	recovery rate in matrix (%)	grades	reproducibility in dimethylation (%)	reproducibility in matrix (%)	rrades
42	CCTESLVNR	7	33.3	M	102.25	95.70	H	92.89	95.08	H	2.97	2.81	H
43	RPCFSALTPDETYVPK	0	55.8	H	101.31	90.20	M	98.38	93.05	H	5.06	2.23	M
44	AFDEK	60	19.3	L	98.29	97.53	H	91.04	89.08	M	3.24	2.09	H
45	LFTFHADICTLPDTEK	0	56.2	H	97.47	96.61	H	85.09	100.20	M	6.74	2.73	M
46	QTALVELLK	4	30.3	M	99.60	97.01	H	97.54	94.51	H	2.42	2.69	H
47	ATEEQLK	8	24.8	M	99.64	97.84	H	93.15	89.56	M	1.92	1.34	H
48	TVMENFVAFVDK	1	43.1	M	95.43	93.45	M	93.06	97.37	H	5.03	3.98	M
49	CCAADDK	2	26.5	M	ND	ND	ND	106.07	154.49	L	60.05	77.46	L
50	EACFAVEGPK	2	35.0	M	22.97	10.29	L	94.66	108.06	M	5.25	7.66	M
51	LVVSTQTALA	0	32.0	H	100.12	102.89	H	94.96	102.04	H	4.03	3.57	H

[a]*ND: not detected.

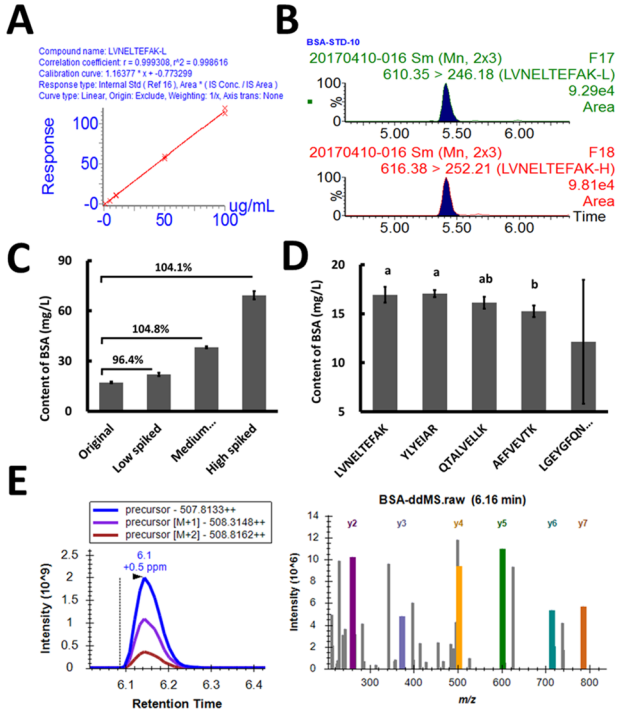

Figure 3. Method validation of proteotypic peptide. (A) Calibration curve of peptide LVNELTEFAK with the correlation coefficient of 0.9993. (B) Quantification chromatography of peptide LVNELTEFAK with multiple reaction monitoring mode (MRM). L quantitative channel was for samples, while H quantitative channel was for the internal standards. (C) The recovery rate of the additional standard. The recovery of the three levels was 96.4% of 5 ppm, 104.8% of 20 ppm, and 104.1% of 50 ppm. (D) Concentration of BSA quantified by different peptides. The concentration of peptide LGEYGFQNALIVR showed a significant deviation. The concentration of BSA using peptide AEFVEVTK was significantly lower than the concentration using peptide LVNELTEFAK. No significant differences in the concentration of BSA were quantified using peptide LVNELTEFAK, YLYEIAR, and QTALVELLK. (E) Chromatography and spectrum of peptide in Skyline software. On the basis of the quality of chromatography and spectrum, peptides were divided into three groups using Skyline software.

The standard addition method was applied to determine the recovery of proteotypic peptide. The raw bovine milk samples were spiked with low, medium, and high standard levels (50, 200, and 500 ppm), which were diluted 10-fold before analysis, and the concentration of the internal standard was 10 ppm. As shown in Figure 3C, the recovery of proteotypic peptide was 96.4% in low, 104.9% in medium, and 104.1% in high spiked levels. This result met the general requirements reported in ISO 5725.

The intraday and interday precision are expressed as relative standard deviation. As shown in Table 3, the intraday precision of proteotypic peptide was no more than 5% and the interday precision was no more than 10%, which met the general requirements reported in ISO 5725.

2.5. Method Comparison. The proteotypic peptide confirmed by our strategy was compared with optimal proteotypic peptides in Skyline. After the analysis of Skyline, peptides were divided into three grades, which were green, yellow, and red.[27] The peptides marked as green were regarded as the proteotypic peptides. Combining with the PeptidePicker, the proteotypic peptides of bovine serum albumin were

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Table 3. Method Validation and Comparison of the Two Peptide Selection Methods (n = 3)

peptides	linearity	LOD (mg/kg)	LOQ (mg/kg)	spiked level (mg/kg)	recovery (%) intraday	RSD (%)	RSD (%) interday	BSA content (mg/kg)
LVNELTEFAK	$Y = 1.27059*X - 1.05696$	0.78	2.59	50	96.43	4.22	8.18	169.3 ± 8.0
				200	104.92	1.78	7.77	
	$R = 0.9995$			500	104.08	2.58	6.74	
YLYEIAR	$Y = 1.11458*X - 0.401474$	0.78	2.59	50	94.77	3.65	9.43	170.5 ± 3.7
				200	104.06	3.18	9.84	
	$R = 0.9999$			500	103.17	4.46	9.83	
QTALVELLK	$Y = 1.4148*X - 1.08762$	1.99	6.63	50	97.25	3.09	9.36	161.2 ± 6.1
				200	96.91	4.73	8.24	
	$R = 0.9996$			500	98.11	4.43	9.36	
AEFVEVTK	$Y = 1.15335*X - 0.689148$	0.86	2.88	50	88.03	4.03	8.44	152.5 ± 6.0
				200	98.22	3.47	7.54	
	$R = 0.9998$			500	102.63	4.73	9.47	
LGEYGFQNALIVR	$Y = 0.239964*X - 0.320657$	15.6	52.0	50	102.21	17.91	46.66	121.4 ± 60.3
				200	101.44	22.40	43.47	
	$R = 0.9969$			500	102.89	16.07	83.21	

YLYEIAR, AEFVEVTK, LGEYGFQNALIVR, and QTALVELLK in Skyline. All proteotypic peptides in the two methods are listed in Table 3, and the method validation was performed following the ISO 5725. As shown in Figure 3D, concentration of peptide LGEYGFQNALIVR showed a significant deviation, which showed a poor capacity of BSA quantification. Among other proteotypic peptides, the concentration of BSA using peptide AEFVEVTK was significantly lower than the concentration using other peptides. It was suggested that the low contents resulted from the different recovery rates of these peptides in the standard and samples. Additionally, the specificity of peptides YLYEIAR and QTALVELLK was poor to meet the quantification of target protein when there was interference from other species. In general, the proteotypic peptide LVNELTEFAK had a better quantification quality of BSA than those peptides confirmed by Skyline software. Skyline is an effective software for peptide evaluation in targeted proteomics, which considered the precursor response characteristics and fragmentation-pattern quality of each peptide. While in our peptide selection strategy, digestion and matrix interference were also considered using dimethylation labeling and enzymolysis model. Therefore, our strategy is an efficient and comprehensive workflow for peptide selection.

In conclusion, the accuracy of protein quantification using peptides as biomarkers is affected by many factors, including peptide specificity, peptide stability, tryptic digestion, and assay matrix. The peptide release ratio was an important factor influencing the accuracy of protein quantification. We have presented a dimethylation high-resolution mass spectrum strategy with a peptide release kinetics model to determine the tryptic digestion release ratio of proteolytic peptides. Combined with specificity, stability, and recovery of fully tryptic peptides, the current strategy can be applied to proteotypic peptide selection for targeted proteins, including biomarkers, bioactive proteins, and food allergens. In this study, proteotypic peptides of bovine serum albumin were determined as LVNELTEFAK, which showed a better performance in method validation than the proteotypic peptides determined by Skyline. Considering the peptide release kinetics as well as mass spectrum property and specificity, this strategy was an efficient and comprehensive workflow for peptide selection in targeted proteomics. The quantification of target proteins using the proteotypic peptides could have the similar result with the protein standard, which facilitated accurate quantification of target proteins in lack of protein standards. This workflow provides a potential common method for marker peptide selection in different food matrices. Different food matrices analyzed through this workflow can obtain corresponding optimal markers for accurate quantification of target proteins.

3. MATERIALS AND METHODS

3.1. Reagents and Samples. BSA was used as the standard for protein quantitation and purchased from Sigma-Aldrich (St. Louis, MO). Sodium bicarbonate ($NaHCO_3$), dithiotheritol (DTT), iodoacetamide (IAA), ammonium hydroxide, sodium cyanoborohydride ($NaBH_3CN$), formaldehyde (CH_2O), and formaldehyde-isotope ($^{13}CD_2O$) were from Sigma-Aldrich (St. Louis, MO) as well. Sequencing grade modified trypsin was from Worthington Biochemical Corporation (Freehold, NJ). Formic acid and acetonitrile of HPLC grade were obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained by a Milli-Q Gradient water purification system (Millipore, Bedford, MA). Raw bovine milk samples were provided by Shiyun Lai in Beingmate Research Institute.

3.2. Protein Digestion. Fifty microliter samples with a certain concentration were diluted using 900 μL of 100 mM $NaHCO_3$ solution, and the final concentration of total protein was kept less than 200 μg/mL. The mixture was reduced by adding 10 μL of 500 mM DTT in a metal bath at 70 °C for 30 min. Alkylation was performed with 30 μL of 500 mM IAA for 30 min at 25 °C in the dark. Subsequently, 10 μL of trypsin (1 mg/mL, trypsin to protein ratio of 1:10, w/w) was added, mixed gently, and incubated for 2 h in the metal bath at 37 °C. The peptide solution from raw bovine milk samples was prepared after passing through a 0.22 μm nylon filter.[28,29] The peptide solution from BSA standards was prepared as described above.

3.3. Dimethylation. Hundred microliters of the peptide solution form raw bovine milk samples was added to 4 μL of

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4% (v/v) CH_2O for light labels, and then 4 μL of 0.6 M $NaBH_3CN$ was added to the solution. The solution was mixed and incubated in a metal bath for 1 h at 25 °C.[30] After terminating the dimethylation reaction by adding 16 μL of 1% (v/v) ammonia, the mixture was acidified by 8 μL of formic acid and centrifuged at 8000*g* for 3 min at 25 °C. Finally, the peptide solution of the sample with the light label was made. In addition, 100 μL of the peptide solution form BSA standards was added to 4 μL of 4% (v/v) CH_2O or $^{13}CD_2O$ respectively for light and heavy labels and then was performed the same steps as above to obtain the peptide solution of BSA with light or heavy label. The peptide solution with the light label was diluted by isometric heavy-labeled peptide solution, which was prepared using the BSA standard solution throughout the experiment.

3.4. High-Resolution Mass Spectrometry. The mixed peptide solution was analyzed using a quadrupole-orbitrap mass spectrometer (Q-Exactive, Thermo) equipped with a UHPLC separation system[31] (Vanquish, Thermo). Each solution of 10 μL was separated on the Acquity BEH 300 C18 column (2.1 mm × 100 mm, 1.7 μm, Waters) at 30 °C. Gradient elution performed with a mixture of 0.1% formic acid-water (mobile phase A) and 0.1% formic acid-acetonitrile (mobile phase B) at the flow rate of 0.3 mL/min: 3% B for 0 min to 1 min; 3% B to 60% B for 1 min to 10 min; 60% B to 100% B for 10 min to 10.5 min; 100% B for 10.5 min to 12.5 min; 100% B to 3% B for 12.5 min to 13 min; and 3% B for 13 min to 15 min. High-resolution mass spectrometry analyses were performed using the quadrupole-orbitrap mass spectrometer with an HESI source in the positive-ion mode.[32] Ionization conditions were set at the sheath gas flow rate of 40 L/min, aux gas flow rate of 10 L/min, spray voltage of 3.5 kV, capillary temperature of 320 °C, s-lens RF level of 50, and aux gas heater temperature of 350 °C. The acquisition mode of quadrupole-orbitrap analyses was set to be the full MS/dd-MS2 (TopN) mode and full MS mode. Full MS/dd-MS2 (TopN) mode is a combination of full MS mode and dd-MS2 mode. Full MS mode employed a mass scan range of 200−2000 *m*/*z*, an orbitrap resolution of 70 000 with maximum latency time of 200 ms, and target AGC values of 1×10^6. The dd-MS2 mode employed an orbitrap resolution of 17 500 with maximum latency time of 50 ms, target AGC values of 1×10^5, loop count of 10, stepped NCE of 25, 30, and 35, and an isolation window of 2.0 *m*/*z*. Data analyses of high-resolution mass spectrometry were performed using Xcalibur (Thermo) and Proteome Discoverer 2.1 (Thermo) with the Sequest algorithm.

3.5. Peptide Selection. *3.5.1. Peptide Specificity.* The specificity of tryptic peptides was confirmed by Basic Local Alignment Search Tool (BLAST) search against the UniProtKB/Swiss-Prot database (https://blast.ncbi.nlm.nih.gov), which required that the peptide length should be more than four amino acids. For setting search criteria on the specific web page, the algorithm was blastp (protein-protein BLAST) and no organism was excluded. After the BLAST search, the hit number and alignment score of each peptide were reported. For specific trypsin peptide, the hit number means the number of interfering peptides involved. It has to be mentioned that the results are data bank dependent; it could be possible that they occur in more organisms, but probably the proteins of these organisms are not investigated, and at the moment, no database entry can be found. According to the hit number, all tryptic peptides were divided into three grades: high (H, hit number = 0), medium (M, 1 ≤ hit number ≤ 10), and low (L, hit number ≥ 11) and the grade "high" means the best peptide specificity. The specific search web page is as follows.

https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

3.5.2. Peptide Digestibility. A mathematical model was established to reflect the time history of peptide production during the tryptic digestion (Note S1). This equation is derived from the Michaelis−Menten eq 1

$$v = \frac{v_{max} \times [S]}{[S] + K_M} \tag{1}$$

where v is the enzyme velocity of the current substrate concentration [S], v_{max} is the maximum enzyme velocity, and K_M is the Michaelis constant.

On the basis of the operation of ordinary differential equation and Taylor series, the function relationship between peptide production (*c*) and digestion time (*t*) could be expressed as eq 2

$$c = A - A \times \exp\left(-\sqrt{\left(1 + \frac{K_M}{A}\right)^2 + \frac{v_{max}}{A} \times t} + \left(1 + \frac{K_M}{A}\right)\right) \tag{2}$$

In protein digestion, it is assumed that the substrate protein would be fully digested into equimolar fully tryptic peptides and the maximum concentration (*A*) of each fully tryptic peptides would be equal to the substrate concentration at the initial time. To simplify the fitting operation, three undetermined parameters were set to be *a*, *k*, and *m*, which were able to reflect the digestibility of peptides as well. The final digestion equation simplified from eq 2 was expressed as eq 3

$$c = a - a \times \exp(-\sqrt{k^2 + m \times t} + k) \tag{3}$$

where *a* equaled *A*, *k* equaled $(1 + K_M/A)$, and *m* equaled v_{max}/A.

For the fitting of digestion curves, the production of fully tryptic peptides from the BSA standard with and without raw milk matrix at different digestion times (2, 4, 6, 8, 10, 20, 40, 60, 80, 100, and 120 min) was determined by high-resolution mass spectrum in full MS mode and quantified by the light-to-heavy isotope ratio, in which the heavy isotopic label was the heavy-labeled peptide solution of BSA standard with 120 min of digestion time. On the basis of eq 3, the digestion curves were fitted using PROC NLIN in SAS (version 9.4) and the iterative method was Gauss−Newton (Figure S1). In the general equation, three variables, *a*, *k*, and *m*, were determined by the experimental data of each peptide and the tryptic hydrolysis rate of each peptide at a specific digestion time was calculated using the fitting equation and eq 4

$$\text{tryptic hydrolysis rate (\%)} = \frac{C_t}{a} \tag{4}$$

where c_t is the relative response value of the peptide at time (*t*) of enzymatic hydrolysis, *a* equaled *A*, is the relative response value of the peptide in complete enzymatic hydrolysis, which predicted by the fitting equation. The tryptic hydrolysis ratio of peptides at the digestion time of 120 min was used as the

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evaluation index for peptide digestibility, and on the basis of the tryptic hydrolysis ratio of each peptide from the BSA standard with and without raw milk matrix, all tryptic peptides were divided into three grades: high (H, both |hydrolysis ratio−100%| ≤ 5%), medium (M, both |hydrolysis ratio−100%| ≤ 10%), and low (L, either |hydrolysis ratio−100%| > 10%). The grade high means the best peptide digestibility in peptide selection.

3.5.3. Peptide Recovery. The efficiency of dimethlation using light or heavy label reagents and the matrix effect of milk matrices could influence the recovery of peptides in mass spectrometry. So the recovery of tryptic peptides was evaluated in two aspects. The first one was the recovery in dimethylation of each peptide, which was evaluated using the light-to-heavy ratio of tryptic peptides from the BSA standard labeled by both light and heavy labels. The specific experimental procedure was to mix equal amounts of peptide solution of BSA with light and heavy label, then perform high-resolution mass spectrometry on the mixture. When the light-to-heavy ratio of tryptic peptide was close to 100%, it was consented that the dimethylation labeling of one tryptic peptide was similar in both light and heavy labeling. The other one was the recovery rate (RR) of each peptide from spiked BSA standard in raw milk matrix. The specific experimental procedure was to mix equal amounts of the peptide solution of BSA with heavy label, and raw bovine milk samples with light label, which spiked the BSA standard. Then, high-resolution mass spectrometry was performed on the mixture. RR was calculated using the following eq 5.

$$\mathrm{RR}=(\text{detection value} - \text{original value})/\text{spiked value} \quad (5)$$

where the detection value is the relative response value of a specific peptide in the mixture, the original value is the relative response value of a specific peptide in the raw bovine milk samples, and the spiked value is the relative response value of a specific peptide in the spiked BSA standard. According to the RR of each peptide in both dimethylation (e.g., light-to-heavy ratio) and raw milk matrix, all tryptic peptides were divided into three grades: high (H, both |RR−100%| ≤ 10%), medium (M, both |RR−100%| ≤ 15%), and low (L, either |RR−100%| > 15%). The grade high means the best peptide recovery in peptide selection.

3.5.4. Peptide Stability. The stability of tryptic peptides was analyzed by the reproducibility of detection values in dimethylation and with raw milk matrix in six parallel tests for 3 days. The reproducibility was evaluated byan RSD of a total of 18 tests, separately in dimethylation and in the matrix. On the basis of the reproducibility of each peptide from the BSA standard with and without raw milk matrix, all tryptic peptides were divided into three grades: high (H, both RSD ≤ 5%), medium (M, both RSD ≤ 10%), and low (L, either RSD > 10%). The grade high means the best peptide stability in peptide selection.

3.6. Peptide Selection Using Skyline Software. Skyline is an application for method creation and data analysis of targeted proteomics. It can also evaluate the quality of the peptide signal and determine the scoring of peptide quality.[27] Raw data files imported in Skyline were acquired using UHPLC-Q-Orbitrap. The FASTA file of BSA was downloaded from Uniprot, and the standard database was downloaded from NIST (http://peptide.nist.gov). The proteotypic peptides were determined using the scoring of peptide quality, which were marked as green (Figure 3E).

3.7. Method Validation. Detection of selected peptides was performed using the multiple reaction monitoring (MRM) method of a Waters TQ-XS mass spectrometer (Waters) equipped with an electrospray ionization (ESI) source in positive-ion mode.[31,33] The parameters of a mass spectrometer were set at a capillary voltage of 3.0 kV, desolvation temperature of 400 °C, desolvation gas flow of 600 L/h, and cone gas flow of 150 L/h. MRM transitions of each peptide are shown in Table 1. The isotope internal standard solution was the peptide solution of the BSA standard with the heavy label, and the concentration of the BSA sample was 10 μg/mL. The isotope internal standard solution was used to eliminate the matrix effect during ionization. In addition, 1, 5, 10, 50, and 100 μg/mL standard solutions using BSA were prepared and the peptide solution with the light label was obtained, respectively, as mentioned in Sections 3.2 and 3.3. Then, the peptide solution of BSA was mixed with the light label and isotope internal standard solution in equal volume. Calibration curves were obtained by using UPLC-MS/MS to analyze the series of the mixture. Raw bovine milk samples were processed using the same steps as above to determine the content of BSA and the liquid-phase conditions are referred to in Section 3.4. The acquired data were processed with MassLynx 4.1 software. This detection method of targeted protein was validated by linearity, sensitivity, recovery, precision, and method comparison. (a) Linearity: The linearity of the standard curve was determined by its linear correlation coefficient (R). (b) Sensitivity: The sensitivity was evaluated by LOD and LOQ, which were the concentrations of the target peptide where their signal-to-noise (S/N) ratio was 3:1 and 10:1, respectively. (c) Recovery: The RR of selected peptide was determined using the standard addition method, in which the three spiked levels of bovine serum albumin were 5 mg BSA/100 g raw milk, 20 mg BSA/100 g raw milk, and 50 mg BSA/100 g raw milk. Then, the RR was calculated by referring to eq 5. (d) Precision: Precision included intraday and interday precision. In general, intraday precision was determined by RSD of six parallel detection values of each sample on the same day. As for the interday precision, the same experiment as above was performed for 3 days and the interday precision was determined using the interday RSD of the entire experiment.

3.8. Statistics. All experiments were performed in triplicates, and results are expressed as mean ± SD. Statistical analysis was performed using SAS 9.4 and Excel 2007, and the fitting code in SAS is shown in Figure S1.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b02002.

Derivation of equation in the digestion model, basic information of fully tryptic peptides with 5−21 amino acids in bovine serum albumin, Michaelis constant (K_M) and maximum reaction rate (v_{max}) of tryptic peptides in BSA, fitting code of PROC NLIN in SAS, quantification results of BSA in raw bovine milk with different peptides, fitting digestion curves of tryptic peptides with 5−21 amino acids in bovine serum albumin, and standard curves of proteotypic peptides selected by Skyline in UPLC-MS/MS with MRM mode (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PSAQ, protein standard absolute quantification; AQUA, absolute quantification; ESP, enhanced signature peptide; BSA, bovine serum albumin; UHPLC-Q-Orbitrap, ultra high-performance liquid chromatography-quadrupole-orbitrap; DTT, dithiotheritol; IAA, iodoacetamide; HESI, heat electronic spray ion; RR, recovery rate; RSD, relative standard deviation; MRM, multiple reaction monitoring; ESI, electronic spray ion; R, linear correlation coefficient; LOD, limit of detection; LOQ, limit of quantitation; SD, standard deviation; ISO, international organization for standardization

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