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Identification of glycated peptides in human serum using LC-MS/MS: A comparison of data-dependent acquisition and Parallel reaction monitoring

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Abstract: Protein glycation is vital to aging and disease. However, glycated proteins are low-abundant in plasma, rendering them difficult to identify using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Many studies have analyzed glycated peptides with high reproducibility. Here, glycated peptides in human serum were analyzed by LC-MS/MS using data-dependent acquisition (DDA) and parallel reaction monitoring (PRM). Boronic acid (BA) enrichment of *in vitro* glycated human serum peptides was performed. BA enrichment identified the most glycated peptides, and the glycated peptides of the more diversified proteins, excluding albumin, were analyzed. In PRM, glycated albumin PSMs were the most common, and this method exhibited the best reproducibility. The results of this study could help compare methods for identifying glycation-related biomarkers.

Key words: Glycation, PTM, PRM, DDA, Enrichment, LC-MS/MS

1. Introduction

Glycation is a spontaneous post-translational modification that occurs at the lysine and arginine residues, and at the N-terminus of proteins.[1] The active carbonyl group of the sugar and the nucleophilic free amino group chemically react to form an unstable Schiff base. The Schiff base is rearranged to form Amadori compounds that form advanced glycation end products (AGEs) through several reactions.[2]

High concentrations of AGEs in the blood cause oxidative stress by activating receptors for advanced glycation end products. The amount of AGEs in the blood is related to degenerative diseases such as Alzheimer's disease.[3,4] Since the AGE level is proportional to the sugar in the serum, studies on their association with diabetes are also being actively conducted.[5]

Glycated proteins are low-abundant in the plasma, so it is difficult to identify them using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with data-dependent acquisition (DDA) without extra steps.[6] Consequently, to increase sensitivity and efficiency, researchers usually perform enrichment of the target peptides prior to LC-MS/MS analysis.[7] Commonly used enrichment methods include two-dimensional gel electrophoresis (2D-GE), affinity chro-

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matography, and two-dimensional online liquid chromatography. However, these methods are not suitable for application to all sample types. For instance, 2D-GE is not appropriate for low-abundance proteins because they would be underrepresented due to being below the detection limit.[8] Furthermore, boronate affinity chromatography (BAC), an example of affinity chromatography, is inefficient, as it captures all variants with 1,2 or 1,3-cis-diol groups.[9] Poor reproducibility is also a major limitation of BAC.

To compensate for this weakness, parallel reaction monitoring (PRM) has been used for glycated hemoglobin (Hb) in samples from patients and healthy donors analyzed at endocrinology departments.[10] The recently developed quadrupole-orbitrap offers specific trapping capacities to enhance the analysis of low-abundance peptides. In addition to proteomics, PRM can also be applied to targeted peptide identification, as it contains all fragment ion information on the preselected precursor with high sensitivity. In this study, a scheduled inclusion list of 100 precursors was used, and the results were estimated by comparison with the standard DDA method. The pseudo-targeted PRM approach had superior sensitivity and reproducibility than was possible when using the DDA method.

To the best of our knowledge, PRM analysis of whole glycated human serum proteins without enrichment has not been sufficiently studied. Herein, we compared the boronic acid (BA) enrichment, PRM acquisition, and DDA methods using *in vitro* incubated human serum samples. We used the information from mass spectrometry results obtained in a previous study[11] on PRM acquisition. The list includes the peptide's *m/z*, charge state, and retention time window, because chromatography is clearer when the elution time is scheduled compared to when it is unscheduled.[12] The PRM results showed high reproducibility and strong performance, especially for the identification of albumin.

2. Experimental

2.1. Chemicals and reagents

Iodoacetamide, ammonium bicarbonate (ABC), $MgCl_2$, urea, tris (hydroxymethyl)aminomethane (Tris), trichloroacetic acid (TCA), and ammonium acetate (NH_4OAc) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tris (2-carboxyethyl) phosphine (TCEP) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Sequencing-grade modified trypsin was acquired from Promega (Madison, WI, USA). Acetic acid (HOAc), methanol, acetonitrile, and high-performance liquid chromatography-grade water were purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid was purchased from Fluka (Charlotte, NC, USA).

2.2. Instrument conditions

Experiments were conducted using a nano liquidchromatography-tandem mass spectrometry (nano-LC-MS/MS) system consisting of a Q Exactive™ Hybrid Quadrupole-Orbitrap MS (Thermo Fisher Scientific) coupled with a Dionex Ultimate 3000 HPLC instrument (Sunnyvale, CA, USA). The mobile phase consisted of 0.1 % Formic Acid (FA) in water (solvent A) and 0.1 % FA in 80 % ACN (solvent B). Peptides were loaded onto an Acclaim™ PepMap™ 100 C18 nano-trap column (75 μm × 2 cm, 3 μm particles, 100 Å pore size) using a mobile phase at a flow rate of 3 μL/min for 10 min. The peptides were separated on a PepMap™ RSLC C18 nanocolumn (2 μm, 100 Å, 75 μm × 5 cm) at a flow rate of 300 nL/min. In DDA, the top 10 precursor peaks were fragmented with higher-energy collisional dissociation, and the normalized collisional energy was 27. Ions were scanned at 70,000 in MS1 and 17,500 in MS2 (the first and second levels of mass analysis, respectively) over an MS scan range of 400-2000 *m/z* for both MS1 and MS2 levels. PRM was analyzed using the glycation inclusion list, and the normalized collisional energy was 27. The ions were scanned at 17,500 in MS2. The concentration of the peptide was measured using a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific), and the injection amount was 1 μg.

2.3. *In vitro* glycation of samples

In vitro glycated human serum was prepared

according to the method of Zhang *et al.*[13] Human serum was incubated with glycation buffer (1M D-(+)-glucose, 50 mM Tris-HCl, pH 7.5) at 37 °C for 48 h before glycation at -20 °C. Free glucose in glycated human serum was removed using 3 kDa Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA, USA).

2.4. Sample preparation using FASP

Glycated standard human serum was digested using filter-aided sample preparation (FASP)[14] on a Microcon YM-30 filter (Millipore). Standard human serum (200 μg) was adjusted to a volume of 100 μL lysis buffer (8 M urea, 0.1 M Tris, and HCl buffer, pH 8.5). Protein was reduced by incubation with TCEP at 37 °C for 30 min and alkylated with iodoacetamide at 25 °C for 1 h in the dark. After washing with 50 mM ABC, the protein was digested with trypsin at an enzyme-to-protein ratio of 1:50 (w/w) at 37 °C for 18 h. The digested protein was collected in new tubes, and trypsin was inactivated by acidification with 20 μL of formic acid. The collected peptides were dried using a HyperVAC-MAX VC2200 centrifugal vacuum concentrator (Hanil Scientific, Inc., Gochon-eup, South Korea) and desalted using ultra-micro spin C18 columns (Harvard Apparatus, Holliston, MA, USA).

2.5. Boronate affinity enrichment

Glycated peptides were enriched using Cellufine PB (JNC Corporation, Tokyo, Japan), which is a phenyl borate affinity ligand. The buffers used were as follows (A) loading buffer: 50 mM $MgCl_2$ and 250 mM NH_4Oac, pH 8.1; (B) washing buffer: 50 mM NH_4HCO_3, pH 8.1; and (C) eluting buffer: 0.1 M HOAc, pH 3.0, all prepared in HPLC-grade water.[15] The 500 μL Cellufine PB was placed on a YM-30 filter and washed five times with buffer (A) for equilibration. The sample was then resuspended in 250 μL of buffer (A) and loaded into a YM-30 filter with Cellufine PB. After loading the sample, it was washed three times with 400 μL of buffer (B). After washing, the retained glycated peptide was eluted twice with 200 μL of buffer (C). Enriched glycated peptides were desalted using ultra-micro spin C18 columns (Harvard Apparatus, Holliston, MA, USA). The desalted glycated peptides were then dried using a centrifugal vacuum concentrator.

2.6. Data analysis and statistical interpretation

The Thermo MS/MS raw files for the analysis were converted to .mzXML[16] format by MSConverter, and then analyzed with COMET[17] against the Uniprot/Swissprot human database FASTA file with 89,838 reviewed protein entries. Search parameters were set as follows: 10 ppm of tolerance of precursor ion masses, 0.02 Da fragment ion mass, and a maximum of two missed cleavages with trypsin enzyme. The modifications made to the peptide sequence were as follows: static carbamidomethylation of cysteine (+57.012 Da), dynamic modifications of methionine oxidation (+15.995 Da), carbamylation of the protein at the N-terminus (+43.006 Da), and acetylation of protein at the N-terminus (+42.011 Da). Glycation of lysine (+162.053 Da) for analysis of glycated proteins was added as a dynamic modification. The search results in pepXML[18] format can be imported into the Trans-Proteomic Pipeline (TPP)[19] and processed using PeptideProphet.[20] A cut-off probability score of 0.99 was used for TPP in this work. It revealed a ≤ 0.01 peptide false discovery rate (FDR) based on a PeptideProphet probability cut-off score of 0.99. Modified peptides were identified with high and medium confidence considering charge-state-specific Xcorr scores (≥ 2 for z = 1.5, ≥ 1.9 for z = 3, ≥ 2.8 for z = 4, and ≥ 3.8 for z = 5)[21].

3. Results and Discussion

In this study, glycated peptides of human serum (HS) were analyzed using *in vitro* glycated HS (*Fig.* 1). We compared three methods to confirm that PRM can be compatible to enrichment; (1) DDA without enrichment, (2) DDA with BA enrichment and (3) PRM analysis. For PRM analysis, data from the DDA or spectral library are required. We constructed the inclusion list with the data which came by our laboratory's experimentation.[11]

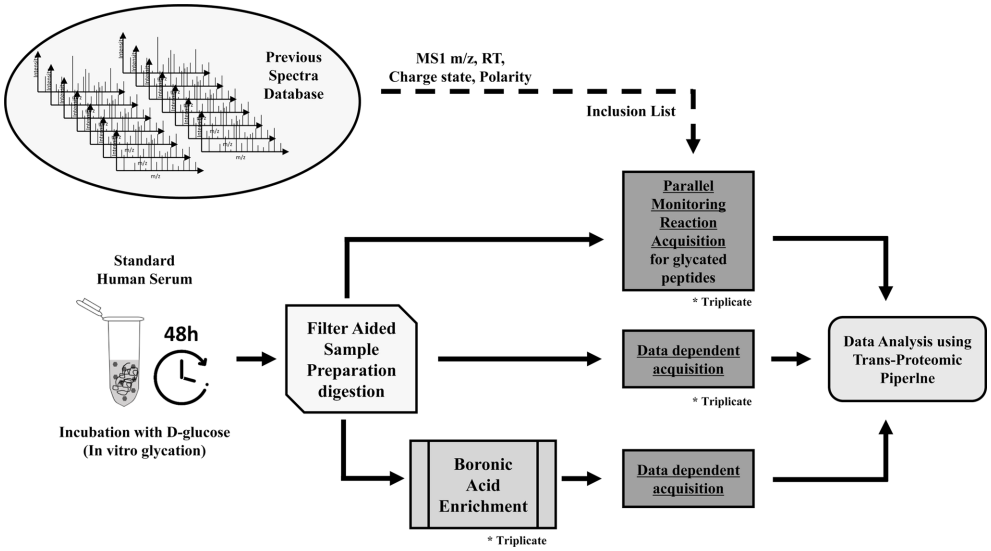

Fig. 1. Protocol comparing each method for identification of glycated peptides.

3.1. Comparison of glycated PSMs

The difference in results was initially confirmed at the PSM (peptide spectrum match) level. The number of glycated PSMs is the total number of identified peptide spectra matched for the glycated peptides. As shown in *Fig.* 2(a), the highest PSM number was acquired when BA enrichment was used. Compared to the other two methods, the reproducibility of BA enrichment was extremely deficient, the means and standard deviations of the triplicate glycated PSMs of each method in *Fig*. 2(b). Furthermore, regardless of the method, the PSMs of glycated albumin is more than half of total PSMs. *Fig.* 3 shows MS/MS spectra of the glycated peptide from LC-MS/MS with PRM mode. Glycated peptide (DGAGDVAFVK* HSTIFENLANK) was identified with Xcorr of 6.283, probability of 1, and a mass accuracy of -0.0249 ppm. The MS/MS fragmentation pattern of a glycated peptide revealed b, y diagnostic product ions. In the Sequest HT algorithm used for data analysis, the Xcorr is a search-dependent score that is critical to peptide identification.[22] As shown in *Fig.* 4, Xcorr represented the highest tendency in PRM.

3.2. Comparison of glycated peptides

We made the unique peptide data by removing the

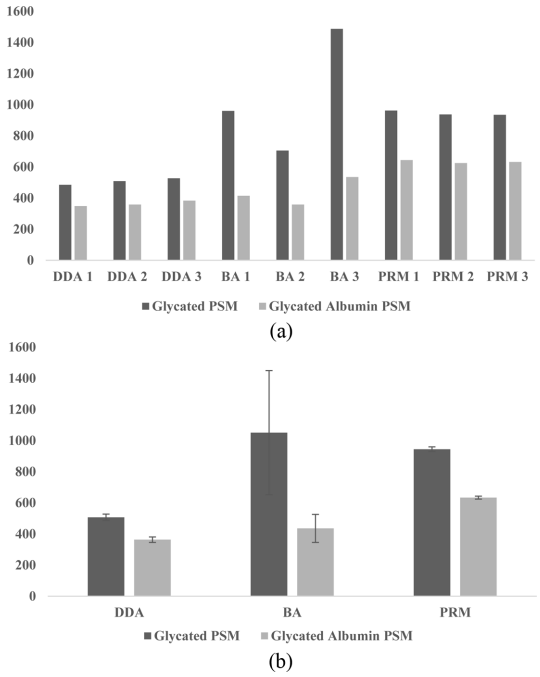

Fig. 2. The glycated PSM results from triplicate liquid chromatography-tandem mass spectrometry (LC-MS/MS) among data-dependent acquisition (DDA), parallel reaction monitoring (PRM), and boronic acid (BA) enrichment of glycated peptides from human serum. The number of identified (a) glycated PSMs and glycated albumin PSMs from each glycated human serum sample. Average number of identified, (b) glycated PSMs and glycated albumin PSMs of DDA, PRM and BA enrichment.

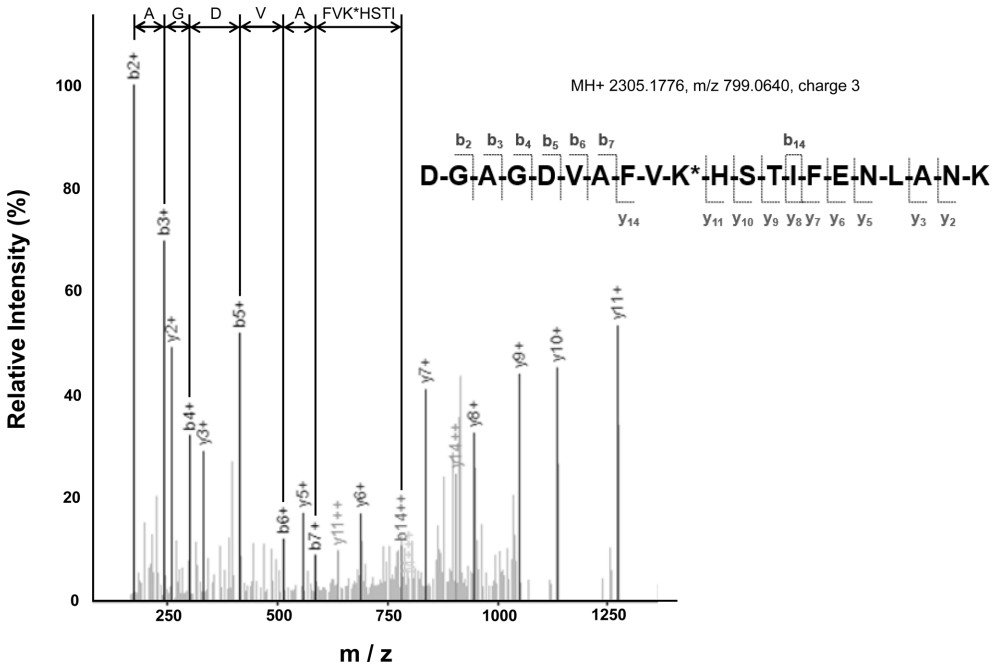

Fig. 3. MS/MS spectra of the triply charged ion (m/z = 799.0640 of glycated peptide DGAGDVAFVK* HSTIFENLANK, MH+ = 2305.1776 (* represents the glycation site).

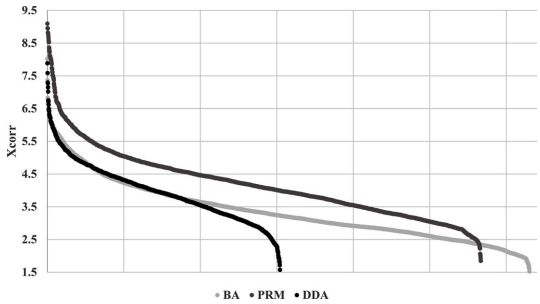

Fig. 4. Xcorr of the glycated PSMs from each method is shown. It was confirmed that Xcorr has the highest tendency in PRM.

overlap of each method. *Table* 1 shows the number of glycated peptides and glycated albumin peptides. Although the smallest PSMs were obtained by DDA, the number of glycated peptides came by PRM was the smallest. The cause for the smaller number of PSMs taken with two other methods compared to PRM is due to a parameter only applicable only to DDA. Xcalibur (Thermo Fisher Scientific), which is software to operate Q-Exactive, has an algorithm called "Dynamic Exclusion^{TM}". This makes a list of some ions that already triggered a DDA scan and prevented them to triggered successive MS2 scan for a certain time of users determined.[23,24] The function helps detect more relatively low-abundant peptides in a complex sample.[25] For this reason that PRM abundant peptides such as albumin were captured instead of relatively low-abundant peptides scheduled in the same window. So, we can propose it would not be appropriate to use PRM when analyzing with no-depletion high dynamic range. However, PRM could be powerful means when quantifying glycated albumin by spectral count as the results of PRM has the highest number of glycated albumins PSMs averages and the smallest standard deviation.

3.3. Venn diagram and String pathway of each method

The Venn diagram of the unique glycated peptides for the three methods is shown in *Fig.* 5(a). Most glycated peptides could be found in BA enrichment method. This suggests that the enrichment is more acceptable for *de-novo* glycated peptide and glycated site discovery.[26,27] Even so, as mentioned above, the enrichment method has tremendously insufficient

Table 1. The numbers of glycated peptides identified in human serum. Comparison of triplicate liquid chromatography-tandem mass spectrometry (LC-MS/MS) results among data-dependent acquisition (DDA), parallel reaction monitoring (PRM), and boronic acid (BA) enrichment of glycated peptides from human serum

	Number of glycated peptides	Avg. ± SD	Number of glycated albumin peptides	Avg. ± SD
DDA 1	127		64	
DDA 2	142	136 ± 8	66	66 ± 2
DDA 3	140		68	
BA 1	122		36	
BA 2	76	140 ± 74	25	36 ± 10
BA 3	221		46	
PRM 1	94		44	
PRM 2	93	95 ± 2	44	44 ± 0
PRM 3	98		45	

SD, standard deviation

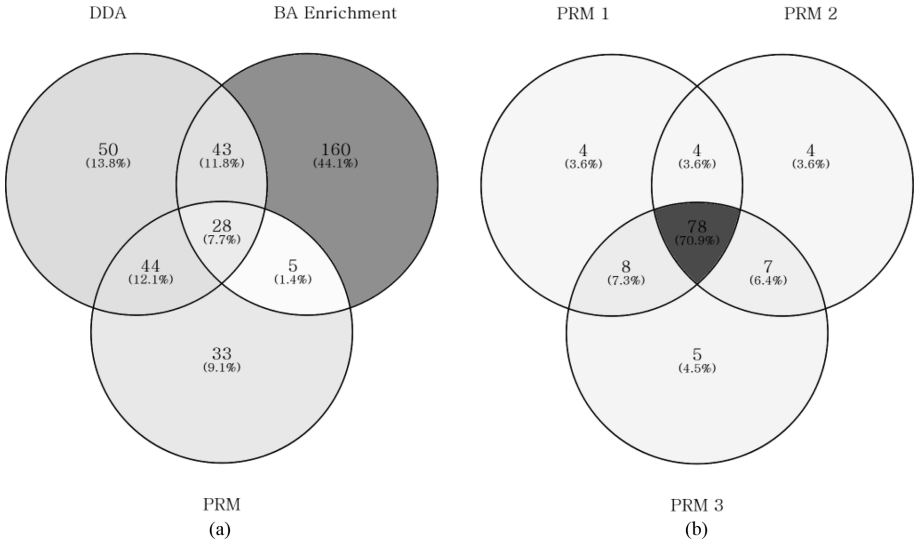

Fig. 5. Venn diagram of overlapping glycated peptides from (a) data-dependent acquisition (DDA), parallel reaction monitoring (PRM), and boronic acid (BA) enrichment and (b) three samples using PRM.

reproducibility and when considered together with the poor ionization efficiency of glycated peptide, we can comment it is improper for quantification.[28] *Fig.* 5(b) shows the Venn diagram of the glycated peptides analyzed by PRM. In PRM, the glycated peptides were repeatedly identified at a rate of 70.9 %, showing high reproducibility. Seventy-eight glycated peptides from triplicate PRM experiments are listed in *Table* 2. Most of these were related to human serum albumin (42 glycated peptides, 53.8 %). The string pathway results of the overlapping glycated proteins analyzed in the PRM are shown in *Fig.* 6. However, no significant

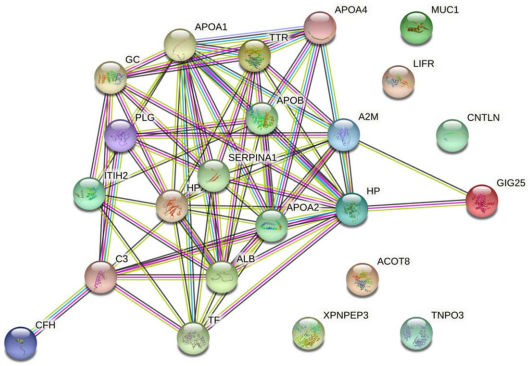

Fig. 6. The string pathway results of the overlapping glycated proteins analyzed in PRM.

Table 2. List of 78 glycated peptides repeated in the triplicate PRM experiment

Protein name	Glycated Peptide Sequence	m/z	Charge state	Total mass	Xcorr
Serum albumin	R.Y**K***AAFTEC**C**QAADK.A	885.6782	4	3538.693306	3.121
Serum albumin	K.TPVSDRVT**K***C**C**TESLVNR.R	571.7781	4	2283.083199	4.297
Serum albumin	K.QEPERNEC**FLQH**K***DDNPNLPR.L	700.3254	4	2797.272408	3.018
Serum albumin	R.NEC**FLQH**K***DDNPNLPR.L	540.501	4	2157.9748	4.131
Ig gamma-3 chain C region (Fragment)	R.VEL**K***TPLGDTTHTC**PR.C	497.5005	4	1985.972847	4.352
Apolipoprotein B-100	K.RPV**K***LLSGGNTLHLVSTTK.T	546.5653	4	2182.232125	5.507
Serotransferrin	K.GDVAFV**K***HQTVPQNTGGKNPDPWAK.N	714.1096	4	2852.409371	5.68
Serum albumin	Y.TYETTLE**K***C**C**AAADPHEC**YAK.V	670.7857	4	2679.113716	4.028
Serotransferrin	R.**K***C**STSSLLEAC**TFR.R	607.9501	3	1820.82839	3.814
Serum albumin	K.**K***VPQVSTPTLVEVSR.N	601.335	3	1800.983237	5.048
Serum albumin	K.TC**VADESAENC**D**K***SLHTLFGDK.L	887.0548	3	2658.142599	3.853
Serum albumin	K.LVTDLT**K***VHTEC**C**HGDLLEC**ADDR.A	755.5915	4	3018.336861	4.467
Serum albumin	R.ADLA**K***YIC**ENQDSISSK.L	701.9965	3	2102.967734	4.615
Haptoglobin	T.n****DIADDGC**P**K***PPEIAHGYVEHSVR.Y	692.5712	4	2766.277779	3.208
Alpha-2-macroglobulin	V.n****EEFVLP**K***FEVQVTVPK.L	524.2806	4	2093.082466	3.059
Serum albumin	K.LVTDLT**K***VHTEC**C**HGDLLEC**ADDRADLAK.Y	1173.213	3	3516.617209	2.924
Alpha-2-macroglobulin	R.**K***P**K***M***C**PQLQQYEMHGPEGLR.V	692.5738	4	2766.277779	5.463
Serotransferrin	K.DLLF**K***DSAHGFLK.V	551.6225	3	1651.845663	4.431
Ig mu chain C region (Fragment)	R.GQPLSPE**K***YVTSAPMPEPQAPGR.Y	867.0951	3	2598.263449	4.87
Alpha-1-antitrypsin	K.**K***LSSWVLLMK.Y	456.2599	3	1365.757834	3.849
Hemopexin	R.W**K***NFPSPVDAAFR.Q	566.2825	3	1695.825583	3.783
Serum albumin	K.AAFTEC**C**QAAD**K***AAC**LLPK.L	763.0183	3	2286.033102	4.007
Vitamin D-binding protein	R.**K***FPSGTFEQVSQLVK.E	619.6595	3	1855.956625	4.173
Serotransferrin (Fragment)	K.DGAGDVAFV**K***HSTIFENLANKADR.D	685.0911	4	2736.335396	6.854
Serum albumin	K.VHTEC**C**HGDLLEC**ADDRADLA**K***YIC**ENQD-SISSK.L	835.1648	5	4170.787592	6.128
Serum albumin	K.LVNEVTEFA**K***TC**VADESAENC**DK.S	931.081	3	2790.221152	4.637
Apolipoprotein A-I	R.V**K***DLATVYVDVLK.D	542.3063	3	1623.896994	4.274
Serum albumin	R.LVRPEVDVM***C**TAFHDNEETFLK**K***.Y	739.8571	4	2955.399361	5.272
Ig lambda-1 chain C regions (Fragment)	K.QSNN**K***YAASSYLSLTPEQWK.S	826.3992	3	2476.17568	5.056
Serum albumin	K.SLHTLFGD**K***LC**TVATLR.E	524.2779	4	2093.082466	4.617
Serum albumin	R.LVRPEVDVMC**TAFHDNEETFLK**K***.Y	735.8584	4	2939.404488	4.441
Serum albumin	R.LVRPEVDVMC**TAFHDNEETFL**K***K.Y	735.8584	4	2939.404488	4.756
Serum albumin	R.LVRPEVDVMC**TAFHDNEETFL**K***K.Y	588.8882	5	2939.404536	3.959
Ig alpha-1 chain C region	K.KGDTFSC**MVGHEALPLAFTQ**K***TIDR.L	746.8678	4	2983.442086	4.908
Serotransferrin (Fragment)	K.DGAGDVAFV**K***HSTIFENLANK.A	799.064	3	2394.170248	6.018
Ig gamma-1 chain C region	V.n****EP**K***SC**D**K***THTC**PPC**PAPELLGGPSV-FLFPPKPK.D	811.9946	5	4054.936762	4.765
Ig alpha-1 chain C	K.**K***GDTFSC**MVGHEALPLAFTQK.T	833.7347	3	2498.182211	4.463
Serum albumin	K.TC**VADESAENC**D**K***SLHTLFGDKLC**TVATLR.E	894.1681	4	3572.643257	5.062
Serum albumin	K.TC**VADESAENC**D**K***SLHTLFGDKLC**TVATLR.E	715.5359	5	3572.643061	3.868
Hemopexin	K.RLE**K***EVGTPHGIILDSVDAAFIC**PGSSR.L	797.4079	4	3185.602486	3.796
Hemopexin	K.RLE**K***EVGTPHGIILDSVDAAFIC**PGSSR.L	1062.8748	3	3185.602438	2.643
Serum albumin	R.RHPYFYAPELLFFA**K***R.Y	739.7212	3	2216.141745	5.693
Serum albumin	R.RHPYFYAPELLFFA**K***R.Y	555.0427	4	2216.141793	4.958
Serum albumin	K.n****VHTEC**C**HGDLLEC**ADDRADLA**K***YIC**ENQDSISSK.L	843.7659	5	4213.793269	4.753

Table 2. Continued

Protein name	Glycated Peptide Sequence	m/z	Charge state	Total mass	Xcorr
Complement C3	R.HQQTVTIPP**K***SSLSVPYVIVPLK.T	674.1336	4	2692.505318	4.495
Serotransferrin (Fragment)	K.LC**MGSGLNLC**EPNN**K***EGYYGYTGAFR.C	1045.1282	3	3132.362692	3.283
Ig gamma-1 chain C region	K.SC**D**K***THTC**PPC**PAPELLGGPSVFLFPPKPK.D	1166.2365	3	3495.687521	3.274
Ig gamma-1 chain C region	K.SC**D**K***THTC**PPC**PAPELLGGPSVFLFPPKPK.D	874.9292	4	3495.687691	4.547
Ig gamma-1 chain C region	K.SC**D**K***THTC**PPC**PAPELLGGPSVFLFPPKPK.D	700.1448	5	3495.687495	6.118
Serum albumin	K.n****TC**VADESAENC**D**K***SLHTLFGDKLC**TVATLR.E	904.9195	4	3615.648873	3.378
Ig alpha-1 chain C region	R.DLC**GC**YSVSSVLPGC**AEPWNHG**K***TFTC**TAAYPESK.T	1028.7009	4	4110.774605	3.58
Alpha-1-antitrypsin	K.LQHLENELTHDIIT**K***FLENEDRR.S	605.9103	5	3024.515009	4.392
Serum albumin	K.LVNEVTEFA**K***TC**VADESAENC**D**K***SLHTLFGDK.L	988.7054	4	3950.792427	3.572
Ig lambda-2 chain C regions (Fragment)	A.GVETTTPS**K***QSNNK*YAASSYLSLTPEQWK.S	885.6782	4	3538.693306	3.121
Serum albumin	R.n****RHPYFYAPELLFFA**K***R.Y	565.7942	4	2259.147652	4.987
Serum albumin	R.HPYFYAPELLFFA**K***R.Y	687.6875	3	2060.040671	3.972
Serum albumin	K.VFDEF**K***PLVEEPQNLIK.Q	736.3876	3	2206.14089	3.594
ITIH4 protein	R.EALI**K***ILDDLSPR.D	548.9733	3	1643.898154	3.825
Hemopexin	K.GYP**K***LLQDEFPGIPSPLDAAVEC**HR.G	743.6181	4	2970.443306	6.362
Apolipoprotein D (Fragment)	R.NPNLPPETVDSL**K***NILTSNNIDVK.K	933.1537	3	2796.43923	3.929
Serum albumin	R.RPC**FSALEVDETYVP**K***EFNAETFTFHADIC**TLSEK.E	863.6037	5	4312.982111	7.928
Serum albumin	K.PLLE**K***SHC**IAEVENDEMPADLPSLAADFVESK.D	929.9444	4	3715.748482	4.776
Serum albumin	K.QNC**ELFEQLGEY**K***FQNALLVR.Y	921.1216	3	2760.342917	3.85
Serum albumin	K.EQL**K***AVMDDFAAFVEK.C	668.3274	3	2001.960348	3.215
Serum albumin	R.LVRPEVDVMC**TAFHDNEETFL**K*****K***YLYEIAR.R	802.9939	5	4009.9331	4.556
Serum albumin	K.RM***PC**AEDYLSVVLNQLC**VLHE**K***TPVSDR.V	877.6784	4	3506.684517	4.765
Serum albumin	K.RMPC**AEDYLSVVLNQLC**VLHE**K***TPVSDR.V	873.6796	4	3490.6894	4.705
Serum albumin	K.RMPC**AEDYLSVVLNQLC**VLHE**K***TPVSDR.V	699.1452	5	3490.689631	4.986
Alpha-2-macroglobulin	R.LLIYAVLPTGDVIGDSA**K***YDVENC**LANK.V	1071.8794	3	3212.616354	3.556
Ig gamma-1 chain C region	K.NQVSLTC**LV**K***GFYPSDIAVEWESNGQPENNYK.T	1283.6035	3	3847.788717	3.641
Apolipoprotein A-IV	K.SELTQQLNALFQD**K***LGEVNTYAGDLQK.K	1062.5331	3	3184.577414	5.88
Serum albumin	K.NYAEA**K***DVFLGMFLYEYAR.R	821.391	3	2461.151144	3.98
Apolipoprotein A-I	K.DLATVYVDVL**K***DSGRDYVSQFEGSALGK.Q	799.3989	4	3193.566597	5.246
Serum albumin	R.MPC**AEDYLSVVLNQLC**VLHE**K***TPVSDR.V	834.6544	4	3334.58857	4.102
Beta-2-glycoprotein 1 (Fragment)	C.TEEG**K***WSPELPVC**APIIC**PPPSIPTFATLR.V	882.1966	4	3524.757271	5.115
Serum albumin	K.VFDEF**K***PLVEEPQNLI**K***QNC**ELFEQLGEYK.F	1002.7394	4	4006.928414	4.622
Ig lambda-2 chain C regions (Fragment)	K.ATLVC**LISDFYPGAVTVAW**K***ADSSPVK.A	765.1427	4	3056.541695	5.055
Serum albumin	E.**K***PLLE**K***SHC**IAEVENDEMPADLPSLAADFVESK.D	1002.4813	4	4006.928414	3.546
Ig kappa chain C region (Fragment)	X.TVAAPSVFIFPPSDEQL**K***SGTASVVC**LLNNFYPR.E	972.4947	4	3885.949654	4.219
Serum albumin	K.DLGEENF**K***ALVLIAFAQYLQQC**PFEDHVK.L	896.9459	4	3583.754586	4.819
Serotransferrin	G.**K***IEC**VSAETTEDC**IA**K***IMNGEADAMSLDGG-FVYIAGK.C	1080.2466	4	4316.957222	3.488
Apolipoprotein A-I	R.QGLLPVLESF**K***VSFLSALEEYTK.K	920.8246	3	2759.451925	3.753
Apolipoprotein A-II	K.**K***AGTELVNFLSYFVELGTQPATQ.-	892.4532	3	2674.337729	6.259

pathway networks were found among them.

4. Conclusions

We analyzed the glycated peptides in human serum through DDA, PRM, and BA enrichment and compared the results. BA enrichment method has the advantage that more diverse peptides can be identified than the other two methods. However, it takes more time and needs a sufficient sample amount for sample preparation. Due to inadequate reproducibility, consideration is required before choosing for quantification. The DDA method was able to obtain results to be ratified with two other methods. It might be for the reason of increased selectivity with the device development. The exclusion list containing mass information is automatically composed. Relatively low-abundant peptides spectra could be acquired since the instrument does not detect peptides in the list for a certain period. The DDA method had a weakness in the data process because of the immense results of data files. The "Dynamic Exclusion™" is not applicable when using PRM. There is a flaw in that more abundant peptides are detected at the overlapped retention time window. It leads to low-abundance peptide spectra cannot be acquired. Nonetheless, PRM can be a powerful substitute on account for high reproducibility and more data points for quantification. When constructing an inclusion list, better results will be obtained by adjusting the number of peptides included in the same retention time window. If the object is to identify or quantify certain peptides in a vastly complex sample like the human serum, additional sample preparation, such as fractionation, might be helpful. We created an inclusion using only the existing experimental result data.[11] Based on the possibility we had confirmed that it would be possible to analyze the glycated peptides without enrichment by adding theoretical spectral data.

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