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Glycomics based biomarkers of the rate of aging : development and applications of high-throughput N-glycan analysis

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Chapter 2.

A HILIC-based high-throughput sample preparation method for N-glycan analysis from total human plasma glycoproteins

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Abstract

Many diseases are associated with changes in the glycosylation of plasma proteins. To search for glycan biomarkers, large sample sets have to be investigated for which high-throughput sample preparation and analysis methods are required. We here describe a 96 well plate-based high-throughput procedure for the rapid preparation of 2-aminobenzoic acid (2-AA)-labeled N-glycans from 10 μ l of human plasma. During this procedure, N-glycans are released from glycoproteins and subsequently labeled with 2-AA without prior purification. A HILIC-based solid phase extraction (SPE) method is then applied to isolate the 2-AA-labeled N-glycans, which can be analyzed by MALDI-TOF-MS, HPLC with fluorescence detection (FL), and CE-MS. The relative standard deviation (RSD) for the intra-batch repeatability and the inter-batch repeatability of the sample preparation method remained below 7% and below 9%, respectively, for all peaks observed by HPLC. Similar results were obtained with MALDI-TOF-MS, where 47 N-glycans could be measured consistently. The 2-AA-labeled N-glycans were additionally analyzed by a CE-ESI-Q-TOF-MS method, which featured high resolution and mass accuracy, allowing the unambiguous determination of the N-glycan compositions. Up to 4 times 96 human plasma samples can be handled in parallel, which, together with the versatility of the 2-AA label, makes this procedure very attractive for glycomics analysis of larger sample cohorts.

Introduction

In recent years, the differential profiling of protein glycosylation characteristics has yielded biomarkers for a number of diseases [9;26-28;30;32;46;166]. This is in line with the role of protein glycosylation in cell-cell and cell-matrix interactions, molecular trafficking, receptor activation and other biological and immunological events [6].

Glycosylation profiling is generally achieved by glycan release, purification, and analysis using mass spectrometry [26;30;46;166], capillary gel electrophoresis with fluorescence detection [28], or high performance liquid chromatography (HPLC) with fluorescence detection (FL) [27;32]. So far, most of the analyses of patient materials have been performed on relatively small numbers of samples. Obviously, for large population studies and thorough testing of glycan biomarker candidates there is a need for high-throughput methods for glycosylation profiling.

To this end, a glycomics platform for the analysis of permethylated oligosaccharide alditols has been introduced by Costello et al. [83], based on graphitized carbon HPLC-electrospray ionization-mass spectrometry (ESI-MS). Additionally, a high-throughput sample preparation method for permethylated glycans from human serum was recently described by Kang et al. [167], while Morelle et al. [75] published on the sample preparation of native N-glycans with non-porous graphitized carbon, followed by permethylation for mass spectrometric analysis. Permethylated glycans give, however, limited resolution in chromatography, and therefore, analytical strategies are restricted. Moreover, for the analysis of native glycans, a sample preparation procedure which makes use of glycoblotting was introduced by Miura et al. [166].

Alternatively, native N-glycans may be analyzed after sample preparation by hydrophilic interaction chromatography (HILIC) SPE. The polysaccharide-type stationary phases cellulose and Sepharose have been used by Wada and coworkers for batch-mode HILIC sample work up of N-glycans and N-glycopeptides for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [53;168]. Recently a microscale HILIC SPE plate was introduced by Yu et al. for the

purification of underivatized glycans for MALDI-MS analysis [95] Royle et al. [80] published on a high-throughput sample preparation method using 2-aminobenzamide (2-AB) labeling and several purification strategies, among which a HILIC SPE method using the above-mentioned plate. Samples are analyzed with HPLC followed by data evaluation using a dedicated database [80]. Furthermore, a HILIC-based N-glycan purification method for 2-aminobenzoic acid 2-AA-labeled N-glycans was reported by Anumula et al. [94]. The same group recently introduced an automated method for N-glycan purification using HILIC [118].

Although useful in certain settings, these methods have several drawbacks: the use of a PhyNexus MEA purification system prohibits the general applicability of the approach published by Anumula et al. [118], as not every laboratory owns this purification system. The protocol introduced by Royle et al. [80] is still labor-intensive and time-consuming. Native, neutral N-glycans can be purified by HILIC SPE [95], yet the analytical possibilities are restricted compared to the analysis of N-glycans labeled with a fluorescent tag. Among the labels used for reductive amination of oligosaccharides, the 2-aminobenzoic acid (2-AA) tag is particularly versatile, as it allows fluorescence detection in HPLC and CE as well as mass spectrometric detection with both MALDI and ESI in positive and negative ionization mode [59;94;96;118]. Importantly, 2-AA labeling of glycans can be performed under aqueous conditions, which allows labeling after glycan release without prior sample work-up [94;169]. It is proposed that the ortho-carboxylic acid functionality of 2-AA participates in stabilizing the Schiff base [90], allowing for labeling of N-glycans under aqueous conditions, whilst other reductive amination protocols require N-glycan work-up after the enzymatic release step and prior to labeling [94;169].

To allow rapid and high throughput sample preparation for glycan profiling of large scale population studies, we here describe a fully automatable, 96-well plate procedure for the sample preparation of 2-AA-labeled N-glycans from human plasma. The protocol is characterized by its simplicity, as only a bench-top vacuum manifold is required. After denaturing of proteins, the N-glycans are released enzymatically and

subsequently labeled without prior purification. Labeled N-glycans are then purified using a HILIC-based SPE method with microcrystalline cellulose as the stationary phase. 2-A-labeled N-glycans are eluted with water prior to analysis by HILIC HPLC-FL, MALDI-TOF-MS or capillary electrophoresis (CE)-ESI-MS.

Materials and Methods

Chemicals

Dimethylsulphoxide (DMSO), ammonium hydroxide, formic acid, Nonidet P-40 (NP-40), 2-aminobenzoic acid (2-AA) and sodium cyanoborohydride were obtained from Sigma (Zwijndrecht, The Netherlands), sodium dodecyl sulphate (SDS) was bought from United States Biochemicals (Cleveland, OH), while PNGase F was obtained from Roche Diagnostics (Mannheim, Germany). Microcrystalline cellulose and glacial acetic acid were purchased from Merck (Darmstadt, Germany). 0.45 µm GHP filter plates were obtained from Pall Corporation (Ann Arbor, MI) and 96-well V-bottom deep well plates from Westburg (Leusden, The Netherlands). Acetonitrile (ACN) was purchased from Biosolve (Valkenswaard, The Netherlands). 2,5-Dihydroxybenzoic acid (DHB) was purchased from Bruker Daltonics (Bremen, Germany).

N-glycan release

(Glyco-)proteins from citrate plasma were denatured using the detergent SDS, and subsequently N-glycans were released enzymatically similar to the procedure described by Morelle et al. [75]. Briefly, 10 µl of citrate plasma was applied per well of a 96-well V-bottom deep well plate. Proteins were denatured after addition of 20 µl 2% SDS by incubation at 60°C for 10 min. Subsequently, 10 µl 4% NP-40 and 0.5 mU of PNGase F in 10 µl 5x PBS was added to the samples. The samples were incubated over night at 37°C for N-glycan release.

2-AA Labeling

The released N-glycans were subsequently labeled with 2-aminobenzoic acid (2-AA) [87]. Labeling mixture was freshly prepared by subsequently dissolving 2-AA and sodium cyanoborohydride in a DMSO: glacial acetic acid mixture (10:3 (v/v))

to a final concentration of 48 mg/ml and 63 mg/ml, respectively. Fifty μ l of labeling mixture was added to each N-glycan release sample in the 96-well plate. Mixing was achieved by shaking for 5 min, followed by 2 h incubation at 65°C. The reaction mixture was allowed to cool down to room temperature. Samples (in a volume of 100 μ l) were brought to 75% ACN by adding 300 μ l ACN.

HILIC SPE

200 μ l of a 200 mg/ml microcrystalline cellulose suspension in water was applied to each well of a 0.45 μ m GHP filter plate. Solvent was removed by application of vacuum using a vacuum manifold (Millipore, Billerica, MA). All wells were pre-washed using 2 x 200 μ l water, followed by equilibration using 3 x 200 μ l ACN:water (80:20 v/v). The samples were loaded to the wells and the wells were subsequently washed using 4 x 150 μ l ACN:water (80:20 v/v). The labeled N-glycans were eluted using 400 μ l water and the eluate was collected in a 96-well V-bottom deep well plate. In all cases maximal 170 mbar vacuum was used to remove solvent from the wells. The pH of the eluate was approximately 6.

HILIC HPLC

Purified 2-AA-labeled N-glycans were separated using normal phase-high performance liquid chromatography (HILIC HPLC) with trapping columns in dual mode. The Ultimate LC system (Dionex, Sunnyvale, CA) consisted of a Famos autosampler, a Switchos module with a loading pump, which was connected to an Ultimate module with two pumps. Using a nanovalve, the system was connected to the detector. The system was controlled by Chromeleon software and equipped with two 2.0 mm x 10 mm TSK gel-Amide 80 trapping columns (Tosoh Biosciences, Stuttgart, Germany) and two 2.0 mm x 250 mm TSK gel-Amide 80 analytical columns (Tosoh Biosciences). A fluorescence detector (FP-2020 plus; Jasco, Easton, MD) was used at excitation wavelength 360 nm and emission wavelength 420 nm.

50 μ l of aqueous eluate from the HILIC SPE were mixed with 150 μ l ACN in a deep-well 96 well plate. The plate was sealed with a silicon cover (Labservices, Breda,

The Netherlands), and placed in the autosampler. A 20 μ l aliquot was injected using a full loop injection procedure. The 2-AA-labeled N-glycans were trapped on the trapping column and washed using ACN:50 mM ammonium formate (pH 4.4; 80:20, by volume) for 3 min. Subsequently the analytical column was switched in line and 2-AA-labeled N-glycans were separated using a linear gradient of ACN (solvent A) and 50 mM ammonium formate (pH 4.4; solvent B). Gradient conditions: 30% to 50% solvent A over 77 min followed by a step to 100% solvent A over 3 min. After 5 min at 100% solvent A, the gradient went back to 30% solvent A over 3 min (hold 15 min). The flow rate was 150 μ l/min. Using this gradient system the total analysis time per column was 106 min.

HILIC HPLC data processing

Data pre-processing was performed using Matlab (version 2007a) software (The Mathworks, Inc., Natick, MA). Files were exported from Chromeleon wp 6.50 as ASCII files and were subsequently loaded into Matlab. The data were normalized, using total intensity, and pre-aligned on the peak of highest intensity. The data were subsequently cropped to the range of 30 to 80 min to reduce the data volume. Correlation optimized warping (COW) was performed according to the method described by Skov et al. [170], which included reference sample generation, segment length and slack size optimization and subsequent alignment.

MALDI-TOF-MS

All experiments were carried out on an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics), equipped with a Smartbeam laser. Two μ l of sample obtained after HILIC SPE was purified by C18 ZipTip (Millipore). ZipTips were pre-washed using 3 x 10 μ l ACN:0.1% TFA (50:50 (v/v)) and subsequently equilibrated using 3 x 10 μ l 0.1% TFA. Samples were applied in 8 μ l 0.1% TFA and the ZipTips were subsequently washed with 3 x 10 μ l 0.1% TFA. 2-AA-labeled N-glycans were eluted using 2 μ l ACN:0.1% TFA (50:50 (v/v)) and immediately spotted on a ground steel target plate and air-dried. 1 μ l of 2,5-dihydroxybenzoic acid (DHB) matrix (20 mg/ml in ACN:water (50:50 v/v)) was then applied to the spots and left to dry. The

Ultraflex was operated in linear negative ionization mode, and calibration was performed on a peptide calibration standard (Bruker Daltonics). Mass spectra from 2000 laser shots were accumulated per spot/sample, originating from eight different positions.

MALDI-TOF-MS data processing

The data was baseline subtracted and the intensities of an identical, defined set of 47 N-glycan peaks were determined for each spectrum by batch processing using FlexAnalysis 3.0 software (Bruker Daltonics). Intensities were subsequently normalized to total intensity of the selected 47 peaks, and total peak intensity percentages were calculated for each peak.

CE-ESI-Q-TOF

The instrumental setup for the analysis of AA-labeled N-glycans with CE-MS consisted of a PrinCE Crystal 310 CE System from Prince Technologies (Emmen, The Netherlands). Fused silica capillaries (Polymicro Technologies, Phoenix, AZ) with an inner diameter of 50 μm and a length of 65 cm were used. New capillaries were preconditioned with NaOH (0.1 mol/L) for 30 minutes and water for 10 minutes (2 bar). A 2 min rinsing step with background electrolyte (BGE) was used for cleaning the capillary between runs.

Samples were dried and reconstituted in 50 μL water. Sample injection was done hydrodynamically at a pressure of 100 mbar for 6 s. The separation electrolyte consisted of an ammonium acetate buffer (10 mmol/L acetic acid and 50 mmol/L NH_3) with a methanol concentration of either 0% or 50% (v/v). Separation was accomplished at 30 kV (+30 kV at the inlet, sprayer on ground) resulting in a current of about 10 μA . The CE was coupled to a quadrupole-time-of-flight mass spectrometer (MicrOTOFQ, Bruker Daltonics) via a coaxial sheath liquid interface (Agilent Technologies, Waldbronn, Germany). The sheath liquid, which consisted of isopropanol:water (1:1 (v/v)) was delivered at a flow rate of 4 $\mu\text{L}/\text{min}$. Ionization can be performed in both positive and negative mode, however, negative mode was chosen in order to achieve higher

selectivity (lower background). An ESI potential of 4500 V was set to the inlet of the MS (sprayer on ground) and the electrospray was assisted by adjusting the nebulizer pressure to 0.2 bar. The dry gas was heated to 170°C, delivered at a flow rate of 4 L/min.

Detection was accomplished between m/z 300 and 3000. Mass transfer was optimized using a 1:100 dilution of “tune mix” from Agilent Technologies. The same solution was also used for calibration (once a day). At the beginning of data processing every analysis was internally calibrated on the basis of five high abundant 2-AA-labeled N-glycan signals according to Table 2 1.

Results

To allow a glycome screening of larger sample populations, a rapid and high-throughput sample preparation method in 96-well plate format was developed for the analysis of N-glycans derived from human plasma. The protocol involves N-glycan release by PNGase F treatment, labeling with 2-aminobenzoic acid (2-AA), and HILIC SPE (Figure 2 1). The 2-AA label was chosen because of its versatility: it allows efficient mass spectrometric analysis of N-glycans with MALDI and ESI in both negative and positive ionization mode. Moreover, this label can be used in chromatography by HILIC, reverse phase, and carbon HPLC, as well as for separating N-glycans by capillary electrophoresis with MS detection.

N-glycan release and labeling

Plasma (glyco-)proteins were denatured, followed by N-glycan release with PNGase F. Glycans were labeled with 2-aminobenzoic acid and purified by solid-phase extraction. Samples were then analyzed by HILIC HPLC FL, and the glycan yield was determined based on the area under the peaks.

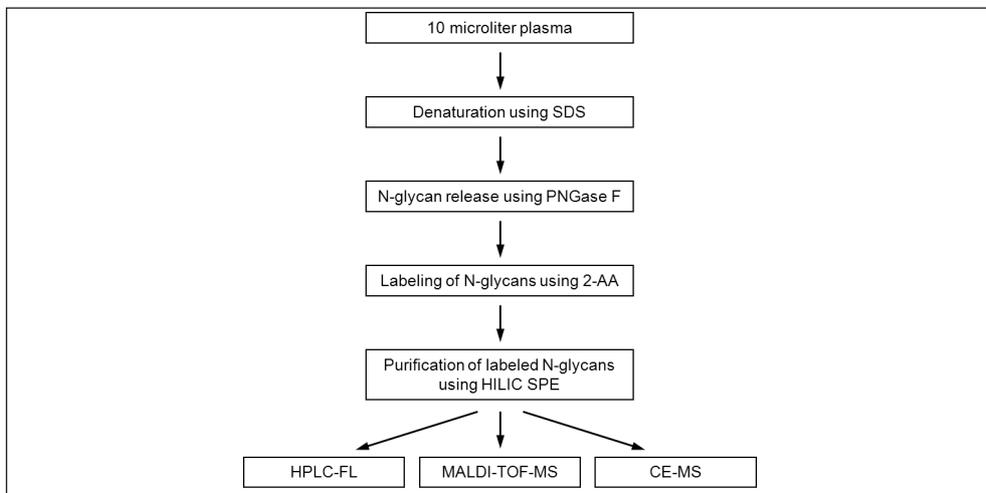


Figure 2 1. Strategy for sample preparation and analysis

Beginning from a default method that uses SDS for heat denaturation followed by addition of NP-40 and N-glycan release by PNGase F, several experiments were performed for method optimization: first, the influence of the denaturation temperature was tested by incubating 10 μ l of plasma with 20 μ l 2% SDS at room temperature, 60°C, and 95°C for 10 min. There was no significant difference between incubation at 95°C and 60°C. The yield at RT, however, was 26 % lower than the yield at 95°C. We chose to apply an incubation temperature of 60°C in the following experiments, as it was compatible with our liquid handling robot. Second, samples were denatured by incubation for 10 min and 30 min at 60°C, in the presence of SDS. As both incubation times resulted in nearly identical yields, an incubation time of 10 min was chosen for further experiments. Third, the influence of NP-40 was assessed by performing N-glycan release (1) after addition of NP-40 (10 μ l of 4% solution in water) as well as (2) without addition of NP-40. The yield without addition of NP-40 was found to be 21 % lower than with addition of NP-40. Fourth, the SDS/NP-40 heat denaturing step was substituted by addition of acetonitrile (20 μ l of 45%) and urea (denaturation by addition of 20 μ l 10 M urea; addition of PBS to achieve final urea concentrations

between 2 and 6.5 M urea, followed by PNGase F treatment), similar to Maley et al. [171]. N-glycan release in the presence of urea resulted in yields which were only 34% (for 2 M urea) and 6% (for 6.5 M urea) of the yields obtained with the SDS/NP-40 procedure. Addition of acetonitrile resulted in precipitates and consequently a very low N-glycan yield. Based on these experiments, the following method was chosen for N-glycan release from 10 μ l of human plasma: the plasma (10 μ l) (glyco) proteins were denatured by addition of 20 μ l 2% SDS followed by 10 min incubation at 60 μ C. Samples were allowed to cool down to room temperature followed by the addition of 10 μ l 4% NP-40, and 0.5 mU PNGase F in 10 μ l of 5x PBS. The N-glycans were cleaved from their carrier proteins during overnight incubation at 37 $^{\circ}$ C.

Released N-glycans were labeled by reductive amination with 2-AA. For this purpose, labeling reagent and sodium cyanoborohydride (reducing agent) in DMSO/acetic acid were directly added to the N-glycan release samples which still contained detergent, buffer and PNGase F as well as all plasma constituents including proteins and lipids. This kind of direct labeling reactions under aqueous conditions have previously been described [94;118;169] and could not be established for other labels such as 2-aminobenzamide (2-AB), which does require prior sample clean-up for efficient labeling [94;169]. The method was optimized with regard to the amount of labeling mixture added. Addition of 50 to 80 μ l of labeling solution resulted in maximal yield, while lower yields of labeled glycans were obtained after addition of lower volumes of labeling solution (data not shown). In the analyses by MALDI-TOF-MS (both prior to and after ZipTip purification) and CE-ESI-Q-TOF-MS, no unlabeled glycans could be observed, indicating complete labeling

HILIC SPE purification

In order to remove salts and detergents from the 2-AA-labeled N-glycan samples, an SPE clean-up procedure was developed. In a pilot experiment, two carbohydrate polymers (microcrystalline cellulose and Sepharose) [53;168] and the synthetic polymer Discovery DPA-6S [94;118;169] were tested for HILIC SPE sample clean-up by packing the stationary phases into 96-well filter plates. These methods

were compared to graphitized carbon [127]. While the obtained 2-AA-labeled N-glycan samples of both carbon and HILIC SPE were of comparable quality as judged by MALDI-TOF-MS and by HILIC HPLC with fluorescence detection, the HILIC SPE methods had several advantages: elution of the 2-AA-labeled N-glycans was achieved with 400 μ l water, and no degradation of the N-glycan in the eluate was observed after several months of storage at -20°C . The graphitized carbon method, in contrast, required elution with 25% acetonitrile containing 0.1% TFA, which made it necessary to remove the solvents and TFA by vacuum centrifugation in order to prevent (partial) loss of sialic acid residues, which occurs during glycan storage in acidic solutions. Moreover, the microcrystalline cellulose is very cheap and easy to handle, which - together with the simplicity of the whole work-up procedure - makes this method attractive.

N-glycan number ^a	N-glycan composition ^b	Calculated m/z ([M-H] ⁻)	Calculated m/z ([M-2H] ²⁻)	Calculated m/z ([M-3H] ³⁻)	Registered m/z ^c	Mass accuracy (ppm)
1	H ₅ N ₂	1354.4789	676.7358	450.8215	1354.4626 ([M-H] ⁻)	-12.05
2	H ₃ N ₃ F ₁	1379.5106	689.2516	459.1653	689.2440 ([M-2H] ²⁻)	-11.09
3	H ₃ N ₄	1436.5320	717.7624	478.1725	717.7585 ([M-2H] ²⁻)	-5.40
4	H ₆ N ₂	1516.5317	757.7622	504.8391	757.7577 ([M-2H] ²⁻)	-5.98
5	H ₃ N ₃ S ₁	1524.5481	761.7704	507.5112	761.7719 ([M-2H] ²⁻)	1.98
6	H ₃ N ₄ F ₁	1582.5899	790.7913 ^d	526.8585	790.7882 ([M-2H] ²⁻)	-3.95
7	H ₄ N ₄	1598.5848	798.7888	532.1901	798.7855 ([M-2H] ²⁻)	-4.11
8	H ₃ N ₅	1639.6114	819.3021	545.8656	819.3022 ([M-2H] ²⁻)	0.17
9	H ₇ N ₂	1678.5846	838.7886	558.8567	838.7867 ([M-2H] ²⁻)	-2.32
10	H ₄ N ₃ S ₁	1686.6009	842.7968	561.5288	842.7934 ([M-2H] ²⁻)	-4.04
11	H ₆ N ₃	1719.6111	859.3019	572.5322	859.3003 ([M-2H] ²⁻)	-1.89
12	H ₄ N ₄ F ₁	1744.6428	871.8177 ^d	580.8761	871.8192 ([M-2H] ²⁻)	1.68
13	H ₅ N ₄	1760.6377	879.8152	586.2077	879.8197 ([M-2H] ²⁻)	5.10
14	H ₃ N ₅ F ₁	1785.6693	892.3310	594.5516	892.3309 ([M-2H] ²⁻)	-0.13
15	H ₄ N ₅	1801.6642	900.3285	599.8832	900.3311 ([M-2H] ²⁻)	2.93
16	H ₄ N ₃ F ₁ S ₁	1832.6588	915.8258	610.2147	915.8256 ([M-2H] ²⁻)	-0.18
17	H ₈ N ₂	1840.6374	919.8151	612.8743	919.8179 ([M-2H] ²⁻)	3.09
18	H ₅ N ₃ S ₁	1848.6537	923.8232	615.5464	923.8222 ([M-2H] ²⁻)	-1.10

19	H ₄ N ₄ S ₁	1889.6803	944.3365	629.2219	944.3320 ([M-2H] ²⁻)	-4.78
20	H ₅ N ₄ F ₁	1906.6956	952.8442	634.8937	952.8477 ([M-2H] ²⁻)	3.71
21	H ₄ N ₅ F ₁	1947.7221	973.3574	648.5692	973.3593 ([M-2H] ²⁻)	1.94
22	H ₅ N ₅	1963.7170	981.3549	653.9008	981.3585 ([M-2H] ²⁻)	3.71
23	H ₅ N ₂	2002.6902	1000.8415	666.8919	1000.847 ([M-2H] ²⁻)	5.63
24	H ₆ N ₃ S ₁	2010.7065	1004.8496	669.5640	1004.851 ([M-2H] ²⁻)	1.38
25	H ₄ N ₄ F ₁ S ₁	2035.7382	1017.3655	677.9079	1017.365 ([M-2H] ²⁻)	-0.75
26	H ₅ N ₄ S ₁	2051.7331	1025.3629	683.2395	1025.365 ([M-2H] ²⁻)	2.23
27	H ₅ N ₅ F ₁	2109.7750	1054.3839	702.5868	1054.380 ([M-2H] ²⁻)	-3.76
28	H ₅ N ₄ F ₁ S ₁	2197.7910	1098.3919 ^d	731.9255	1098.394 ([M-2H] ²⁻)	1.58
29	H ₅ N ₅ S ₁	2254.8125	1126.9026	750.9326	1126.899 ([M-2H] ²⁻)	-3.47
30	H ₆ N ₅ F ₁	2271.8278	1135.4103	756.6044	756.6105 ([M-3H] ³⁻)	8.04
31	H ₅ N ₄ S ₂	2342.8285	1170.9106	780.2713	780.2705 ([M-3H] ³⁻)	-1.05
32	H ₅ N ₅ F ₁ S ₁	2400.8704	1199.9316	799.6186	799.6205 ([M-3H] ³⁻)	2.36
33	H ₆ N ₅ S ₁	2416.8653	1207.9290	804.9502	804.9554 ([M-3H] ³⁻)	6.40
34	H ₅ N ₄ F ₁ S ₂	2488.8864	1243.9396	828.9573 ^d	828.9606 ([M-3H] ³⁻)	4.00
35	H ₅ N ₅ S ₂	2545.9079	1272.4503	847.9644	847.9697 ([M-3H] ³⁻)	6.19
36	H ₆ N ₅ F ₁ S ₁	2562.9232	1280.9580	853.6362	853.6405 ([M-3H] ³⁻)	5.02
37	H ₅ N ₅ F ₁ S ₂	2691.9658	1345.4793	896.6504	896.6574 ([M-3H] ³⁻)	7.79
38	H ₆ N ₅ S ₂	2707.9607	1353.4767	901.9820	901.9820 ([M-3H] ³⁻)	-0.05
39	H ₆ N ₅ F ₁ S ₂	2854.0186	1426.5057	950.6680	950.6735 ([M-3H] ³⁻)	5.77
40	H ₆ N ₅ S ₃	2999.0561	1499.0244	999.0138 ^d	999.0192 ([M-3H] ³⁻)	5.36
41	H ₇ N ₆ S ₂	3073.0929	1536.0428	1023.6928	1023.705 ([M-3H] ³⁻)	11.64
42	H ₆ N ₅ F ₁ S ₃	3145.1140	1572.0534	1047.6998	1047.7050 ([M-3H] ³⁻)	4.95
43	H ₇ N ₆ F ₁ S ₂	3219.1508	1609.0718	1072.3787	1072.3868 ([M-3H] ³⁻)	7.51
44	H ₇ N ₆ S ₃	3364.1883	1681.5905	1120.7246	1120.7244 ([M-3H] ³⁻)	-0.16
45	H ₇ N ₆ F ₁ S ₃	3510.2462	1754.6195	1169.4105	1169.4035 ([M-3H] ³⁻)	-6.03
46	H ₇ N ₆ S ₄	3655.2837	1827.1382	1217.7564	1217.7567 ([M-3H] ³⁻)	0.26
47	H ₇ N ₆ F ₁ S ₄	3801.3417	1900.1672	1266.4424	1266.4347 ([M-3H] ³⁻)	-6.07

Table 2-1. Identification of 47 N-glycans from human plasma glycoproteins, using enzymatic release, 2-AA labeling, HILIC SPE, and detection with MALDI-TOF-MS and CE-ESI-Q-TOF-MS.

^a N-glycan numbers correspond to Figure 3.

^b N-glycan compositions are given in terms of hexose (H), N-acetylhexosamine (N), fucose (F) and sialic acid (S).

^c Masses were registered by CE-ESI-Q-TOF-MS.

^d Signals were used for internal calibration in CE-ESI-Q-TOF-MS.

HILIC HPLC FL

A well-established HILIC HPLC method with fluorescence detection (HILIC HPLC-FL) was chosen for optimization and evaluation of the sample preparation method [80;172]. An average HPLC-FL chromatogram is shown in Figure 2 5 in the supporting information. For the assignment of the N-glycans to the various peaks, literature data on human plasma N-glycans with retention times/elution orders [46;84;172-174] were used together with our own data which were obtained by the analysis of peak fractions by nano-reverse phase LC-MS/MS (data not shown). The repeatability of the HILIC HPLC-FL analysis method was determined by measuring one standard sample (2-AA-labeled N-glycans from plasma glycoproteins) 8 times. The data were processed as described in the experimental section, and relative peak heights were determined. The average relative standard deviation (RSD) of the normalized peak heights was 4% (data not shown). To determine the intra-batch repeatability of the sample preparation method, 10 batches of 4 samples were analyzed with HILIC HPLC-FL, where each batch was run on separate days. For each sample, the relative peak heights were determined and per batch an RSD was calculated. The intra-batch repeatability which was calculated as the average RSD of the 10 batches remained below 7% for all relative peak heights and is depicted by a green line in Figure 2 5. The inter-batch repeatability of the method was determined as the RSD of the relative peak heights of all 40 samples and remained below 9% for all peaks (Figure 2 5).

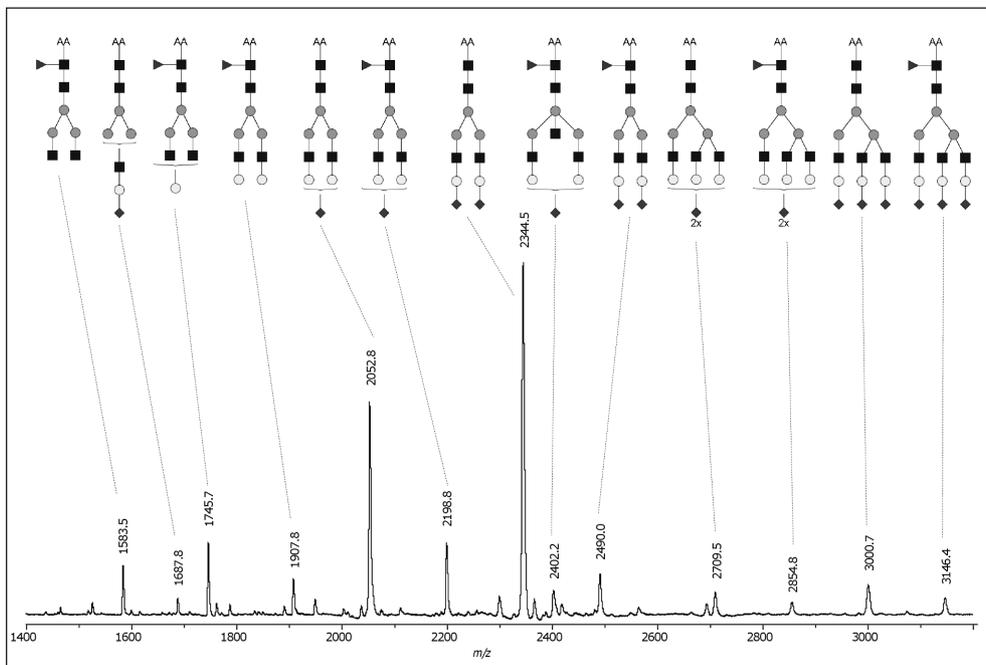


Figure 2-2. MALDI-TOF mass spectrum of 2-AA-labeled N-glycans. A MALDI-TOF mass spectrum of 2-AA-labeled N-glycans from human plasma after HILIC SPE and C18 ZipTip was acquired using DHB matrix in the linear negative ionization mode. Masses are rounded to 1 decimal place. Structures were assigned for the most abundant peaks. Compositions and structural schemes are given in terms of N-acetylglucosamine (square), mannose (dark circle), galactose (light circle), sialic acid (diamond) and fucose (triangle). All N-glycans were labeled with 2-aminobenzoic acid (2-AA).

MALDI-TOF-MS

As a second, complementary analysis method for the profiling of 2-AA-labeled N-glycans from human plasma, negative linear mode MALDI-TOF-MS with DHB matrix was chosen. All samples were desalted using C-18 ZipTips (Millipore) to remove residual sodium before analysis. A characteristic MALDI-TOF mass spectrum is shown in Figure 2 2. Assignments were made based on average masses, measured by MALDI-TOF-MS (data not shown) and accurate masses determined by analysis of a standard sample by CE-ESI-Q-TOF-MS (see below), in combination with literature data [46;84;172-174] on the occurrence of glycans on human plasma glycoproteins and their abundance. Six batches of four standard samples each were analyzed

by MALDI-TOF-MS. Forty-seven N-glycan signals of different compositions were consistently detected in all 24 analyses (listed in Table 2 1) and their average relative peak intensity ratios are given in Figure 2 3. The intra-batch and inter-batch repeatability studies were performed similarly to the HILIC HPLC-FL study, but with 6 batches instead of 10. RSDs for the 47 glycan peaks are shown in Figure 3 and were found to be 9% and 13% on average, respectively. The repeatability of the MALDI-TOF-MS method (analysis of the same sample spotted 8 times) with an average RSD of 7% is likewise shown in Figure 2 3.

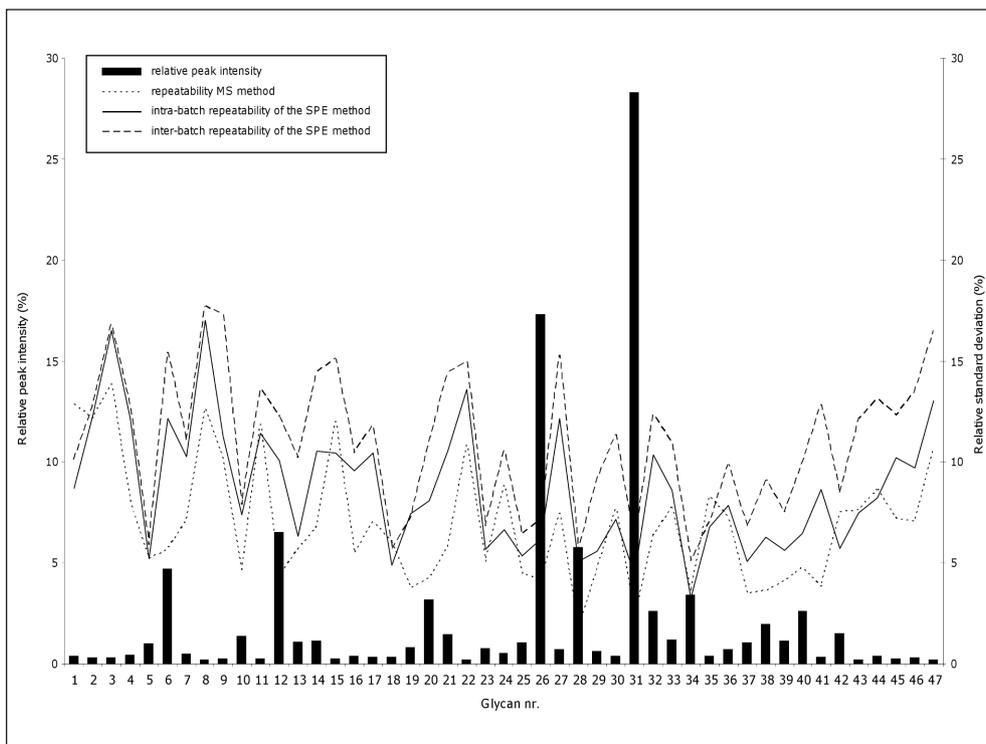


Figure 2 3. Determination of the precision of sample preparation method with MALDI-TOF-MS analysis. Average relative peak intensities of 47 N-glycans that were consistently detected in MALDI-TOF-MS spectra of 20 standard samples. The spectra were acquired using DHB matrix in the linear negative ionization mode. The repeatability of the MALDI-TOF measurements (dashed line), inter-batch variation of the sample preparation method (continuous line) and the intra-batch variation of the sample preparation method (striated line) are added in the graph. N-glycan numbers refer to Table 2 1.

Thus, by both HILIC HPLC fluorescence profiling and MALDI-TOF-MS the chosen sample work-up method comprising PNGase F treatment, 2-AA-labeling, and HILIC SPE with microcrystalline cellulose was found to be suitable for profiling of human plasma N-glycans.

CE-ESI-Q-TOF-MS.

For the fast in-depth analysis of 2-AA-labeled N-glycans from human plasma glycoproteins by high-accuracy electrospray MS, a capillary electrophoresis-ESI-Q-TOF-MS method was developed. An ammonium acetate buffer enabled the fast separation of the N-glycans, which was completed within 5 min (Figure 2 4 A). 2-AA-labeled N-glycans were transported from the inlet (anode) to the mass detector (cathode) by the electroosmotic flow (EOF). Sialylated N-glycans exhibited higher negative charge than non-sialylated N-glycans and, therefore, showed a higher negative electrophoretic mobility resulting in longer migration times. Under these conditions, separation was predominantly based on charge and thus number of sialic acids and size or shape of the N-glycans only played a minor role. After addition of methanol (50%) to the background electrolyte (Figure 2 4 B), the charge of the N-glycans remained the major driving force for the separation. But in addition, size and shape of the N-glycans influenced the separation.

For the determination of accurate masses, mass spectra were averaged for the various charge groups as indicated with horizontal bars in Figure 2 4 B. The obtained masses (Figure 2 6 in the supporting information) were used for deduction of molecular compositions. A total of 47 N-glycans could be identified and corroborated the N-glycan assignments of the MALDI measurements as summarized in Table 2 1.

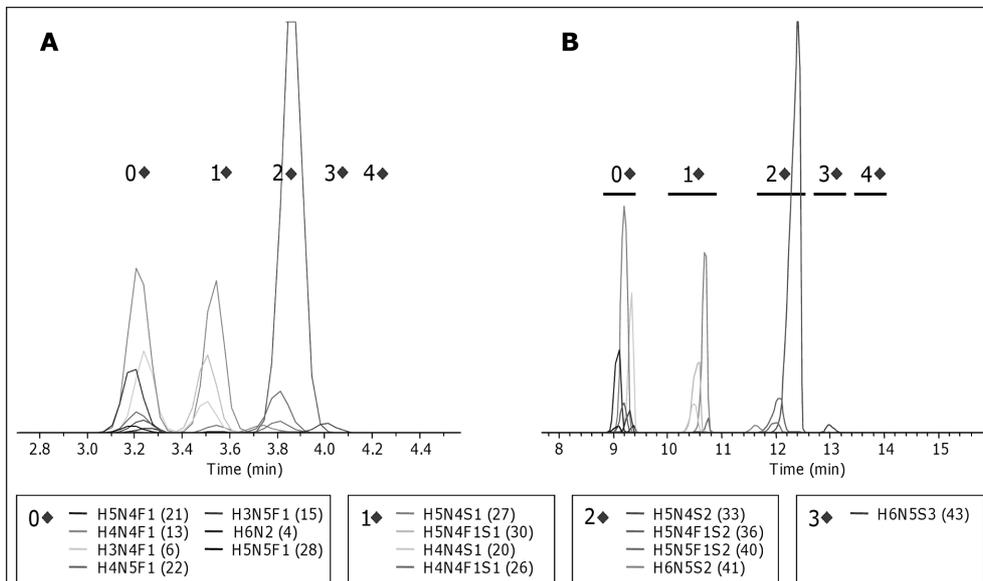


Figure 2 4. Base peak electropherograms of a water based BGE (A) and a 50% methanol containing BGE (B) CE-MS analysis of 2-AA-labeled N-glycans from human plasma. Elution regions of 2-AA-labeled N-glycans with 0, 1, 2, 3 and 4 sialic acids are indicated by horizontal bars. Extracted ion chromatograms are labeled with glycan composition. The N-glycan number referring to Table 2 1 is given in parentheses. For corresponding mass spectra see Figure 2 6.

Discussion

There is an obvious need for high-throughput methods for glycosylation profiling in order to allow a thorough testing of glycan biomarker candidates within population studies. We here describe a method for the fast sample preparation and analysis of N-glycosylation profiles of human plasma proteins. The sample preparation is performed at the 96 well plate format, and up to 384 samples can be handled in parallel, with the entire sample preparation procedure taking 24 hours. The protocol is suitable for automation on a pipetting robot, which would eventually allow an even higher throughput.

N-glycans are fluorescently labeled with 2-AA, enabling labeling under aqueous conditions, without prior sample clean-up, thus decreasing the time needed for the whole procedure. The versatility of the 2-AA label is a second advantage in large population N-glycan profiling, allowing the use of complementary analytical techniques.

Purification of 2-AA-labeled N-glycans was performed with cellulose HILIC SPE, which has several advantages compared to other sample clean-up steps: elution of 2-AA labeled N-glycans is achieved with water, and samples can be stored at -20°C without degradation / loss of sialic acids; moreover, cellulose is cheap and easy to handle. This high-throughput method is thus attractive for the analysis of larger sample populations. Notably, serum N-glycan species with multiple negative charges (N-glycans with three and four sialic acid residues) were successfully eluted from the HILIC SPE material using water as an eluent. This is due to the lack of cationic groups on the cellulose stationary phase, leaving hydrogen bonding as the sole retention principle. In contrast, the commercially available HILIC SPE plate is based on an aminopropyl silica stationary phase, which is expected to undergo ionic interactions with acidic glycan moieties [95]. Suppression of these ionic interactions by the addition of salts or bases is required to use amine stationary phases in the HILIC mode and affords elution of acidic glycan species, as it has been described for HILIC HPLC on amine-bonded silica stationary phases [94;175;176]. Because salt additives are known to interfere with glycan analysis by MALDI-TOF-MS and capillary electrophoresis, we favor the cellulose stationary phase which allows the elution of multiply negatively charged glycans with water.

While 2-AA-labeled glycans could be analyzed by MALDI-TOF-MS immediately after HILIC SPE, a prior reversed phase purification step to remove traces of salts provided better results. Reversed phase SPE which was performed using ZipTips was feasible due to the hydrophobic properties of the 2-aminobenzoic acid tag, while native glycans cannot be retained on C18 material. Notably, this reversed phase micro-SPE method in combination with negative linear mode MALDI-TOF-MS using DHB matrix resulted in peak profiles with good repeatability. Though some variation in peak height ratios was observed between individual spots, we successfully compensated for this effect by accumulating 2000 laser shots. We conclude that the used MALDI-TOF-MS approach is suitable for the determination of relative peak heights of 2-AA-labeled glycans differing in the number of sialic acid residues.

Recently an alternative method for sample preparation and analysis of N-glycans from human glycoprotein samples has been published by Royle et al.[80] In this procedure, (glyco-)proteins are immobilized in 96 well polyvinylidene fluoride (PVDF) filter plates prior to N-glycan release. Salts and reagents can be washed away from the filter plates prior to release and elution of N-glycans and the necessity for a pre-labeling clean up is avoided. The major disadvantage of this method is the limited capacity of the PVDF membrane: Only ~10 pmol (glyco-) proteins are bound per well [99]. An alternative in-gel N-glycan release method has been described [80] in order to be able to handle larger amounts of (glyco-) proteins and obtain larger amounts of N-glycans, which however resulted in a more laborious protocol. In our protocol, the amount of plasma and glycoproteins used can be easily scaled up or down, as an in-solution N-glycan release strategy is used. Royle et al. [80] moreover used 2-aminobenzamide for labeling the N-glycans by reductive amination, which makes it necessary to dry the samples prior to labeling, e.g. by a vacuum centrifugation step, whilst the 2-aminobenzoic acid labeling as used in our procedure is directly performed on the aqueous N-glycan release sample.

Another high-throughput, automated sample preparation method, which includes enzymatic release of N-glycans, fluorescent labeling with 2-AA and HILIC separation was recently published [118]. This method is used only for the release of N-glycans from purified glycoproteins and not for whole human plasma, which is a much more complicated sample matrix. Samples were analyzed by HILIC HPLC, but no mass spectrometry was performed and it was thus not possible to identify the N-glycans. Furthermore, this protocol was based on a PhyNexus MEA Purification System, a platform which is not available in many laboratories.

A variety of routes are available for the analysis of 2-AA-labeled N-glycans. Several analysis methods have been described here: HILIC HPLC-FL, MALDI-TOF-MS, and CE-ESI-Q-TOF-MS. Other analysis methods could include capillary gel electrophoresis with laser induced fluorescence detection (CGE-LIF), possibly using a DNA-sequencer [99], CGE-MS, electrophoretic analysis on microfluidic chips [145],

HPLC-MS using reverse phase or carbon columns or high pH anion exchange chromatography (HPAEC) [94;143].

Conclusion

We here describe a method for the fast sample preparation and analysis of N-glycosylation profiles of human plasma proteins, to allow plasma N-glycan analysis on larger sample populations. The method, which consists of the release of the N-glycans from carrier glycoproteins and subsequent labeling with 2-AA, followed by a HILIC-based solid phase extraction (SPE) method for isolation of the 2-AA-labeled N-glycans, was shown to be highly reproducible. The procedure fulfills the requirements for a robust, high throughput, generally applicable routine for N-glycan analysis from large sets of glycoprotein samples and is suitable for automation.

Acknowledgement

We thank Carolien A.M. Koeleman for excellent assistance.

Supporting information

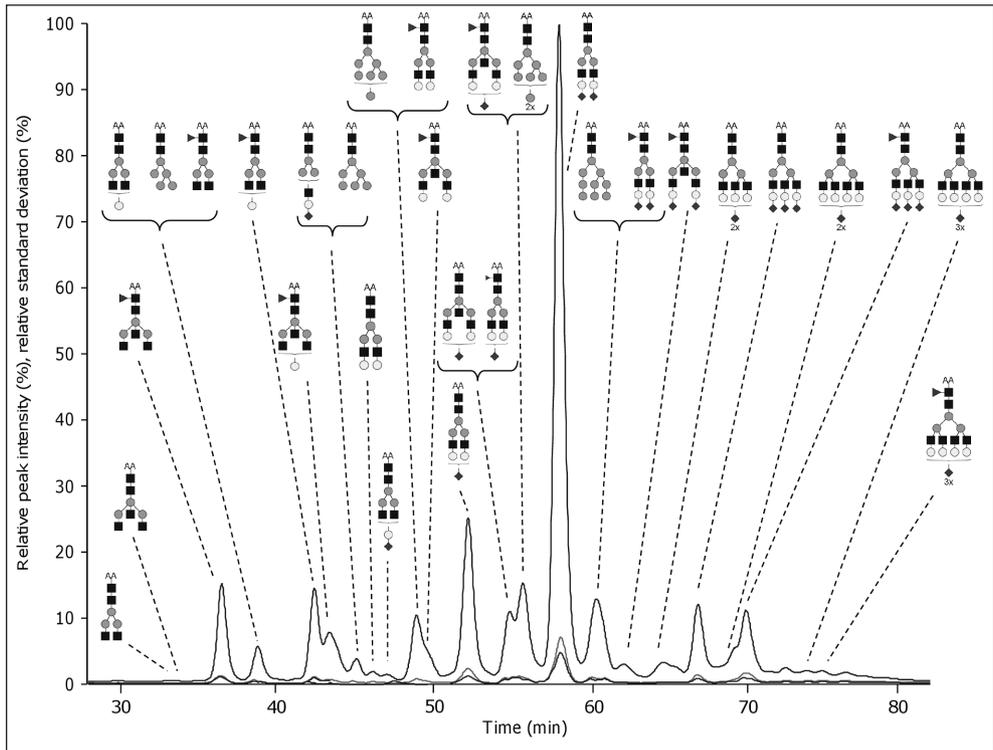


Figure 2 5. Determination of the precision of the sample preparation method with HPLC-FL analysis. Average HPLC-FL chromatogram of 40 measures (blue line), inter-batch variation of the sample preparation method (green line), intra-batch variation of the sample preparation method (brown line). The one or two most abundant N-glycans per peak are assigned. Compositions and structural schemes are given in terms of N-acetylglucosamine (blue square), mannose (green circle), galactose (yellow circle), sialic acid (purple diamond) and fucose (red triangle). All N-glycans were labeled with 2-aminobenzoic acid (2-AA).

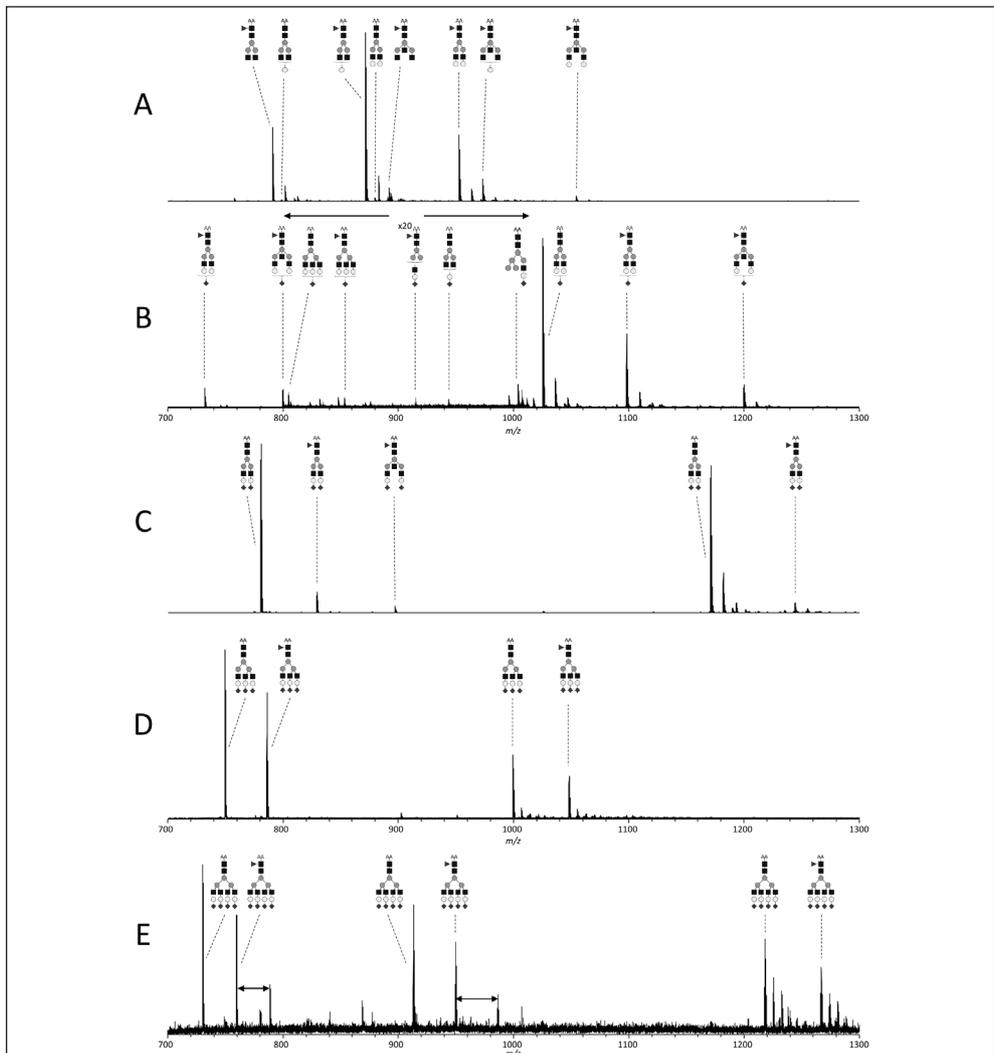


Figure 2 6. Sum mass spectra of CE separated 2-AA-labeled N-glycans from human plasma bearing 0 (A), 1 (B), 2 (C), 3 (D) and 4 (E) sialic acids. Migration time regions were chosen as indicated in Figure 2 4. For key, see Figure 2 5.

