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N-Glycan Profiling of Dried Blood Spots

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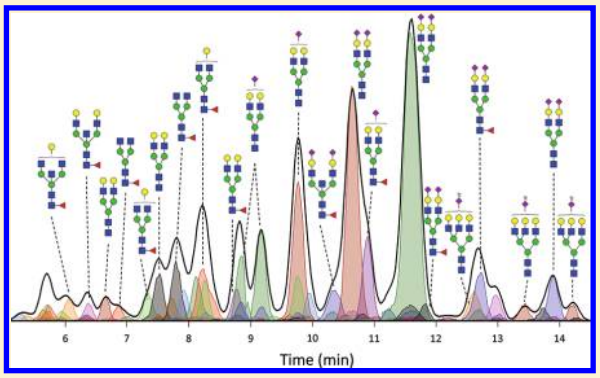
ABSTRACT: Serum *N*-glycan profiles for use as clinical biomarkers of disease(s) is of increasing scientific interest. Promising profiles have already been identified in several diseases, including cancer, Alzheimer's, and diabetes. Venipuncture is routinely performed to collect the blood necessary for this type of analysis, but blood from a fingerstick placed on filter paper (known as dried blood spots (DBS)) is more advantageous. This sampling method is less invasive than "classical" blood drawing, can be performed conveniently at home, and avoids cumbersome shipping and storage procedures. Here, we present a procedure for *N*-glycan profiling of DBS samples consisting of reconstitution of DBS in *N*-glycan release buffer, protein denaturation, enzymatic *N*-glycan release and PGC Solid phase extraction (SPE) for purification. Samples are then analyzed using nanoHPLC-PGC-chip-TOF-MS to generate *N*-glycan profiles. Using this method, ~150 *N*-glycan structures can be monitored, originating from 44 *N*-glycan compositions that can be analyzed with good repeatability (the coefficient of variation (%CV) is ~20%). To assess the stability of the *N*-glycans during storage, DBS samples were stored at room temperature (RT) and −80 °C. No major differences in *N*-glycan composition could be observed. Moreover, upon comparison of the *N*-glycan profile of DBS with profiles obtained from serum, which is a classical matrix for *N*-glycan profiling, similar patterns were observed. The method facilitates large population studies for *N*-glycan profiling, and is especially advantageous for children and the elderly, who have limited blood supplies, as well as animal studies in small mammals.



INTRODUCTION

Over the last five years, the development of high-throughput *N*-glycan profiling methods has enabled researchers to pursue the role of glycomics as potential biomarkers of disease.[1−4] Alterations in *N*-glycan profiles from blood have been observed in cancer (e.g., breast,[5,6] ovarian,[7,8] pancreatic,[9,10] and lung[11]), liver cirrhosis, and fibrosis.[12−14] In addition, large-scale population studies have evaluated the role of aging, sex, smoking behavior, and other environmental factors on blood protein glycosylation.[15−17] Although collection of blood samples is associated with minimal risk to subjects, inconveniences including the requirement of trained phlebotomists, time, and temperature-sensitive blood processing, restricted shipping protocols, and the need for secure refrigerated storage facilities exist. Alternative approaches, such as the analysis of dried blood spots (DBS)—drops of whole blood collected and dried on filter paper—is highly attractive. DBS was first developed in the 1960s, to detect phenylketonuria in newborns.[18] Today, DBS specimens are collected from neonates during their first few days of life to allow screening for a series of genetic in-born errors.[19] While the use of DBS is widely applied in neonatal screening programs, the use of this technique in population-level research is relatively new, as a result of improvement in mass spectrometric assays. For example, the application of DBS in therapeutic drug monitoring is gaining popularity (e.g., refs 20−22).

There are several advantages of DBS:[23]

(1) A finger prick is much less invasive than a regular blood drawing, and can be performed at home, without the need for a medically trained individual.
(2) Only a small amount of blood is necessary, which is advantageous for research involving small mammals, children, and the elderly.
(3) Storage and shipping of DBS specimens is relatively easy. Since the analytes are stabilized on the matrix and are dry, no cold transportation is necessary. Moreover, samples have been reported to remain stable at −80 °C for long periods of time.

For these reasons, DBS specimens are increasingly being utilized in biomarker studies. Thus, a method for the analysis of *N*-glycan patterns from DBS would be valuable.

Current assays for glycosylation profiling are comprised of LC- or CE-based separations, coupled to mass spectrometric or fluorometric analyte detection. The use of HILIC-HPLC with fluorescence detection for the analysis of derivatized glycans is widely accepted,[11,16,17,24] but several studies using CGE-LIF have also been conducted.[14,15,25] We recently developed a strategy to analyze native *N*-glycans using PGC-LC-MS, resulting in the unambiguous identification of over 100 *N*-

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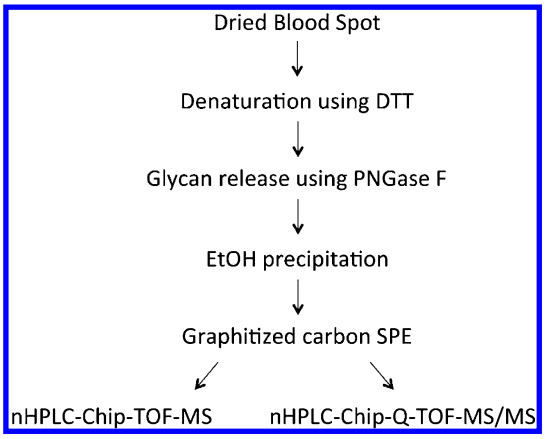

Figure 1. Schematic overview of the procedure for *N*-glycan analysis of dried blood spots (DBS).

glycan compositions and ~300 *N*-glycan species from serum.[26] Below, we describe how we applied this methodology to DBS to achieve a comprehensive profiling of *N*-glycosylation patterns. The procedure involved reconstitution of DBS in *N*-glycan release buffer, protein denaturation, enzymatic *N*-glycan release, and PGC solid-phase extraction (SPE) for purification (Figure 1). Samples are then analyzed using nanoHPLC-PGC-chip-TOF-MS to generate *N*-glycan profiles. The repeatability of the method is assessed, as well as the stability of the *N*-glycans during storage. Moreover, the *N*-glycan profile of DBS is compared to profiles obtained from serum, which is a classical matrix for *N*-glycan profiling.

EXPERIMENTAL SECTION

Dried Blood Spots. Filter paper was obtained from Whatman/GE Healthcare (903 Protein Saver Card, Piscataway, NJ). Blood spots from a healthy volunteer were obtained according to standard procedures.[23] In short, a finger was cleaned with 70% isopropyl alcohol (Fisher Scientific, Waltham, MA) and subsequently pricked with a lancet, similar to those used for monitoring blood glucose levels. The first drop of blood was removed using a sterile wipe, and subsequent drops were collected on the filter paper without the finger touching the paper. Upon air-drying overnight, the sample cards were stored at −80 °C in a closed plastic bag with desiccant.

***N*-Glycan Release from a Dried Blood Spot.** A 3.1-mm disk was punched out of the middle of a dried blood spot using a manual puncher (Whatman/GE Healthcare) and transferred to an Eppendorf tube. Upon addition of 100 μL of 100 mM ammonium bicarbonate (Sigma−Aldrich, St. Louis, MO) with 5 mM dithiothreitol (DTT, Promega, Madison, WI) in water, the samples were vortexed and subsequently sonicated for 5 min. Proteins in the samples were denatured using six cycles alternating between 100 °C and room temperature (RT) for 10 s each. Two μL of PNGaseF (New England Biolabs, Ipswich, MA) was added to the samples, and enzymatic glycan release was performed in a CEM (Matthews, NC) microwave at 20W for 10 min.[27] Deglycosylated proteins were precipitated using 400 μL of ice-cold ethanol, and the samples were chilled at −80 °C for 1 h. Upon centrifugation, the supernatant was transferred to new Eppendorf tubes, and brought to dryness *in vacuo*.

***N*-Glycan Release from a Serum Sample.** Fifty microliters (50 μL) of human standard serum (Sigma−Aldrich) was mixed together with 50 μL of 200 mM ammonium bicarbonate

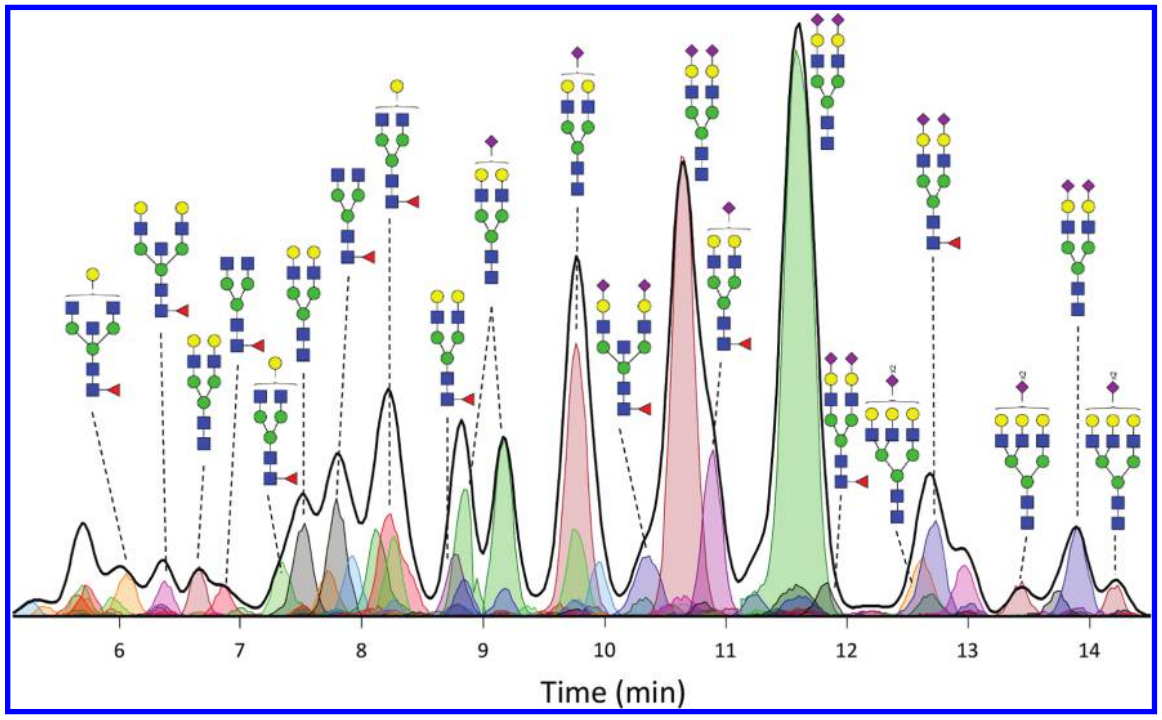

Figure 2. Extracted *N*-glycan chromatogram and extracted compound chromatograms of *N*-glycans derived from dried blood spots (DBS). Extracted *N*-glycan chromatogram (thick black line), as obtained using Masshunter software, depicts the total ion intensity for all *N*-glycan compounds observed. Extracted compound chromatograms for each of the 150 *N*-glycan compositions are automatically extracted (colored lines with integrals, each peak is a different compound) and the most intense signals are annotated with their respective *N*-glycan composition. Key: red triangle, fucose; blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; and purple diamond, sialic acid (NeuAc).

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with 10 mM DTT in water. Upon vortexing, the proteins were denatured and the glycans were released. The proteins were precipitated as described for dried blood spots (DBS).

_N_-Glycan Purification Using Graphitized Carbon SPE. Oligosaccharides released by PNGaseF were purified using graphitized carbon SPE (Grace, Deerfield, IL).[2,26,28] Briefly, cartridges were conditioned using 4 mL of 80% ACN containing 0.05% TFA (EMD Chemicals, Gibbstown, NJ), followed by 4 mL of water containing 0.05% TFA. Oligosaccharide samples were reconstituted in 500 mL of water and subsequently loaded onto the cartridges. Cartridges were washed using 3 × 4 mL of water and *N*-glycans were eluted using 4 mL of 40% ACN containing 0.05% TFA. Samples were dried *in vacuo* prior to analysis.

nanoHPLC-Chip-TOF-MS Analysis. *N*-glycans were analyzed using an Agilent (Santa Clara, CA) 6200 Series nanoHPLC-chip-TOF-MS, consisting of an autosampler (which was maintained at 8 °C), a capillary loading pump, a nanopump, HPLC-chip-MS interface, and an Agilent 6210 Time Of Flight mass spectrometer. The chip (glycan chip II, Agilent) contained a 9 mm × 0.075 mm i.d. enrichment column coupled to a 43 mm × 0.075 mm i.d. analytical column; both packed with 5 μm porous graphitized carbon (PGC). *N*-glycan samples were reconstituted in 45 μL of water and diluted 1:5 with water prior to analysis; 1 μL of sample was used for injection. Upon injection, the sample was loaded onto the enrichment column using 3% ACN containing 0.1% formic acid (FA, Fluka, St. Louis, MO). After the analytical column was switched in-line, the nanopump delivered a gradient of 3% ACN with 0.1% FA (solvent A) and 90% ACN with 0.1% FA (solvent B).

Data Analysis. Data analysis was performed using Masshunter qualitative analysis (version B.03.01, Agilent) and Microsoft Excel for Mac 2011 (version 14.1.3, Microsoft), according to Hua et al.[26] with modifications. Data was loaded into Masshunter qualitative analysis, and glycan features were identified and integrated using the Molecular Feature Extractor algorithm. First, signals above a signal-to-noise threshold of 5.0 were considered. Then, signals are deconvoluted using a tolerance of 0.0025 m/z ± 10 ppm. The resulting deconvoluted masses are subsequently annotated using a retrosynthetic theoretical glycan library,[29] where a 15 ppm mass error was allowed. Glycan compositions, retention times, and volume were exported to csv-format for further evaluation.

nanoHPLC-Chip-Q-TOF-MS/MS Analysis. To allow unambiguous identification of *N*-glycans, samples were analyzed using an Agilent 6200 series nanoHPLC-chip-Q-TOF-MS/MS, consisting of an autosampler (which was maintained at 8 °C), a capillary loading pump, a nanopump, an HPLC-chip-MS interface, and an Agilent 6520 Quadrupole-Time Of Flight mass spectrometer. The chip and gradient were the same as for the nanoHPLC-chip-TOF-MS analysis. *N*-glycans were subjected to collision-induced fragmentation with nitrogen as the collision gas, using a series of collision energies that were dependent on the m/z values of the *N*-glycans. The collision energies correspond to voltages ($V_{collision}$) that were based on the equation

$$V_{collision} = m/z\left(\frac{1.8}{100\ \text{Da}}\right) - 2.4\ \text{V}$$

where the slope and offset of the voltages were set at 1.8/(100 Da) and −2.4, respectively.

Table 1. Glycan Composition from DBS[a]

No.	composition[b]	accurate mass	observed mass	relative abundance in DBS (%)
1	H_3N_2	910.328	910.326	0.13
2	H_3N_3	1113.407	1113.408	0.32
3	$H_3N_3F_1$	1259.465	1259.464	0.23
4	H_3N_4	1316.487	1316.488	0.12
5	$H_3N_4F_1$	1462.544	1462.546	3.10
6	H_3N_5	1519.566	1519.572	0.04
7	$H_3N_5F_1$	1665.624	1665.631	0.92
8	H_4N_2	1072.381	1072.383	0.05
9	H_4N_3	1275.460	1275.460	0.97
10	$H_4N_3S_1$	1566.555	1566.555	1.73
11	$H_4N_3F_1$	1421.518	1421.517	0.09
12	H_4N_4	1478.539	1478.538	0.29
13	$H_4N_4S_1$	1769.635	1769.634	0.38
14	$H_4N_4F_1$	1624.597	1624.606	4.49
15	$H_4N_4F_1S_1$	1915.693	1915.700	0.05
16	H_4N_5	1681.619	1681.621	0.13
17	$H_4N_5S_1$	1972.714	1972.756	0.13
18	$H_4N_5F_1$	1827.677	1827.69	1.42
19	H_5N_2	1234.433	1234.434	0.34
20	H_5N_3	1437.513	1437.509	0.09
21	$H_5N_3S_1$	1728.608	1728.615	0.20
22	H_5N_4	1640.592	1640.599	4.03
23	$H_5N_4S_1$	1931.688	1931.685	18.24
24	$H_5N_4S_2$	2222.783	2222.790	34.64
25	$H_5N_4F_1$	1786.650	1786.652	3.01
26	$H_5N_4F_1S_1$	2077.746	2077.756	5.73
27	$H_5N_4F_1S_2$	2368.841	2368.843	3.68
28	H_5N_5	1843.672	1843.682	0.33
29	$H_5N_5S_1$	2134.767	2134.766	0.69
30	$H_5N_5S_2$	2425.862	2425.845	0.02
31	$H_5N_5F_1$	1989.729	1989.740	1.07
32	$H_5N_5F_1S_1$	2280.825	2280.835	2.96
33	$H_5N_5F_1S_2$	2571.920	2571.932	1.79
34	H_6N_2	1396.486	1396.485	0.52
35	H_6N_3	1599.566	1599.566	0.08
36	$H_6N_5S_1$	2296.820	2296.822	1.11
37	$H_6N_5S_2$	2587.915	2587.927	3.49
38	$H_6N_5F_1S_2$	2733.973	2733.986	0.84
39	$H_6N_5F_1S_3$	3025.069	3025.076	0.71
40	$H_7N_6S_2$	2953.047	2953.052	0.15
41	$H_7N_6S_4$	3535.238	3535.248	0.18
42	H_7N_2	1558.539	1558.543	0.35
43	H_8N_2	1720.592	1720.602	0.56
44	H_9N_2	1882.645	1882.655	0.62

[a]Compositions and observed masses of the glycans identified and integrated by Masshunter software are listed. Since Masshunter automatically deconvolutes the mass spectra, the observed monoisotopic molecular masses are given. Relative abundance percentages were calculated in Excel from the volumes obtained from Masshunter. [b]Key: H = hexose, N = *N*-acetylhexosamine, F = fucose, and S = sialic acid (NeuAc).

RESULTS

A procedure (see Figure 1) is developed for the analysis of *N*-glycan profiles from DBS using enzymatic *N*-glycan release followed by protein precipitation using ethanol and graphitized carbon SPE purification. Oligosaccharide samples are then analyzed using nanoLC-PGC-chip-TOF-MS to obtain isomer-specific *N*-glycan profiles. No derivatization (either oligosaccharide reduction, permethylation, or labeling) was performed,

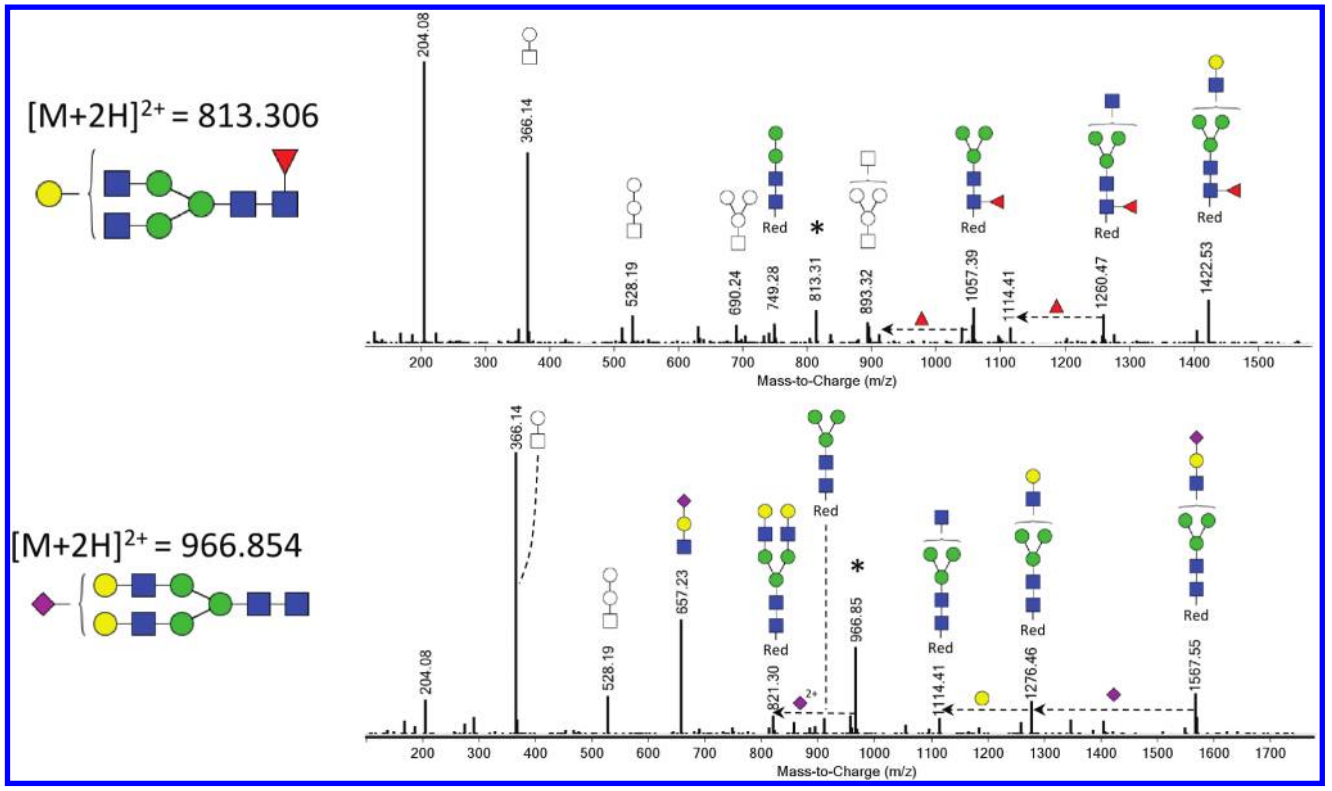

Figure 3. Fragmentation spectra from *N*-glycans derived from DBS. CID fragmentation of *N*-glycans from DBS obtained from nanoHPLC-PGC-chip-Q-TOF-MS. Asterisk (*) indicates the parent ion. Key: square, *N*-acetylhexosamine; circle, hexose; red triangle, fucose; blue square, *N*-acetylglucosamine; green circle, mannose; yellow circle, galactose; and purple diamond, sialic acid, Red: reducing end. The structures represented in color have the reducing end present, facilitating their annotation. When the reducing end is not present, it is not possible to distinguish between galactose and mannose residues; therefore, these structures are represented with symbols only.

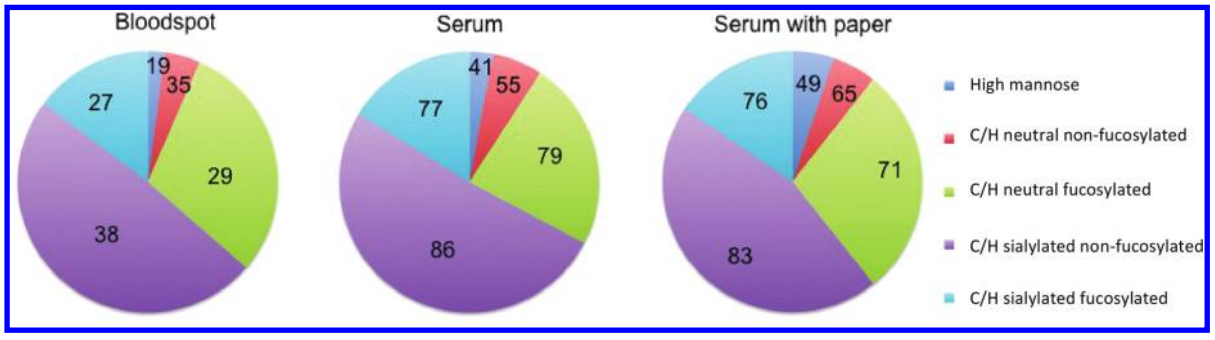

Figure 4. Comparison of the *N*-glycan pattern from serum with and without collection material and DBS. Pie charts from a DBS sample and serum samples with and without collection material. Chart areas represent the relative abundance of *N*-glycan classes, while the numbers represent the number of glycan compounds identified and integrated for each class. Class information is given in terms of high-mannose-type glycans (high mannose), complex/hybrid neutral nonfucosylated glycans, complex/hybrid neutral fucosylated glycans, complex/hybrid sialylated nonfucosylated glycans, and complex/hybrid sialylated and fucosylated glycans.

as such modifications may result in unwanted side products and loss of resolving power during PGC separation. All profiles were obtained in the positive ionization mode.

In Figure 2, an nanoHPLC-PGC-chip-TOF-MS extracted compound chromatogram from *N*-glycans derived from DBS is depicted. Approximately 150 glycan compounds were identified and integrated from each DBS sample, relative to ~330 compounds usually obtained from 50 μL of serum. Fewer compounds are detected from DBS specimens, as the amount of serum in the small (3 mm) disk is much less (~2 μL) than the 50 μL used in serum analysis. Here, note that the analysis is performed on nonreduced glycans, resulting in separation of the α and β anomers. Therefore, the actual number of linkage isomers detected may have to be less. From the 150 *N*-glycan compounds detected in the DBS samples, 44 glycan compositions could be identified and integrated reproducibly using Masshunter, as shown in Table 1. Of these compositions, 7 are high mannose type and 37 are hybrid or complex type; 15 are fucosylated and 19 are sialylated.

To confirm the glycan compositions determined by accurate *m*/*z* signals, fragmentation data was obtained from one DBS sample using nanoLC-PGC-chip-Q-TOF MS/MS. Fragmentation spectra of two randomly chosen *N*-glycans ($H_3N_4F_1$ at *m*/*z* 813.306 and $H_5N_4S_1$ at *m*/*z* 966.854) are depicted in Figure 3. Clear characteristic *N*-glycan fragments were observed (*m*/*z* 366, 528, and 657), confirming the *N*-glycan annotation.

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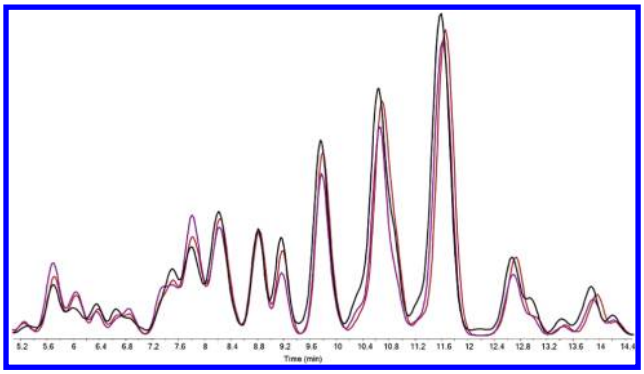

Figure 5. Intraday variability of the analysis of *N*-glycans from dried blood spots. Three DBS collected from the same individual on the same day were prepared and analyzed on the same day. Extracted *N*-glycan chromatograms of the three analyses are overlaid to depict the intraday variability of the analysis.

Effects of Collection Matrix on *N*-Glycosylation Profile. To evaluate the effects of the collection matrix—cellulose-based neonatal screening cards—*N*-glycosylation profiles obtained from 50 μL of standard serum with and without the addition of a full disk (10 mm diameter) of collection paper. Figure 4 (appears later in this paper) shows that no significant changes were obtained between the samples with and without the collection paper, both in the number of compounds determined (344 vs 338, respectively) and compositions.

Comparison between DBS and Serum. Current research on glycan-based biomarker discovery is primarily conducted on serum. To determine if our DBS profile compares favorably with serum, we analyzed commercial serum. Pie charts of the compositional analysis of DBS and serum are depicted in Figure 4. Overall, the *N*-glycan pattern is very similar between the two samples, but a slightly lower amount of high-mannose glycans was observed in the DBS. However, the DBS and serum are not obtained from the same individual, and, because serum *N*-glycosylation patterns show high interindividual variation,[30] the difference observed here could very well be caused by interindividual differences.

Repeatability. Measurement variability was evaluated using one DBS that was prepared in triplicate, according to the procedure described. All three samples were analyzed using nanoLC-PGC-chip-TOF-MS on three consecutive days. Data analysis was performed as described. The average coefficient of variation (%CV) of the measurement variability was obtained by comparing the three daily analyses. Glycan intensities of the 44 glycan compounds consistently observed in all samples (see Table 1) were used to calculate %CV and all of the individual glycan %CVs were averaged to generate the average %CV of the measurement variability, which was 18.8%.

To evaluate the repeatability of the proposed procedure, three dried blood spots were collected from the same individual on three consecutive days. Blood spots were allowed to dry overnight and *N*-glycan release was performed the next day. Samples were analyzed using nanoLC-PGC-chip-TOF-MS and data analysis was performed as previously described. An overlay of the extracted glycan chromatograms obtained from the three analyses on day 1 is depicted in Figure 5. The average within-day %CV was obtained by comparing the analysis of the three samples within one day and was 20.5%; the average interday % CV was obtained by comparing differences in the analysis of the 3 × 3 samples between days and was 20.8%.

Storage of Dried Blood Specimens. It is generally acknowledged that DBS specimens are a good replacement for serum specimens, with the additional advantage of less degradation at ambient temperatures. To evaluate the degradation of *N*-glycans during storage, 18 dried blood spots were collected from the same individual at the same time point. Samples were allowed to dry overnight and *N*-glycans were released the next day (day 0) or stored in a plastic bag with desiccant at RT (25 °C, 8 specimens) or −80 °C (8 specimens). *N*-glycan profiles (n = 2) were obtained after 3, 7, 14, and 31 days of storage. Figure 6 reveals that no significant difference was observed between storage at RT and storage at −80 °C. Also, no difference was observed between day 0 and day 31.

■ CONCLUSIONS

This study describes a new procedure for the analysis of *N*-glycosylation patterns from dried blood spots. Approximately 150 *N*-glycan compounds can be identified from DBS samples using this approach, originating from 44 *N*-glycan compositions. No interference from the collection matrix could be

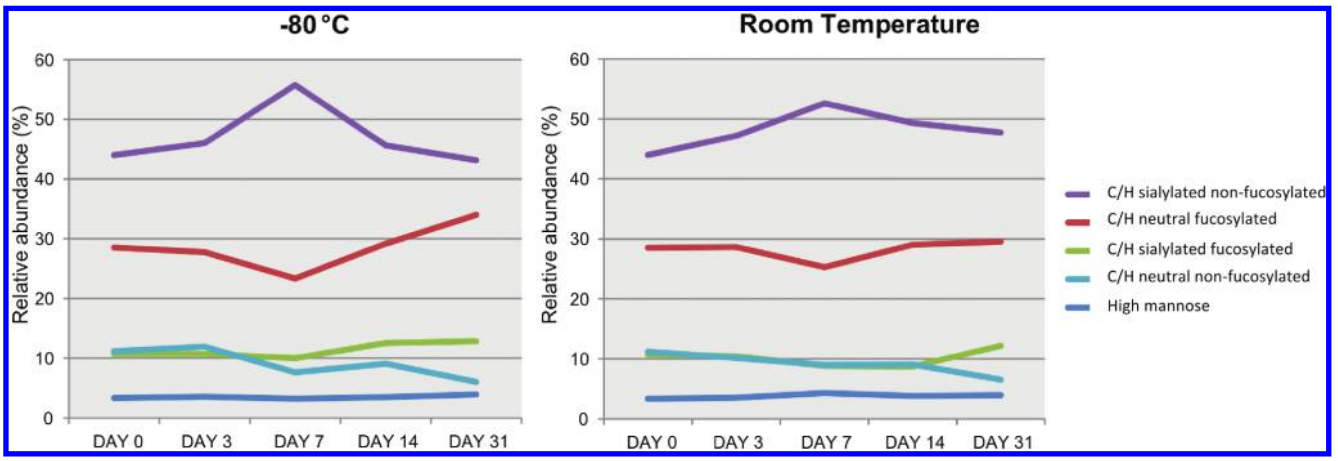

Figure 6. Stability of *N*-glycans in dried blood spots during storage. Dried blood spots were stored either at room temperature or at −80 °C. *N*-glycan profiles (n = 2) were obtained on days 0, 3, 7, 14, and 31 after sample collection. Average relative percentage is depicted for each glycan type. For key, see Figure 4.

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observed. Relative quantitation of the 44 compositions resulted in a %CV of 20.8%, which is similar to serum. Furthermore, we showed that storage of DBS at RT or at −80 °C for at least one month does not affect *N*-glycosylation patterns.

The use of DBS technology for the collection of blood-derived specimens has many advantages, compared to venous blood drawing, which is the current clinical standard. First, sample collection is less invasive and more convenient for the patient. Only a finger prick is required, which is routinely used by diabetic patients, and the sample can be obtained at home on the patient's time schedule. Second, lower volumes are needed. Serum is usually collected in containers of at least 4 mL; a drop of blood is ∼50 μL and is sufficient for a high-quality DBS specimen. Third, there is no need for immediate processing of the sample. Serum must be allowed to clot for a minimum of 60 min, then centrifuged, separated, aliquoted, and frozen. DBS specimens are air-dried prior to RT storage.

These advantages make the use of DBS specimens especially suitable in specific cases: It will be easier to collect DBS specimens from children and elderly, because venipuncture is frequently problematic in these vulnerable patients. Application of DBS specimens in larger-scale population studies will most likely result in better participation numbers. A last group of studies that will benefit of the use of DBS are small mammal studies; currently, larger amounts of blood are needed, which is not required for DBS analysis.

N-glycans are complex structures, comprising several different monosaccharides that can be attached in several different ways. Not only can monosaccharides be linked to different sugars (topoisomers, e.g., triantennary GlcNAc or bisecting GlcNAc), but the monosaccharides may also be linked at different positions (linkage isomers, e.g., α2−3 sialic acid versus α2−6 sialic acid). Over the years, several strategies have been employed for glycan analysis, comprising chromatographic or electromigrative separations as well as mass spectrometry. Recently, especially PGC has been recognized as a good stationary phase for HPLC separation of glycans, because of its efficient separation of both topoisomers, as well as linkage isomers.[2,31,32] Because of the different isomers, the most complete glycan profiles can only be obtained using a combination of separation (here, PGC) and mass spectrometry.[24,31]

With the recent interest for the use of DBS specimens in therapeutic drug monitoring, research is initiated toward optimization of sampling and processing procedures. New coated collection cards have been developed, which precipitate proteins (http://www.whatman.com/DMPK.aspx). The use of these cards for DBS glycomics analysis might be beneficial; however, it is currently unclear whether these cards would be compatible with mass spectrometry (MS)-based studies. Moreover, efforts have been made to automate DBS punching, or even omit punching of DBS. Some manufacturers have developed instruments for controlled DBS punching or immediate extraction (see, e.g., ref 33). Such techniques might also be adapted for automated sample preparation for *N*-glycan analysis from DBS.

Recently, the use of paper spray ionization for direct analysis of metabolites from DBS has been introduced.[34] Several molecules, including epinephrine, serine, cocaine, and imatinib (a small-molecule tyrosine kinase used for the treatment of cancer), have been analyzed by paper spray mass spectrometry, using blood spiked with drugs or metabolites.[35,36] Even though *N*-glycans must be released from their proteins, and chromatographic separation is necessary to separate isomeric *N*-glycans, application of such an approach may be a fast way to obtain global *N*-glycan profiles.

In conclusion, we showed that the analysis of *N*-glycan profiles from DBS is feasible with good repeatability. It is expected that application of the described technology will facilitate further research toward *N*-glycan biology in children, elderly, and large population studies, as well as animal models. Our next steps are to evaluate a large number of serum and DBS collected from the healthy volunteers and subjects with cancer to confirm our findings.

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