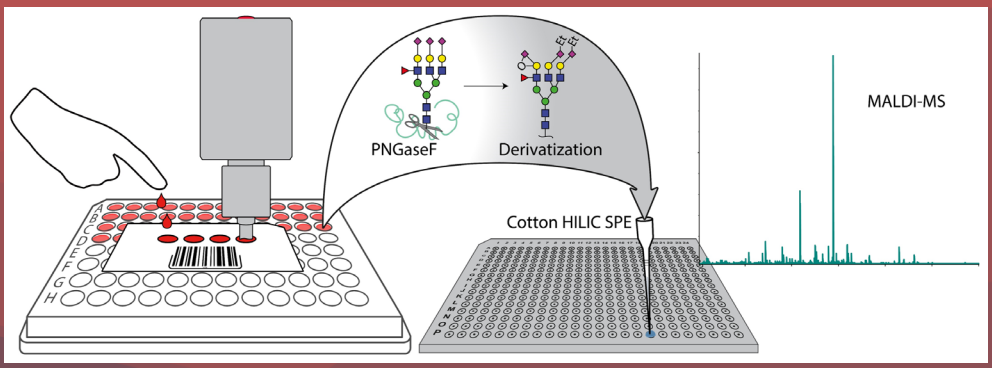
Glycosylation profiling with mass spectrometry: method development and application to cancer biomarker studies

Vreeker, G.C.M.

CHAPTER 3

Dried blood spot *N*-glycome analysis by MALDI mass spectrometry



Based on:

Vreeker, G.C.M.[1,2]*., Bladergroen, M.R.*[1]*, Nicolardi, S.*[1]*, Mesker, W.E.*[1]*, Tollenaar, R.A.E.M.*[1]*, van der Burgt, Y.E.M.*[1,3]*, Wuhrer, M.*[1]*: Dried blood spot N-glycome analysis by MALDI mass spectrometry. Talanta. 205, 120104 (2019). doi:10.1016/j.talanta.2019.06.104. Supplemental info available.*

1 Center for Proteomics and Metabolomics, Leiden University Medical Center, The Netherlands

2 Department of Surgery, Leiden University Medical Center, The Netherlands

3 Department of Clinical Chemistry and Laboratory Medicine, Leiden University Medical Center, The Netherlands

Abstract

Body fluid *N*-glycome analysis as well as glyco-proteoform profiling of existing protein biomarkers potentially provides a stratification layer additional to quantitative, diagnostic protein levels. For clinical omics applications, the collection of a dried blood spot (DBS) is increasingly pursued as an alternative to sampling milliliters of peripheral blood. Here we evaluate DBS cards as a blood collection strategy for protein *N*-glycosylation analysis aiming for high-throughput clinical applications. A protocol for facile *N*-glycosylation profiling from DBS is developed that includes sialic acid linkage differentiation. This protocol is based on a previously established total plasma *N*-glycome mass spectrometry (MS) method, with adjustments for the analysis of DBS specimens. After DBS-punching and protein solubilization *N*-glycans are released, followed by chemical derivatization of sialic acids and MS-measurement of *N*-glycan profiles. With this method, more than 80 different glycan structures are identified from a DBS, with RSDs below 10% for the ten most abundant glycans. *N*-glycan profiles of finger-tip blood and venous blood are compared and short-term stability of DBS is demonstrated. This method for fast *N*-glycosylation profiling of DBS provides a minimally invasive alternative to conventional serum and plasma protein *N*-glycosylation workflows. With simplified blood sampling this DBS approach has vast potential for clinical glycomics applications.

Introduction

Total plasma or serum *N*-glycosylation analysis has been widely performed to study human protein glycosylation in general, and aberrant disease-related glycosylation profiles or alterations specifically.[1–9] Various strategies can be pursued to obtain *N*-glycome readouts from (glyco)proteins present in the circulation[10], however for large-scale evaluations of clinical cohorts throughput numbers become essential. Commonly blood samples (for sequential plasma- or serum-based glycome analysis) are collected by antecubital venipuncture, which needs to be performed by a skilled person. An alternative and less invasive micro blood-sampling procedure to obtain specimens is through collection on a filter paper with a simple finger prick. Additional advantages of such a dried blood spot (DBS) are the ease of transportation and storage of the samples.[11,12] Consequently, DBS collection holds great potential for population-wide biomarker screening programs.[13] Hitherto DBS collections are known from newborn screening programs for metabolic diseases, but dried blood spots are also convenient for pharmacokinetic studies, diagnosis of infectious diseases and monitoring drug therapies.[14–17] Routine measurement of nucleic acids, small molecules and lipids from DBS specimens is pursued in clinical chemistry laboratories and for such determinations mass spectrometry (MS) is the method of choice.[18] Most commonly, MS-analysis is performed after punching a part of the DBS card, however also paper spray- and liquid extraction strategies have been reported that leave the card intact.[19,20]

DBS protein analysis has become feasible as a result of the developments in MS-based proteomics technologies.[21,22] Examples of MS-based protein analysis obtained from DBS are the measurement of glycated and acetylated hemoglobin[23–25] and quantification of ceruloplasmin for newborn screening of Wilson disease.[26]. Current bottom-up proteomics strategies allow for the quantification of 97 proteins.[27] With regard to protein glycosylation analysis, up to now only a couple of examples have demonstrated the analysis of protein glycosylation from DBS, despite its vast biomarker potential.[28] The first one applied enzymatic release of *N*-glycans with subsequent analysis using liquid chromatography(LC)-porous graphitized carbon (PGC)- time-of-flight(TOF)-MS, resulting in the identification and relative quantification of 44 glycan compositions from DBS samples.[29] The second example of DBS protein glycosylation analysis followed an *N*-glycopeptide approach, resulting in the identification of 41 *N*-glycopeptides from 16 major *N*-glycoproteins.[11]

In the current study, we apply a novel high-throughput glycomics method that allows profiling of more than 80 different *N*-glycans from DBS samples with linkage-specific sialic acid analysis using matrix-assisted laser desorption/ionization (MALDI) MS. Furthermore, aiming for a simplified blood collection strategy,

potential stress during sample transportation and storage needs to be considered.[30] Therefore, short-term stability of the *N*-glycome from a DBS will be evaluated at different storage conditions.

Experimental section

Chemicals and enzymes

Milli-Q water was produced with a Q-Gard 2 system at ≥18 MΩ (Millipore). Recombinant peptide-*N*-glycosidase F (PNGase F) was purchased from Roche Diagnostics and potassium dihydrogen phosphate (KH_2PO_4), 50% sodium hydroxide (NaOH), 85% phosphoric acid (H_3PO_4), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \times 2\ H_2O$), sodium chloride (NaCl), super-DHB (9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, sDHB), nonidet P-40 substitute (NP-40) and 1-hydroxybenzotriazole 97% (HOBt) were obtained from Sigma-Aldrich. Potassium hydroxide (KOH), trifluoroacetic acid (TFA), analytical grade ethanol and sodium dodecyl sulfate (SDS), and were obtained from Merck. High performance LC-grade acetonitrile (ACN) was obtained from Biosolve and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC) hydrochloride was purchased from Fluorochem.

Samples

The blood- and plasma samples were collected on Whatman FTA DMPK-C DBS cards from healthy volunteers and fully anonymized. For the stability study at -80 °C, from each volunteer A and B fourteen DBS samples were collected. For the stability study at room temperature sixteen DBS samples were collected from volunteer C. The DBS samples from volunteers A, B and C were, due to the number of blood drops needed for method validation, spotted with a glass pipette from collected EDTA-blood tubes (directly after collection) and will therefore be referred to as DBS (venous). For the stability study at 37 °C eight DBS samples from volunteer D were collected via a finger prick and will therefore be referred to as DBS (finger). The spots from volunteer D were made by washing the hands of the volunteer with water and pressing a lancet (Accu-Chek Safe T-pro plus, 2.3 mm deep) on the fingertip of the volunteer. The finger was gently massaged and the drops of blood were collected. All blood spots were left to dry for 24 hours at room temperature, before they were stored or before further processing. Per DBS two disks of approximately 0.25 cm^2 were punched with an inhouse developed electric puncher.

Enzymatic *N*-glycan release

A punched-out filter paper disk with the DBS was added to 40 µL 2% SDS in an 2 mL Eppendorf tube or microtitration plate (MTP, Nunc 96 wells plate, Thermo Fisher) and incubated for 10 minutes at 60 °C. Subsequently 41 µL of releasing mixture (20 µL acidic 5x PBS (pH 5.6), 20 µL 4% NP-40 and 1 µL PNGase F) was added. The slightly acidic condition in the release reaction was used to rapidly hydrolyze the

glycosylamines which are formed after PNGase F release, to prevent subsequent side reactions of these glycosylamines.[31] The sample was incubated overnight at 37 °C. The samples were directly used for ethyl esterification or stored at -20 °C for later use.

Ethyl esterification

Linkage-specific derivatization of sialic acids through ethylation of α2,6-linked sialic acid residues and via lactone formation of α2,3-linked sialic acids was performed as described before.[32] In short, 2 μL of released glycan sample was added to 20 μL ethyl esterification reagent (0.25 M EDC and 0.25 M HOBt in ethanol). The samples were incubated for one hour at 37 °C. Afterwards 22 μL of ACN was added.

Cotton HILIC purification

Purification of the glycans was performed with cotton hydrophilic interaction liquid chromatography HILIC tips, as described previously.[32,33] In short, 3 mm cotton thread (approximately 180 μg, Pipoos, Utrecht, The Netherlands) was positioned at the bottom of 20 μL tips. The cotton was prewetted with three times 20 μL MQ and subsequently three times conditioned with 85% ACN. The sample was loaded on the cotton by pipetting the sample twenty times up and down. The cotton was washed with first three times 20 μL 85% ACN with 1% TFA and second three times 20 μL 85% ACN. The glycans were eluted from the cotton by pipetting five times up and down in 10 μL MQ. For the analysis 1 μL of sDHB matrix (5 mg mL-1 in 99% ACN with 1 mM NaOH) was spotted with 2 μL of purified glycan sample.

MALDI-TOF and MALDI-FTICR mass measurements

Initially, our standard protocol included MALDI-TOF-MS analysis and such measurements were applied. In the progress of this project, MALDI-Fourier-transform ion cyclotron resonance (FTICR) MS analysis became available and was used for parts of the study.[31] MALDI-TOF-MS and MALDI-FTICR-MS spectra were recorded as described before.[31] In short, for the MALDI-TOF-MS measurements (*m*/*z*-range 1000-5000) an UltrafleXtreme mass spectrometer (Bruker Daltonics) was used in reflectron positive mode with a Smartbeam-II laser. The system was controlled by flexControl software (version 3.4 Build 135). At a frequency of 1000 Hz, 10000 laser shots were shot in a random walking pattern, with 200 shots per raster spot. The MALDI-FTICR-MS spectra were recorded from *m*/*z* 1011 to 5000 on a 15T SolariX XR FTICR mass spectrometer (Bruker Daltonics). The system was operated by ftmsControl (version 2.1.0) and each spectrum consisted of ten scans with 200 laser shots at each raster.

Comparison DBS (venous and from finger) with dried plasma spots (DPS)
From four anonymous volunteers a tube (EDTA) of blood and four DBS (finger) were collected. A drop of blood from the tube was spotted onto each of the marked areas of an additional DBS card. The tubes were subsequently centrifuged at 1000 g for 10 minutes and additionally four plasma spots per volunteer were created. All spots were left to dry for 24 hours, where after the derivatization, purification and measurements were performed.

Short-term stability study
The short-term stability of the glycans in DBS was evaluated for different storage temperatures. Twelve out of fourteen spots of volunteer A and B were stored at -80 °C for two, four and six weeks (per four spots). From the other two spots per volunteer the glycans were directly released. Also, for volunteer C twelve spots were stored at room temperature for two, four and six weeks (per four spots). From the other four spots the glycans were also directly released. Additionally, four spots of volunteer D were stored at 37 °C for two weeks and from four spots of the same volunteer the glycans were again directly released. At the given time points, the glycans were released from the cards that were stored in the freezer. After the release of the glycans from the cards at the last timepoint in each series the derivatization, purification and measurements were performed for all releases from all timepoints.

MS-data preprocessing
The obtained spectra were transformed in text format (x,y) using flexAnalysis version 3.4 and DataAnalysis version 3.1 (both Bruker Daltonics) for respectively the MALDI-TOF-MS and MALDI-FTICR-MS spectra. The resulting text files were calibrated using MassyTools (version 0.1.8.1). In Supporting Information Table S-1 the list of calibration masses is shown. The spectrum curation and analyte curation was performed according to the criteria described previously.[31] For the curation of analytes a list of 134 glycan compositions including 19 dummy signals (Supporting Information Table S-2) was extracted from each spectrum. Analytes were only included in a final extraction of the data if the criteria on S/N ratio, isotopic pattern quality and ppm-error were met. The final analyte lists for MALDI-TOF-MS and MALDI-FTICR-MS spectra can be found in Supporting Information Table S-3. After the final extraction of the signals, the extracted peak-areas are normalized for each individual spectrum to the sum of all signal areas.

Results

DBS sample preparation for *N*-glycome profiles

A protocol was developed that included punching of a disk, solubilization of dried blood with subsequent *N*-glycan release from blood glycoproteins, linkage-specific derivatization of the sialic acids to differentiate between α2,3- and α2,6-linkages, HILIC purification of the released *N*-glycans and MALDI-MS measurement.

The DBS samples were punched out and (glyco)proteins were solubilized in a buffer containing PNGase F to release the *N*-glycans. Thus obtained glycans were subsequently derivatized, purified with cotton HILIC solid phase extraction(SPE) tips, spotted onto a MALDI-target plate and measured with MALDI-MS. All glycans were observed as sodiated species $[M+Na]^+$. MALDI-TOF spectra of *N*-glycans released from DBS were similar with previously reported MALDI-TOF *N*-glycome spectra of serum and plasma (spectra not shown).[4,32,34] In Fig. 1, a typical MALDI-FTICR-MS spectrum of the DBS *N*-glycome is shown, which resembled the earlier reported MALDI-FTICR spectra of plasma protein *N*-glycans.[31] Note that all glycan abundances are reported as relative numbers that are derived after normalization based on the summed glycan intensities. This means that if some signals would increase, other signals will decrease.

The positional and linkage assignments of the glycans were based on previously reported identifications[31,32], except for the linkage of the *N*-acetylneuraminic acid moieties that is inherently determined by the mass shift after derivatization. The reaction scheme of this derivatization is shown in Supporting Information Fig. S-1. From the MALDI-TOF-MS and MALDI-FTICR-MS spectra obtained from DBS respectively 82 and 84 glycan signals were extracted for relative quantification (Supporting Information Table S-3). For both MALDI-TOF-MS and MALDI-FTICR-MS measurements the relative standard deviation (RSD) was below 10% for the ten most abundant glycan signals (accounting for >75% of total analyte area).

Comparison between DBS (venous/from finger) with dried plasma spots (DPS)

The first step in our study concerned the evaluation of overlap as well as potential differences between a dried blood spot and a dried plasma spot. The results are summarized in Fig. 2, and it is demonstrated that glycan profiles obtained from the different samples are highly similar. No differences are observed between the glycan profiles from DBS obtained from a finger prick and from venipuncture, or at least differences are not larger than the technical variation of 10%. In the case of DPS, the glycan profiles slightly differed for the signals annotated as H3N4F1, H4N4F1 and H5N4F1, with a higher signal in the DPS samples and lower corresponding major biantennary glycan H5N4E2

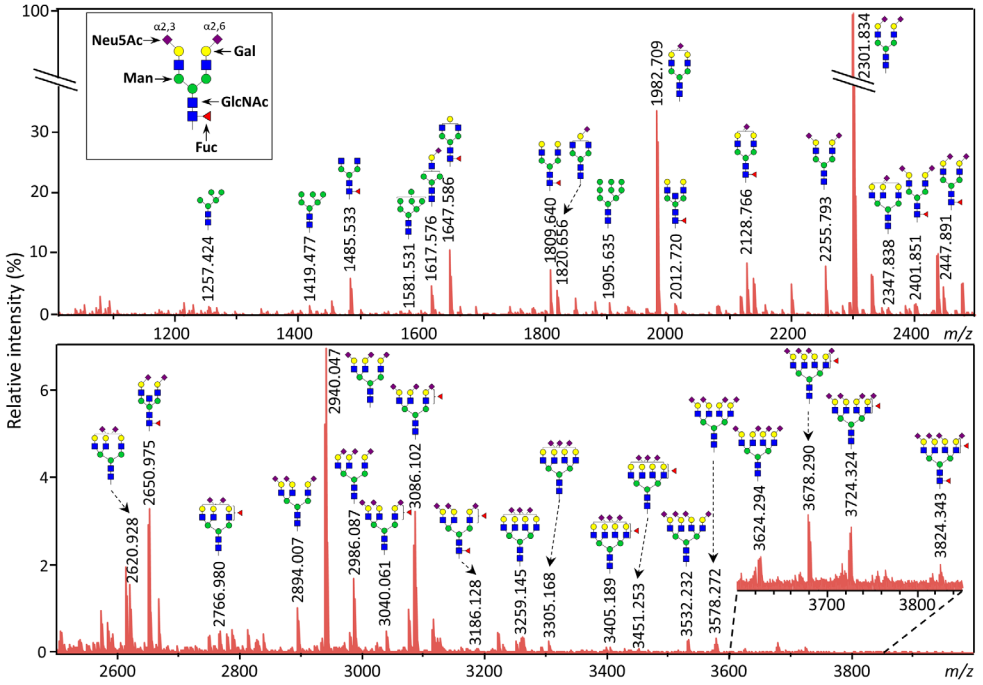


Figure 1. MALDI-FTICR mass spectrum of released *N*-glycome from dried blood spots. Sialic acids are derivatized to allow differentiation between α2,3- and α2,6-linked residues and all glycans are assigned as [M+Na]+. The most intense signals were annotated in the figure, the total list of 84 *N*-glycan compositions which were identified can be found in Supporting Information Table S-3.

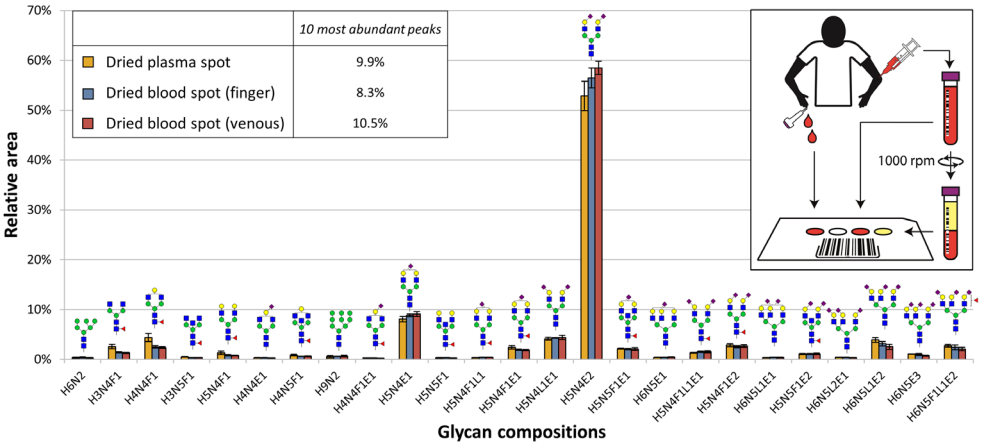


Figure 2. Comparison of MALDI-TOF-MS glycan profiles obtained from plasma, venous blood, and finger-tip blood of a single person. The 25 most abundant glycan compositions are shown. Error bars show standard deviation. H = hexose, N = *N*-acetylhexosamine, F = deoxyhexose (fucose), L = lactonized *N*-acetylneuraminic acid (α2,3-linked), E = ethyl esterified *N*-acetylneuraminic acid (α2,6-linked).

DBS storage conditions

Multiple storage temperatures and time periods were evaluated for the storage of the DBS on the filter paper cards. For the storage at -80 °C and room temperature four time points were measured with a maximum of six weeks storage. The obtained glycan profiles from the spots are constant over time at both storage temperatures, this is shown in Supporting Information Fig. S-2 and S-3. Interestingly, the DBS samples that were stored at 37 °C show similar profiles to the profiles from samples that were directly processed (Fig. 3).

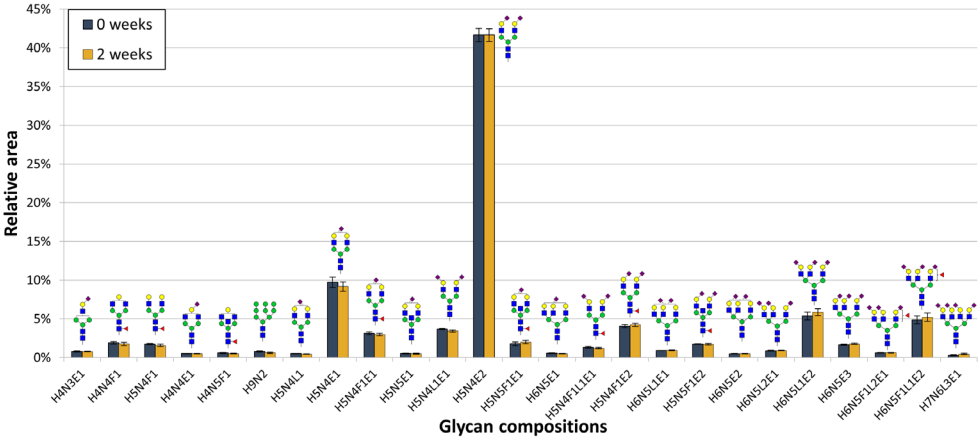


Figure 3. Comparison of MALDI-FTICR-MS glycan profiles from DBS which were stored at 37 °C for two weeks and DBS which, after drying, were directly processed. Error bars show standard deviation. H = hexose, N = *N*-acetylhexosamine, F = deoxyhexose (fucose), L = lactonized *N*-acetylneuraminic acid (α2,3-linked), E = ethyl esterified *N*-acetylneuraminic acid (α2,6-linked).

Discussion

In this study, we present a workflow for *N*-glycan profiling from a DBS with mass measurement precision suitable for clinical cohort analysis. As a starting point our in-house developed plasma glycomics analysis protocol was used.[31] Several changes compared to regular plasma samples were required in order to obtain glycan profiles from a DBS, with the largest adjustment punching out a disk from the DBS. Before punching, the device reads the 2D-barcode of the DBS card ensuring correct track-and-trace of the sample. Such barcode reading prior to punching in combination with fully automated liquid handling of all pipetting step allows screening of large numbers of DBS cards in a standardized way, with throughput numbers up to six MTPs per day (i.e. 576 samples). After the DBS disk is punched and captured in a round-bottom MTP, proteins are solubilized (extracted) from the filter paper using a 2% SDS solution that is identical to the one in the initial plasma protocol. A smaller adjustment involved the total volume of the glycan release reaction that was increased to ensure the disks of the DBS were completely covered with liquid.
MALDI-MS analysis was performed on an FTICR-system for confident glycan identification. The short-term stability experiments were carried out on TOF-MS, which demonstrates that mass measurements are not necessarily limited to an ultrahigh resolution instrument, however it was noted that this slightly decreased the number of identifications. The repertoire of *N*-glycans found in the spectra is highly similar to that obtained from plasma samples using the recently established workflow and exhibits similar repeatability.[31] Furthermore, since quantification of glycans occurs in a relative manner, the analyses are suited for the inherently varying volumes of blood drops on the filter card.

The differences between DPS and DBS glycan profiles were found to be minor (Figure 2). These minor differences comprise three specific immunoglobulin glycans, namely H3N4F1, H4N4F1 and H5N4F1, which is in excellent agreement with a previous report where IgG-specific glycopeptides were lower in abundance in a DBS compared to plasma.[11] Overall it can be concluded that most of the released glycans originate from plasma proteins and that the structural assignment for plasma glycans can be used for the identification of signals in the DBS spectra. Also, the observed signals in spectra from DBS met the criteria for analyte curation with the same list of compositions as the plasma samples.

Glycans are known for their long-term chemical stability. An extreme example hereof is the detection of specific parasite epitopes in ancient mummies.[35] With regard to DBS storage, the stability of glycopeptides was previously evaluated over a period of 180 days and it was found that the summed intensity of 32 *N*-glycopeptides from 14 abundant *N*-glycoproteins did not change.[11] In the current

study, the stability of released *N*-glycans was monitored over a 6-week time-period. Glycosylation profiles did not change after storage at different temperatures (-80 °C, room temperature and 37 °C) over various periods. The confirmation that the storage temperature does not affect the glycan profiles makes it easier to set up a protocol for self-sampling of DBS from patients who have difficulty traveling to a doctor's office or who need regular testing over a longer period.[36,37] Thus obtained DBS sample collections can be shipped to a central facility for *N*-glycome analysis.

In conclusion, this study reports the development and performance of a method for the analysis of the total *N*-glycome from DBS. We have followed up on previous efforts to profile *N*-glycans from DBS and identified and relatively quantified a larger number of glycans. The short-term stability of glycan profiles over time was evaluated for different storage conditions and these results indicated a great potential for facile shipment and storage of DBS cards. The availability of DBS will simplify access to diagnostic tests on the basis of *N*-glycomic analyses since samples can be collected by an individual wherever they are, including at home. We foresee that DBS *N*-glycomic approaches similar to the one outlined here have great potential for clinical biomarker development and implementation.

Acknowledgements

This work was supported by the society "Genootschap ter ondersteuning van de vroege opsporing van kanker" (Lisse, The Netherlands) to further endorse the development of a blood-based test for early detection of cancer (no grant number applicable). The authors thank the anonymous volunteers for their donations and MDs Kiki Vangangelt and Randa Sawires for their assistance in the sample collection.

References

(1) Meany, D. L.; Zhang, Z.; Sokoll, L. J.; Zhang, H.; Chan, D. W. Glycoproteomics for Prostate Cancer Detection: Changes in Serum PSA Glycosylation Patterns. J. Proteome Res. 2009, 8 (2), 613–619. https://doi.org/10.1021/pr8007539.

(2) de Vroome, S. W.; Holst, S.; Girondo, M. R.; van der Burgt, Y. E. M.; Mesker, W. E.; Tollenaar, R. A. E. M.; Wuhrer, M. Serum N-Glycome Alterations in Colorectal Cancer Associate with Survival. Oncotarget 2018, 9 (55), 30610–30623. https://doi.org/10.18632/oncotarget.25753.

(3) Juszczak, A.; Pavić, T.; Vučković, F.; Bennett, A. J.; Shah, N.; Medvidović, E. P.; Groves, C. J.; Šekerija, M.; Chandler, K.; Burrows, C.; et al. Plasma Fucosylated Glycans and C-Reactive Protein As Biomarkers of HNF1A-MODY in Young Adult-Onset Nonautoimmune Diabetes. Diabetes Care 2018, No. 1. https://doi.org/10.2337/dc18-0422.

(4) Reiding, K. R.; Vreeker, G. C. M.; Bondt, A.; Bladergroen, M. R.; Hazes, J. M. W.; van der Burgt, Y. E. M.; Wuhrer, M.; Dolhain, R. J. E. M. Serum Protein N-Glycosylation Changes with Rheumatoid Arthritis Disease Activity during and after Pregnancy. Front. Med. 2018, 4 (241), 1–11. https://doi.org/10.3389/fmed.2017.00241.

(5) Arnold, J. N.; Saldova, R.; Galligan, M. C.; Murphy, T. B.; Mimura-Kimura, Y.; Telford, J. E.; Godwin, A. K.; Rudd, P. M. Novel Glycan Biomarkers for the Detection of Lung Cancer. J. Proteome Res. 2011, 10 (4), 1755–1764. https://doi.org/10.1021/pr101034t.

(6) Kyselova, Z.; Mechref, Y.; Al Bataineh, M. M.; Dobrolecki, L. E.; Hickey, R. J.; Vinson, J.; Sweeney, C. J.; Novotny, M. V. Alterations in the Serum Glycome Due to Metastatic Prostate Cancer. J. Proteome Res. 2007, 6 (5), 1822–1832. https://doi.org/10.1021/pr060664t.

(7) Kirwan, A.; Utratna, M.; O'Dwyer, M. E.; Joshi, L.; Kilcoyne, M. Glycosylation-Based Serum Biomarkers for Cancer Diagnostics and Prognostics. Biomed Res. Int. 2015, 2015, 490531. https://doi.org/10.1155/2015/490531.

(8) Drake, R. R.; Jones, E. E.; Powers, T. W.; Nyalwidhe, J. O. Altered Glycosylation in Prostate Cancer. In Advances in Cancer Research; Elsevier Inc., 2015; Vol. 126, pp 345–382. https://doi.org/10.1016/bs.acr.2014.12.001.

(9) Clerc, F.; Novokmet, M.; Dotz, V.; Reiding, K. R.; de Haan, N.; Kammeijer, G. S. M.; Dalebout, H.; Bladergroen, M. R.; Vukovic, F.; Rapp, E.; et al. Plasma N-Glycan Signatures Are Associated With Features of Inflammatory Bowel Diseases. Gastroenterology 2018, 155 (3), 829–843. https://doi.org/10.1053/j.gastro.2018.05.030.

(10) Reiding, K. R.; Bondt, A.; Hennig, R.; Gardner, R. A.; O'Flaherty, R.; Trbojević-Akmačić, I.; Shubhakar, A.; Hazes, J. M. W.; Reichl, U.; Fernandes, D. L.; et al. High-Throughput Serum N -Glycomics: Method Comparison and Application to Study Rheumatoid Arthritis and Pregnancy-Associated Changes. Mol. Cell. Proteomics 2019, 18 (1), 3–15. https://doi.org/10.1074/mcp.RA117.000454.

(11) Choi, N. Y.; Hwang, H.; Ji, E. S.; Park, G. W.; Lee, J. Y.; Lee, H. K.; Kim, J. Y.; Yoo, J. S. Direct Analysis of Site-Specific N-Glycopeptides of Serological Proteins in Dried Blood Spot Samples. Anal. Bioanal. Chem. 2017, 409 (21), 4971–4981. https://doi.org/10.1007/s00216-017-0438-z.

(12) Freeman, J. D.; Rosman, L. M.; Ratcliff, J. D.; Strickland, P. T.; Graham, D. R.; Silbergeld, E. K. State of the Science in Dried Blood Spots. Clin. Chem. 2018, 64 (4), 656–679. https://doi.org/10.1373/clinchem.2017.275966.

(13) Demirev, P. A. Dried Blood Spots: Analysis and Applications. Anal. Chem. 2013, 85 (2), 779–789. https://doi.org/10.1021/ac303205m.

(14) McDade, T. W.; Williams, S.; Snodgrass, J. J. What a Drop Can Do: Dried Blood Spots as a Minimally Invasive Method for Integrating Biomarkers into Population-Based Research. Demography 2007, 44 (4), 899–925.

(15) Spooner, N.; Lad, R.; Barfield, M. Dried Blood Spots as a Sample Collection Technique for the Determination of Pharmacokinetics in Clinical Studies: Considerations for the Validation of a Quantitative Bioanalytical Method. Anal. Chem. 2009, 81 (4), 1557–1563. https://doi.org/10.1021/ac8022839.

(16) Meesters, R. J.; Hooff, G. P. State-of-the-Art Dried Blood Spot Analysis: An Overview of Recent Advances and Future Trends. Bioanalysis 2013, 5 (17), 2187–2208. https://doi.org/10.4155/bio.13.175.

(17) Müller, M. J.; Stokes, C. S.; Volmer, D. A. Quantification of the 3α and 3β Epimers of 25-Hydroxyvitamin D 3 in Dried Blood Spots by LC-MS/MS Using Artificial Whole Blood Calibration and Chemical Derivatization. Talanta 2017, 165 (October 2016), 398–404. https://doi.org/10.1016/j.talanta.2016.12.081.

(18) Lehmann, S.; Delaby, C.; Vialaret, J.; Ducos, J.; Hirtz, C. Current and Future Use of "Dried Blood Spot" Analyses in Clinical Chemistry. Clin. Chem. Lab. Med. 2013, 51 (10), 1897–1909. https://doi.org/10.1515/cclm-2013-0228.

(19) Ferreira, C. R.; Yannell, K. E.; Jarmusch, A. K.; Pirro, V.; Ouyang, Z.; Graham Cooks, R. Ambient Ionization Mass Spectrometry for Point-of-Care Diagnostics and Other Clinical Measurements. Clin. Chem. 2016, 62 (1), 99–110. https://doi.org/10.1373/clinchem.2014.237164.

(20) Wagner, M.; Tonoli, D.; Varesio, E.; Hopfgartner, G. The Use of Mass Spectrometry to Analyze Dried Blood Spots.

Mass Spectrom. Rev. 2007, 35 (3), 361–438. https://doi.org/10.1002/mas.21441.

(21) Martin, N. J.; Cooper, H. J. Challenges and Opportunities in Mass Spectrometric Analysis of Proteins from Dried Blood Spots. Expert Rev. Proteomics 2014, 11 (6), 685–695. https://doi.org/10.1586/14789450.2014.965158.

(22) van den Broek, I.; Sobhani, K.; Van Eyk, J. E. Advances in Quantifying Apolipoproteins Using LC-MS/MS Technology: Implications for the Clinic. Expert Rev. Proteomics 2017, 14 (10), 869–880. https://doi.org/10.1080/14789450.2017.1374859.

(23) Davison, A. S.; Green, B. N.; Roberts, N. B. Fetal Hemoglobin: Assessment of Glycation and Acetylation Status by Electrospray Ionization Mass Spectrometry. Clin. Chem. Lab. Med. 2008, 46 (9), 1230–1238. https://doi.org/10.1515/CCLM.2008.257.

(24) Daniel, Y. A.; Turner, C.; Haynes, R. M.; Hunt, B. J.; Dalton, R. N. Quantification of Hemoglobin A2by Tandem Mass Spectrometry. Clin. Chem. 2007, 53 (8), 1448–1454. https://doi.org/10.1373/clinchem.2007.088682.

(25) Edwards, R. L.; Creese, A. J.; Baumert, M.; Griffiths, P.; Bunch, J.; Cooper, H. J. Hemoglobin Variant Analysis via Direct Surface Sampling of Dried Blood Spots Coupled with High-Resolution Mass Spectrometry. Anal. Chem. 2011, 83 (6), 2265–2270. https://doi.org/10.1021/ac1030804.

(26) DeWilde, A.; Sadilkova, K.; Sadilek, M.; Vasta, V.; Si, H. H. Tryptic Peptide Analysis of Ceruloplasmin in Dried Blood Spots Using Liquid Chromatography-Tandem Mass Spectrometry: Application to Newborn Screening. Clin. Chem. 2008, 54 (12), 1961–1968. https://doi.org/10.1373/clinchem.2008.111989.

(27) Chambers, A. G.; Percy, A. J.; Yang, J.; Borchers, C. H. Multiple Reaction Monitoring Enables Precise Quantification of 97 Proteins in Dried Blood Spots. Mol. Cell. Proteomics 2015, 14 (11), 3094–3104. https://doi.org/10.1074/mcp.O115.049957.

(28) Glycosciences Committee on Assessing the Importance and Impact of Technology and Glycomics. Transforming Glycoscience: A Roadmap for the Future; National Academies Press: Washington, D.C., 2012. https://doi.org/10.17226/13446.

(29) Ruhaak, L. R.; Miyamoto, S.; Kelly, K.; Lebrilla, C. B. N-Glycan Profiling of Dried Blood Spots. Anal Chem 2012, 84 (1), 396–402. https://doi.org/10.1021/ac202775t.

(30) Han, J.; Higgins, R.; Lim, M. D.; Lin, K.; Yang, J.; Borchers, C. H. Short-Term Stabilities of 21 Amino Acids in Dried Blood Spots. Clin. Chem. 2018, 64 (2), 400–402. https://doi.org/10.1373/clinchem.2017.278457.

(31) Vreeker, G. C. M.; Nicolardi, S.; Bladergroen, M. R.; van der Plas, C. J.; Mesker, W. E.; Tollenaar, R. A. E. M.; van der Burgt, Y. E. M.; Wuhrer, M. Automated Plasma Glycomics with Linkage-Specific Sialic Acid Esterification and Ultrahigh Resolution MS. Anal. Chem. 2018, 90 (20), 11955–11961. https://doi.org/10.1021/acs.analchem.8b02391.

(32) Reiding, K. R.; Blank, D.; Kuijper, D. M.; Deelder, A. M.; Wuhrer, M. High-Throughput Profiling of Protein N-Glycosylation by MALDI-TOF-MS Employing Linkage-Specific Sialic Acid Esterification. Anal. Chem. 2014, 86 (12), 5784–5793. https://doi.org/10.1021/ac500335t.

(33) Selman, M. H. J.; Hemayatkar, M.; Deelder, A. M. A. M.; Wuhrer, M. Cotton HILIC SPE Microtips for Microscale Purification and Enrichment of Glycans and Glycopeptides. Anal. Chem. 2011, 83 (7), 2492–2499. https://doi.org/10.1021/ac1027116.

(34) Bladergroen, M. R.; Reiding, K. R.; Hipgrave Ederveen, A. L.; Vreeker, G. C. M.; Clerc, F.; Holst, S.; Bondt, A.; Wuhrer, M.; van der Burgt, Y. E. M. Automation of High-Throughput Mass Spectrometry-Based Plasma N -Glycome Analysis with Linkage-Specific Sialic Acid Esterification. J. Proteome Res. 2015, 14 (9), 4080–4086. https://doi.org/10.1021/acs.jproteome.5b00538.

(35) Deelder, A. M.; Miller, R. L.; De Jonge, N.; Krijger, F. W. Detection of Schistosome Antigen in Mummies. Lancet 1990, 335 (8691), 724–725. https://doi.org/10.1016/0140-6736(90)90838-V.

(36) Anderson, L. Six Decades Searching for Meaning in the Proteome. J. Proteomics 2014, 107, 24–30. https://doi.org/10.1016/j.jprot.2014.03.005.

(37) Anderson, L. Within Sight of a Rational Pipeline for Development of Protein Diagnostics. Clin. Chem. 2012, 58 (1), 28–30. https://doi.org/10.1373/clinchem.2011.173377.