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Analysis of O-Acetylated Sialic Acids in Dried Blood Spots

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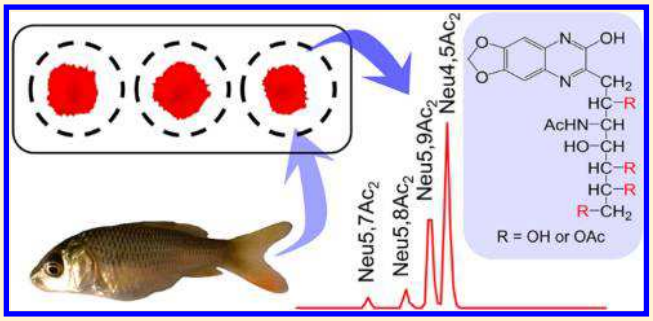
Supporting Information

ABSTRACT: Sialic acid is a family of N- and O-substitutions of neuraminic acid. Plasma or serum sialic acid has been established as a potential disease marker. For example, the presence of 9-*O*-acetyl on the sialic acid of some glycans and glycoconjugates (e.g., 9-*O*-acetyl GD3 ganglioside) could be related to cancer occurrence. A variety of assays are available to measure serum or plasma sialic acid; however, sample preparation and storage can alter the O-acetylation profile due to the loss of *O*-acetyl groups and/or the migration of *O*-acetyl groups. Herein, we report dried blood spot (DBS) sampling, in combination with diamino-4,5-methylenedioxybenzene derivatization, for profiling sialic acids in blood samples with minimal alteration in O-acetylation patterns. The feasibility of the method was first evaluated by analyzing sialic acids in crucian carp blood and comparing with traditional blood/plasma sample preparation procedures. A total of 19 different sialic acids were identified by using liquid chromatography−Orbitrap mass spectrometry, including four mono-O-acetylated *N*-acetylneuraminic acids, four mono-O-acetylated *N*-glycolylneuraminic acids, six di-O-acetylated *N*-acetylneuraminic acids, and three tri-O-acetylated *N*-acetylneuraminic acids. The long-term storage study indicated that DBS sampling could effectively preserve the O-acetylation information for at least 6 weeks. Thus, it is demonstrated that this method is a valuable tool for the study of sialic acid diversity, especially for the characterization of isomeric structures.



The sialic acid family shares the common feature of a nine-carbon backbone and a carboxylic acid residue. They play a variety of physiologically important roles, including intercellular and intermolecular interactions, neuronal outgrowth and memory formation, function of extracellular matrix, binding of viruses and microorganisms, and cancer development.[1−9] Modifications at the C5 position determine the primary sialic acid forms, including *N*-acetylneuraminic acid (NeuAc), *N*-glycolylneuraminic acid (NeuGc), and 2-keto-3-deoxynononic acid (KDN).[5] Further modification with the addition of *O*-acetyl, *O*-sulfo, *O*-lactyl, and/or *O*-methyl groups is found at the hydroxyl groups at the C4, C7, C8, and/or C9 position.[10] Among those modifications, O-acetylation is one of the most common ones for NeuAc and NeuGc.[11] O-Acetylated sialic acids have important functions, such as virus binding and infection, embryonic development, cell apoptosis, immunological processes, cancer development, and stress reaction.[5,12−16]

The O-acetylation of sialic acid has been found in a variety of species, including bacteria, vertebrates, and humans. Because of the differences in number and position of substitutions, more than 20 naturally occurring O-acetylated NeuAcs and NeuGcs have been reported, mainly mono- and di-O-acetylated structures.[17] The O-acetylation has been established as a potential disease marker. For example, the presence of 9-*O*-acetyl group on the sialic acid of some glycans and glycoconjugates could be related to cancer occurrence.[18−21] The profiling of O-acetylation can be performed at the monosaccharide, oligosaccharide, glycopeptide, and glycoconjugate level as well as potential cell level.[10,14,22−26] 1,2-Diamino-4,5-methylenedioxybenzene (DMB) is typically used for labeling released sialic acids, and the derivatives can be analyzed by using reversed-phase chromatography with fluorescence or mass spectrometric detection.[27−29]

The *O*-acetyl groups in sialic acids are labile under both acidic and basic conditions, which may alter the O-acetylation profile due to the loss of *O*-acetyl groups and/or the migration of *O*-acetyl groups. For instance, the *O*-acetyl group at the C7 position can migrate to the C9 hydroxyl group under physiological conditions.[15,30] The loss of *O*-acetyl groups can result from sample storage and purification processes of biological fluids, such as blood and urine. Therefore, it is extremely important to develop a reliable sampling method for

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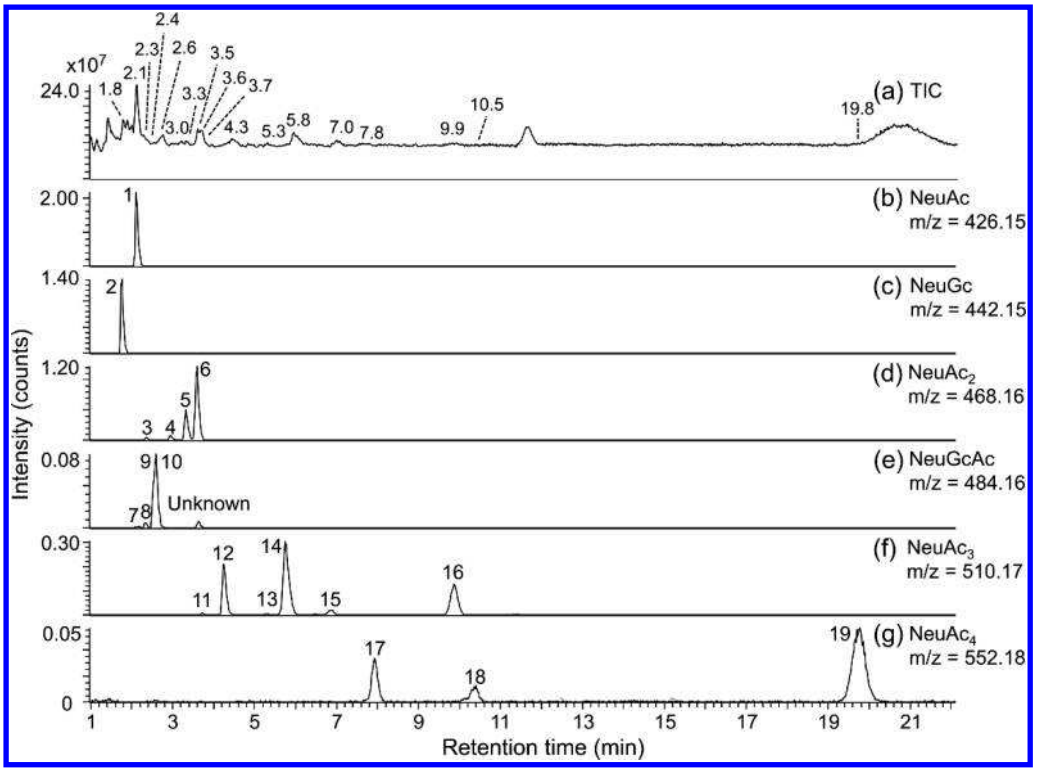

Figure 1. LC−MS analysis of DMB-derivatized sialic acids from crucian carp blood: (a) total ion chromatogram (m/z 200−700), (b) XIC at m/z 426.15 (NeuAc), (c) XIC at m/z 442.15 (NeuGc), (d) XIC at m/z 468.16 ($NeuAc_2$), (e) XIC at m/z 484.16 (NeuGcAc), (f) XIC at m/z 510.17 ($NeuAc_3$), and (g) XIC at m/z 552.18 ($NeuAc_4$).

the analysis of O-acetylated sialic acids. Dried blood spot sampling (DBS) offers several advantages over conventional blood/plasma sampling, including stability at room temperature, requirement of moderate shipment conditions, and a negligible risk of infection with blood-borne viruses.[31−33] In this study, we attempt to develop a DBS-based assay for profiling sialic acids in complex biological samples. Sialic acids were released from DBS and derivatized with DMB, followed by liquid chromatography−Orbitrap mass spectrometry (LC−Orbitrap-MS) analysis. We compared DBS sampling with traditional blood/plasma, including long-term storage stability. The results demonstrated that DBS sampling could effectively preserve the O-acetylation information for at least 6 weeks. Thus, it provides an efficient way for the long-term storage of biological fluid samples that are applicable to clinical diagnosis and disease progression monitoring.

EXPERIMENTAL SECTION

Materials. DMB, acetic acid, 2-mercaptoethanol, sodium hydrosulfite, C18 cartridges, and filter paper were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Methanol (MeOH), acetonitrile (ACN), and trifluoroacetic acid (TFA) were purchased from Thermo Fisher Scientific (Pittsburgh, PA, U.S.A.). Sialic acid reference was purchased from Prozyme (California, U.S.A.). Water was prepared using an Ultrapure Water Systems (Lane End, ELGA LabWater, U.K.). Crucian carp fries (3−4 cm in length) were purchased from Hongxin Aquaculture Farm (Yujiang, China). The sialidase from *Clostridium perfringens* and horse serum were purchased from Sangon Biotech (Shanghai, China). Zebrafish were purchased from China Zebrafish Resource Center (Wuhan, China). Human blood samples were provided by volunteers.

Sample Preparation. Samples were obtained from crucian carp and zebrafish. Typically, 5 μL of blood was blotted on filter paper and subsequently stored at 4 °C. The rest of the samples were placed at −20 °C in the form of blood/plasma. Since the O-acetylated sialic acids show some resistance to sialidase-catalyzed hydrolysis,[34,35] acid hydrolysis was used in this study according to the method described previously.[36] Briefly, 5 μL of blood/plasma was mixed with 195 μL of acetic acid solution with a final concentration of 2 M and incubated at 80 °C for 3 h. For DBS samples, filter paper was immersed into 100 μL of H_2O for 10 min, and then mixed with 100 μL of 4 M acetic acid, followed by incubation at 80 °C for 3 h. After acid hydrolysis, all samples were centrifuged at 12 000g at 4 °C for 40 min and the supernatant was collected and dried immediately under vacuum.

DMB Labeling. The derivatization solution was prepared by dissolving DMB with a final concentration of 7 mM in 1.4 M acetic acid containing 0.75 M 2-mercaptoethanol and 18 mM sodium hydrosulfite.[37] For derivatization, the dried sample was dissolved with 100 μL of DMB solution and incubated at 50 °C for 2.5 h. The solution was then diluted using 1 mL of H_2O and desalted with a C18 cartridge. The C18 cartridge was preconditioned with 2 mL of 80% ACN containing 0.1% TFA (v/v) and 2 mL of H_2O. The loaded sample was washed with 1 mL of H_2O and eluted with 1 mL of 30% ACN (v/v).

LC−Orbitrap-MS Analysis. DMB-derivatized sialic acids were analyzed using an LC−Orbitrap-MS system consisting of an UltiMate 3000 UHPLC coupled to a Q Exactive hybrid quadrupole−Orbitrap mass spectrometer (Thermo Fisher Scientific, U.S.A.). The LC system was equipped with a C18 column (1.7 μm, 2.1 mm × 100 mm, Waters, U.S.A.), which was maintained at 35 °C during analysis. The flow rate was set

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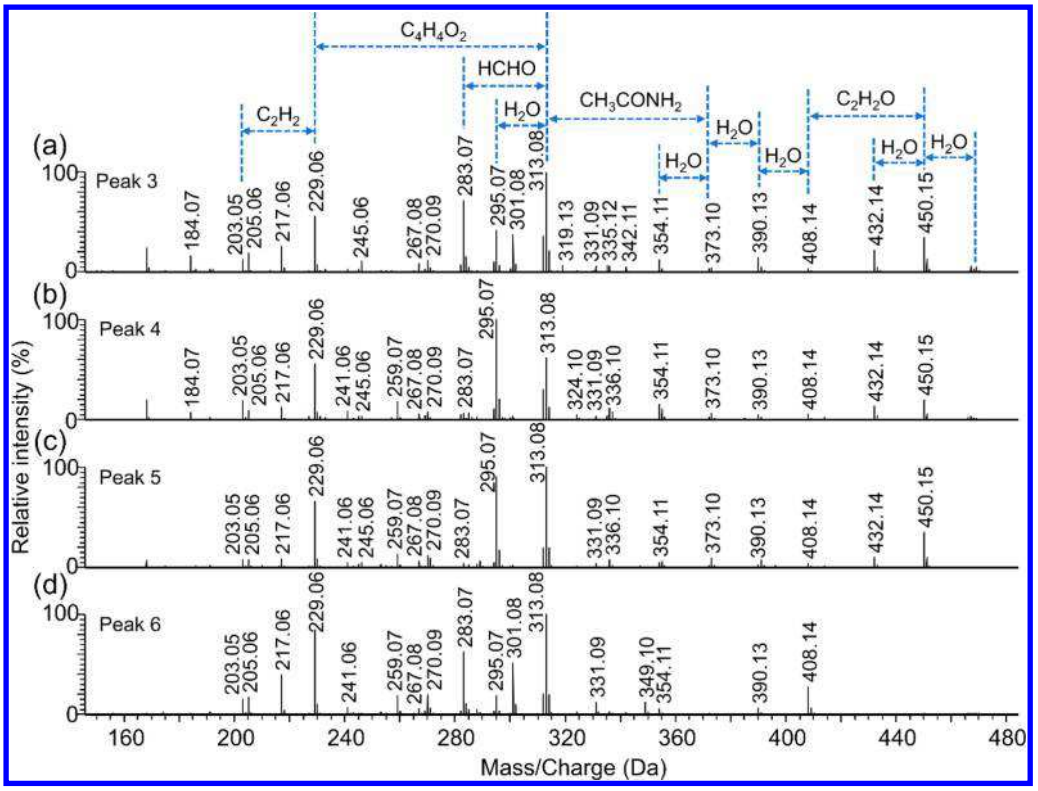

Figure 2. LC−MS/MS analysis of mono-O-acetylated sialic acids: (a) MS/MS spectrum extracted at 2.4 min (peak 3), (b) MS/MS spectrum extracted at 3.0 min (peak 4), (c) MS/MS spectrum extracted at 3.3 min (peak 5), and (d) MS/MS spectrum extracted at 3.6 min (peak 6).

to 0.3 mL/min, and elution was performed in isocratic mode using MeOH/ACN/H_2O (7:9:84, v/v). Mass spectrometry analysis was carried out using positive-ion mode with the following parameters: spray voltage, 3800 V; capillary temperature, 320 °C; sheath gas, 39 unit; auxiliary gas, 15 unit; auxiliary gas heater temperature, 300 °C; collision energy, 25 eV. Data were acquired from m/z 200 to 700 with dd-MS/MS mode at a resolution of 70 000 and subsequently analyzed by Xcalibur software (version 4.0, Thermo Fisher Scientific, U.S.A.) and Skyline software (version 4.1, MacCoss Lab Software, U.S.A.).

All experiments were performed in accordance with the protocols approved by the Ethical Committee of Jianghan University.

RESULTS AND DISCUSSION

LC−MS Analysis of Reference Sialic Acid Mixture. We first validated the DMB derivatization and LC−MS system using a reference standard containing NeuGc, NeuAc, Neu5,7Ac_2, Neu5Gc9Ac, Neu5,9Ac_2, and Neu5,7(8),9Ac_3. As illustrated in Figure S-1 in the Supporting Information, the DMB-labeled sialic acids were well-separated with an eluent containing MeOH/ACN/H_2O (7:9:84, v/v), which makes it possible to identify and quantify sialic acids with various modifications. The number of *O*-acetyl groups and type of the substituent (e.g., NAc or NGc) of each species can be assigned with the detected molecular masses and their corresponding retention times.

Analysis of Sialic Acids in Crucian Carp Blood. The LC−MS analysis of DMB-labeled sialic acids from crucian carp blood is shown in Figure 1. The total ion chromatogram (TIC) is presented in Figure 1a, indicating the presence of more diverse structures compared to the reference standard (Figure S-1). It is well-known that the heterogeneity of sialic acids is due to the combination of the number of *O*-acetyl groups and their positions since O-acetylation can occur at the C4, C7, C8, and/or C9 position of sialic acids. As expected, a single peak was observed in the extracted ion chromatograms (XICs) at m/z 426.15 and m/z 442.15, corresponding to NeuAc and NeuGc, respectively (Figure 1, parts b and c). Comparatively, multiple peaks were detected for O-acetylated species (Figure 1d−g).

Ideally, the location of the different substituents on the sialic acids could be determined by nuclear magnetic resonance (NMR) spectroscopy. Unfortunately, this approach is limited by the available quantity and purity of materials.[10] On the other hand, it has been proved that the identification of sialic acid structures can be achieved by a combination of MS/MS fragment patterns and their corresponding relative retention times (RRTs). As expected, the MS/MS spectra for both NeuAc and NeuGc show the presence of characteristic fragment ions at m/z 313.08, m/z 295.07, and m/z 229.06 (data not shown). Using the retention time of NeuAc as a reference, the calculated RRTs of sialic acid species were used to characterize new family members.[10,38] Figure 1d shows that four isomeric structures were detected when mono-O-acetylation occurs. The extracted MS/MS spectra for four mono-O-acetylated NeuAc (NeuAc_2) species are presented in Figure 2a−d, corresponding to peaks 3−6, respectively. The predominant fragment ions for the four isomers were detected at m/z 313.08, m/z 295.07, and m/z 229.06.[10,39] The comparison of obtained RRTs with those of the reference standard allows us to identify peaks 3 and 5 as Neu5,7Ac_2 and Neu5,9Ac_2, respectively. Because Neu5,8Ac_2 and Neu4,5Ac_2 are not included in the commercial reference, MS/MS spectra and retention orders were used in their identification. For

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example, it was previously reported that 4-O-acetylated sialic acid could not yield $[M + H - 18]^+$ fragment ions, which was significantly different from the modification in the C7, C8, or C9 position of sialic acids.[10] Hence, peaks 4 and 6 were assigned to Neu5,8Ac_2 and Neu4,5Ac_2, respectively. It is worth noting that the calculated RRTs for $NeuAc_2$ species are surprisingly close to that reported in literature (Table S-1 in the Supporting Information).[10] However, the fragment ions at m/z 283.07 were much more abundant in the MS/MS spectra of Neu5,7Ac_2 and Neu4,5Ac_2 than those of Neu5,8Ac_2 and Neu5,9Ac_2. These results confirm that the fragment ion at m/z 283.07 can be a diagnostic ion for the structure that is substituted in C8 or C9 as previously suggested.[10] Meanwhile, the fragment ion at m/z 259.07 is a potential characteristic ion for free C7 since it was detected only if there was no *O*-acetyl group in the C7 position (Figure 2). The XIC indicated that the relative amounts of Neu5,9Ac_2 and Neu4,5Ac_2 are much higher than those of Neu5,7Ac_2 and Neu5,8Ac_2 among the four mono-O-acetylated isomers. This observation may also suggest that the *O*-acetyl group in position C7 and C8 can migrate to C9, and no migration for the C4-acetyl group.

Mono-O-acetylated NeuGc (NeuGcAc) species are also present the four peaks as illustrated in the XICs for m/z 484.16 (Figure 1e). On the basis of the calculated RRTs, it can be derived that peaks 7 and 8 are associated with O-acetylation in positions C7 and C8, respectively (Table S-1). The extracted MS/MS spectrum of peak 7 shows a high abundance of fragment ions at m/z 283.07, indicating no substitution in the C8 or C9 position (Figure S-2a). Furthermore, the fragment ion at m/z 259.07 is absent in the MS/MS spectrum of Neu5Gc7Ac, confirming the diagnostic ion for the substitution in the C7 position. Because their retention times are very close, it is difficult to use RRTs for distinguishing Neu4Ac5Gc and Neu5Gc9Ac.[10] We then compared the retention time and MS/MS fragment pattern between NeuGcAc species from horse serum (i.e., Neu4Ac5Gc) and reference standard (i.e., Neu5Gc9Ac) (Figure S-3). It was found that the LC system is not able to completely separate the two isomers (Figure S-3c). Nevertheless, the extracted MS/MS spectrum at 2.5 min (peak 9) was found to be similar to that of Neu5Gc9Ac (Figures S-2c and S-3e), and the MS/MS spectrum at 2.7 min (peak 10) was similar to that of Neu4Ac5Gc (Figures S-2d and S-3f). These results indicated that the fragmentation pattern and retention order for Neu5Gc7Ac, Neu5Gc8Ac, Neu5Gc9Ac, and Neu4Ac5Gc are comparable to those of their corresponding mono-O-acetylated NeuAc species. Moreover, the RRTs of NeuGcAc species are consistent with the previous report (Table S-1).[10] It is worth noting that we could not derive the structure of the species corresponding to the peak at 3.7 min, which also contains the characteristic fragments of DMB-labeled sialic acids (Figure S-2e).

For di-O-acetylated NeuAc ($NeuAc_3$) species, the XICs revealed the presence of six isomeric structures, i.e., peaks 11−16 (Figure S-4). The six hypothetical isomers consist of three C4-substituted isomers, Neu4,5,7Ac_3, Neu4,5,8Ac_3, and Neu4,5,9Ac_3, and three non-C4-substituted isomers, Neu5,7,8Ac_3, Neu5,7,9Ac_3, and Neu5,8,9Ac_3. Although these isomers were well-separated and the RRTs could be obtained, their accurate identification is still challenging due to the complex fragmentation mechanism and a lack of reference. So far the only report on the RRTs of di-O-acetylated sialic acids proclaimed that the RRT of Neu5,7,9Ac_3 is larger than that of Neu5,8,9Ac_3, but with specification that the assignment might be interchanged.[10] On the other hand, the retention times of four mono-O-acetylated NeuAcs and NeuGcs revealed that the correlation between hydrophobicity and modification position can be ordered as C4 > C9 > C8 > C7. This rank is valid for tri-O-acetylated species, confirmed by the retention order Neu4,5,8,9Ac_4 > Neu4,5,7,8(9)Ac_4 > Neu5,7,8,9Ac_4 (Figure S-5). It is reasonable to assign di-O-acetylated sialic acids with the assumption that the hydrophobicity of a modified sialic acid is associated with the location of *O*-acetyl group and the relative quantity of each species. Hence, we proposed that the retention order for the six isomers is Neu5,7,8Ac_3, Neu5,7,9Ac_3, Neu4,5,7Ac_3, Neu5,8,9Ac_3, Neu4,5,8Ac_3, and Neu4,5,9Ac_3. The tentative identification of each species was then examined with the corresponding MS/MS spectrum. It is worth mentioning that the formation of dehydration fragment ions does not seem applicable to the free C4 position for di-O-acetylated sialic acids. Consequently, the $[M + H - 18]^+$ fragment ion is not a reliable indicator for position assignment of the *O*-acetyl group in a di-O-acetylated structure. On the other hand, the fragment ion at m/z 259.07 can be detected only if there is no substitution in the C7 position. In addition, it was observed that the fragment ions at m/z 283.07 and 301.08 show very low abundances when the position C8 or C9 is substituted (Figure 2 and Figure S-2), agreeing with the MS/MS spectra of peaks 11, 14, 15, and 16. Accordingly, the sialic acids corresponding to peaks 11, 12, and 13 are assigned to Neu5,7,8Ac_3, Neu5,7,9Ac_3, and Neu4,5,7Ac_3, respectively. Similarly, peaks 14, 15, and 16 are assigned to Neu5,8,9Ac_3, Neu4,5,8Ac_3, and Neu4,5,9Ac_3, respectively. Furthermore, the assignment is in agreement with the relative amount of each isomer, with Neu5,7,9Ac_3, Neu5,8,9Ac_3, and Neu4,5,9Ac_3 as the three abundant structures.

The extracted MS/MS spectra for three tri-O-acetylated NeuAc ($NeuAc_4$) species are shown in Figure S-5. In the MS/MS spectra of peaks 17 and 18, the absence of a fragment ion at m/z 259.07 indicates that the position C7 is substituted. Furthermore, it was observed that the structures with free C4 produce detectable fragment ion at m/z 245.06 (Figure 2), thus assigning peak 17 to Neu5,7,8,9Ac_4 and peak 18 to Neu4,5,7,8(9)Ac_4. In the MS/MS spectrum of peak 19, the presence of the fragment ion at m/z 259.07 shows that the position C7 is not substituted, and consequently, peak 19 can be assigned to Neu4,5,8,9Ac_4.

Overall, a total of 19 structures were identified from the analysis of DMB−sialic acids from crucian carp blood, including four isomeric mono-O-acetylated NeuAcs, four isomeric mono-O-acetylated NeuGcs, six isomeric di-O-acetylated NeuAcs, and three isomeric tri-O-acetylated NeuAcs. Surprisingly, an unknown structure with similar mass to that of the NeuGcAc species was also observed (Figure S-2e). To the best of our knowledge, this is the highest number of O-acetylated sialic acids ever detected in a single species. It is worth noting that tri-O-acetylated structures are very rare in nature. Because of the high level of O-acetylation and a rich variety of isomers, it is expected that the collection of sialic acids from crucian carp is an ideal model system for developing assays for profiling O-acetylation of sialic acids.

It has been reported that different sialidases have different substrate specificities, and the enzyme from *C. perfringens* shows similar activities toward Neu5,9Ac_2 from both α2,3- and α2,6-linked to Gal.[40] Thus, in addition to acid hydrolysis, the sialidase from *C. perfringens* was also used to compare the releasing efficiency of different *O*-acetyl sialic acid forms

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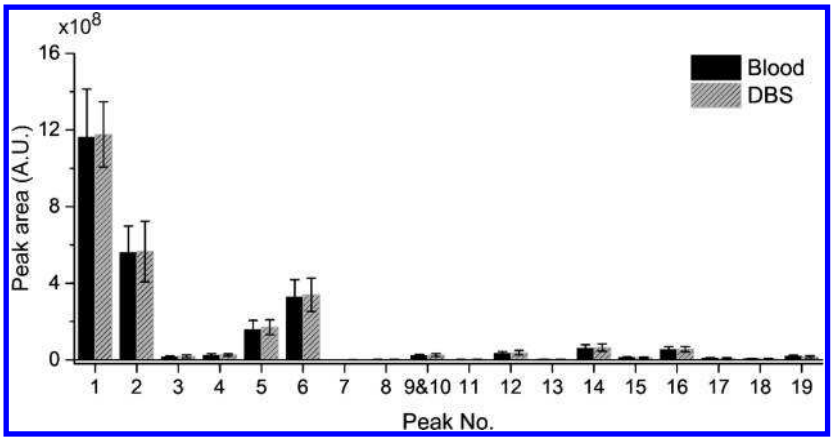

Figure 3. Comparison of the abundance of each species in fresh blood and DBS (n = 5).

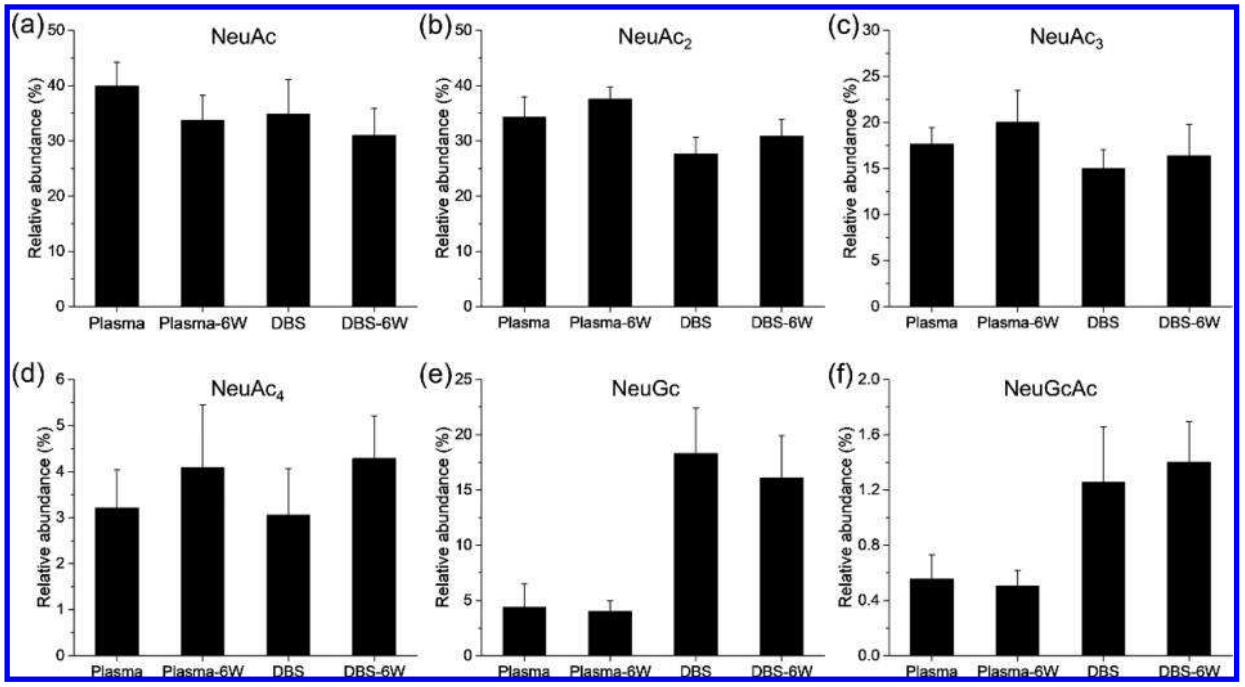

Figure 4. Comparisons of the relative abundances of O-acetylated sialic acids from fresh plasma, plasma with a six-week storage at −20 °C, fresh DBS, and DBS after a six-week storage at 4 °C (n = 10). Normalized abundances for (a) NeuAc, (b) $NeuAc_2$, (c) $NeuAc_3$, (d) $NeuAc_4$, (e) NeuGc, and (f) NeuGcAc. Plasma denotes that fresh plasma samples were analyzed. Plasma-6W means that the aliquots of plasma samples were analyzed after a six-week storage at −20 °C. DBS indicates that fresh prepared DBS samples were analyzed. DBS-6W refers to the DBS samples analyzed after a six-week storage at 4 °C.

(Figure S-6). For NeuAc, NeuGc, $Neu5,8Ac_2$, and $Neu5,9Ac_2$, the differences of peak areas between acid hydrolysis and enzymatic hydrolysis are less than 30%. For $Neu4,5Ac_2$, $NeuAc_3$, and $NeuAc_4$ species, the peak intensities from enzymatic hydrolysis are between 3% and 20% of that from acid hydrolysis. In particular, the sialidase shows very low activity toward $Neu4,5Ac_2$ compared to other $NeuAc_2$ species, which is in line with previous reports.[41]

Comparison of DBS Sampling and Fresh Blood Sample. To evaluate the applicability of DBS sampling to profiling O-acetylation of total sialic acids, we compared the obtained DMB−sialic acids from the freshly prepared blood and DBS. The abundances of these structures are shown in Figure 3, in which the peaks were numbered according to that in Figure 1. The area of each peak from five fresh blood samples as well as five DBS samples was calculated. The ratios of peak area of the same structure from two samples were further calculated, and the average of five ratios are summarized in Table S-2, ranging from 0.8 to 1.1 with relative standard deviation less than 40%. The result confirms that the difference between fresh blood sampling and DBS sampling is insignificant. The results demonstrate that DBS sampling is compatible with LC−MS analysis of O-acetylated sialic acids.

It has been reported that DBS containing unstable compounds should be protected against humidity and stored at a low temperature in order to enhance stability.[42] Some labile compounds (e.g., amino acids, cocaine, diazepam, and acylcarnitines) in DBS can degrade rapidly under ambient conditions.[43−45] Thus, DBS from crucian carp was used to evaluate short-term stability at room temperature. The results indicated that sialic acid degrades at room temperature, revealed by the significant loss of peak intensity, ranging from 20% to 55% for one-day storage and 20% to 75% for two-day storage (Figure S-7). Therefore, the DBS should not be stored

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at room temperature. Recently, a new approach called dried blood spheroids was developed to increase stability for labile compounds under ambient conditions,[45] which may be also applicable for O-acetylated sialic acids.

Effect of Long-Term Storage of Plasma and DBS Sampling on O-Acetylated Sialic Acid. DBS sampling was further evaluated for long-term stability, i.e., determining changes of O-acetylation patterns after storing samples for a period of 6 weeks. Plasma was chosen for comparison with DBS since it is a commonly used form for sample storage. Figure 4 shows the relative abundances of NeuAc, $NeuAc_2$, $NeuAc_3$, $NeuAc_4$, NeuGc, and NeuGcAc from freshly prepared plasma, DBS, and the samples stored for 6 weeks. The relative abundances of NeuAc in fresh plasma and plasma with a six-week storage are 39.9 ± 4.3% and 33.8 ± 4.4%, respectively (n = 10). For DBS, those of the fresh sample and after a six-week storage are 34.8 ± 6.3% and 31.0 ± 4.9% (n = 10), respectively. The data indicate that non-O-acetylated NeuAc and NeuGc are stable in both sampling methods. For $NeuAc_2$ species, their relative abundances are 34.3 ± 3.7% for fresh plasma sample and 37.6 ± 2.1% for the six-week sample, whereas they are 27.6 ± 3.0% for fresh DBS and 30.8 ± 3.1% for the six-week DBS. These results indicate that the relative level of O-acetylation had no significant changes for at least 6 weeks, either when stored as plasma (at −20 °C) or DBS (at 4 °C).

We then investigated the stability of each isomeric O-acetylation sialic acid using Skyline software. It was revealed that the relative abundances of sialic acids corresponding to peaks 6, 16, and 19 had dramatic changes in plasma after long-term storage but no significant changes for DBS (Figure S-8). As illustrated in Figures S-9−S-11, the relative abundance of peak 6 ($Neu4,5Ac_2$), peak 16 ($Neu4,5,9Ac_3$), and peak 19 ($Neu4,5,8,9Ac_4$) decreased by 32.6%, 69.7%, and 59.7% after plasma samples were stored for 6 weeks, respectively. In contrast, the changes of sialic acids from DBS sampling between fresh and six-week storage are less than 25%. These results demonstrate that some isomeric structures significantly degraded in stored plasma, while they were stable for 6 weeks in stored DBS at 4 °C. Since the variation is relatively insignificant compared to that of the total amount of sialic acids, it can be neglected in the analysis of overall level of sialylation.

NeuGc from Blood Cells and Plasma. The total relative abundance of NeuAcs (including all NeuAc species) in fresh plasma is about 15% higher than that in fresh DBS with 95.1 ± 2.2% and 80.4 ± 3.9%, respectively (Figure 4). On the contrary, the relative abundances of NeuGc and NeuGcAc species in plasma samples are 2−4 times lower than those in DBS samples. For example, the relative abundance of NeuGc in DBS samples is 18.3 ± 4.1% but only 4.4 ± 2.1% in plasma samples (Figure 4), whereas the two structures have a similar level in the whole blood and DBS (Figure 3). Since the difference between plasma and DBS is blood cells, we analyzed the sialic acids in blood cells and plasma from the same blood sample. After normalizing total peak areas of the whole-blood sample, the relative abundances of six species were calculated and are shown in Figure 5. The NeuGc in whole blood has an average abundance of 24.3%, 19.2 ± 1.8% in blood cells, and 5.1 ± 0.8% in plasma (n = 5). On the other hand, the relative abundances of NeuAc species have similar distribution between blood cells and plasma. The observation revealed that the ratio of NeuGc to NeuAc varied among tissues of a single species.[46] The specific expression may be associated with the fact that NeuAc and NeuGc have different functions in vivo. For instance, NeuGc has been found to be the predominant sialic acid in porcine peripheral blood lymphocytes, and NeuGc knockout can reduce erythrocyte sequestration and thromboxane elaboration in a pig-to-human xenoperfusion model.[47,48] Nevertheless, the observation confirms that sample preparation is crucial for accurate analysis of sialic acids in a complex biological system.

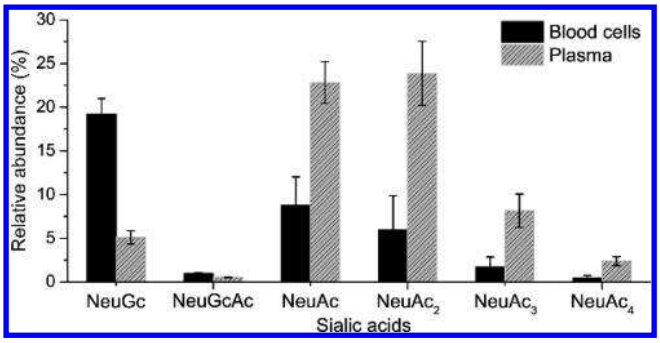

Figure 5. Comparison of the relative abundance of each sialic acid species in isolated blood cells and plasma that were prepared from the same blood samples. The total peak areas of all species were used for normalization.

O-Acetylated Sialic Acids from Zebrafish and Human Blood. Zebrafish (*Danio rerio*) is an important and widely used vertebrate model organism for human health researches. However, the difference of O-acetylation patterns of sialic acids in zebrafish and human has not been well-characterized. Thus, the DBS sampling method was applied to profiling O-acetylation of sialic acid in zebrafish and human blood. As shown in Figure 6, NeuAc is the major sialic acid species in both zebrafish and human blood, with a relative abundance of 93.6 ± 3.0% and 97.3 ± 0.3%, respectively (n = 5). NeuGc is hardly observed in human blood (Figure 6c), and only a small amount is found in zebrafish blood (Figure 6b). $Neu5,9Ac_2$ is the predominant mono-O-acetylated structure in both zebrafish and human blood, with a relative abundance of 78.6 ± 7.9% and 84.8 ± 3.0%, respectively. No multi-O-acetylated structures were detected in zebrafish and human. Overall, zebrafish has a similar pattern of O-acetylation to humans, regardless of the difference in sialylation level.

CONCLUSIONS

We proposed DBS sampling in combination with DMB derivatization and LC−MS/MS for the analysis of O-acetylated sialic acids. The applicability was investigated with fresh samples and samples after a six-week storage by comparing the O-acetylation profiles. The results demonstrated that this method is a valuable tool for the study of sialic acid diversity, especially for the characterization of isomeric structures. Overall, 19 different sialic acids were detected in crucian carp blood, including four mono-O-acetylated *N*-acetylneuraminic acids, four mono-O-acetylated *N*-glycolylneuraminic acids, six di-O-acetylated *N*-acetylneuraminic acids, and three tri-O-acetylated *N*-acetylneuraminic acids. That may also denote that the sialic acids from crucian carp blood are a valuable reference standard for the discovery of new sialic acid members. Interestingly, the relative abundances of NeuGc species are much higher in blood cells than plasma, indicating

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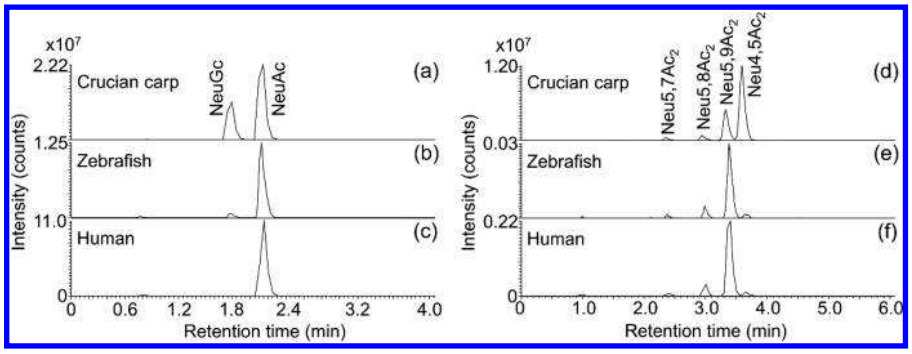

Figure 6. DBS sampling for analysis of sialic acids in crucian carp, zebrafish, and human blood. XICs of NeuAc and NeuGc for the DMB derivatives from (a) crucian carp, (b) zebrafish, and (c) human. XICs of mono-O-acetylated NeuAc for the DMB derivatives from (d) crucian carp, (e) zebrafish, and (f) human.

that the difference between plasma and blood should be considered when studying sialic acids in blood.

The long-term sample stability study showed that DBS is still reliable for profiling O-acetylation after a storage up to 6 weeks. The method was further validated with the analysis of sialic acids in zebrafish and human blood, and the data showed no detectable multi-O-acetylated structures. The results demonstrated that DBS sampling is applicable to the analysis of O-acetylated sialic acids and has a great potential in clinical applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b04420.

Structures and RRTs of 19 sialic acids from crucian carp, MS/MS spectra for NeuGcAc, $NeuAc_3$, and $NeuAc_4$ species, comparisons of acid hydrolysis and enzymatic hydrolysis for the efficiency and retaining of *O*-acetyl sialic acid forms, and O-acetylation profile in fresh plasma and DBS after short-term and long-term storage (PDF)

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Notes

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