Supporting Information

Analysis of *O*-acetylated sialic acids by 3-nitrophenylhydrazine derivatization combined with LC-MS/MS

Chenhao Zhao,[ab] Xingdan Wang,[a] Jing Wu,[a] Yeli Hu,[b] Qiwei Zhang *[a] and Qi Zheng [a]

[a] Key Laboratory of Optoelectronic Chemical Materials and Devices, Ministry of Education, School of Optoelectronic Materials & Technology, Jianghan University, Wuhan 430056, Hubei, People's Republic of China

[b] School of Environmental Ecology and Biological Engineering, Wuhan Institute of Technology, Wuhan 430205, Hubei, People's Republic of China

*Corresponding author: Dr. Qiwei Zhang, School of Optoelectronic Materials and Technology, Jianghan University, Wuhan, 430056, China, E-mail: keewezhang@jhun.edu.cn

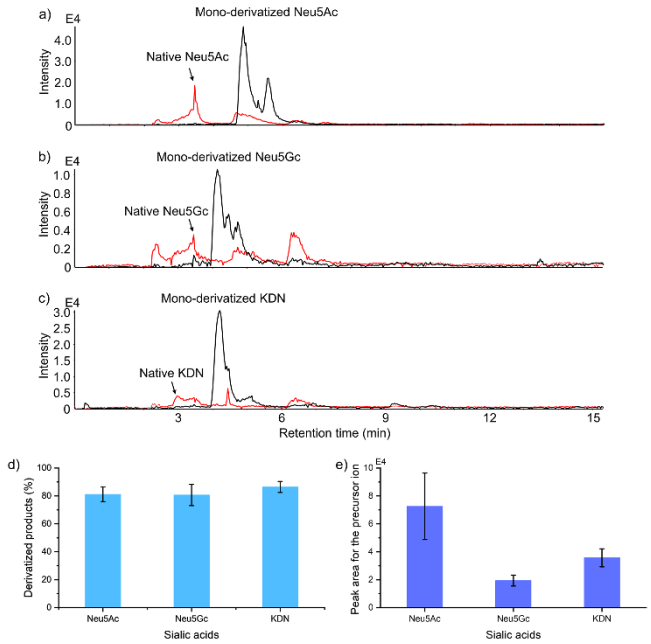


Fig. S1 The extracted ion chromatograms (XICs) display the mono-derivatized and native forms of Neu5Ac (a), Neu5Gc (b), and KDN (c) after the first-step derivatization. To detect native sialic acids, the undesalted samples were analyzed directly by LC-MS. Black line: mono-derivatized products; red line: native sialic acids. (d) The efficiency of the first-step derivatization was calculated based on the peak area of the precursor ion (n = 3). Each efficiency = (mono-derivatized product)/(sum of mono-derivatized product and native sialic acid)×100%. (e) The repeatability of the first-step derivatization was assessed by measuring the peak area of the precursor ion (n = 3).

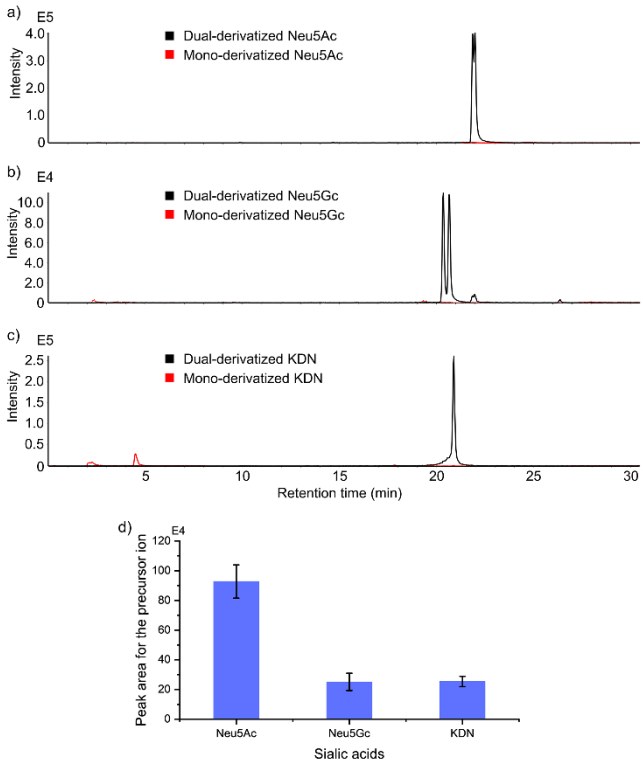


Fig. S2 The XICs display both the di-derivatized and mono-derivatized forms of Neu5Ac (a), Neu5Gc (b), and KDN (c) after undergoing a two-step derivatization. Black line: di-derivatized products; red line: mono-derivatized products. The MS/MS spectrum for the peak with a retention time of 4.6 min in Figure c indicates that it is not a mono-derivatized product. (d) The repeatability of dual derivatization was assessed by measuring the peak area of the precursor ion (n = 3).

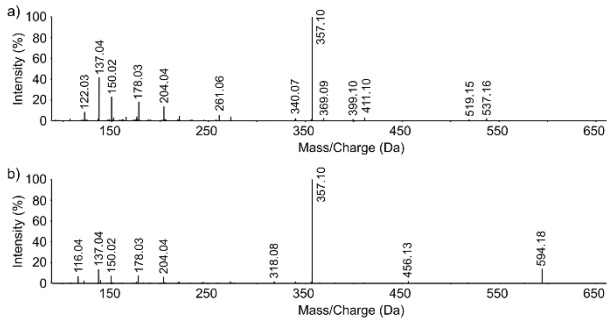
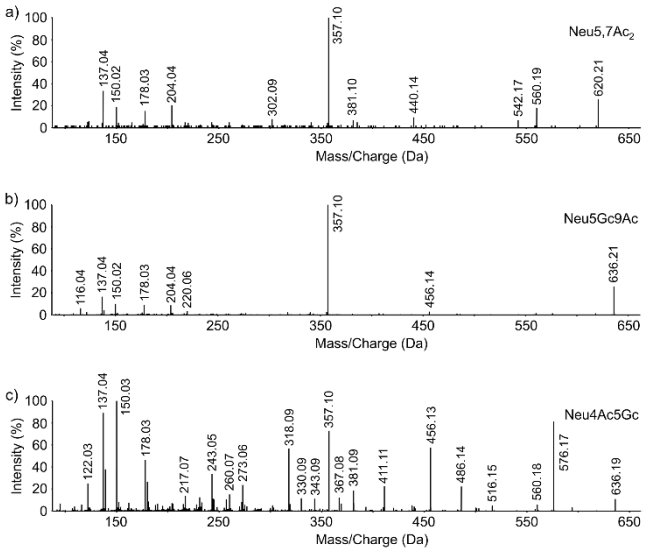


Fig. S3 The MS/MS spectra for 3-NPH-labeled KDN (a) and Neu5Gc (b).



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2 **Fig. S4** The MS/MS spectra for 3-NPH-labeled Neu5,7Ac_2 (a) and Neu5Gc9Ac (b) from a
3 commercial reference; The MS/MS spectrum for 3-NPH-labeled Neu4Ac5Gc (c) from a
4 horse serum sample.

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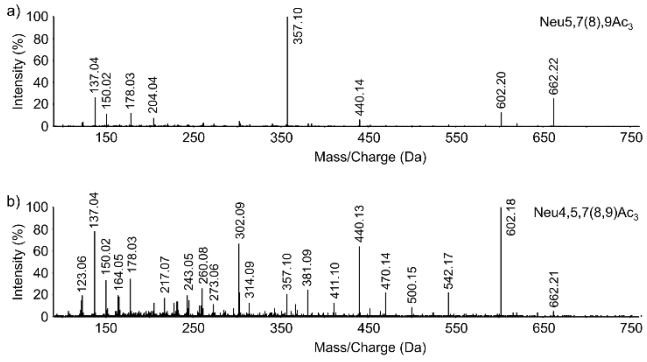

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7 **Fig. S5** (a) The MS/MS spectrum for 3-NPH-labeled Neu5,7(8),9Ac_3 from a commercial
8 reference; (b) The MS/MS spectrum for 3-NPH-labeled Neu4,5,7(8,9)Ac_3 from a horse
9 serum sample.

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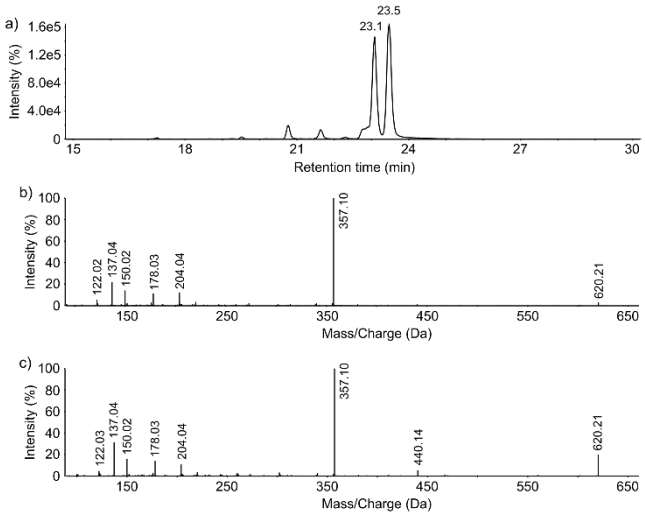

Fig. S6 (a) The XIC at *m/z* 620.20 Da from a commercial reference. The MS/MS spectra for the species at retention times of 23.1 (b) and 23.5 (c) min. Both peaks correspond to Neu5,9Ac$_2$.

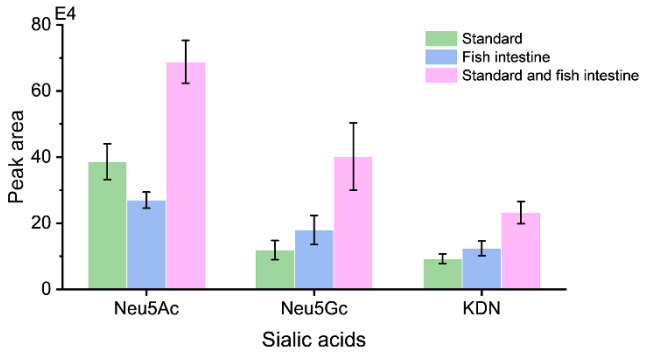

Fig. S7 The peak area for the report ion at *m/z* 357.09 Da was obtained using PRM mode. Three sample groups were analyzed, including sialic acid standards (Neu5Ac, Neu5Gc, and KDN), carp intestinal tissue, and a mixture of standards and tissue. The peak area corresponding to the mixture is between 104% and 135% of the sum of the standard and the tissue.

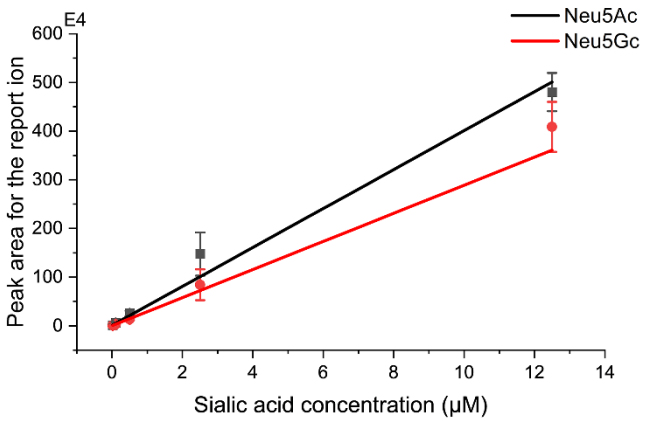

Fig. S8 Linear fitting for 3-NPH-labeled Neu5Ac and Neu5Gc (n = 3). y = peak area for the report ion at *m/z* 357.09 Da; x = concentration.

Table S1. The main product ions for sialic acids labeled with 3-NPH.

No.	Sialic acid	Abbreviation	Source	m/z [M-H]$^-$	Product ions
1	*N*-Acetylneuraminic acid	Neu5Ac	Commercial reference	578.19	178.03, 204.05, 302.09, 357.09, 440.13
2	*N*-Glycolylneuraminic acid	Neu5Gc	Commercial reference	594.18	178.03, 204.05, 318.09, 357.09, 456.13
3	Deaminoneuraminic acid	KDN	Commercial reference	537.16	178.03, 204.05, 261.07, 357.09, 399.11, 411.11
4	4-*O*-Acetyl-*N*-acetylneuraminic acid	Neu4,5Ac$_2$	Horse serum	620.20	178.03, 243.05, 260.08, 302.09, 357.09, 381.09, 440.13, 470.14, 560.19
5	7-*O*-Acetyl-*N*-acetylneuraminic acid	Neu5,7Ac$_2$	Commercial reference	620.20	178.03, 204.05, 302.09, 357.09, 440.13, 560.19

6	9-*O*-Acetyl-*N*-acetylneuraminic acid	Neu5,9Ac_2	Commercial reference	620.20	178.03, 204.05, 357.09, 440.13
7	4-*O*-Acetyl-*N*-glycolylneuraminic acid	Neu4Ac5Gc	Horse serum	636.19	178.03, 243.05, 260.08, 318.09, 357.09, 381.09, 456.13, 486.14, 576.18
8	9-*O*-Acetyl-*N*-glycolylneuraminic acid	Neu5Gc9Ac	Commercial reference	636.19	178.03, 204.05, 357.09, 456.13
9	7(8),9-Di-*O*-acetyl-*N*-acetylneuraminic acid	Neu5,7(8),9Ac_3	Commercial reference	662.21	178.03, 204.05, 302.09, 357.09, 440.13, 602.20
10	4,9-Di-*O*-acetyl-*N*-acetylneuraminic acid	Neu4,5,9Ac_3	Horse serum	662.21	178.03, 260.08, 302.09, 357.09, 381.09, 440.13, 470.14, 542.16, 602.19
11	4,7,8(9)-Tri-*O*-acetyl-*N*-acetylneuraminic acid	Neu4,5,7,8(9)Ac_4	Crucian carp blood	704.22	178.03, 260.08, 302.09, 357.09, 381.09, 440.13, 452.13, 584.18, 644.20
12	4,8,9-Tri-*O*-acetyl-*N*-acetylneuraminic acid	Neu4,5,8,9Ac_4	Crucian carp blood	704.22	178.03, 260.08, 302.09, 357.09, 381.09, 440.13, 470.14, 584.18, 644.20
13	8-*O*-Methyl-*N*-glycolylneuraminic acid	Neu5Gc8Me	Starfish	608.19	178.03, 204.05, 318.09, 357.09, 456.13
14	8-*O*-Methyl-7-*O*-acetyl-*N*-glycolylneuraminic acid	Neu5Gc7Ac8Me	Starfish	650.20	178.03, 357.09, 456.13, 590.18

15	8-*O*-Methyl-9-*O*-acetyl-*N*-glycolylneuraminic acid	Neu5Gc8Me9Ac	Starfish	650.20	178.03, 204.05, 318.09, 357.09, 456.13

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2 **Table S2.** Quantitative calibration curves using the 3-NPH labeling and PRM method.

Sialic acid	Regression equation	R^2	LLOQ[a] (μM)	LOD[b] (μM)
Neu5Ac	$y = 0.93731 + 39.99596x$	0.985	0.02	0.005
Neu5Gc	$y = 0.0084 + 28.87818x$	0.985	0.02	0.01

3 [a]LLOQ: lower limit of quantification; [b]LOD: limit of detection.

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