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Analysis of HMOs in infant formula using Antec Scientific ALEXYS™ Carbohydrate Analyzer and SweetSep™ AEX20 column

November 2025

Contents

Executive summary

This report presents the analysis of seven human milk oligosaccharides (HMOs) in an infant formula sample using the ALEXYS™ Carbohydrate Analyzer. The method was initially tested with the Thermo Scientific™ CarboPac™ PA1 column (2 × 250 mm with pre-column) for a multi-laboratory testing (MLT). To further improve the chromatographic performance, the approach was transferred to and optimized on the Antec Scientific SweetSep™ AEX20 (2.1 × 300 mm) column. The results show that the SweetSep AEX20 column offers excellent separation of the targeted HMOs, with a shorter cycle time than the original method. The SweetSep AEX20 column appears promising as a potential alternative for the official AOAC 2022.04 HMO analysis.

Project details

Project details

Samples received &	On August 21, 2025, the samples were received. Samples: • 2022.04A • 2022.04B • 2022.04C • 2022.04D • 2022.04E • 2022.04F • 2022.04G • 2022.04H • 2022.04J • 2022.04K • 2022.04M • 2022.04N • 2022.04P • 2022.04Q
Sample pretreatment	Weigh 25.00 g ± 10% into a beaker, tare, then add 200.00 g ± 10% of lab water (final mass approximately 225 g). Add a stir bar and stir for 15 minutes or until fully dissolved. Samples of reconstituted materials should be taken while stirring. Transfer the appropriate quantity of reconstituted material or ready-to-feed product to a 25 mL volumetric flask. Typically, this is ~ 1.5 g ±10%. Deliver 100 µL of fructanase D.2.(b), and 100 µL of amyloglucosidase solution (neat, no dilution of amyloglucosidase) to the 25 mL flask. Bring to volume with pH 4.5 acetate buffer D.2.(a). Incubate at 40 °C (±4 °C) for 45 ±5 minutes. Filter through a syringe filter into an autosampler vial. The dilution factor is 2.5, and the samples are stored frozen.
Type of analysis	High-Performance Anion Exchange Chromatography – Pulsed Amperometric Detection (HPAEC-PAD)
Standards	7 HMOs: 2'-Fucosyllactose (2'-FL), 3-Fucosyllactose (3-FL), 6'-Sialyllactose (6'-SL), 3'-Sialyllactose (3'-SL), difucosyllactose (DFL), Lacto-N-Tetraose (LNT), and Lacto-N-neoTetraose (LNnT)
Analysis & Report	Jade van Schaik (Research scientist, Antec Scientific)
Report date	7 November 2025

This project aimed to analyze seven HMOs in infant formula samples using Antec Scientific HPAEC-PAD solutions. For the MLT, we received 14 samples and seven reference standards. The samples were successfully analyzed on the ALEXYS Carbohydrate Analyzer using a 2.1 mm ID × 30 cm SweetSep AEX20 analytical column with an additional pre-column (2.1 mm ID × 5.0 cm AEX20). The initial conditions specified in the MLT protocol were adjusted to improve resolution and reduce cycle time (53 min vs 60 min). Results of the sample analysis with the optimized method are shown later in this report. The conditions used for this approach are listed in Table 1. The chromatographic separation on the CarboPac PA1 reference column was performed as instructed and as summarized in the document's appendix.

HPAEC-PAD Conditions

Table 1 – HPAEC-PAD Conditions

<table>
<tr><td>HPLC system</td><td>ALEXYS Carbohydrate Analyzer</td></tr>
<tr><td>Columns</td><td>SweetSep™ AEX20, 2.1 × 50 mm pre-column, 5 µm
SweetSep™ AEX20, 2.1 × 300 mm analytical column, 5 µm
Borate ion trap, 2.1 × 50 mm column, 10 µm
All columns: Antec Scientific</td></tr>
<tr><td>Mobile phase (MP)</td><td>A: 50 mM NaOH
B: DI water
C: 500 mM NaOH
D: 300 mM NaOAc
Eluents prepared gravimetrically & blanketed with N_2 (5.0)</td></tr>
<tr><td>Gradient program</td><td>
<table>
<tr><th>Time (min)</th><th>Mobile phase</th><th>Description</th></tr>
<tr><td>0 - 15</td><td>20 mM NaOH</td><td>Isocratic elution</td></tr>
<tr><td>15 - 25</td><td>90 mM NaOH + 30 mM NaOAc</td><td>Linear gradient</td></tr>
<tr><td>25 - 30</td><td>90 mM NaOH + 30 mM NaOAc</td><td>Isocratic elution</td></tr>
<tr><td>30 - 34</td><td>90 mM NaOH + 96 mM NaOAc</td><td>Linear gradient</td></tr>
<tr><td>34 - 39</td><td>90 mM NaOH + 96 mM NaOAc</td><td>Isocratic elution</td></tr>
<tr><td>39 - 43</td><td>100 mM NaOH + 240 mM NaOAc</td><td>Rinsing step of the column</td></tr>
<tr><td>43 - 53</td><td>20 mM NaOH</td><td>Equilibration to the starting conditions</td></tr>
</table>
</td></tr>
<tr><td>Flow rate</td><td>0.18 mL/min</td></tr>
<tr><td>Backpressure</td><td>System pressure 200 - 210 bar</td></tr>
<tr><td>Injection loop</td><td>2.5 µL</td></tr>
<tr><td>Temperature</td><td>30 °C for separation, 35 °C for detection</td></tr>
<tr><td>Flow cell</td><td>SenCell with Au WE, stainless steel AE, and HyREF RE, AST 2</td></tr>
<tr><td>Potential waveform (4-step)</td><td>E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V
ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s</td></tr>
<tr><td>I-cell</td><td>about 0.2 µA</td></tr>
<tr><td>ADF</td><td>0.5 Hz</td></tr>
<tr><td>Range</td><td>5 µA/V</td></tr>
</table>

Separation of 7 HMOS Standards

Comparing the chromatographic capabilities of the CarboPac PA1 and the SweetSep AEX20 directly (Figure 1), oligosaccharides — such as 3-FL, DFL, and 2-FL — elute as sharper peaks. The latex agglomeration of the polymeric, monodisperse 5-µm core particles enhances separation, supported by peak focusing achieved through gradient elution, resulting in increased peak capacity. This leads to needle-sharp peaks for LNT, 3-SL, and 6-SL. This improved separation ability of the SweetSep AEX20 allows for distinguishing a peak that previously co-eluted with 6-SL on CarboPac PA1 (see "*" in Figure 1). As a result, the accuracy of 6-SL quantification improves, and the risk of false positives decreases.

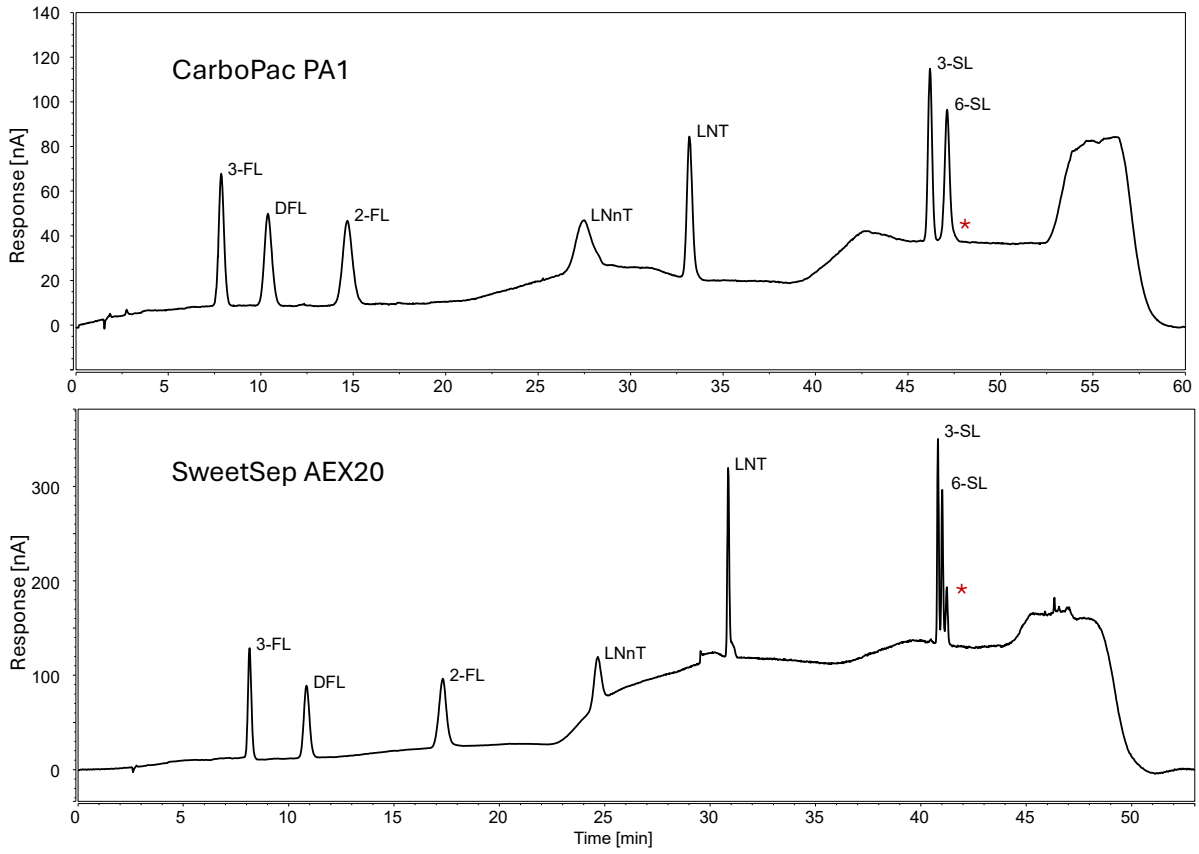

Figure 1 shows the separation of 7 HMO standards on the CarboPac PA1 and the SweetSep AEX20. (Note: the asterisk indicates an additional oligosaccharide chromatographically resolved on the SweetSep AEX20, unlike the reference chromatogram on the CarboPac PA1. See Figure 2.)*

Figure 2 displays the separation performance of the CarboPac PA1 and SweetSep AEX20 for 3-SL and 6-SL. To ensure a fair comparison, the chromatograms were aligned within the specified retention time range using the CDS software. The result clearly shows that the SweetSep AEX20 provides better chromatographic separation, as evidenced by narrower peaks. Additionally, as previously noted, a peak that co-elutes with 6-SL on the CarboPac PA1 is sufficiently separated to allow accurate, bias-free quantification of 6-SL.

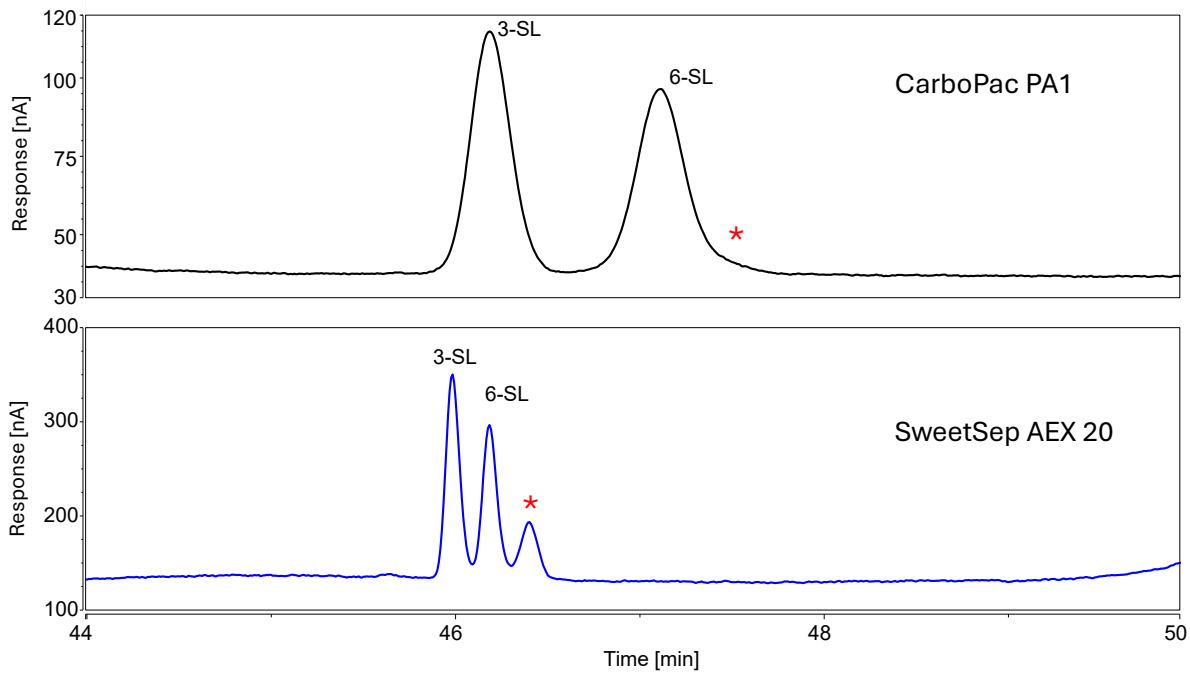

Figure 2. Separation of 3-SL and 6-SL on the CarboPac PA1 and the SweetSep AEX20. (Note: Both chromatograms were standardized based on the retention time of 3-SL). (Note: the asterisk indicates an additional oligosaccharide chromatographically separated on the SweetSep AEX20, in contrast to the reference chromatogram obtained with the CarboPac PA1.)*

Separation of HMO Sample 2022.04A

Figure 3 shows the separation of the seven HMOs in a representative MLT sample. Trace (A) presents the original sample, while trace (B) displays the sample combined with 2.5 µg/mL of each HMO. The excellent separation ability of SweetSep AEX20 is also evident in real samples. All target components are fully resolved from matrix components, allowing interference-free quantification. The improved separation of 3-SL and 6-SL, as previously mentioned, is also evident in the actual samples.

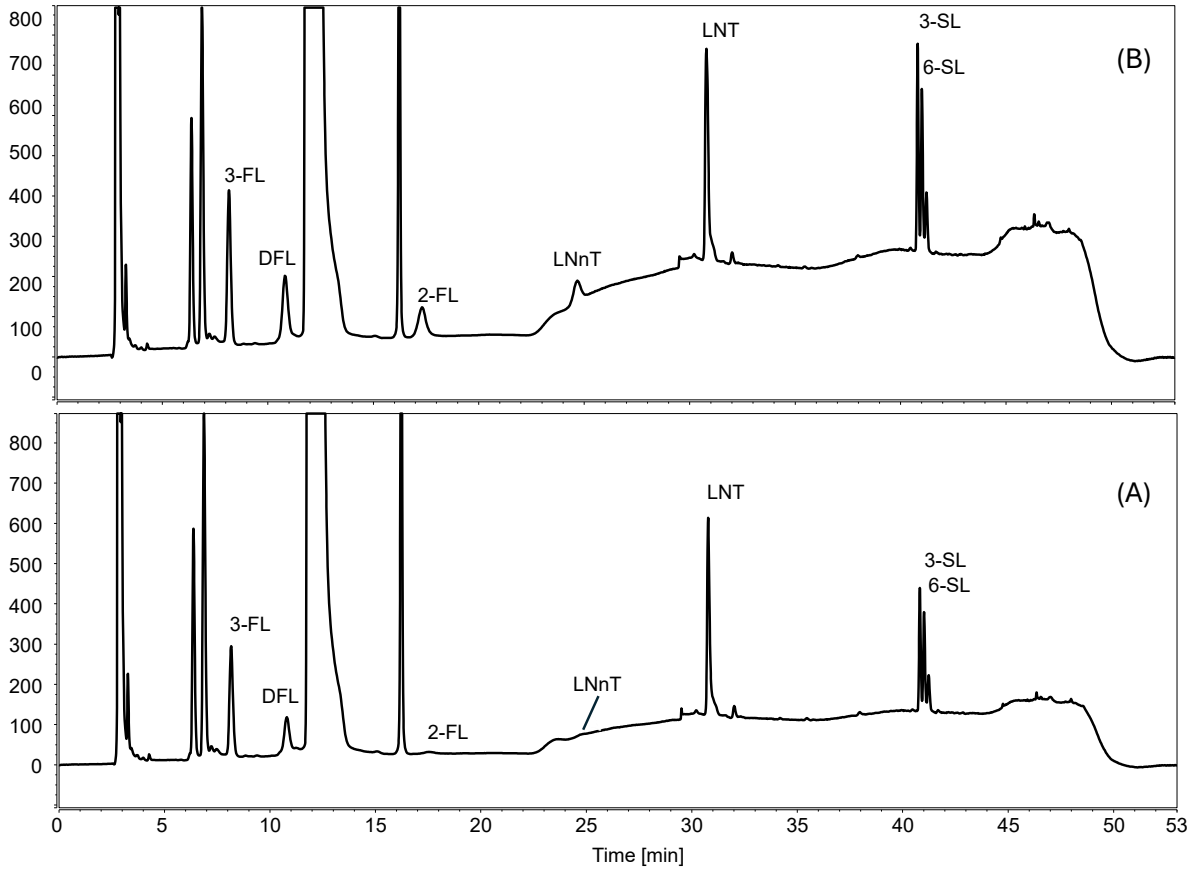

Figure 3. Chromatographic analysis of sample 2022.0A. Trace (A): Original sample. Trace (B): Sum of Sample 2022.04 and a standard solution of 2.5 µg/mL of each of the 7 HMOs.

Comparison of chromatographic performance

A direct comparison between the CarboPac PA1 column, used as a reference column in the AOAC method, and the SweetSep AEX20 column shows not only the advantages discussed earlier but also reveals another potential co-elution on the CarboPac column. While 2-FL appears as a single dominant peak in the CarboPac PA1 chromatogram, it shows as a smaller signal in the SweetSep AEX separation, eluting after a larger peak. Since the superior separation performance of SweetSep AEX20 was evident in separating 3-SL and 6-SL, as well as 3-FL and DFL—early-eluting components that are better separated from the surrounding matrix—it is reasonable to assume that quantifying 2-FL in real samples would benefit from the enhanced separation ability of SweetSep AEX20.

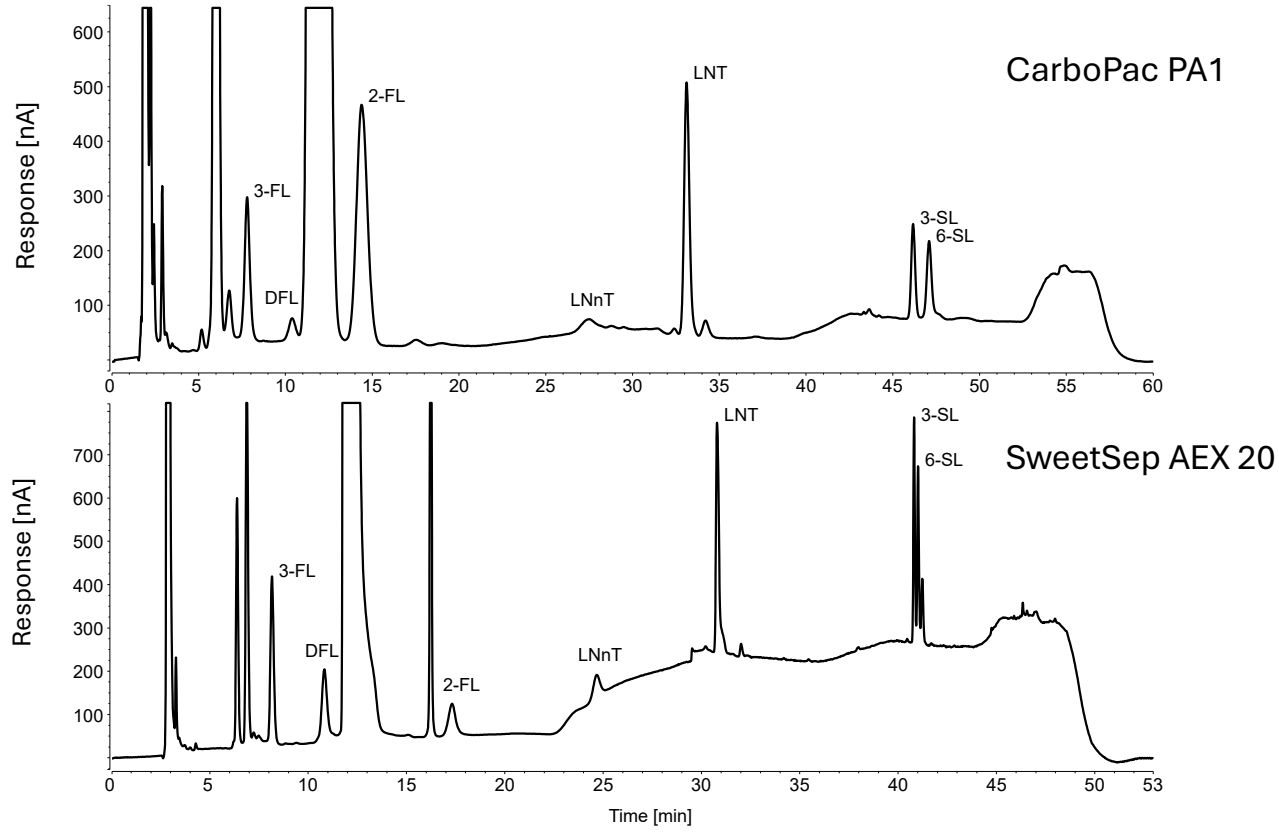

Figure 4: Comparison of separation properties between the conventional reference column and the SweetSep AEX20. Chromatograms show the sum of Sample 2022.04 and a standard solution of 2.5 µg/mL of each of the 7 HMOs. The SweetSep AEX20 improves separation and peak shape for all 7 HMOs.

Comparing the chromatograms of sample 2022.04A reveals not only a sharper LNT signal but also improved separation from closely eluting compounds on the new SweetSep column. The LNT peak on the CarboPac PA1 appears heterogeneous, as indicated by an extra shoulder in the SweetSep chromatogram (see asterisk in Figure 5). This enhanced separation is vital for accurately identifying and quantifying HMOs in real samples. As before, the chromatograms were aligned within the specified retention time range using CDS software to ensure an unbiased comparison of both traces.

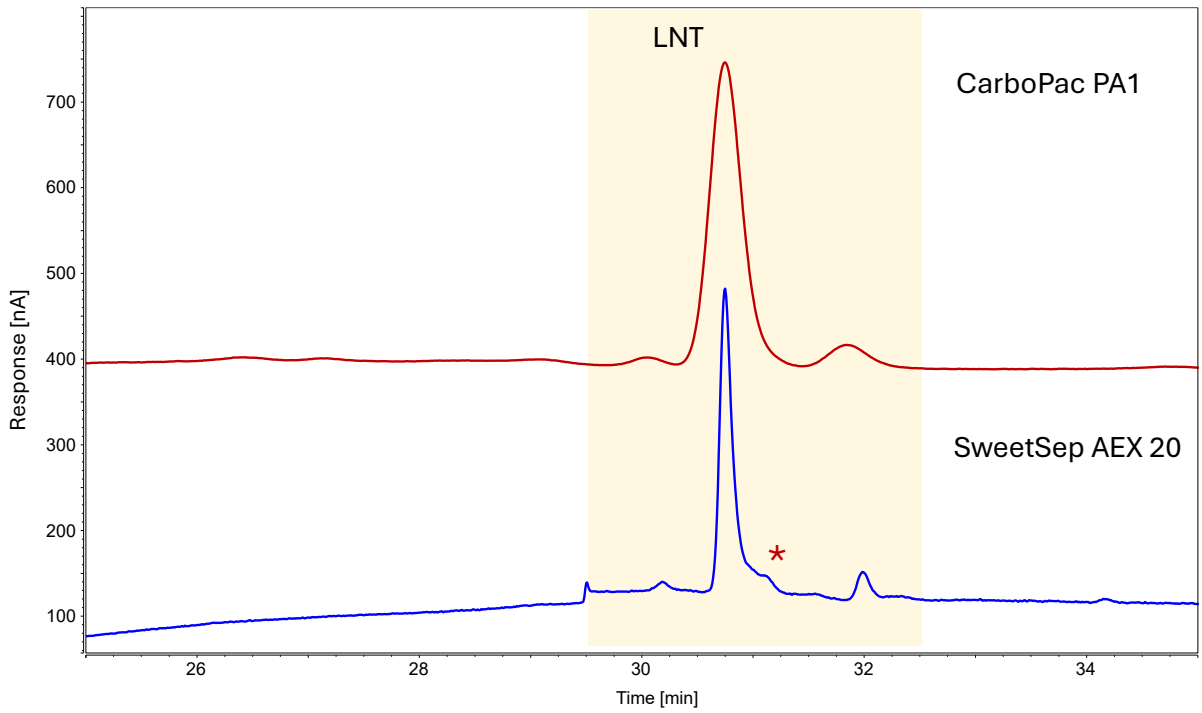

Figure 5 Separation of LNT on the CarboPac PA1 and the SweetSep AEX20. The SweetSep AEX20 not only separates the flanking compounds more effectively from LNT, but also reveals other components not observed on the CarboPac PA1 (see asterisk). (Note: Both chromatograms were standardized based on the retention time of LNT).

Summary

In summary, the objective improvements achieved by using SweetSep AEX20 are shown in Figure 6 and indicated by the color-coded segments: all seven target HMOs are better separated from matrix components on the more advanced phase and elute as sharper peaks — a result of the increased peak capacity of the SweetSep column under gradient operation. An obvious coelution (3-SL and 6SL, area (D)) and a likely coelution (2-FL, area (B)) are resolved on the new 30 cm separation column with a shorter cycle time throughout.

Chromatographic analysis of the different samples and varying conditions did not affect chromatographic performance and critical key parameters such as separation, peak symmetry, and retention. The SweetSep AEX20 column shows promise as a potential alternative for the official AOAC 2022.04 HMO analysis. This higher level of separation indicates considerable progress that may lead to a reanalysis of previously published HMO data.

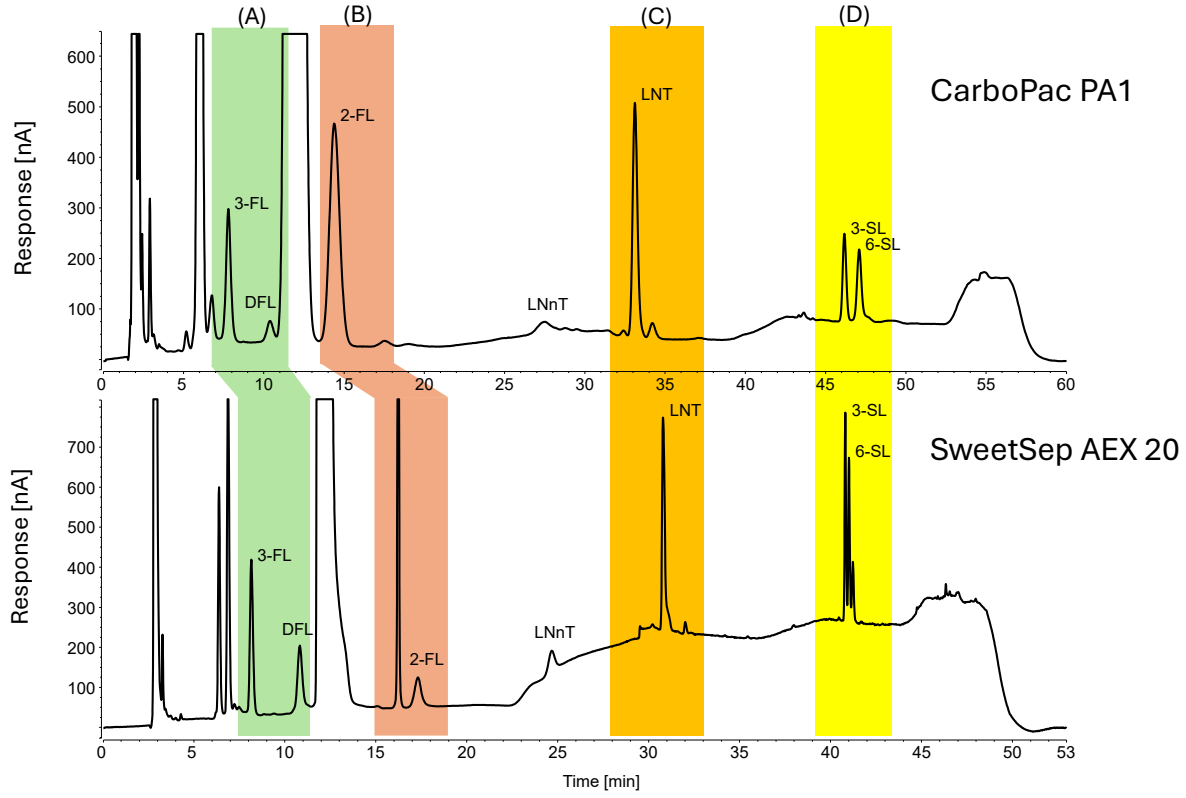

Figure 6: Summary of the achieved improvements with the SweetSep AEX20: (1) Shorter cycle time, improved separation for (2) 3-FL and DFL, (3) 2-FL from matrix interference, (4) LNT from matrix components, and (5) separation of a previously unrecognized co-eluting HMO with 6-SL.

Appendix

System configuration

ALEXYS Carbohydrate Analyzer

The ALEXYS Carbohydrate Analyzer features the ET210 eluent tray (used for storage and nitrogen blanketing of mobile phases during analysis), a P6.1L quaternary LPG pump, an AS6.1L autosampler, a CT2.1 column thermostat, and the DECADE Elite electrochemical detector. The SenCell with an Au working electrode and HyREF (Pd) reference electrode was selected for carbohydrate detection.

Table 2: Ordering Information

Recommended ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Columns	
260.0022	SweetSep™ AEX20, 2.1 x 300 mm column, 5 µm
260.0026	SweetSep™ AEX20, 2.1 x 50 mm precolumn, 5 µm
260.0031	Borate ion trap, 2.1 x 50 mm column, 10 µm

Appendix II – AOAC Experimental Conditions [1]

Instrument settings

Autosampler temperature	10 ± 1°C
Column and detector temperature	20 ± 2°C
Injection volume	4 µL
Injection time	60 min
Flow rate	1.0 mL/min
PAD waveform	Gold, Carbo, Quad—see Waveform setting below.
Reference electrode	AgCl
Data collection	2 Hz
Column pressure	< 2500 psi (≈1400–1800 typical
A 2 × 250 mm CarboPac PA1 was used for the experiments described above. Hence, the flow rate was 0.25 mL/min and the injection volume was 1 µL.	

Elution setting

Time (min)	% Water	%500mM NaOH	%300mM sodiumacetate
0.0	82	18	0
15	82	18	0
25	72	18	10
35	72	18	10
39	50	18	32
49	50	18	32
49.01	0	20	80
53	0	20	80
53.01	82	18	0
60	82	18	0

Waveform settings

Time(s)	Potential(V)	Integration
0.00	0,1	
0.20	0,1	Begin
0.40	0,1	End
0.41	-2	
0.42	-2	
0.43	0,6	
0.44	-0,1	
0.50	-0,1	

References

[1] P. Haselberger, F. Tian, R. Erney, S. Liu, S. Wang, Q. Lin and Ding Yi, "Method for the Determination of 2'-Fucosyllactose (2'-FL), 3-Fucosyllactose (3-FL), 6'-Sialyllactose (6'-SL), 3'-Sialyllactose (3'-SL), Lacto-N-Tetraose (LNT), and Lacto-N-neoTetraose (LNnT) by HPAEC-PAD: First Action 2022.04," *J. AOAC Int.,* pp. 1-9, 21 06 2023.