Separation of human milk oligosaccharides using high-performance anion-exchange chromatography with pulsed amperometric detection

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Separation of human milk oligosaccharides using high-performance anion-exchange chromatography with pulsed amperometric detection

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Introduction

Human Milk Oligosaccharides (HMOs) are composed of 5 different monosaccharides: D-glucose, D-galactose, L-fucose, *N*-acetylneuraminic acid, and *N*-acetylglucosamine. Approximately 200 unique structures have been identified, with the degree of polymerization ranging from 3 to 22. The diversity found among individual mothers is considerable, varying from as few as 23 and up to 130 different oligosaccharides detected. HMOs are known as beneficial for infant health and development, and have received increasing attention in recent years [1].

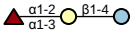
Syntheses of this unique family of lactose-based molecules necessitates analysis methods that can provide separation and quantification of the common structural constituents mentioned, as well as the disaccharide lactose (Galβ1-4Glc) and oligosaccharides such as lacto-*N*-triose II (GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc), and lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), among others (see below).
In the present work, isocratic analyses of various saccharides were performed to serve as a basis for the development of more complex chromatographic methods.

Human milk oligosaccharide structures

Monosaccharide building blocks

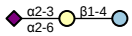
○	Glc	Glucose
○	Gal	Galactose
□	GlcNAc	*N*-Acetylglucosamine
△	Fuc	Fucose
◇	Neu5Ac	*N*-Acetylneuraminic acid

Fucosyllactoses



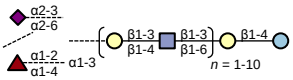
Fucα1-2/3Galβ1-4Glc

Sialyllactoses



Neu5Acα2-3/6Galβ1-4Glc

Complex HMOs



Neu5Acα2-3/6 / Fucα1-2/3/4(Galβ1-3/4GlcNAcβ1-3/6)$_n$Galβ1-4Glc

High-performance anion-exchange chromatography

High-performance anion-exchange chromatography (HPAE) with pulsed amperometric detection (PAD) is a method highly suited for analysis of carbohydrates. HPAE with alkaline eluents results in retention of neutral carbohydrates depending on the number of charged groups in the molecule, pH and concentration of competing anions. The PAD provides sensitivity for carbohydrates in the pmol-range, although the detection response is dependent on eluent pH [2].

Samples prepared from pure standards were eluted using water and aqueous solutions of NaOH (100 mM or 1 M) and NaOAc (10 mM), and a CarboPac PA1 column at 22 °C.

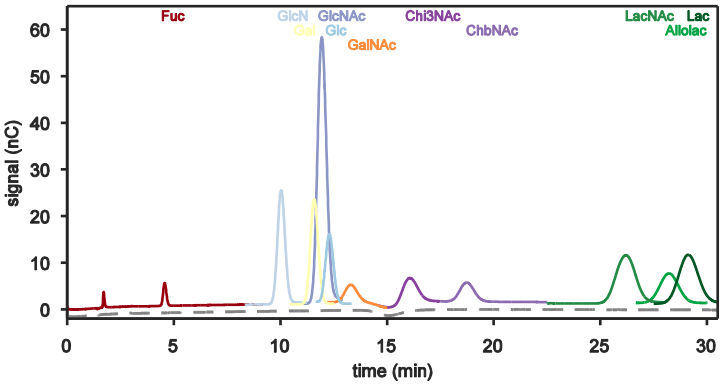


Figure 1: Chromatogram overlay from HPAE analyses of mono- and oligosaccharides.
Isocratic elutions of standards at 25 mM NaOH and 1 mM NaOAc after 10 min equilibration. Injection of pure water subtracted (- - -). See fig. 2 for full names of compounds.

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References
1. Bode L, Jantscher-Krenn E. (2012) Adv. Nutr., 3: 383S
2. Lee YC. (1990) Anal. Biotechnol., 189: 151

Isocratic elutions of mono- and oligosaccharides

Various mono- and oligosaccharides were analysed under isocratic conditions with different eluent concentrations of NaOH in the ranges 5-75 mM and 50-200 mM, while the concentration of NaOAc was maintained at 0, 1, 2 or 4 mM.

The investigated saccharides exhibited different retention properties as an effect of NaOH-concentration, and maximum retention was obtained at different concentrations (see fig. 2). Most of the monosaccharides co-eluted or eluted in close proximity at the upper range of the conditions studied, while variations in retention were observed at lower NaOH-concentrations.

As building blocks for HMOs, the separation of Glc, Gal, and GlcNAc were of particular interest. These saccharides all exhibited increasing retention times over the range from 5 to 14-20 mM NaOH, which indicated that gradient elution with increasing concentration of NaOH in this range and over the range of the retention times of these monosaccharides could be used to optimise the separation and analysis time.

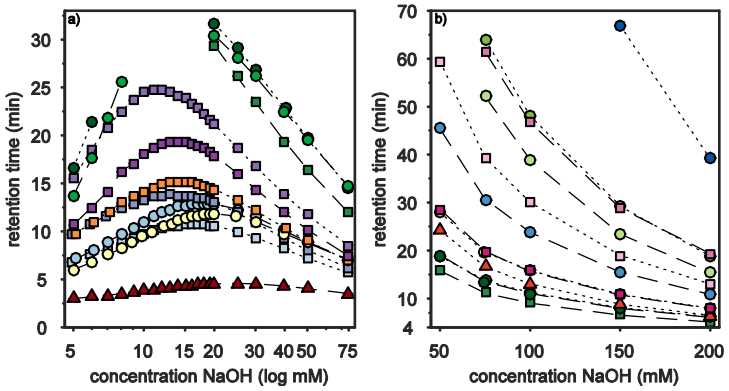

Figure 2: Effect of eluent concentration of NaOH on retention times of mono- and oligosaccharides, with an NaOAc concentration of 1 mM.
a) NaOH concentration range 5-75 mM: ▲ fucose, ○ galactose, □ glucosamine, ● glucose, ■ *N*-acetylglucosamine, ■ *N*-acetylgalactosamine, ■ *N,N′,N″*-triacetylchitotriose, ■ *N,N′*-diacetylchitobiose, ■ *N*-acetyllactosamine, ● lactose, ● allolactose.
b) NaOH concentration range 50-200 mM: ■ *N*-acetyllactosamine, ● lactose, ● allolactose, ▲ 2′-fucosyl-lactose, ○ 6′-galactosyl-lactose, ■ lacto-*N*-triose II, ● maltose, □ lacto-*N*-neotetraose, ○ 4′-galactosyl-lactose, □ lacto-*N*-tetraose, ○ 3′-galactosyl-lactose, ● maltotriose.
Error bars indicate relative standard deviations calculated from fucose as internal standard.
X-axis jitter applied for $\Delta t_R \leq 0.5$.

Increasing concentrations of NaOAc generally decreased retention time, but was observed to improve separation and affect the elution order of some of the saccharides (see fig. 3). For some of the closely eluting di- and oligo-saccharides, this indicated a gradient parameter for optimisation of separation.

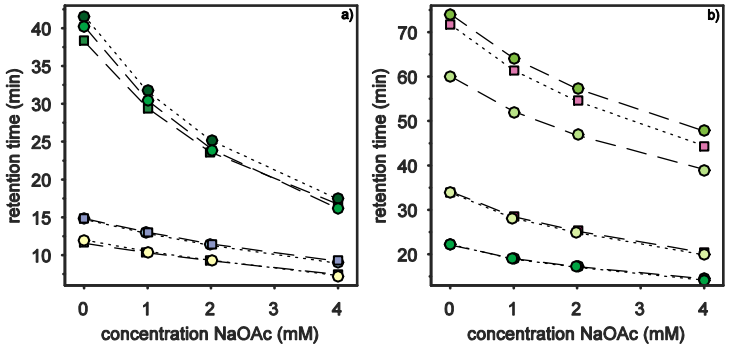

Figure 3: Effect of eluent concentration of NaOAc on retention times of mono- and oligosaccharides, at selected NaOH concentrations.
a) NaOH concentration range 5-75 mM: ○ galactose, □ glucosamine – 12 mM; ● glucose, ■ *N*-acetylglucosamine, ■ *N*-acetyllactosamine, ● lactose, ● allolactose – 20 mM.
b) NaOH concentration range 50-200 mM: ● lactose, ● allolactose, ○ 6′-galactosyl-lactose, ■ lacto-*N*-triose II – 50 mM; ○ 4′-galactosyl-lactose, □ lacto-*N*-tetraose, ○ 3′-galactosyl-lactose – 75 mM.
Error bars indicate relative standard deviations calculated from fucose as internal standard.
X-axis jitter applied for $\Delta t_R \leq 0.5$.

Conclusions

Several of the mono-, di- and oligosaccharides investigated exhibited maximum retention at discrete concentrations of NaOH.

The observed retention properties of the investigated saccharides provide information for designing gradient elution strategies.

Separation of the HMO building blocks glucose, galactose and *N*-acetylglucosamine was indicated as optimisable with gradient methods in the concentration range of 5 to 15 mM NaOH, while disaccharide building blocks could be separated using a NaOAc concentration gradient.