Ultrafast Analysis of Human Milk Oligosaccharides (HMOs) by Capillary Gel Electrophoresis

Andras Guttman[1] Marton Szigeti[2] and Daniel Sarkozi[2]
[1]*SCIEX, Brea, CA 92130, USA;*
[2]*Horváth Csaba Memorial Laboratory of Bioseparation Sciences, University of Debrecen, Hungary*

Introduction

Human milk oligosaccharides (HMOs) are the third most abundant ingredients in human milk [1], and arecritical to child development [2]. Their quantities and structures differ in the various lactation periods of the mother and are population specific [3, 4]. Consequently, there is an increasing demand to produce suitable baby formulas [5], being addressed by both pharma and food manufacturing companies with the goal to develop natural analogue HMO based functional food additives. Academic institutions are also heavily investigating milk samples from various sources to understand the biological role of HMOs in child development [6-8].

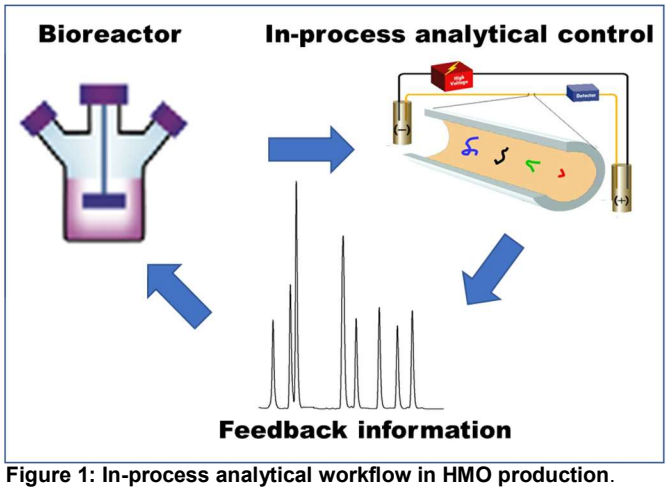


Figure 1: In-process analytical workflow in HMO production.

The building blocks of HMOs are based on lactose that is modified by β-N-acetyllactosamine (LacNAc) or lacto-N-biose (LNB) with the possible addition of fucose (Fuc) or sialic acid (Neu5Ac). Currently, more than 200 HMO structures are known [9]. Their theoretical varied linkage types significantly increase their complexity level, resulting in numerous isomeric structures [10, 11]. Therefore, there is a great demand for high performance analytical methods to address the challenging task of HMO characterization [12]. Capillary electrophoresis (CE) is one of the most effective glycoanalytical methods broadly used in the pharmaceutical, biomedical and food industries [13, 14]. After tagging the sugars with a fluorescent label (e.g., aminopyrenetrisulfonate, APTS), the sensitivity of capillary electrophoresis (CE) based analysis with laser-induced fluorescent detection (LIF) can be as low as 10^{-10} M. APTS also provides charge to the otherwise mostly neutral sugars to produce their electromigration. It is important to note that all reducing sugars are labeled at their reductive end only, making quantification easy due to the one APTS/sugar structure stoichiometry [15]. With the use of borate containing buffers systems, the secondary equilibrium of the borate-diol complexation may further improve resolving power [16]. Utilizing sugar and linkage specific exoglycosidase based digestions, the sequence of the oligosaccharide structures can aslo be determined [17].

During fermentation and enzymatic bioprocessing of HMOs, rapid analytical in-process control is crucial to provide the necessary information for the decision maker to make the appropriate feedback and fine tune the bioreaction parameters. In this Technical Note the analysis of the ten most important HMOs are demonstrated utilizing the SCIEX's Fast Glycan Sample Preparation and Analysis and the SDS-MW kits, this latter with a borate containing background electrolyte. **Food manufacturers currently facing challenges in adopting fast and high-resolution separation methods to the analysis of human milk oligosaccharides will obtain the desired resolution and speed with the use of the methods described in the Technical Note.** Figure 1 shows the suggested workflow of sampling from the bioreactor, rapid analysis by the PA 800 Plus system, and immediate feedback to the bioreactor if any fine tuning is necessary in the fermentation parameters to obtain faster and more precise production of the required HMOs.

Key Features

- An ultrafast glycoanalytical technique to decipher the high complexity of HMOs during the entire bioprocessing workflow, including lot release
- Provides help in faster decision making needed to make the appropriate feedback and fine tune the bioreaction parameters during fermentation and enzymatic bioprocessing of HMOs
- SCIEX's HR-NCHO and SDS-MW separations provided complementary resolving power for all synthetic HMOs analyzed

- Utilization of a secondary equilibrium via borate-diol complexation provided a significant step forward, towards comprehensive in-process control for HMO production
- Fine tuning of the resolving power was possible by mixing the two gel formulations

Experimental

Chemicals

The Fast Glycan Labeling and Analysis Kit (part # B94499PTO) including the separation gel-buffer system and the 8-aminopyrene-1,3,6-trisulfonic acid tagging dye and the separation gel from the SDS-MW kit (part # 390953) were from Sciex (Brea, CA, USA). Acetic acid (glacial), tetrahydrofuran (THF), sodium-cyanoborohydride (1 M in THF) and water (HPLC grade) were from Sigma Aldrich (St. Louis, MO, USA). The HMO standards (Table 1) were from Glycom (Hørsholm, Denmark).

Sample Preparation

Two milligrams of each sugar standards were labeled by the addition of 18 µL of 20 mM APTS (in 20% acetic acid) and 2 µL of $NaBH_3CN$ (1 M in THF), followed by overnight incubation at 37°C with the vial lid open [18]. The labeled samples were reconstituted in 100 µL HPLC grade water (labeled stock) and further diluted by 1,000x to 10,000x prior to capillary gel electrophoresis analysis.

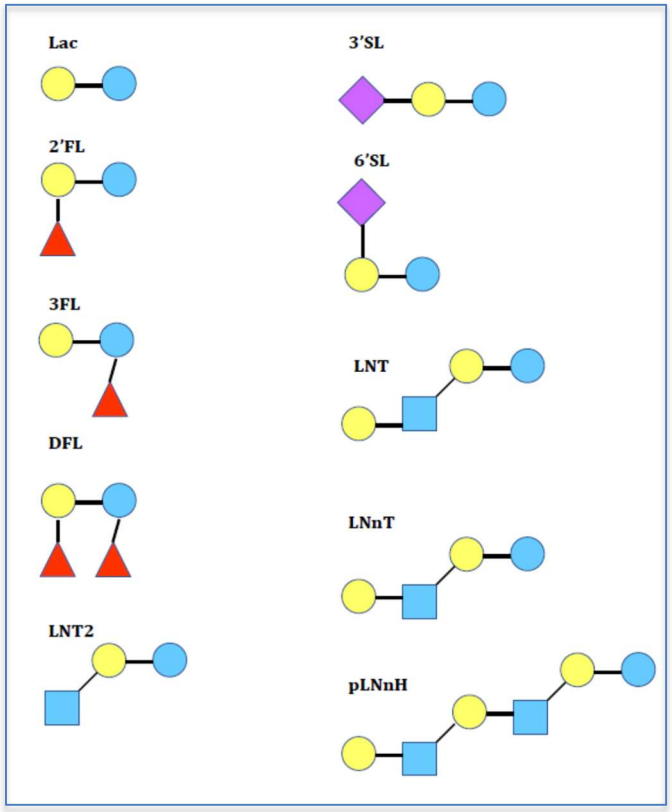
Capillary SDS-Gel Electrophoresis

A PA800 Plus Pharmaceutical Analysis system (Sciex) equipped with a solid state laser-induced fluorescence detector (488 nm excitation wavelength with 520 nm emission filter) was used for all capillary electrophoresis separations with the HR-NCHO and SDS-MW separation gel buffer systems. CGE-LIF analysis of the APTS labeled HMOs were performed in a pre-built EZ-CE cartridge (part # A55625) with 20 cm effective length (30 cm total length), 50 µm ID bare fused silica capillary columns by applying 1,000 V/cm electric field strengths in reversed polarity mode (cathode at the injection side). The electrokinetic sample injection protocol was as follows: 5.0 psi for 5.0 sec water pre-injection, followed by 2.0 kV for 2.0 sec sample injection. Data acquisition and analysis were accomplished using the 32Karat (version 10.1) software package (Sciex).

Results and Discussion

Analysis of the ten most important HMO containing test mixture (Table 1) was accomplished by using the HR-NCHO and SDS-MW gels (Figures 1 and 2, respectively). Closer look at the electropherograms in Figures 1 and 2 exhibited remarkable differences.

Table 1. The components of the HMO test mixture. Lac: lactose, 2'FL: 2'-fucosyllactose, 3FL: 3-fucosyllactose, DFL: difucosyllactose, LNT2: lacto-N-triose, 3'SL: 3'-sialyllactose, 6'SL: 6'-sialyllactose, LNT: lacto-N-tetraose, LNnT: lacto-N-neotetraose, pLNnH: para-lacto-N-neohexaose



With the use of the HR-NCHO gel, the extra charge carrying sialylated structures migrated first (6'-sialyllactose and 3'-sialyllactose) followed by the neutral oligosaccharides according to their hydrodynamic volumes since all of them carried the same APTS associated charges. The disaccharide lactose migrated after the sialylated glycans, followed by the trisaccharides. Foremost, the compact fucosylated ones (2'-fucosyllactose and 3-fucosyllactose) and later the more stretched lacto-N-triose. In this case, due to their very similar mass to hydrodynamic volume ratios, the 2'-fucosyllactose and 3-fucosyllactose structures co-migrated. All tetrasaccharides (difucosyllactose, lacto-N-tetraose and lacto-N-neotetraose) were well separated. Please note that the lacto-N-tetraose and lacto-N-neotetraose structures differed only by their β1-3 and β1-4 galactose linkage at the non-reducing terminal. The large para-lacto-N-neohexaose hexasaccharide migrated last. With the use of this background electrolyte, the entire analysis took less than 3.4 minutes at the optimal separation temperature of 25°C.

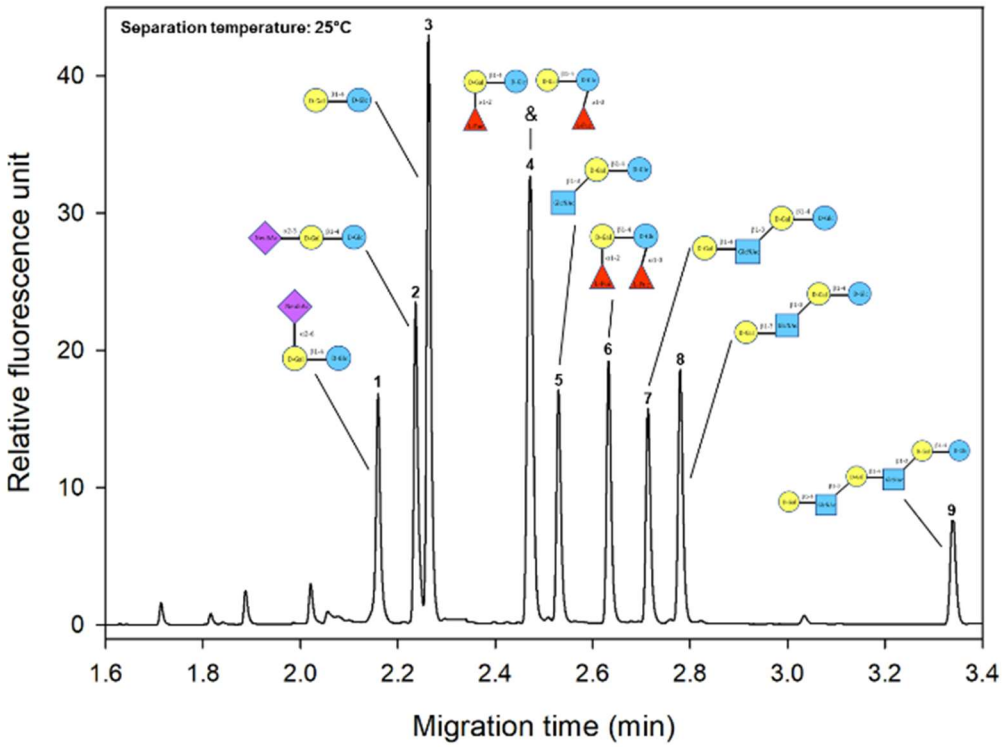


Figure 1. Capillary gel electrophoresis separation of the HMO test mixture using the HR-NCHO gel. The corresponding structures are shown above each peak. Conditions: Bare fused silica capillary with 20 cm effective length (30 cm total length), 50 µm ID; HR-NCHO gel buffer system; Separation temperature: 25°C; Applied electric field strength: 1000 V/cm; Injection sequence: 1) 5.0 psi for 5.0 sec water pre-injection, 2) 2.0 kV for 2.0 sec sample injection.

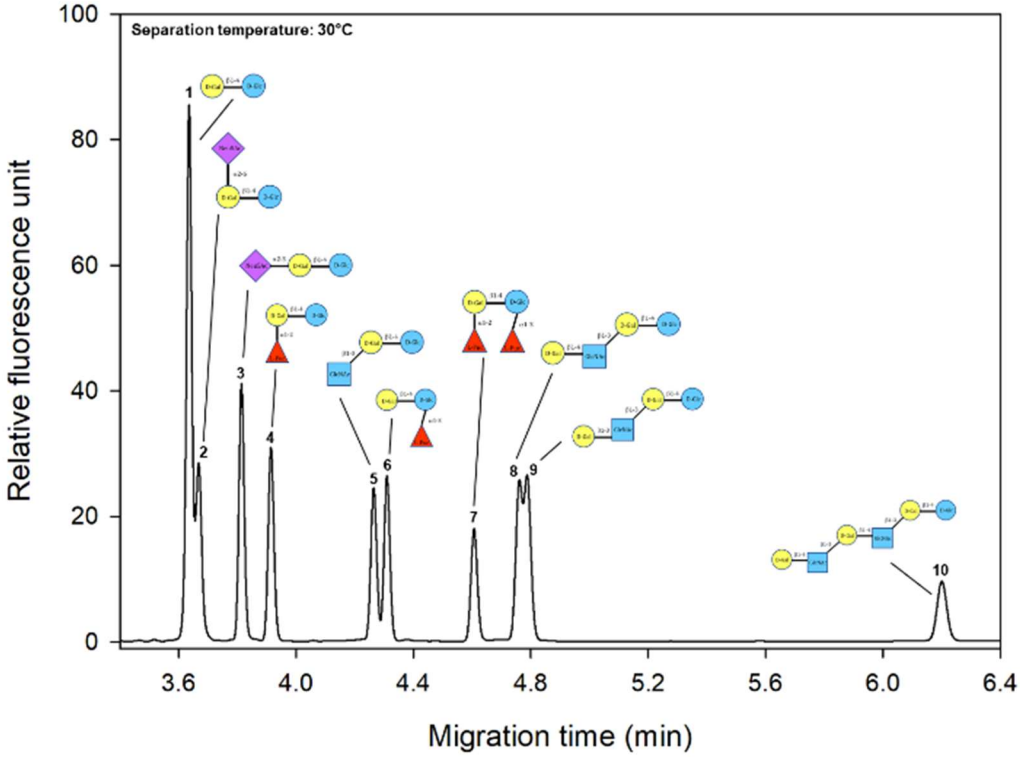


Figure 2. Capillary electrophoresis separation of the HMO test mixture using the SDS-MW gel. The corresponding structures are shown above each peak. Conditions: Bare fused silica capillary with 20 cm effective length (30 cm total length), 50 µm ID; SDS-MW gel buffer system; Separation temperature: 30°C; Applied electric field strength: 1000 V/cm; Injection sequence: 1) 5.0 psi for 5.0 sec water pre-injection, 2) 2.0 kV for 2.0 sec sample injection.

Figure 2 depicts the separation of the HMO test mixture using the SDS-MW gel matrix. As one can observe, the migration order is different in comparison to Figure 1. The total analysis time was slightly longer (6.3 min) at the optimal separation temperature of 30°C. Please note that in this instance the large amount of borate in the gel-buffer system had a strong tendency to complex with the vicinal OH groups of the human milk oligosaccharides. This secondary equilibrium affected the electrophoretic mobilities of the sample components also causing resolution alterations. Lactose, the smallest HMO in the mixture migrated first, faster than the extra charge carrying 6'-sialyllactose and 3'-sialyllactose, probably due to the different borate complexation with these structures. Most notably, excellent separation of the positional isomer trisaccharides of 2'-fucosyllactose and 3-fucosyllactose were obtained with this separation matrix in contrast to their co-migration with the use of the HR-NCHO gel-buffer system. Actually, the separation window was so great that the lacto-N-triose trisaccharide migrated in between them. The tetrasaccharides represent the next separation block. The difucosyllactose structure well separated from the closely migrating lacto-N-tetraose and lacto-N-neotetraose oligosaccharides, which latter two migrated in a split peak. Apparently, the borate complexation very much diminished the hydrodynamic volume differences between the β1-3 and β1-4 linked structures. The hexasaccharide para-lacto-N-neohexaose was well separated from all and migrating last.

Since neither the HR-NCHO nor the SDS-MW gels offered full separation of all 10 important HMOs, mixing of the two gels was attempted. Figure 3 shows the separation of all sample components at 40°C by mixing the two gel formulations in a 1:1 ratio. This example clearly demonstrated that fine tuning by adjusting the proportion of the two gel formulations as well as the separation temperature represented further options to increase the separation power for the sample compontents of interest in hand.

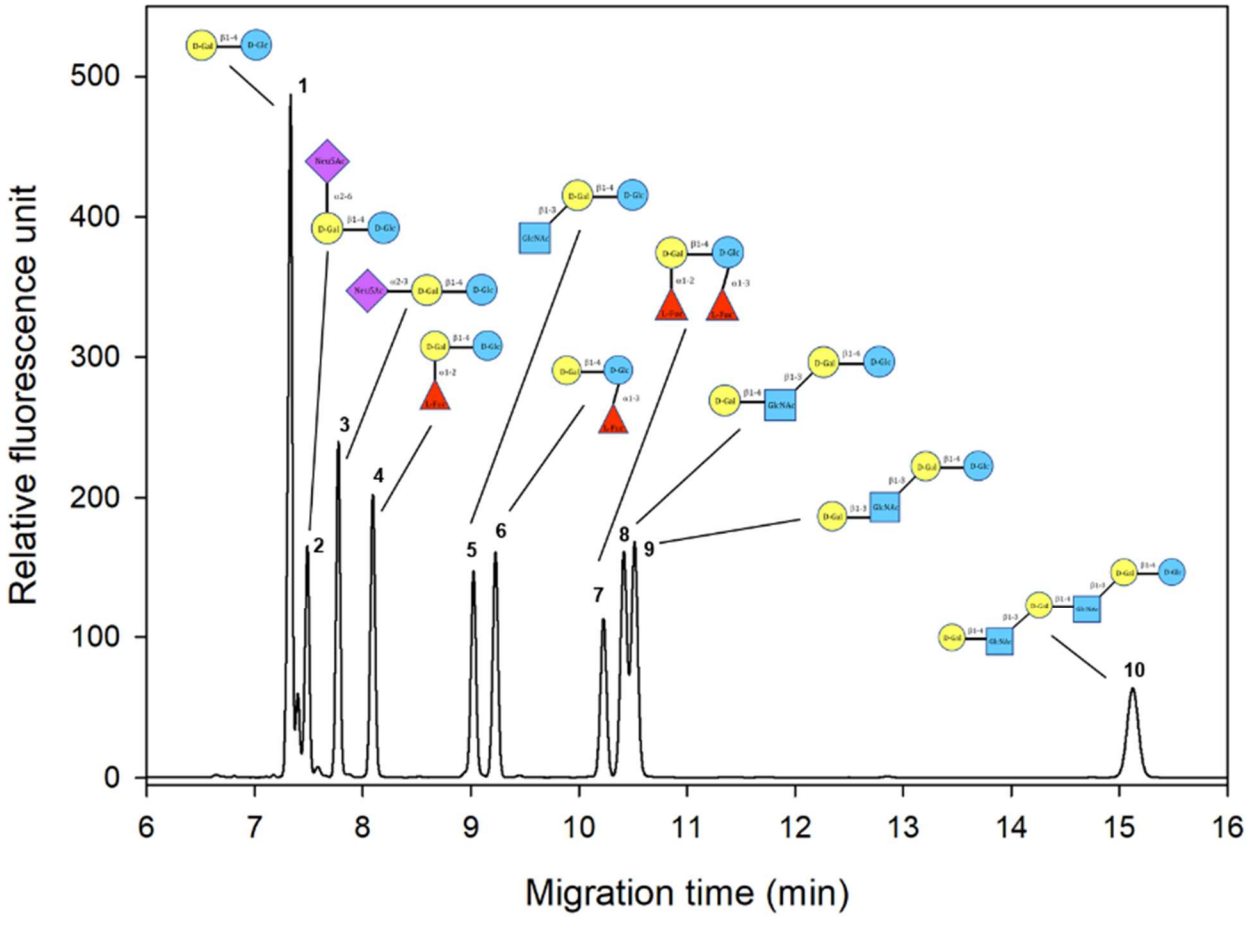


Figure 3. Fine tuning of the resolving power by mixing the HR-NCHO and SDS-MW gels (1:1)

Conclusions

In this Technical Note, two complementary separation matrices the HR-NCHO and SDS-MW gels were compared for ultrafast and high resolution analysis of human milk oligosaccharides (HMOs) by capillary electrophoresis with laser induced fluorescence detection as shown in the workflow of Figure 1. The 2'- and 3-fucosyllactoses were co-migrating with the use of the HR-NCHO gel-buffer system, while well separated in the SDS-MW gel. Conversely, the lacto-N-tetraose and lacto-N-neotetraose, which were well separated by the HR-NCHO gel, resulted only in a split peak with the SDS-MW gel composition. Therefore, the appropriate separation matrix should be chosen to address adequate HMO analysis during production. In a rare case requiring the use of both gels for a sample mixture possibly containing the fucosyllactose and lactotetraose isomers, the total analysis time of the two runs would still be less than 10 minutes. Alternatively, the two gel matrices can be mixed, and the separation temperature can be fine-tuned to accommodate the actual separation problem in hand. **Researchers can now rapidly and efficiently analyze human milk oligosaccharides from production batches, baby formulas or food additives by simply choosing any of these gel-buffer systems or a mixture od both to obtain the desired resolution.**

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