Journal of Pharmaceutical and Biomedical Analysis 233 (2023) 115434

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Short communication

# Capillary electrophoresis analysis of industrial galactooligosaccharides

Daniel Sarkozy [a], Robert Farsang [b], Marton Szigeti [b], Sean Austin [c], Stephen Lock [d], Andras Guttman [a,b,*]

[a] Horváth Csaba Memorial Laboratory of Bioseparation Sciences, Research Center for Molecular Medicine, Faculty of Medicine, Doctoral School of Molecular Medicine, University of Debrecen, Hungary
[b] Translational Glycomics Research Group, Research Institute of Biomolecular and Chemical Engineering, University of Pannonia, Veszprem, Hungary
[c] Nestlé Research, Vers-Chez-Les-Blanc, Lausanne, Switzerland
[d] Sciex, Alderley Park, UK

ARTICLE INFO

*Keywords:*
Capillary electrophoresis
Galactooligosaccharides
Infant formula
Amyloglucosidase
Galactosidase

ABSTRACT

Galactooligosaccharides are added to infant formula to simulate some of the benefits associated with human milk oligosaccharides, in particular to modulate the gut microbiota. During our study the galactooligosaccharide content of an industrial GOS ingredient was determined by differential enzymatic digestion using amyloglucosidase and β-galactosidase. The resulting digests were fluorophore labeled and analyzed by capillary gel electrophoresis with laser induced fluorescence detection. Quantification of the results were based on a lactose calibration curve. Utilizing this approach, the galactooligosaccharide concentration of the sample was determined as 37.23 g/100 g, very similar to earlier HPLC results, but requiring only 20 min separation time. The CGE-LIF method in conjunction with the differential enzymatic digestion protocol demonstrated in this paper offers a rapid and easy to use method to measure galactooligosaccharides and should be applicable to the determination of GOS in infant formulas and other products.

## 1. Introduction

Beta-Galactooligosaccharides (GOS) are non-digestible oligosaccharides industrially synthesised by lactose trans-galactosylation, typically containing between 2 and 8 monosaccharide residues composed of galactose having a glucose or a galactose residue at the reducing end [1]. The galactose residues are typically linked via β1→2, β1→3, β1→4 or β1→6 glycosidic bonds [2–4]. The ratios of the different types of linkages and the final chain length depend on the reaction conditions and the enzymes used for the trans-galactosylation reaction [5]. GOS have been shown to have bifidogenic properties [6,7] and have been added to infant formulas to mimic the effect of human milk oligosaccharides (HMO) [7,8]. They have also been shown to have benefits for the elderly population [9] and may be protective against the so called travelers' diarrhea syndrome [10].

GOS can be analyzed, among other methods, by anion exchange chromatography developed by de Slegte [11], which was approved as an official AOAC method [12]. In their technique, the GOS contents were determined by analyzing the galactose and lactose content before and after enzymatically hydrolyzing the oligosaccharide to glucose and galactose monomers. The galactose released from the GOS represents the actual GOS content. In 2018, Hui et al. improved the de Slegte method to measure both the glucose and galactose contents of GOS ingredients using a similar approach [13]. Unfortunately, neither the de Slegte nor the Hui methods are suitable for the analysis of GOS in high backgrounds of lactose, such as found in infant formula or milk-based products. In order to deal with such matrices, Austin et al. used hydrophilic interaction liquid chromatography (HILIC) with fluorescent and mass spectrometric detection to characterize 2-aminobenzamide (2AB) labeled oligosaccharides and developed a suitable method with good reproducibility for GOS determination in baby formulas as well as adult nutritional products [14]. Besides liquid chromatography, capillary electrophoresis (CE) can also provide high resolution separation of oligosaccharides. Similar to HILIC, CE analyzes fluorophore labeled carbohydrates but utilizes high sensitivity laser induced fluorescent (LIF) detection. The single tag based on structure stoichiometry makes

*Abbreviations:* GOS, galactooligosaccharides; BFS, bare fused silica; CGE-LIF, capillary gel electrophoresis with laser induced fluorescent detection; APTS, 8-Aminopyrene-1,3,6-trisulfonic acid; HMO, human milk oligosaccharides.

* Corresponding author at: Horváth Csaba Memorial Laboratory of Bioseparation Sciences, Research Center for Molecular Medicine, Faculty of Medicine, Doctoral School of Molecular Medicine, University of Debrecen, Hungary.
*E-mail address:* guttmanandras@med.unideb.hu (A. Guttman).

https://doi.org/10.1016/j.jpba.2023.115434
Received 30 March 2023; Received in revised form 28 April 2023; Accepted 29 April 2023
Available online 5 May 2023

quantification easy. The traditionally used 8-aminopyrene-1,3,6-trisulfonic acid (APTS) tag in CE provides three strong negative charges for the oligosaccharide structures enabling their electric field mediated separation with high sensitivity detection [15]. For the time being, there have only been a few attempts to quantify APTS labeled milk oligosaccharides by CE. Albrecht and coworkers demonstrated reproducible galactooligosaccharide extraction with quantitative and high resolution CE-LIF based analysis of complex food samples [16]. Later, Sarkozy et al. reported a high-throughput application using multi-capillary gel electrophoresis optimized for APTS tagged HMO that could be used to quantify low-abundance oligomers from human milk samples [17]. However, satisfactory methodologies to determine the GOS content in matrices with high lactose background are still in high demand.

The objective of our work was to develop a rapid capillary gel electrophoresis based separation technique, which would be applicable for GOS determination in food products including infant formula and consequently in other nutritional products. In conjunction with the amyloglucosidase and galactosidase based differential enzymatic digestion process, our novel approach described in this paper offers a fast complementary method to LC, applicable for the analysis of samples with high lactose background, with additional multiplexing options in the future.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Water (HPLC grade), ammonium acetate, 2-picoline borane and tetrahydrofuran were obtained from Sigma Aldrich (St. Louis, MO, USA). 8-Aminopyrene-1,3,6-trisulfonic acid (APTS) and the N-linked carbohydrate separation buffer (NCHO) of the Fast Glycan Kit were from Bio-Science Kft (Budapest, Hungary). The GOS powder (G99–00019), maltodextrin ladder and lactose were from Nestlé (Lausanne, Switzerland). Glacial acetic acid was purchased from Molar Chemicals Kft (Halásztelek, Hungary). The amyloglucosidase was from Roche (Basel, Switzerland) and the galactosidase from Megazyme (Wicklow, Ireland).

### 2.2. Sample preparation of GOS sample

The GOS powder was dissolved in HPLC grade water (70 °C,15 min) to produce the sample stock solution (10 mg/mL final concentration). To perform amyloglucosidase digestion the GOS sample (50 μL,2 mg/mL) was mixed with amyloglucosidase enzyme solution (20 μL, 60 U/mL in 10 mM ammonium acetate) and HPLC grade water (30 μL). To perform the amyloglucosidase + galactosidase digestion, the GOS sample (50 μL,2 mg/mL) was mixed with 20 μL of amyloglucosidase (as above) and galactosidase (5 μL, 4000 U/mL) and HPLC grade water (20 μL). In both cases, the final concentration of the GOS powder was 1 mg/mL. The digestion reactions were incubated at 60 °C for 2.0 h, followed by inactivation of the enzymes (90 °C, 5.0 min).

### 2.3. Lactose sample preparation for calibration

For quantification, a dilution series based calibration curve of five lactose concentrations were made, covering the tentative GOS concentration range of the sample. The lactose calibration series were made by serial dilution of the 1 mg/mL lactose stock solution to obtain 200; 100; 50; 25 and 12.5 μg/mL concentrations. Lactose spiking of the sample used the 1 mg/mL concentrated solution.

### 2.4. APTS labeling

The fluorophore labeling reaction mixture was prepared by mixing HPLC grade water (2 μL), glacial acetic acid (2.5 μL), tetrahydrofuran (3.5 μL), 2-picoline borane in tetrahydrofuran (1.5 M, 1 μL) and APTS solution (120 mM, 1 μL). Then, the reaction mixtures underwent evaporative labeling (60 °C,1.5 h) with open vial lids, followed by reconstitution in HPLC grade water (1.0 mL). Before CE-LIF measurements, all samples were 100 fold diluted using HPLC grade water and mineral oil (5.0 μL) was placed on top of the samples to prevent evaporation during analysis.

### 2.5. Capillary gel electrophoresis

All CGE measurements were performed on a PA 800 plus capillary electrophoresis system (Beckman Coulter, Brea, CA), equipped with a laser-induced fluorescence (LIF) detector (488 nm excitation and 520 nm emission) using the NCHO gel-buffer in bare fused silica capillary columns (50 μm ID, 50 cm total length with 40 cm effective length). Before the first run, the separation capillary was rinsed with HPLC grade water, 0.5 M NaOH and 0.5 M HCl (90 psi,2 min each). Between the analytical runs, the capillary was only rinsed with the gel–buffer system (3 min, 90 psi). Sample injection was by pressure (5.0 psi,5.0 s) with the temperature of capillary and the sample storage compartment both set at 15 °C. The applied electric field strength during the separations was 600 V/cm with reverse polarity separation mode (anode at the outlet capillary end). Three injections were carried out from each sample type and the calibration solutions.

## 3. Results and discussion

Galactooligosaccharides (GOS) are frequently added to food products such as infant formulas to imitate human milk oligosaccharides and furnish favorable effects via modulating the gut microbiota. During our study of an industrial GOS powder, four differently treated sets as well as one standard calibration set were prepared and analyzed as follows: (1) reference galactooligosaccharide sample, (2) lactose spiked, (3) amyloglucosidase digested, and (4) amyloglucosidase + β-galactosidase digested. The maltodextrins were removed by amyloglucosidase digestion and the GOS content was obtained as the result of the difference of the analyses of step 3 and 4, i.e., with and without β-galactosidase treatment. From each set, three randomly selected samples were measured three times on three different days which included using new buffers and wash solutions.

Fig. 1 shows the capillary gel electrophoresis separation trace of an industrial GOS sample featuring 21 peaks in the degree of polymerization (DP) range up to 8 in less than 11 min. The asterisks indicate APTS related peaks in the identification range of >DP1. The electropherogram apparently has a similar number of peaks as previously seen by HPLC analysis of the same sample, but was obtained 3 times faster (injection to injection [18]). To identify the lactose peak, the sample was spiked by a lactose standard. As the inset in Fig. 1 highlights, peak #3 corresponded to the lactose and was well separated from the other major oligosaccharides in the sample. Statistical evaluation of the separated peaks is depicted in Table 1 showing the average migration time and peak area reproducibilities as 0.276 and 2.496, respectively, from the analysis of 3 parallel samples, 3 separations each during a 3-day period.

The quantitative capability of the method was also thoroughly evaluated using the lactose calibration plot in Fig. 2, that was based on the peak areas of a lactose dilution series shown in Table 2. The lactose concentration was corrected with its purity level. The $r^2$ value of the plot was > 0.999. If the separation capillary and/or instrument is changed, the lactose calibration must be repeated again for downstream use similar to that of other separation methods. The %RSD of the lactose calibration was 2.15.

Similar to earlier HPLC work [18], analysis of the sample was accomplished by applying amyloglucosidase treatment to digest all maltooligomers to their glucose monomer constituents, i.e., to reduce the number of interfering sample components with aldose at their reducing end. During this treatment, apparently all small peaks from the 15–21 range (Fig. 1) shifted to peak 1, proven by the increase in its

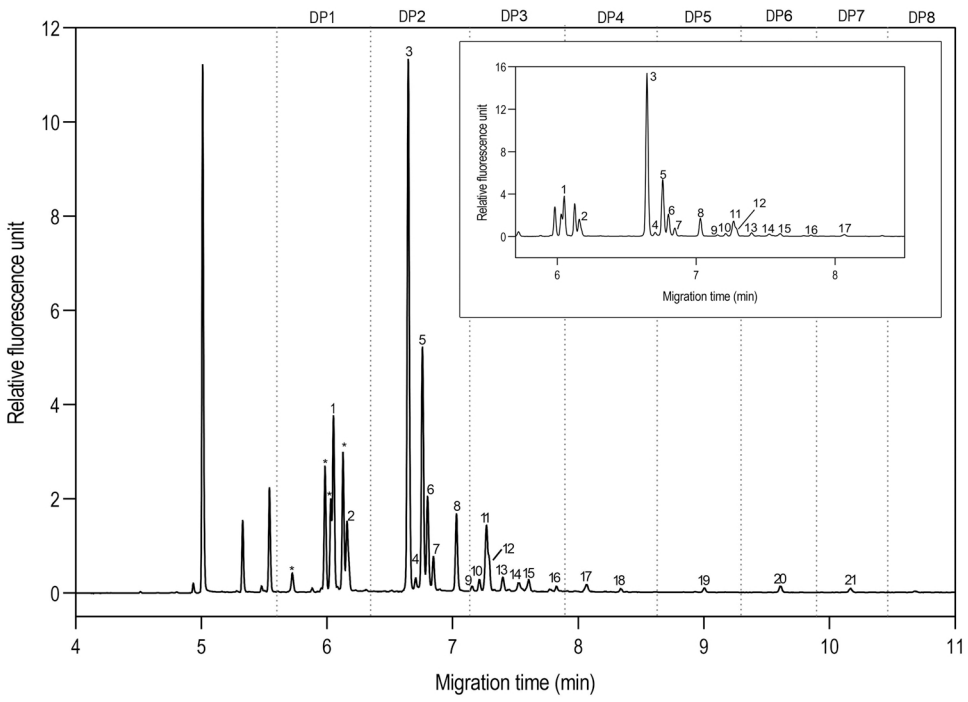


**Fig. 1.** Rapid oligosaccharide profiling of an industrial GOS sample by CE-LIF. The ranges in degree of polymerization (DP) are shown in the upper horizontal axis. The asterisks indicate APTS related peaks in the identification range. The inset depicts the lactose spiked sample showing the increase in peak#3. Separation conditions: 50 cm total length (40 cm effective length, 50 μm ID) BFS capillary, NCHO separation gel buffer, 15 °C separation and sample storage temperature, 30 kV separation voltage in reversed polarity mode, 5.0 psi for 5.0 s sample injection.

**Table 1**
Statistical evaluation of the free oligosaccharides in the industrial GOS sample. The term average represents the consideration of 3 parallel samples with 3 separations each, i.e., 9 measurements during a 3-day period.

| Peak | DP | Migration time (min) | | | Area | | | %Area |
|---|---|---|---|---|---|---|---|---|
| | | Average | Std. Dev. | %RSD | Average | Std. Dev. | %RSD | |
| 1 | 1 | 6.04 | 0.016 | 0.270 | 6.48E-02 | 1.67E-03 | 2.581 | 11.17 |
| 2 | 1 | 6.15 | 0.017 | 0.269 | 2.95E-02 | 4.65E-04 | 1.576 | 5.08 |
| 3 | 2 | 6.64 | 0.018 | 0.272 | 2.17E-01 | 5.34E-03 | 2.459 | 37.49 |
| 4 | 2 | 6.69 | 0.018 | 0.273 | 5.31E-03 | 2.17E-04 | 4.086 | 0.92 |
| 5 | 2 | 6.75 | 0.019 | 0.275 | 1.00E-01 | 2.26E-03 | 2.248 | 17.33 |
| 6 | 2 | 6.79 | 0.018 | 0.272 | 3.89E-02 | 1.03E-03 | 2.648 | 6.72 |
| 7 | 2 | 6.84 | 0.019 | 0.273 | 1.41E-02 | 5.27E-04 | 3.746 | 2.43 |
| 8 | 2 | 7.02 | 0.019 | 0.276 | 3.19E-02 | 7.33E-04 | 2.300 | 5.50 |
| 9 | 3 | 7.14 | 0.020 | 0.274 | 2.09E-03 | 5.99E-05 | 2.864 | 0.36 |
| 10 | 3 | 7.20 | 0.020 | 0.275 | 4.89E-03 | 1.43E-04 | 2.931 | 0.84 |
| 11 | 3 | 7.26 | 0.020 | 0.278 | 2.77E-02 | 5.29E-04 | 1.910 | 4.78 |
| 12 | 3 | 7.28 | 0.020 | 0.278 | 1.39E-02 | 2.92E-04 | 2.099 | 2.40 |
| 13 | 3 | 7.39 | 0.020 | 0.276 | 6.24E-03 | 1.22E-04 | 1.947 | 1.08 |
| 14 | 3 | 7.51 | 0.021 | 0.275 | 4.20E-03 | 1.02E-04 | 2.431 | 0.72 |
| 15 | 3 | 7.59 | 0.021 | 0.274 | 5.10E-03 | 7.57E-05 | 1.482 | 0.88 |
| 16 | 3 | 7.81 | 0.022 | 0.277 | 2.14E-03 | 7.34E-05 | 3.425 | 0.37 |
| 17 | 4 | 8.05 | 0.022 | 0.276 | 3.35E-03 | 6.11E-05 | 1.823 | 0.58 |
| 18 | 4 | 8.33 | 0.023 | 0.275 | 1.30E-03 | 1.72E-05 | 1.322 | 0.22 |
| 19 | 5 | 8.99 | 0.025 | 0.276 | 1.74E-03 | 6.11E-05 | 3.513 | 0.30 |
| 20 | 6 | 9.60 | 0.027 | 0.283 | 3.00E-03 | 5.84E-05 | 1.947 | 0.52 |
| 21 | 7 | 10.15 | 0.029 | 0.288 | 1.91E-03 | 5.86E-05 | 3.074 | 0.33 |
| | | Average | | 0.276 | | | 2.496 | |
| | | | | | Average | | | |
| | | Min | | 0.269 | Min | | 1.322 | |
| | | Max | | 0.288 | Max | | 4.086 | |

relative abundance (Fig. 3, trace A), suggesting that these later migrating peaks were maltooligosaccharides with glucose building blocks. Following the amyloglucosidase treatment, the sample was further treated with β-galactosidase (Fig. 3, trace B), which resulted in shift of all peaks (see inset for smaller peaks) into the DP2–7 range (Fig. 1) to peak 2, i.e., the galactose peak.

The total oligosaccharide content of the GOS powder (excluding lactose) was determined before and after the beta-galactosidase enzyme treatment. The difference between the two analyses was used to calculate the GOS content of the powder resulting in 37.23 g/100 g. This compares well with the 37.0 g/100 g determined by the official HPLC method [12].

## 4. Conclusion

Galactooligosaccharides are important supplements in infant formulas to mimic HMOs and provide beneficial gut microbiota modulating effects. In this paper we reported on a CGE-LIF based analysis of galactooligosaccharides utilizing a differential enzymatic digestion process (amyloglucosidase and galactosidase). The GOS concentration of the

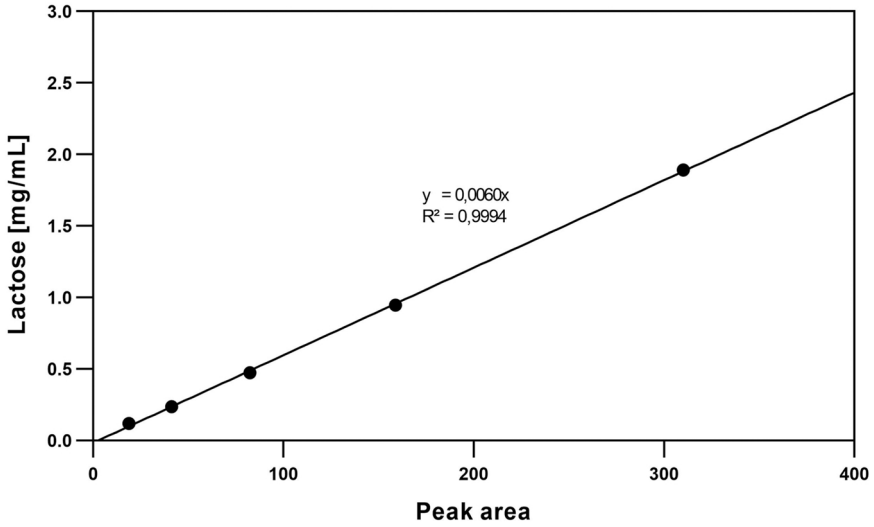


**Fig. 2.** Quantification calibration plot based on the lactose dilution series shown in Table 2.

**Table 2**
Dilution series for lactose calibration. The actually used lactose concentration was corrected with its purity of 94.56 % ($MW_{lactose}$ = 342.3).

| Lactose Conc. [mg/mL] | Corrected Conc. [mg/mL] | Integrated peak area | Measured concentration [mg/mL] | Ratio of measured / Actual concentration |
|---|---|---|---|---|
| 2.000 | 1.891 | 310.210 | 1.888 | 0.998 |
| 1.000 | 0.946 | 158.867 | 0.944 | 0.998 |
| 0.500 | 0.473 | 82.458 | 0.472 | 0.998 |
| 0.250 | 0.236 | 41.311 | 0.236 | 1.000 |
| 0.125 | 0.118 | 18.876 | 0.120 | 1.014 |

sample was determined as 37.23 g/100 g, relating well to the determination by the official (de Slegte) method of 37.00 g/100 g. While the separation step (injection to injection) took only 20 min, the overall sample preparation time was 2.0 h including sample handling, pipetting, reagent preparation and fluorophore labeling/cleanup (100 min). The enzymatic digestions required extra time similar to the HPLC method. The simple capillary gel electrophoresis based method introduced in this paper proved to be appropriate to determine the actual galactooligosaccharide content of a GOS ingredient. Based on our results, we anticipate that CE could replace liquid chromatography methods for rapid analysis of GOS in infant formula and other lactose-containing samples containing typical GOS concentration ranges for such products. Capillary gel electrophoresis based methods also offer the potential of ultrahigh throughput if multicapillary systems are used.

## CRediT authorship contribution statement

**Daniel Sarkozy:** Investigation. **Robert Farsang:** Formal analysis. **Marton Szigeti:** Validation. **Sean Austin:** Conceptualization. **Stephen Lock:** Methodology. **Andras Guttman:** Supervision, Writing – original draft.

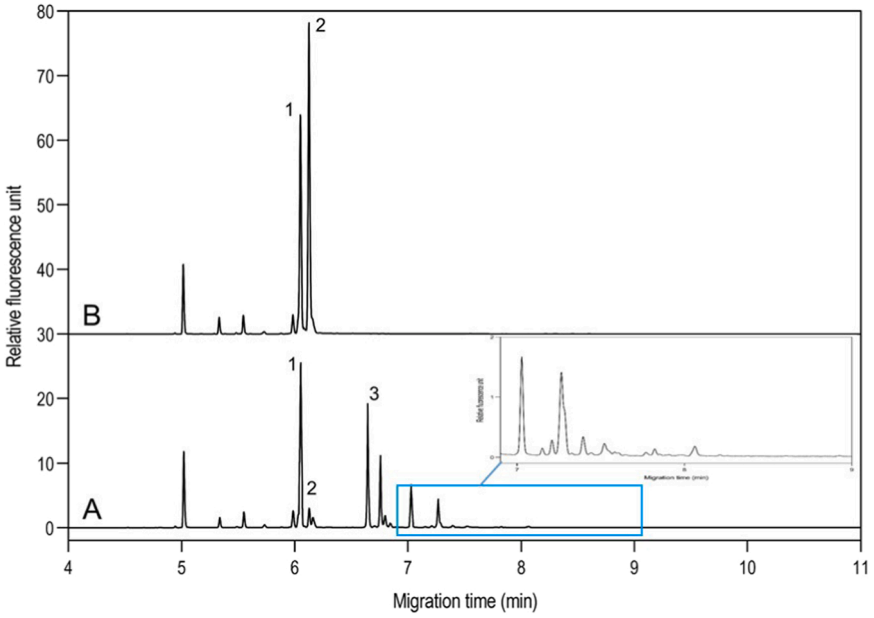


**Fig. 3.** Enzymatic digestion results of the industrial GOS sample analyzed in Fig. 1. Trace A: amyloglucosidase digested GOS powder, Trace B: amyloglucosidase + β-galactosidase digested GOS powder. Peaks of importance: 1 - glucose; 2 - galactose; 3 - lactose. Separation conditions were the same as in Fig. 1. Inset: for better visualization of the smaller peaks in trace A.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Acknowledgment

This is contribution #188 from the Horváth Csaba Memorial Laboratory of Bioseparation Sciences.

## References

[1] D.P.M. Torres, M. do Pilar, F. Goncalves, J.A. Teixeira, L.R. Rodrigues, Galacto-oligosaccharides: production, properties, applications, and significance as prebiotics, Compr. Rev. Food Sci. Food Saf. 9 (2010) 438–454.
[2] S.S. van Leeuwen, B.J.H. Kuipers, L. Dijkhuizen, J.P. Kamerling, Comparative structural characterization of 7 commercial galacto-oligosaccharide (GOS) products, Carbohydr. Res. 425 (2016) 48–58.
[3] O. Hernandez-Hernandez, I. Calvillo, R. Lebron-Aguilar, F.J. Moreno, M.L. Sanz, Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides, J. Chromatogr. A 1220 (2012) 57–67.
[4] L. Coulier, J. Timmermans, R. Bas, D.R. Van Den, I. Haaksman, B. Klarenbeek, T. Slaghek, W. Van Dongen, In-depth characterization of prebiotic galacto-oligosaccharides by a combination of analytical techniques, J. Agric. Food Chem. 57 (18) (2009) 8488–8495.
[5] J. Yang, Y. Xu, Functional carbohydrate polymers: prebiotics, in: T.J. Gutierrez (Ed.), Polymers for Food Applications, Springer International Publishing AG, Switzerland, 2018, pp. 651–691.
[6] M. Ito, Y. Deguchi, A. Miyamori, K. Matsumoto, H. Kikuchi, K. Matsumoto, Y. Kobayashi, T. Yajima, T. Kan, Effects of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation, Microb. Ecol. Health Dis. 3 (6) (1990) 285–292.
[7] G.E. Moro, S. Arslanoglu, Reproducing the bifidogenic effect of human milk in formula-fed infants: why and how? Acta Paediatr. 94 (s449) (2005) 14–17.
[8] Y. Vandenplas, Oligosaccharides in infant formula, Br. J. Nutr. 87 (2002) S293–S296.
[9] A. Surakka, K. Kajander, M. Rajilic-Stojanovic, H. Karjalainen, K. Hatakka, H. Vapaatalo, E.G. Zoetendal, W.M. De Vos, R. Korpela, S. Tynkkynen, Yoghurt containing galactooligosaccharides facilitates defecation among elderly subjects and selectively increases the number of Bifidobacteria, Int. J. Probiot. Prebiot. 4 (1) (2009) 65–74.
[10] A. Drakoularakou, G. Tzortzis, R.A. Rastall, G.R. Gibson, A double-blind, placebo-controlled, randomized human study assessing the capacity of a novel galacto-oligosaccharide mixture in reducing travellers' diarrhoea, Eur. J. Clin. Nutr. 64 (2) (2010) 146–152.
[11] J. de Slegte, Determination of trans-galactooligosaccharides in selected food products by ion-exchange chromatography: collaborative study, J. AOAC Int 85 (2) (2002) 417–423.
[12] Official Methods of Analysis, AOAC International, Rockville, MD, 2019, p. Method 2001.02.
[13] L. Hui, S. Li, C. Xu, M. Pang, S. Wang, Simultaneous determination of galactose, glucose, lactose and galactooligosaccharides in galactooligosaccharides raw materials by highperformance anion-exchange chromatography with pulsed amperometric detection, Food Chem. 263 (2018) 29–36.
[14] D. Cuany, F. Andestion, X. Fontannaz, T. Benet, V. Spichtig, S. Austin, Determination of β-galactooligosaccharides (GOS) in infant formula and adult nutritionals: single laboratory validation, first action 2021.01, J. AOAC Int. 105 (1) (2022) 142–158.
[15] A. Guttman, High-resolution carbohydrate profiling by capillary gel electrophoresis, Nature 380 (6573) (1996) 461–462.
[16] S. Albrecht, H.A. Schols, B. Klarenbeek, A.G. Voragen, H. Gruppen, Introducing capillary electrophoresis with laser-induced fluorescence (CE-LIF) as a potential analysis and quantification tool for galactooligosaccharides extracted from complex food matrices, J. Agric. Food Chem. 58 (5) (2010) 2787–2794.
[17] D. Sarkozy, B. Borza, A. Domokos, E. Varadi, M. Szigeti, A. Meszaros-Matwiejuk, D. Molnar-Gabor, A. Guttman, Ultrafast high-resolution analysis of human milk oligosaccharides by multicapillary gel electrophoresis, Food Chem. 341 (Pt 2) (2021), 128200.
[18] S. Austin, T. Benet, J. Michaud, D. Cuany, P. Rohfritsch, Determination of b-galactooligosaccharides by liquid chromatography, Int. J. Anal. Chem. 2014 (2014), 768406.