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Introducing Capillary Electrophoresis with Laser-Induced Fluorescence (CE−LIF) as a Potential Analysis and Quantification Tool for Galactooligosaccharides Extracted from Complex Food Matrices

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The analysis and quantification of (galacto)oligosaccharides from food matrices demands both a reproducible extraction method as well as a sensitive and accurate analytical method. Three typical matrices, namely, infant formula, fruit juice, and a maltodextrin-rich preparation, to which a commercial galactooligosaccharide mixture was added in a product concentration range from 1.25 to 30%, served as model substrates. Solid-phase extraction on graphitized carbon material upon enzymatic amyloglucosidase pretreatment enabled a good recovery and a selective purification of the different galactooligosaccharide structures from the exceeding amounts of particularly lactose and maltodextrins. With the implementation of capillary electrophoresis in combination with laser-induced fluorescence (CE−LIF) detection, a new possibility facilitating a sensitive qualitative and quantitative determination of the galactooligosaccharide contents in the different food matrices is outlined. Simultaneous monitoring and quantifying prebiotic oligosaccharides embedded in food matrices presents a promising and important step toward an efficient monitoring of individual oligosaccharides and is of interest for research areas dealing with small quantities of oligosaccharides embedded in complex matrices, e.g., body liquids.

KEYWORDS: CE−LIF; galactooligosaccharides; food matrices; quantification

INTRODUCTION

Prebiotic carbohydrates, mainly nondigestible oligosaccharides (NDOs), play a significant role in the fortification of food products with beneficial gut flora components. The amount of prebiotics added to a food matrix is usually low, because studies have revealed already beneficial effects by the ingestion of 2−10 g of NDOs/day (*1*−*3*). Evenmore, a supplementation with doses ≥15 g of NDOs/day may lead to abdominal pain, flatulence, and even diarrhea (*4*, *5*). Maltodextrins, lactose, and monomeric sugars are the common predominating carbohydrates in these food matrices, for example, in powdered milk-based products (*6*). Therefore, quality control of functional food products is a demanding field regarding the sensitivity and accuracy of analytical methods. The presence of such methods is indispensable, and this need has even been reinforced by the recently established European Union (EU) Health Claim Regulation (EG 1924/2006), which demands scientific substantiation of the functional ingredients, concerning both their gastrointestinal functionality and quantity (*7*).

Galactooligosaccharides are prominent representatives of prebiotic carbohydrates, implying their fermentability by beneficial intestinal bacteria and selective stimulation of the latter as well as the resistance to acid hydrolysis, enzymatic digestion, and absorption in the upper gastrointestinal tract (*8*).

The frequent use of galactooligosaccharides in all kind of processed foods, such as acidic beverages, fermented milk products, confectionery, or baby food, can be related to their low-caloric value, bulking capacity, low sweetness, noncariosity, excellent solubility, and heat and storage stability, even in an acidic environment (*9*−*11*). Galactooligosaccharides are usually produced from lactose. The incubation of lactose with fungal, yeast-derived, or bacterial β-galactosidases enables transglycosylation reactions and results in a complex mixture of reducing as well as, in minor amounts, non-reducing galactooligosaccharides or also called *trans*-galactooligosaccharides (DP2−DP≥4). In these mixtures, lactose, glucose, and galactose can be present as well, because of the fact that galactooligosaccharides are kinetic intermediates and also substrates for hydrolysis during production (*10*, *12*). The DP and linkages formed are strongly dependent upon the reaction conditions and enzymes used (*11*).

The existing Association of Official Analytical Chemists (AOAC) method for the determination of *trans*-galactooligosaccharides in food products [AOAC 2001.02 (*6*)] is based on high-performance anion-exchange chromatography−pulsed amperometric detection (HPAEC−PAD) analysis of galactose after β-galactosidase incubation of the galactooligosaccharide-containing matrix, yielding the total amount of galactose present in

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the mixture. According to this AOAC method, the term galactooligosaccharides is defined to include dimers (excluding lactose) and larger oligosaccharides. Although being a robust method, it does not provide any information on the individual oligosaccharides present. Such information would be highly desirable in view of the vast variability in structural features characteristic for galactooligosaccharide mixtures because the composition is used as a quality and identification parameter. Another disadvantage of the AOAC method is that high-lactose contents present in the food matrix tend to influence the measurement of galactose (*6*).

Capillary electrophoresis with laser-induced fluorescence (CE−LIF) detection has been shown to be a powerful and sensitive technique in the separation of 9-aminopyrene-1,4,6-trisulfonate (APTS)-tagged oligosaccharides derived from plant polysaccharides and other natural sources (*13*−*15*). Therefore, this method could also be of great value for the analysis of prebiotic oligosaccharides in complex matrices. Labeling carbohydrates with the fluorescent dye APTS is based on a reductive amination reaction (*16*). APTS carries three negative charges, which provides the neutral carbohydrates with charge, a prerequisite for electrophoretic separation (*17*). Each carbohydrate molecule is attached to one fluorescent molecule. When an internal standard is included, CE−LIF is, therefore, a potential technique for quantitative analysis. Because APTS is only attached to reducing oligosaccharides, non-reducing oligosaccharides are not included in this derivatization method. Galactooligosaccharide mixtures are usually composed of predominantly reducing oligosaccharides. They are often applied in combination with fructooligosaccharides (mainly non-reducing oligosaccharides), e.g., in infant formula (*18*). A labeling of exclusively reducing oligosaccharides thus guarantees a selective detection of galactooligosaccharides in these mixtures. However, the small amount of non-reducing galactooligosaccharides is not included in the analysis and has to be taken into account differently (e.g., by means of a calibration curve).

Besides a preliminary quantitative screening in the beginning of the 1990s, in which APTS was introduced as the fluorescent dye of choice for CE−LIF analysis of carbohydrates (*16*), the quantitative use of CE−LIF has only been studied in detail for monosaccharides and not for oligosaccharides (*14*, *15*).

Characterization and quantification of galactooligosaccharides from food matrices in the first instance demands a reproducible extraction and purification from the predominating and disturbing components present, mainly monomers, lactose, and maltodextrins.

To evaluate the suitability of CE−LIF as an analysis tool for commercially available galactooligosaccharides, the first part of this research is aimed at a reproducible extraction of galactooligosaccharides from complex food matrices. It focuses on the fine-tuning of a solid-phase extraction (SPE) method for oligosaccharides, on the basis of the use of graphitized carbon cartridges (*19*), in combination with an enzymatic amyloglucosidase pretreatment to remove maltodextrins (*20*). Next, CE−LIF is tested for a reproducible quantification of oligosaccharides in general and, more specifically, galactooligosaccharides extracted from food matrices.

MATERIALS AND METHODS

Vivinal GOS, Vivinal GOS-Containing Samples, Enzymes, and Chemicals. Vivinal GOS syrup, Vivinal GOS/maltodextrin preparation, and infant formula were provided by FrieslandCampina DOMO (Zwolle, The Netherlands). The Vivinal GOS syrup (reference material) was prepared by a β-galactosidase from *Bacillus circulans* and specified by the supplier with a dry matter of 75% (w/w), of which 59% (w/w) is galactooligosaccharides, 21% (w/w) is lactose, 19% (w/w) is glucose, and 1% (w/w) is galactose, as determined according to AOAC 2001.02 (*6*). The galactooligosaccharides present in the mixture are predominantly reducing oligosaccharides, with $[\beta$-D-Gal-(1,4)-β-D-Gal-(1,4)$]_n$-Glc being the most abundant structural element for oligosaccharides of DP $\geq$ 3, as determined by nuclear magnetic resonance (NMR) analysis (*21*). Non-reducing oligosaccharides are present predominantely in the form of dimers; β-D-Gal-(1,1)-β-D-Glc and β-D-Gal-(1,1)-α-D-Glc make up about 3% of the oligosaccharide mixture (*21*).

The Vivinal GOS/maltodextrin preparation was specified by the supplier to be composed of 48.5% (w/w) maltodextrins, 28.5% (w/w) Vivinal galactooligosaccharides, 10.1% (w/w) lactose, and 9.7% (w/w) mono- and other disaccharides.

For infant formula, an approximate content of 3% (w/w) Vivinal galactooligosaccharides was given. No further sample specification was provided. Lactose (40%, w/w) and maltodextrin (10%, w/w) were found to be present in this matrix by analysis in our lab, using HPAEC−PAD.

The fruit juice is a commercial product and was obtained from a local shop. It is specified to contain 12.3% (w/v) sugars (glucose, fructose, and saccharose) and 1.25% (w/v) Vivinal galactooligosaccharides.

Amyloglucosidase (source: *Aspergillus niger*) was obtained from Boehringer (B208469, Boehringer, Mannheim, Germany).

Monosaccharides and oligosaccharides used as standards were D-xylose (Sigma-Aldrich, St. Louis, MO), α-L-1,5-arabinooligosaccharides DP3−DP5 (Megazyme, Bray, Ireland), maltodextrin−oligosaccharides (α-D-1,4-glucooligosaccharides) DP3−DP5 (Sigma-Aldrich, St. Louis, MO), and cellodextrin−oligosaccharides (β-D-1,4-glucooligosaccharides) DP3−DP5 (Sigma-Aldrich, St. Louis, MO). All other chemicals used were of analytical grade.

Fractionation of Vivinal GOS. Vivinal GOS was fractionated according to its DP by gel filtration on a BioGel P2 column (600 × 120 mm, 200−400 mesh; Bio-Rad, Hercules, CA). A total of 300 mg of Vivinal GOS syrup dissolved in 10 mL of water was applied to the column and was eluted with Millipore water (1.5 mL/min) at 60 °C using an Akta Explorer system (GE Amersham, Uppsala, Sweden). The column efflux was first led through a refractive index (RI) detector (Shodex RI-72, Showa Denko K.K., Tokyo, Japan) before it was collected in fractions of 5 mL. The fractions were pooled according to the RI profile and freeze-dried.

Oligosaccharide Extraction, Enzymatic Pretreatment, and Purification. For extraction of galactooligosaccharides, 100 mg of Vivinal GOS-containing matrix, respectively, infant formula, fruit juice, or the Vivinal GOS/maltodextrin preparation, was suspended in 50 mL of boiling water and kept at 80 °C for 30 min [AOAC 2001.02 (*6*)]. Fruit juice was adjusted to pH 7 prior to extraction. In the case of dominant levels of maltodextrins being present, specific enzymatic degradation of these maltodextrins using 148 μL of a 1% (w/v) amyloglucosidase solution per 1 mg/mL substrate solution was performed in 0.2 M sodium acetate buffer (pH 4.5, 55 °C, 24 h). The enzyme dosage was chosen according to the best result in the oligosaccharide profile and maltodextrin degradability obtained after testing different substrate/enzyme ratios. The enzyme was inactivated (10 min, 100 °C), and the sample was centrifuged (5 min, 10000*g*, room temperature). High levels of mono- and dimers present in the food matrix or resulting from enzymatic degradation of maltodextrins were removed by SPE on nonporous graphitized carbon cartridges (bed weight,150 mg; tube size, 4 mL; Alltech, Deerfield, IL). The cartridges were washed with 1.5 mL of 80:20 (v/v) acetonitrile (ACN)/water containing 0.1% (v/v) trifluoroacetic acid (TFA) followed by 1.5 mL of Millipore water. A total of 1 mL of the GOS-containing sample was loaded onto the cartridge, which was eluted with 1.5 mL of water. Subsequently, mono- and dimers were eluted with 1.5 mL of 2−5% (v/v) aqueus ACN, depending upon the sample matrix. Sample flowthrough, wash water, and mono- and dimers were trapped as one fraction. The oligosaccharides were eluted with 1.5 mL of 25:75 (v/v) ACN/water containing 0.05% (v/v) TFA and trapped as a separate fraction. The fractions were dried under a stream of air. The fraction containing mono- and dimers was rehydrated with 1 mL of Millipore water, and the oligosaccharide-containing fraction was rehydrated with 500 μL of Millipore water.

The sample matrices (infant formula, fruit juice, and Vivinal GOS/maltodextrin preparation) were extracted in duplicate.

Analytical Methods. *Matrix-Assisted Laser Desorption/Ionization−Time-of-Flight Mass Spectrometry (MALDI−TOF MS).* For

MALDI−TOF MS of oligosaccharides, an Ultraflex workstation (Bruker Daltronics, Bremen, Germany) equipped with a nitrogen laser of 337 nm was used. The measurement was performed in the positive mode. After a delayed extraction time of 200 ns, the ions were accelerated with a 25 kV voltage. The data were collected from averaging 100 laser shots, with the lowest laser energy necessary to obtain sufficient spectra intensity.

The mass spectrometer was calibrated with a mixture of maltodextrins (AVEBE, Veendam, The Netherlands; mass range of 527−2309 Da) as sodium adducts.

Samples (1mg/mL) were desalted with AG 50W-X8 resin (H^+ form; Bio-Rad, Hercules, CA). The desalted samples (1 μL) were mixed directly on a MS target plate with 1 μL of matrix solution consisting of an aqueus solution of 2,5-dihydroxybenzoic acid (10 mg/mL; Bruker Daltonics, Bremen, Germany). The mix was dried under a stream of warm air.

HPAEC. HPAEC of lactose was performed on a Dionex ISC 3000 system (Dionex, Sunnyvale, CA), equipped with a Dionex Carbopac PA-1 column (2 × 250 mm) in combination with a Carbopac PA-1 guard column (2 × 50 mm). Samples (20 μL) were injected by means of a Dionex ISC 3000 autosampler. Elution was performed at 0.3 mL/min at room temperature using a gradient of 0−150 mM sodium acetate in 100 mM NaOH for 15 min. Each elution was followed by a washing step (10 min, 1 M NaOAc in 100 mM NaOH) and an equilibration step (15 min, 100 mM NaOH). Detection was performed using a Dionex ED40 detector in the PAD mode.

CE−LIF. Separation of APTS-derivatized carbohydrates was performed using a ProteomeLab PA 800 characterization system (Beckman Coulter, Fullerton, CA) equipped with a polyvinyl alcohol (N−CHO)-coated capillary (50 μm × 50.2 cm). Detection was performed with a LIF detector (Beckman Coulter, Fullerton, CA) at an excitation wavelength of 488 nm and an emission wavelength of 520 nm after a 40 cm capillary length.

The capillary was pressure-rinsed with water (3 min, 30 psi) and separation buffer (2 min, 30 psi), composed of 25 mM sodium acetate (pH 4.75) containing 0.4% (v/v) polyethylene oxide. The vials of the rinsing solutions were changed every 12 samples. Supplementary to the pressure rinsing, the capillary was rinsed with water by applying current (20 min, 30 kV) to prevent sample carryover. Samples were loaded hydrodynamically (4 s at 0.5 psi, representing approximately 14 nL of sample solution) on the capillary, which was kept at 25 °C. Separation was performed in the reversed polarity mode (20 min, 30 kV) in separation buffer.

Sample Preparation for CE−LIF Analysis. Samples for CE−LIF were prepared using the ProteomeLab Carbohydrate Labeling and Analysis Kit (Beckman Coulter, Fullerton, CA). A total of 2 nmol of xylose was added as an internal standard and mobility marker to the samples (0.02−0.05 mg) instead of the 5 nmol of maltose internal standard proposed by the guidelines of the kit. This mixture was dried using a SpeedVac Concentrator Savant ISS110 (Thermo Electron Corporation, Waltham, MA). The mono- and oligosaccharides present in the dried sample were derivatized with 2 μL of APTS and 2 μL of sodium cyanoborohydride and, afterward, filled up to 50 μL by Millipore water. For CE−LIF, the samples were diluted 10−40 times.

RESULTS AND DISCUSSION

Composition of Vivinal GOS. Vivinal GOS syrup (75% DM) was used as a reference in this study. For characterizing the reference material, pools of Vivinal GOS were prepared by fractionation on Biogel P2 size-exclusion material (**Figure 1A**). The pools each consisted of saccharides of one DP, with minor contamination of the preceding and following DP, as determined by MALDI−TOF MS. Evaluating the proportion (%) of the respective pool areas (RI response) in relation to the total area (representing the total carbohydrate content of Vivinal GOS) resulted in 23% pool 1 (P1; DP1), 39% pool 2 (P2; DP2), 23% pool 3 (P3; DP3), 10% pool 4 (P4; DP4), 4% pool 5 (P5; DP5), and 1% pool 6 (P6; not considered for analysis). HPAEC analysis of Vivinal GOS resulted in a lactose content of 20%, which allowed us to define pool 2 as a mixture consisting of

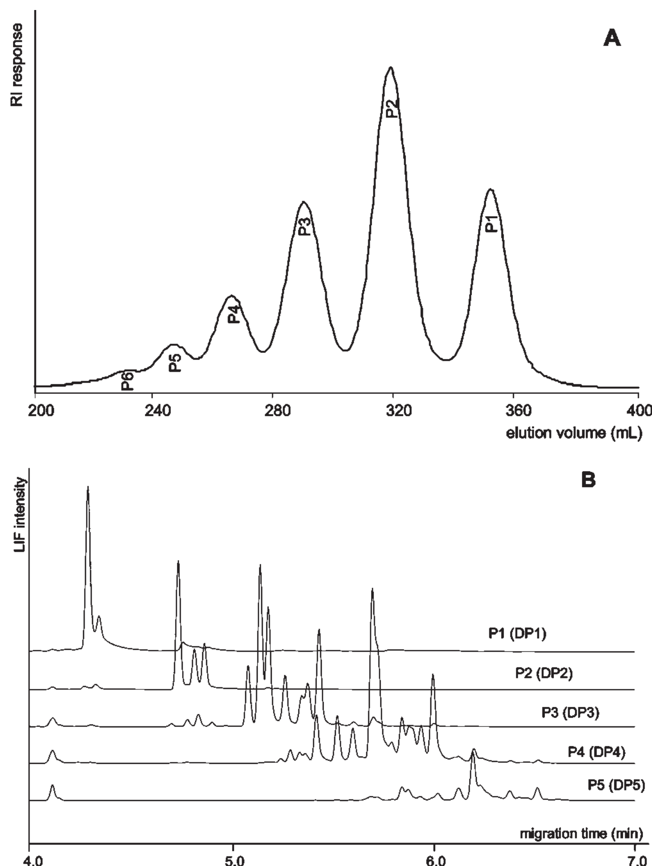

Figure 1. (A) Size-exclusion chromatography profile (RI response) obtained by Biogel P2 column separation of Vivinal GOS. P1−P6 = pool 1−6. (B) CE−LIF electropherograms of Biogel P2 pools. Pools representing DP1 and DP2 were diluted 10 times before CE−LIF analysis.

20% lactose and 19% galactooligosaccharide dimers. The addition of components results in a galactooligosaccharide content of 57%, which is close to what was specified by the supplier (59%).

In view of the subsequent extraction of Vivinal galactooligosaccharides from complex matrices and analysis and quantification by CE−LIF, it is of prime importance to analyze this reference galactooligosaccharide mixture itself by CE−LIF. The electropherograms of the DP1−DP5 pools, derivatized with the fluorescent dye APTS, showed distinct migration times (**Figure 1B**). Although a separation according to the M_w of the molecules is known for CE−LIF (*14*), one has to accept a narrow time window of peak overlap in the beginning and end of the respective DP pools, as is visible for the last abundant peak of the DP3 pool and the first abundant peak of the DP4 pool. This may also slightly influence the subsequent quantification. In the present case, the overlapping peak was accounted for DP3 because the DP3 peak is more abundant than the respective DP4 peak. With increasing DP, increased numbers of peaks were found as was expected for complex transgalactosylation products, indicating the gradual extension of oligosaccharides formed by the β-galactosidase used. A good peak resolution was obtained for the respective DPs. The presence of at least 7 oligosaccharides could be found for the pool representing DP3, with at least 10 for DP4 (pool 4) and at least 8 for DP5 (pool 5) (**Figure 1B**). Our findings are comparable to those by Coulier et al. (*21*). These authors annotated 7 reducing DP3 oligosaccharides by NMR spectroscopy after isolation using size-exclusion chromatography (SEC) and hydrophilic interaction liquid chromatography (HILIC), as well as methylation analysis. In addition,

4 non-reducing minor oligosaccharides were tentatively assigned (*21*).

The standard APTS-labeling conditions advised by the manufacturer (60 °C, 1.5 h) were applied in this study. Testing different labeling conditions (change of derivatization time and temperature and APTS concentration) from those indicated, showed that the conditions set by the manufacturer were most optimal.

Extraction of Galactooligosaccharides from Complex Matrices. Three different matrices (infant formula, fruit juice, and Vivinal GOS/maltodextrin preparation) were chosen to study the extractability and purification of Vivinal galactooligosaccharides added during the production process. The amount of galactooligosaccharides specified for these matrices was determined by the supplier according to the total galactose content after enzymatic hydrolysis of the galactooligosaccharides with β-galactosidase [AOAC 2001.02 (*6*)].

The crude aqueous sample extracts obtained after heat treatment (80 °C, 30 min) yielded electropherograms showing a number of dominating peaks representing monomers (t = 4−4.5 min) and/or lactose (t = 4.8 min), as exemplified for infant formula (curve I in **Figure 2A**). The large peaks caused an unstable baseline, and peaks resulting from oligosaccharides (t = 5−6.5 min) were hardly visible.

For the precise analysis of the galactooligosaccharides, the aqueous sample extracts were loaded on cartridges filled with nonporous graphitized carbon material, for which the capability to separate carbohydrates stepwise after elution by an organic solvent is known (*19*). The concentration of the organic solvent applied (ACN) had to be adjusted for the different types of samples [2% (v/v) ACN for the Vivinal GOS/maltodextrin preparation and 5% (v/v) ACN for infant formula and fruit juice] to remove selectively the high amounts of mono- and/or dimers and not to affect the galactooligosaccharides ≥ DP3, which were used later on for quantification. CE−LIF analysis of the APTS-derivatized galactooligosaccharide fractions showed an improved resolution of oligomeric peaks compared to the electropherograms, resulting from the initial aqueous extracts. However, maltodextrin-containing samples showed clearly the comigration of peaks originating from maltodextrin and galactooligosaccharides, as shown for infant formula (curve II in **Figure 2A**). Maltodextrins in food matrices can be removed by enzymatic incubation with amyloglucosidase (*20*). Applying the enzymatic pretreatment for the removal of maltodextrins prior to mono- and dimer removal in sample matrices containing Vivinal galactooligosaccharides was successful. The peak profile of oligomeric structures was comparable to the Vivinal GOS reference, as shown for infant formula in curve III in **Figure 2A** and curve I **Figure 2B**. As expected, the incubation of the galactooligosaccharide pools with amyloglucosidase did not result in any breakdown of galactooligosaccharides. Similar to the removal of maltodextrins, an appropriate enzymatic incubation would be necessary for matrices containing other disturbing reducing oligosaccharides.

The quantitative recovery of galactooligosaccharides was investigated by standard addition. To the crude aqueous sample extract of infant formula, a known amount of Vivinal GOS was added. The standard addition yielded a recovery of 90% in the CE−LIF electropherograms, which was considered good because of the fact that Vivinal GOS is a multiple component analyte. The fingerprints of the galactooligosaccharides extracted from all three sample matrices are shown in **Figure 2C**. From the matrices tested, galactooligosaccharides could successfully be extracted on the basis of the comparison to the reference substrate. For only the fruit juice, some small irregularities were found in the trimeric (▲ in **Figure 2C**) and tetrameric (■ in **Figure 2C**) structures, which may point to a different batch of Vivinal galactooligosaccharides added to this food matrix.

The mole-based fluorescent derivatization allows for the evaluation of the DP ratios of the galactooligosaccharides extracted. The ratio of DP3/DP4/DP5 was 1:0.6:0.1, 1:0.5:0.1, and 1:0.5:0.1 for galactooligosaccharides extracted from infant formula, fruit juice, and Vivinal GOS/maltodextrin preparation, respectively. The abundancy ratio for the Vivinal GOS reference CE−LIF profile was 1:0.5:0.2 and similar to that found by others (*21*).

Summarizing, the reproducible extraction of Vivinal galactooligosaccharides from complex matrices is nicely demonstrated.

Quantification of Oligosaccharides by CE−LIF. The use of an internal standard carbohydrate and the fact that one APTS molecule is linked to the reducing end of each molecule theoretically enable the quantification of the components present in the oligosaccharide mixtures.

Kabel et al. (*14*) successfully proved the quantifiability of monomers ranging in a concentration from 0.5 to 50 nmol by CE−LIF, dealing with an error of about 10%. Evangelista et al. (*16*) started the relative quantification of oligosaccharides by analyzing different amounts of APTS-derivatized gluco-heptaose and gluco-tetraose, which resulted in a linear correlation between peak area and molar amount of sugar. However, focusing on oligosaccharides, their quantification accuracy on CE−LIF should be validated in more detail with an extended range of different oligosaccharides. Commercially available standards of single DPs (DP3, DP4, and DP5) of maltodextrin−, cellodextrin−, and arabinooligosaccharides were chosen, representing α-D-(1,4)- and β-D-(1,4)-linked hexose−oligosaccharides and α-L-(1,5)-linked pentose−oligosaccharides, respectively.

The CE−LIF response was investigated for stepwise, 2-fold increasing amounts of oligosaccharides, relative to their accurate respective starting concentration at 1 nmol.

A good correlation was found between the relative amount of labeled oligosaccharides and the LIF signal obtained for concentrations up to approximately 30 nmol (**Figure 3**). The single measuring points ranged within an average deviation of ≤10% to the regression line (R^2 = 0.98). Increasing the amounts of labeled carbohydrates above a concentration of 30 nmol showed partly increased deviation values (10−25%), which emphasizes the necessity of operating in low nanomole ranges for accurate quantification purposes.

Quantification of Vivinal Galactooligosaccharides Extracted from Complex Food Matrices. Having established an appropriate extraction method and having proven the quantifiability of oligosaccharrides by CE−LIF allows the quantitative determination of the galactooligosaccharides extracted from food matrices.

To simulate extraction and purification conditions, the amyloglucosidase- and nonamyloglucosidase-incubated Vivinal GOS reference mixtures were subjected to graphitized carbon material for setting up a calibration curve (**Figure 4A**). The mono- and dimeric sugars were partly removed by elution with 2% (v/v) ACN. Solutions containing 30, 50, 90, 120, and 240 μg of galactooligosaccharides [as defined by AOAC (*6*)] were derivatized with APTS and analyzed by CE−LIF. From the electropherograms obtained, the amount (nmoles) of galactooligosaccharides representing DP3−DP5 was calculated on the basis of the internal standard xylose added. Similar to the quantification trial with standard oligosaccharides, the calibration curve exhibits a good linear range for low nanomole amounts (approximately 3−10 nmol) of derivatized oligosaccharides (R^2 = 0.98). One has to take into account that the effective total amount of carbohydrates labeled in these oligosaccharide mixtures was higher, but only oligosaccharides of DP3−DP5 were taken

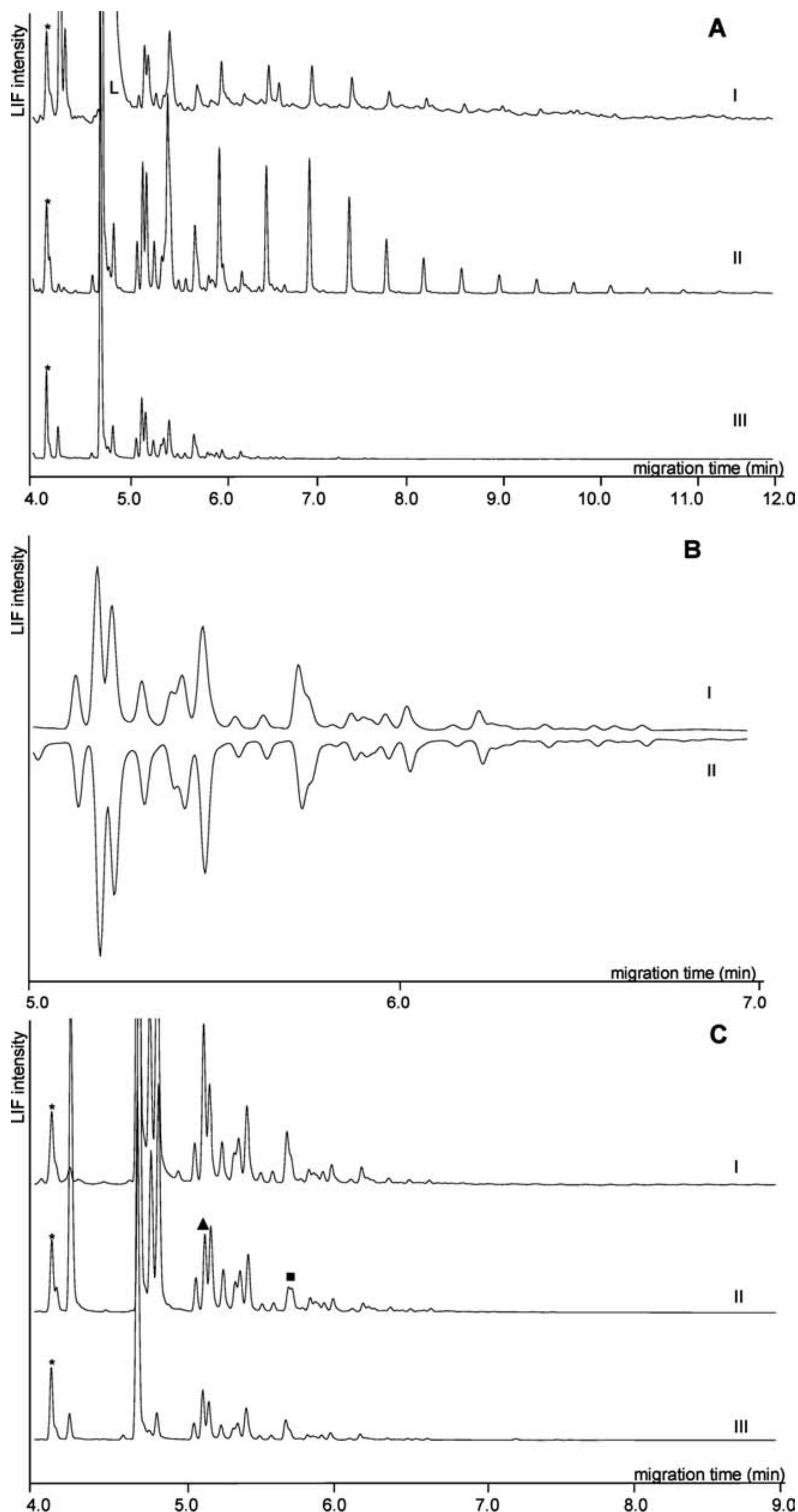

Figure 2. CE−LIF electropherograms of galactooligosaccharides. (A) (I) Aqueus extract infant formula, (II) infant formula after SPE pretreatment, and (III) infant formula after amyloglucosidase and SPE pretreatment. Curve I in panel B and curve III in panel C = curve III in panel A. (B) (II) reference Vivinal GOS (mirrored view), after amyloglucosidase and SPE pretreatment. (C) (I) Vivinal GOS/maltodextrin preparation and (II) fruit juice, both after amyloglucosidase and SPE pretreatment. L = lactose. (∗) = internal standard xylose. (▲/■) = deviating peak ratio in trimeric/tetrameric structures.

into consideration for quantification because of a partial and sample-dependent removal of the mono- and dimers. For 120 μg of reference galactooligosaccharides [according to AOAC (*6*)] approximately 11 nmol of DP3−DP5 was calculated (left scale *y* axes of **Figure 4A**), whereas the total amount of carbohydrates labeled (including residual mono- and dimers) was roughly

calculated to be approximately 30 nmol (right scale y axes of **Figure 4A**). It highlights again that quantification of oligosaccharides should not be performed with amounts of total carbohydrates exceeding 30 nmol. It is the reason for the exclusion of the data points for 240 μg of reference galactooligosaccharides (approximately 55 nmol of total carbohydrate content) for setting up the calibration curve (**Figure 4A**).

Consecutively, the amount of galactooligosaccharides (nanomoles of DP3−DP5) was calculated from the electropherograms of the respective food extracts. The value was then translated to the galactooligosaccharide content (micrograms)

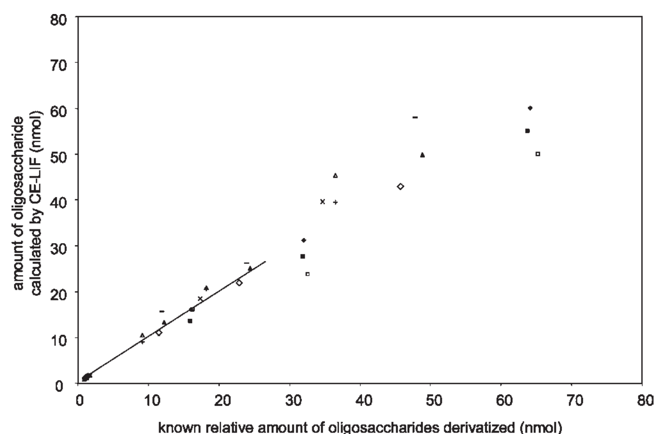

Figure 3. Relative quantification of oligosaccharides by CE−LIF, regression line (R^2 = 0.98). ( ) ara 3, () ara 4, and (■) ara 5 = arabinotri-, tetra-, and penta-ose. (◇) m 3, (△) m 4, and (▲) m 5 = maltotri-, tetra-, and penta-ose. (+) c 3, (×) c 4, and (−) c 5 = cellotri-, tetra-, and penta-ose.

in the product using the calibration curve. Galactooligosaccharides extracted from fruit juice were also quantified using the reference calibration curve because the small structural irregularities were only of intra-DP importance but did not affect the inter-DP ratio of DP3/DP4/DP5 oligosaccharides, as shown (Vivinal GOS reference, 1:0.5:0.2; fruit juice extract, 1:0.5:0.1).

Samples were extracted in duplicate, and mean values were determined (**Table 1**). The results (infant formula, 2.59 ± 0.11 g/100 g, approximately 3 g/100 g specified; fruit juice, 1.20 ± 0.01 g/100 mL, 1.25 g/100 mL specified; and Vivinal GOS/maltodextrin preparation, 27.87 ± 1.84 g/100 mL, 28.5 g/100 g specified) showed that, irrespective of the matrix, the introduced method is a promising way for quantifying galactooligosaccharides with a low intersample deviation. To validate this new method, extensive collaborative studies as described by de Slegte (*6*) would be necessary. This was not performed in the scope of this research.

Vivinal GOS, which was available as a reference, was used as a prebiotic in all food matrices used in this study. However, there may be cases in which quantification should

Table 1. Galactooligosaccharide Contents in Selected Food Products Detected by CE−LIF and According to Product Specifications

	galactooligosaccharides calculated by CE−LIF	product specifications
infant formula (g/100 g)	2.59 ± 0.11	± 3
fruit juice (g/100 mL)	1.20 ± 0.01	1.25
Vivinal GOS/ maltodextrin preparation (g/100 g)	27.87 ± 1.84	28.5

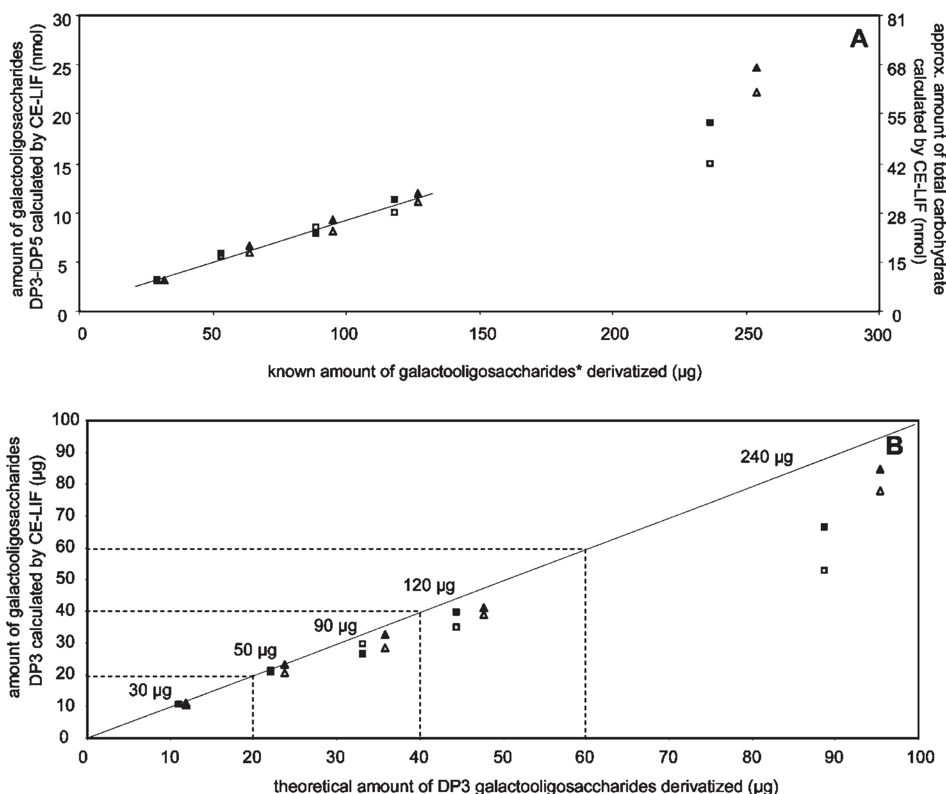

Figure 4. Quantification of Vivinal galactooligosaccharides using CE−LIF. (A) calibration curve (R^2 = 0.98). (B) Recovery of Vivinal galactooligosaccharides DP3 based on content specifications of reference Vivinal GOS. Dotted lines = 100% recovery. Reference values = 30, 50, 90, 120, and 240 μg. (■) AGOS 1 and (□) AGOS 2 = amyloglucosidase-pretreated Vivinal GOS (duplicate measurement). (▲) GOS 1 and (△) GOS 2 = nonamyloglucosidase-pretreated Vivinal GOS (duplicate measurement). (∗) Amount (micrograms) of galactooligosaccharides as defined by AOAC 2001.02 (*6*).

be performed while the prebiotic reference material is not available. This issue can also be of interest for the determination of prebiotic oligosaccharides in complex body liquids, such as gastrointestinal contents and feces, to monitor the fate of prebiotic oligosaccharides in the human gastrointestinal tract.

After unknown oligosaccharides were extracted and purified from the complex matrix, the peak migration times in the electropherogram should be assigned with the respective DP of the oligosaccharide in the first instance. This can be performed by MALDI−TOF MS after fractionation on size-exclusion material or by CE−LIF−MS coupling. The concentration of the respective peaks can subsequently be calculated on the basis of their M_w and CE−LIF area, related to the area of the internal standard used.

As an example, the concentration of the DP3−DP5 structures present in the Vivinal GOS reference was determined, without using the calibration curve. The results were compared to the theoretical concentrations, which were determined on the basis of the Biogel P2 proportions (%) of DP3 (23% on DM), DP4 (10% on DM), and DP5 (4% on DM). The galactooligosaccharide reference solutions were investigated in their amyloglucosidase- and nonamyloglucosidase-pretreated forms, as exemplified for DP3 in **Figure 4B**. The theoretical amount (micrograms) of DP3 galactooligosaccharides calculated is represented by the *x* axis, whereas the DP3 galactooligosaccharides calculated (micrograms) from the CE−LIF response is indicated by the *y* axis in **Figure 4B**. The calculations were based on the internal standard xylose. The dotted line in **Figure 4B** represents the theoretical 100% recovery. DP4 and DP5 were quantified in the same way (data not shown). Remarkably, the data points belonging to the successively increasing concentrations of galactooligosaccharides were deviating to lower values with respect to the theoretical recovery to a small extent (**Figure 4B**). This results from the fact that one should stay in low nanomole ranges ($\leq$30 nmol) for quantification. It may also result from an underestimation of peaks belonging to the respective DPs, because of the small overlap in the beginning and end of the DP pools, as was previously described. Quantifying complex oligosaccharides from scratch is possible, provided an appropriate sample cleanup and dilution is at hand.

Recent studies on the lactose content of Vivinal GOS resulted in a concentration of only 10% lactose, because of the application of an improved gradient for HPAEC analysis (*21*), which allowed for the separation of lactose from other DP2 components. Because dimers were not included for quantification in our study and sample specifications were based on AOAC 2001.02 (*6*), these new data have no effect on our setup. However, the method should be reconsidered if DP2 oligosaccharides are of special interest. The same counts if non-reducing oligosaccharides are of importance, because they are not labeled by the fluorescent APTS.

With the method proposed, galactooligosaccharides could be selectively extracted, despite being embedded in complex food matrices in low amounts. The implementation of CE−LIF showed a promising possibility for the quantification of reducing oligosaccharides. The application of CE−LIF may be of high value for the investigation on residual prebiotic (galacto)oligosaccharides in complex body liquids, providing insight into the fate of these carbohydrates during their fermentation in the human gastrointestinal tract.

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