Synthesis and Characterization of Sialylated Lactose- and Lactulose-Derived Oligosaccharides by *Trypanosoma cruzi* Trans-sialidase

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## ABSTRACT:
Sialylated oligosaccharides contribute 12.6–21.9% of total free oligosaccharides in human milk (hMOS). These acidic hMOS possess probiotic properties and display antiadhesive effects against pathogenic bacteria. Only limited amounts of sialylated hMOS are currently available. The aim of our work is to enzymatically synthesize sialylated oligosaccharides mimicking hMOS functionality. In this study, we tested mixtures of glucosylated-lactose (GL34), galactosylated-lactulose (LGOS), and galacto-oligosaccharide (Vivinal GOS) molecules, as trans-sialylation acceptor substrates. The recombinant trans-sialidase enzyme from *Trypanosoma cruzi* (TcTS) was used for enzymatic decoration, transferring (α2→3)-linked sialic acid from donor substrates to nonreducing terminal β-galactopyranosyl units of these acceptor substrates. The GL34 F2 2-Glc-Lac compound with an accessible terminal galactosyl residue was sialylated efficiently (conversion degree of 47.6%). TcTS sialylated at least 5 LGOS structures and 11 Vivinal GOS DP3→4 compounds. The newly synthesized sialylated oligosaccharides are interesting as potential hMOS mimics for applications in biomedical and functional-food products.

## KEYWORDS:
sialic acid, trans-sialidase, *Trypanosoma cruzi*, trans-sialylation, lactulose, galactooligosaccharides

## INTRODUCTION
In human milk, free oligosaccharides constitute the third-most-abundant component after lactose and fat. Human milk oligosaccharides (hMOS) represent lactose molecules elongated with N-acetylgalactosamine (GlcNAc), galactose (Gal), fucose (Fuc), and N-acetylenuraminic acid (Neu5Ac) with various glycosidic-linkage types. Sialic acid can be coupled to galactose residues in hMOS with (α2→3) or (α2→6) linkages and to GlcNAc with (α2→6) linkages. These sialylated oligosaccharides contribute 12.6–21.9% of total hMOS. There is increasing evidence for positive functional effects from this group of acidic oligosaccharides on human health. Specific hMOS structures, namely, disialyllacto-N-tetraose and 2′-fucosyllactose, prevented and reduced necrotizing enterocolitis (NEC) in neonatal rats and thus may be used to prevent NEC in formula-fed infants. Preventive effects against NEC were also observed with a Sia-GOS mixture, particularly with disialylated GOS. 3′-Sialyllactose stimulates growth of various *Bifidobacterium* strains, including the infant-gut-related *Bifidobacterium longum* subsp. *infantis*. Sialylated oligosaccharides also prevent intestinal attachment of pathogens by acting as receptor analogues, competing with epithelial ligands for bacterial binding. Compared with human milk, free oligosaccharides in the milk of domesticated animals are much less abundant. Bovine milk, for instance, has only trace amounts of milk oligosaccharides. The natural scarcity of these highly bioactive sialylated oligosaccharides stimulated us to study the possible synthesis of mimics via trans-sialylation of β-galactose (β-Gal)-linked compounds in various oligosaccharide mixtures. One example is the Vivinal GOS mixture that is commercially used in infant nutrition.

Recently, we have reported the enzymatic synthesis of two novel oligosaccharide mixtures (GL34 and LGOS) and their structural characterization. GL34 is a mixture of five (α1→2/3/4)-glucosylated lactose molecules, with a degree of polymerization (DP) of 3–4, synthesized from sucrose as the donor substrate by glucansucrases (Gtf180-ΔN and GtfA-ΔN) as biocatalysts. The GL34 mixture exhibits selective stimulatory effects on the growth of various strains of lactobacilli and bifidobacteria. LGOS is a mixture of (β1→2/3/4)-galactosylated lactulose molecules, with one or two galactosyl moieties, synthesized from lactulose as the donor and acceptor substrate by wild-type and mutant β-galactosidase enzymes from *Bacillus circulans* ATCC 31382 (Scheme 1). Previously, oligosaccharides derived from lactulose were shown to promote the growth of bifidobacteria and to exert beneficial effects on the digestive tract.

In view of the potential functional properties of these novel GL34 and LGOS oligosaccharides we decided to try and further develop their structures to better mimic acidic hMOS. In this study, trans-sialidase from *Trypanosoma cruzi* (TcTS) was employed for the trans-sialylation of oligosaccharides in...
the GL34 and LGOS mixtures. Among trans-sialidases (EC 3.2.1.18), T. cruzi trans-sialidase is one of the best-studied enzymes.\(^2\) It plays an important role in host-cell invasion and pathogenicity of T. cruzi because of its ability to scavenge and transfer sialic acid to the pathogen’s extracellular mucins, thereby hiding the pathogen from the host immune system.\(^2\,^3\)\(^,\)\(^4\)

TcTS catalyzes trans-sialylation reactions via a ping-pong mechanism,\(^3\) which starts with formation of a stable sialo-enzyme intermediate through a covalent bond with the nucleophile Tyr342.\(^3\)\(^,\)\(^4\) This is followed by transfer of the sialic acid to a \(\beta\)-Gal-linked acceptor substrate involving a nucleophilic attack of the hydroxyl group at C3 of this \(\beta\)-Gal.\(^3\)\(^,\)\(^4\) When a suitable \(\beta\)-Gal-linked acceptor is absent, this enzyme catalyzes a hydrolysis reaction and sialic acid is released.\(^3\)\(^,\)\(^4\) In case of TcTS, sialyl transfer is catalyzed with much greater efficiency than hydrolysis.\(^3\)\(^,\)\(^4\) TcTS can use glycoproteins or oligosaccharides as acceptor substrates, but it only uses compounds possessing sialic acid (\(\alpha2\rightarrow3\)) linked to a terminal \(\beta\)-Gal as donor substrates.\(^3\)\(^,\)\(^4\) In previous work, we showed that TcTS catalyzes the transfer of sialic acid from a \(\kappa\)-casein-derived glyco-macropeptide (GMP) donor substrate to galacto-oligosaccharides (GOS).\(^6\)\(^,\)\(^7\) However, a detailed analysis of these monosialylated and disialylated GOS structures was not performed. GMP is a byproduct of cheese manufacturing and contains a high levels of \(O\)-glycans, which carry Neu5Ac, including mainly Neu5Ac(\(\alpha2\rightarrow3\))Gal(\(\beta1\rightarrow3\))GalNAc and Neu5Ac(\(\alpha2\rightarrow3\))Gal(\(\beta1\rightarrow3\))[Neu5Ac(\(\alpha2\rightarrow6\))]GalNAc, which can be used as donor substrates.\(^5\)\(^,\)\(^6\)

In this study, we used the GL34 and LGOS mixtures as acceptor substrates and GMP as the donor substrate. The negatively charged products were fractionated using Dowex 1 × 8 chloride. Furthermore, we characterized the sialylated GOS structures that were synthesized in our previous work\(^6\) in more detail. The decorated GL34, GOS, and LGOS structures were identified using high-pH anion-exchange chromatography (HPAEC), and one-dimensional \(\text{^1H NMR spectroscopy (1D \text{^1H NMR spectroscopy).}}\)

### MATERIALS AND METHODS

**Chemicals and Materials.** Bovine \(\kappa\)-casein-derived glyco-macropeptide (GMP) was provided by the FrieslandCampina Innovation Center. N-Acetylneuraminic acid (Neu5Ac), 2-O-(4-methylumbelliferyl)-\(\alpha\)-N-acetylneuraminic acid (4MU-Neu5Ac), and \(N\)-acetylneuraminyl(\(\alpha2\rightarrow3\))lactose (3′-SL) were obtained from Carbo-synth Ltd. Neuraminidase from Clostridium perfringens was obtained from Roche. Synthesis of glucosylated-lactose compounds (GL34),\(^2\) galactosylated-lactulose compounds (LGOS),\(^2\) and sialylated Vivinal GOS (DP3 and DP4) compounds\(^8\) has been reported previously.

**TcTS Expression and Purification.** Escherichia coli BL21 (DE3) (Invitrogen) was used as the host for the expression of the trans-sialidase from Trypanosoma cruzi. Precultures of E. coli BL21 (DE3) harboring pTrcTS611/2 were cultured overnight at 30 °C.\(^5\)\(^,\)\(^6\) Terrific broth (TB) with 12 g of tryptone, 24 g of yeast extract, 4 mL of glycerol, and 100 μg mL\(^{-1}\) ampicillin was inoculated with 1% preculture at 30 °C and 200 rpm. Expression of trans-sialidase was induced using 0.2 mM isopropyl \(\beta\)-d-thiogalactopyranoside (IPTG) when the cell density reached \(A_{600} = 0.4-0.6\). Cultivation was continued at 18 °C for 4 h. The cells were collected by centrifugation (10 min, 4 °C, 10 000g) and washed with 50 mM Tris-HCl buffer
Isolation of Negatively Charged Oligosaccharides by Dowex Chromatography. Dowex X 8 × 8 chloride (Cl\(^{−}\)) (Sigma-Aldrich) was packed in an Econo-column (1.5 × 10 cm, Biorad) and activated with 10 column volumes (10 CV) of NaOH 2 M (at least 1 h contact time). Before injection of samples, the column was equilibrated with 10 CV of water. Elution of the sialylated oligosaccharides was performed at a flow-rate of 1 mL min\(^{−1}\) with Milli-Q water (MQ) and ammonium bicarbonate as eluents. After injection, unbound compounds were removed from the column by washing with MQ for 3 CV. Monoand disialylated and disialylated oligosaccharides were eluted with 3 CV of 50 and 400 mM ammonium bicarbonate, respectively. An extra elution step with 500 mM ammonium bicarbonate was used to wash off all remaining sialylated structures. The collected fractions were lyophilized.

Desialylation of Sialylated Oligosaccharides. Frac-tions of sialylated LGOS were treated with acetic acid (20%) for 1 h at room temperature, which was followed by neutralization with 1 M NaOH. Desialylated fractions were desalted using Carboxyl SPE columns.

Sialylated Vivanal GOS fractions of DP3 and DP4 were desialylated by incubation with 1 U mL\(^{−1}\) neuraminidase (Roche) in 0.1 M acetate buffer (pH 5.0) at 37 °C for 24 h.

HPAEC-PAD Chromatography. Oligosaccharide mixtures were analyzed by HPAEC-PAD profiling on a Dionex ICS-3000 system (Thermo Scientific) equipped with a CarboPac PA-1 column (250 × 2 mm, Dionex) and detected with a pulsed amperometric detector (PAD) using a gold working electrode. Eluting glycans were detected using a standard manufacturer’s quadruple-potential waveform for detecting carbohydrates. Conversion of substrates into sialylated compounds was estimated by comparing converted peaks with unconverted peaks (F1 and F3 for GL34, Fru for LGOS) at different time points, calculating depletion of peaks. A gradient of 30 to 600 mM sodium acetate in 0.1 M NaOH (0.25 mL min\(^{−1}\)) was used for analytical separation of acidic oligosaccharides. Another complex gradient of eluents A (100 mM NaOH), B (600 mM NaOAc in 100 mM NaOH), C (Milli-Q water), and D (50 mM NaOAc) was used for profiling neutral oligosaccharide mixtures as previously described. \(^{20}\)

NMR Spectroscopy. Structures of the transferred compounds were determined by 1D 'H NMR recorded at a probe temperature of 25 °C on a Varian Inova 500 Spectrometer (NMR Center, University of Groningen). The samples were exchanged twice with D\(_2\)O (99.9 atom % D, Cambridge Isotope Laboratories, Inc.) with intermediate lyophilization and then dissolved in 0.65 mL of D\(_2\)O containing acetonitrile as the internal standard (δ \(^{1}\)H 2.225 ppm). Data was recorded at 16k complex data points, and the HOD signal was suppressed using a WET1D pulse (500 MHz spectra). MestReNova 9.1.0 (Mestrelabs Research SL) was used to process NMR spectra, using Whittaker Smoother baseline correction.

**RESULTS AND DISCUSSION**

Previously, N-acetylneuraminic acid (Neu5Ac) was determined to be a major component (>99%) of the 3.6% (w/w) sialic acid in GMP, in comparison with N-glycolylneuraminic acid (Neu5Gc). \(^{34}\) A concentration of 67.5 mg mL\(^{−1}\) GMP, corresponding to 5 mM (α2→3)-linked Neu5Ac, was used as donor substrate for the incubations in this study. At this fixed concentration of GMP as a donor substrate, the
concentrations of the acceptor substrates necessary to obtain their maximal conversion degrees were determined. All the incubations were carried out under the optimal conditions for TcTS as previously reported (in 50 mM sodium citrate buffer pH 5.0 at 25 °C).34,38

Sialylation of GL34 by TcTS. The mixture GL34 (average DP3) was incubated at concentrations of 1, 5, and 10 mM, with 67.5 mg mL−1 GMP and 5 μg mL−1 TcTS at 25 °C and pH 5.0 for 24 h. After incubation, the HPAEC-PAD profiles showed a new peak eluting at a retention time of ∼14.5 min, which is in the retention area of negatively charged oligosaccharides in this gradient (Figure 1, spectrum 2).8 In the HPAEC-PAD profile of neutral oligosaccharides, only the F2-compound peak had a significantly decreased area (Figure 1, spectrum 1). These results suggested that F2 was used as an acceptor substrate for trans-sialylation by TcTS. The signals of Neu5Ac(α2→3) (H-3e at δ 2.755 and H-3a at δ 1.795) were detected in the 1D 1H NMR spectrum of the GL34 mixture after the trans-sialylation reaction (Figure S1). The presence of a new signal at δ 4.212 is fitting with the 3-substitution at the terminal galactosyl residue of F2 with Neu5Ac (Figure S1), confirming the synthesis of Neu5Ac(α2→3)Gal(β1→4)[Glc(α1→2)]Glc (Scheme 2). On the basis of the HPAEC-PAD responses, the maximal conversion of F2 into the corresponding sialylated-F2 was observed with 10 mM GL34 and calculated as 47.6%, on the basis of reduction of the F2 peak. The data shows that only F2 was used as an acceptor substrate for trans-sialylation by TcTS. In the GL34 mixture, F2 is the only compound with an accessible β-Gal residue at a nonreducing terminal position (Scheme 1). TcTS was shown...
to also glycosylate internal $\beta$-Gal residues in specific structures (i.e., in a Gal($\beta$1→6)Gal epitope),\textsuperscript{27,30} but these are absent in GL34. F1 4′-Glc-Lac and F4 2,4′-Glc-Lac, the only other GL34 compounds with nonsubstituted OH-3 positions (on the internal galactose residue, Scheme 1),\textsuperscript{20} were not used as acceptor substrates.

**Sialylation of LGOS by TcTS.** Various concentrations of the LGOS mixture (1, 5, 10, and 15 mM) were incubated with 67.5 mg mL$^{-1}$ GMP as a donor substrate and with TcTS (5 $\mu$g mL$^{-1}$) at 25 °C and pH 5.0. Because of the relatively low stability of this trans-sialidase,\textsuperscript{38} extra TcTS (5 $\mu$g mL$^{-1}$) was added to the incubation mixtures after every 24 h of incubation. The incubation experiments were followed over time, and the highest degree of LGOS conversion into sialylated LGOS was \~52% after 48 h with 1 mM LGOS mixture (Figure 2). Conversion of LGOS was estimated by following nonsialylated-LGOS-peak depletion over time, assuming that all LGOS had similar response factors on the PAD detector. At this LGOS concentration, the conversion degree increased significantly from 37.4 to 52.0% when the incubation lasted from 24 to 48 h (Figure 2). In all cases, the GMP-derived Neu5Ac($\alpha$2→3) as the donor substrate was not completely utilized, with a maximal use of 80% when incubated with 15 mM LGOS for 24 h. Enhanced conversion degrees were not observed when incubating other concentrations of the LGOS mixture longer than 24 h despite renewed addition of TcTS (Figures S2–S4).

The HPAEC-PAD profiles of the incubation mixtures with 1 mM LGOS showed development of several new peaks over time (Figure 3). These new peaks eluted at retention times between 12–22 min, indicating synthesis of a complex mixture of sialylated LGOS. The negatively charged (Sia-LGOS) oligosaccharides were separated from the neutral (LGOS) oligosaccharides by Dowex 1×8 (Cl$^-$) chromatography and...
neutral oligosaccharides in the unbound Dowex fraction eluted during the first 12 min in the HPAEC-PAD profile (Figure 4, spectrum 1). The Dowex fraction that eluted with 50 mM ammonium bicarbonate (Sia-LGOS) eluted between 12 and 18 min in the HPAEC-PAD profile (Figure 4, spectrum 2), fitting with monosialylated structures. The Dowex fraction containing disialylated structures was relatively minor, limiting possibilities for further characterization.

The 1D $^1$H NMR spectrum (Figure S5) of the negatively charged fraction revealed signals at $\delta$ 2.760 and 1.803, which belong to the Neu5Ac H-3e and H-3a atoms, respectively, of the Neu5Ac($\alpha2\rightarrow3$) residues. These NMR-spectroscopy data confirmed the sialylation of LGOS by TcTS. To identify the compounds in the LGOS mixture that were decorated with Neu5Ac, desialylation of these sialylated-LGOS was carried out using 20% acetic acid.

Comparison of the HPAEC-PAD profiles of the desialylated fraction with that of the LGOS mixture allowed identification of at least five structures that were monosialylated by TcTS: LGOS2a or 2b, LGOS4, LGOSS, LGOS6, and LGOS7a or 7b (Figure 5). In the LGOS profile structures, LGOS4 and LGOS5 are the major components. After sialylation and desialylation, the HPAEC-PAD profile showed LGOS6 and LGOS7 to be the predominant structures. In the LGOS mixture, LGOS6 is only a trace peak, but in the sialylated fraction, LGOS6 is the major structure. This indicated that the Gal($\beta1\rightarrow3$)Gal($\beta1\rightarrow4$) epitope is very favorable for sialylation. The LGOS7 peak consisted of two structures, one with a Gal($\beta1\rightarrow3$)Gal($\beta1\rightarrow3$) epitope and one with a Gal($\beta1\rightarrow4$)Gal($\beta1\rightarrow3$) epitope. Although it is not possible to distinguish between the two structures, it is likely that structure LGOS7a, with a terminal Gal($\beta1\rightarrow3$) residue, is the mainly sialylated LGOS7 structure. This fits with previous results on galactosyl-lactose conversions, showing a much higher specificity constant ($k_{cat}/k_{M}$) for the transferase reaction to 3′-galactosyllactose than for the reactions to 4′-galactosyllactose and 6′-galactosyllactose. Closer inspection of the 1D $^1$H NMR profile of the Sia-LGOS fractions revealed the Bf4 signals at $\delta$ 4.200–4.211, which originate from the LGOS4, LGOS5, LGOS6, or LGOS7 structures (slightly shifted). This provided evidence for the presence of the LGOS4, LGOSS,
Figure 6. HPAEC-PAD profiles of the Sia-GOS (A,B) DP3 and (C,D) DP4 fractions. Mono- and disialylated-GOS peaks are marked.

Figure 7. HPAEC-PAD profiles of compounds from sialylated-GOS fractions (A) DP3 and (B) DP4: (1) neutral GOS mixtures at corresponding DP, (2) mono-Sia-GOS, and (3) di-Sia-GOS fractions after being desialylated. Identified GOS peaks are marked with numbers as used by van Leeuwen et al.\textsuperscript{40} corresponding with those in Scheme 3. Peak 6 corresponds to GOS6a or 6b; peak 10 corresponds to GOS10a or 10b; peak 15 corresponds to GOS15a or 15b; peak 16 corresponds to GOS16a, 16b, or 16c; and peak 18 corresponds to GOS18a or 18b.
LGOS6, or LGOS7 compounds in the Sia-LGOS mixture. Moreover, the $^1$H NMR spectrum of this mixture showed anomeric C-1 signals (slightly shifted) at δ 4.694, 4.650, and 4.629 from the structures LGOS4−7.

In LGOS5 (Gal(β1→3)Gal(β1→4)Fru) and LGOS7a (Gal(β1→3)Gal(β1→3)Gal(β1→4)Fru), the O-3 positions of the internal β-Gal residues are already substituted, only the terminal β-Gal residues of LGOSS and LGOS7a are available for (α2→3)-linked decoration with Neu5Ac to yield the corresponding monosialylated oligosaccharides: Neu5Ac(α2→3)Gal(β1→3)Gal(β1→4)Fru and Neu5Ac(α2→3)Gal(β1→3)Gal(β1→3)Gal(β1→4)Fru (Scheme 2). The structure of LGOS4 (Gal(β1→4)Gal(β1→4)Fru) was only monosialylated by TcTS, although it also possesses a nonsubstituted O-3 of the internal β-Gal residue. This was also observed for the similar structure β4′-galactosyl-lactose, of which only the
terminal β-Gal residue was (α2→3)-substituted with Neu5Ac. The disialylated LGOS fraction was too minor to be elucidated. In the LGOS mixture, only LGOS1, LGOS2a, and LGOS3, each with two terminal β-Gal residues, as well as LGOS2b with an internal β-Gal residue (β1→6) linked with a terminal β-Gal residue, are likely disialylated. In this study, the trans-sialidase from T. cruzi was used to transfer sialic acid to oligosaccharides (DP3−4) in GL34, LGOS, and Vivinal GOS mixtures. Decorated structures were identified by HPAEC-PAD chromatography and NMR spectroscopy. As expected, various compounds in these mixtures with one or more accessible β-Gal-OH-3 groups were used as acceptor substrates by TcTS. The F2 (2-Glc-Lac) compound in the GL34 mixture was monosialylated, yielding α3Sia-2-Glc-Lac with a conversion degree of 47.6%. TcTS was able to transfer sialic acid to at least five different compounds in the LGOS mixture with a conversion degree of up to 52%. The conversion of galacto-oligosaccharides (GOS) with DP3−4 (3 mM GOS with 6 mM (α2→3)-linked Neu5Ac) into Sia-GOS by TcTS was clearly lower, at about 35%, but it was obtained under different conditions. The optimal concentrations of the GL34 and LGOS mixtures for maximal conversion by TcTS (10 μg mL−1) in the incubations with 5 mM (α2→3)-linked Neu5Ac (from GMP) were 10 and 1 mM, respectively. In fact, all the structures in the LGOS mixture possess terminal nonreducing β-Gal residues, whereas only the F2 compound of the GL34 mixture has a terminal β-Gal residue. Previously, only lactulose was used as an acceptor substrate for a mutant trans-sialidase Tr13 from T. rangeli. The GOS mixture has been known to provide multiple C-3 hydroxyl groups and to be an easily accessible substrate for trans-sialidase, including TcTS (acceptor) sites. Our study showed that in fact most of the GOS structures of DP3 and DP4 from Vivinal GOS were sialylated by TcTS. Moreover, the results revealed a strong preference for terminal β-Gal residues to be sialylated. Only branched compounds with two nonreducing terminal β-Gal residues were disialylated. The only exception known so far is 6′-galactosyllactose, which is linear with a specific Gal(β1→6)Gal epitope that could be disialylated by TcTS. Moreover, our study showed that structures with a Gal(β1→3) terminal residue were more efficiently sialylated by TcTS.

In conclusion, the data show that enzymatic synthesis of sialylated lactose- and lactulose-derived oligosaccharides, using the TcTS enzyme and (α2→3)-Neu5Ac from GMP as a donor substrate, yields a highly interesting variety of sialylated oligosaccharides. This transfer of sialic acid as a functional group is the first step in developing hMOS-mimicking compounds. In future studies, we aim to optimize their biosynthesis and to evaluate the potential use of these novel compounds for pathogen inhibition and prevention of NEC.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b06974. 1D 1H NMR spectrum and HPAEC-PAD profiles of different samples (PDF)

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ABBREVIATIONS USED

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GOS, galacto-oligosaccharide; GMP, glucosylated-lactose; LGOS, galactosylated-lactulose; Vivinal

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Notes

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ABBREVIATIONS USED

TcTS, trans-sialidase from Trypanosoma cruzi; hMOS, human milk oligosaccharides; GOS, galacto-oligosaccharides; GL34, glucosylated-lactose; LGOS, galactosylated-lactulose; Vivinal GOS, galacto-oligosaccharide; GMP, κ-casein-derived glycomacropeptide; HPAEC-PAD, high-pH anion-exchange chromatography–pulse amperometric detection; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; NMR, nuclear magnetic resonance

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