Final report

Analytical characterization of human milk oligosaccharides

project ID: NKFI 109373 (PD)

prepared and submitted by Szabolcs Béni

The scientific results of the project can be divided into the following sections:

I. Liquid chromatography-based analytical method developments

II. Gas chromatography-based analytical method development

III. NMR-based analytical method development

Results

I/a. LC-MS method development for the detection and quantitation of *N*-acetyllactosamine (LacNAc) and lacto-*N*-biose (LNB) by LC-MS/MS

These two isomeric compounds (see Figure 1.) are the major building blocks of human milk oligosaccharides, however the presence of their unbound form in human milk has not been examined so far. The separation of these highly related structures in their alditol form (see Figure 1.) was accomplished by a gradient LC method and multiple reaction monitoring (MRM) analysis after appropriate sample preparation including size-exclusion chromatography and solid-phase extraction. Baseline separation of the components provides the selectivity for the method.

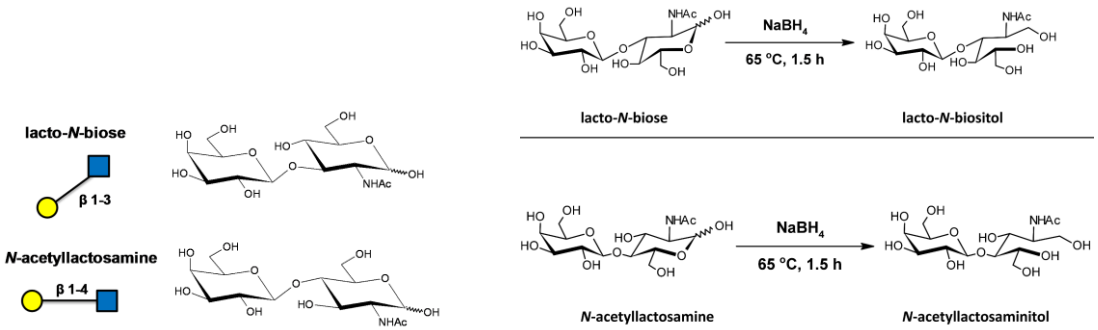

Figure 1. Chemical structure of the two main building blocks of human milk oligosaccahrides lacto lacto-*N*-biose (LNB) and *N*-acetyllactosamine (LacNAc) (left) the mechanism of the reduction with $NaBH_4$ at 65 °C for 1.5 h in the case of both disaccharides resulting N-acetyllactosaminitol and lacto-N-biositol possessing no α/β anomeric form (right).

Validation was performed according to the European Medicines Agency (EMA) Guidelines and the method was found to be precise and accurate. Using our developed and validated method we were able to identify and quantify both saccharides in human milk for the first time.

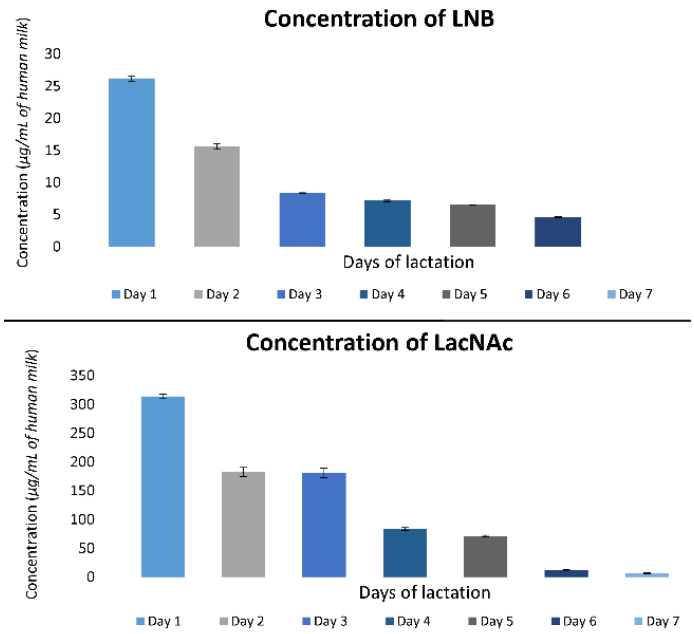

Figure 2. Concentration changes of LNB (top) and LacNAc (bottom) in a human milk sample in the first week of lactation. Concentrations are corrected with the dilution factors, the recovery values and are given to 1 mL of human milk sample.

Based on our results the LacNAc concentration is in the range of 6.7 µg/mL-310 µg/mL while LNB concentration decreased from 26 µg/mL below the detection limit during the first week of lactation (see Figure 2.). The presence of LNB and LacNAc in human milk also implies new biological functions which can lead us closer to the understanding of the various functions of this complex biofluid.

The "*LC-MS method development for the identification and characterization of N-acetyllactosamine (LacNAc) and lacto-N-biose (LNB), the two major building blocks of human milk oligosaccharides in human milk samples by HPLC-MS/MS using a porous graphitic carbon column*" has been published in: Journal of Chromatography A Volume 1422, 2015, Pages 140–146

I/b. LC-MS method development for the Quantitative analysis of 3'-sialyllactose and 6'-sialyllactose in human milk samples by HPLC-MS/MS

As a continuation of our liquid chromatographic method developments for HMO characterization, the two major isomeric anionic trisaccharides (sialyllactose isomers) were subjected to the same procedure: we aimed at determining their concentration in human colostrum as a function of lactation. These sialyllactose isomers (3'-sialyllactose and 6'-sialyllactose) are believed to significantly contribute to brain development, so besides fucosyllactoses, these molecules are also considered as new ingredients for infant formulas. The sample preparation required some modification (including simplification: no size exclusion chromatographic step applied and extra purification with graphite SPE implemented) compared to the previous LC-MS LNB-LacNAc project. Maltotriose was also introduced as the most appropriate standard for the quantitative determination of sialyllactose isomers on a porous graphitic carbon column following reduction by borohydride. Using our validated method the concentration changes of 3'-SL and 6'-SL can be followed from the first day of lactation until the 9th month. The most important method validation data of the new method can be seen in the following table.

Table 1. Method validation data for 3’-SL and 6’-SL: calibration curves and linearity.

3’-SL (n=3)								
Nominal concentration	30 µg/ml	60 µg/ml	120 µg/ml	240 µg/ml	480 µg/ml	960 µg/ml	1920 µg/ml	3000 µg/ml
Back calculated concentration (%)	101.5	99.2	99.9	99.6	101.8	100.9	98.7	100.5
CV %	4.6	1.0	6.5	5.1	2.8	1.3	1.1	0.4
Linearity	$y = -0.1747\ x^2 + 4.9669\ x + 0.0397$				$R^2 = 0.9997$			
6’-SL (n=3)								
Nominal concentration	15 µg/ml	30 µg/ml	60 µg/ml	120 µg/ml	240 µg/ml	480 µg/ml	960 µg/ml	1500 µg/ml
Back calculated concentration (%)	97.4	105.7	96.4	98.8	104.0	95.8	100.0	99.0
CV %	1.6	2.9	3.0	4.8	3.7	3.0	4.2	3.3
Linearity	$y = 0.4560\ x + 0.0058$				$R^2 = 0.9972$			

Measuring several milk samples of a single individual as a course of lactation (see Figure 3.), we have concluded that the concentration of 3’-SL decreased to a small extent during the study period, while a significant decrease of 6’-SL was found after the first week of lactation. The lactation of the same individual after the second delivery resulted slightly lower amounts of both SLs but this difference seemed to disappear after the first month.

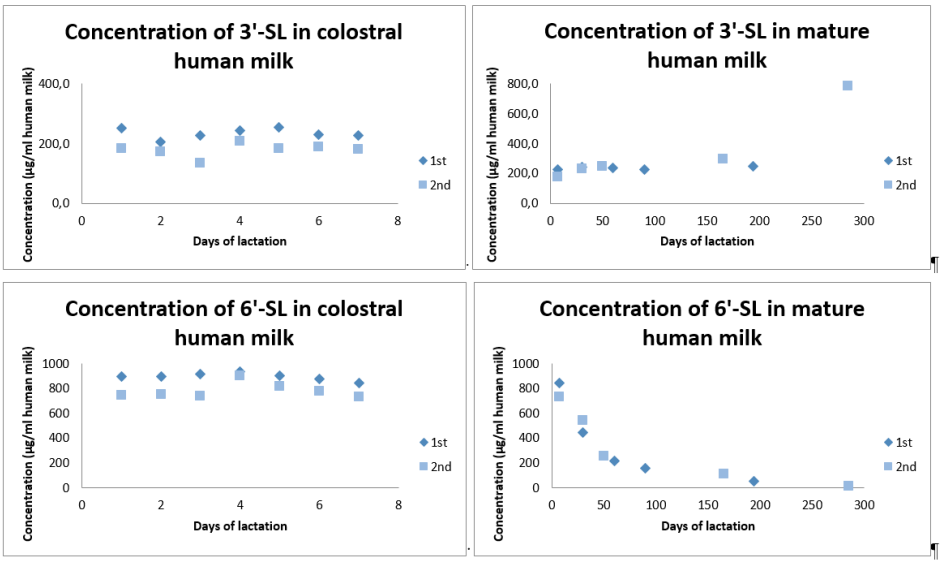

Figure 3. Concentration changes of 3’-SL and 6’-SL in a human milk sample in colostrum and in mature milk.

This bioanalytical protocol could be also applicable for industrial application in case of infant formulas are enriched with 3’-SL and 6’-SL in the near future. The manuscript “Quantitative analysis of 3’- and 6’- sialyllactose in human milk samples by HPLC-MS/MS: a validated method for the comparison of two consecutive lactation periods” is in preparation.

II. Gas chromatography-based analytical method development for the characterization of fucosyllactose isomers

Fucosyllactoses are the main neutral trisaccharides in human milk (Figure 4.). 2'-fucosyllactose (2'-FL), has been officially certified to be used in infant formula in both European and U.S. markets. The ingredient has received EU novel food approval and achieved GRAS (Generally Recognized as Safe) status from the Food and Drug Administration in the USA. As gas chromatography is applied in the analysis of monosaccharides in food industry, therefore we aimed at exploring the capacity of a GC-MS-based method for the quantitation of FL isomer trisaccharides in human milk.

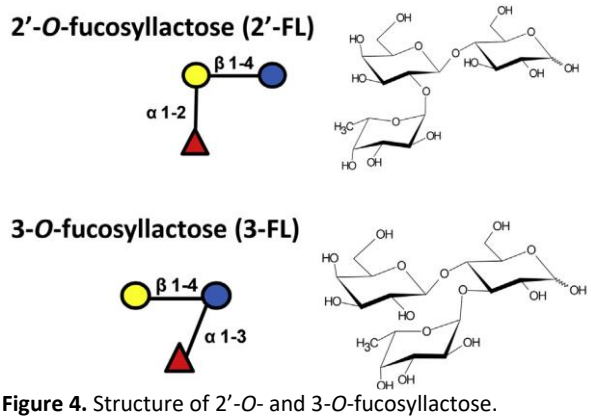

Figure 4. Structure of 2'-*O*- and 3-*O*-fucosyllactose.

Besides the numerous liquid chromatographic methods, to our knowledge no gas chromatographic application has been used so far for the separation and quantification of human milk oligosaccharides from human milk samples. Although gas chromatography is not the first choice in the field of sugar analytics, for the analysis of monosaccharides it is relatively common. The aim of our study was to develop a GC–MS method for the identification and quantification of 2'-FL and 3-FL. 2'-FL is the most abundant oligosaccharide in the human milk. Its linkage isomer 3-FL is found to be in far less amounts, however still contributing to a major part of the total oligosaccharide content of human milk. Besides their significant presence in human milk, their possible biological functions also make them important targets for our studies. As both the chemical and enzymatic synthesis of 2'-FL and 3-FL is relatively straightforward compared to the more complex oligosaccharides, and as 2'-FL can found in significant amounts in the human milk, this compounds is the first HMO infant formulas are now enriched with. Therefore we have developed a GC–MS method for the quantification of the TMS ether oxime derivatives of 2'-*O*-fucosyllactose and 3-*O*-fucosyllactose, the two most abundant trisaccharides in human milk (TMS-oxime derivatization was performed with hydroxylamine and hexamethyldisilazane to stabilize the thermally labile trisaccharides for GC-MS measurements).

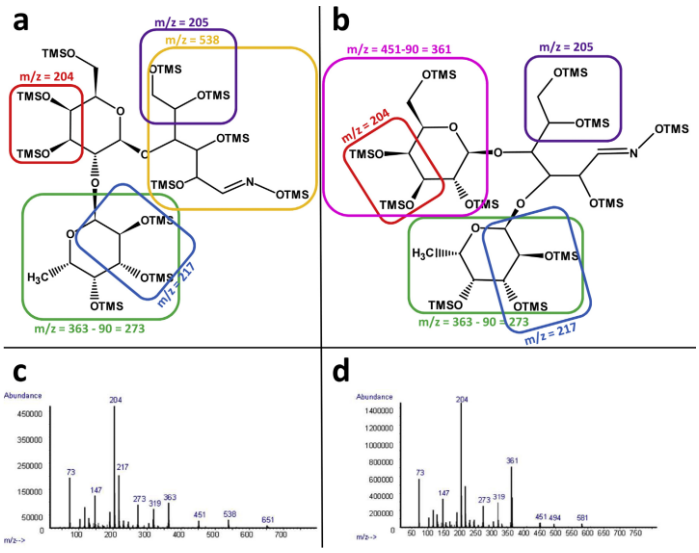

Figure 5. Proposed fragmentation patterns and MS spectra of the trimethylsilyl oxime derivatives of 2'-FL (a,c) and 3-FL (b,d)

The EI fragmentation pattern of the linkage isomers is discussed in details, focusing also on specific fragment ions (see Figure 5.). The GC-MS method with external standard calibration was applied for the monitoring of concentration changes of the trisaccharides throughout the first week of lactation in human milks samples collected from two volunteers. The results showed high concentration of both 2'-FL (4525–6266 µg/mL in donor A and 2694–3551 µg/mL in donor B) and 3-FL (271–441 µg/mL in donor A and 99–208 µg/mL in donor B), while no significant change has been observed throughout the one-week lactation period. The presented GC-MS method can serve as a quality control technique for the infant formulas and also offers an alternative to existing chromatographic methods to investigate HMOs in milk samples.

Following the same sample preparation procedure successfully applied for the non-charged trisaccharides the major charged trisaccharides (sialyllactoses) and non-charged tetrasaccharides (lacto-*N*-tetraose and lacto-*N*-neotetraose) of human milk were subjected for the same GC-derivatization procedure, however all these experiments failed to analyze these saccharides by GC-MS. Surprisingly, based on the literature other tetrasaccharides (and also charged trisaccharides) were successfully investigated by GC, but all those structures lack *N*-acetylglucosamine moiety, one of the building blocks of our analytes. Therefore we concluded that this structural element is responsible for the failure. Applying various derivatization agents and procedure led also to the same result: neither sialyllactoses nor the tertasaccharides could be eluted even applying high upper temperature limit GC columns.

III/a 15N NMR-based method development for the characterization of isomeric HMO structures

Characterizing individual HMO structures are challenging as the separation and isolation of HMOs are laborious and extremely time consuming. Therefore we aimed at developing an NMR-based method in order to characterize HMOs without any prior sample treatment.

Our hypothesis was that *N*-acetylglucosamine (GlcNAc) and sialic acid residues contain the important amide groups detectable by ^{15}N HSQC NMR method. Based on the ^{1}H and ^{15}N NMR chemical shifts of the appropriate amide, one should be able to discriminate individual structures with subtle differences. In order to compile an NMR database for the NH groups having different chemical environment, the following standard compounds were subjected to complete NMR characterization (see Figure 6.). Following complete resonance assignment, the ^{1}H and ^{15}N NMR data on their *N*-acetylglucosamine (GlcNAc) and sialic acid residues were collected (see Table 2.).

Table 2. Complete NMR resonance assignment of GlcNAc moiety in neutral (top) and acidic (bottom) HMO standards.

		^{15}N	NH	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
		(ppm)								
GlcNAc	GlcNAc (α)	123,83	8,12	5,19	3,86	3,75	3,48	3,84	3,77	3,84
	GlcNAc (β)	123,09	8,22	4,70	3,67	3,52	3,45	3,45	3,73	3,90
	LacNac (α)	123,65	8,20	5,20	3,89	3,73	3,89	3,97	3,88	
	LacNac (β)	122,88	8,25	v. a.	3,70	n.a.	3,60	n.a.	3,83	3,96
	LNB (α)	123,19	8,23	5,17	4,06	3,93	3,58	3,89	3,83	
	LNB (β)	122,28	8,33	v. a.	3,80	3,76	3,55	3,49	3,76	3,90
	LNnT / para-LNnH	123,13	8,29	4,70	3,80	3,73	3,58	n.a.	3,85	3,94
	LNT	122,62	8,38	4,72	3,91	3,81	3,57	3,48	3,79	3,88
	LNFP-II	122,49	8,45	4,69	3,95	4,07	3,75	3,53	3,86	3,94
	LNFP-III	122,37	8,42	4,70	3,97	3,87	3,94	3,57	3,87	3,97
	LST-b	122,61	8,35	4,68	3,90	3,79	3,62	3,54	3,77	3,97

		^{15}N	NH	H-3 (ax)	H-3 (eq)	H-4	H-5	H-6	H-7	H-8	H-9	H-9'
		(ppm)										
Neu5Ac	3'-SL	123,06	8,09	1,79	2,75	3,67	3,85	3,62	3,59	3,89	3,64	3,87
	6'-SL	123,14	8,07	1,74	2,71	3,65	3,85	3,71	3,56	3,89	3,64	3,88
	LST-b	123,22	8,10	1,68	2,74	3,67	3,82	3,68	3,58	3,89	3,63	3,86

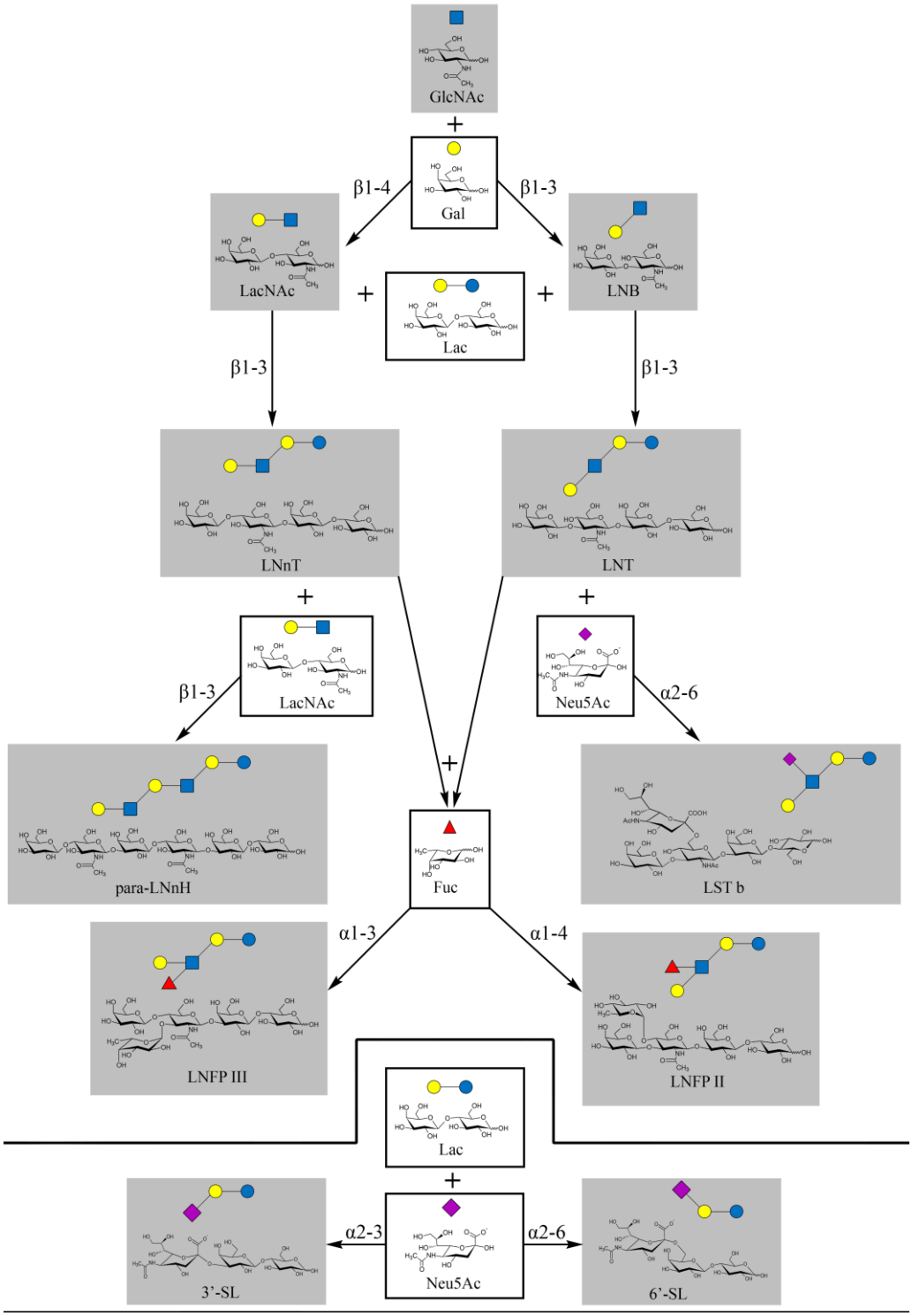

Figure 6. Structures of HMO standards used to construct NH amide (^{1}H and ^{15}N) chemical shift database

The results clearly indicate, that there is a nice separation along the ^{15}N chemical shift scale of various structures. Cross-peak subgroups can be assigned to type I and type II oligosaccharides (according to the linkage (see Figure 7.).

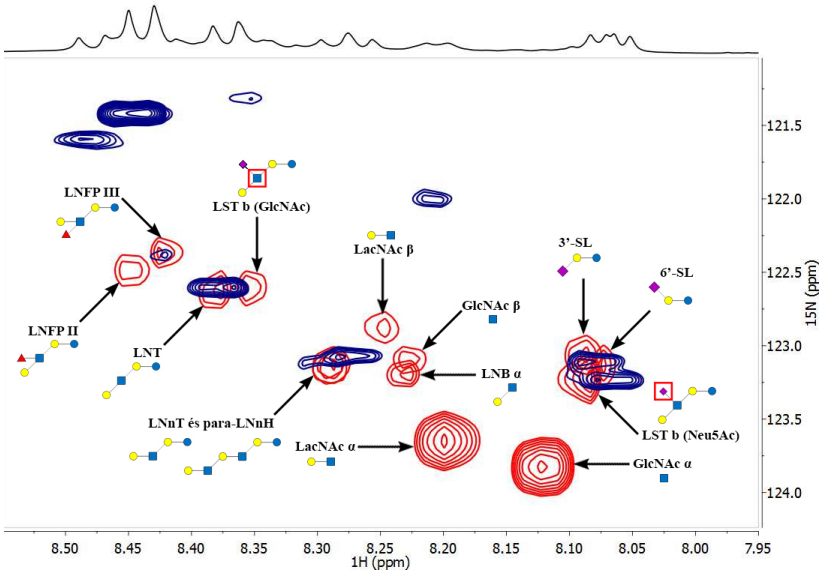

Figure 7. Overlay of ^{1}H-^{15}N HSQC NMR spectra (the amide region) of oligosaccharide mixture isolated from human milk (red) and those of individual standards (blue)

Our results are the first experimental evidences on the applicability of ^{15}N NMR in the characterization of human milk oligosaccharides on crude samples. Utilizing the difference in ^{1}H NMR chemical shifts of the GlcNAc core ^{1}H resonances in various positional isomers, the combination of ^{15}N NMR and ^{1}H-^{1}H TOCSY in a single experiment, the ^{15}N HSQC-TOCSY NMR experiment provided unequivocal resonance assignment of highly related structures such as LNFP II and LNFP III, the two fucosylated pentasaccharides differing in the position of the fucose linkage only (see Figure 8.). This presented NMR method has the advantage of characterizing milk oligosaccharides without laborious sample preparation.

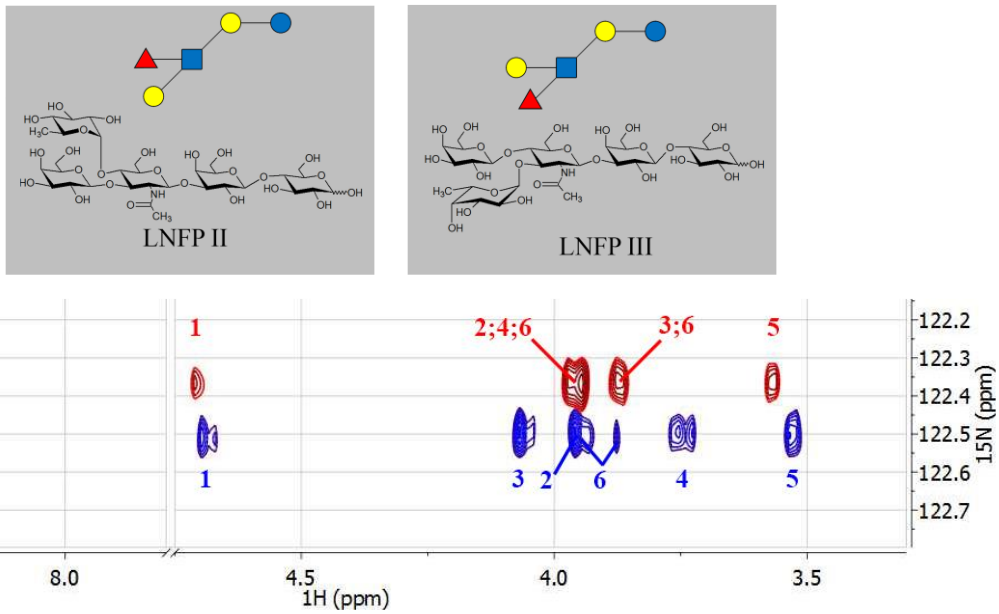

Figure 8. Stuctures of isomeric LNFP-II és LNFP III (top) along with their ^{1}H-^{15}N-HSQC-TOCSY NMR data (bottom). Blue crosspeaks indicate the *N*-acetylglucosamine 1H resonances in LNFP-II, while red crosspeaks correspond to the same moiety in LNFP-III., respectively.

III/b NMR-based method development for the characterization of acetyl migration in saccharides

As very little to no is known about the metabolism of human milk oligosaccharides, a new project was initiated. It is believed that acetylation by bacteria in human colon is one of the most possible metabolic transformation of the non-digestible saccharides. Acetyl-glucose and acetyl-lactose were chosen as model compounds to study their analytical behavior by NMR. First, mono-1-*O*-acetyl-glucose (Figure 9.) and mono-1-*O*-acetyl-lactose (Figure 10.) were synthesized and subjected to NMR studies.

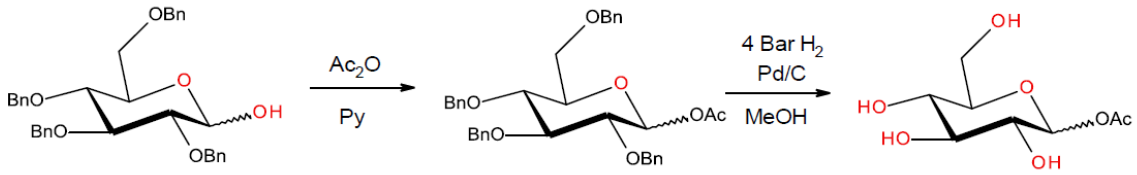

Figure 9. Synthesis of mono-1-*O*-acetyl-glucose.

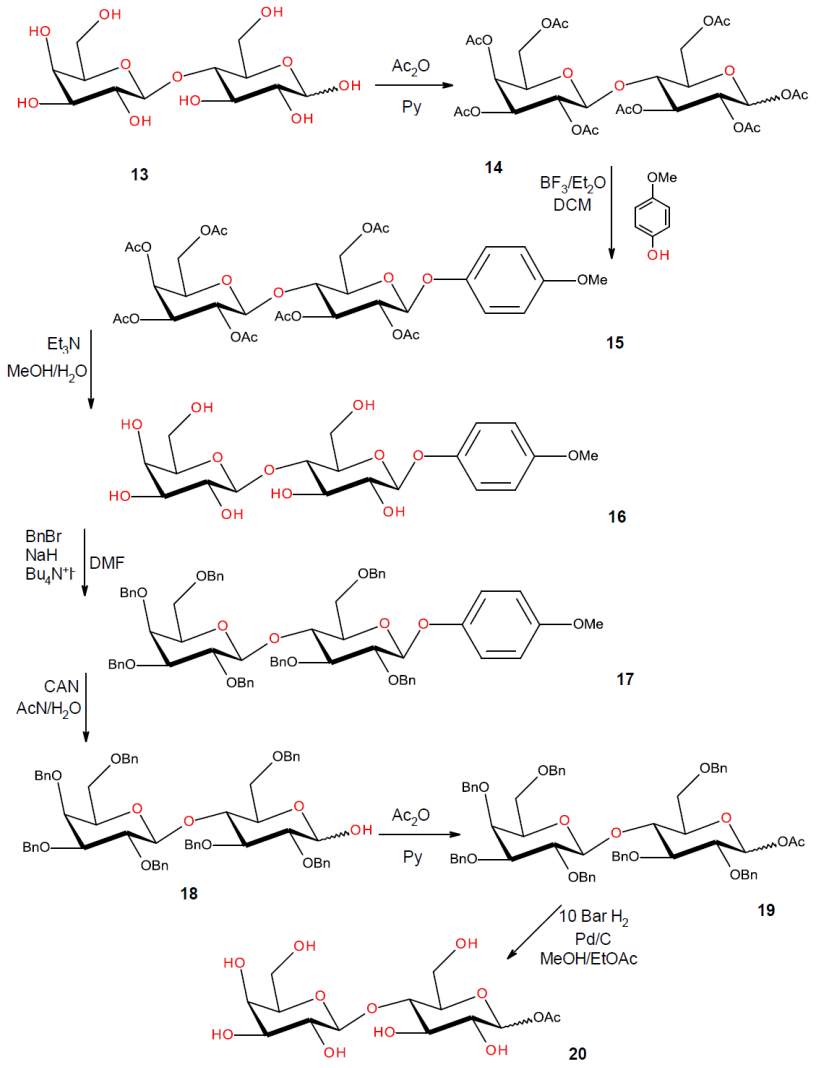

Figure 10. Synthesis of mono-1-*O*-acetyl-lactose.

It was observed, that acyl migration occurred in both cases: 1-*O*-acetyl-Glc converted to the energetically most favored 6-*O*-acetyl-glucose as a function of time (observing the migration from 1-*O*, 2-*O*, 3-*O*, 4-*O* and 6-*O*-sequence), and 4-*O*-acetyl-glucose was found to be the compound with the shortest half-life (see Figure 11.). Besides the synthesis, complete NMR assignments were conducted as well.

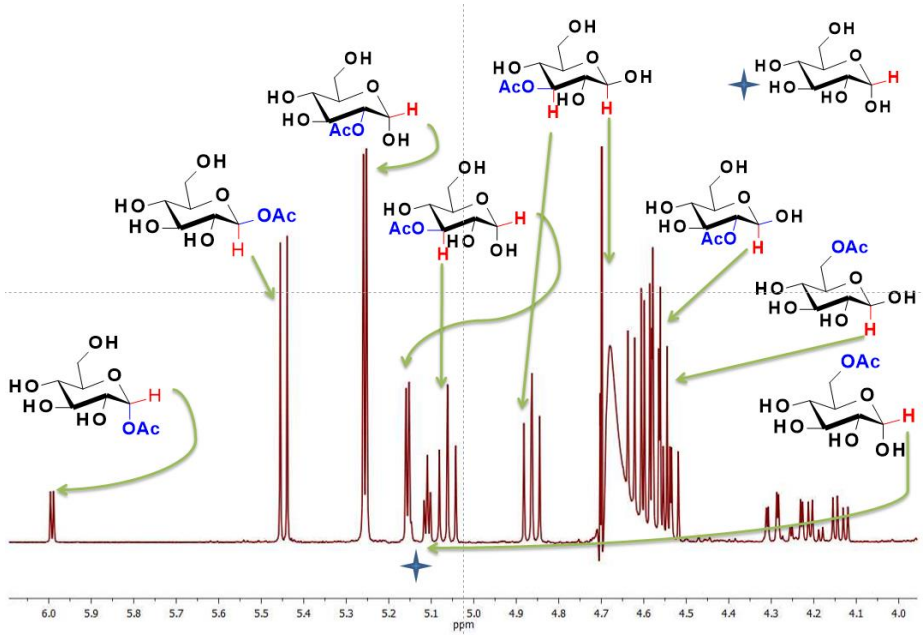

Figure 11. ^{1}H NMR spectrum (anomeric region) investigating the acyl migration in mono-1-*O*-acetyl-glucose (after 190 days of storage at room temperature). Interestingly, no mono-4-*O*-acetyl-glucose was found in the mixture.

In order to prove that 4-*O*-acetyl-glucose can also be identified in the NMR spectrum upon acyl migration, its synthesis was also elaborated (Figure 12.).

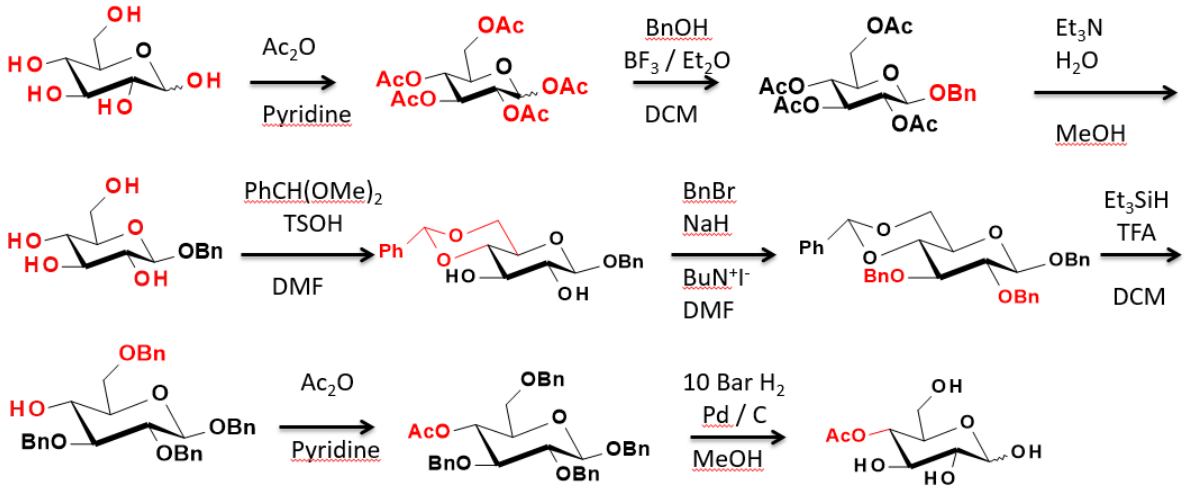

Figure 12. Synthesis of mono-4-*O*-acetyl-glucose.

In the case of 1-O-acetyl-lactose, an 8 steps synthesis was completed and the acyl migration observed for this compound showed the following pattern: 1-*O*-acetyl converted to 2-*O*-acetyl derivative which transformed to the final product of 3-*O*-acetyl-lactose. Therefore no acetyl migration occurred from the glucose to the galactose moiety.

Summary

Chromatographic and NMR-based analytical methods were developed for the qualitative and quantitative characterization of saccharides in breast milk.

- The isomeric disaccharides LacNAc and LNB were identified and quantitated for the first time using LC-MS.
- The isomeric trisaccharides (fucosyllactoses) found in human milk were determined by gas chromatography coupled to mass spectrometry for the first time.
- A validated LC-MS method was developed for the determination of isomeric sialyllactose isomers in human milk.
- A database was constructed containing ^{15}N NMR chemical shifts aiding the structural characterization of human milk oligosaccharide structures without any prior separation.
- Synthetic schemes were successfully designed for the selective 1-*O*-acetylation of glucose and lactose for acetyl-group migration studies.

Publication related to the topic

- R Balogh, P Jankovics, S Béni Qualitative and quantitative analysis of N-acetyllactosamine and lacto-*N*-biose, the two major building blocks of human milk oligosaccharides in human milk samples by high-performance liquid chromatography–tandem mass spectrometry using a porous graphitic carbon column *Journal of Chromatography A* 1422, 140-146 (2015)
- R Balogh, S Szarka, S Béni Determination and quantification of 2'-O-fucosyllactose and 3-O-fucosyllactose in human milk by GC–MS as O-trimethylsilyl-oxime derivatives *Journal of Pharmaceutical and Biomedical Analysis* 115, 450-456 (2015)
- M Grabarics, O Csernák, R Balogh, S Béni Analytical characterization of human milk oligosaccharides–potential applications in pharmaceutical analysis *Journal of Pharmaceutical and Biomedical Analysis* 146, 168-178 (2017)

Three further manuscripts are in preparation.

dr. Réka Balogh (PhD student) will defend her PhD Thesis in this field in 2019 under the supervision of the principal investigator of this project (Sz. Béni).

Zita Boldvai, András Tóth and Márkó Grabarics completed their Pharm D. thesis under the supervision of the principal investigator of this project (Sz. Béni) in this field and also participated in undergraduate student research conference.

Zita Boldvai 1st price (participated at national research competition: OTDK)

András Tóth 2nd price

Márkó Garabarics 1st price (participated at national research competition: OTDK)