A novel method for high-throughput analysis of bioactive oligosaccharides

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Introduction

Milk oligosaccharides (OS) are theorized to promote human health through numerous functional abilities, including modulation of glycan expression on intestinal cells,[1] prevention of necrotizing entercolitis,[2] acting as prebiotics for commensal bacteria,[3] and acting as decoys for pathogen binding.[4] Human milk has high OS concentrations,[5] making it a highly suitable food for developing infants. However, OS are much less abundant in bovine milk and other commercially-available dairy products. Due to the health-promoting nature of these compounds, there is a need to further investigate their individual chemical and microbiological properties. Development of new methods for accurate quantification will enable guidance of industrial scale processing to obtain large amounts of pure oligosaccharides.

The objective of this project was to develop a high-throughput method of chemical analysis for milk OS using liquid chromatography coupled to mass spectrometry (LC-MS). LC-MS techniques can provide a wealth of information and are commonly used to identify, characterize and quantify OS. However, drifts in instrument performance over weeks or months can complicate these analyses for large sample sets. Here we present an LC-MS method that uses commercially-available isobaric tags to allow for sample multiplexing, reducing analysis time by an approximate factor of six. Each tag produces a characteristic fragment ion upon tandem fragmentation that serves as a basis for relative quantification. With this method, we will analyze the OS content of 850 bovine milk samples collected under the Danish-Swedish Milk Genomics Initiative with the goal of identifying breed- and genome-based associations related to OS content. The optimized method will be broadly applicable to the analysis of other free oligosaccharides, as well as protein-linked glycans.

Methods

OS were extracted from bovine milk by adapting techniques used previously by our research group.[6] The unreduced OS were labeled with carbonyl-reactive isobaric tags (Thermo Scientific, Figure 1) according to the manufacturer's instructions.[7] An LC-MS method for OS identification and relative quantification was developed on an Agilent 6520 Accurate Mass quadrupole-time of flight (Q-TOF) mass spectrometer. The most abundant OS were selected for fragmentation through data-dependent analysis. For each precursor ion charge state, a linear trendline was used to link precursor mass-to-charge ratio (m/z) to an optimal collision energy (CE). Trendline slopes were optimized such that relative precursor abundance was consistent across all tandem-MS spectra, and y-intercepts were then adjusted to obtain optimal abundances of the tag fragments (Figure 3). Instrument scan rates and the minimum precursor abundance threshold for fragmentation were adjusted to give the most consistent ratios.

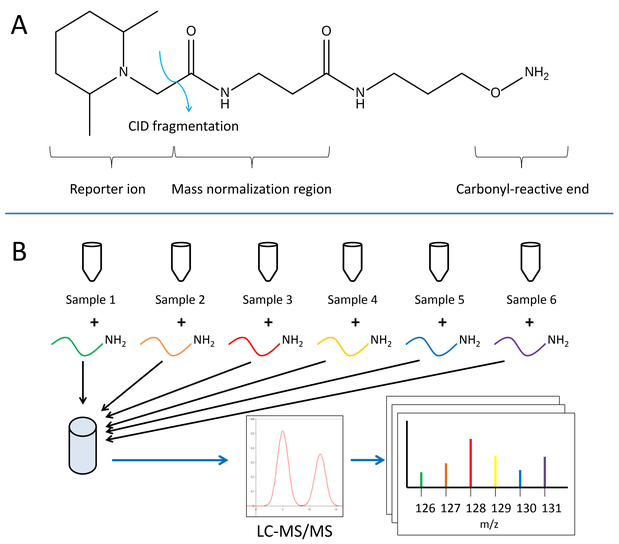

Figure 1. Structure of commercially-available isobaric tags for carbohydrates (A). Each tag has a unique reporter ion mass, allowing a set of tagged samples to be multiplexed. The abundance of each reporter ion is used to measure the amount of each carbohydrate in each sample (B).

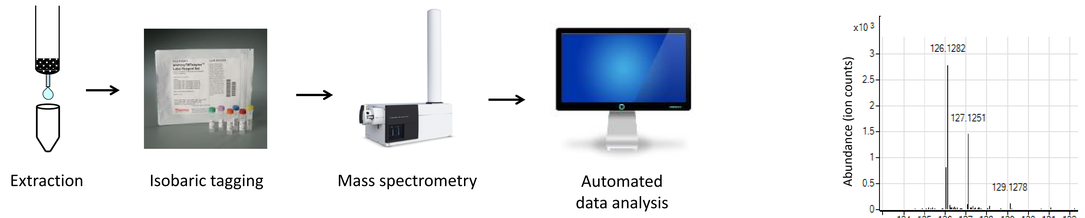

Figure 2. Total high-throughput workflow for the identification and relative quantification of milk OS.

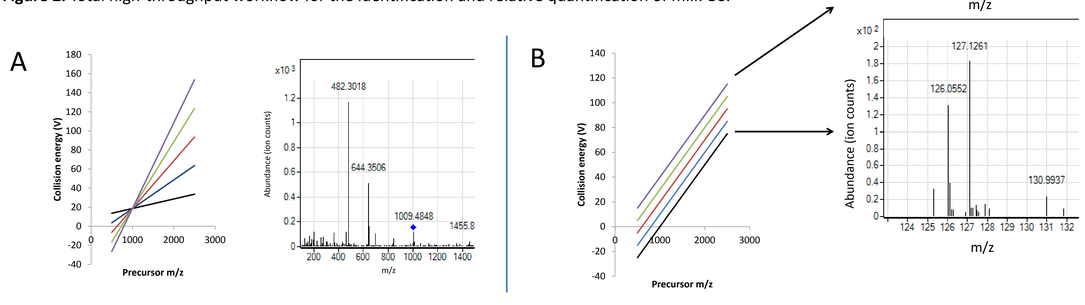

Figure 3. Optimal collision energies were specified by varying the energy linearly with m/z. For each charge state, slopes were optimized such that relative precursor abundances were consistent across the m/z range (A). The y-intercept was then increased until the reporter ions were highly abundant and had accurate ratios (B).

Results

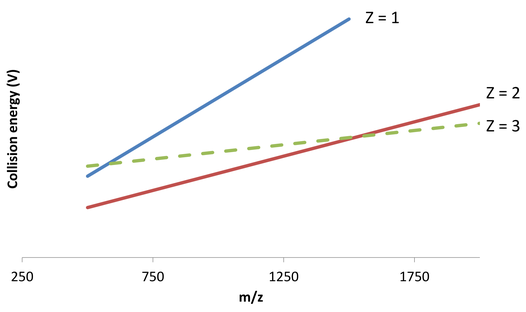

Figure 4. Collision energy parameters for charge states +1 to +3. The y-intercept of the Z = 3 trendline is under final optimization.

- Replicate injections of tagged standards demonstrated improved instrument method repeatability, with a coefficient of variation below 5% for all compounds (Table 1).
- Collison energies were optimized for ions with charges of +1 and +2 (Figure 4). The collision energy trendline for +3 ions will be finalized shortly.
- This novel method can identify and measure the abundance of glycans at trace levels, with a limit of quantification below **5 µg/mL** for the tested standards. Furthermore, multiplexing has an additive effect on OS concentrations, boosting the signal strength for low-abundance OS.
- For high-throughput sample analysis, raw data will be analyzed by SimGlycan (PREMIER Biosoft). A customized milk OS library containing structural information was generated within the software to create a maximally automated data analysis workflow. Example OS profiles with relative quantities are shown in Table 2.

Table 1. Two sets of OS standards were tagged and combined in a 2:1 ratio. Four replicate injections were analyzed using a method optimized for fragmentation of +1 and +2 ions. Average experimental ratios are displayed below.

Standard	Concentration (µg/mL)	Measured abundance ratio	Coefficient of variation
Sialyllactose	45.8	2 : 1.112	4.56%
LNT	8.00	2 : 1.095	4.66%
LNH	8.00	2 : 0.996	3.75%

Table 2. Example results table from high-throughput analysis of bovine milk OS.

Glycan composition	Mass	Relative abundance				Structure
		Sample 1	Sample 2	. . .	Sample 850	
(Gal)2 (Glc)1	504.1690	2	1.2647	. . .	0.5478	
(Hex)2 (HexNAc)1	545.1956	2	1.2074	. . .	0.4993	
(Gal)1 (Glc)1 (NeuAc)1	633.2116	2	1.0132	. . .	0.5736	
(Gal)3 (Glc)1 (GlcNAc)1	869.3012	2	1.1454	. . .	0.5105	
(Gal)3 (Glc)1 (GlcNAc)2	1072.3806	2	1.3044	. . .	0.5001	
⋮	⋮	⋮	⋮	⋮	⋮	⋮

Conclusion

This high-throughput method represents an advancement in the ability of LC-MS techniques to illuminate subtle variations in compound abundances which would otherwise be confounded by drifts that affect large sample sets. Multiplexing further maximizes the information that can be obtained by increasing signal strength for low abundance OS, therefore enabling detection of powerful trace-level bioactive compounds.

This method will be applied to the analysis of several hundred bovine milk samples collected under the Danish-Swedish Milk Genomics Initiative. Although the immediate use of the technique is for milk OS analysis, the method can be extended to identify free- and protein-linked glycans in other food or even biological samples of relevance (*e.g.* blood, urine, saliva) with little to no modification required.

References

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