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Methods for the quantitation of human milk oligosaccharides in bacterial fermentation by mass spectrometry

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Abstract

Oligosaccharides are the third most abundant component in human milk. In the past decades, it became apparent that they would be able to protect against pathogens and participate in the development of the gut microflora for infants. However, their role in infants' nutrition and development remains poorly understood. To better understand this function, it is extremely important to have a quantitative tool for profiling oligosaccharides. In this article, we show the development of a method to quantitatively differentiate the relative amounts of oligosaccharides fermented by different intestinal bacteria. To determine the oligosaccharide consumption, bacteria were grown in a medium using human milk oligosaccharides (HMOs) as the only carbon source purified from breast milk and further analyzed by matrix-assisted laser desorption/ionization–Fourier transform ion cyclotron resonance mass spectrometry (MALDI–FTICR MS). A method using an internal deuterium-labeled standard was developed and compared with an external standard method, with the internal standard method giving better precision and unambiguous measurements than the external standard method and providing to be a novel and robust tool for following bacterial fermentation of milk oligosaccharides.
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The human gastrointestinal tract (GIT)[1] is a complex and efficient system that has the dual role of digestion and absorbing nutrients while protecting the body from bacterial invasion. The GIT supports a large bacterial population of 500–1000 different species that reside in the colon or large intestine, and these have coevolved with humans. The colon has the highest population density of any ecosystem on Earth and has the least diversity [1]. This suggested that there is a strong host selection for specific bacterial species. The bacteria–host relationships are referred to as commensal or mutalistic, meaning that there are both tolerance and benefits to their presence in the colon. It has been long known that *Bifidobacteria* spp. are the predominant microbial species in the infant GIT, but this bacterial population must develop in an appropriate way along with the intestinal immune system during the transition from the sterile environment of the womb, to a breast-feeding infant, to a child, and then to an adult. The colon is the site, where microbial degradation and fermentation of oligosaccharides take place, providing the resident bacterial flora with the required carbon source and energy for growth [2,3]. The question of how the host selects for different intestinal bacteria is not well

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[1] *Abbreviations used*: GIT, gastrointestinal tract; HMO, human milk oligosaccharide; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; TOF, time-of-flight; FTICR, Fourier transform ion cyclotron resonance; DHB, 2,5-dihydroxybenzoic acid; HPLC, high-performance liquid chromatography; GCB–SPE, graphitized carbon black–solid phase extraction; TFA, trifluoroacetic acid; RT, retention time; OS 1, oligosaccharide 1; OS 2, oligosaccharide 2.

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understood, but a clue to the selection process may come from understanding the role of human milk oligosaccharides (HMOs) in the infant gut as prebiotics.

Oligosaccharides are the third most abundant component in human milk and differ in size, structure, and abundance [4]. With few exceptions, all known HMOs have a lactose core and are elongated via linkage of one or more units of galactose, *N*-acetylglucosamine and can be fucosylated and sialylated extensively [5]. It could be estimated at as many as 900 structures, considering all of the monosaccharide combinations and possible linkages [6]. Currently, more than 90 structures have been elucidated and approximately 200 species have been identified [4,7].

Recent studies have shown that *Bifidobacteria* spp. have distinctly higher concentrations in breast-fed infants than in formula-fed infants [4]. The presence of these microorganisms in the infant GIT has been associated with a number of important health-promoting effects, including reduced incidence of diarrheal illnesses, improved lactose digestion, and enhanced immunomodulatory functions [8]. We recently showed that *Bifidobacteria* spp. can grow on purified HMOs as a sole carbon source, supporting the hypothesis that HMOs promote the development of beneficial microorganisms in the infant GIT [9]. However, quantitative measurement of the amount of HMOs fermented by intestinal bacteria has been problematic due to the lack of more reliable and precise quantitation methods for these complex substances.

Matrix-assisted laser desorption/ionization (MALDI) has become a more convenient and sensitive method to analyze complex mixtures of oligosaccharides than have chromatographic techniques [10]. Previous studies on milk oligosaccharide analysis using mass spectrometry (MS) have been mostly qualitative, and more recently a semiquantitative analysis using UV–MALDI–time-of-flight (TOF) was reported on commercial oligosaccharides [7,11–17]. However, the limited resolution capability of the MALDI–TOF technique restricts the application of isotope-labeled internal standards [18]. We previously applied stable isotopic incorporation in the analysis of O-linked mucin-type oligosaccharides from the egg jelly coat of *Xenopus laevis* species using MALDI–Fourier transform ion cyclotron resonance (FTICR) MS [19]. In the current work, two new quantitative methods involving unlabeled external and internal deuterated standards for the analysis of milk oligosaccharides after bacterial fermentation were developed using MALDI–FTICR MS.

Materials and methods

Materials and reagents

Nonporous graphitized carbon cartridges (150 mg bed weight, 4 ml tube size) for desalting were purchased from Alltech (Deerfield, IL, USA). Evaporation of solvent was accomplished using a Centrivap Concentrator (Labconco, Kansas City, MO, USA). Sodium borohydride (98%), sodium borodeuteride (98%), 2,5-dihydroxybenzoic acid (DHB), and β-galactosidase from *Kluyveromyces fragilis* were purchased from Sigma–Aldrich (St. Louis, MO, USA). All bacterial strains were obtained from American Type Culture Collection (Manassas, VA, USA). All other reagents used were of analytical grade or higher. High-performance liquid chromatography (HPLC) analysis was performed on a Hewlett–Packard series 1100 instrument.

Oligosaccharide extraction

Milk samples were obtained from the milk banks of San Jose, California, and Austin, Texas, in the United States. The oligosaccharide extraction procedure was performed according to Ward and coworkers [9]. Briefly, 1 L of milk was centrifuged at 5000*g* for 30 min at 4 °C, and the lipid layer was removed via filtration through glass wool. Then 2 L of ethanol was mixed with the defatted milk, and the solution was kept overnight at 4 °C. Insoluble precipitate was removed by centrifugation at 5000*g* for 30 min at 4 °C, and ethanol was removed by evaporation. Lactose was hydrolyzed using β-galactosidase, and oligosaccharides were isolated from monosaccharides using solid phase extraction with nonporous graphitized carbon.

Bacterial fermentation

Bacterial strains were *Bifidobacterium longum* bv. *infantis* ATCC 15697, *Lactobacillus gasseri* ATCC 33323, *Bifidobacterium longum* bv. *longum* ATCC 15707, and *Escherichia coli* OP50. Bacteria were grown and maintained at 37 °C in modified MRS media supplemented with 0.05% (w/v) L-cysteine, M63 media (*E. coli*), and 2% HMO (w/v) as a sole carbon source. Purified HMOs were filtered (Millex-GV, 0.22 μm, Millipore, Billerica, MA, USA) before being added to the autoclaved cooled media. Media were inoculated directly from frozen stocks, and experiments were performed in triplicates. As negative controls, media were inoculated with bacteria devoid of any carbon source and another control containing HMO but left uninoculated. The culture growth, stopped after 54 h, was followed by measuring the optical density, and supernatant was collected, centrifuged at 2000*g* for 30 min, and used for further analysis.

Oligosaccharide reduction and purification

The oligosaccharides recovered from the supernatant (100 μl) were reduced by adding 100 μl of 2.0 M sodium borohydride or sodium borodeuteride and were incubated at 42 °C for 16 h, desalted, and purified using nonporous graphitized carbon black–solid phase extraction (GCB–SPE). Prior to use, the cartridge was washed with 3 volumes of 80% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA, v/v) and another 3 volumes of deionized water. The oligosaccharide samples were loaded into GCB–SPE cartridge and washed with 7 volumes of deionized water at a

flow rate of 1.0 ml/min, eluted with 2 volumes of 20% acetonitrile in water (v/v), dried in vacuo, and reconstituted with 50 µl deionized water.

HPLC analysis of oligosaccharides

Oligosaccharide profiles were obtained at 206 nm with a Hypercarb Porous Graphitized Carbon column (100 × 2.1 mm, 5 µm particle size, Thermoquest, Hypersil division, Needham, MA, USA). Deionized water (A) and acetonitrile (B) were used as eluents at a flow rate of 0.25 ml/min, and a nonisocratic gradient was applied as follows: 0–44 min, 5–16% B; 44–56 min, 16–28% B; 56–60 min, 28–32% B; 60–71 min, 32% B; 71 min, 82% B; 72–77 min, 5% B; and 78–82 min.

MALDI–FTICR MS analysis

All mass spectra were acquired on an HiRes MALDI–FTICR MS instrument with an external MALDI source, a 355-nm pulsed Nd:YAG laser, a quadrupole ion guide, and a 7.0 Tesla superconducting magnet (IonSpec, Irvine, CA, USA). The analyte–matrix deposit was prepared following the fast evaporation technique on a 100-sample stainless-steel plate, with 2 µl of sample being spotted on the MALDI plate, followed by the addition of 1 µl of 0.01 M NaCl as a dopant and 2 µl of 0.4 M DHB. The spots were allowed to dry under a stream of air before analysis. For detection, ion excitation was performed through an arbitrary waveform with an amplitude of 150.0 V (base to peak) at a rate of 2 MHz for a scan range of *m/z* 216–2500 and 1024 K data points. Six acquisition scans were performed on each sample in the positive ion mode.

Results and discussion

In an attempt to perform a quantitative study of oligosaccharide consumption after bacterial fermentation, three different bacterial samples (*B. longum* bv. *infantis*, *B. longum* bv. *longum*, and *E. coli*) were grown in the presence of purified HMOs. Oligosaccharides were reduced, purified as described in Materials and methods, and first analyzed using HPLC (Fig. 1). The HPLC profiles of recovered oligosaccharides from modified MRS media of *B. longum* bv. *infantis* and *B. longum* bv. *longum* cultures showed large variations from the uninoculated control (Fig. 1A). In the *B. longum* bv. *infantis* chromatogram, several of the major peaks, including the two prominent ones at retention times (RTs) 19 and 21 min, were absent (Fig. 1B). In the *B. longum* bv. *longum* chromatogram, these peaks were also absent and a new peak at approximately RT 28 min was observed (Fig. 1C). These results indicate differential consumption of HMOs, as we reported previously [9]. However, the general glycan profiles and the features of the chromatograms for the uninoculated modified MRS control and *E. coli* sample were similar, indicating that *E. coli* did not consume most, if any, of the oligosaccharides (Fig. 1D).

The ability to resolve a mixture of oligosaccharides is inherent in PGC–HPLC; however, it often is not necessary to completely separate the mixtures into individual components. The sizes of the oligosaccharides and the presence of biologically important residues such as fucose can be determined from simple exact mass profiles to provide valuable insight into potential biological activity [20]. Complete separation creates a number of issues that complicate the analysis. For example, the RT needs to be precise, the individual components need to be baseline resolved, and the chromatogram needs to be annotated. The analysis and the experiment then become prohibitively long. An LC/MS combination would be an appropriate method for resolving these issues, and efforts in our laboratory and elsewhere are focused on developing and evaluating a method with nanoflow LC [21–24]. This method appears to be promising and will be useful when the fate of specific oligosaccharide structures needs to be investigated.

Based on these results, two new methods were developed to obtain sufficient resolution, reproducibility, and accuracy for quantifying bacterial HMO consumption. Observing the intensities of the different oligosaccharides before and after exposure to different intestinal bacteria would provide a measure of the capacity of different bacteria to ferment select oligosaccharide structures. One method used an external standard, and the other method used a deuterium-labeled internal standard; both were run in parallel from a common oligosaccharide source.

Oligosaccharide quantitation using the external standard method

The general strategy followed is outlined in Scheme 1. Briefly, after 54 h, cultures were stopped and oligosaccharides were recovered from the supernatant, reduced by sodium borohydride, purified, and analyzed by MALDI–FTICR MS.

The mass spectrum from the uninoculated control showed a number of oligosaccharides that we previously identified based on exact mass measurements [9]. Although the number of masses that can be tracked is higher, the seven most abundant components were examined. Previous results of these separated components showed that each mass may correspond to approximately between three and seven isomers [24].

The 100% intensity was assigned to the intensity of the uninoculated control, and after the incubation time period intensities at 100% indicated that the oligosaccharides were not consumed.

Escherichia coli showed relatively constant oligosaccharide abundance between 60% and 90% for the various masses measured. Although there have been no previous reports of *E. coli* consuming oligosaccharides, approximately 20% reduction was observed for *m/z* 732.3. A possible explanation for this reduction is the binding of sialylated chains to the surface of *E. coli* as demonstrated previously [20].

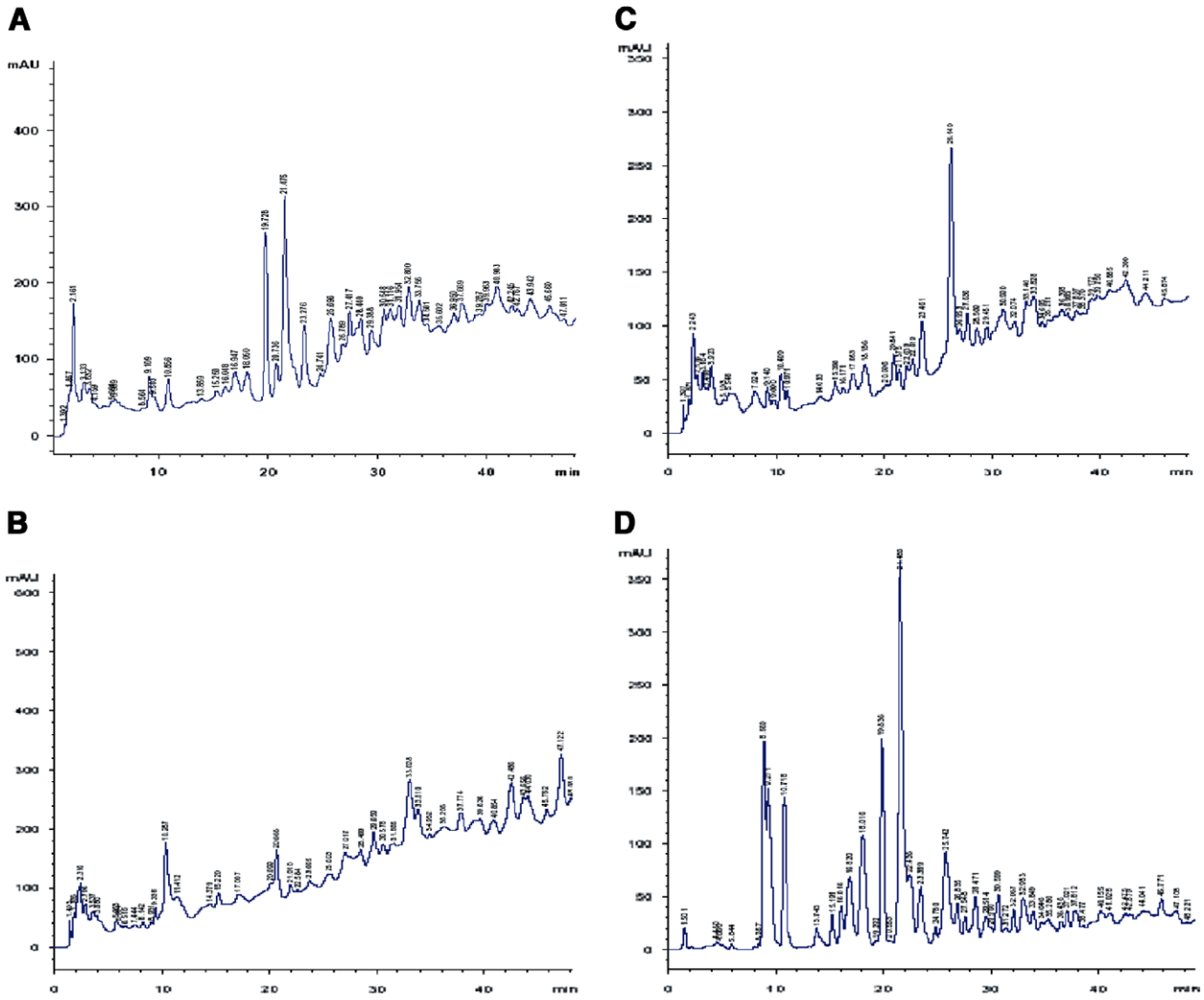
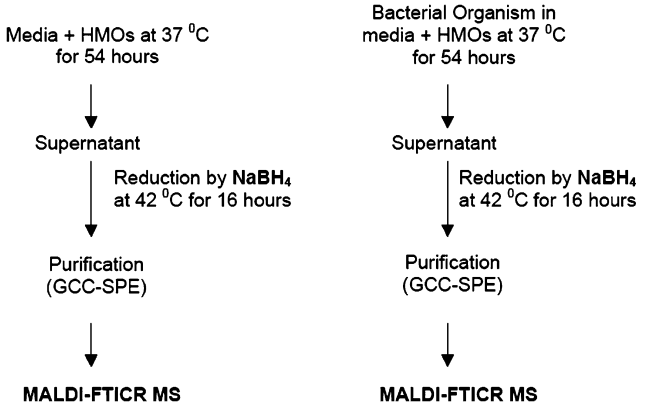


Fig. 1. HPLC analysis of the HMOs remaining after 54 h of culture in an uninoculated media (A) and after growth of *B. longum* bv. *infantis* (B), *B. longum* bv. *longum* (C), and *E. coli* (D).



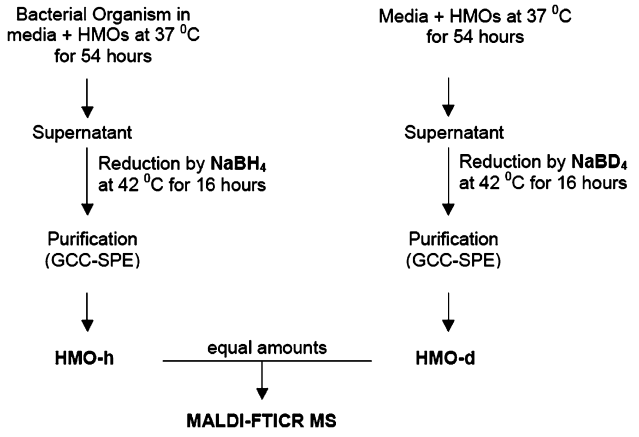
Scheme 1. Quantitation using external standard.

Bifidobacterium longum bv. *infantis* showed more than 50% reduction for nearly all of the oligosaccharides monitored compared with that observed for *E. coli.* There were significant variations between the different oligosaccharides measured; the oligosaccharides at *m*/*z* 1754.6 showed the least consumption at approximately 50%, *m*/*z* 878.3 was consumed down to less than 20%, and *m*/*z* 1243.4 was the most consumed down to less than 10%.

Lactobacillus gasseri, on the other hand, had intensities only slightly more attenuated than those for *E. coli.* The largest change was observed at *m*/*z* 1462.5 with 45% remaining oligosaccharides, and most of the reduction was between 30% and 50% for the other masses measured. The decrease in intensity of specific oligosaccharides after incubation with *B. longum* bv. *infantis* suggested that HMOs were a more viable carbon source for this particular strain than for the *E. coli* strain.

Oligosaccharide quantitation using deuterium-labeled internal standard method

As a more accurate method of quantitation, the addition of an isotopically labeled internal standard was developed. Scheme 2 shows the strategy to analyze HMOs after bacterial fermentation. Bacterial cultures containing the HMOs were grown for 54 h, and oligosaccharides were recovered



Scheme 2. Quantitation using deuterated HMO as internal standard.

from the supernatant, reduced in the presence of sodium borohydride to yield the corresponding alditol, and purified. Uninoculated media containing HMOs were processed as described above, but reduction was performed using sodium borodeuteride (Fig. 2). The HMO consumption analysis was performed by MALDI–FTICR MS. First, the analyses of labeled and unlabeled oligosaccharides were carried out separately. Fig. 3 shows the mass spectra of the unlabeled HMOs from uninoculated M63 media and of the labeled HMOs from inoculated M63 media with *E. coli* (Fig. 3A and B, respectively). No undeuterated species were detected by MALDI–FTICR MS analysis after reduction, showing a 100% efficiency of the labeling reaction. The isotopic distributions of ^{13}C signals were well resolved in both samples, as shown by the insets for *m/z* 732.3 and 733.3 (Fig. 3A and B, respectively). Note that the relative abundance of each isotope in both samples was roughly the same, demonstrating the high efficiency in the reduction and purification processes. The overall mass profile of the deuterium-labeled sample resembled that obtained from the unlabeled oligosaccharide. Moreover, the relative abundances of signals at *m/z* 513, 732, 878, 1097, 1243, 1389, 1462, 1535, 1608, and 1755 were nearly identical, indicating that the ionization efficiency of the singly deuterated species was the same as that of the unlabeled sample. The advantages of this method are that the events of preferential ionization, such as suppression issues and other factors (e.g., possible effects of the media), were avoided.

When the labeled and unlabeled oligosaccharides from the *E. coli* sample were combined and analyzed (Fig. 4), the mass spectrum showed a pattern similar to that from the uncombined analysis (Fig. 3). This result confirmed that deuterium labeling did not alter the mass profile and thus could be used for precise HMO quantitation. ^{13}C isotopic distribution analysis at *m/z* 732.3 from the combined sample is shown in Fig. 4 inset. *A* corresponded to the most abundant isotope of unlabeled oligosaccharide, and *A* + 3 corresponded to the least abundant isotope of labeled oligosaccharide. On the other hand, *A* + 1 and *A* + 2 showed increases in the relative abundance relative to those observed in the uncombined analysis and corresponded to the overlapping isotope signals from unlabeled and labeled oligosaccharides. Therefore, it was possible and necessary to correct for ^{13}C signal contribution from each species. IonSpec exact mass calculator was used to determine the expected intensity of each isotope from unlabeled oligosaccharides, and the ratio of deuterated species to undeuterated species (*D*/*H*) was calculated for each signal. Based on six MALDI–FTICR acquisitions of a single sample from *E. coli* cultures, *D*/*H* ratios for the 12 most abundant HMO signals were determined (Table 1). The ratios varied very little from unity, implying that *E. coli* did not consume any of the oligo-

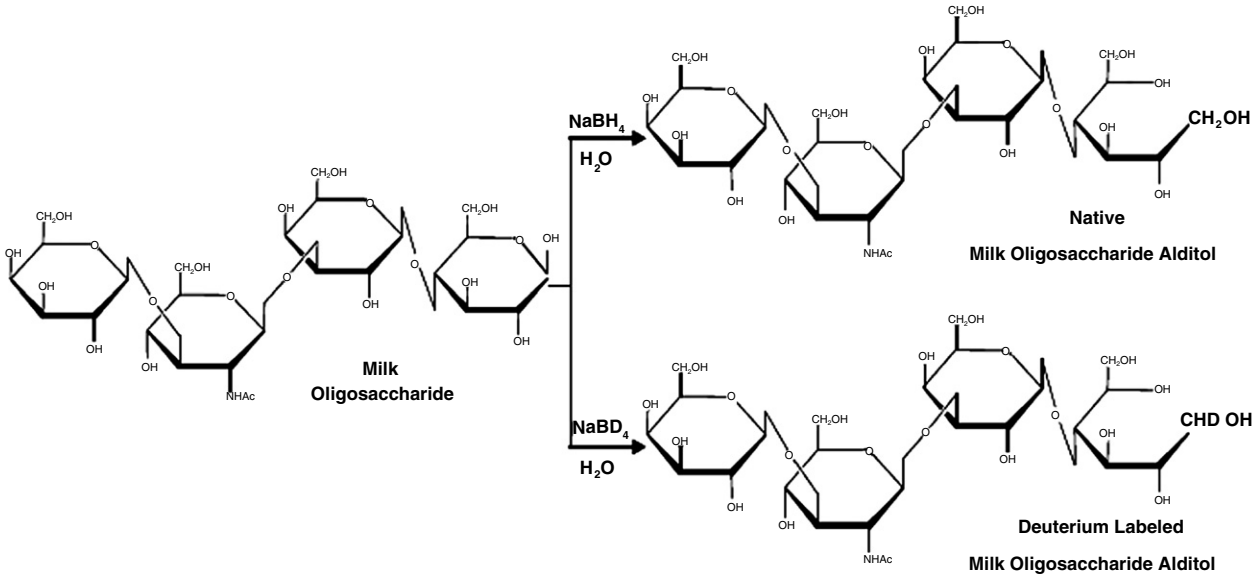


Fig. 2. Scheme of reduction of milk oligosaccharide (*m/z* 732.3) using $NaBH_4$ or $NaBD_4$ yielding the corresponding undeuterated and deuterated alditol forms.

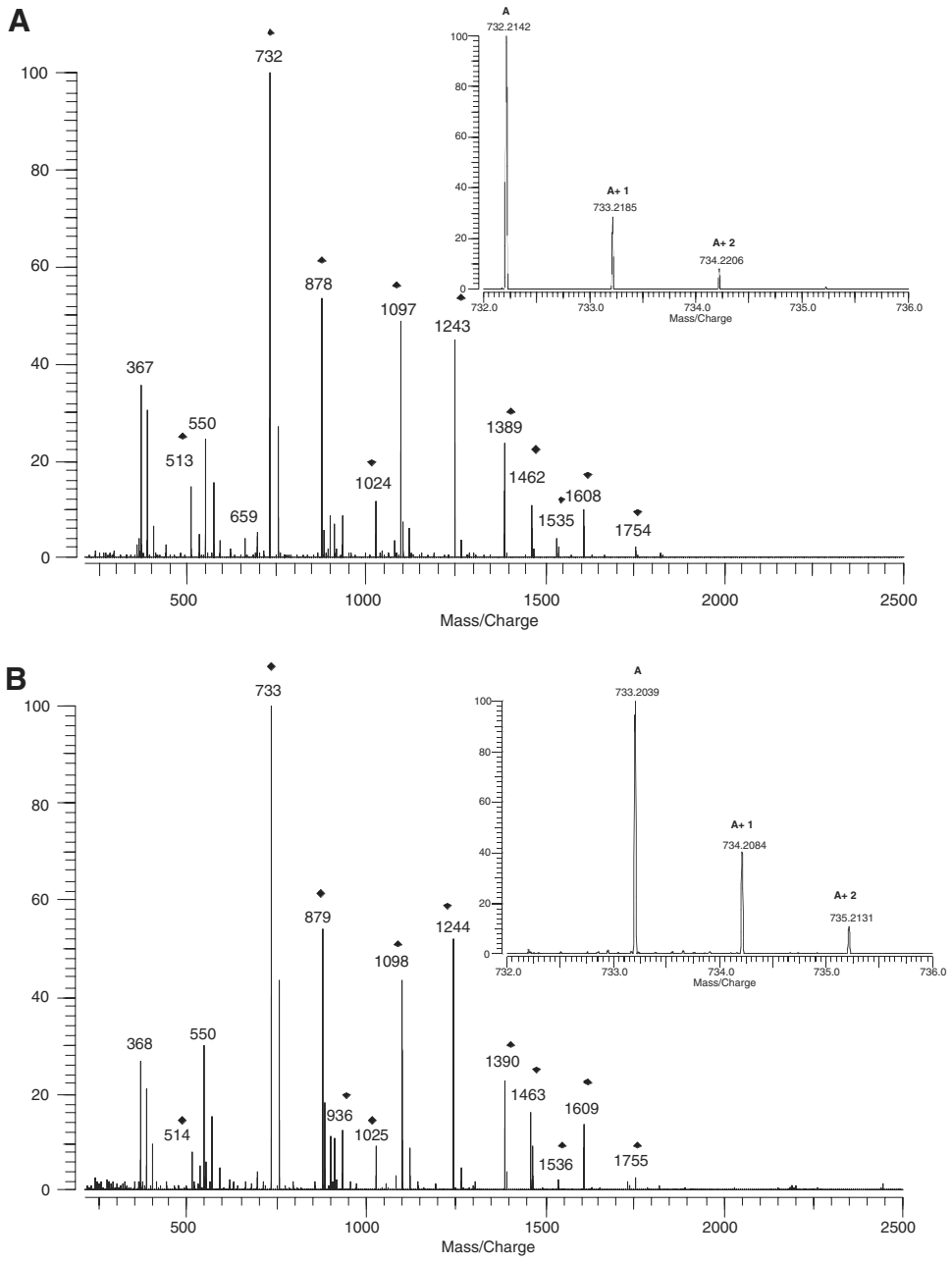


Fig. 3. MALDI–FTICR (+) mass spectra obtained from undeuterated HMOs (A) and deuterated HMOs (B) remaining in medium after inoculation with *E. coli.* The insets show the ^{13}C isotope distributions at signals *m*/*z* 732.3 (A) and 733.3 (B).

saccharides monitored. The low standard deviation for *D*/*H* values included in Table 1 also showed the high precision of the method. These results also provided a general indication of the reproducibility of this method. More variations in the *D*/*H* ratios were observed when the relative intensities of the ions were less than 10%. These could be due to peak coalescence given that the signal of the smaller population often was affected by the significantly larger signal [25,26]. In addition, issues such as the total ion population and the relative ion population between the two masses sometimes affect the ratio of the deuterated and undeuterated species. These came from the damping of the transients of some ions and the coalescence of small ions in the presence of the larger ones, but these were not expected to play a major role here [25–28].

Monitoring the consumption of HMOs by different bacterial species

We recently showed that *Bifidobacteria* spp. can grow on purified HMOs as a sole carbon source, suggesting that HMOs selectively amplify and promote the development of a beneficial microflora in the infant GIT [9]. *B. longum* bv. *infantis* and *B. longum* bv. *longum* were grown in media containing HMOs for 54 h and then were prepared as

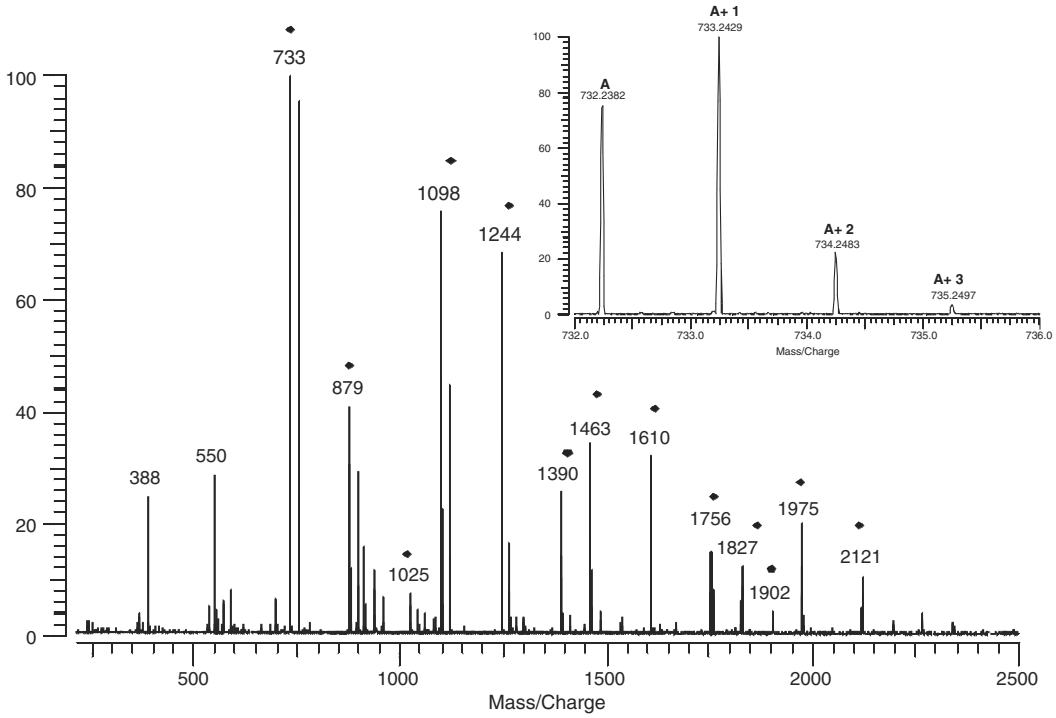


Fig. 4. MALDI–FTICR (+) mass spectrum of HMOs obtained from *E. coli* sample with deuterated HMOs as internal standards.

Table 1
D/*H* calculations from *E. coli* samples

	$[M+Na]^+$	Oligosaccharide composition	*D*/*H*[a]
1	732.3	3Hex 1HexNAc	1.04 ± 0.04
2	878.3	3Hex 1HexNAc 1Fuc	0.99 ± 0.04
3	1024.4	3Hex 1HexNAc 2Fuc	1.00 ± 0.03
4	1097.4	4Hex 2HexNAc	1.10 ± 0.09
5	1243.4	4Hex 2HexNAc 1Fuc	1.11 ± 0.17
6	1389.5	4Hex 2HexNAc 2Fuc	1.14 ± 0.13
7	1462.5	5Hex 3HexNAc	1.16 ± 0.18
8	1535.6	4Hex 2HexNAc 3Fuc	1.17 ± 0.19
9	1608.6	5Hex 3HexNAc 1Fuc	1.27 ± 0.11
10	1754.6	5Hex 3HexNAc 2Fuc	1.15 ± 0.15
11	1827.6	6Hex 4HexNAc	1.36 ± 0.13
12	1900.7	5Hex 3HexNAc 3Fuc	1.14 ± 0.23

[a] $D/H = (n - mq/p)/m$, where $m = A$, $n = A + 1$, $p = A$, $q = A + 1$, m, n = relative intensities, and p, q = expected intensities.

shown in Scheme 2 and analyzed by MALDI–FTICR MS. Both *Bifidobacteria* spp. showed differential consumption of different species of HMOs as it was observed using the external standard method. The *D*/*H* values for each species present in the spectra were calculated. *B. longum* bv. *infantis* showed the highest *D*/*H* ratio of 2.57 for oligosaccharide 1 (OS 1), indicating a preferential consumption of the corresponding oligosaccharide (Fig. 5). The *D*/*H* ratio for oligosaccharide 2 (OS 2) was 1.76, showing that it was moderately consumed. On the contrary, in *B. longum* bv. *longum*, the *D*/*H* ratio for the same signal displayed a small variation from the control for OS 1 and was closer to unity for OS 2, suggesting that the corresponding oligosaccharides were slightly consumed and not consumed, respectively. However, more thorough studies are being undertaken to confirm these results and to determine specific oligosaccharides consumed after bacterial fermentation.

Conclusion

Complex oligosaccharides represent a significant component of human breast milk and are believed to contribute various functional roles in infant health and nutrition [5]. However, to study these important biomolecules, it is essential to develop accurate methods to both differentiate and quantify individual oligosaccharide species. In this work, we have developed two methods for quantifying milk oligosaccharides using MALDI–FTICR MS. The first method used external standards, and the second method used internal deuterated standards. Both methods were employed to characterize the level of oligosaccharide consumption by different *Bifidobacteria* spp. Although we were able to observe the general trend of HMO consumption by different bacteria using the external standard, this method yielded much greater deviations in the absolute intensities of each individual species. The internal standard method gave better precision and high reproducibility, and it was better for quantifying the amount of different oligosaccharide species after bacterial fermentation. Because both the standard and the analyte were ionized from the same sample, it is likely that both would be equally affected even when ionization suppression could occur. The higher variations in the *D*/*H* ratios were observed particularly when the relative abundance of the signals was less than 10%. Thus, it

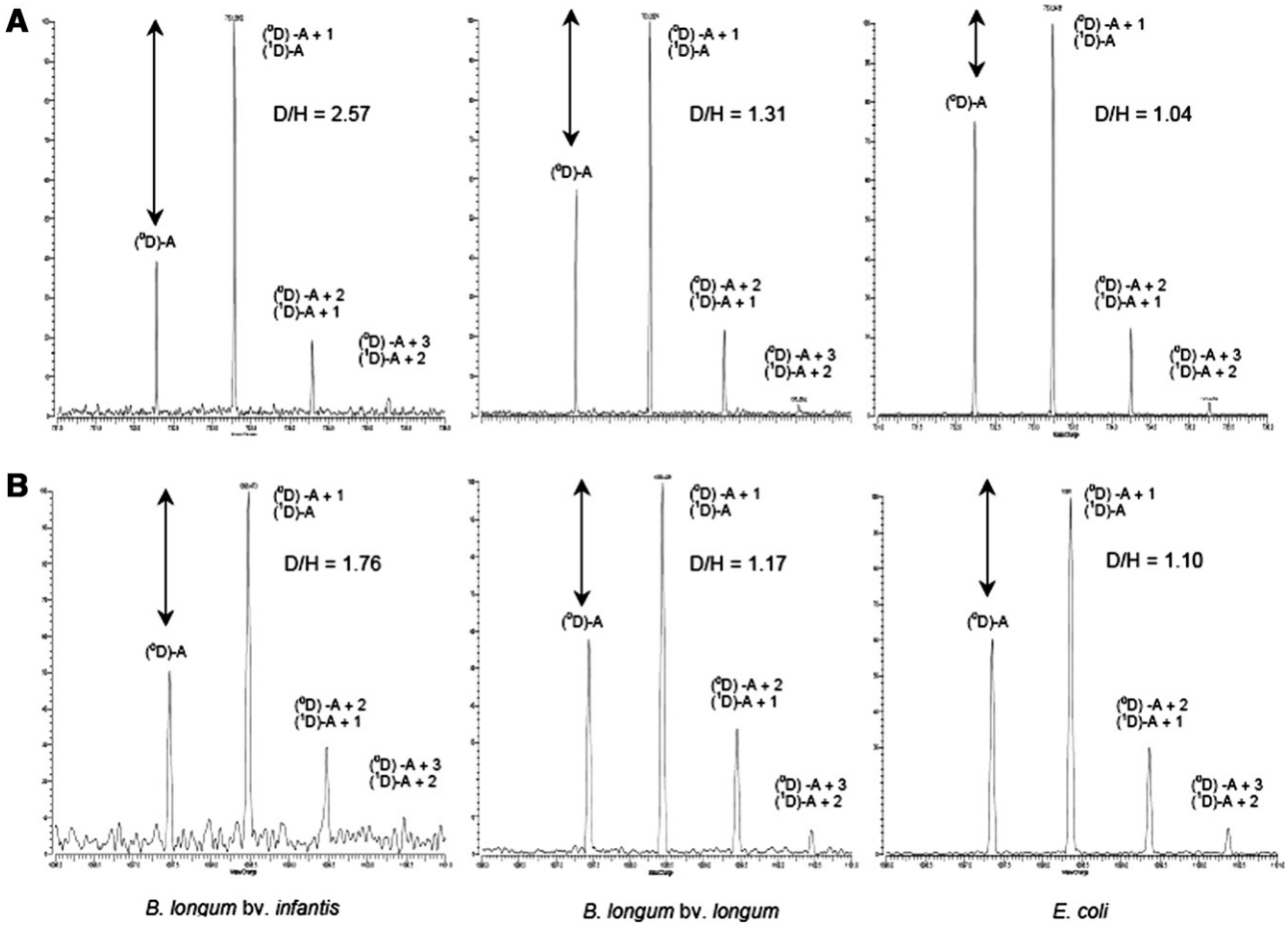


Fig. 5. Comparison of *D*/*H* ratios of oligosaccharide 1 (A) and oligosaccharide 2 (B) after incubation with *B. longum* bv. *infantis*, *B. longum* bv. *infantis*, and *E. coli* (MALDI–FT ICR (+) mass spectra).

may be most useful to apply this method on signals with higher relative abundances. The use of internal deuterated standards allowed the monitoring of complex mixtures of oligosaccharides simultaneously.

The quantitation of differential consumption of oligosaccharides by *Bifidobacteria* spp. observed using these methods proves to be a valuable tool for investigating whether HMOs can act as bifidogenic prebiotics and for understanding this species' role within the complex microbial ecology of the GIT. The oligosaccharide species preferentially consumed by *B. longum* bv. *infantis* demonstrated here gives one possible rationale for this species' predominance within the infant GIT. The strategy followed in the current work provides a quantitative functional assay that enables the assessment of different bacterial strains for their potential growth on HMOs and other substrates.

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References

[1] P.B. Eckburg, E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, D.A. Relman, Diversity of the human intestinal microbial flora, Science 308 (2005) 1635–1638.

[2] F. Crociani, A. Allesandrini, M.M.B. Mucci, B. Biavati, Degradation of complex carbohydrates by *Bifidobacterium* spp., Int. J. Food Microbiol. 24 (1994) 199–210.

[3] C. Shortt, J. O'Brien, Handbook of Functional Dairy Products, CRC Press, Boca Raton, FL, 2004.

[4] C. Kunz, S. Rudloff, W. Baier, N. Klein, S. Strobel, Oligosaccharides in human milk: structural, functional, and metabolic aspects, Annu. Rev. Nutr. 20 (2000) 699–722.

[5] G. Boehm, B. Stahl, Functional dairy products, in: T. Mattila-Sandholm (Ed.), Oligosaccharides, Woodhead, Cambridge, UK, 2003, pp. 203–243.

[6] K.J. Yarema, Handbook of Carbohydrate Engineering, Taylor & Francis, New York, 2005.

[7] B. Finke, B. Stahl, A. Pfenninger, M. Karas, H. Daniel, G. Sawatzki, Analysis of high-molecular-weight oligosaccharides from human milk

by liquid chromatography and MALDI–MS, Anal. Chem. 71 (1999) 3755–3762.
[8] S.C. Leahy, D.G. Higgins, G.F. Fitzgerald, D. van Sinderen, Getting better with *Bifidobacteria*, J. Appl. Microbiol. 98 (2005) 1303–1315.
[9] R.E. Ward, M. Ninonuevo, D.A. Mills, C.B. Lebrilla, J.B. German, In vitro fermentation of breast milk oligosaccharides by *Bifidobacterium infantis* and *Lactobacillus gasseri*, Appl. Environ. Microbiol. 72 (2006) 4497–4499.
[10] D.J. Harvey, Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates, Mass Spectrom. Rev. 18 (1999) 349–450.
[11] T. Yamagaki, H. Suzuki, K. Tachibana, Semiquantitative analysis of isomeric oligosaccharides by negative-ion mode UV–MALDI–TOF postsource decay mass spectrometry and their fragmentation mechanism study at *N*-acetyl hexosamine moiety, J. Mass Spectrom. 41 (2006) 454–462.
[12] B. Stahl, S. Thurl, J.R. Zeng, M. Karas, F. Hillenkamp, M. Steup, G. Sawatzki, Oligosaccharides from human milk as revealed by matrix-assisted laser desorption ionization mass spectrometry, Anal. Biochem. 223 (1994) 218–226.
[13] M.B. Engfer, B. Stahl, B. Finke, G. Sawatzki, H. Daniel, Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract, Am. J. Clin. Nutr. 71 (2000) 1589–1596.
[14] J. Charlwood, D. Tolson, M. Dwek, P. Camilleri, A detailed analysis of neutral and acidic carbohydrates in human milk, Anal. Biochem. 273 (1999) 261–277.
[15] K. Dreisewerd, S. Kolbl, J. Peter-Katalinic, S. Berkenkamp, G. Pohlentz, Analysis of native milk oligosaccharides directly from thin-layer chromatography plates by matrix-assisted laser desorption/ionization orthogonal-time-of-flight mass spectrometry with a glycerol matrix, J. Am. Soc. Mass Spectrom. 17 (2006) 139–150.
[16] T. Yamagaki, H. Suzuki, K. Tachibana, A comparative study of the fragmentation of neutral lactooligosaccharides in negative-ion mode by UV–MALDI–TOF and UV–MALDI ion-trap/TOF mass spectrometry, J. Am. Soc. Mass Spectrom. 17 (2006) 67–74.
[17] Y. Suzuki, M. Suzuki, E. Ito, H. Ishii, K. Miseki, A. Suzuki, Convenient and rapid analysis of linkage isomers of fucose-containing oligosaccharides by matrix-assisted laser desorption/ionization quadrupole ion trap time-of-flight mass spectrometry, Glycoconj. J. 22 (2005) 427–431.
[18] H. Bartsch, W.A. Konig, M. Strassner, U. Hintze, Quantitative determination of native and methylated cyclodextrins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Carbohydr. Res. 286 (1996) 41–53.
[19] Y. Xie, J. Liu, J. Zhang, J.L. Hedrick, C.B. Lebrilla, Method for the comparative glycomic analyses of O-linked, mucin-type oligosaccharides, Anal. Chem. 76 (2004) 5186–5197.
[20] D.S. Newburg, G.M. Ruiz-Palacios, A.L. Morrow, Human milk glycans protect infants against enteric pathogens, Annu. Rev. Nutr. 25 (2005) 37–58.
[21] N.G. Karlsson, N.L. Wilson, H.J. Wirth, P. Dawes, H. Joshi, N.H. Packer, Negative ion graphitised carbon nano-liquid chromatography/mass spectrometry increases sensitivity for glycoprotein oligosaccharide analysis, Rapid Commun. Mass Spectrom. 18 (2004) 2282–2292.
[22] M. Wuhrer, C.A. Koeleman, A.M. Deelder, C.H. Hokke, Normal-phase nanoscale liquid chromatography–mass spectrometry of underivatized oligosaccharides at low-femtomole sensitivity, Anal. Chem. 76 (2004) 833–838.
[23] S. Itoh, N. Kawasaki, M. Ohta, M. Hyuga, S. Hyuga, T. Hayakawa, Simultaneous microanalysis of N-linked oligosaccharides in a glycoprotein using microbore graphitized carbon column liquid chromatography–mass spectrometry, J. Chromatogr. A 968 (2002) 89–100.
[24] M. Ninonuevo, H.J. An, H.F. Yin, K. Killeen, R. Grimm, R. Ward, B. German, C. Lebrilla, Nanoliquid chromatography–mass spectrometry of oligosaccharides employing graphitized carbon chromatography on microchip with a high-accuracy mass analyzer, Electrophoresis 26 (2005) 3641–3649.
[25] E.F. Gordon, D.C. Muddiman, Impact of ion cloud densities on the measurement of relative ion abundances in Fourier transform ion cyclotron resonance mass spectrometry: experimental observations of coulombically induced cyclotron radius perturbations and ion cloud dephasing rates, J. Mass Spectrom. 36 (2001) 195–203.
[26] L.J. de Koning, N.M.M. Nibbering, S.L. van Orden, F.H. Laukien, Mass selection of ions in a Fourier transform ion cyclotron resonance trap using correlated harmonic excitation fields (CHEF), Int. J. Mass Spectrom. 165 (1997) 209–219.
[27] J.D. Hogan, D.A. Laude, Mass discrimination in laser desorption Fourier transform ion cyclotron resonance mass spectrometry cation attachment spectra of polymers, Anal. Chem. 64 (1992) 763–769.
[28] T.C. Farrar, J.W. Elling, M.D. Krahling, Application of linear prediction to Fourier transform ion cyclotron resonance signals for accurate relative ion abundance measurements, Anal. Chem. 64 (1992) 2770–2774.