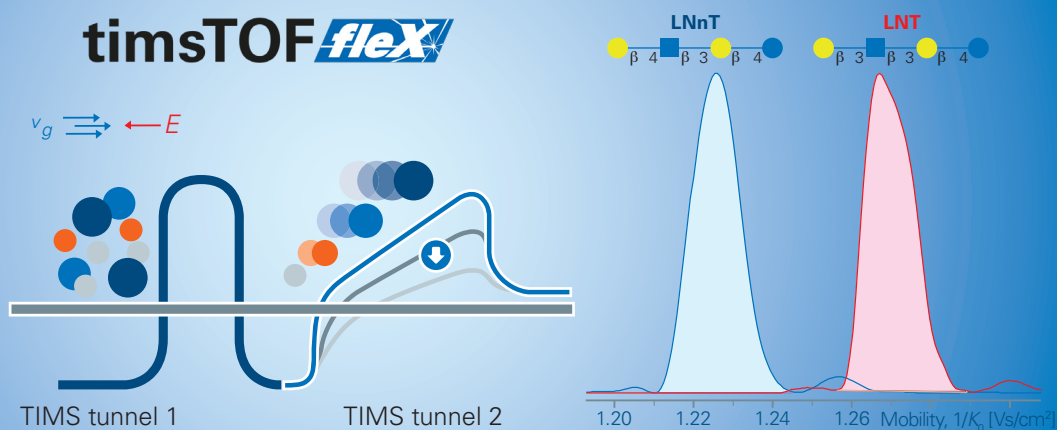




timsTOF *flex*



Rapid profiling of human milk oligosaccharides by TIMS-enabled MALDI mass spectrometry using timsTOF flex

TIMS-enabled MALDI profiling of isomeric human milk oligosaccharides, a class of compounds known for its critical importance to infant health and immune development, enables rapid screening of large cohorts of milk samples and their instant assignment to human milk types I-IV.

Abstract

Here we introduce a TIMS-enabled MALDI method using timsTOF flex providing a highly efficient direct mass spectrometric approach for rapid but comprehensive profiling of human milk oligosaccharides (HMO) including discrimination of structural isomers.

The method allows for isomer-specific detection of acidic and neutral HMOs, such as LSTs, LN(n)T, LNFPs and LNDFHs based on their collisional cross-sections and by complementary utilization of isomer-specific diagnostic fragment ions.

The novel timsTOF flex MALDI method opens up new possibilities for instant assignment of milk samples to human milk types I-IV, as determined by maternal Secretor/Lewis (Se/Le) genotypes, based on presence or absence of specific marker HMOs.

Compared to LC-MS, the TIMS-enabled MALDI method offers high-throughput capability facilitating screening of large sample cohorts at shortest time to result.

Keywords:

Human milk oligosaccharides, HMO, TIMS, timsTOF flex, MALDI, profiling, structural isomers, human milk types, infant health, immune development

Introduction

Human milk oligosaccharides (HMOs) represent the third most abundant solid constituent of human milk and are of critical importance to infant health and immune development [1]. Because of their structural diversity, comprehensive characterization of HMOs by mass spectrometry (MS) till today remains a non-trivial task. The presence of isobaric and isomeric HMO species represents a particular challenge requiring accurate-mass, high MS resolving power and hyphenation to orthogonal separation methods.

Trapped Ion Mobility Spectrometry (TIMS) is capable of separating isobaric and isomeric compounds in the gas phase based on the collisional cross-sections (CCS) of their molecular ions. Hyphenated to time-of-flight mass spectrometry using electrospray ionization (ESI-TOF), TIMS has been successfully applied to the separation of HMO structural isomers [2].

Matrix-assisted laser desorption ionization (MALDI) offers a unique level of analysis speed and sample throughput enabling analysis of large sample cohorts at shortest time to result. The Bruker timsTOF fleX instrument, by featuring an ESI/MALDI dual ion source equipped with ultrafast smartbeam 3D laser, combines the specific merits of MALDI in terms of speed and throughput with the key benefits of timsTOF technology, namely separation of isobars and isomers, providing a unique platform for rapid profiling analyses and, at the same time, delivering more structural insight.

Here we describe a novel TIMS-enabled MALDI-MS method and its application to rapid profiling of human milk oligosaccharides. The method is a promising new tool to rapidly screen HMOs and their structural isomers at high resolution offering new possibilities to instantly assign human milk types I-IV to maternal Secretor/Lewis genotypes.

Experimental

The following abbreviations are used when referring to various HMO compositions:

FL	Fucosyllactose
LNT	Lacto-N-tetraose
LNnT	Lacto-N-neotetraose
LNFP	Lacto-N-fucopentaose
LNDFH	Lacto-N-difucohexaose
LST	Sialyllacto-N-(neo)tetraose
DSLNT	Disialyllacto-N-tetraose

HMOs were isolated from pooled human breast milk by delipidation via centrifugation, protein precipitation and preparative size exclusion chromatography (SEC) to obtain two well-defined HMO fractions:

[Fraction A](#): Short-chain neutral HMOs

[Fraction B](#): Acidic and long-chain neutral HMOs

The sample preparation workflow has been described in detail in [3].

Prefractionated HMO samples and HMO reference standards were prepared on a Bruker MTP Ground Steel MALDI plate at an amount of approximately 50 ng per spot. For positive ion mode analyses, SDHB matrix was applied spiked with NaCl or KCl as cationizing additives. For negative ion mode analyses, SDHB (without salt additives) and 3-aminoquinoline (AQ) spiked with HNO₃ were used as MALDI matrices.

All analyses were performed on a Bruker timsTOF fleX equipped with dual ESI/MALDI ion source and 10 kHz smartbeam 3D laser. The instrument was operated in positive or negative ion polarity in MALDI-TIMS-MS and MALDI-(TIMS-)MS/MS mode, respectively.

MALDI-TIMS-MS data were acquired in two experiments: 1) TIMS overview (wide mobility range, 800 ms TIMS ramp); 2) TIMS highres (narrow mobility range optimized for individual HMO species, 1200 ms TIMS ramp).

For MS/MS experiments, nitrogen was used as a collision gas. Collision energies were optimized for individual HMO species.

m/z calibration was performed in MALDI ionization mode using red phosphorus cluster ions as calibrants.

CCS calibration in TIMS overview experiments was performed in ESI ionization mode using Agilent ESI Low-Concentration Tune Mix.

Results and discussion

Figures 1-2 present the MALDI-TIMS-MS overview profiles, i.e. TIMS heatmaps plotting $1/K_0$ vs. m/z , and the MALDI-MS spectra averaged across the entire $1/K_0$ range covered, resulting from TIMS overview analysis of the two HMO fractions A and B in positive and negative ion mode, respectively.

In positive mode (Figure 1), using SDHB/NaCl as a MALDI matrix, adduct ions with Na^+ as the charging cation were detected as the dominating ion species. In sample fraction A (Figure 1, top), short-chain neutral HMOs, such as lacto-N-(neo)tetraose (LN(n)T), lacto-N-fucopentaose (LNFP) and lacto-N-difucosylhexaose (LNDFH) appeared with highest abundance. In fraction B, short-chain acidic HMOs, such as sialyllacto-N-(neo)tetraose (LST) and disialyllacto-N-tetraose (DSLNT), were detected at high abundance along with a wide range of long-chain neutral and acidic HMOs with up to 18 monosaccharide units comprising up to eight [GalGlcNAc] repeat units modified with varying numbers of fucose and sialic acid residues.

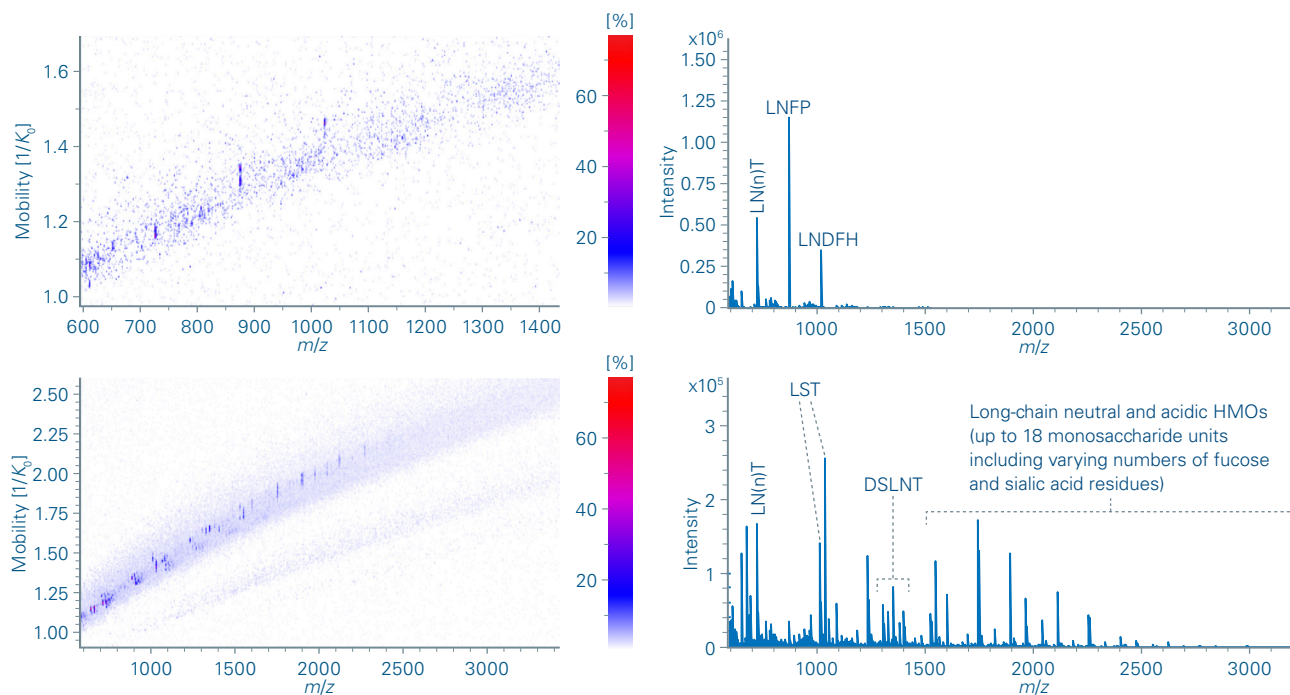


Figure 1. Positive-mode MALDI-TIMS-MS profiles of prefractionated HMO samples.

MALDI matrix: SDHB/NaCl. Top: Fraction A (short-chain neutral HMOs). Bottom: Fraction B (acidic and long-chain neutral HMOs). Left: MALDI-TIMS-MS heatmap $1/K_0$ vs. m/z . Right: MALDI-TIMS-MS average spectra. Peak annotations refer to Na^+ adduct ions (with or without neutral exchanges of acidic protons by sodium cations).

In negative ion mode, using 3-AQ/HNO₃ as a MALDI matrix (Figure 2), neutral HMOs appeared in the shape of adduct ions [M+NO₃]⁻, whereas acidic HMO species were detected as deprotonated molecular ions [M-H]⁻. Both neutral and acidic HMOs appeared partly converted into their respective 3-AQ-derivatives as a result of 3-AQ being a reactive MALDI matrix.

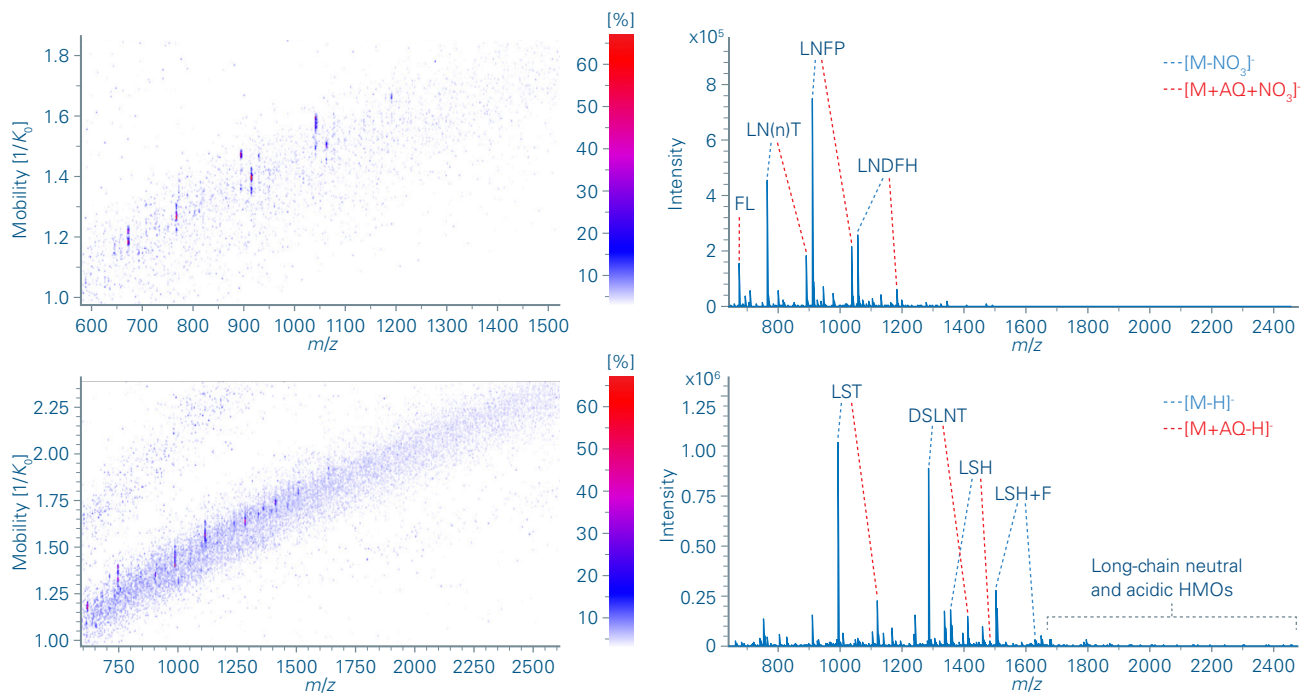


Figure 2. Negative-mode MALDI-TIMS-MS profiles of prefractionated HMO samples.

MALDI matrix: 3-AQ/HNO₃. Top: Fraction A (short-chain neutral HMOs). Bottom: Fraction B (acidic and long-chain neutral HMOs). Left: MALDI-TIMS-MS heatmap $1/K_0$ vs. m/z . Right: MALDI-TIMS-MS average spectra. Color code of annotated signals indicates ion types.

For selected HMO species, isomer separation by MALDI-TIMS-MS was investigated in detail in two experiments applying different TIMS parameters. The TIMS overview experiment used a ramping time of 800 ms and a wide $1/K_0$ window capturing the entire range of HMOs. In the TIMS highres experiment, a ramping time of 1200 ms was used along with a narrow $1/K_0$ window aiming for optimum resolution for a single targeted HMO ion species. Furthermore, various ionization conditions (positive ion mode: [M+Na]⁺, [M+K]⁺; negative ion mode: [M-H]⁻, [M+NO₃]⁻) were investigated regarding their efficiency for TIMS separation of isomeric HMOs. Selected results obtained from these analyses are presented in Figures 3 and 4.

Acidic LST a-c isomers separated best in positive ion mode TIMS, with highest efficiency achieved for ion type [M+K]⁺ (Figure 3 top left). All three LST isomers could be differentiated in HMO sample fraction A based on the shapes of their EIM profiles either directly (LST a and c) or via differential detection (LST b).

Neutral HMOs LN(n)T and LNDFH I/II showed best isomer separation in negative ion mode in the shape of their respective [M+NO₃]⁻ ions. This is shown in the EIM traces given in Figure 3 top right and bottom left.

For the more diverse group of isomeric LNFPs, TIMS achieved partial separation into isomer sub-groups. This is shown in Figure 4 for ion types [M+Na]⁺, [M+K]⁺, [M+NO₃]⁻ and [M+AQ+NO₃]⁻. Best separation efficiency was observed in negative mode for [M+NO₃]⁻ ions allowing for detection of LNFP [I + II] separated from LNFP [III + V].

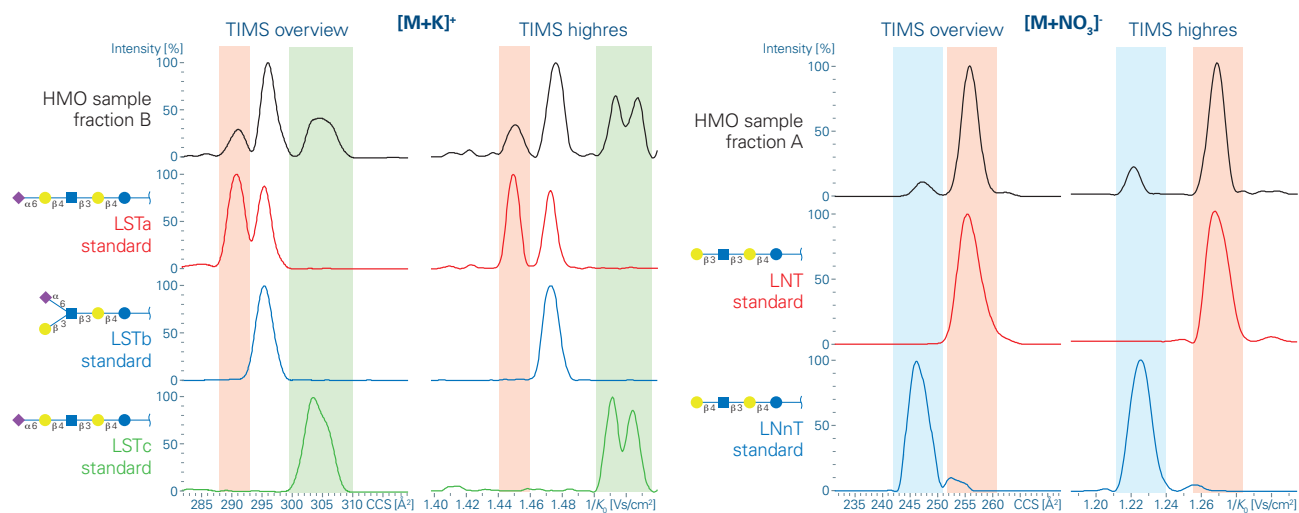


Figure 3. TIMS separation of selected isomeric HMO species under TIMS overview and TIMS highres experiment conditions, respectively. Top left: LST, positive ion mode, $[M+K]^+$; Top right: LN(n)T, negative ion mode, $[M+NO_3]^-$; Bottom left: LNFDH, negative ion mode, $[M+NO_3]^-$; Extracted ion mobigrams (EIM) obtained from isomer reference standards (colored traces) are given in direct comparison with the EIM obtained from HMO fraction A and B (black trace, top panel), respectively.

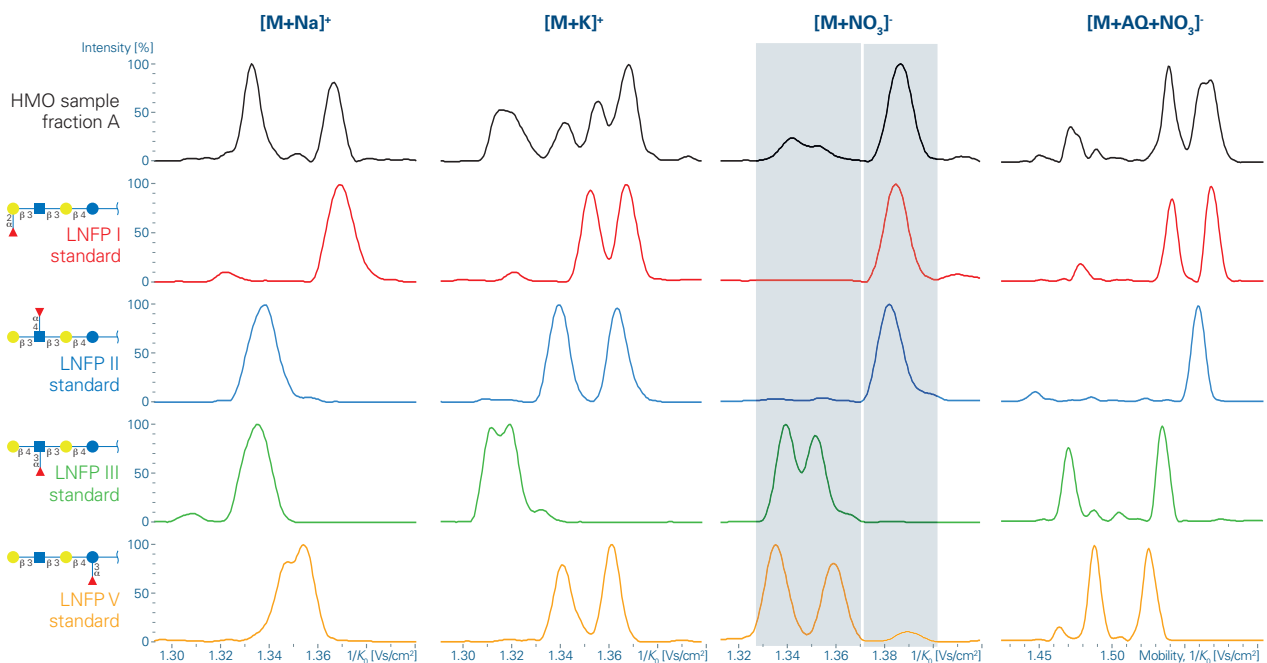
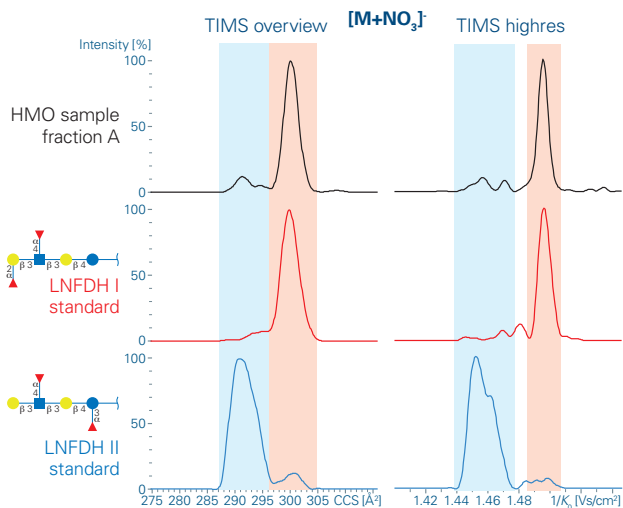


Figure 4. TIMS separation of LNFP I-III, V isomers achieved in TIMS highres experiments for various types of molecular ions. EIMs obtained from LNFP isomer reference standards (colored traces) are given in direct comparison to the EIMs obtained from HMO fraction A (short-chain neutral HMOs, black trace).

Discrimination between individual LNFP isomers was achieved, however, by combining MALDI-TIMS separation with fragmentation analysis by CID-MS/MS. MALDI-CID-MS/MS spectra acquired in negative ion mode from $[M+NO_3]^-$ precursor ions of LNFP isomers I, II, III and V reference standards are given in Figure 5. The colored bars indicate fragment ions that are particularly discriminative of LNFP I versus II and LNFP III versus V, respectively, thus allowing for specific detection of individual LNFP isomers in mixtures such as HMO sample fraction A (short-chain neutral HMOs) by MALDI-TIMS-MS/MS (Figure 6).

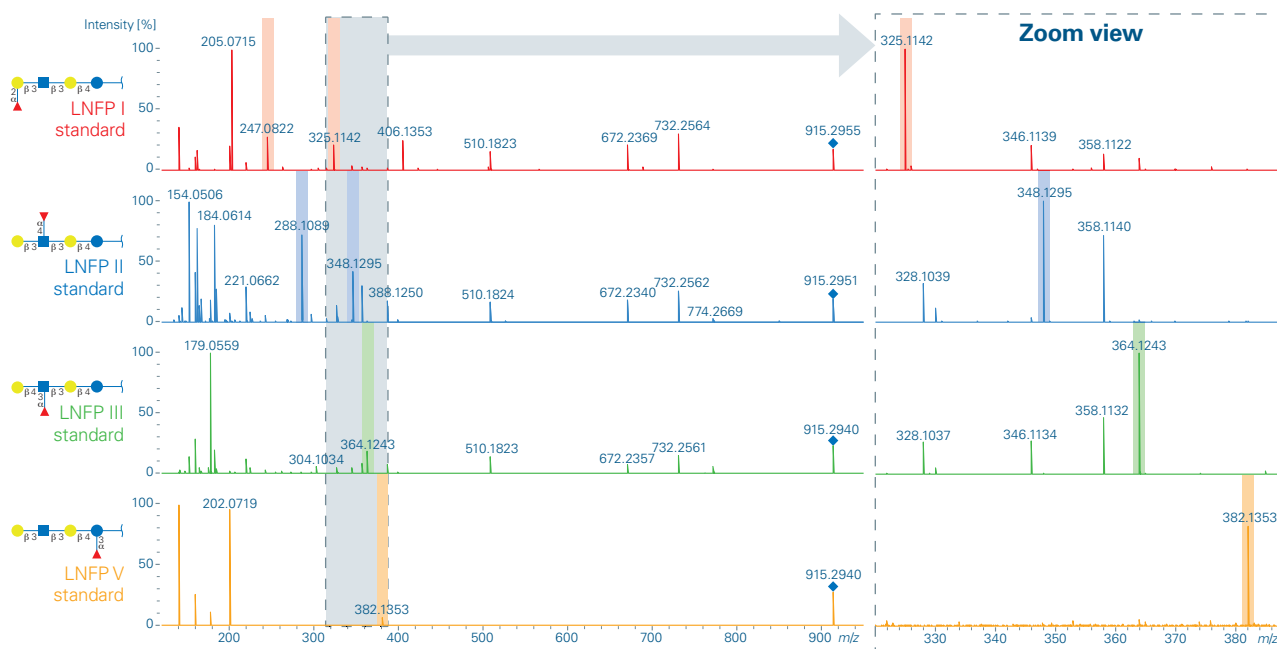


Figure 5. Negative ion mode MALDI-CID-MS/MS spectra of precursor m/z 915.3 representing LNFP $[M+NO_3]^-$. Color bands on the zoomed views indicate diagnostic fragment signals that can serve for discrimination of LNFP I vs. II and LNFP III vs. V, respectively.

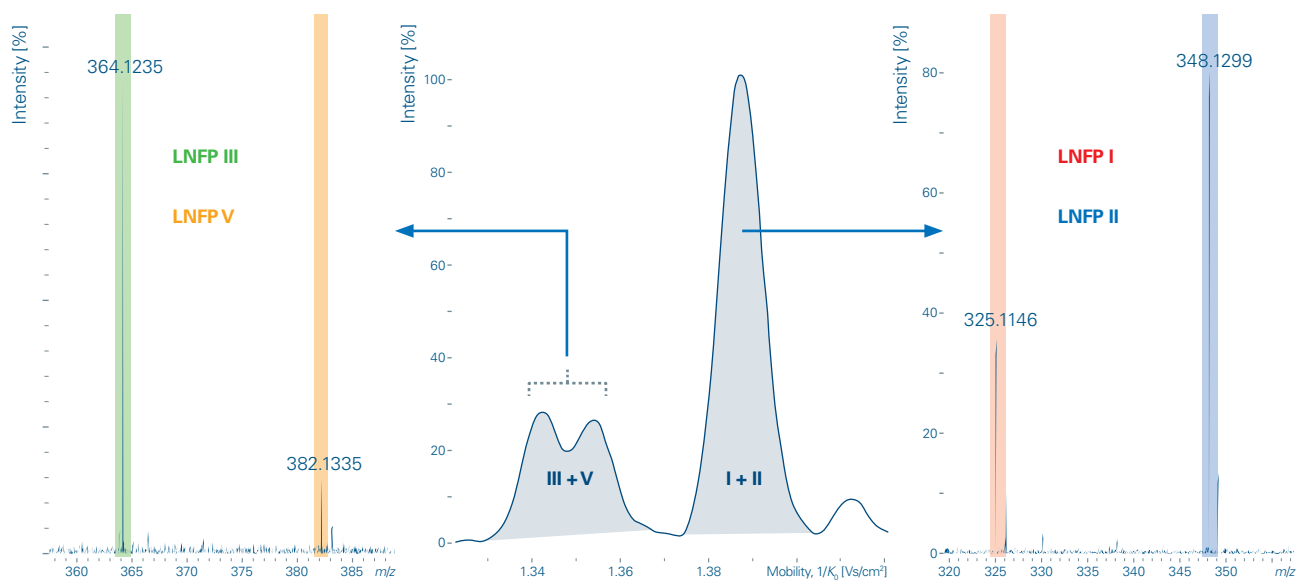


Figure 6. Discrimination of LNFP isomers in HMO sample fraction A (short-chain neutral HMOs) by negative ion mode MALDI-TIMS-MS/MS. Center: Base-peak mobiligram (BPM) obtained from MALDI-TIMS-MS/MS of LNFP precursor ion $[M+NO_3]^-$ m/z 915.3. Left: Zoomed view on MS/MS spectrum extracted from BPM band representing LNFP III+V. Right: Zoomed view on MS/MS spectrum extracted from BPM band representing LNFP I+II. Color coded annotations indicate diagnostic fragments related to individual LNFP isomers.

MALDI-TIMS-MS analysis of long-chain HMO species yielded complex ion mobilograms reflecting their structural and/or ion-conformational diversity. Figure 7 shows this for neutral HMO compositions $\text{GlcGal}(\text{GalGlcNAc})_3\text{Fuc}_{1-4}$, represented as their $[\text{M}+\text{Na}]^+$ molecular ions. An increasing number of fucose residues present in these HMO compositions correlates with increasing CCS. The number of fucose residues was unambiguously verified by MALDI-CID-MS/MS (Figure 7, bottom) based on fragment signals appearing in distances of 146 m/z as a result of consecutive losses of fucose residues.

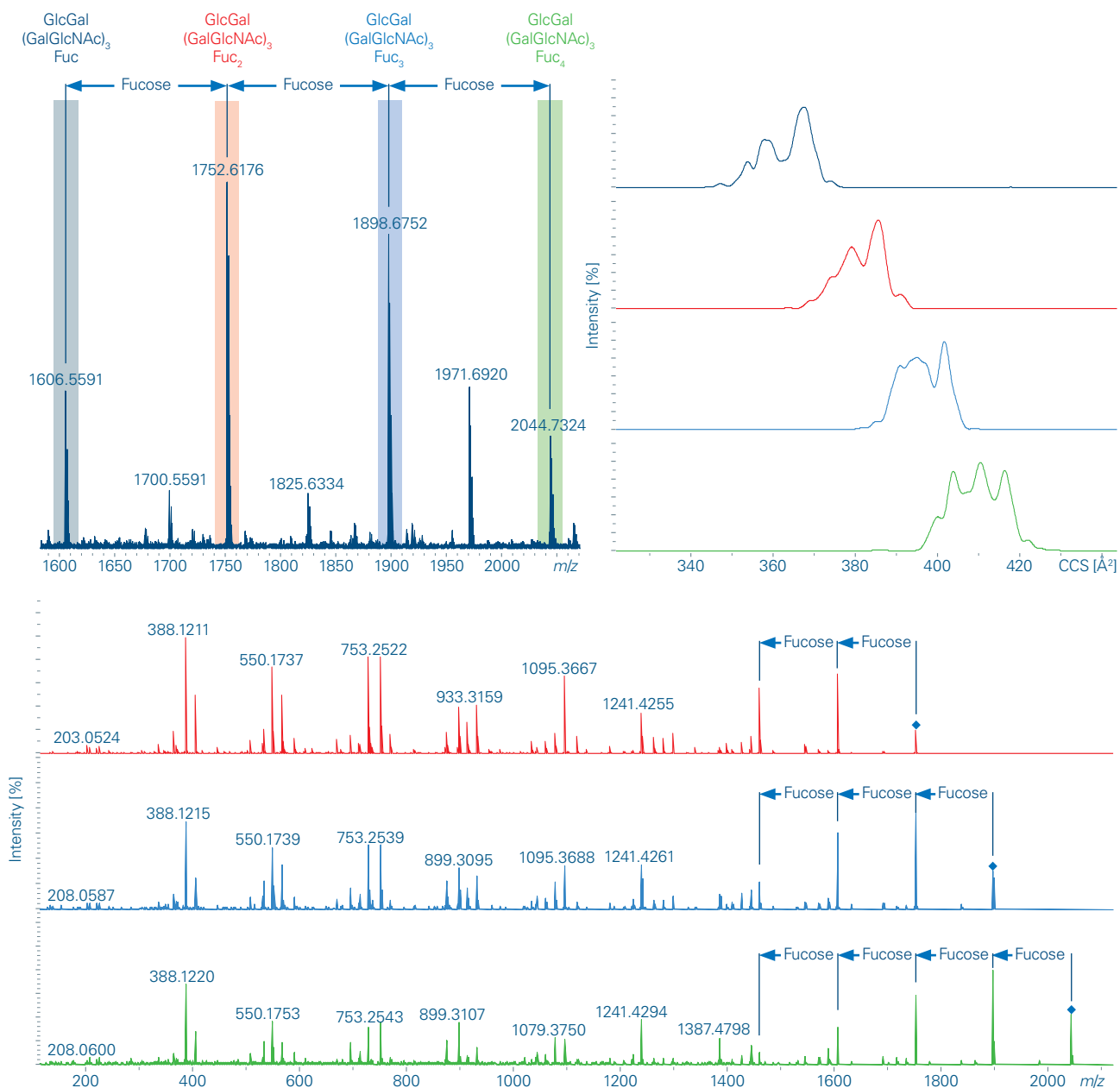


Figure 7. Positive ion mode MALDI-TIMS-MS and -MS/MS analysis of neutral long-chain HMOs in sample fraction B.

MALDI matrix: SDHB/NaCl. Top left: Total-TIMS average MS spectrum extracted from MALDI-TIMS-MS overview analysis. Color bands indicate $[\text{M}+\text{Na}]^+$ molecular ions representing HMO compositions $\text{GlcGal}(\text{GalGlcNAc})_3\text{Fuc}_{1-4}$. Top right: EIMS of $[\text{M}+\text{Na}]^+$ molecular ions of HMO compositions $\text{GlcGal}(\text{GalGlcNAc})_3\text{Fuc}_{1-4}$. Bottom: MALDI-CID-MS/MS spectra of $[\text{M}+\text{Na}]^+$ precursors of HMO compositions $\text{GlcGal}(\text{GalGlcNAc})_3\text{Fuc}_{2-4}$.

Conclusions

- TIMS-enabled MALDI-MS performed on timsTOF fleX provides a highly efficient direct MS approach for rapid but comprehensive HMO profiling including identification of structural isomers.
- The TIMS-enabled MALDI method allows for isomer-specific detection of neutral and acidic HMOs, such as LSTs, LN(n)T, LNFPs and LNDFHs, based on their collisional cross-sections and isomer-specific diagnostic fragment ions observed in negative ion mode.
- The timsTOF fleX method described here offers new possibilities to rapidly assign milk samples to human milk types I-IV, as determined by maternal Secretor/Lewis (Se/Le) genotypes, based on presence or absence of specific marker HMOs (e.g., 2'-FL, LNFP I, LNFP II, LNDFH I).
- Compared to LC-MS methods, TIMS-enabled MALDI profiling offers true high-throughput capability facilitating screening of large sample cohorts with shortest turnaround time.

References

- [1] Slater AS, Hickey RM, Davey GP (2024). *Front. Immunol.* **15**:1523829. <https://doi.org/10.3389/fimmu.2024.1523829>
- [2] Rathahao-Paris E, Delvaux A, Li M, Guillon B, Venot E, Fenaille F, Adel-Patient K, Alves S (2022). *J. Mass Spectrom.* **57**:e4885. <https://doi.org/10.1002/jms.4885>
- [3] Siziba LP, Mank M, Stahl B, Gonsalves J, Blijenberg B, Rothenbacher D, Genuneit J (2021). *Nutrients.* **13**:1973. <https://doi.org/10.3390/nu13061973>

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