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# Rapid structural characterization of human milk oligosaccharides and distinction of their isomers using trapped ion mobility spectrometry time-of-flight mass spectrometry

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## Abstract

Oligosaccharides have multiple functions essential for health. Derived from the condensation of two to several monosaccharides, they are structurally diverse with many co-occurring structural isomer families, which make their characterization difficult. Thanks to its ability to separate small molecules based on their mass, size, shape, and charge, ion mobility-mass spectrometry (IM-MS) has emerged as a powerful tool for separating glycan isomers. Here, the potential of such a technique for the rapid characterization of main human milk oligosaccharides (HMOs) was investigated. Our study focused on 18 HMO standards. The IM-MS analysis enabled to distinguish almost all the HMOs studied, in particular thanks to the single ion mobility monitoring acquisition using the trapped ion mobility spectrometry device, providing high ion mobility resolution and enhanced ion mobility separation. Alternatively, the combination of IM-MS separation with MS/MS experiments has proven to increase performance in identifying HMOs and especially isomers poorly separated by ion mobility alone. Finally, collision cross-section values are provided for each species generated from the 18 HMOs standards, which can serve as an additional identifier to characterize HMOs.

**KEYWORDS**
human milk oligosaccharide, ion mobility, isomer characterization, mass spectrometry, MS/MS experiments

## 1 | INTRODUCTION

Oligosaccharides are involved in many biological processes, including cell recognition and immune response. Carbohydrate chains result from the condensation of two to up to 20 monosaccharides by glycosidic bonds. There are glycoproteins, glycosphingolipids, and free oligosaccharides such as human milk oligosaccharides (HMOs) that are very abundant in breast milk and known to play a crucial role as prebiotics, allowing the development of the infant's microbiota.[1]

HMOs are structurally complex glycans, each HMO being composed of a lactose unit (galactosyl-β1–4-glucose), condensed with one or more monosaccharides among the five basic monosaccharides including D-glucose (Glc), D-galactose (Gal), L-fucose (Fuc), N-acetylglucosamine (GlcNAc), and N-acetylneuraminic acid or sialic

acid (SA) (see Figure 1). Lactose can be fucosylated or sialylated to form, for example, 2′-fucosyllactose (2′FL) and 3-fucosyllactose (3FL) or 3′-sialyllactose (3′SL) and 6′-sialyllactose (6′SL), respectively. It can also be elongated by a disaccharide unit leading for example to lacto-N-tetraose (LNT) and/or lacto-N-neotetraose (LNnT). Additional monosaccharide and/or disaccharide units can be added, generating linear (e.g., Lacto-N-fucopentaose I [LNFP I] and sialyllacto-N-neotetraose a [LST a]) or branched HMOs (e.g., lacto-N-hexaose [LNH] and difucosyllacto-N-hexaose [DFLNH]). Therefore, HMOs consist of monosaccharides linked by various types of

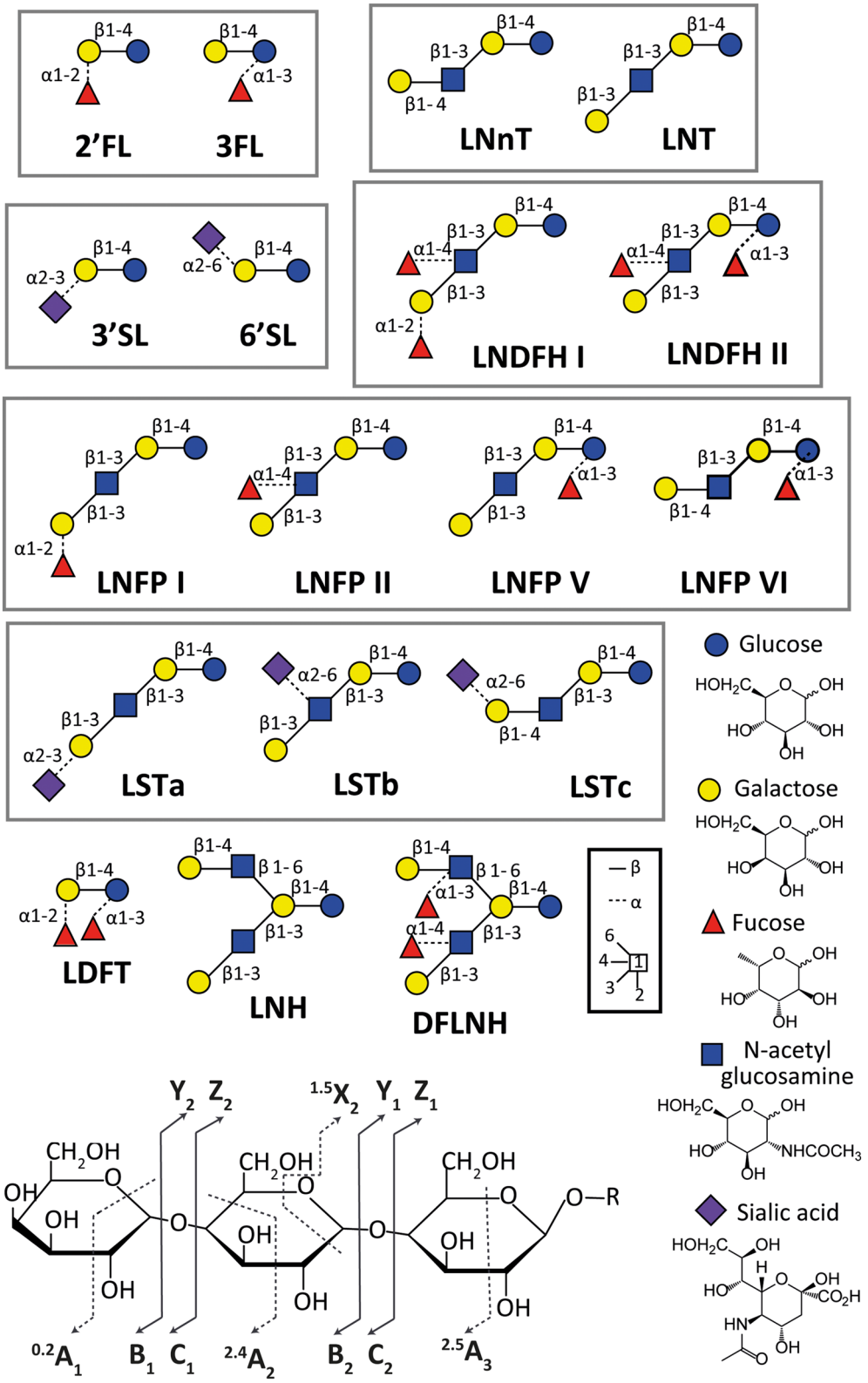


**FIGURE 1** Structures of the studied HMOs (according to the guidelines of the symbol nomenclature for the depiction of glycans[2]) and nomenclature used for oligosaccharide fragmentation in tandem mass spectrometry following that proposed by Domon and Costello.[3] HMOs, human milk oligosaccharides

bonds (i.e., linear or branched structures and α or β linkages via 1–2,3,4 or 2–3,6 linkages), resulting in diverse structures and numerous families of structural isomers; for example, over 200 different HMO structures have been reported in pooled human milk samples.[6]

Various analytical techniques have been developed for carbohydrate analysis.[7,8] Most of them are often based on mass spectrometry (MS) allowing sensitive and specific detection.[9] In addition, tandem mass spectrometry (MS/MS) provides access to structural details essential to unambiguously characterize a given structure. The coupling of MS with a hyphenated method such as liquid chromatography (LC, using hydrophilic-interaction chromatography or porous graphitized carbon columns) or capillary electrophoresis has been widely used to analyze oligosaccharides in complex mixtures.[9] However, such hyphenated techniques are not suitable for high throughput assays, because of the time need to separate complex oligosaccharide mixtures before MS detection. In addition, reduction and permethylation of oligosaccharides are often performed before the LC/MS analysis, requiring additional sample preparation steps that lengthen the overall analysis time.[10]

Alternatively, ion mobility (IM) technology based on the ion separation in the gas phase has proven to be a promising tool for the rapid characterization of isomeric compounds.[11] Its coupling with mass spectrometry (IM-MS) offers an additional dimension of separation without lengthening the acquisition time unlike traditional gas chromatography-mass spectrometry (GC/MS) or LC/MS analysis because the IM separation is done on a millisecond scale. In addition to its ability to separate ions based on their size, shape, mass, and charge, IM-MS provides a relevant intrinsic parameter, that is, the collision cross-section (CCS), which can be used as an additional descriptor to aid in assigning the identity of a given compound. Few studies have focused on the structural characterization of HMOs by IM-MS.[12–14] In a previous work, we have evaluated the potential of direct introduction-trapped ion mobility spectrometry time-of-flight mass spectrometry (TIMS-TOF) for the rapid characterization of HMO isomers and demonstrated its ability to distinguish four pairs of HMO isomers that are 2′FL/3FL, 3′SL/6′SL, LNT/LNnT, and LNFP I/LNFP V.[15] A major advantage of the TIMS-TOF instrument is the possibility of modifying the scan mode allowing to increase the resolving power of the TIMS analyzer and therefore to improve the IM separation.

The objective of this work is to extend the previous IM-MS based method to a rapid identification of a large number of HMOs. Eighteen HMO standards (Figure 1) were analyzed using a full ion mobility scan and single ion mobility monitoring ($SIM^2$) method to assess IM separation, providing a CCS library of HMOs for various positively and negatively charged adduct ions. The $SIM^2$ method implies the detection of a narrow ion mobility range centered on the studied species, providing a high mobility resolving power (up to 200) that facilitates the separation and/or the distinction of very closed mobility peaks. Complementary MS/MS experiments were conducted to obtain essential data for the subsequent high throughput structural characterization of isomers.

## 2 | MATERIAL AND METHODS

### 2.1 | Chemicals and milk samples

Ultrapure water with a resistance of 18.2 MΩ.cm was produced by a Select HP water purification system (Purite France eau, Lormont, France). Methanol high-performance liquid chromatography grade) was acquired from VWR Chemicals (Fontenay-sous-Bois, France). The calibration solution called ESI-L Low Concentration Tuning Mix (G1969–85000) was purchased from Agilent Technologies (Santa Clara, CA, USA) for mass and ion mobility calibration of the TIMS-TOF instrument. HMO standards (Figure 1) were purchased from Carbosynth (Slovakia, 2′FL, 3FL, 3′SL, 6′SL, LNFP I and II, LST b and c, LNH, LDFT, LNDFH I, and DFLNH) and Elicityl (Crolles, France, LNT, LNnT, LST a, LNFP V and VI, and LNDFH II). HMO standards have a purity ≥ 90%, except for LDFT and LNFP V whose purity was about 80%. Among the 18 HMO standards, there are four isomer pairs, that is 2′FL/3FL, 3′SL/6′SL, LNDFH I/II, LNT/LNnT; two isomer families, that is, LST a/b/c and LNFP I/II/ V/VI; and three individual HMOs, that is, LNH, LDFT, and DFLNH.

All HMO standards were dissolved in water–methanol (1:1, *v*/*v*) at final concentrations ranging from 1 to 5 ng/μl depending on the experiment performed. For positive ionization experiments, formic acid was added at low level (0.1% in volume) to the HMO standard solutions to promote the formation of protonated ions.

### 2.2 | Ion mobility spectrometry–mass spectrometry analysis

All IM-MS experiments were performed on a trapped ion mobility spectrometer - quadrupole - time of flight (Bruker Daltonics, Bremen, Germany)[16] equipped with an electrospray ionization (ESI) source using direct infusion at a flow rate of 3 mL $min^{-1}$. The instrumental parameters were optimized for the detection of HMOs using the oTof control software (Bruker Daltonics). The end plate offset was set at 500 V and the electrospray voltage at −4500 V and +3500 V in positive and negative ionization, respectively. The capillary temperature was maintained at 250°C. Nitrogen was used as the spray and drift gas. The dry gas and the nebulizer gas were fixed at 3.0 L $min^{-1}$ and 0.3 bar, respectively. In the TIMS analyzer, the Funnel 1 RF, Funnel 2 RF, and deflection delta were set at 260 Vpp, 250 Vpp, and ±80 V, respectively. The ion charge control was kept at 1.5 Mio to avoid TIMS saturation.

Mass spectra were recorded in a range of *m*/*z* 100–1650 with a transfer time of 70 μs and a pre-pulse storage of 5 μs. Two ion mobility detection modes were used: (i) a full ion mobility scan where ion mobility spectra are collected within a large ion mobility range, that is, reduced mobilities ($1/K_0$) from 0.55 to 1.90 V.s.$cm^{-2}$ with a scan rate of 9.52 Hz, and (ii) a $SIM^2$ method using a narrow ion mobility range detection, typically a $1/K_0$ window of about 0.10 V.s.$cm^{-2}$ with a scan rate between 2 and 5 Hz.

CCS values were obtained from the reduced mobilities measured, $K_0$, requiring a calibration step. Therefore, external calibrations in *m*/*z*

(in quadratic mode) and reduced mobility values (in linear mode) were carried out using the ESI-L low concentration tuning mix solution.[15] Note that the ΔCCS value, corresponding to the difference in CCS between two ion mobility peaks A and B, is systematically calculated (in %) in order to evaluate the separation capacity of the TIMS device. We also used ΔCCS (in percent) to compare the measurements with the values reported in the literature.

MS/MS experiments were performed after ion mobility separation within a full ion mobility scan using CID (collision-induced dissociation) conditions. The following MS/MS conditions were used: 2 u for the isolation width of precursor ions, an activation energy between 18 and 80 eV (the exact value used is indicated on the MS/MS spectra, see Figure S1 in the supporting information) depending on the selected precursor ions in order to obtain high abundant fragment ions while maintaining sufficient precursor intensity into MS/MS spectra. Nitrogen was used as the collision gas.

## 3 | RESULT AND DISCUSSION

### 3.1 | Full ion mobility scan for analysis of HMOs and distinction of their isomers

IM-MS analysis of the 18 HMO standards was first conducted by applying a full ion mobility scan in order to detect a maximum of ionic species in a wide ion mobility range where $1/K_0$ ranged from 0.55 to 1.90 V.s.cm$^{-2}$ (Figure S2 in the supporting information). All IM-MS analyses were carried out in triplicate, and the averaged $^{TIMS}CCS_{N2}$ values for both positively and negatively charged species generated from every HMOs are reported in Table S1 in the supporting information.

In the negative ionization mode, all HMOs studied were detected as deprotonated species $[M-H]^-$. $[M+Cl]^-$ and $[M+HCOO]^-$ adducts were also observed, except for 3′SL, 6′SL and LST a, b, and c (Table S1 in the supporting information). In positive ionization mode, many species like $[M+H]^+$, $[M+Ca]^{2+}$, $[M+Na]^+$, $[M+K]^+$, and/or $[M-H+Ca]^+$ were produced. However, the $[M+H]^+$ ions were not observed for 2′FL and 3FL, and they were weakly detected for 3′SL. It should be noted that the isobaric $[M+K]^+$ and $[M-H+Ca]^+$ ions are detected together under the same ion mobility spectrum extracted at a given *m/z* value. Indeed, their distinction is not achieved using a TOF analyzer with a resolving power of 50,000 even if their annotation can be done from accurate mass measurements and/or salt addition for unambiguous identification (Figure S3 in the supporting information). Additionally, multiple mobility peaks could be observed for a given HMO ion (Figure 2), which is likely because of (i) isobaric contaminants from solvent or consumables, and/or more probably to (ii) the presence of different oligosaccharide conformations and/or ion structures (e.g., protomers).[17,18] Note that multiple mobility peaks are often observed for both protonated and deprotonated compounds, especially under high-resolution mobility, certainly because of the existence of protomers or deprotomers.[17]

Examination of CCS values recorded for all the species detected in three replicate analyses indicates good repeatability of the CCS

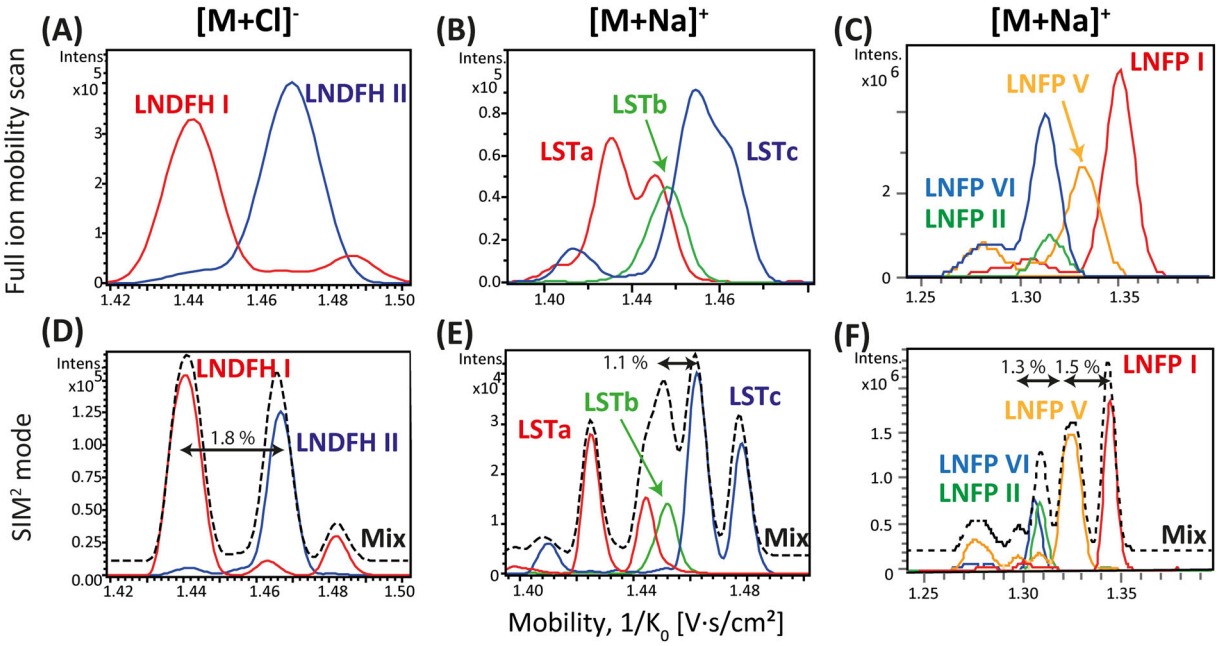


**FIGURE 2** Ion mobility spectra extracted for the $[M+Cl]^-$ (A and D) and $[M+Na]^+$ (B, C, E, and F) ions from IM-MS analysis of isolated (full lines) or equimolar mixtures (mix, dotted lines) of LNDFH I, LNDFH II (A and D); LSTa, LSTb, and LSTc (B and E); and LNFP I, LNFP II, LNFP V, and LNFP VI (C and F) using full ion mobility scan ($1/K_0$ from 0.55 to 1.90 V.s.cm$^{-2}$, for Figure 2A–C) and single ion mobility monitoring (SIM$^2$) method ($1/K_0$ window of about 0.15 V.s.cm$^{-2}$, in Figure 2D–F) for the same HMO species. Note that ΔCCS (in percent) are reported for some HMO isomers. IM-MS, ion mobility-mass spectrometry; CCS, collision cross-section; HMO, human milk oligosaccharide

**TABLE 1** Optimal isomer separation (or distinction) conditions using TIMS instrument

| Acquisition mode | Isomer separation | Studied HMOs | Considered ions |
|---|---|---|---|
| Full ion mobility scan | Full separation with ΔCCS% > 3.5% | LNT/LNnT | $[M-H]^-$, $[M+H]^+$ |
| $SIM^2$ mode | Full separation with 1.5% < ΔCCS% < 3.5% | 2′FL/3FL | $[M+Cl]^-$, $[M+Na]^+$, $[M+Ca]^{2+}$, $[M+K]^+$ |
| | | LNDFH I/LNDFH II | $[M+Cl]^-$, $[M+Na]^+$, $[M+K]^+$ |
| | Isomer distinction 1% < ΔCCS% < 1.5% | 3′SL/6′SL | $[M-H]^-$, $[M+Na]^+$ |
| | | LST a/LST b/LST c | $[M+Na]^+$, $[M+K]^+$ |
| | No separation ΔCCS% < 1% | LNFP I/LNFP II/LNFP V/LNFP VI | All species |

*Note*: $SIM^2$, single ion mobility monitoring; HMOs, human milk oligosaccharides; CCS, collision cross-section; TIMS, trapped ion mobility spectrometry.

measurements with RSD < 0.5%. This high precision is consistent with our previous work.[15,19] Some of experimental CCS values obtained in the present study were also compared with those found in the studies from Zheng et al.[20] and May et al.[21] that assessed cross-platform measurement accuracy (Table S2 in the supporting information). Small deviations, that is, ΔCCS% < 2%, were observed, which are within the accepted tolerance for cross-platform measurements.[22] Thus, the TIMS-TOF instrument provides reliable $^{TIMS}CCS_{N2}$ values that can be used for further characterization of the 18 HMOs.

The ability of TIMS-TOF to separate isomers can be evaluated based on the ΔCCS% between the isomers considered. As previously reported,[15,19] a baseline separation between two mobility peaks is achieved in full ion mobility scan of TIMS device (i.e., using a wide ion mobility detection range from 0.55 to 1.90 $V.s.cm^{-2}$) for ΔCCS% > 3.5%, whereas a $SIM^2$ mode (i.e., using a narrow ion mobility range) is necessary to baseline-separate mobility peaks with 1.5% < ΔCCS% < 3.5%. Only partially separated peaks could be obtained with $SIM^2$ for 1% < ΔCCS% < 1.5%. Based on this criteria, only LNT/LNnT isomer pair can be easily separated in full ion mobility scan at moderate resolving power ($50 < R_p < 80$) when considering the $[M\text{-}H]^-$ or $[M+H]^+$ species (Table 1). Although the other isomer pairs could not be well separated in full ion mobility scan, the distinction of between 2′FL and 3FL or between LNDFH I and LNDFH II could be achieved for the $[M+Cl]^-$ and $[M+K]^+$/$[M-H+Ca]^+$ species (Figure 2A). Other adduct ions could also be used to distinguish isomers like $[M+Na]^+$ ions for 3′SL/6′SL, 2′FL/3FL, and LNDFH I/II pairs.

Although baseline separation could not be achieved for all HMO isomers studied using full ion mobility scan, their distinction is possible for most of them, except for the four LNFP isomers that could not be individually characterized whatever the species considered. To improve the ion mobility separation and the distinction of HMO isomers, the $SIM^2$ method was then applied (Table 1).

## 3.2 | $SIM^2$ for enhanced isomer separation

A prior IM-MS analysis in full ion mobility scan is necessary to determine the $1/K_0$ value of relevant species. Then, $SIM^2$ was performed to monitor a specific species by narrowing the detection range of ion mobility centered on the targeted HMO ion. Indeed, considering the TIMS principle, the use of a narrower voltage range ($V_{ramp}$) and a larger ramp time ($T_{ramp}$) during the elution step allows enhancing the ion mobility separation with resolving power up to 200.[23] Using $SIM^2$ method with TIMS-TOF instrument, the baseline separation of isomers with differences in CCS values smaller than 2% has been demonstrated.[19]

$SIM^2$ method was applied for the analysis of all HMO standards in positive and negative ion modes in order to improve isomer separation. Thus, all species generated from the 18 HMO standards could be detected at high mobility resolving power ($R_p > 100$). For example, we show comparison of the extracted ion mobility spectra for the $[M+Cl]^-$ or $[M+Na]^+$ adducts from IM-MS analysis in full ion mobility scan versus $SIM^2$ mode for LNDFH I and LNDFH II pair (Figure 2A and D); LSTa, LSTb, and LSTc (Figure 2B and E); and LNFP I, LNFP II, LNFP V, and LNFP VI (Figure 2C and F). In full ion mobility scan, LNDFH I and LNDFH II could be separated as $[M+Cl]^-$ ions but with peak overlapping at 20% valley (Figure 2A), whereas the three isomers of LST could not be well separated as $[M+Na]^+$ ions. (Figure 2B). Obviously, the use of $SIM^2$ method allows obtaining high mobility resolution detection and reducing the ion mobility peak width (Figure 2D, E, and F) compared to that from a full ion mobility scan (Figure 2A, B, and C). Therefore, the separation between LNDFH I and LNDFH II is enhanced (Figure 2D). In addition, the broad mobility peak of LSTa as well as that of LSTc are split into two peaks, and these two isomers could be well separated (Figure 2E). Although the LSTb peak still overlaps the signals of LSTa and LSTc, its presence could be detected with the $SIM^2$ method. Nevertheless, the distinction of LSTb could be achieved by considering another species such as $[M+K]^+$ (Figure S2 in the supporting information). By increasing the ion mobility resolution, the $SIM^2$ method improved the HMO separation, making easier the distinction of isomers with ΔCCS% > 1%.

However, the LNFP isomer family could not be well characterized whatever the species considered. Indeed, even using $SIM^2$ method offering high ion mobility resolution, individual isomer separation could not be obtained. For the best ion mobility separation conditions, the $[M+Na]^+$ species of LNFP isomers only allowed the separation of LNFP I and LNFP V isomers from the others, but the LNFP II and LNFP VI signals are still overlapping (Figure 2E). In contrast, for all negatively charged species, the four isomers of LNFP could only be

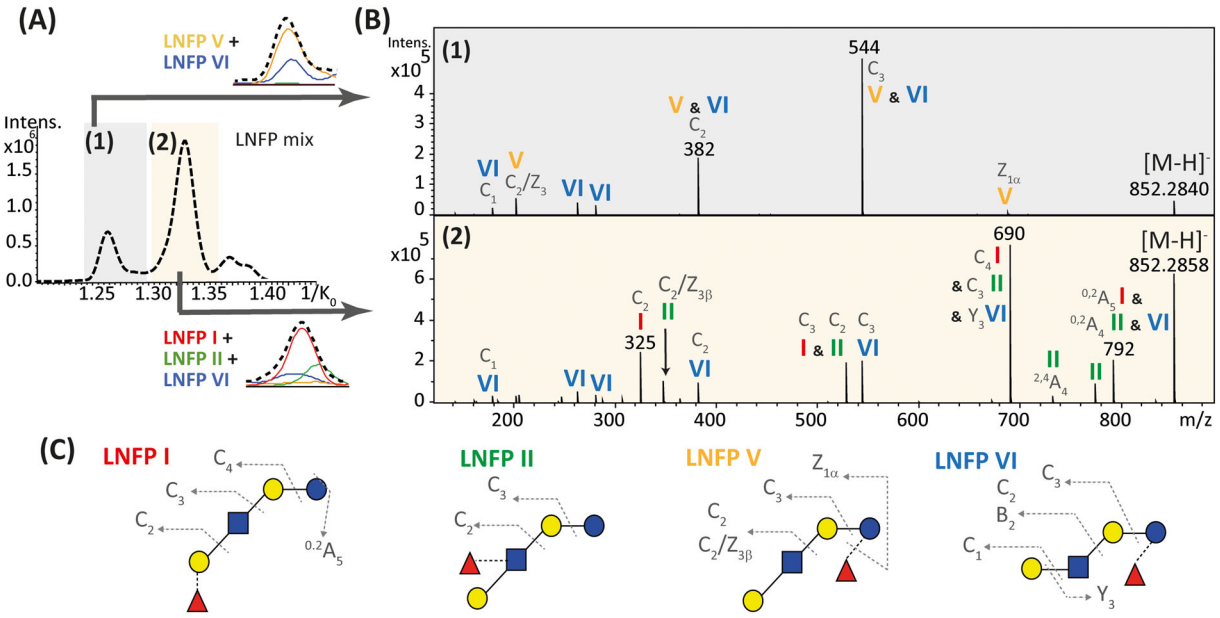


**FIGURE 3** Combination of ion mobility separation and MS/MS experiments to improve LNFP isomer distinction. (A) Extracted ion mobility spectra of $[M - H]^-$ species from the IM-MS analysis of equimolar mixture of the four LNFP isomers using full ion mobility scan. (B) Extracted MS/MS spectra obtained for a mobility range of (B.1) $1/K_0 = 1.24–1.30$ and (B.2) $1/K_0 = 1.30–1.36$ V.s.cm$^{-2}$. The diagnostic ions detected indicated overlaid MS/MS spectra of LNFP V and VI (B.1), and overlaid MS/MS spectra of the four isomers of LNFP (B.2). (C) Structure of the four LNFP isomers with main decompositions. IM-MS, ion mobility-mass spectrometry

separated into two sub-groups as exemplified in Figure 3A. The extracted ion mobility spectra of the $[M - H]^-$ species generated by the four isomers of LNFP display two main mobility peaks: (i) the first one containing the mixture of LNFP V and VI was detected at $1/K_0$ between 1.25 and 1.30 V.s.cm$^{-2}$ (Figure 3A), and (ii) the second that contains LNFP I, II, and VI was detected at $1/K_0$ between 1.30 and 1.35 V.s.cm$^{-2}$ (Figure 3A). Therefore, complementary tandem mass spectrometry (MS/MS) experiments were performed to help in isomer structural characterization.

### 3.3 | Ion mobility-tandem mass spectrometry to improve structural characterization of HMOs

Complementary to ion mobility separation, tandem mass spectrometry experiments (MS/MS) were carried out on all species detected in positive and negative ionization modes for each studied HMO in order to get structural information essential for further HMO characterization, in particular to distinguish isomers not well separated by ion mobility. As already reported,[15,24] specific fragmentation patterns of HMO isomers can be obtained from negatively charged species (Figure S1 in the supporting information), which was not the case from MS/MS experiments conducted in positive ionization mode. The nomenclature introduced by Domon and Costello[3] (Figure 1) was used to annotate the two main types of produced ions resulting from (i) glycosidic bond cleavage (B, Y, C, and Z) and (ii) cross-ring fragmentation (A and X). Therefore, the detection of corresponding ions reflects the presence of a given isomer in the analyzed sample.

Despite the partial ion mobility separation in full ion mobility scan of deprotonated ions (Figure 3A), the four isomers of LNFP could be distinguished thanks to the combination with the MS/MS experiments allowing the detection of the diagnostic product ions characteristic of each isomer (Figure 3B and C). Decomposition of deprotonated species results in the detection of diagnostic product ions: at *m/z* 325 for LNFP I; *m/z* 348, *m/z* 288, and *m/z* 184 for LNFP II; *m/z* 202 for LNFP V; and *m/z* 179, *m/z* 263, and *m/z* 281 for LNFP VI (Figures 3B and C and S1). Similarly, the characterization of the other HMO isomers was obtained thanks to diagnostic fragment ions generated from negatively charged precursor ions under IM-MS/MS analysis, with the exception of LST set (Figure S1 in the supporting information).

## 4 | CONCLUSION

High ion mobility resolution is essential for the separation of structurally related compounds with very close ion mobility, in particular for the characterization of isomers. In our study, all the studied HMO isomers could be distinguished by considering at least one specific ionic species generated under positive or negative ion mode thanks to the ion mobility separation and, for those poorly separated by ion mobility, complementary MS/MS experiments are able to provide diagnostic product ions. Therefore, the combination of ion mobility separation and MS/MS provides optimal performance for in-depth characterization of HMOs and isomer distinction. Such an approach appears very useful for the rapid analysis of breastmilk samples and, more importantly, for the characterization of the HMO profiles of

breastmilk samples, which varies from one individual to another and especially between individuals from different regions.

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## DATA AVAILABILITY STATEMENT

Data available in article supplementary material

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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