Identification of structurally closely related monosaccharide and disaccharide isomers by PMP labeling in conjunction with IM-MS/MS

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It remains particularly difficult for gaining unambiguous information on anomer, linkage, and position isomers of oligosaccharides using conventional mass spectrometry (MS) methods. In our laboratory, an ion mobility (IM) shift strategy was employed to improve confidence in the identification of structurally closely related disaccharide and monosaccharide isomers using IMMS. Higher separation between structural isomers was achieved using 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization in comparison with phenylhydrazine (PHN) derivatization. Furthermore, the combination of pre-IM fragmentation of PMP derivatives provided sufficient resolution to separate the isomers not resolved in the IMMS. To chart the structural variation observed in IMMS, the collision cross sections (CCSs) for the corresponding ions were measured. We analyzed nine disaccharide and three monosaccharide isomers that differ in composition, linkages, or configuration. Our data show that coexisting carbohydrate isomers can be identified by the PMP labeling technique in conjunction with ion-mobility separation and tandem mass spectrometry. The practical application of this rapid and effective method that requires only small amounts of sample is demonstrated by the successful analysis of water-soluble ginseng extract. This demonstrated the potential of this method to measure a variety of heterogeneous sample mixtures, which may have an important impact on the field of glycomics.

Carbohydrates play critical roles in a large number of biological processes such as protein conformation, molecular recognition, and cellular interaction1–3. While their structural elucidation is an essential prerequisite for understanding their many functions at the molecular level, the diversity of the constituent monosaccharides, anomeric configuration, and glycosidic linkages makes this task analytically demanding4,5. This is one reason why glycomics lags behind the advances in genomics and proteomics. Moreover, due to the difficulty in the separation and purification of carbohydrates, the preparation from biological sources is frequently accompanied by complex mixtures, where isomers must be distinguished in order to achieve complete identification.

Many strategies have been employed for analysis of carbohydrates, such as NMR spectroscopy6 and high-performance liquid chromatography (HPLC)7, often with the goal of recognizing isomers. The NMR-based approach is efficient for evaluating isomeric heterogeneity, and for structural elucidation, but has the limitation of needing considerable amounts of the analytes and obtaining single molecular species. In comparison, HPLC is time-consuming, and unambiguous identification of isomers is often not possible. Mass spectrometry (MS) plays an important role in structural elucidation of carbohydrates due to its high sensitivity and analysis speed8–13. However, analysis of carbohydrates by MS has been challenging in part due to the frequent presence of large amounts of oligosaccharide isomers, which often display very similar collision-induced dissociation (CID) mass spectra.

Ion mobility mass spectrometry (IMMS) is a unique gas phase ion separation technique on the basis of the collision cross section (CCS), charge, mass, drift gas polarizability and lifetimes of ion-neutral

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gaseous complexes14,15. IMMS is a promising approach to overcoming the above mentioned limitations, making it an ideal candidate for differentiation of isomers. The Synapt G2 high definition mass spectrometry (HDMS)16,17, traveling wave ion mobility mass spectrometry (TWIMMS)18,19, is a hybrid quadrupole/ion mobility separator/orthogonal time-of-flight (TOF) MS instrument. More recently, IMMS has been increasingly applied to the separation and analysis of small molecules and biomacromolecules in the gas phase based on measuring their arrival time distributions (ATD) and their CCSs20–23. TWIMMS has been applied in the field of carbohydrate research, and has been reported to unambiguously distinguish both simple standards and biological mixtures of isomeric oligosaccharides24–33. Identifying oligosaccharides by TWIMMS was demonstrated24–27 by both pre- and/or post-IM fragmentation prior to MS analysis, enhancing confidence in carbohydrate identification. In most of the cases, the separation of carbohydrates by IMMS has been performed for the sodiated precursor ions in the positive mode. However, the ionic radii, valence of cations, and number of metal ion adducts will distinctly affect the conformation and separation of carbohydrate isomers in IMMS28–30. In addition, better separation among oligosaccharide isomers can be achieved in the negative ion mode 31–35, with or without addition of anion salts. However, it was recently demonstrated that compositional isomeric carbohydrates could not be differentiated by IMMS in the recent article in Nature33. Harvey et al.34 reported the use of TWIMMS combined with negative ion fragmentation, for determining the structures of high-mannose glycans. Analysis of high-mannose N-glycans by TWIMMS revealed the presence of distinctive gas-phase conformers exclusive to [M–H]− ions35. Isomer separation of small carbohydrates by IMMS has also been reported22,36, but the analytes are not fully resolved. To increase the CCSs of the oligosaccharide isomers, Fenn and McLean37 have employed boronic acid derivatization as an ion mobility shift strategy, but no arrival time distributions of the derivatized isomers were reported.

Recently, Both et al.38 reported IMMS separation of isobaric monosaccharides and differentiation of CID fragment ions from disaccharides and polysaccharides, yet not all isomers were distinguishable. 1-Phenyl-3-methyl-5-pyrazolone (PMP), initially reported as a labeling reagent for reducing carbohydrates by Honda's group39, has been widely used for derivatization of reducing carbohydrates because derivatization reduces the derivatization of reducing carbohydrates because the derivatization is fast, mild, and has a simple clean-up procedure. Here, we present a novel method using PMP derivatization followed by IMMS for the simultaneous structural analysis of carbohydrate isomers. In an effort to obtain better ion mobility separation, we investigated factors including wave velocities, wave heights, and derivatization reagents. Water-soluble ginseng monosaccharides (WGOS-1) and water-soluble ginseng disaccharides (WGOS-2) were used to evaluate this method, demonstrating the powerful applicability of this approach for analysis of mixtures.

Results and Discussion
Arrival time distributions (ATDs) of 10 disaccharide and 4 monosaccharide isomers. The structures of all the 10 disaccharides and 4 monosaccharides used in this study.
Two commonly used derivatization reagents of carbohydrates, PMP and PHN, were used to covalently modify saccharides. The generalized schemes for the reaction of carbohydrates with PMP and PHN were shown in Supplementary Fig. S2, with cellobiose used as an example. The mass shifts resulting from carbohydrate derivatization with PMP and PHN are 330 and 88 Da, respectively (Supplementary Fig. S3). The CCSs for the underivatized and derivatized species were calculated to determine the effect of derivatization on the resulting structural shift in IMMS analysis. For the PMP-derivatized species, the preponderance (99%) of the signal was for proto-saccharides. The generalized schemes for the reaction of carbohydrates with PMP and PHN were shown in Supplementary Fig. S2, with cellobiose used as an example. The mass shifts resulting from carbohydrate derivatization with PMP and PHN are 330 and 88 Da, respectively (Supplementary Fig. S3). The CCSs for the underivatized and derivatized species were calculated to determine the effect of derivatization on the resulting structural shift in IMMS analysis. For the PMP-derivatized species, the preponderance (99%) of the signal was for proto-saccharides, and also had very broad peak width (Supplementary Fig. S6). This observation was in agreement with a previous report38.

As observed in Supplementary Fig. S1, the drift times of sucrose and the other disaccharides differ by at least 0.11 ms; those of glucose and its isomers differ by 0.11 ms. Consequently, sucrose and glucose were more readily distinguished with their respective isomers by their drift times. However, the drift times of the rest of the studied saccharides were essentially indistinguishable. These results for the monosaccharide isomers were in agreement with the previous report38.

**Effect of derivatization reagents.** The ion mobility shift reagent strategy was employed to increase the CCSs of the above mentioned disaccharides and monosaccharides, with the aim of distinguishing the isomers. As observed in Supplementary Fig. S1, the drift times of sucrose and the other disaccharides differ by at least 0.11 ms; those of glucose and its isomers differ by 0.11 ms. Consequently, sucrose and glucose were more readily distinguished with their respective isomers by their drift times. However, the drift times of the rest of the studied saccharides were essentially indistinguishable. These results for the monosaccharide isomers were in agreement with the previous report38.

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As expected, the saccharides showed different drift times by the ion mobility shift reagent strategy. As illustrated by the ATDs presented in Fig. 3, the three disaccharide isomers maltose (t_D = 8.73 ms), isomaltose (t_D = 8.03 ms), and laminaribiose (t_D = 9.01 ms) as well as the three monosaccharide isomers galactose (t_D = 6.18 ms), mannose (t_D = 6.29 ms), and fructose (t_D = 6.4 ms) were more readily distinguished by the drift times of their PMP derivatives. The leading edge (i.e. laminaribiose) may arise due to the bisPMP bind-

Figure 2. A plot of CCS vs. m/z for (a) [M + Na]^+ of underivatized carbohydrates, and (b) [M + Na]^+ of PMP- and (c) [M + Na]^+ of PHN-derivatized carbohydrate isomers. RE (relative error) <3%. Refer to Table 1 for tabulated values.
mass, which caused an IM peak broadening of γ-CD. Here, it was inferred that the width differences of ATDs for some disaccharides resulted from their different spatial configurations.

In short, the results showed that the majority of disaccharide and monosaccharide structural isomers exhibited unique mobility drift times, even though not all of them were fully resolved.

Tandem mass spectrometric analysis of PMP derivatives of disaccharides. The MS² strategy was utilized to further enhance the identification of specific disaccharides. The mobility and mass selected ions could be introduced into the trap cell installed in front of a TWIMS, which enables the isomeric heterogeneity of product ions to be evaluated. The product ions in the tandem mass spectra of PHN derivatives of disaccharides (Supplementary Fig. S3) were the corresponding native disaccharide ions. Thus, it was impossible to differentiate the six unresolved disaccharide isomers mentioned above by the mobility spectra extracted for the product ions at m/z 365.11, as shown in Supplementary Fig. S1. To solve this problem, we took recourse to MS² analysis of PMP derivatives of disaccharides (Fig. 4). The mobilities of the product ions, [monoPMP-disaccharide + H]^+ ions at m/z 499.19 (Fig. 4, panel a) and [monoPMP-disaccharide + Na]^+ ions at m/z 521.17 (Fig. 4, panel b), were examined and compared. The only difference between the two specific ions is the ionized form. From Fig. 4, it is clearly seen that the ionized form exerted a significant influence on the drift times of the product ions. As a general trend, drift times of these ions increased as the size of the ions increased. For example, the drift time of kojibiose varied from t_D = 4.83 ms as the [M + H]^+ ion to t_D = 5.75 ms for the [M + Na]^+ ion.

As presented in Fig. 4a, it became immediately apparent that the product ions of the three pairs of isomers exhibited strikingly different drift times (t_p = 5.48 ms for [monoPMP-kojibiose + H]^+ versus t_p = 5.64 ms for [monoPMP-nigerose + H]^+; t_p = 5.64 ms for [monoPMP-lactose + H]^+ versus t_p = 5.15 ms for [monoPMP-sophoros + H]^+; t_p = 5.75 ms for [monoPMP-cellobiose + H]^+ versus t_p = 5.59 ms for [monoPMP-gentiobiose + H]^+), depending on differences in their linkages. Whereas, as shown in Fig. 4b, some monoPMP disaccharide isomers were separable in the form of sodium ion adducts (different t_p values) while not in the other (same t_p values), which indicated that sodium ion adducts are often not the preferred charge carrier

Figure 3. Overall mobility spectra of the 9 PMP-derivatized disaccharide isomers and 3 PMP-derivatized monosaccharide isomers. All mobility spectra of the disaccharides and monosaccharides were extracted for protonated ions at m/z 673.27 and 511.22, respectively. The arrival times are from three individual measurements, and deviation is ± 0.01 ms.
from the standpoint of IM separation of isomeric carbohydrates. Sodium ion association was disadvantage to the differentiation of the monoPMP derivatives of the linkage isomers cellobiose (β1-4) and gentiobiose (β1-6). In comparison, better separation among structural isomers appeared to be achieved for [M + H]+ cations. The CCSs for the twelve MS2 fragment ions were calculated to determine the structural variation (Supplementary Table S1).

The sodiated monoPMP derivatives of the linkage isomers cellobiose (β1-4) and gentiobiose (β1-6) exhibited CCSs that were almost identical to each other (151.52 Å2 and 151.10 Å2, respectively), which is in good agreement with the results from ATDs. Remarkably, protonated monoPMP derivatives of the six disaccharide isomers exhibited highly diagnostic CCS values that differed by at least 2.1 Å2 for each pair of isomers.

The mixture of the given set of disaccharide and monosaccharide isomers can be differentiated by PMP derivatization in conjunction with ion-mobility separation and MS2. For clarity, the overlaid IMS plots of all the analytes are summarized in Fig. 5.

Application to WGOS isolated from the Panax Ginseng root. As an example of the application of the above technique, Fig. 6 shows the IM spectra of WGOS-1 and WGOS-2 obtained from a warm-water extract of Panax ginseng roots as our previously published procedures41. Direct comparison of Fig. 6a (sodiated WGOS-1 at m/z 203.04 and potassium adduct ions of WGOS-2 at m/z 381.07), 6b (potassium adduct ions of sucrose at m/z 381.07), and 6c (sodiated glucose at m/z 203.04) revealed the presence of sucrose in WGOS-2 and glucose in WGOS-1. The peaks at 2.06 and 3.69 ms in Fig. 6a are ambiguous. The former could be assigned as fructose, mannose, or galactose based on Supplementary Fig. S1, and the later could correspond to kojibiose, nigerose, maltose, sophorose, laminariobiose, or cellobiose (Supplementary Fig. S7). CCS values of the potassium adduct ions of the disaccharide isomers were summarized in Supplementary Table S2.

In order to identify the peaks at 2.06 and 3.69 ms, PMP-derivatized WGOS-1 and WGOS-2 were determined (Fig. 6d). A comparison of the drift times between Fig. 6d, e, and f showed that WGOS-1 was composed of glucose and fructose, indicating the peak at 2.06 ms corresponded to fructose instead of mannose and galactose (Fig. 6a).

Furthermore, taking these results from the tandem mass spectra and full scan mass spectra into consideration, one can conclude that WGOS-2 contains maltose rather than the other disaccharides (Figs 6d,g). Sucrose is a nonreducing disaccharide, thus its PMP derivatives was not detected.

The results showed that the disaccharides in WGOS-2 and monosaccharides in WGOS-1 were identified as sucrose and maltose as well as glucose and fructose, respectively, which were in agreement with the observations made in a previous study42. Thus, the reliability of this method was confirmed.

Conclusions
The differentiation of closely related structural isomers is a serious complication when using mass spectrometry alone. In this study, the coexisting mono- and disaccharide isomers with different linkages, compositions, and configurations were separated by the PMP labeling technique in conjunction with ion-mobility separation and tandem mass spectrometry. The extent of separation was significantly affected by the ionized forms of MS2 fragments, and [M + H]+ cations are the preferred charge carrier from the standpoint of IM separation of isomeric carbohydrates. In addition, our data show that the structural differences between carbohydrate isomers can lead
to distinctly different CCSs. Therefore, carbohydrate isomers can be distinguished not only on the basis of their drift time, but also based on their relative CCS values. All carbohydrates in WGOS-1 and WGOS-2 have been successfully examined using this new method. The simplicity and validity of the method makes it an attractive option for unequivocal differentiation of carbohydrate isomers.

Material & Methods

Chemicals and Reagents. Kojibiose, maltose, isomaltose, nigerose, cellobiose, gentiobiose, melibiose, trehalose, α-cyclodextrin, glucose, fructose, galactose, mannose, and PHN were bought from J&K Chemical Ltd. (Beijing, China). Sophorose was acquired from Shanghai Huicheng Biotechnology Co, Ltd. (Shanghai, China). Laminaribiose was acquired from Beijing Chemsynlab Pharmaceutical Science & Technology Co. Ltd. (Beijing, China). Maltotriose, raffinose, melezitose, sucrose, PMP, and lactose were acquired from Aladdin (Shanghai, China). Methanol (HPLC grade) was obtained from Fisher Chemical Company. All other chemicals used in this study were of analytical grade and were used without further purification. High-purity helium, nitrogen, and argon (99.999%) were supplied by Changchun Juyang Gas Co., Ltd. (Changchun, China). Ultrapure water (specific conductivity, 18.2 MΩ/cm) was produced by a MilliQ device (Millipore, Milford, MA, USA).

Sample Preparation. For ion mobility studies, 2 mM stock solutions of monosaccharides and disaccharides were prepared using ESI solvent (50% aqueous methanol, v/v) and were diluted in 1:20 ESI solvent for individual
lytes were recorded over a range of wave heights and velocities to separate the ions. Under each condition, a 250 mM PMP was prepared by dissolving 60.67 mg of PMP into 10 mL NH$_3$·H$_2$O–methanol solution. PMP and PHN drift gas at a flow rate of 90 mL/min. The traveling wave height was set to 40.0 V. The traveling wave velocity was systematically optimized for maximum resolution. Optimal traveling wave velocities were 550–2500 m/s (the variable IMS wave velocity, start velocity: 550 m/s, end velocity: 2500 m/s) for monosaccharides and disaccharides as well as their derivatives. IM-MS experiments were performed to make an attempt to distinguish barely resolved analytes in the IM-MS. The CID experiments were performed using argon as collision gas at the trap cell temperature at 120 °C, and desolvation temperature at 350 °C. The flow rates of the cone gas and desolvation gas were set to 30 and 450 L h$^{-1}$, respectively.

The major experimental parameters affecting TWIM separation are the drift gas pressure, the TWIM DC traveling wave height, and the TWIM DC traveling wave velocity$^{45,46}$. For all IM experiments, He was introduced at 180 mL/min to the helium cell installed in front of the ion mobility separator, and nitrogen was used as the drift gas at a flow rate of 90 mL/min. The traveling wave height was set to 40.0 V. The traveling wave velocity was systematically optimized for maximum resolution. Optimal traveling wave velocities were 550–2500 m/s (the variable IMS wave velocity, start velocity: 550 m/s, end velocity: 2500 m/s) for monosaccharides and disaccharides as well as their derivatives. IM-MS/MS experiments were performed to make an attempt to distinguish barely resolved analytes in the IM-MS. The CID experiments were performed using argon as collision gas at the trap cell of the instrument at a flow rate of 2 mL/min and a collision energy of 40 V. Data acquisition and processing were conducted using Masslynx 4.1 software (Waters Corp., Manchester, U.K.).

### Collision Cross Sections.

It has been proposed that the CCS is proportional to $t_D^5$ in the traveling-wave IMS system. The exponential factor $X$ depends upon many variables including the traveling wave height and the traveling wave velocity$^{46,47}$. CCS calculations were performed according to previously described protocols$^{47,48}$. The CCSs of the isomeric monosaccharides and disaccharides as well as their derivatives are thus determined according to the calibration curve constructed using the oligosaccharides with known CCSs (cellobiose, 112.4 Å$^2$; maltose, 112.6 Å$^2$; sucrose, 112.6 Å$^2$; lactose, 121.1 Å$^2$; melibiose, 112.2 Å$^2$; trehalose, 110.6 Å$^2$; maltotriose, 142.9 Å$^2$; raffinose, 138.8 Å$^2$; melezitose, 133.5 Å$^2$; $\alpha$-cyclodextrin, 200.7 Å$^2$)$^{28,29}$. IMMS data of the calibrant ions and analytes were recorded over a range of wave heights and velocities to separate the ions. Under each condition, a calibration curve was established to calculate the experimental CCSs.

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| Carbohydrates | [M + Na]$^+$ | [M + PHN + Na]$^+$ | [M + PMP + H]$^+$ |
|---------------|-------------|----------------|----------------|
| lactose       | 121.1$^+$  | 137.3 ± 0.5    | 177.9 ± 0.6    |
| koidiose      | 114.3 ± 0.4| 134.0 ± 0.2    | 180.0 ± 0.5    |
| nigerose      | 112.9 ± 0.5| 126.5 ± 0.6    | 180.1 ± 0.4    |
| maltose       | 112.6 ± 0.4| 138.1 ± 0.2    | 182.5 ± 0.3    |
| isomaltose    | 113.3 ± 0.4| 134.7 ± 0.3    | 174.7 ± 0.2    |
| sophorose     | 114.6 ± 0.6| 133.4 ± 0.7    | 179.2 ± 0.2    |
| laminariobiose| 113.9 ± 0.5| 128.6 ± 0.5    | 184.4 ± 0.3    |
| cellobiose    | 112.4 ± 0.4| 139.4 ± 0.3    | 177.1 ± 0.2    |
| gentiobiose   | 114.7 ± 0.9| 134.3 ± 0.2    | 176.2 ± 0.3    |
| galactose     | 83.5 ± 0.3 | 98.2 ± 0.4     | 152.8 ± 0.1    |
| mannoside     | 83.4 ± 0.5 | 98.0 ± 0.5     | 153.7 ± 0.2    |
| fructose      | 83.7 ± 0.4 | 100.3 ± 0.5    | 154.4 ± 0.2    |

Table 1. The CCSs for underivatized, and PMP- and PHN-derivatized carbohydrate species (n = 16). $^a$The value was reported in reference 28, and $^b$the values were reported in reference 29.
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**Author Contributions**

S.Y.L., H.M.Y. and D.B.W. conceived and designed the research; S.Y.L. and D.B.W. contributed importantly to the discussion of results and manuscript refinement. H.M.Y. performed the experiments with some assistance from X.Y.Z. and R.S. The manuscript was written by H.M.Y. with contributions from L.S., F.R.S. and J.Y.L. All authors discussed and commented on the manuscript and supplementary information.

**Additional Information**

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