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RESEARCH ARTICLE

Complete Hexose Isomer Identification with Mass Spectrometry

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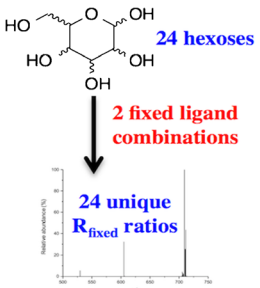

Abstract. The first analytical method is presented for the identification and absolute configuration determination of all 24 aldohexose and 2-ketohexose isomers, including the *D* and *L* enantiomers for allose, altrose, galactose, glucose, gulose, idose, mannose, talose, fructose, psicose, sorbose, and tagatose. Two unique fixed ligand kinetic method combinations were discovered to create significant enough energetic differences to achieve chiral discrimination among all 24 hexoses. Each of these 24 hexoses yields unique ratios of a specific pair of fragment ions that allows for simultaneous determination of identification and absolute configuration. This mass spectrometric-based methodology can be readily employed for accurate identification of any isolated monosaccharide from an unknown biological source. This work provides a key step towards the goal of complete *de novo* carbohydrate analysis.

Keywords: Monosaccharide, Hexose isomers, Sugar sequencing, Mass spectrometry, Kinetic resolution, Carbohydrates, Carbohydrate analysis, Chirality, Configuration determination

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Introduction

Carbohydrates serve key structural and functional roles in nature that include a wide range of biological processes such as cell–cell recognition, protein folding and degradation, immune/host-pathogen responses, and metabolism [1–5]. However, carbohydrate sequencing has significantly lagged capabilities in sequencing nucleic acids and proteins. Compared with these other biomolecules, polymers and oligomers of carbohydrates are more structurally complex because of their large range of stereochemical differences, absolute configuration (*D* or *L*), anomeric configurations (α or β), and linkage positions [2, 4, 6, 7]. This high degree of structural complexity has hindered accurate structural analyses of glycans, so much so that a United States National Academy of Sciences report issued in 2012 has called for the development of technology over the next 10 years to purify, identify, and determine the structures of all the important glycoproteins, glycolipids, and polysaccharides in any biological sample [8].

Currently, structural elucidation projects usually begin with hydrolysis of the glycan into its constituent monosaccharides for their accurate identification, often by a gas chromatography–mass spectrometry (GC-MS) method [9]. Unfortunately, this method is limited by the need for sample derivatization as well as convoluted chromatograms from the resulting mixture of α/β and furanose/pyranose forms [10, 11]. Presently, much attention is given to the structural elucidation of mammalian *N*-glycans and glycosaminoglycans [12]. This task is relatively simpler than *de novo* carbohydrate sequencing because only a small subset of monosaccharide subunits are involved, and all these subunits are believed to be known. However, the presumption of such a limited set of monosaccharide building blocks does not hold up when faced with an unknown biological sample from the many diverse branches of life. The entire set of possible monosaccharide isomers must be taken into account in its analysis. For accurate identification of such unknown glycan structures, reliable high-throughput methods for complete monosaccharide analysis are needed first [2–4, 6, 13]. Monosaccharide constituents are often identified by mass alone into classes such as hexose or amino-hexose as a means of preliminary structural analysis. However, it is unclear whether a single technique such as mass spectrometry could be used to further distinguish between all the possible hexoses, for example. Neutral hexoses (Figure 1) can be described as 12 diastereomers, each composed of two, *D* and *L*, mirror-image enantiomers, for a total of 24 different isomers. Furthermore, these can be divided into the 16 aldohexose isomers (allose, altrose, galactose, glucose, gulose, idose, mannose, and talose),

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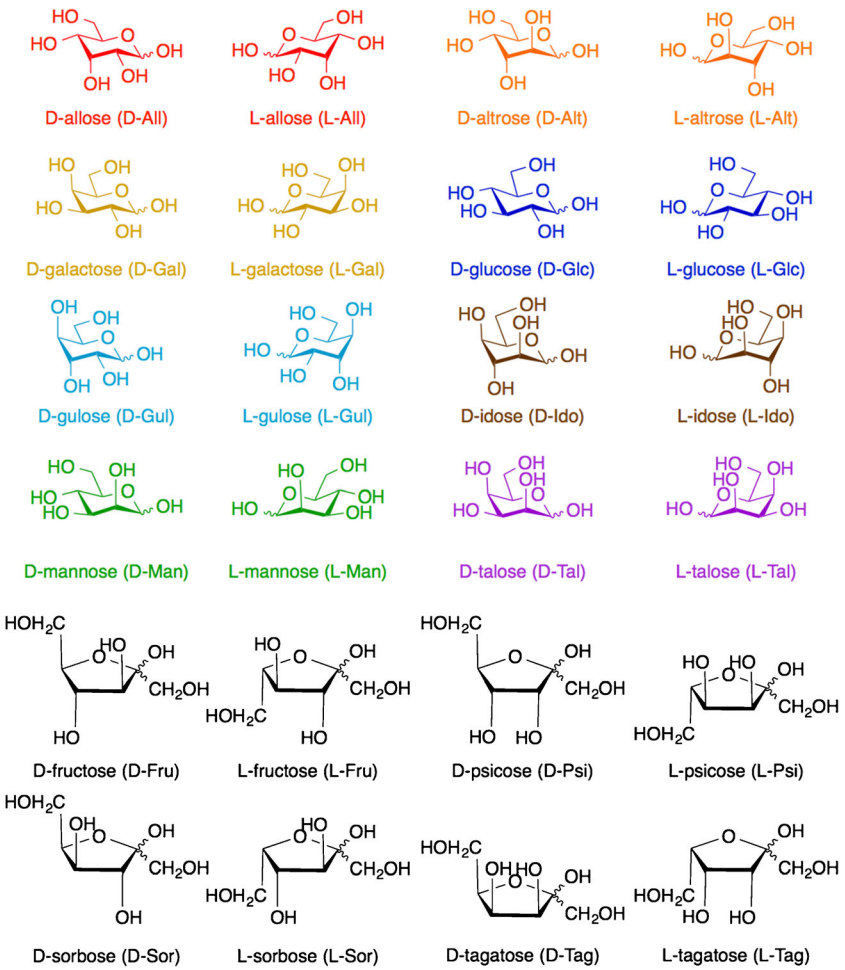

Figure 1. The 24 hexose monosaccharide isomers

and the 8 ketohexose isomers (fructose, psicose, sorbose, and tagatose). Obviously, the major question arises as to whether or not simultaneous discrimination and identification of all 24 neutral monosaccharide isomers is even possible. Their only structural differences (as shown in Figure 1) are the axial or equatorial hydroxyl group permutations at C2, C3, and C4 for the aldohexoses, and C3 and C4 for the ketohexoses. From this structural similarity, it is evident that minimal energetic differences will exist between all 24 isomers. Without sufficient energetic differences between them, discrimination and separation would be impossible. The central challenge then comes down to finding a set of ligands that bind to each of the 24 isomers to create large enough energetic differences to accurately discriminate amongst the isomers within error bars—a daunting task given the incredible difficulty seen in designing artificial receptors that can discriminate amongst sugar stereoisomers. These sugar receptors have had only limited success in achieving stereoselectivity based on the small binding energy differences between stereoisomers [14, 15].

Other analytical techniques employed for monosaccharide analysis include high-performance anion-exchange chromatography (HPAEC), HPLC, and NMR [16–23]. Some disadvantages associated with each include the absence of measurable J values and overlap of proton signals in NMR, and long column regeneration time between runs and inability to determine absolute configuration in HPAEC [16–23]. Although HPLC provides one of the more popular methods for monosaccharide analysis, it has certain limitations [24–30]. Often times, sample preparation or derivatization is needed and, even in the case of chiral columns, there has not been as large or as complete a set of monosaccharides discriminated from one another as presented here. Furthermore, identification of an unknown monosaccharide is limited by overlapping retention times from the α/β and furanose/pyranose forms. The possibility was explored that mass spectrometric methods could overcome these problems and also discriminate monosaccharide identity and absolute configuration. Previous studies have not demonstrated simultaneous discrimination of diastereomers and enantiomers.

Mass spectrometry is often thought of as a chirally blind technique—one that is unable to discriminate between enantiomers, let alone be used for complete analysis of a large isomeric set. However, mass spectrometry has certain inherent advantages compared with the aforementioned analytical techniques previously employed for monosaccharide analysis [1, 17,

18, 22, 31, 32]. Specifically, mass spectrometry does not require any solvent or stationary phase, and also provides fast, accurate, and sensitive sample analysis. To date, monosaccharide analysis techniques in mass spectrometry result in similar MS^1 spectra for diastereomers and similar MS^2 spectra for enantiomers. In other words, no previous mass spectrometric methods have been developed to simultaneously discriminate diastereomers and enantiomers of a complete set of isomers [33–36].

Experimental

Conventional Kinetic Method

Given earlier findings with carbohydrate-active enzymes discriminating amongst different glycan substrates through kinetic rather than purely binding affinity differences [37, 38], it was set out to discover much smaller carbohydrate-binding ligands that could discriminate amongst all the neutral hexoses through kinetic and not just thermodynamic differentiation steps. A related thought process forms the basis of Cooks' kinetic method, perhaps the most widely applied chiral mass spectrometric technique [16–18, 39–45]. This method measures the dissociation rates of cluster ions composed of a metal, chiral reference, and analyte of interest. Fragmentation patterns are affected by the strength of the metal-bound analyte ligand, which varies with chirality of the analyte. Specifically, the basis of the kinetic method is the formation of a singly charged, trimeric ion complex via electrospray ionization (ESI), where M^{II} is the divalent metal cation, reference is the chiral reference, A is the monosaccharide analyte, and k_1 *and* k_2 are the two competitive dissociation pathways (Figure 2). It can be seen that the two diastereomeric fragment ions are either from the neutral loss of an analyte or neutral loss of a reference molecule, both from the initial trimeric ion complex (Figure 2). A branching ratio term, R value, can quantify a relationship between the two diastereomeric fragment ions as a measure of their relative intensities (Equation 1). When an individual enantiomer is present, the R value can become either R_D or R_L, and the chiral discrimination between an enantiomeric pair can be represented by the ratio between R_D and R_L in the form of the R_{chiral} term where a value further from unity is desired (Equation 2).

$$R = \frac{\left[M^{II}(A)(ref)-H\right]^+}{\left[M^{II}(ref)_2-H\right]^+} \quad (1)$$

$$R_{chiral} = \frac{R_D}{R_L} = \frac{\left[M^{II}(A_D)(ref)-H\right]^+ / \left[M^{II}(ref)_2-H\right]^+}{\left[M^{II}(A_L)(ref)-H\right]^+ / \left[M^{II}(ref)_2-H\right]^+} \quad (2)$$

Fixed Ligand Kinetic Method

Although this conventional kinetic method has been widely applied, it is limited by the lack of chiral selectivity in certain cases, as well as isomeric complexes formed in the gas phase from the multiple possible deprotonation (–H) sites. One modification to help alleviate these problems and, furthermore, increase chiral selectivity, is the fixed ligand kinetic method, also developed by Cooks and co-workers [17, 18, 41, 46–48]. Here, the initial trimeric ion complex formed is $[M^{II}(A)(ref)(FL - H)]^+$, while the two diastereomeric fragment ions from the two competitive dissociation pathways are $[M^{II}(A)(FL - H)]^+$ and $[M^{II}(ref)(FL - H)]^+$, where FL is the fixed ligand (Figure 3). It can be seen that an easily deprotonated fixed ligand molecule will replace one of the chiral references in the original trimeric ion complex (Figure 3). More importantly, the fixed ligand is not lost in CID and thus the deprotonation site is confined to it, which avoids the formation of various isomeric structures. Chiral discrimination is improved because more chiral interactions occur in the diastereomeric fragments compared with the conventional kinetic method. Once again, a branching ratio, R_{fixed}, relates the two fragment ions to one another as a measure of their relative intensities (Equation 3). When one specific enantiomer is present, R_{fixed} can become either $R_{D\text{-}fixed}$ or $R_{L\text{-}fixed}$ (Equations 4 and 5). Chiral discrimination between an enantiomeric pair can be quantified

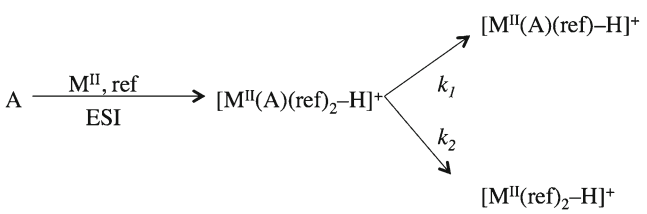

Figure 2. Formation of divalent metal-bound trimeric ion complex via electrospray ionization, and subsequent fragmentation through collision-induced dissociation in conventional kinetic method

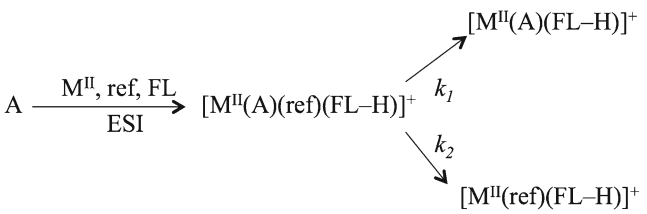

Figure 3. Formation of divalent metal-bound trimeric ion complex via electrospray ionization, and subsequent fragmentation through collision-induced dissociation in fixed ligand kinetic method

(Equation 6) through the $R_{chiral\text{-}fixed}$ term, where a value further from unity is desired.

$$R_{fixed} = \frac{\left[M^{II}(A)(FL{-}H)\right]^{+}}{\left[M^{II}(ref)(FL{-}H)\right]^{+}} \quad (3)$$

$$R_{D\text{-}fixed} = \frac{\left[M^{II}(A_D)(FL{-}H)\right]^{+}}{\left[M^{II}(ref)(FL{-}H)\right]^{+}} \quad (4)$$

$$R_{L\text{-}fixed} = \frac{\left[M^{II}(A_L)(FL{-}H)\right]^{+}}{\left[M^{II}(ref)(FL{-}H)\right]^{+}} \quad (5)$$

$$R_{chiral\text{-}fixed} = \frac{R_{D\text{-}fixed}}{R_{L\text{-}fixed}} \quad (6)$$

Chiral separation in the fixed ligand kinetic method comes from Equation 7, where R_{fixed} is the fixed ligand branching ratio, **R** is the gas constant, $\Delta(\Delta G)$ is the change in Gibbs' free energy, and T_{eff} is the average temperature of the activated complexes [17, 18, 41, 46–48]. Small differences in free energy between the diastereomeric fragment ion complexes will result in larger differences in their branching ratio, R_{fixed}, values. More specifically, differences in the stability (and thus energetics) of the diastereomeric fragment ion complexes will result in different relative intensities and, thus, unique R_{fixed} values for each neutral hexose enantiomer. Figure 4 illustrates a free energy diagram representation of the fixed ligand kinetic method.

$$\ln\left(R_{chiral\text{-}fixed}\right) = \frac{\Delta(\Delta G)}{\boldsymbol{R}T_{eff}} \quad (7)$$

Mass Spectrometric Methodology

Previous monosaccharide work with the Cooks' kinetic method has focused on the $R_{chiral\text{-}fixed}$ term for enantiomeric pairs of the "common" sugars (galactose, glucose, mannose, and fructose) [20, 41, 46, 49]. However, none of these studies have incorporated any of the "rare" sugars (allose, altrose, gulose, idose, talose, psicose, sorbose, and tagatose) into their results. Furthermore, these previous studies have not shown simultaneous discrimination of diastereomers and enantiomers, nor have they included all 24 neutral hexose isomers. It was unclear whether any set of ligands would be able to differentiate amongst so many closely related structures. The challenge was to develop a method that would allow all 24 neutral hexose isomers to be discriminated from one another, with simultaneous absolute configuration determination.

Whereas the Cooks' kinetic method focuses solely on the $R_{chiral\text{-}fixed}$ term to relate one individual pair of *D*/*L* enantiomers, a novel R_{fixed} range term has been developed that allows the simultaneous relation of multiple sets of *D*/*L* enantiomers to one another and determine absolute configuration. From Equations 4 and 5, it can be seen that each monosaccharide will provide a fixed ligand branching ratio, either $R_{D\text{-}fixed}$ or $R_{L\text{-}fixed}$, based on the enantiomer (e.g., $R_{D\text{-}All}$, $R_{L\text{-}All}$, $R_{D\text{-}Alt}$, $R_{L\text{-}Alt}$, $R_{D\text{-}Gal}$, $R_{L\text{-}Gal}$, etc…). Simultaneous monosaccharide identification and absolute configuration determination can be found if each of the 24 hexose isomers has a unique and non-overlapping R_{fixed} range; in other words if $R_{D\text{-}All}$, $R_{L\text{-}All}$, $R_{D\text{-}Alt}$, $R_{L\text{-}Alt}$, $R_{D\text{-}Gal}$, $R_{L\text{-}Gal}$, etc… do not overlap with one another in their R_{fixed} ranges, these 24 hexose isomers are discriminated from one another.

Materials

All neutral monosaccharides, chiral references, fixed ligands, and metal salts were purchased from Carbosynth (Berkshire, UK) and Sigma Aldrich (Milwaukee, WI, USA) without any further purification (≥95%): monosaccharides (*D*/*L*-allose, *D*/*L*-altrose, *D*/*L*-galactose, *D*/*L*-glucose, *D*/*L*-gulose, *D*/*L*-idose, *D*/*L*-mannose, *D*/*L*-talose, *D*/*L*-fructose, *D*/*L*-psicose, *D*/*L*-sorbose, and *D*/*L*-tagatose), metal salts ($CuCl_2$, $NiCl_2$, $MnCl_2$, and

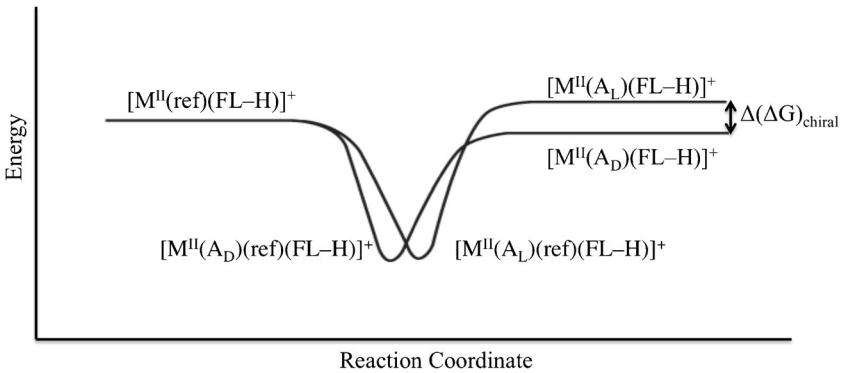

Figure 4. Free energy diagram of fixed ligand kinetic method that illustrates energetic differences of diastereomeric fragment ion complexes

$Na_2B_4O_7$–10 H_2O), chiral references (*L*-Serine and *L*-Aspartic acid), and fixed ligand molecules (*L*-Phe–Gly, 5′CMP, and 5′GMP-Na_2). All stock solutions were prepared with HPLC grade water and HPLC grade methanol in 50:50 (v/v) mixtures. For the conventional kinetic method, the relative concentrations used were 1:4:4, metal:chiral reference:analyte, whereas, with the fixed ligand method, the relative concentrations were 1:4:4:4, metal:chiral reference:fixed ligand:analyte. The final concentrations used in the conventional kinetic method were 5×10^{-5} M divalent metal cation, 2×10^{-4} M chiral reference, and 2×10^{-4} M analyte. In the fixed ligand method, the final concentrations used were 5×10^{-5} M divalent metal cation, 2×10^{-4} M chiral reference, 2×10^{-4} M fixed ligand, and 2×10^{-4} M analyte. It is important to note that traces of formic acid were added to the 5′GMPNa_2 stock solution and allowed to sit at room temperature for 2 wk prior to the experiment [46].

Mass Spectrometry Conditions

All experiments were performed on the ion trap portion of an LTQ Orbitrap XL from Thermo Scientific (San Jose, CA, USA), with an ESI source. The Xcalibur software was used for data acquisition. The source conditions used were: 5 kV spray voltage, 0 V capillary voltage, 150°C capillary temperature, 40 V tube lens voltage, and 6 units sheath gas flow rate. The flow rate used was 5 µL/min with a maximum ion injection time of 500 ms. In MS^2, the parent ion was isolated through the resonance ejection of all other ions, except the *m/z* of interest. Resonance excitation was applied on these selected ions to cause collision-induced dissociation (CID). MS^2 fragmentation and CID conditions were: 10 *m/z* isolation width, 5%–20% normalized collision energy (%NCE), 30 ms activation time with wideband activation. Collision energy in terms of mV applied for collision-induced dissociation was approximately 5–50 mV, and was optimized for each kinetic method combination and kept constant for all of the 24 hexose isomers. Each spectrum represents an average of 32 scans with three microscans included. All results represent triplicate trials with standard deviations included.

Results and Discussion

The goal was to first optimize a set of ligands for the identification and absolute configuration determination of the 16 aldohexose isomers. Once these specific conditions were found, they were then applied to the eight ketohexose isomers. It was decidedly easier to first focus on the larger group of isomers (aldohexoses) and then use these optimized conditions on the smaller group of isomers (ketohexoses) to illustrate the discrimination and absolute configuration determination of all 24 neutral hexose monosaccharide isomers.

Aldohexoses—Conventional Kinetic Method

First, the ability of the conventional kinetic method to resolve all 16 aldohexose isomers was tested. Chiral references and divalent metal cations were initially chosen based on their known ability from previous studies to form the trimeric ion complexes in high abundances, as well as undergo competitive dissociation to form both diastereomeric fragment ions [20, 40, 41, 46, 47, 49]. Furthermore, transition metals contain *d*-electronic orbitals that increase chiral recognition as well as being oxophilic, which is preferred for monosaccharide analytes. Chiral references should have similar size and functional groups (OH) as the analyte of interest [20, 40, 41, 46, 47, 49]. Although boron readily forms *cis*-diols with the hydroxyl groups of monosaccharides [38, 50, 51], B^{III} proved useless as the central metal cation to form the desired trimeric ion complex. Two different conventional kinetic method combinations of metal cations and chiral references, Mn^{II}/*L*-Asp and Cu^{II}/*L*-Ser, were employed for all 16 monosaccharides. It was observed that R values were both too similar from isomer to isomer and irreproducible (i.e., large error bars), caused by the lack of significant energetic differences in the diastereomeric fragment ion complexes formed for each of the 16 different aldohexose isomers with each conventional kinetic method combination applied (see Supporting Information). From these results, no conventional kinetic method combinations were pursued. It is clear that these two combinations cannot resolve the 16 aldohexose isomers, let alone the goal to combine them with the eight ketohexose isomers, for all 24 neutral hexoses. Clearly, a system with the minimum number of steps (combinations) is desired for the development of a robust method amenable to high throughput carbohydrate analysis.

Aldohexoses—Fixed Ligand Kinetic Method

Since the conventional kinetic method was insufficient, the fixed ligand approach was applied. The fixed ligands chosen were *L*-Phe – Gly, 5′CMP, and 5′GMP, based on previous literature [41, 46–48]. Fixed ligands must contain some inherent chirality and, preferably, an aromatic group that could increase chiral discrimination from cation-π interactions with the central, divalent metal cation [32, 46, 47]. More specifically, the exact combinations were: Mn^{II}/ *L*-Asp/*L*-Phe – Gly, Ni^{II}/*L*-Asp/5′CMP, Ni^{II}/*L*-Asp/5′GMP, and Cu^{II}/*L*-Ser/5′GMP. It was determined that if Mn^{II}/*L*-Asp/ *L*-Phe – Gly, and Cu^{II}/*L*-Ser/5′GMP are applied in two different steps to all 16 aldohexose isomers, monosaccharide identification and absolute configuration determination is achieved. Work with the other fixed ligand combinations produced insufficient aldohexose isomer identification (see Supporting Information). A few MS^2 plots of the fragmentation of the parent ion, $[Cu^{II}(A)(L\text{-Ser})(5'GMP - H)]^+$ are shown (Figure 5) to illustrate how R_{fixed} is found, as well as to provide an example of how fragment ion abundances differ from monosaccharide to monosaccharide (see the Supporting Information for MS^1 spectra).

Complete Aldo/Keto-Hexose Discrimination

Since a set of fixed ligand combinations, Mn^{II}/*L*-Asp/*L*-Phe – Gly and Cu^{II}/*L*-Ser/5′GMP, that could resolve the 16

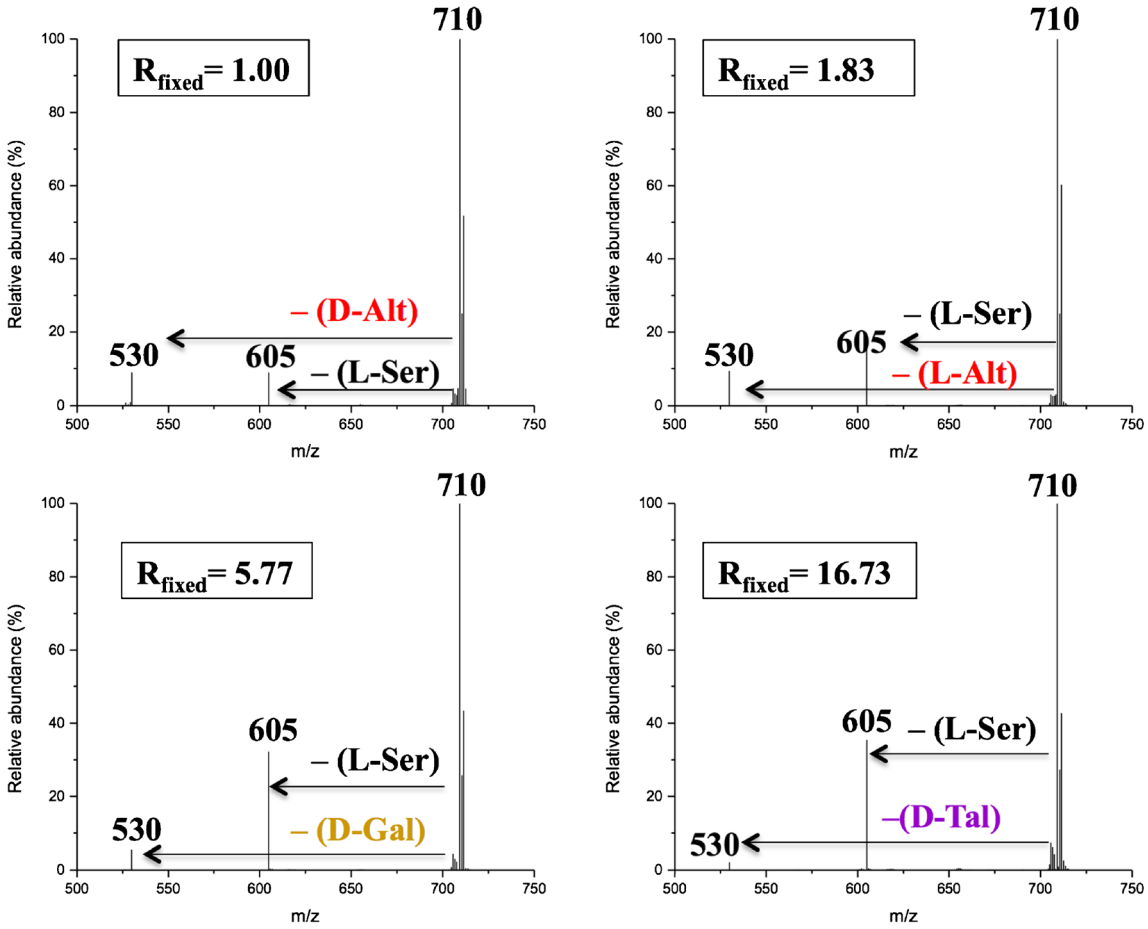

Figure 5. Fixed ligand kinetic method MS^2 spectra with Cu^{II}, *L*-Ser, and 5′GMP, for *D*-Alt, *L*-Alt, *D*-Gal, and *D*-Tal at 10% NCE and 30 ms activation time

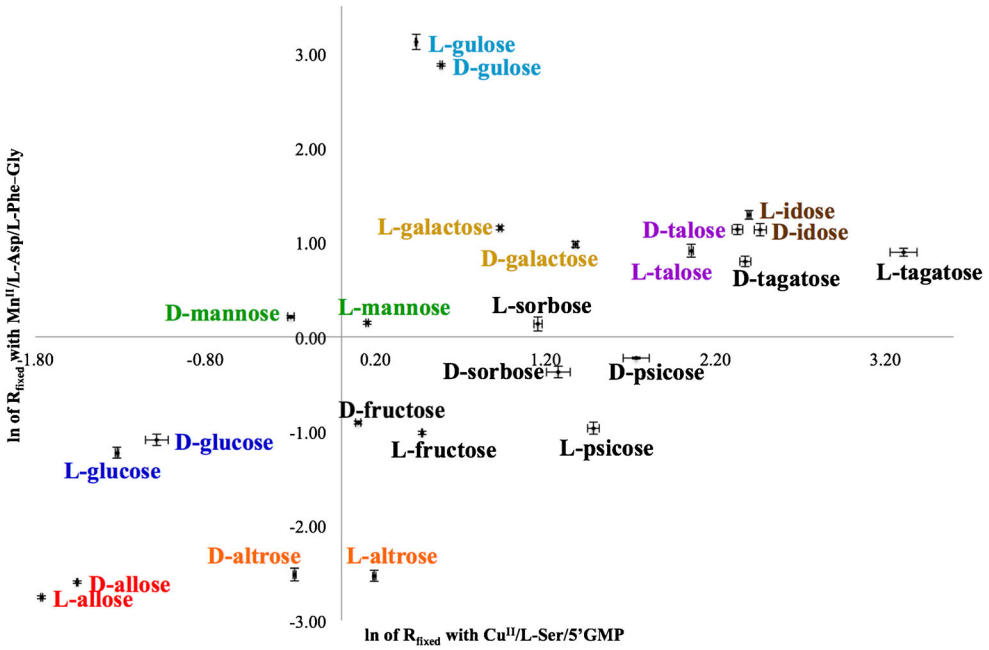

Figure 6. 2-D natural log-natural log plot of R_{fixed} with Cu^{II}/*L*-Ser/5′GMP on the x-axis, and R_{fixed} with Mn^{II}/*L*-Asp/*L*-Phe–Gly on the y-axis; both at 20% NCE and 30 ms activation time. All results represent triplicate trials with standard deviations included

aldohexoses was determined, the next step was to apply those same conditions to the eight ketohexoses. Only these fixed ligand combinations were pursued because they provided the best chiral discrimination among the aldohexose isomers. The thought process was that these two combinations should create significant enough energetic differences among all 24 isomers in order to accurately identify and determine absolute configuration for all the neutral hexose monosaccharides. Optimized mass spectrometric conditions found from the aldohexoses were applied for each of the eight ketohexose isomers with both fixed ligand combinations, Mn^{II}/*L*-Asp/*L*-Phe – Gly and Cu^{II}/*L*-Ser/5′GMP. As previously stated, each isomer will yield a specific R_{fixed} range, for each fixed ligand combination used, based on averaged triplicate trials with standard deviation error bars.

Once again, if each of the 24 isomers has a unique and non-overlapping R_{fixed} range, simultaneous identification and absolute configuration determination is achieved. With data now collected for both the aldohexose and ketohexose isomers, at the same optimized conditions, a novel two-dimensional plot was created. Figure 6 shows the natural logarithm of R_{fixed} values for each isomer with the Mn^{II}/*L*-Asp/*L*-Phe – Gly combination on the y-axis and the natural logarithm of R_{fixed} values for each isomer with the Cu^{II}/*L*-Ser/5′GMP combination on the x-axis. A natural log–natural log plot was chosen because it was found easier to illustrate the data in small numerical ranges, compared with the initial raw data. (For each of the R_{fixed} values in raw data form, see the Supporting Information.) Figure 6 clearly shows all 24 hexose isomers discriminated from one another. Not only is each monosaccharide definitively identified, simultaneous absolute configuration determination is also achieved. This work represents the first such mass spectrometric discrimination, and simultaneous absolute configuration determination, of all 24 neutral hexose monosaccharide isomers.

Comparison with Other Monosaccharide Analysis Techniques

This method matches the low sample size requirements of HPAEC, GC/MS, and HPLC (<5–50 μg) and reduces the amount as compared to NMR (milligram level) [9–11, 16–29, 52, 53]. However, this technique is able to determine absolute configuration, which HPAEC is unable to do, and there is no ambiguity in interpretation since only the relative intensities of two MS^2 peaks are needed, compared with the convoluted chromatograms in GC/MS or HPLC and difficult spectra in NMR. Once the suitable fixed ligand combinations are found, each individual mass spectrometric run takes minutes, which provides a fast and accurate analysis. Lastly, there is no need for sample derivatization or for any home-built instrumentation, as a commercial mass spectrometer without any modifications was used for all the experiments.

Conclusion

Interestingly, in a study of the analysis of epimeric glycans with ion-mobility mass spectrometry, the presence of a single axial hydroxyl substituent on an aldohexose monosaccharide was hypothesized to lead to a lack of discrimination, as in the case of galactose (C4) versus mannose (C2) [6]. This single axial hydroxyl theory, however, does not hold in this fixed ligand method described. The three aldohexose monosaccharides with single axial hydroxyl substituents, allose (C3), galactose (C4), and mannose (C2), are all easily discriminated from one another based on their respective R_{fixed} ranges. Clearly, more studies that do not rely on data from only a small subset of carbohydrates are needed.

Herein the first analytical technique to identify any of the 24 neutral hexose monosaccharides and determine their absolute configuration (Figure 6) has been demonstrated. It can be foreseen that a similar strategy could be applicable for the definitive identification of other monosaccharides, such as the uronic acids, hexosamines, and 2-deoxysugars, which will ultimately allow the construction of mass-spectrometry-based protocols for *de novo* sequencing of glycans. This method can be readily adopted and employed for the identification of any unknown monosaccharide that is isolated from a biological or other source. Sample preparation requirements from one of these biological sources include for the monosaccharide to be free of any salts, which can be accomplished with dialysis followed by lyophilization [54, 55]. In a biological sample, if the precursor ion is in low abundance, mass spectrometry conditions can be optimized to increase its abundance. Furthermore, as long as the precursor ion is in high enough abundance to be successfully trapped, there will be no issues with obtaining the desired pair of fragment ions and, thus, the fixed ligand kinetic method branching ratios. Work is ongoing to interface this mass-spectrometry-based monosaccharide identification method with a microfluidics oligosaccharide hydrolysis/separation platform.

Acknowledgments

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