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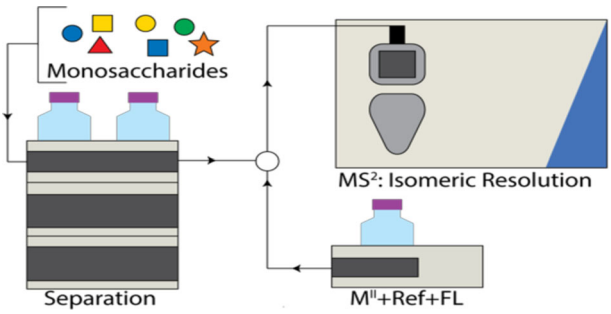
RESEARCH ARTICLE

Development of a Post-Column Liquid Chromatographic Chiral Addition Method for the Separation and Resolution of Common Mammalian Monosaccharides

Zachary Wooke,[1] Gabe Nagy,[1] Lauren F. Barnes,[1] Nicola L. B. Pohl[1,2]

[1]Department of Chemistry, Indiana University, 800 E Kirkwood Ave., Bloomington, IN 47405, USA
[2]Radcliffe Institute for Advanced Study, Harvard University, Cambridge, MA 02318, USA



Abstract. The first solely MS-based methodology for the identification and resolution of the ten common mammalian monosaccharides is presented. Based on Cooks' fixed ligand kinetic method, this technique is effective on multiple classes of monosaccharides and includes the first example of two fixed ligand combinations used in a single multiplexed experiment. Subsequently, a post-HPLC chiral addition method is used in conjunction with this newly developed MS methodology for the separation and identification of mixtures of common neutral mammalian monosaccharides. This proposed technique is able to overcome a limitation of present carbohydrate analysis methods, namely the simultaneous isomeric resolution of multiple monosaccharides in a mixture.

Keywords: Carbohydrates, Monosaccharides, Fixed ligand kinetic method

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Introduction

While other biologically relevant monomers (e.g., amino or nucleic acids) generally have non-isomeric constituents, monosaccharides—with their multiple chiral centers and small set of highly conserved functional groups—provide a tremendous challenge for their accurate, and sensitive, analysis. This, along with the multiple branching points not possible in the other two major biopolymers, results in a high number of possible configurations for the carbohydrates they build. Simply constructing a 6-mer from the ten most common monosaccharides found in mammals results in over 190 billion different chemically possible combinations (the number of actual possibilities likely to be found in nature is of course limited by the available enzymes for synthesis) [1]. Carbohydrates are known to be involved in many biological processes including cell-cell recognition, cellular adhesion, protein folding and solubility, metabolism, and immune/host pathogen responses [2–6]. Because monosaccharides serve as the building blocks to all biologically relevant carbohydrates, their accurate identification is a key part of determining their structure [4–8]. It is of such importance that in 2012, the US National Academy of Sciences 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" [9]. Spectroscopic analytical methods, such as nuclear magnetic resonance spectroscopy [10], are capable of isomeric monosaccharide discrimination but require large (milligram) sample amounts for analysis; additionally, they cannot resolve complex mixtures. To address the issue of sample quantity, a number of mass spectrometry (MS)-based techniques have been developed for the delineation of isomeric monosaccharides. Because of the isobaric and isomeric nature of monosaccharide sets, single-stage MS is incapable of distinguishing isomers, and therefore, analysis is performed in conjunction with another technique or through multi-stage MS experiments. Gas chromatography-mass spectrometry (GC-MS) remains the current state of the art technique

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Correspondence to: Nicola Pohl; *e-mail:* npohl@iu.edu

but is limited by derivatization requirements [11]. Derivatization has proven useful for other techniques as well [12–15], but issues such as incomplete reactions or side reactions can limit the effectiveness of methods that rely on it. Several ion mobility-mass spectrometry (IM-MS) or tandem mass spectrometry (MS^n)-based methods have circumvented these derivatization requirements [16–21]. While these techniques have shown great utility in the rapid identification of individual isomeric monosaccharides, they have remained limited by their inability to resolve complex mixtures of various isomeric and non-isomeric monosaccharides.

Since a mixture of monosaccharides remains challenging to analyze with MS alone, a pre-separation step, such as liquid chromatography (LC), can prove beneficial. Alternatively, such an LC separation need not be perfect (able to completely resolve all monosaccharide components), but rather it needs to resolve enough of the components to aid downstream MS deconvolution. Even conventional LC-MS approaches remain unable to completely identify or resolve all monosaccharide constituents (especially enantiomers) in a potentially complex sample matrix. Chiral chromatographic stationary phases have shown utility in the separation of monosaccharides, including enantiomers, but even those cannot offer complete separation of all isomers. [22] In order to bridge the gap left by a combined imperfect LC separation and imperfect MS strategy, we turned our attention to the development of a post-column addition method that is capable of elucidating isomeric resolution of monosaccharides. It was unclear as to what post-column strategy would be most suitably applied for the resolution of the common mammalian monosaccharides. Based on previous work in our group with the fixed ligand kinetic method (developed by Cooks and co-workers), which has shown potential for the individual identification of a complete isomer set of monosaccharides, we set out to utilize a similar approach for our post-column addition technique [17–19]. Please see the Supplementary Information and these references [17–19, 23] for more information on the fixed ligand kinetic method (FLKM) analysis of carbohydrates. Briefly, a trimeric ion complex is formed containing the analyte, divalent metal cation core, chiral reference (amino acid), and fixed ligand. The complex is activated via collision-induced dissociation (CID), and either the analyte or chiral reference will dissociate. By measuring the ratio of the relative intensities of the diastereomeric product ions, a unique R_{fixed} value is generated. Herein, we present a combined LC-FLKM technique for the simultaneous separation and resolution of monosaccharides.

Experimental

Reagents

All monosaccharides D-xylose, L-fucose, D-glucose, L-glucose, D-galactose, D-mannose, D-glucuronic acid, L-iduronic acid sodium salt, *N*-acetyl-D-glucose, *N*-acetyl-D-galactose, *N*-acetylneuraminic acid), chiral reference molecules (L-aspartic acid, L-glutamic, L-tryptophan), fixed ligand molecules (guanosine monophosphate disodium salt), and metal salts ($NiCl_2$) were purchased from Sigma Aldrich (Milwaulkee, WI, USA) and Carbosynth (Berkshire, UK). Solvents for the screening consisted of a 50/50 (*v/v*) mixture of HPLC-grade water and methanol. Mobile phase used in the LCMS separation was LCMS-grade water and the T-in was a mixture of 25/75 of HPLC-grade water and methanol. Separation was performed on a Rezex RPM-Monosaccharide Pb + 2 Ion exclusion column from Phenomenex (Torrance, CA, USA) using an Agilent 1260 HPLC system with a Diode Array Detector (Santa Clara, CA). Mass spectrometric analysis was performed on a Thermo Scientific LTQ Velos Pro linear ion trap mass spectrometer with an electrospray ionization source in positive ion mode (San Jose, CA, USA). UV-Vis traces were recorded using Agilent's Open Lab CDS Chemstation. Mass spectrometry data was recorded using Thermo Scientific's X-calibur software.

Fixed Ligand Kinetic Method Combination Screening

Solutions of 1 mL 50/50 water/methanol containing 25 μM $NiCl_2$, 100 μM monosaccharide, 100 μM chiral reference, and 100 μM 5′-GMP were prepared for all ten monosaccharides. Solutions were electrosprayed at 5 μL/min and recorded for 50 or 100 scans with 3 microscans per scan. Collision-induced dissociation (CID) was used to produce the product ions at 25% normalized collision energy (NCE). Values for CID voltages applied can be found in Table S1. Each sample was run in triplicate and averaged. Plots were made using the natural logarithm of the averaged R_{fixed} values. Error bars are reported as one standard deviation.

HPLC Separation and Mass Spectrometric Determination

Isocratic liquid chromatography was performed using water as the mobile phase at a flow rate of 1 mL/min. A passive flow split was used to reduce the flow rate to 100 μL/min. The metal, reference, and fixed ligand (500 μM each) were then mixed inline by another HPLC pump at a flow rate of 100 μL/min. Total flow rate into the ESI source was therefore 200 μL/min (Figure 2). UV data was collected at 194 nm with a bandwidth of 8 nm. Mass spectra were collected in a series of five sequential scans repeated during the chromatographic separation. To measure the mixtures and the individual monosaccharides, a selected ion monitoring scan for *m/z* 703, 717, 733, and 774 (mass width 3 *m/z*) and then CID scans at 25% NCE for the same *m/z* values. For the calibration curves, the CID scan at *m/z* 733 used selected reaction monitoring for *m/z* 553 and 600 (mass width 3 *m/z*). R_{fixed} values were determined from the appropriate CID scan using the full width half maximum (FWHM) of a product ion peak. For all experiments, 210 nmol of each monosaccharide was injected unless otherwise indicated. Limits of detection were determined by injecting decreasing amounts of the monosaccharide mixture and analyzing the extracted ion chromatograms of the product ions from their respective CID scan.

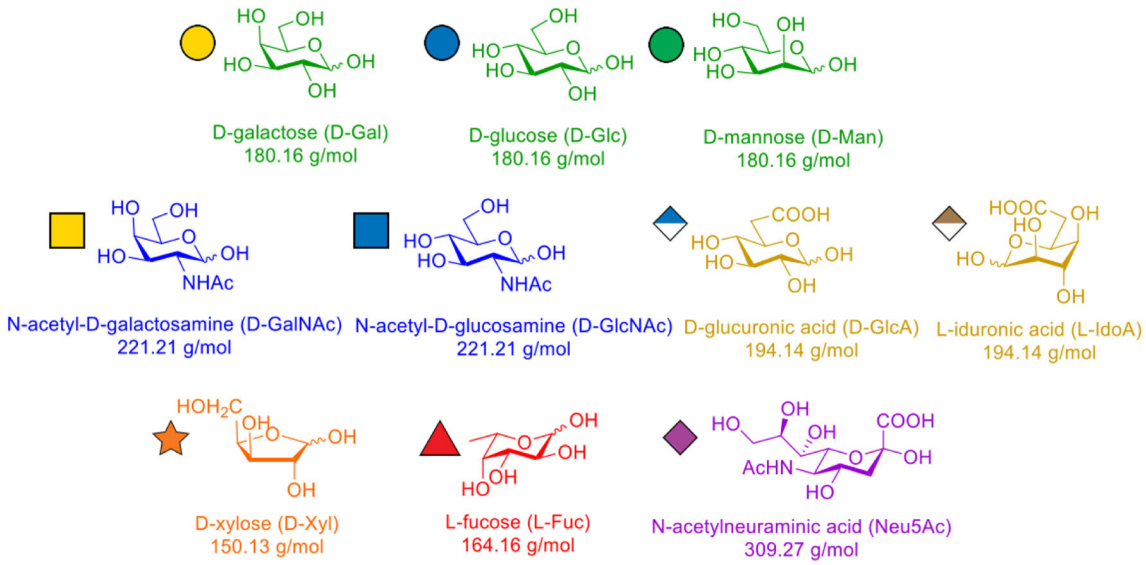

Figure 1. Structures, symbols, and molecular weights of the ten common monosaccharides found in mammalian samples

Correction of Interfering Product Ion

An unknown ion with a *m/z* of 733 is generated from the *N*-acetylhexosamines, which upon activation via CID produces a product ion at *m/z* 600. Because there is coelution between glucose and *N*-acetylglucosamine and between mannose and *N*-acetylgalactosamine, calibration is needed to correct their measurement in solution. Calibration curves for the hexoses are provided in the supplementary information.

D/L-Glucose Mixture Analysis

Five solutions of D- and L-glucose (0, 25, 50, 75, and 100% L-glucose) were prepared. Injections were kept at 210 nmol of total solute. Liquid chromatography and inline mixing were performed as above. Mass spectra were collected in a series of 3 scans. A selected ion monitoring (SIM) scan for *m/z* 203 ($Glc + Na^+$) was followed by two CID scans at *m/z* 733 (Glc-FL complex), a full scan, and then a selected reaction monitoring (SRM) looking at *m/z* 553 (loss of Glc) and *m/z* 600 (loss of reference). A calibration curve was produced using the natural logarithm of the R_{fixed} values at the FWHM of the *m/z* 553 ion chromatogram peak in the SRM scan.

Results and Discussion

Screening for Fixed Ligand Combinations

We hypothesized that if a chiral ligand set could be discovered to individually discriminate among the various mammalian monosaccharides, it could potentially be added in as a chiral modifier, post-column of the LC separation, to further deconvolute components that are unresolved by LC alone. As compared to previous work, where only a single class of monosaccharides was assessed (e.g., hexoses) [18, 19], here, a more universal

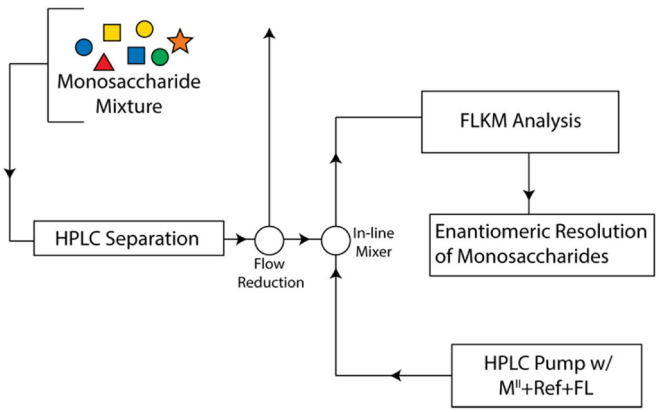

Figure 2. Cartoon depiction of instrumental setup for LC-FLKM. An initial HPLC aids in separation/deconvolution of a mixture. The eluents from the HPLC are then mixed with a fixed ligand (FL) combination before being analyzed via MS^2, where FLKM (FL kinetic method) is used to identify isomeric monosaccharides

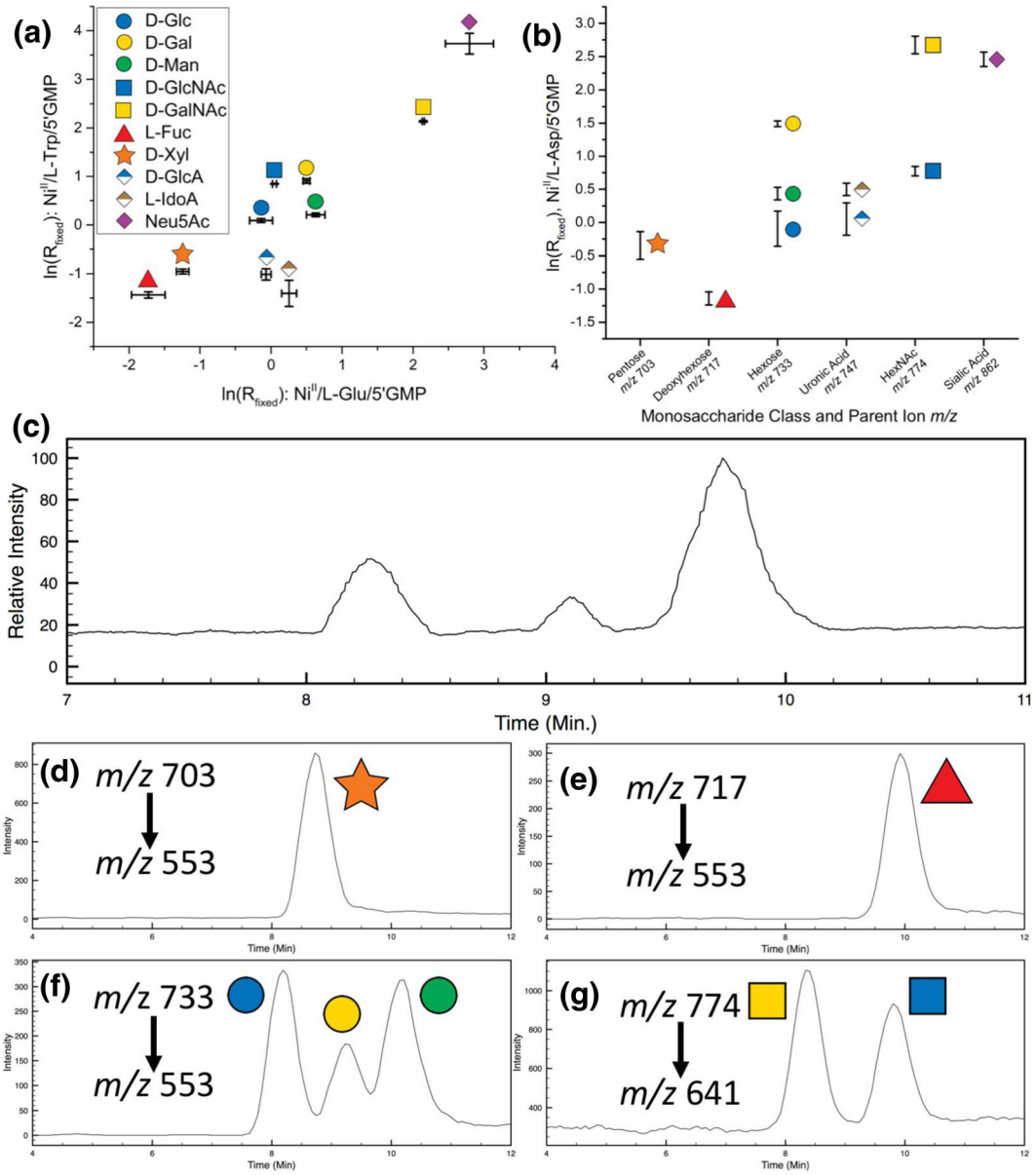

Figure 3. (**a**) Complete monosaccharide discrimination was achieved with the two initial combinations. (**b**) Monosaccharides in the L-Asp complex plotted by the natural log of their R_{fixed} value vs. their parent ion mass-to-charge. (**c**) Total ion chromatogram from a survey of the separation of the seven neutral monosaccharides. (**d**–**g**) Extracted ion chromatograms of one of the product ions from the CID scans for each of the four complexes

fixed ligand combination needed to be found to resolve monosaccharides from multiple classes—in our case, the 10 most common monosaccharides found in mammalian glycans (Figures 1 and 2) [1]. They span seven different classes (pentose, deoxyhexose, hexose, uronic acid, *N*-acetylhexosamine, and sialic acid). Initially, two combinations—Ni^{II}/L-Glu/5'GMP and Ni^{II}/L-Trp/5'GMP—were found to generate distinct two-dimensional data for all 10 monosaccharides, but neither combination was capable of being used individually (Figure 3a). Using two combinations will be less than ideal when the amount of sample is limited due to the fact a separation must be run twice while changing the reference molecule in between them. Multiplexation, or the mixing of two chiral reference molecules for a single metal cation, single fixed ligand, and for a single analyte, in the same solution, yielded exciting results (Figure 4), as a multiplexed fixed ligand analysis has not been previously reported. Thus, identification of the monosaccharides could be performed from a single vial rather than two. However, multiplexing was achieved by using half the amount of each reference as the standard individual trials. Therefore, only half as much of each complex was produced resulting in half as much signal. Because we suspected that post-column introduction of the other fixed ligand components would lower the amount of complex available to sample, due to the severely limited mixing time, we decided to pursue a third combination that could be used individually. Ni^{II}/L-Asp/5′GMP was found to achieve this

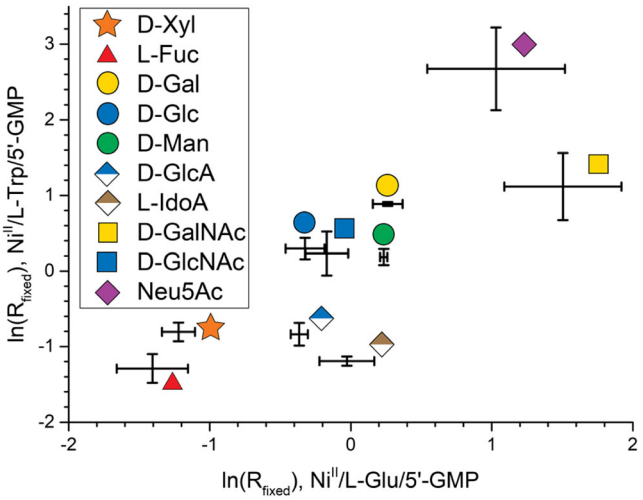

Figure 4. Complete discrimination of the ten monosaccharides is achieved in a multiplexed sample where both L-Trp and L-Glu were present. The overlap between D-Glc and D-GlcNAc does not pose an issue as their parent ions are mass differentiated

by plotting the R_{fixed} values against the parent complexes' *m/z* (Figure 3b). With this combination, all monosaccharides within an individual class (pentose, hexose, etc.) were differentiable. R_{fixed} values do overlap when comparing between different classes, but the classes themselves are mass differentiated. For example, a fixed ligand complex containing D-glucose has a mass-to-charge of 733 while one with L-fucose is *m/z* 717.

LC-FLKM

We chose to perform the chromatographic portion of this work with the seven uncharged monosaccharides as they are harder to separate from each other in a mixture and can typically co-elute. Separation of the monosaccharides was performed using a Rezex RPM-Pb2+ ion-exclusion column. Ion-exclusion chromatography has previously been shown to effectively separate these monosaccharides, and there are multiple hypotheses behind the separation mechanism [24, 25]. In the initial UV absorption chromatogram (SI Figure S3), only the two *N*-acetylhexosamines were observed, due to their higher absorptivity. When looking at the mass spectra, interclass co-elution was observed (e.g., glucose and *N*-acetylglucosamine overlap), but intraclass separation was achieved. (Figure 3d–g) Finally, FLKM can be used to accurately identify the peaks (Figure 5).

As the mechanism by which the complexes are formed is not fully known, it was unclear whether they would be generated with enough efficiency on the timescale of post-column addition mixing. Data was collected as a series of CID scans that were cycled repeatedly throughout the chromatographic separation. Because the parent ion masses are all known, this ensured that data would be collected for all of the complexes. Their R_{fixed} values reasonably agree with those of the individual standards, run under the same analysis, and were clearly differentiable between classes (Figure 6). However, the individual standards for D-Glc and D-Man had to be corrected by adding D-GlcNAc and D-GalNAc, respectively, in equimolar concentrations. It was discovered that the *N*-acetylhexosamines produce ions at *m/z* 733 (potentially in-source fragments) that upon activation produce a product ion with a *m/z* of 600. This product ion interferes with the CID scan which is used to measure R_{fixed} values for the hexoses that also have a product ion at *m/z* 600. Calibration was used to confirm that the interference could be corrected for (SI Figure S5).

For each experiment, only 10% of the monosaccharide separated was sent into the MS for FLKM analysis. The limit of detection (LOD) for the monosaccharides sent to the MS is therefore 10% of the total moles injected to the LC (based on volume loaded onto column and the initial starting concentration). The amount detected varied depending on the class. For example, D-GalNAc had an LOD of ~ 1 µg, but D-Glc was ~

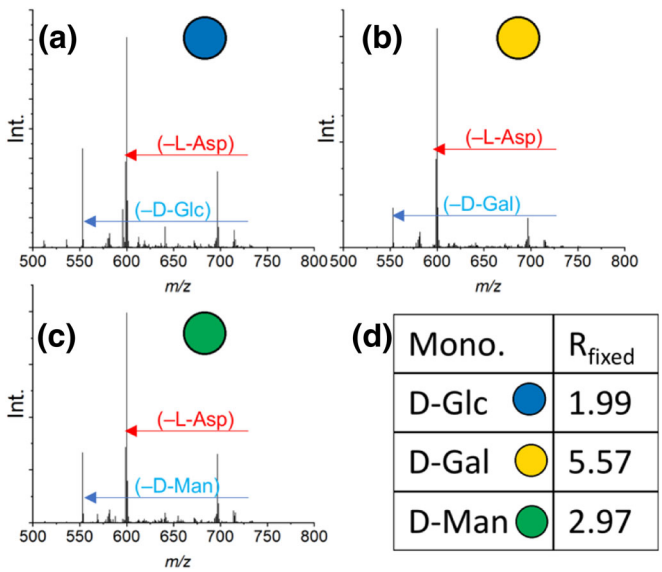

Figure 5. (**a**–**c**) Mass spectra of the FWHM of the peaks from the CID at *m/z* 733 (Figure 2f). (**d**) Table R_{fixed} values for the hexoses

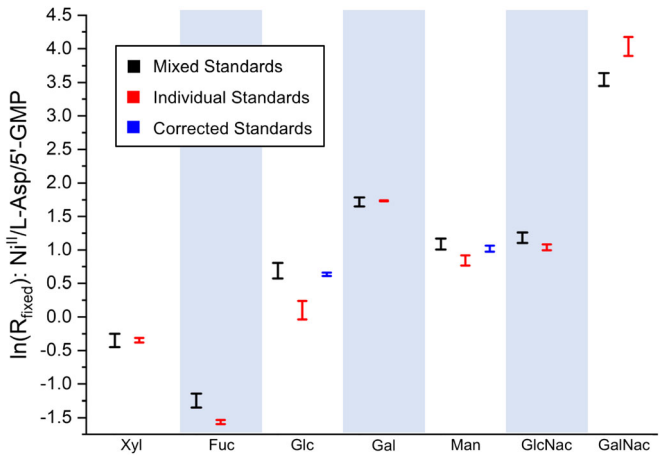

Figure 6. Results from LC-FLKM trials. Bars represent the range from + 1 to – 1 standard deviation. Corrected standards account for the unknown interfering ion produced from the *N*-acetylhexosamines

38 ng. For perspective, a permethylation for a *N*-glycomics workflow typically uses 1–10 µg, meaning that these LODs are analytically relevant [26]. We envision future work to explore the use of smaller particle sizes, and other stationary phases, to potentially further improve upon these LODs.

Enantiomeric Resolution

To further exemplify the power of this method, we wanted to show its capacity for enantiomeric resolution without the need for a chiral LC step. Therefore, we prepared several mixtures of D- and L-glucose and performed LC-FLKM using the same Ni^{II}/L-Asp/5'GMP combination (Figure 7). On the RPM-Pb2+ ion-exclusion column, as expected, D- and L-glucose co-elute and therefore mixtures of the two cannot be discriminated with standard LC-MS techniques. With LC-FLKM, however, mixtures can easily be analyzed and with calibration, the enantiomeric ratio can be measured (SI Figure S6). Thus, LC-FLKM is capable of enantiomeric resolution without the requirement for a chiral stationary phase support. This technique should be easily applicable beyond carbohydrates, as well, based on previous literature FLKM analysis works on a variety of chiral molecules [27–31].

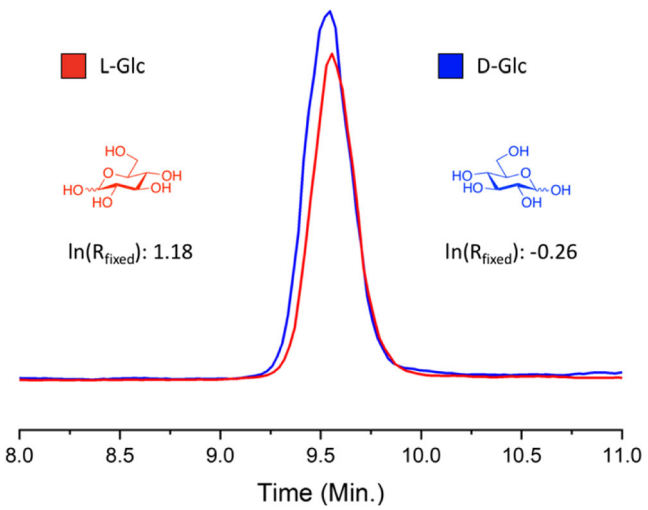

Figure 7. Ion chromatogram at *m/z* 203 $[hexose + Na]^+$ of a series of mixtures of D- and L-glucose. The isomers co-elute and are therefore unable to be discriminated by standard LC-MS. A calibration curve is available in the supplementary information

Conclusion

In summary, Cooks' fixed ligand kinetic method was applied to multiple classes of monosaccharides including deoxy-sugars and uronic acids. The first multiplexation of FLKM was reported. Lastly, FLKM was coupled to HPLC to create a LC-FLKM technique that was used for the simultaneous separation and chiral resolution of monosaccharides. This technique is expected to move beyond carbohydrates though, as molecules for which a set of fixed ligand conditions can be found, are also candidates for LC-FLKM.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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